The Use of Atomic Force Microscopy for Estimating Morphometric Characteristics of Blood Cell

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Abstract—The potential of atomic force microscopy for estimating geometric characteristics of blood cells is demonstrated. Comparison of hemocyte morphometric characteristics recorded using different scanning modes has demonstrated that noncontact and semicontact imaging are adequate for studying the size and geometry of biological objects. A contact scanning of cells leads to their irreversible deformation.

Key words: blood cells, morphometric characteristics, atomic force microscopy

It is known that the cells of various organisms, from bacteria to mammals, are able to regulate their volume [1]. Different systems of intracellular signaling are involved into the regulation of volume [2–6]. The blood leukocytes of higher animals and humans have a membrane reserve as various foldings and expansions [7, 8]. The membrane reserve is involved in implementation of specific types of cell mobility, such as locomotion and deformation. The data on the potential changes in the volume of leukocytes and the range of the membrane reserve have been obtained by light microscopy approaches using the functional samples in the form of hypoosmotic loads or aspiration into a microtube [9, 10].

As many problems connected with specific responses of white blood cells and the functional significance of their volume self-regulation are still unsolved, the search for precise and sensitive methods allowing assessment of the volume of hemocytes is a topical problem. One of the promising methods for studying the cell morphofunctional organization is atomic force microscopy (AFM). AFM has made it possible to record the changes in elastic and viscoelastic properties of connective tissue and epithelial cells [11–17] and study the morphology of human blood leukocytes [18, 19]. However, some researches regard AFM inappropriate for studying cell sizes, because this method gives different morphological parameters for the same cell pools fixed using different techniques [18, 19].

The goal of this work was to study the potential of AFM for estimating cell morphometric characteristics.

Abbreviations: ATM, atomic force microscopy.

MATERIALS AND METHODS

The lymphocytes of laboratory white rats and avian (chicken) erythrocytes were used in the work. Blood was sampled by decapitation of narcotized animals using heparin (20 U/ml) as an anticoagulant. The rat blood was centrifuged for 10 min at 1500 rpm to collect lower part of the plasma, rich with leukocytes, and the leukocyte band. The erythrocyte admixture was destroyed with 0.83% ammonium chloride. The cells were twice washed with isotonic buffer solution (Dulbecco's solution, pH 7.4). Avian erythrocytes were also washed and resuspended in isotonic buffer solution. The cells were fixed with 2% glutaraldehyde (Merck, Germany).

A Video-TesT (St. Petersburg, Russia) hardware–software complex equipped with an Axiostar plus (Carl Zeiss, Germany) microscope and the Video-TesT-Razmer 5.0 program, Quanta 200 3D (FEI, United States) scanning microscope in a low vacuum mode, and INTEGRA Vita (NT MDT, Zelenograd, Russia) atomic force microscope were used in the work. The samples were probed in a single-pass contact, semicontact, or noncontact mode. HA_C1 cantilevers were used for a contact mode and NSG 03 (NT MDT), for semicontact and noncontact modes. The data were processed with the help of the Nova 1.0.26 Build 1397 (NT MTD).

Cell volume was calculated using standard equations for ellipsoid cells $V = 4/3(\pi abc)$, where V is volume and a, b, and c are the large, medium, and small semiaxes, respectively. Cell flattening coefficients were calculated as the ratio of ellipse area (the projection of hemocytes onto the plane of long axes) to the height. Student's test was used for statistical data processing.

Table 1. Morphometric characteristics of lymphocytes at different measuring modes

Measuring mode	Measured parameter, μm	Cells fixed in smear	Cells fixed in suspension
Light microscopy	D	6.46 ± 0.1	5.51 ± 0.1* (88)
Atomic force microscopy (contact mode)	D	9.48 ± 0.5°	6.68 ± 0.3*°
	H	0.92 ± 0.05 (44)	1.87 ± 0.19* (42)

Notes: D, diameter; H, height; cell volume (μm^3) is parenthesized.

* Significance of the differences from cells in smears according to Student's test ($p < 0.01$).

** Significance of the differences from light microscopy differences according to Student's test ($p < 0.01$).

Table 2. Morphometric characteristics of chicken erythrocytes recorded by different methods

Measures parameter, μm	Measurement method	
	Light microscopy	AFM (contact mode)
Length	7.12 ± 0.08	10.01 ± 0.09*
Width	4.05 ± 0.05	6.29 ± 0.12*
Height	2.40 ± 0.04 (37.26 ± 1.4)	2.19 ± 0.06* (70.29 ± 3.1*)

Notes: Cell volume (μm^3) is parenthesized.

* Significance of the differences from the measurement using alight microscopy according to Student's test ($p < 0.01$).

The electron and probe scanning microscopies were performed at the shared access center with the Belgorod State University.

RESULTS AND DISCUSSION

A preliminary set of experiments estimated the effect of glutaraldehyde on the size of rat blood lymphocytes: the diameter of living cells was 8.7 ± 0.1 μm and of fixes cells, 5.9 ± 0.1 μm. The difference in their size, amounting to 32%, fit the earlier data [20] and is explainable by cell wrinkling caused by adding glutaraldehyde to lymphocyte suspension to a concentration of 2% (additional osmolarity, 180 mosmol) [21, 22].

In the second set of experiments, the lymphocyte suspension was divided into two parts. The first part was used to make smears, which were dried and fixed with 2% glutaraldehyde solution. The second part was fixed by supplementing the suspension with glutaraldehyde to a final concentration of 2%. The fixed cells were placed on glass slides. The measurement results obtained using light and atomic force microscopies are listed in Table 1. The differences in cell linear sizes are more pronounced in the case of AFM (29.5%) than in the case of light microscopy (14.7%). As glutaraldehyde preserves an intravital shape of lymphocytes, which is close to a sphere (Fig. 1), the volume was calculated for the cells fixed in suspension and measured using a light microscope. These values exceeded more than twofold the data obtained by AFM (Table 1). However, the volumes of the lymphocytes smeared on slides and fixed in suspension were practically the same, despite that their shapes considerably differed from a

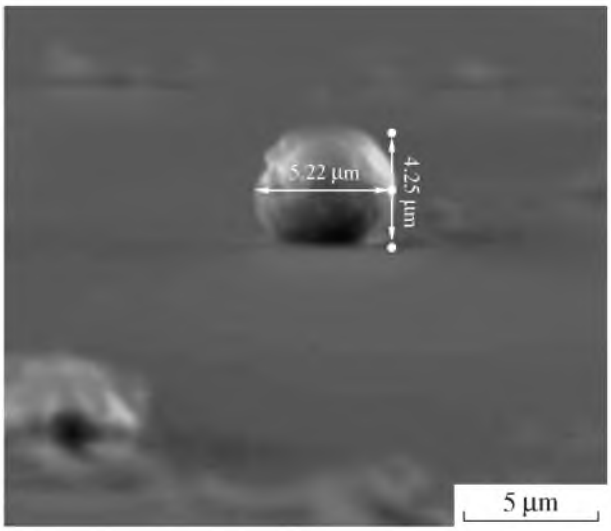

Fig. 1. Electron micrograph of a rat blood lymphocyte fixed in suspension with glutaraldehyde; scanning in a low vacuum mode.

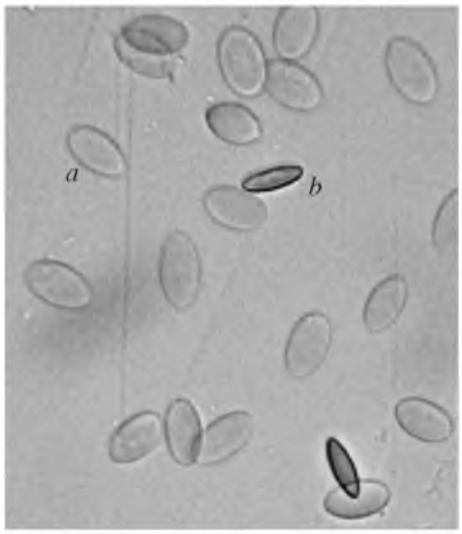

Fig. 2. The avian blood erythrocytes fixed in suspension with glutaraldehyde and placed into a Goryaev chamber (unstained preparation; ×400): (*a*) a flatwise position and (*b*) on the edge.

sphere. One of the possible reasons here could be the "pressing" (deformation) of cells by a cantilever probe. To verify this assumption, we performed an analogous examination using chicken erythrocytes, having a shape of flattened ellipsoids of a small size. The cells fixed with glutaraldehyde in suspension were used. Part of them was placed in a Goryaev chamber, which was gently moved on the object table of microscope, making erythrocytes "tumble". Several photos were taken and three erythrocyte parameters—length, width, and height—were measured with the help of the Video-Test-Razmer 5.0 program (Fig. 2). The other part of the cells was scanned using AFM. The measurement results are listed in Table 2. Similar to the first set of experiments, the scanning of erythrocytes in a contact mode led to their pressing down, thereby to a considerable increase in the long axes (length and width) and a decrease in the short axis (height). The flattening coefficient for erythrocytes calculated based on the light microscopy measurements was 9.4 and based on AFM, 22.6. Lymphocytes displayed the flattening of approximately the same order of magnitude (Table 1). The recalculation for the volume of avian erythrocytes also displayed almost twofold differences. Thus, the measurement in a contact scanning mode demonstrated that even the "softest" cantilevers deformed biological objects, as the probe tip touched the sample surface. Correspondingly, the shape and sizes of the scanned image can differ considerably from the original. Depending on the initial geometric shape of hemocytes, the distortion can lead to either downward or upward bias of the calculated parameter (volume). A set of experiments involving successive cell probing by various methods was performed to solve the stated problem. In the first variant, the same cells were initially scanned in a contact mode and then in a semicontact mode, when the pressure of a cantilever on the sample surface was considerably smaller. This sequence was reversed in the second variant; i.e., the same cells were first scanned in a semicontact and then in a contact mode. In the third variant, we compared a noncontact (the probe did not contact the sample and did not distort

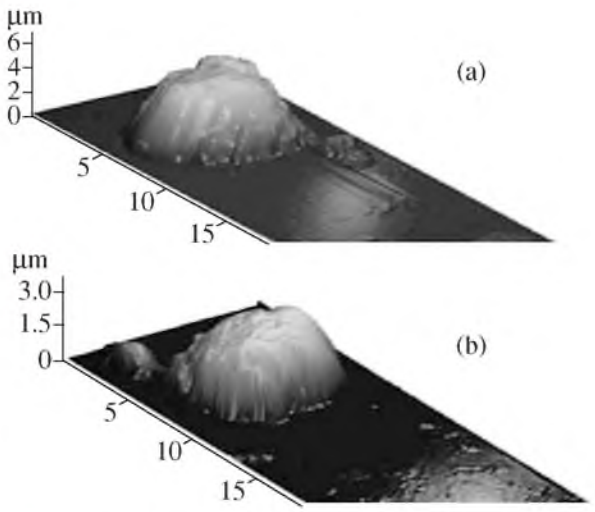

Fig. 3. A rat blood lymphocyte successively scanned by AFM in (a) semicontact and (b) contact modes. A considerable difference in the cell height is evident.

the image) and a semicontact modes by successively scanning the same cells (Table 3). The results demonstrate that the fixed cells are irreversibly deformed in the case of a contact mode. The second scanning in a semicontact mode gave practically the same results. A reverse scanning sequence shows a considerable decrease in the cell vertical size caused by the pressure of a cantilever tip (Fig. 3a, b). The initial scanning in a semicontact or noncontact mode gives almost equal results. The sizes of lymphocytes at the three axes demonstrate that their shape is close a ball, i.e., it is natural but slightly flattened. A small deflection of lymphocytes is also evident in electron micrographs (Fig. 1). The fixation with glutaraldehyde considerably increases the rigidity of cell structures yet does not make cells absolutely inelastic. This is also confirmed by the filtration experiments on the deformation properties of live and fixed leukocytes [23].

Table 3. Morphometric characteristics of lymphocytes for different AFM scanning modes

Examination variants	Sequence of scanning modes	Cell size, μm		
		length	width	height
I	Contact	7.11 ± 0.53	6.12 ± 0.53	3.11 ± 0.47 (71)
	Semicontact	7.26 ± 0.67	6.16 ± 0.67	3.69 ± 0.84 (86)
II	Semicontact	7.63 ± 0.64	7.11 ± 0.75	5.39 ± 0.11 (153)
	Contact	7.51 ± 0.35	6.68 ± 0.53	3.31 ± 0.23* (87)
III	Noncontact	7.64 ± 0.10	6.71 ± 0.15	4.98 ± 0.24 (134)
	Semicontact	7.85 ± 0.18	6.73 ± 0.33	4.77 ± 0.43 (132)

Notes: In each examination variant, the same cells were scanned using different modes: contact, semicontact, and noncontact. Cell volume (μm^3) is parenthesized.

* Significance of the differences from the measurement in a previous scanning mode according to Student's test ($p < 0.01$).

Analysis of our results and their comparison with earlier data demonstrate that both noncontact and semicontact AFM scanning are appropriate for studying the sizes and geometric shapes of biological objects as well as the dynamics of morphometric characteristics influenced by various environmental factors.

REFERENCES

1. R. Kinne, J. Exp. Zool. **265**, 346 (1993).
2. M. E. Chamberlin and K. Strange, Amer. J. Physiol. **257**, 159 (1989).
3. D. Haussinger, Biochem. J. **313**, 697 (1996).
4. J. C. Summers, L. Trais, and R. Lajvardi, Amer. J. Physiol. **273** (2), C360 (1997).
5. A. Tsai, B. Friesenecker, and M. Mc Carthy, Amer. J. Physiol. **275** (6), H2170 (1998).
6. A. A. Mongin and S. N. Orlov, Pathophysiology, No. 8, 77 (2001).
7. U. Bagge and P.-I. Branemark, Adv. Microcirc. **7**, 1 (1977).
8. U. R. Bagge, B. Tohansson, and T. Olofsson, Adv. Microcirc. **7**, 18 (1977).
9. U. Bagge, R. Skalak, and R. Attefors, Adv. Microcirc. **7**, 29 (1977).
10. U. Bagge, B. Amundson, and C. Lauritzen, Acta Physiol. Scand. **180**, 159 (1980).
11. P. Roca-Cusachs, I. Almendros, R. Sunyer, et al., Biophys. J., No. 1, 3508 (2006).
12. F. Rico, P. Roca-Cusachs, N. Gavara, et al., Phys. Rev. **72**, 021914 (2005).
13. G. T. Charras and M. A. Horton, Biophys. J. **83**, 858 (2002).
14. M. J. Rosenbluth, W. A. Lam, and D. A. Fletcher, Biophys. J. **90**, 2994 (2006).
15. J. Alcaraz, L. Buscemi, M. Grabulosa, et al., Biophys. J. **84**, 2071 (2003).
16. M. Radmacher, M. Fritz, C. M. Kacher, et al., Biophys. J. **70**, 556 (1996).
17. S. Park, D. Koch, R. Cardenas, et al., Biophys. J.**89**, 4330 (2005).
18. S. N. Pleskova, Yu. Yu. Gushchina, M. B. Zvonkova, and A. E. Khomutova, Byull. Exp. Biol. Med. **141** (6), 712 (2006).
19. Yu. Yu. Gushchina, S. N. Pleskova, and M. B. Zvonkova, J. Surf. Invest., No. 1, 48 (2005).
20. M. Z. Fedorova and V. P. Levin, Biol. Membrany, No. 4, 310 (2001).
21. F. M. Morel, R. F. Baker, and H. Wayland, J. Cell. Biol. **48**, 91 (1971).
22. G. W. Schmid-Schonbein, Y. Y. Shih, and S. Chicn, Blood **56** (5), 866 (1980).
23. K. Sung, G. Schmid-Schonbein, and R. Skalak, Biophys. J. **39**, 101 (1982).