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Ensemble and single-molecule fluorescence-based assays to monitor DNA binding, translocation, and unwinding by iron-sulfur cluster containing helicases

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Abstract

Many quantitative approaches for analysis of helicase-nucleic acid interactions require a robust and specific signal, which reports on the presence of the helicase and its position on a nucleic acid lattice. Since 2006, iron-sulfur (FeS) clusters have been found in a number of helicases. They serve as endogenous quenchers of Cy3 and Cy5 fluorescence which can be exploited to characterize FeS containing helicases both in ensemble-based assays and at the single-molecule level. Synthetic oligonucleotides site-specifically labeled with either Cy3 or Cy5 can be used to create a variety of DNA substrates that can be used to characterize DNA binding, as well as helicase translocation and unwinding. Equilibrium binding affinities for ssDNA, duplex and branched DNA substrates can be determined using bulk assays. Identification of preferred cognate substrates, and the orientation and position of the helicase when bound to DNA can also be determined by taking advantage of the intrinsic quencher in the helicase. At the single-molecule level, real-time observation of the helicase translocating along DNA either towards the dye or away from the dye can be used to determine the rate of translocation by the helicase on ssDNA and its orientation when bound to DNA. The use of duplex substrates can reveal the rate of unwinding and processivity of the helicase. Finally, the FeS cluster can be used to visualize protein-protein interactions, and to examine the interplay between helicases and other DNA binding proteins on the same DNA substrate.

Keywords

fluorescence; Förster resonance energy transfer (FRET); helicase; iron-sulfur (FeS) cluster; Rad3-family; RPA; single-molecule microscopy; TIRM; translocation; unwinding; XPD

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1. Introduction

The presence of iron-sulfur (FeS) clusters in the Rad3 family helicases [1] led to development of a number of fluorescence based assays to characterize the enzymes' ability to bind to and recognize DNA substrates [2], and observe translocation on a DNA lattice [3]. FeS clusters in these enzymes serve as endogenous quenchers of a wide spectral range of fluorophores which can be used as reporters for monitoring helicase-DNA interactions. This effect has been used to develop new assays for ensemble and single-molecule characterization of Rad3 helicases [2–4]. Moreover, combining FeS cluster-mediated fluorescence quenching with Förster resonance energy transfer (FRET) [5,6] between a pair of fluorophores allows for expansion of these analyses to pseudo three color measurements to monitor complex nucleoprotein interactions. Here we describe the fundamentals of experimental design and data analysis for ensemble and single-molecule assays exploiting the presence of FeS clusters in DNA helicases.

1.1 Rad3 helicases and other FeS-containing translocases

Most of FeS cluster containing helicases identified to date belong to the Rad3 family which comprises a distinct subset of helicase superfamily II [7]. All characterized Rad3 enzymes are *bona fide* DNA helicases that translocate along ssDNA in the 5' to 3' direction [1,8–12]. These enzymes are purported to contain a family-specific domain whose structure is stabilized by an FeS cluster [1,13–15] (Fig. 1a). This auxiliary domain is inserted in the motor core of the helicase between the highly conserved helicase motifs Ia and II of helicase domain I (Fig. 1b). Members of the Rad3 family are found in all domains of life. XPD, Bach1 (FancJ), ChlR1, and Rtel1 have been identified in humans, and have orthologs in other eukaryotes (Fig. 1b). Homologues of XPD helicase can also be found in archaea and bacteria (Fig. 1b). The functional role of the FeS cluster in these enzymes remains unclear. It certainly plays a structural role by stabilizing an auxiliary domain located among the otherwise conserved motor core of the helicase, contains a secondary DNA binding site [2], and is important for helicase activity of several Rad3 family helicases [1,2,16]. A recent study of the *E. coli* DinG helicase demonstrated that this helicase functions under highly oxidative conditions in the presence of H₂O₂. However, upon oxidation of the FeS cluster with nitric oxide, the helicase ceased to function [17]. This finding suggested a regulatory role for the FeS cluster.

1.2 FeS Clusters

FeS clusters are ancient prosthetic groups that are ubiquitously involved in electron transport and complex catalysis that takes advantage of the cationic feature of these clusters [18]. While transporting electrons is unlikely to be useful for a helicase, the overall positive charge of the FeS cluster may facilitate interactions with DNA. Since the cationic nature of FeS clusters can be influenced by solvent exposure and the electrostatic environment, this may provide a means of regulating DNA binding and/or helicase activity under changing cellular conditions. The mechanism by which fluorescence is quenched by FeS clusters has not yet been reported. It is not, however, unprecedented. Quenching of green fluorescence protein (GFP) by the iron atom in hemin was previously observed and attributed to long-range dipole-dipole coupling characteristic to FRET [19]. FRET between small fluorescent dyes and transition metals, such as nickel, has been recently used to visualize changes in protein backbone conformations [20]. Similar induced dipole-dipole coupling may also explain the mechanism by which the FeS cluster in Rad3 helicases quenches fluorescence dyes such as Cy3 and Cy5.

In addition to the Rad3 family helicases, FeS clusters have been recently identified in several other DNA helicases and other DNA-processing enzymes. FeS clusters have been

found in the AddB subunit of bacterial AddAB helicase-nuclease, the DNA replication/repair factor, Dna2 [21], the D subunit of archaeal RNA polymerase [22], and the p58 subunit of human DNA primase [23] [24]. Thus, the approaches described here can be applied beyond analysis of the Rad3 family helicases.

1.3 Helicase characterization assays

The binding affinity of helicases for DNA substrates has traditionally been evaluated using electrophoretic mobility shift assays (EMSA or gel-shift assays). In these assays, DNA substrates are radiolabeled with ^{32}P . Electrophoresis through acrylamide or agarose gels is then used to separate the slower migrating helicase bound DNA from the faster migrating free DNA. Although robust, these assays are not true equilibrium measurements, and can therefore result in significant overestimation of K_d values due to loss of the DNA-helicase bound complex during migration through the gel. Additional disadvantages of these assays typically include being time consuming, and the need for clean up and safety precautions due to the use of radiolabeled DNA substrates. Taking advantage of the endogenous quencher in Rad3 helicases allows monitoring DNA-helicase interactions in solution under true equilibrium conditions. Optimal binding conditions can easily be determined for a broad range of DNA concentrations (starting as low as subnanomolar concentrations of fluorescently-labeled DNA) and helicase concentrations and be varied within the experiment. By strategically positioning the Cy3 or Cy5 fluorophore at various locations on ssDNA or dsDNA substrates, binding positions and modes of the helicase on different DNA substrates can be distinguished [2]. Moreover, replacing the radioactive label with a fluorophore eliminates the time consuming visualization step, as well as clean up and increased health risks associated with handling of radioactive materials. The two approaches, however, are complimentary since it is challenging to discriminate between the number of different protein-DNA species that are simultaneously present in the reaction mixture using the fluorescence-based assay alone. In contrast, many of the distinct complexes can be observed using gel-shift assays, which yield shifted and supershifted products.

Gel-based assays using radiolabeled substrates are also used to analyze helicase translocation and unwinding activities [25–28]. A limitation of the gel-based techniques to monitor DNA unwinding and translocation is that these are “all-or-nothing” measurements in which only the completed product can be observed following unwinding of the substrate. The substrates whose unwinding was not completed before helicase dissociation readily re-anneal back to form the intact product. This “all-or-nothing” approach is advantageous for determining the step size of the helicase [29–33]. These traditional approaches can also be replaced with fluorescence based alternatives [34,35]. These assays, however, are applicable only for monitoring helicase translocation on bare DNA and cannot be used for analysis of complex nucleoprotein assemblies. In contrast, quenching of the fluorescence dye by the FeS cluster reports directly on the distance of the helicase from the dye and is independent of other proteins that may share the lattice with the helicase.

FeS cluster dependent quenching of Cy3 and Cy5 can also be exploited at the single-molecule level using total internal reflection microscopy (TIRM) [3]. The most informative single-molecule TIRM experiments follow FRET between donor and acceptor fluorophores (such as Cy3 and Cy5) site-specifically incorporated into proteins and/or nucleic acids [36,37]. Labeling of the protein for these experiments represents the most challenging step. It is laborious and commonly yields inactive or partially active proteins [38]. When following an iron-containing helicase, the FeS cluster can be used as a proximity signal where quenching of fluorescence indicates the position of the helicase relative to the fluorophore, which negates the need for protein labeling. The rate of translocation by the helicase on naked ssDNA or on ssDNA bound by other proteins can be calculated from the

calibrated quenching signal. Similar to FRET-based experiments only the helicase most proximal to the dye can be monitored.

2. Experimental Design

2.1: Oligonucleotides

There are many commercial sources that provide fluorescently labeled substrates. Oligonucleotides labeled with either Cy3 or Cy5 at the 5' end can be ordered from Eurofins (formerly Operon). Integrated DNA Technologies (IDT) can incorporate Cy3 or Cy5 at the 3' of the oligonucleotide, or internally in addition to placing it at the 5' terminus. Cy3 or Cy5 are preferred because of their excitation and emission in the visible range, long fluorescent lifetime, and resistance to photobleaching. Typically, commercially available oligonucleotides are limited in the lengths and positions of the modifications relative to the 5' terminus of the synthetic DNA. Technical limitations associated with synthesis may be overcome by ligating two separately ordered synthetic oligonucleotides. In order to immobilize oligonucleotide on the neutravidin coated slide for single-molecule visualization, biotin can be incorporated at either the 5' or 3' end of an internally labeled oligonucleotide, or at the end opposite to the fluorophore for end labeled molecules. Substrates are resuspended in H₂O and their concentrations are determined spectrophotometrically using a UV spectrophotometer (e.g. Cary 100/300 from Varian, Inc) and the extinction coefficient provided by the manufacturer.

2.2: Substrate preparation

Duplex DNA substrates should be annealed by mixing the Cy3 or Cy5 labeled oligonucleotide with a complimentary unlabeled oligonucleotide using a 1:1 ratio in annealing buffer (20 mM Tris-HCl pH 7.5; 200 mM KCl; 2 mM potassium phosphate pH 6.8). Reactions are heated to 95°C for 5 minutes and allowed to slowly cool to RT over the course of three hours by placing a heating block on the bench top. After annealing, the reactions are buffer exchanged by passing the reaction through a G-25 column (GE Healthcare) equilibrated in 20 mM Tris-HCl pH 8.0 and stored at 4°C. Substrates left at 4°C for a few days prior to use tend to have less ssDNA than substrates used right away after annealing.

2.2: Ensemble Equilibrium Binding Assays

Equilibrium binding assays are carried out using a fluorescence spectrophotometer (e.g. Cary Eclipse from Varian, Inc.) (Figure 2). Micro-cuvettes with a minimum volume of 150 µL are preferred when protein and DNA are limited. Otherwise, larger volume cuvettes can also be purchased from Starna, Inc. or Helma. Experiments can be carried out using either quartz or optical glass cuvettes because both of these materials are transparent to light in the visible spectra corresponding to excitation and emission spectra of Cy3 and Cy5 dyes. Tris-HCl based reaction buffers can be used with varied salt concentrations (MgCl₂ and NaCl) to optimize conditions. To preserve integrity of the FeS cluster, all reaction buffers should contain a reducing agent (DTT or 2-mercaptoethanol).

Tight binding of the helicase to its substrates in low ionic strength buffers allows for determining binding stoichiometries. In the absence of NaCl, at sub-saturating amounts of helicase, all helicase molecules are assumed to be bound to DNA. Binding stoichiometry or the number of helicases bound per DNA substrate corresponds to the ratio of [helicase]:[DNA] when the signal saturates. At higher salt concentrations (typically above 100 mM NaCl) in conjunction with low DNA substrate concentrations (e.g. 1 nM molecules) binding affinity for ssDNA and duplex DNA substrates can be measured. Bovine Serum Albumin (BSA) (New England Biolabs) (100 µg/mL) is also included in the reaction buffer to prevent

the helicase from adhering to the cuvette walls, which may result in underestimation of binding affinity and overestimation of binding stoichiometry. Depending on the binding stoichiometry, the binding isotherms can be fitted to simple 1:1 binding equation or to more complex binding schemes using competition titration method [39].

Cy3 is excited at 549 nm and its emission is measured at 566 nm. Cy5 is excited at 648 nm and its emission is measured at 668 nm. Excitation and emission slit widths should be adjusted to avoid overlap between excitation and emission wavelengths. Excitation slit width is typically set to 5 nm and the emission slit width is set to 10 nm. The sensitivity of the detector (PMT voltage) is to be adjusted based on the amount of fluorophore labeled DNA molecules in the reaction to avoid oversaturating PMT. For low DNA concentrations of 1 nM or less, the PMT voltage should be set at 900–1000 V. This will allow for a greater change in fluorescence to be observed. Increasing the sensitivity, however, also results in increased noise. For DNA concentrations of 10 nM or more, setting the PMT between 400–600 V is ideal. The greater the fluorescently labeled DNA substrate concentration is, the greater the signal to noise ratio.

Titration reactions are carried out by first recording the baseline fluorescence of the buffer components in the absence of the Cy3 or Cy5 labeled DNA. Recording fluorescence intensity in kinetics mode is advisable because it allows for visual confirmation that the signal has indeed equilibrated before the average is recorded. Once the baseline is recorded, fluorophore-labeled DNA is added to the reaction, fluorescence is allowed to equilibrate and the increase in fluorescence is recorded (Figure 2b). This change in fluorescence is designated as F_0 and is considered maximum fluorescence, which corresponds to 100% free DNA. Titrations with the helicase follow by adding 0.5–2.5 μL aliquots to the desired final concentration. The signal is allowed to equilibrate after addition of each subsequent aliquot and the change in the fluorescence is recorded. Fluorescence intensity is averaged over the course of 1 minute. Each titration is designated $F_1, F_2, \dots F_n$. The relative fluorescence quenching for each concentration is determined as: F_n/F_0 .

3. Experimental design for single-molecule measurements

3.1: Equipment

Conventional prism-based wide-field total internal reflection fluorescence (TIRF) microscopy system can be used to track an FeS cluster containing helicase interacting with fluorescently labeled DNA substrate. This method takes advantage of the distance dependent fluorescent quenching of Cy3 or Cy5 fluorophores as the helicase translocates towards or away from the dye. In addition to prism-based TIRM systems, the FeS cluster mediated quenching can be monitored using objective-based TIRM. The design of a typical prism-based TIR microscope is schematically shown in Figure 3.

To excite Cy3 fluorophores incorporated into surface-tethered DNA molecules, the diode pumped 532 nm laser (50 or 100 mW, Crysta-Laser) is directed through the prism positioned over the microscope flow chamber at a large incident angle (more than 61° relative to surface normal) to generate an evanescent field that propagates beyond the glass/buffer boundary exponentially decaying with the distance. Similarly, the Cy5 fluorophores can be excited using 638 nm stabilized compact laser (50 or 100 mW, Crysta-Laser). The laser polarization and intensity can be modulated by a half-beam waveplate and polarization beam splitter. Fluorescence emission signals emanating from individual Cy3 or Cy5-labeled molecules are collected by a water immersion objective 60 $\times$ or 100 $\times$ (Olympus), passed through a 550 nm long-pass (lp) filter to block out laser scattering, separated by a 630 nm dichroic mirror and detected by EM-CCD camera (Andor) with a time resolution of 30 ms. Fluorescence signals of both dyes are subject to amplification prior to camera readout;

therefore, the recorded fluorescence intensity is reported in arbitrary units (a.u.). The signal is recorded using in house software written in Visual C++ (Microsoft) which is available upon request from the Laboratories of Taekjip Ha and Paul R. Selvin at the University of Illinois at Urbana-Champaign [36]. This IDL (Interface Description Language) software is used to identify independent fluorescence spots and extract kinetics of changes in fluorescence intensity for each fluorophore-tagged molecule. The spots are processed and the combined data of the trajectories can be visualized using MatLab software (The Mathworks, Inc.).

3.2: Sample and buffers

Binding of a Rad3 family helicase to and translocation along the Cy3-labeled ssDNA can be monitored at the level of individual nucleo-protein complexes using Cy3 fluorescence quenching as a proximity indicator. First, the quartz slide (Finkenbeiner) and coverslip are consecutively washed with 10% alconox detergent (ALCONAX), acetone, 1 M KOH and methanol before polyethyleneglycol (PEG) coating in order to eliminate nonspecific surface absorption of proteins. Then, both the slide and coverslip are aminosilanized and reacted with the N-hydroxysuccinimide ester-modified PEG (MW 5,000, Nectar Therapeutics) which also includes a small fraction of biotin-PEG-NHS ester (1–3% w/w of PEG) [6]. Finally, the PEG-coated quartz slide and coverslip are assembled to make the actual flow cell for the single-molecule experiment. The TIR flow chamber is constructed between the slide and coverslip, surrounded by double-sided tape and sealed with epoxy (Fig. 3c). Immobilization of DNA molecules is mediated through biotin-neutravidin interaction between biotinylated oligonucleotides and the neutravidin-coated surface (Fig. 5a). Unbound DNA is removed by washing the microscope slide with reaction buffer. The standard XPD translocation buffer contains: 50 mM Tris-HCl pH 7.5, 3 mM MgCl₂, 1 mM DTT, 100 µg/ml BSA and the oxygen scavenging system consisting of: 1 mg/ml glucose oxidase (Sigma), 0.4% (w/v) D-glucose (Sigma), 0.04 mg/ml catalase (Roche) and 1% v/v 2-mercaptoethanol (Acros). The presence of a reducing agent such as 1% (140 mM) 2-mercaptoethanol, is necessary to prevent oxidation of Fe-S cluster, that could possibly affect the stability and activity of the helicase. When Cy5 fluorophore is used in the quenching experiments or in the single-molecule fluorescence resonance energy transfer (smFRET) assays, it is advantageous to substitute 1mM Trolox for 2-mercaptoethanol to prevent Cy5 blinking [40]. Under these conditions, time trajectories for individual substrates can be recorded for 30 seconds to about 2 minutes before a considerable portion of the fluorophores are subject to photobleaching. Immobilization of 100 pM of fluorescently labeled DNA substrate allows for detection of 100–600 individual molecules per slide. DNA density can be confirmed by counting the surface-tethered Cy3 dyes excited with the 532 nm laser.

3.3: Calibration of the quenching signal

Relationship between Cy3 quenching and the distance between the fluorophore and FeS cluster can be calibrated using an ensemble ssDNA binding assay. Here binding of the FeS-containing helicase to various ssDNA length substrates is monitored under stoichiometric binding conditions (Fig. 4). The Rad3 family helicase, XPD from *Ferroplasma acidarmanus* (*FacXPD*) was used to demonstrate the utility of this approach [3]. We found a change in the helicase (FeS) position by 1 nucleotide can be linearly approximated and corresponded to a $3.0 \pm 0.3\%$ change in Cy3 fluorescence relative to the original signal (Fig. 4). Therefore, Cy3 quenching can be used as a specific and sensitive probe for XPD-ssDNA interaction and for accurately tracking the position of XPD on the DNA lattice. While calibration of the fluorescence quenching shown in Figure 4 is specific to Cy3 dye and to ssDNA under our typical translocation conditions, the change in fluorescence can be calibrated using a similar approach for other systems. The rate of translocation and/or the rate of duplex unwinding are then determined from the initial decrease in fluorescence.

3.4: Measuring ssDNA translocation rate

One hundred fifty pM of *Fac*XPD was added to the flow chamber containing 100 pM of Cy3-labeled ssDNA tethered to the surface. Single-molecule fluorescence trajectories were recorded for 5 minutes at 25°C in the standard translocation buffer in the presence of 1 mM ATP. We observed fluorescence trajectories with distinct quenching patterns that depended on both, the length of the oligonucleotide and its polarity (Figure 5). When Cy3 was incorporated at the 3'-end of a 42-mer ssDNA attached to the slide via biotin moiety at the 5' end of the oligonucleotide, XPD moved with distinct 5' to 3' translocation polarity towards the dye, which manifested in a gradual decrease in Cy3 fluorescence. In most of the translocation events, the helicase translocated off the end of ssDNA lattice, which was observed as an instantaneous recovery of Cy3 fluorescence (upper panel of Fig. 5b). Two step quenching events were also observed and attributed to the helicase binding closer to the fluorophore than the detection limit for fluorescence quenching followed by translocation towards the Cy3 labeled 3'-end (lower panel of Fig. 5b). Portions of individual fluorescence trajectories that showed binding/translocation events were extracted for the each immobilized DNA substrate, sorted according to the quenching pattern (such as those shown in Fig. 5b), fitted to the respective scenario and analyzed. The translocation rates were extracted by fitting the experimental data to the model corresponding to each scenario using GraphPad Prism software. Change in the fluorescence was converted into nucleotides translocated per second using calibration of the FeS-dependent quenching carried out under identical conditions (Figure 4).

Analysis of 200 – 600 independent trajectories is typically used to build a distribution of the translocation rates. Rates collected from all trajectories usually were distributed symmetrically and therefore were fitted to Gaussian distribution to yield average translocation rates. For example, translocation of XPD helicase on a 42 nt ssDNA substrate in the presence of 1 mM ATP yielded an average translocation rate of 13 ± 2 nt/s (Fig. 5c) [3]. The presence of distinct types of translocation events (for example, when ssDNA lattice was coated with ssDNA binding protein), may result in bi-modal distribution, which can be fitted to double Gaussian distribution to yield two translocation rates indicative of two distinct populations of translocation events [3].

3.5: Combining fluorescence quenching by the FeS cluser with FRET to simultaneously monitor ssDNA translocation by a Rad3 helicase and binding of a ssDNA binding protein

Fluorescent labeling of another ssDNA binding protein (for example, RPA2) can be performed by coupling Cy5 monoreactive NHS esters (GE Healthcare) to the N-terminal amine group at pH 7.0 [38,41]. NHS esters react with primary amines, which can be found at the N-terminus of protein or in the side chains of lysines. Due to the high pKa of a typical lysine, only the N-terminal primary amine is reactive at neutral pH. Before labeling, it is extremely important to buffer exchange the protein to remove all traces of Tris or other amine-containing buffers. Briefly, RPA2 was mixed with a 10-fold molar excess of the Cy5 monoreactive NHS esters (GE Healthcare) in buffer L (50 mM potassium phosphate, pH 7.0, 100 mM NaCl and 1 mM DTT) for 30 minutes at RT. Subsequently, the reaction mixtures were incubated for 12 hours at 4°C. The labeling reactions were terminated by addition of 50 mM Tris-HCl, pH 7.5. Cy5-labeled RPA2 was separated from the free dye using a HiTrap Desalting column (GE Healthcare). The ratio of dye incorporated per protein molecule was confirmed to be consistent with a single label per protein molecule, utilizing $\epsilon_{650}(\text{Cy5}) = 250,000 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{280}(\text{Cy3}) = 150,000 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{280}(\text{RPA2}) = 21,890 \text{ M}^{-1}\text{cm}^{-1}$.

Combining the FeS cluster-mediated fluorescence quenching with FRET between a pair of fluorophores allows for expanding these analyses to pseudo three color measurements to

monitor complex nucleoprotein transactions [3]. In the example experiment, the ssDNA binding protein (RPA2) was site-specifically labeled with Cy5 dye at the N-terminus. Binding of the ssDNA binding protein to and its continuous presence on ssDNA was monitored by measuring the FRET between Cy3 (donor) incorporated into 3'-end of ssDNA and Cy5 (acceptor) labeled ssDNA binding protein (RPA2) (Fig. 6a). When 100 nM Cy5 labeled RPA2 was added to the flow chamber containing immobilized Cy3-ssDNA, all recorded trajectories displayed anticorrelated Cy3 and Cy5 signals indicative of intermittent high and low FRET states that reflected RPA2 binding to and dissociation from ssDNA (the "on" and "off" states are indicated for the representative trajectories shown in Figure 6). When the FeS cluster containing helicase, XPD, and 1 mM ATP were added to the reaction buffer along with RPA2, two types of fluorescent trajectories were observed (Fig. 6b and 6c). One type demonstrated gradual Cy3 quenching associated with XPD translocation toward Cy3-labeled ssDNA end to coincide with or to be immediately preceded by a sudden disappearance of the Cy5 signal indicative of dissociation of Cy5-labeled RPA2. Eventual dissociation of XPD resulted in recovery of Cy3 fluorescence but not that of Cy5 indicating that RPA2 has dissociated or was displaced during the XPD translocation event. The other trajectory type showed Cy5 fluorescence decreasing concomitantly with the decrease in Cy3 fluorescence. This synergistic quenching and recovery pattern can be interpreted as RPA2 remaining bound to ssDNA while XPD translocates passed the bound protein. When XPD dissociates from ssDNA, both Cy3 and Cy5 simultaneously recovered their fluorescence (Fig. 6c). This pseudo three color approach can be used to analyze nucleoprotein interactions involving FeS cluster containing helicases and other proteins involved in each of their respective pathways. Translocation of the XPD helicase on ssDNA bound by Cy5-labeled RPA2 protein could be also followed by directly exciting Cy5 fluorophore and following its quenching by approaching helicase or its recovery when the helicase translocates away from the bound Cy5-RPA2 [3].

4. Conclusion

The discovery of the presence of FeS clusters in DNA helicases and other DNA processing proteins has opened the door for novel techniques to characterize DNA substrate recognition and translocation by helicases. The FeS cluster serves as an endogenous reporter eliminating the need for labeling of the purified helicase ensuring that the measurements obtained are true reflections of the helicase and are not affected by potential artifacts associated with labeling. Only a single, simple modification to DNA is needed to have robust assays for monitoring helicase activities. Fluorescence based approaches have the advantage of quantification of true equilibrium binding affinities and provide a means for simple manipulation of assay conditions. Application to single-molecule visualization has provided insight into the fundamental mechanisms of DNA translocation, calculation of the rate of translocation, and the ability to create a pseudo-three color reporter system for monitoring multiple proteins on a single DNA lattice without modifying the helicase itself.

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Appendix 1

General Supplies

Fluorescently Labeled Oligonucleotides:

Integrated DNA Technologies, Inc.

Fluorophores: Cy3, Cy5,
Eurofins
Cy3 and Cy5 (5' end only)

UV Spectrophotometer:

Varian
Agilent Technologies
Eppendorf
Thermo Scientific
Shimadzu
Beckman Coulter
PerkinElmer

Cuvettes (Quartz or Optical Glass):

Starna, Inc.
Helma
VWR

Fluorescence Spectrophotometer:

Varian
ISS
Shimadzu

TIR Microscope:

Olympus
Nikon
Zeiss

EM-CCD Camera

Andor
Hamamatsu

Software:

Visual C++ from Microsoft
IDL from ITT Visual Information Solutions
MatLab from The MathWorks, Inc.
GraphPad Prism from GraphPad Prism Software Inc.

References

1. Rudolf J, Makrantonis V, Ingledew WJ, Stark MJ, White MF. Mol Cell 2006;23:801–808. [PubMed: 16973432]
2. Pugh RA, Honda M, Leesley H, Thomas A, Lin Y, Nilges MJ, Cann IK, Spies M. J Biol Chem 2008;283:1732–1743. [PubMed: 18029358]

3. Honda M, Park J, Pugh RA, Ha T, Spies M. *Mol Cell* 2009;35:694–703. [PubMed: 19748362]
4. Pugh RA, Lin Y, Eller C, Leesley H, Cann IK, Spies M. *J Mol Biol* 2008;383:982–998. [PubMed: 18801373]
5. Clegg RM. *J Biotechnol* 2002;82:177–179. [PubMed: 11999688]
6. Roy R, Hohng S, Ha T. *Nat Methods* 2008;5:507–516. [PubMed: 18511918]
7. Wu Y, Suhasini AN, Brosh RM Jr. *Cell Mol Life Sci* 2009;66:1209–1222. [PubMed: 19099189]
8. Tirode F, Busso D, Coin F, Egly J-M. *Molecular Cell* 1999;3:87–95. [PubMed: 10024882]
9. Sung P, Prakash L, Matson SW, Prakash S. *Proc Natl Acad Sci U S A* 1987;84:8951–8955. [PubMed: 2827162]
10. Liu Z, Hong SW, Escobar M, Vierling E, Mitchell DL, Mount DW, Hall JD. *Plant Physiol* 2003;132:1405–1414. [PubMed: 12857822]
11. Cantor S, Drapkin R, Zhang F, Lin Y, Han J, Pamidi S, Livingston DM. *Proceedings of the National Academy of Sciences* 2004;101:2357–2362.
12. Hirota Y, Lahti JM. *Nucleic Acids Res* 2000;28:917–924. [PubMed: 10648783]
13. Liu H, Rudolf J, Johnson KA, McMahon SA, Oke M, Carter L, McRobbie AM, Brown SE, Naismith JH, White MF. *Cell* 2008;133:801–812. [PubMed: 18510925]
14. Wolski SC, Kuper J, Hanzelmann P, Truglio JJ, Croteau DL, Van Houten B, Kisker C. *PLoS Biol* 2008;6:e149. [PubMed: 18578568]
15. Fan L, Fuss JO, Cheng QJ, Arvai AS, Hammel M, Roberts VA, Cooper PK, Tainer JA. *Cell* 2008;133:789–800. [PubMed: 18510924]
16. Gupta R, Sharma S, Doherty KM, Sommers JA, Cantor SB, Brosh RM Jr. *Nucleic Acids Res* 2006;34:6673–6683. [PubMed: 17145708]
17. Ren B, Ding H. *J. Biol. Chem.* 2008 M807943200.
18. Imlay JA. *Mol Microbiol* 2006;59:1073–1082. [PubMed: 16430685]
19. Takeda S, Kamiya N, Nagamune T. *Anal Biochem* 2003;317:116–119. [PubMed: 12729608]
20. Taraska JW, Puljung MC, Olivier NB, Flynn GE, Zagotta WN. *Nat Methods* 2009;6:532–537. [PubMed: 19525958]
21. Yeeles JT, Cammack R, Dillingham MS. *J Biol Chem* 2009;284:7746–7755. [PubMed: 19129187]
22. Hirata A, Klein BJ, Murakami KS. *Nature* 2008;451:851–854. [PubMed: 18235446]
23. Weiner BE, Huang H, Dattilo BM, Nilges MJ, Fanning E, Chazin WJ. *J Biol Chem* 2007;282:33444–33451. [PubMed: 17893144]
24. Klinge S, Hirst J, Maman JD, Krude T, Pellegrini L. *Nat Struct Mol Biol* 2007;14:875–877. [PubMed: 17704817]
25. Trieu VN, McCarthy D. *J. Bacteriol* 1989;171:2128–2135. [PubMed: 2539361]
26. Petra G-G, Hartmut H-B. *European Journal of Biochemistry* 1990;192:689–693. [PubMed: 2170128]
27. Parsons R, Anderson ME, Tegtmeyer P. *J. Virol* 1990;64:509–518. [PubMed: 2153220]
28. Morris PD, Raney KD. *Biochemistry* 1999;38:5164–5171. [PubMed: 10213622]
29. Serebrov V, Beran RK, Pyle AM. *J Biol Chem* 2009;284:2512–2521. [PubMed: 19010782]
30. Ali JA, Lohman TM. *Science* 1997;275:377–380. [PubMed: 8994032]
31. Ali JA, Maluf NK, Lohman TM. *J Mol Biol* 1999;293:815–834. [PubMed: 10543970]
32. Jankowsky E, Gross CH, Shuman S, Pyle AM. *Nature* 2000;403:447–451. [PubMed: 10667799]
33. Lucius AL, Vindigni A, Gregorian R, Ali JA, Taylor AF, Smith GR, Lohman TM. *J Mol Biol* 2002;324:409–428. [PubMed: 12445778]
34. Dillingham MS, Wigley DB, Webb MR. *Biochemistry* 2002;41:643–651. [PubMed: 11781105]
35. Fischer CJ, Maluf NK, Lohman TM. *J Mol Biol* 2004;344:1287–1309. [PubMed: 15561144]
36. Ha T. *Curr Opin Struct Biol* 2001;11:287–292. [PubMed: 11406376]
37. Joo C, Balci H, Ishitsuka Y, Buranachai C, Ha T. *Annu Rev Biochem* 2008;77:51–76. [PubMed: 18412538]
38. Dillingham MS, Wallace MI. *Org Biomol Chem* 2008;6:3031–3037. [PubMed: 18698457]
39. Jezewska MJ, Bujalowski W. *Biochemistry* 1996;35:2117–2128. [PubMed: 8652554]

40. Rasnik I, McKinney SA, Ha T. Nat Methods 2006;3:891–893. [PubMed: 17013382]
41. Galletto R, Amitani I, Baskin RJ, Kowalczykowski SC. Nature 2006;443:875–878. [PubMed: 16988658]

Figure 1. Rad3 helicases

(a) Crystal structure of a representative Rad3 family helicase, XPD from *Thermoplasma acidophilum* (pdb: 2vsf)[14]. The motor core of XPD helicase consists of two helicase domains, HD2 (green) and HD1 (blue). Two insertions in the motor core form Arch domain (purple) and the FeS cluster containing domain (orange). The FeS cluster is depicted as spheres, and the conserved helicase motifs appear in black. The expected position of the ssDNA substrate is schematically depicted over the crystal structure. (b) Schematic representation of the primary structure of Rad3 helicases. Amino acid sequences of all Rad3 family helicases contain four cysteine residues located between helicase motifs Ia and II. Spacing between these cysteines is conserved between different Rad3 helicases. Shown are the cysteine residues with the number of amino acids separating them from *Thermoplasma acidophilum* (*Tac*), *Ferroplasma acidarmanus* (*Fac*), *Homo sapiens* (*h*), *Mus musculus* (*m*), *Saccharomyces cerevisiae* (*Sce*), *Escherichia coli* (*Ecoli*), *Sulfolobus acidocaldarius* (*Sac*).

Figure 2. Ensemble equilibrium binding assays

(a) DNA is site specifically labeled with a fluorophore (Cy5) (red circle). The dye is excited at 649 nm, which corresponds to maximum of its absorbance. The Cy5 emission is recorded at 668 nm. Initial fluorescence of the Cy5-containing DNA is recorded and attributed to 100% free DNA. Upon binding of the FeS cluster containing helicase in close proximity to the fluorophore results in quenching of Cy3 emission. (b) Raw data from a representative binding experiment, which followed stoichiometric binding of *FacXPD* helicase to the ssDNA-dsDNA junction of a forked DNA substrate. First, baseline fluorescence of the buffer is recorded and averaged over 1 minute. The value of this signal is then subtracted as background from all subsequent measurements. Then fluorescently labeled DNA is added to the cuvette. The observed increase in fluorescence is designated as F_0 . XPD (red ovals) is then titrated into the reaction resulting in stepwise quenching of fluorescence. At each step the fluorescence signal is allowed to equilibrate, and the intensity for each point is averaged over the course of 1 minute. Each titration is labeled as F_n . The collected data are then plotted as F_n/F_0 . (c) Results from a representative assay depicting XPD binding to three different Cy5 labeled substrates under stoichiometric binding conditions. Greater quenching

is observed when XPD binds to a 5'partial duplex (PD) substrate compared to a forked substrate. Little quenching is observed when the helicase binds to a 3'PD substrate indicating that XPD is oriented so that the FeS cluster is directed away from the fluorophore positioned at the ssDNA-dsDNA junction. Data shown in this figure are adapted from Pugh et al.[2]

Figure 3. Prism-type TIRM system

(a) Schematic representation of a typical experimental setup allowing excitation of the tethered Cy3 and Cy5 fluorophores with evanescent field as well as visualization of their fluorescence. In the two-color (FRET) experiments, emission from Cy3 and Cy5 dyes are collected simultaneously but in two separate channels and recorded in two fields on the CCD camera. For each tethered molecule, one can obtain Cy3 and Cy5 fluorescence trajectories. (b) Schematic depiction of the TIR excitation (magnified view of circle region in a). A laser beam directed through the prism placed on the sample quartz slide creates evanescent fields (EF) that selectively excites fluorophores near the slide/buffer interface resulting in a low fluorescence background. (c) TIR flow chamber is made by putting a slide and a coverslip together with double-sided tape and sealing epoxy. The holes on the slide are used for the inlet and outlet for solution exchange.

Figure 4. Calibration of quenching signal

The magnitude of the FeS dependent fluorescence quenching decays linearly with increasing distance between the FeS cluster and fluorophore. Calibration is based on the helicase binding randomly to a series of Cy3 labeled DNA oligonucleotides of varying lengths. When the helicase and Cy3-labeled oligonucleotide are present at a 1:1 ratio, average position of the helicase on the DNA substrate will correspond to the middle of the oligonucleotide. Considering that upon binding, XPD helicase occludes approximately 20 nucleotides of ssDNA, we can calculate the distance from the dye to the front edge of the area on the substrate occluded by bound XPD. Under typical conditions of our single-molecule experiments, change in the helicase position by 1 nucleotide corresponded to a $3.0 \pm 0.3\%$ change in Cy3 fluorescence. Data shown were originally published by Honda, et al [3].

Figure 5. XPD translocation on ssDNA

(a) Schematic depiction of the XPD helicase moving in the 5' to 3' direction on immobilized ssDNA (42-mer) decorated with Cy3 at the 3'-end. (b) Representative single-molecule trajectories of XPD-dependent Cy3-quenching in the presence of ATP. Individual traces demonstrating Cy3 quenching were classified into three types. Trajectory type 1 shows XPD translocation associated with rapid dissociation from Cy3 (upper panel). Calculations of the translocation rate for this type of fluorescence trajectories were done by fitting the experimental data to the 4 segment line as indicated in yellow. Trajectory type 2 shows XPD translocating toward Cy3 and remaining bound for a period of time before dissociating from ssDNA (middle panel). Rate calculations were done by fitting these trajectories to a 3 segment line. Trajectory type 3 shows XPD binding to ssDNA closer to Cy3 than the detection limit and then translocating toward Cy3 (lower panel). Rate calculations were done by fitting these trajectories to a 5 segment line. (c) Each individual translocation event was analyzed by fitting the experimental data to one of the three models shown in (b). Rate in nucleotides per second was determined from conversion of the rate of fluorescence change ($\Delta F/s$) corresponding to XPD translocation to actual translocation rate using distance vs. quenching calibration [3]. 138 individual translocation events were observed in 600 analyzed trajectories. Individual rates were binned in 5% fluorescence change per second intervals, plotted and analyzed by fitting the resulting histogram to Gaussian distribution. The average and standard deviation of the distribution were used to calculate the average translocation rate. Data shown were originally published by Honda, et al [3].

Figure 6. Pseudo tri-color experiment aimed at simultaneous visualization of XPD and ssDNA binding protein RPA2 on the same DNA molecule

(a) Binding of the Cy5-labeled RPA2 to the Cy3-labeled ssDNA (schematically depicted on the left) can be detected by following FRET between the two dyes. Fluorescence trajectories for the Cy3 and Cy5 dyes are shown in green and red, respectively. (b) Uncoupled fluorescence trajectories were observed when RPA2 (100 nM) was displaced by the translocating XPD helicase (150 pM) or spontaneously dissociates from ssDNA immediately before XPD translocation. (c) Synergistic quenching followed by the simultaneous recovery of Cy3 and Cy5 fluorescence was attributed to events where XPD

(150 pM) translocated over the bound RPA2 (100 nM) without displacing it from ssDNA.
Data shown were originally published by Honda, et al [3]