Superresolved digital in-line holographic microscopy for high-resolution lensless biological imaging

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Abstract. Digital in-line holographic microscopy (DIHM) is a modern approach capable of achieving micron-range lateral and depth resolutions in three-dimensional imaging. DIHM in combination with numerical imaging reconstruction uses an extremely simplified setup while retaining the advantages provided by holography with enhanced capabilities derived from algorithmic digital processing. We introduce superresolved DIHM incoming from time and angular multiplexing of the sample spatial frequency information and yielding in the generation of a synthetic aperture (SA). The SA expands the frequency of the imaging system, allowing submicron resolutions in both transversal and axial directions. The proposed approach can be applied when imaging essentially transparent (low-concentration dilutions) and static (slow dynamics) samples. Validation of the method for both a synthetic object (U.S. Air Force resolution test) to quantify the resolution improvement and a biological specimen (sperm cells biosample) are reported showing the generation of high synthetic numerical aperture values working without lenses. © 2010 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3481142]

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1 Introduction

History of digital in-line holographic microscopy (DIHM) (or lensless microscopy) started when Gabor reported on a method to avoid spherical aberration, improving image quality in electron microscopy by preventing lenses in the experimental setup.1 In this in-line configuration, the nondiffracted light acts as reference beam that interferes with the diffracted components generated by the sample. If the object blocks only a small part of the illumination beam (that is, the object can be considered as a weak diffractive sample) then reconstruction of the complex amplitude that is diffracted by the object is feasible by using holographic tools. On the other hand, when the object blocks a high amount of light, reconstruction by Gabor’s concept is not applicable because diffraction dominates the process and other strategies are needed to retrieve the diffracted object’s wavefront information.2,3

Nevertheless, weak diffractive samples are quiet common in biomedical imaging practice because many are low-concentration samples, or single-cell samples, or thick quasi-transparent samples, etc. For this reason, the original idea reported by Gabor1 has been recently revived in the optical range coinciding with the relatively modern developments in both solid-state image sensors and numerical processing capabilities provided by computers.4,5 The use of DIHM in biology has enables 3-D imaging with resolution in the micrometer range in applications such as underwater observations, tracking of moving objects and particles, identification of microorganisms, and the study of erosion processes in coastal sediments.6–9

However, DIHM lacks high numerical aperture (NA) due to both geometrical distortion and the mandatory compromise between the illumination pinhole diameter, the illumination wavelength, and the need to obtain a reasonable light efficiency. Thus, achievable NA values in DIHM are typically around 0.45,10 and ways to improve the NA are of unquestionable interest. One can identify two different types of approaches aimed to improve the NA in DIHM: approaches involving modifications in the DIHM setup (first class) and those involving improvements in the numerical reconstruction procedure (second class).

As an example of first class of methods, Garcia-Sucerquia et al. reported on a clever way to improve the NA of the DIHM setup.11 They replaced the air gap between the input sample and the CCD by a high refractive index medium. This strategy directly increases the NA value because the refractive index of the surrounding medium explicitly defines the NA. Garcia-Sucerquia et al.11 achieved an improvement in the NA value from 0.39 to 0.55. And as second one, Kanka et al. have recently proposed two approaches to provide high-contrast, high-resolution images in DIHM based on the so-called tile superposition propagation (TSP) technique.12,13 Essentially, the TSP method is a fast propagation algorithm involving low-computer-memory consumption for exact scalar wave field propagation. The TSP algorithm divides the recorded ho-
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Fig. 1 Sketch of the SR DIHM setup: (a) on-axis and (b) off-axis illumination cases.

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source–CCD distances are \( d = 400 \, \mu m \) and \( z = 11.4 \, mm \), respectively. The point source is shifted \( 175 \, \mu m \) from the optical axis to allow four off-axis recordings in the vertical (V) and horizontal (H) orthogonal directions.

Note that this distance is lower than the one defined by the Nyquist criterion \( s_m = 0.563 \, \mu m \) and it is the proper distance to transmit quasi-contiguous spectral content in the shorter (V) CCD direction. As result, the NA of the tilted illumination is \( NA_{\text{illum}} = 0.4 \). Figure 2 images both the four recovered bandpass images when tilted beam illumination is performed and after applying the numerical reconstruction procedure, and the generated SA incoming from the addition of the four off-axis images with the central one (on-axis illumination case). Each bandpass image corresponds with an elementary rectangular aperture in the generated SA because they are denoted by different types of lines.

Figure 3 depicts the case of conventional DIHM [Fig. 3(a)] coming from the central rectangular aperture of Fig. 2, and SR DIHM [Fig. 3(b)] coming from the Fourier transformation of the expanded SA. As a result, the NA and SNA values for the V and H directions are improved from \( NA_V = 0.21 \) and \( NA_H = 0.28 \) to \( SNA_V = 0.61 \) and \( SNA_H = 0.68 \), respectively. Obviously, resolution limits are also improved according to those SNA values and can resolve, in both V and H directions, the last element of the USAF test (Group 9, Element 3 having a period of \( 1.55 \, \mu m \)). The three elements corresponding with Group 9 are magnified in Fig. 3(c) while a vertical section along the dashed black line in Fig. 3(c) is plotted in Fig. 3(d) to clearly show the three black lines integrating each one of the elements. However, despite of the generated high SNA value, the new superresolution limits are modest (\( > 1 \, \mu m \)) because of the VCSEL illumination wavelength.

To validate resolution limits of \( < 1 \, \mu m \), we performed a second experiment in which a violet laser \( (\lambda = 0.405 \, \mu m) \) is used. The experimental setup configuration is the same one as before. Once again, the condition imposed by the Nyquist maximum shift \( s_m = 0.268 \, \mu m \) for violet wavelength is still fulfilled. Figure 4(a) depicts the case when conventional DIHM is considered. Because the illumination wavelength of the violet laser is 2.1 times lower than that of VCSEL, the resolution limits become improved while the NA values remained constant. According to the theory, the new superresolution limits are \( SR_V = 0.66 \, \mu m \) and \( SR_H = 0.59 \, \mu m \) incoming from \( SNA_V = 0.61 \) and \( SNA_H = 0.68 \), respectively. However, aside from a global improvement in the image quality, there are not enough elements in the test to quantify the superresolution effect when SR DIHM is applied [Fig. 4(b)]. Thus, we have applied our method to a biological sample: it is an unstained swine sperm biosample that is dried up allowing fixed sperm cells for the experiments. The sperm cells have an ellipsoidal head of \( 6 \times 9 \, \mu m \) and a tail’s width of \( \sim 2 \, \mu m \) on the head side and \( < 1 \, \mu m \) on its end. Figure 5 shows the experimental results. Now, not only four off-axis elementary...
apertures are considered, but eight are, as we can see in the generated SA [Fig. 5(c)]. Thus, full two-dimensional spatial frequency space is covered in the SA. We can observe that the thinner part of the tail, which is not visible under conventional DIHM [Figs. 5(a) and 5(b)], becomes resolved after applying the proposed SR DIHM approach [Figs. 5(d) and 5(e)]. Finally, Figs. 5(f) and 5(g) show a reference image of the same biosample area taken with a 0.8 NA, 60x microscope lens from a BX61 Olympus microscope under spatially coherent (closing the condenser diaphragm) white-light illumination.

4 Conclusions
In this paper, we have introduced and validated SR DIHM as a lensless microscopic imaging method that is based on SA generation incoming from tilted beam illumination over the input sample and holographic recording. The experimental setup becomes extremely simple and verifies the definition of a SNA of being larger than 0.6 in every direction. This NA value obtained when considering the proposed approach is comparable to the maximum achieved 0.7 NA reported in the field of DIHM. The full procedure can be realized in a few seconds by automating the displacement of the source to the off-axis positions and synchronizing the capture of the holograms. We have calibrated and quantified the method using a standard USAF resolution test target achieving a submicron resolution limit.

We have also reported on the application of the proposed approach to imaging of a sperm cell biosample demonstrating its feasibility for the analysis of biological samples. The proposed approach can be applied for submicron resolution lensless imaging purposes when considering essentially transparent (low-concentration dilution) samples. Also, although the proposed approach is limited to samples that are static or has a slow variation in time (a few seconds), extension of the technique to nonstatic (fast dynamic) samples is under consideration.

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