Single Molecule Biophysics Contributed Talks

MEASUREMENTS OF BIOMOLECULAR INTERACTIONS IN PHARMACEUTICAL & IMMUNODIAGNOSTIC SYSTEMS

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Recent advances in the immunodiagnostics and pharmaceutical industries have demanded an improved understanding of the molecular interactions that underpin their technologies. For example, the design of more efficacious therapeutic agents requires a detailed knowledge of the proposed target biomolecule or biomolecular interaction. Techniques with the ability to probe single biomolecular interactions, such as the atomic force microscope (AFM), provide a new approach for the direct investigation of such processes at a fundamental level.

In previous studies, we have employed AFM force measurements to record interaction forces between the biological molecules employed in a commercial immunoassay system[1−3]. More recently, we have employed a similar approach to investigate the influence of the number of epitopes (per protein antigen) in AFM studies of antigen−antibody interactions[4]. In addition, interaction forces have been recorded between an anti−human chorionic gonadotrophin antibody (anti−hCG) and a secondary anti−antihCG antibody, and the effect of molecular orientation on the binding force investigated. Such studies require the covalent attachment of biomolecules to the AFM and substrate surface. To this end we have immobilized proteins using either amino−silane chemistry[1] or via a poly (ethyleneglycol) (PEG) polymer tether.

A similar experimental approach has been employed in our studies in the drug−design & drug−delivery areas. For example, force−measurements have been recorded between complementary strands of DNA before and after treatment with the DNA−binding agent berenil, to investigate its influence on the recorded molecular interaction. In addition, protein coated AFM probes have been employed to investigate specific and non−specific protein interactions with novel biomaterial surfaces for drug−delivery or tissue−engineering.

Thus, results will be presented to illustrate the ability of AFM to provide fundamental data relating to molecular binding processes and surface functionality. The further development of such approaches should impact directly on the range of technologies dependent upon such processes, for example in drug−design and discovery and in the design of improved immunodiagnostics.

[1]Allen S.*et al.*(1996) FEBS Letters, 390, 161
[2]Allen S.*et al.*(1997) Biochemistry 36, 7457
[3]Allen S.*et al.*(1998) Applied Physics A.66, S255
[4]Allen S.*et al.*(1999) Biochemical Journal − in press

Interfacing Novel Detection Technologies in Single Molecule Spectroscopy with Drug Discovery

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Miniaturization is one of the key technology concepts of current high−throughput screening to meet the future needs of fast and cost−effective drug discovery. Confocal fluorescence techniques are amongst the most suitable methodes enabling mechanism−based screening on miniaturized assay formats. In particular, fluorescence correlation spectroscopy (FCS) has already been applied to a high number of assays monitoring molecular interactions where either directly or indirectly a ~ ten−fold change in molecular weight takes place. In a powerful miniaturized HTS environment, however, it is necessary to exploit additional possible single molecule fluorescence detection possibilities. Of essential importance is to resolve populations of molecules based on their respective individual brightness. A new method, FIDA, (Fluorescence Intensity Distribution Analysis), allows the determination of the concentration of fluorescent particle species as function of their specific brightness. The analysis is based on fitting the measured photon count number distribution to a theoretical model. With the assumption that the sample contains a certain number of fluorescent species, the respective concentrations and specific brightness values can be estimated. The theoretically calculated photon count number distributions are sensitive to the spatial brightness function of the equipment, which is therefore carefully characterized before sample analysis. FIDA is an appropriate tool for resolving the molecular mechanism of complex biochemical interactions. Its power can be greatly enhanced by using two photon detectors for simultaneous monitoring different polarization components of fluorescence, or fluorescence of different color, either with a single or two excitation wavelengths. Anisotropy (2D−anisotropy FIDA), 2−color FIDA, burst coincidence, and cross correlation can be analyzed within a single experimental set−up. The strengths and weaknesses of a new method are always revealed with the practical robustness like in assay development for high−throughput screening. The status of practical applications of FCS, but especially FIDA will be reviewed with examples of assay systems monitoring intensity enhancements of one interaction partner, reduction of the fluorescence intensity of one reaction partner by complexation with a protein−ligand conjugated quencher, cell surface receptor binding using vesicles and mRNA quantification

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Fluorescence Intensity Distribution Analysis (FIDA) and its Applications in Biomolecular Detection Technology Kask, P., Palo, K., Ullmann, D., Gall, K. Proc. Natl. Acad. Sci. in press.

Structural dynamics of myosin detected by single molecule fluorescence polarization.

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Muscle contraction is driven at the molecular level by the cyclic interaction of the motor protein myosin with filamentous actin. Novel techniques for single molecule manipulation and fluorescence detection now make it possible to correlate, within the same molecule and in real time, conformational states and mechanical function of motor proteins such as myosin. We have developed a spot−confocal microscope capable of detecting single fluorophore polarization to measure structural dynamics in the smooth muscle myosin light chain domain during active interactions with actin in the *in vitro* motility assay. Smooth muscle myosin from turkey gizzard was fluorescently labeled in the light chain domain by replacing the endogenous regulatory light chain with a modified regulatory light chain labeled at cysteine−108 with the 6−isomer of iodoacetamidotetramethylrhodamine (6−IATR). Unitary fluorescence polarization transients had the same temporal dependence on ATP concentration as unitary mechanical events seen in the optical laser trap, suggesting that the observed events are directly related to the myosin powerstroke. Analysis of the fluorescence polarization data from single myosin molecules indicates that the myosin light chain domain adopts at least two orientational states during the cyclic interaction of myosin with actin, a randomly disordered state, most likely associated the weakly bound actomyosin complex, and an ordered state in which the light chain domain adopts a finite angular orientation when strongly bound to actin following the powerstroke.

Study of the fluorescence fluctuation of highly dilute and semidilutesolutions by photon distribution method.

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We present the study of the fluctuations of the fluorescence coming from a tiny illuminated spot of a solution. We have investigated small dyes like Rhodamine (6G, 101), labeled latex microspheres and proteins (Bovine Serum Albumine and Beta−lactoglobuline). The experimental setup, based on an inverted Nikon microscope and modified to obtain confocal detection, is capable of illuminate about 1fL of the solution allowing the detection of even single molecules.

The fluctuations are studied by a method recently developed by E. Gratton et al. (Biophys. J., Vol 77, Issue 1) and based on the analysis of the photon counts histograms. We have extended here this method to the investigation of highly dilute solutions and to the case of light induced optical trapping. The comparison to the experimental data is satisfactory and validate this kind of analysis which provide directly the average number of molecule in the excitation volume and the molecular quantum efficiency.

A model for DNA base pairs opening

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Recent single molecule experiments have focused on the conformational changes of DNA induced by an external torsional stress. These experiments indicate that a stretched unwound DNA locally denaturate. [see T.R. Strick, V. Croquette, D. Bensimon. Proc. Natl.Acad. Sci. 95,10579 (1998)] We have introduced a model of a DNA molecule that takes into account a geometrical coupling between hydrogen−bond opening and the untwisting of the helicoidal molecular structure. Mechanical statistical calculations allow to obtain a unifying theory of the denaturation transitions of DNA, induced by an external torque (as referred to above) or driven by temperature. The denaturation transition is described in the framework of first order phase transition with control parameters being the temperature and the external torque. >From a dynamical point of view, the distribution of very short relaxation times corresponding to base pairs vibrations and torsion modes can be derived and compared to Raman spectroscopy measurements. Our theoretical results are in good qualitative agreement with experimental measures. http://xxx.lpthe.jussieu.fr/abs/cond−mat/9904277

Spectral diffusion of single hydrocarbons molecules in polycrystalline environment

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Shpol´skii matrices, i.e. polycrystalline n−alkanes have as a characteristic properties that hydrocarbons dissolved in a quickly frozen sample of these materials exhibit a drastic narrowing of the electronic transitions. Existing models for the Shpol´skii effects and the underlying microstructure of the sample are based on observation on bulk systems.

The goal of our work is to perform a systematic study, on the basis on single−molecule experiments, of the microscopic prShpol ski. As a first step we have inestig the spectral diffusion of single mothe matrix of n−tetradecane.Spectral diffusion is a powerful experimental tool to elucidate the dynamics of systems with complicated energy hypersurfaces. By cog the spectr behaviour of three differenTerrylene, diBenzanthanthrene, and for the first time Pentacene) in the same matelucid the dynamical properties of this host matrix.

Kinetics and Mechanisms in Protein Folding

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To understand how proteins fold it is essential to know the time scales and mechanism for several elementary processes. These include the formation of secondary structure (a helices and b sheets), loops, and long−range contacts, as well as global collapse of the unfolded polypeptide chain. We are using nanosecond optical methods for both triggering and observation to investigate these processes. We interpret the results with the aid of statistical mechanical models. One of the surprises from this work is that a simple statistical mechanical model, originally designed to explain the equilibrium and kinetic data on secondary structure formation in isolated peptides, calculates the rates of protein folding from their three−dimensional structure with surprising accuracy. Stochastic kinetic simulations of this model may therefore be useful in suggesting interesting candidates for single molecule investigations of protein folding.

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`Analysis of chromosome translocations, which induce chronic myeloid leukemia and their detection by means of hybridization on oligonucleotide microchips´

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The chromosomal abnormality t (9,22)(q34;q11) , known as the Philadelphia chromosome translocation was first discovered in Chronic myeloid leukemia (CML), where it is found in more than 90% cases. The translocation results in a BCR/ABL chimeric tyrosine kinase oncogene, which contains sequences from the BCR gene upstream of the second exon of the ABL proto−oncogene. The BCR/ABL gene usually transcribes in two types of hybrid transcripts with b3a2 and b2a2 junction.
The detection of these translocations was carried out by hybridization of the transcripts of the fusion gene obtained from the blood sample of the CML patient with oligonucleotide microchip.

The oligonucleotide microchip is a micromatrice of polyacrylamide gel pads (100×100×100 μm) fixed on a glass slide. Oligonucleotides are immobilized in gel pads.
Initially, the experiments were carried out with chimeric transcript obtained from the cell line K562, which has the b3/a2 translocation. Total RNA was isolated by Guanidine−Acid−Phenol extraction . First cDNA strand was synthesized from total RNA in the reverse transcription reaction . Using PCR technique and specific primers a fragment of 200 b.p. was received , which was specific for this translocation. This PCR fragment was absent, when RNA from cell line without translocation was used.
To assure, that the splice site between genes BCR and ABL was really b3/a2 with corresponding sequence, the hybridization with oligonucleotides on microchip was carried out. The b3/a2 translocation−specific oligonucleotide was immobilized in pair with another oligonucleotide with one mismatch in the central position. An analog of antisense strand was hybridized, which was obtained in asymmetric PCR from 200 b.p. BCR/ABL fragment. Very distinct hybridization and well discrimination with the mismatch oligonucleotide were received. So, the identification of the sequence in the splice site was carried out.
The next step was to apply this method to the diagnostics of CML, using the samples of blood of the patients. Total RNA was isolated from blood of the patient, who has the translocation b2/a2. The PCR product was received by RT−PCR technique. It was fluorescently labeled and used in hybridization. The immobilized oligonucleotides were specific to two types of translocation. The distinct hybridization signal with oligonucleotide specific to b2/a2 translocation in comparison to b3/a2 specific oligonucleotide has been received. So, the diagnostics of CML was done.

About the role of microtubule assembly and force generation in the positioning of microtubule asters.

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Microtubules, one of the main components of the cytoskeleton in living cells, are rigid biopolymers that assemble from a protein called tubulin, forming tubular structures of several microns length. The assembly of a single microtubule generates pushing forces on the order of a few picoNewtons (pN), that are important for instance for the motion of chromosomes during mitosis (cell division). In many cells, microtubules are nucleated by one or two microtubule organizing centers, forming so#called microtubule asters. Duringcell division two asters position on opposite sides of the cell forming the `mitotic spindle´ around the chromosomes. We study how single and multiple microtubule asters organize and position themselves in microfabricated chambers that mimic the confined geometry of living cells. Previous experiments have shown that whereas a single aster always positions at the geometrical center of the chamber, for two asters, a repulsive force leads to a steady state separation between them (T. Holy et al. PNAS 94, 28 (1997)). In our current experiment, we are interested in quantifying the relation between microtubule dynamics and aster dynamics on one hand, and in understanding the role of microtubule assembly in aster−aster interaction on the other hand.

We use colloidal spheres coated with microtubule nucleation seeds as artificial microtubule organizing centers. The choice of high index colloids allows us to use optical tweezers to manipulate the position of the organizing centers within the chambers so that aster dynamics can be studied starting from well−controlled initial conditions. Some preliminary results will be presented. Combined with sensitive

position detection of the colloids, this system will eventually be used to measure forces acting on the colloids directly

Versatile Si Sensors for the Nano Scale

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In atomic force microscopy, microfabricated silicon cantilevers are used to measure forces and distances in the pN and subangstroem range, respectively, and to manipulate single biomolecules.[1] In addition, such cantilevers can directly transduce a chemical or physical process into a nanomechanical motion. They are able to measure temperature, heat, and mass changes in the 10^{-5} K, 10^{-15} J, and 10^{-12} g range, respectively. We show that they can monitor the self assembly, adsorption and desorption of molecules on their surface from gas phase or solution. Covering the cantilevers by various metals, polymers or biomolecules, a cantilever array can be used as a chemo− or biosensor.[2]

1) J. Fritz, A. G. Katopodis, F. Kolbinger, D. Anselmetti: Force mediated kinetics of single P−selectin /ligand complexes observed by AFM, PNAS 95 (1998) 12283−12288.
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Kinetics of Single DNA unwinding by Rep Helicase

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DNA helicases are motor proteins that unwind duplex DNA to generate the transient single−stranded DNA intermediates necessary for replication, recombination and repair. We are studying the molecular mechanism of E. coli. Rep helicase using single molecule fluorescence energy transfer method. Rep helicase uses ATP as energy source to move along the DNA. Its processivity, step size, number of ATP consumed per step, existence of backward motion, and its mode of action are not known yet. We have prepared DNA substrates that are labeled with two dyes, donor and acceptor fluorophores. After DNA is specifically immobilized on a surface, Rep helicase is bound to the DNA to form pre−unwinding complex. In this form, two dyes are close to each other and result in 100% energy transfer. As we supply ATP using a flow system, Rep helicase unwinds the DNA and the time averaged separation between the two dyes becomes larger and larger until finally one strand is released. Such distance change is reflected in the gradual decrease of energy tranfer. We clearly see such signal from single DNA unwinding intermediates. Unwinding speed depends on the ATP concentration and is comparable to the speed determined from the bulk solution measurement. Less frequently, we see transient recovery of energy transfer signal, and we are investigating its relation to the helicase function. This single molecule fluorescence approach is likely to yield deeper understanding of the function of this essential enzyme for DNA metabolism.

Ligand induced conformational changes of single RNA molecules

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We present the first demonstration that fluorescence resonance energy transfer can be used to track the motion of a single molecule undergoing conformational changes. As a model system, the conformational changes of individual 3−helix junction RNA molecules induced by the binding of ribosomal protein S15 or Mg^{2+} ions were studied by changes in single molecule fluorescence. The transition from an open to a folded configuration was monitored by the change of fluorescence resonance energy transfer between two different dye molecules attached to the ends of two helices in the RNA junction. Averaged behavior of RNA molecules closely resembles that of unlabeled molecules in solution determined by other bulk assays, proving that this approach is viable and suggesting new opportunities for studying protein−nucleic acids interactions. Surprisingly, we observed an anomalously broad distribution of RNA conformations at intermediate ion concentrations which may be attributed to foldability differences among RNA molecules. In addition, an experimental scheme was developed where the real time response of single molecules can be followed under changing environments. As a demonstration, we repeatedly changed Mg^{2+} concentration in the buffer while monitoring single RNA molecules and showed that individual RNA molecules can measure the instantaneous Mg^{2+} concentration with 20ms time resolution, making it the world smallest Mg^{2+} meter.

Biomolecular processes and structures by AFM

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New biomolecular processes observed by AFM include laminin molecules waving their arms (1) and rapid imaging of DNA degradation. The DNA degradation was imaged with a new prototype small−cantilever AFM capable of capture rates faster than two seconds per image. With this fast capture rate, kinetics of DNase activity were calculated from AFM movies. New biomolecular structures observed by AFM include those of laminin−1, collagen IV, and heparan sulfate proteoglycan (HSPG). These are the major macromolecules of basement membranes, a type of extracellular matrix. The single polysaccharide chains of heparan sulfate in HSPG are visible by AFM.

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SPECTROSCOPY AND MICROSCOPY OF A SINGLE MULTICHROMOPHORIC DENDRIMER MACROMOLECULE.

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Dendrimer macromolecules with chromophores attached at the end of the dendrimer branches allow the study of multichromophoric single molecules where the number and the interaction of the chromophores can be varied in a controlled and defined way.

To this end, different generations of a hexaphenylbenzene dendrimer with peryleneimide as chromophore were synthesized. Single molecule measurements were then performed on the first generation (4 chromophores), the second generation (8 chromophores) and the third generation of the dendrimer (16 chromophores) as well as on a model compound containing 1 chromophore.

The samples where prepared by spin−coating a chloroform solution containing less then 10−9 M of the dendrimer and 3 mg/ml polyvinylbutyral on a clean glass cover slip. The excitation of the dendrimers was performed in an inverted microscope with linear polarized light of 488 nm and 543 nm. The emitted light was detected with APD's operated in the single photon counting mode. The images of all three dendrimers showed emission spots of ca. 300−nm diameter, i.e. near the diffraction limit. Fluorescence intensity traces (transients), spectra and decay times of the single dendrimers were measured. The transients reveal a complex on/off behavior and the existence of several emission levels. The spectra show large spectral jumps as well as changes in the shape of the spectrum. The time evolution of the decay time measured within one dendrimer will be discussed. Furthermore, it will be shown that this data can be used to support a model that was developed (based on picosecond and femtosecond decay times measurements and femtosecond transient absorption measurements) to explain the complex photophysical behavior of this dendrimers in solution.

Simultaneous Measurement of Individual ATPase and Mechanical Reactions by a Single Myosin Molecule at Work

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We have developed a new technique that allows mechanical and ligand−binding events in a single myosin molecule to be monitored simultaneously. We describe how steps in the ATPase reaction are temporally related to mechanical events at the single molecule level. The results show that the force generation does not always coincide with the release of bound nucleotide, presumably ADP. Instead the myosin head produces force several hundreds of milliseconds after bound nucleotide is released. This finding does not support the widely accepted view that force generation is directly coupled to the release of bound ligands. It suggests that myosin has a hysteresis state which stores chemical energy from ATP hydrolysis.

Efficiency and Energy Transduction of Molecular Motors

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We study the energetics of isothermal motors which are driven by a chemical reaction between two states and operate in contact with a single heat bath of constant temperature. We discuss generic aspects of energy transduction such as Onsager relations in the linear response regime as well as the efficiency and dissipation close to and far from equilibrium. In the linear response regime where the system operates reversibly the efficiency is in general nonzero. Studying the properties for specific examples of energy landscapes and transitions, we observe in the linear response regime that the efficiency can have a maximum as a function of temperature. Far from equilibrium in the fully irreversible regime, we find a maximum of the efficiency with values larger than in the linear regime for an optimal choice of the chemical driving force. We show that corresponding efficiencies can be of the order of 50\%. A simple analytic argument allows us to estimate the efficiency in this irreversible regime for small external forces.

VIBRATIONAL−INFRARED NEAR−FIELD MICROSCOPY

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The morphology of nanoscale objects including protein complexes can be imaged by scanning probe microscopes. We show that mid−infrared sensitivity can be added to a scanning probe tip, thus enabling to identify the chemical composition of a nanoscale object.

Essentially we have extended near−field scanning optical microscopy (SNOM) to work at long wavelengths in the range of 3−30 μm, i.e. the mid infrared region, where nearly all substances show a characteristic absorption spectrum which can be used for their identification. Fourier−transform infrared spectrometers are routinely being used in fields such as analytical chemistry down to sample sizes of about 10 μm, limited by
diffraction. Metal−coated infrared fiber tips can in principle be used to obtain better localized illumination. However, the resolution is limited to several μm by the metal waveguide cutoff. Our approach is to use an aperture−less near−field probe which has much better resolution potential. Our experiment uses a commercial cantilevered AFM tip, coated with gold, which is used in tapping mode to record the surface topography. Simultaneously the tip is irradiated with externally applied infrared laser
radiation. The tip serves as antenna structure to present a concentrated infrared field at the tip apex where it interacts with the sample material, and to reradiate the infrared radiation to a detector. The detector signal is lock−in filtered at the tapping frequency in order to extract the near−field information.

Results with metal/semiconductor test samples confirm the very high infrared resolution of 30 nm, given essentially by the tip diameter used. Purely vibrational absorption contrast could be observed with composite polymer samples at about 100 nm resolution, with contrast depending on l being on a vibrational line of one of the materials or not. This contrast is found strongly enhanced compared to what can be expected from simple extrapolation of infrared absorption spectra to small sample size. The enhancement originates from dipolar interaction with the metal tip
electrons, akin to a mechasnism active in SERS (surface enhanced Raman scattering). A widening of

the spectral coverage is foreseen to enable a truely "chemical" microscope.

Reference: B. Knoll and F. Keilmann, Nature 399, 134 (1999)

Mechanochemical Models for Molecular Motors

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A theory of molecular motors is presented that explains how the energy released in single chemical reactions can generate mechanical motion and force. In the simplest case the fluctuating movements of a motor enzyme are well described by a diffusion process on a two−dimensional potential energy surface, where one dimension is a chemical reaction coordinate and the other is the spatial displacement of the motor. The coupling between chemistry and motion results from the shape of the surface, and motor velocities and forces result from diffusion currents on this surface. This microscopic description is shown to posses an equivalent kinetic mechanism in which the rate constants depend on externally applied forces. Using this equivalence, we explore the characteristic properties of several broad classes of motor mechanisms and give general expressions for motor velocity vs. load force for any member of each class. We show that in some cases simple plots of 1/velocity vs. 1/concentration can distinguish between classes of motor mechanisms and may be used to determine the step at which movement occurs.

Coupling between chemistry and motion As a specific example, A motor protein has been animated The specific To illustrate what how these physical concepts work in a concrete case, a short movie that shows a single molecular motor moving on its track according to a simple model based on the stochastic−kinetic theory will be presented.

Molecular motors are single protein molecules that convert chemical energy, usually in the form of adenosine triphosphate, ATP, into mechanical forces and motion. Most organisms have many different motors which are specialized for particular purposes such as cell division, cell crawling, maintaining cell shape, movements of internal organelles, etc. A large number of biological motors and motor−like proteins have been discovered and characterized in recent years. (Spudich, 1994), and there is considerable variation in design and behavior among them, ranging from the two−headed "hand−over−hand" motion of the kinesins and the "rowing" motion of the myosins, to the crawling of DNA and RNA polymerases, to the proton−powered rotary motions of bacterial flagellar motors and F1Fo ATP synthases. Despite this diversity, several lines of evidence suggest that many such "mechanochemical" proteins, which use chemical energy to carry out mechanical processes, share fundamental underlying features that can be understood with the same basic concepts and theories. Together with the discovery of new motor−like systems, a growing body of experimental results has been accumulating, particularly from experiments carried out on single or few motor molecules (Kuo and Sheetz, 1993; Svoboda et al., 1993; Finer et al., 1994; Yin et al., 1995; Coppin et al., 1996, 1997; Higuchi et al., 1997; Hua et al., 1997; Mehta et al., 1997; Schnitzer and Block, 1997; Vugmeyster et al., 1998). The variables most naturally and accurately measured in such single−molecule experiments are force, distance, and time. These are also the variables of greatest functional significance for molecular motors. The availability of distance, force, and velocity as direct experimental observables is beginning to provide a body of basic facts on which well−founded theories of molecular motor function can be built. Recent theoretical efforts

Single−Molecule Mechanochemistry and Molecular Motors

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Molecular motors are single protein molecules that convert chemical energy into mechanical forces and motion. We will present a simple yet general theory of molecular motors in which the motor molecule is thought of as a small machine operating in a thermal bath, subjected to large fluctuations in conformation and chemical state. This physical picture of the motor as a microscopic fluctuating machine corresponds to a random walk or diffusion process on the potential energy surface of the system. The coupling between chemistry and motion results from the shape of the surface, and motor velocities and forces result from diffusion currents on this surface. This microscopic description is equivalent to a kinetic mechanism in which the rate constants depend on externally applied forces. The stochastic theory thus makes the connection between the microscopic view in which protein conformational changes, external forces, and thermal fluctuations are explicitly accounted for, and the macroscopic and phenomenological view of chemical kinetics.

To illustrate the connection between microscopic and macroscopic behavior in molecular motors, we have also carried out an explicit simulation of a single motor molecule subjected to stochastic forces. The simulation was then used to construct a short movie of a single motor molecule moving on its track in the presence of thermal fluctuations. The combination of statistical theory and explicit simulation graphically illustrates the roles played by chemistry, fluctuations, and friction in the overall mechanism of the motor. It also illustrates the great difference between smoothed, macroscopic views of motor proteins, and the highly chaotic situation that actually exists on the single−molecule level.

Activity of DNA polymerase observed by elastic measurements on single DNA molecules

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DNA polymerase catalyses the formation of the complementary strand of a single stranded molecule from the elementary bricks of the DNA. In the course of replication the enzyme changes the elastic properties of DNA. Due to the formation of hairpins and the stronger entropic force, ssDNA is shorter than dsDNA at forces below 5pN. At higher forces ssDNA becomes longer because of the longer contour length.Using a micromechanical setup, we observe the activity of a single polymerase in real time. As the polymerised strand can be ejected by tearing on the template strand at high forces, the experiment can be regarded as a kind of PCR with linear amplification.The sequence of the template and the force acting on the template are shown to influence the velocity of replication.

Dynamics of Extended DNA Molecules

Jens−Christian Meiners

We have studied the dynamics of single DNA molecules with optical tweezers that are equipped for ultra−sensitve force measurements. While the molecule is held at its ends with the optical tweezers, the thermal fluctuations of the restoring force are recorded. A measurement of the amplitude and correlation time of these forces both parallel and perpendicular to the contour line of the molecule shows an anisotropy in the spring constant and the hydrodynamic friction coefficient. This may indicate that an extended polymer can behave hydrodynamically in spite of its flexibility like a ridgid rod.

Positively Charged Vesicles Can Repel Negative Objects

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Charged bilayer vesicles can direct the formation of a surprising new class of colloidal aggregates. Mixed vesicles of neutral and cationic surfactant, when combined with anionic colloidal spheres, spontaneously self−assemble into complexes with close−packed, self−limiting, rafts of spheres floating on tense round vesicles. To understand the phenomenon we show how a positively−charged, mixed bilayer vesicle in the presence of negative surfaces can spontaneously partition into an adhesion zone of definite area, and another zone which repels additional negative objects. Though the membrane itself has nonnegative charge, negative counterions on the interior of the vesicle spontaneously aggregate to the nonadhering zone and present a net negative charge to the exterior.

Binding and hydrolysis of fluorescently labeled nucleotide by F1−ATPase.

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ATP synthase is located on biomembranes, such as the plasma membrane of the bacteria or inner membrane of the mitochondria, and synthesizes/hydrolyses ATP depending on a proton electrochemical potential of the membranes. F1−ATPase is the portion of ATP synthase and shows high hydrolytic activity of ATP (100/s) by itself. Recently Noji et al. designed the system to observe the rotational motion of the central gamma−subunit of F1−ATPase, and directly proved that F1 is the rotary molecular motor. To investigate ATP hydrolysis of F1−ATPase during rotation, we constructed a microscope to visualize a single fluorescently labeled ATP and its direction of polarization. TIRF (total internal reflection fluorescence) was employed to reduce the background noise from fluorophores in a medium and then each spot as a single molecule was distinguished. The intensity of spots was dependent on the direction of polarization of evanescent waves, thus we used epi−illumination with isotropic and non−polarized excitation light. A concentric opaque disk was placed at the conjugate plane of the back focal plane, then a hollow cone illumination passed through the objective with high numerical aperture. These rays were focused at the image plane with a supercritical angle for total reflection, and samples were uniformly illuminated from all directions. F1 was immobilized to a glass surface and binding of

ATP was observed under the microscope. At a low concentration of ATP (2nM), the spots disappeared and the rate was estimated to be about 0.05/s. At high concentrations of ATP (10−25nM), we observed spots that continued to reveal for several minutes on the glass surface, and some of them changed their direction of polarization with time. These results are consistent with repetitive and contiguous binding of fluorescently labeled ATP to F1, which could extend the apparent lifetime of spots and make the direction of polarization variable.

Chemical Transformations in Individual Ultrasmall Biomimetic Containers

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Recent computational studies on networks of biological signaling systems revealed interesting and nonintuitive emergent properties of the networks, including hysterisis, bistability, and oscillatory behaviors. The reductionist approach to understand such behaviors necessitates a detailed understanding of each biochemical reaction and the influence of local nanoenvironment on these enclosed reaction systems.

To meet with these demands, we have developed techniques to study complex biochemical reaction dynamics in three−dimensionally confined nanoenvironments of controlled size, physico−chemical composition, and topology, based on biochemical reaction probing using confocal LIF single−molecule fluorescence microscopy in single phospholipid vesicles, <1−to−5 μm in diameter (1,2). Chemical transformations are initiated either by electroporation (3), electrofusion, or controlled spontaneous fusion.

To create large screening libraries of liposomes with different membrane compositions and contents, fast combinatorial approaches to synthesize these reaction compartments on microchip, using optical trapping, and electrofusion were developed.

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Local environment of single membrane proteins studied by 3D−thermal noise analysis

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To study the environment of single membrane proteins, we have developed three−dimensional single−

particle tracking with microsecond temporal and nanometer spatial resolution. Restricting the motion of the particle using a laser trap enables local diffusion and trapping potential measurements within areas of about (100nm)2. The interference of the forward scattered laser light with the unscattered light is used for the tracking. The method allows to measure the elastic anchorage of the protein and the local viscous drag in the plasma membrane. The drag measurement was performed to investigate the stability, size and mobility of lipid rafts. These membrane microdomains had been implicated in polarized sorting and cellular signaling. Our results agree with a model of a cholesterol stabilized raft, that diffuses as one entity for several minutes over the cell and has a radius of roughly 10−40nm.

Myosin V is a processive actin based motor

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Class V myosins, one of 15 known classes of actin based molecular motors, have been implicated in several forms of organelle transport, perhaps working in concert with microtubule based motors such as kinesin. Such movements may require a motor with mechanochemical properties distinct from those of myosin II, which operates in large ensembles to drive high speed motility as in muscle contraction. A single myosin II molecule can only take one step per diffusional encounter with an actin filament and is therefore called a "non−processive" motor. We have performed in vitro motility and laser tweezer measurements on single myosin V molecules providing direct evidence that myosin V is a "processive" motor, advancing many 35 nm steps per diffusional encounter with its track. Processivity allows single motors to support continuous movement of an organelle along its track. We have investigated myosin V movement under load and at different ATP concentrations. A single myosin V molecule stalls at 2−3 pN and exhibits an extremely high thermodynamic efficiency near stall.

DETECTION, IDENTIFICATION AND HANDLING OF SINGLE BIOMOLECULES USING SEMICONDUCTOR LASERS AND MICROELECTROPHORESIS

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During the last few years several new very promissing detection methods have been developed to monitor the fluorescence characteristics of individual biomolecules in solution and on surfaces. However, the background due to impurities always sets detection limits, especially in biological samples containing buffer, enzymes and other macromolecular compounds. Therefore we decided to use short pulse diode lasers emitting in the red region for the fluorescence excitation of single chromophores in biological relevant fluids and surroundings. In addition, the use of pulsed excitation in combination with time−resolved detection enables a better discrimination between the fluorescence signal of probe molecules and impurities or scattered light. Furthermore, the fluorescence kinetics of single probe molecules can be used as a sensor for microenvironmental changes.

Using pulsed excitation with diode lasers in combination with mechanical manipulation by

functionalized etched optical fibers individual molecules can be handled, detected and identified. For efficient detection of each analyte molecule given in a present sample electrophoresis in submicrometer microchannels is applied. Hence, all molecules passing the detection area are counted and identified with high efficiency. Identification of the labeled analyte molecules is done by time−resolved fluorescence detection (TCSPC) in combination with an efficient maximum likelihood estimator (MLE). In combination with activated optical fibers indivdual molecules can be transferred into the microchannel and analyzed. This technique can be applied for various important applications, i.e. for single molecule DNA sequencing. State of the art and future prospects of this technique will be discussed.

Another possible application of single molecule detection techniques is biodiagnostics, i.e. the early−stage diagnosis of a viral or bacterial infection. Here, we are always confronted with the problem of discrimination between free and bound probe molecule in a relatively large excess of free labeled probe molecules. For discrimination of bound and free probe molecules independent on the change in molecular weigth either single molecule electrophoresis or so−called intelligentˆprobes can be applied. As an example, through small changes in the overall charge of peptides a precise control of the electrophoretic mobility in microchannels can be achieved. Hence, individual bound and free labeled peptide molecules can be separated, even in a 1000fold excess of free labeled peptide molecules. First results on
the development of intelligent probes which directly display information of their microsurrounding, i.e. exhibit a strong increase in fluorescence intensity upon specific binding, will be presented. These probes are ideally suited for in vivo measurements in cells and other biological interessting solvents, such as undiluted serum samples.

Multi−dimensional State−selective Fluorescence Spectroscopy of Single DNA−Molecules in Solution

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Multichannel−detection for simultaneous registration of complete information on single−molecule fluorescence (intensity F, lifetime tau, anisotropy r, and spectral range) is introduced as a new application of the real−time spectroscopic technique BIFL (Burst Integrated Fluorescence Lifetime) [1,2]. This extension of BIFL is carried out by pulsed laser excitation and using a confocal
epi−illuminated microscope with polarizing and dichroic beam−splitters. The time traces of multidimensional parameters [3] are obtained after sliding burst analysis of the registered fluorescence photon bursts generated by a single molecule diffusing through the focus.

At first, three−dimensional measurements of F, tau and r were performed to study the conformational dynamics of DNA−oligonucleotides in aqueous solutions (10^−12 M) which are single− or double−stranded and labeled with rhodamine dyes. Two major conformational states (stable in a ms−range) with characteristic fluorescence properties were found. State−selective analysis of lifetimes and intensities of these conformational states indicates different photophysical parameters, charge−transfer and additional faster conformational dynamics. This is a direct proof for further branching of the energy landscape, which allows a better understanding of dye−nucleobase interactions and DNA−

dynamics.

Finally, first results of fluorescence resonance energy transfer (FRET) between donor−acceptor−dyes investigated by four−channel measurements will be given, in which double−stranded DNA labeled, Rhodamine Green−Cy5 were investigated. Using the four−dimensional BIFL−technique, perspectives for monitoring biomolecular dynamics by FRET studies on single molecule level
will be discussed.

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Polymerization and Mechanical Properties of Single RecA−DNA Filaments

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The polymerization of individual RecA−DNA filaments, containing either ssDNA or dsDNA was followed in real time, and their mechanical properties characterized with force−measuring laser tweezers. It is found that the stretch modulus of a filament is dominated by its (central) DNA component while its bending rigidity is controlled by its (eccentric) protein component. Surprisingly, the longitudinal stiffness of DNA increases 12−fold when contained in the protein helix. Both the stretch modulus and the bending rigidity of a fiber change in the presence of various nucleotide cofactors, e.g. ATP−γS, ATP, and ADP, indicating a substantial re−arrangement of spatial relationships between the nucleic acid and the protein scaffold. In particular, when complexed with ATP a fiber becomes twice as extensible as an ATP−γS fiber, suggesting that 32% of the DNA binding sites have been released in its core. Such release may enable easy rotation of the DNA within the protein helix or slippage of the DNA through the center of the protein helix.

Single−molecule analysis of TopoisomeraseII/DNA interactions

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Topoisomerase II acts by transporting one DNA segment through another, and it can therefore relax DNA supercoils as well as unknot and decatenate DNA. Its activity is essential to cell division, since replicated chromosomes need to be untangled before anaphase. Using a DNA micromanipulation technique, we are able to study the relaxation of supercoils by individual topoisomerase II molecules. These experiments allow us to detect single catalytic cycles of topo II and to decompose the cycle into

its constituent elements. In doing so, we are able to detect mechano−chemical events which cannot be observed in bulk experiments and which help understand the functionning of this remarkable enzyme.

Force production and processivity in mutant kinesins.

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Kinesin is a motor protein which is capable of moving processively against large forces. We have been attempting to characterize the specific regions in the kinesin molecule which are responsible for these properties. It has een suggested that the head−head interaction between loop 8b of one kinesin monomer and loop 10 of the other is involved in the coordination of the two heads required for processive motion (Kozielski et al. 1997). To test this hypothesis, we replaced generated the double mutant K159E/R161E in loop 8b, which disrupts the salt bridge with loop 10. This mutant has a processivity reduced 4−fold from wild type, confirming the hypothesis. We are currently studying an equivalent charge−reversal mutant in loop 10, and hope to restore the loop 8b/loop 10 interaction by combining these two mutants.

We have also been studying force production in a previously characterized set of mutants in the coiled−coil of kinesin. We find that replacement of the neck coiled−coil with an unrelated coiled coil has only a small effect on the stall force of kinesin. Insertion of three glycines into this coiled coil results in a drop in stall force to approximately 50% of wild type. Finally, deletion of this coiled coil results in a dramatic decrease in stall force.

Investigation of energy transfer pathways in single plant antenna complexes

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Pigment protein complexes play an important role in the photosynthesis unit of plants and bacteria. In higher plants the most abundant of these complexes is the light−harvesting chlorophyll a/b protein complex (LHC−II). Confocal microscopy and spectroscopy on single LHC−II has been carried out in a temperature range of T=1.8K to 300K. At low T the emission spectra of single LHC−II show narrow zero phonon lines <2cm^{-1} and spectral jumps in a range of 100 cm^{-1}. Direct observation of single LHC−II emission opens the way to the investigation of the photophysical processes like energy transfer or excitonic coupling. From polarization spectroscopy it is clear that the fluorescence emission is originate from more then one state.

From single molecule biophysics to single molecule biochemistry: Observation of reactions in the light microscope.

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In a non−scanning fluorecence microscope it is possible to study single molecule reactions of biological relevance in an environment similar to conventional biochemistry. One example is the conversion of NAD+ to NADH catalysed by single lactate dehydrogenase enzyme molecules. Femtoliter droplets of substrate and enzyme solutions are micro−injected and mixed on a microscope cover slide. The time course of the fluorescence after the fusion of the droplets corresponds to the reaction kinetics. Since the enzyme solutions are highly diluted, the reactions are catalysed by a countable number of enzyme molecules within the droplets.The actual number of enzyme molecules that catalysed the reaction can be estimated by a statistical approach. In a second type of experiment, the handling and enzymatic cutting of fluorescently stained lambda phage DNA is studied. One end of the DNA molecule is coupled to a microsphere which is held by optical tweezers. The DNA molecule can be stretched by a buffer flow. When the buffer flow is stopped the molecule relaxes. The dynamics of this process can be quantified. The micro−injection of restriction enzymes allows the observation of sequence specific cutting of the DNA molecule in the fluorescence microscope.

Distinct Functional States of E.coli RNA Polymerase Affect its Pausing and Elongation: A Single Molecules Study

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We have observed translocation of single molecules of E. coli RNA polymerase using optical trapping micromanipulation and video microscopy. Stalled transcription complexes are formed with biotinylated DNA and biotinylated RNA polymerase. These complexes are assembled in a microscope flow chamber between two streptavidin−coated beads, one of which is held on the tip of a micropipette. The other bead is extended by a computer−controllable flow; the force load applied to the enzyme is determined by the hydrodynamic drag experienced by this bead. The flow chamber also allows exchange of buffer conditions in which the molecule under study is immersed. Addition of all four NTPs to the tethered transcription complex triggers reinitiation of transcription. Transcription is measured as a shortening of the distance
between the two beads.

Translocation of RNA polymerase as measured by this method is discontinuous. In particular, polymerase molecules pause both reversibly and irreversibly. Comparison of reversible pausing by many individual molecules indicates that pauses occur at specific template positions. Moreover, these positions seem to correspond to pause sites identified biochemically. Thus, we believe that the pausing observed by this method is identical to pausing detected by biochemical methods. In addition, all
molecules eventually pause irreversibly. These irreversible pauses occur at the same template positions as the reversible pauses and appear to be equivalent to transcriptional arrest described in bulk studies. The data also show that reversible pausing is a state off the kinetic path of normal elongation and is a kinetic intermediate between normal elongation and the arrested state. Finally, these single molecule

studies reveal that RNAP molecules exist in alternative substates with different intrinsic transcription rates and different propensities to pause and stop. This conformational metastability of RNAP revealed by our single−molecule views on transcription has direct implications for the mechanisms of gene regulation in both bacteria and eukaryotes.

Folding/unfolding of single protein and RNA molecules observed by fluorescence

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The study of bio−molecules at single molecule level allows one to look beyond the ensemble average and reveals the distributions and time trajectories of the structures and motions of molecules. This approach is particularly important for our understanding of the protein and RNA folding processes. The dynamics of protein and RNA folding are largely unknown. Modern views suggest that proteins and RNAs fold along multiple pathways. Such multiple−pathway−folding dynamics can only be directly confirmed by observing one molecule at a time. Single molecule studies can also identify the kinetic folding intermediates that can not be seen with ensemble measurements. Here, we report our results on single molecule measurements of protein and RNA folding.

We have developed a technique to study the folding of individual protein molecules, using fluorescence self−quenching due to identical dyes. Titin, a large multidomain protein, was used as the model system. Fluorescent dye molecules were covalently attached to the protein. Using an atomic force microscope assay, we have shown that dye−conjugation did not perturb the folding of titin. On native titin, the fluorescence from the dye molecules was quenched due to the proximity of other dye molecules. Unfolding of titin led to a dramatic increase in fluorescence intensity due to a decrease in the proximity of the dye molecules. Such a change is clearly observable at the single molecule level. We have observed the folding and unfolding dynamics of single titin molecules in real time. We are now extending the studies to a single domain protein. Two fluorescence molecules were attached to the two cysteine residues of the protein. Unfolding of this protein also led to a large increase in fluorescence that allows the study of its folding/unfolding processes. Mutagenesis is being carried out on this protein to move the two cysteine residues to specific sites. This will enable us to attach fluorescent labels to different positions on the protein and hopefully will reveal more about conformational changes during folding/unfolding.

The RNA folding experiment is carried out on the Tetrahymena ribozyme. It is derived from a self−splicing group I intron and has served as a valuable model system for understanding RNA folding. This RNA molecule folds into its native state through a few intermediate states. Recently, it has been found that the unfolded ribozyme can fold along different pathways that go to the native state either directly or through a misfolded state. We intend to study the folding/unfolding dynamics of the ribozyme at the single molecule level and obtain more information on the folding pathways and intermediates. To facilitate our single molecule study, the 3′ end of the RNA molecule was modified and immobilized specifically on the surface. We have shown that the immobilization didn′t perturb its enzymatic activity, namely, cleaving the oligonucleotide substrate that base−pairs with the 5′ end of ribozyme. In this experiment, the oligonucleotide substrate is labeled with a fluorescent dye molecule that leaves upon cleavage. The cleavage reaction kinetics of the ribozyme molecule is studied in detail at the single molecule level. The average reaction rates under various conditions are all comparable to the ensemble rates in solution. This is the first time that the activity of an enzyme has been studied in such detail by

single molecule fluorescence. The consistency with solution ensemble measurements shows that surface immobilization and fluorescence labeling, necessary for single molecule fluorescence measurements, does not perturb the system significantly or give distorted results. We are now labeling the ribozyme with two dye molecules to study how it folds/unfolds using single molecule fluorescence energy transfer or fluorescence quenching.

Plastic deformation of a protein

Giovanni Zocchi

We investigate the question of how a globular protein behaves under (mechanical) stress. We follow the deformation of a small number (approx. 10) of albumin molecules at constant load. We observe a slow dynamics similar to creep in ordinary solids.