Investigation of nanolocal fluorescence resonance energy transfer for scanning probe microscopy

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Fluorescence resonance energy transfer (FRET) has been observed between donor dye molecules deposited onto the sample surface and acceptor dye molecules deposited onto the scanning near-field optical microscope (SNOM) or atomic force microscope tip. FRET was observed only when the tip acquired a contact with the sample and took place in a region of few tens of square nanometers in size when thousands (hundreds) of molecules are involved. In view of the obtained results, the perspectives for the construction of a one-atom FRET SNOM are described. © *1999 American Institute of Physics.* [S0003-6951(99)03823-1]

Scanning near-field optical microscopy (SNOM) is now a valuable research tool to image and investigate different samples with a subwavelength spatial resolution. Usually, the spatial resolution of SNOM is limited by the size of an aperture for the light transmission and ranges from 50 to 100 nm, although 20 nm resolution has been demonstrated.[1,2] Further improvement of the resolution seems problematic for the ''classical'' SNOM configurations because the number of photons ''seeping'' through an aperture is rapidly decreasing with the decrease of the aperture size. A number of new approaches to improve the resolution, such as molecular exciton-based SNOM,[3] apertureless SNOM,[4] and SNOM using fluorescence resonance energy transfer (FRET) between a single fluorescence center of the tip and the sample studied[5,6] have been proposed recently.

In the latter case, the idea consists of using the fact that when the distance between donor and acceptor molecules becomes smaller than the characteristic radius of a resonant energy transfer (also named the Förster radius) R_0, the probability of dipole–dipole energy transfer between these molecules is close to unity (see, for instance, Refs. 7 and 8 for a review). One should excite donor fluorescent centers of the tip and monitor fluorescence of the acceptor centers of the sample (or vice versa). A large Stokes shift between absorption and emission spectra in the condensed phase, as well as other optical properties of the system, enable us to eliminate direct excitation of the fluorescence of the studied molecules.[5] The spatial resolution for such an approach is governed not by the size of the aperture of the microscope, but by the value of R_0 which, for typical donor–acceptor pairs, is of the order of 1–5 nm.[7] A detailed analysis shows that not only the spatial resolution, but the sensitivity as well can be improved using a FRET SNOM.[5]

In this letter we present experimental evidence of the applicability of FRET phenomena for near-field optical microscopy. A nanolocal resonant energy-transfer process has been observed between two different dyes. One of them (donor) has been deposited onto the glass sample surface and the other (acceptor) has been deposited onto the surface of a SNOM tip (sharpened optical fiber) or standard atomic force microscope (AFM) silicon nanotip. The FRET process has been observed, *only when the tip acquired contact with the sample*, i.e., in the regions a few tens of square nanometers in size and involved only thousands (probably even hundreds) of dye molecules.

A careful selection of donor and acceptor dye molecule pairs was necessary for the experiments described. DCM dye molecules[9] have been selected as donors because of their excellent fluorescent properties: the fluorescence quantum yield in solutions is close to unity, absorption cross-section value σ at the 488 nm wavelength (which has been selected as an excitation wavelength) is $6\times10^{-17}\,\mathrm{cm}^2$, and high photostability. Different dyes have been tested as an acceptor. The best results have been obtained when using 1-butyl-3,3-dimethyl-2-[5-(1-butyl-3,3-dymethyl-3H-bez[e]indolin-2-yliden)-1,3-pentadienyl]-3H-bez[e]indolium perchlorate molecules (OM57 dye, Al'pha Akonis Company, Moscow): their absorption spectrum corresponds well to the fluorescence spectrum of DCM while their absorption at the 488 nm wavelength is at least three orders of magnitude smaller than at the maximum; these molecules also possess a reasonable fluorescence quantum yield (no smaller than 0.3) and photostability.[10] Calculations show that the characteristic radius R_0 of the dipole–dipole energy transfer for this pair ranges between 3 and 4 nm.

In Fig. 1 we present the spectrum of fluorescence of the two dyes, DCM and OM57, codeposited onto the same glass slide with the submonolayer[11] surface concentrations of $3\times10^{13}\,\mathrm{cm}^{-2}$. From Fig. 1 it is clear that under such conditions the fluorescence of OM57 molecules (spectral range 650–800 nm) is even more prominent than that of DCM (spectral range 550–700 nm) keeping in mind the fact that OM57 molecules do not absorb the excitation wavelength (the fluorescence spectrum of OM57 molecules deposited in the same concentration but without DCM molecules on a

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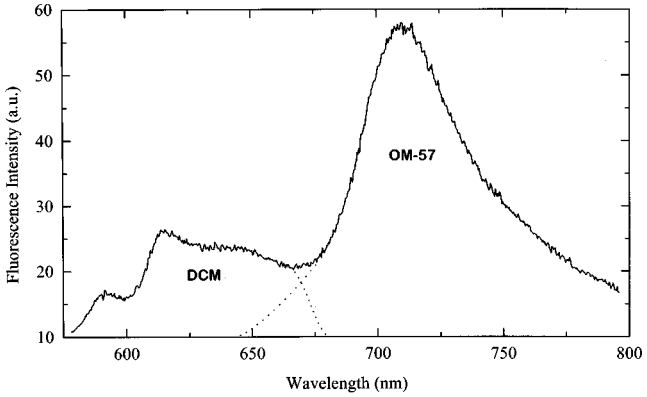

FIG. 1. Fluorescence spectrum of codeposited DCM and OM57 dyes.

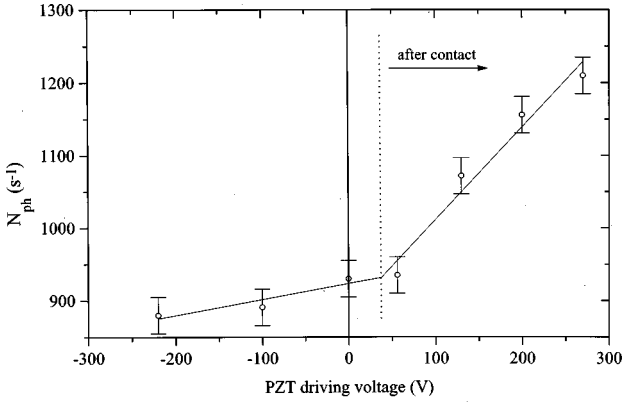

FIG. 3. Dependence of the acceptor's fluorescence signal on the acting force recorded during the SNOM-based FRET experiment. The contact point was determined from the change in oscillation amplitude of the tip.

glass slide barely exceeded the noise level). Thus, Fig. 1 can be regarded as a demonstration of the dipole–dipole resonant energy-transfer process between DCM and OM57 dye molecules.

The scheme of the experiment is shown in Fig. 2. A homemade SNOM using the standard diode laser-based shear force method to regulate the tip–sample distance[1,2] and a homemade electronic unit to control the tip position (described earlier[12]) have been used. Donor dye molecules have been deposited onto the surface of a glass prism by depositing 10 μl of a methanol dye solution with a known concentration and let the drop dry in air at room temperature for some minutes. Acceptor dye molecules have been deposited onto the sharpened fiber by a short-time dipping of the tip into the dye solution; the concentration of the deposited molecules is governed by kinetics.[13]

Light coming out of the opposite side of the sharpened fiber has been detected by a single-photon avalanche diode [(SPAD) EG&G, Canada, noise level-80 s^{-1}] after passing through a number of filters to suppress the stray light and select the light originating from the acceptor molecule fluorescence. The set of filters includes a holographic notch filter for the 488 nm line, a red glass filter with an absorption edge of 660 or 695 nm, and interference filters centered at 750 nm with a width of 70 or 40 nm. From Fig. 1 it can be seen that such a set of filters did enable the separation of the fluorescence coming from the OM57 molecules from that coming from the DCM molecules.

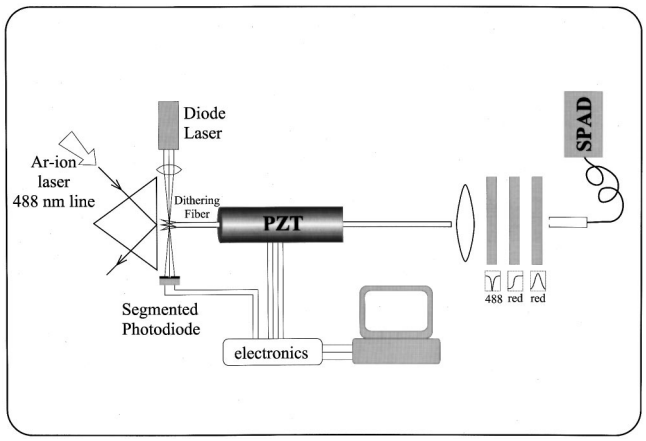

FIG. 2. Schematic of the SNOM-based FRET experiment. The 488 nm spectral line of a cw argon ion laser has been focused onto the glass prism surface in the conditions of total internal reflection (spot diameter ~ 300 μm and laser irradiation intensity I was 15 W/cm^2). SNOM tips with a radius of a curvature of 100–200 nm have been prepared by the usual procedure of etching of optical fibers in concentrated HF solutions.

In Fig. 3 we present the dependence of the fluorescence signal recorded by the SPAD for the case of DCM and OM57 molecules deposited with the surface concentrations of $3\times10^{13}\,cm^{-2}$ as a function of the voltage driving the piezotube in the z direction. The distance Δz between the tip and the sample before the contact can be calculated as a function of the potential difference ΔU applied: $\Delta z=\zeta\Delta U$, where in our case $\zeta=9.5$ nm/V. After contact, it is more reasonable to speak about the change in the force acting between the tip and the sample rather than about the change of a relative distance. An increase of the voltage tends to push the tip (rigidly fixed on the piezo) more strongly against the sample. The acting force F can be calculated using the spring constant k of the sharpened fiber by the relation $F=k\zeta\Delta U$, leading to the flexural bending[14,15] and deformation of the tip.

From Fig. 3 it is easy to see that after contact the acceptor fluorescence signal increases rapidly when the acting force increases. This effect has been well reproduced during at least 20–30 of the cycle contact–out of contact measurements, but an overall slow decrease in the signal due to the photodegradation of the dyes was noticed.

A number of control experiments have been performed using the same tip–sample configuration but in such conditions when both donor and acceptor dyes or one of them were absent. None of these control experiments revealed a behavior analogous to that presented in Fig. 3. Usually, only a very weak change in the fluorescence signal as a function of the driving voltage has been observed and the point of contact did not correspond to any peculiarities of the dependence. Of course, the absolute value of the recorded signal was smaller. Thus, the behavior presented in Fig. 3 was definitely due to the presence of *both* dyes and should be regarded as a demonstration of FRET phenomena in scanning probe microscopy. The increase of the fluorescence signal as a result of the increase of the acting force was due to the corresponding increase of the contact surface, and thus, of the number of molecules involved in the energy-transfer process. It does not seem easy to describe the phenomenon

quantitatively but we hope that the following considerations would be of interest.

Experimental measurements of the spring constant k for the glass fiber tips[16] as well as calculations based on the mechanical properties of a flexural bending of the glass cone[14] show that, for a tip with the radius of curvature of 100 nm, the spring constant should be of the order of 500–1000 N/m. This means that for the experimental displacement of the piezo, $\zeta \Delta U \approx 1.9\ \mu$m the acting force value should be in the range $10^{-4}-10^{-3}$ N. The action of this force will result in an increase of the contact surface. For the rough estimations of the elastic deformation, one can use the known Hertzian expression to describe the contact radius r_c of a sphere pressed against a flat sample surface as a function of an acting force F:[12,17]

$$r_c = \left(\frac{3(1-\nu^2)Fr}{4E} \right)^{1/3}. \tag{1}$$

Here, r is the radius of curvature of the tip, $E = 7 \times 10^{10}\ \mathrm{N/m^2}$ and $\nu = 0.25$ are typical Young's modulus and Poisson's ratio for glass. Use of this expression for the case of $F = 10^{-4}$ N, $r = 100$ nm gives $r_c = 46$ nm, which corresponds to $N_1 \sim 2000$ molecules in the ''FRET active'' contact area for the surface concentration of $3 \times 10^{13}\ \mathrm{cm^{-2}}$.

An absolute value of the fluorescence signal recorded for the sharpest tips used was equal to $N_{\mathrm{ph}} = 80-100\ \mathrm{s^{-1}}$ (with the signal-to-noise ratio of the order of unity). Knowing this value, we can estimate what number of molecules N_2 contributes to the measured signal using the simple relation

$$N_2 = \frac{N_{\mathrm{ph}} h\nu}{I \sigma \eta \Phi}. \tag{2}$$

Here, $h\nu = 4.07 \times 10^{-19}$ J is a photon's energy, Φ is the fluorescence quantum yield of the acceptor molecule, and η is an overall efficiency of the photon collection and detection for our experimental system. The latter can be estimated as follows. The efficiency of the fluorescence photon collection by a sharpened fiber for a geometry similar to ours was reported to be[18] $2-5 \times 10^{-3}$. We estimate the efficiency of the detection of photons coming out of the fiber as 0.1–0.05 (this value is due mainly to the registration only within a rather narrow spectral band of the total acceptor fluorescence because of the strong filtering, see above), and thus, the overall efficiency of the detection is $1-5 \times 10^{-4}$. This means that $N_2 \sim$ 300–1500 acceptor molecules contribute to the measured signal (we assume $\Phi = 0.3$). Both N_1 and N_2 values are in reasonable agreement with each other, strengthening our conclusions about observation of ''nanolocal'' FRET phenomena with only hundreds to thousands of molecules involved. These results were qualitatively confirmed in another series of experiments performed in an AFM-based geometry, which will be published elsewhere.

The results presented in this work should be treated as a demonstration of the FRET phenomenon in scanning near-field optical microscopy. We succeeded in recording a nanolocal ''contact-dependent'' fluorescence signal corresponding to a few thousand or even hundreds of FRET-active molecules. Relying on the experimental results, the sensitivity and noise level, etc., we believe that using a slightly modified approach (for example, based on an illumination-geometry SNOM, where single fluorescent molecules have been indeed observed recently[19]) it will be possible to realize real SNOM based on the FRET process involving only one fluorescent center in the tip apex. This task seems especially timely because the fabrication of suitable ''single fluorescent center-containing'' tips,[5,20] as well as the possibility of nondestructive scanning of the SNOM tips at distances within the Förster radius to the sample,[14] have been recently demonstrated.

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