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spFRET studies of nucleosome dynamics modulated by histone modifications, histone variants and neighboring nucleosomes

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Citation
Buning, R. (2015, January 15). *spFRET studies of nucleosome dynamics modulated by histone modifications, histone variants and neighboring nucleosomes*. *Casimir PhD Series*. Retrieved from https://hdl.handle.net/1887/31477

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Author: Buning, Ruth
Title: spFRET studies of nucleosome dynamics modulated by histone modifications, histone variants and neighboring nucleosomes
Issue Date: 2015-01-15

CHAPTER 5

spFRET reveals changes in nucleosome breathing by neighboring nucleosomes

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Accepted for publication in Journal of Physics: Condensed Matter

Abstract

Chromatin, the structure in which DNA is compacted in eukaryotic cells, plays a key role in regulating DNA accessibility. FRET experiments on single nucleosomes, the basic units in chromatin, have revealed a dynamic nucleosome where spontaneous DNA unwrapping from the ends provides access to the nucleosomal DNA. Here we investigated how this DNA breathing is affected by extension of the linker DNA and by the presence of a neighboring nucleosome. We found that both electrostatic interactions between the entering and exiting linker DNA and nucleosome-nucleosome interactions increase unwrapping. Interactions between neighboring nucleosomes are more likely in dinucleosomes spaced by 55 bp of linker DNA than in dinucleosomes spaced by 50 bp of linker DNA. Such increased unwrapping may not only increase the accessibility of nucleosomal DNA in chromatin fibers, it may also be key to folding of nucleosomes into higher order structures.

5.1 Introduction

Chromatin consists of arrays of nucleosomes connected with 10-90 base pairs (bp) of linker DNA [129]. Interactions between these nucleosomes lead to dense higher order structures. Although the arrangement of nucleosomes and the DNA trajectory in chromatin has been investigated intensively in the past decades, the structure of chromatin remains highly debated [130]. The compact state of native chromatin under physiological salt conditions was visualized by electron microscopy (EM) more than 35 years ago [3]. The DNA trajectory in the fiber was however not visible in the EM pictures of compacted chromatin. The crystal structure of a tetranucleosome, four nucleosomes connected by 20 bp linker DNA, provides more detailed information on the arrangement of nucleosomes and DNA trajectory [131]: next-neighbors interact in a face-to-face configuration and the linker DNA is straight. A similar small array, consisting of three nucleosomes connected by 20 bp linker DNA, has been investigated with FRET (Fluorescence or Förster Resonance Energy Transfer) [54]. The array compacts under influence of magnesium, and next-neighbors appear to interact face-to-face in the same way as in the crystal structure, as demonstrated by a high FRET efficiency between the labels on next-neighboring nucleosomes. Recently, this arrangement was confirmed by cryogenic electron microscopy of 20 and 30 bp linker DNA chromatin fibers that included linker histones [132].

The interactions between nucleosomes are mediated by electrostatics and specific contacts involving flexible histone tails [133]. The unstructured tails allow for alternative orientations between nucleosome pairs that put different constraints on the linker DNA. In native chromatin, the linker length varies between 10 and 90 bp [129], which may lead to interactions between other nucleosome pairs and hence different topologies of the chromatin fiber. Indeed, EM experiments on reconstituted nucleosomal arrays with 30-90 bp linker length suggest interactions between direct neighbors [130]. The force-extension behavior of nucleosomal arrays with 50 bp linker length, as measured on single chromatin fibers using magnetic tweezers, also suggests interactions between direct neighbors [134]. From such force spectroscopy experiments, another interesting feature arises: a large increase in fiber length at a force of 4 pN indicates the disruption of nucleosome-nucleosome interactions [26, unpublished data]. This length increase is larger than what would be expected from the extension of the linker DNA only. The disruption of nucleosome-nucleosome interactions seems to be accompanied by partial unwrapping of the nucleosomal DNA, suggesting that in the condensed fiber, the nucleosomal DNA may already be partly unwrapped.

Partial unwrapping of nucleosomes in a compacted chromatin fiber would have consequences for the accessibility of nucleosomal DNA. It might even make part of the nucleosomal DNA more accessible in a folded chromatin fiber than in an individual nucleosome. Poirier et al. [53] have investigated the accessibility of nucleosomal and

linker DNA to restriction enzymes in both mononucleosomes and in nucleosomal arrays. Interestingly, they found that parts of the nucleosomal DNA in a fiber can be up to 8-fold more accessible than in mononucleosomes.

Individual nucleosomes are dynamic structures. DNA breathing, the transient unwrapping of DNA from the histone core, makes nucleosomal DNA accessible for DNA-binding proteins [25, 58]. Single-pair FRET (spFRET) [135] experiments have quantified this [127], revealing DNA breathing from both nucleosome ends. Nucleosomes are open for about 10 % of the time for tens of milliseconds [42]. DNA breathing is modulated by DNA sequence and by post-translational modifications to the histone proteins [32, 50]. So far, spFRET experiments have mainly focussed on individual nucleosomes, ignoring how nucleosome conformation and dynamics are influenced when the nucleosome is embedded in a chromatin fiber. To address this question, we focus here on (partial) unwrapping of nucleosomal DNA in nucleosomes flanked by linker DNA and/or by a second nucleosome.

We measure unwrapping of nucleosomal DNA in dinucleosomes with 50 bp linker length, because this is the average linker length found *in vivo* [129] and commonly used in chromatin fiber experiments [99, 130, 134]. The mechanical properties of the linker DNA pose constraints on the conformational freedom to position nucleosomes face-to-face, and the energy required to bend the linker DNA could be reduced by partial unwrapping of the nucleosomal DNA. We therefore expect that interactions between nucleosomes will be accompanied by DNA unwrapping from the nucleosome exit, in accordance with observations made by force spectroscopy on single chromatin fibers [26].

The energy required to bend the linker DNA depends on sterical constraints as well as on the linker DNA length. Variations of only a few bp have significant effects on the torsional energy required for positioning neighboring nucleosomes face-to-face, due to the 10.4 bp helical periodicity of DNA. Increasing the linker length with 5 bp from 50 to 55 bp is just a 1.7 nm change in length, but changes the relative orientation of two non-interacting nucleosomes by 180 degrees (see figure 5.2 for an illustration). So far, the effect of the linker length has mostly been studied for multiples of 10 bp. However, a natural preference for 10n+5 bp linker DNA has been suggested, and observed in yeast [136–138]. Such a 'phase offset' may play a crucial role in the formation of chromatin higher order structure.

Not only interactions between nucleosomes determine higher order chromatin structure, the linker DNA itself may also have an effect on the DNA trajectory in the fiber by steric and/or electrostatic interactions with the nucleosome and/or other stretches of linker DNA.

Here, we describe spFRET experiments on single fluorescently labeled nucleosomes flanked by either 300 bp linker DNA or a neighboring nucleosome with 20 or 50 bp linker DNA. Moreover, we compare unwrapping in dinucleosomes with 50 and

with 55 bp linker DNA. We show that the presence of linker DNA and of a neighboring nucleosome results in a significant change in FRET distribution, depending on the linker length. The presence of linker DNA enhances unwrapping of nucleosomal DNA. In dinucleosomes with 55 bp linker DNA, interactions between the nucleosomes appear to further increase the unwrapping probability of the nucleosomal DNA.

5.2 Materials and Methods

5.2.1 Preparation of DNA constructs

A 198 bp DNA template containing a single 601 nucleosome positioning sequence was constructed by PCR. The forward and reverse primers were labeled at a single position with Cy3B and ATTO647N (via amino linker with 6-carbon spacer to the base; IBA. Primer sequences can be found in the supplementary material. The donor and acceptor were separated by 81 bp. After reconstitution, the donor (Cy3B) is located at the second base pair from the nucleosome exit. The acceptor (ATTO647N) is located 10 base pairs from the dyad, leading to a FRET efficiency larger than 0.5 for a fully folded nucleosome. The layout of the mononucleosome construct is shown in figure 5.1. Two non-palindromic restriction sites were included close to the DNA ends: BsaI and BseYI, which were subsequently used to build longer constructs by ligation.

After purification, the 198 bp construct was digested with either BsaI or BseYI and ligated to 300 bp DNA without any nucleosome positioning sequence, or DNA with a second, unlabeled, 601 nucleosome positioning sequence. If necessary, the ligated DNA product was purified from agarose gel to remove unligated product. Figure 5.2 schematically shows all nucleosome constructs described in this paper.

5.2.2 Nucleosome reconstitution

DNA and chicken erythrocyte histone octamers were mixed in various molar ratios in TE (1 mM EDTA, 10 mM Tris.HCl pH 8.0) and 2 M NaCl. Mono- and dinucleosomes were reconstituted by salt gradient dialysis against 0.85, 0.65, 0.5 and finally 0 M NaCl, all buffered with TE in a total volume of 40 µl and a labeled DNA concentration around 50 nM. Competitor DNA, 147 bp unlabeled random sequence DNA (produced with PCR), was included in the reconstitution reaction at concentrations between 150 and 300 nM.

5.2.3 Polyacrylamide gel electrophoresis

Nucleosome reconstitutions were analyzed with 5 % native polyacrylamide gel electrophoresis (PAGE). A sample of 2-8 µl of reconstitution product was loaded on the gel

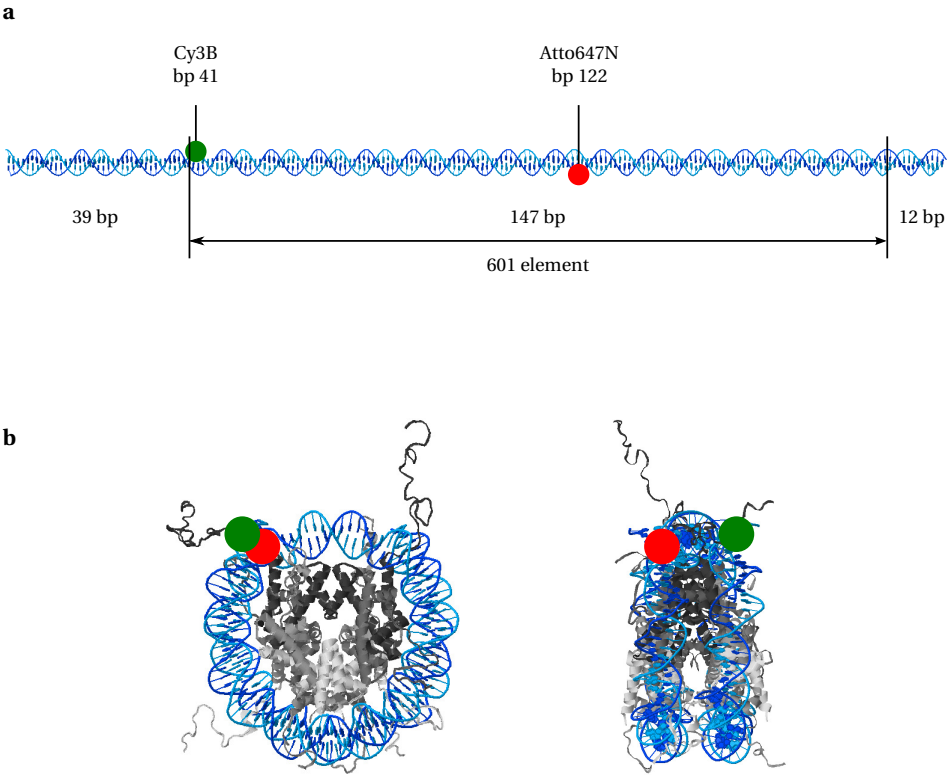


Figure 5.1 – **a:** The 198 bp DNA construct, indicating the position of the 601 positioning element and the fluorescent labels. **b:** Top and side view of the crystal structure of the nucleosome core particle (1KX5, [12]), consisting of 147 bp DNA wrapped around the histone octamer. The positions of the fluorescent labels are indicated.

(29:1 bis:acrylamide, 0.2 × TB, Amersham Bioscience Hoefer SE 400 vertical gel slab unit). The gel was run at 19 V/cm at 7 °C for 90-120 minutes to separate nucleosomes from free DNA. The fluorescence was imaged with a gel imager (Typhoon 9400, GE Healthcare). Red: excitation at 633 nm, emission detected at 670 nm; Green: excitation at 532 nm, emission detected at 580 nm; FRET: excitation at 532 nm, emission detected at 670 nm (all 30 nm bandpass). Gel images were analyzed with ImageJ software to determine the relative intensities of the bands. The uncorrected proximity ratio (E_{PR}^{raw}), as a measure for the FRET efficiency in the bands, was calculated according to equation 5.1 (see section 5.2.6).

5.2.4 Sample preparation

Fluorescently labeled nucleosomes were diluted to a concentration of 50-100 pM in a buffer containing 10 mM Tris.HCl pH 8.0, 0.1 mg/ml BSA, 0.03 % Nonidet-P40 and 2 mM trolox. Where stated, 100 mM KAc and 2 mM $MgAc_2$ were added to the buffer. We used non-stick tubes (Ambion) for nucleosome dilutions. After 15-30 minutes of equilibration to room temperature, a drop of 50 µl was placed on a glass coverslide (#1.5, Menzel) and imaged as described below.

5.2.5 Single-molecule fluorescence microscopy

Single molecules were imaged with a home-built confocal microscope equipped with a 60 × water-immersion objective (NA = 1.2, Olympus), as schematically depicted in figure 5.3a (see also [42]). A 515 nm diode pumped solid state laser (Cobolt) and a

Figure 5.2 ***(facing page)*** – Schematic overview of the constructs described in this chapter. The elementary mononucleosome (MN) contains a single 601 element, flanked by 39 bp on the side closest to the fluorescent labels, and by 12 bp on the opposite side. All other constructs are extensions of the elementary nucleosome. The mononucleosomes with 300 bp linker DNA contain a single 601 element, flanked by 300 bp either on the label side (MNl300), or on the opposite side (MNo300). The dinucleosomes (DN) contain two 601 elements, linked by 20, 50 or 55 bp linker DNA. The two 601 elements are linked via the label side (DNl50 and DNl55), or via the opposite side (DNo20, DNo50 and DNo55). Dinucleosomes linked by 20 bp linker DNA via the label side are missing due to constraints imposed by the positions of restriction sites. The percentages of open nucleosomes ($E_{PR}^{raw} < 0.3$) correspond to single-molecule measurements in 100 mM KAc. Structures shown are based on the nucleosomal DNA from the crystal structure of the nucleosome core particle (1KX5, [12]), which we extended with linear stretches of DNA with the use of [139]. The histone octamers are left out for visual clarity. Note that the images of the constructs just display a linear extension of the linker DNA, and do not reflect actual experimentally determined or theoretically predicted trajectories of the linker DNA. FRET experiments with both labels on the linker DNA rather suggest that the linker DNA is bent [140].

Elementary Mononucleosome				
MN	39 bp 12 bp	open: 11 %		
Mononucleosomes with 300 bp linker DNA	Dinucleosomes 20 bp linker DNA		50 bp linker DNA	55 bp linker DNA
linker DNA attached at the label side	MNl300	open: 22 %	DNl50	open: 13 %	DNl55	open: 21 %
	—					
linker DNA attached at the opposite side	MNo300	open: 32 %	DNo20	open: 12 %	DNo50	open: 31 %	DNo55	open: 26 %

636 nm diode laser (Power Technology) were used as excitation sources. The lasers were alternated at 20 kHz by analog modulation, either directly (636 nm), or with an AOM (515 nm; Isomet). The beams were spatially filtered with a single-mode fiber, and focussed 25 µm above the glass-buffer interface by the objective. The excitation power was 13 µW for 515 nm excitation, and 6 µW for 636 nm excitation. The collected fluorescence was spatially filtered with a 50 µm pinhole in the image plane, and was split into a donor and an acceptor channel by a dichroic mirror (640dcxr, Chroma). The fluorescence was filtered with emission filters (hq570/100m for the donor channel, hq700/75m for the acceptor channel, Chroma) to minimize crosstalk, and was imaged on the active area of single photon avalanche photodiodes (SPCM AQR-14, Perkin-Elmer). The photodiodes were read out with a TimeHarp 200 photon counting board (Picoquant GmbH). In a typical experiment, data was collected for 30 minutes in which 2000-10000 bursts of fluorescence were detected.

5.2.6 Single-molecule data analysis

Bursts of fluorescence were detected using the method described in [66]. A burst was assigned if a minimum of 50 photons were detected with a maximum interphoton time of 100 µs. Photon arrival times in the donor (D) and acceptor (A) channel were sorted according to excitation wavelength, resulting in four photon streams: D-emission upon D-excitation: $I_{D_{ex}}^{D_{em}}$, A-emission upon D-excitation: $I_{D_{ex}}^{A_{em}}$, D-emission upon A-excitation: $I_{A_{ex}}^{D_{em}}$, A-emission upon A-excitation: $I_{A_{ex}}^{A_{em}}$. Example data are shown in figure 5.3b.

For each burst, we estimated the FRET efficiency by the sensitized-acceptor emission method [69, 70]. Following the definitions described by Lee et al. [31], we calculated the uncorrected proximity ratio E_{PR}^{raw} and label stoichiometry S^{raw} from the total number of photons in the burst for the different photon streams:

$$E_{PR}^{raw} = \frac{I_{D_{ex}}^{A_{em}}}{I_{D_{ex}}^{A_{em}} + I_{D_{ex}}^{D_{em}}} \tag{5.1}$$

$$S^{raw} = \frac{I_{D_{ex}}^{A_{em}} + I_{D_{ex}}^{D_{em}}}{I_{D_{ex}}^{A_{em}} + I_{D_{ex}}^{D_{em}} + I_{A_{ex}}^{A_{em}}} \tag{5.2}$$

The excitation powers were chosen such that $I_{D_{ex}}^{A_{em}} + I_{D_{ex}}^{D_{em}} \approx I_{A_{ex}}^{A_{em}}$ for doubly labeled particles, resulting in $S^{raw} \sim 0.5$. An example of a 2D E,S histogram from a typical measurement is shown in figure 5.3c. Nucleosomes containing both donor and acceptor fluorophores were selected for further analysis by taking only bursts with $0.2 < S^{raw} < 0.8$. Histograms of the FRET efficiencies of these bursts show the distribution of FRET efficiencies of the doubly labeled nucleosomes only. Bursts of two

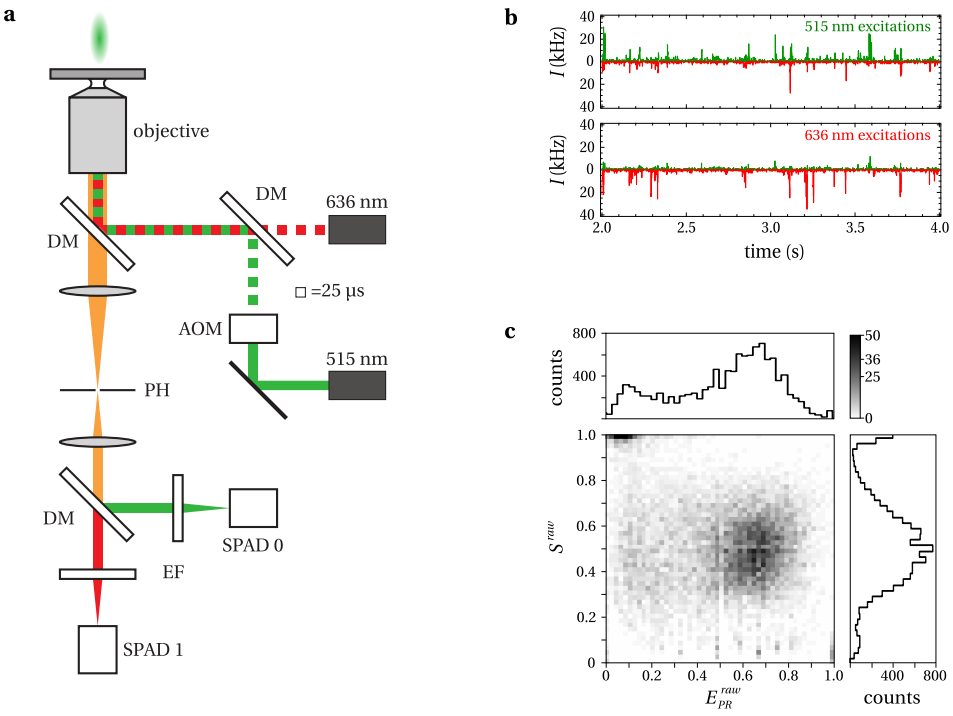


Figure 5.3 – **a:** Schematic overview of the confocal FRET microscope. DM: dichroic mirror; AOM: acousto-optical modulator; PH: pinhole; EF: emission filter; SPAD: single photon avalanche diode. **b:** Typical fluorescence intensity timetraces of the four different photon streams acquired with the setup in a. Photon arrival times are binned to 1 ms. Bursts of fluorescence arise from the passage of a single particle through the excitation volume. **c:** Typical 2D-histogram of FRET efficiency (E_{PR}^{raw}) and label stoichiometry (S^{raw}) for mononucleosomes. Four populations are distinguishable: donor only ($S^{raw} > 0.8$), acceptor only ($S^{raw} < 0.2$), doubly labeled ($0.2 < S^{raw} < 0.8$) with FRET ($E_{PR}^{raw} >\sim 0.3$) and without FRET ($E_{PR}^{raw} <\sim 0.3$).

or three measurements of the same construct and conditions were verified for reproducibility and combined to build one FRET histogram. Histograms were normalized to a total area under the curve of 1 to allow comparison of different constructs. The fraction without FRET (representing open or (partly) dissociated nucleosomes) was determined by taking the area below the histogram for $E_{PR}^{raw} < 0.3$.

Note that we did not attempt to quantify the extent of DNA unwrapping from the histone core. The limited number of photons per burst, the complications with the translation from FRET efficiency to label pair distance [31], and the possibility of conformational changes within a single burst prevent a direct calculation of the label pair distance and from that the nucleosome conformation. Alternatively, one can change the positions of the labels along the DNA to resolve the extent of DNA unwrapping [42, 50], but this requires new DNA constructs. By choosing the location of the FRET pair to be at the very end of the nucleosomal DNA, we ensure that all DNA unwrapping events of over 10 bp DNA are captured in our measurements [127]. In this paper, increased unwrapping therefore refers to more frequent breathing rather than breathing of larger stretches of nucleosomal DNA.

5.3 Results

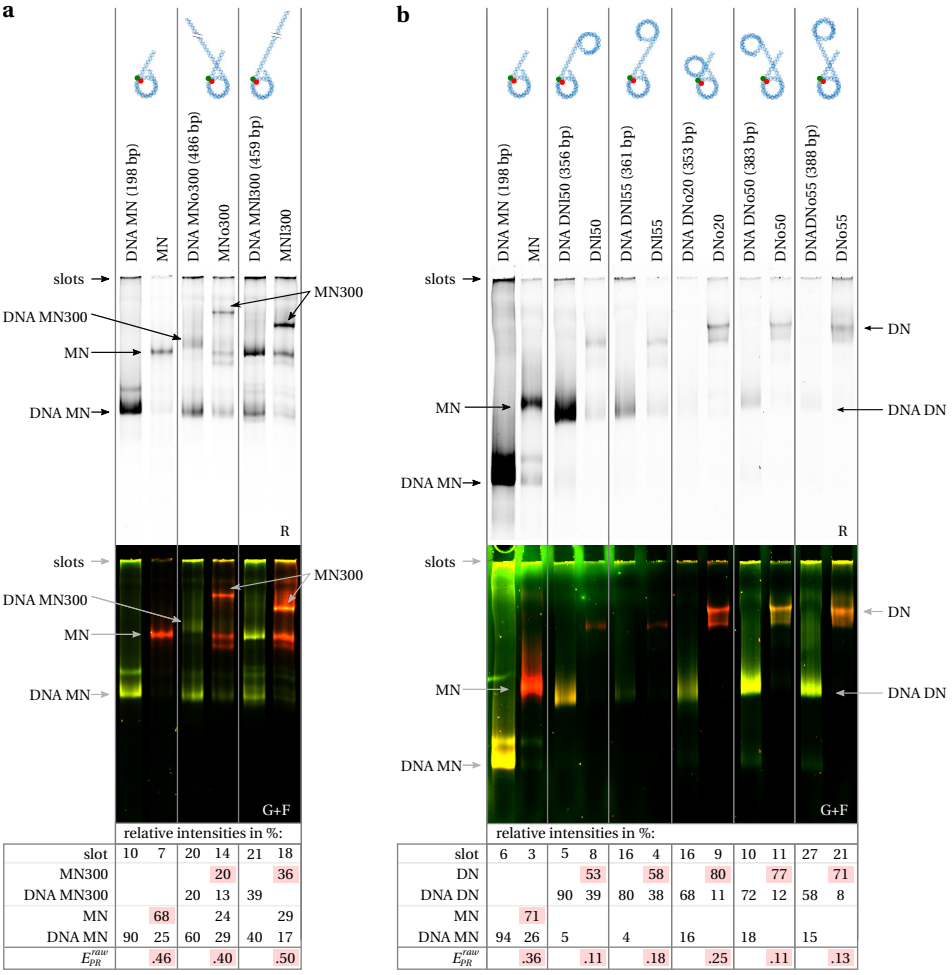
5.3.1 Gel electrophoresis of (di)nucleosome reconstitutions

Figure 5.4 shows the fluorescence in (di)nucleosomes after native gel electrophoresis of all reconstituted nucleosomal constructs, separating free DNA from nucleosomes. The amount of free DNA is generally much smaller than the amount of reconstituted nucleosomes, confirming a high reconstitution yield. As opposed to bare DNA, nucleosomes show significant FRET, indicating reconstitution into fully wrapped nucleosomes, properly positioned on the 601 elements.

In most cases, a single sharp band of nucleosomes is present. However, sometimes a second band is visible just below the main nucleosome band. This minor band can be attributed to incomplete nucleosomes, lacking the H2A/H2B dimer(s). In all cases there is some material left in the slots, originating from aggregates, that show a small FRET signal as well.

FRET differences between constructs are readily visible in the gel: the FRET efficiency of dinucleosomes depends on the linker DNA length. 20 bp linker DNA dinucleosomes yield the highest FRET efficiency and 50 bp linked at the nucleosomal side opposite of the fluorescent labels the lowest.

Since the amount of reconstituted nucleosomes is small (1-2 pmol), we did not purify the nucleosomes after reconstitution. The consequences for the interpretation of single-molecule data will be discussed in the discussion section.



relative intensities in %:

slot	10	7	20	14	21	18
MN300				20		36
DNA MN300			20	13	39	
MN		68		24		29
DNA MN	90	25	60	29	40	17
E_{FR}^{raw}		.46		.40		.50

relative intensities in %:

slot	6	3	5	8	16	4	16	9	10	11	27	21
DN				53		58		80		77		71
DNA DN			90	39	80	38	68	11	72	12	58	8
MN		71										
DNA MN	94	26	5		4		16		18		15	
E_{FR}^{raw}		.36		.11		.18		.25		.11		.13

Figure 5.4 – Fluorescence images of 5 % polyacrylamide gels with reconstituted mononucleosomes (**a**) and dinucleosomes (**b**). Tops (R): acceptor fluorescence upon direct acceptor excitation. Bottoms (G+F): false color overlay of donor and acceptor (FRET) fluorescence upon donor excitation. MN: mononucleosomes. DN: dinucleosomes. Reconstituted nucleosomes show FRET, in contrast to bare DNA. The relative intensities of the bands in each lane from the direct acceptor excitation image are displayed in the table at the bottom, providing a measure for the relative concentrations of the different components (bare DNA, nucleosomes, aggregates). Percentages are approximate within a few percent. Numbers corresponding to mononucleosome bands are highlighted, and the FRET efficiency calculated from the green and FRET intensities of the bands is indicated in the bottom row.

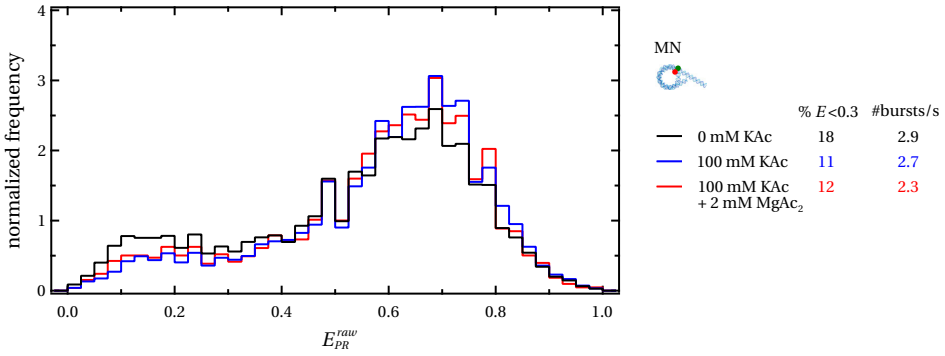


Figure 5.5 – FRET efficiency distributions of mononucleosomes in different salt conditions. The fraction open nucleosomes decreases from 17 to 11 % when increasing the monovalent salt concentration from 0 to 100 mM. Addition of 2 mM magnesium has no effect on the FRET efficiency distribution.

5.3.2 FRET distributions of mononucleosomes

Rather than an average FRET value, as determined from the gel, single-molecule experiments reveal the distribution of FRET values obtained from individual nucleosomes. We attribute bursts with an uncorrected proximity ratio lower than 0.3 to particles without FRET. This can be either bare DNA or nucleosomes that are partly unwrapped ('open').

18 % of the elementary mononucleosomes (MN), shows no FRET at 0 mM salt (figure 5.5). We observed that an increase of monovalent salt to 100 mM decreases the open population to 11 %.

5.3.3 Linker DNA increases DNA breathing

Although DNA breathing has been well established in mononucleosomes, it is unclear how extended DNA linkers affect nucleosome dynamics. Mechanical, hydrodynamic and electrostatic properties of DNA will determine the trajectory and dynamics of the linker DNA, which is linked to the nucleosomal DNA. FRET distributions of mononucleosomes with 300 bp linker DNA are compared with the distribution of mononucleosomes in figure 5.6. 300 bp of linker DNA attached to the label side cause an increase in the population without FRET from 18 % to 26 %. When attached to the opposite side, the population without FRET is more than doubled to 40 %. Increasing the monovalent salt concentration to 100 mM decreased this effect, indicating that electrostatic repulsion is mainly responsible for enhanced opening of the nucleosome.

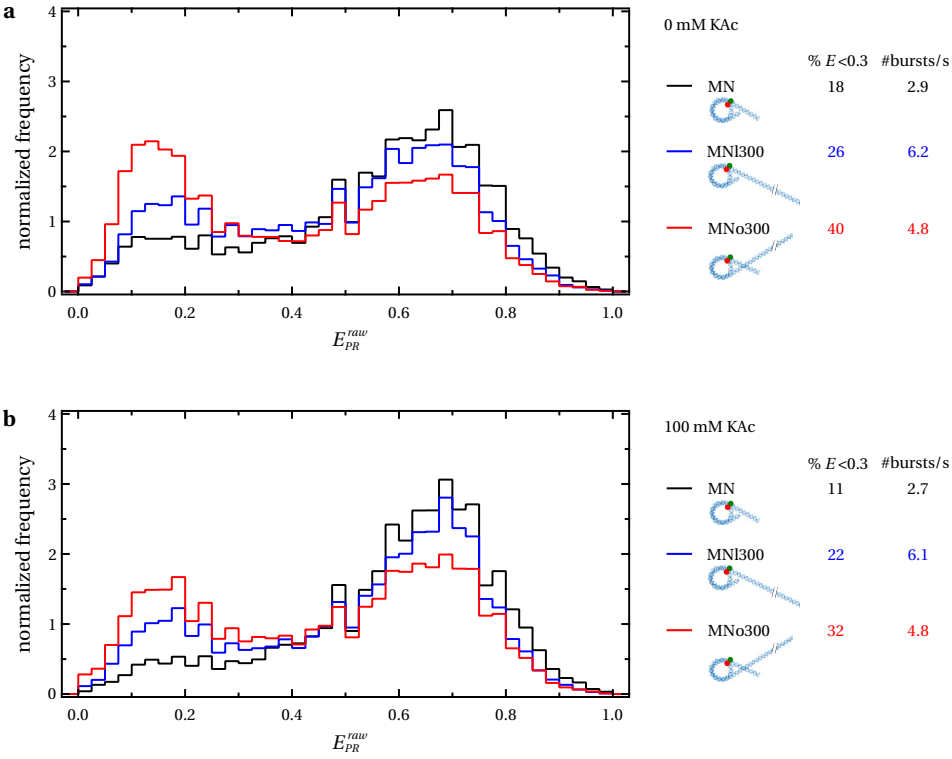


Figure 5.6 – FRET efficiency distributions of mononucleosomes with 300 bp linker DNA, without (**a**) and with (**b**) 100 mM monovalent salt. Nucleosomes with 300 bp linker DNA at the label side (MNl300) or the opposite side (MNo300) have a larger population of open nucleosomes than nucleosomes without the linker DNA. The linker DNA at the side opposite of the label positions has a surprisingly large effect on the population of open nucleosomes. Adding 100 mM monovalent salt decreases this effect about 25 %.

5.3.4 DNA breathing is affected by neighboring nucleosomes

While linker DNA has large effects on nucleosome dynamics, it can be expected that the presence of another nucleosome, connected by linker DNA, will further modulate this, as such a nucleosome is bulkier and carries a high charge density. All dinucleosomes were measured in 100 mM monovalent salt, for proper comparison with the mononucleosomes and in accordance with the conditions used for single-molecule force spectroscopy on chromatin fibers [134]. Moreover, we included 2 mM Mg^{2+}, which is required for folding of nucleosomal arrays into dense 30 nm fibers.

Dinucleosomes with linker DNA at the label side

The distance between non-interacting (straight linker DNA) neighboring nucleosomes with 50 or 55 bp linker DNA is too large for steric or electrostatic effects between the nucleosomes to play a role. Indeed, between the mononucleosomes and the dinucleosomes with 50 bp linker DNA, there are only minor changes in the FRET distribution (see figure 5.7). Dinucleosomes with 55 bp linker DNA, however, show a significantly larger population without FRET. This suggests that a difference in interactions between nucleosomes results in an increased unwrapping probability. We did however not observe an effect of 2 mM magnesium on the FRET distributions.

Dinucleosomes with linker DNA at the side opposite of the label

When the second nucleosome is attached to the side opposite of the labels, we similarly expect no steric or electrostatic effect between non-interacting nucleosomes. However, as can be seen in figure 5.8, dinucleosomes with 50 and with 55 bp linker DNA both show a FRET distribution that is different from that of the mononucleosome. In these constructs, both interactions between the two nucleosomes, between the linker DNAs, and between nucleosomes and linker DNA may play a role, giving rise to a more complex shape of the distribution, including more pronounced intermediate states.

For the dinucleosomes with the linker DNA attached to the side opposite of the fluorescent labels we could also build a construct with 20 bp linker DNA. This distance is too short for the nucleosomes to interact in a face-to-face orientation. The FRET distribution of dinucleosomes with 20 bp linker DNA has a slightly increased population with intermediate FRET values as compared to mononucleosomes, and a similar low FRET fraction. The close proximity of the nucleosomes (~7 nm linker DNA) could allow interactions between the DNA and/or histone octamers of neighboring nucleosomes which may affect nucleosome breathing.

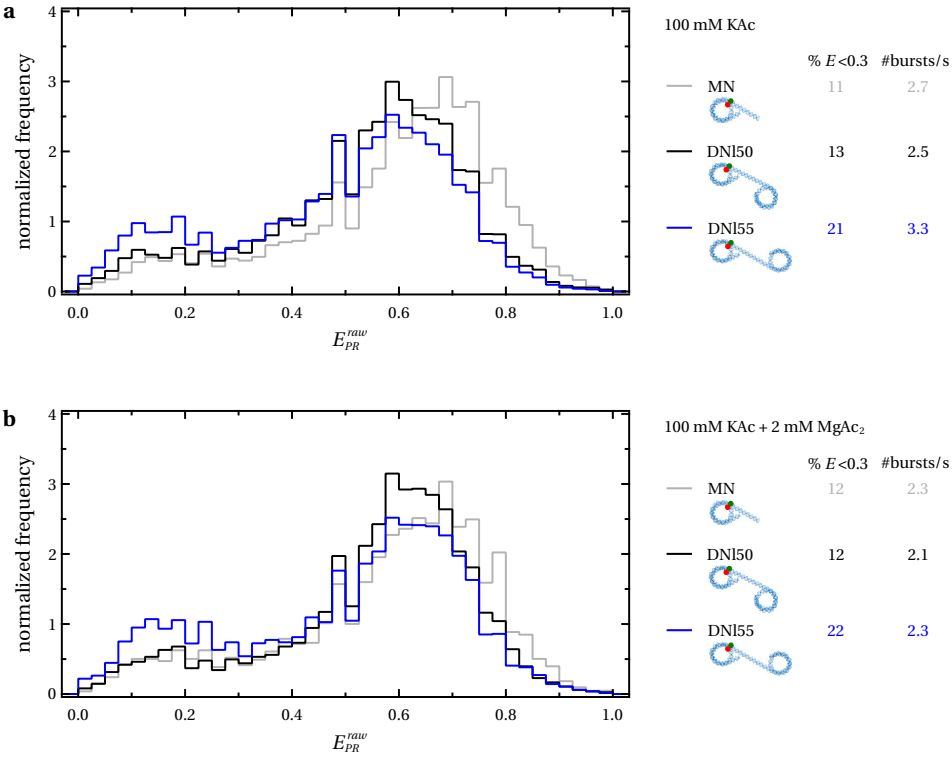


Figure 5.7 – FRET efficiency distributions of dinucleosomes linked via the fluorescent label side with either 50 bp (DNl50) or 55 bp (DNl55) linker DNA, without (**a**) and with (**b**) 2 mM magnesium. Dinucleosomes with 50 bp linker DNA show the same distribution as mononucleosomes. Dinucleosomes with 55 bp linker DNA on the other hand show a larger population of open nucleosomes. Addition of 2 mM Mg^{2+} has no visible effect on the FRET efficiency distributions.

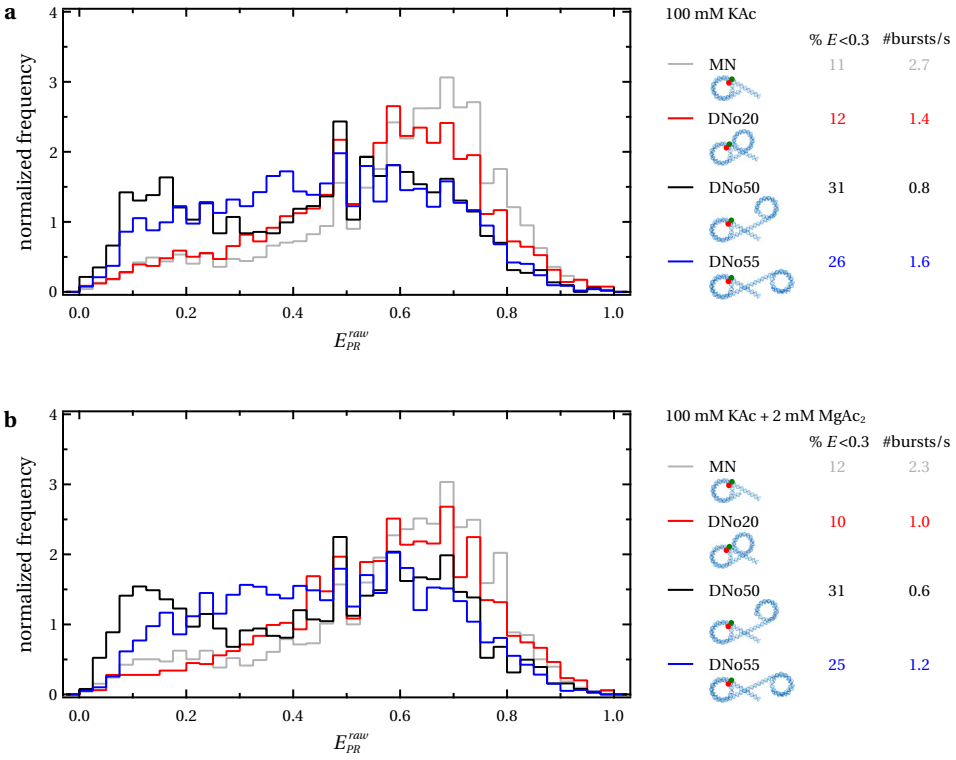


Figure 5.8 – FRET efficiency distributions of dinucleosomes linked via the side opposite of the fluorescent labels with either 20 bp (DNo20), 50 bp (DNo50) or 55 bp (DNo55) linker DNA, without (**a**) and with (**b**) 2 mM magnesium. Dinucleosomes with 20 bp linker DNA show a similar distribution to mononucleosomes. Dinucleosomes with 50 bp linker DNA show a much larger open population. Dinucleosomes with 55 bp linker DNA also show a much larger open population, and in addition a larger fraction with intermediate FRET efficiencies. Addition of 2 mM Mg^{2+} has no visible effect on the FRET efficiency distributions.

5.4 Discussion

Here, we showed that the presence of linker DNA and/or a neighboring nucleosome significantly influences nucleosomal DNA dynamics. Constructs of single nucleosomes flanked by long stretches of linker DNA or by neighboring nucleosomes differ in FRET efficiency distribution from mononucleosomes flanked by short stretches of linker DNA.

5.4.1 Sample handling

Nucleosomes are known to be very sensitive to concentration, buffer conditions and surface exposure [37, 41, 56, 141]. All FRET efficiency distributions that we report here are the result of multiple measurements, that are reproducible within 5 % (fraction E_{PR}^{raw} <0.3). Under suboptimal measurement conditions, e.g. too high dilution and non-passivated surfaces (data not shown), the differences between samples showed the same trend, but generally had a larger fraction of bursts without FRET, indicating that nucleosomes dissociate under these conditions. It is interesting to note that dinucleosomes appeared to be less susceptible to dilution- and surface- driven dissociation than mononucleosomes. This is consistent with cooperative binding of nucleosomes [142].

5.4.2 Sample heterogeneity

Despite careful titration and the use of the strong 601 nucleosome positioning element, gel electrophoresis shows that the reconstitution yield is in many cases not perfect. For some constructs the percentage of nucleosomes in the sample was only about 30 %. Besides nucleosomes, the sample consists of one or more of following components: unreconstituted DNA, unligated DNA, nucleosomes on the unligated DNA and aggregates that remained in the slot. Although this heterogeneity in the sample makes quantification of the results difficult, the qualitative differences between the constructs still hold. The implications for the single-molecule data interpretation are described below.

Mononucleosomes

Quantification of the band intensities of the gel shown in figure 5.4 yields ~70 % of reconstituted nucleosomes and ~25 % of unreconstituted DNA. A significant amount of the material is left in the slots due to aggregation. In the single-molecule experiments however, the fraction without FRET is only 11 % (at 100 mM salt), which sets the maximum on the amount of unreconstituted DNA. The real amount is probably lower, because this population also consists of partly unwrapped nucleosomes. The amount of free DNA present in the gel, which is more than twice as high as the upper limit that

follows from single-molecule measurements, is therefore not representative for the reconstitution yield.

Gel electrophoresis can impose severe disruptions on a nucleosome sample. Conditions in the gel, such as low nucleosome concentration, drive nucleosome dissociation, resulting in a larger fraction of free DNA. Hence, we can not equate the reconstitution yield observed in the gel to the amount of unreconstituted DNA in the single-molecule experiments. The amount of free DNA is however the same in different single-molecule experiments of the same sample, i.e. mononucleosomes in different salt concentrations. The qualitative decrease of the fraction of open nucleosomes with increasing salt concentration can therefore not be explained by differences in reconstitution yield.

The material left in the slots after gel electrophoresis probably consists of aggregates, and can be as high as 20 % or more in some samples. Aggregates can be detected in the single-molecule experiments by bursts with increased duration and intensity. Hardly any such signatures of aggregates were found, indicating that the aggregates found in the slots are artifacts due to the gel electrophoresis, or that these aggregates precipitated before the single-molecule experiments.

Mononucleosomes with linker DNA

Due to incomplete ligation of the DNA construct, a mixture of mononucleosomes with and without 300 bp linker DNA can be present in these samples. Figure 5.4 shows that indeed about half of the nucleosomes lacks the linker. The increase in the fraction of nucleosomes without FRET when 300 bp linker is added is therefore underestimated and the effect of linker DNA on DNA breathing may be larger than measured here.

Dinucleosomes

Unreconstituted DNA is present in the polyacrylamide gels of the dinucleosome reconstitutions in various amounts. For the dinucleosomes with the second nucleosome attached to the label side (DNl50 and DNl55), the amount of unreconstituted DNA is as high as ~40 %, while for the dinucleosomes with the second nucleosome attached to the side opposite of the labels (DNo20, DNo50 and DNo55), it is only ~10 %. The difference between these sets of constructs could be explained by the concentration difference of the nucleosomes loaded into the gel. Comparison with the no-FRET fraction from single-molecule experiments shows that the real fraction of unreconstituted DNA is at most 10 % for both sets of constructs, which is comparable to the amount of unreconstituted DNA in the elementary mononucleosome. Differences should therefore be attributed to (dynamic) changes in nucleosome conformation.

5.4.3 Linker DNA increases breathing in mononucleosomes

The presence of 300 bp linker DNA enhances unwrapping of nucleosomal DNA on both sides of the nucleosome. This could be explained by electrostatic repulsion between the entering and exiting DNA, increasing the unwrapping probability of the nucleosomal DNA. Addition of salt decreases this effect by screening of charges on the DNA. This salt effect was smaller but still measurable in the elementary mononucleosome with 39 and 12 bp of linker DNA.

The increase of FRET between 0 and 100 mM KAc is somewhat counterintuitive, regarding that high salt destabilizes nucleosomes: DNA and histone octamers completely dissociate from each other at 2 M [143, 144]. In a recent paper, Gansen et al. [63] investigated FRET in mononucleosomes over a wide range of nucleosome and salt concentrations. They observed an increase in FRET at moderate salt concentrations as well, but around 400-600 mM. They attribute this to the formation of an intermediate nucleosome conformation towards full dissociation, probably missing one or two dimers. The possibility to observe this intermediate depends on the label positions. With our choice of label positions, at the second bp from the nucleosome exit, dimer loss would result in a complete loss of FRET. We attribute the increase in FRET at 100 mM salt, which is dependent on the length of the linker DNA, to diminished electrostatic repulsion between the linker DNAs. The destabilizing effect of salt on DNA-histone interactions and the electrostatic screening of linker DNAs counteract each other. At modest salt concentrations, the diminished repulsion between linker DNAs apparently dominates over the decreased stability of DNA-histone contacts, resulting in a net increase of the high-FRET population.

5.4.4 Dinucleosomes

Increased unwrapping due to electrostatic repulsion of the linker DNAs is also observed for dinucleosomes with 50 bp linker DNA (DNo50), where the no-FRET fraction is increased compared to mononucleosomes. Dinucleosomes separated by 55 bp linker DNA, however, have a ~10 % larger no-FRET population than with 50 bp linker DNA. Apparently, the neighboring nucleosome increases the unwrapping probability. Interactions between linker DNAs would be the same for both 50 and 55 bp linker DNA. The observed difference in FRET distribution must therefore be attributed to a difference in nucleosome-nucleosome interactions.

Surprisingly, we observe no effect of magnesium in nucleosome-nucleosome interactions. Magnesium is required to induce compaction in nucleosomal arrays with 50 bp linker DNA in [23, 134]. In our dinucleosomes, we do not observe any change in DNA breathing after addition of magnesium, suggesting that the nucleosome is not constrained differently with or without Mg^{2+}. Perhaps a dinucleosome is a too small

unit for mimicking chromatin fiber folding. Magnesium could for example help to bring non-neighboring nucleosomes together, stabilizing two gyres of the 'super helix' in the fiber.

To rationalize the FRET distribution of dinucleosomes with 55 bp linker DNA at the side opposite of the fluorescent labels (DNo55), we need to consider several effects. First, electrostatic repulsion between the linker DNAs favors unwrapping, as for the 50 bp linker DNA construct (DNo50). Second, nucleosome-nucleosome interactions further enhance unwrapping at the side of the linker DNA, similar to the dinucleosomes with 55 bp linker DNA at the label side (DNl55). If such steric constraints are responsible for increased unwrapping, unwrapping at the side of the linker DNA would be anti-correlated to unwrapping at the opposite side, which is observed as a smaller no-FRET population for dinucleosomes with 55 bp rather than 50 bp of linker DNA. Third, if nucleosome-nucleosome interactions occur, the electrostatic interactions between the entering and exiting DNA change due to an altered linker DNA trajectory. The interplay between these effects determines the distribution of FRET efficiencies.

An increase in nucleosomes with intermediate FRET was observed for the dinucleosomes with 55 bp linker DNA attached to the side opposite of the fluorescent labels (DNo55). An intermediate FRET efficiency can result from the average of two conformations when the concentration of labeled nucleosomes in the sample is too high, such that multiple nucleosomes are in the excitation focus at the same time (see [63]). Here, the concentration of labeled nucleosomes is low enough to measure the FRET efficiency in each individual nucleosome separately, as verified by the number of bursts per second. Thus, there must be a fraction of nucleosomes that have a conformation in between open and fully wrapped.

Linker DNA of 20 bp is too short to allow neighboring nucleosomes to interact in a face-to-face manner. The linker DNA and the two nucleosomes are however so close that they are able to interact directly, for example via electrostatic interactions between the DNA and the histone tails. The FRET distribution for dinucleosomes with 20 bp linker DNA (DNo20) is indeed slightly altered compared to mononucleosomes. A small increase in the population with intermediate FRET efficiencies points to a partly unwrapped configuration that is favored, possibly due to interactions between the linker DNA and the neighboring nucleosome.

To probe nucleosome-nucleosome interactions and their dynamics directly, it will be worthwhile to investigate dinucleosomes with various linker lengths, where one FRET label is located at each of the two nucleosomes at well-chosen positions. High FRET will then correspond to nucleosome-nucleosome interactions. However, this requires detailed insight into the structure of dinucleosomes, as the small Förster radius imposes strict constraints on the positions of the pair of labels. Only for 20 bp linker DNA has it been possible to do this [54] with the help of the crystal structure [131].

Such higher order folding remains enigmatic for larger linker lengths.

5.5 Conclusions

We performed spFRET experiments on DNA breathing in nucleosomes flanked by linker DNA and/or a neighboring nucleosome. We observed that the presence of linker DNA and a neighboring nucleosome both influence breathing of nucleosomal DNA. Electrostatic repulsion between the entering and exiting DNA favors unwrapping. An increase of the salt concentration reduces the unwrapping probability by screening electrostatic interactions between the linker DNAs.

Like in previous studies, we observed that interactions between neighboring nucleosomes depend on linker length. Here we showed that not only the linker length but also the phasing relative to the pitch of the DNA affects the interaction between neighboring nucleosomes. A linker length of 50 bp does not seem to favor nucleosome-nucleosome interactions, whereas an increase in linker length with only 5 bp to 55 bp results in increased unwrapping of nucleosomal DNA, probably due to nucleosome-nucleosome interactions in such a dinucleosome.

The conformation and dynamics of nucleosomal DNA has important implications for the structure and dynamics of chromatin fibers. On one hand, the accessibility of nucleosomal DNA, which has mainly been investigated in isolated nucleosomes before, depends strongly on chromatin structure. On the other hand, DNA linker length modulates chromatin structure by allowing interactions between direct neighbors only for specific linker lengths. In this study we contributed to the understanding of these phenomena by carefully monitoring the effects that addition of linker DNA and nucleosome neighbors have on DNA breathing.

Acknowledgements

We thank Guilherme Santos and Prof. Dr. Daniela Rhodes from the MRC Laboratory of Molecular Biology, Cambridge, UK, for providing the chicken erythrocyte histone octamers and Ineke de Boer-Tuyn for technical support.

This work is part of the research program of the Foundation for Fundamental Research on Matter (FOM), which is part of the Netherlands Organization for Scientific Research (NWO).

Supplementary material

The elementary mononucleosome DNA construct was PCR amplified with fluorescently labeled primers (5'→3'):

AGTTCATCCCTTAACCGAGAGACCCTATACGCGGCCGCCC**T**(Cy3B)GGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGA and

TTCAATCCCAGCACAGGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAACG**C**(ATTO647N)GGGGGACA,

with non-palindromic restriction sites (underlined) BsaI (NNNNNGAGACC) and BseYI (CCCAGC), respectively. Modified bases are indicated in bold.

The 300 bp linker DNA segments and unlabeled nucleosome segments were PCR amplified with primers (5'→3'):

MNl300: **A**(biotin)CGCTCAGTGGAACGAAAAC and
GATAAATCTGGAGCCGGTGA

MNo300: CTCCAAGCTGGGCTGTGT and
A(biotin)AACCACCGCTACCAGCG

DNl50: CTGGAGAATCCCGGTGCCGAGGCCGCT and
TATCCGGTCTCCCGGTGCAAGGTCGCTGTTC

DNl55: CTGGAGAATCCCGGTGCCGAGGCCGCT and
AACACGGTCTCACGGTCACCGGCAAGGTCG

DNo20: TGTGATGCTGGGTATACGCGG and
ACAGGATGTATATATCTGACACGTG

DNo50: CATGATGCTGGGCACCGAGTT and
ACAGGATGTATATATCTGACACGTG

DNo55: GAAAGCGCTGGGCTTCACACC and
ACAGGATGTATATATCTGACACGTG

Template for all PCR reactions was the pgem3z plasmid containing a single 601 element [33]. All segments were digested with BsaI (MNl and DNl) or BseYI (MNo and DNo). The elementary nucleosome was digested with either BsaI or BseYI and ligated to one of the segments in the list above to form the corresponding construct.