Local fluorescent probes for the fluorescence resonance energy transfer scanning near-field optical microscopy

G. T. Shubeita, S. K. Sekatskii,[a] and G. Dietler
Institut de Physique de la Matière Condensée Université de Lausanne, BSP, CH1015 Lausanne–Dorigny, Switzerland

V. S. Letokhov
Institute of Spectroscopy, Russian Academy of Sciences, Troitsk, Moscow region, 142190 Russia

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We present fluorescence resonance energy transfer (FRET) images of donor dye molecule clusters recorded using a local fluorescence probe for scanning near-field optical microscopy (SNOM): standard apertured SNOM fiber tip coated with the 30–100-nm-thick polymer layer stained with the acceptor dye molecules. The tip works as a ''self-sharpening pencil'': the apical layers of the FRET-active tip coating are mechanically worn out during scanning thus continuously exposing a fresh active apex to continue imaging. Only a few tens of acceptor molecules are used to form the optical images, and using such an approach spatial resolution better than the size of the aperture is achievable. © *2002 American Institute of Physics.* [DOI: 10.1063/1.1467695]

Among the different approaches to scanning near-field optical microscopy (SNOM; see, for example, Refs. 1 and 2 for a recent review of the field), that based on using local fluorescent probes is especially promising. In this scheme a subwavelength-sized active medium replaces the aperture and is used as a source of light. For a number of SNOM operation modes (e.g., the apertureless one[1,2]) the spectral shift between the excitation light and the local probe fluorescence makes it possible to drastically improve the signal-to-noise ratio (suppress the background). Another advantage is the easier interpretation of the SNOM data because of the well-defined and controllable shape of the excitation source (see, for example, Ref. 3 for a discussion).

Additional advantages exist for the recently proposed[4,5] local fluorescent probes employing the fluorescence resonance energy transfer (FRET), where a donor molecule or other fluorescent center located in the tip apex is used to excite the fluorescence of an acceptor center of the sample (or vice versa). The spatial resolution is governed not by the aperture size 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.[6,7] These advantages can be fully exploited only with FRET active probes containing a single fluorescent center in the apex. Nevertheless, important characteristics of SNOM can be improved when one uses FRET active tips containing hundreds or thousands of fluorescent centers as well.[8–10] In addition to the aforementioned, spatial resolution better than the aperture size can also be achieved.[10] Due to the pseudocontact character of the FRET interaction[6,7] only the molecules located on the sample surface within a circle of a 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; this formula follows from elementary geometrical consideration for a sphere of a radius R contacting a plane and neglecting the tip's deformation upon contact; see Fig. 1). Hence, the spatial resolution is governed by the value of $2r$, which, for the usual case $R_0 \ll R$, can be essentially smaller than R.

Earlier we have selected the dye pair DCM[11]–OM57[12] as the most suitable donor–acceptor pair to work in the geometry of atomic force microscopy (AFM) and SNOM.[10,13] Having a working FRET pair in hand attempts were undertaken to collect FRET SNOM images using the SNOM tip dipped into an OM57 methanol solution (withdrawal deposition method[14,15]) and DCM layers deposited onto a glass slide surface as a sample. These attempts failed due to the fact that for such a method of preparation of the FRET-active tips it turned out that the deposited acceptor layer is eliminated from the tip almost immediately after starting scanning due to mechanical wear and insufficient photostability.

In this paper we present a very simple and straightforward method of preparation of local fluorescent probes for near-field optical microscopy especially suitable for the

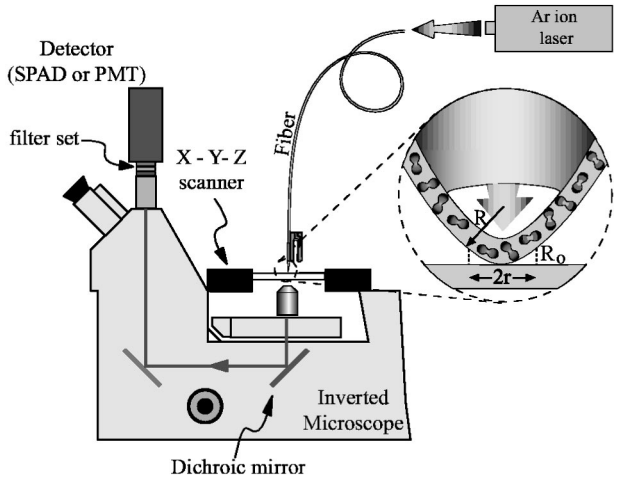

FIG. 1. Schematic of the fluorescence resonance energy transfer scanning near-field optical microscope. The self-sharpening pencil local fluorescent probe in contact with the sample surface is shown in the insert.

[a]Electronic mail: sergey.sekatskii@ipmc.unil.ch

FRET SNOM and capable of subaperture-size resolution. We used the method of dipping the tip into a 3×10^{-5} mol/L chloroform solution of the OM57 dye containing 1 vol % of dissolved polymethyl methacrylate (PMMA). As known from the controllable withdrawal deposition method[15] and confirmed by scanning electron microscopy, thin (30–100-nm-thick) layers of the PMMA stained with the dye at a concentration of $c=1.9\times10^{18}$ cm^{-3} are formed on the tip surface after the solvent dries. This concentration provides enough acceptor molecules capable to contribute to the FRET signal (see below).

In addition to the better photostability and immobilization of the molecules imbedded into the polymer matrices, the self-sharpening pencil behavior could be realized for such a probe (see Fig. 1). The apical layers of the FRET-active tip coating are mechanically worn out during scanning and hence an active apex containing fresh acceptor molecules is continuously exposed to continue imaging. Note that the probe contains acceptor rather than donor molecules. The former do not absorb the laser excitation light which continuously illuminates them through the fiber and hence only the molecules lying within a few nanometers of the apex and which participate in FRET are subject to eventual photobleaching. These molecules are exactly those which are refreshed by the self-sharpening pencil mechanism. Intact acceptor molecules, which also can be worn out of the tip, mix with the donor and rapidly photobleach without a serious influence on the imaging process.

The schematic of the FRET SNOM is shown in Fig. 1. A home-made SNOM built on top of an inverted fluorescence microscope, Carl Zeiss 100M, was operated in the illumination mode to perform the experiments. The tuning fork based shear-force distance regulation[1] was implemented. Commercially available sharpened metal-coated fibers with the nominal aperture size of 100 and 200 nm (Nanonics Supertips, Israel) were used as the SNOM probes. The following filter sets were utilized (these sets were selected earlier as the optimal ones to distinguish the DCM and OM57 fluorescence[10,13]): a holographic notch filter for the 488 nm excitation line (Kaiser, USA) and a long pass 530 nm red glass filter for the detection of the DCM fluorescence, the same filter set in addition to a long pass 695 nm red glass filter was used for the detection of OM57 fluorescence. A dichroic mirror was placed in the optical detection path in the microscope to improve the suppression of the excitation light. The 488 nm cw argon ion laser (Spectra Physics) spectral line was coupled into the fiber probe via a standard fiber coupler (Newport); the light power at the coupler entrance being a few (<10) milliwatts. A single photon avalanche diode (EG&G, Canada) and a channeltron-type photoelectron multiplier tube (Perkin Elmer) were used as the detectors. Further details of the SNOM construction can be found in Refs. 13, 16, and 17.

Two types of samples were studied: DCM methanol solutions spin-coated onto the glass slide surface and small droplets (10–20 μL) of these solutions dropped onto the glass slide surface and then dried in air. An example of the FRET image of one of the dropped-and-dried samples is given in Fig. 2 (the nominal surface concentration of the DCM molecules is 5×10^{14} molecule/cm^2). Prior to OM57 deposition onto the fiber probe, nothing was observed with the same filter set suitable to record the acceptor's fluorescence. Using, for such inactivated fibers, the filter set suitable to detect the DCM fluorescence, we recorded similar pictures. The shear force topography signal, recorded simultaneously with the optical one, reveals that the bright spots are ''hills,'' obviously formed by the DCM molecules ''condensed'' in some places of the sample, where the local dye concentration essentially exceeds the nominal (and average) one. The nonuniformity of the spatial distribution of dye molecules deposited by simple dropping-and-drying at the (sub) micron spatial scale was earlier reported by a number of investigators. No similar structures were observed for clean uncoated glass samples regardless of the SNOM tip and the filter sets used. The near-field optical images were also uniform and structureless when spin-coated DCM samples were studied.

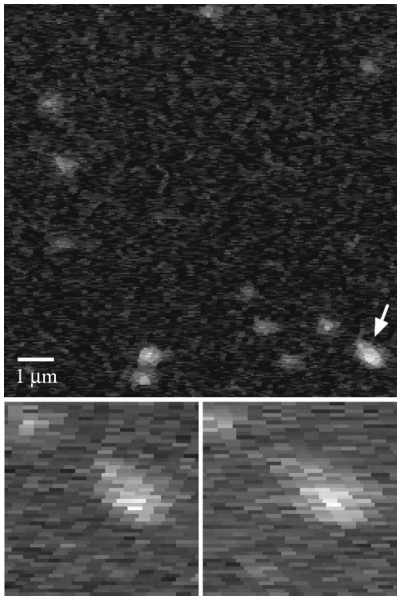

FIG. 2. FRET SNOM image of donor molecule clusters on a glass slide surface. In the insert we show details of the raw images of one of the clusters (indicated by an arrow) recorded for the left-to-right and right-to-left movements of the tip. The size of one pixel is 43 nm, the minimal size of a structure in the scan direction (determined by the photon integration time) is ~170 nm.

The dependencies of the acceptor fluorescence and shear force signal on the applied force recorded for our local fluorescence probes approaching a monolayer of the DCM dye spin-coated onto the glass slide surface are presented in Fig. 3. They demonstrate that the FRET signal appears only after the tip–sample contact is acquired and then it increases with the increase of the tip–sample acting force due to the corresponding increase of the FRET-active surface (contact area). These results are rather similar to those reported by us earlier for the ''standard'' (without a thin polymer film on the apex) FRET fiber probes[10,13] and details of the experiment and further discussion can be found in these papers.

We believe that the SNOM data, taken together with the demonstration of the contact-dependent character of the OM57 fluorescence for the same experimental conditions, constitute sufficient proof that FRET images of the ''clusters'' of the DCM molecules were experimentally observed. The magnitude of the signal S recorded (100–1000 photon/s)

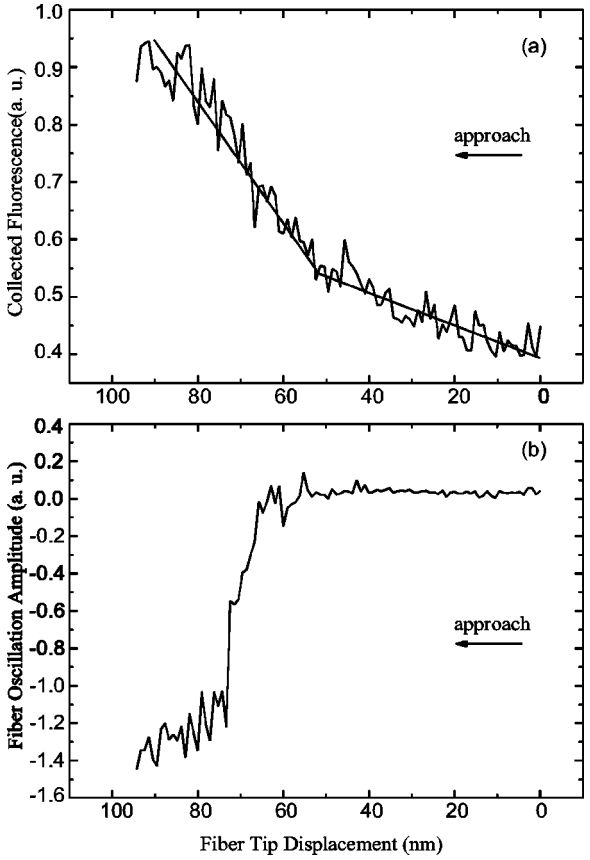

FIG. 3. a) Dependence of the acceptor's fluorescence signal on the acting tip–sample force recorded during one single approach of the SNOM tip coated with the PMMA layer containing OM57 acceptor molecules to the sample (DCM monolayer on the glass slide surface). The maximum acting force is estimated as $\sim 10^{-4}$ N (see Refs. 10, 13). b) Simultaneously recorded dependence of the shear force signal amplitude on the tip–sample relative position.

is also in an order of magnitude agreement with the estimation of the number N_{ac} of the FRET pairs involved. One has $S \cong (\eta I \sigma / h\nu) N_{ac}$, which for the light intensity $I \approx 10^3$ W/cm^2, DCM optical excitation cross-section $\sigma = 6 \times 10^{-17}$ cm^2, photon energy $h\nu = 4.1 \times 10^{-19}$ J and overall detection efficiency[18] $\eta \sim 3 \times 10^{-4} - 10^{-3}$ gives $S \sim 50-150 N_{ac}$ s^{-1}. The value of N_{ac} is defined by the number of acceptor molecules contained in a spherical segment (the apical part of the tip) having the radius r and height R_0 (see Fig. 1 and consideration given above): $N_{ac} = (1/2)\pi r^2 R_0 c = \pi R_0^2 R c$. For $R = 250$ nm and $R_0 = 3$ nm one has $N_{ac} \sim 15$, $S \sim 750-2250$ s^{-1}. A good reproducibility of small details of the light intensity distribution along the DCM clusters (see the insert in Fig. 2) can be considered as an indication of a spatial resolution exceeding the probe aperture (spatial resolution of the order of $2r = 2\sqrt{2RR_0} \cong 80$ nm, is anticipated for our FRET active tip).

Thus, we demonstrate FRET SNOM images obtained using only a few tens of renewable acceptor molecules. The shear force feedback, which is by far the most popular technique in the near-field optics and enables to use the time-gated detection of the optical signal,[17,19] was explored. To conclude, we must note that we were not able to record convincing FRET images using a number of ''recommended'' FRET pairs (see, for example, Ref. 20) including those used in Refs. 8 and 9, where the first FRET SNOM images were reported: fluorescein as a donor and tetramethyl rhodamine or Rhodamine B as an acceptor.[21] The main problem was the too efficient direct excitation of the acceptor by the laser radiation used (488 or 458 nm lines of cw argon ion laser). Not only did these attempts fail, but the attempts to observe unambiguously the contact dependent FRET phenomenon for these pairs on a nanolocal level in a manner analogous to the case of DCM–OM57,[10,13] also failed for the same reason.

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