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Fluorescence standards: Classification, terminology, and recommendations on their selection, use, and production (IUPAC Technical Report)*

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Abstract: Chromophore-based fluorescence standards for the characterization of photoluminescence measuring systems and the determination of relevant fluorometric quantities are classified according to their scope and area of application. General and type-specific requirements for suitable standards are derived for each class of standards. Metrological requirements linked to the realization of comparable measurements are addressed and recommendations on selecting, using, and developing fluorescence standards are given.

Keywords: chromophore-based fluorescence standards; fluorometric quantities; IUPAC Analytical Chemistry Division; IUPAC Organic and Biomolecular Chemistry Division; IUPAC Physical and Biophysical Chemistry Division; photoluminescence; standards.

CONTENTS

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1. INTRODUCTION

Photoluminescence techniques, which yield analyte-specific quantities such as emission and excitation spectra, luminescence quantum yields, luminescence lifetimes, and emission anisotropies, are among the most widely used tools in the materials and life sciences [1–7]. Challenges that presently limit the usefulness and applicability of these techniques include instrument-dependent contributions to otherwise analyte-specific fluorescence signals, a lack of simple methods for measuring absolute luminescence intensities [8–19], and general difficulties in accurately quantifying the properties of analytes from measurements of relative fluorescence intensities. The last of these is closely related to the dependence of the spectroscopic properties of most chromophores (such as absorption and emission spectra, molar absorption coefficient, luminescence quantum yield, luminescence lifetime, and luminescence polarization or anisotropy) on their microenvironment (in terms of temperature, viscosity, solvation, polarity, proticity, pH, ionic strength, presence of quenchers, and attachment to bio- or macromolecules). This situation is further complicated by the existence of very few guidelines, recommendations, and technical notes for the characterization and performance validation of photoluminescence measuring instruments [20–28] and for the performance of measurements of relevant photoluminescence quantities [29]. Moreover, concepts need to be developed, evaluated fluorescence standards need to be made available, and relevant fluorometric quantities (e.g., photoluminescence quantum yield) need to be determined to improve the reliability of quantitative fluorescence analyses [1,2,6,7]. Colorimetry or surface fluorescence [30,31] and, in part, flow cytometry [7,29] are related areas that have been standardized more thoroughly, and serve as examples of what needs to be established for the majority of photoluminescence measuring techniques [1,6,7,32–41].*,†

*Fluorescence standards manufacturers include Invitrogen (formerly Molecular Probes), Starna GmbH, Matech, Labsphere Inc., and LambdaChem GmbH.

†Certain commercial equipment, instruments, or materials are identified in this chapter to foster understanding. Such identification does not imply recommendation or endorsement by IUPAC or the Federal Institute for Materials Research and Testing (BAM) or the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

These limitations, which hamper the reliability and comparability of photoluminescence data [42], can be overcome with simple, well-characterized physical and chemical (i.e., chromophore-based) standards suitable for a reliable and regular instrument characterization, that meet internationally accepted quality criteria. In addition, straightforward recommendations, technical notes, or standard operations are needed for the characterization and performance validation of photoluminescence measuring instruments and for the performance of measurements of relevant luminescence quantities using these standards and reference materials [1,6,43].

The purpose of this document is to classify and derive quality criteria for standards for the characterization and performance validation of photoluminescence measuring systems and for the measurement of relevant fluorometric quantities. In addition, metrological requirements linked to the international infrastructure for realizing world-wide comparable measurements are addressed. Special emphasis is placed on steady-state measurements of photoluminescence spectroscopy. With proper consideration of method-inherent requirements and method-specific limitations, this recommendation can be extended to other photoluminescence techniques including fluorescence microwell plate and microarray technologies, time-resolved photoluminescence spectroscopy, fluorescence microscopy, bio- and chemiluminescence spectroscopy, and flow cytometry.

Division of fluorescence standards into general classes is given in Section 2. General requirements on fluorescence standards as well as on their characterization, documentation, production, and certification are detailed in Section 3. Additional scope-specific requirements for fluorescence standards are covered in Section 4. Standards for fluorometric quantities, such as emission anisotropy, photoluminescence quantum yield, and fluorescence lifetime are only briefly mentioned. These standards are detailed in refs. [44–47]. In the following section, for simplicity, the term *fluorescence* is used not in its strictly photochemical sense, describing the spontaneous emission of radiation (luminescence) from an excited molecular entity with retention of spin multiplicity [48], but rather as a synonym for (photo)luminescence.

2. CLASSIFICATION OF FLUORESCENCE STANDARDS

2.1 Types of fluorescence standards: Scope-specific classification, physical and chemical standards, and traceable measurements

Fluorescence standards can be divided into three general types or classes [49,50] depending on their scope and application:

(i) instrument calibration standards,
(ii) standards for the validation of the performance of fluorescence instruments, and
(iii) application-specific standards.

Depending on the desired application, these standards can be of a physical or chemical nature. *Physical standards* come in the form of devices such as (calibrated) light sources, or (calibrated) detectors [51–54]. Physical standards are often referred to as physical transfer standards (PTSs), thereby underlining their function of transferring known values of a quantity, such as the spectral radiance or the spectral responsivity, to an instrument when used to calibrate measurements of that instrument [51,53,55]. The *spectral responsivity* is the signal output per unit radiant flux incident on a detection system per unit bandwidth, expressed as a function of wavelength. *Calibration* is, in summary, a set of procedures that establishes the relationship between measurements on an instrument and the corresponding quantity values realized by standards [56,57]. PTSs are often used in this way to establish a claim of metrological traceability to a standard quantity for measurements taken on the calibrated instrument. Metrological *traceability* is a property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty [57]. *Unbroken chain of calibrations* refers to the requirement that any inter-

mediate calibrations used to trace the measurement result to the reference must have their values and uncertainties linked to the measurement result as well [57–59]. In radiometry, a calibrated source is also called a *spectral radiance transfer standard*, and a calibrated detector a *spectral responsivity transfer standard* [51].

Traceable measurements are the basis of an international infrastructure for realizing world-wide comparable measurements [60–62]. This metrological requirement is documented, for example, in ISO/IEC 17025 and is relevant for applications such as laboratory accreditation [42]. Traceability does not necessarily require absolute measurements. It can also be realized with relative measurements [51,63]. Procedures and standards to establish a traceability chain for the fluorometric quantities *fluorescence emission spectrum* and *fluorescence excitation spectrum* as well as for the fluorescence quantum yield are illustrated in Fig. 1, thereby linking fluorometry to radiometry with the aid of transfer standards and calibrations.

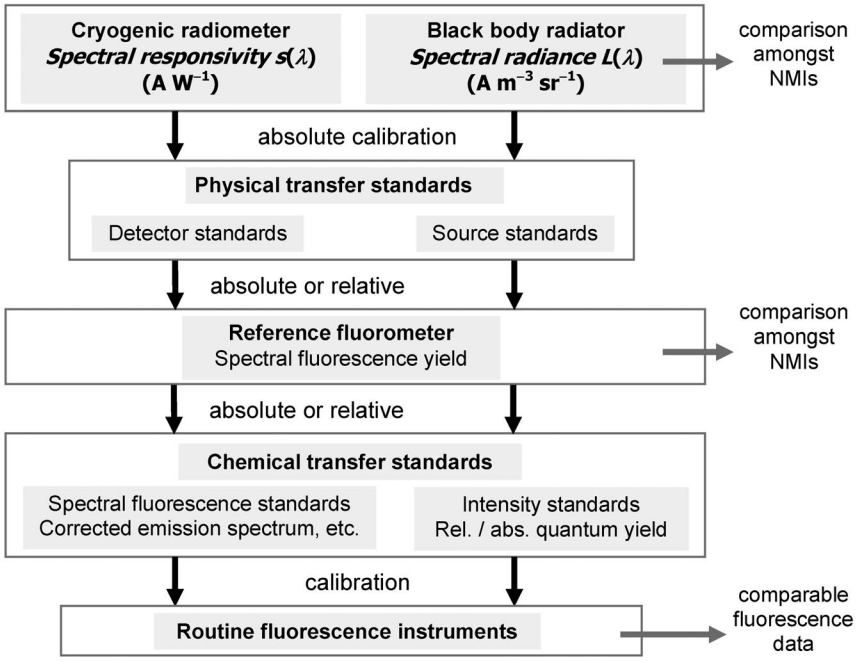

Fig. 1 Procedures and standards to establish a traceability chain for the fluorometric quantities fluorescence emission spectrum and fluorescence excitation spectrum as well as for the fluorescence quantum yield. This chain links fluorometry to radiometry with the aid of transfer standards and calibrations. NMIs: National Metrology Institutes. The working principle of the cryogenic radiometer and the black body radiator and their function as primary standards for spectral responsivity and spectral radiance are detailed in the literature, e.g., [51–55].

Chemical standards are liquid or solid chromophore-based reference materials. In many previous publications, the term *fluorescence standards* has been applied solely to chemical standards. Dependent on their scope and application, chemical fluorescence standards have been further divided into wavelength, emission, and excitation standards [11,50,64], to be used as spectral fluorescence standards, quantum yield standards, luminescence anisotropy standards, or lifetime standards [1,6,7,12,24,25,37–40,46,47,65,66]. As with their physical counterparts, chemical standards can be used to transfer a radiometric quantity like the spectral radiance, but at present, only on a relative, not on an absolute scale [1,6,62]. Their use can also provide traceability if the scope-relevant properties of

the standards have been determined with a traceably characterized fluorescence instrument with a given uncertainty.

2.2 Instrument calibration standards: Calibrated light sources and detectors and spectral fluorescence standards

Instrument calibration standards are standards used for the determination and correction of instrument bias. The scope of these standards, which can be physical devices or reference materials, is to rule out instrumentation as a major source of variability and to yield instrument-independent, comparable fluorescence data. Typical applications are the determination of the wavelength accuracy of wavelength-selecting optical components using, e.g., atomic lamps or the spectral characteristics of fluorescence instruments [11,49,50] using, e.g., certified reference materials (CRMs) in the appropriate sample format at the sample position [67–70], see also Section 4.1.

2.3 Standards for the validation of the performance of fluorescence instruments

These standards represent tools for the periodic verification of instrument performance [49,50]. Such standards can be either physical devices or reference materials and do not necessarily have to mimic the fluorescence properties of typically measured samples. Depending on the instrument parameter(s) to be determined, they can be identical to instrument calibration standards. Typical examples of standards for instrument performance validation are day-to-day intensity standards, which check the instrument's day-to-day performance and long-term stability based on measurements of the (relative) spectral sensitivity. Examples include a sealed cuvette filled with deionized water in the case of the popular Raman test [71,72], or a fluorescent sample that is stable over time and after exposure to light, such as some solid, inorganic fluorophores. Examples of the latter are rare-earth-doped inorganic glasses, e.g., Standard Reference Materials (SRMs) 2940–2943 from the National Institute of Standards and Technology (NIST) [73,74] and the day-to-day intensity standards suggested by BAM [49], and rare-earth-doped poly-tetrafluoroethylene, e.g., those sold by Avian Technologies and Labsphere.

2.4 Application-specific fluorescence standards: Fluorescence intensity standards, fluorescence lifetime, and fluorescence anisotropy standards

The scope of these standards is to aid in the determination of certain photoluminescence quantities such as the luminescence quantum yield or in the determination of fluorophore concentration from comparative measurements of relative fluorescence intensities. These standards should have scope-relevant properties that closely mimic those of the samples to be characterized. Application-specific standards include fluorescence intensity standards like fluorescence quantum yield standards, standards to relate instrument response to chemical concentration or to provide a relative, yet comparable intensity scale, as well as fluorescence lifetime and fluorescence anisotropy standards [1,6,7,65,66,75]. Further details are presented in Section 3.4 and for fluorescence quantum yield, emission anisotropy, and fluorescence lifetime standards also in refs. [1,6,7,11,12,44–47].

3. QUALITY CRITERIA FOR FLUORESCENCE STANDARDS

3.1 General requirements on fluorescence standards

3.1.1 Choice of measurement parameters

Suitable standards must be measurable with routinely used instrument settings. Otherwise, the instrument qualification cannot be reliably used for the correction of measured photoluminescence data for instrument-specific effects. The use of similar instrument settings for instrument characterization and

the actual fluorescence measurements is also a prerequisite for traceable fluorescence measurements [6,42,57,60,61]. Particular attention has to be given to

- slit widths/spectral band-passes,
- detector voltage and detection mode (e.g., analogue-mode measuring photocurrents or photon-counting mode),
- filters,
- polarizer settings (excitation, emission),
- measurement geometry,
- integration (or scanning or averaging) time, and
- pulse duration, delay time, and gate time for instruments equipped with pulsed light sources.

This is also illustrated in refs. [44–47,50,76]. The only exceptions are the determination and checking of the wavelength accuracy of fluorescence instruments, which is typically performed at maximum spectral resolution, and measurements aiming at the comparability of fluorescence signals between different instruments. In the latter case, instrument settings are to be chosen that can be employed for a broad variety of different instruments.

In many cases, this stringent requirement is best met with chemical transfer standards as their chromophore nature guarantees emission characteristics comparable to those of typically measured fluorescent samples. The fulfilment of this criterion can be critical for physical source-based standards, the spectral radiances or emission intensities of which exceed those of common fluorophores by at least two (integrating sphere-type radiator) to four (tungsten strip lamp) orders of magnitude [52].

3.1.2 Properties

The perfect chromophore-based fluorescence standard [50,77] should

- be simple to use,
- be sufficiently stable in solution or as a solid under application-relevant conditions,
- absorb and emit in the same general regions as the compounds under study,
- display a spectral shape for the emission or excitation spectrum suitable for its scope (see Section 4.1 on spectral fluorescence standards),
- have a constant fluorescence quantum yield independent of excitation wavelength and from a single absorption band (and thus emission spectra that are independent of excitation wavelength and excitation spectra that are independent of emission wavelength), see also refs. [44,45],
- have as little overlap as possible between the absorption (excitation) and emission spectrum to minimize dependences on dye concentration and measurement geometry,
- have an isotropic emission (with the exception of fluorescence anisotropy standards, see Section 4.4.3 and ref. [46]),
- reveal a negligible small temperature dependence of its fluorometric properties,
- not be subject to oxygen quenching,
- reveal single exponential decay kinetics, see also ref. [47], and
- be easy to purify.

Many of these properties can be transferred to physical devices. This profile—in conjunction with the instrument parameters listed in the previous section—determine how a reliable fluorescence standard should be characterized and which information should be ideally provided with it.

3.2 Characterization and documentation

The value of a standard is determined by its properties (relevant to the scope of application), the characterization of these properties, the documentation, and the wealth of additional information provided with the standard [6,50,64]. This should ideally include

- scope and limitations of the standard,
- recommended recalibration intervals or shelf life (stability),
- the instrument (including calibration) and instrument settings used for standard characterization, see Section 3.1, as well as the temperature,
- the homogeneity of the fluorophore distribution or the spatial uniformity of the standard's scope-specific properties [6,78,79],
- the standard's "polarization properties", e.g., emission anisotropy (r) or degree of emission polarization (p) [48], and its sensitivity toward the interaction with polarized light (see also ref. [46]), and
- for chemical standards, the chromophore's purity [80], its microenvironment or matrix (type and purity), the chromophore concentration, and preferably the emission lifetime.
- Additional scope-specific requirements on fluorescence standards are detailed in Section 5.

The emission anisotropy of a standard determines whether this standard can be used without polarizers [64]. Nearly isotropic emitters with $r \leq$ ca. 0.05 render polarizers dispensable, whereas for devices or materials revealing a partly or strongly polarized emission, such as organic fluorophores embedded into a solid matrix [64], use without polarizers results in an enlarged calibration uncertainty, the magnitude of which increases with increasing anisotropy of the standard's emission. Knowledge of the standard's emission lifetime is important for chromophores exhibiting luminescence lifetimes in the micro- to millisecond region and particularly for chromophore mixtures. Long lifetimes can in principle induce some limitations in conjunction with instruments equipped with pulsed light sources and can require special care with respect to the choice of parameters like delay, gate, and integration (or scanning) time [11,73,74].

If the fluorescence standards used do not meet these criteria, as well as any additional scope-specific requirements detailed in Section 4, this can result in calibration or measurement uncertainty or, at worst, an instrument characterization that is not reliable.

3.3 Production and certification of standards

The criteria for the production of reference materials are regulated in *ISO Guide 34* [81] and *ISO Guide 35* [82] and the calculation of uncertainties in the *ISO Guide to the Expression of Uncertainty in Measurement* (*GUM*) [83,84]. These criteria should also apply to chromophore-based standards. The values of scope-specific properties of standards can be certified (for chemical standards; yielding CRMs) or calibrated (for physical standards) by certifying bodies and come then with measurement uncertainties. A *certified value* is a value for which the certifying body has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by the certifying body [85]. National Metrology Institutes (NMIs) certify their standards according to the requirements imposed by *ISO Guides 34 and 35* (in the case of most European NMIs, including the Federal Institute for Materials Research and Testing (BAM) [86]) or according to their own documented certification policy (e.g., NIST) [85]). This includes a statement of the uncertainties that apply to the individual item or batch of material. For spectral quantities, such uncertainties are wavelength-dependent [48]. These uncertainties include the calibration uncertainty from the instrument used for the certification of chemical standards, and/or the calibration of physical standards and national primary standards or internationally agreed equivalents (i.e., internationally agreed, traceable transfer standards or reference materials) used for these calibrations. For chemical standards, contributions from homogeneity [87] and stability studies are considered [64] in addition to this. Accordingly, these fluorescence standards are traceable to common references.

Also, certain manufacturers of standards certify their products by different rules. For chromophore-based fluorescence standards from non-NMI sources, if not stated, the production does not necessarily follow the respective ISO guides, and uncertainties are generally not provided. Use of devices

or reference materials with certificates lacking a statement of documented traceability yields measurements whose results are only linked to the respective material. In these cases, the traceability chain ends at the reference material itself.

4. SCOPE-SPECIFIC QUALITY CRITERIA AND REQUIREMENTS

4.1 Spectral fluorescence standards: Wavelength standards, spectral radiance and emission standards, and spectral responsivity and excitation standards

Spectral fluorescence standards are devices or reference materials for the characterization of the spectral characteristics of photoluminescence measuring systems. This includes the wavelength accuracy, spectral resolution and (relative) spectral responsivity of the emission detection system, and the spectral irradiance reaching the sample. Accordingly, the wavelength dependence of the spectral radiance or the spectral responsivity must be known in the case of physical standards. For their chemical counterparts, analogously, the corrected fluorescence emission or excitation spectra must be provided. In this document, the term *corrected spectra* refers to spectra that are corrected for instrument-specific properties, yet not for sample-related effects such as wavelength-dependent pre- and post- or so-called inner filter effects, refraction at the sample boundaries (refractive index of the matrix), and anisotropy of the fluorophore emission [48,64,88–91]. Such effects should be minimized upon proper choice of chromophores and measurement conditions, rendering them negligible within the typical uncertainties of fluorescence measurements [11,64] (see also ref. [46]). Otherwise, these effects need to be considered by additional corrections [88].

Requirements on the spectral shape and structure of the spectra as well as on the number of emission lines or bands are determined by the scope of the respective spectral fluorescence standard, see Sections 4.1.1 and 4.1.2. Methods for the characterization of photoluminescence measuring systems with spectral fluorescence standards and related application-specific details are, e.g., summarized in ref. [76].

4.1.1 Standards for the determination and verification of wavelength accuracy and spectral resolution

Suitable standards must emit a multitude of very narrow emission bands in the UV/visible/NIR spectral region at known spectral positions with a given uncertainty [92]. The wavelength accuracy can be checked by comparison of the band positions of the measured spectra and the known spectral positions [20]. For example, the band positions of atomic discharge lamps have been determined with high precision and accuracy using various types of spectrometers. Wavelength standards with very narrow emission bands, low-pressure atomic discharge lamps in particular, can also be exploited to determine the spectral resolution of photoluminescence measuring systems [20].

The spectral resolution of the instrument to be characterized determines the acceptable width of the spectral lines of the wavelength standard. For the calibration of the wavelength scale of high-precision spectrofluorometers, where typically an accuracy of about 20 cm^{-1} (±0.5 nm at 500 nm) is desired, the most commonly used choice is atomic discharge lamps that display extremely narrow emission lines, see Fig. 2 (panel A). To cover the UV/visible/NIR spectral region, such lamps often contain mixtures of gases such as mercury, argon, and neon [93–97]. As the spectral position of these emission bands is affected by gas pressure, this parameter should be reported by the standard's manufacturer and supplier. Since atomic discharge lamps typically exhibit a very large spectral radiance (emission intensity) as compared to fluorescent samples, the use of an attenuator such as a white standard or a diffuse scatterer is often mandatory to avoid detector saturation. For instruments with a lower spectral resolution, such as microwell plate readers or confocal spectral imaging systems (typically operated with a fixed spectral band-pass between 5 and 30 nm), where the high accuracy provided by atomic discharge lamps is not needed, chromophore-based wavelength standards present a straightforward and simple

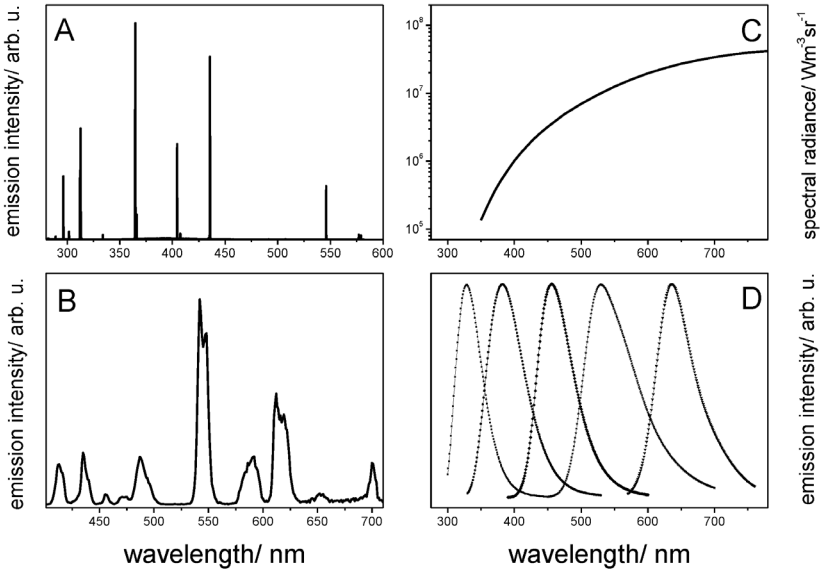

Fig. 2 Emission spectra of physical and chemical standards to determine the spectral characteristics of photoluminescence measuring instruments. Panel A: Atomic discharge lamp containing a mixture of mercury and argon recommended for the validation of the wavelength scale of high-precision spectrofluorometers (emission slit width 0.25 nm). Panel B: Fluorescent glass doped with a multitude of rare earth (RE) metal ions for the determination and verification of the wavelength accuracy of fluorescence measuring systems with low spectral resolution (excitation at 365 nm, emission slit width 2 nm) [102]. Panel C: Integrating sphere-type radiator. The certified spectral radiance of this standard in principle equals its corrected emission spectrum. Panel D: Exemplary set of five emission standards (BAM-F001 to BAM-F005 corresponding to dyes A–E in earlier publications [64,103]).

alternative [6]. Examples include, e.g., $Y_{3-x}Dy_xAl_{15}O_{12}$, a dysprosium-activated yttrium garnet [98,99] and glass-based materials currently tested at BAM and NIST [49,50,100,101], see Fig. 2 (panel B) [102].

4.1.2 Standards for the determination of the (relative) spectral responsivity: Calibrated lamps and emission standards

Devices or reference materials suitable for this purpose must emit a known broad and unstructured spectrum, ideally covering the application-relevant wavelength range [6,11,39,50,64], see Fig. 2 (panels C and D). This is mandatory to minimize the dependence of the shape of the standard's spectrum on instrument resolution/spectral band-pass. The (relative) spectral responsivity is calculated as the quotient of the measured (uncorrected) fluorescence signal and the certified spectral radiance or corrected relative fluorescence intensity of these standards as a function of emission wavelength [39,64,76,103].

Physical spectral radiance transfer standards like tungsten ribbon lamps or integrating sphere-type radiators reveal very broad unstructured emission spectra that cover the UV/visible/NIR spectral region [64], see Fig. 2 (panel C), yet their spectral radiances exceed those of typical fluorescent samples by at least four (for a tungsten ribbon lamp) and two (for an integrating sphere radiator) orders of magnitude [52].

Better suited for the majority of users of photoluminescence techniques are their chemical equivalents or so-called emission standards with certified corrected emission spectra [67–70,103,104]. If the corrected emission spectra of these standards have been determined on a spectrofluorometer, traceably characterized with physical standards, and are provided with (wavelength-dependent) uncertainties, use of these reference materials also yields a traceable instrument characterization [50,51,64,103,104]. The

close match of the spectral radiance and the size and shape of the radiating volume of both standard(s) and samples enables a straightforward determination of the instrument's relative spectral responsivity under application-relevant conditions [64]. Additional scope-specific requirements on emission standards include moderate to high fluorescence quantum yields to enhance the signal-to-noise ratio and to reduce the influence of stray light, solvent emission, and fluorescent impurities on the spectral shape of the standard's fluorescence spectrum [39,62]. In addition, as discussed in Section 3.2, the emission anisotropy (r), which determines whether this standard can be used without polarizers [64], should be preferably minimal as only nearly isotropic emitters with $r \leq$ ca. 0.05 render polarizers dispensable. Nevertheless, correction factors are still dependent on emission polarization settings, due to detection system polarization ratios or G factors [1,6,7], see refs. [46,75]. Since the emission spectrum of a dye is comparatively narrow when compared to the emission spectrum of a calibrated lamp, see Fig. 2 (panels C and D), coverage of a broad spectral region requires the combination of different emission standards in a set [39,49,51,52,64,103]. The reliable determination of the overall spectral responsivity with such a set requires (1) the crossing of spectrally neighboring dye spectra at sufficient fluorescence intensities and (2) the statistically weighted combination of the wavelength-dependent quotients of the measured and the corrected (certified) emission spectra of the set components [64,103]. The crossing of neighboring spectra is desired at an intensity of least 20 % of that of the emission maximum.

4.1.3 Standards for the determination of the relative spectral irradiance at the sample position: Calibrated detectors and excitation standards

Typical examples of standards for the determination of the wavelength dependence of the spectral irradiance reaching the sample are physical detector-based transfer standards such as a silicon photodiode (simple or integrating sphere-type, or trap detector [49–52,100,105]), see Fig. 3, and so-called excitation standards [1,6,64] shown in Fig. 4.

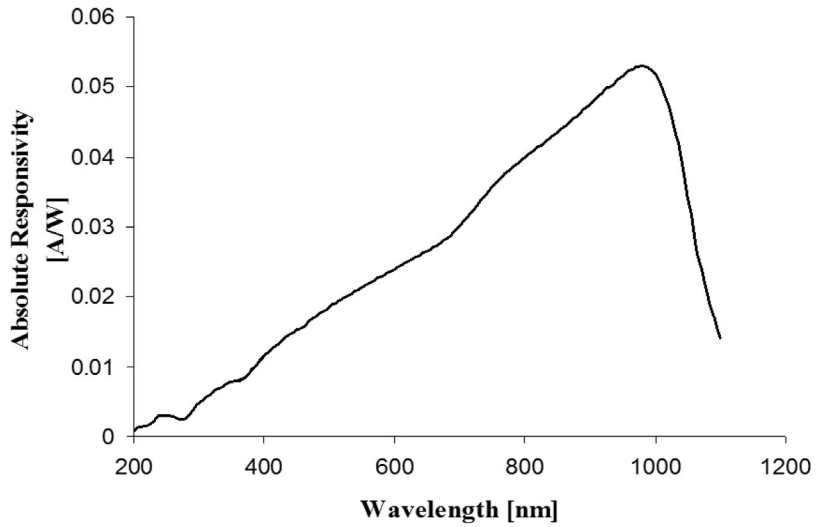

Fig. 3 Spectral responsivity of a calibrated detector, here a Si photodiode (Hamamatsu S2281-01) mounted onto a 51-mm-diameter integrating sphere (Labsphere with Spectraflect⎮ coating). The total uncertainty in each calibrated responsivity value for the calibrated detector is about ±2.0 % at a 95 % confidence level.

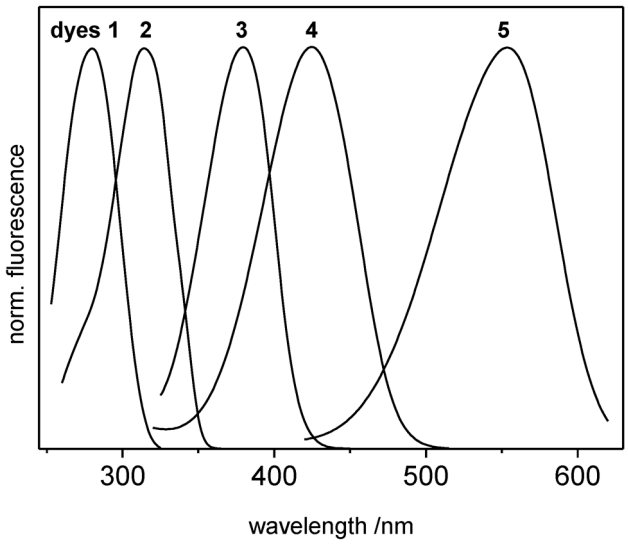

Fig. 4 Corrected excitation spectra of a set of liquid excitation standards, i.e., BAM dyes AX to EX, see also ref. [64].

Scope-specific requirements on such standards are either a known spectral responsivity or a known corrected fluorescence excitation spectrum. In the case of excitation standards that must fulfil similar requirements with respect to the shape of their excitation spectra as emission standards (see Section 4.1.2 and Fig. 4), the use of dilute dye solutions (absorbance $A \leq 0.05$ for a 0°/90° measurement geometry and a 1-cm cell) is mandatory. The proportionality of fluorescence intensity to the absorption factor $f(\lambda_{ex})$, see refs. [1,4,6,76], results in a concentration dependence of the spectral shape of excitation spectra and introduces a dependence on measurement geometry [64]. Coverage of a broad spectral region requires sets of excitation standards, see Fig. 4. The (relative) spectral irradiance reaching the sample position or the (relative) spectral radiant power is calculated as the quotient of the measured signal and the certified spectral responsivity of the detector or the corrected relative fluorescence intensity of the standards as a function of excitation wavelength [64].

As the use of neither quantum counters [1,48,51,106–108] nor actinometers [109–111] is advisable [112] (see also ref. [76]), scope-relevant requirements on these materials were omitted.

4.2 Standards for the validation of instrument performance

The choice of suitable standards for instrument performance validation depends on the instrument parameters to be checked and thus, to a certain extent, on the respective fluorescence technique. The assignment of changes in instrument performance to certain instrument parts, e.g., the clear distinction between drifts arising from changes in the excitation channel and the emission channel, requires tools for the independent measurement of $s(\lambda_{em})$ and $E_\lambda(\lambda_{ex})$, the spectral radiant flux of the excitation radiation at the sample. Standards should be inexpensive and easy-to-use, so they may be applied frequently to detect any drift in instrument performance under application-relevant conditions. Such standards, which may be developed in-house and are not necessarily traceable or certified, must be either sufficiently stable under applicable conditions (data from, and parameters of, stability tests should be provided) or, for single-use standards, reveal an excellent reproducibility, preferably in combination with an assigned uncertainty. For chemical standards, stability and reproducibility are both closely linked to fluorophore purity, and in the case of solutions, also to the purity of the solvent [64,80].

The most widely used standards for instrument performance validation are day-to-day intensity standards [50]. Suitable standards are usually spectral fluorescence standards. Such standards can provide a relative intensity scale on a single instrument basis, see also Section 4.3. For this type of standard, a certified quantity is not needed, but tolerance coefficients relating changes in intensity with corresponding changes in experimental conditions should be known or determined. Such tolerance coefficients enable the uncertainty related with the use of the standard to be determined based on the range and uncertainty of experimental conditions (see Section 3.1.1), such as temperature, polarization factors, and light exposure times. These coefficients are routinely considered for and included in the certified uncertainties of CRMs, but are equally important for defining the usefulness of reference materials that may not require rigorous certification, such as those discussed in this and the next two sections.

4.3 Instrument-to-instrument intensity standards to establish a comparable intensity scale

Instrument-to-instrument intensity standards, which are closely related to day-to-day intensity standards, are tools for the comparability of fluorescence intensities across instruments [49,50,113]. They enable a comparable, relative intensity scale for both spectrally resolved and integral fluorescence measurements to be established where the intention is not quantification. Unlike other more application-specific fluorescence intensity standards, they do not need to mimic typically measured samples. Similarly, standards for the performance validation of fluorescence instruments are not necessarily traceable or certified.

The suitability of any material as instrument-to-instrument intensity standards is directly linked to the applicability of identical measurement conditions for the fluorescence instruments to be compared. For instruments equipped with continuous (non-pulsed) excitation sources, this is typically not critical. However, care has to be taken for instruments with pulsed excitation sources and materials containing long-lived emitters, especially mixtures of species varying in lifetime [11]. Additional requirements on such standards are known corrected spectra, if, e.g., their intensities are to be compared with those of other fluorophores or between instruments with different spectral band-passes. Suitable physical or chemical standards should consider the emission range and spectral radiance/fluorescence intensity of typical samples and must be characterized with respect to all parameters that can affect their emission intensity [64].

4.4 Application-specific fluorescence standards

4.4.1 Fluorescence intensity standards: Quantification, comparable intensity scales, and determination of relative fluorescence quantum yields

Fluorescence intensity standards compare the spectral radiance or fluorescence intensity of a sample to that of a standard. Such systems, which are chemical standards in the majority of cases, include

- standards that relate chemical concentration to instrument response for quantifying chromophore concentration from measured fluorescence intensities,
- standards to provide a comparable intensity scale (see also Sections 5.2 and 5.3), and
- fluorescence quantum yield standards.

Standards that relate chemical concentration to instrument response compare the spectral radiance or fluorescence intensity of a sample to that of a standard of known fluorophore concentration under identical measurement conditions, thereby quantifying the concentration or number of fluorophores. This type of intensity standard typically relies on the same fluorophore(s) as those to be quantified. A classical example is the quantitative analysis of fluorescent analytes like polycyclic aromatic hydrocarbons (PAHs) using high-performance liquid chromatography (HPLC) with fluorescence detection,

where the fluorescence intensities from free, i.e., unbound fluorophores, in solutions of identical, or at least very similar, chemical composition are compared [114]. In this case, the chromophore(s) to be specified and the standard are in the same microenvironment and thus reveal identical fluorescence spectra, molar absorption coefficients, and fluorescence quantum yields. Accordingly, absolute numbers of fluorophores in the sample can be derived.

For the comparison of free and immobilized fluorophores, for example, dyes attached to beads, particles, or macro- and biomolecules, where the microenvironment of the dye in the sample and in the standard differ, other concepts for fluorescence intensity standards have been developed. These concepts all aim at the provision of a straightforward, yet relative intensity scale that is comparable across instruments, laboratories, and for the same instrument, over time. Strategies based on chemical standards like the widely used concept of molecules of equivalent soluble fluorophore (MESF) developed for flow cytometry [11,29,49,115,116] often try to consider and minimize the effect of dye microenvironment on fluorophore quantification by using the same fluorophores as used in the samples (e.g., as fluorescent labels) in a well-defined microenvironment. Nevertheless, these approaches do not provide the absolute number of fluorophores in the sample, but only an approximate number at best.

Fluorescence quantum yield standards frequently used in fluorometry are employed as a reference for the determination of the (relative) fluorescence quantum yield of an analyte [1,4,6,7,117–121]. These standards, which are detailed in refs. [44,45], are typically not based on the same fluorophore(s) as the measured samples, but should absorb and emit within the same spectral regions. The luminescence quantum yield of these standards should be reliably known, preferably including its uncertainty. Because fluorescence quantum yields can be sensitive to factors such as oxygen concentration (as well as to the presence of other quenchers), temperature, excitation wavelength, and chromophore concentration, these parameters should be given [1,3,4,6,7,65,122–126]. Preferably, the magnitude of the quantum yields of standard and sample should be similar, to circumvent problems related to nonlinearities of the detection system, or dilution errors.

4.4.2 Luminescence lifetime standards

Luminescence lifetime standards that are detailed in ref. [47] are used to calibrate or test the resolution of time- and frequency-domain instrumentation employed for luminescence lifetime measurements [1,6,7,66,127–129]. For time- and frequency-domain fluorescence lifetime spectroscopy in the picosecond to lower nanosecond temporal range, they can also be valuable to determine the (wavelength-dependent) time response of the detection system at the same emission wavelength as used for the sample, thus eliminating any color shift [1,6,7]. Use of these standards to assess other method-inherent sources of error is addressed in ref. [47].

Suitable lifetime standards must reveal mono-exponential decays of constant lifetime independent of excitation and emission wavelength at typically used emission wavelengths, and their lifetimes should be within the lifetime range of typically measured samples. For modern pico- and nanosecond temporal range or mega- to gigahertz frequency domain instrumentation, the luminescence lifetimes of such standards should be on the order of a few tens of picoseconds up to several tens of nanoseconds. Suitable approaches can include here the use of a single mono-exponentially decaying fluorophore or mixtures of fluorophore-quencher pairs of known dye and quencher concentration for tuning the dye's emission lifetime. Care has to be taken with fluorophores that show a charge transfer (CT) emission, in slowly relaxing solvents such as ethanol. Due to the relaxation of the solvent molecules around the CT state, such chromophores show a time-dependent shift of the emission spectrum to longer wavelength (referred to as dynamic Stokes shift) within the picosecond temporal range [1,130]. For lifetime measurements in the micro- and millisecond temporal range, standards with lifetimes within the lifetime range of typically measured samples are required.

As many samples display bi-, multi-, or nonexponential decays, it can be valuable to have standards with more complex decay behavior and known emission decay times and known relative contributions [1,127]. Suitable standards can be best produced by combining two or more dye solutions, each

with a known single exponential fluorescence decay to create bi- or multiexponential decays. The dyes should not interact, quench, react, transfer energy, or form complexes. Such standards would enable both instrument hardware (optics and electronics) and data analysis software to be tested simultaneously as to the system's effectiveness at determining complex time-decay behavior. Standard decay data can also be used to determine the effectiveness of the data analysis software, independent of the instrument performance.

4.4.3 Standards for fluorescence polarization

Fluorescence polarization standards with a known emission anisotropy or degree of polarization (p), which are detailed in ref. [47], can be used to calibrate or test instrumentation used for measurements of polarization [1,75] relying on the photoselective excitation of fluorophores by polarized light. Generally, suitable standards should cover the polarization range from $p = 0$ (isotropic emission) to $p = 0.5$ (anisotropic emission). Standards with a high anisotropy (r) or a high degree of polarization (p) are valuable to identify artifacts that depolarize the emission, whereas isotropic emitters enable the verification of whether the G-factor has been measured accurately. As the anisotropy of a chromophore can depend on both excitation and emission wavelength, these dependences should be provided and the standard should be used only in a wavelength range where its polarization is largely independent of wavelength. Only dilute dye solutions should be used to avoid energy transfer-corruption of fluorescence polarization.

5. ADAPTATION OF FLUORESCENCE STANDARDS TO DIFFERENT FLUORESCENCE TECHNIQUES

The transfer and adaptation of evaluated and established procedures and standards for instrument characterization and instrument performance validation from one fluorescence technique to another requires proper consideration of method-inherent requirements on standards, and of scope-specific limitations of methods and standards [6,50,131]. This includes, for instance, adaptation of measurement parameters, measurement geometry, sample or standard format, excitation wavelength(s), and (photochemical and thermal) stability [49,50]. The latter is of special importance for techniques using lasers as excitation sources with their strongly enhanced excitation intensity or spectral radiance and fixed excitation wavelength [131]. Also, the standard's luminescence lifetime can be critical as this parameter controls the standard's suitability for techniques that use pulsed excitation light sources, or that employ short measurement or integration times (pixel times) such as fluorescence microscopy [6,131].

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