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OS-FRET: A New One-Sample Method for Improved FRET Measurements[†]

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ABSTRACT: Fluorescence resonance energy transfer (FRET) is a powerful tool for studying macromolecular assemblies in vitro under near-physiological conditions. Here we present a new type of one-sample FRET (OS-FRET) method employing a novel, nonfluorescent methanethiosulfonate-linked acceptor that can be reversibly coupled to a target sulfhydryl residue via a disulfide bond. After the quenched donor emission is quantitated, the acceptor is removed by reduction, allowing measurement of unquenched donor emission in the same sample. Previous one-sample methods provide distinct advantages in specific FRET applications. The new OS-FRET method is a generalizable spectrochemical approach that can be applied to macromolecular systems lacking essential disulfide bonds and eliminates the potential systematic errors of some earlier one-sample methods. In addition, OS-FRET enables quantitative FRET measurements in virtually any fluorescence spectrometer or detection device. Compared to conventional multisample FRET methods, OS-FRET conserves sample, increases the precision of data, and shortens the time per measurement. The utility of the method is illustrated by its application to a protein complex of known structure formed by CheW and the P4−P5 fragment of CheA, both from *Thermotoga maritima*. The findings confirm the practicality and advantages of OS-FRET. Anticipated applications of OS-FRET include analysis of macromolecular structure, binding and conformational dynamics, and high-throughput screening for interactions and inhibitors.

Fluorescence resonance energy transfer (FRET)[1] is widely used to study protein−protein interactions and conformational changes in soluble and membrane-associated complexes. Its resolution is lower than that of crystallography or NMR, but it can be applied to systems of arbitrary size and often can be conducted using near-physiological protein concentrations and buffer conditions.

FRET is a nonradiative energy transfer process in which the excitation energy of an excited donor fluorophore is transferred to a nearby ground state acceptor chromophore, thereby returning the donor to the ground state while exciting the acceptor. To a first approximation, the efficiency of energy transfer E is given by

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

and

$$R_0 \propto [\kappa^2 \eta^{-4} Q_D J(\lambda)]^{1/6} \quad (2)$$

where r is the distance between the chromophores and R_0 is the Förster distance at which the transfer efficiency (E) equals $^1/_2$. For a given donor−acceptor pair, κ^2 is the orientation factor describing the relative geometries of the donor and acceptor transition dipoles, η is the refractive index of the medium, Q_D is the fluorescence quantum yield of the donor in the absence of FRET, and $J(\lambda)$ is the integrated overlap of the donor and acceptor emission and absorption spectra, respectively (*1*−*3*).

FRET efficiency is often quantitated as the loss of donor fluorescence intensity in the presence of an acceptor fluor, which requires up to three samples (Figure 1a, top panel): (1) a donor-only sample to measure the initial donor fluorescence, (2) a donor−acceptor sample to measure the donor fluorescence in the presence of FRET, and (3) an acceptor-only sample to quantitate fluorescence at the donor emission wavelength arising from direct acceptor excitation. In recent years, some studies have employed a nonfluorescent acceptor, termed a dark acceptor or quencher, which eliminates the need for the acceptor-only sample, but such studies still require both donor-only and donor−quencher samples to measure FRET efficiency (reviewed in ref *4*). When multiple samples are used to measure FRET, small variations in sample composition or cuvette positioning decrease the precision. Moreover, when a donor-only sample is employed, acceptor inner-filter effects may not be adequately quantitated. Measurement of FRET by donor lifetime analysis can overcome some of these weaknesses but generally requires two samples (reviewed in ref *2*).

Several specialized FRET methods requiring just one sample have been developed, each with unique advantages. However, some of these approaches may introduce systematic errors into FRET quantitation, while others may be difficult to generalize to different macromolecular systems or to macro-scale fluorescence detection devices (including fluorescence spectrometers). For example, one approach uncouples the donor−acceptor pair by proteolytically degrading the complex or by chemically releasing the donor (*5*, *6*), which may trigger significant changes in donor environment and spectral properties. A second approach bleaches or photoswitches the acceptor on−off using ultra-high-intensity irradiation (*7*−*13*), but this method alters acceptor

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[1]Abbreviations: FRET, fluorescence resonance energy transfer; OS-FRET, one-sample FRET; NFQ1, nonfluorescent quencher 1; MTS, methanethiosulfonate; DTT, dithiothreitol; Ni-NTA, Ni(II)-nitrilotriacetic acid; BSA, bovine serum albumin; TCEP, tris(2-carboxyethyl)phosphine; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; PCR, polymerase chain reaction.

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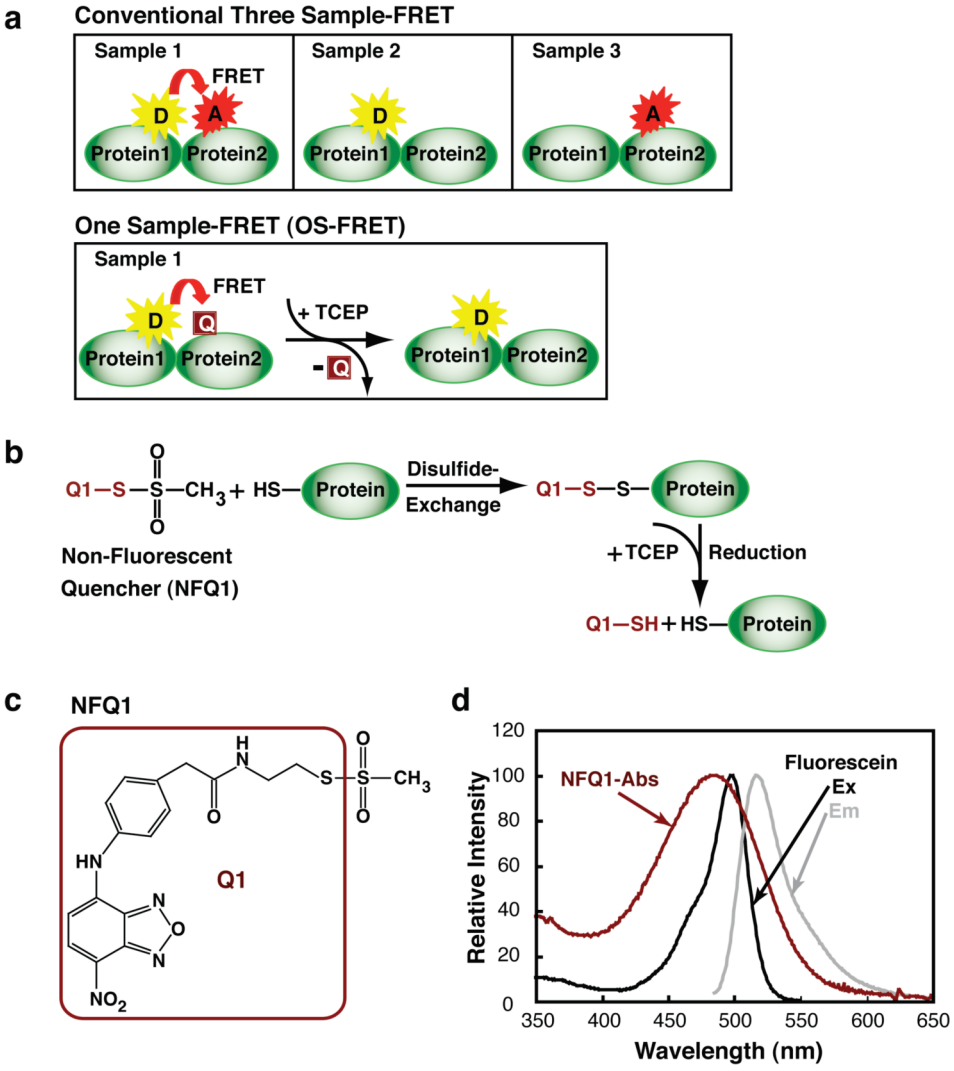

FIGURE 1: Principle of OS-FRET. (a) Comparison of the conventional three-sample FRET method employing a fluorescent donor−acceptor pair (top) to the new OS-FRET method utilizing a fluorescent donor and a reversible, nonfluorescent acceptor (or quencher, bottom). (b) Illustration of the reversible coupling chemistry used in OS-FRET. The label is attached via disulfide formation and can be quantitatively released by addition of a reducing agent like TCEP. (c) Chemical structure of the new nonfluorescent quencher 1 (NFQ1). (d) Spectral overlay of the NFQ1 absorption spectrum (brown) with the fluorescein excitation (black) and emission (gray) spectra.

inner-filter effects and may photodamage the sample. A third approach switches the acceptor on or off via a chemical modification; however, such a modification again alters acceptor inner filtering, and a second donor-only sample is typically required (*14*, *15*). A fourth approach utilizes a transition metal ion bound to a pair of engineered His residues as a reversible quencher released by addition of a metal chelator (*16*); however, the R_0 of ≤10 Å for such quenching is too short for many applications, and the need for two closely spaced His residues complicates the design of nonperturbing labeling sites. Finally, some single-molecule or nanoscale FRET methods (*13*, *17*−*21*) utilize only one sample but require highly specialized instrumentation and are not suitable for bulk samples or high-throughput assays.

Here we present a novel one-sample FRET (OS-FRET) method designed to minimize systematic errors while at the same time eliminating the need for multiple samples, thereby improving the precision of data, reducing the measurement time, and conserving materials relative to multisample approaches. The OS-FRET strategy quantitates FRET from a standard donor to a new type of acceptor combining two existing technologies into a single molecule: (i) the chromophore is a dark acceptor or quencher, thereby eliminating possible acceptor fluorescence at the donor emission wavelength because of direct acceptor excitation, and (ii) the coupling chemistry is a reversible disulfide, allowing measurement of quenched donor fluorescence, reductive release of quencher into solution, and quantitation of unquenched donor fluorescence all in the same sample (Figure 1a, bottom panel). The utility of the new method is tested in a well-characterized protein complex formed between the CheA and CheW proteins of bacterial chemotaxis. These proteins normally associate in the ultrastable, membrane-bound chemosensory signaling lattice (*22*), but the isolated P4−P5 fragment of CheA (CheA$_F$) yields a soluble bimolecular complex with CheW. The high-resolution crystal structure of the CheA$_F$−CheW complex has been determined for the *Thermotoga maritima* proteins [Protein Data Bank (PDB) entry 2CH4] (*23*). This representative application of OS-FRET confirms its simplicity and utility in the analysis of binding interactions, its suitability for standard fluorescence detection systems (including spectrofluorimeters), and its advantages over conventional multisample FRET methods.

MATERIALS AND METHODS

Materials. Reagents were obtained from the following sources: mutagenic oligonucleotides from Integrated DNA Technologies, sulfhydryl-specific probes fluorescein 5-maleimide (5FM) and Alexa 750 from Invitrogen Molecular Probes, Ni-NTA from Qiagen, BSA and TCEP from Thermo Fisher, and spectroscopic grade DMSO and DMF from Sigma. All other chemicals were analytical grade and from Sigma unless noted otherwise.

Mutagenesis. The plasmids encoding N-terminal six-His-tagged *T. maritima* CheW in the pET28a vector and the N-terminal six-His-tagged *T. maritima* CheA P4−P5 domain ($CheA_F$) in pET28a were a generous gift from B. Crane (Cornell University, Ithaca, NY) (*23*). Site-directed Cys mutants were generated using the PCR-based QuickChange XLII mutagenesis kit (Agilent). All mutants were confirmed by sequencing the entire coding region.

Protein Purification. His-tagged proteins were isolated by Ni-NTA affinity chromatography as described previously (*24*). Purified proteins were stored at −80 °C in buffer A [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 10 μM DTT, and 10% glycerol]. Protein concentrations were measured by the Bradford assay using BSA as a standard. Purity was quantitated by SDS−PAGE and Coomassie staining and was generally >98%.

NFQ1 Labeling. We prepared NFQ1 stock solutions by dissolving 20 mM NFQ1 in DMSO. Single-Cys mutant proteins were first incubated in buffer A with 1 mM DTT for 60 min at room temperature to reduce possible disulfides. DTT was then removed by size exclusion chromatography over a PD10 desalting column (GE Healthcare). Protein was collected directly in sample vials holding buffer B [50 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and free label. Final concentrations during labeling reactions were 5 μM protein and 25 μM label. The reaction was allowed to proceed for 2 h at room temperature in the dark while the mixture was gently stirred. Subsequently, protein was bound to a Ni-NTA column and the remaining free label removed when the column was extensively washed with 10 column volumes of buffer B. The labeled protein was eluted with buffer B containing 500 mM imidazole and dialyzed twice against buffer A without DTT. Finally, the samples were incubated with 5 mM *N*-ethylmaleimide for 30 min to block unlabeled Cys residues. Samples were aliquoted, snap-frozen in liquid N_2, and stored at −80 °C. Each aliquot was used only once and not refrozen. Labeling efficiency was quantitated as described previously (*25*), and no unlabeled protein was detected by electrospray mass spectrometry on an Applied Biosystems QStar Pulsar instrument (Figure S1 of the Supporting Information).

5FM and Alexa 750 Labeling. Labeling of single-Cys mutant proteins with 5FM or Alexa 750 was conducted as described for NFQ1 labeling with the following modifications. Stock solutions consisted of 20 mM 5FM in DMF and 40 mM Alexa 750 in DMSO. Labeling reactions were conducted for 30 min at room temperature in the dark. The masses of labeled proteins were verified by mass spectrometry on a PerSeptive Voyager DE-STR MALDI-TOF instrument.

FRET Measurements. (*i*) *Experimental Conditions*. Steady state fluorescence experiments utilized a Photon Technology International QM-2000-6SE fluorescence spectrometer and were conducted at 25 °C in buffer C [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.2 μM BSA, and 10% glycerol]. The excitation wavelength was 492 nm (slit width of 4 nm), and the emission wavelength was 515 nm (slit width of 8 nm). All fluorescence measurements were taken in ratiometric mode, in which the sample fluorescence is ratioed to a reference channel monitoring lamp fluctuations, thereby eliminating the contributions of lamp noise and intensity drift.

Because of the hydrophobicity of chromophores, particularly NFQ1, BSA (0.2 μM) was included in all samples to minimize nonspecific binding of labeled proteins to glass surfaces and to minimize nonspecific binding of free probe to protein, particularly after release of NFQ1 by reduction. We pretreated all pipet tips used for the FRET experiment by rinsing them with a 2 mg/mL BSA solution, pipetting up and down several times and then drying, to prevent nonspecific binding of labeled protein or free probe.

(*ii*) *Complex Formation*. Complexes for the different experiments were formed by incubation of 0.1 μM donor-labeled CheW mutant (or the unlabeled, NEM-treated protein) with 0.5 μM acceptor-labeled $CheA_F$ S660C (or the unlabeled, NEM-treated protein) for 15 min at room temperature.

(*iii*) *OS-FRET*. First, the donor fluorescence intensity of the donor−quencher complex between a 5FM-labeled CheW mutant and NFQ1-labeled $CheA_F$ S660C was measured. Subsequently, TCEP was added to a final concentration of 1 mM in the cuvette, still in the sample holder, and the sample was gently stirred in place for 15 min. This reduction step releases NFQ1 from the complex into solution out of FRET range, allowing measurement of the unquenched donor fluorescence intensity. Electrospray mass spectrometric analysis of the products confirmed that NFQ1 release was quantitative within the limit of detection (Figure S1 of the Supporting Information). The apparent FRET efficiency was calculated as

$$E_{app} = 1 - \frac{F_{DQ}}{F_{DQ+TCEP}} \quad (3)$$

where F_{DQ} is the donor fluorescence intensity of the donor−quencher complex and $F_{DQ+TCEP}$ is the unquenched donor fluorescence intensity after release of the quencher by TCEP.

(*iv*) *Two-Sample FRET Using NFQ1 as the Acceptor*. Two different complexes with identical protein concentrations were formed in two separate samples. One (donor−quencher) contained the 5FM-labeled CheW mutant and NFQ1-labeled $CheA_F$ S660C, while the other (donor-only) contained the 5FM-labeled CheW mutant and unlabeled $CheA_F$ S660C. The donor fluorescence intensity of both samples was measured, and the apparent FRET efficiency was calculated as

$$E_{app} = 1 - \frac{F_{DQ}}{F_D} \quad (4)$$

where F_{DQ} is the donor fluorescence intensity of the donor−quencher complex and F_D is the donor fluorescence intensity of the donor-only complex.

(*v*) *Three-Sample FRET Using Alexa 750 as the Acceptor*. Three different complexes with identical protein concentrations were formed in three separate samples. One (donor−acceptor) contained the 5FM-labeled CheW mutant and Alexa 750-labeled $CheA_F$ S660C; one (donor-only) contained the 5FM-labeled CheW mutant and unlabeled $CheA_F$ S660C, and the other (acceptor-only) contained the unlabeled CheW mutant and Alexa 750-labeled $CheA_F$ S660C. The donor fluorescence intensity of all three samples was measured, and the apparent FRET efficiency was calculated as

$$E_{app} = 1 - \frac{F_{DA} - F_{A(D)}}{F_D} \quad (5)$$

where F_{DA} is the donor fluorescence intensity of the donor−acceptor complex, $F_{A(D)}$ is the acceptor fluorescence at the donor emission wavelength due to both direct acceptor excitation and FRET, and F_D is the donor fluorescence intensity of the donor-only complex. In practice, it is not possible to measure $F_{A(D)}$ directly; however, the contribution due to direct acceptor fluorescence can be determined as the fluorescence intensity of the acceptor-only complex (F_A). In our system, due to the Stokes shifts of fluorescein and Alexa 750, the acceptor fluorescence at the donor emission wavelength arising from FRET must be vanishingly small and can be neglected; thus, the approximation $F_{A(D)} \sim F_A$ is used in eq 5. Note this approximation will not be valid for all FRET pairs.

Determination of K_D Values for Formation of the Complex and Correction of FRET Efficiencies. For each donor−acceptor complex, the equilibrium dissociation constant (K_D) was determined via titration of the donor-labeled CheW mutant with quencher- or fluorescent acceptor-labeled $CheA_F$ S660C. The growing FRET signal was used to quantitate complex formation. NFQ1-labeled or Alexa 750-labeled $CheA_F$ S660C was titrated into a cuvette containing 0.1 μM 5FM-labeled CheW. Following each addition, the sample was incubated at 25 °C for 10 min to reach equilibrium, and then the donor fluorescence intensity was measured. FRET efficiency at each titration point was calculated as

$$E = 1 - \frac{F_T}{F_D} \quad (6)$$

where F_T is the donor fluorescence intensity at the titration point and F_D is the donor fluorescence intensity of the donor-only sample prior to titration. Normalized FRET efficiency was plotted against the total CheW concentration, and K_D was determined using KaleidaGraph version 4.0 to best fit the quadratic binding equation, which accounts for the distribution of total CheW between bound and free populations:

$$E = E_{max} P_B = E_{max} \left[\frac{[CheA_F]_{total} + [CheW]_{total} + K_D}{2} - \sqrt{\frac{([CheA_F]_{total} + [CheW]_{total} + K_D)^2}{4} - [CheA_F]_{total}[CheW]_{total}} \right] / ([CheW]_{total}) \quad (7)$$

where E is the measured FRET efficiency, E_{max} is the FRET efficiency at saturation, P_B is the fraction of CheW bound in the complex, $[CheA_F]_{total}$ is the total concentration of labeled $CheA_F$, $[CheW]_{total}$ is the total concentration of labeled CheW, and K_D is the equilibrium dissociation constant.

The resulting K_D values were used to correct the apparent FRET efficiencies of one-, two-, and three-sample FRET measurements for incomplete complex formation at the protein concentrations employed. When complex formation was incomplete, a small percentage of donor-labeled CheW molecules were not bound to quencher- or acceptor-labeled $CheA_F$, thereby artificially decreasing the apparent FRET efficiency (E_{app}). The fraction of the donor-labeled CheW population successfully bound to $CheA_F$ (P_B) was determined from the measured K_D of the complex and the total concentration of labeled $CheA_F$ ($[CheA_F]_{total}$) using eq 7. The corrected FRET efficiency (E_{cor}) was calculated as

$$E_{cor} = \frac{E_{app}}{P_B} \quad (8)$$

which fully accounts for the effects of incomplete complex formation.

Determination of the Quantum Yield of 5FM-Labeled CheW. The quantum yield was determined as described at http://www.jobinyvon.com/usadivisions/Fluorescence/applications/quantumyieldstrad.pdf . Fluorescein in 0.1 M NaOH was used as a standard with a quantum yield of 0.93 (*2*). Samples of individual 5FM-labeled CheW mutants were measured in buffer A without DTT. To minimize errors, the absorbance of each solution was measured in a 5 cm path length cuvette.

RESULTS

A New Reversible Dark Acceptor. To implement the OS-FRET strategy, we designed and synthesized a novel, reversibly coupled dark acceptor (or quencher) by combining the nonfluorescent chromophore of QSY35 (Invitrogen) (*26*), an NBD derivative, with the methanethiosulfonate functionality. Currently, methanethiosulfonate derivatives are widely employed to couple spectroscopic probes to specific protein Cys residues via disulfide exchange as Figure 1b illustrates (*26*−*28*). Figure 1c shows the chemical structure of the new probe, nonfluorescent quencher 1 (NFQ1). The structure of NFQ1 was confirmed by NMR (data not shown). NFQ1 has an extinction coefficient ε_{485} of 10030 M^{-1} cm^{-1} in aqueous buffer at pH 7.5. Because NFQ1 has limited solubility in aqueous buffer, stock solutions were made in DMSO. Figure 1d shows the donor fluorescein 5-maleimide excitation and emission spectra, and the NFQ1 absorption spectrum, where both probes are coupled to free Cys in buffer at pH 7.5. We estimate the Förster distance (R_0) for the fluorescein−NFQ1 pair to be ~39 Å on the basis of (i) a quantum yield (Q_D) of 0.69 measured for fluorescein-coupled CheW (see Materials and Methods) and (ii) an average orientation factor (κ^2) of $\sim^2/_3$ assuming rapid, isotropic tumbling of one or both transition dipoles. Notably, the unusually broad absorption spectrum of NFQ1 ensures its utility as a dark acceptor for many donors.

Preparing the Protein Complex for FRET Studies. Figure 2a illustrates the known structure of the soluble complex formed by association of the $CheA_F$ fragment (residues 289−671 of CheA) with CheW (*23*). Both proteins from *T. maritima* lack Cys residues, and the K_D for their association is in the submicromolar range, making the complex well-suited for FRET measurements. Engineered Cys residues were introduced as labeling sites (Figure 2a): five CheW sites (yellow spheres) were selected for donor fluorescein and one $CheA_F$ site (S660C, brown sphere) selected for dark quencher NFQ1. In the crystal structure, the selected donor−quencher pairings exhibit Cβ−Cβ distances from 8 to 48 Å, designed to yield a large range of FRET efficiencies.

Standard methods were employed to couple donor fluorescein to the five CheW Cys mutants (*25*). The labeling reaction used to couple the new NFQ1 probe to $CheA_F$ S660C yielding an NFQ1 labeling efficiency of $\geq 93 \pm 1\%$ as determined biochemically (see Materials and Methods) and by mass spectrometry analysis (Figure S1 of the Supporting Information). Thus, nearly all available Cys residues were labeled with a quencher. The high labeling efficiency is especially important for the quencher, because on average each complex containing a donor-labeled CheW must also contain quencher-labeled $CheA_F$ for accurate FRET efficiency measurements.

FRET Efficiencies Measured by OS-FRET. Panels b and c of Figure 2 present spectra for two representative donor−quencher pairs, and Table 1 summarizes the OS-FRET results for

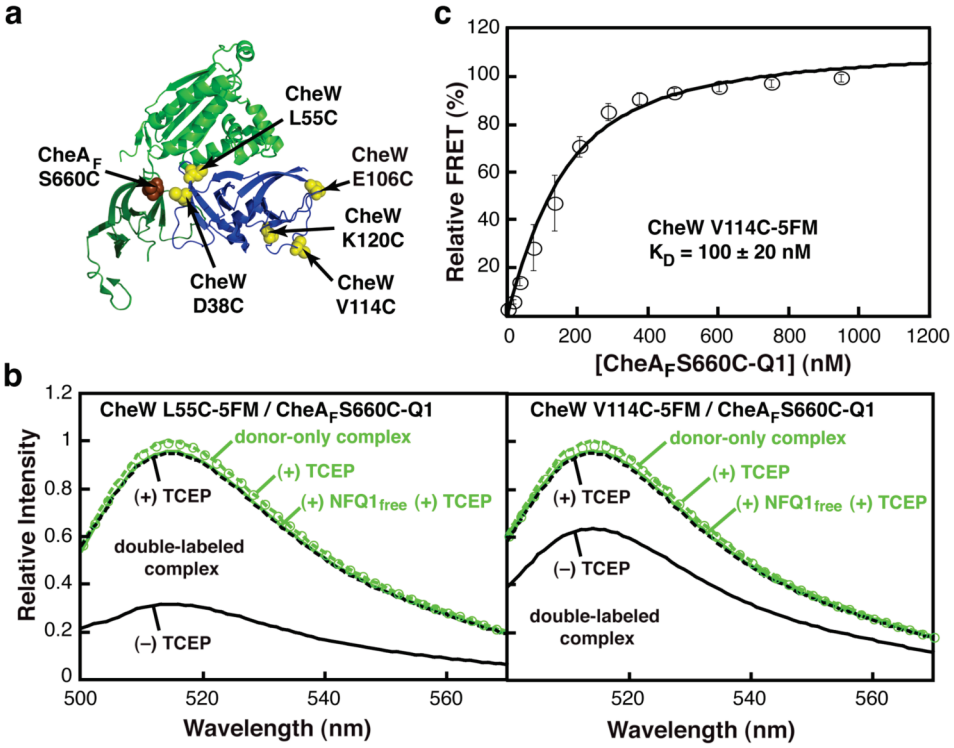

FIGURE 2: Application of OS-FRET to a complex with a known structure. (a) Complex between CheW (blue) and the P4−P5 domain fragment of CheA (CheA$_F$, green), both from *T. maritima*. Positions chosen for single Cys mutations are highlighted as spheres: brown for the NFQ1 labeling site and yellow for the fluorescein labeling sites. This panel was created in PyMol (DeLano Scientific LLC) using PDB entry 2CH4. (b) Emission spectra of the doubly labeled complex [(−)TCEP, solid black line] and of the singly labeled complex after reductive release of the quencher [(+)TCEP, dashed black line]. Also shown are the emission spectra of the donor-only complex lacking quencher altogether (dashed green line) and of the donor-only complex after addition of reducing agent [(+)TCEP, green circles] or free quencher and reducing agent [(+)NFQ1 (+)TCEP, solid green line under the dashed black line]. The left panel presents data for a short donor−acceptor separation (CheW L55C−5FM/CheA$_F$ S660C−NFQ1, Cβ−Cβ distance of 16 Å), while the right panel presents data for a longer separation (CheW V114C−5FM/CheA$_F$ S660C−NFQ1, Cβ−Cβ distance of 47 Å). (c) FRET titration to determine the equilibrium dissociation constant for a representative complex (CheW V114C−5FM/CheA$_F$ S660C−NFQ1). Titration points (○) were fitted to the quadratic binding equation (eq 7, fitted in KaleidaGraph version 4.0), yielding the best-fit curve (—) and K_D.

all five pairs. As detailed in Materials and Methods, complexes were formed via incubation of donor-labeled CheW and quencher-labeled CheA$_F$ for 15 min, and then the quenched donor emission spectrum was recorded (solid line in Figure 2b). Subsequently, to release the quencher, reducing agent (TCEP) was added and the sample incubated 15 min without removing it from the spectrometer. Finally, the unquenched emission spectrum of the donor-only complex was recorded (dashed black line in Figure 2b). Release of the quencher out of FRET range yielded a significant donor emission increase, and the increase was greater for the closer representative pair (in Figure 2b, compare the left and right panels). This procedure was used to measure the uncorrected FRET efficiency for each donor−quencher pair at sufficiently high concentrations of quencher-labeled CheA$_F$ to nearly saturate donor-labeled CheW. In a separate titration experiment to measure the K_D of each donor−quencher pair, FRET efficiencies were measured as quencher-labeled CheA$_F$ was titrated into the sample (Figure 2c; see Materials and Methods). The final, corrected FRET efficiencies listed in Table 1 include corrections for incomplete saturation of the donor-labeled protein with the quencher-labeled protein.

Separate control studies were conducted to ensure the OS-FRET method was properly optimized for the CheA$_F$−CheW complex. Addition of TCEP to the donor-only complex had no measurable effect on donor fluorescence (Figure 2b), indicating that the effect of TCEP on the donor−quencher complex arose simply from quencher release as intended. Addition of NFQ1 and TCEP to the donor-only complex yielded the same level of donor fluorescence as TCEP-triggered release of NFQ1 in donor−quencher samples (Figure 2b), providing optical evidence that TCEP quantitatively releases the quencher and that quencher inner filtering (see Discussion) is unaltered by quencher release. Finally, mass spectrometry analysis of products provided chemical evidence that the TCEP treatment yielded quantitative quencher release (Figure S1 of the Supporting Information).

FRET Efficiencies Measured by Multisample FRET. For comparison, we also measured FRET efficiencies for the same five donor−quencher pairs using conventional two- and three-sample methods, again employing fluorescein as a donor. The two-sample method used NFQ1 as a nonfluorescent acceptor and calculated FRET efficiencies from the emissions of donor−quencher and donor-only samples. The three-sample method employed Alexa 750 as a fluorescent acceptor and determined FRET efficiencies from donor−acceptor, acceptor-only, and donor-only samples. The estimated Förster distance (R_0) for the fluorescein−Alexa 750 donor−acceptor pair is ~42 Å, similar to that of the fluorescein−NFQ1 donor−quencher pair. Table 1 compares the one-, two-, and three-sample FRET efficiencies, all corrected for incomplete saturation of CheW with CheA$_F$.

DISCUSSION

Examination of the results reveals that the OS-FRET method is superior to the conventional two- and three-sample methods in several ways. OS-FRET uses 50 and 67% less sample than the two- and three-sample methods, respectively, and reduces the

Table 1: Comparison of OS-FRET with Two- and Three-Sample FRET Methods[a]

donor, CheW probe	acceptor, $CheA_F$ S660C	K_D (nM)	$C_\beta-C_\beta$ distance (Å)	FRET efficiency[b] (%)		
				predicted	OS-FRET	multisample FRET
D38C-5FM	NFQ1	80 ± 10	9	99.9	83.7 ± 0.5	86.6 ± 0.8[c]
L55C-5FM		110 ± 30	16	99.6	93.5 ± 0.5	95.6 ± 0.8[c]
K120C-5FM		80 ± 20	40	48	34.0 ± 0.6	38.7 ± 1.2[c]
V114C-5FM		100 ± 20	47	26	31.8 ± 0.7	38.9 ± 1.2[c]
E106C-5FM		100 ± 10	48	24	28.3 ± 1.0	32.5 ± 1.5[c]
D38C-5FM	Alexa 750	150 ± 20	9	99.9	−	74.1 ± 2.7[d]
L55C-5FM		20 ± 10	16	99.7	−	65.8 ± 3.2[d]
K120C-5FM		90 ± 30	40	56	−	35.4 ± 2.0[d]
V114C-5FM		130 ± 30	47	33	−	34.7 ± 2.8[d]
E106C-5FM		110 ± 20	48	30	−	32.2 ± 3.0[d]

[a]All errors are standard errors of the mean, calculated for an average of three means, where each mean is an average of triplicate samples. [b]Predicted FRET efficiencies calculated using R_0 values of 39.7 Å for the 5FM−NFQ1 pair and 41.7 Å for the 5FM−Alexa 750 pair, estimated using approximations indicated in the text. Experimental FRET efficiencies are corrected for incomplete saturation of donor-labeled protein with acceptor-labeled protein as described in Materials and Methods. [c]Two samples: donor-only complex and donor−acceptor complex. [d]Three samples: donor-only complex, acceptor-only complex, and donor−acceptor complex.

time per measurement approximately 30−70% (time estimates are based on optimized protocols for triplicate measurements using a four-sample cuvette holder and include the time required for reductive quencher release, but do not include additional time savings provided by simpler data analysis and fewer protein purifications needed for OS-FRET). Table 1 further shows that the precision of OS-FRET is on average 1.7- and 4.2-fold better than the two- and three-sample methods, respectively, because of the error introduced by multiple samples. Moreover, in contrast to OS-FRET, the accuracy of two- and three-sample methods can be compromised by acceptor inner-filter effects. As illustrated in Figure 2b for the fluorescein−NFQ1 pairs, reduced donor−quencher samples exhibit donor emission intensities slightly lower than those of donor-only samples. This is due to NFQ1 absorbance, or inner filtering, at the donor excitation and emission wavelengths as directly confirmed via addition of free NFQ1 to the donor-only labeled sample (Figure 2b). In OS-FRET, the quencher and its inner filtering are present in both the FRET and non-FRET states; thus, inner filtering is automatically divided out in OS-FRET efficiency calculations (eq 3). As a result, OS-FRET yields more accurate FRET efficiencies that are slightly lower than those measured by methods employing donor-only samples. This explains the small but reproducible differences between OS-FRET and two-sample FRET efficiencies for the 5FM−NFQ1 pair in Table 1, where the OS-FRET efficiencies are 1−7% lower.

Not surprisingly, the FRET efficiencies measured by all approaches deviate somewhat from the values predicted by standard Förster theory for the crystal $C\beta-C\beta$ distances. The measured OS-FRET efficiencies, for example, roughly follow the predicted r^{-6} dependence on donor−quencher separation (Table 1), but deviations are observed. Assuming the crystal structure is an accurate representation of the solution structure, the observed deviations could arise from multiple factors. For a given donor−acceptor pair, the true donor−acceptor distance typically differs from the crystal $C\beta-C\beta$ separation because of the large sizes of the probes and the flexibilities of their linkers. In addition, the assumption of isotropic, rapid probe motion ($\kappa^2 = {}^2/_3$) could be unjustified if the local environments of the probes produce strong steric hindrance or binding interactions. Finally, for small donor−acceptor separations approaching the dimensions of the probes, the point-dipole approximation employed by Förster theory could break down (*29*, *30*). Thus, as for other FRET methods, the structural information provided by OS-FRET distance measurements is of a low resolution (Table 1). It follows that OS-FRET is most useful for monitoring macromolecular binding titrations (Figure 2C and Table 1), for low-resolution structural analysis, and for detection of function-associated conformational changes.

Overall, OS-FRET provides a unique combination of six useful properties. OS-FRET (i) measures FRET efficiency in just one sample, (ii) typically preserves the same donor local environment when the quencher is released (assuming that the quencher is neither contacting nor allosterically coupled to the donor), (iii) ensures that quencher inner filtering does not introduce systematic error, (iv) provides R_0 values on a useful macromolecular distance scale, (v) utilizes a sulfhydryl-specific, reversible coupling technology and engineered Cys residues known to be useful in protein studies, and (vi) can be implemented using a conventional fluorimeter or other macro-scale fluorescence detector without potentially damaging, ultra-high-intensity irradiation. Our study directly demonstrates the practicality of OS-FRET and its advantages relative to multisample FRET in studies of a representative macromolecular complex.

The limitations of OS-FRET are relatively minor. (i) NFQ1 is quite hydrophobic, which can cause it to bind to a protein surface rather than freely tumbling while coupled or to remain noncovalently bound to the protein after reductive release. The latter concern is eliminated by the present experimental design that ensures complete quencher dissociation following reductive release (see Materials and Methods). The development of new-generation, more hydrophilic probes may provide an alternative solution. (ii) The use of a dark quencher prevents direct confirmation that FRET is the quenching mechanism; if desired, one can test whether quenching exhibits the r^{-6} distance dependence characteristic of FRET within practical constraints (Table 1; discussed above). (iii) The use of a dark quencher prevents full fluorescence anisotropy analysis of donor and acceptor tumbling useful in minimizing uncertainty in κ^2. This limitation is deemed minor because many FRET applications, such as qualitative characterization of conformational changes, do not require full knowledge of κ^2 (most published FRET studies, even those employing fluorescent acceptors, do not investigate probe tumbling).

In summary, the OS-FRET method employing the novel dark quencher NFQ1 allows precise, accurate measurement of FRET in a single sample. The method optimizes sample efficiency and reduces measurement time while minimizing errors relative to conventional multisample FRET methods. We expect that OS-FRET will be useful in a wide range of experiments probing macromolecules and their complexes and will be successfully measured in an array of fluorescence detection devices. OS-FRET is especially useful in systems in which sample availability is limited and small changes in FRET must be quantitated with great accuracy. In our laboratory, for example, OS-FRET is proving useful in studies of on−off switching in membrane-associated signaling complexes (A. H. Erbse, A. J. Berlinberg, and J. J. Falke, unpublished observations). An important future application of OS-FRET will be high-throughput studies of protein−protein binding or inhibitors that block binding, because the OS-FRET method can be easily extended to multiwell plate formats.

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SUPPORTING INFORMATION AVAILABLE

Mass graphs for unlabeled CheW, the labeled CheW−NFQ1 pair, and the labeled CheW−NFQ1 pair treated with TCEP to reductively release the NFQ1 probe (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

NOTE ADDED IN PROOF

NFQ1 is now available commercially from Biotium.

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