Digital in-line holographic microscopy with partially coherent light: micrometer resolution

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Using a blue light-emitting diode (LED) it is shown that lensless Digital In-line Holography Microscopy (DIHM) with spherical waves can yield micrometer resolution, even when large objects, such as the head of a fruit fly (Drosophila melanogaster), are imaged. By changing the size of the pinhole, the influence of spatial coherence of the spherical wave at the plane of the sample on the resolution is analysed. Although the achieved resolution is less than that ultimately obtained with a fully coherent laser and a numerical aperture of over 0.5, the use of a LED allows for a very compact and low cost implementation of DIHM. Experiments with micrometer-size beads support the claim of micrometer resolution.

Keywords: Digital in-line holographic microscopy; coherence; resolution.

Con el uso de un diodo emisor de luz (LED) se muestra que la microscopia holográfica digital en línea sin lentes (DIHM), puede lograr resolución micrométrica aun cuando se observan objetos extensos, como la cabeza de una mosca de la especie Drosophila melanogaster. Por medio del cambio del tamaño del pinhole, se analiza el efecto que tiene coherencia espacial de las ondas esféricas en el plano de la muestra, sobre la resolución del microscopio. Aunque la resolución alcanzada es menor que la se obtiene con un láser completamente coherente iluminando un sistema con apertura mayor a 0.5, el uso del LED permite la implementación de un sistema DIHM de bajo costo. Experimentos realizados con esferas de tamaño micrométrico validan el alcance de resolución micrométrica.

Descriptores: Microscopía holográfica digital en línea; coherencia; resolución.

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1. Introduction

With the invention of white light and Fourier holography in the off-line geometry it has been demonstrated that partially coherent light is under certain restrictive circumstances sufficient for holographic reconstruction. As a result, some efforts have been made to examine the usefulness and limitations of using partially coherent light sources in digital holographic microscopy. Low coherence sources have been used to implement optical coherence tomography with numerical reconstruction of phase and amplitude [1,2]; depth resolution equal to the coherence length of the source (25 μm) and lateral resolution given by the diffraction limit of the microscope objective (2.2 μm) were achieved. In particular, it has been recognized that reconstructed images obtained by digital holography with lenses (in the off-line geometry, mainly) suffer in quality due to the presence of speckle or coherent noise inherent to laser sources. In order to reduce the phase noise introduced by the highly coherent sources, Stürwald *et al.* [3] have employed a light emitting diode (LED) as a source with reduced coherence for imaging pancreas tumor cells. Dubois *et al.* [4,5] have proposed several ways to overcome this problem; namely, by reducing the spatial coherence of the illuminating light, therefore improving the quality of the reconstructed images from 3D samples and flow analysis systems. The same idea of short coherence sources has been utilized to do optical sectioning. Based upon the limited optical path difference allows for producing a steady interference pattern, Pedrini and Tiziani [6] and Martinez-Leon *et al.* [7] have produced 3D images obtained from numerical reconstruction of holograms recorded without and with lenses, respectively; all these efforts make use of the temporal coherence of light either to reduce nuisance effects of laser sources or to get improved 3D images of bulk samples for particle flow analysis.

The problem of coherence noise is overcomed automatically in Digital In-line Holographic Microscopy (DIHM) as the pinhole, the source of spherical waves, acts as a spatial filter; this fact was pointed out by our group [8] and by Repetto *et al.* [9]. The latter used a LED focussed down onto a 10 μm pinhole as a source to image 10 μm beads and also showed that DIHM has other important advantages in terms of signal-to-noise ratio and alignment simplification, as it is also pointed in many papers by the present authors.

In a recent paper, Gopinathan *et al.* [10] have analysed via the second order coherence theory the influence of tem-

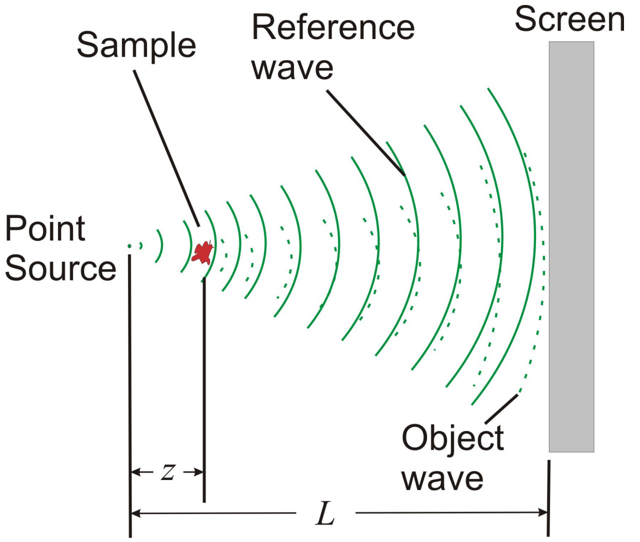

FIGURE 1. Schematic setup Digital In-line Holographic Microscopy.

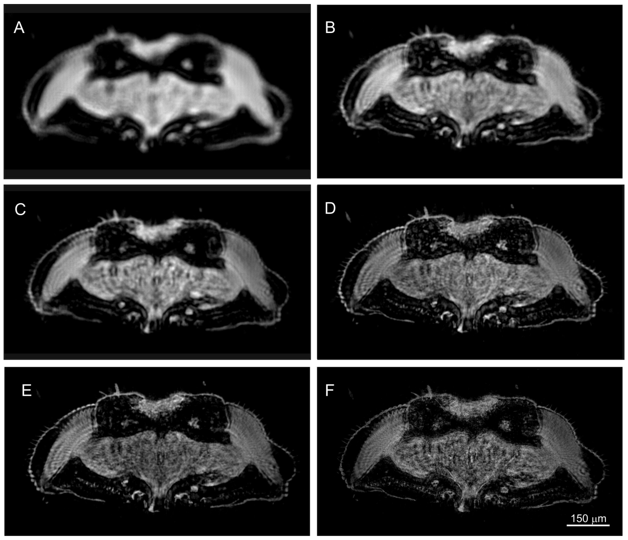

FIGURE 2. Effect of the spatial coherence in DIHM. The diameter of the circular area that is almost coherently illuminated is D_{coh}=6.2 μm, 9.4 μm, 20.1 μm, 40.8 μm and 115 μm for panels A, B, C, D and E for pinholes of diameters d_p = 30 μm, 20 μm, 10 μm, 5 μm and 2 μm, respectively; the mean wavelength was $\bar{\lambda}$=450 nm. The image in panel F corresponds to fully coherent illumination and wavelength λ=405 nm. For all of the experiments the numerical aperture was set up to 0.41 and the pinhole-object distance varying between z = 400 and 900 μm.

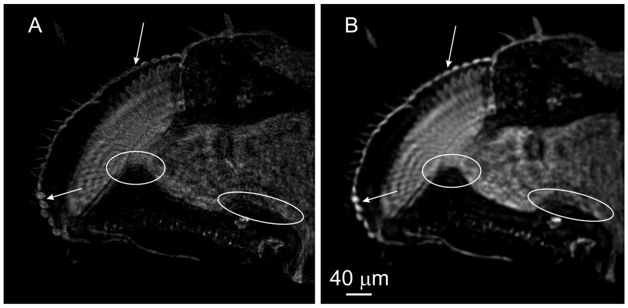

FIGURE 3. High resolution DIHM with biological samples. Panels A and B show reconstructed images of paraffin wax section of drosophila melanogaster head stained by the Bodian method, obtained with fully coherent (panel A) and partially coherent (panel B) DIHM; the numerical aperture for both experiments was 0.48.

poral and spatial coherence on DIHM. By means of numerical modeling and experimental results on imaging latex beads, they show that the reduction of the coherence of light leads to broadening of the impulse response and therefore smear the details of the reconstructed image.

Here we report a more extensive experimental study of the effects of limited spatial coherence on the quality of the reconstructed images from DIHM. Light emerging from a super luminescence blue LED is focussed down onto a pinhole so that the spatial coherence is controlled by simply varying the size of the pinhole. The ultimate resolution of light-emitting diode DIHM is obtained when the LED is focussed down to the point where illumination is almost as coherent as using a blue laser. The best-achieved resolution is comparable but not quite equal to that obtained with fully coherent illumination: while LASER-DIHM (fully coherent DIHM) provides sub-micrometer lateral resolution, LED-DIHM (partially coherent DIHM) achieves resolution in the micrometer range.

2. Dihm with partially coherent illumination

DIHM has now been perfected to the point that submicrometer lateral resolution is routinely achieved [11-13]. In LASER-DIHM, a highly coherent optical source (laser) of wavelength λ is focussed onto a pinhole of diameter of order λ, to generate highly coherent spherical waves that illuminate a sample placed at a distance z from the pinhole. The weak wave scattered by the sample interferes with the strong unscattered reference wave from the pinhole producing an interference pattern on the surface of a two-dimensional screen (CMOS or CCD camera) and the recorded intensity, the inline hologram, is transferred to a PC for further processing and numerical reconstruction (see Fig. 1).

The scattered wave, which carries the information of the object, is retrieved from the in-line hologram in a three steps process: i) the intensity impinging upon the screen when the object is removed, called reference intensity, is recorded; ii) one performs a pixel-wise subtraction between the in-line hologram and the reference intensity to get the contrast inline hologram $\tilde{I}$; and iii) through numerical diffraction of the conjugate unscattered reference wave, when it illuminates the contrast hologram, one retrieves the information of the sample. Since in DIHM the reference wave is spherical, this process of reconstruction is given by the Kirchhoff-Helmholtz transform [14]

$$K(\mathbf{r}) = \int_{\text{screen}} d^2\xi \; \tilde{I}(\xi) \exp[ik\mathbf{r}\cdot\xi/|\xi|]. \qquad (1)$$

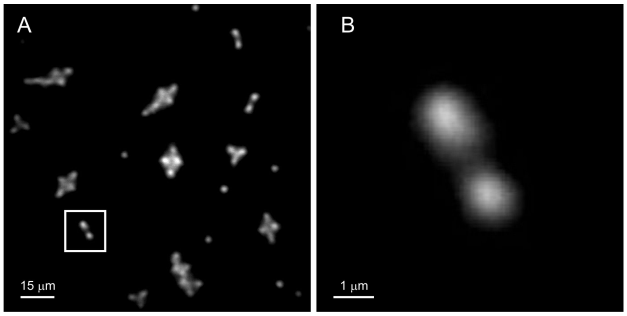

FIGURE 4. Spatially partially coherent DIHM of microspheres. Panel A shows the reconstructed hologram of 2 μm diameter polystyrene beads. Panel B shows an enlarged picture of the highlighted area (white square) of panel A.

In Eq. (1) the integration extends over the surface of the screen with coordinates ξ=(X, Y, L) with L the distance from the pinhole to the center of the screen; $k = 2\pi/\lambda$ is the wave number. $K(\mathbf{r})$ is a complex quantity that can be calculated on a number of planes at various distances z_r from the point source (pinhole) in order to recover the three-dimensional information of the sample from a single two-dimensional in-line hologram. This fact constitutes the main advantage of DIHM over optical microscopy, since it allows one to obtain information in three-dimensions without the need of any mechanical refocusing at sub-micrometer resolution [10,14].

The numerical implementation of Eq. (1) uses a coordinate transformation that rewrites the integral in the form of a scalable convolution, which is solved by three successive, two-dimensional, fast Fourier transforms [16]. The latest implementation of this algorithm [17], running on a laptop with an Intel Core DUO processor, reconstructs in-line holograms of 2048×2048 pixels and 15 bits depth in seconds.

DIHM with partially coherent illumination has been implemented by focusing the light from an ultra-bright LED onto a pinhole of diameter d_p. The incoherent source is a blue LED with a mean wavelength $\bar{\lambda}$=450 nm and a FWHM of 25 nmk, which can record in-line holograms of samples with optical thickness up to 8 μm. The spatial coherence over the sample plane is determine by the van Cittert-Zernike theorem [18], which states that the diameter of the area that is almost coherently illuminated at the plane of the sample is given by $D_{coh} = 0.32\bar{\lambda}z/d_p$, where z is the distance between the pinhole and the sample. Thus, for a fixed pinhole-sample distance one can vary the spatial coherence in DIHM by simply adjusting the diameter d_p of the pinhole. A complementary discussion about the effects of the partially coherence illumination in DIHM can be found in Ref. 10.

3. Experimental method and results

The most devastating effects of the highly coherent illumination in digital holography can be considered when biological samples are studied; this fact has been recognised by many authors, and consequently, different ways of tackling this problem have been proposed [2-7,19]. Here we have chosen the reduction of the spatial coherence and therefore to test the effect of the spatial coherence on the performance of DIHM; a series of experiments have been carried out on relatively large biological samples with dimensions of tens and hundreds of micrometers, but with micron-sized internal structure. Such large objects block out a substantial part of the illumination cone with the result that the scattered wave is actually longer comparable in intensity to the unscattered wave. Recall that the conventional argument is that for holography to work the scattered wave must be small compared to the unscattered wave, although the term "small" is rarely quantified [18]. It has been proven repeatedly (quote some earlier papers by the authors) that numerical reconstruction works well even when the two waves are of the same magnitude because the noise introduced by classical diffraction (the term in the quadratic amplitude of the scattered wave) remains a fairly uniform background, see for instance [8,11,15].

The first experiments were done on a paraffin wax section of the head of a fruit fly (Drosophila melanogaster) stained by the Bodian method [18]. The head is about 1000 micron wide and the sample has a thickness of the order of 10 μm. The panels of Fig. 2 show the reconstructed images when the spatial coherence over the plane of the sample is changed. The diameter of the circular area that is almost coherently illuminated (D_{coh}) is varied from 6.2 μm for Panel A to 115 μm for panel E, see figure caption for the other parameters, and panel F is the reconstructed image for fully coherent illumination; the latter is obtained with a violet laser of wavelength λ=405 nm illuminating a 0.5 μm diameter pinhole. For panels A and B, the low spatial coherence of the source results in reconstructed images in which only the overall shape of the sample is visible and very few details of its internal structure are recognizable, *i.e.* the resolution deteriorates due to the reduced spatial coherence that broadens the impulse response of the microscope [9]. Increasing the spatial coherence over the sample plane, renders more internal details of the sample, a visible and a sharper contrast of the boundaries of the overall sample is obtained, see panel C, D and E. Panels D, E and F show comparable resolution but the fully coherent DIHM exhibits lower signal-to-noise ratio (SNR) than the partially coherent illumination. The latter also used a smaller pinhole of diameter 0.5 μm.

For a more detailed comparison we show in panels A and B of Fig. 3 an enlarged section taken at a slightly larger numerical aperture of 0.48. Note however, that the resolution is controlled by the smaller of the numerical apertures of the pinhole ($NA = 1.22\lambda/d_p$) and that it is given by the half-angle under which the CCD detector is seen from the pinhole. Panel A shows the reconstructed image from LASER-DIHM and panel B for LED-DIHM. The arrows on both panels show typical spots where the difference in spatial resolution is noticeable. Note however, that the limited spatial coherence of LED-DIHM leads to some signal averaging so that the resulting image has less coherent noise than that of LASER-DIHM; the encircle spots support this statement. From this point of view, the performance of LED-DIHM is quite remarkable considering the simplicity and cost reduction by a factor of a thousand when using a LED instead of a LASER.

For a quantitative assessment of the achievable lateral resolution with LED-DIHM, experiments with polystyrene beads were carried out. The best achieved results are shown in Fig. 4. There, reconstructed images of 2 μm diameter polystyrene beads are shown when they are imaged with the LED-DIHM. The experiments were carried out with a blue LED focused down onto a 2 μm diameter pinhole, *i.e.* with a numerical aperture of 0.28. On the other hand, the numerical aperture of the optical system was set at 0.35 (by adjusting the pinhole-screen distance) which would give a theoretical resolution [10] of 0.8 μm. From the pictures in Fig. 4, panels A and B, we note that the beads (of diameter 2 μm) are resolved according to the estimate based on the smaller numerical aperture of the pinhole. This is clearly shown in panel A where doublets, triples and quadruplets of beads are fully resolved. We note in passing that Laser-DIHM with a 2 μm pinhole shows similar (low) resolution.

4. Conclusion

It has been shown that it is possible to achieve micrometer resolution when a partially coherent and inexpensive light source, such as an LED, is used in DIHM. It is important to emphasize the requirement that the area of almost coherent illumination at the object plane is comparable to the size of the object itself. LED-DIHM is the most cost-effective and mechanically the simplest implementation of in-line holography with spherical waves, and should be a useful addition to the tools to study larger biological organisms in the millimetre range, being LASER-DIHM the preferred method for the study of micrometer and submicrometer structures.

The current constraint on LED-DIHM is the fact that the power presently available for LEDs is too low to get enough light through a pinhole smaller than 2 μm in diameter. When more powerful LEDs become available it will be straightforward to get better resolution while maintaining the advantage of the LED-DIHM to improve the overall signal to noise ratio.

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