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Scanning near-field optical microscopy using semiconductor nanocrystals as a local fluorescence and fluorescence resonance energy transfer source

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Summary

Local fluorescence probes based on CdSe semiconductor nanocrystals were prepared and tested by recording scanning near-field optical microscopy (SNOM) images of calibration samples and fluorescence resonance energy transfer SNOM (FRET SNOM) images of acceptor dye molecules inhomogeneously deposited onto a glass substrate. Thousands of nanocrystals contribute to the signal when this probe is used as a local fluorescence source while only tens of those (the most apical) are involved in imaging for the FRET SNOM operation mode. The dip-coating method used to make the probe enables diminishing the number of active fluorescent nanocrystals easily. Prospects to realize FRET SNOM based on a single fluorescence centre using such an approach are briefly described.

Introduction

Semiconductor nanocrystals, CdSe in particular, are considered as very useful chromophores for all kinds of fluorescence microscopy owing to their excellent photophysical properties, in addition to the possibility of tuning their fluorescence spectra by simply changing the size of the nanocrystals through varying their synthesis conditions (Yoffe, 2001). For these reasons they have been extensively studied using different optical microscopy methods. The overwhelming majority of such studies were devoted to studying CdSe and other nanocrystals as samples, including some in which the nanocrystals were used as labels of biological samples. Here, we report the results of experiments where CdSe nanocrystals are used as an instrument (light source); namely, as a local fluorescent probe for SNOM. In this scheme a subwavelength-sized active medium replaces the aperture and the near-field image is formed by recording changes in the collected fluorescence of this medium. The spectral shift between the excitation light and the fluorescence of the local probe allows for a drastically enhanced signal-to-noise ratio through suppressing the background, in a number of SNOM operation modes (notably, apertureless SNOM). Another advantage is the easier interpretation of SNOM data because of the well-defined and controllable shape of the excitation source (see Sandoghdar & Mlynek, 1999, for example, for a recent discussion of microscopy based on a local fluorescence probe).

Another area in which the use of semiconductor nanocrystals could be especially important is the fluorescence resonance energy-transfer scanning near-field optical microscopy (FRET SNOM) proposed a few years ago (Sekatskii & Letokhov, 1996). Here, a donor molecule or other fluorescent centre located in the tip apex is used to excite the fluorescence of an acceptor centre of the sample (or vice versa). The spatial resolution of FRET SNOM is no longer limited by the size of the aperture of the microscope but rather by the value of the characteristic Förster radius, R_0, which for typical FRET pairs ranges from 2 to 8 nm (Wu & Brandt, 1994; Clegg, 1996). The first experimental FRET SNOM images (Vickery & Dunn, 1999, 2001; Shubeita *et al.*, 2002) have been recently obtained using different FRET pairs of laser dyes for the donor and the acceptor. Despite the fact that plenty of molecules contributed to the optical signal, subaperture-sized spatial resolution (Shubeita *et al.*, 2002) and some other interesting peculiarities of the method were demonstrated. Nevertheless, it is obvious that the full advantages of the FRET SNOM can be achieved only when a single fluorescence centre is used as a light source (as an 'electron excitation' source). The aforementioned experiments showed that dye molecules are not photostable enough to attain this goal. Essentially, better photostability of semiconductor nanocrystals makes them very promising for

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such a study. This circumstance was the main motivation of our work, and the first FRET SNOM images obtained using CdSe nanocrystals as donors (not yet at the single molecule level) are reported in this paper.

Experimental

Local fluorescence probes

Nearly monodisperse ZnS-coated CdSe nanocrystals were synthesized in Mainz University following the procedure of Murray *et al.* (1993) and Hines & Guyot-Sionnest (1996), with some modifications. The overlayer of ZnS essentially increases both the fluorescence quantum yield and the stability of CdSe nanocrystals. At the same time, the thickness of this overlayer is smaller than the characteristic Förster radius and therefore does not affect the FRET process significantly.

Initial solutions of CdSe nanocrystals in toluene had a concentration of 10^{-5}–10^{-4} mol L^{-1}. These solutions were used to prepare the semiconductor nanocrystal-based local fluorescence probes exploiting the dip-coating method (withdrawal deposition) (Yang *et al.*, 1980; Garoff *et al.*, 1982) in a manner similar to that used by us earlier to prepare local fluorescence probes based on dye molecules (Shubeita *et al.*, 2002). To that end, we first mixed initial CdSe solutions with a solution of polystyrene in toluene to have CdSe nanocrystal solutions containing 1–2 wt% polystyrene. Subsequently, commercially available metal-coated SNOM fibre probes having a nominal aperture size for light transmission of 100–200 nm (Nanonics Supertips, Israel) were briefly dipped and then withdrawn from these solutions. As known from the controllable dip-coating method (Yang *et al.*, 1980) a thin (30–100 nm thick) layer of the polymer stained with CdSe nanocrystals is formed on the tip surface after the solvent dries off. This was also confirmed by us through analysing scanning electron microscope images of the probes and is in accordance with our SNOM data obtained using those probes (Shubeita *et al.*, 2002). The concentration, c, of the nanocrystals in the polymer layer depends on their initial concentration in solution and on the content of dissolved polystyrene; c ranged from 1 to 2×10^{18} cm^{-3} in our experiments. An apertured SNOM fibre tip modified in such a manner serves as a local fluorescence probe: the active area has subwavelength dimensions (100–200 nm lateral and 30–100 nm axial dimensions) and contains typically from 1000 to 10 000 fluorescent semiconductor nanocrystals.

A schematic diagram of the SNOM used in the experiments at hand is shown in Fig. 1 (see Sekatskii *et al.*, 2000; Shubeita *et al.*, 2002, for further description of this home-made SNOM built atop an inverted fluorescence microscope Carl Zeiss 100M). The tuning-fork-based shear force distance regulation scheme was implemented. SNOM was operated in the illumination mode: the 458-nm spectral line of a cw argon ion laser was passed through a special narrow-band interferometric filter before coupling to the fibre to excite the fluorescence of

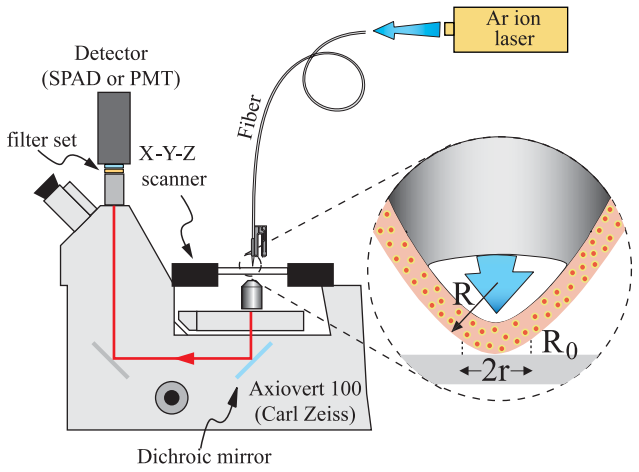

Fig. 1. Schematic diagram of the SNOM. The local fluorescence probe formed from a thin polymer layer stained with CdSe nanocrystals deposited onto the tip of an apertured fibre probe is shown in the insert while in contact with the sample surface.

CdSe nanocrystals on the tip. A channeltron-type photomultiplier tube working in the photon counting mode (Perkin Elmer) was used to detect light.

CdSe nanocrystals having different optical absorption maxima (eight samples with maxima ranging between 530 and 650 nm) were investigated. The most interesting results were obtained for two samples with absorption maxima at 584 and 586 nm, and these results are reported here. The filter set used to suppress the excitation light consisted of a notch filter (Kaiser, U.S.A.) and a number of glass low-pass optical filters with absorption threshold of 490, 520 and 530 nm. In addition, a dichroic mirror in the optical path of the inverted fluorescence microscope was also used. Those filters were removed when optical images formed by the excitation light rather than the local fluorescence source were recorded.

To illustrate the performance of this local fluorescence probe-based near-field microscope, SNOM images of two types of samples are presented in Fig. 2: closely packed polystyrene beads, 500 nm in diameter, deposited onto a glass slide (Fig. 2a) and the so-called Fischer shadow samples (Fig. 2b). Fischer shadow samples were fabricated by evaporating gold on a closely packed arrangement of 500-nm polystyrene spheres which were then washed out leaving holes in the 20-nm gold film (Fischer & Zingsheim, 1981). For comparison, shear force topographical images are presented in these figures as well.

From Fig. 2(a) one can see that the near-field optical images of polystyrene beads have a ring-like shape. This shape was not observed in images of the same sample obtained when the excitation light was recorded and is a characteristic feature of local fluorescence probe-based images. This ring-like shape is a result of the dependence of the fluorescence yield of a single molecule (semiconductor nanocrystal) on the local refraction index of the medium (see, for example, Novotny, 1996; Klimov

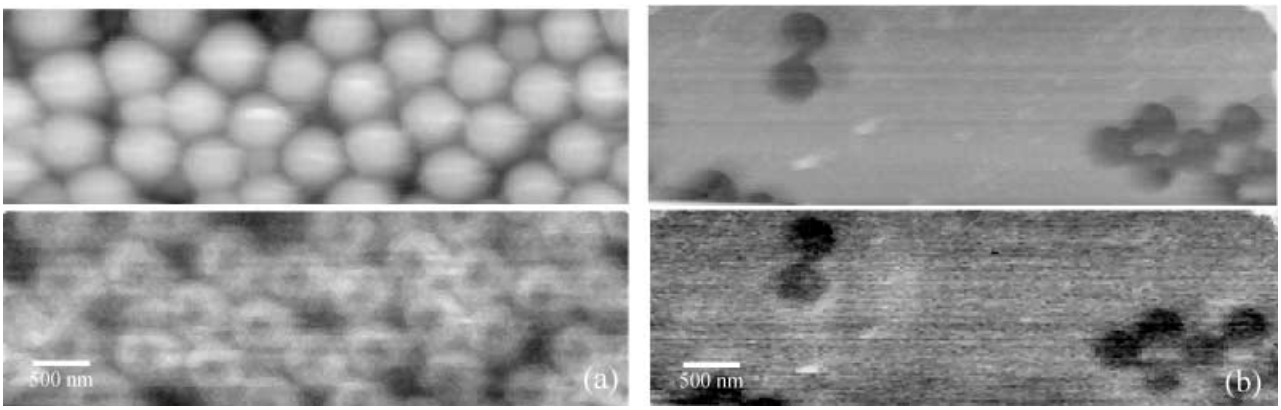

Fig. 2. Near-field optical (bottom) and shear force topographical (top) images obtained using a local fluorescence probe based on CdSe nanocrystals. The samples are: (a) closely packed polystyrene beads, 500 nm in diameter; (b) Fischer shadow sample forming 500-nm holes in a 20-nm-thick gold film.

et al., 2001; Rahmani *et al.*, 2001). Analysis shows that for different orientations of a fluorescent molecule located close to a dielectric object, the normalized lifetime of the molecule is larger when it is placed closer to the centre of the object than when it is placed near its edge (figure 4 from Novotny, 1996, and others). Clearly, transparent dielectric beads will be imaged as ring-like structures as a result of that. This situation is somewhat complementary to the process of excitation of a single fluorescent centre by SNOM, where ring-like images were earlier observed in a number of experiments for molecules (starting from the famous paper of Betzig & Chichester, 1993) and quantum dots (Geller *et al.*, 2001).

'Inverted' optical contrast is seen in Fig. 2(b): the image of the intact gold film is brighter than that of the holes in the film while the brightest areas are the edges of the metal film. This is a demonstration of the local electromagnetic field enhancement by the gold nanostructures ('lightning rod' effect and surface plasmon resonance) due to the surface roughness.

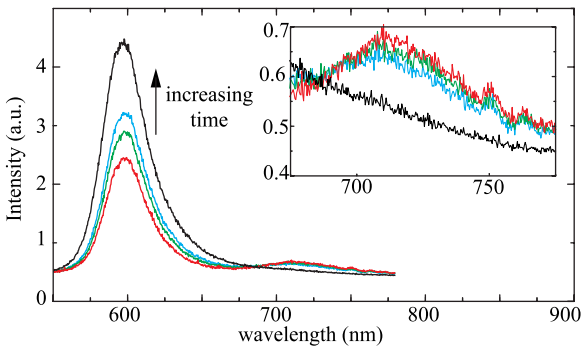

Fig. 3. Dynamics of the fluorescence of co-deposited layers of CdSe nanocrystals and the dye OM57. The nanocrystals were deposited by spin-coating and had a nominal surface concentration of 10^{13} cm^{-2} estimated as for dye molecules spin coated under the same conditions. OM57 was deposited by drying a droplet of the dye solution on the surface (nominal surface concentration of 3×10^{13} cm^{-2}). The integration time to obtain one spectrum is 0.2 s and the time interval between two subsequent spectra is about 30 s. The insert is an enlargement of the acceptor fluorescence.

FRET SNOM using CdSe nanocrystals

Prior to attempting the observation of FRET SNOM images using CdSe nanocrystals, we performed a number of experiments to study the fluorescence of (sub)monolayers of different CdSe nanocrystals and dye molecules co-deposited onto a glass slide. The experimental method was similar to that used by us earlier with dye pairs co-deposited on a glass slide (Shubeita *et al.*, 1999) and the aim of these experiments was to find the most appropriate FRET pair of CdSe nanocrystals and dye molecules. Both the spin-coating technique and simple dropping of 10–20 μL of dye solution onto the glass slide with the subsequent drying of the solvent were used to prepare the samples. The glass slide was irradiated with the 458-nm argon ion laser line focused by a 40× micro-objective to an estimated intensity of about 200 W cm^{-2}; the same objective was used to collect fluorescence. A dichroic mirror and an appropriate filter set were used to suppress the excitation light and fluorescence spectra were measured using a Jobin–Yvone spectrometer equipped with a CCD camera.

The most convincing results demonstrating FRET between CdSe nanocrystals and dye molecules were obtained using nanocrystals with the absorption maximum at 584 nm and the laser dye OM57 (Al'pha Akonis, Moscow; see e.g. Shubeita *et al.*, 1999, for the chemical formula of this dye). Those results are presented in Fig. 3. From this succession of spectra it is clear that the fluorescence of nanocrystals increases as a function of irradiation time while the fluorescence of dye molecules decreases as a function of that time. This is one of the well-known FRET fingerprints: the donor fluorescence increases owing to the photobleaching of acceptor molecules which makes the FRET process progressively less effective. Based on the data given in Fig. 3 we can give a rough estimate

of the efficiency, E, of the FRET process: $E = (I_D - I_{DA})I_D$ (cf. for example, Ha *et al.*, 1996). Here I_D is the donor fluorescence intensity after acceptor photobleaching and I_{DA} is the donor fluorescence intensity in the presence of the acceptor. Such an estimation gives $E \approx 0.7$ which is in reasonable agreement with the fact that the mean donor–acceptor distance (~2 nm for the surface concentrations used) is less than the calculated value of Förster radius (~4 nm). Figure 3 clearly demonstrates, as well, the essentially larger fluorescence yield of CdSe nanocrystals in comparison with that of the OM57 dye molecules.

This 'most efficient' FRET pair of semiconductor nanocrystals and dye molecules, and the optical filter set selected as the most appropriate to distinguish between donor's and acceptor's fluorescence, were used to record FRET SNOM images. At this stage, we managed to obtain reproducible images recorded using tens of FRET active pairs, i.e. images similar to the dye–dye FRET SNOM images recently reported by us (Shubeita *et al.*, 2002). The discussion of the results and the experimental technique are quite similar for both cases, so here we only give a brief description of the work; see the aforementioned paper for further details. In particular, there, as well as in the earlier work (Shubeita *et al.*, 1999), we demonstrated both the extremely inefficient direct excitation of acceptor (OM57) molecules by the laser light and the 'contact' character of FRET leading to the acceptor fluorescence.

A FRET SNOM image of clusters of the acceptor molecule OM57 is presented in Fig. 4. Those inhomogeneously distributed dye islands were formed by depositing a droplet of a solution of the dye on a glass slide and letting it dry in air. Analysing the fine details of the near-field optical images of the dye molecule clusters (principally excellent reproducibility of images recorded for the left-to-right and right-to-left movements of the tip along the surface) we can give an estimation of the spatial resolution as ~100 nm. This subaperture-size resolution is explained as follows. Owing to the 'pseudocontact' character of the FRET interaction, only the molecules located on the sample surface within a circle of diameter of $2r = 2\sqrt{(2RR_0)}$ from the point of contact will be visualized. Here, R is the radius of curvature of the tip. As schematized in Fig. 1, this formula follows from elementary geometrical consideration for a sphere of radius R (SNOM tip) touching a plane and neglecting its deformation upon contact. Hence, the spatial resolution is governed by the value of $2r$, which is essentially smaller than the aperture, R. For our case of $R_0 = 4$ nm and $R = 250$ nm we have $2r \approx 90$ nm.

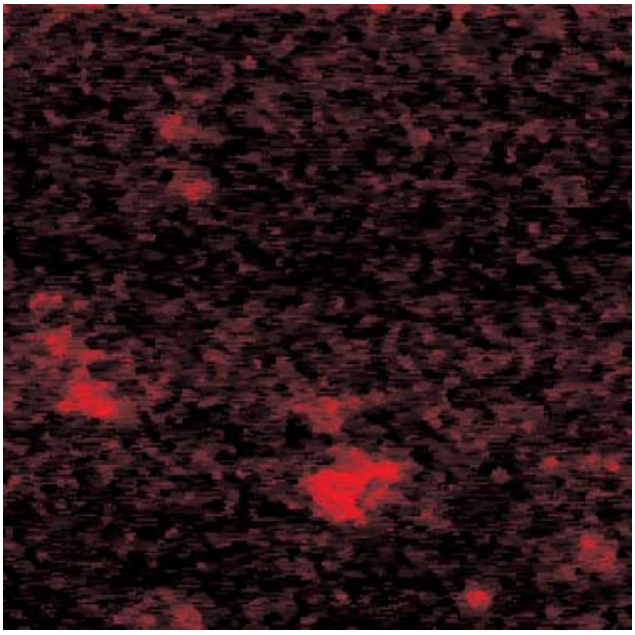

Fig. 4. FRET SNOM image (size: 11×11 μm) of OM57 dye clusters on a glass substrate (nominal surface concentration of 3×10^{14} cm^{-2}) obtained using the local fluorescence probe based on CdSe nanocrystals.

The number, N, of FRET pairs which contribute to the fluorescence signal is defined by the number of nanocrystals contained in a spherical segment (the apical part of the tip) having a radius r and height R_0 (see Fig. 1): $N = 1/2\pi r^2 R_0 c = \pi R_0^2 R c$, which gives $N \sim 10$–15. This estimation is in agreement with the magnitude of the recorded signal, $S \sim 200$–1500 counts s^{-1}. Given the overall detection efficiency η of our microscope of about 3×10^{-4} (Shubeita *et al.*, 2002), CdSe nanocrystal absorption cross-section $\sigma \approx 10^{-16}$ cm^2, photon energy $h\nu = 4.1 \times 10^{-19}$ J and light intensity $I \approx 10^3$ W cm^{-2} one expects $S = (\eta I \sigma)/(h\nu)N \cong 1000$ s^{-1}. The acceptor's excitation probability decreases as the sixth power of the distance between the donor and the acceptor when it exceeds the value of R_0, which means that FRET from all nanocrystals located out of the segment considered can contribute with no more than 20% to the acceptor's fluorescence signal. The process of excitation of an acceptor molecule by donor fluorescence rather than FRET can also be neglected for our probe. For our concentration of donors, c, and the thickness of the donor-containing polymer layer, l, its efficiency can attain the value of only $\sim\sigma cl \approx 2 \times 10^{-3}$ in comparison to the efficiency of the FRET excitation of an acceptor molecule by a single nanocrystal located within a Förster distance from it.

Conclusions

Our experimental data demonstrate the usefulness of the local fluorescence probe based on CdSe semiconductor nanocrystals. At this stage, we have a light source of a few thousands of fluorescence centres when used as an active probe for near-field fluorescence microscopy and a few tens of fluorescence centres when used as a FRET SNOM active probe. The very simple and easy-to-use dip-coating preparation method enables us easily to decerease the concentration of nanocrystals in the probe down to the level at which only one fluorescence centre will contribute to the FRET image.

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