MASTER

Characterization of the double stranded DNA stain SYTOX orange

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Characterization of the double stranded DNA stain SYTOX Orange

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Abstract

We present a study of the interaction of the highly sensitive nucleic acid stain SYTOX Orange with double stranded DNA and its dependence on the experimental conditions. Combining double optical tweezers with single molecule fluorescence microscopy, we demonstrate that SYTOX is greatly superior to the far more often used YOYO-1 in terms of alteration of the DNA properties. Thus, in low to medium concentration it does not prevent DNA unwinding, and therefore constitutes an excellent reporter of B-DNA structure. Results from single molecule experiments show that probably a greatly increased off-rate compared to YOYO is responsible for the decreased distortion of the DNA structure.

In difference to what has been reported for YOYO, we observe for SYTOX that the salt conditions do have a great influence on SYTOX its affinity for DNA, but do not have an effect on SYTOX its off-rate. We quantified the binding as a function of the stretching force applied to DNA, and find that it is in accordance with a mono-intercalation interaction at low concentrations. However, we provide clear evidence for the presence of a second binding mode with a strongly reduced DNA-affinity which dominates at SYTOX concentrations in excess of 1µM. Finally, it is shown that for high fractional binding significant fluorescence quenching due to electronic dye-dye coupling takes place, and that photobleached SYTOX does not display significant intercalating properties. Altogether, our results demonstrate that our experimental approach provides an excellent tool for the detailed study of fluorescent DNA intercalators.
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1 Introduction

1.1 The carrier of genetic information

The genetic traits like human skin colour, blood type and hair colour are encoded in the genes and each gene codes for a single trait. Organisms have thousands of genes and humans are estimated to have between 20,000 and 25,000 of them (1). Genes are encoded in the 46 deoxyribonucleic acid (DNA) molecules present in the 23 human chromosome pairs. Consequently, many genes are encoded in a single chromosome pair. For instance, the DNA molecule present in the largest human chromosome, chromosome1, codes for approximately 4300 genes (2).

A DNA molecule consists of two complementary chains of many simple units, called nucleotides. A single nucleotide is composed of a nucleobase, the five-carbon sugar deoxyribose and a phosphate group. Deoxyribose together with the phosphate group forms the negatively charged backbone of the DNA molecule (Figure 1-1). Adenine, Thymine, Guanine and Cytosine are the four nucleobases contained in DNA molecule which are commonly abbreviated by A, T, G and C respectively. A and T, and C and G are complementary bases and to form a base pair they from a connection via two and a more stronger connection via three H-bonds respectively (Figure 1-1). It is the order of these four nucleobases along the DNA molecules backbone that encodes information. The DNA molecule present in chromosome 1 consists out of ~240million base pairs (2) and the size of a gene varies substantially, but it is typically around a thousand base pairs long (3).

![Figure 1-1 – A DNA molecule is composed of nucleotides which are connected via H-bonds. The order of these nucleotides encodes genetic information.](image)

As shortly discussed above, a DNA molecule can be seen as the carrier of genetic information. At the same time, this genetic information is packed together in a unique and highly ordered structure. A DNA molecule has a width of 2nm (4) and its double-helical structure results in a distance of 0.34nm between adjacent base pairs (5). To compare it with
something macroscopic, one might imagine a DNA molecule as a very long flexible and extensible string. A DNA molecule gets its helical structure by winding its two chains of nucleotides together with one turn every 10.5 base pairs. That is, the molecule has a twist of 100.8°/nm (4). Therefore, DNA can as well be seen as a mechanical object and described by properties like contour length and elasticity (Section 2.1).
1.2 **DNA-protein interactions**

At some moment, a cell decides that it needs a certain protein corresponding to a certain genetic trait. Subsequently, the RNA polymerase enzyme binds to the DNA molecule at the position where the gene coding for the desired protein resides. This enzyme locally melts the double stranded DNA (dsDNA) molecule into two single strands and the enzyme subsequently starts to move in the 5' direction. A complementary nucleotide is bound to each nucleotide that the RNA polymerase enzyme passes (Figure 1-2). This process results in a messenger RNA (mRNA) strand, which is composed of the nucleotide sequence of the gene.

![Figure 1-2 – The process of DNA transcription is schematically shown in this figure. The black part of the dsDNA molecule (grey) is the part that codes for one single gene. The RNA polymerase enzyme moves in the 5' direction along one of both strands and it simultaneously produces the complementary strand; a piece of mRNA strand (blue).](image)

The mRNA molecule is detached from the DNA molecule during the process and it is finally completely detached and thus able to freely move throughout the cell. This process of mRNA creation by the RNA polymerase enzyme is called transcription. The final step in protein synthesis is called translation. During this step, a ribosome binds to the mRNA molecule and decodes this polynucleotide chain into a chain of amino acids, which is called a protein. In this process, each three consecutive nucleotides of the mRNA molecule code for a single amino acid. Depending on which amino acids are bound together, the resulting protein gets its function and a variation in a gene results in a different protein and thus in a different genetic trait.

In case a cell decides to divide and thus clone itself, it needs to duplicate all its parts, including the DNA. This process of DNA copying is called DNA replication and is initiated by a helicase enzyme which first unwinds the helical dsDNA molecule and subsequently separates the two strands of the dsDNA molecule by breaking the hydrogen bonds between them. Next, a DNA polymerase enzyme binds at each strand and it adds the complementary nucleotide to each nucleotide it passes.
1.3 Biophysical properties of DNA

The two biological processes just described are examples of DNA-protein interactions. During these and many other (6) (7) processes the protein in question continuously bends and stretches the DNA molecule. On a larger scale, the compaction of DNA is bounded by its elastic properties. Hence, the elasticity of the DNA molecule affects a wide variety of cellular processes and to understand these processes, knowledge about the elasticity of DNA is important.

In this work optical tweezers (Section 3.1.2) will be used to acquire force distance data on dsDNA molecules. Ever since the first stretching experiments were performed on single dsDNA molecules, many efforts have been put in understanding its elastic behaviour (8). As a result, the elastic behaviour below the overstretching force of 65pN is now well understood in terms of the worm like chain model. This model describes the end-to-end length of a dsDNA molecule as a function of the stretching force applied to this molecule in terms of three mechanical properties: The contour length, the persistence length and the elastic modulus (Section 2.1). At a stretching force of 65pN the dsDNA molecule undergoes a cooperative phase transition (“overstretching plateau”): Its end-to-end length increases by approximately a factor 1.7 while the stretching force increases over only a very narrow range (9) as shown by the force distance curve of Figure 1-3.

![Figure 1-3](image-url)

**Figure 1-3** – First report of the overstretching transition of dsDNA (9) which was extensively investigated and characterized by P. Gross et al (10) (11).

The structural change which a dsDNA molecule undergoes during the overstretching transition is as well extensively studied; the overstretching transition is a reversible process and it comprises a gradual force-induced unwinding and subsequently melting of a dsDNA molecule from its double helical structure into single stranded DNA (ssDNA) (10). During the overstretching transition the DNA molecule can have three compositions, depending on the amount of ions available in the surrounding buffer.

Dissolved ions shield the strong, negative charge (2e per base pair) of a dsDNA molecule and thus decrease the electrostatic repulsion between the two backbones, stabilizing the double helix (12). In the case of a high salt buffer the electrostatic repulsion is greatly reduced, and
during the overstretching transition parts of unwound DNA, named S-DNA (Figure 1-4b) or small melting bubbles (Figure 1-4c) are created all over the dsDNA molecule. Thus, the dsDNA molecule starts to unwind at multiple points simultaneously and the many small parts of S-DNA lengthen in a *continuous* way as the end-to-end length of the DNA molecule is increased. This melting process results in a featureless overstretching plateau (11).

In a buffer containing only a small amount of ions, too little ions are available to overcome the repulsive force between both backbones and now unpeeling (Figure 1-4d) is the major process occurring during the overstretching transition. This process occurs in a *non-continuous* way because the dsDNA nucleotide sequence effects the strand separation (AT-rich regions separate more easily). This stepwise unpeeling of a dsDNA molecule results in an overstretching plateau containing many small force steps (12).

![Figure 1-4](image)

**Figure 1-4** – (a) an unstretched dsDNA molecule with its double helical structure named B-DNA. A dsDNA molecule surrounded by a high salt buffer deforms during the overstretching transition mostly via the formation of (b) S-DNA (unwound DNA with intact hydrogen bonds) or the creation of (c) melting bubbles (partial ssDNA sections). In case of a low salt buffer (d) unpeeling (melting from the end or nicks) is preferred.

Because of this characteristic “fingerprint” a dsDNA stretching curve displays during stretching, optical tweezers are a powerful technique to study DNA-protein interactions, e.g. by measuring the distortions bound proteins induce on the DNA. However, they are limited for the application of single molecule interaction, because then the distortions are only marginal; furthermore, they do not allow to study how and where proteins are localized on the DNA. One powerful possibility to overcome these limitations therefore is to employ fluorescently labelled molecules and use fluorescence microscopy to follow them.
1.4 DNA visualization by intercalation

A molecule labelled by a fluorescent dye essentially acts like a microscopic light-bulb which is switched on with the excitation light. Yanagida et al first successfully observed single DNA molecules labelled with the fluorescent dye DAPI in 1981 (13). In order to act as a good fluorescent label one needs to have a dye which binds tightly to the molecule of interest. In the case of a dsDNA molecule, intercalators are such dyes with a high binding affinity. A typical intercalator normally contains a positively charged aromatic system which can “slip” between adjacent base pairs, thus binding via dispersive forces. Furthermore, intercalators have the huge advantage that their chromophoric system can be tuned to display a strong increase in quantum yield upon binding to a dsDNA molecule; this means a single dsDNA molecule can be virtually imaged against zero background, because free intercalators do basically not fluoresce. Some cellular processes like replication and transcription can be studied by using dyes which are able to specifically bind to a ssDNA molecule or to a dsDNA molecule. For instance, when a helicase is melting a stained dsDNA molecule into two single strands, the dye molecule can no longer bind and consequently an increasing part of the DNA molecule is no longer stained and consequently visible. In this way, Bianco et al (14) were for the first time able to directly visualize the unwinding activity of a single helicase on a dsDNA molecule (Figure 1-5).

Figure 1-5 – A dsDNA molecule is stained with the dsDNA intercalator YOYO-1 and fixed at one of its ends. The flow stretched dsDNA molecule becomes visible when excitation is started (second image). The subsequent images show a shortening of the dsDNA part of the DNA molecule due to the unwinding activity of a single helicase (Image taken from (14)).
However, a major drawback of using a dye molecule to visualize a dsDNA molecule is that it binds (Section 2.2) to this very dsDNA molecule. In doing so, the three dimensional structure and hereby the mechanical properties of this very DNA molecule are modified. This modification might influence the activity of DNA binding enzymes, resulting in the acquisition of artefacts (15). Logically, an ideal “reporter” molecule does not influence any of the mechanical properties of a dsDNA molecule by binding to it.
1.5 Overview of the project
The high affinity nucleic acid stain SYTOX Orange is characterized in this work as it specifically binds to dsDNA molecules. Moreover, it appeared during our first test experiments that this dye influences the mechanical properties of a dsDNA molecule by a relative small amount (Section 4.1). It is preferred to have a dye which causes only few photocleavage events, as cleavage of the investigated dsDNA molecule marks the end of the experiment. Preference is given to a dye with a low photobleaching rate as this makes longer imaging possible. As already mentioned, a third major point of concern is to find a dye molecule which has a big increase of its fluorescence intensity upon binding to a dsDNA molecule.

Research questions

- What are the advantages of SYTOX Orange compared to the far more often used dye YOYO-1?
- Via which binding mechanism does SYTOX Orange bind to a dsDNA molecule?
- Can we estimate the number of SYTOX Orange molecule bound to a dsDNA molecule?
- What is the photobleaching time of SYTOX Orange?
- How long does a SYTOX Orange molecule on average bind to a dsDNA molecule?
- How do the length and the elasticity of a dsDNA molecule change due to binding of SYTOX Orange?
2 Theory

2.1 Mechanical description of a DNA molecule

A DNA molecule experiences a force in case it is grasped by its ends which are subsequently pulled apart. At all times, a DNA molecule is as well exposed to thermal forces because it is surrounded by a fluid. The influences of these forces on a DNA molecule will be discussed below and combined into the so-called worm like chain model (WLC-model). This model mechanically describes a dsDNA molecule by presenting the stretching force $F$ as a function of the end-to-end length $L$. The freely joint chain model (FJC-model) has been used to successfully describe the elasticity of single stranded DNA molecules (9).

2.1.1 Thermal forces in the WLC-model

The shape of a free floating dsDNA molecule in solution changes continuously due to the influence of thermal forces. More curvature will be present in the dsDNA molecule when there is more thermal energy available. However, the maximum amount of curvature is limited by the intrinsic stiffness of the dsDNA molecule. So there will be a balance between these two. To be able to quantitatively describe the amount of curvature present in the dsDNA molecule, it might be imagined as a thin inextensible elastic rod (Figure 2-1).

Figure 2-1 – Small part of a dsDNA molecule in solution with a length $L_c$ which is locally bent due to thermal forces. The variables shown are used to describe the amount of curvature present in the entire DNA molecule.

The $s$–coordinate represents the contour distance with respect to one end. In case no external stretching force is applied to the dsDNA molecule, integration over $ds$ from one end to the other end results in the contour length of the dsDNA molecule, $L_c$. The distance from one end to the other end of the dsDNA molecule $L$ will be named the end-to-end length. It is related to the contour length via

$$L_c = \int_0^L ds = \int_0^L \sqrt{1 + \left(\frac{dy}{dx}\right)^2} \, dx$$

and for a free floating dsDNA molecule it will be much smaller than the contour length because the dsDNA molecule contains a lot of bends. In the limiting case of no thermal
energy at zero temperature, the end-to-end length equals the contour length as the dsDNA molecule will never be curved.

Let $\mathbf{r}(s)$ be the radius vector representing the curvature at an arbitrary point of the curve.

Then, the tangent vector $\mathbf{t}(s)$ is given by

$$
\mathbf{t} = \frac{\partial \mathbf{r}}{\partial s}
$$

To determine the amount of bending $|\boldsymbol{\beta}|$ present in the curve, the derivative of the $\mathbf{t}(s)$ is calculated. To do this, the tangent vector $\mathbf{t}(s)$ is moved over a small distance $ds$ along the curve to the nearby point $s+ds$ and compared to the tangent vector $\mathbf{t}(s+ds)$ at this point. The difference between both vectors equals $d\mathbf{t}$, resulting in

$$
\mathbf{\beta} = \frac{d\mathbf{t}}{ds}
$$

with magnitude

$$
|\boldsymbol{\beta}| = \frac{d\theta}{ds} = \frac{ds/|\mathbf{r}|}{ds} = |\mathbf{r}|^{-1}
$$

The energy present in the entire rod due to bending, $E_b$, scales quadratic with the inverse of the radius of curvature and it depends linearly on the persistence length of the dsDNA molecule, resulting in (16)

$$
E_b = \frac{1}{2} k_B T \int_0^{L_c} L_p \left( \frac{d\mathbf{t}}{ds} \right)^2 ds
$$

where $k_B$ is the Boltzmann constant and $T$ is the temperature. It can be assumed that the persistence length is constant along the dsDNA molecule.

The persistence length of a one dimensional molecule, like a dsDNA molecule, is defined as the distance over which the correlation of the orientation of the tangent vector $\mathbf{t}$ is lost due to thermal fluctuations. So it is a measure of the molecules stiffness and its value decreases with an increasing temperature. This means that in case a filament has $L_c < L_p$ or $L_c > L_p$ it appears rigid or flexible respectively. So only in the latter case bending energy will be present in the dsDNA molecule. Experimental values found for the persistence length at room temperature vary from 50nm for dsDNA in physiological salt (9) up to a couple of millimetres for microtubules (17).

### 2.1.2 Stretching forces in the WLC-model

Now imaging the dsDNA molecule is no longer free floating in solution but instead grasped by both its ends. These ends are subsequently pulled apart to achieve an end-to-end length $L$ at a stretching force $F$. The tensile energy introduced into the dsDNA molecule is given by

$$
E_t = -F \cdot (L - L_c)
$$

In case a relative small stretching force ($F \leq 10 pN$) is applied to the dsDNA molecule, the end-to-end length will still be smaller than the contour length. The origin of this elastic
behaviour is purely entropic and at these small forces there will be a balance between the stretching force and thermal force.

The exact force $F$ needed to elongate a dsDNA molecule over a distance $\Delta L \equiv L - L_c$ has to be computed numerically from the two previous equations, but a useful approximation is given by

$$F = \frac{k_B T}{L_p} \left[ \frac{1}{4(1 - L/L_c)^2} - \frac{1}{4} + \frac{L}{L_c} \right]$$

This model is named the inextensible worm like chain model (18); In this model it is assumed that the dsDNA molecule behaves as an inextensible flexible rod and thus only its bending energy is included in this model and twisting and elongation energies are not. Consequently, at forces above 10pN this model does not fit the data properly because dsDNA is an extensible and not an inextensible molecule (Figure 1-3). To improve this model, the dsDNA molecule should be modelled as a thin extensible flexible rod.

At moderate forces, the backbone of the dsDNA becomes increasingly stretched with an increasing stretching force and the elastic response is no longer merely entropic, but more and more enthalpic. This stretching of the dsDNA backbone is taken into account in the extensible worm like chain model (16). In a harmonic approximation the energy balance for the dsDNA molecule is given by

$$E = \frac{1}{2} k_B T \int_0^L \left( \frac{c^2}{2} \right) ds - F\Delta L + \frac{1}{2} \frac{K}{L_c} \Delta L^2$$

In this equation, only the last term is extra as compared to the energy balance valid for the inextensible WLC-model. It represents the energy stored in a Hookean spring or, to say it in a different way, it measures the resistance against longitudinal extension of the dsDNA backbone. A typical value for the elastic modulus $K$ of dsDNA is 1500pN. To again obtain an expression for the end-to-end length of the dsDNA molecule as a function of the force, the above expression is harmonically approximated (16), leading to

$$L = L_c \left( 1 - \frac{1}{2} \frac{k_B T}{L_p F} + \frac{F}{K} \right)$$

This expression is valid between 5pN and 50pN and it is very powerful. Just by increasing the end-to-end length of a dsDNA molecule with a known contour length to a length $L$ and by simultaneously monitoring the corresponding force, it gives access to three physical properties of the dsDNA molecule. Below the inextensible and extensible worm like chain models are plotted for changing parameter values. The contour length is held constant at 16.5μm as it only shifts the entire curve to the left or to the right in case its value is changed.
Inextensible WLCmodel

Extensible WLCmodel

Lp=50nm, K=20000pN
Lp=50nm, K=1000pN
Lp=30nm, K=1000pN
Lp=70nm, K=1000pN

Worm like chain model

Figure 2-2 – Inextensible (black solid line) and extensible WLC-model to model dsDNA as presented by Equation 7 and 9 respectively for varying values of the persistence length \( L_p \) and the elastic modulus \( K \). The contour length, indicated by the dashed line, is held constant at 16.5μm.

In contrast with the inextensible WLC-model which approaches the contour length asymptotically, the slope of the extensible WLC-model for higher forces is determined by the elastic modulus. The extensible WLC-model approaches the inextensible WLC-model for very large values of \( K \). The persistence length is of major influence in the entropic regime; however its impact reduces drastically in the enthalpic regime.
2.2 Binding modes of staining agents to dsDNA

Dye molecules (as well as other ligands) tend to interact non-covalently with a dsDNA molecule through two general modes. Surface binding can occur at both the major and the minor groove (for instance DAPI and the Hoechst dyes (19)) or external as shown in Figure 2-3. Stabilization of these binding modes is achieved via a mixture of hydrophobic forces, electrostatic forces and hydrogen bonds (20).

![Figure 2-3](image)

Figure 2-3 – Schematic diagram which shows the different ways a dye molecule can interact with a dsDNA molecule (Image taken from (21)).

The second interaction mode is intercalation, first suggested by Lerman in 1961 (22) (23). A molecule intercalates when its small planar aromatic moiety slides between two adjacent base pairs of a dsDNA molecule. The planar moiety will become oriented perpendicular to the helical axis and after intercalation there will be a substantial structural overlap between the base pairs and the intercalator. Structural formulas of two intercalating dyes are presented in Figure 2-4.

![Figure 2-4](image)

Figure 2-4 – The structural formulas of two nucleic acid stains which interact with a dsDNA molecule through intercalation are shown. a) The mono-intercalator ethidium bromide. b) The bis-intercalator YOYO-1 (Molecular Probes, Invitrogen).
The ethidium bromide molecule is called a mono-intercalator as it only contains a single aromatic moiety and this molecule will slide entirely between two base pairs when it intercalates. In the case of the dimer YOYO-1, which consists of two aromatic moieties connected with a linker, both moieties intercalate between base pairs (bis-intercalation) while the linker remains outside of the double helical structure as indicated by Figure 2-3. During intercalation each second binding site remains unoccupied, resulting for mono-intercalation and bis-intercalation in a maximum staining ratio, i.e. the number of dye molecules per base pair, of 1:2 (24) (25) and 1:4 (26) (27) respectively. These values correspond to a binding site size (in base pairs), i.e. the number of base pairs which is made inaccessible to another ligand molecule, of \( n=2 \) and \( n=4 \) respectively.

The space needed for a single intercalation event is provided by the dsDNA molecule via local unwinding of its helical structure which results in an increase of the mutual distance between two consecutive base pairs from 0.34nm to 0.68nm (24) (28). This results in an increase of its contour length of \( \Delta L_c = 0.34\text{nm} \) and \( \Delta L_c = 0.68\text{nm} \) for a mono-intercalator and a bis-intercalator respectively and, moreover, its persistence length and its elastic modulus elasticity are also affected.

All dyes mentioned so far in this section have widespread use; however three of them have a relative low affinity for dsDNA and also only a small increase of their quantum efficiency upon binding to a dsDNA molecule, resulting in a high background intensity. The quantum efficiency is defined by Equation 10 and in order to have a strong increase of the quantum efficiency of an intercalating dye upon binding to a dsDNA molecule, \( k_{\text{radiative}} \) of the intercalator should be much smaller compared to \( k_{\text{radiative}} \) of the intercalator-dsDNA complex.

\[
\Phi = \frac{k_{\text{radiative}}}{k_{\text{radiative}} + k_{\text{non-radiative}}} \tag{10}
\]

YOYO-1, as well as TOTO, YO and TO (YO and TO are the monomeric counterparts of YOYO-1 and TOTO respectively) belong to the more recently discovered cyanine family of nucleic acid stains which typically have a high quantum yield increase (1000 fold for YOYO-1) upon intercalating to a dsDNA molecule due to interactions between the base pairs and the intercalator (29). However, like many other cyanine dyes also these dyes have shown a change in the preferred binding mode when the dye concentration is sufficiently high: For low dye concentration they do bind to a dsDNA molecule via intercalation, however, for an increasing dye concentration minor groove binding is preferred (30) (31).
3 Experimental methods

In this chapter the setup used to perform single molecule experiments is discussed (Section 3.1). A Labview program (National Instruments) is used to control the setup. In Section 3.2 the standard procedure followed to capture two beads with a single dsDNA molecule in between is presented. Section 3.3 will focus on the ‘force clamp plug-in’ of the Labview program which is frequently used during the performed experiments.

3.1 Experimental setup

Single molecule experiments are performed using the experimental approach shown in Figure 3-1. It combines the techniques of Micro fluidics (Section 3.1.1), Optical tweezers (Section 3.1.2) and Epifluorescence microscopy (Section 3.1.3).

Figure 3-1 – A schematic representation of our experimental approach. Two micrometer sized beads with a dsDNA molecule attached to it, are trapped in the focal points of two laser beams. Subsequently, tension is applied to this dsDNA molecule by moving both laser beams apart. Via epifluorescence microscopy the stained dsDNA molecule is visualized.
3.1.1 Micro fluidics

By using a multichannel flow system (Micronit) as shown in Figure 3-2, interactions between a single DNA molecule and a protein or a straining agent can be studied in different chemical environments with good temporal control. The interior of the flowcell has a height of 100µm and the x and y dimensions are shown in the lower drawing of Figure 3-2. To be able to quickly change (0.3mm/s) between the five different environments, the entire flow system is mounted on top of a movable stage (Applied Scientific Instruments). The five sample solutions are stored in containers which are all in direct contact with the pressure chamber. The nitrogen pressure inside this pressure chamber is controlled by valves. A manual switch at the bottom of each container enables quick starting and stopping of each flow separately. Via micrometer sized flexible tubing, the fluid enters the flowcell (Figure 3-2). Three of the five input channels enter the centre part of the flowcell parallel. This results in three laminar flows which barely mix. Even a few minutes after the flow is turned off, mixing of the different environments will only occur in a small extent. The fourth and the fifth input channel enter the centre part of the flowcell perpendicular to the main flow. Eventually all fluid leaves the flowcell via a single flexible tube which exits into the waste container.

![Image of laminar multichannel flow system](image)

Figure 3-2 – The top figure shows a schematic representation of the laminar multichannel flow system. The tubing (red) transports five different fluids into the flowcell (light blue) and a single tube guides all fluid out of the flowcell into a waste container. The flowcell is made of glass and it is fixed inside a holder. The five input channels converge into a larger common channel and all fluid (dark blue) leaves the flowcell via a single output channel. The lower figure shows the dimensions of the interior of the flowcell – a single box is 1x1mm.

At any time air bubbles may leak into the flowcell and get stuck there. Frequently these bubbles do drastically disturb the flow, so they have to be removed if they are present. Some of these bubbles are simply removed by temporarily increasing the speed of the flow. However, the maximum pressure which can be reached inside the pressure chamber is limited. That is why a syringe is added to the end of the output tube to apply an extra negative pressure to the flowcell and increase the speed of the flow even more. In this way even the smallest bubbles are easily removed. Moreover, the tubing might become (partially) blocked due to clustering of material and the syringe is frequently useful here as well.
Before any reagents of interest are flushed into the flowcell, the entire multichannel flow system (containers, tubing and flowcell) is extensively cleaned to get rid of any leftover from previous experiments. First all five containers are topped up with deionised water and 200µl is quickly flushed through each channel. Next, 1ml of bleach solution per channel is rinsed through the system during a period of 30 minutes and subsequently the system is again briefly flushed with deionised water. The bleach solution contains about 0.7M NaOCl and has strongly oxidizing properties, thus it will degrade any present biological material. This is especially important for our use of intercalators, since they display high non-specific binding properties to the tubes, and are virtually impossible to completely remove from the system in any other way. As a third step, 200µl of 1M HCl is rinsed through each of the channels within five minutes time and again this step is followed with a quick flush of deionised water. Next, the system is rinsed for ten minutes with a 10mM Na₂S₂O₃ solution which effectively destroys any residual bleach amounts. Finally the flow system is thoroughly flushed with deionised water.

Additional to the cleaning procedure, the multichannel flow system is as well passivated before experiments are performed. The protein casein is used for passivation. Casein binds to the interior of the flow system and it prevents binding of any other material. Consequently, the reagents of interest are not (partially) lost due to sidewall binding. The flow system is passivated as follows: (i) flush all channels with PBS buffer for 5 minutes (ii) flush slowly 1µl of 1:50 1% casein solution for 30 minutes, (ii) flush again thoroughly with PBS buffer to remove any residual casein.

During this research only four channels are used. The channels 1, 2, 3 and 4 are respectively employed to flush beads, DNA, buffer and staining agent into the flowcell and they will be discussed in the subsequent subparagraphs. A few millilitres of each reagent is a suitable amount for one day of experiments.
3.1.1.1 Channel I & II; beads and DNA

The dsDNA molecule used is bacteriophage lambda DNA; A linear dsDNA molecule of 48,502 base pairs with two complementary 12-nucleotide single-stranded 5’-overhangs. In terms of the WLC-model (Section 2.1) these dsDNA molecules are characterized by $L_C=16.49\mu m$, $L_p=56nm$ and $S=1500pN$. In order to have a high affinity binding of these dsDNA molecules, streptavidin-coated polystyrene spheres (3.18μm diameter, Spherotech Inc., IL) are used and the overhangs of the dsDNA molecules are labelled with biotinylated nucleotides by Klenow DNA polymerase exo’. The biotin-streptavidin system is one of the strongest non-covalent interactions known in nature, and its affinity is expressed by the equilibrium binding constant which is as high as $K_{eq,B} = 2.5 \cdot 10^{13} M^{-1}$ (32).

3.1.1.2 Channel III; buffer

In this subparagraph the five buffers used are summarized. The streptavidin-coated beads of channel I and the biotinylated dsDNA molecules of channel II are diluted in PBS buffer. Besides this buffer, a low salt buffer with and without casein, a moderate salt buffer and a high salt buffer are used as reagents for channel III:

Low salt buffer with casein:
- 10mM TRIS, pH 7.5
- 20mM NaCl
- (0.02% casein)

Low salt buffer without casein:
- 10mM TRIS, pH 7.5
- 20mM NaCl

Medium salt buffer:
- 10mM TRIS, pH 7.5
- 50mM NaCl
- 2mM MgCl$_2$

High salt buffer:
- 10mM TRIS, pH 7.5
- 150mM NaCl
- 10mM MgCl$_2$

In physiological conditions the concentration of NaCl is typically between 50mM and 150mM and the concentration of MgCl$_2$ is typically between 0mM and 5mM. In this work more extreme buffers are used as well to investigate some limiting cases.
3.1.1.3 Channel IV; SYTOX Orange Nucleic Acid Stain

The high affinity nucleic acid stain SYTOX Orange (Molecular Probes, Invitrogen) is used to stain dsDNA. This cyanine dye undergoes an approximate 500 fold fluorescence enhancement upon binding to dsDNA and its exact molecular structure is the proprietary information of Molecular Probes (21) and not available to the public. However, SYTOX is believed to be a mono-intercalator (25). The SYTOX Orange/dsDNA complex has its absorbance maximum at 547nm, its emission maximum is at 570nm (Figure 3-3) and it has a quantum yield of 0.9.

![Absorption and fluorescence emission spectrum of the SYTOX Orange nucleic acid stain bound to dsDNA](image)

Figure 3-3 – Absorption and fluorescence emission spectrum of the SYTOX Orange nucleic acid stain bound to dsDNA (Data from Product Information sheet).

The stock solution contains 5mM SYTOX Orange in DMSO and it is stored at -20°C. DMSO is used as a solvent for the stock solution instead of water because degradation of SYTOX happens less fast in DMSO. The DMSO solution may be subjected to many freeze-thaw cycles without reagent degradation. Several dilutions of the stock solution in the buffer in question (Section 3.1.1.2) are used as reagents for channel IV. With the intention to prevent any possible influence of DMSO on our experiments it was decided to allow only up to 5% of DMSO in this reagent. This means that a SYTOX concentration of at most 250µM can be achieved.

Before starting an experiment approximately 100µl of this reagent was flushed through channel IV. This proved to be necessary because due to the hydrophobic nature of dye molecules (Figure 2-4) they partially stick to the wall of the flowcell which results in a decrease of the SYTOX concentration. Moreover, during experiments it was as well experienced that these dye molecules cannot be removed by only thoroughly flushing the flowcell. Even after flushing all the channels with multiple millilitres of a non-dye containing reagent, dye molecules remained present inside the flowcell. Consequently, when experiments had to be performed at a lower SYTOX concentration the entire flow system had to be cleaned.
3.1.2 Optical tweezers

3.1.2.1 Physical interpretation

Obviously a stable trap requires a potential minimum, such that a small shift of the trapped object results in a restoring force back to the centre of the trap. To create a three dimensional optical trap a light source which produces a high flux of photons is essential. A laser is a suitable source for this purpose. The Mie solution to the Maxwell equations describes scattering of electromagnetic radiation from spheres (33) and it is used to explain optical trapping of spherical objects. The solution includes the diameter of the trapped sphere, \(2r\), and the wavelength of light used to trap it, \(\lambda_0\). In most biophysical trapping experiments \(2r\) and \(\lambda_0\) have the same order of magnitude. In this regime the Mie solution is quite complex. However, the two limiting cases with \(2r\ll\lambda_0\) or \(2r\gg\lambda_0\) can be understood more easily. Moreover, by using ray optics the latter one allows for gaining a good physical intuition of optical trapping of spheres.

The gradient force attracts an object with a different refractive index (beads with \(n_2=1.57\)) than the surrounding medium (mostly water with \(n_1=1.33\)) towards the focal point of the laser beam as schematically explained in Figure 3-4.

![Figure 3-4](image-url)

**Figure 3-4** – A transparent micrometer sized sphere \((2r>>\lambda_0)\) is trapped near the focal point of a highly focused laser beam due to momentum transfer in both the lateral and an axial direction caused by refraction, as depicted in a) and b) respectively. The white arrows indicate the net restoring force.

A bead that has made a little shift in the lateral direction with respect to the potential minimum is depicted in Figure 3-4a. Due to the Gaussian profile of the laser beam the bead experiences a lateral gradient force. More photons will enter at the left side of this bead compared to the right side \((p_1>p_2)\) causing a net momentum transfer to the refracting medium directed to the centre of the beam. The trapped bead might as well be moved in the axial direction as depicted Figure 3-4b. In this case the incident light coming from large angles is responsible for the restoring axial gradient force. Moreover, the bead is only stably trapped in case the scattering force which pushes the object along the positive z-direction is compensated by the axial gradient force which pulls the object back in the negative z-
direction. Obviously the high NA objective is essential here. Due to the scattering force, the potential minimum is not situated exactly at the focal point, but instead at slightly higher $z$.

3.1.2.2 Setup

A commercial inverted microscope (Eclipse TE2000-U, Nikon) forms the centre part of the setup and it holds a water immersion objective (60x/1.2 WI Plan Apo, Nikon) and two dichroic mirrors, DM2 and DM3 (Figure 3-5).

![Figure 3-5 – Schematic representation of the setup in which a dual optical trap and a fluorescence microscope are combined with a multichannel flow system. The abbreviations used: DM – dichroic mirror, EM – emission filter, IR – infrared short pass filter, PSD – position sensitive detector, LED – light emitting diode, (EM-)CCD – (electron multiplied) charged coupled device.]

Micrometer sized polystyrene spheres (Section 3.1.1.1) are trapped using a continuous wave 3W Nd:YVO₄ laser (Ventus 1,064nm, Laser Quantum). This laser emits a satisfying amount of photons as in this way a maximum force of approximately 100pN can be exerted on a bead before it is able to escape the optical trap. Such forces are sufficient to study the elastic behaviour of a DNA molecule. The choice for a wavelength of 1064nm is mainly governed by photo-damage on biological material, which is minimal in the near-infrared (34).

The beam of trapping laser first passes a Faraday isolator (1030-1080nm ISO-FR Dy-OPT, Newport) to prevent any laser light from reflecting back into the laser cavity. Using a polarizing beam splitter cube (10BC16PC.9, Newport), two laser beams are created with an identical power and an opposite polarization state. Expansion of these beams using a 1:2.67 telescope system is necessary to slightly overfill the entrance of the objective and in this way fully exploit the objectives high NA. The first lens can be move in the lateral direction as indicated by the arrows in Figure 3-5, resulting in a movable and a fixed trap. The mutual separation of the two trapping beams is in the order of micrometers after they are
combined via the second beam splitter. Using a dichroic mirror (‘DM2’: z950dcs-puv-3p, Chroma) both beams are coupled into the water immersion objective. This objective focuses both beams at the interior part of the flowcell, 50μm away from both its top and its bottom.

At this point we are able to catch two beads with a dsDNA molecule bound to it. The catching procedure will extensively be described and discussed in Section 3.2. In the following two sections it will be explained how the end-to-end length $L$ of a caught dsDNA molecule (Section 3.1.2.3) and the stretching force $F$ exerted on this very molecule (Section 3.1.2.4) are calculated.

### 3.1.2.3 Distance

The distance between the centres of the two trapped beads, $D$, is calculated from a bright field microscope image (Figure 3-6) which is recorded at 75Hz by using blue LED illumination (LXHL-NB98 Luxeon Star/O, Lumileds) and an at 91.1nm/pixel calibrated CCD camera (DCC1545M, Thorlabs). To avoid any spectral overlap, bandpass filters (‘EM2’: D440/20, Chroma) are positioned directly in front of both the LED and the CCD camera. The LED light is directed towards the flowcell via a dichroic mirror (‘DM1’: 520dcr, Chroma) and after passing the condenser, the flowcell and the objective, it enters the CCD camera via a second dichroic mirror (‘DM4’: z488r dc xr, Chroma) which separates this light from the fluorescent light. In front of this dichroic mirror an infrared short pass filter (‘IR1’: E750SP, Chroma) is positioned to block any reflected trapping laser light. The Labview program (IMAQ Find Pattern 2 VI) matches the recorded image with a previously recorded template image of a trapped bead and from this information it can calculate the distance $D$. The end-to-end length of a caught dsDNA molecule equals $L=D-2r$.

**Figure 3-6 – Bright field microscope image of two trapped 3.18µm polystyrene beads**

The standard deviation of $D$, $\sigma_D$, is important to know because it tells something about the reliability of acquired data. To determine $\sigma_D$, two beads are trapped and $D$ is held constant at 12μm, according to the software. Subsequently the value of $D$ is recorded during four minutes and plotted in a histogram with intervals of 0.5nm.
Figure 3-7 – Histogram showing the distance between the centres of two trapped beads in case a fixed distance is imposed during four minutes. The curve is a Gaussian fit to the histogram.

The average distance is found to be 11.965μm with a standard deviation of 1nm. So the position of both beads is known with a relative high accuracy as the distance between both beads is varied over tens of micrometers as already shown in Figure 1-3.

3.1.2.4 Stretching force

Next to the trapping force (Section 3.1.2.1) another force is exerted on both beads in case a dsDNA molecule is caught and subsequently stretched. Now both beads experience half of the stretching force $F$ which is applied to the caught dsDNA molecule. Via monitoring the stretching force exerted on one of both beads, the stretching force $F$ applied to the dsDNA molecule can easily be calculated.

Due to the stretching force, a bead is pulled slightly in the lateral direction away from the trap its potential minima. This causes the trapping laser beam to become deflected by an amount which is linear proportional to the shift of the bead (35). This deviation is measured in both the $x$ and $y$ direction using a duolateral position sensitive detector (PSD) (DL-100-7PCBA, Pacific Silicon Sensor Inc.). As shown in Figure 3-5, before the transmitted laser light reaches the PSD it passes an oil immersion condenser with NA=1.4 (Achr Apl, Nikon) to collimate the transmitted trapping light, a polarizer (03 FPI 003, CVI Melles Griot) to block the beam originating from the movable trap, a lens to focus the other beam on the PSD and a neutral density filter (NE03A-B) (not shown in Figure 3-5). The PSD senses the position of a light spot on its surface with a rate of 20kHz and it provides the corresponding voltages in both $x$ and $y$ direction; Acquired voltages scale linearly with the $x$ and $y$-position at the photodiode.

To determine which stretching force corresponds to which voltage, and thus which stretching force corresponds to which bead displacement, the stiffness of the optical trap, $\kappa$.
[N/m], has to be determined and the PSD has to be calibrated. This is achieved by measuring the effect of the trap on the Brownian motion of a trapped bead. The force balance for a trapped particle in a viscous fluid is given by the Langevin equation

$$\gamma \frac{dx}{dt} + \kappa x = F(t)$$

In the above equation it is assumed that the trapping force is linearly proportional to the displacement away from the centres trap with proportionality constant $\kappa$. The drag coefficient of the spherical bead $f$ is a known variable because it is related via stokes law to the dynamic viscosity $\eta (\eta_{water} = 1.002 \times 10^{-3} \text{ Pa s})$ and the bead diameter $d$.

$$\gamma = 3\pi \eta d$$

The random thermal force $F(t)$ represents the Brownian motion, which averages to zero over time. The Fourier transform of equation 11 is given by

$$2\pi f \xi(f) + \kappa \xi(f) = \tilde{F}(f)$$

In this equation $\xi(f)$ and $\tilde{F}(f)$ are the Fourier transforms of $x(t)$ and $F(t)$ respectively. Using the fluctuation dissipation theorem it can be derived that for idealized Brownian motion $|\tilde{F}(f)| = 4\gamma k_B T$ (36). Subsequently the power spectrum $S_x(f)$ [m$^2$/Hz] is obtained by also taking the modulus of the left part of equation 13 and solving the resulting equation for $\xi(f)$.

$$S_x(f) = |\xi|^2(f) = \frac{k_B T}{\gamma \pi^2 (\frac{f_c^2}{f_c^2} + f^2)}$$

The corner frequency is defined as $f_c = \kappa / 2\pi \gamma$ and thereby the stiffness of the optical trap $\kappa$, is calculated by fitting the data acquired by the PSD (Figure 3-8).

However, not a displacement in nanometres but rather a voltage in volts is measured by the PSD. So before any stretching forces can be calculated, the measured uncalibrated power spectrum, $S_v(f)$ [V$^2$/Hz], has to be calibrated to obtain $S_x(f)$. In the limit of the lowest frequencies the power spectrum equals $S_x^0$

$$\lim_{f \to 0} S_x(f) = S_x^0 = \frac{k_B T}{\gamma \pi^2 f_c^2}$$

and the uncalibrated power spectrum is related to the calibrated one via

$$S_x(f) = R^2 \cdot S_v(f)$$

where $R$ [m/V] represents the distance response. Via knowledge about the asymptotic behaviour at low frequencies (Equation 15), $R$ can be derived

$$R = \left[ \frac{k_B T}{\gamma \pi^2 S_v^0 f_c^2} \right]^{1/2}$$

$S_v^0$ is the value of the uncalibrated power spectrum at low frequencies and it is the second parameter whose value is calculated by fitting as shown in Figure 3-8. To calibrate the data
to actual forces, i.e. to find the force response \( R' \) [N/V], the distance response \( R \) should simply be multiplied by the trap stiffness \( \kappa \).

Figure 3-8 – Power spectrum of a 3.18µm bead in water, trapped in an optical trap (black line). Data was acquired by the PSD for 4 seconds at 50kHz. The fit to the data (red line) provides a corner frequency of \( f_c = 1437.9 \) Hz and a low frequency limit of \( S_V^0 = 8.1 \times 10^{-10} V^2/Hz \), resulting in a trap stiffness \( \kappa = 271 \) pN/µm and a force response of \( 776 \) pN/V. The two vertical lines indicated the frequency regime used during fitting.

In the regime where the corner frequency is much smaller compared to the frequency \( f \), the power spectrum falls off like \( 1/f^2 \). In this regime the trap stiffness is too small to influence the high frequency motion of the bead; i.e. its high frequency motion of a trapped bead is identical to that of a free floating bead. An increase of the trapping laser power will result in an increase of the trap stiffness and thus an increase of the corner frequency. Moreover, according to Equation 15 and 16, the value of \( S_V^0 \) will decrease.

In the case of optical pushing the laser power has to be changed to increase the force on a caught dsDNA molecule (37). In this technique the dsDNA molecule is bound to a substrate at one end and the other end of the dsDNA molecule is bound to a bead. Subsequently the trapping laser is directed parallel to the dsDNA molecule and pushes the bead a couple of micrometers away from the substrate, depending on the dsDNA length and the laser power used. Due to changing the laser power, the stiffness of the trap experienced by the bead changes and for each laser power a new calibration has to be performed. Optical tweezers used in this work do not suffer from this issue because different forces can be applied to a dsDNA molecule by changes the mutual distance between the traps.
3.1.3 Epifluorescence microscopy

Fluorescence is the emission of a photon by a molecule. It is caused by the preceding absorption of a photon with a shorter wavelength by this very molecule. The red shift of the fluorescent light is exploited in epifluorescence microscopy to selectively collect only the emitted fluorescence and not the reflected excitation light.

The excitation light source is a 532nm laser (GCL-025-L, 25mW cw, CrystaLaser) which approximately matches the maximum of the absorption spectrum of SYTOX (Figure 3-3). A cylindrical instead of a spherical lens is chosen to expand the laser beam because this lens focuses the light which passes through on to a line. In this way homogeneous illumination of a caught dsDNA molecule is achieved. Before the laser light enters the objective, it also first passes one of the optical density (OD) filters (not shown in Figure 3-5) to control the laser power entering the flowcell and a dichroic mirror (‘DM3’: z532rdc, Chroma) which reflects only in the region of 532nm and is transparent for all other wavelengths. So it is transparent for the fluorescent light origination from the SYTOX molecules as well as for the LED light. It is noted that the objective used to focus the excitation light inside the flowcell is the same one used to focus the infrared laser (Section 3.1.2) and to collect the LED light (Figure 3-5).

SYTOX molecules absorb excitation light and subsequently reemit fluorescence light in all directions. This emitted light is then partially collected by the same objective. This technique is named epifluorescence microscopy. Subsequently the reflected excitation light is blocked by DM3 and the collected fluorescent light is transmitted by this dichroic mirror. Next, the light beam passes the infrared short pass filter (IR1) and the emission bandpass filter (‘EM1’: 582/75, Chroma) to block any residual unwanted light. Although the fluorescence intensity of an individual fluorophore is very faint, nowadays detection instruments are sufficiently sensitive to pick up these amounts light. The fluorescence is imaged onto a very sensitive (QE > 90%) EM-CCD camera (‘EM-CCD’: Cascade 512B, Princeton Instruments) which has a readout time of 64ms and a minimum integration time of 100ms and consequently the shortest integration time achievable with this camera is 164ms.

All dyes mentioned in this work do have the advantage of a huge fluorescence increase by binding to a dsDNA molecule as already mentioned for SYTOX in Section 3.1.1.3. This is what makes the dsDNA molecule visible with respect to the background, even when the dsDNA molecule still resides inside the dye channel. However, fluorophores which do not have a fluorescence increase after they are bound to a dsDNA molecule can be used as well. After incubation in the dye channel, the stained dsDNA molecule is moved out of this channel into the buffer channel. In this way no unbound fluorophores are present and the dsDNA molecule is again visible. A limitation of fluorescence microscopy is the finite lifetime of a fluorophore due to photobleaching (Section 4.3). This limits the total number of emitted photons per fluorophore to typically $10^5$-$10^6$ per dye molecule (38) (39).
3.2 Catching procedure

The streptavidin-coated beads (Section 3.1.1.1) are diluted in PBS to get a suitable concentration. In case the bead concentration is too low, it takes a long time before two beads are trapped and when the concentration is too high, trapped beads get kicked out of the trap by other beads or multiple beads are caught in a single trap. Directly after capture of a second bead, the Labview program starts plotting the $F$ in the x-direction as a function of $D$ in real time. Plotting occurs at a rate which is equal to that of the CCD camera because the PSD acquires data at a much higher rate. At this moment the stage is moved 0.5mm perpendicular to the flow direction to position the beads inside the laminar dsDNA flow.

By use of the movable lens (Figure 3-5) the distance $D$ is varied between 8µm and 21µm with a frequency of 0.5Hz. A dsDNA molecule binds in the first instance only to a single bead and when it does it gets flow stretched. In case the dsDNA molecule is bound to the upper stream bead and when at the same time $D < L$, the dsDNA molecule is able to bind to the second bead as well. Due to use of the ping-pong option it is directly seen in the force distance graph when a dsDNA molecule is caught. After capture the stage is immediately again moved by 0.5mm to prevent catching of multiple dsDNA molecules. The beads with the caught dsDNA molecule(s) are now located inside the laminar buffer flow (Section 3.1.1.2). At this time the switches at the bottom of all four containers are closed to prevent a force offset due to flow. Now the force exerted on the beads is calibrated as described in Section 3.1.2.4. Subsequently the desired experiments can be performed. A new calibration is performed after each movement of the beads to a new position inside the flowcell.

Before introducing the biotinylated dsDNA molecules (Section 3.1.1.1) to the flowcell they are also diluted in PBS, approximately by a factor of 500. Logically it will take a long time to catch a single dsDNA molecule in case the dsDNA concentration is very low. However a concentration which is very high will result the capture of multiple dsDNA molecules, even when the beads are only shortly positioned in the dsDNA channel. The capture of multiple dsDNA molecules at once is not necessarily bad because it might be possible to break the abundant caught dsDNA molecules as shown by the force distance curve of Figure 3-9.

![Figure 3-9](image-url) – By sufficient stretching, three out of four caught dsDNA molecules broke and the most stable one remained. In the force distance curve (left) three overstretching plateaus are clearly visible with a mutual separation around 65pN. The right-hand graph shows the end-to-end length of this very dsDNA molecule versus time.
In this figure three dsDNA molecules are broken after sufficient stretching and moreover, the most stable dsDNA molecule of all remained. When a single dsDNA molecule is caught, the recorded force distance curve will overlay the theoretical curve of lambda DNA (Section 3.1.1.1). In case breaking of abundant dsDNA molecules appears to be impossible, shutters are shortly used to block both trapping beams or a strong flow is temporarily applied. In this way both beads with the dsDNA molecules attached, are lost. Subsequently new beads and a new dsDNA molecule have to be caught. When the correct bead and dsDNA concentrations are found after playing around a bit, two beads with a single dsDNA molecule are usually caught within twenty seconds.
3.3 Force clamp

With the intention to apply a constant stretching force \( F(t) \) on a dsDNA molecule, a proportional-integral-derivate controller (PID controller) is incorporated in the Labview program. In this way \( F \) can be clamped at a targeted value. Fluctuations between the targeted stretching force \( F_t \) and the actual stretching force \( F(t) \), \( \Delta F(t) \equiv F_t - F(t) \) are minimized by tuning the proportional gain constant \( K_p \), the integral gain constant \( K_i \), and the derivative gain constant \( K_d \), which are incorporated in the following equation

\[
F(t) = K_p \Delta F(t) + K_i \int_0^t \Delta F(\tau) d\tau + K_d \frac{d}{dt} \Delta F(t)
\]

The proportional term changes the stretching force by an amount which is proportional to \( \Delta F(t) \). The contribution of the integral term is both proportional to \( \Delta F(t) \) and the duration of \( \Delta F(t) \). To obtain a working force clamp, the last two terms can be chosen zero. However, to reduce fluctuations in and overshoots of \( F(t) \), the integral gain constant is chosen nonzero as well. The force clamp is acting at 75Hz and \( K_p = 1 \), \( K_i = 10 \) and \( K_d = 0 \). To prevent sudden large changes in the end-to-end length of the dsDNA molecule, the speed at which the beads are allowed to move is limited by the software. In the example below, the force is clamped at 35pN and subsequently the force is monitored during five minutes at a frequency of only 10Hz. Subsequently, the force is plotted in a histogram with intervals of 0.04pN.

![Figure 3-10 – A histogram obtained from clamping a dsDNA molecule for 300 seconds at F=35.0pN. Data as acquired at a frequency of 10Hz. The line shown is a Gaussian fit to the histogram.](image)

The average stretching force equalled the targeted force of 35.0pN. The standard deviation in this data set was \( \sigma = 0.3pN \) which is a little less than 1% of the applied stretching force.
4 Results and discussion

Before presenting the results obtained from stained dsDNA molecules, first the influence of the four buffers used in this work (Section 3.1.1.2) on a bare dsDNA molecule will be shown.

Single dsDNA molecules were caught and stretched in one of the buffers. Stretching and relaxation were in succession performed for $8 \mu m < L < 28 \mu m$ at a speed of $1 \mu m/s$. For all four buffers a typical force distance curve of a single dsDNA molecule is shown in Figure 4-1.

Figure 4-1 – Typical force distance curves of a single dsDNA molecule surrounded by the low salt with casein (red), the low salt without casein (green), the medium salt (blue) and the high salt (cyan) buffer. The stretching and relaxation part of a force distance curve are represented by a solid and a dotted line respectively. Theoretical curves for a 48.502bp ssDNA molecule (9) and a 48.502bp dsDNA molecule (Section 2.1) are shown for comparison.

The stretching parts of the force distance curves look similar, which means that the mechanical properties of the dsDNA molecule do not depend on the salt concentration of the surrounding buffer. Also the overstretching plateaus look quite similar however, when fewer ions present in the buffer, the more force variations are observed during the overstretching transition due to the preferred melting via unpeeling (Section 1.2).

Larger differences are observed in the relaxation curves because S-DNA and melting bubbles recover quicker compared to unpeeled regions. This is explained by the fact that the three dimensional structure is more disturbed in the case of unpeeling compared to when S-DNA or melting bubbles are created (Figure 1-4). So the more unpeeling has occurred during stretching, the longer it will take for the single stranded parts of the DNA molecule to turn back into the double helical structure while the DNA molecules end-to-end length is shortened again. As a result more hysteresis is observed when a dsDNA molecule is relaxed in the low salt buffer compared to when it is relaxed in the high salt buffer.

Finally it is noted that casein does neither influence the stretching curve, nor influence the relaxation curve of a dsDNA molecule.
4.1 Comparison between YOYO-1 and SYTOX Orange

While YOYO-1 because of its high dsDNA-affinity and superior brightness is an excellent tool for the visualization of single dsDNA molecules (Figure 1-5), there is a concern that its binding significantly alters the properties of this very dsDNA molecule. Thus, some indications exist that the helicase activity is negatively affected in case a dsDNA molecule is stained using YOYO-1 because of strong stabilization due to intercalation (40). Since a helicase basically melts a dsDNA molecule, we can probe for such an effect of a DNA binding agent by studying the force response of the stained dsDNA molecule in the overstretching region (Figure 1-4), where we expect melting to occur.

4.1.1 YOYO-1

Accordingly, a stretching curve of a single dsDNA molecule and the corresponding fluorescence signal were recorded in the presence of the high salt buffer with 1nM of YOYO-1 (Figure 4-2). This concentration was used to obtain a fairly low, yet still sufficient (= homogeneous) staining along the dsDNA molecule. For comparison, the stretching curve of an unstained dsDNA molecule is also shown.

Figure 4-2 – (left) A comparison between the stretching curves of a dsDNA molecule in the absence (yellow) and presence (blue) of 1nM YOYO-1. The inset displays a frame of the fluorescence movie at an extension around 16.5um (~30pN) displaying fairly homogeneous YOYO staining. (right) The corresponding kymograph of the stretching curve in 1nM YOYO. In a kymograph all the frames of the movie are stacked in a single image. Note that the bright band to the right is the bead in the movable trap and that its displacement marks the end-to-end length of the dsDNA molecule.

Compared to an unstained dsDNA molecule, the dsDNA molecule stained with YOYO displays two major differences. First the stretching curve shows an increasing deviation to higher end-to-end lengths in the force range from 0pN up to 65pN which can be explained via a comparison with the kymograph. It shows that the fluorescence intensity increases strongly.
in this force range which is caused by more intercalated YOYO molecules and each of these increases the contour length of the dsDNA molecule by 0.68nm (Section 2.2). More important, however, is the change in the section of the overstretching transition: while for an unstained dsDNA molecule this section is marked by a flat plateau, in the presence of YOYO, the “plateau” displays a significant force increase. This vanishing of the overstretching transition can also be deduced from the kymograph which shows a virtually constant staining in the overstretching section. This is in contrast to our expectation for stretching a dsDNA molecule because unwound or melted parts of the dsDNA molecule should not be stained by a reporter for dsDNA. Therefore we can conclude that YOYO-1 does not allow the dsDNA molecule to melt and that the mechanical properties of a dsDNA molecule are strongly influenced by intercalated YOYO-1 molecules.

4.1.2 SYTOX Orange

In our search for a valid alternative for YOYO-1 as an efficient dsDNA stain, we settled on SYTOX Orange (Section 3.1.1.3), inspired by its recent use in a single molecule assay by Van Oijen et al (41) which had indicated negligible effect on polymerase activity. The experiments just presented using YOYO-1, are repeated by using SYTOX Orange. Figure 4-3 displays both stretching and fluorescence data of a dsDNA molecule in the presence of 5nM SYTOX Orange, selected to give roughly identical staining as the 1nM YOYO of Figure 4-2.

![Figure 4-3](image)

Figure 4-3 – (left) The stretching curve of a dsDNA molecule in the absence (yellow) and presence (blue) of 5nM SYTOX Orange. (right) The corresponding kymograph. Note that the horizontal stripes in the kymograph are caused by inhomogeneities in the illumination; however, they do not affect the major conclusion that dark sections (=unwound/melted DNA) appear at different strand positions upon DNA overstretching.

The stretching curve of SYTOX looks fairly similar to the stretching curve obtained using YOYO-1 (Figure 4-2) in the force range from 0pN up to 65pN in the sense that higher stretching forces result in larger deviations in the end-to-end length compared to the
stretching curve of an unstained dsDNA molecule. This can again be accounted for by a higher staining ratio at higher forces, as demonstrated by the kymograph. However, the fluorescence response in the overstretching regime demonstrates that the melting characteristics of unstained dsDNA are retained. Thus, during overstretching, dark patches representing unstained DNA appear in the kymograph and they steadily increase in size, demonstrating a gradual transition from the double helical structure into a completely melted structure which obviously does not permit SYTOX binding. This is confirmed in the stretching curve which shows a retained overstretching plateau. From this data we can already conclude that SYTOX Orange is a much better reporter on the structure of a dsDNA molecule compared to YOYO-1, because in its presence the double stranded structure is changed only a little and melting is still allowed. As noticed in the Introduction, an ideal dye does not influence the mechanical properties of a dsDNA at all and thus SYTOX in low concentration comes very close to an ideal reporter dye. In contrast, YOYO-1 strongly alters the overstretching transition, indicating that in this regime the stretched strand should be rather regarded as a novel DNA-YOYO configuration than as a stained dsDNA molecule.

4.1.3 Concentration dependence of SYTOX binding

Since it was established that SYTOX has superior binding characteristics compared to YOYO, we first wanted to study its effect on dsDNA at different concentrations. Figure 4-4 compares stretching curves of dsDNA recorded in the high salt buffer in presence of 20nM, 100nM and 500nM SYTOX and the curves of Figure 4-3 are shown again.

![Variation of the SYTOX concentration in the high salt buffer](image)

Figure 4-4 – dsDNA molecules stretched in the high salt buffer with various SYTOX concentrations.

The comparison demonstrates that for concentrations exceeding 5nM also SYTOX significantly alters the mechanical properties of a dsDNA molecule. The most remarkable change observed here, is that the start of the overstretching plateau is shifted to longer end-to-end lengths with higher concentrations, resulting in a shortening of the overstretching
plateau until it eventually vanishes for SYTOX concentrations exceeding 20nM. A quantitative analysis of these types of curves is presented in Section 4.5.

To better understand the observed transitions in the overstretching plateau, we recorded also fluorescence data for 20nM and 100nM of SYTOX and these results together with the corresponding force distance data are presented in Figure 4-5.

![Figure 4-5](image)

**Figure 4-5** – The distance (black line) and the force (red line) are plotted as a function of time and the recorded kymograph is superimposed on the corresponding graph.

The appearance of the first dark patches coincides with the start of the overstretching plateau when 20nM SYTOX is used. A comparison between the 5nM and the 20nM curve (Figure 4-4) shows that the overstretching plateau (and hence also the first dark patches) appears later; nevertheless, also for 20nM SYTOX the SYTOX stain has completely disappeared near the end of the overstretching regime. On the other hand, in the case of 100nM the overstretching plateau is completely vanished and the fluorescence does no longer vanish, although there are still some darker stripes observed at high stretching forces. This indicates that for such high SYTOX concentrations the structure of the dsDNA molecule is affected so drastically that the dsDNA molecule is no longer able to melt. Thus, a new DNA-SYTOX structure is formed which widely differs from the B-DNA structure.

The deviation of the end-to-end length just before the overstretching starts was approximately three times smaller when 1nM of YOYO-1 was used compared to when 20nM of SYTOX was used as indicated by the red bars in Figure 4-2 and Figure 4-4. This is most likely explained by the fact that less dye molecules are bound to the dsDNA molecule as less dye molecules have a smaller influence on the mechanical properties of the dsDNA molecule and hence produce a smaller deviation. However, while in the case of 1nM YOYO-1 the overstretching “plateau” displays a significant force increase, for 20nM of SYTOX it still is virtually flat. This demonstrates once again that a small amount of YOYO-1 molecules has a stronger influence on a dsDNA molecule than a fairly large amount of SYTOX molecules.

In summary, in contrast to YOYO-1, SYTOX Orange is able to both visualize a single dsDNA molecule and, while doing this affect the mechanical properties of the dsDNA molecule only very little. Consequently, YOYO-1 does not and SYTOX Orange does allow a dsDNA molecule to melt. Therefore, SYTOX Orange is a promising staining agent for dsDNA molecules and in the following sections SYTOX Orange will be further characterized.
4.2 Binding of SYTOX Orange in equilibrium

Next to melting of a dsDNA molecule, the kymographs of Figure 4-5 revealed a fluorescence intensity which increased when a higher stretching force was applied to the dsDNA molecule. With the intention to quantify this behaviour, a dsDNA molecule was caught in the high salt buffer with 5nM SYTOX (equal conditions to the ones used in Figure 4-3). By use of the force clamp (Section 3.3) the stretching force was in succession held constant at values ranging from 10pN to 55pN with a stepsize of 5pN. The fluorescence signal was simultaneously recorded for 200 seconds using an integration time of 1 second (Figure 4-6).

Figure 4-6 – Here, a stretching force of 45pN is applied to a dsDNA molecule in the high salt buffer with 5nM SYTOX. The green area includes the middle 33% of the dsDNA molecule and together with the blue area they mark the region of interest of the signal and the background respectively.

To exclude any fluorescence originating from the beads, the region of interest does not cover the entire dsDNA molecule, but instead only the middle third. The background-subtracted fluorescence signal as a function of time was calculated by subtracting the background intensity (blue area in Figure 4-6) from the signal intensity (green area in Figure 4-6) for all frames contained in the movie. The sum of signals was plotted as a function of the stretching force (Figure 4-7). The averaged fluorescence movies are also presented.

Figure 4-7 – The fluorescence intensity (left) shows an exponential increase as a function of force. The red line is a single exponential fit to the data. For comparison, the corresponding fluorescence images are shown (right).
The fluorescence intensity exhibits seemingly exponential dependence which is verified by an excellent fit with a single exponential function. This is accounted for by the fact that when a dsDNA molecule is exposed to a stretching force, the base pairs are further apart compared to the ones of a non-stretched dsDNA molecule. Consequently intercalation is easier at higher stretching forces. The free energy needed for a SYTOX molecule to intercalate, $\Delta_r G$, is then reduced by an amount equal to the work done upon lengthening the dsDNA molecule at a given force, $F\Delta x$, where $\Delta x$ is the dsDNA elongation upon the intercalation of a single aromatic group:

$$\Delta_r G(F) = \Delta_r G_0 - F\Delta x$$  \hspace{1cm} (19)

In the above equation, $\Delta_r G_0$ represents the free energy needed for a SYTOX molecule to intercalate when no stretching force is applied (42). Moreover, the process of intercalation is not static but dynamic, i.e. SYTOX molecules continuously associate to and dissociate from the dsDNA molecule. Consequently there is a dynamic equilibrium between free SYTOX molecules and intercalated ones characterised by the on-rate $k_{\text{on}}$ and the off-rate $k_{\text{off}}$ of SYTOX molecules.

$$[\text{SYTOX}] + \frac{k_{\text{on}}}{k_{\text{off}}} \text{DNA}_{\text{free}} \rightleftharpoons \text{DNA}_{\text{occupied}}$$  \hspace{1cm} (20)

Here $[\text{SYTOX}]$ represents the SYTOX concentration and the total number of potential binding sites on the dsDNA molecule $N$, is the sum of $\text{DNA}_{\text{free}}$ and $\text{DNA}_{\text{occupied}}$ which are the number of available and occupied binding sites respectively. Because the system is in equilibrium, both numbers are on average constant over time. The latter one equals the number of intercalated SYTOX molecules $N_{\text{sytox}}$ and $k_{\text{on}}$ is linear proportional to the number of intercalation events per second, $N_f$, via

$$k_{\text{on}} = \frac{N_f}{[\text{SYTOX}]}$$  \hspace{1cm} (21)

In a chemical equilibrium the net free energy change due to SYTOX intercalation equals (43)

$$\Delta_r G = -k_B T \ln K_{\text{eq.B}}$$  \hspace{1cm} (22)

where $k_B$ is the Boltzmann constant, $T$ is the temperature and $K_{\text{eq.B}}$ is the equilibrium binding constant which equals (44)

$$K_{\text{eq.B}} = \frac{k_{\text{on}}}{k_{\text{off}}} = \frac{\text{DNA}_{\text{occupied}}}{[\text{SYTOX}] \cdot \text{DNA}_{\text{free}}}$$  \hspace{1cm} (23)

Hence $K_{\text{eq.B}}$ scales linearly with $N_{\text{sytox}}$ and since each intercalated SYTOX molecule will - in contrast to an unbound molecule - fluoresce upon excitation, $K_{\text{eq.B}}$ scales also linearly with the fluorescence intensity $I$. In combination with the substitution of Equation 22 into Equation 19, an exponential expression for the fluorescence intensity of intercalated SYTOX molecules versus the force is then obtained as

$$K_{\text{eq.B}} \propto N_{\text{sytox}} \propto I = I_0 e^{F\Delta x/k_BT} + I_1$$  \hspace{1cm} (24)
where $I_0 \propto K_{eq,B,0} = e^{-\Delta G_0/k_BT}$ and $I_0 + I_1$ represents the fluorescence intensity when no stretching force is applied. Via the fit of Figure 4-7 characteristic force of $k_B T/\Delta x = 11.8 \pm 0.3 \text{pN}$ was calculated which corresponds to $\Delta x = 0.34 \pm 0.01 \text{nm}$. This value is in agreement with the expected value for mono-intercalator as presented in Section 2.2 (24) (28), moreover, it means that the contour length of the dsDNA molecule gets increased by 0.34nm upon the intercalation of a single SYTOX molecule. Both from the excellent fit as well as from the calculated value for $\Delta x$, it is concluded that each SYTOX molecule which does fluoresce is bound to the dsDNA molecule via intercalation.

Based on Equation 24, also an exponential increase of the end-to-end length increase as a function of force is expected as each intercalated SYTOX molecule affects the end-to-end length the same. As mentioned in Section 4.2, the stretching curves obtained for 0nM and 5nM SYTOX (Figure 4-3) differ only very little, meaning that the mechanical properties of the dsDNA molecule do not change drastically. However, if we take a closer look to the deviation between these curves a different result is observed. In Figure 4-8 the mutual difference between the end-to-end lengths of the dsDNA molecules is plotted as a function of the applied stretching force.

![Figure 4-8](image)

**Figure 4-8** – The end-to-end length increase between the stretching curves recorded in the absence and presence of 5nM SYTOX (Figure 4-3) increases as a function of the force (black points). A characteristic force of $14.0 \pm 0.1 \text{pN}$ was calculated via fitting the data to a single exponential function (red line).

Indeed, this data was as well nicely fitted with a single exponential function and a characteristic force of $F_0 = 14.0 \pm 0.1 \text{pN}$ was calculated. This value differs near 20% from the characteristic force calculated from the fluorescence data. We stress that the *end-to-end length increase* is not solely due to the increase of the *contour length* by 0.34nm upon the intercalation of a single SYTOX molecule. First, intercalated sytox molecules change as well the persistence length and the elastic modulus (Figure 2-2) and second, SYTOX molecules...
might be bound to the dsDNA molecule via another force dependent and non-fluorescent binding mechanism, both influencing the end-to-end length increase. However it will be shown in Section 4.5 that only binding via intercalation was occurring here. Thus, the characteristic force corresponds to an end-to-end length increase due to a single intercalation event of \( \Delta L = 0.29 \pm 0.01 \text{nm} \). From this value it can be estimated that at 60pN, corresponding to \( L = 350 \text{nm} \), about 1200 SYTOX molecules were intercalated. Also an exponential dependence of the end-to-end length increase versus force was observed between the 0nM and 1nM YOYO-1 stretching curves of Figure 4-2 and via a single exponential fit \( \Delta L = 0.16 \pm 0.02 \text{nm} \) was calculated. This value is about half of the value calculated for the mono-intercalator SYTOX. Thus, the reduction of the free energy needed for a YOYO-1 molecule to bis-intercalate, i.e. twice \( F \Delta x \), is equal to \( F \Delta x \) for a mono-intercalator. Thus, the force dependence of the end-to-end length increase upon the intercalation of a single aromatic group was halved. This observation can be further checked by measuring the fluorescence intensity versus force for YOYO-1 as has been done for SYTOX in Figure 4-7. Similar reduced force dependent binding of bis-intercalators was also observed by Murade et al for the bis-intercalator YOYO-1 compared to monomeric counterpart YO-PRO-1 and (45).

As can be seen by eye via comparing the 0nM curve and for instance the 500nM curve of Figure 4-4, the exponential behaviour of the end-to-end length increase is lost and instead a slower increase of the end-to-end length increase is observed. This is due the reduced number of binding sites available after the SYTOX concentration and or the stretching force is raised.

One of the things shown in this section so far is that the number of intercalated SYTOX molecules increases exponentially with the applied stretching force when the system is in a dynamic equilibrium as described by Equation 23. While this has already been well established, we can use our single molecule resolution to demonstrate that the increase in the number of intercalated SYTOX molecules as a function of the applied stretching force is mainly caused by a decreased off-rate, whereas the on-rate is largely unaffected.

A dsDNA molecule in the low salt buffer (3.1.1.2) was stained with very little SYTOX molecules with the intention to visualize single intercalation events. Such a small amount of staining is achieved by positioning the dsDNA molecule close to, but not inside the fourth channel of the flowcell. In this way only diffused SYTOX molecules will reach and bind to the dsDNA molecule. It is noted that the sytox concentration is unknown at this point, but that it is constant. Subsequently the dsDNA molecule is in succession exposed to a stretching force of 30pN and 50pN and the fluorescence is simultaneously recorded. Approximately 450 frames were recorded for both stretching forces by using the shortest integration time possible, \( \tau_{\text{int}} = 164 \text{ms} \). The obtained kymograph is shown in Figure 4-9.
The interaction time of a SYTOX molecule with the dsDNA molecule does depend on the stretching force and a single intercalation event takes hundreds of milliseconds. The kymograph contains approximately 1000 frames which were recorded using an integration time of 164 ms. The red boxes indicated the time intervals of constant force.

By counting the number of frames a SYTOX molecule fluoresces, the interaction time of this molecule with the dsDNA molecule, $\tau_0$, is known. These interaction times are combined in a cumulative distribution in which the number of events longer than a certain time is plotted versus the time (Figure 4-10).

![Figure 4-9](image)

**Figure 4-9** – The interaction time of a SYTOX molecules with the dsDNA molecule does depend on the stretching force and a single intercalation event takes hundreds of milliseconds. The kymograph contains approximately 1000 frames which were recorded using an integration time of 164 ms. The red boxes indicated the time intervals of constant force.

![Figure 4-10](image)

**Figure 4-10** – Cumulative distribution of the interaction times of single SYTOX molecules with a dsDNA molecule of which the kymograph was shown in Figure 4-9. The lines are single exponential fits to the data.

To calculate the characteristic interaction time, this cumulative distribution is fitted to a single exponential function. This results in $\tau_0(F = 30) = 0.21 \pm 0.02$ s and $\tau_0(F = 50) = 0.60 \pm 0.08$ s, so the average time of an intercalation event increases when the stretching force is raised, which corresponds to a decreased off-rate as it equals $k_{off} = 1/\tau_0$.

By counting the number of intercalation events visible in the kymograph, $N_j(30\ pN) = 1\ s^{-1}$ and $N_j(50\ pN) = 1.8\ s^{-1}$ were determined. However, to properly assess these results, it has
to be considered that events shorter than one frame produce very little photons, and therefore likely will be missed. This missed fraction equals $f_m(F) = 1 - \exp(-\tau_{\text{int}}/\tau_0)$ resulting in $f_m(30\ pN) = 0.54$ and $f_m(50\ pN) = 0.24$. Thus, we obtain $N_f(30\ pN) = 2.2\ s^{-1}$ compared to $N_f(50\ pN) = 2.4\ s^{-1}$, which is well within the estimated error. In summary, we can conclude that a higher stretching force does not facilitate intercalation, but it renders the SYTOX interaction between the base pairs of the dsDNA molecule more stable.

The obtained stabilization factor from the interaction times upon a force increase from 30pN to 50pN is $0.60/0.21\approx2.9$, which is significantly less than the 5.5fold fluorescence intensity increase observed in the same force range (Figure 4-7). The discrepancy can likely be attributed to photobleaching (Section 4.3), which is a force independent process. Therefore longer interaction times will be more shortened due to photobleaching, and as a result the stabilization factor calculated by using the bleach-corrected data is expected to be closer to 5.5.

As presented in Section 4.1, YOYO-1 changes the mechanical properties of a dsDNA molecule much more than SYTOX Orange does. Our single molecule imaging capability allows us to demonstrate that further. To this end, a dsDNA molecule was imaged in the presence of a very low amount of YOYO-1 molecules while it was exposed to different stretching forces (Figure 4-11).

Figure 4-11 – The interaction time of a YOYO-1 molecule with the dsDNA molecule does depend on the stretching force and a single intercalation event takes multiple seconds. The kymograph contains 600 frames which were recorded using an integration time of 1 second. The red boxes indicated the time intervals of constant force.

While not enough data was obtained to produce sufficient statistics, it again seems by eye that the number of intercalation events does not drastically change with the force, whereas the off-rate clearly decreases. In particular, the interaction time at high forces is at least two orders of magnitude larger than the interaction time of SYTOX since it exceeds 100 seconds at a stretching force of 60pN. This relative long interaction time of YOYO-1 molecules compared to SYTOX molecules accounts for the vanishing of the overstretching transition as presented in Figure 4-2.
4.3 Photobleaching of SYTOX Orange

A dye molecule which leaves the dsDNA via dissociation leaves on average after an interaction time $\tau_i$. However, a SYTOX molecule could undergo similar to most other fluorophores irreversible photobleaching events after the molecule has been excited by a laser and consequently the average time an intercalated dye molecule fluoresces, $\tau_0$, is reduced compared to $\tau_i$. Since dissociation and photobleaching are independent processes, the rates of both processes will add up to give the observed rate as their sum

$$\frac{1}{\tau_0} = \frac{1}{\tau_i} + \frac{1}{\tau_B}$$

where $1/\tau_B$ is the average photobleaching rate, which equals zero when the excitation laser is turned off. In the excitation power range used in this work photobleaching can be considered to be a linear process, i.e., the photobleaching rate increases linear with the excitation intensity. Different excitation laser powers inside the flowcell are achieved by the use of a variety of OD filters.

A dsDNA molecule was exposed to a constant stretching force of 40pN and it is again stained with very little SYTOX molecules in the low salt buffer to visualize single SYTOX molecules. A low salt buffer was used since previous YOYO data indicated that kinetics slow down at lower ionic strength (46), thereby leading to a longer (i.e., experimentally better accessible) interaction time. A movie containing 2000 frames was recorded using an integration time of 164 ms. The optical density filter 0.6 was used to reduce the excitation laser power to 25% of its initial value. The first 600 frames of the movie are shown in the kymograph of Figure 4.12.

Figure 4.12 – The kymograph shows single SYTOX molecules which are bound to the dsDNA molecule. The SYTOX molecules are visible for hundreds of milliseconds.

By counting the number of frames a single SYTOX molecule is visible, its interaction time $\tau_0$ is easily determined. The interaction times of all SYTOX molecules intercalated during the movie were combined in a cumulative distribution (left-hand graph in Figure 4.13). Results which were obtained by using other OD filters (OD1.0, OD0.4 and OD0.2) and, furthermore, data from measurements in the high salt buffer were also included. When the excitation power was reduced to only 10% by using OD1.0, the integration time was increased to 264 ms because due to the decreased fluorescence intensity of the intercalated SYTOX molecules, they were no longer visible when an integration time of only 164 ms was used.
All six data sets have been fitted with a single exponential function and it this way the characteristic observation time $\tau_0$ was calculated. In right-hand graph of Figure 4-13 the characteristic interaction rate, $1/\tau_0$, is plotted as a function of the excitation power for both the low salt and the high salt buffer.

Figure 4-13 – Observed interaction times of single SYTOX molecules with the dsDNA molecule are combined in a cumulative distribution shown in the left-hand figure. The lines in this graph are single exponential fits to the data and the calculated characteristic interaction rates are plotted as a function of the excitation power in the right-hand graph (black points). The red solid line is a fit to the data measured in the low salt buffer and the dashed red line serves as a guide to the eye.

The linear behaviour of photobleaching is clearly visible in the low salt buffer and via a linear fit to the data an interaction time of $\tau_i = 1.0 \pm 0.2 \text{s}$ was calculated. In all experiments presented in this work, OD 1.6 was used which corresponds to an interaction time of $\tau_0(T = 0.025) = 0.9 \pm 0.2 \text{s}$. So when the dsDNA molecule is surrounded by the low salt buffer only a small fraction, about 10%, of the SYTOX molecules gets photobleached at low excitation powers and the major part of the intercalated SYTOX molecules stops to fluorescence due to dissociation.

On the other hand the observation times measured in the high salt buffer, which was used for the experiments described in the Sections 4.1 and 4.2, are shorter compared to the ones measured in the low salt buffer. Nevertheless, two characteristic times could be determined in this buffer. The dashed red line in right-hand graph of Figure 4-13 serves as a guide to the eye and it shows that also in the high salt buffer, the majority of the bound SYTOX molecules stops to fluorescence due to dissociation when OD 1.6 is used. Comparison of the data from high with low salt reveals two slightly surprising facts: first, the interaction rates, $1/\tau_i$, are virtually identical, i.e. it seems that the interaction time does not depend on the salt concentration, in contrast to what was observed for YOYO (46). Moreover, the observation time is much more sensitive to the excitation power when more ions are present in the buffer, i.e. the bleaching rate of SYTOX increases with the salt concentration. In order to rationalize the latter finding, it has to be considered that high sensitivity intercalators such as SYTOX and YOYO are designed to display a strong fluorescence increase upon intercalation; as a result, their fluorescence properties are expected to be highly sensitive to slight changes in the intercalation. For example, it could be imagined that at high salt, the
aromatic stacking of the intercalator between the base pairs becomes more flexible, and as a result the non-radiative decay rate of the excited dye increases. If such a non-radiative relaxation pathway could lead to a photo-oxidative process for instance by reacting with oxygen which in turn destroys the chromophoric system, then this should result in a higher bleaching rate. However, this increased non-radiative rate should be visible by a lower quantum yield. Indeed, under identical excitation and imaging conditions, we observed a significantly lower signal from single SYTOX molecules in high salt compared to single SYTOX molecules in low salt (Appendix). Thus the above proposed hypothesis would be in accordance with our experimental findings.

Because a photobleached SYTOX molecule does not fluoresce it can be concluded that a photobleached SYTOX molecule interacts differently with the dsDNA molecule compared to before it was photobleached. In order to investigate this effect, a dsDNA molecule was stretched in 100nM SYTOX while the excitation laser was alternately turned on and off. Subsequently we compared stretching curves of dsDNA molecules in 100nM SYTOX obtained during excitation with stretching curves recorded in the dark (Figure 4-14).

![Figure 4-14](image_url)

**Figure 4-14** – (left) The force (red line) and the distance (black line) are plotted versus time and the corresponding kymograph is superimposed on this data. (right) A deviation between stretching curves recorded during excitation (blue line) and in the dark (yellow line, already presented in Figure 4-4) is observed. The stretching curve of an unstained dsDNA molecule is shown as a reference (black line, already presented in Figure 4-2).

The results presented in the left graph show an increase of the force simultaneously with excitation. This results in a slight but significant shift of the stretching curves recorded during excitation to shorter end-to-end lengths (right graph). From this we conclude that a SYTOX molecule does not only stop to fluoresce after it has been photobleached, but the photobleached fraction of intercalator will also instantly detach from the dsDNA molecule and shift the equilibrium to a lower staining ratio, and thus a shorter end-to-end length is measured. Nevertheless, because there is only a small deviation between both force distance curves, the staining ratio is only influenced by a limited amount. This is due to the continuous and fast association of new SYTOX molecules (Section 4.4). The stretching curves show the effect of photocleavage as well. The dsDNA molecules which were stretched while the excitation laser was turned on broke earlier compared to the ones which were stretched while the excitation laser was turned off.
4.4 Association and dissociation of SYTOX Orange

One of the research questions that motivated this project is how SYTOX affects the mechanical properties of a dsDNA molecule. In other words, how do the contour length, the persistence length and the elastic modulus of a dsDNA molecule change, having a certain staining ratio. As explained in Section 2.1, these mechanical properties are calculated by fitting an acquired force distance curve up to 60pN to the WLC-model. So to be able to calculate the effect due to intercalated SYTOX molecules on the mechanical properties of a dsDNA molecule, force distance data is required of a dsDNA molecule with a constant staining ratio. However, all the force distance curves of stained dsDNA molecules presented so far (Figure 4-2 - Figure 4-5 and Figure 4-14) were recorded using a stretching speed of 0.1µm/s resulting in a system (Equation 20) which was continuously in equilibrium meaning that due to the force dependent amount of intercalated SYTOX molecules, the staining ratio was not constant during the data acquisition.

Similar to all chemical equilibriums, also this system needs a certain amount of time to reach its equilibrium. In case a dsDNA molecule is stretched much faster than 0.1µm/s, the system cannot fully equilibrate during the stretching and consequently it equilibrates after the stretching is finished. So it will only be possible to acquire force distance data on a dsDNA molecule with a constant staining ratio when the time needed for data acquisition can be shortened by such an amount that it becomes much shorter compared to the time the system needs to reach equilibrium. Thus, the maximum speed at which the movable trap can be moved is approximately 10µm/s and data has to be acquired from 0pN up to 60pN which corresponds to at least an end-to-end length increase of 3µm as was shown in Figure 4-4 resulting in a minimal data acquisition time of 300ms.

The time to reach equilibrium depends on the on-rate and the off-rate of SYTOX molecules and their quotient defines the equilibrium binding constant (Equation 23) which expresses the affinity. To check for salt dependent kinetics which has been observed by Paik et al for YOYO-1 (46), we checked the influence of the salt concentration on the staining ratio as in this case a higher staining ratio corresponds to an increased affinity. To do this, force distance curves of a dsDNA molecule in the high salt, in the medium salt and in the low salt + casein buffer were recorded at a speed of 0.1µm/s (Figure 4-15) while an equal amount 5nM SYTOX was added to each buffer.

Both the effect of the salt concentration as well as the effect of casein on the stretching curve are significant. When the dsDNA molecule is surrounded by fewer ions, its backbone is less screened which causes an increased affinity of SYTOX, i.e., most likely a higher on-rate as it was shown in Figure 4-13 that the off-rate is largely unaffected by the salt concentration. This is expressed by the 5nM curve as a bigger shift towards bigger end-to-end lengths compared to the 0nM curve. Casein has detergent properties (30) (47). Therefore it probably breaks down H-aggregates of SYTOX molecules resulting in an increased effective SYTOX concentration. This, together with the low salt buffer accounts for the observed shift towards even bigger end-to-end lengths.
Figure 4-15 – Stretching curves recorded in the high salt buffer (Figure 4-4), in the medium salt buffer and in the low salt + casein buffer are presented. The stretching curve of an unstained dsDNA molecule in the high salt buffer (black line) is shown as a reference (Figure 4-2).

To quantitatively determine how long it takes for the system to equilibrate, a dsDNA molecule in the low and in the high salt buffer with 50nM SYTOX was subjected to a distance jump cycle. In this procedure the end-to-end length was increased using the maximum stretching speed of 10µm/s and subsequently the molecule was stretched back to the original length. These end-to-end length jumps are repeated several times, and the force response resulting from each jump is recorded (Figure 4-16).

Figure 4-16 – The stretching force experienced by a stained dsDNA molecule is plotted versus time. A similar relaxation of the force is observed in both buffers after a quick end-to-end length increase or decrease is performed. By fitting the relaxation curves with a single exponential function (red lines) a time constant between 150ms and 250ms was calculated.
After each jump to a higher end-to-end length, a force relaxation was observed which indicates binding of SYTOX molecules. Thus, additional intercalating SYTOX molecules will result in a lengthening of the DNA and subsequently a lower force at the same end-to-end length. If a jump is performed to a smaller end-to-end length, we observed a force increase, accounted for by detachment of SYTOX molecules from the dsDNA molecule. By fitting the data sets with a single exponential function a time constant between 150ms and 250ms was calculated for both the force decrease as well as for the force increase. It is stressed that for both the low salt as well as for the high salt buffer the end-to-end length increase (decrease) was chosen such that the force equilibrated at 50pN (20pN). This force value corresponds due to the salt concentration dependent affinity of SYTOX, to different end-to-end lengths as was shown for 5nM SYTOX in Figure 4-15. Consequently the force relaxation cannot be related to the on-rate (off-rate) of SYTOX molecules. However, it can be concluded that these rates are high compared to the minimal data acquisition time of 300ms in both the low as well as in the high salt buffer.

The fast on-rate and the fast off-rate could qualitatively also be reproduced by looking at the fluorescence intensity, using the minimum integration time of 164ms. The experiment was repeated for the reverse end-to-end length change. Both in the case of association as well in the case of dissociation, a clear change in the fluorescence intensity is observed in each consecutive frame, confirming that SYTOX intake and release occurs at least on the same timescale as the used time resolution of 164ms.

Figure 4-17 – The right and the left panel show the increase and the decrease at a speed of 10µm/s of the end-to-end length respectively of a dsDNA molecule in the high salt buffer with 50nM SYTOX. The frames shown are consecutive frames and they are recorded using an integration time of 164ms. A changed amount of staining was observed in each consecutive frame.
Due to the force dependent binding of SYTOX (Section 4.2) and due to its fast association and fast dissociation in both low and high salt, it is impossible to record a force distance curve of a dsDNA molecule with a constant staining ratio, giving the time resolution of the employed instrument. This means that the contour length, the persistence length and the elastic modulus of a dsDNA molecule cannot be directly deduced from the staining ratio.

We therefore sought another way of obtaining a stretching curve at constant staining ratio by achieving SYTOX saturation. The idea behind that is that if a sufficiently high SYTOX concentration is used, then binding should already be saturated at low tension, thus the force dependent SYTOX binding will be eliminated, and stretching curves in equilibrium could be obtained. When the force distance curve of a saturated dsDNA molecule is known, this curve can be fitted to the WLC-model to calculate the contour length, persistence length and the elastic modulus of a saturated dsDNA molecule. Subsequently all force distance curves obtained from dsDNA molecules with a force dependent amount of staining could be analyzed by describing them as a linear combination of the curves obtained from a bare dsDNA molecule and a saturated dsDNA molecule.
4.5 Increasing the SYTOX Orange concentration

With the intention to reach the maximum staining ratio and thus saturation the low salt + casein buffer was used as the environment where dsDNA molecules were stretched as the affinity of SYTOX is the highest in this buffer as was shown in Figure 4-15.

Force distance curves were recorded using a stretching speed of 0.1µm/s and the SYTOX concentration is gradually increased up to 200µM. The latter is the highest concentration which could be practically achieved, given that the commercially available stock solution of SYTOX is only 5mM in DMSO. The measured curves are presented by the solid lines in Figure 4-18 and the SYTOX concentrations used are listed in the graph as well. Comparable to the curves of Figure 4-4, first a shortening and raising of the overstretching plateau is observed and at SYTOX concentrations exceeding 5nM the melting transition is totally vanished.

![Variation of the SYTOX concentration](image)

Figure 4-18 – The shift compared to the 0nM curve is bigger when a higher SYTOX concentration is used. The dashed lines are fast relaxation curves of a dsDNA molecule which was incubated at 100pN and 210pN in 200µM SYTOX. The curves show that even when a SYTOX concentration as high as 200µM is used, saturation of a dsDNA molecule with SYTOX molecules is not reached.

The acquired set of stretching curves shows that when at higher concentration range the distance between adjacent curves diminishes. However, proper saturation cannot be achieved within the available SYTOX concentration range as none of the recorded curves overlays with another one. This is furthermore demonstrated by closer investigating the stretch behaviour in the highest concentration of 200µM SYTOX. Thus, the dashed lines in Figure 4-18 show relaxation curves of a dsDNA molecule stained with 200µM SYTOX starting at a stretching force of 100pN and 210pN and going back to 0pN. Relaxation is performed as quickly as possible, i.e., at a speed around 10µm/s. If saturation would have been achieved, then binding should be force-independent, accordingly these curves should superimpose with the stretching curve; however, both are significantly shifted to higher end-to-end lengths, indicating additional SYTOX intake upon stretching. In case the relaxation would
have been performed slowly the relaxation curve would have superimposed with the stretching curve because in that case the system would always have been in equilibrium.

The fact that the saturation of a dsDNA molecule with SYTOX molecules is not achieved means that the in Section 4.4 proposed analysis of dsDNA molecule with a force dependent amount of staining is not possible. Consequently, a detailed analysis from the force distance data of how SYTOX influences the contour length, the persistence length and the elastic modulus of a dsDNA molecule cannot be achieved. The only thing which can be concluded is that SYTOX does influence them, as a change in the end-to-end length is observed.

An additional way of demonstrating that saturation had not been reached is to plot the fractional end-to-end length increase at a certain force as a function of the SYTOX concentration. The fractional end-to-end length increase, $\gamma$, is defined as

$$\gamma = \frac{L_{\text{sytox}} - L_0}{L_0}$$

where $L$ is the end-to-end length of the stained dsDNA molecule which depends on the SYTOX concentration and $L_0$ is the end-to-end length of the unstained dsDNA molecule. In Figure 4-19 $\gamma([\text{SYTOX}])$ at a stretching force of 30pN is plotted and the inset shows the same data on a logarithmic x-axis.

![Figure 4-19](image)

**Figure 4-19** – The fractional end-to-end length increase of a dsDNA molecule which was exposed to a stretching force of 30pN is plotted as a function of the SYTOX concentration. The inset shows the same data on a logarithmic x-axis and the straight red lines serve as a guide for the eye.

In particular from the semi-log depiction it is clear that $\gamma([\text{SYTOX}])$ does not converge to saturation, but instead keeps on increasing steadily. Furthermore, this graph reveals two striking regimes in which the end-to-end length increase is very well defined by a logarithmic dependence; however, in the second regime which seems to dominate at concentrations $>1\mu\text{M}$, the end-to-end length increase is strongly slowed down. This finding gives already a first hint that we might be looking here at two binding regimes with distinct properties.
Two other important parameters regarding the SYTOX DNA interaction can be calculated from the force distance curves presented in Figure 4-18. Starting with equation 23, McGhee and Von Hippel derived an equation to describe binding of a ligand to a monodimensional lattice with N identical potential binding sites (i.e. to a dsDNA molecule); The staining ratio, which is linear proportional to the fractional end-to-end length increase, versus the SYTOX concentration, was derived in terms of the ligand its binding site size n (Section 2.2) and the equilibrium binding constant \( K_{eq,B} \) of the system (44). The fact that for a gap, \( g \) residues long, between adjacent bound ligands, the number of binding sites left equals zero when \( g < n \), was taken into account. The McGhee-Von Hippel binding isotherm is given by

\[
\gamma = K_{eq,B}(1-\gamma)(\frac{1-n\gamma}{1-n\gamma+\gamma})^{n-1}
\]

Only these data points which belong to the first regime were fitted (Figure 4-19). The fitted curves together with the data for a stretching force of 10pN, 20pN, 30pN, 40pN and 50pN are presented in left-hand graph of Figure 4-20.

**Figure 4-20** – The data points shown in the left-hand graph are taken directly from the force distance curves of Figure 4-18 at the specified force and the fractional end-to-end length increase is fitted to the McGhee-Von Hippel binding isotherm (solid lines). The calculated equilibrium binding constant is plotted in the right-hand graph as a function of force. The red solid line is an exponential fit to the data.

This observation is a further confirmation that SYTOX can bind to a dsDNA molecule via two binding modes. The first binding mode is intercalation as discussed in Section 4.2. This is also in excellent agreement with the McGhee-Von Hippel fitting, since \( n = 2 \) was calculated irrespective of the force and this is the value theoretically predicted for an intercalator (Section 2.2). The equilibrium binding constant presented by the right-hand graph of Figure 4-20 indeed shows an exponential force dependence as expected based on Equation 24. A single exponential fit to this data resulted in a characteristic force of \( F_0 = 13 \pm 1 \) pN, which is within the error margin compared to the value calculated in Figure 4-8. The equilibrium binding constant at zero force equals \( K_{eq,B,0} = 4.8 \cdot 10^5 M^{-1} \), which is in good agreement
with values found for other mono-intercalators such as ethidium ($K_{eq,R,0} = 4.6 \cdot 10^5 M^{-1}$) (42) and YO ($K_{eq,R,0} = 5.8 \cdot 10^5 M^{-1}$) (15) (24).

At concentrations exceeding 1µM (this concentration is force dependent), the intercalative binding mode gets saturated and a second binding mode seems to dominate, thus causing the deviation from the McGhee-Von Hippel binding isotherm at higher concentration. The second binding mode has a much lower affinity compared to the first one, because it is relevant only at elevated concentrations, thus also explaining that saturation is not yet achieved at 200µM.

To investigate the second binding mode further, also fluorescence data was collected at high staining ratios. In the left panel of Figure 4-21 the distance and the force of a dsDNA molecule which was stretched in the high salt with 500nM SYTOX are plotted as a function of time and the corresponding kymograph is superimposed on this data. A linear cross section of the kymograph is shown in the right-hand graph of Figure 4-21 as a function of the end-to-end length.

Figure 4-21 – (left) The distance (black line) and the force (red line) are shown versus time and the kymograph is superimposed on these curves. The horizontal lines are an imaging artefact and can be ignored. (right) A linear cross section of the kymograph shows a decreasing fluorescence signal for L>~20.5µm. The red lines serve as a guide for the eye.

The decrease of the fluorescence intensity starts at an end-to-end length around 20.5µm corresponding to an end-to-end length increase of 4.8µm (Figure 4-4). This corresponds to a staining ratio of roughly a third as the number of intercalated SYTOX molecules equals $L/\Delta L = 4.1 \mu m/0.29 nm \approx 1.4 \cdot 10^4$. The decreased fluorescence intensity is not due to melting of the dsDNA molecule as was the case in Figure 4-3. This can be check from the 500nM stretching curve (Figure 4-4) which is drastically shifted compared to the 0nM curve. However it is true that the fluorescence decrease is explained by quenching of the SYTOX molecules which has been reported for other cyanine dyes to be caused by minor groove binding (31). Minor groove binding would also qualitatively explain why the second binding mode displays a slower end-to-end length increase compared to the first intercalative binding mode (right-hand graph in Figure 4-20). However, further evaluation of the detailed nature of this binding mode is beyond the scope of this project.
5 Conclusions

Here we presented a thorough study of the interaction of the high affinity nucleic acid stain SYTOX Orange with dsDNA, and its dependence on the experimental conditions. Using both fluorescence (Figure 4-7) and also force distance data (Figure 4-8), we demonstrated that SYTOX binding is highly force dependent, and that this dependence can be quantitatively described by a mono-intercalation model, in which each SYTOX molecule increases the dsDNA molecule its contour length by a length of one base pair. More precisely, our single molecule studies provide a good indication that the force dependence is dominated by a decrease of the off-rate with force while the on-rate seems to be largely unaffected by force (Figure 4-9).

Moreover, in contrast to the frequently used dsDNA stain YOYO-1 (Figure 4-2), our results in the DNA overstretching regime demonstrate that SYTOX is an excellent reporter on the normal B-DNA structure in the low concentration regime (Figure 4-3, Figure 4-5). That is, it does not alter the general unwinding/melting characteristics of B-DNA, since it stabilizes the double stranded structure only little. This is further demonstrated by our finding that SYTOX displays short binding events (even at high force only on the second scale, Figure 4-10), compared to YOYO-1 molecules which under higher forces can tightly bind for minutes (Figure 4-11). The length of these binding events was only slightly influenced by photobleaching in the excitation power range used. We furthermore established somewhat unexpectedly - both by fluorescence and stretching results (Figure 4-16) - that the interaction time seems to be mainly salt independent (Figure 4-13). On the other hand, we clearly show that low salt increases SYTOX affinity to DNA (Figure 4-15), from which we can conclude that this dependence has to be triggered by the on-rate of SYTOX. From the observed differences in photobleaching we also propose that the nature of SYTOX intercalation also depends on the salt concentration.

In addition, we provide clear evidence that the photobleached SYTOX molecules are also greatly affected in their intercalation capability, thus a greatly reduced amount of intercalative binding was observed (Figure 4-14). Finally, we provide strong evidence that at high staining ratios intermolecular coupling of the chromophoric systems occurs which provides efficient non-radiative decay pathways, and therefore results in a nearly complete quenching of the fluorescing SYTOX molecules (Figure 4-21).

We characterized SYTOX binding via stretching DNA in a low salt buffer quantitatively over more than five orders of magnitude (Figure 4-18) and could demonstrate that at least two binding modes have to be present to sufficiently describe the observed binding characteristics (Figure 4-19). Our McGhee-Von Hippel isotherm fitting (Figure 4-20) provides clear evidence that up to a concentration of 1μM, intercalation is the dominant binding mode. This also shows that the maximum amount of intercalative binding occurs with one SYTOX molecule every two base pairs. At higher concentration, a second binding mode of much lower DNA affinity takes over and accounts for our failure to reach binding saturation at 200μM SYTOX. Based on our preliminary modes, this second binding mode most likely is due to a minor groove binding, thus in accord with other known intercalators.
References


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7 Appendix

A comparison between the two movies recorded using OD0.6 (one was recorded in the low salt and one in the high salt buffer) is made to check for an increased non-radiative decay rate of an excited SYTOX molecule in a higher salt buffers. This increased non-radiative rate should now be visible by a lower quantum yield. In the cumulative distribution of Figure 7-1 the normalized and background corrected fluorescence intensity of single SYTOX molecules is plotted for both the low salt and the high salt buffer. Both data sets are fitted to a Gaussian curve.

Figure 7-1 – The cumulative distribution of the fluorescence signal from single SYTOX molecules in both the low and the high salt buffer is shown. The lines are Gaussian fits to the data.

For the low salt buffer the fit resulted in a mean intensity of 16.9±0.3 and it the high salt buffer a mean intensity of 15.2±0.2 was calculated. Indeed, we observed a significantly lower signal from SYTOX in high salt compared to SYTOX in low salt.