

Intravital Imaging of Blood Cells Under Stress to Assess the Organism Condition

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Abstract

Biological specimens are the most difficult to study as almost exclusively light elements are considered, a little visibility yielded and structures are sparsely distinguishable. In addition, cell structures are easily damaged on any stage of microscopic exploration. The optimal solution is to study their phase changes using laser interference microscopy. The method is based on the analysis of refractive index of intracellular medium of cells with subsequent computer analysis and processing of phase images followed by three-dimensional reconstruction of the object. The obtained images are digitally recorded, and any point of view can be introduced for viewing. This work uses laser interference microscopy followed by 3D reconstruction of cell images to study blood cells: neutrophils and erythrocytes by 3D reconstruction of neutrophils have demonstrated changes of their functional activity under technological stress, while 3D reconstruction of erythrocytes revealed a violation of the normal discoidal cell shape. Interpretation of the obtained data indicates a restructuring of the cellular component of nonspecific resistance and deterioration of the oxygen transport function of erythrocytes under technological stress. Visualization of interference patterns of blood cells through 3D image processing allows obtaining a vital analysis of cells, the technique that is not available for other methods of microscopy, in particular light microscopy, which is traditionally used as a prognostic method of the state of the body. This direction represents the most important condition for further development of works in the field of cell diagnostics. The obtained results prove the applicability of 3D reconstruction of cell images for bioscanning of technological stress.

Keywords: laser interference microscopy, 3D image, blood cells, technological stress.

1. Introduction

The modern level of development of optical electronics and computer technology allows creating and implementing the new tools for study of biological objects. The method of interference microscopy is one of the most promising. It allows noninvasive studies of cell morphology and dynamics with ultrahigh spatial resolution [1, 2]. Unlike many other microscopy methods, the method of interference microscopy provides possibility of a native object study without preliminary fixation and staining, [3]. The recorded value of the optical difference in

the interferometer allows obtaining quantitative information on the volume distribution of the refractive index of the object and its morphology which is an important feature of interference methods [4]. Estimation of the phase height distribution reconstructs a true three-dimensional surface of the object [5]. In our opinion, a study of optical density and reproduction of cell geometry provides additional information about blood cells, which are traditionally studied by methods of light microscopy.

The aim of the work is to discuss and demonstrate the new methodological approaches to the analysis of neutrophils and erythrocytes state in norm and under stress using interference microscopy.

2. Laser Interference Microscopy

The method of interference microscopy is based on the principle of measuring phases which are normalized quantities and determine the optical parameters of the object. The method of phase steps at control of polarization registers phase images of object [3]. The phase difference of the object is calculated by the phase-step method and is schematically represented as follows:

$$\begin{cases} I_0(x, y) = A(x, y) + B(x, y) \cos(\Phi(x, y)) \\ I_1(x, y) = A(x, y) + B(x, y) \cos(\Phi(x, y + kd)) \\ I_2(x, y) = A(x, y) + B(x, y) \cos(\Phi(x, y + 2kd)) \\ I_3(x, y, t) = A(x, y) + B(x, y) \cos(\Phi(x, y + 3kd(t))) \end{cases}$$

Where $I_{0-3}(x, y)$ – the distribution of radiation intensity in the field of view of the photodetector, k – the wave number, d – the shift value of the reference mirror.

The required value of the phase difference is determined by taking into account the intensity distribution in the field of view of the photodetector:

$$\Phi(x, y) = \arctg \left[\frac{\sqrt{[(I_1 - I_2) + (I_0 - I_3(t_0))] \cdot [3(I_1 - I_2) - (I_0 - I_3(t_0))]} }{I_1 + I_2 - I_0 - I_3(t_0)} \right]$$

Where $I_3(t_0)$ – the instantaneous value of intensity determined by the exposure time of the photodetector.

Interference images of 1280x1024 pixels include 3 frames per second and 128x128 pixels up to 250 frames per second. This variant of phase step counting in phase images achieves spatial superresolution [6] which gives additional information compared to classical images obtained by light microscopy. The advantages of measuring phase steps using interference microscopy can be clearly demonstrated by Figure 1.

Figure 1 shows the interpretation of the phase step measurement, taking into account the individual pixels.

The solid line in Figure 1 (a) shows the interferogram obtained for the pixel highlighted in red with coordinates (1,1). The interferogram in this case represents the time dependence of the radiation intensity value in the field of view of one pixel of the photodetector and reflects the sequence of single-pixel fragments of thousands of 1024x1024 pixel interferograms. Then, as highlighted with a dotted line n in Figure 1(a) we obtain the dependence of the radiation intensity for the adjacent (blue) pixel with coordinates (1, 2) and calculate the phase value for this area using the relation $\delta\varphi = \varphi(1, 2) - \varphi(1, 1)$. We obtain a complete phase image of the object by repeating this sequence for all pixels.

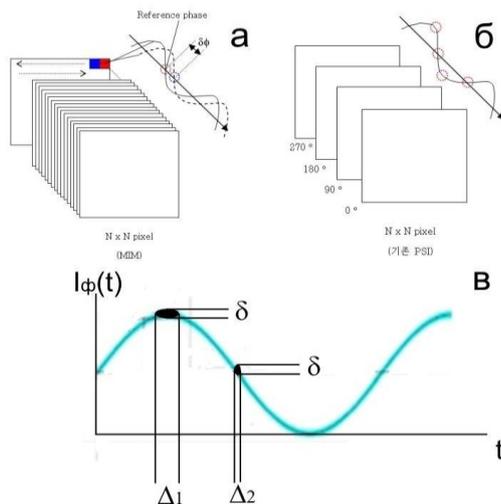


Figure 1 – Measuring phase steps using interference microscopy

Thus high spatial resolution of the microscope is determined by decrease of phase steps (Figure 1a, 1b), whereas with increase of phase steps the confidence interval from Δ_1 to Δ_2 of phase value in different points of sinusoid is calculated rather roughly (Figure 1c). As a result of the construction of interferograms the resolution of interference microscopy reaches 0.1 nm in the vertical and 15 - 100 nm in the plane of the object.

The optical scheme of the laser channel is a modification of the Mach-Zehnder interferometer based on independent polarization control in the object and reference arms of the interferometer.

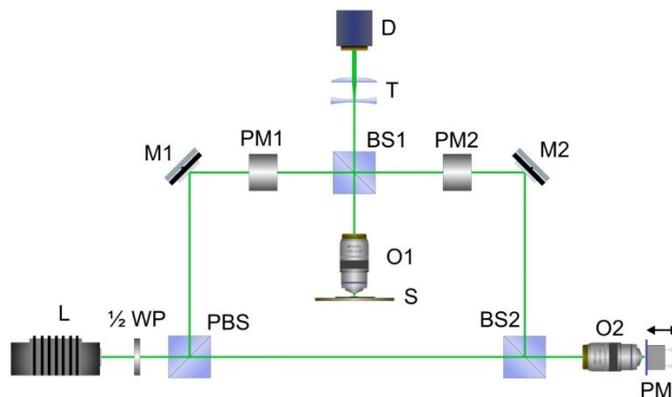


Figure 2 – Optical scheme of the laser channel of the interference microscope.

As shown in Figure 2 the laser beam from the laser (L) is divided into two at the polarizing beam splitter PBS. One beam (objective) is focused by the objective O1 on the object which is placed on the stage S and reflecting from the mirror substrate through the light splitter BS1 and the telescopic system T gets to the photodetector D - camera Silicon Imaging model SI - 128of. Another beam (reference) does not pass through the object and is focused by the lens O2 and reflected from the mirror of the piezoelectric transducer (PM) and the same falls into the photodetector, where the interference of beams occurs and a phase image is formed. At construction of the phase image the signal is normalized by wavelength and the optical difference of a course of beams which characterizes value of phase height of object (F) in the given point is determined:

$$\Phi = \frac{(\varphi_0 - \varphi_{obj}) \lambda}{2\pi} - \Phi_0$$

Where φ_0 – the initial phase, φ_{obj} – the phase shift by the object, λ – the wavelength of radiation, Φ_0 – the constant shift which is determined by the choice of the initial phase reference point.

Registration of phase height (Φ) in all points of the object forms 3D images. Processing of the received data is carried out with LabView software. Dynamic phase images are obtained and processed in the Airyscan V.6 software. The user interface of Airyscan V.6 consists of two parts: the panel of parameters determining correct operation of the program (the left part) and the main elements of the Tab Control (the right part) which display phase images (Figure 3).

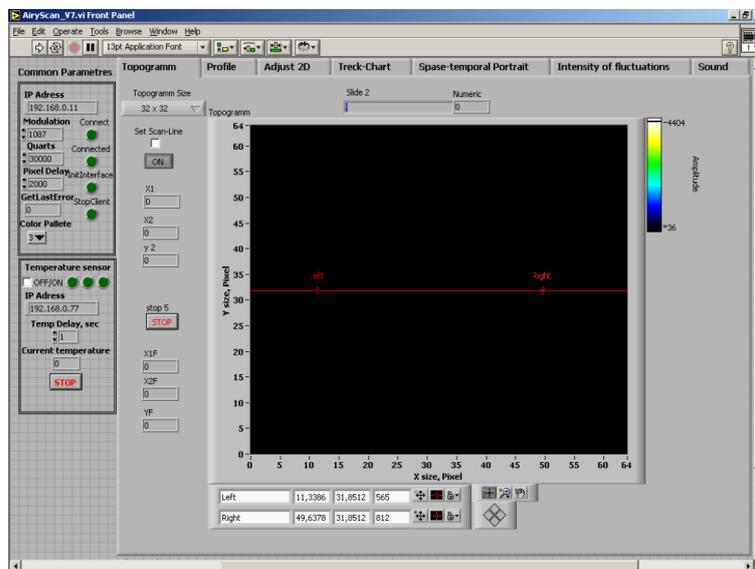


Figure 3 – Airyscan program interface

Topo3D software is used to playback 3D images. The program sequentially reproduces a series of static phase images and displays a 3D image with a possible cross section of phase images.

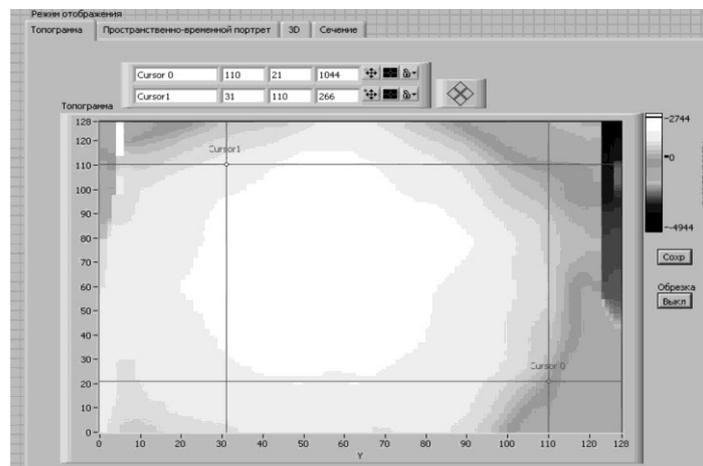


Figure 4 – Toro3D program interface

Playback modes include a phase image playback page with the ability to crop images using two cursors that define the clipping area, allowing you to maintain the dimensionality of the clipped images.

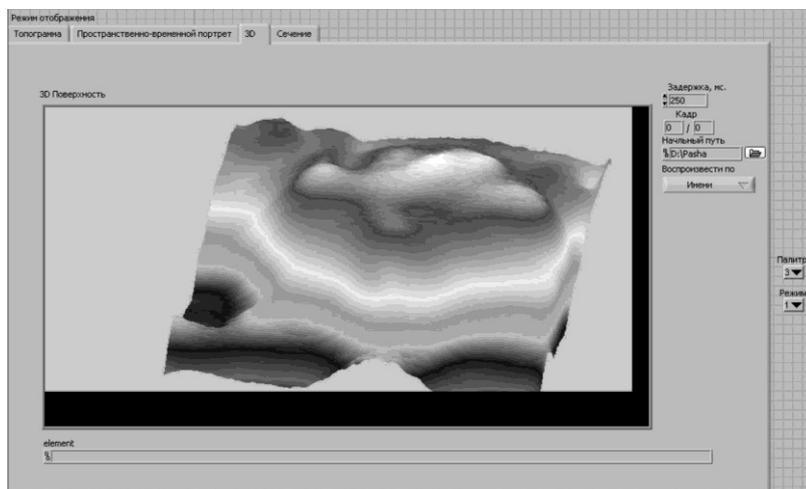


Figure 5 – 3D phase images mode window

3. Object of the study

Analysis of neutrophils and erythrocytes under technological stress was carried out by interference microscopy with further 3D reconstruction according to the purpose of the study. 30 clinically healthy high-yielding black and gray cows were the object of study. The choice of the research object was dictated by the strategic task of modern animal breeding associated with the reduction of losses caused by technological stress. Technological stress causes higher susceptibility of animals to pathogens and reduction of animal productivity. A combination of factors: regrouping, change of service personnel and veterinary and sanitary manipulations were technological stress for animals. During the study, blood sampling in all experimental animals was carried out before the technological stress and after the action of the combination of factors causing technological stress.

Neutrophils were isolated by the standard method on a double density gradient (1.077 and 1.093). The cells were washed by centrifugation in Hanks's solution. The supernatant was poured off, and neutrophils were diluted with Hanks' solution to a concentration of 2×10^6 . Erythrocytes were examined in whole blood.

The morphofunctional state of cells was assessed by computer phase morphometry based on a domestic computer laser phase-interference microscope MIM-340 (Yekaterinburg, Russia) [7].

Leukocytes and erythrocytes were additionally examined in smears stained by Romanovsky-Giemsa. The morphology of leukocytes and erythrocytes was examined on a light microscope Micromed C-11 (Russia) with MECOS-C software.

4. Visualization of blood cells by interference microscopy

Interference microscopy of neutrophils in intact animals not subjected to technological stress allows us to identify the two most distinct cell populations (Figure 6).

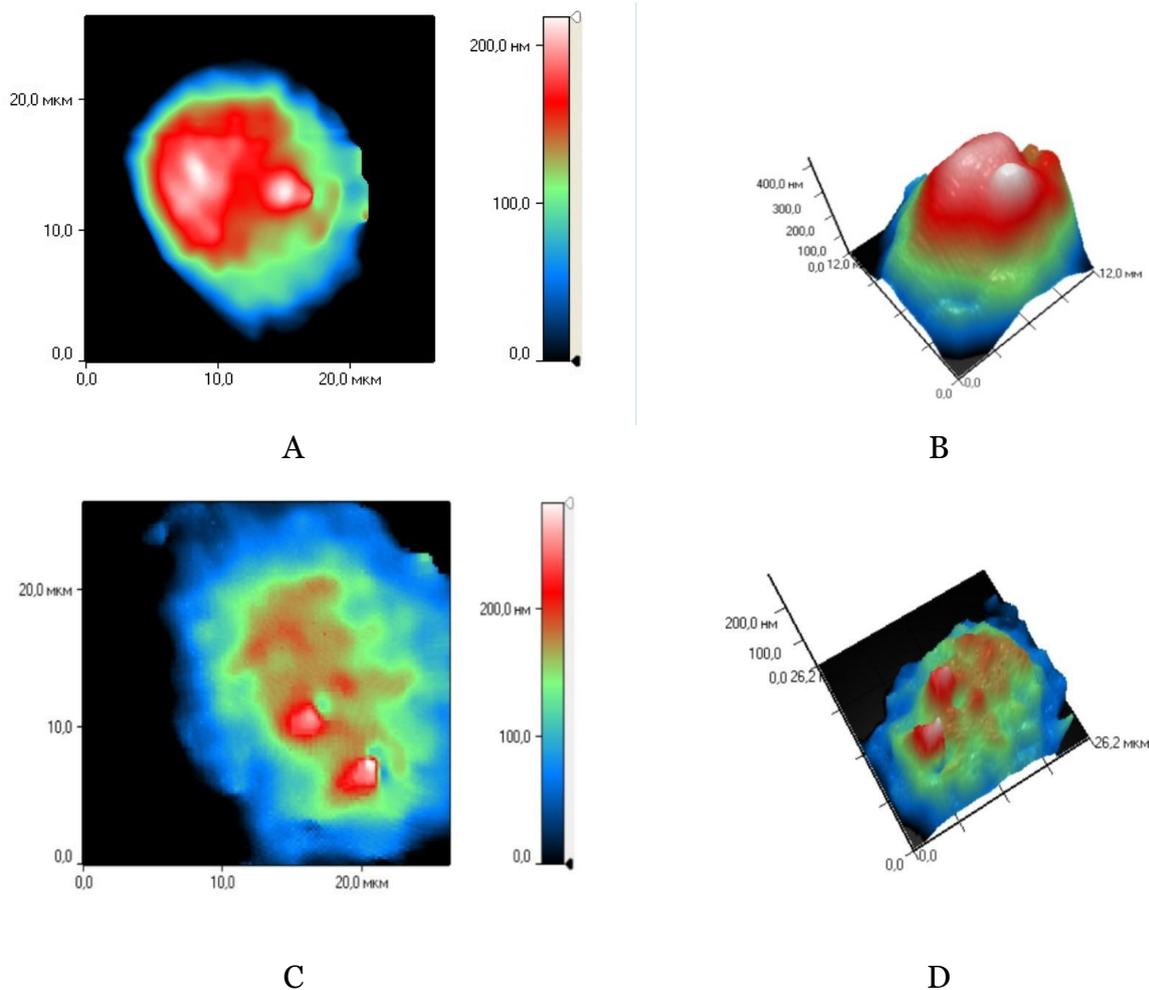


Figure 6 – Different morphological types of neutrophils. Phase image (topogram) (A) and 3D reconstruction of cell image (B) of morphological type I. Phase image (topogram) (C) and 3D reconstruction of cell image (D) of morphological type II.

The first population of neutrophils is represented by the 1st morphological cell type [8]: 3D reconstruction of the cell image clearly demonstrates that these are round-shaped cells with a clearly distinguished nucleus and uniform distribution of intracellular contents (Figure 6 A, B). 3D reconstruction of the second cell population displays an uneven surface with many convexities and depressions. This is due to the spatial redistribution of the cytoplasm, intracellular organelles and nucleus. This is morphological type II (Figure 6 C, D).

Counting the I and II morphological cell types showed that under technological stress, the I morphological type decreased by 3 times and the II morphological type of neutrophils increased by 2 times.

Analysis of erythrocyte morphology by interference laser microscopy revealed that before technological stress, erythrocytes were characterized by a normal biconvex cell shape (Figure 7 A, B). Technological stress caused a change in the shape of the erythrocytes. 3D reconstruction of the cell images revealed spikes, ridges, and protrusions on the cell surface (Figure 7 C, D).

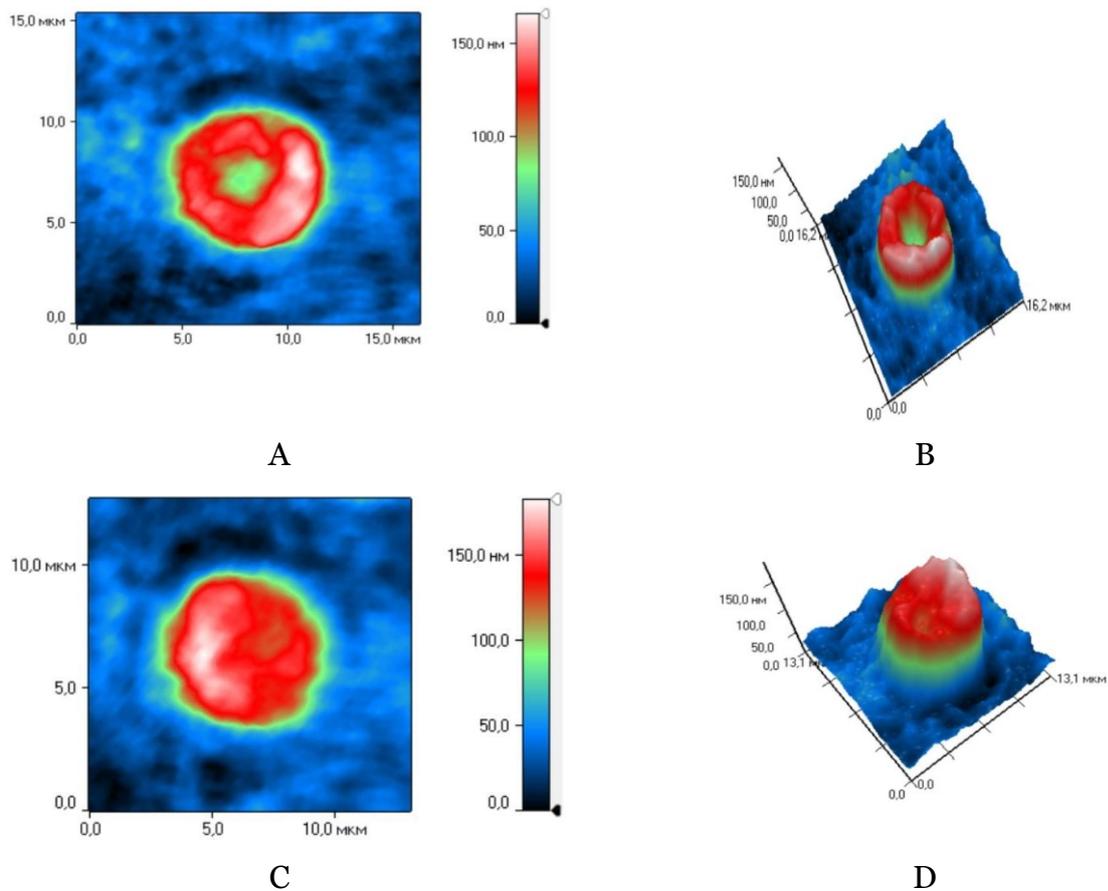


Figure 7 – Different morphological forms of erythrocytes. Phase image (topogram) (A) and 3D reconstruction of cell images (B) of erythrocytes before technological stress. Phase image (topogram) (C) and 3D reconstruction of cellular images (D) of erythrocytes after stress.

It should be noted that unlike 3D reconstruction of cell images, the use of light microscopy does not provide an opportunity to determine the change in the shape of neutrophils and erythrocytes (Figure 8). All cells have a rounded shape.

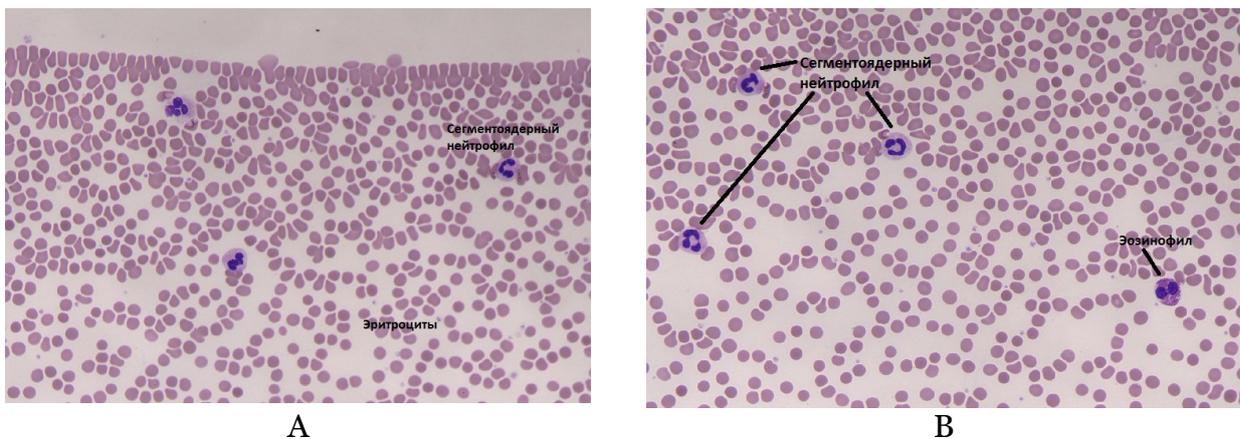


Figure 8 – Cell morphology under the light microscope.
A – before stress, B – after stress

Based on the results obtained, we can conclude that the reconstruction of 3D images allows cell morphology analysis and significantly complements representation obtained with the light microscopy data. In addition, imaging is the basis for the analysis of the mechanism of stress exposure.

Neutrophils are the first protective cell barrier against infections, the most numerous phagocytes in the human body which are quickly mobilized from the bloodstream to the infectious focus or site of injury [9]. Deformed cell contours indicate a certain degree of cell activity [10]. An increase in morphologically altered erythrocytes is of key importance. Changes in erythrocyte morphology are reflected in the oxygen-transporting function of blood [11] which leads to impaired blood supply to tissues [12].

The study reveals the possibility of using laser interference microscopy to assess cell morphology within nanometer scale range. Analysis of the results reveals changes in the cellular link of nonspecific resistance and deterioration of the oxygen transport function of erythrocytes under technological stress.

5. Conclusion

3D imaging using interference microscopy allows us to quickly identify and obtain the most informative data on the geometric parameters of cells. Blood cells in this method of analysis are not subjected to additional sample preparation before the study (fixation, staining, treatment with contrasting agents), which minimizes the possibility of artifacts. In addition, 3D computer images are lifetime visualization of cellular processes and can be obtained in a very short time. The mentioned facts represent the most important condition for further development of works in the field of cell diagnostics. Using computer methods of cytodiagnostics in the work revealed new aspects of functional morphology of neutrophils and erythrocytes under stress. The obtained data are of fundamental importance for the development of new methods for rapid diagnosis of the adaptation reserve at the cellular level.

Acknowledgements

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References

- [1] Lue N., Popescu G., Ikeda T., Dasari R., Badizadegan K., Feld M. Live cell refractometry using microfluidic devices // *Opt. Lett.*, 2006, 31(18), P. 2759-2761 doi: 10.1364/ol.31.002759
- [2] Park Y., Popescu G., Badizadegan K., Dasari R., Feld M. Diffraction phase and fluorescence microscopy // *Opt. Expr.* 2006, 14(18), P.8263-8268. doi: 10.1364/oe.14.008263
- [3] Vishnyakov G.N., Levin G.G, Minaev V.L., Pickalov V.V., Likhachev A.V. Tomographic interference microscopy of living cells // *Microscopy and Analysis*, 2004, № 87, P. 19-23.
- [4] Zagubizhenko M. V., Yusipovich A. I., Pirutin S. K., Minaev V. L., Kudryashov Yu. B. Use of laser interference microscopy to study the state of mouse peritoneal macrophages irradiated with ultraviolet light // *Radiation Biology. Radioecology*, 2011, V. 51. № 6, P. 715. [in Russian].
- [5] Deryugina A.V., Ivashchenko M.N., Ignatiev P.S., Lodyanoy M.S., Samodelkin A.G. Alterations in the phase portrait and electrophoretic mobility of erythrocytes in various diseases // *Modern Technologies in Medicine*, 2019, T. 11. № 2, C. 63-68. doi: 10.17691/stm2019.11.2.09.

[6] Tychinsky V.P., Kretushev A.V., Vyshenskaya T. V., Tikhonov A.N. Coherent phase microscopy in cell biology: visualization of metabolic states // *Biochim. Biophys. Acta*, 2005, № 1708, P. 362-366. doi: 10.1016/j.bbabi.2005.04.002

[7] Deryugina A.V., Ivashchenko M.N., Ignatyev P.S., Belov A.A., Petrov V.A. Diagnostics possibilities of erythrocytes analysis by the method of laser interference microscopy // *Klinicheskaya Laboratornaya Diagnostika*, 2021, V. 66, № 1. P. 22-25. doi: 10.18821/0869-2084-2021-66-1-22-25

[8] Vasilenko I.A., Nikitin A.A., Malichenko N.V., Ivanjuta I.A., Metelin V.B., Agadzhanyan B.J. Cytometry of neutrophils for evaluation of complex treatment efficiency in patients with mandibular osteomyelitis // *Clinical Medicine Almanac*, 2008, №.18, P. 63-68. [in Russian].

[9] Mantovani A., Cassatella M.A., Costantini C., Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity // *Nat. Rev. Immunol*, 2011, № 11, P. 519-531. doi: 10.1038/nri3024

[10] Tuzlukov I.I. Features of the morphology of neutrophilic leukocytes in the postpartum period // *Pavlov Russian Medical and Biological Bulletin*, 2004, № 3-4, P. 113-117. [in Russian].

[11] Kobayashi S.D., Malachowa N., DeLeo F.R. Influence of microbes on neutrophil life and death // *Front. Cell. Infect. Microbiol*, 2017, № 7, P. 159. doi: 10.3389/fcimb.2017.00159

[12] Emelianov V.V., Leontev D.V., Ishchenko A.V., Bulavintseva T.S., Savateeva E.A., Danilova I.G.. Atomic force microscopy imaging of red blood cells and metabolic disorders in experimental diabetes mellitus and its correction with lipoic acid // *Biophysics*, 2016, V. 61. № 5, P. 922-926. [in Russian].