Beyond nanosizing: an approach to shape analysis of fluorescent nanostructures by SMI-microscopy

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Abstract

Using spatially modulated illumination (SMI) light microscopy it is possible to measure the sizes of fluorescent structures that have an extension far below the conventional optical resolution limit ("subresolution size"). Presently, the sizes are determined as the object extension along the optical axis of the SMI microscope. For this, however, "a priori" assumptions on the fluorochrome distribution ("shape") within the examined fluorescent structure have to be made. Usually it is assumed that the fluorochrome follows a Gauss-distribution or a spherical distribution. In this report we overcome the necessity to make an assumption on the shape of the fluorochrome distribution. We introduce two new experimentally obtained parameters which allow the determination of a shape measure to describe the spatial distribution of the fluorescent dye. This becomes possible by independent measurements with different excitation wavelengths. As an example, we present shape parameter measurements on individual fluorescent microspheres with a nominal geometrical diameter ("size") of 190 nm. In the case investigated, the experimental shape correlated well with a homogeneous fluorochrome distribution ("spherical shape") but not with a variety of other "shapes".

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1. Introduction

The principle approach in spatially modulated illumination (SMI) microscopy is to measure the intensity of a fluorescent small structure while it is moved in steps of some tens of nanometers through an exciting "standing wave" field that is generated from the interference of two recombined, collimated laserbeams [1–3]. The resulting signal shows a modulation due to the excitation fringes of the wavefield and a non-modulating background that correlates with the size (i.e. geometrical extension) of the object [3–5]. The calibration function required to deduce the object size from this nonmodulating "background" may be determined experimentally with objects of known size, or determined by "virtual SMI microscopy" calculations [5]. Using excitation wavelengths in the visible range, this method presently allows size determination from a maximum of several
hundred nanometers down to about 40 nm on fluorescent microspheres [6,7], measurements on the size of specifically labelled gene regions in intact cell nuclei [8] as well as investigations on the size of transcription factories, both at a diameter around 50–70 nm [9]. Since the first reports on SMI-nanosizing, the method was improved significantly by usage of reference objects (objects with known size and dye distribution), which allow to measure the axial point spread function of the SMI microscope [7]. Using reference objects, disturbances of the standing wavefield could be taken into account. Until now, however, SMI-nanosizing required some basic assumptions concerning the “shape” of the examined fluorochrome distributions. In the beginning, it was assumed that the dye is Gaussian-like distributed over the unknown object and the FWHM₀ of the fluorochrome distribution (full width at half maximum) was considered to be equal to the size of the structure. Later on, different assumptions on the shape were considered for the size evaluation (e.g. spherical dye distribution). It has been shown that the assumed shape of the structure has some influence on the measured size [10]. Here, we show a further improvement in SMI-nanosizing. In this report, we present an approach which makes it possible to determine a shape parameter describing the global fluorochrome distribution. Thus, shape parameter measurements on sub-resolution sized fluorescent objects become possible without additional assumptions on the fluorochrome distribution. The measured shape value may then be used to select the appropriate calibration function for the determination of the geometrical size, i.e. the spatial extension of the object containing the fluorochromes.

2. Materials and methods

2.1. SMI-microscope

The SMI-microscope used for the measurements has been described in detail elsewhere [2,4]. Briefly, an interferometric setup produces a standing wave illumination pattern for fluorescence excitation. In this interferometer, the specimen is illuminated via two opposing objective lenses, yielding an axially modulated illumination along which the specimen is moved in precise steps with a minimum of 20 nm. One of the two oil immersion objective lenses of high numerical aperture is used to collect the fluorescence light, which is then imaged onto a CCD camera. For a complete 3D image, the specimen is moved through the focal plane, and thus also through the standing wavefield. Hence, the SMI point-spread-function (PSF) can be described as the product of the illumination pattern, represented by a \( \cos^2 \)-function, and the detection PSF of a conventional widefield microscope.

2.2. Specimen

SMI-microscope measurements were performed using fluorescent microspheres with a nominal (geometrical) diameter of 190 nm. These microspheres (F-8807) were manufactured by Molecular Probes (Eugene, Oregon, www.probes.com). They can be excited with \( \lambda_{\text{ex}} = 488 \) and 647 nm and emit red light in both cases. To obtain an information about the wavefield disturbances we used fluorescent microspheres with a nominal (geometrical) diameter of 100 nm as reference objects [7]. Two kinds of microspheres were used: G-100-microspheres from Duke Scientific Corporation (Palo Alto, CA, www.dukescientific.com), which can be excited by \( \lambda_{\text{ex}} = 488 \) nm and T-8878-microspheres from Molecular Probes, which can be excited by \( \lambda_{\text{ex}} = 647 \) nm. As embedding medium VECTASHIELD from Vector Laboratories (Burlingame, CA, www.vectorlabs.com) was used; this reduces the bleaching of the fluorescent dye and has a refraction index of approximately \( n = 1.440 \).

2.3. Preparation of the object slides

First, a dilution of all three kinds of microspheres in distilled water was produced. This dilution was put in an ultrasonic bath for approximately 10 min to eliminate all clusters of microspheres. Ten microliters from this dilution were distributed over an object slide. After a few minutes the water was evaporated and the microspheres adhered on the glass. Then a drop of VECTASHIELD was applied to the object slide and a cover slip was used for sealing. Fig. 1 shows a preparation of such an object slide.

2.4. Axial intensity distribution and modulation contrast \( R \)

The dependency of the detected intensity from the axial position (position along the optical axis) of the object is given by the axial intensity distribution (AID or \( I_{\text{AID}}(z) \)). As the excitation wavefield shows intensity
maxima and minima this is also true for the AID. The ratio between the interior, non-modulating “background” of the AID and the absolute intensity maximum of the AID is called the modulation contrast \( R \). It relates to the axial extension of the object. Fig. 2 schematically shows a calculated AID. In Fig. 3 a measured AID of a 190 nm microsphere with an excitation wavelength of 488 nm is shown. The AID can be calculated by the convolution of the axial projection of the fluorochrome distribution \( \rho(z) \) with the Point Spread Function (PSF or \( I_{PSF}(z) \)):

\[
I_{AID}(z) = \int_{-\infty}^{\infty} \rho(a)I_{PSF}(z-a) \, da.
\]

Using objects for which the dye distribution \( \rho(z) \) is known, one can reconstruct the microscope PSF by applying the FOURIER-transformation on the measured AID. It is

\[
\text{FT}(I_{AID}(z)) = \text{FT}(\rho(z)) \text{FT}(I_{PSF}(z))
\]

\[
\Rightarrow I_{PSF}(z) = \text{IFT}\left\{ \text{FT}(I_{AID}(z)) / \text{FT}(\rho(z)) \right\}.
\]

where FT denotes the FOURIER-transformation and IFT is the inverse FOURIER-transformation. This reconstructed reference PSF allows to calculate the modulation contrast \( R_0 \) of the PSF for this condition. \( R_0 \) relates to the disturbances in the wavefield. If the wavefield follows an ideal \( \cos^2 \)-modulated distribution, \( R_0 \) is zero. As the disturbances become larger, \( R_0 \) increases and takes values between zero and one (see also [5,7,10]). Using various “virtual SMI microscopy” approaches, for a given fluorochrome distribution \( \rho(z) \) (denoted as “shape”), the modulation contrast can be calculated [5,7].

### 2.5. Determination of shape measures

For objects that are small compared to the FWHM of the axial PSF of a widefield microscope and for symmetrical dye distributions, the modulation contrast \( R \) can be approximated by

\[
R = \frac{\int_{-\infty}^{\infty} \rho_0(z)(\sin^2(kz) + c) \, dz}{\int_{-\infty}^{\infty} \rho_0(z)(\cos^2(kz) + c) \, dz},
\]

where \( \rho_0(z) \) is the normalized axial projection of the dye distribution to be investigated: \( \rho_0(z) = m \int_{-\infty}^{\infty} \rho(x,y,z) \, dx \, dy \). \( m \) is the normalization constant, such that \( \int_{-\infty}^{\infty} \rho_0(z) \, dz = 1 \). \( c \) describes a constant (not modulating) intensity of the excitation light field and relates to the modulation contrast \( R_0 \) of the PSF:

\[
R_0 = \frac{c}{1+c} \quad \Rightarrow \quad c = \frac{R_0}{1-R_0}.
\]

\( k \) depends on the refraction index of the embedding medium and the excitation wavelength: \( k = \frac{2\pi n}{\lambda} \).

Using the polynomial approximation \( \cos^2(z) \approx 1 + a_1 z^2 + a_2 z^4 \) one obtains

\[
R = \frac{\int_{-\infty}^{\infty} \rho_0(z)(-a_1 k^2 z^2 - a_2 k^4 z^4 + c) \, dz}{\int_{-\infty}^{\infty} \rho_0(z)(1 + a_1 k^2 z^2 + a_2 k^4 z^4 + c) \, dz}
\]

\[
= \frac{-a_1 k^2 \int_{-\infty}^{\infty} \rho_0(z) z^2 \, dz - a_2 k^4 \int_{-\infty}^{\infty} \rho_0(z) z^4 \, dz + c}{1 + a_1 k^2 \int_{-\infty}^{\infty} \rho_0(z) z^2 \, dz + a_2 k^4 \int_{-\infty}^{\infty} \rho_0(z) z^4 \, dz + c}
\]

\[
= \frac{-a_1 k^2 s_2 - a_2 k^4 s_4 + c}{1 + a_1 k^2 s_2 + a_2 k^4 s_4 + c}.
\]

In the equation above the abbreviations \( s_2 := \left( \int_{-\infty}^{\infty} \rho_0(z) z^2 \, dz \right)^{1/2} \) and \( s_4 := \left( \int_{-\infty}^{\infty} \rho_0(z) z^4 \, dz \right)^{1/4} \) were used. \( s_2 \) and \( s_4 \) can be interpreted as size related parameters. To avoid confusion with the conventional (geometrical) end-to-end size, we shall call both, \( s_2 \) and \( s_4 \) a size momentum. As the polynomial expansion of \( R \) was truncated after the \( z^4 \)-term, the approximation gives only reasonable values if the dye distribution represented by \( \rho(z) \) is small enough. Then, the modulation

Fig. 2. Schematic axial intensity distribution (AID). The modulation contrast, which is related to the object size, is defined by \( R = \frac{l_{\text{min}}}{l_{\text{max}}} \).

Fig. 3. Measured axial intensity distribution (AID) of a microsphere with a diameter of 190 nm using an excitation wavelength of 488 nm.
contrast of the examined structure can be determined only by knowledge of its $s_2$ and $s_4$-values. For even smaller objects the modulation contrast $R$ can be calculated from the $s_4$-value alone. In general, all values $s_n := \left( \int_{-\infty}^{\infty} \rho_0(z) |z|^n \, dz \right)^{1/n}$ can be considered as size related parameters. This is shown by simple scaling considerations: If $s_n$ is a size related parameter, then it is expected that $s_n$ changes by a factor of $1/r$ if $\rho_0(z)$ is changed to $\rho'_0(z) := r \rho_0(rz)$. $\rho'_0$ has the same shape as $\rho_0$, but is smaller by a factor of $r$.

$$s'_n = \left( \int_{-\infty}^{\infty} \rho'_0(z) |z|^n \, dz \right)^{1/n}$$
$$= \left( \int_{-\infty}^{\infty} r \rho_0(rz) |z|^n \, dz \right)^{1/n}$$
$$= \left( \int_{-\infty}^{\infty} r \rho_0(t) |t|^n \, dt \right)^{1/n}$$
$$= \frac{1}{r} \left( \int_{-\infty}^{\infty} \rho_0(t) |t|^n \, dt \right)^{1/n}$$
$$= \frac{1}{r} s_n.$$  

By dividing two size momenta, one obtains a measure whose important characteristic is its independence from the object size. Hence we call this ratio a shape measure. In this article we use the shape measure $s := \frac{s_2}{s_4}$. The shape measures of some important dye distributions are shown in Table 1. In Figs. 4b and c the projections of a homogeneously stained sphere (b) and a surface-stained sphere (c) onto the optical axis ($z$-axis) are shown together with their $s$-values. Figs. 4a, d and e show some other axial dye distributions and their $s$-values.

### Table 1. Important axial dye distributions and their shape value $s$

<table>
<thead>
<tr>
<th>Shape</th>
<th>$s$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaussian dye distribution</td>
<td>0.7598</td>
</tr>
<tr>
<td>Sphere (homogeneously dyed)</td>
<td>0.8265</td>
</tr>
<tr>
<td>Sphere (dye only on surface)</td>
<td>0.8633</td>
</tr>
</tbody>
</table>

The coefficients $a_1$ and $a_2$ depend on the polynomial approximation for $\cos^2(z)$. If the Taylor-expansion is chosen, it is $a_1 = -1$ and $a_2 = \frac{1}{2}$. However, the Taylor-expansion gives a polynomial that agrees only well with $\cos^2(z)$ near $z = 0$, but for larger $z$-values the error becomes too large for our purpose. Instead, we determined the coefficients by fitting a polynomial to $\cos^2(kz)$ in the range from $-100$ to $100$ nm. By this procedure we obtained the following coefficients for $\cos(z)^2$: $a_1 = -0.924379$ and $a_2 = 0.21625$. Solving the system of linear Eqs. (3) gives unknowns $s_2$ and $s_4$.

$$a_1 k^2 s_2^2 + a_2 k^4 s_4^4 = \frac{c(1 - R) - R}{R + 1}.$$  

By measuring two modulation contrasts with different excitation wavelengths one obtains a system of two linear equations which can be used to determine the two

### 3. Results

#### 3.1. Measurements

An object slide as shown in Fig. 1 was localized in the standing fieldwave of the SMI-microscope and was moved in steps of 20 nm along the optical axis. After each step a two-dimensional (2D) image was registered by means of a CCD-camera. A total of 250 images was
All distributions have a size momentum important. In case of a scaling of the dye distribution the scaling of the horizontal axis is not values on the horizontal and the vertical axis are in a.u. As the diameter of 69 nm. sphere (b) has a diameter of 89 nm and the sphere (c) a diameter of 69 nm.

Fig. 4. Different axial fluorochrome distributions (“shapes”) and their s-values. In (b) the axial projection of a homogeneously stained sphere is shown, (c) shows the axial projection of a surface-stained sphere. These distributions can be described by the model $\rho(z) = (z^2 - \frac{1}{4})^n \quad (|z| \leq \frac{1}{2})$ with $n = 1$ (homogeneous sphere) or $n = 0$ (surface-stained sphere). All distributions have a size momentum $s_2 = 20$ nm. The values on the horizontal and the vertical axis are in a.u. As the s-value depends only on the shape and not on the size of the dye distribution the scaling of the horizontal axis is not important. In case of a scaling of the z-axis in nanometers, the sphere (b) has a diameter of 89 nm and the sphere (c) a diameter of 69 nm.

3.2. Determination of parameters

In the next step, the AID of all objects were extracted from the data stacks. The automated background subtraction algorithm of the SMI-software was applied. From the AIDs of the reference objects a mean AID was calculated for each excitation wavelength. These mean AIDs were used to calculate the axial reference PSF for each excitation wavelength by usage of formula (1). The modulation contrasts $R_{0,l}$ of these reference PSFs were determined. They specify the disturbances in the standing wavefield. $R_{0,l} = 0$ means that there is no constant not modulating intensity part in the standing wave field. Then the modulation contrasts of the 190 nm-spheres were experimentally determined from their AIDs for both excitation wavelengths. By use of Eq. (4) the size momenta $s_2, s_4$ and the shape parameter $s = \frac{s_2}{s_4}$ of the 190 nm-spheres were calculated from the experimentally obtained modulation contrasts.

The modulation contrasts and shape parameter obtained are shown in Tables 2–4. Table 2 shows the modulation contrasts of the point spread functions that

Table 2. Experimental modulation contrast $R_0$ of the reference PSF determined from the AIDs of the reference objects (100 nm nominal diameter)

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>$R_{0,647}$</th>
<th>$R_{0,488}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.106 ± 0.014</td>
<td>0.371 ± 0.032</td>
</tr>
<tr>
<td>2</td>
<td>0.152 ± 0.028</td>
<td>0.221 ± 0.030</td>
</tr>
</tbody>
</table>

Approximately 10 AIDs were used to calculate a mean AID. From this mean AID the PSF was calculated. The error is the standard deviation of the single measurement.

Table 3. Experimental modulation contrasts $R$ of the 190 nm-spheres

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>$N$</th>
<th>$R_{647}$</th>
<th>$R_{488}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>0.422 ± 0.049</td>
<td>0.785 ± 0.033</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0.427 ± 0.039</td>
<td>0.758 ± 0.024</td>
</tr>
</tbody>
</table>

$N$ is the number of 190 nm-spheres evaluated. The error is the standard deviation of the single measurement.

Table 4. Measured size momenta and shape parameter value of the 190 nm-spheres

<table>
<thead>
<tr>
<th></th>
<th>$s_2$ [nm]</th>
<th>$s_4$ [nm]</th>
<th>$s := \frac{s_2}{s_4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretically calculated</td>
<td>42.50</td>
<td>51.42</td>
<td>0.8265</td>
</tr>
<tr>
<td>Experimental results</td>
<td>41.11</td>
<td>49.15</td>
<td>0.8363</td>
</tr>
<tr>
<td>Errors (SDM*)</td>
<td>±1.07</td>
<td>±2.53</td>
<td>±0.0220</td>
</tr>
</tbody>
</table>

*The error is the standard deviation of the mean.

taken. This procedure was first performed with an excitation wavelength of $\lambda_{ex} = 488$ nm and then repeated with $\lambda_{ex} = 647$ nm. Between the acquisition of the two data stacks, the object slide was not moved in the lateral direction. This procedure was repeated once at a different lateral location of the object slide, resulting in a total of four data stacks of 2D images.
were calculated from the AIDs of the reference objects by Eq. (1). Table 3 shows the modulation contrasts of the "unknown" objects (190 nm-spheres) for the excitation wavelengths $\lambda_{ex} = 488$ and 647 nm. Using the values from Tables 2 and 3 and Eq. (4) one obtains the values $s_2$, $s_4$ and $s$ in Table 4.

### 3.3. Comparison with theoretical values

The $s_2$ and $s_4$ values determined in Table 4 are not identical with the geometrical diameter of the objects but represent some sort of "normalized" size parameter. To find out how accurate the measured parameters $s_2$, $s_4$ and $s$ are, they were compared with the values expected from certain geometrical assumptions. The parameters can be obtained by using the definitions $s_2 := (\int_{-\infty}^{\infty} \rho_0(z) z^2 dz)^{1/2}$, $s_4 := (\int_{-\infty}^{\infty} \rho_0(z) z^4 dz)^{1/4}$ and assumptions about the shape of the objects that were examined. For example, if one assumes a fluorochrome distribution corresponding to that of a homogeneously stained sphere, a shape parameter value $s = 0.8265$ is obtained. Other assumptions about the fluorochrome distribution yield other shape parameter values (Fig. 4). The experimentally obtained value $s_{exp} = 0.8363$ obviously fits well to that of a homogeneous, spherical dye distribution.

For many considerations it is important to have an information also about the geometrical extension of the object. By use of a model for the axial dye distribution it is possible to estimate the geometrical diameter of the fluorescent structure. We applied the following model ($d, n \in \mathbb{R}^+$):

$$\rho(z) = \begin{cases} \left(\frac{d}{2} - |z|\right)^n, & |z| \leq \frac{d}{2}, \\ 0, & \text{otherwise}. \end{cases}$$

In this model, $d$ describes the geometrical extension of the object and $n$ relates to the shape of the axial dye distribution. For example, if it is $n = 1$ (see Fig. 4b), then the model describes the axial projection of a homogeneous, spherical dye distribution. For $n = 0$ (see Fig. 4c) the model describes the axial projection of a sphere which is stained only on its surface. From the experimentally obtained values $s_2 = 41.11$ nm and $s = 0.8363$ it is then possible to calculate the parameters $n$ and $d$ of the model. We obtained $n = 0.64$ and $d = 170.1$ nm for the microspheres with a nominal diameter of 190 nm. As the nominal value is given by the manufacturer with an error of $\pm 10\%$, both values are in good agreement.

Using the assumption of a homogeneously stained sphere with a diameter of 190 nm we obtained $s_2 = 42.50$ nm and $s_4 = 51.42$. These theoretical values agree very well with the measured values $s_2 = 41.11$ nm and $s_4 = 49.15$ nm.

### 4. Discussion

In conventional light microscopy (including conventional laser scanning confocal devices), a reliable determination of the geometrical extension of single fluorescent objects is limited to ranges larger than 100–200 nm [11–13]. To allow the light optical measurement of interior structure details given by the "shape" of the fluorochrome distribution within the object, various approaches to increase the optical resolution have been developed, such as 4Pi-microscopy [14,15], or methods of patterned illumination microscopy [16,17,19]. Here, we show that in addition to an appropriate increase in optical resolution (as defined by the full width at half maximum of the PSF), also SMI microscopy measurements may be used. In this latter case, an improved "shape" estimate allows also a more reliable determination of the geometrical size of the fluorochrome labelled object. Since each of the light "nanoscopy" methods mentioned has its specific advantages, we anticipate that a combined use will eventually allow highly accurate measurements of geometrical object sizes and interior structural details of individual nanostructures hitherto thought to be not accessible to fluorescence light microscopy.

The results presented in this report indicate that an SMI-Nanosizing evaluation module based on the determination of $s_2$, $s_4$ and $s$-parameters as defined here is feasible and allows an experimentally based estimate about the fluorochromes distribution in a subwavelength sized object. A basic condition for this method is that the fluorochromes in the "unknown" object must be excitable at two different wavelengths. We used $\lambda_{ex} = 488$ and 647 nm. Instead of using two different excitation wavelengths one might in future only change the "effective" wavelength (i.e. twice the distance between two intensity maxima of the standing wave field [1]) by varying the angle between the two interfering laser beams. This would have the additional advantage that more than two effective wavelengths could be used to examine the object; this would allow to determine more than two shape related parameters.

The implication of the presented method for biological research is evident as for many large macromolecule clusters ("biomolecular machines") their structure and interaction between each other is determined by their inner organization. For example, it is widely accepted that the interior structure of individual gene domains in cell nuclei (having a typical diameter in the hundred nanometer range) is important for gene regulation [20,18,21]. Using appropriate molecular labelling techniques, such differences may be translated into different fluorochrome distributions which can be discriminated by SMI-"Nanoshaping".
Acknowledgements

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