ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(6): 391–395 **CN** 31-1940/Q

Non-invasive *in Situ* Simultaneous Measurement of Multi-parameter Mechanical Properties of Red Blood Cell Membrane

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Abstract The purpose of this study was to develop a new dynamic image analyzing technique that will give us the ability to measure the viscoelastic parameters of individual living red blood cells non-invasively, *in situ* and in real time. With this technique, the bending modulus K_C, the shear elasticity μ and their ratio ε were measured under different temperatures, oxygen partial pressures and osmotic pressures. The results not only show the effects of external conditions on mechanical properties of cell membranes including deformability, flexibility, adhesive ability and plasticity, but also demonstrate that the technique can be used to measure cell membrane parameters continuously under several physiological and pathological conditions.

Key words fast image analyzing; red blood cell (RBC); cell membrane elastic property

A wide variety of cell membrane mechanical properties reflect life-cycle processes, including metabolism, respiration, photosynthesis, information exchange, transportation of substances through cell membranes, and so on. The red blood cell (RBC), normally having the appearance of a biconcave dish, can carry oxygen everywhere *in vivo* and has some special properties, such as deformability, flexibility, adhesive ability and plasticity. The study on the RBC's shape changes is important for clinical diagnosis and treatment of diseases. The interest in this field of research is focused not only on the changes in shape caused by external forces, but also on those caused by the distribution of proteins in both the cell membrane and cytoskeleton under different physiological and biochemical conditions. To study the former, that is, the passive shape changes, three main methods can be used: adsorption, probe pressure and micro-pinhole, all of which are invasive. The study on the latter, also called independent changes, is focused particularly on the mechanism of the RBC's shape change, theories of shape change, such as the constitutive equations, and the biochemical and energy translation processes during the shape change. Because the techniques used to study the independent changes should be non-invasive, few appropriate methods have been developed to measure them [1–3].

The purpose of this study was to develop a novel method to perform non-invasive, *in situ*, real-time and dynamic measurement of the elastic properties of the RBC under various physiological and biochemical conditions. The method is based on the flicker phenomenon, which was discovered almost 100 years ago. It is a kind of glitter arising from the fluctuation of directions and intensities of diffractive or reflective light caused by the cell membrane's fluctuation. This phenomenon has been regarded as a spontaneous activity of the RBC, and it provides mechanical information related to the cell membranes. The image technique for analyzing the flicker phenomenon can not only measure multiple mechanical parameters, but also analyze the structural parameters of the RBC non-invasively and in real time, using simple experimental instruments. The experimental method in this paper was developed based on these advantages. We designed software according to previous theoretical models [3–5] to perform continuous measurements of the bending modulus K_C, the shear elasticity μ, and their ratio ε of the RBC under various external conditions, such as temperature, osmotic pressure and oxygen partial pressure.

Received: November 26, 2004 Accepted: March 28, 2005
This work was supported by a grant from the National Natural Science Foundation of China (No. 60377043)
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DOI: 10.1111/j.1745-7270.2005.00056.x

Materials and Methods

Experimental set-up

We developed a multidimensional micro-image analysis system, the schematic diagram of which is shown in **Fig. 1**. It consists of two parts: the hardware, which consists of an inverted microscope (TE300; Nikon, Tokyo, Japan), a cell culture mini-chamber, a linear array charge-coupled device (IMAC-CCD30 768×572; Altlussheim, Germany), and an image capture card (MATROX-METEORII PCI; San Diego, CA, USA). And the software consists of a multifunctional micro-image processor and analyzer. The system is capable of capturing hundreds of cell frames in a few seconds, and performing non-invasive, *in situ*, real-time measurements of multiple parameters of a single cell with high sensitivity.

Cell culture mini-chamber

In order to conduct the continuous dynamic measurements of a single cell, we designed a cell culture mini-chamber [6], which is capable of maintaining a constant temperature (range: 5–85 °C; precision: ±0.5 °C, adjustable) and a constant gas flow (CO_2), with a controllable liquid culture circulating through the chamber. The structure of the cell culture mini-chamber is shown in **Fig. 2**. With the mini-chamber, we were able to observe one cell under the microscope for a long time. That made it possible to measure continuously the structural and functional changes of the RBC under various external conditions.

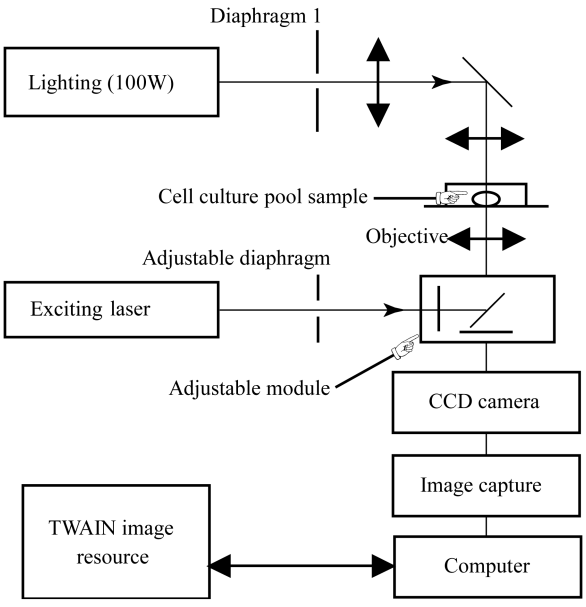

Fig. 1 The experimental set-up

TWAIN, standard for image acquisition devices.

Preparation of RBCs

The samples were prepared according to the method described by Huang *et al.* [7]. The RBCs were collected from the heparinized vein blood of a healthy male adult. RBCs of various ages were separated using the sugar density gradient centrifugal method. Forty percent sucrose solution was diluted with saline water to different volume percentage concentrations (27%, 26%, 25%, 24% and 23%), and 0.5 ml diluted sucrose solutions were added in succession, with increasing concentration, to a centrifugal tube to form a medium with a density gradient. Then, 0.5 ml of RBCs was added slowly to the medium, and the cells were delaminated by centrifugation (3000 *g*, 12–25 min). The top cells, which were the youngest RBCs, were collected and washed three times with PBS (pH 7.4) before measurements were taken.

Principle

Using a phase contrast microscope, the gray image of a single RBC with a refraction fringe ("halo") was recorded for 100 s (30 frames/s). After a threshold was determined, the edge of the cell in four directions (up, down, left and right) was captured by fast digital video image processing, which is shown in **Fig. 3**. The software automatically calculated the fluctuation of the cell membrane in four directions. After acquiring 4×3000 values, we calculated the mean square errors and multiple elastic parameters of the RBC based on the following formulae, which have been verified by the RBC decomposition model [3].

Equation (1) is the Fourier transform of four fluctuation amplitudes with respect to the azimuthal mode number m for each time and n expresses dimension of Fourier transform. **Equation (2)** calculates ε, the ratio of the bending modulus K_C to the shear elasticity μ, with two azimuthal modes. In **Equation (3)**, R is the radius of the RBC, K_B is the Boltzmann constant (1.38×10^{-23} J·K^{-1}) and T expresses temperature. The bending modulus K_C expresses the transfiguration capability of the cells. The smaller the K_C value, the better the transfiguration capability of the cells, and the more difficult it is for the cells to be broken, and vice versa. The shear elasticity μ is calculated by **Equation (4)**, and it expresses the adhesive capability of the cells. Based on this method, we can estimate the

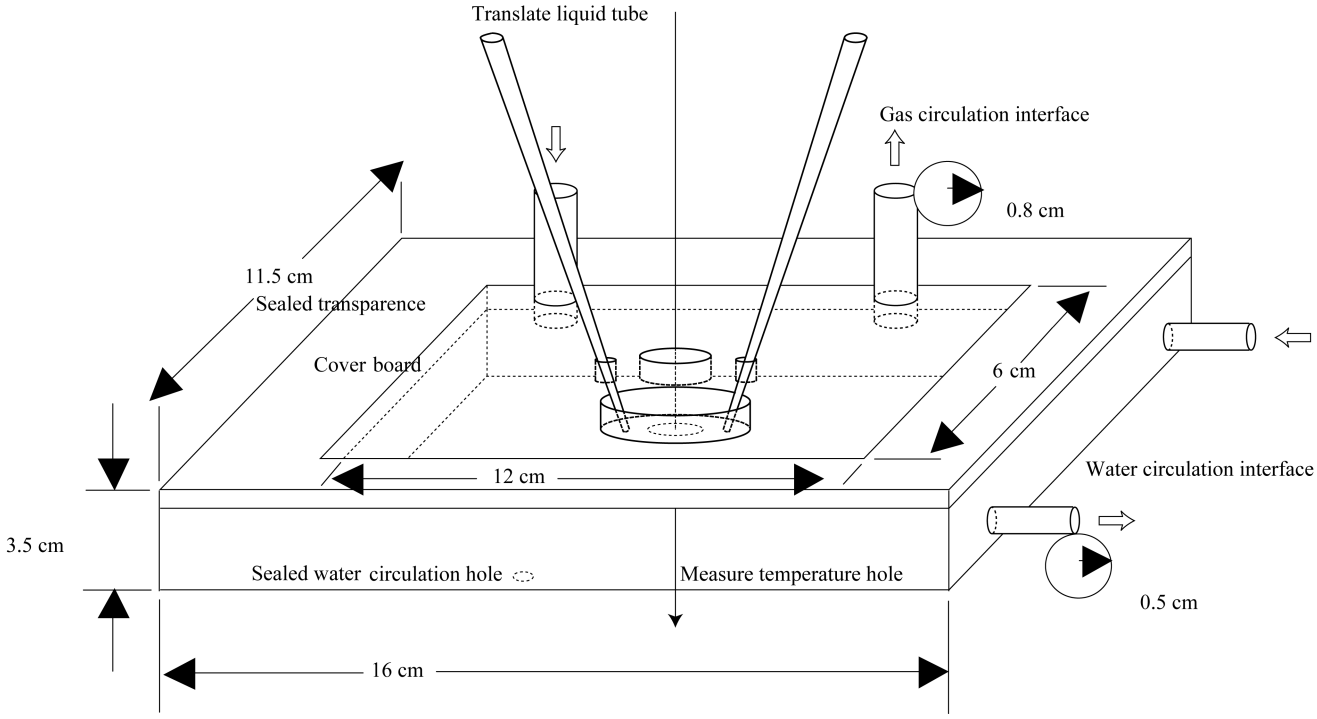

Fig. 2 The structure of mini-chamber for cell culture

fluctuation of the cell membrane according to the algorithm of the center of gravity (COG) [8] with a measurement accuracy of around 5 nm.

$$\delta n_m = \frac{1}{4}\sum_{n=0}^{3}\delta n(n)e^{i2\pi nm/4} \quad (1)$$

$$\varepsilon = \frac{\delta n_{m=1}^2}{\delta n_{m=2}^2} \quad (2)$$

$$\delta n_{m=2}^2 = \frac{R^2 K_B T}{K_C} \quad (3)$$

$$\varepsilon = \frac{\mu R^2}{K_C} \quad (4)$$

Results

The graphic user interface (GUI) of the software used for measuring RBCs is shown in **Fig. 3**. The phase contrast micrograph shows an RBC with the flicker phenomenon. Four scanning lines track the fluctuation of the edges of the cell in four directions respectively, and record the different positions of the cell membrane. The halo fluctuation of the cell membrane in four directions with

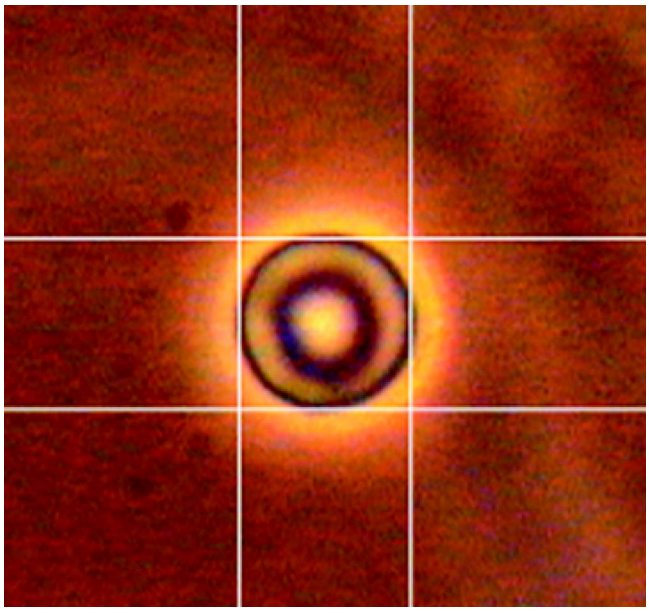

Fig. 3 Illumination software interface

Magnification, ×200.

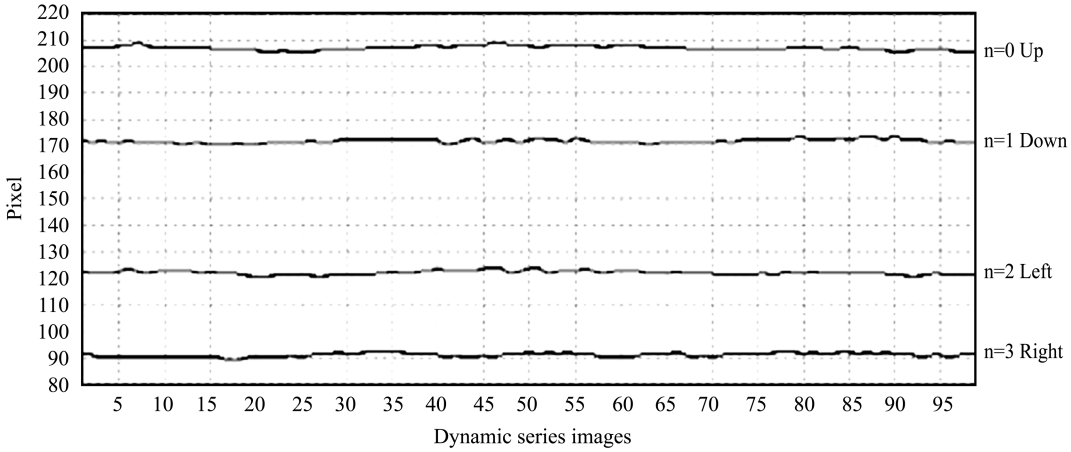

Fig. 4 The halo variation of cell membrane position versus dynamic series images

dynamic series images is shown in **Fig. 4**. According to the principle discussed above, K_C, μ, and ε can be calculated. In our experiment, 30 RBCs were measured under various external conditions. According to statistical theory [9], the average measurement aberrance coefficient is 0.25, which means the statistical sample number should be no less than 23. The sample number in our experiment is up to the standard, so the statistical results are reliable. The variation of the three RBC elastic parameters with temperature is shown in **Fig. 5**, with the temperature varying from 33 °C to 41 °C. Initially, K_C decreases with increasing temperature. The minimum value of K_C occurs at 37 °C, where the RBC has the best transfiguration capability. After that point, the K_C gradually increases with increasing temperature in the range of 37–41 °C. The variation of the other two parameters, μ and ε, with temperature is similar to that of K_C. In addition, at 37 °C, the K_C in the oxygen-free atmosphere is larger than the K_C in the oxygenated one, as shown in **Table 1**. The variation of the three RBC elastic parameters with osmotic pressure is shown in **Fig. 6**. With the osmotic pressure varying from 150 to 450 msm/Kg H_2O, K_C initially decreases with increasing osmotic pressure. The minimum value of K_C occurs at 288 msm/Kg H_2O, where the RBC has the best transfiguration capability. After that point, K_C gradually

Table 1 Variation of K_C, μ and ε with oxygen partial pressure

Atmosphere	K_C ($\times 10^{-19}$ J)	μ ($\times 10^{-7}$ N/m)	ε
Oxygen-free	3.52±0.12	0.71±0.27	1.82±0.08
Oxygenated	2.78±0.21	0.53±0.31	1.73±0.12

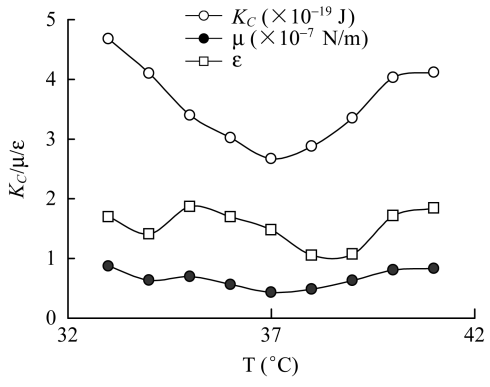

Fig. 5 Variation of K_C, μ and ε with temperature

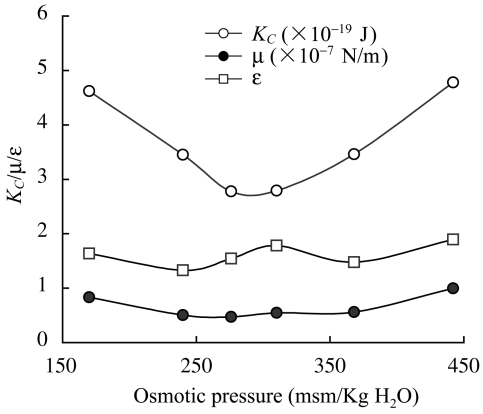

Fig. 6 Variation of K_C, μ and ε with osmotic pressure

increases with increasing osmotic pressure in the range of 288–450 msm/Kg H_2O. The variation of the other two parameters, μ and ε, with osmotic pressure is similar to that of K_C.

Discussion

The bending modulus K_C is an important parameter that indicates the transfiguration capability of the RBC. The smaller its value, the better the transfiguration capability of the RBC. Our experimental results show that the minimum value of the RBC bending modulus occurs at 37 °C, where the RBC has the best transfiguration capability, which is beneficial for exchanging substances with the environment. The result coincides with that obtained from the quasi-elastic laser light scattering instrument (BI2000, BI9000 AT model; Brookhaven Company, NY, USA) [10]. The μ value reflects the adhesive ability of the RBC. The smaller it is, the stronger the adhesive ability of the RBC. Previous results have shown that the adhesive ability can be weakened by both unusually high (such as 42 °C) and low (such as 32 °C) temperatures. The cell shows better adhesive ability when the temperature is moderate (such as 37 °C). With lower osmotic pressure, the appearance of the RBC is nearly spherical, which results in its lower adhesive ability because of the surface tension effect caused by the inflation. When the osmotic pressure is higher, the RBC shrinks because it loses water, and the size of the intracellular particles increase, which will reduce the spontaneous activities of the cell membrane, and the adhesive ability declines. Previous results have shown that the shear elasticity μ of the RBC is larger at either low (<250 msm/Kg H_2O) or high (>350 msm/Kg H_2O) osmotic pressure, and it reaches the minimum value at medium osmotic pressure (about 288 msm/Kg H_2O). The results also show that the changes in the transfiguration ability are larger than those in the adhesive ability under various external conditions. ε depends on the fluctuation of the cell membrane. The ratio indicates the relationship between the bending modulus K_C and the adhesive ability μ, and it also reflects the effect of various external conditions. The results suggest that when the external conditions are normal, its value is nearly 1; otherwise, its value is far from 1.

All previous methods for measuring the bending modulus have an obvious disadvantage; that is, they are invasive. The quasi-elastic laser light scattering method is an improvement on the previous methods [10,11]. In this study, we have developed a fast dynamic image analysis method to perform multi-parameter dynamic measurements of the elastic properties of the red blood cell membrane. The method is simple, fast and convenient. It may become a complementary method for laboratory research work, and a useful tool to measure the elastic properties of living cell membranes. It may also be used to study the correlation between the structures and functions of cells.

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