Impact of metal-modified AFM tips on the fluorescence of single nanocrystals

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ABSTRACT

The influence of metal surfaces and nanoparticles on the fluorescence emission of fluorophores in close proximity is of particular interest for biophysical applications, near field optics and biosensing. For instance, the quenching of fluorophores by gold nanoparticles can be used for the investigation of biomolecular conformational changes or interactions and silver coated metal tips are potent scanning near field optical microscopy tips. Apart from the quenching effects, nanoparticles are used for fluorescence enhancement in biosensor applications.
Here we use a setup combining total internal reflection fluorescence microscopy (TIRFM) with the piezo-controlled nanometer-sensitive movement of an atomic force microscope (AFM) in order to measure and quantify the fluorescence emission as a function of distance between single fluorophores and metal nanoparticles or tiny metal tips. By using CdSe/ZnS nanocrystals as fluorophores and gold as metal we observed significant fluorescence quenching as well as enhancement due to exciton-plasmon coupling. In the future, these experiments will be extended to metal nanoparticles of different elements, alloys, sizes and shapes, giving insight into the related energy transfer processes and quenching mechanisms.

Keywords: Single Molecule Manipulation; TIRF; AFM; Quantum Dots

1. INTRODUCTION

In recent years, various methods to manipulate and characterize single molecules have been developed allowing to investigate for example mechanical and optical properties as well as conformation dynamics. A recent and very important task is to measure several complementary physical properties at a time by the combination of conceptually different techniques. In particular, the combination of mechanical and optical approaches namely atomic force microscopy or spectroscopy and laser induced single molecule fluorescence microscopy holds great promises for future investigation.
The evolution of scanning probe microscopy (SPM) techniques especially AFM[1] had great impact on nanobiophysical assays. Single molecule force techniques aim on mechanical properties like dissociation forces of single ligand-receptor complexes[2-7] or structural rearrangements[8-10]. The basic concept of mechanical approaches is the usage of a small transducer attached to an individual molecule. Whereas optical[11] or magnetic tweezers[12] use micro beads trapped in an external field, micro-fabricated cantilevers are used in AFM. The intrinsic advantages of AFM allowing topographical and force measurements makes it a favorable candidate for the embedding in a mechano-optical setup.
The optical observation of single molecules has profited from evolutions in fluorescence imaging and spectroscopy techniques[13; 14]. Minimizing the excitation or detection volume had great impact on the enhancement of the signal-to-noise ratio. This confinement can be achieved by different approaches, the most important being confocal laser scanning microscopy (CLSM), where the fluorescent light emanating from the sample is focused on a pinhole, filtering scattered

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light not deriving from the focal plane. A method limiting the excitation volume is two photon laser scanning microscopy[15] (2PLSM) where a laser with twice the excitation wavelength is focused through a high numerical aperture objective lens. The sample is excited by a two photon process which is only likely in the focal volume due to the required intensity. Scanning near-field optical microscopy[16] (SNOM) is a scanning method with exploits the evanescent wave field emanating from a small aperture or the illumination of a tiny apertureless metal tip[17]. Total internal reflection fluorescence microscopy (TIRFM) also uses an evanescent field generated by the total reflection of light at a high refraction index to low refraction index boundary. The excitation volume is confined in z-direction by the penetration depth of the evanescent wave, resulting in a significantly reduced fluorescence background. This technique, if applied to study single molecules[18; 19], has the advantage that complete illumination can be observed, hence the imaging of many fluorophores at a time is possible; a slight disadvantage is the necessity to use comparably slow array detectors like CCD cameras.

The information gained in single molecule fluorescence measurements allows direct insight in energy changes of a single fluorophore or energy transfer between individual fluorophores or fluorophores and metallic objects. Compared to ensemble experiments it is possible to discover and investigate energetic subpopulations and intermediate states. Procedures using radiationless energy transfer between individual fluorophores or fluorophores and metallic interfaces like fluorescent resonant energy transfer (FRET)[20] or quenching show strong distance dependency and can therefore serve as a ruler on the nanometer scale. These provide supplementary information e.g. characteristic length scales of molecular reactions or folding pathways. In this context, semiconductor nanocrystals[21-23] (quantum dots) as a novel type of fluorescent probe has evoked considerable attention. Their remarkable resistance to photo-bleaching and high excitation cross sections in combination with the possibility to functionalize in a biocompatible way and attach to biomolecules make them a promising alternative for optical nanoscale experiments.

The complementarity of single molecule force and fluorescence techniques strongly advises a combination of these approaches. The concurrent measurement of mechanical and optical properties will undoubtedly open a new perspective on the forces, energies and conformational changes that go together with biomolecular interaction. So far only few mechano-optical experiments have been established; thereunder are the combination of AFM and CLSM[24-26], an optical trap with combined TIRF detection[27] or a combined AFM-TIRFM setup[28; 29.]

In this paper we will report on the distance dependent fluorescence emission characteristics of a gold-modified AFM tip using a combined AFM-TIRFM setup.

2. INSTRUMENTATION

For our approach of combining atomic force and total internal reflection microscopy it is essential to maintain highest possible versatility for both hard- and software. Therefore we use a home-built system (Fig 1). The AFM head has a perpendicular beam geometry i.e. a laser beam is directed towards a polarizing beam splitter where the incident is separated into two linearly but orthogonally polarized beams. The perpendicular diverted beam passes a $\lambda/4$ wave plate (Linos, Göttingen, Germany) where the polarisation is changed to circular. The incident laser beam is focussed on the AFM cantilever by a microscope objective lens (Zeiss LD Achroplan 20x, Carl Zeiss, Jena, Germany) whereas the reflected beam is recollimated. By passing the wave plate for a second time the polarisation is again changed to linear but tilted by $\pi/2$ referring to the incident beam ensuring the undiverted pass through the beam splitter. A dichroic mirror (XF2021, Omega Optical Inc., Brattleboro, USA) allows observation of cantilever and sample but leaves the laser beam unaffected. The following band pass filter (3RD640-690, Omega Optical Inc., Brattleboro, USA) inhibits optical crosstalk between the AFM laser and stray light from the optical excited sample. A quadrant photodiode (QD50-5T, Centronic, Croydon Surrey, England) serves as position sensitive device. The current from each segment is converted to a voltage by single high speed operational amplifiers (Burr-Brown OPA655, Texas Instruments, Dallas Texas, USA). These show excellent performance for high bandwidths as well as reasonable signals even for poor cantilever reflectivity. A quadrant detector electronics is used for both generating deflection, lateral and sum signals and further amplification.

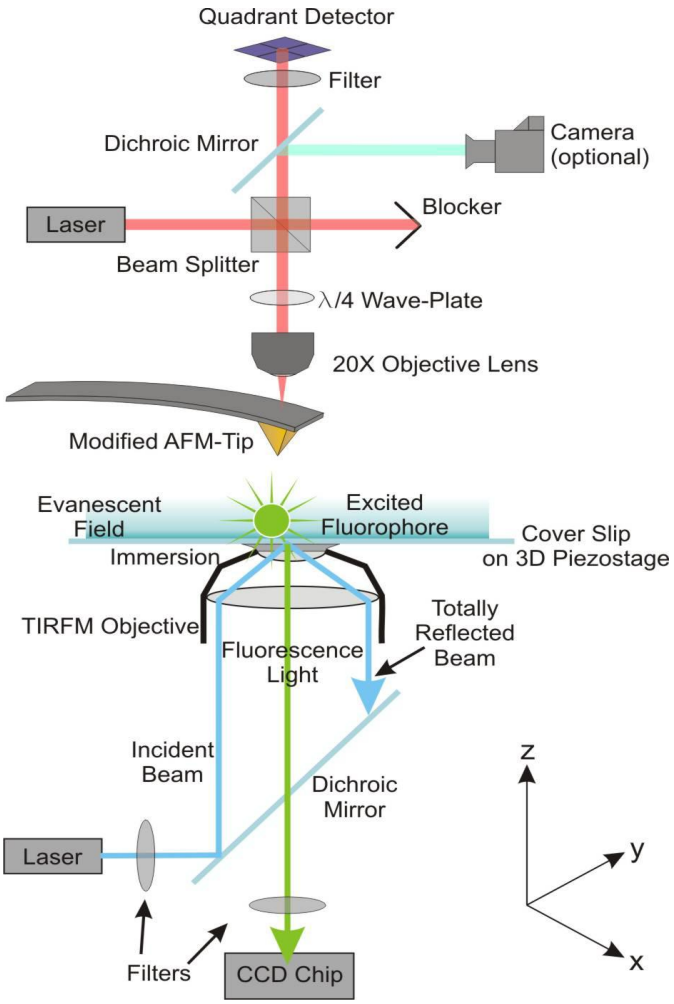

Fig. 1: Schematic of the combined AFM-TIRF setup
A laser beam is directed via a TIRF objective lens at an angle of total reflection onto a glass cover slip. An evanescent wave field protruding some tens of nanometers beyond the surface excites immobilized fluorophores. Fluorescence emission of individual fluorophores is recorded by a CCD camera. The AFM head mounted on top of the microscope enables the modulation of fluorescence emission by the means of gold modified AFM tips brought into close proximity to a fluorophore.

The data acquisition and experiment control is done by an ADbasic program running on an ADwin Gold real-time system (Jäger Messtechnik GmbH, Lorsch, Germany) combined with a MATLAB (MathWorks, Natick, Massachusetts, USA) graphical user interface.
The AFM head is mounted on a frame bearing a piezo driven 3D sample stage (PI 517.3CL, Physik Instrumente GmbH & Co. KG, Karlsruhe, Germany) with travel ranges of 100x100x20 µm. The stage is accessed digitally via an adequate controller (E516, Physik Instrumente GmbH & Co. KG, Karlsruhe, Germany) to enhance resolution reproducibility.
The complete stage is mounted on an inverted microscope (Zeiss Axiovert S100, Carl Zeiss, Jena, Germany).
The sample is optically excited by an argon ion laser (cw 10 mW @ 488 nm). For excitation power control neutral density filters with an attenuation ranging from 0.5 to 0.05 were installed in the laser path. To avoid excitation by other laser lines a filter is inserted into the optical path (XF1073 475/40, Omega Optical Inc. Brattleboro, USA). The sample is excited from underneath by an objective lens (Olympus Plapo 100X TIRFM, NA=1,45; Olympus, Tokyo, Japan)

either in the bright field illumination or by the evanescent field emanating from the totally reflected laser beam at the glass (cover slip) air interface. The presented results were achieved in the TIRF (total internal reflection fluorescence) mode since it has a superior signal-to-noise ratio due to the confinement of the excitational volume in z-direction. Fluorescent light is directed from the microscope lens through a band pass filter (HQ 580/80, AHF Analysentechnik, Tübingen, Germany) and an additional ten fold magnification to a highly sensitive CCD-Camera (I-PentaMAX, Roper Scientific, Trenton, New Jersey, USA) with integrated micro channel plate intensifier. AD-conversion is done by a 5 MHz 12-bit converter coming to a sampling rate of 20 Hz for a full 512X512 pixel frame. Reducing the read out area higher frame rates can be achieved.

3. MATERIALS AND METHODS

Fluorescent CdSe/ZnS nanocrystal quantum dots were prepared following a previously published protocol[30]. They showed an emission maximum around 585 nm. For immobilization, the semiconductor nanocrystals were diluted in heptane and subsequently dried on a cleaned (treatment with caroic acid, acetone, ethanol, Milli-Q filtered water, UVO cleaner) glass coverslip.
Silicon AFM tips (Budget Sensors Tap300Al, Innovative Solutions Bulgaria Ltd., Sofia, Bulgaria) were coated with a 20 nm gold layer by evaporation. It has been demonstrated that the fluorescence emission of the semiconductor nanocrystals is effectively quenched if brought into contact with gold surfaces[28; 29].

4. RESULTS AND DISCUSSION

For the investigation of the dependence of the quantum dot fluorescence intensity on the distance of the tip, measurements at numerous z-distances were performed. Figure 2a shows the mean fluorescence emission of an individual CdSe nanocrystal averaged over 400 frames with 50 ms exposure time each as a function of the tip-surface distance. Figure 2b shows the probability of finding the quantum dot in the emitting state. For small distances between tip and surface ($z \approx 0$) the luminescence decreases rapidly until it is quenched which is in complete agreement with previous findings.[29]

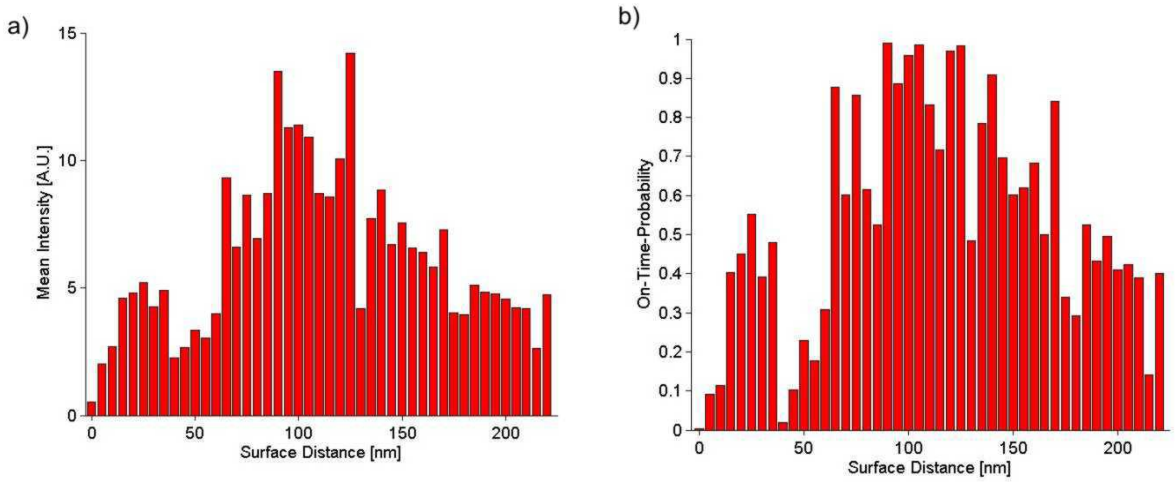

Fig. 2: Mean intensity (a) and probability of finding the quantum dot in the on-state (b) as a function of distance.

When increasing the distance between quantum dot and tip the integrated intensity does not increase strictly monotonically. Instead, at z ≈ 25 nm, the total emission reaches a relative maximum followed by a minimum at z ≈ 40 nm. This luminescence enhancement has been found and discussed previously in the distance-dependent interaction of metal nanoparticles and organic dye molecules[31] and can be attributed to resonant exciton-plasmon coupling. At z ≈ 120 nm, the mean emission of the nanocrystal reaches a maximum.[32] The coupling efficiency, however, is rather small (a factor of two, if the values at 120 and 220 nm are compared), a finding that can also be inferred from the overlap between the emission spectrum of the CdSe nanocrystal and the gold absorption spectrum.
The apparent similarity of the distance dependency of the mean fluorescence intensity (Fig 2a) and the probability to find the nanocrystal in the bright state (Fig 2b) suggests that within the temporal resolution of the CCD camera the blinking frequency was modulated.

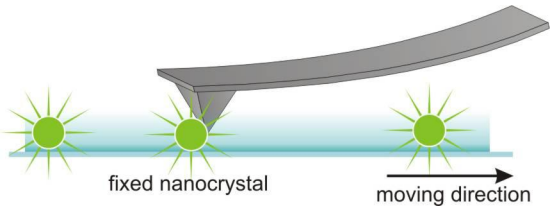

Fig. 3: Scheme of the control experiment
A single quantum dot is pinned down to the surface by an unmodified cantilever while the cover slip underneath is moved.

To rule out mechanical artifacts between tip and fluorophore control experiments were performed using an unmodified Si cantilever. Figure 3 shows a sketch of the control experiment. A single quantum dot is pinned down to the sample surface by the cantilever. The surface is shifted stepwise for several hundred nanometers as the luminescence of the confined quantum dot and some close by is measured. The subsequent fluorescence images (Fig. 4) show an area of 3.5 x 3.5 µm where each image is the average of 200 frames with an exposure time of 50 ms. It can clearly be seen that the fluorescence of the fixed quantum dot is not reduced. This proves that the quenching is not due to mechanical artifacts but due to the interaction with the gold.

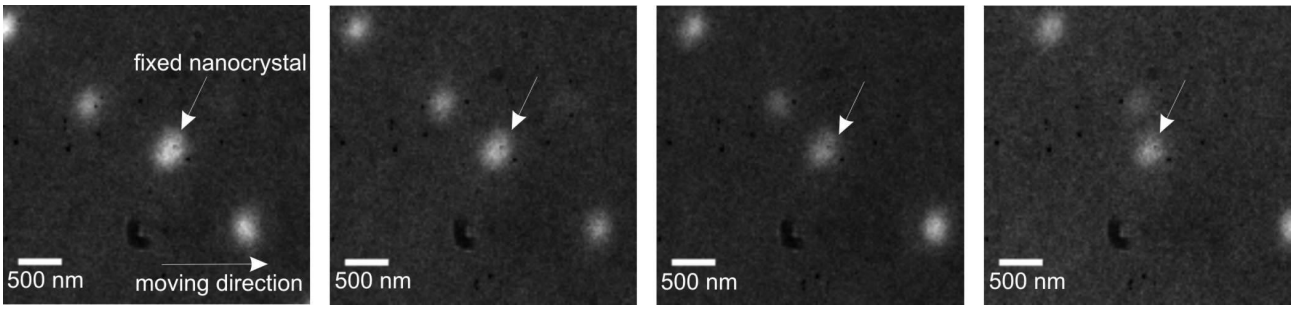

Fig. 4: Control experiment
Subsequent fluorescence images of single quantum dots, one is pinned down at a fixed position whereas the quantum dots in vicinity are shifted roughly 700 nm with the surface. Every image is the average of 200 frames with exposure time of 50 ms each.

5. CONCLUSION

In summary, we presented a combined AFM-TIRF setup facilitating new means of modulating fluorescence emission from a single semiconductor quantum dot by intervention of gold coated AFM tips. It was possible to observe the luminescence characteristics as a function of distance between fluorophore and tip. Additionally fluorescence enhancement due to exciton-plasmon coupling was detected. In future experiments the distance dependency will be investigated more detailed e.g. using different polarization angles or various metals and single nanoparticles attached to the AFM tip.

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