Digital holographic multi-focus quantitative phase contrast microscopy

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A modular setup for digital holography microscopy that allows non-destructive, marker free and quantitative high resolution full field measurements of transparent objects is described. The applied reconstruction algorithms enable the reconstruction of multiple image planes. Results from investigations of biological specimen show the systems applicability for numerical focus tracking and quantitative 3d life cell imaging.

1 Introduction

The lateral resolution of digital holography is in common restricted by the pixel pitch of the applied imaging sensor. For this reason a modular setup for digital holography is integrated into a commercial microscope to improve the lateral resolution up to the diffraction limit. The interferometric determination of the object wave phase provides an axial resolution that amounts to less than ten nanometers (quantified by the analysis of noise) [1]. The application of microscope lenses with high numerical aperture decrease the depth of field. Therefore, for common microscope techniques focus tracking during the measurement is necessary. Digital holographic microscopy enables the advantage of multiple focus plane reconstruction from a single hologram. Thus, focus adjustment can be carried out numerically after the measurement and mechanical auto focus tracking during the measurement is avoided. Measurement results of technical objects for system characterization are shown as well as investigations on biological specimen.

2 Setup and Hologram Reconstruction

Fig. 1 shows the schematic of the modular digital holographic microscopy setup that was integrated into a Zeiss axioplan 2 fluorescence microscope. The emitted light of a frequency doubled Nd:YAG laser (λ = 532 nm) is divided into object wave and reference wave which are coupled into single mode polarization maintaining glass fibers. The coherent illumination of the probe is realized in transmission light arrangement. For that purpose the object wave is integrated into the optical path of the microscope by a polarizing beam splitter cube. The condenser is adjusted as for conventional white light illumination. Standard water immersion microscope objectives are used for investigations of living cells in culture medium to magnify the object wave. To arrange holographic "off-axis" geometry a slightly tilted reference wave is superimposed to the object wave by a second beam splitter cube. As a result, an interferogram with a high carrier fringe pattern is generated. The digital hologram is captured by an 8 bit CCD array with IEEE1394 interface.

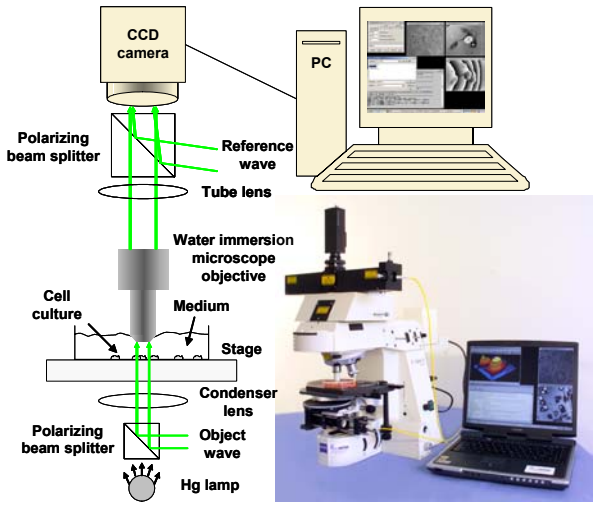

Fig. 1: *Modular digital holographic microscopy setup in transmission light arrangement adapted to a Zeiss axioplan 2 fluorescence microscope.*

Numerical reconstruction of the object wave (amplitude and phase) is performed in two steps. In a first step the complex object wave is reconstructed by solving the interferogram equation within the hologram plane. For that purpose a mathematical model of the difference phase distribution is required. If necessary due to defocused object structures the complex object wave is propagated in a second step. Numerical propagation is performed by evaluation of the Fresnel-Kirchhoff diffraction integral, e. g. with the discrete Fresnel transform. The combination of image plane holography with wave front propagation enables the reconstruction of multiple focus planes without the disturbing terms: twin image and zero order. As a result, the full pixel resolution of the CCD sensor is available for the reconstructed object wave [1].

3 Results and Discussion

Fig. 2a shows the microscopic white light image of a part of a fly wing captured with a 20x magnification microscope objective (NA=0.4). The numerical aperture restricts the depth of field (DOF) that amounts: $z_{DOF} \approx 3.5$ µm. For this reason objects whose thickness/topology exceeds the DOF cannot be focused. In Fig. 2a the upper left part of the image is in focus while the lower right part (see magnified section) is not in focus. Fig. 2b shows a digital hologram captured at the same focus position. The reconstructed amplitude of the object wave within the hologram plane is depicted in Fig. 2c and is comparable to Fig. 2a. Fig. 2d shows the numerically refocused image of the same hologram (Fig. 2b). The tiny hairs at the edge of the fly wing are clearly visible and imaged separately (compare magnified sections).

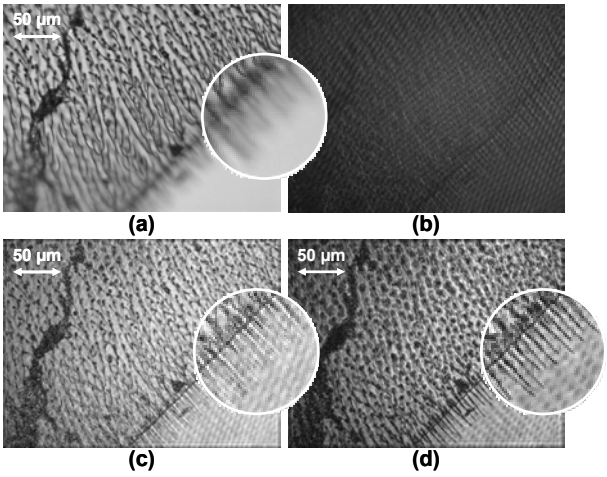

Fig. 2: *Part of a fly wing magnified with 20x microscope lens: (a) white light image, (b) digital hologram, (c) reconstructed amplitude (hologram plane), (d) reconstructed amplitude (numerically focused).*

Fig. 3 shows the digitally captured hologram (object wave magnified with 20x water immersion microscope lens) of two living pancreas carcinoma cells (PaTu 8988T). The reconstructed numerically focused spatial phase distribution of the cells is depicted in Fig. 3b. The gray values represent quantitative phase data. As a result the thickness and in case of adherent cells the morphology can be calculated. For that purpose determination of the refractive index of the cells and the surrounding medium is necessary. A method for determination of the integral refractive index of cells in transmission light arrangement by digital holographic microscopy is described in [2] ($n_c = 1.38 \pm 0.015$). Fig. 3c shows a 3D image of the cells with cross section. Even small lamellipodia of the cell are resolved (white circle).

The presented results show that digital holographic microscopy allows high resolution multi focus reconstruction of amplitude and quantitative phase data. Therefore, the technique is of particular advantage for marker free, non destructive and full-field recording of living biological samples. Due to recording is only restricted by the frame rate of the applied imaging sensor digital holographic microscopy can also be applied for the analysis of (fast) dynamic processes like effects of drug delivery or cell migration. In conclusion the technique has the potential to form a versatile tool for microscopy applications in the life sciences and biophotonics.

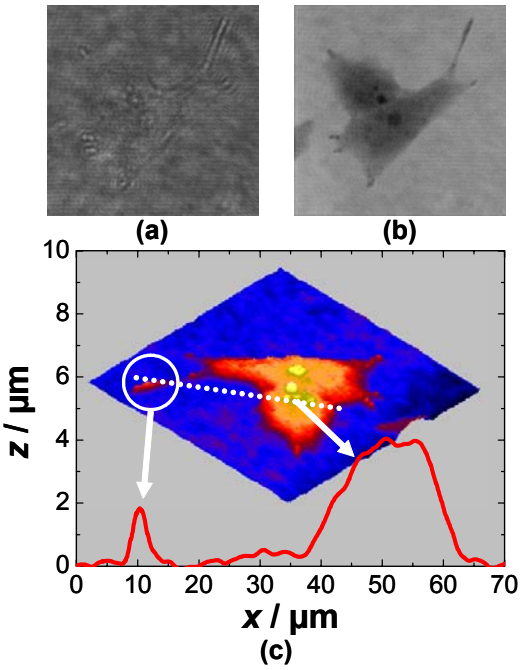

Fig. 3: *(a) Digital hologram of two pancreas carcinoma-cells (PaTu 8988T) captured with 20x microscope lens, (b) reconstructed and numerically focused quantitative phase contrast image of the cells, (c) 3D image with cross section (white line) through cell body and lamellipodia.*

4 Acknowledgements

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Literature

[1] D. Carl, B. Kemper, G. Wernicke, G. von Bally, "Parameter Optimized Digital Holographic Microscope for High Resolution Living Cell Analysis", *Appl. Opt*. Vol. 43, No. 36, pp. 6536-6544 (2004).

[2] B. Kemper, D. Carl, J. Schnekenburger, I. Bredebusch, M. Schäfer, W. Domschke, G. von Bally, "Marker free dynamic 3D-Shape Measurement of Living Pancreas Tumor Cells by Digital Holographic Microscopy", *J. Biomed. Opt.* (submitted).