Combining TIRF-FRET and magnetic tweezers to study and manipulate nucleosomes.

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Abstract

TIRF-FRET and magnetic tweezers (MT) are commonly used devices to research DNA mechanics and dynamics. We equipped a TIRF-FRET setup with a magnetic tweezer. The combination of TIRF-FRET and MT makes a lot of experiments involving simultaneous force-spectroscopy and fluorescence possible. For example measurements on dinucleosome-dynamics, which is a step on the road to unravelling the mechanics of chromatin. Several calibrations and control-experiments are done, which show the correct working of both tweezer alone, and combination of tweezer and TIRF-FRET. Furthermore, we found that the position above the evanescent field of the TIRF where we can distinguish between magnetic bead and fluorophore is more than 2 μm from the surface, so the probed DNA-molecule has to be longer than this.

Introduction

In the beginning of the past decade the Human Genome Project showed the world the complete sequence of the human genome. Although we now know this sequence, we do not know in detail how it is structurally organised and how this affects the regulation of the genes. For example; each cell holds a copy of the same chromosomes, yet cells differ and have different functions depending on their place in the body. This cannot be established by DNA alone; there must a mechanism that manages which genes are expressed and which are not. It is a long road to unravel the mysteries of these mechanisms, but this study provides a powerful tool to lift up a tip of the veil.

A human has approximately two meters of DNA in each cell, this is about 6 billion base pairs! In order to fit in a cell nucleus in an organised way, this DNA has to be folded. As figure 1 shows; the first step in this folding is provided by nucleosomes. These nucleosomes consist of histone octamers around which a DNA-strand folds 1.7 times [1]. Arrays of nucleosomes form a higher order structure which we know as chromatin. Even more steps eventually form the famous chromosomes. The nucleosomes play a crucial role in modulating the accessibility of DNA to DNA-processing enzymes.

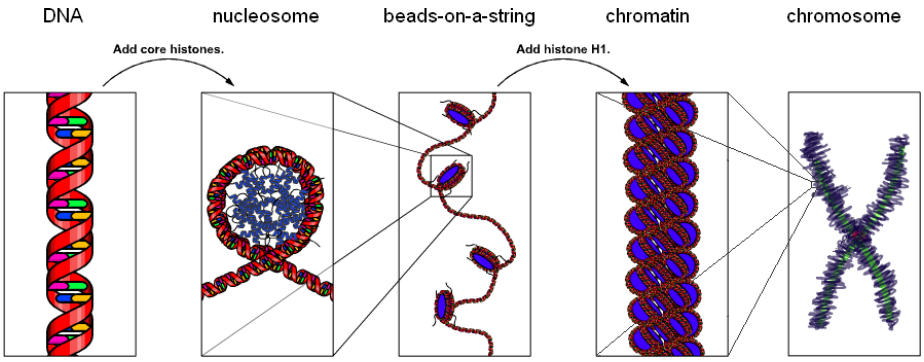

Figure 1: Different levels of DNA folding. The folding starts with DNA on the left. The first step in folding is provided by nucleosomes: histone octamers around which the DNA folds 1.7 times. After several steps the DNA is folded to the chromosome on the right. *Source: wikipedia.org*

Experiments have shown that nucleosomes provide access to the DNA by a process we call DNA-breathing [2]: a part of the DNA unwinds transiently from the histone octamer and can be accessed by enzymes. DNA breathing in single nucleosomes has been investigated with Förster Resonance Energy Transfer (FRET) and with magnetic tweezers (MT). FRET, a fluorescence technique used to measure nanometer-scale distances, uses a donor fluorophore, which transmits its energy to an acceptor fluorophore when near enough, usually within 5 nm. Combined with Total Internal Reflection Fluorescence (TIRF) microscopy on immobilised molecules, FRET showed transient unwrapping of DNA at biological relevant time-scales [3]. Magnetic tweezers are used to apply force to single molecules by attaching a paramagnetic bead to the molecule and exerting a force on the bead by external magnets. A study involving MT revealed part of the folding mechanism of chromatin, showing unwrapping and partial unwrapping of the DNA from the histone octamer [4].

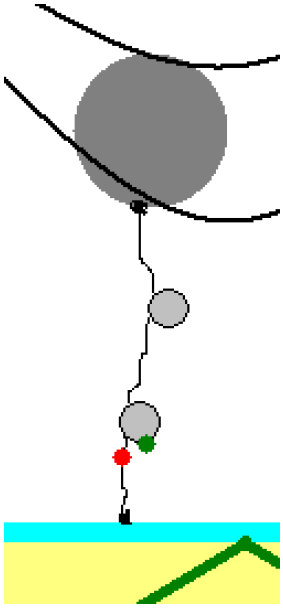

Figure 2: Measurement on two nucleosomes. Breathing is detected by an increase in the length of the construct. FRET discloses which nucleosome breathes.

These experiments all focus on single nucleosomes. However, in vivo, nucleosomes are never alone. We would like to study nucleosome-nucleosome interactions. By combining TIRF-FRET with magnetic tweezers, measurements on breathing in two nucleosomes on the same DNA-strand (dinucleosomes) are possible. Since the DNA partially unwraps from the histone octamer when breathing, this breathing will result in an elongation of the construct. This elongation is measurable using a magnetic tweezer (MT) which has the ability to track the magnetic bead tethered to one end of the DNA. The other end is attached to the glass. Breathing is also measurable by use of FRET-labels. We can measure breathing in dinucleosomes by magnetic tweezers, while FRET-labels, attached to one of the nucleosomes show us which of the nucleosomes breathes.

For this experiment to become possible, we equipped an existing TIRF microscope, used to do FRET experiments with, with a MT. We investigated its capabilities by reproducing results on force-spectroscopy on a strand of DNA. Because a bright bead can obscure measurements on faint fluorophores, we examined the fluorescence of the magnetic bead compared to FRET-fluorophores. First, this thesis describes the working of TIRF, FRET and MT and specifically the way tracking of the magnetic bead and force-calibration is done. Also it explains in detail the way the combined TIRF-FRET MT device is built.

TIRF-FRET

Förster Resonance Energy Transfer (FRET) is used in single molecule microscopy to measure distances. FRET makes use of two fluorophores. A laser is tuned to excite one of the fluorophores, called donor, which will normally emit a photon of its characteristic color. When the distance between donor and acceptor fluorophores is small, the donor can transfer its excitation-energy to the acceptor. The acceptor then emits a photon with a longer wavelength. The probability for this non-radiative transfer is given by [5]:

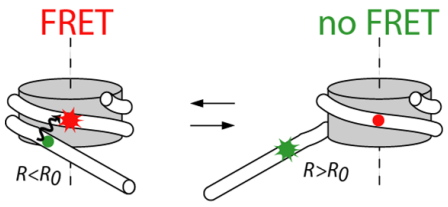

Figure 3: FRET and nucleosome breathing. In the closed state the nucleosomal DNA is inaccessible, the FRET-labels on the DNA are near to each other and can transfer energy; we can detect this state by a high FRET-efficiency (left). In the open state the DNA is accessible and the FRET-labels are not near to each other; we can detect this state by a low FRET-efficiency.

$$E = \frac{1}{1 + (\frac{r}{R_0})^6}$$

where r is the distance between both fluorophores, and R_0 is the distance when $E = 0.5$, which is called the Förster radius. A typical value for R_0 is 5 nm which makes FRET very suitable for experiments on the 11 nm diameter nucleosome. Figure 3 shows how breathing of nucleosomes is detected using FRET.

TIRF stands for Total Internal Reflection Fluorescence. This phenomenon is used in fluorescence microscopy to create an evanescent field, which then only excites fluorophores near to the surface. When light passes from an optical dense medium to a less dense medium, the light gets refracted. If the angle of incidence is greater than the critical angle given by Snell's law;

$$\theta_c = \arcsin \frac{n_-}{n_+}$$

al the light gets reflected, known as total internal reflection, as in figure 4. However, boundary conditions on the Maxwell equations dictate a residue field at the other side of the interface [6]. This field is called an evanescent field and since its strength decays exponentially with increasing distance, it is used in TIRF microscopy to selectively illuminate fluorophores near the glass-sample interface.

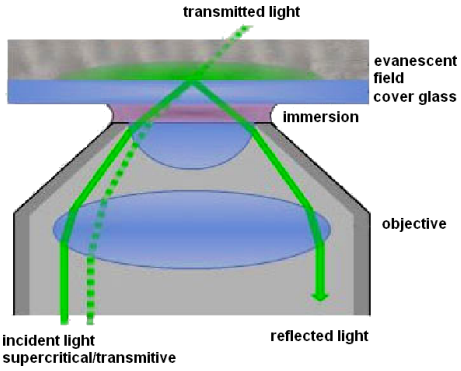

Figure 4: Objective-type TIRF. When light hits the glass-water interface at an angle greater than the critical angle it gets reflected. Boundary conditions on the Maxwell equations dictate an evanescent field.

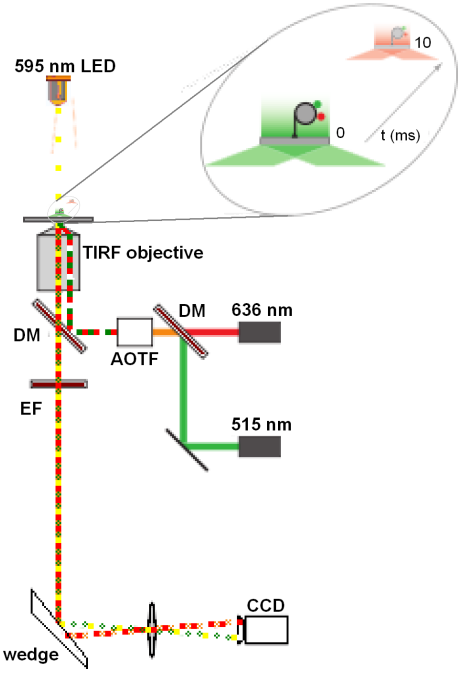

Figure 5: Light-rays in the setup. Green (515 nm) and red (636 nm) lasers illuminate the sample. These are bundled by a dichroic mirror. ALEX is taken care of by an AOTF. Another dichroic mirror directs laser-light to the sample but lets returning fluorescent light through. This light passes an extra filter which filters out the residue laser-light. A wedge splits green and red channels to make a two-color image. The tweezer adds a 595 nm LED to the light-path.

The TIRF setup is an homebuilt device that consists of an inverted microscope, using an 100× 1.45 NA Nikon oil immersion objective and an 150 mm tube lens. In infinity space are mirrors and dichroic filters located to direct the laser light to the sample, and fluorescence light to a Roper Scientific Cascade 512B CCD camera. Figure 5 shows a picture of the light-paths in the setup. To make a twocolor-image, a wedge geometrically splits red and green fluorescence images to be measured separately. FRET donor and acceptor fluorophores are illuminated by alternating laser excitation (ALEX), with a typical illumination time of about 10 ms per color.

Magnetic tweezers

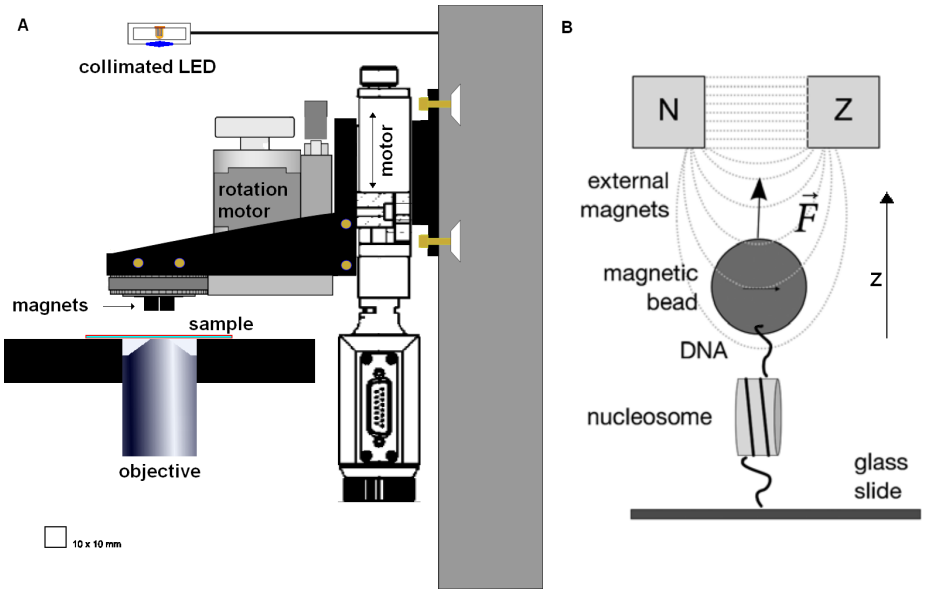

Figure 6: Magnetic tweezer. **A.** The tweezer-part of the setup, the magnets are positioned above the sample and movable up and down, and rotatable. A LED illuminates the sample from above. **B.** The force on the super-paramagnetic bead is in the direction of, and proportional to the gradient in the magnetic field. Moving the magnets away results in a lower force, moving the magnets closer results in higher force. *Source: [4]*

The extension of a molecule as a function of the applied force gives us information on its mechanical properties and dynamics. A magnetic tweezer is a tool to apply such force and measure the extension. A DNA-molecule attached to the glass on one end, and to a super-paramagnetic bead on the other, can be stretched by applying force via the magnetic bead on the DNA by means of external magnets. We use two magnets, spaced about 1 mm apart, to create a magnetic field, as in figure 6. The magnets can rotate and move up and down by stepper-motors.

The bead is pulled towards the highest gradient in the magnetic field. The force can be controlled by moving the magnets. Moving them closer to the sample results in a higher force, moving them away decreases the force. Depending on the size of the magnetic bead our setup can apply forces up to 20 pN.

Bead-Tracking

We measure the stretching behaviour by tracking the magnetic bead. To do that, the sample in our magnetic tweezers setup is illuminated by a 595 nm collimated point-source LED. Tracking in x and y-directions is simply done by correlating the image to an image where the bead is in the center. More important is the tracking in the z-direction. The pictures in figure 7A show typical diffraction patterns a bead creates, on different positions above the focal plane. The diffraction pattern is used to track the height (z) of the bead. Each pattern can be characterised with a certain phase-offset, which then becomes a measure for the distance between bead and focal plane. Each image is correlated with a stack of images with a certain known pattern.

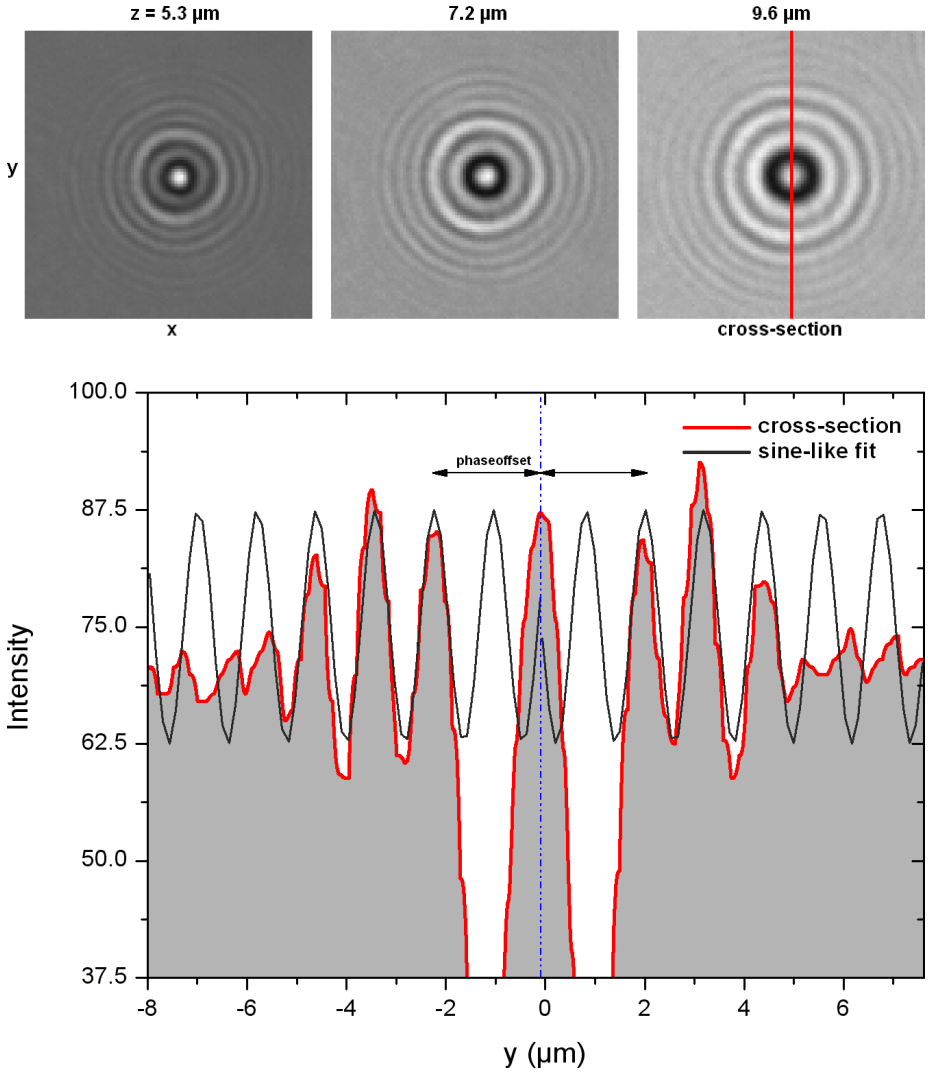

Figure 7: A. Typical image of a 2 µm bead out of focus at different heights. **B.** Cross-cut of the third bead in A. A sine-like function is fitted to the pattern to demonstrate the phase-offset which is a measure for the distance between bead and focal plane.

Since the picture of the diffraction pattern is a measure for the distance between bead and focal plane, it has to be calibrated. The usual way to do this is to make a lookup-table (LUT) of pictures at different known distances. This LUT is then used to compare measured images with, thus to find the measured distance. However, this method is not very fast. Since we can calculate a phase-offset for every image, the only thing we need to do, is to calibrate the phase-distance relationship. This is done by moving the objective, and thus the focal plane, a certain known distance; in the meanwhile calculating the phase of the captured images. Our calibration of a 1 µm bead is shown in figure 8. Determining the phase sometimes goes wrong when the distance between bead and focal plane is small, because then the bead is in focus and the image does not show many fringes. Furthermore, in a certain region near the focal plane, there is more than one height for a given phase. This means we only can track the bead if there is some distance between bead and focal plane, more than 1 µm above the focal plane or more than 2.5 µm below the focal plane. Before every measurement we move the bead a few µm above or below the focal plane to make sure it never gets in the region where it becomes untrackable. The trackable regime is fitted with a polynomial, which is then used to calculate the height from the phase, this is what we call a lookup-table (LUT) in our experiments.

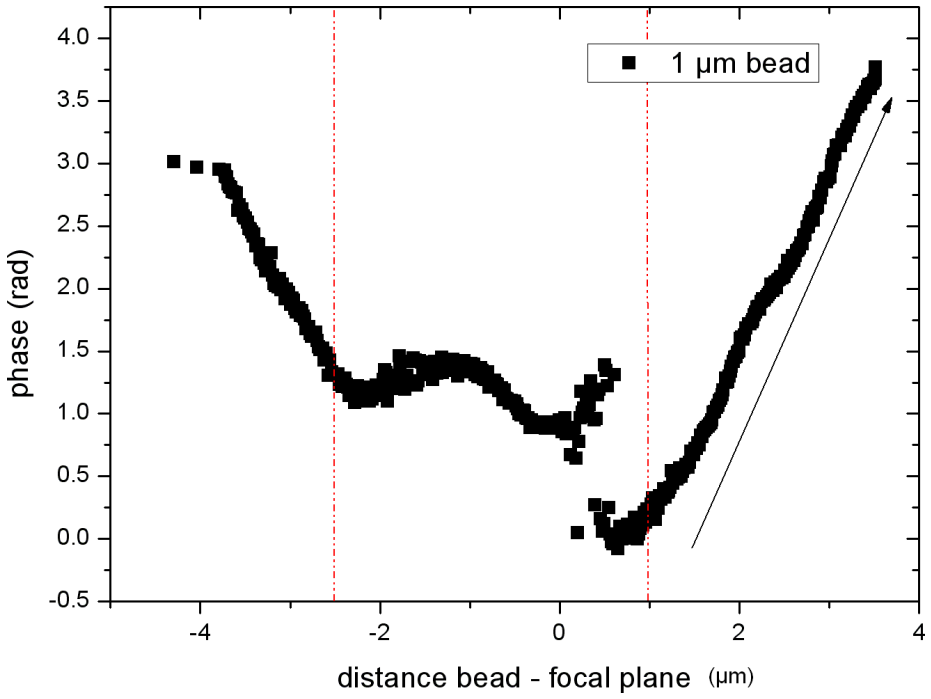

Figure 8: Lookuptable (LUT). Calibrating the bead tracking in z-position: the objective is moved several micrometers while tracking a bead (which in this stage only gives the phase of the diffraction pattern) stuck to the surface. This bead thus moves from under the focal plane, through the focal plane to above the focal plane. One of the linear regimes is then fitted with a polynomial which is used for tracking. The interval between the red lines is not usable for tracking because multiple heights correspond to the same phase in that regime.

To check our phase-height relation, we did an experiment in which we moved the focus and in the meanwhile tracked an immobile bead. Figure 9 shows the result. The movement of the focus should give the bead an apparent motion 0.88 as fast due to the difference in refractive indices of glass and water. The slope in the figure is about 0.93. This difference is caused by inaccuracies in the movement of the stepper-motor to which the objective is attached. Also the hysteresis is caused by inaccuracies in the stepper-motor movement. To achieve a higher accuracy we should add a piezo-motor to the setup to be able to move the focus more reliably.

In order to be able to track beads it is necessary to define a zero-position. It is logical to use the position where the bead is lying on the glass as zero. Because of drift in the system we have to measure this position right before and after every measurement. We let the bead descent onto the glass under gravity. To do this we apply no force on the bead with the magnets by moving them away.

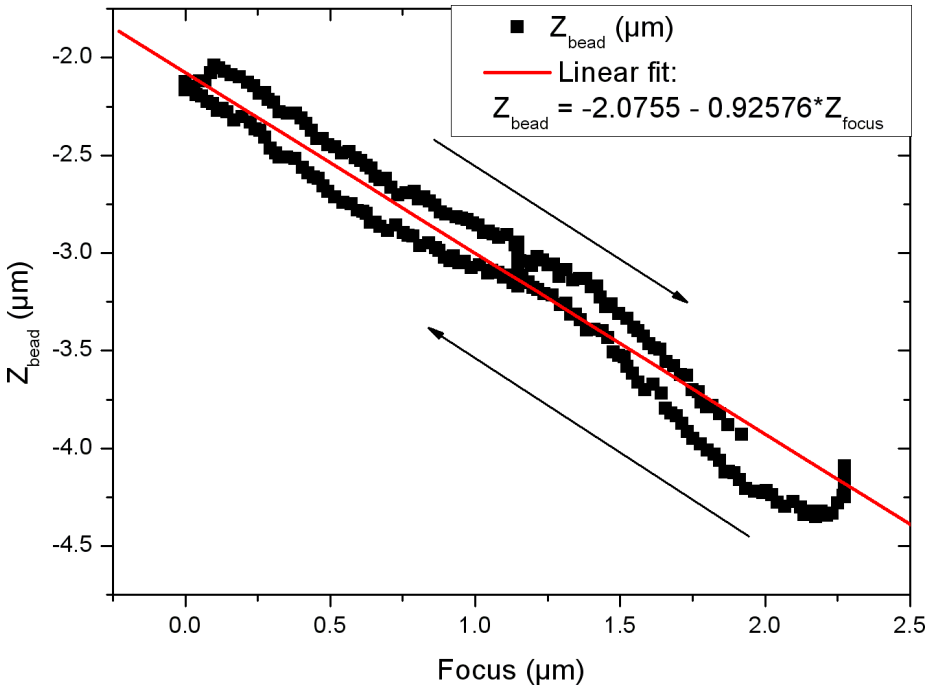

Figure 9: LUT-control experiment. Movement of the focus results in the apparent movement of the bead in the opposite direction and 0.88 times as fast. The fitted value (0.93) differs from this mainly because of inaccuracies in the stepper-motor movement focus, this is also the cause of the hysteresis.

Force calibration

We apply a force to the tethered DNA via a magnetic bead. As said, the closer the magnets are to the sample, the higher the force on the bead is. To do force-spectroscopy it is necessary to calibrate this force. Because the bead is a tiny object in a solution, it undergoes brownian motion. The amplitude of this motion is limited because the bead is tethered. Its amplitude is a measure for

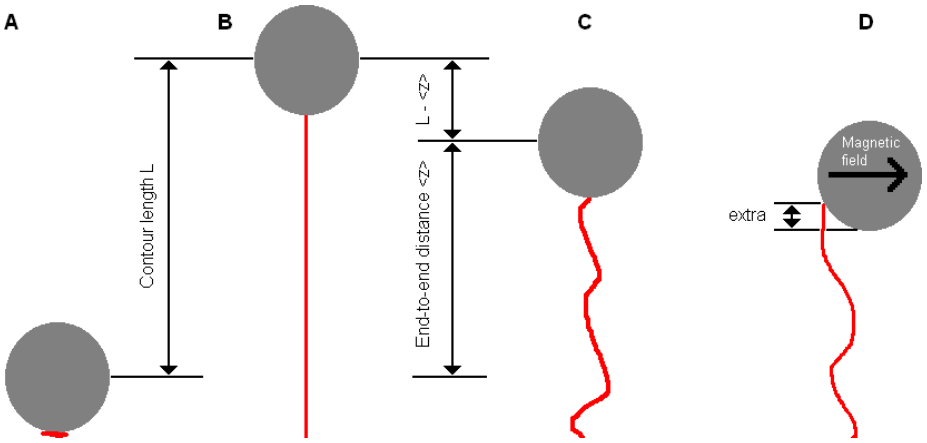

Figure 10: Beads tethered to DNA under different tension. **A.** Very low tension, the bead is on the glass. **B.** Very high tension, the DNA is fully stretched. **C & D.** Intermediate tension, in D. an extra correction is needed to account for the fact that the DNA is not attached to the bottom of the bead.

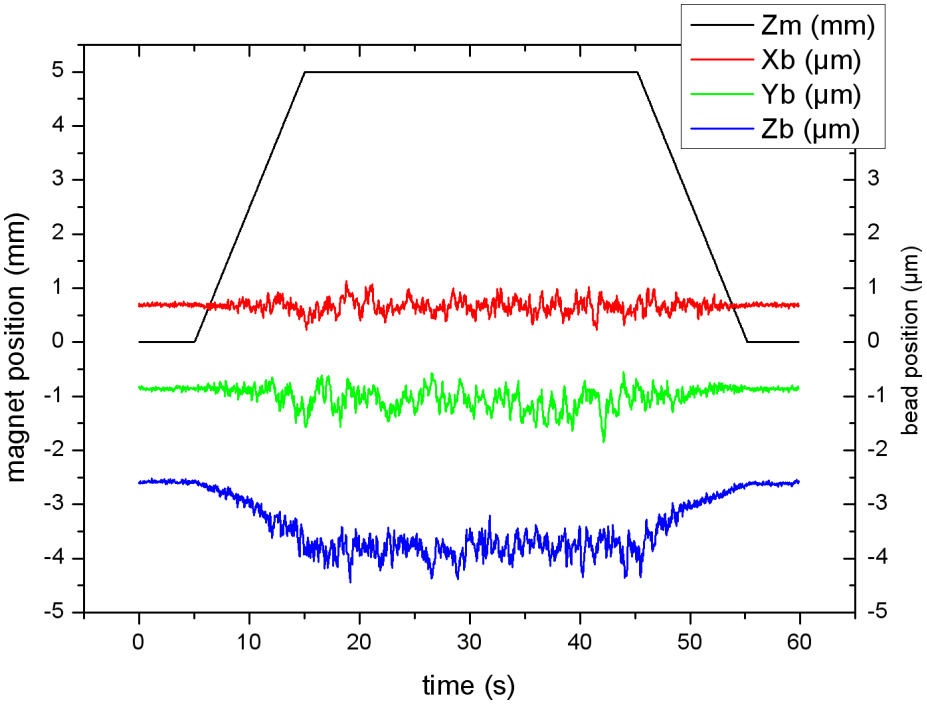

Figure 11: Force-calibration for a magnet-position of 5 mm from the sample on a 1 µm super-paramagnetic bead tethered to 2 µm DNA. The variance in position in x and y are measures for the force on the bead. To measure the end-to-end ditance, we take the position where the bead is under high force as an offset.

the force F applied to the DNA attached to it in the following way:

$$F(z) = \frac{k_B T}{\langle \delta x^2 \rangle} \langle z \rangle \tag{1}$$

where k_BT is the usual Boltzmann-factor-temperature-combination, $\langle z \rangle$ is the end-to-end distance of the DNA, and $\langle \delta x^2 \rangle = \langle (x - \langle x \rangle)^2 \rangle$ is the mean squared displacement from the mean position. The end-to-end distance $\langle z \rangle$ of the DNA can be determined by the difference between the current bead-position and the bead-position at zero force, as in figure 10A & C. To find the mentioned zero-position we should let the bead descent to the glass, before and after every measurement. But, because some beads very much like to stick to the glass, we do not do this. In stead we apply a high force to the bead, like in figure 10B, and track the bead at that position. Because we measured the distance the bead travels between high and low force before the start of the calibration, we now know our zero-position where the bead would be lying on the glass.

We calibrate the force by tracking a bead tethered to a DNA molecule for half a minute. Figure 11 gives an example of a time-trace to calibrate the force on a bead when the magnets are 5 mm away from the top glass of the sample. Figure 12 shows force-calibrations for 1 and 2.8 µm beads. The force is dependent on the size of the beads and decays exponentially. This force usually is calibrated once per bead-size and type, fitted with an exponential and then used until changes are made to the setup.

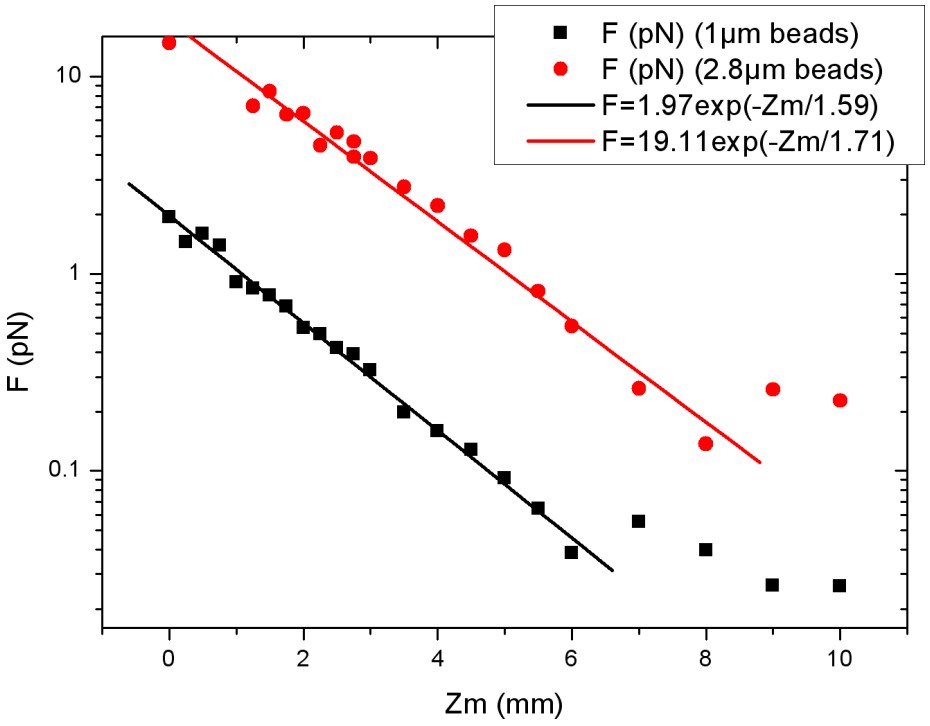

Figure 12: Force-calibration; the force is calculated for each magnet distance from the sample (z_m) by measuring a beads Brownian motion and the DNA's end-to-end-distance. The force decays exponentially and is dependent on the bead size. Black: 1 µm beads, red: 2.8 µm beads. Fits through these points are used as calibration. Points in the low-force regime are excluded because of volume-excluding effects.

Combining TIRF with magnetic tweezers

Our device combines TIRF, FRET and MT. We have to design our tweezer such that it does not interfere with the fluorescence measurements, but still is able to work. In figure 13 the transmission of the set of filters used for FRET experiments is shown. The light of our LED used to illuminate the magnetic beads must pass these filters. We choose a 595 nm collimated point-source LED (IMM Photonics LED-1115-ELC-595-29-5). Its light passes the filters but does not excite the FRET-fluorophores Cy3B and ATTO647N we use.

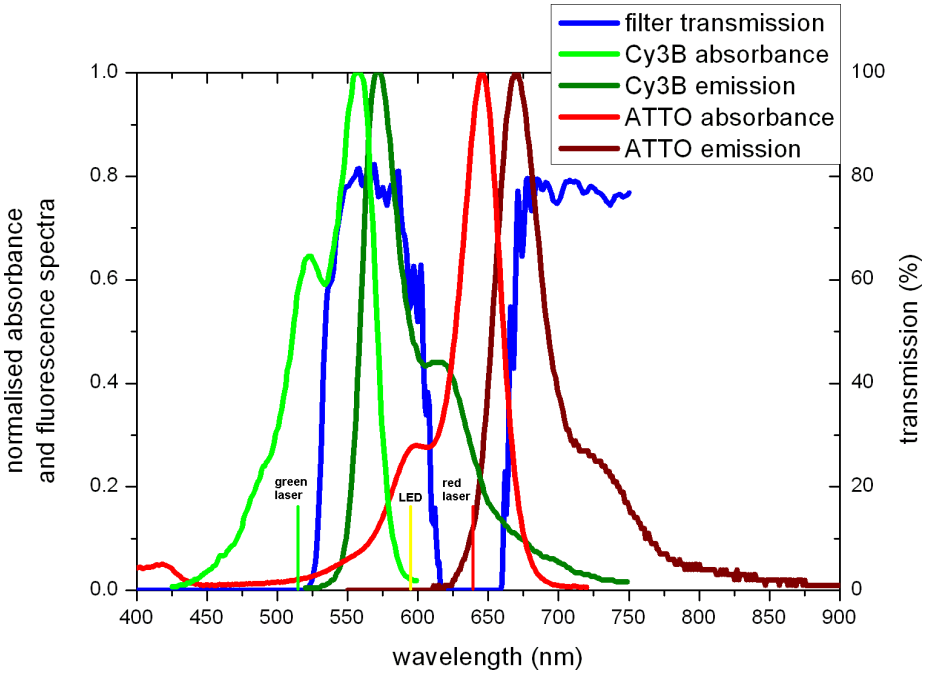

Figure 13: Spectra of FRET-donor (Cy3B) and acceptor (ATTO647N) labels, and the transmission of the filters between sample and CCD.

We want to capture fluorescence and transmission images. We make use of alternating laser excitation (ALEX) in our FRET-experiments. This excites green and red fluorophores by turns and synchronises the camera to these frames. In stead of collecting frames with either green or red excitation, we now also capture frames with only LED illumination. Thus every frame gets its own illumination.

To measure dynamics, we want an as fast as possible framerate. Our camera can measure fast dynamics up to 10 ms short, depending on the region of interest we choose. In overlap-mode every frame is read out while the CCD is already capturing the next frame. This allows for the highest framerate with the maximum of exposure time per frame. From relatively faint fluorophores, we want to collect as much light as possible per frame. The transmission image, however is much brighter, and the camera gain and exposure time that are optimal for the fluorophores are not ideal for the transmission images. Shorter exposure times per frame are useful in this case. The bulb-mode of the camera allows for this. In this mode, we trigger the exposure simultaneously with the

AOTF and the LED by a hardware clock on our DAQ-card. In overlap-mode we only trigger the start of the camera. The camera then triggers the AOTF and the LED. Figure 14 shows how the triggering is done in these two modes.

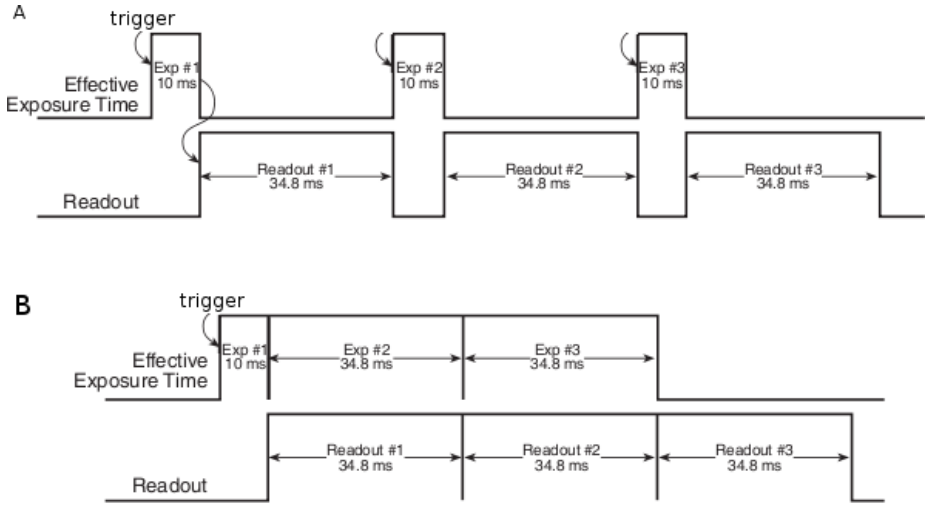

Figure 14: Camera modes & triggering. **A.** If the camera operates in bulb-mode, the CCD is exposed as long as the trigger is high, after that the frame is read out. **B.** If the camera operates in overlap-mode, the CCD is exposed for a pre-set time, which can never be shorter as the readout-time. Every frame is read out when the next frame is integrated. *Source: camera manual Roper Scientific 512B.*

Results

Worm-like chain

To test the magnetic tweezers, we did a force-spectroscopy experiment on a 2 µm long piece of DNA, (a 25-times 601-repeat), tethered to 1 µm beads. DNA-molecules under low tension can be described by the Worm-Like-Chain model (WLC) [7]:

$$\frac{FP}{k_bT} = \frac{1}{4(1-z/L)^2} + \frac{z}{L} + \frac{1}{4} \tag{2}$$

in which F is the force applied to the DNA and z is the end-to-end distance. P is the persistence length of the DNA, usually around 50 nm, and L is the contour length. Figure 15 shows the result of this experiment. The points in this graph are all constant-force measurements, also used to make the force-calibration in figure 12. Equation is fitted to these points. This fits the values of 2.42 µm for the contour length, which corresponds to the expected value of somewhere around 2 µm, and 37 nm for the persistence length. The 37 nm is small compared to the theoretical value of 50 nm for the persistence length of DNA. However we might be underestimating the length of the DNA, and thus the persistence length. Figure 10D shows the cause of this underestimating. If the DNA is not attached to the bottom of the bead, but somewhere to the side, we underestimate the length. To correct for this effect we have to determine the attach point of the DNA to the bead by rotating the bead. We can do this by

rotating the magnets. However, because many beads formed a cloud and blocked our view when we tried to do this, we were not able to do this correction.

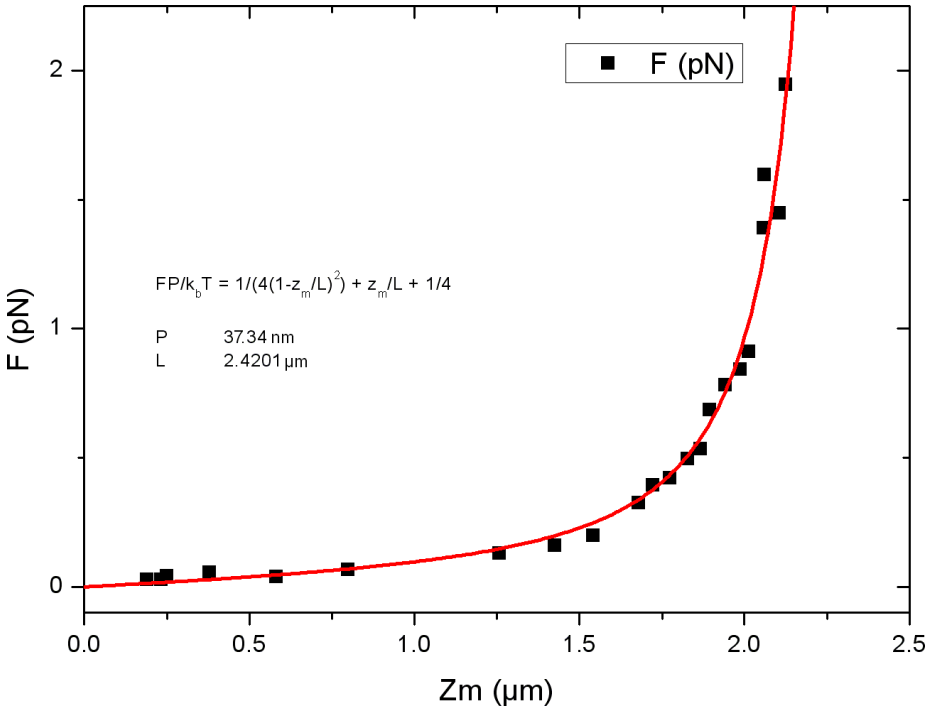

Figure 15: Force-extension curve. An approximately 2 µm long DNA molecule, tethered to a 1 µm paramagnetic bead is fitted to a WLC which gives a contour length of 2.42 µm and a persistence length of 37 nm.

Bead-fluorescence

Autofluorescent light from the bead can obscure the fluorescence from single fluorophores. As can be seen in the spectrum of scattered and autofluorescent light off beads, multiplied by the filters transmissions, the bead is autofluorescent. Figure 17, shows the bead becomes less bright when farther from the surface. So the bead must be out of the evanescent field. We want to know how high a bead has to be so it does not interfere with single nucleosome fluorescence. A simple experiment to test this is as follows: tether a bead to a DNA-molecule and stretch the DNA with the tweezers. If we illuminate the beads with green and red lasers used for FRET experiments we can measure the fluorescence and scattering of the bead on different heights above the glass.

We want to measure the scattering and autofluorescence of light off the bead on different heights above the glass. In experiments with fluorophores, one needs to focus on the fluorophore which is to be measured. In TIRF-experiments these fluorophores are near to the glass. So the focal plane in our experiment has to be near to the glass. But when we do that, the bead is too near to the focal plane to be trackable. We can get around this if we first calibrate the position of the bead for every magnet position. Such a position-calibration curve is shown in figure 18. A 3rd order polynomial is fitted to this curve to

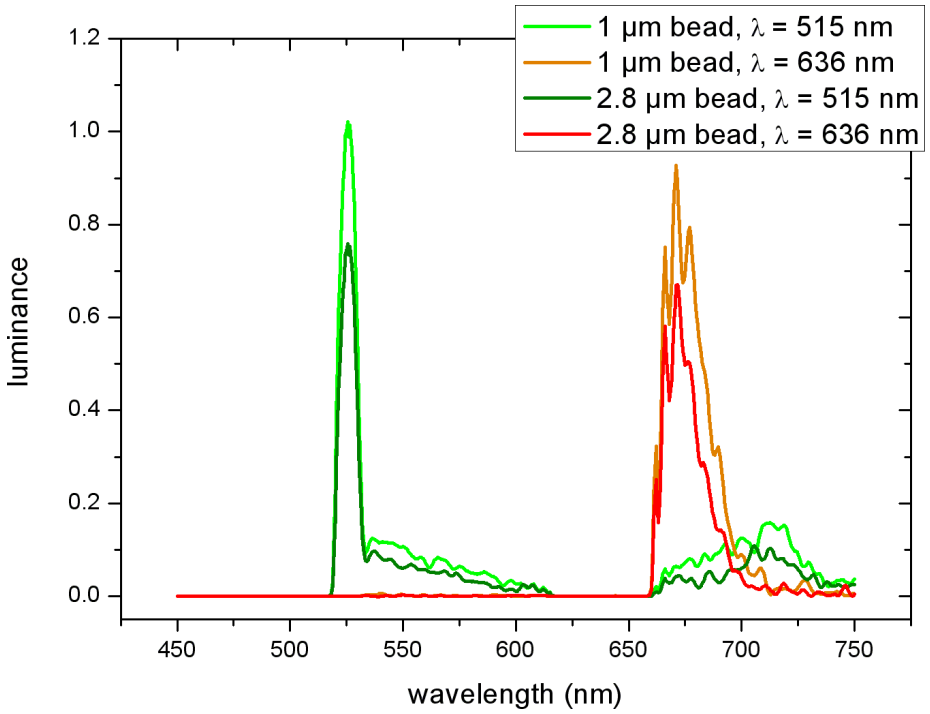

Figure 16: Autofluorescence of the magnetic beads, multiplied by the transmission of the filters in the setup. Scattered light is filtered out. The 50.000 times diluted beads are illuminated by 515 and 636 nm sources and the scattering and auto-fluorescence are measured in a spectrophotometer.

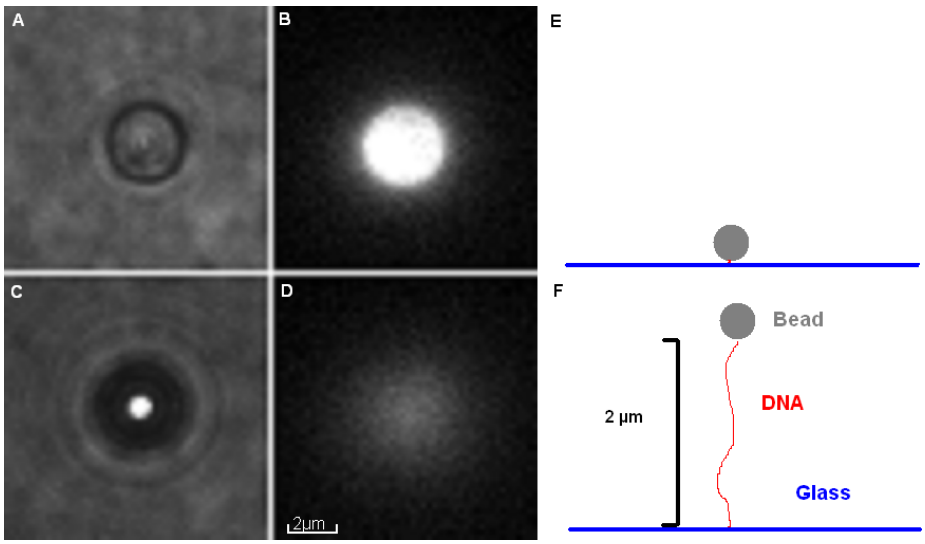

Figure 17: Visible decrease in fluorescence of 1 µm beads. **A.** & **B.** Transmission and fluorescence images of a bead in focus and on the glass. **C.** & **D.** Transmission and fluorescence images of the same bead out of focus and approximately 2 µm above the glass. The bead in C is trackable, the bead in A is not, while only the fluorescence in B is useful for measurements. In **E.** & **F.** the positions of the bead in respectively A & B and C & D. E & F are not to scale.

filter out unwanted fluctuations. After this we measure the fluorescence of a bead with the focus on the surface. The fluorescence intensity is determined by integrating the counts in a 30×30 square around the bead, and subtracting the background from squares above and below the image of the bead. We used the position-calibration to calculate the distance from the bottom of the bead to the glass. Figure 19 shows the measured autofluorescence for different magnet positions with an illumination of 0.18 kW/cm^2 of green, and 0.24 kW/cm^2 of red laser-light. Because the evanescent field roughly decays exponentially with increasing distance from the glass-water interface, we would expect a tiny probe to reproduce this exponential. Because beads are big compared to the typical decay length of 200 nm of the evanescent field we can at best approximate the exponential behaviour of the field with this probe.

A single Cy3B fluorophore label excited by green (515 nm) 0.1 kW/cm^2 laser-light gives about 2160 counts/36 ms in the green channel, the bead gives about 3250 counts/pixel/36 ms in the same channel when 2 µm above the glass. Given the fact that these counts can be distributed over several pixels, we would need at least a DNA-molecule longer than 2 µm.

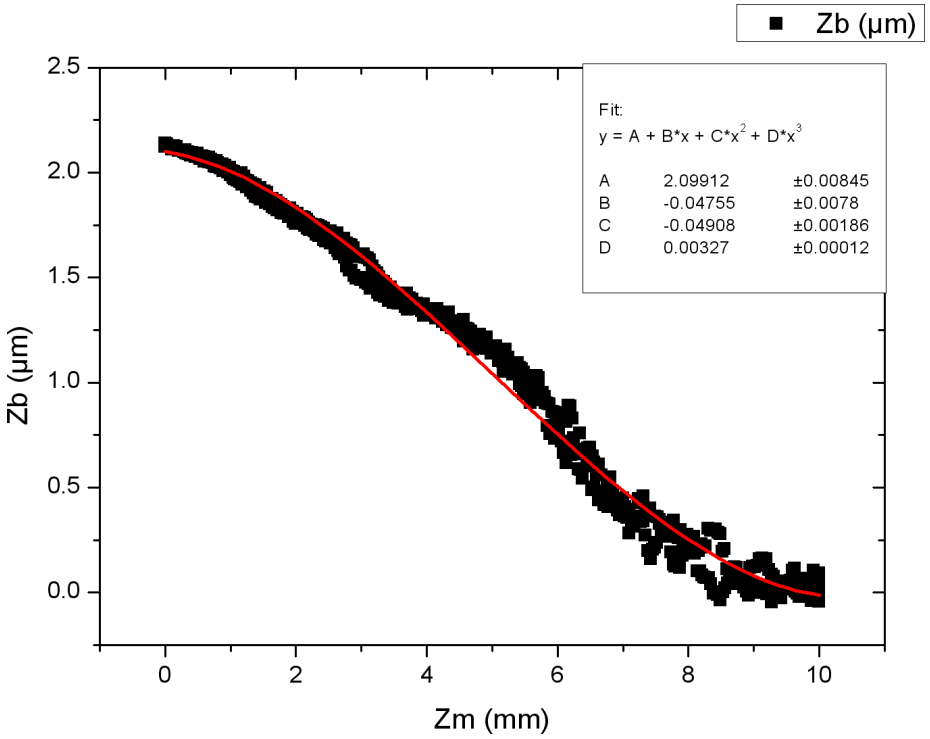

Figure 18: Magnet-bead-position calibration; because simultaneous tracking and fluorescence measurements on a bead are difficult we make use of a calibration which gives the distance of the bottom of the bead to the glass (z_b) for a given magnet position (z_m). This way we don't have to track a bead tethered to DNA when measuring fluorescence.

Because a bead illuminates many pixels compared to a single fluorophore, we might be able to view the bead as a background. This way the fluorescence will be measurable if the signal from the fluorophore is bigger as the noise in the bead-intensity. To compare the number of counts from a single fluorophore

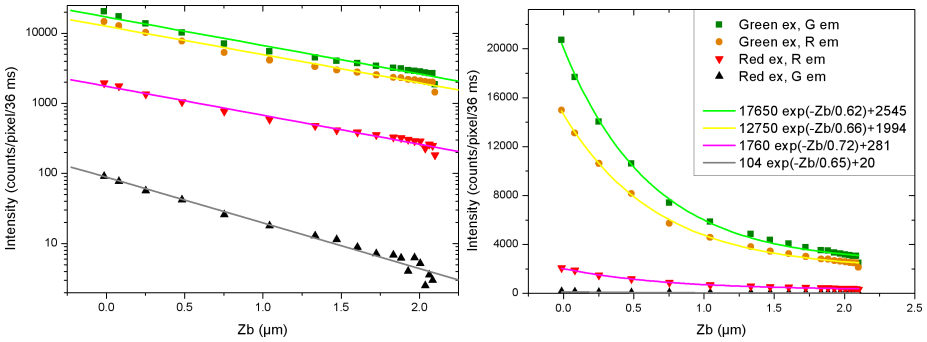

Figure 19: 1 µm Bead fluorescence for different distances from bead to glass (z_b), and colors. Red: Green excitation, green fluorescence, Orange: Green excitation, red fluorescence, Black: red excitation, green fluorescence, Red: red excitation, red fluorescence. Theory predicts an evanescent field decays exponentially with increasing distance from the glass, which these curves nearly match. On the measured distances between bead and glass (0 - 2 µm), the bead is still too bright.

with the noise, we calculated the noise of one pixel in the center of the bead over time. The noise is caused by photon noise in the bead and fluorophore fluorescence and by the motion of the bead. The result is shown in figure 20. Here we see that the noise eventually gets down to about the fluorophore noise of 300 counts/pixel/36 ms. A bead at 2 µm above the surface has a noise of 440 counts /pixel/36 ms. Even at 1 µm above the surface the signal from the fluorophore is twice as big as the noise. So a 2 µm long DNA-molecule is long enough to get a decent signal to noise ratio as long as the bead is kept from descending towards the surface.

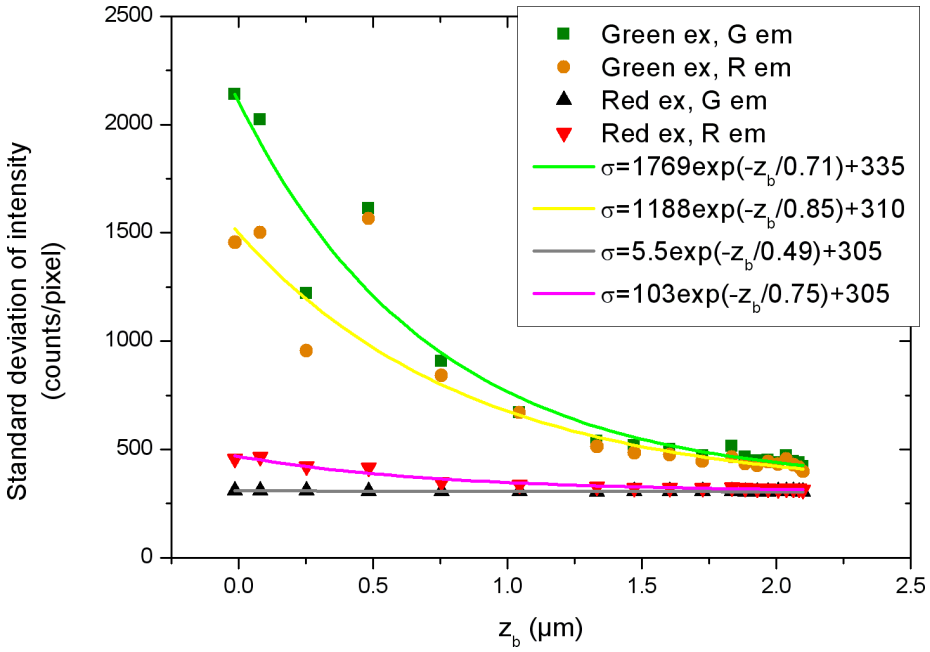

Figure 20: Noise over time in bead fluorescence and fluorophore fluorescence for different distances from bead to glass (z_b), excited and integrated for 36 ms per frame, and colors. Red: Green excitation, green fluorescence, Orange: Green excitation, red fluorescence, Black: red excitation, green fluorescence, Red: red excitation, red fluorescence. A Cy3B FRET-donor gives about 2160 counts.

In combined measurements we want to track the bead when it is above the focal plane. According to figure 8 a 1 µm bead should be more than 1 µm above the focal plane to be trackable. According to figure 20, the bead should be more than about 1 µm above the glass in order not to obscure the fluorescence signal. When measuring with 1 or 2 µm DNA, the DNA always has to be kept high under some force, or it will approach the focal plane and descent into the evanescent field. This high force on a DNA molecule can have unwanted side-effects when measuring other things on the DNA. To be able to apply much lower forces to the DNA one needs to use much longer DNA, so stretching to 2 µm requires less force.

Conclusion

We combined an existing TIRF-setup with a magnetic tweezer. Except for the usual software bugs, the combined device is ready for use now. We showed that doing simultaneous measurements on single DNA-molecules with FRET and tweezers is possible with this combined setup if some things are kept in mind. We have to use DNA-strands longer than 1 µm for the magnetic bead to be out of the evanescent field and to stay trackable. Also the forces on the magnetic bead cannot be too low because it then would descent into the evanescent field. The fluorophores used in experiments done with this combined setup need to be attached near to the glass, to be as bright as possible, so to have a good S/N-ratio. However if we use for example histone octamers and try to attach them on DNA near to the glass, we might get problems with nucleosomes sticking to the glass. Also, it will become more difficult to reconstitute nucleosomes at the right spot on a very long strand of DNA.

We tested the device and found it gives reproducible results. A number of corrections possible on the measurements involving the DNA-strands length, can be done to improve the results even further. Calibration of the force on magnetic beads would improve if we use a piezomotor in stead of a stepper-motor to move the focal plane when making a LUT.

We now have a device which gives us great opportunities to explore the world of chromatin even further. Even now, when there are many improvements and upgrades possible to the combined setup, this combined setup is a promising device for fulfilling its first goal in giving us detailed insight in breathing of dinucleosomes.

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