Real Time 3D Cytomorphological Imaging using Digital Holographic Microscopy and Fluorescence Microscopy for Space Biology

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Abstract: A microscope operating in Digital Holographic Microscopy (DHM) and classical widefield epi-fluorescence microscopy in a time sequential manner is developed to study morphological alterations of mouse myoblast cells under simulated microgravity in real time.
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1. Introduction

Spaceflight causes alterations in living organisms that is associated to reduced gravity. Previous research works focusing on muscle tissue have shown that spaceflights cause muscle atrophy and associated decline in peak power and force [1-3]. In order to better understand the underlying biological mechanisms, these findings directed researchers from tissue level to cellular level investigations. Organization and stabilization of cytoskeleton can be explained by considering cell as a tensegrity structure that preserves the integrity based on self balancing of tension and compression on the structural elements such as microtubules and microfilaments [4]. Cell morphology experiments conducted in parabolic flight missions showed that exposure to microgravity for 20 seconds is sufficient to observe slight change on cell shape [5]. Cell spreading, lamellipodia formation and actin filament redistribution were observed at longer duration parabolic flight experiments [6]. Random positioning machine (RPM), which is composed of two randomly rotating concentric frames that accommodate biological sample at the center of the inner frame, was proven to be valid ground based microgravity simulation tool suited for such biological studies[7,8].

In this work, a microscope is constructed for the real time imaging of living mouse myoblast cells (C2C12) exposed to simulated microgravity on a RPM using digital holographic microscopy (DHM) and fluorescence microscopy. DHM has proven itself as a robust technique for real time 3D microscopy and attracted the attention for microgravity studies [9]. In this technique, both sample thickness and refractive index information are accessible in real time with diffraction limited lateral resolution and nanometer level axial accuracy for thickness [10]. Selective imaging of transfected cytoskeletal sites is carried out by fluorescence microscopy. Taking advantage of these capabilities of both methods, cell contour variation in three dimensions and actin filament arrangement are imaged in real time to access more information on the cytoskeletal dynamics under microgravity.

2. Method

Holography has been a well known method for a long time to encode the complex field of an object in form of intensity. It basically relies on the recording of the interference pattern of two waves, one of which experiences an unknown distortion caused by an object (also called as object wave), emerging from the same coherent source. Later, the recorded interference pattern is illuminated by a wave that is identical to the known wave (reference wave) of recording procedure. This illumination results in the construction of a perfect replicate of object wave and eventually a 3D image of the object. In digital holography, holograms are recorded on a charge coupled device (CCD) camera, and the complex object field is numerically reconstructed. Reconstructed complex object field is decomposed into amplitude and phase images that provide absorption and optical path length information of the object in the full field [11]. Optical path length (OPL) is considered to be either topology or optical thickness (absolute thickness times mean refractive index) of the sample, according to the recording scheme (reflection or transmission).

Depending on the relative axes orientation of reference and object waves, hologram recording is classified as in-line or off-axis. In the case of off-axis recording, object and reference waves reach to the CCD camera with a small angle between their axes. Thanks to this tilt angle, spatial frequency information related to the object wave can be filtered from the unnecessary frequency content at the beginning of the numerical treatment of the hologram [12]. With the numerical corrections any aberration can be easily compensated [13]. DHM in transmission mode has become an attractive tool for the non-invasive marker free imaging of 3D cell morphology with the advantage of speed and digital refocusing over other methods [14].

3. Experimental Setup

Experimental microscope that is fixed on RPM encloses DHM and widefield epi-fluorescence microscopy that operate in time sequential manner in the same housing. For DHM operation, a collimated diode laser beam is split into two arms using a beam splitter. One of the arms is expanded and directed to a dichroic beam splitter, while the other arm first passes through a condenser lens, cell sample, and a 60X microscope objective. Both arms are combined by the dichroic beam splitter and form the hologram on the Andor Luca electron multiplying CCD (EMCCD) camera located at the image plane. This dichroic beam splitter is selected to have a suitable spectral response (cut-off wavelength at 500nm, 85/15 at 653nm) to be used for both fluorescence and DHM. In the fluorescence microscopy mode, a light emitting diode (LED) with the central wavelength of 470nm and width of 30nm that coincides with the excitation band for the EGFP transfected actin filaments in C2C12 cells is used as the excitation source. A lens and a diaphragm are placed on the excitation path to provide uniform illumination. Shaped illumination light passes through the excitation filter and dichroic beam splitter and impends on the cell sample through the microscope objective. Emitted fluorescence signal is imaged on the camera after being directed by the dichroic beam splitter and passed through emission filter. Fig. 1 shows the 3D model of the microscope housing and optical scheme.

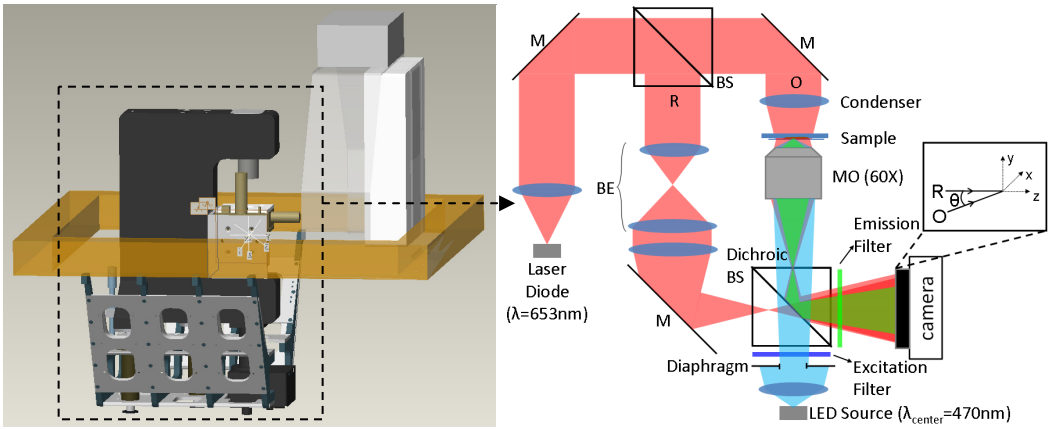
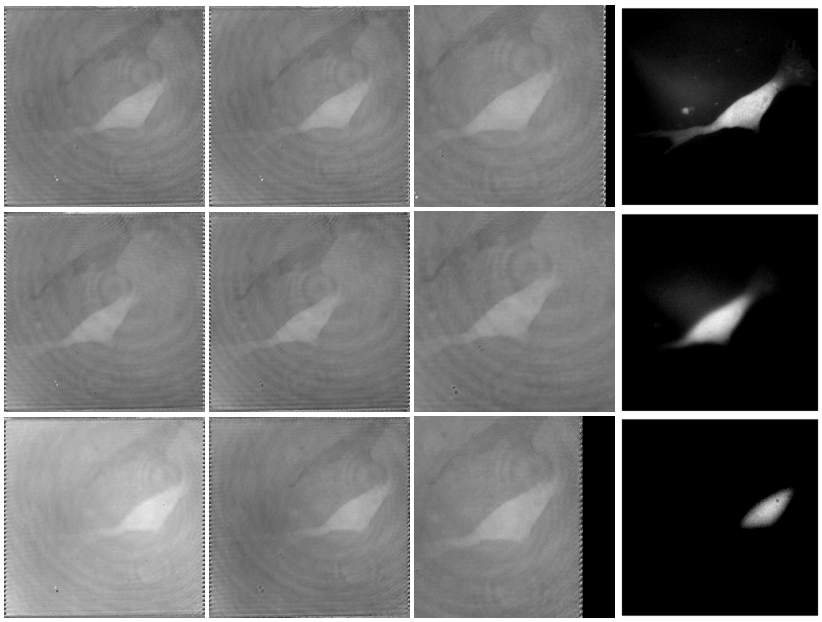


Fig. 1. 3D model and simplified optical schematic of the microscope: *M* mirror, *BS* beam splitter, *BE* beam expander, *MO* microscope objective, *R* reference wave, *O* object wave

Experimental platform of RPM is large enough to house a small computer and several other small sized instruments as well as the microscope with limited number of inputs. Experimental computer on the platform is responsible from the image acquisition and source switching between the laser of DHM and LED of fluorescence microscopy mode sequentially. However, computational power of this computer is not sufficient to post process captured holograms and reconstruct the phase images. Experimental computer is linked to an external computer via Wi-Fi over TCP-IP protocol and streams lossless compressed forms of captured holograms and fluorescence images to the external computer. External computer hosts software to reconstruct phases from streamed holograms. In this way, OPL variations on the cells are observable in real time. Furthermore, in case of sample shift in axial direction it is possible to refocus phase images digitally.

4. Results

In order to evaluate the performance of the microscope, a complete automated operation setting is constructed with all required elements to image fixed C2C12 cells with EGFP transfected actin filaments on a 1D rotational frame. As an extremely important advantage of DHM in such a case, tilt of beams and defocusing of sample due to stability problems can be digitally compensated. Following figure shows phase and fluorescence images from this automated setup. Fig.2a has phase images for three different rotational positions. Because of the slight movement of reference beam from position to position, original reconstructed phase images express tilt like linear phase increase. These images are numerically corrected for this effect. However, defocusing and shift of the cell are observable on these images. After application of digital focusing procedure to every hologram independently, all phase can be reconstructed at best focus plane as shown in fig.2b. Residual shift of the cell is corrected using a simple tracking code fig.2c.



(a) (b) (c) (d)

Fig. 2. A set of (a-c) phase (d) fluorescence images of a fixed C2C12 cell during microscope rotation. Phase images with tilt (a), digital focus (b), and shift (c) correction.

5. Conclusion

A microscope operating in DHM and widefield epi-fluorescence microscopy was built to observe cytomorphological modifications of living cells under simulated microgravity by RPM in real time. Microscope sequentially operates in DHM and fluorescence microscopy modes under the control of an on-board computer, and numerical hologram reconstruction is carried out on a remote server. Initial results from the microscope on a 1D rotational frame with fixed cells confirms preliminary DHM robustness for such imaging conditions thanks to numerical corrections and focusing. Future work involves actual experiments carried out for living cells to observe cytomorphological modification and further improvement of post processing methods.

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