

# Excited state processes in individual multichromophoric systems

Johan Hofkens<sup>a\*</sup>, Tom Vosch<sup>a</sup>, Mircea Cotlet<sup>a</sup>, Satoshi Habuchi<sup>a</sup>, Koen Van Der Biest<sup>a</sup>, Klaus Müllen<sup>b</sup>, Gunter Dirix<sup>c</sup>, Jan Michiels<sup>c</sup>, Jos Vanderleyden<sup>c</sup>, Markus Sauer<sup>d</sup>, Frans C. De Schryver<sup>a\*</sup>

<sup>a</sup>Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200 F, B-3001 Heverlee Belgium

<sup>b</sup>Max-Planck-Institut für Polymerforschung, Ackermannweg 10, D-55128 Mainz, Germany

<sup>c</sup>Center of Microbial and Plant Genetics, Kasteelpark Arenberg 20, B-3001 Heverlee Belgium

<sup>d</sup>Physikalisch-Chemisches Institut, Universität Heidelberg, Im Neuenheimer Feld 253, 69120 Heidelberg, Germany

## ABSTRACT

Multichromophoric systems play a key role in biological systems (light harvesting antenna complexes, fluorescent proteins...) and are equally important in material science applications (e.g. light emitting devices (LED) based on conjugated polymers). Our approach to get insight in the excited state processes of such systems is to make use of dendrimers labeled with photostable perylene dyes. Dendrimers synthesis indeed allows changing the number, relative position and orientation of attached chromophores in a controlled way. In the present contribution, excited state processes such as energy hopping, singlet-singlet annihilation, singlet-triplet annihilation are identified in individual tetrachromophoric dendrimers immobilized in a polymer matrix. Similar processes are then demonstrated to occur as well in immobilized tetramers of a red fluorescent protein from a coral of the *Discosoma* genus (DsRed).

**Keywords:** Single molecules, excited state processes, dendrimers, DsRed fluorescent protein

## 1. INTRODUCTION

Multichromophoric systems are the object of study in different branches of science like biology (LH2 light harvesting antenna system), material science (light emitting devices) and fundamental photophysics<sup>1-5</sup>. Both conducting polymers and LH2 are inhomogeneous systems at the molecular level. In case of the polymer this is due to poly-dispersity. Conformational fluctuations lead to inhomogeneity in physical parameters for the protein. Furthermore, such multichromophoric systems usually display complex photophysical behavior. Because of the control over size, shape and number of attached chromophores, dendrimers are ideal model systems to study the fundamental aspects of the photophysical processes that occur in multichromophoric compounds. Processes such as energy hopping (seen as a decay component in time resolved fluorescence anisotropy measurements) and singlet-singlet annihilation (seen as a 10 ps intensity dependent decay component in upconversion measurements) have been shown in ensemble measurements on multichromophoric dendrimers<sup>6,7</sup>. A method that has advanced the understanding of photophysics in heterogeneous samples is single molecule spectroscopy (SMS). It has been argued and demonstrated that SMS provides direct information about the molecular distribution of physical quantities like the emission maximum or decay times rather than just the averaged values delivered by ensemble measurements<sup>8</sup>. SMS also allows following fluctuations in the physical properties of one single entity<sup>8</sup>. We report here on the different excited state processes that could be identified in tetrachromophoric dendrimers at the single molecule level. The tetrameric dendrimer was chosen because it forms an ideal model compound for the recently identified red fluorescent protein DsRed (*Discosoma* genus)<sup>9</sup>. It was reported that even at single molecule concentrations this protein tends to aggregate to form tetramers<sup>10,11</sup>. A detailed photophysical insight in the protein is of great interest for biology as it is one of the most popular red-absorbing and red-emitting fluorescent proteins known at present.

## 2. MATERIALS AND METHODS

For the single molecule measurements the tetrameric dendrimer, compound 1, was dispersed in a thin polymer film and investigated by confocal fluorescence microscopy at room temperature. Samples were prepared by spincoating at 1000 RPM on a glass cover slip a toluene solution of 3 mg/ml Zeonex (polynorborene) containing a  $10^{-10}$  M concentration of compound 1. AFM measurements showed a film thickness between 200 to 300 nm. The confocal fluorescence

microscope that works in the epilluminescence mode is described elsewhere in more detail<sup>12</sup>. As excitation light we used the 488-nm line of a continuous wave Argon-Ion-Laser (Stabilite, Spectra-Physics) or the frequency doubled output of a Ti-Saf laser (Tsunami, Spectra Physics, 8.18 MHz, 1.2 ps FWHM). A 488nm-notch plus filter (Kaiser Optics) was used in the detection path to suppress remaining excitation light. The applied power was 400-600 W/cm<sup>2</sup> at the sample for CW light and 2.1KW/cm<sup>2</sup> for pulsed light experiments. To record single-molecule fluorescence transients, single dendrimers/proteins were selected from an image scan and positioned in the laser focus of the confocal microscope. Measurements were always performed at ambient temperature and atmosphere. For each examined molecule a spectrum was recorded to make sure that no impurities were investigated. Spectra were recorded with a liquid nitrogen cooled CCD-camera (Princeton Instruments) that was coupled to a polychromator (Acton Spectra Pro 150). The recorded spectra were first background corrected by subtraction of a spectrum from a blank sample and then corrected for the response of the CCD-camera and the intrinsic properties of the optical elements.

In order to obtain the red fluorescent protein DsRed (compound 2), standard methods were used for in vitro DNA manipulations<sup>13</sup>. The gene coding for DsRed was amplified by PCR using the primers Rhi-40 (5'-accgctcgagaccatgaggtctccaagaatgttatcaagg-3') and Rhi-41 (5'-agagctgcagccgctaaaggaacagatgg-3') and pDsRed (Clontech) as a template. The amplified fragment carries XhoI and PstI restriction sites at the 5' and 3' ends, respectively. PCR reactions were performed with PWO DNA-polymerase as previously described<sup>14</sup>. The 800-bp PCR fragment was digested with XhoI and PstI and ligated in the corresponding sites of pBAD/HisA. The DsRed sequence was confirmed by DNA sequence analysis. Expression of DsRed was induced for 12 hours in Escherichia coli Top10 induced with 0.2 % arabinose and grown at an optical density at 600 nm of 0.5. The DsRed proteins carrying the N-terminal polyhistidine tag were purified under native conditions by Ni-affinity chromatography using the Xpress Protein Purification System and concentrated in a Vivaspin 6 concentrator. Polyacrylamide gel electrophoresis was used to confirm the samples' purity. The protein was stored in a phosphate buffered saline solution (PBS, pH 7.5, Sigma). For single molecule experiments, the solution of DsRed was mixed with a PBS solution containing 1 wt.-% polyvinylalcohol (PVA, Agfa, M<sub>w</sub> 25000) resulting in a final concentration of 10<sup>-10</sup> M for the protein and then spin coated on clean cover glasses. The 543-nm laser line from a He-Ne laser was used as excitation light. The excitation power at the sample in all experiments was about 350 W/cm<sup>2</sup>.

### 3. RESULTS AND DISCUSSION FOR THE DENDRIMER

The structure of the dendritic compound 1 is shown in Figure 1. The dendrimer consists of a sp<sup>3</sup> carbon atom as core and polyphenylene arms with strongly twisted benzene units bearing four chromophores at the rim, attached in the para position (compound 1, see Fig. 1). This type of polyphenylene dendrimers are well soluble and are thermally and photophysically stable as well as highly shape persistent<sup>15-17</sup>. The synthesis is based on a Diels-Alder reaction and allows the controlled attachment of a well-defined number (in this case four) of chromophores to the outer rim of the dendritic structure (Fig. 1). The chromophore perylene imide was chosen because of the high photostability, absorption wavelength (around 500nm), absorption coefficient ( $\epsilon = 40000 \text{ cm}^{-1} \text{ mol}^{-1}$  in toluene) and high fluorescence quantum yield ( $\phi_f > 0.9$  in toluene). Moreover the polyphenylene building block does not absorb at the excitation wavelengths of interest (above 450nm) used to excite the perylene imide chromophore.



Figure 1: 2 and 3 dimensional representation of the tetrameric dendrimer 1.

#### 3.1 Energy hopping

Energy hopping is the term used to describe the energy transfer process between identical chromophores in a multichromophoric system<sup>6</sup>. This energy transfer results from the spectral overlap between absorption and emission spectrum of a chromophore. It has been shown previously that when multichromophoric compounds are immobilized in

a polymer matrix, the different chromophores feel slightly different environments<sup>5</sup>. As a result, at any given moment in time, one of the chromophores will be the lowest in energy. If the inter-chromophore distance is such that energy hopping is an efficient process, the low-energetic chromophore can act as a fluorescent trap as all excitation energy will be transferred to that chromophore. If such a process occurs in the investigated system it should show up in single molecule polarization measurements (vide infra). In these measurements the fluorescence is divided over two detectors using a polarizing beam splitter. From the resulting trajectories a degree of polarization ( $p$ ) can be calculated using equation 1:

$$p = \frac{I_{par} - G \times I_{per}}{I_{par} + G \times I_{per}} \quad \text{equation 1}$$

where  $G$  is a factor that corrects for the different detection efficiencies of both detection channels.

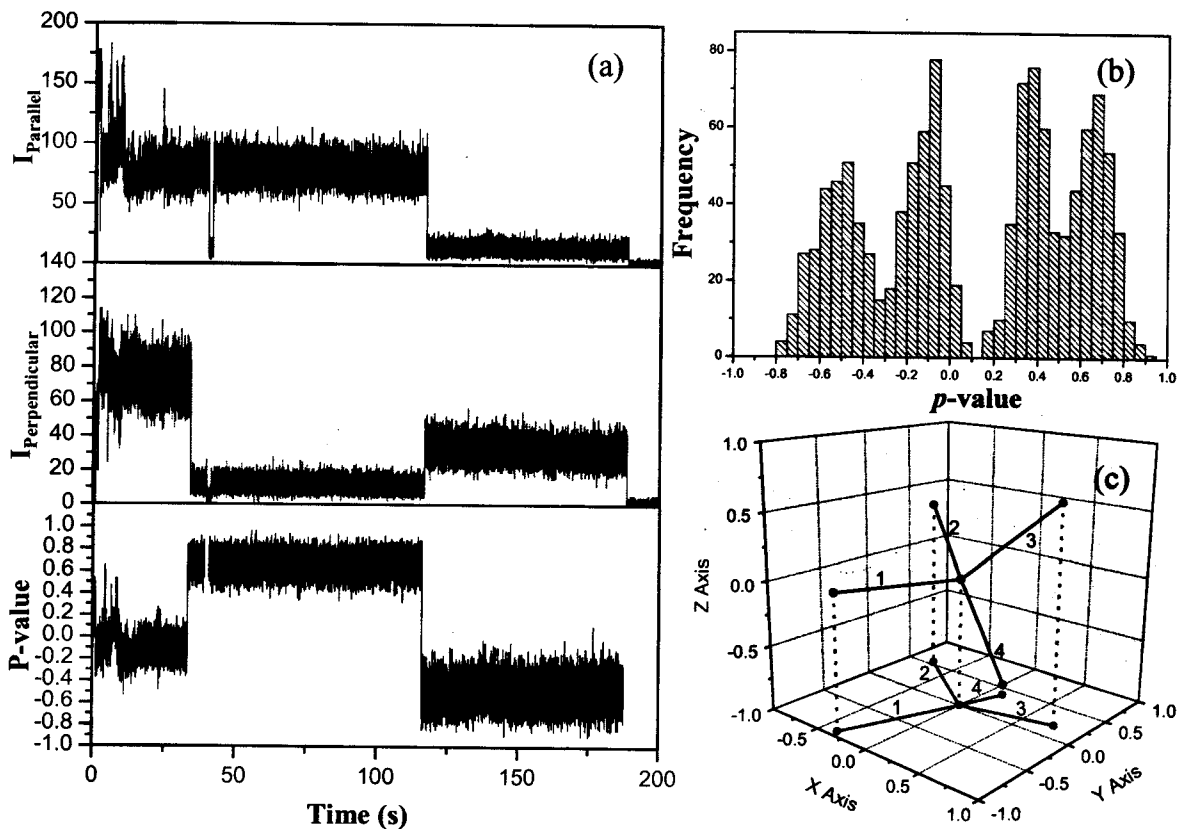


Figure 2: a) Example of a fluorescence transient where the parallel and perpendicular polarized components were detected separately. The polarization value is given in the lower graph. b) Histogram of the  $p$ -values (300 data points out of each level). c) Representation of the chromophores of a random oriented molecule and its projection in the XY-plane.

The  $p$ -value of an immobilized single molecule can have extreme values of 1 and  $-1$ <sup>5,18</sup>. Fluorescence intensity trajectories of 81 individual molecules of compound 1 were recorded using circular excitation light to avoid photo-selection. In Figure 2a-b one sees an example of a molecule of 1 showing the parallel and perpendicular component of the emission and four distinctive values for the polarization. The change of polarization as a function of time, coupled with variations in the fluorescence intensity suggests that there is stepwise photobleaching and energy hopping among the chromophores to a fluorescent trap. As argued, at any given time only one chromophore acts as a fluorescent trap. Upon bleaching of the dye molecule that acts as the fluorescent trap, the chromophore second lowest in energy becomes the fluorescent trap. Furthermore the bleached chromophore does not act as a trap for the fluorescence. An example of a histogram of the  $p$ -values is given in Figure 2b. The figure was made by histogramming 300 data points from each  $p$ -

value level in the lower trace of Figure 2a. Histogramming all data points of the  $p$ -value trace of Figure 2a does not clearly show the four levels since the first level is short and would not be clearly seen in the overall histogram. The histogram shows clearly four peaks situated around -0.51, -0.12, 0.38, 0.68. The  $p$ -values obtained from the measurements can be detected as the projections of the emission dipole moments in the experimental XY-plane (Fig. 2c). Figure 2c shows four unit vectors arranged in a tetrahedral fashion. From this random in space oriented molecule, a projection in the XY-plane was made. The calculated  $p$ -values for this example are -0.21, 0.70, 0.51, and -0.92 for respectively vector 1, 2, 3 and 4. From the example given above it is clear that the jumps in the polarized fluorescence trajectories proof the presence of an energy hopping process.

### 3.2 Singlet-singlet annihilation

Having a system with four closely spaced chromophores, it is expected that each of the chromophores can absorb light, but due to excited state energy hopping, it is not necessarily true that each of the four emits light due to singlet singlet annihilation. We confirmed that multiple chromophores on a single dendrimer absorb light using rotating linearly polarized excitation light and analyzing the total emitted fluorescence in time<sup>19</sup>. A single PI chromophore has a well-defined absorption dipole, and the emitted fluorescence drops to the background when the linear polarization of the excitation beam is oriented perpendicular to the absorption dipole. For multiple chromophores with nonparallel absorption dipoles, the fluorescence will not drop to the background regardless of the polarization angle of excitation. The number of independent emitters present in the detection volume can be determined by measuring the distribution of inter-photon times in a manner similar to classical anti-bunching experiments. For a single emitter, the probability of emitting two consecutive photons drops to zero for time intervals shorter than the excited state lifetime. In simple terms, the molecule cannot emit two photons simultaneously. After photon emission, a molecule must be re-excited and wait, on average, one fluorescence lifetime before another photon can be emitted. For sufficiently short laser pulses the number of photon-pairs detected per laser pulse can be used to determine whether the emission is from one or more independently emitting quantum systems. A recently developed method for the analysis of interphoton times using the classical Hanbury-Twiss and Brown setup<sup>20,21</sup> was applied to determine the number of emitting chromophores on single dendrimers. The fluorescence intensity and the interphoton-distances were measured for the same dendrimer molecule (Fig. 3a). Several intensity levels in the transient indicate successive photobleaching of individual chromophores. Figure 3b shows the distribution of interphoton times collected during the first intensity level. In Figure 3a, the central peak of the distribution at a delay time of 0 ns corresponds to photon pairs induced by the same laser pulse. In all other cases, interphoton-times are distributed around a multiple of the repetition rate, i.e. one peak every ~122 ns. For clarity, only the central three peaks are shown in Fig. 3b. Previously, it was shown that the ratio of number of photon pairs contributing to the central peak,  $N_c$ , to the average number of counts in the lateral peaks,  $\overline{N}_l$ , can be used to estimate the number of independently emitting chromophores. Neglecting background,  $N_c/\overline{N}_l$  ratios of 0.0, 0.5, 0.67, and 0.75 are expected for 1-4 molecules, respectively. From the distribution shown in Figure 3b, we determined an  $N_c/\overline{N}_l$  ratio of  $185/2981 = 0.062 \pm 0.006$ .

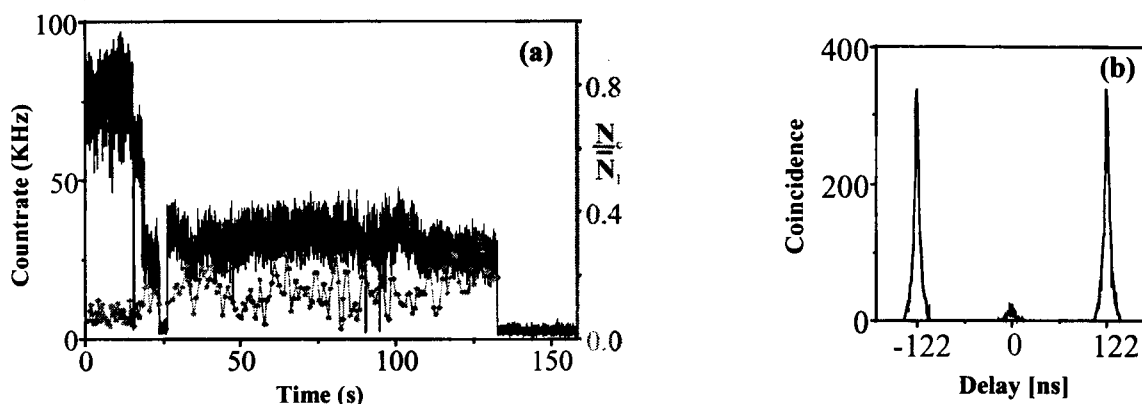


Figure 3: (a) Fluorescence intensity transient (black) and  $N_c/\overline{N}_l$  ratio transient (gray) of compound 1 using circular polarized excitation ( $2.1 \text{ KW/cm}^2$ ). (b) Interphoton-distance (coincidence) histogram corresponding to the first intensity level (15 seconds) of the transient depicted in (a).

The non-zero background in SM measurements always produces a small contribution of detection events that fall in the central  $N_c$  peak. These can originate from background-background, signal-background and background-signal detection pairs. Considering our experimental conditions ( $S/B \sim 80$ ) the determined  $N_c/\overline{N}_i$  ratio of 0.062 corresponds to the expected ratio for a single emitter<sup>20</sup>. Figure 3a shows a sliding window analysis of the  $N_c/\overline{N}_i$  ratio including 50000 photons for each data point while progressing in steps of 25000 photons (gray dots in Figure 3a). The low value of the ratio indicates that the system behaves as a single emitter throughout the transient. The increase of the  $N_c/\overline{N}_i$  ratio as the fluorescence intensity drops during the course of the transient is expected as the  $S/B$ -ratio decreases with the successive photobleaching of the chromophores. From these experiments, it is clear that multiple chromophores of the dendrimer 1 can absorb light, but that only one of the chromophores emits light at any given time. To explain why the system behaves as a single quantum system, an efficient non-radiative pathway has to be present. A recent ensemble fluorescence upconversion study revealed a power dependent 10 ps time constant, which was only present in multi-chromophoric dendrimers but not in the single chromophore reference compound<sup>22</sup>. Accordingly, singlet-singlet annihilation was concluded to be the source of the power dependent decay component. As this process is more than two orders of magnitude faster than the fluorescence decay ( $\sim 4.3$  ns), annihilation of multiple excitons is practically complete before fluorescence photons are emitted. Exciton annihilation is conceived as a Förster-type incoherent energy transfer from the excited donor to the excited acceptor molecule with the result of a doubly excited acceptor state. The latter can quickly relax to the singlet excited state. Observing a single multichromophoric system, efficient singlet-singlet annihilation is reflected by the fact that only one photon is emitted per laser pulse.

### 3.3 Singlet-triplet annihilation

All of the fluorescence transients of dendrimer 1 show several intensity levels, usually four. Reversible jumps between high levels and lower levels or the off level are seen. Even the transients recorded using circular polarized light, exciting all four chromophores in the dendrimer, show reversible jumps between the high level and the off level as can be seen in Figure 4a. The reversible jumps (back to the initial intensity level) also observed in the beginning of the transient, corresponding to a situation in which the four chromophore are still intact, are referred to as collective on/off jumps. Several types of off-periods, related to different types of off-processes can be discriminated. Long off periods of seconds to tens of seconds can be seen, usually in the lower intensity levels of the transient. A second off-process has a typical length of 1 to 100 ms. The transient shown in Figure 4a is a transient recorded of dendrimer 1 immobilized in Zeonex and coated with aluminum. Zooming in the first high intensity level reveals an additional off process with an average length of around 0.3 ms (Fig. 4b). Clearly, reversible jumps from the high intensity level to background and back can be seen. We refer to these on/off jumps as collective on/off jumps. These collective on/off jumps molecules were reported before in literature by several groups,<sup>1-5</sup> for different multichromophoric systems. The four chromophores being simultaneously in the off-state is an implausible explanation for the observed collective on-off behavior in the transients of compound 1. Another explanation could be that the four chromophores are strongly coupled and hence act as one quantum system. If this system went to an off-state, such as the triplet state, this would account for the collective phenomena observed for compound 1. No support can be found for this model from the solution data as the absorption spectra of compound 1 and the PI chromophore hardly differ. However, coulombic interaction in the excited state is possible. Calculations show that all chromophores are well within the Förster radius for energy transfer<sup>7</sup>. As stated above, this implies that fluorescence will occur from the chromophoric site in the dendrimer that at a given point in the trajectory is lowest in energy and hence acts as a trapping site from which fluorescence will occur. From this it can be deduced that off states have to be explained by radiationless deactivation channels that are opened via the energetically lowest chromophoric site. We suggest that the triplet state of the energetically lowest lying chromophoric site is a possible candidate. Excitation energy transfer from the first singlet excited state to the first triplet state resulting in the singlet ground state and a higher lying triplet state is a spin allowed process as the spin multiplicity on each of the chromophores is maintained. It can occur in multichromophoric dendrimer systems like compound 1, if the rate constant of energy transfer from the singlet excited state to the triplet excited state is sufficiently high. The good overlap between the triplet absorption spectrum of compound 1, measured in solution via the transient absorption technique<sup>17</sup>, and the emission spectra dendrimer 1 in solution further supports this hypothesis. The relaxation of the higher triplet state to the first triplet state is a very fast, spin allowed non-radiative process. The competition between singlet/triplet energy transfer and fluorescence from  $S_1$  might then account for the occurrence of both off levels and low levels within the binning time. Note that the fast on/off process can not be resolved in uncoated Zeonex samples and that an average off-time of 0.18ms is measured when dendrimer 1 is immobilized in PMMA<sup>23</sup> (polymethylmetacrylate).

As triplet lifetimes of several seconds or hundreds of milliseconds are unlikely, other deactivation channels have to be considered for the other off-times. The formation of a radical/cation or radical/anion pair was suggested for a different multichromophoric system<sup>3-5</sup> and might play a role in this system as well.

To calculate the intersystem crossing rate, one needs first to calculate the quantum yield of intersystem crossing. When the quantum yield of fluorescence is close to one, like is the case for perylene imide, one can calculate the quantum yield of intersystem crossing by dividing the number of off periods by the number of emitted photons (this is the number of detected photons divided by the detection efficiency of the experimental setup, which is approximately 10 percent)<sup>5</sup>. The value for the quantum yield obtained from this molecule is then divided by the fluorescence life time, resulting in the rate of intersystem crossing. The value used for the fluorescence lifetime, 4.3 ns, was obtained from ensemble measurements. A  $k_{ISC}$  of  $4 \times 10^4 \text{ s}^{-1}$  was calculated in this way<sup>24</sup>. Summarizing, singlet-triplet energy transfer or annihilation can be seen in multichromophoric systems as collective on/off blinking in the fluorescence intensity traces.

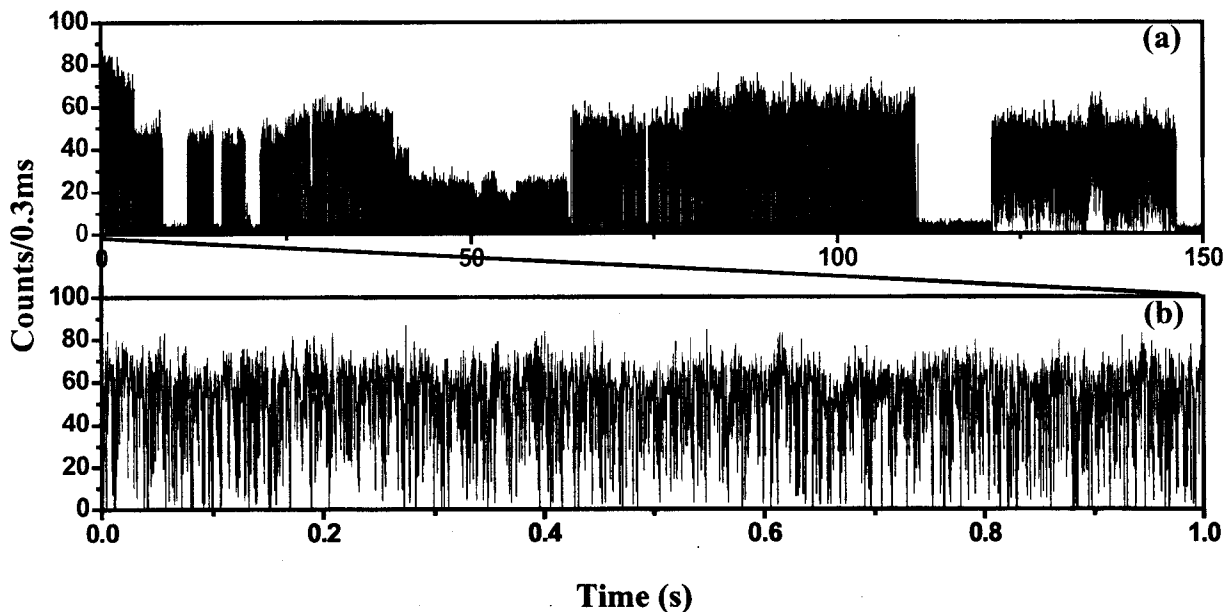


Figure 4: (a) Fluorescence transient of an individual molecule of compound 1 immobilized in Zeonex and coated with aluminum. Excitation occurred at 488 nm and circularly polarized light was used. (b) Zoom in the first high intensity level of the transient depicted in (a) showing a fast on/off process.

## 4. RESULTS AND DISCUSSIONS FOR THE DSRED PROTEIN.

### 4.1 Energy hopping.

The crystal structure of DsRed showed that DsRed exists as well-defined tetramers<sup>25</sup>. Such a tetramer, based on crystallographic data, is shown in Figure 5a. The position of the chromophores inside the barrel structures is also shown. In Figure 5b the orientation of the transition dipole moments of the four chromophores in a randomly oriented tetramer are depicted, together with their projection in the xy plane. As the tetramers consist of two nearly parallel pairs of monomeric units, this symmetry is also found back in the projection of the transition dipole moments. Similar as for dendrimer 1, DsRed displays jumps in the  $I_{par}$  and  $I_{perp}$  components of the detected fluorescence and, as a consequence, jumps in the values of  $p$  are observed. Figure 5c, left panel, presents, as an example, time traces for the  $I_{par}$  and  $I_{perp}$  components as well as for  $p$ . Discrete jumps from positive to negative values and back to the positive can be observed in the time evolution of  $p$ , in particular at the beginning of the time trace. Frequency histogramming of the values of  $p$  for the first intense region from 3.5 to 5 s results in a bimodal distribution, consistent with the picture described above, with two distinct Gaussian peaks located at  $-0.25$  and  $0.16$ . Similar behavior was found for 50 % of the investigated DsRed molecules. It was shown in DsRed that the chromophore is rigidly encapsulated inside the barrel structure<sup>11</sup>. Thus, the discrete jumps observed for DsRed cannot be attributed to only one emitting chromophore. A change in polarization from  $-0.25$  to  $0.16$  corresponds to a change in the orientation of the transition dipole moment of about 15

degrees. We correlate the multimodal distribution of the values of  $P$  for DsRed with emission from different chromophores, one at a time. In combination with the different intensity levels observed for the fluorescence transients (vide infra), the presence of the discrete jumps are a clear indication for the tetramerization of DsRed, even at single molecule level. Just as in the dendrimer 1 model system, by exciting a DsRed tetramer, energy hopping among the chromophores occurs, one of them acting as an energy trap and finally emitting the fluorescence. After bleaching or going into a dark state, its place will be taken by another chromophore that can act as the fluorescing trap, i.e. being at that moment the lowest in energy. However, 50 % of the investigated DsRed molecules do not show discrete jumps in the values of  $P$  during the time of experiment, but only different intensity levels. This can be due to the more complex photophysics of DsRed as compared with dendrimer 1. We recently showed that, additionally, photoconversion to a more red-absorbing and red-emitting species takes place in individual DsRed tetramers<sup>10</sup>. One photoconverted chromophore can then eventually act as a fluorescent trap with a fixed orientation during the recording of the polarized fluorescence intensity trajectory. Alternatively, also the presence of immature chromophores in some of the tetramers can be the reason for the absence of the jumps in  $p$ <sup>10</sup>.

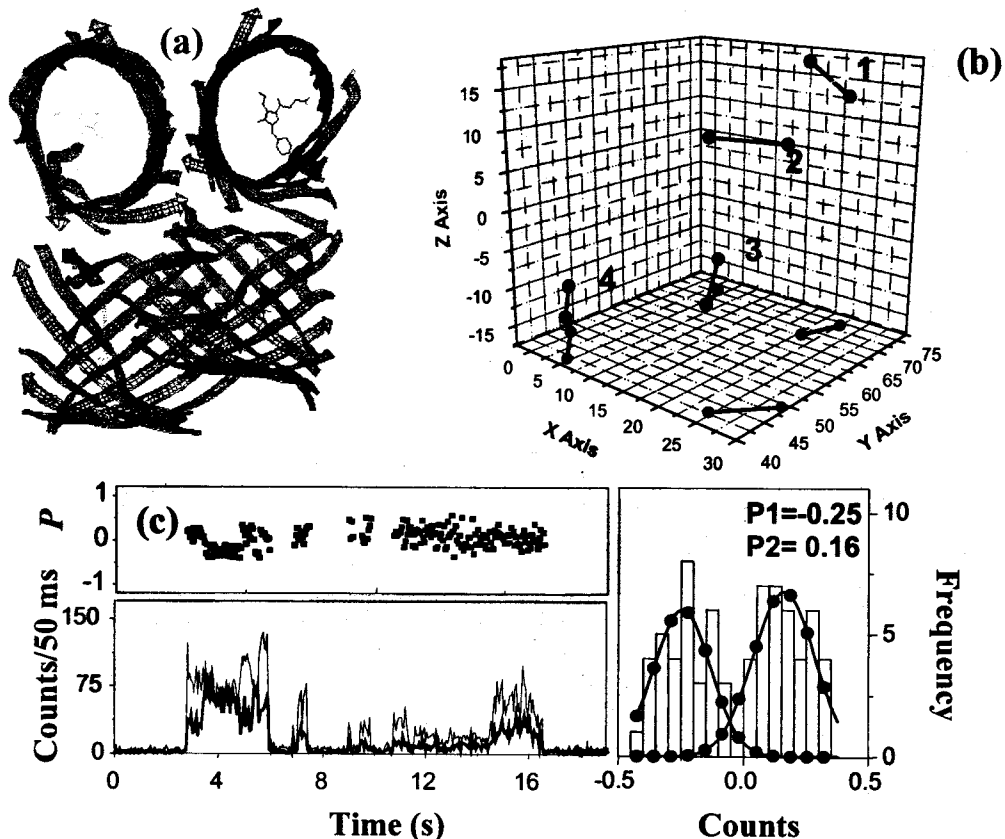


Figure 5: (a) Model of a DsRed tetramer based on crystallographic data. (b) Orientation of the transition dipole moments of the four chromophores (indicated by numbers 1 to 4) in a randomly oriented tetramer, together with their projection in the xy-plane. (c) the parallel-polarized (black line) and perpendicular-polarized (gray line) components of the fluorescence transient together with the corresponding  $P$  trace, background subtracted, for an individual DsRed tetramer in PVA. Right panel: the frequency histograms of the values of  $P$  together with the Gaussian fits for the corresponding traces depicted in the left panel. A two Gaussian fit yielding -0.25 and 0.16 for the mean  $P$  values was necessary.

#### 4.2 Singlet-triplet annihilation.

The fluorescence transients recorded from individual DsRed tetramers (64 for circular and 45 for linear polarization) show more than one intensity level. An example is depicted in Figure 6a, left panel. Although the fluorescence intensity traces were recorded with 0.5 ms bin time, they were rebinned to 70 ms in order to clearly show the different levels. As an additional proof for the presence of more than one intensity level, the corresponding frequency histogram of the

detected counts, after background subtraction is presented in Figure 6a, right panel. Histograms for both circular as well as for linear polarized excitation could be fitted with four Gaussians, yielding peaks at 80, 325, 465 and 720 counts per 70 ms. Of the total number of the individual DsRed molecules excited with circular polarized light, 40 % display four intensity levels, the rest three or two levels. The maximum number of counts detected in 5 ms ranges from 55 to 160. For a total observation time of 100 s, individual DsRed molecules emit, for an excitation power of  $350 \text{ W/cm}^2$ , for at least 10 seconds, the most intense level being in the beginning of the fluorescence transient. Dark periods in the time range of seconds were also observed. As previously obtained for compound 1, the existence of stepwise changes in the fluorescence intensity and jumps between different intensity levels is typically for multichromophoric systems. The presence of several intensity levels, stepwise changes and jumps in all the investigated fluorescence transients indicates that, even at subnanomolar concentration used to prepare the single molecule samples, DsRed indeed exists as tetramer. Due to incomplete maturation of the chromophores in DsRed, not all transients show four intensity levels<sup>10</sup>. A zoom (Fig. 6b) in the fluorescence transients of DsRed depicted in Figure 6a reveals similar collective on-off behavior as for dendrimer 1 (in all the intensity levels). A fit of the histogram of the off-times of the selected regions yielded an off-time of 1.6ms for this particular tetramer. The presence of fast collective on-off blinking in all intensity levels, even in the highest which is related to the emission of the intact tetramer, is again a demonstration of the singlet-triplet annihilation process taking place in this multichromophoric system as well<sup>26</sup>. The triplet lifetime of 1.6 ms is much longer than the triplet lifetime recovered in solution for similar fluorescent proteins<sup>27</sup>. PVA, having a permeability for molecular oxygen that is a factor of about 1000 lower than for polymers like polystyrene, will have a considerable influence on the triplet lifetimes of the investigated molecules<sup>28</sup>.

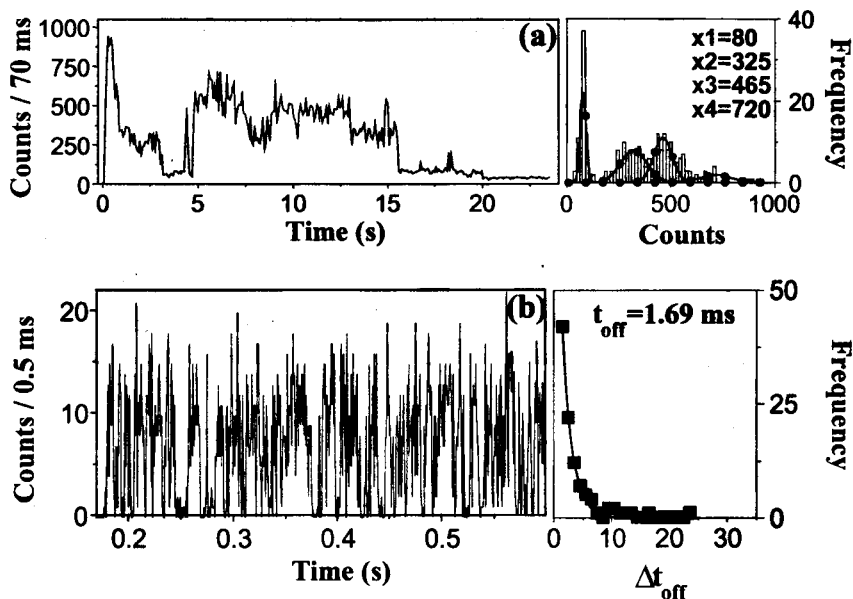


Figure 6: (a) Left panel: fluorescence transients of DsRed in PVA excited at 543 nm with circular polarized light. Right panel; the corresponding frequency histograms, background subtracted, of the fluorescence transients depicted in the left panel. The Gaussian fits of these frequency histograms (solid line + dots) are depicted in the right panel, too. (b) Zooms in the fluorescence transient depicted in (a), left panel, and the corresponding frequency histograms (full square) of the duration of the off times. The corresponding single exponential fits of this histogram (solid line) together with the obtained  $\Delta\tau_{\text{off}}$  are also depicted.

## 5. CONCLUSIONS

In this paper photophysical processes such as energy hopping, singlet singlet annihilation and singlet-triplet annihilation were identified in a tetrachromophoric dendrimer system. Energy hopping can be deduced from single molecule polarization measurements. Singlet-singlet annihilation at the single molecule level is seen from the antibunching in the emission of multichromophoric compounds. Singlet-triplet annihilation results in collective on/off jumps in individual multichromophoric systems. These fundamental processes will show up in all multichromophoric entities and we

established how they can be recognized. As an example, we show some of the processes in individual DsRed tetramers, where otherwise they would be obscured by photoconversion of the chromophores and photo-induced proton transfer. Thus, single molecule fluorescence detection and spectroscopy lead to a better understanding of how excitation energy is dissipated in the multichromophoric entities studied here.

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