NANOMETRY OF BIO-MOLECULAR COMPLEXES USING PSF RECONSTRUCTION IN SMI MICROSCOPY

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We report an improved size measurement method using Spatially Modulated Illumination Microscopy (SMI) [1] in combination with reference objects of known size and shape to reconstruct the microscope PSF. This method is applied to determine the size of individual fluorescent objects far below the optical resolution limit.

1 Introduction

Although light microscopy and three-dimensional (3D-) image analysis have made considerable progress during the last decade, it is still challenging to analyze the genome nano-architecture of specific gene domains in 3D-conserved cell nuclei by fluorescence microscopy. Structured Illumination has been applied, e.g. in Spatially Modulated Illumination (SMI) [1] microscopy, to circumvent the limited optical resolution of visible light microscopy. The calibration of SMI intensities within the interferometric illumination allows high precision nanosizing. This is done be a reconstruction of the axial PSF, which allows to overcome assumptions previously made on the shape of the SMI-PSF. These assumptions were necessary in former SMI nanosizing measurements [2]. In the improved method, reference objects with known dye distribution have to be put additionally to the unknown objects on the object slide or on the cover slip.

In quantitative molecular cytogenetics this “nanosizing” method allows a variety of applications. Experimental SMI measurements of fluorescent labeled objects inside of cryosections and 3D conserved cells were performed using 488 nm excitation. Questions of gene expression analysis, transcription factories analysis; protein cluster analysis, and gene compaction analysis have been addressed.

2 SMI Microscope

The SMI microscope combines an epifluorescence microscope with structured illumination. A detailed description of the instrument can be found in [1]. Presently, two objective lenses are situated inside a Mach-Zehnder interferometer streched from a collimated laser light source by a 50:50 beam splitter. In the two interferometer arms, the collimated laser beams are focused into the back focal plane of two opposing objective lenses, resulting in a standing wavefield. The high precision size and distance measurements with the SMI microscope are based on the exact knowledge of the wavelength of the standing wavefield as well as on its angle with respect to the optical axis. This can be controlled by aligning the orientation of the standing wavefield with the object slide, and the measurement of the standing wavefield frequency can be used to estimate the refractive index at the position of the object. As the present standing wavefield provides a modulation only in this direction, additional spatial frequencies propagated into the image space are available only in the axial direction. Superpositioning three or more laser beams at the position of the probe could also provide for lateral modulation in the future.

An important practical feature of the SMI-nanosizing method described is that such studies may be conducted using conventional microscope object slides and cover slips. The volume values obtained are about two orders of magnitude smaller than the illumination or observation volume, respectively, in a confocal laser scanning microscope using a high numerical aperture objective lens [3].

3 Nanosizing

First, the SMI microscope PSF has to be determined. This can be done prior to the measurement by using a numerical calculation of the detection PSF and multiplying the result with the standing wave illumination pattern (c.f. Fig 1a)

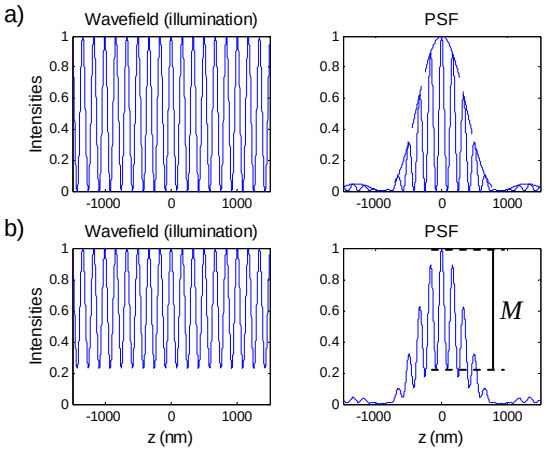

Fig 1. *The calculated axial PSF of the SMI microscope. a) for optimal alignment the PSF modulation goes down to zero. b) If the intensities in both interferometer arms are not equal, a non-modulating contribution to the PSF is found.*

Using a convolution with the fluorochrome distribution ρ within the objects of interest,

$$AID = \rho(z) \otimes PSF_{\mathrm{SMI}}(z) \qquad (1)$$

one can obtain the so called Axial Intensity Distribution (AID). This AID can be calculated using eq. (1) for different object sizes, thus producing a functional S-M-relationship between the object size S and the modulation M (see fig. 1).

For unknown objects, the AID can be measured in the SMI by registering the fluorescence intensity while moving the object along the optical axis. After determination of the modulation M for the unknown objects, one uses the S-M-relation to obtain the size S for this object.

However, in general, the interference of the light from both interferometer arms is not optimal, e.g. due to misalignment (c.f. Fig 1b). Hence, the modulation depth of the SMI-PSF is not maximal. In this case, also the modulation M of the AID is reduced. By measuring the AID for calibration objects of known size, the axial PSF of the SMI can be reconstructed, allowing to obtain a true S-M-relationship for the given SMI interferometer alignment.

4 Image acquisition

The fluorescent specimen were placed into the space between the two opposite objective lenses and mounted between a conventional object slide (76 mm x 25 mm x 1 mm) and a cover slip (thickness ~ 170 µm). For the performance of the measurements they were moved along the axial direction (providing optical sectioning), using a piezoelectric stage (Physik Instrumente, Waldbronn, Germany) with a typical step size of 40 nm.

5 Extraction of the size dependent modulation

The modulation M for a given objects AID can be obtained by a non-linear least square fit, e.g. using the following fit function:

$$AID = \left(M \cos^2(k_{wf} z) + A\right)\mathrm{sinc}^2(k_{\mathrm{det}} z), \quad (2)$$

where A is maximum of the the non-modulating inner part, k_{wf} is the wave vector of the standing wave field, k_{det} is given by the axial resolution of the detection objective lens. In eq. (2) it is assumed that the phase of the wavefield is zero in the focal plane. Alternatively, if the positions of the object are not important, also a fit to the amplitudes in the Fourier domain is possible.

6 Results

Size measurements on 140 and 200nm (nominal diameter) beads using reference objects on the object slide yielded 140.0±2.2 and 197.2±2.7 nm. The error given is the standard deviation of the mean of N=80 selected objects (beads). The results show that when performing many measurements on the same kind of objects one gets mean values very near to the true values with an accuracy in the range of a few nanometers.

In a comparative study of transcription factories [4], size values very close to those obtained from Electron Microscopy were obtained. When applying Fluorescence in Situ Hybridization, also the sizes of labeled gene regions could be measured well below the conventional resolution limit [5].

References

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