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Nucleosome Dynamics as Studied by Single-pair Fluorescence Resonance Energy Transfer: A Reevaluation

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Abstract Accessibility of nucleosomal DNA to protein factor binding is ensured by at least three mechanisms: post-synthetic modifications to the histones, chromatin remodeling, and spontaneous unwrapping of the DNA from the histone core. We have previously used single-pair fluorescence resonance energy transfer (spFRET) experiments to investigate long-range conformational fluctuations in nucleosomal DNA (Tomschik M, Zheng H, van Holde K, Zlatanova J, Leuba SH in Proc Natl Acad Sci USA 102 (9):3278–3283, 2005). Recent work has drawn attention to a major artifact in such studies due to photoblinking of the acceptor fluorophore. We have now used formaldehyde-crosslinked nucleosomes and imaging in the presence of Trolox, an efficient triplet-state quencher that suppresses photoblinking, to reevaluate our previous conclusions. Careful analysis of the data indicates that most of the events previously characterized as nucleosome 'opening' must have corresponded to photoblinking. There is, nevertheless, evidence for the existence of infrequent, rapid opening events.

Keywords Evanescent field fluorescence microscope · Nucleosome DNA unwrapping · nucleosome opening · photoblinking

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Introduction

The accessibility of nucleosomal DNA to protein factors that perform and regulate DNA transactions in the eukaryotic nucleus is of prime importance to the life of a cell. The thinking in the field has been dominated by the hypothesis that accessibility is controlled by histone post-translational modifications (the "histone code" hypothesis, [1, 2]) and by the activity of chromatin remodelling factors that use the energy of ATP hydrolysis to make chromatin structure more 'fluid'. These two mechanisms implicitly assume that chromatin is a passive entity, waiting to be appropriately marked or remodeled to function.

Recent years have witnessed the emergence of a new paradigm suggesting a third (nonexclusive) mechanism for ensuring site accessibility in nucleosomal DNA (reviewed in [3]). Experiments from both Widom's laboratory (e.g., [4–6]) and our own [7] reported evidence for spontaneous unwrapping of nucleosomal DNA from around the histone core, thus temporarily releasing portions of nucleosomal DNA from the histone grip to allow protein binding to occur. The Tomschik et al. paper [7] used results from single-pair Fluorescence Resonance Energy Transfer (spFRET) experiments to deduce the existence of long-range, all-or-none transitions, in which as much as 70–80 bp of DNA are unwrapped from around the histone core. Such motions, which we term 'opening' to distinguish them from shorter-range DNA unwrapping ('breathing') are supported by theory [8–10]; they are also consistent with results from Optical Tweezers stretching of nucleosome arrays [11].

However, spFRET experiments are prone to artifacts stemming from the photophysical properties of the fluorescent probes used for labeling. Blinking of the acceptor dye is especially problematic since the spontaneous transitions

of the dye into non-emitting states, accompanied by an increase in the donor dye emission, may be mistaken as evidence for structural transitions in the molecules/molecular complexes under investigation. Although the existence of blinking has been described more than a decade ago [12, 13], only very recently has a more in-depth investigation into the phenomenon been undertaken. The major events underlying blinking on microsecond to millisecond time scales have been identified as transitions to non-emitting triplet states, photo-induced *trans-cis* isomerization reactions that lead to non-emitting *cis*-conformations, slow polarization effects, or the formation of radical species [14–16]. The events leading to the long Cy5 dark states lasting for seconds have not been rigorously identified. Sabanayagam et al. [17] have postulated an involvement of an excited state transition to the triplet state that ultimately leads to the formation of a long-lived radical. In any case, the intermittency of the acceptor fluorophore is a complex phenomenon and is influenced by the chemical environment, the proximity and the structure of the donor, the presence of shorter-wavelength illumination without the actual donor being present, and the occurrence of FRET (e.g., [17–20]). Of special concern to studying biomolecular processes are exactly these intermittent states that can last milliseconds to seconds, since this is the time scale for structural transitions in biomacromolecules and biochemical reactions.

Realizing the dangers created by photoblinking of the acceptor dye, we felt it essential to reinvestigate the presumed 'opening' transitions in the nucleosome [7]. This was accomplished by: (1) using formaldehyde-crosslinked nucleosomes in which opening transitions cannot occur because of the covalent bonds between the histones and the DNA; and (2) performing the experiments in the presence of Trolox, a water-soluble derivative of vitamin E that suppresses photoblinking [21]. These studies lead us to believe that many of the 'opening' events were caused by blinking. Nevertheless, we believe that true opening events still occur, as evidenced by statistically significant differences between controlled and crosslinked particles imaged in Trolox.

Materials and methods

DNA, histones, and nucleosomes

All common chemicals were obtained from Sigma (St. Louis, MO). Preparation of histone octamers and labeled GUB DNA was described earlier [7]. Briefly, the 164 bp long GUB DNA used for spFRET imaging was prepared by PCR using plasmid pGUB [22, 23] as template, and Cy3 and Cy5 labeled 50-mer oligonucleotides (IDT DNA, Coralville, IA, amino-link labels at positions 47 and 122, respectively, Cy3- and Cy5-NHS esters from GE Healthcare, Piscataway, NJ) as primers; the Cy3 primer was also labeled with biotin on the 5′ end. 208 bp long DNA (208 DNA) was prepared by digestion of plasmid p208–35 carrying multiple 208 bp repeats of the 5S DNA from *L. variegatus* [24] with AvaI (New England BioLabs, Ipswich, MA), followed by gel filtration purification. Histone octamers were isolated from chicken erythrocytes as in [25].

Nucleosomes were reconstituted as described [7]: 10 ng of fluorescently labeled GUB DNA were mixed with 10 μg of 208 DNA and the solution was adjusted to 2 M NaCl. 10 μg of chicken octamers were added and the mixture was incubated at 37 °C in the dark for 30 min. Stepwise dilution with TE (10 mM Tris–HCl/0.5 mM EDTA, pH 7.5) to 1 M, 0.75 M, and 0.5 M NaCl with 30 min incubation periods was followed by dialysis to 10 mM Tris–HCl, pH 7.5, for at least 3 h, but usually overnight (using Slide-A-Lyzer, Pierce, Rockford, IL). Reconstitutes were checked on 1% agarose gels; only those with ~90% of DNA reconstituted into nucleosomes were used for analysis.

Nucleosome crosslinking and testing for crosslinking efficiency

Nucleosomes were crosslinked by 30 min incubation of nucleosome solution (50–200 μg/ml) with 2% formaldehyde at 37 °C (20 mM potassium phosphate, pH 7.0 was substituted for the Tris buffer in dilution and dialysis steps). Mock reactions were performed in parallel, omitting the formaldehyde. Crosslinking was quenched by adding excess of 250 mM glycine and dialysis if further biochemical experiments were planned; samples used directly for imaging were introduced into the flow cell and excess solution was washed away. For experiment with "on-slide" crosslinking, crosslinking was for 60 min.

To test for efficiency of crosslinking, crosslinked and control samples were mixed with either native DNA loading buffer or SDS-loading buffer and incubated at 37 °C for 15 minutes before loading onto 1% agarose gels. An additional test was based on restriction endonuclease accessibility. 2 μg of nucleosomes were digested with different restriction endonucleases in commercially provided buffers (New England BioLabs) for 2 h at 37 °C. Digested samples were phenol-chloroform extracted, ethanol precipitated, and analyzed on 10% PAGE in TBE. Gels were stained with SYBR Green I (Invitrogen, Carlsbad, CA) and analyzed using ImageQuant software (BioRad, Hercules, CA).

Single-molecule fluorescence microscopy

Flow cells were prepared and used as in [7, 26]. Cleaned quartz slides (Finkenbeiner, Waltham, MA) with two 1-mm holes were sealed with 3M double-sided tape as spacer and

cleaned 24×60 mm no/ 1 coverslips (Fisher, Pittsburgh, PA), to form the bottom of the flow cell.

Most of the time, we used biotinylated BSA (Sigma) in a passive-attachment method: solution of biotinylated BSA (1 mg/ml in PBS) was injected into the flow cell for 10 min, followed by a wash with T150 (10 mM Tris-HCI, 150 mM NaCl, pH 7.5) and 10 min incubation with 0.2 mg/ml solution of streptavidin (Invitrogen). After a thorough wash with T150, the flow cell was ready for sample injection. The nucleosome solution was typically diluted 10–20-fold with T150 to an approximate concentration of 10–20 μg/ml of the carrier nucleosomes. One to two minutes of incubation were sufficient for a good sample density in the flow cell. Unattached nucleosomes were washed out with T150 containing oxygen scavenger system [27] with either 140 mM β-mercaptoethanol (BME) or 1.6–1.8 mM Trolox (Sigma).

In a few cases, the slides were alternatively treated with (3-Aminopropyl)triethoxysilane (APTES, Sigma) followed by mixture of polyethylene glycol (PEG) and Biotin–PEG–NHS esters (Nektar Therapeutics, Birmingham, AL) in order to coat the surface with a hydrophilic layer [28–30]. The results were the same as when using the BSA-method.

Imaging setup

The imaging setup was essentially the same as in [7]. We used a 532 nm laser (CrystaLaser, Reno, NV) for total internal reflection illumination through a pellin broca prism (CVI Laser, Albuquerque, NM) placed on top of the slide. Imaging was by the inverted IX-71 Olympus microscope (Leeds Precision Instruments, Minneapolis, MN), using a 60x water immersion objective and a Dual-View filter set (Optical Insights/Photometrics, Tucson, AZ) consisting of a 610 nm long-pass dichroic mirror, a 580/40 band-pass filter for the Cy3 signal and a 645 long-pass filter (Chroma, Rockingham, VT) for the Cy5 emission. Images were collected with Cascade 512B (Photometrics) camera and IPLab software (BD Biosciences, Rockville, MD) at 90 or 51 ms time resolution (50 or 30 ms exposure times, respectively).

Data processing and analysis was performed in IPLab essentially as described [7]. We have also used home-written procedures in IDL (ITT, Boulder, CO) and MATLAB (MathWorks, Natick, MA) software, courtesy of Dr. Rasnik [31]. Peak fitting was done using Origin (OriginLab, Northampton, MA). The latter analysis offers more automated processing; it still contains an element of subjectivity due to the need for initial manual trace sorting. To be able to do a more rigorous comparison with our previously published data, we kept the dwell time analysis the same [7].

Results

Creating a nucleosome particle refractive to DNA unwrapping

In 2005 we published the first spFRET investigation on the dynamic properties of individual nucleosome particles [7]. Figure 1a depicts the structure of the nucleosome, with the locations of the donor and acceptor dyes on the DNA (see "Materials and methods"). The locations of the fluorophores were chosen so that no FRET was expected to occur on naked DNA, whereas the formation of a nucleosome was expected to lead to high FRET efficiency. This is what we observed [7]. We reported highly dynamic changes in the FRET signal with time, which we interpreted as nucleosomal opening-closing transitions.

To further explore the dynamical behavior of nucleosomes under various conditions we needed a true negative control, i.e. nucleosome particles that are not expected to be dynamic. The need for such a negative control became even more obvious by numerous publications from physics laboratories that appeared following our publication (see "Introduction"). We turned to using formaldehyde crosslinking, one of the most frequently used crosslinking procedures in the chromatin field. Formaldehyde covalently joins side-chain nitrogens in proteins and the exocyclic amino and the endocyclic imino groups of DNA [32–34] when they are ~2 Å apart.

Because the goal was to prevent spontaneous opening of the nucleosomes, we needed to estimate the degree of crosslinking required. For the crosslinking experiments we used a different DNA positioning sequence, 208 DNA, which was unlabeled and was available in larger quantities. 208 nucleosomes were prepared by the salt-jump method with dialysis against 20 mM KPi and checked on 1% native agarose gels (Fig. 1b). As expected, the fixed particle is more compact, and moves faster than the control nucleosome.

Samples with optimal histone-to-DNA ratio were crosslinked with different concentrations of formaldehyde or for different amounts of time. The ability of SDS to dissociate the particle into its DNA and histone constituents was used as a qualitative criterion for successful crosslinking. Samples were loaded in SDS-containing buffer and electrophoresed in either SDS-PAGE or agarose gels. Concentration of formaldehyde of 1% and crosslinking times between 10 and 15 min were sufficient to prevent any histone dissociation from the DNA (not shown). To be on the safe side, we chose longer (30 min) crosslinking times and a higher (2%) formaldehyde concentration (Fig. 1b, lanes marked Fx) for all further experiments.

A somewhat more quantitative test for crosslinking compares the degree of accessibility to restriction enzymes

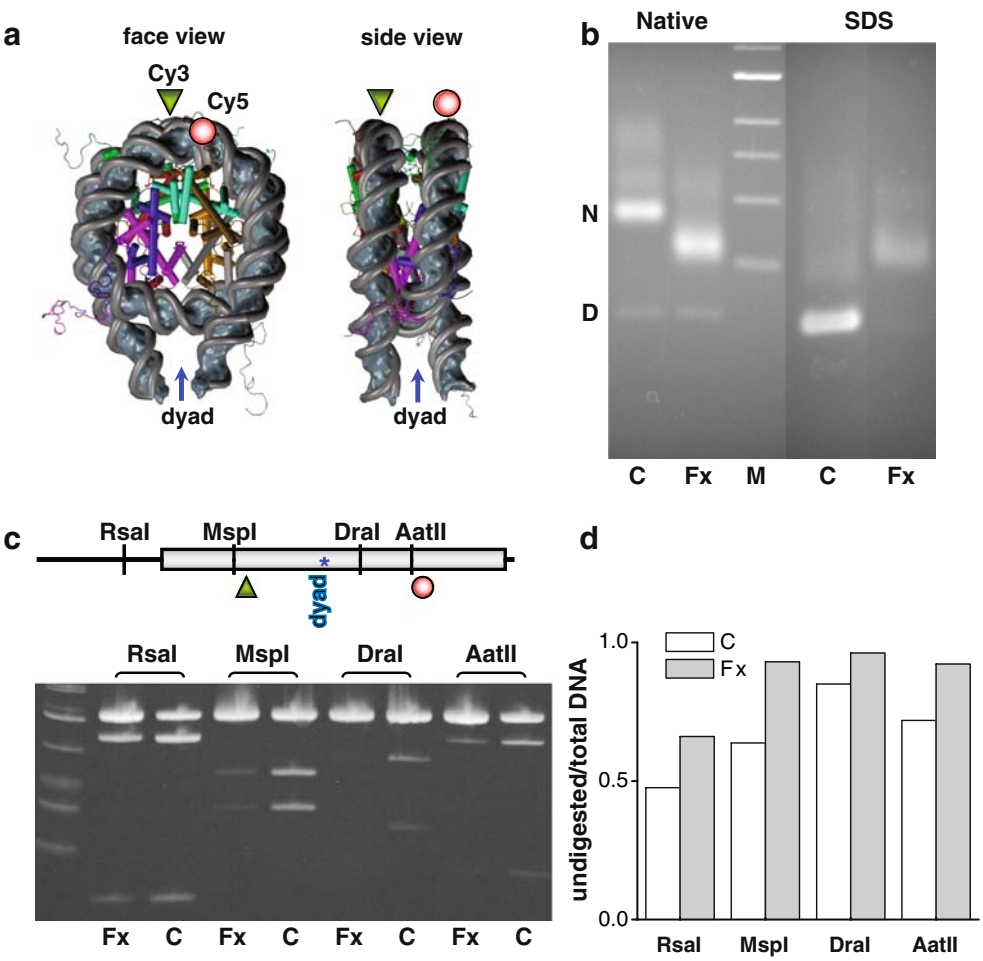

Fig. 1 Location of the donor and acceptor fluorophores in the nucleosome and formaldehyde crosslinking. **a** Schematic of locations of donor (*Cy3*) and acceptor (*Cy5*) fluorophores on the nucleosome formed on 164 bp GUB DNA. Nucleosome model courtesy of J.-M. Victor. **b** Nucleosome crosslinking tested by agarose gel electrophoresis. Control (*C*) and crosslinked (*Fx*) nucleosomes were loaded with native DNA loading buffer (*left side*) or SDS-loading buffer (*right side*). *N*, nucleosome; *D*, naked DNA; *M*, 1 kb marker (New England BioLabs). **c** Restriction enzyme sites on 208 DNA; *gray box* represents nucleosome position, *asterisk* the nucleosome dyad axis, and the *triangle* and the *circle* the donor and acceptor dyes, respectively. Comparison of different restriction enzyme digestion patterns of fixed (*Fx*) and control (*C*) nucleosomes on 10% PAGE. *Left lane*, low MW marker (New England BioLabs). **d** Quantitative analysis of the degree of 208 DNA digestibility from **c**

in the control and fixed material. This method has an additional advantage, since by using restriction enzymes whose sites are located at different positions along the DNA one can get an idea of whether the sites expected to be involved in nucleosome opening are actually crosslinked to the histones.

We performed restriction digests with about 2 μg of reconstituted material (crosslinked and non-crosslinked control) and 10 U of each enzyme for 2 h at 37 °C and analyzed the DNA on a native PAGE. The ratio of undigested DNA fragment to total DNA was used as a measure of digestibility. An example gel (Fig. 1c) shows quite clear change in the degree of digestion by DraI, MspI, and AatII upon crosslinking. The quantitative analysis plotted in Fig. 1d shows that these enzymes exhibit much more limited accessibility on crosslinked samples. The degree of digestion in the crosslinked samples, 5–10%, corresponds to ~5–10% of naked DNA present in the samples after reconstitution (see Fig. 1b); the crosslinking efficiency was therefore estimated to be at least 90–95%. RsaI also exhibits a decreased level of digestion on crosslinked material, yet the location of its cleavage site outside the major nucleosome position grants higher accessibility in both the crosslinked and the control material. Note that the control material alone shows decreasing digestibility towards the dyad axis, but upon crosslinking the difference between MspI, DraI and AatII levels off. Based on these observations we are confident that a 30 min 2% formaldehyde crosslinking yields a nucleosome particle that is not expected to undergo spontaneous DNA unwrapping.

spFRET on crosslinked nucleosomes imaged in BME

We then compared the behavior of crosslinked and non-crosslinked nucleosomes in spFRET experiments. The DNA sequence used for these experiments was the 164 bp GUB nucleosome positioning sequence that contained a Cy3 (donor) and a Cy5 (acceptor) fluorophores located 80 bps apart, on the DNA gyres across from the dyad axis (see Fig. 1a). Labeled GUB nucleosomes were immobilized on streptavidin-coated quartz slides through their 5' biotin moiety and wide-field TIR imaging was first performed in buffer containing 1% BME and an oxygen scavenger

system, to replicate the conditions in Tomschik et al. [7]. Upon excitation with a 532 nm laser numerous fluorescent spots were observed in both the donor and acceptor channels. The donor channel image had more spots than the acceptor one; we attributed this difference to (1) acceptor dye inactivity, and (2) possible permanent nucleosome unwrapping (accompanied by loss of FRET) upon surface attachment. Example time trajectories of the fluorescence intensities of the donor and acceptor dyes are presented in Fig. 2.

The image analysis described in [26] produced an apparent FRET efficiency (E_{app}) distribution with maxima around 0.9 in both control (0.94±0.12) and crosslinked (0.92±0.12) nucleosomes (Fig. 3*A* and *B*; Table 1). We attribute the breadth of the distribution at least partially to existence of real nucleosomal subpopulations (see "Discussion").

The analysis of individual fluorescence intensity vs. time traces (Fig. 2) and dwell times was performed as in [7, 26]. Sorting through about a thousand traces for each sample we found at least 30% of traces exhibiting multiple anti-correlated transitions of the Cy3 and Cy5 fluorescence intensities. We also observed numerous uncorrelated transitions often interspersed with the anti-correlated ones; the uncorrelated transitions were not included in the analysis. Most of the anti-correlated transitions were from high-FRET (E_{app}=0.9) to zero-FRET, were very brief, and reversible. Such high-FRET to zero-FRET transitions were earlier interpreted as long-range unwrapping of the DNA from the histone core [7]; this interpretation was supported by theory [8, 9] and single-molecule nucleosomal array stretching experiments [11] (for more detailed discussion, see [7]).

Figure 4a and b portray the resulting comparisons of the dwell time distributions for the zero-FRET and high-FRET states (τ_{ON}=1.61 and 1.66 s, τ_{OFF}=0.17 and 0.19 s for control and crosslinked samples, respectively). The results for the crosslinked nucleosomes originate from two different types of measurements, one from nucleosome samples crosslinked in bulk solution prior to imaging, and the second one from "on-slide"-crosslinked nucleosomes. We had expected that formaldehyde crosslinking would result in a loss of fluorescence fluctuations in individual fluorescence intensity vs. time traces. The lack of differences betweens the control and crosslinked samples suggests that the prevalent majority of measured fluorescence intensity fluctuations under these conditions does not originate from nucleosomal dynamics.

spFRET measurements in the presence of Trolox

Recently, Rasnik et al. [21] have reported that substitution of the triplet state quencher BME for Trolox (water-soluble vitamin E derivative) in the imaging buffer (also containing oxygen-scavenger system to prolong the life-time of the

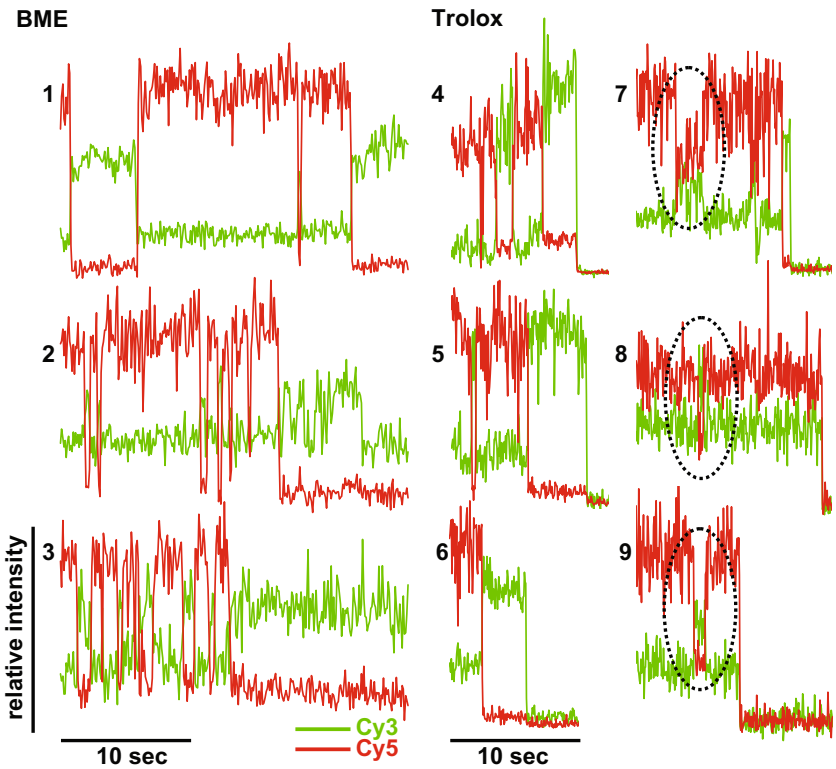

Fig. 2 Example time trajectories displaying fluctuations between high and low fluorescence intensity of the donor (*green*) and acceptor (*red*) fluorophores. Trajectories obtained from samples measured in BME on the *left* (*1–3*), from Trolox (*4–9*) on the *right*. Traces 1–5 display typical cross-correlated high- to no-FRET transitions (blinking or opening), traces 7–9 exhibit smaller-range FRET changes (partial unwrapping, *circled*). Trace 6 shows the most frequent situation with no event happening

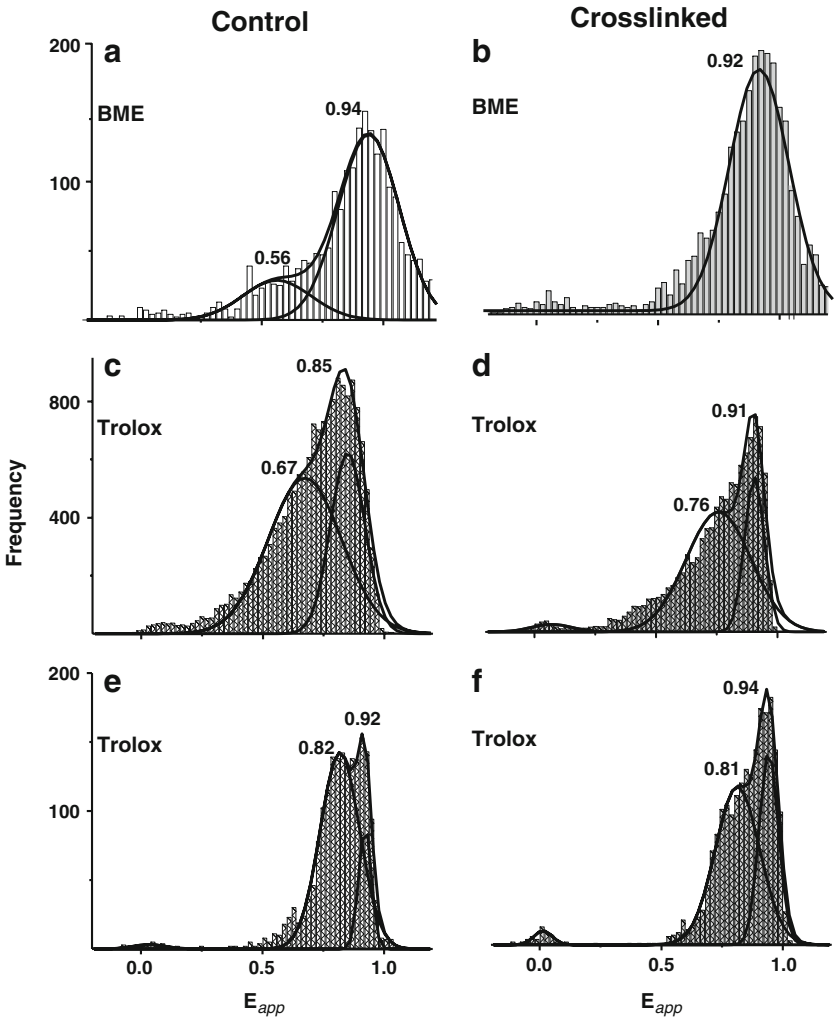

Fig. 3 Summary of E_{app} analyses. Distributions of E_{app} from control (*left panel*) and crosslinked (*right panel*) samples were fitted with single or multiple Gaussians for optimal fit. Results obtained from image analysis of video frames numbers 3, 8 and 13 (combined): (**a**, **b**), measurements in 140 mM BME; (**c**, **d**), measurements in 1.6–1.8 mM Trolox; (**e**, **f**) distributions of E_{app} calculated from individual time trajectories. 50 data points were used from each of ~40 time traces from control (**e**) and crosslinked (**f**) nucleosomes, after correction for donor leakage β

dyes) resulted in quite efficient suppression of Cy5 blinking behavior.

Thus, we repeated our experiments in the presence of ~1.8 mM Trolox. We have also adapted the data analysis procedure from Dr. Rasnik (e.g. [31]) to maximize the quality of image alignment between the donor and acceptor channels. The new analysis yielded FRET efficiency distributions similar to the ones obtained using our previous analysis (Fig. 3c and d); importantly, the control and crosslinked samples were very similar to each other. If fitted with single Gaussian peak, the E_{app} maxima are 0.76 and 0.83. The experimental distribution is much better fitted with two Gaussians, having maxima at 0.67 and 0.85 for the control, and 0.76 and 0.91 for the crosslinked samples, respectively. We have also calculated E_{app} from individual traces from which we could extract the leakage factor β (β is a measure of the leakage of donor signal into the acceptor channel) and use it for more precise FRET efficiency calculations [35]. The distributions presented in Fig. 3e and f are sums of 50 data points each from about 20 trajectories per sample, to equalize the input from individual molecules. Indeed, the distributions were narrower; however, they indicated the presence of two subpopulations.

In the measurements conducted in the presence of Trolox, the fluorescence fluctuation frequency dropped dramatically (Table 1), as expected if the majority of transitions observed in the presence of BME were due to photoblinking of the acceptor dye. The percentage of traces showing anti-correlated events was 5.4% and 4.6% for control and crosslinked samples, respectively. Importantly, the frequency of such transitions per trace was lower in the crosslinked samples (1.7 events/trace in control vs. 1.2 events/trace in crosslinked samples, Table 1).

By fitting the distributions of the ON and OFF times with exponential decay functions (Fig. 4) we extracted τ_{ON}=1.6 s and τ_{OFF}=0.25 s for the control samples, and τ_{ON}=3.7 s and τ_{OFF}=0.55 s for the crosslinked ones. The total time the molecules spent in zero-FRET state was 1.4% for the control and 2.1% for the crosslinked samples.

Table 1 Summary of spFRET parameters obtained on control and crosslinked nucleosomes using BME or Trolox as triplet state quenchers

	BME		Trolox	
	Control	Crosslinked	Control	Crosslinked
E_{app} (from time frames)	0.56±0.14	0.92±0.12	0.67±0.15	0.76±0.13
	0.94±0.12		0.85±0.07	0.91±0.04
E_{app} (from individual traces)			0.82±0.08	0.81±0.09
			0.92±0.02	0.94±0.04
τ_{ON} (s)	1.61±0.04	1.66±0.04	1.59±0.18	3.68±0.16
Mean (s)			2.97±3.77	3.76±4.06
τ_{OFF} (s)	0.17±0.002	0.19±0.003	0.25±0.01	0.55±0.08 !
Mean (s)			0.98±2.54	2.06±2.83
Total traces	1452	1071	2999	1903
Traces w events (%)	32.5	49.4	5.3	4.6
Total number of events/total number of traces with events	1819/472	2146/529	269/159	102/88
Number of events per trace	3.85	4.06	1.69	1.16
Number of events per second (from event traces)			0.13±0.001	0.11±0.002
Time spent OFF (%)			1.4	2.1

Discussion

Soon after we published our spFRET investigation of nucleosomal DNA dynamics [7], a number of papers from physics laboratories made it clear that the acceptor dye Cy5 could experience dark, non-emitting states that could last for seconds, even minutes (see "Introduction"). These dark states have to be taken into account to ensure proper interpretation of spFRET data.

As a first step towards a better understanding of whether and to what degree spFRET measurements reflect nucleosomal dynamics, we decided to use crosslinked mononucleosomes. After optimizing the conditions for crosslinking that produced 'frozen' particles, i.e. particles that cannot undergo DNA unwrapping, we performed spFRET imaging and data analysis. The comparison between control and crosslinked nucleosomes revealed no significant differences between the two samples when measured in the presence of BME, the triplet-state quencher widely used by us and others in the field. The only noticeable difference was in the overall frequency of anti-correlated fluorescence intensity transitions, which was actually slightly higher in the crosslinked samples (Table 1). These observations were very surprising and meant that the observed fluorescence dynamics originated from photoblinking artifacts in the vast majority of cases. This interpretation is in agreement with recently published data [21, 36]. These papers also reported that the presence of BME may partly cause the photoblinking and that having 1–2 mM Trolox in the imaging solution minimized (yet not completely eliminated) photoblinking of Cy5 attached to DNA molecules or mononucleosomes [21, 36].

We have therefore repeated the imaging of crosslinked and non-crosslinked nucleosomes in 1.6–1.8 mM Trolox. The frequency of transition events per trace, as well as the percentage of traces containing transitions dropped significantly compared to measurements in the presence of BME (Table 1). This observation alone confirms that the majority of fluorescence dynamic events measured in the presence of BME both here and in our previous work [7] are of photophysical origin.

We attempted to extract as much information from our current measurements as we could to clarify the origin of the observed dynamics. Dwell time analyses of the Trolox experiments suggest a difference between crosslinked and control mononucleosomes (Fig. 4, Table 1). What seems counterintuitive is the difference itself: the dwell time of the zero-FRET state is longer for the crosslinked samples than for the control ones; we attribute this to eliminating shorter-lived nucleosomal open states in the crosslinked material. The longer τ_{ON} from crosslinked samples might also reflect elimination of short lived opening events as well. It is clear that we should consider the dwell times for crosslinked nucleosomes as 'baseline' values representing the residual photoblinking in the presence of Trolox. If the time trajectories were the composite of spontaneous nucleosome opening/closing events and photoblinking, we should observe difference in the dwell times, which we do. Needles to say, the dwell time statistics are quite poor in view of the rarity of events, despite the huge number of traces analyzed; still the difference between the two dwell times is ~two fold. In view of the poor binning we calculated also mean values of the lifetimes (Table 1); large standard deviation, however, prevents us to make any clearer conclusion.

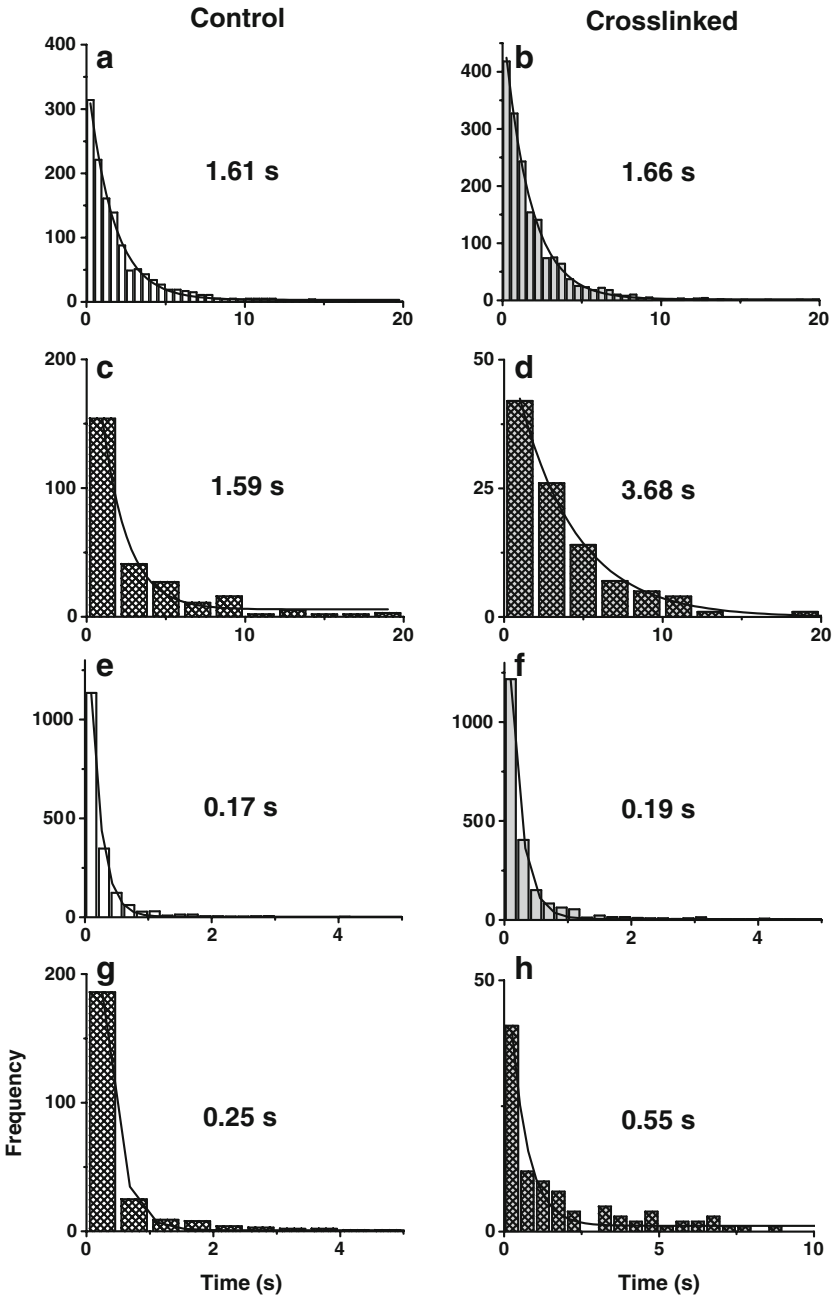

Fig. 4 Dwell time histograms summary. (**a**–**d**), dwell times of high FRET state (ON) between transitions; (**e**–**h**), dwell times of low FRET state (OFF). The frequencies of dwell times were plotted and fitted with a single exponential decay function. Derived τs are shown for each plot. Panels (**a**), (**b**), (**e**), and (**f**) are from measurements in BME; panels (**c**), (**d**), (**g**), and (**h**) from measurements in Trolox. (**a**), (**c**), (**e**), and (**g**), control samples; (**b**), (**d**), (**f**), and (**h**), crosslinked samples

Another factor that supports the conclusion that some of the transitions represent true nucleosome opening events is the number of events per trace, and the number of events per second, both of which are higher for the control than for the crosslinked material.

Thus, the only reasonable explanation for the small proportion of events that do not originate from photoblinking is an opening transition as postulated in Tomschik et al. [7]. Only such a long-range unwrapping of ~70–80 bp could result in the high-FRET to zero-FRET transitions that we observed with our placement of the dyes. In addition to the theoretical support for such a transition referred to earlier [8, 9], there are now new theoretical considerations that explain why the DNA stops to unpeel further, once it encounters the dyad and why the nucleosome does not fall apart altogether. Schiessel [10] refers to the reason as the 'first-second round difference': once one turn of nucleosomal DNA has unwrapped, the remaining turn gets a strong grip on the octamer, since this residual turn does not feel the repulsion from the first turn. We would like to point out that the very low frequency of opening events deduced from the present measurements conform to the earlier biochemical measurements from Widom's laboratory. Polach and Widom [6] reported that the equilibrium

restriction site accessibility decreased from the end of the nucleosomal DNA toward the dyad axis by more than two orders of magnitude (see also [3]). This also states that the true opening events must be very rare, in agreement with data presented here.

There seems to be a clearer indication of long-range opening events in some traces (Fig. 2, traces 7–9) which escaped the all-none requirement of our earlier event selection. Although the range of FRET change is smaller, meaning the dyes did not move away from each other to a non-FRET distance, we interpret these events as partial unwrapping of at least 40–50 bps from the histone surface. We would like to point out that these partial FRET changes were not found in crosslinked sample traces; their frequency was also very low in the non-crosslinked samples (estimated to be less than 0.5%).

Finally, we would like to address the 'doubleness' of the peak in the E_{app} distributions from the Trolox experiments. If we assume that these two poorly-resolved peaks represent two subpopulations of nucleosomes, the lower FRET peak could be due to the so-called 'gaped' particle, theoretically predicted by Victor's laboratory [37, 38]. The gaped particle could reflect a hinge opening of ~30° around an axis at the H3C110/H3C110 interface and would involve breaking of H2A/H2B contacts with H3′/H4′ and H2A′/H2B′. The relative magnitude of the two peaks may reflect a dynamic equilibrium between the conventional closed particle and its gaped counterpart.

The only known experimental solution to differentiate between opening and blinking events is the use of ALEX (Alternating Laser EXcitation) [39, 40]. The principle is based on a periodic alternation of donor and acceptor excitation by donor-specific and acceptor-specific lasers within one observation. The donor excitation serves the same purpose as in 'classic' spFRET, the acceptor excitation works to 'police' the acceptor dyes photophysical status. At this stage, we are in the beginning of setting-up this system.

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References

1. Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403(6765):41–45
2. Turner BM (1993) Decoding the nucleosome. Cell 75(1):5–8
3. van Holde K, Zlatanova J (2006) Scanning chromatin: a new paradigm? J Biol Chem 281(18):12197–12200
4. Li G, Levitus M, Bustamante C, Widom J (2005) Rapid spontaneous accessibility of nucleosomal DNA. Nat Struct Mol Biol 12(1):46–53
5. Li G, Widom J (2004) Nucleosomes facilitate their own invasion. Nat Struct Mol Biol 11(8):763–769
6. Polach KJ, Widom J (1995) Mechanism of protein access to specific DNA sequences in chromatin: a dynamic equilibrium model for gene regulation. J Mol Biol 254(2):130–149
7. Tomschik M, Zheng H, van Holde K, Zlatanova J, Leuba SH (2005) Fast, long-range, reversible conformational fluctuations in nucleosomes revealed by single-pair fluorescence resonance energy transfer. Proc Natl Acad Sci USA 102(9):3278–3283
8. Marky NL, Manning GS (1991) The elastic resilience of DNA can induce all-or-none structural transitions in the nucleosome core particle. Biopolymers 31(13):1543–1557
9. Marky NL, Manning GS (1995) A theory of DNA dissociation from the nucleosome. J Mol Biol 254(1):50–61
10. Schiessel H (2006) The nucleosome: a transparent, slippery, sticky and yet stable DNA-protein complex. Eur Phys J E Soft Matter 19 (3):251–262
11. Brower-Toland BD, Smith CL, Yeh RC, Lis JT, Peterson CL, Wang MD (2002) Mechanical disruption of individual nucleosomes reveals a reversible multistage release of DNA. Proc Natl Acad Sci USA 99(4):1960–1965
12. Ambrose WP, Goodwin PM, Keller RA, Martin JC (1994) Alterations of single molecule fluorescence lifetimes in near-field optical microscopy. Science 265(5170):364–367
13. Dunn RC, Xie X, Simon JD (1993) Real-time spectroscopic techniques for probing conformational dynamics of heme proteins. Methods Enzymol 226:177–198
14. Bagshaw CR, Cherny D (2006) Blinking fluorophores: what do they tell us about protein dynamics? Biochem Soc Trans 34(Pt 5):979–982
15. Schuster J, Cichos F, von Borczykowski C (2005) Blinking of single dye molecules in various environments. Opt Spectrosc 98:712–717
16. Widengren J, Schwille P (2000) Characterization of photoinduced isomerization and back-isomerization of the cyanine dye Cy5 by fluorescence correlation spectroscopy. J Phys Chem 104:6416–6428
17. Sabanayagam CR, Eid JS, Meller A (2005) Long time scale blinking kinetics of cyanine fluorophores conjugated to DNA and its effect on Forster resonance energy transfer. J Chem Phys 123 (22):224708
18. Bates M, Blosser TR, Zhuang X (2005) Short-range spectroscopic ruler based on a single-molecule optical switch. Phys Rev Lett 94 (10):108101
19. Heilemann M, Margeat E, Kasper R, Sauer M, Tinnefeld P (2005) Carbocyanine dyes as efficient reversible single-molecule optical switch. J Am Chem Soc 127(11):3801–3806
20. Sabanayagam CR, Eid JS, Meller A (2005) Using fluorescence resonance energy transfer to measure distances along individual DNA molecules: corrections due to nonideal transfer. J Chem Phys 122(6):061103
21. Rasnik I, McKinney SA, Ha T (2006) Nonblinking and long-lasting single-molecule fluorescence imaging. Nat Methods 3 (11):891–893
22. Adams CC, Workman JL (1995) Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative. Mol Cell Biol 15(3):1405–1421
23. An W, Leuba SH, van Holde K, Zlatanova J (1998) Linker histone protects linker DNA on only one side of the core particle and in a sequence-dependent manner. Proc Natl Acad Sci USA 95 (7):3396–3401
24. Simpson RT, Thoma F, Brubaker JM (1985) Chromatin reconstituted from tandemly repeated cloned DNA fragments and core histones: a model system for study of higher order structure. Cell 42(3):799–808

25. Tomschik M, Karymov MA, Zlatanova J, Leuba SH (2001) The archaeal histone-fold protein HMf organizes DNA into bona fide chromatin fibers. Structure 9(12):1201–1211
26. Zheng H, Tomschik M, Zlatanova J, Leuba SH (2005) In: Golemis E, Adams P (eds) Protein-protein interactions, a molecular cloning manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 1–16
27. Harada Y, Sakurada K, Aoki T, Thomas DD, Yanagida T (1990) Mechanochemical coupling in actomyosin energy transduction studied by in vitro movement assay. J Mol Biol 216(1):49–68
28. Ha T, Rasnik I, Cheng W, Babcock HP, Gauss GH, Lohman TM, Chu S (2002) Initiation and re-initiation of DNA unwinding by the Escherichia coli Rep helicase. Nature 419(6907):638–641
29. Rasnik I, McKinney SA, Ha T (2005) Surfaces and orientations: much to FRET about? Acc Chem Res 38(7):542–548
30. Sofia SJ, Premnath VV, Merrill EW (1998) Poly(ethylene oxide) Grafted to silicon surfaces: grafting density and protein adsorption. Macromolecules 31(15):5059–5070
31. Rasnik I, Myong S, Cheng W, Lohman TM, Ha T (2004) DNA-binding orientation and domain conformation of the E. coli rep helicase monomer bound to a partial duplex junction: single-molecule studies of fluorescently labeled enzymes. J Mol Biol 336 (2):395–408
32. Jackson V (1999) Formaldehyde cross-linking for studying nucleosomal dynamics. Methods 17(2):125–139
33. McGhee JD, von Hippel PH (1975) Formaldehyde as a probe of DNA structure. II. Reaction with endocyclic imino groups of DNA bases. Biochemistry 14(6):1297–1303
34. McGhee JD, von Hippel PH (1975) Formaldehyde as a probe of DNA structure. I. Reaction with exocyclic amino groups of DNA bases. Biochemistry 14(6):1281–1296
35. Ha T (2001) Single-molecule fluorescence resonance energy transfer. Methods 25(1):78–86
36. Koopmans WJ, Brehm A, Logie C, Schmidt T, van Noort J (2007) Single-pair FRET microscopy reveals mononucleosome dynamics. J Fluoresc 17(6):785–795
37. Mozziconacci J, Lavelle C, Barbi M, Lesne A, Victor JM (2006) A physical model for the condensation and decondensation of eukaryotic chromosomes. FEBS Lett 580(2):368–372
38. Mozziconacci J, Victor JM (2003) Nucleosome gaping supports a functional structure for the 30 nm chromatin fiber. J Struct Biol 143(1):72–76
39. Kapanidis AN, Lee NK, Laurence TA, Doose S, Margeat E, Weiss S (2004) Fluorescence-aided molecule sorting: analysis of structure and interactions by alternating-laser excitation of single molecules. Proc Natl Acad Sci USA 101(24):8936–8941
40. Margeat E, Kapanidis AN, Tinnefeld P, Wang Y, Mukhopadhyay J, Ebright RH, Weiss S (2006) Direct observation of abortive initiation and promoter escape within single immobilized transcription complexes. Biophys J 90(4):1419–1431