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Spectrum Instruments

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Application Notes

Life Sciences

BLOOD CELLS STUDY

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The Aim of the Study

The aim of the study is to examine deformation of erythrocytes and changes in membrane structure during virus-erythrocyte interaction. This investigation shows the potential of AFM when used to examine blood cells in different physiological conditions (disease, drug action etc.)

Getting ready the measurements

Preparation of the Sample

The procedure of biological sample preparation is of critical importance. Two basic procedures are used for examination in the air.

Smear method. A simple blood smear is suitable for examination. It is prepared on a slide with a standard clinical laboratory method. A 5 mkl-aliquot of blood is placed onto a slide and smeared with another slide. The smear thickness decreases along a direction of smearing. The atomic force microscope SolverP47BIO has light inverting microscope as an integral part, the latter can be used for preliminary analysis of the sample. We apply it to find the areas, where blood cells are arranged in one layer and are accessible for studying. The sample is ready to research and does not require additional manipulations.

Cell sedimentation from a suspension. We use the suspension of erythrocytes in a buffer (phosphate or anyone) with pH 7.0. Cells are fixed by adding paraformaldehyde to a final concentration of 2% for 2 or more hours; then they are put into distilled water up to the concentration of 10^8 cell/ml. A 5 mkl-aliquot is placed onto a slide and dried in the air. The slide is attached to the microscope stage by double-sided stick tape.

Preparation for Measurement

RBC can be examined in the air or a liquid buffer. Imaging erythrocytes in buffer under physiological conditions is a more complex method. According to [1, 2] drying of erythrocytes practically does not change their shape and membrane structure. It is in dried samples that the spectrin membrane skeleton was imaged [1]. Dry mode allows registering the distortion of fine membrane structure while spatial resolution in a liquid medium is limited [3]. That's why we use the dry mode.

Both IC mode and contact mode are suitable for measurements in the air. The error mode can be used in both cases with similar results. IC mode allows simultaneous imaging in phase contrast, which gives data on the object's elasticity, and the contact mode allows imaging in the lateral force (friction force) mode and measuring the object's elasticity (in the mode of Force-distance curves). In this case, IC mode is employed to examine erythrocytes in the air.

When the membrane images with high resolution are obtained, it is useful to examine two images:

In HIGH and MAG (error mode).In our case, the phase contrast mode did not give additional information and was not employed in the air.

Cantilevers

We use NSG11cantilever in the intermittent contact mode and CSC11 cantilever in the contact mode. Dry erythrocytes are stable samples and not very sensitive to the tip chosen. Results with similar quality were obtained in the intermittent contact and contact mode with different cantilevers. For the liquid medium, it is very important to choose the softest cantilever to decrease the interaction forces between the tip and the sample. Erythrocytes can break away in scanning.

Special Measurement Conditions

We use a few virus-RBC pairs with viruses of different families and erythrocytes obtained from different sources. Viral suspension was mixed with erythrocytes at 4-6^{o}C to reduce the rate of cell-virus interaction. The reaction was stopped by addition of paraformaldehyde to a final concentration of 2%.

Measurement Procedure

IC mode in the air was used. The quality of cells was estimated in a Biolam light inverted microscope that is a part of SOLVERP47BIO. The scan size was 70x70 mkm, therefore, the first scanning showed some tens of erythrocytes. This enabled to estimate deformations of RBC and to choose the object for detailed examination. We performed simultaneous registration of HIGHT and MAG signals, when the fine membrane structure was studied.

Results: Analysis, Processing, Problems and Prospects

Overall scans in IC mode show that the smear method of preparation can deform the cells. Nevertheless this is a fast and simple method. It displays adequate results of size distribution of cells. We can see other sorts of blood cells in the smears, observe the so-called “rouleaus” described in low circulating blood and are seen in a light microscope. Sedimentation method is more flexible in use, but its application can lead to Artifacts of preparation. Comparison of overall scans of control and experimental samples gives an overview of deformation of erythrocytes during their interaction with viral particles.

For instance, avian erythrocytes do not vary during interaction with parvovirus and influenza virus while simian erythrocytes deform very fast and very strongly. The Error mode allows detecting small details of the membrane structure against an abrupt slope of the erythrocyte surface. This mode enables to identify defects of the membrane surface after the virus action. On a control sample, we can see the net of the membrane peptide described [1, 2]. Influenza virus particles are visualized on the surface of chicken erythrocytes. According to publish data, the sorption on a substrate and drying of erythrocytes do not change the shape of erythrocytes and their surface structure. The comparison of deformations of RBC induced by different effects (chemicals, ionic strength or pH of solutions, disease, action of viral particles, mechanical deformation) (see [8]) enables to understand physical and chemical reactions that take place in each specific case. This information helps to develop the therapies and to create effective drugs and vaccines.

AFM study of blood can be used for direct diagnostics of blood diseases [9].

References

1.Minoru Takeuchi, Hiroshi Miyamoto, Yasushi Sako, Hideo Komizu, and Akihiro Kusumi Structure of the Erythrocyte Membrane Skeleton as Observed by Atomic Force Microscopy Biophys J, May 1998, p. 2171-2183, Vol. 74, No. 5.

2. Zhang, P. C., Bai, C., Huang, Y. M., Zhao, H., Fang, Y., Wang, N. X. and Li, Q. (1995). "Atomic force microscopy study of fine structures of the entire surface of red blood cells." Scanning Microsc. 9(4): 981-989; discussion 1009-1010.

3. Nowakowski R, Luckham P, Winlove P Imaging erythrocytes under physiological conditions by atomic force microscopy. Biochim Biophys Acta 2001 Oct 1;1514(2):170-6.

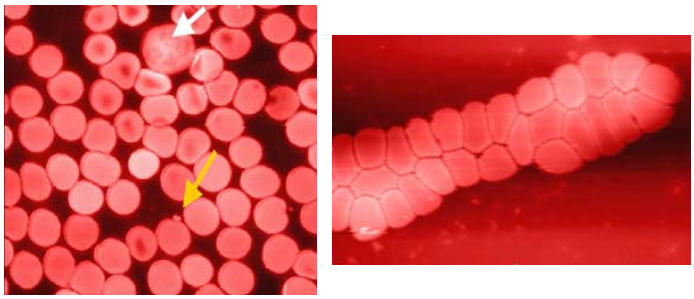
4. Zaitsev B.N., Durymanov A.G., Generalov V.M. Atomic Force Microscopy of the Interaction of Erythrocyte Membrane and Virus Particles. Proc. Intern. Workshop "Scanning Probe Microscopy-2002", p. 211-213. Nizhny Novgorod, 03.03-06.03.2002.

5. Ohta Y, Okamoto H, Kanno M, Okuda T Atomic force microscopic observation of mechanically traumatized erythrocytes.. Artif Organs 2002 Jan;26(1):10-7.

6. Cheng Y, Liu M, Li R, Wang C, Bai C, Wang K. Gadolinium induces domain and pore formation of human erythrocyte membrane: an atomic force microscopic study. Biochim Biophys Acta 1999 Oct 15;1421(2):249-60.
7. Girasole, M., Cricenti, A., Generosi, R., Congiu-Castellano, A., Boffi, F., Arcovito, A., Boumis, G. and Amiconi, G. (2000). "Atomic force microscopy study of erythrocyte shape and membrane structure after treatment with a dihydropyridinic drug." Appl. Phys. Letters. 76(24): 3650-3652.
8. Girasole M, Cricenti A, Generosi R, Congiu-Castellano A, Boumis G, Amiconi G. Artificially induced unusual shape of erythrocytes: an atomic force microscopy study. J Microsc 2001 Oct;204(Pt 1):46-52.
9. O'Reilly M, McDonnell L, O'Mullane J. Quantification of red blood cells using atomic force microscopy. Ultramicroscopy 2001 Jan;86(1-2):107-12

Blood Smear. Overall View. Intermittent Contact Mode

Blood smear is the simplest biological sample. It can be prepared within a few minutes (see Preparation of the Sample). Many peptides, which are present in blood, block salt crystallization in drying, and removal of salt becomes unnecessary. We can see many interesting objects in a blood smear in all ranges of magnification - from tens of microns (blood cell) to a few nanometers (blood peptides and features of membrane). The material for the preparation is always with you. You can to use it to check your microscope, only observe sterility.



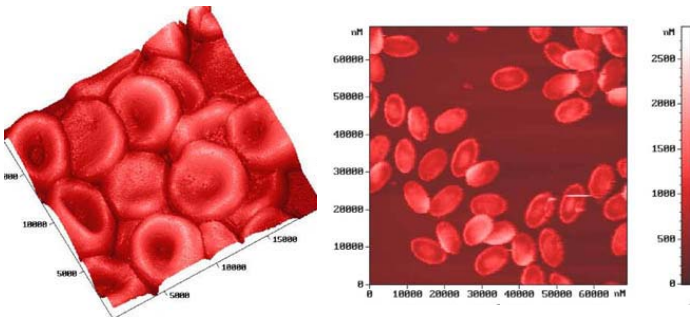
a) b)

Fig. 1 NSG11 cantilever was applied in the intermittent contact mode.

a)– 70x70 mkm scan of a human blood smear on a slide. Most cells are red blood cells (RBC), or erythrocytes. They are similar in size, but their shapes change easily (for instance, for better movement through capillaries). Their shapes on the scan are deformed by pressure of neighboring cells. White arrow – monocyte; yellow arrow – platelet.
b) – 70x45 mkm scan of an erythrocyte rouleau. These rouleaus can be observed by light microscopy in slow circulating blood.

Suspension. Overall view. Intermittent Contact Mode

Cell sedimentation from an erythrocyte suspension is a more equilibrium process than preparation of a blood smear. We use this process when we have to obtain a size and form distribution of blood cells without mechanical deformation. Model experiments are another advantage of cell sedimentation from a suspension. It is possible to study physical, chemical and other actions on the cell membrane, for example, that of viral particles or drugs (see Virus-cell Interaction).
Cell sedimentation from a suspension is a more complex process than blood smear preparation. There is a risk of introducing changes of the shape and the structure of the cell membrane. This occurs when pH or ionic strength of the buffer change (see Artifacts of Preparation). Suspension of blood cells is prepared by adding fresh blood to a buffer containing anticoagulant (EDTA, sodium citrate, heparin) in order to avoid blood coagulation.
Inevitable disbalance between blood proteins and buffer salts results in salt crystallization that occurs in drying and deformation of blood cells in the scan picture. Removal of salts with distilled water causes detachment of adsorbed cells from the support. That's why, when working with suspensions, cells should be either immobilized on the support with special agents (such as polylisine) or released from the support into distilled water. Cells should be chemically fixed before being released into distilled water to avoid their damage and deformation caused by low ionic force. We used the paraformaldehyde fixation method followed by release into distilled water. Erythrocytes can be also deformed in a suspension when contact of two or more cells occurs. Therefore, single objects should be chosen for deformation studies.



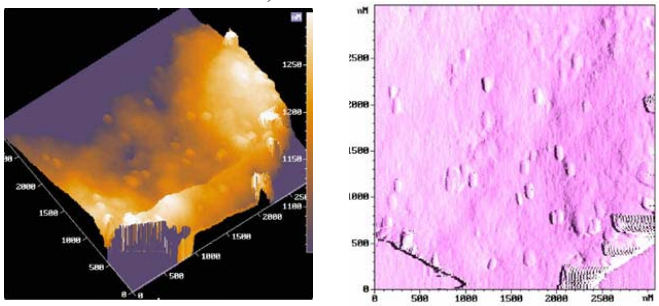
a) b)

Fig. 2 (a, b)

a) – erythrocytes of rhesus monkey that deposited from a suspension. IC mode. The shapes of soft red blood cells of mammals are deformed by neighboring cells.
b) - chicken erythrocytes. IC mode. Nucleated avian erythrocytes are more rigid than mammalian erythrocytes and keep the shape during mutual contact.

Error Mode

Error mode is intended for examination of fine features of a surface with too different heights. We use the error mode for detection of 10-20 nm Z features on a sharp slope of an erythrocyte (height difference of 1 mkm).

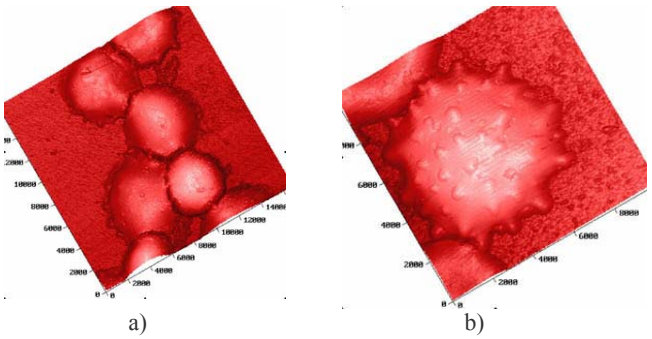


a) b)

Fig. 3 The surface of a chicken erythrocyte with adsorbed influenza virus particles: a – intermittent contact mode, three-dimensional view; b – error mode.

a) In the first case, small surface details (influenza virions) are either indistinguishable or visible only on the marked portion of the figure.
b) In the error mode, virions are observed on the whole surfaces of erythrocytes. Besides, artifacts caused by the probe excitation at sharp boundaries during scanning are clearly seen.

Artifacts of Preparation

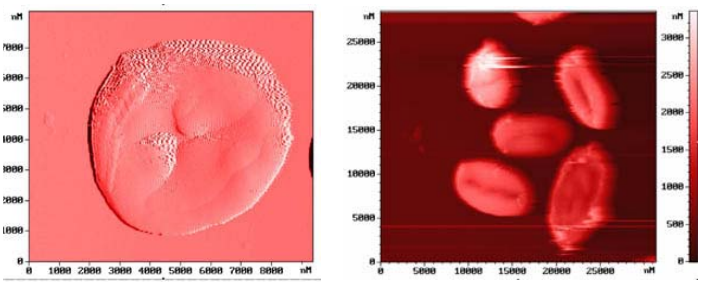


a) b)

Fig. 4 Deformation of erythrocytes generated by defects of preparation: a) change of pH during fixation and washing; b) effect of hypertonic buffer.

Careless handling of a suspension can cause deformation. Changes of pH (in this case, acidity of a solution) lead to distortion of membrane integrity followed by spherocytosis and induction of fusing process. Increase in ionic strength causes dehydration of RBC and, as a consequence, deformation.

Scanning Artifacts



a) b)

Fig. 5 (a, b)

a) - Scanning artifacts associated with probe excitation at erythrocyte edges when differences in height are very sharp.
They are indistinguishable in HIGH mode, but are visible in the error mode and phase mode.
b) - Scanning artifacts associated with distortion of erythrocyte membrane and appearance of sites to which the probe clings. Truly, this effect cannot be related to artifacts (phenomena bearing no relation to the object of research). Membrane distortions during scanning are associated with membrane structure and can be used to detect changes occurring in the membrane.

Virus-cell Interaction

Virus-cell interaction can be considered as an example of special action on the membrane. This is a more complex process than chemical or mechanical action. Its results depend on the family of viruses and the source of erythrocytes. Moreover, the virus-erythrocyte reaction can differ for two different strains of the same virus. Virus-erythrocyte interaction changes the fine structure of the membrane and the shape of red blood cells. We can observe viral particles on the membrane surface when virus-erythrocyte interaction is lacking and there is only sorption process. Virus-erythrocyte interaction is described in [4]. Samples were prepared by cell sedimentation from a suspension (see Suspension. Overall View). The intermittent contact mode was used. Two signals were determined simultaneously. The latter signal corresponded to the ERROR MODE. It was used to localize fine features that corresponded to defects on the membrane or adsorbed viral particles (10-20 nm) against the background of sharp changes of height associated with the shape of RBC (difference about 1 micron).

Change of the Shape

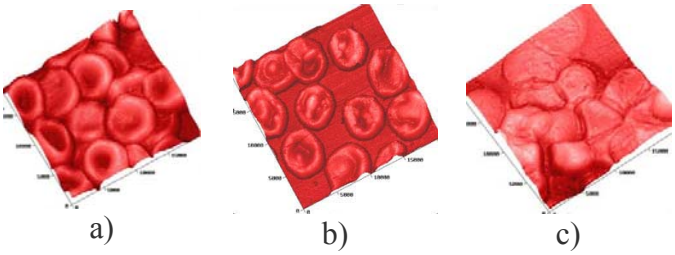


Fig. 6 Virus-induced consecutive stages of deformation of rhesus monkey erythrocytes(a, b, c)

a) – control sample. Deformations were caused by cell-to-cell interaction;
b) - rhesus monkey erythrocytes after interaction with influenza virus;
c) – final degradation of rhesus monkey erythrocytes after 90 minute interaction with canine parvovirus.
Changes of the shape of RBC are polymorphous and depend on the nature of action. Deformation associated with cell-virus interaction depended on the family of viruses and the source of erythrocytes. For example, some viruses interact with of rhesus monkey erythrocytes and are neutral to goose and chicken erythrocytes and vice versa

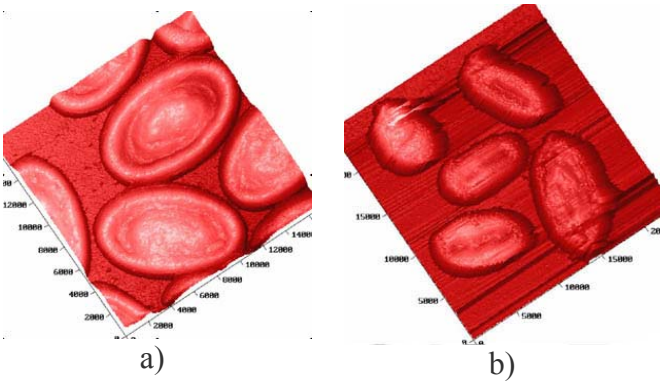


Fig.7 These scans show the shape of chicken erythrocytes before (a) and after (b) interaction with rubella virus.

Changes of the shape of RBC arise also after mechanical deformation [5] and chemical action [6,7]. Biochemical causes of these changes are not clear yet [8].

Membrane Structure

We examined simultaneously fine membrane structure and deformation.

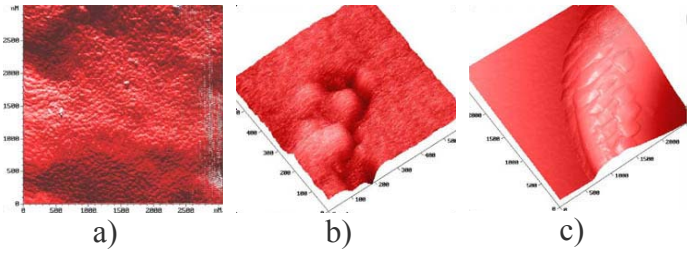


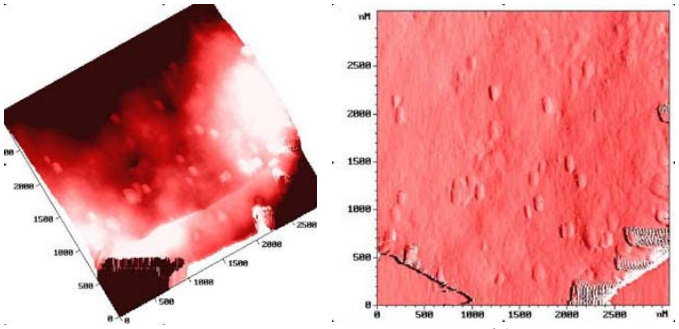
Fig. 8 (a, b, c)

a) – control sample. The membrane has classical structure that was described previously [1,2]. Fine net features can be interpreted as a spectrin membrane skeleton [1].
b) – damages on rhesus monkey erythrocytes membrane after addition of canine parvovirus.

c) – clusters on the edge of erythrocytes. These structures are frequently observed on erythrocytes after their interaction with viruses. They are not observed on native cells. We cannot explain their nature yet. Parallelism of lines suggests their artificial origin and relation to the scanning process, however, the angle between the parallel structures in clusters and the scanning direction changes from cell to cell.

Viral Particles on Membrane

Viral particles can be imaged on the surface of RBC when virus-erythrocyte reaction is absent or very slow.



a) b)

Fig. 9 (a, b)

a) and b) – images of influenza virus particles on the surface of a chicken erythrocyte obtained simultaneously in HIGH (a) and MAG (b) mode. We can see the advantage of ERROR MODE (MAG) for studying small details (20 nm high virus particles) on an abrupt slope of erythrocyte surface (about 1 mkm high). MAG mode shows the whole surface of the erythrocyte while HIGH mode shows only a part of the cell surface that is located between two horizontal sections. Artifacts in the right low corner of the MAG-image are caused by the probe excitation during scanning. The outlines of virions extended in the same direction are caused by the form of the tip used (influenza virions have spherical form).

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