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Novel proposals in widefield 3D microscopy

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ABSTRACT

Patterned illumination is a successful set of techniques in high resolution 3D microscopy. In particular, structured illumination microscopy is based on the projection of 1D periodic patterns onto the 3D sample under study. In this research we propose the implementation of a very simple method for the flexible production of 1D structured illumination. Specifically, we propose the insertion of a Fresnel biprism after a monochromatic point source. The biprism produces a pair of twin, fully coherent, virtual point sources. After imaging the virtual sources onto the objective aperture stop, the expected 1D periodic pattern is produced into the 3D sample. The main advantage of using the Fresnel biprism is that by simply varying the distance between the biprism and the point source one can tune the period of the fringes while keeping their contrast.

Keywords: Optical microscopy, optical sectioning, superresolution, structured illumination

1. INTRODUCTION

Conventional light microscopes present fundamental limitations regarding lateral spatial resolution and optical sectioning capability. These restrictions are imposed by the structure of the optical transfer function (OTF) relating the 3D spatial-frequency contents of both the object and the image irradiance distributions. On the one hand, this transfer function presents a finite extent in both lateral and axial directions, being most elongated along the lateral one. The maximum lateral spatial-frequency allowed to pass throughout the imaging system is given by

$$|\mathbf{u}_0| = 2\frac{NA}{\lambda}, \tag{1}$$

where λ is the illumination wavelength (in vacuum) and $NA = n_i \sin\alpha$ is the numerical aperture of the objective lens, with α its semiaperture angle and n_i the refraction index of the immersion medium. This upper limit for the lateral spatial-frequency that can be imaged is the reason for the finite lateral resolution of conventional microscopes. On the other hand, the transfer function has a rotationally symmetrical shape respect to the axial-frequency axis, showing a *hollow* conical structure around this symmetry direction. This *missing* cone is in fact the responsible for the lack of optical sectioning capability of conventional microscopy instruments. A view of this transfer function for a circular clear pupil microscope is shown in Fig. 1 as a function of the normalized spatial-frequencies

$$\bar{\mathbf{u}} = \frac{\lambda}{n_i \sin\alpha}\mathbf{u} \text{ and } \bar{w} = \frac{\lambda}{2n_i \sin^2(\alpha/2)}w \tag{2}$$

where $\mathbf{u} = (u, v)$ and w stand for lateral and axial spatial-frequencies, respectively.

Many efforts have been applied in the last decades to overcome these limitations.[1] Non-uniform illumination proposals are between the most promising techniques nowadays. In the so-called structured illumination (SI) microscopy a periodic pattern, transverse to the optical axis, is projected onto the specimen and a stack of 2D images are recorded after scanning the object axially.[2–4] This illumination structure acts as a *carrier* periodic pattern, which generates several spectrally-shifted replicas of the original spatial-frequency content of the object. Through a phase-shift method, an extended spectrum of the object can be recovered over the image. This completion not only extends the lateral spatial resolution but also provides the system with optical sectioning capabilities by fulfilling the *missing* cone in the transfer function.

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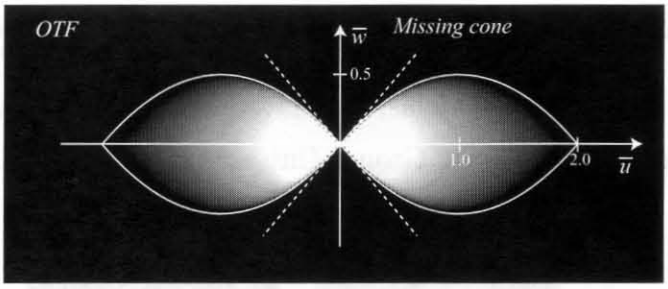

Figure 1. Meridian section of the 3D OTF for a clear circular pupil system. $\bar{u}$ and $\bar{w}$ stand for normalized lateral and axial spatial-frequencies, respectively.

The main advantages of structured illumination over other 3D scanning techniques are the imaging speed and the light efficiency. It is thus a very interesting technique since it permits to achieve the same optical sectioning capacity as, for instance, with confocal microscopy, but working in the wide-field regime, providing a fast, optical sectioning, high resolution imaging technique. Additionally, it is possible to use large working distances in opposition to other more sophisticated techniques like 4Pi microscopy.

Two different configurations in SI microscopy are currently used, both easily obtained from the standard microscope scheme. In the coherent brightfield SI the illumination plane wave is substituted by two tilted ones, whose interference generates the desired periodic pattern, as shown in Sect. 2. Our proposal here is to suggest the implementation of a novel flexible method to produced suitable periodic patterns onto the sample under issue. In Sect. 3 we present our simple optical setup, that can provide easily 1D constant-contrast tunable-frequency fringes for use in SI.[4] This setup has been implemented and successfully tested, as shown in Sect. 4, providing a flexible scheme that can be easily adapted to different goals in SI microscopy, namely, optimum sectioning capability or maximum lateral resolution improvement.

2. CONVENTIONAL STRUCTURED ILLUMINATION

Let us now present briefly the conventional brightfield SI microscope setup. We assume in the following an spatially-incoherent response of the 3D samples as, e.g., in fluorescence. As previously pointed out, the 1D dimensional grid pattern is produced by the interference of two collimated beams, as illustrated in Fig. 2.

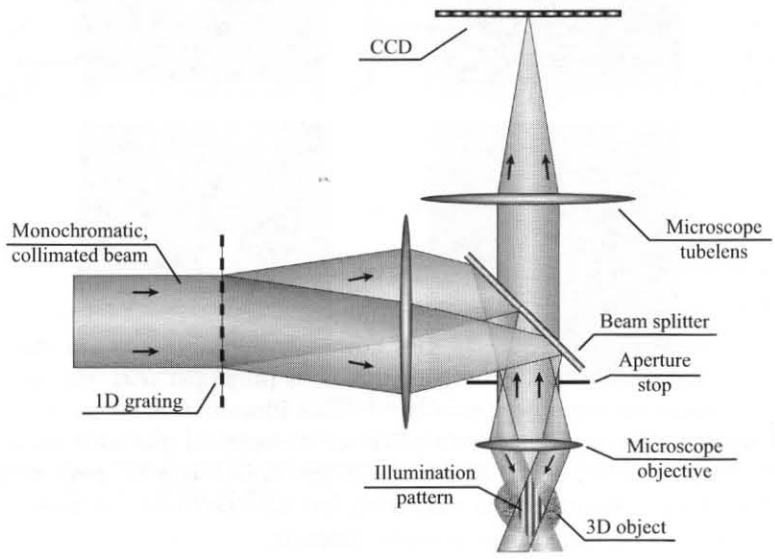

Figure 2. Conventional coherent SI microscope scheme.

The intensity illumination pattern that impinges the 3D sample can be written as

$$G(\mathbf{x}) = 2\left[1 + \cos\left(\frac{2\pi \sin\alpha}{\lambda} Ax + \phi_i\right)\right], \tag{3}$$

where $A = 2\sin\sigma/\sin\alpha$, being 2σ the angle between the interfering beams. For the illuminated sample, we assume a response proportional to the excitation intensity $G(\mathbf{x})$ times a characteristic function $O(x,z)$ of the sample —irradiance reflectivity (or transmittance in transmission schemes) or density of dye molecules in fluorescence—. In such a case, the 3D intensity distribution in the image is given by

$$I(\mathbf{x},z) = 2\left[1 + \cos\left(\frac{2\pi \sin\alpha}{\lambda} Ax + \phi_i\right)\right] O(\mathbf{x},z) \otimes_3 |h(\mathbf{x},z)|^2, \tag{4}$$

being $|h(\mathbf{x},z)|^2$ the irradiance point spread function (PSF) of the collection arm of the microscope and $\otimes_3$ the 3D convolution operation. To obtain an image with increased resolution it is necessary to transfer the grid pattern from the object side to the PSF side in the above equation. This can be done by computational reconstruction after recording, at every axial scanning step, three images I_0, I_1, and I_2, obtained by adjusting the illuminating-beam phase differences to $\phi_i = 0,\ \pi/2,\ and\ \pi$, respectively*. By linear superposition of such intensities one can build the following functions

$$A_0(\mathbf{x},z) = \frac{1}{2}\left[I_0(\mathbf{x},z) + I_2(\mathbf{x},z)\right], \tag{5}$$

$$A_1(\mathbf{x},z) = \frac{1}{4}\left[(1-\mathrm{i})\, I_0(\mathbf{x},z) + 2\mathrm{i} I_1(\mathbf{x},z) - (1+\mathrm{i})\, I_2(\mathbf{x},z)\right], \tag{6}$$

and

$$A_2(\mathbf{x},z) = \frac{1}{4}\left[(1+\mathrm{i})\, I_0(\mathbf{x},z) - 2\mathrm{i} I_1(\mathbf{x},z) - (1-\mathrm{i})\, I_2(\mathbf{x},z)\right]. \tag{7}$$

that can be combined to obtain the optically sectioned synthetic image as

$$I_S(\mathbf{x},z) = A_0(\mathbf{x},z) + \exp(\mathrm{i}2\pi Ax)\, A_1(\mathbf{x},z) + \exp(-\mathrm{i}2\pi Ax)\, A_2(\mathbf{x},z) = O(\mathbf{x},z) \otimes_3 |h_S(\mathbf{x},z)|^2, \tag{8}$$

where

$$|h_S(\mathbf{x},z)|^2 = |h(\mathbf{x},z)|^2 \cos^2\left(\frac{\pi \sin\alpha}{\lambda} Ax\right). \tag{9}$$

It is very illustrative to analyze this synthetic imaging process in the frequency domain. The synthetic OTF is obtained as the 3D Fourier transform of the synthetic PSF, that is

$$H_S(\bar{\mathbf{u}},\bar{w}) = H(\bar{\mathbf{u}},\bar{w}) \otimes_3 \left[\delta(\bar{\mathbf{u}},\bar{w}) + \frac{1}{2}\delta(\bar{u}-A,\bar{v},\bar{w}) + \frac{1}{2}\delta(\bar{u}+A,\bar{v},\bar{w})\right]. \tag{10}$$

In Fig. 3 we have represented, in a greytone draw, two sections of the 3D synthetic OTF for the maximum value for the grating frequency, namely, $A = 2$ (corresponding to $\sigma = \alpha$). In this case, the maximum achievable improvement in lateral resolution is reached since the cutoff lateral spatial-frequency is extended at most. Note that this effect is applied only along the x direction, but the same improvement along other lateral directions can be obtained with the grating properly oriented.

Note, however, that this increase in lateral resolution is not accompanied by any improvement in optical sectioning capacity. In fact, the missing cone is still present so that no optical sectioning is achieved. It is easy to realize, however, that a smart use of the SI procedure would permit, by use of proper values for the grating normalized frequency A, to fill the missing cone and therefore to obtain simultaneously an improvement in lateral resolution and a good optical sectioning capacity. As an example of this, in Fig. 4 we show the synthetic OTF

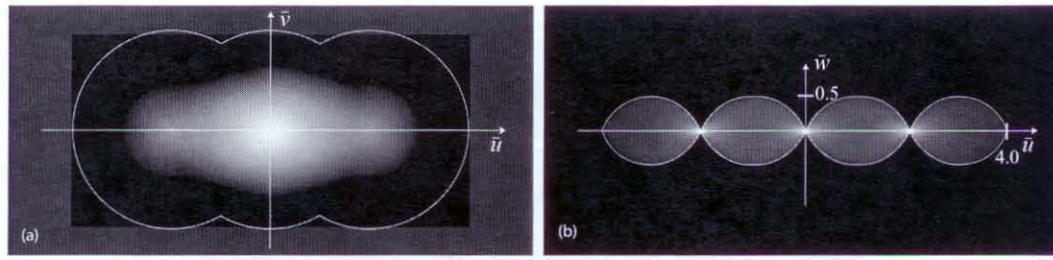

Figure 3. Two views of the 3D OTF obtained under SI for maximum lateral resolution improvement.

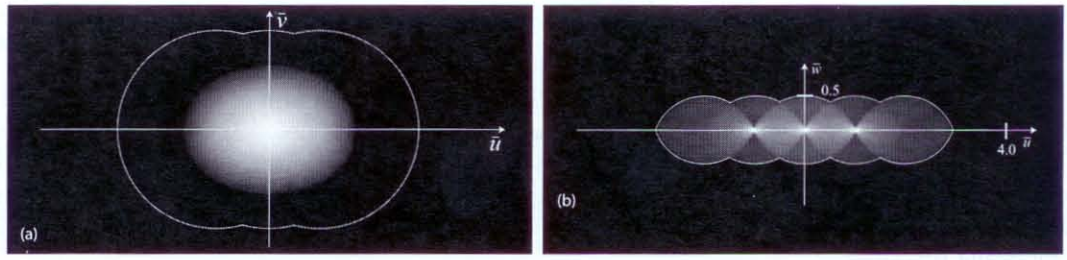

Figure 4. Two views of the 3D OTF obtained under SI for optimum optical sectioning capability.

of an SI microscope designed for the achievement of optimum optical sectioning capacity, which is obtained for $A = \sin\alpha$ ($\sin\sigma = 0.5\sin^2\alpha$).

It is worth to note that different goals in SI miroscopy, namely, optimum optical sectioning or maximum lateral resolution, require different values of the frequency A of the illumination pattern. On the other hand, this frequency depends not only on the period of the grating used to split the original illuminating plane wave, but also on the optical power of the microscope objective. Thus, a change in this inspection objective would require a readjustment of the period of the grating to achieve the proposed optimization of the features of the SI microscope. This desirable tunability is not easily reached in the classic SI scheme, unless sophisticated variable-frequency diffraction gratings are used. In next section we propose the use of a very simple element, a Fresnel biprism, to easily produce constant-contrast variable-period fringes onto the sample. Our proposal implies very small changes in the classic scheme of the SI microscope, in such a way that its implementation is suitable even into commercially available SI instruments.

3. TUNABLE-FREQUENCY STRUCTURED ILLUMINATION

Let us now propose a much more flexible setup for the generation of a periodic 1D pattern onto the sample of the microscope. The frequency A in the classic SI illumination scheme is directly related with the angle between the interfering plane waves after passage through the objective lens. For a given power of the optical elements in the setup, this angle is set by the angle of the diffracted waves at the grating. This angle, ultimately, is fixed by the grating period. However, a pair of interfering plane waves can be also obtained from two fully coherent point sources *collimated* by an spherical lens. In this case, the angle between the exiting waves is controlled by the focal length of the lens and, remarkably, by the distance between the point sources. Our proposal is to generate a pair of fully coherent point sources with easily tunable distance between them. Further collimation of the resulting spherical waves produces the desirable tunability in the frequency of the resulting interference fringes.

To achieve this goal, we propose the use of a Fresnel biprism, which consists of two thin prisms joined by their bases, as shown in Fig. 5. If this element is illuminated by a point source, two virtual point sources are generated after passage through it. These two sources lay on the same transverse plane as the original one,

*The value of ϕ_i can be modified at will very easily by lateral displacement of the 1D grating.

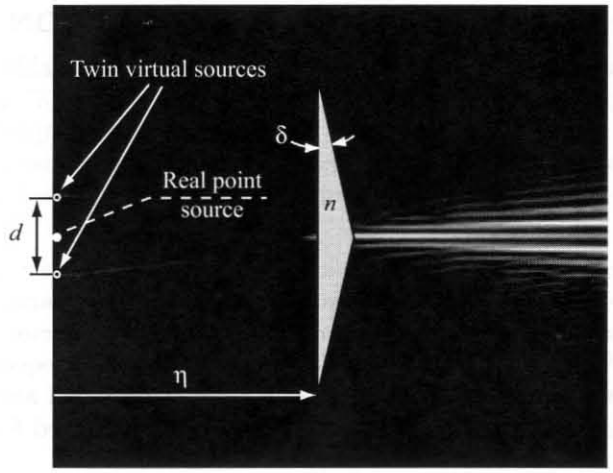

Figure 5. Diffracted field by a Fresnel biprism. The original point source is splitted in two fully-coherent virtual emissors.

symmetrically located around it, with a separation proportional to the distance η between the biprism and the real source. Mathematically, the gap between the virtual sources is given by

$$d = 2(n-1)\tan\delta\,\eta \tag{11}$$

where n and δ are the refractive index and the refringence angle of the biprism, respectively. Note that simply by shifting the biprism respect to the real point source one can change the separation between the virtual sources. These sources are fully coherent since the light *emerging* from them comes in fact from the same original point source. Thus, their interaction produce interferences, as can be seen in Fig. 5.

If we consider now an spherical lens with its back focal plane laying on the virtual twin sources location, a pair of interfering plane waves is generated, whose relative angle can be varied simply by changing the gap between the sources. This tunability can be added to the SI microscope in Fig. 2 by replacing the grating and the plane-wave illumination by a point source, a Fresnel biprism and a collimating lens, as sketched in Fig. 6. The axial displacement of the biprism in this new setup generate a continuous variation in the frequency A of the 1D periodic illumination pattern onto the sample. Moreover, the lateral displacement of the biprism produce also a phase delay between the virtual sources that can be used in the phase-shifting procedure required in SI microscopy.

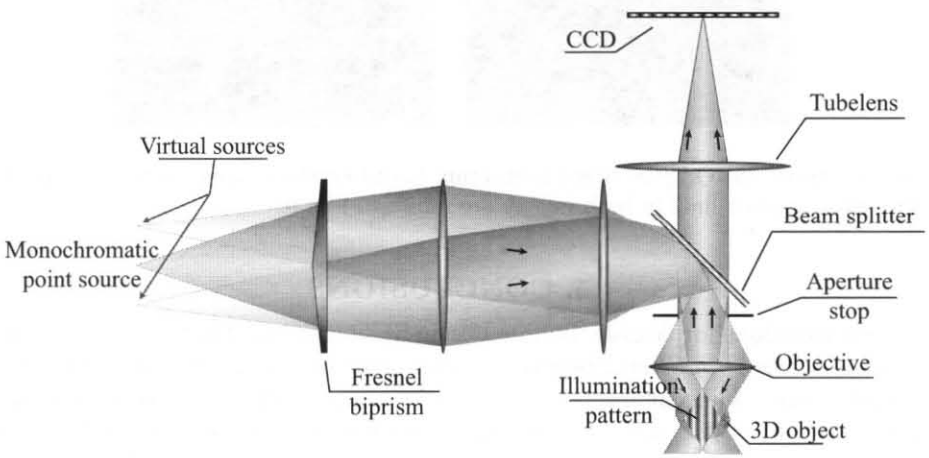

Figure 6. Tunable-frequency coherent SI microscope scheme.

4. EXPERIMENTAL VERIFICATION

As a test for our proposal, we use the scheme in Fig. 6 to implement a tunable-frequency SI microscope. For our implementation we use a Fresnel biprism with refringence angle $\delta = 0.5°$ and refraction index $n = 1.51$. We generate the real point source from the output of an optical fiber coupled with a standard HeNe laser ($\lambda = 632.8$ nm). For the collimation of the twin spherical wavefronts we use an achromat with focal length $f = 200$ mm. This setup was followed by a standard microscope with a $f = 150$ mm tubelens and a low-NA (0.10) low-magnification ($5\times$) objective.

We present in Fig. 7 two images of the illuminated sample plane for two different axial positions of the biprism. As a target we use a *USAF 1951* test, that allows a direct comparison of the periodic illumination and the callibrated spatial-frequency sections of the test. A variation by a factor 5 in the frequency A is clearly shown in this two pictures. We use also the highest of these *carrier* spatial-frequencies to obtain a superresolved synthetic image of the target under issue. Figure 8 shows both the original and synthetic images obtained in this case. In Fig. 9 a profile of the 181 lp/mm group of the target is presented for both images, showing clearly the improvement in the lateral resolution predicted by the SI technique.

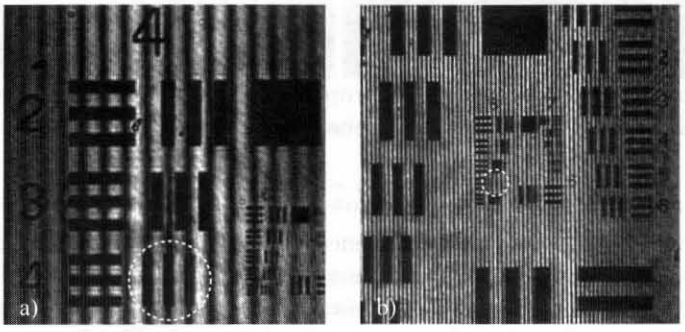

Figure 7. Illuminated target for two different axial locations of the biprism in the tunable-frequency SI microscope. The dashed circles correspond to the groups in the target with: (a) 22 lp/mm; (b) 102 lp/mm.

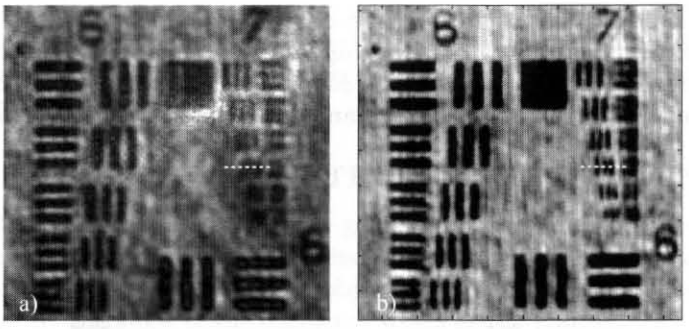

Figure 8. Images of the target: (a) Original brightfield setup; (b) SI synthetic image with a 102 lp/mm 1D illumination pattern. The profiles along dashed lines in both images are shown in Fig. 9.

5. CONCLUSIONS

We present here a new technique for flexible illumination in SI microscopy. On the basis of a very simple concept, the splitting of a point source by a Fresnel biprism, we can produce patterned illumination with variable period onto the sample under issue. This tunability, achievable by simply shifting the biprism along the optical axis, makes this proposal specially useful when optimizing for transverse or axial superresolution, or when different objectives are used to inspect the sample. Some experimental verifications of the technique have been also presented. Finally, it is noticeable that our setup implies only a slight modification of the conventional SI scheme and, thus, can be easily adapted even to commercially available SI microscopes.

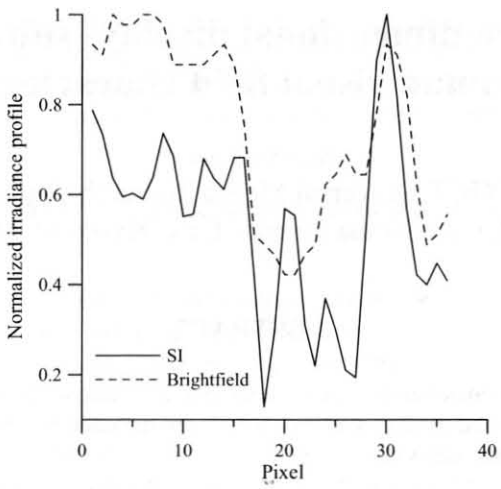

Figure 9. Profile of the images in Fig. 8 for the 181 lp/mm group in the target.

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