NANOBIOLOGY: IMAGING, MANIPULATION AND FUNCTIONAL PROBING AT THE SINGLE MOLECULE LEVEL

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Abstract

In nanobiology and nanobiotechnology, characterization of biological structures, their interaction, functional processes and dynamical phenomena are investigated at the single molecule level. In this chapter, latest aspects in biophysical single-molecule methodology for molecular imaging, manipulation and functional probing are summarized. Experimental results from atomic force microscopy (AFM), single-molecule dynamic force spectroscopy (DFS), optical single molecule detection (OSMD, TIRF), scanning near-field optical microscopy (SNOM), and optical tweezers (OT) are given with implications to genomic, proteomic, and bioanalytical research.

1 Introduction

Recent developments in ultrasensitive biophysical instrumentation allow optomechanical investigation and characterization of biomolecules, complexes, and cells at the level of single molecules. This gives quantitative and direct insights into the physical mechanisms of structural and functional properties of biomaterials without ensemble and averaging effects that can be used in basic research as well as in biotechnological applications. In addition, these new optomechanical tools not only allow observation but also give access to single molecule manipulation and engineering.

There are several reasons to investigate and characterize the physical mechanisms of biomolecules, complexes and cells at the single molecule level. Beside the very pragmatic reason to save expensive material (biomolecules are expensive and often only available in tiny amounts), more important, their properties are not averaged out in an ensemble, information about distribution functions and subpopoulations are accessible, they do not need to be synchronized to be in the identical state, and they can be driven or manipulated into exotic states which will be occupied only in rare cases.

It is hoped, that the interdisciplinary character of nanobiology and nanobiotechnology to study the physics of life processes will lead to a better understanding of complex phenomena like gene regulation, molecular signalling and molecular transport in cells. Furthermore, new technologies for ultrasensitive biosensors and biodiagnostic devices will contribute to a new quantitative understanding of cell biology in the future and foster research within the framework of synthetic biology1, systems nanobiology2 and single cell analytics3.

2 Experimental

Atomic force microscopy (AFM): We used commercial AFM instruments (Nanoscope IIIa, Multimode and Bioscope, Veeco Instruments) with commercial standard Si-cantilevers (Pointprobe, Nanosensors) in tapping mode of operation for imaging. Surface topographs and phase signals were simultaneously recorded. Single molecule AFM force spectroscopy experiments were recorded with Si_3N_4-AFM cantilevers under buffer solution (Veeco Instruments and Olympus) either by the commercial built-in algorithms for force-distance measurements or a home-built force spectroscopy electronics (16bit AD/DA) combining a dedicated Labview code (National Instruments) with analogue high voltage electronics (NanoTechTools, Echandens)[4].

Optical tweezers (OT): Our home-built single-beam OT set-up is integrated into an inverted optical (bright-field & fluorescence) microscope (Axiovert 135, Zeiss) and equipped with a Nd:YAG laser (1 W, 1064 nm, Laser 2000). Additional instrumental details describing optics, electronics and liquid handling have been published recently[5].

Scanning near-field optical microscopy (SNOM): A modification of a commercial SNOM (Triple-O) that was integrated into an inverted optical microscope was used for these experiments. Fiberoptic near-field probes were prepared with a dedicated dry-etching process[3] and used with a diode pumped solid-state laser (λ=532 nm, CrystaLaser). The distance control was performed using a tuning fork as a shear force detector, where the fork was dithered by an excitation piezo. Additional information describing the mechanical set-up, optics, detectors and experimental control has been published recently[6].

Total internal reflection fluorescence microscopy (TIRFM): A TIRFM was integrated into an inverted optical microscope (Zeiss) with a TIRF objective (60x, Olympus), an Ar^+-ion laser, and a sensitive CCD camera (intensified CCD, Roper Scientific)[7].

3 Experimental Results & Discussion

The following paragraph is divided in two parts covering aspects from single molecule imaging (3.1) and functional probing of single molecules (3.2). Both sections deal with optomechanical single-molecule techniques applied to fundamental as well as applied problems.

3.1 Imaging at the Single Molecule Level

3.1.1 Imaging Single Molecules with Atomic Force Microscopy

In atomic force microscopy (AFM) [8] a sharp probing tip attached to a cantilever is scanned point- and line-wise by means of piezoelectric transducers over a surface of interest. The minute deflections as well as the change of oscillating amplitude and frequency of this mechanical force transducer are measured in the quasistatic deflection mode and in the dynamic force or tapping mode of operation, and are directly related to the force (or the force gradient) between the tip and the probed surface [9]. These cantilever deflections are nowadays optically measured by laser beam deflection [10] or laser interferometry [11] techniques (Figure 1).

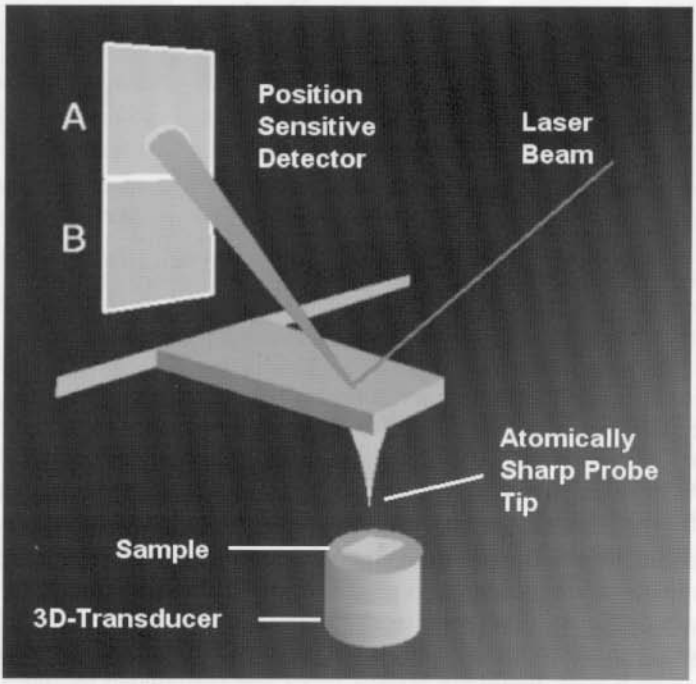


Figure 1. Scheme of AFM (Image courtesy of Basel University)

An electronic feedback loop controls and maintains these forces during the scanning process that allows reconstruction and visualization of the sample topography with a computer. The advent of this technique lies in the broad applicability to nearly all surfaces at a spatial resolution of nanometers (0.1 nm) and a force sensitivity of piconewtons (10 pN) with a temporal dynamics in the milliseconds (1 ms). This allows addressing and measurement of individual atoms and (bio)molecules without any additional staining or labelling procedures. In addition, since this technique is

immanently insensitive to the experimental environment allowing physical experiments in ultra-high vacuum at low temperatures as well as biochemical experiments under physiological buffer conditions, this technique has revolutionized our imagination and visualization capabilities from the atomistic and molecular world. In order to investigate single biomolecules with AFM they have to be immobilised on a flat surface like e.g. mica, glass, gold, or silicon. In order to maintain their functionality and to prevent them from denaturation, standard immobilization procedures have been conceived over the last ten years via bivalent counter ions (physically), thin organic self-assembled monolayer films like thiols and silanes (chemically) and/or specific ligand-receptor interactions (biologically) (Figure 2).

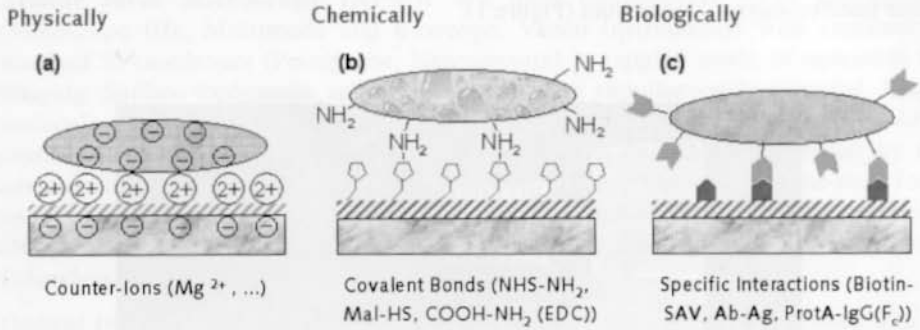


Figure 2. Molecular immobilisation schemes via physical, chemical and biological strategies.

In this book chapter we mostly made use of an immobilisation strategy where the biomolecules of interest were immobilized on aminopropylethoxysilane (APTES) terminated mica from gas phase silanisation [12] and imaging was conducted in AFM tapping mode of operation.

This surface immobilisation is, if conducted in an appropriate functional way, by no means a problem when compared to physicochemical free-solution experiments, since we obviously found no deviations from established free-solution data. Interestingly, under in-vivo conditions biomolecules like chromatin, proteins or carbohydrates in a living cell or in a body liquid are always faced and surrounded by inner surfaces like cell and organelle membranes, ribosome and macromolecular surfaces, which in turn make conventional free-solution experiments more questionable.

In figure 3, three experimental images from DNA are shown which underline the imaging capabilities of in-situ AFM applied to genomic systems, and make use of a topview representation style where the topographical variations are colour-encoded to visualize the measured features. Whereas in figure 3a circular DNA plasmids (pGEM, Promega) can be readily identified, figure 3b exhibits supercoiled circular DNA plasmids with a higher degree of conformational topology. Interestingly, this topological supercoiling can differentiate between active and non-active isoforms of the same molecule. In figure 3c, two protein-DNA complexes are shown, where two

restriction endonucleases HaeIII specifically bind to the binding sequence (GGCC) of pre-digested double-stranded lambda-DNA fragments [13].

Specific protein-nucleic acid interaction is of central importance for many cellular processes like replication, transcription, translation, expression regulation, repair, chromatin packing and many more and therefore of ongoing interest in future experiments.

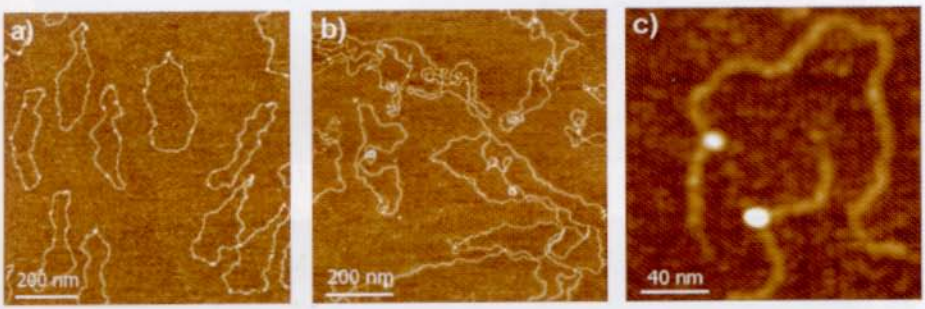


Figure 3. AFM images of circular DNA-plasmids, supercoiled DNA plasmids and DNA-protein complexes.

In figure 4, AFM is applied to immunological proteins like immunoglobulin G (IgG) and immunoglobulin M (IgM) for elucidating the structural differences between the Y-shaped IgG (150 kDa) and the pentameric IgM (700kDa). Both image galleries show an overview image on the left and subsequent 8 high-resolution images from the same field together with a schematic model on the right. Although the molecular topographs are affected by the randomness of the immobilisation, a clear difference in size and in the structural appearance is visible. Namely, the Y-shaped structure of the IgG´s and the more complex, circular-arranged domain structure of the IgM´s can readily be discerned. In contrast to earlier experiments under low-temperature vacuum conditions [14], these results nicely show the imaging capabilities of AFM on these systems also at room temperature.

Protein aggregation and fibrillogenesis of proteins is an important issue in many cellular processes like mitosis (aggregation of microtubules to mitotic spindles) or cell migration (polymerisation of actin), however, also accompanies the development of neurodegenerative diseases like Alzheimer's disease (AD). The understanding of the fibrillogenesis of _-amyloids to protofibrils and fibrils plays a central role in the plaque formation in the brain of AD patients. Current research strategies aim for developing new therapeutic drugs that interfere with the process of amyloid fibrillogenesis in order to influence and control the development of AD. The fibrillogenesis of the 40 amino acid long _-amyloid can be investigated by AFM under in-vitro conditions in time lapse AFM [15]. In figure 5, three AFM-images of _-amyloid (1-40) are shown, showing the amyloid nucleation and protofibrillogensis (figure 5a), and the aggregation of structured amyloid fibrils to protein plaques (figures 5b). In figure 5c a detailed view of an individual amyloid fibril with an axial-helical periodicity of ~ 53 nm is shown.

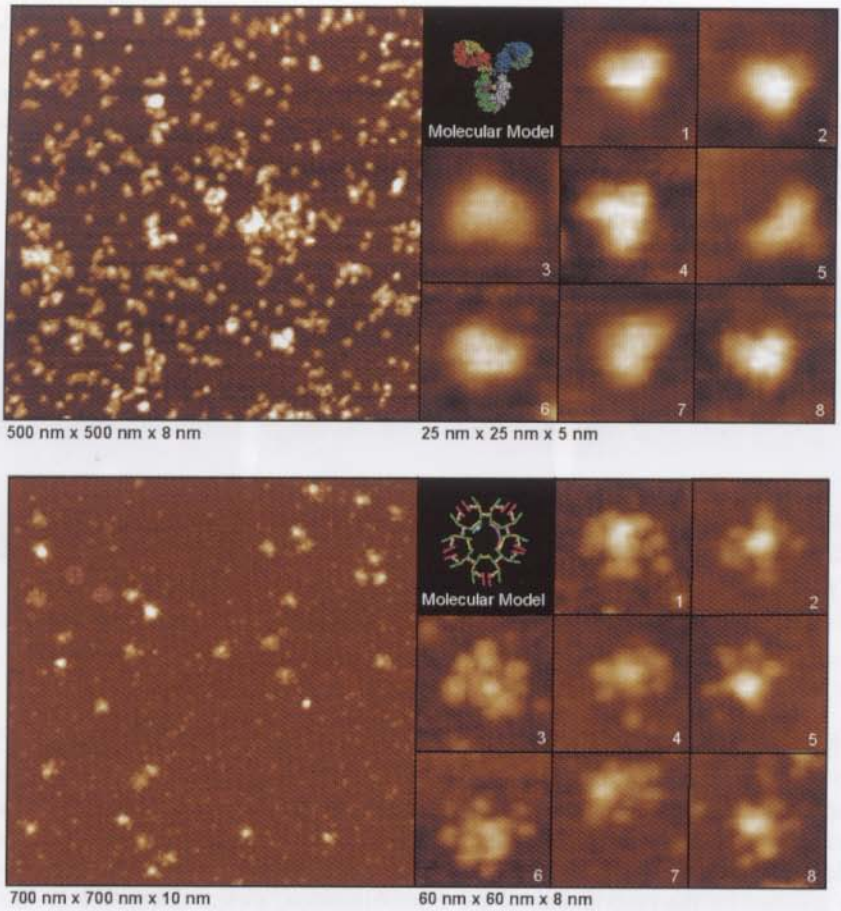


Figure 4. Serie of AFM images from antibodies. IgG´s (top) IgM´s (bottom).

The investigation of structural properties from individual biomolecules at nanometer resolution was historically covered by transmission electron microscopy (TEM) that successfully demonstrated for many decades the impressive capabilities of this high-vacuum technology which made use of sophisticated staining procedures. AFM added a new and complementary value, since it allows the investigation of native and functional biomolecules under physiological conditions without molecular staining at nanometer resolution. From this point of view, the development of AFM from an in-vitro microscopy method to an in-vivo method in order to directly measure living cells was not that far fetched. In figure 6, first molecularly resolved AFM experiments on living bacterial cells are shown [16]. The bacterium *Corynebacterium glutamicum* is a gram-positive bacterium of high industrial relevance, since it is used for amino acid production. Its cell wall consists of a number of different lipid, protein and glycocalix layers. From a structural point of view, its paracrystalline surface layer (S-Layer) composed of regularly, two-dimensionally arranged proteins is probably one of the most striking topological cell wall feature [17]. A perspective representation of a

topography image of a living bacterium is shown in figure 6a, which was measured in AFM tapping mode of operation where the cantilever oscillates with amplitude of a few nanometers close to its eigenfrequency. It turned out, that the simultaneously recorded phase signal (between excitation and real oscillation of the cantilever) carries subtle and detailed information about the surface structure of these bacterial surfaces [16] (Figure 6b). Therefore, it can clearly be discerned that the measured bacterium is currently undergoing a cell division process. A more closer look to the nanometer scale structure by AFM phase imaging even reveals molecular details like the periodic, hexagonally-packed surface structure with a periodicity of 18 nm, which fully complies with the mentioned S-layer structure [17]. These results demonstrate that scanning probe microscopy is not only limited to in-vitro experiments, but can also be applied to study living cells at the single macromolecular scale.

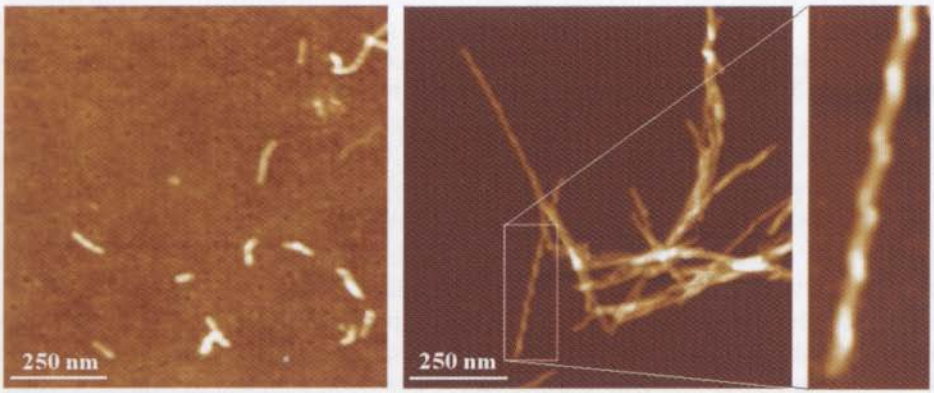


Figure 5. AFM images of Alzheimer _-amyloid (1-40) protofibrils (left) and fibrils (middle, and right). Nucleation of _-amyloids (1-40) to protofibrils and fibrils is an essential process related to plaque formation in the brain of Alzheimer´s patients.

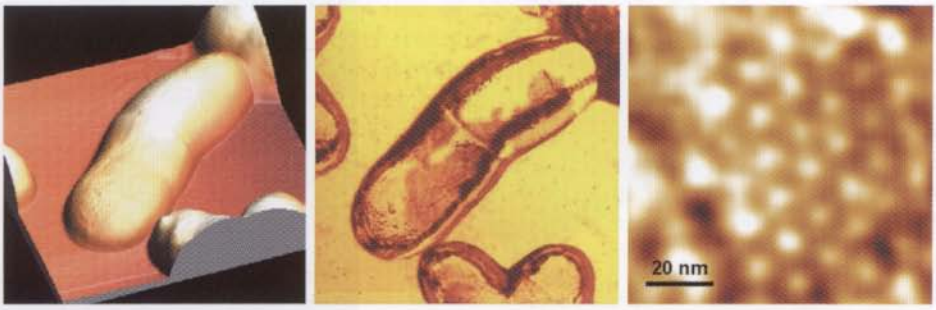


Figure 6. AFM tapping mode images from a living bacterial cell (Corynebacterium glutamicum). (a) AFM surface topography (5_m scan), (b) simultaneously recorded phase image with detailed surface structures, and (c) molecularly resolved S-layer structure in AFM phase image.

3.1.2 Imaging Single Molecules with Optical Methods

Since AFM is a purely surface sensitive technique and often limited in its time resolution for studying dynamic processes, electron microscopy limited to vacuum conditions and statically preserved molecules, there is lots of demand for a complementary microscopy with access to spectroscopic information and to dynamics at high temporal resolution. Optical microscopy is certainly the method of choice, especially, since new sensitive, single photon-counting detectors and cameras allow fluorescence detection of single molecules. In addition, optical phenomena like fluorescence resonant energy transfer (FRET) [18], single molecule dye tracing (SMDT) [19], evanescent fields and planar wave-guide technologies (PWG) [20], time-resolved lifetime imaging, statistical analysis of single molecule emission [21] or fluorescence correlation spectroscopy (FCS) [22] contribute to the renaissance of optics or photonics in biology. Furthermore, the strong rivality between the different microscopy techniques has created new striking and breathtaking concepts for improving the resolution in optical microscopy that go beyond the diffraction limits of Ernst Abbe. Namely, near-field optical concepts like scanning near-field optical microscopy [23] and far-field concepts like 4-Pi microscopy (4πM) [24,25] and stimulated emission depletion microscopy (STED) [26,27] will revolutionize optical microscopy within the next years.

Since in this chapter the main emphasis is put on single molecule detection, two examples of optical single molecule detection are given. Since most biomolecules can not directly be detected in the visible spectral range they have to be labelled with appropriate labels (organic fluorophores, green-fluorescent proteins, semiconductor nanocrystals - quantum dots) Although, this additional labelling can be regarded as a drawback, it allows spectral discrimination and identification of molecular species in a highly complex environment like a living cell. Especially, the development of new dye molecules exhibiting excellent brightness at a better photobleaching stability will foster this emerging field of single molecule optical microscopy.

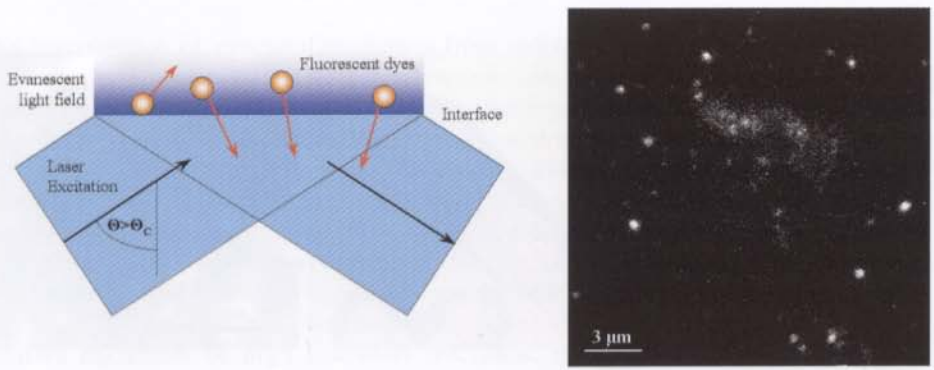


Figure 7. Optical single molecule detection with TIRFM. TIRFM principle (left) and optical single molecule detection of Alexa 488 dye molecules in polymer matrix (right).

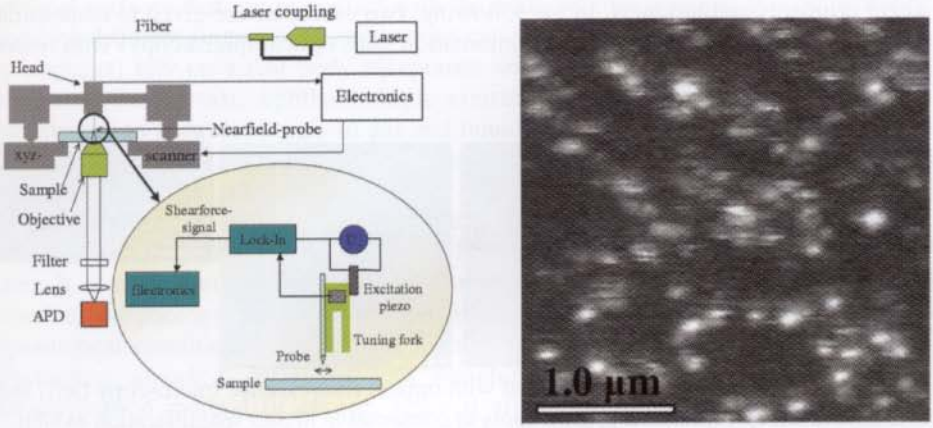


Figure 8. Single dye molecules (R6G) embedded in PVA polymer matrix imaged with SNOM.

In total internal reflexion fluorescence microscopy (TIRFM) only a very thin surface evanescent layer is optically probed by an appropriate microscopical geometry (figure 7a). This allows spatial discrimination from background fluorescence and imaging of individual fluorophores as indicated in figures 7b-d. Here, individual and well separated fluorescent dye molecules (Alexa 488, Molecular Probes) are excited with an Ar^+-ion-laser at 488 nm and detected with a sensitive CCD camera (Pentamax, ICCD, Roper Scientific) [7].

The high sensitivity of the camera combined with a fast camera electronics allows image sequences of this field of view at a repetition rate of 25 ms, exhibiting the known blinking dynamics of single molecules [28]. Although this far-field technique is diffraction limited, which results in a intensity distribution of each fluorescence maximum of _/2, individual dye molecules can readily be discerned and the position of each molecule can be calculated via fitting a Gauss-distribution to the measurement within an accuracy of some nanometers.

In contrast, the theoretical resolution of SNOM exceeds the diffraction limit and accessing the nanometer world by scanning local nanometer-sized light sources like small apertures and laser activated metallic tips to optically probe surfaces of interest. In this chapter, fiber-based SNOM is presented, where individual dye molecules (rhodamine 6G) are embedded in a solid poly(vinyl alcohol) (PVA) matrix and imaged by scanning a metallized and sharpened quartz fiber which carries a 80nm aperture in its tip [4] (Figure 8). The actual resolution of this SNOM experiment is given by the full width at half maximum of the measured fluorescence intensities of ~100 nm which is clearly favourable to the criterion of Abbe.

3.1.3 Imaging for Proteomic, Bioanalytical and Biomedical Applications

Beside these fundamental experiments which proved the optomechanical capabilities to image and measure individual molecules, these techniques can to be applied to real-

world problems and products. In the following, two examples are given to demonstrate the unique capabilities of AFM (in combination with optical microscopy) with respect of resolution and versality.

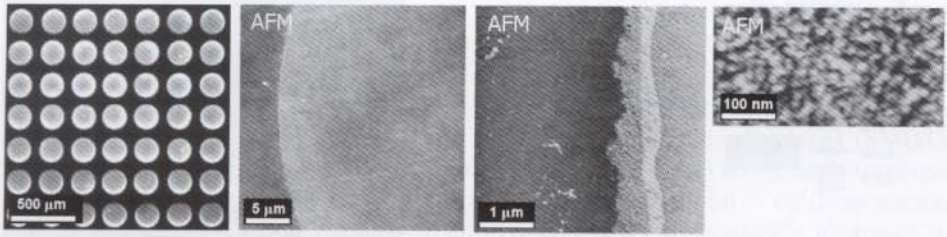


Figure 9. Protein array surface imaged with optical fluorescence microscopy (left) and AFM (others). Individual protein spots at consecutive higher magnification exhibit detailed structural information until individual proteins can be identified (right) and their density can be estimated.

Protein biochips and protein array technologies are promising candidates for future diagnostic screenings and proteomic assays. The controlled immobilisation of different proteins to artificial array architectures has to be controlled and optimized with respect of functional immobilisation strategies, protein density and orientation, reduction of unspecific binding, (fluorescence) chip readout, minimizing molecular spot crosstalk, quantification and sensitivity of readout signal. In that sense, biochip array surfaces have to be characterized and controlled from the macroscopic to the microscopic length scale.

In figure 9, a serie of fluorescence and AFM micrographs of a regular protein array (fluorescently labelled proteins) on a glass chip is shown. The fluorescence image of the protein array (figure 9, left) gives a nice overview about the homogeneity and possible edge effects that can influence the quantitativeness and reproducibility of the chip readout. Subsequent AFM images at higher resolutions give access to the topographical properties and variations in the thickness of the protein array spots that carry information about its protein coverage and homogeneity. At last, individual IgG-molecules can be identified and allow a direct estimate of the protein density.

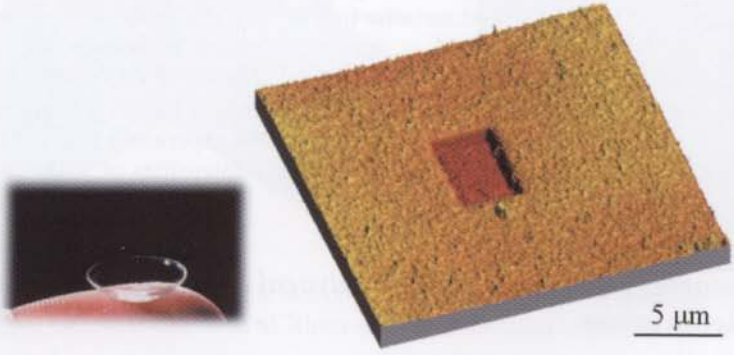


Figure 10. Contact lens surface with surface modification measured by AFM in artificial tear liquid.

Artificial surfaces that are in contact with the body rely on a proper biocompatibility for which different strategies of integrations can be pursued. Biomedical implants (e.g. hip implants) rely on a fast body acceptance and a seamless integration via cellular settlement. In contrast, ophthalmologic contact lenses strive for less interaction guaranteeing an adequate supply of gas and liquid to the eye, preventing from bacterial adhesion, and allowing a high wearing comfort. Contact lens materials are often made from hydrophobic polymers with dedicated high gas and water permeability properties that have to be coated in a hydrophilic manner. The surface of latest generation contact lenses are engineered as extremely hydrophilic hydrogels containing more than 70 % of water. It is clear that an appropriate characterization of this hydrogel coating with respect of morphology, thickness, and coating homogeneity is only meaningful under physiological conditions since characterization in dehydrated state would only give information about a collapsed and denaturated system. Here, AFM is the characterization method of choice, since it allows investigation of these delicate surfaces under artificial (and real) tear liquid. In figure 10, an AFM image of a contact lens system is taken in artificial tear liquid shown, revealing the homogeneous hydrogel coating. In order to fulfil the requirements of regulative affair issues many physicochemical properties like the coating thickness have to be delivered at the stage product "anmeldung". Since AFM is not only a microscopical method and can be used to manipulate and engineer molecules [13] and surfaces, it allows scratching and removing of a soft coating in a controlled way by increasing the partially interaction force, and reimaging of the modified surface again under low force. The squared depression in figure 10 is such a surface modification where the hydrogel coating has been removed by AFM, which allows a careful determination of the coating thickness under physiological conditions.

3.2 Probing Molecular Function and Affinity at the Single Molecule Level

Biomolecules are characterized by their molecular structure and their structure-related function, which is embedded in hierarchical processes at the cellular level and beyond. The selectivity and specificity of the possible interactions is governed by the phenomenon of molecular recognition and the key-lock principle, coined by Emil Fischer in 1896 [29], describing a non-covalent, multibinding phenomenon of chemically different and spatially arranged weak bonds like hydrogen bridges and weak electrostatic bonds like van-der-Waaals bonds. This selectivity and specificity is fundamental for an efficient cellular organisation and the manifold aspects of cellular self-organisation and regulation. The molecular recognition forces are characterized by subtle and adequate affinities, which describe the "quality" of the binding and can be characterized either by reaction equilibrium constants, binding energies, or bond lifetimes. Historically, these binding properties were defined and have always been measured on a molecular ensemble. Over the last ten years, single molecule experiments not only contributed with structural imaging to this field, but also introduced, what is commonly referred to as dynamic force spectroscopy of single molecules, and which is measurement of these binding properties at the level of single molecules. The theoretical description and experimental verification proved the seamless integration between the physicochemical ensemble and force-based single molecule concepts, which in turn is a nice verification of the ergodic principle from statistical mechanics.

In the following, two aspects of single molecule force spectroscopy are described, which should give a brief overview about current possibilities and limits to functionally probe single biomolecules.

3.2.1 *Dynamic Force Spectroscopy with AFM*

In AFM dynamic force spectroscopy (AFM-FS), binding forces between two molecules of interest are measured by functionally immobilizing them on two different surfaces (i.e. the tip of an AFM and an appropriate counter surface) that are brought in mechanical contact under physiological conditions (see figure 11). Controlled approaching and withdrawing of the two surfaces allows molecular association and forces dissociation between the molecules in contact [30]. The control of the molecular surface density, its appropriate functional immobilization (see also paragraph 3.1.1) and the introduction of convenient heterobifunctional molecular spacers to reduce unspecific background and to increase steric flexibility [31], allow functional experiments and measurements with single molecule complexes with a force sensitivity of about 10 pN [38-43].

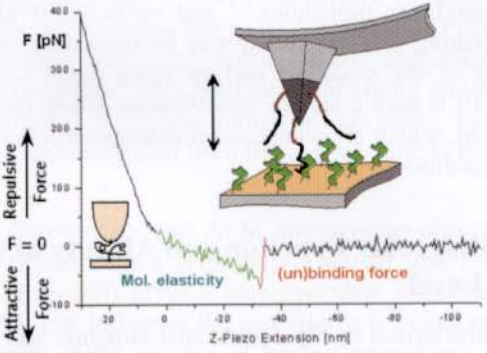


Figure 11. AFM single molecule force spectroscopy (dynamic force spectroscopy)

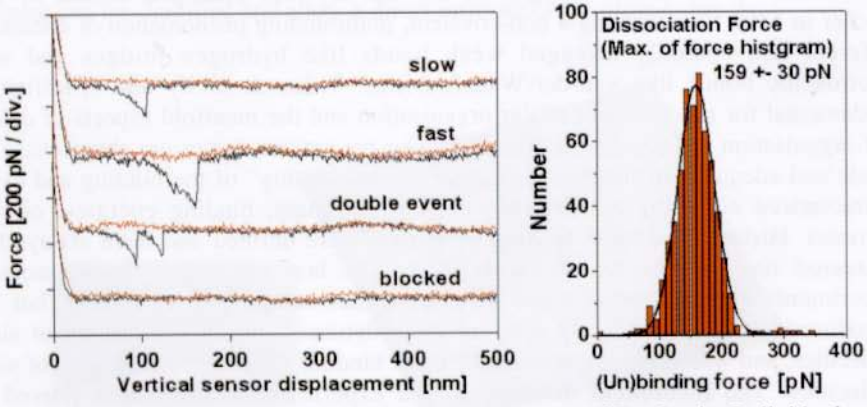


Figure 12. AFM single molecule force spectroscopy of P-selectin. Individual molecular dissociation events between P-selectin and PSGL-1 (left) and corresponding dissociation force histogram (right).

Since its introduction about 10 years ago [32-35], AFM-FS has evolved into a valuable tool for measuring intermolecular binding forces, molecular elasticities, binding specificity and reaction rate constants [38-43]. In figure 12a, three examples of single molecule force-distance curves are given from cell-adhesion molecules P-selectin that bind to their glycoprotein ligand (PSGL-1) [36]. This important cell recognition process plays an important role in the leukocyte recruitment of the inflammatory cascade and essentially relies on the mechanical force which is build-up between P-selectin activated endothelium and PSGL-1 carrying leucocytes in the blood stream at the single molecule level. Since molecular unbinding or dissociation is a statistical process of stochastic nature, many single molecule dissociation events (typically 100 or more) have to be monitored which are commonly plotted in a force histogram (figure 12). It was found experimentally that the maximum of this force distribution, commonly referred to as the dissociation force, is not a fundamental value but varies with the experimental velocity or with the molecular loading rate. The development of an adequate theory for thermal dissociation under an external force in 1997 [37] allowed quantitative interpretation of these experimental data with respect of kinetic reaction rates and binding energy landscapes. A first detailed analysis of this theoretical concept for P-selectin allowed the determination of the kinetic off-rates under zero-force and forced conditions, as well as the corresponding reaction lengths (binding potential width) measured on single complexes (see figure 13) [36].

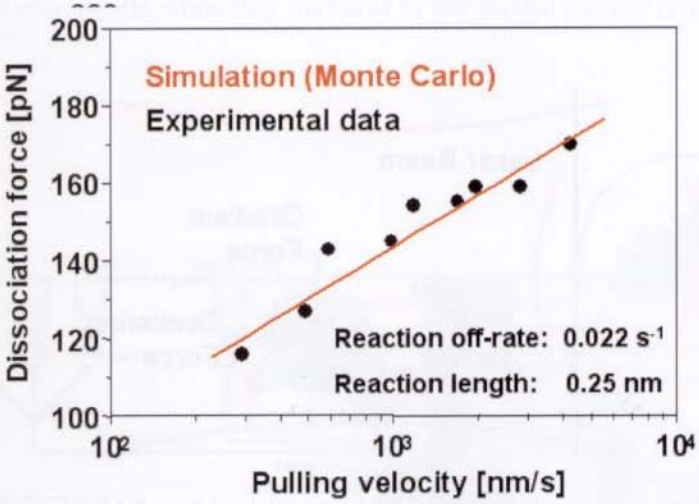


Figure 13. Dynamic force spectroscopy of P-selectin-PSGL-1 interaction. From velocity dependent experiments kinetic off-rate and the reaction length can be determined. For details see Ref.[38-43].

In the following, many specific receptor-ligand systems have been quantitatively investigated within this theoretical framework from immunological antigen-single chain antibody fragments [38], DNA-protein interactions from bacterial transcription regulation [39-41], DNA-peptide interaction in synthetic biology [42], or synthetic host-guest calixarene receptors from supramolecular chemistry [43]. It was very important to see,

that the quantitative binding analysis of single molecules via AFM-FS fully agrees with data from established physicochemical ensemble experiments from free-solution and allowed a novel and complementary approach of molecular affinity ranking [42,43]. Furthermore, the complementary approach of single molecule force spectroscopy allows an affinity ranking with the sensitivity of single point mutations in a broad affinity range of 10^{-4}–10^{-15} M without being essentially affected by solubility effects [42,43]. This novel access to the molecular mechanisms of binding, reaction off-rates, energy landscapes, equilibrium constants and binding energies will open new fascinating possibilities for analyzing complex and regulated biomolecular interaction processes in the future.

3.2.2 *Optical Tweezers*

Since its introduction in 1971 by Arthur Ashkin [44], the manipulation of micron-sized objects by light in optical tweezers (OT) has stimulated fascinating experiments in single molecule biophysics [45]. Briefly, an object, preferentially a sphere of higher refractive index, can be trapped (and therefore steered) in an electric field gradient in an viscous environment of lower refractive index by photonic forces, which are the result of the transfer of momentum during refractive light propagation through the sphere (figure 14). Since the photonic momentum is small, appropriate powerful lasers, preferentially at 1064 nm for minimal optical absorption in biological systems and with Gaussian beam profile, are commonly used in OT-technology.

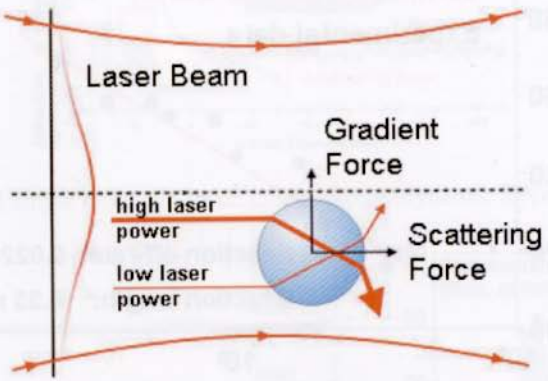


Figure 14. Optical tweezers – principle (Image adapted from S.M. Block, Stanford University)

The goal is a harmonic, optical trapping potential of adjustable depth that allows force experiments with a force sensitivity far better than 1 pN. Recently, we set up a high-stability, single-beam optical tweezers system which was integrated into an inverted optical microscope and allowed manipulation of single polystyrene (PS) beads with a force sensitivity of 0.1 pN in a frequency range of 0-10kHz up to a maximum force of more than 140 pN [5] (Figure 14).

OT can be used in single molecule experiments with DNA, where e.g. the linear 16 μm long DNA of the lambda bacteriophage can be immobilized between two PS-microbeads, one held with a micropipette and the other in an optical trap. By a controlled and mechanical separation the two beads, the molecule is extended and overstretched and has to respond via its natural molecular elasticity, which can be measured by an appropriate detection of the bead position within the optical trap potential [5,46]. The result is a molecular elasticity curve of DNA under physiological conditions exhibiting a very prominent plateau that reflects the double-stranded structure of B-DNA during its cooperative structural phase transition to S-DNA [47] (Figure 16, black curve). Interestingly, this single molecule force response can be investigated in the presence of DNA-binding ligands like dystamycin A (an antitumor peptide drug which is known to bind in the minor groove of DNA), alpha-helical peptides (major groove binders), or intercalating agents like ethidium bromide, YOYO or others. From figure 16, it can directly been concluded that binding of molecules to DNA immediately affects the molecular elasticity of the system and can be used as a single molecule biosensor to probe the binding of small molecules to DNA in single shot experiments. Most interestingly, these single molecule force elasticity fingerprints even allow discrimination of the different binding mechanisms [47].

As in AFM, OT can also be used to probe individual living cells at the single molecule level in a functional way *in-vivo* [16]. The interaction force between IgM antibodies immobilized on PS microbeads and cell membrane bound B-cell receptors (BCR) was investigated on single B-cells. The living cells were optically trapped and transferred to a dedicated micropipette, where they were hold by low suction pressure (Figure 17).

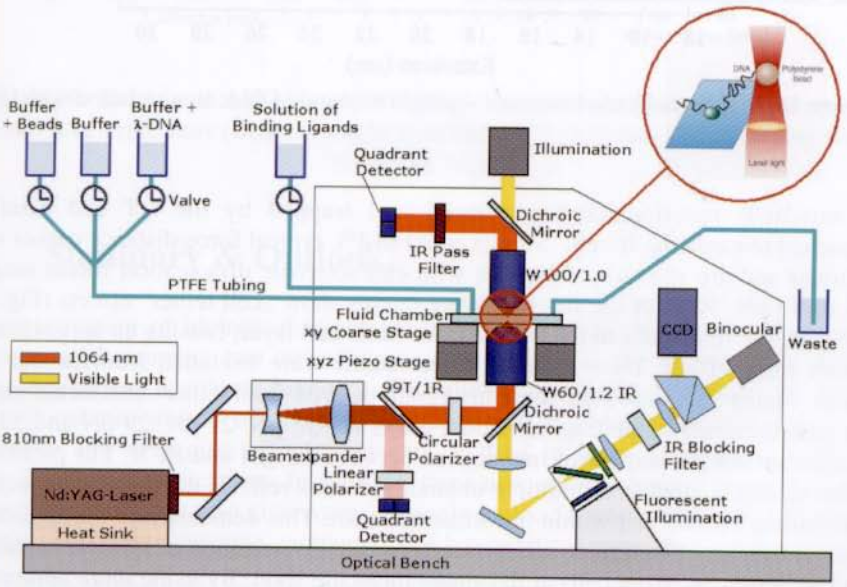
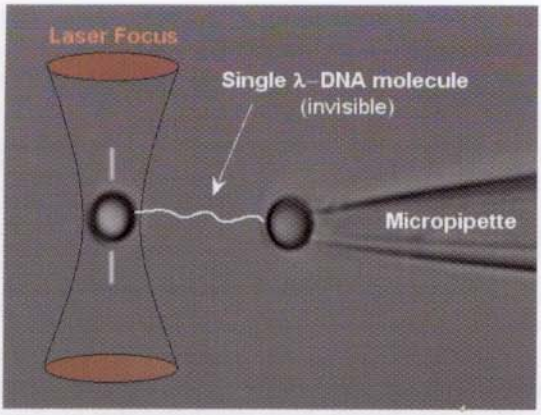


Figure 15. Optical tweezers set-up at Bielefeld University



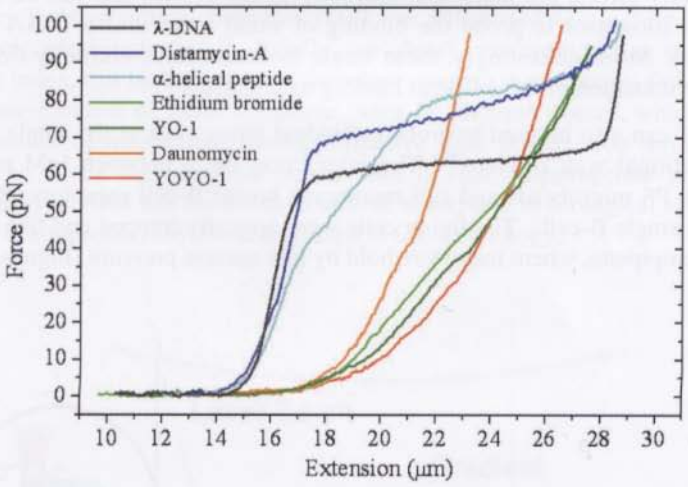


Figure 16. Single molecule biosensor – single molecule DNA manipulation with OT.

The anti-BCR functionalized microbead was trapped by the OT and carefully approached towards the B-cell. Similar to AFM-FS, typical force-distance curves were monitored and are shown in figure 18 with characteristic dissociation forces ranging from 10-40 pN. 30 % of the force-distance curves show „cell tether" effects (Fig. 18a (2)), where the IgM binds to the high viscous outer cell layer, causing an approximately constant force effect. These binding force results were excluded from further data analysis. Figure 18b shows the force histogram derived from tether- unaffected rupture force measurements exhibiting individual peaks at ~13 pN, 21 pN, 29 pN and 39 pN, indicating an integer number of interactions between msIgM and BCR. The probability to observe single, double or multiple unbinding events reflects the membrane receptor density of the probed cell within the area of contact. This demonstrates the application of optical tweezers based force spectroscopy for the investigation of specific membrane bound receptors on living cells at the single molecule level. By using more appropriate immobilization schemes in the future, dynamic force spectroscopy experiments have the potential to measure the kinetics and thermodynamics of single membrane-bound

receptors *in-vivo* and to investigate structurally hierarchic and more complex phenomena like receptor aggregation in biomembranes.

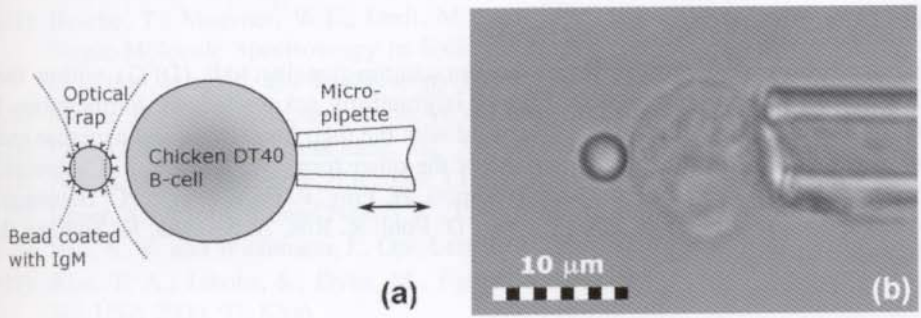


Figure 17. Single cell analysis with OT. Chicken B-cell immobilizes with micropipette is functionally probed by antibody coated microbead steered with optical tweezers.

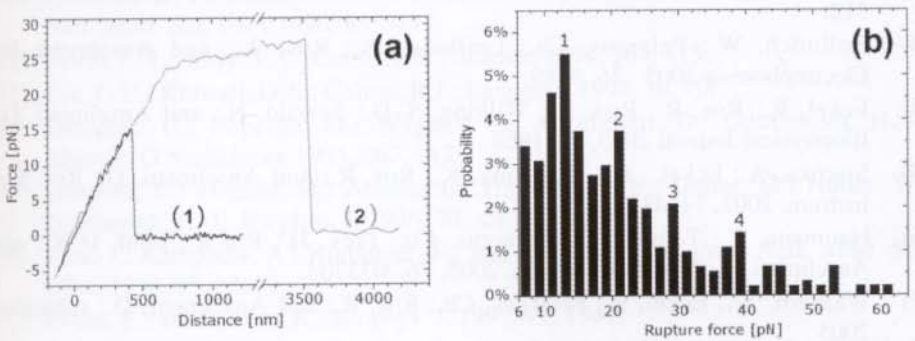


Figure 18. Single cell analysis with OT. Single rupture events between of membrane bound B-cell receptors (BCR) and IgM-type anti-BCR (left) and corresponding force histogram (right).

4 Summary & Outlook

Characterization of biological structures, their interaction, functional processes and dynamical phenomena can nowadays be investigated at the single molecule level (nanobiology and nanobiotechnology). This allows quantitative and direct insights into the physical mechanisms of structural and functional properties of biomaterials without ensemble and averaging effects that can be used in basic research as well as in biotechnological applications. In addition, these new optomechanical concepts not only allow observation but also give access to single molecule manipulation and engineering with implications to genomic, proteomic, and bioanalytical research.

Recent developments in ultrasensitive biophysical instrumentation that allow optomechanical investigation and characterization of biomolecules, complexes, and

cells at the level of single molecules were summarized and discussed on the basis of latest experimental results.

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