THE JOURNAL OF PHYSICAL CHEMISTRY Letters

Letter

pubs.acs.org/JPCL

Photon Antibunching in Complex Intermolecular Fluorescence Quenching Kinetics

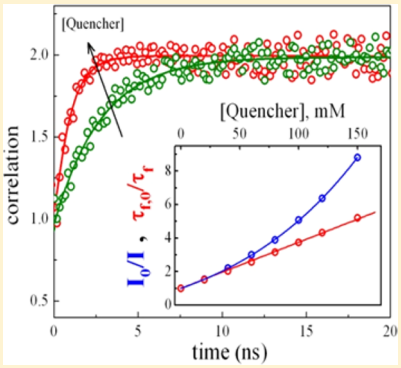
Arjun Sharma,[†] Jörg Enderlein,[‡] and Manoj Kumbhakar*[,†]

[†]Radiation & Photochemistry Division, Bhabha Atomic Research Center, Mumbai 400085, India

[‡]III Institute of Physics, Georg August University, 37077 Göttingen, Germany

Supporting Information

ABSTRACT: We present a novel fluorescence spectroscopic method, which combines fluorescence antibunching, time-correlated single-photon counting (TCSPC), and steady-state emission spectroscopy, to study chemical reactions at the single molecule level. We exemplify our method on investigating intermolecular fluorescence quenching of Rhodamine110 by aniline. We demonstrate that the combination of measurements of fluorescence antibunching, fluorescence lifetime, and fluorescence steady state intensity, captures the full picture of the complex quenching kinetics, which involves static and dynamics quenching, and which cannot be seen by steady-state or lifetime measurements alone.

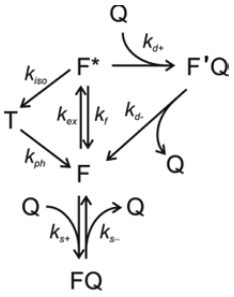


Fluorescence antibunching is observed in fluorescence correlation spectroscopy (FCS) curves when the correlation time becomes equal or smaller than the excited state decay time of the studied fluorescent emitter. It is a purely quantum-optical phenomenon and reflects the fact that a single fluorescent molecule cannot emit more than one photon per excitation cycle, which reduces the chance to observe two consecutive photons from one and the same molecule at very short correlation times. This leads to a typical antibunching dip in the fluorescence correlation curve. For a simple two-state system (ground and excited state), the correlation curve at very short lag times follows an inverse exponential law, $1 - \exp(-t/\tau_{ab})$, where the antibunching relaxation rate $1/\tau_{ab}$ is given by

$$\tau_{ab}^{-1} = k_e + k_f \quad (1)$$

Here, k_e is the excitation rate from the ground to the excited state, and k_f is the decay rate from the excited to the ground state. Thus, antibunching experiments provide direct access to the de-excitation rate $1/k_f$, which would be the fluorescence lifetime for an unperturbed fluorescent molecule. However, it includes the excitation rate k_e, which is equal to the excitation power P times the absorption cross section σ_{abs} of the studied molecule. However, for more complex systems such as the reaction scheme shown in Chart 1, which involves static and dynamic quenching of a fluorescent dye by a quencher moiety Q (and additional triplet state photophysics), the antibunching part of an FCS curve becomes much more complex, but also contains much more information than conventional ensemble measurements (cf. Supporting Information). In the present paper, we study the dynamic and static quenching kinetics of the fluorescent dye Rhodamine 110 by the quencher aniline. The assumed fluorescence and quenching kinetics scheme is shown in Chart 1, which is based on the ensemble spectroscopy measurements.

Chart 1. Schematic of Fluorescence and Reaction Scheme



A fluorescent molecule is excited, with rate k_{ex}, from its singlet ground state (F) to its first excited singlet state (F*). From there, it can either relax to the ground state (with rate k_f), switch into its triplet state T (with intersystem crossing rate k_{iso}), or associated with a quencher molecule to form an encounter complex F′Q (dynamics quenching, rate constant k_d). The encounter complex F′Q dissociates, with rate k_{d-}, into F and Q. Alternatively, the fluorophore can form with the quencher a nonfluorescent complex FQ (static quenching) while it is in the ground state (rate constant k_{s+}), which then dissociates back into F and Q with rate k_{s-}. Finally, the relaxation from the triplet state to the ground state is described by the phosphorescence rate k_{ph}. In the above scheme, F′Q and

Received: July 4, 2016
Accepted: July 28, 2016
Published: July 28, 2016

DOI: 10.1021/acs.jpclett.6b01467
J. Phys. Chem. Lett. 2016, 7, 3137−3141

FQ are different, as the former is formed via dynamic quenching by collision (i.e., encounter complex formation), while the latter is formed via static quenching by ground state nonfluorescent complex formation and the presence of two different quenching interactions is clearly indicated from Stern−Volmer analysis of ensemble results, as discussed later.

Conventional studies of fluorescence quenching use conventional time-resolved (TR) and/or steady-state (SS) ensemble spectroscopy and Stern−Volmer (SV) analysis,[1,2] but these measurements cannot disentangle all the described mechanism and quantify all the involved reaction rates and rate constants. Moreover, for slow reactant diffusion, as commonly observed in high-viscous media like organized assemblies, ionic liquids, or cellular environments, a simple SV analysis for extracting precise values of the quenching rate k_q and its relation with reaction free energy ($-\Delta G^0$) often leads to erroneous results.[3] Here, we will add FCS and, in particular, fluorescence antibunching for fully elucidating the complex reaction scheme. It should be noted that fluorescence quenching of organic dyes with tryptophan has been studied earlier with conventional FCS measurements,[4] although a reliable fit of the experimental data has been difficult with slow complexation kinetics, which overlaps in time with intersystem crossing and triplet state decay kinetics.

The reaction scheme of Chart 1 involves 5 states: F, F*, T, F′Q, and FQ. The corresponding reaction rate equations read, in matrix notation,

$$\frac{\mathrm{d}}{\mathrm{d}t}\begin{pmatrix} \mathrm{F} \\ \mathrm{F}^* \\ \mathrm{T} \\ \mathrm{F'Q} \\ \mathrm{FQ} \end{pmatrix} = \hat{M}(q)\cdot\begin{pmatrix} \mathrm{F} \\ \mathrm{F}^* \\ \mathrm{T} \\ \mathrm{F'Q} \\ \mathrm{FQ} \end{pmatrix} \tag{2}$$

where $\hat{\mathbf{M}}$ is the reaction rate matrix:

$$\hat{\mathbf{M}}(q) = \begin{pmatrix} -k_{ex} - k_{s+}q & k_f & k_{ph} & k_{d-} & k_{s-} \\ k_{ex} & -k_f - k_{iso} - k_{d+}q & 0 & 0 & 0 \\ 0 & k_{iso} & -k_{ph} & 0 & 0 \\ 0 & k_{d+}q & 0 & -k_{d-} & 0 \\ k_{s+}q & 0 & 0 & 0 & -k_{s-} \end{pmatrix} \tag{3}$$

which is a function of quencher concentration q. With this reaction scheme, we can obtain all measurable quantities of interest. First, the fluorescence decay follows a simple monoexponential behavior with fluorescence decay time

$$\tau(q) = (k_f + k_{iso} + k_{d+}q)^{-1} \tag{4}$$

Second, from solving the steady sate equation by setting the left-hand side in eq 2 to zero, we find that the inverse of the steady state intensity $I(q)$ is a second order polynomial of q,[5]

$$\frac{I_0}{I(q)} = 1 + aq + bq^2 \tag{5}$$

with coefficients

$$a = \frac{k_{ph}[k_{d+}(k_{ex} + k_{d-}) + k_{d-}(k_{s+}/k_{s-})(k_f + k_{iso})]}{k_{d-}[k_{ex}k_{iso} + (k_{ex} + k_f + k_{iso})k_{ph}]} \tag{6}$$

and

$$b = \frac{k_{s+}}{k_{s-}}\cdot\frac{k_{ph}k_{d+}}{k_{ex}k_{iso} + k_{ph}(k_{ex} + k_f + k_{iso})} \tag{7}$$

This allows us to express the unknown reaction rate constants k_{d-} and k_{s-} through the other constants and the coefficient values a and b as,

$$k_{d-} = \frac{k_{d+}^2 k_{ex} k_{ph}}{[ak_{d+} - b(k_f + k_{iso})][k_{ex}k_{iso} + (k_{ex} + k_f + k_{iso})k_{ph}] - k_{d+}^2 k_{ph}} \tag{8}$$

and

$$k_{s-} = \frac{k_{d+}k_{ph}k_{s+}}{b[k_{ex}k_{iso} + k_{ph}(k_{ex} + k_f + k_{iso})]} \tag{9}$$

Finally, the short lag-time part $g_{ab}(t|q)$ of the FCS curve (the antibunching-dominated part, where the impact of diffusion is still negligible) is given by the expression

$$g_{ab}(t|q) \propto \begin{pmatrix} 0 \\ 1 \\ 0 \\ 0 \\ 0 \end{pmatrix}^{\mathrm{T}} \cdot\exp[t\hat{M}(q)]\cdot\begin{pmatrix} 1 \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix} \tag{10}$$

which describes the probability to find the molecule back in the excited state at time t when it just relaxed back to the ground state at time zero. Here, the superscript T on the column vector indicates matrix transposition, and the exponent is understood as a matrix exponentiation. This expression for $g_{ab}(t|q)$ cannot be further simplified and has to be computed numerically.

Our experimental setup for photon antibunching experiments is based on an epi-fluorescence confocal microscope with CW argon ion laser excitation at 488 nm wavelength (LSM 710, Carl Zeiss GmbH). Collected fluorescence photons are split, with a polarizing beam splitter, toward two Hybrid PMT detectors (HPM-100-40, Becker & Hickl GmbH). The detected photons are registered with high temporal resolution (165 ps), from which second-order correlation functions ($G_{ab}(\tau)$) are generated using a DPC-230 correlator card (Becker & Hickl GmbH).

Measurements were performed on aqueous solutions of Rhodamine-110, Rh-110 (F), a common probe for single molecule spectroscopy and imaging. We recorded a series of FCS curves for varying excitation power, from 5 kW/cm^2 to 275 kW/cm^2. In the absence of any quencher, the antibunching part of the FCS curve, as shown in Figure 1, is described by a simple exponential law, $1 - \exp(-t/\tau_{ab})$, where the inverse antibunching relaxation time, $1/\tau_{ab}$, is given by the sum of the excited state lifetime τ (=$1/k_f$) and the absorption cross section σ_{abs} times the excitation power P. A linear fit of the antibunching relaxation rate $1/\tau_{ab}$ as a function of excitation intensity (excluding the triplet-state induced saturation region at moderately high excitation powers) yields a k_f value of 2.49 ± 0.06 × 10^8 s^{-1}, which corresponds to a lifetime value of τ = 4.0 ns. This lifetime value matches perfectly to the fluorescence decay time of Rh-110 as measured with time-correlated single-photon counting (TCSPC) (4.0 ± 0.1 ns), as well as other values reported in the literature. The slope of the linear fit corresponds to an absorption cross section of σ_{abs} = 2.5 ± 0.2 × 10^{-16} cm^2, which is also very similar to the value of 2.6 × 10^{-16} cm^2 reported by Ringemann et al.[6]

DOI: 10.1021/acs.jpclett.6b01467
J. Phys. Chem. Lett. 2016, 7, 3137−3141

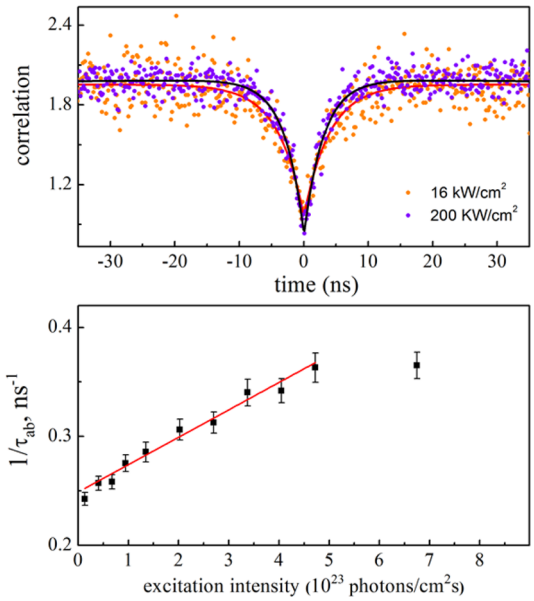


Figure 1. Nanosecond correlation at different excitation powers (top). Plot of relaxation rate as a function of excitation intensity (bottom).

This result demonstrates the capability of fluorescence antibunching measurements for measuring excitation and de-excitation times on a nanosecond time-scale with high accuracy. Next, we studied the quenching behavior of Rh-110 fluorescence in the presence of the quencher aniline (Q) in water.[7] For this purpose, we measured the steady-state intensity, and recorded TCSPC fluorescence lifetime curves and antibunching curves, $g_{ab}(\tau|q)$, at various concentration values q of aniline. To avoid any nonlinearity effects connected with triplet-state (or higher excited state) pumping, all quenching experiments were performed with moderate excitation powers below 200 kW/cm^2, which is the intensity range where we observed a linear dependence between $1/\tau_{ab}$ and excitation intensity (see Figure 1). For this excitation intensity, we used standard FCS for determining values for the intersystem crossing rate k_{iso} and the triplet state de-excitation rate k_{ph}, which occurred to be $k_{iso} = 8.9 \times 10^5$ s^{-1} and $k_{ph} = 2.1 \times 10^5$ s^{-1},[6] respectively. These values were then used for all subsequent data analysis.

The recorded TCSPC curves could be perfectly fit with monoexponential decay curves (see Figure 2). This indicates that there is no reverse rate from the F′Q state to F* + Q, which would instantly lead to a multiexponential decay behavior of the TCSPC curves. Furthermore, the inverse of the fitted fluorescence decay times, τ_0/τ, shows a perfectly linear dependence on quencher concentration q, as expected from eq 4. Fitting this curve with a linear fit (see Figure 4) yields a value for the rate constant of excited-dye/quencher complex formation as $k_{d+} = 5.98 \times 10^9$ M^{-1} s^{-1}.

Next, we recorded steady-state absorption spectra and fluorescence intensities as a function of quencher concentration. The steady-state absorption spectra of Rh-110 show merely ~1 nm bathochromic shift in the presence of a very high aniline concentration of 150 mM, indicating a very weak ground state complex formation (see Figure 3). The SS fluorescence intensity gradually decreases with increasing quencher concentration, without any change in the spectral shape, indicating fluorescence quenching without exciplex formation. As expected from eq 5, the inverse of the recorded

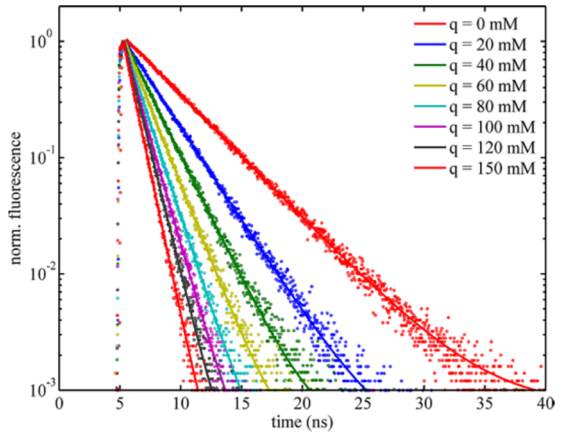


Figure 2. Measured TCSPC curves (open circles) at various quencher concentrations, together with monoexponential fit curves (solid lines).

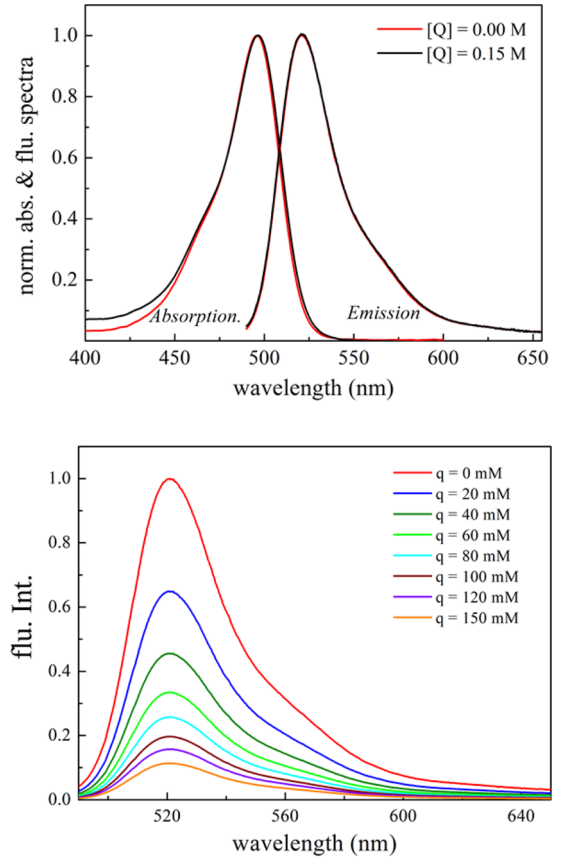


Figure 3. SS absorption spectra of Rh-110 for zero and for 150 mM quencher concentration (top). Fluorescence emission spectra of Rh-110 at different quencher concentrations (bottom).

steady state fluorescence, $I_0/I(q)$, as a function of quencher concentration can be perfectly fitted with a quadratic polynomial in q, see Figure 4, which fixes the values of the constants a and b. Knowing these values, the rate constants k_{d-} and k_{s-} can be calculated if one knows all the other rate constants, see eqs 8 and 9. However, the value of k_{ex} and k_{s+} are still unknown, and they cannot be found from TCSPC and steady state intensity measurements alone.

However, at zero quencher concentration, the fluorescence antibunching relaxation follows a simple monoexponential behavior (see Figure 5), and the relaxation rate is equal to k_{ex} +

DOI: 10.1021/acs.jpclett.6b01467
J. Phys. Chem. Lett. 2016, 7, 3137−3141

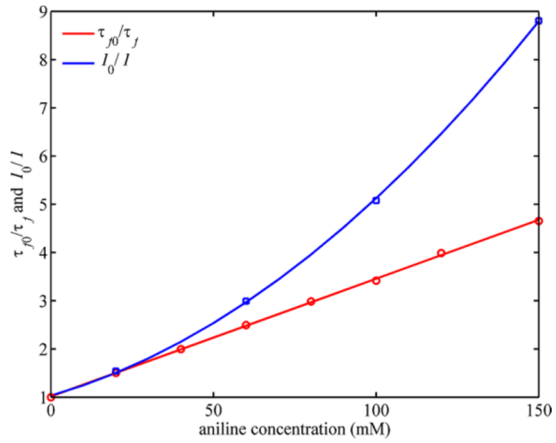


Figure 4. Dependence of the inverse fluorescence decay time, τ_{f0}/τ_f (red circle), and inverse of the steady-state fluorescence intensity, I_0/I (blue squares), as a function of quencher concentration q. The inverse lifetime curve is fitted by a linear fit (red line), and the inverse intensity curve is fitted with a quadratic polynomial (blue line).

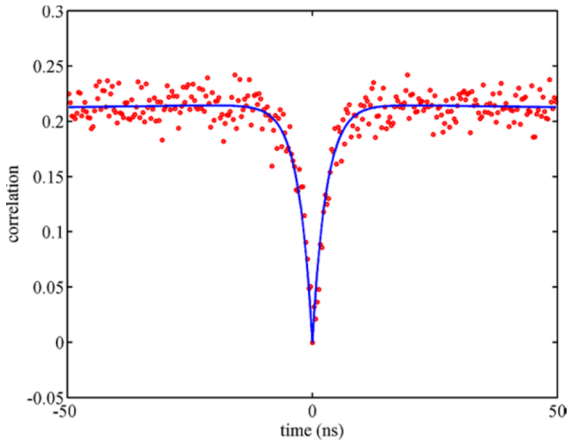


Figure 5. Fluorescence antibunching curve of Rh-110 at zero quencher concentration (red circles). The blue line represents a fit with a monoexponential relaxation function of the form $1-\exp[-(k_{ex} + k_f + k_{iso})t]$.

$k_f + k_{iso}$. Thus, already knowing k_f and k_{iso}, we could determine excitation rate for our excitation conditions as $k_{ex} = 3.34 \times 10^8$ s^{-1}. For all subsequent antibunching measurements, we used identical excitation conditions, so that this excitation rate was the same for all measurements.

Next, we recorded antibunching curves of Rh-110 for increasing aniline concentrations (see Figure 6). These curves were globally fitted with the model, eq 10, having only the value of k_{s+}, which is the only left unknown rate constant, as the free fit parameter. The model can globally fit all antibunching curves very well (see Figure 6). However, it occurs that the static quenching kinetics is by orders of magnitude smaller than the dynamic quenching kinetics, and repeating the fitting yields widely varying values for k_{s+} smaller than $\sim 5 \times 10^5$ M^{-1} s^{-1}. For such small rate constants, the fit quality of the antibunching curves depends only on the ratio of k_{s+}/k_{s-}, which is found to be equal to 29.4 M^{-1}, and the value of k_{d-}, which is found to be $k_{d-} = 2.62 \times 10^8$ s^{-1}.

Thus, we find a very slow static quenching kinetics, which is by 5 orders of magnitude slower than the dynamic quenching kinetics. However, the equilibrium constants are very similar,

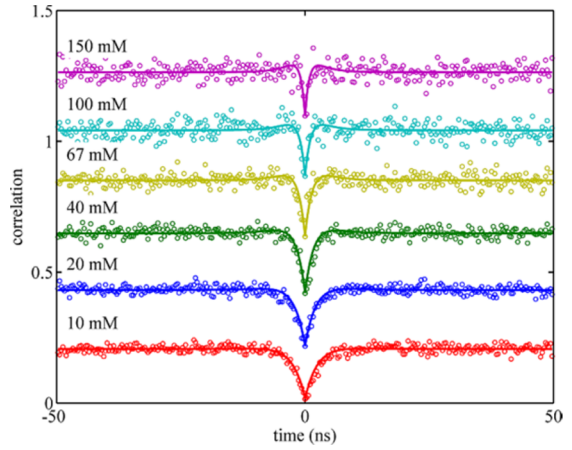


Figure 6. Measured antibunching curves (circles) at increasing quencher concentration (as indicated left above each curve). For better visibility, subsequent antibunching curves are shifted by a value of 0.2 vertically. The solid lines show a global fit of all curves with the model given by eq 10. The apparently damped oscillatory behavior visible at higher quencher concentrations is the direct result of the complex multistate kinetics shown in Chart 1 and described by eq 2. It should be emphasized that the presented analysis, represented by eq 2, is rigorous and does not involve any approximations.

$k_{d+}/k_{d-} = 22.8$ M^{-1} and $k_{s+}/k_{s-} = 29.4$ M^{-1}. This explains why we see a strongly nonlinear dependence of I_0/I in the steady-state intensity measurements. Antibunching, in principle, can precisely determine the k_{s+} and k_{s-} rates individually, but the slow complexation kinetics of the Rh110/aniline system does not show up on the nanosecond temporal window of antibunching.

It should be emphasized that only the combination of steady-state fluorescence, TCSPC measurements, and antibunching FCS measurements allowed us to determine all the essential rates and rate constants that describe the dynamics and static quenching of Rhodamine-110 by aniline, a task that would have been impossible without this combination. Static quenching escapes detection by TCSPC, so that time-resolved Stern–Volmer plots reflect only dynamic quenching.[1,2] Our results demonstrate that photon antibunching is a promising and powerful tool for studying the excited state dynamics of complex systems at the molecular level, and that it is capable of determining the total ensemble of rates and rate constants, in contrast to TCSPC and/or steady-state measurements alone (Figure S4 and S5). Our method can also be employed to study reaction rates in viscous media (e.g., organized assemblies, ionic liquids, cellular environment, etc.) where the conventional TCSPC-SV approach for extracting photoinduced reaction rates becomes questionable.

The first prediction of photon antibunching in fluorescence was made by Ehrenberg and Rigler[8] in their treatment of rotational diffusion in fluorescence correlation spectroscopy (FCS) and was experimentally measured by Kask et al.[9] for fluorescent dye molecules in water way back in 1980s. Ambrose et al.[10] first demonstrated fluorescence antibunching on silica surface. Over the years, photon antibunching has been employed to explore stoichiometry of aggregates and complexes,[11−15] resolving subpopulations in heterogeneous ensembles, investigate photophysics of dyes[16] and semiconductor nanocrystals,[17,18] investigation of ground-state proton transfer within the photocycle of a photoacid[19,20] and even subdiffraction limited quantum imaging.[21] Yet, the

DOI: 10.1021/acs.jpclett.6b01467
J. Phys. Chem. Lett. 2016, 7, 3137−3141

impending competence of photon antibunching to investigate excited state chemical reactions; more specifically intermolecular fluorescence quenching, has not been endeavored before. Present results highlight the possibility of exploring complex quenching kinetics in chemical and biological sciences at the molecular level.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.6b01467.

Experimental methods and supplementary figures showing the differences in quenching information obtained from antibunching and conventional means (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: manojk@barc.gov.in.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported by DAE, India. A.S. acknowledges DAE-BARC for his Ph.D. fellowship. Encouragement and support from Dr. D. K. Palit, Head, RPC Division, BARC & Dr. H. Pal, Head, MP Section, RPCD, BARC, are gratefully acknowledged.

■ REFERENCES

(1) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Spinger: New York, 2006.

(2) Valeur, B. *Molecular Fluorescence: Principles and Applications*; Wiley-VCH Verlag GmbH: Weinheim, Germany, 2001.

(3) Kumbhakar, M.; Manna, A.; Sayed, M.; Kumar, A.; Pal, H. Observation of the Marcus inverted region for bimolecular photo-induced electron ransfer reactions in viscous media. *J. Phys. Chem. B* **2014**, *118*, 10704−10715.

(4) Doose, S.; Neuweiler, H.; Sauer, M. A close look at fluorescence quenching of organic dyes by tryptophan. *ChemPhysChem* **2005**, *6*, 2277−2285.

(5) This is an exact equation derived from the general kinetic scheme of eq 2 and is used to estimate complex formation constants, similar to conventional ensemble Stern-Volmer analysis.

(6) Ringemann, C.; Schonle, A.; Giske, A.; von Middendorff, C.; Hell, S. W.; Eggeling, C. Enhancing fluorescence brightness: Effect of reverse intersystem crossing studied by fluorescence fluctuation spectroscopy. *ChemPhysChem* **2008**, *9*, 612−624.

(7) In analogy with the fluorescence quenching reported for several rhodamine derivatives with various amines, we recognize photo-induced electron transfer (PET) as the quecheing mechanism. The free energy for PET reaction of Rh-110 and aniline is −1.21 eV. Fluorescence quenching by energy transfer from excited Rh-110 to aniline is energetically unfavorable. Aniline is soluble in water up to 36 g/L at 25 °C (CAS Data Base), and in the present study we used a maximum aniline concentration of 14 g/L (= 150 mM).

(8) Ehrenberg, M.; Rigler, R. Rotational Brownian motion and fluorescence intensity fluctuations. *Chem. Phys.* **1974**, *4*, 390−401.

(9) Kask, P.; Piksarv, P.; Mets, Ü. Fluorescence correlation spectroscopy in the nanosecond time range: Photon antibunching in dye fluorescence. *Eur. Biophys. J.* **1985**, *12*, 163−166.

(10) Ambrose, W. P.; Goodwin, P. M.; Enderlein, J.; Semin, D. J.; Martin, J. C.; Keller, R. A. Fluorescence photon antibunching from single molecules on a surface. *Chem. Phys. Lett.* **1997**, *269*, 365−370.

(11) Hu, D.; Lu, H. P. Single-molecule triplet-state photon antibunching at room temperature. *J. Phys. Chem. B* **2005**, *109*, 9861−9864.

(12) Tinnefeld, P.; Weston, K. D.; Vosch, T.; Cotlet, M.; Weil, T.; Hofkens, J.; Müllen, K.; De Schryver, F. C.; Sauer, M. Antibunching in the emission of a single tetrachromophoric dendritic system. *J. Am. Chem. Soc.* **2002**, *124*, 14310−14311.

(13) Sýkora, J.; Kaiser, K.; Gregor, I.; Bönigk, W.; Schmalzing, G.; Enderlein, J. Exploring fluorescence antibunching in solution to determine the stoichiometry of molecular complexes. *Anal. Chem.* **2007**, *79*, 4040−4049.

(14) Bussian, D. A.; Malko, A. V.; Htoon, H.; Chen, Y.; Hollingsworth, J. A.; Klimov, V. I. Quantum optics with nanocrystal quantum dots in solution: Quantitative study of clustering. *J. Phys. Chem. C* **2009**, *113*, 2241−2246.

(15) Ta, H.; Kiel, A.; Wahl, M.; Herten, D.-P. Experimental approach to extend the range for counting fluorescent molecules based on photon-antibunching. *Phys. Chem. Chem. Phys.* **2010**, *12*, 10295−10300.

(16) Mets, Ü.; Widengren, J.; Rigler, R. Application of the antibunching in dye fluorescence: measuring excitation rates in solution. *Chem. Phys.* **1997**, *218*, 191−198.

(17) Nair, G.; Zhao, J.; Bawendi, M. G. Biexciton quantum yield of single semiconductor nanocrystals from photon statistics. *Nano Lett.* **2011**, *11*, 1136−1140.

(18) LeBlanc, S. J.; McClanahan, M. R.; Jones, M.; Moyer, P. J. Enhancement of multiphoton emission from single CdSe quantum dots coupled to gold films. *Nano Lett.* **2013**, *13*, 1662−1669.

(19) Vester, M.; Staut, T.; Enderlein, J.; Jung, G. Photon antibunching in a cyclic chemical reaction scheme. *J. Phys. Chem. Lett.* **2015**, *6*, 1149−1154.

(20) Vester, M.; Grueter, A.; Finkler, B.; Becker, R.; Jung, G. Biexponential photon antibunching: Recombination kinetics with the Förster-cycle in DMSO. *Phys. Chem. Chem. Phys.* **2016**, *18*, 10281−10288.

(21) Schwartz, O.; Levitt, J. M.; Tenne, R.; Itzhakov, S.; Deutsch, Z.; Oron, D. Superresolution microscopy with quantum emitters. *Nano Lett.* **2013**, *13*, 5832−5836.

DOI: 10.1021/acs.jpclett.6b01467
J. Phys. Chem. Lett. 2016, 7, 3137−3141