DIGITAL HOLOGRAPHIC MICROSCOPY , A NEW IMAGING TECHNOLOGY APPLIED TO LIFE SCIENCES

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Abstract: Digital Holographic Microscopy (DHM) is a new imaging technique offering high resolution and real time observation capabilities: longitudinal resolutions of a few nanometers in air and a few tens of nanometers in liquids are achievable, provided that optical signals diffracted by the object can be rendered sufficiently large. Different biological objects, living biological cells in culture, organelles, nuclei have been observed. It is shown that very small movements and deformations are observable, which are produced, in particular, by stimulation of excitable cells (neurons) and their metabolic activities. This new imaging approach is based on the coherent propagation of light in dielectric matter, biological matter in particular. The originality of our approach is to provide both a slightly modified microscope design, yielding digital holograms of microscopic objects and an interactive computer environment to easily reconstruct object shape from digital holograms at the nanoscale. Real time image reconstruction and 3D rendering is henceforth possible, providing a new tool in the hands of biologists. Direct imaging of living cells and tissues structures are realized i*n vitro*.

Introduction

DHM (Digital Holographic Microscopy) is an imaging modality that meets a growing interest in 3D microscopy of small biological objects like isolated cells, or tissue specimens [1-9]. Interest has been put recently to the fore for biological applications [10-13]. It is characterized by the fact that the wavefront is reconstructed in a numerical form, directly from the digitalized hologram, in the form of a set of complex numbers describing the wavefront. Accuracy in the sub-micron-range along the optical axis can be achieved, down to the nanometer. Applied to microscopy, this technique, that we have called Digital Holographic Microscopy (DHM) [3], comprises a microscope objective to adapt the sampling capacity of the camera to the information content of the hologram.

Materials and Methods

Digital Holographic Microscopy is a new imaging technique with high resolution and real time observation capabilities. Digital holography appears as an imaging modality, which offers large and appealing perspectives in microscopy. The method brings quantitative data that are derived from the digitally reconstructed complex wavefront. Simultaneous amplitude and absolute phase are computed from a single hologram, acquired digitally with a video camera and used to determine precisely the optical pathlength of the beam propagating in the micro-optical device or reflected by it. Highly resolved images of the refractive indices and/or of the shape of the object can be derived from these data. The method requires the adjustment of several reconstruction parameters [3], adjustment, which can be performed easily with a computer-aided method developed in our group. Using a high numerical aperture, sub-micron transverse resolution has been achieved: 600nm lateral resolution. Accuracies of approximately half a degree have been estimated for phase measurements. In reflection geometry, this corresponds to a vertical resolution less than 1 nanometer at a wavelength of 632 nanometers. In the transmission geometry, the resolution is limited around 10nm for transparent objects with refractive index around 1.5 in air.

Digital Holographic Microscopy (DHM) comprises the recording of a digital hologram of the specimen by means of a standard CCD camera inserted at the exit of a Mach-Zhender type interferometer (see figure 1) . The hologram is formed on the CCD camera, as the result of the interference of the object wave *O*, scattered by the specimen, and of the reference wave *R,* which is beamed independently on the other arm of the interferometer. The setup has been designed for imaging specimen in transmission, which is the most easy

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configuration for the observation of transparent biological objects. Several other configurations are possible, according to the targeted applications, but will not be discussed here. The reference beam is controllable both in intensity and polarization in order to optimize contrast and signal.

For the investigation of microscopic specimen, a microscope objective (MO) is introduced in the interferometer to adapt the sampling capacity of the camera to the information content of the hologram.

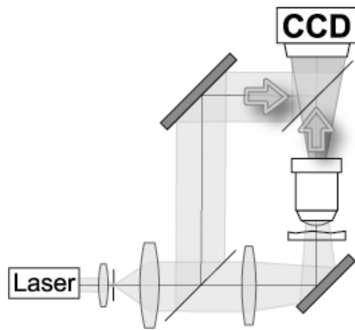

Figure 1:Optical setup for transmission DHM

The hologram intensity is given by expression (1) :

$$I_H(x,y) = (R+O)^* \cdot (R+O) = |R|^2 + |O|^2 + R^*O + RO^* \quad (1)$$

Where O and R are respectively, on one side, the wavefield of the wave scattered by the object and collected by the MO, and, on the other side, the wave field of the reference wave R. The reconstruction method is based on the restoration of the object wavefield O, which is usually achieved in the hologram plane x-y by forming the product of the hologram intensity by the reference wave R:

$$O(x,y) = I_{H\ filtered}(x,y) \cdot R(x,y) \Big/ \left| R \cdot R^* \right| \quad (2)$$

The intensity of the hologram $I_H(x,y)$, must be filtered by selecting, in the Fourier domain of the hologram, the signal corresponding to the third term in equation (1): R^*O, which will be the only one, capable to restore uniquely the wavefront O.

Then, the wavefield must be propagated in the plane of the object. This can be simply achieved by computing the Fresnel transform of the wavefield. The mathematical expression (3) is used for that computation.

$$O(\xi,\eta) = -i \cdot \exp(ikd) \cdot \mathbb{F}^{\sigma}_{Fresnel}\left[O(x,y)\right] \quad (3)$$

which, in the paraxial approximation, can be put in form (4), which will be computed after discretization.

$$\mathbb{F}^{\sigma}_{Fresnel}\left[O(x,y)\right] = \\ = \frac{1}{\sigma^2} \exp\left[\frac{i\pi}{\sigma^2}\left(\xi^2+\eta^2\right)\right] \cdot \quad (4) \\ \cdot \mathbb{F}_{Fourier}\left\{O(x,y) \cdot \exp\left[\frac{i\pi}{\sigma^2}\left(x^2+y^2\right)\right]\right\}$$

with: $\sigma = \sqrt{\lambda d} = \sqrt{2\pi \frac{d}{k}}$ (5)

The reconstructed wavefront simultaneously delivers the phase information, which reveals the 3D topography of an object surface and the intensity image, as obtained by conventional optical microscope. In the transmission geometry (that is considered in this paper), the phase information yields the differential Optical Pathlength: OPL of the beam crossing the biological specimen, compared to the reference beam crossing the perfusion medium.

The originality of our approach is to provide ultra-high accuracy in the reconstructed images, both by using a slightly modified microscope design yielding digital holograms of microscopic objects, and by taking advantage of an interactive computer environment to reconstruct easily object shape from digital holograms. The use of a slightly off-axis configuration, enables to capture the whole image information by a single hologram acquisition [3]. By using a time gated camera or illumination sources, it is possible to avoid perturbations originating from parasitic movements or vibrations or perturbing ambient light. On the other side, wavefront reconstruction rate may be as high as 15 frames/second, making DHM an ideal solution to perform real time measurements on living specimens.

Digitalized hologram are taken in a slightly off axis geometry. In our DHM implementation, no time heterodyning or moving mirrors are required and the microscope design is therefore simple and robust. DHM brings quantitative data derived simultaneously from the amplitude and phase of the complex reconstructed wave front diffracted by the object. Microscopic objects can be imaged in transmission geometry. DHM provides an absolute phase image, which can be directly interpreted in term of refractive index and/or profile of the object. Very high accuracies can be achieved, which are comparable to that provided by high quality interferometers, but DHM offers a better flexibility and the capability of adjusting the reference plane with the computer, i.e. without positioning the beam or the object. This computerized procedure adds much flexibility and all adjustment procedures can even be made transparent to the user.

The holograms are acquired with a CCD or MOS camera and then digitized. A digital expression of the wavefront is formed in the hologram plane x-y and then propagated in the object plane ξ–η according to the Huyghens-Fresnel diffraction law.

Results

The method described provides absolute phase-contrast images of living cells in culture, for which the reconstructed phase distribution provides a quantitative and precise measurement of the 2D distribution of optical pathlengths at the surface of the specimen. In biology, quantitative information concerning cell

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morphology and volume, as well as the protein content potentially can be derived from DHM images, provided that adequate processing of data will be chosen. Using a high numerical aperture, a sub-micron transverse resolution is achieved. An accuracy of a fraction of a degree (λ/1800) [13] has been estimated for phase measurements. In the transmission geometry, the resolution for thickness measurements depends on the refractive index of the specimen and a resolution in the range of a few tens of nanometers has been estimated for living cells in cultures.

Figure 3: shows an example of a 3D image of a preparation of neuron cells in culture, obtained by Digital Holographic Microscopy: thickness of sample has been estimated to 7 microns, with an optical pathlength resolution of around 40 nm. The field of view is around 30 x 30 µm.

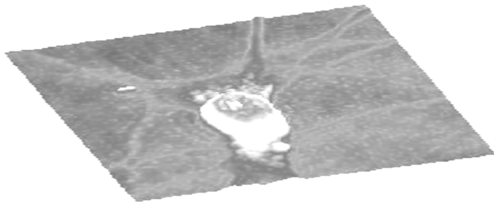

Fig.2: Image of living preparation of one neuron cell in culture obtained by Digital Holographic Microscopy: thickness of sample has been estimated to 7 microns, with a resolution of 40 nm . The field of view is around 30 x 30µm

Discussion

Living neurons in culture have been imaged with unrivalled resolutions [10]. Minute deformations and movements of living cells could be detected and monitored. Both morphology and functional studies can be carried out on living preparations [13]. The method is completely non invasive, because the illumination levels are very low, much less than in fluorescence microscopy. Cultures can be kept alive for several hours and even days. Therefore, long-term evolution of cells can be studied. On the other hand, fast acquisition (at video rate or even faster) is feasible, because the method is a non-scanning one and acquisition is made in parallel with a large bandwidth, showing tiny movements of the membranes or cytoplasm.

Conclusions

The interest of Digital Holography applied to Microscopy can now be considered as established in its theoretical fundaments and in a variety of practical applications.

Several biological applications have been already proved: cell and tissue observations, visualisation of protein deposit, surface structuring and patterning in view of biocompatibility and biomaterials developments. The quantitative study of cytoarchitecture of cells and more complex arrangement of cells is essential in the understanding of biophysical mechanisms. In particular, the genesis of pathology: cancer, neuro-degeneration, indirect effects of inflammation, oedema, viral infections can benefit from cell morphology studies. Cell dynamics is a preferential domain where DHM technology will bring new input: fast imaging capabilities, non-scanning and non-touch features of DHM appear as determinant advantages in the context of biology. Demonstration that minute deformations and movements of living cells can be detected and monitored has been already brought[13]. Both morphology and functional studies can be carried out on living preparations. The method is completely non invasive, because the illumination levels are much lower than fluorescence based imaging techniques, including multi-photon microscopy. Cultures can be kept alive for several hours and days.

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