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ORIGINAL PAPER

Two-Photon Excited Fluorescence Energy Transfer: A Study Based on Oligonucleotide Rulers

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Abstract The use of two-photon excitation of fluorescence for detection of fluorescence resonance energy transfer (FRET) was studied for a selected fluorescent donor–acceptor pair. A method based on labeled DNA was developed for controlling the distance between the donor and the acceptor molecules. The method consists of hybridization of fluorescent oligonucleotides to a complementary single-stranded target DNA. As the efficiency of FRET is strongly distance dependent, energy transfer does not occur unless the fluorescent oligonucleotides and the target DNA are hybridized. A high degree of DNA hybridization and an excellent FRET efficiency were verified with one-photon excited fluorescence studies. Excitation spectra of fluorophores are usually wider in case of two-photon excitation than in the case of one-photon excitation [1]. This makes the selective excitation of donor difficult and might cause errors in detection of FRET with two-photon excited fluorescence. Different techniques to analyze the FRET efficiency from two-photon excited fluorescence data are discussed. The quenching of the donor fluorescence intensity turned to be the most consistent way to detect the FRET efficiency. The two-photon excited FRET is shown to give a good response to the distance between the donor and the acceptor molecules.

Keywords Two-photon excitation · FRET · Spectroscopy · Fluorescent labels · DNA · Oligonucleotide · Hybridization

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Introduction

Fluorescence resonance energy transfer (FRET) is one of the few techniques that enables the detection of molecular interaction and molecular movements within the cell with true molecular resolution. FRET is a nonradiative process between two molecules. Energy is transferred by dipole–dipole interaction from excited molecule (donor) to another molecule (acceptor), creating an excited state of the acceptor. The dipole–dipole interaction is a short-range effect and FRET can be used as "spectroscopic ruler" [2] in the range of 10–100 Å [3, 4]. FRET is strongly distance dependent and commonly used for studying molecular interactions. The efficiency of resonance energy transfer is proportional to the inverse sixth power (r^{-6}) of the distance between donor and acceptor molecules. The efficiency of a FRET pair is defined by a Förster radius, R_0, that is a distance, where 50% of the donor excitation energy is transferred to the acceptor. The Förster distance is defined as

$$R_0 = 9.78 \times 10^3 \left[\kappa^2 \eta^{-4} \Phi_D J(\lambda)\right]^{1/6} \quad (1)$$

where Φ_D is the fluorescent quantum yield of the donor, η is the refractive index of the medium, and κ^2 is the orientation factor [5]. $J(\lambda)$ is an overlap integral of the donor absorption spectrum and the acceptor emission spectrum, and is given by

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\, \varepsilon_A(\lambda)\, \lambda^4\, d\lambda}{\int_0^\infty F_D(\lambda)\, d\lambda} \quad (2)$$

where $F_D(\lambda)$ is the fluorescence spectrum of the donor, $\varepsilon_A(\lambda)$ is the absorption spectrum of the acceptor, and λ is the

wavelength [5]. To achieve an efficient FRET the emission spectrum of the donor and the absorption spectrum of the acceptor must overlap sufficiently.

The detection of fluorescence energy transfer has been demonstrated with different methods. The methods are based on detection of donor quenching [6], acceptor enhancement or changes in anisotropy [7, 8]. The simplest method of detecting the donor quenching is the observation of changes in the fluorescence intensity [9–15]. Another approach is the observation of the changes in the fluorescence lifetime of the donor [9, 10, 16–18]. Thus, the FRET efficiency can be determined either from the fluorescence intensities

$$E = 1 - \frac{F_{\mathrm{DA}}}{F_{\mathrm{D}}} \tag{3}$$

where F_{DA} is the fluorescence intensity of the donor in the presence of acceptor, and F_{D} is the fluorescence intensity of the donor in the absence of acceptor. Or from the fluorescence lifetimes as follows:

$$E = 1 - \frac{\tau_{\mathrm{DA}}}{\tau_{\mathrm{D}}} \tag{4}$$

where τ_{D} is the fluorescence lifetime of the donor in the absence of acceptor, and τ_{DA} is the fluorescence lifetime of the donor in the presence of the acceptor.

Fluorescence resonance energy transfer has numerous applications in research. FRET has been used for investigation of DNA's functions and properties [12, 13, 18–23], and to observe RNA from living cells [12, 13, 21]. Protein interactions and protein folding has been studied with FRET [7, 24–26], and fluorescent sensors [6, 27–29] and biomedical assays [27, 30] that exploit fluorescence energy transfer have also been developed.

Two-photon excited (TPE) fluorescence is created, when two near-infrared photons are absorbed simultaneously by a fluorescent molecule. Two-photon excited fluorescence spectroscopy and imaging have some known advantages compared to one-photon excited (OPE) fluorescence: reduced total photobleaching and low background level [31]. Two-photon excitation has been utilized in microscopic imaging [32], biomedical assays [33] and spectroscopy [34].

Studies of FRET have been published utilizing labeled oligonucleotides. These studies base on two different approaches: the most common method is based on two complementary oligonucleotides, which have been labeled with different fluorescent molecules [8–11, 18, 20, 22, 35–37]. The second method consists of two labeled noncomplementary oligonucleotides and a target DNA [12, 13, 19, 21, 22, 38]. We chose the latter approach and developed a method on the basis of labeled DNA to study the two-photon excited FRET. The developed method is based on two labeled oligonucleotides (acceptor and donor) and specific modulator oligonucleotide and target DNA as shown in Fig. 1. FRET can be detected after the modulator and two labeled oligonucleotides are hybridized to the target DNA. The developed method enables also to measure FRET efficiency in different distances by changing the length of the modulator oligonucleotide. Different techniques to analyze the FRET efficiency from two-photon excited fluorescence data are demonstrated.

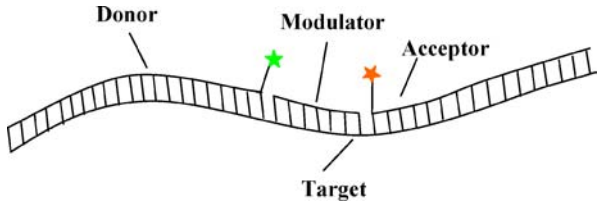

Fig. 1 DNA method used in FRET measurements. The method consist of donor, acceptor, and modulator oligonucleotides that are noncomplementary to each other. FRET is detected after the hybridization of the oligonucleotides to a target oligonucleotide

Experimental

Oligonucleotides

Oligonucleotides were purchased from MWG-Biotech AG, Ebersberg, Germany. The acceptor oligonucleotide was purchased ready-conjugated with Cy5 (Amersham Bioscience Ltd., Buckinghamshire, UK) at the 5′-end via spacer. The donor oligonucleotide had aminomodification via C6-spacer at the 3′-end. The donor oligonucleotide was conjugated with BF530, a label that has absorption maxima at 530 nm and fluorescence emission maxima at 556 nm as an oligonucleotide conjugate. The synthesis of BF530 has been described previously [39], and the labeling of the donor oligonucleotide was performed as reported earlier [40]. Briefly, the donor oligonucleotide in water (3.5 mM, 10 μl, and 35 nmol) and BF530 with succinimidyl ester in dry dimethylformamide (23 mM, 30 μl, and 700 nmol) were combined in sodium borate buffer (100 mM, 110 μl, and pH 8.5) and incubated over night. The unreacted BF530-label was eliminated by precipitation (ethanol and sodium chloride). All oligonucleotides were dissolved in RIOS-water and the concentrations of the oligonucleotides were determined spectrofotometrically (SD-2000 Ocean Optics single beam fiber optic diode array spectrometer). The molar ratios of dye to oligonucleotide were determined to be 0.9–1.2 in both cases; the acceptor oligonucleotide and the donor oligonucleotide.

Oligonucleotide sequences: Donor 20 base pairs (bp) 5′-GGTCTTTTGGCATTACCGAT-BF530-3′; Acceptor 17 bp 5′-CY5-TGA GGGTGAACTTGCGC-3′; Target 37 bp 5′-GCGCAAGTTCACCCTCAATCGGTAATGCCAAAAGA CC-3′; Modulator 4 bp 5′-ACCA-3′; Target 41 bp 5′-GCGC

AAGTTCACCCTCATGGTATC GGTAATGCCAAAAGA CC-3′; Modulator 8 bp 5′-AAGCCGTA-3'; Target 45 bp 5′-GCGCAAGTTCACCCTC ATACGGCTTATCGGTAAT GCCAAAAG ACC-3′; Modulator 14 bp 5′-ATGAACAT AGTCAA-3′; Target 51 bp 5′-GCGCAAGTTCACCCT CATTGACTATGTTCATATCGG TAATGCCAAAAGAC C-3′; Modulator 18 bp 5′-AAGTGAACATAGT CAG TA-3′; Target 55 bp 5′-GCGCAAGTTCACCCTCATACTG ACTATGTTCACTTAT CGGTAATGCCAAAAGACC-3′; Modulator 28 bp 5′-AAGTGAACATATCATTGAGCATGAC GTA-3′; Target 65 bp 5′-GCGCAAGTTCACCCTCAT ACGTCAT GCTCAATGATATGTTCACTTATCGGTAAT-GCCAAAA GACC-3′

Hybridization

Hybridizations were performed as follows: 7.5 pmol BF530 labeled donor oligonucleotide, 7.5 pmol CY5 labeled acceptor oligonucleotide, 7.5 pmol target oligonucleotide and 15 pmol modulator oligonucleotide were mixed together in 148 μl hybridization buffer (10 mM Tris–HCl, 150 mM NaCl, 15 mM $MgCl_2$, 0.01% Tween and 5 vol.% dextran sulfate) and the mixture was incubated with continuous shaking (600 rpm, Thermomixer, Eppendorf, Hamburg, Germany) at $+40^{\circ}$C for 1.5 h. After the incubation the mixture was allowed to cool down to room temperature before the fluorescence measurements were performed.

Fluorescence measurements

The spectrum and lifetime measurements in single-photon excitation mode were measured with a self-built spectrometer using an excitation wavelength of 532 nm. A pulsing Nd:YAG laser (Time Bandwidth Products Picolo, Zürich, Switzerland) was used as a light source. The wavelength of the laser was 1,064 nm with a nominal pulse width of 250 ps, a nominal repetition rate of 1 MHz and an average power of 115 mW. The 532 nm wavelength was generated by nonlinear crystal (LBO, 3 mm) and the primary wavelength was filtered out using bandpass filter (BG39, 3 mm, Schott, Maintz, Germany). Sample volume of 100 μl in semi-micro Plastibrand UV-cuvette (Brand GMBH, Wertheim, Germany) and conventional fluorometry set-up with a 90° angle between excitation and detection were used. Spectra were measured with monocromator (DM 150, Bentham Instruments Limited, Berkshire, UK), and a photomultiplier tube (R5600, Hamamatsu Photonics K.K., Sunayama-cho, Japan) was used as a detector in single-photon counting mode. Fluorescence signal was recorded with a time-to-amplitude converter (TimeHarp 200, PicoQuant Gmbh, Berlin, Germany).

The two-photon fluorescence spectra were measured with a set-up built in an optical module of ArcDia TPX-microfluorometer [33] (ArcDia Ltd., Turku, Finland). A mode-locked femtosecond diode pumped Nd:glass laser (Time-Bandwidth Products GLX-200, Zürich, Switzerland) was used as a light source. The wavelength of the laser was 1,057 nm with a nominal pulse width of 140 fs (sech2), a repetition rate of 110 MHz and an average power of 150 mW. The laser beam at 1,057 nm was reflected by a dichroic mirror through a beam scanner and focused with a microscope objective lens (Leica C-Plan 40 × 0.65, Leica Microsystems, Bensheim, Germany) through the cuvette bottom to the sample. A plastic bottom 384-well plate (Greiner Bio-one GmbH, Frickenhausen, Germany) was used with sample volumes of 15 μl. The two-photon process was confirmed by measuring the power dependence of the fluorescence intensity, and the power of the fitting was determined to be 1.98. The fluorescence light from the sample was collected with the same microscope objective lens and directed within a range from 530 to 700 nm through the dichroic mirror and an optical fiber to a monocromator (Oriel 77250, Grating 77911, Stratford, USA). A photomultiplier tube (R5600, Hamamatsu Photonics K.K., Sunayama-cho, Japan) was used as a detector in single-photon counting mode. Fluorescence signal was recorded with a time-to-amplitude converter (TimeHarp 200, PicoQuant Gmbh, Berlin, Germany).

The fluorescence spectra were recorded in wavelength range of 540–700 nm and the fluorescence lifetimes were recorded at centre wavelength 556 nm for donor, and at 665 nm for acceptor. The same wavelengths were used both in one-photon and in two-photon excited fluorescence measurements. The background signal, so. emission spectrum from the buffer, were subtracted from all reported data.

Results and discussion

Förster distance

The use of BF530–Cy5 pair in FRET experiments has several benefits. The fluorescence emission of BF530 and Cy5 are well separated and the excitation of Cy5 is negligible when 532 nm excitation is used for the excitation of BF530 (Fig. 2). The fluorescence decay lifetimes are also favorable. The use of an acceptor with short lifetime [29] and a donor with longer lifetime has been shown to give good signal-to-noise ratios, when FRET is detected from changes in the lifetimes [13]. The characteristic Förster distance, R_0, for the BF530–Cy5 pair was calculated from the fluorescence spectra of BF530–oligonucleotide conjugate and from absorption spectra of Cy5–oligonucleotide conjugate. In the Förster distance calculation the orientation factor, κ^2, was assumed for a free rotor, and therefore $\kappa^2 = 2/3$ [9, 41]. The quantum yield of the donor, Φ_D, is 0.25 as an oligonucleotide conjugate [40]. The refractive index was chosen to be that of

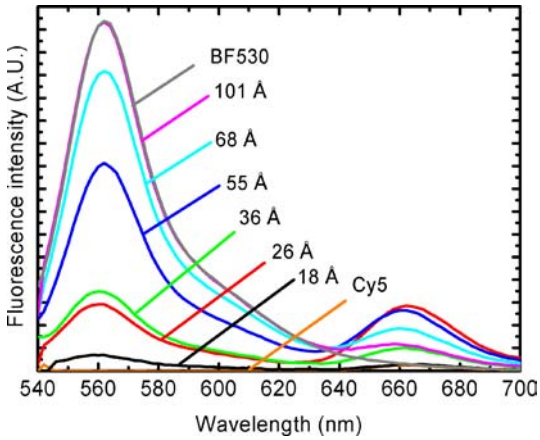

Fig. 2 One-photon excited emission spectra showing energy transfer between donor and acceptor molecules. The donor is BF530 labeled oligonucleotide, and the acceptor is Cy5 labeled oligonucleotide. Samples: the emission spectra of the hybridizated donor oligonucleotide, the emission spectra of the hybridizated acceptor oligonucleotide, and the emission spectra from the hybridization of the FRET samples with different distances between the donor and the acceptor in Ångströms. The conversion was done by DNA model [9, 37], and the modulator lengths were corresponding to the base pair separation: 28 bp – 101 Å; 18 bp – 68 Å; 14 bp – 55 Å; 8 bp – 36 Å; 4 bp – 26 Å; 0 bp – 18 Å

water ($\eta = 1.333$), and the overlap integral [15, 42] was calculated to be 5.6×10^{-13} cm^3 M^{-1} for BF530–Cy5 FRET pair. The Förster distance, R_0, was determined to be 51 Å. This theoretical value of Förster distance was used to compare the fluorescence resonance energy transfer efficiency in the case of OPE and TPE.

Oligonucleotide method

To investigate FRET efficiency, a specific oligonucleotide method was developed. The oligonucleotide method is based on two fluorescent oligonucleotides and on a modulator oligonucleotide, which hybridize to complementary target oligonucleotide. The fluorescent donor and acceptor oligonucleotides, and the modulator oligonucleotides were non-complementary with respect to each other. In this scheme FRET can be detected after the hybridization of the acceptor, donor, modulator, and target oligonucleotide. The modulator oligonucleotide strand was used for two reasons: (i) to reduce the uncertainties in the distance estimations, by stiffening the double strand and (ii) to assure that the double strand will pose a helical form despite of the long base pair separation of the FRET pair. Previous studies have shown that fully double stranded structure gives more reliable results than a structure that consist of incomplete double strand [13].

A schematic figure of the method is shown in Fig. 1. Two labeled oligonucleotides were used as a donor and as an acceptor. The donor oligonucleotide was labeled with a fluorescent BF530 in the 3′-end, and had a sequence that was complementary to the 5′-end of the target. The acceptor oligonucleotide had a fluorescent Cy5 in the 5′-end and had a sequence that was complementary to the 3′-end of the target, respectively. This method circumvents the problems of fluorescence quenching [9] and through-helix transfer [14] that are caused by the stacking of labels to the end of the double helix [8, 38]. The FRET samples were made by mixing molar equivalents of donor oligonucleotide, acceptor oligonucleotide, target oligonucleotide and an excess of modulator oligonucleotide. The hybridization efficiency was improved by adding dextran sulfate. The use of dextran sulfate has proven to accelerate the hybridization kinetics, and to improve the hybridization efficiency with picomolar concentrations [43, 44]. Different dextran sulfate concentrations, in the range of 0–10 vol.%, were tested in the hybridizations. A relative hybridization efficiency was determined from the enhanced FRET efficiency in the hybridized samples that contained different amounts of dextran sulfate. The highest degree of hybridization was achieved by using 5 vol.% dextran sulfate in the hybridization buffer. The FRET efficiency without dextran sulfate was 26% for the donor–acceptor distance of 55 Å, and after the addition of the dextran sulfate the FRET efficiency for the donor–acceptor distance of 55 Å was improved to 40%. Thus, the achieved hybridization efficiency was excellent without purification. The developed method is suitable and useful to study several FRET pair candidates in different distances without a need to manufacture and purify several different acceptor and donor oligonucleotides [8, 12, 13]. This method also minimizes the errors rising from the difference in acceptor and donor concentrations, oligonucleotide sequences and hybridizations.

Fluorescence measurements

The fluorescence measurements were carried out with the hybridized samples. The measured samples, including the only donor and the only acceptor samples were always hybridized with their complementary oligonucleotides to exclude the fluorescence intensity changes after hybridization [14]. One-photon excited emission spectra were measured to evaluate the developed oligonucleotide method. Figure 2 shows clearly observable FRET in the one-photon excited fluorescence spectra, when the acceptor and the donor oligonucleotides are hybridized to the target. When the distance between FRET pairs is changed the OPE emission spectra of the FRET pairs are showing the expected decrease in donor fluorescence and the increase of the acceptor fluorescence. In addition, there is a clear correlation between the decreased and the increased signals in each individual spectrum. However, in the shortest distances the donor and the acceptor signals are not following the Förster theory, which indicates other than FRET behavior. One reason for this variation can be the linker between the DNA strand

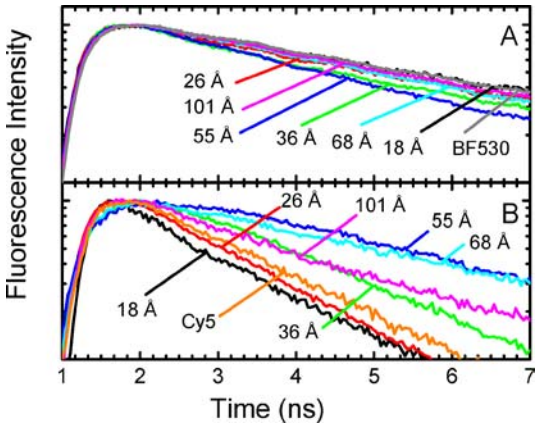

Fig. 3 Time-resolved fluorescence intensity using one-photon excitation. (A) Fluorescence emission intensity of the donor at 556 nm and (B) fluorescence emission intensity of the acceptor at 665 nm with different distances between the donor and the acceptor in Ångströms [9, 37]

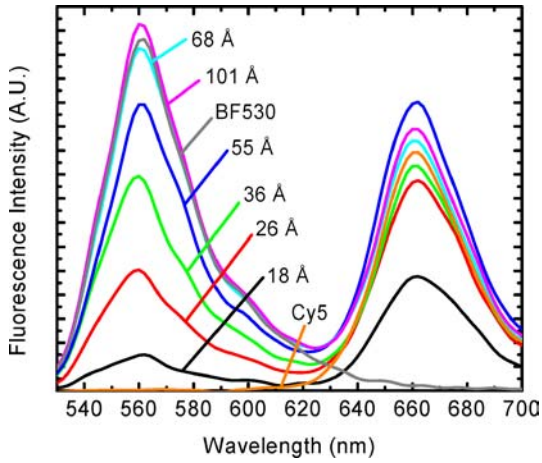

Fig. 4 Two-photon excited emission spectra showing energy transfer between donor and acceptor molecules. The donor is BF530 labeled oligonucleotide and the acceptor is Cy5 labeled oligonucleotide. Samples: the emission spectra of the hybridized donor oligonucleotide, the emission spectra of the hybridized acceptor oligonucleotide, and the emission spectra from the hybridization of the FRET samples with different distances between the donor and the acceptor in Ångströms [9, 37]

and the fluorescent molecule. The linkers may enable some movement for the fluorescent molecules, and therefore the distance between the donor and the acceptor can differ from the calculated base pair separations. Also, at short distances between the donor and the acceptor the FRET theory no longer holds [3]. With the very short distances (<25 Å), despite the donor intensity has been decreased, the energy has not been transferred to the acceptor in the same proportion; this phenomenon has also has been reported earlier [9]. In addition to emission spectra, the time-resolved fluorescence intensity was measured from the samples. Figure 3 shows the influence of the energy transfer to OPE fluorescence lifetimes of the donor and the acceptor. The FRET can be detected from the fluorescence lifetimes: the donor fluorescence lifetime was shortened from 5.0 to 3.6 ns and the acceptor fluorescence lifetime became longer from 1.8 to 4.1 ns in the samples, where the donor and the acceptor are closer to each other.

The same samples were measured using two-photon excitation. TPE fluorescence spectra of the hybridized samples are shown in Fig. 4, and FRET can be observed from the spectra. There is a clear decrease in the donor intensity, when the distance between the donor and the acceptor is shortened. In contrast to OPE, there is no significant difference in the acceptor emission intensities. Instead, the acceptor emission intensities were almost the same regardless of the distance between the donor and the acceptor. A likely reason for the unchanged acceptor intensity is the strong direct excitation of the acceptor. Figure 5 shows the time-resolved fluorescence intensity using two-photon excitation. The changes in the fluorescence emission lifetimes appear much smaller than when using one-photon excitation. The donor lifetime was varied from 6.1 to 4.2 ns and the acceptor lifetime was changed from 1.5 to 1.6 ns. It is likely that the phenomenon is in part due to the direct excitation of the acceptor and thus, the possible effect of energy transfer to the emission lifetime is not resolvable because of frustrated energy transfer. Another reason lies in the detection system. With the currently used laser of high repetition rate (110 MHz), the observation is limited to a window where the changes have least contrast. However, the reason for the reduced change in the TPE donor emission lifetime is not fully explained by these factors: the FRET is still clearly observable from the TPE donor emission intensities and also OPE measurements proved that the samples are showing a good FRET signal. On the other hand, there is a clear correlation between the small changes in the TPE donor emission lifetime and the

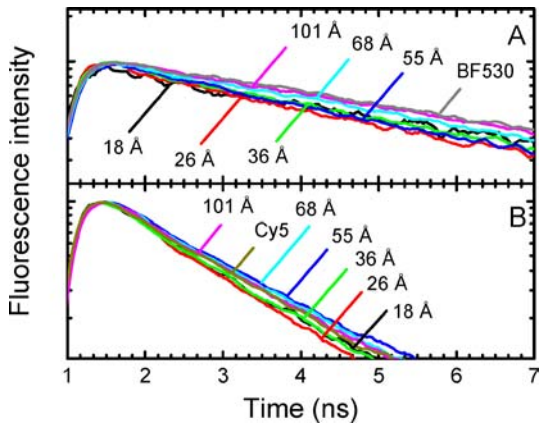

Fig. 5 Time-resolved fluorescence intensity using two-photon excitation. (A) Fluorescence emission intensity of the donor at 556 nm and (B) fluorescence emission intensity of the acceptor at 665 nm with different distances between the donor and the acceptor

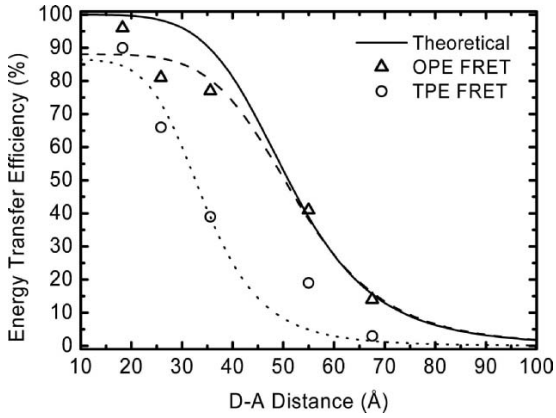

Fig. 6 FRET efficiency determined from the quenching of donor at different distances between donor and acceptor. Triangles and circles represent the obtained energy transfer efficiencies of the one-photon and two-photon excited FRET, respectively. The solid line is the theoretical prediction of the FRET efficiency and the dashed lines are Förster theory fits to the experimental data

distance between the donor and the acceptor, the magnitude of the lifetime change is just reduced compared to the change in one-photon excited emission lifetime.

The FRET efficiency was determined from the fluorescence emission spectrum. The FRET efficiency was calculated from the donor intensity decrease in the OPE and TPE fluorescence emission spectra. The measured FRET efficiencies were compared to the theoretical FRET values calculated using the Förster theory. The base pair separations of the donor and acceptor were converted to distance using the commonly used model for DNA-FRET efficiencies [9, 37]. Figure 6 shows the calculated and measured FRET efficiencies as a function of the distance between the donor and the acceptor. The measured OPE FRET efficiencies are in good agreement with the theoretical prediction. The Förster distance, R_0, for the measured OPE FRET was determined by fitting the Förster theory to the experimental data and a value of 51 Å was obtained for the R_0. It is the very same value that was obtained by Eq. 3, indicating that our DNA method is working and the samples are excellent for FRET study. However, at the short distances the experimental OPE FRET efficiencies are a little lower than the theory predicts. This deviation might be due to an incomplete hybridization or quenching mechanisms that take place at short distances. One reason for the difference in the FRET efficiency may also be found in the process of determining the Förster distance. The donor and acceptor molecules were assumed as free rotors, and at short distances this may not be the situation. The measured TPE FRET efficiencies are not in as good agreement with the theoretical prediction as in the OPE FRET efficiencies. The TPE FRET efficiency values remain smaller and a Förster distance of 34 Å was obtained by the fitting. However, the TPE FRET efficiency data gives a strong and consistent response to the distance between the donor and the acceptor.

The OPE and TPE FRET efficiencies were also determined from the fluorescence lifetimes. The overall FRET efficiencies determined from the emission lifetime data were evidently lower than expected by the total fluorescence intensities. Hence, the fluorescence lifetime measurements indicate that the donor is efficiently quenched also by other mechanisms as earlier supposed by Dietrich *et al*. [9]. According to our fluorescence intensity and lifetime data, these mechanisms may cause static quenching, whose strength is dependent on the distance between the donor and the acceptor or on the length of the used DNA. And in addition, the static quenching appears to be more effective when two-photon excitation is used. However, to be able to give reliable information about these mechanisms, further experiments need to be done.

Conclusion

In this paper, we have compared one-photon excited (OPE) FRET to two-photon excited (TPE) FRET, and described the methods to analyze the phenomenon. The energy transfer was studied with the developed DNA method for controlling the distance between the donor and the acceptor. An excellent correlation between the measured OPE FRET efficiency and the theoretical prediction was found by observing the quenching of donor intensity. The quenching of the donor intensity also turned to be the most reliable way to detect the TPE FRET efficiency and a good response to the distance between the donor and the acceptor was found with TPE FRET. The determined Förster distances are in the range of typical distances that has been utilized in biological applications of FRET. Therefore, the TPE FRET can be expected to be applicable and useful for such biological applications.

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