3rd Micro and Nano Flows Conference
Thessaloniki, Greece, 22-24 August 2011
Keynote Contribution

Digital optical and scanning probe microscopy for biocells inspection and manipulation

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Abstract Nanobiotechnology and scanning probe microscopy (SPM) are new fields that are of interest to modern medical science. Nowadays atomic force microscopy (AFM) is widely used in medical-biological researches from the all variety of SPM. Without special methods of preparation AFM gives an opportunity to investigate the morphology of the surface of different biological objects with nanoresolution. Also this method allows to analyze the physical and mechanical properties at micro- and nanoscale. Our experimental complex with the functions of scanning probe and optical microscopy is intended for different materials investigation including biological cells. A special optical system makes it possible to visualize the objects position of the probe in the microscale. AFM is used for visualization and identification of the local adhesion and viscoelastic properties of biological cells. Dynamic laser speckle (DLS) is used for real-time monitoring of cells motility in living tissues. Another opportunity of this complex is some manipulation with the cell by means of the applied load variation. This technique greatly enhances the possibilities and opens a new field of experiments in cell biology. The purpose of this work is to show the possibility of AFM and DLS for studies of biological cells, namely measurement of the general cells motility in living tissues, the elastic modulus of the single cell membrane, as well as to identify the forces causing damage of the membrane.

Keywords: Atomic Force Microscopy, Biological Cells, Dynamic Laser Speckle, Nanoresolution

1. Introduction

Nowadays AFM is widely used in medical-biological researches from all variety of SPM (see e.g. Fischer-Cripps 2004). It is one of the instruments that enables receiving the spatial images of the surface with a resolution close to atomic one. Besides, as shown by Burnham and Colton 1989, Ueda et al 2001, Ikai, and Afrin 2003, AFM is successfully used to estimate the local elastic and adhesive properties of the surface. Another important development in this field is the direct measurement of interaction forces between ligands and receptors using an atomic force microscope (see e.g. Sako et al 2000, Fujiwara et al 2002, Byassee 2000). When we scan soft and delicate object like biological cell in a contact mode the surface may be damaged by the probe if a loading force is too large. Deducing a guiding principle for the magnitude of the force required for cell manipulation aimed at cellular level surgery. The objects of the present investigation are erythrocytes due to their availability. At the same time, erythrocyte membranes have similar principles of organization as biological membranes. Therefore, it is convenient to use them for the cell elastography methods developing as a natural model for studying the general structural and functional characteristics of membranes (see e.g. Lee and Lim 2007).

The use of laser light for illumination opens additional possibilities for bio-tissue monitoring. Coherent light scattered from a diffuse object produces a random granular interference structure some distance away from the object, which is called a speckle pattern (see e.g. Fercher and Briers 1981, Fomin 1998, Asseban et al 2000). Such a pattern can also be observed when a laser light illuminates a living semitransparent tissue. These biospeckles play a dual role: as a source of noise in tissue images, and as a carrier of useful information on the biological or

physiological activity of living tissues, such as subskin blood flow and general tissue-structure motility, see e.g. Bazylev et al 2002, Bazylev et al 2003.

The purpose of this work is to show the possibility of AFM and DLS for studying biological cells, namely measurement of the general cells motility in living tissues, the elastic modulus of the single cell membrane, as well as to identify the forces causing damage of the membrane. The purposeful use of the latest information can be used for cutting the cell membrane i.e. for the so-called cell surgery.

2. Force spectroscopy

The function of force spectroscopy is a standard mode of AFM. The method consists of the realization of the contact deformation of the specimen using the probe and of the measurement of the dependence between the probe interaction force and the distance (see e.g. Burnham and Colton 1989). By recording the cantilever deflection while the sample stage moves up, reaches the tip and retracted (fig. 1). The force curve is obtained by the monitoring of the movement of the reflected laser beam from the back of the cantilever. The force curves are the relation between the bend of the cantilever and the position of the probe. Knowing the displacement of the sample in the vertical direction and the amplitude of cantilever bending, then it is possible to calculate the total external force that has been applied to it and the resulting deformation of the sample. The force curve is applied to the calculation of Young's modulus (fig. 2). Fig. 2 shows different parts of the indentation process. At the beginning of the force curve measurement, the tip is far away from the sample and only approaches to it (a). Since the tip is not in contact with the sample yet, the cantilever deflection is constant. At the moment that the tip touches the sample, the cantilever moves upward (b). Then the tip indents the sample, and the bending of the cantilever moves downward (c). The sample is then retracted (d), as indicated by the curve, which shows the deflection during retraction. However, if there is an adhesion between sample and tip, the tip will stick to the sample beyond the point of contact (e), until it finally breaks free again and the deflection returns to zero (f). The approach curve exactly retraces the returns to point (e) if there is no any piezo hysteresis.

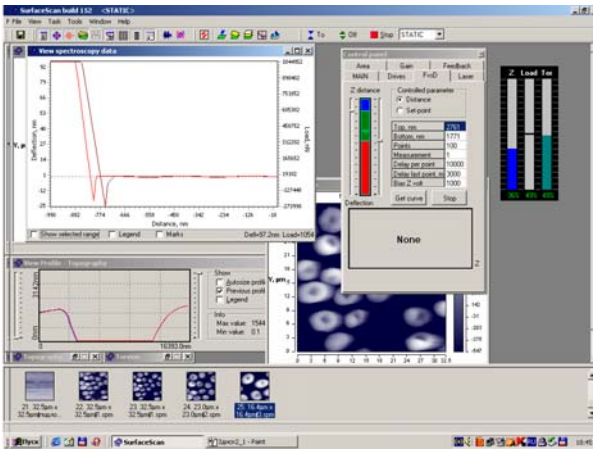


Fig. 1. Window of the AFM controlling program. The regime of static force spectroscopy.

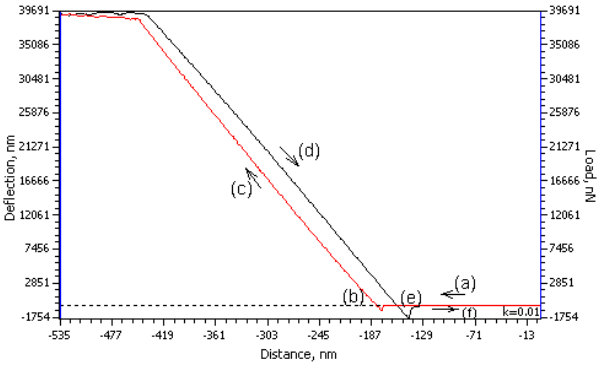


Fig. 2. Typical force curve for erythrocytes.

3. Metodology

3.1 Sample's preparation

The blood of healthy persons surveyed in the Republican scientific practical centre "Cardiology" (Minsk, Belarus) was used. A drop of venous blood stabilized by heparin was fixed in a 1.5% wt/vol glutaraldehyde solution (Fluka AG). Fixation was carried out during 30 min at room temperature. Then the blood solution was centrifuged at 1500 revolutions per minute during 3 min. The

supernatant was discarded and the erythrocytes were washed twice in a buffer solution (PBS) and twice in H_2O. The cells were put on the glass surface and dried at room temperature.

3.2 Atomic force microscopy

AFM researches were carried out using atomic-force microscopy NT-206 ("MicroTestMachines", Belarus) working in contact mode. The standard cantilevers NSC11 ("MikroMasch" Co., Estonia) with the spring constant of 3 N/m were used. The tip radius was checked by using a standard TGT01 silicon grating (NT-MDT, Moscow) and was in the range from 40 nm for topography visualization to 60 nm for cell stiffness determination.

Local elastic properties of red blood cells were quantitatively determined due to force spectroscopy regime. By recording the cantilever deflection while the tip is brought in contact at the fixed point and retracted, we obtain force curve. The Young's modulus was calculated using the Hertz model describing the elastic deformation of the two bodies in contact under load (see e.g. Hertz 1881). We consider that the indented sample is assumed to be extremely thick in comparison to the indentation depth. In this case the elastic modulus can be calculated as described earlier (see e.g. Chizhik et al 1998, Kuznetsova et al 2007).

3.3 Statistical analysis

The data are presented as mean ± standard deviation of the mean and analyzed by the Student's t-test. A p-value of less than 0.05 was considered to be statistically significant.

4. Different forces action

The definition of the cells elasticity modulus can be considered as the first example of the cell's manipulation. Our earlier researches showed that erythrocyte elasticity modulus hasn't depended on the area of indentation (see e.g. Drozd and Chizhik 2009).

The value of this parameter in the centre of the cell hasn't different from the value at the periphery. At the same time the elasticity modulus depends on time of loading. It has been shown that the choice of the indenter contact velocity effects on the erythrocyte membrane surface for its elasticity estimations is very important. Based on the received data, it is evident that the estimation of cell membrane properties largely depends on the ratio between the length of the relaxation transitions and the time of term effects in the tests (see e.g. Drozd et al 2009). In this work we compared the action of different forces on the cell's membrane. The erythrocytes elastic modulus has been determined at the undamaged load (fig.3.).

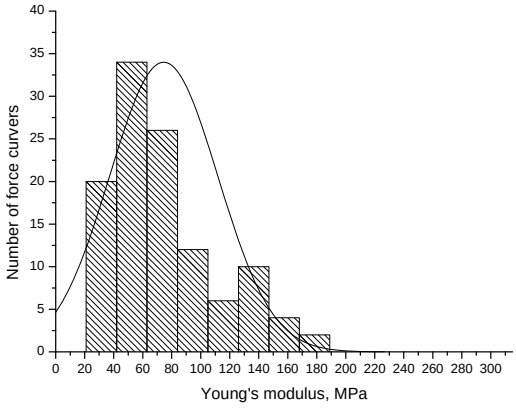
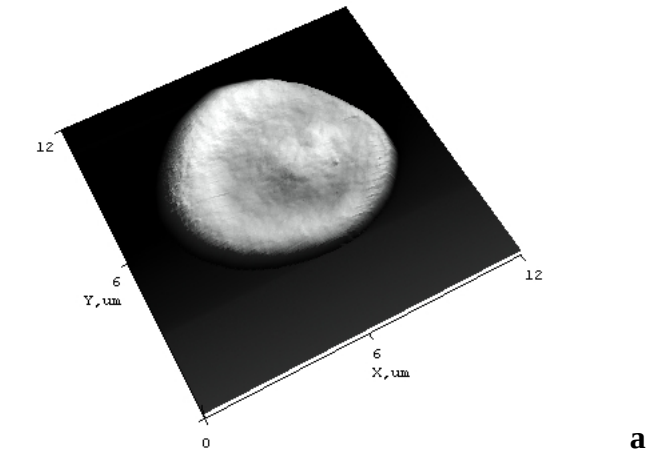


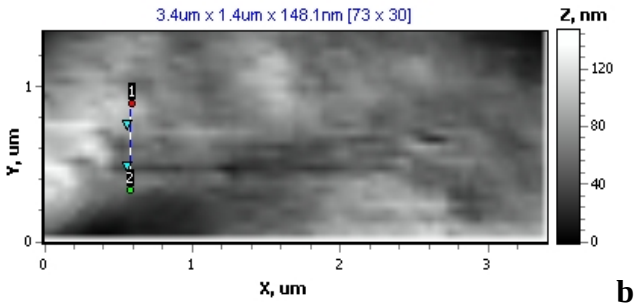
Fig. 3. Diagram of the distribution of the modulus of elasticity of erythrocyte membranes in healthy persons.

The second example of the cell's manipulation is the cutting of the cell. This may be achieved by the loading of the certain force to the membrane. In our experiments four values of load parameters were used. These parameters are non-dimensional and show the load applied to the console (30, 50, 70 and 80%). Real values of the load may be determined after calculations on the bases of the force spectroscopy data. Fig. 4. shows the results of erythrocytes scanning before (a) and after (b) the load increase (from 30% till 70%).

At this load the membrane of red blood cells was scratched and the depth of the hollowing was near 10 nm.



a



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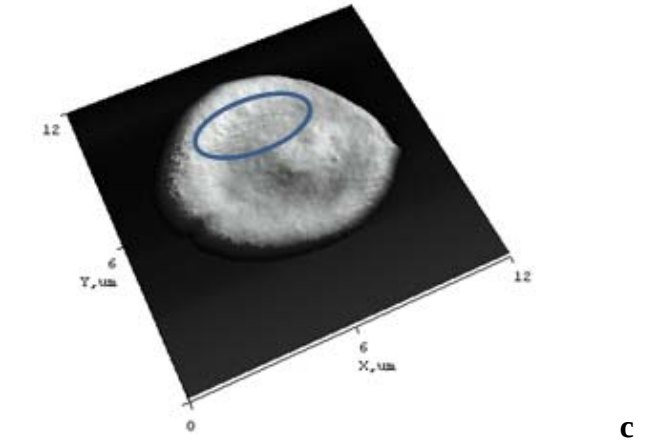

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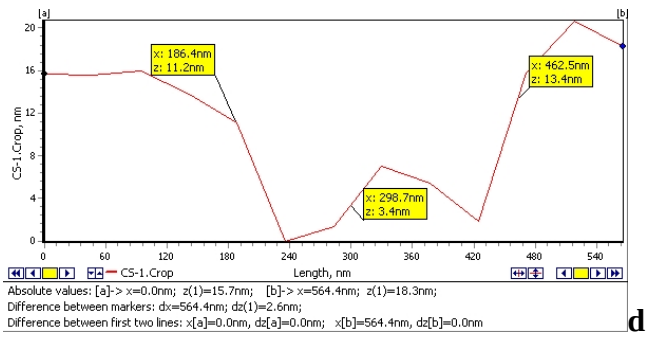
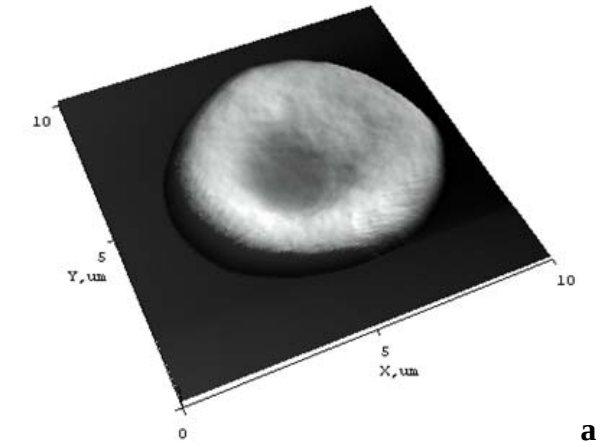
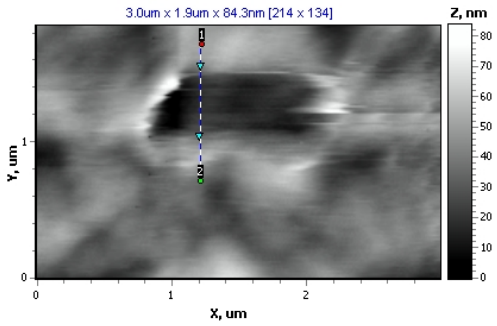


d

Fig. 4. AFM image of erythrocytes: a – normal cell, scanning area 12.0 x 12.0 μm; b - erythrocyte after load increase (70%), scanning area 12.0 x 12.0 μm; c - hollowing, scanning area 1.4 x 3.4 μm; d – profile of the surface section.



a



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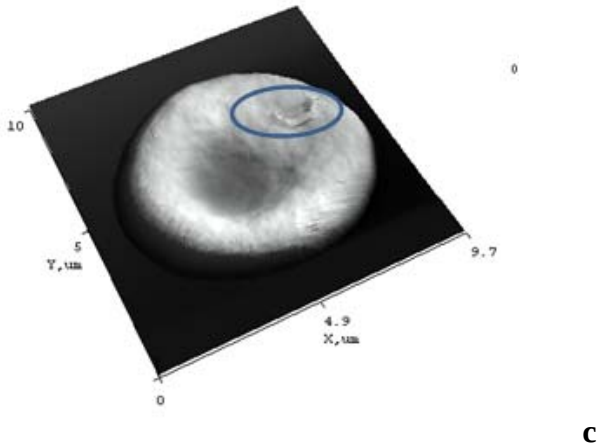

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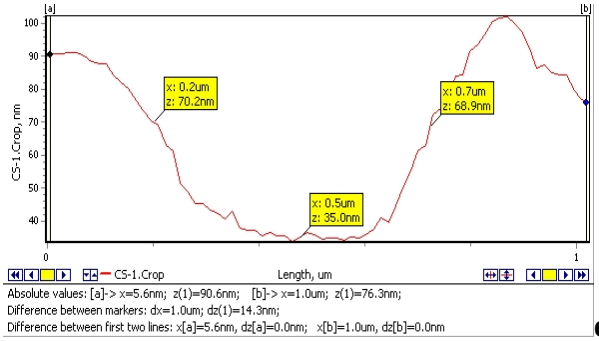

d

Fig. 5. AFM image of erythrocytes: a – normal cell, scanning area 10.0 x 10.0 μm; b - erythrocyte after load increase (80%), scanning area 10.0 x 10.0 μm; c - hollowing, scanning area 3.0 x 1.9 μm; d – profile of the surface section.

This is comparable with the thickness of the erythrocyte's membrane. Thus this load allows cutting up the cell's membrane.

Fig. 5. show red blood cell before (a) and after (b) the force loading (from 30% till 70%). At this load the membrane of red blood cells was damaged strongly and the depth of the hollowing was near 40 nm. This is comparable with the thickness of the erythrocyte's membrane together with the cytoskeleton.

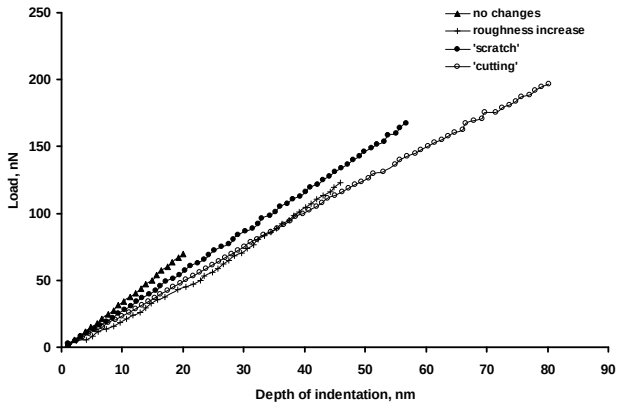

Fig. 6. Diagram of the relation between the applying load and the indentation depth for the erythrocyte membrane at different loading regime.

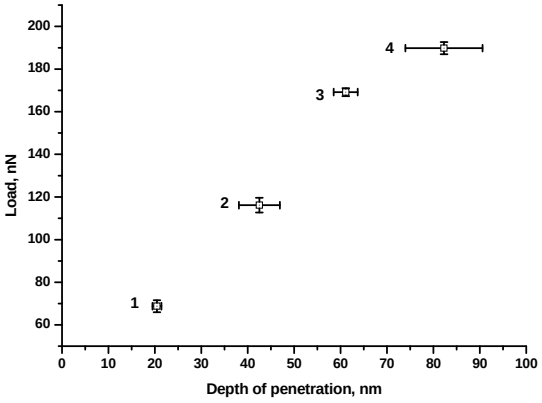

Fig. 7. Diagram of the average values of the applying load at maximum of the penetration depth (different loading regime).

Using this force the cell may be cut strongly for the inner content investigation. We calculated the value of the force at these four load parameters. There were the linear dependence between the applying load and the depth of indentation (fig. 6). But the values of the maximum forces were different and the depth of penetration was greater than the force was larger. The average values of the maximum forces reached at the four different parameters of loading (30, 50, 70 and 80%) and the maximum values of the penetration depth are presented in Fig 7. When the force was equal to 20 nN the roughness of the cell surface was increased during the scanning in a contact mode. If the loading force was from 130 to 170 nN the tip scratched the cell damaging the membrane. And when the force was more than 190 nN the deeper penetration of the tip took place.

5. Cells motility monitoring

5.1. Biospeckle formation

Visible laser light penetrates into the bio tissue at a depth of about 200–1000 μm and is multiply scattered by the erythrocytes flowing inside the living tissues. So, the image of a tissue illuminated with laser light differs from an image taken under white light illumination in the speckle pattern being superimposed on the surface features of the tissue. As the scatterers (erythrocytes) move, the speckles also move and change their shape. The dynamic (time-dependent) biospeckle pattern is formed as a superposition of some moving speckles with different dynamics, including static speckles, see fig. 8.

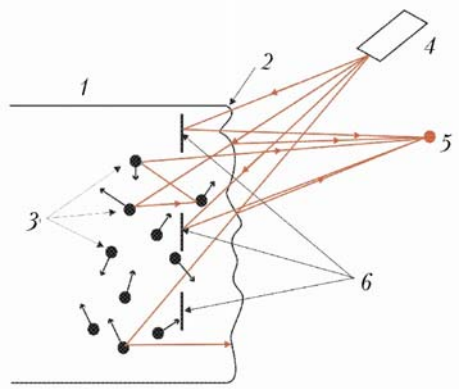

Fig. 8. Biospeckle formation under laser tissue probing. 1 – living biotissue; 2 – biotissue surface; 3 – moving erythrocytes; 4 – laser; 5 – speckle; 6 – unmoved scatterers.

For single-point measurements, the intensity fluctuations measured at the point are characterized by the time-correlation length defined by the time at which the normalized temporal autocorrelation function of intensity fluctuations falls to 1/e. This statistical quantity is inversely proportional to the fluctuating speed of the speckle intensity. Its reciprocal value measures the velocity of a diffuse object at least for speckles scattered once. More general description of dynamic speckle patterns is based on the use of multidimensional space–time cross-correlation functions.

5.2. Biospeckle image formation and treatment

The schematic diagram for experimental setup used in this study is shown in Fig. 9. A low power He–Ne laser is used as a light source.

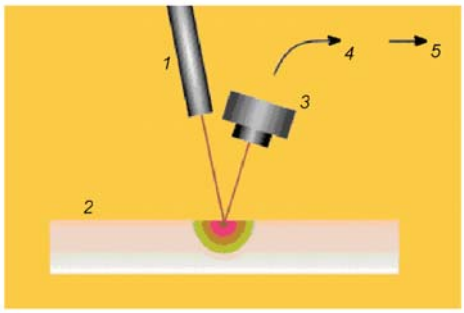

Fig. 9. Schematic diagram od DLS technique. 1 –probing laser;; 2 –living biotissue 3 – CCD camera; 4 –digital image memory; 5 – PC.

The collimated laser beam is focused onto the tissue under study through a thin transparent glass used to prevent mechanical movement of the tissue. Light scattered by moving erythrocytes in the illuminated volume is collected by camera lens onto the CCD matrice, where the speckle pattern is formed. Since the erythrocytes are moving, the speckles are also moving, thus forming a dynamic speckle field. The exposure time varied from 10 μs (for cross-correlation analysis of subsequent frames) to 1/60 s (for a single exposure mode). Speckle patterns are recorded as a distribution of grey values I(m, n) in digital form for each pixel (m, n) of the CCD matrix. In real-time operation, the image analysis is performed during the time interval between subsequent (two or more) frames, see fig. 10.

Three methods of the dynamic speckle patterns evaluation were tested. Both decorrelation and auto-correlation analysis were realized in a near-to-real time mode, when all digital specklegram treatment was performed during the time interval between subsequent frames (40 ms), and results in the form of 2D maps of subskin blood flux were visualized on the PC monitor with frequencies being 10-25 Hz. The full cross-correlation analysis of the dynamic bio-speckle pattern needs a little more PC time and only quasi-real time operation with present hardware was achieved at a frequency of about 10 maps/s.

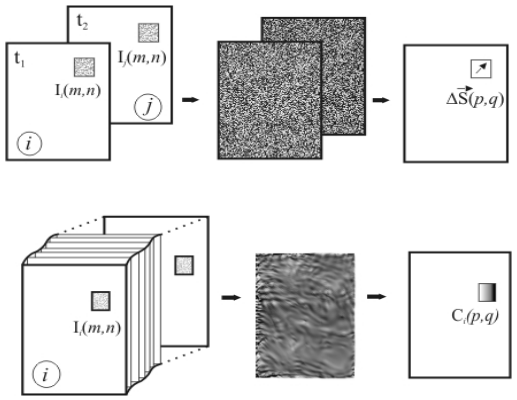

Fig. 10. Illustration of the cross-correlation analysis of a succession of images and autocorrelation analysis of a single-exposure specklogram with the use of fast Fourier transform.

5.3. Erythrocytes dynamics

Detailed analysis of multiple scattering on bio-speckle formation and its dynamics shows that the time-space cross-correlation analysis of the temporal evaluation of the bio-speckle patterns is an effective means of real time flow and stress visualization of a living tissue. Digital processing of bio-speckle patterns records yields 2D maps exhibiting the

erythrocytes flow temporal and spatial variations, see fig.11.

Dynamic speckles have two fundamental motions of speckles. For the first type of speckle motion, called "translation," the speckles move as a whole and their shape remains unchanged for a considerable displacement. For the second type of speckle motion, speckles deform, disappear, and reappear without appreciable displacement of their positions. This type of speckle motion is called speckle "boiling". In both cases, the speckle behavior depends not only on the motion of the scatterers but also on the parameters of the optical scheme used for speckle observation. In most cases, dynamic biospeckle mode is mixed and speckles translate gradually, changing the structure.

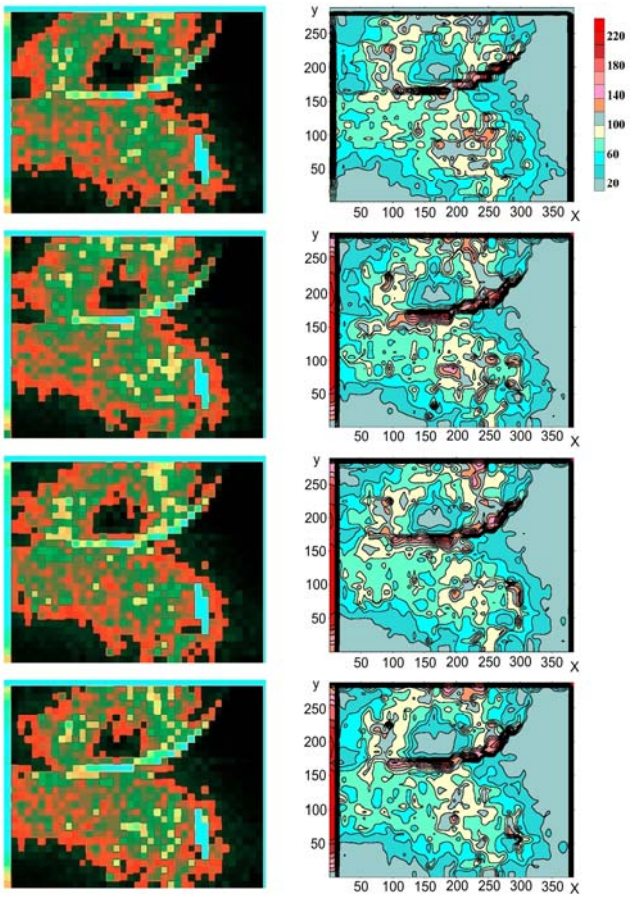

Fig. 11. Real-time maps showing the intensity of the erythrocytes motions reconstructed by the contrast variation in single (prolonged) exposure speckle photography (left) and isolines of these maps (right).

The information obtained with cross-correlation analysis seems to be a little excessive for the present task as contains the direction of the averaged bio-speckle displacement. For such random fields as erythrocytes flow maps it seems that decorrelation and/or auto-correlation analysis is faster and sufficient to extract only the value of the averaged blood flux intensity.

CONCLUSIONS

This investigation reveals the opportunity of AFM for the mechanical properties of cells measurements and certain actions on them. There are two main method of living cells manipulation using AFM: elasticity measurement and some force action. We have determined the forces under the influence of which the cell's membrane was cut. Thus the principal of experimental results of the force measurements was survey for the deducing a guiding principle for the magnitude of the force required for cell manipulation aimed at cellular level surgery.

In addition, quantitative monitoring of erythrocytes motions in living tissues with the use of digital dynamic laser speckle photography is demonstrated. The proposed software makes it possible to recover up to 250,000 motion vectors in a two-dimensional flow region of size 20x30 mm, providing spatial resolution of about10 μm.

Acknowledgements

The authors wish to thank Professor C.A. Greated (The University of Edinburgh, UK) for valuable discussions and recommendations, as well as the National Academy of Sciences and Foundation for Basic Research of Belarus for partial financial support of the work with grants and projects "ЭЭ 1.6.1", T11MC-023, T10P-029.

References

Asseban, A., Lallemand, M., Saulnier, J.-B., Fomin, N., Lavinskaya, E., Merzkirch, W., Vitkin, D., 2000. Digital speckle photography and speckle tomography in heat transfer studies, Opt. Laser Technol. 32, 583–592.

Bazylev, N., Fomin, N.A., Fuentes, C., Hirano, T., Lavinskaya, E., Martemianov, S., Mizukaki, T., Nakagava, N., Rubnikovich, S., Saulnier, J.-B., . Takayama, K., Tuhault, J.-L., 2003. Laser Monitor for Soft and Hard Biotissue Analysis Using Dynamic Speckle Ptotography, Laser Physics, 13, 1–10.

Bazylev, N.B., Fomin, N.A., Lavinskaya, E.I., Rubnikovich, S.P., 2002. Real-time blood micro-circulation analysis in living tissues by dynamic speckle technique, Acta Bioeng. Biomech. 4, 510–511.

Burnham, N. and Colton, R.J., 1989. Measuring the nanomechanical properties and surface forces of materials using an atomic force microscope, J. Vac. Sci. Technol. A7, 2906–2913.

Byassee, T.A., Chan, W.C., Nie, S., 2000. Probing single molecules in single living cells, Anal. Chem. 72**,** 5606–5611.

Chizhik, S.A., Huang, Z., Gorbunov, V.V., Myshkin, N.K., Tsukruk, V.V., 1998. Micromechanical properties of elastic polymeric materials as probed by scanning force microscopy, Langmuir. 14, 2606-2609.

Drozd, E.S. and Chizhik, S.A., 2009. Combined atomic force microscopy and optical microscopy measurements as a method of erythrocyte investigation. Proc. of SPIE. Intern. Soc. for Optical Eng. 7377, 73770E-73770E-8.

Drozd, E.S., Chizhik, S.A., Konstantinova, E.E., 2009. Probing mechanical and structural properties of the erythrocyte membrane by atomic force microscopy, Russian J. of Biomechanics 4 (46), 22-30. (in Russian).

Fercher, A.F. and Briers, J.D., 1981. Flow visualization by means of single-exposure speckle photography, Opt. Commun. 37, 326–329.

Fischer-Cripps, A.C., 2004. Nanoindentation. 2nd Ed. New York: Springer-Verlag.

Fomin, N., 1998. Speckle Photography for Fluid Mechanics Measurements, Springer-Verlag, Berlin.

Fujiwara, T., Ritchie, K., Murakoshi, H., Jacobson, K., Kusumi, A., 2002. Phospholipids undergo hop diffusion in compartmentalized cell membrane, J. Cell Biol. 157**,** 1071–1081.

Hertz, H., 1881. Ueber den kontakt elastischer koerper, J. fuer die Reine Angewandte Mathematik. 92, 156.

Ikai, A. and Afrin, R., 2003. Toward mechanical manipulations of cell membranes and membrane proteins using an atomic force microscope, Cell Biochemistry and Biophysics39, 257-277.

Kuznetsova, T.G., Starodubtseva, M.N., Yegorenkov, N.I., Chizhik, S.A., Zhdanov, R.I., 2007. Atomic force microscopy probing of cell elasticity, Micron. 38, 824-833.

Lee, G.Y.H. and Lim C.T., 2007. Biomechanics approaches to studying human diseases, Trends Biotechnol. 25 (3), 111–118.

Sako, Y., Minoghchi, S., Yanagida, T., 2000. Single-molecule imaging of EGFR signaling on the surface of living cells, Nat. Cell Biol. 2**,** 168–172.

Ueda, M., Sako, Y., Tanaka, T., Devreotes, P., Yanagida, T., 2001. Single-molecule analysis of chemotactic signaling in Dictyostelium cells, Science 294**,** 864–867.