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

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CHAPTER 3

OPTIMIZING DNA SYNTHESIS, PURIFICATION, AND HANDLING FOR SINGLE-PAIR FRET EXPERIMENTS ON NUCLEOSOMES

3.1 Introduction

Recent years have seen a flux in chemical and biological methods and equipment for more reliable synthesis, purification and identification of biomolecules[148][149]. The emergence of bioinformatics, computational biology and -omics sciences have simplified and made accessible computational tools to design and predict biochemical compounds[150][151]. Smaller detection volumes facilitated by technologies like nanoholes[152], nanojets[153], nano-antennas[154] and sub-microfluidic channels[155], have decreased the need for large amounts of samples and hence the need for bulk production of such samples.

An ongoing issue in biological chemistry is that protocols of others are difficult to reproduce, as some information, knowledge or expertise is assumed to be, but in fact is not, trivial[156][157][158][159]. For parameter settings of computational predictions[160], temperature steps and cycles for synthesis through PCR[161] or biochemical reaction steps for surface functionalization[162][163] one sometimes needs to take into account the impact of something as trivial as room temperature [164].

In this chapter we expand on the detailed protocols used for synthesis and purification of relatively short DNA strands ranging between 200 to 500 base pairs from 80 bp long primers containing fluorophores and either a protein recognition element at specific positions, or elongated or shortened DNA arms. The unusual length of the primers and the DNA construct, the reconstitution into nucleosomes and the application of spFRET required adjustments of standard protocols. Here, we have optimized PCR, ligation, PEG and ethanol precipitation protocols to acquire high yields of the desired end product. We will show the consequences of non-optimized steps or the absence of some of these steps on single-molecule fluorescence microscopy and spectroscopy data and the conclusions one might erroneously draw from these kind of results.

3.2 Construct design

The Widom 601 sequence was incorporated in all DNA and subsequent nucleosome constructs used in this thesis (with the exception of DNA-FRET) and was chosen because of its high affinity to, as well as its positional accuracy of the histone octamer[41]. The 601 sequence was inserted into a plasmid (pGem3z) which was used as the template in PCR amplification.

Also included in the plasmid, flanking the 601 sequence, were two recognition sites for restriction enzymes. After synthesis through PCR, the base DNA construct (Figure **3.1a**) was 198 base pairs long and included fluorophores ATTO647N (@T41) and Cy3B (@C122), recognition sites for BsaI (bp19-24, magenta) and BseYI (bp187-192, blue). Additional DNA was included in the hinges (capital letters, bp 1-40 and 187-198) to ensure high yields for restriction. In order to investigate the binding efficiency of the Glucocorticoid Receptor (GR), as well as to quantify the effect on nucleosome dynamics of the protein's recognition site (GRE), as we have done in Chapters 6 and 4 of this thesis, we inserted the GRE into the Widom601 sequence at 4 different sequences (Fig. **3.1c**).

FIGURE 3.1: **a)** Blueprint of the 198 basepair DNA construct containing Widom 601 sequence. **b)** The forward primer contains label ATTO647N, a recognition site for BsaI and is 80 bp long. The reverse primer contains label Cy3B, a recognition site for BseYI and is 85 bp long. **c)** GRE sequence substitutions inserted in the forward primer.

3.3 DNA synthesis and purification

DNA strands were synthesized via Polymerase Chain Reaction (PCR). During PCR a mix of nucleotides, primers (single-stranded DNA / oligonucleotides), template (double-stranded DNA) and a DNA polymerase enzyme were

cyclically heated and cooled to amplify the template. The first step was activation of the DNA polymerase; after this step, each cycle started at at high temperature to melt the double stranded DNA template. The temperature was then lowered to allow for annealing of the primers to the melted DNA. For the final step in the cycle the temperature was changed to an intermediate of the melting and annealing steps to elongate the primers bound to the strands of the DNA template by the DNA polymerase. The details of the program will be discussed below.
After PCR synthesis, the double-stranded DNA needed to be extracted from the PCR solution to properly determine the yield, as well as omit effects of the solution on subsequent single-molecule experiments. Also, the product needed to be separated from unused primers, as free, labeled primers would affect the fluorescence signal.

3.3.1 Decelerated PCR to optimize synthesis of DNA constructs from long primers

As the length and the high GC-content of the primers for constructing the DNA containing the 601 sequence required a relatively high annealing temperature[165][166], bringing it close to the elongation temperature, the PCR protocol was optimized by trying a range of annealing temperatures between 60-72°C. Also, compared to the previous protocol used by Buning[104], we increased the times of the amplification steps as well as the number of cycles[167]. To further optimize the PCR protocol the ratio of the forward and reverse primers was also investigated. The optimized PCR protocol was as follows:

· Activation: 4 minutes at 95°C
  Followed by 50 cycles of
· Denaturation: 45 seconds at 95°C
· Annealing: 30 seconds at 68°C
· Elongation: 1 minute at 72°C.

The protocol was concluded with an additional 5 minutes at 72°C, and cooled down (on hold) for infinite time at 4°C. The optimized mixture for the primers was 1:3 or 1:4, regardless of which primer is in excess for synthesizing double-labeled constructs, and a ratio 1:6 of forward (ATTO647N) : reverse (unlabeled) to produce single-labeled constructs,

see figure **3.2b**. Final concentrations of the DNA constructs were between 150-400 ng/ml.

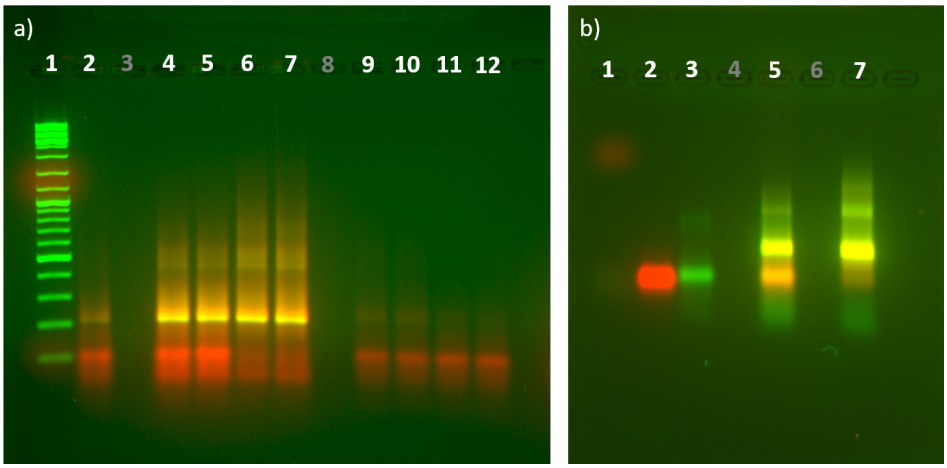

FIGURE 3.2: **Optimization of PEG-NaCl precipitation showed that the protocol is optimal at room temperature and subsequent ethanol washing. a)** After PCR (lane 2) DNA product is visible at 200 bp (GeneRuler DNA Ladder (Thermo Fisher Scientific) in lane 1) as well as residual forward primer (red band at 100 bp), which was added in excess (forward:reverse 8:1) for testing the PEG-NaCl protocol. Incubating the PCR product overnight in 24% PEG and 1.8 M NaCl at 0°C resulted in resulted in incomplete separation of product and primer; there was still primer present in the pellets, and DNA product in the supernatant (pellets: lanes 4 and 5, supernatants: lanes 9 and 10). Performing the protocol at 20°C resulted in pellets practically free of primer, and less than 10% of the product left in the supernatant (pellets: lanes 6 and 7, supernatants: lanes 11 and 12). The 1.5% agarose gel was stained for 2 hrs with 1% EtBr solution, image is an overlay of fluorescence channels 515 nm and 647 nm. **b)** When primers are added in lower excess (ratio forward:reverse 3:1) after PCR the ratio product to primers was 1:1 (lane 5). After PEG-NaCl precipitation, less than 5% of the product was primer. Moreover, both primers did not seem to be present in a clear band. This gel was not stained with EtBr, so the DNA ladder was only visible in lane 1 through red marker 1200bp. The image is an overlay of fluorescence channels 515 nm and 647 nm.

3.3.2 PEG and salt-induced precipitation optimized for long primers and short DNA strands

With the length of the primers (of at least 80 nucleotides) being relatively close to the length of their product (198 base pairs), purification became a non-trivial exercise. As excision from agarose gel and subsequent ethanol purification proved a time-intensive and low-yield method[104], separation through PEG precipitation was investigated. The principle of this method is that DNA can be precipitated from a solution by neutralizing its charge with salt ions. The polymer, here polyethylene glycol (PEG) 6000 ($H(OCH_2CH_2)_nOH$, n=6000), functions as a crowding agent that facilitates the aggregation of DNA. This technique works best when the sizes of the fragments to be separated differ at least by a factor of two. This separation threshold can be modulated by altering the percentage of PEG in the solution; smaller DNA-fragments will precipitate at a higher PEG percentage, larger DNA-fragments at a lower PEG percentage. Titrating PEG percentages, we determined the separation threshold for our 198 bp DNA strand is around 24% PEG. Other parameters influencing the efficiency of the precipitation include salt valency and concentration, incubation temperature, as well as centrifuge time and rotor speed. Based on the previous findings of Schleif[168] and Ran[169] titrations of NaCl (up to 2 M) and $MgCl_2$ (up to 40 mM) were tested; we found monovalent NaCl at a final concentration of 1.8 M to be most optimal for our construct. Incubation temperature was tested at 0, 20 (RT) and 37°C (fig. **3.2a**: lanes 4, 5, 9, 10 at 0°C, lanes 6, 7, 11, 12 at 20°C. 37C not shown); between 20 and 37 degrees no significant differences were observed in efficiency. Between 0 and 20 degrees Celsius we quantified the concentration differences by assessment of the fluorescent intensity in ImageJ. The ratio DNA:primer in the PCR mix is 2:7 (Fig. **3.2a**, lane 2) after precipitation; at 0°C this ratio is 2:3 (lanes 4 and 5), at 20°C it is 1:1 (lanes 6 and 7). At 20°C the primer is not present at one specific size (no band visible), which might indicate the presence of hair-pinned (non-functional) primers[170]. Moreover, in the supernatant the 198 DNA product was visibly present at 0°C (lanes 9 and 10) at a ratio of 2:5, whereas the ratio at 20°C was 1:10 (lanes 11 and 12). The optimized PEG-NaCl protocol was as follows:

· Mix PCR sample (up to 400 μl) with PEG (to 24%) and NaCl (to 1.8M) to a total volume of 1 ml

· Incubate overnight at 20°C (RT)
· Centrifuge for 2 hours at 14.000 rpm
· Remove supernatant and resuspend pellet in MilliQ (50-100 μl)

Using this protocol it was possible to obtain products with concentrations up to 700ng/ml. Figure **3.2b** shows the results after PCR (with primers in ratio 1:3) (lane 5), and after PEG-NaCl precipitation (lane 7). Based on fluorescence intensity assessed with ImageJ we estimated less than 5% of the DNA in the sample after PEG-NaCl was primers. Ethanol precipitation was necessary as an additional cleaning step due to remnant PEG; the yield after ethanol washing (with Promega DNA Purification Kit) was between 50-70%, with concentrations of 350 ng/ml. Compared to extraction from gel and subsequent ethanol precipitation, where yields were on average 40%, with concentrations of $\sim$100ng/ml, PEG-NaCl precipitation proved a clear improvement. Compared to loading and excising constructs from gel, PEG-NaCl is significantly less time and labour consuming.

3.4 Nucleosome reconstitution

Nucleosomes were reconstituted from DNA and histones (recombinant human, EpiCypher) via salt-gradient dialysis. DNA substrate containing the 601 sequence and histones were mixed in molar ratios ranging from 1:0.5 (Fig. **3.3a** lane 4) to 1:2.5 (Fig. **3.3a** lane 8) and complemented with competitor DNA; the latter was added to prevent aggregate formation from a surplus of histones. Final concentrations of the DNA construct and competitor DNA were 100 nM and 40 nM. TE (Tris/EDTA) and NaCl were added to final concentrations of 1x (TE) and 2 M (NaCl) and the samples were diluted with MilliQ to 50 μl. They were then loaded in mini dialysis tubes (Thermo Scientific, Slide-A-Lyzer mini dialysis tubes 3500 MWCO) and secured in a home-made tube holder. The holder was placed in 200 ml high-salt buffer (2 M NaCl, 1x TE, MilliQ) in a 1L glass beaker, containing a magnetic stirrer. The beaker was placed in a cold room (4°C) and connected via an Econo gradient pump (Bio-Rad) to 1 L of low-salt buffer (1x TE, MilliQ). The magnetic stirrer was set to low speed to mix inflowing low-salt buffer with the high-salt buffer. Depending on how fast reconstituted nucleosomes were needed, the flow rate was set to 1.2 ml/min for overnight, or to 1.9-2.2 ml/min for daytime reconstitutions. After dialysis was completed, samples

were collected in low-binding 1 mL Eppendorf tubes and stored in 4°C.

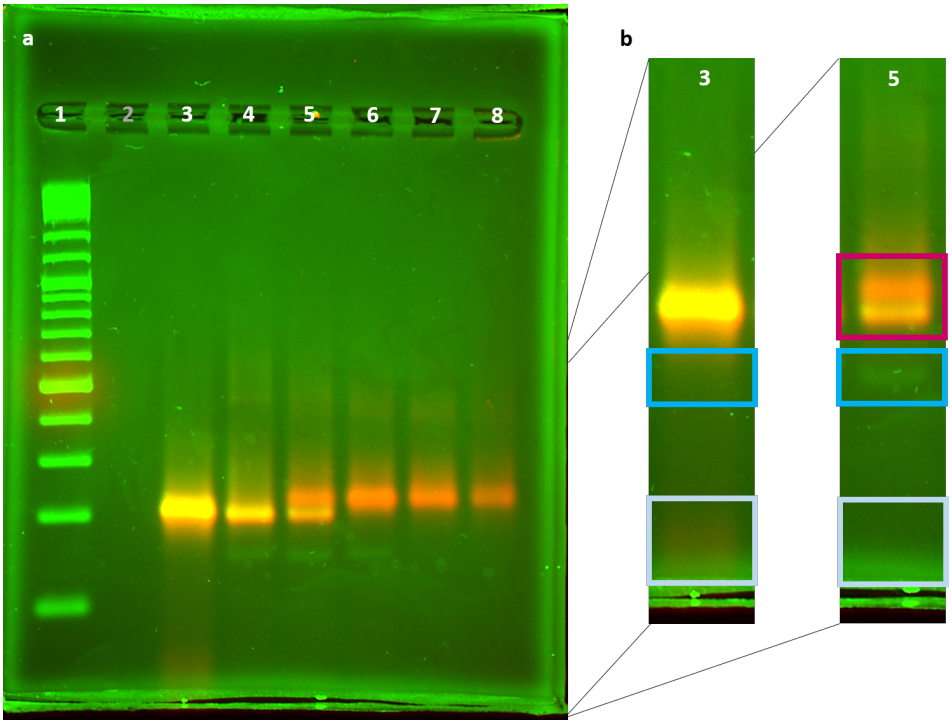

FIGURE 3.3: **DNA reconstitution through automated dialysis showed robust results from histone titration. a)**: gel with DNA ladder (lane 1), EtBr stained. lane 3: DNA, lane 4: DNA:histones (HO) 1:0.5, lane 5: DNA:HO 1:1, lane 6: DNA:HO 1:1.5, lane 7: DNA:HO 1:2, lane 8: DNA:HO 1:2.5. **b)** excerpts of lane 3 and lane 5; grey squares = left over primers (lane 3) disappeared (lane 5) through dialysis, blue squares = competitor DNA (lane 5), red square = incomplete reconstitution.

Mononucleosomes reconstituted via this protocol can be stored at 4°C for years. The reconstitution protocol described here was used in this thesis and is an optimization of an earlier protocol used by Buning[104], where the salt-gradient was applied in discrete steps. The protocol also differs from the one used for the reconstitution of chromatin fibers by Kaczmarczyk et al.[171], which used 10.000 MWCO mini dialysis tubes and maintained a dialysis flow rate of 0.9 ml/min.
Results of a nucleosome reconstitution were assessed on gel (0.2 TB) as depicted in figure **3.3**. Despite the small size difference a clear distinction

can be made between free (lane 3) and reconstituted (lanes 5-8) DNA. The difference was most obvious in lane 5, where both forms were present due to an incomplete reconstitution. Next to gel shift, the difference was also visible from fluorescence. Nucleosomes appear as an orange band, caused by a decrease of green emission from green excitation due to FRET (fig. **3.3b**, right lane (5), yellow and orange band in red square).
Another indicator of incomplete reconstitution is the presence of unreconstituted competitor DNA (fig. **3.3b**, lanes 3 and 5, blue square) around 150 bp. This band, which is visible through EtBr staining of DNA, disappears in higher reconstitutions due to reconstitution of the competitor DNA as nucleosomes don't stain well with EtBr. Lastly, it was observed that leftover primers were removed in the reconstitution process (fig. **3.3b**, lane 5, grey square) despite that their molecular weight ($\sim$ 25 kDa) was larger than the cut-off of the dialysis membrane.

3.5 Surface passivation and molecule immobilization

Scanning confocal microscopy (SCM) has been used to study nucleosomes dynamics[172][126] and DNA/nucleosome interactions with proteins by immobilizing (one of) the molecules on a passivated surface[95]. In principle, SCM allows for tracking of separate, single molecules. However, surface passivation and immobilization puts high demands on the used protocols.
During sample preparations in our lab, it was observed that chemicals needed to be fresh (APTES), well-mixed (PEG-bPEG), fresh, uncontaminated and well-solvated (Neutravidin) and at appropriate concentrations (molecule of interest, several picomolar) to ensure a certain homogeneous surface cleaning, surface coverage and evenly separated binding positions. Even an optimized passivation and immobilization protocol for nucleosomes can however cause non-optimal results; some of the molecules are immobilized too close to one another for optical resolving (figure **3.4a**, light blue circle). A subsequent issue is bleaching of fluorophores by long exposure times: in figures **3.4a-b** the fluorophores on the nucleosome tracked in the white circle were bleached after measuring for 180 seconds. However, the timetraces in figure **3.4c** showed that the signal from Cy3B already disappeared after 22 seconds; the red emission from red excitation appeared quite unstable over time; this was likely caused by

small movements of the sample stage.

The surface passivation protocol consisted of four steps:
1) Cover slide cleaning: performed in a beaker glass that fits a cover slide holder. Typical volume needed: ∼350 ml. Sonicate glass cover slide in MilliQ (10 minutes), 1M KOH (10 minutes), MilliQ (10 minutes), 1M KOH (10 minutes), then MilliQ (10 minutes) again.
2) Aminosilanization: prepare a 4% solution of 3-aminopropyltriethoxysilane (APTES) in acetone (Volume should be sufficiently large to cover surface if slide is not on flow cell yet). Immerse cover slide in reagent for 3-5 minutes. Rinse surface with acetone, MilliQ. Blow dry, attach to flow cell, rinse with NaHCO3.
3) PEG incubation: treat surface for 20-30 minutes with 100:1 methoxyPEG-succinimidyl succinate : biotin-PEG-OCH2CH2-CO2-NHS (PEG:bPEG) in 100 mM NaHCO3 (pH 8.3). Rinse with Tris 10 mM.
4) Sample preparation: treat surface for 20-40 minutes with Neutravidin (1 μM) in buffer of 10 mM Tris-HCL (pH 8.0) and 10% BSA. Rinse with MilliQ. Add molecule of biotinylated DNA (10 pM) in a buffer containing at least 10 mM Tris and 10% BSA. NP-40 and Trolox can also be added; however, when added during our experiments no improvement in signal stability was observed.

Typical results of this protocol are shown in figure **3.4a-b** (double-labeled nucleosomes) and figure **3.5a-d** (single-labeled DNA, red). Examining label stoichiometry and FRET efficiency of immobilized nucleosomes showed the effect of immobilization on the ratio of populations, as discussed in the previous chapter. Although an oxygen scavenger system as well as a photobleaching reductor (Trolox) were added, scanning immobilized nucleosomes showed 64% of the acceptor fluorophores was quenched or bleached, compared to 14% in spFRET burst experiments. Only 9% of nucleosomes lost their donor fluorescence through immobilization in SCM. In figure **3.4a** the the donor- and acceptor-only nucleosomes show as green, respectively red blots. Nucleosomes containing both fluorescent

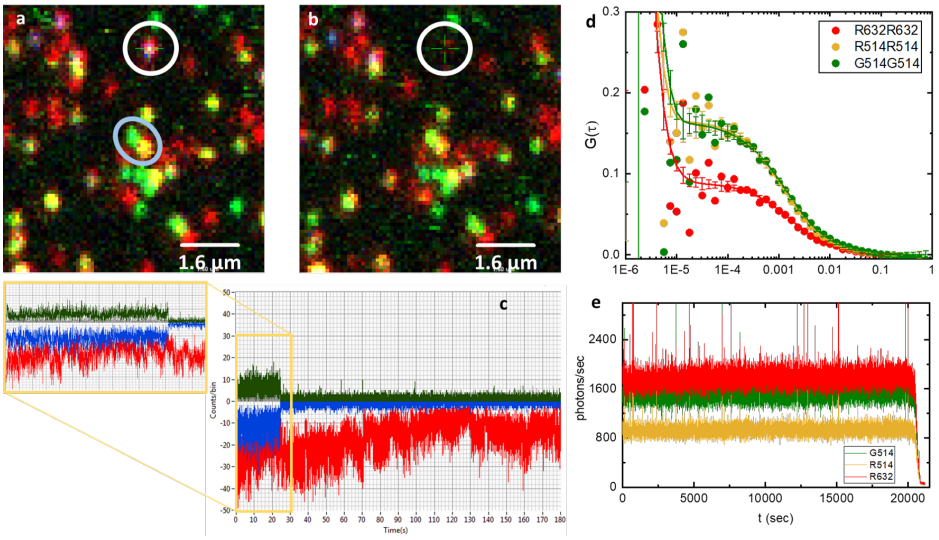

FIGURE 3.4: **Surface passivation with PEG/bPEG conserves DNA compaction in nucleosomes but makes separation of single molecule difficult for further analysis. a)** Surface passivation resulted in intact immobilized nucleosomes (white circle), visible from the overlay of the red, green and FRET (blue colored) channels. Overlay showed nucleosomes having a pink hue, whereas dissociated nucleosomes showed up as yellow/orange dots (lower dot in light blue circle). **b)** Imaging caused however irreversible bleaching of both fluorophores (white circle). **c)** In the first 23 seconds of the point scan (inset), it was not clear from the timetraces if nucleosome dynamics in the order of seconds was occurring. Also, from investigating its time-trace, it was uncertain if the red fluorophore had survived the scan. **d)** Although not as visually direct as SCM images, FCS correlation curves from nucleosomes contain similar information on nucleosome breathing dynamics. **e)** FCS measurements were performed on diffusing nucleosomes in solution, which lowered bleaching events to practically zero. In solution, nucleosomes could be measured for more than 22.000 seconds (= 6.1 hours) without changes in fluorescence intensity; the measurement was only stopped because the water droplet between the objective and glass slide had evaporated.

labels made up only 27% when immobilized, compared to 80% of the total population when freely diffusing. Of the population of double-labeled nucleosomes, about half was observed having a FRET signal (pink blots in fig. **3.4a**, designated as high-FRET fraction), and the other half having no or a minimal FRET signal (yellow blots in fig. **3.4a**, designated as low-FRET fraction). Performing a point scan on a high-FRET nucleosome (fig. **3.4a**,

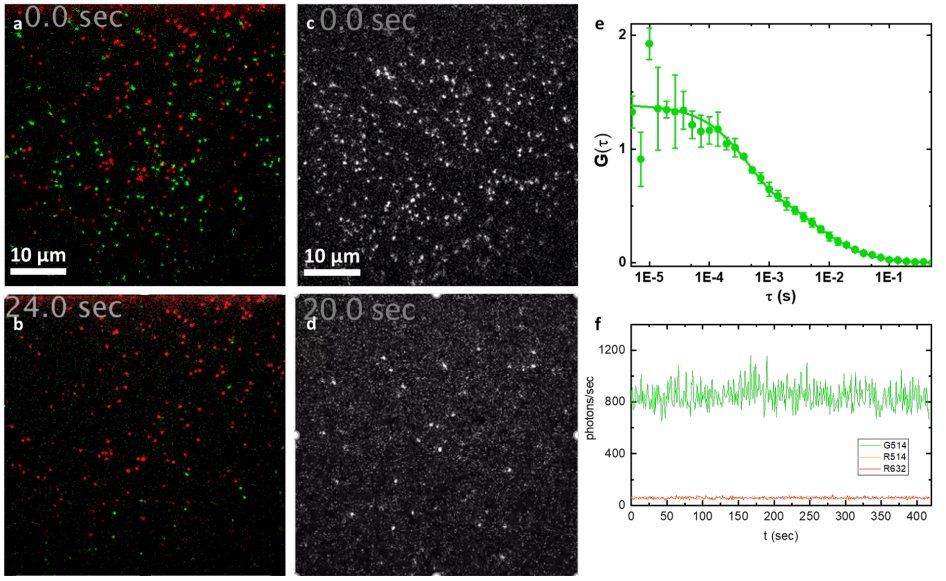

FIGURE 3.5: **Surface passivation with PEG/bPEG causes nonspecific binding of the Glucocorticoid Receptor to the surface and not to specific DNA. a)** Investigating binding of the GR protein (green, labeled with EYFP) to DNA containing a GR specific binding sequence (red, labeled with ATTO647N) in TIRF did not result in colocalization (3x) of the red ($\sim$ 250x) and green ($\sim$ 180x) signals. **b)** After several seconds, the green signals decreased, implying bleaching of EYFP, due to the GR not dissociating, nor swapping with other GR from the solution, but staying bound to the surface, likely saturating it so no new GR was able to bind. **c)** When GR was added to a surface not containing any DNA (either containing a specific binding sequence or not) similar binding **d)** and staying bound was observed. **e)** Using FCS it was possible to create a robust correlation curve, **f)** from a stable fluorescent signal not showing any signs of non-specific sticking and staying bound to the glass surface.

white circle)as described in the previous chapter resulted in time traces of donor (forest green), acceptor (red) and FRET (dark blue) fluorescence, plotted in figure **3.4c**. For 23 seconds, until bleaching of the donor fluorophore terminated the donor as well as FRET signal, the three time traces were clearly distinguishable from background. However, unlike observations from TIRF measurements on immobilized nucleosomes by Koopmans et al.[172] intensity switching in the order of seconds in the donor and FRET channel was not observed in our experiments. Apart

from one of the fluorophores (most often the donor) bleaching after 5-25 seconds of point scanning (fig. **3.4b**, **3.4c**, inset) time traces also showed fluctuations, likely originating from not only movement of the nucleosome around the immobilization point, but also vibrations/instabilities in the confocal setup (fig. **3.4c**, red time trace). In addition, in SCM nucleosomes were designated manually, as our search algorithm was unable to distinguish between molecules too close to eachother (fig. **3.4a**, blue circle). Compared to the highly automated FCS data acquisition and analysis this was a very time-consuming step.
Surface passivation is also used in interaction experiments with immobilized DNA or nucleosomes and proteins from solution[50][95][100].

3.6 Conclusions

In this chapter we have shown that optimizing the synthesis, separation and purification of DNA constructs resulted in higher yields, higher purity of the end product, were less labor intensive and less prone to errors; i.e. more control over DNA substrates. It is however prudent to keep in mind that product optimization is still very construct dependent.
Surface passivation and immobilization protocols were optimized as well and resulted in fluorescent signal clearly distinguishable from background noise. However, signal from single nucleosomes were still difficult to attain due to clustering. Interactions with the surface also seemed to decrease the high-FRET population, compared to nucleosomes measured in solution, and irreversible bleaching together with clustering made finding suitable nucleosome very labor and time intensive. Also, when nucleosomes survived surface immobilization no dynamics in order of seconds was observed. The absence of dynamics might be caused by the addition of stabilising agent Nonidet P40 (NP-40), which is known to prevent surface adsorption[173]. It is also associated with nucleosome dissociation at higher concentrations of NaCl. We will show in chapter 4 addition of NP-40 slows down nucleosome dynamics significantly.
For DNA/nucleosome - protein interactions, minimal specific interactions and predominantly nonspecific interactions were observed when samples were deposited on PEG/bPEG coated surfaces. These interactions were not observed during spFRET burst and PIE-F(C)CS measurements in samples on untreated glass (data shown in next chapters).