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Characterization of photoluminescence measuring systems (IUPAC Technical Report)*

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Abstract: Procedures for the characterization of photoluminescence measuring systems are discussed, focusing on spectrofluorometers and fit-for-purpose methods including suitable standards. The aim here is to increase the awareness for the importance of a reliable instrument characterization and to improve the reliability and comparability of measurements of photoluminescence.

Keywords: fluorescence intensities; instrument calibration; IUPAC Analytical Chemistry Division; IUPAC Organic and Biomolecular Chemistry Division; IUPAC Physical and Biophysical Chemistry Division; performance validation; photoluminescence; photoluminescence measuring systems; relative fluorescence quantum yields.

CONTENTS

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1. INTRODUCTION

Despite the widespread and increasing use of photoluminescence measuring techniques in materials science, (bio)analytical chemistry, medical diagnostics, and biotechnology [1,5–7], many method-inherent problems are often neglected, resulting in measurements that are unreliable and of poor quality. These problems include, e.g., the nonlinearity of the detection system, and spectral bandpass-, detector voltage- and polarization-dependent effects. Furthermore, the general need for correction of measured signals for unwanted contributions from instrument-dependent effects that are wavelength-, polarization-, and time-dependent is frequently underestimated despite the significant distortions in spectral shape and intensity that are often introduced [8–12]. These luminescence-inherent drawbacks hamper the reliability of photoluminescence data, the comparability of measurements between instruments, and quantification from measured fluorescence intensities [6,13] as well as the determination of relative fluorescence quantum yields [14], see also refs. [15,16]. These demands enhance the need for internationally accepted procedures for instrument calibration and instrument performance validation (IPV) in conjunction with suitable standards [7,17,18]. However, with the exception of colorimetry or surface fluorescence [19,20], at present, there are only a few guidelines, recommendations, and technical notes for the characterization and performance validation of photoluminescence measuring instruments [13,21–29] including choice of standards [1,6,10,11,17,21,24,30–33], see also ref. [34]. This can result in considerable calibration uncertainties and, subsequently, measurement uncertainties.

To improve the overall reliability and comparability of fluorescence measurements, the purpose of this document is to present and discuss procedures for the characterization and performance validation of photoluminescence measuring systems. Special emphasis is dedicated to steady-state measurements of photoluminescence in solution and monochromator-based systems such as are most commonly found in research-grade instrumentation. This document does not cover the use of charge-coupled device (CCD) detectors and acousto-optical tunable filters (AOTFs). But, with proper consideration of method-inherent requirements and method-specific limitations, this recommendation can be extended to other photoluminescence techniques and instrumentation.

2. QUANTITIES AFFECTING THE MEASUREMENT OF PHOTOLUMINESCENCE

In the following, for simplicity, the term "fluorescence" is used not in its strictly photochemical sense describing the spontaneous emission of radiation (luminescence) from an excited molecular entity with retention of spin multiplicity [35], but rather as a synonym for photoluminescence. The choice of suitable standards including requirements on physical and chemical, i.e., chromophore-based, standards, which are both closely related to instrument characterization, are detailed in ref. [34].

As illustrated in eq. 1, for each luminescence technique, the measured fluorescence signal or photocurrent per unit bandwidth of the excitation light ($d\lambda_{ex}$) and per unit bandwidth of the emitted and detected fluorescence light ($d\lambda_{em}$), $\frac{d^2 I_m(\lambda_{ex},\lambda_{em})}{d\lambda_{ex} d\lambda_{em}}$ is determined by both instrument- and analyte-specific quantities [17,18]. Integration over the excitation and emission spectral bandpasses used yields the measured fluorescence signal at a certain emission wavelength λ_{em} (for a given excitation wavelength λ_{ex}). Instrument-specific quantities include the spectral radiant power P_λ at the wavelength λ_{ex} per unit bandwidth reaching the sample, i.e., $dP(\lambda_{ex})/d\lambda_{ex}$, and the spectral responsivity $s(\lambda_{em})$ of the emission or detection channel. $P_\lambda(\lambda_{ex})$ is controlled by the spectral radiance $L_\lambda(\lambda_{ex})$ of the excitation light source and the transmittance of optical components like lenses, mirrors, filters, monochromator gratings, beam splitters, and polarizers in the excitation channel. $P_\lambda(\lambda_{ex})$ equals the quotient of the spectral irradiance E_λ at the wavelength λ_{ex} reaching the sample, i.e., $E_\lambda(\lambda_{ex})$, multiplied with the cross-sectional area of the sample in the detection region irradiated by the excitation beam, i.e., S_{ex} [$P_\lambda(\lambda_{ex}) = E_\lambda(\lambda_{ex}) \times S_{ex}$]. The spectral responsivity $s(\lambda_{em})^{\dagger\dagger}$ that is occasionally termed "spectral sensitivity", equals the quotient of the detector output (photocurrent) by the detector input (incident radiant power)

at wavelength λ_{em} [32,36]. $s(\lambda_{em})$ is determined by the wavelength-dependent transmittance or reflectance of the optical components in the emission channel and the wavelength-dependent responsivity of the detector.

$$\frac{d^2 I_m(\lambda_{ex},\lambda_{em})}{d\lambda_{ex} d\lambda_{em}} = f(\lambda_{ex}) \times P_\lambda(\lambda_{ex}) \times \frac{\lambda_{ex}}{hc} \times F_{p,\lambda}(\lambda_{em}) \times \frac{hc}{\lambda_{em}} \times \Phi_f \times K \times s(\lambda_{em}) \quad (1)$$

Analyte-specific quantities that control measured fluorescence signals from the material side are the analyte's absorption factor $f(\lambda_{ex})$, formerly known as absorptance, see eq. 2, and $F_{p,\lambda}(\lambda_{em})$ [14,17,32], see eq. 1. $f(\lambda_{ex})$ is nonlinearly linked to absorbance and thus, to the concentration C by the Beer–Lambert law, see eq. 2. $F_{p,\lambda}(\lambda_{em})$ represents the normalized photon emission spectrum, i.e., the probability density that an emitted photon has the wavelength λ_{em}. The integral of $F_{p,\lambda}(\lambda_{em})$ over the complete emission spectrum equals unity. Φ_f is the fluorescence quantum yield, which equals the number of emitted photons per number of absorbed photons, see eq. 3. K is a factor that takes into account the geometry of the instrument, including the overlap between the excitation and emission volumes within the sample [37]; K includes the probability for detection of a photon emitted from the illuminated volume. The factors λ_{ex}/hc and hc/λ_{em} in eq. 1 convert radiometric units to photonic units (λ_{ex}/hc) and vice versa (hc/λ_{em}) as the fluorescence quantum yield Φ_f in eq. 1 is a photon ratio and $P_\lambda(\lambda_{ex})$ and $s(\lambda_{em})$ are expressed in radiometric units. $P_\lambda(\lambda_{ex}) \times \lambda_{ex}/hc$ equals the spectral photon flux $q_{p,\lambda}(\lambda_{ex})$ reaching the sample.

$$f(\lambda_{ex}) = 1 - 10^{-A(\lambda_{ex})} = 1 - 10^{-\varepsilon\,(\lambda_{ex}) \times C \times l} \quad (2)$$

$$\Phi_f = N_{em}/N_{abs} \quad (3)$$

3. INSTRUMENT CHARACTERIZATION

The characterization of a photoluminescence measuring instrument must include the determination of the following quantities and parameters, which can affect the analyte-specific spectral position, spectral shape, and intensity of signals measured in steady-state fluorometry:

i. the range of linearity of the instrument's detection system,
ii. the wavelength accuracy of the instrument's excitation and/or emission channel,
iii. the instrument's spectral resolution,
iv. the (relative) spectral responsivity of its emission channel, $s(\lambda_{em})$ [38], and
v. the (relative) spectral irradiance $E_\lambda(\lambda_{ex})$ at the sample position, corresponding to the (relative) spectral radiance of the excitation channel reaching the sample.

A fit-for-purpose instrument characterization consists of two steps. Firstly, the level of uncertainty desired for fluorescence measurements and accordingly, for instrument characterization, needs to be decided on to rationalize efforts. Secondly, it needs to be performed with consideration of the samples to be measured and corrected and thus strictly at the measurement conditions employed, i.e., at the same instrument settings and measurement geometry as used for the samples. Relevant instrument parameters to be considered and kept constant here include:

i. the spectral bandpass of the excitation and emission monochromator;
ii. the settings of the excitation and emission polarizer, see also ref. [39];
iii. additional optical components such as filters or attenuators used in the excitation and emission channel;
iv. the detector voltage; and
v. integration time and scan speed (less stringent).

vi. In the case of pulsed light sources, for certain calibrations and standards, also the delay and the gate can play a role, see refs. 41 and 69 in ref. [34].

Generally, the uncertainty of instrument characterization depends on the reliability and suitability of the calibration procedures chosen, as well as on the suitability of the transfer standard(s), see ref. [34], and on the uncertainty of the standard's certified or reported radiometric and fluorometric quantities [29]. A *transfer standard* is a reference standard used to transfer the value of one reference standard to a measurement or to another reference standard. In the vast majority of cases, a relative instrument characterization is sufficient. If very challenging measurements of absolute fluorescence intensities are desired, additionally, a geometry factor (K in eq. 1) needs to be considered, which accounts for the (instrument- and sample-dependent) ratio of the solid angles of fluorescence emission and detection, and the ratio of the size of the illuminated and detected volumes [18,40]. In the following, different procedures for the characterization of the above-listed relevant quantities of a photoluminescence measuring instrument are presented and discussed.

3.1 Linearity of the detection system

The first step toward a reliable instrument characterization and reliable fluorescence measurements is the determination of the linear range of the instrument's detection system(s) under routine operating conditions. This is required prior to each instrument (re)calibration, i.e., the (re)determination of $s(\lambda_{em})$ and/or $E_\lambda(\lambda_{ex})$. Here, particular attention has to be given to slit widths (and therefore spectral bandpass), filters, detector voltage, detection mode, and sample containers, as well as proper choice of procedures. For fluorescence instruments equipped with a reference detector, which accounts for fluctuations of the excitation light intensity [incident radiant power $P(\lambda_{ex})$], the linearity of this detection system must also be considered [17,29]. The range of linearity of the reference detection system can only be determined for instruments with emission and reference detector signals that can be read out separately, typically high-end research-grade fluorometers. Even though ratio-only instruments are typically designed to reduce such effects, it is generally recommended that fluorescence measurements be collected at signal levels far from detector saturation to minimize contributions from nonlinearities of the detection system to the overall uncertainty of spectral correction and fluorescence measurements. If such effects cannot be excluded, as, for instance, in certain cases of quantitative fluorometry with unknown analyte concentrations or for compounds with unknown fluorescence quantum yields, classical N-point calibration procedures have to be additionally performed, thereby considering the eventually reached maximum fluorescence intensity.

Typical methods for the determination of the linear range of a detection system include:

i. the variation of the spectral radiance of a lamp by means of attenuators, such as optical filters with known transmission characteristics or polarizers (via polarizer settings),
ii. the double aperture method [41,42],
iii. the variation of the light intensity via chromophore concentration [29], and
iv. the measurement of ratios of signals [17].

Drawbacks of methods i and ii are the costs for the necessary optical components as well as often an enhanced uncertainty owing to the introduction of additional spectral distortions and the non-ideal reproducibility of positioning of the optical components [17]. Both methods are only recommendable for expert laboratories. The reliability of method iii depends on the chosen chromophore(s). A suitable dye should display well-separated absorption and emission bands to minimize inner filter effects and should not be prone to quenching and aggregation or dimerization [17,29]. Also, use of very dilute dye solutions, e.g., with absorbances preferably below 0.05 or at least below 0.1 for 1-cm cells, is recommended, as only then can a linear dependence of fluorescence intensity on dye concentration be anticipated [17,43]. Method iii is likely the most commonly used and is recommended in

ASTM E 578-01 in conjunction with quinine sulfate dihydrate, emitting in the visible region and displaying little spectral overlap between its absorption and emission band [22,29]. An elegant and recommendable procedure is method iv, which is illustrated in Fig. 1. This method is robust, simple, and not very susceptible to additional measurement uncertainties [17]. It can be realized, for example, via controlled modulation of the spectral radiance of the instrument's excitation light source or a second lamp with attenuators in front of the light source, a white standard at the sample position and an emission polarizer. The results from such an approach are shown in Fig. 1. Here, the spectral radiance/intensity of the fluorometer's excitation light at 400 nm, which was varied by the use of neutral density filters, was scattered by a white standard at sample position toward the emission channel and measured at emission polarizer settings of 0° and 90°. Operation of the detector in its linear range, from zero to thousands of counts per second (cps) in this case, should yield a constant intensity ratio within the measurement uncertainty illustrated by the bars. The solid line represents a modified exponential fit (quotient of two exponential saturation curves) to the overall measured data. The dotted line is the corresponding statistical uncertainty. Deviations from a constant value exceeding the uncertainty of fluorescence measurements (indicated by the bars) reveal the upper limit of the linearity of the emission detection system, here 10 000 cps. For this method, the attenuation factor should be on the order of 2 to 5. In addition, this method requires knowledge of the positioning uncertainty of the optical components, here the polarizers.

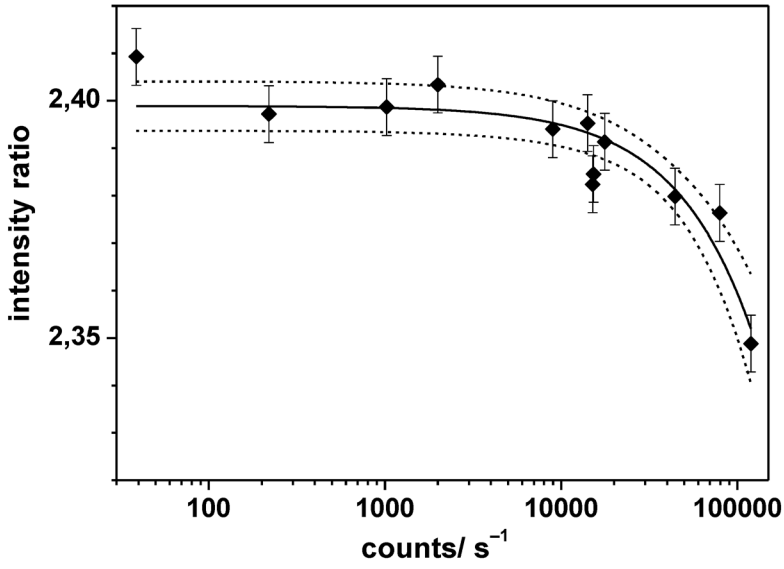

Fig. 1 Example for the method of signal ratioing developed by BAM for the determination of the linear range of detection systems: Ratio of the spectral radiances or light intensities recorded at two settings of the emission polarizer vs. registered photon counts of the detector for an increasing spectral irradiance.

A straightforward alternative for the broad majority of fluorescence users is the use of dilute dye solutions (absorbances at the excitation wavelength preferably at maximum 0.05 or at least below 0.1) and the variation of the light intensity reaching the detector via dye concentration for method iv. Signal ratioing can be achieved, e.g., by using different settings of an emission polarizer, an attenuator in the emission channel or different emission wavelengths.

3.2 Wavelength accuracy and spectral resolution of emission

Each instrument qualification must include the verification and control of the wavelength accuracy of the excitation and/or emission channel at multiple wavelengths within the spectral region used for fluorescence measurements [18]. This check should be performed at regular intervals, typically every six months (these intervals also depend on the procedures used for the regular validation of instrument performance, see Section 3.5) or at least once per year. For high-precision fluorometers, as a rule of thumb, deviations in the wavelength accuracy in the UV/vis region should not exceed 0.5 nm. Control of the wavelength accuracy is mandatory prior to each determination of $s(\lambda_{em})$ and/or $E_\lambda(\lambda_{ex})$.

The spectral resolution of the instrument to be characterized determines the acceptable width and spacing of the spectral lines of the wavelength standard. These narrow emission bands must cover the desired UV/vis/NIR spectral region at known spectral positions with a given uncertainty [10,44], see ref. [34]. The wavelength accuracy can be checked by comparing the measured line positions with the known line positions of the standard [21].

3.2.1 Wavelength accuracy of the emission channel

The wavelength standards of choice are typically:

i. an atomic discharge lamp containing mixtures of gases such as mercury, argon, and neon that all emit extremely narrow emission lines within the UV/vis/NIR spectral region [21,29,34,45–51];
ii. a fluorescent material revealing several very narrow emission bands within the UV/vis region placed at sample position [10,34,45,52–54];
iii. the instrument's xenon excitation source, e.g., in conjunction with a white standard or scatterer at sample position with the excitation monochromator set at zero order running an emission scan; and
iv. a chromophore solution revealing several very narrow absorption bands within the UV/vis region in conjunction with a specific calibration accessory [55].

Atomic lamp-based methods are most commonly used as wavelength standards and can achieve greater precision and accuracy than other methods. Typically, an accuracy of about 20 cm^{-1} (±0.5 nm at 500 nm) is desired for research-grade benchtop spectrofluorometers. This level of accuracy is easily attainable using method i, but methods ii–iv can also achieve this in principle, e.g., in conjunction with a fitting routine. Since atomic discharge lamps exhibit a very large spectral radiance as compared to fluorescent samples, the use of an attenuator, such as a white standard or a diffuse scatterer at the sample position, or a neutral density filter, in conjunction with this device is often necessary to avoid detector saturation. This also simplifies coupling of the lamp's emission into the detection system.

Method ii presents a very attractive, simple, and straightforward alternative for less stringent requirements on spectral resolution or for instruments providing a lower spectral resolution, such as microplate readers or confocal spectral imaging systems (typically operated with a fixed spectral bandpass between 5 and 30 nm), chromophore-based wavelength standards such as a dysprosium-activated yttrium garnet and cuvette-shaped glasses doped with different rare earth (RE) or transition-metal ions currently tested at the Federal Institute for Materials Research and Testing (BAM) the National Institute of Standards and Technology (NIST) [10,34,44].

Methods iii and iv are not offhand recommendable, although different manufacturers of steady-state fluorometers recommend the determination of the wavelength accuracy via scanning of the emission peaks in the instrument's xenon excitation source. Xenon lamps emit narrow lines only in the spectral region between ca. 400 and 500 nm and a broad spectrum at shorter and longer wavelength. Moreover, the xenon source peaks are composed of multiple emission lines. This can cause these peak positions to change with time and particularly between lamps [28]. Alternatively, the well-known (often certified) absorption spectrum of a compound revealing several narrow lines such as a solution of holmium oxide in perchloric acid [56], with absorption lines between 240 and 641 nm, can be exploited.

In the case of the emission channel, however, this requires specific calibration accessories, such as a diffuse reflector and a special sample holder, which hampers the widespread use of this procedure [28].

3.2.2 Wavelength accuracy of the excitation channel

The wavelength accuracy of the excitation channel can be, in principle, determined analogously to that of the emission channel. The wavelength standards of choice are typically:

i. an atomic discharge lamp [18,21,29,34] or
ii. a chromophore solution revealing several very narrow absorption bands within the UV/vis region.

For method i, an atomic discharge lamp is, e.g., coupled into the excitation channel via an optical fiber or placed inside the housing of the (switched off) instrument's excitation source. Critical here are the tedious alignment and the detection schemes that can both significantly decrease the accuracy of such a determination in comparison to the emission channel. Also, misalignment of the lamp can result in spectral deviations of the emission lines. This method is thus only recommended for applications that rely on a very high spectral resolution.

Otherwise, method ii using, e.g., a solution of holmium oxide in perchloric acid, see Section 3.2.1, in a conventional fluorescent cell presents a very attractive and simple alternative. This cell is placed at sample position. The wavelength accuracy of the excitation channel can then be controlled under typically used measurement conditions with a detector placed behind the cell via a simple absorption measurement independent from the emission channel.

If the accuracy of the emission wavelength selector, typically a monochromator, is already known, a diffuse scatterer, e.g., a scattering solution or a diffuse reflector, at the sample position can be used to scatter a fraction of the excitation beam into the detection system to determine the wavelength accuracy of the excitation channel. One wavelength selector is tuned over the other, which is fixed. The wavelength bias between the two wavelength selectors is equal to the difference between the set wavelength position and the observed peak position of the collected spectrum. This method [18,21] can be used at any wavelength, unlike many other methods that depend on a limited number of set excitation wavelengths determined by the reference material chosen.

3.2.3 Spectral resolution of monochromators

The spectral bandwidth of the monochromator(s) of fluorescence instruments and the instrument's spectral resolution follow from measurements of the full width at half height of the maximum (FWHM) of selected emission bands of a wavelength standard, typically at a very narrow slit width or at the most narrow one provided by the instrument. This is described in ASTM E 388-04 for spectrofluorometers using an atomic discharge lamp [21] and illustrated in Fig. 2 for the Hg doublet at 577 and 579 nm. The spectral resolution follows from the FWHM of the emission bands. As the spectral bandpass is usually wavelength-dependent, the instrument's spectral resolution should be obtained within the typically measured wavelength region. Chromophore-based wavelength standards, which reveal emission spectra consisting of several narrow bands, such as fluorescent RE-doped glasses or an absorbing holmium oxide solution, can be used for this purpose, in principle, but are not well suited for the determination of the spectral resolution of high-precision spectrofluorometers.

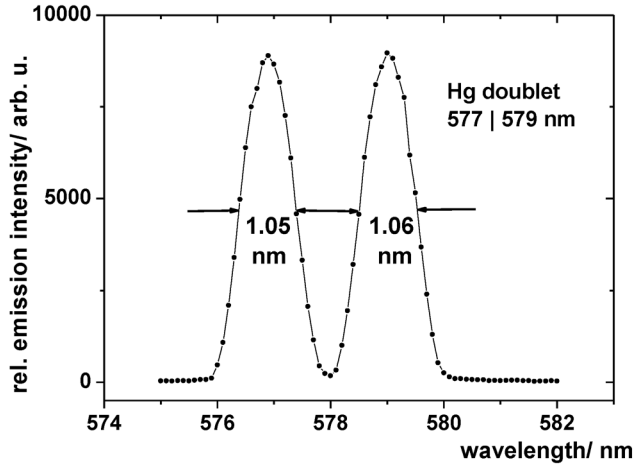

Fig. 2 Determination of the spectral resolution of the emission monochromator of a high-end precision fluorometer with an atomic discharge lamp in conjunction with a diffuser (emission slit width 1 nm).

3.3 Determination of the (relative) spectral responsivity: Calibrated lamps and emission standards

The spectral responsivity of the emission channel of a fluorescence instrument, see eq. 1, can be obtained with a source-based standard like a lamp or chromophore-based emission standards that emit a broad, preferably unstructured spectrum in the UV/vis/NIR spectral region [8,9,11,12,17,18,29]. The wavelength-dependent spectral radiance or corrected emission spectrum of these standards must be known and should be preferably certified with a stated uncertainty. A *certified value* is a value for which the certifying body has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by the certifying body [57]. Further requirements on suitable standards are detailed in ref. [34]. For the spectral correction of emission spectra, typically only the knowledge of the relative wavelength dependence of s is sufficient that is termed here relative spectral responsivity. The relative spectral responsivity equals the ratio of the spectral responsivity $s(\lambda)$ at wavelength λ to the spectral responsivity at a reference wavelength λ_0 [36]. The instrument's (relative) spectral responsivity equals the quotient of the measured (uncorrected) emission spectrum and the certified spectral radiance or corrected emission spectrum of the standard, see Fig. 3 [17,33,40,58].

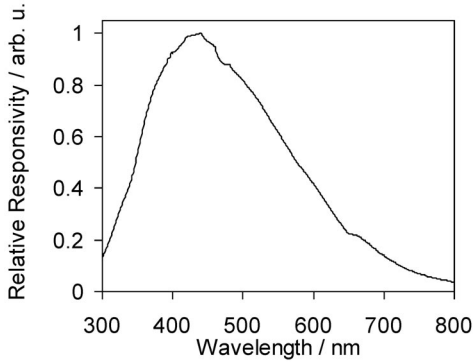

Fig. 3 Example of the relative spectral responsivity of an emission detection system (grating monochromator-PMT based) [18], for which a correction needs to be applied to a measured emission spectrum to obtain its true spectral shape (relative intensities) [59].

Knowledge and consideration of $s(\lambda_{em})$ is the prerequisite for reliable and comparable emission spectra and emission-excitation matrices (EEM) as well as for fluorescence quantum yields [1,6,8,10,11,14], see also refs. [15,16] and the majority of quantitative fluorescence measurements [10]. Accordingly, the reliability of the determination of $s(\lambda_{em})$ is of utmost importance and should be regularly controlled. A recalibration of the emission channel is recommended once per year (this depends also on the procedures used for the validation of instrument performance) [17,27,29]. Regular checks on changes of the relative shape of $s(\lambda_{em})$ should be performed every three months either with emission standards [17,34] or by a comparison of the uncorrected instrument-specific spectra of a set of dyes covering the typically used emission range. These dyes should preferably not show structured emission spectra, see also requirements on suitable emission standards given in [34]. Changes are expected to occur especially in the UV region owing to aging of optical instrument components. Redetermination of $s(\lambda_{em})$ is required after each change of instrument components in the emission channel.

Typical methods for the determination of the relative spectral shape of the spectral responsivity of the emission channel include the following procedures:

i. use of a calibrated physical transfer standard for spectral radiance such as a tungsten ribbon lamp or an integrating sphere-type radiator [17,18,29,32],
ii. use of the previously characterized excitation channel (with a calibrated detector, see Section 3.4), as calibrated light source in a synchronous scan of the excitation and emission channel with a calibrated white (diffuse reflector) standard at the sample position [18], or
iii. use of chromophore-based spectral fluorescence or so-called emission standards [17,29,33,34,60–69].

All of these approaches are in principle traceable to a radiometric scale [17,18,40], see also ref. [34] for a definition of traceability. In this case, traceability implies a linking of fluorescence intensities to either the spectral radiance scale or the spectral responsivity scale using physical or chemical transfer standards. As detailed in Section 3.5, for certain applications like the determination of the fluorescence quantum yield, traceability to a photonic scale like the spectral photon flux is mandatory. In any case, the radiometric or photonic quantity to which traceability was established during instrument characterization should be explicitly stated, as this can affect the comparability of corrected fluorescence spectra, see also Section 3.4.

For approach i, the very broad unstructured emission spectra of tungsten ribbon lamps and integrating sphere-type spectral radiance transfer standards that cover the UV/vis/NIR spectral region are attractive choices [29,32,40,68]. Drawbacks of these calibrated source standards are, however, a tedious alignment, regular and expensive recalibrations, restrictions on measurement geometry, and a considerable size that can hamper their application for compact fluorescence instruments. Moreover, their spectral radiances usually exceed those of typical fluorescent samples by at least four (tungsten ribbon lamp) to two (integrating sphere radiator) orders of magnitude [40]. Accordingly, such source standards can only be used in conjunction with sophisticated attenuation procedures [17], which decrease the spectral radiance to a level within the linear range of the fluorometer's detection system.

Method ii relies on the use of a white standard, the wavelength dependence of the spectral radiance factor of which was determined (and certified) for the measurement geometry employed, and on the synchronized behavior of the emission and excitation monochromators. The simplest chromophore-based method iii relies on the availability of suitable sets of emission standards, which have become easier to obtain commercially [6,11,17,29,70–75].

Generally, the use of methods i and ii is only recommended for expert laboratories. Method i can yield the relative shape of $s(\lambda_{em})$ with an uncertainty of 5–10 %, using a confidence interval of 95 % [68,69,76]. In a recent study of four National Metrology Institutes (NMIs) on the state-of-the art comparability of corrected emission spectra of several test dyes using different calibration procedures, a comparability of corrected emission spectra within a relative standard uncertainty of 4.2 % was reported for the use of physical transfer standards (PTS) like calibrated lamps when performing measurements

and calibrations under identical conditions [68]. Owing to the smaller calibration uncertainties of detector-based, compared to light source-based, transfer standards [18,32], method ii can in principle yield a smaller calibration uncertainty, if uncertainties related to fluctuations of the spectral radiance of the excitation light source, and the different radiating volumes and spectral radiances of the calibrated excitation channel and typically measured samples, can be minimized [18]. However, the often faced inability of method ii to totally compensate for the spectral structure (peaks) of the xenon excitation source can introduce an additional uncertainty, resulting in an overall uncertainty of 10 % (confidence interval of 95 %) [17,18]. Thus, among methods i and ii, method i is typically favored. For the determination of $s(\lambda_{em})$ in the NIR region, i.e., for wavelength above 800 nm, at present, method i is the only option. Method ii can be useful for emission wavelengths below 380 nm where the spectral radiance of source-based standards strongly decreases, and thus their calibration uncertainty correspondingly increases [17,18,32], and as a tool to check on method i [18].

Method iii is the recommended one for the UV/vis region for the broad community of fluorescence users as it is less prone to errors. For nonexpert users, generally, the many sources of error inherent to the reliable operation of the devices required for methods i and ii can result in high uncertainties as stated above. In the case of method iii, however, the close match of the spectral radiance and the size and shape of the radiating volume of both standard(s) and samples enables a straightforward determination of the instrument's relative spectral responsivity under application-relevant conditions [17], thereby elegantly circumventing most sources of uncertainty related to method i. An inter-instrument comparability of emission spectra of better than 5 %, i.e., less than a 5 % difference between spectra taken on different instruments, is principally achievable using this method, as revealed in Fig. 4 [40,58,68]. In a recent assessment of the calibration performance of 12 field laboratories with the use

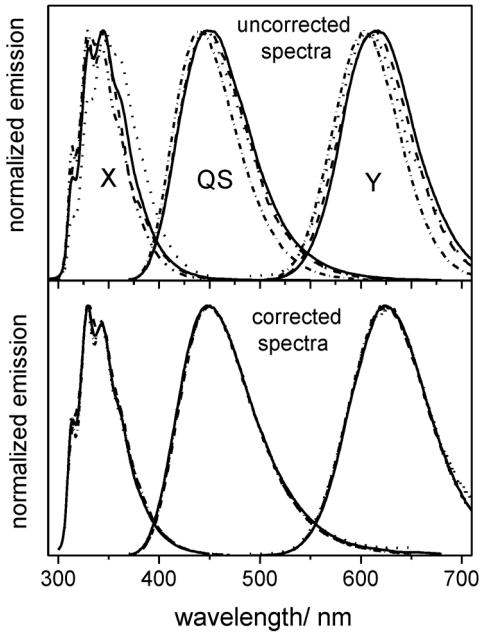

Fig. 4 Spectral correction and achievable interlaboratory comparability of fluorescence spectra with a dye-based emission correction (here using BAM-F001 to BAM-F005 [17,58]) as determined for four different commercial spectrofluorometers (different line types representing different instruments). Comparison of uncorrected emission spectra of the dyes *p*-terphenyl (dye X), quinine sulfate (dye QS), and 4-dicyanomethylene-2-methyl-6-(*p*-dimethylaminostyryl)-4*H*-pyran (DCM; dye Y) measured with four different commercial spectrofluorometers (upper panel) and the resulting corrected spectra (lower panel).

of spectral fluorescence standards, a comparability of the corrected emission spectra of 3 test dyes within a relative standard uncertainty of 6.8 % was reported [69].

3.4 Determination of the (relative) spectral radiant power or the (relative) spectral irradiance at sample position: Calibrated detectors and excitation standards

Instrument-independent excitation spectra and the comparison of (integral) emission intensities measured at different excitation wavelengths require knowledge and consideration of the spectral radiant power $P_\lambda(\lambda_{ex})$ or the spectral irradiance at the sample position $E_\lambda(\lambda_{ex})$ [17,29,76,77]. Both quantities are closely linked [35], see Table 1. For simplicity's sake, in the following, we solely refer to $E_\lambda(\lambda_{ex})$, as previously used by us [17,29,32], although for more recent publications on the determination of fluorescence quantum yields using two different excitation wavelengths for standard and sample, the spectral radiant power $P_\lambda(\lambda_{ex})$ was used [76], see also eq. 1. "Relative" implies here relative wavelength dependence.

For the determination of the relative spectral shape of $E_\lambda(\lambda_{ex})$, which is sufficient in the majority of cases, the wavelength and polarization dependence of the radiant power reaching the sample (in relative units) needs to be obtained [18]. Here, it is typically assumed that the illuminated volume does not change between instrument characterization and measurement of fluorescent samples to be corrected [17]. For the few cases where the absolute values of $E_\lambda(\lambda_{ex})$ are desired, such as the direct comparison of fluorescence intensities generated by different instruments or the determination of absolute fluorescence quantum yields, additional knowledge of the illuminated volume of the spectral responsivity transfer standard (and the sample) is required [18].

Knowledge and consideration of $E_\lambda(\lambda_{ex})$ is the prerequisite for reliable and comparable excitation spectra and EEM [18] as well as for the determination of fluorescence quantum yields employing different excitation wavelengths for standard and sample, see also refs. [15,16,76–78]. A recalibration of the excitation channel is recommended once per year (this depends also on the procedures used for the validation of instrument performance). In addition, regular measurements with day-to-day intensity standards can provide a hint for changes of $E_\lambda(\lambda_{ex})$, see ref. [34] and Section 3.5. Redetermination of $E_\lambda(\lambda_{ex})$ is mandatory after each change of instrument components in the excitation channel such as a change of the excitation light source.

The following procedures have been described for the determination of $E_\lambda(\lambda_{ex})$:

i. use of a calibrated spectral responsivity transfer standard such as a calibrated detector, typically a silicon photodiode (simple or integrating sphere-type, trap detector [29,32,76–80]) placed at sample position,
ii. application of the previously characterized emission channel, see Section 3.3, as "calibrated detector" in a synchronous scan of the excitation and emission channel with a white standard at sample position [17,29],
iii. use of chromophore-based so-called excitation standards with known corrected excitation spectra [11,17,25,61],
iv. use of a pyroelectric detector [81],
v. use of a quantum counter [1,29,82–85],
vi. use of an actinometer [85–87], and
vii. the comparison of the absorption and excitation spectrum of a chromophore [17].

The most common method for the traceable measurement of the spectral shape of $E_\lambda(\lambda_{ex})$ is method i [18]. In the case of a radiant power-calibrated detector, this method can yield absolute values of $E_\lambda(\lambda_{ex})$ in combination with a known volume or cross-section of the illuminated detection area.

Also, method ii can in principle lead to the relative spectral shape of $E_\lambda(\lambda_{ex})$ and to absolute values of $E_\lambda(\lambda_{ex})$. A prerequisite here is that for both the characterization of the spectral responsivity of the emission channel and the spectral irradiance at sample position, the angle under which the white

standard is illuminated (either with a spectral radiance transfer standard or the excitation channel), the illuminated area of the white standard, and the angle of detection are either kept constant or are known and accordingly considered. Knowledge of the illuminated area is required for absolute measurements of $E_\lambda(\lambda_{ex})$.

Table 1 SI-consistent terminology and symbols used, see ref. [34]. The subscript λ denotes per nanometer or spectral quantities (derivative with respect to wavelength). The only exception presents the spectral responsivity where spectral implies wavelength-dependent [36]. The subscript p, see e.g., ref. [15], denotes photonic quantities. They differ from radiometric quantities in the replacement of power (energy per unit time) by quanta per unit time ("quanta" per second has units of s^{-1}). Conversion factors involve Plank constant, h, and the velocity of light, c. The indices ex, em, and abs symbolize excitation, emission, and absorption. The term "intensity", traditionally used for photon flux, fluence rate, irradiance, or radiant power [34], will be retained here when its usage does not lead to misunderstandings.

Symbol	Name	Unit
$I_m(\lambda_{ex},\lambda_{em})$	measured fluorescence signal*	A
$I_u(\lambda_{ex},\lambda_{em})$	uncorrected spectrum*	$A{\cdot}nm^{-2}$
$I_b(\lambda_{ex},\lambda_{em})$	blank spectrum*	$A{\cdot}nm^{-2}$
$I_c(\lambda_{ex},\lambda_{em})$	corrected spectrum*	$A{\cdot}nm^{-2}$
$P(\lambda_{ex})^\dagger$	radiant power	W
$P_\lambda(\lambda_{ex})$	spectral radiant power	$W{\cdot}nm^{-1}$
$E(\lambda_{ex})^\ddagger$	irradiance	$W{\cdot}m^{-2}$
$E_\lambda(\lambda_{ex})^\S$	spectral irradiance	$W{\cdot}m^{-2}{\cdot}nm^{-1}$
$E_{p,\lambda}(\lambda_{ex})$	spectral photon irradiance, = $E_\lambda(\lambda_{ex}) \times \lambda/(hc)$	$m^{-2}{\cdot}s^{-1}{\cdot}nm^{-1}$
$q_{p,\lambda}(\lambda_{ex})$	spectral photon flux**	$s^{-1}{\cdot}nm^{-1}$
$L_\lambda(\lambda_{ex})$	spectral radiance	$W{\cdot}m^{-2}{\cdot}sr^{-1}{\cdot}nm^{-1}$
$L_{p,\lambda}(\lambda_{ex})$	spectral photon radiance = $L_\lambda(\lambda_{ex}) \times \lambda/(hc)$	$m^{-2}{\cdot}sr^{-1}{\cdot}s^{-1}{\cdot}nm^{-1}$
$s(\lambda_{em})^{\dagger\dagger}$	spectral responsivity	$A{\cdot}W^{-1}$
$d\lambda_{ex}$, $d\lambda_{em}$	spectral bandwidth (excitation, emission)	nm
$F_{p,\lambda}(\lambda_{em})$	normalized photon emission spectrum	nm^{-1}
Φ_f	fluorescence quantum yield	1
N_{abs}, N_{em}	number of photons (absorbed, emitted)	1
K	optical geometry factor	1
$f(\lambda_{ex})$	absorption factor	1
$A(\lambda_{ex})$	absorbance	1
$\varepsilon(\lambda_{ex})$	molar (decadic) absorption coefficient	$dm^3{\cdot}cm^{-1}{\cdot}mol^{-1}$
$C^{\ddagger\ddagger}$	analyte concentration	$mol{\cdot}dm^{-3}$
l	optical pathlength	cm
S_{ex}	irradiated area of sample	m^2
c	velocity of light	$m{\cdot}s^{-2}$
h	Planck constant [6.626 0693(11) × 10^{-34}]	J·s

*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 eq. 1. Blank- or background-corrected spectra obtained upon subtraction of the blank spectrum measured for the fluorophore-free solvent or matrix (under identical measurement conditions as the sample) from the measured 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.

†In radiometry, *flux* (Φ) is used with the same units as radiant power P. The symbol Φ is reserved for quantum yield in photochemistry. Here, we typically use radiant power.

(*continues on next page*)

Table 1 (*Continued*).

‡The irradiance E is the radiant power P (radiant energy per time) received on a surface, i.e., the radiant power P of all wavelengths incident from all upward directions on a small element of surface containing the point under consideration divided by the area of the element [35].
§The spectral irradiance E_λ is the derivative of the irradiance, E, with respect to wavelength, λ [35].
**The photon flux $q_p(\lambda)$ is the integral of the photon irradiance $E_p(\lambda)$ over the illuminated area.
††Note that in the previously published IUPAC Technical Report, "Fluorescence standards: Classification, terminology, and recommendations on their selection, use, and production" [34], we erroneously defined the spectral responsivity as the signal output per radiant power (equaling radiant flux) incident on a detection system per unit bandwidth of the incident light expressed as a function of wavelength and not as the quotient of the detector output (photocurrent) by the detector input (incident radiant power) at wavelength λ as used here [32,36].
‡‡We use here C as symbol for concentration in mol·dm^{-3} that is typically abbreviated with c in order to avoid confusion with the velocity of light, c.

Method iii, which requires suitable sets of excitation standards and dilute dye solutions [17], see also ref. [34], and assumes comparable illuminated and detected volumes for standard(s) and samples, yields only the relative values of $E_\lambda(\lambda_{ex})$. The need for dilute dye solutions is related to the proportionality of fluorescence intensity to the absorption factor f, see eq. 2. This results in a concentration dependence of the spectral shape of excitation spectra and introduces a dependence on measurement geometry. For instance, for a 0°/90° measurement geometry, the chromophore absorbance should not exceed 0.05. Drawbacks of method iii are the lack of certified excitation standards, with the first set of excitation standards being only recently presented [17], and more generally, the limited reliability of literature data. In all these cases, the (relative) spectral irradiance reaching the sample position equals the quotient of the measured signal and the certified spectral responsivity of the detector or the corrected spectra of the standards [17,18,76].

Of much less importance and not advisable is the application of methods iv–vii. A pyroelectric detector reveals a drastically reduced sensitivity and accuracy compared to, e.g., a silicon photodiode. A quantum counter is prone to polarization and geometry effects, and the reliability of an actinometer relies on a sufficiently well characterized photochemical reaction [14]. In addition, the most simple method vii can lead to a comparatively high calibration uncertainty if the dye photophysics are not very well known. For example, the fluorescence quantum yield of the dye may depend on excitation wavelength for excitation at two different electronic transitions [17]. This is, however, more an exception than a rule as there are very few compounds that do not comply with Kasha's rule about emission from the lowest excited singlet state [1,6].

The best-suited method for the measurement of the spectral shape of $E_\lambda(\lambda_{ex})$ is method i [18,76]. This can be performed with an uncertainty of about 5 % (confidence interval of 95 %). The results from the determination of the relative spectral irradiance of the excitation channel of a spectrofluorometer employing method i and a calibrated silicon photodiode integrating sphere assembly (known wavelength dependence of the spectral responsivity) at the sample position, are revealed in Fig. 5 [17]. During this calibration, two signals are measured: (a) the signal from the calibrated detector is recorded (as photocurrent) to obtain the wavelength-dependent and polarization-dependent spectral irradiance at sample position and (b) the signal monitored by the spectrofluorometer's reference detector (the spectral responsivity of which is typically not known) at a certain emission wavelength, that provides a measure for the relative spectral irradiance reaching the reference channel, $E_{ref,\lambda}(\lambda_{ex})$ [17,76]. The signal from the spectrofluorometer's reference detector is used to account for fluctuations of the spectral radiance of the excitation light source during fluorescence measurements and instrument calibration [76,88], that would otherwise affect measured emission intensities and measured excitation spectra. Accordingly, for this procedure, the correspondingly obtained excitation correction curve $EX(\lambda_{ex})$, see Fig. 5 (dotted line), equals the quotient of the relative spectral irradiance of the excitation channel reach-

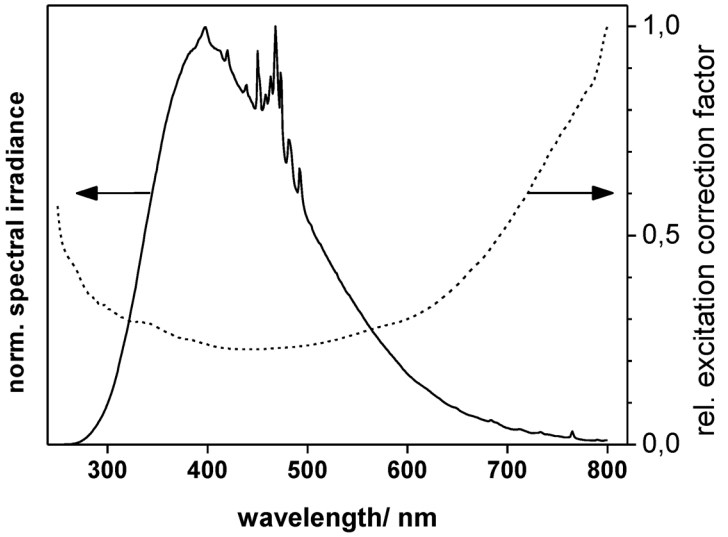

Fig. 5 Relative spectral irradiance at sample position $E_{ex,\lambda}(\lambda_{ex})$ of the excitation channel of a spectrofluorometer equipped with a separately addressable detection and reference channel measured with a calibrated silicon photodiode integrating sphere assembly of known spectral responsivity at sample position (solid line: signal recorded from calibrated detector) and the corresponding excitation correction curve $EX(\lambda_{ex}) = E_{ex,\lambda}(\lambda_{ex})/E_{ref,\lambda}(\lambda_{ex})$ (dotted line). The signal recorded by the spectrofluorometer's reference channel is omitted for better clarity. Corrected excitation spectra $I_c(\lambda_{ex},\lambda_{em})$ are calculated by division of the uncorrected spectrum by the excitation correction curve $EX(\lambda_{ex})$ obtained under identical conditions (see eq. 5).

ing the sample, $E_{ex,\lambda}(\lambda_{ex})$, and the signal of the reference channel, $E_{ref,\lambda}(\lambda_{ex})$, respectively, at the time of the instrument calibration.

During the subsequently performed measurement of the fluorescence excitation spectrum of a sample, the signal of the emission channel, which actually represents the sample's uncorrected excitation spectrum, $I_u(\lambda_{ex},\lambda_{em})$, and the signal monitored by the reference channel, $E'_{ref,\lambda}(\lambda_{ex})$ are recorded. $E_{ref,\lambda}(\lambda_{ex})$ provides a measure of the relative spectral irradiance reaching the reference channel at the time of the measurement of the excitation scan. Depending on the photoluminescence measuring instrument used, this yields either two signals (for spectrofluorometers reading out the emission and the reference channel separately) or a single signal that presents the quotient of the signal from the emission detector and the reference detector $[I_u(\lambda_{ex},\lambda_{em})/E'_{ref,\lambda}(\lambda_{ex})]$. If the same instrument settings are used for the instrument characterization with the calibrated detector and the excitation scan with the sample, and the reference detector is operated in the linear range, $E_{ex,\lambda}(\lambda_{ex})$ and $E'_{ref,\lambda}(\lambda_{ex})$ can be expected to be identical [17,74,87]. As follows from eq. 4, with this procedure, corrected excitation spectra $I_c(\lambda_{ex},\lambda_{em})$ are calculated by division of the uncorrected excitation spectrum (typically measured as quotient $I_c(\lambda_{ex},\lambda_{em})/E'_{ref,\lambda}(\lambda_{ex})$ by the excitation correction curve $EX(\lambda_{ex}) = E_\lambda(\lambda_{ex})/E_{ref,\lambda}(\lambda_{ex})$, obtained under identical conditions.

$$I_c(\lambda_{ex},\lambda_{em}) = \frac{I_u(\lambda_{ex},\lambda_{em})}{E'_{ref,\lambda}(\lambda_{ex})} \times \frac{E_{ref,\lambda}(\lambda_{ex})}{E_{ex,\lambda}(\lambda_{ex})} \quad (4)$$

3.5 Instrument characterization and consideration of the photonic nature of light

As derived in the previous sections, an instrument characterization should be performed traceable to radiometric units, i.e., to the spectral radiance and the spectral responsivity, as done in colorimetry, see ref. [34]. However, for the determination of certain spectroscopic quantities like the photoluminescence

quantum yield [90], see ref. [15], the energy of the emitted photons must be taken into account [29,76–78] as the fluorescence quantum yield represents the ratio of the number of emitted photons and absorbed photons and not a ratio of emitted and absorbed radiant powers. Accordingly, corrected emission spectra referenced to L_λ have to be multiplied by the wavelength λ_{em} prior to integration on a wavelength scale to consider the photonic nature of the emitted light. This principally establishes traceability to the spectral photon radiance $L_{p,\lambda} = L_\lambda \times \lambda/(hc)$ [90,91]. Otherwise, the fluorescence quantum yields of chromophores emitting in the vis and especially in the NIR spectral region are underestimated as compared to dyes fluorescing in the UV [17].

In Fig. 6, the relative (top) and normalized (bottom) corrected emission spectra of three dyes emitting at different wavelength regions and the ratios of the integral fluorescence intensities (weighted by the dye's absorption factor at the excitation wavelength, top) are compared. This underlines that if the energy of the emitted photons is not considered, for two solutions having the same fluorescence quantum yield, the one with the longer wavelength emission gives a lower value.

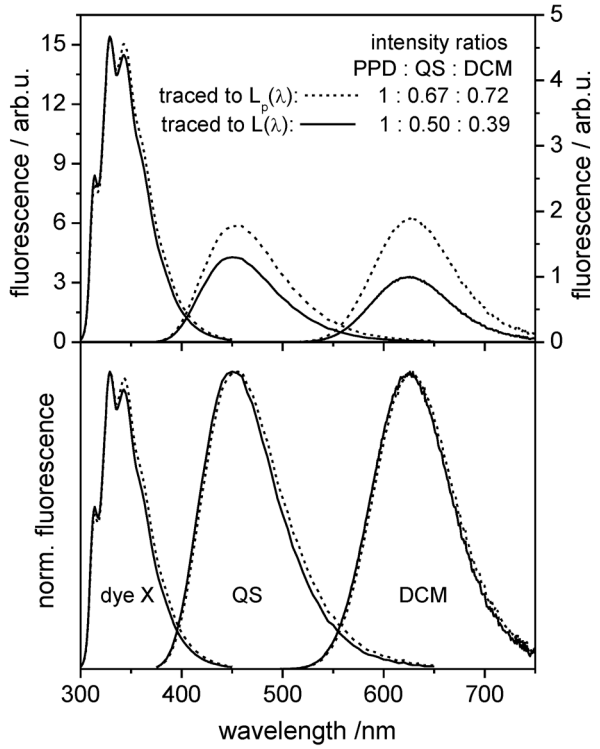

Fig. 6 Comparison of the relative (top) and normalized (bottom) corrected emission spectra of the three dyes *p*-terphenyl (dye x), QS (quinine sulfate), and DCM [4-dicyanomethylene-2-methyl-6-(*p*-dimethylaminostyryl)-4*H*-pyran] referenced to the spectral radiance L_λ (solid lines, right axes) and to the spectral photon radiance $L_{p,\lambda}$ (dotted lines, left axis), respectively. The ratios given refer to integral fluorescence intensities (weighted by the dye's absorption factor at the excitation wavelength).

Consideration of the energy of the absorbed photons is mandatory for the determination of the fluorescence quantum yield of a sample relative to a standard using different excitation wavelengths λ_{ex} for sample and standard. This procedure compares the (integral) fluorescence intensities resulting at different excitation wavelengths [29,76–78]. Consideration of the photonic nature of the excitation light establishes the traceability to the spectral photon flux $q_{p,\lambda} = P_\lambda(\lambda) \times \lambda/(hc)$ or the spectral photon irra-

diance $E_{p,\lambda} = E_\lambda(\lambda) \times \lambda/(hc)$ [35,76]. This is also required for the comparison of corrected excitation spectra with measured absorption spectra, i.e., the wavelength dependence of the absorption factor $f(\lambda)$.

3.6 Validation of instrument performance

Generally, control and validation of the instrument performance are recommended on a daily or weekly basis. The choice of suitable procedures and standards for the validation of instrument performance depends on the instrument parameters to be checked and thus, to a certain extent, on the respective fluorescence technique. These measurements must always be performed with similar instrument settings and with either very robust or easily reproduced standards with a minimum, preferably known, uncertainty, see also ref. [34] for requirements on suitable standards. The assignment of changes in instrument performance to certain instrument parts, i.e., the clear distinction between drifts arising from changes of the excitation and the emission channel, requires tools for the independent measurement of $s(\lambda_{em})$ and $E_\lambda(\lambda_{ex})$.

Different manufacturers of steady-state fluorometers recommend the so-called Raman test (excitation at 350 nm, Raman band recorded at 397 nm [29,93,94]) for the control of the instrument's long-term stability and sensitivity employing a sealed cuvette of nonfluorescent water. In principle, different excitation wavelengths can be used, with the difference in energy between excitation and Raman band always equaling 3400 cm^{-1}. Although very convenient and well suited for daily use, this method, which is often integrated into the software of fluorescence instruments, is effectively limited to excitation wavelengths below 400 nm owing to the λ^{-4}-dependence of the intensity of scattered light. More attractive candidates that cover the UV/vis/NIR spectral region are compounds that emit multiple peaks over a broad spectral range [10,29,44,72,95,96], see also ref. [34]. Measurement of these standards provides simultaneous information on instrument drift, spectral sensitivity (via changes in the relative emission intensity), and changes in the relative shape of the spectral responsivity of the emission channel.

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