

Multiparameter Single Molecule Spectroscopy gives insight into the complex Photophysics of Fluorescence Energy Transfer (FRET) coupled Biosystems

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ABSTRACT

Since the discovery of the technique in the early 1990s, single molecule spectroscopy has been used as a powerful tool to investigate and characterize fluorescent molecules, revealing insights into molecular behavior far beyond the information content that can be obtained by conventional ensemble studies. Several spectroscopic techniques have been established at the single molecule level, including spectrally resolved fluorescence, fluorescence lifetime investigations, or single molecule Raman measurements. However, the combination of two or more of these spectroscopies applied to the same individual molecule in multiparameter approaches yields a deeper understanding of molecular systems. In this contribution, we present our results of combined spectrally- and time-resolved fluorescence microscopy of the intrinsic fluorescence energy transfer (FRET) system of the red fluorescent protein DsRed. Correlating the results obtained from the two spectroscopic techniques, we are able to determine all relevant parameters to describe the energy transfer processes within the DsRed system without any further assumptions. We further discuss fluorescence and surface enhanced Raman scattering (SERS) spectroscopy of the same individual DsRed unit, which can help to propose mechanisms for photodegeneration of the distinct chromophores involved.

Keywords: autofluorescent protein, DsRed, SERS, FRET, single molecule

1. INTRODUCTION

Since their establishment in the 1990s, single molecule techniques have gained more and more scientific interest and developed as a standard tool in many fields of applications^[1]. One major reason for this wide-spread success is due to the fact that single molecule studies overcome averaging inherent in ensemble measurements. This way, more detailed insights into the target system or its interaction with the local chemical environment can be gained, which typically reveal pronounced deviations from the averaged behavior known from the ensemble^[2]. Besides the mere recording of the emission intensity, properties such as fluorescence spectra, surface enhanced Raman scattering, or fluorescence decay characteristics can be analyzed in single molecule studies^[3-8]. As in the case for the analysis of ensembles of molecules^[9], the information content of single molecule analysis can be further increased by applying a combination of two or more spectroscopic techniques on the same individual molecule^[10]. In this contribution, we present such multiparameter approaches for the Discosoma reef coral red fluorescent protein DsRed and its variants, revealing new insights into the molecular photophysics of this fascinating system. DsRed is a rewarding subject of study for several reasons. Firstly, as for all autofluorescent proteins known so far, the chromophores of this tetrameric protein are located inside a beta-barrel structure^[11] (Fig. 1a). Thus the chromophore is shielded within a well-defined nano-environment and the emission is expected to be independent of the matrix surrounding the protein. Accordingly, if spectral dynamics are observed they must be attributed to intrinsic photophysics of the system rather than to interactions with the matrix^[12]. Secondly, DsRed forms obligate tetramers composed of four of these monomeric barrel-subunits which are arranged as a perpendicularly oriented dimer pair. Each of the subunits contains either a green or a red emitting chromophore with a

statistical composition within the tetramer. These two chromophoric species do not act independently of each other but are dipole coupled via a fluorescence resonance energy transfer (FRET) mechanism, resulting in dominant red emission upon excitation in the blue spectral regime (Fig. 1b). Together with the well-known steric arrangement, DsRed exhibits a rigid FRET system which is shielded from environmental influences^[13].

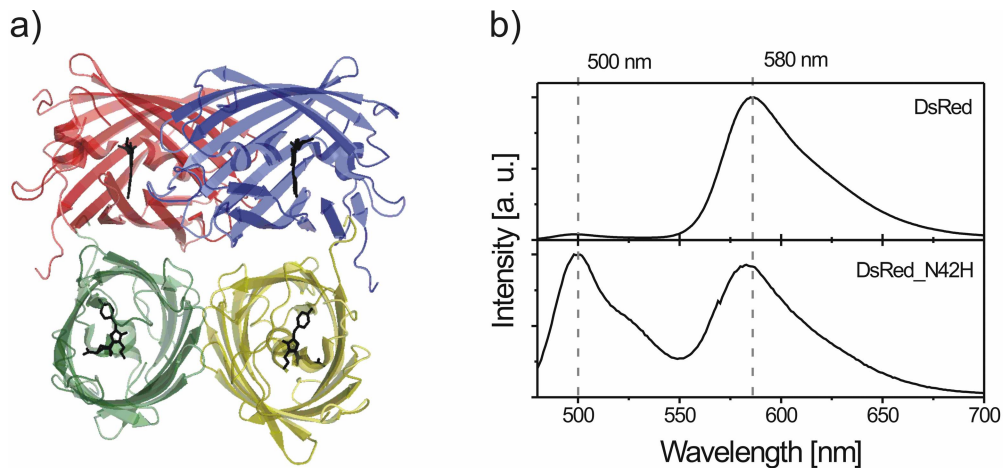


Figure 1: a) Tetrameric structure of DsRed as obtained from X-ray studies^[14]. The chromophores depicted in black are shielded from the environment by a protein shell built from amino acid β -sheets. b) Fluorescence spectra of DsRed (top panel) and DsRed_N42H (bottom panel). The excitation wavelength was $\lambda_{exc} = 457$ nm for both spectra.

Due to the tetrameric nature with coupled chromophores of DsRed, a precise determination of fundamental spectroscopic parameters such as fluorescence lifetimes or fluorescence quantum yields for the different chromophores is difficult if not impossible to obtain using conventional methods. While these parameters can be accessed quite easily for the red emitting chromophore by long-wavelength excitation ($\lambda_{exc} > 550$ nm)^[8], severe problems arise in case of the green emitting donor as the dipole-coupling of the two chromophores frustrates an isolated investigation of the green chromophore. Furthermore, a disassembly of the tetramers into functional monomers containing only one emitting species by chemical or biochemical means is impossible. To overcome these restrictions, the spectroscopic parameters obtained from the green fluorescent proteins (GFP and E(nhanced)GFP) have been used to quantitatively describe the energy transfer system of DsRed (see^[15,16] and references therein). The use of GFP-type proteins as an equivalent of the green emitting chromophore of DsRed seemed to be valid as it turned out that the chromophore of GFP was both a structural and a spectral analogue to the green emitting species in DsRed^[15].

Single molecule studies combining different spectroscopic methods can be an appropriate tool to verify this assumption. Recent single molecule studies revealed that DsRed tetramers are randomly composed of green or red emitting monomeric subunits with an equal occurrence probability^[17]. Hence, there exists a finite probability to observe a solely green emitting tetramer with no red emitting acceptor chromophore present. In this contribution we show that, if one can identify these subpopulations spectroscopically on the single molecule level, successive time resolved investigations can provide access to the missing fundamental data such as the fluorescence lifetime of the donor in the absence of an acceptor or the fluorescence quantum efficiency of the green emitting chromophore.

After the potential of this temporally and spectrally resolved multiparameter approach is discussed in the first part of this article, we discuss how the combination of spectroscopic methods such as fluorescence and Raman scattering on the single molecule level can help to elucidate photochemical reactions. In short, first spectrally resolved fluorescence measurements are used to discriminate the two spectral forms of DsRed. Then, surface enhanced Raman spectroscopy is performed on the same individual molecules, allowing for the assignment of distinct Raman bands to distinct bonds in the chemical structure of the two respective chromophores. Now, one can distinguish the different emitting species solely by their Raman spectra, opening the possibility to monitor potential structural changes of the chromophores.

2. RESULTS AND DISCUSSION

2.1 Combined time and spectrally resolved single molecule microscopy

The shape of the fluorescence intensity decay recorded from DsRed is influenced by a variety of different emitting species present in DsRed and its variants. In addition to the two dipole coupled major emitting forms ($\lambda_{\max, \text{green}} = 505 \text{ nm}$, $\lambda_{\max, \text{red}} = 580 \text{ nm}$), further spectral forms have recently been found [18]. Hence, recording emission intensity decays in the ensemble results in complex decay trajectories, revealing for example at least two time constants for excitation in the blue and detection in the red spectral region [19]. Although these components can be assigned to chromophoric subensembles as identified by single molecule studies relatively easy [8,20,21], the decay curves become even more complex for detection of light emitted in the green spectral region. Here, the curves obtained for ensemble studies can be fitted to a three-exponential decay function, revealing three distinct decay constants. Besides two short-lived contributions ($\tau_1 = 0.12 \text{ ns}$ and $\tau_2 = 1.1 \text{ ns}$), an additional long lived component ($\tau_3 = 5.1 \text{ ns}$) with a contribution of about 3% is found (Fig. 2a). According to $\tau_{DA} = \tau_D - \tau_D \cdot E$ with E as the energy transfer efficiency, assuming an additional decay channel in a FRET system, one expects a shortened lifetime of the donor in presence of an acceptor (τ_{DA}) relative to the donor lifetime when no acceptor is present (τ_D). Hence, the short time constants can be assigned to the (partly) quenched donor emission. However, the assignment of the long lived component to a chromophoric species in DsRed is difficult without making any assumptions.

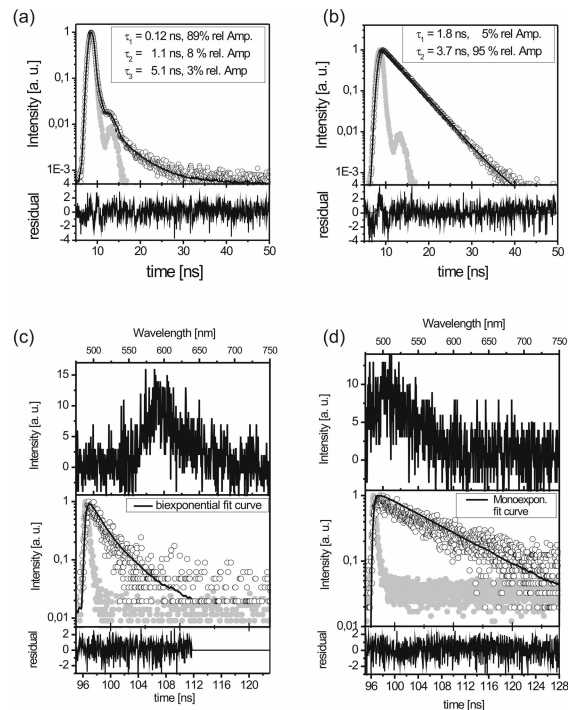


Figure 2: a) Fluorescence decay curve of an ensemble of DsRed upon excitation in the blue ($\lambda_{\text{exc}} = 467 \text{ nm}$) and detection in the green ($\lambda_{\text{det}} = 505 \text{ nm}$) spectral region. Besides two short lived components a long lived decay constant of $\tau = 5.1 \text{ ns}$ could be fitted to the experimental data. b) Fluorescence decay curve of an ensemble of DsRed upon excitation in the blue ($\lambda_{\text{exc}} = 467 \text{ nm}$) and detection in the red ($\lambda_{\text{det}} = 600 \text{ nm}$) spectral region. The decay constants are in good agreement with the data reported in the literature and the single molecule results discussed in the text. c) Top: Fluorescence spectrum of a single DsRed tetramer, showing dominant red emission. Center: Fluorescence intensity decay (circles), biexponential fit-curve (solid line) and instrument response function (grey dots). d) Top: Fluorescence spectrum of a single DsRed tetramer, showing solely green emission. Center: Fluorescence intensity decay (circles), biexponential fit-curve (solid line) and instrument response function (grey dots). Bottom of all plots: residuals of the fitted decay.

Here, single molecule studies can help to shed more light onto the distinct behavior of the individual chromophoric species in DsRed as it is possible to identify and choose tetramers containing only one chromophoric species for investigation. To accomplish multiparameter time- and spectrally resolved single molecule microscopy we prepared samples of ultra-low concentrations of DsRed ($c = 10^{-9}$ M) in 2% aqueous polyvinyl alcohol (PVA) solution which was then spincoated onto commercially available low-fluorescent microscope cover-slips at 8000 rpm. This procedure is known to form homogeneous thin polymer films with isolated dispersed DsRed proteins which can be independently addressed using confocal fluorescence microscopy^[3]. We then recorded 2D fluorescence raster scan images to locate the DsRed tetramers and first acquired fluorescence spectra at distinct bright spots. Afterwards, the detection-lightpath was switched to a spectrally integrating avalanche photodiode (APD) connected to a time correlated single photon counting (TCSPC) board to record fluorescence intensity decays.

For our studies, we investigated a total of 154 DsRed tetramers and found a majority of 100 entities with dominant red emission whereas 40 proteins exhibited mixed green and red emission. 14 tetramers emitted exclusively green fluorescence, representing a fraction of 9% of all investigated proteins in good agreement with numbers reported in the literature^[17,22]. For the dominant population of red emitting proteins we consistently observed intensity decay curves which could be fitted to a biexponential decay function. Here, we found time constants for the longer component around 3.7 ns, being in good agreement with values reported in the literature for bulk measurements where exclusively the red emitting chromophore was excited^[8,20] and our own ensemble results for the red emitting chromophore, which revealed a biexponential intensity decay upon excitation at $\lambda_{exc} = 561$ nm (Fig. 2 a and b) with a major contribution (95 %) of 3.7 ns. Therefore, we assign the longer decay component to originate from the red emitting chromophore in DsRed. However, for all our single molecule studies with excitation in the blue spectral regime, we found an additional short decay component with time constants ranging from 0.13 ns to 1.7 ns. These values correspond to numbers found in the ensemble for blue excitation and can be assigned to residual emission of the green emitting chromophore, which is partly quenched due to energy transfer to the red emitting acceptor.

A completely different scenario was observed for all tetramers showing solely green emission. Here, we measured pure monoexponential decay curves. As both spectral and structural data of DsRed suggest a highly efficient energy transfer from the green to the red emitting chromophore (spectral overlap, dipole orientation, interchromophoric distances), we expect red emission as well as a multiexponential intensity decay as soon as at least one red emitting chromophore is present in the tetramer. Hence, the observation of pure green emission combined with a strictly monoexponential intensity decay lends credence to the conclusion that we exclusively investigate tetramers solely composed of the green emitting chromophore of DsRed. Accordingly, the spectral parameters gained from these single molecule studies of the green emitting entities refer to the undisturbed green emitting chromophore when no energy transfer to an acceptor occurs. We are thus able to determine the fluorescence lifetime of the green emitting chromophore without interference by a red emitting species, even if several identical chromophores are present in the tetramer^[23]. Strikingly, for all investigated green emitting species we found exceptional long fluorescence lifetimes with decay constants in the range of 5 ns to 7.5 ns, respectively, with an average value of 6.2 ns (Fig. 2 d). This finding is astonishing as these numbers exceed the fluorescence lifetime of EGFP ($\tau_{EGFP} = 3.3$ ns^[24]) by almost a factor of two. The chromophore of (E)GFP is a structural and spectral homologue to the green emitting chromophore of DsRed^[15,16] and references therein). However, the protein shell forming the local chemical environment differs substantially, and likely strongly impacts the photophysics of the respective chromophores.

The knowledge of the unquenched fluorescence lifetime of the green emitting chromophore of DsRed is the missing link to a robust and quantitative description of the energy transfer processes within the tetramer. Considering a DsRed system composed of one green emitting donor interacting with three red emitting acceptors, we expect the most efficient donor quenching, represented by the shortest donor fluorescence lifetime. Hence, for the shortest observed decay constant of $\tau_{DA} = 0.13$ ns we calculate a Förster distance R_0 , defined as the interchromophoric distance where half of the excitation energy is transferred, for a system of one donor and three acceptors according to

$$R_0^6 = \left(\frac{\tau_D}{\tau_{DA}} - 1 \right) \cdot \left(\frac{r_1^6 \cdot r_2^6 \cdot r_3^6}{\kappa_1^2 \cdot r_2^6 r_3^6 + \kappa_2^2 \cdot r_1^6 r_3^6 + \kappa_3^2 \cdot r_1^6 r_2^6} \right) \quad (1)$$

and obtain an value of $R_0 = 4.65$ nm. The effective interchromophoric distances r_i as well as the orientation factors κ_i^2 for each pair of chromophores were obtained from X-ray data [22]. The knowledge of the R_0 value allows for the determination of the fluorescence quantum yield of the donor chromophore $\Phi_D = 0.18$ according to equation 2.

$$\Phi_D = \frac{R_0^6}{8.785 \times 10^{-5} n^{-4} J(\lambda)} \cdot \frac{\sum_{i=1}^3 (r_i^6)^{-1}}{\sum_{i=1}^3 \frac{\kappa_i^2}{r_i^6}} \quad (2).$$

Here $J(\lambda)$ denotes the spectral overlap integral between donor emission spectrum (derived from the ensemble data of the DsRed mutant AG4 [31]) and the acceptor absorbance spectrum (derived from the shifted fluorescence-excitation spectrum of the monomeric red fluorescent protein mRFP [19]) while n is the refractive index of the surrounding PVA matrix. With the knowledge of all relevant spectroscopic parameters, now the Förster radii $R_{0,i}$, the fluorescence lifetime of the quenched donor $\tau_{DA,i}$, and the energy transfer efficiencies of each steric arrangement of two chromophores can be calculated as summarized in table 1 according to:

$$E_i = \frac{R_{0,i}^6}{R_{0,i}^6 + r_i^6} = 1 - \frac{\tau_{DA,i}}{\tau_D}$$

$$R_{0,i}^6 = \frac{r_{0,i}^6 \tau_D}{\tau_{DA,i}} - r_i^6 \quad (3).$$

$$\tau_{DA,i} = \tau_D - \frac{R_{0,i}^6}{R_{0,i}^6 + r_i^6}$$

| i | r_i | κ_i^2 | $\tau_{DA,i}$ | $R_{0,i}$ | E_i |
|-----|---------|--------------|----------------|-----------|------------|
| AB | 3.36 nm | 0.56 | 1.62 ns | 3.99 nm | 74% |
| DB | 2.73 nm | 1.73 | 0.19 ns | 4.82 nm | 97% |
| CA | 2.74 nm | 1.54 | 0.23 ns | 4.73 nm | 96% |
| CD | 3.36 nm | 0.58 | 1.58 ns | 4.02 nm | 75% |
| AD | 4.29 nm | 0.01 | 6.13 ns | 2.04 nm | 1% |
| BC | 4.29 nm | 0.01 | 6.13 ns | 2.04 nm | 1% |

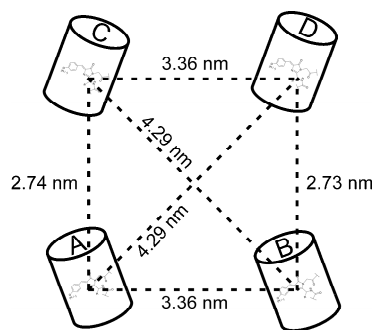


Table 1: Calculated Förster radii $R_{0,i}$, donor lifetimes $\tau_{DA,i}$ and energy transfer efficiencies E_i for the different energy transfer pairs i within a DsRed tetrameric unit. Letters A-D refer to the monomers sketched on the right. Figure on the right: interchromophoric distances as derived from X-ray structural data [22].

The outlined data are valid for FRET systems composed of exactly two chromophores. Considering a native (tetrameric) system composed of four coupled chromophores, one obtains τ_{DA} values in the range of $\tau_{DA} = 0.12 - 0.14$ ns for a complete energy migration system in good agreement with the decay constants reported in the literature for the quenched green emitting chromophore of DsRed [8,20].

2.2 Fluorescence and Raman spectroscopy on the same individual DsRed tetramer

In the previous section we demonstrated how the combination of spectrally- and time-resolved fluorescence studies can help to clarify intrinsic photophysical processes of complex biosystems such as the red fluorescent protein DsRed. In addition to fluorescence based methods, surface enhanced Raman scattering (SERS) techniques have gained importance in single molecule studies over the past decade and several reports have been published^[6,7,25-32]. The main advantage of this technique is based on the fact that Raman spectroscopy monitors vibrations of chemical bonds. Thus one obtains a molecular fingerprint of the investigated species which gives direct information about the chemical structure of the molecules. However, besides ongoing discussions about definitive demonstrations of single molecule observation by SERS, the technique suffers from two major restrictions. Firstly, the molecular fingerprint, which is provided by SERS spectroscopy, can only be assigned to distinct molecular features for small molecules but does succeed for complex molecules such as proteins only in exceptional cases. Moreover, in SERS the signal enhancement is achieved by a noble metal particle adjacent to a target molecule. Commonly, noble metal nano-particles dispersed over the sample surface are used as a Raman active medium, resulting in a low spatial density of Raman active hot spots. Hence, Raman imaging was impossible since the obtained Raman image always samples the arrangement of the metal particles rather than the assembly of the target molecules that might be located between isolated particles and hence are not accessible for Raman investigations.

One way to overcome the restrictions mentioned above is given by multiparameter single molecule spectroscopy combining fluorescence and Raman spectroscopy for example on the same individual fluorescent protein. In short, after fluorescence spectroscopy has been carried out until photobleaching occurred with the proteins directly dispersed on the glass surface, the sample is covered with a thin silver metal film evaporated under high vacuum conditions. Silver films with a thickness < 5 nm are known to form island films with alternating hills and holes of about 50 nm in size^[33] resulting in the formation of a high spatial density of Raman active hot spots. These structures are dispersed homogeneously over the entire sample surface, rendering Raman imaging possible. However, to record both fluorescence emission and Raman scattering of the same individual molecule, the sample has to be marked in a way that a relocalization of distinct molecules with high precision is possible. This can be achieved by evaporating a macroscopic silver structure on the sample surface prior to fluorescence measurements. Recording the elastically backscattered light then enables the relocalization of identical sample areas.

For autofluorescent proteins it has been shown before that the recorded surface enhanced Raman spectra mainly originate from the chromophoric units of the tetramer rather than from the outer protein barrel^[34]. Accordingly, the obtained Raman spectra reveal a structure considerably less complex than one would expect for a macro-molecule such as an autofluorescent protein and the assignment of distinct bands to structural properties of the chromophore is possible^[34,35].

As Raman spectra of autofluorescent proteins mainly refer to the vibronic signature of the chromophore, correlating the fluorescence spectra and the Raman spectra obtained from the photobleached DsRed proteins even allows for an assignment of distinct spectral bands in the Raman spectra to the two different chromophores of the protein. Accordingly, the two different chromophoric species of DsRed can be discriminated by the presence of distinct Raman bands which consequently facilitates the assignment of Raman spectra derived from unbleached DsRed tetramers recorded without prior fluorescence studies to originate from either the green or the red emitting chromophore. As Raman spectra reveal a fingerprint of the molecular structure of a target molecule, this way it is possible to investigate and compare Raman spectra of bleached and unbleached chromophores, allowing for the determination of photo-degeneration processes separately for the distinct chromophoric species of DsRed. A detailed description of the experimental work as well as of the obtained results can be found in ref.^[36].

3. CONCLUSION

In this article, we have presented two examples of multiparameter single molecule spectroscopy. We could show that such combined approaches have the potential to give more insight into molecular features than the sum of the isolated methods is capable of. In detail, we have demonstrated how combined spectrally and temporally resolved microscopy of individual DsRed tetramers reveal fundamental spectroscopic parameters which have been the missing link to a quantitative description of the energy transfer processes within the DsRed system. In the second part of the contribution,

we discussed how combined Raman and fluorescence spectroscopy on the same individual DsRed protein can be performed and how a careful analysis of the recorded spectra can help to determine different photoproducts of the protein.

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