

Single-Molecule Fluorescence Studies of Nucleosome Dynamics

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Abstract: Single-molecule techniques have transformed dramatically the way we think about biophysics, making it possible to address questions about the dynamics of systems in equilibrium that were practically unthinkable just a decade ago. This review focuses on how single-molecule fluorescence and fluorescence correlation techniques have allowed the investigation of the mechanisms by which nucleosomes allow enzymes and other proteins to access DNA regions that are buried within the nucleosome structure. It has been established that DNA-protein and protein-protein interactions in nucleosomes are very dynamic. The dynamics of the interactions between the DNA and the histone proteins have been investigated by single-molecule FRET and fluorescence correlation spectroscopy. Results are consistent with the so-called site exposure model, in which DNA transiently and spontaneously unwraps from the histone core. DNA accessibility is greatest for sites close to the DNA termini, and decreases towards the nucleosome dyad. Evidence also suggests that DNA sequence plays an important role. The dynamics of the H2A-H2B dimers within the nucleosome has also been addressed by several groups in terms of their implications in determining nucleosome stability and DNA dynamics.

1. INTRODUCTION

A. The Nucleosome Core Particle

Eukaryotic DNA is compacted at multiple levels to allow long molecules of DNA to fit in the cellular nucleus. This is achieved by a hierarchical scheme of folding that involves the formation of a nucleoprotein complex called chromatin [1]. Nucleosomes constitute the most fundamental packing level of eukaryotic chromatin. Each individual nucleosome core particle consists of a complex of eight positively charged histone proteins and approximately 147 base pairs of double stranded DNA (Fig. (1)). The protein core contains two molecules each of histones H2A, H2B, H3 and H4 forming an octameric protein core around which the DNA wraps in roughly 1.6 superhelical turns [2, 3]. The persistence length of double stranded DNA under physiological conditions is approximately 50 nm (~150 bp) [4]. Tight interactions at various contact points between the DNA and the proteins facilitate the distortion required to wrap this stiff DNA around the protein core [5]. An inside look at the structure of the nucleosome reveals that the H3 and H4 proteins form two heterodimers and then together form an (H3-H4)₂ tetramer, flanked on either side by an (H2A-H2B) dimer. The middle region of the DNA wraps around the tetramer, while the remainder interacts with the H2A-H2B dimers. In the absence of DNA, contacts between the H2A-H2B dimers and the tetramer are weak, leading to disassembly of the protein core [6].

The accessibility of DNA in nucleosomes differs significantly from that of naked (isolated) DNA. This has deep consequences in all biological processes that use DNA as a substrate, including transcription, regulation, recombination

and repair. The mechanisms by which nucleosomal DNA becomes accessible to the many enzymes and proteins that need to bind to it during these biological activities are dynamic in nature. Crystallographic data has shown that contacts with the histone octamer are weaker for the terminal ~20 DNA base pairs [7], in support with models that postulate that nucleosomes are not static entities.

B. Nucleosome Dynamics

There are several important aspects of the dynamical nature of nucleosomes that are critical for understanding how the nucleosome structure gives accessibility to the enzymes involved in transcription, replication, gene regulation and repair. Although traditional biochemical techniques have provided evidence of nucleosome dynamics [8], these methods are indirect, and are limited in their capabilities to measure kinetic rates and lifetimes. Single-molecule methods, on the other hand, are uniquely suitable to explore the dynamics of systems in equilibrium, and have opened up new ways of investigating the dynamical aspects of conformational transitions in nucleosomes.

Studies have shown that although nucleosomes are stable, they exhibit reversible and spontaneous conformational fluctuations in which a portion of the DNA transiently unwraps from the protein core (Fig. (2)). This model, called the "site exposure model" [8, 9], plays an important role in exposing target DNA sites to the protein machinery, which are otherwise buried inside the nucleosomes. Equilibrium constants for the site exposure model have been measured by J. Widom and coworkers using conventional biochemical experiments that compare the activities or restriction enzymes on nucleosomes and naked DNA [8]. These studies demonstrated that accessibility of target sequences is largest for sites close to the DNA termini, and decreases for the more internal sites. Although enzymatic methods have proved to be extremely powerful in the investigation of the equilibrium

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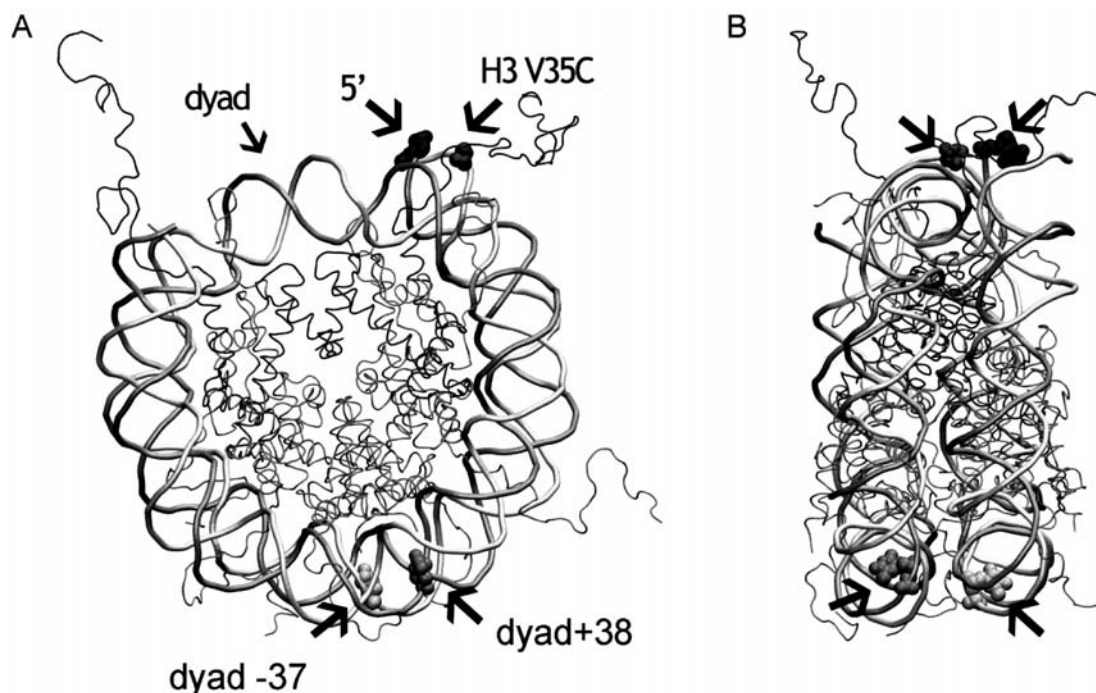


Fig. (1). Two perpendicular views of the nucleosome structure showing the locations of the dyes used in references [29, 30]. The location of the dyes used in the studies by Li *et al.* [29] is shown by the top arrows (donor on one DNA 5' end and acceptor on the H3 histone). The location of the dyes used in the studies by Tomschik *et al.* [30] is shown by the arrows on the bottom. The donor and acceptor dyes are attached to bases on separate strands of DNA separated by 75 bp, located at almost the same distance from the dyad.

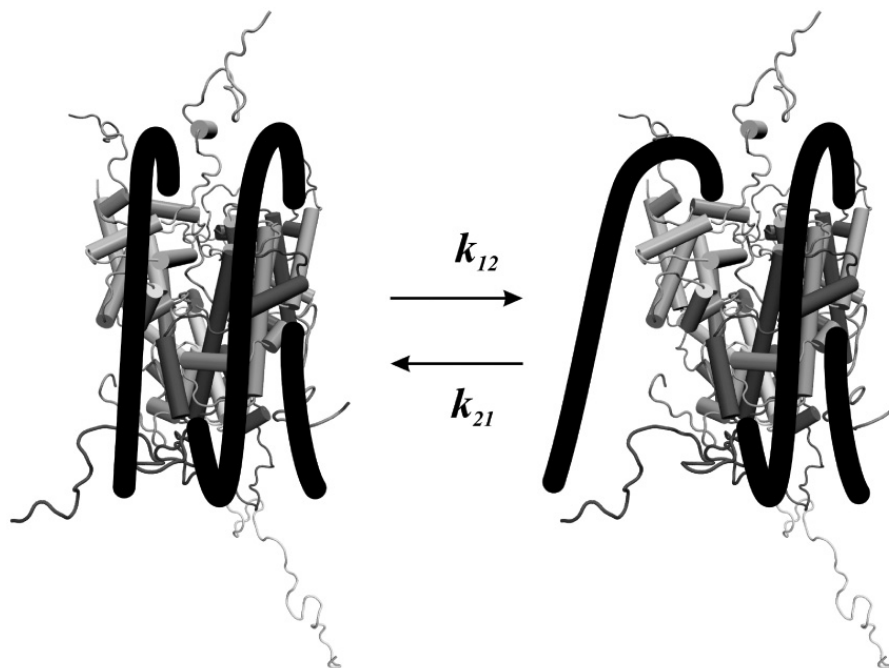


Fig. (2). Schematic representation of the site exposure model, in which DNA transiently unwraps from the nucleosome exposing target sites that were previously inaccessible due to tight DNA-protein interactions [8, 9]. The DNA is represented as a thick black line superimposed to the protein core. The closed and open states are referred to as “state 1” and “state 2” respectively, with k_{12} and k_{21} representing the kinetic rates of the interconversion process.

properties of the site exposure model, they do not provide information about its dynamical aspects. It is also important to consider the role of the H2A-H2B dimers in nucleosome

dynamics as they have been shown to be released during replication and transcription [10]. The investigation of the rates of conformational dynamics has been made possible

thanks to the advent of single-molecule techniques, and constitutes the focus of this review.

C. Single-Molecule Förster Resonance Energy Transfer (smFRET)

FRET is a spectroscopic technique that has been widely used in a variety of life sciences applications, including the study of ligand-receptor interactions, protein-protein interactions, protein folding kinetics, enzyme activity, nucleic acid structure and dynamics, immunoassays, biosensors, etc. The widespread use of the technique is based on its appropriateness to measure distances in the 10-100 Å range, which are on the order of the typical sizes of proteins and membranes. The most common application of FRET in the biological sciences is to measure the distance between two sites of a macromolecule. This was pioneered by Stryer, who coined the term “spectroscopic ruler” in 1967 [11].

In FRET, a donor fluorescent molecule is excited at its specific excitation wavelength, and the excitation energy can be partially transferred to the acceptor, which then emits photons at its characteristic wavelength range. The efficiency of transfer (E) depends on the sixth power of the distance between the two fluorophores (r):

$$E = \left[1 + (r/R_0)^6 \right]^{-1} \quad (1)$$

where R_0 is the Förster distance, a parameter that depends on the spectroscopic properties of the two fluorophores and their relative orientation. Equation 1 provides the basis for the applications of FRET as a tool to measure distances in macromolecules. However, it is important to mention that a number of factors prevent FRET from being a precise technique for distance determination. These include uncertainties in donor-acceptor distance due to the presence of the chemical linkers used in order to label the macromolecule with fluorescent probes, and uncertainties in donor-acceptor orientation [12, 13].

While historically FRET experiments were done with samples containing a large number of molecules (“bulk” FRET measurements), since its inception nearly a decade ago, smFRET has revolutionized the field of biophysics [14, 15]. Single-molecule methods have multiple advantages, including the ability to obtain detailed information about the presence of sub-populations within a sample, and dynamic information that would otherwise be lost in the ensemble average. In the case of single-molecule FRET, experiments are most commonly performed either on molecules diffusing freely in solution by means of single-molecule confocal microscopy, or on molecules that have been immobilized on a surface using total internal reflection fluorescence microscopy (TIRF).

In confocal microscopy (Fig. (3A)), a laser is focused through a high numerical aperture (NA) objective lens and the emitted fluorescence is subsequently collected using the same objective. The emitted light is filtered through a pinhole assembly creating a small observation volume (~few fL). A dichroic mirror is then used to spectrally separate the photons from the donor and acceptor fluorophores, which are detected using single photon counting devices such as avalanche photodiodes (APDs). The average number of mole-

cules observed at a time is kept lower than one by using very dilute solutions of the biological sample (typically around 100 pM).

In TIRF microscopy, a laser is used to illuminate the sample either using a prism (Fig. (3B)), or through an objective. The laser is directed into the sample at an angle larger than the critical angle, leading to total internal reflection. This creates an evanescent field that excites only those molecules within a few hundred nm from the surface. The emitted fluorescence is then collected through an objective and separated into the donor and acceptor images using a dichroic mirror, and then detected onto two halves of a CCD camera [16].

In either method of acquisition, a time series of the donor and the acceptor intensities are collected, from which a FRET trajectory can be calculated as

$$E = \frac{I_A}{I_A + \gamma I_D} \quad (2)$$

where I_A and I_D are the intensities in the acceptor and donor channels respectively, and γ is a correction factor used to account for wavelength-dependent differences in detection efficiency and the difference in the fluorescence quantum yields of the fluorophores.

FRET experiments suffer from a variety of potential sources of artifacts, some of which are accentuated in single-molecule experiments where excitation powers per molecule are much larger than in typical bulk assays. Of particular concern is photobleaching, defined as the irreversible loss of fluorescence efficiency due to photochemical processes occurring from the excited state. Photobleaching limits the total amount of photons that can be emitted by a single-fluorophore, and it is usually a major limiting factor in any time-based fluorescence measurement that requires high excitation powers. Photoblinking is a term used when loss of fluorescence is reversible [17, 18], and appears as intermittent interruptions in fluorescence intensity. In the context of FRET, photobleaching and photoblinking represent a major problem because the fluorescent molecules used as acceptors are usually less photostable than those used as donors. A sudden decrease in acceptor signal due to these photophysical phenomena will be accompanied by a simultaneous increase in donor intensity, and can be erroneously interpreted as decrease in FRET efficiency due to conformational dynamics. Photostability can be largely increased by reducing the concentration of oxygen in the solution by means of a combination of enzymes and chemicals commonly referred to as “oxygen scavenging systems” [19, 20]. In addition, the use of alternating excitation (ALEX) has proven to be a valuable tool to distinguish between true low FRET (i.e. absence of acceptor emission due to low FRET) from photobleaching or blinking (i.e. absence of acceptor emission due to a photochemical reaction) [21, 22]. The technique is based on the alternation between two excitation wavelengths, one that excites the donor (and thus the acceptor through FRET), and the other one that only excites the acceptor directly. Photobleaching will result in a complete loss of acceptor fluorescence, so no signal is expected in either case. However, if the acceptor is intact, but just too far from the donor to result in significant FRET, fluorescence will be

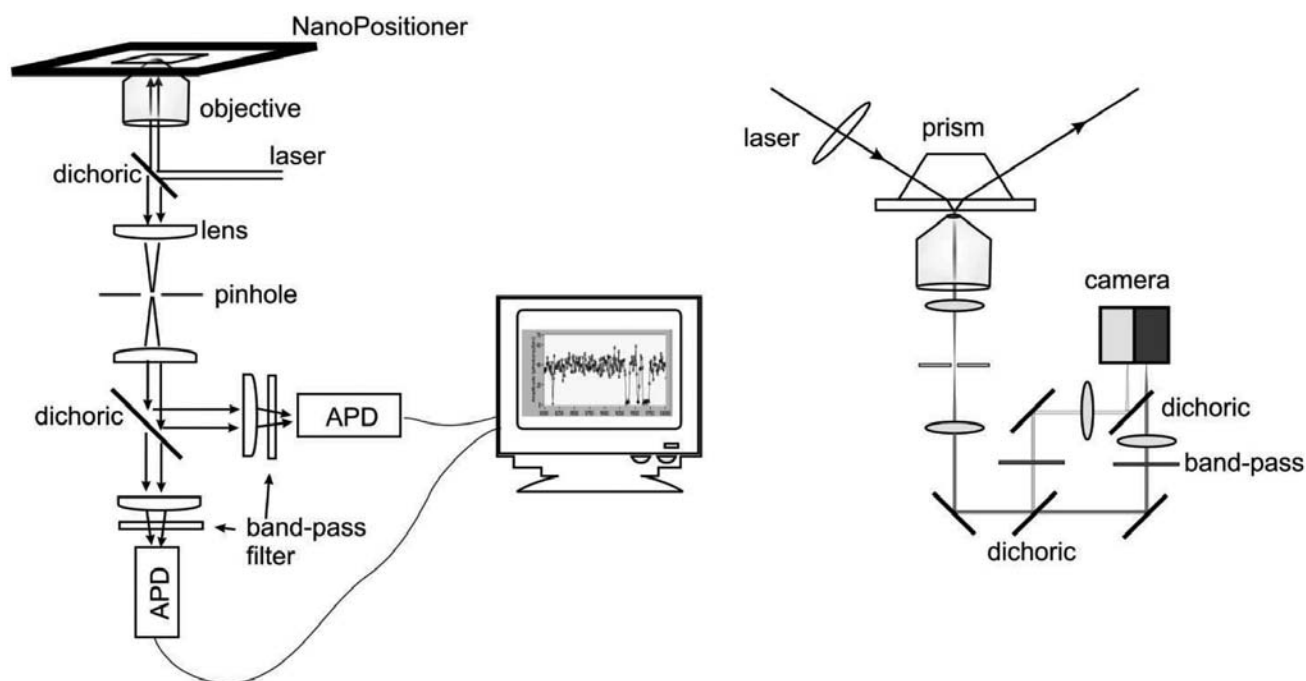


Fig. (3). Schematic representation of the instrumentation used to measure smFRET and FCS (see text). Left: Confocal setup with two detectors for FRET and FCS measurements. Right: Prism-based TIRF setup using two different regions of the same camera for donor and acceptor imaging (see reference [15] for more details).

observed when a laser that matches its characteristic absorption wavelength is used for excitation.

D. Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) is an experimental technique in which fluctuations in fluorescence intensity are analyzed statistically. These fluctuations can arise from different processes, including diffusion of biomolecules in and out the observation volume, conformational dynamics within the system or fluorophore photophysics. When applied to the study of conformational dynamics, the technique takes advantage of the fact that changes in conformation produce fluctuations in fluorescence intensity provided that the system is labeled with a FRET pair or a fluorophore-quencher system. For example, consider a mononucleosome in equilibrium between two conformations as shown in Fig. (2), which has been fluorescently labeled with a donor-acceptor pair so that the efficiency of FRET is different in the two states. A variety of labeling schemes has been used for such studies, and will be discussed in section II. Fluctuations in both donor and acceptor intensities are expected as nucleosomes undergo conformational changes. In FCS, these fluctuations are measured, quantified and analyzed, with the goal of recovering the relevant timescales of conformational dynamics.

In FCS, the pertinent variable is the fluctuation in intensity and not the actual fluorescence intensity by itself. Fluctuations, which can be either positive or negative, represent deviations from the mean and are given by $\delta I(t) = I(t) - \langle I(t) \rangle$, where $\langle I(t) \rangle$ represents the average intensity. Correlation functions are then calculated in order to quantify these fluctuations and provide information about their relevant timescales.

The normalized correlation functions are defined as:

$$G_{xy}(\tau) = \frac{\langle \delta I_x(t) \delta I_y(t + \tau) \rangle}{\langle I_x(t) \rangle \langle I_y(t) \rangle} \quad (3)$$

where the angular brackets represent the time average over the data accumulation time and I_x and I_y represent the intensities measured at detectors x and y . If $x = y$ the resulting function is called autocorrelation whereas if $x \neq y$, it is called cross-correlation (Fig. (4)). Once the correlation functions are obtained, they are fitted with the appropriate models in order to obtain physical parameters related to the processes that give rise to the intensity fluctuations. An accessible introduction to the physical meaning of the correlation functions defined in eq. 3 and FCS in general can be found in reference [23].

Although it was first described in 1972 [24] and demonstrated experimentally in 1974 [25], the technique has skyrocketed in popularity in the past 15 years thanks to advances in instrumentation. The instrumentation needed for performing FCS involves using the same confocal microscope described above for smFRET, and acquiring data using a commercial hardware correlator or through a digital counting card with high time resolution.

Diffusion in and out of the observation volume represents a source of fluctuations that is present in all experiments dealing with systems that diffuse free in solution (i.e. that have not been immobilized). If the system is undergoing any process that alters the fluorescence signal, such as triplet state formation or conformational dynamics which alters intensity through FRET or quenching, additional components need to be added to the correlation function (see refs.

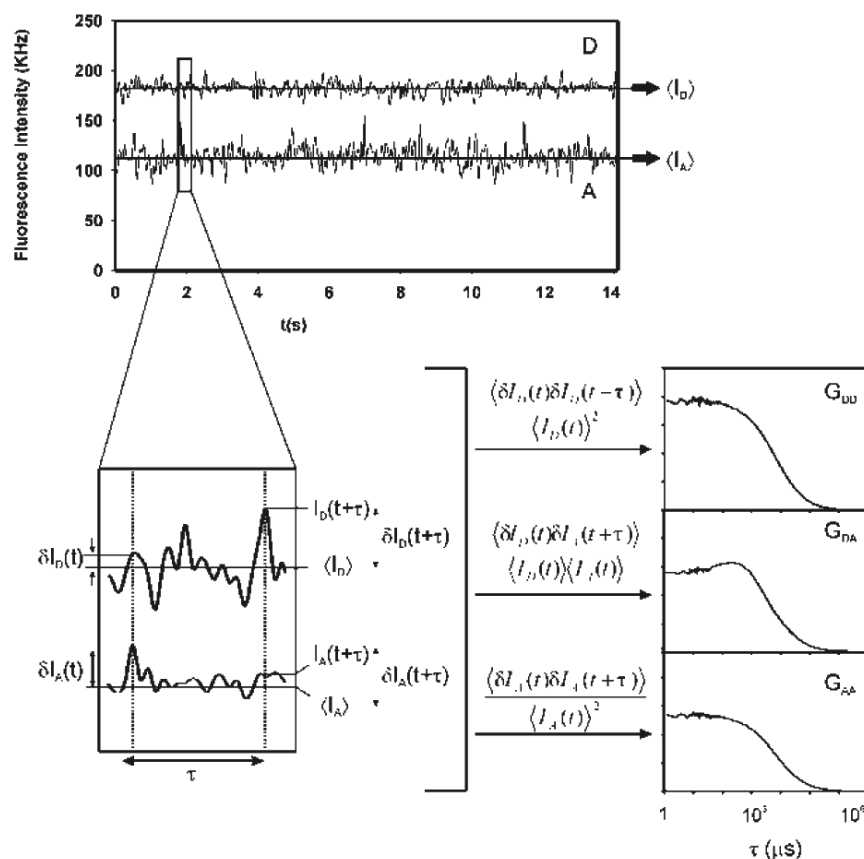


Fig. (4). Schematic representation of how the correlation functions (G_{DD} , G_{AA} and G_{DA}) are calculated from the experimentally acquired donor and acceptor intensities. Intensities at each detector are measured as a function of time (top). Fluctuations in fluorescence intensity in each detector are then calculated as the difference between the actual intensity and the average intensity (bottom-left). Three correlation decays can then be calculated according to equation 3 (bottom-right).

[26-28] for an in-depth review of the use of FCS to study conformational dynamics).

It should be noted that strictly speaking, FCS is not a technique that requires the observation of single-molecules. Yet, the technique shares many characteristics with true single-molecule techniques, including common instrumentation and some aspects of data analysis. As in the case of single-molecule fluorescence, FCS allows the study of conformational fluctuations that would be hidden in the average in studies using very large number of molecules. For this reason, FCS is commonly regarded as a methodology very closely related to single-molecule techniques, and will be considered in this review in conjunction with smFRET studies.

2. SMFRET AND FCS STUDIES OF NUCLEOSOMES DYNAMICS

The first study of nucleosome dynamics was carried out by Li *et al.* in 2004 using FCS [29], and a variety of studies addressing different aspects of the dynamic properties of nucleosomes has been published since then. In this review, we aim to discuss the most significant results of these studies, which have been organized based on common experimental approaches.

A. Single-Molecule FRET Studies on Immobilized Nucleosomes

The first single-molecule study on immobilized mononucleosomes was published by S. H. Leuba and coworkers in 2005 [30]. In this work, the authors used a 164-bp DNA GUB nucleosome positioning sequence modified with both a Cy3-Cy5 FRET pair and a biotin for surface immobilization. The fluorescent dyes were attached covalently to amino-modified bases located on opposite strands so that their final location on the double helix was equidistant from the nucleosome dyad, and 75 bp apart from each other. This distance is more than four times the Förster distance for this FRET pair, and therefore the efficiency of FRET is expected to be negligible unless the two dyes are brought close together by the interaction between the DNA and the histones (see Fig. (1)). Therefore, changes in FRET efficiency between a high- and a low- value are expected to be indicative of wrapping-unwrapping transitions. Using this approach, the authors reported a mean lifetime for the wrapped conformation of about 2-5 seconds depending on ionic strength, and a lifetime for the unwrapped conformation of about 100-200 ms. However, a very careful study by Koopmans *et al.* demonstrated that most of the apparent FRET transitions reported in this paper were not due to nucleosomal dynamics, but to acceptor photophysics [31]. In other words, most

of the events that led to a decrease in Cy5 fluorescence intensity (and concomitant increase in Cy3 intensity) were of photophysical origin, and had been erroneously interpreted as changes in nucleosome conformation. Leuba and co-workers published a subsequent correction [32] confirming the observations of Koopmans *et al.*, and indicating that true events existed, but were rarer than previously reported. The results of this reevaluation were published in an independent manuscript by Zlatanova and coworkers [33].

This incident demonstrates the importance of taking into account and ruling out potential sources of artifacts in single-molecule experiments. Koopmans *et al.* [31] demonstrated the importance of Cy5 blinking in these experiments by using Trolox, an antioxidant that has been shown to dramatically reduce Cy5 blinking and photobleaching [19]. Once experiments were performed in the presence of Trolox, only 3% of the intact nucleosomes showed events with reduced FRET efficiency that were clearly distinguishable from blinking, which leads to apparent changes in FRET (Fig. (5)). If the sample is excited at 514 nm, the excitation wavelength of the donor, the intensities of the donor and the acceptor show anticorrelated changes (top panel) that translate into variations in apparent FRET efficiency (bottom). However, Cy5 blinking becomes evident when samples are excited directly with a 633 nm laser: the same intensity fluctuations are observed for Cy5 (middle), indicating that they are not related to true distance-dependent FRET changes, but to acceptor excursions to a dark state (blinking).

In this work, Koopmans *et al.* used nucleosomes prepared from recombinant histone octamers and a 177 bp DNA construct based on the 601 nucleosome positioning sequence developed by the Widom lab [34]. As in Leuba's original approach, the authors also used a biotinylated DNA construct containing a FRET pair. However, a key difference in this case was the choice of fluorophore location: the acceptor was

located at the nucleosome exit, and the donor 80 bp apart near the dyad axis. The distance between the fluorophores in the reconstituted nucleosome was estimated as 4 nm, so that efficient FRET was expected in the closed conformation. Unwrapping of the DNA from the nucleosome was expected to increase the donor-acceptor distance, resulting in a decrease in FRET. In Leuba's approach, both the donor and acceptor were located inside the nucleosome close to the dyad, so only long-range fluctuations would lead to measurable changes in FRET. Here, the acceptor was located close to the nucleosome exit so the detachment of 10-20 bp of DNA would be enough to cause a complete loss of FRET. Another consequence of this experimental design is the ability to detect the loss of the H2A-H2B dimer, which can be a consequence of nucleosome interactions with the surface. Because the end of the DNA is in close contact with the H2A-H2B dimer, the loss of these subunits would result in the detachment of a stretch of DNA and a concomitant loss of FRET. In contrast, the labeled part of the DNA in the nucleosomes used by Leuba's group is in contact with the more stable H3-H4 tetramer, so it is likely that the fluorescence intensities would remain unchanged even if nucleosomes lose the H2A-H2B dimer. In fact, surface immobilization has proved to be one of the most important challenges in the study of nucleosome dynamics on surfaces. Koopmans *et al.* reported that 90% of the nucleosomes appeared as dissociated complexes, and 97% of the intact nucleosomes did not show any significant change in FRET during the accessible observation time (10 ms-10's of seconds). The same group carried out a subsequent study of the effect of immobilization strategy on the results of nucleosome smFRET studies with the goal of characterizing possible surface artifacts and determining appropriate surfaces for these studies [35]. The conclusion of this work is that a cross-linked star polyethylene glycol coating performs best in terms of nucleosome integrity. These surfaces were prepared by first creating an

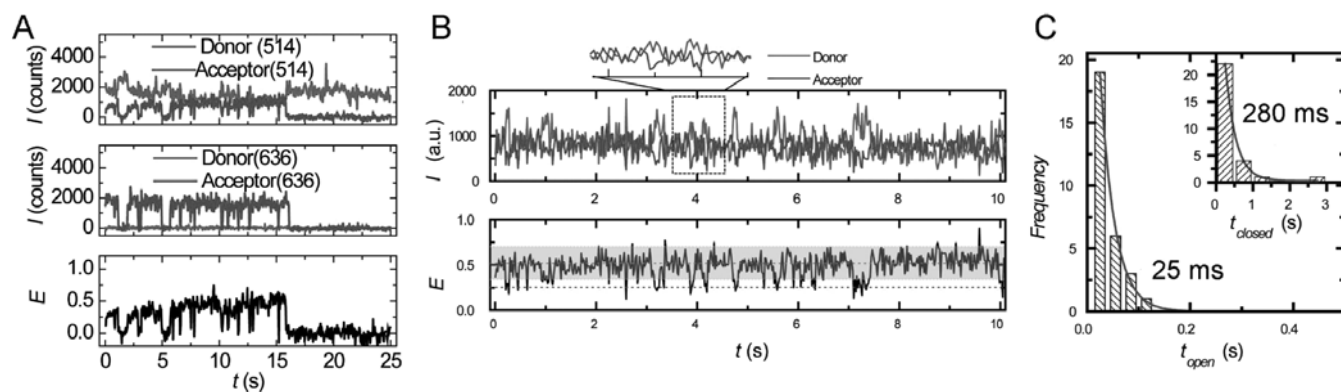


Fig. (5). **A:** sm-FRET traces from individual nucleosomes showing artifacts due to Cy5 blinking [31]. The top panel shows the donor (black) and acceptor (gray) intensities detected with 514 nm excitation. The middle panel shows the intensities measured with 636 nm excitation, providing evidence of acceptor blinking. The calculated FRET efficiency fluctuates between two values (bottom panel), which can be misinterpreted as nucleosome dynamics when only 514 nm excitation is used. **B:** evidence of nucleosome dynamics as studied by smFRET on molecules immobilized on star-PEG-coated surfaces [35]. The FRET efficiency (bottom) is calculated from the measured donor and acceptor fluorescence intensities (black and gray respectively). The shaded bar indicates 96% confidence interval for the theoretical photon and instrument noise. The FRET efficiency fluctuates between a high FRET state (closed conformation) and a low FRET state (open conformation). **C:** Histogram of the open state, from which a mean lifetime of 25 ms is calculated. A similar analysis yields a lifetime of the close state of 280 ms (inset). [Reproduced from reference [35], with permission of the publisher. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA]

amino-functionalized slide with poly-D-lysine, followed by spin-coating a solution of biocytin (a molecule consisting of biotin conjugated to the epsilon amine of lysine) and star PEG (six-arm NCO PEG stars). The iso-cyanate (NCO-) terminated star molecules react with the amino group of biocytin, leaving a biotin available for subsequent reaction with neutravidin and final binding of the biotinylated nucleosomes. Using this surface, Koopmans and coworkers obtained 90% specificity and 78% nucleosome integrity. These values represent a significant improvement over the commonly used BSA-biotin surface (28% integrity) and linear PEG (53% integrity). Nucleosome integrity was measured using alternating-laser excitation (ALEX) [21, 22], so that donor- and acceptor-only species can be distinguished from doubly labeled nucleosomes. In these experiments, samples are excited alternately by a green and red laser. The first excites the donor, and thus the acceptor through energy transfer only in intact nucleosomes. The red laser excites the acceptor directly, and is therefore used to verify the presence of the acceptor in a fluorescent state. The measured open state and closed state lifetimes using the star PEG surfaces were 25 ms and 280 ms respectively, in excellent agreement with previous studies in solution by Li *et al.* [29].

B. Nucleosome Dynamics by Fluorescence Correlation Spectroscopy (FCS)

The first paper to report on the wrapping-unwrapping dynamics of nucleosomes was published by the groups of Bustamante and Widom in 2004 [29]. In this work, we used FCS, in conjunction with stopped-flow FRET, to characterize the timescales of the wrapping-unwrapping process in mononucleosomes diffusing freely in solution. This approach has several advantages over studies on immobilized molecules, but on the downside it requires the assumption of a kinetic model (e.g. a two-state system) and is therefore likely to miss the existence of subpopulations in the sample. On the positive side, the sample does not need to be immobilized, which is particularly advantageous in the case of nucleosomes in light of the results obtained by the van Noort lab described above [35]. Furthermore, FCS can be used to investigate dynamics at much shorter timescales than those accessible by single-molecule studies (~100 ns - 1 s vs. 10 ms-10s), and can be performed at higher concentrations (<100 nM) thus reducing the likelihood of nucleosome dissociation.

In our first studies, we used nucleosomes centered on a 147-bp nucleosome positioning DNA containing a Cy3 molecule attached to the 5' end. The acceptor (Cy5) was attached to a unique cysteine on histone H3 V35C located on the histone core nearby the 5' end of the wrapped DNA (Fig. (1)). It is important to note that while all the other studies reviewed in this paper contain both the donor and acceptor molecules bound to the DNA, this is the only study in which one of the fluorophores is bound to a histone protein. As a consequence of the histone octamer containing two copies of each histone protein, nucleosomes might be labeled with two acceptors. However, only one of these molecules is significantly close to the donor, therefore dominating the observed FRET changes. Experiments were performed in the presence of a large excess of unlabeled core particles to decrease the likelihood of nucleosome dissociation.

FCS was used in this study to monitor conformational fluctuations in a small number of nucleosomes. The strategy was based on earlier work by Bonnet *et al.*, who used fluorescence fluctuation techniques to investigate conformational fluctuations in DNA hairpins [36]. For nucleosomes labeled with donor alone, fluctuations in fluorescence intensity arise from diffusion of the labeled particles into and out of the confocal volume. However, for donor-acceptor double-labeled nucleosomes, both diffusion and intramolecular dynamics contribute to the fluctuations in donor intensity. The ratio of the donor autocorrelation functions of the donor-only sample (G_{DD}^0) and the donor-acceptor sample (G_{DD}) isolates the contributions due to nucleosome dynamics from those due to diffusion. For a two-state mechanism (Fig. (2)) with a completely quenched wrapped state ($E_1 = 1$):

$$\frac{G_{DD}}{G_{DD}^0} = 1 + \frac{k_{21}}{k_{12}} e^{-(k_{12} + k_{21})\tau} \quad (4)$$

where k_{12} , and k_{21} are the unwrapping and wrapping rate constants defined in Fig. (2). Fitting the experimentally obtained G_{DD}/G_{DD}^0 ratio yielded $k_{21} = 20 \text{ s}^{-1}$ and $k_{12} = 3.6 \text{ s}^{-1}$.

It should be noted that the ratio k_{12}/k_{21} is obtained from the amplitude of the observed exponential relaxation in G_{DD}/G_{DD}^0 , and is thus particularly sensitive to experimental variables that are hard to control precisely, including sample concentration, size and shape of the confocal volume, inhomogeneities in cover slip thickness, etc. On the other hand, the relaxation time of the reaction, $(k_{12} + k_{21})^{-1}$, is much less sensitive to experimental artifacts, and is thus determined with more precision in these experiments. We later demonstrated that the same kinetic information can be obtained from the measurements of the donor-acceptor sample only, without the need of using a donor-only sample as a reference [37]. Because all the information is obtained from the same stream of photons, the uncertainties related to exchanging samples mentioned above are eliminated. In this work, we showed that although the individual correlation functions (G_{DD} , G_{AA} and G_{DA}) depend on translational diffusion ($T(\tau)$) and intramolecular dynamics ($R(\tau)$), the ratio of any two decays (G_{DD}/G_{DA} , G_{AA}/G_{DA} , or G_{DD}/G_{AA}) cancels out the diffusion term thus isolating terms that depend on the kinetic rates and FRET efficiencies of the open and closed states:

$$G_{xy}(\tau) = T(\tau)R_{xy}(\tau) \quad (5)$$

where the functions $R_{xy}(\tau)$ represent the contributions of intramolecular dynamics to the autocorrelation and crosscorrelation functions. These functions depend on the FRET efficiencies of the closed and open states (Fig (2)) and the kinetic rates of the opening and closing transitions, k_{12} and k_{21} (see reference [37] for the analytical expressions).

As a proof-of-principle, we reevaluated our previous measurements [29] according to this new methodology, obtaining $k_{12} = 0.8 \text{ s}^{-1}$, $k_{21} = 20 \text{ s}^{-1}$, $E_1 = 0.89$ and $E_2 = 0$, in excellent agreement with our previous work (Fig. (6)). Note that the relaxation time is dominated by the fastest reaction, so k_{21} can be determined with better precision than k_{12} .

Although the ratio between any two correlation functions does not depend on the diffusion term, the signal-to-noise

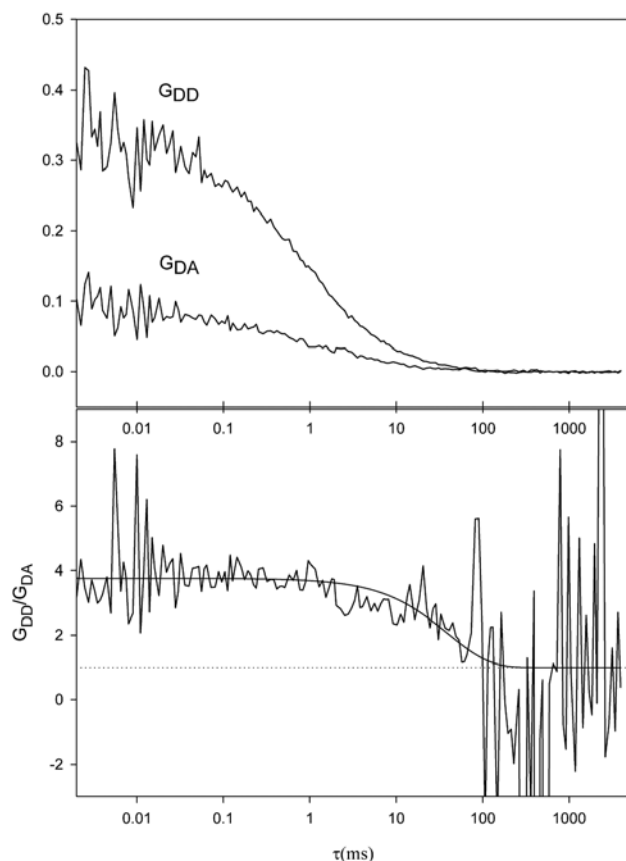


Fig. (6). Top: Experimental donor autocorrelation and donor-acceptor cross-correlation (G_{DD} and G_{DA}) decays obtained with a donor-acceptor double-labeled nucleosome. Bottom: the ratio G_{DD}/G_{DA} isolates the contributions due to conformational dynamics from those due to diffusion (see text). [Reproduced from reference [37], with permission of the publisher. Copyright 2007 American Chemical Society]

ratio is very poor if the residence time of the molecule in the confocal volume is smaller than the relaxation time of the process under study. In other words, diffusion dominates the correlation decays, so the ‘useful’ information lies at the tails, where statistics is poor. Kelbauskas *et al.* circumvented this problem by embedding the nucleosome particles in 3% agarose gels [38]. Using this approach, they increased the residence time by approximately an order of magnitude, obtaining less noisy measurements for a series of nucleosomes reconstituted on different DNA sequences. In this work, the authors investigated the role of DNA sequence on nucleosome stability and dynamics using a combination of FCS measurements and smFRET in solution. The studied sequences included a TATA-containing sequence from the yeast GAL10 promoter, a regulatory sequence from the MMTV promoter, and a fragment from the well-studied sea urchin 5S rDNA gene [38, 39]. All samples contained Cy3-Cy5 double-labeled DNA so that the dyes were at a 30bp distance from each terminus. Significant changes were observed between 5S and the two promoter nucleosomes, including interesting differences when nucleosomes were diluted to sub-nM concentrations. In the first of a series of papers, the authors used FCS to characterize the diffusion coef-

ficient of the three samples, observing that nucleosomes reconstituted on the 5S sequence diffused about 40% faster than the other two [39]. In addition, these nucleosomes appeared less stable at low concentrations, as judged by their FRET efficiencies. These differences were interpreted as being a consequence of sequence-dependent stability variations associated with H2A/H2B loss. This was confirmed in later work [38] with the use of γ NAP-1, a histone chaperone that has been shown to cause H2A/H2B release from nucleosomes *in vitro* [40]. Furthermore, the terminal region of the DNA is in closer contact with the H2A/H2B histones than the internal regions, suggesting that the apparent stability and dynamics probed by these methods should depend on the particular location of the fluorophores on the DNA. In fact, it was shown that nucleosomes appear less stable and more dynamic when they are probed with fluorophores attached to the terminal regions compared to the internal regions [41]. This was demonstrated by Kelbauskas *et al.* who compared nucleosomes reconstituted from 160 bp MMTV DNA fragments containing the fluorophores at positions 45 and 125 (internally labeled nucleosomes) or positions 12 and 88 (end-middle labeled nucleosomes). Similar labeling strategies were also carried out with 5S nucleosomes. In both cases, the relaxation times measured by FCS were consistent with a model in which the residence time in the open state is longer for end-middle labeled nucleosomes than for internally labeled nucleosomes.

C. Single-Molecule FRET Studies on Freely Diffusing Nucleosomes

Gansen *et al.* investigated the influence of dye photo-physics and nucleosome stability in smFRET studies on freely diffusing nucleosomes [42, 43]. Sample concentrations of ~ 40 pM, equivalent to an average of 0.03 molecules per observation volume, were employed so that the probability of detecting more than one particle was negligible. Nucleosome integrity at these low concentrations is a concern, but these conditions are necessary since it has been suggested that even a sample concentration as low as 0.1 molecules per observation volume on average many not be small enough for smFRET measurements on diffusing molecules [44]. This issue has been also addressed in reference [45].

In this work, the authors demonstrated that nucleosome stability is an issue when subnanomolar concentrations are used, and that dissociation is further enhanced at higher ionic strengths. To circumvent this issue, the authors tested the effect of adding inert proteins to the solution, and observed that the addition of BSA (~ 0.2 mg/mL) maintained nucleosome integrity even at 300 mM NaCl [42]. Unlabeled nucleosomes have been also used with the same goal by Li *et al.* [29], but BSA proved to be more efficient and contributes less to the background signal.

Studies with freely diffusing nucleosomes have also provided a way of resolving different populations within a nucleosome preparation [42]. Nucleosomes prepared with the 601, 612 [34] and 5S positioning sequences show different smFRET results, consistent with the 612 sequence having a more defined positioning on the nucleosome particle [44], and the 5S being the most conformationally heterogeneous [43]. Further studies by the same group demonstrated that

histone acetylation decreases nucleosome stability, probably because the neutralization of the positive lysine residues decreases the interaction of the tails with the DNA [43]. These studies are based on the analysis of the so-called FRET histograms, which are obtained by measuring a large number of molecules, calculating the FRET efficiency of each individual observation, and constructing a histogram of the results. A detailed discussion of the optimum way of constructing and analyzing such data has been published elsewhere [46].

CONCLUDING REMARKS

The last five years witnessed a growing number of studies on the dynamic properties of nucleosomes thanks to new advances in single-molecule fluorescence and fluorescence correlation techniques. Several studies have established that nucleosomes are dynamic in nature, even when they are isolated from the cellular machinery. Evidently, nucleosomes in the interior of the cell are not isolated, and a myriad of chromatin remodeling factors and other proteins are known to be involved in cellular processes involving nucleosomes [47, 48]. What is then the relevance of these *in vitro* studies on isolated nucleosomes? On one hand, these experiments have allowed researchers to establish a 'baseline' for future studies on chromatin remodeling using similar approaches. On the other hand, the study of spontaneous fluctuations in nucleosomes allows the investigation of the role of these spontaneous conformational changes in the mechanisms by which remodeling factors and enzymes that need to move along nucleosomal DNA function. For instance, the question of how eukaryotic RNA polymerases transcribe through nucleosomes has been a field of active research in the last years [49-51]. Recent studies by Bustamante and collaborators have shown that RNA polymerase II, the enzyme responsible for transcribing all messenger RNAs in eukaryotic cells, can exert about 7 pN of force [52]. Is this force used by the enzyme to make its way through the nucleosome, or does the enzyme take advantage of wrapping-unwrapping fluctuations that occur spontaneously? Questions like this require that spontaneous fluctuations are investigated and understood, and the single-molecule experiments reviewed in this manuscript suggest that spontaneous fluctuations are likely to be taken advantage of by enzymes and other proteins that need to bind to nucleosomal DNA during biological activity.

Current evidence suggests that DNA sequence plays an important role in determining the equilibrium constants and reaction rates of DNA wrapping and unwrapping, and that sites closer to the DNA termini are more accessible than those closer to the nucleosome dyad. Furthermore, it has been demonstrated that these measurements are prone to experimental artifacts that are still being characterized and understood. It is expected that the quality and quantity of these studies will increase in the near future as techniques and methods of analysis are being constantly improved.

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