High-resolution fluorescence microscopy using three-dimensional structured illumination

P.F. Gardeazábal Rodríguez, P. Blandin, I. Maksimovic, E. Sepulveda, E. Muro, B. Dubertret, and V. Loriette
Laboratoire Photons et Matière, UPR 5 du C.N.R.S., Ecole Supérieure de Physique et Chimie Industrielles de la ville de Paris, 10 rue Vauquelin, 75005 Paris, France

ABSTRACT

We developed a high-resolution microscope based on three-dimensional structured illumination generated with two spatial light modulators. This setup enables both lateral resolution improvement by a factor two and axial localization of point like objects with nanometric precision.

Keywords: three-dimensional microscopy, fluorescence microscopy, superresolution

1. INTRODUCTION

Improvement of optical resolution has ever been a burning issue in microscopy. Indeed, users of microscopy setups, like biologists or physicians, need for some applications to image single molecule with subcellular resolution. During the past few years, the emergence of new imaging techniques in far-field optical microscopy has enabled to go beyond the traditional diffraction limit described by Abbe. In fluorescence microscopy, we can distinguish between two classes of techniques: one achieving real resolution improvement (SIM [1], STED [2] …), the other one relying on a very accurate localization of individual emitters (PALM [3], STORM [4], PALMIRA [5] …). All these approaches give complementary results; their choice depends on the studied sample or process and the type of fluorophores used.

Structured illumination to improve resolution in microscopy has been stated approximately ten years ago [6,7]. The principle of this technique is to pass high spatial frequencies through the optical transfer function of the microscope using moiré effect. By superimposing an illumination pattern on the sample, the pattern and sample spatial frequencies combine to allow the information of the sample carried by the high frequencies to be codified in the low frequencies, henceforth the high-resolution information is not cut by the optical transfer function. With this technique, enhancement of the lateral resolution by a factor two has been theoretically and experimentally demonstrated [1]. Furthermore, by saturating the fluorescence, unlimited resolution can be theoretically achieved. However this Saturated Structured Illumination Microscopy (SSIM) is difficult to implement, because it requires high excitation powers, acquisition of a lot of images, and heavy data treatment. Structured Illumination Microscopy (SIM) is probably the superresolution technique with the best photonic yield since just one excitation laser is required and wide field excitation and detection are performed. Moreover, the optical design of such a setup is relatively simple, concerning the excitation as well as the detection. The performances obtained with this technique are really attractive, but all the samples cannot be observed. Too aberrating or diffusing samples degrade the excitation pattern created in the focus plane and thus don’t permit to use SIM.

In this paper, we present a technique that associates the improvement of lateral resolution by a factor two with an axial super-localization with a 50 nm precision. This localization is based on a method that codes axial information using three-dimensional structured illumination with a single objective in a commercial microscope [8]. Based on Gustafsson's set-up [1], we use three interfering beams to introduce a spatial modulated intensity pattern both in the lateral plane and along the axial direction. The pattern is displaced laterally and axially with no mechanical elements.
2. THEORY OF 3D STRUCTURED ILLUMINATION MICROSCOPY

We consider the first three diffraction orders \((-1, 0, +1)\) resulting from the diffraction of a laser beam by a phase grating. When these three beams, considered as plane waves in the objective’s front focal plane, interfere inside the sample, for a given lateral position of the diffraction grating, the intensity pattern is described by:

\[
I(x, z) = I_0 \left[ \alpha^2 + 4 \cos \left( \frac{2\pi x}{2p_x} + \phi_x \right) ^2 + 4 \alpha \cos \left( \frac{2\pi x}{2p_x} + \phi_x \right) \cos \left( \frac{2\pi z}{p_z} + \phi_z \right) \right]
\]

where \(\alpha\) is the ratio between the 0 order amplitude and the +1 or -1 order amplitude, \(2p_x\) and \(p_z\) are lateral and axial periods (figure 1), \(\phi_x\) is the lateral position offset of the grating, and \(\phi_z\) is the phase shift applied to the zeroth order to move the illumination structure in the axial direction. Denoting by \(n\) the refraction index of the immersion medium, by \(\lambda_{exc}\) the excitation wavelength, and by \(\mu\) the incidence angle of the +1 and -1 orders, we have:

\[
2p_x = \frac{\lambda_{exc}}{n \sin(\mu)}, \quad p_z = \frac{\lambda_{exc}}{n(1-\cos(\mu))}
\]

The illumination pattern has two contributions: a term with a lateral period \(p_x\) and no axial dependency, which gives rise to lateral superresolution, and a term with a lateral period \(2p_x\) and a cosine axial dependency. For a given value of \(\phi_z\), the \(p_z\) term can be isolated by summing three images with three different illumination axial positions corresponding to \(\phi_z = \left[-\frac{2\pi}{3}, 0, \frac{2\pi}{3}\right]\). In the general case, the axial position of an object inside this structure can be obtained by taking three images with three different phase shifts applied to the zeroth order. If the phase shifts are \(\{-\beta, 0, \beta\}\) and the corresponding three images noted \(\{I_{-1}, I_0, I_1\}\), the following combination permits extraction of the axial position of the object:

\[
\frac{I_{-1} - I_1}{2I_0 - (I_{-1} + I_1)} = \sin(\beta) \tan \left( \frac{2\pi z}{p_z} \right)
\]

This three-image algorithm has already been used for improving the axial localization in optical microscopes [9]. The value of \(\beta\) is usually fixed to \(2\pi/3\) in order to preserve lateral superresolution.

Figure 1: Three-dimensional structured illumination in the x, y and z directions demonstrated in a qdots fluorescent plane.
The use of three diffraction orders through a single objective has, however, some important drawbacks: first, the angle between the plane waves inside the sample is twice as small as in the classical two-beam structured illumination. This generates a pattern with a lateral period twice as large as the one generated by two-beam illumination. To preserve the lateral resolution enhancement of structured illumination while gaining axial localization information, this pattern has to be eliminated. Twenty-seven images are thus required when using three beams: nine images, as in the standard two-beam configuration [7], for three different axial positions of the illumination. The axial localization accuracy is also not as much enhanced as in two-objective setups [reference of 4Pi microscopy], because the angles between interfering plane waves is not 180° but at most one half of the objective angular aperture. Then the relative weight of the three beams must be carefully adjusted to obtain a good contrast.

3. EXPERIMENTAL SETUP

An easy way to produce structured illumination both axially and laterally through a single objective is to use interfering beams arriving with different incidence angles on the sample. The simplest setup makes use of three beams, which interfere in the focal plane of the objective to generate a 3D structure of the electromagnetic field. To create these diffraction orders, we propose a setup based on programmable diffractive optical elements. With such devices, no mechanical moving parts are required and the stability, the reproducibility, and the precision of the illumination displacement are increased (setup reported in figure 2). Moreover, the use of programmable diffractive optical elements enables to tune finely the grating properties (period, contrast, amplitude…).

The excitation laser beam passes through a beam expander and is reflected by a first programmable diffractive optical element (SLM 1) to generate and select the three beams (the zeroth and two complementary diffraction orders are selected). The diffraction angles are adapted to the microscope pupil diameter with a 3.7mm telescope (lenses L1 and L2). A programmable diaphragm and a phase shifter (SLM 2) are placed in the Fourier plane of the first lens (L1), and permit to displace the pattern along the axial direction. A periscope (not shown) directs the selected beams through the microscope tube lens (L3), and the beams are focused on the back focal plane of the microscope objective lens. The signal is recovered by epifluorescence and directed to an EM-CCD camera using a dichroic filter and the camera tube lens (LC). The acquisition rate is limited to 2 Hz for a 27 images set by the refresh rate of the two programmable diffractive optical elements, which is limited to 60 Hz.

Figure 2: experimental setup.
4. IMAGES RECONSTRUCTION

An important and precise work is required to reconstruct the final images. For each plane of the sample, we make 27 acquisitions to be able to achieve lateral superresolution and axial superlocalization. Data processing is quite different in these two cases.

4.1 Lateral superresolution

In the acquisition algorithm presented above, we acquire three images for each of the nine classical pattern positions, used in traditional lateral (2D) structured illumination microscopy [1]. Indeed, for each position, we apply 3 different phase shifts to the zeroth diffraction order \( \phi_z = 2\pi/3, 0, 2\pi/3 \) to move axially the structured illumination. So, to obtain the nine classical images, we just add the three images with different phase shifts for the corresponding lateral position of the pattern.

For each pattern orientation \( d \), the three images obtained at different illumination phases \( \phi_s = 2\pi/3, 0, 2\pi/3 \) are Fourier transformed spatially. With linear combinations on these three images, it is possible to obtain three information components that have been moved in frequency space, through mixing with the illumination pattern, by vectors \( -kp \), where \( k \) is the spatial frequency of the illumination pattern and \( p = \{-1,0,+1\} \) for the three components (figure 3). Once this vector and the relative phase shift between the components are known by data processing, we move the different spectral components in the Fourier domain at their right place.

By doing this with the three different orientations of the pattern, and by adding calculations to fit the different subspectra (normalization by the OTF, Wiener filter, …), we can build a new spatial spectrum of our image, which is twice larger (figure 4) than the original. The superresolved image is then obtained performing the inverse Fourier transform of the reconstructed spectrum.

![Figure 3: shifts of the different subspectra obtained using linear combination in the reciprocal space to reconstruct a larger spatial spectrum.](image)
4.2 Axial superlocalization

As seen previously, with such a three-dimension (3D) structured illumination, it is also possible to extract some information about the axial localization of the fluorescent objects. To obtain this axial localization, the three images can be acquired either by moving the sample or the objective or by axially translating the illumination pattern, in which case the sample and the objective are fixed. Nearly all setups aiming at obtaining axial localization, with the exception of 4Pi setups [10], employ the first solution [11, 12]. The latter solution has many advantages, because the object point-spread function (PSF) remains unchanged during the 27-image acquisition set; only the excitation intensity changes. This allows performing a pixel-by-pixel combination of images and makes the axial localization independent of the PSF sampling on the CCD. This differs fundamentally from other axial localization approaches where the axial structure of the objective PSF is probed, which requires to sample the PSF on many pixels and thus reduces the microscope field of view. As an advantage, the axial localization being independent on the PSF shape, it will be independent on the axial position of the object under the microscope objective.

To do so, we need three different acquisitions, for a given lateral position of the pattern, with axial phase shifts $\{-2\pi/3, 0, +2\pi/3\}$, then we perform the calculation pixel by pixel for the entire image field. By noting the corresponding three taken images $\{I_{-1}, I_0, I_{+1}\}$, we obtain the axial position of the object using the following combination:

$$\frac{z}{p_z} = \frac{1}{2\pi} \tan^{-1} \left[ \frac{\sqrt{3}(I_{-1}(x,z) - I_{+1}(x,z))}{2I_0(x,z) - I_{-1}(x,z) - I_{+1}(x,z)} \right]$$

It is important to notice, that with this simple relation, we have not access to the absolute value of $z$: we estimate the ratio $\frac{z}{p_z}$. With other and more complicated algorithms, it would be surely possible to exploit differently the axial structure of the pattern.
5. RESULTS

First, we have verified that with our setup, we obtain the super resolution, despite we use three diffractions orders: to do that, we have compared an image field calculated with a 27-images set recorded using three-beam illumination to a nine-images set taken using only two beams, for the last one we have blocked the zeroth-order beam. The results show that the lateral resolution enhancement is unaffected by the presence of the third beam: the resolution enhancement is 1.8 with two beams and 1.9 using three beams. This result was obtained using a 488 nm excitation laser and a NA=1.2 water immersion objective lens, and we had \( p_z = 1.7 \mu m \) and \( p_x = 390 nm \).

Then, the set-up was applied to observe biological samples. We present here the first images obtained on cells. The samples observed are HeLa cells where the tubulin was labelled with Alexa 555. We show that using our setup, we can obtain superresolved images of the cell (figure 5b) and at the same time extract axial information of the subcellular structures (figure 5c).

![Figure 3](image)

Figure 3: images obtained with a biological sample. a) Image in conventional fluorescence microscopy. b) Image obtained with our setup: the lateral resolution is increased by a factor two. c) Axial reconstruction of the sample using our axial coding.

6. CONCLUSION

We have presented the development of an original fluorescence microscope based on a three dimensional structured illumination. This setup enables to obtain simultaneously a lateral resolution improvement of factor two, with respect to a classical microscope, and to localize axially fluorescent emitters with accuracy of the order of 50 nanometers. We have also presented images obtained in biological samples.
REFERENCES


