Manipulation of the local density of photonic states to elucidate fluorescent protein emission rates

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We present experiments to determine the quantum efficiency and emission oscillator strength of exclusively the emitting states of the widely used enhanced green fluorescent protein (EGFP). We positioned the emitters at precisely defined distances from a mirror to control the local density of optical states, resulting in characteristic changes in the fluorescence decay rate that we monitored by fluorescence lifetime microscopy. To the best of our knowledge, this is the first emission lifetime control of a biological emitter. From the oscillation of the observed emission lifetimes as a function of the emitter to mirror distance, we determined the radiative and nonradiative decay rates of the fluorophore. Since only the emitting species contribute to the change in emission lifetimes, the rates determined characterize specifically the quantum efficiency and oscillator strength of the on-states of the emitter, in contrast to other methods that do not differentiate between emitting and dark states. The method reported is especially interesting for photophysically complex systems like fluorescent proteins, where a range of emitting and dark forms has been observed. We have validated the analysis method using Rhodamine 6G dye, obtaining results in very good agreement with the literature. For EGFP we determine the quantum efficiency of the on-states to be 72%. As expected for this complex system, our value is higher than that determined by methods that average over on- and off-states.

1. Introduction

The discovery, development, and use of genetically-encodable visible fluorescent proteins (VFPs) has provided revolutionary new capabilities to visualize molecular and cellular biological processes using fluorescence microscopy. 1–3 Despite the widespread use of VFPs as reporters and sensors in cellular environments the versatile photophysics of fluorescent proteins is still subject to intense research. Understanding the photophysics of fluorescent proteins is essential for accurate interpretation of the biological and biochemical processes illuminated by the fluorescent proteins as well as for the development of biosensors based on fluorescent proteins.

A multitude of studies has addressed the spectral complexity of fluorescent proteins. Different chromophores can form within one protein,4,5 intrinsic and photoinduced spectral shifts have been observed,6–8 and it has been demonstrated that VFPs exhibit rapid blinking as a result of transitions between emitting and non-emitting states.9–11 This photophysical complexity influences the determination of fundamental emission properties such as the radiative and nonradiative decay rates, fluorescence quantum efficiency and oscillator strength, since most approaches average over the different subensembles exhibiting different photophysical properties in the analyzed sample.

The most widely-used approach determines the quantum efficiency of an unknown fluorophore by comparing the wavelength integrated emission intensity of the unknown sample to a known reference having the same absorbance at the chosen excitation wavelength.12 Using this value for quantum efficiency and the measured fluorescence lifetime, the radiative and non-radiative decay rates and emission oscillator strengths can be calculated. However, this approach is rigorously correct only for systems of identical emitters, and averages over all spectral species. It does not discriminate between (a) molecules in emitting states and those in (b) absorbing, but non-emitting states or (c) in photoinduced dark states created by the measurement itself. These dark states bias the determined quantum efficiency and hence affect the calculated values for the decay rates and oscillator strength.

Fluorescent proteins exhibit blinking attributed to spontaneous as well as photoinduced transitions between emitting to non-emitting states,9–11 and exhibit intrinsic spectral fluctuations due to chemical or environmental influences upon the chromophore.6,13 Consequently, the values for fluorescent protein quantum efficiencies derived from different studies vary considerably. A typical example is the red emitting protein DsRed, for which values for the quantum efficiency in the literature range from the first pioneering results reporting 23%,14 to the much higher consensus values currently, but which still range from 68%,15,16 to 79%.17 Another example is...
the green emitting protein Azami green, for which quantum efficiencies of 67%\textsuperscript{18} and 90%\textsuperscript{19} were reported. It is therefore evident that a method is needed for the determination of quantum efficiencies that is independent of relative measurements and the presence of non-emitting states.

We present here the first such experiments on fluorescent proteins using a method to determine radiative and nonradiative decay rates of an emitter, and thus quantum efficiency and emission oscillator strength, that characterize the emitting states of the chromophore and is not dependent on spectral integration. This method has been applied in other research fields to erbium ions,\textsuperscript{20} semiconductor colloidal nanocrystals,\textsuperscript{21,22} silicon nanocrystals\textsuperscript{23} and self-assembled quantum dots.\textsuperscript{24} We control the local density of optical states by positioning the fluorescent protein at precisely defined distances from a metallic mirror. From a classical point of view, if an emitter is placed in front of a metallic mirror the field emitted directly by the emitter interferes with the field from the emitter’s mirror image. If the distance from the mirror is such that the interference is constructive, the local density of states, and hence the emission rate, will be increased. If on the other hand, the distance is such that the interference is destructive, the local density of states, and hence the emission rate, will be reduced. In this way control over the distance from the emitters to the mirror corresponds to control of the local density of states. A classical model taking full account of material properties of the mirror and dielectric environment was developed by Chance \textit{et al.}\textsuperscript{25}

The distance-dependent modification of the local density of states results in a characteristic oscillation in the fluorescence decay rate\textsuperscript{25–28} that we monitored by fluorescence lifetime microscopy. The results were modeled using the single mirror model\textsuperscript{25} yielding the radiative and non-radiative decay rates of the emitting states, and by extension, a set of relevant photophysical parameters of the protein including quantum efficiency and emission oscillator strength. This approach has the clear advantage that it is based on measurements of emission properties reflecting purely the characteristics of the emission transition, rather than those of the absorption as is the case for other standard techniques to determine these parameters. In the present approach, no prior knowledge of absorption characteristics is required, representing a direct determination of the emission band properties.

We first studied the well-characterized laser dye Rhodamine 6G (Fig. 1a) to validate our sample preparation and measurement method and to measure the complete set of photophysical parameters of this important dye. We then demonstrated that this approach of controlling the local density of states is suitable for the analysis of photophysically complex systems like fluorescent proteins. We analyzed the emission from the widely-used fluorescent protein EGFP (Fig. 1b) to sensibly measure the decay rates, the quantum efficiency of the on-states, and the emission oscillator strength.

\section*{2. Methods}

To control the local density of states of the emitters, we chose to work in a single mirror configuration. The emitters are embedded in an isotropic medium and localized at a well-defined distance from the metallic mirror. To realize these conditions we used the sample configuration shown in Fig. 2a.

\subsection*{Mirror and spacer layer fabrication}

The mirrors used in this work were fabricated by multilayer e-gun deposition on a silicon wafer. First a layer of 50 nm of chromium was deposited to improve the adhesion of the silver layer that acts as the mirror. The thickness of the silver layer deposited over the chromium was 200 nm. Finally the SiO\textsubscript{2} layer that acts as spacer was deposited with a controlled thickness to obtain the selected distance between the emitters and the silver mirror. The whole deposition process was carried out in a Balzers BAK 600 e-gun evaporation machine. The multideposition can be done keeping the vacuum in the chamber between different material depositions while optimizing the conditions of the surfaces for the following deposition. This approach helps to improve the adherence, especially between the easy to oxidize silver layer and the SiO\textsubscript{2}.

The mirrors and spacer layers were characterized by scanning electron microscopy (SEM), imaging different positions of a cross section of each mirror-spacer. A typical cross section SEM image of the mirror and covering SiO\textsubscript{2} layer is shown in Fig. 2b. The thickness of the silver mirror and of the SiO\textsubscript{2} spacer layer was found to be homogeneous over the analyzed cross sections. The refractive index of the SiO\textsubscript{2} layer was measured with ellipsometry, giving a value of \( n = 1.46 \pm 0.05 \) for the different SiO\textsubscript{2} spacer thicknesses.

\subsection*{Sample film and cover}

To form a thin layer of the fluorescent molecules over the SiO\textsubscript{2} spacer, the emitting molecules (Rhodamine 6G from Lambda Physics; EGFP obtained as reported in ref. 6) were embedded in a polymer matrix to obtain a nearly isotropic orientation of the molecules in the layer. 1% PVA was selected for this purpose. A solution of PVA and the corresponding molecule at low concentrations to exclude dye–dye interactions was spin coated over the surface. From the original wafer 2 cm \times 2 cm samples were cut and 100 \( \mu \)l of the PVA solution was spin
coated onto the sample at 6000 rpm for 10 s. The thickness of the resulting PVA layer was characterized by atomic force microscopy spanning a region containing a scratch made on the layer with a Teflon tweezer, yielding a homogeneous layer of 17 ± 3 nm. A typical AFM image with the corresponding averaged height profile is presented in Fig. 2c. The distance between the emitters and the mirrors, used in the analysis, is defined by the nominal spacer thickness plus half the PVA layer thickness, which is held constant for all samples.

Finally a thick layer of PMMA ($n = 1.489$) was deposited onto the fluorescent layer to match the refractive index of the spacer and reproduce the conditions of a single mirror. A drop of ∼2% PMMA in toluene solution was deposited and dried over the sample to form a layer $> 1 \mu m$.

To determine the lifetime of the respective emitter in a homogeneous medium with the same refractive index of the spacer used in the mirror samples, we followed the same sample film and cover production procedure but used a quartz coverslip as substrate (replacing the mirror) which has a refractive index ($n = 1.46$).

Emission lifetime determination

The fluorescence decay curves were measured with a time correlated single photon counting (TCSPC) system. The sample was illuminated with a pulsed diode laser with a pulse duration of 50 ps (FWHM) and a repetition rate 20 MHz (BDL475, 469 nm, B&H, Germany). Epi illumination (40°, NA 0.6) was used, collecting the emission through the same objective. A 15 nm band pass emission filter centered at 556 nm was used for Rhodamine 6G and a 10 nm band pass emission filter centered at 510 nm was used for EGFP in the detection path to limit the detection wavelength to the peak of the emission spectrum. To eliminate any influence by scattered or back-reflected excitation light, an additional long pass filter (razor edge 473.0 nm, Semrock, USA) was inserted into the detection path. The emission is focused to the active area of a single photon avalanche diode (PDM series, MPD, Italy, peak quantum efficiency of ∼50% at 500 nm) connected to the TCSPC module (SPC-830, Becker & Hickl, Germany) to perform time resolved fluorescence measurements. Integration times per decay curve were chosen to yield a total of approximately 20 000 counts per decay curve to assure accurate determination of the characteristic lifetime ($\tau$) by the TCSPC card software (SPCimage, B&H, Germany).

3. Results and discussion

Rhodamine 6G

We first tested our approach by measuring a series of Rhodamine 6G samples. A typical fluorescence decay curve and fit for a Rhodamine 6G sample on a mirror with 150 nm emitter–mirror distance is presented in Fig. 3. The data obtained were fitted by a monoeponential decay function. The quality of the fit was judged by the $\chi^2$ parameter, where a $\chi^2 = 1$ is characteristic of an accurate fit. The residuals plotted at the bottom of Fig. 3 show that the fitting is good in the whole range of the decay.

Since small variations in the thickness of the layers defining the distance to the mirror, as well as variations in the fits of the data will give rise to changes in the recorded lifetimes, it is necessary to collect a statistically relevant number of decay curves for each sample. To achieve this, we collected five separate fluorescence lifetime images at different positions of each sample representing one emitter–mirror distance, thus
accounting for any possible inhomogeneities in the emitter–mirror distance within one sample. Each image contained 32 decay curves measured on an area of 16 \times 16 \text{m}, see Fig. 3b. The decay times extracted from the mono-exponential fitting of the 5120 constituent single pixel decay curves were collected in a single histogram that represents the measured lifetime distribution for each distance to the mirror, see Fig. 3c. From the distribution we can ascertain the most frequent lifetime as well as the width of the distribution as a measure of the quality of the lifetime determination for each dye-mirror distance.

Fig. 3c shows a narrow distribution of measured lifetimes that can be fitted by a Gaussian distribution. The width of the distribution is 0.087 ns FWHM, which is very small compared to the lifetime of the emitter. The small FWHM shows the good quality of our sample preparation and measurement. The most frequent lifetime of 3.33 ns is the peak of the Gaussian distribution for a Rhodamine 6G–mirror distance of 150 nm. This value is used to calculate the decay rate for the respective distance. For all the distances measured, the lifetime presents a similar Gaussian distribution, with a FWHM of 0.090 \pm 0.009 ns. The peak positions vary as expected with the emitter–mirror distance, corresponding to the expected modulation of the decay rate.

Using the same measurement procedure described above, we obtained an emission lifetime of 3.3 ns, corresponding to a decay rate of 0.30 ns\(^{-1}\), for Rhodamine 6G in a homogeneous medium of refractive index of \(n = 1.46\). This rate was then used as a normalization factor for the analysis. The normalized decay rates for Rhodamine 6G are plotted as a function of the distance to the mirror in Fig. 4a. The error bars on the abscissa (the distance to the mirror) are defined as the sum of the uncertainties in SiO\(_2\) and PVA layer thicknesses (\(\Delta z = \pm 6 \text{ nm}\)) while the error bars for the decay rate were calculated using the half-width of the lifetime (\(\Delta \tau = 0.045 \text{ ns}\)).

In Fig. 4a we present the normalized decay rate of Rhodamine 6G versus the distance to the mirror. The results are modelled by the single mirror configuration model. Two main effects...
are expected. First, the decay rates oscillate with the increase of the distance to the mirror, since the phase of the reflected field changes with this distance. Second, the oscillation amplitude decreases with increasing distance, since the radiation field of the dipole decreases with the distance between the emitter and its mirror image. Similar behaviour is predicted for different transition dipole orientations with respect to the mirror. Indeed a damped oscillation of the normalized decay rate as a function of the distance to the mirror is observed in Fig. 4a, consistent with the prediction by theory for a single mirror configuration model.

For our preparation method of embedding emitters in a polymer film we expect an isotropic distribution of the emission dipole orientations with respect to the mirror. Fig. 4 shows that for emitter-mirror distances ≥ 110 nm, the oscillation in measured data indeed follows the values predicted for isotropically distributed emitters (solid line). Fig. 4 also depicts the normalized decay rates calculated for the parallel (long dashed) and perpendicular (short dashed) orientations. The calculation was performed for the wavelength corresponding to the Rhodamine 6G emission peak (555 nm), the refractive indexes for silver at the corresponding wavelength (n_Ag(λ = 555 nm) = 0.129 + i3.25^{10}) and a quantum efficiency Q = 95% (value obtained from analysis below). For emitter–mirror distances less than 110 nm the measured decay rates are below the predicted values (grey data points in Fig. 4a), even considering a deviation from the isotropic distribution orientation and all transition moments being oriented parallel to the mirror. For these emitter–mirror distances, we repeated the experiment and reproduced the same decay rates. The observed deviation from the model was only found for Rhodamine6G, and not for the fluorescent protein EGFP (see below). While no explanation has been found for this discrepancy yet, these two data points were tentatively excluded for the following analysis.

The measured decay rate γ(w,z) is the sum of two contributions, the non-radiative decay rate γ_{nrad}(ω) and the radiative decay rate γ_{rad}(w,z). The radiative decay rate, according to Fermi’s golden rule, is proportional to the projected local density of optical states ρ(w,z) (LDOS) and depends explicitly on the distance to the mirror z, and the transition frequency ω. Therefore the total decay rate can be expressed as

\[ γ(ω, z) = γ_{nrad}(ω) + γ_{hom}(ω) \frac{ρ(ω, z)}{ρ_{hom}(ω)} \]  

where γ_{nrad}(ω) and ρ_{hom}(ω) are the decay rate and corresponding LDOS respectively in a homogeneous medium of the corresponding refractive index. The total decay rate is plotted as a function of the normalized LDOS in Fig. 4b. The error bars for the decay rate are based on an uncertainty in the lifetime of half-width of the lifetime distribution (Δτ = 0.045 ns) and for the normalized LDOS are based on the interval corresponding to the uncertainty Δz in the distance to the mirror.

A linear relation was found between the measured decay rate γ(w,z) for Rhodamine 6G and the calculated normalized LDOS as expected from Fermi’s golden rule. The data were fitted by a linear function (Fig. 4b), yielding for the non-radiative decay rate a value of γ_{nrad} = 0.015 ± 0.02 ns\(^{-1}\) and for the decay rate in a homogeneous medium γ_{hom} = 0.29 ± 0.02 ns\(^{-1}\). This results in a quantum efficiency of Q = 95% ± 5%. Although Rhodamine 6G was embedded in polyvinylalcohol in our study, the determined quantum efficiency can be compared with values determined for Rhodamine 6G in ethanol, since ethanol and polyvinylalcohol provide a chemically very similar environment to the emitter. Indeed our value and the values reported in the literature for the quantum efficiency of Rhodamine 6G in ethanol determined with different methods, ranging from 0.94 to 0.96, agree very well.

Inasmuch as our method only addresses emitting or “on” states of the fluorophore, the correspondence of our result for Q with the literature reports reflects the relatively non-complex photophysics of Rhodamine 6G. Although dark states have been observed for Rhodamine 6G, these states have been found to be linked to the triplet state and a dark state formed through the triplet state. Although the intersystem crossing rates to the triplet state were found to vary depending on the solvent used, the probability for inter-system crossing to the triplet state for Rhodamine 6G is generally low enough to exclude a noticeable buildup of molecules in the triplet state, and thus a formation of dark states, under the applied conditions.

From the homogeneous decay rate γ_{hom} and the emission oscillator strength f_{emiss} of the transition can be calculated

\[ f_{emiss}(ω) = \frac{2 m_e q_0 e^2}{q^2 \hbar^2 c^2} γ_{rad}(ω) \]  

where m and q are the electron mass and charge respectively, ε_0 is the vacuum permittivity, c is the speed of light and n is the refractive index of the surrounding media. Here we use the frequency corresponding to the peak of the emission (555 nm), in the center of the detection band to calculate the effective emission oscillator strength of the transition.

The effective emission oscillator strength for Rhodamine 6G is thus determined to be f_{emiss} = 0.92 ± 0.03. To the best of our knowledge, no value for the emission oscillator strength has been reported for Rhodamine 6G. The absorption oscillator strength has been determined before to be f_{abs} = 0.69,\(^{36}\)

Enhanced green fluorescent protein (EGFP)

We show the decay curves from the fluorescent protein EGFP for two sample–mirror distances in Fig. 5a. The data correspond to distances to the mirror of 170 nm and 60 nm. The decay of EGFP emission at 60 nm distance to the mirror is decidedly faster than the EGFP decay recorded for a EGFP–mirror distance of 170 nm. Both decay curves were well-fitted with a monoeponential function (τ = 2.26 ns, γ^2 = 1.02 for 170 nm and τ = 1.36 ns, γ^2 = 1.6 for 60 nm). Clearly, placing the EGFP molecules at different distances from the silver mirror induced a change in the emission lifetimes, as expected from theory. To accurately determine the characteristic lifetime of EGFP for each distance to the mirror, fluorescence lifetime imaging was used to determine the most frequent lifetime and the width of the lifetime distribution for each distance. The width of the
decay rates the quantum efficiency is calculated to be 72% in the literature on the modulation depth of the decay rate as influence of its moderate quantum efficiency of 60% reported sample–mirror distance. For EGFP we expect to see the most frequent lifetime observed, as a function of the FWHM and could be fitted by Gaussian functions.

The calculated normalized decay rate for the distance to the mirror a shorter lifetime is observed (\(\tau = 2.26\) ns, \(\chi^2 = 1.02\) for 170 nm and \(\tau = 1.36\) ns, \(\chi^2 = 1.6\) for 60 nm), as predicted by the theory. The grey circles correspond to the IRF of the system. (b) Normalized decay rate vs. the distance to the interface for EGFP. The measured values (circles) follow the predicted behaviour for \(Q = 72\%\) and isotropic dipole orientation (solid line), which is clearly different from the expected modulation for \(Q = 100\%\) (dashed line). (c) Total decay rate as a function of the normalized LDOS. The linear fit (solid line) yields values of the nonradiative decay rate \(\gamma_{\text{rad}} = 0.14 \pm 0.03\) ns\(^{-1}\) and the radiative decay rate \(\gamma_{\text{rad}} = 0.36 \pm 0.03\) ns\(^{-1}\). From these decay rates the quantum efficiency is calculated to be 72% ± 6%.

**Fig. 5** (a) Typical decay curve measured for EGFP for distances to the mirror of 60 nm (circles) and 170 nm (triangles). Curves are normalized to unity to clearly visualize the difference in lifetime. The black lines correspond to mono-exponential fits. At shorter distances to the mirror a shorter lifetime is observed (\(\tau = 2.26\) ns, \(\chi^2 = 1.02\) for 170 nm and \(\tau = 1.36\) ns, \(\chi^2 = 1.6\) for 60 nm), as predicted by the theory. The grey circles correspond to the IRF of the system. (b) Normalized decay rate vs. the distance to the interface for EGFP. The measured values (circles) follow the predicted behaviour for \(Q = 72\%\) and isotropic dipole orientation (solid line), which is clearly different from the expected modulation for \(Q = 100\%\) (dashed line). (c) Total decay rate as a function of the normalized LDOS. The linear fit (solid line) yields values of the nonradiative decay rate \(\gamma_{\text{rad}} = 0.14 \pm 0.03\) ns\(^{-1}\) and the radiative decay rate \(\gamma_{\text{rad}} = 0.36 \pm 0.03\) ns\(^{-1}\). From these decay rates the quantum efficiency is calculated to be 72% ± 6%.

Lifetime distributions was found to be narrow (0.08 ± 0.01 ns FWHM) and could be fitted by Gaussian functions.

In Fig. 5b we present the observed decay rate, derived from the most frequent lifetime observed, as a function of the sample–mirror distance. For EGFP we expect to see the influence of its moderate quantum efficiency of 60% reported in the literature on the modulation depth of the decay rate as a function of distance to the mirror. A reduced quantum efficiency results in a lower modulation depth of the decay rate. Indeed, we observe this behaviour. The normalized decay rates show less modulation than expected for unity quantum efficiency, but agree very well with calculations considering a quantum efficiency of 72% (derived from analysis below). The calculated normalized decay rate for unity quantum efficiency is also shown in Fig. 5b to illustrate the change in modulation depth, clearly noticeable for shorter distances. The calculations were performed using the wavelength corresponding to the EGFP emission peak (512 nm), and the refractive index for silver at this wavelength (\(n_{\text{Ag}(512\ \text{nm})} = 0.129 + i3.02\)).

The total decay rate is plotted as function of the normalized LDOS (Fig. 5c) and fitted with a linear function from which the non-radiative decay rate of \(\gamma_{\text{rad}} = 0.14 \pm 0.03\) ns\(^{-1}\) and the decay rate in a homogeneous medium \(\gamma_{\text{hom}} = 0.36 \pm 0.03\) ns\(^{-1}\) are derived. Using these values we calculate a quantum efficiency of \(Q = 72\% ± 6\%\) and effective emission oscillator strength (eqn (2)) of \(f_{\text{emiss}} = 0.97 ± 0.06\).

By modulating the local density of states and observing the change in emission lifetime, one obtains the photophysical properties characteristic of the emitting states of the fluorophore. Non-emitting states do not contribute to the observed emission lifetime and are thus not accounted for. Since conventional measurements are adversely affected by absorbing and non-emitting as well as non-absorbing states photoinduced during the measurement process, we expected to find an increased value for the on state quantum efficiency. Indeed the value for the quantum efficiency we obtain (72%) is markedly higher than the value of \(Q = 60\%\) reported before, consistent with reports that a significant fraction of green emitting proteins can reside in dark states even at very low excitation intensities. The reported values for the radiative and nonradiative decay rate for a green fluorescent protein mutant closely related to the EGFP analyzed in this study were smaller than the values we determine, which is a consequence of the lower quantum efficiency used to calculate the rates in this report. The effective emission oscillator strength of EGFP we determine is \(f = 0.97\). To the best of our knowledge no emission or absorbance oscillator strength has been experimentally determined for any fluorescent protein to date. The anionic chromophore of the green fluorescent protein, which is also the chromophore of EGFP studied here, has been analyzed by computational methods. These studies have shown that the HOMO–LUMO coupling has the largest absorbance oscillator strength and thus is the source of the absorption. The calculated strong HOMO–LUMO coupling agrees well with the effective emission oscillator strength that we determined.

4. Conclusions

We have presented for the first time the determination of fundamental photophysical parameters of the on states of a fluorescent protein by systematically changing the local density of optical states. The local density of states is changed by controlling the spacing between the emitters and a metallic mirror, which results in characteristic changes of the emission lifetime. Analyzing the change of emission lifetime as a function of the emitter distance to the mirror gives access to the decay rates, quantum efficiency and emission oscillator strength. Since only emitting states contribute to the emission lifetime, only the properties of the emitting states are determined, in contrast to other methods that average over...
emitting and non-emitting states. The method presented does not require measurements relative to a reference molecule. We show that changing the local density of optical states is an especially potent method to analyze complex photophysical systems like fluorescent proteins, a class of molecules which are known to exhibit significant residence times in intrinsic or photoinduced dark states. We validated our approach with the photophysically simple dye Rhodamine 6G, and recovered a quantum efficiency in excellent agreement with literature. In contrast, for the complex biological fluorescent protein EGFP, we determined a clearly higher value for the quantum efficiency of the emitting states exclusively ($Q = 72\%$) than reported values averaged over on- and off-states ($Q = 60\%$). The increased value agrees well with reports that a significant fraction of green emitting proteins can reside in dark states. Theoretical studies predict a strong HOMO–LUMO coupling, which agrees well with our measurement.

The consistency between our measurements and previously reported values from both experimental as well as theoretical determinations, serve to validate the method as a new and potentially very powerful manner to obtain fundamental insights into the complex fluorescent protein emission properties. We even envision the individual characterisation of different emitting forms coexisting in many fluorescent proteins. In this case, the decay characteristics are multieponential, and the influence of a modified local density of photonic states on each individual decay component can be analyzed.

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