# Single Molecule Techniques for Biomedicine and Pharmacology

K.O. Greulich*

*Inst. Mol. Biotech., Jena Beütenbergstr.11 D07745 Jena, Germany*

**Abstract:** The present review gives a short summary on techniques useful for single molecule research, describes experiments on *in vitro* single molecule detection and reactions of single molecules and finally reports on the behavior of single molecules and single virus particles in living cells. One experiment on single molecule enzyme kinetics of lactate dehydrogenase, an enzyme used in the diagnosis of heart attacks and one experiment on restriction analysis of individual DNa molecules are described in some detail. Where it is possible, the relevance to pharmacology and biomedicine is emphasized, often as a perspective or suggestion for experiments, since in this young field of science a not too large variety of experiments have indeed already been devoted directly to drug action.

**Key Words:** Techniques for single molecule studies, single molecule biophysics, single molecule biochemistry, proteins, DNA.

## INTRODUCTION

The history of single molecule research begins as early as 1961 with studies on the reaction catalysed by a single beta - galactosidase molecule held in a microdroplet [1]. Thus, surprisingly, single molecule enzymology, and not, for example, single molecule spectroscopy, marks the early beginnings of this exciting research field. Apart from this single isolated experiment, the first years of single molecule research were devoted to the development of techniques and to experiments in basic research. The 1990ies were the decade of high resolution single molecule spectroscopy on small molecules with lower relevance for life processes and have been recently summarised in several reviews [2-4]. From the mid 1990 ies, research on single biomolecules gained impetus, thus preparing the field for diagnostic applications in biomedicine and pharmacology. By 2003, in addition to a number of reviews on basic aspects of single molecule research, a few special issues of regular journals [5-8] and two books [9, 10] on single molecules were available in literature. A recent review has reported on the basic aspects of detection and characterization of single biomolecules and their reactions [11]. Since some of the experiments mentioned in that paper are also of interest for biomedicine and pharmacology, part of the information given there is reproduced in the present review in order to provide a sufficiently broad account on the field from the viewpoint of drug research. Also, techniques and equipment are shortly addressed which are described in more detail in other contributions of this volume. For details, the reader is referred to these articles presented by the specialists in the respective field. Note that this is not a classical review but a sketchy collection of single molecule techniques with potential use for pharmacology. Therefore, the citation list is far from complete

With the advent of single molecule techniques the ultimate limit of analytical (bio-)chemistry has been reached. What this means may be illustrated in a simple example: provided one has a single molecule sensor which can distinguish a specific type of molecule from all other molecules in the environment (human taste and smell sensors are probably of that quality), one can detect a source of molecules which emits moderate amounts of material at a distance of hundreds of kilometers, for example, a person exhaling air containing specifically smelling molecules. For quantitative details see [12]. This can explain why some people believe to "smell" and perceive events over large distances and it also may explain part of the enormous abilities of some animals to find their way over long distances. A whole class of observations may thus be transferred from the realm of parapsychology to strict single molecule biochemistry. To achieve this, knowledge on single molecule reactions of taste or smell sensors would be required which is so far not yet available. However, as a first step towards this goal the single molecule study of more commonly known enzyme reactions is now possible and one experiment on lactate dedydrogenase, an enzyme relevant for the diagnosis of heart infarction, will be reported to some detail in the present contribution.

A second type of reactions aims even more at molecular individuality: when fluorescence - labeled DNA is cut by sequence-specific enzymes (restriction endonucleases), a specific cutting pattern will be generated. Modifications (mutations) of the recognition site will result in a different cutting (restriction) pattern by which two individual molecules can be distinguished. As a bulk technique using a large number of molecules, such a restriction analysis is the basis for the identification of individuals, for example in paternity tests or in crime cases. Using it as a single molecule technique the true individuality of DNA molecules can be addressed. How important it is to regard DNA as a molecular individual becomes clear when one realizes that, in order to synthesize only one copy of each possible short DNA molecule of 120 base pairs in length, more than the visible mass of the universe would be needed (for quantitative details see [13], page 162). Knowledge on the sequence of a human "standard"- genome is only a first step

*Address correspondence to this author at the Inst. Mol. Biotech., Jena Beütenbergstr.11 D07745 Jena, Germany; E-mail : kog@imb-jena.de

1389-2010/04 $45.00+.00 © 2004 Bentham Science Publishers Ltd.

for understanding inter - individual differences. In order to understand diseases throughout large populations, single DNA molecule techniques are urgently needed.

One may ask if single molecule techniques indeed are relevant for biomedicine and pharmacology. Certainly, *in vitro* single molecules experiments can help to understand molecular processes underlying drug action in more detail than bulk experiments can do. Surprisingly, however, single molecule studies are also relevant for the understanding of the action of drugs in living cells. A short calculation makes this point clear. Typical doses in a therapy are of the order of 100 mg per dragee, tablet etc. At a typical molecular weight of a few hundred daltons this corresponds to approximately $10^{20}$ molecules (for other amounts one can scale up or down this calculation). The human body consists of approximately $10^{14}$ cells. Thus, in total, $10^{6}$ drug molecules per cell are available. Assuming that the duration of drug action is 1 hr, only a few hundred drug molecules per second are available for each cell. In practice, this number is probably much lower, due to non - ideal pharmacokinetics. In this sense, drug action is indeed a process involving, at a given time, only a small number of, albeit perhaps not exactly single, molecules.

## MATERIAL AND METHODS

### Optical Spectroscopy

Several classical techniques of spectroscopy have been developed into single molecule techniques. The most sensitive one is fluorescence spectroscopy, since it works, at least in principle, against a dark background. Thus, every photon emitted by the molecule of interest can be used to obtain information on the latter. A number of variants of fluorescence spectroscopy will be discussed below in some detail. However, other optical techniques have also been developed into single molecule techniques. The most surprising achievement has been seen in Raman spectroscopy, an initially notoriously non - sensitive technique ( see for example [14] and a contribution of this group in the present volumes). The trick is that signal enhancement at rough metallic (silver) surfaces is used, an effect which works best when the surface has a self - similar fractal topology [15]. Surface enhanced Raman spectroscopy (SERS) now allows single molecule studies.

### Near Field Microscopy

While spectroscopy allows to look into the bond structure of molecules, many questions of drug research require information on the spatial structure of molecules and complexes thereof. Such information can be obtained with high resolution microscopy. Extremely high resolution can be achieved with near field techniques. The near field microscopes all work according to a common basic principle: A sort of ultra fine stylus scans an object line by line and subsequently assembles an image from the data it has obtained. This data assembly takes some time and therefore the temporal resolution of these microscopes is limited to seconds or somewhat below seconds (however, later on in this review a recent development is described which allows to combine high temporal with high spatial resolution). The spatial resolution is better than with other tools available in molecular pharmacology and biomedicine. The reason for this is that the stylus comes very close to the object under observation. It is the same principle as the stethoscope of a medical doctor: For example, acoustic waves of a beating heart with wavelengths of metres can be exploited to localise the heart with an accuracy of centimetres. Therefore, the optical version of the near field microscopes, the SNOM (scanning near field optical microscope) has also been also called "optical stethoscope". It has a typical spatial resolution of 60 to 80 nm and can, among others, be used as a highly localised spectroscope. The second near field microscope used in the analysis of single biomolecules is the atomic force microscope (AFM). Here, a mixture of van der Waals- , electrostatic- and other forces is used to detect the molecule. Since these are all short range forces, one has to get closer to the molecule under observation and the spatial resolution with the AFM is better than with the SNOM, for ideal (hard) objects in the subnanometer range. Even the combination of an AFM with a confocal laser scanning microscope is available [16]. The best resolution among the near field microscopes is obtained with the scanning tunnelling microscope (STM) which, under favourable conditions, can detect sub - molecular structures. It needs electrically conducting objects and is therefore not generally suitable for single bio - molecule studies. Interestingly, the most successful use of near field microscopes is meanwhile not imaging but manipulation. The tip of an atomic force microscope is used as an extremely fine manipulator for handling individual molecules and for measuring forces in macromolecules such as proteins, RNA and DNA during stretching the latter. If one exerts such a force on molecules bound to each other, force - extension curves can be obtained which show a break at some specific force. This force, however, is not sharply defined. In fact one obtains a probability curve with a most probable force of a few hundred piconewtons and a FWHM of 50-100 piconewtons (see for example [17]). For a recent report on a technical variant of this force spectroscopy see [18]. AFM tips can now be prepared with an individual atom as the active tip.

### Fluorescence Microscopy, Dyes and Detectors

The spatial resolution of light microscopes is limited by the wavelength of the light which is used for observation. Thus, for a long period, single molecule detection was thought to contradict the Abbe criterion which states that the resolution of a conventional far field microscope is not better than 50 % of the wavelength (500 nanometres or 0.5 micrometres for green light). However, this was too pessimistic since spatial resolution is not always needed in single molecule research. When the molecules are prepared with sufficiently small concentration onto a surface, they have an average distance of several micrometres from each other. They have no longer to be spatially resolved but have just to be visualised. Visualisation of individual molecules is possible down to sizes of a few nanometres (see for example [13], page 274). The trade off one has to accept is that the molecules appear too large, but for many applications this drawback is acceptable. The conventional fluorescence microscope has two significant advantages compared to the high resolution near field techniques described above. It is

fast, i.e dynamics of molecules and reactions becomes experimentally accessible and preparation techniques can be easily adapted from bulk analytical methods. Even imaging by lifetimes, not by intensity, of single molecules is possible [19].

Detecting single molecules by a fluorescence microscope is not so much a problem of the number of emitted photons. Since a molecule can, in principle, emit one photon every 10 nanoseconds, up to 100 million photons per second might be expected from a single molecule. Two problems hamper this process: First, a dye molecule will decay after a few hundred up to a million of cycles of excitation and emission into non - fluorescing derivative. Second, in order to excite a dye molecule at practical concentrations some hundred million photons of the exciting light will pass the detection volume. In other words, one has a large number of unwanted photons per useful photon for the detection of the molecule. Optical filtering techniques are not completely sufficient to identify the useful photon. This is the major challenge for classical light microscopy in single molecule analysis. Part of these problems may be overcome by replacing classical fluorescence dyes by nanocrystals or quantum dots [20]. These are nanometre sized clusters of, for example, zinc sulfide capped cadmium selenide. Their absorption and emission behaviour is mainly determined by their size: small clusters are blue, larger ones are red. An advantage of these quantum dots is their very good absorption and emission behaviour, a disadvantage is their bulkiness.

When a fluorescence microscope is equipped with a standard TV CCD camera, one can obtain 25 images per second. This corresponds to a temporal resolution of 40 milliseconds. Special cameras are much faster, millisecond resolution is easily available when one is willing to go a step beyond standard CCD recording. For low level light, as in single molecule research, cooled CCD cameras integrating over several image frames can be used, but then the temporal resolution is decreased correspondingly. When extreme sensitivities at high temporal resolution are required, single photon counting cameras are available. These are in principle arrays of micro-photo-multipliers, often with 250 x 250 pixel elements. In almost all cases the reduced (as compared to standard CCD cameras with 700 x 500 pixels) image size is completely sufficient, since single molecule observation requires only parts of a full visual field in a light microscope. Colour information can be added either by using a true colour camera. They do usually not have the sensitivity described above. More efficient is the use of optical filters. Fast switching filter wheels and computer programs for exactly overlaying of the different colours are available on the market.

## FRET

FRET (Foerster / fluorescence resonance energy transfer) can increase the resolution beyond the diffraction limit of conventional microscopy. Here a pair of fluorescent molecules is used to measure distances of the order of 10 nm. One molecule of this pair, the donor, absorbs light and usually re-emits it as fluorescence at a somewhat larger wavelength. If, however, the second molecule, the acceptor, with an absorption maximum close to the emission maximum of the donor, comes closer to the donor than the "Foerster" distance, the absorbed energy in the donor is transmitted to the acceptor which finally emits light at an even larger (more red) wavelength. Thus, a red shift heralds the mutual approaching of the donor / acceptor pair to a distance closer than the Foerster distance, which, for example, is approximately 10 nm for the dye pair Cy3 / Cy5. Since typical diameters of proteins are between 5 and 15 nm, FRET experiments with this donor / acceptor pair can be used to test if a protein molecule is folded or denatured, i.e to study protein folding on a single molecule basis. Cy3 / Cy5 or other donor / acceptor pairs can also be used to study protein-protein or protein-DNA interactions as well as RNA folding on a single molecule basis.

## TIRF: Total Internal Reflection Fluorescence-Excitation

If one wants to look at the action of a few molecules close to the surface of a cell, for example to study the influence of a pharmacologically active substance on the surface of a given cell type, it is often helpful to visualize only a small layer at the cell surface. Total internal reflection [21] allows such a detailed look. If one shines (laser-) light into a microscope slide, the light will not penetrate the large faces but will be transported through the slide until it leaves it via the opposite small face. A similar effect is also known from light transmitting fibres. If one looks into the microscope, the slide will appear dark. For a small fraction of the light there is, however, an exception. Due to quantum mechanical principles, the light field leaks through the large faces of the microscope slide, approximately one wavelength deep, i.e. half a micrometre. Usually, one cannot see this. If , however, a potentially fluorescent molecule happens to be in this small volume, it will be excited and reveal fluorescence. Thus, it is possible to study single molecules in a thin layer above and below the microscope slide without illuminating all the impurity molecules in the solution. Conventional fluorescence microscopy and TIRF microscopy in single molecule research have been compared by Paige *et al*. [22].

## FCS: Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy is explicitely discussed in other chapters of this issue. Therefore, here only a short qualitative insight is given. In contrast to TIRF, FCS illuminates a diffraction limited three dimensional volume of interest, just by sharp focussing of a laser into a solution containing the highly diluted molecules to be studied. At a time " t " one detects a single molecule in a diffraction limited volume (linear distances 0.5 micrometre), and after a certan time " T ", one looks if it is still there. Slow particles will be found several times, fast particles will be found only two or three times. Mathematically, this is described by the correlation function

$G(t)$ = Sum over $I(t) * I(t + T)$, where $T$ is the waiting time

$G(t)$ is related to the speed of the molecule which can be used to calculate the diffusion constant of the molecule. The latter can be used to estimate the molecular weight. Such experiments are often used to measure the binding of two individual molecules. One example is the binding of an antigen to an antibody or the hybridisation of a small DNA or RNA oligonucleotide to a long DNA or RNA molecule [23]. Binding sites of single glucocorticoid molecules on cell

membranes have been studied by FCS by Maier *et al.* [24]. In such experiments, the smaller binding partner is made fluorescent. One will see the small partner as fast diffusing object and the complex of small with large partner as slow object. Two colour techniques [25-27], occasionally even combined with FRET [28] have now overcome an early limitation of FCS, namely the fact that the two binding partners had to differ in molecular weight by almost an order of magnitude.

## Nanotweezers for the Handling of Single Molecules

In many single molecule investigations it is mandatory to find techniques for single molecule handling, i.e. to apply a sort of gentle tweezers which allow to manipulate individual molecules. Such a tool is the tip of an AFM. It can exert and measure forces of a few hundred piconewtons up to a nanonewton. For comparison: a million of such AFM tips would be required to lift 100 mg at the surface of the earth. If more gentle handling or more sensitive force measurement is required, optical tweezers are the tools of choice. Optical tweezers (see for example [13, 29]) are made of a continuous (often infrared) laser of moderate power, which is focussed into a microscope to the theoretical (diffraction) limit. Light pressure and gradient forces push and pull microscopic objects in the focus of the laser. By moving the focus, these objects can be moved along complex trajectories. Since only forces exerted by light are acting, no sticking of individual molecules to micro-tools such as AFM tips or disruption by the latter occurs. As with AFM tips, mechanical properties of biomolecules can be measured with optical tweezers. Optical tweezers are also used as stylus, similarly as in the near field microscopy described above, to detect force landscapes on the surfaces of soft objects such as cells. This technique has been dubbed "Photonic Force Microscope" [30].

## From Concentrations to Intermolecular Distances

For discussions on single molecule reactions it is often more convenient to use the microscopic intermolecular distance "d” for a concentration instead of the macroscopic concentration value "c". The equation for conversion can be derived as follows:

$$c' = c\ [mol / l] * 0.001\ [l / cm^{3}] * 6 * 10^{23}\ [molecules / mol] = 6 * 10^{20} * c \quad (1)$$

where c' is now expressed in molecules / $cm^3$ when c is given in mol / l. The intermolecular distance can then be calculated as the inverse cubic root and becomes

$$d = 1.185 / c^{1/3} \quad (2)$$

where d is the average intermolecular distance in nanometres when c is given in mol / l. For example, the intermolecular distance at a concentration of 1 mol / l is 1.185 nm. Correspondingly, for a concentration of 1 µmol / l it is 118.5 nm.

## Femtodroplets and the Poisson Statistics

Single molecule enzyme reactions can be best interpreted, when the experiments are performed at conditions as close as possible to those in bulk reactions, i.e. when the experiments just represent a downscaling of conventional biochemistry. Particularly then, for example single molecule kinetic parameters such as reaction rates or "Michaelis Menten constants" (see later on in this review) can be directly compared with values known from conventional biochemistry. The most straightforward strategy to do that is to perform the experiments at low enzyme concentrations and in minute reaction volumes so that only one or a few enzyme molecules are in the observed volume. For example, a 1 nanomolar ($6*10^{14}$ molecules per litre) enzyme concentration corresponds to somewhat less than one molecule per femtolitre. The latter is a droplet with linear dimensions of approximately one micrometre and can be generated by pipetting with commercially available equipment well known from patch clamp or microinjection techniques.

The technique has some disadvantage, which however can be alleviated by suitable data evaluation. If for determination of the kinetic constants of an enzyme a substrate concentration in the low micromolar range is required, only a few thousand substrate molecules per enzyme molecule are available, i.e substrate consumption during the reaction has to be taken into consideration. A second disadvantage of such a strategy is, that the true number of enzyme molecules in a femtodroplet is given by Poisson statistics. If, on average, one molecule is expected to be present in a droplet, the true distribution is

$$P(n) = 1*n / n! * e^{1} \quad (3)$$

where n is the number of molecules in the droplet, e = 2.71 and P(n) is the probabilty of occurence of such a number of molecules in a droplet. The percentage is 100 * P(n). The "faculty" n ! is defined as n ! = 1 * 2 * ... * n, with 0 ! = 1.

The probability to find no or one molecule in the respective droplet is 37 % each (1 / e = 0.369). In 26 % of the droplets, more than one molecule will be found. Similarly, if on average two molecules are expected, the corresponding distribution is

$$P(n) = 2*n / n! * e^{-2} \quad (4)$$

For a mean value of two molecules per droplet the probability of "empty" droplets is 13.6 %. Droplets with one and two molecules are found with 27.2 % each. Three molecules are found in 18.1 %, four molecules still in 9 % of the droplets. In approx. 5 % of all droplets, five or more molecules are expected.

This Poisson statistics on the one hand complicates the evaluation of single molecule experiments. In turn, if one measures reaction rates for, say, 10 reactions under identical conditions and finds Poisson statistics verified, one is on the safe side to assume true single molecule conditions. This has not been considered in a number of single molecule enzyme reactions published so far, and enormous differences in individual reaction rates have been invoked. Probably, at least part of these large differences in individual reaction rates can be attributed to Poisson statistics.

# RESULTS: EXAMPLES OF APPLICATION

## Proteins

For some biological macromolecules the individual differences are not only just interesting for reasons of basic

research. Such differences may be important for health and disease. For example, the malfunction of an enzyme may cause irregularities. Malfunction may start with the occurrence of a specific variant of a molecule type, develop by a sort of evolution and may only gradually turn into a true disease form. Early detection of such changes on the single molecule basis will probably allow early diagnosis and thus may help to prevent the outbreak of a disease.

**Examples of Spectroscopic Applications**

A quite obvious step towards single molecule research is just to increase the sensitivity down to the single molecule limit [31]. One protein molecule of particular interest is the green fluorescent protein GFP, since it's mRNA can be fused with the mRNA of another protein. After *in vitro* translation, the combined protein often preserves the function of the fused protein, but is now fluorescing and its action, for example inside a living cell, can be studied. Therefore, the spectral properties of GFP are of special interest and have been studied correspondingly on a single molecule basis [32]) An interesting class of proteins are light harvesting molecules which convert the energy of light into chemical energy. Complex energy conversion processes have now been understood on a single molecule basis [33, 34]. The most straightforward study of macromolecular structure is possible if these molecules change their spectroscopic behaviour as a function of conformation. Such a molecule is the (non - biological) MEH - PPV, a conjugated phenylene - vinylene polymer. Huser *et al.* [35] could demonstrate that single MEH - PPV molecules have a conformational memory when cast from toluene, but not when cast from chloroform, where they behave as multi - chromophore systems. Unfortunately, biomolecules do not reveal such a intrinsic structure - spectrum relationship and here dye molecules have to be attached for folding studies. FRET (see corresponding chapter above) may be the technique of choice. While a number of proteins, such as crystalline from the eye lens spontaneously fold into their correct three dimensional structure, many others need the support from partner proteins called chaperones. GroES and GroEL are such proteins, and their interaction is essential for protein folding in the bacteria E.coli. Using Cy3 and Cy5 as FRET partners, Taguchi *et al*. [36] could show that both molecules bind with kinetic constants known from bulk experiments. However, only the single molecule experiments could reveal that the two molecules remain in contact for approx. 3 sec. This is the time a target protein has left in order to fold into its correct structure.

**Force Spectroscopy of Selected Proteins and Protein DNA Interactions**

A field which appears at a first glance quite unrelated to pharmacology or biomedicine is "force spectroscopy". As already mentioned above, the tip of an atomic force microscope is used as a nano - mechanical tool. One end of the molecule is attached to a surface, the other end to the AFM tip. Then the tip - bound end is pulled and the force is measured which is required to pull the molecule apart. Also, the force acting between two molecules can be studied. Essentially, the force acting between two inteacting molecules is measured at different loads and at different speeds of pulling. Surprisingly, experiments of this type indeed have relevance for drug research. For example, when leukocytes search for a region of inflammation inside the body, they move through a blood vessel in a rolling manner. In a region of inflammation, this rolling motion is stopped by capture molecules and the leukocyte is attached to the vessel walls, which it penetrates to migrate finally to the site of disease. An important molecular interaction is the binding of a selectin molecule to a P-selectin glycoprotein ligand-1 molecule. This binding process has been studied by Marshall *et al*. [37]. The surprising result was that the bond lifetime is prolonged by moderate forces (catch force) but shortened by larger forces (slip force). It is immediately clear that drugs which can modify this binding force will also be able to modify recognition of disease sites by lymphocytes.

P-selectin - ligand binding, also mediates adhesive interactions in immune recognition but also in metastasis of several cancer types. Forces up to 175 piconewton, i.e. 1 / 5 th of a covalent bond, can act [38]. The rate constant for unbinding may vary from 0.2 $s^{-1}$ up to 2.8 $s^{-1}$. Such a pronounced difference explains different adhesion behaviour of the cells which use P-selectin. A molecule with similar function is fibronectin. Its mechanical properties are critical for the elasticity of the extra - cellular matrix and connective tissue. Oberhauser *et al*. [39] have found an extreme extensibility of this molecule. The molecule has a hierarchical force profile, indicating that it has different building blocks with different mechanical stability, particularly its FNIII region.

The von Willebrandt factor is a protein involved in many aspects of protein clotting in blood. Occasionally it comes in ultra - large aggregates (ULVWF), particularly in catastrophic micro - angiopathic disorders. Using optical tweezers, Arya *et al*. could show that specific conformations, and not so much the size of the aggregates, is critical for the interaction of ULVWF with platelets, cells which mediate the generation of thrombi in blood [40].

Titin, a tandem array of fibronectin (see below) and a sort of immunoglubulin, is anchoring the motor protein myosin of muscle (see below) to elements of the skeleton. If a muscle is slightly over - stretched, titin dampens the forces and reversibly unfolds. Only after strong over - stretching the muscle is hurt. Due to this importance titin is among the molecule best investigated by single molecule force spectroscopy. Just to mention one work, Grama *et al.* [41] have labelled titin with the fluorescing rhodamine which, due to self quenching when two rhodamines are approaching each other, act similarly as the donor / acceptor pairs described above. Surprisingly, chemical denaturation of titin leaves the titin molecule intact, perhaps since acidified muscles need to keep their mechanical stability.

Force spectroscopy has also been used to study the binding of restriction enzymes (see later on in this review) to their specific binding sites on DNA. An activation barrier for the disruption of protein DNA binding has been found [42].

**The Interaction of Antibodies with Their Antigens**

The binding of an antibody to its antigen is a crucial step in the response of the immune systeme to infection.

Modifying this process by drugs means getting influence on the progress of disease and modifying the efficiency of the immune response. Tetin *et al*. [43] have shown that the equilibrium dissociation constant of an antibody against the test antigen digoxin yields the same value if fluorescence polarisation or FCS is used, i.e. both techniques are suited to determine such constants. A disadvantage of these techniques is, that they are performed in solution, while typical antigen antibody reactions occur at surfaces. Such a surface studies were performed by Seeger *et al*. [44] and Li *et al*. [45]. With tapping mode atomic force microscopy they monitored the binding constant of two different antigen/antibody pairs at near physiological conditions in molecular monolayer consisting of the antibodies. The binding constants were essentially determined by counting the ratio of antibodies with and without bound antigen. This technique is obviously also suited to evaluate antibody micro - arrays. Since the Y shaped antibody molecule has two binding sites for antigen, it is interesting to know if both act independently or co - operate. This question has been tackled by using an AFM simultaneously as microscope and a tool to measure forces. The unbinding force for an antibody against serum albumin has been measured as 244 + / - 22 piconewton and an action of both binding sites independent on each other has been detected [46]. The binding to individually addressed antibodies has been measured by Ros *et al*. [47]. Jermutus *et al*. [48] have co - expressed and co - translated single chain antibody Fc fragments with ribosomal mRNA, i.e. they displayed them on these large molecules. Then they could study antigen binding on a single molecule basis using FCS and determine kinetic constants of that process with good statistics.

## Molecules Generating Motion (and Life Processes) : Kinesin, Myosin, Dynein

Molecular motors are molecules generally converting chemical energy into motion. Some small organic molecules are known to act as motors. They will not be discussed here. (Bio-) molecular motors govern processes characteristic for life of cells and organisms, such as generation of motion in eukaryotic cells, cell division, cell movement, segregation of genetic material or positioning of organelles. Due to their fundamental function biomolecular motors are used as therapeutic targets (for example taxol treatment in cancer chemotherapy.) Linear motors move along filamentous macromolecules or macromolecule assemblies which act as a kind of track. Prominent examples are kinesins and dyneins, that belong to motor families with more than one hundred members generating motion along microtubules by hydrolysing ATP as a fuel. Another motor is myosin in its numerous variants, well known for generating motion along actin micro - filaments and thus contributing critically to muscle contraction.

The motor proteins kinesin, dynein and myosin consist of an elongated stalk-like tail and one or two globular heads binding ATP as well as microtubules respectively actin micro - filaments. Upon ATP consumption they undergo conformational transitions connected with walking along the filamentous tracks. Thereby, the angle between the tail and the head is changed causing, if bound, a stroke driving the head forward in relation to the track. After such a stroke the head is released, swings back to its "starting” position, binds to a new site on the track, and is loaded by new ATP. The process is ready for repetition. In principle, most of the related questions to motor function and regulation can be answered by bulk experiments comprising some or many motor molecules. For example, the force generated by a muscle filament can be measured macroscopically. The number of myosin molecules involved in this process can be determined and allows to calculate force generation per myosin molecule. But, one problem arises immediately: The duty ratio, i.e. the ration of the active period of the motor to the whole molecular cycle. It represents the fraction of molecules of a collective which is involved in force generation at any given instant. Depending on the model used, the duty ratio can be estimated in the range between 5 and 90 % - resulting in a factor of 20 in uncertainty. Therefore it is essential to measure these mechano-chemical processes on a single molecule basis. One way to measure single molecule mechanics is to use fine glass microtools, contributing, however, inherent problems such as adhesion and perturbation of the tiny forces involved. Optical tweezers are a second tool for single molecule motility research, allowing to evaluate both techniques against each other. Due to the high interest in these questions , a large number of single molecule experiments have been described in early literature by the groups of Block, Molloy, Schliwa, Sheetz, Simmons, Spudich, Warshaw, Yanagida. These work are reviewed, for example, in [13], chapter 4.3, by Vale and Milligan, [49] and by Ishii and Yanagida [50].

More recently, Nishiyama *et al.* [51] have studied the chemo - mechanical coupling of forward and backward stepping of kinesin. They formulated a model where the driving force for motion along the microtubule is Brownian motion and where the energy is primarily used to decrease of entropy, i.e. by giving this motion a direction. In similar experiments, [52] dynein was investigated and the related molecule dynactin which can move bidirectionally along microtubules. The authors postulated that dynactin enables dynein to participate efficiently in this bidirectional transport. Rock *et al.* [53] reported that Myosin VI is a highly processive motor with very large step size. The maximum step size reported so far is 35 nm for higher plant myosin XI [54]. Baker *et al.* [55]) have given the basis for understanding the ATP reaction which provides the energy under minimal load. They found that the dissociation on one phosphate group is more tightly related to the generation of motion than could be demonstrated before. An idea how single molecule studies may be related to the multi - molecule interaction in real life, was given for kinesin by the work of Peterman *et al.* [56]. These authors compared the relative orientations of monomeric and dimeric kinesin and showed that both kinesin molecules have to bind with similar orientation in order to reveal optimal activity. Among others, such investigations predict that biomolecular motors are active to transport artificial loads, that they can be regulated and tuned under cell free conditions and that they are able to work in predetermined directions [57].

As for technical motors, rotatory molecular motors also exist. F1 ATP ase is a representative member of this class of molecular motors and converts chemical energy of ATP into rotation. Interestingly, it can perform also the reverse task:

when rotation is induced by exerting force on the molecule, it converts the low-energy -ADP into high-energy-ATP, i.e. it converts mechanical into chemical energy. In a typical experiment, a fluorescence labelled actin filament is bound to one of the subunits of the F1 ATP ase. The ATP ase is fixed to a surface. The actin filament can be easily observed by fluorescence microscopy and manipulated by micromechnical tools or optical tweezers. If left free and supplied with ATP, the ATP ase rotates. This can be observed by rotation of the actin filament. A study of this type is for example that of Kato -Yamada [58].

### Visualising the Action of Individual Dynein and Myosin V Molecules in Real Time

The probably most exciting recent development in single molecule research results from the possibility to obtain movies from working individual molecules. This does not mean the somewhat indirect visualisation of molecule action as described in the previous chapter. Indeed, meanwhile it is possible to have a direct look on the dynamics of individual biomolecular motors. Burgess *et al*. [59] have organised a temporal series of electron microscopic snapshots of dynein c molecules into a sequence of events which allows to reveal how dynein generates motion along microtubules. They could show, that the stalk like tail performs structural changes which may be required to generate motion. Even more exciting are the results of Ando *et al*. [60] using a home made atomic force microscope with a temporal resolution of 80 ms, i.e. half the frequency of commercial TV video. These studies with myosin V directly reveal that the stalk of the myosin molecule is very flexible and that there are changes in lengths of this arm, indicating, similarly as the dynein data, that structural changes of this part of the molecule, and not so much changes in the relative orientation of the heads of these molecules, generate motion. Thus, the mechanisms developed in the more indirect experiments described in the previous chapter may need some modification in order to allow for these recent observations.

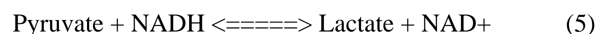
### Single Molecule Enzyme Kinetics

While the experiments just described give a detailed insight into basic processes of life and may thus indirectly be used in drug research, scaling down common enzyme kinetics to the single molecule level has the potiential to be used directly in diagnosis and in the search for new drugs. From protein structure considerations one expects that individual enzyme molecules of a species would differ slightly but distinctly from each other and thus may show different reaction behaviour [61]. This may be reflected in different kinetic constants such a maximum velocity or Michaelis constant. If the variety of kinetic constants can be ordered into a few groups one speaks of isoforms of the enzyme, but one should be aware, that a much larger variety of individual forms of the enzyme exists, which may only be detectable with single molecule enzyme kinetics.

From conventional biochemistry (enzyme concentrations $10^{-9}$ mol / ml or $10^{13}$- $10^{14}$ enzyme molecules) the step to single molecule enzyme kinetics involves an increase in sensitivity of at least 13 to 14 orders of magnitude. In recent years, enzyme kinetics has been developed down to the single molecule level. The conformational dynamics and cleaving mechanisms of enzymes has been directly observed on surfaces [62]. The group of Xie has used the natural fluorescence of cholesterol oxidase, which is fluorescent in its oxidized form. As the reaction proceeds the enzyme oscillates between the oxidised and reduced states, i.e. it blinks. Each blinking corresponds to one enzymatic turnover, which was followed with time (see for example [63]). Also, fluctuation of reaction rates of one cholesterol oxidase molecule was observed. Earlier, Dovichi and co - workers [64] had used a similar approach to confine single molecules of alkaline phosphatase and to measure the reaction products completely inside a capillary. A clinically interesting enzyme is lactate dehydrogenase(LDH). Xue and Yeung [61] had studied this reaction at selected substrate concentrations in a capillary tube equipped with a LIF detector and found different activities for individual molecules. As an alternative, the "droplet in substrate” technique, where the reaction proceeds at the surface of a highly viscous droplet containing a few LDH molecules which was previously injected into a substrate solution allowed very detailed studies on this reaction [65]. Quantised reaction rates have been found. The rates were multiple integers of a basic reaction rate. Using equation (3) and (4), above, this was interpreted as the reaction catalysed by one, two ....up to six enzyme molecules [66], Since the LDH reaction is exploited in the diagnosis of heart attacks, the following chapter shows in detail how techniques of classical biochemistry can be scaled down to (almost) the single molecule level:

### Classical Enzyme Kinetics in the Single Molecule Range

The LDH reaction is particularly suitable for single molecule research, since it involves a fluorescent substrate which is converted into a non fluorescent product, i.e the reaction can be observed by monitoring the disappearance of fluorescence. NADH, which fluoresces at 440 nm when excited at 360 nm, is converted into almost non-fluorescent NAD+ in the overall reaction of lactate dehydrogenase:



Pyruvate + NADH <=====> Lactate + NAD+ (5)

In the here used "femtodroplet pipetting method" (in contrast to the "droplet in substrate technique" mentioned above), the reaction proceeds in a capillary. In equidistant time intervals, typically every few minutes, femtodroplets (a few μm in diameter, i.e $10^{20}$ femtoliters) are pipetted onto a glass surface and the fluorescence is measured. In reactions, where fluorescence is reduced, the fluorescence intensity of the droplets decreases with time. The fluorescence can be measured by image analysis with a suitable program, here the program ”NIH image” (Fig. **1**).

A check with no enzyme present showed that the decrease in fluorescence is not caused by photobleaching. The brightness of the spots is translated into an intensity vs. time curve. The slope is interpreted as the reaction rates v. When the reaction rates v thus determined at different substrate concentrations [S] is plotted according to eq. (6) (i.e 1 / v versus 1 / [S], see Fig. **2** below) two typical parameters of biochemistry, the maximum rate $v_{max}$ and the Michaelis Menten constant Km can be determined:

$$1 / v = 1 / v_{max} + Km / (v_{max} * [S]) \quad (6)$$

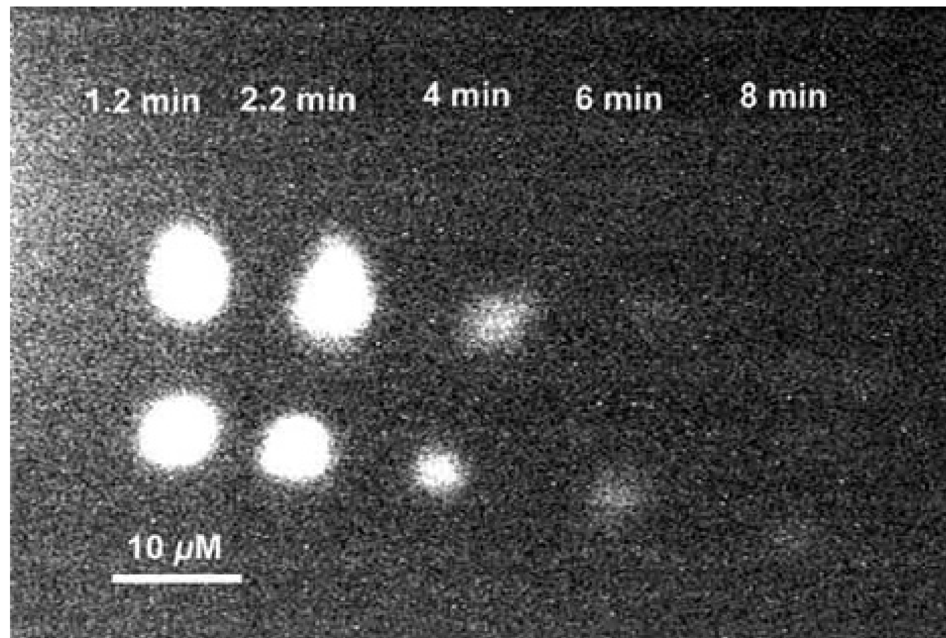



**Fig. (1).** Two rows of femtodroplets. An obvious decrease in fluorescence intensity can be observed which is not caused by bleaching, since in the absence of enzyme the fluorescence would not change with time

Equation (6) and the plot in fig. **2** follow from the Michaelis-Menten equation are called the Lineweaver Burk plot. Its slope gives Km / $v_{max}$, its intercept with the x-axis gives 1/Km, from which Km can be immediately calculated.

The result of this experiment is that the Michaelis Menten constant can be determined for single molecules with reasonable accuracy. However, the pattern seen in Fig. **2** indicates some so far not yet understood non-competitive inhibition of the single molecule reaction by some so far unidentified impurity. Another result could have been, that all lines have a common intersection point on the y axis instead the x axis (competitive inhibition) or that all lines are parallel (uncompetitive inhibition).

Fig. **3** shows the result of a series of experiments covering several weeks. Though the Michaelis Menten constant reveals considerable day to day differences, such a variation is also seen with conventional enzyme kinetic experiments and is within the range of Michaelis Menten constants published in literature. It should be stressed here again that more than ten orders of magnitude in the number of enzyme molecules lies between the bulk data and those obtained with single molecules.

**Biophysical Properties of RNA, DNA and Chromatin**

As mentioned in the introduction, the variability of RNA and DNA is virtually unlimited. Textbooks show DNA molecules as extended filamentous structures. Single molecule experiments show that in physiological environment, i.e. at salt concentrations around 150 mM, this is not the case. DNA tends to collapse into a globular structure [66]. Fig. **4** shows micrographs of this process.

A plot ot the apparent length of the collapsing DNA

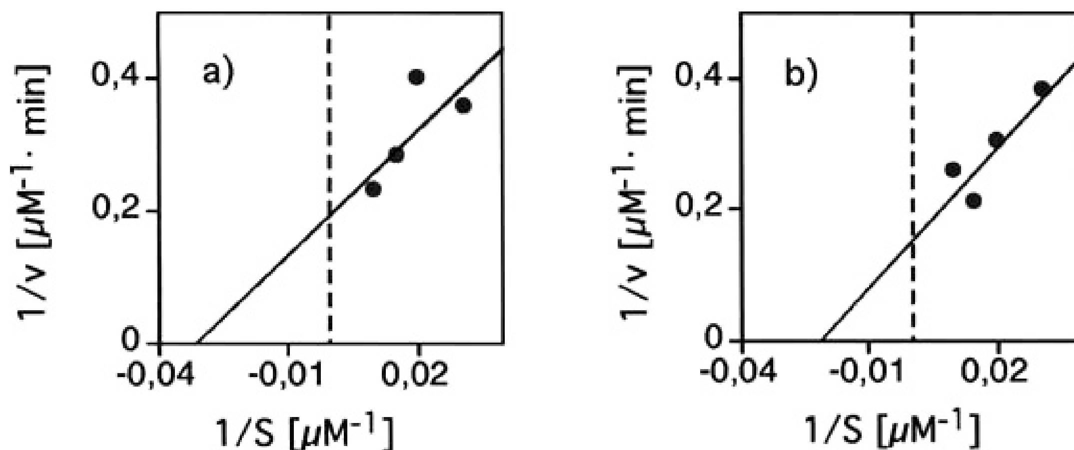



**Fig. (2).** Examples of Lineweaver-Burk plots obtained using femtodroplet pipetting. The LDH concentration was approximately 244pM or 1,5 enzyme molecules per 10 fl. The plot in a) yields Km=31μM, the one in b) Km=45,9μM.

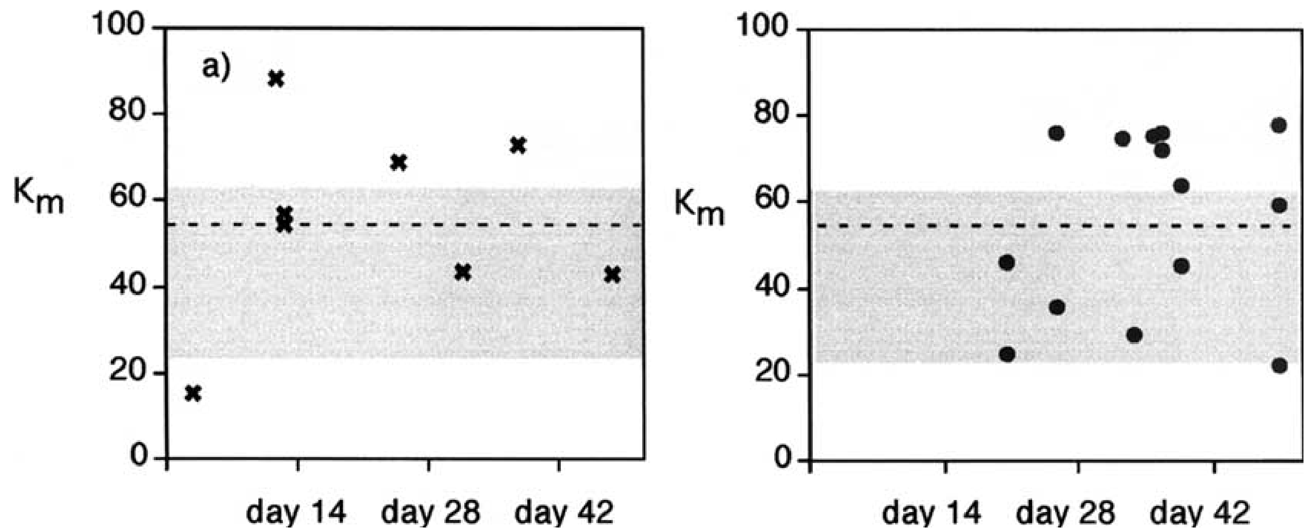



**Fig. (3).** a) Bulk Km values obtained on different calendar days by conventional analysis b) Km values determined on different calendar days by femtodroplet pipetting. The grey areas indicate the range of bulk Michaelis constants given in literature.

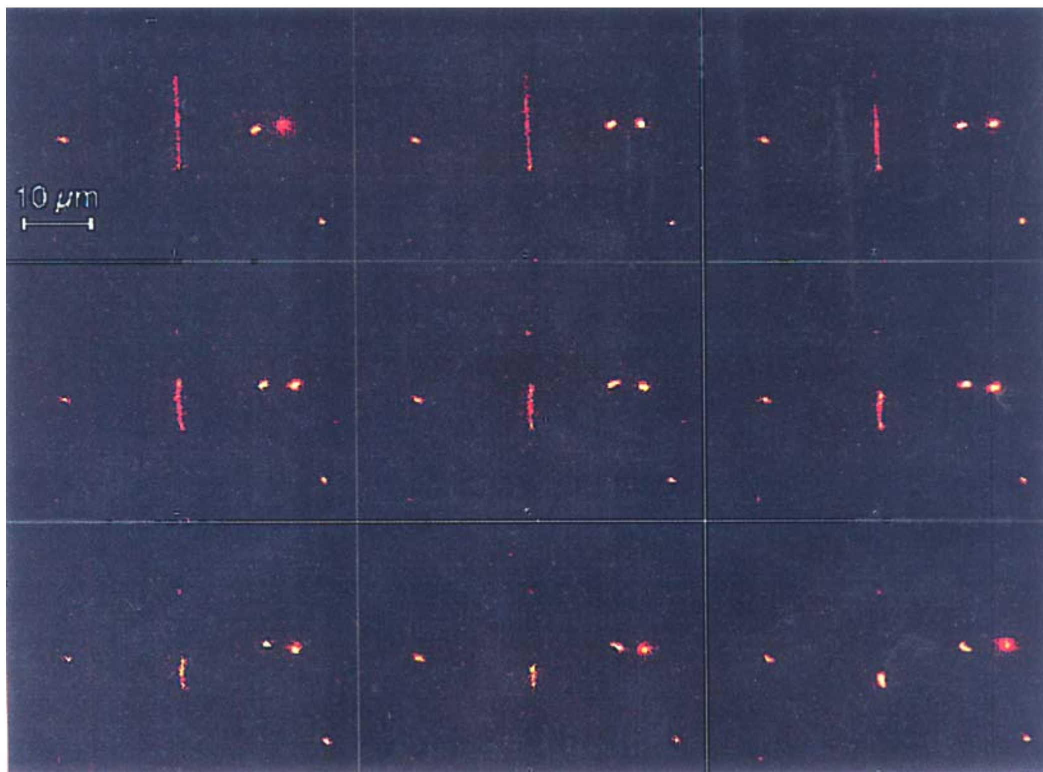



**Fig. (4).** Collpse of a single, SYBRGreen stained DNA molecule, after the contact to the surface of the microscope slide has been released.

molecule versus time is shown in Fig. **5**. Also shown is the time course of the apparent length in the presence of the antibiotic netropsin and SN6113, a substance which is presently tested as anticancer drug. Both substances are minor groove binders and it is evident that they stiffen the DNA molecule. Such an effect has also been observed after binding of histone like proteins to DNA [67]. So far, this effect is just an interesting observation. It is not clear if it can be exploited for drug action. As a speculation, stiffening DNA may make it more accessible for a second group of drugs which either enhance degradation of DNA in cancer cells, or it may increase access of repair enzymes to DNA.

The inset of Fig. **5** gives a double logarithmic representation of the data which can be represented approximately as a straight line. This is not too surprising, since double logarithmic plots often yield straight lines. The interesting detail is that this line has a slope of - 1 / 2, exactly what one expects if the DNA molecule has the biomechanics of a

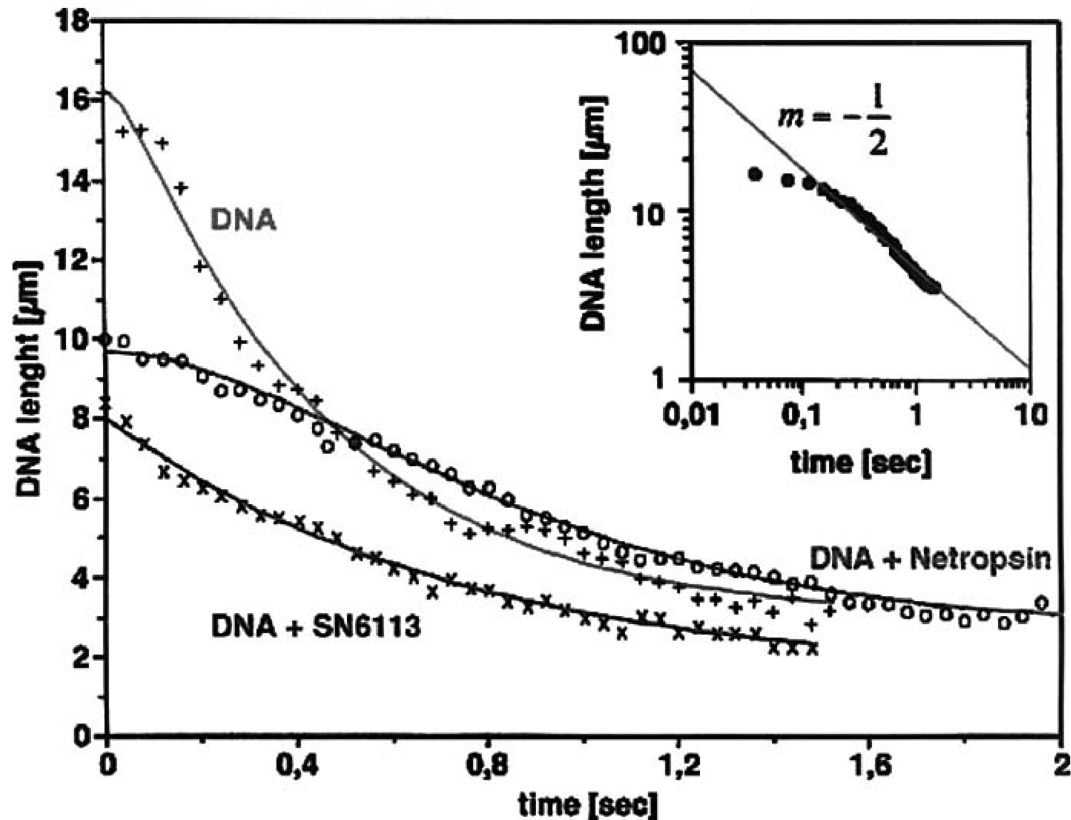



**Fig. (5).** Graphical representation of the decrease of length of the molecule in fig. 4. Inset: Double logarithmic plot of these data. For details see text.

classical harmonic spring. The harmonic spring description is a simple method to describe the action of potential drugs such as the two substances mentioned above just by two parameters, the spring constant and a damping factor.

The collapse of a DNA molecule can be reversed by appliying external force and even be used to stretch or over - stretch individual DNA molecules. With forces of the order of 10 - 20 piconewtons, the DNA molecule can be extended into the normal structure with a length of about 0.3 nm per base pair. At forces between 20 and 60 piconewtons the DNA molecule is reversibly over - stretched. up to the 1.7. fold of its "natural" length and is occasionally termed S-DNA. Above 60 piconewtons, irreversible over - stretching will occur. Similar effects are seen with double stranded RNA. Using the tip of an AFM , Bonin *et al*. [68] have investigated the flexibility of dsRNA. They have found a conformational change similar to the DNA B - S transition but over - stretching is possible up to a factor of 2.

The folding of single stranded RNA is different and comparable rather to protein folding. Indeed , some single stranded RNA molecules fold into structures with enzymatic activity (ribozymes). Using single molecule FRET, Russell *et al*. [69] have shown that folding occurs in deep valleys of the folding landscape, i.e along very stable pathways. Interestingly, the temporal sequence of folding is very critical. If long range contacts are made at the right time, they stabilise a structure, otherwise they may destabilise it. With similar techniques [70] it could be shown that the *Tetrahymena thermophilia* ribozyme has eight different folding barriers. Forces of 10 to 30 piconewtons are required to overcome these barriers. A recent review on biophysical properties of DNA is available [71].

**Chromatin, the Open form of Chromosomes**

Of particular interest are the mechanical properties of chromatin , the complex of DNA with histones which in its most compact form is known as the chromosome. Knowledge on forces acting in chromatin and on structural transitions of chromatin under the influence of a force is essential to understand the dynamics inside a cell nucleus. There, 2 meters of DNA have one the one hand to be tightly packed in order to fit into the cell nucleus with linear dimensions of a few micrometers, on the other hand regions of chromatin have to be unpacked in order to make the DNA available for transcription. It is reasonable to assume that strong forces are acting. Two types of single chromatin experiments can be envisioned to give insight into such processes. When a piece of chromatin consisting of DNA is wound around nucleosomes is stretched, in a first step 76 base pairs are released at low stretching force. When the force is increased, additional 80 base pairs are released in two stages [72]. In a reverse experiment Leuba *et al.* [73] studied the assembly of nucleosomes and chromatin. They found a strong inhibition of assembly at forces as low as 10 piconewton, indicating that DNA needs its full flexibility to wrap around the nucleosomes. Two recent reviews [74] ,[75] have summarised such experiments.

## DNA Melting and Zipping : Separating and Joining the Two Strands

A considerable wealth of information on the double stranded DNA duplex can be obtained by pulling apart the two strands. To achieve that, one end of the molecule is attached to a surface. Of the other end, one strand is also fixed to some point while the second strand is attached to an AFM tip or a bead which may be manipulated by magnetic or optical tweezers. Force can then be used to separate the two strands of DNA [76]. Stretched unwound DNA denatures locally even at comparably low forces of 2 piconewtons [77]. However, for complete separation of short double helices, 17 - 40 piconewtons are required to overcome the energy barrier for rupture of 9 -13 kcal per mol [78]. Unzipping (or melting) can also be induced by high temperature. In combination with pulling the molecule, such a melting can be actively induced, and the DNA denaturation - renaturation kinetics can be studied. The T4 gene 32 protein pg32 specifically binds to single stranded , i.e. molten DNA. With single molecule force spectroscopy it was shown that, surprisingly, gp32 does not reduce, as expected, the melting temperature, but affects the kinetics of this process [79]. Multiple metastable intermediates were found when lambda phage DNA molecules were unzipped [80]. Unzipping changes pace in a sequence dependent manner. Not only unzipping has been observed on a single molecule basis. Also the rejoining of individual DNA strands can be observed on a single molecule basis. A single nanopore, made of alpha hemolysin, is required. By this approach, the binding of an octanucleotide to its complementary DNA strand has been observed with techniques derived from patch clamping. In spite of the complex environment, kinetics and binding constants for duplex formation were comparable to those of bulk experiments [81]. This is an example, where single molecule experiments do not provide essentially new information, but confirm that bulk experiments provide useful data to understand the single molecule process.

## Replication, Transcription DNA Repair and DNA Mapping

So far, biophysical DNA studies were reported. The step to biochemistry or molecular biology is represented by single molecule studies on enzymes working on DNA, particularly polymerases, enzymes involved in DNA replication and repair. A second sort of enzymes, endonucleases, will be discussed separately below. Occasionally, it is single molecule research which detects the true inherent complexity of polymerases, the enzyme class which synthesizes RNA from the DNA blueprint [82]. Individual molecules of RNA polymerase reveal a surprisingly uniform kinetics [83], in contrast to some predictions derived from protein folding theories, from which significant differences were forecasted on the basis of structural heterogeneity. Obviously, evolution has selected, from the variety of possible structures, only those which reveal optimal function. Such a polymerase does not polymerise the RNA continuously, but pausing can be detected. When the template DNA molecule is stretched, this pausing can be modified and thus studied in more detail [84]. In a similar way, it has been shown that the rate of DNA replication slows down when the DNA molecule is stretched with a force of up to 20 piconewton. Above this force, replication completely ceases [85]. In order to observe reactions, also combed DNA can be used, i.e. DNA stretched on the surface of a microscope slide. Topisomerases , i.e. enzymes which are involved in the unwinding of DNA have been studied on a single molecule basis by Strick *et al*. [86] and Dekker *et al*. [87]), nucleotide excision repair by Segers - Nolten [88]. The question on how enzyme molecules find DNA and keep contact during catalysis is of major importance for maintenance and replication of the genome. The binding domain of Tc3 transposase, for example, when stained with the fluorescence dye tetramethyl rhodamine (TMR) flucutates between a fluorescent and a quenched form, when bound to DNA [89]. Also, a so far unsolved or at least heavily disputed question asks for the mechanism of enzymes at work. Jiao *et al*. [90] have detected, by time lapse atomic force microscopy, two modes of target recognition between the tumour suppressor protein p53 and a DNA fragment containing a recognition site for this protein : direct binding and initial non specific binding with subsequent one dimensional diffusion of the protein along the DNA to the specific site. Even more important, polymerases or nucleases work on long DNA molecules, repeating a catalytic step many times. Polymerases have to add nucleotides step by step, nucleases have to remove or cut them. The question is then, for how many steps on enzyme molecule repeats this task, i.e. how long does it stay attached to the individual DNA molecule. This has been discussed under the aspect of processivity. A number of these questions have been solved with ingenious interpretations of bulk experiments, but the final answer can only be obtained by direct observation of single molecule reactions. Harada *et al*. [91] have shown in real time how a RNA polymerase molecule proceeds along a stretched DNA molecule for approx. 1 micrometer. In these studies, the DNA molecule remained unstained and the enzyme molecule was fluorescence labelled, so that one could observe a fluorescent spot running along an invisible linear track. In a similar study, however with restriction endonucleases, the opposite strategy was employed: The DNA molecule was fluorescence labelled and the action of the enzymes was observed by the cuts they induced. Since the cuts in Fig. **6**, taken from [92], are consecutive, one can conclude that one enzyme molecule remains attached for several micrometers and induces sequentially all visible cuts.

## Single DNA Molecule Sequencing

One of the probably most exciting potential applications of single molecule research is the sequencing of DNA. The idea was fostered by the early success to observe the enzymatic degradation of the molecule [93]. Unfortunately, full single DNA molecule sequencing , after a decade of research and even after human genome sequencing has been completed, has not lived up to its promises. What has been achieved recently is the reading of 5 bp fragments in single DNA molecules [94]. The principle idea is straightforward: Using an exonuclease which digests DNA from one of its end base by base, a flow of single nucleotides through a detection volume is generated. By identification of the sequence of the single individual DNA bases flowing through this volume, the sequence of the DNA macromolecule, held in position by optical tweezers or an AFM tip, can be

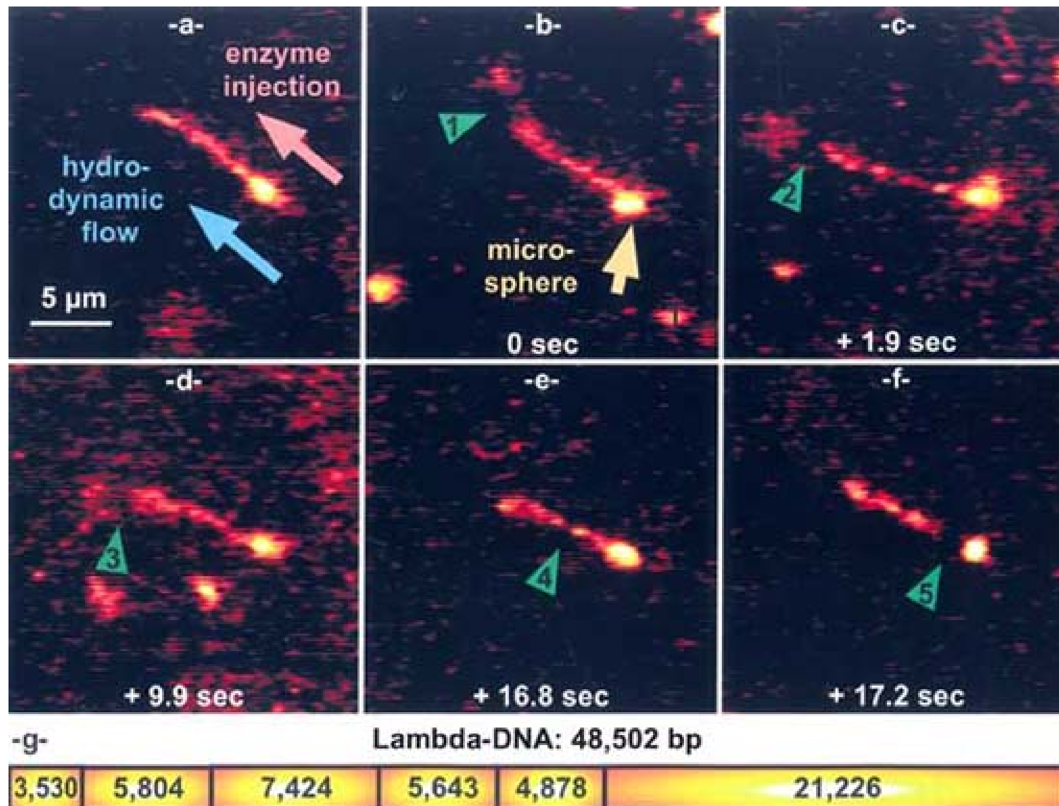



**Fig. (6).** Cutting of a single Lambda phage" DNA molecule which is held by optical tweezers, into a sequence specific pattern, The DNA molecule is stretched by hydrodynamic flow, otherwise it would collapse into a globular structure. The Eco R1 molecule attaches with highest probability downstream", which can be explained by a geometric shadow effect. Then it searches for its first recognition sequence and cuts. It remains on the molecule and continues search until all five restriction sites are consecutively cut. Cases where the enzyme molecule binds not absolutely downstream, and cases where the enzyme molecule falls off with the DNA fragment, are also observed but represent the minority of events.

determined. Two problems have so far hampered this approach. As for all other optical detection techniques in single molecule research, fluorescence is needed. Unfortunately, at room temperature, DNA bases reveal a notoriously low fluorescence yield, 4 to 5 orders of magnitude lower than typical fluorescence dyes which have a yield of some tens percent. A few attempts to work in the cold at nitrogen or even helium temperatures have so far not been successful, since the digestion-enzyme reaction has to be performed at room temperature while detection has to be performed in the cold, and the sharp change of temperature has so far not yet been managed. The alternative, to attach sequence specific fluorescence dyes, is exceedingly more difficult than in the examples described above, since for DNA sequencing, each DNA base has to be labelled and the overall yield of labelling has to be 99.9 % in order to be competitive with conventional DNA sequencing. The labelling reaction has to be template directed, i.e. to be a sort of DNA amplification. Once such a reaction is required, one can, however, easily amplify the single DNA molecule a million of times and the perform conventional sequencing. Such a strategy has been used for single molecule profiling of pre -mRNA splicing [95], after amplification of DNA [96]) and, in a slight modification, with the BEAM (bead, emulsion, amplification, magnetics) technique [97]. Individual DNA molecules are captured with a magnetic microbead, amplified by PCR so that each bead carries thousands copies of the same DNA molecule which can then be sequenced. DNA sequencing after amplification of an individual DNA molecule has been successful) and is occasionally referred to as single molecule DNA sequencing. The original approach of direct sequencing, however, has remained just a concept.

## Single Molecule DNA Mapping

In the analysis of some diseases it it important to know the order of genes or sequence elements along a given region of the genome. On a 10 to 100 megabase scale, fluorescence *in situ* hybridisation (FISH) of metaphase chromosomes can reveal this order. If, however, such information is required on a kilobase or low megabase scale , single DNA moleculs replace the metaphase chromosomes as template. Individual kilobase or megabase sized DNA molecules are stretched and attached at the surface of a microscope slide and short, kilobase sized fluorescent DNA molecules are bound (hybridised) in a sequence specific manner to the long target DNA [98]. One will find a fingerprint like sequence dependent pattern of fluorescence spots. For the preparation of a large number of target molecules, molecular DNA combing has been developed, a technique where many DNA molecules are stretched and parallelised by the receding meniscus of an evaporating microdroplet [99]. Combed DNA can be used for mapping of a large number of parallel molecules [100]. These authors could quantify the number of

single gene copies on genomic DNA. By hybridization of 300 kb probes against human chromosome 11, the structural organization of this chromosome could be established on a single molecule basis [101]. A fluid flow technique, similar to the one described in the preceding chapter, has been used to characterise DNA fragments after application of ionising radiation. The fragments are flowing away and can be quantified in a distant observation volume as fluorescent bursts [102, 103]. Anazawa *et al*. [104] have presented a study on electrophoretic quantification of single DNA molecules.

### Restriction Fragment Sizing of Individual DNA Molecules

Foquet *et al*. [105] have performed fragment sizing in submicrometre sized fluidic channels. Fragment sizing can, however, also be used to get sequence information and thus to characterise individual DNA molecules. As in sequencing, the individual DNA molecule is held in position by optical tweezers and is stretched hydrodynamically or electrostatically. Then the molecule is cut into different fragments by sequence specific restriction endonucleases, quite similar as it is done in bulk DNA typing to solve, for example, criminal cases or to perform paternity tests. The fragments have sizes which reflect the sequence of the individual DNA molecule.

Fig. **7** shows, for DNA from the bacteriophage Lambda, the sizes of the DNA fragments detected by this approach (green columns) as compared to the sizes expected from the sequence. The accuracy of individual fragment sizes is lower but comparable to those from conventional bulk experiments using 10 - 15 orders of magnitude more molecules. Individual DNA molecules can be safely characterised.

Interestingly, almost identical results can be achieved by light induced molecular cutting [107]. Here, restriction enzyme molecules were first allowed to bind to their sequence specific site on a DNA molecule and then to act as a sort of photo - sensitisers. With unspecific illumination by light, the DNA was then fragmented.

A particularly original strategy for DNA restriction uses unzipping of the double stranded DNA molecule. If the two strands of a double stranded DNA molecule are pulled apart with AFM tips, force is needed. At positions where restriction endonuclease molecules have bound to their recognition site on the respective DNA molecule, the force required abruptly increases and then decreases. This pattern of force peaks can be used to assemble a restriction pattern with an accuracy of approx. 25 base pairs [106].

### The Step from *In Vitro* to *In Situ* and *In Vivo* Single Molecule Research: Single Molecules in Cells

As a first step towards observation of single molecules in cells, the insertion of dye molecules into model membranes has been reported by Milhiet [108]. Protein molecules which usually reside in the cell membrane, have been incorporated into artificial membranes and their behaviour could be studied there [109, 110]. Sub - wavelength resolution co - localisation of individual dye molecules was reported by Heilemann [111]. Also, on living cells the binding strength of single fibrinogen-integrin molecule pairs has been measured by allowing cells to get into contact and then measuring the acting forces [112]. With optical tweezers, the binding strength was measured to range from 60 to 100 piconewtons. Since the cells used in these studies, platelets, play a pivotal role in blood clotting, such information is of importance for understanding basic features of the generation of heart attacks and possibly may give hints for strategies for prevention of these catastrophic events.

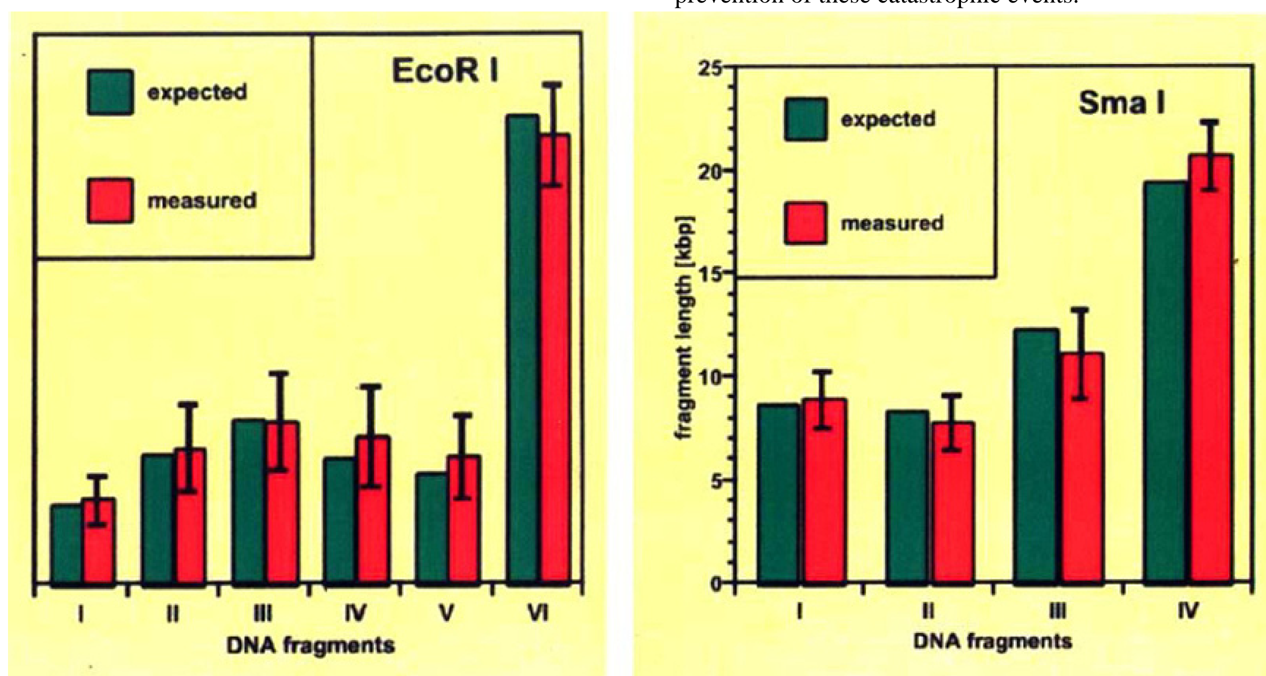



**Fig. (7).** Determination of the size of the restriction fragments: The fluorescence of the fragment is set in relation to the fluorescence of the whole molecule and then multiplied with the length of the latter, 48, 502 base pairs. The respective left columns of the left and right panels are the values expected theoretically on the basis of the sequence of the molecule and the recongition sequences of the two enzyme molecules used, Eco R1 (left) and Sma 1 (right).

Smith *et al*. [113] have shown how the Phi 29 portal motor protein packs the 6.6 micrometer long double stranded DNA molecule which contains the genome of this virus against a force of more than 50 piconewtons into the protein capsid of this virus. A corresponding theory on these processes is meanwhile also available [114].

Also, with the aim to study how the mono - cellular slime mold *Dictyostelium discoideum* reacts to chemotactic attraction, the binding of c-AMP molecules to different sites on the surface of these cells has been studied. The probability of individual association and dissociation events was greater for receptors at the anterior end of the cell, i.e. the side into which the cells migrate [115]. Particularly exciting is the possibility to "harvest" molecules which are just passing a pore between cell nucleus and cytoplasma using an AFM tip. This has been accomplished by Oberleithner *et al*. [116] with eggs from the frog *Xenopus laevis*. The motion of a beta-galactosidase molecules through 3T3 fibroblasts has been reported by Kues and Kubitscheck [117]. This working group has also shown how to observe the motion of single molecules perpendicularly to the observation plane (the z-direction) of a microscope [118], a very important technique to observe single molecule signalling in a cell from its surface through the nuclear membrane into the cell nucleus or vice versa.

From the viewpoint of molecular pharmacology, the study of the efflux pump machinery in *Pseudomonas aeruginosa* is particularly interesting. This bacterium causes problems in many hospital and are deadly to old patients, where they cause lung infection as well as for cystic fibrosis patients. In a model study, Xu *et al*. [119] have studied the transport of material through the cell membrane of *Pseudomonas aeruginosa* on a single molecule basis.

A look into a cell with unprecedented detail is possible with a technique presented by Knemeyer *et al*. [120]. Fluorescence labelled oligonucleotides were injected in cells and their binding to m RNA was observed with spectrally resolved fluorescence lifetime imaging microscopy (SFLIM). Essentially, individual mRNA molecules could thus be visualized.

### Single Molecule Gene Expression Detection

A particularly interesting strategy of intervention by drugs is to take influence on gene expression and transport of genes into the cytoplasm, where the information is translated into proteins. Essentially, when a cell expresses a gene, corresponding mRNA can be found in it's cytoplasma. In order to find out if a specific gene is expressed or if it's expression is changed in the presence of a drug, one has to attach a fluorescence label to the corresponding RNA. This fluorescent label can, for example, be a gene specific RNA probe. If the cell content is suspended and observed in a small detection volume of an FCS device, one can identify mRNA which has bound a partner molecule. This allows to count the number of mRNA molecules corresponding to the gene of interest. So far the feasibility of the method has been shown. A number of details will have to be solved until the experiment can become routine. However, the first step has been made [121].

### Observing the Infection of Individual Cells by Individual Virus Particles

The probably most exciting perspective of the application of single molecule techniques in biomedicine and pharmaceutical technology is the observation of the action of individual virus particles while they just infect a target cell. The technique of choice for such studies is fluorescence microscopy, since living cells are involved in such experiments. It is clear that such a detailed analysis of cell infection will allow a much better fine tuned development of pharmaceuticals than classical techniques, where the drug developer is blind in the most critical moment of infection. As a perspective, galenics and drug delivery may be further improved to prevent cell infection of individual virus particles which otherwise undermine the action of drugs developed without the detailed knowledge of the very instant of cell infection.

In a classical work laying the foundation of this exciting field of research, the group of Christof Bräuchle [122] studied infection of individual cells by individual adeno - associated virus particles. Comparing the reaction paths and speeds of many individual virus particles from the surface of the target cells into the nucleus it could be shown that there are preferential reaction paths and that the average infection time is approx. 12 min, however with a large deviation among individual virus particles

A similar approach was used to observe the infection of Chinese hamster ovary cells by influenza viruses [123]. Here, the influenza virus X 31 was first incubated with DiD (Molecular Probes) and then with the amino reactive dyes Cy3 and CypHer5 (Amersham). This was sufficient to attach a fluorescence label to the virus and still retain it's infectivity. The virus entered the cell in a three stage active transport process. First, the virus moved, in an actin dependent manner, in the cell periphery. In a second step it is translocated to the perinuclear region via dynein transport and finally the virus moves on microtubules in the perinuclear region. Each of these stages might be a target for drug intervention and thus such a single particle technique may turn out to become an invaluable tool in basic drug research.

## CONCLUDING REMARK

Single molecule detection and manipulation, though still in an early stage of application in pharmacology and biomedicine, promise to open the path for a completely new analytics , requiring orders of magnitude less material and providing information on pharmacological processes with unprecedented information content.

## ACKNOWLEDGEMENT

This work was supported by the Deutsche Forschungsgemeinschaft, Grant Uh 93, 2-3 and the Stiftung Volkswagenwerk, Grant I 75099.

## REFERENCES

[1] Rothman, B. Measurement of activity of single molecules of beta D galactosidase (1961) *Proc. Natl. Acad. Sci.* **47**, 1981-1991.

[2] Basche T. (1999) Quantum optical experiments with single molecules (in German) *Phys. Bl.* **52**, 456-459.

[3] Tamarat, P.H.; Maali, A.; Lounis, B.; Orrit, M. Ten years of single molecule spectroscopy *J. Phys. Chem.* **104.1**, 1-16.

[4] Moerner W.E. A dozen years of single molecule spectroscopy in physics, chemistry and biophysics (2002) *J. Phys. Chem.* **106.5**, 910 - 927.

[5] Greulich, K.O.; Klose, E.; Neumann, H. eds. Single molecule detection (1995) *Exp. Tech. Phys.* **41** (2), 157 - 311.

[6] Erdmann, R. and Seidel, C. eds. Single molecule detection: Basics and applications in the life sciences (1997) *Bioimaging* **5** (1).

[7] Erdmann, R.; Enderlein, J.; Seidel, C.eds. Single molecule detection: Basics and applications in the life sciences (1998) *Bioimaging* **6** (3).

[8] Erdmann, R.; Enderlein, J.; Seidel, C. eds. Single molecule detection and ultrasensitive analysis in the life sciences (1999) *Cytometry* **36** (3), 162-264.

[9] Basche,T.; Moerner, W.E.; Orrit, M.; Wild, U.P. Single molecule optical detection, imaging and spectroscopy (1997) Wiley VCH Weinheim, New York, Basel, Cambridge Tokio.

[10] Zander, C.; Enderlein, J.; Keller, R.A (eds). Single molecule detection in solution (2002) Wiley VCH Berlin

[11] Greulich, K.O. (2003) Single biomolecule detection and characterization. In: Encyclopedia of Analytical Chemistry R.A. Meyers, ed. pp 654 -662 John Wiley and Sons Chichester

[12] Nasanshargal, B.; Schäfer, B.; Greulich, K.O. Single molecule reactions of the enzyme LDH and of restriction endonucleases in the fluorescence microscope in: 2002 Fluorescence Spectroscopy, Imaging and Probes, R. Kraayenhoff, A.J.W.G. Visser, H.C. Gerritsen eds. Springer Berlin Heidelberg New York

[13] Greulich, K.O. (1999) Birkhäuser Basel Wien Boston (Monography) Micromanipulation by light in biology and medicine: The laser microbeam and optical tweezers.

[14] Kneipp, K.; Wang,Y.; Kneipp, H.; Perelman, LT.; Itzkan, I.; Dasari, R.R.; Feld, M.S. Single molecule detection using surface enhanced Raman scattering (SERS) (1997) *Phys. Rev. Lett.* **78**, 1667-1670.

[15] Wang, Z, Pan S, Krauss T D, Du H, Rothberg L J The structural basis for giant enhancement enabling single -molecule Raman scattering (2003) *Proc. Natl. Acad. Sci.* **100**, 8638 -8643.

[16] Horton, M.; Charras, G.; Ballestrem, C.; Lehenkari, P. Integration of atomic force and confocal microscopy. (2000) *Sing. Mol.* **1**, 135 -138.

[17] Grange, W.; Strunz, T.; Schumakovich, I.,Güntherodt, H.J,Hegner, M. Molecular recognition and adhesion of individual DNA strands studied by dynamic force microscopy (2001) *Sing. Mol.* **2**, 75-78.

[18] Stark, M.; Heckl, W.; Guckenberger, R. Inverting dynamic force microscopy: From signals to time resolved interaction forces (2002) *Proc. Natl. Acad. Sci.* **99.13**, 8473-8478.

[19] Tinnefeld, P.; Buschmann, V.; Herten, D.P.; Han, K.T.; Sauer, M. Confocal fluorescence lifetime imaging micrsocopy (FLIM) at the single molecule level (2000) *Sing. Mol.* **1**, 215-223.

[20] Gao, X. and Nie, S. Molecular profiling of single cells and tissue specimens with qunatum dots (2003) *Trends in Biotechnology* 21.9, 371-374.

[21] Sako, Y.; Uyemura, T. Total internal reflection microscopy for single-molecule imaging in living cells (2002) *Cell Struct. Funct.* **27**, 357-365.

[22] Paige, M.F.; Bjerneld,E.J.; Moerner, WE. A comparison of through the objective total internal reflection microscopy and epifluorescence microscopy for single molecule research (2001) *Sing. Mol.* **2**, 191-203.

[23] Kinjo, M.; Rigler, R. Ultrasensitive hybridization analysis using fluorescence correlation spectroscopy (1995) *Nucl. Acids Res.* **23**, 1795-1799.

[24] Maier, C.; Rünzle, D.; Wagner, L.; Grabner, G.; Köhler, G.; Luger, A. Evidence for specific glucocorticoid binding sites on the cell membrane by fluorescence correlation spectroscopy (2002) *Sing. Mol*. **3**, 211 -217.

[25] Kohl, T.; Heinze, K.G.; Kuhlemann, R.; Koltermann, A.; Schwille, P. A protease assay for two photon cross correlation and FRET analysis based solely on fluorescent proteins (2002) *Proc. Natl. Acad. Sci.* **99.19, 12161-12166.

[26] Weidemann, T.; Wachsmuth, M.; Tewes, M.; Rippe, K.; Langowski, J. Analysis of ligand binding by two colour fluorescence cross correlation spectroscopy (2002) *Sing. Mol*. **3**, 49-62.

[27] Li, H.; Ying, L.; Green, J J.; Balasubramanian, S.; Klenerman, D Ultrasensitive coincidence fluorescence detection of single DNA molecules (2003) *Anal. Chem.* **75,** 1664 -1670.

[28] Amediek, A.; Haustein, E.; Scherfeld, D.; Schwille, P. Scanning dual-color cross correlation analysis for dynamic co- localization studies of immobile molecules (2002) *Sing. Mol.* **3**, 201-210.

[29] Greulich, K.O.; Pilarczyk, G.; Hoffmann, A.; Meyer zu Hörste, G.; Schäfer, B.; Uhl, V.; Monajembashi, S. Micromanipulation by laser microbeam and optical tweezers: from plant cells to single molecules (2000) *J. Microscopy* **198,** 182-187

[30] Pralle, A.; Florin, E.L.; Stelzer, E. Hörber, H. Photonic force microscopy: A new tool providing new methods to study membranes at the molecular level (2000) *Sing.Mol.* **1**, 129 -133.

[31] Bach, H.; Renn, A.; Wild, U.P. Spectral imaging of single molecules (2000) *Sing. Mol.* **1**, 73-78.

[32] Zumbusch, A.; Jung, G. Single molecule spectroscopy of the green fluorescent protein: a critical assessment. (2000) *Sing. Mol.* **1**, 261-270.

[33] Köhler J. Optical spectroscopy of individual objects (2001) *Naturw*. **88**, 514-521.

[34] Tietz, C.; Gehrken, U.; Jelezko, F.; Wrachtrup, J. Polarization measurements of single pigment protein complexes. (2000) *Sing. Mol.* **1**, 67-72

[35] Huser, T.; Yang,M.; Rothberg, L.J. Single chain spectroscopy of conformational dependence of conjugated polymer photophysics (2000) *Proc. Natl. Acad. Sci.* **97**, 11187-11191.

[36] Taguchi, H.; Ueono, T.; Tadakuma, H.; Yoshida, M.; Funatsu, T. Single molecule observation of protein - protein interactions in the chaperonin system (2001) *Nat. Biotech.* **19**, 861-865.

[37] Marshall, B T, Long, M, Piper, J W, Yago, T, McEver, R P, Zhu, C Direct observation of catch bonds involving cell-adhesion molecules (2003) *Nature* **423**, 190 - 193.

[38] Hanley, W.; McCarty,O.; Jadhav, S.; Tseng, Y.; Wirtz, D.; Konstanopoulos K. Single molecule characterization of P-Selectin/Ligand binding (2003) *J. Biol. Chem.* **278**, 10556 -10561.

[39] Oberhauser, A.F.; Badilla-Fernandez, C.; Carrion-Vasquez, M.; Fernandez, J.M. The mechanical hierarchies of fibronectin observed with single molecule AFM (2003) *J. Mol. Biol.* **319.2** , 433-447.

[40] Arya, M.; Anvari, B.; Romo, G.M.; Cruz,M.A.; Dong,J-F.; McIntire, L.V.; Moake, J.L.; Lopez, J.A. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bond with the platelet glycoprotein Ib-IX complex: studies using optical tweezers (2002) *Blood* **99.11**, 3971-3977.

[41] Grama, L.; Somogy, B.; Kellermayer, M.S.Z. Global configuration of single Titin molecules observed through chain-associated rhodamine dimers (2001) *Proc. Natl. Acad. Sci.* **98.25**, 14362-4367.

[42] Koch, S.J. and Wang, M.D. Dynamic force spectroscopy of protein - DNA interactions by unzipping DNA. (2003) *Phys. Rev. Lett.* **91,** 28103-.1 - 28103.4

[43] Tetin, S.Y.; Swift, K.M.; Matayoshi, E.D. Measuring antibody affinity and performing immunoassay at the single molecule level (2002) *Analyt. Biochem.* **307.1**, 84-91.

[44] Seeger, S. Single molecule fluorescence: high performance molecular diagnosis and screening (1998) *Bioforum* **4**, 179 -185.

[45] Li, L.; Chen, S.; Oh, S.; Jiang, S. *In situ* single-molecules detection of antibody-antigen binding by tapping mode atomic force microscopy (2002) *Anal. Chem***.74**, 6017-6022.

[46] Hinterdorfer, P.; Baumgartner, W.; Gruber, H.J.; Schilcher.; K., Schindler, H.G. Detection and localization of individual antibody-antigen recognition events by atomic force microscopy (1996) *Proc. Natl. Acad. Sci.* **93**, 3477-3481.

[47] Ros, R.; Schwenger, F.; Anselmetti, D.; Kubon, M.; Schäfer, R.; Plückthun, A.; Tiefenauer, L. Antigen binding forces of individually addressed single chain Fv antibody molecules (1998) *Proc. Natl. Acad. Sci.* **95**, 7402-7405.

[48] Jermutus, L.; Kolly, R.; Földes-Papp, Z.; Hanes, J.; Rigler, R.; Plückthun, A. Ligand binding of a ribosome-displayed protein detected in solution at the single molecule level by fluorescence correlation spectroscopy (2002) *Eur. Bioph. J.* **31**, 179 -184.

[49] Vale, R.D and Milligan, R.A. The way things move: looking under the hood of molecular motor proteins. (2000) *Science* **288**, 88-95.

[50] Ishii ,Y and Yanagida, T. Single molecule detection in the life sciences (2000) *Single Molecules* **1**, 5-13.

[51] Nishiyama, M.; Higuchi, H.; Yanagida, T. Chemomechanical coupling of the forward and backward steps of single kinesin molecules (2002) *Nat. Cell Biol.* **4**, 790-795.

[52] Gross, S.P.; Welte, M.A.; Block, S.M.; Wieschaus, E.F. Coordination of opposite polarity microtubule motors (2002) *J. Cell. Biol.* **156.4**, 715 -724.

[53] Rock, R.S.; Rice, S.E.; Willes, A.L.; Purcell, T.J.; Spudich J.A.; Sweeney, H.L. Myosin VI is a processive motor with a large step size (2001) *Proc. Natl. Acad. Sci.* **98 ,** 13655 -13659.

[54] Tomigawa, M.; Kojima, H.; Yokato, E.; Orii, H.; Nakamori, R.; Katayama, E.; Anson M.; Shimmen T.; Oiwa K. Higher plant myosin XI moves processively on actin with 35 nm steps at high velocity (2003) *EMBO J.* **22.6**, 1263-1272.

[55] Baker, J.E.; Brosseau, C.; Jole, P.B.; Warshaw, D.M. The biochemical kinetics underlying actin movement generated by one and many skeletal muscle myosin molecules. (2002) *Biophys. J*. **82.4**, 2134 -2147.

[56] Peterman, E.G.; Sosa, H.; Goldstein, L.S.B.; Moerner, W.E. Polarized fluorescence microscopy of individual and many kinesin motors bound to axonemal microtubules (2001) *Bioph. J.* **81**, 2851 - 2863.

[57] Böhm, K.J.; Stracke, R.; Mühlig, P.; Unger,E. Motor protein-driven unidirectional transport of micrometer-sized car goes across isopolar microtubule arr ays. (2001) *Nanotechnology* **12** 238-244.

[58] Kato - Yamada, Y.; Noji,H.; Yasuda, R.; Kinosita jr, K.; Yoshida, M. Direct observation of the rotation of the epsilon subunit in F1 - ATPase (1998) *J. Biol. Chem*, 19375 -19377.

[59] Burgess, S.A.; Walker, M.L.; Sakakibara, H.; Knight, P.J.; Oiwa, K Dynein structure and power stroke (2003) *Nature* **421**, 715 -718

[60] Ando, T.; Kodera, N.; Naito, Y.; Furuta, K.; Toyoshima, Y.Y. A high speed atomic force microscope for studyingbiological macromolecules in action (2003) *Chem. Phys. Chem*, in press

[61] Xue, Q.; Yeung, E.S. Difference in the chemical reactivity of individual molecules of an enzyme reaction (1995) *Nature* **373**, 681-83.

[62] Ha, T.; Ting, A.Y.; Liang, J.; Caldwell, W.B.; Deniz, A.A.; Chemla, D.S.; Schultz, P.G.; Weiss, S. Single molecule fluorescence spectroscopy of enzyme conformational dynamics and cleavage mechanism (1999) *Proc. Natl. Acad. Sci.* **96,** 893-898.

[63] Xie,X.S. Single molecule approach to enzymology (2001) *Sing. Mol.* **2**, **4**, 229-236.

[64] Dovichi, N.J.; Martin, J.C.; Jett, J.H.; Trukula, M.; Keller, R.A. (1984) *Anal. Chem.* **56**, 348-54.

[65] Uhl, V.; Pilarczyk, G.; Greulich, K. O. Fluorescence microscopic observation of catalysis by single or few LDH 1 enzyme molecules (1998) *Biol. Chem.* **379**, 1175-1180.

[66] Schäfer, B.; Nasanshargal, B.; Monajembashi, S.; Gemeinhardt, H.; Greulich, K. O.; Uhl, V. Study of single molecule dynamics and reactions with classic light microscopy (1999) *Cytometry* **36.3**, 209-216.

[67] Amit, R, Oppenheim, A B, Stavans, J Increased bending rigidity of single DNA molecules by H-NS, a temperature and osmolarity sensor (2003) *Bioph. J.* **84**, 2467 -2473.

[68] Bonin, M.; Zhu, R.; Klaue, Y.; Oberstrass J.; Oesterschulze E.; Nellen W. Analysis of RNA flexibility by scanning force spectroscopy (2002) *Nucl. Acids Res.* **30. 16**, e81.

[69] Russell, R.; Zhuang, X.; Babcock, H.P.; Millett, I.S.; Doniach ,S.; Chu ,S.; Herschlag, D. Exploring the folding landscape of structured RNA (2002) *Proc. Natl. Acad. Sci.* **99.1**, 155-160.

[70] Onoa, B.; Dumont, S.; Liphardt, J.; Smith, S.B, Tinoco, I.; Bustamante C. Identifying kinetic barriers to mechanical unfolding of the T.thermophila ribozyme (2003) *Science* **299**, 1892- 1895.

[71] Bustamante,C: Braynt, Z, Smith, S. B. Ten years of tension: single molecule DNA mechanics (2003) *Nature* **421**, 423 -427

[72] Brower-Toland, B.B.; Smith, C.L.; Yeh, R.C.; Lis, J.T.; Peterson, C.L.; Wang, M.D. Mechanical disruption of individual nucleosomes reveals a reversible multistage release of DNA (2002) *Proc. Natl. Acad. Sci.* **99.4**, 1960-1965.

[73] Leuba, S.H.; Karymov, M.A.; Tomschik, M.; Ramjit, R.; Smith, P.; Zlatanova, J. Assembly of single chromatin fibers depends on the tension in the DNA molecule: Magnetic tweezers study (2003) *Proc. Natl. Acad. Sci.* **100.2,** 495-500.

[74] Zlatanova, J, and Leuba, S.H. Chromatin fibres, One-at- a- time (2003) *J. Mol. Biol.* 331, 1-19.

[75] Zlatanova, J, Forcing chromatin (2003) *J. Biol. Chem.* **278**, 23213 -23216.

[76] Essevaz-Roulet, B.; Bockelmann, U.; Heslot, F. Mechanical separation of the complementary strands of DNA" (1997) *Proc. Natl. Acad. Sci.* **94**, 11935-11940.

[77] Strick, T.R.; Croquette,V.; Bensimon, D. Homologous pairing in stretched supercoiled DNA (1998) *Proc. Natl. Acad. Sci.* **95**, 10579-10583.

[78] Pope.L.H.; Davies,M.C.; Laughton,C.A., Roberts, C.J., Tendler, S.J.B.; Williams, P.M. Force induced melting of a short double helix (2001) *Eur. Bioph. J.* **30**, 53 -62.

[79] Pant, K.; Karpel, R.L.; Williams, M.C. Kinetic regulation of single DNA molecule denaturation by T4 gene 32 protein structural domains (2003) *J. Mol. Biol.* **327.3**, 571-57.

[80] Danilowicz, C.; Coljee, V.W.; Bouzigues, C.; Lubensky, D.K.; Nelson , D.R.; Prentiss, M. DNA unzipped under a constant force exhibits multiple meatastable intermediates (2003) *Proc. Natl. Acad. Sci.* **100.4**, 1694-1699.

[81] Howorka, S.; Movileanu, L.; Braha, O.; Bayley, H. Kinetics of duplex formation for individual DNA strands within a single protein nanopore (2001) *Proc. Natl Acad. Sci.* **98.23**, 12996-13001.

[82] Guthold, M and Erie, D.A. Single molecule study reveals a complex E.coli RNA polymerase (2001) *ChemBioChem.* **2**, 167-170.

[83] Adelman, K.; La Porta, A.; Santangelo, T.J.; Lis, J.T.; Roberts, J.W.; Wang, M.D.Single molecule analysis of RNA polymerase elongation reveals uniform kinetic behaviour (2002) *Proc. Natl. Acad. Sci.* **99.21**, 13538-13543.

[84] Forde, N.R.; Izhaky, D.; Woodcock, G.R.; Wuite, G.J.L.; Bustamante, C. Using mechanical force to probe the mechanism of pausing and arrest during continuous elongation by Escherichia coli RNA polymerase. (2002) *Proc. Natl. Acad. Sci.* **99.18**, 11682-11687.

[85] Goel ,A.; Frank-Kamenetskii, M.D.; Ellenberger, T.; Herschbach, D. Tuning DNA strings: Modulating the rate of DNA replication with mechanical tension (2001) *Proc. Natl. Acad. Sci.* **98.15**, 8485-8489.

[86] Strick, T.R.; Croquette,V.; Bensimon, D. Single molecule analyis of DNA uncoiling by a type II topoisomerase (2000) *Nature* **404**, 901-904.

[87] Dekker, N.H.; Rybenkov, V.V.; Duguet, M.; Crisona, N.J.; Cozzarrelli, N.R.; Bensimon, D.; Croquette, V. The mechanism of type IA topoisomerases (2002) *Proc. Natl. Acad. Sci.* **99.19**, 12126-12131.

[88] Segers-Nolten, G.M.J.; Wyman, C.; Wijgers, N.; Vermeulen,W.; Lenferink A.T.M.; Hoeijmakers J.H.G. Greve, J.; Otto, C. Scanning confocal microscopy for single molecule analysis of nucleotide excision repair complexes (2002) *Nucl. Acids Res.* **30.21,** 4720-4727.

[89] Daniel, D.C.; Thompson, M.; Woodbury, N.W. DNA-binding interactions and conformational fluctuations of Tc3 transposase DNA binding domain examined with single molecule fluorescence spectroscopy (2002) *Bioph. J.* **82.3**, 1654-1666.

[90] Jiao, Y.; Cherny, D.; Heim,, G.; Jovin, T.M.; Schäffer, T.E. Dynamic interactions of p53 with DNA in solution by time lapse atomic force microscopy (2001) *J. Mol. Biol.* **314**, 233-243.

[91] Harada, Y, Funatsu, T.; Murakami, K.; Nonoyama, Y.; Ishihama, A.; Yanagida, T. Single molecule imaging of RNA polymerase - DNA interactions in real time (1999) *Bioph. J.* **76**, 709-715.

[92] Schäfer, B.; Gemeinhardt, H.; Greulich, K.O. Direct microscopic observation of the time course of single molecule DNA restriction reactions (2001) *Angew. Chem. Int. Ed.* 4663-4666.

[93] Bezanilla, M.; Drake, B.; Nudler, E.; Kashlev, M.; Hansma, P.K.; Hansma, H.G. Motion and enzymatic degradation of DNA in the atomic force microscope (1994) *Bioph. J.* **67**, 2454-2458.

[94] Braslavsky, I, Hebert, B, Kartalov, E, Quake, S R Sequence information can be obtained from single DNA molecules (2003) *Proc. Natl. Acad. Sci.* 1**00**, 3960 3964.

[95] Zhu, J, Shendure, J, Mitra,R D, Church, G M Single molecule profiling of alternative pre mRNA splicing (2003) *Science* **301**, 836 -838.

[96] Wabuyele, M.B and Soper, S.A. PCR amplification and sequencing of single copy DNA molecules (2001) *Sing. Mol.* **2**, 13-22.

[97] Dressman, D.; Yan, H.; Traverso, G.; Kinzler, K W.; Vogelstein, B Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations (2003) *Proc. Natl. Acad. Sci.* **100**, 8817- 8822.

[98] Wang, Y.K.; Huff, E.; Schwartz, D.C. Optical mapping of site directed cleavages on single DNA molecules by the recA assisted restriction endonuclease technique (1995) *Proc. Natl. Acad. Sci.* 165-169.

[99] Bensimon, D.; Simon, A.J.; Croquette, V.; Bensimon, A. Stretching DNA with a receding meniscus: Experiments and models (1995) *Phys. Rev. Lett.* **74**, 4754 -4757.

[100] Gueroui, N.; Place, C.; Freyssingeas, E.; Berge, B. Observation by fluorescence microscopy of transcription on single combed DNA (2002) *Proc. Natl. Acad. Sci.* **99.9**, 6005-6010.

[101] Conti, C and Bensimon, A A combinatorial approach for fast, high resolution mapping (2002) *Genomics* **80**, 135 -137.

[102] Haab, B.B and Mathies, R. Single molecule fluorescence burst detection of DNA fragments separated by capillary electrophoresis (1995) *Anal. Chem.* 3235-3260.

[103] Filippova, E.M.; Monteleone, D.C.; Trunk, J.G.; Sutherland, B.M.; Quake, S.R.; Sutherland, J.C. Quantifying double-strand breaks and clustered damages in DNA by single-molecule laser fluorescence sizing (2003) *Bioph. J.* **84.2**, 1281-1290.

[104] Anazawa, T.; Matsunaga, H.; Yeung, E.S. Electrophoretic quantitation of nucleic acids without amplification by single molecule imaging (2002) *Anal. Chem.* **74**, 5033-5038.

[105] Foquet, M.; Korlach, J.; Zipfel, W.; Webb, W.W.; Craighead, H.G. DNA fragment sizing by single molecule detection in submicrometer-sized closed fluidic channels (2002) *Anal. Chem.* **74,** 1415-1422.

[106] Koch S.J.; Shundrovsky A.; Jantzen B.C.; Wang M.D. Probing Protein - DNA interactions by unzipping a single DNA double helix (2002) *Bioph. J.* **83.2** , 1098-1105.

[107] Namasivayam, V, Larson, R G, Burke D T, Burns, M A Light induced molecular cutting: Localized reaction on a single DNA molecule (2003) *Anal. Chem.* **75,** 4188 - 4194.

[108] Milhiet, P.E.; Giocondi, M.C.; Baghdadi, O.; Ronzon F. Le Grimmellec, C.; Roux, B. AFM detection of GPI protein insertion into DOPC / DPPC model membranes (2002) *Sing. Mol.* **3**, 135-140.

[109] Schütz, G.J.; Kada, G.; Pastushenko, V.P.; Schimndler, H Properties of lipid microdomains in a muscle cell membrane visualized by single molecule spectroscopy (2000) *EMBO J.* **19**, 892 -90

[110] Hinterdorfer,P.; Baumgartner,W.; Gruber, H.J.; Schilcher,K.; Schindler,H. Detection and localization of individual antibody-antigen recognition events by atomic force microscopy (1996) *Proc. Natl. Acad. Sci.* 93, 3477 - 3481

[111] Heilemann, M.; Herten, D.P.; Heintzmann, R.; Cremer, C.; Müller C.; Tinnefeld, P.; Weston, K.D.; Wolfrum, J.; Sauer M. High resolution colocalization of single dye molecules by fluorescence lifetime imaging microscopy. (2002) *Anal. Chem.* **74.14,** 3511-3517.

[112] Litvinov, R.I.; Shuman, H.; Bennett, J.S.; Weisel, J.W. Binding strength and activation state of single fibrinogen-integrin pairs on living cells (2002) *Proc. Natl. Acad. Sci.* **99.11**, 7426-7431.

[113] Smith, D.E.; Tans, S.J.; Smith, S.B.; Grimes, S.; Anderson ,D.L.; Bustamante, C. The bacteriophage Phi29 portal motor can package DNA against a large internal force (2001) *Nature* **413**, 748-752.

[114] Purohit, P.P.; Kondev, J.; Phillips, R. Mechanics of DNA packaging in viruses (2003) *Proc. Natl. Acad. Sci.* **100.6**, 3173-3178

[115] Ueda, V.; Sako, Y.; Tanaka, T.; Devreotes, P.; Yanagida, T. Single-molecule analysis of chemotactic signaling in *Dictyostelium* cells (2001) *Science* **294**, 864-867.

[116] Oberleithner, H.; Schäfer, C.; Shahin,V.; Schlune,A.; Schillers, H.; Reinhardt, J. Nuclear plug harvesting using atomic force microscopy (2001) *Sing. Mol.* **2**,117 -120.

[117] Kues, T.; Peters, R.; Kubitscheck, U. Visualization and tracking of single protein molecules in the cell nucleus (2001) *Bioph. J.* **80**, 2954 - 2967.

[118] Kues, T. and Kubitschek, U. Single molecule motion perpendicular to the focal plane of a microscope: Application to splicing factor dynamics within the cell nucleus (2002) *Sing. Mol.* **3**, 218 - 224.

[119] Xu, X.; Brownlow, W.; Huang, S.; Chen, J Single molecule detection o f efflux pump machinery in *Pseudomonas aeruginosa* (2003) *Biochem. Biophys. Res. Commun.* **305**, 79 -86

[120] Knemeyer, J P, Herten, D P, Sauer, M Detection and identification of single molecules in living cells using spectrally resolved fluorescence lifetime imaging microscopy (2003) *Anal. Chem.* **75**, 2147 - 2153.

[121] Korn K.; Gardellin P.; Liao B.; Amacker M.; Bergström A.; Björkmann H.; Camacho A.; Dörhöfer S.; Dörre K.; Enström J,.; Ericson T.; Favez T.; Gösch M,.; Honegger A.; Jaccoud S.; Lapczyna M.; Litborn E.; Thyberg P.; Winter H.; Rigler R Gene expression anaylsis using single molecule detection (2003) *Nucl. Acids Res.* **31** (**16**) e 89

[122] Seisenberger, G.; Ried, M.U.; Endreß, T.; Büning, H.; Hallek, M.; Bräuchle, C. Real-time single molecules imaging of the infection pathway of an adeno-associate virus (2001) *Science* **294**, 1929-1932.

[123] Lakadamyali M.; Rust M J.; Babcock H P.; Zhuang X Visualizing infection of individual influenza viruses (2003) *Proc. Natl. Acad. Sci.* **100.** 9280 - 9285.