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Traceability in Fluorometry: Part II. Spectral Fluorescence Standards

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The need for the traceable characterization of fluorescence instruments is emphasized from a chemist's point of view, focusing on spectral fluorescence standards for the determination of the wavelength- and polarization-dependent relative spectral responsivity and relative spectral irradiance of fluorescence measuring systems, respectively. In a first step, major sources of error of fluorescence measurements and instrument calibration are revealed to underline the importance of this issue and to illustrate advantages and disadvantages of physical and chemical transfer standards for generation of spectral correction curves. Secondly, examples for sets of traceable chemical emission and excitation standards are shown that cover a broad spectral region and simple procedures for the determination of corrected emission spectra with acceptable uncertainties are presented. With proper consideration of the respective measurement principle and geometry, these dye-based characterization procedures can be not only applied to spectrofluorometers but also to other types of fluorescence measuring systems and even to Raman spectrometers.

KEY WORDS: Fluorescence; standard; spectral correction; emission; excitation; traceability.

INTRODUCTION

Photoluminescence forms the basis of a wide variety of applications in material sciences, (bio)analytical chemistry, medical diagnostics, and biotechnology [1–5]. Despite of the widespread and ever increasing use of fluorescence techniques, many method-inherent problems and their influence on calibration and performance validation of fluorescence instruments and accordingly quality and reliability of measurements are still often neglected. This includes, for instance, nonlinearities of the detection system, effect of spectral bandpass and detector voltage as well as instrument- and sample-related polarization effects. Furthermore, the need for correction of measured fluorescence data for instrument-specific characteristics is frequently underestimated.

Generally, comparability of fluorescence data across instruments, laboratories, and over time relies on the knowledge of the relative spectral variations of the instrument's optical and opto-electronical components. These variations can be static in nature, when inherent properties of single components are concerned, or they can show systematic trends, e.g., aging-related drifts. Circumvention and control of such variations thus require determination of the spectral characteristics of fluorescence instruments at regular intervals [6,7].[5] This eventually implies measurement of the wavelength- and polarization-dependent relative spectral irradiance at sample position and determination of the wavelength- and polarization-dependent relative spectral responsivity of the excitation

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[5] The discussion or development of fluorescence intensity standards is not within the scope of this article, cf. [7].

and emission channel, termed excitation and emission correction curves. This can be accomplished with calibrated physical transfer standards [8] or chromophore-based certified reference materials. The latter are typically referred to as *spectral fluorescence standards* or *emission* and *excitation standards* [9,10]. Aside from the general desire for comparable fluorescence spectra, correction of measured data for specific instrument effects is a prerequisite for fluorescence methods that rely on the comparison of two fluorophores with different absorption and emission features, e.g. standard and sample with nonmatching emission spectra or measured at two different excitation wavelengths, respectively [11]. Examples are the determination of relative fluorescence quantum yields or particular issues in quantitative fluorometry. Furthermore, with the increasing trend in analytical chemistry, bioanalysis, and medical diagnostics of using spectral information from fluorescence measurements for the identification of analytes [12,13], and concerning the impact of spectra matching for certain fluorescence techniques like for instance flow cytometry [14,15], determination and availability of reliably corrected fluorescence spectra are gaining importance. This simultaneously enhances the need for internationally accepted procedures for instrument calibration and performance validation (IPV) as well as easy-to-operate and commercially available secondary standards. Such standards and procedures eventually enable fulfillment of the globalization-imposed trends of quality assurance, traceability, and accreditation also for fluorometry [16,17].

At present, these demands are poorly met for photoluminescence techniques. Up to now, there exists only a limited number of recommendations on the characterization of fluorescence instruments [18–20], and surveys on instrument performance and comparability of data have been barely performed for fluorescence techniques [21–23]. Moreover—aside from classical physical transfer standards described in Part I of this series [8], and the many different potential emission and excitation standards reported in the literature [1,9,11,24,25]—there are only very few spectral fluorescence standards commercially available [26].[6,7,8] At present, there exists a single certified reference material [27],[9] quinine sulfate dihydrate (SRM 936a) developed by the National Institute of Standards and Technology (NIST) [26]. This traceable emission standard can be, however, used for the determination of the relative spectral responsivity of fluorescence measuring systems only in the spectral region of ca. 395–565 nm.[10] All the other fluorescence standards available or recommended are typically not traceable to a primary standard and are often of insufficient quality with respect to their radiometric/spectroscopic and analytical characterization. Also, in most cases, uncertainties of the fluorescence quantities that are relevant for calibration are missing. This situation renders a reliable instrument characterization difficult, especially for unexperienced users of fluorescence techniques who desire and need certified, yet easy-to-operate reference materials and standard operation procedures for their use.

To improve the comparability and reliability of fluorescence measurements, in this paper, spectral correction of fluorescence data and realization of traceable photoluminescence measurements are discussed from a chemist's point of view, thereby also referring to Part I of this series focusing on the traceability chain of fluorometry and classical physical transfer standards [8]. To underline the importance of purpose-fit instrument calibrations that take into account the type of samples to be corrected, in a first step, sources of systematic error inherent to all types of photoluminescence measurements are illustrated that affect the uncertainty of the characterization of fluorescence instruments and the choice of suited secondary standards. In a second step, approaches to instrument characterization employing classical physical transfer standards and spectral fluorescence standards are revealed and compared. Special emphasis is dedicated to requirements on and development of easy-to-use emission and excitation standards, and simple, yet traceable procedures for spectral correction of fluorescence spectra with uncertainties that are acceptable for today's state-of-the-art instrumentation. Principally, with proper consideration of the underlying measurement principle and geometry, these standards and procedures can be applied not only to spectrofluorometers but also to other types of fluorescence measuring systems such as, for instance, laser setups and monochromator-type microplate readers and, with more restrictions, to fluorescence microscopes [28],[11] fluorescence detectors for

[6] Reference dye sampler kit (R-14782), Molecular Probes Inc.

[7] 6BF fluorescent reference materials set, emission and excitation standards, Starna GmbH.

[8] Perylene-type dyes FM 1 and FM 2, LambdaChem GmbH.

[9] For clear definition of the metrological hierarchy of reference materials, see [30].

[10] Fluorescence intensities of $\geq$10% of the emission at the maximum of the band are required at least for spectral calibration with acceptable uncertainties.

[11] The use of macrofluorescence standards for microfluorometry can for instance imply consideration of the impact of microscope parameters defining the volume illuminated and observed such as objective magnification and numerical aperture, emission and excitation aperture size, and focal point as well as consideration of complications due to pre-filter and post-filter effects. Furthermore, the increased spectral

chromatography that record emission spectra as well as Raman spectrometers [29].

EXPERIMENTAL

Terminology

In fluorescence spectroscopy, terminology is often not consistent and can be misleading. We, thus, decided to define the terms that are important for spectral correction within the context of this paper. For the symbols used here, the subscripts ex, em, λ, and p denote *excitation*, *emission*, *per nanometer* or *spectral*, and *photon*, respectively.

Measured fluorescence spectra, $I_m(\lambda, \lambda_{em})$, contain sample- and instrument-specific contributions. Removal of background signals such as scattering and fluorescence from the solvent and dark counts at the detector is obtained by subtraction of a *background spectrum*, $I_b(\lambda_{ex}, \lambda_{em})$, that was recorded under identical measurement conditions for a blank solvent sample. This procedure yields spectrally *uncorrected spectra*, $I_u(\lambda_{ex}, \lambda_{em}) = I_m(\lambda_{ex}, \lambda_{em}) - I_b(\lambda_{ex}, \lambda_{em})$. Spectra which are additionally corrected for the spectral characteristics of the respective instrument are termed *corrected spectra*, $I_c(\lambda_{ex}, \lambda_{em})$. *Corrected emission* and *excitation spectra* are obtained from $I_u(\lambda_{ex}, \lambda_{em})$ by application of experimentally determined *emission* or *excitation correction curves*. These curves represent the (wavelength- and polarization-dependent) relative spectral responsivity of the emission channel and the (wavelength- and polarization-dependent) relative spectral irradiance of the excitation channel at sample position, respectively. $I_c(\lambda_{ex}, \lambda_{em})$ are not corrected for sample-related effects such as pre- and post- or so-called inner filter effects, quenching by oxygen, and refractive index [30–32]. For the spectral fluorescence standards presented here, such effects are minimized on proper choice of chromophores and measurement conditions and are negligible within the reported uncertainties [33].[12]

The *spectral fluorescence yield* $F_\lambda(\lambda_{ex}, \lambda_{em})$ used in Part I of this series [8] is the ratio of the spectral radiant power (flux) of the emitted radiation divided by the spectral radiant power (flux) of the absorbed radiation. Division of the numerator and the denominator by the energy of the emitted and absorbed photons, respectively, yields the *spectral photon yield of fluorescence*. This quantity is the ratio of the number of emitted photons per number of absorbed photons per wavelength [31]. The term *fluorescence quantum yield* that is frequently used in fluorometry is the integral of the spectral photon yield of fluorescence over the whole emission spectrum.

Solvents and Reagents

All the organic solvents used were of spectroscopic grade and were purchased from Fluka GmbH. Perchloric acid was purchased from Merck. The dyes are of highest purity commercially available and were obtained from Fluka AG, Lambda Physics GmbH, and LambdaChem GmbH. *p*-Terphenyl embedded into a cuvette-shaped polymethylmethacrylate (PMMA) block was purchased from Starna GmbH. The purity of the neat dyes was typically ≥99.5% as has been determined by HPLC using a diode array detector and a fluorescence detector.

The presented set of emission standards consisting of dyes A, B, C, D, and E will be soon certified by BAM. This set of emission standards and its individual components will then be commercially available from BAM as Kit Spectral Fluorescence Standards or BAM certified reference materials (CRM) BAM-F001 to BAM-F05 equaling dyes A, B, C, D, and E as well as through all subsidiaries of Sigma-Aldrich with Fluka product number 72594 equaling dye A, 23923 (dye B), 96158 (dye C), 74245 (dye D), and 94053 (dye E), respectively. The software for generation of spectral correction curves from measured and corrected fluorescence spectra of these dyes that was developed by BAM will be provided with the emission standards as well as a standard operation procedure for their use tested with different types of common fluorescence instruments.

Equipment/Procedures

Equipment

Absorption spectra were recorded on a Carl Zeiss Specord M400/M500 and a Bruins Instruments Omega 10 spectrophotometer. Measurement of emission and excitation spectra was carried out with a Perkin Elmer LS50B spectrofluorometer (single monochromator of Monk-Gillison type, PMT R928 from Hamamatsu, analog detection mode, generated signal equals ratio of signals measured with emission and reference channel) and a Spectronics Instruments 8100 spectrofluorometer operated in the photon counting mode. The latter is of T-type design with a double monochromator (Seya Namioka type with holographic gratings) and a Peltier cooled PMT (R928, Hamamatsu) for measurements in the UV/Vis and a single monochromator (Czerney-Turner, 500 mm) and

irradiance under microscopic illumination of the standard can lead to enhanced photobleaching.

[12] In the literature, also the terms *apparent spectra* for S_{meas} and *technical spectra* for S_{corr} are used, cf. [33].

a silicon (Si) avalanche photodiode for measurements in the Vis/NIR spectral region. The reference channel of fluorometer 8100 is equipped with a Peltier cooled PMT (R928, Hamamatsu) that can be adjusted in its spectral responsivity and read out separately from the emission detector. Typical fluorescence measurements with fluorometer 8100 were performed with Glan-Thompson polarizers placed in the excitation channel and the two emission channels, respectively.

The wavelength accuracy of the monochromators of both fluorometers was controlled with a custom-built cuvette-shaped low pressure neon discharge lamp and a pen-type mercury lamp from UVP Inc., placed at sample position. For fluorometer 8100, emission correction curves were determined with an integration sphere-type spectral radiance transfer standard (quartz halogen lamp placed inside an integrating sphere, Gigahertz-Optik GmbH; BN9701, see Part I of this series [8]) and a non-fluorescent diffuse reflectance or white standard (Gigahertz-Optik GmbH), both calibrated by the Physikalisch-Technische Bundesanstalt (PTB). The standard lamp was equipped with an aperture (8 mm × 10 mm) and its spectral radiance was accordingly calibrated for this modification. Excitation correction curves were obtained with a PTB-calibrated Si photodiode that is mounted inside an integrating sphere (Gigahertz-Optik GmbH) placed at sample position. Emission and excitation correction curves were measured in relative intensities for all the spectral bandpasses and polarizer settings used for typical fluorescence experiments, i.e., 0°, 90°, and 54.7° as well as without polarizer. In the case of fluorometer LS50B, emission and excitation correction curves were determined for each bandpass setting with a set of spectral fluorescence standards, the corrected emission and excitation spectra of which were previously obtained with fluorometer 8100. Polarizers were not used with this fluorometer. The calibration procedures are described in more detail in a separate paragraph.

Procedures

All the absorption and emission measurements were performed at a temperature of 25 ± 1°C. Unless otherwise stated, only dilute dye solutions with absorbances $A \leq 0.04$ at the excitation wavelength (emission spectra) or at the maximum of the low energy absorption band (excitation spectra) were used. Prior to spectral correction, a background spectrum that was recorded under identical measurement conditions for a blank solvent sample was subtracted from the measured spectrum, see also "Terminology" section. Spectral correction was then achieved by division of $I_u(\lambda_{ex}, \lambda_{em})$ by the corresponding spectral correction curves, yielding the corrected fluorescence spectrum $I_c(\lambda_{ex}, \lambda_{em})$.

RESULTS/DISCUSSION

Need for Determination of the Spectral Characteristics of Fluorescence Instruments

Independent of fluorescence technique and type of measurement, e.g. spectrally resolved or integral at fixed wavelengths, the fluorescence signal, a spectral intensity distribution, that originates from the fluorescent analyte is distorted by the instrument yielding uncorrected data $I_u(\lambda_{ex}, \lambda_{em})$ [34], see Eq. (1). Equation (1), which is the integrated form of Eq. (1) presented in Part I of this series [8], assumes very dilute solutions, negligible inner filter effects, and validity of Beer–Lambert law as is typically fulfilled for the majority of fluorescence measurements. Signal-relevant analyte properties are the chromophore's absorptance at the excitation wavelength $\alpha(\lambda_{ex})$, which is nonlinearly linked to absorbance $A(\lambda_{ex})$ by Beer–Lambert law,[13] see Eqs. (2) and (3), and its spectral fluorescence yield $F_\lambda(\lambda_{ex},\lambda_{em})$ [31]. $\varepsilon(\lambda_{ex})$ equals the chromophore's molar absorption coefficient, l the optical pathlength, and c the chromophore's concentration. Instrument-specific effects are linked to the spectral irradiance reaching the sample $E_{ex,\lambda}(\lambda_{ex})$ and the spectral responsivity of the emission channel $s(\lambda_{em})$,[14] see Eq. (1). The former includes the wavelength-, polarization- and time-dependent spectral radiance of the excitation light source and transmittance of optical components like lenses, mirrors, filters, monochromator gratings or polarizers in the excitation channel. The latter depends on the wavelength-, polarization-, and time-dependent spectral sensitivity of the detection system and transmittance of optical components in the emission channel.

$$I_u(\lambda_{ex}, \lambda_{em}) = \alpha(\lambda_{ex}) \times F_\lambda(\lambda_{ex}, \lambda_{em}) \times E_{ex,\lambda}(\lambda_{ex}) \times s(\lambda_{em}) \quad (1)$$

$$\alpha(\lambda_{ex}) = 1 - 10^{-A(\lambda_{ex})} = 1 - 10^{-c \times \varepsilon(\lambda_{ex}) \times l} \quad (2)$$

$$\alpha(\lambda_{ex}) = 1 - e^{-\ln(10) \times A(\lambda_{ex})} = 1 - 1 + \ln(10) \times A(\lambda_{ex}) - \frac{(\ln(10) \times A(\lambda_{ex}))^2}{2!} + \cdots \quad (3)$$

$$\alpha(\lambda_{ex}) \approx \ln(10) \times A(\lambda_{ex}) \quad (4)$$

[13] Only for very dilute solutions, e.g. for an absorbance below 0.05, the exponential term in Eqs. (2) and (3) can be expanded and orders higher than 1 can be neglected with an uncertainty of ≤5%.

[14] Due to radiometric convention, $s(\lambda)$ always implies $s_\lambda(\lambda)$.

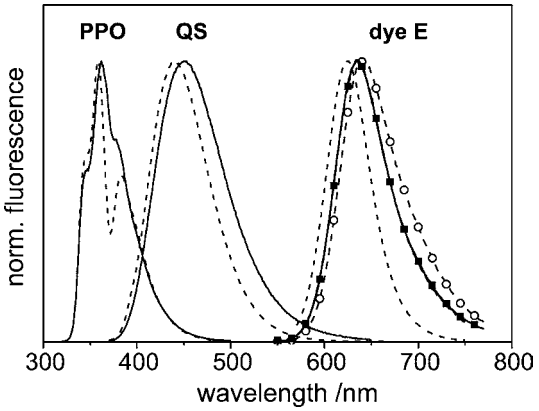

Fig. 1. Normalized uncorrected (dashed lines; circles) and corrected (solid lines; squares) emission spectra of ethanolic solutions of PPO and dye E and quinine sulfate (QS) in 0.1 M $HClO_4$. Dashed and solid lines correspond to measurements that were performed with the UV/Vis detection system, circles and squares to those where the Vis/NIR channel of fluorometer 8100 was employed. For dye E, the corrected spectra obtained with both detection systems are virtually identical. The uncorrected emission spectrum of PPO was measured with an emission polarizer set to 90°.

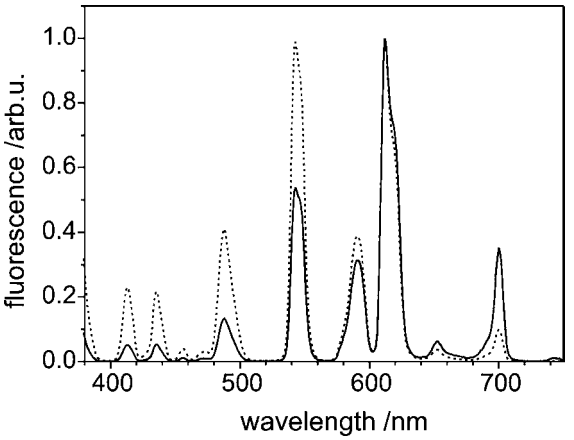

Fig. 2. Uncorrected (dotted) and corrected (solid line) emission spectra of a glass block doped with a mixture of rare-earth metal ions. Excitation was at 370 nm. The spectra were normalized at 612 nm for better comparison of the changes in fluorescence intensity related to spectral correction.

As fluorescence emission or excitation spectra are typically recorded at fixed excitation and emission wavelengths, correction of measured data for the spectral characteristics of the emission and excitation channel can be performed separately. Principally, as is mentioned in Part I of this series [8], this can be achieved by absolute measurements of fluorescence intensities can be performed. However, for elimination of instrument-specific wavelength and polarization dependences, determination of the instrument's relative spectral responsivity termed emission correction curve and its relative spectral irradiance at sample position or so-called excitation correction curve are sufficient and by far more easy to realize [34]. For comparison of fluorescence intensities, additional use of application-specific standards with known fluorescence yields, and excitation and emission spectra that closely match those of the fluorescent analyte to be quantified is purpose-fit in most cases [35].

The influence of these instrument-specific spectral effects on fluorescence data and the according need for spectral correction are illustrated in Figs. 1–3, respectively. Figure 1 depicts the normalized uncorrected emission spectra of three typical organic fluorophores and the corresponding corrected spectra. The indentation at ca. 370 nm seen in the uncorrected spectrum of PPO results from diffraction effects (Wood anomalies) of the instrument's monochromator gratings. The occurrence and size of such effects depends on the degree of the polarization of the incident emission, the type of monochromator gratings, and the monochromator design and thus varies amongst fluorescence instruments. Furthermore, as is highlighted for dye E, uncorrected emission spectra obtained with different detection systems/fluorometers can considerably deviate. A similar comparison of uncorrected and corrected spectra is shown in Fig. 2 for a mixture of rare-earth metal ions displaying very narrow emission bands. Due to the comparatively small width of the emission bands, basically no changes in the spectral position and line shape occur on spectral correction. However, the ratios of the relative intensities of the emission bands are clearly instrument dependent. Similar effects,

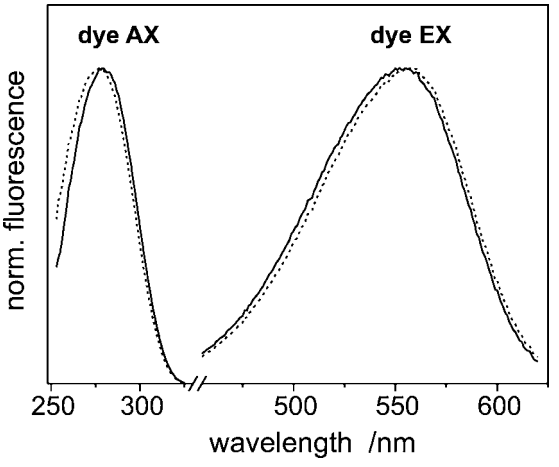

Fig. 3. Normalized uncorrected (dotted) and corrected (solid line) excitation spectra of two typical organic chromophores AX and EX dissolved in ethanol.

i.e., instrument-specific differences between uncorrected and corrected spectra result also for excitation spectra as is exemplary shown in Fig. 3 for the two typical organic fluorophores dye AX and dye EX.

Emission and Excitation Correction Curves

Principally, emission and excitation correction curves are obtained on division of the signal measured for a suited secondary standard with the instrument to be calibrated by the standard's certified/reported radiometric/fluorometric quantity. Emission correction curves are determined with a source of known spectral radiance. This can be either a certified spectral radiance transfer standard like a tungsten strip or an integrating sphere-type standard lamp or a chemical fluorescence standard with a known—preferably certified—corrected emission spectrum [36–38].[15,16] Both approaches can be made traceable to the spectral radiance scale realized by the primary radiometric standard *blackbody radiator* [8]. Excitation correction curve can be measured for instance with a calibrated detector, typically a Si photodiode (simple or integrating sphere-type, trap detector [39]) or, less common, a pyroelectric detector.[17] A chemical approach employs fluorescent compounds termed excitation standards with known and preferably certified corrected excitation spectra [1,9,11]. For both types of secondary standards, these measurements can be made traceable to the spectral responsivity scale realized via the primary radiometric standard *cryogenic radiometer* [8]. Other suggested, though not advisable approaches to excitation correction curves include quantum counters with an excitation wavelength-independent fluorescence quantum yield [34], actinometers that rely on the wavelength-independent quantum yield of a photochemical reaction, yielding a measurable and well-characterized product, and comparison of the absorption and excitation spectrum of a chromophore [9,31]. The former is not recommended because of the sensitivity of quantum counters to dye concentration, measurement geometry, polarization, and temperature and actinometers cover only a limited wavelength region. The latter procedure is rather comfortable yet not very accurate as it assumes matching absorption and excitation spectra. This is only valid for very dilute solutions of a pure compound that possesses an excitation wavelength-independent emission spectrum and quantum yield and is accordingly not suitable for compounds with rich excited-state photochemistry. Moreover, the excitation channel can be calibrated with a previously characterized emission channel and vice versa. This principally traceable procedure includes performance of a synchronous scan with a calibrated non-fluorescent white standard at sample position.

Parameters Affecting Instrument Characterization and Fluorescence Measurements

Generally, each a purpose-fit instrument characterization consists of three steps. At first, the level of uncertainty desired for fluorescence measurements and accordingly, for instrument calibration, needs to be decided on to rationalize efforts. Secondly, as is demonstrated in this section, spectral correction curves should be always determined with consideration of samples to be corrected and thus measurement conditions commonly employed, i.e., instrument settings and geometry. Thirdly, the wavelength-dependent uncertainty of spectral correction curves depends on the reliability and suitability of the transfer standard(s) and calibration procedures chosen as well as on the uncertainty of the standard's certified/reported radiometric/fluorometric quantities. For example, the calibration-relevant properties of the transfer standards have to be characterized for the chosen measurement geometry. Otherwise, the traceability chain is interrupted and reliability of the standard is not given anymore. To minimize calibration uncertainties, all these steps require knowledge and consideration of typical sources of error that are inherent to fluorescence measurements, such as nonlinearities of the detection system, effects of spectral bandpass and detector voltage as well as instrument- and sample-related polarization effects.

Linearity of the Detection System

Aside from control of the wavelength accuracy of the wavelength selecting optical components, a prerequisite for determination of emission and excitation correction curves and generally for accurate fluorescence measurements is knowledge of the linear range of the detection system(s) for common instrument settings/measurement conditions. The influence of the linearity of the detection system on the recorded signal is illustrated in Fig. 4 for

[15] The use of a fluorescent dye, quinine sulfate in 1 M H_2SO_4, as a chemical transfer standard was first proposed by Kortüm and Finckh [36].

[16] The strategy to employ a number of fluorescent dyes with different emission features as standards for the spectral range from 330–950 nm has first been applied by Lippert *et al*. [37].

[17] A pyroelectric detector measures the energy of absorbed photon with a wavelength independent responsivity (grey detector), but with a drastically reduced sensitivity and accuracy compared to, for instance, a Si photodiode.

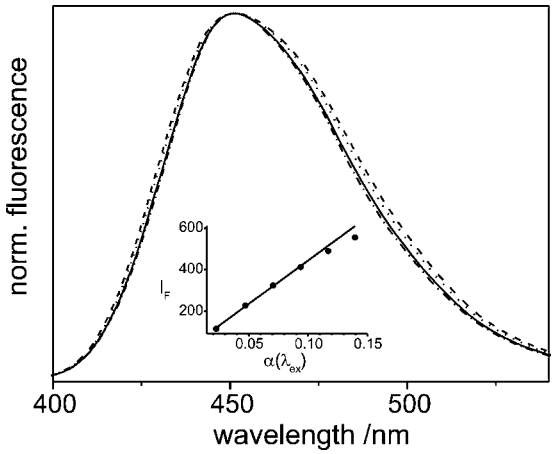

Fig. 4. Normalized uncorrected emission spectra of dye C in ethanol as a function of dye concentration—corresponding to $\alpha(\lambda_{ex}) = 1 - 10^{-A(\lambda_{ex}:378\,nm)} = 0.023$ (dashed-dotted), 0.045, 0.067 (solid), 0.088, 0.109 (dotted-dotted), and 0.129 (dashed-dashed line)—measured with fluorometer LS50B using an excitation wavelength of 378 nm and a PMT voltage of 775 V, respectively. Inset: relative fluorescence intensity at the emission maximum as a function of $\alpha(\lambda_{ex})$ of dye C.

the variation of signal intensity via dye concentration. To minimize contributions from inner filter effects, a charge transfer (CT)-type chromophore, dye C, with minimum overlap between absorption and emission bands and absorbances in the range of 0.01 up to 0.06 at the excitation wavelength is used. As follows from comparison of the corresponding uncorrected normalized emission spectra, flattening of spectra occurs at high signal intensities. This is a clear indication for the onset of saturation of the detection system. Aside from such spectral effects—depending on the selected emission wavelength—this also leads to systematic errors of measured fluorescence intensities as illustrated for the plot of the relative fluorescence intensity of dye C versus absorptance at the excitation wavelength in the inset in Fig. 4. The deviation from linearity is caused here by the nonlinear response of the detection system, see also Eq. (2).

To avoid systematic errors due to such nonlinearities, the limits of the linear range of the detection system(s) have to be determined under routine operating conditions prior to spectral calibration and fluorescence analysis. Here, particular attention has to be given to slit widths/spectral bandpass, filters, detector voltage, detection mode, sample containers as well as proper choice of procedure used. Furthermore, as the majority of fluorescence instruments reports signal ratios, i.e., only signal ratios are stored in the data files and not individual readings of the emission and reference channel, respectively, not only the linearity of the emission detection system but similarly that of the reference detection system can play a role. However, the range of linearity of the reference detection system can be determined only for high-end research fluorometers, where emission and reference detector can be addressed and read out separately. Although ratioing-type routine instruments are typically designed to reduce such effects, it is generally recommended to conduct fluorescence measurements at signal intensities/counting rates 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 avoided 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.

The range of linearity of the emission detection system of a fluorescence instrument can be measured on defined physical or chemical variation of the amount of light reaching the detector. The former typically implies control of a (standard) lamp's spectral radiance, for instance, by means of attenuators such as optical filters with certified transmission characteristics, sieve-type attenuators or, less frequent, polarizers (via polarizer settings) [40]. The latter takes advantage of the polarization-dependent transmittance of the emission monochromator. All these approaches to light attenuation introduce an additional spectral component to the lamp's emission spectrum. Whenever optical components with certified spectral characteristics—and thus a certified degree of homogeneity of the material—are not available,[18] in-house determination of the transmittance of the light-attenuating component(s) employed is required. However, this can introduce an additional uncertainty due to (potential) inhomogeneity and/or reproducible positioning of these component(s). In the case of sieve-type attenuators, the position within the emission channel can strongly influence the calibration results as diffraction is not negligible and transmittance, thus, becomes wavelength dependent. The by far most common procedure for the determination of the range of linearity of the detection system is the variation of the light intensity/spectral radiance via chromophore concentration [20] as shown in Fig. 4. Here, however, sample-related contributions to nonlinearities such as, for instance, inner filter effects, concentration-quenching or aggregation need to be avoided. Best suited for this purpose is a strongly fluorescent dye with well-separated absorption and emission bands as well as a

[18] At present, neutral density filters as certified secondary standards are only available for wavelengths above ca. 300 nm.

concentration-independent emission spectrum and fluorescence quantum yield within the concentration range used. For this approach, basically two effects have to be considered. First, the measured fluorescence intensity I is proportional to the fluorophore's absorptance and not to absorbance, see Eqs. (1)–(3). To linearize this relation, the exponential term in Eqs. (2) and (3) has to be expanded. Only for very dilute solutions, e.g. for an absorbance $A \leq 0.05$, orders higher than 1 can be neglected with an uncertainty of $\leq$5%, yielding a linear dependence of the measured fluorescence intensity on fluorophore concentration, see Eq. (4). Secondly, the nonlinearity of the I-to-A response can be affected by the geometry of the spectral bandpass/monochromator/detector ensemble [41]. To avoid systematic errors, for a dye-based approach, it is thus recommended to use the concentration dependence of both the fluorescence intensity and the shape of the normalized uncorrected emission spectrum for determination of the range of linearity of the detection system.

Effect of Spectral Bandpass

Generally, a measured fluorescence spectrum is a convolution of the pure spectrum and the bandpass function of the monochromator [42]. The influence of the spectral bandpass is illustrated in Fig. 5 for the very narrow emission bands of a neon lamp used for calibration of the wavelength scale, the comparatively broad emission spectrum of a particular type of organic fluorophore, and the extremely broad emission spectrum of a spectral radiance transfer standard. The structure or bumps observed for the latter is caused by Wood anomalies of the emission monochromator gratings of the instrument used. As to be expected, the size of effect imposed by spectral bandpass depends on the width and structure of the respective spectrum, with the strongest influence occurring for very narrow bands and structured spectra. Due to the typically extremely broad and unstructured emission spectrum of a spectral radiance transfer standard, the influence of spectral bandpass on source-based emission correction curves is commonly negligible. Contrary, for dye-based correction curves, such effects can play a role, as follows from the middle part of Fig. 5, with the actual size of the observed effects depending on the width and structure of the fluorescence bands of the spectral fluorescence standards employed, see also "Approaches to the Determination of the Spectral Characteristics of Fluorescence Instruments" section. Accordingly, for determination of dye-based spectral correction curves with minimum uncertainty, similar bandpass settings for calibration and routine measurements are recommended. Otherwise,

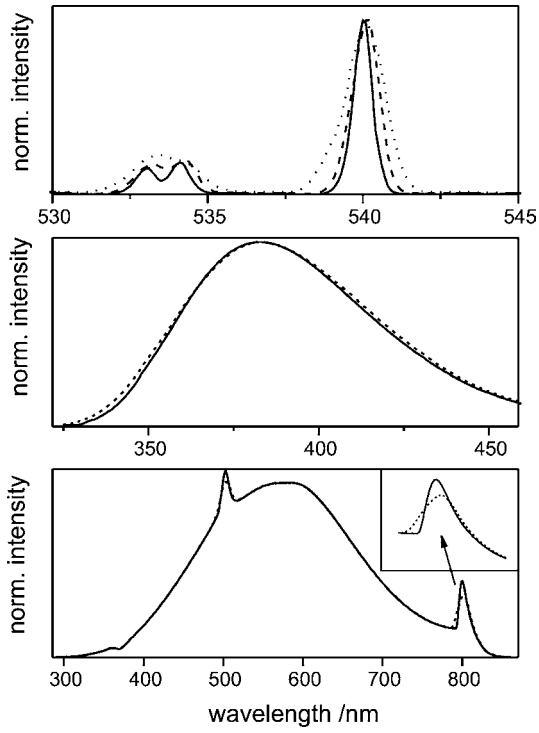

Fig. 5. Effect of spectral bandpass on the emission spectra of a narrow emitter, i.e. a pen-type lamp (top), a typical organic chromophore (middle), and an integrating sphere-type spectral radiance transfer standard (bottom). Top: fluorometer 8100, spectral bandpasses of the emission monochromator of 0.5 nm (solid), 1 nm (dashed) and 2 nm (dotted line). Middle: fluorometer LS50B, spectral bandpasses of 2.5 nm (dotted) and 15 nm (solid line). Bottom: fluorometer 8100, spectral bandpass of 1 nm (dotted) and 8 nm (solid line), no emission polarizer.

the influence of this parameter needs to be determined and accordingly included into the uncertainty budget of spectral correction.

Effect of Detector Voltage

As an example for many other parameters that can bias the uncertainty of fluorescence measurements, the influence of detector voltage is illustrated. The detector voltage affects both the spectral responsivity of the detector and its range of linearity and can introduce spectral distortions similarly to those depicted in Fig. 4. Moreover, to control the influence of this parameter, the principle of signal generation of the respective type of fluorescence instrument needs to be known. For the majority of fluorescence instruments used for routine analyses such as LS50B, the reported signal represents the ratio of the signals measured by the emission and reference detector. In such cases, either the same voltage is applied to

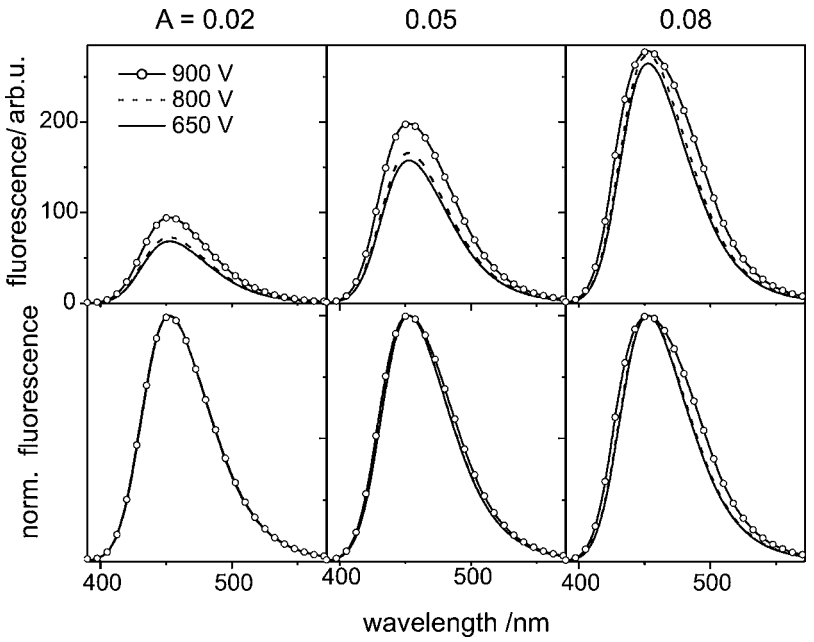

Fig. 6. Uncorrected non-normalized (top) and normalized (bottom) emission spectra of dye C in ethanol as a function of PMT voltage measured for three dye concentrations with fluorometer LS50B. $A(\lambda_{ex}) = 0.02$ (left), 0.05 (middle), 0.08 (right). Excitation was at 378 nm.

both detectors (LS50B) or only the voltage of the emission detector can be varied and the voltage at the reference detector is kept constant.

In Fig. 6, the influence of detector voltage on fluorescence measurements is examplary revealed for the uncorrected non-normalized (upper panels) and normalized (lower panels) fluorescence spectra of three solutions of dye C varying in dye concentration and fluorometer LS50B. Aside from the PMT voltage, identical conditions as for the spectra shown in Fig. 4 were employed. The rather small enhancement in signal intensity on increasing PMT voltage observed for each dye concentration, see upper panels, reflects the simultaneous increase of the voltages at both detectors. For $A = 0.08$, see left panel, the comparatively small differences in intensity for 800 and 900 V indicates the onset of saturation of the detection system at this level of dye concentration/fluorescence intensity. A comparison of the corresponding normalized emission spectra depicted in the lower panels reveals the spectral components of these effects: Matching spectra are found in the case of $A = 0.02$, slight flattening for $A = 0.05$, and considerable spectral broadening for $A = 0.08$. The actual size of these voltage effects that cannot be neglected depends on the type of detector and principle of signal generation and thus needs to be determined for each fluorescence instrument. Accordingly, to minimize uncertainties, the detector voltage should be kept identical for the determination of spectral correction curves and fluorescence measurements.

Instrument- and Sample-Related Polarization Effects

Fluorescence measurements and spectral calibration can be affected by instrument- and sample-related polarization effects [43]. The former include the degree of polarization of the spectral irradiance at sample position and the polarization-dependent responsivity of the emission channel, i.e. the ratio of its responsivities to vertically and horizontally polarized light.[19] The magnitude of sample-related polarization effects reflects the fluorescence anisotropy or (de)polarization of the sample. This is the polarization response of the sample typically measured with plane polarized excitation light [1].

Instrument-Related Polarization Effects

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 [44]. Polarization effects of detectors are typically only

[19] The viewing angle is the angle between the direction of the propagation of the exciting light and the direction from which the emission is detected.

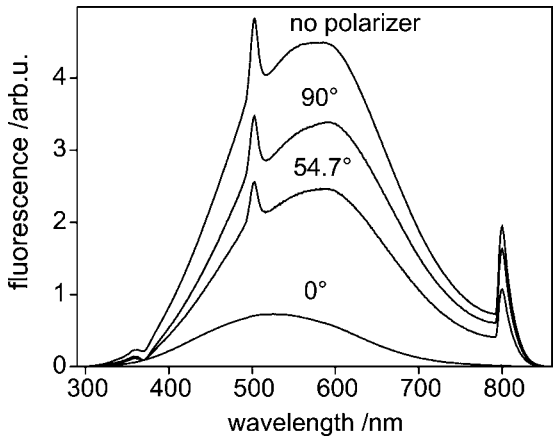

Fig. 7. Emission spectrum of a spectral radiance transfer standard emitting unpolarized light measured without and with emission polarizers at different settings, i.e. 0° (horizontal), 90° (vertical), and 54.7° ("magic angle"), respectively.

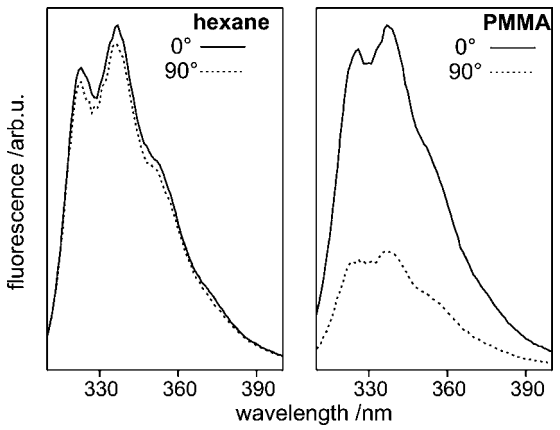

Fig. 8. Corrected emission spectra of the principally isotropic emitter *p*-terphenyl in a fluid solvent (hexane) and embedded in a cuvette-shaped polymer block (PMMA), measured with excitation polarizer at 0° and emission polarizer at 0° and 90°. For both samples, the intensity differences reflect the degree of fluorescence polarization of *p*-terphenyl in the two media.

in the range of a few percent. This has basically two consequences. The excitation light is always at least partly polarized with the degree of polarization depending on instrument. In addition, the spectral responsivity of the emission channel does not only depend on wavelength but also on polarization. The latter effect is highlighted in Fig. 7 for a spectral radiance transfer standard the emission of which was measured without and with emission polarizers set to 0° (vertical), 90° (horizontal), and 54.7°, respectively.[20] The emission of this standard lamp is not polarized. As follows from a comparison of the resulting uncorrected spectra, the settings of the emission polarizer influence not only strongly the intensity of the emission signal but, to a lesser extent, also its spectral shape, especially at wavelengths in the vicinity of the Wood anomalies. This reveals the polarization dependence of the transmittance of the emission monochromator which is strongly reduced for vertically polarized light compared to horizontally polarized light. The intensities of the emission measured at 54.7° and without polarizer reflect the respective contributions from both polarization directions. The magnitude of instrument-related polarization effects depends on the type of monochromator and gratings employed. For the instrument used here, a smooth uncorrected emission spectrum results only for vertical polarization. With emission correction curves recorded for the respective polarizer settings, instrument-related polarization effects can be eliminated.

Sample-Related Polarization Effects

Due to photoselection, only chromophores with transition dipole moments parallel to the polarization direction of the incident light absorb light. The anisotropy of the subsequently emitted light depends on the extent to which the excited chromophores rotate during their excited-state lifetime. Isotropic emission commonly requires small molecules with a comparatively long excited-state lifetime and a short rotational correlation time [1],[21] i.e., a fast rotational diffusion of the molecules during their excited-state lifetime [45]. This is supported by non-viscous fluid solvents that do not undergo specific solvent–solute interactions with the dissolved chromophores. Nearly isotropic emission can be also observed for luminescent metal ions embedded into solid matrices. Sample-related polarization effects, i.e. a significant anisotropy, typically occur for large chromophores with short fluorescence lifetimes such as, for instance, many NIR dyes, fluorescent macromolecules as well as organic dyes or luminescent metal complexes in a confined environment or viscous solvents.

Figure 8 illustrates sample-related polarization effects examplary for the corrected emission spectra of the principally nearly isotropic emitter *p*-terphenyl dissolved in the fluid solvent hexane (left) embedded into a

[20] An angle of 0° implies vertical, e.g. parallel to the grating groves, 90° horizontal, e.g. normal to the plane of the excitation and emission direction, respectively, and 54.7° equals the magic angle where similar contributions of horizontally and vertically polarized light are obtained for unpolarized light.

[21] The rotation correlation time that describes the rotational rate of a molecule depends on viscosity, molecular volume, and temperature.

cuvette-shaped PMMA block (right). The latter converts this dye into an anisotropic emitter. Both experiment were performed with the excitation polarizer set to 0° and emission polarizer settings of 0° and 90°, respectively. For the unobstructed dye, i.e., *p*-terphenyl in hexane, the intensities of the corrected emission spectra are very similar for both polarizer settings, whereas for the dye in a confined environment, these intensities differ significantly. These differences in intensity basically reveal the fluorescence anisotropy *r* of *p*-terphenyl in hexane and PMMA, respectively, with the increase in *r* from 0.026 to 0.38, reflecting the transition from "nearly isotropic emission" to "almost perfectly anisotropic emission." The emission spectra that would result without use of an emission polarizer can be calculated from the sum of the emission spectra obtained for 0° and 90°, divided by two. Consideration of the latter is a striking evidence for the carefulness that has to be devoted to the choice of adequate measurement conditions for samples other than dilute fluid solutions of small chromophores. Analogously, in the case of corrected excitation spectra, defined polarizer settings are mandatory for the respective measurements with non-isotropically emitting samples and accordingly, determination of suited correction curves.

When deciding on a suitable fluorescence standard for instrument calibration, to minimize uncertainties of instrument characterization, users of fluorescence techniques also need to be aware of polarization-related effects. Generally, if no polarizers are to be employed for measurement of anisotropic emitters, the measurement uncertainty can considerably increase, with the size of such systematic errors depending on the sensitivity of the respective fluorescence instrument towards polarization effects. Typical (intensity and spectral) errors can be in the range of ca. 20% [9].

Recommendations for Purpose-Fit Spectral Correction with Minimized Uncertainty

As has been illustrated in this section, aside from control of the wavelength accuracy of the wavelength selecting optical components and consideration of the (upper limit of the) linearity of the detection system(s), purpose-fit characterization of the spectral characteristics of fluorescence instruments and performance validation should be carried out with commonly used, i.e., routine instrumental parameters and measurement conditions to minimize calibration and accordingly measurement uncertainties. This refers especially to choice of polarizer settings, detector voltage, comparable signal intensities or counting rates, and spectral bandpasses as well as measurement geometries. To rationalize calibration efforts, generation of universal correction curves for selected sets of measurement conditions is sometimes desirable. In this case, influences of the varied parameters within a set have to be determined and accordingly included into the wavelength-dependent uncertainty budget of the respective spectral correction curves. However, for the use of polarizers, correction curves for each polarizer setting are mandatory. Here, spectral correction curves that represent normalized and not relative spectral irradiances and spectral responsivities are to be avoided as the former still enable spectral correction but lead to loss of information on fluorescence (de)polarization. Furthermore, whenever measurements and calibration experiments are conducted without polarizers, careful consideration of the fluorescence anisotropy of the standard and the sample is essential prior to application of a correction step.

Approaches to the Determination of the Spectral Characteristics of Fluorescence Instruments

Physical vs. Chemical Transfer Standards

Execution of instrument characterization under identical conditions as employed for measurements of fluorescent samples imposes strong restrictions on transfer standards. Classical physical transfer standards[22] like calibrated standard lamps and detectors [8]—although traceable, typically certified by National Metrological Institutes (NMIs), and not restricted to photoluminescence measuring systems only [46]—require a certain background in optics for proper use, can be tedious to operate, and need regular and expensive recalibrations. Aging effects also can be critical, e.g. changes in the wavelength-dependent spectral radiance of standard lamps with burning time [47], or deviations from certified values due to improper maintenance or frequent transport. The latter issues render regular control of the standard's certified radiometric quantity a prerequisite for accurate calibration. Furthermore, classical radiometric standards often impose restrictions on measurement geometry, presumably because they are not specifically designed for luminescence and fluorescence applications and often do not fit into compact fluorescence instruments.

For source-based instrument calibrations, major sources of error originate from the strongly different emission characteristics and spectral radiances of standard

[22] Here, it is distinguished between classical physical transfer standards such as standard lamps and detectors and physical standards that are designed for specific applications in fluorometry like for instance the GLOWELL standards for the calibration of luminometers, fluorometers, and CCD systems, see www.luxbiotech.com. The latter are beyond the scope of this article.

lamps and luminescent samples.[23] As a rule of thumb, the spectral radiances of a tungsten strip lamp and an integrating sphere-type radiator exceed that of a fluorescent sample by ca. six and three orders of magnitude, respectively. In the worst case, emission correction curves are obtained that are not suited for correction of the spectra of fluorescent samples. To avoid systematic errors due to nonlinearities of the detection system, taking into account typical linear ranges of four orders of magnitude, this intensity mismatch requires controlled attenuation of the standard lamp. However, as has been detailed in "Parameters Affecting Instrument Characterization and Fluorescene Measurement" section and Figs. 5–7, application of conventional attenuation procedures as well as employment of different measurement parameters like e.g. spectral bandpasses and detector voltages for standard lamp and fluorescent samples harbor various potential errors and introduce additional uncertainties.

A detector-based calibration is a frequent approach for the traceable characterization of the excitation channel, but less common for the determination of emission correction curves. However, this approach is tempting due to the smaller calibration uncertainty of spectral responsivity transfer standards compared to that achievable, for instance, for integrating sphere-type spectral radiance transfer standards [8]. A detector-based emission correction relies on the previously characterized spectral irradiance at sample position (excitation correction) and a synchronous scan of excitation and emission channel with a non-fluorescent white standard at sample position. Aside from being tedious, major sources of error can originate here from a non-synchronized behavior of emission and excitation monochromators. Additionally, its accuracy relies on the reproducibility of the position of the white standard. For instruments equipped with a reference channel, changes of the spectral radiance of the excitation light source in between calibration and measurements can be accounted for by the reference channel as is described in "Development of Sets of Emission and Excitation Standards" section. With proper consideration of these influences, this approach also leads to a traceable instrument characterization.

Moreover, independent of the type of sample measured and method used, for proper quality control, suitability and accuracy of source- or detector-based correction curves should be generally controlled prior to use. This is also strongly recommended for emission and/or excitation correction curves implemented into fluorescence instruments by the instrument's manufacturers that are commonly obtained with classical physical transfer standards. Such control, however, is critically linked to the availability of well-characterized and, ideally certified emission and excitation standards that closely match typically measured samples.

Generally, application of physical transfer standards for instrument characterization and determination of emission and excitation correction curves is appropriate for NMIs and—with restrictions—for instrument manufacturers. However, it is not recommended for the broad community of users of fluorescence techniques. To improve quality assurance in fluorometry and comparability of fluorescence data on a broad level, simple, yet traceable approaches need to be established. This can be easiest realized with emission and excitation standards covering the UV/Vis/NIR spectral region. Such spectral fluorescence standards with certified corrected emission and excitation spectra that are, however, restricted to the characterization of photoluminescence measuring systems are by far more comfortable to operate and closer match commonly analyzed samples. If properly designed, these principally traceable chemical-type standards can allow for an instrument calibration under identical conditions, e.g. measurement geometry, instrument settings, sample containers/formate, comparable signal intensities or counting rates as used for routine analysis. Thus, sources of systematic error such as, for instance, different measurement geometries or emission characteristics and spectral radiances of standard and fluorescent samples can be elegantly avoided. Mimicking of the illuminated and detected volume of a sample by a standard is automatically realized, and, if required, the emitted intensity can be to a certain extent controlled by variation of dye concentration, at least for liquid standards.

Need for and Requirements on Emission and Excitation Standards

To broadly establish spectral correction and comparability of fluorescence data, commercially available, easy-to-operate and ideally traceable sets of emission standards for the spectral region of ca. 250–900 nm and sets of excitation standards for the spectral region of ca. 220–850 nm are desired. Requirements on suited candidates, that can be either of liquid type, i.e., a very dilute solution of a chromophore, or a solid consisting of a chromophore incorporated into a polymer or glass, have been frequently discussed. Basically, there are two types of categories: photophysical and photochemical properties and ease of use [9,11,37,38].

Standards suited for spectral correction must have broad and unstructured emission and/or excitation spectra

[23] In this context, spectral radiance equals emission intensity.

with no vibronic structure to avoid a strong dependence of the spectral shape of the fluorescence spectrum and the corresponding correction curve on instrument resolution/ spectral bandpass, see also Fig. 5. Furthermore, for the use of emission and excitation standards with structured spectra, also uncertainties in the determination of the wavelength lead to more severe uncertainties in fluorescence intensity compared to standards with broad and unstructured spectra. The need to fulfill this essential requirement on spectral shape is illustrated in Fig. 9 by comparison of the corrected emission spectrum of dye KR2 obtained with two potential emission standards, i.e., dye FM1 with a structured and dye D with a broad and unstructured emission spectrum, respectively. The corrected emission spectra of dyes FM1 and D as obtained with fluorometer 8100 and a source-based correction curve and the corresponding uncorrected emission spectra measured with LS50B using two different spectral bandpasses are depicted in the upper part of Fig. 9. The quotients of the corrected and uncorrected spectra, i.e., the emission correction curves shown in the middle part of the figure, are structured for dye FM1 and show a strong dependence on spectral bandpass. In contrast, dye D yields unstructured correction curves with a comparatively small dependence on spectral bandpass. Accordingly, application of FM2-based correction curves to the uncorrected spectra of dye KR2 results in corrected spectra that considerably deviate from the emission spectrum obtained with a source-based correction curve in both shape and spectral position of the emission maximum, especially for a spectral bandpass of 15 nm, see lower part of Fig. 9. Contrary, only a small broadening of the respective emission spectra occurs for dye D-based correction due to the different monochromator settings used.

Other requirements on spectral fluorescence standards are little overlap between absorption and emission to circumvent inner filter effects, and moderate to strong fluorescence quantum yields to increase the signal-to-noise ratio and to minimize the influence of stray light, solvent emission, and fluorescent impurities on the shape of the standard's fluorescence spectrum. Within the spectral region employed for spectral correction, the shape of the emission spectra of the standards should be independent of concentration and excitation wavelength and the excitation line shape of excitation standards should be independent of emission wavelength, respectively. This implies that the fluorescence quantum yields of the standards are independent of excitation wavelength and concentration within this spectral region which accordingly needs to be reported. The concentration dependence of excitation spectra is detailed in "Development of Sets of Emission and Excitation Standards" section. The fluorescence anisotropy should be small, e.g. $r \leq 0.05$ within the analytically relevant room temperature region of ca. 20–30°C, yielding a virtually isotropic fluorescence to avoid additional polarization effects. Only application of such standards guarantees negligible additional uncertainties under measurement conditions that can dispense with or for instruments that lack polarizers.[24] In the UV/Vis spectral region, the fulfillment of this requirement that also imposes strong restrictions on suited solvents for liquid standards is not problematic. However, in the NIR spectral region, this criterion can be difficult to meet. Here, realistically, slightly higher anisotropies of e.g. $r \leq 0.10$ might

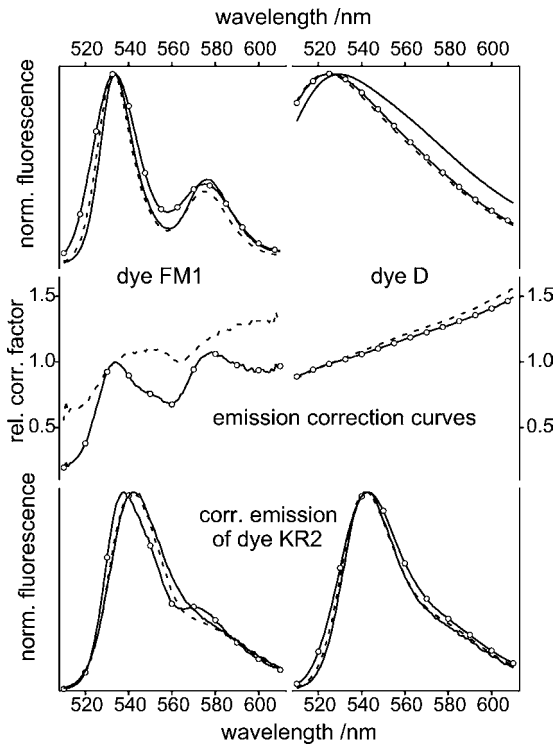

Fig. 9. Comparison of the corrected emission spectra of dye KR2 obtained with two potential emission standards, dye FM1 with a structured and dye D with a broad and unstructured emission spectrum, respectively. Top: corrected emission spectra I_c of dyes FM1 and D (solid lines) as obtained with fluorometer 8100 (spectral bandpass 1 nm) and a source-based correction curve, and the corresponding uncorrected emission spectra I_u measured with fluorometer LS50B and spectral bandpasses in emission of 5 nm (dashed lines) and 15 nm (circles). Middle: emission correction curves obtained upon division of I_c by the corresponding I_u. Bottom: I_c of dye KR2 obtained with fluorometer 8100 and a source-based correction curve (solid line) and the corresponding I_c obtained with fluorometer LS50B and the dye-based emission correction curves shown in the middle section.

[24] Exceptions that are beyond the scope of this article are here the spectral correction of fluorescence spectra of anisotropically emitting samples measured without polarizers.

have to be accepted and accordingly, a small increase in uncertainty for instrument characterization without polarizers. The temperature dependence of the shape of the fluorescence spectra in the temperature region of ca. 20–30°C should be ideally negligible or at least very small and accordingly reported. In the latter case, temperature control is recommended or temperature-related fluorescence effects have to be included into the uncertainty budget of spectral correction. Also, fluorescence quenching by oxygen should be small. A sufficient thermal and photochemical stability is mandatory and formation of fluorescent photoproducts is to be avoided. For the design of standard sets from components that all have to meet these requirements, the fluorescence spectra of spectrally neighboring chromophores must cross at points of sufficient fluorescence intensity, e.g. at least at 20% of the relative maximum fluorescence intensity.

Criteria for the Choice of Emission and Excitation Standards

Similar to other reference materials, the value of a spectral fluorescence standard depends on its suitability and reliability. To minimize standard-related uncertainties for spectral correction of fluorescence data, that can easily reach values of $\geq$50%, there are three types of categories of criteria to be considered.

First, the previously discussed requirements should be fulfilled. Secondly, the reliability of a fluorescence standard is determined by the characterization of its calibration-relevant properties, the stability under application-relevant conditions, the uncertainty of the certified/characterized quantity, and the supply of additional information relevant for proper application. This also includes a tested standard operation procedure. To assure the accuracy of the standard's reported fluorescence properties such as corrected emission or excitation spectra, these properties have to be measured with a reliably and traceably characterized fluorescence instrument. To enable evaluation of the latter, the procedure used for instrument calibration including instrument settings and calibration uncertainty should be provided. Additionally, to minimize standard-related uncertainties, e.g. characterization of the wavelength, temperature, and concentration dependence of the application relevant fluorescence properties, fluorescence anisotropy, and thermal and photochemical stability is desired as well as information on storage conditions, scope, and limitations for use. Furthermore, for chromophore-based fluorescence standards, the compound's purity should be reported including method of analysis and respective uncertainty as dye purity can affect its spectroscopic properties, photochemical and thermal stability and—via variation in impurity content for instance on a batch-to-batch basis—reproducibility. Thus, it needs to be eventually considered for determination of the uncertainty of the certified fluorescence property. Furthermore, due to the sensitivity of fluorescence to microenvironment, for liquid standards, the solvent to be employed needs to be specified with respect to properties that can potentially lead to artifacts such as for instance pH for aqueous solutions [7] or water content in the case of polar hygroscopic solvents. An alternative is provision of solvent of known and reported quality with the standard. Solid standards require additional characterization of the homogeneity of the dye's distribution in the matrix to guarantee a uniform fluorescence. The third criterion holds only for sets of fluorescence standards. Here, tested procedures for spectra/curve linking for generation of an overall spectral correction curve from measured uncorrected and supplied corrected spectra of the set components are mandatory, preferably as a software. In addition, procedures for the determination of the fluorescence standard-based calibration uncertainty are eventually desired.

To the best of our knowledge, at present, the first two criteria are only met by the liquid emission standard quinine sulfate dihydrate [22]. The development of this reference material included not only construction and calibration of a reference fluorometer for the characterization and according certification of the dye's normalized corrected emission spectrum but also a detailed study of its spectroscopic behavior, including temperature dependence and fluorescence anisotropy, thermal and photochemical stability as well as purity and homogeneity. Moreover, the wavelength-dependent uncertainty of the respective fluorescence quantity has been reported. However, this standard can be used only for spectral correction in the region of *ca*. 395–565 nm [26,31]. All the other commercially available spectral fluorescence standards[2,3,4] and the huge majority of potential fluorescence standards described [1,9,11,22,24] do not meet these criteria. Typical standard-inherent sources of systematic error are here the now and then questionable determination of the standard's fluorescence spectra, sample-related polarization effects, insufficient stability (irreproducible) presence of unknown fluorescence impurities, an inhomogeneous chromophore distribution for solid systems as well as use of unsuited standard combinations and linking procedures. This strongly limits the otherwise straightforward determination of fluorescence standard-based spectral correction curves at present and introduces unnecessarily high uncertainties. This situation is been currently overcome by the development of sets of emission and excitation standards at BAM that meet all of the aforementioned criteria.

Development of Sets of Emission and Excitation Standards

Traceable Procedures for Spectrofluorometer Characterization

Aside from the challenging combination of suited emission and excitation standards to standard sets, the desired small uncertainty of fluorescence standard-based calibrations requires minimization of the uncertainty of the characterization of the standard's fluorescence properties and accordingly, of the spectrofluorometer calibration. At BAM, this is eventually met by the design of an optimized reference instrument. Furthermore, it implies development of traceable and very accurate procedures for instrument characterization. For characterization of the emission and excitation standards presented here as well as for certification, we used a modified spectrofluorometer 8100 from Spectronics Instruments. To make the illuminated volume of measured dye solutions independent of excitation and emission wavelength, the conventionally used condensor lenses are replaced by custom-designed achromats to minimize achromatic aberrations. The reference channel is equipped with a custom-designed Teflon scatterer and a variable aperture between beam splitter and scatterer. The latter enables controlled attenuation of the reference signal thereby avoiding nonlinearity problems of the reference detector that can be adjusted in its spectral responsivity and read out separately from the emission detection system. Instrument characterization included control of the wavelength accuracy of the excitation and emission monochromators and the linear range of the emission and reference detection systems as well as determination of the relative spectral responsivity and relative spectral irradiance of the instrument's emission and excitation channel and the overall calibration uncertainty. For the former, at first, the wavelength accuracy of the two emission monochromators is controlled within the range of 250–810 nm employing several emission lines of a neon and a mercury lamp placed at sample position. In a second step, the wavelength accuracy of the excitation monochromator is checked at several wavelength (intervals) within this spectral range with the previously calibrated UV/Vis emission channel and a Teflon scatterer at sample position. The upper limit of linearity of the detection systems is determined by measuring the ratio of the (unpolarized) light/spectral radiance scattered from a non-fluorescent white standard at sample position upon illumination with the fluorometer's excitation light source at 400 nm at two different emission polarizer settings, e.g. 0° and 90°, as a function of increasing spectral irradiance with the emission monochromator set to 400 nm and the spectral irradiance varied via neutral density filters (C. Monte, private communication), see Fig. 10. Within the linear range of the detection system, the ratio of the two recorded intensities should be constant. The significant deviation from a constant value occurring at counting rates of $\geq$10,000 s^{-1} indicates the upper limit of the detector's linearity.

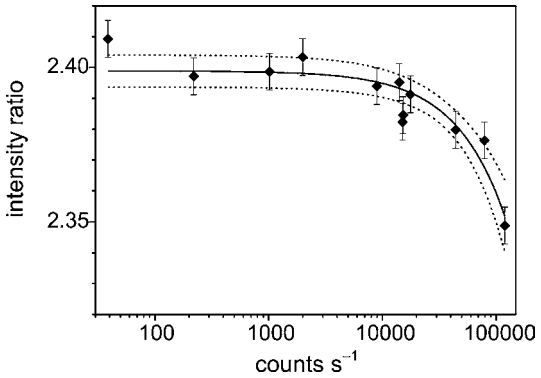

Fig. 10. Ratio of the spectral radiance scattered from a non-fluorescent white standard at sample position measured at two different emission polarizer settings, here 0° and 90°, as a function of increasing spectral irradiance at 400 nm. The significant deviation from a constant value at counting rates $\geq$10,000 s^{-1} indicates the upper limit of the linearity of the emission detection system.

To minimize uncertainties of corrected emission spectra, we perform the source-based calibration of the two emission channels not only at similar instrument settings as used for measurement of dye solutions but also at spectral radiances comparable to those emitted by typical fluorescent samples. This requires strong reduction of the spectral radiance of the integrating sphere-type spectral radiance transfer standard without affecting its emission characteristics and without interrupting the traceability chain. Thus, we exploited the quadratic distance dependence of diffuse illumination. Accordingly, a non-fluorescent white standard, the reflectance of which is calibrated for 0°/45° measurement geometry, is placed at sample position normal to the direction of detection of the emission monochromator and illuminated with the standard lamp at an angle of 45°. The standard lamp is mounted on an optical bench attached to the fluorometer's sample compartment at an angle of 45°. With this set up, the spectral irradiance at the white standard and thus the spectral radiance reaching the emission detection system can be controlled simply by variation of the distance between lamp and white standard. To avoid additional contributions from nonlinearities of the detection system to the overall calibration and measurement uncertainty, photon counting rates well below the upper limit of linearity of the detection system are used.

For determination of corrected excitation spectra, in a first step, the wavelength- and polarization-dependent

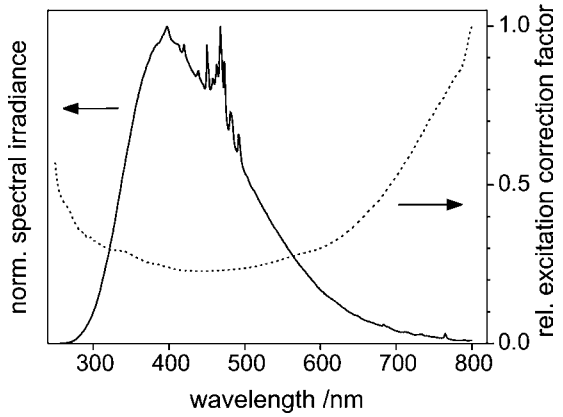

Fig. 11. Relative spectral irradiance at sample position $E_{ex}(\lambda_{ex})$ of the excitation channel of fluorometer 8100 (solid) and the corresponding excitation correction curve $F(\lambda_{ex})$ (dotted line). $E_{ex}(\lambda_{ex})$ is determined with a PTB-calibrated Si photodiode of known spectral responsivity. $F(\lambda_{ex})$ is calculated from $E_{ex}(\lambda_{ex})$ and the corresponding signal of the reference channel, recorded simultaneously during calibration.

relative spectral irradiance of the excitation channel of fluorometer 8100 shown in Fig. 11 is measured with a PTB-calibrated Si photodiode integrating sphere assembly at sample position. Fluctuations of the spectral radiance of the excitation light source during instrument characterization that would otherwise affect the correction of excitation spectra are taken into account via the reference detector. The latter measures the wavelength- and polarization-dependent relative spectral irradiance of the reference channel, $E_{ref,\lambda}(\lambda_{ex})$ which is omitted in Fig. 11 for better clarity. The excitation correction curve depicted in Fig. 11 accordingly equals the quotient of the relative spectral irradiances of the excitation channel reaching the sample, $E_{ex,\lambda}(\lambda_{ex})$, and the reference channel, $E_{ref,\lambda}(\lambda_{ex})$ at the time of instrument calibration. During an excitation scan with a sample, the emission detector measures the respective uncorrected excitation spectrum $I_u(\lambda_{ex},\lambda_{em})$ and the reference detector records fluctuations of the spectral radiance of the excitation light source at the time of measurement, i.e., $E'_{ref,\lambda}(\lambda_{ex})$. Corrected excitation spectra $I_c(\lambda_{ex},\lambda_{em})$ are then calculated by division of the latter quotient by the excitation correction curve obtained under identical measurement conditions as illustrated in Eq. (5). This straightforward procedure assumes that the spectral characteristics of the reference channel remain constant in between calibration and measurement, and operation of the reference detector in the linear range.

$$I_c(\lambda_{ex}, \lambda_{em}) = \frac{I_u(\lambda_{ex}, \lambda_{em})}{E'_{ref,\lambda}(\lambda_{ex})} \times \frac{E_{ref,\lambda}(\lambda_{ex})}{E_{ex,\lambda}(\lambda_{ex})} \qquad (5)$$

Set of Traceable Emission Standards

The second step to liquid emission standards covering a broad spectral region involves combination of suited chromophores to a set, determination of the wavelength-dependent uncertainty of the corrected emission spectra of the set components, and development of a linking procedure of the correction curves obtained for the individual standards to a global emission correction curve. A set of traceable and isotropically emitting emission standards that will be soon certified and released by BAM is shown in Fig. 12 [48]. This set consists of dyes A, B, C, D, and E, each dissolved in ethanol, and covers the spectral range from ca. 310–730 nm. The combined, wavelength-dependent uncertainties (95% confidence interval) of the corrected emission spectra of these dyes are illustrated in the middle part of Fig. 12. These uncertainties result from uncertainties of the respective fluorescence measurements (standard deviation of a sequence of measurements) with the set components and the overall uncertainty of the wavelength- and polarization-dependent relative spectral responsivity of the emission channel. The latter includes the respective calibration uncertainties of the physical transfer standards used for fluorometer characterization, e.g. the spectral radiance transfer standard and the white standard, the nonlinearity of the detection system, the wavelength deviation of the emission monochromator, the reproducibility of positioning of the emission polarizer, and the uncertainty of the measurement of the standard lamp's emission spectrum. The latter is again determined from the standard deviation of a sequence of measurements. Also governed by the uncertainty of the measurement of the standard lamp's emission spectrum are the reproducibility of positioning of the white standard and the detection statistics. Contribution of each dye to the overall wavelength-dependent uncertainty of the set shown in the lower part of Fig. 12 is obtained from the former by using contributions of each dye only within the spectral region defined by the crossing points (cf. dotted vertical lines in Fig. 12) of the emission spectra of spectrally neighboring set components. With this procedure, only regions of considerably high fluorescence intensity of the dye spectra are considered, thereby minimizing contributions of each dye to the combined, wavelength-dependent uncertainty of emission correction. The uncertainty at the crossing points is calculated from the relative contributions of the uncertainties of two neighboring dyes.

The main contribution to the overall uncertainty of the emission correction curve originates from the calibration uncertainty of the spectral radiance transfer standard as follows from Part I of this series [8]. Currently, a new calibration facility has been successfully tested at

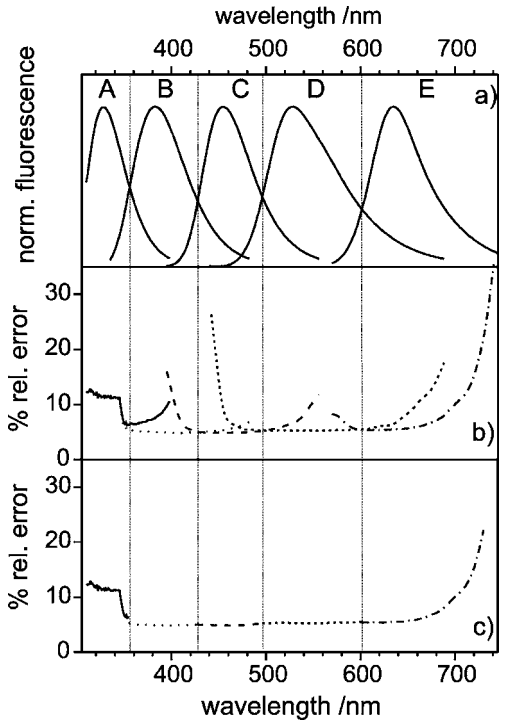

Fig. 12. Normalized corrected emission spectra I_c including wavelength-dependent uncertainty (95% confidence interval) of a set of five liquid emission standards. Top: normalized I_c of dyes A–E. Middle: corresponding wavelength-dependent uncertainties (95% confidence interval). Bottom: combined uncertainty contributing to the dye-based global emission correction curve.

PTB to further decrease the overall calibration uncertainty (95% confidence interval) from currently maximum 10–4% in the UV region. With this only recently reached calibration uncertainty of the standard lamp, the wavelength-dependent uncertainty of the corrected emission spectra of the set components depicted in Fig. 12 can be most likely reduced to maximum 10% in the critical UV region that is relevant for dyes A and B. These optimized data will then be used for certification of the set's corrected emission spectra.

Aside from our set of isotropically emitting emission standards, up to now there have been only five examples for combinations of emission standards reported so far [10,37,38,49].[2,25] However, the sets of liquid standards available from Molecular Probes[2] and presented by Velapoldi and Tonnesen [10] both contain fluorophores like e.g. fluorescein and one or two rhodamine dyes with comparatively small and structured emission spectra that do not meet the previously discussed requirements on this type of spectral fluorescence standards. Moreover, use of different solvents for set components can easily lead to systematic errors. Lippert's early introduced set also contains a structured emitter, β-naphthol, and various chromophores with only moderate to rather low fluorescence yields such as 4-dimethylamino-4′-nitrostilbene or *m*-dimethylamino-nitrobenzene [37]. SRM 1931, a set of four phosphors mixed with a polytetrafluorethylene resin, not available any more, imposes severe restrictions on measurement geometry, e.g. performance of front surface fluorescence measurements, and requires the use of polarizers [49]. The set of liquid standards developed by Gardecki and Maroncelli [38] includes dyes of insufficient photostability such as tryptophan and coumarin 102, α-NPO with a structured emission spectrum, and LDS 751 with a fluorescence anisotropy $\geq$5%. Also, the purity of the dyes is not reported and different solvents for the set components are required. Furthermore, with the exception of Gardecki's approach [38], there are no procedures provided for generation of an overall correction curve from individual correction curves obtained for each standard.

Calculation of Spectral Correction Curves

Broad establishment of spectral correction requires not only suited and commercially available sets of spectral fluorescence standards including standard operation procedures for use but also a procedure or preferably a software to generate an overall spectral correction curve from measured and corrected fluorescence spectra of the set components. A procedure that has been successfully tested by BAM for different fluorescence measuring systems is illustrated in Fig. 13 for the BAM emission standards introduced in Fig. 12. The normalized corrected emission spectra $I_c^X(\lambda_{em})$ of the set components (X), e.g. dyes A, B, C, D, and E that will be certified by BAM, and the corresponding uncorrected emission spectra $I_u^X(\lambda_{em})$ measured with the fluorometer to be calibrated, here fluorometer LS50B, are shown in the lower part of Fig. 13. The individual correction curves $F^X(\lambda)$ that are calculated for each set component by division of its background-subtracted, uncorrected spectrum $I_u^X(\lambda_{em})$ by the corresponding certified standard spectrum, are depicted in the upper part of Fig. 13. The global correction curve $F(\lambda)$, which represents the wavelength- and polarization-dependent relative spectral responsivity of the emission channel, is obtained from $F^X(\lambda)$ by a linking procedure developed by us based on the method of Gardecki and Maroncelli [38,50]. Corrected emission spectra are accordingly obtained by division of measured data by $F(\lambda)$.

With this set of emission standards and our software, up to now, emission correction curves have been

[25] The emission and excitation standards from Starna GmbH are not designed as a set of standards to completely cover a very broad spectral region.

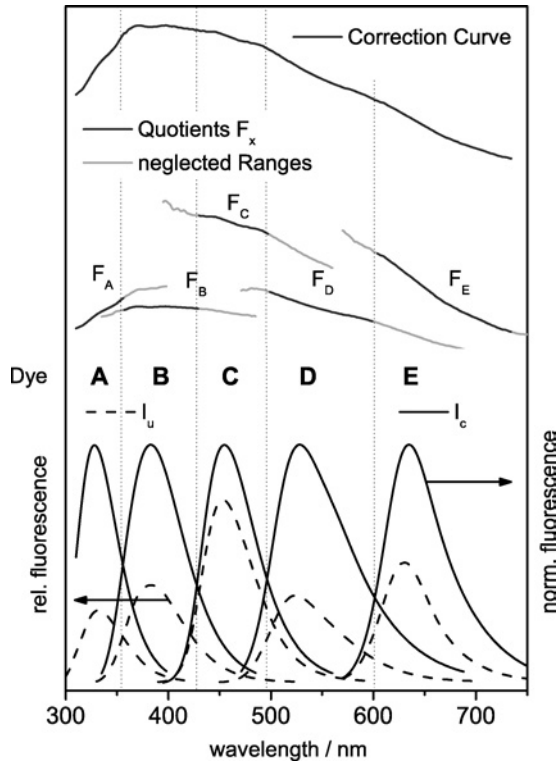

Fig. 13. Determination of the emission correction curve with a set of emission standards. Bottom: uncorrected emission spectra $I_u^X(\lambda)$ (dashed lines) measured with the fluorescence instrument to be calibrated, here LS50B, and corresponding normalized corrected emission spectra $I_c^X(\lambda)$ (solid lines) of the set components (X = dyes A–E). Middle: individual emission correction curves $F^X(\lambda)$ of dyes A–E equaling $I_u^X(\lambda)$ divided by $I_c^X(\lambda)$. Top: overall emission correction curve $F(\lambda)$ obtained from $F^X(\lambda)$ by a linking procedure developed by BAM based on the approach of Gardecki. $F(\lambda)$ equals the relative spectral responsivity of the instrument's emission channel. Division of measured data by $F(\lambda)$ accordingly yields corrected emission spectra.

successfully determined for several types of common fluorescence instruments like spectrofluorometers and a microplate reader. This approach is been currently tested for laser setups and fluorescence microscopes. Moreover, this procedure that can be adapted to different formats and measurement geometries is not only simple, fast, and traceable but also presents an elegant way of obtaining the wavelength-dependent uncertainty budget of fluorescence measurements. Aside from the data provided by us that enable calculation of the uncertainty of the dye-based correction curve, this requires consideration of the measurement uncertainty of the respective fluorescence instrument by the user/operator via the standard deviation of a sequence of fluorescence measurements with these dyes. Here, seven replicates per dye are recommended. To extend this elegant calibration procedure up to ca. 950 nm, ongoing research activities at BAM are dedicated to testing of one or two additional set components already identified. Furthermore, based on the reduced calibration uncertainty of the spectral radiance transfer standard realized presently by PTB, the uncertainty of the corrected emission spectra of the set will be reduced to maximum 10% (95% confidence interval) before release. With the reference fluorometer that will be presumably available by the end of 2005, we aim at a further reduction of this uncertainty to 2%.

Traceability of Emission Measurements

As already discussed, traceable characterization of the spectral features of fluorescence instruments can be established via measurements of relative spectral responsivities and irradiances. However, as is exemplary illustrated for emission correction, the radiometric quantity to which traceability is established, i.e., spectral radiance $L(\lambda)$ or spectral photon radiance $L_p(\lambda)$, plays a non-negligible role for comparability of corrected emission spectra and eventually also for fluorescence quantum yields. The influence of the reference quantity which has been debated for many years [51,52], is depicted in Fig. 14 for three dyes, i.e., PPD, quinine sulfate dihydrate, and dye G. As follows from a comparison of the corrected emission spectra referenced to spectral radiance and spectral photon radiance, the size of the respective spectral (bottom) and intensity (top) differences depend on both the spectral position of the dye's emission maximum and the width of its emission band. The strongest spectral deviations that are, however, small compared to the intensity effects, occur in the UV region whereas the effect of the respective reference quantity on the resulting intensity of the corrected emission spectrum and thus fluorescence quantum yield increases with decreasing photon energy. Moreover—aside from affecting the comparability of corrected emission spectra and fluorescence quantum yields—the not yet defined reference quantity of emission also renders the use of typically dimensionless emission correction curves problematic that were implemented into fluorescence instruments by the manufacturers. Accordingly, the radiometric reference quantity used for determination of corrected emission spectra and spectral correction curves has to be documented in the certificate of emission standards and instrument manuals.

As follows from Fig. 14, for determination of fluorescence quantum yields the radiometric reference quantity of emission measurements needs to be considered in any case. As the fluorescence quantum yield is defined as the ratio of the number of emitted photons to the number of absorbed photons, use of an emission correction referring to spectral radiance requires consideration of the

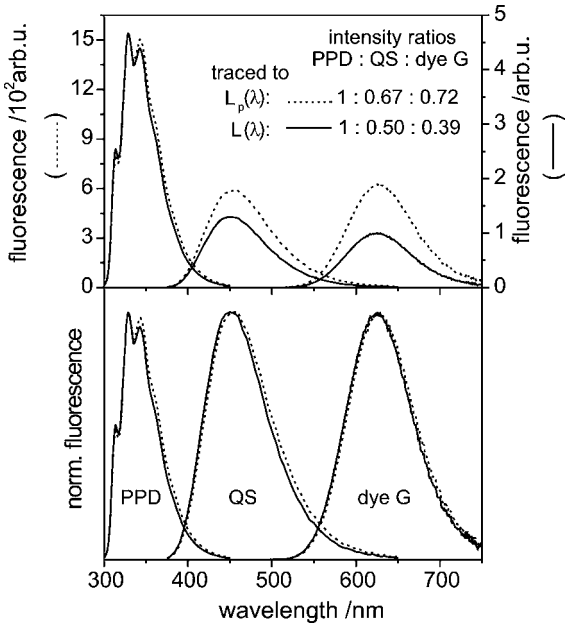

Fig. 14. Comparison of the relative (top) and normalized (bottom) corrected emission spectra of the three dyes PPD, quinine sulfate (QS), and dye G, referenced to spectral radiance $L(\lambda)$ (solid, right axis) and spectral photon radiance $L_p(\lambda)$ (dotted line, left axis), respectively. The ratios given refer to integral fluorescence intensities.

photonic nature of the emitted light by division of the accordingly corrected emission spectrum by photon energy. Otherwise, as is depicted in the upper part of Fig. 14 and the given intensity ratios, the fluorescence quantum yields of chromophores emitting in the Vis and especially in the NIR region are underestimated compared to those of UV dyes, e.g. for two solutions having actually the same fluorescence quantum yield, the one with the longer wavelength emission gives a lower value.

In our opinion, an internationally accepted agreement on the reference quantity of emission spectra by NMIs, standardization bodies like the American Society for Testing of Materials (ASTM International), relevant scientific associations such as, for instance, the International Union of Pure and Applied Spectroscopy (IUPAC), and instrument manufacturers is needed. Here, it needs to be kept in mind that the reference quantity spectral radiance is used by the majority of fluorescence spectroscopists as well as in closely related colorimetry. Moreover, most corrected emission spectra reported [34,38] or certified [26] are referenced to this quantity. However, with the use of the reference quantity spectral photon radiance that takes the quantum nature of the emitted photons into account, the integrated area under the corrected emission spectrum is proportional to fluorescence quantum yield. Analogously, also for excitation correction, the respective reference quantity, i.e., spectral irradiance or spectral photon irradiance plays a role and needs to be defined here as well. For instance, comparison of fluorescence intensities/emission spectra measured at two different excitation wavelength, requires consideration of the photonic nature of the exciting light. Accordingly, for use of an excitation correction curve that is typically referenced to spectral irradiance, these values have to be divided by the corresponding photon energies.

Excitation Standards

For determination of a dye-based excitation correction curve, principally a similar procedure can be employed as for generation of an emission correction curve. However, the proportionality of fluorescence to absorptance leading to a concentration-dependent shape of excitation spectra, see Eqs. (1)–(4), needs to be additionally considered as well as fluctuations of the spectral radiance of the excitation light source that affect the spectral irradiance reaching the sample and thus the relative intensity of the measured excitation spectra. The former is illustrated in Fig. 15 exemplary for CT-type dye BX with minimum overlap between absorption/excitation and emission spectra and absorbances at the low energy absorption maximum between 0.02 and 0.20 and the latter can be simply accounted for with a reference detector, see Eq. (5). As follows from Eqs. (2)–(4) and the concentration dependence of the normalized absorptance of dye BX shown in the lower part of Fig. 15, spectral broadening of excitation spectra with increasing dye concentration is related to the fact that the detected fluorescence signal is not directly proportional to absorbance but to absorptance. Thus, for a measurement geometry of 0°/90° and determination of excitation spectra with an additional uncertainty of ca. $\leq$5%, the chromophore's absorbance should not exceed 0.05. This concentration dependence can be different for other measurement geometries. Moreover, use of mirror-type cuvette holders is to be avoided as variations in optical pathlength account for similar effects as variations in concentration. In contrast to the previously introduced set of emission standards, that tolerates fluctuations in dye concentration of at least 50%, determination of a fluorescence standard-based excitation correction curve with minimized uncertainty imposes strict requirements on dye concentrations/absorbances, optical pathlength in the cuvette, and measurement geometry. An elegant approach here is calculation of these concentration and pathlength-related spectral effects and their inclusion into the uncertainty budget of excitation correction. The concentration dependence of excitation spectra is a general problem for samples with low fluorescence

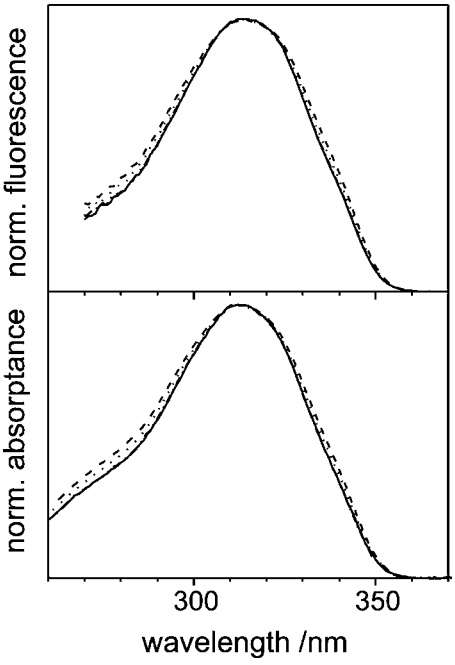

Fig. 15. Top: concentration dependence of the normalized excitation spectrum of a typical fluorophore (dye BX) in ethanol for $\alpha(\lambda_{abs}) =$ 0.045 (solid), 0.109 (dash-dotted), 0.241 (dotted), and 0.369 (dashed line), respectively. Detection was at 378 nm, the lowest energy absorption maximum at 315 nm, respectively. The solid and the dash-dotted line are virtually identical. Bottom: concentration dependence of the corresponding normalized absorptances.

quantum yields that typically require absorbances >0.05 for an acceptable signal-to-noise ratio or for compounds which show two optical transitions, i.e. S^0–S^1 and S^0–S^2 with considerable differences in oscillator strengths. In the latter case, measurement of excitation spectra at two different concentrations for the wavelength regions of the respective optical transitions is recommended.

A first—and to the best of our knowledge only—example for a set of excitation standards is presented in Fig. 16. These excitation standards supposedly enable determination of the relative spectral irradiance of the excitation channel at sample position traceable to the primary standard cryogenic radiometer. Aside from stability tests and determination of the combined uncertainty of the corrected excitation spectra, the suitability of this approach is been currently tested at BAM for several types of common fluorescence instruments employing the BAM software developed for emission standards.

CONCLUSION AND OUTLOOK

To improve quality assurance in fluorometry and establish traceability on a broad level, commercially available, easy-to-operate, and traceable fluorescence standards are mandatory that allow for determination of specific spectral characteristics and performance validation of common types of fluorescence instruments under routine measurement conditions. A first step into this direction are the sets of emission and excitation standards developed by us in combination with a tested software for generation of spectral correction curves from measured and corrected fluorescence spectra. A first set of emission standards covering the spectral region of 310–730 nm will soon be certified by BAM and will be commercially available from BAM as well as through all subsidiaries of Sigma-Aldrich. Due to the resemblance between these spectral fluorescence standards and typically measured fluorescent samples, many of the problems of calibration of fluorescence instruments, that are related to use of physical transfer standards, can be elegantly circumvented. Moreover, due to the liquid nature and the nearly isotropic emission of the set components, these standards can be employed for a broad variety of measurement geometies and formates. For the equally desired standardization of measurements of fluorescence intensity, however, either fluorescence intensity standards that spectrally match commonly used fluorescent labels or measurements of absolute fluorescence intensities or quantum yields are required. To realize the latter, a reference instrument is been currently built at BAM that will be eventually used for dissemination of absolute fluorescence spectra and quantum yields.

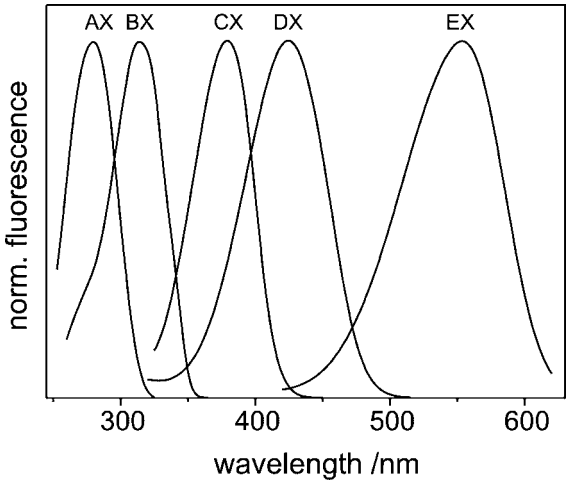

Fig. 16. Set of liquid excitation standards.

Aside from the need for sets of emission and excitation standards for the UV/Vis/NIR spectral region addressed here, there is also a strong demand for properly characterized and ideally certified fluorescence quantum yield, lifetime, and (de)polarization standards for this spectral region. For the latter two types of standards, at

first requirements on suited candidates should be defined thereby also taking current fluorescence instrumentation and future developments into account. Moreover, e.g. fluorescence lifetime standards additionally require consideration of typically used software for data evaluation. In addition to fluorescence standard-focused research activities and spectral correction, generally reliable and purpose-fit procedures for characterization of common types of fluorescence instruments including, for instance, range of linearity of detection systems, dynamic range, and instrument long-term stability need to be developed and documented that are accepted by standardization bodies like ASTM International, regulatory agencies, scientific associations such as for instance IUPAC, instrument manufacturers as well as the broad community of fluorescence users. Furthermore, the necessary level of traceability in fluorometry needs to be discussed for frequently used fluorescence techniques. Also, Round Robin tests for fluorometric quantities relevant to a broad community of users should be performed to clarify the actual level of competence and accordingly improvement in quality assurance required.

ACKNOWLEDGMENTS

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