Published in final edited form as:

J Am Chem Soc. 2012 April 4; 134(13): 5734–5737. doi:10.1021/ja2119964.

Non-fluorescent Quenchers to Correlate Single-Molecule Conformational and Compositional Dynamics

Jin Chen^{1,2}, Albert Tsai^{1,2}, Alexey Petrov², and Joseph D. Puglisi^{2,*}

¹Department of Applied Physics, Stanford University, Stanford, CA 94305-4090, USA

²Department of Structural Biology, Stanford University School of Medicine, Stanford, CA 94305-5126, USA

Abstract

Single-molecule Förster resonance energy transfer (smFRET) is a powerful method to study the conformational dynamics of a biomolecule in real-time. However, studying how interacting ligands correlate with and regulate the conformational dynamics of the biomolecule is extremely challenging due to the availability of a limited number of fluorescent dyes with both high quantum yield and minimal spectral overlap. Here, we report the use of a non-fluorescent quencher (Black Hole Quencher, BHQ) as an acceptor for smFRET. Using a Cy3/BHQ pair, we accurately follow conformational changes of the ribosome during elongation in real time. We demonstrate the application of single-color FRET to correlate the conformational dynamics of the ribosome with the compositional dynamics of tRNA. We use the normal Cy5 FRET acceptor to observe arrival of a fluorescently-labeled tRNA with a concomitant transition of the ribosome from the locked to the unlocked conformation. Our results illustrate the potential of non-fluorescent quenchers in single-molecule correlation studies.

Single-molecule Förster resonance energy transfer (smFRET) is a powerful technique to study biomolecular dynamics and interactions, providing conformational information regarding the system that is free from ensemble averaging. smFRET studies on transcription, translation, replication, recombination, RNA folding, and protein folding have revealed many rare, transient interactions and conformational subpopulations that are undetectable by bulk biochemical methods^{1–4}. Recent single-molecule studies on translation allowed us to follow, in real-time, ribosome conformation and tRNA dynamics^{5–13}.

FRET efficiency between two fluorescent dye molecules (termed donor and acceptor) depends strongly on the distance between the two dyes¹⁴. The energy transfer efficiency is given by

Corresponding Author. puglisi@stanford.edu.

ASSOCIATED CONTENT

Supplementary figures, information, and data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

J.C. conducted experiments and analyzed the data. J.C., A.T., A.P., and J.D.P. designed the experiments. J.C. and J.D.P. wrote the paper.

$$E = \frac{1}{1 + (r/R_0)^6} \quad (1)$$

where r is the inter-dye distance and R_0 is the Förster radius, the inter-dye distance with a FRET efficiency of 0.5¹⁵.

The Cy3 and Cy5 dye pair is commonly employed in single molecule systems. In a smFRET experiment, Cy3 (the donor) is excited with a 532 nm laser, and the energy is transferred to a nearby Cy5 (the acceptor) dye, which then fluoresces in the red region of the spectrum. Because FRET alters the time-averaged fluorescence intensities of the donor and acceptor, FRET efficiency can be simply calculated with:

$$E = \frac{I_{Cy5}}{I_{Cy3} + I_{Cy5}} \quad (2)$$

The intensities used here have to be corrected for detection efficiencies, quantum yields, and background.

Even though smFRET reports on conformational dynamics in real-time with angstrom resolution, how these conformational dynamics correlate with the interactions of other compositions and ligands remain invisible. However, the small number of dyes suitable for single-molecule fluorescence hinders studies of such multicomponent systems by limiting the number of components that can be observed simultaneously. Other issues include that the photo-physical behavior of acceptor dyes in FRET is often suboptimal, marred by short photobleaching times and long-lived non-fluorescent dark states. Also, the concentration of fluorescent acceptors in solution often cannot exceed 50 nM in fluorescence microscopy due to excessive background (because of cross-talk from donor excitation), hampering investigation of many biological systems.

Here we report the use of a non-fluorescent quencher as an acceptor in FRET, thus mitigating the aforementioned problems. We replaced the acceptor dye Cy5 with the non-radiative acceptor Black Hole Quencher (BHQ-2, Figure 1b). The energy transferred from the donor dye to BHQ is released as heat instead of photons. By measuring the quenched intensity of the donor and comparing with the unquenched intensity, we can calculate the single-dye FRET efficiency:

$$E = 1 - \frac{I_{Cy3}}{I_{Cy3,MAX}} \quad (3)$$

Using BHQ as the non-fluorescent FRET acceptor lacks the traditional anti-correlated intensity signal of the acceptor dye, but conformational information can still be extracted by examining the donor dye signal. This approach has several advantages. Most importantly, the use of BHQ frees the spectral region of the acceptor dye for labeling other components of the system in multiplexed experiments. The use of BHQ further eliminates the poor behavior of the acceptor fluorescence, extending the observation time by removing the

problem of the acceptor dye photobleaching (Supplementary Figure 1). The high spectral overlap between Cy3 and BHQ results in efficient energy transfer for FRET¹⁶ (Figure 1a). Since BHQ itself is not fluorescent, background noise is reduced, resulting in an increased signal-to-noise ratio at high concentrations of BHQ-labeled molecules. We demonstrated the utility of FRET with non-fluorescent quenchers for single-molecule dynamics and time-correlation measurements by observing conformational changes of the ribosome during translation in real time.

Previous work on non-fluorescent quenchers has shown their applications in a wide variety of systems. Schwartz *et al.* labeled Cy3 and BHQ-2 on opposite ends of a DNA hairpin to measure the replication through the hairpin by a DNA polymerase¹⁷. Takada *et al.* employed a TAMRA/BHQ system to investigate DNA hole transfer¹⁸. Other work also demonstrated the use of non-fluorescent quenchers in FRET assays and microscopy^{19–22}. However, in this work, we demonstrate the use of BHQ to correlate the conformational dynamics of a biomolecule in interest with the interactions of other factors and ligands. This is of particular importance since the conformational dynamics of a biomolecule are often regulated through the interactions of other ligands.

The behavior of the donor dye-BHQ pair was first characterized by using oligonucleotide standards with varying distances between Cy3 and BHQ. We measured Cy3 intensity of five duplex oligonucleotides with increasing distances between Cy3 and BHQ-2 ranging from 41 to 68 Å, assuming a B-form DNA conformation with a rise of 3.4 Å per base pair. A 5'-biotinylated and 3'-Cy3-labeled oligonucleotide was annealed to a series of 3'-BHQ-labeled oligonucleotides of varying length, providing a defined set of FRET distances between Cy3 and BHQ. The duplexes were immobilized on biotin-PEG-covered quartz surfaces through a neutravidin-biotin interaction and imaged using a prism-based total internal reflection fluorescence (TIRF) microscope with 532-nm excitation at 100 ms exposure time per frame⁵.

We observed the expected decrease of Cy3 emission intensity as the inter-dye distance decreased, in excellent agreement with the theoretical FRET trace (Figure 1c), calculated using a R_0 for Cy3-BHQ of 50.2 Å¹⁷. These results confirm that the Cy3 BHQ system works as a one-color FRET pair for single-molecule experiments with expected quenching efficiencies.

Having shown that BHQ works as expected in static systems, we next characterized BHQ in a system with dynamic conformational changes, where most of the potential applications of BHQ lie. We characterized the behavior of donor-BHQ pair using ratcheting *Escherichia coli* ribosomes that are site-specifically labeled with fluorescent dyes on the 30S and 50S subunits at positions that result in inter-subunit FRET, a system that has been well characterized previously^{5,23}.

Cy3-labeled 30S pre-initiation complexes (PICs) containing fMet-tRNA^{fMet} were immobilized through biotinylated mRNA on a PEG-derivatized quartz surface. The 6(FK) mRNA contains the 5' untranslated region (UTR) and Shine-Dalgarno sequence from phage T4 gene 32, followed by six alternating Phe and Lys codon, UAA stop codon, and four

additional Phe codons. BHQ-labeled 50S subunits, elongator ternary complex (aa-tRNA•EF-Tu•GTP), EF-G, and IF2 were delivered to the surface (Figure 2a). Initiation occurs upon IF2-mediated 50S subunit joining to 30S subunits containing an initiator tRNA. The fluorescence intensity of Cy3 drops upon assembly of the 70S complex during initiation as Cy3 is quenched by BHQ. During elongation, fluorescence intensity alternates between a high and low intensity state, which is consistent with the locking and unlocking of the ribosome upon translocation (Figure 2b). The high intensity state (similar to the low-FRET state for Cy3-Cy5 FRET) corresponds to the unlocked conformation, and the low intensity state corresponds to the locked conformation of the ribosome. Each cycle of low-high-low intensity states represents one round of elongation, as confirmed previously⁵. We can calculate the FRET efficiency of the two states with Equation 3.

Using the intensity before 50S subunit joining as I_{MAX} , the FRET efficiencies of the two states are ~0.30 and ~0.49 (Supplementary Figure 1). Given the R_0 of Cy3 and BHQ (50.2 Å), the Cy3-BHQ FRET efficiencies match well with theoretical calculations (Supplementary Figure 4). Thus, we were able to use BHQ to observe subunit joining and ribosomal conformational changes in real time that occur during translation initiation and elongation.

Cy3/BHQ-labeled ribosomes were next used to follow ribosome dynamics through multiple cycles of elongation. In the presence of 80 nM each of Phe and Lys ternary complexes (TC), and EF-G, we observed ribosomes translating the entire 6(FK) mRNA (Figure 2b). The number of observed elongation cycles for ~300 ribosomes is shown as histograms in Figure 2c and 2d. The main challenge for observation of all the elongation cycles is fluorophore photobleaching. Withholding Lys tRNA TC correctly prevents the ribosome from proceeding beyond the first codon (Figure 2c).

To test that our measured elongation rates depend properly on ternary complex and EF-G concentrations as previously reported⁵, we varied their concentrations. At 160 nM Phe TC and Lys TC, but 80 nM EF-G, the apparent translation efficiency is higher than at 80 nM TC and 80 nM EF-G (Figure 3a), which is attributable to a faster elongation rate translating more codons before Cy3 photobleaches. The mean lifetimes of the two elongation states is determined by fitting lifetimes to a single exponential distribution. By doubling the concentration of TC from 80 nM to 160 nM, the lifetime of the high FRET state (locked conformation of the ribosome waiting for peptide bond formation) is decreased (Figure 3c). The lifetimes of the low FRET state (unlocked conformation of the ribosome waiting to translocate) does not change, since the lifetime depends on the concentration of EF-G (Figure 3d). Moreover, the rate of 50S subunit joining depends only on the concentration of 50S subunit, and not on the concentration of ternary complex (Supplementary Figure 3). Using Cy3 and BHQ labeled ribosomes and using our statistical analytical methods, we can accurately identify all the ratcheting signals as well as calculate mean lifetimes that agree well with previous reports⁵. This shows that BHQ is effective as a non-fluorescent FRET acceptor for studying conformational dynamics.

We next extended the Cy3-BHQ FRET system by exploiting the vacant Cy5 spectral region, re-introducing Cy5 as a reporter for another molecule rather than as a Cy3 FRET acceptor.

To improve our signal under dual illumination at 532 nm and 647 nm, we replaced the Cy3 fluorophore used to label the 30S subunit with Cy3B, which has an increased quantum yield. FRET distributions and lifetimes for ribosomes labeled with Cy3B are identical to those obtained with Cy3-labeled ribosomes, except that their FRET efficiencies are shifted to higher values (~0.4 and ~0.6) (Supplementary Figure 5). From this, we calculated that R_0 for Cy3B and BHQ is ~54 Å. As a result of the increased brightness of Cy3B and signal-to-noise ratio (3.43 compared to 1.93 of Cy3), we could shorten the exposure time for data collection to 25 ms from the original 100 ms. This permitted increasing TC to 200 nM and EF-G to 500 nM, approaching concentrations found *in vivo*. At these conditions we were able to identify accurately ratcheting signals, obtaining lifetimes that matched expected values (Supplementary Figure 6).

We followed translation of the 6(FK) mRNA in the presence of Phe-(Cy5)tRNA^{Phe} and unlabeled Lys-tRNA^{Lys} to correlate the compositional signals of the arrival of tRNAs with the conformational dynamics of Cy3B/BHQ labeled ribosomes. Arrival of a Phe-tRNA^{Phe}, shown as a red fluorescent pulse, is concomitant with the transition from low to high donor intensity of the ratcheting signal, but sensitive to the position of the ribosome on the mRNA (Figure 4a). Post-synchronizing both ribosome conformational signals and normalized red fluorescent pulses to the first and second high-low FRET transitions (low-high intensity transition) reveals that Phe-(Cy5)tRNA^{Phe} arrives only in the first FRET transition, consistent with the mRNA sequence. Within the time resolution of this measurement (100 ms), the arrival of Phe-(Cy5)tRNA^{Phe} occurs simultaneously with ribosome unlocking.

The observed mean Cy5 tRNA lifetime of 20.8 seconds (Supplementary Figure 7) and a signal-to-noise ratio of 1.59 (compared to a mean lifetime of < 4 seconds and signal-to-noise ratio of < 1.0 for Cy2)⁵, allowed following the arrival and departure of tRNAs. Our tRNA correlation experiment shows that tRNA^{Phe} arrival to the ribosome and peptide bond formation is correlated with conformational changes of the ribosomal subunits, consistent with previous findings^{9–11}. tRNA^{Phe} arrival and selection leads to the simultaneous unlocking of the ribosomal subunit. This tRNA then transits through the ribosome, passing from the A/P hybrid state into the P site upon one round of translocation. The subsequent tRNA^{Lys} then arrives, placing the tRNA^{Phe} in the P/E hybrid state. The final round of translocation, driven by EF-G, causes the locking of the ribosome and release of tRNA^{Phe} from the E site. With this signal, we were able to follow the ribosome in real-time as it transitions between the locked and unlocked conformations concurrent with tRNA transits. This method can be extended to other translation factors to study the correlation between the ribosomal conformational changes and these factors, or even to other biomolecular systems.

In summary, this study shows the promise of non-fluorescent FRET acceptors for correlating single-molecule conformational and compositional dynamics. We have used Cy3-(or Cy3B)-labeled 30S ribosomal subunits with BHQ-labeled 50S subunits to observe translation initiation and elongation in real-time with single-codon resolution, and our results are comparable with previous data obtained using Cy3-Cy5 FRET. By using the BHQ as the FRET acceptor, additional spectral channels are freed for multiplexed single-molecule experiments and correlation studies. This allows not only the study of conformational dynamics, but also the interactions of multiple biomolecules and how they

regulate conformation. BHQ thus provides a powerful way to investigate correlated dynamics of different factors that were not previously accessible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank Seán O'Leary (Stanford), Guy Kornberg (Stanford), and Thomas Noriega (UCSF) for valuable discussions. This work is funded by NIH grant GM51266.

ABBREVIATIONS

smFRET	single molecule Förster resonance energy transfer
BHQ	Black Hole Quencher
TC	ternary complex
EF-G	elongation factor G

REFERENCES

1. Joo C, Balci H, Ishitsuka Y, Buranachai C, Ha T. *Annu. Rev. Biochem.* 2008; 77:51. [PubMed: 18412538]
2. Weiss S. *Science.* 1999; 283:1676. [PubMed: 10073925]
3. Weiss S. *Nat. Struct. Biol.* 2000; 7:724. [PubMed: 10966638]
4. Tinoco I Jr, Gonzalez RL Jr. *Genes Dev.* 2011; 25:1205. [PubMed: 21685361]
5. Aitken CE, Puglisi JD. *Nat. Struct. Mol. Biol.* 2010; 17:793. [PubMed: 20562856]
6. Blanchard SC, Gonzalez RL, Kim HD, Chu S, Puglisi JD. *Nat. Struct. Mol. Biol.* 2004; 11:1008. [PubMed: 15448679]
7. Blanchard SC, Kim HD, Gonzalez RL Jr, Puglisi JD, Chu S. *Proc. Natl. Acad. Sci. U.S.A.* 2004; 101:12893. [PubMed: 15317937]
8. Cornish PV, Ermolenko DN, Noller HF, Ha T. *Mol. Cell.* 2008; 30:578. [PubMed: 18538656]
9. Fei J, Bronson JE, Hofman JM, Srinivas RL, Wiggins CH, Gonzalez RL Jr. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106:15702. [PubMed: 19717422]
10. Fei J, Kosuri P, MacDougall DD, Gonzalez RL Jr. *Mol. Cell.* 2008; 30:348. [PubMed: 18471980]
11. Marshall RA, Dorywalska M, Puglisi JD. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:15364. [PubMed: 18824686]
12. Uemura S, Aitken CE, Korlach J, Flusberg BA, Turner SW, Puglisi JD. *Nature.* 2010; 464:1012. [PubMed: 20393556]
13. Wen JD, Lancaster L, Hodges C, Zeri AC, Yoshimura SH, Noller HF, Bustamante C, Tinoco I. *Nature.* 2008; 452:598. [PubMed: 18327250]
14. Roy R, Hohng S, Ha T. *Nat. Methods.* 2008; 5:507. [PubMed: 18511918]
15. Lakowicz, JR. *Principles of fluorescence spectroscopy.* 3rd ed.. New York: Springer; 2006.
16. Marras SA, Kramer FR, Tyagi S. *Nucleic Acids Res.* 2002; 30:e122. [PubMed: 12409481]
17. Schwartz JJ, Quake SR. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106:20294. [PubMed: 19906998]
18. Takada T, Takeda Y, Fujitsuka M, Majima T. *J. Am. Chem. Soc.* 2009; 131:6656. [PubMed: 19388695]
19. Orte A, Clarke RW, Klenerman D. *Anal. Chem.* 2008; 80:8389. [PubMed: 18855410]

20. Youn HJ, Terpetschnig E, Szmecinski H, Lakowicz JR. Anal. Biochem. 1995; 232:24. [PubMed: 8600827]
21. Tahmassebi DC, Millar DP. Biochem. Biophys. Res. Commun. 2009; 380:277. [PubMed: 19167347]
22. Ganesan S, Ameer-Beg SM, Ng TT, Vojnovic B, Wouters FS. Proc. Natl. Acad. Sci. U.S.A. 2006; 103:4089. [PubMed: 16537489]
23. Dorywalska M, Blanchard SC, Gonzalez RL, Kim HD, Chu S, Puglisi JD. Nucleic Acids Res. 2005; 33:182. [PubMed: 15647501]

Figure 1.

(a) Chemical structure of BHQ-2 conjugated to an oligonucleotide. The emission spectrum of Cy3 with absorption spectrum of BHQ-2 shows large spectral overlap for efficient energy transfer during FRET. (b) Intensity histogram of doubly-labeled oligonucleotides of varying inter-dye distance between Cy3 and BHQ. (c) Mean Cy3 fluorescence intensity as a function of inter-dye distance, along with the theoretical FRET curve. Error bars are 1 standard deviation. The mean intensity increases with increasing inter-dye distance, following the theoretical FRET curve within 1 standard deviation of error (resulting from the error in estimating I_{MAX} when calculating fluorescence intensity from FRET efficiency).

Figure 2.

(a) Schematic of delivering BHQ-labeled 50S subunits with EF-G and ternary complex to pre-assembled PIC immobilized on PEG-covered quartz surface. (b) Representative time trace of fluorescence intensity for elongating ribosome translating mRNA 6(FK) (alternating Phe and Lys). On the right shows the histogram of the normalized number of elongation cycles observed for single ribosomes. (c). Representative time trace for elongating ribosome translating 6(FK) without Lys tRNA. Translation is stalled after the first codon. The small number of additional events beyond the first codon shown in the histogram is likely due to statistical errors in the identification of transitions by our analytical method.

Figure 3.

(a) Comparison histogram of apparent translation efficiency at 80 nM TC (red) and 160 nM TC (blue). At higher [TC], the apparent translation efficiency is higher. (b) FRET state of the two conformational states, with a mean corresponding to 0.3 for the unlocked state and a mean of 0.49 for the locked state. (c) Mean lifetime (sec) of the locked state. The lifetime is lowered as [TC] is increased. (d) Mean lifetime (sec) of unlocked state. The lifetime is not dependent on [TC].

Figure 4.

(a) Representative time trace of a correlation experiment, with the green trace (Cy3B) showing the ribosome locking and unlocking during elongation, and the red (Cy5) showing the arrival and departure of Phe-tRNA^{Phe}. (b) Post-synchronized time trace of the arrival of Phe-(Cy5)tRNA^{Phe} to the conformation change of the ribosome from locked to unlocked state. The arrival of tRNA is directly correlated with the unlocking of the ribosome. The

unlocking of the ribosome at the second codon is not correlated with a Cy5 pulse, consistent with the mRNA sequence.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript