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The Development of Segmentation Algorithms in Holographic Microscopy and Tomography for Determination of Morphological Parameters of Cells

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Abstract—Algorithms of cell segmentation on two-dimensional phase images and three-dimensional distributions of a refractive index obtained by means of digital holographic microscopy and tomography are developed. The proposed algorithms are optimized for determining cell morphology characteristics including the cell volume, projection area, and surface area. A comparative analysis of the error of cell volume determination by holographic methods using the proposed cell segmentation algorithms and the standard method of confocal fluorescence microscopy has been performed.

Keywords: holographic microscopy, holographic tomography, morphological characteristics of cells, cell segmentation.

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At present, investigations of many intracellular processes are carried out by means of fluorescence microscopy techniques, which are capable of detecting and determining the regions of localization of many organic compounds using immunofluorescence analysis [1]. A large number of fluorophore markers specific to various proteins involved in important biological processes, along with the rapid development of technologies improving spatial resolution of fluorescence microscopy [2], makes these methods of living cell analysis irreplaceable tools in the investigation of many biological processes. At the same time, approaches based on the analysis of morphological characteristics of cells still remain topical and informative in both clinical practice [3] and fundamental biological investigations [4].

The commonly accepted and most reliable method of determining the volume, membrane surface area, sphericity index and some other morphological parameters of cells is offered by the confocal fluorescence microscopy. Measurements are usually performed using a fluorescent dye (acridine orange) that is capable of binding to DNA and RNA in living cells and exhibits intense fluorescence under irradiation in a spectral range of 460–500 nm. Accumulation of this dye in juxtamembrane area and inside the cell allows construction of a three-dimensional (3D) intracellular distribution of dye molecules and, determination of the morphological parameters of cells [5]. However, the need to introduce fluorophores into cells and their exposure to intense laser radiation used in the confocal fluorescence microscopy for fluorophore excitation do not allow long-term monitoring of living cells [6].

In the present work, we have developed algorithms of cell segmentation on two-dimensional phase images and 3D distributions of refractive index obtained, respectively, by digital holographic microscopy and tomography. The proposed algorithms are optimized for determining the morphological characteristics of cells.

Digital holographic microscopy, allows one to obtain phase images of individual cells and to perform their noninvasive monitoring for a prolonged period of time. The morphological characteristics of cells can be determined from their phase images if assuming that the average refractive index inside the cell is constant. For attached cells, it can be suggested that the lower part of the cell membrane surface coincides with the $Z = 0$ plane and the upper part can be described by the following equation:

$$Z(x, y) = \frac{\lambda}{2\pi}(n_{media} - n_{cell})\varphi(x, y), \qquad (1)$$

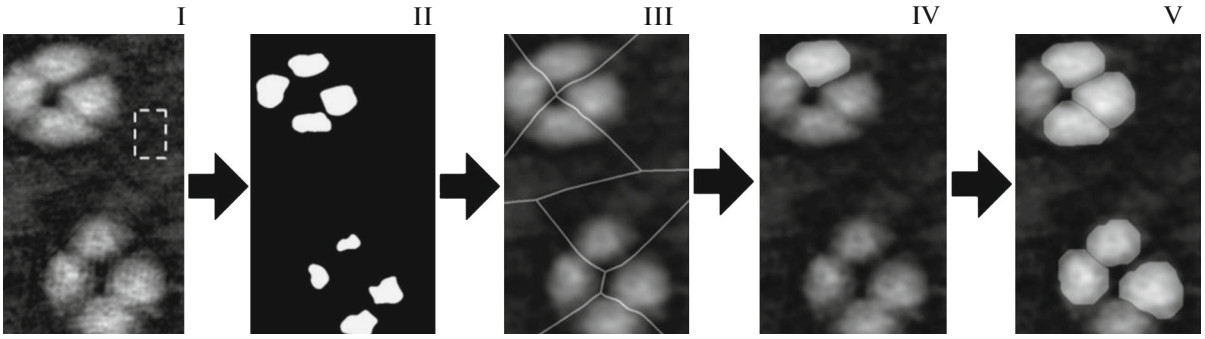

Fig. 1. Scheme of the procedure of cell segmentation on 2D phase images.

where n_{cell} is the cell refractive index, which is assumed to be constant (for fixed cells, $n_{cell} = 1.478$ [7]), and n_{media} is the refractive index of the medium. Using microscopic data on the distribution of phase shift $\varphi(x, y)$ introduced by cells into the wave front, one can determine the main morphological characteristics of a cell, including its volume,

$$V_{cell} = \int_{cell} \frac{\lambda}{2\pi}(n_{media} - n_{cell})\varphi(x, y)dxdy \qquad (2)$$

and the total area of the membrane surface,

$$S_{membrane} = \int_{cell} \sqrt{1 + \frac{\partial Z(x,y)^2}{\partial x} + \frac{\partial Z(x,y)^2}{\partial y}}\, dxdy + \int_{cell} dxdy. \qquad (3)$$

The first term in Eq. (3) represents the area of the upper part of the membrane surface, while the second term determines the area of the lower (attached) part.

In order to perform automated segmentation of cells on their phase images obtained in a holographic microscope, we have developed a special procedure comprising five steps (Fig. 1). First (step I), sequential scanning of the entire phase image is used to find an area with minimal gradient of the phase shift, which corresponds to the area containing no cells. Then (step II), the positions of local maxima are determined and surrounding masks are formed to indicate approximate positions of cells and (step III) transformations described in [8] are used to draw boundary lines separating adjacent cells. Finally, exact boundaries of cells are determined (step IV) by expanding each local maximum (with allowance for the separating lines) until the average phase shift from added area equals to to that in the normalization area determined at the first step and (step V) cells in contact with the boundary of the phase image are removed.

In the case of holographic tomography based on the detection and reconstruction of a set of several dozen digital holograms recorded at different angles of incidence of the probe wave onto the object, a data set on 3D distribution of refractive index in the sample can be obtained [9, 10]. An important difference of digital holographic tomography from traditional confocal fluorescence microscopy is that the former method requires measuring several dozens of digital holograms, while the total scanning of a cell culture sample by fluorescence microscopy requires a large number of separate measurements. As a result, the time needed to obtain a necessary set of data in the holographic tomography does not exceed several seconds whereas the measurement of 3D distribution of fluorophore in the sample takes about several minutes. However, use of the holographic tomography implies numerical processing of the obtained data and solving the inverse problem for reconstruction of the 3D data array from the set of 2D projections, while the spatial distribution of fluorophore can be obtained directly, without additional calculations. This circumstance leads to a much simpler procedure of cell segmentation based on the processing of data obtained by confocal fluorescence microscopy [5]. In addition, the numerical processing of tomographic data frequently leads to distortions in the obtained data arrays, particularly in images in the *XZ* and *YZ* cross sections. For increasing the accuracy of cell segmentation on 3D distributions of refractive index, we propose using a segmentation algorithm based on refractive index gradient rather than on its absolute value.

The proposed algorithm operates as follows.

1. First, a cell is roughly isolated in *XY* and *XZ* cross sections and the exact determination of cell boundaries is based on the refractive index gradient, which is especially important for determining cell edges on the *Z* axis.

2. Then, additional pixels are included in the neighboring area, which possess a rather high refractive index that is definitely characteristic for cell structures only.

3. In addition, the class of objects belonging to the cell must also include cavities completely or partly (by 70–80%) surrounded by segmented structures. This is due to the fact that a cell membrane at normal conditions has the form of a concave shell.

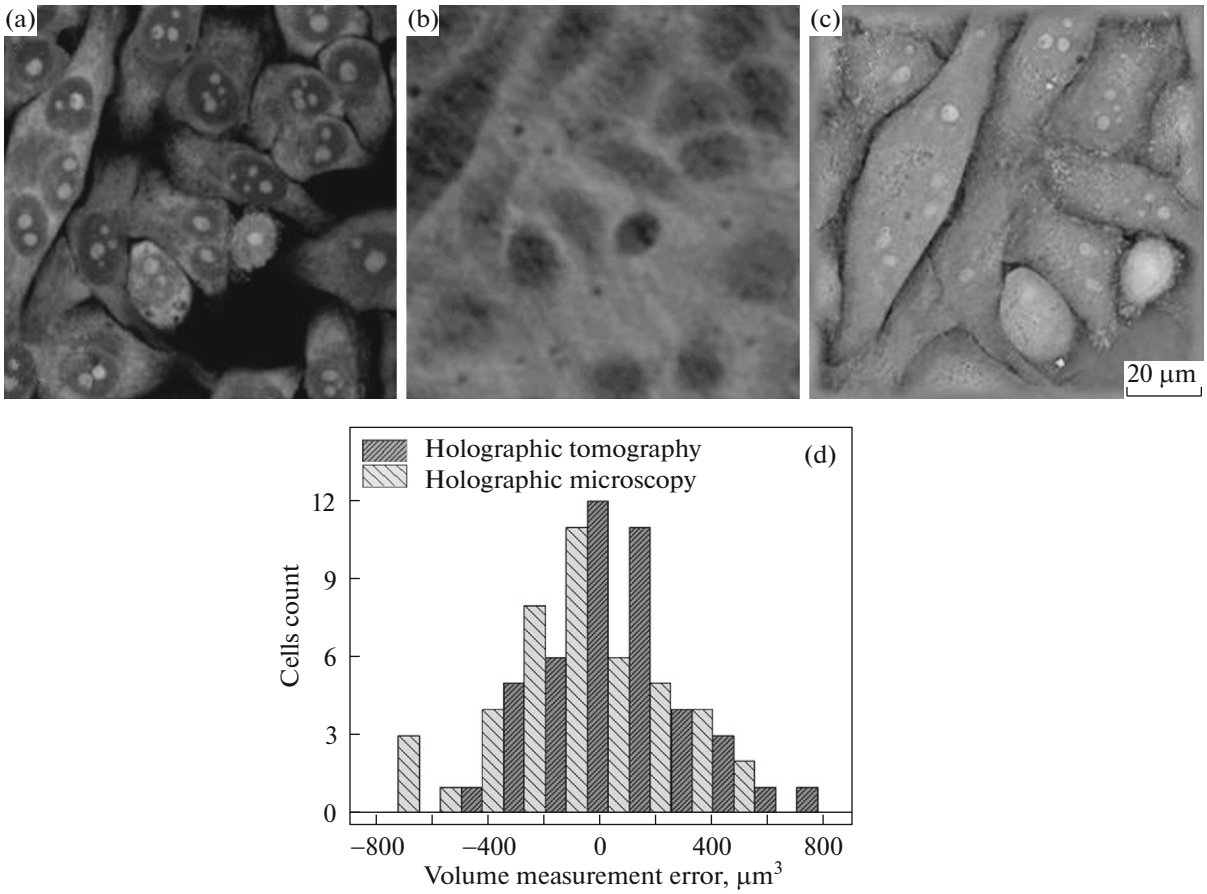

Fig. 2. Examples of the same group of cell images by (a) confocal fluorescence microscopy, (b) digital holographic microscopy, and (c) holographic tomography. (d) Statistical distributions of the errors of determination of HeLa cell volume measured by methods of holographic microscopy and holographic tomography.

The last (third) step of the algorithm can be excluded from the segmentation process, provided that the morphology of cells is significantly distorted, e.g., during cell mitosis or necrosis.

The algorithms of cell segmentation and cell volume determination were verified in an experiment, in which the same group of cells in a fixed sample of HeLa cells was studied by holographic microscopy and holographic tomography (Figs. 2a–2c). The measurements were performed using a home-made holographic microscope (see [4, 7]), 3D Cell Explorer holographic tomographic microscope (Nanolive), and Leica TCS SP5 confocal fluorescence microscope.

Results of the cell volume determination by holographic methods were compared to the data obtained by means of confocal fluorescence microscopy, for which the error is known not to exceed 5% [5]. The obtained statistical distribution of the deviation of cell volume measured by both holographic methods from the results obtained by fluorescence microscopy is presented in Fig. 2d. A comparative analysis of these results gives errors of cell volume determination by holographic microscopy $\sigma_{DHM} = 278$ μm^3 and by holographic tomography $\sigma_{DHT} = 321$ μm^3, with corresponding relative errors of $\delta_{DHM} = 12.8\%$ and $\delta_{DHT} = 14.3\%$. The somewhat greater value of error in the case of holographic tomography is probably related to more complicated processes of cell segmentation in the 3D space. On the other hand, exact determination of the morphological parameters of cells in the case of holographic microscopy requires knowledge of the average refractive index of cells, which somewhat restricts the applicability of this method.

Thus, we have developed algorithms for automated cell segmentation on two-dimensional phase images and three-dimensional distributions of refractive index. The errors of determination of HeLa cell volume using these algorithms do not exceed 15%, which indicates that both digital holographic microscopy and tomography can be successfully used for determining the morphological characteristics of cells. The significant advantages of holographic methods are their noninvasive ness and possibility of prolonged monitoring of the dynamics of morphological changes in living cells.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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