HIGH-RESOLUTION SCANNING ION-CONDUCTANCE MICROSCOPY FOR THE STUDY OF BLOOD CELL MORPHOLOGY AND RIGIDITY

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Abstract. The methods of scanning microscopy were used to study blood cells (neutrophil granulocytes, lymphocytes, and erythrocytes) morphology and rigidity. Differences between morphology and adhesion properties of fixed and living cells were shown. The Young's modulus in various lymphocytes and erythrocytes populations was obtained. Scanning ion-conductance microscopy was compared to the most widespread biological scanning probe methods: atomic force microscopy and scanning electron microscopy. It was established that scanning ion-conductance microscopy is capable of obtaining the morphology and stiffness of living cells at one scan.

Keywords: atomic force microscopy, scanning ion-conductance microscopy, scanning electron microscopy, neutrophil granulocytes, lymphocytes, erythrocytes, morphology, rigidity.

List of Abbreviations

AFM – atomic force microscopy
LGL – large granular lymphocyte
NG – neutrophil granulocyte
PBS – phosphate-buffered saline
RBC – red blood cell
SEM – scanning electron microscopy
SICM – scanning ion-conductance microscopy

Introduction

Deciphering the connection between the structure and function of human cells remains the "holy grail" of biology and medicine. The Human Cell Atlas Project (Regev *et al*., 2017) aims to achieve this understanding by starting at the molecular level and systematizing and describing all types of cells in detail. This project is comparable to the Human Genome Program, which led to the decoding of the genome. It is supported by the Horizon Europe program. Establishing a relationship between the structure and function of different types of cells at the molecular level will lead to a better understanding of human physiology in normal and pathological conditions.

However, there are many aspects that need to be considered when creating an atlas for any cell type, ranging from structure and biochemistry, transcriptome and proteome, to a wide range of cellular activities such as secretion, metabolism and growth. The creation of a reference atlas of blood cells that are actively involved in the development of any infection greatly facilitate the diseases of various origins diagnosis and the search for effective therapy against these diseases.

The main approach to the modern view of the problem was the use of animal models for the study of diseases and testing of various medicinal and prophylactic (including vaccines) drugs for the treatment and prevention of these diseases. These models have provided a wealth of information (Xiong *et al*., 2013; Jucker, M., 2010), however it turns out that more than 70% of drugs have shown excellent results in animals are inefficient in human studies (Perel *et al*., 2007; Curry, 2003). In addition, unforeseen side effects are another danger of animal-tested drugs. For example, after a couple of decades of research and huge spendings on developing about ninety HIV drugs showed excellent results in animal models (including primates) turned out to be untenable in humans (Bailey, 2008). Similarly, in 2003 Elan Pharmaceutical prematurely terminated a phase two of clinical trial when their Alzheimer's disease vaccine caused brain swelling in humans, although no such side effects were ever found in mice or primates (Lemere, 2009).

To exclude these animal model limitations cellular mechanobiology became a new trend in cell research. This direction has become extremely popular in two breakthrough areas: (1) the development of the atomic force microscopy (AFM) method, which made it possible to mechanically influence cells and tissues directly with immediate monitoring of the results of such an impact; (2) the development of new medical areas that required a thorough study of the influence of not only the structure and biochemistry of the cell, but also its shape, size and mechanical properties for the successful creation of scaffolds (Perea-Gil *et al.*, 2018, Prüller et al., 2018, Calhoun *et al.*, 2019) and 3D conduits (Yurie *et al*.. 2020, Fukunishi *et al.*, 2016). However, while the mechanical properties of cells used in 3D printing of organs are actively studied, cause without knowledge of mechanics it is not possible to create a full-fledged construct, the complex study of many other cells taking part in various life-important cellular mechanisms of man's body is still not prepare.

So, to create a complete atlas of the cellular mechanisms, the study of the mechanical properties of many types of cells is the most important task. In this work we used new scanning ion-conductance microscopy (SICM) method for the morphology and rigidity study of living blood cells: lymphocytes, neutrophil granulocytes (NG) and red blood cells (RBC). Also, SICM method was compared to existing AFM and scanning electron microscopy (SEM).

Materials and Methods

Ethical approval

Permission for the study was obtained from the Commission on Bioethics of the Lobachevsky State University of Nizhny Novgorod (Protocol № 9 dated July 17, 2017).

Isolation of NGs and lymphocytes

Venous blood of healthy volunteer donors of both sexes aged 20–40 years was used. Blood sampling was carried out in the morning after the information form signing by donor. Blood was stabilized with sodium heparin.

To isolate NGs and lymphocytes, blood was fractionated on a double ficoll-trazograph gradient (ρ = 1.077 g/ml, ρ = 1.100 g/ml, 400 g, 40 min). Then both NGs and lymphocytes were washed with buffered saline containing 0.1137 M NaCl and 0.0027 M KCl, pH 7.20–7.40 (400 g, 3 min) and observed at a final concentration of 1 × 106 cells/ml in phosphate-buffered saline (PBS). Cells were taken into the experiment immediately after isolation. To prevent neutrophil priming, sample preparation was carried out in siliconized dishes (Pleskova *et al*., 2020).

Cell morphology studying by AFM and SEM

To prepare AFM samples of NGs and lymphocytes, 100 µl of cell suspension of NGs or lymphocytes were added to glass slides previously defatted with 1:1 96% ethanol / 99% diethyl ether. The samples were fixed after 15 minutes with an equal volume of 0.56 M glutaraldehyde, then after 20 minutes were washed four times by immersion in distilled water and dried in air at room temperature for 48 hours.

A similar technique was used for preparing SEM samples besides glass slides were changed by Au/GaAs-coated substrate, and the volume of introduced liquids was reduced by a factor of 10 (Pleskova *et al*., 2019).

Cell morphology was visualized by AFM using NTegra microscope (NT-MDT, Russia) with C-MSCT probes (Bruker, USA). All scanning were performed using resonance scanning mode.

For the SEM visualization JSM-IT300LV (JEOL, Japan), operating at an accelerating voltage of 20 kV and a probe current of 0.25 nA was used. The diameter of the electron probe was no more than 5 nm. The studies were carried out in high vacuum, the signal from the sample was simultaneously detected in two channels of low-energy secondary electron and reflected electron detector (Pleskova *et al*., 2020).

Cell morphology and rigidity studying by SICM

A Petri dish was loaded with 2 ml of a suspension of NGs or lymphocytes. The sample

was incubated (20 min, 37 °C) for spontaneous cell adhesion, then washed with PBS, and studied in the experiment alive in a similar medium (Pleskova *et al*., 2020).

For the studying of erythrocytes, 2 drops of heparinized blood were added to 2 ml of PBS in the Petri dish with an adhesive coating. The sample was incubated (120 min, 37 °C), then washed with PBS and scanned in a similar manner to NGs and lymphocytes.

The borosilicate glass capillaries with diameter 1.0 mm (outer) and 0.58 mm (inner) were used as blanks for nanopipettes for low-stress SICM. Nanopipettes with 25–40 nm tip inner radius were drawn on a P-2000 laser puller (Sutter Instruments, USA). The ion current was measured using a MultiClamp 700 B amplifier (Molecular Devices, Wokingham, United Kingdom). Scanning was performed using "hopping" mode at a fall rate of 120–150 nm/ms with bias potential of 200 mV. The stress that the nanopipette induces on a cell was calculated from the gap size in terms of the ion current decrease at two different set points 0.6% and 2%. ICAPPIC scanning software was used for imaging (Pleskova *et al*., 2020).

Statistical analysis

The Origin 8.0 Server Package was used for statistical analysis.

Results

Lymphocytes scanned by all three methods had similar morphology despite the fact of fixation by glutaraldehyde in AFM and SEM experiments. Usual diameter of cells varies between 6 and 15 μm, and lymphocytes could be divided into two groups: large granular lymphocytes (LGLs) with 9–15 μm diameter and small lymphocytes (6–9 μm). All types had folded surface structure due to high density of receptor-fields which clearly visualizes by any method.

There were two usual types of lymphocytes: typical half-sphere cells (Fig. 1) from subpopulation of αβT-lymphocytes and cells with different adhesion properties (Fig. 2). The latter were relatively less common. They contained a larger amount of cytoplasm (the nuclear-cytoplasmic ratio is lower), had a higher ability to adhere and, as a result, a larger adhesion area than the half-sphere cells. The edges of these lymphocytes were more uneven: cells took various shapes and forms on the surface (Fig. 2). These features are characteristic of cells belonging to the subpopulation of γδT-lymphocytes.

Cells with large adhesion area and diameter were more common in AFM experiments either due to adhesion on another surface (defatted glass instead of TC-treated polystyrene in SICM study) or different time of adhesion because of cells fixation. Same time height of these two types differed significantly: αβT-lymphocytes were usually higher than γδT (2,3 μm (Fig. 1B) and 1,4 μm (Fig. 2B) with mean max height in experiment $1{,}86 \pm 0{,}16$ μm, n = 16). So, despite the differences in morphology and diameter, the volume of cells most likely was close to similar (mean $56{,}83 \pm \pm 6{,}45$ μm^3, n = 16).

Also, some intermediate form of two above types was seen rarely (Fig. 3). Their adhesion area and size were closer to the half-spheres while the edges didn't have a clear outline like γδT-lymphocytes. This character of adhesion can be explained by high content of the cytoplasm in comparison with the cells forming a half-sphere.

When rigidity measurement of the cells was performed by SICM, another difference between αβT and γδT-lymphocytes was found. The latter had relatively higher Young's modulus in comparison with half-spheres. While Young's modulus of αβT-cells central part lied mostly in range of 20–50 Pa (Fig. 4A), same characteristic of forms with large adhesion zone was 140–400 Pa (Fig. 4B). Cytoplasm adhesion zone's figures are controversial because of high opportunity of substrate influence on rigidity measurement due to thickness of these zones (less than 0,1 μm) so they weren't counted. Although Young's modulus of substrate was much higher (about $8{,}6 \times 10^4$ Pa) than registered ones, usual median rigidity of any lymphocyte population in any conditions rarely exceeds 1500 Pa with median number about 85 Pa for naive cells (Bufi *et al.*, 2015). Returns to the rigidity dif-

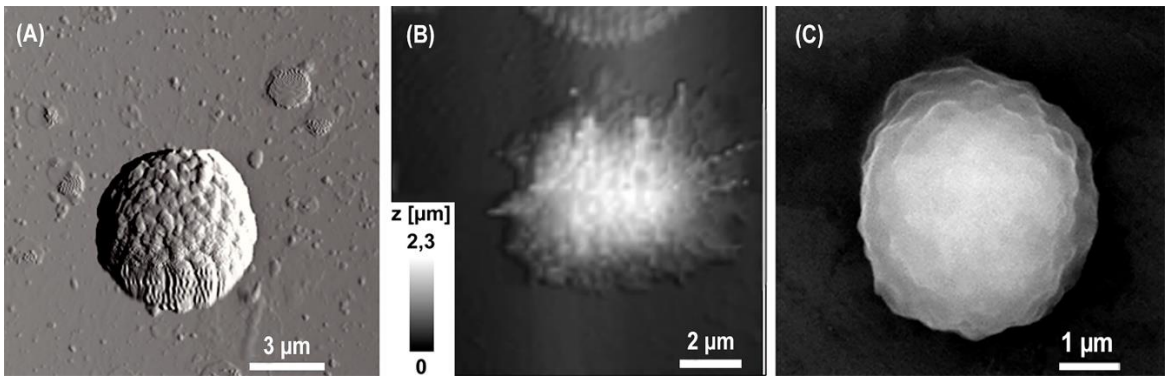

Fig. 1. Morphology of sphere-liked lymphocytes on the substrate: A, AFM, cell fixed with 2.5% glutaraldehyde; B, SICM, living cell in PBS; C, SEM, cell fixed with 2.5% glutaraldehyde

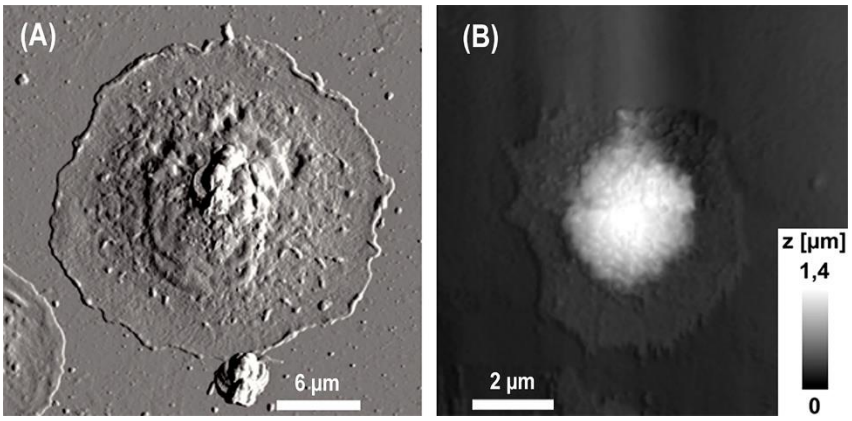

Fig. 2. Morphology of lymphocytes with large adhesion zone: A, AFM, cell fixed with 2.5% glutaraldehyde; B, SICM, living cell in PBS

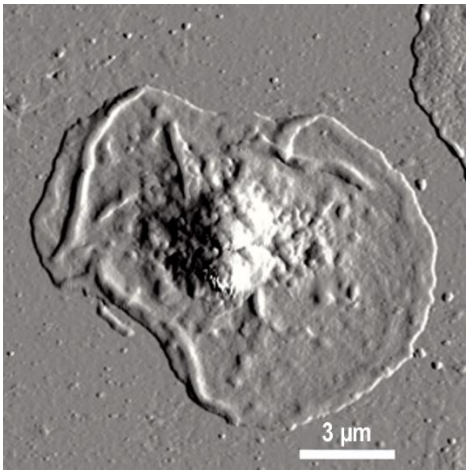

Fig. 3. Morphology of fixed with 2.5% glutaraldehyde lymphocyte with hybrid structure between half-sphere and cell with large adhesion zone scanned by AFM. The cell has form of dome with gentle slopes

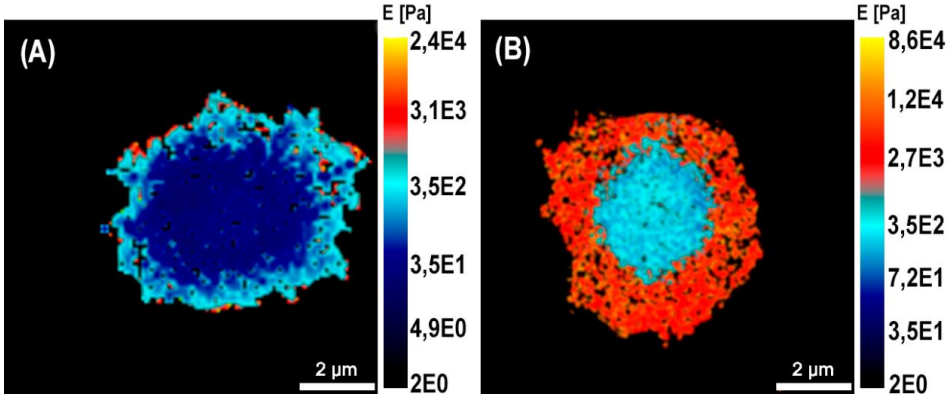

Fig. 4. Low-stress stiffness mapping of living lymphocytes, visualized by SICM: A, alf-sphere cell, contains soft central part and slightly stiffer frontier; B, stiffer cell with large adhesion zone

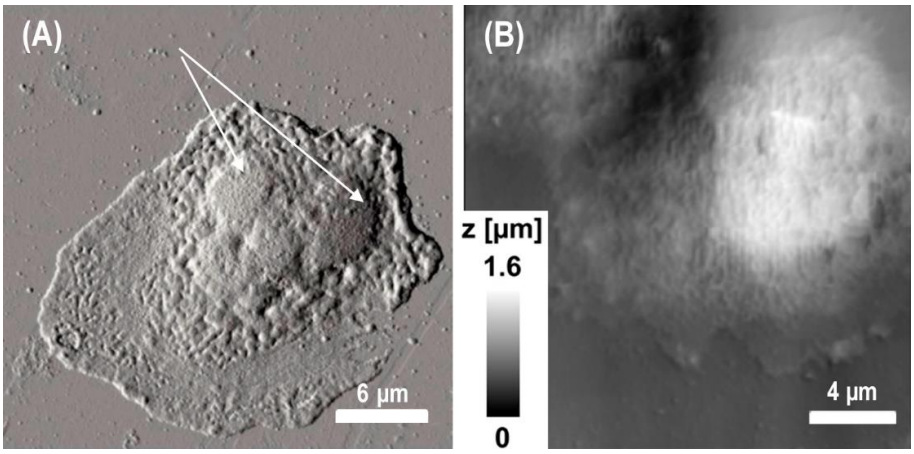

Fig. 5. Morphology of NGs on the substrate: A, AFM, cell fixed with 2.5% glutaralde-hyde, arrows indicate lobes of cell; B, SICM, living cell in PBS, adhesion zone is much lesser than AFM one

ference, while the mechanical properties of cells depend on their actomyosin cytoskeleton (Stricker *et al.*, 2010), mostly on F-actin (Bufi *et al.*, 2015) and vimentin intermediate fila-ments (IFs) (Brown *et al.*, 2001) containing, it could be a reason of obtained results. Most in-teresting that low stiffness off cell helps itself in tissue migration process, so there can be a correlation between adhesion properties and ri-gidity: high adhesion to surface prevents active motion and migration of stiff cell while soft cell should keep its mobility. So, typical half-sphere cell could be younger and more functional ac-tive than cell with higher adhesion, which is also proved by their numbers.

NGs scanned by AFM and SICM meanwhile didn't have many population differences but cell morphology varied between methods. In AFM scans it were rather flat cells with height no more than 2 µm (mean max height 1,84 ± ± 0,41 µm, n = 10) in lobes area and large visi-ble cytoplasm area (Fig. 5A). This form can be explained by dehydration while fixation (Pleskova *et al.*, 2005) and adherence proper-ties. These cells didn't have pseudopodia so most likely wasn't primed. Similar cells were seen in SICM scanning too (Fig. 5B) although cytoplasm adhesion zone was much smaller than AFM one. But there were mostly high cells (up to 4,5 µm) without large cytoplasm zone (Fig. 6, Fig. 7A).

This phenomenon can be explained by three major differences between studies: (1) in SICM experiments cells were alive; (2) prepared sur-

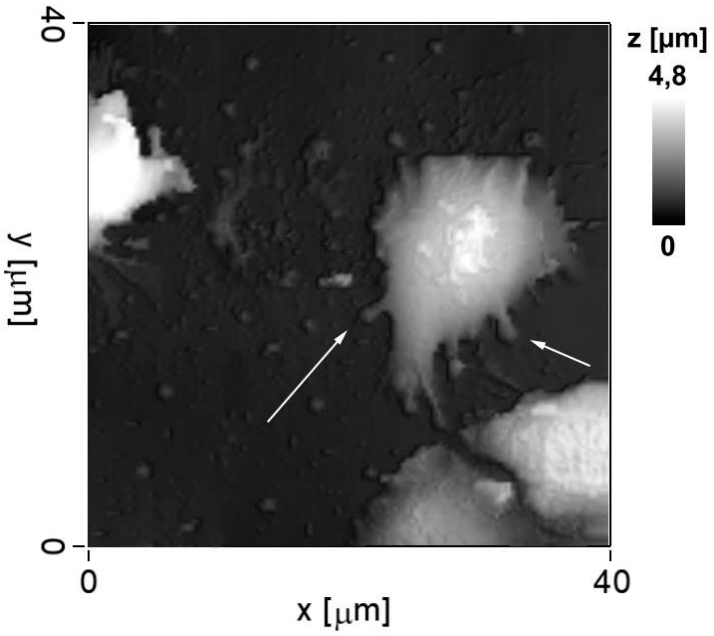

Fig. 6. The beginning of the NG's apoptosis (central cell). Apoptotic bodies are visualized around all cell surface (indicated by arrows)

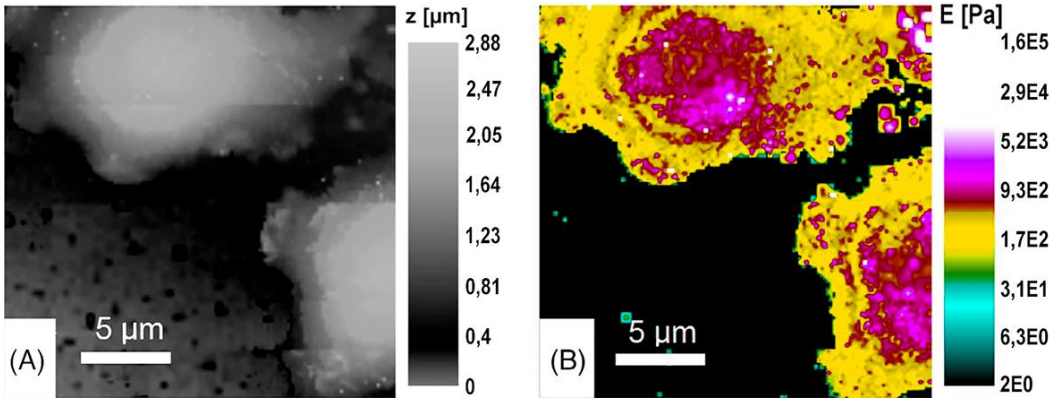

Fig. 7. Low-stress stiffness mapping of living NGs, visualized by SICM: A, topography of NG; B, rigidity map, central part is slightly stiffer than periphery

face on Petri dishes should be more suitable to cell adhesion than glass; (3) there was no organic agents which could change NG's membrane properties (Graham and Orenstein, 2007). As we described before, half-sphere lymphocytes can save its form and prevent adhesion to keeps mobility. So, most likely living neutrophils in SICM scanning were primed by PBS-medium, temperature conditions or combination of these factors. Due to that most of NGs minimized adherence with the surface. Indirect proof of this was obtained when apoptosis of the cell was visualized on the second hour of scanning (Fig. 6). While other cells on the scan were relatively flat with typical thin cytoplasm edges, dying cell were closer to half-sphere form with apoptotic bodies detached from the cell. Another explanation – while biomechanical properties of the cell changes drastically during adhesion (primarily due to cytoskeleton changes), which was already shown for NGs and ECs (Wang *et al.*, 2001), differences between cells with different adhesion nature don't look strange either for NGs or lymphocytes. It again reminds about the role of adhesion in any cell's interaction. Interesting fact, there were no

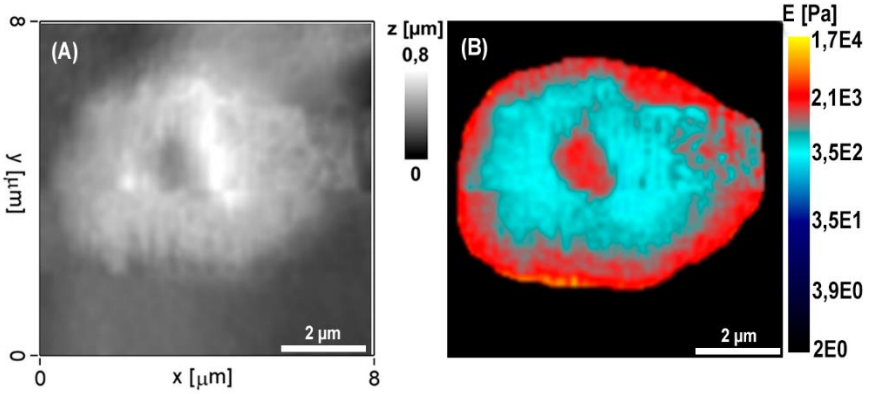

Fig. 8. Low-stress stiffness mapping of typical biconcave living RBC, visualized by SICM: A, topography of RBC; B, rigidity map, central part is under influence of substrate stiffness

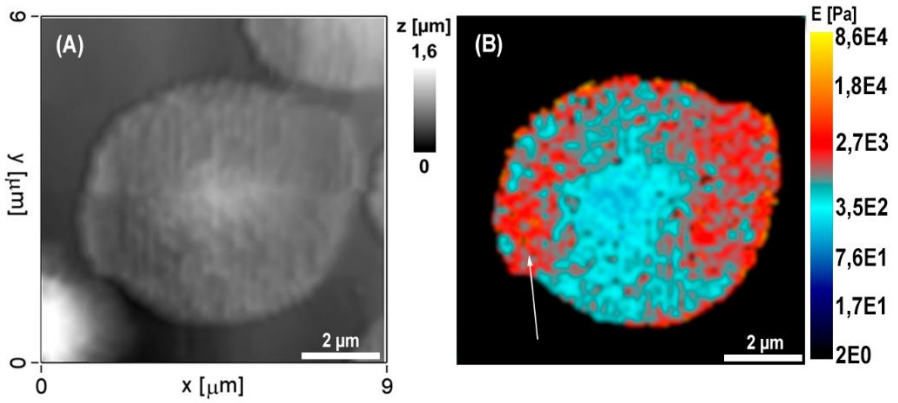

Fig. 9. Low-stress stiffness mapping of living spherocyte form, visualized by SICM: A, topography of RBC; B, rigidity map, the distribution of the rigidity is not even

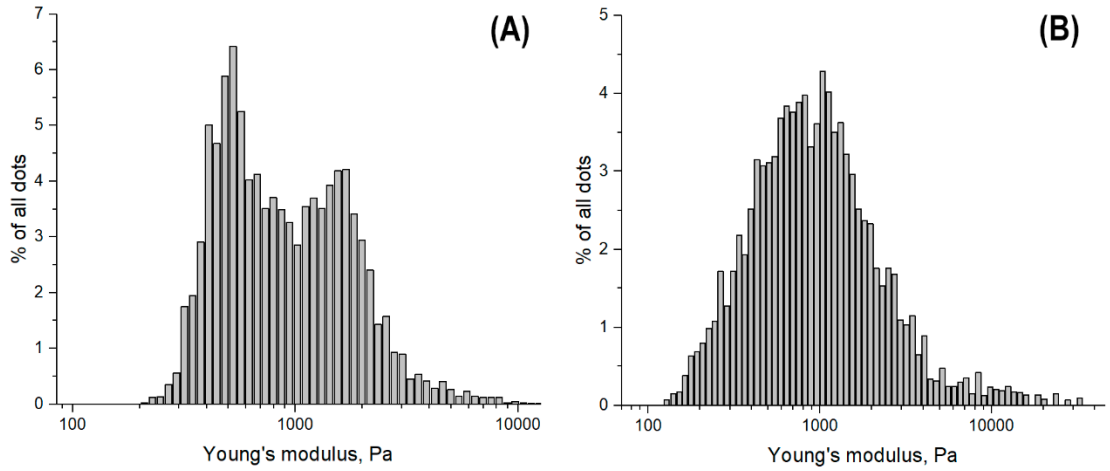

Fig. 10. Histograms of Young's modulus distribution on RBCs: A, biconcave RBC; B, spherocyte

remarkable changes between lymphocytes in AFM and SICM scans. The reason can be different sensitiveness to activation of lymphocytes and NGs or age of cells.

NGs rigidity (Fig. 7) obtained in SICM scanning was relatively higher than lymphocytes with Young's modulus 765 ± 149 Pa in central part and 434 ± 150 Pa on periphery. This is in

line with previous experiments where T-lymphocytes were found to be the softest leucocytes (Bufi *et al.*, 2015).

Erythrocytes in experiment couldn't be fixed successfully to provide AFM scanning so only SICM images were obtained. Two types of RBC were found: classical round and biconcave cells (Fig. 8A) and spherocytes with some elevation in the center which could be cluster of hemoglobin (Fig. 9A). Latter type was slightly more common.

Rigidity analysis (Fig. 8B and Fig. 9B) also showed differences between cell types. While typical RBC was mostly soft (Young's modulus is around 350 Pa on most surface) and only stiff on frontier parts and center, where cell is thin and substrate influence is significant, spherocytes didn't have even distribution of soft and stiff sites.

The histogram of rigidity was produced (Fig. 10) and mean Young's modulus was calculated for both cells: 1144 Pa for typical RBC and 1516 Pa for spherocyte.

As can be seen on histograms, Young's modulus distribution of spherocyte is closer to normal although some especially high numbers are most likely substrate-influenced stiffness, scanned at the edge of the cell. Despite this there are also stiff areas (E > 1500 Pa) within cell (indicated by arrow in Fig. 9B). Because spherocytes are abnormal form of RBC losing part of its membrane (Huisjes *et al.*, 2020), it can be same explanations as for leucocytes are on the way: either experimental medium and conditions providing stress to cells or there are aged cells changed their properties.

Discussion

As long mechanical properties of the cell affect wide circle of its interactions in whole organism, there is still a request to determine the rigidity of living cells in a closer to native environment. Mechanical properties of RBCs take key part in development of many blood diseases affecting adhesion of leukocytes (Gutierrez *et al.*, 2018), rigidity of neutrophils nucleus is one of the main indexes of their migration ability (Manley et al., 2018) and deforming of lymphocytes accompanies chronic lymphocytic leukemia (Zheng *et al.*, 2015). Nowadays it's already possible to determine these changes in rigidity for average blood populations quite accurate (Zheng *et al.*, 2015; Gutierrez *et al.*, 2021). However, it can be used as good diagnostics but can't help to investigate the reasons of these changes or potential influence on specific cells interactions in particular mechanisms.

Same time existing microscopy methods allow researching in areas of individual cell rigidity, morphology and structures localization and also modulating required research conditions in vitro. The great advantage of scanning methods over optical ones is a higher image resolution and the possibility of interacting with an object. Although there are some limitations: SEM provides only one-time high-resolution morphology scan of fixed cell and AFM which is able to research morphology and rigidity in dynamic additionally deform the object of study, which affects the reproducibility of the results obtained in physiological conditions. The SICM method, on the other hand, should facilitate the process of studying cell mechanics in the dynamics of cell vital activity with minimal physical impact on the system and morphology scanning same time.

Compare cell morphology visualized by AFM, SEM and SICM in this study we obtained rather similar results with different cell types, although complete reproducibility was not ensured. The main reason of this was the complexity of the study of living cells: while fixed preparations do not change over time and can be studied by any methods, the study by the SICM should additionally include the stages of selecting optimal conditions for both cell adhesion and cell survival. For example, when we tried to isolate the erythrocyte fraction by centrifugation, and then examine it by analogy with leukocytes, we observed flat "shadows" of dead cells during scanning, and the results did not reflect reality. Thus, the study of living cells is much more labor-intensive and time-consuming, and the influence of the study conditions on the result is much more significant.

On the other hand, the SICM results reflect the real state of affairs much more accurately in comparison with the study of fixed samples and

provide more data. Using SICM for individual blood cells rigidity measurement and morphology imaging at one scan we found differences in mechanical properties of different cells population with possibility of object identification by structure. It allows to exclude additional fluorescent imaging of scanning zone using in AFM rigidity measurement (Thomas *et al*., 2013; Hayashi & Iwata, 2015) and increase numbers of samples at one cell. Comparing rigidity and morphology maps it's possible to understand the nature of numbers changing as it seen at neutrophils lobe part. Same was seen for erythrocyte's caves and γδt-lymphocyte where higher rigidity of edges was the consequence of thinner cytoplasm at adhesive zone. Influence of the surface on thin objects although can be corrected using additional calculations (Rheinlaender & Schäffer, 2020). Thus, by SCIM method we get more accurate rigidity data with clear structures visualization.

While ability of imaging live changes of living cell was shown using NG apoptosis as example, rigidity changes in simulated systems also can be measured. As has been shown before, Staphylococcus aureus can decrease neutrophil's membrane stiffness (Pleskova et al., 2020). So, recreating physiological and pathological processes it's possible to get morphological and mechanical data of this interactions in dynamic which is impossible by SEM and difficult to perform by AFM due to cell's deforming and additional imaging needed. Influence of experiment conditions on cells clearly seen on the example of visualization of spherocytes. Obtained differences in Young's modulus between normal RBC and spherocytes can be applied at blood diseases researches as well similar figures for lymphocytes populations. And controlling conditions more accurate, any cell form and functional state can be obtained and studied. It also become possible by minimization the stress exerted on the cell. In our study we can scan one cell for hours without its deformation and breakaway from the substrate.

Since the SICM microscope is capable of examining cell morphology and rigidity at the same time with minimal impact on system, it is one of the most versatile tools for the comprehensive study of biological objects. Recreating multicomponent interactions between cells with parallel high-resolution scanning in the ways of morphology and rigidity, brand new information of organism systems working will be achieved. Making complex three-dimensional structures as tissue models for all-round study at ones will allow to modulate cellular mechanisms in vitro with the precision if it was in situ, by minimization either time of scanning or physical intervention. Therefore, by developing the methodology, it will be possible to complete studies on the creation of an atlas of cellular mechanisms using the SICM method, which will serve as the basis for creating real clinical methods.

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