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

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Chapter 2

Engineering Mononucleosomes for Single-Pair FRET Experiments[1]

Abstract In DNA nanotechnology, DNA is used as a structural material, rather than as an information carrier. The structural organization of the DNA itself determines accessibility to its underlying information content *in vivo*. Nucleosomes form the basic level of DNA compaction in eukaryotic nuclei. Nucleosomes sterically hinder enzymes that must 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. Exploiting bottom-up control, we designed and constructed nucleosomes with fluorescent labels at strategically chosen locations to study nucleosome structure and dynamics in molecular detail with single-pair Fluorescence Resonance Energy Transfer (spFRET) microscopy. 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 allows a fast characterization of nucleosome conformational distributions.

[1]Part of this chapter is to appear as a contribution to protocols in DNA nanotechnology (ed. G. Zuccheri & B. Samori) in the Methods in Molecular Biology series, Humana Press

2.1 Introduction

DNA nanotechnology uses DNA molecules as smart building blocks for construction at the nanoscale [1]. It exploits the unique molecular recognition properties of DNA to direct the self-assembly of new nanostructures and devices with bottom-up control. A major goal of DNA nanotechnology is to use DNA as a scaffold to structure other molecules (e.g. proteins or electronic components), relying on its chemical stability, rigidity, and predictable structure [2]. To date, DNA has been successfully used to create a variety of complex 2D and 3D architectures, ranging from cubes [3] and tetrahedrons [4] to elaborate world-maps [5]. For this purpose, DNA is used as a structural material, rather than as an information carrier.

At a higher level, it is the structural organization of the DNA itself that determines accessibility to its underlying information content *in vivo*. The basic unit of DNA organization in eukaryotic nuclei is the nucleosome. A nucleosome core particle consists of 50 nm of DNA wrapped in nearly two turns around a histone-octamer core [6]. Arrays of nucleosomes can fold into fibers, which in turn can condense into higher-order structures. Since nucleosomes sterically hinder enzymes that bind the nucleosomal DNA, they play an important role in gene regulation. To understand the mechanisms underlying gene regulation, it is essential to resolve the structural and dynamic properties of nucleosomes in detail [7].

We use a bottom-up approach similar to DNA nanotechnology to study DNA organization in the nucleosome using single-pair Fluorescence Resonance Energy Transfer (spFRET) microscopy [8, 9]. We designed and chose individual DNA and histone components exactly such that DNA folding can be studied at any desired location in the nucleosome.

Nucleosomes are assembled on a DNA template through a salt dialysis reconstitution with purified core histones [10]. The fluorescently labeled DNA template contains a strong nucleosome positioning element [11], so that nucleosomes are exactly positioned at a specific location on the DNA. This level of control ensures that the fluorescent labels in the DNA are incorporated at the desired location in the nucleosome, resulting in efficient FRET. Further modifications of the DNA allow specific immobilization of the nucleosomes to a surface. The FRET efficiency of individual nucleosomes can be monitored in detail with single-molecule fluorescence microscopy. For these experiments, nucleosomes are diluted to single-molecule concentrations in optimized buffer conditions. Widefield total internal reflection fluorescence (TIRF) microscopy on immobilized molecules allows the monitoring of individual nucleosomes for tens of seconds to minutes, revealing their dynamic behavior in time [8, 9]. Alternatively, fluorescence microscopy on freely diffusing molecules in a femtoliter confocal detection volume allows a fast characterization of nucleosome conformational distributions [12, 13].

2.2 Materials

2.2.1 DNA preparation and purification

1. Template DNA containing the 601 nucleosome positioning sequence.
2. HPLC grade fluorescently labeled forward and reverse primer (IBA GmbH) dissolved at 50 µM in 1X TE (10 mM Tris.HCl pH 8, 1 mM EDTA). Store in aliquots at -20 °C.
3. FastStart PCR kit (Roche) with thermostable hot start polymerase (5 U/µl), nucleotide mix (10 mM of each dNTP), and 10X reaction buffer. Store at -20 °C.
4. Thin-walled PCR tubes (Eppendorf).
5. QIAquick PCR purification kit, or QIAquick Gel Extraction kit (Qiagen).

2.2.2 Mononucleosome reconstitution

1. TE dialysis buffer: 50X stock solution (500 mM Tris.HCl pH 8, 50 mM EDTA).
2. 5 M NaCl stock solution.
3. 5-20 µM recombinant histone octamers in 1X TE, 2 M NaCl, and 5 mM 2-mercapto-ethanol; 2 µM mixed sequence competitor DNA (~147 bp) in 1X TE, 2 M NaCl, or
4. 3-10 µM micrococcal nuclease digested nucleosome core particles.
5. Dialysis tubes: Slide-A-Lyzer MINI dialysis units (10K MWCO, Pierce).

2.2.3 Polyacrylamide Gel Electrophoresis (PAGE)

1. Running buffer: 5X TB (450 mM Tris, 450 mM Boric Acid). Store at room temperature.
2. 40% acrylamide:bisacrylamide solution (29:1, Bio-Rad) (this is a neurotoxin when unpolymerized, so avoid exposure). Store at 4 °C.
3. N,N,N,N'-Tetramethyl-ethylenediamine (TEMED, Bio-Rad).
4. Ammonium persulfate (APS): 10% solution in water (*see* **Note 1**), stored in aliquots at -20 °C.
5. Loading buffer (6X): 10 mM Tris.HCl pH 8, 60 mM EDTA, 60% glycerol.

2.2.4 Single-molecule FRET measurements

Cover slide preparation

1. Microscope cover slips (24 x 60 mm # 1.5, Menzel).
2. Cleaning agents: RBS-50 detergent (Fluka), 96% AR grade ethanol (Biosolve).
3. Poly-D-lysine (Sigma) dissolved at 0.1 mg/ml in water, and stored at 4 °C.
4. NCO star PEGs (kind gift of Dr. Groll, RWTH Aachen, *see* **Note 2**).
5. Tetrahydrofuran (THF) (Sigma)
6. Biocytin (Sigma) dissolved at 1 mg/ml in water, and stored at -20 °C.

Single-molecule imaging

1. T50 buffer (*see* **Note 3**): 10 mM Tris.HCl (pH 8), 50 mM NaCl.
2. Oxygen scavenger system: Catalase (Fluka) stored at 4 °C, glucose oxidase (Sigma) stored at -20 °C.
3. β-D-glucose (Sigma).
4. Triplet quencher (trolox): tetramethylchroman-2-carboxylic acid (Sigma).
5. Sodium hydroxide solution (120 mM NaOH)
6. 100X bovine serum albumin (BSA) solution: 10 mg/ml in T50.
7. 10 mM Tris.HCl (pH 8).
8. Neutravidin (Pierce), dissolved at 5 mg/ml in water and 10% glycerol, stored in aliquots at -80 °C.
9. CoverWell perfusion chamber gaskets (Invitrogen).

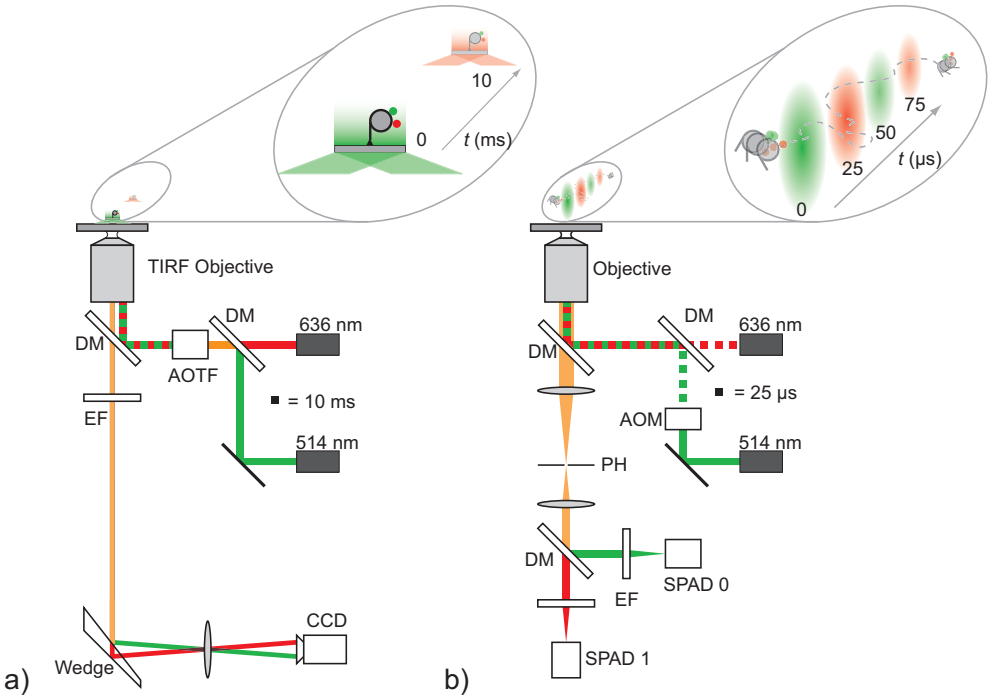

Figure 2.1: Single-pair FRET microscopes. a) Widefield TIRF microscope. DM, dichroic mirror; AOTF, Acousto Optical Tunable Filter; EF, Emission Filter; CCD, Charge Coupled Device. The combined lasers are alternated (green and red boxes) at 20-100 Hz with an AOTF that is synchronized with the CCD camera. TIR excitation is achieved by displacing the excitation beams relative to the optical axis. The resulting fluorescence (orange) from immobilized molecules is collected by the objective, and filtered through an emission filter. Donor (green) and acceptor (red) fluorescence are simultaneously imaged on separate areas of the CCD chip using a dichroic mirror wedge. b) Fluorescence microscope with confocal geometry. AOM, Acousto Optical Modulator; PH, Pinhole; SPAD, Single-Photon Avalanche Diode. The lasers are alternated at 20 kHz by analog modulation either directly (636 nm laser) or with an AOM (515 nm laser), synchronized with the 10 MHz clock on the photon counting board. The resulting fluorescence from freely diffusing molecules in the excitation volume is collected by the objective, filtered through an emission filter, and spatially filtered through a pinhole. Donor and acceptor fluorescence are imaged on different SPADs using a dichroic mirror.

2.3 Single-molecule fluorescence microscopes

We used two complementary approaches to image individual nucleosomes using single-molecule fluorescence microscopy, as depicted in figure 2.1.

We imaged nucleosomes tethered to a polymer-coated cover slide with a widefield TIRF microscope. In this configuration, 10-100 molecules can be observed in parallel for seconds or even minutes. It is even possible to perform multiple experiments on an individual molecule, e.g. by varying the buffer conditions while observing a specific molecule. The time resolution is given by the readout time of the CCD camera, typically 10 ms in our experiments. Single-molecule sensitivity is achieved by using an electron multiplication gain CCD with 90% quantum efficiency, an evanescent excitation volume to reduce background fluorescence and a high NA objective that increases the overall photon collection efficiency of the microscope.

Alternatively, we imaged molecules in free solution with a confocal microscope. In this configuration, thousands of molecules can be sampled rapidly to extract the conformational distribution of the sample. Additionally, there is no need for immobilization, which is a potential source of artifacts. The observation time is limited to the diffusion time through the excitation volume, typically a few milliseconds. Single-molecule sensitivity is achieved by using single photon avalanche diodes with >60% quantum efficiency, a pinhole in combination with a diffraction-limited excitation volume to reject background fluorescence, and a high NA objective that increases the overall photon collection efficiency of the microscope.

In both setups, we use Alternating Laser Excitation (ALEX) to monitor the label stoichiometry. This allows us to direct the data analysis to properly folded, doubly labeled molecules, to reject molecules with only a donor or acceptor fluorophore, and to discriminate nucleosome conformational dynamics from photobleaching and photoblinking of the dyes.

2.3.1 Widefield TIRF setup

Immobilized nucleosomes were imaged with a home-built TIRF microscope equipped with a 100X oil-immersion TIRF microscope objective (NA = 1.45, NIKON), as schematically depicted in figure 2.1.a. The 514 nm line of an Ar-ion laser (Coherent) and a 636 nm diode laser (Power Technology) were used as excitation sources. The beams were spatially filtered with a single-mode polarization maintaining fiber (O.Z. Optics) or a pinhole, and were combined with a dichroic mirror (z514bcm). ALEX was achieved through an Acousto Optical Tunable Filter (AOTF, A.A. Opto–Electronics), alternating at 20-100 Hz. The alternating beams were then expanded and focused in the back focal plane of the objective. Both beams were circularly polarized and were displaced parallel to the optical axis of the objective, so that an evanescent excitation field (0.1-1 kW/cm^2) was generated by total internal reflection of the light at the glass-water interface. The fluorescence was collected by the objective and filtered through a

custom-made dual color band pass filter (z514-639m) that rejects scattered laser light, and a long pass filter (OG530, Schott). The fluorescence was further split into a donor and an acceptor channel by a custom-made dichroic wedge mirror (0.5° angle interferometer flat wedge (CVI), with 640dcxr and 640dcspxr coatings) placed in the infinity path of the microscope. A +150 mm achromatic lens projected the separate images on a multiplication gain CCD camera (Cascade 512B, Roper Scientific) operating at a frame rate of 20 to 100 Hz. The microscope was enclosed in light-tight containers, to prevent ambient light from illuminating the CCD. CCD and AOTF were synchronized, so that each captured frame was illuminated either with the 514 nm laser or with the 636 nm laser. Subsequent data-analysis allowed sorting of the images based on excitation and emission wavelength.

2.3.2 Confocal setup

Freely diffusing molecules were imaged in solution with a home-built confocal microscope equipped with a 60X water-immersion microscope objective (NA = 1.2, Olympus), as schematically depicted in figure 2.1.b. A 515 nm diode pumped solid state laser (Cobolt) and a 636 nm diode laser (Power Technology) were used as excitation sources. The lasers were alternated at 20 kHz by analog modulation, either directly (636 nm laser) or with an AOM (515 nm laser; Isomet). The alternation was synchronized with the 10 MHz clock on the TimeHarp 200 photon counting board. The linearly polarized beams were combined with a dichroic mirror (z514bcm) and were spatially filtered with a single mode optical fiber (O.Z. Optics). The beams were collimated with a +45 mm achromatic lens, projected into the objective with a dichroic mirror (z514/640rpc), and focused to a tight spot by the objective, with an intensity of ~1 kW/cm^{2}at the minimal beam waist. The fluorescence was collected by the objective and filtered through a custom-made dual color band pass filter (514/639m), that rejects scattered laser light. The fluorescence emission was imaged with a +150 mm achromatic lens and spatially filtered with a 50 µm pinhole in the image plane. The fluorescence was collimated with a +75 mm achromatic lens and was split into a donor and an acceptor channel by a dichroic mirror (640dcxr). The fluorescence was filtered with emission filters (hq570/100m for the donor channel, hq700/75m for the acceptor channel) to minimize crosstalk, and was imaged on the active area of single photon avalanche photodiodes (SPCM AQR-14, EG&G) with +75 mm achromatic lenses. The photodiodes were enclosed in a light-tight container, to reduce background illumination. The photodiodes were connected to a TimeHarp 200 photon counting board (Picoquant GmbH) through a router. Subsequent data-analysis allowed sorting of the photon arrival times based on excitation and emission wavelength. Unless stated otherwise, all filters and dichroic mirrors were purchased from Chroma; all posts, mounts, mirrors, and lenses were purchased from Thorlabs.

2.4 Methods

To obtain exact positioning of a FRET pair at a specific location in the nucleosome, the use of a nucleosome positioning DNA sequence is necessary. We use the 601 sequence, which has a single dominant position for nucleosome formation [11]. Strategic locations for labeling the DNA can be deduced from a high resolution crystal structure [14]. Donor and acceptor fluorophores are then incorporated in the template DNA through a PCR with fluorescently labeled primers. Long, ~80 base pair (bp), primers are needed to label the DNA at internal positions in the nucleosome.

Mononucleosomes are then assembled on the DNA with a salt dialysis reconstitution [10]. It is important to mix DNA and histone proteins in the right stoichiometry: a too low octamer-to-DNA ratio results in a sub-saturated reconstitution, a too high ratio results in the formation of DNA-histone aggregates. The optimal stoichiometry is found by titrating the DNA with increasing amounts of histone octamers. To prevent formation of aggregates, mixed sequence competitor DNA can be included in the reaction. Nucleosomes will preferentially form on the labeled DNA containing the nucleosome positioning element. Excess histones will bind to the competitor DNA.

Alternatively, an exchange reconstitution is used. Histone octamers are then supplied in the right stoichiometry in the form of micrococcal nuclease digested nucleosome core particles (NCPs). In a reconstitution with a 5-10 fold excess of NCPs over the fluorescently labeled DNA, nucleosomes will first form on the nucleosome positioning DNA. The reconstitution yield in both cases is checked with polyacrylamide gel electrophoresis (PAGE). The FRET efficiency is obtained from a bulk fluorescence emission spectrum.

In order to image single nucleosomes, the concentration should be sufficiently low (10-100 pM). To prevent dilution-driven dissociation [15], nucleosomes are diluted in a buffer containing BSA and 10-100 nM unlabeled nucleosomes. Imaging takes place in a buffer containing an oxygen scavenger system, to prevent photobleaching and -blinking (*see* **Note 4**). Widefield TIRF microscopy is performed on nucleosomes that are immobilized to the microscope cover slide through biotin-neutravidin linkage. In this way, individual nucleosomes can be monitored for tens of seconds to minutes. The cover slide has to be treated with a special starPEG coating to prevent non-specific adsorption of the -sticky- histone proteins to the glass [9]. Alternatively, short bursts of fluorescence from freely diffusing molecules can be used to determine the conformational distribution. With this approach, immobilization to a coated cover slide is not necessary.

2.4.1 Choice of label positions and primer design

1. A good indication for where each base is located in the nucleosome core particle, can be derived from high-resolution nucleosome crystal structures [14]. We mapped out the base-to-base distance for each possible combination of bases on opposite DNA strands, as shown in figure 2.2.a. Bases separated a full nucleosomal turn (~80 bp) are spaced less than 2 nm apart, so that efficient FRET can occur when these locations are labeled with a FRET pair. Note that the average distance between the fluorophores is slightly different than depicted in figure 2.2.a, since they are attached to the bases with a short carbon linker.

2. The forward strand is labeled with a donor fluorophore, the reverse strand with an acceptor. We chose Cy3B as donor and ATTO647N as acceptor, because of the photostability, high extinction coefficients, and high quantum yields of these dyes. The Förster radius is approximately 5.5 nm for this pair.

3. Fluorescent labeling is done with modified bases. Thymine bases are optimal targets for this, since they can easily be replaced with a modified dUTP. Cytosine bases are an alternative; they can be replaced with a modified dCTP. The dyes are attached to the base with a carbon linker, so that the fluorophore can rotate freely. Furthermore, it is important to only label bases that face outward from the nucleosome to prevent interactions of the fluorophores with the histone protein core.

4. The fluorescent labels are inserted in the DNA with labeled single-stranded DNA primers through a PCR reaction. A biotin modification can be applied at one of the 5' ends to allow for immobilization of the DNA to a neutravidin-coated surface. To label the DNA deep inside the nucleosome, long (> 80 bp) primers are needed. It is important to place the label not too close (<5 bp) to the primer end, to prevent stalling of the polymerase reaction. We successfully used the following primers on the 601 nucleosome positioning sequence: forward primer: 5'-TTGGC<u>T</u>GGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGCGCTG-3', reverse primer 5'-biotin-TTGGACAGGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCCCTTGGCGGTTAAAACG<u>C</u>GGGGGACAGC-3'. Label positions are underlined. This set of primers places one label at the nucleosome exit, and the other internally at the nucleosome dyad axis, to monitor DNA unwrapping starting from one nucleosome end. If different label positions are chosen, the same primers can be used to monitor a variety of positions inside the nucleosome.

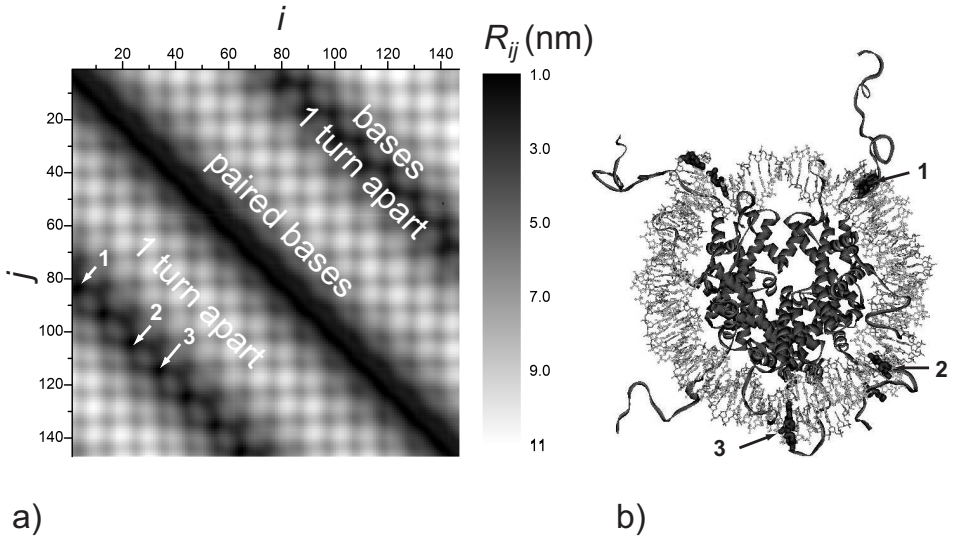

Figure 2.2: Choice of labeling positions. a) Map of the distance R_{ij} between DNA base *i* and base *j* on the complementary strand for crystal structure 1kx5 [14]. The bases come within 2 nm proximity when they are separated by a full nucleosomal turn (~80 bp). When facing outward from the nucleosome, these are ideal locations for placing a FRET pair (e.g. locations 1-3). The diagonal represents paired bases. b) Strategic positions for placing a FRET pair indicating in the nucleosome crystal structure: at the nucleosome exit (1), or reporting on internal sites in the nucleosome (2,3).

2.4.2 DNA preparation and purification

1. In a 0.5 ml PCR tube, mix 5 µl PCR buffer containing $MgCl_2$, 1 µl solution of dNTPs, 1.5 µl fluorescently labeled forward primer, 1.5 µl fluorescently labeled reverse primer, 0.5 µl faststart Taq polymerase, 200 ng template DNA containing the 601 sequence, and water in a total volume of 50 µl.

2. Place the tube in a thermal cycler equipped with a heated lid, such as the Techgene (Techne, Cambridge, UK).

3. Amplify the DNA with 35 cycles of the following two-step PCR cycle: 30 sec denaturation at 95 °C, 1 min annealing and polymerization at 72 °C. The first cycle is preceded by a 5 min initial denaturation step at 95 °C. The last cycle is followed by a final 10 min polymerization step at 72 °C. We use a two-step cycle because of the long, ~80 bp, primers involved.

4. Purify the DNA with a Qiagen PCR purification kit according to the instructions supplied in the manual. We observed that it is difficult to remove all free primer DNA in this way. Alternatively, for a higher degree of purity, analyze the PCR reaction on a 1% agarose gel, excise the desired DNA band, and purify with a Qiagen Gel Extraction kit according to the instructions supplied in the manual.

2.4.3 Mononucleosome reconstitution

Titration with stoichiometric DNA-to-octamer ratios

1. Titrations are carried out by varying the DNA-to-octamer ratio, typically from 1:0.5, 1:1, 1:1.5, 1:2, to 1:2.5, or as high as necessary. We use a minimum reaction volume of 30 µl for the dialysis tubes. The initial salt concentration should be 2 M NaCl, adjusted with a 5 M NaCl solution.

2. In a 1.5 ml centrifuge tube, mix 2 µg of labeled DNA, and 2 µg of unlabeled competitor DNA in 30 µl of 2 M NaCl buffered with 1X TE (pH 8).

3. Add the appropriate amount of histone octamers, and mix.

4. Incubate 30 min on ice.

5. Transfer the sample to a presoaked dialysis tube. Cap the tube and place the tube in floating device in a beaker containing the buffer for the first dialysis step (1X TE with 0.85 M NaCl).

6. Dialyze at 4 °C against 500 ml of 1X TE containing 0.85, 0.65, 0.5, and finally 0 M NaCl for at least 60 min per step. Continuously stir the buffer with a magnetic stirrer at a low speed setting.

7. Recover the contents from the dialysis tube, and analyze with bulk fluorescence spectroscopy and 5% PAGE as described below. An example result is shown in figure 2.3.a. The reconstituted material can be stored at 4 °C for several weeks (*see* **Note 5**). The titration point with the highest reconstitution yield (typically 80%) is subsequently used for single-molecule experiments.

Nucleosome Exchange Reconstitution

1. Exchange reconstitutions are carried out by transferring nucleosome cores from micrococcal nuclease digested nucleosome core particles to the fluorescently labeled DNA containing the 601 sequence. We use a minimum reaction volume of 30 µl for the dialysis tubes. It is important to completely dissociate the nucleosome core particles. Therefore, the initial salt concentration should be 2 M NaCl, adjusted with a 5 M NaCl solution.

2. In a 1.5 ml centrifuge tube, mix 2 µg of labeled DNA, and a 5-fold excess of nucleosome core particles in 30 µl of 2 M NaCl buffered with 1X TE (pH 8).

3. Incubate 30 min on ice.

4. Transfer the sample to a presoaked dialysis tube. Cap the tube and place the tube in floating device in a beaker containing the buffer for the first dialysis step.

5. Dialyze at 4 °C against 500 ml of 1X TE containing 0.85, 0.65, 0.5, and finally 0 M NaCl (for at least 60 min per step). Continuously stir the buffer with a magnetic stirrer at a low speed setting.

6. Recover the contents from the dialysis tube, and analyze with bulk fluorescence spectroscopy and 5% PAGE as described below. The reconstituted material can be stored at 4 °C for several weeks (*see* **Note 5**). The reconstitution yield is typically >90%. An example result is shown in figure 2.3.b.

2.4.4 Polyacrylamide Gel Electrophoresis

1. We use a Amersham Bioscience Hoefer SE 400 vertical gel slab unit (14 cm wide, 14 cm high), with a custom made pump unit for buffer recirculation. Buffer recirculation is necessary to prevent depletion of the low ionic strength running buffer (0.2X TB). Low ionic strengths are needed to prevent dissociation of nucleosomes.

2. The glass plates should be scrubbed clean with a detergent and rinsed extensively with distilled water. Rinse with 70% ethanol and air-dry.

3. Prepare a 1.5 mm thick 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide, 0.2X TB) by mixing 5 ml acrylamide, 1.6 ml 5X TB, 33 ml water. Degas for 10 min and mix with 200 µl APS and 80 µl TEMED to initiate polymerization. Pour the gel, insert the comb, and allow to polymerize for 30 min.

4. Remove the comb and rinse the wells with 0.2X TB buffer. Add 0.2X TB running buffer to the upper and lower chambers of the gel unit.

5. Prerun the gel for at least 60 min at 4 °C at 19 V/cm, while continuously recirculating the buffer.

6. Load 0.2-1 pmol of reconstituted nucleosome core particles in 6 µl 1X loading buffer. The use of dyes such as xylene cyanol and bromophenol blue is not recommended, since their autofluorescence may interfere with the fluorescence from the nucleosomes. To track the migration of DNA in the gel, load the dye in a separate lane of the gel.

7. Run the gel for 75 min at 4 °C at 19 V/cm, while continuously recirculating the buffer.

8. Image the fluorescence with a gel imager such as the Typhoon 9400 (GE). To assess the reconstitution yield, excite the acceptor fluorophore (as shown in figure 2.3.a and b.) since this is a direct reporter of the amount of DNA in each band. To obtain an indication of the FRET efficiency, the donor and acceptor fluorescence should be recorded separately while exciting the donor fluorophore. If the reconstitution yield is known, the FRET efficiency can be further quantified with a bulk fluorescence emission spectrum, as shown in figure 2.3.c. We use the $ratio_A$ method as described in detail by Clegg [16].

2.4.5 Single-molecule FRET measurements

Microscope slide cleaning

1. Place the microscope slides in a rack. Use clean tweezers.

2. Sonicate 15 min in 1% anionic detergent (RBS 50) at 90 °C. Rinse with water.

3. Sonicate 60 min in ethanol. Rinse with water

4. Flame dry slides with a Bunsen burner to remove any remaining traces of organic impurities. Hold the slides with a reverse action tweezers, and gently move the slide through the flame.

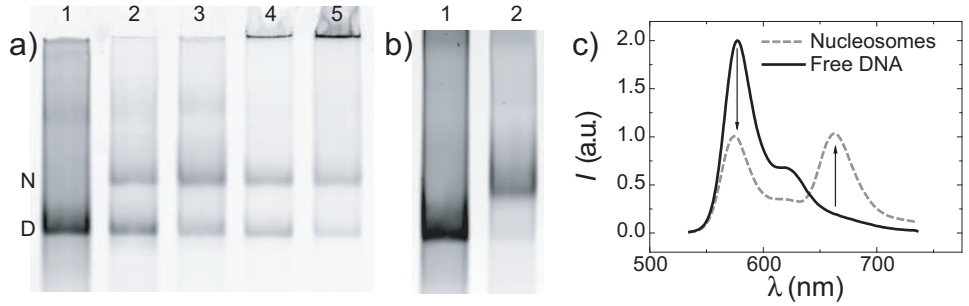

Figure 2.3: Nucleosomes analyzed on 5% PAGE (acceptor fluorescence with excitation at 633 nm) and with bulk fluorescence spectroscopy. a) Octamer titration reconstitution. Lane 1: free DNA, Lane 2-5: DNA:octamer ratios ranging from 1:2 to 1:3.5. All lanes show a band of nucleosomes (N) which migrate slower through the gel than the DNA (D). Lane 4 and 5 show fluorescence in the slots, originating from DNA-histone aggregates due to an excess of histone octamer proteins. b) Exchange reconstitution. Lane 1: free DNA. Lane 2: nucleosome reconstitution. The reconstitution yield in this case is >95%. c) Fluorescence emission spectra of free DNA and reconstituted nucleosomes. Excitation was at 515 nm, the emission was recorded from 535-735 nm. Reconstituted nucleosomes show efficient (>60%) FRET, indicated by the decrease in donor fluorescence around 560 nm, and the increase in acceptor fluorescence at 670 nm.

5. Clean the slides with an UVO ozone cleaning device (Jelight) for at least 60 min to obtain hydrophilic slides without any fluorescent impurities.

Passivation and functionalization

1. Cover the clean slides with 100 µl poly-D-lysine (0.01 mg/ml, Sigma), and incubate for 1 min. This step is needed for amino-functionalization of the slides. Rinse with water and blow dry in a nitrogen stream.

2. Dissolve six-arm NCO PEG stars (MW 12 kDa) in THF at a concentration of 20 mg/ml, and dilute in water to a concentration of 2 mg/ml. This will initiate a crosslinking reaction between the PEGs. Add biocytin (Sigma) to a final concentration of 1 µg/ml to obtain sparse biotinylation.

3. Sterile filtrate the solution through a 0.22 µm syringe filter (Milli-Q) five minutes after mixing, onto the amino-functionalized cover slide.

4. Spincoat the fully covered slide for 45 seconds at 2500 rpm.

5. Incubate the slides at room temperature overnight to complete the crosslinking reaction. Slides can be stored in the dark for up to one week.

Oxygen scavenger system

1. A 1X oxygen scavenger system consists of 0.4-4% glucose, 2.2 µg/ml (2170 U/ml) catalase, 0.92 mg/ml (165 U/ml) glucose oxidase, and 2 mM trolox.

2. Mix 11 µl catalase with 1 ml T50, sterile filtrate with a 0.22 µm syringe filter and centrifuge for 15 min (at 4 °C, 13.2k rpm) in a table-top centrifuge. Take 200 µl of the supernatant.

3. Dissolve 92 mg glucose oxidase in 1 ml T50, sterile filtrate with a 0.22 µm syringe filter and centrifuge for 15 min (at 4 °C, 13.2k rpm) in a table-top centrifuge. Take 200 µl of the supernatant.

4. Mix the catalase and glucose oxidase to obtain a 50X stock solution, and keep on ice. This is more than sufficient for a day of measurements. Further storage is also possible (*see* **Note 6**).

5. Prepare a 40% w/v solution of β-D-glucose and sterile filtrate with a 0.22 µm syringe filter. Store at 4 °C.

6. Dissolve 250 mg trolox in 1 ml 120 mM NaOH (by vortexing and sonicating) to obtain a 100 mM (50X) trolox stock solution. Sterile filtrate with a 0.02 µm syringe filter. Store in aliquots at -80 °C.

Single-pair FRET microscopy with widefield microscopy

1. Assemble a flow channel by placing a CoverWell perfusion chamber gasket on a functionalized slide.

2. Fill the flow chamber with T50 buffer and hydrate the slide for 5 min.

3. Inject a neutravidin solution (0.01-0.1 mg/ml) and incubate for 5 min. Wash excess neutravidin away with 2-3 flow chamber volumes of T50.

4. Dilute the labeled, biotinylated nucleosomes to 10-100 pM in a buffer containing 0.1 mg/ml BSA, 10-100 nM unlabeled nucleosomes, and 1X oxygen scavenger (*see* **Note 7** and the previous paragraph). Inject the sample in the flow chamber and seal by covering the holes with a glass slide. Immobilization is virtually instantaneous. Record the fluorescence with a widefield microscope. Example data is shown in figure 2.4.a-c.

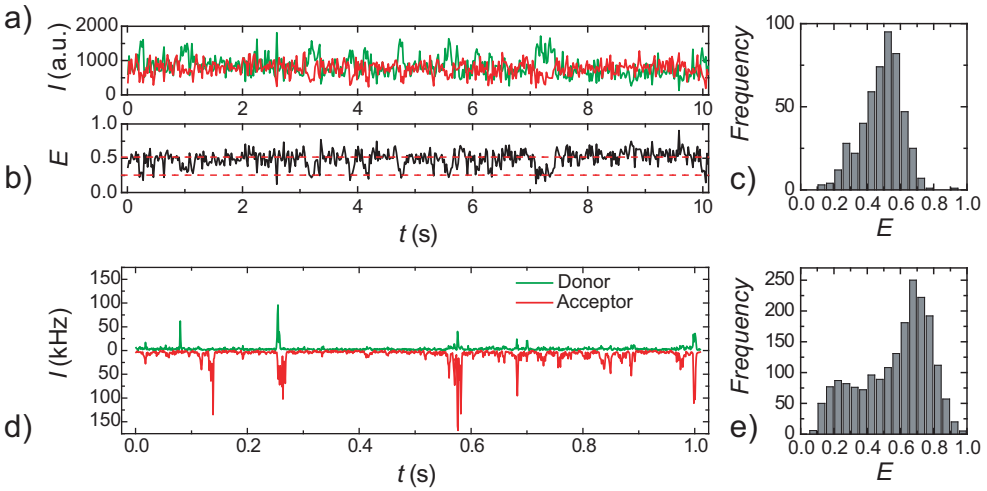

Figure 2.4: Single-pair FRET microscopy on nucleosomes. a) Fluorescence intensity time trace of donor and acceptor fluorescence of a single immobilized nucleosome. b) Resulting FRET efficiency E as a function of time. Fluctuations in E are caused by breathing dynamics of the nucleosome. c) FRET efficiency histogram of the time trace in b). d) Fluorescence intensity time trace of fluorescence originating from freely diffusing molecules. Bursts of fluorescence arise from the passage of a single molecule through the excitation volume. e) Histogram of FRET efficiencies calculated from bursts as in d). A high and a low FRET population can clearly be distinguished. These reflect different conformations of the nucleosome.

Single-pair FRET confocal microscopy

1. Alternatively, image freely diffusing molecules using confocal microscopy. Immobilization to a functionalized cover slide is not needed in this case, so the surface passivation and functionalization steps described in section 2.4.5 can be omitted. The observation time is limited to a few ms per molecule.

2. Mount a clean cover slide on the microscope, and place a droplet (~50 µl) of 10-100 pM labeled nucleosomes in a buffer containing 0.1 mg/ml BSA, and 10-100 nM unlabeled nucleosomes. Focus 25 µm above the glass surface, and record the fluorescence. Example data is shown in figure 2.4.d and e.

2.5 Notes

1. Unless stated otherwise, all solutions are prepared with 18.2 MΩ·cm MilliQ water. This standard is referred to as "water" in the text.

2. StarPEGs are not commercially available. With commercially available linear PEGs, we observed non-specific adsorption and dissociation of nucleosomes [9]. It has been reported that a repeated PEGylation step may increase the specificity and reduce non-specific adsorption of slides coated with linear PEGs however [17].

3. Care must be taken to avoid autofluorescent contamination of buffers, samples and microscope slides. Work at a clean lab bench, using clean glassware, and always wear gloves. Buffers for single molecule imaging are sterile filtrated with a 0.02 µm syringe filter (Whatman Anotop 25). This removes virtually all autofluorescent impurities.

4. We recommend to use Alternating Laser EXcitation (ALEX) [18] to virtually sort molecules based on their label stoichiometry, and to discriminate residual bleaching and blinking events from real conformational transitions.

5. Store the nucleosomes at the highest concentration possible (preferably >100 nM) to prevent dilution-driven dissociation. The use of low protein-binding tubes is recommended to minimize sticking to the tube.

6. If desired, 50 µl aliquots 50X glucose oxidase and catalase can be stored at -80 °C for several weeks. Although it is recommended not to freeze the catalase, we did not observe a major loss in oxygen scavenging activity from these aliquots.

7. Buffers for single molecule imaging are degassed prior to use, to remove the oxygen already in solution. When using oxygen scavenger the sample can be imaged for 30 min;

after that the pH will drop as a result of the production of gluconic acid as a byproduct of the oxygen scavenging reaction.

2.6 Conclusion

The methods presented here provide a simple and straightforward way to generate mononucleosomes with a FRET pair at various locations in the nucleosome. A careful design of the DNA template leads to exactly positioned nucleosomes, with fluorescent labels at the desired location. Salt dialysis reconstitution with stoichiometric amounts of histones directs the assembly of a homogeneous sample of nucleosomes, as deduced from PAGE and bulk fluorescence measurements. These well-characterized nucleosome constructs allow detailed single-molecule measurements of nucleosome structure and dynamics. Widefield TIRF microscopy on immobilized molecules allows the monitoring of individual nucleosomes for tens of seconds to minutes. Fluorescence microscopy on freely diffusing molecules in a femtoliter confocal detection volume allows a fast characterization of nucleosome conformational distributions free from possible interactions with the cover slide. The bottom-up approach for studying mononucleosome structure and dynamics presented here can pave the way for understanding the physical mechanisms underlying gene regulation.

The nucleosome is the fundamental building block of DNA organization *in vivo*. DNA programmability is of crucial importance for designing and constructing nucleosomes as a model system of this higher-order DNA structure: modified nucleotides allow the incorporation of fluorescent and biotin labels in the nucleosome, while the use of a nucleosome positioning sequence directs the formation of the nucleosome to a specific position on the DNA. The predictable structure of the resulting nucleosome could facilitate the architecture of new DNA-protein scaffolds and higher-order structures. Therefore, it could be exploited as a structural motif in DNA nanotechnology.

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