

Traceability in Fluorometry—Part I: Physical Standards

J. Hollandt,^{1,4} R. D. Taubert,¹ J. Seidel,¹ U. Resch-Genger,³ A. Gugg-Helminger,²
D. Pfeifer,³ C. Monte,³ and W. Pilz³

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The inter-instrument, inter-laboratory, and long-term comparability of fluorescence data requires the correction of the measured emission and excitation spectra for the wavelength- and polarization-dependent spectral irradiance of the excitation channel at the sample position and the spectral responsivity of the emission channel employing procedures that guarantee traceability to the respective primary standards. In this respect the traceability chain of fluorometry is discussed from a radiometrist's point of view. This involves, in a first step, the realization of the spectral radiance scale, based on the blackbody radiator and electron storage ring, and the spectral responsivity scale, based on the cryogenic radiometer and their control via key comparisons of the national metrology institutes. In a second step, the characterization including state-of-the art uncertainties of the respective source and detector transfer standards such as tungsten strip lamps, integrating sphere radiators, and trap detectors used to disseminate these radiometric quantities to users of spectroscopic techniques is presented.

KEY WORDS: Fluorometry; traceability; radiometry; spectral radiance; spectral responsivity.

INTRODUCTION

Luminescence techniques are of widespread and ever-increasing use in material sciences, chemical and bio-analysis, forensics, and medical diagnostics due to their high sensitivity, intrinsic selectivity of communication, ease of use, speed of analysis, non-invasive character, and suitability for remote access with fibre optics and for miniaturization [1]. Despite many advantages of luminescence measurements, it is often overlooked that each luminescence technique yields a signal that is not only determined by the spectroscopic properties of the analyte of interest, i.e. its absorption and emission spectrum, molar absorption coefficient, and luminescence quantum

yield, but always contain instrument-related contributions. The latter originate from the wavelength- and polarization-dependent spectral irradiance of the excitation channel and the spectral responsivity of the emission channel of the employed fluorescence instrument. These effects are determined by the spectral characteristics of the optical components of the instrument such as excitation light source, monochromators, polarizers, lenses, mirrors and filters as well as the spectral responsivity of the detector. The excitation channel determines the spectral irradiance at the sample, and thus, together with the analyte concentration, its molar absorption coefficient, and luminescence quantum yield determines the intensity of the emission from the sample. The spectral responsivity of the emission channel determines the measured signal caused by the detected sample emission. As fluorescence emission and excitation spectra are typically recorded at fixed excitation and emission wavelengths, respectively, correction for the spectral characteristics of the emission and excitation channel can be, however, performed separately. For luminescence techniques such as for instance chemiluminescence, which do not require absorption of

¹ Physikalisch-Technische Bundesanstalt, Abbestrasse 2-12, D-10587 Berlin, Germany.

² Gigahertz-Optik GmbH, Postfach 1445, D-82170 Puchheim, Germany.

³ Federal Institute for Materials Research and Testing, Working Group Optical Spectroscopy, Division I.3, BAM, Berlin, Germany.

⁴ To whom correspondence should be addressed. E-mail: joerg.hollandt@ptb.de.

photons, commonly only the spectral response of the emission channel has to be considered.

For inter-instrument and inter-laboratory comparisons of luminescence data, it is necessary to eliminate the instrumental contributions to receive the truly sample related and hence comparison of information of the fluorescence experiment. As aging of optical components and detection systems affects their spectral characteristics, these instrumental effects also complicate the long-term comparability of luminescence data measured on the same instrument. For methods comparing the emission of two fluorophores with different emission spectra, such as for instance the determination of luminescence quantum yields or the fluorescence intensities resulting from excitation at two different wavelengths, the removal of the instrument-specific contributions from the measured raw data is mandatory. Furthermore, the knowledge of the instrument's spectral characteristics may be helpful for the optimization of luminescence-based analytical techniques, for instance via proper choice of fluorophores with absorption and emission spectra that fit with the highest spectral irradiance at the sample position and optimum responsivity of the detector, respectively. Additionally, comparability of data, and thus proper characterization of fluorescence measuring systems, is required to meet the globalization-imposed trends of quality assurance and traceability as given in EN ISO/IEC 17025.

In principle, two approaches are possible for the traceable radiometric characterization of a fluorescence instrument: by applying physical transfer standards calibrated traceable to the primary radiometric standards, e.g. a detector transfer standard for the characterization of the excitation channel and a spectral radiance transfer standard for the emission channel or by using chromophore-based certified fluorometric reference materials. The latter

are referred to as spectral fluorescence standards or excitation and emission standards. They have certified corrected fluorescence excitation and emission spectra, free of instrument-specific wavelength dependencies, that have been measured with a spectrofluorometer traceably characterized to the primary radiometric standards. The first method is subject of part I of this series whereas the second method is presented in part II.

RADIOMETRIC CHARACTERIZATION OF A FLUORESCENCE INSTRUMENT

A schematic of a steady state fluorescence experiment is shown in Fig. 1. The sample under investigation is irradiated by an irradiance E_{ex} from the excitation channel of the fluorescence instrument set to a wavelength λ_{ex} . The emitted fluorescence radiation is then measured by the emission channel with the spectral responsivity $s(\lambda_{\text{em}})$ under an angle of e.g. 90° to the excitation channel. The illuminated and detected volume is defined by the optical systems of the excitation and emission channel, respectively.

The measured signal $I_m(\lambda_{\text{ex}}, \lambda_{\text{em}})$ by the emission channel set to a fixed wavelength λ_{em} is given by

$$I_m(\lambda_{\text{ex}}, \lambda_{\text{em}}) = \int_{\Delta\lambda} s(\lambda)\Phi_\lambda(\lambda) d\lambda \quad (1)$$

where $s(\lambda)$ is the spectral responsivity and $\Delta\lambda$ is the spectral bandwidth of the emission channel. $\Phi_\lambda(\lambda)$ denotes the spectral radiant power radiated from the illuminated volume into the emission channel and is given by

$$\Phi_\lambda(\lambda) = \varepsilon(\lambda_{\text{ex}})cF_\lambda(\lambda_{\text{ex}}, \lambda) \int E_{\text{ex}}(\vec{r})P(\vec{r}) dV \quad (2)$$

with $\varepsilon(\lambda_{\text{ex}})$ being the molar absorption coefficient at the excitation wavelength λ_{ex} , c the concentration of the

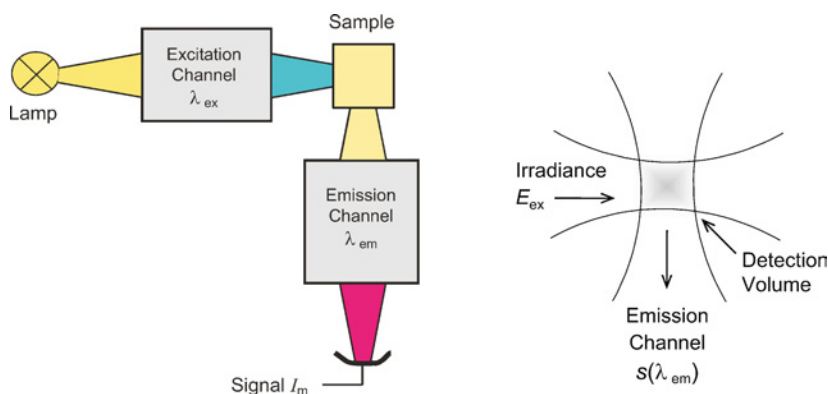


Fig. 1. Schematics of the experimental setup used in fluorescence techniques (left) and of the distribution of the excitation and emission beam in the sample (right).

Table I. Physical Quantities in Fluorometry and Their Units

Symbol	Physical quantity	Unit
Φ_λ	Spectral radiant power	W m^{-1}
E	Irradiance	W m^{-2}
E_λ	Spectral irradiance	W m^{-3}
L	Radiance	$\text{W m}^{-2} \text{sr}^{-1}$
L_λ	Spectral radiance	$\text{W m}^{-3} \text{sr}^{-1}$
s	Spectral responsivity to radiant power	A W^{-1}
s_E	Spectral responsivity to irradiance	$\text{A W}^{-1} \text{m}^2$
I	Photocurrent of detector	A
c	Concentration	mol m^{-3}
$\varepsilon(\lambda_{\text{ex}})$	Molar absorption coefficient	$\text{m}^2 \text{mol}^{-1}$
F_λ	Spectral fluorescence yield	m^{-1}

fluorophor in the sample, $F_\lambda(\lambda_{\text{ex}}, \lambda)$ the spectral fluorescence yield, $E_{\text{ex}}(\vec{r})$ the irradiance at the position \vec{r} in the sample and $P(\vec{r})$ the detection probability of a photon emitted at the position \vec{r} (see Table I).

According to Eqs. (1) and (2), the measured raw measured signal of a fluorescence experiment depends on two types of parameters: the sample-specific quantities, i.e. the concentration of the fluorophore, the spectral fluorescence yield and the molar absorption coefficient, and the instrument-specific quantities, i.e. the irradiance of the excitation channel and the spectral responsivity of the emission channel. To extract the desired sample-specific information from the measured data, the instrumental effects need to be eliminated. This can be achieved by a radiometric characterization of the excitation and emission channel with calibrated physical transfer standards in a two-step procedure shown in Fig. 2. In a first step, measurement of the spectral irradiance E_λ of the excitation channel is performed by replacing the fluorescent

sample by a transfer detector standard, calibrated in terms of *spectral responsivity to radiant power* by comparison with an electrical substitution cryogenic radiometer as a primary detector standard for high-accuracy optical power measurement (see chapter on ‘Primary Radiometric Standards’). This transfer detector, in the most accurate case a so-called trap-detector (see chapter on ‘Radiometric Transfer Standards’), is used with an optical precision aperture of known area to transform it into a standard of *spectral responsivity to irradiance*. Measurement of the detector signal I_{Det} with the over-illuminated detector aperture area yields the required *spectral irradiance* at the sample position. In a second step, the sample is replaced by a transfer source standard calibrated in terms of *spectral radiance* by comparison to an electron storage ring or a high-temperature blackbody radiator as primary source standards of known radiant power (see chapter on ‘Primary Radiometric Standards’). This transfer source standard, e.g. a tungsten ribbon lamp or an integrating sphere radiator (see chapter on ‘Radiometric Transfer Standards’), emits a known spectral radiant power per area and per solid angle. The *spectral responsivity* of the emission channel results from the ratio between the measured detector signal I_m and the accepted radiant power of the emission channel.

PRIMARY RADIOMETRIC STANDARDS

To achieve a traceable radiometric calibration of a fluorescence instrument with lowest possible uncertainties, the applied radiometric transfer standards have to be calibrated in terms of spectral radiance and spectral responsivity against a primary source-based or

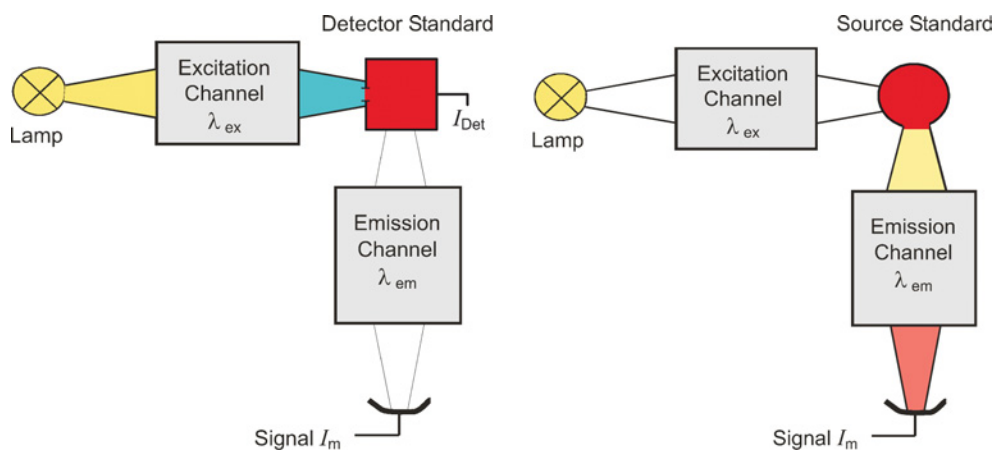


Fig. 2. Calibration procedures of a fluorescence instrument by means of physical transfer standards: detector-based calibration of the excitation channel (left) and source-based calibration of the emission channel (right).

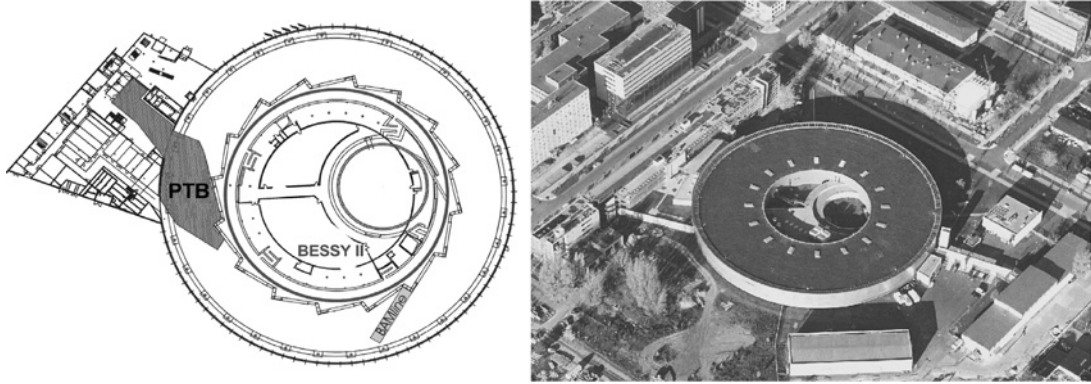


Fig. 3. Ground plan and aerial photograph of the electron storage ring BESSY II in Berlin-Adlershof, a primary radiometric standard from the X-ray to the UV spectral range. The highlighted areas denote the PTB radiometry laboratory and the beam line used by BAM.

detector-based radiometric standard through an unbroken chain of comparisons with given uncertainties.

SOURCE-BASED PRIMARY STANDARDS

The PTB source-based primary standards for the realization and dissemination of the spectral radiance from the vacuum ultraviolet to the near infrared spectral range are the electron storage ring BESSY II (40–400 nm) (Figs. 3 and 4) [2,3] and the high-temperature blackbody HTBB 3200 pg (220 nm–2.5 μm) (Figs. 4 and 5) [4]. The unique property of these primary radiometric sources is that their absolute radiant power and derived radiometric quantities like the spectral radiance can be determined from the measurement of basic source parameters on the basis of fundamental physical equations.

For an electron storage ring like BESSY II, the spectral radiant power Φ_λ of the synchrotron radiation is given

by Schwinger’s formula (Eq. 3) [5] and can be calculated from a few basic storage ring parameters: the energy of the stored electrons W , the electron ring current I , the magnetic induction of the bending magnet B , the radius R of the electron trajectory (it follows from W and B), and the geometry parameters d , r , and Ψ (Fig. 4).

$$\begin{aligned} \Phi_\lambda^{\text{SR}}(\lambda) &= \Phi_\lambda^{\text{SR},\text{II}}(\lambda) + \Phi_\lambda^{\text{SR},\perp}(\lambda) \\ &= \int_{-\varphi_m}^{\varphi_m} \int_{\psi_0 - \sqrt{\psi_m^2 - \varphi^2}}^{\psi_0 + \sqrt{\psi_m^2 - \varphi^2}} I_\lambda^{\text{SR}}(\lambda) \cos \psi \, d\psi \, d\varphi \\ &= \frac{2eR^2I}{3\varepsilon_0\lambda^4\gamma^4} \int_{-\varphi_m}^{\varphi_m} \int_{\psi_0 - \sqrt{\psi_m^2 - \varphi^2}}^{\psi_0 + \sqrt{\psi_m^2 - \varphi^2}} \\ &\quad \times \left\{ [1 + (\gamma\psi)^2]^2 K_{2/3}^2(\xi) + [1 + (\gamma\psi)^2] (\gamma\psi)^2 K_{1/3}^2(\xi) \right\} d\psi \, d\varphi \end{aligned} \quad (3)$$

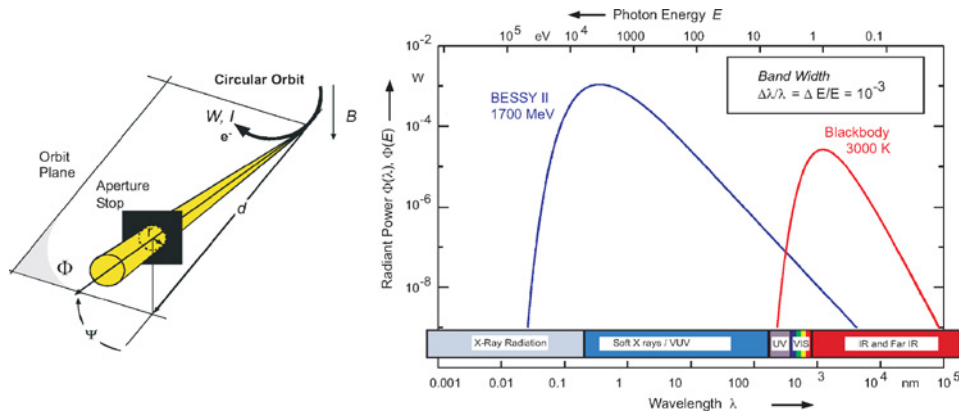


Fig. 4. Schematics of the required parameters for the calculation of the spectral radiant power of an electron storage ring (left). A synchrotron radiation spectrum of BESSY II at an electron energy of 1700 MeV compared with a spectrum of a blackbody at a temperature of 3000 K (right).

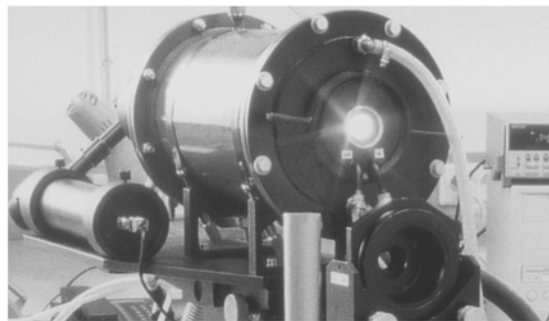
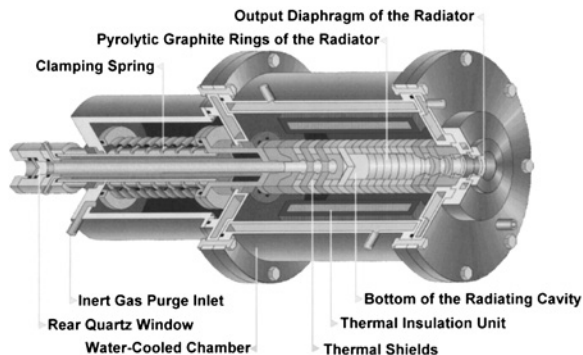


Fig. 5. The high-temperature blackbody HTBB 3200 pg, the primary spectral radiance standard from the ultraviolet to the infrared spectral range at PTB.

with

$$\gamma = \frac{W}{m_0 c^2}, \quad \xi = \frac{2\pi R}{3\gamma^3 \lambda} [1 + (\gamma \psi)^2]^{3/2}, \quad R = \frac{W}{ecB},$$

$$\cos \psi_0 \approx 1, \quad \cos \psi \approx 1,$$

$$\psi_m = \varphi_m = \arctan(r/d^{SR}) \approx r/d^{SR}$$

m_0 : mass of electron; c : velocity of light; $K_{1/3}K_{2/3}$: modified BESSEL-Functions.

The calibration of radiometric transfer standards with BESSY II in the wavelengths range from 40 to 400 nm, in particular the spectral radiance of radiation sources and the spectral responsivity of detectors, is performed with typical standard uncertainties of 2% [6] and 0.2% [3], respectively.

Analogously to the electron storage ring the absolute spectral radiance L_λ of the blackbody HTBB 3200 pg (Fig. 5) [7] can be calculated according to Planck's law [8] if its temperature T and, since it is not an ideal blackbody, its emissivity ε is known

$$L_\lambda(\varepsilon, \lambda, T) = \varepsilon \frac{2hc^2}{n^2 \lambda^5} \left(\exp\left(\frac{hc}{n\lambda kT}\right) - 1 \right)^{-1} \quad (4)$$

with h being the Planck constant, c the velocity of light, n the refractive index of air and k the Boltzmann constant. Operating at temperatures as high as 3200 K, the HTBB has a sufficiently high radiance to serve as a primary standard from the ultraviolet to the infrared, complementing ideally the operating wavelength range of the electron storage ring BESSY II (Fig. 4). The calibration of spectral radiance transfer standards by comparison to the high-temperature blackbody is typically performed with a standard uncertainty of below 0.5% in the visible and near infrared spectral range (Fig. 6) [4].

DETECTOR-BASED PRIMARY STANDARD

A major improvement in the detector-based radiometry was the introduction of the electrical substitution radiometer operated at cryogenic temperatures (typically below 20 K), the so-called cryogenic radiometer, in the late 1970s [9]. A schematic view of the operating principle is given in Fig. 7 (left). A cavity with a very high absorptivity serves as a black detector (inverse blackbody). Ideally, incoming radiation is completely absorbed by the cavity. The resulting temperature increase of the cavity is measured. Then the radiation is switched off and the same temperature increase is brought about by electrical heating. By assuming perfect equivalence of optical and electrical heating the radiation power is measured traceable to electrical units. A detailed description can be found in [10]. Achieving outstandingly small relative uncertainties of the order of 10^{-5} for optical power measurement, this instrument has become an established tool in the national metrology laboratories for the high-accuracy realization and dissemination of spectral responsivity.

PTB operates several cryogenic radiometers using the electron storage ring BESSY II, lasers, and lamp/monochromator combinations as radiation sources. The laser-based cryogenic radiometer of PTB, the radiation thermometry cryogenic radiometer (RTCR) [11], is shown in Fig. 7 (right). By using laser lines from an Ar⁺-laser, a Kr⁺-laser, a tuneable Ti:sapphire laser system, a Nd:YAG laser and two infrared diode lasers, the RTCR facility covers the wavelength range from 238 nm to 1.55 μ m. The relative standard uncertainty of optical power measurement (50–500 μ W) with the RTCR is 2×10^{-4} to 8×10^{-4} for the UV, and 7×10^{-5} to 4×10^{-4} in the visible and near infrared, respectively. These small uncertainties have been confirmed in the CCPR⁵

⁵ CCPR: Consultative Committee for Photometry and Radiometry.

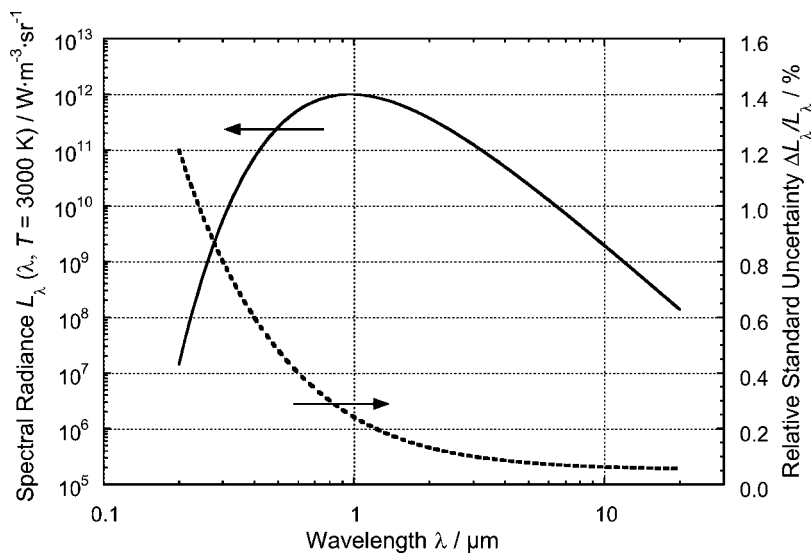


Fig. 6. Spectral radiance L_λ of an ideal blackbody operated at a temperature of 3000 K from 200 to 20 μm (solid line, left scale) and its relative standard uncertainty resulting from a standard uncertainty of 1.5 K in the temperature determination of the blackbody (dotted line, right scale).

S3 comparison of cryogenic radiometers (Fig. 8) [12]. Due to the large number of participating laboratories and the limited transportability of the cryogenic radiometers, this international comparison was performed indirectly by means of trap-detectors as high-accuracy transfer standards. The spectral responsivity of trap-detectors has been proven to be highly stable. For the comparison at up to six selected laser lines of Ar^+ -, Kr^+ - and HeNe-lasers the spectral responsivity of trap-detectors has been calibrated

by direct comparison to a cryogenic radiometer by each participant.

RADIOMETRIC TRANSFER STANDARDS

Radiometric transfer standards are used to disseminate radiometric quantities from the primary radiometric standards to the user.

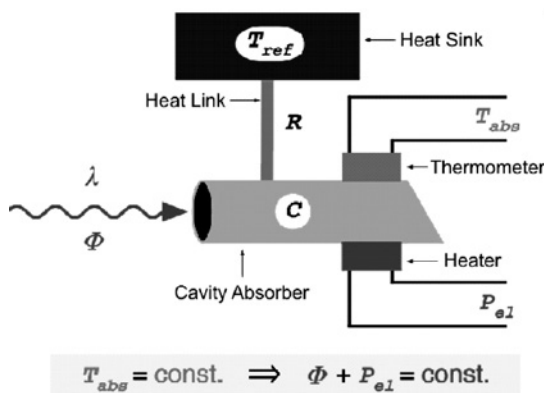


Fig. 7. Operating principle of a cryogenic electrical substitution radiometer (left) and the PTB radiation thermometry cryogenic radiometer (right).

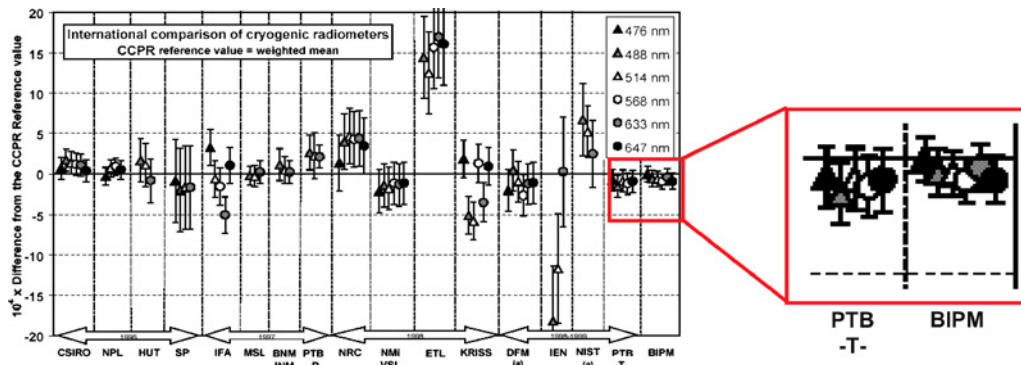


Fig. 8. Results of the international comparison for cryogenic radiometers (1996–1999). The enlarged area displays the results from the RTCR of PTB in comparison to the results of the BIPM and the key comparison reference value [12].

SOURCE-BASED TRANSFER STANDARDS

Up to now, due to its very good long-term stability, the conventional source-based transfer standard for the high-accuracy dissemination of the spectral radiance in the wavelength range from 220 to 2500 nm is the gas-filled tungsten ribbon filament lamp operated at radiance temperatures from 2300 to 2500 K ($\lambda = 650$ nm) (Fig. 9). To achieve highest accuracies, this transfer standard is calibrated at the Spectral Radiance Comparator I of PTB against the primary radiance standard, the high-temperature blackbody [4]. The lowest achievable relative standard uncertainty for the radiance calibration of tung-

sten ribbon lamps is about 0.3% in the near infrared and visible spectral range. Due to a poorer signal-to-noise ratio the uncertainty increases in the ultraviolet spectral range to 4% (220 nm).

To avoid possible non-linearities of the emission channel during calibration, a suitable radiance standard should be of a radiance comparable in magnitude to the fluorescent sample. Or a procedure needs to be developed for the controlled attenuation of its spectral radiance without affecting its spectral emission characteristics and without interrupting the traceability chain. Furthermore, the emitted radiation should be unpolarized and the standard source should have a large and



Fig. 9. Spectral radiance transfer standards: a tungsten ribbon filament lamp (OSRAM Wi17/G) operated at radiance temperatures ($\lambda = 650$ nm) from 2300–2500 K (left) and an integrating sphere type, low-intensity spectral radiance standard (Gigahertz BN9701) (right).

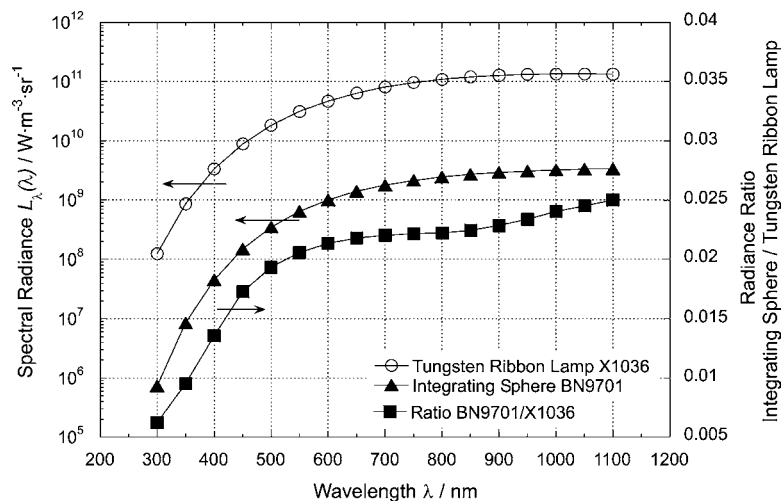


Fig. 10. Spectral radiance of a tungsten ribbon filament lamp (OSRAM Wi17/G) operated at a radiance temperature of 2300 K ($\lambda = 650$ nm) and of an integrating sphere type, low-intensity spectral radiance standard (Gigahertz BN 9701).

homogeneous radiating area, which allows to observe a radiating area comparable in size with the observed fluorescing area of the sample. Finally, the source should be easy to align and its radiance should be insensitive to slight misalignment in angle and position.

Even though tungsten ribbon filament lamps are state-of-the-art source-based transfer standards for spectral radiance at the highest metrological level due to their excellent stability, they do not fulfill all these special requirements for the traceable calibration of the emission channel of fluorimeters. For instance, the spectral radiance of a tungsten ribbon lamp is several orders of magnitude higher than that of the illuminated fluorescent sample and alignment is tedious. To better meet the requirements of fluorometry, user-friendly compact integrating sphere-based spectral radiance standards (Figs. 9–11) are currently investigated at PTB and calibrated for this purpose. Due to their lower spectral radiance however, the calibration uncertainty of their spectral radiance is increased compared to that of a tungsten ribbon lamp (Fig. 10). However, only recently at PTB the overall standard uncertainty of this calibration could be reduced to 2% in the UV region. Furthermore, first investigations of the long-term stability of these integrating sphere radiators are encouraging with changes in spectral radiance of below 2% in the visible and below 5% in the ultraviolet spectral range after 800 h of operation. However, even integrating sphere radiators are still two to three orders of magnitude brighter than a typical fluorescent sample and thus require a reliable method of wavelength-independent attenuation to enable the characterization of a fluorescence instrument under measurement conditions, i.e. with instrument set-

tings such as for instance spectral bandpass or detector voltage, as used for typical fluorescence measurements within the linear range of the emission detection system [13]. Here, either the development of a second generation of integrating sphere radiators with further reduced spectral radiances that can be still calibrated with an acceptable uncertainty or the development of an elegant procedure for the controlled attenuation of the spectral radiance of an integrating sphere type standard without affecting its emission characteristics and without interrupting the traceability chain is required. This will be presented in part II of this series [13].

DETECTOR-BASED TRANSFER STANDARD

Silicon and InGaAs semiconductor photodiodes calibrated against the cryogenic electrical substitution radiometer serve as transfer standards for the realization and dissemination of the spectral responsivity in the wavelength range from 200 to 1600 nm. For detector-based high-accuracy radiometry in the range from 200 to 1000 nm, PTB uses detectors with a special design, so-called trap detectors, consisting of a set of three windowless silicon photodiodes with a spatial orientation as shown in the schematic drawing in Fig. 12. The incoming radiation undergoes five reflections inside the detector and is therefore almost completely absorbed before it leaves the detector, the reflected optical power being less than 0.5% for wavelengths above 500 nm. Moreover, trap-detectors have proven to be very stable, nearly insensitive to polarization, and show an excellent homogeneity

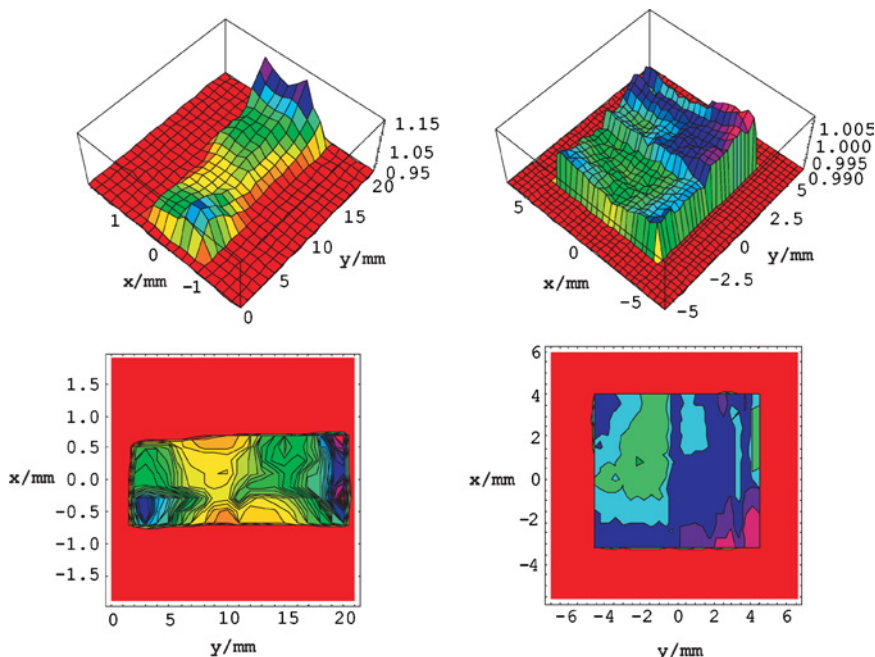


Fig. 11. The spatial radiance distribution over the radiating area at 650 nm of a tungsten ribbon filament lamp (OSRAM Wi17/G) (left) and an integrating sphere type spectral radiance standard (Gigahertz BN9701) (right). One change of greyscale corresponds to a relative change of the spectral radiance of 5×10^{-3} (tungsten ribbon lamp) and 2×10^{-3} (integrating sphere).

regarding their spectral responsivity over the detector area [14]. With these properties, trap detectors are ideal candidates for the embodiment of the spectral responsivity scale.

To establish a continuous scale of spectral responsivity at PTB, the trap-detector is calibrated in a first step against the primary detector standard, the cryogenic ra-

diometer RTCR at discrete laser lines [11,15]. Then the spectral responsivity between the laser lines is interpolated with a physical model based on the internal quantum efficiency and the reflectivity of silicon photodiodes [16]. The interpolated spectral responsivity of a silicon-photodiode trap-detector and its relative standard uncertainty is given in Fig. 13.

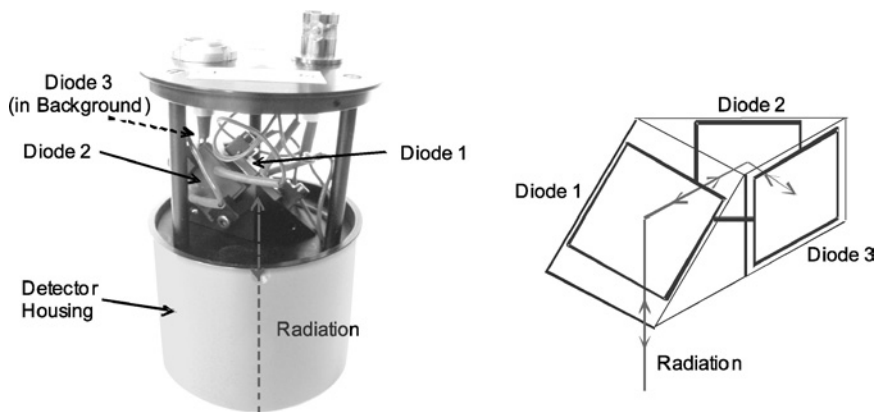


Fig. 12. The PTB-design trap-detector, a transfer standard for spectral responsivity in the wavelength range from 238 to 1015 nm (the detector housing is partly removed for better visualization purposes) (left) and a schematic view of the photodiodes positioning within the detector (right).

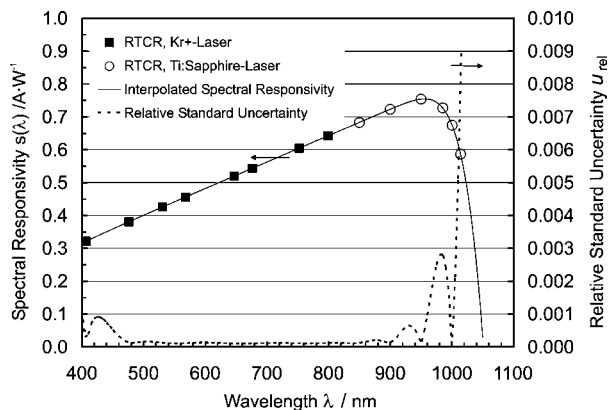


Fig. 13. Interpolated spectral responsivity $s(\lambda)$ of a silicon photodiodes trap-detector (solid line, left scale) and the corresponding relative standard uncertainty u_{rel} (dotted line, right scale). The data points result from the calibration of the trap-detector against the cryogenic radiometer RTCR at discrete laser lines.

EXAMPLE OF THE RADIOMETRIC CHARACTERIZATION OF A FLUORESCENCE INSTRUMENT

In principle, radiometric transfer standards allow a radiometric characterization of a fluorescence instrument in such a way that the system can perform quantitative measurements traceable to the SI units, i.e. absolute measurements of fluorescence intensities. However, aside from the wavelength- and polarization-dependent spectral irradiance of the excitation channel at the sample and the spectral responsivity of the emission channel, such a challenging absolute radiometric characterization requires additional knowledge of geometric quantities like the observed sample volume and the solid angle of accepted radiation by the emission channel. This is for instance mandatory for the measurement of absolute fluorescence quantum yields. For the majority of fluorescence techniques, however, it is sufficient to characterize the relative (spectral distribution of the) spectral irradiance at the sample and the relative (spectral distribution of the) spectral responsivity of the emission channel. Such a relative radiometric characterization that yields so-called emission and excitation correction curves in relative units is much easier to perform but allows only to correct the measured fluorescence data for relative instrument specific wavelength and polarization dependencies [17–19]. The corrected emission and excitation spectra that are obtained on application of these spectral correction curves on the measured data are comparable across instruments, laboratories and over time. For the comparison of relative fluorescence intensities, the additional use of application-

specific standards with excitation and emission spectra closely matching that of the analyte to be quantified is purpose-fit in most cases [18,19].

As an example for a relative radiometric characterization of a fluorescence instrument by means of physical transfer standards, the accordingly determined wavelength- and polarization-dependent relative spectral irradiance of the excitation channel or excitation correction curve and the relative spectral responsivity of the emission channel of the BAM spectrofluorometer 8100 (Spectronics Instruments), the emission correction curve, are given in Figs. 14 and 15, respectively. The calibration procedures are described in more detail in part II of this series [13]. Corrected spectra free of instrument specific distortions are obtained by division of the measured uncorrected spectra by the spectral correction curves determined at identical instrument settings (e.g. spectral bandpass, detector voltage, and polarizer settings). The relative spectral irradiance of the excitation channel at sample position that is exemplarily illustrated for the BAM fluorometer in Fig. 14 is typically determined with a calibrated detector transfer standard. Here, a silicon photodiode-integrating sphere assembly calibrated by PTB in terms of spectral responsivity to radiant power, see Fig. 14, is used. As is discussed in part II of this series [13], for the spectral correction of measured excitation spectra fluctuations of the spectral radiance of the excitation light source, that would otherwise affect the spectral correction, are taken into account via a reference detector. Principally, corrected excitation spectra free of instrument specific distortions are obtained by division of the measured uncorrected spectra by the spectral correction curve determined at identical instrument settings (e.g. spectral bandpass, detector voltage, and polarizer settings).

The wavelength- and polarization-dependent relative spectral responsivity of the emission channel of the 8100 spectrofluorometer is determined with the PTB-calibrated integrating sphere type spectral radiance transfer standard BN9701. To perform the source-based calibration of the emission channel in the linear range of the emission detection system at identical instrument settings as used for the measurement of typical fluorescent samples, the spectral radiance of the standard lamp has to be strongly reduced without influencing its spectral characteristics and without interrupting the traceability chain. Matching of the magnitude of the spectral radiance of the standard to those of fluorescent samples is achieved by illuminating a PTB-calibrated non-fluorescent diffuse reflectance or white standard at the sample position from an adjustable distance with the transfer standard lamp. This calibration procedure which exploits the quadratic distance dependence of the diffuse illumination is described

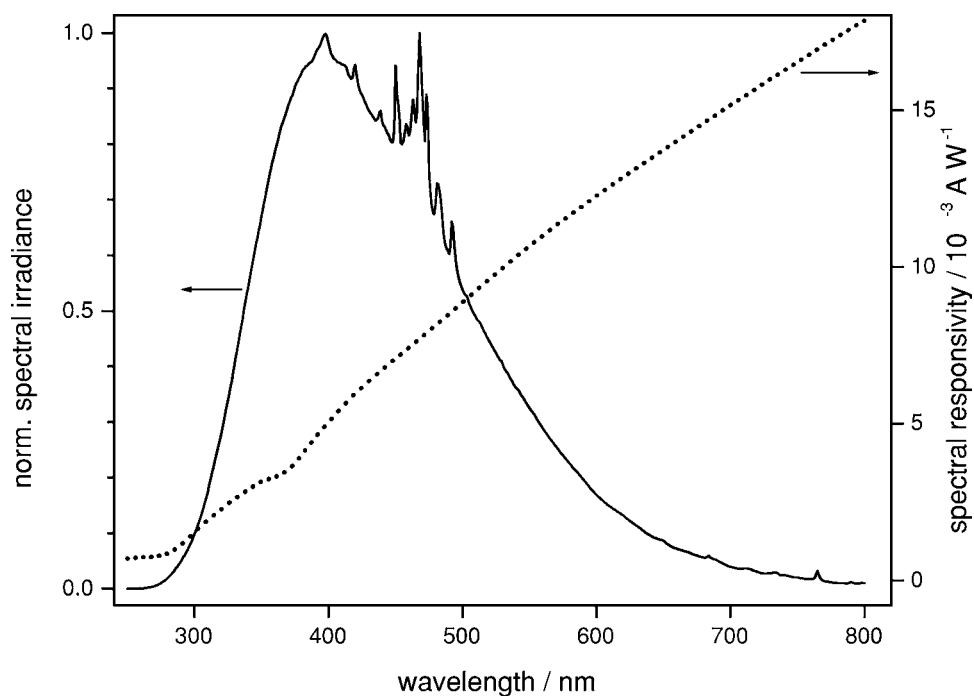


Fig. 14. Normalized spectral irradiance of the excitation channel of a fluorometer (solid line) measured with a PTB-calibrated Si photodiode of known spectral responsivity (dotted line) at sample position.

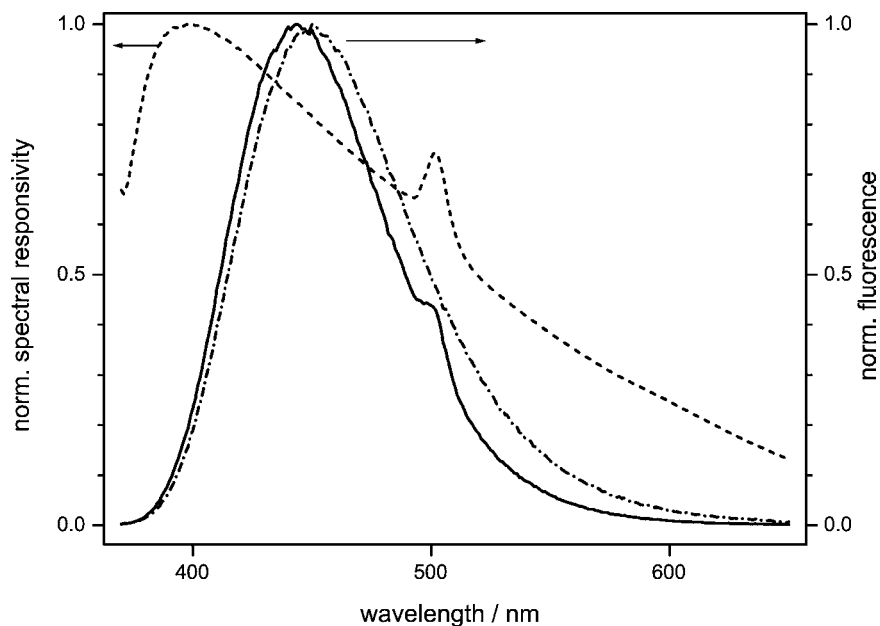


Fig. 15. Normalized spectral responsivity of the emission channel of a fluorometer (dashed line) determined with a PTB-calibrated integrating sphere radiator and a PTB-calibrated non-fluorescent white standard. To exemplify visualize the need for the correction of fluorescence spectra for instrument specific wavelength dependencies, the normalized uncorrected (solid line) and corrected emission spectra (dash dotted line) of quinine sulfate dihydrate in 0.105 M perchloric acid are shown. The corrected emission spectrum of the dye is obtained by division of the measured uncorrected spectrum by the emission correction curve.

in more detail in part II of this series [13]. The accordingly obtained normalized relative spectral responsivity of the emission channel is given in Fig. 15 (dashed line). To visualize the need for the correction of fluorescence spectra for instrument specific wavelength dependencies, in Fig. 15 also the normalized uncorrected (solid line) and corrected emission spectra (dash dotted line) of quinine sulfate dihydrate in 0.105 M perchloric acid are exemplary shown. The corrected emission spectrum of the dye is obtained by division of the measured uncorrected spectrum by the emission correction curve. The bump seen in the uncorrected emission spectrum results from diffraction effects (Wood anomalies) of the monochromator gratings used and is eliminated by spectral correction.

SUMMARY AND OUTLOOK

The presented approach for the traceable radiometric calibration of the excitation and emission channel of a fluorescence instrument with highest possible accuracy by the use of physical standards forms the baseline for the establishment of radiometric traceability in fluorometry. However, due to the complexity of this method and the expensive instrumentation involved it is recommended only to a limited number of users, i.e. national metrology laboratories operating reference fluorescence instruments or colorimeters, and laboratories needing an extensive radiometric characterization of their high-accuracy fluorescence instrument. Furthermore, the physical size of the transfer standards usually exceeds the size of a standard cuvette and inhibits their application in many compact fluorometer systems. Therefore, using chemical standards, that can e.g. be adapted to different measurement geometries, with certified fluorescence excitation and emission spectra for the routine calibration of fluorescence instruments, as explained in Part II of this series, seems more appropriate and convenient for the majority of users.

To overcome the above mentioned limitations of physical radiometric transfer standards in fluorometry the basic outline of a compact physical transfer standard which might allow user-friendly routine calibrations of fluorescence instruments is presented in Fig. 16. The can- or cuvette-shaped standard consists of a diffuser plate mounted inside a cavity with diffuse reflecting inner walls, having an outer size that fits well into the sample holder of a common fluorescence instrument. The spectral irradiance from the excitation channel of the fluorescence instrument to be calibrated enters the cavity, is diffusely reflected by the diffuser plate and the cavity walls, and simultaneously detected by a built-in high-stability silicon photodiode. The inner geometry of

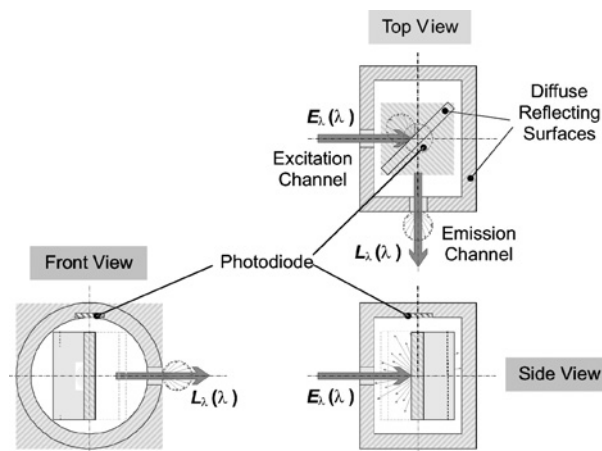


Fig. 16. Schematic of a new type, easy-to-use physical transfer standard for the simultaneous radiometric characterization of the excitation and emission channel of a fluorescence instrument.

the cavity and the reflectivity of the diffuser material can be chosen such that the radiance of this Lambertian radiator which leaves the standard and is detected by the emission channel of the fluorescence instrument matches well with the intensity of a typical fluorescence signal. The advantage of the presented standard is that the complete standard (cavity and diode as one unit) can be calibrated for its absolute spectral responsivity and the cavity for its spectral transmission. With such a standard the characterization of the excitation and emission channel can be performed simultaneously, using the radiation from the excitation channel in combination with the standard for the calibration of the emission channel. Such an approach can be also of considerable interest for colorimetry.

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