Combined TIRF-AFM Setup: Controlled Quenching of Individual Quantum Dots

Rainer Eckel[1*], Volker Walhorn[1*], Christoph Pelargus[1], Jörg Martini[1], Thomas Nann[2], Dario Anselmetti[1], and Robert Ros[1†]

[1]Experimental Biophysics and Applied Nanoscience, Bielefeld University, Universitätsstraße 25, 33615 Bielefeld, Germany
[2]Freiburg Materials Research Center (FMF), University of Freiburg, Stefan-Maier-Straße 21, 79104 Freiburg, Germany

ABSTRACT

Single molecules can nowadays be investigated by means of optical, mechanical and electrical methods. Fluorescence imaging and spectroscopy yield valuable and quantitative information about the optical properties and the spatial distribution of single molecules. Force spectroscopy by atomic force microscopy (AFM) or optical tweezers allows addressing, manipulation and quantitative probing of the nanomechanical properties of individual macromolecules.
We present a combined AFM and total internal reflection fluorescence (TIRF) microscopy setup that enables ultrasensitive laser induced fluorescence detection of individual fluorophores, control of the AFM probe position in x, y and z-direction with nanometer precision, and simultaneous investigation of optical and mechanical properties at the single molecule level. Here, we present the distance-controlled quenching of semiconductor quantum dot clusters with an AFM tip. In future applications, fluorescence resonant energy transfer between single donor and acceptor molecules will be investigated.

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

1. INTRODUCTION

An important challenge for nanoscale science is the direct manipulation of individual nanoobjects, combined with simultaneous observation of the system's response which can manifest itself in properties like force, conformational change and optical properties. The ambition to measure several physical properties of single nanoobjects at a time requires the combination of conceptually different techniques which have evolved in the past decade. Especially the combination of single molecule fluorescence detection and atomic force microscopy or spectroscopy holds great promises for future investigation.
The observation of individual nanoscale behavior has greatly benefitted from the development of single molecule fluorescence imaging and spectroscopy techniques[1; 2]. Optical single molecule detection with low signal-to-noise ratios is facilitated by the illumination of small excitational volumes with laser light and collection of the emitted fluorescence light via a high numerical aperture objective lens which is coupled to a suitable, highly sensitive detector such as an avalanche photodiode, a photomultiplier tube, or an intensified charge-coupled device (CCD) camera. The confinement of the excitational volume can be achieved via different approaches, the most important being laser scanning confocal microscopy, where the fluorescence emission is focussed by a microscope lens through a pinhole aperture at the image plane, scanning near-field optical microscopy[3], another scanning method which exploits the evanescent wave field

* Contributed equally to this work.
† rros@physik.uni-bielefeld.de; phone +49-(0)521 1065388; fax +49-(0)521 1062959; www.physik.uni-bielefeld.de/biophysik

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emanating from a nanoscale aperture, and total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy also makes use of an evanescent wave field which is generated by total internal reflection of an incident laser beam at a high refraction index to low refraction index boundary. The illumination in TIRF microscopy is confined to a small area within the penetration depth of the evanescent wave, resulting in a significantly reduced fluorescence background. The technique, if applied to study single molecules[4; 5], has the advantage that a complete illumination area enables the observation of many single fluorophores at a time; a slight disadvantage is the necessity to recur to comparably slow array detectors like CCD cameras.

The data obtained in single molecule fluorescence experiments yield direct information about energy changes in a single fluorophore and the transfer of energy which accompanies the interaction between different fluorophores. This information can indicate subpopulations or intermediates that are difficult or impossible to observe in a classical ensemble measurement. Specialized techniques like FRET (fluorescence resonant energy transfer) which require the radiationless interaction of two fluorophores and exhibit a strong distance dependence provide additional data, e.g. about folding pathways or the characteristic length scale of molecular recognition reactions. In this context, semiconductor nanocrystals (quantum dots) as a novel type of fluorescent probe have evoked considerable attention. Their long-term photostability and high excitation cross sections in combination with the possibility to functionalize them in a biocompatible way and attach them to biomolecules make them a promising alternative for optical nanoscale experiments[6-8].

Single molecule force techniques focus on the mechanical properties of individual nanoobjects and can be regarded as a conceptually different approach which is complementary to optical experiments. They provide important information about the forces which accompany structural rearrangements within a single macromolecule[9-11], or the forces and kinetics involved in the breaking of single ligand-receptor bonds[12-17]. Whereas in optical[18] or magnetic[19] tweezers the force is tranduced via a microbead exposed to an external field, the atomic force microscope (AFM)[20] uses a microfabricated cantilever as mechanical force transducer to which a sharp nano-probe is attached. This technique also be used to address and manipulate single nanoobjects.

The complementarity of single molecule fluorescence and force techniques strongly advises for a combination of the different approaches. The simultaneous measurement of optical and mechanical properties will certainly provide new mechanistic insight into the forces, energies and conformational changes that accompany biomolecular interaction. Up to now, only few experiments which combine mechanical manipulation and fluorescence imaging or spectroscopy have been reported. Lang et al. presented a setup combining TIRF detection and optical trapping of single biomolecules[21]. Recently, also the combination of AFM and confocal microscopy has been reported[22; 23].

In this work, a combined AFM-TIRF microscopy setup for the manipulation of single nanoobjects and the quantitative probing of biomolecular interactions is introduced which allows simultaneous mechanical manipulation and fluorescence imaging. The controlled quenching of semiconductor quantum dot fluorescence emission by mechanical intervention of an AFM tip is presented.

2. INSTRUMENTATION

The experiments are performed on a home-built AFM-head (Fig. 1) using the beam deflection method[24] to read out the cantilever deflection. A laser diode module (HL11, Lisa Laser, Katlenburg, Lindau, Germany, optical power <1mW @ 670 nm) with an integrated collimation optics is directed on a polarizing beam splitter (Linos, Göttingen, Germany). The incident beam is divided into two orthogonally linear polarized beams from which the undiverted is blocked. The other beam passes a $\lambda/4$ wave plate (Linos, Göttingen, Germany) where the polarisation is changed to circular. A focussing lens f = 8 mm serves to direct the laser on the cantilever as well as to collimate the reflected part of the beam. Passing again the $\lambda/4$ wave plate the polarisation is changed to linear polarisation which is tilted by $\pi/2$ referring to the incident beam. This enables the passing of the reflected beam through the beam splitter with virtually no loss of intensity. A dichroic mirror (XF2021, Omega Optical Inc., Brattleboro, USA) allows observation of the cantilever and sample but leaves the deflected laser beam unaffected. A quadrant photodiode (QD50-5T, Centronic, Croydon Surrey, England) serves as position sensitive device. For each segment the induced current is converted to voltage by a single high speed operational amplifier (Burr-Brown OPA655, Texas Instruments, Dallas Texas, USA). These yield high bandwidth performance as well as reasonable signals even for poor cantilever reflectivity. A home-made quadrant detector electronics is used for both generating deflection and lateral signals and further amplification. Optionally an analog 8th order Tchebycheff low pass filter (f_{cutoff} = 4 kHz) can be inserted before the data acquisition hardware. 16-bit

multifunction AD/DA boards and a 16-bit transient recorder are used, respectively, for experiment control and data acquisition (PCI-6036E and PCI-6704, National Instruments, Austin Texas, USA and Spectrum MI.4540, Spectrum GmbH, Grosshandorf, Germany). The AFM control software is a self-coded LabVIEW program (National Instruments, Austin Texas, USA).

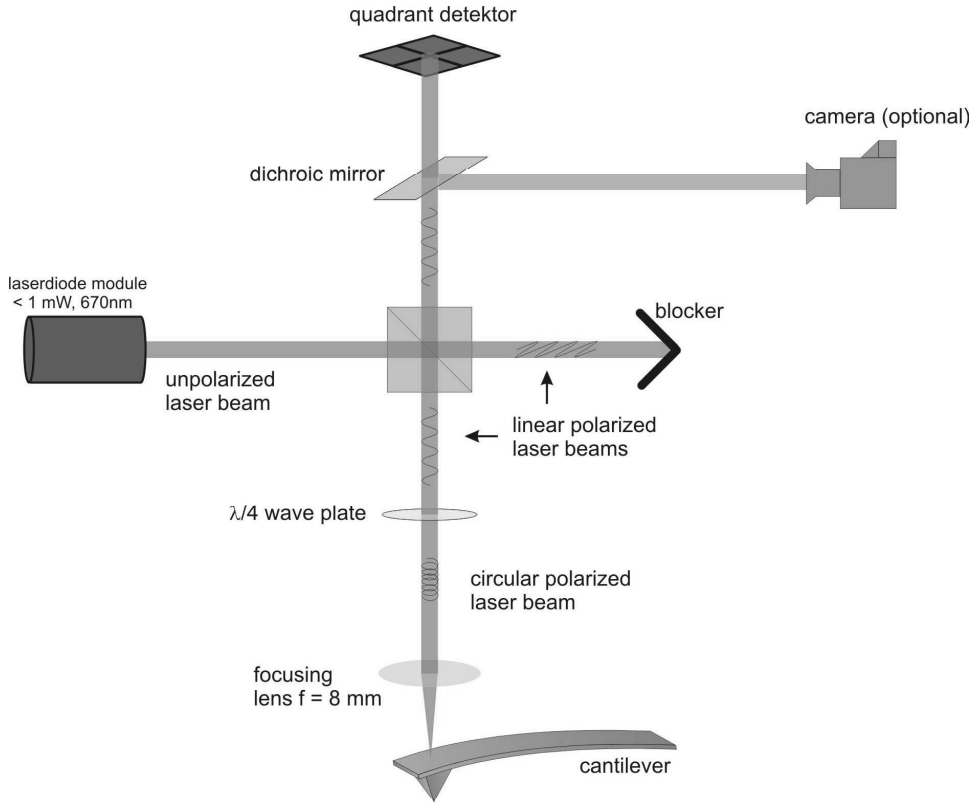

Fig. 1: AFM head setup
Schematic of the optical components of the home-built AFM. A collimated laser beam is linearly polarized and redirected by a beam splitter. Polarisation is changed to circular by a λ/4 wave plate. A lens focuses the incident and collimates the reflected laser beam. The λ/4 wave plate changes polarisation back to linear. The beam splitter and the dichroic mirror are passed. The cantilever deflection is read out by a quadrant photodiode.

The AFM-head is mounted on a frame bearing the sample stage. This stage consists of a sample holder for 24x24 mm coverslips on a manually driven 2D stage which itself is mounted on a 3D piezo stage (PI 517.3CL, Physik Instrumente GmbH & Co. KG, Karlsruhe, Germany) with travel ranges of 100x100x20 μm. The piezo stage can be operated in open loop (analog) and closed loop mode (analog or digital) via an adequate controller (E516, Physik Instrumente GmbH & Co. KG, Karlsruhe, Germany). The sample stage is mounted on an inverting microscope (Zeiss Axiovert S100, Carl Zeiss, Jena, Germany) such that the microscope lens points to the sample (Fig. 2).
The sample is optically excited by an argon ion laser (cw 50 mW @ 488 nm). 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 illuminated from underneath by an objective lens (Olympus Plapo 100X TIRFM, NA=1,45; Olympus, Tokyo, Japan) either in EF (epifluorescence) or TIRF (total internal reflection fluorescence) mode. Fluorescent light is directed from the microscope lens through a band pass filter (HQ 580/80, AHF Analysentechnik, Tübingen, Germany) to a high speed CCD-Camera (I-PentaMAX, Roper Scientific, Trenton, New Jersey, USA). The acquired fluorescence signal is intensified by a micro channel plate intensifier and directed to a 512X512 pixel CCD chip. A 5 MHz 12-bit AD-

converter realizes a frame rate of approximately 20Hz for a full frame. By choosing a region of interest higher frame rates can be achieved.

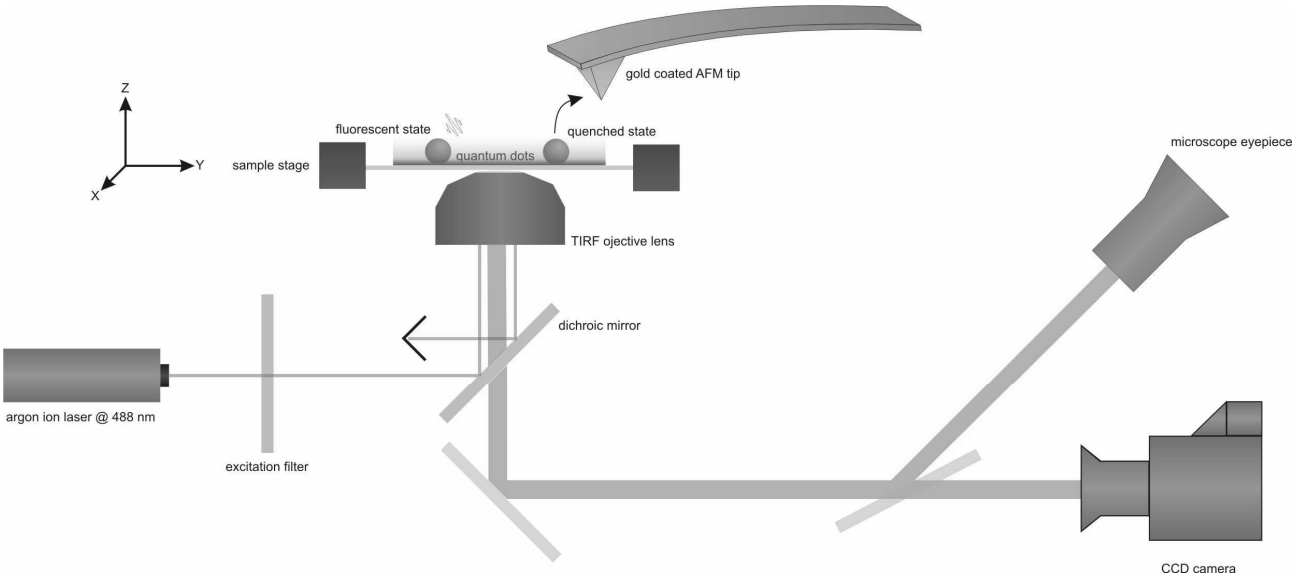

Fig. 2: TIRF setup
Quantum dots on a coverslip are illuminated by an argon ion laser. Fluorescent light is directed via a dichroic mirror to a CCD camera. Switching between the fluorescent and extinguished state is induced by retracting and approaching a gold coated cantilever.

3. MATERIALS AND METHODS

Fluorescent CdSe/ZnS nanocrystal quantum dots were prepared following a previously published protocol[25]. In short, a solution of cadmium stearate and trioctylselenide in trioctylphosphine was swiftly injected into a hot mixture of trioctylphosphineoxide and hexadecylamine. The mixture was stirred for several minutes and then quenched by adding cold butanol. The resulting nanocrystals were resuspended in trioctylphosphineoxide. To this dispersion, a solution of diethylzinc and hexamethyldisilathiane in trioctylphosphine was added dropwise at elevated temperature for one day. The CdSe/ZnS nanocrystals obtained in this reaction were purified and redispersed in chloroform. 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, UVO cleaner) glass coverslip.
Silicon nitride AFM tips (Microlevers, Veeco Metrology LLC, S. Barbara, California,USA) were coated with a 10 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[26].

4. RESULTS AND CONCLUSION

To test the TIRF microscopy setup, single quantum dots were detected and their fluorescence emission and fluorescence intermittency (blinking) observed. Fig. 3 shows a time series, taken with a frame rate of 20 Hz, of a 12x12 μm^2 frame from a quantum dot experiment. As is evident from the flares in fluorescence intensity, one of the bright spots (termed b) in the frame exhibits the characteristic blinking and can be unambiguously identified as single nanocrystals, the other two spots (a and c) being probably a bunch of several ones.

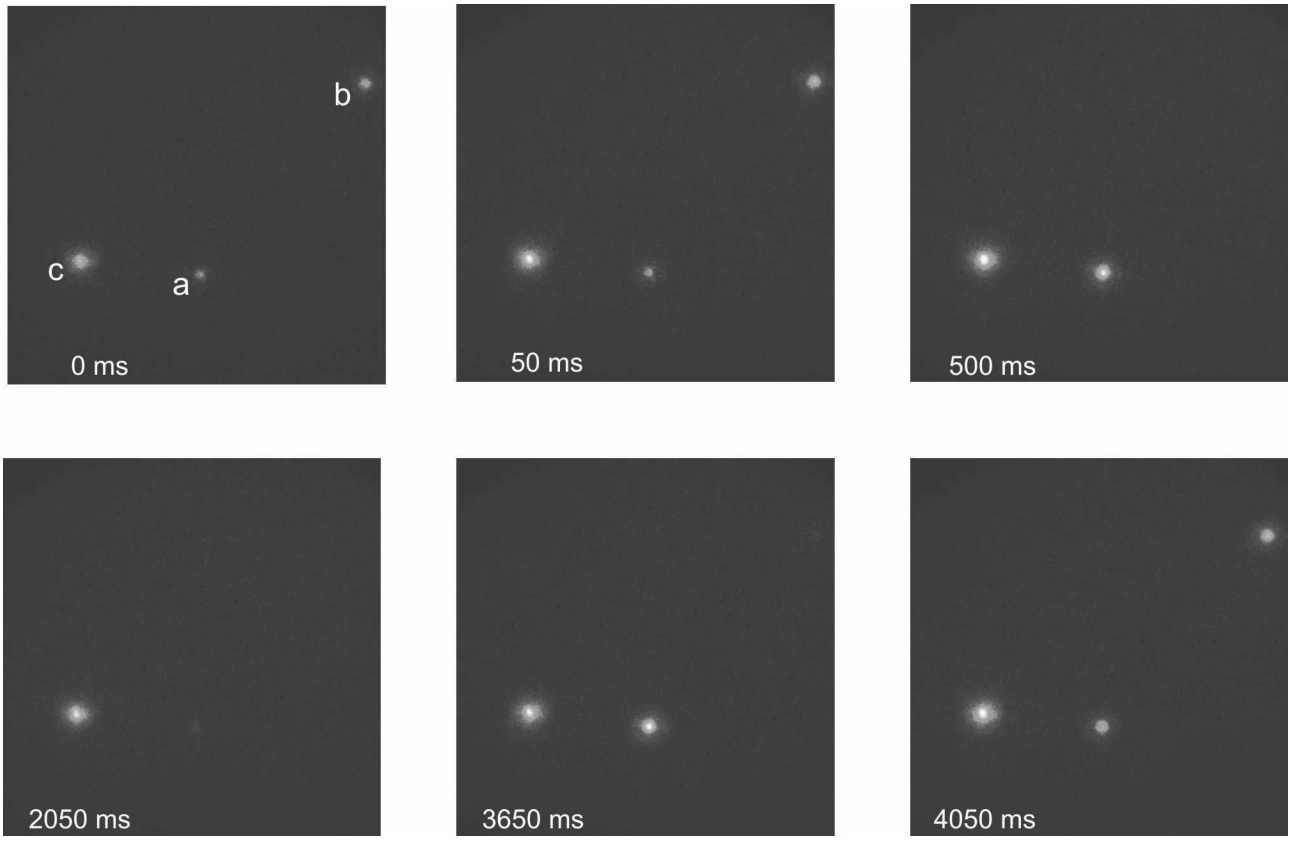

Fig. 3: Optical imaging of semiconductor quantum dots
Successive images of semiconductor quantum dots on a coverslip taken with a frame rate of 20 Hz. The spot marked with (b) represents a single nanocrystal; this can be inferred from the typical blinking behaviour. The spots (a) and (c), however, exhibit only slight fluctuations of intensity and therefore might represent rather an aggregate of several semiconductor quantum dots.

Using the combined TIRF-AFM setup, the mechanical emission control of a small cluster of single quantum dots by means of gold-coated AFM probe has been achieved. In a series of mechano-optical experiments, fluorescence images of the aggregate were taken at a frame rate of 20 Hz while the lateral position of the tip was varied. The results are presented in Fig. 4. The first row demonstrates the respective position of the AFM tip: At (a), which corresponds to the tip position during the first five seconds of the experiment, the tip is separated from the nanocrystal fluorophores by a lateral distance of a half micron; after five seconds (b), the tip has moved laterally into the near proximity of the cluster; the gold surface is now very close to the quantum dots; after another five seconds (c), the tip is removed again. The second row shows fluorescence images of the quantum dot aggregate (frame: 600x600 nm^2) from the time intervals (a), (b) and (c), respectively. The third row presents the plot of intensity against time. It is evident from (b) that approximation of the tip causes effective quenching of the quantum dot fluorescence. Upon withdrawal of the tip (c), the original fluorescence emission from the cluster can only be partly regained; from the corresponding intensity trace and the image, it can be concluded that one of the presumably three single dots has been removed or bleached by the tip. Nevertheless, this experiment clearly proves the capability of the combined TIRF-AFM setup to control the optical properties of single nanoobjects by external, mechanical intervention.

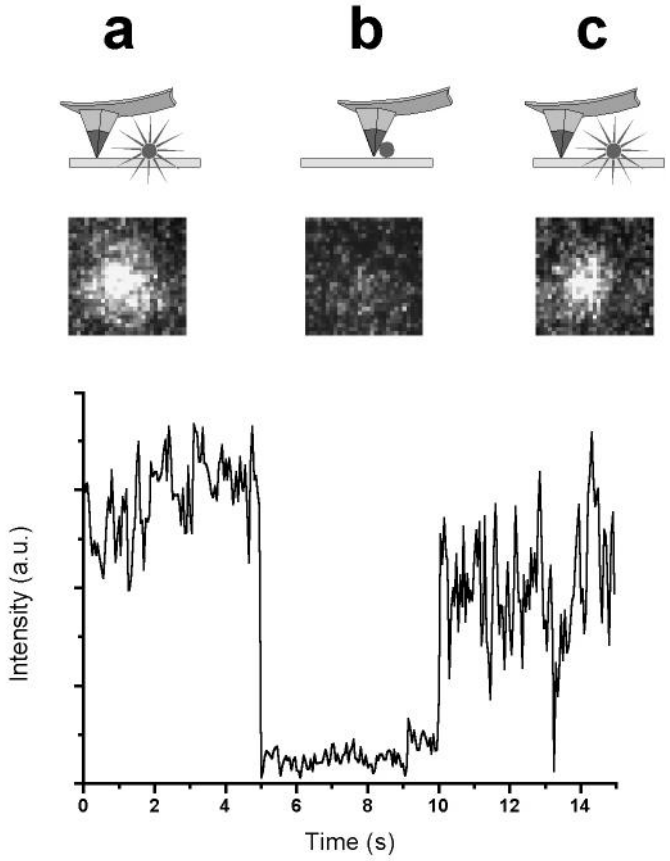

Fig. 4: External emission control of a semiconductor quantum dot cluster.
The fluorescence emission from a bunch of semiconductor nanocrystals (probably three) could be effectively quenched by moving a gold-coated AFM tip into the proximity of the aggregate. The emission of at least some (probably two) nanocrystals could be re-activated upon lateral retraction of the tip. The first row of images shows a schematic of the lateral tip movement, the second a fluorescence image of the aggregate, the third a plot of the fluorescence intensity against time. During the first five seconds of the experiment (a), the tip was not in contact with the aggregate. For the next five seconds (b), the tip was moved laterally and brought into contact with the aggragate. Subsequently, it was removed again (c).

Further improvements of the setup will facilitate the simultaneous AFM topography and fluorescence imaging of the sample. It is planned to address individual fluorophores with different receptors and to perform affinity experiments with these. The next step on the way to simultaneous fluorescence and force spectroscopy on single biomolecules will be the addition of an acceptor fluorophore (instead of a quenching agent) to the AFM tip and, subsequently, the simultaneous measurement of tip position and fluorescence resonant energy transfer between the donor and acceptor. Furthermore, the experiment discussed here resembles an off-switching of the fluorophore and hence a “negative” control; the inverse approach, i.e., a FRET setup with an acceptor fluorophore attached to the sample surface and a donor at the AFM tip, would correspond to a “positive” control of fluorophore emission, i.e., the fluorescence signal is switched on if the external stimulus (the donor fluorophore at the tip) is brought close.

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REFERENCES

1. Moerner,W.E. and Fromm,D.P.: "Methods of single-molecule fluorescence spectroscopy and microscopy", Rev.Sci.Instr., **74**, 3597-3619, 2003.

2. Orrit,M.: "Single-molecule spectroscopy: The road ahead", J.Chem.Phys., **117**, 10938-10946, 2002.

3. Pohl,D.W., Denk,W., and Lanz,M.: "Optical stethoscopy: Image recording with resolution lambda/20", Appl.Phys.Lett., **44**, 651-653, 1984.

4. Funatsu,T., Harada,Y., Tokunaga,M., Saito,K., and Yanagida,T.: "Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution", Nature, **374**, 555-559, 1995.

5. Dickson,R.M., Norris,D.J., Tzeng,Y.-L., and Moerner,W.E.: "Three-dimensional imaging of single molecules solvated in pores of poly(acrylamide) gels", Science, **274**, 966-968, 1996.

6. Larson,D.R., Zipfel,W.R., Williams,R., Clark,S.W., Bruchez,M.P., Wise,F.W., and Webb,W.W.: "Water-soluble quantum dots for multiphoton fluorescence imaging in vivo", Science, **300**, 1434-1436, 2003.

7. Parak,W.J., Gerion,D., Pellegrino,T., Zanchet,D., Micheel,C., Williams,S.C., Boudreau,R., Le Gros,M.A., Larabell,C.A., and Alivisatos,A.P.: "Biological applications of colloidal nanocrystals", Nanotechnology, **14**, R15-R27, 2003.

8. Bruchez Jr.,M., Moronne,M., Gin,P., Weiss,S., and Alivisatos,A.P.: "Semiconductor nanocrystals as fluorescent biological labels", Science, **281**, 2013-2016, 1998.

9. Rief,M., Oesterhelt,F., Heymann,B., and Gaub,H.E.: "Single molecule force spectroscopy on polysaccharides by atomic force microscopy", Science, **275**, 1295-1297, 1997.

10. Rief,M., Gautel,M., Oesterhelt,F., Fernandez,J.M., and Gaub,H.E.: "Reversible unfolding of individual titin immunoglobulin domains by AFM", Science, **276**, 1109-1112, 1997.

11. Eckel,R., Ros,R., Ros,A., Wilking,S.D., Sewald,N., and Anselmetti,D.: "Identification of binding mechanisms in single molecule-DNA complexes", Biophys.J., **85**, 1968-1973, 2003.

12. Florin,E.-L., Moy,V.T., and Gaub,H.E.: "Adhesion forces between individual ligand-receptor pairs", Science, **264**, 415-417, 1994.

13. Lee,G.U., Chrisey,L.A., and Colton,R.J.: "Direct measurement of the forces between complementary strands of DNA", Science, **266**, 771-773, 1994.

14. Ros,R., Schwesinger,F., Anselmetti,D., Kubon,M., Schäfer,R., Plückthun,A., and Tiefenauer,L.: "Antigen binding forces of individually addressed single-chain Fv antibody molecules", Proc.Natl.Acad.Sci.USA, **95**, 7402-7405, 1998.

15. Bartels,F.W., Baumgarth,B., Anselmetti,D., Ros,R., and Becker,A.: "Specific binding of the regulatory protein ExpG to promoter regions of the galactoglucan biosynthesis gene cluster of Sinorhizobium meliloti - a combined molecular biology and force spectroscopy investigation", J.Struct.Biol., **143**, 145-152, 2003.

16. Eckel,R., Ros,R., Decker,B., Mattay,J., and Anselmetti,D.: "Supramolecular chemistry at the single-molecule level", Angew.Chem.Int.Ed., **44**, 484-488, 2005.

17. Eckel,R., Wilking,S.D., Becker,A., Sewald,N., Ros,R., and Anselmetti,D.: "Single-molecule experiments in synthetic biology: An approach to the affinity ranking of DNA-binding peptides", Angew.Chem.Int.Ed., **44**, 3921-3924, 2005.

18. Ashkin,A.: "Optical laser trapping and manipulation of neutral particles using lasers", Proc.Natl.Acad.Sci.USA, **94**, 4853-4860, 1997.

19. Smith,S.B., Finzi,L., and Bustamante,C.: "Direct mechanical measurements of the elasticity of single DNA molecules by using magnetic beads", Science, **258**, 1122-1126, 1992.

20. Binnig,G., Quate,C.F., and Gerber,C.: "Atomic Force Microscope", Phys.Rev.Lett., **56**, 930-933, 1986.

21. Lang,M.J., Fordyce,P.M., and Block,S.M.: "Combined optical trapping and single-molecule fluorescence", J.Biol., **2** , 2003.

22. Kassies,R., van der Werf,K.O., Lenferink,A., Hunter,C.N., Olsen,J.D., Subramaniam,V., and Otto,C.: "Combined AFM and confocal fluorescence microscope for applications in bio-nanotechnology", J.Microsc., **217**, 109-116, 2005.

23. Owen,R.J., Heyes,C.D., Knebel,D., Rocker,C., and Nienhaus,G.U.: "An integrated instrumental setup for the combination of atomic force microscopy with optical spectroscopy", Biopolymers, **(electronic publication ahead of print)**, 2005.

24. Meyer,G. and Amer,N.M.: "Novel optical approach to atomic force microscopy", Appl.Phys.Lett., **53**, 1045-1047, 1988.

25. Nann,T.: "Phase-transfer of CdSe@ZnS quantum dots using amphiphilic hyperbranched polyethylenimine", Chem.Commun., **2005**, 1735-1736, 2005.

26. Nikoobakht,B., Burda,C., Braun,M., Hun,M., and El-Sayed,M.A.: "The quenching of CdSe quantum dots photolumiescence by gold nanoparticles in solution", Photochem.Photobiol., **75**, 591-597, 2002.