Nanoscale instruments for the positioning of molecules, for the measurement of molecular forces, and for the quantification of conformational equilibria

Dissertation by

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Nanoscale instruments for the positioning of molecules, for the measurement of molecular forces, and for the quantification of conformational equilibria

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List of peer-reviewed publications

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Placing molecules with Bohr radius resolution using DNA origami

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Uncovering the forces between nucleosomes using DNA origami

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Exploring nucleosome unwrapping using DNA origami
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Scientific instruments made from DNA

Unraveling the mechanisms of biological processes at the nanoscale is one of the main challenges in current biophysical research. Numerous tools and methods have been developed to help expand the understanding of biological processes at ever smaller scales. For instance, structure determination methods such as X-ray crystallography, nuclear magnetic resonance spectroscopy, or electron microscopy supply high-resolution structures of proteins, nucleic acids, and their assemblies. Microscopy methods such as super-resolution microscopy or electron microscopy provide direct insights into the spatial arrangement of these biological components in close-to-native environments. In addition, single-molecule methods such as single-molecule force spectroscopy or single-molecule fluorescence spectroscopy enable studying the mechanical properties and conformational dynamics of biomolecules on the single-particle level. Most of these methods enable the user to passively observe or image a system with minimal external interference from the experiment, whereas the ability to control or actively manipulate the nanoscopic system under study is often limited. Actively manipulating or perturbing biological systems at the nanoscale, while monitoring their responses, may however lead to a deeper understanding and to new insights.

Enabled by their intricate three-dimensional structures, natural proteins perform remarkable functions, such as catalyzing biochemical reactions, transporting cargo, constructing scaffolding networks, or sensing molecules. Scientific tools that operate on the same length scale and with similar capabilities as natural proteins would enable the user to precisely control and manipulate experiments at the nanoscale and thus to develop novel approaches for studying biophysical processes. Programmable self-assembly with nucleic acids [1–15], in particular scaffolded DNA origami [4–7, 10, 13–18], allows creating almost arbitrary nanoscale shapes which can serve as addressable molecular breadboards for arranging biological components or as mechanical parts for nanoscale devices.

The self-assembly of DNA origami structures is a highly parallel process, producing $\sim 10^{12}$ identical discrete DNA objects in a single reaction. Furthermore, recent advance-
ments in the design, assembly, and purification process of DNA origami objects [19–22] as well as in the design and structure prediction software [23–25] make DNA origami a powerful technique for constructing nanoscale scientific instruments. Indeed, DNA-origami-based tools were recently used to align membrane proteins for nuclear magnetic resonance structure determination [26], to arrange molecular motors for studying their collective behaviour [27,28], to augment stochastic sensing with nanopores [29,30], to arrange membrane receptors for studying their organization-dependent signalling [31], to arrange SNARE proteins for studying membrane fusion [32], to position nucleic acid motifs for studying their interactions and conformations [33–35], and to perform measurements of weak base-pair-stacking interactions [36].

These studies highlight the potential for using DNA-based devices as scientific instruments to study nanoscale biological processes, but they mainly exploit the mechanically rigid global structure of DNA origami objects or their ability to position molecular components in nanometer-spaced rigid spatial arrangements. The goal of this thesis was to construct a nanoscopic scientific instrument using DNA origami which enables the precise positioning of molecular groups and biomolecules in user-defined distances and orientations (i) and which can report on the conformational states of mounted biomolecules while applying molecular forces (ii).

(i) Placing molecules

Natural and designed proteins achieve atomic-scale positional and orientational control over, for instance, residue side-chains [37] or bound ligands [38], which enable a set of remarkable functions [39–41]. Few artificial structures made from nucleic acids, however, have been shown to achieve structural accuracies smaller than 5 nm [10,42–45], and subnanometer positional control has only been demonstrated in the unit cells of DNA crystals [46]. To both test and expand the placement capabilities of artificial self-assembled DNA origami objects, we constructed a molecular positioning apparatus and explored its positioning properties in the first part of this thesis (chapter 1). The positioning apparatus allowed us to adjust the average distance between two molecules with subnanometer accuracy over a wide range of distances (1.4–9.9 nm) and it exhibited subnanometer structural fluctuation amplitudes, approaching the structural properties of natural proteins.

(ii) Molecular forces and state readout

Single-molecule force-spectroscopy methods have led to important insights into the functions and mechanical properties of proteins and nucleic acids [47–50]. This is mainly because these methods combine the ability to read out the specimen’s extension (and thereby its conformational state) with the ability to apply forces to populate otherwise
rarely occupied states. Conventional force-spectroscopy methods are, however, challenged by their low throughput and by their requirement for connecting microscopic specimens to macroscopic instrumentation. Furthermore, these methods do not allow the user to control the orientation of mounted target molecules. To overcome these challenges, we developed a nanoscopic DNA origami force spectrometer which combines the two key aspects of force-spectroscopy methods. It reports on the state of the mounted biomolecular system and it allows to apply weak molecular forces. We used our DNA origami force spectrometer to measure quantitatively the distance-, orientation-, and modification-dependent interactions between two nucleosome core particles (nucleosomes) (chapter 2). Finally, we demonstrated that the spectrometer can be used to follow the conformational transition from a bent to a straight nucleosomal DNA state upon salt-induced nucleosome disassembly (chapter 3).

During these studies, we also establish a quantitative state readout of our device using transmission electron microscopy (TEM) and a semi-quantitative state readout in solution using Förster resonance energy transfer spectroscopy in combination with gel electrophoresis (chapter A).
Scientific instruments made from DNA
Chapter 1

Placing molecules using DNA origami

The function and performance of a nanoscale scientific instrument will rely on the accuracy and precision with which molecular components such as functional groups, fluorescent molecules, or biological macromolecules may be arranged and positioned within the reference frame of the DNA origami scaffold. Thus, testing and improving the accuracy and precision, with which a molecular distance between two molecules can be adjusted is an important task for constructing molecular-scale tools. In addition to these technological challenges, precisely controlling the relative positions of molecular groups may allow for studying and optimizing distance dependent molecular interactions such as Förster resonance energy transfer (FRET) [51].

Remark: parts of this chapter have been published in reference [52]. DNA objects in this thesis were created using the scaffolded DNA origami technique [4–7, 13, 14, 16, 17]. Since this technique was merely applied as described in the references, it was refrained from giving an extensive introduction. The interested reader can find an excellent introduction to the scaffolded DNA origami technique in reference [53] or [54].

1.1 The DNA origami-based positioning apparatus

Double-stranded B-form DNA has on average an axial rise of 0.335 nm per base pair that is associated with a 34° rotation of the DNA backbone [55,56]. A simple double-stranded DNA duplex would therefore provide limited spatial sampling as a positioning system. To overcome this limitation, we devised a positioning unit consisting of three double-stranded DNA helices that form an isosceles triangle (Fig. 1.1A). The length of the triangle’s base determines the angle between the triangle’s legs. We can adjust the distance between two points on opposing legs of the triangle by adding or removing base pairs from the
Figure 1.1: Concept of the positioning apparatus. (A) Schematic representation of three double-stranded DNA helices forming the fundamental positioning unit. Icons indicate mounting locations for fluorescent molecules and thiol groups used in this study. (B) Schematic representation of the positioning apparatus. Each cylindrical segment represents 10.5 bp of double-stranded DNA. Surface representations highlight the fundamental positioning unit shown in (A) and adjuster helices. Mounting positions are indicated by the icons as shown in (A). (C, left) Backbone-backbone distances for the three mounting locations derived from a structural model of the positioning apparatus. (C, right) Average backbone-backbone distance increments and backbone-backbone ranges for the three mounting locations.
triangle’s base helix (adjuster helix). The length of the adjuster helix can be adjusted in increments of one base pair, whereas the separation between two points on opposing legs of the triangle are adjusted in smaller increments that depend on the distance of the mounting sites from the triangle’s vertex (icons in Fig. 1.1A). At room temperature in solution, a simple triangle of double-stranded DNA molecules would exhibit large thermal fluctuations and out-of-plane movement. We therefore inserted the positioning unit into a DNA origami scaffold (positioning apparatus) (Fig. 1.1B and Fig. B.1), realizing four positioning units in parallel which are connected to and supported mechanically by the DNA origami framework. In addition, we implemented hinge flexibility with two-bases-long DNA connections at the triangle’s vertex (Fig. B.2). We developed a geometrical model (see below) which considers the helicity of the adjuster helix, the offset due to the hinge mechanism, and the location of the leg-helix backbone in three dimensions to calculate distances between three pairs of mounting locations used in this work (Fig. 1.1A-C and Fig. 1.2).

We used the theorem of intersecting lines to predict the positioner’s opening angle $\theta$ from the number of base pairs in the adjuster helix $n$ (Fig. 1.2A),

$$\theta(n) = 2 \arcsin \left( \frac{d_{\text{adjuster}}(n) - d_0}{2 d_{\text{leg}}} \right)$$  \hspace{1cm} (1.1)$$

where $d_{\text{leg}} \approx 15.6 \text{ nm}$ is the average length of the triangle’s legs (46.5 bp, assuming 0.335 nm rise per base pair), $d_0$ is an offset that accounts for the two single-stranded DNA bases at the hinge, and $d_{\text{adjuster}}$ is the effective length of the adjuster helix. The effective length of the adjuster helix accounts for its helicity as well as for its flexibility due to thermal fluctuations. To determine this effective length, we first calculated the distance between terminal C5 carbon atoms in a double-stranded DNA molecule $d_{C5,C5}$, assuming straight B-form DNA (Fig. 1.2B inset), and then scaled this distance with a factor $\alpha_{\text{bending}}$ to account for DNA fluctuations in solution:

$$d_{\text{adjuster}}(n) \approx d_{C5,C5}(n) \cdot \alpha_{\text{bending}}(n)$$  \hspace{1cm} (1.2)$$

We extracted $d_{C5,C5}$ distances from a structural model of straight B-form DNA [57] and approximated the double-stranded adjuster helices as semiflexible polymers to calculate the bending factor $\alpha_{\text{bending}}$. We used the radial end-to-end distribution function $G$ for a semiflexible polymer from reference [58] to compute the average end-to-end distance $l_{sfp}$ of $j$ DNA molecules in parallel (Fig. 1.2B):

$$\langle l_{sfp}(n) \rangle = \int_0^\infty r \left[ G(\vec{r}, n) 4\pi r^2 \right]^j dr$$  \hspace{1cm} (1.3)$$
Figure 1.2: Dependence of the opening angle $\theta$ on the number of base pairs $n$ in the adjuster helix. (A) Schematic of the positioning apparatus (top) and the hinge region (bottom) with dimensions used in the geometrical model. (B) Effective length of the adjuster helix $d_{\text{adjuster}}$ from terminal C5-C5 atoms as a function of the number of base pairs. Orange line: contour length of double-stranded DNA as $l_c(n) = n \cdot \cot 335\,\text{nm}$; red circles: C5-C5 distances of a straight B-form DNA molecule [57]; black crosses: C5-C5 distances of a straight B-form DNA molecule [57] corrected for DNA flexibility according to equation 1.2 and 1.4; inset: schematic of the C5-C5 distances (black lines) in a double-stranded B-form DNA molecule.

where we used a persistence length of 150 bp for double-stranded DNA [50]. The correction factor $\alpha_{\text{bending}}$ can be obtained by normalizing the average end-to-end distance to the contour length of straight B-form DNA ($l_c(n) = n \cdot 0.335\,\text{nm}$).

\[
\alpha_{\text{bending}}(n) = \frac{l_{sfp}(n)}{l_c} \quad (1.4)
\]

1.2 Structure validation of the positioner with direct imaging

We validated the self-assembled structure of the positioning apparatus (positioner) by direct imaging of purified assembly reaction products using negative-staining transmission electron microscopy (TEM). The layer of adjuster helices, the distinct patterns due to the projected DNA honeycomb lattice, and the overall shape were discernible in single-particle micrographs (Fig. B.3, Fig. B.4). Reference-free 2D classification yielded classes
with opening angles that increase as a function of the number of base pairs in the adjuster helix (Fig. 1.3).

Our structural model of the positioning apparatus (equation 1.1) relates the number of base pairs in the adjuster helix \( n \) to the opening angle \( \theta \). To validate this relationship and along with it the structural model of the positioning apparatus, we measured the opening angle of individual TEM particles and computed opening-angle histograms for each positioner variant (Fig. 1.4, Tab. B.2). Since the shapes of the opening-angle histograms followed normal distributions, we calculated the mean opening angle and the standard deviation for each variant. To test our structural model and to determine the hinge offset \( d_0 \), we fitted the offset to the mean opening angles using equation 1.1, resulting in a best fit for \( d_0 = 0.4 \pm 0.1 \text{ nm} \) (Fig. 1.5). The structural model of our positioning apparatus captures the relationship between the mean opening angle and number of base pairs in the adjuster helix (Fig. 1.5). Systematic deviations of the predicted opening angle from the experimental mean opening angle occur only below \( \sim 16 \text{ bp} \) adjuster-helix length. Comparison to the average micrographs in Fig. 1.3 indicates an increased offset at the hinge for average particles with small opening angles, suggesting an extension of the single-stranded DNA connections at the hinge or deformations of the double-stranded DNA parts at the hinge. These deformations might occur due to a torque that is caused by an increased electrostatic repulsion of the beams of the positioner at small opening angles. The best fit offset \( (d_0 = 0.4 \pm 0.1 \text{ nm}) \) is smaller than what would be expected from a 3-bases-long stretch of single-stranded DNA (1.9 nm contour length, assuming a
Figure 1.4: Positioner opening-angle distributions from single-particle negative-staining TEM micrographs. Red bars: angle histograms from single-particle micrographs (bin with of 3°) for positioners with adjuster helix length of 10-50 bp. Black lines: normal distributions with means and standard deviations from the single-particle angles. For each positioner variant, 100-233 single particles were measured (Tab. B.2).

0.63 nm distance between DNA phosphates [59]). Stacking of single-stranded DNA bases to terminal base pairs in combination with strong bending of these single-stranded elements might cause a smaller distance $d_0$.

The opening-angle standard deviations range from 3.7° to 5.8° and increase slightly with the length of the adjuster helix. We hypothesize that three mechanisms contribute to the width of the measured distributions: (i) statistical variations due to inaccurate manual opening-angle measurements of single TEM particles ($\sigma_{\text{man}}$), (ii) angle variations of 2D-projected angles from out-of-plane tilted particles due to TEM-grid surface roughness ($\sigma_{\text{proj}}$), and (iii) intrinsic flexibility of the positioning apparatus ($\sigma_{\text{flex}}$). If all sources of errors are normally distributed, the final width of the distribution would be given by $\sigma^2_{\text{tot}} = \sigma^2_{\text{man}} + \sigma^2_{\text{proj}} + \sigma^2_{\text{flex}}$. We estimated the error due to manual angle measurement by repeatedly measuring the opening angle of a single TEM particle to $\sigma_{\text{man}} \sim 1°-2°$. Simulations that deposit positioners on tilted surfaces and calculate 2D-projected angles
suggest an increased standard deviation of projected 2D angles, but no shift of the mean opening angle. Projections of the positioner beam’s DNA honeycomb lattice should yield three distinct intensity maxima only if particles are in the correct orientation [6]. Since only particles with three discernible intensity maxima of the DNA honeycomb lattice were analyzed, the maximal surface tilt can be estimated to $\sim 10^\circ$. On the basis of this maximal tilt angle, we estimate the projection standard deviation to $\sigma_{\text{proj}} \approx 0.1^\circ$ for particles with a $10^\circ$ opening angle and up to $\sigma_d \approx 0.5^\circ$ for particles with a $60^\circ$ opening angle. Finally, assuming normally distributed errors, we would expect intrinsic positioner flexibilities of $\sigma_{\text{flex}} \approx 3^\circ$-$5^\circ$.

### 1.3 Placement validation using fluorescence spectroscopy

Negative-staining TEM allowed us to validate the overall structure of the positioning apparatus and to establish the relation between the number of base pairs in the adjuster helix and the opening angle of the positioning apparatus. Because the photophysical properties of fluorescent molecules depend on their molecular environment [60–62], fluorescence spectroscopy may report on the distance between two fluorescent molecules with greater resolution than negative-staining TEM [63]. To exploit these distance-dependent fluorescence phenomena, we mounted fluorescent dye pairs at three positions along the legs of the positioning unit (Fig. 1.1A and Fig. 1.6A) and assembled positioner variants with four parallel adjuster helices with lengths ranging from 10 bp to 50 bp in one base-pair increments. Gel electrophoresis of these samples showed a decreasing electrophoretic mobility with the number of base pairs in the adjuster helix (Fig. 1.6B), presumably due to the larger cross section of positioners with larger opening angles. We imaged the re-
Figure 1.6: Verification of the structural model by placing fluorescent molecules. (A) Schematic of the positioning unit indicating dye mounting locations (D=ATTO550, A=ATTO647N) either 4-6 bp (red circles), 15 bp (orange triangles), or 25-26 bp (cyan squares) away from the hinge (Fig. 1.7). (B) Areas of laser-scanned gel-electrophoresis experiments corresponding to monomeric positioners with four parallel adjuster helices and with fluorescent dyes mounted as indicated by the icons. Gels were laser-scanned in three fluorescence channels ((A$_{em}$|D$_{ex}$): acceptor emission upon donor excitation, (D$_{em}$|D$_{ex}$): donor emission upon donor excitation, (A$_{em}$|A$_{ex}$): acceptor emission upon acceptor excitation). Brightness levels were adjusted for presentation. Depicted bands correspond to monomer bands (see Fig. B.5 for complete gels). Numbers indicate the number of base pairs in the adjuster helices.
resulting gels with a laser scanner, which allowed us to quantify the fluorescence intensity of the gel bands corresponding to the monomeric species. Specifically, we scanned gels with three different combinations of excitation wavelengths and emission filters (channels): donor emission upon donor excitation ($D_{em}|D_{ex}$), acceptor emission upon donor excitation ($A_{em}|D_{ex}$), and acceptor emission upon acceptor excitation ($A_{em}|A_{ex}$) (Fig. 1.6B, Fig. B.5, see chapter A for detailed methods). Next, we integrated the fluorescence intensity of each band and normalized it to a reference sample’s intensity that was included in each electrophoresis experiment to account for gel-to-gel intensity variations (chapter A). This procedure yielded three distinct curves for each mounting position (Fig. 1.8A).

Our structural model allows us to calculate the opening angle of the positioner for a given number of base pairs in the adjuster helices. The fluorescent properties of the dye molecules will however depend on the distance between the two dyes. To calculate this dye-dye distance, we built a structural model of the dye attachment (Fig. 1.7A-C). Specifically, we consider the helicity of the dye-mounting DNA helices to determine the exact DNA backbone positions to which the fluorescent dyes are attached. Since dye molecules were attached via flexible $C_6$-linkers with contour lengths of 1-2 nm to a terminal 3′ phosphate or to a C5-methyl group of a thymine base, we assume the average dye positions to protrude radially by 0.6 nm from the DNA backbone (Fig. 1.7D,E).

The structural model that considers the average dye positions allows us to compute the dye-dye distance for a given number of base pairs in the adjuster helix and for a given mounting position of the fluorescent molecules. When we plot the measured fluorescent brightness for each mounting variant versus the calculated dye-dye distance, the three distinct curves collapse to one curve (master curve) in each fluorescence channel (Fig. 1.8B). This simultaneous collapse of three distinct curves to one master curve in each fluorescence channel validates our structural model independently of the photophysical phenomena causing the fluorescence brightness variations.

The fluorescent properties of a dye pair change with the dye-dye distance and their relative orientation. We were able to fit the three master curves simultaneously with a photophysical model that accounts for Förster resonance energy transfer (FRET) and contact quenching. FRET is a mechanism in which an excited molecule (donor) transfers its energy non-radiatively through dipole-dipole interactions to another molecule (acceptor) in its vicinity [51,64]. The rate of energy transfer depends on the distance, on the relative orientation, and on the spectral overlap of the donor and acceptor molecule. Contact quenching is a phenomenon where the fluorescence brightness and extinction coefficient of a dye molecule is altered by the presence of another molecule and is usually due to direct overlap of molecular orbitals [61,62,65]. The quenching efficiency depends on the dye-dye distance, on the dimensions of the dye molecule, and on a molecule-specific relaxation rate. The variations in the fluorescence signals are due to a combination of contact quenching
Figure 1.7: Structural model for dye-dye distance calculation. (A) Schematic cross-section of the two helices on which fluorescent dyes are mounted. The two helices are connected with a 2-bases-long DNA strand. Radial lines give the base orientation of the fluorescently modified DNA base as a function of the distance from the hinge in base pairs. (B) Schematic representation of the two helices on which fluorescent dyes are mounted. Dyes are attached to the DNA backbone via C₆-linkers. The average locations are depicted by colored spheres. (C) Side view of the structural model in (B). (D) Schematics of the average dye positions. Dyes mounted 4 bp and 6 bp away from the hinge (red) were attached using a modified thymine base. (E) Scheme of the distance calculation. The length of the adjuster helix \( d_{\text{adjuster}} \) determines the angle between the mounting helices. \( d_0 \): effective length of the 2-bases long DNA connection. \( d \): average dye-dye distance.
Figure 1.8: Verification of the structural model by placing fluorescent molecules. (A) Mean and standard deviation of integrated monomer-band intensities of at least three independent experiments for each detection channel versus the number of base pairs in the adjuster helices (see Fig. 1.6 for exemplary gels). (B) As (A), but plotted versus the dye-dye distance calculated with our structural model (Fig. 1.7). Solid line: fit of a photophysical model to the three curves simultaneously.
and FRET; contact quenching occurs at small average dye-dye distances (\(\lesssim 3.5\, \text{nm}\)) whereas FRET occurs at intermediate average dye-dye distances (3-9 nm).

To understand the photophysical model, we need to consider the contributions of FRET and contact quenching to the fluorescence signals in each fluorescence channel separately:

**\(A_{\text{em}}|A_{\text{ex}}\) channel:** The fluorescence brightness in the \(A_{\text{em}}|A_{\text{ex}}\) channel varies due to distant dependent contact quenching. Objects that carry an intact donor molecule and an intact acceptor molecule will yield a quenched signal from the acceptor dye for small dye-dye distances. Objects that carry only an acceptor molecule will contribute unquenched fluorescence of this dye in the \(A_{\text{em}}|A_{\text{ex}}\) channel regardless of the positioner’s opening angle. The brightness of a given sample is therefore due to contributions from intact particles and defect particles, and can be expressed using the quenching efficiency \(Q\) and probabilities, \(p_A\) and \(p_D\), that an object carries an intact acceptor or donor dye respectively.

\[
I_{A|A} = I_{A|A}^0 p_A p_D (1 - Q) + I_{A|A}^0 p_A (1 - p_D) \quad (1.5)
\]

\[
= I_{A|A}^0 p_A (1 - p_D Q) \quad (1.6)
\]

where \(I_{A|A}^0\) is a proportionality constant. For large distances (\(> 8\, \text{nm}\)), the contact quenching vanishes (\(Q \approx 0\)) and we therefore normalize the master curve in the \(A_{\text{em}}|A_{\text{ex}}\) channel to one. This normalization effectively eliminates the prefactors (\(Q \approx 0 \Rightarrow I_{A|A}^0 p_A \approx 1\)).

**\(D_{\text{em}}|D_{\text{ex}}\) channel:** Objects with an intact donor and an intact acceptor dye show fluorescence of the donor upon donor excitation that is quenched due to contact quenching (quenching efficiency \(Q\)) and Förster resonance energy transfer (FRET efficiency \(E\)). Objects that only incorporate an intact donor dye, but no acceptor dye, will yield unquenched fluorescence. Objects that only incorporate an acceptor dye will not be detected in the \(D_{\text{em}}|D_{\text{ex}}\) channel:

\[
I_{D|D} = I_{D|D}^0 p_A p_D (1 - E) (1 - Q) + I_{D|D}^0 p_D (1 - p_A) \quad (1.7)
\]

\[
= I_{D|D}^0 p_D (1 + p_A (E Q - E - Q)) \quad (1.8)
\]

Again, \(p_A\) and \(p_D\) refer to the probabilities of carrying an intact acceptor or donor molecule respectively. We normalize the master curve in the \(D_{\text{em}}|D_{\text{ex}}\) channel to one at large distances (\(> 9\, \text{nm}\)), where neither FRET nor contact quenching occurs. This normalization effectively eliminates the prefactors (\(Q \approx E \approx 0 \Rightarrow I_{D|D}^0 p_D \approx 1\)).

**\(A_{\text{em}}|D_{\text{ex}}\) channel:** Fluorescence brightness in the \(A_{\text{em}}|D_{\text{ex}}\) channel originates from positioner objects with an intact FRET-dye pair where the dyes are close enough to
transfer energy by FRET. Thus, only objects that carry an intact acceptor and an intact donor molecule contribute fluorescence brightness:

\[ I_{A|D} = I^0_{A|D} p_A p_D E (1 - Q) \]  

(1.9)

Fluorescence that is lost in the \((D_{em}|D_{ex})\) signal due to FRET should appear in the \((A_{em}|D_{ex})\) signal. Because of different quantum yields of the fluorescent dyes and different detection efficiencies in the detection channels only a fraction \(\gamma\) of this fluorescence is detected. This fraction can be computed by imposing a conservation of fluorescence intensity \((I^\text{norm}_{D|D} + I^\text{norm}_{A|D} = 1)\) for dye-dye distances where no contact quenching occurs:

1 = \(I^\text{norm}_{D|D} + I^\text{norm}_{A|D}\)  

(1.10)

\[ = 1 - p_A E + \gamma I^0_{A|D} p_A p_D E \]  

(1.11)

\[ \Leftrightarrow \gamma = \frac{1}{I^0_{D\rightarrow A|PD}} \]  

(1.12)

The constant of proportionality \(\gamma\) between the two signal types was determined experimentally to \(\gamma \approx 0.48\) for the data set at large distances where no contact quenching occurs using a sample of known FRET efficiency.

In summary, the fluorescence signals are described by the following set of equations:

\[ I_{A|A} = 1 - p_D Q \]  

(1.13)

\[ I_{A|D} = p_A E (1 - Q) \]  

(1.14)

\[ I_{D|D} = 1 + p_A (E Q - E - Q) \]  

(1.15)

The quenching efficiency \(Q\) and the FRET efficiency \(E\) depend on the dye-dye distance, whereas the probabilities of carrying an acceptor or donor molecule, \(p_A\) and \(p_D\), are assumed to be independent of dye-dye distance. We assumed equal intact dye frequencies for the three different mounting locations, neglecting different synthesis yields or incorporation frequencies of fluoroescently modified DNA oligonucleotides. Furthermore, we assumed maximum FRET efficiency and quenching efficiency at small dye-dye distances \((Q \approx 1\) and \(E \approx 1)\). Residual fluorescence is therefore due to DNA objects that carry only one instead of two dyes; and we can estimate the probabilities of carrying an intact fluorophore from equations 1.13 that simplify at maximum FRET and quenching efficiencies to

\[ I_{A|A} = 1 - p_D \]  

\[ I_{A|D} = 0 \]  

\[ I_{D|D} = 1 - p_A \]  

(1.16)

Using these relations in combination with our three master curves, we obtain fluorophore
incorporation frequencies of \( p_D \approx 0.90 \) and \( p_A \approx 0.95 \). Both a finite frequency of fluorescence modifications at the DNA oligonucleotides, as well as a finite incorporation yield of oligonucleotides into the DNA origami object may result in a limited frequency of intact fluorophores on the assembled positioning apparatus.

To fit the fluorescence master curves, we need to express the FRET efficiency \( E \) and the quenching efficiency \( Q \) as functions of the average dye-dye distance \( d \). Quenching is a short-ranged phenomenon that describes multiple physical mechanisms, such as intersystem crossing, electron exchange quenching, or photoinduced electron transfer [65]. These mechanisms have in common that they require contact of electron orbits to relax the excited molecule non-radiatively. The quenching rate \( k_E \) therefore decreases with the electron density as \( k_E = B \exp(-\beta (d-r_c)) \). \( r_c \) is the distance where molecular contact occurs, \( \beta \) describes the decay of the electronic density and is close to 1 Å\(^{-1} \) [65], and \( B \) is a time constant on the order of 10\(^{-13} \) s [65]. With the decay rate of the donor molecule \( \tau_D^{-1} \), we can use the quenching rate to compute the quenching efficiency:

\[
Q(d) = \frac{k_E}{\tau_D^{-1} + k_E} = \frac{1}{1 + A e^{\beta d}} \tag{1.17}
\]

where \( A = \tau_D^{-1} B^{-1} \exp(-\beta r_c) \). \( A \) and \( \beta \) are not known for our system and are therefore fit parameters.

During Förster resonance energy transfer an excited molecule (donor) transfers its energy non-radiatively via dipole interactions to an acceptor molecule. Since the electric field of an electric dipole decays with the distance \( r \) as \( r^{-3} \), dipole-dipole interactions decay as \( r^{-6} \) and the resonance energy transfer rate \( k_T \) is given by

\[
k_T = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6 \tag{1.18}
\]

where \( r \) is the distance between the molecules and \( \tau_D \) is the donor lifetime in the absence of the acceptor molecule. \( R_0 \) is the Förster radius that depends on the quantum yield of the donor \( \phi_D \), on the refractive index of the medium \( n \), on the spectral overlap between the normalized donor emission spectrum \( f_D(\lambda) \) and the acceptor extinction spectrum \( \epsilon_A(\lambda) \), and on the relative orientation of the dipole moments \( \kappa^2 \) [51, 64]

\[
R_0^6 = \frac{9 \phi_D \ln(10) \kappa^2}{128 \pi^5 n^4 N_A} \int f_D(\lambda) \epsilon_A(\lambda) \lambda^4 \, d\lambda \tag{1.19}
\]

\( \kappa^2 \) can assume values from 0 to 4, but is 2/3 for freely rotating molecules that can sample all possible dipole-dipole orientations. The FRET efficiency of a given dye configuration
$E'$ is the transfer rate normalized to all decay channels of the donor

$$E'(r) = \frac{k_T}{k_T + \tau_D^{-1}} = \frac{1}{1 + (r/R_0)^6} \quad (1.20)$$

This relation holds for a specific dye-dye distance $r$ and dye-dye orientation. During a fluorescence bulk measurement, the fluorescence of different particles is averaged and, since the fluorescent dyes are mounted via flexible C6-linkers (contour length of 1-2 nm), a FRET dye pair exhibits different dye-dye distances and orientations. We are therefore actually measuring the average FRET efficiency $E = \langle E' \rangle = \langle 1/(1 + (r/R_0)^6) \rangle$. It is important to note that averaging over FRET efficiencies is not the same as calculating the FRET efficiency from an average distance $1/(1 + \langle r/R_0 \rangle^6) \neq 1/(1 + \langle r/R_0 \rangle^6)$ [63, 66]. Since our structural model allows us to calculate the average dye-dye distance $d = \langle r \rangle$ we developed an heuristic model that treats this averaging effectively:

$$E(d) = \langle \frac{1}{1 + (r/R_0)^6} \rangle_d \approx \frac{1}{1 + ((d - a)/R_0)^6} \quad (1.21)$$

where $a$ describes an effective offset that is generated by the dye fluctuations.

Having established relations for the FRET and quenching efficiency as functions of the average dye-dye distances, we fitted the set of parameters $\{A, \beta, R_0, a\}$ simultaneously to the three master curves using constant probabilities of donor and acceptor incorporation ($p_D = 0.90$ and $p_A = 0.95$):

$$A \approx 0.023 \pm 0.004, \quad \beta \approx (0.143 \pm 0.007) \text{Å}^{-1}, \quad R_0 \approx (79 \pm 4) \text{Å}, \quad a \approx (-29 \pm 4) \text{Å}$$

The fitted inverse decay length of the electron density $\beta$ is smaller than literature values suggest (0.1 Å$^{-1}$ versus 1 Å$^{-1}$) [65], which may be caused by fluctuations of the dye molecules that effectively increase the decay length. Similarly, because dye fluctuations are treated effectively (see above) the fitted Förster radius is larger than expected (7.9 nm versus 6.5 nm) in our heuristic model.

For the mounting position that is closest to the hinge ($\sim$5 bp), we observe signal changes upon changing the length of the adjuster helix by one base pair. A one-base-pair increment in the adjuster helix would correspond to a change in the average dye-dye distance of $\sim$ 0.04 nm. In order to validate this signal change, we mounted two dyes of the same type (ATTO647N) and attached a reporter dye molecule (ATTO550) at a distal site of the positioning apparatus, where it can not interact with the other dye pair (Fig. 1.9A). The reporter dye allowed us to normalize the fluorescence intensity of the dye pair $I_{A|A}/I_R$ for each gel band individually (see chapter A) and thus reduce signal variations (Fig. 1.9B,C and Fig. B.6). We also normalized each gel-experiment curve.
to a reference sample that was included in each gel. The resulting relative fluorescence intensity confirmed a signal change upon changing the length of the adjuster helix by one base pair, thus controlling the average dye-dye distance by increments of 0.03-0.04 nm (Fig. 1.9B,C).

The normalized fluorescence intensities ought to be described using only contact quenching. Considering, that the collected signal can be attributed to objects with two intact acceptor molecules that result in quenched fluorescence and to objects that carry only one intact acceptor dye, we can write the normalized intensity:

$$\frac{I_A}{I_R} = I^0 p_A (1 - p_A Q) = I^0 p_A \left(1 - p_A \frac{1}{1 + A e^{\beta d}}\right) \quad (1.22)$$

where $I^0$ is a constant of proportionality and $p_A$ is the probability that an object carries an intact acceptor molecule. For large distances, where no contact quenching occurs, the signal should approach a constant. We therefore normalized the signal to one at large dye-dye distances ($I^0 p_A = 1$) and fitted the set of parameters $\{A, \beta, p_A\}$ to the data using the following expressions:

$$A \approx 0.0008 \pm 0.0005, \quad \beta \approx (0.24 \pm 0.02) \text{Å}^{-1}, \quad p_A = (82 \pm 2)\%$$

The best fit parameters are similar to the parameters that were obtained from the experiments with two different fluorescent dye molecules, except for the dye-specific parameter $A$ that depends on the distance of molecular contact and on the dye-specific lifetimes.
1.4 Quantification of molecular fluctuations using crosslinking

We observed a difference in fluorescence signal between positioner samples whose adjuster-helix length differed by only one base pair, suggesting a positioning accuracy of \(~0.04\text{ nm}\) in the average dye-dye distance. The measurements described in the previous section report on the controllability of the average distance between two fluorescent dyes, because conformational fluctuations are averaged out in bulk fluorescence experiments. We developed a crosslinking assay to study the molecular fluctuations of the positioning apparatus (Fig. 1.10A) [67]. We attached thiol groups to the legs of the positioning unit at two mounting locations that were used previously (Fig. 1.1), and mixed positioners with mounted thiol groups with bismaleimide crosslinkers. The crosslinking reaction proceeds in two steps. In the first reaction step, a bismaleimide crosslinker attaches to a positioner from solution when one of the crosslinker’s maleimide groups reacts with one of the positioner’s thiol groups. In the second reaction step, the free maleimide group of the bound crosslinking can react with the free thiol group at the opposing positioner beam with a rate constant \(k_i\); thus crosslinking the thiol groups of the positioning apparatus (Fig. 1.10 and Fig. 1.11A). Alternatively, a free crosslinker can bind from solution to the unreacted thiol group with a rate constant \(k_{sol}\), resulting in a non-crosslinked positioner object with two bound crosslinkers. Upon completion of the crosslinking reaction, we add denaturing agents that disassemble the positioning apparatus into its single-stranded DNA components and separate crosslinked from non-crosslinked products using polyacrylamide gel electrophoresis (Fig. 1.11B). Thiol-modified DNA strands were modified additionally with a fluorescent dye, allowing us to distinguish thiol-modified DNA strands from other DNA in fluorescence laser-scans of polyacrylamide gels. Thiol strands from opposing beams of the positioner had different lengths and were modified with different fluorescent dyes (Cy3 and Cy5). Thus, gel bands corresponding to crosslinked DNA strands (ALB) show fluorescence of both dyes and migrate with smaller electrophoretic mobility, whereas gel bands corresponding to non-crosslinked DNA strands (LB, B, AL, A) show fluorescence of only one dye and migrate with larger electrophoretic mobility. We integrated the fluorescence signal of each gel band and quantified the crosslinking yield by normalizing the fluorescence brightnesses of the crosslinked species to the sum of the fluorescence brightness of crosslinked and non-crosslinked species.

Using the assay described above, we measured the crosslinking yield for positioners with adjuster helix length from 10bp to 50bp for two thiol-mounting positions (4.5bp and 15bp away from the hinge). We plotted the crosslinking yield versus the distance between the mounting positions of the thiol groups on the DNA backbone as calculated with our structural model of the positioning apparatus (Fig. 1.12A). In addition, we used
Figure 1.10: Crosslinking reaction to measure molecular fluctuations. Top: Schematic of the positioning unit with a crosslinking molecule (dumbbell spring) attached to the bottom helix. A bismaleimide crosslinker can bind from solution with the rate constant $k_{sol}$ competing with the internal crosslinking reaction (rate constant $k_i$). The internal crosslinking rate constant depends on the extent of thermal fluctuations $\sigma_d$ and on the amount of crosslinker fluctuations. Orange spheres represent reactive groups (thiol or maleimide). Bottom: Chemical structure and reaction of a pPDM crosslinker molecule [68] with a thiol group that is attached to the 3'-end of a DNA oligonucleotide producing a covalently linked molecule.

Figure 1.11: Crosslinking assay to measure molecular fluctuations. (A) Scheme of the crosslinking reaction pathways. Thiol groups (orange spheres on positioner) react with a bismaleimide crosslinker (dumbbell springs). P: unreacted positioner, PL*: positioner with one crosslinker bound, PL: crosslinked positioner, PL2: positioner with two crosslinkers bound. $k_{sol}$: rate constant for binding from solution, $c_L$: concentration of crosslinker in solution, $k_i$: crosslinking rate constant. (B) Left: Schematic representation of crosslinked (top) and non-crosslinked DNA single strands with fluorescent dyes (red=Cy5, green=Cy3). Right: Laser-scanned image of a 20% polyacrylamide gel on which denatured crosslinking reactions with different crosslinker molecules were electrophoresed. A,B: Fluorescently labeled DNA strands (A=Cy5, B=Cy3); AL,BL: Fluorescently labeled DNA strands with a covalently bound crosslinker molecule (L); ALB: Fluorescently labeled DNA strands covalently crosslinked with a crosslinker molecule.
five different bismaleimide crosslinkers which, owing to their different chemical structures, have different contour lengths and flexibilities [68] (Tab. B.2). Crosslinking yields are high at small backbone-backbone mounting distances (< 3 nm), gradually decrease at intermediate distances (3 nm - 5 nm), and vanish at large distances (> 5 nm). The shape of the curves can be understood by considering a gedankenexperiment in which the extent of fluctuation is varied. In the fluctuation-free case, we would observe a crosslinked species only if the length of the crosslinker exactly matches the distance between the two thiol groups. If the crosslinker molecule, but not the thiol groups, exhibited fluctuations, we would observe crosslinking only if the distance between the thiol groups was in the range of possible linker end-to-end distances (Fig. 1.13), and we would expect a step-like drop-off if the thiol-thiol distance is larger than the contour length of the crosslinker molecule. If, however, the thiol group positions exhibit fluctuations as well, we will expect a shift in the drop-off to thiol-thiol distances greater than the crosslinker’s contour length, because fluctuations will lead to conformations in which the linker can span the distance between the thiol groups. In addition, fluctuations will increase the conformational space accessible to the thiol groups and we would expect the drop-off to occur more gradually. In order to test this hypothesis, we introduced additional flexibility by mounting one or both thiol groups with flexible pentathymine linkers (Fig. 1.12B, C). If one thiol group is mounted with increased flexibility, the yield drop-off occurs at larger backbone-backbone distances and proceeds more gradually. If both thiol groups are mounted with increased flexibility, no yield drop-off is detected within the range of tested backbone-backbone distances.

To determine molecular fluctuation parameters of the positioning apparatus from the experimentally measured yield curves, we need to first solve the chemical rate equations that describe the crosslinking reaction. The rate constants will then depend on the molecular parameters and report on the amplitude of fluctuations.

The crosslinking reaction can be described with a set of chemical rate equations. Our chemical network (Fig. 1.13) considers four positioner states: the unreacted positioner (P), the positioner with one attached bismaleimide crosslinker (PL∗), the crosslinked positioner (PL), and the positioner with two attached crosslinkers (PL₂). Upon addition of the crosslinker (L) to the positioner solution, the reaction proceeds in two steps. First, a crosslinker from solution binds to one of the two free thiol groups on the positioner with the apparent rate 2k_{sol}c_L, where k_{sol} is the chemical rate constant for binding from solution and c_L is the concentration of crosslinker molecules in solution (typically ≈ 10 μM). The factor of two accounts for the fact that two thiol groups are mounted on each positioner object. In the second step, the positioner can either be crosslinked with an internal reaction rate constant k_i or a second crosslinker can bind from solution with the rate k_{sol}c_L. This
Figure 1.12: Quantifying molecular fluctuations. (A) Top: crosslinker structures used in this study, sorted according to their contour lengths. Bottom: normalized mean crosslinking yield of positioners with four adjuster helices versus the distance between the thiol-mounting locations on the DNA backbone. Error bars: one standard deviation from three independent yield measurements. Circles: thiol groups were mounted ~5 bp away from the hinge. Triangles: thiol groups were mounted 15 bp away from the hinge. Colors indicate the bismaleimide crosslinker used in the reaction. Solid lines: fit of the fluctuation model to all five yield curves simultaneously. Yield curves were normalized to 1 at small (< 3 nm) backbone-backbone distances. (B) Normalized crosslinking yield for positioners where one thiol group were mounted using flexible pentathymine linkers. (C) Normalized crosslinking yield for positioners where both thiol groups was mounted using a flexible pentathymine linker.
system can be described with the following set of chemical rate equations:

\[
\begin{align*}
P : \quad & \frac{dc_P}{dt} = -2 k_{sol} c_L c_P \\
PL^* : \quad & \frac{dc_{PL^*}}{dt} = 2 k_{sol} c_L c_P - k_{sol} c_L c_{PL^*} - k_1 c_{PL^*} \\
PL_2 : \quad & \frac{dc_{PL_2}}{dt} = k_{sol} c_L c_{PL^*} \\
PL : \quad & \frac{dc_{PL}}{dt} = k_1 c_{PL^*} \\
L : \quad & \frac{dc_L}{dt} = -2 k_{sol} c_L c_P - k_{sol} c_L c_{PL^*}
\end{align*}
\]

In the limit of crosslinker excess over positioner objects \((c_L \gg c_P)\), the concentration of crosslinkers in solution can be regarded as constant and we can describe the state evolution
of a single positioning apparatus with an already bound crosslinker molecule as follows:

\[
P_{L^*} : \quad \frac{dp_{PL^*}}{dt} = -(k_{sol}c_L + k_i)p_{PL^*}
\]

\[
P_{L_2} : \quad \frac{dp_{PL_2}}{dt} = k_{sol}c_Lp_{PL^*}
\]

\[
P_{L} : \quad \frac{dp_{L}}{dt} = k_i p_{PL^*}
\]

where \(p_{PL^*}, p_{PL_2},\) and \(p_{PL}\) denote the probabilities of finding the positioning apparatus in the respective states. The set of rate equations above can be solved analytically and with the initial conditions \(p_{PL^*}(0) = 1, p_{PL}(0) = 0, p_{PL_2}(0) = 0\) we obtain:

\[
p_{PL^*}(t) = e^{-\kappa t}
\]

\[
p_{PL_2}(t) = \frac{k_{sol}c_L}{\kappa} (1 - e^{-\kappa t})
\]

\[
p_{PL}(t) = \frac{k_i}{\kappa} (1 - e^{-\kappa t})
\]

with the sum of the two rates \(\kappa = k_i + k_{sol}c_L\). In the limit of reaction completion \((t \to \infty)\), the positioner is in the crosslinked state with probability \(k_i/\kappa\) or in the non-crosslinked state with probability \(k_{sol}c_L/\kappa\). Since this result holds true for all positioners in a crosslinking reaction, we can calculate the fraction of positioner objects in the crosslinked state after reaction completion \(\alpha_\infty\) (crosslinking yield):

\[
\alpha_\infty = \frac{p_{PL}(t \to \infty)}{p_{PL}(t \to \infty) + p_{PL_2}(t \to \infty) + p_{PL^*}(t \to \infty)} = \frac{1}{1 + \frac{k_{sol}c_L}{k_i}}
\]  

(1.23)

The final crosslinking yield depends on the ratio between the internal crosslinking rate \(k_i\) and the apparent rate of crosslinker binding from solution \(k_{sol}c_L\). The internal crosslinking rate constant \(k_i\) in turn reports on the molecular details of the positioner structure. In particular, it depends on the colocalization probability \(p_{coloc}\) of the free maleimide group of the already bound crosslinker with the free thiol group on the positioner:

\[
k_i = k_0 p_{coloc}
\]  

(1.24)

where \(k_0\) is the crosslinking rate at perfect colocalization. The colocalization probability depends on the mean distance and distance fluctuations between the thiol groups as well as the mechanical properties of the crosslinker. In order to determine the molecular fluctuations, we assumed normally distributed localization probabilities for the two thiol
groups $A$ and $B$ with mean distance $d$ and standard deviation $\sigma_{\text{thiol}}$:

$$p_{\text{thiol}, A} \sim \mathcal{N} \left( \begin{pmatrix} 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} \sigma_{\text{thiol}} & 0 & 0 \\ 0 & \sigma_{\text{thiol}} & 0 \\ 0 & 0 & \sigma_{\text{thiol}} \end{pmatrix} \right)$$

$$p_{\text{thiol}, B} \sim \mathcal{N} \left( \begin{pmatrix} 0 \\ 0 \\ d \end{pmatrix}, \begin{pmatrix} \sigma_{\text{thiol}} & 0 & 0 \\ 0 & \sigma_{\text{thiol}} & 0 \\ 0 & 0 & \sigma_{\text{thiol}} \end{pmatrix} \right)$$

The distribution of the sum of two normally distributed random variables is again a normal distribution. Hence, the distance vector between the thiol groups $\vec{r}_{AB} = \vec{r}_B - \vec{r}_A$ follows a normal distribution with standard deviation $\sigma_d = \sqrt{\sigma_{\text{thiol}}^2 + \sigma_{\text{thiol}}^2}$:

$$p_d(\vec{r}_{AB}) \sim \mathcal{N} \left( \begin{pmatrix} 0 \\ 0 \\ d \end{pmatrix}, \begin{pmatrix} \sigma_d & 0 & 0 \\ 0 & \sigma_d & 0 \\ 0 & 0 & \sigma_d \end{pmatrix} \right)$$

This may also be interpreted as placing the origin of the coordinate system in one of the thiol groups. $p_d(\vec{r}_{AB})$ is the probability density of the two thiol groups having a particular distance vector $\vec{r}_{AB}$. The free maleimide group and the free thiol group are colocalized if the maleimide crosslinker can span the distance $\vec{r}_{AB}$:

$$p_{\text{coloc}}(d, \sigma_d) = \int 3 d^1 \sqrt{2\pi \sigma_d^3} e^{-\frac{x^2+y^2+(z-d)^2}{2\sigma_d^2}} p_{L, \text{end-to-end}}(\sqrt{x^2+y^2+z^2}) \cdot N$$

where $p_L(\vec{r})$ is the probability density of the end-to-end vector of the bismaleimide crosslinker. The probability density of the end-to-end vector can be computed from the sulfur-to-sulfur distance distribution $p_{L, \text{end-to-end}}(r)$ for each linker type reported in reference [68]:

$$p_L(\vec{r}) = \frac{p_{L, \text{end-to-end}}(r)}{4\pi r^2 \cdot N}$$

with a normalization constant $N = \int p_{L, \text{end-to-end}}(r)/(4\pi r^2) \, dr^3$. In summary, the colocalization probability is given by:

$$p_{\text{coloc}}(d, \sigma_d) = \int dV \frac{1}{\sqrt{2\pi \sigma_d^3}} e^{-\frac{x^2+y^2+(z-d)^2}{2\sigma_d^2}} \frac{p_{L, \text{end-to-end}}(\sqrt{x^2+y^2+z^2})}{4\pi (x^2+y^2+z^2) \cdot N} \tag{1.25}$$

We evaluate the integral in equation 1.25 numerically using the end-to-end distance distribution from reference [68]. As an example, one can consider the limit of a crosslinker molecule of zero length ($p_L(\vec{r}) = \delta^3(\vec{0})$). In this case, the colocalization probability reduces to a normal distribution:

$$p_{\text{coloc}}(d, \sigma_d) = \frac{1}{\sqrt{2\pi \sigma_d^3}} e^{-\frac{d^2}{2\sigma_d^2}}$$
As another example, one can consider the limit of a positioner without thermal fluctuations \( p_d(\vec{r}) = \delta(x) \delta(y) \delta(z - d) \). The positioner then samples the end-to-end distance distribution of the crosslinker:

\[
p_{\text{coloc}}(d, \sigma_d) = \frac{p_{L, \text{end-to-end}}(d)}{4\pi d^2 N}
\]

Combining equation 1.23 and equation 1.24, we can describe the experimentally measured yield as function of the backbone-backbone distance \( d \):

\[
\alpha_\infty(d) = \left[ 1 + \frac{p_0}{p_{\text{coloc}}(d, \sigma_d)} \right]^{-1}
\]  \hspace{1cm} (1.26)

with the constant \( p_0 = k_{\text{sol}} c_L / k_0 \) and the distance fluctuations \( \sigma_d \). Hence, for each linker type the yield curve can be described with two parameters: \( \sigma_d \) and \( p_0 \). As we increase the mean thiol-thiol distance \( d \), the colocalization probability \( p_{\text{coloc}} \), and thus the internal crosslinking rate \( k_i \), decreases.

We assume that the fluctuations of the thiol-thiol distance \( \sigma_d \) do not depend on the linker type, whereas the constant \( p_0 \) can vary from crosslinker to crosslinker. Since the samples were purified using PEG precipitation, which is not selective for defective objects [21], a fraction \( \beta_{\text{defect}} \) of defective particles with fluctuations \( \sigma_{d,\text{defect}} \) is expected. Samples with flexible pentathymine linkers between the DNA backbone and the thiol group are expected to show larger fluctuations and can therefore be described with separate standard deviations \( \sigma_{d,5T} \) and \( \sigma_{d,5T,5T} \). We fitted the set of six parameters \( \{ \sigma_d, \sigma_{d,\text{defect}}, \beta_{\text{defect}}, \sigma_{d,5T}, \sigma_{d,5T,5T}, p_0 \} \) to all yield curves simultaneously using \( \chi^2 \)-minimization (solid lines in Fig. 1.12):

<table>
<thead>
<tr>
<th>Positioner variant</th>
<th>Distance fluctuations ( \sigma_d ) [Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact positioners, (80 ± 7)%</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>Defective positioners, (20 ± 7)%</td>
<td>10.1 ± 0.8</td>
</tr>
<tr>
<td>Top thiol group mounted with additional flexibility</td>
<td>13.1 ± 0.5</td>
</tr>
<tr>
<td>Both thiol groups mounted with additional flexibility</td>
<td>30 ± 17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Crosslinker molecule</th>
<th>BMOE</th>
<th>mPDM</th>
<th>pPDM</th>
<th>BMH</th>
<th>BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p_0 = k_{\text{sol}} c_L / k_0 ) [10^{-}9]</td>
<td>9 ± 4</td>
<td>9 ± 4</td>
<td>9 ± 3</td>
<td>4 ± 2</td>
<td>9 ± 4</td>
</tr>
</tbody>
</table>

where errors are standard errors of the parameters. Most positioner particles (80%) exhibit thiol-thiol distance fluctuations of 0.7 nm, corresponding to thiol root mean square fluctuations (RMSF) of 0.5 nm, while a smaller population (20%) of presumably defective positioners show larger distance fluctuations (1.0 nm). Since thiol groups are attached to the DNA 3′-end via a three-carbon-long linker with a contour length of \(~0.4\) nm (Fig. 1.10), the backbone DNA RMSF in the non-defective positioners might be smaller than
0.5 nm. Indeed, all-atom molecular dynamics simulations are in agreement with these small structural fluctuations (RMSF of 0.4 nm) \[25, 69\]. The fit of the model to the data with positioners where one thiol group was mounted with a flexible pentathyime linker yielded thiol-thiol distance fluctuations of 1.3 nm. If two thiol groups were mounted with flexible pentathyime linkers, we find that the fluctuation amplitude in the thiol-thiol distance coordinate exceeds 3 nm.

1.5 Summary

In this chapter we have introduced a DNA origami positioning apparatus that uses an embedded isosceles triangle of connected DNA double helices as a positioning unit. Adjusting the length of the triangle’s base (adjuster helix) allows to finely tune the angle and therefore the distance between two points on the triangle’s legs. We verified this principle using direct imaging (negative staining TEM) and verified a structural model that relates the number of base pairs in the adjuster helix to the opening angle of the positioning apparatus. We then mounted fluorescent molecules on the positioner and studied their fluorescence brightness variations upon changing the opening angle of the positioner and as a function of the fluorophore mounting location. The distinct intensity curves collapsed on three master curves when we used our structural model to plot fluorescence intensities versus calculate dye-dye distances. This collapse validated the structural model that predicts average dye-dye distance incrementation of \(\sim 0.04\) nm. Finally, we studied the extent of structural fluctuations using quantitative crosslinking experiments in combination with a reaction model. Mounted thiol groups exhibited structural fluctuations with RMSF of 0.5 nm. In summary, our positioning apparatus enables us to precisely tune the average distance between attached molecules in defined increments and with small fluctuations in the position coordinate.
Chapter 2

Force spectroscopy using DNA origami

Applying forces to biomolecules or biomolecular interaction partners while following their conformational state has led to new insights into the functions and mechanical properties of nucleic acids and proteins [47–50]. State of the art force spectroscopy methods rely on different force generating mechanisms. For example, an atomic force microscope generates forces by moving an elastic microscopic cantilever, optical tweezers generate forces on micrometer-sized dielectric beads with focused laser beams, and magnetic tweezers generate forces on micrometer-sized magnetic beads with an inhomogeneous magnetic field. To study nanometer-sized target molecules, the forces that are created at the micrometer scale need to be propagated to the nanoscale. This manipulation of nanometer-sized objects with micrometer- to millimeter-sized instruments poses a technical challenge and requires great experimental expertise. Molecular instruments that generate forces on the same length scale as the target molecule could improve or complement existing force spectroscopy methods. We therefore converted our DNA origami positioning apparatus into a force spectrometer that uses the mechanical properties of DNA to generate weak forces on the nanoscale.

Remark: parts of this chapter have been published in reference [70] and the research was done in close collaboration with Philip Ketterer¹, Corinna Lieleg², Sarah Schunter², Philipp Korber² and Hendrik Dietz¹. In particular, Corinna Lieleg and Sarah Schunter assembled, tested, and modified nucleosomes under the supervision of Philipp Korber. Philip Ketterer and Corinna Lieleg developed nucleosomes with single-stranded DNA attachment handles.

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2.1 The DNA origami force spectrometer

A DNA origami force spectrometer should allow placing and orienting interaction partners in three-dimensional space, applying forces that counteract attractive interactions, and reading out the conformational state of the molecular system under study. We implemented these capabilities by modifying the design of our positioning apparatus (chapter 1). The force spectrometer consist of two beams that are connected by a hinge mechanism (Fig. 2.1A). Each beam consists of 24 interconnected double-stranded DNA helices. The hinge mechanism features both four 2-bases-long connections at the vertex of the beams that constrain the rotation axis, as well as four 30-bases-long DNA loops at a more distal position that act as entropic springs (see Fig. B.2 and Fig. C.1 for details of the design). This hinge mechanism, in combination with electrostatic and steric interactions, generates an angle-dependent torque. Two interacting partners, placed on the beams of this DNA origami device, or, alternatively, a molecule that is connected to both beams of the device, will experience a force depending on the angle between the beams. The state of the interacting molecules, i.e. their distance, or alternatively the extension of a mounted molecule can be read out by measuring the device’s opening angle using TEM imaging or by measuring the FRET efficiency using fluorescent dyes that are mounted on opposing beams. Because our modified positioning apparatus allows to position target molecules, to apply forces to these target molecules, and to readout the system’s state, it becomes a force spectrometer.

To calibrate the force response of the force spectrometer (spectrometer), we imaged spectrometer particles using negative-staining TEM and measured the opening angles of individual particles (Fig. 2.1A and Fig. C.3). We used kernel density estimation [71, 72] to calculate the opening-angle distribution from the single-particle angles (Fig. 2.1A,B). Spectrometer particles sample a wide range of opening angles between $0^\circ$-110$^\circ$ with a maximum at 70$^\circ$-80$^\circ$. Assuming that all TEM particles are identical but sample different conformations, the angle distribution is a result of the angle-dependent Boltzmann weighted energy landscape of the force spectrometer (Fig. 2.1B, bottom). Thus, the probability of measuring a particular opening angle $\theta$ is given by the Boltzmann factor:

$$p(\theta) = \frac{1}{Z} e^{-\frac{E(\theta)}{k_B T}}$$

where $p(\theta)$ is the probability density of the opening angle $\theta$, $Z = \int_0^{2\pi} e^{-E(\theta)/k_B T} d\theta$ is the partition function, and $E(\theta)$ is the angle-dependent free energy of the force spectrometer. We assume that this energy landscape is caused by the single-stranded DNA elements of the hinge mechanism that act as entropic springs and by the electrostatic repulsion of the spectrometer’s beams at small opening angles. Since the persistence length of single-
Figure 2.1: Calibration of the DNA origami force spectrometer. (A) Top: Schematic of the DNA origami force spectrometer with a FRET dye pair (red and green spheres). Bottom: Exemplary negative-staining single-particle TEM micrographs. Scale bar: 30 nm. (B) Top: Uniform kernel densities estimates (bandwidth $3^\circ$, from 3991 individual particles) of the opening-angle probability density of the empty spectrometer (blue line). Maximum likelihood fit of an energetic model that accounts for a spring-loaded hinge and electrostatic repulsion between the beams of the spectrometer (black line). Bottom: Free energy landscape (solid lines) and torque (dashed lines) of the empty spectrometer calculated from the kernel density estimates in the top graph (blue lines) or from the maximum likelihood fit (black lines). The torque was calculated from a normal kernel density estimate (bandwidth $3^\circ$) of the angle distribution.
stranded DNA ($\approx 1.5 \text{ nm}$ [59]) is much smaller than the contour length of a 30-bases-long DNA single strand ($\approx 19 \text{ nm}$, assuming a contour length per base of 0.63 nm [59]), we can model these single-stranded DNA loops as entropic springs. Hence, the contribution of the entropic springs to the spectrometer potential is

$$E_k(\theta) = \frac{1}{2} k_{\text{eff}} \left[ 2a \cos \left( \frac{\theta}{2} \right) - L_0 \right]^2$$

where $k_{\text{eff}}$ is an effective spring constant, $L_0$ is the mean end-to-end distance of the spring, and $a \approx 3.3 \text{ nm}$ is the distance from the hinge axis to the location where the single-stranded DNA loops exit the force spectrometer (Fig. B.2 and Fig. C.2). As the DNA backbone carries negative charges, we expect an electrostatic repulsion of the two beams of the force spectrometer, which should contribute to the energy landscape, especially at small opening angles. As a simplification, we assume a constant charge density at the surface of the beams and use the Debye-Hückel approximation [73] to compute the electrostatic energy (Fig. C.2)

$$E_{\text{DH}}(\theta) = c \int_0^{L_{\text{beam}}} \int_0^{L_{\text{beam}}} \frac{e^{-r_{q_1,q_2}/l_{\text{Debye}}}}{r_{q_1,q_2}} \, dq_1 \, dq_2$$

where $c$ is a constant that accounts for the charge density, $r_{q_1,q_2}$ is the distance between two point charges, $q_1$ and $q_2$, on opposing beams, and $l_{\text{Debye}} \approx 1.6 \text{ nm}$ is the Debye length at our experimental conditions (10 mM MgCl$_2$). We fitted the effective spring constant $k_{\text{eff}}$, the equilibrium length $L_0$, and the constant $c$ of our model to the angle distribution of our spectrometer using maximum likelihood estimation (Fig. 2.1B and Fig. C.2). The fitted model describes the angle distribution with an effective spring constant of $k_{\text{eff}} \approx 17 \pm 1 \text{ pNnm}^{-1}$, a equilibrium length of $L_0 \approx 5.37 \pm 0.02 \text{ nm}$, and an electrostatic repulsion constant of $c \approx 0.02 \pm 0.01 \text{ nm} k_B T C^{-2}$. The fitted equilibrium length is comparable to the expected end-to-end distance of a 30-bases-long single-stranded DNA molecule (6.7 nm, using the end-to-end distance distribution from reference [59]), whereas the effective stiffness is larger than expected for four 30-bases-long single-stranded DNA strands in parallel ($k \approx 0.8 \text{ pN/nm}$, assuming four entropic springs each with a spring constant of $k \approx 3 k_B T/2P L = 0.2 \text{ pN/nm}$ using $P = 1.5 \text{ nm}$ and $L = 19 \text{ nm}$). The latter discrepancy could point to a contribution from the four 2-bases-long hinge connections to the torque or to secondary structures of the 30-bases-long loops that increase the effective stiffness of the entropic spring. In addition, we neglected steric clashes of the top beam with the bottom beam at large opening angles. Finally, an angle-dependent torque that can be altered by changing the length of the single-stranded DNA loops at the hinge has been demonstrated in a similar hinge design [74].

Regardless of origin of the the energy landscape, two interacting molecules placed
on opposing beams of the spectrometer or a molecule that is connected to both beams of the spectrometers will diffuse in the energy landscape generated by the spectrometer. Conformational states or interactions of the target molecules will therefore be biased by the energetic penalty of forcing the spectrometer from an equilibrium angle of $\sim 75^\circ$ to the opening angle of the conformational state. For instance, closing the spectrometer to occupy a bound state with an opening angle of $20^\circ$ will require an energy $\sim 1.5 \text{ kcal/mol}$, whereas closing the spectrometer to occupy a bound state with an opening angle of $50^\circ$ will require an energy of $\sim 0.5 \text{ kcal/mol}$. The spectrometer offers two possibilities to adjust the force bias: the distance from the hinge of the interacting molecules may be adjusted to realize different energetic penalties for forming a bound state, or alternatively the single-stranded DNA springs at the hinge may be shortened or lengthened to carry more or less load respectively. Finally, the position of the fluorescent dyes may be selected to tune the FRET sensitivity according to the range of sampled opening angles (Fig. B.10).

### 2.2 Resolving nucleosome interactions with the force spectrometer

As a first target for our force spectrometer, we studied the interaction between two nucleosome core particles (nucleosomes) as a function of nucleosome-nucleosome distance, relative nucleosome orientation, and nucleosome modification. Nucleosomes are DNA-protein complexes which contribute to the compaction and regulation of genomic DNA [76]. In a nucleosome, 147 base pairs of double-stranded DNA are wrapped in 1.65 turns in a left-handed super helix around a histone protein octamer resulting in a cylindrical-shaped particle with a diameter of $\sim 10.5 \text{ nm}$ and a height of $\sim 4.5 \text{ nm}$ [75, 77–83]. Two copies of each histone protein (H2A, H2B, H3, H4) form the protein octamer core. The nucleosome is stabilized by electrostatic interactions between the positively charged histone proteins and the negatively charged DNA backbones [84]. Multiple nucleosomes that are sequentially positioned on double-stranded DNA, similar to beads on a string, have been shown to assemble into higher-ordered structures with a diameter of $\sim 30 \text{ nm}$ \textit{in vitro} [85–90]. This compaction is believed to be driven by attractive forces between adjacent nucleosomes [85]. N-terminal histone tails protruding from the nucleosome are known to participate in this interaction; for instance, compaction of the chromatin fibre is lost upon histone tail acetylation [89,91–93]. Studies of long ($\sim 2000$ nucleosomes) [94], intermediate ($\sim 30$ nucleosomes) [95], and short ($\sim 12$ nucleosomes) [96] chromatin fibers using optical or magnetic tweezer experiments required extensive modelling [97] to calculate the nucleosome interaction strength and yielded a wide range of interaction energies ($-0.3$ to $-8 \text{ kcal/mol}$ [94–96]). Furthermore, a preferred orientation between adjacent nucleosomes has been suggested on the basis of single-molecule FRET experiments [98] and on cryo
Figure 2.2: Studying nucleosome pair interactions with a DNA origami force spectrometer. (A) Schematic of two nucleosomes (adapted from 3MVD.pdb [75]) with nucleosomal template DNA (yellow), histone protein octamers (blue), and schematic N-terminal histone tails (red spheres). (B) Schematic of the DNA origami force spectrometer with nucleosomes attached at the distal site (31.2 nm away from the hinge). The torque generation of the hinge mechanism is depicted schematically as a red torsional spring. The location of the FRET dyes is indicated by the green (ATTO550) and red (ATTO647N) sphere.
CHAPTER 2. FORCE SPECTROSCOPY USING DNA ORIGAMI

electron microscopy reconstructions [85, 87]. We therefore mounted nucleosomes on the beams of our force spectrometer to study their interaction.

In order to attach two nucleosomes to the force spectrometer, Corinna Lieleg$^3$ and Philip Ketterer$^4$ developed a method to assemble nucleosomes with DNA single-strands that radially protrude at defined positions from the nucleosomal DNA (Fig. C.4). Briefly, a 147-bp-long DNA single-strand is annealed with partly complementary DNA single-strands forming a 147-bp-long DNA double-strand with DNA single-strands protruding from branching points along the DNA backbone. We used a template DNA sequence, the Widom 601 sequence, that is known to yield thermodynamically stable nucleosomes [99]. The nucleosomes were assembled from a histone protein mix and branched template DNA at high salt by gradually reducing the ionic strength of the solution using salt gradient dialysis [100]. The assembled nucleosomes with DNA single-strands were characterized using TEM imaging and native and denaturing gel electrophoresis for correct histone protein stoichiometry and nucleosome stability in the buffer conditions used in the consecutive studies [70]. Finally, we assembled force spectrometers with two DNA single-strands protruding from each beam of the spectrometer that were complementary to the nucleosome single-strand DNA handles. Nucleosome-loaded force spectrometers will thus self-assemble through DNA strand hybridization when mixing the DNA-single-strand-labeled nucleosomes with spectrometers presenting the complementary DNA single-strands (Fig. C.6). This allowed us to place nucleosomes in defined orientations along the beams of the spectrometer.

In the first set of experiments, we placed nucleosomes 15.6 nm (proximal) or 31.2 nm (distal) away from the hinge, collected negative-staining TEM particles (Fig. 2.3, Fig. C.8, and Fig. C.9), and measured opening angles of individual particles (Fig. 2.4). We started with a set of particles that also included spectrometers with less than two or incorrectly attached nucleosomes (Fig. 2.3). In particular, some particles showed nucleosome adhesion to the TEM grid surface resulting in an incorrectly attached nucleosome with disc-shaped appearance. Defective particles occur since all eight DNA single-strands must be present in the right conformation to yield an intact particle and a finite incorporation yield of staple-oligonucleotides has been observed [22]. In order to avoid selection bias, we first randomized particles from different experiments (see following sections), then selected for spectrometer particles with straight beams and two nucleosomes in the correct orientation, and finally de-randomized the particle set to obtain selection-bias-free sets of intact particles (Fig. 2.3, Fig. 2.4, and Fig. C.7).

The angle distributions of the nucleosome-loaded spectrometers shifted with respect

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Figure 2.3: TEM particle selection. Exemplary field of view of nucleosome-loaded force spectrometers in negative-staining TEM (wildtype *Xenopus laevis* nucleosomes). Green check marks: correctly attached nucleosomes, red crosses: incorrectly attached nucleosomes. Scale bars: 50 nm.
Figure 2.4: Proximal versus distal nucleosome placement position analyzed with TEM. (A) Exemplary negative-staining TEM particles of spectrometers with two nucleosomes at the proximal site (15.6 nm away from the hinge). Scale bar: 30 nm. Orange curve: uniform kernel density estimate (bandwidth 3°) of the opening angle distribution from 1301 spectrometer particles with two nucleosomes at the proximal site. Dashed black line: uniform kernel density estimate (bandwidth 3°) of the empty spectrometer (see Fig. 2.1). (B) Same as (A), but with nucleosomes at the distal site (31.2 nm away from the hinge). The uniform kernel density estimate was calculated from 158 particles. Wild type nucleosomes from recombinantly expressed Xenopus laevis histones were used and the nucleosome dyad axis intersect at 78°.
to the empty spectrometer (Fig. 2.4). A new peak occurs at \( \sim 42^\circ \) for the proximal variant and at \( \sim 23^\circ \) for the distal variant corresponding to TEM particles where the nucleosome surfaces are in close contact. The occurrence of a new species indicates the existence of attractive forces between nucleosomes. Assuming a simplified two state model, we estimated that 60% of the particles are in an apparent bound state for the proximal variant, whereas only 8% are in an apparent bound state for the distal variant. This reduced occupancy is due to the increased energy that is required for realizing a smaller opening angle of the force spectrometer. On the basis of these estimated occupancies \( p_c \), the free energy difference between the closed (nucleosomes bound) and open state would correspond to \( \Delta G_{\text{distal}} = -\log(p_c/(1-p_c)) \approx 2.4 \ k_B T \) for the distal variant or \( \Delta G_{\text{proximal}} = -\log(p_c/(1-p_c)) \approx -0.4 \ k_B T \) for the proximal variant. The energetic bias of the spectrometer at the distal site would be greater than at the proximal site \( (\Delta \Delta G \approx 2.8 \ k_B T \approx 1.7 \ \text{kcal/mol}) \). These energetic values are valid as a first approximation, but since the bound state at the proximal variant overlaps considerably with the angle distribution of the empty spectrometer a more sophisticated analysis is required.

As before, we assume identical particles and use Boltzmann statistics to compute the free energy landscape of the nucleosome-loaded spectrometer for the distal and proximal variants from the TEM angle distributions (Fig. 2.5A). The resulting energy landscapes reflect contributions from both the spectrometer and the nucleosome-nucleosome interaction. We need to subtract the free energy landscape of the empty spectrometer from the free energy landscape of the nucleosome-loaded spectrometers to remove the spectrometer’s contribution to the energy landscape and therefore determine the unbiased nucleosome pair potential. To compare the energy landscape of the calibration (of the empty spectrometer) with the energy landscapes of the nucleosome-loaded spectrometers, we need to shift the energy landscapes by a constant \( \ln(Z) \) that depends on the overall shape of the free energy landscape:

\[
\frac{E(\theta)}{k_B T} = -\ln(p(\theta)) - \ln(Z)
\]

The interaction between nucleosomes is expected to vanish for large nucleosome separations. We can use this physical assumption to shift each energy landscape by a constant \( \Delta \) that depends on the shape of the distribution at large nucleosome distances, i.e. larger opening angles:

\[
\frac{E_{\text{nuc}}(\theta)}{k_B T} = -\ln(p_{\text{nuc}}(\theta)) + \Delta
\]

with \( \Delta = \ln \left( \frac{\int_0^{2\pi} p_{\text{nuc}}(\theta) \ d\theta}{\int_0^{2\pi} p_{\text{empty}}(\theta) \ d\theta} \right) \).

where \( p_{\text{empty}} \) is the density estimate of the empty spectrometer, \( p_{\text{nuc}} \) is the density estimate of the nucleosome loaded spectrometer, and \( \theta_0 \) is a cut off at which the nucleosome interaction is expected to vanish. We chose \( \theta_0 = 75^\circ \), which corresponds to a nucleosome-nucleosome distance (center-of-mass to center-of-mass, see below) of \( \approx 15.4 \) nm. Furthermore, we approximated the integrals as sums over the kernel density estimates for the probability densities with an upper integration limit of \( 120^\circ \). Thus, the nucleosome pair potential can be calculated:

\[
E_{\text{pot}}(\theta) = E_{\text{nuc}}(\theta) - E_{\text{empty}}(\theta) = k_BT \left[ -\ln(p_{\text{nuc}}(\theta)) + \Delta + \ln(p_{\text{empty}}(\theta)) \right]
\]

In addition, we developed a molecular model of the two nucleosomes attached on the beams of the spectrometer that allowed us to plot the pair potential versus the center-of-mass (CM) to center-of-mass (CM) distance between two mounted nucleosomes (Fig. 2.5B and Fig. 2.12). The nucleosome-nucleosome interaction shows typical characteristics of pair potentials. The interaction vanishes for large nucleosome-nucleosome distances (\( \gtrsim 12 \) nm), has a minimum of \(-1.4\) kcal/mol at intermediate distances (6.5 nm), and is repulsive for small distances (\( \lesssim 3 \) nm). Indeed, our structural model predicts steric clashes of the nucleosome pair for nucleosome-nucleosome distances smaller than \( \sim 7 \) nm (Fig. 2.12). The long interaction range (5-6 nm) might point at histone-tail mediated interactions, since it matches roughly the contour length of the histone H4 tail (\( \sim 9.5 \) nm assuming 26 amino acids with a length of 0.365 nm per amino acid [101]). Additionally, the equilibrium length of 6-7 nm agrees well with nucleosome separations observed in a tetranucleosome crystal structure (\( \sim 7 \) nm) [85], in tetranucleosome cryo-EM reconstructions (5.4 nm) [87], and in single-molecule FRET studies (\( \sim 6.5 \) nm) [98]. The distal and the proximal variant yield similar nucleosome pair potentials. Given the same number of TEM particles, the proximal variant offers higher angular sampling resolution. We thus used this variant for further experiments.

The orientation of the nucleosomes relative to the force spectrometer is defined by the two attachment handles. It would however be conceivable that the nucleosome might rotate around the axis defined by the two attachment points, which would allow for two possible nucleosome orientations on the spectrometer. We designed the attachment handles to protrude at \( \sim 1 \) nm height from the cylindrical-shaped nucleosome that has a total height of \( \sim 4.5 \) nm, thus biasing the nucleosome to attach in a defined orientation to the spectrometer (Fig. C.6). In order to confirm the correct nucleosome orientation on the force spectrometer, we divided the set of TEM particles of nucleosome-loaded spectrometers into groups of particles with similar opening angle (within a range of 5\(^\circ\)) and calculated average particles of these subsets (Fig. 2.6A). The resulting average micrographs showed
densities at the designed nucleosome positions and matched the dimensions of a nucleosome. The nucleosome configurations predicted by our structural model compare well with the average particle geometry (Fig. 2.6B). In fact, some averaged micrographs resolve more detailed structural features of the nucleosome (subsets with opening angles around 38°, 43°, 48°, and 68°). These nucleosome density features match well to the prediction of our structural model (Fig. 2.6C), confirming the designed nucleosome orientation on the force spectrometer.

We relied on direct imaging of our spectrometers using negative staining TEM for determining the nucleosome pair potentials. In order to corroborate these findings in more native conditions, we performed TEM imaging of empty and nucleosome-loaded spectrometer samples in amorphous ice (cryo-EM). During cryo-EM grid preparation, samples were adsorbed on a thin charged carbon film to yield particles in the side-view configuration, and frozen rapidly using liquid nitrogen to trap the spectrometer particles in their current conformational state. Not considering the inverted contrast, cryo-EM particles have similar appearance to negative-stained particles (Fig. 2.7 and Fig.2.8). The force spectrometer produces enough contrast that the typical pattern due to the DNA honeycomb lattice is well visible in single particles. In contrast, the nucleosome density has much reduced contrast in cryo-EM particles. Selecting intact particles, that is, spectrometer particles with two successfully attached nucleosomes presents a greater difficulty (Fig. C.7).
Figure 2.6: Comparison of structural nucleosome-positioning model to average TEM particles. (A) Top row: average TEM micrograph of nucleosome-loaded spectrometer particles that had an opening angle within a window of 5° centred around the indicated angle. Scale bar: 25 nm. Bottom row: magnified areas of micrographs in top row. Scale bar: 10 nm. (B) Top row: nucleosome configuration computed with our structural nucleosome-positioning model. Yellow: nucleosomal DNA, blue: histone proteins. Nucleosome structure adapted from 3MVD.pdb [75]. Bottom row: overlay of structural model and average TEM micrographs from bottom row in (A). Scale bar: 10 nm. (C) Overlays of structural model and average TEM micrograph for an opening angle of 38°. Opacity from structural model from left to right: 0%, 40%, and 100%. Scale bar: 5 nm
Despite this difficulty, we collected 1704 cryo-EM particles of the empty spectrometer and 179 cryo-EM particles of the nucleosome-loaded spectrometer and estimated their angle distributions (Fig. 2.7 and Fig. 2.9A,B). Empty spectrometers from cryo-EM and negative-staining TEM sample similar distributions that peak at a similar opening angles ($70^\circ$-$80^\circ$). Intermediate opening angles ($20^\circ$-$60^\circ$) are however less frequently observed for the cryo-EM data set, which suggest a decreased energetic bias in negative-staining TEM with respect to cryo-EM. The cryo-EM opening-angle data of the nucleosome-loaded spectrometers indicate similar deviations from the negative-staining distribution. Specifically, the cryo-EM distribution has a maximum at $\sim 62^\circ$, whereas the negative-staining angle distribution peaks at $\sim 42^\circ$. An additional constant bias of the force spectrometer in cryo-EM would cancel out, if empty and nucleosome-loaded spectrometer data are compared. We therefore computed the free energy landscapes for the cryo-EM distributions (Fig. 2.9C) and determined the nucleosome pair potential by subtracting the energy landscape of the empty spectrometer from the energy landscape of the nucleosome-loaded spectrometer. The resulting cryo-EM nucleosome pair potential resembles the nucleosome pair potential determined from negative-staining TEM. The reduced interaction strength ($-1.2$ kcal/mol versus $-1.4$ kcal/mol) could be due to incorrectly selected particles, since intact particles are difficult to identify in cryo-EM, or due to the small sample-size of the cryo-EM data set. The negative-staining procedure therefore alters the energy landscape of the force spectrometer.
Figure 2.8: Comparison of single particles from negative-staining TEM and from cryo-EM. Exemplary cryo-EM (top rows) and negative-staining TEM (bottom rows) single-particle micrographs with different opening angles of empty spectrometers (A) and of nucleosome-loaded spectrometers (wild type nucleosomes from *Xenopus laevis*) (B). Scale bars: 50 nm.
Figure 2.9: Quantitative comparison of negative-staining versus cryo-EM angle distributions and energy landscapes. (A) Uniform kernel density estimates (bandwidth $3^\circ$) of empty-spectrometer angle distributions in negative-staining TEM (red line, 3091 particles) or in cryo-EM (blue line, from 1704 particles). (B) Uniform kernel density estimates (bandwidth $3^\circ$) of nucleosome-loaded-spectrometer angle distributions in negative-staining TEM (red line, 1301 particles) or in cryo-EM (blue line, from 179 particles). Wild type nucleosomes from Xenopus laevis were used. Cryo-EM data is shown in blue. Negative-staining TEM data is shown in red. (C) Free energy landscapes derived from the angle distributions shown in (A) and (B). Dashed lines: empty spectrometer, solid lines: nucleosome-loaded spectrometer. (C) Nucleosome pair potential derived from the energy landscapes shown in (C).
spectrometer, but does not affect the nucleosome-nucleosome interaction. The change in angle occupancies can be accounted for using a spectrometer-only calibration acquired at the same experimental conditions as the sample-loaded spectrometers.

Direct imaging using TEM allows counting the frequencies with which single particles occupy different opening-angle states, thus enabling to infer the underlying energy landscapes. Since this method requires adsorbing particles to the TEM-grid surface, a complementary solution-based experiment that confirms the attractive nucleosome interactions in a solution-based environment is desirable. Previously, we mounted two fluorescent dye molecules on the beams of the positioning apparatus and used the changes in the dye’s fluorescence intensities to validate the structure of our positioning apparatus. These experiments exploited the sensitivity of the dye’s fluorescence brightness to the conformational state of the positioning apparatus. The fluorescence brightness of a FRET dye pair mounted on opposing beams of the force spectrometer will therefore report on the opening angle of the spectrometer. We chose to mount fluorescent dyes 15 bp away from the hinge, since this position offers angular sensitivity starting from $\sim 20^\circ$ to up to $\sim 90^\circ$ (Fig. B.10).

Finally, we used the previously introduced method that combines gel electrophoresis with fluorescence imaging to determine the FRET efficiency of the monomer species in solution (Fig. 2.10 and chapter A).

Empty spectrometers without attachment handles had slightly larger FRET efficiencies than empty spectrometers with attachment handles (0.36 versus 0.34), which might be due to the additional charge or volume of the protruding DNA single-strands (Fig. 2.10). Spectrometers with one nucleosome at the proximal site had an increased FRET efficiency of 0.39 in comparison to the empty spectrometer. This could point to a weak interaction of the positively charged histone proteins with the negatively charged surface of the spectrometer. The FRET efficiency of the spectrometer with one nucleosome depended slightly on the attachment site (top beam versus bottom beam), which might indicate different nucleosome attachment yields. Two attached nucleosomes give an even higher FRET efficiency than spectrometers with one attached nucleosome (0.45), confirming the attractive interaction between two nucleosomes in solution. Nucleosomes attached at the distal position yielded similar trends: a slight FRET efficiency increase when attaching one nucleosome, but a larger increase in FRET efficiency if two nucleosomes are attached. The overall FRET efficiency of the distal variant with two nucleosomes is smaller (0.39) than the FRET efficiency of the proximal variant with two nucleosomes (0.45). Although the distal variant should give a higher FRET efficiency, because of the smaller opening angle of the bound state, the amount of spectrometers in the bound state will be smaller for the distal variant (8% versus 60%), because forming a bound state requires a larger energetic penalty. Finally, cryo- and negative-staining TEM imaging showed that a considerable fraction of particles were defective, i.e. did not contain two attached nucleosomes.
Figure 2.10: Proximal versus distal nucleosome placement position analyzed with ensemble FRET. Top: pixelated FRET efficiency calculated from laser-scanned images for an area of an exemplary agarose gel on which nucleosome-loaded spectrometer variants (indicated by icon) were electrophoresed (see chapter A for data-analysis method). Bands correspond to monomeric spectrometer species. * = empty spectrometer with single-stranded DNA attachment handles. Bottom: mean and standard deviation of monomer band FRET efficiencies from 5 independent gel-electrophoresis experiments. Wild type nucleosome from recombinantly expressed Xenopus laevis histones were used and the nucleosome dyad axis intersect at 78°.

in the correct configuration. The ensemble FRET signal obtained from the spectrometer monomer band is therefore a population average over both intact and defect spectrometer species as well as a conformational average over the angular states sampled by a spectrometer species. Nonetheless, the results of the ensemble FRET based assay are in qualitative agreement with the data collected with the TEM-based assay and support the quantitative TEM-based pair potentials.

2.3 Orientation- and modification-dependent nucleosome pair potentials

Relative nucleosome orientation

The force spectrometer enables us to control the nucleosome position (proximal and distal) and the nucleosome orientation. We studied a sample where we rotated the bottom nucleosome by 180° by interchanging the DNA handles that protrude from the bottom beam of the spectrometer, yielding a 258° angle between the nucleosome’s dyad axes. Single-particle TEM statistics yield an angle distribution that resembles the angle distribution of the non-rotated variant, whereas the gel-based fluorescence essay yields a slightly reduced FRET efficiency for the rotated variant (0.45 versus 0.43) (Fig. 2.11A,D). We used the TEM angle distributions to compute nucleosome pair potentials for the two nucleosome orientations tested (Fig. 2.13A). The two nucleosome orientations yield similar
A nucleosome interacts with other nucleosomes depending on the orientation of the nucleosome and its modifications. (A-C) Uniform kernel density estimates (bandwidth $3^\circ$) of opening angle distributions from spectrometer particles with two nucleosomes at the proximal site. Dashed black line: uniform kernel density estimates (bandwidth $3^\circ$) of the empty spectrometer (Fig. 2.1). (A) The bottom nucleosome was rotated by $180^\circ$ with respect to the previous measurements (Fig. 2.4) yielding an angle between the nucleosome dyad axis of $258^\circ$. Recombinantly expressed wild type Xenopus laevis histones were used to assemble nucleosomes. Angle distribution estimated from 979 particles. (B) Angle distribution estimated from 818 particles. Recombinantly expressed Xenopus laevis histones lacking N-terminal histone tails were used. (C) Angle distribution estimated from 846 particles. Recombinantly expressed Xenopus laevis histones were used to assemble nucleosomes and assembled nucleosomes were treated with MOF acetyltransferase which mainly acetylates lysine 16 in the H4 tail. (D) Top: pixelated FRET efficiency calculated from laser-scanned images for an area of an exemplary agarose gel on which nucleosome-loaded spectrometers with different nucleosome variants (indicated by icon as in (A-C)) and dyad axis orientation were electrophoresed. Bands correspond to monomeric spectrometer species. Bottom: mean and standard deviation of monomer band FRET efficiencies from 5 independent gel-electrophoresis experiments.
pair potentials, that differ slightly in the location and in the depth of the minimum. The rotated variant (258° between the dyad axes) exhibits a minimum at ~7 nm with a depth of −1.6 kcal/mol, whereas the non-rotated variant (78° between the dyad axes) exhibited a minimum at ~6 nm with a depth of −1.4 kcal/mol. Our structural model of the nucleosomes on the beams of the spectrometer predicts the onset of steric clashes for nucleosome distances from 6.7 nm for the non-rotated variant and from 6.9 nm for the rotated variant. Because of the structural deviations of the nucleosomes from a perfect cylindrical-shaped particle (Fig. 2.12B, C), differences in the onset of steric clashes could lead to a shift in the minimum of the pair potential.

**Nucleosome modifications**

N-terminal histone tails have been shown to contribute to interactions between nucleosomes, specifically the H4 tail is believed to mediate attractive interactions. In particular, acetylation of the lysine 16 in the H4 tail has been shown to reduce chromatin compaction [89, 91–93]. We therefore used our DNA origami force spectrometer to study quantitatively the contribution of H4-K16 acetylation and of histone tail removal on the nucleosome pair potential. We assembled tail-less nucleosomes from recombinantly expressed histones from *Xenopus laevis* that lacked N-terminal tails and acetylated wild type histones from *Xenopus laevis* using MOF acetyltransferase, which is largely specific for K16 in H4 [102]. We analyzed these samples using angle distributions from negative-staining TEM (Fig. 2.11B,C) and gel-based ensemble FRET efficiencies (Fig. 2.11D). Angle distributions revealed a strongly reduced frequency of particles corresponding to the bound state for the tail-less variant and reduced occupancies of the bound state for
the variant with acetylated H4-K16 with respect to the wild type nucleosome samples. In agreement with these distributions, the FRET efficiency of the tail-less variant was also strongly reduced (0.33), whereas the FRET efficiency of the acetylated variant was reduced to 0.39 with respect to the wild type nucleosome variant (0.44). We calculated the nucleosome pair potentials for all nucleosome variants (Fig. 2.13B). Removing the N-terminal histone tails reduced interaction strength to $-0.4 \text{kcal/mol}$, indicating the importance of the histone tails in mediating the attractive forces or shielding repulsive forces. Interestingly, acetylation of the H4-K16 reduced the interaction strength to a similar level ($-0.6 \text{kcal/mol}$) while maintaining a larger range than the tail-less variant. This finding corroborates the role of the H4 tail in mediating the attractive forces between nucleosomes and quantifies the free energy difference between acetylated and modification-free nucleosomes.

### 2.4 Implications of the pair potential for chromatin

The structure and dynamics of chromatin are much debated [103–107]. Defined structures have been observed only in vitro using for instance electron microscopy [87], X-ray crystallography [85], or crosslinking studies [88]. In the absence of other protein co-factors, a chromatin structure and its dynamics would depend on the attractive nucleosome interactions that we quantified, and the mechanical properties of the linker-DNA (double-stranded DNA stretch between two nucleosomes). Based on our pair potentials, the energy gained from forming one nucleosome stack could for instance balance a 70° bend or up to 50° twist in a 47-bp-long linker DNA [108]. We estimated the stiffness of one nucleosome stack by fitting a harmonic potential to the minimum of the nucleosome pair potential. The fitted spring constant of $1.2 \text{pN/\text{nm}}$ compares well to mechanical stretching experiments of long chromatin fibers ($0.5 \text{pN/\text{nm}} - 2 \text{pN/\text{nm}}$). Based on this stiffness, we would expect root mean squared fluctuations of $1.8 \text{nm}$ in the nucleosome-nucleosome distance coordinate. If the exact shape of the potential is considered these fluctuations might be even larger ($\sim 3 \text{nm}$). In the context of a structured chromatin fiber however, a stiff DNA scaffold might reduce these fluctuations. Taken together, our findings would favour a more dynamic and more liquid-like chromatin organization [109].

### 2.5 Summary

We introduced a DNA origami force spectrometer that provided us positional control to constrain the motion of two nucleosomes along a defined arc-shaped coordinate, to control their orientations, and to read out the frequency of adopted nucleosome distances. Single-stranded DNA elements at the hinge of the spectrometer serve as weak entropic
Figure 2.13: Quantifying the influence of nucleosome orientation and nucleosome modifications on the nucleosome interaction. (A) Left: free energy landscapes derived from uniform (thin lines) and normal (thick lines) kernel density estimates (bandwidth 3°) of the angle distributions from empty spectrometers (black) and nucleosome-loaded spectrometers with wildtype nucleosomes from Xenopus laevis in two orientations (red: dyad axes intersect at 78°, blue: dyad axes intersect at 258°). Right: nucleosome pair potentials derived from energy landscapes shown on the left. (B) Free energy landscapes (left) and nucleosome pair potentials (right) as in (A) for nucleosomes from Xenopus laevis whose dyad axes intersect at 78°, but which were modified. Red lines: wildtype nucleosomes; cyan lines: wild type nucleosomes that were treated with MOF acetyltransferase, which is largely specific for the K16 in histone H4; orange lines: nucleosomes from histones lacking N-terminal tails. Dashed black lines in pair potentials indicate a fit of the data to a quadratic potential.
springs that counterbalance the attractive forces between the nucleosomes. In order to quantify an interaction strength, both the bound and unbound states need to be observed. In the case of nucleosome interactions, the energetic bias of the force spectrometer was in a range where both states were visited by the system. In general however, biomolecules might exhibit much stronger or weaker interactions. The energetic bias of the force spectrometer may be adjusted accordingly by either placing the interaction partners closer or farther away from the hinge, or by shortening or lengthening the single-stranded DNA loops at the hinge. We calibrated the force bias of our spectrometer and determined the distance-dependent nucleosome pair potential. The negative-staining TEM findings were reproduced quantitatively with cryo-EM measurements and qualitatively with ensemble FRET measurements.

We have applied our force spectrometer to quantify the distance- and orientation-dependent interactions between wild type, tail-less, and acetylated nucleosomes. We found a weak (−1.4 to −1.6 kcal/mol) but long-ranged (~6 nm) interaction which did not change strongly when changing the nucleosome orientation by 180°. Upon removing the histone tails, the interaction strength was decreased by a factor of 3.5, demonstrating the importance of the N-terminal histone tails for mediating the attractive interactions. Specifically, the H4 tail carries most of the interaction, as acetylation of its N-terminal tail at amino acid K16 reduces the interaction strength by a similar amount (a factor of 2.3). These quantified nucleosome pair potentials may for instance be used as input potentials or benchmarks for chromatin simulations [110] and suggest a dynamic chromatin organization, because thermal fluctuations might disrupt the weak nucleosome-nucleosome attraction and no strong orientational bias was found.

We envision our force spectrometer to contribute resolving other weak biomolecular interactions, because it offers a modifiable platform for attaching other biomolecules, a calibrated TEM and FRET readout, and an adjustable force bias. Future experimental targets could include DNA hybridization, hairpin formation in single-stranded DNA or RNA molecules, protein conformation studies, and structure determination under load.
Chapter 3

Quantifying conformational equilibria using DNA origami

In the previous chapter (chapter 2), we have introduced a DNA origami force spectrometer and used it to study the interactions between two nucleosome core particles (nucleosomes) as a function of an internucleosomal distance coordinate. In this chapter, we will use the force spectrometer to study the salt-induced nucleosome-disassembly transition, where the state of the force spectrometer reports on an intranucleosomal extension coordinate. By measuring changes of the spectrometer’s opening-angle frequencies with TEM imaging and FRET spectroscopy, we will demonstrate the capabilities of the force spectrometer to follow and to report on a biomolecule’s conformational state.

Remark: parts of this chapter have been published [111] and the research was done in close collaboration with Philip Ketterer\textsuperscript{1}, Corinna Lieleg\textsuperscript{2}, Philipp Korber\textsuperscript{2} and Hendrik Dietz\textsuperscript{3}. In particular, Corinna Lieleg assembled, tested, and modified nucleosomes under the supervision of Philipp Korber. Philip Ketterer and Corinna Lieleg developed nucleosomes with single-stranded DNA overhangs. Nucleosomes assembled from Drosophila embryo histones were used in the experiments presented in this chapter.

3.1 Resolving nucleosome unwrapping with the force spectrometer

As described before (chapter 2), nucleosomes are protein-DNA complexes, in which 147 base pairs of double-stranded DNA are wrapped in 1.65 turns in a left-handed super helix around a histone protein octamer resulting in a cylindrical-shaped particle with a diameter of $\sim 10.5\ \text{nm}$ and a height of $\sim 4.5\ \text{nm}$. Two copies of each histone protein (H2A, H2B, H3,
H4) form the protein octamer [75, 77–83]. The nucleosome is stabilized by electrostatic interactions between the positively charged histone proteins and the negatively charged DNA backbone [84, 112]. In vitro, the nucleosome can therefore be reconstituted by salt-gradient dialysis, i.e. gradually reducing the NaCl concentration from 2 M to 50 mM [100]. The nucleosome assembly proceeds in three major steps during the salt-gradient dialysis [77, 113–115]: at \(\sim 1\) M NaCl, H3-H4 dimers associate to tetrasomes \((\text{H3-H4})_2\) that bind to the nucleosomal DNA; at \(\sim 0.8\) M NaCl, H2A-H2B dimers associate to the tetrasome forming a partly wrapped nucleosome; at \(\sim 0.6\) M, the nucleosome relaxes into its final wrapped state. The disassembly process has been shown to follow the reverse pathway [113]. Thus, starting from an assembled nucleosome where the nucleosomal DNA is wrapped completely around the histone core, we expect the nucleosomal DNA to unwrap from the histone core as the ionic strength of the solution is increases.

### Mounting multiple nucleosomes on the spectrometer

To monitor the salt-induced nucleosome unwrapping transition, we mounted nucleosomes between the beams of the spectrometer such that the opening angle of the force spectrometer reports on the conformational state of a nucleosomal DNA segment (Fig. 3.1A). To assemble spectrometers with two-point attached nucleosomes, we first assembled nucleosomes with two single-stranded DNA handles protruding at two distinct sites radially from the nucleosomal DNA (see chapter 2 and Fig. C.4) and then mixed these single-strand labeled nucleosomes with spectrometers that displayed complementary single-stranded DNA handles (Fig. 3.1B). The branching points of the nucleosomal template DNA therefore determine the nucleosomal arc-segment on which the spectrometer reports. Our spectrometer provides four parallel mounting helices on each beam, therefore allowing us to mount up to four nucleosomes in parallel; or in total \(2^4\) different combinations, as each nucleosome attachment site can be activated (by displaying the single-stranded DNA handles) or can be deactivated (by not displaying single-stranded DNA handles). We designed each nucleosome attachment site to display a distinct sequence and assembled nucleosomes with complementary sequences to ensure site-specific nucleosome attachment.

We imaged spectrometers with mounted nucleosomes in different configurations using negative-staining TEM and performed 2D classification of single-particles (Fig. 3.1C). In top-view class averages, nucleosome density is apparent at positions where the designed nucleosome attachment sites are located and side-view class averages show disc-shaped densities with nucleosome dimensions at the designed nucleosomes attachment sites. In addition, the brightness of the nucleosome density correlates with the number of parallel attached nucleosomes (Fig. 3.1D). These data thus demonstrates the site-specific attachment of nucleosomes into the force spectrometer. In principle, the attachment should allow the nucleosomes to rotate around two attachment sites at the opposing beams. Interest-
Figure 3.1: Mounting nucleosomes between the beams of the force spectrometer. (A) Schematic of the force spectrometer with mounted nucleosomes at two out of four possible positions as indicated by the icon (solid element = nucleosome attachment site) (B) Schematic illustration of the nucleosome attachment. Left: Spectrometer with single-stranded DNA attachment handles protruding from both beams. Hinge flexibility is indicated by the blur of the top beam. Right: Spectrometer with attached nucleosome. (C) Schematic in top view, average TEM micrograph in top view, and average TEM micrograph in side view from spectrometers with multiple mounted nucleosomes. Designed nucleosome attachment-sites are indicated by the icons (solid element = nucleosome attachment site). White arrows indicate density corresponding to nucleosomes. NaCl was added to a final concentration of 500 mM immediately prior to TEM grid preparation to obtain single-particles in top-view. Scale bar: 25 nm (D) Areas of side-view TEM averages shown in (C) highlighting the nucleosome density. Grayscale levels were adjusted for equal brightness of the spectrometer beams. Scale bar: 10 nm
ingly, we find an asymmetric nucleosome density for the spectrometer variant with one nucleosome (Fig. 3.1D left, reduced density top-left arc segment), indicating a preferred orientation of the nucleosome in the spectrometer. These asymmetric features vanish however if more than one nucleosome is attached, indicating randomly oriented nucleosomes if more than one nucleosome is attached.

Salt-induced nucleosome unwrapping studied with TEM

As described above, increasing the ionic strength of the solution will destabilize the interaction between histone proteins and nucleosomal DNA and thereby inducing the unwrapping transition (Fig. 3.2A). We therefore incubated nucleosome-loaded spectrometers with different amounts of additional NaCl and imaged these samples with negative-staining TEM (Fig. 3.2B, see Fig. D.3 and Fig. D.4 for exemplary field of views at different concentrations of NaCl and with different nucleosome configurations). Indeed, the number of spectrometer particles with disc-shapes nucleosome densities decreased with increasing concentration of NaCl. Density corresponding to correctly mounted nucleosomes was discernible in single-particle micrographs. Thus allowing us to divide our data set of TEM particles into classes with or without a correctly mounted nucleosome (Fig. 3.2B). The fraction of spectrometer particles with a correctly mounted nucleosome was similar without additional NaCl and with 0.5 M additional NaCl (52% and 58%, respectively), but decreased when 1.0 M NaCl was added (32%) and vanished when 1.5 M NaCl was added (5%). Incorporation yields of nucleosomes have been small for this set of experiments, presumably due to a lack of nucleosomes in solutions, as indicated by the lack of free nucleosomes on the TEM grid. Indeed, spectrometers with more than one nucleosome mounting site and larger nucleosome excess exhibited higher incorporation yields, approaching ~100% (Fig. D.4).

Furthermore, the ability to differentiate between spectrometers with attached nucleosomes and spectrometers without attached nucleosomes enabled us to calculate angle distributions for each set of TEM particles (Fig. 3.2C). Without additional NaCl, the opening-angle distribution of spectrometers without nucleosomes resembles the opening-angle distribution of the empty spectrometer (see chapter 2), whereas the angle distributions of spectrometers with nucleosomes shows a broad maximum at 45°-55°. Assuming a length of 14.5 nm between the backbone attachment sites on the beams of the spectrometer (nucleosome diameter of 10.5 nm and two DNA double helices, each 2 nm), we would expect an opening angle of 55°. 2D classification of all TEM particles yield classes with elliptical shaped nucleosome densities at small opening angles (≤ 52°) and circular shaped nucleosome densities at larger opening angles (≥ 52°) (Fig. 3.3), indicating that the nucleosome attachment with two DNA-double-helix linkers provides enough flexibility to the nucleosome to assume tilted orientations. The frequency of particles with smaller
Figure 3.2: Salt-induced nucleosome unwrapping studied with TEM. (A) Schematic of the nucleosome disassembly induced conformational change of the spectrometer. Left: Spectrometer with mounted and wrapped nucleosome 15.6 nm away from the hinge. Colored stars indicate FRET-pair dye positions 5.4 nm away from the hinge (D=ATTO550, A=ATTO647N). Right: Spectrometer after salt-induced nucleosome disassembly. The histone octamer (blue) dissociated from the nucleosome DNA (yellow). (B) Exemplary negative-staining TEM field of views of spectrometers with one mounted nucleosome at the site indicated by the icon (solid element = activated nucleosome attachment site) at two concentrations of additional NaCl. Green circles indicate nucleosomes with correctly mounted nucleosomes. Scale bar: 100 nm. (C) Uniform kernel density estimates (bandwidth 3°) of opening-angle distributions of spectrometers with (green) or without (red) correctly mounted nucleosomes. (D) Exemplary negative-staining TEM class averages of spectrometers and magnified subareas for 0.0 M, 0.5 M, 1.0 M, and 1.5 M additional NaCl (from left to right). Circles indicate nucleosome dimensions (diameter of 10.5 nm). Scale bars: 10 nm.
angles ($\lesssim 50^\circ$) decreases upon addition of 0.5 M NaCl, resulting in a more narrow angle distribution with a maximum at 55°. We hypothesize that increased interactions with the TEM-grid surface at increased salt concentrations force the nucleosome into a flat orientation. The amount of spectrometer particles with a visible nucleosome decreases at 1.0 M additional NaCl, whereas the amount of spectrometer particles lacking a nucleosome increases. Especially the frequency of spectrometer particles with an opening angle of 60°-70° increases in the population of particles without a nucleosome. At 1.5 M additional NaCl, the particles without nucleosomes sample a broad distribution with a maximum at 77°. As the nucleosome unwraps, a 41 bp stretch of the nucleosomal DNA changes from a bend into an extended state and the distance between the backbone attachment sites on the beams of the spectrometer should increase to 17.7 nm (41 bp stretch of double-stranded DNA, assuming a rise per base pair of 0.335 nm, and two DNA duplex linkers, each 2 nm in diameter). According to our geometric model of the spectrometer, a 17.7 nm distance in the sample coordinate would result in an opening angle of 69°. Finally, we do not find particles with correctly attached nucleosomes having large opening angles ($\gtrsim 90^\circ$) at NaCl concentrations from 0.0 M-0.5 M, which indicates that the force bias of the spectrometer is too small to unwrap the nucleosome at these solution conditions.

2D classification of the complete set of single-particles yielded classes with and without disc-shaped nucleosome features. Corroborating the opening-angle distributions, the amount of particles that fall into a class without nucleosome features increases with the concentration of additional NaCl (Fig. 3.3 and Fig. 3.2D). Whenever nucleosome density was visible, the density was disc-shaped, indicating a one-step transition within the resolution provided by the data.

Salt-induced nucleosome unwrapping studied with FRET spectroscopy

In the previous section we have established that the state of the force spectrometer, specifically its opening angle as measured using direct TEM imaging, reports on the conformational state of the mounted nucleosomes. Alternatively to TEM, the opening angle can be measured using FRET dyes that we mounted on the beams of the force spectrometer (Fig. 3.2A). We placed the FRET dye pair 5.4 nm away from the hinge, thereby obtaining a change in FRET efficiency upon nucleosome unwrapping from $\sim$0.6 to $\sim$0.4. To study the salt-induced nucleosome unwrapping in solution with our spectrometer, we incubated nucleosome-loaded spectrometers with different amounts of additional NaCl at 25° and analyzed these incubations using our gel-based FRET method (chapter A). Our gel-based FRET method allowed us to compare electrophoretic mobility and FRET efficiency in one stroke and at identical solution conditions among samples that were previously subjected to different concentrations of NaCl. Low temperature (15°-20°) and low-salt gel-electrophoresis conditions inhibit nucleosome dissociation, whereas sample dilution
Figure 3.3: Classification of spectrometer particles at different salt concentrations. (A) Reference-free negative-staining TEM classes from spectrometers with nucleosome configuration indicated by the icon. Nucleosomes were attached to spectrometers followed by incubation at 11 mM MgCl₂ and different concentrations of NaCl. Classification was performed with the complete set of particles, regardless of visible nucleosome density. Scale bar: 50 nm. (B) Magnified and brightness adjusted areas of corresponding class averages from (A). Scale bar: 10 nm.
during gel electrophoreses prevents de novo nucleosome formation. Prior to incubation with NaCl, we expect samples to consist of two spectrometer species: spectrometers with nucleosomes bridging the beams of the spectrometer (closed species), or spectrometers without nucleosomes bridging the beams of the spectrometer (empty species). The empty species may consist of empty spectrometers, spectrometers where one nucleosome is bound to each beam, or spectrometers where a nucleosome is bound to only one beam. After incubation with NaCl, histone proteins may have dissociated from the nucleosomes, inducing the unwrapping of the nucleosomal DNA, and we expect samples to consist of three spectrometer species: spectrometers with nucleosomes bridging the beams of the spectrometer as before (closed species), spectrometers without nucleosomes bridging the beams of the spectrometer (empty species), or spectrometers with unwrapped nucleosome DNA bridging the beams of the spectrometer (open species). We can track quantitatively the fraction of wrapped nucleosomes in solution by comparing the FRET efficiency of a given sample to the FRET efficiencies of samples with known conformation (closed ≈ 0.6, open ≈ 0.4, and open/empty ≈ 0.36).

We conducted two sets of experiments: in the first set of experiments, we analyzed a spectrometer sample with a particular nucleosome arrangement that was incubated at different concentrations of additional NaCl on the same gel (salt screen) (Fig. 3.4A); whereas in the second set of experiments, we analyzed spectrometer samples of all possible nucleosome arrangements that were incubated at the same concentration of additional NaCl on the same gel (nucleosome-configuration screen) (Fig. 3.4B).

For the first set of experiments, we assembled spectrometers with nucleosomes in different configurations, added NaCl from 0 M to 2.5 M, and incubated the samples for 1-3 days at room temperature. Gel electrophoresis of spectrometer variants with one nucleosome at low NaCl concentrations yield to overlapping bands with different FRET efficiencies (Fig. 3.4A, top gel). The leading band vanishes (at ~0.5 M additional NaCl) as the ionic strength of the incubation increases, identifying the faster migrating species as spectrometers with wrapped nucleosomes bridging both beams of the spectrometer (closed species). Spectrometers without a bridging nucleosome and spectrometers with nucleosomal DNA bridging the beams exhibit smaller electrophoretic mobility, presumably because of the increased spectrometer cross section. Spectrometers with more than one active nucleosome attachment site show unwrapping transitions at 0.9 M-1.1 M additional NaCl and less spectrometers without a bridging element (empty species), since the spectrometer will appear closed as long as one nucleosome is bridging the spectrometer (Fig. 3.4A).

In the second set of experiments, we assembled spectrometers with all nucleosome configurations, incubated these samples at salt concentrations where the unwrapping transition occurs (0.9 M, 1.0 M, and 1.1 M additional NaCl), and analyzed the reactions using the gel-based FRET assay (Fig. 3.4B). Separating the closed and open conformation of the
Figure 3.4: Salt-induced nucleosome unwrapping studied with gel electrophoresis and FRET spectroscopy. (A) FRET image calculated from laser-scanned images of 3% agarose gels on which nucleosome-loaded spectrometers (nucleosome configuration indicated by the icons) were electrophoresed for ~7 h at 70 V (see chapter A for FRET image calculation). Nucleosome-loaded spectrometers were incubated for 1-3 days with additional NaCl, as indicated by the numbers (0.0 M - 2.5 M). P: gel pocket; D: spectrometer dimer band; c: spectrometer with assembled nucleosome; o: spectrometer with nucleosomal DNA bridging the two beams or empty spectrometer. (B) FRET image calculated from laser-scanned images of 2% agarose gels on which nucleosome-loaded spectrometers (nucleosome configuration indicated by the icons and numbers 1-4) were electrophoresed for 2.5 h at 70 V (see chapter A for FRET image calculation). Nucleosome-loaded spectrometers were incubated for 1-3 days with additional NaCl (0.9 M, 1.0 M, or 1.1 M). Band description as in (A).
spectrometer in the previous gel-electrophoresis experiments required using high agarose concentrations (3%) and long electrophoresis times (∼7 h). Since increased agarose concentrations typically yield aggregates in the gel pockets, and since extended electrophoresis might cause shifts in the equilibrium distribution, gel electrophoresis experiments were performed on 2% agarose gels for 2.5 h yielding less separated species. However, the FRET signal reports on the distribution of closed versus open (or empty) spectrometers. For all three NaCl concentrations tested, FRET efficiency increased as the number of nucleosomes in the spectrometer increased, whereas the specific nucleosome configurations had little influence.

3.2 Quantifying nucleosome-unwrapping equilibria

To evaluate the gel-based FRET experiments quantitatively, we determined the FRET efficiency of each spectrometer monomer band, averaging closed and open (or empty) species (see chapter A). Since the measured FRET efficiency is an average of the three species mentioned above, different analysis methods, depending on the type of the experiment, are required to correct for FRET signals from defect objects (empty species).

Correction of FRET efficiencies for salt-titration experiments with the same nucleosome configuration

To compute the fraction of closed spectrometer particles $p_c$ from experiments where different amounts of salt was added to the same nucleosome configuration, we subtracted the FRET efficiency at high ionic strength $E_o$, where the nucleosome is disassembled (open and empty species) and normalized to values at low ionic strength, where the nucleosome is assembled $E_c$ (closed species). For each band, the fraction of closed particles in solution is

$$p_c = \frac{E - E_o}{E_c - E_o}$$

with $E_o = E(2 \text{ M NaCl})$ and $E_c = (E(0 \text{ M NaCl}) + E(0.1 \text{ M NaCl}))/2$. Empty spectrometers yield FRET efficiencies of 0.36, spectrometers with nucleosomal DNA bridging the beams yield FRET efficiencies of 0.4, and spectrometer with wrapped nucleosomes bridging the beams yield FRET efficiencies of 0.6.

Correction of FRET efficiencies for nucleosome-configuration experiments at constant additional NaCl

To evaluate experiments where we added the same amount of salt to samples with different nucleosome configurations, we need to account for a finite occupation probability of each
nucleosome attachment site. The measured FRET efficiency \( \hat{E} \) for each nucleosome configuration results from objects with correctly incorporated nucleosomes and from objects with unoccupied nucleosome binding sites. Each designed nucleosome attachment site can be occupied or unoccupied because of defect spectrometers or nucleosomes. For example, the measured FRET efficiency for the variant with one nucleosome at the right-most site (variant 0001, we label the variant using four binary numbers, where the location of the number indicates the nucleosome attachment site (see icons in figures) and 0 corresponds to no bridging element present, whereas 1 corresponds to a bridging element (nucleosome or nucleosomal DNA) present.) is given by:

\[
\hat{E}_{0001} = p_1 E_{0001} + (1 - p_1) E_{no}
\]

where \( p_1 \) is the incorporation probability for the right-most site, \( E_{0001} \) is the FRET efficiency of the object with a bridging nucleosome, and \( E_{no} \) is the FRET efficiency for the spectrometer without a bridging nucleosome. Similar expressions hold for the other variants with one nucleosome. Measured FRET efficiencies for variants with more than one nucleosome thus originate from a mixtures of correctly assembled objects and objects with less, partially, or no nucleosomes attached. For instance, the measured FRET efficiency for the variant 1001 is given by the following expression, if we assume independent nucleosome incorporation:

\[
\hat{E}_{1001} = p_1 p_4 E_{1001} + p_1 (1 - p_4) E_{0001} + (1 - p_1) p_4 E_{1000} + (1 - p_1)(1 - p_4) E_{no}
\]

with \( p_i \) being the incorporation probability at site \( i \) (we count the sites as follows: left most=4, middle left =3, middle right=2, right most = 1 ) and \( E \) being the FRET efficiency of the correctly assembled objects. The measured FRET efficiency is therefore a linear combination of the FRET efficiencies from the different species. We measured the occupation probabilities \( p_i \) independently at low-salt conditions and calculated the true FRET efficiency by decomposing the set of linear equations for the measured FRET efficiencies (see chapter E for detailed calculations). Using the decomposed FRET efficiencies \( E \), we can calculate the fraction of closed spectrometers:

\[
p_c = \frac{E - E_o}{E_c - E_o}
\]

Two-state model of nucleosome unwrapping

In order to describe the experimental data quantitatively, we consider a simplified reaction network with the following reaction species:
Figure 3.5: Closed fraction of spectrometers in solution as a function of additional NaCl and of the number of nucleosomes. (A) Fraction of closed spectrometers from salt-screen experiments with constant nucleosome configuration but different salt concentrations prior to gel electrophoresis. Results from spectrometers with one (blue circles), two (red circles), and four (purple circles) nucleosomes and the global fit to the reaction model (lines) are shown. (B) Fraction of closed spectrometers from nucleosome configuration-screen experiments with different nucleosome configurations but constant salt concentrations prior to gel electrophoresis. Results from spectrometers with one (blue triangles), two (red triangles), three (yellow triangles), and four (purple triangles) nucleosomes are shown. Lines indicate closed fraction from the fit of the reaction model. (C) Combined data from (A) (circles) and (B) (triangles) with the fitted reaction model (lines).
In this simplified model, we treat the step-wise (dis)assembly of the nucleosome as a one-step reaction, since the change in the reaction coordinate monitored in our experiments is assumed to report on one of the multiple steps in the nucleosome disassembly process. Classification of TEM particles corroborated this simplification to an apparent two-state transition from an assembled nucleosome with wrapped nucleosomal DNA to a disassembled nucleosomes with unwrapped nucleosomal DNA, since no classes corresponding to partially assembled nucleosomes were found. In addition, we assume independent binding of histone octamers in a sequential fashion. Depending on the total number of nucleosome binding sites on the spectrometer, different reactions may proceed:

One binding site: \( S + H \xrightleftharpoons{K_D} SH \)

Two binding sites: \( S + 2H \xrightleftharpoons{K_{D/2}} SH + H \xrightleftharpoons{K_D} SH_2 \)

Three binding sites: \( S + 3H \xrightleftharpoons{K_{D/3}} SH + 2H \xrightleftharpoons{K_{D/2}} SH_2 + H \xrightleftharpoons{K_D} SH_3 \)

Four binding sites: \( S + 4H \xrightleftharpoons{K_{D/4}} SH + 3H \xrightleftharpoons{K_{D/3}} SH_2 + 2H \xrightleftharpoons{K_{D/2}} SH_3 + H \xrightleftharpoons{K_D} SH_4 \)

Side reaction: \( T + H \xrightleftharpoons{K_D} TH \)

Because of the energetic gain of the force spectrometer upon transitioning from the closed to the open state, unbinding of the last nucleosome releases an extra free energy \( \Delta G_0 \), which we included as \( K_S = \exp(\Delta G_0/k_B T) \) in the model above. For unwrapping of a nucleosome, the free energy \( \Delta G_{\text{wrap}} \) is required. Because this wrapping free energy depends on the ionic strength of the solution we included an effective nucleosome dissociation constant \( K_D \) that depends on the amount of additional NaCl in solution \( K_D = \exp((\Delta G_{\text{wrap}} + c_{NaCl} \Delta \Delta G_{\text{wrap}}/\Delta c_{NaCl})/k_B T) M \) in the model. The side reaction accounts for excess nucleosomes in solution and for nucleosomes that are bound to only one beam of a spectrometer. Given a set of parameters \{\( \Delta G_0, \Delta G_{\text{wrap}}, \Delta \Delta G_{\text{wrap}}/\Delta c_{NaCl} \}\) and initial concentrations \([H]_0 = 0, [S]_0 = 0, [SH_i]_0 = 40 \text{ nM}, [T]_0 = 0, \text{ and } [TH]_0 = 70 \text{ nM}\), we can solve the corresponding rate equations (numerically) assuming equilibrium (steady state) for the concentrations of each species and calculate the fraction of closed spectrometer objects in solution:

\[
p_c = \frac{[SH_1] + \cdots + [SH_i]}{[S] + [SH_1] + \cdots + [SH_i]}\]
where \(i\) denotes the number of nucleosome attachment sites on the spectrometer for a particular sample. We fitted the parameters of our model to the combined data from salt-titration experiments and nucleosome-configuration screen experiments (Fig. 3.5C).

\[
\begin{array}{cccc}
\Delta G_0 [\text{kcal/mol}] & \Delta G_{\text{wrap}} [\text{kcal/mol}] & \Delta \Delta G_{\text{wrap}}/\Delta c_{\text{NaCl}} [\text{kcal/mol}] \\
1.2 \pm 0.3 & -14.5 \pm 0.6 & 4.3 \pm 0.4
\end{array}
\]

The energetic penalty for closing the spectrometer (1.2 kcal/mol) is consistent with an energetic penalty for closing the spectrometer to opening angles between 30° to 50° (1.2-0.5 kcal/mol as expected from TEM-based free energy landscapes. Using the best-fit parameters we can calculate approximate nucleosome dissociation constants at different concentrations of NaCl:

<table>
<thead>
<tr>
<th>Additional NaCl [M]</th>
<th>Nucleosome dissociation constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>10-70 pM</td>
</tr>
<tr>
<td>0.5</td>
<td>0.3-3.6 nM</td>
</tr>
<tr>
<td>1.0</td>
<td>7-180 nM</td>
</tr>
<tr>
<td>1.5</td>
<td>0.2-9 µM</td>
</tr>
</tbody>
</table>

These dissociation constant agree well with reported values at 500 mM NaCl (0.03 nM to 3 nM [116,117]).

**Nucleosome next-neighbour interactions at nucleosome unwrapping conditions**

To test if next-neighbour interactions (e.g. weak nucleosome pair interactions as measured in chapter 2) contribute to the overall stability of adjacent nucleosomes in the force spectrometer, we analyzed the gel-based FRET experiments which were performed at constant salt concentration for different nucleosome configurations in more detail. Specifically, spectrometers with the same number of attached nucleosomes, but with different nucleosome configurations and therefore different next-neighbour interactions should show different unwrapping transitions. First we calculated the free energy \(\Delta G\) assuming a two state system from the fraction of closed spectrometers \(p_c\):

\[
p_c = \frac{1 + e^{-\Delta G/k_B T}}{e^{-\Delta G/k_B T}}
\]

\[
\Leftrightarrow \Delta G = \ln \left( \frac{1 - p_c}{p_c} \right)
\]

and plotted the free energy versus the configuration of nucleosomes for spectrometer variants with two or three attached nucleosomes (Fig. 3.6). If the nucleosome-nucleosome interaction contributes to the overall stability of the spectrometer-nucleosome-system, we would expect the free energy to show two levels that differ by the free energy \(\Delta \Delta G_{\text{stack}}\). We computed the two free energy levels from the number of nucleosome stacks for each
nucleosome configuration:

\[
\Delta \Delta G_{\text{stack}} = \Delta G(n + 1) - \Delta G(n)
\]

where \(\Delta G(n + 1)\) is the mean free energy of the variants with \(n+1\) nucleosome stacks (Fig. 3.6). A pair of adjacent nucleosomes weakly destabilizes the system, which could point to a slight steric hindrance or entropic exclusion of nucleosomes. In fact, a single nucleosome could potentially rotate around its two attachment sites, whereas a neighbouring nucleosome would constrain the rotational freedom. Thus requiring an entropic penalty for incorporating a next-neighbour nucleosome. Since the distance between adjacent nucleosome-attachment sites is slightly less than the height of a nucleosome (~4.4 nm, assuming an effective helix diameter of 2.5 nm within a DNA origami object, versus ~4.5 nm nucleosome height), steric hinderance might add an additional energetic penalty. Finally, the correction for a finite nucleosome attachment yield might bias the next-neighbour free energy, since independent binding of nucleosomes is assumed.

### 3.3 Summary

We have used a DNA origami force spectrometer to study the salt induced disassembly of nucleosome core particles. Direct imaging using negative staining TEM confirmed the site-specific attachment of up to four nucleosomes in a user-defined configuration and demonstrated the conformational change of the spectrometer upon salt-induced nucleosome disassembly. Gel electrophoretic mobility experiments in combination with a quantitative ensemble FRET study complemented the findings from direct TEM imaging and enabled us to efficiently screen various salt conditions and nucleosome configurations. Based on these experiments, we find nucleosome dissociation constants in the picomolar range at low ionic strength (11 mM MgCl\(_2\)), in the nanomolar range at intermediate ionic strength (11 mM MgCl\(_2\) with 0.5-1 M NaCl) and in the micromolar range at larger ionic strength (11 mM MgCl\(_2\) and more than 1 M of NaCl).

We have used our force spectrometer to track the conformational change of a macromolecular assembly in solution and quantified the conformational equilibria as a function of the solution conditions. Depending on the target molecule, the spectrometers range of sensitivity can be adjusted by carefully choosing the fluorescent dye-pair positions and the nucleosome attachment sites along the spectrometer beams; thus extending the range of FRET, which is typically limited to distances of 1-10 nm [118]. Indeed, our force spectrometer enabled us to monitor the nucleosome unwrapping along an intranucleosomal coordinate that changes from 10 nm to 15 nm upon nucleosome unwrapping. We envision our force spectrometer to resolve structural changes within other biomolecules and
Figure 3.6: Contribution of nucleosome-pair interactions to the unwrapping free energy. Free energy of the spectrometer-nucleosome-system as computed from eq. 3.4 for three different concentrations of sodium chloride and for nucleosome configurations with two (A) or three nucleosomes (B). Data points: mean from four independent measurements; error bars: error of mean, computed using gaussian error propagation; dashed lines: model assuming no dependence of the free energy on nucleosome next-neighbour interactions; solid lines: model assuming a linear dependence of the free energy on the number of nucleosome next-neighbour interactions. Our system relies on the unwrapping transition for reading out the state of the spectrometers in solution and is therefore only sensitive at the transition between the open and closed conformation. In our reaction conditions, spectrometers with three nucleosomes at 0.9 M NaCl are stable and the spectrometer is closed. Hence, free energies for these experimental conditions can not be determined with our system.
assemblies. For example, due to the high structural stability of DNA origami objects in the presence of chaotropic agents [119], protein-unfolding studies, where our spectrometer reports on the separation between two sites on the protein surface, are conceivable. In conclusion, our spectrometer provides a modular and adjustable platform with a readout to study and quantify conformational equilibria for targets from small biomolecules up to large macromolecular assemblies.
Chapter 4

Summary and outlook

In this work, we have introduced a hinged DNA origami device for (i) precisely positioning two molecular groups relative to each other, for (ii) quantitatively studying distance- and orientation-dependent bimolecular free-energy landscapes under forces, and for (iii) tracking conformational changes of macromolecular assemblies.

During these three studies we established and optimized experimental methods and data-analysis procedures, such as the determination of force-spectrometer free-energy landscapes from single-particle TEM measurements, the quantification of fluorescence signals from gel-electrophoresis experiments, the force-generation with molecular components, or the attachment and arrangement of biological target molecules to DNA origami objects. Thus, the three studies serve as proof-of-concept studies and established the methodology for future experiments.

(i) Positioning of molecular groups

We placed fluorescent molecules or thiol-groups on a DNA origami positioning apparatus and used a DNA-based adjuster-screw mechanism to vary their average intramolecular distances from 1.4 nm up to 9.9 nm in subnanometer increments of 0.04 nm, 0.10 nm, or 0.17 nm. In addition to the capability of adjusting precisely average molecular distances, we found small molecular fluctuations of mounted thiol groups using a quantitative crosslinking assay (RMSF 0.5 nm). The ability to fine-tune molecular distances in combination with reduced molecular fluctuations (as found in protein structures) suggest the possibility to design and build molecular structures, such as small-molecule binding pockets or active sites with subnanometer accuracy using DNA origami. For instance, we already control molecular distances through the global shape of our positioning apparatus, in a similar fashion as to how a small active site in a protein is supported by the protein’s global shape. To realize active sites within a DNA origami support structure, one could place multiple functional groups in close proximity and control their arrangement using
multiple positioning-unit-like structural elements. Besides designing molecular structures, the positioning apparatus could be used to study the distance-dependent properties of molecules, similar how we studied the distance-dependent fluorescence brightness variations of fluorescent dyes. Alternatively the positioner could be used to template chemical reactions or measure molecular distances. In fact, the positioner already templates the crosslinking reaction to yield hetero-crosslinked versus homo-crosslinked DNA strands (Fig. 4.1A) and we could estimate the crosslinker contour length from the crosslinking yield curves (Fig. 4.1B).

(ii) Quantifying bimolecular interactions under force

We transformed the positioning apparatus into a force spectrometer by removing the angular constrains between the two beams; thus allowing the spectrometer to rotate around its hinge and realize different opening-angles. Because the hinge generates an angle-dependent torque, interaction partners placed on opposing beams of the force spectrometer will experience a weak force that counteracts an attractive interaction. We calibrated the force response of our spectrometer, mounted two nucleosomes on the spectrometer beams, and measured the distance-dependent nucleosome pair potential for different nucleosome variants and different nucleosome orientations. This study illustrates the potential for quantifying bimolecular interactions; future target systems could include hybridization of DNA or RNA, binding of proteins to DNA, or protein-protein interactions. The single-particle TEM readout enabled us to precisely measure the force spectrometer’s opening-angle distributions and the FRET readout complemented the experiments with qualitative in-solution data. Ensemble FRET measurements in solution were limited to a qualitative analysis, because defect objects produce considerable background signal. Single-molecule FRET experiments with our force spectrometer, where intact objects could be identified on the basis of their dynamical behaviour, could allow determining energetics and dynamics of molecular interactions under force and in solution. Indeed, first DNA-hybridization experiments, where short complementary DNA single-strands protrude from the spectrometer beams, showed two-state-like transitions in single-molecule FRET experiments (Fig. 4.1C). Finally, the current design allows for destabilizing a bound state with up to 2 kcal/mol, but modification of the hinge should allow for increasing or decreasing this energetic bias in future designs.

(iii) Quantifying conformational transitions under force

The force-spectrometer’s opening angle can report on the extension of a molecule that is attached to both spectrometer beams simultaneously. As a proof-of-concept, we mounted up to four nucleosomes in parallel, such that the spectrometer’s opening angle reports on
Figure 4.1: Templated crosslinking reaction, crosslinker contour-length estimation, and single-molecule FRET readout. (A) Laser scanned image of a polyacrylamide gel of denatured crosslinking reactions that were either templated by the positioner (+) or proceeded in solution (-). Numbers indicate concentration of thiol-modified DNA strands in solution or on the positioner in nanomolar. ALB, ALA, BLB = crosslinked DNA strands, LB and AL = DNA strands with one attached bismaleimide crosslinker, A and B = DNA strands. (B) Molecular extension of crosslinker molecules. Squares: contour length from [68]. Triangles: 90 percentile of the sulfur-to-sulfur distance from [68]. Circles: Length of crosslinker molecules derived from the measured distance where the yield reaches 50%. An offset of 1.35 nm (mean contour length of used crosslinkers) was subtracted from each distance to account for the fluctuations of the positioner. (C) Image of a 3% agarose gel on which spectrometers were electrophoresed. A DNA single-strands with complementary sequences protruded from the top and the bottom spectrometer beam (similar to adjuster helices). Numbers give the length of the single-strands in bases. P: gel pocket; o: open conformation of the spectrometer; c: closed conformation of the spectrometer upon hybridization of the two DNA strands. (D) Exemplary intensity traces of a single spot from a single-molecule TIRF experiment versus time of spectrometers with 10-bases-long DNA strands protruding from each beam. Two fluorescence channels and the FRET efficiency (E) are shown. Scale bar: 1 min.

the conformational transition of a nucleosome DNA arc-segment from a bend to a straight state upon salt-induced nucleosome disassembly. Assuming a two-state unwrapping transition and using the FRET readout to quantify the amount of wrapped versus unwrapped nucleosomes in solution, we determined free energies parameters of the nucleosome disassembly. A single-molecule FRET assay with a carefully adjusted force bias could allow for studying nucleosome unwrapping in more detail. In addition, the energetics and dynamics of conformational transitions of other targets such as secondary structure motifs of DNA or RNA or protein conformations could be studied with our spectrometer. The spectrometer system allows for applying molecular forces without further external manipulation.
Appendix A

Quantifying fluorescence in gel-electrophoresis experiments

This method section describes the developed materials and methods to analyze and quantify laser-scanned gel-electrophoresis experiments.

Remark: parts of this section have been published in reference [52, 70, 111]. The analysis software was developed in collaboration with Jean-Philippe Jacques Sobczak\(^1\) and the repository with the corresponding MATLAB implementation (MATLAB 2015b, The MathWorks, Inc., Natick, Massachusetts, United States) is publicly available under https://github.com/JPSS/MATLAB_TOOLBOX.git or https://github.com/jonasfunke/MY_MATLAB_TOOLS.

Gel-electrophoresis and gel imaging

Typically 5-10 µl of sample solution that was mixed 5:1 with a loading dye (15% Ficoll 400, 5 mM Tris-HCl, 0.15% bromophenol blue, pH 8.5) were applied to 2-3% (w/w) agarose gels. For the DNA origami object studied in this thesis, a higher agarose concentration in combination with a longer run time yielded better separation of objects with different opening angles. Samples were typically electrophoresed for 2-3 h at 70 V in a water bath or at 90 V in an ice bath. Gels and running buffer contained 0.5xTBE (1 mM EDTA, 44.5 mM Tris base, 44.5 mM mM boric acid, pH 8.3). Gels were laser-scanned (Typhoon Fla 9500, GE Healthcare) with a resolution of 50 µm/pixel in three fluorescence channels, that were selected to match the excitation and emission spectra of the donor (ATTO550 or Cy3) and the acceptor (ATTO647N or Cy5):

\(^1\)PhD student in the laboratory of Prof. Dr. Hendrik Dietz, Physik Department and Institute for Advanced Study, Technische Universität München, Garching bei München
**APPENDIX A. QUANTIFYING FLUORESCENCE IN GEL-ELECTROPHORESIS**

**EXPERIMENTS**

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In general, comparability of band fluorescence between gels is increased by maintaining gel electrophoresis and scanning parameters, i.e. agarose concentration, gel volume, voltage, run time, running buffer, pixel size, PMT-voltage, throughout a set of experiments. Similar to how DNA ladders are used as molecular weight references to compare electrophoretic mobility among gel electrophoretic experiments, we included fluorescent samples in each gel experiment that serve as fluorescence references. For instance, when studying relative fluorescence intensities, we included a sample that carried one donor and one acceptor molecule and a sample that carried one donor and two acceptor molecules; thus realizing two relative brightness references. For FRET experiments, we additionally included samples with only one donor or only one acceptor molecule to correct for crosstalk between fluorescence channels.

**Correction for background fluorescence**

In order to correct for an offset in fluorescence intensity and long-ranged variations in the fluorescence background, four sample-free areas where selected manually and their mean intensity was calculated. A two dimensional second order polynomial \(I_{i,j} = c_0 + c_1 i + c_2 j + c_3 i j\) was fitted to these mean intensities and then subtracted from each image. The parameter of the fitted polynomial that corresponds to a constant offset \(c_0\), was typically much larger than the parameters corresponding to linear and quadratic variations \(c_1, c_2, c_3\); therefore indicating an almost constant background fluorescence.

**Correction for image shift and fluorescence crosstalk**

A gel is scanned consecutively in each fluorescence channel. Since the gel might slightly move during consecutive scans, an alignment of laser-scans between the different detection channels is sometimes required. We therefore aligned images of different channels by maximizing the cross-correlation of an area where samples with signals in both fluorescence channels were present. Typically, image shifts ranged between 0 to 5 pixels \((0 - 250 \mu m)\).

Crosstalk between fluorescence channels was only detected in the \(A_{em}|D_{ex}\) channel (Fig. A.1A,B). The source for this crosstalk can be attributed to leakage of donor fluorescence into the acceptor emission window \(\beta_{leak}\) and direct excitation of the acceptor dye with the excitation wavelength of the donor molecule \(\beta_{dir}\). The fraction of leaked fluorescence \(\beta_{leak}\) was determined from a sample that contained only a donor dye by plotting for each pixel of the selected monomer band area the signal \(I_{A|D}\) versus \(I_{D|D}\) (Fig.
Figure A.1: Fluorescence crosstalk correction. (A,B) Laser-scanned 2% agarose gel in three fluorescence channels ((D<sub>xem</sub>|D<sub>xex</sub>), (A<sub>xem</sub>|A<sub>xex</sub>), and (A<sub>xem</sub>|D<sub>xex</sub>) corrected for fluorescence crosstalk) on which positioner samples with only one donor dye (D) or with only one acceptor dye (A) or with both donor and acceptor dyes (D+A) were electrophoresed. Image brightnesses were adjusted to highlight crosstalk. P: pocket, D: dimer band, M: monomer band. Scat-terplots of pixel grayscale values from a donor-only sample (C) and from an acceptor-only sample (D). Areas in (A) and (B) indicate pixels selected for determining the crosstalk parameters.

A.1C). This resulted in a scatterplot, which could be fitted with a linear relation, where the slope is the leakage constant $\beta_{\text{leak}}$. In analogy, the fraction of fluorescence due to direct excitation of the acceptor $\beta_{\text{dir}}$ was determined from a sample that contained only an acceptor dye, by plotting for each pixel of the selected monomer band area the signal $I_{A|D}$ versus $I_{A|A}$ (Fig. A.1D). The signal in channel (A<sub>xem</sub>|D<sub>xex</sub>) can be corrected for crosstalk as follows:

$$I_{A|D,\text{corrected}}^{i,j} = I_{A|D}^{i,j} - \beta_{\text{leak}} I_{D|D}^{i,j} - \beta_{\text{dir}} I_{A|A}^{i,j}$$

where $I_{A|D}^{i,j}$, $I_{D|D}^{i,j}$, and $I_{A|A}^{i,j}$ refer to the background corrected intensities at each pixel $(i, j)$. 
Selection of band areas

Band areas were selected semi-automatically in two steps. First, a custom made semi-automated detection algorithm determined the location of all lanes based on a horizontal profile that integrated out the migration direction. Typical band widths ranged from 60 pixel (3 mm) for small gel pockets up to 170 pixel (8.5 mm) for larger gel pockets. Second, the height of the band was selected manually since the band shape depended on experimental conditions, such as agarose concentration or running time. Typically, we chose an area with a height of ±10-20 pixel (0.5-1 mm) around the maximum intensity. In order to compare fluorescence band intensities, the band height and band width was kept constant during a set of experiments.

Absolute band intensities

The total intensity of a monomer band was calculated by summing up background and crosstalk-corrected fluorescence intensities of all pixels in the selected area. Typically, absolute fluorescence intensities can vary slightly from gel to gel by a constant factor, presumably because of band shape, gel thickness, and errors in background or crosstalk correction. To account for these variations, when absolute intensities were evaluated, we included the same reference samples in each gel throughout all experiments. We repeated each gel electrophoresis experiment multiple times (typically 3-5 times), and for each gel we normalized the fluorescence intensities of the sample bands to the signal of the reference sample that was present the respective gel. Normalized intensities were then averaged and the standard deviation as used as a measure of variability among consecutive measurements. Repeating the experiment and averaging the resulting normalized fluorescence is necessary, since the actual amount of loaded sample and therefore the absolute intensity varies due to experimental errors.

Relative band brightness

Absolute band brightnesses determination requires experimental effort, since absolute band intensities can vary due to experimental conditions (see above) and since the absolute brightness depends on the actual amount of sample in the band. Relative band brightnesses, that is, the ratio of fluorescence from two different detection channels of a sample which carries two different fluorescent dye molecules, is more robust against variations in sample concentration, sample amount, and gel conditions. We tested two methods to compute relative fluorescence brightnesses. The first method uses the summed pixel
intensity from fluorescence channels of each band to compute the ratio $R$:

$$R_{ch1/ch2} = \frac{\sum_{i,j} I_{ch1}(i,j)}{\sum_{i,j} I_{ch2}(i,j)}$$

where $I_{ch1}(i,j)$ and $I_{ch2}(i,j)$ are the background and crosstalk corrected intensities of pixel $(i,j)$ and where the summation is carried out over the selected band area. The second method uses a scatterplot, where for each pixel of the selected band area the background and crosstalk corrected intensity in one channel is plotted versus the background and crosstalk corrected intensity in the other channel. The resulting scatterplot can be fitted with a linear relation, where the slope corresponds to the ratio $R$ (similar as to how the brightnesses were corrected for fluorescence crosstalk, Fig. A.1). The first method performs better when the signal is very small, but is biased by the background correction of the gel and therefore shows larger gel-to-gel variations than the second method. We therefore typically used the second method.

To calibrate for different detection efficiencies in the fluorescent channels and different properties of the fluorescent dyes, we included a reference sample with a known fluorescence ratio (e.g. one donor molecule and one acceptor molecule) on each gel that allowed us to normalize the experimental ratios to a reference ratio.

Calculating FRET efficiencies

We estimate the FRET efficiency of a gel band as follows:

$$E = \frac{\frac{I_{A}}{I_{A}}}{I_{D}} \approx \left(1 + \frac{R_{D}}{\eta \eta}ight)^{-1}$$

where $R_{D} = \frac{I_{D}}{I_{D}}$ is the ratio of fluorescence from channel $(D_{em}|D_{ex})$ to $(A_{em}|D_{ex})$. $\gamma$ is a normalization constant that accounts for differences in detection efficiency of the fluorescence channels and quantum yield of the donor and acceptor molecule:

$$\gamma = \frac{\phi A \eta A \eta A}{\phi D \eta D \eta A}$$

where $\phi A$ and $\phi D$ are quantum yields of the acceptor and donor molecules. $\eta A \eta A (\eta D \eta D)$ is the photomultiplier voltage dependent detection efficiency that is proportional to the probability of detecting a photon that is emitted by the acceptor (donor) molecule in the acceptor (donor) channel. We determined this $\gamma$ factor for each gel separately by comparing the measured fluorescence ratio of a reference sample to the reference sample’s known FRET efficiency ($\sim 0.5$). $\gamma$ values typically ranged from 0.5 to 1.5, depending on the selected photomultiplier voltages.
**Depiction of laser-scanned gels as FRET images**

To visualize the FRET efficiencies of gel electrophoretic experiments, the FRET efficiency of each pixel $E_{i,j}$ was determined from background and crosstalk corrected laser-scanned images:

$$E_{i,j} = \frac{I_{i,j}^{A|D}}{I_{i,j}^{A|D} + \gamma I_{i,j}^{D|D}}$$

where $I_{i,j}^{A|D}$ denotes the background and crosstalk corrected intensity of pixel $(i, j)$ in the indicated fluorescence channel. Areas where the fluorescence signal is on the same scale as the fluorescence noise, i.e. areas where no or little sample is present, yield FRET efficiencies with large errors. We therefore applied a transparency mask $T_{i,j}$ to the FRET image:

$$T_{i,j} = \min \left( \frac{I_{i,j}^{A|D}}{c}, 1 \right)$$

Since the signal in the $(A_{em}|A_{ex})$ channel is proportional to the amount of sample (if contact quenching is neglected), gel areas with sample will have a high transparency, whereas gel areas without sample will have a low transparency (see Fig. A.2 for exemplary images).
Figure A.2: Depiction of laser-scanned gels as FRET images. (A) Background and crosstalk corrected laser-scanned images in three fluorescence channels of a 2% agarose gel on which positioner samples with a FRET dye pair (ATTO647N, ATTO550, 26 bp away from the hinge) and different adjuster helix length were electrophoresed. (B) E: FRET image calculated from background and crosstalk corrected pixel intensities. T: Transparency mask that is applied to the FRET image E. TxE: FRET image with the applied transparency mask T. P: gel pocket, D: dimer band, M: monomer band.
Appendix B

Supporting information: Placing molecules using DNA origami
Figure B.1: Design of the positioning apparatus. Design diagram of the positioning apparatus prepared using caDNAno [23]. Black circles, triangles and squares indicate mounting locations of fluorescent molecules. Adjuster helices are depicted as purple oligonucleotides and adjuster helix sequences were designed with NUPACK [120].
Figure B.2: Design elements of the positioning apparatus. (A) Cross section of the top beam (helices 0 to 23) and bottom beam (helix 28 to 51). Four two-bases-long single-stranded DNA elements connect the top and bottom beam to form a hinge (solid black lines). Four 30-bases-long single-stranded DNA loops are required by design (dotted black lines). (B) Schematic of the positioning apparatus with highlighted design elements. (C) Schematic of the two hinge elements. Solid line: hinge formed by two single-stranded DNA bases. Dotted line: loop of 30 single-stranded bases. (D) Schematic for one adjuster DNA. Two protruding DNA overhangs on opposite beams form the adjuster helix. Triangles indicate 3’ ends. Up to four double stranded adjuster helices in parallel can be implemented. In order to prevent undesired cross-binding between neighbouring adjuster helices, four sets of two complementary sequences were designed for sequence orthogonality using NUPACK [120].
Figure B.3: Exemplary field of views. Exemplary field of views of negative-stained TEM micrographs of the positioning apparatus with three adjuster helices in parallel for four length variants. Samples were purified using PEG precipitation [21]. Length of adjuster helices is indicated in each image. Scale bars: 100 nm.
Figure B.4: Exemplary single-particle libraries. Exemplary particle libraries of the positioning apparatus with three adjuster helices in parallel used to compute class averages. Length of adjuster helices is indicated in each image. Scale bars: 100 nm.
Table B.1: Mean opening angle $\bar{\Theta}$ as a function of number of base pairs $n$ in the adjuster helices. Standard deviation $\sigma_\Theta$, error of mean $\sigma_\Theta/\sqrt{N}$ and number of measured particles $N$. 

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Figure B.5: Fluorescence data of positioners with two different interacting dyes. EXEMPLARY laser-scanned images of agarose gels on which positioner samples with four parallel adjuster helices were electrophoresed. Donor (D=ATTO550) and acceptor (A=ATTO647) molecules were mounted ∼5 bp (A), 15 bp (B), or ∼26 bp (C) away from the hinge. Brightness levels were scaled for presentation. Reference samples (Ref1=high FRET efficiency, Ref2=low FRET efficiency, D-only=only a donor molecule was attached, A-only=only an acceptor molecule was attached) were included in each gel to enable cross-gel comparison. P = gel pocket, Po = positioner monomers, S = excess staples.
Figure B.6: Fluorescence data of positioners with two equal interacting dyes. Exemplary laser-scanned images of agarose gels on which positioner samples with four parallel adjuster helices were electrophoresed. Reference (R=ATTO550) and interacting (A=ATTO647) molecules were mounted ∼5 bp (A), 15 bp (B), or ∼26 bp (C) away from the hinge. Brightness levels were scaled for presentation. Reference samples (Ref1=positioner with one reference and one acceptor dye, Ref2=positioner with one reference and two acceptor dyes, open=no adjuster helix) were included in each gel to enable cross-gel comparison. P = gel pocket, Po = positioner monomers, S = excess staples.
APPENDIX B. SUPPORTING INFORMATION: PLACING MOLECULES USING DNA ORIGAMI

Table B.2: Bismaleimide crosslinker properties as reported in [68]. Molecular distances are given in Å.

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Figure B.7: Analysis gels of crosslinking reactions. Exemplary laser-scanned images of denatured crosslinking reactions with different bismaleimide crosslinker molecules where thiols were mounted 4bp and 5bp away from the hinge on positioners with four adjuster helices. open: positioner without adjuster helices, ALB: crosslinked DNA strands, AL: fluorescently-labled thiol-modified DNA strand that has reacted with a crosslinker. A: thiol-modified DNA strands (presumably broken). DNA strand A was fluorescently modified with cy5.
Figure B.8: Analysis gels of crosslinking reactions. Exemplary laser-scanned images of denatured crosslinking reactions with different bismaleimide crosslinker molecules. Thiols were mounted 15 bp away from the hinge on positioners with four adjuster helices. open: positioner without adjuster helices, ALB: crosslinked DNA strands, AL, BL: fluorescently-labeled thiol-modified DNA strands that have reacted with a crosslinker. A, B: fluorescently-labeled thiol-modified DNA strands (presumably broken). DNA strand A was fluorescently modified with cy5. DNA strand B was fluorescently modified with cy3.
Figure B.9: Analysis gels of crosslinking reactions. Exemplary laser-scanned images of denatured crosslinking reactions with different bismaleimide crosslinker molecules. Thiols were mounted 15 bp away from the hinge on positioners with four adjuster helices. open: positioner without adjuster helices, ALB: crosslinked DNA strands, AL, BL: fluorescently-labeled thiol-modified DNA strands that have reacted with a crosslinker. A, B: fluorescently-labeled thiol-modified DNA strands (presumably broken). DNA strand A was fluorescently modified with cy5. DNA strand B was fluorescently modified with cy3. (A) One thiol group was mounted with an additional pentathymine linker. (B) Both thiol groups were mounted with an additional pentathymine linker.
Table B.3: Distances between backbone locations where thiols were mounted for each length of adjuster helices $n$ used in this study. 

$d_1$: Thiol groups were mounted four (top helix) and five (bottom helix) base pairs away from the hinge. 

$d_2$: Thiol groups were mounted 15 base pairs away from the hinge. 

$d_3$: Thiol groups were mounted 25 (top helix) and 26 (bottom helix) base pairs away from the hinge.
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Table B.4: Adjuster helix sequences. Sequences (5' to 3') of adjuster helices protruding from the indicated helix (see Fig. B.1). Sequences were designed with NUPACK [120].
Table B.5: Adjuster helix sequences. Sequences (5′ to 3′) of adjuster helices protruding from the indicated helix (see Fig. B.1). Sequences were designed with NUPACK [120]
Figure B.10: **Dynamic range of FRET efficiencies.** Left: schematic representation of the positioning apparatus (top) and magnified view depicting the fluorophore mounting positions (bottom). Right: FRET efficiency of positioners with four parallel adjuster helices with length ranging from 10 bp to 50 bp calculated from laser-scanned gel-electrophoresis experiments. The opening angle was determined from single-particle TEM data (see Fig. 1.5). Error bars indicate one standard deviation from four independent experiments.
Appendix C

Supporting information: Force spectroscopy using DNA origami
Figure C.1: Design of the force spectrometer. Design diagram of the force spectrometer prepared using caDNA2 [23]. Black circles indicate mounting locations of fluorescent molecules. Nucleosome attachment strands are depicted as purple (proximal) and green (distal) oligonucleotides. Nucleosome attachment strand sequences were designed with NUPACK [120].
Figure C.2: Characterization of the force spectrometer. (A) Cross section of the force spectrometer (top left), where the single-stranded DNA connections between the top beam (helix 0-23) and the bottom beam (helix 28-51) are depicted as solid lines (2 bases) and dashed lines (30 bases). Schematic of the force spectrometer (bottom right), where the section shown in (B) is highlighted. (B) Schematic model of the hinge mechanism. The 30-bases-long loops are depicted as a spring with energy $E_k$. Surface charges ($q_1, q_2$ on the beams) generate electrostatic interactions $E_{DH}$ that are described with the Debye-Hueckel-approximation. (C) Blue line: uniform kernel density estimate of the probability density of the opening angle with bandwidth $3^\circ$. Black line: maximum-likelihood estimation of the probability density with a model that considers the energetic contribution from the entropic springs at the hinge and the electrostatic repulsion of the two beams. (D) Kernel density estimates of the energy landscape of the force spectrometer with a uniform kernel (blue line) and a normal kernel (orange line) with a bandwidth of $3^\circ$. Solid black line: energy landscape of the maximum-likelihood estimation shown in (C). Black dashed line: energetic contribution of the electrostatic repulsion. Black dotted line: energetic contribution of the spring potential. (E) Torque of the force spectrometer calculated from the normal kernel density estimation and maximum-likelihood estimation shown in (D).
Figure C.3: Exemplary particles of the force spectrometer. 210 (out of 3091) exemplary negative-staining TEM particles of the empty force spectrometer. Scale: 50 nm. This figure was reproduced from reference [70].
Figure C.4: Design, preparation, and characterization of nucleosomes with radially protruding DNA single strands. (A) Top left: schematic of branched variants of the 601 nucleosome positioning sequence [99] with up to four (positions A1 to A4) protruding DNA single strands. Bottom left: schematic of purified histone octamer. Right: schematics of nucleosomes with radially protruding DNA single strands at positions A1 and A3 that are produced by salt gradient dialysis from the components on the left. The bottom schematic is based on 3MVD.pdb [75]. (B) Native ethidium bromide stained 4.5 % polyacrylamide gel electrophoresis of various samples: Lane 1: continuous template DNA. Lane 2: nucleosomes assembled using continuous template DNA as in lane 1. Lane 3: template DNA with four nicks at positions A1-A4. Lane 4: nucleosomes assembled using nicked template DNA as in lane 3. Lane 5: template DNA with two protruding single strands at positions A1 and A3 (see A). Lane 6: nucleosomes assembled using template DNA with two protruding single strands as in lane 5. Lane 7: template DNA with four protruding single strands at positions A1-A4. Lane 8: nucleosomes assembled using the template DNA as in lane 7. Nucleosomes were assembled by salt gradient dialysis reconstitution with Drosophila embryo histones. See reference [70] for detailed protocols. (C), Representative electron micrograph of nucleosomes with two protruding DNA single strands (A1, A3) and recombinant tail-less histones from Xenopus laevis. Scale bar: 50 nm. (D) Projection of a nucleosome crystal structure (3MVD.pdb) (top) and average electron micrograph from nucleosomes shown in C (bottom). Black arrows indicate radial intensity signatures stemming from grooves in the DNA template. Red arrows indicate the dyad axis in the arc segment having only one DNA turn. This figure was reproduced from reference [70].
APPENDIX C. SUPPORTING INFORMATION: FORCE SPECTROSCOPY USING DNA ORIGAMI

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Divided Widom 601 sequence

A1 - CTCAATTGGTCGTAGACAGC
A2 - TCTAGCACCGCTTAAACGCAC
A3 - GTACGCGCTGTCCCCCGCGT
A4 - TTTTAACCGCCAAGGGGATTACTCCCTAGTCTC
CAGGCACGTGTCAGATATATACATCCGAT

Figure C.5: Sequences of single-stranded DNA handles protruding from the nucleosome. (A) Top: tailed template DNA with up to four single-stranded DNA handles. Bottom: divided Widom 601 sequence with indicated handle placeholders (A1-A4). (B) Top: schematic nucleosome with up to four potential single-stranded DNA handles. Bottom: two sets of orthogonal sequences which can protrude at indicated positions (A1-A4) from the nucleosome. (C) Set of sequences protruding from the beams of the spectrometer. Due to the advantageous geometry (see Fig. C.6), only position A1 and A3 were used. This figure was reproduced from reference [70].

Figure C.6: Attachment of nucleosomes to force spectrometer. (A) Left: Schematic representation of the force spectrometer with single-strand DNA attachment handles at the distal position and of a nucleosome with complementary single-stranded DNA handles. Right: Schematic representation of the force spectrometer with two attached nucleosomes at the distal position. (B) Schematic representation of a nucleosome with attachment handles at positions A1 and A3 (left) and at position A2 and A4 (right). Dashed lines indicate geometric height of the attachment handles with respect to the nucleosome surface. This figure was reproduced from reference [70].
Figure C.7: Comparison of negative-staining TEM and cryo-EM field of views. (A) Exemplary field of views of empty force spectrometers in negative-staining TEM (left) and in cryo-EM (right). (B) Exemplary field of views of nucleosome-loaded (wildtype Xenopus laevis) force spectrometers in negative-staining TEM (left) and in cryo-EM (right). Cryo-EM sample was a 1:1 mixture of empty spectrometers and nucleosome-loaded spectrometers. Green check marks: correctly attached nucleosomes; red crosses: incorrectly attached nucleosomes; white arrows: attached nucleosomes. Scale bars: 50 nm. This figure was reproduced from reference [70].
Figure C.8: Exemplary particles of the force spectrometer with attached nucleosomes at the proximal position. 203 (out of 1301) exemplary negative-staining TEM particles of the force spectrometer with two bound nucleosomes (wildtype, *Xenopus laevis*) at the proximal position. Scale bar: 50 nm. This figure was reproduced from reference [70].
Figure C.9: Exemplary particles of the force spectrometer with attached nucleosomes at the distal position. 124 (out of 158) exemplary negative-staining TEM particles of the force spectrometer with two bound nucleosomes (wildtype, *Xenopus laevis*) at the distal position. Scale bar: 50 nm. This figure was reproduced from reference [70].
Appendix D

Supporting information: Quantifying conformational equilibria using DNA origami
APPENDIX D. SUPPORTING INFORMATION: QUANTIFYING CONFORMATIONAL EQUILIBRIA USING DNA ORIGAMI

Figure D.1: Nucleosome with single-stranded DNA handles. (A) Scheme of the branched DNA template used to assemble the nucleosome [70]. (B) Three views of the nucleosome based on 3MVD.pdb [75]. Colors of DNA strands correspond to colors in scheme (A). The bend segment of the branched template DNA, which induces the conformational change of the spectrometer upon nucleosome disassembly, is shown at low transparency. The expected distance between the two attachment sites is 10 nm. Dashed line indicates the dyad axis of the nucleosome. (C) Sequences used to assemble the branched template DNA. Colors correspond to colors in scheme (A). Sequences of single-stranded DNA handles that branch from the branched single-strands at the 5′-ends are not shown (see Fig. D.2). This figure was reproduced from reference [111].

Figure D.2: Attachment of nucleosomes to the force spectrometer. (A) Scheme of the force spectrometer cross-section with helices numbered as in Fig. C.1. Black dots indicate positions where single-stranded DNA attachment handles protrude. (B) Sequences protruding from the spectrometer as indicated by the labels of the black dots in (A). (C) Schematic representation of the nucleosome with two single-stranded DNA handles. (D) Four sets of sequences of the single-stranded DNA handles protruding from a nucleosome. This figure was reproduced from reference [111].
Figure D.3: TEM field of views of salt-titration experiments with one nucleosome. Exemplary negative staining TEM field of views of spectrometers with nucleosome configuration as indicated by the icon (solid element indicates nucleosome position). Nucleosomes were attached to spectrometers and subsequently incubated at 11 mM MgCl$_2$ with different concentrations of NaCl as indicated in each micrograph. Micrographs were high-pass filtered with a cut-off radius of 25 nm. Scale bar: 100 nm.
Figure D.4: TEM field of views of salt-titration experiments with two nucleosomes. (A) and (B): Exemplary negative staining TEM field of views of spectrometers with nucleosome configuration as indicated by the icon (solid elements indicate nucleosome positions). Nucleosomes were attached to spectrometers and subsequently incubated at 11 mM MgCl₂ with different concentrations of NaCl as indicated in each micrograph. Micrographs were high-pass filtered with a cut-off radius of 25 nm. Scale bar: 100 nm.
Figure D.5: Equilibration test. (A) Fraction of closed objects as a function of additional NaCl for spectrometers with two attached nucleosomes. Red lines: samples were incubated for 3 days prior to gel electrophoresis. Blue lines: samples were incubated for 1 day prior to gel electrophoresis. (B) Fraction of closed objects as a function of additional NaCl for spectrometers with four attached nucleosomes. Red lines: samples were incubated for 3 days prior to gel electrophoresis. Blue lines: samples were incubated for 1 day prior to gel electrophoresis. This figure was reproduced from reference [111].
Appendix E

Decomposition of mixed FRET efficiencies from nucleosome-configuration experiments

The measured FRET efficiency $\tilde{E}$ for each nucleosome configuration results from objects with correctly incorporated nucleosomes and from objects with unoccupied nucleosome binding sites. Each designed nucleosome attachment site can be occupied or unoccupied because of defect spectrometers or nucleosomes. For example, the measured FRET efficiency for the variant with one nucleosome at the right-most site (variant 0001, we label the variant using four binary numbers, where the location of the number indicates the nucleosome attachment site (see icons in figures) and 0 corresponds to no bridging element present, whereas 1 corresponds to a bridging element (nucleosome or nucleosome DNA) present.) is given by:

$$\tilde{E}_{0001} = p_1 E_{0001} + (1 - p_1) E_{no}$$

where $p_1$ is the incorporation probability for the right-most site, $E_{0001}$ is the FRET efficiency of the object with a bridging nucleosome, and $E_{no}$ is the FRET efficiency for the spectrometer without a bridging nucleosome. Similar expressions hold for variants with one nucleosome. Measured FRET efficiencies for variants with more than one nucleosome thus consist of a mixture of correctly assembled objects and objects with less, partially or no nucleosomes attached. For instance, the measured FRET efficiency for the variant 1001
is given by the following statement, if we assume independent nucleosome incorporation:

\[ \tilde{E}_{1001} = p_1 p_4 E_{1001} + p_1 (1 - p_4) E_{0001} + (1 - p_1) p_4 E_{1000} + (1 - p_1)(1 - p_4) E_{no} \]

with \( p_i \) being the incorporation probability at site \( i \) (we count the sites as follows: left most=4, middle left =3, middle right=2, right most = 1 ) and \( E \) being the FRET efficiencies of the correctly assembled objects. Similar expression hold true for variants with three or four nucleosomes. In total, 15 combinations are possible (excluding variant 0000).

These equations can be written in vector form:

\[ \tilde{E} = M E + E_{no} b \] (E.1)

where \( \tilde{E} \) is a row vector of measured FRET efficiencies and \( E \) is a row vector of FRET efficiencies of correctly assembled objects. \( M \) is a matrix that mixes the FRET efficiencies and is given by:

\[
M = \begin{pmatrix}
p_1 & p_2 & \cdots & \\
p_2 & p_3 & \cdots & \\
p_1(1 - p_4) & (1 - p_1)p_4 & p_4 & \cdots \\
p_2(1 - p_4) & (1 - p_3)p_4 & p_2p_4 & \cdots \\
p_1(1 - p_3) & (1 - p_1)p_3 & & \\
\vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\
\end{pmatrix}
\]

\( b \) is a row vector that is given by:

\[
b = \begin{pmatrix}
(1 - p_1) \\
(1 - p_2) \\
(1 - p_3) \\
(1 - p_4) \\
(1 - p_1)(1 - p_4) \\
(1 - p_2)(1 - p_4) \\
(1 - p_1)(1 - p_3) \\
\vdots \\
(1 - p_1)(1 - p_2)(1 - p_3)(1 - p_4)
\end{pmatrix}, \quad \tilde{E} = \begin{pmatrix}
\tilde{E}_{0001} \\
\tilde{E}_{0010} \\
\tilde{E}_{0100} \\
\tilde{E}_{1000} \\
\tilde{E}_{1001} \\
\tilde{E}_{1100} \\
\tilde{E}_{0101} \\
\vdots \\
\tilde{E}_{1111}
\end{pmatrix}, \quad E = \begin{pmatrix}
E_{0001} \\
E_{0010} \\
E_{0100} \\
E_{1000} \\
E_{1001} \\
E_{1100} \\
E_{0101} \\
\vdots \\
E_{1111}
\end{pmatrix}
\]

Given the incorporation probabilities \( (p_i) \) and the FRET efficiency for the object with a nucleosome \( (E_{no}) \), equation E.1 can be solved for the true (decomposed) FRET efficiencies.
E (Fig. E.1B).

**Determination of incorporation probabilities**

We determined $E_{no} \approx 0.35$ using an empty spectrometer without attached nucleosomes. At low ionic strength, all spectrometers with correctly attached nucleosomes are assumed to be in a closed conformation. Hence $E_{0001} = E_{0010} = \cdots = E_c$, with a constant FRET efficiency $E_c$ of the closed object. We determined the FRET efficiency of the objects 0001, 0010, 0100, 1000, and 0000 at additional 0 M NaCl. The equations from above simplify to

\begin{align}
\tilde{E}_{0001} &= p_1 E_c + (1 - p_1) E_{no} \\
\tilde{E}_{0010} &= p_2 E_c + (1 - p_2) E_{no} \\
\tilde{E}_{0100} &= p_3 E_c + (1 - p_3) E_{no} \\
\tilde{E}_{1000} &= p_4 E_c + (1 - p_4) E_{no} \\
\tilde{E}_{1111} &= p_1 p_2 p_3 p_4 E_c + (1 - (1 - p_1)(1 - p_2)(1 - p_3)(1 - p_4)) E_{no}
\end{align}

These are five equations for five variables ($p_1$, $p_2$, $p_3$, $p_4$, and $E_c$) that can be solved to:

$$p_1 \approx 0.97, \quad p_2 \approx 0.77, \quad p_3 \approx 0.71, \quad p_4 \approx 0.89, \quad E_c \approx 0.57$$

**Fraction of closed spectrometers from nucleosome-screen experiments**

Using the determined incorporation probabilities, the FRET efficiencies for the closed object, and the FRET efficiencies of the object without a nucleosome, it is possible to invert equation E.1. At ionic strength conditions, where the nucleosome is unwrapping the corrected FRET efficiency is an equilibrium between open and closed states:

$$E = p_o E_o + (1 - p_o) E_c$$

where $E$ is the vector of true FRET efficiencies, $p_o$ is a vector of probabilities that variants are in the open state and $E_o$ is the FRET efficiency of the open state. $E_o \approx 0.39$ was determined from samples where the nucleosome was disassembled with high ionic strength (at 2.5 M NaCl). The probability for the closed state is then given by (Fig. E.1C):

$$p_c = \frac{E - E_o}{E_c - E_o}$$
Figure E.1: Decomposition of FRET efficiencies in nucleosome-configuration screens. Mean FRET efficiency of gel band (A), mean decomposed FRET efficiency (B), and mean closed fraction of spectrometers (C) for three different sodium chloride concentrations prior to gel electrophoresis for each nucleosome configuration, as indicated by the icon in the bottom row (solid elements indicate occupied nucleosome binding sites). Colors indicate total number of occupied nucleosome binding sites (blue = one, red = two, yellow = three, purple = four). Error bars indicate one standard deviation based on four independent measurements. (A-B) Dashed lines indicate FRET levels of the closed ($E_c$), the open ($E_o$), and the free ($E_{no}$) spectrometer. (C) Dashed line indicates 100% spectrometers in closed conformation.
Appendix F

Materials and methods

F.1 Design, self-assembly, and purification of DNA origami objects

**Design:** The DNA origami positioning apparatus and the force spectrometer were designed using caDNAno [23] and CanDo [24] software. Sequences of adjuster helices and nucleosome handles were designed for sequence orthogonality in NUPACK [120].

**Assembly:** The self assembly of the DNA origami positioning apparatus and the force spectrometer was performed as described in [52, 53, 70] with reaction mixtures containing 40 nM scaffold DNA (p7704) [121], 200 nM of each DNA oligonucleotide strand, 20 mM MgCl₂, 5 mM TRIS base, 1 mM EDTA, and 5 mM NaCl (pH ~ 8). Fluorescently modified DNA oligonucleotides were included in folding reactions, while thiol-modified oligonucleotides were added after folding. Oligonucleotides were obtained from Eurofins MWG (Ebersberg, Germany). Reaction mixtures were annealed with a TETRAD (MJ Research, now Biorad) thermal cycling device using an annealing protocol that was optimized according to [20]:

<table>
<thead>
<tr>
<th>Temperature [°]</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>15 min</td>
</tr>
<tr>
<td>58-55</td>
<td>3 h/°</td>
</tr>
<tr>
<td>52-50</td>
<td>3 h/°</td>
</tr>
<tr>
<td>12</td>
<td>∞</td>
</tr>
</tbody>
</table>

**PEG purification:** Self-assembly reactions were purified by means of two rounds of PEG precipitation according to [21].
F.2 Negative-staining TEM

TEM-grid preparation, TEM imaging, and TEM image analysis was performed as described in [52, 70, 111].

**TEM-grid preparation:** Typically, 3 µl of purified sample at ∼10 nM was adsorbed for 30 seconds on a glow-discharged formvar-supported carbon-coated Cu400 TEM grid (Science Services, Munich, Germany) and stained using a 2% aqueous uranyl formate solution containing 25 mM sodium hydroxide.

**TEM imaging:** Grids were imaged with a AMT 4 Megapixel CCD camera at 28500-fold magnification using a Philips CM100 electron microscope operated at 100 kV.

**Image analysis:** Particles were either picked manually in Xmipp 3 [122] or in Eman2 [123]. Positioner particles with adjuster helices were identified and extracted from micrographs with a custom made software implemented in MATLAB 2014b (The MathWorks, Inc., Natick, Massachusetts, United States). 2D class averaging was performed with Xmipp 3 using the mlf2d protocol [124]. Opening angles were measured along the inner helices of the DNA origami object in single-particle micrographs with ImageJ64 [125] and Fiji [126] using the arrow tool.

F.3 Cryo-EM

Cryo-EM-grid preparation, cryo-EM imaging, and cryo-EM image analysis was performed as described in [70].

**Cryo-EM-grid preparation:** Samples of force spectrometers with or without attached nucleosomes (in 11 mM MgCl₂, 5 mM TRIS base, 1 mM EDTA and 35 mM NaCl) were incubated for 120 s on glow-discharged lacey carbon grids with ultrathin carbon film (TED PELLA, 01824) and vitrified using a freeze-plunging device (Vitrobot Mark IV, FEI).

**Cryo-EM imaging:** Samples were imaged at liquid nitrogen temperatures using a Tecnai Spirit TEM (FEI) operated at 120 kV with a 4x4k Eagle CCD Detector (FEI) at 26000x (pixel size 4.188 Å) or 30000x (pixel size 3.574 Å) magnification with a defocus of -2 µm to -1 µm.

F.4 Crosslinking reactions

Crosslinking experiments with the positioner apparatus were performed as described in [52].
Sample preparation: Positioner objects were assembled without thiol-modified DNA oligonucleotides. Purified DNA structures were then incubated over night at room temperature with thiol modified DNA oligonucleotides at 200 nM in buffer containing 50 mM HEPES, 10 mM MgCl$_2$, pH 6.7, and 10 mM TCEP. Subsequently, samples were subjected to two rounds of purification (using PEG precipitation) to remove excess oligonucleotides and TCEP.

Crosslinking reaction: Positioner samples were reacted immediately after reduction over night at room temperature with freshly weighed and dissolved bismaleimide crosslinkers. A typical reaction contained 10 µM bismaleimide crosslinker, 50 nM positioner in reaction buffer (50 mM HEPES, 10 mM MgCl$_2$, pH 6.7). Prior to the reaction, bismaleimide crosslinkers were dissolved to 20 mM in DMSO and then further diluted to 100 µM in reaction buffer (50 mM HEPES, 10 mM MgCl$_2$, pH 6.7). Bismaleimide crosslinkers were obtained from Sigma-Aldrich (St. Louis, Missouri, USA), and Life Technologies (Darmstadt, Germany).

Denaturation: Reacted samples were mixed 1:5 with denaturing loading dye (8 M Urea, 32 mM EDTA, 84 mM TRIS base, pH ~8, 12% Ficoll, 0.08% Bromphenol blue) containing 10 mM TCEP and incubated for one hour at room temperature.

Polyacrylamide gel electrophoresis: Denatured samples were gel electrophoresed for 2 h and 30 min at 100 V in 20 % polyacrylamide gels. 0.5xTBE (1 mM EDTA, 44.5 mM Tris base, 44.5 mM boric acid, pH 8.3) was used as running and gel buffer. Gels were laser scanned (Typhoon Fla 9500, GE Healthcare) with a resolution of 50 µm/pixel in two channels: 532 nm excitation with an emission window of 560-580 nm for detection of Cy3-modified strands and 635 nm excitation with an emission larger than 665 nm for detection of Cy5-modified DNA oligonucleotides.

Image analysis: Laser-scanned images were corrected for background fluorescence as described above (section A). Areas of the crosslinked and not crosslinked bands were selected by hand. Fluorescence brightness of each band was determined by summing pixel intensities within these areas. Finally, the yield for each sample was calculated from the summed intensities $I$ as $I_{\text{crosslinked}}/(I_{\text{crosslinked}} + I_{\text{monomer}})$. The resulting yield was consistent in both detection channels (Cy3 and Cy5). We used the signal in the Cy5 channel, since the fluorescence in the Cy5 detection channel had better signal-to-noise ratio.

Normalization of yield curves: Defective thiol-groups, defective bismaleimide crosslinkers, and defective DNA objects present a background of non-crosslinked product. Indeed, the measured yield curves show a plateau at very small distances between the thiol groups with a maximum yield of $\sim$ 35-50%. We therefore normalized the yield
curves to 1 using the median of the six data points corresponding to the smallest thiol distances.

F.5 Attachment of nucleosomes to the force spectrometer

40 nM of spectrometers featuring single-stranded DNA attachment handles (A1 and A3) were incubated with 70-135 nM of nucleosomes for each attachment site on the spectrometer in buffer containing 11 mM MgCl₂, 5 mM Tris base, 1 mM EDTA, and 35 mM NaCl (pH 8) at 4°C overnight. Samples were used without further purification for the preparation of TEM grids, for gel electrophoresis experiments, or for salt-titration experiments.

F.6 Salt-induced nucleosome unwrapping protocols

Incubation of nucleosome-loaded spectrometers with additional NaCl: Salt-screen experiments were performed in 11 mM MgCl₂, 5 mM Tris base, 1 mM EDTA, and 35 mM NaCl, (pH ~8) for 1-2 days at room temperature (25°C) with different amounts of additional NaCl. We confirmed that our systems are equilibrated after a 1 day incubation with NaCl at room temperature by comparing salt-screen experiments that were incubated for 1 or 3 days (Fig. D.5).

Salt-screen experiments: Gel electrophoresis of nucleosome-loaded force spectrometers with different nucleosome configurations at constant NaCl concentration was performed in 2% agarose gels for 2.5 h at 70 V.

Nucleosome-configuration screen experiments: Gel electrophoresis of nucleosome-loaded force spectrometers with the same nucleosome configuration but varying NaCl concentrations was performed in 3% agarose gels for 7.5 h at 70 V.

Gel-electrophoresis conditions: Gel-electrophoresis experiments were performed buffer contained 11 mM MgCl₂ and 0.5xTBE (1 mM EDTA, 44.5 mM Tris base, 44.5 mM boric acid, pH 8.3).
Bibliography


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