Improving Autofluorescent Proteins: Comparative Studies of the Effective Brightness of Green Fluorescent Protein (GFP) Mutants

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ABSTRACT We study the photophysical behavior of 8 mutants of Green Fluorescent Protein (GFP) using fluorescence correlation spectroscopy (FCS) on the single molecule level and double resonance excitation of bulk samples. Experimental data reported here and the previously published data on the RH/R- equilibrium and fluorescence quantum yields \( \Phi_F \) (Jung et al., 2005; Biophys J 88:1932–1947) are analyzed with respect to single molecule as well as conventional fluorescence microscopy. The fraction of GFP molecules in a dark state, \([D]\), reduces the effective absorption cross section under photostationary conditions. The determination of the excitable fraction \([B]\) and its fluorescence quantum yield \( \Phi_{FL} \) gives the effective brightness \( \Phi_{eff} \). Our results show that in its wavelength range, eGFP is, among the GFPs, the best fluorophore for most microscopic applications. However, in the red shifted YFP-proteins, there is still potential for improvement, since a pronounced dark state population is detectable in all mutants investigated so far. We propose to use the mutant T203Y/E222Q in imaging studies, whenever the expression yield is not a limiting factor. In FCS experiments, where the useful concentration range of the expressed molecules is restricted to concentrations below micromolarity, our data suggest the use of wt-GFP or mutant T205Y, as these represent photochemical buffers. Both mutants might surpass the limitations given by out-of-focus bleaching in live cell microscopy. Microsc. Res. Tech. 69:175–185, 2006.

KEY WORDS fluorescence microscopy; single molecule spectroscopy; cytometry; FCS; PCH

INTRODUCTION

In the past decade, Green Fluorescent Protein (GFP) of the Pacific jellyfish, Aequorea victoria, and similar proteins from reef animals became universal labeling tools for fluorescence microscopy of live cells (Lippincott-Schwartz et al., 2001; Tsien, 1998; Zimmer, 2002). Their widespread use is owed to the unusual origin of the fluorescence. All proteins of this type are lacking an external cofactor as chromophore. Instead, the fluorophore is formed in the protein’s interior in an autocatalytic reaction (Chalfie et al., 1994). Tagging of almost arbitrary gene products is established by fusion of the gene code to that of the target protein. This enables localization studies and tracking of protein movements by means of fluorescence microscopy. Interaction of differently marked proteins can be studied qualitatively by observation of Förster-resonance energy transfer (FRET) (Weiss, 2000).

Quantitative conclusions in cytometry and microscopy, as drawn e.g., in expression studies (Blake et al., 2003), depend on a careful characterization of photophysical parameters of the marker proteins (Patterson et al., 1997; Piston et al., 1999). GFP, the best characterized protein of this new class of fluorescent proteins, consists of 238 amino acids. These form a barrel-like structure with an approximate dimension of 4 nm in height and 2 nm in diameter (Brejc et al., 1997; Ormö et al., 1996; Palm et al., 1997; Yang et al., 1996). Fluorescence originates from a p-hydroxybenzylidene-imidazolinone-chromophore, which is held tightly by the surrounding protein barrel. The cyclization of three amino acids and the subsequent reaction steps during fluorophore formation rely on a proper alignment of amino acids in their vicinity (Barondeau et al., 2003; Branchini et al., 1998). The chromophore environment, however, does not only affect the kinetics of the chromophore formation but also other physicochemical properties. An important example of this is the influence exhibited by the \( pK_a \) values of the phenolic OH-group, i.e., the equilibrium between the neutral (RH) and the anionic chromophore state (R-). Both forms differ in their electronic properties and give rise to absorbances at \( \sim 400 \) nm and \( \sim 480 \) nm, respectively (Brejc et al., 1997). Further shifts in the respective absorption and fluorescence spectra can be induced by mutations (Heim and Tsien, 1996; Wachtler et al., 1998). While shifts in the spectra are readily observed, the less accessible but equally important fluorescence quantum yields are also influenced by the interaction between chromophore and protein scaffold. Actually,
fluorescence quantum yields $\Phi_F$ of the isolated chromophore are low in highly viscous media like polyvinylalcohol, but can be as high as 80% in wild-type GFP (Kummer et al., 2002; Litvinenko et al., 2003). This impressively demonstrates that the relaxation pathways of the chromophore’s excited electronic state and, thus, the apparent brightness of GFP mutants depend on the local protein environment of the chromophore. Several internal conversion modes, which lead to a reduction of $\Phi_F$, were analyzed both theoretically and experimentally (Kummer et al., 1998, 2000; Martin et al., 2004; Weber et al., 1999). We recently studied the influence of four mutations and their combinations in close vicinity of the chromophore on $\Phi_F$. Our results indicated that fluorescence quantum yields $\Phi_F$ of up to 100% are possible with an appropriate chromophore environment (Jung et al., 2005).

This contradicts the common observation in ultra-sensitive fluorescence microscopy that GFP and its derivatives are not as bright as it would be expected from the above mentioned bulk data (Garcia-Parajo et al., 2000). Indeed, experiments with individual molecules revealed numerous dark states, i.e., nonfluorescent or nonexcitable isomeric states of the chromophore (Moerner, 2002). Their population can be light-induced or spontaneous, and the resulting “off-states” possess lifetimes between microseconds and hours. In only a few cases, it was possible to assign a mechanism for the fluorescence intermittency. These examples include external and internal protonations as well as putative cis–trans isomerizations (Haupts et al., 1998; Nifosi et al., 2003; Schwille et al., 2000; Widengren et al., 1999b). Experiments with double resonance excitation revealed the RH-state as a dark state, which was formed as a photoproduct after excitation of R– as the educt state (Jung et al., 2000, 2001a,b). Although the analysis of the nature of other dark states is impeded by a strong heterogeneity in their lifetimes (Garcia-Parajo et al., 2000), a quantification of the extent to which dark states are populated in autofluorescent proteins is important for microscopical applications. This concerns bulk fluorescence microscopy, in which lower expression values and thus a smaller concentration of the native protein of a biological sample are possible with brighter GFP mutants, as well as single molecule microscopy. Knowledge of the dark state population enables the users to calculate the effective brightness up to the ms-scale. This is the typical upper limit of pixel dwell times in confocal fluorescence microscopy.

In our contribution, we report experimental data obtained by fluorescence correlation spectroscopy (FCS) on the single molecule level and double resonance bulk excitation (Jung et al., 2000). Most often, FCS is applied in microscopy to study the diffusion of tagged proteins (Brock et al., 1998). In addition, FCS is an important tool for spectroscopic investigations of photodynamics. It allows the characterization of the fraction of molecules in a dark state under the influence of a constant irradiation intensity (Widengren et al., 1995). Such photostationary conditions are already reached within microseconds after the onset of the illumination.

The chromophore equilibria between the RH and the R– chromophore state in the chosen eight mutants of GFP are shifted to the anionic R– form of the chromophore. Our selection is based on the better photophysical properties of R–, when compared with those of RH, such as higher extinction coefficients (Tsien, 1998), higher overall fluorescence quantum yields (Tsien, 1998), lower tendency to photoconversion (Bell et al., 2003) and, due to their red-shifted absorption spectra, less spectral overlap with cellular autofluorescence at typical excitation wavelengths (Blab et al., 2001). Here, we determine the effective brightness $\Phi_{eff}$ of these mutants as the product of the fluorescence quantum yield $\Phi_F$ and the fraction of molecules in a fluorescent state, $B_l$, and show how efficiently the fluorescence can be enhanced by simultaneous two-color irradiation. Our results are discussed with respect to different microscopical applications of fluorescence spectroscopy, and with a focus on the detection of single molecule as the technique that is most demanding for the high quality of the employed fluorophores.

MATERIALS AND METHODS
Sample Preparation

Protein samples were a kind gift of J. Wiehler (Gene Center, LMU Munich), and were prepared as described recently (Jung et al., 2005). They were stored at 4°C in a physiological buffer solution at pH 7.4. Typical concentrations of the stock solutions were 10⁻¹⁰ M. Mutants used in this study were wt-GFP, S65G, E222Q, and S65G/E222Q and their combinations with the mutation T203Y. The latter mutants are also referred to as yellow fluorescent proteins (YFPs). In addition to these eight mutants, eGFP (Clontech, Mountain View, CA) was used as a reference.

For the spectroscopic experiments, 100 μL of the stock solutions were diluted with pure water (HPLC-grade, Riedel-de-Haen, Seelze, Germany), filtered with a 0.22-μm filter (Millex GV13, Millipore, Schwalbach, Germany), and then diluted with pH 10 buffer solutions (HPCE-grade, Fluka, Buchs, Switzerland) to 10⁻⁷ M for the double resonance experiments and between 10⁻⁸ and 10⁻⁹ M for the FCS experiments. Thorough sonification after each dilution step prevented protein aggregation. Observation of fluorescence at 10⁻⁷ M even after prolonged sonification indicated that this procedure did not affect the protein’s photophysical behavior.

The high pH value was chosen for two practical reasons: firstly, a decrease in the concentration of GFP was observed in FCS at a pH value 8 and below. This was detrimental to the analysis in long FCS-runs. We attributed this phenomenon to absorption of the proteins to the negatively charged substrate surface. Secondly, experiments at pH values above the published $pK_a$ values were we able to a priori exclude external protonation as an origin of the dark state population. Further reasons for the chosen high pH value, which concern the ease of the analysis, are given in the Discussion section.

Spectral Characterization

Absorption spectra were recorded with a standard UV-VIS spectrometer (330, Perkin–Elmer, Wellesley, MA) at pH 10. Fluorescence excitation and emission spectra were taken with a commercial fluorescence spectrometer (nF 900, Edinburgh Instruments, Livingston, UK) in 90° geometry. The values of $\lambda_{max}$ negligibly differed to the previously published values, only
the ratio of the RH to R\(^{-}\) bands in the absorbance respective excitation spectra changed due to the higher pH value in this study.

**Microscopic Setup**

The setup, used in this study, is based on a homebuilt confocal microscope designed for single molecule experiments, as described previously in detail (Jung et al., 2001a). Briefly, light from a krypton-ion and an argon-ion laser (Innova 200 and Innova 90-5, Coherent, Santa Clara, CA) is simultaneously focused onto the sample using a dichroic mirror (485 DCXR, Chroma, for GFPs, Rockingham, VT or 505DCLP02, Omega, for YFPs, Brattleboro, VT) and a Nikon 60\(\times\), NA 1.2 water immersion objective on an inverted Nikon TE 300 microscope. Main operating wavelengths were 407 and 476 nm for GFPs or 496 nm for YFPs, respectively. These wavelengths were chosen to address the absorption bands of RH and R\(^{-}\) in GFPs or YFPs. The beams of both lasers were combined and made collinear using a dichroic beamsplitter (420DCLP02, Omega). Emitted light is collected in backscattering geometry and directed through a confocal pinhole (diameter of 50 µm). Remaining excitation light is removed with bandpass filters (535DF55, Omega, for YFPs or, HQ 510/50, Chroma, for GFPs). For ensemble experiments, the signal is then detected with a single avalanche photodiode module. The autocorrelation functions were recorded using a commercial correlator (ALV Langen, Germany, ALV-5000/F).

**Analysis of FCS Data**

The principles of FCS have been described in several review articles (Eigen and Rigler, 1994; Hess et al., 2002). Briefly, FCS allows the calculation of the autocorrelation function \(g^2(\tau)\) from fluorescence fluctuations. These are caused either by a change in the number, \(N\), of molecules in the detection volume or by intramolecular on-off dynamics of individual molecules such as transitions into the triplet state and other dark states. Hence, the analysis of \(g^2(\tau)\) can supply information about the concentration of the solution, the diffusion coefficients, and the molecular rate constants into and out of the dark states, \(k_{\text{off}}\) and \(k_{\text{on}}\) (Eq. (1b)) (Widengren et al., 1995).

\[
g^2(\tau) - 1 = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_{\text{D}}} \right)^{-1} \left( 1 + \sum_i C_i \exp(\lambda_i \tau_i) \right) \quad (1a)
\]

\[
\approx \frac{1}{N} \left( 1 + \frac{\tau}{\tau_{\text{D}}} \right)^{-1} \left( 1 + \sum_i \frac{k_{\text{off}}}{k_{\text{on}}} \exp(-k_{\text{off}} + k_{\text{on}} \tau_i) \right)
\]

As the observed molecules are not immobilized, photophysical transition can only be investigated on a time scale given by the residence time of the molecules in the detection volume. Typical transit time in FCS, as given by the decay time constant \(\tau_p\) of \(g^2(\tau)\) due to diffusion, are up to 1 ms in our setup. This covers pixel dwell times typically encountered in confocal fluorescence microscopy. As our experiments were performed at varying, but moderate, intensities photobleaching was negligible and a constant \(\tau_p\) was assumed, where global fitting was performed (Origin 6.0, Microcal). A two-dimensional diffusion model was sufficient for the analysis of our data and restricted the number of fit parameters to a minimum (Rigler et al., 1993). The omission of a term describing the axial diffusion out of the detection volume is convenient at the pinhole diameter which we used.

In the case of multiple dark states, Eq. (1b) is only an approximation. The analysis of the population dynamics of three or more dark states with FCS is tedious and oftentimes impossible (Widengren et al., 1999a; Widengren and Schwille, 2000). In the case of two dark states, the analysis of the dynamics of the resulting four-level system leads to mathematical expressions for the decay amplitudes \(C_i\) and for the decay constants \(\lambda_i\), which are not suited for fitting purposes (Eq. (1a)) (Boiron et al., 1996; Jung et al., 2001a). The sum of the amplitudes \(\Sigma C_i\), however, remains constant independent of the fitting model. In the following, \(\Sigma C_i\) is called the contrast for the internal dynamics. It can be converted to \(|D|\), the fraction of molecules in dark states under photostationary conditions (Eq. (2)), which is the relevant quantity for the determination of the effective brightness \(\Phi_{\text{eff}}\) (see results).

\[
\frac{|D|}{1 - |D|} = \sum C_i \rightarrow |D| = \frac{\sum C_i}{1 + \sum C_i}
\]

**Analysis of the Double Resonance Excitation Data**

Excitation spectroscopy with simultaneous 407 and 476 nm irradiation was previously used to characterize \(|D|\) in a photoconvertible dark state (Jung et al., 2000). Here, 476 and 496 nm light was used to excite R\(^{-}\) in GFP and YFPs, respectively. The relative fluorescence enhancement \(E_{\text{FL}}\) by additional 407 nm excitation was calculated by a saturation model (Eq. (3)).

\[
E_{\text{FL}}(\text{at } 407\text{ nm}) = E_{\text{max}} \frac{I}{I + I_s}
\]

In this model, the observed increase in fluorescence intensity, caused by illumination with 407 nm light, saturates when the intensity of 407 nm light is increased. Since it is directly related to \(|D|\) under one-color photostationary conditions (Eq. (4)), not the saturation intensity \(I_s\) but the maximum fluorescence enhancement \(E_{\text{max}}\) is of interest with respect to \(\Phi_{\text{eff}}\) of fluorescent proteins.

\[
|D| = \frac{E_{\text{max}}}{1 + E_{\text{max}}}
\]

In our model, \(D\) is a photoproduct state of the initially excited R\(^{-}\). The anionic state is then efficiently repopulated by the addition short-wavelength irradi-
tion (Jung et al., 2001a). To avoid obscuration of this well-characterized effect by the photoconversion of a prevailing RH, we restricted our experiments to mutants that were predominantly existing as R\(^{-}\) in the ground state. For this reason, wt-GFP and T203Y were excluded from our study (Fig. 1).

**RESULTS**

**Absorption and Excitation Spectra**

Figure 1 shows the absorption spectra of the T203-containing (GFPs, Fig. 1a) and the Y203-containing fluorescent proteins (YFPs, Fig. 1b) at pH 10. Except from wt-GFP and the mutant T203Y, which both show a dominant absorption band at \(\sim 400\) nm, R\(^{-}\) preponderates in all other mutants. Absorption maxima were found at \(\sim 475\) nm for GFPs (with the exception of S65G) and \(\sim 510\) nm for all YFPs. Among these mutants, residual absorbance of RH was only detected in the mutants possessing the double mutation T203Y/S65G. However, the RH state in these two proteins could not be excited to fluoresce above 500 nm (Fig. 1c). In agreement with previous work, fluorescence was detected near 505 nm for GFPs, and near 525 nm for YFPs. The relative population of R\(^{-}\) in wt-GFP as well as in T203Y is hardly affected by lowering the pH to 7.4 (Jung et al., 2005). Hence, the chromophore's protonation equilibrium is in both mutants merely susceptible to the external concentration of protons at these pH values.

**Fluorescence Correlation Spectroscopy**

FCS was performed with excitation of R\(^{-}\) at 476 nm (GFPs) or 496 nm (YFPs). Only in the case of S65G/E222Q, two-color excitation under saturation conditions (see below) was necessary to obtain FCS curves of sufficient quality. FCS curves were fitted by a monoexponential or—where required after examination of the fit residuals—by a biexponential approach for the internal dynamics. The curves were normalized by the number of molecules \(N\) in the detection volume (Fig. 2), which facilitates the visual comparison of data of different intensities or of different mutants. Classification of the obtained curves, in dependence of the applied excitation intensity, leads to four different types of internal dynamics. The intensity-independent flickering between a bright and a dark state (type I) was observed only once (Fig. 2a, mutant T203Y). Intensity-dependent on-off dynamics (type II) with a saturating contrast \(C\) was observed for wt-GFP, S65G, and T203Y/S65G (Figs. 2b and 2c). Type III behavior designates a linearly growing contrast \(C\) with increasing intensities (Fig. 2d). It was solely observed in T203Y/E222Q. The remaining three mutants, all containing E222Q, exhibited a combination of type II and III dynamics (Fig. 2e and 2f). In the latter mutants, an interpretation of the intensity-dependent decay constants \(k_{i}\) by FCS is hampered by the intrinsic inaccuracy of the fit function (Eq. (1b)) and requires a tedious analysis, which requires two-

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[Fig. 1. Normalized absorption and excitation spectra of the investigated mutants at pH 10. S65 containing mutants are printed in black, whereas S65G mutated proteins are shown in gray. Full lines represent proteins with E222, and broken lines indicate E222Q mutants. (a) The relative population of R\(^{-}\) in wt-GFP is hardly affected by lowering the pH to 7.4 in GFP mutants. (b) YFP mutants containing the additional mutation T203Y. Again, the R\(^{-}\) population in T203Y is marginally affected by a physiological pH value. (c) Excitation spectra of the investigated YFP mutants (\(\lambda_{\text{exc}} = 535\) nm). The main difference between (b) and (c) concerns the RH species that are nonfluorescent in all species except a subpopulation in T203Y. We assign this fraction to the dark state observed by FCS.]
Fig. 2. FCS experiments at pH 10 at different excitation intensities. The correlation functions are normalized to the number of molecules $N$. $\lambda_{\text{exc}} = 476 \text{ nm}$ for GFP, and $\lambda_{\text{exc}} = 496 \text{ nm}$ for YFPs. (a) In T203Y no intensity dependence is observed (type I dynamics). The dark state must be seen in the absorption spectrum. (b) Light-driven flickering in wt-GFP, similar intensity dependent curves are observed in S65G and T203Y/S65G (type II). Only in S65G, the underlying dynamics can be correlated to the fluorescence enhancement shown in Figure 3. (c) Analysis of the contrast $C$ in the normalized FCS curves of wt-GFP (Fig. 2b). Saturation of the contrast $C$ is observed. The saturation behavior of the contrast $C$ is explained by a photo-induced depopulation competing with a thermal relaxation channel in the dark state's dynamics. (d) Linear increase of the contrast with higher intensities in mutant T203Y/E222Q (FCS curves not shown) (type III). (e) FCS curves of the triple mutant T203Y/S65G/E222Q. Strong intensity dependence is observed (cf. Fig. 2f) (f) Only a plot of the yielded contrast $C$ against the applied intensity reveals that the intensity dependent curves in Figure 2e is indicative of a combination of type II and type III dynamics. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].
color experiments (E222Q, see Jung et al., 2001a). In the special case of S65G/E222Q, experimental data (FCS and double resonance data in combination) suggest the existence of at least three dark states in the accessible time regime. For this mutant, a conclusive picture of the underlying dynamics cannot be obtained. Note, however, that for the evaluation of $[D]$, it is only necessary to determine $\Sigma C_i$.

Table 1 summarizes the outcome of the analysis according to the classification. The fourth category is therefore found in the rows of both type II and III. A constant contrast $C$, be it intensity dependent or not (type I and II), can easily be converted to $[D]$. In the case of type III, $[D]$ changes as the excitation intensity changes. The same situation is met in the triplet state population of common fluorescent dyes. Under these conditions, $[D]$ can be calculated for a given excitation intensity from the slope $dC_i/dI$ (Wildengren et al., 1995).

### Double Resonance Excitation

Saturation experiments of the fluorescence enhancement by adding short-wavelength light (407 nm) was performed for various excitation intensities of 476 nm light, as described previously in more detail for the mutant E222Q (Jung et al., 2000). $E_{\text{max}}$ was observed in each case already at low intensities of 476 nm light. It is a measure of the amount of reversibly photoconverted protein under photostationary conditions. Please note that the experimentally observed value of the enhancement represents only a lower limit as the enhancement sensitively depends on the accurate overlap of 407 and 476 nm (or 496 nm) laser light in the detection volume. Further complication of the interpretation arises by the wavelength-dependence of $E_{\text{max}}$ (data not shown).

Table 3a displays $E_{\text{max}}$ for GFPs (excitation at 476 nm) and YFPs (excitation at 496 nm). In direct comparison of the mutation pattern, GFPs always shows a higher $E_{\text{max}}$ by two-color excitation than YFPs. The small effect in YFPs, which did not exceed 25% even

### Calculation of the Effective Brightness $\Phi_{\text{eff}}$

For the calculation of $\Phi_{\text{eff}}$ one has to determine $\Phi_{\text{fl}}$ and $[D]$. Additionally, our results obtained at pH 10 should be transferred to physiological conditions where the equilibrium between RH and $R^*$ might be shifted. With the recently published equilibrium data and $\Phi_{\text{fl}}$, we now have access to the $\Phi_{\text{eff}}$ of various variants of fluorescent proteins at physiological conditions. $\Phi_{\text{eff}}$, therefore, not only allows for all fluorescence quenching processes, which occur on a sub-ms timescale, but also gives an estimate of the effective absorption cross section. The data are summarized in Table 2 and partly visualized in Figure 4.

$\Phi_{\text{fl}}$, which is the ratio of the number of fluorescence photons and the number of absorbed photons, can be calculated from the fluorescence lifetime, $t_{\text{fl}}$, and the radiative lifetime (Strickler and Berg, 1962). The latter quantity is accessible from the absorption and fluorescence spectra of the fluorescent compounds. We previously compared the values derived by this method with values obtained by referencing the fluorescence intensity to that of wt-GFP (Jung et al., 2005). The same tendencies were displayed by both ways of deduction, although the absolute values differed slightly. Consistently higher $\Phi_{\text{fl}}$ was found by the method based on measuring $t_{\text{fl}}$. The advantage of the determination of

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**Table 1. Classification of observed dark state dynamics in FCS experiments and obtained contrasts according to Eq. (1)**

<table>
<thead>
<tr>
<th>Mutant Type</th>
<th>Contrast $C$</th>
<th>Sensitivity to 407 nm light</th>
<th>$dC_i/dI$ (kW$^{-1}$ cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I and Type II* (with respect to wt-GFP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt-GFP</td>
<td>0.25 ± 0.02b</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>S65G</td>
<td>0.45 ± 0.04b</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E222Q</td>
<td>1.54 ± 0.09</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E222Q/S65G</td>
<td>0.33 ± 0.05</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>T203Y</td>
<td>1.1 ± 0.1b</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>T203Y/S65G</td>
<td>0.62 ± 0.02b</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>T203Y/S65G/E222Q</td>
<td>0.89 ± 0.09</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Type III*</td>
<td>E222Q</td>
<td>0.28b</td>
<td>0.014 ± 0.002</td>
</tr>
<tr>
<td>E222Q/S65G</td>
<td>0.42b</td>
<td>0.021 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>T203Y/E222Q</td>
<td>0.60b</td>
<td>0.030 ± 0.002e</td>
<td></td>
</tr>
<tr>
<td>T203Y/S65G/E222Q</td>
<td>1.08b</td>
<td>0.054 ± 0.004</td>
<td></td>
</tr>
</tbody>
</table>

* $\lambda_{\text{max}}$ = 476 nm for GFP; $\lambda_{\text{max}}$ = 496 nm for YFPs.
* Constant contrast $C$, (Figs. 2a–2c, 2e, and 2f). The saturation of the contrast—shown in Figure 2b and 2c—is neglected, but leads to a higher population of the bright state, $[B]$, at lower intensities.
* Type II.
* With additional 407 nm irradiation at saturation.
* Type I.
* Intensity-dependent contrast $C$, (Figs. 2d–2f). For the evaluation of the contrast $C$, an excitation intensity of 20 kW/cm$^2$ is used. This roughly corresponds to 10 excitation cycles $\mu$s$^{-1}$ for the listed mutants.
* Contrast $C$ at 20 kW/cm$^2$.
* Type III.

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**Fig. 3.** Maximal fluorescence enhancement $E_{\text{max}}$ under two-color irradiation. For comparison, the value of eGFP, taken from reference (Jung et al., 2000) is also depicted. Strong enhancement is visible only in GFP variants. The small values in YFPs might be related to a vanishing population of RH-species. For wt-GFP and T203Y, such ensemble-type experiments cannot be performed because of the prevailing RH population.
TABLE 2. Effective brightness \( \Phi_{ef} \) calculated at 10 excitation cycles \( \mu s^{-1} \)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>( \Sigma C )</th>
<th>( E_{\text{max}}(%) )</th>
<th>([D]) at pH 10</th>
<th>([B]) at pH 10</th>
<th>([R^-]) at pH 7</th>
<th>( \tau_{\text{FL}} ) (ns)</th>
<th>( \Phi_{\text{FL}} )</th>
<th>( \Phi_{\text{ef}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-type</td>
<td>0.25</td>
<td>-</td>
<td>0.20</td>
<td>0.80</td>
<td>0.20</td>
<td>3.4</td>
<td>0.80</td>
<td>0.13</td>
</tr>
<tr>
<td>S65G</td>
<td>0.40</td>
<td>55</td>
<td>0.29</td>
<td>0.71</td>
<td>0.91</td>
<td>1.6</td>
<td>0.39</td>
<td>0.25</td>
</tr>
<tr>
<td>E222Q</td>
<td>1.82</td>
<td>175</td>
<td>0.65</td>
<td>0.35</td>
<td>0.96</td>
<td>2.5</td>
<td>0.60</td>
<td>0.20</td>
</tr>
<tr>
<td>S65G/E222Q</td>
<td>0.75</td>
<td>400</td>
<td>0.89</td>
<td>0.11</td>
<td>0.89</td>
<td>1.5</td>
<td>0.37</td>
<td>0.04</td>
</tr>
<tr>
<td>T203Y</td>
<td>1.1</td>
<td>-</td>
<td>0.52</td>
<td>0.48</td>
<td>0.11</td>
<td>4.4</td>
<td>0.98</td>
<td>0.05</td>
</tr>
<tr>
<td>T203Y/S65G</td>
<td>0.62</td>
<td>10</td>
<td>0.38</td>
<td>0.62</td>
<td>0.68</td>
<td>3.8</td>
<td>0.84</td>
<td>0.35</td>
</tr>
<tr>
<td>T203Y/E222Q</td>
<td>0.60</td>
<td>10</td>
<td>0.37</td>
<td>0.63</td>
<td>0.87</td>
<td>4.2</td>
<td>0.92</td>
<td>0.50</td>
</tr>
<tr>
<td>T203Y/S65G/E222Q</td>
<td>1.77</td>
<td>15</td>
<td>0.64</td>
<td>0.36</td>
<td>0.59</td>
<td>3.6</td>
<td>0.79</td>
<td>0.17</td>
</tr>
</tbody>
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The sum of contrast \( \Sigma C \) is taken from Table 1 and \( E_{\text{max}} \) is taken from Figure 3. The population in the anionic state \( R^- \), the fluorescence lifetime \( \tau_{\text{FL}} \) and the fluorescence quantum yield \( \Phi_{\text{FL}} \) are taken from Reference (Jung et al., 2005). The fraction of molecules in some dark state \([D]\) is calculated from \( \Sigma C \) and \( E_{\text{max}} \), depending on whether \( C_i \) can be correlated to \( E_{\text{max}} \) or not. The small \( E_{\text{max}} \) in YFPs is more likely related to the remaining population in RH and is therefore neglected. The bright fraction \([B]\) at pH 10 is \( 1 - [D] \), \([B]\) and its product with \([R^-]\) are depicted in Figure 4b. The product of \([B]\), \([R^-]\), and \( \Phi_{\text{FL}} \) is the effective brightness \( \Phi_{\text{ef}} \) (cf. Fig. 4c), the product of \([B]\) and \( \Phi_{\text{FL}} \) (cf. Fig. 4d) is related to the molecular brightness in photon counting histogram analysis (Chen et al., 2002).

Fig. 4. Visualization of the data in Table 2. (a) Fluorescence quantum yield \( \Phi_{\text{FL}} \) on the basis of fluorescence lifetime measurements (Jung et al., 2005). The longer lifetime component was used when decay heterogeneity was observed. (b) The bright fraction \([B]\) at pH 10 (hatched bars) that was obtained by the FCS experiments (Fig. 2) or by the double resonance experiments (Fig. 3). Smaller values of \([B]\) are determined if the RH/R\(^-\) equilibrium at physiological pH is considered (full bars). (c) The effective brightness \( \Phi_{\text{ef}} \) at physiological pH value as the product of \([B]\), \([R^-]\) and \( \Phi_{\text{FL}} \) For comparison, a value of \(~50\%\) is calculated for eGFP. (d) The relative molecular brightness \( e_{\text{rel}} \), as it appears in FCS experiments or in photon counting histogram analysis. The difference with (c) is that the slow interconversion of RH and R\(^-\), which occurs in wt-GFP as well as in T203Y on a longer time scale than the diffusion in FCS, does not reduce the actual brightness. Moreover, slow interconverting molecules act as a pool of unbleached molecules. \( e_{\text{rel}} \) is the product of \( \Phi_{\text{FL}} \) and the values are represented by the hatched bars in Figure 4b.
**DISCUSSION**

**Nature of the Dark States**

FCS is a spectroscopic method that registers fluorescence fluctuations caused by diffusion or intrinsic photodynamics. The nature of the dark states, which lead to fluorescence intermittency, is a priori unknown when they are photochemically populated. Numerous attempts, based on chemical intuition, were made to unravel the structural and electronic background of these dark states: addition of heavy-atoms to the solvent and removal of the quencher oxygen from the solvent increased the population of a photochemically populated triplet state (Widengren et al., 1995); theoretical work supported the assignment of photochemically populated and depopulated dark states to cis/trans isomeric states (Schwille et al., 2000; Widengren and Schwille, 2000); double resonance excitation was performed and gave strong evidence for a protonated chromophore species in GFP mutants (Jung et al., 2001a).

The situation is different for nonphotochemically driven flickering. Here, the dark state is a ground-state species and should, therefore, be visible in the absorption spectrum, although its spectrum might be overlapping with that of the fluorescent species. A prominent example is the protonation of fluorescein and GFP variants by buffer molecules, which in both cases leads to only a weak excitable fluorophore (Widengren et al., 1999b). From the rate constants for protonation and deprotonation at different pH values, $pK_a$ values can determined. These are intrinsically identical to the $pK_a$ values obtained by bulk measurements. In the case of fluorescent proteins, where the absorption maximum of RH is clearly shifted to $\sim$400 nm, the fraction of molecules that is not excited by 476 nm light can therefore be deduced by the absorption spectrum at the relevant pH value. Hence, more than the pure practical reasons given in the spectral characterization led us to choose pH 10 for our experiments: avoiding further complications of the dynamic FCS data by an additional decay of $g^2(\tau)$; simplification of the interpretation of the double-resonance data; transferability of the data to physiological conditions. It was previously shown for the mutant E22Q that $[D]$ is not affected by changes in the pH value between pH 7 and 10 (Jung et al., 2001a), and we assume that this also holds true for the other here investigated mutants. An influence of the pH value on light-induced dynamics was never observed before for any GFP mutant by FCS. A higher fluorescence increase at lower pH values, as observed in another mutant (Jung et al., 2001b), would correspond to an even higher $[D]$ and consequently further reduced $[B]$ and $\Phi_{ef}$.

At pH 10, a light-independent process (type I) was only observed for the mutant T203Y with $\sim$50% dark molecules (Fig. 2a). A high $\Phi_{eff}$ of R$^*$ clearly above 50% in the bulk excludes an isomeric, non, or weakly fluorescent R$^*$ state as an explanation. Inspection of the absorption spectrum instead suggests a RH state. The fraction of nonexcitable molecules $[D]$, deduced from FCS (Table 2), disagrees however with the RH-population, which is derived from the absorption spectrum ($\sim$8%). A tentative explanation is given by the excitation spectrum in T203Y (Fig. 1c). By comparison with the absorption spectrum (Fig. 1b), we deduce that 15% to 20% of the RH fraction in T203Y can be excited to green fluorescence. This excitable fraction corresponds to a fraction of $\sim$15% of all proteins, which is similar to the amount of R$^*$ species. Flickering might correspond to the rearrangement between two fluorescent proteins form. The vast majority of the protein however would be an actually nonfluorescent RH conformer (Kummer et al., 2000). Its interconversion occurs on a longer timescale than $\tau_D$ which is not covered by FCS. Though, other origins of the obvious discrepancy between the absorption spectra and the FCS data are imaginable.

Type II dynamics comprises the light-driven interconversion between a bright and a dark state similar to those found in eYFP and related mutants (Fig. 2b and 2c, Schwille et al., 2000). In this study, such behavior is detected for 6 out of 8 mutants, but the underlying mechanism most likely varies between the mutants. In
GFPs, at least some of the resulting dark states are sensitive to 407 nm light, as can be seen by the bulk-saturation data. This might also be true for the dark state, which is observed in wt-GFP (Fig. 3). The investigated YFP-mutants however do not show a pronounced response to 407 nm irradiation. Hence, this observation suggests that the chemical nature of the dark state differs between GFPs and YFPs. We however cannot exclude by our FCS experiments that dark states in YFPs, which are populated with a quantum yield below 10⁻⁴, are sensitive to violet or near UV light. Such photoconvertable isomeric states were found by experiments on individual molecules (Chirico et al., 2005; Dickson et al., 1997). Noteworthy is the case of E222Q/S65G, where a fluorescence enhancement of ~400% by simultaneous two-color excitation was monitored (Fig. 3). From this value, one can directly derive that at least 80% of the molecules are dark at a given point in time. This also explains why reliable FCS data could only be obtained under the saturating conditions of two-color excitation. Whether the remaining light-induced constant contrast C, which was detected in the FCS experiments, corresponds to the putative cis/trans-isomerization in YFPs, cannot be answered by our experiments.

The third class of dark states in our set of mutants is clearly correlated to the mutation E222Q (Figs. 2d–2f; Jung et al., 2001a). An increasing [D] with increasing power was previously observed in common fluorescent dyes, and was attributed to the intersystem crossing from the excited singlet state to the triplet state (Widengren et al., 1995). Experiments which show that the lifetime of the triplet state is limited by a diffusion-controlled quenching by solute oxygen and an enhanced intersystem crossing by solvated heavy atoms supported this assignment. Such experiments, although not attempted in the course of this study, presumably fail as the chromophore in GFPs is well protected from the outer solvent by the protein scaffold. It is however difficult to imagine how the E222Q mutation with the simple outer solvent by the protein scaffold. It is however difficult to imagine how the E222Q mutation with the simple outer solvent by the protein scaffold. It is however difficult to imagine how the E222Q mutation with the simple outer solvent by the protein scaffold. It is however difficult to imagine how the E222Q mutation with the simple outer solvent by the protein scaffold.
those used in single molecule imaging microscopy are applied. Usage of E222Q containing fluorescent proteins should then be avoided as in these mutants dark states are very efficiently populated. For these experiments however, we propose usage of T203Y or wt-GFP, although $\Phi_{\text{eff}}$ of these mutants is very low.

FCS is a microscopic method that uses low concentrations of analytes. $\Phi_{\text{eff}}$ of individual molecules therefore is a key factor in FCS, as it contributes quadratically to the amplitude of the correlation function. Thus, discrimination of highly fluorescent molecules against the background can be achieved (Broek et al., 1998). $\Phi_{\text{eff}}$ on the time scale of a FCS experiments (i.e., $\sim 1$ ms), which is related to the molecular brightness in photon-counting histogram (PCH) analysis (Chen et al., 2002), is only influenced by on-off kinetics on that timescale. Rearrangements on a longer timescale, which are the only plausible explanation of the disagreement of the dark fraction obtained by FCS to the preponderance of RH in the absorption spectrum in wt-GFP and T203Y, do not affect this quantity. Consequently, wt-GFP is superior to eGFP and T203Y superior to the other investigated YFP-mutants in FCS experiments (Fig. 4d). Additionally, one can benefit from the yet neglected reservoir of RH species by using these mutants. Slow conversion of the main part of the protein delivers R$^\text{reservoir}$ of RH species by using these mutants. Slow investigations YFP-mutants in FCS experiments (Fig. 4d).

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REFERENCES


