The Nucleolus and Cellular Stress: Analysis by Coherent Phase Microscopy

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ABSTRACT

Using the original method of coherent phase microscopy (CPM), we demonstrate that: 1) optically dense subcellular structures, in particular, the nucleoli, are detectable by CPM in cultured mammalian cells or freshly isolated human tumor specimens, and 2) the key parameters of CPM, i.e., the optical thickness, optical diameter and refractivity index can be used for quantitative analysis of the nucleolar response to pharmacological inhibitors of vital cellular processes such as gene transcription. CPM allows for direct visualization of non-fixed, non-stained cells followed by computer assisted data analysis. Inhibition of gene transcription with actinomycin D and olivomycin led to a dramatic decrease of the optical thickness and refractivity index of the nucleoli within the initial minutes of treatment. Furthermore, an indirect interference with transcription by ATP depletion with uncoupling agents resulted in a decreased optical thickness of the nucleoli. In contrast, drugs that do not alter transcription had no effect on the optical parameters of the nucleoli. We conclude that CPM detects early changes of nucleolar structure, most probably reflecting the segregation of its components in response to transcriptional poisons. CPM can be used for rapid, informative and inexpensive screening of novel anticancer drugs that target gene transcription machinery.

Key words: optics, coherent phase microscopy, gene transcription, tumor cells.

1. INTRODUCTION

Dynamic processes that comprise the reaction of biological objects to stress are associated with major structural changes detectable as altered physical state. In particular, any microobject can be regarded as structurally complex entity, so that the lazer irradiation of the object would cause deformation of the beam's wavefront in the image plane. Registration of optical path difference (or 'phase thickness') allows for generating a 3D optical plot of the microobject in which differential optical density of its parts is represented in artificial colors. This principle underlies coherent phase microscopy (CPM), a method originally used by us to monitor living biological microobjects [1,2]. This non-invasive method provided rapid quantitative data on the response to metabolic poisons in prokaryotes and isolated eukaryotic cell organelles.

We sought to extrapolate CPM on the analysis of eukaryotic cells, namely, to evaluate the changes of subcellular structures in response to a variety of environmental stress stimuli. We focused on the nucleoli, the sites of ribosomal biogenesis, as major targets of many harmful insults [3]. Indeed, these dynamic structures are highly sensitive to toxins, and the altered nucleolar morphology and physiology is a hallmark of cellular damage by cytotoxic agents [3, 4].

2. MATERIALS AND METHODS

HCT116, MCF-7 and NIH 3T3 murine fibroblast cell lines were propagated in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2 мМ L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37^0C, 5% CO_2 in humidified atmosphere. Cells in logarithmic phase of growth were plated on glass coverslips to reach ~ 50% confluence by the day of the experiment. Actinomycin D, rotenone, SF6847, vincristine (Sigma-Aldrich) and olivomycin (gift of M.Preobrazhenskaya, Gause Institute of New Antibiotics, Moscow, Russia) were added to cell cultures for 5-60 min. The concentrations of each agent in our experiments were the highest commonly used to block transcription, deplete the intracellular ATP pool, inhibit Ca^{2+} channels or depolymerize microtubules, respectively. After the completion of exposure cell monolayers were immediately subjected to CPM (see below). In some experiments cells were isolated from patients' biopsies by careful dissection of tumor tissue and mild washing in saline.

Cultured cells and the ex vivo cells were examined under "Airyscan" microscope equipped with a He-Ne lazer (λ = 633 nm), a dissector for registering the interference signal and an electronic device for computer assisted cell imaging. The Olympus 20*/0.4 objective with a 12 mm viewfield was used. Individual cells were visualized in the optical channel of the microscope, then their topograms and phase thickness profiles were analyzed. About 20 randomly chosen cells were examined in each specimen. Horizontal sections of the nucleolar images were made at ½ height. The sizes of the nucleoli and phase thickness Δh were measured with ± 100 nm and 3 nm accuracy, respectively.

3. RESULTS AND DISCUSSION

3.1. Principle of CPM

The basic principle of CPM (Fig.1, *upper panel*) is the measurement of local optical thickness $h(x, y)$ that is linked with refractivities of the object $n(x,y,z)$ and external medium n_0 by the equation:

$$h(x, y) = \int [n(x,y,z) - n_0] dz \,. \qquad (1)$$

The following parameters were used to interpret the images: the profile $h(x)$ of phase thickness, its maximal

value Δh and the horizontal size (or diameter) of the structural element *d*. The physical model is shown in Fig. 1, *upper panel*, where n(x,y,z) is the index of refractivity of the object, H is physical (geometrical) thickness, and n_0 is the index of refractivity of external medium.

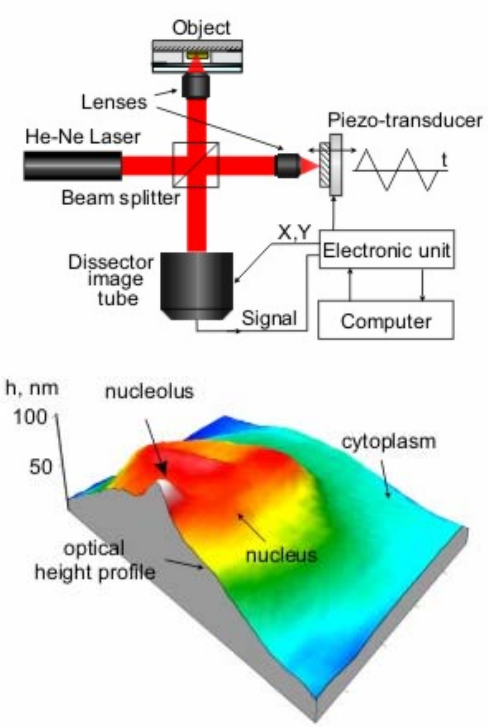

Figure 1. Visualization of subcellular structures by CPM.
upper panel: physical principle of CPM; *lower panel*: a 3D image of human colon carcinoma cell (HCT116 cell line).

The optical path difference *h(x,y)* in the object's image and the maximal value of *h(x,y)* dependent on physical thickness and the difference between the indexes of refractivity of the object and medium $\Delta h = H[\langle n(x,y,z)\rangle - n_0]$. In the phase image of the cell (the topogram, Fig.1, *lower panel*) the optically dense nucleolus was identified by local increase of phase thickness within the nucleus. Nucleolar refractivity Δn that reflects the contrast of the nucleolus in the phase image, depends on the difference between mean refractivities $\langle n(x,y,z)\rangle$ and $\langle n_N\rangle$ of the nucleus $\Delta n = \Delta h/H = \langle n(x,y,z)\rangle - \langle n_N\rangle \approx \Delta h/d$ given the shape of the nucleus is spherical ($H \approx d$).

3.2. Optical effects of the direct inhibitors of gene transcription.

Single cell analysis by CPM allowed us to obtain 3D images of the nucleoli and the adjacent areas of the nucleoplasm. In untreated HCT116 cells the nucleolus visualized as optically dense structure (Fig. 2, *upper panel*), becomes flattened and less dense rapidly after the addition of transcriptional inhibitor actinomycin D (Fig.2, *lower panel*). By 30 min. with actinomycin D the phase thickness and refractivity index of the nucleoli were markedly decreased, as presented by overall shift of the histograms of distribution of respective parameters (Fig. 3). Similar effects were observed with another inhibitor of gene transcription, the antibiotic olivomycin [5] Both actinomycin D and olivomycin caused a decrease of the phase thickness and refractivity index of the nucleoli in MCF-7 breast carcinoma epithelium and NIH 3T3 fibroblasts (not shown). Thus, pharmacological agents known to directly target gene transcription machinery alter the major optical parameters of the nucleoli in different mammalian cell types, and these changes are detectable by CPM.

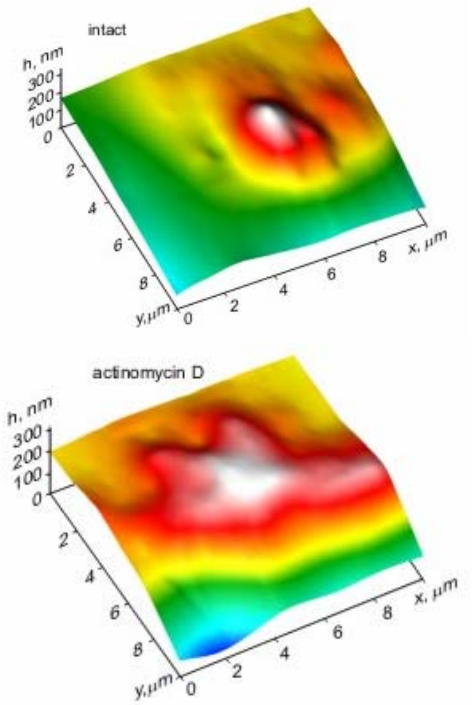

Figure 2. Single cell analysis by CPM.
Three-D images of HCT116 cells before (*upper panel*) and 15 min. after (*lower panel*) addition of 1 μM actinomycin D.

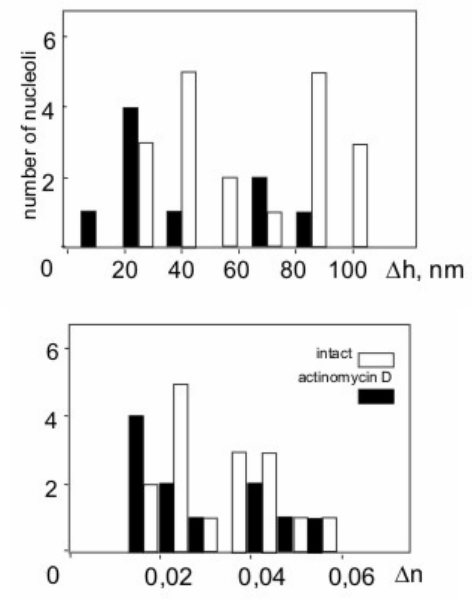

Figure 3. Decreased optical parameters of the nucleoli in actinomycin D treated cells. Phase optical thickness (*upper panel*) and refractivity (*lower panel*) of the nucleoli in HCT116 cells treated with actinomycin D

(1 μM, 15 min.).

However, the detailed analysis revealed that the response of the nucleoli is more complex than merely a uniform drop of the optical density. After 10-20 min. with actinomycin D, the dense zones were found in the nucleoli along with optically sparse areas. The time course of optical thickness of individual nucleolus in actinomycin D treated HCT116 cells is given in Fig. 4 These results show that the nucleoli not simply become less dense but the phase thickness is rather re-distributed throughout the nucleolus. Nevertheless, the major trend of the effect of the transcriptional inhibitor is a decreased optical density of the nucleoli.

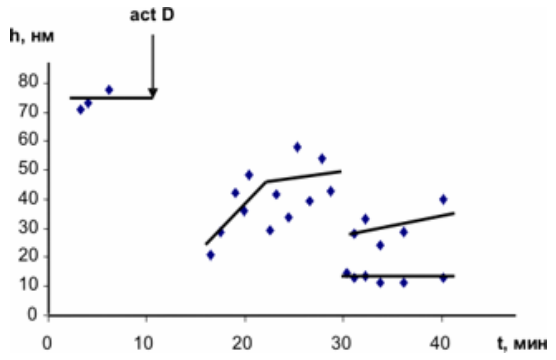

Figure 4. Time course of phase thickness of the nucleoli of HCT116 cells after the addition of actinomycin D (*arrow*).

This effect is not limited to cultured cells but can also be detected in tumor specimens isolated from patients' biopsies.

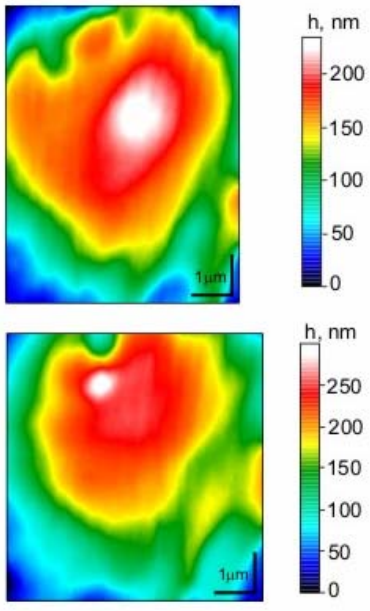

Figure 5. CPM of the breast carcinoma cell isolated from patient's biopsy. See text for details.

Fig. 5 shows a topogram of the breast carcinoma cell before (*upper panel*) or 5 min. after (*lower panel*) the addition of 1 μM actinomycin D. Note the optically dense nucleolus in the untreated cell and a decrease of phase thickness after treatment with actinomycin D.

3.3. Optical effects in the nucleoli upon an indirect impairment of transcription.

Direct inhibitors of transcription such as actinomycin D act by physical interaction with the components of this process. However, transcription and other important events in the nucleoli can be impaired by treatments not associated with direct interference with the nucleolar structures. Given that transcription is an ATP dependent process, depletion of cytoplasmic ATP pool should result in altered transcription. We treated HCT116 cells with the combination of the mitochondrial complex I inhibitor rotenone plus SF6847, the agent that uncouples oxidation and phosphorylation. As shown in Fig. 6, the phase thickness of the nucleoli decreased within 15 min. of treatment with rotenone+SF6847.

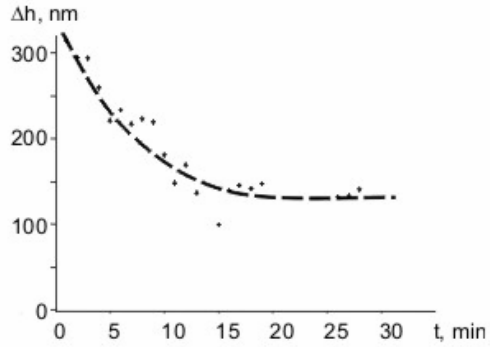

Figure 6. Decreased phase thickness of the nucleoli in energy depleted cells.
Time course of phase thickness of the nucleoli after addition of 50 μM rotenone+10 μM SF6847.

Finally, a decrease of the phase thickness of the nucleoli was found when HCT116 cells were treated with vincristine (1 μM, 30 min.), an anticancer drug that blocks polymerization of microtubules and therefore impairs the traffic between the cytoplasm and the nucleus (not shown). In contrast, drugs that do not alter transcription (such as Ca^{2+} channel blocker verapamil or the aglycon of olivomycin) had no effect on the optical parameters of the nucleoli. Thus, the nucleoli respond to the stimuli that alter, directly and/or indirectly, their morpho-physiological state by overall decrease of optical density.

The mechanism of the alteration of density in the nucleoli of stressed cells remains to be elucidated. The main problem is to identify the physical origin of the parameters detectable by CPM. One can hypothesize that the optical density is associated with proteins and water. Changes in the amount or physical state of proteins in the nucleoli of cells treated with transcriptional inhibitors are termed 'segregation of the nucleolar components' [6]. This phenomenon largely comprises a bi-directional transport of proteins and ions within the nucleolus and between the nucleoli and the nucleoplasm. Importantly, the volume of the granular component decreases, and it is this decrease that can well be associated with lower phase thickness and refractivity of the nucleoli. Thus, upon inhibition of transcription the nucleolar structures change their optical

characteristics and relocalize, and the nucleolar segregation is detectable by CPM.

In summary, we demonstrate that CPM, a method originally used for the analysis of living prokaryotes and eukaryotic organelles, provides information about the response of mammalian cells to a variety of stress stimuli. The nucleoli, a major target of these stimuli, can be clearly visualized by CPM in cultured mammalian cells or freshly isolated human tumor specimens as distinct optically dense subcellular structures. The quantitative data generated in CPM, i.e., the optical thickness, optical diameter and refractivity, reflect the changes in nucleolar morphology and function upon treatment of individual cells with the agents that directly interact with the transcriptional machinery as well as with the drugs that alter the nucleolar morphology and physiology by energy depletion or damage of cytoskeleton. CPM based analysis requires no fixation or staining of cells, and therefore allows for detection of rapid (within the initial minutes of treatment) changes in the nucleoli. Although more work in needed to attribute the parameters of CPM to the complex processes that occur in stressed nucleoli, the decreased phase thickness of the nucleoli most probably reflects the segregation of its components. The practical application of CPM can be rapid, informative and inexpensive screening of novel anticancer drugs that target gene transcription.

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