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Experimental techniques for single cell and single molecule biomechanics

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Abstract

Stresses and strains that act on the human body can arise either from external physical forces or internal physiological environmental conditions. These biophysical interactions can occur not only at the musculoskeletal but also cellular and molecular levels and can determine the health and function of the human body. Here, we seek to investigate the structure-property-function relationship of cells and biomolecules so as to understand their important physiological functions as well as establish possible connections to human diseases. With the recent advancements in cell and molecular biology, biophysics and nanotechnology, several innovative and state-of-the-art experimental techniques and equipment have been developed to probe the structural and mechanical properties of biostructures from the micro- down to picoscale. Some of these experimental techniques include the optical or laser trap method, micropipette aspiration, step-pressure technique, atomic force microscopy and molecular force spectroscopy. In this article, we will review the basic principles and usage of these techniques to conduct single cell and single molecule biomechanics research.

Keywords: Single cell mechanics; Single molecule biomechanics; Biophysical interactions; Optical or laser traps; Micropipette aspiration; Atomic force microscopy

1. Introduction

Studying the biomechanics of human cells and biomolecules is important for several reasons. Firstly, our human body is constantly exposed to physical stresses and strains. Such biophysical interactions can occur not only at the musculoskeletal but also cellular and molecular levels and can determine the health and function of the human body [1–3]. These biomechanical stresses can arise either from external physical forces acting on the body or physiological environmental conditions occurring within the body.

Secondly, biomechanical investigation can provide quantitative study on the change in the physical properties of cells and biomolecules with the progression of certain human diseases such as malaria, sickle cell anemia and cancer. Such deviation in the structural and mechanical properties can be detrimental as they can result in breakdown of their physiological functions at both the tissue and organ levels.

Stresses and strains have also been known to produce certain biological and biochemical responses in cells leading to events such as cell motility, cell differentiation and even apoptosis (or programmed cell death). Hence, the study of cell and biomolecular mechanics is a first step towards understanding the important process of mechanostranduction, where the conversion of mechanical signals to biological and biochemical responses in the cell can be better understood [4].

Apart from mechanical loadings, certain chemicals and drugs are known to affect the mechanical properties of cells and biomolecules. For example, the chemotactic agent f-Met-Leu-Phe (fMLP) can lead to increased stiffness of neutrophils [5,6] and cytochalasin D and latrunculin B can disrupt the actin filaments and significantly affect the stiffness of cells [7].

Finally, the biomechanical properties of cells and biomolecules can potentially be used to quantitatively reflect the state of their health. This may in fact lead to possible useful applications in clinical diagnostics and even provide suitable strategies towards effective therapeutic treatments of human diseases.

We seek to investigate the structure–property–function relationship of living cells and biomolecules so as to understand their important physiological functions as well as establish possible connections to certain human diseases. We have developed as well as used several cutting edge biophysical experimental techniques to investigate a variety of

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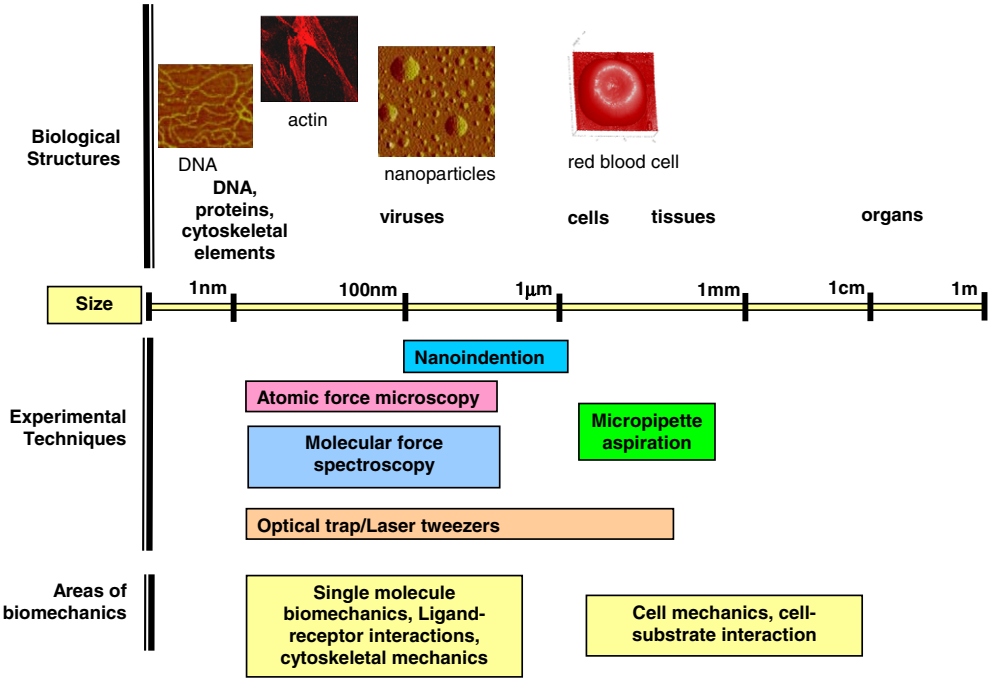

Fig. 1. Experimental techniques for conducting mechanical tests in single cell and single molecule biomechanics.

problems relating to single cell and single molecule biomechanics and these will be highlighted in this paper.

2. Experimental techniques

With the recent advancements in molecular and cell biology, biophysics and nanotechnology, several innovative and state-of-the-art experimental techniques and equipment have been developed to probe the structural and mechanical properties of biostructures from the micro down to the picoscale [8–11]. These techniques cannot only allow us to perform direct mechanical probing and manipulation of single cells and biomolecules, but also allow such tests to be conducted under physiological conditions.

Arising from the availability of numerous techniques, selecting the right type of experimental technique and apparatus to use will depend on the type, size and mechanical properties of biological structure and the biomechanical and

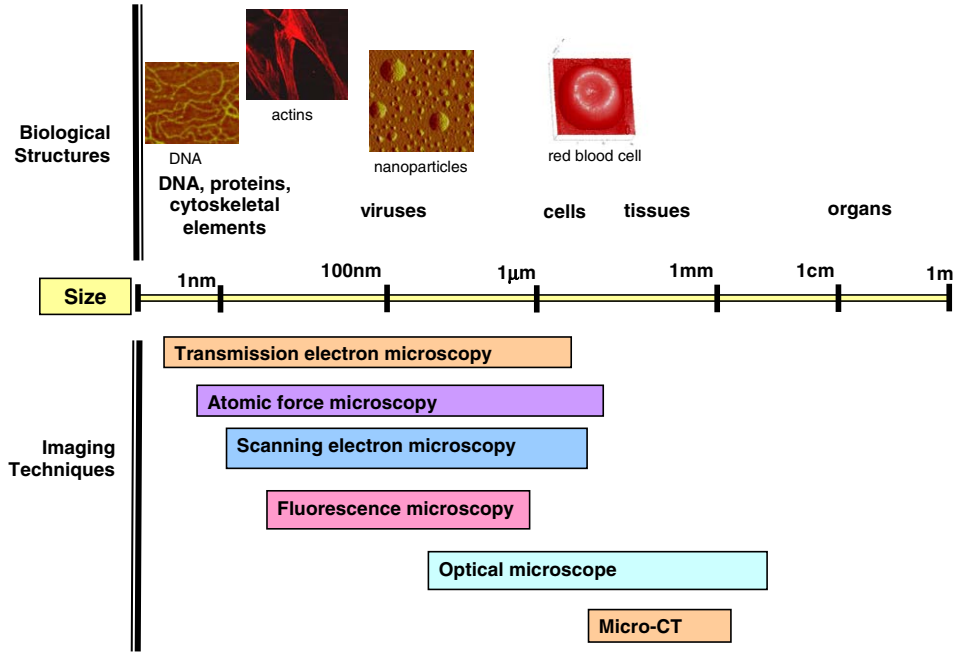

Fig. 2. Imaging techniques that can be used to observe physical, biological and biochemical changes occurring in biological structures during biomechanical tests of cells and biomolecules.

biophysical event of interest. Essentially, we need to be able to conduct the appropriate biomechanical test as well as conduct in situ observation of the physical, biological and biochemical responses occurring in the sample. Fig. 1 shows some of the experimental techniques used for conducting biomechanical tests in single cells and single molecules while Fig. 2 shows the imaging techniques available for use in such tests. Note that selection of appropriate techniques is predominantly dependent on the size of the biological structure of interest. For example, atomic force microscopy and optical tweezers techniques are appropriate for use on nanoscale biostructures such as DNA, actins and viruses while micropipette aspiration is more suited for use on microscale biostructures such as red blood cells and neutrophils. Similarly, atomic force microscopy and fluorescence microscopy are suitable for observing physical, biological and even biochemical responses occurring at the nanoscale while optical microscopy and micro-CT (computer tomography) are more suited for observing similar events occurring at the microscale.

Table 1 summarizes some of the biomechanical tests as well as testing and imaging instruments/techniques employed to study biological structures ranging from single biomolecules, cells to tissues.

In our laboratory, the experimental techniques that we have used include the optical or laser trap method, micropipette aspiration, step-pressure technique, atomic force microscopy and molecular force spectroscopy. We will review the basic principles as well as uses of these techniques to conduct a variety of single cell and single molecule biomechanics research.

2.1. Optical trap method

The optical or laser trap (also termed laser tweezers) uses a laser to trap, controls and manipulates minute objects or particles in a medium. When a laser light is made to shine on a dielectric particle whose refractive index is higher than the medium, there occurs light pressure (or gradient force) moving the particle towards the focal point rather than that (scattering force) moving the particle away from it. As a result, a net force eventually pushes the particle towards the focal point of the laser (Fig. 3). The optical trap has now become an essential tool in conducting research in both physics and biology.

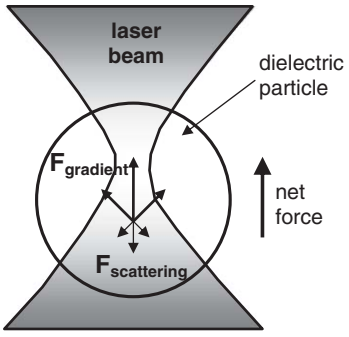

Fig. 3. Optical trapping of a dielectric particle — the balance of the gradient and scattering forces pushes the particle towards the focal point of the laser.

2.1.1. Study of single cell and single molecule biomechanics using optical traps

Arthur Ashkin, regarded as the pioneer of the optical trap method, was the first to conduct a wide range of experiments from the cooling and trapping of neutral atoms to trapping individual viruses and *E. coli* bacteria [12,13]. Since then, the optical tweezers have been used to mechanically probe the membrane properties of cells [14–19], elasticity of single DNA molecules [20] and protein–protein interaction forces [21].

2.1.1.1. Probing the mechanical properties of malaria infected human red blood cells (RBCs). Conducting deformation study of living cells can yield important understanding of the relation between biomechanical states and the onset and progression of diseases such as cancer and malaria. In fact, the mechanical properties of cells are reflective of their molecular structures and organization [22] and can act as a useful indication of such cellular diseases.

We first conducted study of single cell mechanics of healthy RBCs by using the optical tweezers technique as well as numerical simulation [19,23,24] as shown in Figs. 4 and 5. New results on the elastic properties and viscoelastic response of human RBCs when subjected to large elastic deformation were obtained as compared to that for small elastic deformation performed by previous researchers [15,17].

Malaria is one of the deadliest diseases on earth. There are about 500 million clinical cases with about 2–3 million deaths each year arising from malarial infection. The erythrocytic stages in the life cycle of the parasite, *Plasmodium falciparum*, are closely related to the pathogenesis of malaria. It is now

Table 1
Summary of biomechanical testing and imaging methodologies for biomolecules, cells and tissues

Biological structure	Order of magnitude	Mechanical tests	Mechanical testing apparatus	Imaging techniques
Biomolecules	~nm ~pN	Tension, twist	AFM, optical tweezers, magnetic tweezers	AFM, TEM, fluorescence microscopy
Living cells	~10 μm ~pN, nN	Tension, compression, shear, adhesion, aspiration, nanoindentation	AFM, optical trap or laser tweezers, magnetic tweezers, micropipettes, micromanipulators, flow based apparatus	AFM, TEM, SEM, optical, fluorescence and laser confocal microscopy
Soft tissues	>100 μm ~nN, mN	Tension, biaxial stretching, bending, shear, indentation	Micromanipulators, microtester	AFM, TEM, SEM, optical, fluorescence and laser confocal microscopy
Hard tissues	>100 μm ~nN, mN	Tension, compression, bending, shear, torsion, indentation	Nanoindenter, microindenter, microtester	AFM, TEM, SEM, micro-CT, optical, fluorescence and laser confocal microscopy

AFM—atomic force microscopy, TEM—transmission electron microscopy, SEM—scanning electron microscope, CT—computer tomography.

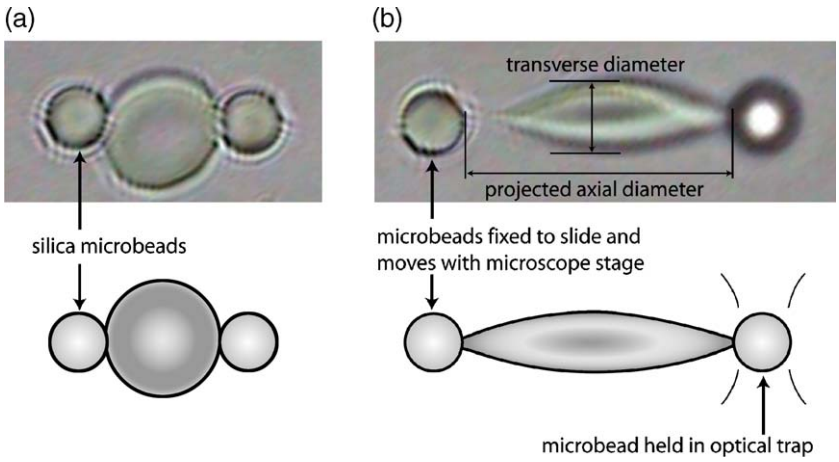

Fig. 4. Stretching of a healthy RBC using the optical trap method. Two silica microbeads of 4.12 μm in diameter are non-specifically attached to the RBC at diametrically opposite points. (a) The optical image shows an unstretched RBC with the left bead adhered to the surface of the glass slide. (b) The right bead is then trapped using the laser as shown by the lighted up bead. Here, the trapped bead remains stationary while the glass slide is moved towards the left thereby stretching the cell. The optical image shows large deformation of the RBC at stretching force of 193 pN [23].

known that when red cells are invaded by the malaria parasites, there is significant reduction in the deformability of the red cells and this can lead to blockage of microvasculature [25] as well as severe anemia. However, the understanding of the mechanism by which RBCs become rigid still remains incomplete. Prior work [26] on the aspiration of a malaria infected cell into a micropipette showed that the malaria parasite, *P. falciparum,* could result in the significant stiffening of infected human RBCs.

For the first time, we conducted optical trap study to observe the progressive changes on the mechanical response of infected RBCs at the different developmental stages of *P. falciparum* infection [27,28]. From the early ring stage to late trophozoite and schizont stages, about an order of magnitude increase in the shear modulus was observed. Fig. 6 shows optical images of the infected RBCs (*P. falciparum* ring stage parasitized RBC (Pf-R-pRBC), *P. falciparum* trophozite parasitized RBC (Pf-T-pRBC) and *P. falciparum* schizont parasitized RBC (Pf-S-pRBC)) and the two control conditions (Healthy RBC (H-RBC) and *P. falciparum* uninfected RBC (Pf-U-RBC)) being stretched by optical tweezers up to 151pN at room temperature and in PBS solution. This study provides quantitative results on the changes in the deformability of the infected RBC at the different stages of parasite development. There is noted

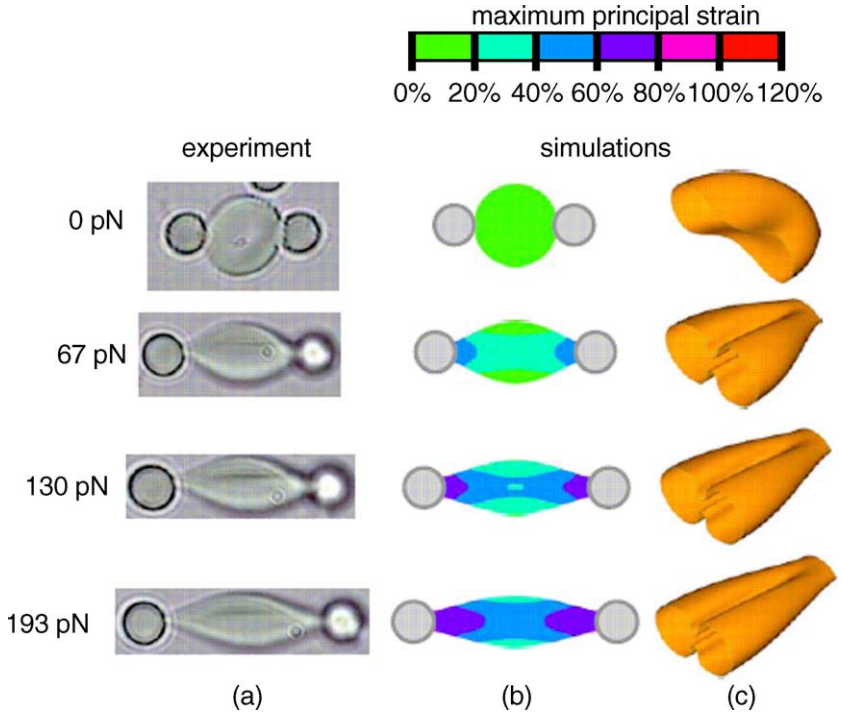

Fig. 5. Images of an RBC being stretched from 0 to 193 pN. (a) The left column shows images obtained from experiment while (b) the center column and (c) right column show top view and three-dimensional view of the half model corresponding to the large deformation simulation of the RBC, respectively. The color contours in the middle column shows distribution of constant maximum principal strains [23].

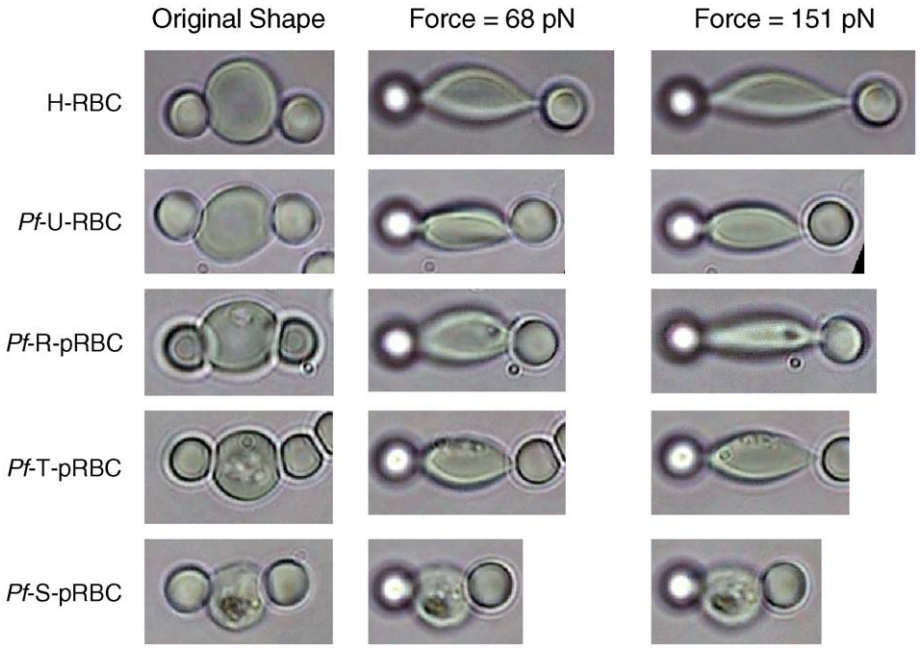

Fig. 6. Optical images of H-RBC, Pf-U-RBC, Pf-R-pRBC, Pf-T-pRBC and Pf-S-pRBC in PBS solution at room temperature (left column) before stretching, (middle column) at stretch force of about 68 pN (middle column) and at stretch force of about 151 pN (right column) [28].

significant stiffening of the RBC due to *P. falciparum* infection.

2.1.1.2. Stretching of single DNA molecules. The elastic properties of DNA molecules are important and essential for many biological behaviors. These include DNA binding to proteins, packaging in bacteriophage capsids, and wrapping around nucleosomes. In the last decade, many experimental techniques have emerged to manipulate single DNA molecule, e.g., optical tweezers [29–31], atomic force microscopy [32,33], and magnetic tweezers [34]. Studies have shown that overstretching of B-DNA will transform it into S-DNA and is about 1.7 times of the contour length of B-DNA [29,35]. Also, since DNA molecules have negatively charged phosphate groups along the double helix, the overstretching transition from B-DNA to S-DNA is found to be sensitive to the medium conditions [36].

Ions can strongly affect the biological behaviors of DNA. While this effect is often explained by the simple ion exchange equilibrium, in many cases, ions appear to exert their influence by modifying the structure and mechanical properties of DNA in terms of its bending and torsional rigidity [36,37]. The study on the effect of ionic concentration on the elasticity of DNA is therefore essential in understanding the mechanisms involved. Here, we seek to measure the effect of Na^+ concentration on the overstretching transition of B-DNA molecule at physiological temperature using both experimental and numerical methods [38,39]. Using optical tweezers (Fig. 7), the relationship of stretching force and relative extension is obtained by stretching single B-DNA molecule. As the concentration increases from 0.909 to 909 mM, the overstretching transition force increases from about 43 to about 65.65 pN (Fig. 8). However, the different ionic

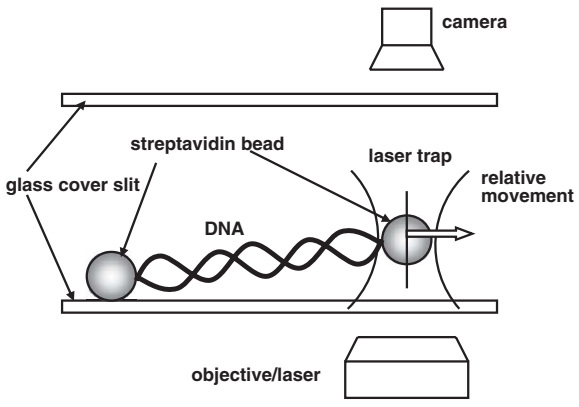

Fig. 7. Schematic of the optical trap setup for the stretching of a double stranded DNA molecule.

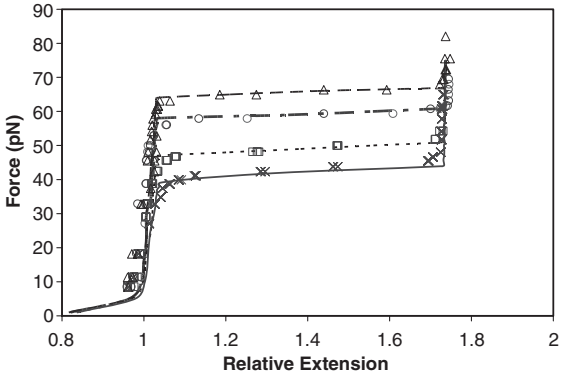

Fig. 8. Plots of stretching force against relative extension of the single DNA molecule. Experimental results in 909 mM (Δ), 150 mM (○), 9.09 mM (□), 0.909 mM (×) NaCl solutions and numerical results in 909 mM (–), 150 mM (-.-), 9.09 mM (. . .), 0.909 mM (—) NaCl solutions [38].

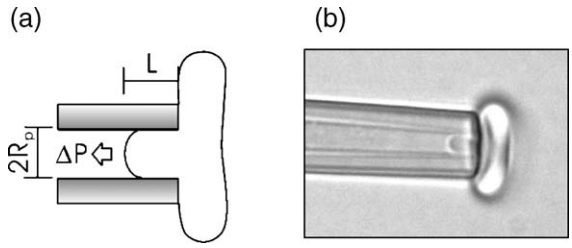

Fig. 9. (a) Schematic of a micropipette aspiration (MA) technique and (b) image on the aspiration of a RBC.

strength does not have much influence on the elasticity of the DNA molecule before and after this overstretching transition. The width of each transition is also about the same. Also, the overstretching transition force is found to vary linearly with the natural logarithm of Na^+ concentration.

Based on a model by Zhou et al. [40], we proposed a 3-D model with combination of bending deformations of DNA backbones, cooperativity of base-stacking interactions, electrostatic interactions, and spatial effects of DNA double helix structure [39]. Metropolis Monte Carlo method was also used to study this model [38]. The numerical results obtained are found to be supported by experiments.

2.1.2. Comments

An advantage of this optical trap method is the use of laser which ensures that there is no physical contact with the sample. Also, the ability to sense forces down to sub pN, which is hard to achieve by other techniques, makes the optical trap an excellent tool for studying cell and molecular mechanics. However, the current limitation on the maximum force achievable which is a few hundred pN, and the possible photo damage effect on biological sample at high laser power limits its broader applications.

2.2. Micropipette aspiration

Micropipette aspiration (MA) provides a simple method to probe the mechanical properties of single cells. A simple illustration is shown in Fig. 9(a). This technique uses a suction pressure to partially or wholly suck a single cell into a micropipette whose diameter may range from less than 1 μm to 10 μm and uses video microscopy to record the shape change of the cell. By measuring the cell elongation into the pipette as a result of the suction pressure, we can evaluate the mechanical properties of the cell. An image of red blood cell being sucked into a 1 μm diameter micropipette is shown in Fig. 9(b).

Mitchison and Swann was the first to use MA to measure the elastic properties of sea urchin eggs in 1954 [41]. Since then, this method has been used to measure the membrane elasticity of many types of cells including red blood cells and leukocytes [42,43]; [reviewed by 44,45]. Besides directly measuring the membrane deformation, MA can also be combined with fluorescence technique to determine the lipid or protein distribution under stress [46,47].

2.2.1. Micropipette aspiration of fibroblasts

Fig. 10 shows the deformation sequence of a fibroblast being sucked into a 6 μm micropipette. At $t=0$ s, a sucking pressure of 1 cm H_2O (98 Pa) was applied to the suspended cell and maintained. The cell was found to deform increasingly over a period of 500 s. This creep response can be used to infer the viscoelastic properties of the cytoskeleton within the cell [48]. We have modeled the fibroblasts with a standard linear solid model and performed finite element simulation of the viscoelastic deformation process [49]. The standard linear solid model was composed of three elements—a spring (k_1) parallels another spring (k_2) and a dashpot (μ) in series [50]. The elastic moduli of the normal NIH3T3 fibroblasts at 37 °C were found to be $k_1=13.6\pm4.9$ (mean±standard deviation) Pa and $k_2=26.4\pm9.2$ Pa; the viscous modulus was found to be $\mu=2208\pm812$ Pa·s. Treatment of the fibroblasts with 2 μM cytochalasin D resulted in significant decrease in the stiffness to $k_1=8.1\pm2.2$ Pa, $k_2=15.7\pm4.5$ Pa and 936 ± 338 Pa·s [48], indicating that the actin cytoskeleton is an important load bearing structure of the cells as was also suggested by other authors [e.g., Ref. 51].

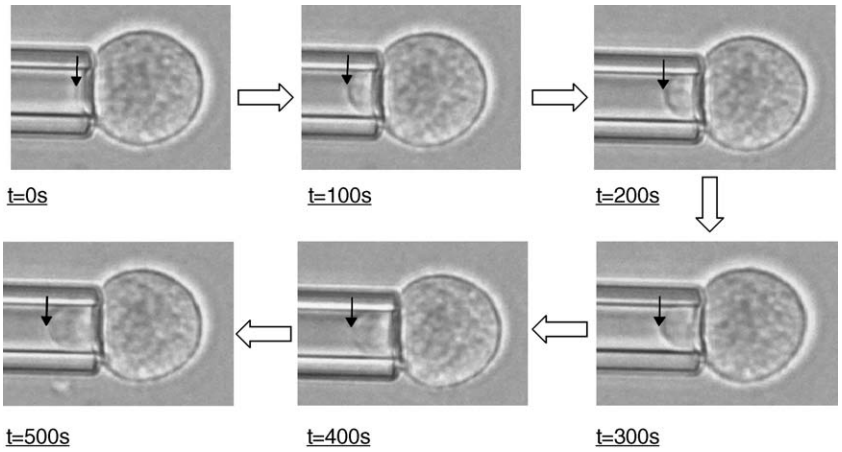

Fig. 10. Sequence of an NIH 3T3 fibroblast being aspirated into a micropipette.

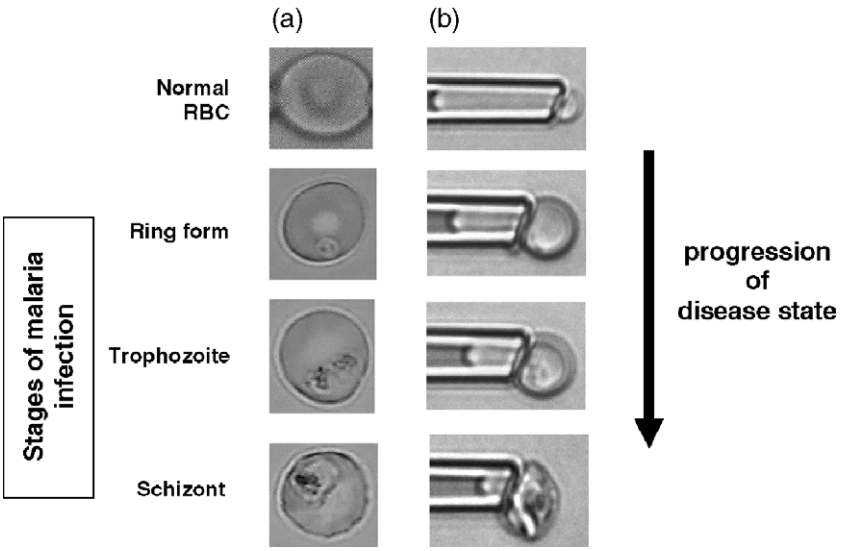

Fig. 11. Mechanically probing the various disease states of a malaria infected RBC using the micropipette aspiration technique [10].

2.2.2. Micropipette aspiration of malaria infected RBCs

We have also used micropipette aspiration to measure the deformability of individual RBCs at the different stages of malaria infection [52]. Again, it is found that the deformability of RBCs decrease progressively with maturation of the parasite (Fig. 11). From the early ring form to the late schizont stage, the red cells are observed to behave from a membrane-bounded liquid drop to that of a viscoelastic solid, respectively. Several reasons for this include the multiplication of the parasites within the cell and the internal structural and molecular changes that the cell undergoes as the parasite matures within the cell. Arising from this solid-like behavior, a homogeneous incompressible standard neo-Hookean solid model was proposed to model the deformability of the whole cell and analyzed with the finite element method (Fig. 12). The apparent bulk shear modulus of the late schizont stage infected RBC was found to be about 50 Pa [53,54].

2.2.3. Comments

Some of the main disadvantages of the MA technique are the stress concentration at the pipette edge and the friction existing between the micropipette surface and the cell membrane. These may complicate the force calibration process and interfere with the mechanical response of the cell during aspiration.

2.3. Step pressure technique using micropipettes

Intercellular adhesion is important in a number of cellular processes. For example, the proteins involved in intercellular adhesion play a significant role not only in keeping an intact architecture of tissues but also in regulating the growth and differentiation of the cells [55]. To understand the role and contribution of different proteins to intercellular adhesion is hence an important area of research. To this end, a number of methods have been developed to quantify the intercellular adhesion forces so as to gain a deeper understanding of these forces [44].

Step-pressure technique utilizing micropipettes developed by Sung et al. is one of the simplest methods that can be used to quantify the intercellular adhesion forces due to different proteins [56,57]. Here, water columns connected to the micropipettes are used to regulate the pressure in the pipettes. The small area of the pipette allows us to apply forces as small as several hundred piconewtons and micromanipulators allow a precise movement of the pipettes. Here, we demonstrate the principal steps of this technique as illustrated in Fig. 13.

The separation force or mean adhesion force is calculated by multiplying the pressure needed to separate the cells with the area of the micropipette. Transfection of cells with specific proteins (which otherwise do not express the proteins),

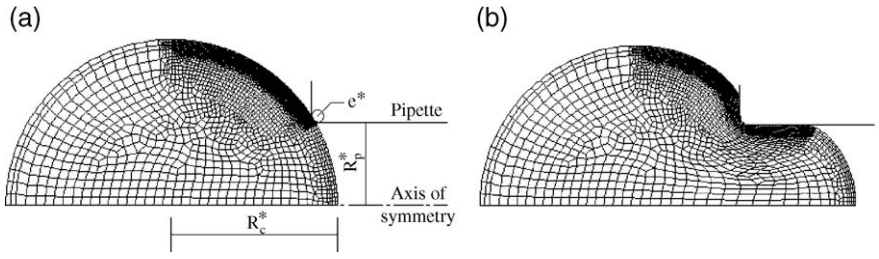

Fig. 12. Finite element modeling of the micropipette aspiration of the late schizont stage infected RBC. (a) The finite element model which modeled the late stage infected cell as a homogeneous viscoelastic solid. (b) The deformed finite element mesh simulates the aspiration of the infected cell [53].

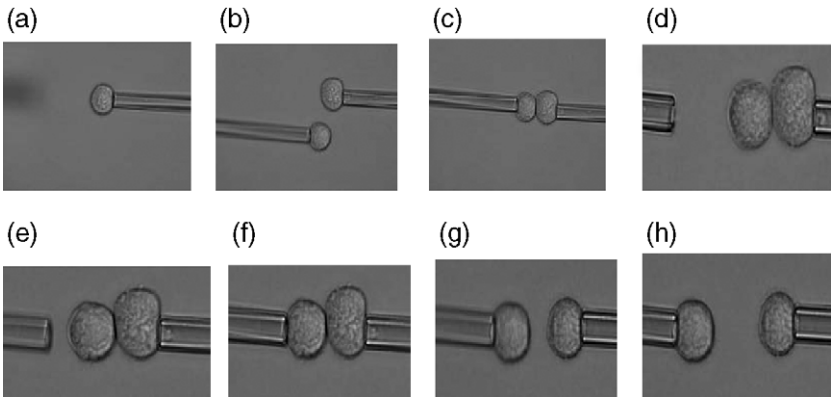

Fig. 13. Procedural steps in the "step-pressure technique" of measuring cell–cell adhesion: (a) a cell is aspirated into the right pipette with a large pressure; (b, c) a second cell is moved close to the first till a contact is made; (d) the cell–cell adhesion is allowed to form for a specified amount of time; (e–h) pressure in the left pipette is increased step wise till the two cells detach from each other.

antibodies to the interacting protein domains and mutated protein domains can be used as positive and negative controls [58,59].

We have transfected fibroblasts with different intercellular adhesion proteins and used the step-pressure technique to quantify the contribution of different proteins to intercellular adhesion force (Fig. 14). Our results indicate that the step pressure technique is a simple and reliable method for quantifying intercellular adhesion forces.

2.4. Atomic Force Microscopy (AFM)

AFM can act as both a powerful imaging tool and a force sensor with piconewton force resolution. The technique involves a very sharp tiny tip mounted at the end of a flexible cantilever which directly 'senses' a sample surface. Very precise relative lateral and vertical displacement between the sample and AFM tip is achieved via a computer-controlled piezoelectric stage or cantilever holder. The interaction force between the tip and sample surface induces deflection of the cantilever and this is recorded by a laser beam reflected off the back of the cantilever. A sensitive photodiode is then used to capture the change in the movement and direction of the reflected laser beam (Fig. 15).

2.4.1. Using AFM to image and probe the elasticity of cells

AFM has now become an essential imaging apparatus and complements the traditional electronic microscopy equipment

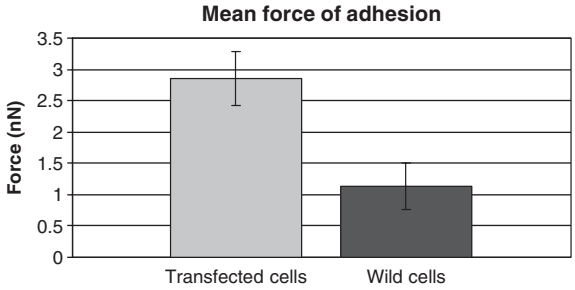

Fig. 14. Mean adhesion force of cells transfected with cell adhesion proteins is higher than that of wild type fibroblast cells.

used since being first built by Binnig et al. [60]. It has also become an indispensable tool for measuring forces associated with cellular and molecular biomechanical events.

The most important characteristic of the AFM is its ability to image samples in fluids and this is of special importance for biological samples because it allows imaging of biological materials in their native and physiological environment. As such, study of soft biological samples has now made AFM one of the most popular imaging tools in biology.

Here, we have developed a set of AFM procedures to image and probe the mechanical properties of soft living cells in their physiological conditions, as illustrated in Fig. 16. With these procedures, we hope to conduct further studies on the mechanical properties of other types of cells and how these quantitative studies can aid in the detection, diagnosis and treatment of diseases.

A detailed set of guidelines was developed for both the imaging and elastic mapping of human dermal fibroblasts (HDF). The Hertz's Theory of Contact was then used to obtain the elastic modulus on the various locations of the cell surface as shown in Fig. 16(a). The elastic moduli of the cell were found to vary from 0.5 to 9 kPa. These values were dependent on the positions where the cell was being probed, e.g., on a weakly supported membrane, on a stiff cytoskeletal actin fiber or on the nucleus region.

From this study, the AFM not only enables study of the surface morphology of living cells, but also allows calculation of the spatial distribution of the stiffness of the cell surface [61,62]. As such, it provides essential information on the stiffness characteristics of the cytoskeletal fibers underlying the cell membrane [63]. However, this approach is still limited by the Hertz's contact theory as well as the complexity of the molecular structures which make up the cell membrane as well as the underlying structures and cytoskeletal organization and careful analysis needs to be done.

2.4.2. Using AFM to probe the molecular interaction between gram-negative bacteria and white blood cells

AFM has also now been increasingly used for molecular force spectroscopy. One such application is the study of molecular interaction between gram-negative bacteria and

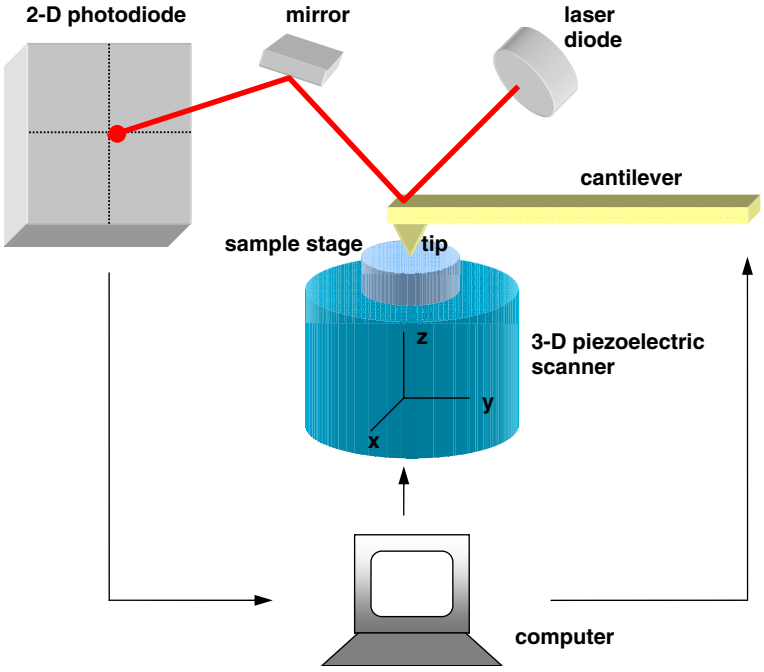

Fig. 15. Schematic of an AFM set up.

white blood cells that we have performed in our laboratory. It is known that this interaction has led to a condition known as septic shock, or systemic inflammatory response to microbial infection, and is a clinical syndrome that can lead to multiple organ failure and mortality in intensive care units in hospitals. This occurs due to gram-negative infection [64] following major surgeries and procedures such as insertion of catheters or intravenous equipment.

Lipopolysaccharide (LPS) and CD14 have been identified as the key molecules in the pathogenesis of septic shock [65]. LPS is the main constituent of the outer membrane of gram-negative bacteria while CD14 is a receptor protein on the

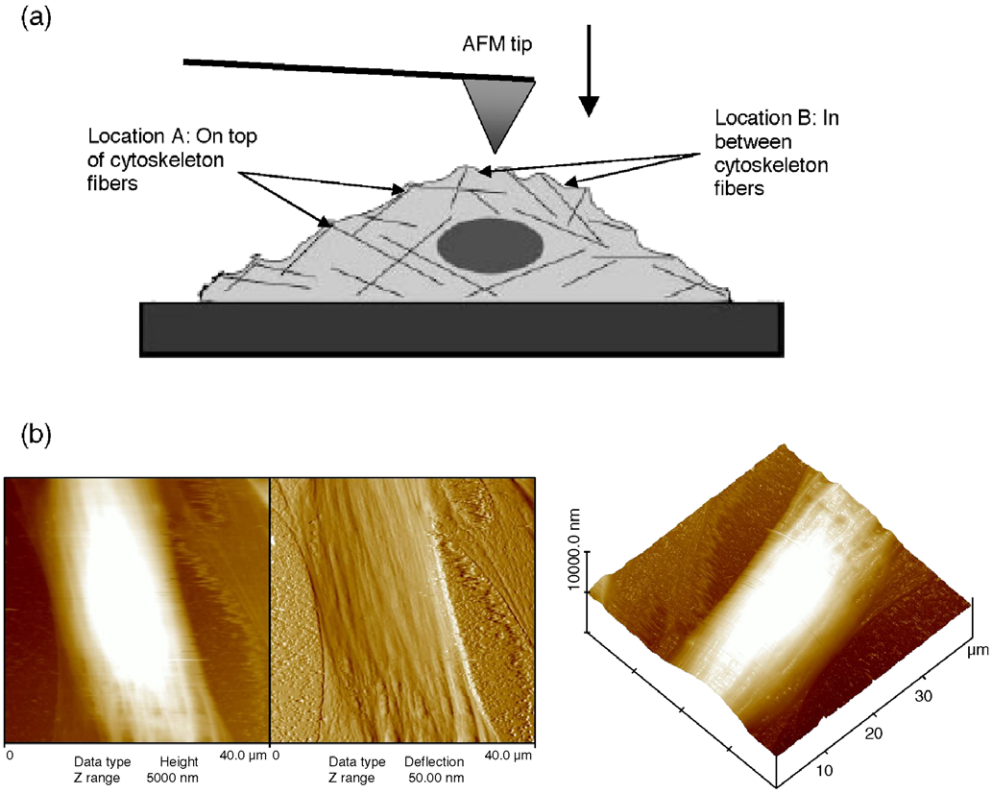

Fig. 16. AFM imaging and indentation of human dermal fibroblasts by an AFM tip. (a) Schematic drawing of the mechanical probing of a fibroblast cell by an AFM tip. (b) AFM imaging of a fibroblast surface which clearly elucidates the actin filaments.

membrane of white blood cells. Studies have shown that the Lipid A portion of the LPS, especially its two phosphate groups are responsible for the toxicity of LPS [66].

Here, we seek to investigate the molecular interactions between white blood cells and different forms of Gram-negative bacteria [67]. AFM is used to study the interactions between CD14 proteins and LPS molecules. Fig. 17(a) shows a schematic of an AFM tip functionalized with CD14 probing LPS molecules that have been deposited on a substrate. The functionalized AFM tip is first brought into contact with the LPS molecules until molecular adhesion and interaction is formed between both sets of proteins. The AFM tip is then retracted slowly until the tip is detached from the LPS deposited substrate.

Fig. 17(b) shows a typical force spectroscopy plot arising from this CD14-LPS interaction. Its shape reflects an initial large peak followed by the characteristic non-linear, parabolic-like curve. The initial large peak indicates the presence of non-specific interactions, the parabolic region corresponds to the extension of the PEG linker and the last peak indicates the single molecular bond strength between CD14 and LPS. By studying this interaction, the actual mechanism and specific location of LPS which interacts with CD14 is determined. It is hoped that this will assist researchers in developing inhibitor drugs by indicating the specific location that they should target. In addition, this technique can be useful in helping to quantitatively determine the effectiveness of developing drugs as inhibitors of CD14-LPS interaction.

3. Conclusions

The experimental study on the mechanics of cells and biomolecules will continue to play an important role in helping us not only to better understand the physiological functions, but also the effects of certain diseases on the human body. With the advancement of nanotechnology, various innovative and state of the art techniques have been developed which can now allow us to probe the physical properties of biostructures from the micro down to the picoscale. This area of research will

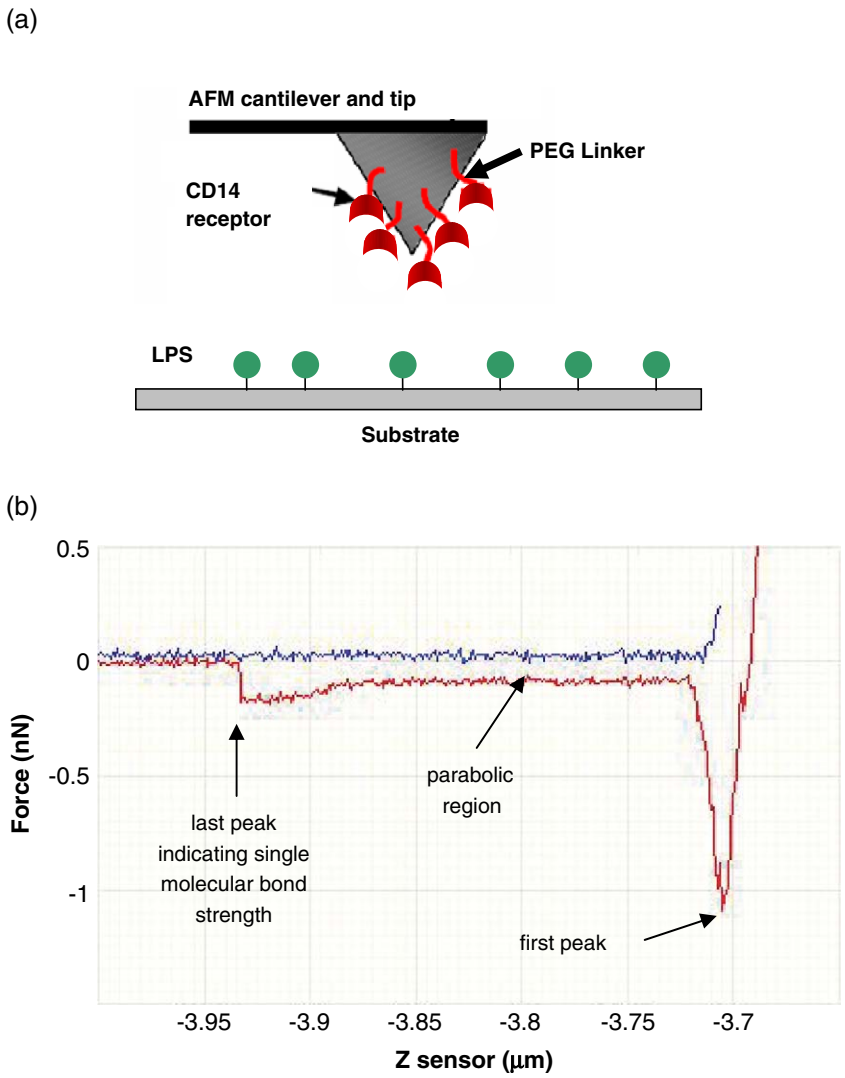

Fig. 17. (a) Schematic showing an AFM tip functionalized with CD14 probing LPS molecules that have been deposited on a substrate. (b) A typical force spectroscopy plot arising from this CD14-LPS interaction.

certainly hold great potential for further breakthroughs in the understanding of the complexities of life.

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