Enhanced quantitative phase imaging in self-interference digital holographic microscopy using an electrically focus tunable lens

Robin Schubert,1,2 Angelika Vollmer,1 Steffi Ketelhut,3 Björn Kemper3,*
1 Center for Biomedical Optics and Photonics, University of Muenster, Robert-Koch-Str. 45, D-48149 Muenster, Germany
2 George Huntington Institute, Johann-Krane-Weg 27, D-48149 Muenster, Germany
3 Biomedical Technology Center, University of Muenster, Mendelstr. 17, D-48149 Muenster, Germany
*bkemper@uni-muenster.de

Abstract: Self-interference digital holographic microscopy (DHM) has been found particular suitable for simplified quantitative phase imaging of living cells. However, a main drawback of the self-interference DHM principle are scattering patterns that are induced by the coherent nature of the laser light which affect the resolution for detection of optical path length changes. We present a simple and efficient technique for the reduction of coherent disturbances in quantitative phase images. Therefore, amplitude and phase of the sample illumination are modulated by an electrically focus tunable lens. The proposed method is in particular convenient with the self-interference DHM concept. Results from the characterization of the method show that a reduction of coherence induced disturbances up to 70 percent can be achieved. Finally, the performance for enhanced quantitative imaging of living cells is demonstrated.

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1. Introduction

Digital holographic microscopy (DHM) and related techniques have been shown to be versatile tools for non-destructive quantitative phase imaging of reflective technical surfaces and label-free minimally invasive investigations of almost transparent phase objects like living biological cells as for example reviewed in [1–5]. In DHM the object wave is recorded in amplitude and phase by digital capturing of the interference pattern that is obtained from the superimposition with a coherent reference wave [6]. This allows the detection of optical
path length (OPL) changes with high precision and subsequent numerical refocusing [6] or autofocus[7].

However, if a coherent light source like a laser is used to achieve interference, the resolution for OPL changes is affected by scattering patterns in the optical path. Thus, many attempts for capturing digital holograms with low-coherent light sources (LCLS) [8–17] or by using laser light with reduced coherence, for example, generated by a rotating diffusor [18] were reported. Other approaches have been described in which a reduction of speckle noise is achieved by recording multiple off-axis holograms at different polarizations of object and reference wave in combination with subsequently averaging of the reconstructed amplitude distributions [19]. A reduction of coherence induced disturbances could also be achieved by averaging the reconstructed object waves from hologram series that were acquired at slightly different incident angles of the illumination [20]. This effect was also observed as a positive side effect in laser-based digital holographic implementations of tomographic phase microscopy [21–24] or synthetic aperture microscopy approaches for enhanced spatial resolution [23, 24] in which the object illumination is tilted during the hologram acquisition. Such approaches have been demonstrated to suppress coherence-induced noise patterns significantly but typically require very accurate alignment, special common path interferometer arrangements, or the integration of precise mechanical beam tilting devises that need subsequent numerical correction of the continuous object wave tilt in the reconstructed quantitative phase images. The usage of spectrally broadened LCLSs can also affect precise post processing of the reconstructed complex wave fronts. For example, short coherence lengths limit refocusing by numerical propagation, or complex deconvolution algorithms can be necessary for accurate quantitative phase imaging.

Here, we present a simple and cost efficient approach for reduction of coherence-induced noise patterns in laser light-based DHM. In our concept, the object illumination is modulated in amplitude and phase with an electrically focus tunable lens during the recording of a sequence of off-axis holograms. Series of amplitude and phase distributions are reconstructed by existing numerical procedures and subsequently averaged to achieve enhanced amplitude and quantitative phase images. The method is demonstrated and found particularly convenient for a recently reported self-interference DHM principle [25]. However, the concept may also be used with other experimental setups like for example Mach-Zehnder interferometer-based DHM implementations.

2. Setup for self-interference digital holographic microscopy and principle for reduction of coherence induced disturbances

Figure 1 shows the used experimental setup that is based on an inverted research microscope (Zeiss Axio observer A1, Carl Zeiss Micro Imaging GmbH, Goettingen, Germany) with an attached self-interference DHM unit as already presented with details in [25]. A frequency-doubled Nd:yttrium aluminium garnet (YAG) laser (Laser-Compact Co. Ltd, Moscow, Russia), emitting light at \( \lambda = 532 \) nm with a coherence length of \( l_c =250 \) μm is utilized for sample illumination via a single mode optical fiber (SM). For imaging of the sample (S) the microscope’s objective lenses (OL) are applied.
The object wave, leaving the microscope after passing the tube lens (TL), is coupled via aelay lens \( (f_R = 100 \text{ mm}) \) into a Michelson interferometer with a slightly tilted mirror (M1). Due to the tilt of M1, the sheared waves create conventional off-axis holograms which are recorded with a charge coupled device sensor (CCD, The Imaging Source DMK 41BU02, Bremen, Germany). The Michelson interferometer-like setup achieves high interferometric stability [25] and easy coherence length matching. Since the wave front curvatures of object and reference wave are almost spherical and nearly identical, the resulting off-axis carrier fringes consist of a highly parallel pattern. For modulation of the object illumination a focus tunable lens (FL, Optotune EL-10-30, Switzerland, tunable focal length range: \( f_{FL} = 100-150 \text{ mm} \)) is implemented as an additional optical element between the end of the optical fiber (SM) and the Zeiss microscope’s condenser lens (CL, \( NA = 0.55 \)). Changing the focal length of the FL causes a variation of statistically distributed scattering patterns that are induced by surfaces inside the optical path or dust particles outside the focal plane. In contrast, the optical path length changes caused by the investigated object are not affected. Furthermore, thanks to the shearographic self-interference setup, the off-axis carrier fringe pattern is almost not altered during the modulation of the illumination. The numerical reconstruction of the resulting series of off-axis holograms is performed by spatial phase shifting with optional numerical refocusing using a variant of the convolution method as described with details elsewhere [26, 27]. However, also other common numerical reconstruction methods may be applicable. From the resulting complex object wave, beside the amplitude, the phase is retrieved modulo \( 2\pi \). After removal of the \( 2\pi \) ambiguity the unwrapped phase distribution can be used for quantitative phase imaging. In order to eliminate coherence induced disturbances, series of holograms of a specimen are recorded at fixed position of the CL while the object illumination is modulated with the FL. To ensure interferometric stability, each hologram of a sequence is acquired with an exposure time in the range of a few milliseconds. Afterwards, from every hologram of a captured series, sharply focused amplitude and phase images of the sample in the image plane are reconstructed separately and subsequently averaged.
3. Results

An optimum modulation of the object illumination in the configuration shown in Fig. 1 requires a maximized change of the focus point near the sample. The parameters of the optical path of the utilized Zeiss microscope were only available with limited accuracy. Thus, we first calculated the maximum change of the focus point near the sample roughly. Therefore, a thin lens system was assumed by estimating a focal length \( f_{CL} \approx 30 \text{ mm} \) for the microscope condenser (CL) and using different values for the distance between CL and the FL. In further empirical investigations these parameters were optimized experimentally. We finally retrieved for a maximum illumination modulation a distance of 50 mm between the SM’s end and the FL and 250 mm for the distance between CL and FL. This resulted in a variation of the distance range between the focus point of the illumination near the sample to the CL from 31.5 mm to 35.5 mm for the overall focal length change of the FL (\( f_{FL} = 100-150 \text{ mm} \)). We also analyzed the influence of the modulated illumination on the off-axis carrier fringes of the digital holograms in spatial domain and two-dimensional frequency space for different microscope lenses (10x: Zeiss 10x/EC Plan-Neofluar, 40x: Zeiss 40x/0.6 Korr LD Plan-Neofluar, 63x: Zeiss 63x/0.75 Korr LD Plan-Neofluar). An overall focal length change of the FL induced maximum global lateral shifts of the off-axis carrier fringe pattern up to one fringe within the field of view (for illustration see left panel of Media 1) while a corresponding change < 0.02\( \pi \)/pixel of the spatial phase gradient of the carrier fringes was detected. It has been demonstrated previously that such spatial phase gradient changes, near an optimum value of 0.5\( \pi \)/pixel for optimized phase retrieval as used in our experiments, does not affect the accuracy of phase retrieval [28] and can be considered in the utilized numerical reconstruction procedures [26, 27]. However, these parameters may differ for implementations of DHM with other optical imaging configurations.

Next, we experimentally analyzed the performance of the method. Therefore, the maximum number of acquired holograms with fully modulated illumination was limited to \( N = 15 \). This turned out to be a practical compromise between the image acquisition time of a hologram series (in our configuration \( \approx 4 \text{ s} \), with respect to image stabilization after altering the focus of FL) that should allow live cell imaging, noise reduction (see Fig. 3) and limit data storage requirements. An USAF 1951 target with absorbing test structures was used to illustrate the lateral resolution of our setup, the capabilities of the method for imaging of structures with sharp edges and to determine the signal to noise ratio (SNR) in the reconstructed amplitude distributions. Figure 2 shows amplitude images of the test target that were achieved with a 10x microscope lens (Zeiss EC Plan-Neofluar 10x 0.3, \( NA = 0.3 \)). The underlying measurement procedure is illustrated in Media 1. The smallest lateral structures of the test chart (Group 7.6, width of the smallest lateral structure: 2.1 \( \mu \text{m} \)) are clearly resolved for both, the single amplitude image (Fig. 2(a)), reconstructed from a single hologram (\( N = 1 \)), and for the averaged amplitude distribution (Fig. 2(b)) that was achieved from \( N = 15 \) holograms acquired with modulated object illumination. However, due to the lack of suitable test chart structures the maximum lateral resolution of 1.1 \( \mu \text{m} \), predicted by the numerical aperture of the 10x OL, could not be validated.

Parasitic noise patterns that are visible in Fig. 2(a) appear to be significantly reduced in Fig. 2(b). The cross-sections in Fig. 2(c) through in magnified areas in Figs. 2(c) and 2(d) support this finding. To quantify the quality of the amplitude distributions the SNR of the image structures in the USAF test chart was determined in 10 areas for different numbers \( N \) of acquired holograms and objective lenses (OLs) with different magnifications (10x: Zeiss 10x/EC Plan-Neofluar, 40x: Zeiss 40x/0.6 Korr LD Plan-Neofluar, 63x: Zeiss 63x/0.75 Korr LD Plan-Neofluar). We also quantified the corresponding phase noise \( \sigma \) and the related optical path length variations \( \Delta OPL = (\sigma/2\pi)\lambda \) in the simultaneously reconstructed phase distributions in 10 areas without test chart structures.
Fig. 2. Amplitude images of an USAF 1951 test target coded to 256 gray levels, acquired with a 10x microscope lens ($NA = 0.3$). (a) Amplitude from a single hologram. (b) averaged amplitude reconstructed from a sequence of $N = 15$ holograms acquired under illumination modulation. (c), (d) magnified areas of the smallest test chart structures. (e) cross-sections through the magnified areas along the white lines in (c) and (d). The operation principle of the method, the underlying series of off-axis holograms and the corresponding series of single reconstructed amplitude images are illustrated in Media 1.

Figures 3(a), 3(c) and 3(e) show an enhancement of SNR with an increased number of acquired holograms for all microscope lenses, up to 185% for the 10x OL. In agreement with these finding the corresponding values for $\sigma$ that are plotted in Figs. 3(b), 3(d) and 3(f) appear significantly reduced. The relative degree of noise reduction is found in agreement with previously reported data for speckle noise reduction [19, 20, 24] but is lower than for approaches in which a rotating diffuser was applied [18]. We also compared the experimentally obtained phase noise decrease to the course of noise reduction that is theoretically predicted for averaging of uncorrelated laser speckle patterns (solid lines in Figs. 3(b), 3(d), 3(f)) [29]. Therefore, following approaches in [19, 24] the relation between $\sigma$ and the numbers of averaged phase images was estimated to be proportional to $1/\sqrt{N}$ plus an additional constant that considers the contribution of fixed pattern noise like parasitic interferences due to internal reflections within optical components [30]. The resulting fitted curves match the experimental data within the error range and indicate that for a number $N > 15$ a slight further phase noise reduction can be expected. Slight deviations between theoretical and experimental data may be explained by the different optical parameters of the OLs that individually influence the change of the fluctuations of the coherence induced scattering pattern during the modulation with the FL. The minimum phase noise was determined for the 10x OL to $\sigma_{\text{min}} = 0.03$ rad (Fig. 3(a)). This corresponds to a minimum OLP variation $\Delta OPL_{\text{min}} = 2.9$ nm and a noise reduction of $>70\%$ compared with the noise from a single hologram ($N = 1$). However, for the 40x and 63x OLs only lower SNR values and slightly higher phase noise levels than with 10x OL could be achieved. This may be explained by the different individual scattering properties of the objectives. In addition, the OLs with higher magnifications also magnify the coherence induced disturbances that are induced in the previous optical path and thus cause noise patterns with lower spatial frequencies in the
digital holograms. A further efficient reduction of such noise patterns requires a higher degree of modulation of the illumination that could not be achieved with the presented experimental configuration.

Fig. 3. (a), (c), (e) Signal to noise ratio (SNR) of the USAF test target structures in amplitude images for microscope lenses with different magnifications (10x: Zeiss 10x/ EC Plan-Neofluar, 40x: Zeiss 40x/0.6 Korr LD Plan-Neofluar, 63x: Zeiss 63x/0.75 Korr LD Plan-Neofluar); (b), (d), (f) phase noise $\sigma$ and related OPL variations in the corresponding quantitative phase images; $N$: number of off-axis holograms that were recorded during modulated illumination with the FL. Data points and error bars represent the mean values for SNR and $\sigma$ that were acquired in ten different areas of the averaged amplitude and phase images as well as the corresponding standard deviations. The solid curves in (b), (d), (f) present the theoretically expected phase noise decrease.

Finally, we analyzed the feasibility of our approach for quantitative live cell imaging by observing human fibro sarcoma cells (HT-1080, purchased from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in a time-lapse experiment. For DHM analysis the cells were cultured in Petri dishes with a glass lid (ibidi µ-Dish with glass lid, ibidi GmbH, Munich, Germany) and observed in HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) buffered cell culture medium. A heating chamber (HT200, ibidi GmbH, Munich, Germany) provided temperature stabilization at $37^\circ$ C. Series of $N = 15$
holograms with fully modulated object illumination were acquired every 5 minutes for 11.5 hours. For imaging, a 20x microscope lens (Zeiss LD Acroplan 20x/0.4 Korr) was used.

![Figure 4](image)

Fig. 4. (a) Representative segmented quantitative phase images of reconstructed holograms of human fibrosarcoma cells (HT-1080), acquired at different time points during a time-lapse experiment ($\Delta t = 5$ min, $t_{\text{max}} = 11.5$ h). Left column: phase images reconstructed from single holograms ($N = 1$). Right column: Averaged phase images retrieved from $N = 15$ holograms that were acquired during modulated illumination with the FL. Arrows mark differences of the detected cell covered surface. Cross-sections through a cell along the dashed lines are plotted in Fig. 5(a). (b) Corresponding numerically simulated differential interference contrast images calculated by the first derivative of the images in (a) in $x$-direction (Media 2).

Figure 4(a) shows representative single ($N = 1$) and averaged ($N = 15$) quantitative phase images of HT-1080 cells at different time points. For further performance evaluation the images were segmented using the free software cell profiler (www.cellprofiler.org [31]). The averaged phase images reveal considerably enhanced information about intracellular structures like nucleoli and vacuoles and in particular about thin extrusions and the cell borders (see arrows in Fig. 4(a)) which could be also detected by the utilized image segmentation algorithm. In this measurement, the OPL variations could be reduced by 65%, from 15.5 nm ($N = 1$) to 5.5 nm ($N = 15$). This indicates a slightly higher noise level than for the USAF test target (Fig. 3) which may be explained by a higher number of optical interfaces in the sample chamber and dissolved particles in the cell culture medium. Figure 4(b) shows images that were retrieved from the phase distributions in Fig. 4(a) by calculating the first derivative in $x$-direction following [32, 33] in order to simulate images that are similar to differential interference contrast (DIC). The achieved image qualities reflect the reduction of coherent disturbances of the images in Fig. 4(a).
Figure 5(a) illustrates the reduction of coherence induced disturbances by cross-sections through single \( (N = 1) \) and averaged \( (N = 15) \) quantitative phase images of a cell at \( t = 11.3 \) h (see dashed white lines in the last column of Fig. 4(a)). The corresponding cell thickness \( d \) was calculated from the phase values \( \Delta \phi \) as described in [27]. Therefore, an integral cellular refractive index \( n_c = 1.373 \pm 0.005 \) that was retrieved from DHM measurements on suspended HT-1080 cells (see detailed description of the procedure in [34]), and a refractive index of the cell culture medium \( n_{med} = 1.337 \pm 0.001 \), were used. To further quantify the impact of enhanced quantitative phase imaging by object wave modulation with the FL on the analysis of cellular growth, the area \( S_c \) covered by the cells was determined in the segmented quantitative DHM phase images. In addition, the averaged phase contrast \( \Delta \bar{\phi} \) caused by the cells in the area \( S_c \) and the corresponding average cell thickness \( \bar{d} \) was retrieved. Finally, from the parameters \( S_c \) and \( \Delta \bar{\phi} \) the change of the cellular dry mass DM, which is related to the intracellular protein content [35], was retrieved as described in [36] following an approach in [37]. Figure 5(b) shows that the area \( S_c \) covered by the cells is underestimated for the quantitative phase images from single holograms in comparison to the averaged phase images from modulated illumination for periods in which the cells adhere thin on the Petri dish bottom. This leads to an overestimation of the average phase contrast and the corresponding average thickness of very thin cells (Fig. 5(c)) as for \( N = 1 \) due to the higher phase noise thinner parts at the outer cell borders are not correctly considered by the image segmentation procedure (for illustration see arrows in Fig. 4(a) and Media 2). Finally, the results in Fig. 5(d) show, that for \( N = 1 \) due to the contribution of a globally higher background noise level, the cellular dry mass is also slightly overestimated compared to \( N = 15 \) (see also illustration in Fig. 5(a)).
4. Conclusions

In conclusion our results demonstrate that coherence induced disturbances in self-interference DHM are efficiently reduced by amplitude and phase modulation of the object illumination which is achieved by an electrically focus tunable lens. The proposed concept is capable to be integrated into existing DHM setups with only few modifications. The evaluation of the retrieved off-axis holograms can be performed with common numerical reconstruction procedures. The maximum degree for reduction of coherence induced disturbances depends on the specific optical parameters of the utilized experimental arrangement as well as on the used optical magnification and thus has to be individually optimized. The numerical aperture of the object illumination changed during the hologram acquisition due to the operation principle of the proposed method. However, for the presented experimental configuration no influence on the lateral resolution in the reconstructed images was observed. The applicability of our approach for enhanced quantitative live cell imaging was demonstrated by time-lapse observation of human fibro sarcoma cells. Intracellular structures were recovered in averaged phase images of migrating HT-1080 cells that could not be revealed in phase maps from single holograms. Furthermore, improved quantitative phase imaging by modulation of the object illumination with the electrically focus tunable lens allowed improved imaging of thin cell borders. This enables image segmentation with increased precision and thus led to enhanced accuracy in the retrieval of cell growth parameters like cell covered area, average cell thickness and dry mass. The main limitation of our approach is currently the hologram acquisition time of ≈4s which restricts the application for observing fast cellular processes. Data acquisition times may be accelerated in future by optimized synchronization and increased hologram acquisition rates but still are significantly higher than for single-shot-techniques. Nevertheless, in its current configuration the method prospects to be a versatile label-free tool for morphology observation and growth analysis of very thin single cells with enhanced accuracy.

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