

# **PhD Project**

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**“Single-molecule DNA biochemistry, genetic  
circuits and chromatin”**

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## **Abstract**

Recent years in research have witnessed a revolution in experimental (bio)chemistry. Tools like optical tweezers, atomic force microscopes and evanescent wave optics have been applied to detect and manipulate single molecules.

These techniques are especially valuable to study biochemical reactions which naturally involve only a limited number of molecules. A chemical reaction involving only a limited number of reaction centers like transcription has a dynamic behavior that cannot be described in classical terms derived from bulk measurements. Instead, transcription of a gene is a highly stochastic process. In order to understand the stochastic nature of the transcription reaction it is necessary to study this process on the single molecule level.

Chromatin has long been thought as a static, non-participating structural element. However, it is now clear that chromatin is a dynamic structure and histones are integral and dynamic components of the machinery responsible for regulating transcription in eukaryotes. Yet, the different levels of chromatin structure and its variations due to histone modifications are poorly characterized.

Using single-molecule micromanipulation and detection techniques I plan to study different *in vitro* reconstituted and remodeled chromatin conformations. These studies might also lead to new insights on higher order structures of chromatin.

I further plan to develop a functional assay for transcription by using a fluorescence based technique to directly measure transcription rates. This technique might be sensitive enough to detect single RNA transcripts. In combination with a micromanipulation setup this should be a valuable tool to study how DNA conformation influences the dynamics of transcription.

### Outline of experiments to study chromatin conformation on the single molecule level

1. *In vitro* reconstitution of chromatin on a single lambda DNA using crude and recombinant assembly systems. Characterization of the assembly/disassembly kinetics on the single DNA molecule level.
2. Force-extension measurements with an optical tweezer or an atomic force microscope (AFM) setup to probe the forces necessary to destabilize different levels of the chromatin structure. The force extension measurements should also reveal the degree of regularity of the assembled nucleosomal array.
3. Characterization of different conformations of chromatin: after remodeling with ATP-dependent activities, after acetylation, methylation, poly-ADP-ribosylation of histone tails.
4. Use DNA tethered bead as mechanosensor to probe for changes in elasticity during chromatin assembly and remodeling.
5. Develop a sensitive single DNA molecule assay to study single nucleosome binding by observation of the introduced supercoils
6. Visualize assembled chromatin with AFM (as control experiment)
7. Combine these mechanical measurements with a fluorescence based transcription assay to study the influence of chromatin conformation on transcriptional activity (see below)

### Outline of experiments to study dynamics of transcription on the single molecule level

1. It is necessary to develop a simple *in vitro* reconstituted transcription assay to measure rates of transcription from a limited number of molecules, e.g. using a combined evanescent wave/fluorescence resonance energy transfer (FRET) setup
2. If possible extend the sensitivity of this transcription assay to monitor a single DNA templated transcription reaction.
3. Combine the transcription assay with micromanipulation of single DNA molecules (optical tweezer setup) in order to study the influence of DNA conformation (looping, chromatin) on transcription initiation and elongation rates.
4. Combine a small number of genes in a genetic circuit on a microchip to study the design principles of a robust network which is able to perform a small computational task.

# Background and significance

## ***Single DNA molecule biochemistry, genetic circuits and chromatin***

### **Introduction: What can physics learn from biology when it comes to transcription**

My research interest is based on two different ideas, both formulated about 40 years ago:

At the Cold Spring Harbor Symposium 1961, Jacob and Monod commented that “it is obvious from the analysis of these [bacterial genetic regulatory] mechanisms that their known elements could be connected into a wide variety of ‘circuits’ endowed with any desired degree of stability”. (Jacob, 1962)

In a talk given in 1959, Richard Feynman was the first scientist to suggest that devices and materials could someday be fabricated to atomic specifications: "The principles of physics, as far as I can see, do not speak against the possibility of maneuvering things atom by atom." (Feynman, 1961)

Both ideas seem to have nothing in common and I have to explain why they do.

Jacob and Monod were the first to express the idea of building genetic circuits. Genetic circuits are biochemical reactions where the expression of one gene often regulates the expression of other genes. These circuits are the computational units coded in the genome of cells which are used to program the cells function.

Every cell contains only one genome which is built from individual genes present in low copy numbers, typically one or two. From a chemical point of view it is interesting that a very complex, time sustaining nonequilibrium reaction (the functioning cell) can be generated with essentially a single molecule: the genome, coded in the DNA, wrapped up in the chromosome(s). The DNA is coding for all the structures of the living cell which are produced from this single molecule. Thus, with the genetic circuits coded on a single DNA molecule the cell is capable of assembling molecular machines like polymerases, ribosomes, motors, phages ... atom by atom.

To enable assembly of (in this case self assemble) structures atom by atom is one goal of nanotechnology, which started with Feynmans idea 1959. If we can understand how the cell uses a single DNA molecule as template in the transcription/translation reaction to finally produce self assembling proteins, we might derive general principles how to create self assembly systems (Merkle, 1999).

A chemical reaction involving only a limited number of molecules (in the limit only one DNA molecule) is obviously a very special kind of reaction. Since there is only one molecule involved, the kinetics will be stochastic/probabilistic.

The stochastic behavior of a single molecule reaction cannot be measured with a conventional biochemical assay in bulk: only average values of e.g. transcription rates are accessible.

In recent years techniques have been developed which allow to detect and manipulate individual molecules. This revolution in experimental (bio)chemistry allows for the first time to look at properties of a single molecule instead of the ensemble average of thousands of copies of this molecule.

Measurements with single molecules allow construction of a frequency histogram of the actual distribution of values for an experimental parameter, rather than just the average (mean) value of the distribution. Experiments in the single-molecule regime further remove the need for synchronization of many single molecules undergoing a time-dependent process. This should be extremely useful to study complex biochemical reactions where often many components are needed to form a molecular machine like the transcription or replication complex or to study dynamic biochemical structures difficult to synchronize like chromatin.

## **The dynamic chromatin structure**

DNA in most eukaryotic cells is organized in the form of chromatin. Chromatin forms the basic structural matrix of the chromosomes, which represent the largest and most visible physical structure involved in the transfer of genetic information from one generation to its progeny (Wolffe, 1998). The genetic information of a typical human cell consists of  $3 \times 10^9$  bp. Fully extended as a linear DNA molecule the human genome would extend more than a meter. In order to package this highly negatively charged polymer into a cell nucleus of only  $10^{-5}$  meter in diameter the DNA has to be highly compacted.

The principal unit of DNA packaging in chromosomes is the core nucleosome and linker DNA. The core nucleosome is a DNA-protein complex in which 146 bp of DNA is bent around an octamer of histone proteins to form  $\sim 1.7$  turns of a left-handed superhelix. The core nucleosome octamer consists of two of each core histones H2A, H2B, H3 and H4. The chromatosome includes this core nucleosome and about 20 bp of linker DNA (Bednar et al., 1995) which is associated to a linker histone (H1 or H5).

The crystal structure of the core nucleosome (Luger et al., 1997) revealed much about this basic unit of the chromosome although there is still some discussion about the position of the linker histone and the location of the flexible H3 and H4 histone tails.

The array of nucleosomes along the DNA molecule represents the first level of compaction of DNA into the nucleus. The condensation ratio of 1:6 gained by formation

of this linear nucleosomal array cannot account for the thousand fold compaction ratio that must exist in vivo. Some higher order folding of the nucleosomal fiber must therefore exist.

Many models for the next level of chromatin organization have been proposed, the most prominent one is the solenoid model (Finch and Klug, 1976). The solenoid model has imposed the idea that some kind of regular structure must describe the condensed fiber under physiological salt conditions. Recently, new methods of visualization and reinterpretation of older data however show that it is unlikely that any regular helical model describes significant portions of the chromatin fiber (van Holde and Zlatanova, 1995). There is even evidence that the amount of DNA wrapped around the nucleosome might vary (van Holde and Zlatanova, 1999). Practically nothing is known about higher order chromatin structures.

Chromatin has long been thought as a static, non-participating structural element. However, it is now clear that chromatin is a dynamic structure and histones are integral and dynamic components of the machinery responsible for regulating gene transcription (Workman and Kingston, 1998) (Felsenfeld, 1992) as well as replication, repair, recombination and chromosome segregation.

The dynamic chromatin structure can be altered by covalent and non-covalent modifications (Zlatanova et al., 1998) (van Holde and Zlatanova, 1996).

The chromatin structure is covalently modulated by different posttranslational modifications of histones. The remarkable diversity and biological specificity associated with distinct patterns of covalent histone modifications let them appear like a histone code that is read by other proteins (Strahl and Allis, 2000).

Especially histone acetylation is in the limelight of covalent histone modifications since this modification seems to be correlated to transcriptional activity (Struhl, 1998). It is speculated that histone tail acetylation by HATs (histone acetyl transferases) changes nucleosomal structure, for example by unwinding/overwinding (Norton et al., 1989).

Non-covalent histone modifications involve regulatory proteins like ATP-dependent chromatin remodeling factors that bind directly to nucleosomal histones to create altered chromatin conformations (Muchardt and Yaniv, 1999) or alter the position of nucleosomes (Whitehouse et al., 1999).

Dependent on the chromatin conformation, DNA binding proteins like activators or general transcription factors can gain or loose access to their binding sites (Kwon et al., 1994) thereby activating or repressing transcription. This is a model to explain regulation of transcription by chromatin remodeling factors. It is proposed for genes activated by steroid hormone receptors (Jenster, 1998).

Chromatin is essential for compaction of our genetic code into the cell nucleus and different chromatin structures are involved in the regulation of basic cellular processes

like eukaryotic transcription. Yet, the different levels of chromatin structure and its variations due to histone modifications are poorly characterized.

## **Techniques to study chromatin on the single DNA molecule level**

New experimental techniques in single DNA molecule biochemistry (van Holde, 1999) (Mehta et al., 1999) open up the door to study chromatin conformations in unprecedented detail on the single molecule level.

One of the breakthroughs in instrumentation allowing single molecule experiments was the invention of the optical tweezer by Arthur Ashkin 1970 at AT & T Bell Laboratories (Ashkin, 1970) (Ashkin, 1998). By focusing a laser beam into a diffraction limited spot Ashkin found that small particles can be trapped in the center of the focused beam (Block, 1992). If the particle is displaced a small distance from the center of the focused laser beam the particle feels a linear restoring force pulling it back to its equilibrium position in the beam center. This setup thus allows a trapped particle to be steered in three dimensions.

Micron sized polystyrene beads (Ashkin, 1986), viruses (Ashkin and Dziedzic, 1987), cells (Ashkin et al., 1987) and organelles of cells are examples for particles which can be micromanipulated by an optical tweezer.

By combining an optical tweezer with a position detector for the trapped particle (e.g. a micron sized bead) quantitative measurements of nanometer displacements of the particle and piconewtons forces acting on the particle can be obtained with millisecond resolution (Simmons et al., 1996).

The force needed to stretch a single molecules like DNA can be measured if a trapped bead is tethered to a surface via a single DNA polymer (e.g. the lambda genome). By micromanipulation of the bead the DNA can be stretched and the force versus extension can be measured (Cluzel et al., 1996) (Smith et al., 1996) (Smith et al., 1992). For the first time entropic and intrinsic elasticity of the DNA molecule could be directly measured. An unexpected conformation of overstretched DNA was also found.

DNA is not the only molecule being studied. The optical tweezer (or similar setups) can also be used to study molecule-sized machines like the RNA polymerase (Davenport et al., 2000), DNA polymerase (Wuite et al., 2000) or topoisomerase acting on a tethered piece of DNA.

In general, DNA stretching experiments (force-extension measurements) can be used to not only to measure mechanical properties of a polymer but also to probe the energy involved in protein-DNA interactions (Marko and Siggia, 1997). Proteins that bind to DNA dissociate under the force applied along the DNA. Measuring this force and the extension gained by dissociation of the protein allows to infer thermodynamic parameters like binding enthalpy from biophysical measurements. These measurements can further

be used to follow binding kinetics, e.g. the assembly of RecA on a single lambda DNA molecule (Hegner et al., 1999; Shivashankar et al., 1999).

Force-extension measurements also give information on the structure of the stretched polymer. When stretching the Titin molecule, unfolding of single immunoglobulin and fibronectin domains every 20 nm can be monitored (Tskhovrebova et al., 1997) (Trinick and Tskhovrebova, 1999).

Recently, force-extension measurements with purified chromosomes from erythrocytes revealed the average forces stabilizing higher order structures (Cui and Bustamante, 2000). The Young modulus has been measured by folding and unfolding of whole newt chromosomes (Poirier et al., 2000) (Houchmantzadeh et al., 1997).

Another approach to study single DNA molecules or even atoms uses nanometer scale interactions with sharp tips in scanning tunneling microscopy or atomic force microscopy (AFM) (Hansma and Pietrasanta, 1998). The current resolution limit with AFM is about 10 nm, so that DNA and Proteins interacting with DNA can be visualized. It is now also possible to image biological macromolecules with AFM in solution (Bustamante et al., 1997). The ability to image unfixed biological samples in buffer makes it possible to follow conformational changes in real time such as dynamic protein/DNA interactions (Guthold et al., 1999; Kasas et al., 1997).

AFM is a powerful tool in visualization and analysis of chromatin (Bustamante et al., 1997; Leuba and Bustamante, 1999).

AFM can also be used to make force-extension measurements with forces exceeding the capabilities of an optical tweezer ( $> 100$  pN) (Fisher et al., 1999).

**Using the techniques outlined above I propose the following experiments at the single molecule level to study different chromatin conformations and their function in transcription**

- 8. *In vitro* reconstitution of chromatin on a single lambda DNA using crude and recombinant assembly systems. Characterization of the assembly/disassembly kinetics on the single DNA molecule level.**
- 9. Force-extension measurements with an optical tweezer or an AFM setup to probe the forces necessary to destabilize different levels of the chromatin structure. The force extension measurements should also reveal the degree of regularity of the assembled nucleosomal array.**
- 10. Characterization of different conformations of chromatin: after remodeling with ATP-dependent activities, after acetylation, methylation, poly-ADP-ribosylation of histone tails.**
- 11. Use DNA tethered bead as mechanosensor to probe for changes in elasticity during chromatin assembly and remodeling.**

- 12. Develop a sensitive single DNA molecule assay to study single nucleosome binding by observation of the introduced supercoils (every DNA bound nucleosome introduces one supercoil).**
- 13. Visualize assembled chromatin with AFM (as control experiment)**
- 14. Combine these mechanical measurements with a fluorescence based transcription assay to study the influence of chromatin conformation on transcriptional activity (see below)**

## **Bottom up approach to study genetic circuits *in vitro* and the influence of DNA conformation on transcription regulation**

The cell is a micron scale chemical reactor. Although the cell is packed with DNA, proteins and metabolites, many reactions still occur at low concentrations.

Transcription, the heart of the regulatory processes in the cell, is such a reaction which involves a small number of reaction centers (copies of a gene) and slow reaction rates.

Due to the small number of reaction centers and the slow reaction rates, a single transcription reaction shows a noisy behavior due to stochastic fluctuations of the transcription rate (McAdams and Arkin, 1999).

Indeed, simulations (McAdams and Arkin, 1997) as well as experimental data (Ross et al., 1994) support the idea that the transcription of a single gene is a probabilistic process.

In order to achieve robustness (deterministic behavior) of a whole genetic circuit, which is a genetically controlled pathway where the protein product encoded by one gene often regulates expression of other genes, requires certain design features.

In order to understand the design principle of naturally occurring genetic circuits it is necessary to understand the dynamics of the biochemical reactions involved. The dynamics of conventional enzymatic reactions involving large copy number of individual molecules is reasonable well understood whereas the probabilistic description and measurement of a transcription reaction involving a limited copy number of individual molecules is still in its infancy.

The dynamics of the transcription reaction can be studied either by simulation or direct measurement *in vivo* or through reconstitution of the transcription reaction *in vitro*.

Recently, two genetic circuits have been constructed in *E.coli*: A bistable switch (Gardner et al., 2000) and an oscillator (Elowitz and Leibler, 2000).

Simulations of the dynamic behavior of multigene systems can help to identify such design principles (McAdams and Arkin, 1998).

These simulations still lack experimental data on regulation of transcription rates of single genes against which predictions can be tested and parameters can be fit. This requires the quantitative understanding of the stochastic *in vitro* reconstituted transcription reaction from a single or very limited number of DNA molecules in kinetic terms.

Transcription seems to be a random and slow process (Ross et al., 1994) thus it is necessary to reduce the ensemble averaging in an *in vitro* measurement in order to understand the probabilistic nature of the process. **The ideal reconstituted *in vitro* assay for the process of transcription would thus be to monitor a single DNA templated transcription reaction.**

A first step towards this ideal reconstituted *in vitro* assay would be the development of a simple assay to measure transcription rates. There are only a few examples where transcription rates have been directly measured (Ishiguro et al., 1996) (Dunkak et al., 1996) and all of these measurements represent rather a technical challenge than a widely applicable method.

If the sensitivity of this assay can be extended to the single molecule level it would be interesting to study the influence of DNA conformation on the transcription reaction (Davenport et al., 2000). By combining the transcription assay (which will probably be fluorescence based because of its required sensitivity) with a setup to manipulate single DNA molecules (optical tweezer), it will be possible to directly measure effects of DNA conformation on the fidelity of the transcription reaction.

By combining several transcription/translation reactions in a genetic circuit on a microchip the dynamic behavior of small genetic circuits could be monitored *in vitro*. These studies would help to understand how the cell achieves deterministic and stochastic behavior *in vivo*. These studies would also contribute to understand how to program genetic circuits (biocomputing).

The first challenge for biocomputing was the introduction of new metabolic pathways into existing cells in order to produce or degrade an organic substance (metabolic engineering). (Remark: The difference between metabolic engineering and engineering a genetic circuit is that none of the genes of a metabolic engineered pathway regulate the expression of any other. Thus, these pathways are not able to perform computational tasks since they only remain in one state).

The tools of metabolic engineering have been well developed and are now being applied for the optimization of biocatalysts used in the production of a wide range of pharmaceutically important molecules (Chartrain et al., 2000).

The next step of biocomputing is to build genetic circuits *in vivo* and *in vitro* able to perform simple computational tasks as e.g. a switch or an oscillator (McAdams and Arkin, 2000). The further next step in biocomputing will be to design new molecular

machines and genetic circuits by using the DNA code. This requires that protein folding is reasonably well understood.

The final goal would be to interface the biological and engineering world by creating a solely artificial automata made of biological and mechanical/electrical components.

### **Techniques relevant to setup a fluorescence based transcription assay with a possible single molecule detection limit**

There have been experiments to monitor transcription on the single molecule level (Davenport et al., 2000; Schafer et al., 1991; Wang et al., 1998; Yin et al., 1995). None of these experiments used a fluorescence based method for detection although recent advances in single-molecule spectroscopy offer new tools for the study of individual macromolecules (Weiss, 1999) (Bonnet et al., 1998; Bonnet et al., 1999).

Optical detection of single molecules has been achieved by both frequency-modulated absorption and laser-induced fluorescence. Because of the low background and high signal-to-noise ratio, laser-induced fluorescence has become the most widely used method.

Under favorable conditions, individual fluorophore molecules like fluorescein, rhodamine or cyanine can emit an average of  $10^5$ - $10^6$  fluorescence photons before photobleaching. Current ultrasensitive instrumentation using single-photon counting avalanche photodiodes allows approximately 5% overall detection efficiency. Thus, 5000 to 50 000 photons can be observed from a typical fluorophore. This number is sufficient not only for single-molecule detection, but it is also sufficient for spectroscopic identification and real-time monitoring over an extended period of time (Nie and Zare, 1997). Recently it has become even possible to use CCD cameras to image single molecule fluorescence (Unger et al., 1999).

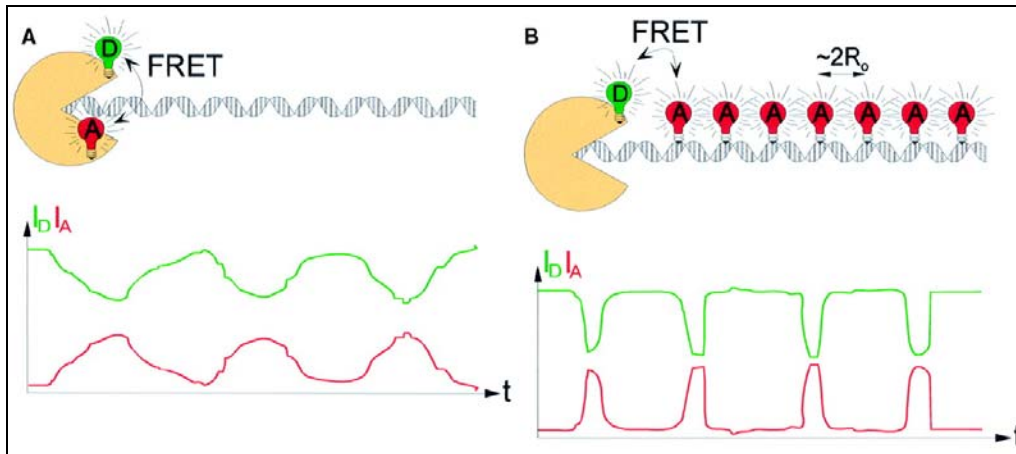
The key challenge is to reduce the background interference. This background interference has gradually been overcome by the use of high-performance optical filters, ultrapure solvents, and the reduction of illuminated-sample volume through the use of laser excitation in the confocal, near-field, microdroplet and evanescent wave configurations.

The evanescent method has certain advantages. First, the background signal is generally low because only a thin layer of about 200 nm is probed. Second, the dynamics of molecules on a surface like binding or polymerization can be studied at the single-molecule level because the fluorescence signal is only detected when molecules move into the evanescent field.

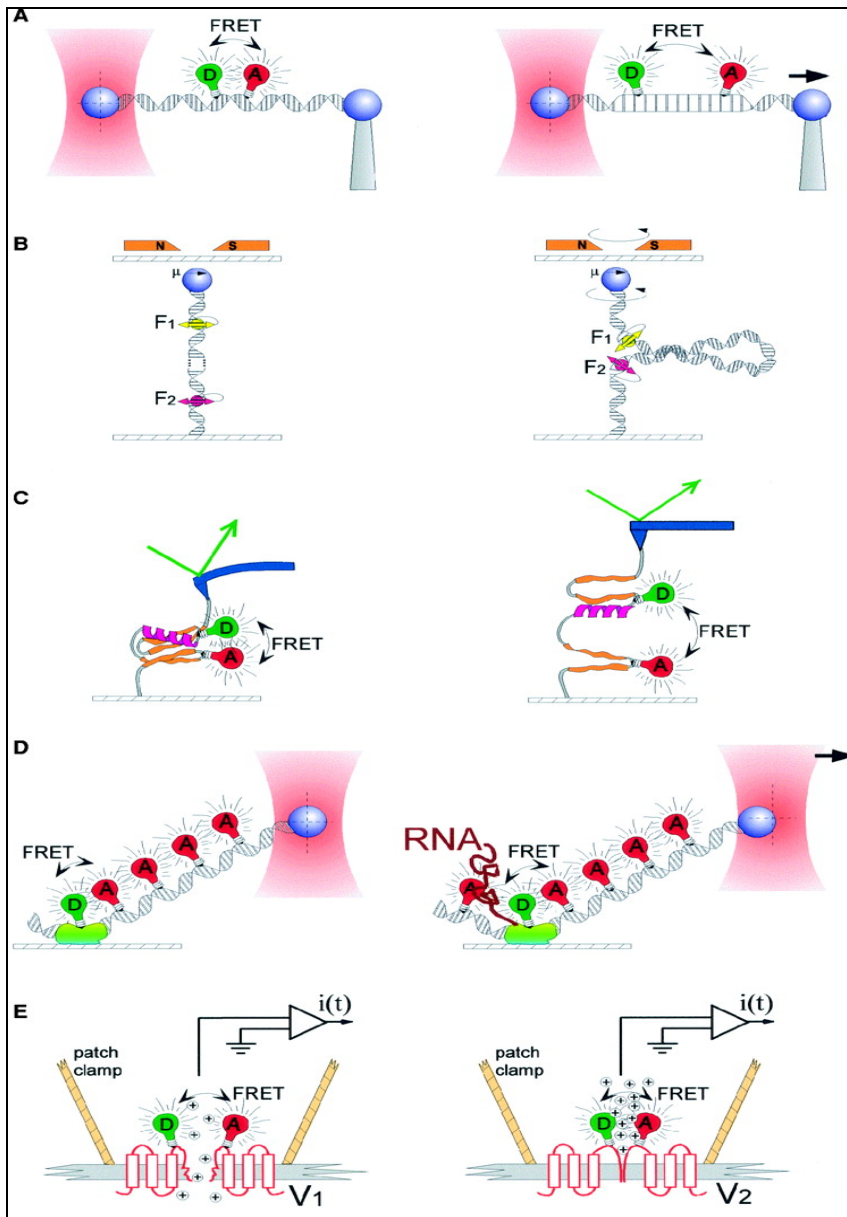
Evanescent wave excitation is achieved by total internal reflection at the glass-liquid interface (Axelrod et al., 1983). Recently this method (in combination with other methods) has been used successfully to study biochemical processes on the single-molecule level. This approach has been used to image single fluorescently labeled

myosin molecules and detect individual ATP turnover reactions (Funatsu et al., 1995). Also, individual interactions of single RNA polymerase molecules with a single molecule of lambda-phage DNA have been directly observed (Harada et al., 1999).

Another valuable tool to study single molecule by laser-induced fluorescence is fluorescence resonance energy transfer (FRET) (Foerster, 1948). This technique, capable of measuring distances on the 2- to 8-nm scale, relies on the distance-dependent energy transfer between a donor fluorophore and acceptor fluorophore. The following cartoons show types of experiments one could think of in order to study single-molecules using FRET (from (Weiss, 1999)):



**Figure 1** A cartoon illustrating (A) intramolecular and (B) intermolecular FRET nuclelease-DNA interactions. Intramolecular FRET measures conformational dynamics of the enzyme during catalysis. Intermolecular FRET measures association, catalysis, and dissociation of substrate molecules. Multiple acceptors at equal distances on the DNA act as a “ruler”.  $R_0$  is the Foerster radius (distance at which 50% of the energy is transferred). This scheme can be generalized to many other protein-DNA interactions. Courtesy S.Weiss.



**Figure 2** An outlook to possible future experiments that combine single-molecule manipulation and single-molecule fluorescence spectroscopy techniques. Such measurements will allow correlation of local structural changes with global macromolecule function or response to an external stimulus. Left and right panels show two different time points in the experiment. (A) DNA mechanical stretching together with FRET. (B) DNA mechanical coiling together with dipole orientation measurement. (C) Protein mechanical unfolding together with FRET. (D) Monitoring movements and forces during transcription by laser tweezers and FRET. (E) Single-channel recording by patch-clamp together with FRET. The ionic current measured by the patch-clamp is represented by  $i(t)$ . Courtesy S.Weiss.

A combination of an evanescent wave setup with a FRET measurement would be a valuable tool to study the kinetics of a transcription reaction from surface immobilized DNA (or RNA polymerase) molecules. A combined optical tweezer and FRET experiment could also be conceived.

With the background and techniques described above, I propose a bottom up approach to study genetic circuits *in vitro* and learn how transcription is regulated by different DNA conformations:

5. It is necessary to develop a simple *in vitro* reconstituted transcription assay to measure rates of transcription from a limited number of molecules, e.g. using a combined evanescent wave/FRET setup
6. If possible extend the sensitivity of this transcription assay to monitor a single DNA templated transcription reaction.
7. Combine the transcription assay with micromanipulation of single DNA molecules (optical tweezer setup) in order to study the influence of DNA conformation (looping, chromatin) on transcription initiation and elongation rates.
8. Combine a small number of genes in a genetic circuit on a microchip to study the design principles of a robust network which is able to perform a small computational task.

## Preliminary data and planned experiments

### *Study of chromatin conformations*

#### Assembly of chromatin in bulk on lambda DNA

The first level in the hierarchy of chromatin folding, the association of DNA with the core histones to form regularly spaced nucleosomes, can be reconstituted *in vitro* using different assembly systems. There are a variety of procedures for the preparation of chromatin *in vitro*, each of which has specific advantages and drawbacks. Some methods employ purified components, whereas other techniques involve the use of crude cell extracts. One criteria for successful reconstitution of functional chromatin is the regular spacing of the nucleosomes on the DNA template. The regularity of the nucleosomal spacing of the reconstituted chromatin can be observed by micrococcal nuclease digestion analysis. Micrococcal nuclease catalyzes double-stranded DNA cleavage in the linker DNA between nucleosomal cores in chromatin, and partial digestion of chromatin by micrococcal nuclease will generate mono- and oligonucleosomal fragments. If the chromatin contains regularly spaced nucleosomes, then the DNA derived from the micrococcal nuclease-digested chromatin will appear as a distinct ladder on an agarose gel (Noll and Kornberg, 1977).

In collaboration with Prof. R.G.Roeder's Laboratory (The Rockefeller University, New York) I used *Drosophila* chromatin assembly extracts to reconstitute chromatin with lambda DNA. The crude *Drosophila* chromatin assembly extracts are essentially cytoplasmatic supernatants generated by centrifugation of embryo homogenates of preblastoderm embryos (Becker et al., 1994; Becker and Wu, 1992).

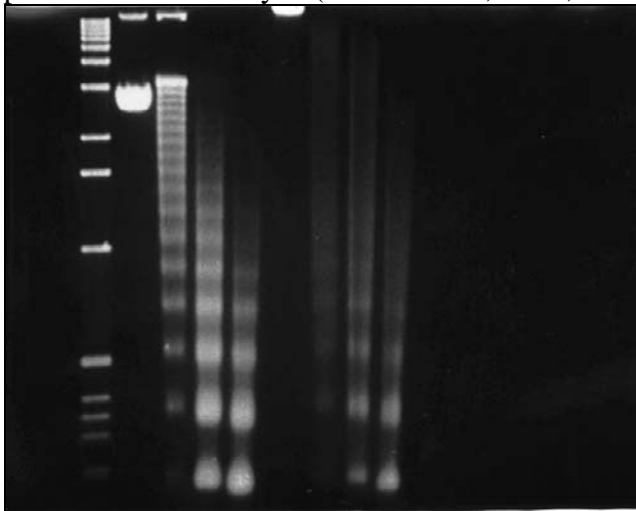


Figure 3 Micrococcal analysis of chromatin assembled with *Drosophila* extract on a plasmid versus assembly on lambda DNA. Lanes 1: Boehringer Mannheim Marker VI (220, 298, 344, 396, 517...) 2: Input Plasmid pBSKS(+), 3-5: Timecourse Micrococcal digestion 45'', 1'30'', 5' 6: Input lambda DNA 7-9: Timecourse Micrococcal digestion 45'', 1'30'', 5'

Figure 3 shows that regularly spaced chromatin can be assembled even on a long DNA molecule like lambda DNA (about 48 kb). As a control I show the assembly of chromatin on a 3 kb plasmid (pBSKS(+)-tRNA).

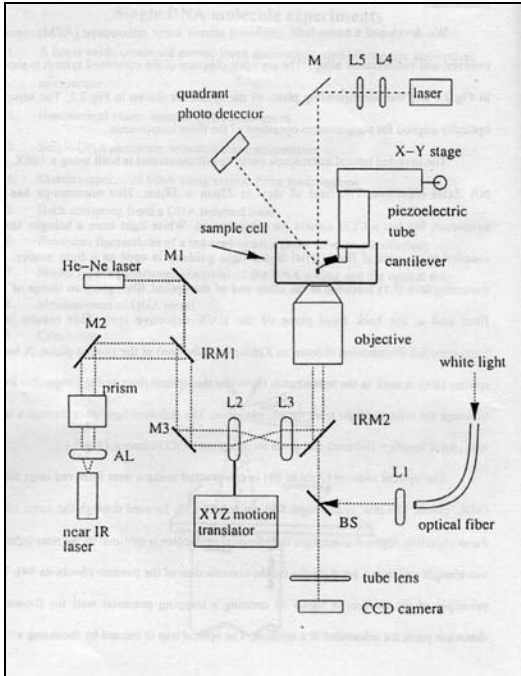
Also in collaboration with Prof.R.G.Roeder's Laboratory I started to assemble chromatin on lambda DNA using purified components. It has been shown that periodic nucleosomal arrays can be assembled with ACF1, an ISWI-containing factor, and NAP-1, a core histone chaperone, in an ATP-dependent process (Ito et al., 1997) (Ito et al., 1999). All three *Drosophila* proteins are expressed in Sf9 cells using the Baculovirus expression system. The Flag tagged proteins are purified using affinity chromatography (M2 agarose).

With less efficiency, recombinant *Drosophila* nucleosome assembly protein (NAP-1) is sufficient to assemble nucleosomes with no additional factors or metabolites required (McQuibban et al., 1998) .

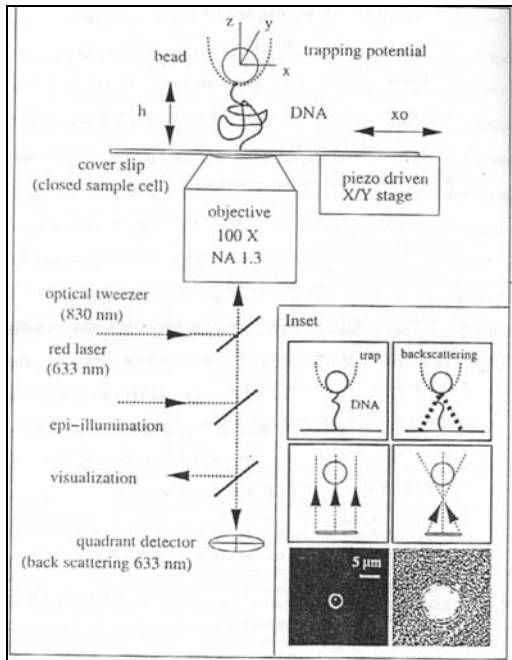
### **Instrumentation: Setup to manipulate single lambda DNA molecules with an optical tweezer**

In Prof. Libchaber's Laboratory I started working together with Dr. G.V.Shivashankar on his home-built optical tweezer setup (Shivashankar, 1998; Shivashankar, 1997). The figures below show the schematic of the setup. The relevant parts for this experiment are the following:

The setup consists of an inverted optical microscope with epi-illumination from a white light source, a piezostage to move the coverslip relative to the fixed objective and an optical tweezer. The inverted optical microscope is constructed using a 100X, 1.3 NA Zeiss objective. The field of view of the optical microscope is 73  $\mu\text{m}$  x 58  $\mu\text{m}$ . A near infrared laser diode (SDL, power 150 mW, wavelength 830 nm), focused through the same objective, is used to construct the optical tweezer. With the telescopic lens system (lens L2 and L3) the focus of the infrared laser can be steered in three dimensions by moving lens L2. Trapping forces are in the order of 0-15 picon. The sample cell is a polyvinyl O ring (diameter 22 mm) adhered with paraffin to a cleaned coverslip .



**Figure 4 Ray optics of the setup.** The inverted optical microscope is built using a 100X 1.3 NA Zeiss objective. White light coupled through an optical fiber is focused through lens L1 thus giving an image of the fiber end at the back of the 100X objective. This results in homogeneous (Kohler) illumination at the sample plane. A CCD camera is used for visualization. The optical tweezer is constructed using a near infra red laser diode (SDL, 150 mW, 830 nm) focused through the same 100X objective. The laser is collimated to a spherical beam of 8 mm in diameter using an aspheric lens (AL) and an anamorphic prism. A red laser is aligned collinear with the infrared laser. The beams pass through a telescopic lens system (L2 and L3) before entering the objective lens. The telescopic lens set is used to steer the optical trap. The atomic force microscope construction is mounted on top of the objective. Courtesy G.V.Shivashankar



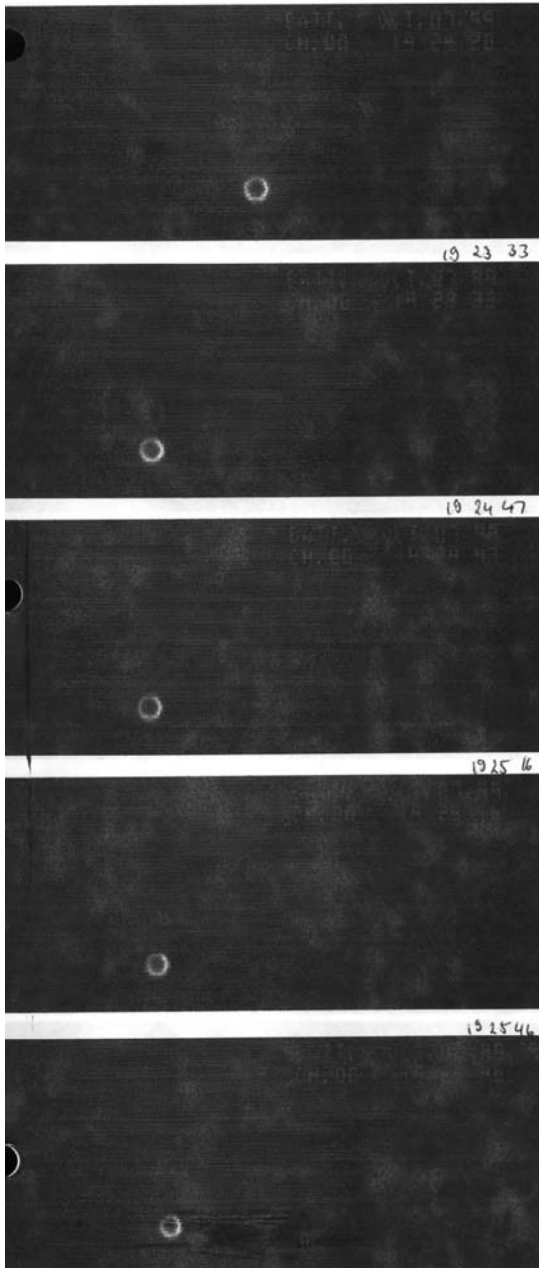
**Figure 5 Schematic of the setup:** The optical tweezer uses a custom made inverted optical microscope. A red laser, collinear with the optical tweezer, is used to scatter light from a tethered bead in the trap. Backscattered light is projected onto the quadrant detector at the image plane. DNA stretching is achieved by moving the piezo-driven stage along the x-axis, while the tethered bead is confined in the optical trap. Inset: bright field image of a DNA tethered bead in the optical trap and the backscattered light image of the same bead illuminated with a red laser and the corresponding types of illumination. Both images are visualized using a CCD camera. Courtesy G.V.Shivashankar

## Measuring of the DNA contour length during chromatin assembly

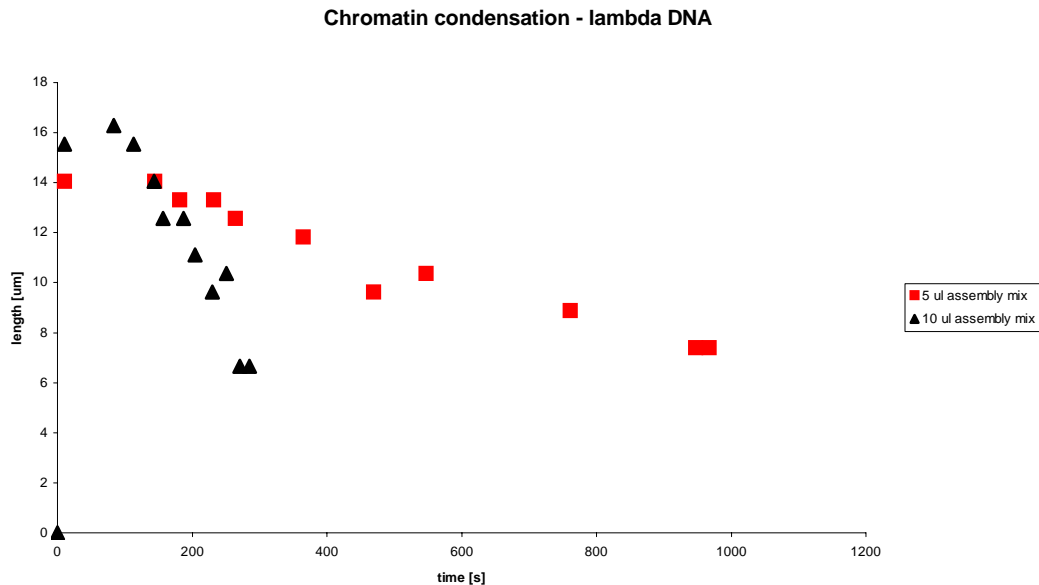
A DNA tethered bead is trapped with an optical tweezer. For the following experiment I was interested in measuring the contour length of the lambda DNA decreasing during chromatin assembly. The DNA can be stretched by steering the trapped bead with the optical tweezer from the initial equilibrium position in one direction until the DNA is fully stretched. Then the bead escapes the trap and returns to its initial equilibrium position since the DNA recoils. The bead has a diameter of 3  $\mu\text{m}$ , the DNA has a initial contour length of 16.5  $\mu\text{m}$  when fully extended. The bead is visualized on a CCD camera with an 100X objective. Thus by simply visualizing the position of the trapped bead when the DNA is fully extended versus the equilibrium position of the non-trapped bead the contour length of the lambda DNA can be measured with an accuracy of  $\pm 1.5 \mu\text{m}$  (the bead diameter).

The DNA attachment to the bead and coverslip is done unspecifically by resuspending the bead and the DNA in PBS buffer pH 6.0 and deposition of this mix on the coverslip over night (Allemand et al., 1997). The next day one can select for one bead which is tethered to the coverslip by a single lambda DNA molecule. To be sure that the bead is tethered to the coverslip by only one molecule the tethering can be characterized by a force-extension measurement (see also below). The force-extension data for a single

lambda molecule is characterized practically and theoretically and can be used as a reference (Wang et al., 1997) (Bustamante et al., 1994; Smith et al., 1996).



**Figure 6** Illustrates part of a timecourse of lambda DNA contour length measurement during chromatin assembly with *Drosophila* extract. A 3  $\mu\text{m}$  polystyrene bead is tethered to the coverslip with a single lambda DNA molecule. The top picture shows the equilibrium position of the bead when the DNA forms a random coil. The following pictures are taken 5, 21, 50 and 80 seconds after onset of assembly. They show the bead position when the DNA is fully extended by steering the bead with the optical tweezer. The difference between the extended and the equilibrium position of the bead is the contour length of the DNA.



**Figure 7** Change of contour length of a single lambda DNA molecule versus time during chromatin assembly with *Drosophila* extract. The kinetics for two different concentrations of *Drosophila* extract are shown.

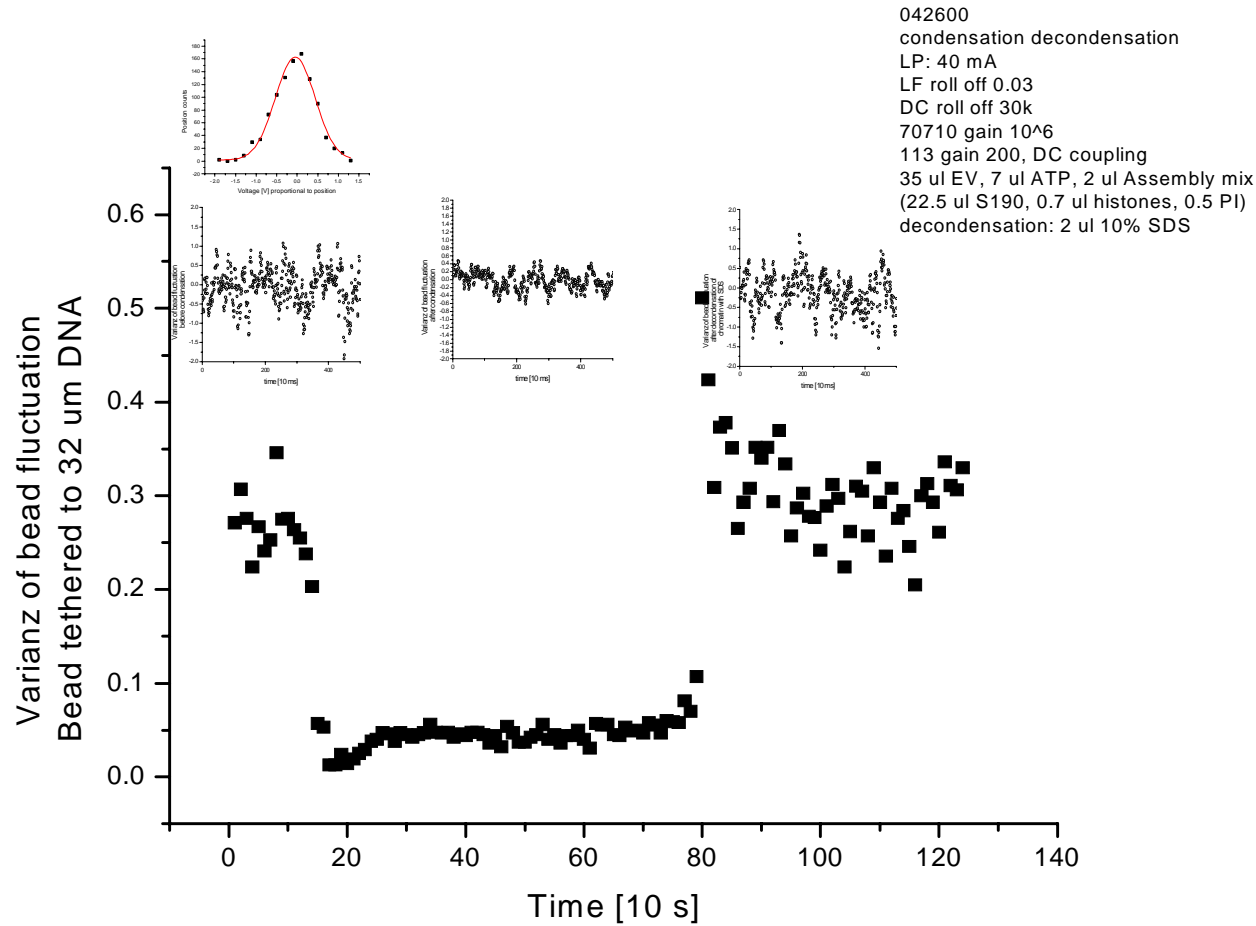
## Elasticity measurements of assembled chromatin

The tweezer setup is also equipped with a He-Ne laser (633 nm, 8mW) as shown in the schematic. Collinear with the infrared laser beam, light from this red laser is focused through the 100X objective and used to scatter light from a single DNA tethered polystyrene bead. The backscattered light is collected using the same 100X objective and projected onto a quadrant detector (UDT, Spot 4D), placed in the image plane. With the quadrant detector the position of the bead can be measured with an accuracy of less than 10 nm.

The bead is fluctuating in a potential well described by a superposition of two potentials: one corresponding to the optical trap and the other representing the action of the DNA polymer. The optical trap has a harmonic core and can be modeled with only one parameter (the trap stiffness) which can be measured by Stokes drag method or by measuring the corner frequency of the power spectrum of the free bead fluctuations. Thus, it is possible to measure the contribution to the trapping potential due to the tethered DNA polymer (Shivashankar, 1998). The DNA tethered bead is now a measure of the mechanical response of a single DNA molecule. This method has been used to study the kinetics of RecA assembly on lambda DNA (Shivashankar et al., 1999).

I started to measure the kinetics of the elasticity of lambda DNA during chromatin assembly with *Drosophila* assembly extract. The elasticity of the DNA is a function of persistence length and stretch modulus. The persistence length is an important parameter

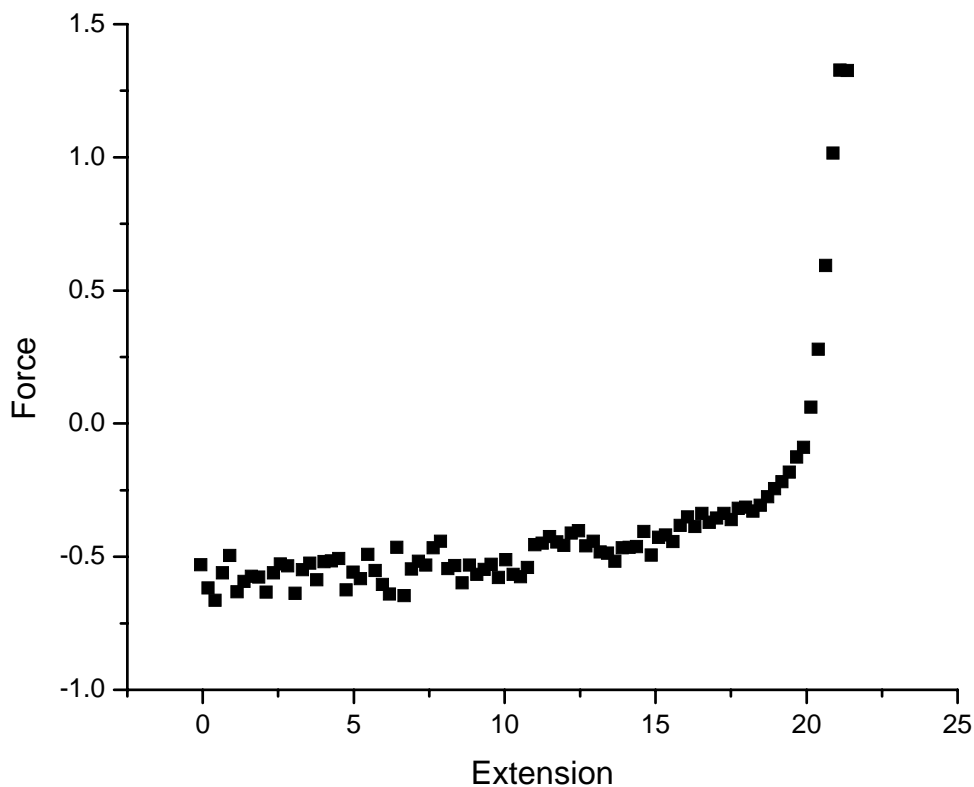
in order to predict the looping probability of DNA. Since DNA looping is thought to be involved in many cellular processes like activation of transcription (Ptashne, 1988) (Dillon et al., 1997), it is potentially interesting to directly measure changes in persistence length in chromatin (Ringrose et al., 1999) especially if these studies can be extended to study chromatin remodeling activities (see below).



**Figure 8** The bead is tethered with one lambda DNA molecule to the coverslip. Position of the bead is detected as described in the text. Change in the variance of bead fluctuation (which is a function of the persistence length) before addition of *Drosophila* extract (0-150 s), after assembly (200-800 s) and after disassembly with SDS (Sodiumdodecylsulfate) buffer (800-1200 s). Inset: Three timecourses of bead position along one axis versus time. From this raw data a histogram is plotted. The Variance is calculated by fitting the histogram to a Gaussian curve (red).

## Force extension measurements on chromatin

The details of the measurement setup and calibration of the optical trap have been described. In summary, light from a red laser, collinear with the infrared laser beam is used to scatter light from a DNA tethered polystyrene bead confined in the trap. The backscattered light from the bead is collected using the same objective and is then focused onto a quadrant detector. Measuring the displacement of the bead position relative to the center of the focused IR laser allows to determine the force acting on the bead. Force extension measurements are recorded by moving the piezo-stage and simultaneously recording the displacement of the tethered bead relative to the focus of the collinear red and infrared laser. As mentioned, force-extension measurements for single lambda DNA are well characterized. The picture below shows the typical response measured when the bead is tethered through a single lambda DNA molecule to the coverslip.



**Figure 9** Force-extension measurement with single lambda DNA. The scale of force and extension are not normalized. Before reaching the contour length, small forces are needed to remove the random coil when stretching the single DNA molecule (entropic regime). When the contour length is reached, much higher forces are needed to stretch the DNA beyond 16.5 um through unstacking of bases (enthalpic regime).

I am planning to do force-extension measurements during assembly and after remodeling of the chromatin structure. This should provide valuable information about the change of contour and persistence length during assembly and remodeling of chromatin.

### **AFM measurements to reveal (higher order) structure of chromatin and forces involved stabilizing different conformations**

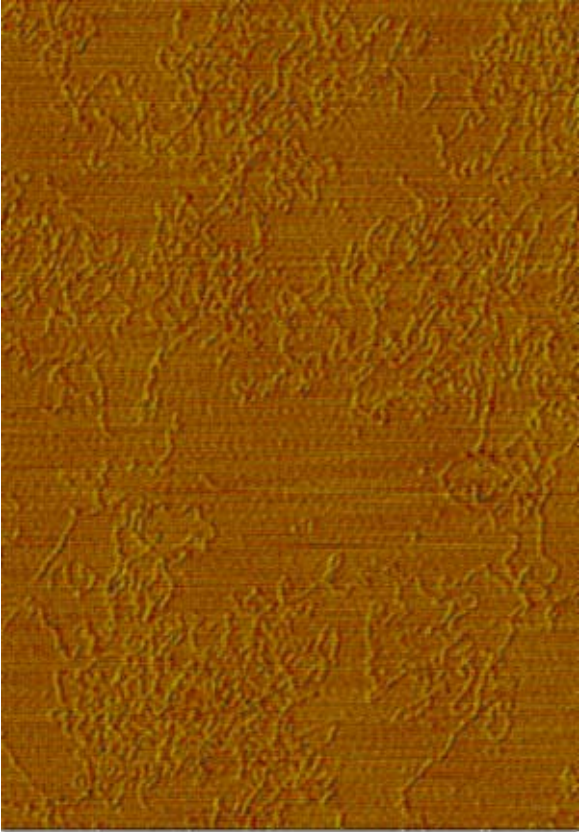
The maximum force of the optical tweezer (about 15 pN) is not high enough to decondense the chromatin once it is assembled. I plan to use the AFM setup (Shivashankar, 1997) to couple the assembled chromatin fiber to the tip of the cantilever of an AFM. A force-extension measurement should reveal the process of dissociation of the nucleosomes. This might reveal the forces involved in stabilizing different levels of the chromatin structure. With this measurement I might also be able to probe for the regularity of the nucleosomal array.

### **Sensitive assay to monitor nucleosomal binding and possible remodeling**

The binding of every nucleosome on the lambda DNA introduces one supercoil. If the chromatin is assembled with a purified system using ACF and NAP-1, there should be no topoisomerase present to chemically relax the introduced supercoil. Instead, the supercoil should mechanically relax by spinning the DNA tethered bead. I might be able to simply visualize this process by using an anisotropic bead. This promises to be a very sensitive assay which might also be capable of monitoring the winding/unwinding of DNA around the nucleosome due to remodeling activities.

### **Scanning force microscopy to visualize chromatin structures**

I started to use a Nanoscope III AFM at the NEC Research Institute Princeton to visualize DNA deposited on a mica slide.



**Figure 10 Imaging of lambda DNA molecules in air on mica. The picture shows several lambda DNA molecules deposited on a mica slide.**

I am planning to extend this experiment to visualize the chromatin I am using in my other experiments. This might be a good control to proof that I am really assembling functional chromatin.

### ***Toward a fluorescence based single-molecule transcription assay***

#### **Fluorescence based transcription assay from surface immobilized DNA**

In Prof. Libchaber's laboratory we developed a chip to control gene expression from surface immobilized DNA templates by heating of ITO pads (in press). Gene expression is monitored by measuring the activity of the expressed luciferase gene. Currently we are combining this method with an evanescent wave setup. A large prism is used to create an evanescent wave with a polarized argon laser. A coverslip can be mounted on

the prism. Fluorescence light within 300 nm of the surface of the coverslip is collected using two photomultipliers, one for each polarization, which allows to measure the anisotropy of the collected light. We are planning to use this anisotropy measurement of fluorescence to develop an assay for transcription e.g. by simply using fluorescence labeled ribonucleotides. The anisotropy of the labeled ribonucleotide incorporated in the nascent RNA polymer is much higher than for the freely diffusing monomer. The sensitivity of this setup can be improved further by using FRET for detection of the transcript. This might enable to detect a single RNA transcript.

This transcription assay might also enable to study the dynamics of genetic circuits capable of performing small computational tasks. Different genes can be deposited at fixed position on the coverslip (biochip). Using an *in vitro* transcription/translation system one could design an artificial circuit where the expression of one gene regulates the expression another gene. A small bistable switch or oscillator could be a first computational task.

## Summary of graduate coursework relevant to the proposed research

Gene Expression (Rockefeller University, New York)  
Physics for Biology (Rockefeller University, New York)  
Modern Optics (Polytech University, Brooklyn)

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