Using digital inline holographic microscopy and quantitative phase contrast imaging to assess viability of cultured mammalian cells

Sergey Missan\textsuperscript{a}, Olga Hrytsenko\textsuperscript{b}

\textsuperscript{a}4Deep inwater imaging, Halifax, NS, Canada B3L 1L9; \textsuperscript{b}Dept. of Biology, Dalhousie Univ., Halifax, NS, Canada B3H 4R2

ABSTRACT

Digital inline holographic microscopy was used to record holograms of mammalian cells (HEK293, B16, and E0771) in culture. The holograms have been reconstructed using Octopus software (4Deep inwater imaging) and phase shift maps were unwrapped using the FFT-based phase unwrapping algorithm. The unwrapped phase shifts were used to determine the maximum phase shifts in individual cells. Addition of 0.5 mM H\textsubscript{2}O\textsubscript{2} to cell media produced rapid rounding of cultured cells, followed by cell membrane rupture. The cell morphology changes and cell membrane ruptures were detected in real time and were apparent in the unwrapped phase shift images. The results indicate that quantitative phase contrast imaging produced by the digital inline holographic microscope can be used for the label-free real time automated determination of cell viability and confluence in mammalian cell cultures.

Keywords: digital inline holography, cell viability, quantitative phase contrast, mammalian cell culture

1. INTRODUCTION

The ideal cell viability assay should be accurate, be able to provide results in real time, not use chemical labels that can be cytotoxic and adversely affect cell physiology, and be able to count cells in situ using standard cell culture dishes and plates. Unfortunately, none of the existing and commonly used methods satisfy the above requirements. Dye exclusion assays (Propidium Iodide\textsuperscript{1}, Trypan Blue\textsuperscript{2}) use chemicals and require cell resuspension for counting, fluorescence-based assays (MTT\textsuperscript{3,4}, Resazurin reduction\textsuperscript{5}, GF-AFC\textsuperscript{6}) require long incubation times and use toxic labels, and cell impedance methods\textsuperscript{7} require the use of special titration plates with incorporated electrodes.

Recently, new optical methods became available; those that precisely measure the phase shift of light as it passes through cells. The phase shift is directly proportional to the cell refractive index (RI) and cell thickness\textsuperscript{8}. The integral RI of a cell is determined by the concentration of the intracellular ions and macromolecules\textsuperscript{9}. Metabolic changes associated with cell death are known to change the intracellular solute concentration and, therefore, the RI\textsuperscript{10}. The added benefit of phase images is an increased contrast of semitransparent objects compared to bright field microscopy, which helps with label-free cell image segmentation and morphological analysis\textsuperscript{5}. Several methods have been developed that allow for the quantitative phase measurements, including ptychography\textsuperscript{11,12}, transport-of-intensity equation (TIE) methods\textsuperscript{13,14}, and digital holography\textsuperscript{15,16}.

Digital inline holographic microscopy (DIHM) with a point source is a simple, lens-free implementation of Gabor\textsuperscript{17}-style holography\textsuperscript{18-20}. Unlike the off axis holography, DIHM is self-referencing and does not require the use of beam splitters and precise alignment of optical components. Unlike TIE or ptychography, the phase information is obtained from a single image frame (hologram). Owing to its simplicity, DIHM can easily be incorporated into various cell imaging
configurations and offers good quality phase images with up to 0.5 μm lateral resolution\textsuperscript{21}. We have investigated whether quantitative phase contrast images obtained with DIHM can be used to distinguish between dead and living cells and can, therefore, form the basis for the in situ, automated, label-free assay for viability and confluence of mammalian cells in culture.

2. METHODS

2.1 Cell cultures

HEK293T cell line (Human Embryonic Kidney) was maintained in DMEM (GE Healthcare, Mississauga, ON, Canada) supplemented with 10% fetal bovine serum (GE Healthcare), 2 mM L-glutamine (Sigma-Aldrich, Oakville, ON, Canada), 100 units/ml penicillin (Sigma-Aldrich), 100 μg/ml streptomycin (Sigma-Aldrich), 0.1 mM MEM Non-Essential Amino Acids (Sigma-Aldrich), and 1 mM Sodium Pyruvate (Sigma-Aldrich).

B16-F10 cell line (Mus musculus skin melanoma) was maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

E0771 cell line (Mus musculus medullary breast adenocarcinoma) was maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin.

Twenty-four hours before the experiments, 2x10\textsuperscript{5} cells/well were seeded in 6 well culture plates (BD Falcon, Mississauga, ON, Canada) with 2 ml of corresponding media for imaging with DIHM and Zernike phase contrast.

2.2 DIHM microscope

A desktop model of the digital inline holographic microscope (4Deep inwater imaging, Halifax, NS, Canada) was used for DIHM imaging of the cultured cells. The basic schematic of the DIHM is shown in Figure 1. Light from a constant wave 405 nm solid state laser is focused on a 0.5 μm pinhole, producing spherical waves that travel through the imaged volume. A digital camera sensor is placed 20 to 40 mm away from the pinhole, aligned with the optical path of the microscope. Light scattered by the objects within the imaged volume interfere with the reference waves which travel unobstructed between the pinhole and the digital imaging sensor. An interference pattern, a hologram, is recorded by a camera and stored as a BMP image for further numerical reconstruction.
2.3 Hologram reconstructions and 2D phase unwrapping
Holograms were numerically reconstructed with the Octopus software (4Deep inwater imaging) using the previously described phase reconstruction algorithm\cite{18,21}. A FFT-based 2D phase unwrapping algorithm\cite{22} was used and implemented, together with the hologram reconstructions, using the NVIDIA CUDA GPGPU toolkit\cite{23}. Phase reconstruction combined with 2D phase unwrapping time was in the order of 300 ms for a 2048x2048 hologram on the NVIDIA Quadro K2000M GPU.

2.4 Phase analysis
The maximum absolute phase shifts for each reconstructed cell image have been determined from the unwrapped phase reconstructions and have been monitored over time for further statistical analysis.

2.5 Calculation of integral cell RI
The integral RI of a cell was determined from the Eq. (1):

$$\Delta \phi = \frac{2\pi}{\lambda} (n_{cell} - n_{medium})h$$  (1)
where $\Delta \phi$ is the maximum phase shift in radians, $\lambda$ is the laser wavelength, $n_{\text{cell}}$ is RI of a cell, $n_{\text{medium}}$ is RI of cell medium (1.339)\(^{24}\) and $h$ is the optical thickness of a cell (in $\mu$m). For spherical cells, the diameter of a cell = $2h$.

2.6 Induction of cell death with hydrogen peroxide
The cytotoxic effects of H\(_2\)O\(_2\) in cell cultures have been extensively described\(^{25}\). Stabilized 30% H\(_2\)O\(_2\) (Sigma-Aldrich) was added directly to the cell culture medium to obtain a final concentration of 0.5 mM.

2.7 Zernike phase contrast
Zernike phase contrast images were obtained with a Leica DMIRE2 inverted phase contrast microscope (Leica Microsystems GmbH, Wetzlar, Germany), equipped with HC PL Fluotar 10X Zernik phase objective (Leica). Images were captured with a Hamamatsu ORCA C4742-95 digital camera (Hamamatsu Photonics K.K., Hamamatsu, Japan) and saved as TIFF images for further analysis.

2.8 Statistics
Experimental data are expressed as means ± SEM. Statistical comparisons were made using Student's paired $t$ test. Differences were considered significant when $P \leq 0.05$. 
3. RESULTS

Holograms of HEK293 cells growing in a cell culture dish have been recorded at the rate of 0.2 Hz. For each cell measured, maximum absolute phase shift values (in radians) have been determined from the unwrapped phase reconstructions and plotted as a function of time. The representative results are shown in Figure 2a: addition of 0.5 mM H$_2$O$_2$ to cell culture medium rapidly induced rounding and shrinking of cells (image panels 2 and 3). The change in cell morphology was accompanied by a slight gradual increase in the maximum phase shift values. Approximately 20 min after H$_2$O$_2$ addition, the cell membrane ruptured that was evident as both the loss of sharp cell edges and overall intensity in phase images (image panels 4 and 5), as well as the abrupt decrease in the maximum phase shift to 26% of the pre-H$_2$O$_2$ values.

In the control experiment without the addition of H$_2$O$_2$, there were no noticeable changes in cell morphology or in the maximum phase shift values (Figure 2b).
The integral cell RI was calculated according to the Eq. (1), based on the assumption that HEK293 cells exposed to H\textsubscript{2}O\textsubscript{2} are changing their shapes to those of a perfect sphere. For H\textsubscript{2}O\textsubscript{2}-treated HEK293 cells, the mean RI was $n_{\text{cell}} = 1.3713 \pm 0.0009$; $N = 35$ cells.

Figure 3. Effects of 0.5 mM H\textsubscript{2}O\textsubscript{2} on B16 cell morphology and phase. The moment of H\textsubscript{2}O\textsubscript{2} addition to cell medium is marked with the arrow. The numbers in the time course correspond to the times when the representative reconstructed phase images in the panels above were captured. Scale bar = 20 $\mu$m.

Murine melanoma B16 cells are pigmented and, therefore, can pose a challenge to acquiring good phase images due to light absorbance within the cytoplasm. Experiments were performed using the same protocol as with HEK293 cells. Even though the maximum phase shift at the beginning of the experiment (5 rad) was smaller than that recorded in HEK293 cells (6 rad), addition of 0.5 mM H\textsubscript{2}O\textsubscript{2} caused cell rounding and an initial phase shift increase followed by membrane rupture (Figure 3), that was apparent both in the phase images and also by the variation of the phase shift values over time.
Murine breast cancer cells E0771 are commonly used in cancer drug testing and cytotoxicity assays\textsuperscript{26}. To determine whether quantitative phase imaging can be used to assess the viability of these cells, E0771 cells were studied under the same protocol as HEK293 and B16 cells. There were less pronounced changes in cell shape over the course of the experiment (Figure 4). The maximum phase shifts initially increased from 4 to 5.8 rad and then decreased gradually to 2.1 rad 53 min after application of H\textsubscript{2}O\textsubscript{2}. No prominent moment of membrane rupture was detected in these experiments.
To compare quantitative phase contrast recordings obtained with DIHM to those imaged with Zernike phase contrast, we have repeated the experiments with HEK293 cells using the inverted phase contrast microscope.

Images of cells were captured at the rate of 0.2 Hz and maximum intensity values were determined for each individual cell. Due to the “halo” artefact around the cell edges, care has been taken to analyze pixels that lay within the cell boundaries. The intensity values have been plotted as a function of time and representative frames and a time course are shown in Figure 5. The initial maximum pixel intensity of 56 quickly increased to 212 after the addition of 0.5 mM H$_2$O$_2$ to the cell culture medium. Cell morphology at the same time changed to the characteristic round and compact cell body appearance previously observed with DIHM. Membrane rupture has been detected after 25 min of incubation in the presence of H$_2$O$_2$ (image panel 5) and maximum pixel intensity afterwards decreased to 43 (77% of the pre-H$_2$O$_2$ values).
A summary of the quantitative phase shift data is shown in Figure 6a. For each pair of results, measurements were taken at the beginning and at the end of each experiment. There was no significant change in phase shift values obtained for HEK293 cells in the absence of H$_2$O$_2$ (HEK Ctrl). Incubation of HEK293, B16 and E0771 cells with H$_2$O$_2$ resulted in decrease of the maximum phase shifts to 32%, 43% and 51% control, respectively.

For HEK293 cells imaged with Zernike phase contrast (Figure 6b), the pixel intensity values at the end of the experiment decreased to 68% of the control intensity.

4. DISCUSSION

The results of the present study clearly indicate that quantitative phase images obtained with DIHM can be used to investigate the morphology of mammalian cells in culture and to distinguish between dead and alive cells. For all cell types measured (HEK293, B16, E0771) there was a strong decrease of the phase shift values after incubation in the presence of H$_2$O$_2$. The cell images obtained with DIHM showed good contrast, making them suitable for the automated label-free morphology analysis and cell confluence estimation.
Unlike off-axis holography, point source DIHM is extremely simple, does not require the precise alignment of optical components and contains no moving parts. Digital refocusing is possible to accommodate various cell incubation plates and dishes and different recording conditions. Unlike TEI and ptychography, where multiple exposures are required to calculate phase contrast images, in DIHM a single exposure contains all the necessary information for the reconstruction of the quantitative phase contrast signal, thus allowing for the high speed data acquisition limited only by the frame rate of the digital camera.

In our experiments, Zernike phase contrast was also able to distinguish between dead and living cells. However, the difference in phase shifts obtained with Zernike phase contrast was much smaller than that obtained with DIHM. In addition, in images obtained with Zernike phase contrast, cells exhibited the characteristic “halo” artefacts, making automated cell segmentation and measurement of the cell phase much more difficult.

Quantitative phase contrast imaging obtained with off-axis holography has previously been used to monitor cell counts in adherent mammalian cell cultures, measure cell RIs in flow channels and in the adherent cell culture, detect L-glutamate induced cell death in mouse cortical neurons, measure morphological changes in HeLa cells incubated with methanol, and monitor cell volume changes in KB cells undergoing apoptosis. To our knowledge, the present report is the first attempt to use quantitative phase shifts recorded with the point source DIHM for the assessment of cell viability in culture. The present study demonstrates that the acute cytotoxic events can be detected in real time and with great accuracy by means of monitoring of the maximum phase shift values with DIHM.

The integral RI of HEK293 cells determined here with DIHM (1.3713) was in good agreement with values previously measured with other methods in Caco-2 cells (1.3713), airway smooth muscle cells (1.36), mouse cortical neurons (1.3774), 3T3 fibroblasts (1.358-1.374), and HeLa cells (1.371). Traditionally, cell death is categorized into the two main forms – necrosis and apoptosis. Necrosis is characterized by initial cell swelling, followed by a loss of cell integrity due to membrane rupture. On the other hand, apoptosis is characterized by initial cell rounding and volume reduction, followed by the formation of apoptotic bodies. Interestingly, exposure of HEK293 and B16 cells to H$_2$O$_2$ showed the early morphological signs of the apoptotic cell death (rounding and shrinkage) followed by cell membrane rupture characteristic of necrosis. It was previously shown that incubation of T-lymphoma Jurkat cells with 50 μM H$_2$O$_2$ induced apoptosis, whereas incubation with 0.5 mM H$_2$O$_2$ induced necrosis, and 0.2 mM H$_2$O$_2$ induced apoptosis whereas 2 mM induced necrosis in U-937 human promonocytic cells. It is possible therefore, that 0.5 mM H$_2$O$_2$ can induce a “mixed” program of cell death that combines morphological characteristics of both apoptosis and necrosis. In the case of E0771 cells, phase changes where much slower than those measured in HEK293 and B16 cells, there was less pronounced cell rounding and shrinkage as well as less abrupt changes in phase shifts detected. While these morphological changes are difficult to precisely attribute to either apoptosis or necrosis, there was still a significant change in phase shift values detected in E0771 cells incubated with H$_2$O$_2$. It is now recognized that the simple morphological analysis is not accurate enough to describe all possible cell death modalities, and classification based on biochemical assays can yield more precise information. Nevertheless, we feel that the DIHM-based quantitative phase analysis can provide accurate rapid determination of the number of cells that are either dead or are in the state of metabolic stress.

In summary, DIHM is capable of providing good quality label-free in situ phase images of mammalian cells in culture, and is compatible with standard cell culture plates and dishes. A clear differentiation between dead and living cells based on phase shift measurements is possible. Future work will include the incorporation of a microsecond-pulsed laser light source into DIHM design to reduce the cell photo damage in long-term experiments and implementation of the fully automated cell counting and viability analysis software.
ACKNOWLEDGEMENTS

The authors thank Dr. Manfred Jericho for his help with Zernike phase contrast imaging and Drs. Hans Juergen Kreuzer and Stephen Jones for many stimulating discussions and help with manuscript preparation. This work was partially supported by the Canadian National Research Council Industrial Research Assistance Program (NRC IRAP).

REFERENCES


