Nucleosome dynamics resolved with single-pair fluorescence resonance energy transfer spectroscopy

Koopmans, W.J.A.

Chapter 1

Introduction

The work described in this thesis addresses an intriguing question at the heart of many processes that govern life: how is accessibility to the genetic information in the DNA achieved in its very tightly folded context? This chapter introduces the main subjects in this study: the nucleosome and single-pair FRET spectroscopy. It concludes with an outline of the scope of this thesis.

1.1 The nucleosome

Eukaryotic DNA is organized in arrays of nucleosomes. The hereditary information in the human genome is encoded in more than 3 billion base pairs (bp), equivalent to 1 m of DNA. Somatic cells contain two copies of the complete genome, distributed over 46 chromosomes. If not condensed, the DNA in a chromosome would form a swollen coil of ~100 μm in diameter [1]; yet all the DNA is stored in the nucleus, which is only about 5 μm in size! The challenging task of packaging eukaryotic DNA to make it fit in the nucleus is achieved by specialized proteins that bind and fold the DNA in higher and higher levels of condensation, as schematically depicted in figure 1.1.a. The resulting DNA-protein complex is termed chromatin. In the hierarchy of chromatin, condensation ranges from 10^5-fold linear compaction in the mitotic chromosome down to 5-fold compaction in the fundamental repeating unit of DNA organization, a structure called the nucleosome.

A detailed description of the structure of the nucleosome can be obtained from high-resolution X-ray crystal structures [2, 3], as shown in figure 1.1.b. The nucleosome core particle consists of 147 bp of DNA wrapped around a histone octamer protein core in 1.7 left-handed superhelical turns. The histone octamer core has a modular design: it is composed of a $(H3\text{-}H4)_2$ tetramer at the center, and two H2A-H2B dimers at the ends of the DNA path. DNA binding

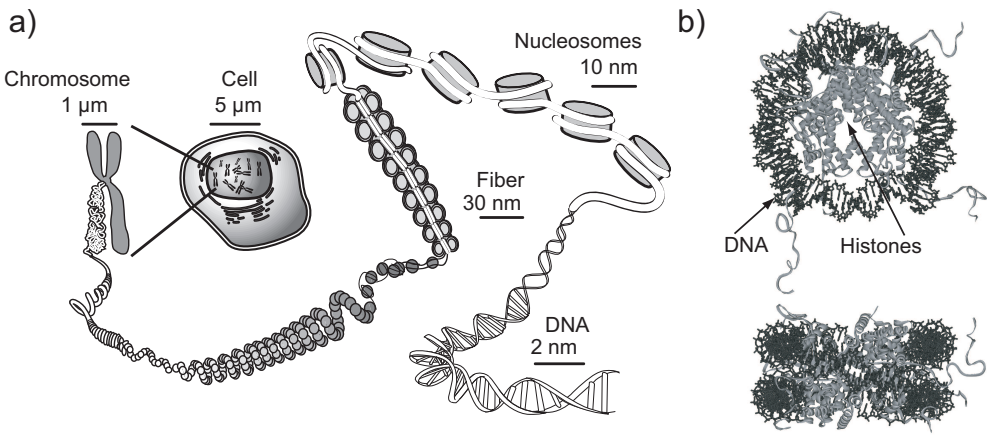

Figure 1.1: Eukaryotic DNA is organized in arrays of nucleosomes. a) DNA in the nucleus is compacted in chromatin, of which the nucleosome is the basic unit. Arrays of nucleosomes form higher-order structures, ultimately giving rise to the highly condensed mitotic chromosome. b) Top view and side view of a high resolution crystal structure (*1kx5* [2]) of the nucleosome core particle. The nucleosome core particle consists of ~50 nm of DNA wrapped in 1.7 turns around a histone octamer protein core.

occurs primarily to the DNA backbone facing the histone core: electrostatic interactions and hydrogen bonds form contacts every 10 bp when the DNA minor groove faces inwards. This lack of sequence specificity ensures that almost any DNA can be incorporated in a nucleosome. Nucleosome core particles are separated from each other by 10-50 bp linker DNA, forming a "beads-on-a-string" chain. In this array nucleosomes interact with neighboring nucleosomes, resulting in higher-order structure to achieve more DNA compaction.

The nucleosome is a remarkable structure, for several reasons:

- The stiffness of DNA is characterized by a persistence length of 50 nm, which means that DNA is essentially straight on that length scale. Yet one persistence length of DNA is wrapped in nearly two full turns in the nucleosome, effectively making it a loaded spring [1].

- Nucleosomes favor particular sequences over others, even though most histone-DNA interactions are not sequence specific. This presumably reflects the ability of particular sequence motifs to more easily accommodate the bending and twisting required for wrapping DNA along the histone octamer perimeter [4]. It has been proposed that this sequence dependence acts as a nucleosome positioning code, that drive nucleosomes to strategic positions in regulatory processes [5].

- The nucleosome has a pronounced charge distribution, with a highly negatively charged

DNA chain that repels itself, and positively charged lysine and arginine groups on the histones. Nucleosome structure is sensitive to salt conditions [6] and to modifications of the charge distribution (e.g. by acetylation of lysine groups [7]).

- Flexible, unstructured histone tails protrude from the core and are exposed. Covalent modifications to residues on these tails, such as phosphorylation and methylation, play a crucial role in regulating nucleosome structure. This may reveal a “histone code" that regulates the genetic information [8].

It is of vital importance that nucleosomes reconcile two conflicting demands: nucleosomes have to package DNA, but also have to ensure that the encoded information in the DNA can be accessed at appropriate times. This leads to the complex and exciting interplay of nucleosome structure and function described in the above.

Nucleosome dynamics are the key for understanding gene regulation. All transactions on DNA in the nucleus take place on nucleosome substrates. The nucleosome is intimately involved in transcription control, and therefore lies at the heart of gene regulation. For example, histones serve as general gene repressors [9], because DNA wrapped in nucleosomes is sterically occluded from enzymes in the transcription machinery. Chromatin structure has to be substantially remodeled to accommodate transcription of the DNA to mRNA. Therefore, in order to understand physical aspects of gene regulation, it is of key importance to understand the conformational dynamics and structural plasticity of nucleosomes that underlie accessibility to the wrapped DNA.

Several mechanisms that ensure nucleosome accessibility have been identified (reviewed for example by Luger [10] or Flaus and Owen-Hughes [11, 12]). These mechanisms can be divided into two broad classes: i) Actively driven accessibility to nucleosomal DNA is catalyzed by chromatin remodelling enzymes, large protein machines that change the position, structure or composition of nucleosomes. In doing so, they consume energy in the form of ATP [12, 13]. ii) Spontaneous accessibility does not require ATP, but relies on intrinsic conformational changes, such as thermal repositioning [14], histone dimer exchange [15], and transient site-exposure by DNA binding and rebinding at the nucleosome ends, in a process termed breathing [16, 17](schematically depicted in figure 1.2.a-c). How these different mechanisms are coupled to each other and how they are employed in transcription, repair, and replication is an intriguing and important question. For example, unwrapping of nucleosomal DNA by breathing may be captured by a site-specific DNA-binding protein, that in turn recruits a remodeling factor to a particular nucleosome [18]. However, many details of the underlying conformational changes in these mechanisms remain to be resolved. For example, little is known about the kinetics of processes such as DNA breathing. Also, the large number

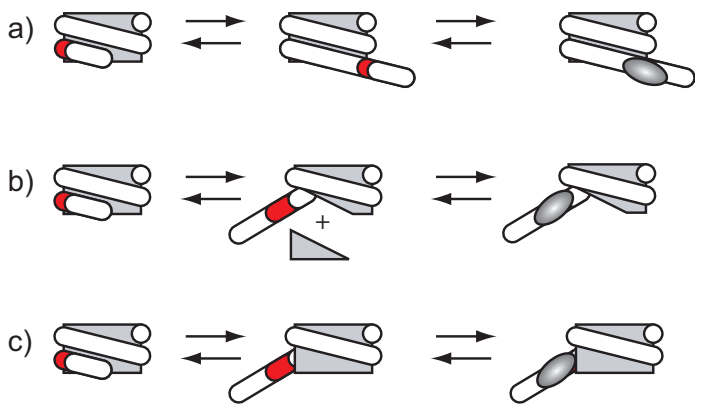

Figure 1.2: Mechanisms for spontaneous enzyme accessibility of nucleosomal DNA. a) Thermal repositioning. b) H2A-H2B dimer exchange. c) Site exposure by DNA breathing from the nucleosome ends. Each mechanism exposes a previously occluded stretch of DNA. In this way, regulatory proteins (dark gray) may bind their recognition site (red) in the nucleosomal DNA.

of DNA-histone contacts raise the questions which bonds are broken in what order for achieving DNA accessibility. Finally, each mechanism probably involves a wealth of closely related intermediate states, and a subtle free energy landscape which is difficult to probe experimentally. Yet for a complete understanding of regulated DNA accessibility at any given site, it is necessary to understand the structure of the underlying chromatin at molecular detail [10].

1.2 Single-pair Fluorescence Resonance Energy Transfer Spectroscopy

FRET is a sensitive tool for studying conformational changes in bio-molecules. Fluorescence (or Förster) Resonance Energy Transfer (FRET) is a process in which excitation energy from a donor fluorophore is transferred non-radiatively to an acceptor molecule via an induced dipole-induced dipole interaction [19]. The efficiency of energy transfer, E, is given by

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}, \tag{1.1}$$

where R is the distance between donor and acceptor and R_0 is the Förster radius, at which 50% energy transfer occurs (typically 5 nm). Because of this strong distance dependence, FRET can be applied as a molecular ruler in the 2-8 nm range [20]: a small change in distance is converted to a shift in fluorescence emission which can easily be detected. The dimensions

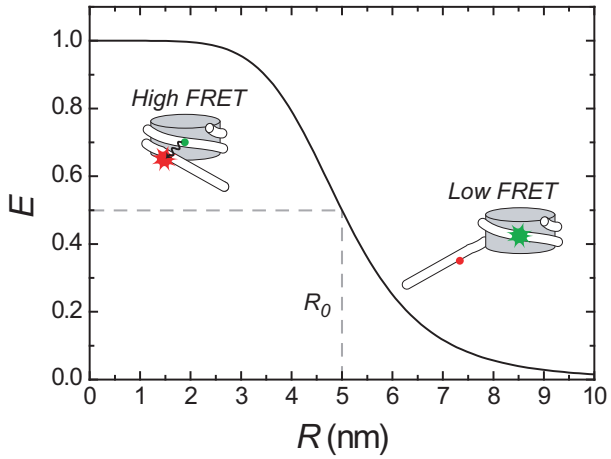

Figure 1.3: FRET as a molecular ruler for detecting conformational changes in the nucleosome. A wrapped nucleosome with a FRET pair at a strategic location brings the fluorophores in close proximity, resulting in efficient energy transfer. A transiently unwrapped nucleosome, as a result of DNA breathing for example, temporarily shows a loss in FRET.

of many biologically relevant structures, such as the Holliday junction or the nucleosome, are accessible by FRET. Furthermore, specific fluorescence labeling of strategic sites on DNA or proteins is possible in many instances. Therefore, FRET is a technique well-suited to studying conformational changes in bio-molecules [21] that govern life at its molecular basis, such as nucleosome dynamics (see figure 1.3).

spFRET spectroscopy reveals the conformational distribution and dynamics of single molecules. In bulk FRET experiments, the fluorescence emission of an ensemble of molecules is recorded, yielding an ensemble-averaged FRET efficiency of the system under study. Information about the conformational heterogeneity or kinetic processes that occur in the ensemble is lost in this way. This information can be obtained with single-pair FRET (spFRET) spectroscopy, in which FRET is applied at the single-molecule level. This was first demonstrated over a decade ago by Ha *et al.* [22]. Using spFRET, the conformational distribution can be reconstructed from the FRET footprint of many individual molecules, and conformational dynamics can be monitored by following a single molecule in time.

To detect the fluorescence of a single pair of fluorophores, it is crucial to collect as many photons as possible. This is a challenging task, which can only be achieved at an acceptable signal to noise ratio because of recent technical advances that resulted in superior quality microscope objectives, photostable fluorophores, optimized fluorescence emission filters, and

sensitive single-photon avalanche photodiodes and multiplication gain CCD cameras. Two detection schemes are frequently employed in spFRET experiments [23]: widefield TIRF microscopy on molecules immobilized to a surface, and confocal microscopy on molecules that freely diffuse in solution. Surface immobilization provides an extended observation time, limited only by photobleaching (a light-induced reaction that results in irreversible loss of the fluorescent state of a fluorophore). Immobilization therefore is a great tool for studying slow processes that occur on timescales longer than 10 ms. A drawback of this method is that the surface may interact with the molecule of interest, so that great care must be taken to employ an optimized immobilization scheme. In-solution experiments do no suffer from these surface-induced artifacts. In this case, the observation time is limited by diffusion through the confocal spot, which is typically on the order of 1 ms. Hence this detection scheme is better suitable for following fast processes or obtaining snapshots of the conformational distribution. Both detection schemes have been successfully employed to unravel subtle conformational changes in a variety of bio-molecules, such as DNA [24, 25], RNA [26] and DNA-protein interactions [27].

Conceptually, spFRET is a versatile, simple and elegant tool in molecular biophysics. Great care has to be taken to interpret spFRET data correctly, though. For example, it is not straightforward to convert a FRET efficiency to an accurate value for distance [28]. Also, photochemical processes that strongly affect the fluorescence emission of the dyes, such as photoblinking and photobleaching, interfere with fluctuations in FRET due to conformational changes. A more advanced alternating laser excitation scheme (ALEX), that simultaneously reports on the FRET efficiency and label stoichiometry [29, 30], is needed to filter out all blinking and bleaching events. Finally, immobilization and dilution to the picomolar concentration needed to resolve single molecules may induce artifacts. In this thesis, I describe how we addressed these issues and how we were able to use spFRET as a sensitive reporter on nucleosome conformation and dynamics.

1.3 Scope of this thesis

This thesis reports experimental work on nucleosome structure and dynamics, using spFRET as a reporter of nucleosome conformation at the single-molecule level. Each chapter was written as a separate research article focusing on specific aspects of nucleosome conformational changes and the experimental methodology used to study these.

Chapter 2 is the materials and methods section of this thesis. It gives a detailed overview of the procedures that were established to reconstitute nucleosomes with a FRET pair exactly at the desired location, and how to analyze them with ensemble and single-molecule techniques. We describe the microscope setups that were constructed to perform experiments on immobilized nucleosomes and on nucleosomes in solution, and present example data.

Chapter 3 describes spFRET experiments on immobilized nucleosomes, that resolve DNA breathing in individual nucleosomes. Immobilization results in dissociation of a large fraction of the nucleosomes, which are excluded from further data-analysis. We report how photoblinking interferes with determination of breathing kinetics, and how this issue is resolved using alternating excitation and a special triplet quencher. We observe that most of the properly immobilized, non-blinking nucleosomes show stable FRET on timescales between 0.01-10 s, while 3% show dynamics with a dwell time of 120 ms that we attribute to conformational changes in the nucleosome. Our findings illustrate not only the merits but also typical caveats encountered in single-molecule FRET studies on complex biological systems.

Chapter 4 further explores the issue of nucleosome immobilization. We report on various nucleosome immobilization strategies, such as single point attachment to polyethylene glycol or bovine serum albumin coated surfaces, and confinement in porous agarose or polyacrylamide gels. We compared the immobilization specificity and structural integrity of immobilized nucleosomes. A crosslinked star polyethylene glycol coating performed best with respect to tethering specificity and nucleosome integrity, and enabled us for the first time to reproduce bulk nucleosome unwrapping kinetics in single nucleosomes without immobilization artifacts.

Chapter 5 reports on spFRET experiments on diffusing nucleosomes, either in free solution or after PAGE separation. We combined spFRET and alternating excitation with a correlation analysis on selected bursts of fluorescence, to resolve a variety of progressively unwrapped nucleosome conformations. The experiments reveal that nucleosomes are considerably unwrapped, but yet remains stably associated. Our findings quantify the delicate interplay between accessibility and condensation in nucleosomes using a powerful combination of single-molecule fluorescence techniques and gel electrophoresis to resolve the resulting conformational heterogeneity.

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