

Relative and absolute determination of fluorescence quantum yields of transparent samples

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Abstract — Luminescence techniques are amongst the most widely used detection methods in the life and material sciences. At the core of these methods are 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 –labeled polymeric particles. A key parameter for fluorophore comparison is the fluorescence quantum yield (Φ_f): 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 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 *ca.* 350 nm to 950 nm assessed by us with commercial and custom-designed instrumentation. With these protocols and standards, uncertainties of 5% to 10% can be achieved within 2 hours.

INTRODUCTION

In the last decades, luminescence techniques evolved into some of the most popular analytical and detection tools in the life and material sciences due to their sensitivity, comparable ease of use, relatively inexpensive instrumentation, and their suitability for multiplexed analysis, combined spectrally, temporally, and spatially resolved measurements as well as for remote sensing¹⁻⁵. The toolbox of fluorophores for a broad variety of applications and targets in the ultraviolet (UV), visible (vis), and lately, also the near-infrared (NIR) spectral region is ever growing⁶⁻¹², with especially fluorescent proteins^{7, 13}, semiconductor nanocrystals (so-called quantum dots)^{10, 14, 15} and 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}, and fluorophore-labeled biomolecules like 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, there exist an ever increasing number of sophisticated probes and sensors, the optical properties of which can be modulated selectively by chemical or biological inputs^{11, 25-28}. Other examples are multichromophoric reporters like fluorophore-doped or –labeled polymeric particles and multimodal systems for the readout with different detection methods or suspension assays²⁹⁻³². Dye classes commonly employed 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}, fluorophore-doped or -labeled polymeric particles²⁹⁻³¹ and recently, also upconversion nanocrystals^{16, 17}.

The design of such functional fluorophores as well as their application and the validation of analytical methods relying on their use require 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 for 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 ($\varepsilon(\lambda_{ex})$) gives the fluorophore's brightness B ($B = \Phi_f \times \varepsilon(\lambda_{ex})$) 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 (FRET) 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 like 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 yield³⁶⁻⁴¹ or absolutely with an integrating sphere setup⁴²⁻⁴⁹. 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 section*, Option A).^{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, the quantum yield is obtained directly in a single measurement without the need for a quantum yield standard (see *PROCEDURE section*, Option B).⁴⁷⁻⁴⁹ This principally very attractive method is currently gaining in 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. For materials like upconversion nanocrystals excited at 980 nm⁵², which absorb two or more photons prior to light emission and thus reveal excitation-power density-dependent photoluminescence quantum yields, and for quantum yield measurements of all scattering samples like nanoparticles or certain bioconjugates and for solid samples like films or powders used as converter materials^{45, 53, 54}, the absolute measurement of Φ_f is mandatory.

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 like 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 on the sample geometry and 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 to the number of absorbed photons. The number of absorbed photons follows from the decrease in the incident excitation light intensity (that is 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 be also obtained indirectly from measurements of the dissipated heat 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 an 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}. Amongst 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 to reliably measure 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 due 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 that 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 in the order of $\pm 4\%$ (for $\Phi_f > 0.10$) in the wavelength region of ca. 400 nm to 950 nm^{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 like rhodamine 101, fluorescein, and rhodamine 6G⁶⁰ 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 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 region⁴⁷⁻⁴⁹. The working principle of transfer chains follows principally e.g., from section *Reagent setup - Dyes*. For example, we linked quinine sulfate dihydrate via coumarin 153 (C153), fluorescein (F) and rhodamine 6G (R6G1) to R101⁴⁹, and the NIR dye IR125 vs. 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/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 like small organic dyes or fluorescent proteins are not or only barely sensitive to the presence of oxygen. The classical example for a small organic dye, which reveals an oxygen-sensitive quantum yield and lifetime is pyrene with its for such an organic dye extremely long fluorescence lifetime exceeding 100 ns. Also e.g., a small oxygen dependence of the quantum yield of diphenylanthracene (DPA) was reported⁶²⁻⁶⁴. Special care has to be taken for emitters like certain transition metal ion complexes (e.g., Ru(II) or Ir(III) complexes etc.) displaying partly or completely forbidden optical transitions, and thus, very long lifetimes of their excited states, as well as the measurement of phosphorescence quantum yields, Φ_p of organic dyes. The intrinsically longer luminescence or phosphorescence lifetimes of these emitters in 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, during the luminescence measurements, the purging should be stopped in order to avoid intensity fluctuations in the emission caused by light scattering and refraction at 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 as employed for fluorescence measurements in 0°/90° measurement geometry and integrating sphere measurements. Moreover, e.g., 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 employing 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 in a separate section in the *TROUBLESHOOTING* section.

Critical can be also 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. 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 absorbances of sample and standard or the use of an attenuator (with an ideally wavelength-independent transmission profile in the emission channel that needs to be assessed with an absorption spectrometer). All these procedures may result in enhanced measurement uncertainties.

Quantum yield standards

We introduce recently evaluated quantum yield standards for the spectral region of *ca.* 350 nm to 950 nm^{48, 49, 57} that meet the requirements on such standards recently defined in a technical note by the International Union of Pure and Applied Chemistry (IUPAC)⁶⁶ (see *Materials, Dyes*). Here, we deliberately chose dyes and solvents that are commercially available at a reasonable price in a reliable purity and measurement conditions (e.g., air-saturated solutions, no additional dye purification steps) which enable 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 like coumarins^{46, 60}. Reasons for these deviations may be the determination of the instrument's spectral responsivity required for the spectral correction of the measured emission spectra for instrument-specific effects, see e.g., next section, and 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 like the temperature may have a significant impact 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 non-radiative deactivation of the excited state. The most prominent example is here rhodamine B^{68, 69}. Two reviews summarizing potential quantum yield standards from the literature were published by IUPAC^{60, 70}. Other frequently discussed quantum yield standards for the red wavelength region are rhodamine 6G^{71, 72}, rhodamine 101^{60, 72}, and cresyl violet⁷³. Cresyl violet should be used with care as for this dye, we observed slight stability problems which we, however, never studied systematically.

Still existing limitations are the lack of evaluated quantum yield standards for the ultraviolet region, i.e., for the spectral region from *ca.* 250 nm to 400 nm and for the near infrared, i.e., for the spectral range > 700 nm. The former is also related to the fact that in the UV region for wavelengths below *ca.* < 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 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 oxygen⁶²⁻⁶⁴. Moreover, their structured absorption and emission spectra render measurements with these dyes very sensitive to possible uncertainties of the wavelength scales of the different spectrometers employed. For fluorophores absorbing and/or emitting at shorter wavelengths like tryptophan or tyrosine, our protocol for relative measurements can be used to create a transfer chain of dyes (see *Method Development*).

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 ^{75, 76}. Subtraction of a blank spectrum (I_b) obtained for the fluorophore-free solvent or matrix (under identical measurement conditions as used the sample) from the measured fluorescence spectrum of the sample yields a blank- or background corrected fluorescence spectrum (consideration of e.g., scattering and fluorescence from the solvent and dark counts at the detector). Subsequent correction for the fluorometer's wavelength- and polarization-dependent responsivity (spectral responsivity $s(\lambda)$), which is an instrument-specific quantity, leads to a corrected spectrum I_c that are instrument-independent (see equation 1)^{75, 76}. This

procedure is termed spectral correction or in the case of emission spectra, emission correction.

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

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 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 the velocity of light in vacuo (see 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 comprise also the wavelength-region of the excitation providing the relative number of absorbed photons.

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

Care has to be taken, when employing an emission correction implemented by the spectrometer manufacturer into the fluorescence spectrometer or generally for the use of 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. Due to the calibration described in the *Supplementary Methods* of the manuscript, always relative quantities are 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 method of detection, i.e., photon counting versus analog (photocurrent) detection mode. For example, a photo diode measures a photocurrent and provides a signal in Ampere (A) which is, for a given wavelength (and measured within the linear range of the detector) proportional to the photon flux (unit 1/s). Contrary, 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 number of photons collected within the integration time. For this reason, we used the general 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.

Table 1: Symbols and units

Symbol	Name	Units
$I_u(\lambda_{em})$	uncorrected spectrum	count nm ⁻¹
$I_c(\lambda_{em})$	corrected spectrum	counts nm ⁻¹
$F(\lambda_{em})$	relative integrated photon flux	s ⁻¹
$s(\lambda_{em})$	relative spectral responsivity	counts W ⁻¹
Φ_f	fluorescence quantum yield	1
N_{abs}, N_{em}	number of photons (absorbed, emitted)	1
$f(\lambda_{ex})$	absorption factor	1
$A(\lambda_{ex})$	absorbance	1
$\epsilon(\lambda_{ex})$	molar (decadic) absorption coefficient	dm ³ cm ⁻¹ mol ⁻¹
n	refractive index	1

EXPERIMENTAL DESIGN

In the following sections, suitable procedures for the relative and absolute determination of fluorescence quantum yields of transparent fluorophore solutions are described, including the most critical steps, with detailed protocols and recommended quantum yield standards as well as common pitfalls (see section *TROUBLESHOOTING*) being highlighted in the *PROCEDURE* section. For relative measurements, we describe here only the case of identical excitation wavelengths for sample and quantum yield standard as 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 (see *EQUIPMENT AND MATERIALS*) presented here, which cover the wavelength region of *ca.* 350 nm to 950 nm, there is no need for the application of different excitation wavelengths for sample and standard for the characterization of the vast majority of fluorophores employed 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 spectrometer, for absolute measurements integrating sphere setup), ii.) operation of the detectors of these instruments in their linear range (that should be previously determined^{76, 77}), and iii.) knowledge of the (relative) spectral responsivity (emission correction curve) of the spectrometer's detection channel (see *Supplementary Methods*). This quantity can be determined with e.g., spectral fluorescence standards which are meanwhile commercially available^{41, 48, 58, 59, 75, 77, 78}, see also section on *Supplementary Methods*. Suitable quantum yield standards assessed and recommended by us for relative measurements of fluorescence quantum yields in the wavelength region of *ca.* 350 nm to 950 nm are summarized in section *EQUIPMENT AND MATERIALS* and detailed in the *Supporting Data*^{47-49, 57, 77}.

Sample preparation. For the preparation of a suitable sample, some basic principles have to be considered. For the determination of fluorescence quantum yields, always fresh solutions made from high purity dyes and solvents should be used which should be prepared with care using clean equipment (pipettes, sample containers, measurement cells etc.). It needs to be made sure that the dye is completely dissolved without adsorption onto the cell windows. Samples should be always stored in the dark, either at room temperature, 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: 1) 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 to solvent only)? If yes, scattering centres are present. Scattering centres 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. 2) Concentration: Is the solution strongly colored? If yes, the concentration may be too high and the solution should thus be diluted. 3) Complete dissolution: are there particle/granular residues? If yes, wait, dilute the solution or carefully increase the temperature slightly or use an ultrasonic bath. Use of the latter two methods depends also on the stability of the respective compound. More hints to avoid sample-related problems and examples for measurement data indicating pitfalls are presented in the protocol and the trouble shooting section.

Handling. Always take special care to avoid contact with the surface of the integrating sphere and make sure 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 contaminations from the environment. Contaminations may influence the calibration and reduce the sensitivity of the measurement system. Especially 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 cautiously.

Data evaluation. For data evaluation, software is needed, which can perform basic mathematical operations like multiplication, division, summation, and subtraction. We recommend e.g., Excel, Origin, Matlab, or software from the spectrometer manufacturer. Formulas which 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 prior to the summation.

MATERIALS

Equipment Setup

Cuvettes. For absorption and fluorescence measurements, the same 10 mm × 10 mm cuvettes should be used, preferentially made from quartz, e.g., from Hellma. In the case of commercial stand-alone integration sphere setups, special cuvettes like the 10 mm × 10 mm long-necked quartz cuvettes from Hamamatsu Photonics K.K. developed for their setup, must be employed. Here, special care should be taken to use always the same volume for the dye solutions and the blank (solvent only).

Absorption spectrometer

Absorption spectra were recorded on a CARY 5000 absorption spectrometer from Varian Inc. The accuracy of the intensity and wavelength scale of this instrument is regularly controlled with certified absorption standards from Hellma GmbH. The working principle of this spectrometer is shown in Figure 1.

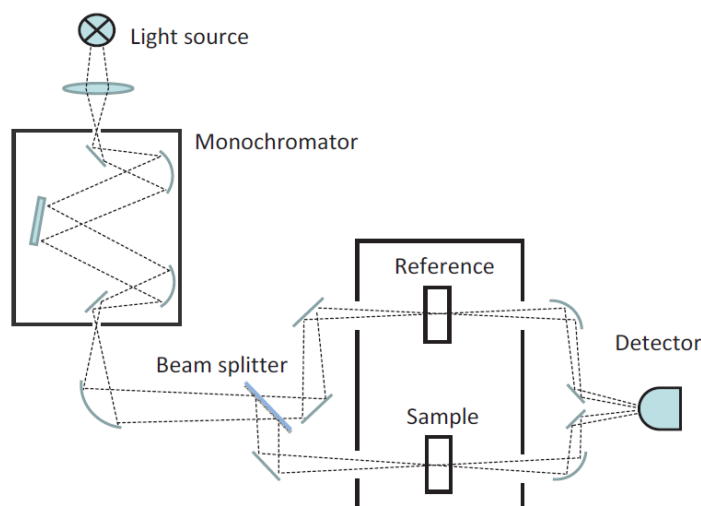


Figure 1: Scheme of a double beam absorption spectrometer.

Fluorescence spectrometer

A fluorescence spectrometer (Figure 2, 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 a UV/vis- and a vis/NIR detection channel and a separately addressable reference channel, respectively, all operated in the photon counting mode, using a conventional $0/90^\circ$ measurement geometry^{41, 58, 75}. All fluorescence measurements were performed 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 demonstrated 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, 79}.

Integrating sphere setup

An integrating sphere setup is used for the absolute determination of quantum yields (Figure 2, configuration B). We performed absolute measurements of the quantum yields with a calibrated custom-built integrating sphere setup previously described. These measurements were evaluated by comparison with fluorescence quantum yields determined relatively with optical methods⁴⁹. Moreover, comparative measurements were performed 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 chosen as representative examples for a charge transfer dye with a large Stokes shift in polar solvents (minimum spectral overlap between absorption and emission) and a dye with a small Stokes shift (considerable spectral overlap between absorption and emission; typical for fluorophores with resonant emission¹⁰), respectively, we compared the results obtained with our procedure and the Φ_f values derived with the emission correction curve implemented by Hamamatsu and the data evaluation software from Hamamatsu (formulas not provided; no reabsorption

correction). These comparisons underlined the reliability of the implemented emission correction curve in the vis region and the need for a reabsorption correction⁴⁷.

The smallest quantum yield, which we measured with our equipment (absolutely and relatively) until now was 0.016 for cryptocyanine in ethanol.

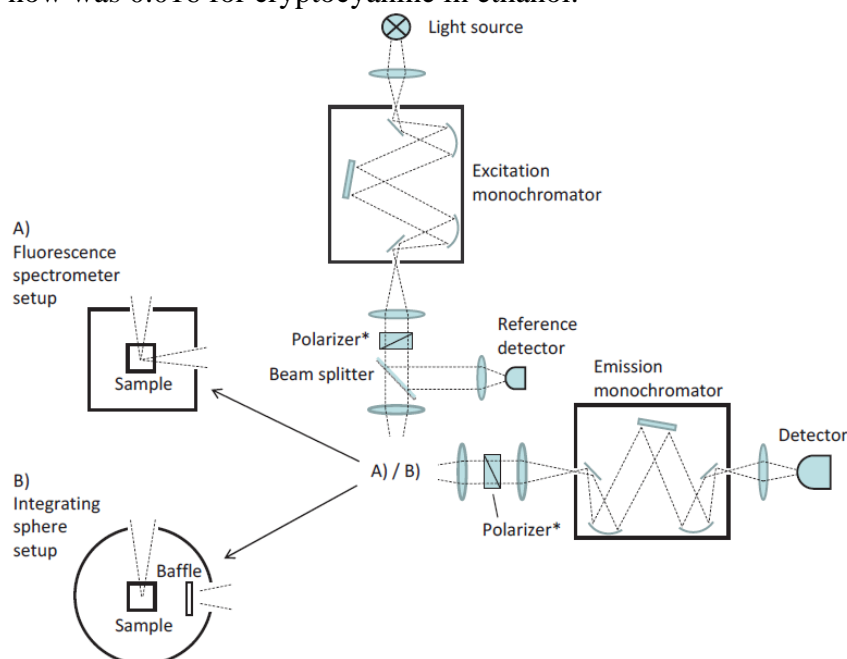


Figure 2: Scheme of a fluorescence spectrometer (configuration A) and an integrating sphere setup (configuration B). *In the case of the integrating sphere setup, polarizers are not necessary.

Instrument calibration and instrumental prerequisites

The calibration of the detection system is a prerequisite to determine 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 like the spectral fluorescence standards F001 to F005^{47, 58, 59, 62} are a comparably inexpensive option to obtain the emission correction curve in the spectral range from 300 to 770 nm^{58, 59, 75, 80}. Physical transfer standards like calibrated lamps have to be used for high precision calibrations and especially for wavelengths > 750 nm, due 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, 81, 82}. As the uncertainty of the measurement of the spectral responsivity of the fluorescence instrument directly affects the overall measurement uncertainty of Φ_f ^{48, 49}, special attention has to be paid to the calibration procedure(s) and reliable reference data from all standards employed.

Higher measurement uncertainties may be also encountered for the use of fluorescence spectrometers that are not equipped with polarizers due to polarization effects resulting from the sample or/and the standard, especially for NIR emitters due to their larger size and shortened fluorescence lifetimes, for fluorophore-labeled (bio)macromolecules and for fluorophores dissolved in solvents of high viscosity like glycerol or entrapped in solid materials (only exceptions are here certain glasses doped with transition and rare earth metal ions)⁷⁵. The size 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 due to multiple scattering and reflection events that result in a complete loss of polarization information of the detected photons.

For integrating sphere setups, in addition, enhanced measurement uncertainties can arise from inaccurately considered reabsorption effects that are accordingly addressed by us in the *PROCEDURES*' section and in the *Supplementary Methods*. Other possible sources of uncertainty are inappropriate and inhomogeneous surface coatings used for sample holders or the sphere surface itself and non-reproducible sample positioning.

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

Reagent Setup

Dyes. Generally, use only commercial fluorophores of the highest purity available. For molecular fluorophores, control of dye purity by e.g., thin layer chromatography (TLC) or high performance liquid chromatography (HPLC; preferably use of diode array detector and fluorescence detector; at least, measurement of absorbance at a typical / the chosen excitation wavelength and at an exemplarily chosen wavelength in the UV below 300 nm for a very stringent purity check) as used by us for the fluorescence standards summarized in Table 3 and Figure 3^{48, 49} is strongly recommended prior to use. The reference dyes employed by us and their purity obtained by HPLC (HPLC system from Knauer equipped with a diode array detector) are detailed in Table 2^{48, 49} (Only data obtained at selected detection wavelengths are shown.). Figure 3 shows the absorption and emission spectra of these standards.

Table 2: Reference dyes used as standards and dye purity assessed by high performance liquid chromatography shown for selected representative detection wavelengths. ^a Detection wavelength; ^b follows from the NIST certificate and certification report. ^c follows from the certificate of analysis.

Dye	Abbreviation	Source	λ (nm) ^a	Purity (%)
quinine sulfate dihydrate	QS ^b	National Institute of Standards and Technology (SRM936a) ⁷⁴		≥ 98
coumarin 153	C153	Lambda Physik GmbH (batch number 029303)	422	99
			455	99.5
fluorescein	F	Sigma-Aldrich (batch number BCBG1058V)	280	> 99
rhodamine 6G	R6G	Lambda Physik GmbH (batch number 119202)	480, 530	98.5
rhodamine 101	R101	Lambda Physik GmbH (batch number 019502)	525	95.5
			565	97.4
oxazine 1	OXA1	Lambda Physik GmbH (batch number 090214)	665	98.2
HITCI	HITCI	Lambda Physik GmbH (batch number 029006)	760	97.9
IR125	IR125	Lambda Physik GmbH (batch number 10970; counter anion perchlorate)	800	99.1

For the recommendation of suitable quantum yield standards, we intended to provide examples for dyes, which are commercially available at a reasonable price, thereby also avoiding additional purification procedures like TLC or HPLC that require a certain chemical expertise and lab equipment. This enables the broad community of users of fluorescence techniques to employ these materials as long as there are no certified fluorescence quantum yield standards available. Although dyes of a purity of 99 % or higher are principally ideal, such materials are difficult to obtain for the complete wavelength region covered by our recommended standards. To minimize purity-related uncertainties, we provided information on the materials used by us including dye manufacturer and batch number and on dye purity including our measurement conditions used.

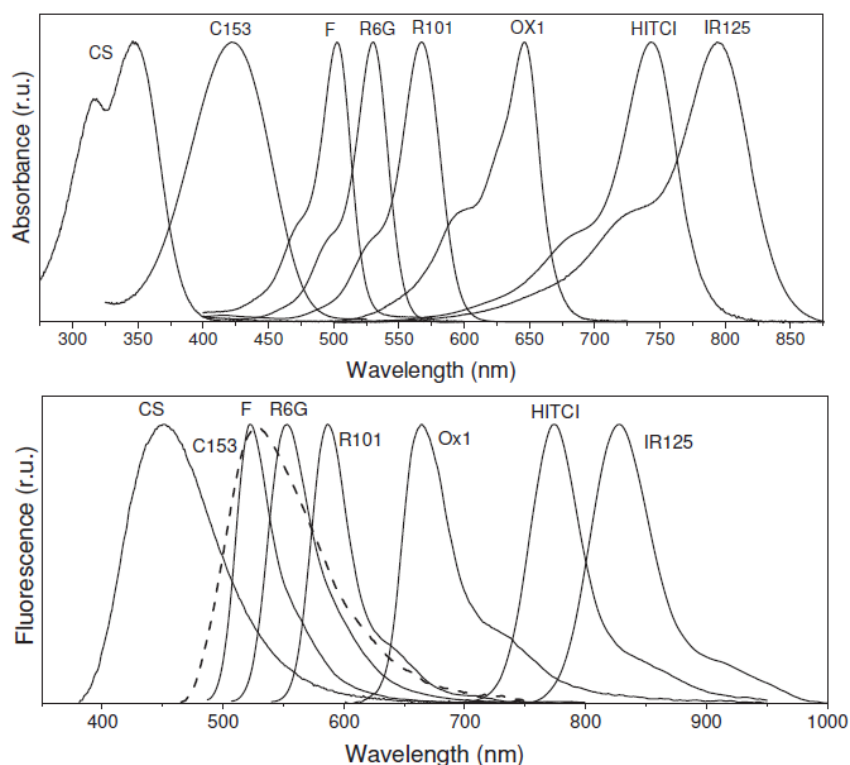


Figure 3: Absorption- (top) and emission (bottom) spectra of the recommended quantum yield standards.

The fluorescence quantum yields (Φ_f) of the recommended reference dyes (Table 3) in the manuscript were calculated from integrated, blank- and spectrally corrected emission spectra employing different fluorescence standards in the case of the relative measurements (use of a chain of Φ_f transfer standards and identical excitation wavelengths for the respective sample/standard pair) or an integrating sphere setup^{48, 49}. For each compound-solvent pair, the quantum yield was always determined at least twice, often using concentration series for dyes with a small Stokes shift to enable a reabsorption correction⁴⁷⁻⁴⁹. For the determination of relative standard deviations, at least six independent measurements per dye were performed. In all cases, oxygen-saturated solutions were used (air-pressure 101 kPa, temperature 25 °C) to ease the reproducibility of these measurements.

Table 3 Fluorescence quantum yields and absorption and emission ranges of the dyes recommended as quantum yield standards. * Relative determination of fluorescence quantum yield using the absolutely measured fluorescence quantum yield of R101 as reference.^{48, 49}

Dye	QS	C153	F	R6G	R101	OX1	HITCI	IR125
Solvent	0.105 M HClO ₄	EtOH	0.1 M NaOH	EtOH	EtOH	EtOH	EtOH	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	750-1000
Φ	0.59*	0.53*	0.89*	0.91*	0.915	0.15	0.30	0.23
$\Delta\Phi$	0.04	0.04	0.04	0.04	0.028	0.01	0.01	0.01

Solvents. Generally, only solvents of the highest purity commercially available are recommended that should be free of fluorescent impurities. This is typically the case for spectroscopic grade solvents or for solvents for liquid chromatography. Check on solvent purity first by measurement of an emission spectrum at the excitation wavelength to be used for subsequent fluorescence quantum yield measurements. For a very stringent control of solvent purity, excitation in the ultraviolet region below 300 nm can be performed. Avoid the use of “old” solvents as e.g., traces of water in hygroscopic solvents like ethanol or DMSO can affect the spectral shape and intensity of the absorption and emission spectra of many fluorophores, their molar absorption coefficients, and especially their fluorescence quantum yields. The solvents employed by us for these quantum yield standards, i.e., ethanol in the case of R101, R6G, C153, OXA1, and HITCI, dimethylsulfoxide (DMSO) for IR125 and 0.105 M perchloric acid for QS, were of spectroscopic grade and purchased from Sigma Aldrich Inc. and Merck KGaA, respectively. NaOH used for the preparation of the 0.1 M NaOH solution required for fluorescein was purchased from Merck KGaA. Prior to use, all solvents were checked 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 like certain NIR emitters. The absorption spectra of the dye solutions should be measured regularly to control dye stability and the uptake of water in the case of hygroscopic solvents like 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 like 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. Also the presence of decomposition products from the solvent needs to be avoided. For example, ethers are prone to autooxidation in the presence of light and air yielding strongly oxidizing peroxides that should be removed e.g., by addition of potassium hydroxide prior to use. Chlorinated solvents like CH₂Cl₂ and CHCl₃ can contain hydrochloric acid formed upon partial hydrolysis of the solvent, which can result in dye protonation. Dimethylformamide (DMF) can decompose into dimethylamine and formaldehyde which can affect certain dyes. Oxygen can quench the emission of fluorophores with longer fluorescence lifetimes, see also section on *Limitations*. Be always aware of the relevant application conditions of your sample, if necessary e.g., remove oxygen as described.

In general, fresh preparation of all sample solutions for each measurement is recommended.

PROCEDURES

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

A Relative determination of fluorescence quantum yields approximately 90 min. for moderately to strongly emissive samples.

- i. Clean and dry four 10 mm × 10 mm cuvettes, make sure that the cuvettes are clean and no residual solvent is present as already small traces of the solvent(s) used for cell cleaning (e.g. acetone, ethanol, water) can possibly influence measured quantum yields. **<CRITICAL STEP> For measurements in the UV – spectral region (< 400 nm), use quartz cells only.**
- ii. *Measurement and adjustment of the absorbance A of the quantum yield standard and the sample (steps 1A(ii)-(iii))* 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

- iii. Fill a cuvette with the sample and measure its absorption spectrum (absorbance A as a function of wavelength); if necessary, dilute the dye solution until the absorbance reaches at maximum 0.1 at the longest wavelength absorption band to minimize possible reabsorption effects.
- iv. *Test measurements (steps 1A(iv)-(v))* After sample preparation, measure the absorption spectrum, wait for 5 to 10 minutes, and re-measure the absorption spectrum, thus making sure it remains constant over time.

?Troubleshooting.

- v. Dilute the sample by a factor of at least 5, wait for 5 to 10 minutes, 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 provide a hint for dye aggregation.

?Troubleshooting.

- vi. Choose a quantum yield standard from Table 3 and Figure 3 which 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 sample and standard are excited at an almost plateau-like region of their absorption spectra or at least at a wavelength with only little slope in the absorption spectrum (see Figure 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 (see Table 3).

- ix. Adjust the standard's absorbance to match that of the sample at the chosen excitation wavelength (see Figure 4). Make sure that the absorbance of the standard does not exceed values of 0.1 at the longest wavelength absorption maxima of the fluorophore to reduce possible reabsorption effects, especially for dyes with a small Stokes shift. If this impedes to match the absorbances at the excitation wavelength, try to keep the differences as small as possible.
- x. *Measurement of the emission spectra (steps IA(x)-(xiv)).* Choose instrument settings of your fluorescence spectrometer such that sample and 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 (magic angle conditions) to render detected emission intensities independent of a possible emission anisotropy of the sample and the standard^{1, 48}. Make sure 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 measurements. (steps IA(xi)-(xii)).* Measure the fluorescence intensity at the emission maximum of the sample for these instrument settings over a period of 5 to 10 min.. Make sure no increase or decrease can be observed.

?Troubleshooting

- xii. Measure the emission spectrum of the sample after dilution by a factor of ca. 5 (see also absorption measurements, step IA(v)). Make sure the measured fluorescence intensity diminishes by approximately the factor used for dilution and 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 longterm illumination (30 min. or more) of the sample during multiple fluorescence measurements, measure the absorption spectrum after completion of the emission measurements to check on sample stability.
- xv. *Calculation of the relative quantum yield (step IA(xv)-(xix))* Calculate the absorption factors f_{st} and f_x of the standard and the sample from the measured absorbances (A) at the excitation wavelength using equation 3. The index x denotes the sample, the index st the standard. If the absorbances determined in Step $IA(iii)$ and $IA(ix)$ match, the quotient f_{st}/f_x equals 1 and this step ($IA(xv)$) can be omitted; for non-matching absorbances, the quotient f_{st}/f_x must be considered.

$$f(\lambda_{ex}) = 1 - 10^{-A(\lambda_{ex})} \quad (\text{eq. 3})$$

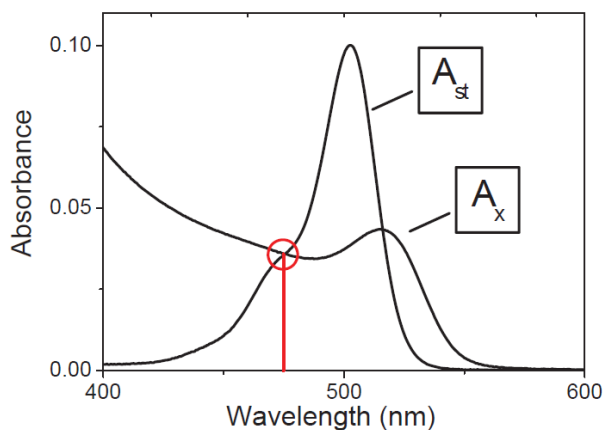


Figure 4: Absorption spectra of sample (A_x) and standard (A_{st}). The red circle/line indicates the optimal wavelength for excitation.

For higher precision, the bandpass $\Delta\lambda_{ex}$ used for excitation can be considered according to equation 4.

$$f(\lambda_{ex}) = \int_{\lambda_{ex} - \frac{1}{2}\Delta\lambda_{ex}}^{\lambda_{ex} + \frac{1}{2}\Delta\lambda_{ex}} 1 - 10^{-A(\lambda_{ex})} d\lambda_{ex} \quad (\text{eq. 4})$$

- xvi.** Subtract the solvent spectra from the measured emission spectra of sample and 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 *GENERAL REMARKS*).
- xvii.** Correct the blank-corrected emission spectra of sample and standard for the spectral responsivity of the emission channel of the fluorometer (see *Supplementary Methods* for more details). **Prior to the next step**, make sure 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 *GENERAL REMARKS in the 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 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, the index st 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.

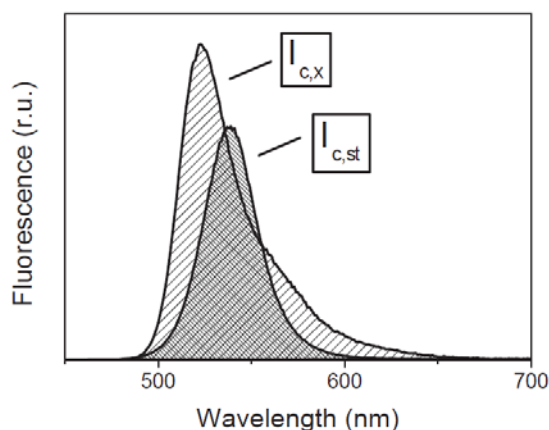


Figure 5: Examples for emission spectra of sample ($I_{c,x}$) and standard ($I_{c,st}$).

$$F = \int_{\lambda_{em}} I_c \cdot \lambda_{em} d\lambda_{em} \quad (\text{eq. 5})$$

<**CRITICAL STEP**> Equation 5 considers the photonic nature of the emitted light by multiplication with λ_{em} ⁸³ (hc_0 is omitted, see section *GENERAL REMARKS in the Introduction and Supplementary Methods*).

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

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

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

F is the integral photon flux (*step 1A(xvii)*), f the absorption factor (*step 1A(xv)*), n the refractive index of the solvent, and Φ_f the quantum yield. The index x denotes the sample, the index st the standard. To be strictly correct the refractive indices at the mean/average emission wavelength should be used. These values are often difficult to obtain from literature for certain solvents. Use instead the values given for the standard wavelengths (e.g. sodium D-line at 589 nm).⁸⁴

B Absolute determination of fluorescence quantum yields (approximately 40 min. for moderately to strongly emissive species).

- i.** Clean and dry two 10 mm × 10 mm cuvettes or the cuvettes required for these measurements, make sure that the cuvettes are clean and no residual solvent is present as already small traces of the solvent(s) used for cell cleaning (e.g. acetone, ethanol, water) can possibly 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 (steps 1B(ii)-(vii))* After sample preparation, measure the absorption spectrum, wait for 5 to 10 minutes and re-measure

the absorption spectrum, making sure 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 to 10 minutes, 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.
- v. Measure the fluorescence intensity at the emission maximum of the sample for these instrument settings over a period of 5 to 10 minutes. Make sure no increase or decrease can be observed.

?Troubleshooting

- vi. Measure the emission spectrum of the sample after dilution by a factor of ca. 5 (see also absorption measurements, *step 1B(iii)*). Make sure the measured fluorescence intensity diminishes by approximately the factor used for dilution and 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}, 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 (see Figure 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 (steps 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 bandpass, wavelength region for signal detection covering excitation and emission, emission bandpass, and integration time). The most important criterion here is that the intensity of the excitation peak should be as high as possible, yet 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. Make sure that the cell position equals the position of the blank. If necessary, use e.g., a laser for cell adjustment.
- xvi.** Measure the transmitted (not-absorbed) excitation light and the fluorescence using identical measurement conditions and instrument settings as employed for the blank.
- xvii.** *Calculation of the quantum yield (steps IB(xvii)-(xxi))* Correct the recorded signals obtained from the sample and the blank for the instrument-specific spectral responsivity.
- xviii.** **Prior to the next step**, make sure 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 section *GENERAL REMARKS* and *Supplementary Methods*.)
- xix.** Calculate the absorbed photon flux F_{abs} and the emitted photon flux F separating the measured spectra of sample and blank in an excitation and an emission region as shown in Figure 6 and subsequently calculating 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 signals of the sample and the blank in the spectral region of the emission according to equations 7 and 8. Measurements of a blank are indicated with the index b, measurements of the sample 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.

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

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

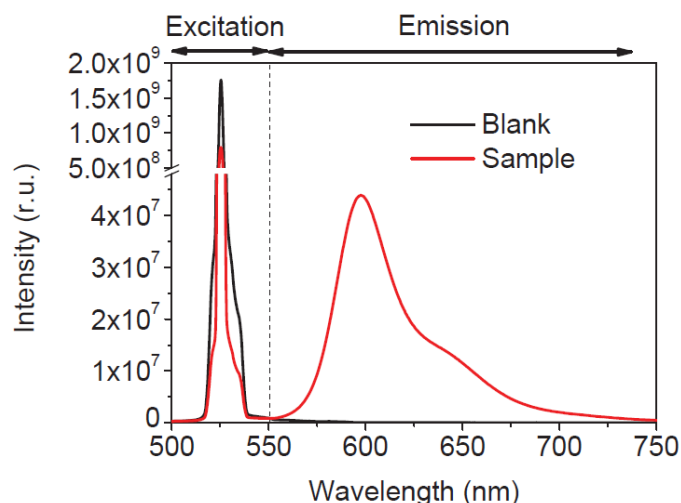


Figure 6: Example for the signals of an integrating sphere measurement for sample and blank and position of the separation between excitation and emission (dotted vertical line).

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

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

- xxi. Correct reabsorption effects as described by us and others^{47, 48, 85}, see also *TROUBLESHOOTING* and *Supplementary Methods*.

ANTICIPATED RESULTS

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 *ca.* 350 nm to 950 nm will lead to an improved reliability of fluorescence quantum yield measurements. The most error-prone steps for the determination of fluorescence quantum yields, which are the 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, a procedure for a reabsorption correction (see *Supplementary Methods*) is given that is required e.g., for the accurate absolute measurement of fluorescence quantum yields of dyes with a very small Stokes shift using an integrating sphere setup. It is to be anticipated 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.

TROUBLESHOOTING

See Table 4 for Troubleshooting advice.

Table 4: Troubleshooting

Problem	Step	Possible reason	Solution
Fluorescence spectrum distorted at short wavelength side of the emission band; shift of emission maximum to longer wavelengths.	1A(xii) 1B(vi)	Reabsorption caused by high dye concentration (see <i>Troubleshooting c</i>).	Dilute the sample solution.
Untypical features in the emission spectrum, e.g., narrow peaks.	1A(xiii) 1B(iv)	Contribution of scattered excitation light to the measured emission spectrum.	Change 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).
Untypical or concentration-dependent absorption spectrum, especially at the short wavelength side of the long wavelength absorption band.	1A(v) 1B(iii)	Dye aggregation caused by high dye concentration (see <i>Troubleshooting d</i>).	Dilute the solution.
Negative or positive off-set in the absorption and/or inclined spectrum.	1A(ii)	Dirty cells, incorrect baseline correction.	Make sure the cells are clean and repeat baseline correction.
Time-dependent increase in absorbance.	1A(iv) 1B(ii)	Incomplete fluorophore dissolution.	Wait until all fluorophores in the sample solution are completely dissolved.
Time-dependent decrease in absorbance.	1A(iv) 1B(ii)	Fluorophore adsorption on cell walls. Decomposition of dye.	Change measurement cell. Change solvent.
Positive off-set in the absorption spectrum and/or untypically high absorbance at short wavelengths.	1A(iii) 1B(ii)	Scattering samples (see <i>Troubleshooting b</i>).	Use an integrating sphere setup.
Increase of emission intensity during illumination.	1A(xi) 1B(v)	Photobrightening.	Sample-specific problem, i.e., for quantum dots ^{10,91} ; use of pre-illuminated samples can be an alternative, yet only 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.
Decrease of emission intensity during illumination.	1A(xi) 1A(xiv) 1B(v)	Photodecomposition.	Reduce excitation light intensity.
Measurement of fluorescence spectra of sample and	1A(xiii)	Fluorescence quantum yields of sample and standard	Dilute the standard solution until its emission spectrum can be recorded under the same

standard with identical instrument settings impossible.		largely deviate.	measurement conditions as used for the measurement of the emission spectrum of the sample. Then, measure the standard's absorption spectrum, see also <i>Procedure A, step 4A</i> .
Decrease in the absorption factor-weighted emission after dilution.	<i>IA(xii)</i> <i>IB(vi)</i>	Concentration-dependent changed of the equilibrium between of surface-bound molecules and dissolved molecules in solution, i.e., ligand desorption in the case of quantum dots upon dilution ⁴¹ (see <i>Troubleshooting e</i>).	Sample-specific problem; one possible alternative can be here the use of other cuvettes with different optical pathlengths to minimize reabsorption effects while maintain a high fluorophore concentration ⁴¹ .
Increase in the absorption factor-weighted emission after dilution.	<i>IA(xii)</i> <i>IB(vi)</i>	Dye aggregation (see above) 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 <i>Troubleshooting d</i>).	Further dilution of the fluorophore solution.

a) Spectral correction of fluorescence spectra for the instrument-specific spectral responsivity

Without spectral correction of measured instrument-specific fluorescence spectra, significant deviations in shape and intensity and thus, also in the resulting fluorescence quantum yields, can occur as exemplarily 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.

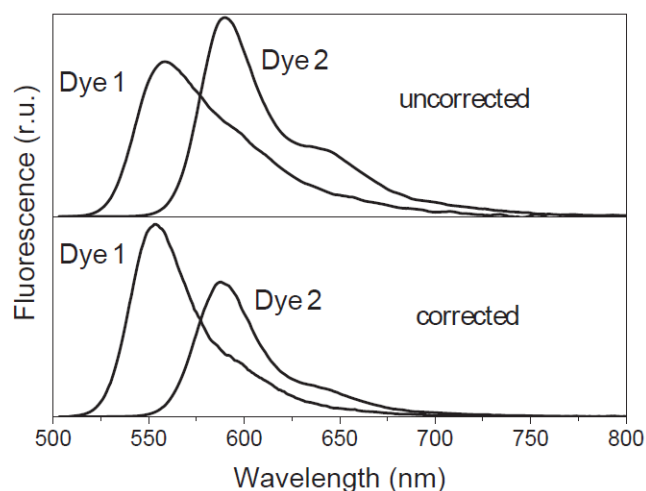


Figure 7: Effect of the spectral correction on the shape and relative intensities of the emission spectra of two dyes.

b) Scattering samples (relative determination of Φ_f)

For solutions of large fluorophore-labeled biomolecules or other macro-molecules as well as for colloids and suspensions of particles, liposomes etc., 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 Figure 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). 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 intensity⁸⁶. The fluorescence quantum yields of such samples can be reliably measured only with an integrating sphere setup^{49, 52, 87-89}.

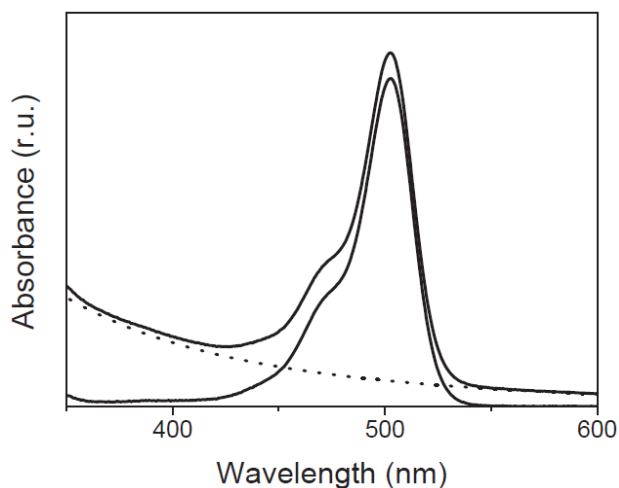


Figure 8: Visualization of the effect of a scattering background (dotted line) on the dye's absorption spectrum, thereby yielding an overestimation of the absorption especially at the short wavelength region.

c) Reabsorption

At high dye concentration, the emitted light can be reabsorbed by the fluorophore in the region of the spectral overlap between absorption and emission as shown in Figure 9 exemplarily for R6G in water. Subsequently, the measured quantum yield is reduced as compared to 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 (indicated by the arrow in Figure 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 ($>$ ca. 600 nm). Reabsorption effects are especially pronounced for integrating sphere setups⁴⁷⁻⁴⁹ 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. The performance of a reabsorption correction is described in the *Supporting Information* and has been reported by us and others^{47, 48, 85}.

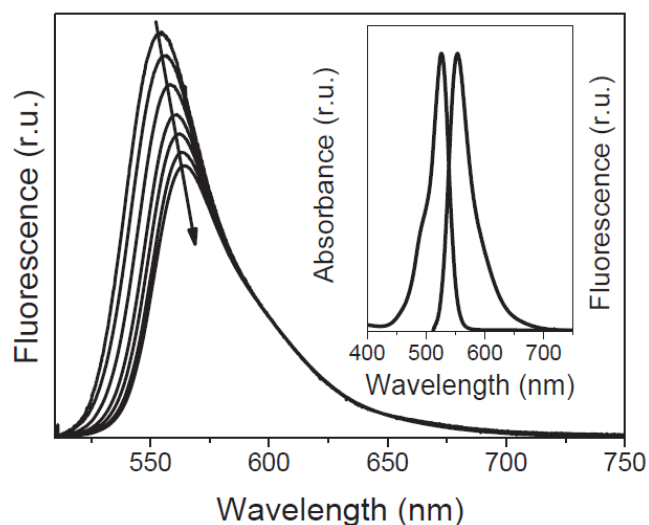


Figure 9: Distortion of the emission spectrum due to fluorescence reabsorption. The inset shows the overlap of the absorption (left curve) with the emission spectrum (right curve).

d) Dye aggregation

At high concentration, some dyes like xanthenes, cyanines, and porphyrines can form non-fluorescent or only weakly emissive aggregates^{10, 57, 90}. This is favored e.g., for hydrophobic dyes in aqueous solution. Typically, dye aggregation results in an enhanced absorption at the short wavelength shoulder of the longest wavelength absorption band (H-type aggregates) as exemplarily displayed in Figure 10 for R6G in water. The inset shows the absorption spectra of the pure monomeric and aggregated form of this dye. The formation of dye aggregates hampers the correct determination of the absorption factor of the non-aggregated dyes at wavelengths in the region of dimer absorption, e.g., at the vibronic shoulder of the 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 considered⁹⁰.

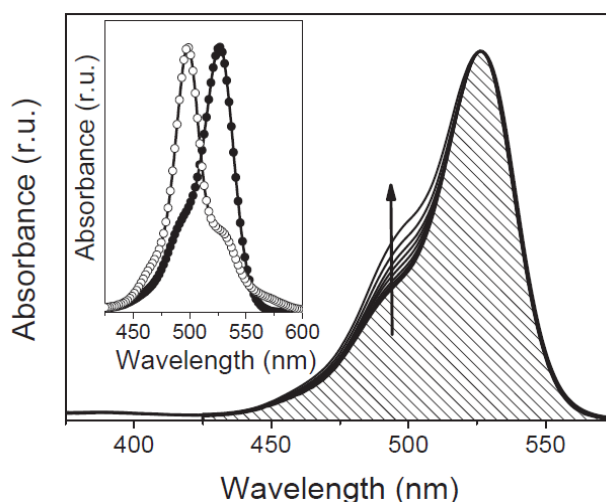


Figure 10: Typical changes in the absorption spectrum caused by dye aggregation (H-type aggregates). The inset shows the pure spectra of the monomer (filled circles) and the aggregate (open circles).

e) Dilution of samples with surface bound-ligands – ligand desorption

The optical properties of fluorophores like quantum dots, which consist of a semiconductor core with surface-bound ligands which are coordinatively, yet 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 or loss in fluorescence. 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.

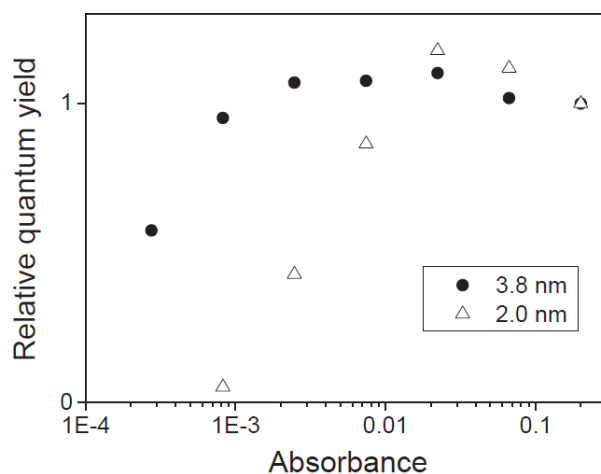


Figure 11: Concentration dependence of the fluorescence quantum yield of two CdTe quantum dots differing in particle size. The absorbance of the sample (x-axis) is proportional to particle concentration.

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Author Contributions

All authors contributed to the concept of the manuscript and to the chapters `experimental design` and `materials section`. The general remarks and troubleshooting section was written by Christian Würth and Markus Grabolle with aid from Ute Resch-Genger. The procedure for relative quantum yield measurements were developed and written by Markus Grabolle, Jutta Pauli, and Christian Würth. The procedure for absolute quantum yield measurements was developed, simplified and written by Christian Würth. Monika Spieles contributed technically, with e.g. new measurements of dye purity. Ute Resch-Genger initiated this research, wrote the introduction, and gave an overview of different techniques for the determination of photoluminescence quantum yields including limitations, method development and fluorescence standards.

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