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Applications of Holographic Microscopy in Life Sciences

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<http://dx.doi.org/10.5772/56176>

1. Introduction

Imaging of microscopic objects is an essential art, especially in life sciences. Rapid progress in electronic detection and control, digital imaging, image processing, and numerical computation has been crucial in advancing modern microscopy. At present the 3D imaging of biological samples is done by confocal microscopes. Their ability to image biological events in real time is limited by the time necessary to capture stacks of images taken through a certain plane in cells or tissues from which a 3D view is calculated. Digital holographic microscopy is a new imaging technology applied to optical microscopy. The digital holographic microscopy is a very advanced imaging technique because it yields a 3D volume image from a single image capture.

Holography is a technique by which a wavefront can be recorded and subsequently reconstructed in the absence of the original wavefront i.e. a 3D image is observed just as if the object was still present and being illuminated in the same way as when the holographic recording was made [1]. In conventional holography, invented by Gabor [2], the holograms are photographically recorded and optically reconstructed. Both the amplitude and phase information of the light wave are recorded in a hologram. Because the holographic image retains the phase as well as the amplitude information, a variety of interference experiments can be performed, and this is the basis of many interferometric applications in metrology.

Digital holography does not require wet chemical processing of a photographic plate, although at some expense of resolution. However, once the amplitude and phase of the light wave are recorded numerically, one can easily subject these data to a variety of manipulations, and so digital holography offers capabilities not available in conventional holography. The remarkable aspect of the digital reconstruction – its possibility to refocus at different depths inside a transparent object, depending on the reconstruction distance, makes this technique very suitable for biological cells studies and could have many applications in life sciences.

In digital holography the reconstruction process is implemented by multiplication of the CCD captured and PC stored digital hologram by the numerical description of the reference wave, and convolution of the result with the impulse response function of the optical system. The diffracted field in the image plane is given by the Rayleigh-Sommerfield diffraction formula [3]

$$\psi(x', y') = \frac{1}{i\lambda} \iint h(\xi, \eta) r(\xi, \eta) f(x', y', \xi, \eta) \cos \Theta dx dy \quad (1)$$

with $f(x', y', \xi, \eta) = \frac{\exp(ik\rho)}{\rho}$ and $\rho = \sqrt{d'^2 + (\xi - x')^2 + (\eta - y')^2}$

where d' is the reconstruction distance, i.e. the distance measured from the hologram plane ξ - η to the image plane; $h(\xi, \eta)$ is the recorded hologram; $r(\xi, \eta)$ represents the reference wave field; k denotes the wave number and λ is the wavelength of the laser source. Due to the small angles between the hologram normal and the rays from the hologram to the image points, the obliquity factor $\cos \Theta$ can normally be set = 1.

Equation (1) is the basis for numerical hologram reconstruction. Because the reconstructed wavefield $\Psi(x', y')$ is a complex function, both the intensity as well as the phase can be calculated [1].

Digital holographic microscopy (DHM) can provide quantitative marker-free imaging that is suitable for high resolving investigations of transparent and reflective surfaces as well as for fast analysis of living cells under usual laboratory conditions. One of many interesting applications of DHM is to study cells without staining or labeling them and without affecting them in any way.

This chapter is divided in two parts. The first part reviews the recent advances in the application of digital holographic microscopy to biological specimen. The second part of the chapter describes the development of a digital holographic microscope at the Agricultural University of Plovdiv and reports some of its life science applications.

2. Recent advances in the application of digital holographic microscopy to biological specimen

Kim M. K. [4] has proposed a novel digital holographic method that allows axial resolution of objects by superposition of a number of numerically reconstructed optical diffraction fields of digital holograms that are optically recorded with a number of wavelengths. The principle of wavelength-scanning digital interference holography is applied to imaging of 3D objects with diffuse surfaces, such as a biological specimen. The head of a small insect of a few millimeters in size is imaged with 120 μm axial resolution and ~20 μm lateral resolution. An animated 3D numerical model of the object surface structure is generated from the tomographic data with good fidelity. The experiments are performed using a standard holographic apparatus. Approximately 50 mW of ring dye laser output is expanded to about 10 mm diameter and

spatially filtered. The object beam is apertured to about 5 mm diameter and illuminates the sample object. The scattered light from the object is combined with the reference beam. The magnifying lens images the optical field at infinity. The digital camera is focused at infinity, so that it records a magnified image of the optical intensity. It is important to aperture the object beam so that it only illuminates the area of the object that is to be imaged, otherwise spurious scattering can seriously affect the quality of the picture.

A digital holographic microscope (DHM) in a transmission mode has been developed in Rappaz, B. et al. [5], adapted to the quantitative study of cellular dynamics. Living cells in culture are optically explored by calculating the phase shift they produce on the transmitted wave front. The high temporal stability of the phase signal and the low acquisition time makes it possible to monitor cellular dynamics processes. An experimental procedure allowing calculating both the integral refractive index and the cellular thickness (morphometry) from the measured phase shift is presented. Specifically, the method has been applied to study the dynamics of neurons in culture during a hypotonic stress. Such stress produces a decrease of the phase which can be entirely resolved by applying the methodological approach described in the article; in fact the method allows determining independently the thickness and the integral refractive index of cells. The phase signal depends on both the thickness and the refractive index of the specimen. To decouple these two contributions, a procedure named "decoupling procedure" is applied. The 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. Quantitatively, the local cellular thickness measurement can be performed with accuracy of 1 μm . Spatial averaging allows measuring mean thickness of cellular regions corresponding to the area of typical neuronal bodies, i.e. 170 μm^2 , with an accuracy of a few tens of nanometers. On the other hand, the spatial variations of the integral refractive index have been estimated at 0.005 and the mean integral refractive index can be measured with an accuracy of 0.0003. The cellular refractive index is a poorly documented parameter which is related to the intracellular content and which is relevant for the interpretation of the functional light imaging signal resulting from a multiple scattering process in biological tissues.

Marquet, P. et al. [6] present for the first time DHM images of living cells in culture. They represent the distribution of the optical path length over the cell, which has been measured with subwavelength accuracy. These DHM images are compared with those obtained by use of the widely exploited phase contrast and Nomarski differential interference contrast techniques. The developed digital holographic microscope presents a simplified and easy-to-operate technique compared with classical interferometry. The authors show that digital recording and numerical reconstruction of holograms, offers new perspectives in imaging, because numerical processing of a complex wave front allows one to compute simultaneously the intensity and the phase distribution of the propagated wave. Digital holography has made it possible to focus numerically on different object planes without using any opto-mechanical movement. Moreover, different lens aberrations can be corrected by a numerical procedure. In the article for the first time absolute phase distribution images of living neurons in a culture are obtained by use of DHM with accuracy in the 2–4° range. To compare the DHM with the

standard and widely used techniques of phase contrast (PhC) and differential interference contrast (DIC) microscopy in biology, images of living neurons obtained with PhC, DIC, and DHM are presented.

In an article of Garcia-Sucerquia J. et al. [7] some significant characteristics of (digital in-line holographic microscopy) DIHM and the underwater DIHM have been emphasized:

1. Simplicity of the microscope: DIHM is microscopy without objective lenses. The hardware necessary for the desktop version is a laser, a pinhole, and a CCD camera. For the underwater DIHM version, they use the same elements contained in a submersible hermetic shell.
2. Maximum information: a single hologram contains all the information about the 3D structure of the object. A set of multiple holograms can be properly added to provide information about 4D trajectories of samples.
3. Maximum resolution: optimal resolution, of the order of the wavelength of the laser, can be obtained easily with both versions.
4. Simplicity of sample preparation: this is mainly true for biological samples, because no sectioning or staining is required, so that living cells and specimens can be observed in depth. Indeed, for the underwater DIHM, there is no sample preparation at all, and real-time information of living organisms can be retrieved.

It is shown [7] that high-resolution tracking of many particles in 4D can be obtained from just one difference hologram. Since resolution of the order of a wavelength of the light has been achieved with DIHM, tracking of organisms as small as bacteria is possible, as would be the motion of plankton in water or, at lower resolution, the trajectories of flying insects. DIHM can also be used on macroscopic biological specimens, prepared by standard histological procedures, as for a histological section of the head of the fruit fly, *Drosophila melanogaster*. Such images reveal the structure of the pigmented compound eye and different neuropile regions of the brain within the head cuticle, including the optic neuropiles underlying the compound eye. DIHM, with its inherent capability of obtaining magnified images of objects, unlike conventional off-axis holography, is therefore a powerful new tool for a large variety of research fields.

The optical arrangement described by Parshall D. et al. [8] provides a straightforward means for high-resolution holographic microscopic imaging. There is no need for elaborate processing such as magnification by using a reconstruction wavelength that is compared with the recording wavelength, which inevitably introduces aberration, or using an aperture array in front of the camera and scanning it to artificially increase the CCD resolution. Also the phase image appears less noisy than the amplitude image. The amplitude image reflects the intensity variations in the reference wave, whereas the phase noise comes mostly from the quality of the optical surfaces in the imaging system. The former is much more difficult to control. The phase images have less noise than the amplitude image, and one can readily discern the index variation over the nucleus as it is done for an onion cell.

In this article [8] a number of experimental results has been presented that reveal the effectiveness of digital holography in high-resolution biological microscopy. In particular, phase-imaging digital holography offers a highly sensitive and versatile means to measure and monitor optical path variations. The authors have offered biological microscopy by two-wavelength phase-imaging digital holography and proposed its extension to three-wavelength phase imaging for longer axial ranges with undiminished resolution.

Jeong, K. et al. [9] show that coherence-gated digital holography detects motility as deep as 10 optical thickness lengths inside tissue. This opens prospects to use motility as a contrast agent when imaging at depths inaccessible to conventional motility assay approaches. Coherence-gated digital holography is an interferometric imaging approach that measures motility with displacement sensitivity below a fraction of a wavelength, over a macroscopic lateral field of view up to 1 mm. Motility at depth appears in real-time holograms as dynamic speckle. Furthermore, the authors define a motility metric based on the coefficient of intensity variance per pixel that becomes a novel imaging contrast agent. The authors demonstrate that the motility metric enables direct visualization of the effect of cytoskeletal anti-cancer drugs on tissue inside its natural three-dimensional environment, allowing measurements of tissue and cellular response to drugs. The reconstructed *en face* image of the rat tumor spheroid is striking. The rat osteogenic sarcoma tumor spheroids are grown *in vitro* in a rotating bioreactor. The spheroids may be grown up to several mm in diameter, and thus are large enough to simulate the thickness of different mammalian tissue. As tumor spheroids are cultured, they undergo cell apoptosis and necrosis in their center and so consist of an inner necrotic core with low activity and an outer shell with a thickness of 100 to 200 μm of viable proliferating cells with high motility. The speckle images of the tumor spheroids shimmer due to motility in tissue, and statistical properties of the dynamic speckle are obtained by capturing successive images at a fixed depth.

A method of quantitative phase microscopy with asynchronous digital holography has been suggested by Chalut K. J. et al. [10]. An essential requirement that must be met to apply a phase microscopy system to imaging the dynamics of live cells is that the system can acquire quantitative phase images of the sample at a high rate (>100 Hz). Although modern CCDs are capable of >100 Hz image acquisition rates, multiple interferograms are often necessary to extract the phase information, which reduces the acquisition rate considerably. Additionally, if multiple interferograms are used, they must be recorded fast enough so that instabilities in the system and the dynamics of the cells themselves do not vary appreciably during acquisition. The authors [10] demonstrated that the system is capable of obtaining quantitative phase measurements on millisecond time scales. The inclusion of acousto-optic modulators in each arm of the interferometer permits to use phase-shifting interferometry. The system is innovative in the field of digital holography, because the phase shift is easily evaluated, which greatly simplifies the experimental setup. In addition, the algorithm requires only two phase-shifted interferograms, compared to the usual 4 interferograms required in most phase shifting algorithms. A potential increase in speed can be realized by utilizing frame transfer CCD devices, which can record two images on a microsecond time scale. By transferring frames without reading them out, the latency between two interferograms is greatly reduced and the

quantitative phase imaging frame rate is then nearly the frame rate of the camera, of the order of 100 Hz. It is demonstrated, with a red blood cell sample, and a smooth muscle cell sample, that this system is capable of obtaining quantitative phase images of live cells.

Kemper, B. & von Bally, G. [11] carried out an analysis of living pancreas tumor cells (Patu8988T) to reveal the prospective of digital holographic microscopy for the visualization of drug induced morphology changes. The authors exposed the tumor cells to an anticancer drug (Taxol). Digital holograms of selected cells were recorded continuously every 120 s over 16 h in a temperature stabilized environment with an inverse digital holographic microscopy setup. DHM has clearly shown that Taxol first induces morphological changes as cell rounding that effects an increase in cell thickness. Afterward, for all the specimens, the final cell collapse is detected precisely by a significant decrease of the phase contrast. The authors also demonstrate that the subsequent numerical focus adjustment reduces unwrapping artifacts that are caused by diffraction patterns in the defocused phase contrast images. For investigation of suspension cells this feature is of particular advantage because cells in different focal planes can be investigated by the evaluation of a single captured hologram. The results show that digital holographic phase contrast microscopy can be applied for quantitative long-term observation of living cells. The studies show new ways for marker-free dynamic monitoring of cell morphology changes to access new parameters, e.g., for quantitative observation of the time-dependent reactions of cells to drugs. In addition, for investigation of cells, the scattering properties of the cell culture medium and the optical quality of the cell handling equipment (e.g., glass carrier, coverslip, or Petri dishes) must be considered. In summary, the presented results demonstrate that digital holographic microscopy can be applied for noncontact, marker-free, and quantitative phase contrast imaging. The method allows a high resolution multifocus reconstruction of amplitude and phase data from a single recorded digital hologram. It enables hologram capture time in the millisecond range. The hologram acquisition rate is limited by the digital recording device.

A phase-imaging technique to quantitatively study the three-dimensional structure of cells is presented by Khmaladze A. et al. [12]. The method, based on the simultaneous dual-wavelength digital holography, allows for higher axial range at which the phase imaging can be performed. The technique is capable of nanometer axial resolution. The method compares favorably to software unwrapping, as the technique does not produce non-existent phase steps. Curvature mismatches between the reference and an object beam is numerically compensated. The 3D images of SKOV-3 ovarian cancer cells are presented. The measurements of the optical thickness of cells can then be performed. One also needs to make an assumption of the cells refractive index, which is taken to be 1.375. While it may not be possible to precisely determine the refractive index of the cell at each individual point, this number is always close to the refractive index of water and unlikely to deviate by more than a few percent. As a result, the accuracy and the level of details of the dual-wavelength images of cells, presented here, are superior to what has been previously demonstrated. In comparison to the software unwrapping, dual-wavelength optical unwrapping method is advantageous, as it requires no intensive computation procedures and can handle complex phase topologies. The proposed method of curvature correction is simple and effective to easily implement the experiment

without the microscope objectives in the reference arms of the Michelson interferometer. This greatly simplifies the optical setup and makes it much easier to achieve the initial adjustments of the apparatus. Simultaneous dual wavelength setup utilized together with the angular spectrum algorithm provides an easy way to acquire single frame images in real time, which can be used to study cell migration.

Langehanenberg P. et al. [13] propose autofocusing in digital holographic phase contrast microscopy on pure phase objects for live cell imaging. Common passive optical autofocus techniques are based on axial scanning of the image space by mechanical adjustment of a lens element or a stage to find the maximum image definition. In digital holography, this scanning process is performed numerically by variation of the propagation distance in the convolution-based propagation. The main task in passive autofocusing is the determination and maximization of the image sharpness. Pure phase objects with negligible absorption such as technical reflective specimens or biological cells are sharply focused at the setting with the least contours in the amplitude distributions. In contrast to the bright-field case, in digital holography this setting is of particular interest, as the amplitude and phase distributions are accessible simultaneously, and the focal setting with the least-contrasted amplitude image corresponds to the best-resolved structures in the quantitative phase contrast distribution. Four numerical methods are compared in order to identify best autofocus method for application in digital holographic microscopy.

Remmersmann C. et al. [14] present research for the optimization of a temporal phase-shifting (TPS) - based digital holographic microscopy setup. In order to enable a phase-shift-dependent investigation a variable three-step algorithm is applied. First, the phase error of the reconstructed object wave is evaluated theoretically. In a second step the results obtained from the calculations are compared to the measured phase noise. Finally, the applicability for noise reduction is demonstrated by quantitative phase contrast imaging of a pancreas tumor cell sample. Theoretical and experimental investigations on phase errors in temporal phase-shifting-based digital holographic reconstruction have been performed in order to minimize the noise within the reconstructed object wave. Coherent as well as partially coherent light sources were applied and compared. The application example of LED and laser-based digital holographic microscopy on fixed pancreas tumor cells demonstrates that disturbances in the reconstructed amplitude and phase distributions due to multiple reflections within the experimental setup can be effectively reduced by partially coherent light sources.

Choi Y. S. and Lee S. J. [15] apply digital holographic microscopy (DHM) for three-dimensional volumetric measurement of red blood cells in motion. Currently, various particle image velocimetry (PIV) measurement techniques have been applied to numerous hemorheological studies. Standard PIV methods provide two-dimensional (2D) planar information confined in a thin depth of field. Holography is capable of recording 3D volumetric field information in a single hologram. The recent development of digital holography enables the volumetric measurement of particle fields without the use of any chemical or physical processes. In this technique, a digital hologram of the particles distributed in a flow is directly recorded digitally. The 3D flow information can be subsequently obtained through the numerical reconstruction and the particle tracking procedure. The authors applied DHM to measure the 3D motion of

human red blood cells (RBCs) in a microtube flow. DHM requires only a pair of particle hologram images to get complete 3D flow information and this is of great advantage in motion analysis of individual blood cells. The viability and uncertainty of the established DHM system in the detection of 3D RBC position were evaluated by a planar test target. The position in depth of a RBC was located by applying focus functions that quantify the sharpness of its reconstructed image. Five focus functions were evaluated to find the suitable function that provides minimum uncertainty. Finally, the sample trajectories as well as the 3D velocity profiles of RBCs inside the microtube flow are presented and the measurement uncertainties are discussed.

Warnasooriya N. et al. [16] captured pictures of gold nanoparticles in living cell environments using heterodyne digital holographic microscopy. With recent developments in the fields of nanotechnology and modern optical microscopy, the use of nanometric particles as biomarkers in biological specimens has been rapidly increased. The paper describes an imaging microscopic technique based on heterodyne digital holography where subwavelength-sized gold colloids can be imaged in cell environments. Surface cellular receptors of 3T3 mouse fibroblasts were labeled with 40 nm gold nanoparticles, and the biological specimen is imaged in a total internal reflection configuration with holographic microscopy. Due to a higher scattering efficiency of the gold nanoparticles versus that of cellular structures, accurate localization of a gold marker is obtained within a 3D mapping of the entire sample's scattered field, with a lateral precision of 5 nm and 100 nm in the x, y and in the z directions respectively, demonstrating the ability of holographic microscopy to locate nanoparticles in living cell environments. However, in order to apply these techniques to biological specimens, important issues must be considered. In biological samples, the particle holographic signal is superimposed with the light scattered by cell refractive index fluctuations, which yields a speckle field. This paper studies the possibility of 3D holographic imaging in a biological context. Since the cell-scattered speckle field cannot be avoided, it is important for future cell labeling applications, to scale the particle signal with respect to the scattered speckle. The authors show that the amplitude of the 40 nm gold particle signal is much larger than the cell-scattered field. NIH 3T3 mouse fibroblasts are used (quoted as 3T3 cells) with integrin surface receptors labeled with 40 nm gold particles. Streptavidin-coated gold nanoparticles were attached to the cell surface integrin receptors via biotin and fibronectin proteins. Fibronectin proteins were labeled with biotin. The illumination source is a single-mode near infrared laser diode. A polarizing beam splitter cube (PBS) is used to split the original illumination laser light into two beams, a reference beam and an object illumination beam forming the two arms of a Mach-Zehnder interferometer. A CCD camera detects the interference pattern (hologram) and sends it to a computer. The hologram is then reconstructed numerically. Using a parabolic approximation for the local field, the location of the gold particle can be calculated by fitting the data points that are above half maximum. The accuracy of the measurement made by this method is ± 5 nm in the x and y directions. The authors show that the acquisition of a single image is sufficient to localize in 3D the nanoparticle within a 90 micrometer thick sample, with localization accuracy similar to that obtained in conventional light microscopy. This method provides significant progress towards the development of 3D microscopy in living cell environments,

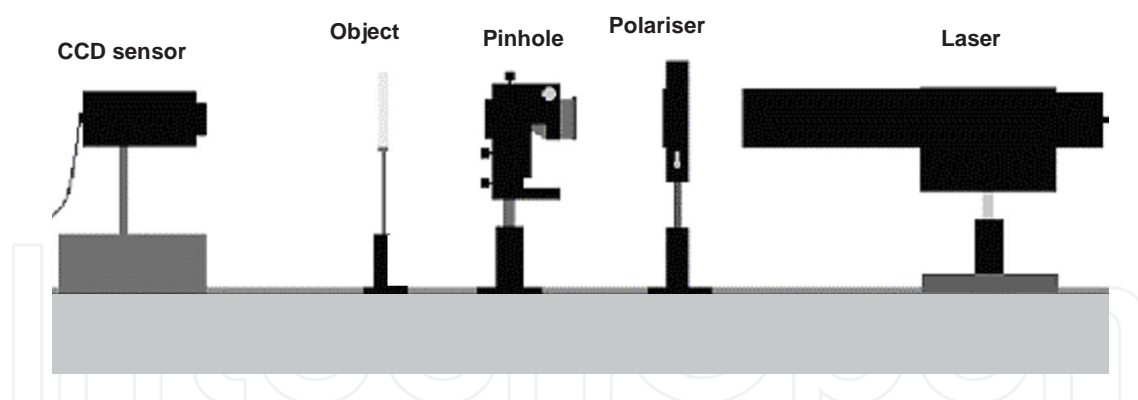


Figure 1. Optical setup of the digital in-line holographic microscope

since the 3D reconstruction of such a thick sample by conventional light microscopy would require the acquisition of a stack of hundreds of slices.

The traction force produced by biological cells has been visualized by Yu X. et al. [17]. Quantitative phase microscopy by digital holography (DH-QPM) has been utilized to study the wrinkling of a silicone rubber film by motile fibroblasts. Surface deformation and the cellular traction force have been measured from phase profiles in a direct and straightforward manner. DH-QPM is shown to provide highly efficient and versatile means for quantitatively analyzing cellular motility. The traction force has been measured as $\sim 4 \times 10^{-3}$ dyn/cell based on the degree of wrinkling determined from phase information. Fourier transformation and the angular spectrum methods were applied to the complex hologram obtained to calculate the phase-contrast, dark-field, Zernike and differential interference contrast (DIC) images. The basic principles of DH have been applied to quantitative imaging of wrinkles on silicone rubber due to cell adhesion and motility. The approach is sensitive to cellular forces and it can detect and quantify variations in force within the adhesion area of a cell over time. DH-QPM is shown to be an effective approach for measuring the traction forces of cells. A time-lapse phase movie of the migration of cells was recorded every 3 min over a period of 2 hours. The traction force for NHDFs is a factor of five smaller than for chick heart fibroblast cited in literature. This is a substantial achievement in the quantitative profiling of substrate deformation and wrinkling under cellular traction force achieved by the quantitative phase microscopy of digital holography.

3. Digital in-line holographic microscopy for life science applications at the Agricultural University Plovdiv

A digital in-line holographic microscope (DIHM) was developed at the Agricultural University of Plovdiv. The light source is 20 mW He-Ne laser. The emerging spherical wave illuminates the object, and the hologram is recorded on a CCD sensor and stored in a computer.

DIHM was applied to visualise live algae cells of two different species (*Pseudokirchneriella subcapitata* and *Chlorella vulgaris*) without any preliminary preparation. Digital reconstruction of the recorded interference patterns is performed using the “HoloVision 2.2” software [18].

Figure 2 and Figure 3 show the holograms and the reconstructed intensities to represent the object. Four wavefront intensities of each digital hologram (2a and 2b) are reconstructed at different consecutive planes with the distance between them changing by 2 μm . The reconstructed intensities illustrate the possibility of observation of different layers in a live cell obtained from one digital hologram only. In that way many cuts of one live object can be done and observed from one hologram of the whole object.

These experiments illustrate the capability of DHM for non-invasively visualizing and quantifying biological cells and tissues. That's why DHM can be successfully used for:

- cell counting
- measuring cell viability directly in the cell culture
- label-free viability analysis of adherent cell cultures etc.

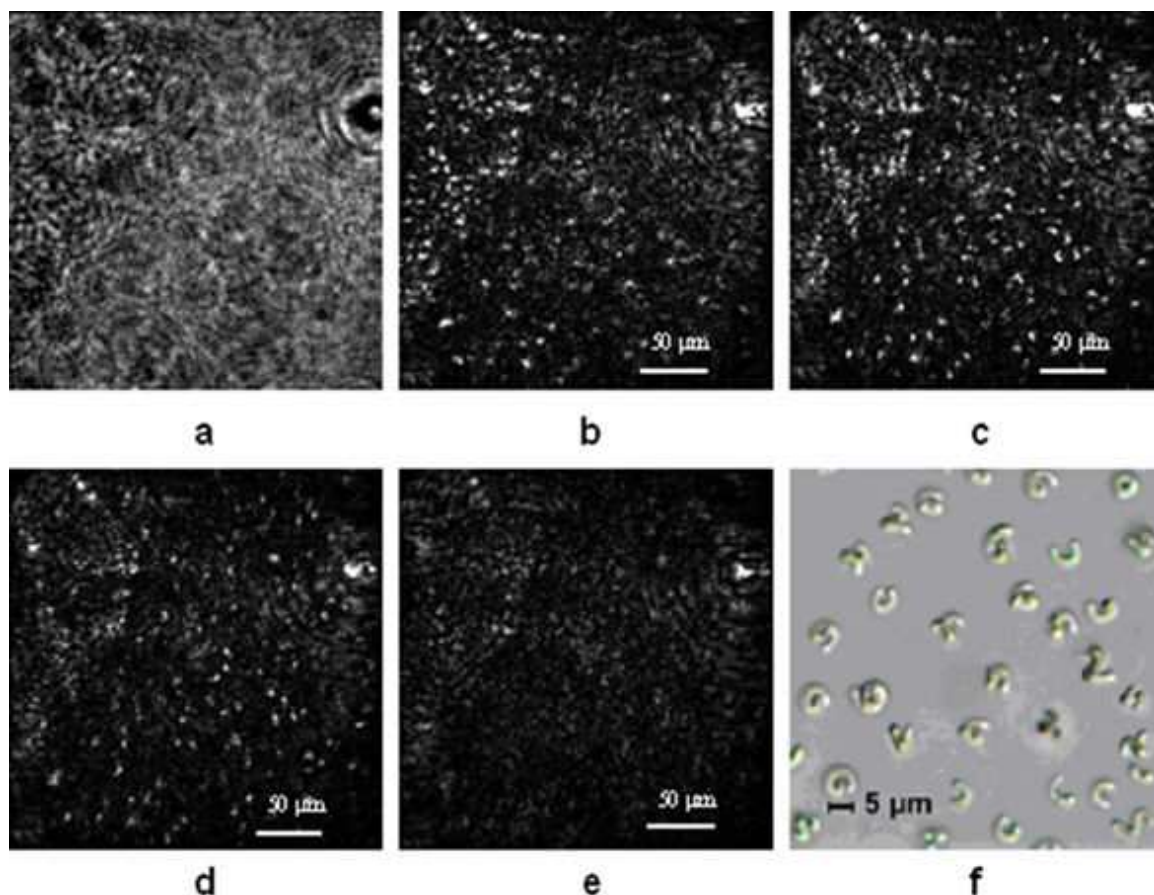


Figure 2. Images of algae *Pseudokirchneriella subcapitata*: a) digital hologram; b-e) the wavefront intensity at four consecutive planes with the distance between them changing by 2 μm ; f – image from electron microscope

Clearly, DIHM is capable of visualising live cells with dimensions 5 – 10 μm without any preliminary preparation. It can be applied to dynamic quantitative visualisation of live cell deformations to study their interactions with other particles as well as the surrounding environment. This makes the DIHM a valuable technique for many life science applications. Further development of the technique is envisaged in order to overcome the limited pixel resolution of a CCD sensor, which is the major drawback of DIHM at present.

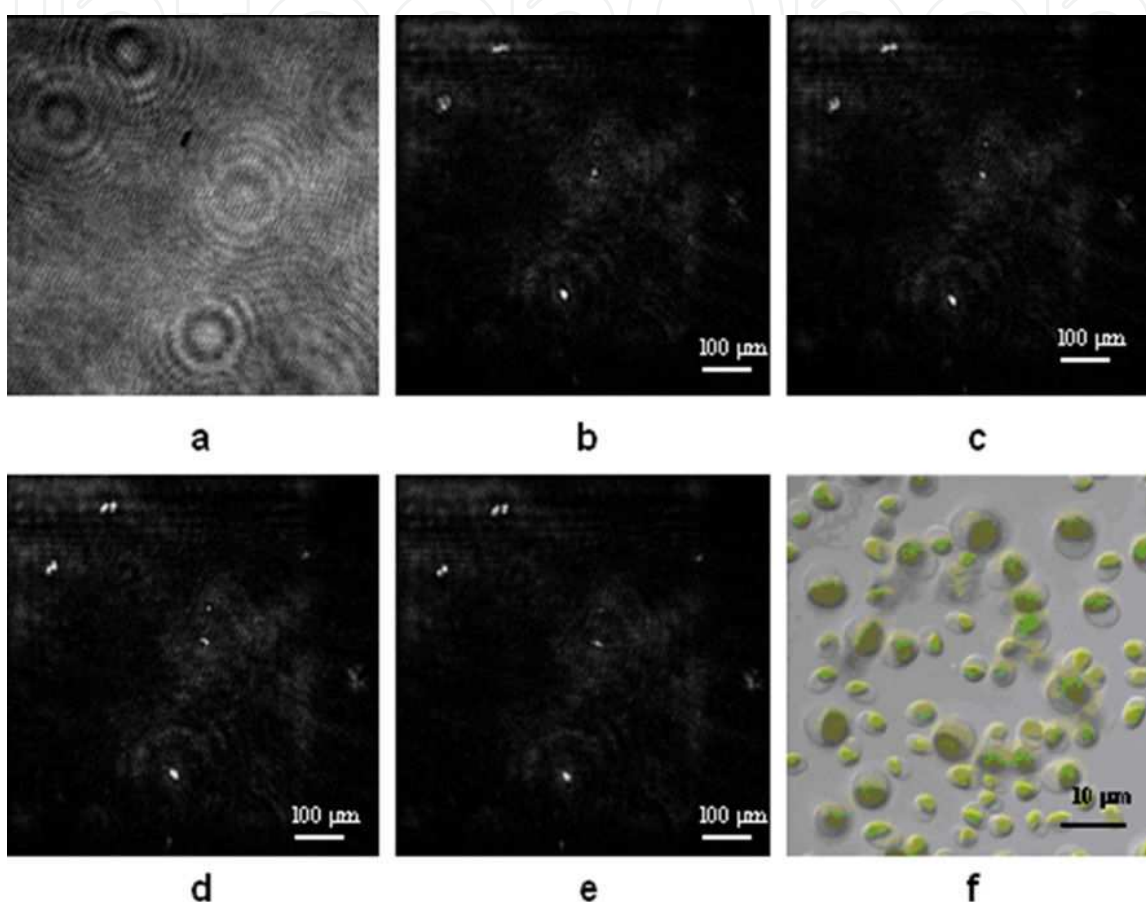


Figure 3. Images of algae *Chlorella vulgaris*: a) digital hologram; b-e) the wavefront intensity at four consecutive planes with the distance between them changing by 2 μm ; f – image from electron microscope

Figure 4 and Figure 5 present digital holograms of algae cells *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* taken at different stages of their life cycle and the reconstructed intensities of these holograms. The cells morphology is visible on the images showing the reconstructed intensities.

These experiments illustrate the capability of DHM for:

- label free morphology analysis of cells
- label free studies of cell division and migration
- label-free analysis of subcellular motion in living tissues etc.

By combining several images reconstructed from the same digital hologram, but at different focal planes, an increased depth of field can be obtained, which is vastly superior to the depth of field achieved with traditional light microscopy.

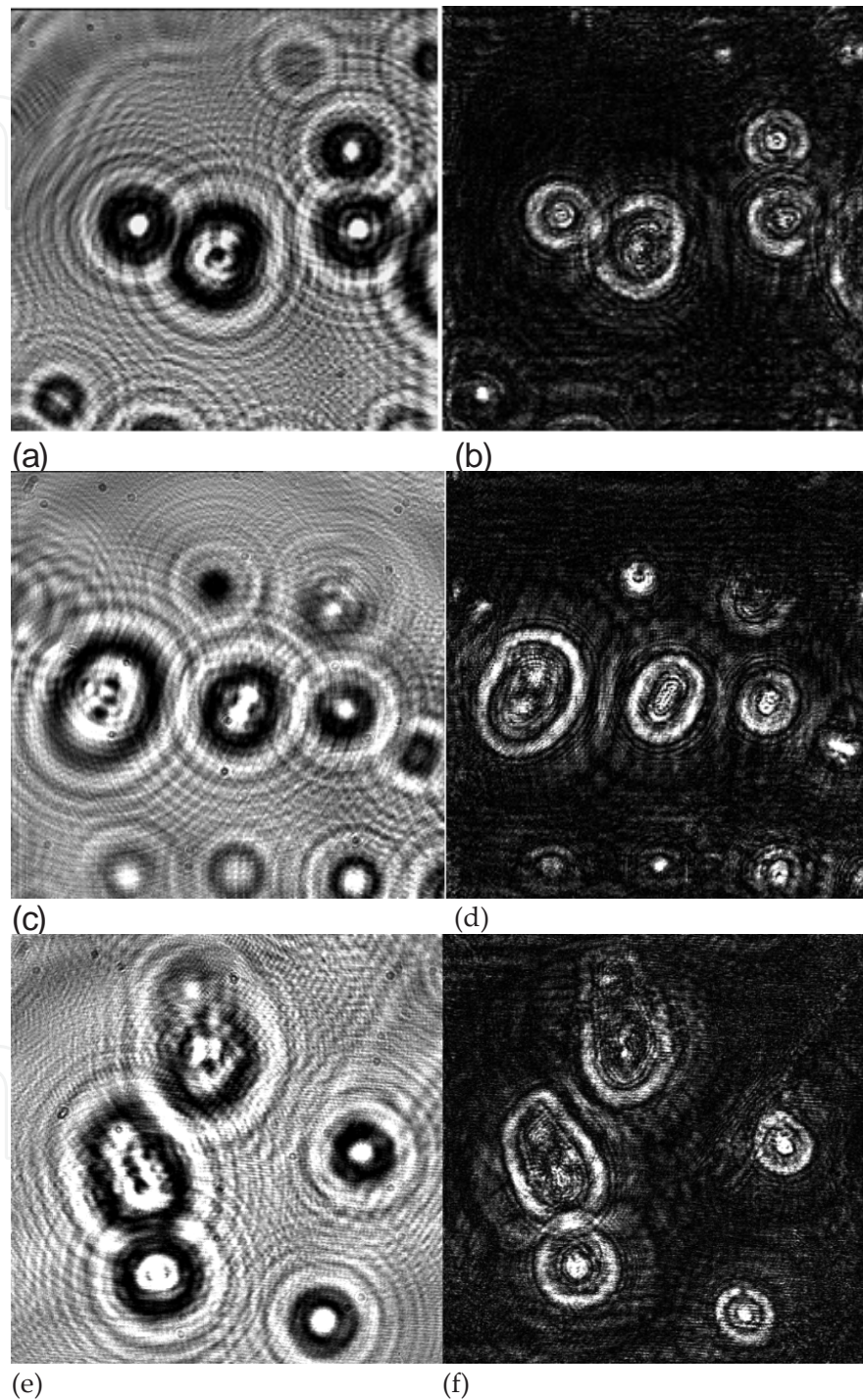


Figure 4. Images of algae *Pseudokirchneriella subcapitata*, approximately 10 μm in a sickle: a) digital hologram of two days old cells; b) the wavefront intensity of a) c) digital hologram of 4 days old cells; d) the wavefront intensity of c) e) digital hologram of 9 days old cells; f) the wavefront intensity of e).

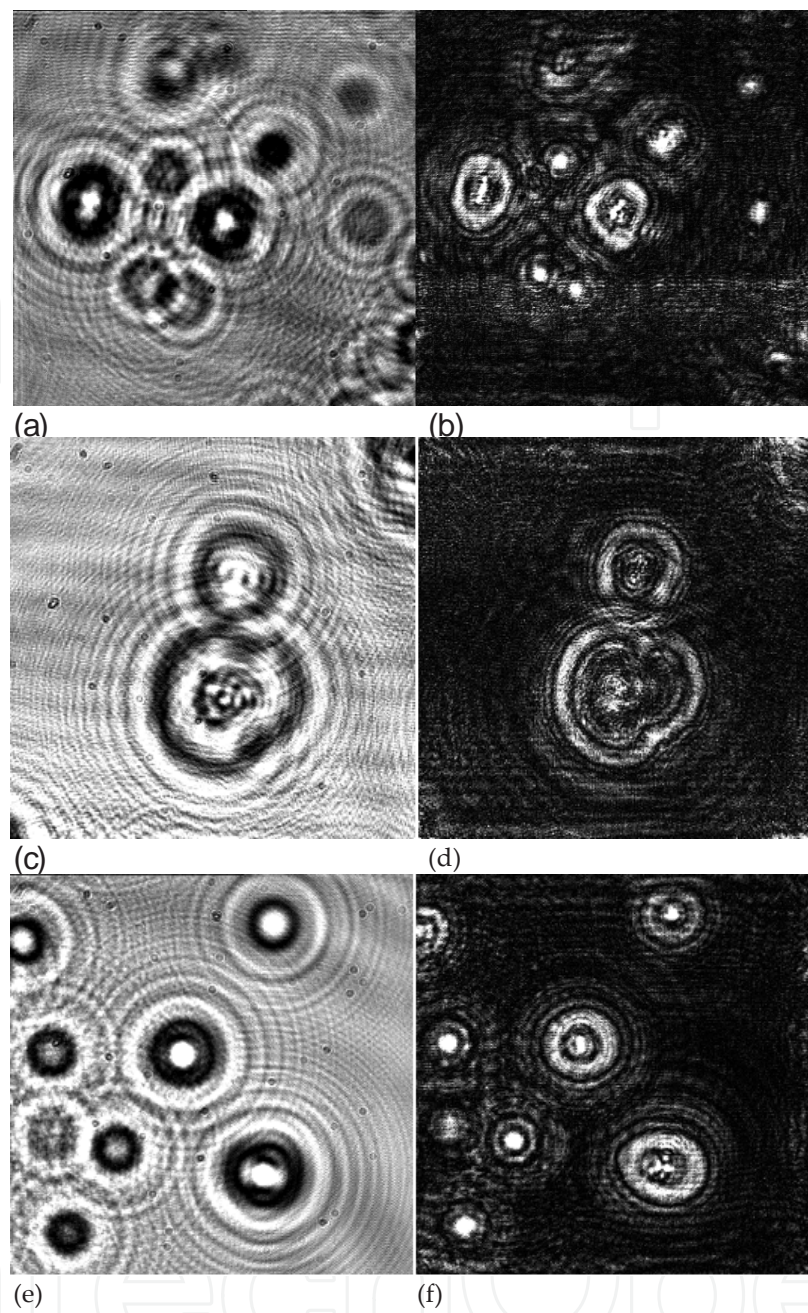


Figure 5. Images of algae *Chlorella vulgaris*, approximately 3 μm in diameter: a) digital hologram of two days old cells; b) the wavefront intensity of a) c) digital hologram of 4 days old cells; d) the wavefront intensity of c) e) digital hologram of 9 days old cells; f) the wavefront intensity of e).

4. Conclusion

DIHM imaging is very advanced method because digital holography yields a 3D volume image from a single interferogram capture. This makes the development of a dynamic microscope capable of fast 3D imaging an achievable objective.

The attractive features of DHM are: a very high acquisition rate (limited only by the video acquisition frequency), monitoring of physiological and pathological activity of cell and tissue culture, non contact, non destructive, marker free in vivo imaging.

DIHM is capable of label free morphology analysis of cells and label free studies of cell division and migration. It is a very attractive technique for application in biological research and in the agricultural science. Other life science and medical applications are also envisaged.

Further development of this technique will involve the use of lasers with shorter wavelength and CCD cameras with higher resolution. These developments will allow the application of DIHM for study of cell features having dimensions below 100 nm.

The work on the development of the Digital In-line Holographic Microscope was financially supported by the Agricultural University of Plovdiv.

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References

- [1] Jones, R, & Wykes, C. *Holographic and Speckle Interferometry*, Cambridge University Press, Cambridge, UK (1989).
- [2] Gabor, D. *Nature* 161, 777-778 (1948).
- [3] Palacios, J, Ricardo, D, Palacios, E, Goncalves, J, & Valin, R. De Souza, "3D image reconstruction of transparent microscopic objects using digital holography", *Optics Communications* 248, 41-50 (2005).
- [4] Kim, M. K. Tomographic three-dimensional imaging of a biological specimen using wavelength-scanning digital interference holography", *Optics Express* 7 (9), 305-310 (2000).
- [5] Rappaz, B, Marquet, P, Cuche, E, Emery, Y, Depeursinge, C, & Magistretti, P. J. Measurement of the integral refractive index and dynamic cell morphometry of living cells with digital holographic microscopy", *Optics Express* 13, 9361-9373 (2005).
- [6] Marquet, P, Rappaz, B, & Magistretti, P. J. Digital holographic microscopy: a noninvasive contrast imaging technique allowing quantitative visualization of living cells with subwavelength axial accuracy", *Optics Letters* 30, 468-470 (2005).

- [7] Garcia-sucerquia, J, Xu, W, Jericho, S. K, Klages, P, Jericho, M. H, & Kreuzer, H. J. Digital in-line holographic microscopy", *Applied Optics* 45 (5), 836-850 (2006).
- [8] Parshall, D, & Kim, M. K. Digital holographic microscopy with dual-wavelength phase unwrapping", *Applied Optics* 45 (3), 451-459 (2006).
- [9] Jeong, K, Turek, J. J, & Nolte, D. D. Volumetric motility-contrast imaging of tissue response to cytoskeletal anti-cancer drugs", *Optics Express*, 15 (21), 14057-14065 (2007).
- [10] Chalut, K. J, Brown, W. J, & Wax, A. Quantitative phase microscopy with asynchronous digital holography", *Optics Express* 15 (6), 3047-3052 (2007).
- [11] Kemper, B, & Von Bally, G. Digital holographic microscopy for live cell applications and technical inspection", *Applied Optics* 47 (4), AA61(2008). , 52.
- [12] Khmaladze, A, Kim, M, & Lo, C. M. Phase imaging of cells by simultaneous dual-wavelength reflection digital holography", *Optics Express* 16 (15), 10900-10911 (2008).
- [13] Langehanenberg, P, Kemper, B, Dirksen, D, & Von Bally, G. Autofocusing in digital holographic phase contrast microscopy on pure phase objects for live cell imaging", *Applied Optics* 47(19), DD182 (2008). , 176.
- [14] Remmersmann, C, Stüwald, S, Kemper, B, Langehanenberg, G, & Von Bally, G. Phase noise optimization in temporal phase-shifting digital holography with partial coherence light sources and its application in quantitative cell imaging", *Applied Optics* 48 (8), 1463-1472 (2009).
- [15] Choi, Y. S, & Lee, S. J. Three-dimensional volumetric measurement of red blood cell motion using digital holographic microscopy", *Applied Optics* 48 (16), 2983-2990 (2009).
- [16] Warnasooriy, N, Joud, F, Bun, P, Tessier, G, Coppey-moisan, M, Desbiolles, P, Atlan, M, Abboud, M, & Gross, M. Imaging gold nanoparticles in living cell environments using heterodyne digital holographic microscopy", *Optics Express* 18 (4), 3264-3273, (2010).
- [17] Yu, X, Cross, M, Liu, C, Clark, D. C, Haynie, D. T, & Kim, M. K. Measurement of the traction force of biological cells by digital holography", *Biomedical Optics Express* 3 (1), 153-159 (2012).
- [18] Oysen Skotheim and Vegard LTuft, "HoloVision 2.2.1" software package for numerical reconstruction and analysis of digitally sampled holograms, Norwegian University of Science and Technology, Norway (2001).

