

Advantages of digital holographic microscopy for real-time full field absolute phase imaging

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ABSTRACT

Different interferometric techniques were developed last decade to obtain full field, quantitative, and absolute phase imaging, such as phase-shifting, Fourier phase microscopy, Hilbert phase microscopy or digital holographic microscopy (DHM). Although, these techniques are very similar, DHM combines several advantages. In contrast, to phase shifting, DHM is indeed capable of single-shot hologram recording allowing a real-time absolute phase imaging. On the other hand, unlike to Fourier phase or Hilbert phase microscopy, DHM does not require to record in focus images of the specimen on the digital detector (CCD or CMOS camera), because a numerical focalization adjustment can be performed by a numerical wavefront propagation. Consequently, the depth of view of high NA microscope objectives is numerically extended. For example, two different biological cells, floating at different depths in a liquid, can be focalized numerically from the same digital hologram. Moreover, the numerical propagation associated to digital optics and automatic fitting procedures, permits vibrations insensitive full-field phase imaging and the complete compensation for a priori any image distortion or/and phase aberrations introduced for example by imperfections of holders or perfusion chamber. Examples of real-time full field phase images of biological cells have been demonstrated.

Keywords: Digital holography, absolute phase measurement, aberration compensation, microscopy

1. INTRODUCTION

The phase imaging technique such as phase contrast or Differential Interference Contrast (DIC) microscopies increased the apparent contrast of the transparent or semi-transparent objects such as a lot of biological specimens or cells. But if these types of microscopes give high resolved qualitative contrast image, they do not deliver quantitative data of the phase. Therefore different techniques were developed in the last decade to obtain full field, absolute and quantitative phase imaging, especially in life sciences applications. Even, different denomination exists for the different techniques such as phase-shifting,¹⁻⁵ Fourier phase microscopy,^{6,7} Hilbert phase microscopy,⁸⁻¹¹ or digital holographic microscopy (DHM),¹²⁻¹⁵ these techniques are very similar, as it will be demonstrated in this paper. But it will be shown that DHM is clearly advantageous compared to phase-shifting for real-time monitoring of biological or metrological applications, due to a single acquisition need. Furthermore, DHM can use digital optics in order to increase digitally the depth of field and to compensate for phase aberrations and image distortion introduced by the optical optics of the setup (microscope objective, holder, lenses...) that is not possible with Fourier phase or Hilbert phase microscopy.

2. DIGITAL HOLOGRAPHIC MICROSCOPE

Digital holographic microscopes (DHM) allow retrieving quantitative information of object wavefront by the numerical reconstruction of its amplitude and phase from a single digital hologram recorded in an off-axis geometry by a CCD or CMOS camera.^{13,14,16}

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2.1. DHM Setup

The setup is based on a classical Mach-Zehnder off-axis holographic interferometer [Fig. 1(a) in reflection and (b) in transmission]. The beam coming from the laser source is split. On one side, the object beam \mathbf{O} illuminates the sample and a microscope objective (MO) is used to increase the lateral resolution. Then, the object wave interferes on the CCD with the reference beam \mathbf{R} to produce a hologram [upper image of Fig. 1(c)]:

$$I_H(x, y) = |\mathbf{R}|^2 + |\mathbf{O}|^2 + \mathbf{R}\mathbf{O}^* + \mathbf{R}^*\mathbf{O}, \quad (1)$$

where, the two first terms are the zero order term and the third and fourth are the real and virtual images.

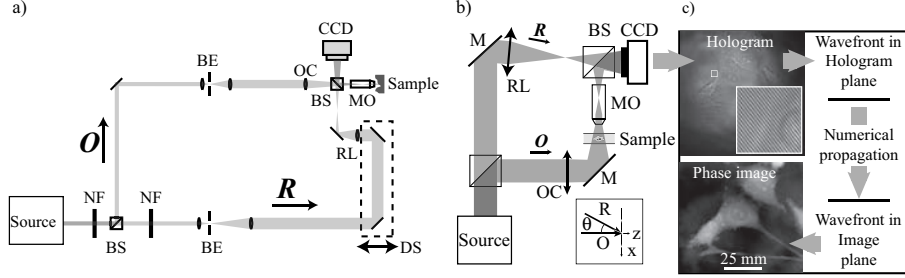


Figure 1. Experimental setups in reflection (a) and transmission (b) and reconstruction procedure (c). \mathbf{O} : object wave, \mathbf{R} : reference wave, NF: neutral filter, BS: beam splitters, BE: Beam Expander, MO: Microscope Objective, OC: Object Beam Condenser, RL: Reference Lens, CCD: Charged Coupled Device Camera, DS: Delay System.

In contrary of digital in-line holography, the hologram is recorded in an off-axis geometry, meaning that a small angle θ is introduced between both waves. As shown in the reflection setup [Fig. 1(a)] a delay system (DS) is sometimes used to adjust the optical path length of the reference wave to the one of the object wave when a short coherence length source is used; for example to perform time-domain optical coherence tomography with DHM^{17,18}. Two more lenses are used: one is the object beam condenser (OC) that focuses the object beam at the back focal length of the microscope objective in order to have a collimated beam illuminating the sample, and the second is the reference lens (RL) that curves the reference beam to match approximately the curvature introduced by the microscope objective on the object beam in the CCD plane. This curvature matching is approximative and does not require an exact and delicate adjustment of RL position, because the residual curvature difference can be easily compensated numerically by a digital optics presented in details in Refs. 19–21. It is to note that the image of the specimen through the MO is not focused on the CCD camera but about 5cm behind the CCD camera.

2.2. Reconstruction

2.2.1. Spatial frequency filtering

Due to the off-axis geometry, the zero order and the real and virtual image frequencies are separated in the hologram spectrum. The first step of the reconstruction procedure consists therefore to apply a spatial frequency filter on the hologram spectrum in order to propagate only the virtual images ($\mathbf{R}^*\mathbf{O}$) of Eq. 1.²² The filtered digital hologram is computed as:

$$I_H^F(k, l) = \text{FFT}^{-1} [\text{FFT}(I_H)P(\nu_k, \nu_l)], \quad (2)$$

where $P(\nu_k, \nu_l)$ is a mask defining the spatial filtering, that can be seen as a numerical pinhole; and ν_k, ν_l are the spatial frequencies coordinates.

2.2.2. Numerical propagation

Three different methods are used to reconstruct the wavefront [Fig. 1(c)] : the convolution formalism (CF),^{20, 23, 24} the angular spectrum (AS)^{25, 26} and the Fresnel algorithm that needs a single Fourier transform (SFFT).^{22, 27} Without losing generality, only the CF method is presented.

As shown in Ref. 20, in CF method, the reconstructed wavefront is written:

$$\Psi_{CF}(m, n) = \Gamma^I A \times \text{FFT}^{-1} [\text{FFT} \{ \Gamma^H I_H^F \} \cdot G_{CF}], \quad (3)$$

where $A = \exp(i2\pi d/\lambda)/(i\lambda d)$, d the reconstruction distance, λ the wavelength, Γ^i are numerical lenses in the hologram (i=H) and image (i=I) planes used to compensate for aberration or/and distortion²⁰ (usually, $\Gamma^H = R$ is simply the digital reference wave), FFT the Fourier transform, FFT^{-1} the inverse Fourier transform, and the kernel is

$$G_{CF}(k, l) = \exp[-i\pi\lambda d(\nu_k^2 + \nu_l^2)] \quad (4)$$

$$= \exp\left[-i\frac{\pi\lambda d}{N^2\Delta x^2}(k^2 + l^2)\right], \quad (5)$$

where $\nu_k = k/(N\Delta x)$, $\nu_l = l/(N\Delta y)$ are the spatial frequencies coordinates; N the number of pixels, Δx the pixel size and k, l entire number $-N/2 \leq k, l < N/2$.

2.3. Digital Optics

Because the reconstructed wavefront is digital, the usual optical transformation such as modification of focusing distance, the image magnification or the compensation for aberrations by the introduction of lenses can be performed numerically by using a digital optics applied from a unique recorded hologram.

2.3.1. Extended depth of focus and digital refocusing

As described in Eqs. 3 and 5, the reconstruction can be performed at different distances to extend digitally the depth of field as demonstrated for example in Ref. 28. Another advantage is the possibility to focalize from the same hologram several specimen that are in different planes or to compensate for a movement of the specimen in the z-direction (for example for a cell swimming in an immersion liquid) as demonstrated in Fig. 2.

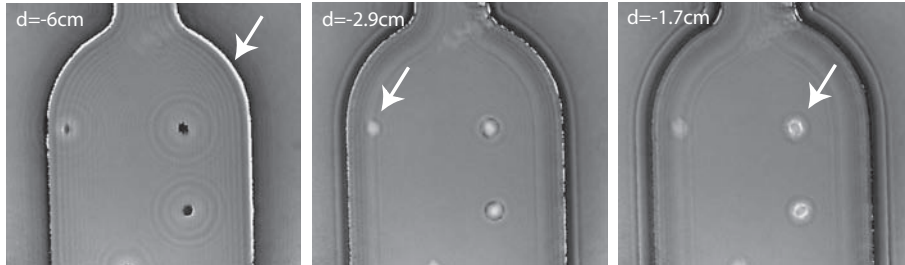


Figure 2. Example of the extended depth of focus on erythrocytes swimming in liquid inside micro-channels. The reconstruction distances allows the focalization of the channel border ($d=-6\text{cm}$), the left erythrocyte cell ($d=-2.9\text{cm}$) and the right cells ($d=-1.7\text{cm}$) from the same recorded hologram. Arrows indicates the focused edge or cell.

2.3.2. Compensation for phase aberration and distortion

The introduction of numerical lenses, described extensively in Refs 19–21, 24, allows the compensation for phase aberrations and image distortion introduced for example by unperfect MO, lenses; coverslip; beam-splitter; or specimen holder. Furthermore, these numerical lenses defined by polynomials (standard, Zernike...) can be computed semi-automatically by fitting the phase value in the hologram plane in areas assumed to be flat in the specimen (for example area surrounding the specimen).^{20, 21} An example where digital optics compensate for phase aberration and image distortion produced by a lens ball used as microscope objective is presented in Fig.3

Miccio *et al.* presents in Ref. 29 a particular case of this method by applying the fitting on the entire field of view. But as told by the authors, the error introduced by fitting also the specimen area is about 1 rad, that corresponds to a precision of $\lambda/6$, that is much more than the precision obtain for example by Charrière *et al.* for micro-lenses shape measurement (spatial RMS phase value about 4 degrees: $\lambda/40$ in transmission and $\lambda/175$ in reflection).³⁰ The method using selected areas is clearly supposed to give better results.

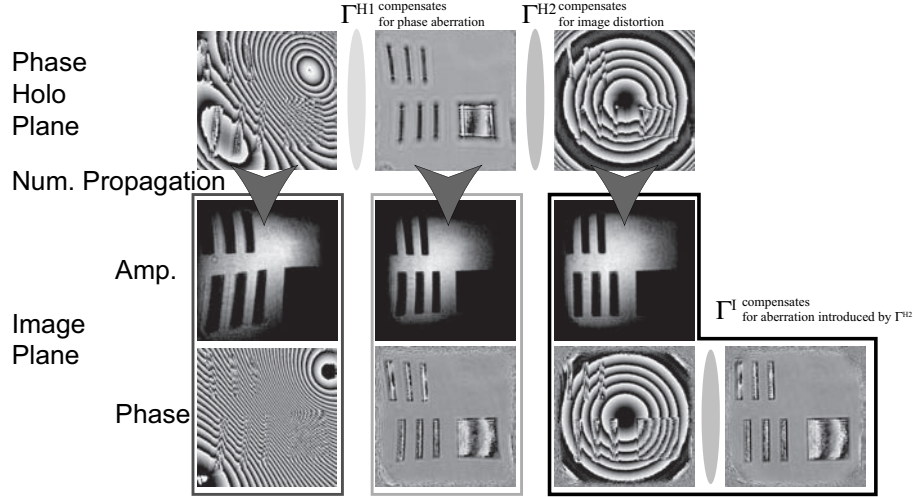


Figure 3. Complete numerical compensation for image distortion and phase aberration induced by a lens ball used as MO. First column, the reconstruction without compensation; second column after the reference hologram method correction; and third column, the manual compensation for residual image distortion.

2.3.3. Compensation for chromatic aberration

Finally, the digital optics allows to compensate for chromatic aberrations²⁰ when multi-wavelengths are used to perform synthetic wavelength DHM³¹ or tomography.^{32,33} Indeed, the chromatic aberration introduces a shift in z-direction of the focus plane, compensated by the adjustment of the reconstruction distance; and the compensation for the different magnifications for the different wavelengths is done with a magnification lens placed in the hologram plane.²⁰

2.3.4. Insensitiveness to vibrations

Vibrations are a problem in interferometric measurements because they modify strongly the optical path length of the light. In the context of DHM, these vibrations induce a random phase on the reconstructed phase image, that is not judicious when dynamical measurements are done on biological specimen for example.³⁴ A simple numerical procedure consists in measuring this phase in an area of the field of view where no time modification of the phase occurs (areas of the coverslip without presence of biological specimen). Then this computed phase defined the offset value to subtract to the entire phase in order to compensate for the vibrations.

3. PHASE-SHIFTING METHOD

3.1. Digital in-line holography

The digital in-line holography¹⁻⁵ differs from DHM by the configuration of the hologram recording. Indeed, for this technique, holograms are acquired in in-line geometry ($\theta = 0$ in Fig. 1). Therefore, the three orders terms are superposed in the hologram spectrum, and therefore the spatial frequency filtering is not possible. In order to reconstruct the complex wavefront, the phase-shifting method, involving the acquisition of several holograms (I_{Hn}) with different phase shifts of the reference wave $R_n = R \exp(in\pi/2)$ (typically four holograms with $n=0,1,2$ and 3), is applied.¹ Then, the phase in the hologram plane is computed from the relation:

$$\Phi(m, n) = \tan^{-1} \left(\frac{I_{H3} - I_{H1}}{I_{H0} - I_{H2}} \right) \quad (6)$$

The amplitude of the object wave in the hologram plane is computed from the intensity that results from the blockage of the reference wave. Then, CF, AS or SFFT could be used for the wavefront propagation.

The evident advantage of DHM compared to digital in-line holography is the single hologram acquisition and therefore an easy way to perform real-time imaging,^{31, 34-36} even if different complicated techniques were developed to achieve real-time imaging also in-line geometry such as the use of several CCD cameras,⁵ polarization phase-shifting² or micro-retarder array.⁴ Furthermore, the precision on the phase measurement is strongly influenced by the error on the phase shift (error on the position of the piezo-electrical motor or precision of the phase delay induced by spatial light modulator...). This error is often compensated by more recorded phase shift³⁷ or by using more complex algorithms.³⁸

3.2. Fourier phase microscopy

The Fourier phase microscopy^{6,7} developed by Feld *et al.* (FPM) uses also a phase-shifting method to recover the wavefront but the image recorded on the CCD plane is quite different. First, the object image is focalized on the CCD camera by using a 4f lenses system (no numerical propagation needed). Secondly, instead of applying the phase shifting on a separated reference wave as in in-line holography, a phase contrast filter was placed in the Fourier plane of the 4f system. This phase contrast filter introduces a phase shift delay for all the object frequencies (ac term), excepting for the zero order (dc term) that passes through a pinhole. On other words, the ac term corresponds to the object wave that is phase-shifted, and the dc term to the reference wave, that interfere on the CCD camera.

An advantage of this technique is the phase stability due to the common path method (the reference and object waves passes through the same optics). But, the pinhole size of the phase contrast filter is a limitation of the technique in term of resolution and field of view (See Ref. 7 for details). On the other hand, the phase-shift technique is also a limitation for real-time imaging even if fast phase shifts at 100ms interval can be done to achieve 10Hz reconstruction rate.⁷ Finally, if the suppression of the computation of the numerical propagation seems to be an advantage in term of time consuming, it is negligible with the capacity of the nowadays computers.

4. HILBERT PHASE MICROSCOPY

The Hilbert phase microscopy as described in Refs. 8,9 is strongly similar to off-axis DHM in the recording and on the reconstruction procedure. Indeed, the setup is very similar to Fig. 1(b), excepting that a MO (identical to the MO in the object arm) replaced the lens condenser OC, in order to match the wavefront curvature of the object wave; and that the image of the specimen is formed in the CCD plane to avoid numerical propagation. In this configuration, the interference between the object wave and reference wave, called "spatially varying irradiance"⁸ or "spatial irradiance"⁹ is

$$I(x) = I_R + I_S(x) + 2\sqrt{I_R I_S(x)} \cos[qx + \Phi(x)] \quad (7)$$

and is the same expression as the digital hologram (Eq. 1) in DHM, with $I(x) = I_H$, $I_R = |\mathbf{R}|^2$, $I_S = |\mathbf{O}|^2$ and writing $\mathbf{R} = \sqrt{I_R} \exp(iqx)$ as a plane wave and $\mathbf{O} = \sqrt{I_S} \exp[i\Phi(x)]$, it is easily demonstrated that $2\sqrt{I_R I_S(x)} \cos[qx + \Phi(x)] = \mathbf{R}\mathbf{O}^* + \mathbf{R}^*\mathbf{O}$.

In Ref. 8, the reconstruction procedure is explained in details. First, "the interferogram recorded by the CCD is Fourier transformed and high-pass filtered, such that the sinusoidal signal is obtained. To obtain the complex analytic signal associated with this real signal we compute the two-dimensional Fourier transform and suppress the negative spatial frequencies." The "high-pass filtering" selects the virtual and real image frequencies and the suppression of the negative frequencies select only one of the twin image frequencies. The filtered interferogram can be therefore expressed as two steps:

$$I^{F1}(x) = \text{FFT}^{-1} [\text{FFT}[I(x)]HP] \quad (8)$$

$$I^F(x) = \text{FFT}^{-1} [\text{FFT}[I^{F1}(x)]NFS], \quad (9)$$

where *HP* is the High-pass filtering and *NFS* the negative frequencies suppression filter. This two steps can easily simplified by using only one filter:

$$I^F(x) = \text{FFT}^{-1} [\text{FFT}[I(x)](HP \cdot NFS)], \quad (10)$$

where one see that $HP \cdot NFS$ is equivalent to $P(\nu_k, \nu_l)$ of Eq. 1. This filtering method is therefore totally equivalent to the filtering method presented in Ref. 22 and in the section 2.2.1.

Then, "from an inverse Fourier-transform operation [of $\text{FFT}(I^F(x))$] a complex two-dimensional signal is obtained that can provide uniquely the information about the phase of the object". Finally, "the quantitative phase image is obtained by subtraction of the linear phase". This subtraction is necessary to compensate for the tilt aberration introduced by the off-axis geometry. Briefly, the phase Hilbert algorithm (PH) can be written as:

$$\Psi_{\text{PH}}(m, n) = LP \times \text{FFT}^{-1} [\text{FFT} [I^F(x)]] . \quad (11)$$

where LP is a complex array that subtracts the linear phase. One see that Eqs. 11 and 3 are equal when:

$$\Gamma^H = 1, \quad (12)$$

$$\Gamma^I = LP, \quad (13)$$

$$G_{CF} = 1 \Leftrightarrow d = 0. \quad (14)$$

In other words Hilbert phase microscopy is a particular case of DHM where no numerical propagation is necessary because the specimen object is formed on the CCD plane. Basically it is the same technique as DHM with the disadvantage not to be able to compensate for image distortion with numerical lenses and to modify the focus position with the digital reconstruction distance.

5. ADVANTAGES AND RESULTS OF DHM

The comparison of DHM with the phase-shifting methods such as in-line digital holography and Fourier phase microscopy, and Hilbert phase microscopy demonstrates the different techniques are very similar even if their denomination are different. In particular we show that the Hilbert phase microscopy is a particular case of DHM for which the image is focalized on the CCD camera. Nevertheless, DHM as numerous advantages. First of all, because a single image acquisition is needed, real-time imaging at high frequency can be performed. The temporal resolution depends only on the specifications of the recording device; in contrary of phase-shifting that depends of the rate of the phase shift modification (mechanical displacement of mirror, or modification of voltage in a light modulator,...). Secondly, the digital focusing is a crucial advantage of the technique, especially in life sciences applications where specimen are not always attached but could swim in a surrounding medium. It is also possible to experiment a phenomena during a long time without modification of the setup and to compensate numerically for specimen movements afterwards. Furthermore, the argument of time consuming of the numerical propagation is now obsolete because of the computer performance (15fps with 1024x1024 pixels hologram are easily achieved).

5.1. Erythrocytes

Specifically, DHM allows one to observe non-invasively and in real time the 3D cellular dynamics with an axial resolution of a few tens of nanometers. As an example, this high-resolution capability has permitted to quantitatively measure, without using any time consuming staining, the volume and shape of red blood cells (Fig. 4), pathognomonic parameters for diagnosing various blood and parasitic diseases including thalassemia and malaria. Furthermore, the DHM capability of measuring intracellular refractive index³⁴ provides an accurate measurement of the mean cell hemoglobin concentration (MCHC), another relevant clinical parameters for a variety of pathological states.

5.2. Drug effects

Another illustrative example (Fig. 5 concerns the DHM capability to reveal the early alterations in cell morphology induced by apoptosis. Beyond the possibility to allow the dynamic study of fundamental mechanisms involved in the apoptosis process, the DHM nanoscale cell visualization permits the high throughput screening of various apoptosis-inducing drugs consider as a class of promising new anti-cancerous agents.

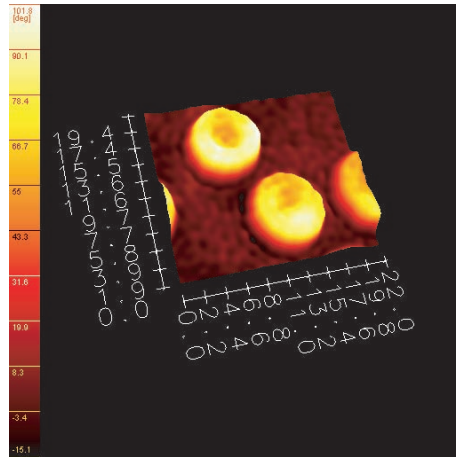


Figure 4. 3D visualization of erythrocytes.

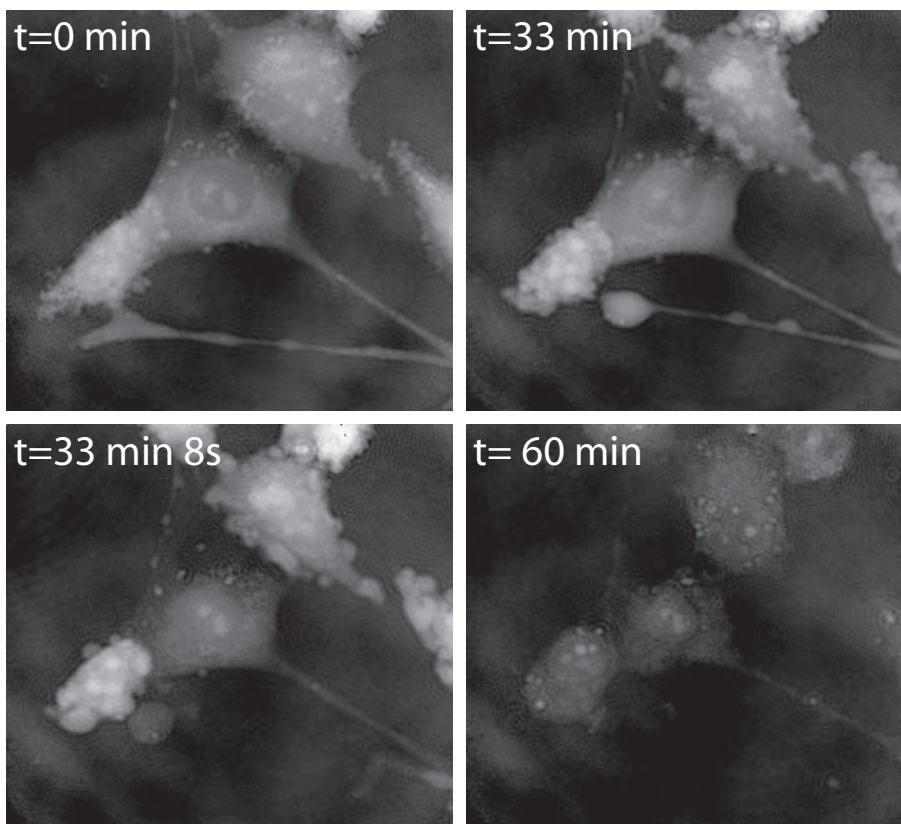


Figure 5. Early alterations of Prostate cell morphology associated with drug-induced apoptosis, including protrusion apparitions, cells swelling in bubble form as well as nuclear condensation, can be clearly observe.

6. CONCLUSION

We demonstrate in this paper that DHM has various advantages compared to similar full-field interferometric methods. Compared to phase-shifting method used in in-line digital holography and Fourier phase microscopy, DHM needs only a single hologram acquisition and therefore real-time imaging depends only on acquisition rate of the recording device. Furthermore, the numerical propagation and digital optics offers to DHM unique tools able to increase the depth of focus, compensate for aberrations and image distortion, in contrary of the

Fourier phase and Hilbert phase microscopy. These advantages makes DHM a perfect instrument for precise, quantitative, full-field and real-time imaging, in particular in life sciences applications.

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