Quantifying nucleosome dynamics and protein binding with PIE-FCCS and spFRET

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Summary

Nucleosomes are the first level of DNA compaction in the nucleus of eukaryotic cells. In human cells, about two meters of DNA is condensed in this way into a sphere with a diameter of six micrometres. Although it is in this highly condensed state, called chromatin, the DNA in chromatin is involved in processes such as transcription and DNA repair, for which the DNA must be accessible. To this end, the structure of chromatin is modulated by histone tail modifications, protein recognition elements in the DNA sequence and a large number of protein-DNA interactions. Aforementioned processes often require direct DNA access and are therefore dependent on a change in nucleosome compaction. In this thesis I describe the results of Pulsed Interleaved Excitation and Fluorescence (Cross) Correlation Spectroscopy (PIE-F(C)CS) combined with single-pair Förster Resonance Energy Transfer (spFRET) used to study dynamics in single nucleosomes, which depends on subtle differences in the length of DNA ends, DNA sequence, histone variants and specific and non-specific protein interactions. This technique, which can resolve distances between two fluorophores of only a few nanometers, is an excellent technique to monitor changes in nucleosomal compaction, as the nucleosome is only ten nanometers in diameter. In combination with F(C)CS and PIE, spFRET makes it possible to monitor conformational dynamics on a timescale of micro to milliseconds.

Chapter 1 provides an overview of previous studies of the nucleosome, its role in chromatin compaction *in vitro* and *in vivo* and the effects of DNA sequence, histone modifications and protein interactions on stability and dynamics of nucleosomes. Studies show a dual role for the nucleosome; as a stable structure that is recognized by transcription factors that selectively bind to specific histone modifications or DNA sequences, and as a dynamic entity that is able to temporarily release DNA, thereby regulating processes on the DNA. All of this happens while DNA is folded in the cells in a seemingly disorganized way. In **Chapters 2 and 3** we take a closer look at the optical, analytical and biological tools we developed and used to reveal conformational changes in a single nucleosome. In **Chapter 2** we describe the microscope setup and analysis process for single-molecule experiments.

We show how quantifying the subtleties of fluorophore signals in the microscope setup results in reliable correlation curves of those fluorescent signals after signal processing. The synthesis, purification and processing of DNA, nucleosomes and proteins were optimized, as described in **Chapter 3**. In addition, we present a new algorithm to exclude effects of aggregation in long measurements. With this algorithm one does not have to measure 'around the aggregates' as is usual. PIE-F(C)CS with spFRET was used to obtain the concentration, diffusion times and reaction kinetics of multiple populations from a single measurement. By optimizing the data analysis, a high degree of accuracy for the physical parameters can be achieved. This means that PIE-FCCS in combination with spFRET can elucidate small differences between two very similar biological samples. Both methods chapters are concluded with validations of the experimental methods.
In **Chapter 4** we show the effects of changes in DNA sequence, linker DNA and buffer composition on nucleosomes. In salt concentrations below physiological conditions, nucleosomes prefer to be in a closed conformation. With increasing NaCl concentration, the opening rate of the nucleosome increases while the closing rate remains the same. DNA-histone interactions become stronger with the length of linker DNA. The insertion of the Glucocorticoid Response Element (GRE) into the Widom 601 DNA sequence reduces nucleosome stability more as the GRE was introduced deeper into the nucleosome (i.e., toward the dyad). Except for the nucleosome in which the GRE faces the histone core, all GRE nucleosomes increase their opening rates with increasing NaCl concentration. Positioning the GRE toward the histone core does not increase opening speed, but rather decreases closing speed. The GRE could increase the stiffness of the DNA strand, making it energetically less favorable to bend towards the histone core. Positioning the GRE towards the dyad reduces the critical NaCl concentration at which the dynamic equilibrium changes. Comparing PIE-FCS results with findings from burst analysis for nucleosomes with variable linker DNA lengths in low NaCl concentration provides insight into the effect of linker DNA on compaction. The nucleosome with the shortest linkers has a high FRET population similar to the nucleosome with the crossing linkers in burst analysis, while PIE-FCS shows a closed fraction that was similar to nucleosomes without crossing DNA. Addition of stabilizer compounds and oxygen scavengers significantly slow down nucleosomal dynamics and nucleosomes are then more in a closed conformation. It could be argued that adding stabilizers resembles

more *in vivo* packed environments, and we want to emphasize that the effect of additives on dynamics and stability should be considered when comparing experiments.

In **Chapter 5** we quantify the effect of post-translational histone modification (PTM) H3K36me3 on nucleosome stability and dynamics. Nucleosomes containing H3K36 trimethylation appear more open, based on mean FRET and equilibrium constants. The FRET signal shows that the trimethylation does not inhibit bending of the nucleosomal DNA to the histone core. However, it reduces the number of electrostatic interactions by a factor of two. This decrease in DNA-histone interactions also leads to faster dynamics of H3K36me3 nucleosomes in both low and high salt conditions. Increased kinetics due to fewer interactions between DNA arms and histone core may be the reason how the trimethylation facilitates binding to nucleosomal DNA in processes such as transcription and DNA repair. The use of PIE-FCCS on single-labeled nucleosomes and labeled variants of the LEDGF protein (wild-type and AT-hook deficient) shows that H3K36me3 increases LEDGF affinity for nucleosomes. The diffusion times found for different LEDGF-nucleosome complexes differ by more than a millisecond, indicating different modes of interaction, depending on the LEDGF variant, and whether or not H3K36me3 was present in the nucleosome. The slowest complex, the combination of LEDGF-WT and nucleosomes without H3K36me3, still diffuses faster than open nucleosomes. The results for fractions and diffusion times for experiments on WT nucleosomes appear to be more accurate than those for experiments on H3K36me3 nucleosomes; since these values are based on the correlation curve, which represents the nucleosome in certain conformational states, large errors may be caused by transition states due to the altered interactions of the H3 tail with nucleosomal DNA. Despite the loss of closed nucleosomes at the start of an experiment, the loss of FRET per nucleosome is minimal, depending on the protein and nucleosome variants, implying that LEDGF binding is primarily via free open nucleosomes and suggesting that LEDGF binding stabilizes open nucleosomes.

In **Chapter 6** we make the transition from *in vitro* with purified proteins and DNA to *ex vivo* experiments with GR in nuclear extract. Addition of c-Jun slightly increases DNA diffusion time, after exclusion of condensates. Increasing the concentration of c-Jun does not increase DNA diffusion time when the signal is filtered from condensate contributions. These observa-

tions indicate non-specific interactions of c-Jun with DNA and may imply a DNA condensing role for c-Jun during transcription. Explaining the interaction of GR in a nuclear extract with DNA or nucleosomes has been less straightforward; in agarose gel, activated GR in the nuclear extract is visible as a smeared band in the high molecular weight regions. This smearing may be due to non-specific, transient interactions with other proteins in the extract, which has been observed previously. It may also be an intrinsic property of the GR being in a disordered state, which is often correlated to a protein's activity level. These results are in agreement with those of FCCS experiments, where complexes of GR with either DNA or nucleosome are barely visible, implying a low concentration of a complex. For all DNA and nucleosome constructs, binding affinities are 20 nM and greater, and there is no significant difference in dissociation energy for DNA constructs GREh, GRE2 and GRE3. These results differ from previous results, where the compaction of DNA into nucleosomes, as well as the embedding of the GRE position in nucleosomes, were shown to increase the affinity of GR. Here, GR had the highest affinity for nucleosomes containing the GRE closest to the exit (GREh). Although differences in affinity were small, it appears that *ex vivo*, dissociation depends on accessibility of the GRE in nucleosome, i.e. a GRE placed closer to the nucleosomal exit increases access for GR.
In conclusion, the combination of these findings shows that small changes to the nucleosome, either structurally or electrostatically, can have a significant effect on its stability and breathing dynamics *in vitro*. In thesis we have shown that differences as small as changing 7 base pairs can be detected with our combination of microscopic techniques. Extending to *in vivo* environments, these observations imply only very small differences in energy are necessary to initiate changes in chromatin compaction, allowing DNA-protein interactions and subsequent processes such as transcription. PIE-FCCS combined with spFRET is an excellent tool for resolving these energetically subtle differences in structural as well as kinetic parameters, and with small adaptations could also be used to measure in *in vivo* systems to investigate which changes in DNA sequence, DNA linker length or histone tails is decisive factor for the stabiliteit and dynamics of chromatin.