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Point-spread function synthesis in scanning holographic microscopy

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Abstract

Scanning holographic microscopy is a two-pupil synthesis method allowing the capture of single-sideband inline holograms of noncoherent (e.g., fluorescent) three-dimensional specimens in a single two-dimensional scan. The flexibility offered by the two-pupil method in synthesizing unusual point-spread functions is discussed. We illustrate and compare three examples of holographic recording, using computer simulations. The first example is the classical hologram in which each object point is encoded as a spherical wave. The second example uses pupils with spherical phase distributions having opposite curvatures, leading to reconstructed images with a resolution limit that is half that of the objective. In the third example, axicon pupils are used to obtain axially sectioned images.

1. INTRODUCTION

Holography was invented by Gabor in 1948 as an attempt to improve the resolution of the electron microscopes available at the time.¹ With the availability of laser sources in the 1960's and the spatial carrier method introduced by Leith and Upatnieks,² holography became an area of active research in the 1970's and 1980's. Numerous applications in metrology, three-dimensional (3D) sensing, image processing, and data storage, to name only a few, were proposed. Many of these ideas were successfully implemented, but a number remained at the laboratory demonstration stage. In particular, holographic microscopy had little success until the recent advances in high-resolution solid-state array sensors based on charge-coupled device technology (e.g., the CCD and complementary metal-oxide semiconductor), as well as in digital computational and data storage capacities. Since then, digital holography, which was first proposed by Goodman and Lawrence³ and in which the hologram is recorded on a 2D pixelated array sensor and is reconstructed by digital backpropagation using diffraction theory, has become a practical tool with an increasing number of applications.⁴ In particular, digital holographic microscopy of biological specimens was successfully demonstrated^{5,6} and shown to offer unique possibilities such as the acquisition of quantitative phase information⁷ and *a posteriori* compensation of aberrations.⁸

One attractive advantage of holography in biological studies is that the information of an entire 3D volume of scatterers is acquired in a single 2D scan of the array detector. The advantage of speed is crucial to capture 3D dynamics of cellular activities, for example. Modern microscopic methods produce 3D images of incomparable spatial resolution, but the axial sections are collected sequentially, which hampers an accurate acquisition of the 3D dynamics. In spite of its successes, digital holography has an important limitation due to the spatial and temporal coherence needed to record the interference pattern of the scattered field with a reference wave from the same source. This precludes the use of holography with fluorescent specimens, which scatter incoherent light. The study of dynamical cellular processes, however, requires both high-spatial-resolution imaging of structural information and the capture of temporally resolved activities revealed by fluorescence-labeling methods. Scanning holographic microscopy is a two-pupil synthesis method allowing the capture of single-sideband in-line holograms of incoherent or fluorescent 3D specimens.⁹

The recording of an in-line Gabor hologram, whether analog or digital, can be described in the Fresnel approximation as a coherent imaging process with a point-spread function (PSF), which is a complex spherical wave. Unfortunately, the PSF of incoherent optical imaging systems is constrained to be real positive.¹⁰ This is imposed by the fact that the incoherent transfer function is the autocorrelation of the pupil of the imaging system.⁹ Two-pupil interaction methods (also called two-pupil synthesis) have been proposed to overcome this limitation.¹¹ In a two-pupil system, the transfer function is the cross correlation of two different pupils, with the consequence that the associated PSF is, in general, complex. To extract the desired information unambiguously by demodulation of the signal from a two-pupil imaging system, the two pupils must be distinguishable in the frequency domain. This distinguishability is also required in conventional off-axis holography, where the distinction is made in the spatial frequency domain.² With the two-pupil method, the distinction can be made either in the spatial frequency domain¹² or in the temporal frequency domain, with the latter scheme offering greatly reduced coherence requirements.¹¹ It was recognized early by Poon and Korpel that the two-pupil method could provide a means of implementing the holographic principle of Gabor with incoherent and self-luminous objects.¹³ The proposed scheme, later called scanning holography,¹⁴ was to scan the object in a 2D raster with the desired temporally modulated PSF (a spherical wave for a Gabor hologram) and demodulate the signal in the temporal frequency domain by heterodyning, or filtering in Fourier space.^{14–16}

The application of scanning holography to high-resolution microscopy opens up the possibility of capturing the structure of thick 3D specimens labeled with fluorescence markers in a single 2D scan. The method has been studied in some detail,^{15,16} and preliminary results have been published.¹⁷ More recent results have shown experimentally that the reconstruction of scanning holographic micrograms can lead to a spatial resolution exceeding the resolution limit of the objective.¹⁸ High-resolution holographic reconstructions of 3D fluorescent specimens have also been recently demonstrated.⁹

The goals of this paper are to demonstrate that not only can scanning holographic microscopy reconstruct images with a resolution comparable with or better than that of a wide-field microscope using the same objective but also that the two-pupil interaction method on which it is based opens up the possibility of synthesizing PSFs that are not accessible to conventional imaging systems, whether coherent or incoherent. In Section 2, we briefly review the principle of two-pupil interaction imaging in order to establish the notations. Section 3 outlines the use of the method to obtain holograms of incoherent objects. This information is not new but is needed for comparison with the two examples of unconventional PSFs described in Sections 4 and 5. These comparisons are supported by numerical simulations. A summary is given in Section 6.

The two-pupil method offers a broad range of possibilities for synthesizing unconventional PSFs. Once the desired imaging properties are clearly defined, one can search for pupil distributions capable of synthesizing the desired PSF. We illustrate these possibilities in Sections 4 and 5, with two examples requiring pupil functions that can be implemented with simple hardware. The first example requires pupils with spherical phase fronts, which are easily obtained with spherical lenses. The second example requires conical phase fronts, which can be obtained using commercially available spatial light modulators. In the example of Section 4, the goal is to obtain the highest possible transverse resolution with a given objective and in a single acquisition step. In conventional wide-field imaging, the transverse resolution is limited by the numerical aperture of the objective ($NA = \sin \alpha$, where α is the angle of the steepest ray entering the objective) to $\Delta x \approx \lambda/2 \sin \alpha$ (λ is the wavelength of the radiation). We show in Section 4 that, with two pupils having spherical phase fronts of opposite curvatures, it is possible to reduce the transverse resolution limit to $\Delta x \approx \lambda/4 \sin \alpha$. Experimental results showing this improvement have already been published,¹⁸ but a clear physical explanation was not

provided. The reason for the factor-of-2 improvement in transverse resolution is that each pupil produces in object space a spherical wave with half-cone angle α , the acceptance angle of the objective. The interference of the two wavefronts, if they have opposite curvatures, leads to a hologram in which each object point is encoded as a spherical wave with an acceptance angle 2α , thus leading to a gain of resolution of a factor of 2 on reconstruction. It is also shown in Section 4 that the arrangement with two spherical wave pupils with opposite curvatures produces an interference pattern in object space that, to first order, is invariant with defocusing. Because of this, the images reconstructed from holograms recorded with these pupils exhibit an extended depth of focus. Images with extended depth of focus are desirable in high-resolution microscopy of thick specimens because these images are not corrupted by the out-of-focus flare that severely degrades the contrast and resolution in wide-field microscopy.¹⁹

In the example discussed in Section 5, the goal is to obtain a hologram in which only the information from a chosen thin axial section of the sample is recorded. Clearly, the holographic advantage is lost in this scheme, since the axial sections must be recorded sequentially. Nevertheless, the method offers an interesting alternative to the confocal methods for rejecting the out-of-focus information. We show in Section 5 that sectioning can be achieved with two pupils having conical wavefronts of opposite phases (we later refer to these as axicon pupils). The scanning distribution in this case is the superposition of two thin rings, and the modulated signal arises only from the regions of the sample where the two rings overlap. If the two axicons have opposite phases, the effect of defocusing is to increase the radius of one of the rings and decrease the radius of the other. Their overlap is thus limited to a thin axial section of the specimen. The out-of-focus information is illuminated by nonoverlapping rings and does not contribute to the modulated signal (although it will contribute to the noise). This mode of operation, where the sectioning is due to diffraction and interferences, is to be contrasted with other methods of optical sectioning and confocal imaging,^{20–23} in which pinholes are used to physically prevent the out-of-focus information from reaching the detector. The confocal method is very direct but signal wasting. It is also quite sensitive to misalignment and noise.

2. TWO-PUPIL SYNTHESIS

Figure 1 is a sketch of the generic two-pupil synthesis setup. Two pupils with complex amplitude distributions $P_1(u, v)$, $P_2(u, v)$, created, for example, by two spatial light modulators, are illuminated by two plane waves from the same coherent source. In one of the channels, the radiation is given a temporal frequency offset Ω by using, for example, an electro-optic phase modulator or an acousto-optic device. The tilde indicates pupil distributions expressed as functions of the spatial frequency coordinates (u, v) . The objective projects two diffraction distributions in object space with spatial coordinates (x, y, z) . The specimen is scanned in the x - y plane, and spatially integrating detectors collect the transmitted, scattered, or fluorescent radiation. The amplitude distribution projected on the specimen is $P_1(x, y, z) + P_2(x, y, z)\exp(-i\Omega t)$, where, in the Fresnel approximation limit,

$$P_j(x, y, z) = F^{-2} \left\{ \tilde{P}_j(u, v) \exp[-i\pi\lambda z(u^2 + v^2)] \right\} \quad (1)$$

is the 3D diffraction distribution due to pupil j .²⁴ F^{-2} indicates a 2D inverse Fourier transform of the defocused pupil. For a weakly scattering specimen (first Born approximation) with scattering (or fluorescence) radiance $I(x, y, z)$, the signal from the detector is

$$\int dz \left| P_1(x_s, y_s, z) + P_2(x_s, y_s, z) \exp(-i\Omega t) \right|^2 I(x - x_s, y - y_s, z), \quad (2)$$

where (x_s, y_s) is the instantaneous position of the 2D scanning stage. As seen in this expression, the 2D scan effects a 2D spatial convolution recorded in the time domain. After demodulation

and bandpass filtering of the signal around the carrier frequency Ω , we obtain a 2D distribution of complex amplitudes that is a generalized hologram. It has the form

$$H(x_s, y_s) = \int dx dy dz P_1(x, y, z) P_2^*(x, y, z) I(x - x_s, y - y_s, z). \quad (3)$$

The function

$$S(x, y, z) = P_1(x, y, z) P_2^*(x, y, z) \quad (4)$$

is called the scanning distribution. In short notation, Eq. (3) becomes

$$H(x, y, z) = \int dz S(x, y, z) \otimes I(x, y, z), \quad (5)$$

where the symbol $\otimes$ indicates a 2D convolution. Equation (3) or (5) represents the output of an incoherent imaging system. It is incoherent in the sense that it is linear in object intensity and insensitive to its phase, but its spread function $S(x,y,z)$ is, in general, complex. As will be reviewed in Section 3, if $S(x,y,z)$ is a spherical wave, $H(x,y)$ is a single-sideband in-line Gabor hologram of the 3D object intensity distribution $I(x,y,z)$.

3. SCANNING HOLOGRAPHIC MICROSCOPY

Holograms of fluorescent specimens are obtained by using a scanning distribution at the excitation wavelength and collecting the spatially integrated scattered fluorescence signal at the emission wavelength.⁹ A single-sideband inline Gabor hologram results from using a small aperture as one of the pupils (so that its spread function in object space is a quasi-planar wave with limited extent) and a spherical wave as the other pupil.¹⁵ The pupil amplitude distributions are

$$\begin{aligned} P_1(u, v) &\sim \delta(u, v), \\ P_2(u, v) &= \exp[-i\pi\lambda z_0(u^2 + v^2)] P_0(u, v). \end{aligned} \quad (6)$$

$P_0(u, v) = \text{circ}(\sqrt{u^2 + v^2} / \rho_{\max})$ is the support of the pupil. $\text{Circ}(x) = 1$ for $x < 1$ and $= 0$ otherwise. $\rho_{\max} = \sin \alpha / \lambda$ is the spatial frequency cutoff of the objective. With expressions (1), (4), and (6), the scanning distribution is found to be, in the Fresnel approximation,

$$S(x, y, z) = F^2 \left\{ \exp[-i\pi\lambda(z_0 + z)(u^2 + v^2)] P_0(u, v) \right\}. \quad (7)$$

This amplitude distribution is approximately (in the paraxial approximation) a spherical wave with a numerical aperture $\sin \alpha$ coming to a focus at a distance z_0 from the focal plane of the objective, as shown in Fig. 1. The transverse radius of the scanning distribution is given by $a = z_0 \sin \alpha$. This radius is a free parameter that can be varied by changing the distance z_0 , provided, in practice, that the Fresnel number ($N_F = a^2 / \lambda z_0$) is not too small.²⁵

The hologram is reconstructed by correlation with the cross section of the scanning distribution matched to the desired plane of focus z_R . Thus, with Eq. (5), the reconstruction amplitude is

$$\begin{aligned} I_R(x, y, z_R) &= H(x, y) \otimes S^*(x, y, z_R) \\ &= \int dz I(x, y, z) \otimes Q(x, y, z; z_R), \end{aligned} \quad (8)$$

where the asterisk indicates a complex conjugate and

$$Q(x, y, z; z_R) = S(x, y, z) \otimes S^*(x, y, z_R) \quad (9)$$

represents the 3D PS of the holographic imaging process. In Fourier space, the reconstruction algorithm reads as

$$I_R(u, v; z_R) = H(u, v) S^*(u, v; z_R), \quad (10)$$

and the transfer function is, using Eqs. (1), (6), and (7),

$$\begin{aligned} Q(u, v; z) &= S(u, v; 0) S^*(u, v; z) \\ &= \exp[-i\pi\lambda z(u^2 + v^2)] |P_0(u, v)|^2. \end{aligned} \quad (11)$$

Equation (11) represents the transfer function of a coherent imaging system with defocus z . It is important to realize that although the holographic process described here is incoherent in the sense that it is sensitive to object intensity only (a requirement to accommodate fluorescent specimens), the characteristics of the reconstructed images are those of a coherent system. Specifically, the transfer function is the pupil itself. Thus, for example, the in-focus transfer function ($z=0$) is unity up to the cutoff frequency of the objective. In three dimensions, the transfer function is confined to the surface of the McCutchen generalized pupil $\delta(\lambda^{-2} - u^2 - v^2 - w^2)$, where (u, v, w) are the spatial frequency coordinates in 3D space for a monochromatic radiation with wavelength λ .²⁴

The expected transverse and axial resolution limits of the reconstructed images are identical to the limits of a coherent imaging system having the same numerical aperture but with the difference that these limits refer to an intensity distribution rather than to a complex amplitude distribution. Using the Marechal criterion, the transverse resolution limit is defined as the distance at which a point source must be displaced in the transverse plane to produce a phase shift of π at the edge of the scanning distribution. Similarly, the axial resolution limit is the distance a point source must be displaced axially to produce the same phase shift of π at the edge of the scanning distribution. This definition leads to the usual expressions $\Delta x = \lambda/2 \sin \alpha$ and $\Delta z = \lambda/4 \sin^2(\alpha/2)$.

The result of this analysis is illustrated by digitally simulating the recording and reconstruction of simple objects in 3D space. We chose an objective with a numerical aperture $\sin \alpha = 0.25$ and a focal length of 2 cm. The wavelength of the excitation radiation is 0.5 μm (the emission wavelength plays no role in this analysis). The distance z_0 is chosen to be 80 μm so that the radius of the scanning distribution is approximately $a \sim 20 \mu\text{m}$ in object space. The expected transverse and axial resolution limits of the reconstructed images are $\Delta x \sim 1 \mu\text{m}$ and $\Delta z \sim 8 \mu\text{m}$.

Figure 2 shows the magnitude of the single-sideband hologram of seven point sources 7.5 μm apart transversally and with axial positions ranging from -12 to $+12 \mu\text{m}$, in steps of 4 μm , from the focal plane of the objective. The axial distance between two adjacent points is thus one half the objective's axial resolution limit (or depth of focus). The reconstruction of the hologram, focused on the central point source ($z=0$), is shown in Fig. 3. We chose to represent the amplitude of the reconstruction rather than the physically more meaningful intensity (since we are dealing with an incoherent object) in order to artificially emphasize the sidelobes of the reconstruction. The full width at half-maximum amplitude (FWHM) is $\sim 1.4 \mu\text{m}$ for the central point in focus. The FWHM intensity is $\sim 1 \mu\text{m}$. The intensity maximum is measured to drop by ~ 4 dB at a defocus distance equal to one depth of focus ($\sim 8 \mu\text{m}$). It is apparent in Fig. 2 that

each point source is uniquely encoded in the hologram as a spherical wave with a radius of curvature and size proportional to its axial position. Thus, each section can be extracted from the hologram by backpropagation to the desired depth (or correlation with the cross section of the scanning distribution at the chosen depth). For example, Fig. 4 shows the reconstruction of the hologram of Fig. 2 focused in the plane of the first point source, at 12 μm from the focal plane.

The reconstructions of point objects give a good measure of the PSF, its size, and its sidelobes, but it is also important to visualize how the broad, quasi-uniform areas of an object are represented, as it gives a qualitative idea of the severity of the background buildup due to the out-of-focus contributions. Figure 5 shows the reconstruction of two self-luminous cross bars 5 μm wide and 16 μm (two depths of focus) apart axially. The reconstruction is focused on the vertical bar. The horizontal bar contributes to the out-of-focus noise that degrades the image.

4. TWO-PUPIL IMAGING WITH TRANSVERSE RESOLUTION EXCEEDING THE RAYLEIGH LIMIT OF THE OBJECTIVE

In all forms of microscopic imaging, the numerical aperture of the objective limits the transverse spatial frequencies transmitted by the system. The way this limitation occurs, however, is quite different in a single-pupil imaging system and in a two-pupil scanning system. In the single-pupil system, the largest spatial frequency transmitted in coherent mode is $\rho_{\text{max}} = \sin \alpha / \lambda$ because α is the entrance angle of the steepest ray transmitted by the objective. In the two-pupil scanning holographic system, the transverse resolution is determined by the highest spatial frequency of the scanning distribution. As an example of how the two-pupil synthesis method can be exploited to obtain unconventional imaging characteristics, we show in this section that, by using two pupils with opposite curvatures, it is possible to synthesize a scanning distribution with a spatial frequency cutoff twice as large as that of the objective. Consequently, the transverse resolution of the reconstructed images is expected to be half the resolution limit of the objective.

With the two pupil distributions

$$\begin{aligned} P_1(u, v) &= \exp[-i\pi\lambda z_0(u^2 + v^2)]P_0(u, v), \\ P_2(u, v) &= \exp[+i\pi\lambda z_0(u^2 + v^2)]P_0(u, v), \end{aligned} \quad (12)$$

the scanning distribution is, from Eq. (4),

$$\begin{aligned} S(x, y, z) &= (F^{-2}\{\exp[-i\pi\lambda(z_0 + z)(u^2 + v^2)]P_0(u, v)\}) \\ &\times (F^{-2}\{\exp[-i\pi\lambda(-z_0 + z)(u^2 + v^2)]P_0(u, v)\}). \end{aligned} \quad (13)$$

Further analysis of this system could be performed numerically. However, it is instructive to make use of reasonable approximations in order to gain a qualitative understanding of the system's performances by analytical means. We make the assumption that the inverse Fourier transform of a pupil with quadratic phase and a cutoff frequency ρ_{max} is approximately a spherical wave with limited support. This approximation is reasonably valid if the Fresnel number is sufficiently large.²⁴ Specifically, we assume the following approximation to be valid:

$$\begin{aligned}
 F^{-2} \{ \exp [- i \pi \lambda z_0 (u^2 + v^2)] \text{circ}(\sqrt{u^2 + v^2} / \rho_{\max}) \} \\
 \sim \exp [i \pi (x^2 + y^2) / \lambda z_0] \text{circ}(\sqrt{x^2 + y^2} / \lambda z_0 \rho_{\max}).
 \end{aligned}
 \quad (14)$$

With this approximation, the scanning distribution and the out-of-focus transfer function are, respectively,

$$\begin{aligned}
 S(x, y, z) \sim \exp [i \pi (x^2 + y^2) / \lambda (z_0 / 2) (1 - z^2 / z_0^2)] \\
 \times \text{circ}(\sqrt{x^2 + y^2} / a),
 \end{aligned}
 \quad (15)$$

where the radius of the distribution is

$$a \approx (z_0 - |z|) \lambda \rho_{\max} = (z_0 - |z|) \sin \alpha, \quad (16)$$

and

$$\mathcal{Q}(u, v; z) \sim \exp [i \pi \lambda (u^2 + v^2) z^2 / 2 z_0] \text{circ}(\sqrt{u^2 + v^2} / 2 \rho_{\max}). \quad (17)$$

By comparison with the result of Section 3 [Eqs. (7) and (11)], two remarkable characteristics are immediately apparent. As expected, expression (17) shows that the transfer function has a cutoff frequency twice as large as that of the objective. The in-focus transfer function ($z=0$) is unity up to the cutoff $2\rho_{\max}$. Consequently, we expect the transverse resolution of the reconstructed images to be half that of the objective, at least within the limit of validity of the approximation used. It should be noted that, in incoherent mode, the cutoff frequency of the optical transfer function is also $2\rho_{\max}$, but there is an important difference. In an incoherent imaging system, the transfer function decreases monotonically to zero at the cutoff frequency. Consequently, the higher spatial frequencies are represented with a greatly reduced contrast, compared with the low frequencies. In the system just described, the transfer function is unity up to the cutoff. The reason for the transverse resolution of the scanning holographic method using pupils with opposite curvatures to be twice that of the objective is simple. The scanning distribution of expression (15) has the same radius ($a=z_0 \sin \alpha$) as that obtained in Section 3, but its spherical curvature is twice as large ($2/z_0$ rather than $1/z_0$). Therefore its Fresnel number is also twice as large, leading to the stated gain in resolution. We expect this gain in transverse resolution to be close to a factor of 2 for relatively small numerical apertures (<0.5). With higher numerical apertures, the approximation of expression (15) becomes invalid, and the gain in resolution depends on the distance z_0 , which is the free parameter that can be chosen to obtain a scanning distribution of desired size.

The second remarkable attribute of the transfer function of expression (17) is that the out-of-focus phase error varies quadratically with the defocus distance z . This is to be contrasted with conventional coherent imaging and with the two-pupil scheme of Section 3 [Eq. (11)] where the out-of-focus phase error varies linearly with the defocus distance. As stated earlier, the depth of focus (DOF) is defined as the axial displacement of a point object leading to a phase error of π at the cutoff frequency. With the transfer function of Eq. (11), the depth of focus is that of a conventional coherent imaging system, namely,

$$\text{DOF} = (\Delta z)_1 = \lambda^{-1} \rho_{\max}^2 \sim \lambda / \sin^2 \alpha, \quad (18)$$

With the transfer function of expression (17), the defocus distance leading to a phase error of π at the cutoff frequency is found to be

$$\text{DOF} = (\Delta z)_2 = (z_0 / 2\lambda)^{1/2} \rho_{\max}^{-1} = (\lambda z_0 / 2)^{1/2} / \sin \alpha. \quad (19)$$

Comparing expressions (18) and (19), we observe that the depth of focus with two spherical pupils with opposite curvatures is extended by a factor $(\Delta z)_2 / (\Delta z)_1 = \sqrt{N_F / 2}$, where $N_F = z_0 \sin^2(\alpha) / \lambda$ is the Fresnel number of the scanning distribution. Since z_0 (and thus N_F) is a free parameter, the degree of extension of the depth of focus can be tailored to accommodate specific specimen's depths.

As noted in the Introduction, images with extended depth of focus are devoid of out-of-focus flare and, for that reason, are of interest in 3D microscopic imaging of thick specimens.¹⁹ The images reconstructed with the scanning holographic method described in this section do not only exhibit a tunable extended depth of focus but they also have a transverse resolution exceeding the Rayleigh limit of the objective, which makes them doubly attractive.

Figure 6 shows the hologram of the seven point sources used as a test object. It is apparent that the hologram of each point is nearly independent of its axial position. This is, of course, a result of the reduced sensitivity to defocus of the scanning distribution created by the two pupils with opposite curvatures. With the parameter chosen for the simulation ($\sin \alpha = 0.25$, $z_0 = 80 \mu\text{m}$, $\lambda = 0.5 \mu\text{m}$), the Fresnel number is found to be $N_F \sim 20$, and the depth of focus is $\sim 18 \mu\text{m}$ (compared with the depth of focus of the objective, which was $\sim 8 \mu\text{m}$). The depth of focus can be varied by adjusting the distance z_0 without affecting the transverse resolution. The reconstruction of the hologram of the seven point sources with extended depth of focus is shown in Fig. 7. The reconstruction is focused on the central point source. We again chose to plot the amplitude rather than the intensity to enhance the sidelobes. The seven points are $4 \mu\text{m}$ apart axially and range from -12 to $+12 \mu\text{m}$ on both sides of the focal plane. The widths of the reconstructed points (FWHM amplitude $\sim 0.8 \mu\text{m}$, and FWHM intensity $\sim 0.5 \mu\text{m}$) show that the transverse resolution limit is indeed half that of the objective. It is also remarkable that the width of the reconstructed point sources is independent of their axial position, showing that the depth-of-focus extension is nearly perfect. The peak intensity has dropped by only approximately -2 dB at a defocus distance of one depth of focus ($\sim 8 \mu\text{m}$).

The intensity of the reconstruction of two crossed bars $5 \mu\text{m}$ wide and $16 \mu\text{m}$ apart axially is shown in Fig. 8. The reconstruction is focused on the vertical bar (as in Fig. 5), but the horizontal bar is now in reasonable focus as well. Of course, the price to pay for an extended depth of focus is that the information concerning the axial position of the bars is lost.

5. SCANNING HOLOGRAPHIC MICROSCOPY WITH AXIAL SECTIONING

Optical sectioning is essential to reduce or eliminate the out-of-focus flare in microscopic images of 3D thick specimens, when quantitative information of axial positions is needed. Two methods are commonly used to achieve this goal. The first is the confocal method, in which the out-of-focus information is physically prevented from reaching the detector by the use of pinholes.²² This requires a 3D scan of the specimen. The second method is the deconvolution method in which a series of axial sections are recorded in wide-field mode and processed digitally using blind or constrained deconvolution techniques.^{20,26} Critical comparisons of the two methods have been made,^{26,27} but their relative merits are, as one could expect, strongly specimen dependent. In this section, we illustrate how the two-pupil interaction scheme of scanning holographic microscopy can be exploited to synthesize a scanning distribution that captures selectively the specimen information from a thin axial section only.

The pupils needed to realize axial sectioning must be such that the interference of their respective diffraction distributions in object space is localized in a thin axial section of the specimen. This will happen if the spread functions overlap only in that axial section.

Localization along the z axis in the spatial domain can be obtained with pupils that exhibit a high degree of axial invariance in the frequency domain. An example of such pupils is the linear axicon^{28,29} with a conical wavefront of the form $\exp[i2\pi a(u^2+v^2)^{1/2}]$. Such axicon pupils can easily be generated with available spatial light modulators. The two pupils used to demonstrate axial sectioning are

$$\begin{aligned} \mathcal{P}_1(u, v) &= \exp(+i2\pi a\sqrt{u^2+v^2})\mathcal{P}_0(u, v), \\ \mathcal{P}_2(u, v) &= \exp(-i2\pi a\sqrt{u^2+v^2})\mathcal{P}_0(u, v). \end{aligned} \quad (20)$$

The corresponding spread functions from Eq. (1) are, in the plane $z=0$, two overlapping rings with radii equal to a . The feature of interest that explains how sectioning occurs is that the two ring-shaped distributions overlap exactly only in the plane $z=0$. Away from that plane, one of the rings spreads inward, while the other spreads outward. This effect is readily apparent from simple ray tracing and is due to the fact that the phases of the two axicon pupils have opposite signs. Since the spread functions of the two pupils overlap only in a thin axial section, the modulated part of the scanning distribution $S(x,y,z)$ is also confined to the same axial section. Consequently, only the specimen features located in that section contribute to the modulated signal from which the hologram is constructed. Features from other axial planes do not contribute to the modulated signal because the overlap of the two ring distributions decreases rapidly with defocus. Of course, these features are illuminated as well but with nonmodulated light. They may contribute to the signal background, and eventually to the noise, but they do not contribute to the information of the chosen section. To be fair, it must be emphasized that to build a 3D representation of the specimen, a series of axial sections must be recorded sequentially, as is required with confocal and deconvolution methods. In a sense, this defeats one of the purposes of holography, which is to capture 3D information in a single 2D scan. Nevertheless, the reconstruction of each section obtained as described is devoid of the infamous out-of-focus flare due to all the other sections. In contrast with the confocal method where the undesired information is rejected by using precisely positioned pinholes, here the rejection of the undesired information is due to the fact that the two pupils interact only in one chosen axial plane of the specimen where their diffraction distributions overlap and interfere.

The hologram of the seven point sources obtained with the two axicon pupils of Eqs. (20) is shown in Fig. 9. We used the same objective with numerical aperture 0.25 and focal length 2 cm, and the radius of the ring-shaped scanning distribution was chosen to be $a=20\text{ }\mu\text{m}$ to match the size of the scanning distributions in the two previous examples. Figure 10 shows the reconstruction of the seven point sources. The scanning ring distribution is focused in the focal plane where the central point source ($z=0$) is located. The hologram should capture the object information of that section only and suppress the rest. As before, we chose to represent the reconstruction amplitude rather than the intensity to emphasize the sidelobes. The seven point sources are $4\text{ }\mu\text{m}$ apart axially (one half the depth of focus of the objective). Figure 10 shows that the peak intensity of the reconstruction of a point source located one depth of focus away from the chosen section has dropped by approximately -10 dB . However, the most remarkable features seen in Fig. 10 are not only that the peak intensity of the reconstruction of out-of-focus features decreases dramatically with defocus but that the total energy of the out-of-focus reconstruction decreases rapidly as well with defocus. This is, of course, what is expected for true axial sectioning. Namely, the out-of-focus information should not appear in the reconstruction of the chosen section. This point is illustrated by comparing Figs. 10 and 3. In Fig. 3, which illustrates the holographic reconstruction with the attributes of a conventional imaging system, the peak intensity of the out-of-focus reconstruction also drops with defocus. However, the total energy of the reconstruction of an out-of-focus point source remains constant. The energy is simply redistributed over a larger area. It is precisely this redistributed energy that is responsible for the degrading haze and flare in wide-field images. In contrast,

Fig. 10 shows that beyond a distance of about one depth of focus on both sides of the chosen section, the contributing energy of the out-of-focus information becomes negligible. The degree of sectioning can be varied by using different axicons, leading to ring distributions with different aspect ratios. The main lobe of the in-focus PSF shown by the reconstruction of the central point in Fig. 10 has a FWHM amplitude $\sim 0.8 \mu\text{m}$ and FWHM intensity $0.5 \mu\text{m}$. This is comparable to the FWHM of the improved resolution system described in Section 4 and is about half the FWHM of the conventional image obtained with the system described in Section 3.

Figure 11 shows the reconstruction of the two bars, with the axial section chosen to coincide with the plane of the vertical bar. The horizontal bar, which is two focal distances away, is almost entirely suppressed, demonstrating the sectioning effect. The reconstruction shows strong edge enhancement. This is due to the fact that the transfer function $\mathcal{Q}(u, v)$ obtained with the axicon pupils has the nature of a high-pass filter with strong attenuation of the low spatial frequencies. This is evidenced by the strong sidelobe of the PSF seen in Fig. 10. These sidelobes are negative, which makes the total area under the PSF amplitude small, a feature leading to edge enhancement. This edge enhancement effect may or may not be desirable. For example, it can be useful for metrological purposes, as seen by the trace through the reconstruction of the $5 \mu\text{m}$ wide vertical bar shown in Fig. 12. The exact positions of the edges of the bar, which are identified by the two arrows, can be located with an accuracy far exceeding the resolution limit of the system (which itself already exceeds the resolution limit of the objective, as discussed above). If the edge enhancement is undesirable, it is possible to compensate for it in the reconstruction process by restoring the low spatial frequencies that have been attenuated. For example, instead of the reconstruction algorithm of Eq. (8), we can include an inverse filtering term in the algorithm:

$$I_g(u, v) = H(u, v) \frac{\mathcal{S}^*(u, v)}{|\mathcal{S}(u, v)|^2 + \Gamma}, \quad (21)$$

where the parameter Γ is adjusted to implement a simple form of regularization. Figure 13 shows the reconstruction of the bars using this algorithm with a particular value of Γ to achieve partial equalization of the transfer function. The reconstruction is more faithful in the sense that some of the lower spatial frequencies have been restored in the vertical bar, but, as a consequence, the sharpness of the axial sectioning has also been reduced. Since the recovery of low spatial frequencies is an easy task, compared with the recovery of high spatial frequencies, it appears that the balance between the degree of sectioning and the degree of edge enhancement can be adjusted at will.

6. SUMMARY

We have discussed how the two-pupil synthesis method on which scanning holographic microscopy is based can be used to synthesize unusual point-spread functions. In the conventional scanning hologram using a point pupil and a spherical pupil, each object point is encoded as a spherical wave having a radius of curvature proportional to its axial position. The maximum numerical aperture of that wave, and therefore the best resolution achievable, is that of the objective. We have shown that, by recording the hologram with two spherical pupils having opposite curvatures, it was possible to improve the resolution limit to half that of the objective. This improvement is due to the fact that both pupils can have numerical apertures equal to that of the objective, so that, if the pupils have opposite curvatures, their interaction term has twice that numerical aperture. The scanning distribution obtained with such pupils is, to first order, invariant with axial position. Consequently, the reconstructions exhibit an extended depth of focus and are devoid of the infamous out-of-focus flares that plague conventional wide-field microscopic images of thick specimens. As a third example, we

illustrated the use of axicon pupils to obtain edge-enhanced and sectioned reconstructions. This property is due to the fact that if the axicon pupils have opposite phases, their interaction is confined to a thin axial plane in object space. Consequently, the holograms obtained with such pupils contain only the information of the specimen section located in that particular plane. These are only a few examples of all the possibilities that scanning holographic microscopy based on two-pupil synthesis can offer.

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Fig. 1. Generic arrangement of a two-pupil synthesis setup in scanning mode, implementing an incoherent holographic system with a complex spread function. SLM, spatial light modulator; EO, electro-optic.

Fig. 2.

Amplitude of the hologram of seven incoherent point sources $7.5\ \mu\text{m}$ apart transversally and $4\ \mu\text{m}$ ($1/2$ depth of focus) apart axially. Axial positions range from -12 to $+12\ \mu\text{m}$ from the objective's focal plane. Hologram obtained with one pointlike pupil and one pupil with spherical phase front. System parameters: numerical aperture $\sin \alpha = 0.25$, $z_0 = 80\ \mu\text{m}$, scanning distribution radius $a = 20\ \mu\text{m}$.

Fig. 3. (a) Amplitude of the reconstruction of the hologram of Fig. 2 focused on the central point source at $z=0$. (b) Profile of the reconstructed amplitude of the seven point sources. Note: the amplitude is shown rather than the intensity to emphasize the sidelobes.

Fig. 4.
(a) Amplitude of the reconstruction of the hologram of Fig. 2 focused on the first point source at $z=-12\text{ }\mu\text{m}$. (b) Profile of the reconstructed amplitude.

Fig. 5. Intensity of the reconstruction of two bars using the same parameters as described in Fig. 2. The bars are 16 μm (two depths of focus) apart axially. Reconstruction is focused on the vertical bar.

Fig. 6.

Amplitude of the hologram of seven incoherent point sources $7.5\text{ }\mu\text{m}$ apart transversally and $4\text{ }\mu\text{m}$ apart axially. Axial positions range from -12 to $+12\text{ }\mu\text{m}$ from the objective's focal plane. Hologram obtained with two pupils having spherical phase fronts with opposite curvatures. System parameters: numerical aperture $\sin\alpha=0.25$, $z_0 = \pm 80\text{ }\mu\text{m}$, scanning distribution radius $a=20\text{ }\mu\text{m}$.

Fig. 7.
 (a) Amplitude of the reconstruction of the hologram of Fig. 6 focused on the central point source at $z=0$. (b) Profile of the reconstructed amplitude of the seven point sources, demonstrating a gain of a factor of 2 in transverse resolution and an extended depth of focus.

Fig. 8.

Intensity of the reconstruction of two bars using the same parameters as described in Fig. 5. The bars are 16 μm apart axially. The reconstruction is focused on the vertical bar, but both are in reasonable focus.

Fig. 9.

Amplitude of the hologram of seven incoherent point sources $7.5\text{ }\mu\text{m}$ apart transversally and $4\text{ }\mu\text{m}$ apart axially. Axial positions range from -12 to $+12\text{ }\mu\text{m}$ from the objective's focal plane. Hologram obtained with two pupils having linear phase fronts (axicons) with opposite phases. System parameters: numerical aperture $\sin\alpha=0.25$, ring scanning distribution radius $a=20\text{ }\mu\text{m}$.

Fig. 10.
 (a) Amplitude of the reconstruction of the hologram of Fig. 9 focused on the central point source at $z=0$. (b) Profile of the reconstructed amplitude of the seven point sources, demonstrating axial sectioning and suppression of the out-of-focus information.

Fig. 11.

Intensity of the reconstruction of two bars using the same parameters as described in Fig. 9. The bars are 16 μm apart axially. The plane containing the vertical bar was selected for sectioning. The axicon pupils lead to a transfer function suppressing the low spatial frequencies and exhibiting a high-pass filtering behavior.

Fig. 12.

Trace through the reconstruction of the vertical bar in Fig. 11, illustrating the possibility of locating spatial features with an accuracy far exceeding the resolution of the objective. The arrows indicate the precise location of the edges of the bar.

Fig. 13. Reconstruction of the hologram of Fig. 9 using the algorithm described in the text to recover, to a controllable degree, the low spatial frequencies that have been suppressed in the hologram.