RESEARCH ARTICLE

Evaluation by Experimentation and Simulation of a FRET Pair Comprising Fluorescent Nucleobase Analogs in Nucleosomes

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Abstract: Förster resonance energy transfer (FRET) is an attractive tool for understanding biomolecular dynamics. FRET-based analysis of nucleosomes has the potential to fill the knowledge gaps between static structures and dynamic cellular behaviors. Compared with typical FRET pairs using bulky fluorophores introduced by flexible linkers, fluorescent nucleoside-based FRET pair has great potential since it can be fitted within the helical structures of nucleic acids. Herein we report on the construction of nucleosomes containing a nucleobase FRET pair and the investigation of experimental and theoretical FRET efficiencies through steady-state fluorescence spectroscopy and calculation based on molecular dynamics simulations, respectively. Distinguishable experimental FRET efficiencies were observed depending on the positions of FRET pairs in nucleosomal DNA. The tendency could be supported by theoretical study. This work suggests the possibility of our approach to analyze structural changes of nucleosomes by epigenetic modifications or internucleosomal interactions.

Introduction

Förster resonance energy transfer (FRET) is one of the attractive tools for understanding the dynamics of biomolecules.[1,2] FRET is a distance- and orientation-dependent energy migration from a donor to an acceptor.[3–5] Distance dependency has been mainly used for analysis of biomolecules regarding orientation factors as an average value of 2/3 due to free rotation of fluorophores. FRET analysis is not suitable for comprehensive analysis of target molecules because it only produces information on the arrangement of two molecules. Although the information is limited compared with X-ray crystallography, it enables real-time observation in solution even at single-molecule resolution. We envisage that integrations of multiple methods for structural analysis such as X-ray crystallography, cryo-electron microscopy, NMR spectroscopy, FRET, and simulations will facilitate our understanding of biology at an atomic scale.[6]

A nucleosome is the most basic component of chromatin.[7] It is formed by wrapping a histone octamer with approximately 145 bp of DNA strand.[8,9] The nucleosome itself and its assemblies are known to play important roles in the regulation of gene expression[10,11] and formation of chromatin structures.[12] Research on dynamic behaviors of nucleosomes is indispensable to understanding the mechanisms of biological processes related to genomic DNA. To date, various structures of mono- and multi-nucleosomes have been resolved by X-ray crystallography[9,13–16] and cryogenic electron microscopy.[17–19] In research on cellular nucleosomes, live-cell fluorescence imaging has revealed the dynamics and localization of chromatin[20–22] and related proteins. Next-generation sequencing has facilitated our understanding of nucleosome positioning and translocation of nucleosomal DNA.[10] To fill the knowledge gaps between static structures and cellular behavior, real-time observation of nucleosomes in solution by FRET should be useful.[23] In this context, FRET-based assays have revealed reversible conformational fluctuations,[24] disassemblies in high salt concentration,[25,26] and sliding caused by various kinds of chromatin remodeling enzymes.[27–29]

Recently, FRET pairs comprising fluorescent nucleobases have been investigated.[30–33] Compared with nucleotides tethering fluorophores, fluorescent nucleobases[34] have compact structures because they were developed from structures of native nucleobases. They are located within the double-helix structure of DNA and thus minimize alterations to molecules of interest. Considering that interactions between nucleic acids and fluorophores affect higher-order structures,[35] small modifications are favored for observing the dynamics of nucleic acids and protein-nucleic acid complexes. Our group has reported the incorporation of a FRET pair into a nucleosome and observation of the FRET.[36] For two-position combinations of a donor and an acceptor, experimental FRET efficiencies were calculated, and the orientation factors, κ^2, were estimated using distance values obtained by an energy-minimization model. We demonstrated the applicability of a FRET pair comprising fluorescent nucleobase analogs in a nucleosome. However, the FRET pair showed overlapping fluorescence spectra of the donor and acceptor, which made the analysis of experimental results complex and required deconvolution of the spectra. To solve this problem

chemically, we developed a fluorescent thymine analog, ^{diox}T, which has a shorter fluorescent wavelength.[37] Using it as a donor, we created a new FRET pair and evaluated it in canonically right-handed DNA.[38] A comparison of experimental and theoretical values showed better consistency than the previous work, suggesting the utility of this pair in structural analysis.

Herein, we report on the evaluation of our new FRET pair in nucleosomes by experimentation and simulation (**Figure 1a**). After the preparation of nucleosomal DNA containing the FRET-pairable fluorescent nucleosides, the modified nucleosomes were reconstituted. Fluorescence spectra were then recorded to calculate experimental FRET efficiencies. We compared the FRET efficiencies of modified nucleosomes with three acceptor positions. To verify the data, we calculated theoretical FRET efficiencies based on all-atom molecular dynamics (MD) simulations over 10 ns intervals. Experimental and theoretical values showed a good correlation, even though experimental values generally showed lower values than theoretical values. It suggests that our system has the potential to analyze structural changes of nucleosomes caused by epigenetic modifications or inter-nucleosomal interactions.

Results and Discussion

Preparation of nucleosome containing a nucleobase–analog FRET pair

We prepared a nucleosome containing the ^{diox}T–tC FRET pair (**Figure 1b, c**). One of the most representative nucleosome-forming sequences, the Widom 601 sequence[39] with 145 bp, was selected as the nucleosomal DNA. Initially, we performed enzymatic reactions described in previous studies,[36,40] such as ligation and PCR to obtain modified long DNA strands. However, it was difficult to obtain a sufficient quantity of pure DNA for fluorescence spectroscopy. The yield from ligation was low and PCR generated an impurity that was difficult to remove (data not shown). We thus decided to chemically synthesize the 145-mer oligonucleotide by solid-phase phosphoramidite chemistry. (Details of synthesis are described in the Experimental Section.) The phosphoramidite of ^{diox}T was synthesized as previously reported[37] whereas the phosphoramidite of tC[41] was purchased. In addition to avoiding the problems involved in the enzymatic synthesis, we were able to incorporate the fluorescent nucleobases into desirable arbitrary positions because PCR restricts positioning to relatively terminal positions in the products. Based on our previous research,[37,38] the donor was incorporated into positions whose adjacent bases are adenine or thymine for the higher quantum yield of the donor to maximize the Förster radius. In this work, we incorporated the acceptor into three positions to compare FRET efficiencies depending on distance and orientation factors (**Figure 2a**, **Table S1**).

We conducted nucleosome reconstitution (**Figure 2b**) by a conventional salt dialysis method.[42] We purchased histone octamer (Histone Octamer, Recombinant Human) produced by EpiCypher, NC, USA.[43] The formation of the nucleosome was confirmed by native polyacrylamide gel electrophoresis (**Figure 2c, d**). The ratio between DNA and histone octamer was optimized; it required adding 1.2 eq. of histone octamer in our experiments (**Figure S3**). Nucleosomes containing only the donor or acceptor and both the donor and acceptor were prepared for three different positions of the acceptor. We evaluated FRET efficiency in two independent ways: quenching of the donor and enhancement of fluorescence of the acceptor.

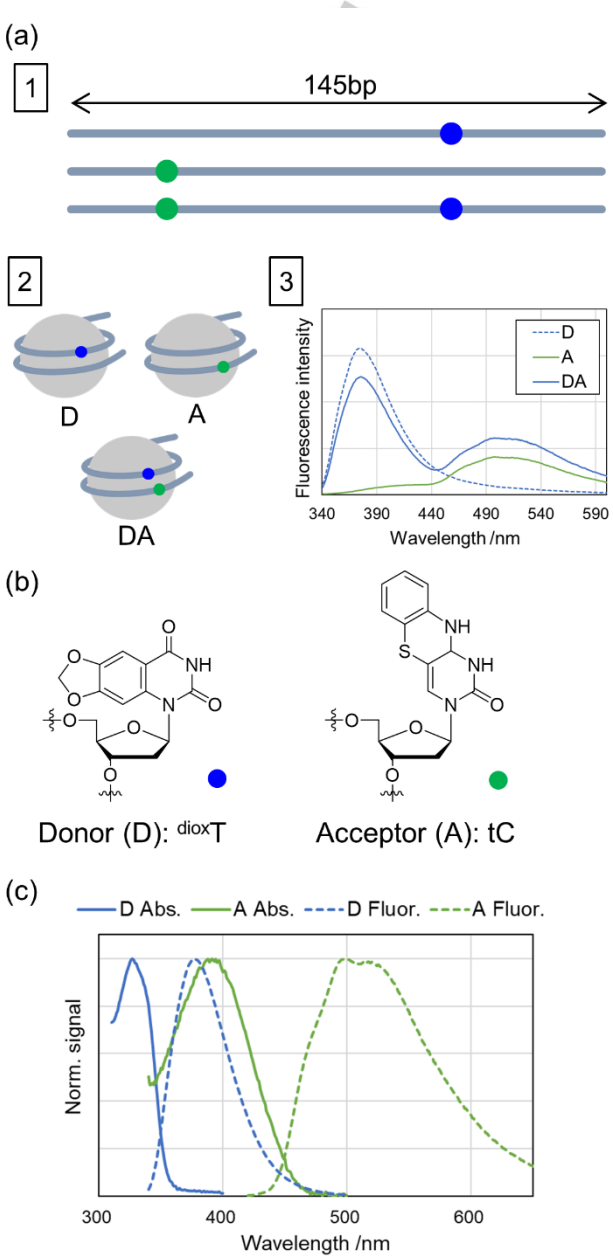

Figure 1 Design of the work. **(a)** Scheme of this research. Chemical synthesis of nucleosomal DNA, nucleosome reconstitution, and fluorescence spectroscopy were used to measure FRET efficiency. **(b)** Chemical structures of the donor (^{diox}T) and acceptor (tC) used in this study. **(c)** Normalized absorption and fluorescence spectra of the donor and acceptor in double-stranded DNA. Each oligonucleotide was prepared in our previous work.

Fluorescence spectrometry to measure FRET efficiency in nucleosomes

Fluorescence spectra were taken for the nucleosomes whose positions of the acceptor differed. We observed quenching of the donor around 380 nm (**Figure 3a**) and enhanced fluorescence of the acceptor around 530 nm (**Figure 3b**) by FRET. The signals from 340 to presumably 500 nm observed in the spectra of nucleosomes containing only the acceptor are scattering light. The spectra visually suggested the highest FRET efficiency of

nucleosome 2 since it showed the greatest changes in fluorescence intensity. There is a concern about the effect of concentrations of the nucleosomes on the changes in fluorescence intensity. It was excluded by the constant fluorescence intensity of the acceptor excited at absorption maximum (380 nm) with or without the donor (**Figure S4**). FRET efficiency (E) was calculated based on the fluorescence of the donor (**Equation 1**) or the fluorescence of the acceptor (**Equation 2**). In **Equation 1**, $I_{D,380}$ and $I_{DA,380}$ are the fluorescence intensity of the donor at 380 nm in the nucleosomes containing only the donor or both the donor and acceptor, respectively. In **Equation 2**, $A_{A,325}$ and $A_{D,325}$ are the absorptions of the acceptor and the donor at 325 nm, respectively. $I_{DA,530}$ is the fluorescence intensity of the acceptor at 530 nm in the nucleosomes containing only the donor or both the donor and the acceptor, respectively.

$$E_1 = 1 - I_{DA,380}/I_{D,380} \quad (1)$$

$$E_2 = [A_{A,325}/A_{D,325}][(I_{DA,530}/I_{A,530}) - 1] \quad (2)$$

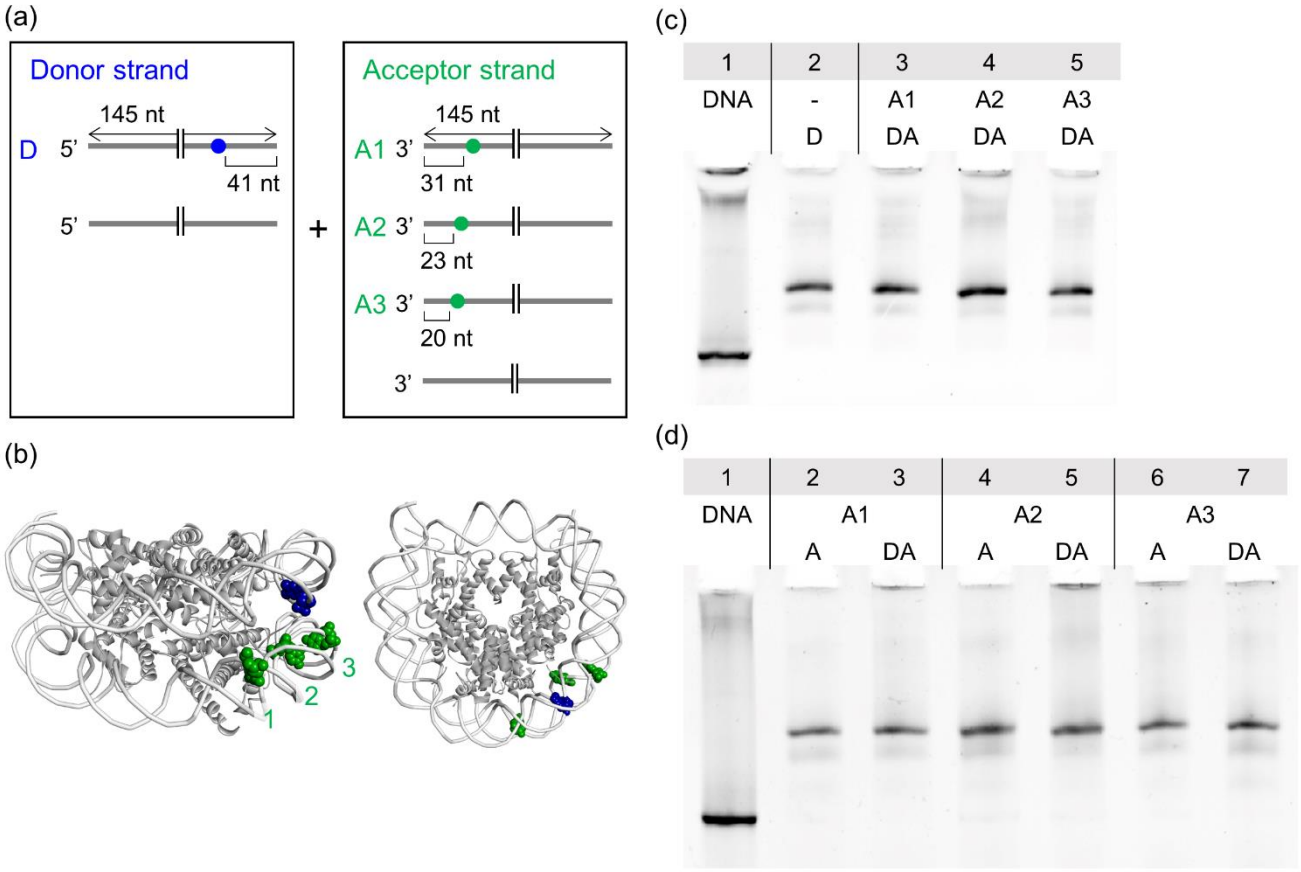

Figure 2. Preparation of nucleosomes containing the FRET pair. **(a)** Schematic illustration of the DNA strands used in this study. The blue and green dots represent the donor and the acceptor, respectively. **(b)** Illustration to show the positions of the FRET pair. The thymidine and cytidine which were substituted with the donor and the acceptor are labeled with blue and green, respectively. A reported crystal structure of nucleosome core particle was used (3LZ0). Left: side view. Right: top view. Confirmation of nucleosome reconstitution by 6% native PAGE. **(c)** From left to right: (1) 145 bp free DNA; (2) nucleosome with D; (3) nucleosome with D and A1; (4) nucleosome with D and A2; (5) nucleosome with D and A3. **(d)** From left to right: (1) 145 bp free DNA; (2) nucleosome with A1, (3) nucleosome with D and A1; (4) nucleosome with A2, (5) nucleosome with D and A2; (6) nucleosome with A3, (7) nucleosome with D and A3.

Duplicate spectra were recorded, and the average values were calculated (**Figure 4**). They showed different FRET efficiencies depending on the positions of the acceptor, although the distances between the donor and acceptor were similar based on the reported crystal structures (3LZ0).[9] To investigate these efficiencies in more detail, we conducted all-atom MD simulations to evaluate our experimental outcomes.

MD simulation for theoretical FRET efficiencies

Theoretical values based on MD simulations should be able to provide more promising data than those based on single structures because MD simulation can take thermal vibrations and structural deviations into account. In our previous paper[38], only one idealized model of DNA duplex was used for the calculation of theoretical FRET efficiencies, but MD simulation can treat nonidealities in structures of nucleosomes as research on FRET in DNA brick-based nanostructures.[44] Several groups have reported that MD simulations can work for the prediction of FRET efficiencies.[45] Thus, we performed all-atom MD simulations including explicit solvent molecules over 10 ns intervals. Our FRET pair clearly showed orientation dependency in the canonical duplex structure.[38] Therefore, an estimation of an orientation factor based on simulation independent of experimentation is valuable to help understand the experimental results.

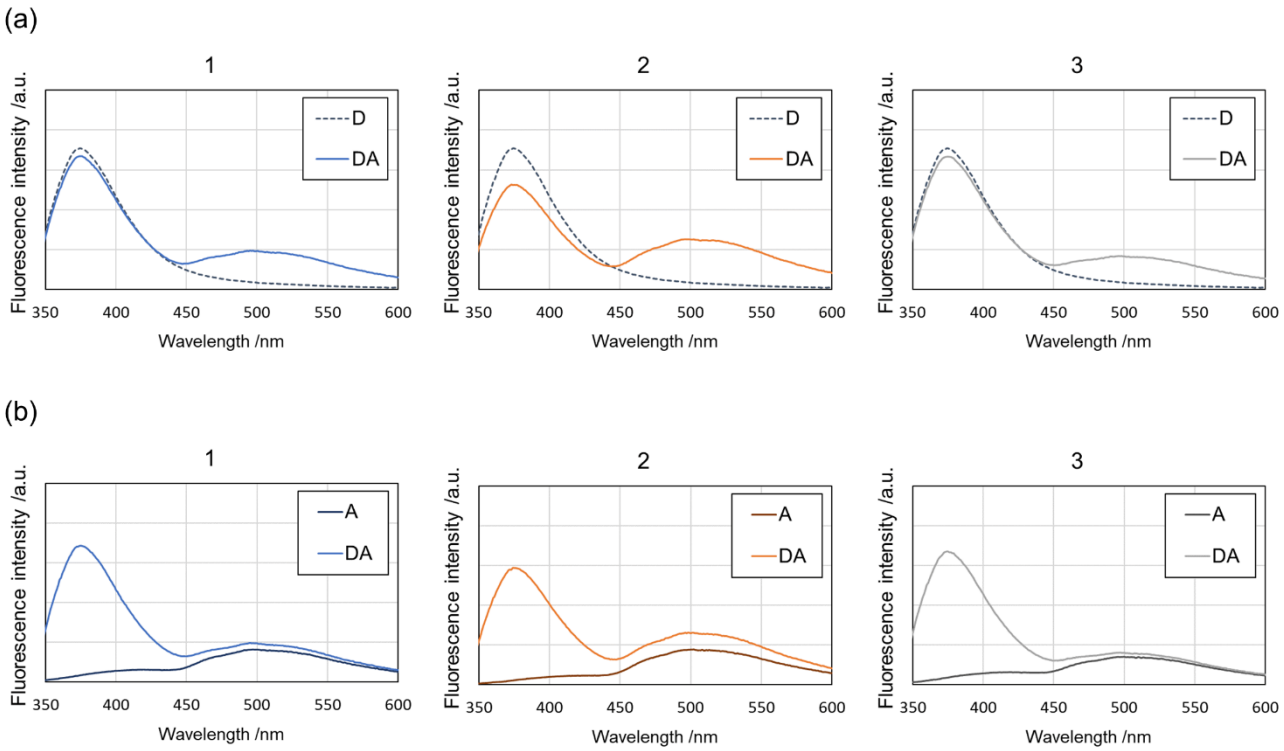

Figure 3. Steady-state fluorescence spectrometry of nucleosomes to evaluate FRET efficiencies for three different positions of the acceptor. **(a)** Fluorescence spectra of nucleosomes containing only the donor (dotted) and both the donor and acceptor (solid) to observe quenching of the donor by FRET. λex = 325 nm (absorption maximum of the donor). **(b)** Fluorescence spectra of nucleosomes containing only the acceptor (dark) or both the donor and acceptor (bright) to observe enhanced fluorescence intensity of the acceptor by FRET. λex = 380 nm (absorption maximum of the acceptor). Blue, orange, and gray spectra are those of nucleosomes whose acceptor strands are A1, A2, and A3, respectively. All spectra were corrected using Rhodamine B and a halogen lamp as standards.

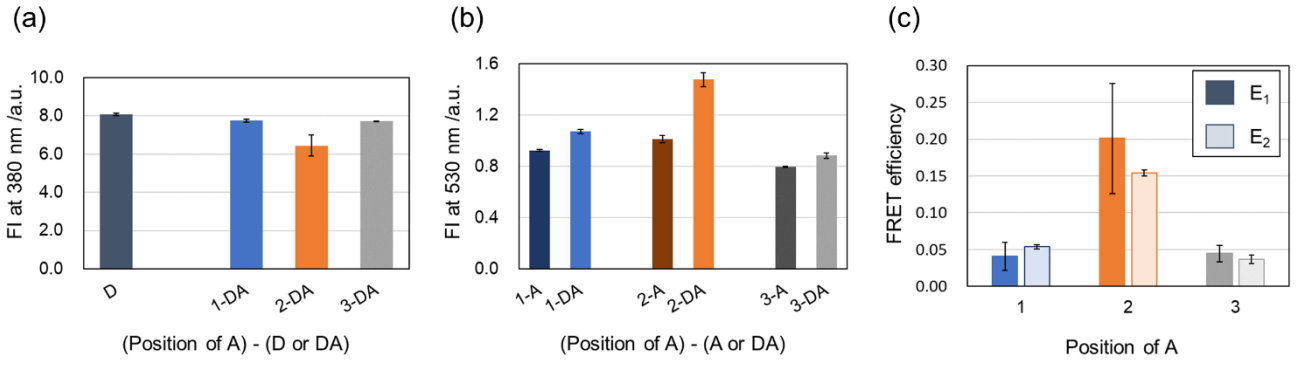

Figure 4. Analysis of steady-state fluorescence spectra to calculate FRET efficiency. Fluorescence intensity (FI) of **(a)** the donor at 325 nm and **(b)** the acceptor at 380 nm in the steady-state fluorescence spectra. **(c)** Calculated FRET efficiencies based on quenching of the donor (E_1) and enhanced fluorescence of the acceptor (E_2). In all figures, each value reflects the average of duplicate measurements for the experimental values, and the bars represent standard deviations.

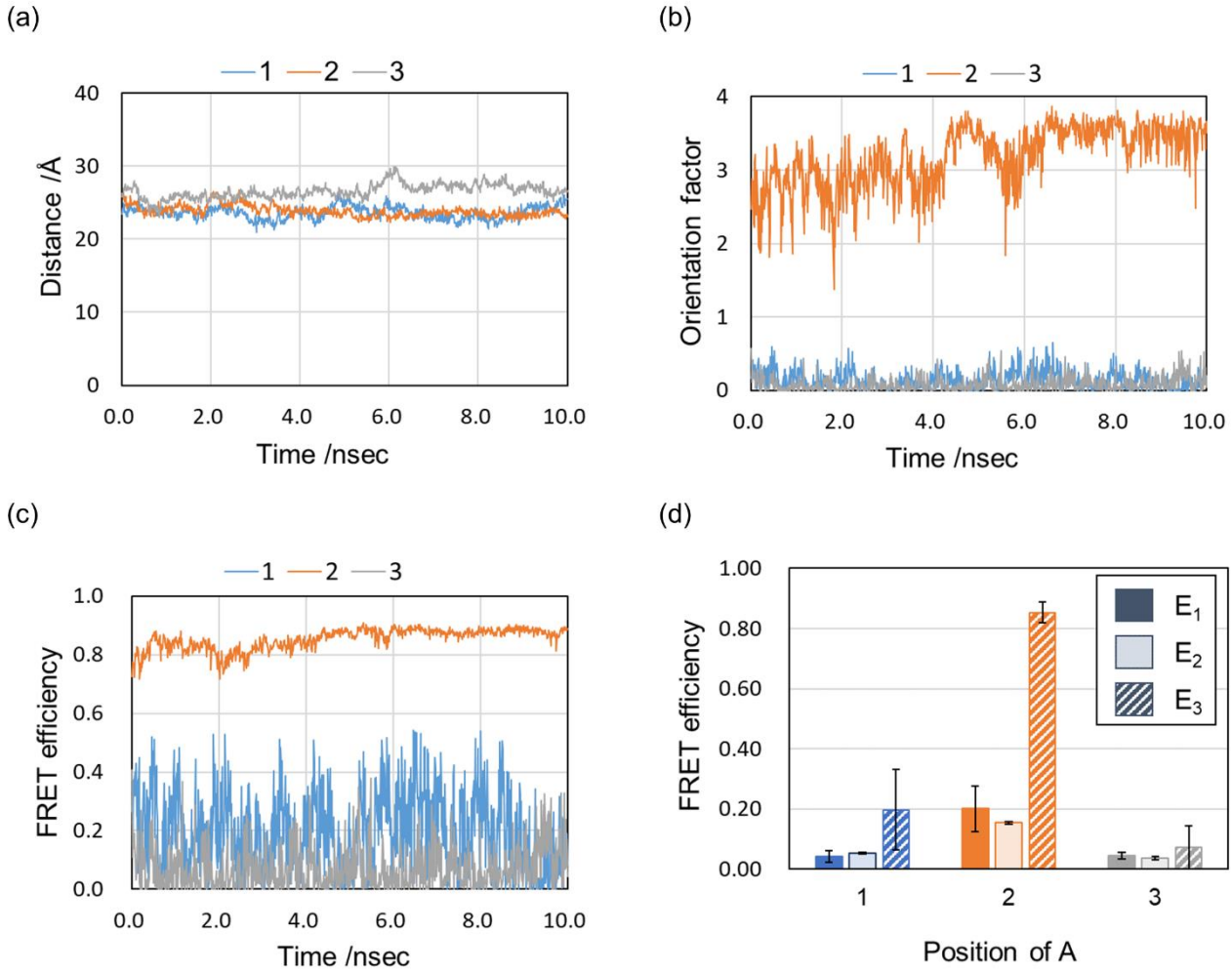

Figure 5 MD simulation to calculate theoretical FRET efficiency. **(a)** Distance between donor and acceptor. **(b)** Orientation factor. **(c)** Theoretical FRET efficiency. Blue, orange, and gray trajectories represent values of nucleosomes whose positions of acceptor are 1, 2, and 3, respectively. **(d)** Comparison of experimental values and theoretical values. Theoretical values are average values through 10 ns. Error bars of experimental and theoretical values show standard deviations for duplicate measurements and the simulations for 10 ns, respectively.

In our simulations, force field parameters of native amino acids and nucleotides were assigned by parm10 supplemented by ff14sb and bsc1 force fields. Fluorescent nucleosides were parameterized by extended Hückel theory. Other conditions are described in Materials and Methods. We analyzed the trajectory data and calculated the distances between the donor and acceptor, the orientation factors, and the theoretical FRET efficiencies (**Figure 5a-c**). Duplicate simulations were conducted to confirm reproducibility. As expected from the crystal structures, the distances between the donor and acceptor were similar within the range from 20 to 30 Å. In contrast, orientation factors showed significant differences; nucleosome 2 showed much higher values compared with nucleosome 1 and 3. This difference caused markedly different theoretical FRET efficiencies (**Figure 5d**). For further analysis of the orientations, the cosine values determining the factors were plotted (**Figure S5**). The graph suggested that the favorable orientation of the nucleosome 2 is mainly attributed to θ_A, an angle between transition dipole moments of the acceptor and a line between the donor and acceptor.

In comparing experimental and theoretical FRET efficiencies, we found a consistent tendency as listed in order of the values 2 > 1 > 3, however, the values of experimental results were generally much lower than that of theoretical results. The similarity supports our expectation that MD simulation can be useful to evaluate experimental FRET efficiencies of orientation-dependent FRET pairs. Orientation factors of such FRET pairs are fixed within double-stranded DNA by hydrogen bonding and stacking interactions, but thermal vibration could have significant effects on FRET efficiency especially when orientation factors are close to zero.

Nonetheless, we cannot dismiss inconsistencies in experimental and theoretical values. We confirmed that nucleosome reconstitution did not cause quenching of the donor (**Figure S6**). In the calculation of the theoretical FRET efficiencies, the refractive index (n) is regarded as 1.4 for biomolecules. In microenvironments, refractive indices can be different from 1.4, but measurements of local refractive indices are practically impossible. We would like to suggest the possibility of subpopulations that have a potential to reduce FRET efficiency (**Figure 6a**). Single-molecule FRET measurement in which both donor and acceptor were incorporated into the entry-exit site of nucleosomal DNA suggested the presence of a considerable amount (90%) of dissociated states and their results were distinct from blinking.[46] In this context, we conducted MNase digestion to investigate subpopulation. MNase, micrococcal nuclease has exo- and endo-nuclease activities and preferentially digests free

DNA compared with DNA bound to protein. The preference has been used to digest linker DNA in relation to nucleosome positioning. DNA strands recovered from nucleosomes with or without MNase treatment showed significantly different band patterns (**Figure 6b**). After MNase treatment, the bands were mainly observed at approximately 100 bp indicating cleavage of approximately 20 bp in the entry-exit sites. The positions of the acceptor did not show significant effects on digestion (Data not shown). The digestion result would support our supposition explaining low experimental FRET efficiencies. A follow-up study on the formation of chromatosome by binding of histone H1 to nucleosomes might provide a clue regarding this issue.[47] Further study is required for valid evaluation of experiments by simulations, but our results will be a pilot to use nucleoside-based FRET pairs in the structural analysis of epigenetic modifications to nucleosomes or of inter-nucleosomal interactions.

Conclusion

In this work, we established a system in which a nucleoside-based FRET pair is incorporated into a mono-nucleosome. The modified nucleosomal DNA strands were chemically synthesized and nucleosomes were reconstituted in vitro. Chemical synthesis of modified nucleosomes enables the incorporation of the FRET pair into arbitrary positions. Steady-state fluorescence spectrometry of the nucleosomes showed different FRET efficiencies depending on the positions of the donor and acceptor. Based on MD simulations, the theoretical FRET efficiencies showed good correlations between FRET efficiency and positions of the FRET pair to the experiment demonstrating the contributions of orientation factors. Although it requires further study to improve relatively lower experimental value than theoretical values, our results suggest the possibility of rational design of FRET-based approach based on experimental analysis and MD simulation. Because our FRET pair was located within the helical structure of nucleic acids, DNA in this case, it will be exploited without unwanted interactions of fluorophores compared with conventional FRET-based assays. With this technique, we envisage that it will be possible to analyze structural change of nucleosomes caused by epigenetic modifications or inter-nucleosomal interactions. In future, single-molecule FRET analysis[48] of nucleosomes using a FRET pair comprising of a fluorescent nucleobase that has red-shifted spectra is expected.

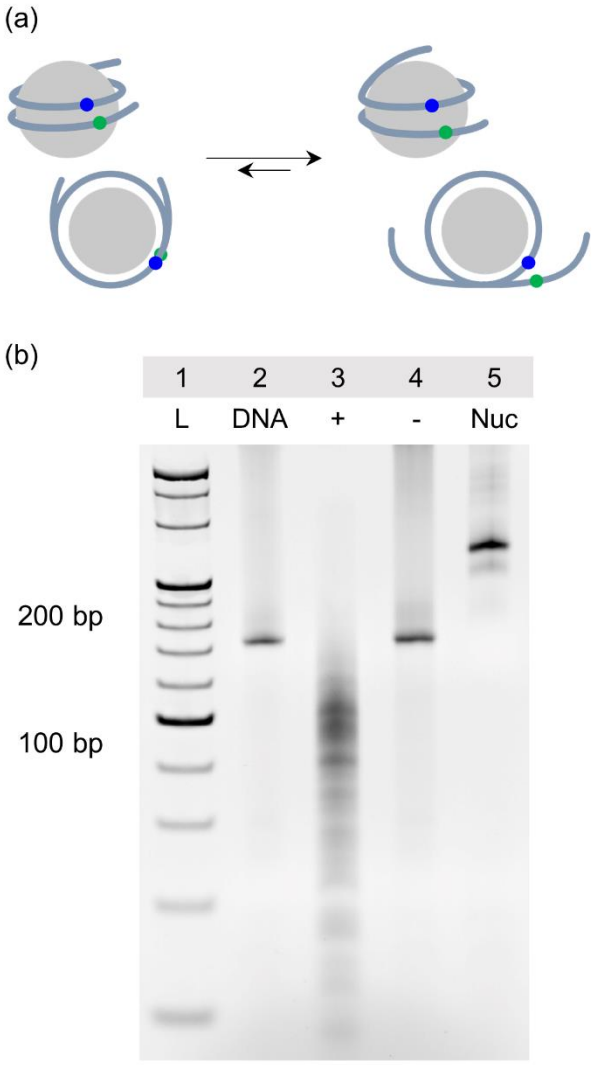

Figure 6. Partially disassembled nucleosomes that could cause lower experimental FRET efficiencies than theoretical values. **(a)** Illustration of unwrapping of DNA in entry-exit sites. This dissociation can increase physical distances between donor and acceptor and decrease FRET efficiency. **(b)** MNase digestion to evaluate the flexibility of terminal DNA in nucleosome core particles. 6% native PAGE. From left to right: (1) 20bp ladder; (2) 145 bp free DNA; (3) DNA recovered from nucleosome 2 with donor and acceptor treated with MNase; (4) DNA recovered from nucleosome 2 with donor and acceptor without MNase treatment; (5) nucleosome 2 with donor and acceptor without any treatment.

Experimental Section

Materials

The donor, ^{dioxo}T[37], and its phosphoramidite were synthesized as reported and a phosphoramidite of tC[49] was purchased from Glen Research. Oligonucleotides which consist of only native nucleotides were purchased from Sigma-Aldrich. Histone octamers for nucleosome reconstitution were purchased from EpiCypher (Histone octamers, Recombinant Human) and used without further purification. A dialysis cup for a small amount (MWCO 8000, Bio-Tech) was used for dialysis. MNase was purchased from New England Biolabs and proteinase K was purchased from Invitrogen. All the other chemicals and solvents were purchased from Aldrich, Wako, and applied biosystems. All of those were used without further purification. Water was deionized (specific resistance of > 18.0 MΩ cm^{-1} at 25 °C) by a Milli-Q system (Millipore Corp.).

Preparation of modified long oligonucleotides.

The modified 145nt oligonucleotides were synthesized by automated solid-phase synthesizer (M-2MX, NIHON TECHNO SERVICE CO., LTD) in 1 µmol scale. As solid support, CPG with larger pore size (3000 Å) than the general one (1000 Å) was used. DNA synthesis was performed with the support of NIHON TECHNO SERVICE CO., LTD. After partially synthesizing designed sequences, including the incorporation of fluorescent nucleoside in our laboratory, the activable resin was delivered to the company and the remaining sequences were synthesized. After cleavage and purification with general protocols, the amount of oligonucleotide was used for nucleosome reconstitution. The overall yield of the donor strand and the acceptor strands were approximately estimated by NanoDrop as 2% (420 µg) and 4% (950 µg), respectively.

Nucleosome reconstitution

Nucleosomes were reconstituted following the reported method.[42] Samples contained 2.5 µM of double-stranded nucleosomal DNA, 3.0 µM of histone octamer, 2 M of NaCl, and 20 mM of HEPES-KOH buffer (pH 7.5). For simplification of the procedure, we conducted reconstitution in two dialysis steps: 1st dialysis against a buffer containing 20 mM HEPES buffer (pH 7.5) and 2 M NaCl and 2nd dialysis against a buffer containing 20 mM HEPES buffer (pH 7.5) without NaCl. Other conditions are similar to the report. The formation of nucleosomes was confirmed by 6% native polyacrylamide gel electrophoresis (100 V, 60 min, 4 °C, TBE running buffer). As a sample buffer, Novex™ Hi-Density TBE Sample Buffer (5X) (Thermo Fisher Scientific) was used. The gel was visualized by ethidium bromide. The gel images were taken by GelDoc Go Gel Imaging System (Bio-Rad Laboratories, Inc.).

Fluorescence spectrometry

Fluorescence measurements were conducted using fluorescence cells with a 0.5 cm path length on an FP-8300 Spectrofluorometer (JASCO Corp.). The emission spectra were recorded from 220 to 600 nm with an excitation wavelength of 325 nm. The spectra were corrected by using Rhodamine B and a halogen lamp as standards. The correction solved anomalous diffraction by gratings observed around 510 nm in the raw spectra.

Molecular dynamics simulation

Molecular Operating Environment (MOE)[50] was used for the construction of initial structures of the modified nucleosomes and preparation of input files for simulations. MD simulations were done by NAMD 2.14[51] using parm 10 force field supplemented by ff14sb and bsc1 force field. Fluorescent nucleosides were parameterized by Extended Hückel theory. The nucleosomes were solvated with water molecules with 10 Å of margin in a box shape. 20 mM of potassium chloride was added to the system. The simulation was done for 10 ns at 300 K. All simulations were replicated twice to confirm the reproducibility of tendency in the trajectories. The trajectory data was analyzed by MOE.

Calculation of theoretical FRET efficiencies

Basic equations for FRET efficiency were described in our previous report[38] on the basis of established theories[5,52]. We calculated the orientation factor by Equation 2 since the cylinder model of canonical DNA cannot be used for nucleosomes. In the equation, e_1 and e_2 are unit vectors of the transition dipole moments of the donor and acceptor, respectively, and e_{12} is the unit vector from donor to acceptor.

$$\kappa = e_1 \cdot e_2 - 3(e_1 \cdot e_{12})(e_{12} \cdot e_2) \quad (3)$$

Vector components of the e_1, e_2, and e_{12} were obtained as follows. The transition dipole moments of the donor and the acceptor have been calculated by time-dependent density functional theory (TDDFT).[38] The vector components of the transition dipole moments were approximately expressed by a linear combination of N1C2 and N1C5 vectors. Finally, the vector components in the geometry of nucleosomes were obtained by the coordinate of N1, C2, and C5 atoms in MD trajectories. Distances between the donor and the acceptor and e_{12} were calculated by the center of the mass of the fluorescent nucleobases. The center of mass was obtained by an in-house-made SVL program. The cosine values were calculated by definition of inner product and vector components.

MNase digestion

We followed a reported method.[53] After MNase treatment of nucleosomes for 30 min at 37 °C, the reaction was stopped by the addition of EGTA to 10 mM. The reaction mixture was treated with Proteinase K for 2 h at 65 °C. DNA was purified by phenol/chloroform extraction and ethanol precipitation and analyzed by 6% native PAGE.

Supporting Information.

A complete list of DNA sequences used in this study (Table S1), Native PAGE to examine the equivalence of histone octamer to nucleosomal DNA (Figure S1), fluorescence spectra excited at the absorption maximum of the acceptor (380 nm) (Figure S2), cosine values determining orientation factor (Figure S3), comparison of the fluorescence spectra of the donor in 145 bp DNA or nucleosome (Figure S4).

Acknowledgments

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Keywords: Fluorescent nucleic acid • DNA • FRET • Nucleosome

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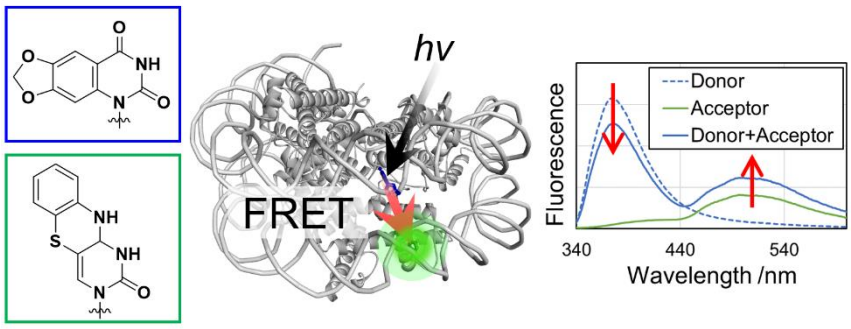

Minimal modification and orientation dependency: Nucleobase FRET pair was incorporated into nucleosome and its ability was examined by experimentation and simulation. Experimental and theoretical FRET efficiencies showed similar trends suggesting orientation dependency of the FRET pair.