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Accurate Fluorescence Quantum Yield Determination by Fluorescence Correlation Spectroscopy

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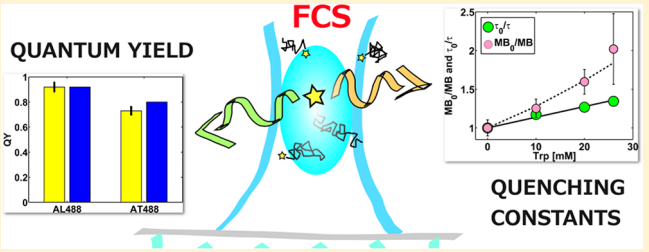
Supporting Information

ABSTRACT: Here, we present a comparative method for the accurate determination of fluorescence quantum yields (QYs) by fluorescence correlation spectroscopy. By exploiting the high sensitivity of single-molecule spectroscopy, we obtain the QYs of samples in the microliter range and at (sub)nanomolar concentrations. Additionally, in combination with fluorescence lifetime measurements, our method allows the quantification of both static and collisional quenching constants. Thus, besides being simple and fast, our method opens up the possibility to photophysically characterize labeled biomolecules under application-relevant conditions and with low sample consumption, which is often important in single-molecule studies.



INTRODUCTION

In the past two decades, the offspring of single-molecule spectroscopy and super-resolution imaging prompted a revolution in the realm of life sciences. Indeed, these techniques allow(ed) the real-time observation of a wealth of biochemical and cellular processes with unprecedented clarity and precision both *in vitro*[1] and *in vivo*.[2,3] In this regard, a common thread is the requirement to specifically couple fluorescence probes to the macromolecule of interest, e.g., oligonucleotides or proteins. The optical properties of the most commonly used organic fluorophores dramatically depend on the fluorophore's local environment. Thus, the validation of any analytical method employing fluorescent molecules requires the spectroscopic characterization of these probes under application-relevant conditions. In this respect, the fluorescence quantum yield (QY) is one of the key parameters in single-molecule fluorescence spectroscopy. Indeed, the QY determines the molecular brightness (MB) of a fluorophore, which is proportional to the number of photons emitted by a fluorophore per unit time, assessing its suitability for ultrasensitive spectroscopy.[4,5] Moreover, accurate QYs are required for the quantitative analysis of single-molecule Förster resonance energy transfer experiments.[5,6]

In this Article, we report on accurate QY determination by confocal microscopy. The QYs of diffusing molecules are measured relative to those of reference fluorophores. This new method relies on precise MBs supplied by correlation analysis of the fluorescence intensity fluctuations recorded with a confocal microscope at nanomolar concentrations.[7] Thus, besides an intrinsically low sample consumption (≤400 pmol), the method is simple and rapidly performed without the extra need of any custom-built device.[8−10] Beyond that, with respect to previously published results on the single-molecule level,[11] our approach circumvents the necessity for surface immobilization. Finally, in combination with fluorescence lifetime measurements, our method allows the quantification of both static and collisional quenching, unlike other approaches which are only sensitive to collisional quenching.[8]

MATERIALS AND METHODS

Materials. *Preparation of Fluorophore Samples.* Fluorescein (FL) from a reference dye kit (Life Technologies, Carlsbad, USA) was delivered in dimethylsulfoxide (DMSO) by the manufacturer and dissolved in 0.1 M NaOH for the measurements. Alexa 488 (AL488), Atto 488 (AT488), and Alexa 647 (AL647) functionalized with a succinimidyl ester group were used for preparing the solutions of freely diffusing fluorophores. Following the manufacturers' instructions (Atto-Tec, Siegen, Germany, and Life Technologies, Carlsbad, USA), the lyophilized fluorophore powder was dissolved in anhydrous DMSO (Sigma-Aldrich, St. Louis, USA) and diluted in the specific aqueous solvent. AL488 and AL647 were diluted in phosphate-buffered saline (50 mM potassium phosphate, 150 mM NaCl, pH 7.2); AT488 was diluted in ultrapure water. For the quenching experiments, solutions of 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) and 150 mM NaCl at pH 7.4 were enriched with the required amount of the quencher, L-tryptophan (Sigma-Aldrich, St. Louis, USA).

Preparation of Single-Labeled Protein Molecules. Details on the preparation of single-cysteine phosphoglycerate kinase (PGK) mutants, as well as the protein labeling procedure, are

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described elsewhere.[12] AL488 and AL647, with a maleimide reactive group, were used to site-specifically label PGK. Labeling of cysteine residues was performed following the manufacturers' instructions (Atto-Tec, Siegen, Germany, and Life Technologies, Carlsbad, USA). All measurements of labeled protein were performed in a buffer containing 50 mM MOPS and 50 mM NaCl at pH 7.4.

Instrumentation. *Confocal Microscopy.* All time-resolved measurements were performed using a MicroTime 200 confocal microscope (PicoQuant, Berlin, Germany). Fifty microliter droplets of sample solutions were deposited on the cover slides. Measurements of freely diffusing fluorophores were performed using an untreated cover slide, whereas protein solutions were placed on surfaces that were plasma-cleaned (PDC-32G, Harrick Plasma, Ithaca, USA) and subsequently treated with a 10 mg/mL bovine serum albumin solution (incubation time 30 min), rinsed with water, and dried with nitrogen. Depending on the spectral properties of the sample, laser light of wavelength λ_{ex} = 481/633 nm for pulsed excitation (20 MHz) or λ_{ex} = 487/637 nm for continuous excitation (LDH-D-C 485 and LDH-D-C 640, PicoQuant, Berlin, Germany) was used for irradiation. The linearly polarized excitation light was focused by a water immersion objective (UplanSApo, 60×, NA 1.2, Olympus Deutschland, Hamburg, Germany) to a point approximately 10 μm above the glass surface. After collection by the objective, the fluorescence emission passing through a dual-band dichroic mirror (XF2401, Omega Optical, Brattleboro, USA) was focused on a 30 μm pinhole. After being split by a 50/50 beam splitter cube (Olympus Deutschland, Hamburg, Germany) or a polarizing beam splitter cube (Linos Photonics, Göttingen, Germany), and after passing the respective emission filter (FF01 530/55 or ET658/80 M, Semrock, Rochester, USA), the emitted light was finally detected by single-photon avalanche diodes (τ-SPAD, PicoQuant, Berlin, Germany). The arrival time of each photon was recorded with a time-correlated single-photon counting module (PicoHarp300, PicoQuant, Berlin, Germany). Correlation analysis of the intensity−time traces was performed using the Symphotime software (Picoquant, Berlin, Germany). All other data sets were analyzed using Origin (OriginLab Corp., Northampton, USA) or self-written Matlab (Mathworks, Natick, USA) routines.

Absorption and Fluorescence Spectroscopy. Absorption spectra were recorded in 1 cm path length quartz cuvettes (104F-QS, Hellma, Mühlheim, Germany) by using a double-beam UV-2600 UV−vis spectrophotometer (Shimadzu, Kyoto, Japan). All fluorescence emission spectra were recorded with the QuantaMaster40 spectrofluorometer (PTI, Birmingham, USA) using 3 mm path length quartz cuvettes (105.253-QS, Hellma, Mühlheim, Germany) with a sample volume of ∼100 μL. All spectra were corrected for background intensities by subtracting the spectra of pure solvent measured under identical conditions. Additionally, a correction function provided by the manufacturer was applied to the fluorescence spectra to account for the detection efficiency of the photomultiplier tube. To avoid possible distortion of the spectra due to inner-filter effects, all sample solutions were diluted to optical densities below 0.01.

Theoretical Foundation of the Method. The fluorescence QY defines the efficiency of conversion of absorbed photons into emitted quanta of light. Thus,

$$\mathrm{QY} \equiv \frac{N_{em}}{N_{abs}} \tag{1}$$

where N_{em} and N_{abs} represent the numbers of emitted and absorbed photons, respectively. Despite its apparent simplicity, this equation is not easily applicable for measuring accurate QYs because of the inherent experimental difficulties in determining absolute numbers of photons.[13] To overcome this problem, we developed a comparative method,[14,15] further on called the MB method, that compares the MB of a fluorophore with unknown QY to that of a reference sample with known QY. The working equation used by the MB method is derived in the following.

The MB defines the fluorescence count rate per molecule $k_f(\vec{r})$ recorded by a given optical setup with a defined excitation intensity distribution. For a sample concentration which is spatially homogeneous throughout the detection volume, the MB is obtained as[16]

$$\mathrm{MB} = g \int_V \mathrm{CEF}(\vec{r})\, k_f(\vec{r})\, \mathrm{d}V \tag{2}$$

Here, $\mathrm{CEF}(\vec{r})$ is the collection efficiency function defined by the properties of the objective and by the pinhole.[17] The parameter g accounts for the quantum efficiency of the detectors, as well as for attenuation of the fluorescence emission by optical elements in the light path. For continuous-wave (cw) excitation, $k_f(\vec{r})$ is calculated as[18]

$$k_f(\vec{r}) = \frac{\mathrm{QY}\ \sigma\ I_0\ \mathrm{EID}(\vec{r})}{1 + \tau\ \sigma\ I_0\ \mathrm{EID}(\vec{r})\ (1 + k_{isc}/k_T)} \tag{3}$$

where σ is the absorption cross-section, τ is the fluorescence lifetime of the fluorophore, $\mathrm{EID}(\vec{r})$ is the normalized excitation intensity distribution, I_0 is the applied excitation intensity, k_{isc} is the intersystem crossing rate, and k_T is the triplet state depletion rate. In the limit of low excitation intensities (see Supporting Information), $k_f(\vec{r})$ depends linearly on I_0:

$$k_f(\vec{r}) \simeq \mathrm{QY}\ \sigma\ \mathrm{EID}(\vec{r})\ I_0 \tag{4}$$

Consequently, also the MB linearly depends on the excitation intensity:

$$\mathrm{MB} \simeq [g\ \mathrm{QY}\ \sigma \int_V \mathrm{CEF}(\vec{r})\ \mathrm{EID}(\vec{r})\ \mathrm{d}V] I_0 \tag{5}$$

with a slope

$$m \simeq g\ \mathrm{QY}\ \sigma \int_V \mathrm{CEF}(\vec{r})\ \mathrm{EID}(\vec{r})\ \mathrm{d}V \tag{6}$$

For convenience, the absorption cross-section σ can be replaced by the molar absorption coefficient ϵ by exploting the proportionality (σ = 2.303ϵ/N_A) between these two quantities.[19] Thus, from the ratio between the slopes m of the "unknown" (S) and a reference (R) sample, and after some rearrangements, the following expression is obtained:

$$\mathrm{QY_S} \simeq \frac{g_R\ \epsilon_R(\lambda_{ex})}{g_S\ \epsilon_S(\lambda_{ex})} \frac{m_S}{m_R} \mathrm{QY_R} \tag{7}$$

Here, the integrals appearing in eq 6 cancel out for spectrally similar samples and a fixed setup. This equation is the foundation of the MB method. Obviously, g is calculated from the transmission efficiency of the optical elements of the microscope as well as from the quantum efficiency of the detectors. The molar absorption coefficient $\epsilon(\lambda_{ex})$ at the

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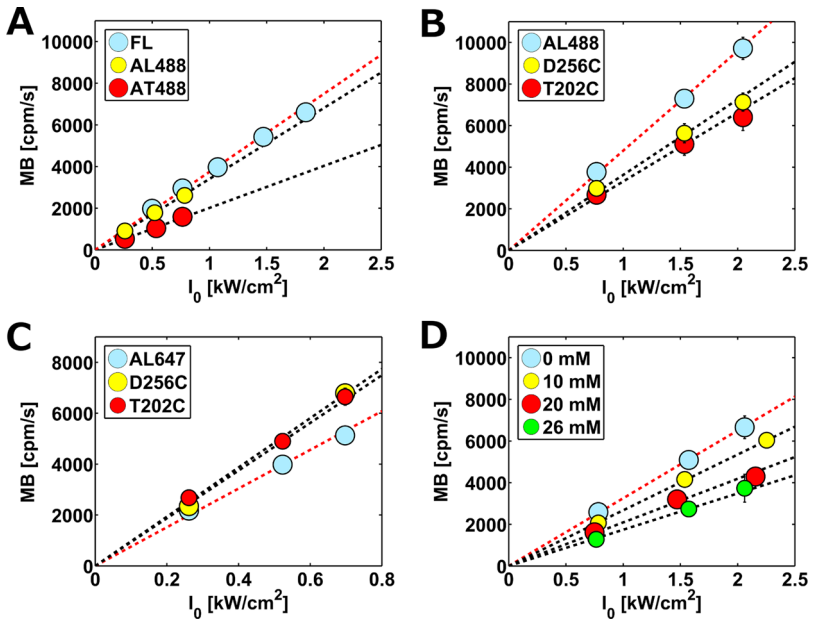

Figure 1. Linear regression of the MBs measured at different excitation intensities for (A) free fluorophores; (B) AL488 single-labeled PGK; (C) AL647 single-labeled PGK; and (D) free AL488 with Trp. The slope m is proportional to the QY. The red dashed lines identify the reference samples. Error bars are shown if larger than the marker size. The larger MB of AL488 in panel (B) with respect to panels (A) and (D) is due to realignment of the microscope.

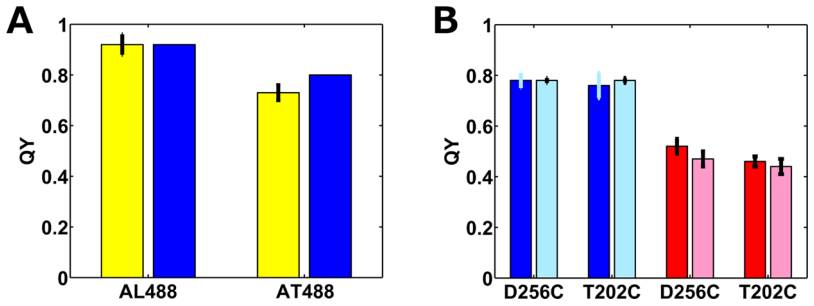

Figure 2. (A) Measured (yellow) and commonly accepted[20,21] (blue) QYs of free AL488 and AT488. (B) QYs measured with the MB method (blue and red) and the τ method (light blue and pink) for PGK-bound AL488 (shades of blue) and PGK-bound AL647 (shades of red). All QYs are listed in Tables S2 and S3.

excitation wavelength is determined by multiplying the value of the normalized absorption spectrum at λ_{ex} with the maximum absorption coefficient ϵ_{max} known from the literature. Finally, the slope m is obtained from linear regression of the measured MBs plotted as a function of the excitation intensity. Thus, according to eq 7, the QYs are obtained by measurements in solution determining the MBs of the "unknown" and the reference sample at different excitation intensities. This equation is applicable if three conditions are fulfilled: (i) low excitation intensities, (ii) spectrally similar S and R fluorophores, and (iii) singly labeled molecules. The MB method works for both cw and pulsed excitation (see Supporting Information). Further details for the implementation of the method are given in the Supporting Information.

To complement our results with an independent estimate of the QYs, and to quantify static and collisional quenching constants, we utilized a second comparative approach exploting the linear relationship between the QY and the fluorescence lifetime[19] (see Supporting Information). This method, named "τ method", is based on the following equation:

$$QY_S \simeq \frac{\tau_S}{\tau_R} QY_R \tag{8}$$

where τ is the amplitude-averaged fluorescence lifetime. The τ method is limited to fluorophores with similar natural lifetimes.

RESULTS AND DISCUSSION

To test the accuracy and the sensitivity of the MB method, we conducted experiments on freely diffusing fluorophores. Three well-characterized organic fluorophores were used: (1) Fluorescein (FL), (2) Alexa 488 (AL488), and (3) Atto 488 (AT488). Here FL in a 0.1 M NaOH/water solution was used as the reference sample. AL488 and AT488 in aqueous solutions were considered as the "unknown" samples. From the slopes of the data shown in Figure 1A, values of QY = 0.92 ± 0.04 (for AL488) and 0.73 ± 0.03 (for AT488) were obtained. Relative deviations of 0.0% and 8.8% from the commonly accepted values of 0.92[20] and 0.80[21] were observed (see Figure 2A). Interestingly, the QY of AT488 measured with the MB method perfectly agrees with the value of 0.74 ± 0.06 recently obtained by Chizhik and co-workers with a nanocavity-based approach.[8] Thus, the accuracy of the MB method is

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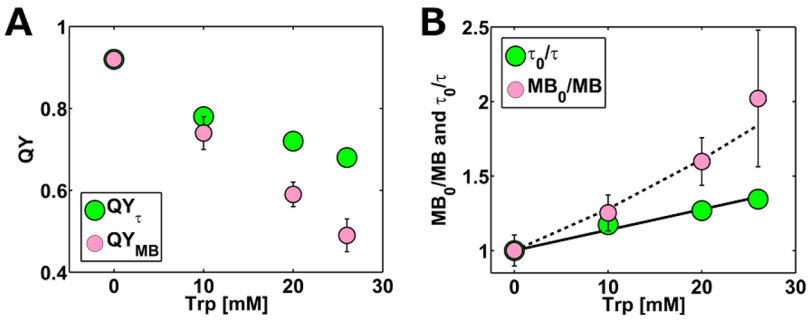

Figure 3. Solutions of free AL488 and Trp at different concentrations. (A) Measured QYs and (B) Stern–Volmer plot. The τ_0/τ and MB_0/MB data were globally fitted with eqs S29 (dashed line) and S30 (continuous line) to determine the static (K_s) and collisional (K_c) quenching constants.

comparable to, if not higher than, those of the mostly commonly used optical methods, which lie between 5% and 10%.[14]

To test whether the MB method is also applicable to protein-bound fluorophores, we conducted experiments on single-labeled proteins. For this purpose, single mutants (D256C and T202C) of phosphoglycerate kinase (PGK) from yeast were site-specifically labeled with either AL488 or Alexa 647 (AL647) as previously described.[12] Here, free AL488 and AL647 in aqueous solutions were used as the reference fluorophores. The fluorescence of AL488 attached to PGK is quenched, as readily observed from the smaller slopes in Figure 1B. Conversely, the QY of AL647 bound to PGK increases with respect to free AL647 (see Figure 1C). For both attached fluorophores, AL488 as well as AL647, similar QYs are observed for different labeling positions (see the blue and red bars in Figure 2B). This indicates analogous local environments surrounding the fluorophores on the PGK surface with respect to quenching. Moreover, the sensitivity of the MB method was estimated to be ~0.05, from the largest absolute experimental error obtained from the data. Besides that, to assess the reliability of the MB method, we measured the QYs of single-labeled PGK with the τ method and compared the results. The independently determined QYs coincide for both mutants and for both fluorophores in the limit of error (see Figure 2B).

To verify the sensitivity of the MB method to quenchers, we added different concentrations (in the millimolar range) of L-tryptophan (Trp) to the solution containing free AL488. Since Trp is a quencher of fluorescence displaying static as well as collisional quenching,[22,23] we measured the QYs with both the MB method (QY_{MB}) and the τ method (QY_τ). In fact, the fluorescence lifetime, unlike the MB, is not affected by static quenching.[19] Thus, in the presence of static quenchers, QY_τ must be larger than QY_{MB}. A combination of collisional and static quenching is clearly observed, as shown in Figure 3A. Indeed, both the QY_τ and the QY_{MB} values decrease at higher concentrations, and the QY_τ values are larger than the QY_{MB} values. This observation is confirmed by a Stern–Volmer plot (see Figure 3B), as indicated by the upward curvature of the MB_0/MB data (collisional + static) and the linear increase of the τ_0/τ data (collisional).[19]

To determine the collisional and static quenching constants, the data in Figure 3B were globally fitted. The collisional quenching constant $K_c = 13.8 \pm 0.3\ M^{-1}$ and the bimolecular quenching rate $k_c = K_c/\tau_0 = 3.4 \pm 0.1\ M^{-1}\ ns^{-1}$ perfectly agree with the literature values of 14.2 M^{-1} and 3.5 $M^{-1}\ ns^{-1}$.[22,24] Additionally, the static quenching constant $K_s = 12 \pm 4\ M^{-1}$ allows us to calculate a "sphere-of-action"[19,25] of radius ~17 Å, which is comparable to the value of ~19 Å measured for a fluorophore similar in size to AL488.[26]

CONCLUSION

Today, a comparative method[14]—the so-called optical method—is used for QY determination in a lot of laboratories. This method compares the integral fluorescence emission of the "unknown" and the reference samples under identical measuring conditions for solutions of known absorbances. Practically, the set of absorption and emission spectra is obtained by varying the fluorophore concentrations. Thus, only an absorption spectrophotometer and a fluorescence spectrophotometer are needed, which makes this method attractive to most groups. To achieve an adequate accuracy with the optical method, cuvettes with long path length are often required resulting in a large sample consumption.[15] This limitation is intrinsically overcome by the MB method. This method relies on the MBs calculated from the precise photon count rates and concentrations supplied by fluorescence correlation spectroscopy. Here, the excitation intensity is changed instead of the sample concentration. By employing ultrasensitive confocal detection of diffusing molecules, the MB method allows QY measurements at low concentrations (~1 nM) with a small sample consumption, ≤400 pmol (in the worst case scenario of a fluorophore with low molar absorption coefficient, e.g., AL488 with ϵ = 73 000 $M^{-1}\ cm^{-1}$), which is ~3.5 smaller than that required by the optical method (see Supporting Information). The sample consumption may further be lowered below 14 pmol (~100 times smaller than the optical method) by using a microvolume spectrophotometer for measuring absorption spectra. Consequently, the MB method is perfectly suited for repetitive QY measurements of biomolecules with low expression and/or labeling yields. Besides that, advantages with lipidic vesicles and crowding agents can potentially be envisioned. In addition, the sensitivity of the MB method to the different quenching mechanisms may allow the characterization of fluorophores surrounded by complex environments with respect to quenching.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, theoretical background, and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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