Nunchuck Nanostructures for Dynamic Measurement of dsDNA Bending

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by

Deborah Clayton-Warwick

Thesis Advisor:
Dr. Deborah K. Fygenson
Associate Professor of Physics

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The Dissertation of Deborah Clayton-Warwick is Approved by:

Faculty Mentor

Faculty Advisor

University of California
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Abstract

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Chapter 1

Introduction

DNA performs a wide variety of functions that are vital to the growth and maintenance of life. In particular, the bending of DNA facilitates many biological processes by directing the expression and storage of genetic information. Examples of such processes include transcription, DNA packing, replication, recombination, and chromatin organization. DNA bending can occur either through the constructive addition of small bends associated with repeated special base sequences\textsuperscript{1}, known as intrinsic bending, or through the binding of a DNA-binding protein to its recognition sequence\textsuperscript{2}, known as induced bending.

Intrinsically bent DNA was discovered more than 30 years ago when kinetoplast fragments (concentrated regions of DNA outside of the nucleus) were found to migrate anomalously slowly on polyacrylamide gels\textsuperscript{3}. Since its discovery, gel mobility experiments have provided crucial insight into the nature of DNA bending, including the direction of bending for intrinsic bends\textsuperscript{4, 5, 6} and the relative binding constants in induced bending\textsuperscript{7}. Bent DNA has also been extensively studied through X-ray crystallography\textsuperscript{8}, which provides structural detail with angstrom-level resolution. However, this method can be exceedingly tedious as crystallization is unpredictable. Additionally, the crystal
imposes packing effects that may heavily influence DNA bending\textsuperscript{9}. Studies of the atomistic structure of DNA oligomers are also performed in solution using nuclear magnetic resonance (NMR) spectroscopy. Due to the short-range nature of NMR, however, it is usually not possible to study global features of DNA bending this way. Methods, such as cryo-transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM), have also been used to study DNA bending, but like crystallography and NMR, these methods provide only static, population-averaged bend angles, thus revealing little of the variability and nothing of the dynamics of DNA bending.

Forster resonance energy transfer (FRET), a method of measuring distance based on the photophysical interaction between a ‘donor’ and ‘acceptor’ dye, has been used to make dynamic, single-molecule measurements of DNA bending. By using FRET-based sensors, several groups\textsuperscript{10, 11} have been able to study dynamic changes in donor-acceptor distance due to protein-induced DNA bending. Although this method provides insight into the dynamics of induced bending, it is limited to the study of very bent DNA as the donor-acceptor distance must be no greater than 10 nm. A new method that not only allows for a wider range in the study of DNA bending, but is also accessible and affordable is needed.

This thesis introduces a new tool for measuring DNA bend angles, the DNA ‘munchuck’, and describes work aimed at calibrating the tool on intrinsically bent sequences of double-stranded DNA (dsDNA). A DNA munchuck consists of a double-stranded DNA linker, 58 base pairs in length, flanked on either end by DNA nanotubes $\sim 3 \ \mu$m long. These nanotubes act as extensions of the linker to mechanically magnify the bend angle. For the purpose of calibration, linker sequences (Section 2.1.1) were designed to have intrinsic bends of varying degree. It is noted that the linker can also be easily redesigned to contain a binding protein’s recognition sequence for future studies.
regarding induced DNA bending. Measurement involves observing a nunchuck via fluorescence microscopy as it diffuses through an imaging solution designed to restrict its motion to a two-dimensional imaging plane. The angular dynamics and bend angle distributions of the nunchuck are then analyzed as a function of linker sequence. By doing so, we found that external effects of the method design, such as imaging conditions and nunchuck structure, obscure the distinction between linkers. However, some signature of the linkers is still visible, suggesting that, with further development, the nunchuck will be an effective tool to measure DNA bending dynamics.
Chapter 2

Materials and Methods

DNA nunchucks are made by linking two cylindrical DNA origami seeds (Figure 2.1a) endwise with a synthetic stretch of double stranded DNA (Figure 2.1b) and then nucleating nanotubes from opposing ends of the seeds (Figure 2.1c-d).
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Figure 2.1: Hierarchical assembly of DNA nunchucks (Ref.[12]).
2.1 DNA Origami Seed Structure Design

DNA origami is formed by folding a long scaffold strand of DNA into specified shapes using many short staple strands\textsuperscript{13}. Here, the scaffold strand (ssM13mp18) was folded into a cylindrical structure with facets designed to match the size and shape of a nanotube. With an appropriate set of single stranded sequences protruding from one of its end (‘adapter tiles’\textsuperscript{14}), the origami tube can serve as a seed, nucleating a nanotube from that end (see Section 2.2). Approximately one-third of the scaffold strand was used to construct the designed seed. The remaining unused scaffold was left as a single-stranded loop that protrudes from the midpoint of the seed (Figure 2.2a-b)\textsuperscript{15}. To label the seeds, ATTO647N-conjugated fluorophore strands (sequences in appendix) were hybridized to this unused scaffold loop (Figure 2.2c). The munchuck seed is composed of two separate nanotube seeds (monomers) linked together through a synthetic stretch of dsDNA. These dimers are formed by mixing the two monomer seeds, each having one of two complementary linker strands protruding from one end (Figure 2.1a).

2.1.1 DNA linker strands

Studies have shown that appreciable intrinsic DNA bending occurs when A tracts, defined as runs of at least four consecutive A-T base pairs, are imbedded within a DNA sequence\textsuperscript{16, 17}. Koo et al. found that a single A-tract six base pairs in length produces a maximal degree of bending: $17 - 21^\circ$\textsuperscript{17}. They further showed that A-tracts can be concatenated to produce greater bends if their spacing matches the pitch of the DNA double-helix. With such appropriate phasing, as the number of A-tracts in a sequence increases, the degree of bending increases proportionally. For the purpose of calibrating the DNA munchucks, we designed three different linker sequences with 0, 1, and 4 A tracts to produce $0^\circ$, $19^\circ$ and $76^\circ$ bends, respectively. As a control, we also designed a linker
with tracts of twelve non-complimentary T's at the center on each linker strand. This 'poly-T bubble', having no preferred bend state, should allow the nun-chuck to explore all possible angular configurations with equal probability, thus resulting in a time-averaged bend angle of 0°.

2.2 DNA Nanotube Arm Design

DNA nanotubes are composed of versatile, nanoscale building blocks called DNA tiles[18, 19]. The DNA double-crossover molecules used as tiles in this study are of a type known as DAE-E tiles. They consist of five short DNA strands that self-assemble to form a well-defined tile structure (Figure 2.3a) based on preferential complimentary binding. These tile structures present specific sequences, called sticky ends, at each of four corners in a plane (Figure 2.3b). Sticky ends can be programmed to hybridize so that tiles form extended structures. Here, the tiles assemble into diagonally striped
lattices (Figure 2.3c) which then cyclize into nanotubes (Figure 2.3d) due to intrinsic curvature with an angle of $150 \pm 10^\circ$ between neighboring helix-pairs\textsuperscript{13}. We label each tile with a Cy3 fluorophore on the 5' end of its central strand, which is well removed from the sticky ends that are hybridizing to form the nanotube structure (Figure 2.3b). At the sticky-end melting temperature, nanotubes grow from seeds more rapidly than they nucleate spontaneously\textsuperscript{12}. Extended incubation at the sticky-end melting temperature and a proper ratio of tiles to seeds produce mchucks with nanotube arm lengths of $3 \pm 1 \mu$m, as described in the next section. These nanotube arms are long and stiff enough (persistence length $\sim 10 \mu$m) to mechanically magnify the bend angle of the dsDNA linker.
2.3 Nunchuck Preparation

2.3.1 DNA strands and reagents

DNA tile, adapter, staple, and linker strand sequences are listed in the appendix. All strands, except the scaffold, were purchased from Integrated DNA Technologies, Inc. Tile, adapter, and linker strands were HPLC purified after synthesis while staple strands were provided with standard desalting. Scaffold strand, M13mp18, was purchased from Bayou Biolabs (cat. no. P-107). Reactions were performed in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) with 12.5 mM magnesium acetate (TAE/\(Mg^{2+}\) buffer) unless mentioned otherwise. To prevent adsorption of DNA to the walls of the PCR tubes in which mixtures were incubated, 0.15 mg/ml BSA (DNase and RNase free) (A8549, Sigma-Aldrich Co.) was added during nanotube nunchuck growth. T4 polynucleotide kinase (M0202S), T4 DNA ligase (M0202S) and T4 DNA ligase reaction buffer (B0202S) from New England Biolabs, Inc. were used for ligation.

2.3.2 Nunchuck seed structure synthesis

Two solutions, each containing M13mp18 (10 nM), staple strands (100 nM), adapter strands (100 nM), kinase (0.6 units/\(\mu l\)), and one of the linker strands (10 nM), were incubated separately at 37°C for 2 hours to promote kinase activity, then annealed from 90°C to 20°C at −1°C/min to allow the origami seeds to assemble. The two solutions were then combined in a single PCR tube and held at 32°C for 12 hours to promote dimerization via the (complimentary) linker strands. Finally, ligase (20 units/\(\mu l\)) was added and the mixture was incubated overnight at room temperature. The addition of kinase and ligase creates a more stable dimer bond. Kinase phosphorylates the DNA and ligase catalyzes the formation of a phosphodiester bond to seal the DNA strands together.
Finally, Proteinase K (100 μg/ml) was added at 37°C and samples were incubated for one hour to digest unused proteins. The mixture was then run through a 1% agarose gel, pre-stained with 1.25 μg/ml Ethidium Bromide, at 100 V for ~1 hour to separate and the dimer band was extracted using a Freeze N’ Squeeze DNA Gel Extraction Spin Column (Biorad).

2.3.3 Growing nanotube nunchucks

Tile strands (50 nM), attachment strand mix (0.6 nM), seed labeling strands (50 nM), and excess adapters (0.4 nM) were mixed in TAE/Mg\(^{2+}\) buffer with BSA and held at 90°C for 5 mins. The sample was then cooled from 90°C to 45°C at –1°C/min, held at 45°C for 60 mins to allow for DAE-E tile assembly, and then cooled further from 45°C to 34°C at –0.1°C/min. Gel-purified nunchuck seeds were added at 40°C. Samples were then held at 34°C for 15 hours to allow for tile sticky-end hybridization and nanotube growth.

2.3.4 Fluorescence microscopy

Guard strands

To prevent continued nucleation and spontaneous growth during room-temperature imaging, guard strands were added to solutions prior to imaging. Guard strands are complimentary in sequence to the short, sticky-end bearing strands in the DAE-E tiles. When present in excess, they effectively remove the sticky-end bearing strands from free DAE-E tiles and nanotube ends\(^{[15, 20]}\). Specifically, 0.2 μl of 4 μM guard strands were added to 2 μl of annealed nunchuck mixture at 34°C and incubated for 1 minute just before imaging. On the time scale of a few hours, this procedure inhibits new nanotube growth without destabilizing existing nanotubes.
Oxygen Scavenging System (OSS)

To prevent oxidation and improve fluorescent dye stability, an oxygen scavenging system\[^{23}\] was used. First, the prepared nunchuck - guard strand mixture was diluted into 10 μl of a dilution buffer made of 1 mg/ml BSA, 0.54% methyl cellulose (MeCe), and TAE buffer. Here, MeCe acts as a crowding agent that confines nunchucks against the imaging surfaces (see Section 3.5.1). 10 μl of the diluted nunchuck mixture was then added to 0.5 μl of 1 μM protocatechuic acid (PCA) and 0.12 μl of 83.33 mM Trolox.

Surface passivation

To prevent nunchucks from sticking to glass surfaces during imaging, two methods of surface passivation were used:

i. **PEG surface passivation:** Silane functionalized polyethylene glycol (PEG) is a small, highly-entropic molecule that adheres to surfaces through a silane linkage. A covering of these molecules creates a surface that entropically excludes other molecules from binding to it. To passivate the imaging surfaces, a mixture of 0.5% PEG in ethanol with 1% acetic acid was placed on a glass slide and covered with a coverslip. This was then dried in an oven at 70°C for 30 minutes, and then stored in a laminar flow hood. Immediately prior to use, the slide and coverslip were separated, rinsed with DI water to remove excess PEG, and dried using nitrogen air.

ii. **BSA surface passivation:** Bovine serum albumin (BSA) is a globular protein that passivates glass surfaces by creating an adsorption layer that is less positively charged, making the DNA less likely to stick. To passivate surfaces using this
Materials and Methods

method, glass slides and coverslips were soaked in BSA (10 mg/ml) for 12 hours and dried using nitrogen air prior to imaging. BSA passivation was the primary method used in imaging (see Section 4.2).

2.3.5 Imaging

![Images](image_url)

Figure 2.4: Fluorescence images of (a) Nanotube arms using a Cy3 filter and (b) DNA origami seeds using an ATTO647N filter; (c) Overlay showing one nunchuck (labeled with arrow) and spontaneously nucleated nanotubes. Note: The seed is not exactly at the vertex due to movement of the nunchuck between images. Scale bar is 2 μm.

5 µl of sample was deposited on a glass coverslip (22 x 30 mm Corning cat. no. 2935-223), placed on a slide (75 x 25 mm Gold Seal cat. no. 3010), and sealed with paraffin wax. Imaging was done with epi-fluorescence using a 100x/1.4NA oil-immersion objective on an inverted microscope (Olympus IX-70), a cooled CCD camera (Hamamatsu, Orca 2e), and a filter set optimized for Cy3 and ATTO647N dyes (Omega Optical, XF100). First, two images were taken, one capturing the nanotubes using the Cy3 filter and the other capturing the seeds using the ATTO647N filter. An example of a color-enhanced overlay of these two images can be seen in Figure 2.4. By taking these first two images, we were able to distinguish nunchucks (labeled with an arrow in Figure 2.4c) from spontaneously nucleated nanotubes. Having verified that a given nanotube structure was a nunchuck, a sequence of images of the nunchuck was taken using the Cy3 filter with a 0.8 second exposure time and an interval of 1.5 seconds between frames. Image sequences were
terminated when either (i) the nunchuck diffused out of the field of view, (ii) the nunchuck became stuck to the surface or to another nanostructure or, most commonly, (iii) the image quality diminished due to photobleaching of the fluorescent dye. Image sequences were approximately 600 frames on average.

2.4 Image Analysis

2.4.1 Data extraction

Image sequences were first contrast enhanced to 0.4% pixel saturation and then cropped to contain a single nunchuck. Contours were then extracted from each image using the JFilament plugin for image processing program ImageJ\textsuperscript{22}. This plugin traces the nunchuck in each frame and outputs x-y positions for each pixel along the trace. The output data was then run through a custom MATLAB code. This code first "guesses" at the location of the nunchuck vertex in each contour by calculating the position of maximum excursion from the end-to-end vector. It then refines the guess by requiring consistency in vertex location and arm lengths throughout the image sequence. The vertex location was correctly identified in each frame with < 5% error. This error rate was determined by labeling the calculated vertex in each frame and manually checking for, and ignoring, all incorrectly labeled frames.

Finally, a measure of the vertex angle was extracted by creating a set of 0.5 μm tangent vectors at varying distances from the vertex starting at ±1 μm. The angles between all possible pairs of vectors on opposite sides of the vertex were computed using the cross product and averaged to produce a single bend angle for a given contour. The sign of the cross product was also extracted to determine the direction, or handedness, of the bend with respect to the longer of the two arms (Figure 2.5).
2.4.2 Analysis methods

All plots were created using the graphing and data analysis application KaleidaGraph (Synergy Software). Angular dynamics were analyzed by plotting bend angle as a function of time (Figure 3.2) and trends in bending were analyzed by creating polar histograms of cumulative data for each linker (Figure 3.7).

Correlation measurements were made to determine the relationship between frames over time using:

$$\xi(\Delta t) = \left\langle \frac{(\theta(t) - \bar{\theta}) \cdot (\theta(t + \Delta t) - \bar{\theta})}{\sigma_\theta^2} \right\rangle$$  \hspace{1cm} (2.1)

Using a custom MATLAB code, the deviation from the average bend angle of pairs of bend angles separated by a time interval $\Delta t$ were calculated and their product was normalized by the square of the standard deviation. This calculation, when averaged over all possible pairs for a given $\Delta t$, is the so-called correlation function, $\xi(\Delta t)$.

Figures 3.3 and 3.4 present plots of $\xi(\Delta t)$, from which the correlation time ($t_{cor}$) of a data set is determined, defined as the time interval over which pairs of points first appear uncorrelated (e.g. $\xi(\Delta t = t_{cor}) = 0$) (marked in Figure 3.3-3.4).
Chapter 3

Results

3.1 Angular Dynamics

Figure 3.2 presents the angular dynamics of four nunchucks for comparison. All four nunchucks were identically prepared with 1 A-tract linkers. In these plots, 0° corresponds to a straight nunchuck, or fully open bend angle and 180° corresponds to an folded nunchuck, or fully closed angle (Figure 3.1). Qualitatively, the bending dynamics of these four nunchucks are very different. The nunchuck in Figure 3.2a shows persistent bending with a sudden switch in handedness; the nunchuck in Figure 3.2b shows gradual transitions between short-lived persistent angles; the nunchuck in Figure 3.2c shows large fluctuations about a gradually decreasing mean bend angle; and the nunchuck in Figure 3.2d shows not only a persistent bend, but also a strong preference in handedness.
Figure 3.1: Images of the same nunchuck with an (a) open (0°) and (b) closed (180°) bend angle. The two arms are labeled with different colored lines for clarity. Scale bar is 1μm.

Figure 3.2: Plots demonstrating varied angular dynamics for identically prepared nunchucks (here with 1 A-tract linkers): (a) Persistent mean angle of approximately −60° that switches handedness suddenly for the remaining ~ 30% of the image sequence; (b) Gradual transitions between short-lived persistent angles (frames 40-150 at 90° and frames 590-650 at −130°); (c) Large fluctuations that are comparable to the global variation in angle. No persistent bends; (d) Persistent mean angle of approximately 90° with a strong preference in handedness, switching only from frames 570-620.
Results

If the observed angular dynamics were caused by intrinsic bending of the linker, we would expect all nunchucks made with the same linker strand to have similar bend angle time series. The variation in Figure 3.2 therefore suggests that the bend angle dynamics being observed are not a direct reflection of the intrinsic bend in the linker, but rather of some dominant external factor. One possibility is that a variety of structural configurations exist due to the relatively large nanotube arms. For example, torsional fluctuations of the arms of one nunchuck may cause the linker to twist, resulting in one type of bending, while the longer arms of another nunchuck may have more drag, resulting in a different type of bending.

Alternatively, such variation may arise if the individual image sequences do not accurately reflect the full range of a single nunchuck’s dynamic behavior. This would result from undersampling, or only collecting enough measurements to observe a portion of the full range of dynamics. Since photobleaching often limited the duration of a data set, undersampling, to the extent that it occurred, might be corrected by increasing the time interval between frames. To identify the appropriate sampling rate, we looked at correlation times in the bend angle series.
3.2 Correlation Times

![Graphs showing correlation times for different linkers: (a) 0 A-tract, (b) 1 A-tract, (c) 4 A-tract, and (d) poly-T nunchucks.]

Figure 3.3: Examples of “typical” correlation behavior for (a) 0 A-tract, (b) 1 A-tract, (c) 4 A-tract, and (d) poly-T nunchucks. The standard deviation of equation (2.1) gives the error bars for each point. The time at which the bend angle measurements become uncorrelated (zero crossing of the correlation function) is defined as the correlation time ($t_{cor}$) and is labeled in the above plots. The total time for the image sequence ($t_{tot}$) and the total length of the nunchuck $L_{tot}$ are also noted.

Figure 3.3 presents examples of the time-dependence of angular correlations for each of the four linker types. “Typical” behavior is indicative of most data sets ($\geq 76\%$) in which $t_{cor}$ was less than $10\%$ of the image sequence length. Comparison of correlation times with time series (Figure 3.4) shows that correlation times are dependent on angular dynamics. Figure 3.4a-b show two examples of dynamics that result in a low
$t_{cor}$ (less than 10% of the image sequence length). The nunchuck in Figure 3.4a appears to have a gradually changing mean angle, and should thus have a long $t_{cor}$. However, the fluctuations are large enough that the time spent on either side of the mean is similar and thus the angle decorrelates quickly. By similar reasoning, the small fluctuations about the persistent bend in Figure 3.4b also result in a low $t_{cor}$.

Examples of dynamics that result in a high $t_{cor}$ can be seen in Figure 3.4c and d.1-2. Although the nunchuck in Figure 3.4d.2 is also persistently bent, the switch in handedness for $\sim$ 30% of the image sequence causes the mean bend angle to shift toward $0^\circ$. Because the nunchuck spends disproportionate amounts of time away from $0^\circ$, $t_{cor}$ is falsely prolonged. To correct such data sets, the absolute value of angular data was used to determine $t_{cor}$ (Figure 3.4d.3). Some data sets, such as in Figure 3.4c, remained highly correlated due to relatively small oscillations about gradually changing angles. Because of their high $t_{cor}$, and consequently small number of independent frames, these data sets were ignored. All other data sets were down-sampled to contain only independent frames that were separated by their relevant $t_{cor}$. Although the size of data sets drastically decreased from this down-sampling (Figure 3.8), reliable angle measurements could now be sampled from an ergodic system.
Figure 3.4: Comparison of correlation behavior with time series for various nunchucks: (a)-(b) Large fluctuations compared to the global variation in angle results in a low \( t_{cor} \); (c) Small fluctuations about a gradually changing angle results in a high \( t_{cor} \). These type of data sets were ignored; (d.1)-(d.2) The switch in handedness for 30% of the image sequence results in a falsely prolonged correlation between measurements. These data sets were corrected by finding \( t_{cor} \) from the absolute value of the time series (d.3).
3.2.1 Dependence on arm length

Nanotube arm length was found to affect angular dynamics in that the crowding agent, MeCe, seemed to restrict movement for long-armed munchucks (see Section 3.5.1). Due to the sensitivity of $t_{cor}$ to angular dynamics, we hypothesized that arm length would also affect $t_{cor}$. To test this hypothesis, $t_{cor}$ was plotted against the shortest, average, and longest arm length for each linker (Figure 3.5).

While the majority of the linkers (0 A-tract, 1 A-tract, and poly-T) appear to show no correlation, 4 A-tract has a distinct linear relation between $t_{cor}$ and arm length. This anomalous behavior may be explained by atypical imaging conditions for the 4 A-tract linker. Prior to data collection for this thesis, surface passivation was consistently done using BSA. However, when sticking first appeared to be limiting data collection, poly-ethylene glycol (PEG) passivation was tested as an alternative. While munchucks imaged on freshly made PEG-treated glass showed significantly less sticking (for 4 A-tract), the amount of sticking dramatically increased when using PEG-treated glass that had been stored for only one day. Because preparing PEG-passivated glass is an elaborate procedure requiring more than one day’s work, we went back to using BSA passivation. The disparity in method for 4 A-tract seems to have escaped attention until the current analysis. The data nevertheless offers insight into the much larger effect that surface passivation may have on dynamics than previously thought.
Figure 3.5: Plots used to determine what type of, if any, relationship there is between arm length and $t_{cor}$ for the four linker types. It is seen that most of the linkers ((a) 0 A-tract, (b) 1 A-tract, and (d) Poly-T) show no correlation between these two factors, yet (c) 4 A-tract shows a distinct linear relation.

3.3 Persistently Bent States

Persistently bent states, defined by an average bend angle that persists for more than 100 frames (150 seconds), are observed in each linker type (Figure 3.6). The cause of these states is currently unknown, but the fact that they occur for every linker tested suggests that it is not the linker but some common aspect of nunchuck structure that causes them (see Section 4.3). Figure 3.6 illustrates the qualitatively different behavior
of the fluctuations exhibited by each linker in a persistently bent state. The 0 A-tract nunchuck seems to gradually oscillate about its persistent bend (evidenced by the sort of 'hilly' behavior in Figure 3.6a), whereas the 1 A-tract nunchuck in Figure 3.6b fluctuates about a clearly defined persistent bend. In fact, the 1 A-tract data contained more persistently bent states than any other linker (Section 4.3). Conversely, the 4 A-tract and poly-T nunchucks have persistent angles that are much less clearly defined because of large fluctuations about the mean angle (Figure 3.6c and d). These unique responses by each linker type to the cause of persistent bends may suggest some sort of underlying signature of the linkers.

Figure 3.6: The way in which each linker fluctuates about its persistently bent state may be a signature of the linker. (a) 0 A-tract shows gradual oscillations about its persistent state; (b) 1 A-tract has the most well defined (consistent, small fluctuations) persistent bends; (c)-(d) 4 A-tract and poly-T both have relatively large fluctuations.
3.4 Bend Angle Distributions

Down-sampled data sets were compiled for each linker and plotted on polar histograms (Figure 3.7). In these histograms, data is binned every 40° and the radius depicts the fraction of angles in that sector. The top half of the histogram represents bend angles from 0° (fully open) to 180° (fully closed) with positive handedness, and the bottom half represents identical bend angles with negative handedness. Because there is currently no way to orient the handedness of bend angles relative to one another, data sets with a dominant preferred angle were oriented such that the dominant angle had a positive handedness. Comparison of the histograms reveals a preference for open angles (−90°−0° and 0°−90°) in all linkers. Of these two open bend states, there also appears to be a preference for one handedness over the other. However, because of the reorientation of dominant angles, the particular handedness that is preferred cannot be determined.

As the control, poly-T is expected to have no preference in bend angle, resulting in a rotationally symmetric distribution. The reorientation of dominant angles would skew the distribution toward positive angles, but would not account for the preference for open angles seen in Figure 3.7d. This suggests that there may be some external factor that interferes with bending by allowing particular bend states and excluding others. Comparison of the distributions in Figure 3.7 suggests that an underlying effect of linker bending may still be visible though. For example, the median bend angle for intrinsically bent linkers, 1 A-tract and 4 A-tract, is ~30° greater than that of non-intrinsically bent linkers, 0 A-tract and poly-T (Figure 3.8). However, due to the dominance of the external factor, it is difficult to distinguish what effects might be caused by the linker.
Figure 3.7: Polar histograms of down-sampled bend angle distributions for each linker. Total number of measurements used in these histograms were (a) 0 A-tract: 129, (b) 1 A-tract: 274, (c) 4 A-tract: 314 and (d) Poly-T: 244. Comparison of these histograms shows that every linker appears to prefer open angles (right quadrants) and one handedness over the other (upper right quadrant). Intrinsically bent linkers (b and c) have a higher median bend than non-intrinsically bent linkers (a and d) (Figure 3.8).
3.5 Possible Factors Affecting Nunchuck Dynamics

3.5.1 Imaging conditions

To obtain reliable bend angle measurements, the concentration of the crowding agent, MeCe, was required to be high enough to confine nunchucks to the two-dimensional imaging plane, but also low enough that nunchucks were not restricted in movement. A final concentration of 0.45% MeCe was found to appropriately balance these two factors for nunchucks with targeted arm length of ~3 μm. Because all samples were prepared using 10 μl of the same dilution buffer (Section 2.3.4), the only uncertainty in MeCe concentration arose from pipetting error, which was within the standard deviation (0.1 mg) of the electronic balance used (Shimadzu AY220). It is therefore unlikely that fluctuations in MeCe concentration were great enough to affect nunchuck dynamics for the targeted arm length. However, it was found that nunchucks with arms that deviate from 3 μm by ~±1 μm do experience adverse effects of the crowding agent. For example, nunchucks with short arms (~2 μm) behave as if MeCe concentration is too low and
can move out of the imaging plane. This results in a measurement of the projection of the bend angle onto the imaging plane rather than the bend angle itself. Conversely, if a nunchuck has arms that are greater than \( \sim 4 \, \mu m \), it behaves as if the MeCe concentration is too high and movement is restricted by the crowding of MeCe pores.

![Images](image)

Figure 3.9: All frames were averaged for various image sequences to produce single images showing the cumulative movement for several nunchucks. (a)-(b) show the repetitive back-and-forth motion of long 0 A-tract nunchucks; (c) A short-armed 0 A-tract nunchuck has freer rotational and translational movement demonstrated by a broad, dim shape; (d)-(e) Nunchucks with different linkers but of similar length do not appear to be as restricted in movement as 0 A-tract.

By taking the average of all frames in an image sequence, the entirety of a nunchuck’s movement can be viewed in a single image (Figure 3.9). The elongated, bright shapes in Figures 3.9a and b represent repetitive back-and-forth movement of long 0 A-tract
nunchucks (7.3 μm and 6.8 μm total length respectively). This type of snake-like motion, termed ‘reptation’, is typical of a polymer entangled in a mesh, where the polymer can only move along its long axis once its ends have thoroughly explored the configurational space through thermal fluctuations. The dimmer, broad shape in Figure 3.9c shows the movement of a short (4.1 μm total length) 0 A-tract nunchuck that does not have the same restrictions in movement as the previous two longer nunchucks. Comparison of 0 A-tract with other linkers of similar lengths (Figure 3.9d-f) reveals that this level of reptation may also be a function of linker. Linkers that are expected to prefer open angles (0 and 1 A-tract) appear to be more susceptible to reptation while linkers that have an expected preference for bent states (4 A-tract and poly-T) show little to no reptation. Therefore, MeCe not only appears to affect angular dynamics as a function of arm length but of linker type as well.

3.5.2 Nunchuck structure

The observed preference for open angles and particular handedness may also be explained by the interference of nunchuck structure. Recalling the seed structure design, only one-third of the single stranded scaffold is used in the construction of monomer seeds (Figure 2.2). When the dimer seed is constructed, it is possible that these two identical relatively large loops interact by repelling each other and/or becoming entangled and binding together through complimentary tracts. Another structural component that may affect angular dynamics is the relative size of the nanotube diameter compared with linker length. Although nunchucks are designed with maximum separation between nanotubes by placing a half integer number of helical turns between them\textsuperscript{12}, the nanotubes may still prevent very closed angles by interfering with one another (Section 4.4.3).
Chapter 4

Discussion

4.1 Imaging Optimization

In the current analysis, data sets were drastically reduced in size to eliminate correlated measurements which resulted from an insufficient time interval between frames (or delay time). To maximize the number of independent measurements, therefore, the delay time should be equal to $t_{cor}$. However, $t_{cor}$ can reach up to 60 seconds in some cases, making it difficult to manually track a nunchuck between frames.

The analysis presented here suggests that $t_{cor}$ may be minimized by using PEG passivation (which may be the more effective of the two passivation techniques) and imaging only short-armed nunchucks (which were found to be less susceptible to MeCe concentration). It is possible that even with short-armed nunchucks and PEG-passivated surfaces, the correlations would still require too long a time interval between frames to reliably track nunchucks. Therefore, some down-sampling will still be required, but to a much lesser extent. This limitation might be eliminated altogether through the use of a computer-controlled microscope platform which would track the movement of the nunchuck and re-position itself so that the nunchuck is in the center of the frame.
after each measurement. With the current stationary platform, the delay time will be maximized, producing larger independent data sets, ultimately facilitating more reliable identification and correction of external effects on dynamics.

4.2 Surface Passivation

The unique correlation between $t_{cor}$ and arm length for the 4 A-tract data was likely a result of using PEG rather than BSA passivation during imaging. The relationship seen for 4 A-tract is expected here because angular dynamics were found to be dependent on the diffusion of nanotube arms. It is hypothesized that data collected using BSA passivation does not show this correlation because it is not as effective as PEG passivation. BSA passivates by creating an adsorption layer that may be crrugated, which would create barriers to and localize munchuck movement. Conversely, PEG attaches to the surface through a chemical silane bond, resulting in a molecularly smooth, more uniformly passivated surface. In addition, PEG-passivation may be more effective because the PEG-silane molecules entropically exclude other molecules from the surface whereas BSA prevents sticking by creating a less strongly-charged surface than glass.

To test the hypothesis that passivation technique affects dynamics, we will collect 4 A-tract data using BSA passivation and look to see whether the relationship seen with PEG is reproducible. If PEG does in fact prove to be more efficient, we will also collect data for 0 A-tract, 1 A-tract, and poly-T linkers on PEG-passivated glass.

4.3 Persistent Bends

The observed persistent bends may exist for a variety of reasons. First, these bends may represent energy minimums about the steady state average. In this case,
the fluctuations about a persistent bend would signify the exploration of the potential well minimum, which in turn provides insight into the stiffness of bending. However, because angular dynamics were found to be heavily influenced by external factors, this would not necessarily tell us about the stiffness of the linker, but rather whatever causes the bending. Qualitatively, 1 A-tract has small fluctuations about very persistent angles (Figure 3.6b), suggesting stiff bending, while 4 A-tract and poly-T have large fluctuations, suggesting more flexible bending (Figure 3.6c-d). The slow oscillations in 0 A-tract (Figure 3.6a) suggest that reptation may be disrupting the otherwise persistent bend. It is also possible that fluctuations about persistently bent states are a result of the vertex moving in and out of the imaging plane. To distinguish between the two possible causes for fluctuations and rule out the latter effect, much stiffer nanotube arms are needed. Typically, nanotubes are made stiffer by increasing their diameter\(^{24}\), but this could be problematic here because larger nanotubes are more likely to interfere with each other at very bent states (Section 4.4.3).

It is also interesting to note that the 1 A-tract data has many more clearly defined persistent bends than any other linker. This is particularly interesting in regards to the small heterodimeric protein, integration host factor (IHF), which specifically binds to DNA through A-tracts and play a vital architectural role in many prokaryotes\(^{25}\). Studies have therefore been conducted to determine whether the binding of the IHF causes bending or if the pre-bent DNA makes it easier for the IHF to bind. This is currently unresolved, but it is interesting to note the persistent bends occur in our 1 A-tract nunchucks in the absence of IHF, suggesting that the bending may come first. However, because of the likely interference of external factors, nothing can be said conclusively about the linker bending itself.
4.4 Method Improvements

4.4.1 Scaffold loops

Currently, only one-third of the M13 DNA scaffold is used in the construction of a monomer seed and the remaining two-thirds (~4500 bp or 1.5 μm) is left as a single stranded loop (Figure 2.2). In the dimer seed, it is likely that the two relatively large loops heavily influence bending dynamics of the nunchuck. Current work is being done to remove this excess scaffold through asymmetric polymerase chain reaction (aPCR). This method preferentially amplifies one part of the DNA scaffold (the region used to construct the monomer seed) through a great excess of one primer, and all excess scaffold can be removed through electrophoresis gel purification.

4.4.2 Seed design

The excess scaffold may also be eliminated by using the entire scaffold in the construction of the monomer seed. However, this cannot be accomplished by simply extending the length of the current cylindrical seeds, as these can develop a curvature that could produce adverse torsional forces on the linker. Because the redesign of a seed comes with an entirely new set of complications, this approach will not be explored until all reasonable approaches to removing the excess scaffold have been exhausted.

4.4.3 Steric hindrance

Steric effects arise because each atom within a molecule occupies a certain amount of space, and when atoms are brought too close together, there is a cost in energy due to the Pauli exclusion principle. In our system, as the nunchuck moves toward more closed angles, the areas occupied by each nanotube arm are more likely to interact through
steric hindrance. This is the effect in which the two areas cannot overlap, and past some angle (Figure 4.1), all other bend states are prohibited. With a linker of 58 bp or 20 nm (1 bp ~ 340 pm) and nanotubes that are 12 nm in diameter\textsuperscript{[15]} (all numbers are rounded to 2 significant figures), and making the simplifying assumption that the linker is exactly folded in half, this number was calculated using the law of cosines to be:

$$\cos(\theta) = \frac{10^2 + 10^2 - 12^2}{2(10)(10)}$$

(4.1)

$$\theta = 74^\circ$$

(4.2)

Figure 4.1: Side view of the smallest possible angle at which a nunchuck experiences steric hindrance determined by the diameter of the seed ($D_{seed}$) and the length of the linker ($L_{linker}$). Here we assume an ideal case where the linker is exactly bent in half.

If the nunchucks were exactly confined to a two dimensional plane, all angles smaller than this would be prohibited. While infrequent, we do see states that are more bent than this, suggesting that the nanotubes can move in three-dimensions to avoid each other to some extent.
Chapter 5

Conclusions

Although DNA bending has been studied extensively, not much is currently understood about its dynamics. Here, we have created a bend angle measurement tool, the DNA nunchuck, which allows for the analysis of bending dynamics via fluorescence microscopy. Through our analysis, we have discovered several issues with our method though. Primarily, angular dynamics were not found to be determined by the linker sequence as designed, but rather by some external factor. By analyzing bend angle distributions, we found a preference for open angles and a particular handedness in all linkers, suggesting a common external factor, such as imaging conditions and/or nunchuck structure. In addition, the interval between frames was found to be insufficient to produce independent frames, resulting in a large decrease in sample size. Finally, by using PEG rather than BSA passivation for 4 A-tract, we found that dynamics may also be affected by passivation technique. While these issues may seem numerous, they offer an excellent opportunity to optimize the DNA nunchuck with increasing speed and efficiency. Our current approach to accomplish this includes removing the excess scaffold through aPCR, imaging only short-armed nunchucks using PEG passivation, and maximizing the interval between frames. These three efforts will allow us to eliminate known structural and imag-
ing effects, collect much larger data sets, and ultimately identify and correct issues with much more ease. With more development, this technique will not only allow for robust dynamic DNA bend angle measurements, but it will make these types of measurements affordable and accessible to any lab with a fluorescence microscope.
Chapter 6

Appendix

Labeling strand sequences
Labeling_strand_ATTO488 /5ATTO488N/AAGCGTAGTCGGATCTC
Labeling_strand_ATTO647N /5ATTO647NN/AAGCGTAGTCGGATCTC

Attachment strand sequences
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REdSED nanotube tile sequences

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RE-3Cy3: /Cy3/TCTACGGAATGTTGCGAATAATCAATGAAAGGACGATGG
RE-4: CAGACCGAGAGGTTGAGTGTTGGATGATCC
RE-5: CCACATACCTGCTTATGATTTGATCTGCTGCTGAG
SE-1: CTCAAGGGCACAGGCTTCCGAGAGGAGGAAACT
SE-2DIAG: GTCTTGGTACGAGCACTCGAGAAGTA
SE-3Cy3: /Cy3/CCAGAACGGCGCTGCTGCTGCTGAGGAAACT
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/Cy3/ denotes a Cy3 fluorophore covalently attached to the 5' end of the DNA.

**REdSEd adapter tile sequences**

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Appendix

Chapter 6

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T.3R4E_HP: ATATATTCTTCCTCGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.3R6E_HP: CTCTATCGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.3R8E_HP: AGATAGTCTTATGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.3R10E_HP: CAGTCATTTTTAACAGCTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R4F_HP: CGTTAAACAACTGTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R6F_HP: ATACAGGAATGCGTGGCTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R8F_HP: CTCTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R10F_HP: CGTTAAACAACTGTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R2F_HP: AGGTGACTATAATAGTCTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R4F_HP: GAGCCGCGCCAGGTGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R6F_HP: AATACGACATTTGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R8F_HP: ATACAGGAATGCGTGGCTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R10F_HP: TAACGACTAGATGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R1E_HP: GACGTCATTTTTAACAGCTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R2E_HP: TTAACGACTAGATGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R4E_HP: AACGTCATTTTTAACAGCTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R6E_HP: GACTGGGTGAGCGTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R8E_HP: TTAACGACTAGATGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.1R10E_HP: TAACGACTAGATGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.3R2F_HP: TGCTTGATGCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.3R4F_HP: GCCACCCAGCTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.3R6F_HP: CCGGAAACTAAAAGGAGGACGCTTCTGCTGAGGTTTTTGCCGATCGTAAGCTGCTACAGGA
T.3R8F_HP: AAGAGGTACAGCGACGGAGCTTTCGCTGAGTTTCACTCCGACCT
T.3R10F_HP: GCCCAATAGACCGGAGCACGCGCTTTTACCCATGCGTGTTTTGAGTTACCTACGAAAA
T3R2E_HP: GGAAAGCTCACTGCGCGGCTTTTGCTGCCACTTGCTGCTGGTATCCGAGGGTTT
T3R4E_HP: GCTTCTCAGCGACGAGACCTGCGGCTTTTGCTGCCACTTGCTGCTGGTATCCGAGGGTTT
T3R6E_HP: TATCTTATCAGCCTGCCCTCTGCTGGTATCCGAGGGTTT
T3R8E_HP: ATACCCCAACACCAACCGGCCTACCCGCTTGTGCGGTTAATAGGAGCAAAGA
T3R10E_HP: CCACCAAGTTGCTGCTGGTATCCGAGGGTTT
T5R2F_HP: AAAGGCACTTCTCAGGCTGCTGCTGGTATCCGAGGGTTT
T5R4F_HP: TATCTTATCAGCCTGCCCTCTGCTGGTATCCGAGGGTTT
T5R6F_HP: AGCAAGTGCTACCTGCTGCTGCTGGTATCCGAGGGTTT
T5R8F_HP: ATACCCCAACACCAACCGGCCTACCCGCTTGTGCGGTTAATAGGAGCAAAGA
T5R10F_HP: CCACCAAGTTGCTGCTGGTATCCGAGGGTTT
T5R2E_HP: AAAGGCACTTCTCAGGCTGCTGCTGGTATCCGAGGGTTT
T5R4E_HP: TATCTTATCAGCCTGCCCTCTGCTGGTATCCGAGGGTTT
T5R6E_HP: AAAGGCACTTCTCAGGCTGCTGCTGGTATCCGAGGGTTT
T5R8E_HP: ATACCCCAACACCAACCGGCCTACCCGCTTGTGCGGTTAATAGGAGCAAAGA

**Linker sequences**

0 A-tract:
Linker 1: GGGGCACTTCTCAGGCTGCTGCTGGTATCCGAGGGTTT
Linker 2: ATACCCCAACACCAACCGGCCTACCCGCTTGTGCGGTTAATAGGAGCAAAGA

1 A-tract:
Linker 1: GGGGCACTTCTCAGGCTGCTGCTGGTATCCGAGGGTTT
Linker 2: ATACCCCAACACCAACCGGCCTACCCGCTTGTGCGGTTAATAGGAGCAAAGA

4 A-tract:
Linker 1: GGGGCACTTCTCAGGCTGCTGCTGGTATCCGAGGGTTT
Linker 2: ATACCCCAACACCAACCGGCCTACCCGCTTGTGCGGTTAATAGGAGCAAAGA

Poly-T:
Linker 1: GGGGCACTTCTCAGGCTGCTGCTGGTATCCGAGGGTTT
Linker 2: ATACCCCAACACCAACCGGCCTACCCGCTTGTGCGGTTAATAGGAGCAAAGA

ACACCATCGAGAACCCCTCTACGCGAGGAAATTTTCACCGATCAC

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Bibliography


