Rotary mechanisms and scientific devices based on DNA

Dissertation

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Rotary mechanisms and scientific devices based on DNA

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How we make DNA origami

DNA origami scaffold for studying intrinsically disordered proteins of the nuclear pore complex
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Acknowledgement
1. Introduction

Nature provides countless examples of intriguing nanoscale machinery with fascinating capabilities. Examples include phages and their infection mechanisms [1], enzymes, linear and rotary molecular motors [2–4], and membrane machinery such as the nuclear pore complex [5]. Reproducing and expanding these functionalities by rational design has remained very challenging using current technologies. Being able to build similarly sophisticated devices as nature does would greatly benefit many fields. For instance, nanorobots could carry out biomedical tasks such as encapsulating a drug and actively delivering it to specific target cells or synthetic enzymes could catalyze chemical reactions and thus help to produce or digest a wide array of substances.

In nature, most of these objects consist of heteropolymeric chains of amino acids which fold into intricate three-dimensional shapes called proteins. The shapes and dynamics of proteins depend on many complex interactions that occur between the amino acids. The complexity of these interactions in solution makes predicting the folded shape of a given amino acid sequence challenging. Hence, it is even more difficult to actively design a sequence that folds into a desired shape. Recent advances in the field of protein design allow designing such sequences for some desired non-natural shapes. However, designing conformational changes and heteropolymeric assemblies of these designed proteins remains very challenging [6,7].

Compared to chains of amino acids, chains of nucleotides forming deoxyribonucleic acids (DNA) exhibit more predictable and chemically stable interactions. While nature uses DNA primarily for information storage, a field known as DNA Nanotechnology developed various approaches to use DNA as a programmable building material to fabricate a wide variety of three-dimensional shapes at the nanoscale. This field of DNA nanotechnology has seen tremendous success over the last decade and reported several nanodevices even capable of controlled conformational changes [8–11]. DNA has, for example, been utilized to build simple molecular machines which are inspired by linear molecular motors such as myosin [12]. Typically, in these devices that are called ‘walkers’ a small DNA strand is able to ‘walk’ along a DNA track through toehold-mediated strand displacement [13,14].
The research field currently pursues several distinct directions: (1) extending the space of potential shapes and conformations that can be made from DNA by developing novel design methods [15, 16], (2) building nanometer-sized tools based on the positioning capabilities which studying certain biological objects and processes [17, 20], (3) testing DNA origami objects for in-vivo applications such as targeted drug delivery [21] and (4) exploring novel inter-disciplinary applications in e.g. semiconductor research [22–24] or plasmonics [25, 26]. This thesis describes three different projects coarsely located in the areas (1) and (2).

Scaffolded DNA origami [27] has shown significant success in building nanometer-sized objects using molecular self-assembly. Three unique features make this technology a prime candidate to create sophisticated objects: (1) A vast space of desired three-dimensional shapes can be achieved [28–32]. (2) Chemical modification of individual nucleotides allow the precise placement of functional chemical groups with Bohr radius precision within a DNA origami object [17]. (3) Flexible parts, such as joints or hinges, can be integrated and enable the design of conformational changes [33–37]. Recently, a significant advance in this technology enabled the assembly of large structures and the control of conformational dynamics [38]. The improvements were possible through the use of weak base-stacking interactions [39, 40] in combination with shape-complementarity.

The first part of this thesis describes the assembly of DNA origami structures which reproduce some of the functionalities of natural rotary molecular motors such as the ATP synthase. This type of motors has remained particularly hard to be reproduced synthetically [2, 4, 41]. Despite their structural complexity, these devices need an energy source in order to perform active rotary motion. In nature, the latter is often achieved by either harvesting the chemical energy of a certain reaction (e.g. hydrolyzing ATP to ADP and P) or using entropic energies that are available in different ion concentrations across a membrane.

To date, synthetic chemistry has developed the most impressive examples of designed driven molecular motors [42–45], which were recently recognized with the Nobel Prize in Chemistry.

Chapter 2 presents a rotary apparatus made from DNA origami components that is able to perform diffusive rotary motion. The first part describes the design and assembly of this apparatus. The second part explains single-molecule fluorescence microscopy experiments carried out to directly observe the rotary motion. Chapter 3 describes the design and assembly of an extended version of this rotary apparatus designed to be actively driven by an electroosmotic flow.
The third chapter reports a DNA origami object used to position and study the properties of proteins of the nuclear pore complex (NPC). NPCs are located in the nuclear envelope in eukaryotic cells and represent an intriguing example of a complex assembly consisting of hundreds of proteins. The permeability of this pore is highly selective and mediated by intrinsically disordered proteins called FG-Nucleoporins (FG-NUPs) located inside the transport channel. The selection mechanism of the NPCs are currently poorly understood. Chapter 4 describes the design and assembly of a DNA ring matching the radial dimensions of NPCs and the successful attachment of FG-NUPs to the ring.

The last chapter summarizes the use of a DNA origami object as a passive force spectrometer to measure weak interactions between two biomolecules. The biomolecules chosen as a model system are nucleosome core particles whose interaction is important for the structure of chromatin which in turn is crucial for the regulation for many genomic processes such as transcription and replication.
2. Rotary apparatus made from 3D DNA origami components

The ATP synthase [46][47] is a molecular motor and an enzyme at the same time. It can either pump protons through a membrane by obtaining energy from hydrolyzing ATP or it can function reversely, synthesizing ATP by using the entropic energy contained in a proton gradient over a membrane. In both cases the ATP synthase performs a rotary motion. Reproducing the functionality of such a machine on the nanoscale remains out of reach for current technologies. A first step towards replicating the ATP synthase’s functionalities is building an apparatus on a similar length scale that features a freely-rotating part. This chapter describes the assembly of such a rotary apparatus from 3D DNA origami components that can perform rotary motion driven by thermal energy. Section 2.1 describes the design and assembly of an initial version of the rotary apparatus which was partially modified in a second version (Sec. 2.2). Single-molecule fluorescence microscopy was subsequently used to study the dynamics of the apparatus (Sec. 2.4). Direct observation of the rotational motion was achieved using an extended version of the apparatus (Sec. 2.5).

Except for section 2.1 most parts of this chapter have been published in [48]. Elena Willner supported me as a working student by preparing some of the gels for the electrophoretic mobility measurements as well as acquiring some of the single-molecule fluorescence microscopy data under my supervision. Since the DNA origami technique has been well-established and described in the literature an extensive introduction was omitted. Excellent introductions can be found in references [49, 50].

2.1. Design and assembly of rotary apparatus version 1

A molecular rotary apparatus should feature a part that can freely rotate and provide a possibility to directly observe the rotary motion in solution. To build such a mechanism using scaffolded DNA origami several aspects have to be taken into account. (1) The size of a single object is limited by the length of the circular scaffold DNA strand. (2) The
Figure 2.1.: **Schematic design of rotary apparatus v1.** a, Rotor (blue) and clamps (white) with shape-complementary features and brackets (red). See Figs. 3.1 and 3.2 for full design schemes. b, Cross section of the body of the assembled rotary apparatus with clamps (light and dark gray) and rotor (blue) with central rotation axis (red). c, Rotor bound to one clamp (right), yellow arrows indicate positions of complementary single-strands for connecting the clamps. d, Assembled rotary apparatus with bound rotor. e, Assembled rotary apparatus with unbound rotor. f, Scheme of rotor - clamp connection with toehold (red). See table B.1 for sequences. g, Scheme of clamp - clamp connection. See table B.2 for sequences.
spatial arrangement of neighboring helices on a honeycomb lattice limits the approximation of a rotationally symmetric cross section to a hexagonal shape. (3) The smallest mechanically rigid rotation axis in honeycomb configuration is a bundle consisting of 6 double-stranded DNA helices.

With these limitations in mind an initial design was devised (Fig. 2.1). The rotary apparatus consists of three separate DNA origami structures: two clamps (clamp A and clamp B) serve as an axle bearing which encloses a central rotor (Fig. 2.1a). The body of the rotor consists of 64 parallel DNA helices arranged on a honeycomb lattice cross section. The central 6 helices protrude out of the body serving as a rotation axis. This axis is bend by a 90° corner expanding to a 70 nm long lever arm which consists of 10 helices. The lever arm is intended to allow reading out the rotary motion by modifying it with fluorophores which can be tracked by single-molecule fluorescence microscopy. The body has a height of 32 nm which was chosen to ensure mechanical rigidity by having on average four Holliday junctions connecting neighboring helices along the height (Fig. 2.2).

Each clamp consists of 60 helices forming a 2-layer honeycomb compartment with two protruding brackets bend by 90°, each consisting of 6 helices (Fig. 2.3). The brackets are meant to spatially confine the rotor inside the clamp dimer. The two clamps have exactly the same shape but are rotated by 180° around the central rotation axis in the assembled apparatus. The resulting cross section of the full assembly provides enough spatial freedom for the rotor body to perform rotational motion (Fig. 2.1b).

The assembly of the full rotary apparatus is meant to be sequential: (1) The rotor binds to clamp A; (2) clamp B binds to clamp A thereby completing the bearing and confining the rotor inside; (3) the rotor unbinds from clamp A and can thus perform one-dimensional rotational motion (Fig. 2.1c-e).

A critical component is the interface between the rotor and clamp A since it needs to be reversible. During the assembly the rotor should bind to the clamp, whereas after assembly it needs to be released. Toehold-mediated strand displacement has been used extensively in DNA nanotechnology to create such reversible interfaces [51,52]. Here, it is employed to allow binding and unbinding of the rotor (Fig. 2.1f). To this end, the rotor and the clamp feature in total 8 protruding DNA single-strands with a sequence of 10 nt on the rotor side. These strands serve as a toehold to bind additional oligonucleotides

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\[1\] The alternative square lattice configuration was not further considered in this project due to its rectangular cross sections.
Figure 2.2: Design scheme of rotor v1. Individual parts as indicated. Generated with caDNA2o v0.2.
2.1. Design and assembly of rotary apparatus version 1

Figure 2.3.: Design scheme of clamp v1. Individual parts as indicated. Generated with caDNAno v0.2.
Figure 2.4: Schematic representations of shape-complementary binding pocket in unbound (a) and bound (b) position. Scaffold DNA strand is depicted in blue, orange background highlights blunt ends that are stacking with each other.

which will displace the shorter strands protruding from the rotor. After successful strand displacement, double-strands protruding from the rotor at all 8 positions will remain. These remaining strands may be problematic since they could inhibit the rotary motion by sterical hindrance.

At the time of the first design of the rotary apparatus independently performed experiments by Klaus Wagenbauer and Thomas Gerling in the Dietz lab established the usage of base-stacking interactions combined with the principle of shape-complementarity for inter- and intra-monomer interaction interfaces in DNA origami objects [38]. Base-stacking is a weak interaction [40] that occurs between neighboring bases in a double helix perpendicular to the hydrogen bonds formed by Watson-Crick base-pairing. The corresponding interaction interfaces on DNA origami objects are implemented via protrusions and shape-complementary recessions (Fig. 2.4). Typically, multiple neighboring helices are selected to form a protrusion and helices with the same geometric arrangement are interrupted to form a shape-complementary recession. The interaction between DNA origami objects with these features can be controlled via the mono- or divalent cation concentration present in solution or by changing the temperature of the solution [38]. The same experiments also suggested that combining shape-complementarity with strand hybridization for non-reversible polymerization interfaces can achieve higher yields than using strand hybridization alone.

The interface between the two clamps features such a shape-complementary interface as well as complementary single-strands protruding at the top and bottom of each clamp.
2.1. Design and assembly of rotary apparatus version 1

at the interface (Fig. 2.1g). The single-strands are intended to irreversibly hybridize and thus to reinforce the connection between the two clamps. The sequences of these single-strands as well as the presence of the protruding single-strands for rotor - clamp dimerization are the only differences between clamp A and clamp B.

The design of the 90° corners is based on Thomas Martin’s design strategy described in [53]. Since the cross section of the honeycomb lattice is not symmetrical under rotations of 90° the design scheme of the corner has to be adjusted depending on the bend direction. The corner design was improved iteratively using three-dimensional models predicted by CanDo [54].

Electrophoretic mobility analysis (EMA) was used to determine optimal self-assembly reaction conditions for the two components (see B.9). Successfully folded DNA origami objects typically exhibit a higher mobility than the scaffold DNA. The rotor and the clamp yield well-defined leading bands indicating successful assembly of both components. All protruding single-strands designed for polymerizing the individual components were included in the self-assembly reaction mixture. EMA was subsequently used again to study the polymerization reactions. Prior to mixing the individual parts, excess staple strands from the self-assembly reactions were removed using PEG precipitation [55]. For polymerization, rotor and clamp A were mixed at a 1:1 ratio and incubated at various temperatures and MgCl₂ concentrations for 12 hours. EMA of the resulting samples (Fig. 2.5) revealed the appearance of a new band with less mobility than the two monomeric components. Since a significantly reduced mobility is expected for a dimeric species this band can likely be attributed to the clamp A - rotor dimer. The intensity of this band gets weaker for incubation conditions with increasing MgCl₂ concentration and temperatures above 40° C. Bands corresponding to clamp A and rotor monomers were present for all tested polymerization conditions indicating that the polymerization reaction did not succeed for all objects.

Similar experiments were performed for the dimerization of clamp A and B (Fig. 2.6). Two different lengths of the protruding single-strands were tested because longer overhangs are expected to strengthen the connection while increasing the chance of undesired interactions between the overhangs. Clamps with 15 nt long overhangs yield more defined dimer bands than with 10 nt. The dimerization yield decreases below 40° C and appears not to depend strongly on the MgCl₂ concentration in the tested range. No clamp monomers are left in solution indicating a high dimerization yield. The tested conditions were chosen based on previous polymerization experiments performed in the Dietz lab.
2. Rotary apparatus made from 3D DNA origami components

Figure 2.5.: EMA of clamp - rotor dimerization. Laser-scanned image of a 2% agarose gel with 11 mM MgCl$_2$. S = scaffold DNA, A = clamp A, R = rotor. Polymerization conditions as indicated, concentrations refer to the MgCl$_2$ concentration present during the incubation. Incubation time was 10 hours. The area inside the white dashed line has been auto-leveled.

Based on the obtained results the assembly of the full rotary apparatus was performed along this protocol:

1. Incubate clamp A with the rotor at 40$^\circ$C in the presence of 30 mM MgCl$_2$ for 24 hours.

2. Add clamp B and incubate at 40$^\circ$C in the presence of 30 mM MgCl$_2$ for 24 hours.

3. Add remover strands in excess at a ratio of 5:1 per binding site and incubate at room temperature for 24 hours.

EMA of the resulting samples (Fig. 2.7) revealed the appearance of two new bands corresponding to the expected mobilities of empty clamp dimers and fully assembled trimers. However, upon addition of the remover strand in the third step, the trimer band disappeared suggesting that the rotor was successfully unbound from clamp A as intended by the designed strand displacement scheme but it is able to diffuse out of the closed clamp dimer.

Negative-stain transmission electron microscopy (TEM) was used to verify the band assignments (Fig. 2.8) and the designed shape of all objects. The 90$^\circ$ corners of the brackets appear to be very flexible (Fig. 2.8a-b).

Taken together, the rotary apparatus successfully self-assembled and the rotor could be released from clamp A using strand displacement. However, the clamp dimer does not
2.1. Design and assembly of rotary apparatus version 1

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<th>10 bp overhangs</th>
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Figure 2.6.: **EMA of clamp - clamp dimerization.** Laser-scanned image of a 2% agarose gel with 11 mM MgCl$_2$. S = scaffold DNA, A = clamp A, B = clamp B. Polymerization conditions as indicated, concentrations refer to the MgCl$_2$ concentration present during the incubation. Incubation time was 10 hours.

Figure 2.7.: **EMA of rotary apparatus trimerization.** Laser-scanned image of a 2% agarose gel with 11 mM MgCl$_2$. S = scaffold DNA, A = clamp A, B = clamp B, R = rotor, 1 = rotor and clamp dimerization, 2 = bearing closure reaction in which the second clamp was added to reaction mixture in 1, 3 = rotor release reaction in which remover strands were added to the reaction mixture in 2, T = trimer, C-C = clamp dimer. All lanes have been auto-leveled below the pocket. The area inside the white dashed line has been separately auto-leveled.
2. Rotary apparatus made from 3D DNA origami components

Figure 2.8.: TEM of rotary apparatus v1. Exemplary negative-stain TEM micrographs of: a, Rotor and clamp unit. b, Clamp A - clamp B dimers. c, Fully assembled rotary apparatuses in side and top view (left and right). Scale bar is 50 nm.
Figure 2.9.: Schematic design of rotary apparatus v2. a. View of all components. Shape-complementary protrusions and recessions of the clamp - rotor interface are colored in red and magnified in insets. See Figs. B.3 and B.4 for full design schemes. b, First assembly step: rotor is docked into one clamp. Shape-complementary features of the clamp - clamp interface are colored in red and magnified in insets. Yellow arrows indicate positions of complementary sticky ends. The same sequences as in version 1 (see table B.2) are used. c, Second assembly step: Second clamp is bound. Brackets are colored in red. Insets highlight the bracket - clamp interface. Additional oligonucleotides (depicted in yellow) can hybridize to small single-stranded loops at the end of the brackets and the clamp (depicted in yellow). d, Fully assembled rotary apparatus with indicated dimensions. e, Rotary apparatus with unbound rotor. Figure as published in [48].

seem to spatially confine the rotor sufficiently after its release from the clamp. In addition, the overall assembly yield is low, largely limited by the clamp - rotor dimerization step. In order to address both issues, a second version was designed which is presented in the following chapter.

2.2. Design of rotary apparatus version 2

Figure 2.9 shows the components and assembly steps of the second version of the rotary apparatus. The sequential assembly strategy is generally similar to version 1 but version
2 differs from the first version in four aspects: (a) the bottom brackets are removed and the top brackets are reinforced, (b) the top brackets can be connected to the opposite clamp by additional oligonucleotides, (c) the interface between the clamp and the rotor is implemented using a shape-complementary protrusion and recession and (d) the lever arm cross section was reduced to a 6 helix-bundle and extended to a length of 100 nm.

Aspects (a) and (b) were both implemented to confine the rotor stronger inside the bearing. The limited length of the scaffold DNA required removing the bottom bracket in order to gain enough material to broaden and therefore strengthen the top brackets (Fig. 2.10). A potential caveat of this modification could be that the rotor can diffuse out through the open bottom side of the bearing in case the 90° corner between the rotor body and the lever arm is not sufficiently stiff to withstand thermal fluctuations. However, the assembled rotary apparatus will be docked onto a glass slide to perform single-molecule fluorescence microscopy experiments. The glass slide would seal the apparatus at the bottom and undocking of the rotor prior to surface attachment may be avoided by performing the entire assembly at high salt conditions which increases the binding affinity between rotor and bearing (Sec. 4.5 and [38]).

The interface between the clamp and the rotor was altered to increase the clamp - rotor dimerization yield. The clamp - clamp dimerization experiments of version 1 (Sec. 2.1) suggest that shape-complementary binding pockets allow dimerization with high yield. Other experiments in the Dietz lab performed at the same time suggested a significantly higher polymerization yield using shape-complementary protrusions and recessions compared to sequence-complementary single-strands ('sticky ends') as well [38]. In addition, avoiding the toehold-mediated strand displacement mechanism used in version 1, might
Figure 2.11: Design scheme of clamp v2. Individual parts as indicated. Protrusions and recessions are highlighted in red and blue respectively. Generated with caDNAno v0.2.
2. Rotary apparatus made from 3D DNA origami components

Figure 2.12: Design scheme of rotor v2. Individual parts as indicated. Generated with caDNAno v0.2.
reduce the risk of sterical hindrance caused by the remaining protruding double-strands after release of the rotor (see also Sec. 2.1).

In order to accommodate the binding pockets for the rotor, the scaffold routing in the clamp design had to be modified accordingly (Fig. 2.11). As in version 1, clamp A and B are similar except for the protruding single-strands used for clamp - clamp dimerization. Thus, two binding pockets for the rotor, one in each clamp, are present. The design of the rotor was only slightly altered by adding the protrusion for binding to the clamp and extending the lever arm (Fig. 2.12). The lever arm was extended to facilitate single-molecule fluorescence microscopy measurements by requiring less spatial resolution (see below).

### 2.3. Assembly and characterization

Various dimerization conditions were analyzed using EMA (Figs. B.10 and B.11) and suggested a similar polymerization protocol as for version 1 (Sec. 2.1). Instead of adding the remover strands in the last step of the polymerization protocol, in version 2, the oligonucleotides intended to connect the brackets to the opposing clamp are added. The resulting EMA of the individual assembly steps of the full rotary apparatus is shown in Fig. 2.13a.

The lanes containing the monomeric parts all yield a well-defined leading band (Fig. 2.13a, lanes 'A', 'B', and 'R') suggesting successful self-assembly of all parts. The monomer band of the rotor exhibits significantly higher mobility than the monomer bands of the two clamps since the rotor structure is more compact per design as the wider clamps.

The first dimerization step results in two independent bands with the same mobilities as the monomers when the gel is run in the presence of 11 mM MgCl$_2$ whereas a slower band appears in the presence of 22 mM MgCl$_2$ (Fig. 2.13 lane '1'). This suggests that the rotor - clamp dimer falls apart at lower MgCl$_2$ concentrations whereas it is stable at higher MgCl$_2$ concentrations thus confirming the typical binding behavior of shape-complementary protrusions and recessions.

Upon addition of clamp B two slower bands appear. The faster band can be attributed to empty clamp - clamp dimers by comparing it to a sample of just clamp A and B (Fig. 2.13 lane 'C-C' and '2') while the slower band presumably contains correct trimers.

Upon addition of the oligonucleotides to close the brackets, both bands show a slightly
2. Rotary apparatus made from 3D DNA origami components

Figure 2.13.: EMA of trimer assembly. **a**, Laser-scanned image of a 2% agarose gel with 11 mM MgCl₂. L = 1 kb ladder, S = scaffold DNA, A = clamp A, B = clamp B, R = rotor, C-C = clamp dimer, 1 = first assembly step containing rotor and clamp A, 2 = second assembly step where clamp B was added to reaction mixture in 1, 3 = third assembly step where bracket closing oligos were added to reaction mixture in 2. **b**, Same as in **a** but gel was run in the presence of 22 MgCl₂.
higher mobility (Fig. 2.13 lane ‘2’ and ‘3’). Since closed brackets should make the overall structure more compact this result supports the band assignment.
While the rotor - clamp A dimer falls apart in the presence of 11 mM MgCl$_2$, the trimer, even with open brackets, seems to be stable in the same conditions. The 90° corner of the lever arm is thus stable enough to prevent the rotor from diffusing out through the bottom of the axle bearing.

![Figure 2.14: Negative-stain TEM of assembly steps.](image)

**Figure 2.14.: Negative-stain TEM of assembly steps.** a-g. Schematic representation (top) and average TEM micrograph (bottom) of clamp (a), rotor (b), rotor - clamp dimer (c), trimer with open brackets (d), trimer with closed brackets (e), clamp - rotor dimer top view (f), and trimer with closed brackets top view (g). White arrows indicate rotor binding pocket in clamp and brackets. White circles indicate the approximate circular shape of the hexagonal cross section of the rotor in docked (dashed circle) and undocked (solid circle) position. h, Exemplary TEM micrograph of the final reaction mixture shown in 2.13 lane 3. All scale bars are 50 nm.
2. Rotary apparatus made from 3D DNA origami components

Negative-stain TEM confirmed that all components and assembly steps are in agreement with the design (Fig. 2.14). The rotor binding pocket inside the clamp is clearly visible (Fig. 2.14a) and at the position of the brackets a higher density is visible after addition of the oligonucleotides closing the brackets suggesting a higher stiffness of the brackets (Fig. 2.14d-e). The influence of connecting the brackets to the opposite clamp was further analyzed by imaging samples of clamp dimers with and without closing oligonucleotides using TEM (Fig. 2.15). The exemplary micrographs of single particles as well as average images indicate a significantly enhanced rigidity of the entire clamp dimer after closing the brackets. With open brackets the clamp dimer seems to be flexible enough to get flattened during staining and drying the sample on a TEM grid.

Figure 2.15. Electron microscopy of clamp dimers. a, Exemplary TEM micrograph of clamp - clamp dimers with open brackets and corresponding schematic representation (inset). b, Exemplary TEM micrograph of clamp - clamp dimers with closed brackets and corresponding schematic representation (inset). c, Average TEM micrograph of the sample shown in a. d, Multiple classes of average TEM micrographs of the sample shown in b. All scale bars are 50 nm.
2.4. Characterization of rotary motion

Nearly 20 years ago Kazuhiko Kinoshita and coworkers performed renowned single-molecule fluorescence microscopy experiments to study the rotary motion of the ATP synthase \[56, 57\]. In one set of experiments they attached micrometer-long fluorescently labeled actin filaments to the F1-ATP synthase. By immobilizing the modified F1-ATP synthases on a surface they were thus able to observe the rotary motion by recording and tracking the fluorescence signal of the labelled actin filament in a single-molecule fluorescence microscope. The experiments presented in the following section are inspired by the work of Kinoshita and coworkers.

Figure 2.16.: Fluorescence microscopy measurement of rotary apparatus. a, Schematic view of rotary apparatus attached to a glass slide via 10 biotin - NeutrAvidin - biotin connections. b, Top: schematic view of rotary apparatus overlayed in the two docked and intermediate positions. Red star indicates positions of 6 cyanine-5 dyes. Bottom left: exemplary single frames of a fluorescence microscopy movie of the cyanine-5 emission wavelength acquired in total internal reflection (TIRF) mode in the presence of 5 mM MgCl\(_2\) (see Fig. B.16 for all frames). Bottom right: sum over all 750 frames of the movie. All scale bars are 0.5 µm. c, Scatterplot of tip coordinates of the movie shown in b using centroid tracking. d, Time evolution (top) and corresponding histogram (bottom) of the rotation angle. The rotation angle is defined as the angle between (i) a line connecting the origin of the scatterplot and the mean of the bottom right cluster (scatterplot) of fitted coordinates and (ii) a line connecting the origin with the fitted coordinates.
In order to rigidly attach the rotary apparatus to glass slides a common protocol based on biotin - NeutrAvidin - biotin interactions which had been used in the Dietz lab before [38] was utilized. To this end, five staple oligonucleotides protruding at the bottom of each clamp were modified with a biotin group (Fig. 2.16 a). These oligonucleotides were chosen such that they cover roughly equally spaced positions along the clamp. In addition, six oligonucleotides in the tip of the lever arm were labeled with cyanine-5 dyes.

All fluorescence microscopy recordings were acquired on a home-built two-color total internal reflection fluorescence (TIRF) microscope available in the Dietz lab (see Sec. B.1.2 for details) and centroid tracking [58] was employed to quantitatively evaluate the measurements.

Measurements in the presence of 5 mM MgCl$_2$ revealed many spots that switch between two positions with a distance of 200 - 250 nm (Fig. 2.16 b-d). This observation may be interpreted as the rotor changing between the two docking sites in the axle bearing thus causing the lever arm to rotate by 180°.

To verify this hypothesis two additional rotor variants with different docking affinities were prepared (Fig. 2.17 a, design schemes: Figs. B.5 and B.6): The ‘static’ rotor version is bound to the clamp via sequence-complementary single-strands (4 nucleotides on each helix) protruding at each helix of the shape-complementary protrusion. The corresponding strands in the recession of the clamp are shortened such that the protruding single-strands from the rotor can directly bind to the scaffold strand in the clamp. In the ‘weak’ rotor version six oligonucleotides in the protrusion forming base-stacks with the clamp are shortened such that the stacking interaction between the blunt ends is deactivated due to their spatial separation.

EMA confirmed the successful assembly of trimers containing both rotor variants (Fig. B.12) and revealed the different binding affinities. While the rotor - clamp dimer with the normal rotor variant (’strong’) is bound in a gel with 22 mM MgCl$_2$ and falls apart in a gel with 11 mM MgCl$_2$ (Sec. 2.3), the static rotor variant was bound to the clamp in a gel with 11 mM MgCl$_2$ and the weak rotor variant undocks from the clamp even in the presence of 22 mM MgCl$_2$.

Single-molecule fluorescence microscopy recordings of all variants at different MgCl$_2$ concentrations were obtained and sorted into ’switching’ and ’stationary’ spots (Fig. 2.17 b). A spot was classified as ’switching’ when it exhibited at least one back-and-forth switching event during the time of the measurement. All measurements were independently per-
2.4. Characterization of rotary motion

**Figure 2.17.** Statistics of fluorescence microscopy measurements. 

**a.** Schematic representation of three rotor variants with different docking affinities (see Figs. 3.5 and 4.6 for design schemes).

**b.** Statistical ratio of switching vs. stationary spots. Unclassifiable recordings have been neglected. The total number of evaluated spots is 9490.

**c.** Histograms of docking dwell times in the presence of 5 mM MgCl₂ for the weak and strong rotor version (30 and 25 particles analyzed) and in the presence of 10 mM MgCl₂ for the weak rotor version (17 particles analyzed). Solid lines are double-exponential fits; dashed lines are single-exponential fits. Mean durations of docking dwells are: weak version 5 mM MgCl₂, 0.6 s (standard deviation (SD) 1.8 s); strong version 5 mM MgCl₂, 1.6 s (SD 3.6 s); and weak version 10 mM MgCl₂, 1.7 s (SD 2.4 s).
formed three times using separately assembled samples to account for sample-to-sample variations.
The static rotor variant is predominantly stationary in all tested MgCl$_2$ concentrations. Around 40% of the stationary rotary apparatuses containing the strong rotor variant exhibit switching behavior at 5 mM MgCl$_2$ but mostly stationary behavior at higher concentrations. The weak rotor variant is stationary at high MgCl$_2$ concentrations but switches frequently at 5 and 10 mM MgCl$_2$. These findings are in agreement with expectation since the static rotor variant should be rigidly attached to the clamp while the base-stacking interaction can be stabilized via the MgCl$_2$ concentration. The switching behavior of the rotor can thus be attributed to the docking sites in the clamps.

The particles that don’t show switching behavior even at low MgCl$_2$ concentrations (e.g. 60% in case of the strong rotor variant at 5 mM MgCl$_2$) can likely be attributed to non-specific binding of the lever arm to the surface or defective particles. In the filament-labeled ATP synthase experiments a high influence of the surface was observed as well. Around 80% of the particles were immobile, 20% of the particles showed some movement but only 1 out of 70 particles exhibited rotation [56].

To analyze the kinetics of the rotor docking reaction, average lifetimes of the bound state were extracted from the fluorescence microscopy data (Fig. 2.17c). The lifetimes of all rotor variants are on the scale of seconds and increase with the MgCl$_2$ concentrations and the number of blunt ends stabilizing the bound position via the rotor protrusion. A double-exponential distribution approximates the experimental lifetimes well whereas a single-exponential does not accurately represent the measured lifetimes. If both docking sites were exactly equal a single-exponential distribution of the lifetimes would be expected. The double-exponential distribution indicates that different lifetimes are present which may be attributed to surface or attachment-induced deformations of the rotary apparatus as well as defects such as truncated staple oligonucleotide inside the binding pockets.

To demonstrate the modularity of the rotary apparatus, a clamp version with three docking sites was prepared (Fig. 2.18a, design scheme: Fig. B.7). In order to accommodate three docking sites the clamp design described above needs to be modified since each clamp only contains two out of six complete outer walls (Fig. 2.1b) thus allowing only two docking sites per clamp. Consequently, the connection between the two clamps was moved such that it is positioned in two opposing corners of the hexagonal cross section of the clamp dimer.
2.4. Characterization of rotary motion

Figure 2.18.: Fluorescence microscopy measurements rotary apparatus with 6 docking sites. a, Schematic representation (top) and average negative-stain TEM micrograph (bottom) of clamp with three docking sites. White arrows indicate positions of docking sites. Scale bar is 50 nm. b, Scatterplot of exemplary single-particle fluorescence microscopy recording of a rotary apparatus with three docking sites. 750 frames with an exposure time of 50 ms were recorded in total. c, Average rotation angle histogram of 11 single-particle recordings in which the $0^\circ$ position of each recording was assigned to the cluster with the highest occupation frequency.
2. Rotary apparatus made from 3D DNA origami components

Another aspect of scaffolded DNA origami objects affects this design: neighboring helices are always anti-parallel, i.e. in the clamp one could define for each helix in the wall an 'up' or 'down' direction. The shape-complementary protrusions and recession were so far designed such that the blunt ends which are supposed to stack to each other are oriented as in natural B-form DNA following the 5' to 3' direction, i.e. a 'down' helix would meet a 'down' helix in the rotor in the stacked conformation. The pattern of 'up' and 'down' does however alternate from side to side in the hexagonal shape of the clamp dimer. It is thus not possible to design all three binding pockets such that the stacks are oriented in the natural direction. The middle binding pocket in the modified clamp version is therefore reversed. Although it is likely that there is some interaction between the aromatic rings also in the reversed orientation, the strength of this stacking interaction has not been measured to date and will likely be different compared to the natural orientation. In addition, the binding affinity of the rotor protrusion to the other binding pockets will also be different since no scaffold permutation could be found such that the identity of the bases at the blunt ends are exactly equal (see also Sec. A.1).

EMA confirmed the successful polymerization of rotary apparatuses using the clamp with three docking sites (Fig. B.13) and negative-stain TEM confirmed the shape of the clamp by clearly showing three docking sites in average micrographs (Fig. 2.18b).

Single-molecule fluorescence microscopy experiments revealed many recordings in which the labeled rotor tip seems to frequently occupy multiple but defined positions which are roughly positioned on a circle with a radius of 100 nm (Fig. 2.18c). Typically three to four clusters of positions are visible with different occupation frequencies. The clusters may be attributed to the docking sites and different occupation frequencies are presumably caused by different binding affinities. A few recordings showed up to 5 clustered positions but one cluster was typically occupied in only a few frames. The latter may be attributed to the docking sites with flipped orientations of the blunt ends. The asymmetry of some recordings (3 or 5 clustered positions) might also indicate slight deformations in the rotary apparatus since by design each docking site occurs twice which should result in even numbers of clustered positions.

To further analyze the pattern of clustered positions, the rotation angle histograms of 11 individual single-particle recordings were averaged by setting the 0° position of each spot to the cluster position with the highest occupation frequency (Fig. 2.18c). The thus obtained average rotation angle histogram contains three peaks with different amplitudes which are roughly spaced by 60°. Each peak may be attributed to a docking site and the amplitude to the binding affinity.
2.5. Direct observation of rotary motion

The previous section presented experiments demonstrating that the mobility of the rotor inside the axle bearing can be rationally controlled. A direct observation of the rotary motion in a single fluorescence microscopy movie frame was not obtained though, presumably because the motion of the rotor is too fast for the time resolution of the microscope.

Kazuhiko Kinosita and coworkers were facing a similar problem with the ATP synthase when they attached the labeled actin filament \[56, 57\] in order to use fluorescence microscopy. The rotation of the ATP synthase was too fast to discriminate sub-steps between the observed 120° steps. They switched to dark-field microscopy which required attaching a gold particle of around 40 nm diameter to the ATP synthase allowing a much faster readout with exposure times around 100 µs \[59\]. With this setup they were able to discriminate additional sub-steps between the 120° steps.
A similar idea was applied to the rotary apparatus described in the remainder of this chapter. Instead of changing the type of microscopy, the rotor was extended with a 430 nm-long 6 helix bundle (Fig. 2.19a and B.8) in order to increase the drag and slow down the rotation. The extension is connected to the rotor via 6 sequence-complementary sticky ends each containing 3 G-C basepairs. Even though the persistence length of a six-helix bundle is around 2 \( \mu \text{m} \) [60] and thus significantly smaller than the persistence length of an actin filament (16-17\( \mu \text{m} \) [61, 62]), the extended lever arm should be sufficiently stiff to serve as an indicator.

The diffusion coefficient can be used to estimate the differences in rotation speed between the short and the extended rotor. According to [63] the rotational diffusion coefficient of a cylinder orthogonal to its long axis can be written as:

\[
D^\perp_R = \frac{3k_B T}{4\pi \eta L^3} (\ln \frac{L}{d} + X^\perp_R(p))
\]

where \( \eta \) is the viscosity of the fluid and \( X^\perp_R \) is a correction term for the geometry of the caps of the cylinder which is in first-order approximation independent of \( p \). The lever arm can be approximated as a cylinder with diameter \( d = 8 \text{ nm} \) and with length \( L_s =120 \text{ nm} \) for the short version and \( L_l = 500 \text{ nm} \) for the long version. The calculated diffusion constant is thus around 47-fold smaller for the long rotor version than for the rotor with the short lever arm.

EMA was used to verify successful polymerization and test various assembly strategies (Fig. B.14). Negative-stain TEM confirmed the expected shape of the extended rotor with a lever arm length of in total 0.5 \( \mu \text{m} \) (Fig. 2.19 b).

Single-molecule fluorescence microscopy measurements of rotary apparatuses containing the extended rotor with 6 cyanine-5 dyes at the tip of the extended lever arm were obtained in a similar fashion as above at 5 mM MgCl\(_2\). Single frames of individual spots frequently showed arc-like signatures (Fig. 2.20 left) of up to 270° with a radius of around 0.5 \( \mu \text{m} \). These images thus directly confirm the rotary motion of the tip of the lever arm. The largest arc-like signatures of 270° with an exposure time of 50 ms correspond to an angular velocity of 94 rad \( \text{s}^{-1} \).

Two corresponding types of sum-image signatures were observed: (a) double spots with a spacing of around 1 \( \mu \text{m} \) and (b) donut-shaped circles with a diameter of around 1 \( \mu \text{m} \) (Fig. 2.20 right). The double spot signatures can be directly related to the results obtained with the short lever arm. These particles presumably switch between the two opposing


2.5. Direct observation of rotary motion

Figure 2.20.: Exemplary single-molecule recording of extended rotary apparatus. a-b, Exemplary frames of two single-particle fluorescence microscopy recordings acquired in total internal reflection (TIRF) mode (left) and corresponding sum over all 1500 frames (right). Scale bar is 0.5 \( \mu \)m. See Figs. B.19 and B.20 for all frames.

docking sites present in the bearing. Particles with the donut-shaped signatures appear to freely rotate without a preferential binding site. This behavior may be attributed to deformations of the docking sites induced by distortions of the clamp caused by non-matching surface distributions of NeutrAvidin.

A question that remains is why the latter category was not observed with the short lever arm. Since the motion of the short rotor will be significantly faster individual frames would likely predominantly feature a donut-shaped signature. Applying centroid tracking to these donut-shaped images will result in a fitted position in the center of the donut thus letting these particles essentially appear stationary in scatterplots of the fitted coordinates of the lever arm tip.

Besides these two types of recordings several other sum-image signatures were observed (Fig. B.21a). For instance, sum-image signatures resembling a half circle indicate a deformed rotary apparatus with sterically confined rotor mobility. Filled circle sum-image signatures can be caused by either a defect 90° corner of the lever arm which causes the lever arm to freely fluctuate or attachment of only one biotin anchor to the glass slide allowing the entire apparatus to rotate. Around 90% of all particles appear to be stationary which can likely be attributed to strong non-specific surface attachment of the lever arm which is expected to be significantly stronger than for the short lever arm due to its length.
To verify that the donut-shaped sum signatures are not a mere effect of the evanescent illumination mode, additional recordings using epi-fluorescence illumination were obtained (Fig. 3.21b). Sum-images of these recordings showed donut-shaped sum signatures and thus confirmed the results obtained in evanescent-illumination mode.

2.6. Conclusions and Outlook

Taken together, the experiments presented in this chapter demonstrate the successful assembly and validation of a rotary mechanism which is driven by Brownian motion of surrounding water molecules and ions. The design of this mechanism allows control over the mobility of the rotor, the number of docking sites and the dwell times at these docking sites. It thus resembles some of the functionality of natural molecular motors but it is not capable of driven rotary motion using an energy source.

A candidate theory to describe biological molecular motors is the concept of Brownian motors [64–67]. In Brownian motors directional motion is generated by thermal fluctuations of the moving objects in an asymmetric ratchet-shaped potential which periodically changes between different levels. These periodical fluctuations can be loosely coupled to a chemical reaction such as ATP binding and subsequent hydrolysis. The fluctuations can be described either by changes in the surrounding potential landscape or by changes of the energies of the mobile objects themselves. In both options the relative difference between the energies of the mobile objects and the potential landscape changes. Such periodic fluctuations thus alter the mobility of the moving objects between a state of high and low mobility.

In the design presented here, the mobile object is the rotor and the potential landscape is formed by the two clamps. An asymmetric ratchet potential could be implemented by inserting ratchet-like protrusions on the inside of the clamps. The strength of these ratchets could determine the depth of the resulting ratchet potential. Several ways to implement fluctuations to this potential can be envisioned. (1) Fast heating and subsequent equilibration of the surrounding water with an infrared (IR) laser inside a fluorescence microscope [68], or (2) coupling the strength of the ratchets to an enzymatic reaction, such as DNA cleavage by DNAzymes [69].

Measuring such directed rotary motion might require switching from fluorescence microscopy to dark-field microscopy which allows a much faster exposure time due to a higher photon flux. A short exposure time would avoid the arc-like signatures in sin-
gle frames observed above which are difficult to handle when using automated centroid tracking.

The rotary apparatus presented here may be seen as a platform to test various concepts. Apart from Brownian motors, the next chapter describes an extension of the rotary apparatus which aims at using the energy in an electroosmotic flow.
3. Extended rotary apparatus for directed rotation by an electroosmotic flow

The following chapter presents the design and experiments to characterize the assembly of an extended rotary apparatus which is meant to be driven by an electroosmotic flow in a solid-state nanopore. This project is a collaboration with the Dekker lab at TU Delft. Daniel Verscheuren will perform the single-molecule microscopy of the DNA nanostructure in solid-state nanopores in the Dekker lab.

3.1. Introduction

A rotary apparatus which is driven by an electroosmotic flow (eof) should feature an asymmetric part that resembles the properties of rotor blades at the macroscopic level. Collisions of ions and water molecules with the blades would exert a force driving a directed rotary motion. To accommodate such rotor blades in the rotary apparatus presented in the previous chapter, the rotor unit was extended with a twisted bottom part (Fig. 3.1a-b). This bottom part is intended to be inside the nanopore once it is docked since the strongest flow occurs there [70]. In addition, the two clamps were also extended to prevent undesired interactions of the twisted rotor bottom and the membrane in which the nanopore is located. Each clamp features a double-stranded leash of around 400 bp at the bottom (similar as the ring in chapter 4) to support pre-orientation of the apparatus while docking it to the nanopore (Fig. 3.1c-d). Two flaps pointing outward are meant to dock the apparatus in the nanopore and prevent it from being dragged through. The thus obtained ∼30 MDa object requires a total of 6 circular scaffold DNA molecules. It will be referred to as extended rotary apparatus in the remainder of this chapter.

Florian Praetorius and Christian Wachauf recently designed and produced a 7560 bp long circular DNA sequence in the Dietz lab as part of a separate project. The sequence was chosen such that no sequence of 7 consecutive bases occurs twice within in the strand. If this circular DNA strand is used as a scaffold DNA for DNA origami objects, it can be self-assembled together with a conventional m13mp18-derived scaffold DNA in a one-pot
3. Extended rotary apparatus for directed rotation by an electroosmotic flow

Figure 3.1.: Schematic design of extended rotary apparatus (eRA).

- **a.** View of all components with twisted rotor extension (purple) and brackets, docking sites and outer flaps (all red).
- **b.** Cross section of the body of the assembled eRA with clamps (light and dark gray), rotor (blue) with central rotation axis (red), twisted part (orange), and the docking sites in the clamps (purple).
- **c.** Assembled eRA and solid-state nanopore with applied voltage between both sides.
- **d.** eRA docked into a solid-state nanopore. Black arrows indicate flow of ions which is meant to cause rotation of the rotor (indicated by red arrow).
3.2. Design and assembly of the rotor

Figure 3.2.: Schematic scaffold DNA arrangement of eof-rotor versions 1-3. The two scaffold DNA strands are depicted in blue and red. See Figs. C.1, C.2, and C.3 for full design schemes including sequences.

reaction into one object. It thus essentially allows the design of objects which are twice as large as conventional objects. This method was used to extend each component of the rotary apparatus by adding a second scaffold DNA molecule to form the extended bottom part.

A fast readout will be provided by a custom-built dark-field microscopy setup which can contain flow cells with nanopores at TU Delft (see also Sec. 2.5). To generate a sufficiently strong signal in the microscope, a gold nanoparticle (AuNp) will be attached to the tip of the rotor. Besides a fast readout, a rigid attachment of the clamps to the membrane may be necessary to avoid rotation of the entire rotary apparatus which would not allow to discriminate and control the rotor rotation. The rigid surface attachment will also enable reversing the flow after docking.

3.2. Design and assembly of the rotor

Four design iterations of the extended rotor with blades (referred to as eof-rotor in the remainder) were necessary to obtain well-folded objects. All versions were designed sequentially based on the results of the previous version. Version 1 - 3 differ in the relative arrangement of the two scaffold DNA strands within the object (Fig. 3.2). In version 1, one scaffold strand forms the twisted bottom part and half of the central rotation axis including the lever arm. The second scaffold strand thus forms the upper body of the eof-rotor around the central axis including the shape-complementary protrusions as well as the other half of the central axis and the lever arm. In version 2, the two scaffold strands
are separated into an upper (lever arm) and a lower part (body and twisted blades). The scaffold strand forming the lever arm protrudes around 30 nm into the eof-rotor body to provide enough overlap for a rigid connection of both parts. In version 3, all parts of the object contain both scaffold strands in roughly equal parts.

EMA was used to evaluate different thermal annealing ramps for self-assembly reactions of version 1 (Fig. C.7a). A successfully folded object containing two scaffold strands should exhibit a similar mobility as two dimerized objects containing one scaffold strand each. All tested annealing ramps of version 1 yield only very faint bands while high intensities occur in the pocket suggesting that aggregates form during self-assembly. A faint leading band with a similar mobility as the scaffold DNA appears in long folding ramps such as 60-40 °C with 2 h/° indicating that it does not contain fully assembled eof-rotors. A slower, equally faint band might correspond to fully assembled objects. Negative-stain TEM imaging after thermal annealing confirmed many aggregates and defective objects (Fig. 3.3a) in agreement with the EMA data. Imaging of the fast leading band (Fig. 3.3b) shows folded rotor bodies with an additional undefined tail. The images suggest the following interpretation: The arrangement of the two scaffold strands supports the formation of kinetic traps during the thermal annealing ramp since one scaffold strand forms a part (the body) that fully circumvents the central axis which is formed by the other scaffold strand. If the body forms first during the thermal annealing ramp, the second scaffold strand will likely not be able to fold the central axis inside the body.

In order to solve this issue the scaffold arrangement was altered in version 2 such that the two scaffolds are significantly less intertwined. EMA of the reaction mixtures after thermal annealing (Fig. C.7b) revealed two strong bands with a similar mobility as a single-scaffold object indicating that both scaffold strands form their half of the full object independently. EMA of self-assembly reactions using short temperature intervals (Fig. C.7, left) suggests that the self-assembly of the two objects happens at different temperatures. The faster band appears below 60 °C while the slightly slower part appears below 52 °C.

This behavior may be attributed to the geometric differences of the designed shapes of the two parts. Assuming that the shape of the object correlates with the temperature interval in which the folding reaction takes place a significant geometric difference might lead to different folding temperatures. During an annealing from e.g. 60 to 40 °C one part might form first at a higher temperature while the second part folds independently at a lower temperature. Since the staple oligonucleotides are present in excess over scaffold DNA, those staple oligonucleotides which are intended to connect both parts can independently
bind to both folded parts and will inhibit any connection.

Based on these findings the scaffold arrangement of version 3 was modified such that both scaffolds cover an equal geometry. EMA (Fig. C.8a) confirmed successful assembly into a monomeric object of the expected size and negative-stain TEM imaging confirmed the overall shape of the eof-rotor (Fig. 3.3c). However, the images also indicate that the bottom part containing the twisted rotor blades did not fold into a well-defined shape.

![Figure 3.3.](image)

**Figure 3.3.:** Exemplary negative-stain TEM micrographs of eof-rotor versions. **a.** Unpurified eof-rotor v1 (annealing ramp: 60-40°C with 1 h/° in the presence of 20 mM MgCl₂). **b.** Gel cutout (see Fig. C.7 for gel) of eof-rotor v1. **c.** Non-purified sample of eof-rotor v3 (annealing ramp: 60-40°C with 1 h/° in the presence of 20 mM MgCl₂). **d.** PEG-purified sample of eof-rotor v4. Scale bar is 100 nm.

The first three versions are designed such that the twisted rotor blades feature a left-handed twist by stretching and over-twisting the DNA helix to 240° every 6 basepairs instead of 7 as described in [29]. The typical sequence-complementary binding domains of staple strands to the scaffold strand are thus reduced to multiples of 6 instead of 7 in the bottom part. An alternative is to induce a right-handed twist by compressing...
and under-twisting the DNA to 240° every 8 basepairs. The typical binding domain of a staple is 2 bases longer in this case compared to the left-handed twist which might enhance folding quality.

This assumption was tested in eof-rotor v4 by inserting 1 basepair every 7 bases. EMA (Fig. C.8 b) confirmed successful assembly into a monomeric object of the expected size and negative-stain TEM imaging confirmed the overall shape of the eof-rotor (Fig. 3.3 c) including the twisted rotor blades.

3.3. Design and assembly of the clamp

Two versions of the extended clamp (referred to as eof-clamp in the remainder) were designed (Fig. 3.4): (1) with an additional bracket in the middle to reinforce the bearing and (2) without the middle bracket. Both brackets can be connected to the opposite clamp with additional oligonucleotides. To this end, a small opening was inserted in the first clamp version serving as a binding pocket for the middle bracket of the opposing clamp. Two versions were designed since the additional bracket in the middle might sterically hinder attachment of the rotor. Both versions feature a flap consisting of 10 helices that is pointing outward which is meant to dock the rotary apparatus in the nanopore by preventing it from being dragged through. The flaps are implemented using 90° corners in the corresponding helices in the upper part of the clamp. Four protruding single-strands are placed at the bottom of each of these flaps which can be used as attachment anchors for complementary strands with a chemical modification for rigid surface attachment. The two scaffold strands are separated into the top and bottom half in both clamp ver-
3.4. Trimer assembly

sions (Figs. C.5 and C.6). Based on the findings presented in section 3.2, this arrangement should support successful self-assembly reactions since the geometries of the upper and lower part are very similar.

EMA confirmed successful self-assembly of both eof-clamp versions (Fig. C.9). To test the influence of the middle brackets both versions were trimerized with rotor v2 (see Sec. 2.3) and analyzed using EMA (Fig 3.5a).

Trimerization reactions with eof-clamp v1 yield a leading band consisting of rotor monomers and a very faint slower band which can be attributed to clamp dimers by comparing it to a sample with just clamp A and B (lane C-C v2). The result suggests that the clamps strongly aggregate, either as monomers or dimers, since no corresponding bands are visible.

Trimerization reactions with clamp v2 yield three bands which can be attributed to (from fast to slow) rotor v2 monomers, clamp v2 dimers, and trimers consisting of clamp A, rotor v2, and clamp B. The trimer band appears to be the strongest at incubation conditions of 30 mM MgCl₂ and 35°C. Negative-stain TEM imaging supports the band assignment (Fig. 3.5b).

Besides the trimerization reactions, dimerizing the two clamp versions directly without a rotor (Fig 3.5a lane C-C v1 vs. v2), reveals that the dimer band of version 2 is significantly stronger and faster suggesting a more compact structure and a higher yield. The difference in mobility between the two dimers may be explained by the middle bracket which widens the dimer in case it does not bind into the corresponding opening in the opposing clamp.

Negative-stain TEM was used to confirm the shape of the two clamp versions and their dimers (Fig 3.6). The clamp v2 dimers appear to be significantly more compact thus confirming the lower mobility of clamp v2 dimers in EMA compared to clamp v1 dimers which may be attributed to non-bound middle brackets.

To summarize, the middle brackets seem to inhibit binding of the rotor and version 2 was correspondingly used for all experiments in the remainder of this chapter.

3.4. Trimer assembly

The trimer assembly protocol was iteratively optimized using EMA resulting in the following steps:
Figure 3.5.: Experimental comparison of eof-clamp version 1 and 2. a, Laser-scanned image of a 1.5% agarose gel with 11 mM MgCl₂. L = 1 kb ladder, R = rotor, A = eof-clamp A v1, B = eof-clamp B v1, C-C v1 = eof-clamp dimer v1, C-C v2 = eof-clamp dimer v2. All other lanes: Trimerizations of eof-clamp v1 and v2 with rotor v2 including bracket closing oligonucleotides. Trimerization conditions as indicated, concentrations refer to the MgCl₂ concentration present during the incubation. All lanes have been globally auto-leveled below the pockets. b, Exemplary negative-stain TEM micrograph of trimerization sample with eof-clamp v2 incubated in the presence of 30 mM MgCl₂ at 35° C. Scale bar is 50 nm.
Figure 3.6.: Negative-stain TEM micrographs of eof-clamp version 1 and 2. a, PEG-purified eof-clamp v1. b, Eof-clamp v1 dimers including bracket closing oligonucleotides. c, PEG-purified eof-clamp v2. d, Eof-clamp v2 dimers including bracket closing oligonucleotides. Scale bar is 50 nm.
1. Incubate eof-clamp A with the eof-rotor at 45° C in the presence of 20 mM MgCl₂ for 24 hours.

2. Add eof-clamp B and incubate at 45° C in the presence of 20 mM MgCl₂ for 24 hours.

3. Add bracket closing oligonucleotides in excess at a ratio of 5:1 per binding site and incubate at room temperature for 24 hours.

An exemplary screen of various assembly reactions analyzed by EMA is shown in Fig. 3.7a. The trimerization reactions yield four bands which can be attributed to (from fast to slow): rotor monomers, empty clamp dimers, correct trimers, and higher order structures presumably consisting of four or more clamps since it is also present in samples containing clamp A and clamp B. Negative-stain TEM imaging yielded a particle distribution which supports this band assignment (Fig. 3.7b).

Average images of assembled extended rotary apparatuses obtained from negative-stain TEM micrographs confirmed that the shape agrees with the design (Fig. 3.8a). A tomosogram of a single particle calculated from a negative-stain TEM tilt series (Fig. 3.8b) was calculated to verify the shape of the extended rotary apparatus in the third dimension. The outer flap and one leash at the bottom of one clamp are clearly visible and match the expected positions.

3.5. Conclusions and Outlook

In summary, this chapter presents the successful assembly of an extended version of the rotary apparatus aiming at directed rotary motion driven by an electroosmotic flow through a nanopore. Several design iterations of the components were necessary to obtain a reasonable folding yield. According to these experiments, the folding yield for objects containing two scaffold DNA strands appears to be significantly enhanced when the parts formed by the two scaffolds are similar regarding their geometric shape. Further designs containing two scaffold strands in the future will have to validate this finding.

Several further steps are necessary before the extended rotary apparatus can be tested in the Dekker lab at TU Delft:

- The attachment of gold nanoparticles (AuNp) to the tip of the lever arm needs to be verified and optimized.
3.5. Conclusions and Outlook

Figure 3.7.: EMA and negative-stain TEM of trimer assembly. a, Laser-scanned image of a 1.5% agarose gel with 11 mM MgCl₂. R = eof-rotor v4, A = eof-clamp A v2, B = eof-clamp B v2, C-C = ext. clamp v2 dimers. All other lanes: Trimerizations of eof-clamp v2 with eof-rotor v4 including bracket closing oligonucleotides. Trimerization conditions as indicated, concentrations refer to the MgCl₂ concentration present during the incubation. Area inside the white dashed line has been separately auto-leveled. b, Exemplary negative-stain TEM micrograph of a trimerized sample in the presence of 20 mM MgCl₂ at 45° C. Red arrows indicate fully assembled trimers. Scale bar is 100 nm.
3. Extended rotary apparatus for directed rotation by an electroosmotic flow

Figure 3.8.: Average negative-stain TEM micrograph and tomogram of trimer. a, Schematic view (top) and average negative-stain TEM micrograph (bottom) of trimer consisting of two eof-clamps and an eof-rotor. b, Top: Schematic side view of the trimer with indicated positions of slices, bottom: Corresponding tomogram slices (ordered 1 to 4 from top left to bottom right). White arrow indicates visible leash. Scale bars are 50 nm.

- The surface attachment of the outer flaps of the clamps to the membrane (typically silicon nitride (SiN₂) in the Dekker lab) has to be tested with various chemical reaction partners on both sides. The surface charge of the modification on the membrane is expected to play an important role. A positive surface charge might repel the rotary apparatus while a negative surface-charge could lead to unspecific binding in a non-docked position.

The most critical question is whether the asymmetry of the bottom part of the rotor is sufficiently strong such that collisions with water molecules and ions are strong enough to drive the rotor. A significant part of the electroosmotic flow might be non-usable since DNA origami objects are known to be permeable to ions [71]. Depending on the experimental results of the dark-field microscopy measurements with an applied electroosmotic flow the design of the bottom part of the rotor may need to be adjusted.
4. Positioning FG-NUPs using a DNA origami scaffold

This chapter presents the assembly and characterization of a DNA origami ring designed to match the radial dimension of the inner channel of nuclear pore complexes (NPCs) [5, 72–74]. NPCs are located in the nuclear envelope in eukaryotic cells and mediate transportation between the nucleus and the cytoplasm. The permeability of NPCs is controlled by FG-Nucleoporin proteins (FG-NUPs) which are located inside the central channel. The DNA ring presented here allows attachment of up to 32 copies of these intrinsically disordered proteins inside the ring and can thus be used as a tool to position and study the properties of these proteins.

After a short introduction to NPCs (Sec. 4.1), multiple design iterations to assemble the DNA ring are described (Sec. 4.2). Section 4.3 presents experiments to confirm the successful attachment of NSP1 and a mutated variant (NSP1-S) to the ring. Cryo-electron microscopy (cryo-EM) was subsequently used to quantitatively study the density of the proteins inside the rings (Sec. 4.4). Section 4.5 concludes with initial experiments to stack multiple rings together aiming at more complex assemblies.

The research was performed in collaboration with the Dekker lab at TU Delft, the Onck lab at University of Groningen and the Görlich lab at the Max Planck Institute for Biophysical Chemistry in Göttingen. Adithya Ananth performed the electrophoretic conductivity measurements on solid-state nanopores (not shown in this thesis) as well as the fluorescence microscopy measurements in the Dekker lab. Ankur Mishra performed coarse-grained molecular dynamics (MD) simulations of the proteins in the Onck lab. Dirk Görlich provided the NSP1 and NSP1-S proteins. Eva Bertosin operated the cryo-EM microscope and acquired the raw data for the cryo-EM measurements in the Dietz lab at TU Munich. Except for section 4.1 and 4.3 most parts of this chapter together with additional data obtained by Adithya Ananth and Ankur Mishra were submitted for publication.
4. Positioning FG-NUPs using a DNA origami scaffold

Figure 4.1.: Schematics of nuclear pore complex and DNA ring. a, Illustration of a nuclear pore complex. Taken with permission from [75]. b, DNA ring with attached FG-NUPs proteins. c, DNA ring with attached proteins and a solid-state nanopore.

4.1. Introduction

The genetic information in eukaryotic cells is stored in the nucleus which is separated from the cytoplasm by the nuclear envelope. This separation protects the nucleus but also requires efficient and selective transport through the envelope. Besides many types of RNA, larger objects, such as ribosome subunits need to be able to get from the nucleus to the cytoplasm. Nuclear pore complexes (NPCs) are located in the nuclear envelope and fulfill this task [5,72–74]. NPCs are large complexes (60 MDa in yeast and 120 MDa in humans) comprised of many copies of around 30 different proteins called nucleoporins (NUPs). The inside of the transport channel is filled with intrinsically disordered FG-nucleoporins (FG-NUPs), named after the FG-repeats (Phenylalanine (Phe) - Glycine (Gly) residues) or FXFG-repeats\(^1\) (Fig. 4.1a). Understanding the structural details as well as the corresponding transport mechanism of NPCs is an active field of research. While the understanding of the structured outer part of the NPCs is increasing, the structural details and the corresponding selective permeability of the FG-NUPs in the inner channel remain under debate.

While NPCs are permeable for ions and molecules smaller than \(\sim 30\,\text{kDa}\), larger molecules such as RNA and proteins need to attach to specialized nuclear transport receptors (NTRs) in order to pass through the pores [76]. The NTRs can shuttle cargo objects of a diameter of up to 40 nm through the NPCs [77]. The selective permeability as well as the transport mechanism are known to be mediated by interactions of the FG do-

\(^1\)X can be any arbitrary residue.
mains of the FG-NUPs among each other and with the NTRs but the exact mechanism is poorly understood. Several models explaining the transport mechanism have been proposed [78–81].

Understanding the role of different FG-NUPs and distinguishing which residues are critical for the transport mechanism remains challenging. *In vivo* studies suffer from the complexity of NPCs and missing in situ methods that allow studying the structure at the nanoscale. Reconstituting the NPC complex for *in vitro* studies has not been feasible so far.

A simplified system, in which a single or a few variants of FG-NUPs are positioned in a similar arrangement as in NPCs would facilitate studying the influence of individual FG-NUPs as well as the influence of mutations. Such minimal artificial NPCs have been realized by attaching a single type of FG-NUP to nanopores within a polymeric or solid-state SiN membrane [82,83]. However, this approach does not allow controlling the number and position of the FG-NUPs as well as combining multiple types of FG-NUPs.

The remainder of this chapter describes the assembly and characterization of a ring using the DNA origami technique. The ring matches the diameter of NPCs and can thus serve as a scaffold for positioning FG-NUPs (Fig. 4.1b) featuring up to 32 protein attachment sites pointing radially inward. The DNA origami technique allows precise control over the number and positions of attachment sites. A leash at the bottom of the ring is intended to facilitate docking to solid-state nanopores in order to perform conductivity measurements as in [84] (Fig. 4.1c). The FG-NUP NSP1 as well as a mutated version NSP1-S are used to demonstrate the successful attachment.

### 4.2. Design and assembly of the DNA ring

A DNA ring which is intended to be a scaffold to arrange FG-NUPs similar to the central channel of NPCs which allows conductivity measurements should meet the following criteria:

- Match the inner diameter (~35 nm) of NPCs.
- Provide many attachment positions for proteins pointing inward.
- Feature a double-stranded leash at the bottom to facilitate docking to solid-state nanopores in the desired orientation.
4. Positioning FG-NUPs using a DNA origami scaffold

Figure 4.2.: Geometry of DNA ring. a, Schematic top view of DNA ring with indicated dimensions. b, Schematic cross section of the ring with indicated dimensions, start of the leash (green) and location of SiN₂ membrane (grey) in the docked position.

Based on these requirements a design outline of the DNA ring was devised (Fig. 4.2). It features a leash of around 500 bp and can host a total of 32 attachment sites at two different height levels inside the ring. The precise design of the attachment anchors will be discussed in section 4.3. Only one similar design of a DNA origami ring was published recently in the context of a different application [18]. The inner diameter of this ring is 29 nm but the cross section consists only of 6 helices presumably causing this ring to be mechanically less rigid.

Curvature in DNA origami objects can be induced by adding DNA basepairs in the outer (‘insertions’) and removing DNA basepairs (‘deletions’) in the inner helices [29, 30]. This effectively stretches or shortens the helices respectively. However, the presence of insertions and deletions in neighboring helices requires over- and undertwisting of the respective helix when Holliday junctions are placed at regular intervals between the helices. Due to the negative twist-stretch coupling in DNA [85, 86] an equal number of insertions in the outer helices and deletions in the inner helices results in a global left-handed twist. This global twist can potentially be compensated by placing additional insertions, which induce a global right-handed twist. The twist-compensation is specifically important when the helices are intended to be connected to themselves at the two ends as it is necessary in a ring.

2The precise length of the leash varies slightly between the different versions.
4.2. Design and assembly of the DNA ring

Figure 4.3.: Distribution of deletions and insertions in ring v1 and v2. Schematic cross section with number of additional basepairs per 7 bases indicated below for ring version 1 (a) and version 2 (b). See Figs. D.1 and D.2 for full design schemes incl. sequences.

The design schemes were optimized in a tedious iterative procedure using CanDo [54]. In order to optimize the pattern of insertions and deletions, such that the radius of curvature is correct and no global twist occurs, the staple oligonucleotides connecting the helices at the ends which close the ring were removed before submission to CanDo. In this way two initial versions were conceived which only differ in their arrangement of insertions and deletions (Fig. 4.3). In version 1, additional insertions to compensate the global twist are placed on the outer helices. This required the insertion of 2 additional basepairs every 7 basepairs on the outermost helices which strongly alters the number of basepairs per full turn of these helices. In contrast, in version 2 the 'neutral' middle line was shifted such that more insertions are present. Two versions were created since version 1 may require passing high energy barriers during the self-assembly reaction due to the strong distortion of the outer helices.

Electrophoretic mobility analysis (EMA) was used to analyze the self-assembly reactions of version 1 and 2. Using various thermal annealing ramps only very faint bands were observed with a leading band with slightly lower mobility then the circular scaffold DNA (Fig. 4.4) suggesting that the objects did not fully assemble. The results of the self-assembly reactions were imaged using negative-stain transmission electron microscopy (TEM) (Fig. 4.5). The images show objects that coarsely resemble the shape of a ring with the designed dimensions but are significantly distorted and thus verify that the rings
4. Positioning FG-NUPs using a DNA origami scaffold

Figure 4.4.: EMA of self-assembly of ring v1 (a) and v2 (b). Laser-scanned images of a 2% agarose gels with 11 mM MgCl$_2$. S = scaffold DNA. Folding ramps as indicated in the presence of 20 mM MgCl$_2$. Areas inside the white dashed lines have been auto-leveled.
4.2. Design and assembly of the DNA ring

Figure 4.5.: Negative-stain electron microscopy of ring v1 and v2. Exemplary negative-stain TEM micrographs of ring version 1 (a) and version 2 (b). The thermal annealing ramp for both versions is 48-45°C with 1 h/°. Scale bar is 50 nm.

are not correctly assembled.

Due to the negative results of the assembly of version 1 and 2, a further design iteration was performed. Version 3 (Fig. 4.6) is based on version 1 but differs in two aspects:

- Inspired by the design schemes for curved DNA origami objects reported in [30], the distance between consecutive cross-overs connecting selected neighboring helices was reduced from 21 bp to 42 bp (Fig. 4.7a). The pairs of helices were selected based on the difference of their number of insertions or deletions per 7 basepairs.

- The presence of a scaffold seam (Fig. 4.7b) was omitted since it might represent a potential breaking point of the object.

EMA of self-assembly reactions of version 3 yields well-defined leading bands with slightly higher mobility than the scaffold DNA indicating successful assembly (Fig. 4.8). Negative-stain TEM images confirmed that the shape of the rings is in agreement with the design (Fig. 4.9).
4. Positioning FG-NUPs using a DNA origami scaffold

**Figure 4.6.: Design scheme of ring v3.** Generated with caDNAo v0.2. See Fig. D.3 for full design scheme incl. sequences.
4.2. Design and assembly of the DNA ring

Figure 4.7.: Schematic design patterns in ring v3. a. Cross section of DNA ring. Red lines indicate connections between neighboring helices which feature a reduced cross-over density. b. Simplified scheme of a scaffold crossover seam. Blue lines represent the scaffold strand, gray lines represent staple oligonucleotides.

Figure 4.8.: EMA of self-assembly of ring v3. Laser-scanned images of a 2% agarose gel with 11 mM MgCl₂. S = scaffold DNA. Folding ramps as indicated in the presence of 20 mM MgCl₂. All lanes have been auto-leveled below the pockets.
4. Positioning FG-NUPs using a DNA origami scaffold

Figure 4.9: Negative-stain TEM of ring v3. a, Exemplary negative-stain TEM micrograph of an unpurified ring v3 self-assembly reaction. b, Exemplary negative-stain TEM micrograph of PEG-purified self-assembly reaction with average micrograph (inset). Scale bar is 50 nm.

Figure 4.10: EMA of ring v4 and v5. Laser-scanned images of a 2% agarose gel with 11 mM MgCl₂. S = scaffold DNA. Folding ramps as indicated in the presence of 20 mM MgCl₂. White arrow indicates two samples that were unintentionally interchanged.

Two additional versions were prepared in order to probe which of the two changes between version 1 and 3 were critical for correct assembly. Version 4 (Fig. D.4) features a reduced cross-over density between the same helices, as version 3, but contains a scaffold seam. Accordingly, version 5 (Fig. D.5) has no scaffold seam but full cross-over density.
EMAs of self-assembly reactions of these two versions (Fig. 4.10) show that for version 5 no bands are visible while for version 4 a fast leading band and a slower second band appear. Comparison to the EMA result of version 3 (Fig. 4.8) suggests that these bands can be attributed to successfully folded monomers and dimers.

Taken together, these findings indicate that the critical design modification is the reduction of the cross-over density. The dimer band of version 4 appears to be significantly stronger than for version 3 indicating that the presence of the seam might favor the assembly of unwanted dimers. However, it cannot be excluded that the different self-assembly results are influenced by the changes of the staple oligonucleotide sequences which cannot be avoided upon changing the scaffold routing as well as the pattern of insertions and deletions.

Compared to other DNA origami objects presented in this thesis as well as in other published results [87] the rings appear to self-assemble at relatively low temperatures. This may be attributed to additional energy barriers present due to the curvature.

4.3. Attachment of NSP1 and NSP1-S

In order to attach the FG-NUP proteins to the DNA ring, they were conjugated with a DNA oligonucleotide and the attachment is achieved via binding to complementary single-strands protruding radially inward from the ring (Fig. 4.11 a-b). The design of each attachment site consists of two single-strands protruding from the DNA ring at the same location which can form a duplex consisting of 4 G-C basepairs and a poly-T linker to allow extension of the two strands to match the DNA diameter (Fig. 4.11 c-d). This design is based on other experiments separately performed by Anna-Katharina Pumm in the Dietz lab as part of a different project. Her results indicated higher accessibility of the attachment sequence when the G-C duplex is present instead of extending only one of the staple oligonucleotides. This effect may be caused by the attachment sequence being hidden inside the object if only one staple oligonucleotide is elongated with the attachment sequence.

Three different versions with 8, 16 and 32 attachment sites of the ring were prepared (Fig. 4.11 e). EMAs of these samples after removal of excess staple strands through PEG precipitation yields well-defined monomer bands exhibiting decreasing mobility with increasing number of attachment sites (Fig. D.7).

The conjugation was performed in the Dekker lab at TU Delft.
Figure 4.11: Schematic attachment of FG-NUPs to DNA ring. a, DNA ring with 8 attachment anchors and single FG-NUP protein (yellow) with attached single-stranded DNA (purple). b, NSP1 attached to DNA ring. c, Complementary sequences on DNA ring and attached FG-NUP. d, Scheme of single attachment anchor. Red lines indicated unpaired thymine bases. e, View onto inside of the ring. Light blue dots mark positions of attachment anchors used in the ring variant with 8 attachment anchors. Light blue and dark blue dots are used in the ring with 32 attachment anchors. Dimensions as indicated.
To verify the presence of the attachment sites, rings with 8 and 32 anchors were incubated with cyanine-5 (Cy5) labeled oligonucleotides and analyzed using EMA (Fig. 4.12a). The sample with 32 attachment anchors yields a 3.2-fold higher fluorescence signal than a sample with 8 anchors. The measured value is lower than the ideal value of 4-fold higher fluorescence because presumably not all rings bound 32 complementary oligonucleotides. To quantify the number of attached oligos, total internal reflection microscopy (TIRFM) was used (Fig. 4.12b). To this end, rings with 8 attachment anchors and three additional biotin-modified staple strands at the bottom of the rings were incubated with the cyanine-5 labeled oligonucleotides and attached to glass slides via biotin-streptavidin-biotin interactions. Analysis of the number of observed bleaching steps in the intensity traces of 240 single-particles recordings in the cyanine-5 channel yields a histogram with a clear peak around 7-8. While almost no recordings indicated more than 8 attached oligonucleotides around 30% of rings yield 3-6 bleaching steps. These rings may have bound fewer oligonucleotides due to assembly defects in the DNA rings.

The FG-NUP protein tested in this thesis is NSP1, which occurs in NPCs in yeast cells and a mutated version, NSP1-S, where all the hydrophobic amino acids leucine (L),
iso-leucine (I), valine (V) and phenylalanine (F) are replaced by the hydrophilic amino acid serine (S) to test the influence of the hydrophobic interactions. These proteins need to be stored in the presence of 5 M Guanidinium chloride (GuHCl) to prevent aggregation due to their hydrophobicity. Therefore, a significant concentration of GuHCl will be present if the protein is incubated with the DNA rings at high excess ratios which may degrade the ring. In order to verify the stability of the ring in the presence of GuHCl, rings were incubated at different GuHCl concentrations and analyzed using EMA (Fig. D.8). The thus obtained band pattern does not alter significantly from samples without GuHCl suggesting that the rings are stable in the tested GuHCl concentration range.

In order to test the protein attachment DNA rings with 32 attachment sites were incubated with NSP1 in excess at two different ratios and subsequently analyzed using EMA (Fig. 4.13a). These samples remain mostly in the gel pocket, except for a faint smear in which the fastest objects exhibit a much slower mobility than rings without protein. This result indicates interaction of the rings with NSP1 but also suggests that large aggregates are forming.

Negative-stain TEM imaging of the unpurified sample with lower NSP1 excess yields micrographs with some monomeric rings which appear to be blurry and no intensity difference between the inside of the rings and the background can be observed (Fig. 4.13b).
The blurriness may be attributed to excess proteins covering the entire TEM grid causing additional electron scattering on top of the rings. If correct, an additional purification step to remove excess protein will be necessary.

Since gel extraction as a potential purification method is challenging because there is no clear gel band which can be attributed to a species of ring monomers with attached proteins, PEG precipitation was utilized. Similar to the non-purified samples, most of the purified samples remain in the pockets of the EMA (Fig. 4.13a). A faint leading band appears for samples incubated at lower protein excess with a mobility similar to the ring monomers, which lack the protein, which may be attributed to rings with no attached protein. However, in negative-stain TEM images of the purified samples the rings appear much clearer and a higher density inside the rings can be discriminated from the background (Fig. 4.14b), suggesting that the excess protein was successfully removed.

Figure 4.14: Negative-stain classification of DNA rings with proteins. a-c, Top: exemplary field-of-view micrographs of respective sample. Bottom: average histogram of two manual classifications of particles of respective sample into categories low, medium and high density inside the ring. Samples: DNA origami ring without attached protein (a), with up to 32 NSP1 proteins attached (b) and with up to 32 NSP1-S proteins attached (c). See Fig. D.10 for exemplary particles. Scale bar is 50 nm.

The micrographs of rings with and without protein obtained by negative-stain TEM imaging were manually sorted into three different classes depending on the densities inside the rings (Fig. 4.14). Particles of all three samples of rings without protein, with NSP1 and with NSP1-S were randomly mixed before the classification to avoid any human bias. The classification was independently performed twice, by Eva Bertosin and me, and the
resulting values were averaged.
The observed distribution of densities reveals a clear difference between samples of rings without protein and rings incubated with NSP1. Rings without protein were almost entirely classified with low filling density, while 80% of rings with NSP1 contain either medium or high filling. The classification of rings incubated with NSP1-S also differs from rings without protein but almost 60% of them contain low density. The lower densities of NSP1-S compared to NSP1 can be either explained by a lower attachment yield or by a weaker interaction between NSP1-S with itself.

To gain a qualitative impression of the three-dimensional shape of the NSP1 proteins inside the DNA ring, a tomogram of the DNA ring with 32 attachment sites without protein and with NSP1 was calculated from a negative-stain TEM tilt series (Fig. 4.15). The thus obtained reconstruction indicates intensities inside the ring through various slices along the symmetry axis of the ring which can be interpreted as a homogeneous filling with NSP1.

In summary, this section qualitatively shows the successful attachment of NSP1 and NSP1-S to the DNA rings using negative-stain TEM. However, quantitative comparison of the obtained data is difficult due to the unknown influence of the staining agent. The next section presents data obtained using cryo-electron microscopy (cryo-EM) which does not require the use of a staining agent.

4.4. Analysis of FG-NUPs density using cryo-EM

To analyze the protein densities inside the rings, three samples as shown in Fig. 4.14 were imaged using cryo-electron microscopy (cryo-EM). The resulting average micrographs clearly indicate different average densities inside the rings (Fig. 4.16 a-c). While the sample containing DNA rings without proteins does not indicate any density inside the rings higher than the background at large radii outside the ring, the average micrographs of samples with rings with NSP1 and NSP1-S yield higher densities inside the rings, suggesting that the proteins are present. The protein density appears to be significantly stronger for NSP1 than for NSP1-S.

In order to quantitatively compare the cryo-EM average micrographs the circularly averaged density profiles (Fig. 4.16c) were integrated over radii that correspond to the inside of the ring. The resulting integrated values ($I$) should be proportional to the total mass inside the ring. Dividing $I$ of the sample of DNA rings with NSP1-S by $I$ of the sample
Figure 4.15.: Negative-stain tomography of DNA ring with and without attached NSP1 proteins. Top: schematic representation of DNA ring without (a) and with (b) up to 32 attached NSP1 proteins. Numbers indicate positions of slices of reconstruction shown below. Bottom: exemplary slices of reconstructed 3D volume of corresponding sample. Red arrows indicate leash of DNA rings. Scale bar is 50 nm.
Figure 4.16: Cryo-EM of DNA rings with NSP1 and NSP1-S. 

- **a.** Schematic illustration of (top to bottom) (1) DNA ring without protein, (2) DNA ring with up to 32 NSP1 proteins attached and (3) DNA ring with up to 32 NSP1-S proteins attached. 
- **b.** Corresponding average cryo-EM micrographs. Scale bar is 50 nm. See Fig. D.11 for exemplary particles. 
- **c.** Circularly averaged intensity profiles of the three average micrographs shown in **b.** 
- **d-e.** Mean mass densities of proteins from CG simulations in top and side view.
with NSP1 yields a ratio of $I_{\text{NSP1-S}}/I_{\text{NSP1}} = 0.7$.

Coarse-grained (CG) simulations of the proteins were performed by Ankur Mishra in the Onck lab to complement the experimentally obtained densities (Fig. 4.16d-e). Details about the simulations can be found in [88]. Calculating the same ratio as mentioned above using data from the simulations, resulted in a value of 0.82 and is thus in reasonable agreement with the experimental data. The main uncertainty in this calculation is that the average cryo-EM micrographs have only been normalized by a constant offset and no multiplicative factors. Such factors are challenging to calculate due to a missing reference point that should exhibit equal intensity in all three images.

Taken together, the results obtained from cryo-EM suggest that NSP1 forms a denser network inside the ring than NSP1-S, which is in agreement with the CG simulations. The FG-repeats thus appear to increase the interaction among multiple NSP1 proteins. However, it cannot be fully excluded that these results are caused by a lower attachment yield for NSP1-S compared to NSP1. Fluorescently labeling the proteins might allow analyzing the attachment yield using TIRF microscopy as described in section 4.3.

As a by-product of the cryo-EM measurements, rotationally aligned average micrographs of the DNA rings without proteins were calculated (Fig. D.12). Comparison of these images to the shape of the DNA ring predicted by CanDo reveals that circularly distributed density spokes along the ring can be attributed to cross-overs between layers of the DNA ring confirming that the DNA ring matches the design.

In future experiments, different types of FG-NUPs could be combined on one DNA ring or on multiple DNA rings which are polymerized along the symmetry axis of the ring to form longer tubes. The next chapter describes a modified design of the DNA ring aiming at building such tubes.

### 4.5. Polymerization of the DNA ring

Shape-complementary protrusions and recessions (Sec. 2.1) had so far not been tested on curved DNA nanostructures. In order to validate whether these interfaces can also be used on curved objects, the design of the DNA ring version 3 was modified to accommodate such features (Figs. 4.17 and 4.18). EMA of various thermal annealing ramps (Fig. D.9) yields a clear monomer band indicat-
4. Positioning FG-NUPs using a DNA origami scaffold

Figure 4.17.: Schematics of ring with shape-complementary features. a, Two rings unbound. b, Two rings bound via shape-complementary interfaces. c, Cross section of ring with shape-complementary interfaces. Helices containing protrusions and recessions are marked in red and blue respectively.

ing successful self-assembly of this version of the ring. In order to test whether the shape-complementary features lead to polymerization, an initial test was performed with objects whose excess staple strand were removed by PEG precipitation [55]. Negative-stain TEM imaging of a corresponding sample, incubated for 12 hours in the presence of 60 mM MgCl$_2$, yields many dimers and a few trimers (Fig. 4.19). The shape-complementary features thus appear to successfully support polymerization. Further experiments will have to show whether similar yields as with non-curved objects can be achieved.

4.6. Conclusions and Outlook

In summary, this chapter demonstrated the assembly of a DNA ring consisting of 18 parallel helices and an inner diameter of $\sim$ 34 nm. Reducing the density of cross-overs between selected neighboring helices was found to be pivotal for a successful self-assembly reaction. NSP1 and NSP1-S conjugated to a DNA single-strand can be attached inside the ring via hybridization to sequence-complementary single-strands protruding radially inward. The number of attached proteins may thus be rationally controlled.

2D average micrographs of the rings with attached NSP1 and NSP1-S obtained by cryo-EM revealed significantly different densities inside the rings for NSP1 and NSP1-S in accordance with the predictions of CG simulations. This study was submitted for publication together with conductivity measurements obtained by A. Ananth which revealed a lower ionic conductivity of rings with NSP1 than with NSP1-S.
Figure 4.18.: Design scheme of ring with shape-complementary features. Protrusions and recessions are highlighted in red and blue respectively. See Fig. D.6 for full design scheme incl. sequences. Generated with caDNAzo v0.2.
Figure 4.19: Negative-stain TEM micrographs of polymerized ring. Exemplary negative-stain TEM micrographs of the ring with shape-complementary features after incubation at 60 mM MgCl$_2$ for 8 hours. Scale bar is 50 nm.

The addition of an asymmetric marker outside the ring in a future version might enable calculations of 3D reconstructions from single particles to obtain structural information about the interactions between FG-NUPs. If reliable 3D reconstructions are possible, the addition of NTRs such as karyopherins might enable a more detailed understanding of the interaction between NTRs and FG-NUPs.

Shape-complementary protrusion and recession were implemented in a modified DNA ring version. Initial experiments yielded successful polymerization indicating that such interaction interfaces can also be used on curved objects. Using strand hybridization in addition to the base-stacking interaction of the shape-complementary interfaces might increase the polymerization yield.

More sophisticated assemblies consisting of multiple stacked rings with different types of FG-NUP proteins attached can be envisioned in the future.
5. Uncovering the forces between nucleosomes using DNA origami

This chapter presents measurements of the energy landscapes between two nucleosomes using a passive force spectrometer assembled via the DNA origami technique. The interaction between nucleosomes is considered to be important for the formation and structure of the so-called chromatin fiber which folds and compacts DNA in the nucleus of eukaryotic cells and is therefore crucial for the regulation of many genomic processes.

The first part provides a short introduction and describes the design and assembly of modified nucleosomes with single-stranded DNA handles for attachment to the force spectrometer. The second part explains the attachment of the nucleosomes to the force spectrometer and presents the obtained energy landscapes.

The research presented in this chapter was performed in close collaboration with Jonas Funke from the Dietz lab and Corinna Lieleg from the Korber lab at LMU Munich. Corinna Lieleg performed the salt gradient dialysis to assemble the nucleosomes and prepared the histone proteins. Jonas Funke prepared the force spectrometers and collected the negative-stain TEM data together with me. All parts of this chapter have been published in [89] together with additional data obtained by Jonas Funke and Corinna Lieleg.

5.1. Introduction

The DNA in the nucleus of eukaryotic cells is tightly folded into large complexes called chromosomes. A fundamental building block of chromosomes are nucleosomes (also referred to as 'nucleosome core particles'). A nucleosome consists of 147 bp double-stranded DNA that is wrapped in 1.65 left-handed turns around a protein octamer consisting of two copies of four different proteins called histones (Fig. 5.1a). Various high-resolution structures of nucleosomes have been reported [90–93]. The entire genomic DNA of a eukaryotic cell that is wrapped around many nucleosomes is referred to as 'chromatin'. The existence of a fixed structure of chromatin remains debated [94] and the interaction between two nucleosomes may have a strong influence on the formation of a particular structure.
Previous mechanical stretching experiments (e.g. [95]) as well as the crystal structure of a tetranucleosome [96] hinted at an attractive stacking interaction between the nucleosomes. The N-terminal histone tails and especially acetylation of the lysine 16 in the tail of histone H4 have been reported to strongly influence this interaction [97–99]. Absolute values of the interaction energy between nucleosomes have been inferred from the stretching experiments but the reported values vary greatly between -0.3 to -8 kcal/mol [95,100–102].

A previously published DNA origami structure consisting of two beams that are connected via a hinge mechanism [17] was used as a passive force spectrometer (Fig. 5.1b). A detailed description and characterization of the force spectrometer can be found in [103]. The main characteristic used in this study is that the hinge mechanism is influenced by electrostatic and sterical interactions resulting in a particular vertex angle distribution which can be read out using negative-stain transmission electron microscopy (TEM) (Fig. 5.2a-b).

The Boltzmann distribution links the probability to find a force spectrometer in a particular state, in this case characterized by the vortex angle Θ, to the free energy \( E(\Theta) \):

\[
p(\Theta) = \frac{1}{Z} e^{-\frac{E(\Theta)}{k_B T}}
\]

\[(5.1)\]
where $k_B$ is the Boltzmann constant, $T$ the temperature and $Z$ the canonical partition function. This connection allows the calculation of the underlying energy landscape (Fig. 5.2c). The curve yields a clear minimum at vertex angles of 70 - 75° and increases at lower and higher angles. The interaction between two biomolecules mounted on the two beams (see Fig. 5.1b) will thus experience an angle dependent torque due to the force spectrometer.

5.2. Assembly of nucleosomes and attachment to force spectrometer

Attachment to DNA origami objects is commonly achieved by hybridisation to a specific sequence that is protruding from the DNA structure at the desired location. Therefore, the nucleosomes need to be modified such that they feature single-stranded DNA handles (Fig. 5.3) which can hybridize to complementary sequences protruding from the force spectrometer.
spectrometer. To this end, one of the strands of the 147bp long template DNA was divided into 6 shorter strands. The positions of the corresponding nicks were chosen in a way that single-stranded overhangs at these nicks point radially outward from the nucleosome. The corresponding neighboring nucleotides are not involved in histone binding and they are roughly separated by 90° angles around the central axis of the nucleosome. The Widom-601 sequence [93] is known for its high affinity to form nucleosomes and was therefore used for the template DNA in this study. The experimental nucleosome assembly was performed as follows: (1) The template DNA was first self-assembled by thermal annealing and (2) the nucleosomes were reconstituted using salt gradient dialysis (see [89,105] for details).

Electrophoretic mobility analysis (EMA) was used to analyze the annealing of the template DNA as well as the assembly of the nucleosomes with the corresponding template DNA (Fig. 5.4a). The template DNA yields clear monomeric bands with decreasing mobility with higher number of protruding DNA handles. Similarly, after salt gradient dialysis with histone octamers the samples yield monomeric bands with the same mobility trend suggesting successful assembly of the nucleosomes. All assembled nucleosomes run slower than the corresponding template DNA which can be attributed to the increased mass of the complex.

TEM imaging was utilized to verify the shape of the assembled nucleosomes (Fig. 5.4b-c). The images show disc-shaped particles which match the dimensions of nucleosomes. Comparison of average TEM micrographs with a low pass-filtered transmission projection of a crystal structure suggests that the nucleosomes assembled completely. For instance,
5.2. Assembly of nucleosomes and attachment to force spectrometer

Figure 5.4: Experimental assembly of nucleosomes with DNA handles. a, Native ethidium bromide–stained 4.5% polyacrylamide gel electrophoresis of various samples: 1 = continuous non-nicked template DNA, 2 = nucleosomes assembled using continuous template DNA of lane 1, 3 = template DNA with four nicks at positions A1 to A4, 4 = nucleosomes assembled using nicked template DNA of lane 3, 5 = template DNA with two protruding single-strands at positions A1 and A3, 6 = nucleosomes assembled using template DNA with two protruding single-strands of lane 5, 7 = template DNA with four protruding single-strands at positions A1 to A4, 8 = nucleosomes assembled using template DNA of lane 7. This measurement has been performed by Corinna Lieleg. See [89] for details on the nucleosome assembly. b, Exemplary negative-stain TEM micrograph of nucleosomes with wild-type histones. Scale bar is 50 nm. c, Top: low pass filtered projection of nucleosome crystal structure pdb 3MVD. Bottom: negative-stain TEM reference-free class average of nucleosomes with wild-type histones. Circularly distributed density spokes indicated by arrows. See Fig. E.2 for additional class averages.

The arc segment formed by the histones is clearly visible in both images. Several other experiments to verify the nucleosome quality can be found in [89].

Only nucleosomes with two attachment handles at positions A1 and A3 are used in the remainder of this chapter since the two handles are protruding at a similar height from the nucleosome which is favorable for attachment to the force spectrometer (Fig. 5.5a). In order to attach the nucleosomes, force spectrometers were assembled such that DNA single-strands with complementary sequences to the DNA handles on the nucleosomes protrude from the top and the bottom arm (Fig. 5.5b). The dyad axes of the two nucleosomes will thus enclose an angle of 78° (Fig. E.1). Two samples of nucleosomes with different sets of attachment sequences were prepared to be able to change the relative orientation of the nucleosomes (Sec. E.1.2).

TEM was used to verify the attachment of the nucleosomes to the force spectrometer (Fig. 5.6). The obtained average micrographs clearly reveal an object with the dimensions of a nucleosome attached to both arms of the force spectrometer for different vertex angles of the spectrometer.
Figure 5.5.: Schematic attachment of nucleosomes to force spectrometer. a, Top: nucleosome with single-stranded handles at positions A1 and A3. Bottom: nucleosome with single-stranded handles at positions A2 and A4. b, Left: force spectrometer with single-stranded attachment handles and a single nucleosome with complementary attachment handles at positions A1 and A3. Right: force spectrometer with two attached nucleosomes in distal position.

Figure 5.6.: Average TEM micrographs of force spectrometer with attached nucleosomes with wild-type histones. Vertex angles as indicated. Scale bar is 25 nm.
5.3. Vertex angle distributions of force spectrometer with nucleosomes

As before for spectrometers without attached nucleosomes, the vertex angle distribution of force spectrometers with mounted nucleosomes were obtained from TEM images. To this end, only force spectrometers with two bound nucleosomes were selected manually (Fig. 5.7) and the vertex angles of the remaining particles were subsequently measured manually. Two samples with different mounting positions of the nucleosomes (at either 15 nm (‘proximal’) or 30 nm (‘distal’) from the hinge axis) were analyzed (Fig. 5.8).

The vertex angle distribution of force spectrometers with nucleosomes mounted in the proximal position reveals a clear peak at \(\sim 42^\circ\) corresponding to force spectrometers where the nucleosomes are in close contact (see Fig. 5.6 for comparison). These small opening angles occurred much more frequently than in the sample containing force spectrometers without nucleosomes which suggests an attractive interaction between the nucleosomes.

**Figure 5.7.: Particle selection from negative-stain TEM micrographs.** Two exemplary field-of-view negative-stain TEM micrographs of force spectrometers incubated with nucleosomes with wild-type histones. Particles labeled with a green circle were selected as particles with two bound nucleosomes. The other particles were neglected due to the following reasons (as indicated by red numeric label): 1 = No nucleosome bound, 2 = Only one nucleosome bound, 3 = Force spectrometer or at least one nucleosome distorted. Scale bar is 100 nm.
Figure 5.8.: Vertex angle distribution of force spectrometers with nucleosomes at different positions. a-b, Left: exemplary negative-stain TEM micrographs of force spectrometers. See Figs. E.4 and E.5 for additional particles. Right: corresponding kernel density estimation (bandwidth 3°) of vertex angle distribution. Samples: force spectrometer with two attached nucleosomes in proximal position (a, N = 1301) and with two attached nucleosomes in distal position (b, N = 158). Dashed black line in a and b is the vertex angle distribution of the force spectrometer without nucleosomes (see Fig. 5.2). All scale bars are 30 nm.

For spectrometers with mounted nucleosomes in the distal position the closed state corresponds to a vertex angle of \( \sim 23° \). The angle distribution of the corresponding sample yields a clear peak at this value but the fraction of particles in this closed state is around 8-fold lower than in the sample containing force spectrometers with nucleosomes at the proximal position. The smaller fraction of closed spectrometers may be attributed to the higher energy barrier that needs to be overcome to close the spectrometer up to an angle of 23° compared to 42°. The proximal position appears to be favorable for characterizing the interaction of nucleosomes and was therefore used for all measurements in the remainder of this chapter.

To test the influence of the relative orientation of the nucleosomes on their interaction one nucleosome was rotated by 180° by interchanging the sequences of the corresponding attachment handles on the spectrometer. The resulting vertex angle distribution (Fig. 5.9a) does not deviate strongly from the distribution of non-rotated nucleosomes suggest-
5.4. Energy landscapes of nucleosome-nucleosome interactions

Figure 5.9.: Vertex angle distribution of force spectrometers with nucleosomes in different relative positions and modified nucleosomes. a-c, Kernel density estimation (bandwidth 3°) of vertex angle distribution of force spectrometer with two nucleosomes where one nucleosome was rotated by 180° (a, N = 979), with two nucleosome with histones with acetylated H4 tails (b, N = 846) and with two nucleosomes lacking N-terminal histone tails (c, N = 818). Dashed black line in (a-c) is the vertex angle distribution of the force spectrometer without nucleosomes (see Fig. 5.2).

The N-terminal histone tails and especially acetylation of the lysine 16 in histone H4 have previously been shown to have a significant influence on the internucleosomal interaction \([97, 99]\). To test these findings with the force spectrometer, nucleosomes with acetylated lysine 16 in histone H4 and nucleosomes with histones without N-terminal tails were prepared and attached to the spectrometer. The vertex angle distributions of these samples (Fig. 5.9 b-c) both reveal a significantly reduced fraction of closed spectrometers.

5.4. Energy landscapes of nucleosome-nucleosome interactions

As described above (Sec. 5.1) energy landscapes were calculated from the angle distributions shown in Figs. 5.8 and 5.9 (Fig. 5.10 inset). The energy landscape of the force spectrometer alone can now be subtracted from the energy landscapes of force spectrometers with mounted nucleosomes in order to obtain the internucleosomal interaction potentials (Fig. 5.10 main).

The resulting energy landscapes reveal a weak internucleosomal interaction of around \(-1.6\) kcal/mol at the minimum and a spatial range of approx. 6 nm. Rotating one of the nucleosomes by 180° alters the interaction only slightly. In contrast, acetylation of the
5. Uncovering the forces between nucleosomes using DNA origami

Figure 5.10: Energy landscapes of internucleosomal interactions. Normal (thick lines) and uniform (thin lines) kernel density estimates (kernel density bandwidth of 3°) of the internucleosomal interaction energy landscapes as a function of the distance between the centers of mass of the nucleosomes (CM; computed from pdb 3MVD using Co-atoms for histones and C4-atoms for nucleosomal DNA). Samples: nucleosomes with wild-type histones, oriented such that their dyad axes enclose an angle of 78° (red) or 258° (dark blue), nucleosome with acetylated lysine 16 on the H4 histone (light blue), and nucleosome without N-terminal histone tails (yellow). Inset: free energy calculated from angle distributions shown in Figs. 5.8 and 5.9. See Sec. E.4 for details on the calculation and normalization of the energy landscapes.
lysine 16 on the N-terminal tail of the H4 histone weakens the interaction almost as much as removing all N-terminal histone tails. The lysine 16 on the H4 histone thus appears to be almost entirely responsible for the internucleosomal interaction. The 6 nm range of the interaction corresponds to approximately 2/3 of the overall contour length of the histone H4 tail (26 amino acids, assuming 0.365 nm for each amino acid) which agrees with previous studies reporting that the internucleosomal interaction is based on binding of the H4 tail to an acidic patch on the surface of the second nucleosome [97].

The Gay-Berne potential [106] has previously been used to model internucleosomal interactions [107]. The potential was fitted to the energy landscapes presented here in order to obtain the corresponding parameters which can be used in existing models of chromatin (Sec. E.5).

5.5. Conclusions and Outlook

This chapter describes the application of a DNA origami object with a hinge mechanism ('force spectrometer') to measure weak interactions between two biomolecules. The design of the force spectrometer allows measuring interactions on the order of a few kcal/mol. The mounting position of the molecules may be adjusted depending on the interaction strength.

Nucleosomes were chosen as a model system for studying interactions with the force spectrometer. To this end, modified nucleosomes featuring single-stranded DNA handles were assembled and successfully attached to the force spectrometer. The nucleosomes were found to be weakly interacting on the order of $-1.6$ kcal/mol. Acetylation of the lysine 16 on the N-terminal tail of the histone H4 switches the interaction off almost entirely in a similar way as by removing all N-terminal histone tails. The weak interaction strength suggests a flexible chromatin structure which may adopt several conformations in-vivo. The obtained energy landscapes may support more accurate models of chromatin.

This study was published together with additional in-solution data obtained by J. Funke. For these measurements, a pair of dyes which can undergo Förster resonance energy transfer (FRET) was mounted on the force spectrometer providing a fluorescent readout of the vertex angle. These measurements confirmed the trend of vertex angle distributions obtained by TEM imaging.
In future experiments the force spectrometer could be used to study other weakly interacting pairs of biomolecules given that those can be attached to DNA nanostructures. Single-molecule fluorescence measurements using a pair of dyes which can undergo FRET as described above might allow measuring interaction kinetics of the attached targets. Cryo-electron microscopy of the force spectrometer might enable measuring conformational changes of the targets under an external force.
Appendices
A. Materials and Methods

A.1. Design of DNA origami structures

All DNA origami structures were designed in an iterative procedure using caDNAno v0.2 [108] and CanDo [54]. All structures containing shape-complementary protrusions and recessions with blunt ends where optimized for strong base-stacking by choosing the optimal circular scaffold DNA permutation using a custom script written in MathWorks MATLAB (R2013b) which is based on the base-stacking interaction strengths reported in [109].

A.2. Folding protocols

The self-assembly of DNA origami objects containing one circular scaffold DNA strand was performed as described in [48,49] with reaction mixtures containing 50 nM scaffold DNA, 200 nM of each DNA oligonucleotide ‘staple’ strand, 20 mM MgCl₂, 5 mM Tris, 1 mM EDTA, and 5 mM NaCl (pH 8). Reaction mixtures were annealed with a TETRAD (Biorad) thermal cycling device. Annealing ramp temperature screens to determine the optimal folding conditions were frozen in liquid-nitrogen (LN₂) immediately after completion of the folding reaction and thawed directly before analysis. DNA origami objects containing two circular scaffold DNA strands were annealed with 40 nM scaffold DNA, 250 nM of each DNA oligonucleotide ‘staple’ strand apart from the above. All oligonucleotides with and without chemical modifications were obtained from Eurofins MWG (Ebersberg, Germany). Scaffold DNA was prepared by Florian Preatorious as described in [110].

A.3. Agarose gel electrophoresis

The detailed Dietz lab protocol for casting agarose gels containing MgCl₂ can be found in [53]. The conditions used for the gels shown in this thesis are:

- Gels were cooled by an iced water-bath.
A. Materials and Methods

- Gels were stained using Ethidium Bromide (EtBr).
- Gels were run in a 0.5x TBE buffer (1 mM EDTA, 44.5 mM Tris, 44.5 mM boric acid, pH 8.3) with varying MgCl$_2$ concentrations (as indicated in the gel description).
- Gels were laser-scanned (Typhoon Fla 9500, GE Healthcare) with a resolution of 50 µm/pixel.

A.4. Agarose gel extraction

The desired band was cut out of the gel, placed in a conventional Eppendorf tube and smashed with a small stick. The lower part of the tube was cut, placed upside down in a Freeze ’N Squeeze gel extraction column (Biorad), and centrifuged for 5 minutes at 5 krcf and 25° C.

A.5. PEG precipitation

The protocol for purification by polyethylene glycol (PEG) precipitation was first established by Evi Stahl and can be found in [55]. In case that the PEG precipitation was performed twice, the first pellet was dissolved in 1x folding buffer containing 20 mM MgCl$_2$.

A.6. TEM grid preparation and imaging

Purified reaction products were adsorbed on glow-discharged formvar-supported carbon-coated Cu400 TEM grids (Science Services, Munich, Germany) and stained using a 2% aqueous uranyl formate solution containing 25 mM sodium hydroxide. Imaging was performed using a Philips CM100 electron microscope operated at 100 kV. Images were acquired using an AMT 4 Megapixel CCD camera. Imaging was performed at a magnification of 28,500.

A.7. 2D class averaging

For image processing, libraries of individual particle micrographs were created by particle picking using the EMAN2 [111] boxing routine. Generation of average particle micro-
graphs was performed using Xmipp \cite{112,113} mlf–2Dalign routine.

A.8. Tomography imaging and analysis

Tomography tilt series were acquired using a FEI Tecnai Spirit electron microscope operated at 120 kV using a TemCam-F416 (Tietz) camera. Imaging was performed at a magnification of 42,000 between $-50^\circ$ and $50^\circ$ in steps of $1^\circ$. Tomography calculations were performed using IMOD \cite{114} using a SIRT-like filter equivalent to 5 iterations.
B. Supp. material: Rotary apparatus made from 3D DNA origami components

B.1. Protocols

B.1.1. Polymerization of rotary apparatus

The polymerization protocol was published in [48] and is reprinted here:

The protocol used for polymerization of the rotary apparatus is as follows: (1) incubation of the rotor with the first clamp element in a stoichiometry of 1:1 in the presence of 30 mM MgCl$_2$ at 40°C for 24 hours, (2) addition of the second clamp element in a stoichiometry of 1:2 and increasing the MgCl$_2$ concentration to 40 mM and incubation at 40°C for 24 hours, and (3) addition of auxiliary oligonucleotides for fixing the brackets in a stoichiometry of 5:1 excess per strand and binding site and incubation at 20°C for 24 hours.

B.1.2. Single-molecule fluorescence microscopy of rotary apparatus

The preparation of PEGylated microscope cover slides as well as the sample preparation and the protocol for performing the single-molecule microscopy experiments is described in [48]. The sample preparation and microscopy protocol is reprinted here:

All samples used for single-molecule microscopy experiments were purified from agarose gels after polymerization (Sec. A.4). To create a small-sample chamber, a glass coverslip was attached to the PEGylated cover slider by vacuum grease. This chamber was washed with a T50 buffer (10 mM Tris-HCl, 50 mM NaCl), incubated with a solution of NeutrAvidin (0.05 mg/ml) for 10 min, and subsequently washed with the T50 buffer solution. For immobilization purposes, the sample was incubated in the chamber for 10 min and was subsequently washed with single-molecule imaging buffer (2 mM Trolox, 1.6 D-Glucose, 100 mM Tris-HCl (pH 8), 108500 U/ml Catalase, 22100 U/ml Glucose...
Oxydase, 1 mM EDTA, 5 mM NaCl and different MgCl₂ concentrations between 5 and 20 mM). Experiments with the extended rotor were performed at 20°C using a home-built objective-type total internal reflection single-molecule setup. The samples were excited with a red diode laser (640 nm, Oxxius). Fluorescence signals of the cyanine-5 dyes were collected through a 100x numerical aperture, 1.49 oil-immersion objective (Nikon Apochromat) and recorded by an electron-multiplying CCD (EMCCD) camera (Andor iXon+). Videos were taken with a frame rate of 50 ms. Experiments with the rotor with the short lever arm were performed using the same setup in ALEX mode \[115\]. An acousto-optical tunable filter (Pegasus Optics) was used to alternately excite the sample with a green diode laser (532 nm, Oxxius) and with a red diode laser (640 nm, Oxxius). Fluorescence signals of the cyanine-5 and cyanine-3 dyes were collected and split by wavelength with a dichroic mirror into two detection channels. The two detection channels were recorded by two separate EMCCD cameras (Andor iXon+). Videos were taken with a frame rate of 50 ms, which results in an effective time of 100 ms per frame due to ALEX. The videos were processed and analyzed using custom software written in MathWorks MATLAB (R2013b).
### B.2. Design schemes

<table>
<thead>
<tr>
<th>Number</th>
<th>Sequence on rotor side</th>
<th>Sequence on clamp side</th>
<th>Corresponding remover sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AGCCAACTCT-TGCAAGCTCC-TT-(R)</td>
<td>(C)-TT-AGAGTTGGCT</td>
<td>GGGAGCTGCA-AGAGTTGGCT</td>
</tr>
<tr>
<td>2</td>
<td>AGTGCTTTAC-GCTGGGTCC-TT-(R)</td>
<td>(C)-TT-GTAAAGCAGCT</td>
<td>GGACCCAAGCGTAAAGCAGCT</td>
</tr>
<tr>
<td>3</td>
<td>TCCGACAGGC-CACTACTCGG-TT-(R)</td>
<td>(C)-TT-GCCTGTGGGA</td>
<td>CCGAGTAGTG-GCCTGTGGGA</td>
</tr>
<tr>
<td>4</td>
<td>GATATATTGAGTGGGTAC-TT-(R)</td>
<td>(C)-TT-TCAATATATC</td>
<td>GTATGAAACATCAATATATC</td>
</tr>
<tr>
<td>5</td>
<td>TTAAGGAATAT-TCATGATGAG-TT-(R)</td>
<td>(C)-TT-TATTGGCTAA</td>
<td>TCCATGCAAGATATTGGCTAA</td>
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<tr>
<td>6</td>
<td>CTCTTATGGC-GAGTGACTCA-TT-(R)</td>
<td>(C)-TT-GCCTAAGAG</td>
<td>TGAGTCACTC-GCCTAAGAG</td>
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<tr>
<td>7</td>
<td>GGTATGGTGACGTAAAC-TT-(R)</td>
<td>(C)-TT-GCCAGTAACC</td>
<td>GTTTGATCAG-GCCAGTAACC</td>
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<tr>
<td>8</td>
<td>TAGCCCTACGACAGTTGCT-TT-(R)</td>
<td>(C)-TT-TGTAGGGCTA</td>
<td>ACAGCAACGCT-TGTAGGGCTA</td>
</tr>
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</table>

**Table B.1.: Clamp - rotor dimerization sequences.** Sequences were designed using NuPack [116]. (R) and (C) indicate sequence parts that bind to the scaffold DNA in the rotor and clamp respectively. Sequence and corresponding reverse complementary sequence are indicated by the same color.
Table B.2.: Clamp dimerization sequences. Sequences were designed using NuPack [116].

<table>
<thead>
<tr>
<th>Number</th>
<th>Sequence</th>
<th>Reverse complementary sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATGCGGCCTTCGTCG</td>
<td>CGACGAAAGGCCGCAT</td>
</tr>
<tr>
<td>2</td>
<td>AGTTTAACCTTACCT</td>
<td>AGGTAAGTTAAAACCT</td>
</tr>
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<td>3</td>
<td>TAACGATGAATTCCTT</td>
<td>AAGAATTCATCGTTA</td>
</tr>
<tr>
<td>4</td>
<td>TCTTTCCCTGTCACG</td>
<td>CTGCACCCAGGAAAAGA</td>
</tr>
<tr>
<td>5</td>
<td>ACGTTCTGTTCGAT</td>
<td>ATCGAACAGGAACGT</td>
</tr>
<tr>
<td>6</td>
<td>GGGATAGCAGGACGG</td>
<td>CCGTCTGCTGCTATCCC</td>
</tr>
<tr>
<td>7</td>
<td>CCTACCCGCCCCTACA</td>
<td>TGTGAGGGCGGCTAGG</td>
</tr>
<tr>
<td>8</td>
<td>TCTAGTGGTTGGCTG</td>
<td>AAGAATTCATCGTTA</td>
</tr>
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</table>
B.2. Design schemes

Figure B.1.: Design scheme including sequences of rotor v1. Generated with caDNAno v0.2.
Figure B.2.: Design scheme including sequences of clamp v1. Generated with caDNAno v0.2.
Figure B.3.: Design scheme including sequences of rotor v2. Generated with caDNAno v0.2
Figure B.4.: Design scheme including sequences of clamp v2. Generated with caDNAno v0.2
Figure B.5.: Design scheme including sequences of static version of rotor v2. Generated with caDNAo v0.2
Figure B.6.: Design scheme including sequences of rotor v2. Generated with caDNAno v0.2
Figure B.7.: Design scheme including sequences of clamp with three docking sites.
Generated with caDNAo v0.2
Figure B.8.: Design scheme including sequences of lever extension. Generated with caDNAano v0.2. The scheme was split into two halves (a and b), black arrows indicate the same position.
B.3. Electrophoretic mobility analysis data

Figure B.9.: EMA of folding reactions of clamp v1 and rotor v1. Laser-scanned images of 2% agarose gels with 11 mM MgCl$_2$ in which folding reactions of rotor (a) and clamp (b) were electrophoresed. S = scaffold DNA, all folding ramps in the presence of 20 mM MgCl$_2$. 
B. Supp. material: Rotary apparatus made from 3D DNA origami components

Figure B.10.: EMA of clamp v1 dimerization. L = 1 kb ladder, S = scaffold DNA, A = clamp A, B = clamp B. Concentrations refer to the MgCl$_2$ concentration during the folding reaction. Polymerized samples were incubated for 12 hours. All lanes expect S have been auto-leveled globally below the pocket. L has been auto-leveled separately.

![EMA of clamp v1 dimerization](image)

Figure B.11.: EMA of clamp v1 - rotor v1 dimerization. Laser-scanned image of a 2% agarose gel with 11 mM MgCl$_2$. L = 1 kb ladder, S = scaffold DNA, A = clamp A, R = rotor, C-C = clamp dimer. Concentrations refer to the MgCl$_2$ concentration during the folding reaction. Polymerized samples were incubated for 12 hours. All lanes expect S have been auto-leveled below the pocket.

![EMA of clamp v1 - rotor v1 dimerization](image)
Figure B.12: EMA of trimer assembly with static (a) and weak (b) rotor variant. 

a, Laser-scanned image of a 2% agarose gel with 11 mM MgCl₂. L = 1 kb ladder, S = scaffold DNA, A = clamp A, B = clamp B, R = strong rotor, C-C = clamp dimer, 1 = first assembly step containing rotor and clamp A, 2 = second assembly step where clamp B was added to reaction mixture in 1, 3 = third assembly step where bracket closing oligos where added to reaction mixture in 2. b, Same as a but gel was run in the presence of 22 MgCl₂ and the rotor is the weak variant.
Figure B.13.: EMA of trimer assembly with clamp with 3 docking sites. 

a. Laser-scanned image of a 2% agarose gel with 11 mM MgCl$_2$. L = 1 kb ladder, S = scaffold DNA, A = clamp A, B = clamp B, R = rotor, C-C = clamp dimer, 1 = first assembly step containing rotor and clamp A, 2 = second assembly step where clamp B was added to reaction mixture in 1, 3 = third assembly step where bracket closing oligos were added to reaction mixture in 2. 

b. Same as a but gel was run in the presence of 22 mM MgCl$_2$. 

---

**Table 1:**

<table>
<thead>
<tr>
<th>Monomers</th>
<th>Assembly steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>S</td>
</tr>
<tr>
<td>L</td>
<td>S</td>
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Figure B.14.: EMA of rotary apparatus assembly with crank lever extension. Laser-scanned image of a 1.5% agarose gel with 22 mM MgCl₂. L = 1 kb ladder, S = scaffold DNA, A = clamp A, B = clamp B, R = rotor, LE = lever extension, C-C = clamp dimer. S1 = assembly path 1: 1 = rotor and lever extension dimerization, 2 = rotor and clamp A dimerization, 3 = clamp B added to the mixture in 2, 4 = bracket closing oligos added to reaction mixture in 3. S2 = assembly path 2: 1'-3' = trimerization as in Fig. 2.13, 4' = lever extension added to the mixture in 3'. S3 = assembly path 3: 3'' = lever extension added to the mixture in 2', 4'' = bracket closing oligos added to reaction mixture in 3''. R-LL denotes the dimer consisting of rotor and lever extension, fT the trimer consisting of rotor and both clamps with fixed brackets, and fT-LL the tetramer consisting of fT and the lever extension. S3 was used for the experimental TEM and fluorescence microscopy data presented in chapter 2.
B.4. Electron microscopy data

Figure B.15.: Exemplary negative-stain TEM micrographs of rotary apparatus with extended crank lever. Scale bar is 50 nm.

B.5. Fluorescence microscopy data
Figure B.16.: All 750 frames of single-particle recording shown in Fig. 2.16b. Scale bar is 1\,\mu m.
Figure B.17.: Additional single-particle recordings of the rotary apparatus with the strong rotor variant in the presence of 5 mM MgCl₂. Processed as in Fig. 2.16d.
Figure B.18: Additional single-particle recordings of the rotary apparatus with the weak rotor variant in the presence of 5 mM MgCl$_2$ (a) and 10 mM MgCl$_2$ (b). Processed as in Fig. 2.16d.
Figure B.19: All 1500 frames of single-particle recording shown in Fig. 2.20a. Scale bar is 5 µm.
Figure B.20.: All 1500 frames of single-particle recording shown in Fig. 2.20b. Scale bar is 5 \( \mu m \).
Figure B.21.: Comparison of evanescent (a) and epi-illumination excitation (b). Exemplary sum over all 1500 frames of single-particle recordings acquired in the presence of 5 mM MgCl$_2$ sorted into different classes as indicated. Scale bar is 5 µm.
C. Supp. material: Extended rotary apparatus for directed rotation by an electroosmotic flow
C. Supp. material: Extended rotary apparatus for directed rotation by an electroosmotic flow

C.1. Design schemes

Figure C.1.: Design scheme including sequences of eof-rotor v1. Generated with caDNAo v0.2. The scheme was split into two halves (a and b), black arrows indicate the same position.
Figure C.2.: Design scheme including sequences of eol-rotor v2. Generated with caDNAv0 v0.2. The scheme was split into two halves (a and b), black arrows indicate the same position.
Figure C.3.: Design scheme including sequences of eof-rotor v3. Generated with caDNAo v0.2. The scheme was split into two halves (a and b), black arrows indicate the same position.
Figure C.4.: Design scheme including sequences of eol-rotor v4. Generated with caDNAno v0.2. The scheme was split into two halves (a and b), black arrows indicate the same position.
Figure C.5.: Design scheme including sequences of eof-clamp v1 Generated with caD-NAno v0.2. Green dots indicate positions of attachment anchors for surface attachment. Dashed black line marks middle brackets and orange dashed line marks leash.
Figure C.6.: Design scheme including sequences of cof-clamp v2. Generated with caDNAo v0.2. Green dots indicate positions of attachment anchors for surface attachment. Orange dashed line marks leash.
C. Supp. material: Extended rotary apparatus for directed rotation by an electroosmotic flow

C.2. Electrophoretic mobility analysis data

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<th>Folding ramp °</th>
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<th>S</th>
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<tr>
<td>15mM</td>
<td>60-40° 3h</td>
<td>L</td>
<td>S*</td>
</tr>
<tr>
<td>20mM</td>
<td>60-40° 1h</td>
<td>L</td>
<td>S</td>
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<tr>
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<td>60-40° 2h</td>
<td>L</td>
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</tr>
<tr>
<td>20mM</td>
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<td>L</td>
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</tbody>
</table>

**Figure C.7.** EMA of eof-rotor v1 and v2. Laser-scanned images of 1.5% agarose gels with 5 mM MgCl₂ of eof-rotor v1 (a) and eof-rotor v2 (b) folding reactions. L = 1 kb ladder, S = scaffold DNA. Folding ramps and MgCl₂ concentrations as indicated. Red dashed line indicates gel cutout used for negative-stain TEM imaging shown in Fig. 3.3. All gels were globally auto-leveled below the pockets.
C.2. Electrophoretic mobility analysis data

Figure C.8.: EMA of eof-rotor v3 and v4. Laser-scanned images of 1.5% agarose gels with 5 mM MgCl$_2$ of eof-rotor v3 (a) and eof-rotor v4 (b) folding reactions. L = 1 kb ladder, S = scaffold DNA. Folding ramps and MgCl$_2$ concentrations as indicated. All gels were globally auto-leveled below the pockets.
Figure C.9.: EMA of eof-clamp v1 and v2. Laser-scanned images of 1.5% agarose gels with 5 mM MgCl$_2$ of eof-clamp v1 (a) and eof-clamp v2 (b) folding reactions. L = 1 kb ladder, S = scaffold DNA. Folding ramps and MgCl$_2$ concentrations as indicated. All gels were globally auto-leveled below the pockets. Area inside the white dashed line was separately auto-leveled. Lanes that contain ladders have been separately auto-leveled.
D. Supp. material: Positioning FG-Nups using a DNA origami scaffold

D.1. Protocols

D.1.1. Cryo-EM grid preparation and imaging

Samples of DNA origami rings with or without attached proteins (in 20 mM MgCl2, 5 mM Tris, 1 mM EDTA, and 5 mM NaCl) were incubated for 120 s on glow-discharged lacy carbon grids with ultrathin carbon film (Ted Pella, 01824) and vitrified using a freezeplunging device (Vitrobot Mark IV, FEI). Samples were imaged at liquid nitrogen temperatures using a Titan Krios TEM (FEI) operated at 300 kV with a Falcon II detector (FEI) at a magnification of 29,000 (pixel size, 2.319 Å/nm) with a defocus of -2 µm.

D.1.2. Cryo-EM 2D class averaging

2D averages shown in Fig. 4.16 were calculated using a custom script written in MathWorks MATLAB (R2013b). Rotationally aligned 2D class averages (Fig. D.12) were calculated using Relion2 [117] and ctf-correction was performed with Ctffind4.0 [118].

D.1.3. Single-molecule fluorescence microscopy of DNA ring

The protocol is part of [119] and is reprinted here:

Cyanine-5 (Cy5) labelled oligonucleotides were purchased from IDT technologies (IDT; Coralville, IA). Cy5-labelled oligonucleotides were annealed on DNA origami rings by incubating at a 1:10 ratio of attachment sites to oligonucleotides in 250 mM KCl, 10 mM Tris, 1 mM EDTA, and 10 mM MgCl2 at 37 °C for 60 min. The DNA origami rings were not purified from excess Cy5-labelled oligonucleotides as they would be removed during buffer exchange.

Flow cells were assembled by sandwiching double-sided tape between PEG passivated
microscope quartz slides and cover slips. A small fraction (1:100 ratio) of PEG molecules contained a biotin moiety to facilitate the immobilization and imaging of biotin-labelled DNA origami rings (see figure). Flow cell was first incubated with 0.1 mg/ml streptavidin in buffer A (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM MgCl2) for 1 min. Excess streptavidin was removed with 100 µl of buffer A. Then the DNA origami rings with attached labelled oligonucleotides at a concentration of around 50 pM were introduced into the flow cell and incubated for one minute before removing the excess with 100 µl of buffer A. The imaging buffer consisting of 40 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl2, 2 mM trolox was introduced afterwards. An oxygen scavenging system (0.3 mg/ml glucose oxidase and 40 µg/ml catalase with 5% (w/v) glucose as a substrate) was included for obtaining stable fluorescence from the dye molecules. The TIRF-microscope setup was described previously in [120]. The fluorescence of Cy5 molecules was recorded using a 640 nm laser with 30 mW power at a frame rate of 2 Hz until the bright spots reached the background level. The fluorescence intensity from each spot was plotted over time. Bleaching of individual fluorophores resulted in a clear step-wise decrease in fluorescence which facilitated us to count the total number of fluorophores in each origami ring.

D.2. Design schemes
Figure D.1.: Design scheme including sequences of ring v1. Generated with caDNAno v0.2.
Figure D.2.: Design scheme including sequences of ring v2. Generated with caDNAno v0.2.
Figure D.3.: Design scheme including sequences of ring v3. Generated with caDNA
no v0.2.
Figure D.4.: Design scheme including sequences of ring v4. Generated with caDNA2o v0.2.
Figure D.5.: Design scheme including sequences of ring v5. Generated with caDNAo v0.2.
Figure D.6.: Design scheme including sequences of ring with shape-complementary features. Generated with caDNAno v0.2.
D.3. Electrophoretic mobility analysis data

Figure D.7.: EMA of purified DNA rings. Laser-scanned image of 2% agarose gel with 11 mM MgCl$_2$. L = 1 kb ladder, S = scaffold DNA. Numbers correspond to the number of attachment anchors. All lanes have been auto-leveled below the pockets. Lanes L and S have been separately auto-leveled.

Figure D.8.: EMA of DNA ring incubated with GuHCl. Laser-scanned image of 2% agarose gel with 11 mM MgCl$_2$. S = scaffold DNA. Concentrations refer to the GuHCl concentration present during the incubation. Incubation time is 12 hours.
Figure D.9: EMA of folding reactions of ring with shape-complementary features. Laser-scanned image of 2% agarose gel with 11 mM MgCl$_2$. S = scaffold DNA, folding ramps as indicated. Concentrations refer to the MgCl$_2$ concentration during the folding reaction. If no concentration is indicated, 20 mM were used.
Figure D.10: Exemplary particles of negative-stain TEM classification. a, with high density filling, b, with medium density filling and c, with low density filling. Frame colors indicate sample of framed particle: orange = ring with NSP1, blue = ring with NSP1-S, gray = ring only. Scale bar is 50 nm.
Figure D.11.: Exemplary cryo-EM micrographs of DNA rings with up to 32 NSP1 proteins attached. Scale bar is 50 nm.

Figure D.12.: Comparison of cryo-EM average micrograph and CanDo structure prediction. a, Average cryo-EM micrograph of DNA ring without protein. White arrows indicate density spokes. Scale bar is 50 nm. b, Structure of DNA ring without leash calculated by CanDo [54]. Black arrows indicate positions of crossovers.
E. Supp. material: Uncovering the forces between nucleosomes using DNA origami

E.1. Protocols

E.1.1. Assembly of branched nucleosome template DNA

The protocol was published in [89] and is reprinted here:

For NCPs with nicked or nicked and branched templates, the templates for reconstitution were generated by hybridization of respective oligonucleotides to a continuous 147 nt single DNA strand, which was synthesized by Integrated DNA Technologies (IDT), Leuven, Belgium and PAGE purified. All other oligonucleotides were synthesized in HPSF grade by Eurofins Genomics, Ebersberg, Germany. Hybridization of the oligonucleotides was performed by heating to 60°C and cooling to 40°C in steps of 1°C/h in 10 mM Tris-HCL, pH 7.6, 2 M NaCl and 1 mM EDTA. Complete annealing of all oligonucleotides to dsDNA was confirmed by agarose gel electrophoresis prior to nucleosome reconstitution.

E.1.2. Attachment of nucleosomes to the force spectrometer

The protocol was published in [89] and is reprinted here:

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, 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.
E.2. Nucleosome template DNA design

One strand of the Widom 601 sequence was divided into six sequences such that the attachment handles protrude radially from the NCP in 90° steps. The sequences are:

<table>
<thead>
<tr>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCGAGAATCCCCGGTGCCGAGGCCG</td>
</tr>
<tr>
<td>(A1)-CTCAATTGGTGCTAGACAGC</td>
</tr>
<tr>
<td>(A2)-TCTAGCACCAGCTAAACGCAC</td>
</tr>
<tr>
<td>(A3)-GTACCGCCTGTCCCAGCGGT</td>
</tr>
<tr>
<td>(A4)-TTTAACGCAAGGGGATTACTCCCTAGTCTC</td>
</tr>
<tr>
<td>CAGGCACGTGTGATATATACATCCGAT</td>
</tr>
</tbody>
</table>

The single-stranded attachment handles were always placed at the 5'-end of the respective strand. The sequences used for the attachments handles were designed with NuPack [116]:

<table>
<thead>
<tr>
<th>Handle</th>
<th>Sequence set 1 (bottom beam)</th>
<th>Sequence set 2 (top beam)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>AACTCGTGCTGGGGTTGGGCG</td>
<td>AGTGTCAGGCACACCTGCGGCG</td>
</tr>
<tr>
<td>A2</td>
<td>CTCCTCCTCCCCGAGGTC</td>
<td>GCAGAACGTGGAGAAGTTTG</td>
</tr>
<tr>
<td>A3</td>
<td>GCCGCAGGATACAGAATACG</td>
<td>CGTAGAGTCGCTAATATAT</td>
</tr>
<tr>
<td>A4</td>
<td>TCCTCAGACTTAACACAGGG</td>
<td>AGATCTCTCTACGCGAGGATA</td>
</tr>
</tbody>
</table>
Figure E.1.: Relative orientation of nucleosomes on force spectrometer. a, Ribbon view of 147 bp DNA template wrapped around the histone octamer (not shown) taken from pdb 3MVD. Oligonucleotides used in the branched template are shown in different colours. Dashed line indicates the dyad axis. Solid line indicates attachment handle positions A1 and A3. b, Schematic representation of force spectrometer with two attached nucleosomes on top and bottom arm in distal position. Red cylinders indicate dyad axis for each nucleosome. Positions of attachment handles as indicated.
Figure E.2.: Additional reference-free class averages of nucleosomes with wildtype histones. Scale bar is 10 nm.

E.3. Electron microscopy data
Figure E.3.: Exemplary negative-stain TEM micrographs of force spectrometers without attached nucleosomes. 210 (out of 3091) particles. Scale bar is 50 nm.
Figure E.4.: Exemplary negative-stain TEM micrographs of force spectrometers with two attached nucleosomes in proximal position. 203 (out of 1301) particles. Scale bar is 50 nm.
Figure E.5.: Exemplary negative-stain TEM micrographs of force spectrometers with two attached wt NCPs in distal location. 124 (out of 158) particles. Scale bar is 50 nm.
E. Calculation of nucleosome-nucleosome energy landscapes

The calculation was published in [89] and is reprinted here:

In order to extract the energy landscape of two nucleosomes from the set of measured vertex angles, we first estimate the probability density \( p(\theta) \) of each variant using kernel density estimation with a uniform or normal kernel and a bandwidth of 3°. Assuming Boltzmann statistics, the probability density is given by:

\[
p(\theta) = \frac{1}{Z} e^{-\frac{E(\theta)}{k_BT}}
\]

with the partition function \( Z = \int_{0}^{2\pi} e^{-\frac{E(\theta)}{k_BT}} d\theta \). Hence, the free energy landscape of a given sample can be calculated (Fig. E.6 a, b):

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

where \( \ln(Z) \) is a constant that depends on the underlying energy landscape, i.e. the nucleosome-variant. In order to compare different samples, we assume that the interaction between nucleosomes vanishes at large distances. We can therefore shift each energy landscape by a variant-dependent constant \( \Delta_i \):

\[
\frac{E_i(\theta)}{k_BT} = -\ln(p_i(\theta)) + \Delta_i
\]

(5.1)

with \( \Delta_i = \ln \left( \frac{\int_{\theta_0}^{2\pi} p_i(\theta) \, d\theta}{\int_{\theta_0}^{2\pi} p_{noNCP}(\theta) \, d\theta} \right) \)

(5.2)

where \( p_{noNCP} \) is the density estimate of the bare spectrometer and \( \theta_0 = 75^\circ \) (\( \approx 15.4 \) nm CM-CM distance) is a cut off at which the interaction vanishes (Fig. E.6 c, d). The integrals were approximated by sums over the kernel density estimates of the probability densities with an upper integration limit of 120°.

The pair-potential of two nucleosomes can then be calculated:

\[
E_i_{NCP-NCP}(\theta) = E_i(\theta) - E_{noNCP}(\theta)
\]

\[
= k_BT \left[ -\ln(p_i(\theta)) + \Delta_i + \ln(p_{noNCP}(\theta)) \right]
\]
E.4. Calculation of nucleosome-nucleosome energy landscapes

Figure E.6.: Calculation of free energy landscapes. a, Uniform kernel density estimates $p_i(\theta)$ (bandwidth 3°) of probability densities for constructs with different nucleosome variants and nucleosome orientations. b, Free energy computed as $\Delta G = -\ln(p_i(\theta))$. c, Scaled uniform kernel density estimates $\bar{p}_i(\theta) = p_i(\theta) e^{-\Delta_i}$, where the offset $\Delta_i$ was calculated according to equation 5.2 with a cut off at 75° (dashed line). d, Shifted energy landscapes $\Delta G = -\ln(p_i(\theta)) + \Delta_i$. 
Figure E.7.: Gay-Berne potentials fits to nucleosome-nucleosome energy landscapes. Fits (dashed lines) of Gay-Berne potentials to energy landscapes of nucleosome-nucleosome interactions (see Fig. 5.10) lacking N-terminal histone tails (a), where the lysine 16 at the H4-tail is acetylated (b), nucleosome with wildtype histones where the two dyad axes enclose an angle of 78° (c) and nucleosomes with wildtype histones where the two dyad axes enclose an angle of 258° (d).
E.5. Gay-Berne potentials fitted to energy landscapes

The energy landscapes were fitted with Gay-Berne-potentials that were used previously for modeling nucleosome-nucleosome interactions [107]. The Gay-Berne potential [106] used here is written as follows:

\[ V(x) = 4e\left(\frac{s_0}{x - x_0 + s_0}\right)^2 - \left(\frac{s_0}{x - x_0 + s_0}\right)^6 \] (5.3)

The fits were performed between CM-CM distances of 3.15 and 18.8 nm. The resulting curves are shown in Fig. E.7. The obtained fit parameters are:

<table>
<thead>
<tr>
<th>Variant</th>
<th>( e ) ([k_B T])</th>
<th>( s_0 ) ([nm])</th>
<th>( x_0 ) ([nm])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleosomes lacking N-terminal tails</td>
<td>0.59 ± 0.03</td>
<td>16.14 ± 0.65</td>
<td>4.23 ± 0.04</td>
</tr>
<tr>
<td>WT nucleosomes where dyad axes</td>
<td>2.29 ± 0.05</td>
<td>17.43 ± 0.48</td>
<td>3.24 ± 0.04</td>
</tr>
<tr>
<td>enclose an angle of 78°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleosomes where the lysine 16 at the H4-Tail is acetylated</td>
<td>0.93 ± 0.03</td>
<td>16.65 ± 0.48</td>
<td>4.25 ± 0.03</td>
</tr>
<tr>
<td>WT nucleosomes where dyad axes</td>
<td>2.16 ± 0.06</td>
<td>20.21 ± 0.68</td>
<td>3.07 ± 0.04</td>
</tr>
<tr>
<td>enclose an angle of 258°</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


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