Label-free observation of three-dimensional morphology change of a single PC12 cell by digital holographic microscopy

Tanveer Ahamd Mir a,⇑, Hiroaki Shinohara a,b,*

a Major of Biological Information Systems Science, Graduate School of Innovative Life Science for Education, University of Toyama, 3190 Gofuku, Toyama 930-8555, Japan
b Field of Life Information and System Sciences, Graduate School of Science and Engineering for Research, University of Toyama, Toyama 930-8555, Japan

Article info

Article history:
Received 21 April 2012
Received in revised form 5 June 2012
Accepted 3 July 2012
Available online 14 July 2012

Keywords:
Digital holographic microscopy
PC12 cell
KCl
3D morphology
Cell height

ABSTRACT

Observation of three-dimensional (3D) morphology changes of a single mammalian cell is very useful to understand cell response for various stimuli. Conventional techniques to evaluate morphology changes with sufficient precision and high temporal resolution are limited. For example, the confocal fluorescence microscope is available to take 3D morphology changes, whereas fluorescence microscopic observation requires labeling the cells with fluorescence dye. Recently, a novel imaging method based on digital holography was developed for nonlabeling microscopic observation of 3D morphology. Digital holographic microscopy has high potentiality in digital focusing properties, video-frequency capability, non-invasive operation, and so forth. It obtains a quantitative phase image of a living cell from a single recorded hologram, with interferometric accuracy, and surveys the rapid morphology change of a single cell. In this study, digital holographic microscopy was applied to monitor the 3D morphology change of an individual PC12 cell, a nerve model cell, subjected to high K+ stimulation. Phase images of the rapidly swelling cell were acquired, and time lapse reconstruction of 3D cell morphology was performed from phase images. Our results demonstrate that digital holographic imaging is a powerful new tool for evaluation of cell response against various stimulants without any labeling reagent.

© 2012 Elsevier Inc. All rights reserved.

Living cells possess structural and physical properties that enable them to withstand the physiological environment as well as mechanical stimuli occurring within and outside the body. Any deviation from these properties will undermine not only the physiological integrity of the cells but also their biological functions. Nowadays, more and more scientists are interested in the study of cell structure, function, and characteristics of living cells at the single cell level. Measurement of the volume of intact viable cells presents challenging problems in many areas of experimental and diagnostic science involved with the investigation of cellular morphology, growth, and function. Currently, there are a few conventional systems that enable direct intrinsic studies of a single cell, including capillary electrophoresis and flow cytometry [1]. By these systems, it is impossible to observe three-dimensional (3D) images of cell morphology. However, these conventional methodologies give only limited information about the cell size. Still, 3D imaging of complex microscopic and submicroscopic processes in living cells and tissues is still a challenging issue.

Recent developments in morphological observations of living cells have been limited to differential interference contrast (DIC) microscopy, confocal laser scanning microscopy (CLSM), atomic force microscopy (AFM) with cantilever, and scanning ion conductance microscopy (SICM) [2–4]. CLSM needs fluorescent dye for observation. AFM and SICM need time to get one frame due to using the contact mode, where the scanned cells inevitably suffer various degrees of structural deformation or damage because of their intrinsic softness, which may provoke significant biological changes inside the cell. In DIC microscopy, the phase-to-amplitude conversion is nonlinear and there are significant artifacts in the images such as the halo in phase contrast and the disappearance of contrast along the direction perpendicular to shear in DIC; hence, quantitative phase imaging is not feasible with DIC. Because of these limitations, all of these techniques have limited applicability to monitor 3D morphology of single mammalian cells quantitatively under physiological and dynamic conditions. Therefore, rapid, sensitive, and noninvasive methods with high-resolution capability are needed for the measurements of 3D structural changes of living cells and tissues. Moreover, understanding the origin of cell membrane fluctuations and 3D shape measurement of living cells may provide information about the functional status of living cells under normal and pathological conditions.

* Corresponding authors. Address: Major of Biological Information Systems Science, Graduate School of Innovative Life Science for Education, University of Toyama, Toyama 930-8555, Japan. Fax: +81 76 445 6832 (T.A. Mir).
E-mail addresses: mirtanveer0699@gmail.com (T.A. Mir), hshinoha@eng.u-toyama.ac.jp (H. Shinohara).

1 Abbreviations used: 3D, three-dimensional; DIC, differential interference contrast; CLSM, confocal laser scanning microscopy; AFM, atomic force microscopy; SICM, scanning ion conductance microscopy; DHM, digital holographic microscopy; CCD, charge-coupled device; DMEM, Dulbecco’s modified Eagle’s medium.

Here we demonstrate a novel approach of digital holographic microscopy (DHM) that can be aimed at monitoring and quantifying cellular processes in a noninvasive and nonlabeling way. It is an attractive tool for 3D bioimaging because it allows the simultaneous observation of both the amplitude and quantitative phase contrast images with an interferometer resolution from a single recorded hologram [5–8]. Simultaneously, many holograms can be recorded with a charge-coupled device (CCD) camera in less than 1 s, and the images of the object are reconstructed numerically afterward. Because the focusing can be adjusted during the reconstruction process, digital holography is free from the process of mechanical focusing and can be used to monitor dynamic change of objects, which makes it an interesting alternative to conventional microscopy [9,10]. DHM not only allows visualization of transparent biological specimens but also provides information about morphological changes associated with exogenous stimulus. Using this method, it is possible to perform time-resolved quantitative microscopic measurements of changes in the lateral and axial shapes under different experimental conditions.

In this study, we investigated the effect of high K+ stimulation on morphological changes of PC12 cells with DHM. The PC12 cell line was used in the experiment because these cells are widely used, in biomedical research, as model system for studying cellular functions of neurons. We expected that the experimental results would provide some direction for further studies of dynamic cellular events of the living cells by the digital holographic technique.

Materials and methods

PC12 cells (RCB0009) were purchased from the cell bank of RIKEN Bio Resources Center (Tsukuba, Japan). Dulbecco’s modified Eagle’s medium (DMEM), penicillin–streptomycin, and horse serum were purchased from Gibco (Tokyo, Japan). Fetal bovine serum was obtained from ICN Biomedicals (Tokyo, Japan). KCl was obtained from Wako Pure Chemical Industries (Tokyo, Japan). Modified Hank’s balanced salt powders were purchased from Sigma–Aldrich (Tokyo, Japan).

Cell culture

PC12 cells were cultured in culture flasks (25 cm²) and were maintained in DMEM (Gibco) supplemented with 10% horse serum, 5% fetal bovine serum (ICN Biomedicals), and 1% penicillin–streptomycin antibiotics (Sigma) and incubated at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide. Then, 24 h prior to conducting DHM experiments, PC12 cells were plated on glass base culture dishes (35 mm) and incubated. On removal from the incubator, the samples were mounted to a DHM stand and experiments were then conducted under ambient laboratory conditions.

Experimental setup

We performed our experiments on a transmission DHM setup (DHM T1000 from Lyncée Tec.) [9]. A scheme of DHM is shown in Fig. 1. The polarized laser diode emitting at 635 nm is used as a light source. Data were collected by an Olympus microscope objective (20× magnification, NA = 0.4). A charge-coupled device (CCD camera) (512 × 512 pixels, 8 bit), provided with square pixels of size 6.7 μm and a maximal frame rate up to 25 Hz, detects the interference pattern (hologram) and sends it to a computer. The hologram is then numerically treated, and the complex field is reconstructed numerically. All experiments were performed without any specific vibration-insulating system; because a single hologram is required to reconstruct the complex diffracted wavefront, this is performed in a short time (down to 20 μs with CCD camera), leading to high measurement stability. The phase reconstruction rate is 15 frames per second.

Reconstruction of digital holograms

The working principle of DHM relied on the acquisition of a hologram generated by the interference between an objective wave and a reference wave. For the holographic recording, the emitted source from the collimated laser diode split into two parts: the object beam illuminated the specimen and combined with the reference beam on the measurement plane in the so-called off-axis configuration [7]. The reference beam and sample beam interfered with each other and formed a hologram of the specimen that contains the amplitude and phase information of the sample. The detailed reconstruction process was performed by re-illumination of the hologram by a digital reference wave, and a numerical correction of the wavefront aberrations induced by the objective and by the off-axis geometry was reported previously [5]. To calculate the phase holograms recorded by the CCD camera, we used KOALA

![Fig.1. Experimental setup configurations of the transmission DHM. A laser diode produces the coherent light, which is divided by a beam splitter. The orange color beam shows the DHM laser beam path with object and reference arm, and the specimen is illuminated by one beam through a condenser. A microscope objective collects the transmitted light and forms the object wave, which interferes with a reference beam to produce the hologram recorded by the digital CCD camera.](image-url)
Fig. 2. Digital holographic response of an individual PC12 cell. (A) Quantitative digital holographic phase images of a single living PC12 cell after 100 mM K⁺ stimulation. The image marked as 20 s was analyzed after injecting Hanks’ solution as a control stimulant. K⁺ solution was injected at 24 s after control stimulation, and the images marked as 27, 30, 40, 60, and 80 s were analyzed after high K⁺ stimulation. (B) Appearance of a single living PC12 cell in the quantitative 3D holographic phase images before and after high K⁺ stimulation. The image marked as 20 s (7.1 µm) shows the initial height of the cell after Hanks’ solution stimulation. The images marked as 27 s (height 8.2 µm), 30 s (8.6 µm), 40 s (9.4 µm), 60 s (9.5 µm), and 80 s (9.6 µm) were analyzed after stimulating high K⁺ solution. (C) Time course of PC12 cell height change at the cross section of panel A before and after stimulation with 100 mM K⁺.
Results

DHM can monitor in situ and rapid change in 3D morphology of a single PC12 cell

We monitored 3D morphology change of a single PC12 cell on K⁺ stimulation, which induced depolarization following intracellular Ca²⁺ elevation. High K⁺ solution was ejected from a micropipette pointed toward the cells, and cells responded with a robust intracellular Ca²⁺ rise, as reported by a rapid increase of Ca²⁺ by the increase of fluorescence intensity [11]. The cell sample is measured from the phase images, and the 3D images and phase profile line constructed from phase images provide the morphological identity to the cell. From the phase measurement, we can determine the physical thickness of the PC12 cells for indefinite periods of time. Fig 2A illustrates the appearance of a single living PC12 cell in the quantitative digital holographic phase images before and after injection of high (100 mM) K⁺ solution. The phase images were reconstructed for the measurement of height increase observation by either plotting line profiles or constructing 3D images. Fig. 2B illustrates the appearance of a single living PC12 cell in the quantitative 3D holographic images before and after 100 mM K⁺ stimulation. The shape transformation of PC12 cells attached on the bottom surface of a glass base dish was clearly observed. The image marked as 20 s was analyzed after injecting Hanks’ solution as a control stimulant. The initial height of the cell was measured as 7.1 μm. After stimulating high K⁺ solution at 24 s, a clear change in 3D morphology of the cell was observed. As can be seen, a sudden increase occurred in cell morphology within a few seconds from the application of high K⁺ solution to the PC12 cell. The images marked as 27 s (8.2 μm), 30 s (8.6 μm), 40 s (9.4 μm), 60 s (9.5 μm), and 80 s (9.6 μm) were analyzed after high K⁺ stimulation. Results showed that application of high K⁺ solution initiated a rapid increase in the time-dependent signal for height change. From the Ca²⁺ measurement system, it was known that the intracellular Ca²⁺ increase in PC12 cells began within 3 to 5 s after K⁺ solution application (data not shown). As a consequence, the 3D shape of the cells changed dramatically, resulting in the appearance of sharp spines. As depicted in Fig. 2C, administration of high K⁺ induced a time-dependent increase in the cell morphology and line profiles. The increase in cell shape/volume began within 3 to 5 s of depolarizing solution application.

It is well known that 30 mM K⁺ is enough to excite PC12 cells [12,13]. Therefore, additional experiments were performed to validate the standard experimental protocol by the use of 30 mM K⁺ solution to observe 3D morphology change in a concentration-dependent manner. Typical phase distribution changes of PC12 cell bodies were induced by 30 mM K⁺ stimulation, and the images were acquired (data not shown). The representation of the phase distribution by stimulating 30 mM K⁺ solution showed a phase increase in a similar fashion, as previously shown in Fig. 2B for 100 mM K⁺ stimulation. After stimulating 30 mM K⁺ solution, the reconstructed images for height change measurement showed a clear change in both 3D morphology and line profile (data not shown). Furthermore, we endeavored to monitor height change via 3D morphology change of a single PC12 cell after stimulating K⁺ at low concentration (10 mM). From previous references, it was known that PC12 cells cannot be depolarized by 10 mM K⁺ stimulation [13]. On stimulating the PC12 cell with a low concentration of K⁺, no response was observed (data not shown). Our comparative results obtained by digital holographic measurement for PC12 cell height/width change at different points of time confirmed that 3D morphological change observed by stimulant stimulation was concentration dependent as well as time dependent. Fig. 3A depicts the comparative DHM response obtained in single PC12 cells after stimulating different concentrations of K⁺ stimulation. Results showed that once the cells were stimulated with a high enough concentration of their respective stimulant, significant changes in the cell morphology might be observed. Fig. 3B shows the cellular height increase of individual PC12 cells within 15 s after stimulating different concentrations of K⁺ solution.

Discussion

In this study, we have demonstrated a novel approach based on digital holography microscopy by which 3D morphology of a nerve model cell could be imaged on K⁺ stimulation for each time point. No special treatment of PC12 cells was required, and cells could be maintained viable under physiological conditions throughout the experiment. The digital holographic system allows the digital recording and reconstruction of living cell morphology and provides an unprecedented ability to measure cellular height or thickness and to monitor the behavior in response to chemical stimuli. We were interested in assessing rapid 3D morphology changes of intact single PC12 cells, and we found that none of the methods described above could adequately satisfy these requirements. We quantified the influence of exogenous stimulus on the phase measurement in the hologram plane in DHM under the assumption that the cell might change its morphology by depolarization. According to our demonstration, the phase images can be used for 3D morphology observation by either plotting line profiles or constructing 3D images. These parameters can be extracted from a single set of collected data, thereby making it possible to decide.
after the experiment which parameter to use. All information is stored automatically, making it possible to access and reevaluate the information after the experiment. The technique is nondestructive; the same sample can be used for sequential measurements, thereby eliminating the necessity to have separate samples for each time point. By using this technique, we clearly demonstrated thereby eliminating the necessity to have separate samples for tive; the same sample can be used for sequential measurements, the information after the experiment. The technique is nondestruc-
stored automatically, making it possible to access and reevaluate after the experiment which parameter to use. All information is stored automatically, making it possible to access and reevaluate the information after the experiment. The technique is nondestructive; the same sample can be used for sequential measurements, thereby eliminating the necessity to have separate samples for each time point. By using this technique, we clearly demonstrated that extracellular application of high K+ induced height increases of PC12 cells. This study demonstrated that DHM had the potential ability to sense extracellular exogenous stimuli for PC12 cells. The DHM-observed morphological changes of PC12 cells underlying stimulus sensing have not yet been fully determined. The phe-
omenon found in the current study is clearly of physiological importan
t. It is important to notice that the application of this technique allows for the first time to follow such fast shape changes of PC12 cells under chemical stimulus with high temporal resolution. In addition, 3D shape imaging was done to testify whether exogenous stimulus affects the morphology change of the normal PC12 cells or not. Our preliminary experimental results suggest that DHM measurement might be applicable for the real-time monitoring of activation-induced 3D morphology (shape/vol-
ume) change of living cells and tissues in a noninvasive and label-
free manner. Thus, we expect that DHM not only might be used as a new promising tool to analyze possible changes due to drug ac-
tion in a 3D environment but also could be applied as a powerful tool for medical diagnosis and monitoring of diseases.

Conclusion

We have demonstrated a unique noninvasive and label-free dig-
tal holographic technique achieving 3D images of PC12 cells. The major advantage of the holographic technique compared with normal microscopic imaging is that the hologram contains all of the information necessary to reconstruct the object at different positions (e.g., focusing at a certain object plane). Our experimental 3D morphology study showed that PC12 cells exhibited morpho-
logical modifications caused by exogenous stimulation. Our results confirmed that DHM provided the opportunity to easily access information about cell thickness and 3D change in an automatic and noninvasive manner. The possibility to use digital holographic phase image processing to gain information may be very useful in studies of cell condition, differentiation and viability. We expect that the novel DHM technique may soon replace conventional phase contrast methods for biological imaging.

Acknowledgment

We thank Toshikazu Tanida (Digital Micro Systems) for his cooperation in DHM operation.

References