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3-Dimensional super-resolution by spectrally selective imaging

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Abstract

The mutual spatial positions of individual pentacene molecules embedded in a *p*-terphenyl host crystal, residing within the same diffraction-limited volume, are determined with far-field optics with an accuracy far below the Rayleigh distance. This is achieved by spectrally selective imaging, an approach based on the combination of confocal microscopy, single-molecule detection and position-sensitive imaging. It is demonstrated that the molecules can be localized in three dimensions with a precision of 40 nm in the lateral and 100 nm in the axial dimension which represents an enhancement by a factor of 20 and 65, respectively, compared to the Rayleigh distance at the used numerical aperture of the microscope. © 1998 Elsevier Science B.V. All rights reserved.

1. Introduction

Optical microscopy has found applications in many disciplines of science. The spatial resolution of this technique is fundamentally limited by diffraction to dimensions of the wavelength of light, i.e. to several hundreds of nanometers. For a confocal microscope the resolution according to the Rayleigh criterion for the lateral dimension is given by $\delta r = (0.61 \lambda / \text{NA})$, where λ denotes the wavelength and NA the numerical aperture of the objective as defined by $\text{NA} = n \sin \alpha$ with n the refractive index of the propagation medium and α the half-aperture of the imaging optics/objective. The axial resolution is given by $\delta z = 2n\lambda / (\text{NA})^2$. The values δr and δz define a diffraction-limited volume which corre-

sponds to the three-dimensional (3D) resolution of the microscope.

In different experimental approaches, this limitation has been partly circumvented. By axially scanning a sample with respect to the focal volume of a confocal microscope and monitoring the fluorescence intensity of a single molecule, Fleury et al. [1] succeeded in determining the position of an individual molecule in one dimension with an accuracy of 60 nm. Also based on single molecules as fluorescent probes, Schmidt et al. [2] were able to determine the positions of single molecules in two dimensions. In their experiment organic dye molecules were attached to biological systems, and the concentration of the marker molecules was adjusted such that, with highest probability, exactly one or no molecule was present in a diffraction-limited area. Determination of the centre of mass of the photon distribution of the emission of the single molecule allowed to obtain its lateral position with an accuracy of 30 nm and

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thereby to follow two-dimensional diffusion processes on a millisecond time scale. However, information about structural properties from inside the diffraction-limited area was not accessible by this technique because of the requirement that exactly one marker molecule has to be present in the diffraction-limited area. As yet the only optical technique that allows to obtain structural information on a length scale below the Rayleigh distance is near-field optical microscopy providing resolutions down to 20–100 nm by using tapered optical fibers [3,4]. However, this approach is limited also to two dimensions.

Here we show how the 3D spatial position of individual molecules, which are all present within the same diffraction-limited volume, can be obtained by using spectrally selective imaging (SSI). The SSI technique essentially concerns excitation of individual molecules by tuning a narrow-band laser into resonance with the absorption line of a single molecule. Subsequently the 3D photon distribution of the single-molecule emission in the image space is recorded. The spatial position of the molecule is determined from the centroid of the light distribution with an accuracy limited by the signal-to-noise ratio of the recorded photon distribution rather than by the diffraction limit. Tuning the laser into resonance with another molecule within the same diffraction-limited volume, the mutual position of the two molecules can be determined with sub-diffraction-limited accuracy in spite of the fact that both molecules are located closer to each other than the Rayleigh distance. This can be repeated for as many molecules as present in the selected volume. The superresolved 3D image then results from the summation — in computer memory — of the coordinates of the individual molecules. The basic idea of this technique, to use the frequency dimension as an additional parameter for discrimination, has been suggested some years ago [5].

2. Experimental

We used a home-built confocal microscope with an aspheric singlet objective lens with a numerical aperture of 0.55 suitable for operation at liquid-helium temperatures. Operating the microscope at a

wavelength of about 600 nm, $\delta r = 670$ nm and $\delta z = 4$ μm would be theoretically expected. This defines a total diffraction-limited volume of about 2–3 μm^3 . We demonstrate the experimental principle on the model system pentacene in *p*-terphenyl, which allows spectral selection of single molecules [6–8]. Details about the used laser equipment have been published previously [9]. The fluorescence emitted by a selected molecule is collected by the objective lens and separated from the excitation light by appropriate filters. Part of the luminescence is directed towards an avalanche photo diode (SPCM 200, EG&G) which is used to record the fluorescence-excitation spectra of the single molecules. The rest of the signal is directed towards an extremely sensitive image-intensified CCD camera (PentaMax GenIV, Princeton Instruments) which can be displaced axially in the image space to determine the 3D photon distribution of the emission of the molecule (see below).

3. Results and discussion

Fig. 1 shows part of the fluorescence-excitation spectrum of pentacene in *p*-terphenyl, in which the peaks labelled 1–7 correspond to single pentacene molecules. For clarity we discuss the details of the procedures for the lateral and axial position determinations separately. To illustrate this for the lateral

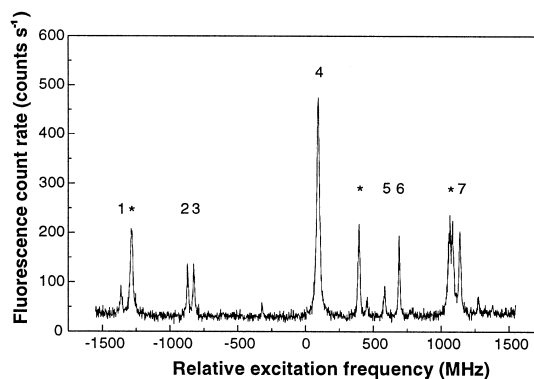


Fig. 1. Part of the fluorescence-excitation spectrum of pentacene in *p*-terphenyl. The laser frequency has been detuned by 20 GHz to the red with respect to the centre of the O₂ ensemble line. The features marked with an asterisk result from two or more molecules and are not considered in the evaluation.

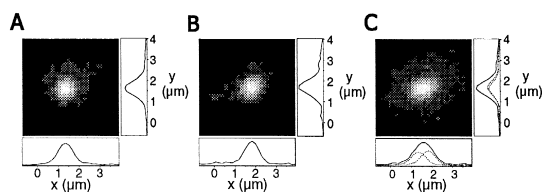


Fig. 2. (a) Diffraction limited image of molecule **3**. Its lateral position is determined as $x_3 = 1.357 \pm 0.040$ and $y_3 = 1.420 \pm 0.040$ μm . (b) Diffraction-limited image of molecule **5**. Here $x_5 = 1.741 \pm 0.040$ and $y_5 = 1.410 \pm 0.040$ μm . Both images have been obtained with an accumulation time of 15 s on the CCD camera. The total image covers 4.7×4.7 μm^2 in object space. (c) Sum of the images shown in part (a) and (b) of the figure.

dimensions we choose molecules **3** and **5** in Fig. 1. First the laser is tuned into resonance with molecule **3**. The resonance is checked continuously by monitoring the fluorescence intensity on the avalanche photo diode. Consequently all light registered by the CCD camera originates exclusively from molecule **3**. In Fig. 2a the corresponding photon distribution on the CCD camera is shown together with two cross-sections along the lateral directions, hereafter called x and y , which show an Airy-function like shape. The characteristic dimensions related to this diffraction pattern are all given by their corresponding values in the object space. The observed photon distribution corresponds to the diffraction-limited image of molecule **3**, for which the actual resolution of the microscope under liquid-helium conditions is found to be $\delta r_{\text{exp}} = 880$ nm. From this pattern the relative x and y positions of molecule **3** have been determined with an accuracy of 40 nm (Fig. 2a) by calculating the centre of mass of the photon distribution. Subsequently the laser is tuned into resonance with molecule **5** and the diffraction pattern used to determine its lateral position is shown in Fig. 2b. For comparison Fig. 2c shows the sum of the patterns of Fig. 2a,b. Because the emission of the molecules overlap, the molecules **3** and **5** obviously could not be resolved in an experiment where spectral selection is not applied.

The axial position is obtained by measuring the radial size of the photon distribution as a function of the detector position along the optical axis. This is illustrated in Fig. 3 for molecules **2** and **3**. Again the horizontal and vertical scales have been converted to object space dimensions. The radial size is defined

by the radius of the area within which 50% of the total intensity of the signal on the CCD camera is contained. To illustrate the changes of the intensity pattern along the optical axis the insets show the photon distribution of molecule **2** when the detector is in front of (left), in (centre), and behind (right) the focal plane of this particular molecule. From the experimental data the axial size of the focus of the microscope is determined to be about $\delta z_{\text{exp}} = 6.5$ μm . The lines in Fig. 3 are fits according to an amplitude profile of a focussed gaussian beam yielding the relative axial positions of the molecules with an accuracy of approximately 100 nm.

The relative positions of the molecules **1–7** are summarized in Table 1 and together with the summed emission graphically depicted in Fig. 4. In a conven-

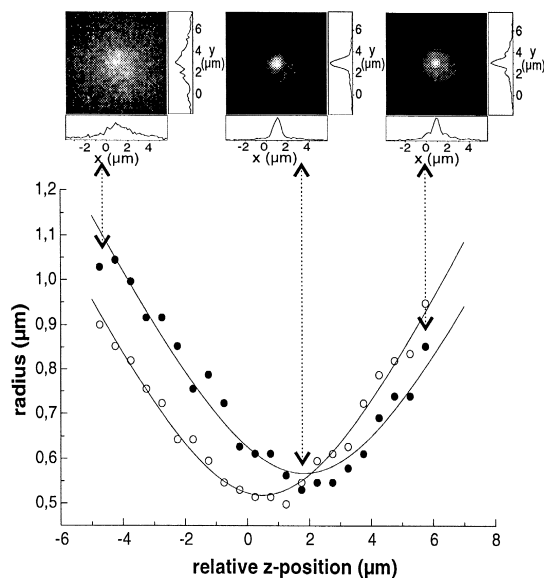


Fig. 3. Dependence of the radial spot size on the axial position of the CCD camera for molecule **2** (filled dots) and molecule **3** (open dots). Horizontal and vertical scales are given in object space dimensions. The spot size $w(z)$ has been obtained by taking the radial average of the photon distribution on the CCD camera with respect to the lateral position of the molecule and determining the radius of the area which contains 50% of the total intensity. The full line corresponds to a fit of a focussed gaussian beam waist according to $w^2(z) = w_0^2 \left(1 + \frac{(z_1 - z)^2 \lambda^2}{\pi^2 w_0^4} \right)$, where w_0 denotes the minimum beam waist and z_i the axial position of molecule 'i'. The axial positions of molecules **2** and **3** are determined to be respectively $z_2 = 1.82 \pm 0.12$ and $z_3 = 0.49 \pm 0.05$ μm . For illustration, three photon distribution patterns are shown for molecule **2** at the indicated focal positions.

Table 1
Relative positions of pentacene molecules 1–7

Molecule	<i>x</i> (μm)	<i>y</i> (μm)	<i>x, y</i> error (μm)	<i>z</i> (μm)	<i>z</i> error (μm)
1	2.320	2.468	0.040	1.71	0.09
2	1.002	2.993	0.040	1.82	0.12
3	1.357	1.420	0.040	0.49	0.05
4	1.304	2.541	0.040	1.51	0.08
5	1.741	1.410	0.040	1.77	0.21
6	1.904	2.371	0.040	1.81	0.09
7	0.803	2.730	0.040	1.29	0.11

tional microscope the spatial resolution is restricted to the size of the distribution of the emission, while this study shows that 3D information well below this limit can be obtained. From a comparison of the various photon distribution patterns we conclude that the lateral accuracy of 40 nm is limited by the mechanical drift of the experimental arrangement during the experiment, rather than by the signal-to-noise ratio of the recorded photon distributions. In the absence of drift the lateral resolution, purely based on the photon statistics, would be 6 nm. Even with the drift the obtained lateral position accuracy is better than 1/20 of the Rayleigh distance. Axially the accuracy is about 100 nm which corresponds to 1/65 of the Rayleigh distance, except for molecule 5 which had a very low fluorescent count rate (< 100 counts/s).

It is noteworthy that the distribution of the molecules shown in Fig. 4 within the excitation volume is not random. From the size of the excitation volume and assuming a random distribution of pentacene molecules in the crystal, one would expect a spread in the lateral positions of about 1 μm and in the axial positions of about 7 μm . While this is indeed observed laterally, in the axial direction all molecules lie within a plane with a thickness of about 1.3 μm . A similar observation was made for two other sets of molecules, within the same sample, that comprise of 4 and 6 molecules, respectively (not shown). The thickness of the sample, about 10 μm , does not explain this observation. It is known that pentacene molecules in *p*-terphenyl may show sudden jumps in their absorption frequency (spectral diffusion) caused by changes of the local environment [10,11]. It is assumed that this affects mainly

pentacene molecules in proximity to domain walls of the host crystal [12,13]. In our experiment we tried to probe highly ordered crystal regions to avoid this effect. To do so we are able to move the sample laterally with respect to the excitation volume on a micrometer length scale [9]. It turned out that molecules stable in frequency could be found only in a very limited region of the crystal. Because we are biased in our experiment to molecules which do not show spectral diffusion it might be that these molecules are present only in a certain crystal region of the studied sample. This structural property of the host crystal on a microscopic scale, undetectable by other optical techniques, is intriguing and deserves further attention.

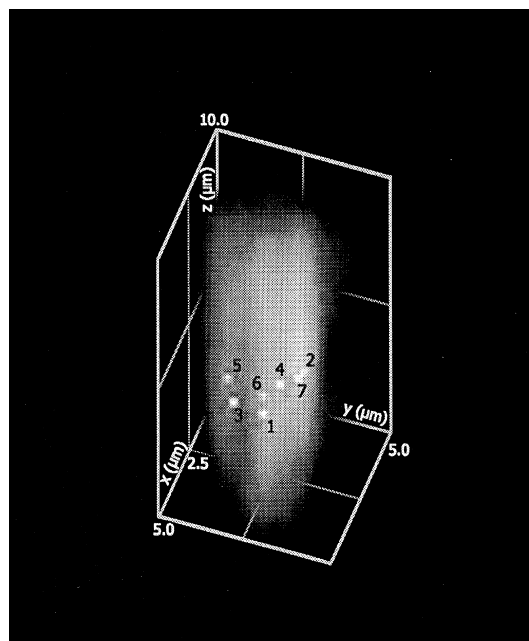


Fig. 4. Relative positions of molecules 1–7 (bright dots) together with the experimentally obtained total 3D photon distribution summed in computer memory and projected into object space. Each molecule contributes one ‘cigar-shaped’ photon distribution whose intensity distribution is given by the greyscale. The size of each single distribution corresponds to the diffraction-limited resolution of the microscope whereas the intensity is determined by the orientation of the transition-dipole moment of the molecule, the saturation characteristics of the optical transition, and the laser intensity at the location of the molecule. In a conventional experiment where all seven molecules are excited simultaneously the total cloud is observed without information on the mutual position of the contributing molecules.

This study demonstrates that the diffraction-limited resolution in far-field optical microscopy can be circumvented by combining single-molecule spectroscopy and position-sensitive imaging. An enhancement by more than one order of magnitude is evident. For pentacene in *p*-terphenyl we verified that a lateral resolution of 50 nm, purely based on photon statistics, requires an illumination time of 300 ms. This makes the method suitable also for molecules that undergo photobleaching or spectral diffusion after a few seconds. The ability to individually address single molecules within the same diffraction-limited volume allows to utilise them as fluorescent markers to determine structural properties on a length scale usually unconceivable with far field optics.

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