Examining live cell cultures during apoptosis by digital holographic phase imaging and Raman spectroscopy

Alexander Khmaladze
SUNY University at Albany, Physics Department, 1400 Washington Avenue, Albany, NY 12222
akhmaladze@albany.edu

Abstract. Cellular apoptosis is a unique, organized series of events, leading to programmed cell death. In this work, we present a combined digital holography/Raman spectroscopy technique to study live cell cultures during apoptosis. Digital holographic microscopy measurements of live cell cultures yield information about cell shape and volume, changes to which are indicative of alterations in cell cycle and initiation of cell death mechanisms. Raman spectroscopic measurements provide complementary information about cells, such as protein, lipid and nucleic acid content, and the spectral signatures associated with structural changes in molecules. Our work indicates that the chemical changes in proteins, which were detected by Raman measurements, preceded morphological changes, which were seen with digital holographic microscopy.

1. Introduction
Digital Holographic Microscopy (DHM) is an imaging technique which is becoming a routine method for three dimension real time imaging and inspection of microstructures and biological systems on a nanometer scale. When the light wave is reflecting from (or propagating through) a microscopic object, it slows down. The resulting wave front changes reveal variations in the optical thickness of the object (see figure 1(a)). This optical path length can be obtained for each point of the object from the phase of light for each pixel of the image \( \varphi(x, y) \). The optical path length can be then converted to physical thickness (assuming that the index of refraction is known), providing the sample height information:

\[
t(x, y) = \frac{\lambda \cdot \varphi(x, y)}{2\pi (n - n_0)} \quad (1),
\]

where \( \lambda \) is the wavelength of light, \( n \) is the index of refraction of the sample, and \( n_0 \) is the index of refraction of the surrounding medium.
Figure 1. The principles of Digital Holographic Microscopy: (a) light wave propagating through a cell, showing the wavefront distortion, which resembles the shape of the cell, (b) the hologram of a reflective resolution target, (c) Fourier transform of (b), showing the first maximum, selected for subsequent reconstruction and (d) 3D reconstruction of the resolution target, showing the height of the steps of 100 nm.

In order to obtain the phase of light propagating through or reflecting from an object, DHM records the interference pattern between an object and a reference waves. The difference between DHM and “regular” holographic setup is that the interference pattern is recorded by a digital camera (see figure 1(b)), which can then be reconstructed numerically. Angular spectrum method, which is used here for numerical reconstruction, is based on performing a Fourier transform of the original hologram, with the subsequent suppression of a DC term (see figure 1(c)). The resulting plane waves are propagated in space numerically by multiplying them by a phase factor. Finally, after performing the inverse Fourier transform, the phase of light for each pixel is obtained as a phase of a complex number. Figure 1(d) shows the height map of the object in figure 1(b), obtained from the phase values.
for each pixel, which is then converted to height values using equation (1). Thus, DHM is a technique that can produce nanometer-scale measurements of height and can be used to image microstructures and biological systems in three-dimensions\(^1\).\(^2\).\(^3\).

Furthermore, DHM provides three-dimensional cell imaging in real-time, enabling monitoring of cell morphological changes during apoptosis\(^4\). Primary signatures of apoptosis include nuclear condensation, fragmentation in internucleosomal DNA, and formation of apoptotic bodies. In addition to that, one of the fundamental apoptosis characteristic is cell volume loss, termed apoptotic volume decrease (AVD), which can be monitored using DHM. Thus, since cell volume is associated with cellular apoptosis, DHM can be used as a great tool to provide information about volume change during this process.

Additionally, Raman spectroscopic studies of cellular cultures have demonstrated significant changes in Raman spectra as a result of cells undergoing apoptosis\(^7\)\(^8\)\(^9\)\(^10\). In this paper, we have monitored apoptotic volume changes of live central nervous system (CNS) cells (C6), while also acquiring its Raman spectral information during several stages of apoptosis to non-invasively monitor the chemical changes within these cells in real time.

2. Experimental

2.1. Digital Holographic Microscopic Measurements

The C6 glial cells were obtained from ATCC (Cat #ATCC® CCL-107™). They were grown to confluency in DMEM/F12 (50:50) media supplemented with 5% FBS and antibiotics. TO induce apoptosis, the cells were treated with 1µM by Doxorubicin (DOX).

Our transmission DHM setup is shown in figure 2. The 633 nm He-Ne laser beam is reflected by plane mirrors M1 and M2 into a microscope objective L1 and a plano-convex lens L2 to obtain a collimated beam with a large cross-section. Beamsplitter B1 then splits the laser beam into the reference and object arms. Lens L3 focuses the beam on the back focal plane of the microscope objectives OBJ1 and OBJ2. The reference beam is reflected by a plane mirror M3, passes through the microscope objective OBJ1, and into the beamsplitter B2, where it recombines with the object beam. Plane mirror M4 directs the object beam through the cell culture, which is kept in the environmental chamber (IBIDI Nanolive Heating System/Temperature Controller/Gas Incubation System), mounted on a three-dimensional translation stage. OBJ2 is also used to forms an image on the CCD camera. Numerical compensation is used to correct the curvature between the reference and object wavefronts\(^6\).

Figure 2. Transmission DHM setup (see text for details).
2.2. **Raman Measurements**

Raman spectra were acquired by a commercial micro-spectroscope (HORIBA XploRA PLUS). This Raman spectrometer is equipped with a 1024×256 TE air cooled CCD chip (pixel size 26 micron, temperature -60°C). HORIBA’s LabSpec6 software was used for data acquisition, fluorescent background removal, baseline correction, and peak fitting. Excitation wavelength was set to 785 nm. Entrance slit of 50 µm produced an approximate spectral resolution of 4 cm⁻¹.

Cells were kept in the incubator and removed for measurements at several time points. For each of the time points, three measurements of three accumulations of 60 seconds each were acquired. To minimize the effects of fluorescence, cells were grown and imaged on quartz substrates. Quartz substrates also provide a known Raman signature that can be used for normalization of Raman signal from different cell cultures.

3. **Results and Discussion**

Our DHM experiments were done on the cultured cells (~5000 cells) that were placed in an environmental chamber, where apoptosis was induced by adding 1µM DOX. The changes in cell volume were monitored in real time over a 3 hour period. Figure 3 shows the percent volume changes in C6 cells as a result of AVD. A substantial decrease in cell volume was observed within 40 mins after DOX treatment. Cellular volume continued to decrease significantly with time. After 150 minutes, the cells were detaching from the bottom of the petri dish, making further volume measurements unreliable. By then, the cell culture volume has decreased to 40% of the original volume.

![Figure 3](image-url)

**Figure 3.** Apoptotic cell culture volume measurements by DHM.

For Raman measurements, the cells were removed from the incubator, placed on the sample stage of the Raman microscope, and their spectra were collected at several time points \( (t=0 \text{ min}, t=50 \text{ min}, t=100 \text{ min} \text{ and } t=150 \text{ min}) \) after the induction of apoptosis with 1µM DOX. Control cell cultures spectra that were taken 200 minutes apart were also acquired.

The spectra were background subtracted, and the following peaks (which are indicative of molecular vibrations in lipids, nucleic acid and proteins), were fitted: 953, 1003, 1092, 1165, 1305,
1338, 1447, 1544, 1611, and 1661 cm$^{-1}$. In addition to that, we have observed peaks at 1054 cm$^{-1}$ and 1220 cm$^{-1}$. These peaks were identified as substrate peaks from our prior measurements of Raman signal from the substrate. Of these peaks, the height of 1220 cm$^{-1}$ peak was used for normalization.

The heights of normalized 1003 cm$^{-1}$, 1165 cm$^{-1}$, 1305 cm$^{-1}$, 1447 cm$^{-1}$ and 1661 cm$^{-1}$ peaks are shown in figure 4. The intensity of phenylalanine peak at 1003 cm$^{-1}$ remained approximately the same through the experiment. The peak at 1165 cm$^{-1}$ (C-C and C-N stretch of proteins) increased in intensity, presumably because of accumulation of proteins and progression of nuclear condensation$^9$. Then, at the last time point at $t=150$ min, when the cells were dying, the intensity decreased, which may indicate the breakdown of proteins. The peak at 1447 cm$^{-1}$ CH$_2$ (bending mode of proteins) and 1661 cm$^{-1}$ Amide I peak also showed similar behaviour. The peak at 1305 cm$^{-1}$ (CH$_2$ deformation in lipids) reduced in intensity, which likely indicates the decreased contribution of lipids to Raman signal obtained from these cells.

![Figure 4](image)

**Figure 4.** Heights of characteristic Raman peaks for apoptotic and control cell cultures (see text for details).

4. **Conclusion**

In this work, the changes in cell volume of C6 glial cells undergoing apoptosis were monitored by digital holographic microscopy, while the chemical changes within these cells were studied by Raman spectroscopy. Glial cells exposed to 1µM DOX exhibited a significant decrease in cell volume, a primary characteristic of apoptosis, which was clearly seen within 40 mins post treatment. A digital
holographic microscope, with the attached environmental chamber, permitted accurate measurements of cell volume for several hundred cells in real time over an extended time period.

Our Raman data indicates that the chemical changes in proteins preceded morphological changes, which were seen with DHM. Based on the results of this study, it is apparent that apoptosis is a unique process, which consists of organized series of events, leading to programmed cell death. This study also emphasizes that DHM and Raman based imaging tools can be developed for non-invasive and simultaneous monitoring of both morphological and chemical changes in cells during apoptosis, which can also be used to monitor other dynamic cell processes.

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