# Quantification of changes in cellular morphology during cell necrosis obtained from 3D refractive index distributions

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# Quantification of changes in cellular morphology during cell necrosis obtained from 3D refractive index distributions

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**Abstract**. In this paper we perform an analysis of changes in cellular morphology in response to photodynamic treatment in vitro. Experiments were performed by means of digital holographic tomography. 3D spatial distributions of refractive index in cells were obtained and main morphological parameters of cells were determined: volume, average height, membrane area. The cellular response to treatment at different doses was studied and changes in morphology were correlated with major cell death pathways, apoptosis and necrosis. The necrosis pathway was studied in dynamics. The data obtained by holographic tomography were validated using a standard test for the integrity of cell membranes, conducted using a confocal fluorescent microscope.

## 1. Introduction

Investigation of living cells response to photodynamic (PD) treatment is an essential task, since photodynamic therapy becomes widely used nowadays for treatment of various cancers [1]. Holographic microscopy and tomography find wide applications in cellular research due to their noninvasiveness and capability to provide quantitative data. Tomographic methods allow for obtaining three-dimensional phase images of intracellular structures with high temporal and spatial resolution. However by now holographic tomography was never applied in studies of cellular response to PD treatment. The most frequently recorded cell death pathway under PD treatment is necrosis, which is characterized by a rapid destruction of cellular membrane and efflux of intracellular content, by development of alterations of surrounding cells and inflammation in vivo [2]. However the processes of autophagy and apoptosis, induced by intracellular generation of reactive oxygen species, were also observed in a number of works [2, 3]. The prevailing cell death pathway strongly depends on PD treatment dose and cell type (see e.g. [4, 5]), therefore an information on various aspects of cellular response to PD treatment is of high importance for activation of the desired pathway depending on the type and purpose of the therapy.

The main goal of this research was to obtain quantitative characteristics of cellular morphology (volume, average height and membrane area) by means of digital holographic tomography and to study living cells response to PD treatment in vitro by monitoring the post-treatment dynamics of these characteristics. Changes in cellular morphology were also determined as function of the applied irradiation dose.

## 2. Research methodology.

In our research morphological changes in cells in vitro occurring in the course of their death were recorded and monitored using the holographic tomographic microscope 3D Cell Explorer (Nanolive, Switzerland). The technique of holographic tomography is based on the principle of holographic microscopy, which allows reconstruction of changes in phase distribution of the wave front passed through the sample. The tomographic technique is implemented by processing of a set of holograms recorded at various angles of the probe beam provided by its rotation around the optical axis [6, 7]. As a result of numerical processing of the obtained set of holograms, the three-dimensional distribution of refractive index in intracellular structures is reconstructed, which is used to assess cell condition, morphology and to identify the cell death pathway.

In our experiments the tomographic technique was applied to studies of the response of human cervical adenocarcinoma HeLa cells to PD treatment with Radachlorin photosensitizer (Radapharma, Russia). Cells were cultivated in Petri dishes in DMEM cell culture medium supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin at 37 $^{o}$C in 5 % CO2 atmosphere. Before conducting the experiments, photosensitizer (PS) was added to the culture medium at the final concentration of 5 μg/ml or 10 μg/ml and cells were incubated in this solution for 4 hours. Then the solution was replaced with the pure culture medium. Cell cultures were irradiated by a semiconductor laser at the wavelength of 660 nm, corresponding to the PS absorption band maximum. The irradiation led to the formation of reactive oxygen species inside the cells and their subsequent death. Cell cultures were subjected to PD treatment with three different irradiation doses: 5 J, 10 J and 20 J, after that Petri dishes with cells were placed back into the incubator. In 24 hours after treatment fixed specimens of cells were prepared and analyzed in the tomographic microscope. Data on morphological parameters of individual cells (volume, height and membrane area) were accumulated and averaged over 50-100 cells in each specimen. As a result of analysis, significant differences were identified in morphological parameters of cells subjected to different treatment doses. These changes correlated with the two main pathways of cell death: apoptosis, which is accompanied by cell rounding, an increase in cytoplasm density and formation of small bubbles (blebbing); and necrosis, characterized by cytoplasm swelling, destruction of organelles, rupture of the cell membrane and efflux of intracellular content.

## 3. Results obtained.

Figure 1 demonstrates typical images of cells obtained in the holographic tomographic microscope at different concentrations of photosensitizer and at different irradiation doses. The analysis of tomographic images and the obtained numerical data on changes in the morphological characteristics of cells allowed us to conclude:

1. At the low treatment dose (irradiation dose 5 J, Radachlorin concentration 5 μg/ml) the cells were able to resist PD treatment, no significant changes in their morphology occurred (figure 1 (1)).
2. At the moderate dose (irradiation dose 10 J, Radachlorin concentration 10 μg/ml), typical signs of apoptosis were observed - blebbing, cell rounding, a noticeable decrease in cell volume and membrane area (figure 1 (2)).
3. At the higher dose (irradiation dose 20 J; Radachlorin concentration 10 μg/ml), evidences of necrosis were apparent - membrane rupture and leakage of the intracellular content (figure 1 (3)).

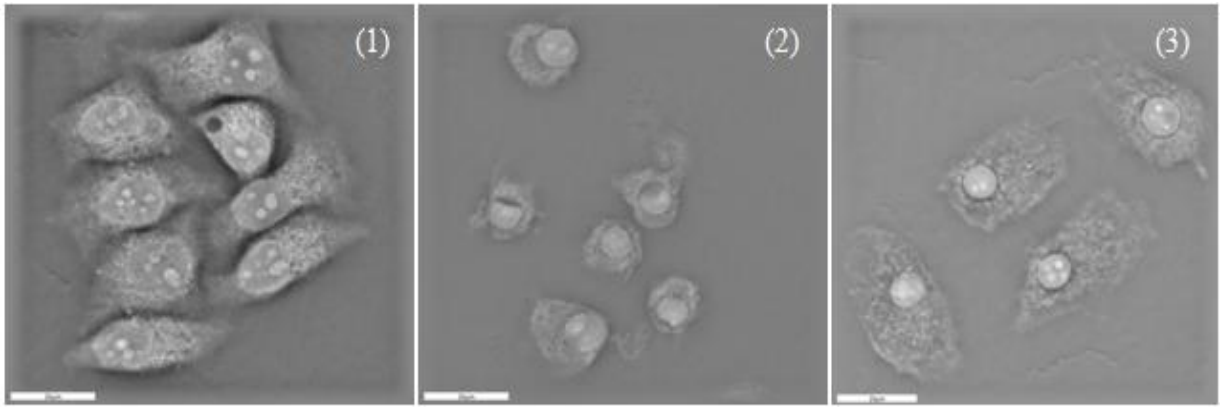


**Figure 1.** Holographic tomography Z-cross sections of HeLa cells subjected to PD treatment at: (1) Irradiation dose 5 J, PS concentration 5 μg/ml; (2) 10 J, 10 μg/ml; (3) 20 J, 10 μg/ml.

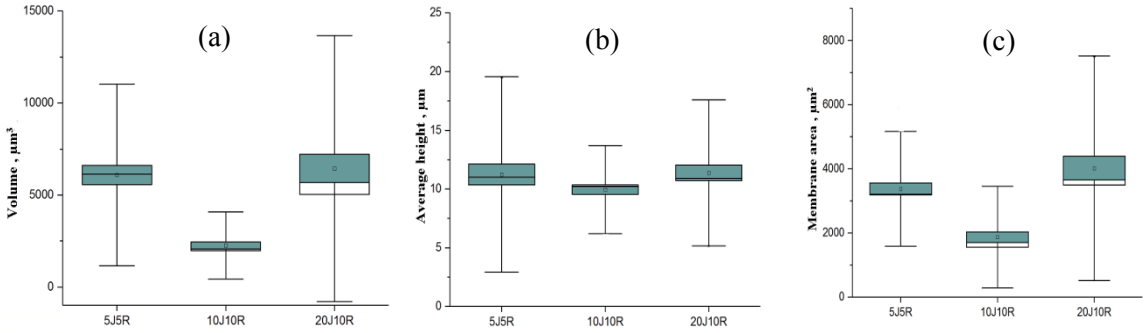


**Figure 2.** Average values of morphological parameters of cells: volume (a), average height (b) and membrane area (c), obtained in 24 hours after PD treatment at different PS concentrations and different irradiation doses (J-irradiation dose (J), R-PS concentration (μg/ml)). Averaging was carried out over 50-100 cells in each specimen.

Thus, the examination of fixed specimens using holographic tomography allowed us to distinguish between the processes of necrosis and apoptosis basing on the analysis of morphological parameters of cells. The prevailing mechanisms of cell death at different doses of PD treatment were identified: high treatment doses led to changes inherent to necrotic pathway, while moderate doses triggered mechanism of apoptosis.

The dynamics of changes in cellular morphology under PD treatment was studied by monitoring of the samples of living cells in tomographic microscope for 2.5 hours after treatment. In this experiment the high treatment dose (PS concentration 10 μg/ml, irradiation dose 20 J) was applied; and cell death through necrotic pathway was expected basing on the results obtained on fixed specimens. In experiments the same set of cellular parameters (volume, average height and membrane area) were determined every 10 minutes and the data were averaged over 15–20 cells in the sample at each time point. Figure 3 demonstrates the obtained temporal variations of these parameters during the observation time. As can be seen from figure 3, significant changes in the morphological parameters of cells occurred during the first 2.5 hours after PD treatment with high dose. All the three parameters demonstrated a maximum at about 60-80 minutes after irradiation. Rise of these parameters was due to the cytoplasm swelling, which was followed by the cell membrane disintegration and efflux of intracellular content. This content then dissolved in the surrounding medium and the refractive index

of this mixture was equalized. At this stage a decrease in the volume (figure 3(b)) and average height (figure 3(c)) of cells was observed.

Thus, it was shown that at the high dose of PD treatment the predominant pathway of cell death was necrosis. The technique of digital holographic tomography allowed us to obtain quantitative information on changes of cellular morphology and to monitor these changes in dynamics over a long period of time.

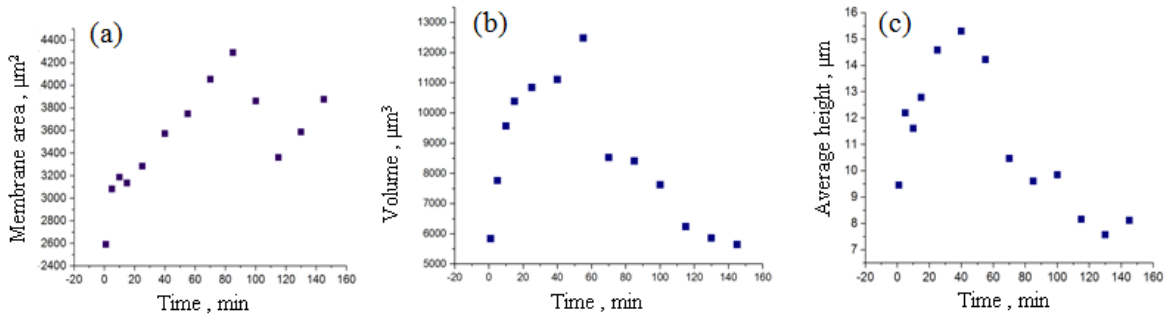


**Figure 3.** Dynamics of morphological parameters of cells during 2.5 hours after PD treatment: (a) - membrane area, (b) - volume, (c) – average height of cells. Each point was obtained by averaging the data over 15–20 cells in the specimen.

The results of the analysis of cellular morphology were compared with the data obtained by means of confocal fluorescence microscopy using a standard test assay containing a mixture of acridine orange and ethidium bromide. Typical fluorescent images of cells recorded in two hours after PD treatment with different doses are shown in figure 4. At low and moderate doses (figure 4(a, b)), only the luminescence of acridine orange was observed, that is indicative of cell membrane integrity. Changes in cellular morphology shown in figure 4(b) are also relevant for apoptosis. At the high treatment dose (figure 4(c)), ethidium bromide fluorescence in cells nuclei was observed. The penetration of this dye into the nucleus indicates the destruction of cellular membrane, and cell death through necrosis pathway.

Thus, the data from confocal fluorescence microscopy validated our results obtained by holographic tomography and confirmed that at high doses of PD treatment the necrotic pathway of cell death prevails while at moderate doses the apoptotic pathway dominates.

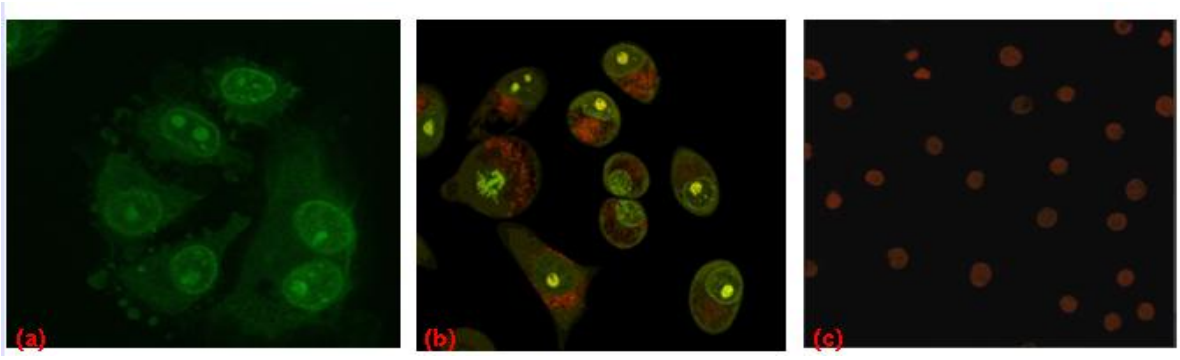


**Figure 4**. AO/EB fluorescent images of HeLa cells taken in two hours after PD treatment at different irradiation doses and different PS concentrations: (a) - 5 J, 5 μg/ml PS, (b) - 10 J, 10 μg/ml PS, (c) - 20 J, 10 μg/ml PS.

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