Digital Holographic Microscopy

A New Method for Surface Analysis and Marker-Free Dynamic Life Cell Imaging

Digital holographic microscopy provides quantitative phase contrast imaging that is suitable for high resolving investigations on reflective surfaces as well as for marker-free analysis of living cells. Results from engineered surfaces and living cells demonstrate applications of digital holographic microscopy for technical inspection and life cell imaging.

Introduction

Holographic interferometric metrology techniques are established tools in many industrial application areas [1, 2]. There are also important application fields in Biophotonics, Life Sciences and Medicine as these techniques can be applied non-destructively, marker-free, "full-field" (no scanning required) and online simultaneously [3-10]. With these features the described digital holographic microscopy concept permits a high resolution, multi focus representation of engineered surfaces and living cells.

In order to establish digital holography in microscopy, the combination with common microscopy techniques is of particular advantage. In this case flexible and compact digital holographic microscopy modules for the integration into modern microscopy systems are required. Furthermore, for an automated evaluation of the measurement data, it is necessary to implement robust algorithms for the numerical reconstruction of digital holograms.

Principle of digital holographic microscopy

Digital holography is based on the classic holographic principle, with the difference that the hologram recording is performed by a digital image sensor, e.g. a CCD or CMOS camera. The subsequent reconstruction of the holographic image that contains the information about the object wave is carried out numerically with a computer.

Fig. 1 depicts the schematics of two "off-axis" setups for digital holographic microscopy that are particularly suitable for the modular integration into commercial microscopy systems. The coherent light of a laser (e.g. a frequency doubled Nd:YAG laser, $\lambda = 532$ nm) is divided into object illumination and reference wave, using singlemode optical fibers. Fig. 1a shows an incident light illumination arrangement for the investigation of reflective samples. The set-up in Fig. 1b is designed to investigate transparent specimen such as living cells. For both setups the coherent laser light for the illumination of the sample is coupled into the optical path of the microscope's condenser by a beam splitter cube. The reference wave is superimposed with the light that is reflected or transmitted by the object by a second beam splitter with a slight tilt against the object wave front in order to generate "off-axis" holograms which are recorded by a CCD camera. After hologram acquisition, the data is transmitted by an IEEE1394 („FireWire") interface to a PC based image processing system, thus avoiding cost intensive frame grabber cards

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with hardware specific software.
The modular add-on approach provides the advantage that common commercial microscope lenses with high numerical aperture (e.g. water and oil immersion) can be used in combination with an optimized (Koehler-like) illumination of the sample. Furthermore, the integration of the additional optical components for digital holography does not restrict the conventional functions of the microscopy systems.

Numerical evaluation of digital holograms by non-diffractive reconstruction

The reconstruction of the digitally recorded holograms is performed numerically with standard computer hardware. In general, Fresnel-transformation based digital holographic reconstruction methods generate not only the information contained in the object wave but also the intensity of the reference wave ("zero order") and a „twin image". Furthermore, the size of the reconstructed holographic image depends on the reconstruction distance to the hologram plane.
A non-diffractive reconstruction algorithm has been developed that is particularly suitable to digital holographic microscopy. In a first step of the reconstruction process the complex object wave is calculated with a spatial phase shifting algorithm in the plane of the CCD image sensor (hologram plane) [6,9]. Afterwards, if necessary, the object wave is propagated towards the focused image plane in frequency space ("convolution method", [9]). The main advantage of the method is that it can also be applied to the evaluation of holograms when the sample is imaged sharply onto the image sensor. In addition, the scale of the holographic amplitude and phase contrast images is constant for the numerical reconstruction in different focus planes and "zero order" and "twin image" are avoided.
Fig. 2 illustrates the evaluation process of digital recorded holograms. Figures 2a and 2b show a digital hologram obtained from a living human pancreas carcinoma cell (Patu8988T) with an inverse microscope arrangement in transmission mode (40x microscope lens, NA = 0.65) and the reconstructed holographic amplitude image that corresponds to a microscopic bright field image at coherent laser light illumination. Fig. 2c depicts the simultaneously reconstructed quantitative phase contrast image modulo 2π. The unwrapped data without 2π ambiguity, are shown in Fig. 2d, representing the optical path length changes that are effected by the sample in comparison to the surrounding medium due to the thickness and the integral refractive index. Fig. 2e depicts a pseudo 3D plot of the data in Fig. 2d. Fig. 2f shows the cell thickness along the marked

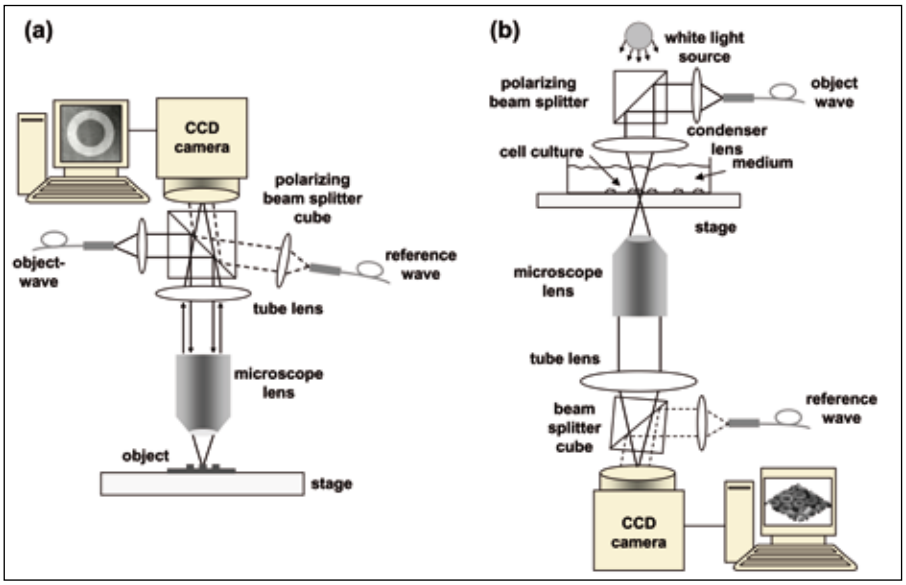
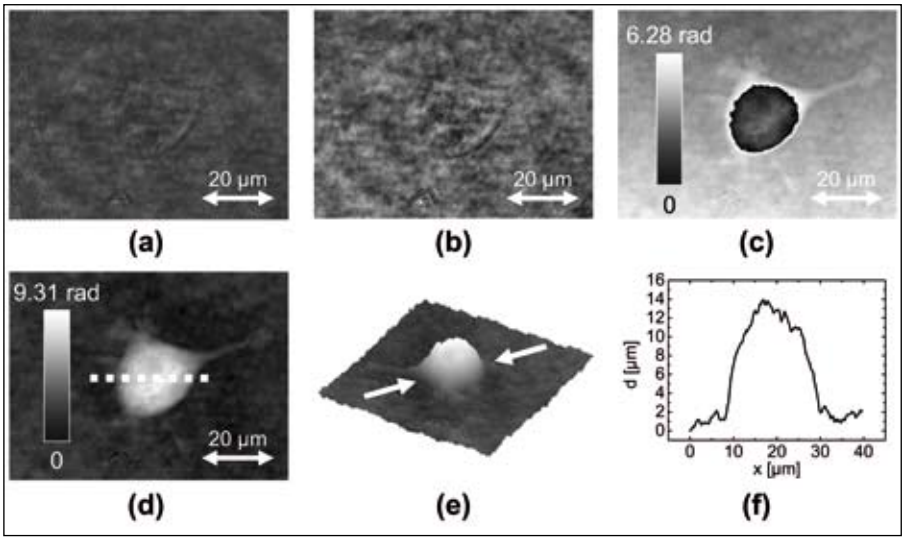


FIGURE 1: **Schematics for digital holographic microscopy using incident light (reflective) (left) and inverse transmission arrangements (right).**

FIGURE 2: **Example for evaluation of digital holograms: (a): digital hologram of a human pancreas carcinoma cell (Patu8988T); (b): reconstructed holographic amplitude image; (c): quantitative phase contrast image (modulo 2π), (d): unwrapped phase distribution; (e): pseudo 3D plot of the unwrapped phase image in gray level representation; (f): calculated cell thickness along the dotted white line in (d). (Cooperation: Dr. Jürgen Schnekenburger, Molecular Gastrointestinal Cell Biology, Department of Medicine B, University of Münster, Germany).**



THE INSTITUTE

Laboratory of Biophysics
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The Laboratory of Biophysics is an interdisciplinary research center of the Medical Faculty of the University of Muenster for research, development and application of coherent and incoherent optical metrology for medical devices and diagnostics. Key topics of current public and industrial funded research projects focus on biophotonics and marker-free life cell imaging.

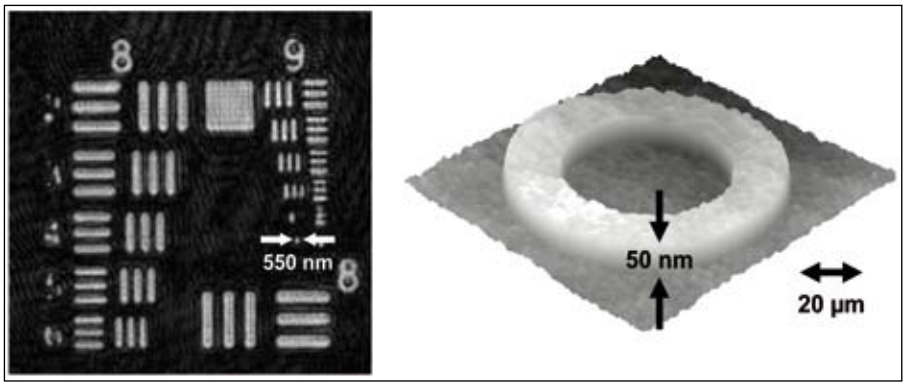


FIGURE 3: **Left: digital holographic reconstructed amplitude image of a semitransparent USAF 1951 resolution test chart. Right: topography calculated from the phase distribution of a reflective metal coated surface (pure phase object, Cooperation: Nano+Bio Center Technical University Kaiserslautern, Germany).**

dotted line in (Fig. 2d) that is determined for a cellular refractive index $n_{cell} = 1.380$ [9] and refractive index of the cell culture medium $n_{medium} = 1.337$.

Resolution and feature of subsequent numerical focus

The left panel of Fig. 3 shows the reconstructed amplitude (holographic image) of a negative USAF 1951 resolution test chart (illumination in transmission), recorded using a 40x microscope lens (NA=0.6). The group 9.6 (resolution limit of the test chart) represents a line width of 550 nm and is resolved clearly. The comparison with the Abbe criterion shows that the lateral resolution is diffraction limited (in correspondence to bright field microscopy) and can be increased by using microscope optics with higher numerical aperture. The right panel of Fig. 3 demonstrates the axial resolution for incident light illumination of a reflective metal surface (nanostructure gold-coated silicon surface), recorded with a 20x microscope lens (NA=0.4). The depicted element represents a height of 50 nm and is resolved clearly in the reconstructed phase distribution. Due to the phase noise the axial resolution is determined to be approx. 5 nm.
The digital reconstruction of different object planes of a single hologram enables a variable (subsequent) numerical focus of digital holographic images ("digital holographic multi focus") without additional mechanical or optical components. Fig. 4 demonstrates the subsequent numerical refocus for the holographic images of a semitransparent USAF 1951 test chart (upper panel) and of a living human liver tumor cell of the type HepG2 (lower panel).

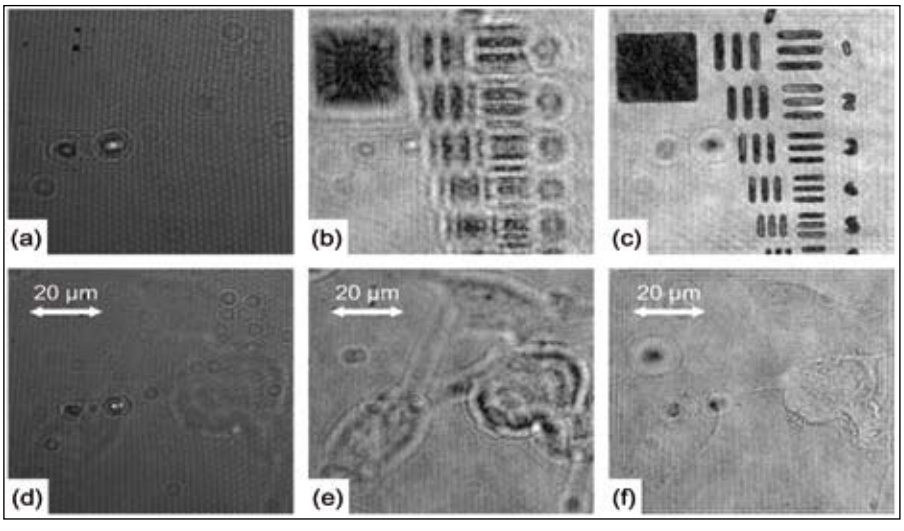
Analysis of dynamic shape variations of living cells

Digital holographic microscopy facilitates automated dynamic multi focus quantitative phase contrast imaging and analysis of living cells using time-lapse techniques. The time interval between the acquisitions of two digital holograms is restricted only by the performance of the image recording sensor. Fig. 5 shows results from investigations of shape variations of a human erythrocyte in suspension (red blood cell) during sedimentation on a coated glass surface. The feature of subsequent digital holographic refocus was applied to compensate defocus effects due the sedimentation process. Figures 5a and 5b show false-color coded pseudo-3D representations of the quantitative phase contrast images of the cell before and directly after the contact with the surface. A shape change from a donut-like structure to a spherical appearance is observed. The cell thickness can be determined from the phase contrast images (representing the optical path length changes effected by the cells in comparison to the surrounding medium) by taking into account the integral cellular refractive index and the refractive index of the cell suspension medium. Fig. 5c quantifies the change of shape by the calculation of the cell thickness before and after the contact with the glass surface at the cross-sections through the holographic phase contrast image that are marked in Figures 5a and 5b by white arrows.

Summary and Perspectives

Digital holographic microscopy provides multi-focus quantitative phase contrast imaging with an axial resolution up to approx. 5 nm. The method represents a versatile extension to established microscopy techniques. In addition to a marker-free topography or morphology analysis, a minimal invasive dynamic detection of deformations and movements is enabled. For these reasons, digital holographic microscopy is particular suitable for investigations of dynamic processes in the field of life cell analysis with high potential for applications in basic research, as well as for commercial utilization e.g. in

FIGURE 4: **(a): Hologram of semitransparent USAF 1951 test chart recorded out of focus in transmission mode (40x microscope lens, NA=0.6); (b): reconstructed object wave intensity in the hologram plane; (c): numerically refocused holographic amplitude image reconstructed by variation of the reconstruction distance; (d), (e), (f): corresponding results obtained from investigations on living human liver tumor cells (HepG2) in culture medium (20x microscope optic, NA=0.4).**



the fields of pharmacy and tissue engineering. The modular approach of digital holographic microscopy opens up prospects for integration into various existing commercial microscopy systems as well as for combined measurements with other established methods such as fluorescence microscopy, Laser Scanning Microscopy (LSM), or Atomic Force Microscopy (AFM).In this way digital holographic microscopy particularly promises access to new parameters and knowledge at the cellular level.

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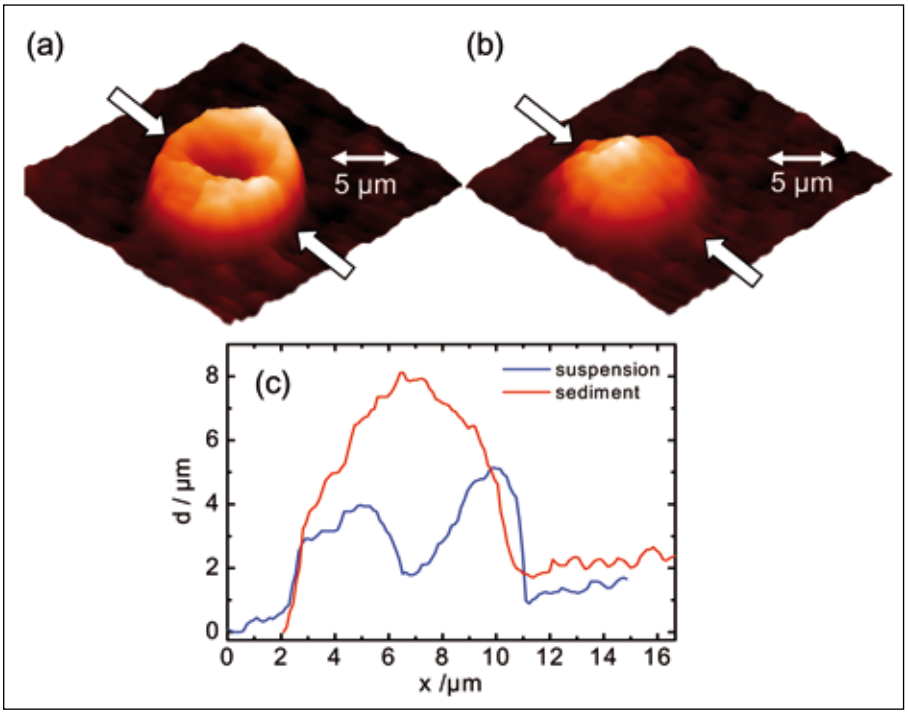

FIGURE 5: **Investigations on shape variations during sedimentation of a human red blood cell (in suspension) on a coated surface (63x microscope lens). (a): pseudo-3d-plot of the digital holographic phase contrast image of a red blood cell before contact with the glass surface, (b): pseudo-3d-plot of the phase contrast image of the same cell after sedimentation, (c): cell thickness before and after the contact with the surface at the cross-sections that are marked in Figures (a) and (b) by white arrows (Cooperation: L. Ivanova and Prof. I. Bernhard, Saarland University, Saarbrücken, Germany).**

References

[1] *T. Kreis*, Holographic Interferometry: Principles and Methods, Akademie-Publishing, Berlin, 1996

[2] *U. Schnars, W. Jüptner*, Digital Holography, Springer-Verlag, Heidelberg, 2005

[3] *B. Kemper, D. Carl, S. Knoche, R. Thien, G. von Bally*, Holographic interferometric microscopy systems for the application on biological samples, Proc. SPIE 5457, 581-589 (2004)

[4] *D. Carl, B. Kemper, G. von Bally*, Digital holographic microscope for living cell analysis, BMT 49 Extension Part 2, 978-979 (2004)

[5] *P. Marquet, B. Rappaz, P. J. Magistretti, E. Cuche, Y. Emery, T. Colomb, C. Depeursinge*, Digital holographic microscopy: a non invasive contrast imaging technique allowing quantitative visualization of living cells with sub wavelength accuracy, Opt. Lett. 30, 468 – 470 (2005)

[6] *M. Liebling, T. Blu, M. Unser*, Complex-wave retrieval from a single off-axis hologram, J. Opt. Soc. Am. A 21, 367 – 377 (2004)

[7] *D. Carl, B. Kemper, G. Wernicke, G. von Bally*, Parameter optimized digital holographic microscope for high resolution living cell analysis, Appl. Opt. 43, 6536-6544 (2004)

[8] *B. Kemper, D. Carl, A. Höink, G. von Bally*, I. Bredebusch, J. Schnekenburger, Modular digital holographic microscopy system for marker-free quantitative phase contrast imaging of living cells, Proc. SPIE 6191(2006)

[9] *B. Kemper, D. Carl, J. Schnekenburger, I. Bredebusch, M. Schäfer, W. Domschke, G. von Bally*, Investigations on living pancreas tumor cells by digital holographic Microscopy, J. Biomed. Opt. 11, 034005 (2006)

[10] *G. von Bally, B. Kemper et al.*: New ways for marker free life cell and tumor analysis (MIKROSO), in: J. Popp, M. Strehle (Eds.): Biophotonics. Visions for a better Health Care, Wiley, 301-360, 2006