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REVIEW

Single-molecule spectroscopy of fluorescent proteins

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Abstract The discovery and use of fluorescent proteins has revolutionized cellular biology. Despite the widespread use of visible fluorescent proteins as reporters and sensors in cellular environments the versatile photophysics of fluorescent proteins is still subject to intense research. Understanding the details of the photophysics of these reporters is essential for accurate interpretation of the biological and biochemical processes illuminated by fluorescent proteins. Some aspects of the complex photophysics of fluorescent proteins can only be observed and understood at the single-molecule level, which removes averaging inherent to ensemble studies. In this paper we review how single-molecule emission detection has helped understanding of the complex photophysics of fluorescent proteins.

Keywords Bioanalytical methods · Fluorescence · Luminescence · Laser spectroscopy · Spectroscopy · Instrumentation · UV–visible

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Introduction

Visualization of the sub-cellular localization of proteins and the dynamics of their transport, trafficking, and interactions with other cellular components is a critical element of understanding complex biological processes. The development of optical microscopy methods, and, in particular, of optical sectioning methods such as confocal microscopy, has been an essential contributor to this goal. Recent advances in optical super-resolution imaging make possible near-molecular resolution optical imaging of biological systems [1–13]. Appropriate fluorescence labels are essential elements of any visualization strategy. Small-molecule fluorophores spanning the entire visible and near-infrared spectrum have been developed, and may be coupled to proteins of interest using various chemical coupling protocols (see, for example, The Handbook—A Guide to Fluorescent Probes and Labeling Technologies, http://probes.invitrogen.com/handbook/). These approaches almost always require external labelling of the protein(s) of interest, followed by re-introduction of the labelled protein into the cell by microinjection or other approaches. Genetically encodable fluorescent markers have the enormous advantage of being capable of expression in a spatially and temporally regulated manner under a choice of different promoters. When fused with a desired protein of interest, such markers enable visualization of the target protein and quantitative microscopy of the biological processes of interest. The discovery[1] [14], subsequent cloning [15], and application of the *Aequoria* green

[1] For a fascinating personal account of the discovery of *Aequoria* GFP, see Shimomura, Journal of Microscopy, 217, 3–15 (2005)

fluorescent protein [16], and the development of new colours of fluorescent proteins by protein engineering of *Aequoria* GFP [17–24], has provided revolutionary new capabilities to visualize molecular and cellular biological processes. The palette of fluorescent proteins has been enormously extended by the discovery of new intrinsically fluorescent visible fluorescent proteins (VFPs) from other marine organisms [25–32], and their optimization [33–38]. The combination of these genetically encodable markers with advanced microscopic and spectroscopic techniques has enabled quantitative measurement of protein–protein interactions. Variants of VFPs exhibiting different colours and photophysical properties such as photoactivation [39–52], photobleaching [53–55], and phototoxicity [56, 57] have provided new windows into the cell. However, many studies have established that VFPs exhibit intrinsically complex photophysical behaviour, and in order to effectively and correctly exploit their enormous power to enable visualization of dynamic biological processes, it is of paramount importance to comprehensively understand the intrinsic photophysical properties of these remarkable fluorophores.

Rapid advances in ultrasensitive optical detection and spectroscopy have made it possible to visualize emitters at the single-molecule level [58–62]. In contrast to ensemble measurements which yield information about the averaged properties of the sample, single-molecule studies yield information about individual molecular entities, bypassing the ensemble averaging effect. These molecular properties vary from molecule to molecule, and with time for individual molecules. As a result, single-molecule studies on a statistically relevant number of molecules yield distributions of data that in the limit of very large numbers of molecules approach ensemble data, but which contain a great deal more detailed information about subensembles and individual properties than ensemble measurements can hope to provide. A particular strength of single-molecule studies is the ability to identify rare forms and subensembles that are hidden or easily overlooked in ensemble studies, and to visualize the evolution of these forms in time, which is impossible in ensemble studies. Single-molecule studies of visible fluorescent proteins yield insights into the details of the complex photophysical landscape of these proteins. At the same time VFPs are proving to be excellent model systems for understanding fundamental photophysics and photochemistry, guest–host interactions, energy-transfer, and the effect of the chromophore nanoenvironment on the photophysical properties of the chromophore.

General structure of VFPs

The many different visible fluorescent proteins (VFPs) now known have been isolated from different marine life forms,

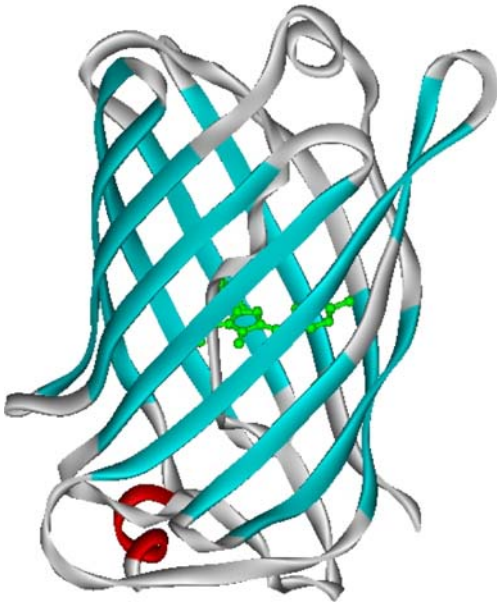


Fig. 1 Members of the class of visible fluorescent proteins share a universal "beta-can" structure of dimensions ~2.4 nm diameter and 4.2 nm height. The autocatalytically formed fluorescing chromophore is encapsulated within the cylinder

but remarkably share the same basic structure elucidated for *Aequoria* GFP [63, 64]. GFP has a molecular weight of 26.9 kDa and forms a barrel-like structure with a diameter of about 2.4 nm and a height of 4.2 nm. Eleven β-sheets form the outer wall of the barrel, and an α-helix runs diagonally through this barrel. The fluorescent chromophore is enclosed in the centre of the β-barrel and is thus shielded from the external environment (Fig. 1).

Chromophore formation—an autocatalytic multi step chemical reaction

The development of fluorescence in fluorescent proteins occurs in a posttranslational process, independent of the host that expresses the protein [16]. Nowadays the mechanism of chromophore formation for a number of different fluorescent proteins showing various emission characteristics is understood in some detail [65–71], although essential questions remain to be answered about the process.

Generally, the fluorescent chromophore in VFPs is the result of a multistep autocatalytic chemical reaction that starts after the folding of the protein into its characteristic β-barrel structure. The common motif of reaction of a tripeptide in the centre of the barrel resulting finally in the fluorescent chromophore is generally found in the whole superfamily of fluorescent proteins. In GFP the essential tripeptide is Ser65-Tyr66-Gly67. First, a cyclization into a

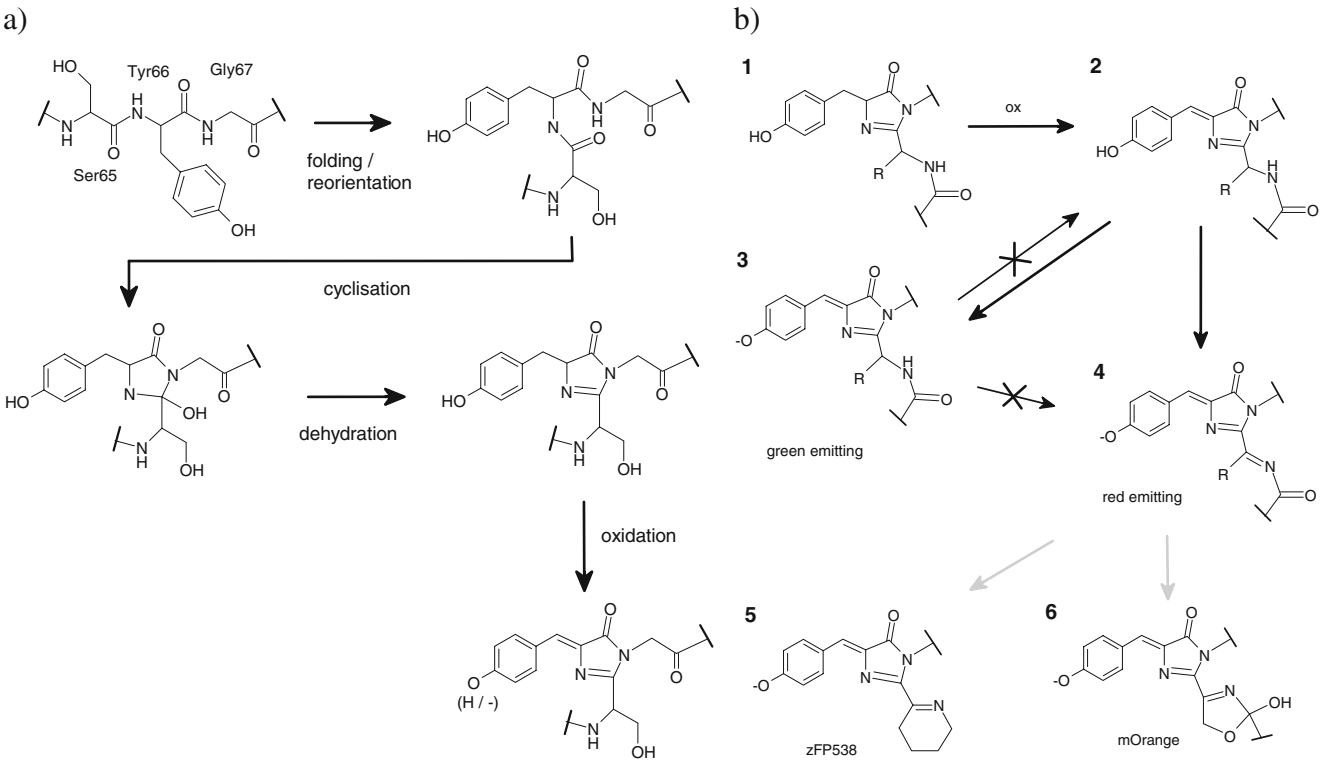


Fig. 2 (**a**) Mechanism proposed by Cubitt et al. [18] for formation of the green-emitting GFP chromophore. (**b**) Mechanism suggested by Verkhusha et al. [65] for formation of the green-emitting GFP-like *3* and the red-emitting chromophore *4* in DsRed and other proteins. The green and the red-emitting chromophores are different end products in a multistep chemical reaction starting with the cyclization of the protein backbone *1* and subsequent oxidation, yielding the protonated form of the green-emitting chromophore *2*. The red-emitting chromophore is an intermediate in the formation of the chromophores in zFP538 *5* and mOrange *6*

five membered heterocycle takes place, followed by an oxidation step that leads to the formation of the green-emitting chromophore in GFP (Fig. 2a). Replacing the central tyrosine by any aromatic group leads to blue and cyan-emitting GFP variants BFP and CFP [67], while modifications of amino acids in the direct vicinity lead to a number of variants with changed optical properties [17–24]. The substitution T203Y, which was rationally designed to result in π-stacking interactions between the chromophore and the tyrosine at position 203, became exceedingly popular because of its red-shifted emission resulting in the first yellow-emitting protein [72]. However, efforts to obtain any further red-shifted fluorescent proteins from GFP failed until very recently [73].

The discovery of further red-emitting fluorescent proteins in nonbioluminescent coral reef organisms filled this gap of orange to red-emitting proteins. The first protein of this group was DsRed [25], followed by many more discovered in other organisms [25–32] and by genetic modification of known proteins [33–38]. Remarkably, in all these proteins the structural motif of a barrel formed by β-sheets is conserved, as is the autocatalytic formation of the chromophore which is encapsulated within the protein barrel. The orange and red emission from these proteins is the result of an additional dehydrogenation which extends the GFP-like chromophore [69, 74, 75]. The mechanism of formation of the chromophore is still debated in the literature. Initially the formation of a GFP-like anionic chromophore was suggested, that then undergoes oxidation and turns into the mature, red-emitting DsRed chromophore [69, 74]. Indeed DsRed and its mutants contain green-emitting proteins [36, 37, 76–78]. An alternative mechanism was suggested by Verkhusha et al. in which the green emitting anionic chromophore is a side-product rather than an intermediate [65] (Fig. 2b), which takes into account that no dynamics between the green and the red-emitting chromophore can be observed. The proposed mechanism explains the generally observed chromophore formation of red-emitting fluorescent proteins. In addition, the red-emitting chromophore itself can be the reactant to yield chromophores with different properties [71, 79–81].

Basis for the spectral complexity of VFPs

Clearly, chromophore formation in fluorescent proteins is a complex, multistage chemical reaction within the protein. The complex chemical reaction scheme that finally leads to

the emitting chromophore implies the possibility of forming side-products that complicate the emission behaviour of the fluorescent proteins, e.g. by resulting in a mixture of emission colours [37, 82, 83]. The multistep chemical reaction resulting in the red-emitting chromophore in DsRed is likely to be responsible for the very rich photophysics of the tetrameric reef coral fluorescent proteins involving different emitting end products and non-emitting side-products. Besides the spontaneous formation of different chromophores, a number of photoinduced modifications of the chromophore and its nanoenvironment are known [84–87]. The possibility of modifying the emission of a fluorescent protein by means of applied light have made the so-called photoactivatable and photoswitchable proteins [39–52, 80, 81, 88] an important tool in many applications.

By virtue of its embedding in the protein scaffold, the chromophore interacts with its local nanoenvironment defined by the surrounding protein. The exact nature of the nanoenvironment of the chromophore influences the photophysical properties of the chromophore [67, 72, 79, 89, 90]. Although fluorescent proteins do not show the major structural rearrangements seen in many enzymes, the proteins are not static, and thermally-driven or photoinduced reorientations in the chromophore vicinity lead to a spread of photophysical properties [89]. Also the change between emitting and non-emitting states has been observed and linked to a structural reorientation in the chromophore environment [53, 54].

Finally, the tendency of many fluorescent proteins to form dimers and tetramers [35, 91–93] can lead to complex energy-transfer interactions between the proteins within the oligomer [86, 92, 94].

Ultrasensitive spectroscopy at the single-molecule level

Principles of SM studies, and instrumentation

Single-molecule spectroscopy enables the analysis of exactly one molecule at a time. Standard ensemble spectroscopy yields an average of a given observable for a large number of presumably identical molecules. By applying single-molecule spectroscopy this averaging effect is removed and variations of data characteristic of the sampled single molecule become visible. Sampling statistically relevant numbers of single molecules gives access to histograms of the relevant observable that describe the distribution of the respective value. Clearly the information content of such a distribution is significantly higher than the averaged value obtained from ensemble studies. The width of such distributions and their shape can be analysed to gain further insight into the photophysics of the analysed systems. Distributions of data are especially expected for complex systems where a multitude of different interactions between the emitting chromophore and its direct environment is possible. For biomolecules like fluorescent proteins, such heterogeneity easily arises because differing conformations or different posttranslational reaction pathways lead to different chromophore end products within the protein. Further, the shape of such distributions can be analysed, e.g. to see if it is unimodal or multimodal, which shows if the system under study is heterogeneous. Finally, single-molecule detection also enables the observation of dynamical changes, either spontaneous or arising from photophysics or photochemistry, without any need for synchronization.

It is evident that single-molecule fluorescence spectroscopy can contribute significantly to the characterization and understanding of complex systems such as fluorescent proteins. For practical realization of single-molecule spectroscopy the reader is referred to papers addressing the technical details of single-molecule emission spectroscopy, reviewed, for example, in Refs. [59, 60, 62, 95]. In short, the key to single-molecule detection is to reduce the sampled volume until there is only one target molecule in the observation volume at a time, to work with extremely clean samples, to reduce the contribution from background noise from the instrumentation or impurities, and to use extremely sensitive detectors. The minimum sample volume is defined by the optical diffraction limit, although recently some techniques have been demonstrated to circumvent this limit [4, 96]. The sampled volume is, in any case, large compared with the size of one molecule, so it is necessary to work with exceedingly high dilutions of target molecules to ensure there is only one fluorescent target molecule embedded in a non-fluorescent matrix or solvent in the observation volume at any given time.

For applications discussed in this article, the most commonly used technique is confocal scanning microscopy, in which the samples are typically raster-scanned to identify and localize emitting single molecules. The single emitters are further analysed by selectively positioning them into the observation volume. Photons emitted by the single molecule are detected by highly sensitive detectors such as avalanche photo diodes (APD) or highly sensitive intensified or back-illuminated and cooled CCD cameras. APDs are spectrally integrating detectors that can give accurate information about the arrival time of single photons, which can be used either to study the evolution of the single molecule emission intensity over time or, when pulsed excitation is used, to determine the emission lifetimes of single emitters. To measure single-molecule emission spectra, the emission is detected via a spectrometer by a very sensitive CCD camera.

To analyse the photophysical properties of fluorescent proteins, these proteins, at very high dilutions (~10^{-11} mol

L^{-1} VFP in polymer solution), are typically immobilized in a very thin film of a non-fluorescent polymer, so that on average less than one protein can be found within the observation volume defined by the optical diffraction limit.

Single emitter intensity trajectories

The intensity trajectory of a single emitter is experimentally the most easily accessible property, and describes the evolution of the emission intensity over time. Changes in the intensity reflect changes affecting the brightness of single-molecule emission, such as changes in molecular orientation or quantum yield, transient transitions into dark states, blinking, or photobleaching. Detailed analysis of intensity trajectories can yield a great deal of information about characteristic on and off times of a fluorophore, and about the details of processes affecting these properties. Systematically varying the excitation power while observing the emission intensity yields information on whether the observed changes into different states are induced by the excitation light. In some experiments two detectors differentiating between different spectral regions, for example, green and red, can broadly discriminate between spectral forms emitting in different parts of the spectrum.

Single VFP intensity trajectories

Most single fluorescent protein studies analyse the change of total single-molecule emission over time. However, especially in photophysically complex systems such as fluorescent proteins, it is very difficult to draw conclusions based on the emission intensity alone.

Analysis of fluorescent proteins at the single-molecule level started as early as 1997 [97]. Dickson et al. recorded intensity trajectories and showed that single GFP proteins undergo repeated cycles of fluorescent emission on a timescale of several seconds, behaviour clearly not observable in ensemble studies because of averaging of the emission from different molecules. Similar blinking behaviour was also observed for other fluorescent proteins [46, 98–100] (Fig. 3) and the duration of the on-times was found to decrease with increasing excitation power [61]. The nature of these dark states is still not clear, although changes in the protonation state of the chromophore have been suggested [46, 97]. In a report using surface-enhanced resonance Raman measurements, the signatures of the protonated and deprotonated chromophore were found to change on time scales similar to that of fluorescence blinking [101]. Nevertheless, the attribution of blinking to changes in the protonation state has been questioned by the finding that the blinking rates on the millisecond to second

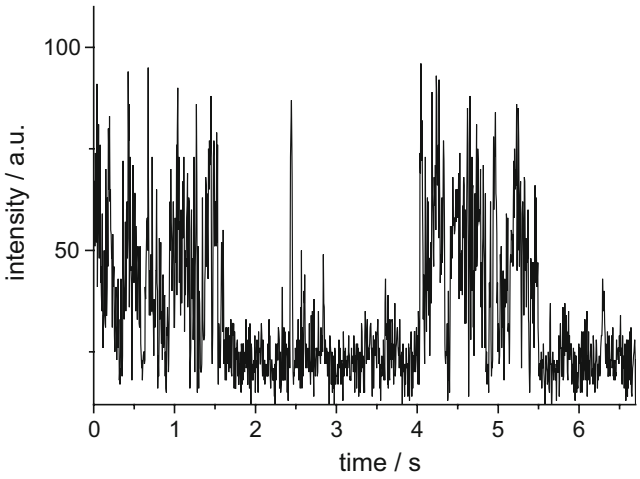


Fig. 3 Single molecule intensity trajectories of the enhanced Green Fluorescent Protein (EGFP). The emission is interrupted by numerous dark intervals. The duration of the on-times was found to decrease with increasing excitation power

time scale were insensitive to pH over a large range [98]. Another explanation for the observed change between emitting and non-emitting states is based on the observation that the GFP chromophore alone does not emit, because of efficient *cis*/*trans* photoisomerization that quenches the fluorescence [102]. It is not until the chromophore is immobilized in one conformation within the protein scaffold that fluorescence occurs. Both *cis* and *trans* conformations of the chromophore can, in principle, emit fluorescence [103, 104]. A reduction of the steric confinement of the chromophore that allows *cis*/*trans* photoisomerization within the protein [105, 106] and rearrangements in the chromophore environment defined by the protein backbone leading to effective alternative deactivation pathways were suggested as the origin of the observed blinking.

In addition, many GFPs exhibit another, long lived, dark state that can be depopulated by illumination with light around 400 nm [46, 97, 107] or by two-photon excitation between 780 and 870 nm [108]. This switching was attributed to spontaneous protonation of the chromophore. The protonated, neutral chromophore has blueshifted absorption and is a very poor emitter. As a result, the protonated chromophore does not absorb at the excitation wavelengths used and is thus dark. Illumination with light of short wavelengths into the absorbance band of the protonated chromophore favours the deprotonation of the chromophore in the excited state. Deprotonation results in the reconstitution of the red-shifted absorption and an efficiently emitting chromophore so that seemingly bleached chromophores were reactivated and regained their fluorescence [46, 97, 107, 108]. The light-induced switching of fluorescent proteins between different emitting or non-emitting states bears great potential for cellular

tracking applications and the newly developed super-resolution microscopy techniques [1, 12, 40, 43, 109]. Nevertheless the use of fluorescent proteins in these applications is limited by slow switching response times and low probability of a switching event after illumination. Recently a number of fluorescent proteins, including, for the first time, monomeric reversibly switchable red fluorescent proteins [110], were engineered that target some of these limitations. Detailed analyses of these switchable fluorescent proteins at the single-molecule level is still at a very nascent stage. Habuchi et al. demonstrated switching between a dim and a bright emitting state on the single-molecule level for the GFP variant Dronpa [111]. They showed that the response to the switching can be of the order of milliseconds and that the photoswitching can be repeated more than 100 times.

Besides fluorescent proteins switching between bright and dim or dark states, proteins switching from a green to a red emitting state have also been discovered [80, 93, 112, 113]. The photoconversion from green to red emission was found to be irreversible and due to cleavage of the backbone of the protein that results in a modified chromophore with extended chromophoric π-system [93, 114]. Again single-molecule emission studies helped to determine the underlying mechanisms of the switching. For the photoswitchable protein Kaede, Schafer et al. found that only 4% of the proteins showed the desired green to red switching after a switching event, and the rest of the proteins regained green emission, showed partial deactivation, or showed partial revival of green fluorescence [113].

Reef coral fluorescent proteins

The tetrameric reef coral fluorescent proteins, of which DsRed was the first isolated representative [25], have also been extensively analysed by single-molecule detection methods.

The analysis of the emission intensity trajectory of single DsRed tetramers showed a decrease of emission in four steps for a large number of analysed tetramers. This stepwise decrease of emission intensity was attributed to the bleaching of individual chromophores within the oligomer (Fig. 4) [115–117], confirming the tetrameric nature of the protein.

Monomeric red-emitting fluorescent proteins derived from the tetrameric DsRed are becoming increasingly available, and some have also been studied on the single-molecule level [100, 106, 118]. mCherry was found to be the most suitable protein for single-molecule studies [118]; the pH-dependent blinking observed for these monomeric red fluorescent proteins was linked to conformational rearrangements that enable radiationless deactivation resulting in dark states [106].

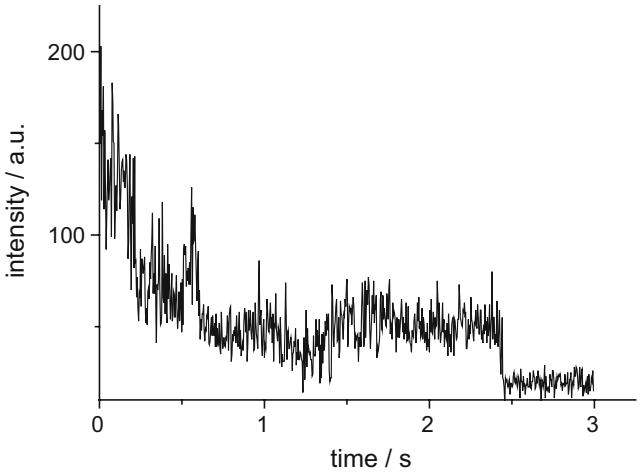


Fig. 4 Representative multi-step intensity trajectory from a single DsRed tetramer. The stepwise bleaching of DsRed has been presented as further evidence that DsRed forms obligate tetramers [115]. The chromophores of one tetramer bleach sequentially and thus give rise to the observed stepwise bleaching

Spectrally resolved single-molecule detection

In addition to spectrally integrating detection or total detection in broad spectral windows by using APDs or multiple APDs separated by appropriate optical filters, the technique of recording full emission spectra from single molecules with high spectral resolution has been established [119–122]. Recording full emission spectra is a very effective way of discriminating between target molecules and unwanted but unavoidable contaminants, because these contaminants usually have a distinctly different emission spectrum. Also, emission spectra are independent of reorientations of the analysed molecule with respect to the excitation light and detection, which change the detected emission intensity. Additional properties, for example the emission maximum position, the spectral shape, the spacing of the vibronic progression or the intensity ratio between different transitions of the vibronic progression, can be derived from the recorded spectrum. As a result, spectrally resolved single-molecule emission spectroscopy is a very sensitive tool for gaining insight into processes inducing small spectral variations, for discriminating between spectrally similar forms, and for following complex spectral behaviour. When sequences of spectra are recorded from a single molecule, possible transitions between spectral forms are directly visible and the comparison between the observed single-molecule spectra and known ensemble spectra can assist in analysis of the spectral behaviour observed at the single-molecule level.

On the downside, recording full emission spectra of single fluorescent molecules is experimentally more complex and results in a much coarser time resolution than when using spectrally integrating detectors.

To record emission spectra the emitted fluorescence of single molecules is dispersed by a spectrometer and imaged on to a highly sensitive, usually cooled, CCD camera. In this way the full emission spectrum is recorded instantaneously and no photons are wasted. Nevertheless, because the emission is dispersed over hundreds of channels, integration times of the order of hundreds of milliseconds to seconds—depending on the excitation power, the extinction coefficient and quantum efficiency of the emitter, and the efficiency of the set-up—are needed to record spectra with acceptable signal-to-noise ratios.

Analysing the emission spectra gives added value to single-emitter studies, and is representative of a general trend toward multiparameter imaging and spectroscopy. Other properties addressed by single-molecule multiparameter detection are the polarization of the emitted light and the fluorescence lifetime [123, 124].

Spectrally resolved single VFP studies

Analysis of the emission spectra of fluorescent proteins is especially interesting because of the vast complexity of this class of emitters. However, the recording of single fluorescent protein emission spectra and series of emission spectra is particularly challenging, because fluorescent proteins are exquisitely sensitive emitters and their photostability is often far worse than that of many synthetic fluorescent dyes [125].

Target molecule identification

The first single-molecule emission spectrum recorded from fluorescent proteins was embedded in studies that actually analysed the emission intensity trajectory of single fluorescent proteins [126]. The single-protein emission spectrum was used to confirm that the bright spots analysed were indeed the targeted GFP variant and not fluorescing impurities or contaminants. In this respect, it was irrelevant if the recorded spectrum was indeed from one emitting protein or from “one or not more than two molecules” as the authors stated [126]. Later Cotlet et al. showed single-protein emission spectra from the GFP variant EGFP and from DsRed to prove that embedding the proteins in a film of poly(vinyl alcohol) to immobilize the protein for analysis does not change their spectra [117].

Identification and characterization of spectral forms

Spectrally resolved single-molecule detection is a powerful tool for gaining insight into the existence of different spectral forms or subensembles. Looking at one emitting system at a time simply removes the influence of predominant forms seen in ensemble measurements. The presence of dominating and rare spectral forms is expressed in a single-molecule experiment by the occurrence of most of the observed molecules showing the signature of the dominating form, whereas a few molecules show the spectral signature from the rare forms. Further, any superposition of characteristics is removed, and as a result one obtains the unaltered spectra of the individual forms that can give direct insight into the physicochemical origin of the characteristic spectra. Another clear advantage justifying the additional experimental complexity of single-molecule detection experiments is the possible direct visualization of transitions between forms, something that is impossible to observe at the ensemble level.

The GFP group of proteins

The systematic analysis of large numbers of single-protein emission spectra were first used to gain detailed insight into the spectral properties and photophysical basis of proteins from the GFP group [127]. For ensembles of fluorescent proteins at cryogenic temperatures Creemers et al. demonstrated the existence of three spectroscopically different, interconvertible forms for a number of GFP variants [87, 128, 129]. These forms are the A-form, with neutral chromophore, the B-form, with deprotonated anionic chromophore, and an I-form, which is an intermediate involved in the transition between these forms. The B-form is the predominant form in the enhanced yellow fluorescent protein (EYFP). Although these cryogenic experiments have established some limits on the heights of the energy barriers between the various forms, the details of these barriers are not fully known and it was unclear if the I-form was of relevance at room temperature.

Spectrally resolved single-molecule detection was used to address the question of different emitting forms of EYFP and of transitions between these forms [127]. At the single-molecule level the properties of emitters are distributed, because the interaction between the individual emitter and its environment or embedding can vary slightly. As a result careful analysis of the obtained data is necessary to discriminate between spectrally distinct forms and variations of the properties from within one form. The most straightforward way to do so is analysis of a statistically relevant number of molecules and subsequent histogramming of the relevant observable. In the histogram different spectral forms become apparent as different distributions of the observed property.

To characterize the individual emission spectra measured, the position of the emission peak maximum was chosen. The emission spectra from 400 single EYFP molecules yielding 1288 single-molecule spectra were recorded and the emission maximum position was determined for each spectrum (Fig. 5a). These maximum positions were then assembled into the histogram presented in Fig. 5b.

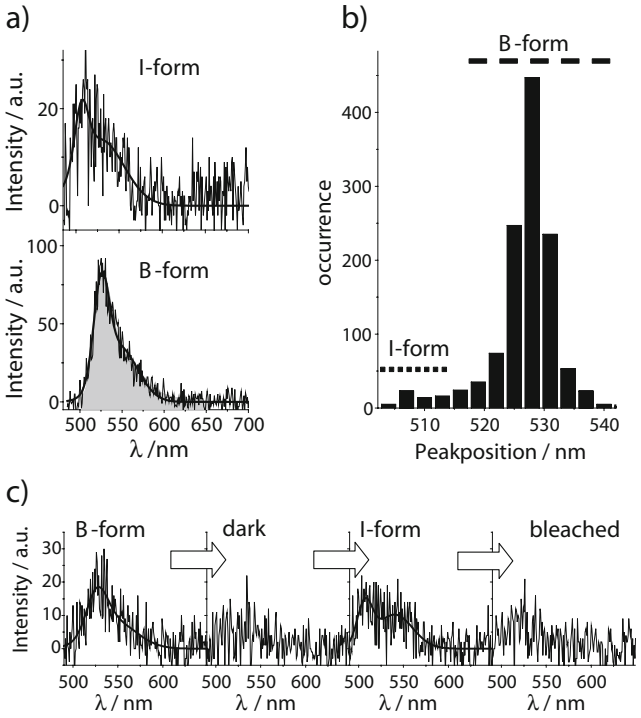


Fig. 5 (**a**) Characteristic single-protein emission spectra of the I and the B-forms of the enhanced yellow fluorescent protein (EYFP); ensemble spectrum in *solid grey*. (**b**) Distribution of emission maximum positions from all analysed single EYFP molecules. The distribution is bimodal, spectra with maxima distributed around 527 nm originate from EYFP molecules in the B-form; spectra with maxima distributed around 507 nm are from proteins in the I-form. (**c**) Spectral sequence showing the transition from the predominant EYFP B-form to the blue-shifted emitting I-form with a dark time of about 1 s between the emission of the different forms. For details see [127]

The histogram of the emission maximum positions from single EYFP molecules clearly does not show a unimodal Gaussian distribution. The distribution is dominated by a peak originating from the B-form with emission maximum ~527 nm, which is in accordance with that expected from ensemble spectroscopy. However, there is a secondary maximum to the blue side of this main distribution which is evidence of a different spectral form. The secondary distribution is centred at ~508 nm and can be associated with the blue-shifted intermediate I-form that had heretofore only been observed at cryogenic temperatures. A key advantage of spectrally resolved single-molecule detection is that one obtains pure spectra of rare forms without any alteration from other, possibly dominating, states. In the case of EYFP the acquisition of the I-form emission spectrum by single-molecule detection gave interesting hints about the underlying molecular basis of this form. The EYFP I-form spectrum was found to be congruent with the well known emission spectrum from the anionic GFP chromophore.

The redshift of the emission of EYFP compared with the anionic GFP chromophore is caused by π-stacking interactions between the amino acid residue of tyrosine introduced at position 203 and the chromophoric π-system [72]. The finding that the EYFP I-form spectrum resembles the emission of the chromophore lacking π-stacking strongly suggests disturbance of the π-stacking by a conformational change in the surroundings of the chromophore, e.g. by a turn or tilt of the phenolic group of the tyrosine residue.

Aside from the static observation of the I-form, direct dynamics between the forms were observed—an observation impossible in ensemble spectroscopy. Interestingly, the transition between the two forms was always accompanied by a dark period between the disappearance of one form and the appearance of the other. This spectral signature is clearly indicative of a two step mechanism with a non-radiative—possibly very effectively quenched—intermediate (Fig. 5c).

The DsRed group of proteins

DsRed was the first red fluorescent protein discovered. DsRed exhibits considerable photophysical complexity, because of its oligomeric nature and the possibility of forming different chromophores. This photophysical complexity and multimeric nature is shared by many of the known red-emitting proteins to-date [27, 130-133], which makes DsRed and its variants a valuable model system for this group of proteins.

DsRed forms obligate tetramers [74], the monomeric subunits of which contain either a green-emitting chromophore analogous to that of GFP or a red-emitting chromophore [69]. The tetrameric structure of the protein makes fluorescence resonance energy transfer coupling of the different chromophores within one tetramer very likely. This possibility, together with the intrinsic complexity seen for all fluorescent proteins, result in markedly rich photophysics.

The first single-molecule emission spectra from DsRed were presented by Cotlet et al. [117] as evidence that embedding of the proteins in a film of PVA to immobilize the protein for analysis does not change the characteristic emission. In another study on DsRed, the same authors combined conventional bulk spectroscopy with multi-property single-molecule detection [134] and the potential to determine the distribution of photophysical properties from a statistically relevant number of analysed single molecules.

Detection from a large number of individual tetramers yields emission spectra with varying spectral positions (Fig. 6). Determination of the position of the emission maximum for each spectrum and histogramming of the results yielded a Gaussian distribution of the emission maximum position with maximum clearly red-shifted relative to the bulk emission maximum position. The histogram clearly shows that on the single-molecule level

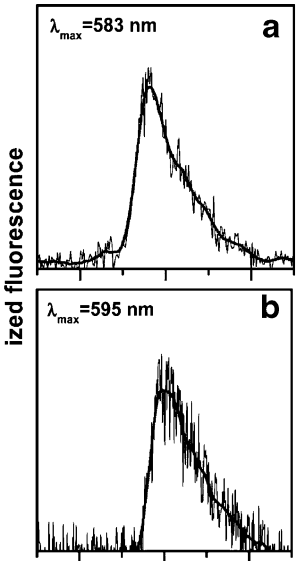


Fig. 6 Emission spectra from single DsRed tetramers. (**a**) Emission with a maximum at 583 nm, as observed for ensemble samples, was only rarely observed. (**b**) Most of the emission spectra showed a distinct shift of the emission spectra to longer wavelength. This red shifted emission was attributed to the photoinduced formation of a "super-red form". From [134], Copyright (2001) National Academy of Sciences, U.S.A.

photophysical properties are not a fixed value but are distributed (Fig. 8), reflecting the individual interactions between single emitters and their embedding. This distribution has its origin in variations in the interaction of the emitting chromophore with the embedding protein scaffold and thus highlights the possibility of microscopic structural variations from protein to protein and the possibility of structural variations with time. These show that on excitation of the red emitting, mature chromophore, the observed spectrum is red-shifted relative to the bulk emission. This super-red form has been detected before [86, 135], but the single-molecule experiments yielded emission spectra of this form for the first time. The use of time-resolved measurements and the variation of excitation wavelength further confirmed the complex photophysical behaviour of DsRed that has to be taken into account when DsRed is used to illuminate biological questions.

A systematic study of the emission spectra of different DsRed mutants at the single-tetramer level further highlighted the spectral complexity of this group of proteins [136]. Amino acid substitutions in these variants (DsRed2: Arg2Ala, Lys5Glu, Lys9Thr, Val105Ala, Ile161Thr, Ser197Ala; Fluorescent Timer: Val105Ala, Ser197Thr; DsRed_N42H: Asn42His; AG4: Val71Met, Val105Ala, Ser197Thr) yield proteins with altered spectral and maturation properties. These substitutions, when situated in the vicinity of the chromophore, also have an effect on the flexibility of the chromophore surroundings and on the chemical nanoenvironment of the chromophore.

This study was the first to unequivocally demonstrate emission from the green-emitting chromophore of DsRed on the single-molecule level. Emission spectra showing emission from the green-emitting chromophore and from the red-emitting chromophore were presented for DsRed (Fig. 7) and for all sampled variants.

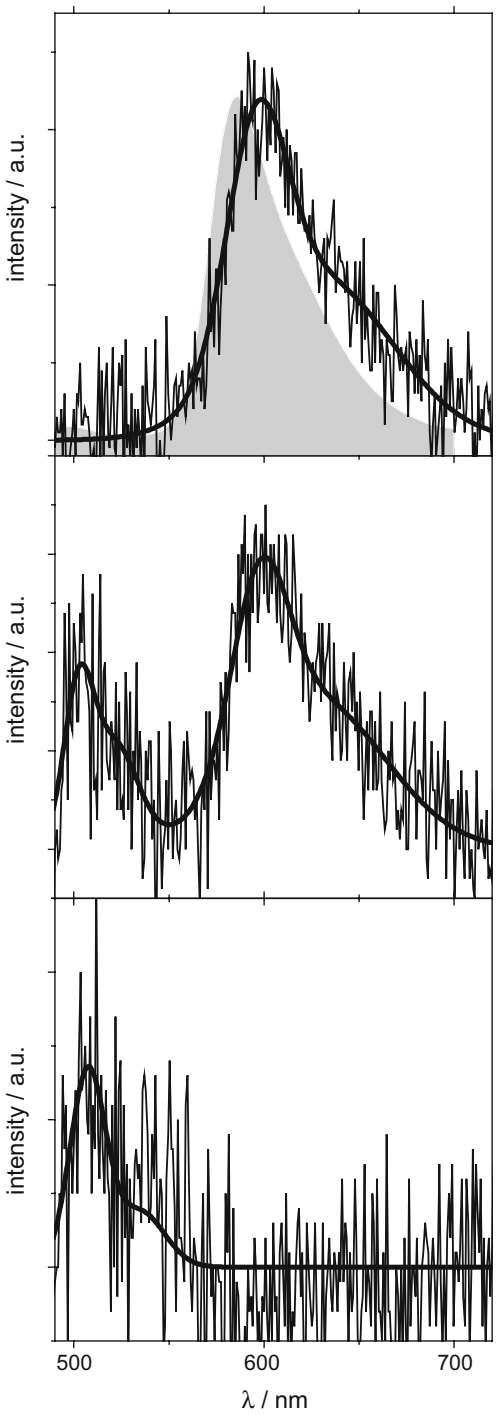


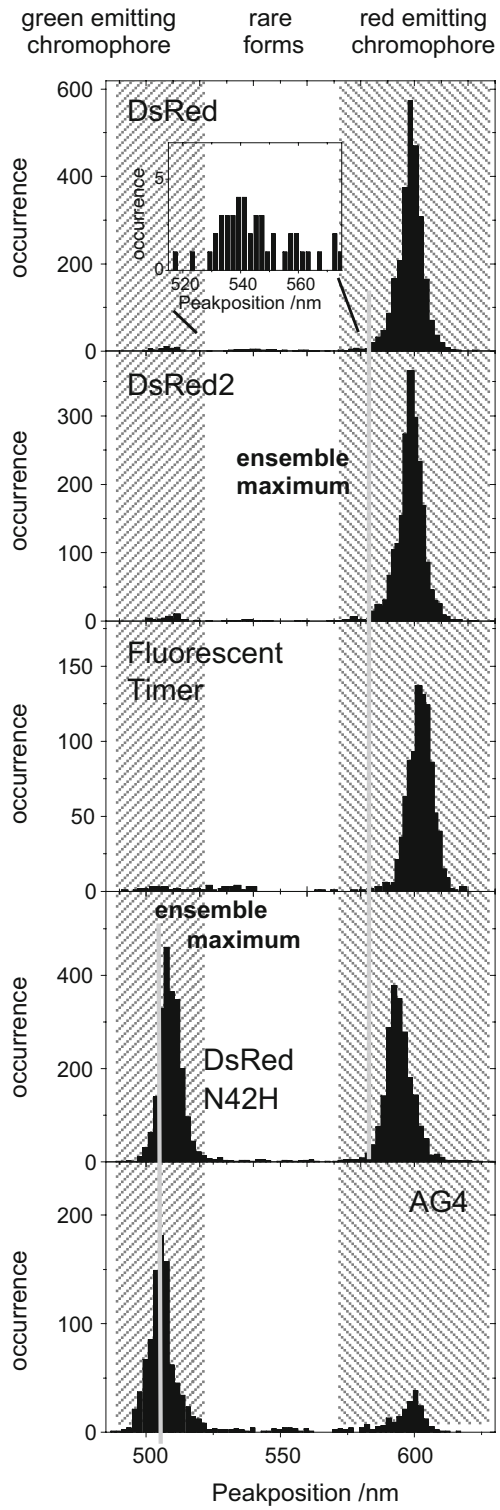
Fig. 7 Single-tetramer spectra of DsRed showing solely red emission (*first panel*), mixed emission (*second panel*), or solely green emission (*third panel*). The observed super-red emission is red shifted with respect to the ensemble spectrum (*grey*). These three types of spectrum were found for all sampled DsRed variants spanning emission colour from green over orange to red. The frequency of occurrence of the respective spectra varied drastically and was consistent with the bulk spectra [136]

Analysis of the emission maximum positions of all single-oligomer spectra of all variants showed that the emission maximum positions of the green-emitting chromophores are distributed around ~503 nm, consistent with bulk spectroscopy (Fig. 8). The emission maximum positions of the red chromophores were found to be distributed ~600 nm (with slight differences for the different variants), a value that is ~16 nm red shifted relative to the bulk emission maximum. This observation verifies the rapid formation of a high-quantum efficiency "super-red" form DsRed.

The super-red form has been observed for DsRed before [86, 134, 135] but its underlying molecular origins are still unclear. Analysis of a number of variants of DsRed made clear that the super-red form is not limited to DsRed but is a general behaviour of reef coral fluorescent proteins derived from DsRed.

Recently it was suggested that the photoconversion to the super-red form is the result of structural changes in the vicinity of the chromophore due to *cis–trans* isomerisation of the chromophore and decarboxylation of a glutamate (Glu-215) in the vicinity of the chromophore [85] resulting in a red-shifted, low-quantum-efficiency form. Indeed, all variants we have analysed so far contain a glutamate residue at position 215; thus the universal observation of the super-red form is consistent with this proposed mechanism. However, the consistent observation of the super-red form at the single-molecule level strongly suggests high quantum efficiency. Also, the reversible photoconversion between a super-red form and other spectral forms observed at cryogenic temperatures [86] contrasts with the proposed mechanism of photoinduced decarboxylation. Taking all the details from ensemble spectroscopy, cryogenic spectroscopy, and single-molecule spectroscopy together it seems likely that two different super-red-emitting forms exist, one with high fluorescence quantum efficiency which reversibly photoconverts to other forms, and which is observed at the single-molecule level and in experiments at cryogenic temperatures. The second super-red form would then be of low quantum efficiency and is potentially formed by irreversible decarboxylation as reported [85].

The histograms of single-molecule emission maximum positions show that for DsRed and all the variants sampled, some oligomers emitting with maximum positions between 530 nm and 570 nm were observed (Fig. 8). In this



◀ **Fig. 8** Histograms of emission peak positions. Consistent with bulk spectroscopy, DsRed and the variants Fluorescent Timer, DsRed2, and AG4 show one main distribution of maximum positions whereas the DsRed_N42H variant shows two distinct distributions originating from the red-emitting and green-emitting chromophores, respectively [136]. The emission from the red-emitting chromophore is systematically red-shifted compared with the bulk emission; this is attributed to the rapid (<1 s) formation of a super-red form of the chromophore. The histogram further shows the emission maximum positions of rare forms emitting in the wavelength area between the typical emission of the green-emitting and the red-emitting chromophores. The *inset* in the top panel is a zoom-in into the spectral region where rare spectral forms are observed. From Ref. [136]

wavelength region the emission cannot be attributed to the main emitting forms. The percentage of tetramers showing this rare form of emission is generally low, but not negligible (DsRed ~3%, Fluorescent Timer ~1%, DsRed2 ~6%, AG4 ~2%, and DsRed_N42H ~2%). For all variants, transitions between the main emitting forms and the rare forms could be observed (Fig. 9), which demonstrates the dynamic formation of the rare spectral forms from the main spectral forms.

The emission observed from the rare forms of DsRed and its variants (Fig. 9) corresponds very well with the emission from the protein zFP538 from coral *Zoanthus sp.* with emission maximum at 538 nm and mOrange with emission maximum at 562 nm. These fluorescent proteins embody a chromophore that resembles a truncated red-emitting DsRed chromophore [71, 79]. The similarity between the emission from zFP538 and mOrange and the rare forms from the DsRed family of fluorescent proteins observed here is suggestive of an analogous, possibly photoactivated, modification of the red-emitting chromophore from an acylimine to an imine resulting in the truncated chromophore. Besides the chemical modification of the chromophore the chromophore nanoenvironment determines the exact emission position and distribution of the emission maximum positions observed at the single-molecule level [89] which accounts for the variances in the emission maximum position of the rare forms. Transitions

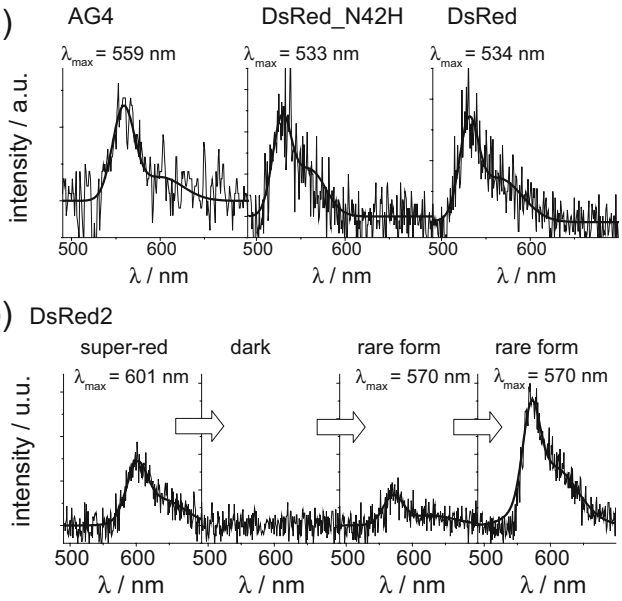


Fig. 9 Emission spectra of rare spectral forms from single reef coral protein oligomers. (**a**) For all proteins rare spectral forms with emission maximum positions between ~530 nm and 575 nm were observed. (**b**) The spectral series shows the transition between the predominant super-red form and a rare spectral form with emission maximum at 570 nm via a dark state [136]

from the main forms to the rare spectral forms were observed, but never transitions back from rare forms to one of the main forms. This observation supports the hypothesis of a modification of the chromophore, because creation of the imine is unlikely to be reversible.

FRET coupling in DsRed proteins by multiparameter spectroscopy

The formation of obligate tetramers and the occurrence of green and red-emitting chromophores within the tetramers of DsRed and a large number of its variants suggests a coupling of the chromophores by FRET. Direct verification is difficult, because it is not possible to directly isolate from the tetramers, monomers containing green and red-emitting chromophores for separate characterization.

The fluorescence decay characteristics recorded on ensemble samples of DsRed are a complex mixture of a number of fast and slow decay components ranging from several hundreds of picoseconds to nanoseconds [94, 134, 137]. This complex decay characteristic is generally attributed to the presence of two different chromophores, one green and one red emitting, and energy-transfer coupling of these chromophores within one tetramer.

Photon antibunching experiments on single DsRed tetramers were used to further elucidate the coupling of fluorophores [138]. Photon antibunching is a clear signature of a nonclassical radiation field, which reflects that a quantum system cannot spontaneously emit two photons successively without some time lag to allow the system to return back to its ground state. For DsRed tetramers photon antibunching was found for most of the tetramers analysed, which emphasizes the effective FRET coupling of chromophores within one tetramer.

Nevertheless, because the green and red emitting monomers in DsRed cannot be isolated for separate analysis, the FRET coupling within the tetramers remained undetermined for a long time. Recently multiparameter single-molecule spectroscopy was used to fill this gap [137]. Initially, single-tetramer spectra of DsRed and then the decay characteristics of the same tetramers were recorded. Recording the emission spectra from single tetramers enabled identification of tetramers containing exclusively green-emitting chromophores. Subsequent determination of the fluorescence lifetimes gave direct access to the fluorescence lifetime of the green-emitting chromophores without any interference from energy transfer to any red-emitting chromophore (Fig. 10). The fluorescence lifetime of the green-emitting chromophore was found to be exceptionally long, ~6 ns, and was the missing link enabling calculation of parameters describing energy transfer within DsRed tetramers.

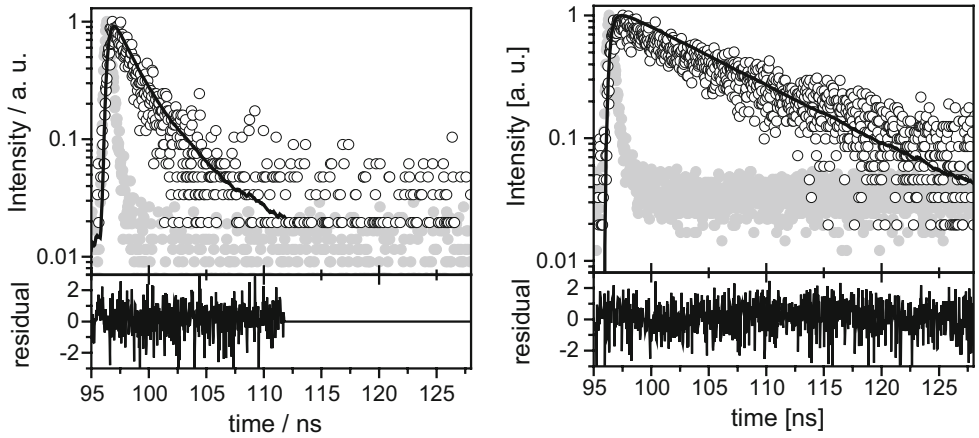


Fig. 10 Emission spectra and fluorescence decay curves were recorded from the same DsRed tetramers. Red emitting tetramers (*top*) showed a biexponential decay of $\tau 1 = 1.2$ ns (37%) and $\tau 2 = 3.7$ ns (63%), while the green emitting tetramers (*bottom*) generally showed monoexponential decays of around 6 ns. Identification of the green-emitting tetramers and measurement of the undisturbed decay characteristics from these green-emitting chromophores enabled full characterization of fluorescence resonance energy transfer coupling in DsRed tetramers [137]

Because the tetramer is composed of a layered dimer of dimers, in principle each monomer has two direct neighbours, one in its layer and one in the other layer of the tetramer. The transfer efficiency between a monomer and its direct neighbour in the other layer of the tetramer is found to be very high (~96%), whereas the transfer efficiency to the other direct neighbour within its layer is found to be a moderate (75%). Energy transfer to the monomer diagonally opposite is found to be unlikely (~1%). The differences in coupling efficiencies to the direct neighbours of 97% and 75% may explain some of the observed dual emission from different chromophores from one tetramer observed in other studies [136, 138].

Disentangling decay pathways by multiparameter single-tetramer studies resulted in the determination of a full set of spectroscopic data describing the energy-transfer coupling within the tetramers of DsRed.

Finally, the range of DsRed variants provides a set of proteins with different chromophore environments, and thus well-defined chromophore–matrix systems. These substitutions influence the flexibility of the chromophore surroundings and the chromophore nanoenvironment, thus enabling comparative studies of the principal factors determining the characteristics of distributions of observables. A detailed study of these variants yielded a correlation between the rigidity of the chromophore environment and the widths of the distribution of emission maximum positions [89].

Summary

Single-molecule emission spectroscopy has proved to be a powerful tool enabling access to aspects of VFP photophysics which can only be seen on the single-molecule level without the averaging inherent to ensemble experiments. Single-molecule spectroscopy of fluorescent proteins is experimentally still challenging, because of the low photostability of fluorescent proteins. Nevertheless, in-depth understanding of the fluorescent protein emission properties is becoming increasingly important, because the applications in which fluorescent proteins are used to quantitatively elucidate biological and biochemical processes are becoming more and more complex.

In particular, techniques that record more data than simply the change of emission intensity over time give detailed insights into the photophysics of fluorescent proteins. These techniques, for example spectrally resolved measurements yielding full emission spectra, or temporally resolved measurements yielding the decay characteristics of single molecules, also give access to the evolution of the observed properties in time, so that spectral dynamics can be followed and their molecular origins can be elucidated. Clearly spectrally and temporally resolved single-molecule emission spectroscopy has added a lot to our understanding of the emission properties of fluorescent proteins by enabling identification of hidden subensembles, giving valuable information about the possible molecular origins of these subensembles, by shedding light on the transitions and dynamics between these subensembles, and by allowing insights into the complex FRET coupling within fluorescent protein tetramers. To date, single-molecule analysis of fluorescent proteins has focused on the most often used proteins from the GFP and the DsRed groups of proteins, but the ever growing number of fluorescent proteins holds further the promise for exciting single-molecule research of this class of complex emitters.

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