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RESEARCH PAPER

Single Molecules

Results and Thoughts on Optical Microscopy Using a Single-molecule Probe

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Abstract

We discuss our recent results as well as planned experiments for obtaining molecular resolution in optical microscopy. By combining single molecule spectroscopy and scanning probe microscopy we have demonstrated optical microscopy with a single-molecule light source. We also sketch a method for the use of an inhomogeneous static electric field for determining the position of a molecule in all three dimensions.

Introduction

The advent of Scanning Near-Field Optical Microscopy (SNOM) has given a large push to ultra high resolution optical microscopy in the past decade. The imaging contrast in the conventional mode of SNOM stems from the scattering of the nonpropagating near field of a subwavelength aperture as it is scanned across the nanostructures of a sample. The spatial resolution that can be achieved depends on the size of the aperture and its separation from the sample since these two parameters determine the degree of coupling between the sample and the high spatial frequency component evanescent waves. Unfortunately, due to the finite skin depth of metals it is impossible to reduce the size of the aperture to an arbitrarily small value [1]. An alternative avenue using a single molecule as a point-like source could, however, fulfill all the requirements for reaching the ultimate limit of SNOM, namely the molecular resolution in optical microscopy [2,4]. The first part of this article will review very briefly our recent demonstration of a microscope based on a single-molecule probe and will discuss the necessary steps for achieving molecular resolution. In the second part we will expand this arrangement and suggest a method in which the combination of high resolution spectroscopy and scanning probe microscopy could be used to resolve single molecules spaced by distances in the order of 1nm even using far-field methods.

Materials and Methods

In our experiment single terrylene molecules embedded in para-terphenyl crystals are optically detected by

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fluorescence excitation spectroscopy [5] performed at T = 1.4K. Light from a narrow-band tunable ring dye laser at a wavelength around 580nm is used for excitation. When the frequency of the laser matches that of the zero-phonon-line of a terrylene molecule its singlet excited state is populated, and subsequently it emits red-shifted photons upon decay to the lower singlet state. By using high quality spectral filters one blocks the residual excitation light as well as the resonant emission of the molecule, giving access to an essentially background-free signal. The linewidth of terrylene transition reaches about 50MHz for T < 2K, corresponding to its natural linewidth. Such a narrow resonance, together with the nanometer size of a single molecule, make it a very sensitive spectral and spatial probe.

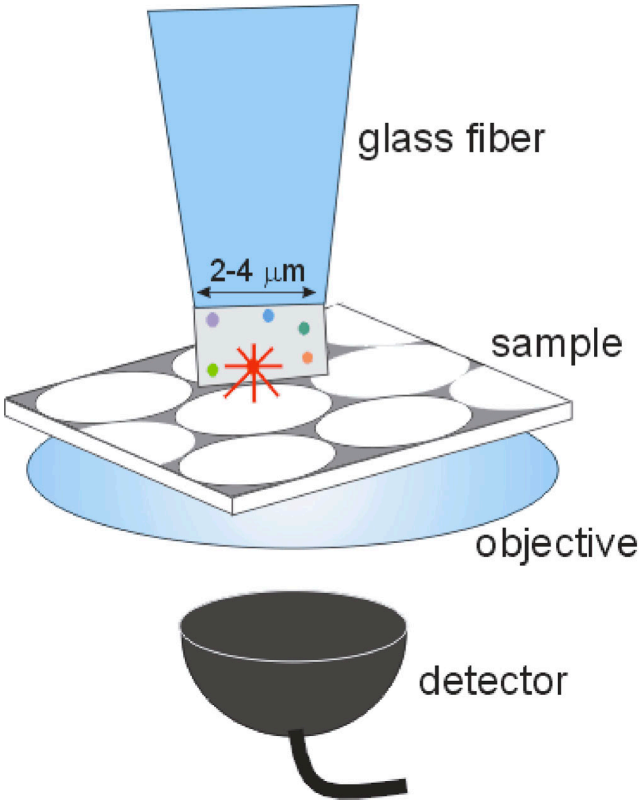

Fig. 1. *The arrangement for performing optical microscopy using a single-molecule probe. A single molecule is detected via fluorescence excitation spectroscopy in a microcrystal at the end of a fiber tip. The fluorescence of the molecule is detected in transmission through a sample and is plotted as the sample is raster scanned. The sample consists of triangular aluminum islands arranged in a hexagonal lattice on glass.*

The schematics of the first experiment we will discuss here is shown in Fig. 1. A micron- sized doped crystal is carefully glued to the end of an optical fiber tip. It is then mounted in the cryogenic SNOM that we have constructed for operation at T = 1.4K so that it can be placed in any desired location in the immediate vicinity of a sample. Using fluorescence excitation spectroscopy we select one of the molecules and detect its fluorescence in transmission through a test sample. By raster scanning the sample and recording the fluorescence intensity at each pixel one obtains an image like in Fig. 2. Contrary to a naive expectation, the metallic islands do not show up as triangles in the image. In order to understand this, one has to remember that a single molecule emits with dipolar characteristics, and therefore its orientation plays an important role in the details of the contrast mechanism. Simulations show that the combination of the sample geometry and the dipolar axis can lead to a wide variety of field intensity distribution on the sample when the boundary conditions for the electromagnetic field are considered [6]. Indeed, this effect has also been observed in conventional aperture SNOM experiments using polarized illumination [7,8].

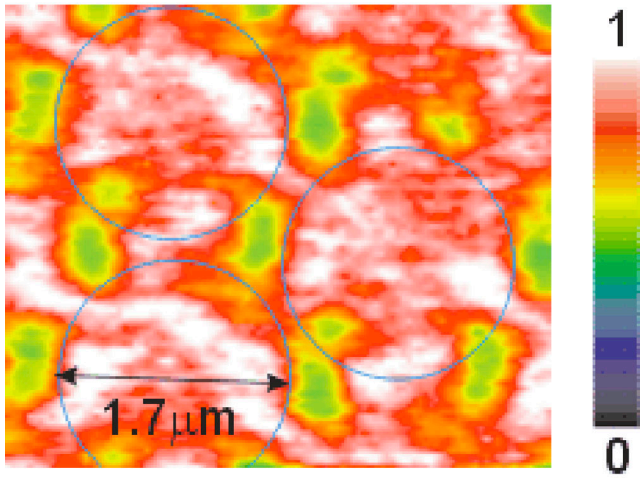

Fig. 2. *An example of an optical image using a single molecule as a light source. The circles show the position of boundaries of the triangular metal islands (see also Fig. 1.) and the color bar on the right shows the scale for the detected intensity.*

Results

The complexity of image formation and image interpretation makes it difficult to assess a value for the obtained spatial resolution in a straightforward manner. Cross-sections of the images hint to a resolution of the order of 150 - 200 nm which is far from what one expects from a single- molecule

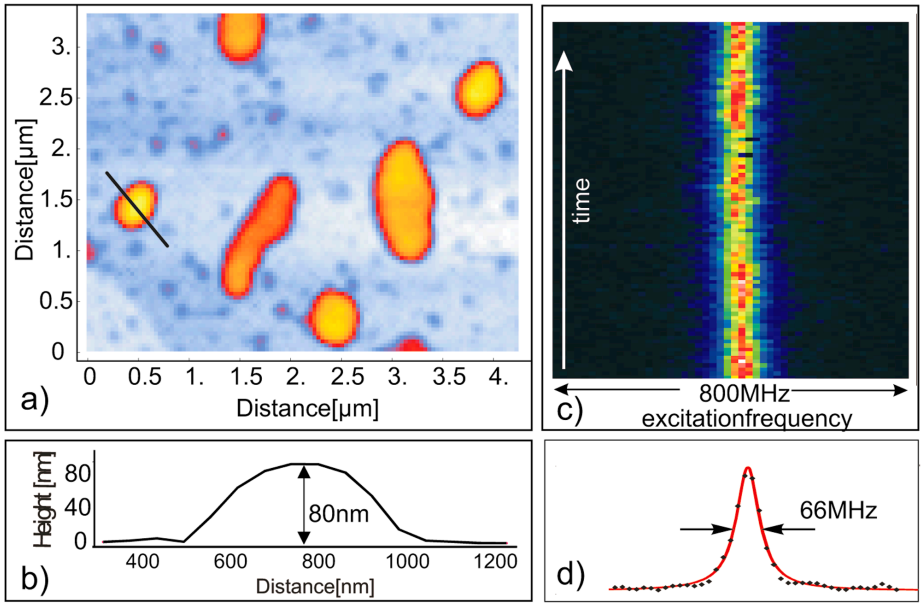

Fig. 3. a) *A shear-force topography image of p-terphenyl nanocrystals doped with terrylene.* b) *A cross-section of the crystal marked in a).* c) *Spectral stability check for a single molecule in a nanocrystal over several minutes of constant illumination.* d) *An excitation fluorescence spectrum of a molecule in a nanocrystal showing a nearly lifetime limited linewidth.*

probe [9]. The explanation is, however, fairly simple. Having used microcrystals of the order to 2-4 µm, it is not surprising that the molecules we have used for imaging were not exactly at the tip edge. One possibility to get access to the molecules very close to the sample is to replace a microcrystal by a nanocrystal so that all molecules that appear in the fluorescence excitation spectrum of the sample are very close to the tip end and can therefore reach the immediate vicinity of the sample. Another possibility is to deposit terrylene molecules on the outer facet of the microcrystal at the tip.

After these first experiments we began to fabricate and examine nanocrystals. Figure 3a) shows a topography image of a cover glass onto which we have grown p-terphenyl nanocrystals doped with terrylene molecules. A cross-section of one of the nanocrystals in Fig. 3b) reveals the lateral and vertical extents of these crystals. As displayed in Figures 3c) and 3d), the spectrum of a single molecule in such a nanocrystal can be as stable and as narrow as that in larger crystals. We note that the fact that despite the vicinity of many interfaces the molecular linewidth remains narrow seems to be in contradiction with previous reports in other systems where a systematic broadening was observed for molecules very close to an interface [10]. A careful study of the influence of interfaces and the nanoscopic matrix surrounding the molecule on its spectrum remains a topic of future work. Having obtained access to stable single molecules in nanocrystals, we can now place them at the end of fibers or as a second alternative we could leave the nanocrystals on a substrate and approach the sample using the machinery of our SNOM.

Before going on to the second part of this paper, let us comment on the nature of the imaging contrast that one would expect from an optical near-field microscope with molecular resolution. Molecular resolution means identification of individual molecules on a sample even if they essentially touch each other. As we pointed out earlier, in order to achieve molecular resolution it is necessary that the probe molecule is brought into molecular contact with the sample. But we also know that under such conditions the spectrum of the probe molecule is strongly modified due to its optical interaction with the sample surface and sample molecules [11]. It will be therefore not sufficient to only record the emission intensity of the molecule, but one should monitor its whole spectrum. Interestingly enough it turns out that the modulation of the molecular linewidth and resonance frequency can both reflect changes in the very local optical contrast of the sample quite efficiently [12]. We also point out that a detailed theoretical treatment of the emission of a molecule that is almost in contact with a surface is very demanding because one can no longer treat the sample using local dielectric functions [13]. To summarize, the resolution and contrast that could be obtained will depend on the details of the interaction between the probe molecule and sample molecules.

We now consider a completely different approach in obtaining optical information from a sample at the molecular level. To set the grounds for what follows, let us refer to some of the recent reports in the literature pointing to the possibility of localizing a single molecule to a great precision using far-field optics [14-16]. The main idea is that although the image of a point-like object such as a single molecule is typically a difrraction-limited large spot on the detector, it is possible to find the center of this spot with subwavelength accuracy if the signal-to-noise ratio is high enough. Using wide field microscopy as well as confocal microscopy this method has reached an accuracy of about ± 30 nm at room temperature, limited by the photostability of available dye molecules. This means that one could "resolve" two molecules separated by a similar distance if

one could only distinguish their image spots. This can be achieved if the two have different absorption/emission spectra, different polarizations or different fluorescence lifetimes. As has been demonstrated by the group in Leiden, this can be achieved very easily when performing fluorescence excitation spectroscopy because each molecule absorbs the excitation light at a different frequency [17]. In addition, the good photostability of the guest molecules under cryogenic conditions increases the localization accuracy because one can collect photons for a longer time and improve the signal-to-noise ratio.

One important advantage of this approach compared to near-field microscopy is that one has access to high resolution information even in the depth of the sample. The axial resolution in far-field microscopy is inherently lower than the lateral resolution due to the slower variation of the point-spread function. To overcome this one can introduce a faster varying intensity function in the axial direction as for example in the case of a standing wave, demonstrated by F. Lanni and coworkers [18]. In our laboratory we have also used this technique in our preliminary measurements and have shown localization of a single molecule with an axial accuracy of ± 24nm, limited by the visibility of the standing wave [19].

Another approach to improving the spatial sensitivity in fluorescence imaging is shown in Fig. 4. Here an external inhomogeneous electric field is applied to the sample including guest molecules. As a molecule is scanned in this field its transition frequency is Stark shifted according to the strength of the local electric field. If the gradient of the electric field is large enough, a spatial map of the Stark shift gives direct information about the three-dimensional location of the molecules under investigation. We note that although the experimental arrangement has a flavor of a near-field experiment, the interaction at hand is of a purely far-field nature and in fact the local electrode is not required to be closer to the sample than a few hundred nanometers. As opposed to sharp tips a microscopic spherical electrode has the great advantage that its fabrication is easy to handle and quite reproducible. Finally, the strength of this method is its sensitivity to axial location of the molecule and at the same time its capability to investigate the depth of a sample. This is not the case when field enhancement is used in the near-field of a metallic tip [20].

Discussion

The methods and the results discussed here point to a revival of optical microscopy as a candidate for ultra high resolution microscopy. While electron microscopy and X-ray diffraction have contributed a tremendous deal to the progress of structural biology, many applications prefer optical investigations. Among the advantages of optical microscopy are the use of low energies, lack of damage to the sample, and therefore the possibility of performing many repeated measurements. Also, when combined with spectroscopic or polarization information fluorescence optical microscopy can provide very rich information about the sample under study, including selectivity to chemical bindings as well as conformational changes. It is important to bear in mind though that at the level of the molecular resolution no microscope serves as an all-purpose device, and the choice of the method has to be made in accord with the specifics of the problem at hand.

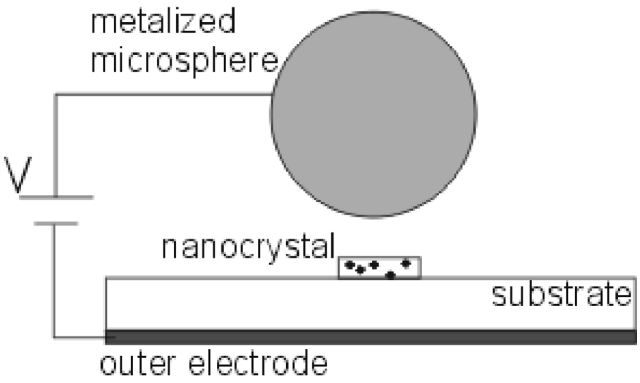

Fig. 4. *The arrangement for three-dimensional localization of a single molecule. A local inhomongeneous static electric field is used to find the center of the molecule by measuring its position-dependent Stark shift.*

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