A Biophotonic Study of Live, Flowing Red Blood Cells in an Optical Trap
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ABSTRACT
We investigate the physics of an optically trapped red blood cell under physiological conditions. When a single, live red blood cell, is placed in an optical trap, the normal biconcave disk shaped cell is observed to undergo a folding action and thereby take up a rod like shape. If such an RBC has any shape anisotropies due to perturbation through malarial infection or hyperosmotic stress, it is observed to rotate in the linearly polarised laser field. Finally when such an optically trapped RBC is exposed to a shear flow, a tank treading like behaviour of the red blood cell membrane is visualised (wherein the RBC membrane revolves around the central body of the cell). The tank treading motion of a red blood cell held stationary in the optical trap allows for the dynamics to be viewed in a prolonged manner without the usage of earlier constraints such fast imaging system.

Optical Trap, Red Blood Cell, Folding, Rotation, Shear Flow, Tank Treading

1. INTRODUCTION

Manipulation of biological matter using light on the microscopic scale is a subject of increasing interest, due to its applicability and relevance to fundamental research. The optical trapping and thereby confining a single cell in a laser field is one such example of how interaction of light and matter affect cellular dynamics. Recent progress in photonics and laser technology now allow applying and sensing forces in nN ranges with pN accuracy and corresponding displacements can be measured with even higher accuracies of nanometer ranges. Even low intensity optical forces that lie between 1-50 pN are capable of physically deforming a cell without causing cell death: this has been demonstrated in trapping experiments involving single red blood cells (RBCs).

RBCs classically are considered to be elastic bags (made of a lipid membrane) filled with an incompressible Newtonian liquid. Just below the lipid lining of these cells is a thin meshwork of proteins called the cytoskeleton. Together the lipid and the protein network form the RBC membrane. The lipid layer imparts a viscous nature to the membrane while the protein layer imparts the elasticity. In equilibrium, such cells take up a biconcave shape (as shown in Figure 1a), which has been hypothesized to be a consequence of minimal membrane bending considerations. This shape is responsible for the RBCs in having a 40% greater surface area than is required to enclose their volume, allowing them to bend and fold without increasing surface area. Such a structure thereby allows the RBCs to squeeze and bend and travel through the narrowest of capillaries which might be as small as 2µm in diameter. Similarly, various other structural aspects of RBCs allow them to carry out their function properly. Thus, the study of such biophysical properties of RBCs is slowly gaining more importance. Biophysical properties of RBCs, like shape, size, and bending modulus, have been extensively investigated.

We report here the optical trapping of healthy and malaria-infected (Plasmodium falciparum) RBCs, we demonstrate that when an RBC is exposed to a highly focussed infrared laser beam (where such a beam is passed through a 100X microscope objective whose NA=1.6) as that of an optical trap, the RBC moves toward the laser focus following which trapping occurs. This is accompanied by buckling of the disk shaped cell which takes a folded rod-like shape. On removal of the laser light, the cell unfolds to its original biconcave shape. We have probed the behaviour of such trapped RBCs in a shear flow. The existence of a tank treading motion of the membrane of these cells is visualised by
attaching a 100nm fluorescent bead on the surface of the RBC. This type of motion has various important implications. Namely, it gives the RBC a lift force while travelling through the blood vessels, thereby not allowing them to settle down and also remain at the centre of flow in the blood vessels and thus in the fastest stream of flow. This ensures efficient transfer of RBC and the oxygen they transport through the blood. We also go on to probe the dynamics of the cytoplasm as the membrane revolves around the cell. Such findings show that the tank treading motion is not only restricted to an ellipsoidal shaped RBC as predicted by the existing theories but have more wider applicability as far as cell shape is concerned.11

Along with folding, two more phenomena are observed when RBCs are kept stationary i.e., in a non flow situation. Firstly, shape-engineered RBCs can be made to rotate using linearly polarized trapping light. Such shape engineering can be brought about by hyperosmotic stress or by the introduction of an intracellular parasite such as *Plasmodium falciparum.*12 Secondly, in the infected RBCs, if there is sufficient amount of the pigment known as hemozoin which is produced during a malarial infection, the RBC seems to undergo bubbling upon trapping.

2. MATERIALS AND METHODS

2.1 Preparation of red blood cell
For obtaining human red blood cells, 100µl of blood was drawn from a finger prick into a vial containing 0.9 mg of anticoagulant powder (where each gram of anticoagulant comprised 450 mg of dextrose, 400 mg of sodium citrate, and 150 mg of citric acid). The RBCs were then separated by centrifugation and resuspended in a physiologically relevant media such as 300mOsm Phosphate buffered saline (PBS). These were considered to be the normal RBCs in all folding and rotation experiments. For Experiments involving tank treading, 100 nm silica beads were attached onto their membrane. To achieve this, a fixed amount RBC suspension with a known concentration was incubated with different bead concentrations for 1 hour at 4 C to allow spontaneous nonspecific adhesion of beads onto the membrane of the RBCs. After this, the cells were microscopically examined for bead attachment. The particular concentration of bead solution at which most RBCs had only one bead attached to its surface was chosen to carry out further experiments. Furthermore some Tank treading experiments involved RBCs with beads within the RBCs rather than on the membrane. For making such RBCs, we induced Heinz bodies within RBCs by incubating them at with 2 mg/ml phenyl hydrazine hydrochloride for 4 hours at 37 C. The hemoglobin within the RBCs partially precipitates and form bead-like opaque structures within the RBCs.

For perturbing RBCs with hyperosmotic stress, the RBCs were incubated in a PBS solution having an Osmolarity of 900mOsm for 15 minutes and then subjected to folding, rotation and tank treading. For malaria infected RBCs, asexual stages of *Plasmodium falciparum 3D7* strain were maintained in *vitro* in Roswell Park Memorial Institute (RPMI) 1640 medium. in human RBCs as per established protocol.13 The identification of such infected RBCs were done by labelling them with a fluorescent marker, DAPI, a membrane-permeable fluorescent nuclear dye, which solely marks the nuclear material of the parasite residing inside the RBC while the uninfected RBCs remain unlabelled. Figure 1b shows such infected cells labelled with DAPI.

All our experiments with human and mice blood were conducted in accordance with rules and procedures adopted by the Tata Institute of Fundamental Research Human and Animal Ethics Committee.

2.2 Instrumentation
Our experimental set-up consisted of an optical tweezers apparatus coupled to a liquid flow cell.13 A schematic representation pertinent to the present set of experiments is shown in Figure 1c. Optical trapping was achieved by focusing a 1064 nm wavelength, continuous wave (cw) light from a Nd:YVO₄ laser through a 100X objective (NA=1.3).A flow cell of dimension 80µm height, 8mm breadth and 48mm length was used for flowing RBCs. The RBCs
suspended in PBS flow through this flow cell at different flow rates or kept stationary and could be trapped in either condition. Such cells were viewed by the same objective and were imaged by a CCD camera operating at either 1250 frames per second or 25 frames per second, coupled to a computer so that observations could be recorded in real time. The individual frames of the recorded movies were analyzed by using the Image J software. The laser power after the objective was kept at 20mW for all our experiments.

2.3 Experimental Procedure
The blood was diluted 1000-fold in the suspending fluid. This suspension was pumped through the flow cell at a constant rate, with values ranging from 0 µl/hr to 750 µl/hr. The corresponding flow velocities were computed in a separate experiment by flowing polystyrene beads at the same rates. For experiments to study folding and rotation of RBCs in an optical trap, after the cells were introduced inside the flow cell, the flow was stopped and the solution was kept stationary. For experiments involving tank treading of RBC, RBCs with beads attached onto them were flow and were trapped while in flow. Such RBCs were brought to a specific height in the flow cell to expose them to a particular shear rate. The shear rates were determined by flowing polystyrene beads and calculating the differences in the velocities of the beads flowing in the different layers in the flow cell.

![Image](image.png)

Figure 1: a) Normal Disk Shaped RBC, b) Labeling of infected RBCs with DAPI. Left hand panel shows a DIC image of RBCs in bright field while the right hand filed shows fluorescence produced by the infected RBCs while the uninfected RBCs appear non-fluorescent. c) Schematic of the setup used for experiments.

3.1 Folding of an RBC in an optical trap
The RBC close to laser focus is seen to be drawn towards the focus, and then undergo a buckling action consequently folding into a rod-like shape. This RBC then aligns itself with the electric vector of the propagating laser. Such a folding action is rationalized by a model based on Euler Buckling. On removing the laser, the RBC is seen to unfold and return to its original biconcave shape. It was found that the time involved for RBC folding (which is in the order of milliseconds) was much less than RBC unfolding (which is in the order of seconds). A series of time lapse images in figure 2 shows the folding behavior of a single RBC measured using a high frame rate camera.

![Image](image.png)

Figure 2: Shows time lapse images of a single RBC folding on being optically. Note that folding time takes ~100 milliseconds while unfolding time is much more, in the order of 10 seconds or more

Although, there is no simple model relating the folding and unfolding times to the shear modulus or any other physical
parameters of the RBC, it can be hypothesized that they are a direct read out of how stiff the cell might be i.e., more stiffness, more will be the folding and unfolding times and vice with the laser power remaining constant. Such folding and unfolding and unfolding times were compared for normal, infected, and osmolarity perturbed (through hyperosmotic stress) RBCs. Folding and unfolding times were found to be minimal for normal RBCs, whereas they increased with infection as well as hyperosmotic stress. It is obvious from the nature of the deformation and the restoration that the RBC undergoes in an optical trap, that it is a viscoelastic phenomenon. As mentioned earlier the lipid membrane of the RBC as well as the cytoplasm is responsible for imparting a viscous nature to the RBC while the cytoskeleton imparts the elastic nature. All three of these components are greatly modified when an RBC is subjected to an infection or to hyperosmotic stress which thus serve as an explanation for the change in the folding and unfolding times the perturbed RBCs show. Such a study establishes a new method for quantification of cell rigidity. Using this technique we have thereby shown that during a malarial infection, the RBCs become much more rigid and explain the reason for the widespread capillary blockages that occur during malaria.

3.2 Tank Treading of a trapped RBC in Shear flow

When the trapped and folded RBC is held in a laminar shear flow that is present in the flow cell, the membrane of the RBC is seen to undergo a Tank Treading (TT) like motion, i.e. it traverses around the RBC akin to treads of a battle tank. Figure 3a depicts such a motion. Various earlier theories attempting to describe TT motion have utilized the Kellar-Skalak (KS) model.\textsuperscript{6,14,15} The KS model utilizes various parameters of the ellipsoidal shape of the RBC that it take up during TT motion namely ellipsoidal axes and angle in the shear plane. In our experimental situation, as shown in Figure 3, such parameters are highly distorted by the folding aligning action of the optical trap. Thus the validity of the KS model in our case becomes questionable. Along with this fact there is another inconsistency with the KS model that comes to light in our setup. TT motion is observed to occur within low viscosities fluids such as PBS, having a viscosity of 0.92 cP, while previous measurements utilized suspending fluid viscosities of at least 10 cP or more.\textsuperscript{16,17} Furthermore, the KS model predicts that at such low viscosities the RBCs would tumble at all values of shear rate, implying that no TT motion would be expected. Therefore, with such inconsistencies with the KS model, the question arises: are we observing tank treading at all? We however are able to show experimental evidence that Tank Treading is indeed taking place in our condition as well.\textsuperscript{11}

We observe that, during TT motion, neither the orientation nor the shape of the optically trapped RBC changes as the bead moves about the cell's cross-section (Figure 3a). This indicates that it is the RBC membrane that is in motion while the shape of the trapped RBC is maintained constant. As already mentioned, the cytoskeleton and the plasma membrane in an RBC form a tightly coupled layer around the cell. However, there are no cytoskeletal elements in the cytoplasm. This allows the membrane and cytoskeleton to revolve around the cytoplasm in coupled fashion.

A salient feature of tank treading behavior in the KS model is the viscous dissipation in the cytoplasm and the cell membrane during each cycle of TT motion. In order to experimentally prove that such dissipation does, indeed, take place, we carried out experiments to explore the presence of viscous lag between consecutive layers of the cytoplasm in the course of TT motion. Following earlier work,\textsuperscript{18} we made measurements on Heinz bodies within RBCs. The RBCs are then made spherical by incubating in a hypotonic medium so as to visualise the Heinz bodies. Figure 3b shows that two bead like Heinz bodies located at different depths with the RBC cytoplasm revolve during TT motion with different velocities thereby proving viscous dissipation within the different layers of the cytoplasm. We also find that the Tank Treading Frequency increases with increase in temperature. Temperature is known to exponentially decrease the viscosity of lipid membrane.\textsuperscript{19} Therefore it can be rationalized that if there is viscous dissipation in the membrane, as membrane viscosity decreases, rate of dissipation also decreases, and therefore more number of tank treading cycles are required to dissipate the same energy, therefore the higher frequencies.

Our work, therefore, seems to indicate that although the orientation and shape of RBCs in an optical trap are not similar
to those considered by the KS model, namely a viscous ellipsoid, the TT motion seems to be faithfully performed, with measured frequencies being in consonance with those predicted for viscous ellipsoids in a shear flow. This indicates that the theories of viscous dissipation and Tank Treading are not necessarily confined to the previously predicted ellipsoidal shape but are more ubiquitous in nature as far as cell shape is concerned.

3.3 RBC Rotation and microbubble formation

During the interaction of the RBC with the optical trap, few other important phenomena are observed. Firstly, in case of a normal RBC, as mentioned earlier, it folds and aligns with the electric vector of the linearly polarized laser. However, when the RBCs are perturbed by osmolarity shock or have early stage infections with the malaria parasite, they start to undergo a rotational movement about the axis of propagation of the laser. (Figure 4) Such observed rotations are modeled by using a langevin-type equation of rotation and are hypothesized to be caused from the shape anisotropies that result in a perturbed RBC.12

Both, malaria parasite and the hyperosmotic stress are known to induce various types of anisotropies such as ionic anisotropy or shape anisotropy. Both these could induce the RBC to rotate in the laser field. Such a study therefore might give more insights of how such anisotropies may be built up within the RBC and to what extent.

Another important feature that was observed in our measurements was the formation of bubbles within the RBC
infected by parasite when held in the trapped, ultimately leading to the damaging of the cells. Such a phenomenon can be rationalized by considering the fact that hemozoin (which is the pigment that is formed inside the RBC when infected with the malaria parasite) has significant absorbance in the infrared wavelength range. Figure 5a shows the absorbance of β-hematin (hemozoin) dissolved in 1N NaOH along with time lapse images (Figure 5c) of an infected RBC forming a bubble.

Based on previous theoretical predictions and results of other workers, it may be hypothesized that such an absorbance causes overheating the hemozoin crystals above critical temperatures (300 °C for water). The majority of the cell constituents inside the RBC are aqueous material. In the foresaid possible way of bubble formation, it involves the vaporization of the water locally inside the cell. This localized vaporized body of water is viewed as the bubble. However, the conditions for generation of laser-induced microbubbles in cells are still unclear due to specific features of cells like very small heated volumes (characteristic size of light-absorbing volumes may vary from 10nm to 10 μm) and confinement by sample chambers with non absorbing fluid.

Figure 5: a) Plot showing the absorbance spectra of hemozoin (solid dark line) and water (dashed line) at the far red and infrared regions. b) Plot shows increasing absorbance of hemozoin with increasing concentration. c) Time lapse images showing a parasitized RBC on being trapped produced a bubble within 80 milliseconds which shrinks and disappears within the next 1 second. The numbers on top of the frames denote the time of capturing the frames.
The formation of microbubbles can be divided into two regimes depending on the size of the bubble. If the microbubble formed is small in size then there is no apparent damage to the cell and it remains trapped. However, as soon as a big microbubble is formed the cell is irreversibly damaged (as shown in figure 5c), often escaping from the trap. Such cells show a reduced and shrivelled shape and do not get trapped again. Although the bubble formation is very fast (with 80 ms) bubble relaxation occurs over a much longer time scale and can take several seconds. Such slow relaxation is a characteristic of dissipative dynamics which again go on to strengthen the hypothesis that the bubbles are formed due to localised heating of the hemozoin crystal within the RBC due to absorption of the infrared laser beam. Laser induced bubbles in living cells have been recently proposed in various applications for molecular diagnostics and therapy.21,22 Given the use of low power lasers in biological applications such as optical trapping, it would be interesting to investigate the possibility of microbubble formation by low power lasers as a mechanism of cell damage as well as investigating the possible applications of the same.

4. CONCLUSION

In summary, we have presented a method whereby the various biophysical properties of individual RBCs can be measured such as membrane rigidity, viscosity without the constraint of mechanical immobilization of the cell. Our technique involves a combination of a liquid flow cell, fluorescence microscopy, and an optical trap such that relatively simple measurements of the shear modulus and buckling properties of single RBCs are facilitated under physiological conditions. Such studies allow greater insights into the properties of these cells, through the various means like folding, rotation or tank treading. Also these could, in a wider perspective serve as tools to detect various biophysical changes that arise within the RBCs.

REFERENCES


