Partially coherent digital in-line holographic microscopy in characterization of a microscopic target

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Digital holographic microscopy enables the capture of large three-dimensional volumes. Instead of using a laser as an illumination source, partially coherent alternatives can be used, such as light-emitting diodes, which produce parasitic reflection and speckle-free holograms. Captured high-contrast holograms are suitable for the characterization of micrometer-sized particles. As the reconstructed phase is not usable in the case of multiple overlapping objects, depth extraction can be conducted on a reconstructed intensity. This work introduces a novel depth extraction algorithm that takes into consideration the possible locations of multiple objects at various depths in the imaged volume. The focus metric, the Tamura coefficient, is applied for each pixel in the reconstructed amplitude throughout the volume. This work also introduces an optimized version of the algorithm, which is run in two stages. During the first stage, coarse positions of the objects are extracted by applying the Tamura coefficient to nonoverlapping window blocks of intensity reconstructions. The second stage produces high-precision characterizations of the objects by calculating the Tamura coefficient with overlapping window blocks around axial positions extracted in the first stage. Experimental results with real-world microscopic objects show the effectiveness of the proposed method. © 2014 Optical Society of America

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1. Introduction
Holography enables the capture of both the amplitude and phase of a wavefront reflected from or transmitted through a real-world three-dimensional (3D) object [1]. In digital holography, the recording medium is a digital sensor, such as a charge-coupled device (CCD) or complementary metal-oxide-semiconductor (CMOS), which enables the capture of holograms in digital format, in turn enabling direct digital processing of the holograms [2–4]. Holography, whether analog or digital, comprises two steps: capturing followed by processing. The processing step in digital holography includes the reconstruction of a hologram, which can be accomplished optoelectronically with the use of a spatial light modulator [5] or numerically by simulating the propagation of light with a computer algorithm [6].

If holography is used in microscopy [digital holographic microscopy (DHM)], the hologram needs to be magnified before capturing it. The magnification can be performed with a microscope objective. This method is suitable for both off-axis and in-line setups. By using an off-axis setup, in which the light is split into object and reference beams with a small separating angle, both reconstructed amplitude and phase can be used without heavy processing [7–9]. In an in-line Gabor interferometer, magnification can
be performed by natural free-space propagation, as discovered by Dennis Gabor, the inventor of holography [1,10].

The Gabor interferometer has been used successfully in the imaging of microspheres [11], biological samples [12–15], microscopic particles [16–18], and micro-organisms [19,20]. The downside of using an in-line setup is the spatially overlapping DC and two twins in the reconstruction, which can lead to difficulties in using the reconstructed phase. However, with weakly scattering objects, the phase is still usable in in-line setups [14]. If the distance between the two twins is large enough, the out-of-focus twin’s energy is spread and will not corrupt the information of the other twin. In hologram recordings of multiple overlapping objects (as in volumes of fibers or particles), the reconstructed phase is not quantitatively usable, and therefore, measurements based on phase typically require a monolayer of objects.

In Gabor interferometry, the magnification changes as a function of sensor–pinhole distance; therefore, for the lateral measurements to be accurate, the axial positions of the objects need to be known. As stated previously, the reconstructed quantitative phase can be used in accurate depth calculations [21–23]; however, if it is not usable, the depth extraction analysis can be conducted on the amplitude/intensity reconstructions.

Various autofocus algorithms have been applied and implemented in the fields of conventional microscopy [24,25] and digital holography [26–33]. These methods cannot be used, as they are for depth extraction of multiple objects or for objects that are tilted along the optical axis, as these algorithms estimate one in-focus depth only. Instead, most can be used as part of shape-from-focus algorithms, which calculate depth value for each pixel. A comprehensive comparative study of different shape-from-focus methods in general has been conducted by Pertuz et al. [34]. In the field of digital holography, shape-from-focus algorithms have been reported in several works. Edge detection using different methods such as variance or wavelet transforms has been tested and applied successfully in [17,35–44]; amplitude analysis has been in active use, especially in particle field holography [13,16,18,45–52]; and other nonconventional methods have been reported in [53–58].

In this work, we introduce a novel shape-from-focus algorithm that can be applied to holograms containing multiple objects at different depths. The algorithm takes advantage of contrast as a measure of focus level. Contrast has experimentally been found to be a solid focus metric for holograms captured with an in-line Gabor interferometer.

The rest of this work is organized as follows: in Section 2, the hologram capture and preprocessing steps are introduced; in Section 3, the developed shape-from-focus algorithm is introduced; in Section 4, the effectiveness of the proposed method with real-world objects is shown; and Section 5 concludes the work.

2. Capture

As the digital holographic reconstructions suffer from speckle noise, which is a consequence of coherent light, numerical reconstructions may require extra processing. One alternative approach is to shorten the coherence length. This can be accomplished by capturing digital holograms with a partially coherent light source [59–63] or by using a rotating diffuser in front of the coherent light source [64]. Partially coherent light enables the capture of speckle and parasitic reflection-free holograms of moving objects. Holograms captured with an LED are free of potentially information-corrupting speckle, as speckle noise is the consequence of long spatial coherence. The short temporal coherence length of an LED enables the capture of parasitic reflection-free holograms, as the two beams, with a path difference greater than the temporal coherence length, will not interfere.

To capture 3D-volume holograms, a similar setup as described in [61] was used with a different LED (center wavelengths $\lambda = 635$ and 470 nm), a 40x microscope objective, a 5 μm pinhole, and a 1280 × 1024 pixel monochrome CMOS camera with a pixel pitch of 5.3 μm (IDS Imaging UI-1242LE) (Fig. 1). The camera performs two-dimensional (2D) sampling of the hologram, which is given by the mutual intensity function of the reference wave and the object-affected wave [65].

To increase the reconstruction quality and to remove possible impurities and unwanted reflections, pixel-wise subtraction between the captured hologram and a reference image is necessary. A reference image is a captured image without the presence of an object. The distance from the pinhole to the camera is crucial for accurate measurements; however, this is not necessarily easily measured. To overcome this
difficulty, a calibration procedure is introduced. This procedure consists of two steps: capturing followed by analyses. In the capturing step, a well-known reference object, the 1951 USAF resolution test chart, is captured by moving the object at different object-to-camera distances (at three different distances in this work). At the next step, captured holograms are numerically reconstructed to the object plane. From the in-focus intensity reconstructions (in this work group 4, element 3 of the 1951 USAF chart is used, which corresponds to 24.8 μm line width), object widths are measured. As the object is captured at various different object-to-camera distances and therefore with different magnifications, a magnification–reconstruction–distance relation can be formed using a simple linear regression.

3. Processing

The reconstruction allows extraction of a diffraction field at any distance \( z \) in the volume. This can be calculated with the Fresnel approximation [66] as

\[
U(x,y;z) = \frac{-i}{\lambda z} \exp(i kz) I_0(x,y) \otimes \exp \left[ \frac{i \pi x^2 + y^2}{\lambda z} \right],
\]

where \( \lambda \) is the wavelength of the light, \( I_0(x,y) \) is the hologram, \( \otimes \) denotes a convolution operation, and \( k = 2\pi/\lambda \). Equation (1) can be solved numerically with the angular spectrum method by using fast Fourier transforms (FFT) as

\[
U(x,y;z) = \mathcal{F}^{-1}[\mathcal{F}[I_0(x,y)] \mathcal{F}[h(x,y;z)]],
\]

where \( \mathcal{F} \) and \( \mathcal{F}^{-1} \) denote the FFT and the inverse FFT, respectively, \( x \) and \( y \) denote the spatial discrete coordinates; and \( h(x,y;z) \) is the diffraction kernel. The FFT of the \( h(x,y;z) \) can be replaced with [66]

\[
\mathcal{F}[h(x,y;z)] = H(f_x,f_y;z)
\]

\[
 = \exp \left\{ -j k z \left[ 1 - (f_x)^2 - (f_y)^2 \right] \right\},
\]

where \( f_x \) and \( f_y \) are the spatial frequencies. From the complex valued reconstruction \( \hat{U}(x,y;z) \), intensity reconstruction can be extracted as

\[
I(x,y;z) = \text{Re}[U(x,y;z)]^2 + \text{Im}[U(x,y;z)]^2
\]

and phase reconstruction can be extracted as

\[
\phi(x,y;z) = \arctan \left( \frac{\text{Im}[U(x,y;z)]}{\text{Re}[U(x,y;z)]} \right).
\]

Our proposed focus metric method is based on the contrast texture measure model [67], which has been used successfully as a focus metric with off-axis holograms [32]. As an approximation of the Tamura coefficient, we calculate the Tamura coefficient as [32,68]

\[
C(I_z) = \sqrt{\frac{\sigma(I_z)}{I_z^2}},
\]

where \( I_z \) is an image or image region, and \( \sigma(I_z) \) is the standard deviation of the image as

\[
\sigma(I_z) = \sqrt{\frac{1}{MN-1} \sum_{i=1}^{M} \sum_{j=1}^{N} (I_z(i,j) - \bar{I}_z)^2},
\]

where \( N \) and \( M \) are image width and height, respectively, and \( \bar{I}_z \) is the average of the image as

\[
\bar{I}_z = \frac{1}{MN} \sum_{i=1}^{M} \sum_{j=1}^{N} I_z(i,j).
\]

In [32], an off-axis hologram is reconstructed in 1 mm steps, and the Tamura coefficient is calculated for each full-size intensity reconstruction. From this set of coefficients, global maximum is searched, which gives the best in-focus position. The global maximum corresponds to a single depth and therefore it cannot be used for scenes which contain multiple objects at different depths. The novelty of the proposed approach is that the scene may contain multiple objects at various different depths. This is taken into account by calculating the Tamura coefficient for each pixel in the reconstruction stack. The reconstruction stack is formed by reconstructing the hologram through a volume with half of the depth of focus of the system to fulfill the sampling theorem. The depth of focus of the system is calculated as

\[
\text{DOF} = \frac{\lambda}{\text{NA}^2},
\]

where \( \text{NA} \) is the numerical aperture of the system as

\[
\text{NA} = n \sin \left[ \tan^{-1} \left( \frac{W}{2L} \right) \right],
\]

where \( n \) is the refractive index, \( W \) is the side length of the camera, and \( L \) is the camera-to-object distance. To calculate Tamura coefficient for each pixel at each depth \( z \) with \( n \times n \) pixel block, the equation becomes

\[
C_z(k,l) = \left( \frac{1}{n^2 - 1} \sum_{i=k-N}^{k+M} \sum_{j=l-N}^{l+M} (I_z(i,j) - \bar{I}_z(k,l))^2 \right)^{1/2} / \bar{I}_z(k,l),
\]

where \( k \in [0, (M - 1)] \), \( l \in [0, (N - 1)] \), and \( \bar{I}_z(k,l) \) is defined as

20 May 2014 / Vol. 53, No. 15 / APPLIED OPTICS
\[ \bar{I}_z(k, l) = \frac{1}{n^2} \sum_{i=k-n/2+1}^{k+n/2-1} \sum_{j=l-n/2+1}^{l+n/2-1} I_z(i, j). \] (12)

The volume \( C_z \) contains 2D Tamura coefficients for each depth \( z \). From this set of 2D coefficients, for each pixel \((k, l)\), we will find the maximum value of \( C_z(k, l) \). Depth \( z \), where the maximum is found, will be stored in a 2D depth map at pixel position \((k, l)\) as

\[ \text{DMap}(k, l) = \arg\max_z C_z(k, l). \] (13)

To find multiple overlapping objects with the same \((k, l)\) coordinates, 2D coefficients can be thresholded with the experimentally chosen threshold, and positions with a coefficient value above the threshold can be stored in a 3D point cloud.

During the experiments it was observed that, by inverting the amplitudes of the reconstructions, the Tamura coefficients were more accurate. This is due to the enhanced contrast of light objects on a dark background [69]. The inversion operation was performed by finding the global maximum of the amplitude reconstruction stack, subtracting that value from each pixel, and taking the modulus of the result.

To shorten the processing time, the algorithm can be optimized and implemented in two steps. In the first step, the reconstruction stack is formed with a larger reconstruction step \( D \) (e.g., 1 mm), and each intensity reconstruction is divided into four equal quadrants. For each of these quadrants, Tamura coefficients are calculated, forming four vectors of equal length, each containing information of spatially different regions of the volume. From each of these vectors, local maxima are extracted with a simple peak-finding algorithm to give a coarse estimation of the object’s position. In the second step, around the found peaks, the Tamura coefficients are calculated for each pixel in overlapping mode as...
defined previously, with new reconstruction distances being around peaks \( \pm D - \text{DOF} \) and with reconstruction step size being half of the depth of focus of the system.

4. Experimental Results

The system was calibrated using the explained calibration procedure which led to the magnification–reconstruction–distance relation as \( M = ad + b \), where constant \( a \) is 43.9489, \( d \) is the reconstruction distance, and constant \( b \) is 0.90063 (Fig. 2). The proposed depth extraction approach was tested with

<table>
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Fig. 5. Reconstructed amplitudes according to the depths from the depth map shown in Fig. 4(d): (a) 57.8 mm, (b) 60.8 mm, (c) 79.8 mm, and (d) 81.4 mm from the hologram plane.

Fig. 6. Point cloud representation of two intersecting fibers. A threshold of 0.4 for Tamura coefficients on each layer was used in creating the point cloud (Media 1).

Fig. 7. Results with 4 \( \mu \)m latex beads; the hologram was captured with blue LED (470 nm center wavelength); coefficients were calculated using a \( 7 \times 7 \) window. (a) Reference subtracted hologram, (b) initial Tamura coefficients, (c) Tamura coefficients above experimentally chosen threshold of 0.18, (d) ground truth of target positions (the lateral and axial positions of different beads were manually estimated), (e) depth map using (b), (f) depth map using (c). Circles inside the region, marked with the white rectangle, are a consequence of strong diffraction pattern around the object. The proposed approach successfully finds all 42 objects. Additionally, two objects are found around the region marked with the white arrow and several objects inside the region marked with the white rectangle. Average absolute error is 1 mm. If the object(s) inside the region marked with the white rectangle are ignored, average absolute error is 0.68 mm.
semitransparent Rayon fibers and 4 μm latex beads that were randomly distributed in water. The sample carrier was an Ibidi μ-Dish 35 mm glass bottom petri dish with 3 mm of sterile water. The reference image was captured before adding the targets [Figs. 3(a)–3(c)]. After adding the targets, the sample was stirred, and holograms of the moving targets were captured.

The initial Tamura coefficients shown in Fig. 4(a) can be thresholded to produce a less noisy depth map. By thresholding and keeping only the values above the threshold, the background can be effectively removed [Fig. 4(c)]. A low-noise depth map can be extracted by placing a depth value for each lateral pixel position, where the axial maximum coefficient is above the threshold [Fig. 4(d)]. Figure 5 shows amplitude reconstructions at different identified depths; the depths of the two intersecting fibers are correctly identified.

Id is the object number as shown in Fig. 4(d), d is the reconstruction distance, and M is the magnification at the d. Lengths were calculated using the regionprops function of MATLAB.

Instead of picking a single depth for each lateral position, a point cloud was created by picking multiple depth values for each lateral position (Fig. 6, Media 1). To remove false positives from the point cloud, Tamura values below 0.4 were not used in the analyses. Figure 7 shows results with 4 μm latex beads together with manually estimated ground truth data. The lateral positions of the beads are accurately estimated. Some of the Tamura coefficient values above the chosen threshold lead to false positives, which is a consequence of noise. This is mostly caused by the diffraction patterns around the out-of-focus objects. Increasing the threshold would lead to missed targets. This trade-off between false positives and missed targets exists with a threshold. Although we have not added numerical noise filtering (such as mean or median filtering) to our reconstructions, this is likely to enhance the accuracy of the proposed method. To test the optimized algorithm, the fiber hologram was reconstructed in 1 mm steps, and each amplitude reconstruction was divided into four quadrants (Fig. 8). Tamura coefficients were calculated for each quadrant (Fig. 9). From the results, it is obvious that all of the objects and their positions cannot be determined by using the Tamura coefficients of full-size amplitude reconstruction. On a regular laptop computer using MATLAB, the optimized methods takes 50 s. Without the optimization, runtime is 120 s.

5. Conclusions

A new depth extraction method that takes advantage of a Tamura contrast texture measure model was introduced. It was shown that amplitude reconstructions can be used effectively to extract depth information of microscopic objects when the reconstructed phase is not available. The proposed approach takes into account that the digital hologram can contain multiple objects at different depths. The optimized version of the algorithm enables fast characterization of volumes containing microscopic objects. Effectiveness and robustness of the method in depth map and point cloud creation was shown with real-world microscopic samples. The holograms were captured with compact, low-cost, LED-illuminated Gabor interferometry, which was shown to produce good-quality, high-contrast digital holograms of moving microscopic objects.

In this work, the used side length of a block was 7 pixels. Using a large block size (compared to the size of the object) may introduce some difficulties. First of all, the large block size enlarges the objects and this would require more processing; using morphological operation such as erosion is needed for the quantitative measurements to be meaningful. The enlargement itself may introduce difficulties if the density of objects in the volume is high. This is because
the coefficient values of different objects may become fused. Using a large block size also leads to increased processing time. Larger block size can also be beneficial if the objects are small; the enlargement may lead to a visually better result. Also, the background information will be diminished with a use of larger block size, because the regions occupied by the objects get higher Tamura coefficient values. These facts also affect the threshold value used. In conclusion, the block size and the threshold should be chosen as appropriate for the application.

It is possible to use the same algorithmic principle and use a different focus metric, such as variance or amplitude. Although we used a four-quadrant approach in the optimized version, the grid in coarse position estimation can be made smaller. Making the grid smaller raises the probability of identifying smaller objects with the cost of calculation time. In addition, the window size in the second stage has an effect on the calculation, as the smaller window size enables the identification of smaller details. These two parameters are object-specific and should be chosen as appropriate for the application.

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