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(dedicated to Prof. Dr. A. M. Brouwer on the occasion of his retirement)

Tarik Aaboub^{1,2,3,4}, Aicha Boukhriss⁴, Said Gmouh³, Martinus H. V. Werts^{1,2*}

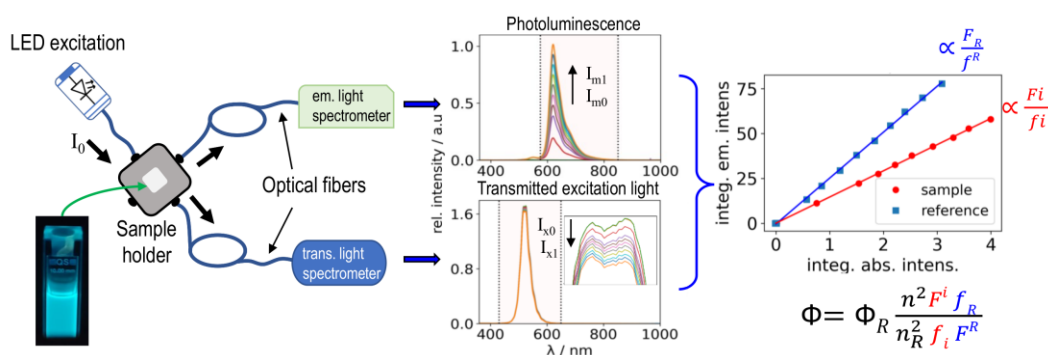
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Abstract

The photoluminescence (PL) quantum yields (QYs) of fluorophores in dilute solutions can be determined fluorimetrically according to the comparative method employing standards of known PLQY. This method has recently been demonstrated to become more robust when the absorption of the excitation light and the PL emission are measured simultaneously using a transmitted light detector integrated in the fluorimeter. Herein, aided by fiber-coupled spectroscopic equipment and computerized data processing, we elaborate on this method by measuring the full corrected intensity spectrum of the excitation light transmitted through the sample. This further releases constraints on the monochromatic character of the excitation light and enables the use of broad-band excitation sources such as light-emitting diodes (LEDs). Furthermore, the protocol includes measurements at increasing dye concentration, rigorously verifying the required proportionality between absorbed and emitted light intensities. The PLQYs of solutions of fluorophores determined using the new method are in close agreement with published values.

Keywords

photoluminescence quantum yield, fluorescent dyes, light-emitting diode, simultaneous absorption and fluorescence emission measurement

1. Introduction

In addition to the photoluminescence emission (PLE) spectrum and the molar absorption coefficient, the photoluminescence quantum yield (PLQY) is an essential photophysical property in the characterization and evaluation of fluorophores and other molecular or nanoscale photoluminescent species in solution. The determination of the PLQY in optically dilute solution using standard “right-angle” spectrofluorimeters has been amply

discussed, documented, and reviewed in the literature [1–6]. The standard comparative fluorimetric method, in which the photoluminescence intensity of a solution of a fluorophore with unknown quantum yield is compared to a standard solution of known PLQY, is now well established. This method (we will refer to it as the “standard method”) is relatively easy to apply, provided that all requirements pertaining to the samples and the apparatus have been strictly satisfied [2–4, 6].

These requirements result from the assumptions and approximations made in the underlying theoretical analysis. The solution to be characterized should contain one single light-absorbing species, typically a pure fluorescent molecular compound. The photoluminescence (PL) of this species is required to have Kasha-Vavilov behavior [7], *i.e.* the excited state from which PL is emitted should always be the same and be reached quasi-instantaneously upon excitation, independently of the excitation wavelength. The solute should be perfectly dissolved, without the formation of aggregates. The sample solutions should be limpid and display no scattering, *i.e.*, the overall light extinction should be equal to the overall light absorption. The PL emission should be isotropic, and light polarization effects should be negligible. Measurements should be carried out on solutions that are optically dilute, *i.e.*, the optical density should be inferior to 0.1.

On the instrumental side, the excitation wavelength should best be the same for the sample and the PLQY standard. If this is not the case, a quantum counter should be used to correct for the differences in excitation light intensity [8]. Furthermore, all PLE spectra used in the measurement should be fully corrected, which means that any electronic dark signal has first been subtracted, followed by the application of suitable wavelength-dependent correction factors [8] such that the spectrum represents the relative spectral photon flux [9] as a function of wavelength¹.

The standard method furthermore relies on the assumption that the excitation light is monochromatic. It also relies on precise measurement of the optical density at the excitation wavelength, which is typically done on a different instrument. This introduces additional experimental uncertainties, a question which has recently been addressed by Nawara and Waluk, who improved on the standard method by carrying out the measurement of sample absorbances simultaneously with the measurement of the PLE spectrum, inside the spectrofluorimeter, using a separate transmittance detector [10]. This method is called the SAFE method referring to “simultaneous absorption and fluorescence emission”. It was demonstrated to be more robust than the standard comparative method. The experimental apparatus was later extended to allow for *in situ* double-beam absorption measurement, further increasing reproducibility [11].

Both the standard and the SAFE methods do not address the case where a relatively broadband light source is used as the excitation source, for example, a light-emitting diode (LED). In such a case, the temptation may exist to consider only the specified (maximum) emission wavelength of the source, or to assume that its spectral photon flux is constant

¹In practice, this is often colloquially referred to as “intensity in photon units”.

over a narrow wavelength range. These approximations are generally not satisfactory for typical LEDs used for fluorescence excitation [12–15] which have relatively broad emission spectra (see **Supplementary Information, Fig. S2**).

Here, we consider a fluorimetric method for comparative measurement of PLQYs in solution in which light absorption and light emission are measured simultaneously, as in the SAFE method, with the modification that the full spectral distribution of the excitation light and its absorption are measured. The method is tentatively called the “SAFER” method and is particularly useful when using LEDs as the fluorescence excitation source. It may also be applied to monochromator-based sources in order to monitor directly the spectrum of the light exiting the excitation monochromator.

As will be shown, the standard method and the SAFE method are approximations of a more general analysis. The mathematical formulation of this underlying analysis is the same for all comparative methods, but the methods differ in the way the light absorption is approximated. The SAFER method presented here fully considers the spectrum of the excitation light in the determination of the amount of absorbed light.

2. Theory

The photoluminescence emission quantum yield, Φ , is given as the ratio of the number of luminescence photons emitted (N_{em}) and the number (N_{abs}) of photons absorbed by the sample. Starting from this definition, and assuming an isotropic emission intensity distribution, we arrive at Eqn. (1) for the experimental measurement of the PLQY in a standard right-angle fluorimetric configuration.

$$\Phi = \frac{N_{\text{em}}}{N_{\text{abs}}} = \frac{K_m \int_0^{\infty} I_m(\lambda) d\lambda}{K_x \int_0^{\infty} I_{\text{abs}}(\lambda) d\lambda} \quad (1)$$

$I_m(\lambda)$ is the corrected emission spectrum of the fluorophore in the solvent. It is corrected such that $I_m(\lambda)$ is proportional to the number of photons emitted per unit of time by the fluorophore at that wavelength *i.e.*, all wavelength dependencies of the spectrometer have been accounted for. As a result, K_m does not depend on wavelength and accounts for geometrical factors in light detection efficiency (including refractive index effects on the detected emission intensity, *vide infra*). It takes also into account any arbitrary scaling that has been applied to the correction factors used for the correction of the emission spectrum. Furthermore, all background signals not coming from the fluorophore (*e.g.*, Rayleigh and Raman scattering by the solvent) have been subtracted from the emission spectrum, as expressed in Eqn. (2).

$$I_m(\lambda) = I_{m1}(\lambda) - I_{m0}(\lambda) \quad (2)$$

Here, $I_{m1}(\lambda)$ is the corrected emission spectrum² of the solution of the fluorescent compound and $I_{m0}(\lambda)$ is the corrected emission spectrum of the illuminated solvent alone. With sufficiently bright fluorophores, we have $I_m(\lambda) \approx I_{m1}(\lambda)$, and contributions from the solvent background due to Rayleigh and Raman scattering will be negligible. For consistent SAFER measurement, the emission spectrum of the solvent alone, $I_{m0}(\lambda)$, is systematically recorded which enables subtraction of weak Rayleigh and Raman signatures in particular for weakly emitting fluorophores.³

The solvent background spectrum $I_{m0}(\lambda)$ is recorded simultaneously with the corrected spectrum of the excitation light that has traversed the pure solvent, $I_{x0}(\lambda)$. Subsequently, the spectrum $I_{m1}(\lambda)$ (solvent with fluorophore added) is recorded simultaneously with the corrected spectrum of the excitation light transmitted through the same solution, $I_{x1}(\lambda)$.

From I_{x0} and I_{x1} , we obtain the spectrum of the absorbed light intensity, I_{abs} , a relative measure of how many photons are absorbed per wavelength (under the condition that the light extinction is due to absorption alone, *i.e.* the sample is a lipid solution).

$$I_{\text{abs}}(\lambda) = I_{x0}(\lambda) - I_{x1}(\lambda) \quad (3)$$

Since $I_{x0}(\lambda)$ is measured with the cuvette only containing the solvent, it already includes all light losses due to the cuvette (reflection losses at the optical interfaces) and losses due to the solvent (residual light absorption, scattering). The contributions of Rayleigh and Raman scattering to these losses are exceedingly small compared to the other losses. The only difference between $I_{x0}(\lambda)$ and $I_{x1}(\lambda)$ being the presence of the fluorophore, $I_{\text{abs}}(\lambda)$ purely represents the light absorption by the fluorophore, just as $I_m(\lambda)$ is purely the light emission from that fluorophore, free from all artifacts already subtracted.

The quantity I_{abs} appears in Eqn. (1) together with the constant K_x which is the (unknown) factor that would convert these relative transmitted excitation photon fluxes into absolute numbers of photons.

At this point, we consider the impact of the refractive index of the solvent on the detection efficiency of the emission channel. In the case of a conventional right-angle fluorimetric set-up, the light collection factor K_m includes a contribution of the refractive index of the solution, which changes the solid angle over which emission light is collected [4]. This refractive index contribution can be factored out of K_m to have it appear explicitly in the expression of the detected emitted light intensity (Eqn. (4)).

$$K_m = n^2 K'_m \quad (4)$$

²A corrected (photon) emission spectrum has the detector dark current subtracted, the correction for wavelength-dependent overall photon detection efficiency applied and represents the relative spectral photon flux as a function of wavelength.

³Introduction of the solute into the solvent increases the absorption of the incoming light. As a result, the Rayleigh and Raman scattering will be somewhat weaker (up to approx. 15%) in $I_{m1}(\lambda)$ compared to $I_{m0}(\lambda)$. The background subtraction will then subtract slightly too much scattering. Such an effect should be carefully considered in the case of very weakly fluorescent species.

With this change, Eqn. (1) becomes Eqn. (5).

$$\Phi = \frac{n^2 K'_m \int_0^\infty I_m(\lambda) d\lambda}{K_x \int_0^\infty I_{\text{abs}}(\lambda) d\lambda} \quad (5)$$

The ratio K'_m/K_x can be determined using a solution of a reference fluorophore with known photoluminescence quantum yield Φ_R [1].

$$\frac{K'_m}{K_x} = \frac{\Phi_R \left[\int_0^\infty I_{Rm}(\lambda) d\lambda \right]^{-1}}{n_R^2 \left[\int_0^\infty I_{Rabs}(\lambda) d\lambda \right]} \quad (6)$$

K'_m/K_x depends only on the instrument and is constant for any given set of experimental settings. We observed that it may even be constant over several days on our fluorimetric set-up (provided that it is left strictly unperturbed). However, the requirement of measuring a fresh reference for each series of measurements is maintained, in order to rigorously validate the determination of the photoluminescence quantum yield.

When plugging the expression for K'_m/K_x of Eqn. (6) back into Eqn. (5), a general expression is obtained, in the form of Eqn. (7), for measuring the photoluminescence quantum yield of a fluorophore with respect to a reference solution using an excitation source having an arbitrary spectrum. This relies on the applicability of the Kasha-Vavilov law and on having a pure fluorophore in the solution. The aim is to use a light source that has a spectrum that is as narrow as possible, but that may be relatively large, such as a light-emitting diode, or a broad-band light source of wavelength-dependent intensity in combination with a band-pass filter.

$$\Phi = \Phi_R \frac{n^2 F^i f_R}{n_R^2 f_i F^R} \quad (7)$$

with

$$F^i = \int_0^\infty I_m(\lambda) d\lambda = \int_0^\infty [I_{m1}(\lambda) - I_{m0}(\lambda)] d\lambda \quad (8)$$

$$f_i = \int_0^\infty I_{\text{abs}}(\lambda) d\lambda = \int_0^\infty [I_{x0}(\lambda) - I_{x1}(\lambda)] d\lambda \quad (9)$$

$$F^R = \int_0^\infty I_{Rm}(\lambda) d\lambda = \int_0^\infty [I_{Rm1}(\lambda) - I_{Rm0}(\lambda)] d\lambda \quad (10)$$

$$f_R = \int_0^\infty I_{Rabs}(\lambda) d\lambda = \int_0^\infty [I_{Rx0}(\lambda) - I_{Rx1}(\lambda)] d\lambda \quad (11)$$

Expression (7) is the formula for the determination of fluorescence quantum yield that is known in the literature. In the context of the present work, there is a difference in how the emission (F) and absorption (f) factors are obtained. The methods described in

previous work use various approximations for the integrated absorbed light intensity (Eqns (9) and (11)). For the completeness of the discussion, we will briefly review these approximations first.

In order to understand the ‘standard’ and SAFE methods, the expression for the absorbed light intensity, I_{abs} in Eqns (9) and (11), can be rearranged by factoring out I_{x_0} . The absorbed light is thus written as the product of the (corrected) spectrum of the relative spectral photon flux of the excitation light that has fully traversed the pure solvent (I_{x_0}) and the fraction of light absorbed specifically by the fluorophore (Eqn. 12).

$$I_{\text{abs}}(\lambda) = I_{x_0}(\lambda) - I_{x_1}(\lambda) = I_{x_0}(\lambda) \left[1 - \frac{I_{x_1}(\lambda)}{I_{x_0}(\lambda)} \right] \quad (12)$$

The term I_{x_1}/I_{x_0} is recognized to be the transmittance of the sample ($T = I_{x_1}/I_{x_0}$). From the transmittance, the optical density is obtained by definition as $\text{OD} = -\log_{10} T$. Thus, I_{abs} may be expressed in terms of optical density (Eqn. 13).

$$I_{\text{abs}}(\lambda) = I_{x_0}(\lambda) - I_{x_1}(\lambda) = I_{x_0}(\lambda) [1 - 10^{-\text{OD}(\lambda)}] \quad (13)$$

At this point, the expressions are still general and do not assume a monochromatic excitation source, nor an excitation light source with a ‘flat’ output spectrum. The factoring out of I_{x_0} , as done in Eqns (12) and (13), is only relevant at wavelengths where the light intensity of the excitation source is not close to zero. To deal with this, the semi-infinite integral over I_{abs} of Eqns (9) and (11) is replaced by a definite integral between the wavelengths where the light source has significant intensity.

In the hypothetical case of perfectly monochromatic light of wavelength λ_{exc} , the excitation light intensity $I_{x_0}(\lambda)$ only has a non-zero value at λ_{exc} and is zero everywhere else. The factor K_x can be scaled such that that I_{x_0} becomes a Dirac delta function (*i.e.* it is only non-zero at λ_{exc} and its integral evaluates to one). Thus, the assumption of perfectly monochromatic light yields Eqn. (14).

$$\int_0^{\infty} I_{\text{abs}}(\lambda) d\lambda = 1 - 10^{-\text{OD}(\lambda_{\text{exc}})} \quad (14)$$

It is seen that with perfectly monochromatic light, the conventionally used expression for the determination of the photoluminescence quantum yield is obtained (*i.e.*, the expression used in the ‘standard’ method)⁴.

The SAFE method by Nawara and Waluk makes a more realistic assumption concerning the properties of excitation light. That method is based on the (implicit) assumption that

⁴ The term, $10^{-\text{OD}(\lambda_{\text{exc}})}$ is sometimes approximated by the first two terms of its Taylor expansion around 0, *i.e.* $10^{-x} = 1 - x \ln(10) + \frac{1}{2}x^2 \ln^2(10) - \dots$. This yields $1 - 10^{-\text{OD}(\lambda_{\text{exc}})} \approx \ln(10) \text{OD}(\lambda_{\text{exc}}) \approx 2.303 \text{OD}(\lambda_{\text{exc}})$, simplifying the appearance of Eqn. (7), since the ratio f_R/f_i then would become the direct ratio of the optical densities. However, the use of this approximation is strongly discouraged since it unnecessarily leads to significant deviations already when $\text{OD} > 0.05$, purely as a result of the truncation of the Taylor series. Electronic calculators readily evaluate 10^{-x} with great precision.

the light intensity of the excitation light exiting the monochromator is constant over a well-defined band from λ_1 to λ_2 . An additional implicit assumption is that the transmitted light detector (*e.g.*, a photodiode) has a flat spectral response. In that case (and with proper scaling of K_x), $I_{x0}(\lambda)$ has the value of one over the interval λ_1 to λ_2 and is zero elsewhere. The absorption integral then becomes Eqn. (15), leading to the expressions used by Nawara and Waluk in the 'SAFE' method.

$$\int_0^{\infty} I_{\text{abs}}(\lambda) d\lambda = \int_{\lambda_1}^{\lambda_2} [1 - 10^{-\text{OD}(\lambda)}] d\lambda \quad (15)$$

The "SAFER" method presented here uses Eqn. (7) with full measurement of the corrected transmitted and emitted light spectra required for direct evaluation of the integrals in Eqns (8...11). It does not use any of the developments in Eqns (12...15). The determination of a single PLQY of a solution thus requires the recording of eight spectra: four spectra each for the sample and the PLQY reference solution, respectively. The four spectra are recorded in pairs, simultaneously measuring the transmitted and emitted light spectra on the same sample in the same sample-holder. In spite of the direct evaluation of the integrals in Eqns (8...11), without explicit use of the optical density in the calculations, the SAFER method is still subject to working at optical densities below 0.1 (at excitation and emission wavelengths), in order to avoid inner filter effects.

A further relevant point to consider is the spectral overlap between the broad-band excitation source and the absorption spectrum of the fluorophore, as implied by Eqn. (13). Since we have a single emitting species with Kasha-Vavilov behavior, every photon absorbed by the species will have the same probability to trigger the emission of a PL photon, independently of the wavelength of that excitation photon. For the PLQY measurement, it is thus necessary and sufficient to measure the number of absorbed photons, integrated over all relevant wavelengths, as expressed by Eqns (9) and (11).

Interestingly, in the case of only partial overlap between the spectrum of the excitation source and the absorption spectrum of the fluorophore, the excitation photons that are outside of the absorption band of the fluorophore, will simply never contribute to f_i (or f_{Ri}). Excitation photons that are within the absorption band may sometimes contribute to f_i (or f_{Ri}). Even within the absorption band of the fluorophores, only a fraction of the incoming excitation photons is absorbed, in line with the requirement to work at $\text{OD} < 0.1$. Those photons whose wavelengths fall inside of the absorption band, but that were not absorbed, do not contribute to f_i (or f_{Ri}), on equal standing with the photons that are outside of the absorption band (and had no chance whatsoever of being absorbed). Of course, there should be sufficient spectral overlap between the excitation source and the fluorophore absorption band to warrant sufficient light absorption for a reliable determination of the integral f_i (or f_{Ri}).

3. Practical realisation

Recording and processing the eight spectra that enter Eqn. (7) may seem daunting, but the method is based on the simultaneous recording of pairs of emission and transmitted light spectra in a digital form followed by automated processing using a microcomputer program. Furthermore, the spectra of the reference only need to be recorded once for a series of measurements.

The experimental set-up (Fig. 1) is based on fiber-coupled spectroscopic equipment, with an excitation light source (choice of LEDs for different excitation wavelengths) and two CCD (or CMOS)-array spectrometers, one for transmitted-light spectra, one for emitted-light spectra. The fiber-coupled LEDs deliver around 50 μW of light to the sample through the illumination optics, which is comparable to the intensity of a Xenon lamp/narrow slit monochromator combination, with the difference that the total intensity is smeared out over a wider range of wavelengths.

The spectral responses of both spectrometers were calibrated using a NIST-traceable incandescent light source and reference solutions of fluorescent compounds with known photon spectra. The dark signal was subtracted from the raw spectra, and the spectra were converted into photon units using the correction factors. We found that LED light sources are highly stable and enable sequential recording of spectra without being affected by intensity fluctuations. Further details on the set-up are given in the Experimental Details.

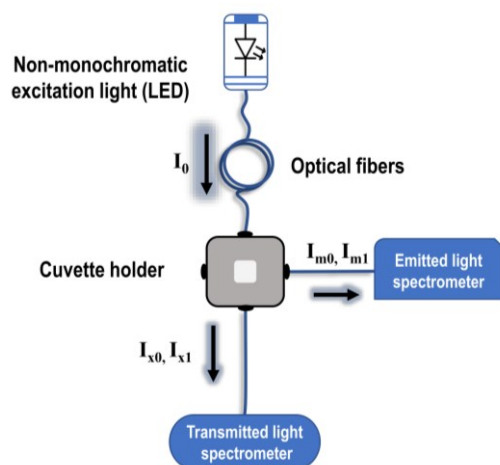


Figure 1. Set-up used for simultaneous light absorption and fluorescence emission measurements of lipid liquid samples for the determination of photoluminescence quantum yields

The ratios F^i/f_i and F^R/f_r in Eqn. (7) can be viewed as the slopes of the plots of the (total) emitted light intensity F as a function of the (total) absorbed light intensity f at different concentrations of the fluorophores. In the practical implementation of the SAFER procedure, we, therefore, measure simultaneously F and f several times with stepwise increases in the concentration of the fluorophore, starting from zero concentration, *i.e.*, pure

solvent (I_{m0} , I_{x0} , I_{Rm0} , I_{Rx0} in Eqns (8...11)). A cuvette containing a known volume of pure solvent and a miniature stir bar is inserted in the cuvette holder, which is equipped with a magnetic stirrer. Small quantities from a concentrated dye stock solution (typically 10^{-4} ... 10^{-3} M in the same solvent) are added, recording transmitted and emitted light spectra after each addition. The cuvette remains in place, avoiding experimental uncertainties related to cuvette replacement. The final concentrations of the dyes in the measured solutions were typically between 10^{-6} M and 10^{-5} M. In all cases the optical density of the solutions did not exceed 0.1 (typically below 0.05).

Interestingly, it is not necessary to precisely know the concentration of the fluorophore for the plot of F vs f , although it may be useful to also study how F and f vary with concentration. For the SAFER measurement, only the plot of F vs f is needed. This must be a straight line going through the origin. If this is not the case, then something is wrong, and a reliable PLQY cannot be determined. In the case of linear plots for both the sample and the reference, the slopes of the lines give the ratios F^i/f_i and F^R/f_r for Eqn. (7). Checking the linearity of F vs f measurements at different concentrations will filter out a number of problems that can arise with PLQY measurements in solution: solubility problems/dye aggregation, (photo)chemical instability, exceeding the limits on optical density, light source problems, etc.

Since the corrected spectra of the excitation light are measured, it is in principle possible to use different excitation sources at different wavelengths between the sample and the reference. In the present work, we mainly used the same excitation for both the sample and the reference. This is preferred since it removes one further source of uncertainty. Also, in the current set-up, changing the excitation is relatively tedious and requires carefully replacing the LEDs while the equipment is in function.

Figure 2 gives an example of a single PLQY measurement for pyranine in 0.01M NaOH_(aq) solution using the SAFER method. The spectra of the excitation light transmitted through the solution and the resulting fluorescence emission are recorded simultaneously at increasing dye concentrations, starting from the pure solvent. All spectra are fully corrected for dark signal and detector response. The spectra are integrated over a band of relevant wavelengths (non-zero intensities) to obtain the integrated intensities for both the transmitted and the emitted light. The integrated absorbed light intensities are calculated from the integrated transmitted light intensities following Eqns (9) and (11), making use of the sum rule of integration. The integrated emitted light intensity is plotted as a function of the integrated absorbed light intensity for both the sample and the reference. Finally, the PLQY is obtained from the ratio of these slopes using Eqn. (7). At least three independent PLQY measurements were made for each dye, in order to obtain an estimate of the experimental uncertainty. Additional examples are given in the **Supplementary Information**.

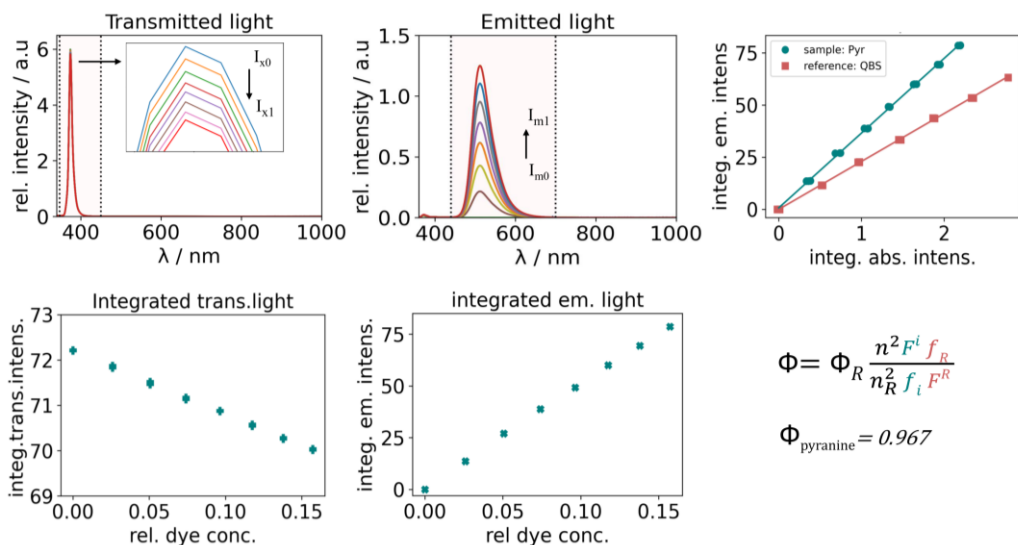


Figure 2. SAFER measurement of the PLQY of pyranine in 0.01M NaOH_(aq) using QBS in 0.1M HClO_{4(aq)} as a reference. Only the spectra of the pyranine sample are shown. Left column: corrected transmitted light spectra and integrated intensities at increasing dye concentration. Center column: corrected emitted light spectra and integrated intensities. The limits of integration are shown as dotted vertical lines. Right column: plots of integrated emitted light vs absorbed light intensities for sample and reference, with the determination of PLQY via Eqn. (7). The value given in the figure is for the particular single measurement shown in the graphs. From four independent measurements, we obtained $\Phi_{\text{pyranine}} = 0.96 \pm 0.08$

4. Results and discussion

Before discussing the actual results of measurements of the PLQYs of a series of fluorophores using the present SAFER method and comparison with values reported in the literature, it is useful to consider the slope of the emitted *vs* absorbed light intensity plot, obtained by simultaneously measuring the transmitted excitation light and emitted PL spectra at increasing dye concentration (**Figure 2**, upper right panel). The measurements at increasing concentration are carried out conveniently by placing a miniature magnetic stir bar in the cuvette initially filled with the solvent and then adding amounts of a concentrated fluorophore stock solution.

For the PLQY determination to be reliable this emitted *vs* absorbed light intensity plot should be linear, for the sample and for the reference. Our method performs this check explicitly. Note that it is the amount of absorbed light that is direct relevance for PLQY determination. The actual dye concentration is only of secondary relevance. The plot of emitted light intensity *vs* concentration loses its linearity at lower dye concentrations than the plot of emitted *vs* absorbed light.

To study the loss of linearity in the emitted *vs* absorbed intensity plot, we performed a series of separate measurements in which the concentration of the dye was deliberately increased above the limit of OD = 0.1. As expected, the dependence generally remains linear until OD ~ 0.1 (**Supplementary Information, Fig. S3, S4, S5**), although there are subtle differences between different fluorophores, depending on the Stokes' shift of the

fluorophore, which changes the strength of the inner-filter effect at higher concentrations (and potentially aggregation behavior or excimer formation). For PLQY determinations, only the linear regions of the data are used, working at low concentrations.

The determination of photoluminescence quantum yields of fluorophores in solution using the SAFER method and broad-band LED excitation was validated by measuring a series of relatively well-known fluorophore solutions whose PLQYs have been reported in the literature (Table 1). The reference solutions used were those recommended by Brouwer [1]: QBS in perchloric acid solution, Flscn in aqueous NaOH, and Rh6G in ethanol. QBS in perchloric acid was preferred over QBS in sulfuric acid, following the work by Nawara and Waluk [16], although we yielded to the temptation to measure the latter as a sample using the former as a reference, confirming the known value. All measurements from this work agree with the literature values. The spectroscopic data were obtained and analyzed as shown in Figure 2, with some further examples given in the **Supplementary Information (Fig. S6, S7)**.

Table 1. Quantum yields of different fluorophores in solution, measured at 296K (+/- 1K) by comparison with recommended PLQY reference standards [1] using the SAFER method. The sample and reference pairs were systematically excited by the same LED light source, except for the Flscn-QBS pair. The last column contains values reported in the literature. The acid/base concentrations of the aqueous solutions are given in the Experimental Details, as well as the abbreviations.

<i>Reference</i>	<i>LED exc. source</i>	<i>Compound</i>	<i>Solvent</i>	Φ <i>(this work)</i>	Φ <i>(literature)</i>
QBS/ HClO ₄ (aq)	340 nm	Anthracene	EtOH	0.26 ± 0.02	0.28 [17, 18]
	380 nm	Pyranine	NaOH(aq)	0.96 ± 0.08	~1.0 [19, 20]
		QBS	H ₂ SO ₄ (aq)	0.55 ± 0.03	0.55 [1, 21–23]
		Flscn ^(a)	NaOH(aq)	0.94 ± 0.06	0.93 [1, 24, 25]
Flscn/ NaOH(aq)	450 nm	Rh6G	EtOH	0.91 ± 0.02	0.95 [1, 26, 27]
		Pyranine	NaOH(aq)	0.99 ± 0.08	~1.0 [19, 20]
		C153	EtOH	0.56 ± 0.03	0.54 [28]
		C153	MeOH	0.48 ± 0.02	0.42 [29, 30]
Rh6G/ EtOH	518 nm	CV	MeOH	0.53 ± 0.03	0.52 [1, 21, 23]
		NR	MeOH	0.43 ± 0.03	0.40 [31]

(a) Flscn excited with 450 nm LED, QBS reference with 380 nm LED

As a first check of consistency, the PLQY of the Flscn reference was also determined using QBS as the reference, and the PLQY of the Rh6G was also measured against Flscn as a reference. Consistent PLQY values were obtained. The measurement for the Flscn/QBS pair required to exceptionally change excitation LEDs between sample (450 nm LED) and reference (380 nm LED), demonstrating that the present SAFER method allows in principle to change excitation LED sources between reference and sample.

Anthracene was measured since it is considered a somewhat challenging case for the standard comparative method because of its narrow vibronic absorption bands [10] that lead to deviations when light absorption and photoluminescence emission are not measured on the same spectrometer, in particular because of different bandwidths between the absorption measurement and the excitation light. Our measured value agrees well with the values reported in the literature [17, 18]. The overlap between the broadband excitation (340 nm LED) and the narrow anthracene absorption peaks is well taken into account by the transmitted light spectroscopic measurement in the SAFER method since it measures the integrated absorbed light intensity

Pyranine, in its fully deprotonated form in basic water, is a well-known strongly fluorescent dye, used in certain fluorescent marker pens. The pK_a of its phenolic group is 7.2, and the protonated and deprotonated forms have different spectral characteristics, which makes pyranine interesting as a ratiometric pH probe for physiological conditions [32, 33]. Pyranine is also a photo-acid, with the pK_a^* of the phenolic group dropping to 1.4 in the excited state [34]. It absorbs and emits light in the same spectral range as the fluorescein dianion, with an emission spectrum that has less structure and better photostability [33].

These observations suggest that pyranine may be a superior fluorescence standard compared to fluorescein. However, there are only a few reports of its PLQY in the literature. A fluorescence quantum yield of 0.82 has been quoted for pyranine in water [35], but neither the pH nor the composition of the aqueous phase, nor the experimental conditions were specified. Two other works agree upon a near-unity (~ 1) PLQY for pyranine in basic water [19, 20]. Our measurements, using both QBS and Flscn as references, confirm this very high value for the fluorescence quantum yield of pyranine in basic water (0.01 M NaOH).

It was observed that, under the experimental conditions used, there are small sample-to-sample variations in the brightness of the pyranine, leading to a somewhat higher uncertainty in the value of the PLQY. We tentatively ascribe this to pH variations of the dilute (10 mM) NaOH solution due to the absorption of CO_2 from the ambient atmosphere. Indeed, upon prolonged exposure of freshly diluted NaOH to air in an open cuvette, the pH may drop to 9.1 at equilibrium, which would lead to the protonation of a small fraction (a few percent) of the pyranine, slightly lowering fluorescence emission. The high dilution for NaOH was chosen following the literature [19, 20], but a suitable buffer, or more concentrated NaOH solution, should be found in which the fluorescence intensity of pyranine is stable, even in equilibrium with ambient air, before pyranine can act as a standard in fluorescence measurements.

Coumarin-153 (C153) was included since it has been suggested as an emission standard for the calibration of spectral responsivity of spectrometers [36]. It is a highly photostable fluorescent (laser) dye. It may have a double use in the standardization of fluorescence measurements, both as a spectral emission standard and a PLQY standard, for which it has favorable properties.

On the red end of the present study, we have Cresyl Violet (CV) and Nile Red (NR). The former has been considered as a standard for red-emitting fluorophores, and its fluorescence quantum yield was determined with photothermal methods [21]. However, its PLQY becomes concentration-dependent already at low concentrations ($> 1 \mu\text{M}$) [37], an observation that is confirmed in the present work (**Supplementary Information, Figure S3**). The PLQY measurements for CV were carried out at concentrations below $0.5 \mu\text{M}$, where the fluorescence emission intensity is proportional to the absorbed light intensity (**Supplementary Information, Figure S6**). NR is an interesting photostable solvatochromic dye [31], that might be considered as a candidate for a future 'red' standard when dissolved in certain polar solvents. Its PLQY in methanol has been reported in the literature [31], but would benefit from further confirmation. Our measured value for the PLQY confirms the published value.

Despite the broad spectral range offered by the emission spectrometer of the present set-up, no measurements of near-infrared fluorescence were made for the present study, focusing on more well-established visible-emitting dyes for validation. PLQY standards for the near-infrared are still less developed [1], even though crucial progress has been made recently [28, 38, 39]. Measurements in the near-infrared are more challenging due to the presence of significant absorption bands of the solvent [39] (vibrational overtones, e.g., water near 980 nm [40]).

The present validation of the SAFER method has concentrated on known fluorophores with sizeable PLQYs. For measurement of weakly emitting species with low PLQYs, the SAFER method will encounter the same limitations as the other relative methods and will depend on the same instrumental capabilities for subtracting any background signals coming from Rayleigh and Raman scattering by the solvent, for providing sufficient linear dynamic range in the emission measurement and for reducing stray light[41] in the spectrograph. The present set-up was not specifically optimized for weakly emitting samples. We estimate conservatively that without any additional precautions the lowest PLQYs to be determined reliably will be on the order of 0.01.

Modifications that could be made to the method in order to gain reliable access to lower PLQYs are: (a) the use of standards that themselves have low quantum yields[1], (b) the use of neutral density filters in the emission channel to diminish the intensity of high PLQY standards, and (c) applying different exposure times between sample and standard[42]. The first modification is preferred. The two latter are instrument-dependent and require further work and validation, e.g., when inserting neutral density filters, an additional correction will be required for the spectral dependence of the optical density of the filter.

5. Conclusion

The experimental results obtained in this work indicate that the proposed SAFER method for measuring PLQYs in solution by comparison with a known PLQY standard can successfully be applied for measurements using non-monochromatic excitation light sources, such as LEDs. It relies on simultaneous measurement of the absorbed and emitted

light intensity spectra using spectrally calibrated spectrometers. Like other methods, it requires pure samples containing only one light-absorbing species. The methodology was explained and illustrated using several well-established dyes for which the PLQY has been measured by different laboratories using different methods. Furthermore, the values of the PLQY of a few less extensively characterized dyes were confirmed by our measurements.

The SAFER method is expected to be useful not only for measurements using LED excitation sources but also for more conventional lamp-monochromator excitation. For instance, xenon lamps have several very narrow and intense peaks in the spectral distribution of their light emission, and with a sufficiently large spectral bandwidth of the monochromator, the excitation light may be spectrally unevenly distributed over this bandwidth. The use of a calibrated transmitted light spectrometer will furthermore ensure strict wavelength calibration and precise monitoring of fluctuations in the excitation light.

Whereas the SAFER method presented here addresses the questions of broad-band excitation and excitation source stability, it keeps other limitations in common with the related standard and SAFE methods for relative measurements of PLQYs in dilute solution. It is affected by inner filter effects at optical densities that exceed 0.1. Also, as discussed, the measurement of weakly emitting fluorophores with low PLQYs poses the same challenges to the SAFER method as to the two other methods.

The automated numerical processing of digitized spectra helps the spectroscopist in avoiding approximations to fundamental expressions for the determination of photophysical parameters of molecular and nanoscale systems from spectroscopic measurements, in the present case the photoluminescence quantum yield.

6. Experimental details

6.1. Chemicals

Fluorescent dyes of spectroscopic purity were obtained from Sigma-Aldrich/Merck or Acros/ThermoFisher and used as received: 2-aminopyridine (2AP), 4-(dicyan omethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran (DCM), quinine bisulfate (QBS), fluorescein (Flscn), rhodamine 6G (Rh6G), pyranine, anthracene (Anth), Cresyl Violet (CV), Coumarin-153 (C153), Nile Red (NR). Solvents and aqueous solutions were supplied by Sigma-Aldrich/Merck and were of analytical or spectroscopic grade: water, perchloric acid (60 wt.% in water), aqueous sulfuric acid (0.5 M), aqueous sodium hydroxide solution (1 M), ethanol (EtOH, 96% with 4% water), methanol (MeOH, 99.8%). The neat solvents were carefully examined using extinction and fluorescence spectroscopies to ensure the absence of any background absorption and fluorescence at the wavelengths used. The acid and base solutions were further diluted with water.

Three recommended [1] PLQY reference solutions were used: QBS in 0.1M HClO_{4(aq)} [16], Flscn in 0.1M NaOH_(aq), and Rh6G in ethanol. Stock solutions were freshly prepared

for each measurement campaign. The sample fluorophores were dissolved in suitable solvents as follows: anthracene in ethanol, QBS in 0.05M H₂SO_{4(aq)}, pyranine in 0.01M NaOH_(aq), C153 in both methanol and ethanol, CV in methanol, and NR in methanol. Measurements were made on air-equilibrated solutions.

6.2. Spectroscopic set-up

The fluorimetric set-up (**Fig. 1** and **Supplementary Information Fig. S1**) consists of fiber-coupled modules. The excitation light source is chosen from a series of fiber-coupled LED modules with nominal peak emissions at 280 nm, 340 nm (Thorlabs), and 380 nm, 450 nm, 518 nm (OceanOptics). These LED sources have their emission in a relatively broadband around the nominal wavelength (**Supplementary Information, Fig. S2**). For the measurement of absorbance spectra alone, a tungsten-halogen (OceanOptics LS1) or Xe flash lamp (Avantes) was used.

The excitation light is fiber-coupled to a thermostated cuvette holder (Quantum Northwest qPod). The light emitted from the solution in the cuvette is collected at a right angle (90°) and coupled into a fiber that is connected to the emitted light spectrometer. The light transmitted through the cuvette (180°) is also coupled into a fiber that is connected to the transmitted light spectrometer.

The emitted light spectrum is measured using an OceanOptics QE65000 spectrometer (Peltier-cooled back-thinned CCD array detector, holographic grating, 350-1050 nm). The transmitted light is measured using an Avantes AvaSpec-ULS2048CL-EVO spectrometer (CMOS array detector, broad-range grating, 250-1100 nm). Corrected (transmitted, emitted) light intensity spectra $I(\lambda)$ in photon units are obtained from the raw spectrometer data $I_{\text{raw}}(\lambda)$ by first subtracting the 'dark' spectrum $I_{\text{dark}}(\lambda)$, measured in the absence of any excitation light, and subsequently applying correction factors $K_{\text{corr}}(\lambda)$.

$$I(\lambda) = K_{\text{corr}}(\lambda)[I_{\text{raw}}(\lambda) - I_{\text{dark}}(\lambda)] \quad (16)$$

The correction factors are obtained from the calibration of the measured spectrum against a reference light source. Two reference sources were used: a NIST traceable incandescent calibration light source (OceanOptics LS1-CAL) and a UV fluorophore emission standard (2-aminopyridine in 0.05M H₂SO_{4(aq)}, [18]). The reference spectra of the calibration sources were represented as relative spectral photon flux *vs* wavelength, so that the obtained wavelength-dependent correction factors $K_{\text{corr}}(\lambda)$ directly convert the dark-subtracted raw intensities $[I_{\text{raw}}(\lambda) - I_{\text{dark}}(\lambda)]$ into corrected spectra of relative spectral photon flux ('photon units'). Specific details of the calibration of $K_{\text{corr}}(\lambda)$ for both spectrometers used is described in the **Supplementary Information (Section S5)**.

For all experiments, standard 10 mm pathlength fluorescence cuvettes are used. These are cleaned by first soaking in 1% Hellmanex solution, followed by washing several times with pure water, spectrograde ethanol, and drying under a gentle flow of filtered air. The cuvette is rinsed with the chosen solvent just before filling it with the solvent. It is then inserted into the cuvette holder (qPod) which offers precise control of temperature, fixed to 23±0.2°C for all experiments. A little magnetic stirrer is used for mixing in the

concentrated analyte solution. Once the cuvette is inserted, it is not removed and re-inserted, further reducing experimental uncertainties.

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Authors contributions

TA prepared the samples, performed spectroscopic measurements, analyzed the data, collected the results, and wrote the original draft. AB and SG planned and supervised the research, provided materials, and validated the results. MHVW planned and supervised the research, formulated the method, set up the instruments, and validated the results. All authors revised and approved the manuscript.

Declarations

Conflict of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors

Consent to participate: Not applicable

Consent to publish: Not applicable

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Supplementary information for

Determination of Photoluminescence Quantum Yields in Dilute Solution using Non-Monochromatic Excitation Light

(dedicated to Prof. Dr. A. M. Brouwer on the occasion of his retirement)

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 - S5.** Spectral correction factors for the spectrometers used
- References

S1. Detailed drawing of the fluorimetric set-up for simultaneous measurement of light absorption and photoluminescence emission

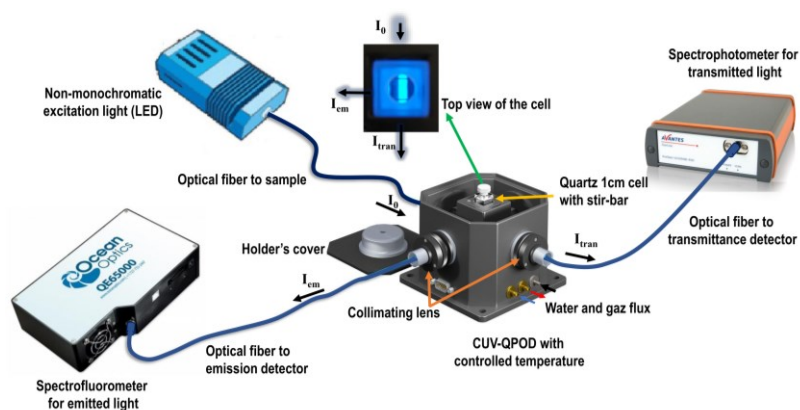


Figure S3. Set-up used for simultaneous light absorption and fluorescence emission measurements of limpid liquid samples for the determination of their photoluminescence quantum yields.

S2. Light emission spectra of LED excitation sources

In this work, different fiber-coupled LED light sources (OceanOptics, Thorlabs) were used to excite the fluorophores. These sources are designated by means of the wavelength of their emission maximum, but contrary to monochromatic light sources (lasers and lamp-monochromator combinations) the LEDs have relatively broad emission spectra, as shown in Fig. S2.

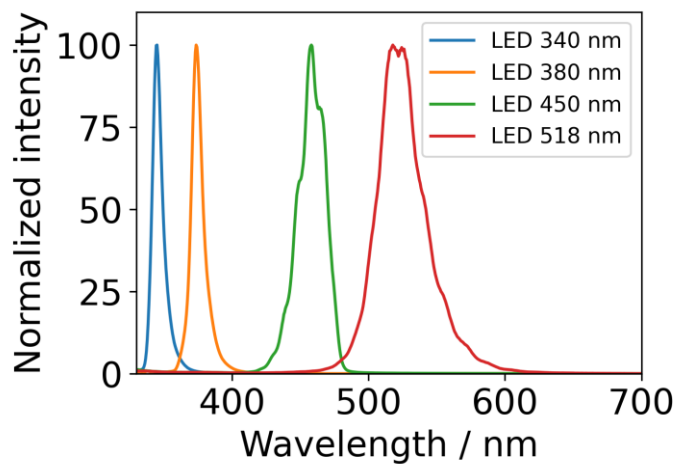


Figure S4. Corrected emission spectra (photon units) of the fiber-coupled LEDs used for excitation.

S3. Proportionality of the emitted light intensity and the integrated absorbed light intensity

Separate fluorescence measurements were performed in which the dye concentration was deliberately increased beyond the limit of OD 0.1, in order to illustrate the usefulness of successive measurements at increasing concentrations for validation of the PLQY result. An issue that may occur in the measurement of photoluminescence quantum yield is the re-absorption of the emitted light by the fluorophore, known as the “inner-filter effect” which leads to distorted emission spectra, and to loss of the proportionality between the emitted light and absorbed light intensities. Furthermore, aggregation or excimer formation might occur for certain compounds as their concentration is increased.

i. Cresyl violet in methanol

Aggregation is known to affect the PLQY of cresyl violet in methanol,[1] at concentrations above 1 μM (corresponding[2] to an optical density of 0.07 at the absorption maximum). Indeed, proportionality between absorbed and emitted light intensities are lost when going beyond this limit (Fig. S3). For PLQY measurement, we worked at concentration below 0.5 μM (Fig. S6)

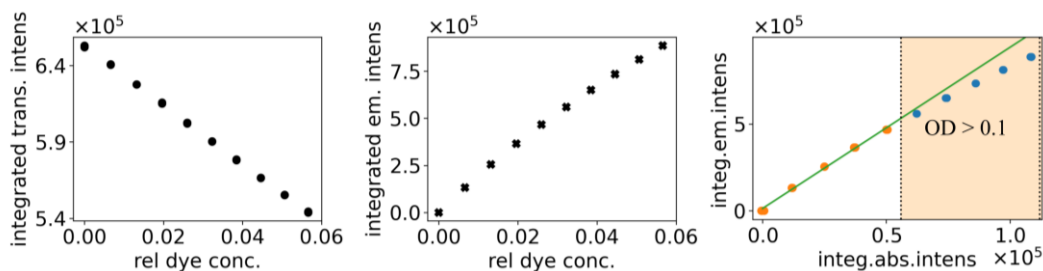


Figure S5. Spectrally-integrated transmitted and emitted light intensities (518 nm LED excitation) as a function of Cresyl Violet concentration in methanol (left and center columns), and the linearity (and loss of linearity) of the plot of the integral emitted light intensity vs the integral absorbed light intensity (right column).

ii. Fluorescein in 0.1 M aqueous NaOH (Fig. S4)

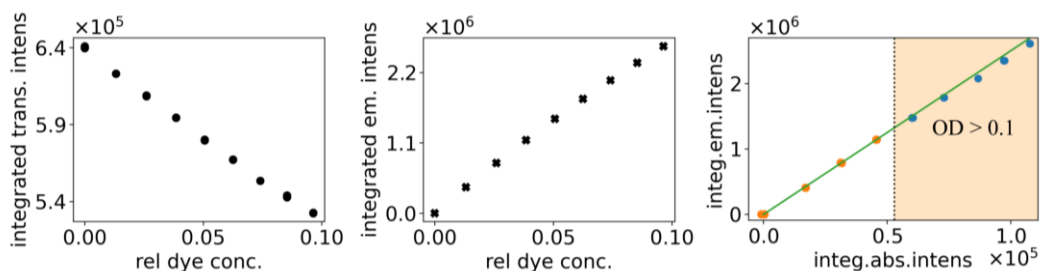


Figure S6. Spectrally-integrated transmitted and emitted light intensities (450 nm LED excitation) as a function of fluorescein concentration in 0.1M NaOH(aq) (left and center columns), and the linearity (and slight loss of linearity) of the plot of the integral emitted light intensity vs the integral absorbed light intensity (right column).

iii. Quinine bisulfate in 0.1M aqueous perchloric acid (Fig. S5)

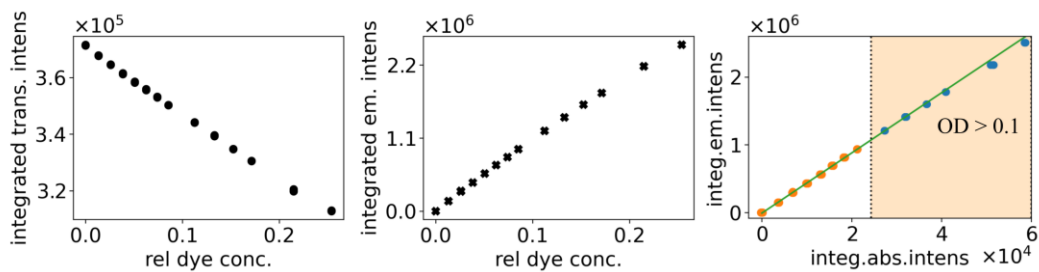


Figure S7. Spectrally-integrated transmitted and emitted light intensities (380 nm LED excitation) as a function of quinine bisulfate concentration in 0.1M $\text{HClO}_4(\text{aq})$ (left and center columns), and the linearity (and slight loss of linearity) of the plot of the integral emitted light intensity vs the integral absorbed light intensity (right column).

S4. Additional illustrations of SAFER PLQY measurements

S4.1. PLQY determination of Cresyl Violet in methanol (Fig. S6)

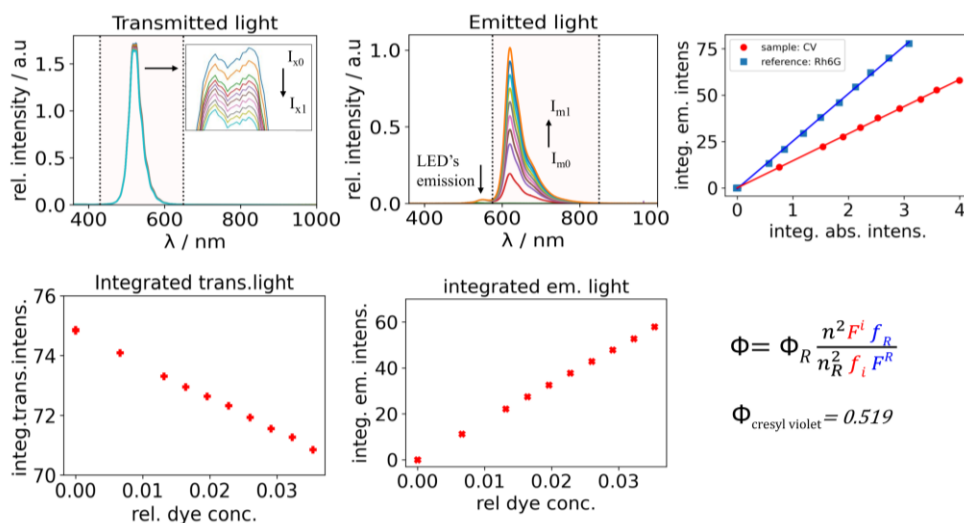


Figure S8. SAFER measurement of the PLQY of Cresyl Violet (CV) in MeOH with Rhodamine 6G in EtOH as a reference. The excitation source was a 518 nm LED. Above $\sim 1 \mu\text{M}$, the PLQY of CV is affected by dye aggregation. Here, we worked at CV concentrations $< 0.5 \mu\text{M}$. Left column: corrected transmitted light spectra and integrated intensities; center column: corrected emitted light spectra and integrated intensities. Only the spectra of the CV sample are shown. Right column: plots of integrated emitted light vs absorbed light intensities for sample and reference, with the determination of PLQY via Eqn. (7). The value given in the figure is for one measurement. From three independent measurements, we obtained $\Phi_{\text{CV}} = 0.53 \pm 0.03$.

S4.2. PLQY determination of anthracene in ethanol (Fig. S7)

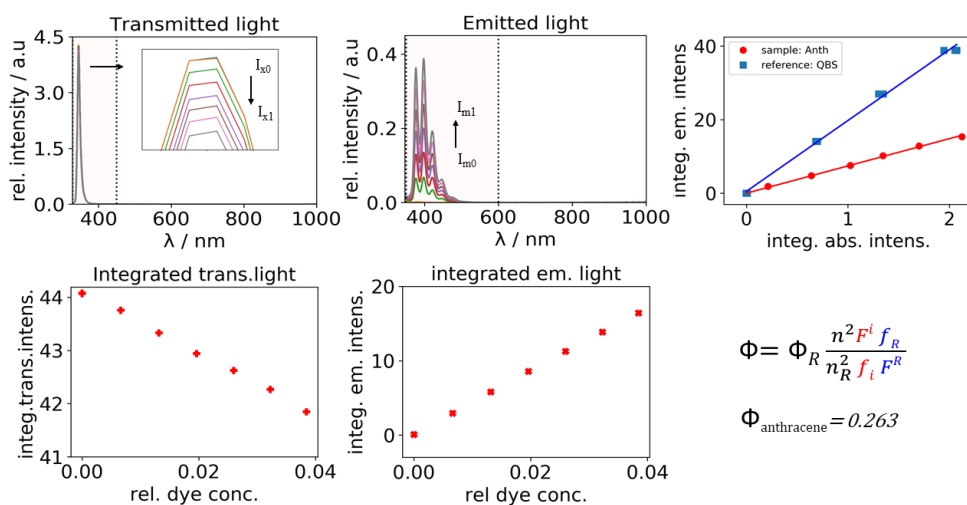


Figure S9. SAFER measurement of the PLQY of anthracene in ethanol with QBS in HClO_4 as a reference. The common excitation source was a 340 nm LED. Left column: corrected transmitted light spectra and integrated intensities; center column: corrected emitted light spectra and integrated intensities. Only the spectra of the anthracene sample are shown. Right column: plots of integrated emitted light vs absorbed light intensities for sample and reference, with the determination of PLQY via Eqn. (7). The value given in the figure is for one measurement. From three independent measurements, we obtained $\Phi_{\text{anth}} = 0.26 \pm 0.02$.

S5. Spectral correction factors for the spectrometers used

S5.1. Emitted light spectrometer (Ocean Optics QE65000)

The correction factors for the fiber-coupled OceanOptics QE65000 spectrometer (HC1 grating, spectral range 350...1050 nm), which is used for measuring the spectra of the emitted light, were determined using two principal emission reference standards. For the range 350...430 nm, the reference standard was a solution of 2-aminopyridine (2AP) in 0.05M H₂SO₄(aq) whose calibrated fluorescence emission spectrum in photon units has been reported in the literature [3]. Its fluorescence was excited using a fiber-coupled 280 nm UV LED (Thorlabs M280F5).

For the range 410...1050 nm, a fiber-coupled incandescent tungsten-halogen calibration lamp (OceanOptics LS1-CAL) was used. The spectral intensity data for this tungsten-halogen light source is traceable to NIST. The supplied spectral irradiance calibration data were converted to relative spectral photon flux ('photon units'). The photon unit values were interpolated between wavelengths using spline interpolation.

The two sets of correction factors obtained *via* Eqn. (16) using the reference sources (2AP resp. LS1-CAL) show very good agreement in the region of spectral overlap. This was used to scale the data sets with respect to each other to make a smooth junction. The resulting merged data set was then scaled such that the minimal value of the set is 1.0. The wavelength of this minimum is where the detection system has the highest overall quantum efficiency. Graphs of the final correction factors are shown in **Fig. S8**.

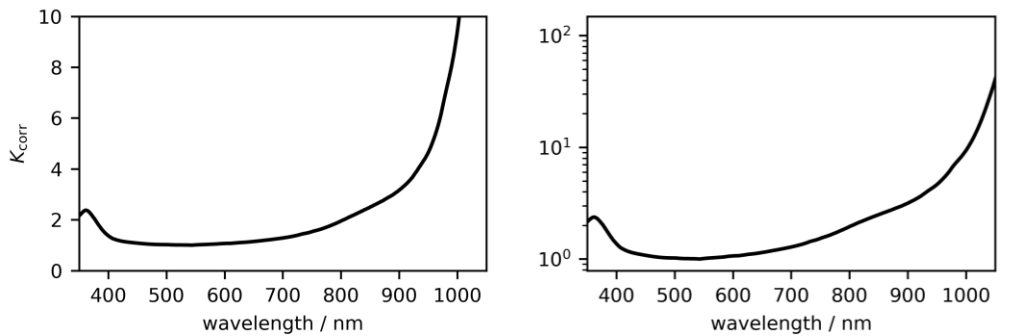


Figure S8. Correction factors $K_{corr}(\lambda)$ for the QE65000 spectrometer (350...1050 nm) on linear (left) and semi-logarithmic (right) scales.

The correction curve for this spectrometer is smooth and reasonably flat up to 920 nm. The spectrometer has good sensitivity in the near-infrared: at 1000 nm the photon detection efficiency is still 10% of the maximum detection efficiency (which is reached near 540 nm)

Additionally, the grating efficiency and detector efficiency data were used to create an alternate set of correction factors. These data were digitized from the documentation supplied by the manufacturer and interpolated using spline interpolation. There was good agreement between these alternate corrections factors and the original correction factors.

Finally, the correction factors were checked by recording fluorescence emission spectra of samples whose spectra have been accurately described in the open literature. For quinine bisulfate in 0.1M HClO₄(aq), a reference emission spectrum has been established and published by the United States National Institute of Standards and Technology (NIST) [4, 5]. The laser dye DCM in methanol is a red-emitting reference. Its corrected emission spectrum has been measured and published by Gardecki and Maroncelli [6].

The agreement between the published spectra and our corrected spectra is very good (**Fig. S9**) indicating the accuracy of the correction factors determined for the QE65000 spectrometer, and consensus among different calibration sources and spectra from the literature.

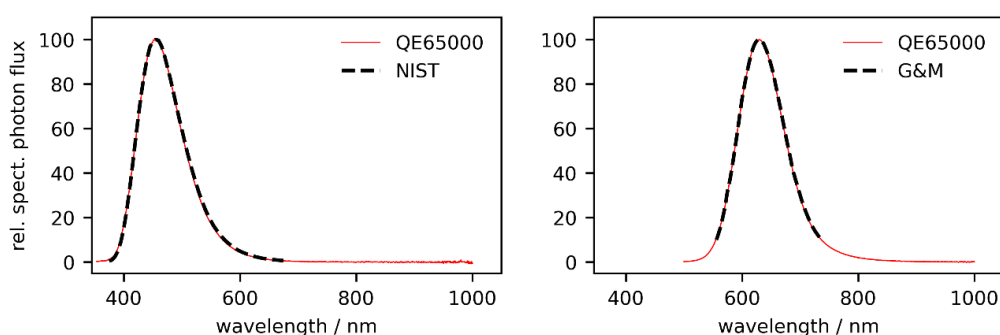


Figure S9. Comparison of corrected fluorescence emission spectra measured using QE65000 spectrometer (solid red line) and spectra from the literature (dashed black line, see Text). Left panel: QBS in 0.1M HClO₄(aq), excited with 380nm LED. Right panel: DCM in methanol, excited with a 450 nm LED.

S5.2. Transmitted light spectrometer (Avantes ULS2048CL-EVO)

The fiber-coupled spectrometer used for measuring the spectrum of the transmitted excitation light (Avantes ULS2048CL-EVO) covers a slightly wider wavelength range (325...1080 nm) than the QE65000 spectrometer. It is equipped with a 25 μ m slit, which leads to higher spectral resolution and lowers sensitivity. The lower sensitivity is a desirable property in this case because of the intensity of the excitation light. The calibration procedure used is very similar to the one described above, using 2AP for the UV and the LS1-CAL tungsten-halogen source for the Vis-NIR window. The correction factors obtained are shown in **Fig. S10**.

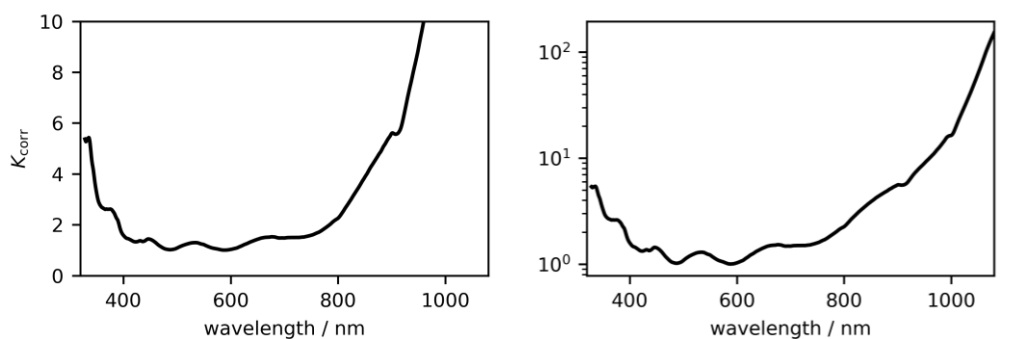


Figure S10. Correction factors $K_{corr}(\lambda)$ for the ULS2048CL spectrometer (325...1080 nm) on linear (left) and semi-logarithmic (right) scales.

The correction factor curve for the ULS2048CL spectrometer shows more structure than the curve for the QE65000 spectrometer. This is due to the ULS2048CL having a CMOS sensor array whereas the QE65000 has a back-thinned CCD array. Over the years CMOS light sensors, in particular image sensors, have been the subject of significant improvement in sensitivity, signal-to-noise ratio, read-out speed, and electronic flexibility, and can now advantageously replace CCD sensors in several spectroscopy applications. However, (back-thinned) CCDs are still superior in terms of homogeneity, dark current, and smoothness of spectral response.

The correction factors determined were checked by measuring corrected emission spectra using the ULS2048CL, like previously done for the QE65000 spectrometer. For this, the spectrometer was temporarily connected to the fiber carrying the emitted light signal. Again, there is very good agreement between our corrected spectra and those reported in the literature (**Fig. S11**). The recorded emission spectra are somewhat noisier due to the limited sensitivity of this spectrometer, which is configured for transmitted light (absorbance) measurements.

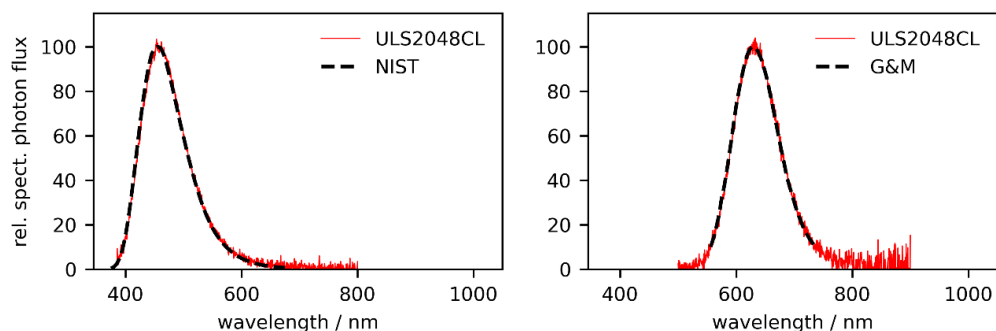


Figure S11. Comparison of corrected fluorescence emission spectra measured using ULS2048CL spectrometer (solid red line) and spectra from the literature (dashed black line, see Text above). Left panel: QBS in 0.1M HClO₄(aq), excited with 380nm LED. Right panel: DCM in methanol, excited with a 450 nm LED.

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