Intramolecular Distances and Dynamics from the Combined Photon Statistics of Single-Molecule FRET and Photoinduced Electron Transfer

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ABSTRACT: Single-molecule Förster resonance energy transfer (FRET) and photoinduced electron transfer (PET) have developed into versatile and complementary methods for probing distances and dynamics in biomolecules. Here we show that the two methods can be combined in one molecule to obtain both accurate distance information and the kinetics of intramolecular contact formation. In a first step, we show that the fluorescent dyes Alexa 488 and Alexa 594, which are frequently used as a donor and acceptor for single-molecule FRET, are also suitable as PET probes with tryptophan as a fluorescence quencher. We then performed combined FRET/PET experiments with FRET donor- and acceptor-labeled polyproline peptides. The placement of a tryptophan residue into the polyglycylserine tail incorporated in the peptides allowed us to measure both FRET efficiencies and the nanosecond dynamics of contact formation between one of the fluorescent dyes and the quencher. Variation of the linker length between the polyproline and the Alexa dyes and in the position of the tryptophan residue demonstrates the sensitivity of this approach. Modeling of the combined photon statistics underlying the combined FRET and PET process enables the accurate analysis of both the resulting transfer efficiency histograms and the nanosecond fluorescence correlation functions. This approach opens up new possibilities for investigating single biomolecules with high spatial and temporal resolution.

INTRODUCTION

Single-molecule spectroscopy has become an integral part of biophysical research,¹ and a wide range of biological questions have been addressed with these methods, including the mechanisms of molecular machines,²−⁴ protein-nucleic acid interactions,⁵,⁶ enzymatic reactions,⁷ and protein or RNA folding,⁸−¹⁰ to name but a few. A particular strength of these methods is the possibility to resolve conformational heterogeneity and to observe dynamics of processes that are difficult to synchronize with the perturbation methods frequently employed in ensemble experiments. A very popular approach has been the use of fluorescence detection, especially in combination with Förster resonance energy transfer (FRET).¹¹ Single-molecule FRET allows distances and distance dynamics to be probed from about 2 to 10 nm, a range dictated by the Förster radii of suitable dye pairs. Advances in methodology, in particular, the use of fluorescence lifetimes and anisotropies in addition to fluorescence count rates from donor and acceptor,¹²,¹³ rigorous data analysis, especially based on the development of comprehensive theoretical concepts¹²,¹⁴−¹²¹ now enables distances,¹² distance distributions,²²,²³ and dynamics to be obtained over a wide range of time scales¹³,²⁴−²⁶ with remarkable accuracy.²⁷

A single-molecule method complementary to FRET that has started to provide important dynamic information on the microsecond and submicrosecond time scale is photoinduced electron transfer (PET) combined with nanosecond-fluorescence correlation spectroscopy (nsFCS), pioneered by Sauer and colleagues.²⁸,²⁹ In this case, static quenching of a fluorophore by tryptophan is used to obtain dynamics based on contact formation within proteins or peptides, conceptually related to some ensemble quenching techniques³⁰−³² and thus complementary to the long-range dynamics accessible with FRET. The on/off behavior resulting from the steep distance dependence of the fluorescence emission in PET can, in principle, be used to probe any process that leads to a change in quenching dynamics, but the method does not afford direct distance information. For instance, PET-FCS allows the determination of loop-closure rates between two points within a polypeptide chain.³⁸ Recent applications include studies of model peptides,²⁸,³³ molecular crowding,³⁴ and fast-folding proteins.³⁵,³⁶

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An important recent goal of single-molecule spectroscopy has been the possibility to monitor more than one distance within a molecule or molecular complex. In the context of protein folding, for example, the simultaneous time-resolved measurement of even a few distances would place much more stringent constraints on the evolution of the native fold than a single distance, and could provide critical tests of theoretical models and simulations. The analysis of the correlation among these distances could also yield information on the width of the microscopic pathway distributions, as pointed out by Onuchic, Wang, and Wolynes.37 One promising approach toward this goal are multicolor FRET experiments, where energy transfer between more than two fluorophores is used to obtain information about more than one distance at the same time.38−41 However, in view of the complementarity of single-molecule FRET and PET, the direct combination of these two methods could be an alternative strategy to enable long-range distances and dynamics based on FRET and more local dynamics of contact formation to be probed in the same molecule at the same time. An additional aspect of interest is the access to submicrosecond time scales, which are of particular relevance for rapid processes such as in very fast-folding proteins42 or unfolded state dynamics, 9 and which provide an important link to theory, simulation, and a better understanding of the physical basis of protein folding in terms of the properties of the underlying free energy landscapes.43−48

Figure 1. Photophysical models of the electronic and molecular transitions of two FRET dye pairs in the presence of donor or acceptor quenching induced by collisions and complex formations with Trp. In a and b, the investigated constructs are illustrated schematically. Both FRET configurations consist of an Alexa 488 dye as a donor (green star) and an Alexa 594 dye (red star) as an acceptor separated by a stiff polyproline sequence indicated as zigzag lines. The PET quencher Trp (gray oval) is attached to flexible (glycine-serine) linkers. The left cartoon (a) represents acceptor quenching; the right cartoon (b) represents donor quenching. The respective other dye is separated too far from the Trp residue to be quenched. The kinetic schemes (c and d) illustrate the two photophysical models. Both models have the states DA, D*A, D*A, and D*A in common, which describe the electronic states of FRET donor (“D”) and acceptor (“A”). The asterisks indicate the electronically excited states from which fluorescence emission is possible. Depending on the construct, each model has to be extended by two additional states: DAQ and D*AQ in the case of acceptor quenching (c), and DQA and DQA in the case of donor quenching (d). DQ and AQ represent the dye-Trp complexes, in which the dyes (D or A) are statically quenched. Fluorescence correlations and mean photon rates can be calculated for both models using the corresponding rate matrices \( K \) and detection matrices \( V_B \) and \( V_B \), respectively.

The state vector for donor quenching is \( p = (p_{DA}, p_{D*A}, p_{DA*}, p_{D*A*}, p_{DQA}, p_{DQA})^T \) and for acceptor quenching \( p = (p_{DAQ}, p_{DQA}, p_{DQ}, p_{AQ}, p_{D*A}, p_{D*A})^T \).
tryptophan (Trp), with the fluorophores Alexa 488 and Alexa 594, a popular FRET pair for single-molecule studies of proteins, to characterize the requisite photophysical properties. In a second step, we incorporated the FRET pair in synthetic peptides consisting of two parts (Figure 1): a polyproline segment acting as a stiff spacer between the dyes, and a flexible glycine-serine linker with Trp incorporated at different positions. FRET between the Alexa dyes thus reports on their intramolecular distance, and the dynamics of quenching of either the donor or the acceptor dye by Trp reports on the intramolecular dynamics of loop formation in the flexible part of the peptide. In a third step, we use a combined kinetic model of the FRET and PET processes (Figure 1) for a quantitative analysis of the nanosecond correlation functions and the transfer efficiency histograms in terms of the underlying photon statistics. Finally, we show that this analysis allows both accurate FRET efficiencies and intramolecular contact dynamics to be extracted.

**MATERIALS AND METHODS**

**Samples.** Alexa 488C2-maleimide and Alexa 594C2-maleimide were synthesized from the corresponding commercially available succinimidyl esters (Invitrogen, Eugene, Oregon, USA). Two solutions of Alexa 488 succinimidyl ester (1.3 mg, 2.0 μmol, 1 equiv) and Alexa 594 succinimidyl ester (1.6 mg, 2.0 μmol, 1 equiv) in dry dimethyl sulfoxide (DMSO) (100 μL) were prepared. N-(2-Aminoethyl)maleimide trifluoroacetate (0.7 mg, 3.0 μmol, 1.5 equiv) and diisopropylethylamine (DIPEA) (10 μL, 58 μmol, 27 equiv) were added successively to each solution. The reactions were quenched after 30 min incubation at room temperature by adding 50 μL water and 10 μL acetic acid (AcOH). The products were purified by reverse phase HPLC chromatography (SunFire Prep C18 5 μm, 19 × 150 mm, Waters, Milford, MA, USA) with a gradient from 0.1% trifluoroacetic acid (TFA) in water to 90% acetonitrile within 30 min at a flow rate of 4 mL/min. The samples were lyophilized, yielding red (1.12 μmol, 53%) and blue (1.05 μmol, 50%) powders for Alexa 488 and Alexa 594, respectively. Both powders were dissolved in dry DMSO.

The polyproline peptides were produced by solid phase synthesis, purified to 80–90% by analytical HPLC, and their intramolecular distance, and the dynamics of quenching of either the donor or the acceptor dye by Trp reports on the intramolecular dynamics of loop formation in the flexible part of the peptide. In a third step, we use a combined kinetic model of the FRET and PET processes (Figure 1) for a quantitative analysis of the nanosecond correlation functions and the transfer efficiency histograms in terms of the underlying photon statistics. Finally, we show that this analysis allows both accurate FRET efficiencies and intramolecular contact dynamics to be extracted.

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The polyproline peptides were produced by solid phase synthesis, purified to 80–90% by analytical HPLC, and their mass confirmed by electrospray ionization mass spectrometry (ESI-MS; PEPTIDE 2.0, Chantilly, VA, USA). Following the protocol supplied by the manufacturer, the cysteines were labeled at the N-terminal glycine using either Alexa Fluor 488 or Alexa Fluor 594 dissolved in a 200 mM pH 9.2 sodium bicarbonate buffer and labeled peptide was purified by multiple cycles of reverse phase chromatography. The labeling success was verified by single-molecule spectroscopy and ESI-MS.

**Fluorescence Spectroscopy.** Fluorescence lifetime decays were measured using a custom-built instrument described previously. Picosecond light pulses from a white light source (SC-450-4, 20 MHz, Fianium, Southampton, U.K.) were used for excitation. The excitation wavelengths were selected by HQ470/40 (Chroma) and z582/15 (Semrock) bandpass filters for Alexa 488 and 594, respectively. Samples (0.1 to 1 μM) were measured in 100 mM potassium phosphate buffer (pH 7.2) containing 143 mM 2-mercaptoethanol and 0.001% (v/v) Tween 20. The instrument was used in magic angle configuration for all measurements.

The recorded fluorescence lifetime decays were fitted with single exponential decays convolved with the instrument response function (IRF) obtained from the measurement of scattered laser light. Fluorescence spectra were recorded at room temperature on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ). The buffer contained 100 mM potassium phosphate pH 7.2, 143 mM 2-mercaptoethanol, and 0.001% Tween 20. A dye concentration of 40 nM was used for both Alexa dyes. For details of the Stern–Volmer analysis of dynamic and static quenching, see the Supporting Information (SI).

**Single Molecule Instrumentation and Measurements.** Single-molecule fluorescence data were recorded using a MicroTime 200 (PicoQuant, Berlin) confocal microscope with four detectors in a configuration described previously. Alexa 488 was excited with a continuous wave (cw) solid-state diode-pumped laser (Coherent Sapphire 488–200). A cw HeNe laser (594 nm, CWI Melles Griot) was used for exciting Alexa 594. The excitation powers, measured at the fully illuminated back-aperture of the water immersion objective (UPLSAPO 60XW, Olympus, Switzerland) of the microscope, were 100 μW and 40 μW for Alexa 488 and Alexa 594, respectively. Transfer efficiency histograms and nsFCS data were recorded at sample concentrations of 50 pM and 1 nM, respectively, in potassium phosphate buffer (see above). For nsFCS data, we recorded both donor and acceptor photons with a pair of detectors each. The photons were distributed by means of a polarizing beam splitter cube followed by dichroic mirrors. The measurement time for each nsFCS measurement was 10–15 hours. A four channel picosecond event timer (HydraHarps 400, PicoQuant, Berlin) recorded the detection times of the photons individually for each detection channel. An autocorrelation, FCS_D(r) or FCS_A(r), was obtained by crosscorrelating the time data of the corresponding detector pair. The donor–acceptor crosscorrelation FCS_Dₐ(r) was determined by crosscorrelating the combined data of the donor channels with those of the acceptor channels. Cross-correlating detector signals makes it possible to obtain FCS data for lag times shorter than the dead times (∼100 ns) of the detectors.

All three correlation curves were determined for positive and negative lag times in order to make optimal use of the data. Note that FCS_Dₐ(r) = FCS_D(r) − FCS_A(r). The nsFCS data were determined with a time binning of one nanosecond if not indicated otherwise. Rotational diffusion of the molecules and dyes would be visible in the subnanosecond part of the FCS curves and thus does not interfere with the dynamics investigated here.
THEORY

Photon Statistics Model. To calculate nsFCS curves, an elegant formalism introduced by Gopich and Szabo was used. A rate matrix $K$ contains the transitions between the states of the kinetic model (see Figure 1). The off-diagonal elements of the detection matrices $V_D$ and $V_A$ contain the radiative rate constants and photon detection efficiencies of the monitored transitions. The time evolution of the state populations $p(t)$ is given by the rate equation:

$$\frac{dp}{dt} = Kp(t)$$

where $p$ is normalized (1T $p = \sum p_i = 1$). FRET donor and acceptor dyes are either in their electronic ground (D, A) or excited states (D*, A*), or they form a complex with Trp, the quencher, Q (DQ, AQ). Without static quenching, our kinetic model consists of four states: DA, D*A, DA*, and D*A*. Donor and acceptor can be excited by irradiation with laser light with the rate constants $k_{exD}$ and $k_{exA}$, respectively, where $\alpha = 0.05$ is the fraction of direct excitation, i.e., the ratio of the extinction coefficients of Alexa 594 and Alexa 488 measured at 488 nm, the wavelength of the exciting laser light. The exited states are depopulated by the ground states with the rate constants $k_{D}$ and $k_{A}$. Energy is transferred from the state D*A to DA* with the rate constant $k_{D}(r) = \frac{k_0}{r^6}$, where $r$ is the intermolecular distance, and $R_0$ is the Förster radius. The double excited state D**A* is depopulated with the rate constants $k_{D}$ and $k_{DSS}$. $k_{DSS}$ describes the effect of singlet–singlet annihilation between excited donor and excited acceptor.

The two types of constructs depicted in Figure 1a,b, either the donor or the acceptor is quenched. Hence, for both cases, the kinetic model needs to be extended by two states, DQA and DQA*, or DAQ and D*AQ, respectively (Figure 1c,d). DQA can be populated by transition from DA and D*A, and DQA* can be populated from DA* and D*A* with the rate constant of complex formation, $\kappa_{DQ}$. The complexed states DQA and DQA* dissociate with the rate constant $\kappa_{off}$ to DA and D*A*, respectively (Figure 1d). Analogously, we introduce $\kappa_{DQ}$ and $\kappa_{off}$ (Figure 1c). It is important to note that energy transfer from D* to the quenched acceptor AQ is possible. However, the absorption spectrum of AQ differs from that of A (Figure 3); as a consequence, the Förster radius for the transfer from D* to AQ is also different. The energy transferred to the AQ complex dissipates by internal conversion. Effectively, there is a nonradiative transition from D*AQ to DAQ with a rate constant $\kappa_{AQ}$ (see Figure 1c).

In summary, the kinetic model in the case of donor quenching consists of the six states DA, D*A, DA*, D*A*, DQA, and DQA*: the system for acceptor quenching consists of DA, D*A, DA*, D*A*, DAQ, and D*AQ. The corresponding rate matrices are shown in Figure 1e,f. Note that the diagonal elements $K_0$ are given by the requirement that the sum over the elements of each column must be zero. The radiative transition matrices are given by $V_D = \xi D_Q k_A V_A$ and $V_A = \xi_A Q k_D V_D$ where $\xi_A$ and $\xi_D$ are the respective detection efficiencies and quantum yields. $V_D$ and $V_A$ are matrices that indicate which transitions of the system are monitored by the photon detection. They are also given in Figure 1e,f for both quenching scenarios.

With the above definitions, the fluorescent intensity correlation functions $g_R(t)$ between photons detected on channels $i$ and $j$ ($i=j=DA$) for a single FRET-labeled species can be calculated as:

$$g_R(t) = \frac{1}{(1 \times p_0)} \left( \sum_{i,j} p_i p_j \right)$$

The symbols $f$ and $d$ indicate the subpopulations with active donor dyes is negligible due to the low direct excitation of the acceptor. We use the equation for FCS of a mixture of freely diffusing species in the limit of short lag time $\tau$ compared to the diffusion time through the confocal volume (i.e., $\tau \ll 1$ ms):

$$G_n(t) = 1 + a_n (c_{i,j} c_{i,j} + c_{i,j} c_{i,j}) (1 + \gamma_{i,j} e^{-t/\tau})$$

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where $G_n(t)$ is calculated from eq 2. The term $(1 + \gamma_{i,j} e^{-t/\tau})$ describes the effect of triplet blinking, and $(1 - e^{-t/\tau})$ is the effect of photon antibunching of a donor-only molecule. These terms are introduced empirically to simplify the computationally demanding analysis. The large time separation between triplet state blinking (1–10 μs) and the other dynamic processes (1–100 ns) justify this procedure. We express the brightness values relative to the brightness of a donor-only molecule as seen by the donor detection channel, i.e., $B_{DA} = 1$, and obtain for the three other values:

$$B_{LD} = 1 - E, \quad B_{LA} = \gamma (E + \alpha) + \beta (1 - E)$$

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where \( N \) is the mean number of fluorescent particles present in the confocal volume, \( n_i \) and \( n_k \) are the mean fluorescence photon detection rates, and \( b_i \) and \( b_j \) are the background rates in detector channels \( i \) and \( j \), respectively. Note that we use \( a_{ij} \) as fit parameters, since the individual components are not of interest for our analysis.

**Global Fitting of the nsFCS Data.** We fitted the model nsFCS curves \( G_{\text{DD}}(\tau) \), \( G_{\text{DA}}(\tau) \), and \( G_{\text{AA}}(\tau) \) (eq 3) globally to the measured auto- and cross-correlation functions, FCS\(_{ij}\) by minimizing the sum of the three corresponding \( \chi^2 \) functions of the form

\[
\chi^2_{ij} = w_{ij} \sum_m (FCS_{ij}(\tau_m) - G_{ij}(\tau_m))^2
\]

The weights are the reciprocal variances for each data point, \( w_{ij} = 1/\sigma^2_{ij} \) (we assume constant variances for each data point in one nsFCS curve). Koppel\(^{58} \) showed that the signal-to-noise ratio of an FCS curve of a single species is proportional to its molecular brightness \( B \) if \( B\Delta \ll 1 \), where \( \Delta \) is the binning interval of the FCS data. (In our case, with a molecular brightness of \( \sim 0.1 \mu s^{-1} \) and \( \Delta = 20 \) ps, this requirement is clearly met.) As the amplitude of the FCS curve itself does not depend on the brightness, we conclude that the variance of the signal is proportional to \( \sigma^2 \sim 1/B^2 \). Generalizing this result to dual color FCS, we obtain \( \sigma^2_{ij} \sim 1/(B_i B_j) \). Therefore, we weight the \( \chi^2 \) functions with \( w_{ij} = B_i B_j \), \( w_{AA} = B_i B_A \), and \( w_{AD} = 4B_A B_d \), where \( B_i \) is the mean relative brightness \( B_i = c_i B_d + c_d B_i \). The factor 4 for \( w_{AD} \) is justified since the cross-correlation was obtained from two donor and two acceptor detection channels (see Materials and Methods). The effective molecular brightness “seen” by a detector pair is twice as large as for a single detector.

Most model parameters are known from independent experiments. The excitation rate constant \( k_{\text{exD}} = 0.02 \text{ ns}^{-1} \) was determined from the relaxation of the antibunching component in the nsFCS of donor-only labeled constructs. \(^{28} \) \( k_0 \) was determined from ensemble fluorescence lifetime decays of donor-only constructs. Corresponding decays for determining \( k_d \) were obtained by acceptor direct excitation of donor- and acceptor-labeled constructs. \( E_i, c_i \) and \( c_d \) were obtained from single-molecule transfer efficiency histograms, and \( k_F \) was calculated according to

\[
k_F = k_T E/(1 - E)
\]

\( k_F \) was calculated using

\[
k_F^{AQ} = k_{T}(R_0^{AQ}/R_0)^6 = k_{D}^{AQ} J
\]

where \( J \) and \( J_{AQ} \) are the spectral overlap integrals\(^{57} \) for the energy transfer to the unquenched and quenched acceptor dyes, respectively. We determined \( J_{AQ}/J = 0.73 \) (see below), \( \alpha \) and \( \beta \) were determined as described.\(^{58} \) The determination of \( \gamma \) is described below. Triplet decay times \( \tau_T \) were determined by fitting the nsFCS curves of Trp-free reference peptides. The remaining free fitting parameters for the data obtained from the constructs with Trp were \( k_{\text{on}} \) and \( k_{\text{off}} \) for describing the static quenching dynamics, the triplet blinking amplitudes \( c_{\tau_T} \), and the amplitudes of the correlation functions, \( a_{ij} \). Note that the values of \( a_{ij} \) do not affect the relative amplitudes of the nanosecond components of \( g_{ij}(\tau) \), but the relative amplitudes of \( g_{ij}(\tau) \) are fully determined by our kinetic model (eq 2). Due to our limited knowledge about singlet–singlet annihilation between Alexa 488 and Alexa 594, we also treated \( k_{SSA} \) as a free fitting parameter, but this process will only affect dynamics on the order of the excited state lifetimes and thus has no significant effect on the contact dynamics of interest here.

**Determination of \( E \) from Photon Detection Rates.** In single-molecule FRET experiments, we aim to obtain the transfer efficiency from the measured donor and acceptor photon detection rates. We calculate their theoretical mean values for our kinetic models as\(^{52} \)

\[
n_D = \frac{1}{2}V_{2D}p_{es} \quad \text{and} \quad n_A = \frac{1}{2}V_{2A}p_{es}
\]

If we assume that the laser intensities are small \( (k_{\text{exA}} \ll k_d) \), such that the population of the double-excited state \( D^* \) is negligible, and if we further assume that \( k_{\text{AQ}} \approx k_{\text{ff}} \), then the following relation can be used to calculate \( E \) from \( n_D \) and \( n_A \):

\[
E = \frac{k_{D}}{k_{D} + k_{F}} = \frac{n_A'}{n_A' + \gamma n_D(1 + k_{\text{AQ}}/k_{\text{AQ}})^{-1}} - \beta n_D
\]

Equation 11 can be verified by inserting \( n_D \) and \( n_A \) calculated from eq 10 for the kinetic model with static acceptor quenching. Static donor quenching has no influence on the formulas. In the absence of static acceptor quenching, the \((1 + k_{\text{AQ}}/k_{\text{AQ}})^{-1}\) terms can be omitted.

In single-molecule experiments, we estimate \( E \) from the numbers of donor and acceptor photons, \( n_D \) and \( n_A \), in a burst of photons. We obtain the rates by \( n_D = N_D/\tau_D - b_D \) and \( n_A = N_A/\tau_A - b_A \), where \( \tau_D \) is the duration of the burst, and \( b_D \) and \( b_A \) are the background rates in the two detection channels. The obtained values for \( n_D \) and \( n_A \) are inserted into eq 11 for calculating \( E \). Because of the limited number of photons present in a burst (at least 30), the obtained values of \( E \) scatter around the true mean transfer efficiency (shot noise). The method described is correct in the limit \( T \gg (k_{\text{AQ}} + k_{\text{ff}})^{-1} \), i.e., if the photon signal is averaged over many cycles of AQ complex formation and dissociation, which is the case here.

**Determination of \( \gamma \).** The value of the correction factor \( \gamma = \xi_{D}(Q_A)/\xi_{D}(Q_D) \) is affected by dynamic quenching of both the donor and the acceptor dyes. We determined its value \( \gamma_0 \) for Alexa 488 and Alexa 594 free in buffer solution for our instrument as described.\(^{58} \) The value for \( \gamma \) when the dyes are bound to our peptides can be calculated according to

\[
\gamma = \frac{\tau_D}{\tau_D + \tau_A}
\]

where \( \tau_D \) and \( \tau_A \) are the fluorescence lifetimes of free donor and acceptor measured in buffer solution, and \( \tau_T = k_T^2 \) and \( \tau_A = k_{\text{ff}}^2 \) are the corresponding fluorescence lifetimes of the dyes while bound to the constructs. They were obtained as described in the Quenching of Alexa 488 and 594 by Tryptophan section.

**RESULTS**

**Quenching of Alexa 488 and 594 by Tryptophan.** A first step toward the combined use of single-molecule FRET and PET is to characterize the quenching of a FRET pair, in this case Alexa 488 and 594, with a suitable quencher. Of the naturally occurring amino acids, L-tryptophan (Trp) is the one with the largest quenching effect on the Alexa 488 and Alexa 594 fluorescent dyes.\(^{59} \) Therefore we characterized the dynamic and static quenching interactions of these dyes with the free
amino acid Trp. To obtain information about dynamic quenching, we performed ensemble lifetime experiments at various concentrations of Trp, as shown in Figure 2c.

Figure 2. (a,b) nsFCS measurements of 1 nM Alexa 488 (a) and Alexa 594 (b) with 40 mM Trp (here a time resolution of 0.1 ns was used). The blue lines represent double exponential fits to the correlation curves used to extract the static quenching rates. (c) Stern–Volmer plots of Alexa 488 (green) and Alexa 594 (red) quenched by Trp based on fluorescence lifetime data. The blue lines represent linear fits used to extract the dynamic quenching Stern–Volmer constants. The statistical uncertainties estimated as standard deviations from two to three independent measurements are within the size of the symbols.

Linear fits to the data reveal a Stern–Volmer constant of $K_{SV} = 14.2 \text{ M}^{-1}$ for Alexa 488 and of 8.2 M$^{-1}$ for Alexa 594. With the mean fluorescence lifetimes $\tau_{DQ} = 4.05 \text{ ns}$ and $\tau_{AQ} = 4.05 \text{ ns}$ of the unquenched dyes, the dynamic quenching rate constants $k_q = K_{SV}/\tau_q$ result as 3.5 ns$^{-1}$ M$^{-1}$ for Alexa 488 and 2.0 ns$^{-1}$ M$^{-1}$ for Alexa 594. To assess whether dynamic quenching is diffusion-limited, we compare these numbers to the corresponding collision rate constants between dyes and quenchers, which can be estimated from

$$k_{\text{coll}} = 4\pi N_A R_{\text{coll}} (D_1 + D_q)$$

with the Avogadro constant $N_A$, the diffusion coefficients of the dye and the quencher, $D_1$ and $D_q$, respectively, were obtained from an estimation method introduced by Wilke-Chang$^{61}$ (see SI), and the collision radius, $R_{\text{coll}}$, was estimated to be 0.5 nm.$^{34,40,62}$ The resulting collision rate constants are 3.9 ns$^{-1}$ M$^{-1}$ for Alexa 488 and 3.6 ns$^{-1}$ M$^{-1}$ for Alexa 594. In view of the remaining uncertainties in estimating $k_{\text{coll}}$, we thus conclude that the quenching process is very close to diffusion-limited in both cases.

Static quenching of Alexa 488 and Alexa 594 was studied by recording FCS curves of 1.0 nM dye in the presence of Trp in the millimolar range. Representative results measured with 40 mM Trp are shown in Figure 2a,b. The decay of the FCS curves in the low nanosecond regime is due to photon antibunching; the decay in the 10 ns range is characteristic of static quenching by complex formation between Trp and the dyes. Triplet blinking leads to a small additional component on the microsecond time scale, barely visible in Figure 2a,b. We fit the nsFCS curves using

$$G(\tau) = 1 + a g(\tau)(1 + c_T e^{-\tau/\tau_p})$$

where $a$ is an overall amplitude, and the term $(1 + c_T e^{-\tau/\tau_p})$ describes triplet blinking, $g(\tau)$ is calculated assuming a three-state system $[(D, D^*, DQ) \text{ or } (A, A^*, AQ)]$ by eq 2 with the rate matrix and the detection matrix of the form

$$K = \begin{pmatrix} -k_{ex} - k_{on}[Q] & k & k_{off} \\ k_{ex} & -k - k_{on}[Q] & 0 \\ k_{on}[Q] & k_{on}[Q] & -k_{off} \end{pmatrix}$$

and

$$\mathbf{V} = \begin{pmatrix} 0 & 1 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \end{pmatrix}$$

$k_{ex}$ and $k$ are the excitation and decay rate constants, and $[Q]$ denotes the quencher concentration. $k_{on}$ and $k_{off}$ are the rate coefficients of Trp-dye complex formation and dissociation, respectively. For Alexa 488 in 40 mM Trp, we obtain $k_{on} = 1.20 \text{ ns}^{-1} \text{ M}^{-1}$ and $k_{off} = 0.071 \text{ ns}^{-1}$, and for Alexa 594 $k_{on} = 1.08 \text{ ns}^{-1} \text{ M}^{-1}$ and $k_{off} = 0.036 \text{ ns}^{-1}$. Comparison of the $k_{on}$ values with the $k_q$ values for dynamic quenching shows that Alexa 488 needs on average about three encounters with Trp and Alexa 594 two encounters with Trp for forming a complex with the quencher in free solution, similar to the behavior of the dye MR121.$^{50}$ Note, however, that for steric reasons, these values might be different when dye and Trp are attached to a polypeptide (see the Efficiency of Dye-Quencher Complex Formation section).

As mentioned before, the absorption spectra of free Alexa 594 (A) and in complex with Trp (AQ) are different (Figure 3). This results in different rates of Förster transfer from $D^*$ to $A^*$.

Figure 3. Absorption spectra of Alexa 594 measured at different Trp concentrations from 0 mM (blue) to 50 mM Trp (red). The black curve is the spectrum $\varepsilon_{AQ}(\lambda)$ calculated for the Alexa 594–Trp complex (eq 16).

A and AQ, $k_q$ and $k_{AQ} = k_{AQ}/J$, respectively. For determining the overlap integrals, we measured the absorption spectra $\varepsilon_A(\lambda)$ and $\varepsilon_{AQ}(\lambda)$ of Alexa 594 without and with 50 mM Trp, respectively (Figure 3). $\varepsilon_A(\lambda)$ can be written as a linear combination of $\varepsilon_A(\lambda)$ for A and the absorption spectrum $\varepsilon_{AQ}(\lambda)$ of the AQ complex:

$$\varepsilon_{AQ}(\lambda) = \frac{1}{1 + K_{1}[Q]} \varepsilon_A(\lambda) + \frac{K_{2}[Q]}{1 + K_{2}[Q]} \varepsilon_{AQ}(\lambda)$$

Here, $K_1 = k_{on}/k_{off} = 30 \text{ M}^{-1}$ is the equilibrium association constant obtained from the corresponding $k_{on}$ and $k_{off}$ values reported above, and $[Q] = 50 \text{ mM}$. By solving the equation for $\varepsilon_{AQ}(\lambda)$, we obtained the absorption spectrum of the AQ complex. Using $\varepsilon_A(\lambda)$ and $\varepsilon_{AQ}(\lambda)$, we calculated $J_{AQ}/J = 0.73$. 

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The analysis of the fluorescence spectra as a function of Trp concentration is in agreement with our assumption that the dye–Trp complexes are dark, as in previously investigated cases. We further analyzed Stern–Volmer plots from the fluorescence data (see SI), and obtained equilibrium constants for static quenching of $K_S = 14.9 \text{ M}^{-1}$ for Alexa 488 and $K_S = 29.6 \text{ M}^{-1}$ for Alexa 594. These values compare well with the ones obtained from the nsFCS data above ($K_S = 17 \text{ M}^{-1}$ for Alexa 488 and $K_S = 30 \text{ M}^{-1}$ for Alexa 594), supporting the consistency of the results.

**FRET and PET Quenching Dynamics in the Same Molecule.** To study FRET combined with PET quenching in a well-defined system, we prepared peptide constructs based on our previous experience with similar systems, where 20 proline residues form a stiff spacer between the FRET dyes (Alexa 488 and Alexa 594) (Figure 4). Even though polyproline peptides have been shown to comprise considerable conformational heterogeneity caused by peptidyl-prolyl cis–trans isomerization, they are the stiffest natural peptide sequences, and the residual distance heterogeneity does not interfere with the requirements for our investigation. For the combination with PET quenching, a flexible (GlySer)$_n$ sequence was included at the C-terminal end of the polyproline peptide, with a Trp residue inserted at different positions. Neuweiler et al. used Gly-Ser-linkers of similar length to study the intramolecular quenching effects of Trp on the oxazine fluorophore MR121. They found pronounced quenching for linker lengths ranging from 4 to 10 residues. We thus prepared peptides with similar sequence separation (three to nine residues) between the Trp residue and the cysteine to which the Alexa dye is attached (Figure 4). To avoid effects on the transfer efficiency from variations in steric constraints introduced by different lengths of the flexible peptide, the number of Gly-Ser repeats was kept constant, with Trp located at different positions in the peptide. The general sequence of the peptide is GP$_n$CGS(W3)GS(W5)GS(W7)GS(W9), where WX indicates the different Trp positions used. To control for nonspecific effects, a reference construct consisting of the polyproline and the GS-sequence but without Trp was produced. For simplicity, we name the constructs Ref, W3, W5, W7, and W9 for the reference and the peptides with Trp at the respective positions. For introducing the fluorescent dyes site-specifically, the peptides where labeled on the amino group of the N-terminal glycine using succinimidyl esters and on the cysteine in position 22 using maleimide derivatives of the Alexa dyes. Besides the commercially available Alexa dyes with a C5 linker, we also synthesized maleimide derivatives of the Alexa dyes with a C2 linker. This allows us to study the influence of the dye linker length on the FRET and PET dynamics. To investigate the effect of PET quenching on both the FRET donor and the acceptor, all constructs were labeled specifically in the two different possible permutations, with either the donor (A488C2/C5) or the acceptor (A594C2/C5) attached to the cysteine close to the Gly-Ser tail containing the Trp (Figure 4). In total, we thus investigated 20 different constructs: five peptide sequences (Ref, W3, W5, W7, W9).
each with two different dye-linkers (C2 and C5) and two labeling permutations.

Figure 4 shows transfer efficiency histograms recorded for the 10 constructs with the C5 dye linkers. Even though the histograms are clearly broadened beyond shot noise owing to residual peptidyl-prolyl cis/trans isomerization,²⁵ they provide a well-defined peak at transfer efficiencies close to 1/2, which allows us assess the effect of PET quenching on the transfer efficiency with high sensitivity. All histograms are shown without and with correction for dynamic and static quenching effects. Not correcting for quenching means that we use eq 11 with $\gamma = \gamma_0$, the value measured for free dyes in the absence of Trp, and that we neglect the $(1 + k_{\text{on}}/k_{\text{off}})^{-1}$ term, a procedure that would correspond to the typical practice in single-molecule FRET experiments. For the peptides with a quenched donor (Figure 4a), the presence of Trp (W3–W9) leads to a slight increase of the uncorrected (or apparent) transfer efficiencies, $E_{\text{app}}$, due to the change in donor quantum yield. For the peptides with a quenched acceptor, the change in $E_{\text{app}}$ is much more drastic (Figure 4c), although the dynamic quenching effect of Trp on Alexa 594 is less pronounced than for Alexa 488 (see the Fluorescence Lifetime Measurements section).

The strong shift of the peak position with Trp present in the sequence is hence induced by the effect of static quenching of the acceptor. The reason for this strong effect is that Alexa 594 in the statically quenched state still accepts energy from Alexa 488 (since its absorption spectrum is only slightly changed in the complex with Trp, Figure 3), but it does not fluoresce, whereas donor emission is virtually unaffected. In contrast, in the case of static quenching of the donor, any excitation of the donor in the DQ state will lead to rapid nonradiative deexcitation, and neither Alexa 488 nor Alexa 594 will emit fluorescence. Static donor quenching thus does not have any effect on the observed transfer efficiency; it just reduces the average molecular brightness of the entire FRET system.

The uncorrected mean transfer efficiencies of all constructs (including those with the short C2 dye linker) are summarized in Figure 5a (see the SI for transfer efficiency histograms of the C2 variants). The transfer efficiencies found for the reference variants are close to $E = 0.6$. In the case of donor quenching, only a slight decrease in $E_{\text{app}}$ results from increasing the sequence separation between Trp and Alexa 488. In the case of acceptor quenching, the much more pronounced effect on $E_{\text{app}}$ is apparent, and a clear increase in $E_{\text{app}}$ with increasing sequence separation is visible, as expected. In all cases, the variants with the shorter C2 dye linker show systematically slightly lower transfer efficiencies, presumably because the shorter linkers do not allow donor and acceptor to approach each other as closely as for the longer dye linkers. In summary, intramolecular PET quenching by Trp of FRET donor and acceptor results in systematic effects on $E_{\text{app}}$. In the following sections, we will demonstrate how the parameters ($t_{\text{D}}, t_{\text{A}}, k_{\text{on}}, k_{\text{off}}$) required for quantifying and understanding these effects based on the kinetic model of the combined FRET/PET process can be obtained from fluorescence lifetime and nsFCS measurements.

**Fluorescence Lifetime Measurements.** To quantify the intramolecular dynamic quenching effect of Trp on the FRET dyes, we determined fluorescence lifetimes with ensemble time-correlated single photon counting. Figure 6a shows two representative fluorescence lifetime measurements. For a peptide labeled only with Alexa 488 at the cysteine, a significant difference is seen in a peptide without (Ref, light green) and with Trp (W3, dark green), as expected from a contribution of dynamic quenching. Both lifetime histograms are well fitted by single exponential decays (see lines in Figure 6a), indicative of a homogeneous sample. For all 20 variants, we determined the mean fluorescence lifetimes of the dyes attached to the cysteine residue, i.e., the dye position affected by Trp quenching (Figure 6b). For all donor lifetimes, we used peptides lacking an acceptor fluorophore.

The comparison of the values obtained for the reference (Ref) variants to the values obtained for the peptides containing Trp (W3–W9) reveals in all cases a significant decrease of the mean lifetime owing to dynamic quenching. The change in mean lifetime is only weakly dependent on the Trp-dye sequence separation. As for the lifetime experiments with free dye and free Trp, a larger dynamic quenching effect was found for Alexa 488 compared to Alexa 594. The dye-linker length (C2 or C5) also has a systematic impact on the fluorescence lifetimes. Whereas the lifetime differences between the C2 and the C5 dye variants are small for the Ref peptide constructs, the differences are larger for the Trp containing constructs. For the peptides with the longer (C5) dye linkers, the observed fluorescence lifetimes are lower compared to the corresponding C2 constructs, possibly due to greater steric constraints or lower accessibility of the Alexa dye imposed by the shorter linker. Altogether, the dynamic quenching effects and their variation with the position of the Trp residue in the peptide are not very large, but they definitely need to be taken

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Figure 5. Transfer efficiencies (averaged over all photon bursts with $E > 0.1$) of all 20 polypeptide constructs. The values are shown without (a) and with (b) correction for quenching effects. Green and red indicate the variants with donor and acceptor quenching, respectively. Values for the variants with C5 dye-linkers (framed symbols) and C2 dye-linkers (unframed symbols) are shown.

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into account for a quantitative analysis of the data. The mean acceptor fluorescence lifetimes, measured for the constructs with quenched donor (A488C5) (see Figure S2), do not deviate from the value found for the corresponding reference without Trp. Therefore we can exclude that the dyes at the N-terminal Gly are dynamically quenched by Trp.

**PET Dynamics from a Global Analysis of nsFCS FRET Curves.** In the next and most important step, we quantified the dynamics of the static intramolecular PET quenching of FRET donor and acceptor by Trp. Using a confocal single-molecule instrument with four synchronized detection channels, we recorded complete sets of nsFCS curves for all 20 constructs, including donor autocorrelations, $G_{DD}(\tau)$, acceptor autocorrelations, $G_{AA}(\tau)$, and donor−acceptor crosscorrelations, $G_{DA}(\tau)$. Figure 7 shows two sets of nsFCS data together with the global fits to the corresponding kinetic models (Figure 1) according to the procedures described in Materials and Methods. Depicted are representative FCS curves for the W3 peptide with quenched donor dye (W3 Alexa 488C5, Figure 7a−c) and for the W3 peptide with quenched acceptor dye (W3 Alexa 594C5, Figure 7d−f), together with data from the corresponding reference peptides without Trp (gray), which only show an antibunching component on the time scale of a few nanoseconds, as expected.

PET quenching of the FRET donor (A488C5) leads to components with a large positive amplitude in all three correlation curves. The formation and dissociation of DQ complexes leads to correlated fluctuations of the photon signal on all detection channels, since neither donor nor acceptor emit fluorescence while the DQ complex is formed. In contrast, in case of acceptor quenching (A594C5), only the acceptor autocorrelation shows a similarly large positive quenching component. In this case, the AQ complex has only very little effect on the fluorescence emission of the donor. However, the crosscorrelation $G_{DA}(\tau)$ does show a small (but significant) component with negative amplitude on the same time scale as the large component in $G_{AA}(\tau)$. Our kinetic model allows us to identify the reason: this small amplitude can be attributed to the change in Förster radius and thus transfer rate constant (from $k_F$ to $k_F^{AQ}$) when Alexa 594 and Trp associate (see the Quenching of Alexa 488 and 594 by Tryptophan section). As a result, donor and acceptor signals are slightly anticorrelated, as

**Figure 6.** (a) Representative fluorescence lifetime decays of donor-only labeled Ref (light green) and W3 (dark green) peptides. Both decays were fitted with single-exponential decays (blue lines). (b) Donor (green) and acceptor (red) mean fluorescence lifetimes of all 20 constructs. Donor fluorescence lifetimes were measured for the variants labeled at the cysteine with Alexa 488. (For these measurements, the constructs were not labeled with acceptor dye.) Acceptor fluorescence lifetimes were measured for the variants labeled at the cysteine with Alexa 594. (c) Collisional quenching rate constants determined from the fluorescence lifetime data (b). Values for the variants with C5 dye-linkers (framed symbols) and C2 dye-linkers (unframed symbols) are shown throughout. The statistical uncertainties estimated as standard deviations from two to three independent measurements are within the size of the symbols.

**Figure 7.** nsFCS data for W3 peptides with quenched donor (A488C5, a-c) and quenched acceptor (A594C5, d-f). For both measurements, the donor−donor (green), donor−acceptor (orange), and acceptor−acceptor (red) nsFCS curves are shown. For each set, model correlation curves $G_{DD}(\tau)$, $G_{DA}(\tau)$, and $G_{AA}(\tau)$ (blue lines) were fitted globally to the data. nsFCS results for the identically labeled reference constructs lacking the Trp residue are shown for comparison (gray).
the donor emission rates increase upon formation of the acceptor–Trp complex owing to the lower rate of energy transfer ($k_{DA}^{\text{rel}} = 0.73k_{p}$). If no energy was transferred from the donor to the AQ complex, we would observe a much stronger anticorrelation in $G_{DA}(\tau)$, and $G_{DD}(\tau)$ would also show a significant component due to acceptor quenching.

We fitted each of the two FCS data sets globally with the appropriate kinetic model including either donor or acceptor quenching (see Figure 2). As described in the Theory section, the only free fitting parameters were the on- and off-rate constants for dye–Trp complex formation, the singlet-single annihilation rate $k_{SSA}$, the triplet blinking amplitudes $c_{ij}$, and the amplitudes $a_{ij}$, which depend only on sample concentration and background signal. All other parameters in the model were determined independently. The values for $E$ used for calculating $k_{p}$ (see eq 8), and the relative values of the molecular brightness (see eq S) were not determined from the transfer efficiency histograms recorded for the corresponding constructs, but from the transfer efficiency histograms recorded for the corresponding reference constructs without Trp. The kinetic model describes the data very well, including the relative amplitudes of all correlation functions from the static quenching component, one of the most stringent requirements for the quantitative description of the process. Figure 8a–f shows the entire series of donor (a,b) and acceptor (e,f) autocorrelation and crosscorrelation (c,d) nsFCS data for the constructs with quenched donor (a,c,e) and quenched acceptor (b,d,f) with C5 dye linkers, illustrating the systematic changes in o and off-rate constants from ensemble lifetime experiments (Figure 6).

As expected, we observe that the amplitudes from static quenching in the FCS curves decrease with increasing Trp–dye sequence separation. Figure 8g shows a comparison of all on- and off-rate constants obtained from the nsFCS fits for all 16 peptides containing Trp. As seen already for the dynamic quenching rate constants (see the Fluorescence Lifetime Measurements section), the on-rates are largely independent of the Trp–dye sequence separation (downward triangles in Figure 8g). More surprisingly, the off-rates increase with increasing dye-quencher separation (upward triangles). The change in off-rates is thus responsible for the decrease in the stability of dye-quencher complexes with increasing sequence separation between the dye and the Trp. While there are no clear differences in the on-rates for the two different dye-linker lengths in case of Alexa 488C2 and Alexa 488CS, the Alexa 594C5 peptides show systematically slightly higher on-rates compared to the corresponding Alexa 594C2 peptides, the same trend as observed for the dynamic quenching rate constants from ensemble lifetime experiments (Figure 6). Consistent with the bimolecular nsFCS experiments with dye and Trp free in solution, we find higher off-rates for peptides where the donor is quenched than where the acceptor is quenched. For comparison, we repeated the measurements for the constructs with quenched acceptor, but by directly exciting the acceptor instead of the donor. The acceptor autocorrelation data obtained were fitted with the corresponding, much simpler, photophysical model. The on- and off-rate constants resulting for the Alexa 594–Trp complex are in good agreement with the results presented above (see Figures S8 and S9 in the SI), confirming that the kinetic model can be used to reliably extract the correct rate constants in the combination of PET with FRET. We confirmed that the dye attached to the N-terminal Gly is not statically quenched by Trp by recording nsFCS data of Alexa 488CS constructs, where we directly excited the acceptor dye with 594 nm laser light (see Figure S10).

**Efficiency of Dye–Quencher Complex Formation.** For the interpretation of PET quenching measurements in terms of contact formation (the quantity that is usually of mechanistic interest), it is important to know the efficiency of complex formation, i.e., how many collisions between Trp and dye are required on average before a stable complex is formed. From our bimolecular measurements with dye and Trp free in solution, we know that the collision rate constant, $k_{coll}$ is
approximately equal to the rate constant of dynamic quenching, \( k_q \). We can thus estimate the mean number of quencher-dye collisions needed before a complex is formed from the on-rate constants, \( k_{\text{on}} \), from the nsFCS measurements as \( k_{\text{on}}/k_{\text{off}} = k_q/k_{\text{on}} \). Figure 9 shows the corresponding ratios calculated from the \( k_q \) and \( k_{\text{on}} \) values given in Figure 6c and Figure 8g, respectively. We observe that, on average, five to nine collisions are required before a complex is formed. For dye and Trp free in solution, we found values for \( k_q/k_{\text{on}} \) ranging from 2 to 3 (data also shown in Figure 9 for comparison).

**Correcting Transfer Efficiency Histograms for Quenching Effects.** Given the robust results obtained from the analysis of the fluorescence lifetime and nsFCS data, the final question is whether we can use these results to account for the transfer efficiency histograms of our peptides in the presence of PET quenching (Figure 4). Direct comparison to the transfer efficiency histograms of the reference peptides (Ref) allows us to test the accuracy of this approach. In addition to the uncorrected histograms already discussed, Figure 4 also shows the corrected histograms from the same experimental data, but corrected according to eqs 8 and 9, i.e., by taking into account the rates of dynamic and static quenching for calculating the effect on the photon count rates and the correction factor \( \chi \). For the peptides with a quenched donor (Alexa 488CS), the distortion of the transfer efficiencies is small, but the correction does improve the agreement with the unquenched reference (Figure 4b). For the peptides with a quenched acceptor (Alexa 594CS), however, the effect of the quenching Trp is dramatic and the correction correspondingly large (Figure 4d). Again, the agreement of the corrected FRET efficiency histograms with the reference is very good. Figure 5b shows the mean transfer efficiencies (\( E > 0.1 \)) of the corrected transfer efficiency histograms for all peptide variants. A comparison of the Ref values to the corrected mean transfer efficiencies reveals differences below \( \sim 0.03 \), which is within the typical uncertainty of single-molecule FRET experiments. The trend in the transfer efficiencies for the different dye-linker lengths with higher transfer efficiencies for the C5 linkers is also well conserved after application of the correction. From this result we can derive three important conclusions. First, the good agreement between the corrected and the reference histograms shows that the correction factors, and therefore the quenching rates obtained, are accurate. Second, these results show that the applied approach and the kinetic model is well suited for quantifying dynamic and static quenching and for correcting single-molecule fluorescence data. Finally, this result highlights how single-molecule FRET can be used as an accurate and precise spectroscopic ruler even in the presence of pronounced quenching effects.

### DISCUSSION

Our results demonstrate the possibility of directly combining quantitative single-molecule FRET and PET in one experimental system. The approach requires a pair of fluorophores that is suitable for single-molecule FRET, and at the same time can be quenched efficiently by a specific additional chromophore incorporated in the polypeptide chain. Our results show that the well-established FRET pair consisting of Alexa 488 and Alexa 594 as donor and acceptor, respectively, together with the natural amino acid Trp, provides a suitable combination of this type. Trp is an efficient fluorescence quencher of both Alexa dyes, with dynamic and static quenching contributions, similar to those in established PET systems, e.g., the oxazine dye MR121, which has been investigated in great detail by Sauer and colleagues. As in the case of MR121, dynamic quenching of Alexa 488 and 594 by Trp is close to the value expected for a diffusion-limited process that can be quantified experimentally by fluorescence lifetime measurements. The two Alexa dyes also form a nonfluorescent complex with Trp with an affinity that is only two- to four-fold lower than for MR121, and that is stable for tens of nanoseconds and can thus be observed by nsFCS.

Given these characteristics, single-molecule FRET between Alexa 488 and 594 can be combined with PET between one of the dyes and Trp in one molecular system. In the example investigated here, the FRET process provides information about the distance between the Alexa dyes spaced by a rigid polyproline linker, and the PET process monitors the contact formation of one of the dyes with Trp incorporated in a flexible peptide tail. However, the two photophysical processes can no longer be treated independently. The most obvious indication is the change in apparent transfer efficiencies caused by the quenching of the FRET dyes (Figure 4). Similarly, the PET process is modulated by FRET, and the resulting nsFCS curves will exhibit signatures of both electron and energy transfer. The simple analysis of nsFCS measurements of PET in terms of single-exponential decays and correlation amplitudes is thus no longer sufficient. A solution to this problem is provided by using a complete kinetic model that takes into account both processes (Figure 1) and can be used to calculate the complete photon statistics of the corresponding measurements according to the procedures developed by Gopich and Szabo, including the photon emission rates and the fluorescence correlation functions. Here, we implemented this approach in a way that allows direct fitting of the entire kinetic model to the nsFCS data, i.e., the donor and acceptor autocorrelation functions and the donor–acceptor crosscorrelation functions, in a global analysis. Since many of the photophysical parameters of the model can be determined independently, a robust analysis of the PET dynamics and the resulting changes in the observed transfer efficiencies is possible. Note that even the amplitudes of the correlation functions are captured quantitatively by the kinetic model, which provides an important constraint for the description of the system.

To enable systematic variation of the quenching efficiency by PET, the position of the Trp residue in the disordered tail of the peptide was varied, with a sequence separation between quenched fluorophore and Trp ranging from three to nine residues.
residues. The use of Gly-Ser peptides allows a direct comparison to previous experiments on contact formation in sequences of this type using PET or triplet–triplet energy transfer. Additionally, the FRET dyes were conjugated in both permutations to probe the effect of selective quenching of either the FRET donor or the acceptor, and the length of the dye linkers was varied. In terms of transfer efficiencies (Figures 4, 5), the effects are most pronounced for quenching of the FRET acceptor, since in this case virtually only the emission of Alexa 594 is affected. In the case of quenching of the donor, Alexa 488, the emission of both FRET dyes is reduced to very similar extents and the apparent FRET efficiencies are only weakly affected (by the dynamic quenching contribution). This behavior is also reflected in the correlation functions (Figure 7). Acceptor quenching is essentially only visible in the acceptor autocorrelation function, but donor quenching affects both autocorrelations and the crosscorrelation function to similar extents. As expected, the effect of quenching and the stability of the Trp–fluorophore complex increases with decreasing sequence separation of fluorophore and Trp (Figure 8). The origin of this trend, however, is somewhat unexpected: while the on-rate for the intramolecular formation of the complex between Trp and FRET dye is essentially independent of their sequence separation over the range investigated here, the off-rate increases with increasing sequence separation. The former observation is in agreement with previous experiments on short flexible peptides and has been attributed to stiffness effects of the chain in a range of segment lengths close to the persistence length, where simple polymer scaling behavior breaks down. The detailed molecular origin of the change in off-rate is currently not clear. However, the pronounced effects of even small changes in linker length of the FRET dyes on the PET process (Figure 8) indicates that steric aspects play an important role, such as the possible relative orientations of dye and Trp and conformational strain from the linker peptide. To our knowledge, no comparable data for the off-rates have been reported in the literature. The on-rates, however, are similar to previously reported values. They range from ~0.02 ns$^{-1}$ to ~0.06 ns$^{-1}$, compared to ~0.08 ns$^{-1}$ for terminal contact formation and ~0.03 ns$^{-1}$ for internal contact formation in Gly-Ser peptides of similar length. This agreement provides additional support for the validity of our analysis. Molecular simulations might allow a more detailed assignment of the possible role of steric effects in the observed behavior.

Finally, the kinetic parameters determined from our analysis can be used to account for the effect of PET quenching on the transfer efficiency histograms (Figure 4, Figure S3), and the agreement of the corrected transfer efficiencies with the histograms in the Trp-free peptides confirms the consistency of the results. We want to point out that PET quenching by Trp (and to a lesser extent by other amino acids) can thus also affect FRET experiments in natural proteins and distort the observed transfer efficiency distributions and fluorescence correlation functions. In the presence of Trp in the sequence, it is therefore essential to exclude the influence of such effects if quantitative information on distances or distance dynamics are to be determined from the experimental data. The most effective way of identifying complications of this type is a quantitative comparison of transfer efficiencies from photon count rates and fluorescence lifetimes in multiparameter analyses and auto- and crosscorrelations of FRET donor and acceptor fluorescence. In the absence of PET quenching, the transfer efficiencies from photon count rates and fluorescence lifetimes should be in agreement. Furthermore, PET quenching leads to characteristic correlated fluctuations of the photon signal apparent in the nsFCS experiments (Figure 7). Note that the effect of PET quenching can be clearly distinguished from distance dynamics between FRET donor and acceptor by comparing auto- and crosscorrelations: FRET-based measurements of distance dynamics lead to correlated fluctuations visible in the donor and acceptor fluorescence autocorrelations, but to anticorrelated behavior in the donor–acceptor crosscorrelation functions (with identical relaxation times). It is thus essential to analyze auto- and crosscorrelation functions globally to ensure a correct interpretation of the results. In case of doubt, it is advisable to prepare a Trp-free variant of the protein or peptide under investigation to allow an unequivocal assignment of the effect. The same concepts and methods used here can of course also be employed in a subpopulation-specific analysis of the correlation functions. However, for dynamics on the fast time scales investigated here, the measurement times would have to be extended significantly to obtain sufficient photon statistics.

**CONCLUSION**

The combination of single-molecule FRET and PET opens up a range of applications and future developments. The FRET/PET approach extends the accessible distance range for the investigation of intra- and intermolecular dynamics in complementarity to multicolor FRET, whose scope is limited by the relatively large Förster radii of current FRET pairs suitable for single-molecule spectroscopy. Note that PET is not limited to the submicrosecond time scale monitored here and could be used for slower processes, e.g. large conformational changes, if fluorophore and quencher come into sufficiently close contact. Since Trp is by far the most efficient quencher of typical FRET dyes, the direct combination of FRET and PET is also highly suitable for nonsynthetic polypeptides; as a natural amino acid, Trp can easily be incorporated at suitable positions in recombinant proteins. An important next step will be to extend the FRET/PET approach in a way that allows information on dynamics to be extracted not only from the PET but also from the FRET process, e.g. to probe correlations between local and nonlocal distance fluctuations within a protein or peptide. The methods and the kinetic model used here can easily be amended for this purpose in analogy to the approach used for extracting long-range distance dynamics from single-molecule FRET and nsFCS. We thus expect that this combined approach will allow previously inaccessible questions in biomolecular dynamics and mechanisms to be answered.

**ASSOCIATED CONTENT**

Supporting Information

Stern–Volmer analysis for Alexa 488C5 and Alexa 594C5. The complete set of the nsFCS data together with the fits for the C2 and C5 dye-linker variants. Transfer efficiency histograms for the C2 dye-linker variants. Alexa 594 direct excitation controls. This material is available free of charge via the Internet at http://pubs.acs.org.
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Supporting Information

Intramolecular Distances and Dynamics from the Combined Photon Statistics of Single-Molecule FRET and Photoinduced Electron Transfer

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Dye-Trp fluorescence emission spectra and Stern-Volmer plots. To analyze the static quenching of Alexa 488 and Alexa 594 by Trp and to test whether the dye-Trp complex is non-fluorescent, we recorded fluorescence emission spectra of free dye in the presence of Trp. The resulting spectra taken for Trp concentrations ranging from 0 to 100 mM are depicted in Figure S1a-c. The ratios $I_0/ I$ of the integrated intensities are shown in the Stern-Volmer plots (Figure S1d-f). $I_0$ is the integrated fluorescence intensity in the absence of Trp. The data could be described assuming a combination of static and dynamic quenching processes:

$$\frac{I_0}{I} = (1 + K_S [Trp]) \cdot (1 + K_D [Trp]),$$

where $K_S$ and $K_D$ are the Stern-Volmer constants for static and dynamic quenching, respectively. We obtained $K_D$ from fluorescence lifetime measurements (see main text) and varied $K_S$ for fitting the data. The fits (red lines) yielded static quenching constants $K_S$ of 15 M$^{-1}$ for Alexa 488 and 30 M$^{-1}$ for Alexa 594. These values are in good agreement with the ones obtained from nsFCS measurements (17 M$^{-1}$ for Alexa 488 and 30 M$^{-1}$ for Alexa 594, see main text). Additionally, we also show the static quenching contribution alone by dividing the data by $(1 + K_D [Trp])$ (blue lines in Figure S1d-f). To confirm that the dye-Trp complex is non-fluorescent, we fit the data with a more general model that includes a term describing a potential residual fluorescence intensity $I_{res}$ of the dye-Trp complex:

$$I = I_0 \frac{1}{1 + K_D [Trp]} + \frac{1}{1 + K_S [Trp]} I_{res} + \frac{K_S [Trp]}{1 + K_S [Trp]}.$$

Data and fits are shown in Figure S1g-i. Again, we used the $K_D$ values from the fluorescence lifetime measurements. $I_{res}$ was constrained to nonnegative values. We obtained essentially the same values for $K_S$ as from the fits above and the found values for $I_{res}$ to be virtually zero (~10$^{-9}$). Since the extrapolation is sensitive to small experimental errors, we repeated the measurements for Alexa 488 at pH 2.5 to increase the solubility of Trp. This enabled us to measure at Trp concentrations up to 100 mM. The resulting value for $K_S$ (26.5 M$^{-1}$) is significantly higher than the value found at pH 7.2. The residual fluorescence intensity of the dye-Trp complex is again virtually zero. We conclude that the data agree within error with our assumption that the dye-Trp complex is non-fluorescent. The assumption is also supported by our observation that with increasing quencher concentration, we observe only a decrease in amplitude, but no change in the shape of the spectra. The spectra are hence not a superposition of two different spectra, as might be expected for a fluorescent dye-Trp complex.

Calculation of diffusion coefficients. The diffusion coefficients of the dyes (including the hydrolyzed linker) and of Trp were calculated using the Wilkie-Chang estimation method combined with the additive method by Schroeder for estimating molar volumes at the normal boiling point. The obtained molar volume for Trp is 217 cm$^3$ mol$^{-1}$ and the corresponding diffusion coefficient in water at 25 °C is 6.7·10$^{-6}$ cm$^2$ s$^{-1}$, which is in good agreement with reported values. For Alexa 488, we obtained a molar volume of 644 cm$^3$ mol$^{-1}$ and for Alexa 594 of 896 cm$^3$ mol$^{-1}$. Using these values to calculate the diffusion coefficients, we obtained 3.5·10$^{-6}$ cm$^2$ s$^{-1}$ for Alexa 488 and 2.9·10$^{-6}$ cm$^2$ s$^{-1}$ for Alexa 594. For Alexa 488, dual-focus FCS measurements performed in our laboratory yielded a diffusion coefficient of 3.7·10$^{-6}$ cm$^2$ s$^{-1}$, in good agreement with the calculated value.
Figure S1. Fluorescence quenching of Alexa 488 and Alexa 594 by free Trp. (a-c) Fluorescence emission spectra of Alexa 488 (a: pH 7.2, b: pH 2.5) and Alexa 594 (c: pH 7.2) for increasing Trp concentrations (blue to red: 0-60 mM Trp (a, c), 0-100 mM Trp (b)). (d-f) Stern-Volmer plots obtained from integrated intensities of the spectra (lighter colored symbols). The data were fitted with the Stern-Volmer equation (Eq. 1) (red curves). The darker points and the blue curves are obtained by division of the data and the fits by \((1 + K_D [Trp])\). The normalized integrated intensities are also represented in (g-i), where the blue curves represent fits with an extended Stern-Volmer model (Eq. 2).
Figure S2. Mean acceptor fluorescence lifetimes measured for the constructs with quenched donor (A488C5). As expected, the lifetimes of the peptides containing Trp (W3-W9) do not differ significantly from the reference (Ref) without Trp. This indicates that there is no dynamic quenching of the fluorescent dye attached to the N-terminal Gly of the peptides. Only the fluorescence of the dye attached to the cysteine is quenched. The statistical uncertainties estimated as standard deviations from two to three independent measurements are within the size of the symbols.
Figure S3. Transfer efficiency histograms measured with the peptide constructs Ref, W3, W5, W7, and W9 with the C2 dye-linker. The left group of histograms (a, b) contain data from peptides with a quenched donor (A488C2), and the right group (c, d) data from peptides with a quenched acceptor (A594C2). Reference histograms (Ref) from constructs lacking Trp are included. The left (a, c) and right (b, d) columns of each group show histograms without and with correction for dynamic and static quenching, respectively. Dashed black lines indicate the positions of the peak maxima of the reference measurements. (Corresponding histograms for the constructs with C5 linkers are shown in the main text.)
Figure S4. nsFCS data obtained from the A488C2-constructs (Ref, W3-W9). For all measurements, the donor-donor (green), donor-acceptor (orange), and acceptor-acceptor (red) nsFCS curves are shown. For each set, model correlation curves $G_{DD} (\tau)$, $G_{DA} (\tau)$, and $G_{AA} (\tau)$ (blue lines) were fitted globally to the data.
Figure S5. nsFCS data obtained from the A488C5-constructs (Ref, W3-W9). For all measurements, the donor-donor (green), donor-acceptor (orange), and acceptor-acceptor (red) nsFCS curves are shown. For each set, model correlation curves $G_{DD}(\tau)$, $G_{DA}(\tau)$, and $G_{AA}(\tau)$ (blue lines) were fitted globally to the data.
Figure S6. nsFCS data obtained from the A594C2-constructs (Ref, W3-W9). For all measurements, the donor-donor (green), donor-acceptor (orange), and acceptor-acceptor (red) nsFCS curves are shown. For each set, model correlation curves $G_{dd}(\tau)$, $G_{da}(\tau)$, and $G_{aa}(\tau)$ (blue lines) were fitted globally to the data.
Figure S7. nsFCS data obtained from the A594C5-constructs (Ref, W3-W9). For all measurements, the donor-donor (green), donor-acceptor (orange), and acceptor-acceptor (red) nsFCS curves are shown. For each set, model correlation curves $G_{DD}(\tau)$, $G_{DA}(\tau)$, and $G_{AA}(\tau)$ (blue lines) were fitted globally to the data.
Figure S8. Acceptor-acceptor nsFCS data of peptides with quenched Alexa 594C2 (a) and Alexa 594C5 (b) upon direct excitation of the acceptor at 595 nm. The colors indicate the position of Trp: red (W3), orange (W5), green (W7), blue (W9), black (Ref without Trp). For easier comparison, the curves are offset by 0.16. (c) On- and off-rate constants obtained from photon statistics fits to the nsFCS data in (a) and (b). Represented are: $k_{\text{on}}^{\text{AQ}}$ (downward triangles) and $k_{\text{off}}^{\text{AQ}}$ (upward triangles). Values for the variants with C5 dye-linkers (framed symbols) and C2 dye-linkers (unframed symbols) are shown. The nsFCS data together with the fits are also presented in Figure S10. Error bars indicate statistical uncertainties estimated as standard deviations from two to three independent measurements. In the cases where the error bars are invisible, they are smaller than the size of the symbols.
Figure S9. Acceptor-acceptor nsFCS data from Alexa 594C2 constructs (left column) and Alexa 594C5 constructs (right column) upon direct excitation of the acceptor at 595 nm. Fits are shown in blue.
Figure S10. Acceptor-acceptor nsFCS data from the peptides W3-W9 with a quenched donor, Alexa 488C2 (a) and Alexa 488C5 (b) upon direct excitation of the acceptor at 595 nm. The colors indicate the position of Trp: red (W3), orange (W5), green (W7), blue (W9). For easier comparison, the curves are offset by 0.16. The difference in the noise levels between (a) and (b) are due to different measurement times. No static quenching component is observed in these correlation data.

References


