

Digital holographic microscopy, a new optical imaging technique to investigate cellular dynamics

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ABSTRACT

We have developed a digital holographic microscope (DHM), in a transmission mode, adapted to the quantitative study of cellular dynamics. Living cells are optically probed by measuring the phase shift they produce on the transmitted wave front. The high temporal stability of the phase signal, equivalent to $\lambda/1800$, and the low acquisition time (down to 20 μ s) enables to monitor cellular dynamics processes. An experimental procedure allowing to calculate both the intracellular refractive index and the cellular thickness (morphometry) from the measured phase shift is presented.

Keywords: Digital Holography Microscopy, 3D cellular dynamics, intracellular refractive index

1. INTRODUCTION

In biology, most microscopy specimens, including living cells, are transparent and differ only slightly from their surroundings in terms of absorbance, thus providing very modest change in the amplitude of the light wave. The examination of such transparent specimens, having the capacity to alter the phase of the detected lightwave and called phase object, led to the development of optical contrast-enhancing imaging techniques.

Among the numerous modalities of contrast-enhancing techniques, which have been developed to visualize non invasively unstained transparent specimens, phase contrast (PhC), as well as Normarski's differential interference contrast (DIC) are available for high-resolution light microscopy and widely used in biology. Although, these techniques reveal the structure of such transparent specimen, the phase information provided is qualitative[1].

Recently, new digital interference microscopy techniques, have demonstrated quantitative phase measurements with interferometric resolutions, i.e. lateral micrometer range and sub-wavelength axial resolutions, of transparent biological specimens, in particular living cells in culture, without the use of any contrast agent [2,3,4].

In this proceeding, we present the real time visualization of cellular dynamics with a digital holographic microscope (DHM). This DHM technique is based on an original numerical reconstruction of holograms [5], which enables the reconstruction of both the amplitude and the quantitative phase contrast images with an interferometric resolution from a single recorded hologram. The numerical reconstruction allows for the correction of lens aberrations; it also enables to compensate for temporal fluctuations induced by environmental and experimental perturbations.

The measured phase shift depends on both the refractive index and the thickness of the specimen, two quantities respectively linked to the nature of the intracellular content and to the morphometry of the specimen. This combined information of phase images does not provide straightforward data about the underlying cellular process.

An experimental protocol called “decoupling procedure” is presented here with the aim of measuring separately the integral refractive index and the cellular thickness from the quantitative phase images of living cells.

2. METHOD

The experimental set-up is a modified Mach-Zender configuration (Fig. 1A). Light transmitted by the specimen is collected by a microscope objective (MO) that forms the object wave O , which interferes with a reference wave R to produce the hologram intensity I_H recorded by the digital camera (Fig. 1A)). Holograms are recorded in an off-axis geometry. Acquisition, digitization and reconstruction of holograms were performed on a 3.2 GHz Pentium IV computer using a video frame grabber. The acquisition time is currently only limited by the speed of the camera (down to $\sim 20\mu\text{s}$). The reconstruction process is achieved in real-time (>10 images/s). A detailed description of the algorithm used for hologram reconstruction has been previously described in [5,6]. We mention that the processing of hologram allows a numerical reshaping of complex wave fronts and of their propagation, thereby replacing the need of complex opto-mechanical adjustment procedures, and fine alignment of the sample along the optical axis. This allows monitoring living material with a great ease of use.

Primary cultures of mouse cortical neurons were prepared according to Brewer and observed in a closed perfusion chamber (Fig.1 A inset) used to apply different solutions.

3. RESULTS AND DISCUSSION

Quantitative phase images of living neurons in culture are obtained by DHM (Fig.1B). The value of each pixel i of the reconstructed phase image can be expressed as:

$$\varphi_i = \frac{2\pi}{\lambda} \int_0^{h_i} n_{c,i}(z) dz + n_m(D - h_i) = \frac{2\pi}{\lambda} \left((\bar{n}_{c,i} - n_m) h_i + n_m D \right) \quad (1)$$

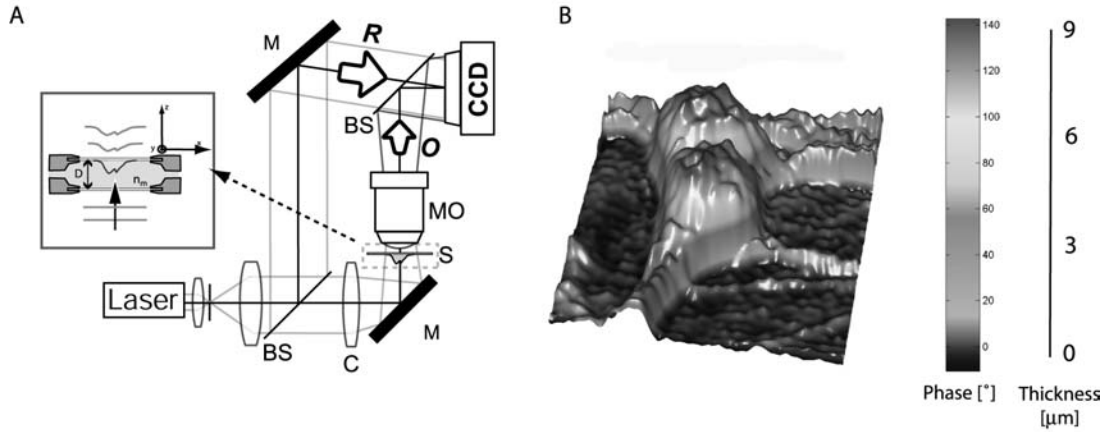


Fig. 1. Basic configuration for digital holographic microscopy (DHM). A VCSEL laser diode produces the coherent light ($\lambda = 658$ nm) which is divided by a beam splitter (BS). The specimen (S) is illuminated by one beam through a condenser (C). A microscope objective (MO) collects the transmitted light and forms the object wave (O) which interferes with a reference beam (R) to produce the hologram recorded by the digital CCD camera (Basler A101f). B. 3D perspective representation of the quantitative phase contrast image of 3 neurons in culture observed with a 63x MO (NA=0.8). Each pixel represents a quantitative measurement of the cellular optical path length (OPL). The scale (at right) relates the OPL (in degree) to the morphology (in μm) using the measured mean value of the neuronal cell body refractive index $\bar{n}_i = 1.3774$. Inset, schematic representation of cultured cells mounted in a closed perfusion chamber. M means mirror others abbreviations define in text

where λ is the wavelength z is the axial coordinate, h_i is the cellular thickness corresponding to the pixel i , $n_{c,i}(z)$ is the function representing the value of the intracellular refractive index along the cellular thickness h_i , $\bar{n}_{c,i} = 1/h_i \int_0^{h_i} n_{c,i}(z) dz$ is the mean value of the intracellular refractive index ($\bar{n}_{c,i}$) along the cellular thickness h_i ,

called the integral refractive index, n_m is the constant refractive index of the perfusion solution and D is the height of the perfusion chamber. The product $n_m D$ is a reference value that can be measured anywhere outside the cell. Monitoring this reference value is important because it enables the compensation of mechanical or thermal instabilities of the set-up during the experiment. For each pixel i , the component of the signal which is specific to the cell, $(\bar{n}_{c,i} - n_m) h_i$, depends on the cell thickness (h_i), the integral refractive index ($\bar{n}_{c,i}$) and the refractive index of extra-cellular medium (n_m), whose values have been measured with an Abbe refractometer at $\lambda = 664$ nm.

Present performances of our set-up enable, with 16 phase image average, a temporal phase stability of 0.2 degree, corresponding to $\lambda/1800$, over several hours, for each pixel of a blank phase image i.e. an experimental configuration corresponding to a perfusion chamber without the presence of any cell in it. As far as cells in culture with a mean refractive index of around 1.375 [8, 9, this study] are considered, this stability corresponds to a vertical sensitivity of ≈ 11 nanometers. For the present experiments achieved with a NA=0.8 and at a wavelength of $\lambda = 664$ nm, we obtain a transverse resolution of ≈ 0.6 μm in good agreement with the Rayleigh's criterion for coherent illumination. The measured irradiance at the neuronal plane was ~ 200 $\mu\text{W}/\text{cm}^2$, which is several orders of magnitude lower than the power used in classical confocal laser scanning microscope.

The phase signal depends on both the thickness and the refractive index of the specimen. To decouple these two contributions, a procedure that we named "decoupling procedure" is applied. It consists in perfusing, consecutively to the standard perfusion solution, a second solution with the same osmolarity (to avoid cell volume variation) but with a different refractive index $n'_m = n_m + \delta n$ and to record the two corresponding holograms. Specifically, the refractive index of the second solution is increased by replacing mannitol (a hydrophilic sugar present in the standard perfusion solution) with equal molarity of the hydrophilic molecule Nycodenz. Typically addition of 4% w/v of Nycodenz increases the refractive index of the solution by $\delta n = 0.006$.

For each pixel i , we obtain two phase recordings ($\varphi_{1,i}, \varphi_{2,i}$). The first one is obtained during the perfusion of the standard solution containing mannitol

$$\varphi_{1,i} = \frac{2\pi}{\lambda} (\bar{n}_{c,i} - n_m) h_i, \quad (1)$$

and the second one when the decoupling solution has completely replaced the standard solution in the perfusion chamber.

$$\varphi_{2,i} = \frac{2\pi}{\lambda} (\bar{n}_{c,i} - (n_m + \delta n)) h_i \quad (2)$$

By solving this equation system, we obtain, for each pixel i , the integral refractive index $\bar{n}_{c,i}$ and the cellular thickness h_i separately.

However, over the 30 seconds solution exchange time of the perfusion chamber, the living cells present micro-movements, which cause artifacts in the calculations of $\bar{n}_{c,i}$ and h_i . Consequently, spatial averaging was used to substantially reduce this micro-movement artifact. Specifically, the mean integral refractive index of a neuronal cell \bar{n}_c , is calculated by taking the average of the integral refractive index over a cellular body. Such a mean integral refractive index \bar{n}_c presents a temporal stability of 0.0003 within 30-second intervals. Subsequently, as a first approximation, \bar{n}_c rather than $\bar{n}_{c,i}$ was used to calculate the cellular thickness of each pixel i . This allows estimating a thickness of 1-3 μm for the neuronal processes and of ~ 9 μm for the cell body (Fig 1B).

The decoupling procedure described here offers an efficient solution to address the question of refractive index measurements on living cells. Only a few reports have appeared in this field. The decoupling procedure has been applied during a hypotonic shock achieved by replacing the standard perfusion solution (229 mOsm/kg H₂O) by a hypotonic solution (144 mOsm/kg H₂O). The decoupling procedure shows the cell body morphometry of two neurons in

culture. Specifically, each image pixel i represents the cellular thickness h_i , thus allowing to visualize the neuronal shape. These morphometry images indicate clearly the expected hypotonic neuronal swelling.

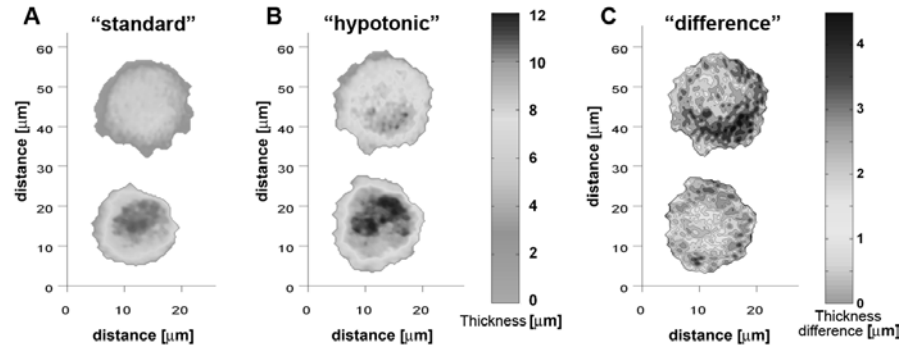


Fig. 2. Morphometry of 2 cell bodies before (panel A) and 3 minutes after the onset (panel B) of a hypotonic shock. Here the z-axis (cellular thickness) is expressed in micrometers. These values were obtained using the results of the decoupling procedure. C. Color-coded distribution of thickness variations resulting from the subtraction of the "standard" image to the "hypotonic" image.

We also mention that the hypotonic shock induces a mean integral refractive index decrease of around 0.002-0.003. This decrease is consistent with a hypotonic water influx, resulting in a dilution of the intracellular protein concentration, the cellular component which largely determines the mean integral refractive index value [6]. This decrease also allows to explain the paradoxical phase signal decline (data not shown) observed during the hypotonic shock.

4. CONCLUSION

Digital holography microscopy (DHM) is an efficient and easy-to-operate technique that allows to obtain, from a single recorded hologram, quantitative phase images of living cell dynamics with interferometric resolution i.e. with an axial sensitivity of ≈ 11 nanometers. These dynamic quantitative phase images, containing information about both the cell morphometry and the integral refractive index, can be unambiguously interpreted thanks to the decoupling procedure presented here. The intracellular refractive index being related to the composition of the intracellular medium, in particular to proteins, spatial refractive index mapping represents a promising way to monitor the local modifications of intracellular proteins distribution occurring during cellular processes.

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