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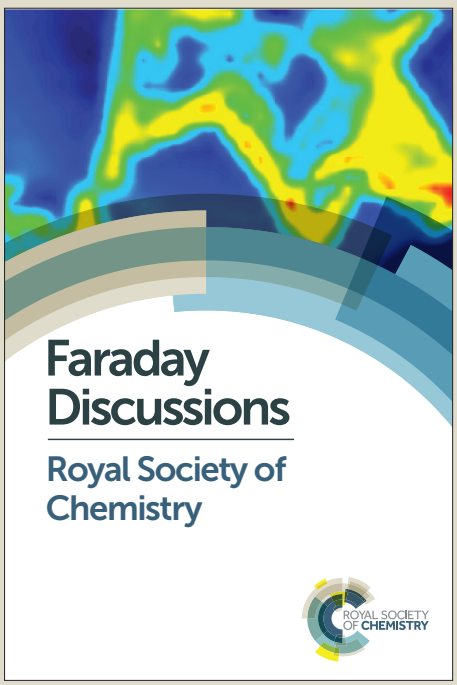

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Quantitative single molecule FRET efficiencies using TIRF microscopy

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Förster Resonance energy transfer (FRET) microscopy at the single molecule level has the potential to yield information on intra and intermolecular distances within the 2-10 nm range of molecules or molecular complexes that undergo frequent conformation changes. A pre-requirement for obtaining accurate distance information is to determine quantitative instrument independent FRET efficiency values. Here, we applied and evaluated a procedure to determine quantitative FRET efficiencies directly from individual fluorescence time traces of surface immobilized DNA molecules without the need for external calibrants. To probe the robustness of the approach over a wide range of FRET efficiencies we used a set of doubly labelled double stranded DNA samples, where the acceptor position was varied systematically. Interestingly, we found that fluorescence contributions arising from direct acceptor excitation following donor excitation are intrinsically taken into account in these conditions as other correction factors can compensate for inaccurate values of these parameters. We give here guidelines, that can be used through tools within the iSMS software (www.isms.au.dk), for determining quantitative FRET and assess uncertainties linked with the procedure. Our results provide insights into the experimental parameters governing quantitative FRET determination, which is essential for obtaining accurate structural information from a wide range of biomolecules.

Introduction

Single molecule Förster resonance energy transfer (smFRET) microscopy is a powerful technique to study conformational changes and dynamics of biomolecules with sub-nanometer resolution.[1] smFRET continues to provide new insights into conformational distributions, dynamic transitions and the kinetics governing diverse biological systems[2, 3] as well as artificial molecular devices.[4, 5] In addition, quantitative smFRET has the potential to report on accurate molecular distances and yield structural information on highly dynamic structures within single molecules, which is difficult to achieve with other techniques.[6, 7] Quantitative Förster resonance energy transfer (FRET) values can indeed be linked to distances with adequate modelling of the donor and acceptor.[7-10] Some of the advantages of using FRET, compared to higher resolution techniques such as Nucleic Magnetic Resonance (NMR) is that very little material is needed and that highly polymorphic and dynamic molecules can be studied. The use of FRET has already successfully provided structural information in a number of systems, ranging from small DNA molecules[11, 12] to proteins and nucleic acids protein complexes.[7, 9, 13]

Single molecule FRET efficiencies are determined from measured single molecule fluorescence intensities obtained with fluorescence microscopy. The measured values are instrument and fluorophore dependent and need to be corrected in order to determine the true FRET efficiency.[14, 15] Instrument-independent FRET efficiencies, which are required in order to use FRET for distance measurements, are given by:

$$E = I_{FRET} / (\gamma \cdot I_{DD} + I_{FRET}) \qquad \text{Eq. 1}$$

where I_{DD} is the measured fluorescence intensity of the donor dye following donor excitation and I_{FRET} is the fluorescence intensity of the acceptor dye resulting from energy transfer, which is given by the measured fluorescence intensity in the acceptor emission channel I_{DA} corrected for contributions from donor fluorescence bleed through into the acceptor emission channel (D-leak) and from direct excitation of acceptor fluorescence (A-Dir) at the donor excitation wavelength. Additionally, the γ factor corrects for differences in detection of the donor and acceptor fluorophores and is given by $\gamma = \eta_A \Phi_A / \eta_D \Phi_D$, where η and Φ are the detection efficiencies and quantum yields of the fluorophores respectively, with the subscript A denoting the acceptor and D the donor fluorophore.

There are a number of methods to determine the three correction factors D-leak, A-Dir and γ used in confocal [14, 16, 17] and widefield single molecule FRET microscopy.[18-20] Total internal reflection fluorescence (TIRF) widefield microscopy allows imaging surface immobilized individual molecules over a long period of time (tens of minutes) with good temporal (~20 ms) and distance (< 0,34 nm corresponding to one DNA base-pair separation) resolutions.[21, 22] Procedures for obtaining

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quantitative FRET values from these experiments have been reported using the alternative laser excitation method (ALEX).[19] In this method, global values of the correction factors are determined and applied to all molecules. In some cases, correction factors can be determined directly from single molecule time traces of immobilized molecules by comparing fluorescence intensities before and after donor and/or acceptor photobleaching of individual molecules.[15, 18, 20, 23] By observing each molecule individually, it becomes possible to determine and take into account correction factor distributions resulting from either molecular or experimental heterogeneities.

Here, we investigated the heterogeneity and accuracy of correction factors in smFRET TIRF microscopy when the factors are determined directly from fluorescence time traces of surface immobilized molecules. To assess the robustness of the approach and the importance of each correction factor for quantitative FRET measurements over a wide range of FRET efficiencies, we used a set of doubly labelled double stranded DNA samples. We found that the value of the direct acceptor correction factor A-Dir, which is often neglected, can affect both γ factor values and the width of the FRET distribution, especially at low FRET value. However, peak FRET efficiencies values were not affected by the A-Dir value when the intensity change during bleaching in single molecule time traces was used to determine the γ factor. Thus, this procedure can provide quantitative FRET, also when the A-Dir correction is neglected as the γ factor can compensate for an inaccurate A-Dir correction. We find a good agreement between measured and calculated FRET efficiencies. Our procedures for determining quantitative smFRET efficiencies using single molecule TIRF microscopy without external calibrants, are implemented within the iSMS software platform and easily applicable.[24]

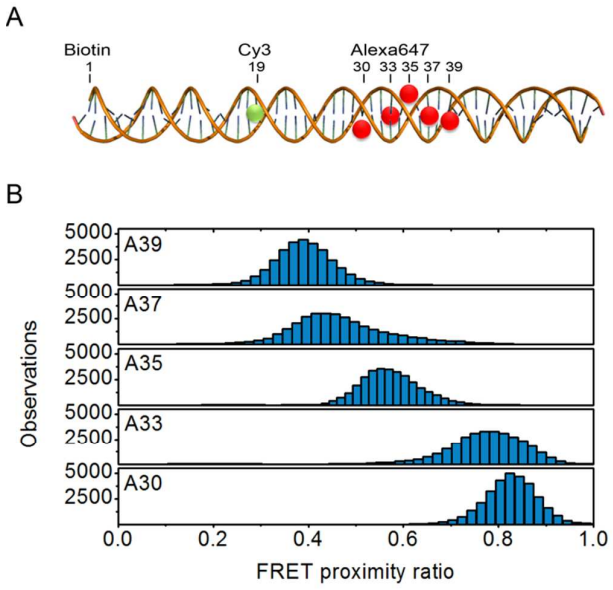

Figure 1: DNA duplex used as a molecular ruler. A) Schematic view of the double stranded DNA samples: One strand contains an internal Cy3, the second strand is labelled with Alexa 647 at one of the indicated positions. B) Single molecule FRET efficiency histograms for each of the 5 DNA samples. The x-axis shows the proximity ratio which is obtained using Eq. 1 where the D-leak and A-Dir correction are set to zero and γ=1.

Experimental

DNA constructs

PAGE grade purified single stranded DNA, labelled with either a donor (D, Cy3) or an acceptor (A, Alexa647), were purchased from IBA (IBA GmbH, Germany). DNA sequences were 5'-CGC GTC GGC AGC ATA CAA **T**AA CCT CAT CGA TAA GAA AGA AAT AAA GAA GAT CGC, where the underlined T base shows the donor position and a biotin was attached on the 5' end. The complementary strand 5'-GCG ATC TTC TTT ATT TCT TTC TTA TCG ATG AGG TTA TTG TAT GCT GCC GAC GCG was synthetized with 5 different acceptor positions at T bases number 16, 18, 20, 22 and 25, which are underlined. Fluorophores were attached using NHS-labeling through 5-C6-amino-2´-dT modifications. Unlabeled strands were also purchased to produce control DNA constructs containing only a donor or acceptor label. Equimolar stoichiometries of donor and acceptor strands were annealed in TAE buffer (20 mM Tris/Acetate/EDTA, pH 8.3) and 100 mM KCl (Sigma-Aldrich) for 5 minutes at 90 C and left to cool down slowly over about one hour. This resulted in five different double stranded samples which are named A39, A37 A35, A33 and A30, where the donor acceptor dye pairs are separated by 20, 18, 16, 14 and 11 base pairs respectively.

Single-molecule FRET microscopy experiments

smFRET experiments were performed using alternating laser excitation as described previously.[15] In short, DNA molecules with a concentration of 20 pM were immobilized via biotin-streptavidin linkage on a quartz coverglass for prism total internal reflection fluorescence microscopy. Fluorescence was measured using an inverted wide-field optical microscope. The sample was illuminated by alternate laser excitation at 514 and 630 nm of the donor and acceptor fluorophore, respectively and fluorescence movies of several minutes were recorded with an EMCCD camera (Andor, iXon3 897) with a 200 ms integration time per frame. Typical excitation intensities were estimated to be ~0.3 and 0.1 kW/cm^2 for the green and red laser, respectively.

The imaging buffer, used in smFRET experiments, was the same as the annealing buffer but contained in addition an enzymatic oxygen scavenging system consisting of glucose oxidase (Sigma-Aldrich, 16.67 units/ml), catalase (Sigma-Aldrich, 260 units/ml), B-D-(+)glucose (Sigma-Aldrich, 4.5 mg/mL), and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, Sigma-Aldrich, 2 mM). Halfway through the acquisition the imaging buffer was substituted with the annealing buffer in order to facilitate fluorescence bleaching.

Data analysis

Data analysis was performed by the smFRET microscopy software package iSMS.[24] Fluorescence time traces of identified FRET-pairs were calculated by aperture photometry and only molecules ending with single-step donor and

acceptor photobleaching were selected for further analysis as this indicated that the source of the signal was a single doubly labelled molecule. Fluorescence intensities were corrected for background signal by subtracting the remaining signal after bleaching of both fluorophores.

Theoretical FRET efficiency determination

Ensemble absorption and fluorescence spectra were measured on control double stranded DNA constructs containing only a donor or an acceptor fluorophore using a Carry 60 absorption spectrometer (Agilent Technologies) and a FluoroMax 3 spectrofluorometer (Horiba Ltd.). Cy3 quantum yield was determined in annealing buffer using 5-carboxytetramethylrhodamine (Life Technologies) in methanol as a reference (with Φ_{ref}=0.68).[25] Based on the results of these measurements, the Förster radius was determined to be R_0=5 nm assuming free fluorophore rotation (κ^2=2/3). Theoretical FRET efficiencies were calculated through the FPS software[7] which takes into account the spatial distributions of donor and acceptor fluorophores using a geometric accessible volume algorithm.

Results and discussion

We studied experimental parameters that influence the accuracy of FRET efficiencies determined using TIRF microscopy on surface-immobilized double-stranded DNA molecules. The DNA-duplexes were labelled with a donor-acceptor FRET pair providing a series of samples with predefined donor acceptor distances varying between ~3 and 7 nm as the acceptor position is systematically varied along the DNA (Fig. 1A). For each of the five DNA samples where donor acceptor fluorophore pairs are positioned at different distances, single molecule fluorescence time traces of the donor and acceptor fluorophores were recorded (Fig. 2) using an alternating laser excitation scheme. The experimental conditions were adapted to have the majority of molecules show donor or acceptor photobleaching within the time frame of the measurements (minutes). Measured raw FRET values were different between the five samples and varied from low to high FRET according to the donor acceptor distance separation (Fig. 1B).

Quantitative FRET efficiency values were determined using Eq.1 with:

$$I_{FRET} = I_{DA} - Lk - Dir \qquad \text{Eq. 2}$$

where *Lk* and *Dir* are the D-leak and A-Dir contributions. These fluorescence correction factors, as well as the γ factor, were determined directly from single molecule time traces (Fig. 2).

Determination of fluorescence correction factors

The D-leak contribution is given by a fraction *l* of the donor fluorescence I_{DD} that leaks through to the acceptor channel with $l = I_{DA}/I_{DD}$ when only the donor fluorophore is active. *l* was determined from individual FRET-pairs, where the acceptor photobleached before the donor fluorophore (Fig. 2A). For the given measurements conditions, the acceptor fluorophore bleached first for ~90% of the molecules and the histogram over all individual *l* values showed a narrow peak (Fig. 2D). Thus *l* could be determined with good precision to a mean value of 0.105±0.018. The obtained value matched theoretical expectations based on the donor fluorescence spectrum and specifications of the filters used in the single molecule experiments.

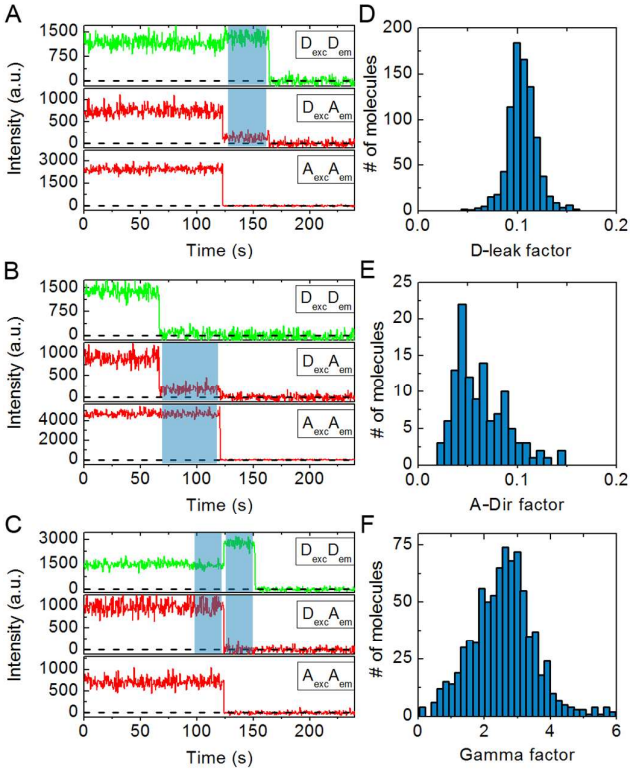

Figure 2: Correction factors are determined from individual molecules fluorescence time traces. A-C) Fluorescence time traces showing from top to bottom I_{DD} (green line), I_{DA} (red ine) and I_{AA} (red line) as a function of time. A) D-leak correction determination from molecules showing acceptor bleaching first. B) A-Dir correction determination for molecules showing donor bleaching first. C) γ factor determination from molecules showing acceptor bleaching first. The frames used when determining correction factors are highlighted in light blue. D-F) Histograms of correction factor obtained from individual molecule time traces on the whole Cy3/Alexa 647 data set (~1000 molecules) for *l* (D), *d* (E) and γ (F). The histograms contain data from 842 (D), 119 (E), and 816 (F) molecules.

The direct acceptor excitation correction factor A-Dir is a fraction *d* of $I_{AA,}$ the acceptor fluorescence following direct acceptor excitation.[14, 19] The correction factor *d* is given by: $d = I_{Dex}/I_{Aex} \cdot \sigma^A_{Dex}/\sigma^A_{Aex}$ including the ratio of acceptor absorption cross sections at the donor and acceptor excitation wavelengths and I_{Dex} and I_{Aex} are the excitation intensities of donor and direct acceptor fluorescence. *d* was determined from single molecule time traces showing donor bleaching as I_{DA}/I_{AA} after donor bleaching which requires two color ALEX experiments (Figure 2B). The distribution of *d* values over all measured DNA samples was broad (Figure 2E) giving a mean value of *d*=0.06±0.03. In comparison, theoretical predictions yielded *d*~0.06.. The large distribution of *d* values arised from the fact that the laser intensities used to excite the donor and acceptor molecules were not completely homogeneous throughout the CCD image field of view. It is to be noted that the A-Dir correction is independent of I_{Aex} and thus should

have a similar distribution width as the D-leak correction factor. Statistics on this correction were however poor as it could be determined for only ~10% of molecules and the A-Dir correction distribution was observed to be wider than D-leak. Thus, using an average A-Dir value on all time traces may introduce an error in quantitative FRET determination.

γ correction factors were determined from single molecule fluorescence time traces as:

$$\gamma = -\Delta I_{FRET}/\Delta I_{DD} \qquad Eq.3$$

where ΔI denotes the difference in the average fluorescence intensities before and after acceptor photobleaching (Fig. 2C). Because E drops to 0 at the acceptor bleaching event, the post bleaching intensities can be used as a reference for the relative brightness of donors and acceptors.[18] The γ factor correction was here determined using average d and I values (Fig. 2F). The histogram of individual values shows a relatively broad distribution and yields an average value of γ=2.6±1.2 with a large uncertainty.

Applying the D-leak and A-Dir corrections will decrease measured energy transfer values. These corrections have proportionally a larger effect for small I_{DA} values and thus will play a larger role for lower energy transfer efficiencies. The more different the γ factor is from 1 the more it will affect the FRET efficiency in a non-linear way. As I_{FRET} is used to determine the γ factor value, it will also depend on the value of the D-leak and A-Dir corrections. Fluctuations of the D-Leak factor were small from molecule to molecule and using either the average value from single molecule measurements or the theoretical value yielded very similar γ factor and FRET efficiency values.

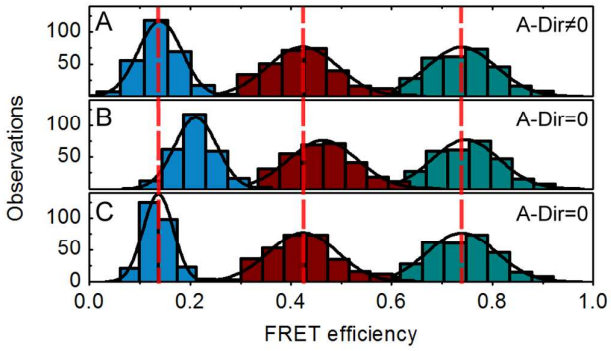

Figure 3: Correction factors for quantitative FRET directly determined for individual molecules. Histograms showing FRET efficiency distributions for 3 representative molecules from samples A39, A35 and A30, respectively, when systematically varying the value of the A-Dir correction. A) d=0.058 and I and γ were individually determined for each molecule: blue curve: I=0.12, γ=1.90, red curve: I=0.09, γ=3.10; green curve: I=0.11, γ=2.54 B) d=0 and the same I and γ values were used as in A). C) d=0 and I and γ were individually determined for each molecule. Blue curve: I=0.12, γ=3.22; red curve: I=0.09, γ=3.64; green curve: I=0.11, γ=2.64.

Effect of direct acceptor excitation

Since determination of the A-Dir correction factor was associated with a large uncertainty, we explored the effect of A-Dir on the value of the γ factor and FRET efficiency. Although direct excitation of the acceptor is a small effect (the ratio of the Alexa647 fluorophore absorption cross sections at donor and acceptor excitation wavelength is 0.02), the A-Dir correction is of about the same order of magnitude as the D-leak correction under our experimental conditions. d factor values depend on excitation spot profiles and thus may well vary from molecule to molecule. Direct determination from single molecule data requires the donor fluorophore to photobleach before the acceptor bleaches. Thus, it is not possible to simultaneously extract A-Dir and γ factor corrections for individual molecules. To gain insight into how the uncertainties for the A-Dir correction factor influence the γ factor, we determined gamma factor corrections assuming different d factor values.

d	γ factor				
	A39	A37	A35	A33	A30
0.0	3.0±0.9	2.9±0.9	2.9±0.6	2.8±0.7	3.3±0.7
0.025	2.7±0.8	2.6±0.9	2.7±0.6	2.7±0.7	3.1±0.7
0.058	2.2±0.8	2.2±0.8	2.5±0.5	2.5±0.7	3.0±0.7
0.091	1.8±0.8	1.8±0.8	2.3±0.5	2.4±0.7	2.9±0.7

Table 1: Average of individual correction factors for quantitative FRET determination, determined from single molecule time traces for samples containing Cy3 and Alexa647

The d factor value influenced average values of γ factors determined using the photobleaching method (vertical comparison in Table 1). γ factor values were observed to decrease for higher d values. . This observation can be understood by the fact that higher values of A-Dir will lead to smaller I_{FRET} signals through Eq. 2, and thus result in smaller values of the γ factor through Eq. 3.

Separate determination of the γ factor for each of the five DNA duplexes (horizontal comparison in Table 1) showed differences between the different constructs. . For the larger A-Dir value (bottom row in Table 1), the average γ factor value increased as the separation between donor and acceptor fluorophores decreased. As the A-Dir correction is a larger fraction of the I_{FRET} signal at low FRET efficiencies, its effect on the γ factor will be larger for large donor acceptor separations and almost negligible at high FRET efficiencies for small donor acceptor separations. γ factor values obtained for the A30 sample were minimally influenced by the value of the A-Dir correction, which is in agreement with the above argument. As the A-Dir correction influences differently γ factor values as a function of donor acceptor distance separation, the γ factor will not necessarily be constant for a given donor acceptor pair. It is also to be noted that a larger uncertainty is associated with individual γ factor determination for larger donor acceptor separations (Table 1). This effect is intrinsic to the use of Eq. 3 for γ factor determination, as large donor acceptor separations result in only small fluorescence changes in both the numerator and denominator of the equation. In order to determine which γ factor value to use for quantitative FRET determination, FRET efficiency histograms were determined for all conditions summarized in Table 1 (data not shown).

We illustrate the effect of *d* and γ on FRET efficiency values by showing FRET efficiency histograms for three individual molecules that have different donor-acceptor separations (Fig. 3). Using γ correction factor values obtained by the photobleach method for a given *d* value: *d*=0.058 (Fig. 3A) and *d*=0 (Fig. 3C) yielded the same FRET efficiencies peak values, despite the use of different γ correction factor values. For each molecule, as illustrated by three examples in Fig. 3, we observed that the same peak FRET efficiencies are obtained throughout the whole FRET range when overestimating, underestimating or even ignoring the A-Dir correction through an adjustment of the γ factor value. However, changing one parameter without adjusting the other resulted in different FRET peak values (Fig. 3B). The dependence of the γ correction factor values on A-Dir can be understood by fully expressing Eq. 3 using Eq. 2. As I_{FRET} after acceptor photobleaching is zero, the larger the A-Dir correction the smaller ΔI_{FRET} and thus smaller γ values are obtained. Thus γ correction factors obtained with the photobleach method can to a large extend compensate for errors in the determination of the A-Dir correction to yield quantitative FRET efficiencies. As such, these γ values include an A-Dir component in addition to the quantum yield and detection efficiency terms. This observation also extends to sample averaged results as peak FRET efficiencies in FRET distribution histograms that include all molecules for a given DNA construct were also observed to be independent of the chosen A-Dir value when using a sample averaged γ factor.

A broadening of FRET efficiency distributions was observed for large *d* values (Fig. 3A). The effect was most visible for molecules showing small FRET efficiencies (E<~0.3), for which the A-Dir correction represents a significant fraction of I_{FRET}. Setting the A-Dir correction to zero and determining γ factor values under this condition resulted in the narrowest FRET efficiency peaks (Fig. 3C). We have thus used this approach for quantitative FRET determination.

Quantitative FRET efficiencies with TIRF microscopy

Quantitative FRET efficiency histograms were determined for each of the five samples (Fig. 4A). Histograms contain only molecules where the acceptor fluorophores bleached first to allow for γ factor determination for individual molecules. FRET values were determined using both a sample averaged (Fig. 4A) and individually determined γ factors (data not shown). Uncertainties on individual γ factors were calculated form Eq. 3 using error propagation and uncertainties linked with the determination of the average fluorescence intensities. We observed that the FRET distribution was broader when using individual γ factors than when using sample averaged γ values. This effect arose due to a large uncertainty on individually determined γ factors for a subset of molecules. Removing these molecules from the FRET histogram yielded narrower FRET distributions with a width comparable to or slightly wider than those obtained using a sample averaged γ factor. Peak FRET values were the same in both cases.

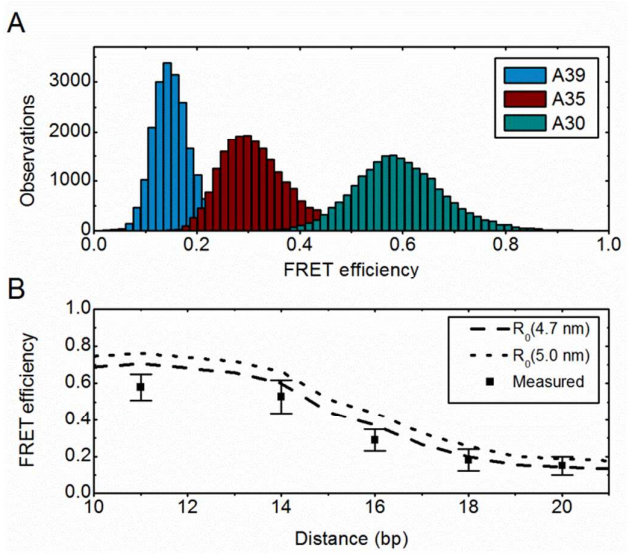

Figure 4: Quantitative FRET efficiency. A) Single molecule FRET histograms showing data for samples A39, A35, A30. Average D-leak and γ-factor corrections were used for each sample. The A-Dir correction was set to zero. B) Comparison between experimentally determined FRET efficiencies and theoretical predictions following procedures from Ref[7] using R_0=4.7 nm and R_0=5 nm. The parameters used to describe the Alexa 647 dye were adapted from Ref.[26] Average γ factors are given in Table 1.

The use of individually determined γ factor values has previously been reported to significantly decrease the width of FRET distribution peaks.[20] This is expected to be the case, provided that the uncertainty of each individually determined γ factor is not larger than the width of the γ factor distribution and that γ factors are not intrinsically heterogeneous. In the present experiments, the uncertainty linked to individual γ factors was in most cases larger than the uncertainty of the average γ factor. Furthermore, the observed γ factor distribution was broad (Fig. 2F). These two factors would contribute to broaden FRET efficiency distribution histograms, and increase the uncertainty of quantitative FRET determination. Broad γ factor distributions have previously been observed for Cy3,[23] and could be intrinsic to the used fluorophore.

FRET efficiency peak values increased with decreasing donor acceptor distance (Fig. 4). Peak FRET efficiencies were compared with theoretical predictions based on the known DNA and fluorophores' structures[7] and a Förster radius R_0 of 4.7 nm and 5 nm (Fig. 4B). Uncertainties in the experimentally determined FRET values were determined from uncertainties on the γ factor (Table 1). Experimental FRET values are in good agreement with theoretical predications, especially when using the lower R_0 value. This R_0 value is slightly lower than our estimate, but is within experimental uncertainty. One source of uncertainty is the used value of κ^2, which may not be completely applicable for immobilized molecules.[22] We conclude that the procedure used here for quantitative FRET determination gives good results.

For the smallest donor acceptor separations, the larger observed FRET efficiency was E~0.6 and the agreement between experimentally determined FRET values and

theoretical predictions was poorer. Comparatively low FRET efficiencies compared to the expected donor acceptor separation were previously reported for Cy3.[23] We observed a larger average γ factor value for the sample with the smallest donor acceptor separation, which could indicate a change in the fluorescence properties of the dyes. Fluorescence quenching effects can influence FRET efficiencies and have been reported to occur for small donor acceptor separations.[27] Although individual single molecule time traces did not show clear evidence of acceptor fluorescence quenching, donor or acceptor fluorescence quenching could well play a role in explaining our observations. This effect can also affect the value of the γ factor so that it depends on donor acceptor separation. Thus procedures for determining γ factors directly for individual samples will give more accurate results than those where a global correction factor is used for a given donor/acceptor fluorophores pair.

Conclusions

In conclusion, we evaluated the robustness of a procedure to determine quantitative FRET efficiencies directly from fluorescence time traces of surface immobilized molecules. We found that the FRET values are not influenced by contributions from direct acceptor excitation following donor excitation in these conditions. Indeed, the correction arising from direct acceptor excitation is intrinsically taken into account and contributes to the value of the γ factor in this procedure. As the correction from direct acceptor excitation is proportionally more important at low FRET values, the γ factor value is expected to vary depending on acceptor donor fluorophore separation if an inaccurate value for the direct acceptor correction is used. A global γ factor for a given donor acceptor fluorophore pair will thus introduce an error for quantitative FRET determination when neglecting the direct acceptor correction. Our results provide insights into the parameters governing quantitative instrument independent FRET determination from TIRF based single molecule experiments. The used procedures are implemented for further use in the iSMS single molecule FRET data analysis software (www.isms.au.dk) that allows determining both average correction factors and their distributions.

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