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

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Summary

Nucleosomes form the first level of DNA compaction in eukaryotic nuclei. Nucleosomes sterically hinder enzymes that bind the nucleosomal DNA, and hence play an important role in gene regulation. In order to understand how accessibility to nucleosomal DNA is regulated, it is necessary to resolve the molecular mechanisms underlying conformational changes in the nucleosome. This thesis presents the results of an experimental study of nucleosome dynamics, using single-pair Fluorescence Resonance Energy Transfer (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 gives a detailed overview of the procedures that were established to reconstitute nucleosomes with a FRET pair exactly at the desired location, and to analyze them with ensemble and single-molecule techniques: we generated a DNA template containing a 601 nucleosome positioning sequence. Donor and acceptor were incorporated at specific locations in the DNA using a polymerase chain reaction (PCR) with fluorescently labeled primers. We reconstituted mononucleosomes from the template DNA and histone octamer proteins with salt dialysis. The reconstitution yield was approximately 90%, as observed with polyacrylamide gel electrophoresis (PAGE). Using widefield Total Internal Reflection Fluorescence (TIRF) microscopy on immobilized molecules, we observed and quantified DNA breathing dynamics on individual nucleosomes. Alternatively, fluorescence microscopy on freely diffusing molecules in a confocal detection volume allowed a fast characterization of nucleosome conformational distributions.

In **Chapter 3** we applied spFRET microscopy for direct observation of intranucleosomal DNA dynamics. Mononucleosomes, containing a FRET pair at the dyad axis and at the exit of the nucleosome core particle, were immobilized through a 30 bp DNA tether on a polyethylene glycol (PEG) functionalized slide and visualized using TIRF microscopy. FRET efficiency time-traces revealed two types of dynamics: acceptor blinking and intramolecular rearrangements. Both Cy5 and ATTO647N acceptor dyes showed severe blinking in a deoxygenated buffer in the presence of 2% β-mercaptoethanol (βME). Replacing the triplet quencher βME

with 1 mM Trolox eliminated most blinking effects. After suppression of blinking three subpopulations were observed: 90% appeared as dissociated complexes; the remaining 10% featured an average FRET efficiency in agreement with that found in intact nucleosomes. In 97% of these intact nucleosomes no significant changes in FRET efficiency were observed in the experimentally accessible time window ranging from 10 ms to 10s of seconds. However, 3% of the intact nucleosomes showed intervals with reduced FRET efficiency, clearly distinct from blinking. These fluctuations with a lifetime of 120 ms, could unambiguously be attributed to DNA breathing. The findings in this chapter illustrate the merits but also typical caveats encountered in single-molecule FRET studies on complex biological systems.

Because we observed that many immobilized molecules appeared as dissociated nucleosomes, we further explored the issue of nucleosome immobilization in **Chapter 4**. Immobilization has several advantages: it allows the extension of observation times to a limit set only by photobleaching, and thus opens the possibility of studying processes occurring on timescales ranging from milliseconds to minutes. It is crucial however, that immobilization itself does not introduce artifacts in the dynamics or affects nucleosome structure. We tested various nucleosome immobilization strategies, such as single point attachment to PEG or bovine serum albumin (BSA) coated surfaces, and confinement in porous agarose or polyacrylamide gels. We compared the immobilization specificity and structural integrity of immobilized nucleosomes for these strategies. A crosslinked star-PEG 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.

In **Chapter 5** we chose a complementary approach to prevent immobilization artifacts and show how DNA unwrapping occurs progressively from both nucleosome ends. We performed spFRET spectroscopy with Alternating Laser Excitation (ALEX) on nucleosomes either in free solution or confined in a gel after PAGE separation. We combined ALEX-spFRET with a Fluorescence Correlation Spectroscopy (FCS) analysis on selected bursts of fluorescence to resolve a variety of unwrapped nucleosome conformations. The experiments revealed that nucleosomes are transiently unwrapped with an equilibrium constant of $\sim$ 0.2 – 0.6 at nucleosome ends, and $\sim$ 0.1 at a location 27 basepairs inside the nucleosome. The DNA and histones yet remain stably associated. Our findings, obtained using a powerful combination of single-molecule fluorescence techniques and gel electrophoresis, emphasize the delicate interplay between DNA accessibility and condensation in chromatin.

In conclusion, we resolved nucleosome dynamics by carefully applying single-pair fluorescence resonance energy transfer spectroscopy in two different microscope setups. We showed that nucleosomal DNA is frequently unwrapped, allowing for interactions with other DNA binding proteins. The versatile techniques presented in this thesis can further be exploited to study the dynamics of other heterogeneous DNA-protein complexes than the nucleosome.