Bulk fluorescence measurements cannot probe the survival-time distribution of single molecules

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Using a straightforward theoretical approach, we show that an ensemble of dyes with a simple first-order photobleaching kinetics yields a power-law decaying emission intensity in a heterogeneous excitation field in bulk fluorescence experiments. Our theoretical considerations are experimentally confirmed for two distinct classes of small organic fluorophores represented by the classical laser dye rhodamine-6G on glass in air and the cyanine dye DiI(C18) in a thin polymethylmethacrylate film. Our results provide evidence that the time course of bleaching in a bulk sample in general does not allow derivation of the properties of survival times of individual quantum systems.

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I. INTRODUCTION

The number of photons emitted before irreversible photobleaching is a key quantity of a fluorophore in single-molecule spectroscopy. Together with the (polarization-dependent) excitation rate it determines the distribution of survival times $p(\tau)$ and thus the photobleaching kinetics. In the presence of a homogeneous and static environment, e.g., at a polymer- or glass-air interface (as compared to biological material), general knowledge predicts that photobleaching of organic dyes is governed by a single rate-limiting transition from the excited state or the triplet state to the nonfluorescent photoproduct. From this simple photophysical argument it is expected that organic fluorophores exhibit a single-exponential intensity decay with an intensity- and polarization-dependent rate at room temperature [1–5]. In contrast, quantum dots with their multiscale blinking, induced by time-varying energy barriers between the excited exciton state and the ionized state, have been reported to display a broad power-law (Lévy-like) distribution that implies $p(\tau) \sim 1/\tau^{\beta}$ with $\beta \leqslant 2$ [6]. While this Lévy-like distribution can still be normalized, the first and all higher moments diverge (in contrast to an exponential distribution, where all moments are finite). This gives rise to an infinite mean lifetime $\langle \tau \rangle$ of the fluorophore. While being remarkable from a theoretical point of view, experimentally one often cannot benefit from this fact as the median of the survival times remains finite.

Assessing the distribution of survival times $p(\tau)$ on the single-molecule level is a demanding exercise as typically more than 10^3 measurements are needed to obtain a proper sampling of the (potentially Lévy-like) distribution. It is therefore tempting to utilize, for example, the temporal decay of bulk fluorescence due to photobleaching as an attractive and robust way to derive $p(\tau)$. Indeed, this approach has recently been applied to a variant of the green fluorescent protein (GFP): based on the power-law decaying bulk fluorescence in confocal microscopy, it has been argued that GFP exhibits a Lévy-like distribution $p(\tau)$ with an infinite mean survival time [7].

Based on simple theoretical considerations, we show here that any two- or three-level fluorophore with a single-exponential distribution of survival times $p(\tau)$, e.g., simple polyaromatic and cyanine dyes, can be expected to exhibit a power-law decaying fluorescence when measuring on bulk samples in a confocal geometry. We argue that inhomogeneous excitation and/or nonlinear bleaching effects are responsible for the intensity-dependent power-law photobleaching decay. We demonstrate also experimentally the generic occurrence of the predicted power-law decay of the bulk fluorescence for two representative classes of chromophores. From our results we conclude that bulk measurements are not suitable for determining the distribution of survival times $p(\tau)$.

II. THEORETICAL CONSIDERATIONS

Simple dyes can approximately be described as a two-level system with a ground state G and an excited state E (Fig. 1). Excitation from the ground state occurs with rate ω which in general depends on the illumination intensity I,

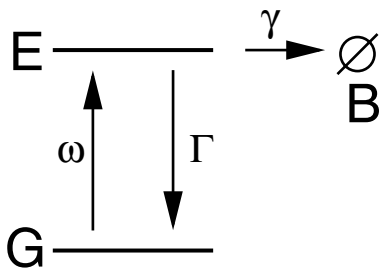

FIG. 1. Simplified Jablonski diagram for the bleaching of a fluorophore. Excitation from the ground state (G) occurs with rate ω which depends on the illumination intensity. From the excited state (E) relaxation to the ground state via spontanous emission of a photon is possible with rate Γ. Alternatively, a transition to the bleached state (B) may occur with rate γ which, in general, also depends on the illumination intensity.

while the spontaneous decay to the ground state via emission of a photon occurs with a fixed rate Γ. From the excited state, the dye can switch to an irreversibly bleached state B with rate γ that, in general, may again depend on the illumination intensity I due to involved energy levels that are only considered implicitly in this simplified two-level approach. A somewhat more sophisticated description of the dye photophysics is obtained by including an additional level T (triplet state) between E and G from which the transition to the bleached state occurs. This additional level T can also account for the (often intensity-dependent) nonradiative relaxation from the excited state to the ground state. For the sake of simplicity, we will neglect T as the temporal behavior of the population kinetics below [Eqs. (1)–(3)] is eventually only modified by a prefactor when including T due to the use of the stationary-state approximation [compare Eq. (4) with the corresponding expression in Ref. [8]].

The normalized population kinetics of the dye is described by

$$\frac{dG}{dt} = \Gamma E - \omega G, \tag{1}$$

$$\frac{dE}{dt} = \omega G - (\Gamma + \gamma)E, \tag{2}$$

$$\frac{dB}{dt} = \gamma E, \tag{3}$$

where the conservation of particles is ensured by demanding $G+E+B=1$. As the spontaneous emission is typically much faster than bleaching ($\Gamma \gg \gamma$), one can use the stationary-state approximation ($dG/dT=0$) which yields $G=E\Gamma/\omega$ and, via the conservation of particles, $E=(1-B)\omega/(\omega+\Gamma)$. Inserting into Eq. (3) finally yields

$$B = 1 - e^{-\lambda \gamma t}, \quad \lambda = \frac{\omega}{\omega + \Gamma}, \tag{4}$$

which implies a population $E=\lambda \exp(-\lambda \gamma t)$ in the excited state. Assuming a homogeneous distribution of dyes and neglecting prefactors that account for detector efficiencies, etc., the total fluorescence recorded from a sample volume Ω at each time t is thus given by

$$F(t) = \int_{\Omega} E\, d^3r = \int_{\Omega} \lambda e^{-\lambda \gamma t} d^3r. \tag{5}$$

For the remainder we will assume that the excitation rate is proportional to the illumination intensity $I(\mathbf{r})$, i.e., $\omega = \omega_0 I(\mathbf{r})$. Clearly, this assumption will fail if the finite residence time $\sim 1/\Gamma$ in the excited state limits the excitability of the dye ("photon antibunching"). In this case, the excitation rate ω has to approach a constant value. We have checked, however, that including this saturation of the excitation rate does not alter the results below. We will therefore use the simple proportionality of ω and $I(\mathbf{r})$. For the bleaching process we will assume that $\gamma = \gamma_0 I(\mathbf{r})^{\mu}$. The empirical free parameter $\mu \geqslant 0$ is introduced to account for nonlinear bleaching and polarization effects as well as the implicit influence of levels that participate in the bleaching reaction but are not considered explicitly in our two-level model.

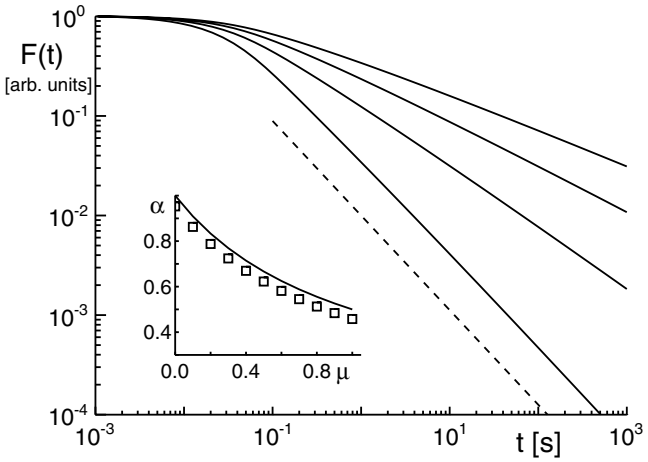

FIG. 2. Equation (5) yields a power-law decay $F(t) \sim 1/t^{\alpha}$ for the fluorescence. The exponent α is near to unity for $\mu=0$ (highlighted by the dashed line) and decreases for increasing values of μ. Inset: the exponent α is approximately given by $\alpha=1/(1+\mu)$ (full line).

When using confocal optics, the light intensity $I(\mathbf{r})$ can be approximated by a three-dimensional Gaussian with typical beam waists r_0 and Sr_0 perpendicular to and along the optical axis, respectively. For simplicity, we assume that the illumination profile is radially symmetric, i.e., $S=1$ and $I(\mathbf{r})=I(r)=I_0 \exp(-r^2/r_0^2)$. We would like to emphasize that our results are not altered when using $S>1$; even assuming only a homogeneous two-dimensional distribution of dyes perpendicular to the optical axis (i.e., neglecting all contributions along the z direction) does not alter the results below.

Solving Eq. (5) numerically in polar coordinates is straightforward and yields a power-law decay $F(t) \sim 1/t^{\alpha}$ for any $\mu \geqslant 0$. The decay exponent is approximately given by $\alpha \approx 1/(1+\mu)$ (Fig. 2). Even in the most naive picture of a constant bleaching rate γ (i.e., $\mu=0$) a power-law decay is observed. The latter result is in fact the same as found by Berglund [9] in a similar, more formal derivation. Following the line of argumentation put forward in Ref. [7], all bulk fluorescence curves with $\alpha \leqslant 1$, i.e., $\mu \geqslant 0$, would imply an infinite average lifetime ($\langle \tau \rangle \to \infty$) of the individual dye molecules despite the fact that our model assumes a simple Poisson process with rate γ for the bleaching process of a single molecule. We would like to note that this behavior is also observed when the stochastic analog to Eq. (5) is considered.

Based on the above theoretical considerations, we predict a generic power-law decay of the bulk fluorescence in confocal microscopy with an illumination-dependent exponent while the distribution of survival times on the single-molecule level is given by a single exponential. We would like to note that the above considerations are valid only for immobilized dyes, while diffusion qualitatively alters the bleaching process since individual fluorophores are subject to a time-varying intensity. In fact, when the dye molecules are subject to diffusion, the bulk fluorescence decay is well described by a single exponential [10].

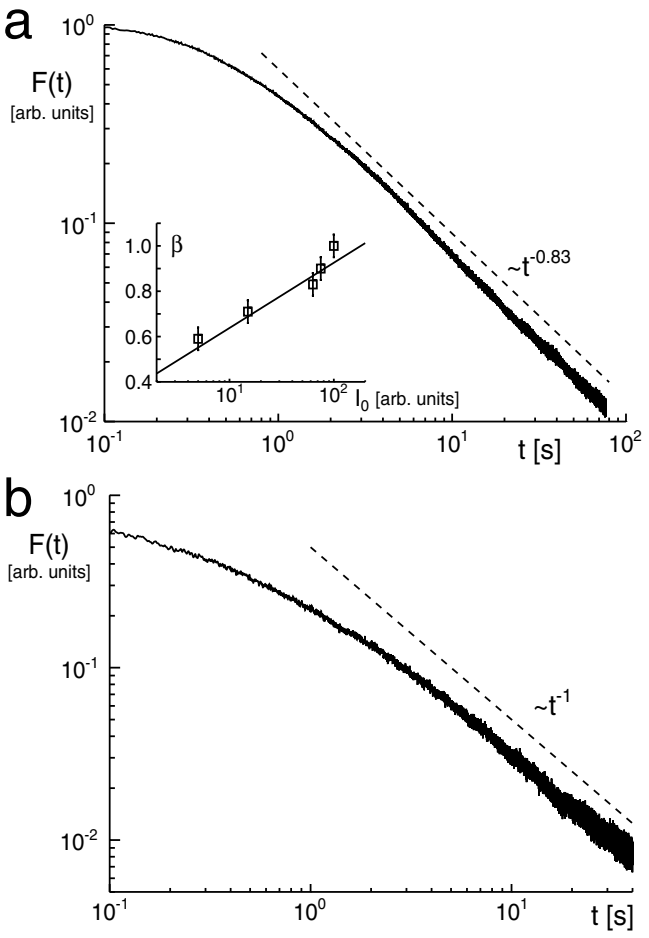

FIG. 3. (a) Representative power-law decay $F(t)\sim 1/t^{\alpha}$ ($\alpha \approx 0.83$) of the bulk fluorescence for immobilized DiI. Inset: The exponent α increases logarithmically with the applied laser intensity I_0 (highlighted by the full line). (b) Representative power-law decay $F(t)\sim 1/t^{\alpha}$ ($\alpha \approx 1$) of the bulk fluorescence for immobilized Rh6G.

III. EXPERIMENTAL RESULTS

To test whether the above predictions are correct, we used a commercial confocal microscope setup, similar to the experimental approach given in [7]. We first monitored the bulk fluorescence decay of the cyanine dye DiI that had been immobilized in a polymethylmethacrylat (PMMA) matrix for several illumination intensities I_0. In fact, DiI embedded in PMMA is a well-characterized guest-host system for single-molecule spectroscopy and has been extensively studied without any suggestion of a nonexponential decay in $P(\tau)$ [1–3,5].

In accordance with our above considerations, we observed a power-law decay $F(t)\sim 1/t^{\alpha}$ for the bulk fluorescence at various laser powers [Fig. 3(a)]. Interestingly, and in agreement with reports on GFP [7], the exponent α showed a logarithmic dependence on the illumination intensity I_0 [Fig. 3(a), inset]. Thus, the confocal geometry imposes a decay of the bulk fluorescence of DiI that appears fundamentally different from its putative single-molecule behavior.

To ensure that the emergence of a power-law decay $F(t)\sim 1/t^{\alpha}$ was not a particular feature of DiI, we also used rhodamine-6G, a well-characterized probe for the bleaching time course [4], as a dried film on glass in air. Again, we observed a power-law decay in the bulk fluorescence [Fig. 3(b)]. Interestingly, and in contrast to DiI, however, rhodamine 6G did not show a variation of the exponent α, indicating that the bleaching process occurs with a fixed rate $\gamma=\text{const}$ (i.e., $\mu=0$). In other words, rhodamine 6G shows an even simpler bleaching mechanism than DiI.

Taken together, our experimental data give support to the theoretical predictions above and highlight the fact that a power-law decaying bulk fluorescence has to be expected even in the case of a dye with a single-exponential distribution of survival times.

IV. DISCUSSION

Recently, a Lévy-like distribution $p(\tau)$ with an infinte mean liftetime $\langle\tau\rangle$ has been postulated for a variant of GFP [7]. In fact, GFP is the most commonly used fluorophore in cell biology as virtually any protein can be expressed in living cells with this fluorescent tag [11]; hence the dynamics of the protein of interest can be studied *in vivo*. The claim of an infinite lifetime for GFP was derived theoretically from the observation that the bulk fluorescence of immobilized GFP molecules showed a power-law decay $F(t)\sim 1/t^{\alpha}$, when monitoring the time course of bleaching with confocal optics. This decay was shown to arise naturally if thermally activated barriers for individual dye molecules are assumed, which eventually leads to a Lévy-like statistics of survival times $p(\tau)\sim 1/\tau^{\beta}$ ($\beta=\alpha+1$) for single molecules. The experimentally found power law in $F(t)$ was thus explained in terms of this dynamic mechanism, i.e., the bulk fluorescence decay was taken as a unique footprint of a Levy distribution $p(\tau)$ on the single-molecule level. It is noteworthy that the experimentally observed exponents $\alpha \leqslant 1$ depended on the illumination intensity (consistent with our observations for DiI, Fig. 3), a feature that was not included and could not be explained within the theory of fluctuating barriers.

Based on our experimental results and the heuristic theoretical consideration we argue that a power-law decaying bulk fluorescence observed with confocal optics cannot be taken as a unique footprint of a Lévy-like distribution of survival times of single molecules. In better words, while a Lévy-like $p(\tau)$ *can* generate a power-law decay in the fluorescence, the inverse conclusion cannot be drawn without further knowledge. We propose that the observed power law is a generic consequence of the inhomogeneous excitation and/or nonlinear bleaching effects in the bulk and may only reflect the Lévy-like distributions of single molecules in rare cases.

Starting from the simple model presented here, more elaborate Jablonski diagrams can be constructed to further improve the description of real experiments. For example, the excited state E from which a fluorescence photon may be emitted is typically reached from the ground state G by exciting an energy level E^* that lies somewhat higher than E which is followed by nonradiative relaxation $E^*\rightarrow E$ via coupling to vibrational modes. This radiationless transition may

be modeled by a distribution of delay times that leads to a renormalization of the rate ω.

While the emergence of a power law in Eq. (5) is due to the confocal geometry, i.e., an inhomogeneous illumination, we would like to note that true nonexponential bleaching kinetics of single molecules and of an ensemble of fluorophores bound to a protein have also been observed experimentally in wide field microscopy [8]. In this case, the observed strong deviations from an exponential behavior arose due to an inhomogeneous distribution of the dipole moments of the immobilized dyes and an imposed polarization-dependent excitation. Thus, these effects may add on the results that we have shown here for inhomogeneous illumination and further stabilize and/or modify the observed power law.

In conclusion, we have presented simple theoretical arguments for the appearance of a power-law-like fluorescence intensity decay in dye molecules with a simple first-order bleaching kinetics due to a spatially heterogeneous excitation in a confocal microscope. Experimental results on two widely used members of common classes of chromophores support our considerations. Taken together, this work confirms the notion that only single-molecule experiments are able to distinguish static heterogeneous ensembles from dynamic ensembles and reveal their true characteristics.

ACKNOWLEDGMENT

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