High-precision distance microscopy of 3D-nanostructures by a spatially modulated excitation fluorescence microscope

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ABSTRACT

To study the 3D-organization and 3D-pathology of the genome in intact cell nuclei, precise and accurate 3D-object localizations and 3D-distance measurements of fluorescent labelled chromatin-nanostructures are required. For this purpose, a high precision fluorescence microscope setup with spatially modulated excitation (SME) has been built up combining advantages of an epifluorescence microscope with interferometric laser illumination in the direction of the optical axis and optical sectioning. The SME allows high precision localization in the nanometer range resulting in a considerable increase of the axial resolution equivalent. This is shown for a configuration of five fluorescent microspheres of 100 nm diameter. Since the optical sections are acquired in an epifluorescent mode, image analysis procedures of high precision spectral distance microscopy were applied to determine the lateral particle localization by the position of the bary center of intensity. From these data, relative axial distances as well as relative 3D-distances were calculated. The results indicate that distance measurements between fluorescent objects can be performed with an accuracy of more than one order of magnitude better than the lateral epifluorescent optical resolution given by the full width half maximum of the central peak of the effective point spread function. Since the definition of the optical resolution in refraction limited optical systems is based on distance measurement, the measure for the accuracy of our results in precision distance calculation is called resolution equivalent.

Keywords: fluorescence light microscopy, standing wave field microscopy, spatially modulated excitation fluorescence microscopy, precision distance measurements, precision localization, spatial resolution

1. INTRODUCTION

To study the functional organization of chromatin in intact interphase cell nuclei[1,2], it is important to analyze small fluorescence labelling sites by quantitative 3D-microscopy. Distances and the topology of fluorescent targets of equal and/or unequal spectral signatures have to be determined. For this purpose, the targets have to be precisely localized[3-5]. From the target loci defined by the bary centers of their fluorescence intensity, the euclidian distances can be calculated by specially designed computer algorithms for digital image analysis. The error of distance measurements strongly depends on the localization error which is especially influenced by the effective optical resolution of the microscope, by the signal to noise ration, by the detector sensitivty, and by the digitization accuracy [4-8]. Using targets of different spectral signature, effects of chromatic shifts have to be considered in addition[7,8]. The well established and most frequently applied fluorescence microscope for 3D-imaging of biological specimen is the confocal laser scanning microscope (CLSM). Under practical conditions, i.e., the suboptimal optical conditions of a biological specimen with fluorescence labelled cell nuclei, the CLSM showed a localization accuracy of about ± 20 nm in lateral and about ± 40 nm in axial direction[3,9] corresponding to about a tenth of the optical resolution defined by the full width at half maximum of the main peak of the effective point spread function. This suggested that 3D-distance measurements can be performed with an accuracy of less than 60 nm[7]. To further increase the localization precision of the given microscope, axial tomography can be applied[6,10]. Approriate rotation results in a localization accuracy of about ± 20 nm for a CLSM independently from the original spatial orientation of the targets[3, 11].

To improve the resolution and thus the localization precision, interferometric illumination and/or detection of the fluorescent targets can be applied[12-16]. However, diffraction limited focussing may result in unwanted optical distortions by refraction index mismatch[17, 18].

Here, we present an epifluorescent laser microscope setup with spatially modulated excitation (SME)[16]. High precision localization and high precision distance measurements are performed. Although optical sections are recorded with an optical standard setup and an 8bit CCD camera, the 3D-distance measurements showed an accuracy of less than ± 40 nm for individual fluorescent microspheres.

2. THE SME-FLUORESCENCE MICROSCOPE

Fig. 1 shows a schematic representation of the SME-fluorescence microscope[16]. Coherent light of an Argon ion laser of 250 - 400 mW at 488 nm was coupled via a single mode, polarization conserving glass fibre into a microscope setup mounted on a thermally stabilized Invar bread board. The light was expanded by means of a collimator and splitted into two beams of equal intensity by means of a beamsplitter. The parallel light of the two beams was focussed into the back focal plane of the two objectives (100 x /0.7 - 1.4 PlanAPO, Leica) using an achromatic lens in front of each objective. Since the objectives worked according to infinite optics, this resulted in parallel light beams of planar wave fronts in the object space. Interference of these two parallel light beams resulted in a standing wave field for SME.

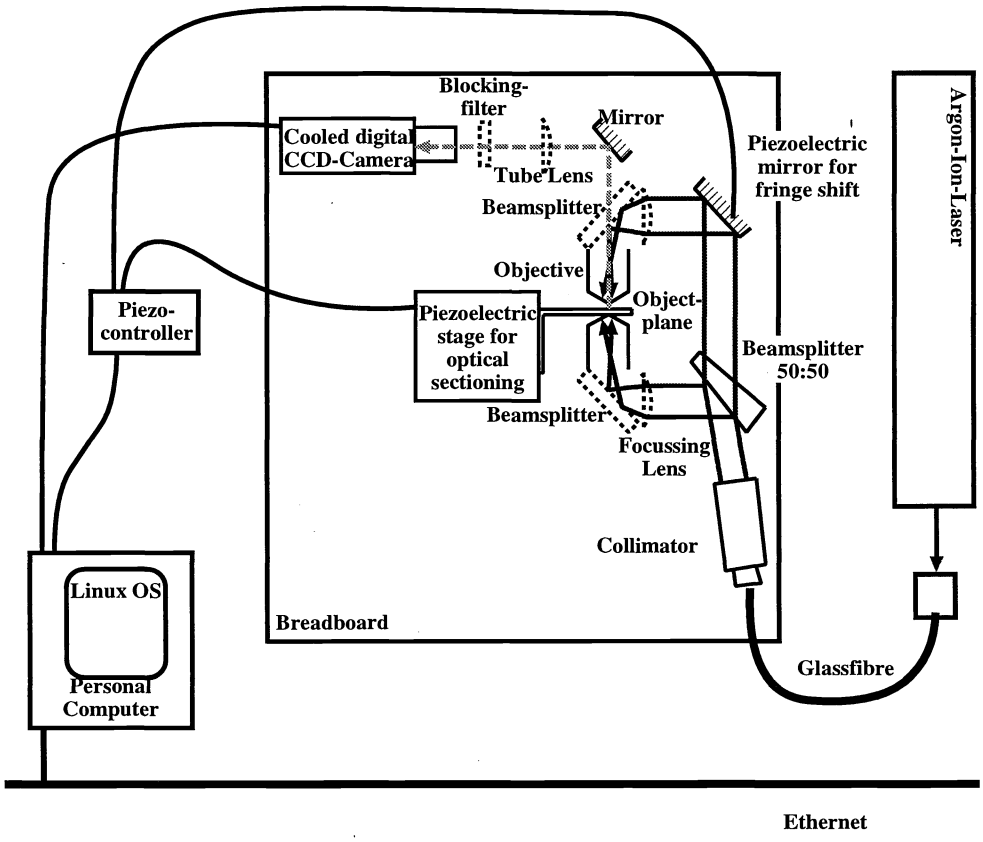


Fig. 1: *Schematic representation of the spatially modulated excitation (SME) fluorescence microscope (for details see text)*

With a lateral move of the focussing lenses, the interference angle ϑ of the two parallel light beams and the optical axis could be varied. The distances d of the interference maxima in the object space were given by[14]:

$$d = \lambda / (2n \cos\vartheta)$$

(λ = laser wave length; n = refraction index of the object; ϑ = interference angle). In the object space, the specimen mounted on standard glass slides was piezoelectrically moved along the direction of the optical axis (z-direction) with a stepsize of 20 nm. For each step, a 2D-image was recorded by a cooled digital CCD-camera (CF20DX; Kappa Messtechnik, GmbH) via an appropriate filter set for FITC (optical sectioning). This resulted in a complete 3D-data stack of up to 400 images. Typically, a volume of up to 62 x 46 x 8 μm^3 can be covered in the object space. The image acquisition time was 2 - 4 sec per optical section resulting in about 25 minutes recording time of a complete 3D-data stack.

The complete SME fluorescence microscope was controlled by a personal computer running under the LINUX operating system. Personal computers connected to the system via Ethernet were used for digital image analysis.

3. HIGH-PRECISION LOCALIZATION

With the SME-fluorescence microscope different configurations of fluorescent microspheres were analyzed. The following results were obtained for five test particles of 100 nm diameter (Polysciences) randomly arranged within an optical depth less than 300 nm (i.e. below the axial optical resolution of a CLSM).

3.1. AXIAL LOCALIZATION

The axial localization (z-localization) was determined from the 3D-data stack using the digital image analysis software KHOROS[19]. Fig. 2 shows an example of a microsphere analyzed by this procedure. From the 3D-data stack axial sections through the beads were extracted. A lateral region of interest (ROI) of 3 x 3 square pixels in each lateral (xy-) image around the central xy-coordinate of each microsphere was determined. The average intensity of each ROI was calculated. Thus, for each 20 nm step along the z-axis an intensity value was obtained resulting in an experimental intensity distribution along the z-axis for each microsphere (Fig. 3).

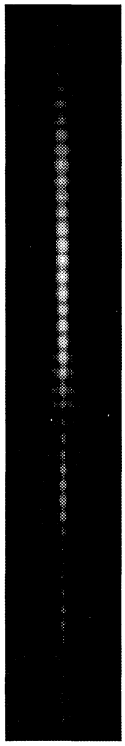
	localization			mean error		relative axial
[nm]	x	y	z	δxy	δz	distance
Bead1	1754,17	4186,66	2943,36	10,37	1,08	0,00
Bead2	2578,68	3966,06	2918,90	11,96	1,20	24,46
Bead3	2811,80	3835,20	3105,00	(0,35)*	1,37	-161,64
Bead4	2940,04	3367,66	3214,58	9,50	1,39	-271,22
Bead5	3708,85	2877,55	3135,18	15,97	2,00	-191,82

Table 1*: x, y, z coordinates (localization) of the five fluorescent microspheres (beads) of 100nm-diameter calculated from a 3D-image stack of 400 optical sections recorded with the SME-fluorescence microscope. The x- and y-coordinates in nm were calculated from the calibrated pixel positions. The z-coordinate was obtained from the fit maximum position. The lateral localization errors δxy were given in nm The relative axial distances were calculated with reference to microsphere 1. These values indicate that the microspheres are located within a depth of less than 300 nm (for further details see text).*
*(*Note: For microsphere 3, only the calibration error was considered, because due to limitations of segmentation relevant thresholding resulted in one pixel only.)*

These intensity distributions were approximated by means of a non linear least-square fit algorithm (Levenberg-Marquardt-Algorithm[20]). This fit procedure was proportional to $sinc^2(z)\ cos^2(z)$ compatible to the axial point spread function of the SME-fluorescence microscope[16]. The z-coordinates of the intensity maximum given in 20 nm step units were rescaled into nm with reference to the image border. Since the equidistant fringe pattern of the illumination field contributed to a large number of fluorescence intensity peaks for each microsphere, a z-localization precision in the range of 1 to 2 nm was obtained (Table 1).

3.2. LATERAL LOCALIZATION

In the optical section closest to the z-locus of each microsphere calculated according to the fit values, image segmentation of the microsphere was performed using the Cavaleri estimator[3,10]. For a series of relevant thresholds[21], the bary center of the segmented intensity distribution was calculated. The variations in x- and y-direction were examined and averaged for the lateral localization error in pixel units. Using a calibration grid, the values for the xy-loci were rescaled into nm with reference to the image border. The calibration error of 0.4 nm in x-direction and 0.3 nm in y-direction was additionally considered for the lateral localization error of the microspheres (Table 1).



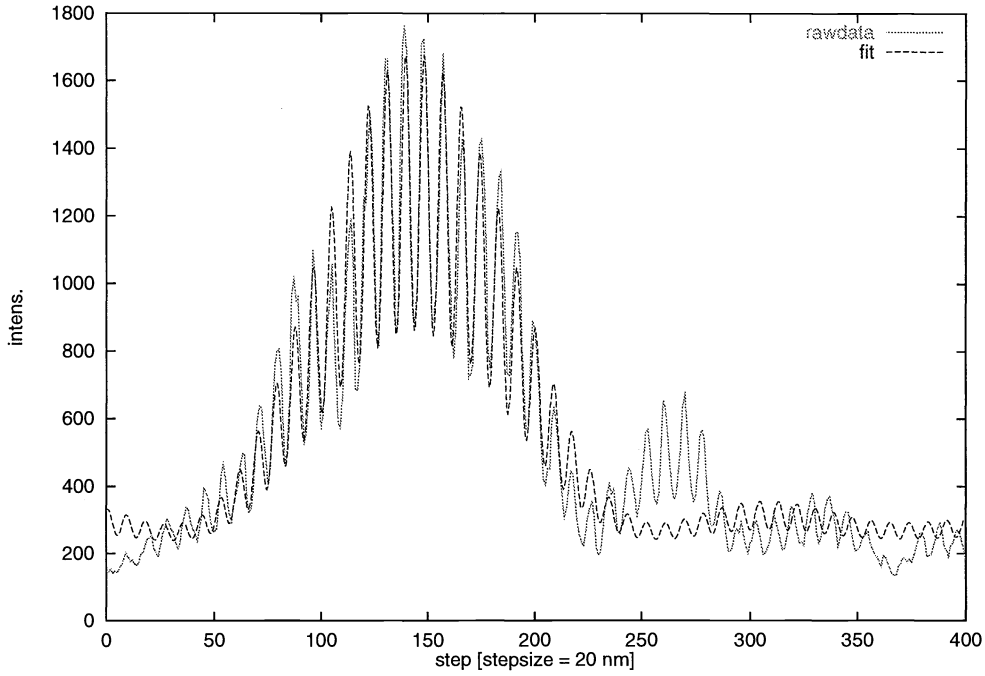


Fig. 2 **Fig. 3**

Example of precise axial (z-) localization of a fluorescent microsphere: **Fig. 2:** *xz-section through the 3D-image stack of a 50 nm fluorescence microsphere. The image stack was obtained from 400 epifluorescent images by optical sectioning with 20 nm stepsize.* **Fig. 3:** *Intensity distribution along the optical axis through the microsphere of Fig 2. The relative fluorescence intensity is shown versus the z-axis in steps of 20 nm (rawdata). The dashed line (fit) represents a non-linear least-square fit from which the maximum localization and the localization error were calculated. For the microsphere shown here, the maximum position was at 148,25 steps corresponding to 2965.06 nm from the image border. The localization accuracy was 0.057 steps corresponding to 1.14 nm.*

4. PRECISION DISTANCE MEASUREMENT

The highly precise localization of the five fluorescent microspheres was used to measure the euclidian distances between them (Table 2). The distance D_{xyz} of microsphere *i* to microsphere *j* is given by

$$D_{xyz} = [(x_i - x_j)^2 + (y_i - y_j)^2 + (z_i - z_j)^2]^{1/2}$$

The distance error δD_{xyz} can be calculated by[11]

$$\delta D_{xyz} = |(x_i - x_j)/ D_{xyz}| (\delta x_i + \delta x_j) + |(y_i - y_j)/D_{xyz}| (\delta y_i + \delta y_j) + |(z_i - z_j)/D_{xyz}| (\delta z_i + \delta z_j)$$

with δx, δy, δz the localization error. In most cases δD_{xyz} was considerably below 40 nm. High precision 3D-distance measurements in the range of 0.3 µm up to about 2.5 µm were possible with an average accuracy of about 27 nm.

[nm]	Bead1	Bead2	Bead3	Bead4	Bead5
Bead1	-------	853,86	1126,16	1466,50	2360,37
Bead2	27,45	-------	325,73	759,00	1583,95
Bead3	16,22	14,04	-------	497,04	1312,52
Bead4	29,53	28,15	9,82	--------	915,19
Bead5	41,32	39,79	22,52	40,47	--------

Dxyz

δDxyz

Table 2: *3D-distanees D_{xyz} in nm of the bary centers of the fire fluorescent microspheres (right up) and the corresponding 3D-distance errors δD_{xyz} (left down) obtained from precision 3D-localization (see Table 1).*

5. DISCUSSION

In this report, the SME-fluorescence microscope[16] was applied for high precision 3D-distance measurements between five fluorescent microspheres of a randomly obtained topology. The approach was based on precise localization[3,7] which can be done with an accuracy typically one order of magnitude below the classical optical resolution determined by the full width at half maximum of the effective point spread function of a given microscope. Since distance measurements are commonly used for resolution definition, the accuracy of the distance measurements obtained from highly precise localization will also be called "optical resolution equivalent". By using targets of different spectral signatures, bary center distances $<< \lambda/2$ can be measured[7].

The optical resolution equivalent of the SME-fluorescence microscope was on average about 27 nm. To our knowledge, this is one of the best values for 3D-microscopes. However, this was mainly controlled by the lateral localization accuracy because SME resulted in an axial localization accuracy of about 1 - 2 nm. To overcome these shortcomings of epifluorescent image acquisition, micro axial tomography [6] may be applied so that each two target points can be tilted into a plane parallel to the optical axis of the system. In addition, other more sophisticated algorithms (e.g. [7,9]) may be used to evaluate the lateral coordinates. By evaluation of the entire data stack (x,y,z) due to an improved photon statistics[7,22] it should be possible to diminish the lateral (x,y) distance error to a few nm only.

The Cavalieri estimator used for segmentation by thresholding was limited in the lateral discrimination of microsphere 2 from microsphere 3, although both were clearly separated in the axial direction. To overcome those problems especially for smaller targets located together, i.e., with a distance below the optical resolution, the targets should carry a different spectral signature. This may be fluorochromes of different emission spectra. Many of these fluorochromes, however, have also different absorption spectra. Therefore, we are going to implement a second laser beam into the SME fluorescence microscope. In this case, also chromatic shifts have to be considered for the accuracy of distance measurement[7,8]. Another possibility may be the use of fluorochromes showing different fluorescence lifetimes.

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