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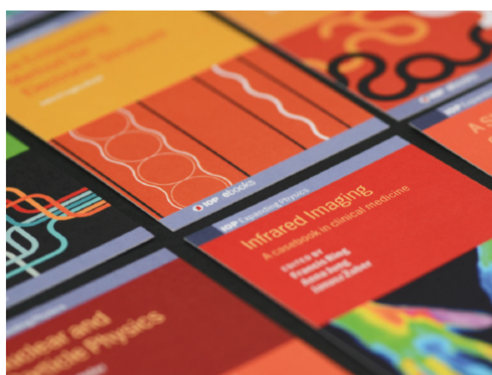

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Opportunities provided by use of Atomic Force Microscopy for studying Invertebrates hemocytes in training biology students

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Abstract. Atomic Force Microscopy provides for probing of cellular characteristics, such as elasticity of cell membranes, adhesion between the probe and the sample, microrelief of cell surfaces, and linear dimensions of cells, including their height. In our study we utilized Atomic Force Microscopy to measure physical parameters of blood cells of invertebrates within the framework of the process of education of biology students. In order to measure elasticity and adhesion, we used an Atomic Force Microscope *Integra Vita NT-MDT*, and cells were scanned in semi-contact mode. We used *NSG3* (*NT-MDT*) silicon tips with hardness of 1.4 N/m, curvature radius of 10 nm. Young's modulus describes elastic properties of the cell membrane. For hemocytes of invertebrates, this parameter computed at different points of the same cell varied significantly. The difference between the greatest and the smallest values suggests that computation of mean elasticity for hemocytes of invertebrates is not informative. It is a well-known fact that Young's moduli computed for central and peripheral portions of a cell are different, and, thus, in this study the values were grouped by their location. The experiment revealed no difference between elastic properties of central and peripheral portions of blood cells of invertebrates.

1. Introduction

Atomic Force Microscopy (AFM) is one of up-to-date methods of measurement of surface topography of cells and physical properties of cell membranes [1-3].

AFM provides ample opportunities for open-air work and measurements in water and buffer solutions with spatial resolution from some to dozens of nanometers [4-6]. AFM is an important method in biology, since it provides for measurement of non-conducting objects in non-vacuum or liquid environment [7] and, at the same time, it does not require staining or spraying of the preparations to be examined [8].

Among multiple morphofunctional cellular parameters, special emphasis should be placed on hardness of the submembrane frame determining cellular functional activity, directed locomotion and

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engagement in phagocytic reactions, which are important criteria describing the adaptation concept, the central concept of biology [9-11].

Chemical composition of the environment of a cell significantly affects the degree of cell membrane deformability, which is mostly determined by values of the plasmalemma elasticity modulus. Mechanical properties of cells are determined by the condition of elements of the cytoskeleton, and physical interaction with the environment. These properties may change in quality in the process of cell division or if the cells die. Vesicle movement, absorption of extracellular materials, signaling and other interactions and motion may further affect these properties. Elastic properties of cells of the same tissue type may change significantly as they are growing older. Membrane elasticity is related to changes in the pattern of arrangement of fibrils in the cytoskeleton, while viscosity is related to cholesterol transfer between the internal lipid layer of the membrane and its outer layer. The elasticity modulus can be used as an indicator of reorganization of cytoskeleton structures in the process of adhesion in which elasticity increases 2 to 3 times [12-13].

2. Object and methods

Sample preparation and scanning of cells: For the purpose of these AFM measurements, hemolymph samples were applied to slides in thin layers (1 μl per slide), and then solutions with different osmolarity were added to the samples (in proportion 1:1). After that the slides were left for 30 min to incubate in a closed chamber with a moisturized sorbent. Upon incubation a lens of liquid was taken (during that time hemocytes had settled on the substrate, and it was highly unlikely that they would be eliminated from the sample together with supernatant liquid), which was scanned in semi-contact mode. If a sample is prepared as described above, cell membranes preserve their intravital properties and, thus, the values measured are certain to be accurate. In this study we used oscillating methods, which are based on parameter recording of interaction between the oscillating cantilever and sample surface. The AFM was operated in contact and semi-contact modes, which differ in the probe tip-to-sample distance. The contact method was used implies direct contact between the probe tip and sample surface in the process of scanning. The semi-contact method provides for partial contact in which the oscillating tip barely touches the sample surface. In the contact methods, adhesion forces may affect the cantilever significantly when the probe is being brought away from the sample. Adhesion force can be calculated if we consider the relationship between the force and probe tip deflection relative to the sample surface along the z-axis as linear. Surface topography of cells and physical properties of cell membranes were measured using the semi-contact method with NSG 03 cantilevers with hardness of 1.4 N/m, curvature radius of 10 nm, and scan frequency of about 0.6 to 0.8 Hz. Linear dimensions of cells were measured in the images obtained. Scanning of cells and analysis and processing of the data obtained with AFM were performed using NT-MDT SPM Software – Nova 1.0.26.1397. The parameters of elasticity and adhesion force were measured by atomic force spectrometry with load applied to 10 local sections on the cell surface. The method is based on plotting "force (force-versus-distance) curves" (DFL (Z)) that reflect deflection of the flexible console of the AFM probe as the probe is brought toward the sample surface at each point of nano-indentation. Deflection of the beam from the equilibrium position is detected by a four-quadrant photodiode and expressed as the current resulting from the error angle between the top and bottom parts of the photodiode. Local Young's modulus was calculated on the basis of a contact problem of Hertz model in Sneddon modification. In handling cells, we assumed that a cell is an elastic isotropic medium with Poisson ratio $\nu = 0.5$, and that the AFM needle tip is a solid cone. The resulting "force-versus-distance curves" were processed using "Ef3" software (NT-MDT, Zelenograd). Analysis of relationship between the sample deformity and applied load enabled us to perform quantitative estimation of the elasticity module and compare results for different sections of the cell surface.

Analysis of parameters of microrelief of cell membranes: Analysis of amplitude (of the so-called "high-rise") average statistical parameters used to describe vertical irregularity of the surface was performed with Image Analysis P9 software. Mean square roughness Sq (Square Roughness, nm) is the critical characteristic of roughness. Parameters Sp (Maximum Peak Height, nm) and Sv (Maximum

Valley Depth, nm) are estimated as the height of the highest peak and the depth of the deepest valley, which are measured from the median plane. Since by its definition Sv is equal to the distance from the lowest point of the surface to the level of the median plane, Sv is equal to the mean thickness of the surface layer. Skewness Ssk describes the profile distribution skewness, where one dip is steep, and the other one is sloping. Kurtosis Sku characterizes the profile distribution length. Parameter Sz (≡ St) is the maximum height of surface relief, calculated as the difference between the highest and the lowest points of the surface within a selected area. This parameter is equal to the thickness of the surface layer confined between planes going through the lowest and highest points of the surface. The solid material is below this layer. Thus, Sz can be considered as a parameter describing the thickness of the perturbed surface layer, which is not filled with the material completely, and in which the relief is changing. Further, we calculated values of one of the morphological parameters describing relief of a local section and the degree of surface evenness, i.e. density of the peaks Sds (1/μm2). This parameter demonstrates the number of peaks per unit of area, which make up the surface.

3. Results

Significant polymorphism in response of different cellular elements is characteristic of leeches *H. medicinalis*, *H. sanguisuga* and *E. octoculata*. If the osmotic pressure is reduced, values of viscoelastic properties of cells of *H. sanguisuga* increase, while those for amoebocytes of the other species tended to decrease. On the whole, hemocytes of leeches demonstrate similar morphophysiological characteristics in isotonia, yet they use different mechanisms to adapt to stress conditions.

Response to osmotic load of the selected five types of cells of oligochaeta differs according to the genus. Representatives of the *Lumbricus* genus are characterized by decrease in force of adhesion to the nanoprobe as osmolarity of the environment changes. At the same time, adhesion force in coelomocytes of many representatives of the *Eisenia* genus increases both in hypotonic and hypertonic solutions. However, response of cells of *E. tetraedra* is different – adhesion force in their coelomocytes decreases under any osmotic load, as is the case with the internal environment of cells of representatives of the *Lumbricus* genus. Coelomocyte membrane elasticity of worms of the *Eisenia* genus increases with changes in salinity of the incubation solution. Cells of *E. rosea* and *E. tetraedra* are the only exception from this rule. Membrane hardness of coelomic fluid cells of representatives of the *Lumbricus* genus (except for *L. castaneus*) increases under osmotic load.

Analysis of viscoelastic properties of hemocytes of shellfish and arthropods demonstrated that changes in adhesion force and membrane elasticity under osmotic load can be related to specific functions of hemocytes. Amoebocytes showed the greatest resistance to osmotic load. In most cases, no significant changes were observed in membrane elasticity and membrane-to-nanoprobe adhesion force.

We used the images obtained with an atomic force microscope to assess the nature of changes in microrelief of hemocyte surfaces in arthropods in normal conditions and under osmotic load. In general, no significant difference was found in parameters of surface topography of all types of hemocytes in all Arthropods in normal conditions and under osmotic load.

Analysis of the data obtained showed no significant changes in parameters of irregularity of surface microrelief under osmotic pressure (table 1).

Table 1. Indicators of amoebocytes, granulocytes, prohemocytes and aggregates surface microrelief inhomogeneity in *Blatella germanica* (*N=30, M±m*).

Indicators of microrelief	amoebocytes	granulocytes	prohemocytes	aggregates
		isotonic solution		
Sq, nm	67.0±18,91	61.8±16.57	34.0±12.09	72.4±19.34
Sa, nm	23.7±9,18	19.4±6.07	11.4±3.36	27.3±5.69
Sp, nm	533.9±126,91	482.5±119.69	202.6±94.79	518.7±128.9
Sv, nm	191.8±19.28	166.3±24.91	94.4±18.09	150.2±27.64
Sds, 1/um·um	0.99±0.11	0.51±0.12	0.55±0.14	0.71±0.19
Ssc, nm	0.52±0.15	0.72±0.18	0.96±0.22	0.51±0.13

hypotonic solution				
Sq, nm	62.5±18.76	64.3±17.08	30.3±11.03	74.9±21.16
Sa, nm	21.4±8.12	22.2±7.58	10.1±2.06	19.5±9.44
Sp, nm	518.5±142.65	400.1±128.79	195.2±99.39	530.1±136.99
Sv, nm	187.1±16.58	160.4±15.61	89.2±10.16	149.3±13.57
Sds, 1/um·um	0.93±0.14	0.47±0.12	0.55±0.17	0.77±0.21
Ssc, nm	0.49±0.19	0.51±0.18	0.87±0.22	0.59±0.14
hypertonic solution				
Sq, nm	69.1±19.65	68.9±19.72	36.5±14.95	85.4±19.41
Sa, nm	27.5±9.87	24.7±9.75	12.7±4.14	29.4±9.22
Sp, nm	595.9±139.24	491.4±147.71	202.3±119.84	564.3±138.23
Sv, nm	197.5±46.9	169.2±58.32	95.7±36.26	168.3±51.24
Sds, 1/um·um	0.97±0.19	0.53±0.14	0.55±0.13	0.76±0.17
Ssc, nm	0.57±0.15	0.65±0.17	0.85±0.19	0.55±0.12

4. Conclusion

Surface roughness, i.e. an aggregate of irregularities forming surface microrelief at a small distance from each other, explains why the actual surface area is greater than the geometrical area. Quantitative characteristics of membrane surface roughness have important practical implications, since they provide for estimation of the extent to which homogeneity or heterogeneity of surfaces affects processes of capturing foreign matters, and resistance of the surface to osmotic load. Examination of the cell surface is a topical problem in many studies. The condition of the cell surface affects some important phenomena in life of a living organism, such as tissue differentiation, organ formation of cells of various tissues, and recognition of foreign inclusions. Some experiments highlight individual aspects of functional activity of cell superficial layers in different contexts. Surface microrelief of hemocyte and coelomocytes, which changes under effects of environmental factors, reflects specific features of their functional status. For instance, some sulci going from the nucleus to the periphery were detected in microrelief of adhesiocytes. These cells are characterized by formation of regular domed elevations and holes that are regular in shape, and this fact gives evidence to the ability of adhesiocytes to eject content of their granules in unfavourable conditions and, thus, to use their internal membrane reserve to maintain the cellular homeostasis.

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