CHAPTER TWO

Single-Molecule Imaging With One Color Fluorescence

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Contents

1.	Protein-Induced Fluorescence Enhancement	34
	1.1 How Does PIFE Work?	35
	1.2 Early Uses of PIFE	36
	1.3 Distance Sensitivity of PIFE at Single-Molecule Resolution	36
2.	Experimental Preparation for PIFE	37
	2.1 Materials for Imaging	38
	2.2 Imaging Surface Preparation	38
	2.3 Oligo Substrate Preparation	39
	2.4 Imaging/Reaction Buffer Preparation	39
	2.5 Single-Molecule Fluorescence Assay	39
	2.6 Substrate Design	40
3.	Static and Transient Protein Binding	41
4.	Filament Formation and Protein Binding to Long DNA Strands	43
5.	Protein Binding and Motility Kinetics	44
6.	PIFE in the Presence of FRET	47
7.	Conclusions	48
References		

Abstract

Single-molecule fluorescence imaging is a powerful tool that enables real-time observation of DNA–protein or RNA–protein interactions with a nanometer precision. Here, we provide a detailed procedure for a previously developed single-molecule fluorescence method, termed "single-molecule protein-induced fluorescence enhancement" (smPIFE). While smFRET (Förster resonance energy transfer) requires both donor and acceptor, protein-induced fluorescence enhancement (PIFE) employs a single dye and measures the increase in fluorescence intensity induced by protein binding near the dye. PIFE displays distance sensitivity within 0–4 nm, making it a powerful complementary or alternative tool to FRET method. In this chapter, we will discuss the various ways that PIFE has been utilized to study protein–nucleic acid interactions.

1. PROTEIN-INDUCED FLUORESCENCE ENHANCEMENT

Single-molecule Förster resonance energy transfer (FRET) assay is widely used to probe nucleic acid-protein interactions that cannot be resolved at an ensemble level (Abbondanzieri et al., 2008; Joo et al., 2006; Myong, Rasnik, Joo, Lohman, & Ha, 2005; Woodside et al., 2006). FRET occurs when an excitation of a donor fluorophore leads to a transfer of its energy to a nearby acceptor, leading to an anticorrelated change between the donor and acceptor intensity. FRET efficiency is calculated as the ratio of intensity emitted by the acceptor over the sum of acceptor and donor, hence can be used as a measure of distance between the two dyes. The two most common ways FRET is used to monitor protein-nucleic acid interactions include (i) an indirect measurement by placing two (FRET pair) dyes directly on DNA/RNA substrate at either end of single-strand DNA, for example and (ii) a direct measurement by applying singly labeling protein (donor, for example) to singly labeled DNA/RNA (acceptor, for example). While FRET is an excellent tool for determining relative distance change between the two dye-labeled molecules or molecular positions, it can be disadvantageous because fluorescent labeling of proteins is often inefficient, time consuming, and sometimes perturbs protein function. In addition, if the equilibrium binding constant of the protein is high, the high concentration of fluorescently labeled molecules may hinder single-molecule detection.

In single-molecule protein-induced fluorescence enhancement (smPIFE) assay, unlabeled protein is applied to fluorescently labeled DNA or RNA substrate. In this regard, the smPIFE presents a clear advantage over FRET because the protein-labeling process can be bypassed and the protein binding kinetic and movement are reported directly through the intensity of fluorescent dye attached to nucleic acid substrate (Hwang, Kim, & Myong, 2011; Myong et al., 2009). The intensity of a fluorophore is enhanced upon binding of a protein in its vicinity and the enhancement scales with the distance between the dye and protein. Due to the distance sensitivity of protein-induced fluorescence enhancement (PIFE) (0–4 nm) which complements that of FRET (3–8 nm) combined with the ease of use, PIFE is a powerful alternative single-molecule detection approach for protein–nucleic acid interactions (Hwang et al., 2011; Hwang & Myong, 2014).

1.1 How Does PIFE Work?

The maximum intensity emitted by a fluorophore is determined by the quantum yield of a particular dye. In 1984, Aramendia et al. observed that the viscosity of the local environment has a direct effect on the quantum yield of cyanine dyes (Aramendia, Negri, & San Roman, 1994). The change in the fluorescence quantum yield was attributed to the *cis-trans* isomerization of the cyanine dye from excited (fluorescent, photoactive) to dark (nonfluorescent) state. The isomerization entails a rotation of one carbon ring with respect to the other carbon ring which is connected through a carbon–carbon double bond (Fig. 1A). Protein binding near the fluorophore results in stabilizing the photoactive *trans* state, thus enhancing the fluorescence intensity, quantum yield, and lifetime (Aramendia et al., 1994; Levitus & Ranjit, 2011; Sanborn, Connolly, Gurunathan, & Levitus, 2007; Stennett, Ciuba, & Levitus, 2014).



Fig. 1 (A) *cis–trans* Isomerization of a Cy3 dye. The *red arrow* indicates the rotation with respect to the carbon–carbon double bonds. (B) Schematic of the *Bam*HI protein binding to sequence-specific restriction sites positioned at five different base pair distances away from the Cy3 fluorophore. The closer the binding site, the brighter the Cy3 intensity. (C) Schematic of RIG-I protein translocates along the dsRNA axis. As RIG-I moves away from the Cy3 dye, the PIFE sensitivity decreases and movement is no longer detected beyond 4 nm. (D) Distance sensitivity of PIFE and FRET.

In addition, the location of the dye can also change the rigidity of *cis–trans* isomerization (Sanborn et al., 2007).

Cy3 is a popular choice of an organic fluorescent dye for PIFE and FRET experiments due to its high absorption coefficient, photostability, and modest quantum yield (Ha, 2001). The PIFE effect has also been observed with several other dyes including DY547, Cy5, and Alexa dyes (Hwang et al., 2011; Myong et al., 2009). In contrast, Cy3B which cannot undergo *cis*-*trans* isomerization exhibits no increase in quantum yield or fluorescent lifetime (Hwang et al., 2011). If a dye has the same type of chemical structure as the cyanine dyes, where two rings are interconnected by carbon–carbon double bonds that can undergo *cis–trans* isomerization, it is expected to exhibit the PIFE effect.

The fluorescence lifetime of the chosen dye can significantly influence the PIFE effect it produces. Sorokina et al. employed time-correlated single photon counting technique to demonstrate a stepwise increase in both the fluorescence intensity and lifetime when T7 RNA polymerase binds to a fluorescently labeled DNA (Sorokina, Koh, Patel, & Ha, 2009). A similar experiment (Fig. 1B) done using a restriction enzyme, *Bam*HI, binding 1–10 bp away from the Cy3 dye also reported corresponding intensity and lifetime changes (Hwang et al., 2011).

1.2 Early Uses of PIFE

Before PIFE was characterized in single-molecule experiments, the same photophysical effect had been utilized in stopped-flow ensemble measurements to investigate kinetics of DNA motor proteins (Fischer & Lohman, 2004; Fischer, Tomko, Wu, & Lohman, 2012; Tomko, Fischer, & Lohman, 2010). Additionally, a similar method was used to probe the binding of T7 Polymerase to DNA by tracking the intensity change (Luo, Wang, Konigsberg, & Xie, 2007). In 2009, we reported translocation activity of a human antiviral protein, RIG-I on double-stranded (ds) RNA, where the movement of RIG-I along dsRNA axis was visualized as gradual intensity increase and decrease in an ATP-dependent manner. Such movement was greatly accelerated on the viral mimic RNA, suggesting a viral recognition mechanism of RIG-I (Myong et al., 2009).

1.3 Distance Sensitivity of PIFE at Single-Molecule Resolution

The distance sensitivity of PIFE was determined by using a restriction enzyme, *Bam*HI, and the RIG-I translocation data (Hwang et al., 2011;

Myong et al., 2009). The binding site for *Bam*HI was specifically engineered to be 1–10 base pairs (bp) away from the Cy3 label positioned on one end of the double-stranded DNA (dsDNA), and the increase in Cy3 intensity was recorded to map out the distance sensitivity of PIFE (Fig. 1B). The PIFE effect was found to be the greatest (2.6 × enhancement) at 1 bp and the lowest ($1.3 \times$ enhancement) at 10 bp, with measurements in between showing an apparent linear relationship between the intensity and distance (Hwang & Myong, 2014). It is interesting to note that the lowest intensity is slightly higher than 1×. The 1.3× enhancement is even observed when *Bam*HI binds 12 and 15 bp away from the fluorophore. We confirmed that this is not due to buffer but due to the protein binding; hence, it raises a possibility about an "action at a distance." This needs to be followed up by future studies.

Single-molecule traces obtained from RIG-I translocation showed repetitive translocation events that can be separated into two phases, the PIFE-sensitive and -insensitive region observed as a linear decline and a plateau in fluorescence intensity, respectively. The linear decrease corresponds to the PIFE-sensitive distance range when RIG-I translocates away from the fluorophore, and the plateaued region is interpreted as the PIFE-insensitive distance range where the protein movement is not detectable by PIFE. Assuming the rate of translocation occurs at a constant velocity, the RIG-I data indicated that the first 10–12 bp of movement was detectable by PIFE, which translates to 0–4 nm distance range (Fig. 1C). In light of the FRET-sensitive distance of 3–8 nm, PIFE fills in the 0–3 nm gap, thereby making it a powerful complementary tool to FRET (Fig. 1D).

2. EXPERIMENTAL PREPARATION FOR PIFE

As with single-molecule FRET measurements, PIFE can be conducted with either the prism- and objective-type total internal reflection fluorescence (TIRF) microscope. A detailed guide on TIRF microscope building has been previously published (Roy, Hohng, & Ha, 2008). Similar to FRET, the laser-excited fluorescence emissions from the DNA molecules on imaging surface are transmitted through the objective lens and captured by an electron multiplying charge-coupled device (EMCCD) camera. One major difference is that a dichroic beam splitter is unnecessary since the only one signal needs to be detected unless two-color PIFE is performed. Twoor multicolor PIFE is feasible if the dyes do not crosstalk or are positioned at a distance from one another. Cy3 (donor) fluorophore on DNA is excited using an Nd:YAG laser (532 n*M*) via total internal reflection. The fluorescence signals from Cy3 are collected through an objective (Olympus Uplan S-Apo, $100 \times$; 1.4 numerical aperture; oil immersion) and detected at 100 ms time resolution using an EMCCD camera (Andor iXon DU-897ECS0-#BV). The EMCCD camera is controlled using custom-made C++ program. Single-molecule traces can be extracted from the recorded video file using IDL software and analyzed with custom-made Matlab codes available for free on the CPLC software website (https://cplc.illinois.edu/software/).

2.1 Materials for Imaging

T50 buffer (a general wash buffer, also used for DNA or RNA immobilization)

10 mM Tris-HCl (pH 8.0) and 50 mM NaCl

NeutrAvidin

10 mg of NeutrAvidin powder is dissolved in 2 mL of T50 Buffer. The stock solution is diluted by adding 20–980 μ L of T50 buffer. This can be store at 4°C for 2–3 months

Glucose oxidase–catalase

1 mg/mL glucose oxidase and 0.03 mg/mL catalase are dissolved in T50 buffer and stored at 4°C

Trolox

3–6 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic) can be used instead of β -mercaptoethanol as shown in Section 2.4. Although the effect of trolox on smPIFE has not been studied, trolox does not seem to interfere with smPIFE imaging

2.2 Imaging Surface Preparation

We follow the same surface passivation protocol used for smFRET (Roy et al., 2008). Quartz slides and coverslips are cleansed by washing steps involving soap, water (Milli-Q), methanol, acetone, KOH, and burning. Then the slides are coated with a biotinylated PEG (1%) and mPEG (methoxypolyethylene glycol) (99%) mixture to form a passivation layer that prevents nonspecific binding of molecules to surface. The slides are then vacuum-packed (a pair of one cover slip and one quartz slide in one 50-mL Corning tube, stored in a plastic sandwich bag vacuum sealed) and stored at -80° C for future use.

On the day of experiment, a pair of cover slip and quartz slide are thawed at room temperature for 15 min, and assembled to create reaction chambers (3–4 chambers with inlet and outlet pores for each chamber) to which all reaction mixtures will be applied (Hwang & Myong, 2014). NeutrAvidin is added to the chamber in preparation for tethering biotinylated and fluorescently labeled nucleic acid substrate.

2.3 Oligo Substrate Preparation

DNA or RNA oligonucleotide labeled with fluorescent dye can be purchased from commercial vendors such as IDT DNA. For single-strand substrate, one end needs to be conjugated with fluorescent dye and the other end with biotin. For an internal labeling, oligos containing internal amino modifier deoxy-thymine (IDT DNA) can be labeled using Cy3 monofunctional NHS esters (Thermo Fisher). Briefly, 10 nmol of aminomodified oligonucleotides in 20 μ L of 50 mM sodium tetraborate buffer (pH 8.5) and 100 nmol of Cy3 NHS ester dissolved in dimethylsulfoxide are mixed and incubated on a rotator overnight at room temperature. Since the dye is light sensitive, the labeling procedure should be performed in a dark room and/or the reaction tubes should be completely covered with aluminum foil. The labeled oligonucleotides are purified by ethanol precipitation repeated at least two times (Joo & Ha, 2012).

In case of preparing double-stranded or partial duplex DNA, the two complementary strands can be annealed by mixing the biotinylated and nonbiotinylated oligonucleotides in a 1:2 molar ratio at 10 μ *M* in T50 buffer. Oligonucleotide mixtures are incubated at 95°C for 2 min followed by slow cooling to room temperature to complete the annealing reaction.

2.4 Imaging/Reaction Buffer Preparation

The imaging solution consists of a salt of choice (commonly used is NaCl or KCl) and a buffering reagent (e.g., TRIS, PBS, MOPS, HEPES). To reduce photobleaching of fluorescent dyes, the imaging buffer is supplemented with the oxygen scavenging system composed of 0.8% (v/v) dextrose, glucose oxidase, catalase, and 1% (v/v) β -mercaptoethanol (Roy et al., 2008).

2.5 Single-Molecule Fluorescence Assay

Annealed DNA or RNA substrates are diluted to 50-100 pM concentration and are applied to the NeutrAvidin coated slide surface. In this concentration range, we observe $\sim 300-500$ fluorescent spots in one field of view of $25 \times 75 \,\mu\text{m}$ area (Joo, Balci, Ishitsuka, Buranachai, & Ha, 2008). If the number of spots is too low (below 100) or too high (above 600), PEG-biotin concentration can be modulated accordingly. It is important to have a clear separation between diffraction limited fluorescent spots for single-molecule detection in general. This is even more critical for smPIFE measurement because the PIFE signal is solely dependent on the fluorescence intensity of single molecules, i.e., even slightly overlapping signal between neighboring molecules may obscure the observable PIFE effect.

FRET value is determined by obtaining a ratio between donor and acceptor signals; hence, the noise in fluorescence signal due to thermal fluctuation or vibration is efficiently canceled out. In contrast, the readout from PIFE is inherently noisier because it is dependent on signals from single fluorophores. Therefore, it is crucial to establish a uniform intensity level before applying proteins for PIFE measurement. This can be best achieved by having an evenly illuminated field of view so that the resulting intensity histogram from all molecules yields one sharp peak. This will allow protein-induced intensity change to be clearly distinguished from DNA or RNA-only signals. We note that depending on the substrate design, the fluorescence signal may be noisier. For example, single-stranded DNA/RNA or structured substrate such as G-quadruplex (GQ) exhibits signal fluctuation due to the conformational flexibility within the substrate is less prone to noise due to the structural rigidity provided by the duplex.

2.6 Substrate Design

As with single-molecule FRET studies, there is a concern that the presence of fluorescent dye on substrate may perturb protein binding and kinetics. Therefore, it is critical to perform an orthogonal functional test to check for the potential dye effect. In the *Bam*HI study used to characterize PIFE distance sensitivity (Hwang et al., 2011), the *Bam*HI bound and cleaved the dsDNA regardless of the dye position. The DNA cleavage, detected by the disappearance of Cy3 dye, occurred immediately following the addition of magnesium. The similar rates of digestion observed in different constructs suggested that the fluorescent dye did not interfere with the function of the protein. Many previous single-molecule FRET studies also showed that the protein's biochemical activity (i.e., translocation, unwinding) is preserved when the DNA/RNA substrates are labeled with dyes Joo et al., 2006; Myong, Bruno, Pyle, & Ha, 2007; Myong et al., 2005;

Qiu et al., 2013; Tang, Roy, Bandwar, Ha, & Patel, 2009). However, it should be noted that the method of dye attachment can be important for the activity of the protein. For example, it is undesirable to attach a dye to the backbone of the DNA/RNA for helicases because many helicases use the phosphate backbone as a walking track (Myong et al., 2007, 2005; Myong & Ha, 2010; Park et al., 2010). In addition, dye can sometimes affect the folding of DNA secondary structures needed for the study. Commonly studied secondary structures such as hairpin junctions and GQ may not fold correctly if the fluorescent dye is located at important positions. This can be checked by using single-molecule FRET, as a control experiment, to see if the desirable FRET level is present. The GQ DNA structure can also be tested by using circular dichroism.

3. STATIC AND TRANSIENT PROTEIN BINDING

The most obvious advantage of PIFE measurement lies in its ability to directly visualize binding of protein to a fluorescently labeled substrate without the need to label or modify the protein in anyway. Any purified protein can be tested simply by applying to the fluorescently labeled substrate immobilized to surface. Once binding is confirmed, protein concentration can be titrated to obtain the protein's binding affinity. Fluorescence intensity histogram generated from collecting thousands of single-molecule traces can be quantified and compared before and after the addition of proteins. If the illumination field is uneven, it is still possible to construct and compare intensity histograms by using the relative value instead of the absolute intensity value. This is done by normalizing the individual protein-bound intensity by their respective protein-unbound intensity found within individual single-molecule traces. Once constructed, the area under the histogram curves can be quantified to estimate the amount bound and unbounded molecules, which can then be used to calculate K_{d} , the protein dissociation constant (Hwang & Myong, 2014).

Recent study employed smPIFE method to study DNA mismatch recognition/repair process (Jeon et al., 2016). Duplexed DNA with a single mismatch was prepared with Cy3, biotin, and digoxigenin (dig) attached as shown (Fig. 2A). In the presence on ATP, the mismatch recognition protein MSH2–MSH6 heterodimer forms a sliding clamp that undergoes diffusion of the dsDNA. The binding of MSH2–MSH6 to the mismatched DNA was visualized by PIFE signal (Fig. 2B) and the lifetime, T_{on} , was determined by fitting the individual dwell times (t_{on}) to exponential decay function



Fig. 2 (A) Schematic of MSH2–MSH6 heterodimer sliding on mismatched DNA. The substrate is labeled with a Cy3 dye near the mismatch as well as a digoxigenin at the one end and biotin on the other end to allow the immobilization of the DNA. (B) The binding of the heterodimer is detected via PIFE. Cy3 intensity increases when protein binds, and the duration of this increase is measured as t_{on} . (C) The overall lifetime T_{on} is determined by fitting all the t_{on} values collected to an exponential decay function.

(Fig. 2C). When the end was blocked with dig–antidig to trap freely diffusing sliding clamps, the $T_{\rm on}$ was extremely long (~509 s). In contrast, sliding clamp rapidly dissociated from DNA substrate containing a single–strand (ss) DNA tail, deduced from the short lifetime (Fig. 2A). The dissociation kinetic was similar regardless of the dig–antidig or RPA blocking, suggesting that the MSH2–MSH6 dissociation is stimulated upon encountering ssDNA–dsDNA junction.

While there are other conventional assays that could also detect the binding of protein to nucleic acid, it is impossible for ensemble technique to detect transient binding of the proteins to their substrate. Since its characterization in 2011, single-molecule PIFE assay has been used as the primary source of measurement for comparing the binding constant of a variety of proteins. Some examples include RIG-I and its truncation mutant (CARDless RIG-I) (Myong et al., 2009), Pol1A and its concentrationdependent binding constant (Markiewicz, Vrtis, Rueda, & Romano, 2012), and the heterodimeric and homodimeric forms of HIV-1 reverse transcriptase protein to DNA–DNA substrates (Marko et al., 2013). It is also often used as a complementary tool to single-molecule FRET, either to confirm the binding of a protein or complex structure to other substrates (Craggs, Hutton, Brenlla, White, & Penedo, 2014), or to estimate base pair distance based on the intensity increase.

4. FILAMENT FORMATION AND PROTEIN BINDING TO LONG DNA STRANDS

We have previously reported that the intensity of the Cy3 dye can be enhanced up to $2.6 \times$ upon the binding of a single protein. This is the most striking advantage of PIFE over FRET, allowing the observation of protein binding kinetics without the need to label the protein. When multiple proteins bind as a group, however, the PIFE effect can reach as high as 3–4 times fluorescence enhancement. This effect was demonstrated when proteins such as RecA and Rad51 assemble into filaments around the DNA substrate (Hwang et al., 2011; Qiu et al., 2013). In our previous study, we used smFRET to determine the step size of Rad51 monomer (3 nt) that leads to filament formation. While stepwise FRET decrease reported on monomer Rad51 binding, it did not inform us about the directionality of filament formation. This was complemented by smPIFE which defined 5' to 3' filament growth direction (Hwang & Myong, 2014; Qiu et al., 2013). A partial duplex DNA with 18 bp of dsDNA with a 3' ssDNA tail composed of polydeoxythymine was labeled with the Cy3 dye at either end of the ssDNA tail. After immobilizing the substrate DNA on single-molecule surface, $1 \mu M$ of Rad51 and 1 m M ATP were added and the rate of PIFE increase was measured. The rate of intensity increase differed between the two dye positions since the Rad51 filament will grow toward the dye in one case and away from the dye in the other. The rate difference can only be measured by PIFE, and allows us to determine the direction of filament growth.

In a recent study by Song et al., smPIFE was utilized to monitor protein binding and DNA remodeling process (Song, Graham, & Loparo, 2016). A 20 kilobase (kb) of dsDNA was sparsely labeled with Cy3 fluorophores and immobilized to single-molecule surface by biotin-streptavidin (Fig. 3A, step 1). Flow was applied to add the DNA binding proteins, which resulted in PIFE effect (Fig. 3A and B, step 2). Subsequently, DNA compaction/shortening was monitored (Fig. 3A and B, step 3) as reduction in DNA length. In contrast, a mutant protein defective in DNA remodeling activity



Fig. 3 (A) Schematic of multiple proteins binding on a long dsDNA sparsely labeled with Cy3 fluorophores. (B) As each protein binds, the Cy3 intensity increases until (C) DNA compaction is finished and maximum PIFE intensity has increased.

resulted in a similar PIFE effect followed by no change in DNA length, in agreement with the known defect in DNA compaction activity (Fig. 3C). This unique imaging capability allowed the authors to determine the protein binding kinetic and the rate of DNA compaction based on the dwell times collected from single-molecule traces.

5. PROTEIN BINDING AND MOTILITY KINETICS

While PIFE is extremely useful as a direct indicator of single or even multiple protein binding, it can also be an efficient tool to measure movement of motor proteins or intrinsically dynamic proteins that slide on DNA/ RNA substrates. Many proteins exhibit sliding activity on DNA/RNA after binding. PIFE signal increases as protein translocates toward the fluorophore. In cases where protein traverses through DNA substrates, different DNA lengths can be tested. If the rate of PIFE fluctuation is correlated with the length of DNA/RNA substrate, it indicates that the protein traverses through the entire length of the substrate rather than a defined length. This rate of translocation or sliding can then be estimated from the speed of fluorescence increase based on the PIFE distance sensitivity. The rate is determined assuming a linear intensity–distance dependence based on our previous calibration, i.e., we assume the maximum and minimum intensity to correspond to 0 and approximately 4 nm from the dye (Hwang et al., 2011; Hwang & Myong, 2014). In cases where the protein activity is stimulated by ATP, the same kinetic experiment can be performed with varying ATP concentrations, which yields the rate of protein translocation at different ATP concentrations. The rate can be fitted to the Michaelis–Menten equation for obtaining $K_{\rm m}$ and $V_{\rm max}$ for the given protein (Hwang & Myong, 2014).

Recent study by Zhou et al. implemented smPIFE in conjunction with smFRET to determine the mechanism of ssDNA translocase, Pif1 (Zhou, Zhang, Bochman, Zakian, & Ha, 2014). The repetitive translocation activity was detected by smFRET which displayed periodic FRET increase and decrease, consistent with repetitive "reeling-in" activity of the protein. By performing smPIFE measurement on the same DNA with Cy3 situated in different locations, i.e., either tip of ssDNA, Pif1 produced a periodic PIFE signal fluctuation on one substrate (Fig. 4A) but not on the alternate substrate (Fig. 4B). Based on this result, the authors confirmed that Pif1 remains at ssDNA/dsDNA junction while periodically reeling in ssDNA powered by ATP hydrolysis.

Our recent study extended the usage of PIFE technique by testing proteins extracted from mammalian cell lysate (Wang, Vukovic, Koh, Schulten, & Myong, 2015). The antibody-based single-molecule pull-down technique was developed by Jain et al. (2011). Previously, we reported that a double-strand RNA binding protein (dsRBP), called Tar RNA binding protein (TRBP), exhibits ATP-independent sliding activity on dsRNA (Koh, Kidwell, Ragunathan, Doudna, & Myong, 2013). To examine if other dsRBPs also display such motility, we conducted a comprehensive study by expanding the array of dsRBPs including ADAD2, Staufen1, and ADAR1 in addition to TRBP. The four dsRBPs were chosen based on the presence of highly conserved dsRBD-I (type I dsRBD) domains even though they differed in total length and overall domain composition. The dsRBP protein was applied to single-molecule surface by adding cell lysate containing overexpressed dsRBP (Fig. 4C). Each protein was tagged with yellow fluorescence protein (YFP), which enabled visualizing the protein expression in cells and pulling down of dsRBP to single-molecule surface coated with anti-YFP antibody (Fig. 4C). Due to an extremely fast photobleaching (<3 s), the YFP does not interfere with the PIFE measurement. On this platform, nonbiotinylated, Cy3-labeled dsRNA (>25 bp) was added to check for the diffusion activity of the four dsRBPs. We performed such experiment on six structural variants of RNA substrates. The PIFE



Fig. 4 (A) Schematic of Pif1 translocating on a DNA substrate that has a Cy3 dye at the free end of the ssDNA. For example, single-molecule trace below shows periodic intensity fluctuations corresponding to Pif1 coming near the dye. (B) Schematic of Pif1 translocating on a DNA substrate that has a Cy3 dye at the junction of the duplex DNA. For example, single-molecule trace below shows no increase in PIFE intensity, suggesting that Pif1 movement is not within the PIFE sensitivity range of the Cy3 dye. (C) Schematic of dsRBP binding experiments using mammalian cell lysate, dsRNA, and detection by PIFE. Two types of single-molecule traces are commonly seen in (D). A static intensity trace (*upper*) indicates protein binding only, and a dynamic intensity trace (*lower*) featuring periodic fluctuations indicates protein diffusion along the dsRNA.

signal fluctuation indicated sliding activity of proteins near the dye. This is a reverse configuration as the protein, instead of the dsRNA, was immobilized to the surface and movement of the RNA along dsRBP was monitored (Fig. 4D). The fraction of sliding molecules was quantified and TRBP showed the highest sliding activity (70%) out of the four, followed by Staufen1 (20–30%). Dwell time analysis for RNA length variants displayed a clear length dependence, indicating that the dsRBP likely slides on the entire length of dsRNA (Wang et al., 2015). Briefly, for each RNA tested, the rate of sliding, or dwell time, can be calculated from the peak-to-peak values of the PIFE fluctuations. The peak-to-peak dwell time represents the time in which the protein undergoes sliding away from the dye. Therefore,

the dwell time indicates how long it takes for the protein to complete one cycle (round trip) of sliding. The dye is positioned at one end of the RNA substrates to minimize interfering with protein binding or sliding and also for comparing dwell times in different RNA lengths.

6. PIFE IN THE PRESENCE OF FRET

If the experiment is designed for FRET measurement, it is the most direct to interpret the result by FRET alone rather than decoupling PIFE and FRET effect. Nevertheless, there are some cases in which PIFE effect can be observed or deduced in FRET experiment. First, the PIFE effect can be seen after the photobleaching of an acceptor dye. This occurs frequently because Cy5 (acceptor) typically undergoes photobleaching more rapidly than Cy3 (donor). If the repetitive translocation activity of a motor protein such as PcrA (Park et al., 2010) and Pif1 (Zhou et al., 2014) was monitored on FRET-DNA construct with Cy3 and Cy5 attached at either end of ssDNA, the reeling-in of ssDNA can be visualized by the periodic FRET fluctuation. After the photobleaching of Cy5 dye at the ssDNA/dsDNA junction, the same activity can be displayed by PIFE signal change (Fig. 5A). In such cases, the confirmatory information obtained from PIFE experiment is that the protein is mediating the reeling-in motion by physically bringing in the end of ssDNA. Second, the PIFE effect can be observed when the protein binding occurs near both donor and acceptor dyes. We took advantage of this effect in our previous study in which RNA helicase A (RHA) protein binding to partially duplexed RNA substrate was visualized as a simultaneous increase in both Cy3 and Cy5 intensity (Fig. 5B). Since both dyes exhibit the *cis-trans* isomerization, it is reasonable to expect PIFE effect on Cy3 and Cy5. We note that we are not comparing PIFE effect between the two dyes quantitatively. We are interpreting the presence of PIFE effect on both dyes as a signature of protein binding near both dyes. This is particularly useful because while the protein binding does not induce FRET change, leaving the PIFE signal as the only means to observe the protein binding (Fig. 5C) (Koh, Xing, Kleiman, & Myong, 2014). The PIFE effect is more pronounced in Cy3 than in Cy5 because RHA binding occurs at the ssRNA/dsRNA junction more proximal to Cy3. Such effect can also be seen in the total intensity (sum of Cy3 and Cy5) trajectory in which the protein binding induces a clear stepwise increase in the total intensity. The time interval between the addition of protein (Fig. 5C, black arrow) and binding of protein (Fig. 5C, red arrow) was collected from many molecules to obtain the



Fig. 5 (A) Schematic of protein interaction with a FRET DNA structure that has the Cy5 dye photobleaches after some time. Corresponding intensity traces show anticorrelated Cy3–Cy5 intensity traces that changes to Cy3 intensity only as Cy5 photobleaches. Note that the periodic intensity fluctuations remain even after Cy5 photobleaches. (B, C) Schematic and single-molecule trace of RHA protein binding to partially duplex RNA substrate labeled with FRET and its eventual unwinding. Binding of protein does not induce change in FRET level but can be seen in the PIFE increase.

binding kinetic. This enabled us to distinguish the binding rate (B) and the off rate (A), which cannot be discerned based on FRET alone (Fig. 5C). PIFE and FRET can be combined as long as the PIFE effect occurs without affecting FRET. In addition to the example given above, one can design a substrate in which two dyes are outside of FRET detection range. PIFE effect on one side can be distinguished from FRET change since only one intensity will increase without the anticorrelated decrease in the other dye.

7. CONCLUSIONS

Since its initial characterization in 2011, the smPIFE has become an increasingly useful tool for researchers to conduct single-molecule protein binding study without protein modification. The multitude of proteins

for which PIFE technique has been applied strongly reflects the universality of PIFE method. Exceptions can be for proteins that possess nickel or manganese metal binding pocket and FeS-containing proteins (Spies, 2014) which may quench the fluorescence when it comes in contact with the fluorophore, although we have never encountered such a case. The high sensitivity of PIFE enables detection of very transient interactions that may not be resolved by biochemical means such as EMSA. In addition, as demonstrated by our previous study on Rad51, the directionally biased intensity increase (always toward dye) makes PIFE an ideal tool for determining the direction of filament growth. Most of the proteins studied so far are involved in the DNA and RNA processing pathways; therefore, it will be exciting to foray into proteins that modify other proteins such as kinases, phosphorylases, and methyl transferases in the near future.

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