

# Determination of the photoluminescence quantum yield of dilute dye solutions (IUPAC Technical Report)\*

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**Abstract:** Procedures for the determination of photoluminescence quantum yields with optical methods are addressed, and challenges associated with these measurements are discussed. Special emphasis is dedicated to relative measurements of fluorescent (i.e., short excited-state lifetime), transparent, dilute dye solutions in conventional cuvettes in a 0°/90° measurement geometry. Recommendations on the selection of suitable quantum yield standards are presented, and requirements for the documentation of photoluminescence quantum yields are derived.

**Keywords:** absorption; dilute dye solutions; fluorescence; IUPAC Analytical Chemistry Division; IUPAC Organic and Biomolecular Chemistry Division; IUPAC Physical and Biophysical Chemistry Division; luminescence; photoluminescence quantum yields.

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## I. INTRODUCTION

Photoluminescence quantum yields are among the most important parameters in luminescence analysis, encompassing both fluorescence and phosphorescence [1,12]. The photoluminescence quantum yield, which is in the following termed “fluorescence quantum yield” ( $\Phi_f$ ), equals the ratio of the number of emitted photons ( $N_{\text{em}}$ ) to the number of absorbed photons ( $N_{\text{abs}}$ ), see eq. 1, and presents thus a direct measure for the fraction of excited molecules that return to the ground state with emission of fluorescence photons [13]. Hence, this quantity is a direct measure for the fluorescence efficiency of a dye and a key parameter in fluorescence measurements. For a single emitter, the photoluminescence quantum yield characterizes a radiative transition in combination with the luminescence lifetime, the luminescence spectrum, and the emission anisotropy. Spectroscopically, together with the molar absorption coefficient,  $\varepsilon(\lambda_{\text{ex}})$ ,  $\Phi_f$  determines the sensitivity for the detection of an analyte or target [1,7,12,13]. Hence, the product  $\Phi_f \times \varepsilon(\lambda_{\text{ex}})$ , which is termed “brightness”, is frequently used as a measure to characterize and compare fluorescent labels and dyes and to assess the performance of new fluorescent probes and sensors and improve their rational design. The  $\Phi_f$  also plays a crucial role for many current and emerging applications of fluorescence techniques in biology and medical diagnostics including fluorescence correlation spectroscopy (FCS) and Förster energy transfer studies (FRET; determination of the Förster radius requires knowledge of the fluorescence quantum yield of the donor in the absence of the acceptor) [14] as well as for the characterization of phosphors and chromophores to be used in light-emitting diodes (LEDs) and organic light-emitting devices (OLEDs), see also [15], as well as for laser and scintillation materials. Moreover, the determination of  $\Phi_f$  is of utmost importance for basic research in photophysics and photochemistry.

$\Phi_f$  can be measured relative to a fluorescent standard material with a known  $\Phi_f$  or as an absolute quantity either directly by optical methods or indirectly by calorimetric methods. The direct method measures the emitted photons, whereas the calorimetric method measures, directly or indirectly, the increase in temperature in the sample [16]. Since Vavilov first introduced both the term “fluorescence yield” and a method for its determination for fluorescent organic dyes in liquid solution [17,18], numerous publications have appeared that deal with measurements of fluorescence quantum yields and/or suggested or recommended quantum yield standards [1,16,19,34–40]. However, despite the obvious and ever increasing importance of  $\Phi_f$  at present, there are only very few overall accepted recommendations for the comparatively simple determination of this quantity even for transparent, dilute solutions available [28,30,36,40] as well as only very few reports on achievable measurement uncertainties [36,38,41,42]. This includes a recent assessment of measurement uncertainties related to each step required for the relative measurement of  $\Phi_f$  employing similar excitation wavelengths for sample and standard, a dye transfer chain approach, and the use of different excitation wavelengths in conjunction with an excitation correction as well as absolute measurements of  $\Phi_f$  with two different integrating spheres, a custom-made and a commercial setup [36,38,42]. In addition, a few application notes have been provided by instrument manufactures [43], yet no approved guidelines or technical notes exist at

present. Only very recently, a detailed protocol describing procedures for the relative and absolute measurement  $\Phi_f$  of transparent dye solutions in the ultraviolet (UV), visible (vis), and near infrared (NIR) spectral region including pitfalls and achievable uncertainties has been reported, which includes also recommendations for suitable reference standards for this wavelength region [44].

The overall goal of this technical note is to provide the broad community of fluorescence spectroscopists with a description of the relative determination of  $\Phi_f$  values. This poses severe challenges in view of the various uncertainties and sources of error in the determinations involving optical methods. We would like to present those sources of error and describe procedures for the reliable determination of  $\Phi_f$ . Special emphasis is dedicated to relative measurements of  $\Phi_f$  of fluorescent (short excited-state lifetimes of typically <10 ns) transparent, dilute dye solutions in conventional cuvettes at room temperature with 0°/90° measurement geometry. Front face measurements and the measurement of  $\Phi_f$  values of scattering solutions are beyond the scope of this document. This holds also true for other methods for the determination of  $\Phi_f$  including calorimetric approaches that have been recently reviewed [16], and integrating sphere-based techniques, which enable measurements of scattering samples and yield absolute fluorescence quantum yields [36,38,40,44,45,47]. The determination of  $\Phi_f$  of solid samples and films is detailed in [15]. Within this focus, recommendations on the selection of suitable quantum yield standards are presented and requirements on the documentation of  $\Phi_f$  are derived. This approach presents the basis for evaluated standard operation procedures recently published [36,38,44]. For the quantification of achievable uncertainties with high-precision instrumentation properly calibrated and moderately to highly emissive dyes, the reader is referred to refs. [36,38,41,44]. Following these procedures using accordingly chosen standards and properly calibrated instrumentation, uncertainties of  $\pm(5\text{--}10)\%$  for the determination of moderate to high  $\Phi_f$  values can be achieved. In a separate technical note [48], the requirements for the minimization of typical calibration and measurements sources of error are discussed. With proper consideration of method-inherent requirements and method-specific limitations, these recommendations can be extended to phosphorescence with its considerably longer lifetime resulting in, for example, an enhanced sensitivity of measured  $\Phi_f$  values to the presence of fluorescence quenchers like oxygen.

## II. TERMINOLOGY

The 2006 update of the recommendations on the use of terms in photochemistry by the Subcommittee on Photochemistry of the IUPAC Organic and Biomolecular Chemistry Division [13] defines the quantum yield ( $\Phi$ ) as number of defined events occurring per photon absorbed by the system.  $\Phi$  can be used for photophysical processes (such as, e.g., *intersystem crossing*, *fluorescence*, and *phosphorescence*) or *photochemical reactions*.

In the older literature, slightly different definitions can be found. Birks [49] defines fluorescence quantum efficiency,  $\eta_f$ —a molecular property—as given above but considers  $\Phi_f$  as a secondary parameter—a sample property—influenced by concentration quenching. Both quantities are related by  $\eta_f = \Phi_f (1 + K C)$ , where  $K$  is the Stern–Volmer coefficient and  $C$  is the concentration of the fluorophore. While  $\Phi_f$  can change with concentration, the molecular parameter  $\eta_f$  does not. The last concept is consistent with the general definition of quantum yield as the number of defined events occurring per photon absorbed by the system.

As stated in the introduction, in the following, the term “quantum yield” will be addressed only in combination with “fluorescence”, with  $\Phi_f$  adopting the definition given in eq. 1. This is the simplest definition of  $\Phi_f$  and eliminates the difficulty arising from the energy difference of absorbed and emitted photons having different wavelengths.

$$\Phi_f = \frac{N_{\text{em}}}{N_{\text{abs}}} \quad (1)$$

If the fluorescence quantum yield of a certain species like a dye is to be determined, this definition refers to chemically identical species and ideally also to spectroscopically identical species (e.g., thereby distinguishing between monomeric and dimeric or aggregated dyes or between dye molecules in different environments). As detailed in Section III.4.6, in this respect, it needs to be distinguished between the fluorescence quantum yield of a solution (e.g., containing different absorbing species of varying  $\Phi_f$  like monomeric and aggregated dye molecules) obtained for an ensemble and the  $\Phi_f$  value of, for example, the species of interest, typically the monomeric dye [44,50,51]. This should be always explained in the measurement procedure.

For a single fluorescent species under ideally dilute conditions,  $\Phi_f$  is identical to the quantum efficiency,  $\eta_f$ , and is intrinsically related to its fluorescence lifetime,  $\tau_f$ , through the radiative and the non-radiative transition probabilities ("rate constants in kinetic terms")  $k_f = \Phi_f \tau_f^{-1}$  and  $k_{nr} = (1 - \Phi_f) \tau_f^{-1}$  [1,49]. This is strictly true only for species revealing mono-exponential decay kinetics [49], where a single lifetime can be related to a single species. For multi-exponential decay kinetics, these relationships are not valid anymore. If  $\Phi_f = \eta_f$ , the fluorescence quantum yield of a certain molecular species is by definition independent of sample absorption, excitation intensity, wavelength, and concentration. As addressed in Section III.4.6, the  $\Phi_f$  value of a sample can vary as a function of fluorophore concentration, for example, in the case of dye aggregation (often termed "self-quenching") [44,50,51]. This is related to the presence of at least two absorbing (and simultaneously excited) species in solution that vary in  $\Phi$ ; yet the  $\Phi$  value of the monomeric dye itself does not change [50]. Whether this can affect the actual  $\Phi_f$  measurement or not depends on the possibility of discriminating spectrally between the different species in excitation and in emission, which is controlled by the type of aggregate formed [52]. Also, the presence of other fluorescence quenchers like heavy- and transition-metal ions and paramagnetic species can affect  $\Phi$ .

### III. RELATIVE DETERMINATION OF FLUORESCENCE QUANTUM YIELDS

With optical methods, the  $\Phi_f$  of an emissive species is typically obtained relative to an appropriate reference with known  $\Phi_f$ , using identical instrument settings/measurement conditions detailed in the following sections including the same excitation wavelength [36,38,41,44]. Less frequently, also different excitation wavelengths for sample and standard and otherwise identical instrument settings/measurement conditions are employed [36].  $\Phi_f$  is then determined according to the formula of Demas and Crosby (eq. 2) [28,30,36,41,44].

$$\Phi_{f,x} = \Phi_{f,st} \frac{F_x}{F_{st}} \frac{f_{st}(\lambda_{ex,st})}{f_x(\lambda_{ex,x})} \frac{n_x^2}{n_{st}^2} \frac{q_{p,st}(\lambda_{ex,st})}{q_{p,x}(\lambda_{ex,x})} \quad (2)$$

In eq. 2, the subscripts x and st denote sample and standard, ex and em excitation and emission wavelength(s),  $\Phi_{f,st}$  equals the standard's  $\Phi_f$ .  $F$  presents the integrated spectral fluorescence photon flux  $q_{p,\lambda}^f(\lambda_{em})$  at the detector that is obtained from the blank and dark-count corrected signal of the emission detector  $I_u(\lambda_{em})$  multiplied by the photon energy  $hc/\lambda_{em}$  ( $h$ : Planck constant;  $c$ : velocity of light) and divided by the spectral responsivity  $s(\lambda_{em})$  of the emission channel (emission correction), see [36,38,41,44]. This quotient is integrated over the complete emission wavelength range of the respective dye [36,38,44].

$$F = \int_{\lambda_{em_1}}^{\lambda_{em_2}} q_{p,\lambda}^f(\lambda_{em}) d\lambda_{em} = (hc)^{-1} \int_{\lambda_{em_1}}^{\lambda_{em_2}} \frac{I_u(\lambda_{em})}{s(\lambda_{em})} \lambda_{em} d\lambda_{em} \quad (3)$$

The absorption factor  $f(\lambda_{ex})$  in eq. 2 provides the fraction of the excitation light absorbed by the chromophore and is equivalent to the obsolete term "absorptance"  $\alpha_x(\lambda_{ex})$ .  $f(\lambda_{ex})$  is linked to

absorbance  $A(\lambda_{\text{ex}}) = \varepsilon(\lambda_{\text{ex}}) C * l$  (cf. Beer–Lambert law), see eq. 4, with  $C$  equaling the concentration of the analyte and  $l$  the optical pathlength.  $f(\lambda_{\text{ex}})$  is given in good approximation by the absorbance  $A(\lambda_{\text{ex}})$  at the excitation wavelength for very dilute and transparent dye solutions. For a more exact calculation of  $f(\lambda_{\text{ex}})$ , the spectral bandwidth of the excitation light  $\Delta\lambda$  should be considered, see eq. 5.

$$f(\lambda_{\text{ex}}) = 1 - 10^{-A(\lambda_{\text{ex}})} = 1 - 10^{-\varepsilon(\lambda_{\text{ex}}) C l} \quad (4)$$

$$f(\lambda_{\text{ex}}) = \frac{\int_{\lambda_{\text{ex}} - \Delta\lambda}^{\lambda_{\text{ex}} + \Delta\lambda} (1 - 10^{-A(\lambda)}) d\lambda}{\int_{\lambda_{\text{ex}} - \Delta\lambda}^{\lambda_{\text{ex}} + \Delta\lambda} d\lambda} \quad (5)$$

$q_{\text{p,st}}(\lambda_{\text{ex,st}})$  and  $q_{\text{p,x}}(\lambda_{\text{ex,x}})$  in eq. 2 are the spectral photon fluxes at sample position for standard and sample at the chosen excitation wavelengths. The photon flux  $q_{\text{p}}(\lambda)$  is the integral of the photon irradiance  $E_{\text{p}}(\lambda)$  over the illuminated area, see [36,44].  $q_{\text{p,st}}(\lambda_{\text{ex,st}})$  and  $q_{\text{p,x}}(\lambda_{\text{ex,x}})$  are identical for use of identical excitation wavelengths for sample and standard and have to be considered only for use of different  $\lambda_{\text{ex}}$  for the sample and the standard [36,48]. The refractive index correction term ( $n_i^2$ ) in eq. 2 has to be applied if different solvents are employed for the sample and the standard [1,36,38,41,44,53,54].

### III.1 Steps and necessary measurements for the relative determination of the fluorescence quantum yield

The procedure to perform relative measurements of  $\Phi_{\text{f}}$  consists of the following steps [36,41,44]:

- i) preliminary measurement of the absorption and emission spectra of the sample,
- ii) choice of a suitable reference (termed also  $\Phi_{\text{f}}$  standard; requirements, see Section III.3) and preliminary measurement of its absorption and emission spectrum,
- iii) choice of measurement conditions (see Section III.4) and measurement of the corresponding absorption and emission spectra of the sample and the standard, and
- iv) data evaluation and calculation of  $\Phi_{\text{f}}$  according to eq. 2.

Protocols for the performance of such measurements, including a set of fluorescence quantum yield standards covering the spectral region from 350 to 1000 nm, have been only recently reported [44]. These protocols also address challenges and critical steps of these measurements.

### III.2 Prerequisites

Prior to the determination of  $\Phi_{\text{f}}$ , the following instrument-specific parameters and quantities must have been determined:

#### III.2.1 Spectrofluorometer

Determination of all quantities and parameters that can affect the spectral position, spectral shape, and intensity of fluorescence signals [36,41,48,55,57]. This includes:

- i) wavelength accuracy of the instrument's excitation and/or emission channel,
- ii) range of linearity of the instrument's detection system, and

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\*We use here  $C$  as symbol for concentration in  $\text{mol dm}^{-3}$ , which is typically abbreviated with  $c$  in order to avoid confusion with the velocity of light,  $c$ .

- iii) wavelength- and polarization-dependent (relative) spectral responsivity of the emission channel [ $s(\lambda_{\text{em}})$ ], termed also “emission correction” [36,41,48], see also eq. 2.

If two different excitation wavelengths are used for sample and standard as described in [36], in addition, knowledge of:

- iv) wavelength- and polarization-dependent (relative) spectral irradiance  $E_{\lambda}(\lambda_{\text{ex}})$  at the sample position or the spectral photon fluxes  $q_{\text{p,st}}(\lambda_{\text{ex,st}})$  and  $q_{\text{p,x}}(\lambda_{\text{ex,x}})$  is mandatory (see also eq. 2 and excitation correction in [48,55]).

The procedure to perform such measurements is detailed in a recent IUPAC technical note [48] and in a technical note from ASTM International [58]. Simple procedures for the determination of the (relative) spectral responsivity were only recently assessed in two international comparisons, one comparison involving four National Metrology Institutes and in another comparison involving several field laboratories randomly chosen from academia and industry [59,60]. The used and evaluated fluorescence standards, the BAM Kit *Spectral Fluorescence Standards* covering the spectral region from 300 to 770 nm, are commercially available.

Moreover, knowledge of the emission anisotropy of the sample and the standard is beneficial (see Section III.4), as this quantity determines the need for the use of excitation and emission polarizers.

### III.2.2 Spectrophotometer

If the absorption of the sample and the standard at the excitation wavelength(s) is measured separately from the fluorescence measurements as is typically the case, all instrument quantities of the spectrophotometer that can affect the spectral position, spectral shape, and intensity of the absorption signals need to be controlled. This includes:

- i) accuracy of the wavelength scale, and
- ii) accuracy of the intensity scale.

### III.2.3 Solvents

Generally, all reagents (dye, solvents, other additives) employed for fluorescence measurements should be of the highest purity available. Accordingly, for the preparation of dye solutions, only solvents of the highest purity should be used. With this respect, it should be kept in mind that commonly used spectroscopic grade solvents are meant for absorption measurements and even such solvents can contain stabilizers that may act as fluorescence quenchers or even emit themselves. Such potential sources of uncertainty can be avoided if the solvents employed for  $\Phi_{\text{f}}$  measurements are freshly distilled following procedures given in [61].

Special care has to be taken in the case of hygroscopic solvents like ethanol, methanol, or dimethylsulfoxide (DMSO), for example, as the presence of water can affect the fluorescence quantum yield of some dyes, especially charge-transfer-operated fluorophores like coumarins or Nile Red. Here, even small amounts of water can result in a decrease of the measured emission intensity, frequently in conjunction with a red shift of the absorption and often a less pronounced shift of the emission maximum. Other critical solvents are ethers (e.g., diethylether and tetrahydrofuran) that can form strongly oxidizing peroxides in the presence of light, air, and water, and the water needs to be removed with potassium hydroxide (KOH). The chlorinated solvents  $\text{CH}_2\text{Cl}_2$  and  $\text{CHCl}_3$  can contain hydrochloric acid, and dimethylformamide (DMF) can decompose into dimethylamine and formaldehyde. For these type of solvents, always the use of fresh solvents (freshly opened or distilled) is strongly recommended. In the case of bidistilled water, pH control is recommended as the pH may vary depending on the water purification procedure used.

### III.2.4 Cuvettes/measurement cells

For the measurement of  $\Phi_{\text{f}}$  it is recommended to use high-quality quartz cells to avoid:

- i) interferences, such as absorption (of exciting and/or emitted light) by the cell (e.g., glass and many polymer cells are unsuitable for measurements in the UV spectral region),
- ii) problems due to a chemical reaction of the solvent with the cell material (rendering the cell windows opaque or even degrading the cuvette, e.g., when certain organic solvents are used with certain polymer cells) or due to unspecific adsorption of the fluorophore at the cell wall,
- iii) differences in optical path lengths of the cuvettes used for sample and standard (e.g., for inexpensive polymer cells, tolerances can be rather large), and
- iv) autofluorescence from the cell material that can be critical for short-wavelength excitation (<500 nm) and for inexpensive polymer cells. This can be controlled prior to use by measuring the emission spectrum of the solvent to be used in such a cell.

Generally, the use of clean glassware and cells and thus, proper cleaning procedures is very critical for all types of fluorescence measurements due to the inherent sensitivity of this method. Even quartz cuvettes can have problems of acidity, adsorption, etc., and a special procedure for cleaning should be adopted. A suitable example is listed in [61].

### III.3 Choice of fluorescence quantum yield standard

Criteria for the choice of suitable  $\Phi_f$  standards were only recently detailed in [36,38,41,44,62]. A suitable  $\Phi_f$  standard should absorb and emit within similar spectral regions as the sample [36,38,41,44]. If standard and sample emit within considerably different spectral regions, the reliability of the spectral correction of the emission spectra is gaining in importance [36]. Also, the standard should be excitable at an excitation wavelength suitable for the sample, see Section III.4. Otherwise, different excitation wavelengths may be used for sample and standard. This requires correction (i.e., consideration of the wavelength dependence of the exciting photon flux, see also Sections III.2.1.iv and III.4.5), which can result in higher uncertainties for non-expert laboratories [36]. The  $\Phi_f$  of the standard must be reliably known, preferably including its uncertainty [36,38,39,41,44], and the standard must be well characterized with respect to all the parameters that can affect its quantum yield such as dye purity, solvent (type and purity), temperature, oxygen concentration (as well as to the presence of other quenchers), excitation wavelength, and chromophore concentration [1–4,16,39,44,63,68], see also [62]. In addition, the measurement conditions (instrument used including calibration, instrument settings, measurement geometry, type of cell used, etc., see Section III.4) should be well documented, see [62] and Section III.9 [9,16,44]. The size of the  $\Phi_f$  values of standard and sample should preferably be not too different to circumvent problems related to nonlinearity of the detection system. This can be the case for variations in  $\Phi_f$  by a factor of at least 5. In this case, either the standard or the sample can be diluted, which, however, results in enhanced measurement uncertainties as highlighted in Section III.4.6, or an attenuator with no or a known wavelength-dependent absorption profile can be used as an alternative. For example, a neutral density filter can be used in the excitation channel for measurement of the fluorescence of the more strongly emitting solution, typically the reference [69]. This may also lead to enhanced measurement uncertainties, see Section III.4.4. The suitability of the attenuation procedure can be controlled by the measurement of absorption and emission spectra for different dye concentrations without and with attenuator. If the attenuator is placed in the emission channel, a comparison of the normalized emission spectra measured without and with attenuator is recommended to make sure that the transmission profile of the attenuator does not affect the spectral shape of the resulting emission spectra. This requires a flat (i.e., wavelength-independent) transmission of the filter within the spectral window of the emission spectrum of the dye or mathematical consideration using, for example, the previously measured transmission spectrum of the attenuator.

The  $\Phi_f$  standards investigated by several independent authors or claimed as reliable standards as well as standards cited in the recent literature have been recently reviewed within IUPAC Project #2004-021-1-300 [39]. The absolute  $\Phi_f$  of some of these commercially available dyes were only very recently

re-measured [36,38,40,42,44], thereby also providing a complete purity analysis. Popular standards include quinine sulfate with all its problematic features—complicated ground-state chemistry [70,71], dependence of emission band position on excitation wavelength [72]—together with fluorescein and rhodamine 6G and rhodamine 101 recommended for the vis region [36,38,41,44]. The wavelength dependence of  $\Phi_f$  of quinine sulfate was only recently re-measured, thereby underlining its potential as quantum yield standard [42]. Fluorescein suffers from its sensitivity to acid–base chemistry and its photochemical instability [24,32]. This must not necessarily present a problem for its use as quantum yield standard if solutions of known and controlled pH are used. Moreover, with the aid of absorption measurements before and after the fluorescence measurements, the photostability of the fluorescein solutions can be controlled. For the rhodamines, rhodamine 6G and rhodamine 101 are probably the best candidates [24,36,39,41,44]. Other popular standards suggested in the literature are anthracene and 9,10-diphenylanthracene (9,10-DPA) despite their susceptibility toward the presence of oxygen, their sharp and narrow bands, and small Stokes shifts. Especially, the latter can be really critical for DPA. In the case of many otherwise attractive coumarins or cyanine dyes (e.g., for use as NIR quantum yield standards), the literature used to disagree on reference values [16]. The relative and absolute fluorescence quantum yield of coumarin 153 in ethanol and dibutylether, however, was recently re-measured [36,41,44,73], thereby also recommending this dye as fluorescence quantum yield standard. Moreover, the absolute fluorescence quantum yields of several NIR dyes that cover the wavelength region from ca. 650 up to 1000 nm have been presented recently [38,44], including uncertainties as well as the  $\Phi_f$  from popular NIR-emissive labels [38].

### III.4 Choice of instrument settings

For the relative determination of  $\Phi_f$ , the emission spectra of the sample and the standard must be obtained with identical spectrofluorometer settings. Also, the emission spectra of the solvents used for the sample and the standard need to be measured with identical instrument settings [blank or blank spectrum  $I_b(\lambda_{\text{ex}}, \lambda_{\text{em}})$ ] for the blank correction of the measured emission spectra  $I_m(\lambda_{\text{ex}}, \lambda_{\text{em}})$  yielding background-corrected, uncorrected (i.e., instrument-dependent) emission spectra  $I_u(\lambda_{\text{ex}}, \lambda_{\text{em}})^*$  with  $I_u(\lambda_{\text{ex}}, \lambda_{\text{em}}) = I_u(\lambda_{\text{em}})$  in equation, see Section III.8 and [36,38,41,44]. As addressed in a recent technical note on the characterization of photoluminescent measuring systems [48], particular attention has to be given to:

- i) slit widths/spectral bandpasses (excitation, emission);
- ii) detector voltage and detection mode (e.g., analogue mode measuring photocurrents or photon-counting mode);
- iii) filters and/or attenuators in the excitation and emission channel;
- iv) polarizers and polarizer settings (excitation and emission channel);
- v) integration (or scanning or averaging) time;
- vi) pulse duration, delay time, and gate time for instruments equipped with pulsed light sources and long-lived chromophores;
- vii) excitation wavelength  $\lambda_{\text{ex}}$ ; and
- viii) type of cell and measurement geometry.

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\*The measured fluorescence signal (excitation and emission) contains instrument-dependent and sample-specific contributions and background signals (e.g., scattering and fluorescence from the solvent and dark counts at the detector; see [48], eq. 1, and [55]). Background-corrected spectra that are not corrected for instrument-specific signal contributions (no spectral correction) are termed “uncorrected spectra”. Additional correction for instrument-specific signal contributions yields corrected spectra that are instrument-independent.

As emphasized in the introduction, for the relative determination of  $\Phi_f$  of transparent dye solutions with a conventional spectrofluorometer, only a  $0^\circ/90^\circ$  measurement geometry should be used.

#### *III.4.1 Spectral bandpass and absorption at the excitation wavelength*

If the absorption of a sample is measured separately, as is typically the case, different monochromator slits in absorption (i.e., those of the spectrophotometer employed) and excitation (i.e., those of the excitation channel/source of the fluorescence instrument), in conjunction with slit functions of different shape, can introduce an additional uncertainty [64,74,75]. To minimize such uncertainties, the excitation slits of the spectrofluorometer should be chosen as narrow as possible. This holds similarly true for the spectrophotometer, yet is typically automatically done (absorption spectra are commonly recorded with a slit width of 1 nm or less).

#### *III.4.2 Spectral bandpass in emission*

The choice of an adequate spectral bandpass in emission is critical when measuring the fluorescence quantum yields of compounds with structured emission spectra like anthracene. If the spectral bandpass is too large, for example, on the order of the width at half height of a sub- or vibronic band of the emission band, the structure becomes smeared and the resulting spectrum appears too smooth. Such effects are even more critical when the spectra are transformed to the energy scale because of the inverse proportionality of wavelength and wavenumber. In general, when fluorescence spectra are converted to the energy scale, this relationship leads to varying spectral bandpasses as a function of the emission wavelength (e.g., 8 nm at 450 nm equals  $395\text{ cm}^{-1}$ , but only  $189\text{ cm}^{-1}$  at 650 nm). The latter is usually taken into account by an empirical  $\lambda^2$  correction [1, p. 53 ff.].

#### *III.4.3 Use of polarizers and instrument- and sample-related polarization effects*

Fluorescence measurements can be affected by instrument- and sample-related polarization effects [76]. The former include the degree of polarization of the spectral irradiance at sample position and the polarization-dependent responsivity of the emission channel, namely, the ratio of its responsivity to vertically and horizontally polarized light [77]. Instrument-related polarization effects are mainly caused by the dependence of the transmittance and reflectance of the instrument's optical components, especially gratings, on the polarization of the incident light [78]. Accordingly, the excitation light is always at least partly polarized, with the degree of polarization depending on the instrument. In the case of conventional lamps like xenon lamps, the main factor controlling the polarization of the excitation light is the monochromator gratings. If lasers are used as excitation sources, the polarization is almost perfect. The spectral responsivity of the emission channel always depends on both wavelength and polarization. Both dependences can be accounted for by excitation and emission correction curves [36,48,55,56,58–60]. Sample-related polarization effects reflect the size of the fluorescence anisotropy or (de)polarization of the sample [1]. Considerable emission anisotropies can be expected, for example, for the vast majority of fluorophores in a confined environment like solid matrices or viscous solvents (restricted or reduced molecular motion and dye rotation in the excited state), for large chromophores with an extended  $\pi$ -system like many NIR dyes as well as for fluorophores attached to (bio)macromolecules, and for dyes with short fluorescence lifetimes ( $<1\text{ ns}$ ) as found for many NIR dyes.

In order to minimize polarization-induced uncertainties, the use of polarizers in the excitation and emission channel is recommended. A maximum signal under defined polarization conditions results for the excitation polarizer set to  $0^\circ$  with respect to the vertical laboratory axis and the emission polarizer set to  $54.7^\circ$  [55]. These measurement conditions, which are also termed “magic angle conditions”, are recommended for very accurate measurements of  $\Phi_f$ . For samples and standards displaying an emission anisotropy  $r$  ca.  $\leq 0.05$  (nearly isotropic emission; most small dyes emitting in the UV and vis spectral region in solvents of low viscosity and in the absence of hydrogen-bonding interactions), polarizers are dispensable, without strongly enhancing the measurement uncertainty. If the fluorescence spectra of anisotropic emitters are measured without polarizers, the measurement uncertainty can considerably increase. The size of such systematic errors depends on the sensitivity of the respective fluorescence

instrument towards polarization effects and on the anisotropy of the sample. Typical (intensity and spectral) errors can be in the range of at least 20 % for each compound [55]. Concerning the relative determination of  $\Phi_f$  values, the largest uncertainties are expected for sample–standard pairs that differ strongly in their emission anisotropy.

#### III.4.4 Use of filters

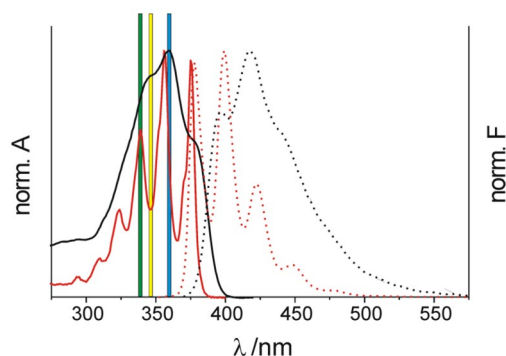
In most cases of dilute and transparent liquid samples, interferences due to stray light or scattering can be removed by subtracting the blank spectrum of the neat solvent or buffer solution measured under identical conditions, see Section III.4. This is especially the case for double monochromators that strongly reduce the level of stray light as compared to single monochromators. For specific purposes, the wavelength chosen for excitation can lie at much shorter wavelengths than the wavelength range scanned when recording the emission spectrum, for example, for fluorophores with pronounced Stokes shifts, when exciting into higher excited states ( $S_2 \leftarrow S_0$ ,  $S_3 \leftarrow S_0$ , etc.) or when studying energy transfer phenomena. In such cases, the use of order sorting or cut-off filters is advisable. As the transmission profile of the filter (i.e., the wavelength dependence of its transmission) can distort the measured fluorescence spectrum, it has to be considered for the spectral correction of measured emission spectra. Moreover, filters are frequently sources of unwanted and unspecific background fluorescence [79]. Thus, before using a filter in a fluorometer, it should be checked for autofluorescence. Typically, the possibility of filter autofluorescence increases with decreasing wavelength. Fluorescent filters are to be strictly avoided.

There are several ways to consider signal contributions from the transmission profile of the filters employed. If the filter is homogeneous (concerning its transmission) and can be placed reproducibly in the fluorometer (minimum and preferably known uncertainty of filter positioning), its transmission spectrum can be measured with a spectrophotometer under similar geometric conditions as employed for the fluorescence measurements. When filters with a known or certified degree of homogeneity of the material (distribution of the absorbing chromophores within the filter material) are not available, the in-house determination of the transmission of the light-attenuating component(s) can be erroneous and can suffer from inhomogeneities and/or irreproducible positioning of the filter [55]. The best way to consider signal contributions from filters is to perform the instrument calibration with these filters at exactly the same position in the optical path of the fluorometer as used for the measurement of the emission spectra. If filter wheels are directly installed in the (inaccessible) housing of the fluorometer, these features have to be taken into account during instrument calibration (see Section III.2.1.iii) and correction curves for all positions of the filter wheel used for subsequent fluorescence measurements have to be recorded.

#### III.4.5 Choice of excitation wavelength

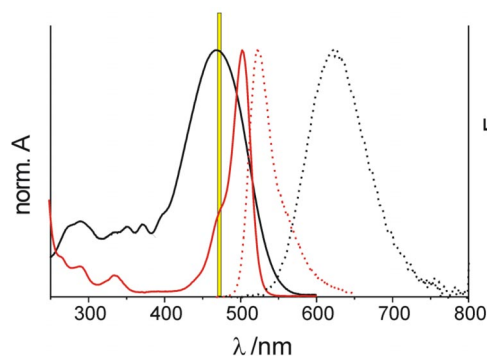
In most cases, the fluorescence of the sample and the standard is excited at the same wavelength. Hence, the choice of a suitable excitation wavelength is of considerable importance for the relative measurement of  $\Phi_f$  as is illustrated for some examples in this section, especially when compounds with narrow and structured absorption bands are concerned. Generally, excitation of the sample and the standard at an almost plateau-like region is recommended or at least at a wavelength where the slope in the absorption spectrum is considerably flat (see also eq. 5). For compounds with a very small Stokes shift, the excitation wavelength must be chosen to be short enough to avoid truncation of the dye's emission spectra at the high-energy side. Excitation into higher excited states (e.g.,  $S_2$  or higher) should be avoided since additional nonradiative deactivation routes might compete with internal conversion from  $S_n$  to  $S_1$ , resulting in a change of the  $\Phi_f$  value of the standard, which can then deviate from the value reported for different measurement conditions. The likelihood of such effects can be controlled by measuring the corrected excitation spectrum at very low absorbance ( $<0.05$ ) and comparing them with the corresponding absorption spectra (wavelength dependence of the absorption factor  $f$ ). Deviations between absorption and excitation are indicative of an excitation wavelength dependence of  $\Phi_f$ . Also, excitation at the red edge of the longest wavelength absorption band can be critical.

Figure 1 illustrates the use of anthracene as reference for the relative determination of the  $\Phi_f$  value of POPOP [1,4-bis(5-phenyloxazol-2-yl)benzene], a well-known scintillator and brightening dye. Anthracene was exemplarily chosen here because of its highly structured absorption spectrum. For anthracene as a dye with a very small Stokes shift, excitation wavelengths shorter than ca. 360 nm should be employed so that the emission spectrum is not truncated on the high-energy side. Second, the narrowness of the anthracene bands renders the choice of the POPOP absorption maximum as the excitation wavelength not very suitable (blue bar in Fig. 1). Small errors in the wavelength accuracy of the spectrophotometer and/or spectrofluorometer as well as differences in the slit functions could easily lead to considerable uncertainties because of the steep slope in the standard's spectrum. The same could be encountered when choosing the shortest wavelength vibronic band of anthracene (green bar, Fig. 1). The best choice for this sample–standard pair presents an excitation wavelength within the yellow bar that crosses both spectra in an almost plateau-like region.



**Fig. 1** Absorption (solid) and fluorescence (dotted) spectra of POPOP (black) and anthracene (red) in ethanol at  $T = 298$  K taken from [16]. The blue bar indicates a 2-nm band centered around the absorption maximum of POPOP, the green bar a 2-nm band centered around the absorption maximum of the third of the vibronic bands of anthracene and the yellow bar a 2-nm band encompassing two rather plateau-like regions in both absorption spectra. The 2-nm band should symbolize the region of absorption or excitation for narrow slit widths.

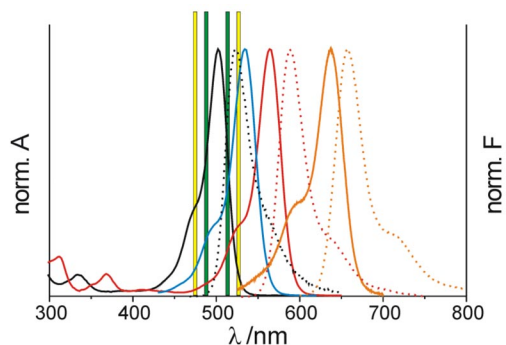
An example of a sample–reference pair with very different absorption and fluorescence properties is DCM [4-dicyanomethylene-2-methyl-6-(*p*-dimethylaminostyryl)-4*H*-pyran] in methanol, referred to as fluorescein in 0.1 N NaOH (Fig. 2). The reference (fluorescein) displays narrow and only



**Fig. 2** Absorption (solid) and fluorescence (dotted) spectra of DCM in methanol (black) and fluorescein in 0.1 N NaOH (red) at  $T = 298$  K taken from [16]. The yellow bar indicates a 2-nm band where excitation would produce minimum errors.

slightly Stokes-shifted spectra, whereas the compound with unknown  $\Phi_f$  (i.e., DCM) shows well-separated broad and structureless absorption and emission bands. Many charge-transfer-operated dyes such as electron donor–electron acceptor-substituted stilbene, styryl, or coumarin dyes show, in solvents of medium or high polarity, absorption and emission properties similar to DCM. As Fig. 2 suggests, for this case, the excitation wavelength should lay at best within the yellow region where the absorption spectrum of DCM is almost in a plateau and where the slope in the spectrum of fluorescein is also considerably flat.

An example for a sample–reference pair where reference and sample cannot be excited at the same wavelength and an extended wavelength region needs to be covered in absorption and in emission is shown in Fig. 3. Here, the absorption spectra of fluorescein (in 0.1 N NaOH) and rhodamine 101 (in ethanol) are compared. Use of fluorescein as reference for rhodamine 101 or vice versa is not possible with the previously described simple pair-wise determination of the  $\Phi_f$  at one excitation wavelength due to the negligible overlap of the absorption bands of both dyes. This situation can be approached with two different strategies as detailed in [16,36], using either different excitation wavelengths for both dyes in conjunction with an excitation correction curve (consideration of the spectral photon fluxes at the chosen excitation wavelengths; see eq. 2), or a so-called chain of  $\Phi_f$  transfer standards. The requirements, advantages, and pitfalls of both methods and the resulting uncertainties are exemplarily summarized for a series of UV/vis emitters in [36]. The former method (i.e., the use of different excitation wavelengths for sample and standard) is indicated by the two yellow bars in Fig. 3. As previously stated, this method can only be recommended for expert laboratories, which can obtain an excitation correction curve with minimum uncertainties [36,56]. Otherwise, the resulting uncertainties are too high compared to the measurement uncertainties achievable with the latter method. An extended wavelength region in absorption and emission can be alternatively covered with the aid of a chain of  $\Phi_f$  standards built up from several dyes with overlapping absorption spectra and excitation wavelength-independent  $\Phi_f$  values. The  $\Phi_f$  values of these dyes are then measured pair-wise at different excitation wavelengths (one excitation wavelength per pair) starting from a golden reference of reliably known  $\Phi_f$  as detailed in [36,38]. This approach is exemplified by the hypothetical blue spectrum and the green bars in Fig. 3, highlighting the build-up of a chain of standard dyes with absorption bands that lie in between the two fluorophores to be measured against each other and that show sufficient overlap with the spectra of both other dyes in a region that can be used for excitation [16,36]. When aiming, for example, at the deter-



**Fig. 3** Absorption (solid) and fluorescence (dotted) spectra of fluorescein in 0.1 N NaOH (black) and rhodamine 101 in ethanol (red) at  $T = 298$  K taken from [16]. Also displayed are the absorption spectrum of a hypothetical chemical transfer standard dye (blue) and the absorption and fluorescence spectra of the Cy5 parent indodicarbocyanine (C5) in methanol (orange) taken from [80]. The yellow bars indicate the 2-nm bands for optimum excitation of fluorescein and rhodamine with two different excitation wavelengths. The green bars indicate the two excitation wavelengths that would be best suitable when first measuring fluorescein with the chemical transfer standard dye and then measuring rhodamine 101 against that dye.

mination of the  $\Phi_f$  of Cy5 (the spectrum of the parent compound is shown in Fig. 3) relative to that of rhodamine 101, it is obvious that a similar procedure as for rhodamine 101 and fluorescein should be employed. With the recent availability of a set of newly recommended quantum yield standards including three new quantum yield standards for the NIR region covering the spectral region from ca. 350 to 1000 nm, all with absolutely measured  $\Phi_f$  values [36,38,41,44], use of such a transfer dye chain approach is only mandatory for certain specific cases.

#### III.4.6 Choice of dye concentration

The relative determination of  $\Phi_f$  relies on the measurement of samples and standards with low absorbances to minimize re-absorption and inner filter effects as well as dye aggregation. Typically, absorbances below 0.1 are used at the excitation wavelength, and generally, the absorbance of the long-wavelength maximum should be kept below 0.1 to minimize such effects. For proper determination of  $\Phi_f$  values using regular spectrofluorometers, the absorbances of sample and standard solutions should be matched. No correction can account for multiple reflections of the incident beam on the cuvette walls when dilute solutions are used, if the divergent beam goes through absorbances that are not identical.

Especially for dyes showing a very small Stokes shift such as fluorescein, rhodamines, anthracene, or most cyanines and BODIPY dyes, the use of very dilute solutions is necessary to eliminate or at least minimize inner-filter or re-absorption effects [1,36,38,41,44,81,82]. Re-absorption effects are typically accompanied by a red shift in emission with increasing dye concentration [36,38,42], whereas the absorption spectra are not affected by dye concentration. The use of very dilute solutions is also necessary for dyes showing self- or concentration quenching when the aim of the measurement is to obtain a molecular parameter characteristic for the monomeric dye [50,51] and not the  $\Phi_f$  value of a solution containing a mixture of monomeric and aggregated dyes. Dyes that are especially prone to aggregation, for example, in aqueous solution or when attached to a bio- or macromolecule are hydrophobic cyanine dyes as well as certain xanthene dyes and porphyrins [50–52,83,86]. Here, the concentration dependence of the spectral shape and intensity of the absorption, fluorescence emission, and fluorescence excitation spectra should be checked prior to the determination of the relative fluorescence quantum yield [51]. A concentration dependence of the absorption spectrum is typical for dye aggregation as well as deviations between the absorption spectrum and the corrected excitation spectrum. For dye solutions or fluorophore-labeled bio- or macromolecules that show dye–dye interactions leading to fluorescence quenching [51,83–85,87], the contribution of the absorption of the nonfluorescent aggregates to the absorbance at the excitation wavelength can be calculated and subsequently considered for the determination of the  $\Phi_f$  values of the monomeric free or bound dyes [50,51,87]. As detailed in Section II, otherwise, the directly measured fluorescence quantum yield represents only the  $\Phi_f$  value of the solution and not the  $\Phi_f$  of the bound dyes.  $\Phi_f$  values obtained for solutions that contain a mixture of species (e.g., fluorophore and quencher of varying concentrations, aggregates of varying sizes, etc.) are only sample-specific quantities and characteristic for a certain dye concentration in a given solvent.

#### III.4.7 Influence of oxygen

Fluorescence is an optically allowed transition. This is implied in the vast majority of cases with short fluorescence lifetimes of 10 ns or less, rendering the quantum yields and lifetimes of such fluorescent molecules not or barely sensitive to the presence of oxygen. Examples for small organic dyes that reveal oxygen-sensitive quantum yields and lifetimes are anthracene and diphenylanthracene. The most prominent exception is pyrene with its exceptionally long fluorescence lifetime exceeding 100 ns.

For measurements in deoxygenated solutions, care must be taken to completely remove oxygen from the dye solution, for example, by pump freeze thaw cycles or by bubbling of inert gases (nitrogen or argon) through the dye. In the latter case, during the luminescence measurements, purging should be stopped, namely, the dye solution should be purged by bubbling through a flask with the solvent used in the determination of  $\Phi_f$ , the purging should be stopped and then the emission should be measured. In this way, continuously enriching the solution with the small amount of oxygen present in the inert

gases is avoided. In any case, it must be clearly stated under which conditions  $\Phi_f$  was measured and how deoxygenation was performed [62].

### III.5 Measurements of sample and standard absorbance

An often underestimated source of uncertainty in the determination of  $\Phi_f$  is the measurement of the absorbance of the sample and the standard at the excitation wavelength. If possible, the same cells should be used for measurements of absorbance and fluorescence. The choice of suitable cells for absorption and fluorescence measurements was already addressed in Section III.2.4. For very accurate absorbance measurements, it is recommended to measure first the blank (solvent in fluorescence cuvette placed in sample beam of the spectrophotometer) against a reference cuvette (placed in the reference beam of the spectrophotometer). Subsequently, an aliquot of the sample or standard stock solution is added to the cuvette containing the blank and also measured against the reference. To minimize uncertainties in the absorbance at the excitation wavelength for very dilute dye solutions (e.g., for absorbances of 0.02 or 0.01), a different procedure can be applied. Here, the absorption is measured in 20-, 50-, or 100-mm cells and a certain amount of this solution is then transferred into a 10-mm cell for the fluorescence measurement. Alternatively, if this is not possible, for example, due to the lack of appropriate material, absorption and fluorescence measurements can be performed at two different dye concentrations using 10-mm cells and the known dilution factor should be used to calculate the actual absorbance at the excitation wavelength of the solution employed for recording the fluorescence.

The case where a highly emissive compound is not very soluble in the chosen solvents can also be critical. This can yield a certain offset that gains in intensity at shorter wavelengths due to scattering or a tailing in absorbance, yet still a high and virtually unperturbed fluorescence signal is observed.

### III.6 Consideration of fluctuations of the spectral irradiance reaching the sample

For each fluorescence technique, the measured emission signal depends on the *spectral irradiance* reaching the sample [9,48,55]. Accordingly, changes of this quantity due to fluctuations of the spectral radiance of the spectrofluorometer's excitation light source can affect measured emission intensities. To take such fluctuations into account, most modern spectrofluorometers are equipped with a reference channel that measures the spectral radiance of the excitation source at the chosen excitation wavelength during the fluorescence measurement. Hence, two photocurrent signals are measured (see [48]), the ratio of them being independent of the fluctuations. Routine instruments typically report only signal ratios, whereas high-end research fluorometers record the signals from each channel separately.

### III.7 Performance of quantum yield measurements

In addition to the procedures and recommendations detailed in the previous sections to circumvent the discussed sources of uncertainty (see also Section III.10), generally at least a double determination of the fluorescence quantum yield (two independent measurements for sample and standard) is recommended. If the quantum yield of a chosen reference is somehow questionable (see Sections III.3 and III.9), for example, because this compound has not yet been really established and its (absolute)  $\Phi_f$  has been given only in a single or very few literature reports, the use of an additional reference [36,41,44] and comparison of the quantum yields of both standards is strongly recommended.

### III.8 Data evaluation and calculation of fluorescence quantum yields

Calculation of the fluorescence quantum yield from absorption and fluorescence measurements of sample and standard, see eq. 2, is comprised of the following steps:

- i) Measurement of the absorbances  $A_x(\lambda_{\text{ex}})$  of the sample and the standard at the respective excitation wavelength  $\lambda_{\text{ex}}$  and calculation of the corresponding absorption factors  $f(\lambda_{\text{ex}})$ , see eqs. 2 and 4. For matched absorbances of sample and reference as recommended in Section III.4, the absorbances or absorption factors in eq. 2 cancel out.
- ii) Blank correction of the emission spectra,  $I_{\text{m}}(\lambda_{\text{ex}}, \lambda_{\text{em}})$ , of the sample and the standard measured at identical measurement conditions. Removal of background signals, such as scattering and fluorescence from the solvent, and dark counts at the detector are obtained by subtraction of a background spectrum that was recorded under identical measurement conditions for a blank solvent sample,  $I_{\text{b}}(\lambda_{\text{ex}}, \lambda_{\text{em}})$  [36,41,44,45], thereby yielding uncorrected spectra,  $I_{\text{u}}(\lambda_{\text{ex}}, \lambda_{\text{em}}) = I_{\text{m}}(\lambda_{\text{ex}}, \lambda_{\text{em}}) - I_{\text{b}}(\lambda_{\text{ex}}, \lambda_{\text{em}})$ .  $I_{\text{u}}(\lambda_{\text{ex}}, \lambda_{\text{em}}) = I_{\text{u}}(\lambda_{\text{em}})$  in eq. 3.
- iii) Correction of the uncorrected spectra of the sample and the standard for instrument-specific signal contributions [48,58]. This includes consideration of the wavelength- and polarization-dependent spectral responsivity of the fluorometer's detection system [emission correction, obtained, e.g., with a spectral radiance transfer standard of known spectral radiance ( $L_{\lambda}$ ), traceable to a radiometric scale], yielding corrected emission spectra,  $I_{\text{c}}(\lambda_{\text{ex}}, \lambda_{\text{em}})$ , see eq. 3 and [36,38,41,44,48,57,58].
- iv) Integration of the corrected emission spectra of sample and standard on a wavelength scale, see eq. 3, followed by consideration of the photonic nature of the emitted light (term  $h c$  in eq. 3). For compounds displaying two emission bands that are not well separated, in addition, spectral deconvolution is required to separate both bands [87].
- v) For the use of two different excitation wavelengths for reference and sample, also the spectral photon fluxes  $q_{\text{p,st}}(\lambda_{\text{ex,st}})$  and  $q_{\text{p,x}}(\lambda_{\text{ex,x}})$  must be determined (see also eq. 2 and excitation correction described in [36,48,55,58]).
- vi) Refractive index correction [term  $(n_{\text{x}}^2/n_{\text{st}}^2)$  in eq. 2] if different solvents are used for the sample and the standard [1,28,36,41,44,54].
- vii) Calculation of the sample's quantum yield relative to the standard following eq. 2.

### III.9 Documentation of fluorescence quantum yields

Fluorescence quantum yields need to be adequately documented as a prerequisite for their reliability. The imposed criteria on documentation similarly hold true for  $\Phi_{\text{f}}$  standards. The following information should be provided, see also [36,44,62]:

- i)  $\Phi_{\text{f}}$  value including uncertainty.
- ii) Sample: for new compounds, chemical structure, dye purity, and method of analysis and, if a purification was performed, also method of purification; for commercial compounds, dye supplier and batch number and preferably also purity as well as solvent or matrix (supplier, type, and purity, possible presence of quenchers).
- iii) Standard: dye supplier and batch number, preferably also dye purity and method of analysis and, if purification was performed, also method of purification, as well as solvent or matrix (supplier, type, and purity); reference value used for fluorescence quantum yield including reference.
- iv) Instrument used including characterization procedures including standards used and uncertainties of the calibration-relevant quantities of the standards.
- v) Measurement conditions used (instrument settings including, e.g., use of polarizers and polarizer settings; excitation wavelength; temperature; presence or absence of oxygen; method of degassing, if used, etc.).

### III.10 Sources of uncertainty and achievable quality of data

Many of the typical sources of uncertainty have been already addressed in the previous sections, for example, Sections III.2 to III.5, in conjunction with procedures to minimize such effects as well as in [36,38,41,44]. The most common sources of uncertainty are as follows:

- i) Instrument characterization: the emission and the excitation correction can be especially critical here (see Section III.2 and [36,38,41,44]).
- ii) Uncertainties in the wavelength accuracy of the spectrophotometer and/or spectrofluorometer as well as differences in the shape of the slit function.
- iii)  $\Phi_f$  standard: reference value used, unsuitable quality (dye with absorbing and/or fluorescing impurities, solvent with fluorescent impurities or containing fluorescence quenchers, unsuitable measurement conditions, e.g., improper excitation wavelength, etc.).
- iv) Performance of absorbance measurements.
- v) Re-absorption, self-quenching, or aggregation affecting the measured absorption at the excitation wavelength and/or the emission spectrum (see Section III.4.6).
- vi) Polarization effects (see Section III.4.3).
- vii) Insufficient thermal or photochemical stability of the sample and/or the standard (stability can be an issue for NIR dyes).
- viii) For weakly and very weakly emitting compounds, very different fluorescence quantum yields of the standard (typically  $\Phi_f \geq 0.5$ ) and the sample can result in enhanced uncertainties either due to operation of the detection system within its nonlinear region or additional uncertainties arising from extra dilution steps.

Major sources of uncertainty present most likely the instrument characterization, the measurement of absorbances, and the still often poor comparability of the reference values of the  $\Phi_f$  standards [16,36,38,41]. To improve the quality of presently available  $\Phi_f$  data, the quality of the correction methods and of the available reference materials including reference values have to be upgraded, evaluated, and established, the latter, for instance, by absolute quantum yield measurements [36,38] and/or round-robin tests of expert laboratories in conjunction with the supply of the accordingly tested fluorescence quantum yield standards. A related procedure has been recently followed for lifetime standards [89] as well as for spectral fluorescence standards [59,60]. In this respect, a set of very well characterized quantum yield standards covering the wavelength region of 350 to 1000 nm has been very recently presented [44].

### III.11 Determination of the uncertainty of fluorescence quantum yields

For the determination of the uncertainty of measured  $\Phi_f$ , typically six independent measurements of the sample and the standard are recommended to account for random errors. A complete uncertainty budget for  $\Phi_f$  values includes additionally methodical uncertainties like the uncertainty budget from the characterization of the spectrophotometer (contributions from the accuracy of wavelength and intensity scale) and the fluorometer (contributions from the accuracy of wavelength scale, nonlinearity of the detection system, emission correction, and excitation correction) and the dye purity [36,38,62,90]. Such an uncertainty budget was only recently reported for recommended quantum yield standards covering the UV/vis/NIR including relative and absolute measurements [36,38,41,44]. A complete uncertainty budget for spectrally corrected emission spectra in the wavelength range from 300 to 770 nm comparing instrument calibration procedures relying on physical and chemical transfer standards follows from [59,60].

The main contributions to the uncertainty of  $\Phi_f$  measurements have been listed in the previous sections. Expert laboratories can achieve a comparability of corrected emission spectra better than 5 % using physical transfer standards [59]. For the majority of fluorescence users, the use of chromophore-

based reference materials is recommended [91] and can yield a similar comparability [60]. The following uncertainties were previously published by us for  $\Phi_f$  values:  $\pm 4\%$  (for  $\Phi_f > 0.4$ ) [41] and  $\pm 10\%$  (for  $0.2 > \Phi_f > 0.02$ ),  $\pm 20\%$  (for  $0.02 > \Phi_f > 0.005$ ), and  $\pm 30\%$  (for  $0.005 > \Phi_f$ ) [92]. Recent improvements in spectral correction as a result of an improved uncertainty of the spectral radiance transfer standard used and improved calibration strategies allowed for a reduction of these values to  $\pm 2\%$  for  $\Phi_f > 0.10$ ,  $\pm 7\%$  for  $0.10 > \Phi_f > 0.01$ , and  $\pm 15\%$  for  $\Phi_f < 0.01$  [36,38,44]. Well-trained personnel can reach repeat accuracies of  $\pm 0.5\%$  ( $\Phi_f > 0.20$ ),  $\pm 2\%$  ( $0.20 > \Phi_f > 0.05$ ), and  $\pm 7\%$  ( $\Phi_f < 0.05$ ) for six independent measurements [93].

#### IV. TEMPERATURE DEPENDENCE OF FLUORESCENCE QUANTUM YIELDS

The elucidation of the processes that lead to the partial quenching of the fluorescence of most chromophores in solution at room temperature such as photoisomerization, charge-shift, or charge-transfer processes requires measurements of the fluorescence quantum yield (and lifetime, not to be discussed here [94]) as a function of temperature. Typically, the temperature range from room temperature to 77 K is scanned. In addition to the sources of uncertainty already addressed for room-temperature measurements (see Sections III.2–III.5 and III.10), the following issues have to be considered:

- i) Avoidance of the interference of increased scattering due to cracks in the solid matrix at low temperature: use of a solvent or solvent mixtures that form optically transparent glasses below the glass temperature [95,97].
- ii) Consideration of the temperature dependence of the refractive index of the solvent [98].
- iii) Determination of the actual absorbance at every point of the temperature run as for most dyes, the shape of the absorption spectrum also changes as a function of temperature—often the bands are bathochromically shifted due to an increase of the solvent's dielectric constant and narrowed due to a freezing of vibronic modes. At best, the absorption of the sample is directly measured in a cryostat mounted in the fluorometer. Otherwise, at least for fluorophores with a well-separated  $S_1 \leftarrow S_0$  transition and matching absorption and fluorescence excitation spectra, the (change in) absorbance at the excitation wavelength can be obtained from room-temperature absorption spectra and room- and low-temperature fluorescence excitation spectra, since the oscillator strength of an electronic transition does not change as a function of temperature [16].
- iv) Consideration of the temperature-induced changes in the density of the solvent and the corresponding change in dye concentration.
- v) Consideration of polarization effects (see Section III.4.3).

#### V. PHOSPHORESCENCE QUANTUM YIELDS

The determination of phosphorescence quantum yields,  $\Phi_p$ , or the measurement of quantum yields of emitters like certain transition metal ion complexes [e.g., Ru(II) or Ir(III) complexes, etc.] displaying partly or completely forbidden optical transitions (e.g., transitions with a considerable triplet character, etc.), and thus, very long lifetimes of their excited states, is experimentally very similar to the determination of  $\Phi_f$  for dyes with short-lived excited states [99,100]. However, due to the intrinsically longer lifetime of the phosphorescence or luminescence decays, which can be in the order of several hundred ns up to a few ms, special attention has to be paid to the exclusion of quenchers (e.g., exclusion of oxygen) and the use of pulsed excitation sources (e.g., see Section III.2). The complete removal of oxygen is important, for example, by pump freeze cycles or bubbling of inert gases (nitrogen or argon) through the dye solutions as previously described in Section III.4.7. In any case, the procedure used should be clearly stated [62]. Difficulties in the determination of  $\Phi_p$  can arise when a compound is able to decay through both channels, phosphorescence and fluorescence, as is the case for (poly)cyclic aromatic hydrocarbons in a glass at low temperatures [101]. If the two luminescence spectra are not well sepa-

rated, spectral deconvolution in connection with lifetime measurements or time-gated detection can be used to separate the slowly decaying from the fast decaying species.

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