Extended focused imaging of a microparticle field with digital holographic microscopy

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We present a numerical technique for extended focused imaging and three-dimensional analysis of a microparticle field observed in a digital holographic microscope working in transmission. The three-dimensional localization of objects is performed using the local focus plane determination method based on the integrated amplitude modulus. We apply the refocusing criterion locally for each pixel, using small overlapping windows, to obtain the depth map and a synthetic image in which all objects are refocused independent from their refocusing distance. A successful application of this technique in the analysis of the microgravity particle flow experiment is presented. © 2008 Optical Society of America

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In traditional optical microscopy depth of focus is a limiting factor in many applications. In-focus observation of the entire volume of an experimental cell can be achieved by mechanical scanning and repositioning of the sample in such a way that slices of the sample are in focus one by one. However, this time-consuming procedure does not allow for observation of rapid phenomena, such as particle flow. Recently, digital holographic microscopy (DHM) proved to provide, at the same time, a quick acquisition rate and one-shot recording of the complete holographic image of the observed volume [1–3]. The plane-by-plane refocusing can be done numerically in postprocessing giving in-focus images of objects at different focusing distances, which enables the following of dynamic processes in the whole volume of the experimental cell.

The numerical refocusing in digital holography (DH) replaces the manual focusing performed in a traditional microscopy. However, it requires a numerical criterion to decide at which distance the investigated object is in focus. A number of various focus metrics have been proposed using an intensity gradient [4], self-entropy [5], local intensity variance [6], spectral l_1 norms [7], and wavelet theory [8]. The best-focus detection has also been used to achieve an extended depth of field in images of macroscopic objects [9,10], while in DHM an extended depth of field has been achieved using the phase map retrieved from a hologram of a large continuous object [11]. Holographic methods have also been used to detect and track microscopic objects without focus depth extension [12–16].

We have recently proposed [17] a method to find the refocusing distance of an object investigated by digital holographic microscopy working in transmission. We analyzed the integrated modulus M_d of the numerically propagated complex amplitude u_d as a function of the propagation distance d,

$$M_d = \int_{-\infty}^{\infty}\int_{-\infty}^{\infty} |u_d(x,y)|\mathrm{d}x\mathrm{d}y. \tag{1}$$

When the best-focus distance is reached, the integrated amplitude M_d reaches minimum for the pure amplitude object and maximum for the pure phase object. However, this criterion works well when all objects in the scene are at the same distance. When there are a number of objects placed at different refocusing planes, function $M_d(d)$ has a number of corresponding local minima, and it is not possible to bring all objects into focus at once. This fact is particularly limiting in particle flow experiments where dozens or hundreds of particles are present in the field of view. Therefore it is necessary to apply the criterion locally in such a way that a correct refocusing distance is found for each object.

The global criterion is low bounded by the square root of the total energy $\sqrt{E}$ and requires the assumption that the total light intensity is conserved during digital propagation. While this can be assumed when the whole image is taken into account and the refocusing distance is sufficiently short, it will not hold when we take into consideration a small region of the image. As a result the extremum of M_d may not necessarily indicate the actual best-focus plane. However, we can define a local focus criterion normalized by the total light intensity in the selected region of interest (ROI), which is low bounded by 1 in all propagation planes,

$$M_{dL} = \frac{\iint_{\mathrm{ROI}} |u_d(x,y)|\mathrm{d}x\mathrm{d}y}{\sqrt{\iint_{\mathrm{ROI}} |u_d(x,y)|^2\mathrm{d}x\mathrm{d}y}}. \tag{2}$$

This criterion can be applied locally, and it can be shown that its global extremum is reached at the best-focus plane [18]. We create a synthetic extended focused image (EFI) in which all particles are in focus. This is achieved by repropagating the whole image over a range of distances, which should include the correct best-focus distances of all objects, and we calculate the local refocusing criterion in overlapping ROIs around each point. As a result we obtain a depth map $DMap(x,y)$ containing the best-focus distance for each pixel of the image. The synthetic EFI is created by replacing each pixel with the corresponding value of intensity of that pixel at its best-focus distance. The result of this procedure is presented in Fig. 1. It shows that the EFI gives us a very good approximation of the circular shape of the opaque particles with a satisfying contrast between dark objects and bright background. As the best-focus criterion does not return valid information when there is no object in the ROI the background contains some numerical artifacts. If an EFI with a uniform background is required for visualization purposes, these artifacts can be removed using the information about the variance of $M_{dL}(d)$.

Figure 2 presents a magnification of two microparticles with a diameter of 7 μm refocused manually using classical DH propagation [Figs. 2(b) and 2(c)] and reconstructed in the EFI [Figs. 2(d) and 2(e)] created using various sizes of ROIs. The choice of the local ROI is crucial for the quality of the EFI. If it is small, the numerical artifacts around the particles decrease the contrast between object and background [Fig. 2(d)], while too large a ROI may result in incorrect reconstruction [right particle in Fig. 2(f)] when objects are too close to each other. An optimal size of the ROI [in this case 51×51 pixels; Fig. 2(e)] is case dependent and must be chosen based on the size of objects and the density of the particle field.

The obtained image allows us to perform object detection and image segmentation to extract the position and size of each separate particle. Combining it with the depth map we obtain a three-dimensional position and diameter of each detected particle. Although the accuracy of lateral coordinates depends on the optical resolution of the system, the precision of the vertical position (refocusing distance) is limited by the depth of focus of the optical system, low-pass filtering of the Fourier method, and the distance between the neighboring refocusing planes used in the algorithm. Unfortunately, the propagation and calculation of the local focus criterion for each pixel in the repropagated plane is by far the most time consuming part of our algorithm. Therefore it is not feasible to use a very short distance between two consecutive refocusing planes. We propose a significantly more time-efficient method to improve the vertical localization accuracy. First, we take a relatively large (e.g., 10 μm) distance between refocusing planes to create

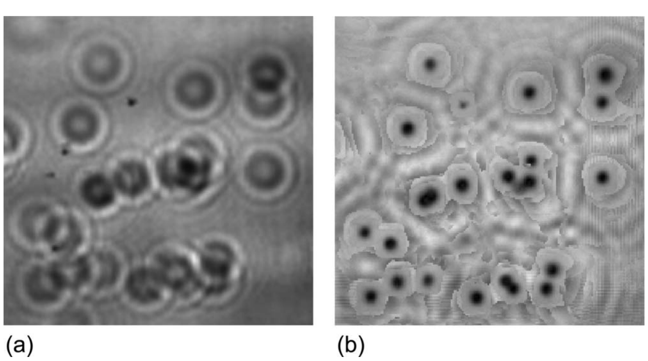

Fig. 1. Demonstration of the local refocusing algorithm: (a) original out-of-focus intensity retrieved from the digital hologram, (b) synthetic EFI. In that example particles have a diameter of 5 μm, pixel size is 0.33 μm, and the ROI is 31×31 pixels large.

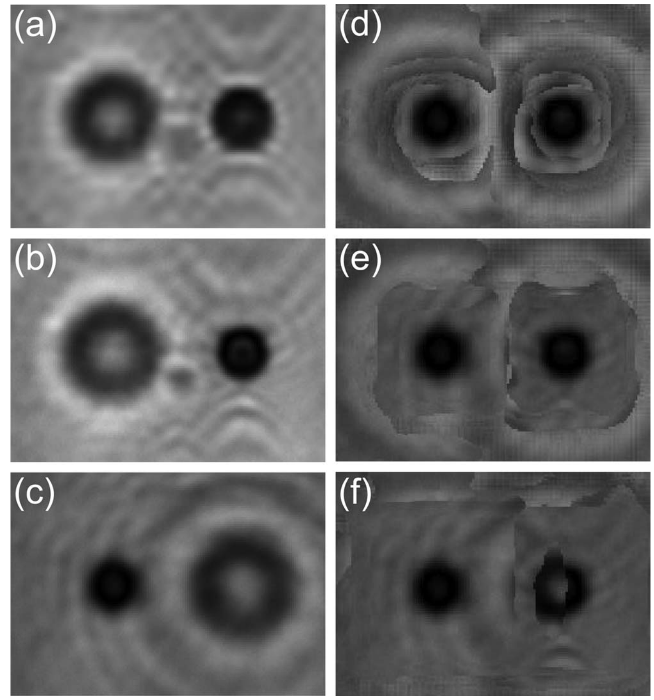

Fig. 2. Comparison of the digitally refocused DHM images of 7 μm latex particles with a synthetic EFI created using various sizes of ROIs. (a) Original intensity image, (b) right particle in focus ($d=-18$ μm), (c) left particle in focus ($d=115$ μm), EFI images with a ROI of (d) 25×25, (e) 51×51, and (f) 75×75 pixels. Pixel size is 0.33 μm.

an approximate synthetic EFI of particles. In this image we localize the particles and then perform fine refocusing of each particle separately. For each particle we take a rectangular ROI that is slightly bigger than the particle in such a way that it completely contains this particle as well as a small margin around it. Then, using a nonlinear optimization algorithm (simplex search method—MATLAB fminsearch function), we find within a predefined accuracy the refocusing distance by applying the focus criterion to the whole ROI. Using this combined approach we can increase the precision of the vertical localization at a time cost proportional to the number of objects, performing the propagation and calculation of the focus criterion only on a small ROI. As the final step of processing we eliminate false detections, which are usually caused by particle aggregates, overlapping particles, and dust on optical elements, using additional criteria. Aggregates can be easily rejected by checking the shape of the detected object, while dust does not produce a deep minimum of the focus criterion $M_{dL}(d)$.

We have tested this algorithm on a number of various synthetic and experimental images. Typically the full analysis of a 1024×1024 hologram containing dozens of particles takes less than 2 min on an Intel Core2Duo 2 GHz portable processor using a ROI of 31×31 pixels, a 10 μm refocusing step, and a required position accuracy of 1 μm.

In our earlier work [3] we demonstrated that DHM enables in-focus visualization of silica microparticles [monodisperse microspheres with a diameter of 5 μm and a density of 2.5 g/cm^3 (Duke Scientific)] in the whole volume of a 234 μm thick step split-flow lateral-transport thin (Step-SPLITT) separation cell [19]. Here we analyze this microparticle field using the synthetic EFI. In our experiment we use a Mach–Zehnder configuration with partially spatially coherent illumination [3] and the Fourier method for the extraction of complex amplitude from the single exposition digital holograms [1]. We observe the resuspension of particles originally placed on the bottom wall of the cell in Poiseuille flow of mean velocity of 17.8 cm/s.

The synthetic EFI [Fig. 3(b)] obtained with a ROI of 31×31 pixels shows all particles in-focus even though the background illumination is not uniform. It is possible to localize almost all particles [Fig. 3(c)] using simple thresholding and, in agreement with theoretical predictions, they are found at a similar distance from the bottom wall of the channel [Fig. 3(d)]. The refocusing distances are Gaussian distributed with a relatively small standard deviation of 7.8 μm. The distribution of measured diameters is also Gaussian with the mean value of 5.33 μm and a standard deviation of 0.29 μm, which is in line with the real diameter of particles. However, it shows that although the described algorithm correctly approximates the average value of diameter, every single measurement is burdened with a relatively high uncertainty. The measurement of the diameter is very sensitive to the parameters of the detection, in particular the threshold. A way to improve the accuracy of diameter measurement would be to use a more sophisticated segmentation method, which would take into account the local characteristics of the background illumination.

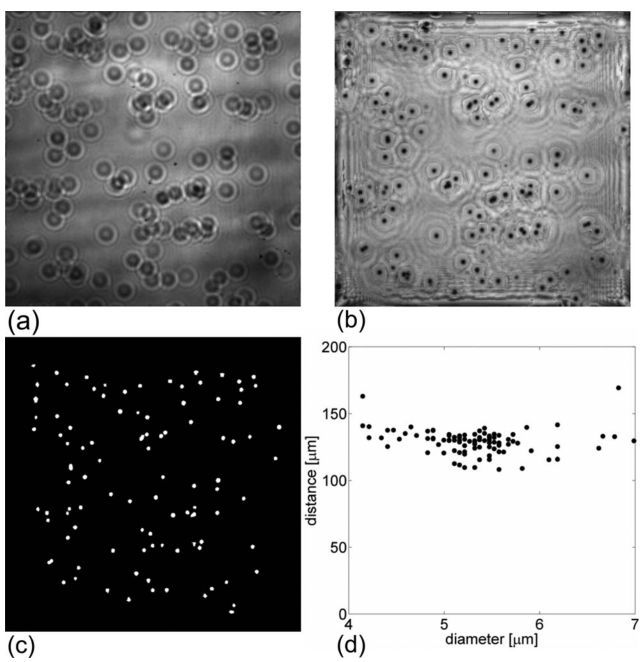

Fig. 3. Particle detection in the hologram recorded in a microparticle resuspension experiment under microgravity conditions [19]: (a) original intensity reconstructed from the hologram (field of view: 330 μm $\times$ 330 μm), (b) synthetic EFI, (c) result of image segmentation and detection of particles, (d) refocusing distance versus diameter of the detected particles.

The presented technique of EFI creation enables automatic particle detection and tracking in a series of holograms recorded in particle flow experiments. Compared to other techniques of focus depth extension it seems to be most attractive when there is a large number of particles in the experimental volume under investigation.

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