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Relative and absolute determination of fluorescence quantum yields of transparent samples

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Luminescence techniques are among the most widely used detection methods in the life and material sciences. At the core of these methods is an ever-increasing variety of fluorescent reporters (i.e., simple dyes, fluorescent labels, probes, sensors and switches) from different fluorophore classes ranging from small organic dyes and metal ion complexes, quantum dots and upconversion nanocrystals to differently sized fluorophore-doped or fluorophore-labeled polymeric particles. A key parameter for fluorophore comparison is the fluorescence quantum yield (Φ_f), which is the direct measure for the efficiency of the conversion of absorbed light into emitted light. In this protocol, we describe procedures for relative and absolute determinations of Φ_f values of fluorophores in transparent solution using optical methods, and we address typical sources of uncertainty and fluorophore class-specific challenges. For relative determinations of Φ_f , the sample is analyzed using a conventional fluorescence spectrometer. For absolute determinations of Φ_f , a calibrated stand-alone integrating sphere setup is used. To reduce standard-related uncertainties for relative measurements, we introduce a series of eight candidate quantum yield standards for the wavelength region of ~350–950 nm, which we have assessed with commercial and custom-designed instrumentation. With these protocols and standards, uncertainties of 5–10% can be achieved within 2 h.

INTRODUCTION

In the last few decades, luminescence techniques evolved into some of the most popular analytical and detection tools in the life and material sciences owing to their sensitivity, comparable ease of use, relatively inexpensive instrumentation, as well as for their suitability for multiplexed analysis; for combined spectrally, temporally and spatially resolved measurements; and for remote sensing^{1–5}. The toolbox of fluorophores for a broad variety of applications and targets in the UV-visible (vis) and in the near-IR (NIR) spectral region is ever growing^{6–12}, especially with fluorescent proteins^{7,13}, semiconductor nanocrystals (so-called quantum dots)^{10,14,15} and, more recently, upconversion nanocrystals^{16,17} gaining in importance. Typical examples for popular fluorophores include laser dyes and reactive dyes for the labeling of peptides, proteins and (oligo)nucleotides^{10,18}; fluorescent proteins for cell studies^{7,13,19}; fluorophore-labeled biomolecules such as secondary antibodies for immunohistochemistry and flow cytometry; as well as DNA hybridization probes^{18,20–22} and contrast agents for *in vivo* NIR fluorescence imaging of pathological changes^{12,23,24}. In addition, the optical properties of an ever-increasing number of sophisticated probes and sensors can be modulated selectively by chemical or biological inputs^{11,25–28}. Other examples are multichromophoric reporters such as fluorophore-doped or fluorophore-labeled polymeric particles and multimodal systems for the readout with different detection methods or suspension assays^{29–32}. Dye classes commonly used for such applications include small organic dyes^{6,10} and metal ligand complexes³³ (e.g., transition and rare earth metal ion complexes), fluorescent proteins^{7,13,19}, semiconductor nanocrystals^{10,14,15,34,35}, and fluorophore-doped or fluorophore-labeled polymeric particles^{29–31}; recently upconversion nanocrystals have also been used^{16,17}.

The design of such functional fluorophores, as well as their application and the validation of analytical methods relying

on their use, requires the spectroscopic characterization of the optical properties of these materials under application-relevant conditions. This comprises the measurement of absorption and emission spectra, as well as the determination of molar absorption coefficients ϵ (at the absorption maximum and the excitation wavelength of choice, λ_{ex}) and photoluminescence quantum yields (termed here fluorescence quantum yields, Φ_f) in representative environments.

One of the most important parameters for comparing fluorophores is the fluorescence quantum yield. Φ_f is the direct measure of the efficiency of the conversion of absorbed photons into emitted photons. The product of Φ_f and the molar absorption coefficient at the excitation wavelength ($\epsilon(\lambda_{\text{ex}})$) gives the fluorophore's brightness B ($B = \Phi_f \times \epsilon(\lambda_{\text{ex}})$), which determines the analytical sensitivity from the fluorophore side^{10,36}. Moreover, knowledge of Φ_f is required for the calculation of the efficiencies of fluorescence energy transfer processes²⁰. Hence, Φ_f values of commonly used and newly developed fluorophores are of considerable interest for the bioanalytical and medicinal community, as well as for researchers in the materials sciences.

Relative versus absolute measurement of quantum yields

The fluorescence quantum yield of transparent samples such as solutions of molecular fluorophores, most fluorophore-labeled biomolecules and small-sized quantum dots can be determined with optical methods either relative to a fluorescent standard of known fluorescence quantum yield^{36–41} or absolutely with an integrating sphere setup^{42–49}. The most widely used relative optical method relies on the comparison of integral emission spectra of the sample and the standard obtained under identical measurement conditions for solutions of known absorbances or absorption factors⁵⁰ at the excitation wavelength

(see PROCEDURE Step 1A)^{37,38,41,48}. This necessitates only common laboratory equipment, i.e., a conventional absorption spectrometer and a conventional fluorescence spectrometer^{41,48}. In the case of absolute measurements, Φ_f is obtained directly in a single measurement without the need for a quantum yield standard (see PROCEDURE Step 1B)^{47–49}. This principally very attractive method is currently gaining importance, as stand-alone integrating sphere setups are increasingly becoming commercially available^{43,47}. Moreover, it is the only option for the measurement of fluorescence quantum yields of transparent samples absorbing or emitting in wavelength regions for which no reliable quantum yield standards are available, as is the case for IR emitters (emission wavelengths > 950 nm)⁵¹. It is mandatory to take an absolute measurement of Φ_f for materials such as upconversion nanocrystals excited at 980 nm (ref. 52), which absorb two or more photons before light emission and thus reveal excitation-power density-dependent photoluminescence quantum yields; for quantum yield measurements of all scattering samples such as nanoparticles or certain bioconjugates; as well as for solid samples, such as films or powders used as converter materials^{45,53,54}.

The main differences between the relative and absolute determination of fluorescence quantum yields originate from the fact that a conventional fluorescence spectrometer can detect only a certain fraction of the emitted light. The size of this fraction depends on many different factors, including the numerical apertures for excitation and the solid angle for detection, the emission wavelength, the emission anisotropy, the refractive index of the solvent, the scattering of the sample and the sample geometry, and it is thus impossible to quantify. The use of a fluorescence spectrometer therefore requires a standard with known Φ_f and with optical properties closely matching those of the investigated sample. An integrating sphere, however, detects all light emitted from the excited sample, and hence allows for the absolute measurement of the fluorescence quantum yield by simply comparing the number of emitted photons with the number of absorbed photons. The number of absorbed photons follows from the decrease in the incident excitation light intensity (measured with a blank at the sample position) caused by the absorbing sample in the integrating sphere. Photon numbers in this respect always refer to relative photon numbers that are sufficient for the calculation of the fluorescence quantum yield. In the following sections, we omit the term 'relative' for reasons of readability^{47,48}.

Alternative methods

The fluorescence quantum yields of transparent fluorophore solutions can also be obtained indirectly from measurements of the dissipated heat by applying photoacoustic spectroscopy (PAS) and thermal lensing techniques or other calorimetric methods^{46,55,56}. PAS and thermal lensing techniques, which commonly use intense lasers as excitation light sources and require a non-emissive reference with absorption and thermal properties of the solvent matching those of the sample, rely on more specialized, more expensive and typically custom-designed equipment and are thus not as popular as optical methods^{46,57}. Among these photothermal methods, only PAS is suitable for the determination of the fluorescence quantum yields of scattering samples⁵⁵.

Method development

The general importance of Φ_f data in conjunction with often-encountered difficulties in reliably measuring such values motivated us to assess procedures and achievable uncertainties for the straightforward determination of this quantity. In doing so, we focused on optical methods, owing to their widespread application and the comparatively simple and commercially available equipment required.

To address common measurement difficulties, we designed and developed protocols for the relative and absolute determination of fluorescence quantum yields of transparent fluorophore solutions that minimize the most relevant and common sources of uncertainty such as the accurate consideration of the wavelength-dependent instrument responsivity, reabsorption effects and the Φ_f value of the quantum yield standard. Simple procedures for the determination of the spectral responsivity of fluorescence spectrometers relying on commercialized spectral fluorescence standards are provided, which were assessed by several National Metrology Institutes and field laboratories^{58,59}. With these optimized protocols and new standards, we could measure fluorescence quantum yields relatively and absolutely with measurement uncertainties on the order of $\pm 4\%$ (for $\Phi_f > 0.10$) in the wavelength region of $\sim 400\text{--}950$ nm (refs. 48,49).

For relative measurements of Φ_f , the limited reliability of the fluorescence quantum yields of fluorophores, which absorb and emit outside the wavelength region of relatively well-established quantum yield standards such as rhodamine 101, fluorescein and rhodamine 6G (ref. 60), can be circumvented with the aid of a chain of Φ_f transfer standards made from several dyes. Such a chain of transfer standards can be created by measuring the fluorescence quantum yields of several dyes pairwise, starting from a standard of reliably known Φ_f (ref. 48). This approach can be used to cover an extended wavelength region in absorption and emission. Transfer chains were demonstrated by us for relative measurements of Φ_f in the vis and NIR region^{47–49}. The working principle of transfer chains follows principally, e.g., from Reagent Setup (see 'Dyes'). For example, we linked quinine sulfate dihydrate via coumarin 153 (C153), fluorescein (F) and rhodamine 6G (R6G) to R101 (ref. 49), and the NIR dye IR125 versus HITCI, oxazine 1 (OX1) to rhodamine 101 (R101)⁴⁸, respectively. Prerequisites for such a transfer chain approach are dyes with excitation wavelength-independent quantum yields that can be excited pairwise at the same wavelength.

Limitations

Despite the broad applications of these protocols for transparent fluorophore solutions, care is required for the determination of the fluorescence quantum yields of luminescent lanthanide complexes and chelates, as the excitation and emission processes in these materials involve several steps^{33,61}.

Quantum yields of fluorescent molecules can be measured in air-saturated solutions, as is the case for most measurements, or in deoxygenated solution. As fluorescence is an optically allowed transition, yielding short fluorescence lifetimes of 10 ns or less in the vast majority of cases, the quantum yields and lifetimes of fluorescent molecules such as small organic dyes or fluorescent proteins are not (or only barely) sensitive to the presence of oxygen. The classical example, which reveals an oxygen-sensitive

quantum yield and lifetime, is pyrene, which for such an organic dye has an extremely long fluorescence lifetime, exceeding 100 ns. In addition, a small oxygen dependence of the quantum yield of diphenylanthracene has been reported^{62–64}. Special care has to be taken for emitters such as certain transition metal ion complexes (e.g., Ru(II) or Ir(III) complexes) that have partly or completely forbidden optical transitions and thus very long lifetimes of their excited states, as well as for the measurement of phosphorescence quantum yields (Φ_p) of organic dyes. The intrinsically longer luminescence or phosphorescence lifetimes of these emitters (on the order of several hundred ns up to a few ms) favor collisional luminescence quenching by oxygen and thus result in oxygen-dependent quantum yields. In this respect, it needs to be also considered that the solubility of oxygen is solvent dependent. For measurements in deoxygenated solution, care must be taken to completely remove oxygen from the dye solution, e.g., by pump-freeze-thaw cycles or by bubbling of inert gases (nitrogen or argon) through the dye solution. In the latter case, purging should be stopped during the luminescence measurements in order to avoid intensity fluctuations in the emission caused by light scattering and refraction by the gas bubbles. In any case, it must be clearly stated under which conditions the fluorescence quantum yield was measured and how deoxygenation was performed.

We do not recommend front-face measurements for the determination of photoluminescence quantum yields, because for this measurement geometry the determination of the number of absorbed photons is too error prone. Such measurements typically require different (i.e., higher) dye concentrations than those used for fluorescence measurements in 0°/90° measurement geometry and integrating sphere measurements. Moreover, the penetration depth of the excitation light depends on dye absorption and thus dye concentration. Front-face measurements should be used only for the determination of emission profiles and at maximum for a rough estimate of relative fluorescence quantum yields using dye and standard concentrations of high and matching absorbances. In addition, the use of higher concentrated dye solutions always requires careful control of aggregation phenomena that are detailed separately in the TROUBLESHOOTING section.

The measurement of very small quantum yields (e.g., <0.01) relative to a moderately to highly emissive standard (quantum yields >0.2) using identical fluorometer settings can also be crucial. A recently reported procedure introduces the use of a neutral density filter in the excitation pathway for the reference, but not for the sample⁶⁵. Other alternatives may be the use of different sample and standard absorbances or the use of an attenuator (ideally with a wavelength-independent transmission profile in the emission channel that needs to be assessed with an absorption spectrometer). In the latter case, the transmission profile of the attenuator can also affect the spectral shape of the dye's emission band, which needs to be subsequently considered. All these procedures may result in enhanced measurement uncertainties.

Quantum yield standards

We introduce recently evaluated quantum yield standards for the spectral region of ~350–950 nm (refs. 48,49,57) that meet the requirements on such standards recently defined in a technical note by the IUPAC⁶⁶ (see 'Dyes' in Reagent Setup). Here we deliberately chose dyes and solvents that are commercially available at a reasonable price in reliable purity and measurement

conditions (e.g., air-saturated solutions, no additional dye purification steps), which enables the straightforward use of these protocols and tools.

As recently discussed and demonstrated, literature values for quantum yields of dye solutions often differ considerably even for well-established dye classes such as coumarins^{46,60}. Reasons for these deviations may be the determination of the instrument's spectral responsivity, which is required for the spectral correction of measured emission spectra for instrument-specific effects. In addition, these deviations can be affected by the purity of the dyes and/or solvents used (or in the case of, e.g., water, the pH). Also, the concentration of the dye and the solvent itself can influence recorded signals and may lead to misinterpretation⁶⁷. Moreover, environmental conditions such as the temperature may have a major effect on the determined quantum yield of a dye solution. A pronounced temperature dependence is frequently observed for dyes containing freely rotatable groups, which are involved in processes affecting the nonradiative deactivation of the excited state. The most prominent example here is rhodamine B^{68,69}. Two reviews summarizing potential quantum yield standards from the literature were published by the International Union of Pure and Applied Chemistry (IUPAC)^{60,70}. Other frequently discussed quantum yield standards for the red wavelength region are rhodamine 6G (refs. 71,72), rhodamine 101 (refs. 60,72) and cresyl violet⁷³. Cresyl violet should be used with care, because for this dye we observed slight stability problems, although we have not studied this issue systematically.

Still-existing limitations include the lack of evaluated quantum yield standards for the UV region (i.e., for the spectral region from ~250 to 400 nm) and for the NIR (i.e., for the spectral range >700 nm). The former is also related to the fact that in the UV region for wavelengths below ~300 nm, the absolute determination of fluorescence quantum yields becomes extremely difficult, and most manufacturers of integrating sphere setups and accessories face problems with measurements in this so-called 'naphthalene wavelength region.' For excitation wavelengths >300 nm, we recommend quinine sulfate dihydrate as the quantum yield standard^{47,48,74}. We do not recommend anthracene and diphenylanthracene because of the slight sensitivity of their quantum yields to the presence of oxygen^{62–64}. Moreover, their structured absorption and emission spectra render measurements with these dyes very sensitive to possible uncertainties in the wavelength scales of the different spectrometers used. For fluorophores absorbing and/or emitting at shorter wavelengths, such as tryptophan or tyrosine, our protocol for relative measurements can be used to create a transfer chain of dyes (see 'Method development' above).

General remarks

Radiometric and spectroscopic basics. Measured fluorescence spectra always contain instrument-dependent and sample-specific contributions and are thus termed uncorrected spectra I_u (refs. 75,76). Subtraction of a blank spectrum (I_b) obtained for the fluorophore-free solvent or matrix (under identical measurement conditions to those used for the sample) from the measured fluorescence spectrum of the sample yields a blank- or background-corrected fluorescence spectrum (consideration of, for example, scattering and fluorescence from the solvent and dark counts at the detector). Subsequent correction for the fluorometer's

PROTOCOL

wavelength- and polarization-dependent responsivity (spectral responsivity $s(\lambda)$), which is an instrument-specific quantity, leads to a corrected spectrum I_c that is instrument-independent (equation 1)^{75,76}. This procedure is termed spectral correction or, in the case of emission spectra, emission correction.

$$I_c(\lambda) = \frac{I_u(\lambda) - I_b(\lambda)}{s(\lambda)} \quad (1)$$

As the fluorescence quantum yield is the ratio of the number of emitted to the number of absorbed photons, all fluorescence spectra have to be converted to a (relative) number of photons per unit time (i.e., a photon flux). To transform a radiometric quantity X (i.e., power; energy per unit time) into the corresponding photonic quantity X_p (i.e., photons per unit time; unit is s^{-1}), the radiometric quantity has to be multiplied with $\lambda/(hc_0)$, where h is the Planck constant and c_0 is the velocity of light *in vacuo* (equation 2), respectively. As the term hc_0 cancels out in the subsequent calculation of the quantum yield (absolute and relative determination), it is omitted in the equations used in this protocol. For integrating sphere measurements, the procedures described above must also comprise the wavelength region of the excitation providing the relative number of absorbed photons.

$$X_p = X \cdot \frac{\lambda}{hc_0} \quad (2)$$

Care has to be taken when you use an emission correction implemented by the spectrometer manufacturer into the fluorescence spectrometer, and in general when you use all kinds of built-in automatic correction procedures. Some manufacturers already included the multiplication with λ in their emission correction curves (i.e., their emission correction curve refers to the spectral photon flux of the reference light source used for instrument calibration^{75,76}; see also **Supplementary Methods**). Thus, after performance of the spectral correction, the resulting corrected emission spectrum is already given in photonic quantities (photons per unit time). Here, multiplication with λ must be omitted. The expression of 'photons per unit time' originates from the physical units of the photon flux. Owing to the calibration described in the **Supplementary Methods** of the manuscript, relative quantities are always determined. To determine the total number of photons, an absolute calibration is necessary, which is even more challenging than the procedures described here and not necessary for the determination of photoluminescence quantum yields. In fact, the calculation depends on the detector and the method of detection (i.e., photon counting versus analog (photocurrent) detection mode). For example, a photodiode measures a photocurrent and provides a signal in Amperes (A), which is, for a given wavelength (and measured within the linear range of the detector), proportional to the photon flux (unit $1 s^{-1}$). In contrast, a photomultiplier operated in the photon-counting mode and a CCD array yield relative counts. The number of counts depends on the integration time (and threshold settings) used for signal detection. This quantity (counts) may be converted into the number of photons collected within the integration time. For this reason, we used the general

TABLE 1 | Symbols and units.

Symbol	Name	Units
$I_u(\lambda_{em})$	Uncorrected spectrum	Counts per nm
$I_c(\lambda_{em})$	Corrected spectrum	Counts per nm
F	Relative integrated photon flux	s^{-1}
$s(\lambda_{em})$	Relative spectral responsivity	Counts per W
Φ_f	Fluorescence quantum yield	1
N_{abs}, N_{em}	Number of photons (absorbed, emitted)	1
f	Absorption factor	1
$A(\lambda_{ex})$	Absorbance	1
$\varepsilon(\lambda_{ex})$	Molar (decadic) absorption coefficient	$dm^3 cm^{-1} mol^{-1}$
N	Refractive index	1

expressions for the quantum yield determination in terms of photons per unit time or photon flux.

Symbols and terminology. Table 1 summarizes the symbols and units that are used in this protocol.

Experimental design

In the following sections, we describe suitable procedures for the relative and absolute determination of fluorescence quantum yields of transparent fluorophore solutions; the most crucial steps, with detailed protocols and recommended quantum yield standards, as well as common pitfalls (see TROUBLESHOOTING), are highlighted in the PROCEDURE. For relative measurements, we describe here only the use of identical excitation wavelengths for sample and quantum yield standards; this is because the otherwise-mandatory excitation correction, which considers the different spectral radiant fluxes at the chosen excitation wavelengths⁴⁸, can introduce high uncertainties for inexperienced users of fluorescence techniques. Moreover, with the fluorescence quantum yield standards (Equipment Setup) presented here, which cover the wavelength region of ~350–950 nm, there is no need for the application of different excitation wavelengths for the sample and the standard in order to characterize the vast majority of fluorophores used in the life and material sciences.

Prerequisites for the relative and absolute determination of fluorescence quantum yields following these procedures are (i) control of the wavelength accuracy of the excitation and emission channel of all instruments used (i.e., in the case of relative measurements, absorption and fluorescence spectrometers, and for absolute measurements, an integrating sphere setup), (ii) operation of the detectors of these instruments in their linear range (which should be previously determined^{76,77}), and (iii) knowledge of the (relative) spectral responsivity (emission correction curve) of the spectrometer's detection channel (**Supplementary Methods**). This quantity can be determined with, e.g., spectral fluorescence

standards that are commercially available^{41,48,58,59,75–77}; also see **Supplementary Methods**. Suitable quantum yield standards that we have assessed and recommended for relative measurements of fluorescence quantum yields in the wavelength region of ~350–950 nm are summarized in the Equipment Setup section and detailed in the **Supplementary Data**^{47–49,57,77}.

Sample preparation. To prepare a suitable sample, some basic principles have to be considered. To determine fluorescence quantum yields, always use fresh solutions made from high-purity dyes and solvents, which should be prepared with care using clean equipment (pipettes, sample containers, measurement cells and so on). Ensure that the dye is completely dissolved without adsorption onto the cell windows. Samples should always be stored in the dark, either at room temperature (25 °C), when they are sufficiently thermally stable, or in the refrigerator. Only fresh solvents of the highest purity (spectroscopic grade) should be used; see also Reagent Setup ('Dye solutions').

Quick tests indicating an improper preparation of the samples absorbing in the visible spectral range without the use of spectroscopic equipment are as follows.

- **Transparency.** Is the solution not completely clear when light is shining through the measurement cell? Is the original color of a beam (no fluorescence) of, e.g., a red laser pointer visible (compare with solvent only)? If yes, scattering centers are present. Scattering centers point to the possible presence of dye aggregates. Size-exclusion filters can be used to remove aggregates (e.g., PVDF HPLC filters are available in various sizes).
- **Concentration.** Is the solution strongly colored? If yes, the concentration may be too high and the solution should thus be diluted.

- **Complete dissolution.** Are there particle or granular residues? If yes, wait, dilute the solution or carefully increase the temperature slightly or use an ultrasonic bath.

The use of the latter two methods depends also on the stability of the respective compound. More hints to avoid sample-related problems and examples of measurement data indicating pitfalls are presented in the PROCEDURE and TROUBLESHOOTING sections.

Handling. Always take special care to avoid contact with the surface of the integrating sphere, and ensure that the integrating sphere is not contaminated with the sample. Always store the integrating sphere properly closed in between measurements to avoid the intake of dust particles or other possible environmental contamination. Contamination may influence the calibration and reduce the sensitivity of the measurement system. In particular, fluorescent contaminants are difficult to remove and can ruin the integrating sphere.

Always handle the standards used for calibration exactly as described in the manuals, and follow each step carefully.

Data evaluation. For data evaluation, software that can perform basic mathematical operations such as multiplication, division, summation and subtraction is needed. We recommend, e.g., Excel, Origin, MATLAB or software from the spectrometer manufacturer. Formulas that include integration of a function or spectrum can be executed as a summation of the wavelength-dependent data normally plotted as ordinate data (*y* axis). For the calculation of the integral as a sum, it is necessary to use spectra with equally spaced data points. Otherwise, an interpolation is necessary to obtain equally separated data points before the summation.

MATERIALS

REAGENTS

- Ethanol
- DMSO
- NaOH (Merck)
- Fluorescence reference standards (see **Table 2**)

EQUIPMENT

- Thin-layer chromatography (TLC) or HPLC system (preferably use diode array detector and fluorescence detector)
- Quartz cuvettes, 10 mm × 10 mm (e.g., Hellma, Hamamatsu)
- Absorption spectrometer (e.g., Varian, CARY 5000)
- Fluorescence spectrometer (e.g., Spectronics Instruments 8100)
- Integrating sphere setup (e.g., custom-made from BAM or from Hamamatsu)
- Software for data analysis (e.g., Excel, MATLAB)
- Pipettes (e.g., Eppendorf)

REAGENT SETUP

Dyes Generally, use only commercial fluorophores of the highest purity available. For molecular fluorophores, we strongly recommend control of dye purity before use by, e.g., thin-layer chromatography (TLC) or HPLC (preferably, use a diode array detector and a fluorescence detector; at a minimum, measure absorbance at a typical wavelength and at an example chosen wavelength in the UV range below 300 nm for a very stringent purity check) for the fluorescence standards summarized in **Table 3** and **Figure 1** (refs. 48,49). The reference dyes we used and their purity measures we obtained by HPLC (HPLC system from Knauer equipped with a diode array detector) are detailed in **Table 2** (refs. 48,49) (only data obtained at selected detection wavelengths are shown). **Figure 1** shows the absorption and emission spectra of these standards.

As the recommended and suitable quantum yield standards, we provide examples for dyes that are commercially available at a reasonable price, thereby also avoiding the need for additional purification procedures such as TLC or HPLC, which require specific chemical expertise and laboratory equipment. This enables the broad community of users of fluorescence techniques to use these materials when there are no certified fluorescence quantum yield standards available. Although dyes of a purity of 99% or higher are ideal in principle, such materials are difficult to obtain for the complete wavelength region covered by our recommended standards. To minimize purity-related uncertainties, we provide information on the materials we have used, including dye manufacturer and batch number, as well as on dye purity under the measurement conditions we used.

The fluorescence quantum yields (Φ_f) of the recommended reference dyes (**Table 3**) were calculated from integrated, blank-corrected, and spectrally corrected emission spectra using different fluorescence standards for the relative measurements (use of a chain of Φ_f transfer standards and identical excitation wavelengths for the respective sample/standard pair) or for an integrating sphere setup^{48,49}. For each compound-solvent pair, we always determined the quantum yield at least twice, often using concentration series for dyes with a small Stokes shift to enable a reabsorption correction^{47–49}. To determine relative s.d., we made at least six independent measurements per dye. In all cases, we used oxygen-saturated solutions (air pressure 101 kPa, temperature 25 °C) to ease measurement reproducibility.

Solvents Generally, only solvents of the highest purity commercially available are recommended; these solvents should be free of fluorescent impurities. This is typically the case for spectroscopy- or liquid chromatography-grade solvents. Check solvent purity first by measurement of an emission spectrum at



TABLE 2 | Reference dyes used as standards and dye purity assessed by HPLC shown for selected representative detection wavelengths.

Dye	Abbreviation	Source	λ (nm) ^a	Purity (%)
Quinine sulfate dihydrate	QS ^b	National Institute of Standards and Technology (NIST; SRM936a) ⁷⁴	347	≥98
Coumarin 153	C153	Lambda Physik (batch no. 029303)	422 455	99 99.5
Fluorescein	F	Sigma-Aldrich (batch no. BCBG1058V)	280	>99
Rhodamine 6G	R6G	Lambda Physik (batch no. 119202)	480, 530	98.5
Rhodamine 101	R101	Lambda Physik (batch no. 019502)	525 565	95.5 97.4
Oxazine 1	OX1	Lambda Physik (batch no. 090214)	665	98.2
HITCI	HITCI	Lambda Physik (batch no. 029006)	760	97.9
IR125	IR125	Lambda Physik (batch no. 10970; counter anion perchlorate)	800	99.1

Follows from the certificate of analysis.

^aDetection wavelength. ^bFollows from the NIST certificate and certification report.

the excitation wavelength to be used for subsequent fluorescence quantum yield measurements. For a very stringent control of solvent purity, excitation in the UV region below 300 nm can be performed. Avoid the use of ‘old’ solvents, as traces of water in hygroscopic solvents such as ethanol or DMSO can affect the spectral shape and intensity of the absorption and emission spectra of many fluorophores, as well as their molar absorption coefficients and their fluorescence quantum yields. The solvents we used for these quantum yield standards (i.e., ethanol in the case of R101, R6G, C153, OX1 and HITCI; DMSO for IR125; and 0.105 M perchloric acid for QS), were of spectroscopic grade and were purchased from Sigma-Aldrich and Merck, respectively. We used NaOH purchased from Merck for the preparation of the 0.1 M NaOH solution required for fluorescein. Before use, we checked all solvents for fluorescent impurities.

Dye solutions Solutions of the standard dyes should be freshly prepared for each measurement, either from the solid dye or from stock solutions (e.g., concentrations in the range of $(1-5) \times 10^{-4}$ M) stored in the dark at room temperature or in the refrigerator at 4 °C for less stable dyes such as certain NIR emitters. The absorption spectra of the dye solutions should be measured regularly to control dye stability and the uptake of water in hygroscopic solvents such as ethanol, methanol and DMSO. Water uptake, which can result in a change in fluorescence quantum yield (i.e., a decrease, especially for charge transfer-operated dyes such as coumarins), is often also indicated by changes in the spectral position of the absorption and/or emission bands,

as well as by a shortening of the dye’s fluorescence lifetime. In addition, the presence of decomposition products from the solvent needs to be avoided. For example, ethers are prone to auto-oxidation in the presence of light and air, yielding strongly oxidizing peroxides that should be removed (e.g., by the addition of potassium hydroxide before use). Chlorinated solvents such as CH₂Cl₂ and CHCl₃ can contain hydrochloric acid formed upon partial hydrolysis of the solvent, which can result in dye protonation. Dimethylformamide can decompose into dimethylamine and formaldehyde, which can affect certain dyes. Oxygen can quench the emission of fluorophores with longer fluorescence lifetimes (see ‘Limitations’ in the INTRODUCTION). Always be aware of the relevant application conditions of your sample; if necessary, remove oxygen as described. In general, fresh preparation of all sample solutions for each measurement is recommended.

EQUIPMENT SETUP

Cuvettes For absorption and fluorescence measurements, the same 10 mm × 10 mm cuvettes should be used, preferentially made from quartz. For commercial stand-alone integration sphere setups, special cuvettes such as the 10 mm × 10 mm long-necked quartz cuvettes from Hamamatsu Photonics must be used. Special care should be taken to always use the same volume for the dye solutions and the blank (solvent only).

Absorption spectrometer We recorded absorption spectra on a CARY 5000 absorption spectrometer from Varian. The accuracy of the intensity

TABLE 3 | Fluorescence quantum yields and absorption and emission ranges of the recommended quantum yield standards.

Dye	QS	C153	F	R6G	R101	OX1	HITCI	IR125
Solvent	0.105 M HClO ₄	Ethanol	0.1 M NaOH	Ethanol	Ethanol	Ethanol	Ethanol	DMSO
Absorbance (nm)	270–400	350–500	400–550	425–575	475–620	500–710	535–825	550–875
Emission (nm)	385–700	465–750	490–690	505–750	540–750	615–950	700–950	750–1,000
Φ_f	0.59 ^a	0.53 ^a	0.89 ^a	0.91 ^a	0.915	0.15	0.30	0.23
$\Delta\Phi_f$	0.04	0.04	0.04	0.04	0.028	0.01	0.01	0.01

^aRelative determination of fluorescence quantum yield using the absolutely measured fluorescence quantum yield of R101 (refs. 48,49).

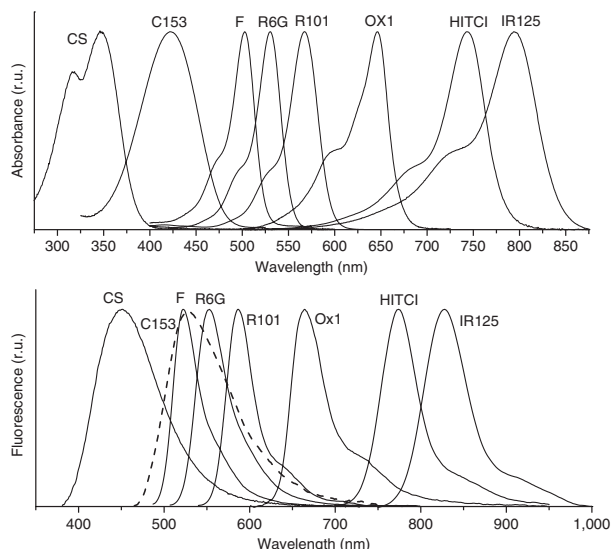


Figure 1 | Absorption (top) and emission (bottom) spectra of the recommended quantum yield standards. r.u., relative units.

and wavelength scale of this instrument is regularly controlled with certified absorption standards from Hellma. A schematic of the working principles of this spectrometer is shown in **Figure 2**.

Fluorescence spectrometer A fluorescence spectrometer (**Fig. 3**, configuration A) is used for the relative determination of quantum yields. Fluorescence spectra were measured with a previously described calibrated Spectronics Instruments 8100 fluorescence spectrometer of T-type design equipped with UV-vis and a vis-NIR detection channels and a separately addressable reference channel, all operated in photon-counting mode using a conventional $0^\circ/90^\circ$ measurement geometry^{41,58,75}. We performed all fluorescence measurements with Glan-Thompson polarizers placed in the excitation channel and the emission channels set to 0° and 54.7° (magic-angle conditions)¹. The calibration of this fluorescence spectrometer with physical transfer standards (i.e., the determination of the wavelength accuracy, range of linearity, emission correction and excitation correction curves) has been previously reported^{41,48,75}. The reliability of these calibration procedures was only recently shown in an international interlaboratory comparison⁵⁸. All fluorescence emission spectra presented are corrected for the wavelength- and polarization-dependent spectral responsivity of the detection system traceable to the spectral radiance scale^{48,49,58,75,78}.

Integrating sphere setup An integrating sphere setup is used for the absolute determination of quantum yields (**Fig. 3**, configuration B). We performed absolute measurements of the quantum yields with a calibrated, custom-built integrating sphere setup that was previously described⁴⁹. We evaluated these measurements by comparison with fluorescence quantum yields determined relatively with optical methods⁴⁹. Moreover, we performed comparative measurements with the integrating sphere setup C9920-02 from Hamamatsu, using a reabsorption correction^{47,48} (**Supplementary Methods**). For the dyes quinine sulfate dihydrate and rhodamine 101, which we chose as representative examples, respectively, of a charge-transfer dye with a large Stokes shift in polar solvents (minimum spectral overlap between absorption and emission) and of a dye with a small Stokes shift (considerable spectral overlap between absorption and emission; typical for fluorophores with resonant emission¹⁰), we compared the results obtained with our procedure and the Φ_f values derived with the emission correction curve implemented by and data evaluation software from Hamamatsu (formulas not provided; no reabsorption correction). These comparisons underlined the reliability of the implemented emission correction curve in the visible region and the need for a reabsorption correction⁴⁷.

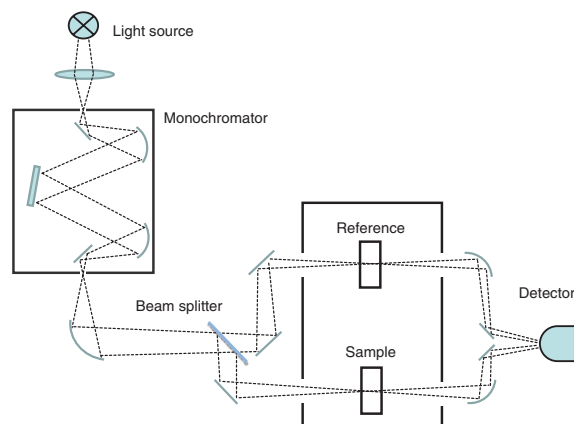


Figure 2 | Scheme of a double-beam absorption spectrometer.

The smallest quantum yield, which we measured with our equipment (absolutely and relatively) until now, was 0.016 for cryptocyanine in ethanol. **Instrument calibration and instrumental prerequisites** The calibration of the detection system is a prerequisite for determining the photoluminescence quantum yield correctly. The (relative) spectral responsivity (or the emission correction curve equaling the inverse (relative) spectral responsivity) has to be obtained with physical or chemical transfer standards. Both procedures are detailed in the **Supplementary Methods**. Chemical transfer standards such as the spectral fluorescence standards F001 to F005 (refs. 47,58,59,62) are a comparably inexpensive option for obtaining the emission correction curve in the spectral range from 300 to 770 nm (refs. 58,59,75,79). Physical transfer standards such as calibrated lamps must be used for high-precision calibrations and especially for wavelengths > 750 nm, owing to the lack of certified and evaluated spectral fluorescence standards for the NIR region and the debatable reliability of literature data of fluorescence reference materials recommended for this wavelength region^{1,80,81}. As the uncertainty of the measurement of the spectral responsivity of the fluorescence instrument directly affects the overall measurement uncertainty of Φ_f (refs. 48,49), special attention has to be paid to the calibration procedure(s) and reliable reference data from all standards used.

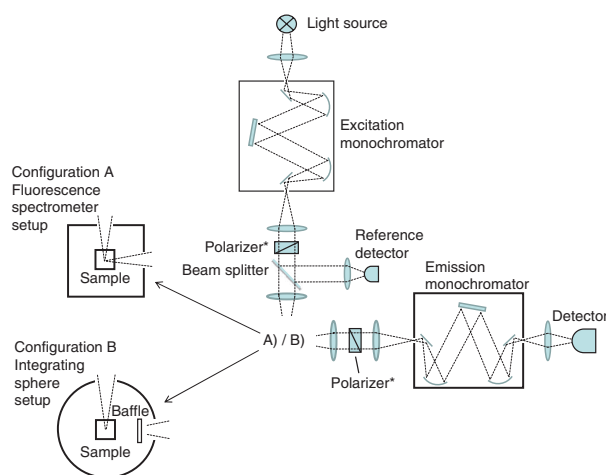


Figure 3 | Scheme of a fluorescence spectrometer (configuration A) and an integrating sphere setup (configuration B). *In the case of the integrating sphere setup, polarizers are not necessary.

PROTOCOL

Higher measurement uncertainties may be also encountered in fluorescence spectrometers that are not equipped with polarizers because of polarization effects resulting from the sample or/and the standard, especially for NIR emitters, which are larger in size and have shortened fluorescence lifetimes. This can also occur in fluorophore-labeled (bio)macromolecules and in fluorophores dissolved in solvents that have high viscosity (such as glycerol) or are entrapped in solid materials (the only exceptions are certain glasses that have been doped with transition and rare earth metal ions)⁷⁵. The extent of such uncertainty contributions depends on the difference between the emission anisotropy of the standard and sample for relative measurements of fluorescence quantum yields. Polarization effects do not

affect measurements with integrating sphere setups because of multiple scattering and reflection events that result in a complete loss of polarization information of the detected photons.

When integrating sphere setups, enhanced measurement uncertainties can also arise from inaccurately considered reabsorption effects; we address these accordingly in the PROCEDURE and in the **Supplementary Methods**. Other possible sources of uncertainty are inappropriate and non-homogeneous surface coatings used for sample holders or the sphere surface itself and nonreproducible sample positioning.

In any case, measurements should be performed at least in duplicate; we recommend several replicates to reduce the measurement uncertainty.

PROCEDURE

1| Perform the steps according to option A for the relative determination or option B for the absolute determination of fluorescence quantum yields.

(A) Relative determination of fluorescence quantum yields ● TIMING ~90 min for moderately to strongly emissive samples

- (i) Clean and dry four cuvettes (10 mm × 10 mm); ensure that no residual solvent is present, as small traces of the solvent(s) used for cell cleaning (e.g., acetone, ethanol, water) can influence measured quantum yields.
▲ **CRITICAL STEP** For measurements in the UV-spectral region (<400 nm), use quartz cells only.
- (ii) *Measure and adjust the absorbance (A) of the quantum yield standard and the sample (Step 1A(ii–ix)).* Perform a baseline correction.

Double-beam absorption spectrometer	Use two solvent-filled cuvettes and remove one cell from the spectrometer
Single-beam absorption spectrometer	Use a single solvent-filled cuvette and remove the cell

? TROUBLESHOOTING

- (iii) Fill a cuvette with the sample and measure its absorption spectrum (*A* as a function of wavelength); if necessary to minimize possible reabsorption effects, dilute the dye solution until the absorbance reaches a maximum value of 0.1 at the longest wavelength absorption band.
? **TROUBLESHOOTING**
- (iv) *Test the measurements (Step 1A(iv,v)).* After sample preparation, measure the absorption spectrum, wait for 5 to 10 min and re-measure the absorption spectrum, thus making sure that it remains constant over time.
? **TROUBLESHOOTING**
- (v) Dilute the sample by a factor of at least 5, wait for 5–10 min, measure the resulting absorption spectrum and compare its shape with that of the previously measured absorption spectrum of the more concentrated sample solution (normalization of the determined spectra may be necessary). Spectral deviations suggest dye aggregation.
? **TROUBLESHOOTING**
- (vi) Choose a quantum yield standard (from **Table 3** and **Fig. 1**) that absorbs in the similar wavelength region as the sample. Use the corresponding solvent of highest purity.
- (vii) Prepare a fresh solution of the quantum yield standard from a stock solution and measure its absorption spectrum.
- (viii) Choose an excitation wavelength such that the sample and the standard are excited at an almost plateau-like region of their absorption spectra or at least at a wavelength with little slope in the absorption spectrum (**Fig. 4**). Avoid fluorophore excitation in the red tail of the longest wavelength absorption band, which can result in a spectral overlap of the scattered excitation light with the emission spectrum (**Table 3**).
- (ix) Adjust the standard's absorbance to match that of the sample at the chosen excitation wavelength (**Fig. 4**). To reduce possible reabsorption effects, ensure that the absorbance of the standard does not exceed values of 0.1 at the longest wavelength absorption maxima of the fluorophore, especially for dyes with a small Stokes shift. If it is difficult to match the absorbances at the excitation wavelength, try to keep the differences as small as possible.
- (x) *Measure the emission spectra (Step 1A(x–xiv)).* Choose fluorescence spectrometer settings such that the sample and the standard can be measured with identical instrument settings (excitation wavelength, slit widths of excitation and emission monochromator, scan speed, integration time). For instruments equipped with polarizers, set the excitation polarizer to 0° and the emission polarizer to 54.7° measured from the vertical axis (magic-angle conditions) in order to render detected emission intensities independently of a possible emission anisotropy of the sample and the standard^{1,48}. Ensure that the detection system is always operated within its linear range and that the recorded fluorescence signals

are smooth. If necessary, carry out multiple measurements and average the resulting spectra to reduce noise.

- (xi) *Test the measurements (Step 1A(xi, xii)).* Measure the fluorescence intensity at the emission maximum of the sample for these instrument settings over a period of 5–10 min. Ensure that no increase or decrease can be observed.

? TROUBLESHOOTING

- (xii) Measure the emission spectrum of the sample after dilution by a factor of ~5 (see also Step 1A(v)). Ensure that the measured fluorescence intensity diminishes by approximately the factor used for dilution and that the spectral shape of the emission band does not change (normalization of the determined spectra may be necessary).

? TROUBLESHOOTING

- (xiii) Measure the emission spectrum of the sample and a blank spectrum using an identical cuvette filled with the pure solvent under identical measurement conditions.

? TROUBLESHOOTING

- (xiv) For emission measurements with weakly emissive samples, which can require long-term illumination (30 min or more) of the sample during multiple fluorescence measurements, measure the absorption spectrum after completing the emission measurements in order to check sample stability.
- (xv) *Calculation of the relative quantum yield (Step 1A(xv–xix)).* Calculate the absorption factors f_{st} and f_x of the standard and the sample from the measured A value at the excitation wavelength using equation (3). The index x denotes the sample, and the index st denotes the standard. If the absorbances determined in Step 1A(iii) and (ix) match, the quotient f_{st}/f_x equals 1, and this step (i.e., Step 1A(xv)) can be omitted; for non-matching absorbances, the quotient f_{st}/f_x must be considered.

$$f = 1 - 10^{-A(\lambda_{ex})} \tag{3}$$

For higher precision, the band-pass $\Delta\lambda_{ex}$ used for excitation can be considered according to equation (4).

$$f = \frac{\lambda_{ex} + 1/2\Delta\lambda_{ex}}{\lambda_{ex} - 1/2\Delta\lambda_{ex}} \int_{\lambda_{ex} - 1/2\Delta\lambda_{ex}}^{\lambda_{ex} + 1/2\Delta\lambda_{ex}} 1 - 10^{-A(\lambda_{ex})} d\lambda_{ex} \tag{4}$$

- (xvi) Subtract the solvent spectra from the measured emission spectra of the sample and the standard to obtain blank-corrected emission spectra. This accounts for signal contributions from scattered light and fluorescent impurities in the solvent, as well as for dark noise of the detector (see also INTRODUCTION).
- (xvii) Correct the blank-corrected sample and standard emission spectra for the spectral responsivity of the emission channel of the fluorometer (see **Supplementary Methods**). Before the next step, ensure that the emission correction curve was determined in reference to the spectral radiance, i.e., multiplication with λ is not included in the emission correction curve (see INTRODUCTION and **Supplementary Methods**).
- (xviii) Calculate the relative integral photon fluxes F_x and F_{st} emitted from the sample and the quantum yield standard from the spectrally corrected and blank-corrected spectra of the sample (I_c , see equation 1) according to equation (5); see **Figure 5**. The index x denotes the sample, and the index st denotes the standard. If the emission correction curve was not determined in reference to the spectral radiance, but in reference to the spectral photon flux, omit multiplication with λ_{em} here.

$$F = \int_{\lambda_{em}} I_c \cdot \lambda_{em} d\lambda_{em} \tag{5}$$

The upper and lower limit of the integration of the emission spectra of the sample and the standard should be chosen to cover the complete emission band of the fluorophore.

▲ CRITICAL STEP Equation (5) considers the photonic nature of the emitted light by multiplication with λ_{em} (ref. 82) (hc_0 is omitted; see the INTRODUCTION and **Supplementary Methods**).

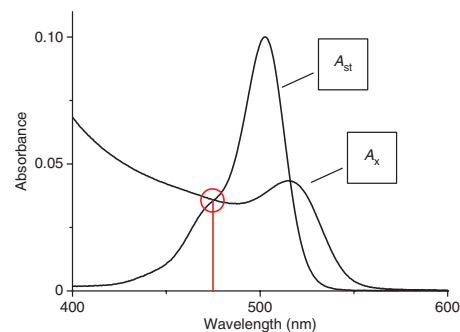


Figure 4 | Absorption spectra of the sample (A_x) and the standard (A_{st}). The red circle and line indicate the optimal wavelength for excitation.

PROTOCOL

- (xix) Calculate the photoluminescence quantum yield according to equation (6).

$$\Phi_{f,x} = \Phi_{f,st} \cdot \frac{F_x}{F_{st}} \cdot \frac{f_{st}}{f_x} \cdot \frac{n_x^2(\lambda_{em})}{n_{st}^2(\lambda_{em})} \quad (6)$$

F is the integral photon flux (Step 1A(xvii)), f is the absorption factor (Step 1A(xv)), n is the refractive index of the solvent and Φ_f is the quantum yield. The index x denotes the sample, and the index st denotes the standard. To be strictly correct, the refractive indices at the mean or average emission wavelength should be used. These values are often difficult to obtain from literature for certain solvents. Instead, you can use the values given for the standard wavelengths (e.g., sodium D-line at 589 nm)⁸³.

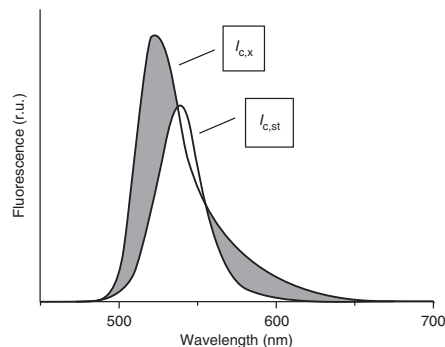


Figure 5 | Example emission spectra of the sample ($I_{c,x}$) and the standard ($I_{c,st}$). r.u., relative units.

(B) Absolute determination of fluorescence quantum yields ● TIMING ~40 min for moderately to strongly emissive species

- (i) Clean and dry two cuvettes (10 mm × 10 mm or as required for these measurements). Ensure that no residual solvent is present, as small traces of the solvent(s) used for cell cleaning (e.g., acetone, ethanol, water) can influence the quantum yields of the investigated samples.
▲ **CRITICAL STEP** For measurements in the UV spectral region (<400 nm), use quartz cells only.
- (ii) *Sample preparation and test measurements (Step 1B(ii–vii))*. After sample preparation, measure the absorption spectrum, wait for 5–10 min and re-measure the absorption spectrum, ensuring that it remains constant and that no time-dependent changes occur.
? **TROUBLESHOOTING**
- (iii) Dilute the sample by a factor of at least 5, wait for 5–10 min, measure the resulting absorption spectrum and compare it with the previously measured absorption spectrum of the more-concentrated sample (normalization of the determined spectra may be necessary). Spectral deviations provide a hint for dye aggregation.
? **TROUBLESHOOTING**
- (iv) Measure the sample's emission spectrum with a fluorometer.
? **TROUBLESHOOTING**
- (v) Measure the fluorescence intensity at the emission maximum of the sample for these instrument settings over a period of 5–10 min. Ensure that no increase or decrease can be observed.
? **TROUBLESHOOTING**
- (vi) Measure the emission spectrum of the sample after dilution by a factor of ~5 (see also absorption measurements, Step 1B(iii)). Ensure that the measured fluorescence intensity diminishes by approximately the factor used for dilution and that the spectral shape of the emission band does not change (normalization of the determined spectra may be necessary).
? **TROUBLESHOOTING**
- (vii) Adjust the absorbance of the sample at the excitation wavelength to minimize inner filter effects that are especially pronounced for integrating spheres^{47,49}, and try to keep absorbances low, especially for fluorophores with a small Stokes shift.
- (viii) Choose a suitable excitation wavelength for the integrating sphere measurements, thereby making sure that the excitation peak can be clearly separated from the fluorophore's emission (**Fig. 6**). Avoid fluorophore excitation in the red tail of the fluorophore's longest-wavelength absorption band for a straightforward separation of excitation and emission.
- (ix) Fill a second cuvette with solvent only; use the same volume as used for the sample⁴⁷.
- (x) *Sample and blank measurements (Step 1B(x–xvi))*. Place the solvent-filled cuvette (blank) in the sample holder.
- (xi) Place the sample holder with the blank inside the integrating sphere. If necessary, adjust the position of the cell.
- (xii) Choose the measurement parameters (excitation wavelength, excitation band-pass, wavelength region for signal detection covering excitation and emission, emission band-pass and integration time). The most important criterion here is that the intensity of the excitation peak should be as high as possible, although care must be taken to operate the detector within its linear range.
- (xiii) Measure a blank spectrum within the spectral range of the excitation peak and of the sample emission, preferably with a single scan. In the case of a poor signal-to-noise ratio, repeat the measurement and average the resulting spectra until the quality of the data has been sufficiently improved (smooth spectra).
- (xiv) Remove the sample holder with the blank from the integrating sphere and place the sample in the sample holder.



- (xv) Place the sample holder inside the integrating sphere. Ensure that the cell position equals the position of the blank. If necessary, use, e.g., a laser for cell adjustment.
- (xvi) Measure both the transmitted (not absorbed) excitation light and the fluorescence, by using identical measurement conditions and instrument settings to those used for the blank.
- (xvii) *Calculation of the quantum yield (Step 1B(xvii–xxi)).* Correct the recorded signals obtained from the sample and the blank for the instrument-specific spectral responsivity.
- (xviii) Before the next step, make sure that the emission correction curve was determined in reference to the spectral radiance. (If the emission correction curve was not determined in reference to the spectral radiance, but in reference to the spectral photon flux, omit multiplication with λ_{em} here; see the INTRODUCTION and **Supplementary Methods.**)
- (xix) Calculate the absorbed photon flux F_{abs} and the emitted photon flux F , separating the measured spectra of the sample and the blank into an excitation and an emission region as shown in **Figure 6**. Subsequently, calculate (i) the absorbed photon flux (F_{abs}) from the integrated difference of the spectrally corrected signals of the blank and the sample in the spectral range of the excitation, and (ii) the emitted photon flux (F) from the integrated difference of the spectrally corrected sample and blank signals in the spectral region of the emission according to equations (7 and 8). Blank measurements are indicated with the index b, and sample measurements are indicated with the index x, respectively. If the emission correction curve was not determined in reference to the spectral radiance, but in reference to the spectral photon flux, omit multiplication with λ_{em} here

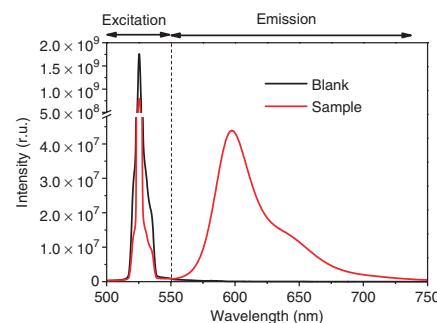


Figure 6 | Example of the signals of an integrating sphere measurement for the sample and the blank. The example shows the position of the separation between excitation and emission (dashed vertical line). r.u., relative units.

$$F = \int_{\lambda_{em}} \frac{I_x(\lambda_{em}) - I_b(\lambda_{em})}{s(\lambda_{em})} \lambda_{em} d\lambda_{em} \quad (7)$$

$$F_{abs} = \int_{\lambda_{ex}} \frac{I_b(\lambda_{ex}) - I_x(\lambda_{ex})}{s(\lambda_{ex})} \lambda_{ex} d\lambda_{ex} \quad (8)$$

- (xx) Calculate the absolute fluorescence quantum yield as a quotient of the photon flux emitted from the sample (F) and the absorbed photon flux (F_{abs}).

$$\Phi_f = \frac{F}{F_{abs}} \quad (9)$$

- (xxi) Correct reabsorption effects as described by us and others^{47,48,84}; see also TROUBLESHOOTING and **Supplementary Methods.**

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(ii)	Negative or positive offset in the absorption and/or inclined spectrum	Dirty cells, incorrect baseline correction	Make sure the cells are clean and repeat the baseline correction
1A(iii), 1B(ii)	Positive offset in the absorption spectrum and/or untypically high absorbance at short wavelengths	Scattering samples (see ‘Scattering samples’ troubleshooting note)	Use an integrating sphere setup

(continued)

TABLE 4 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
1A(iv), 1B(ii)	Time-dependent increase in absorbance	Incomplete fluorophore dissolution	Wait until all fluorophores in the sample solution are completely dissolved
	Time-dependent decrease in absorbance	Fluorophore adsorption on cell walls; decomposition of dye	Change the measurement cell; change the solvent
1A(v), 1B(iii)	Atypical or concentration-dependent absorption spectrum, especially at the short-wavelength side of the long-wavelength absorption band	Dye aggregation caused by high dye concentration (see 'Dye aggregation' troubleshooting note)	Dilute the solution
1A(xi), 1B(v)	Increase of emission intensity during illumination	Photobrightening	Sample-specific problem, i.e., for quantum dots ^{10,90} . The use of preilluminated samples can be an alternative; they must be used with care, as the occurrence of photobrightening can depend on excitation wavelength. Also, the light-induced increase in emission intensity can disappear after storage of the sample in the dark
1A(xi), 1A(xiv), 1B(v)	Decrease of emission intensity during illumination	Photodecomposition	Reduce the excitation light intensity
1A(xii), 1B(vi)	Decrease in the absorption factor-weighted emission after dilution	Concentration-dependent change in the equilibrium between surface-bound molecules and dissolved molecules in solution, i.e., ligand desorption in the case of quantum dots upon dilution ⁴¹ (see 'Ligand desorption' troubleshooting note)	Sample-specific problem; one possible alternative is the use of other cuvettes with different optical path lengths to minimize reabsorption effects while maintaining a high fluorophore concentration ⁴¹
	Increase in the absorption factor-weighted emission after dilution	Dye aggregation or incomplete dissolution of the fluorophores or reduction in reabsorption. In the latter case, a blue shift in the spectral position of the emission peak should be observed (see 'Dye aggregation' troubleshooting note)	Further dilution of the fluorophore solution
	Fluorescence spectrum distorted at the short-wavelength side of the emission band; shift of the emission maximum to longer wavelengths	Reabsorption caused by high dye concentration (see 'Reabsorption' troubleshooting note)	Dilute the sample solution
1A(xiii)	Measurement of sample and standard fluorescence spectra with identical instrument settings is impossible	Fluorescence quantum yields of the sample and standard deviate substantially	Dilute the standard solution until its emission spectrum can be recorded under the same measurement conditions as those used for the emission spectrum of the sample. Next, measure the standard's absorption spectrum
1A(xiii), 1B(iv)	Atypical features in the emission spectrum, e.g., narrow peaks	Contribution of scattered excitation light to the measured emission spectrum	Change the excitation wavelength such that the scattered excitation light can be easily spectrally separated from the fluorophore's emission. Scattered light can also appear at the doubled excitation wavelength (second-order effect)

Spectral correction of fluorescence spectra for instrument-specific spectral responsivity

Without spectral correction of measured instrument-specific fluorescence spectra, substantial deviations can occur in shape and intensity, and thus also in the resulting fluorescence quantum yields, as illustrated for two dyes in **Figure 7**. In this example, the quantum yield of dye 2 measured relative to dye 1, obtained from the spectrally corrected fluorescence spectra of both dyes, differs by a factor of 1.54 from the value obtained using the corresponding uncorrected spectra.

Scattering samples (relative determination of Φ_f)

For solutions of large fluorophore-labeled biomolecules or other macromolecules, as well as for colloids and suspensions of particles, liposomes and so on, scattering from the sample can distort the absorption spectrum of the dye. This is illustrated in **Figure 8**. In this case, the measured spectrum (solid line in **Fig. 8**) cannot be used to correctly determine the absorption factor, because an unknown fraction of the measured absorption is caused by the scattering background (dotted line in **Fig. 8**). Furthermore, the propagation of excitation and fluorescence light is altered in scattering media, which results in changes in the illuminated and the detected volume within the cuvette and thus in changes in the measured fluorescence intensity⁸⁵. The fluorescence quantum yields of such samples can be reliably measured only with an integrating sphere setup^{49,52,86–88}.

Reabsorption

At high dye concentrations, emitted light can be reabsorbed by the fluorophore in the region of the spectral overlap between absorption and emission as shown in **Figure 9** for R6G in water. Subsequently, the measured quantum yield is reduced as compared with a fluorescence quantum yield obtained for less-concentrated solutions of this dye, revealing less or no reabsorption. Indicative of reabsorption is a distortion of the emission spectrum at its short-wavelength side, with the emission maximum undergoing an apparent red shift with increasing dye concentration (arrow in **Fig. 9**). Moreover, the resulting concentration-normalized integral fluorescence intensity is diminished with increasing dye concentration. To visualize the effect of reabsorption on emission spectra, the emission spectra in **Figure 9** were normalized at the long-wavelength side of the emission band, where no absorption occurs (>600 nm). Reabsorption effects are especially pronounced for integrating sphere setups^{47–49} and for dyes with a large overlap of absorption and emission as found for, e.g., xanthene dyes, cyanines, BODIPY dyes and quantum dots¹⁰. Such effects can be overcome by the measurement of concentration series and the measurement of an undisturbed emission spectrum for a very dilute dye solution either with an integrating sphere setup or with a fluorescence spectrometer, followed by a reabsorption correction. A reabsorption correction is described in the **Supplementary Data** and has been reported by us and others^{47,48,84}.

Dye aggregation

At high concentrations, some dyes such as xanthenes, cyanines and porphyrines can form nonfluorescent or only weakly emissive aggregates^{10,57,89}. This is favored, e.g., for hydrophobic dyes in aqueous solutions. Typically, dye aggregation results in an enhanced absorption at the short-wavelength shoulder of the longest-wavelength absorption band (H-type aggregates), as shown in **Figure 10** for R6G in water. The inset shows the absorption spectra of the pure monomeric and aggregated forms of this dye. The formation of dye aggregates hampers the correct determination of the absorption factor of the nonaggregated dyes at wavelengths in the region of dimer absorption, such as at the vibronic shoulder of the

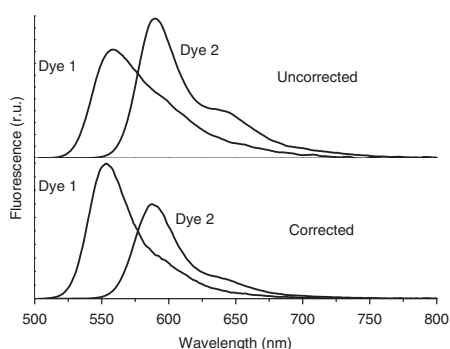


Figure 7 | The effect of spectral correction on the shape and relative intensities of the emission spectra of two dyes. r.u., relative units.

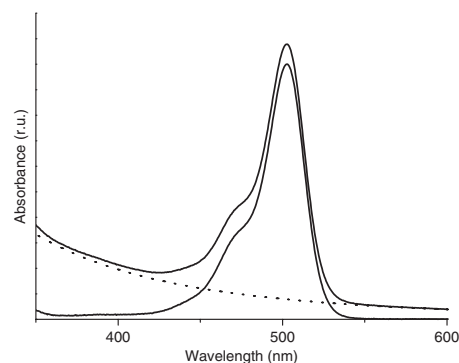


Figure 8 | Visualization of the effect of a scattering background on a dye's absorption spectrum. The scattering background (dotted line) leads to an overestimation of the absorption, especially in the short-wavelength region. r.u., relative units.

PROTOCOL

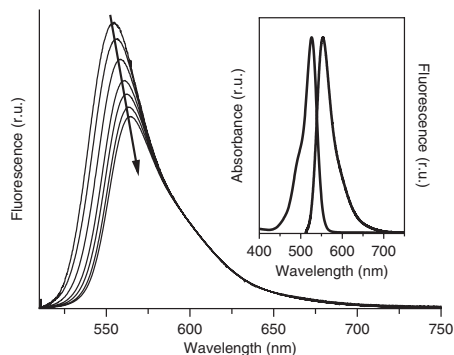


Figure 9 | Distortion of the emission spectrum as a result of fluorescence reabsorption. The inset shows the overlap of the absorption (left curve) with the emission spectrum (right curve). r.u., relative units.

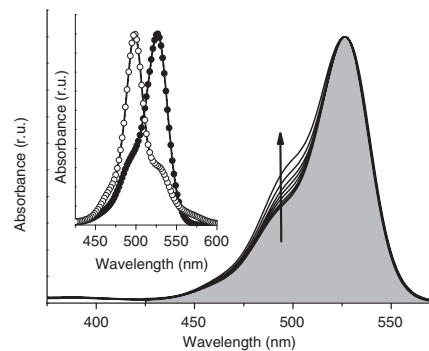


Figure 10 | Typical changes in the absorption spectrum caused by dye aggregation (H-type aggregates). The inset shows the pure spectra of the monomer (filled circles) and the aggregate (open circles). r.u., relative units.

longest-wavelength absorption band, where these fluorophores are typically excited for fluorescence studies. To account for this effect, only dilute dye solutions should be used. If this is not possible, the contribution of dye aggregates to the measured absorbance at the excitation wavelength needs to be mathematically considered⁸⁹.

Ligand desorption

The optical properties of fluorophores such as quantum dots, which consist of a semiconductor core with surface-bound ligands that are coordinatively but not covalently bound to the particle surface, can be affected by ligand adsorption–desorption equilibria^{10,41}. Sample dilution can shift these equilibria, leading to the desorption of surface-bound ligands, which results in a decrease in fluorescence quantum yield (**Fig. 11**). The occurrence and size of such effects depends on the binding strength of the ligands to the particle surface, on the particle size and on the solvent⁴¹.

● TIMING

Step 1A, relative determination of fluorescence quantum yields: ~90 min for moderately to strongly emissive samples
Step 1B, absolute determination of fluorescence quantum yields: ~40 min for moderately to strongly emissive species

ANTICIPATED RESULTS

The use of these protocols for the relative and absolute determination of fluorescence quantum yields of transparent dye solutions and the recommended quantum yield standards for the wavelength region of ~350–950 nm will lead to an improved reliability of fluorescence quantum yield measurements. The most error-prone steps in determining fluorescence quantum yields, which involve emission correction (spectral correction of the measured emission spectra for the instrument-specific wavelength- and polarization-dependent spectral responsivity) and the reliability of the Φ_f value of the standard, can be minimized. In addition, we provide a procedure for a reabsorption correction (**Supplementary Methods**) that is required, when you use an integrating sphere setup, for the accurate absolute measurement of fluorescence quantum yields of dyes with a very small Stokes shift. We anticipate that with these procedures, in combination with the recommended standards and methods for the determination of the emission correction curve (**Supplementary Methods**), uncertainties between $\pm 5\%$ and $\pm 10\%$ are achievable for the determination of fluorescence quantum yields.

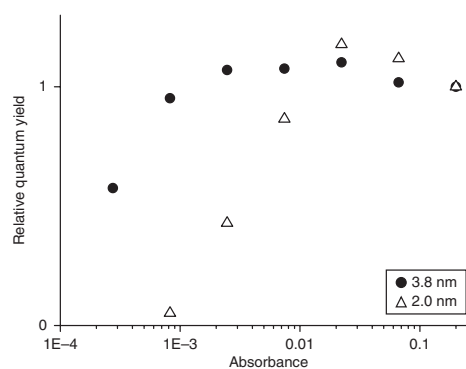


Figure 11 | Concentration dependence of the fluorescence quantum yield of two CdTe quantum dots differing in particle size. The absorbance of the sample (x axis) is proportional to particle concentration.

Note: Supplementary information is available in the online version of the paper.

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