

# Need for and Metrological Approaches Towards Standardization of Fluorescence Measurements from the View of National Metrology Institutes

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**Abstract** The need for standardization in fluorescence measurements to improve quality assurance and to meet regulatory demands is addressed from the viewpoint of National Metrology Institutes (NMIs). Classes of fluorescence standards are defined, including instrument calibration standards for the determination and correction of instrument bias, application-specific standards based on commonly used fluorescent labels, and instrument validation standards for periodic checks of instrument performance. The need for each class of standard is addressed and on-going efforts by NMIs and others are described. Several certified reference materials (CRMs) that have recently been developed by NMIs are highlighted. These include spectral correction standards, developed independently by both NIST and BAM (Germany), and fluorescence intensity standards for flow cytometry, developed by NIST. In addition, future activities at both institutes are addressed such as the development of day-to-day intensity standards.

**Keywords** Calibration · Emission standards · Fluorescence intensity standards · Fluorescence standards · Quality assurance

### Abbreviations

ABC	Antibody binding capacity
ASTM	ASTM International
BAM	Federal Institute for Materials Research and Testing, Germany
CRM	Certified reference material
EEM	Excitation-emission matrix
FDA	United States Food and Drug Administration
FL1	First fluorescence channel of a flow cytometer
ISO	International Organization for Standardization, Geneva
IUPAC	Union of Pure and Applied Chemistry
LED	Light emitting diode
MESF	Molecules of equivalent soluble fluorophore
NBS	National Bureau of Standards, USA
NIST	National Institute of Standards and Technology, USA
NMI	National Metrology Institute
NPL	National Physical Laboratory, UK
NRC	National Research Council, Canada
OLED	Organic light emitting diode
PTB	Physikalisch-Technische Bundesanstalt, Germany
R	Measured reference signal
S	Measured fluorescence signal, uncorrected
S <sub>cor</sub>	Measured fluorescence signal, corrected for detection system responsivity
SOP	Standard operation procedure
SRM®	Standard Reference Material®

## 1

### Introduction

The use of fluorescence continues to increase in the life and material sciences with many techniques having matured to a state where quantification is desired [1–6]. Compared to other prominent analytical methods, however, standardization of fluorescence measurements is still in its infancy, despite the fact that it is not a new technique. Its birth was marked by Stokes' study of quinine sulfate in 1852 [7], where he used what can today be recognized as a basic fluorometric setup, but it was not until the appearance of the first commercial instruments in the late 1950s that fluorescence became a common analytical tool [8]. Since this time, the range of fluorescence-based techniques has expanded greatly with parameters measured including (integral) fluorescence intensities at selected excitation and emission wavelengths and polarization conditions, often in combination with spatial and temporal resolution, and increasingly at the single-molecule-detection level. However, what is often overlooked is that all types of fluorescence measurements yield

signals that contain both analyte- and instrument-specific contributions, and the resulting demand to remove the latter from the former to obtain fluorescence data comparable across instruments, laboratories, and over time is increasing [9]. Further fluorescence-inherent drawbacks relate to difficulties in accurately measuring absolute fluorescence intensities and hence in realizing true quantitative measurements [10]. In addition, the sensitivity of the absorption and fluorescence properties of most chromophores to their microenvironment can hamper quantification based on measurements of relative fluorescence intensities. Despite these problems and the ever-increasing complexity of fluorescence instrumentation, there are still very few commercially available reference materials to aid in the qualification of fluorescence instruments and the validation of related measurements or assays. Moreover, guidelines for the characterization of fluorescence instruments and for the performance and evaluation of selected measurements that were developed, mostly in the 1980s, e.g., by ASTM International, the Ultraviolet Spectrometry Group, a UK-based forum of spectrometer users from industry, academia and government, and the International Union of Pure and Applied Chemistry (IUPAC), focus mainly on steady-state fluorometry and do not include more modern fluorescence instrumentation and methods [11–16].

The present state of standardization of fluorescence measurements is closely linked to past and ongoing efforts of National Metrology Institutes (NMIs) in the development of fluorescence standards. In the late 1960s, the National Bureau of Standards (NBS), now known as the National Institute of Standards and Technology (NIST), became involved in fluorescence spectrometry to characterize “television” phosphors provided as NBS Standard Sample Phosphors [17]. By the early 1970s, NBS as the most active NMI in the fluorescence area<sup>1</sup> started to develop material standards for the calibration of fluorescence spectrometers and held related workshops to meet the demands of the clinical chemistry community [18, 19]. In addition, a high-accuracy fluorescence spectrophotometer was built and qualified to achieve the levels of precision and accuracy required for certified reference materials (CRMs) [20–22]. This instrument was used to certify the corrected emission spectrum, i.e., the instrument-independent spectrum, of Standard Reference Material® (SRM®) 936 Quinine Sulfate Dihydrate in 1979 [23], which covers the spectral region from about 395 to 565 nm.<sup>2</sup> This material was the first spectral correction standard for fluorescence to be released by an NMI and is still available as SRM 936a [9, 24]. In 1989, SRM 1931 [25] was released as a set of four solid spectral fluorescence standards in a cuvette format, but was restricted in measurement geometry and certified using polarizers [26]. These

<sup>1</sup> BAM (Federal Institute of Materials Research and Testing, Germany), NRC (National research Council, Canada), NPL (National Physics Laboratory, UK), and TKK (Metrology Resrach Institute, Finland) were mainly active in the area of colorimetry or so-called bispectral fluorescence at that time.

<sup>2</sup> Fluorescence intensities of  $\geq 10\%$  of the emission at the maximum of the band are required at least for spectral calibration with acceptable uncertainties.

efforts provided the basis to establish how and why users of fluorescence techniques should regularly calibrate their instruments and initiated the development of physical and chemical transfer standards of varying quality by a number of companies [9].

Despite the existing literature on instrument calibration [1, 5, 14, 19, 22], pressures from regulatory agencies, and the global trends of quality assurance, traceability, and accreditation [27, 28], many users of fluorescence techniques continue to restrict instrument characterization to calibration curves of fluorescence intensity versus concentration for quantifying analyte concentrations of unknowns. The influence of other instrument parameters on measured fluorescence quantities, such as emission spectra, is typically neglected, or the necessary corrections are expected to be included in the instrument manufacturer's software. At the same time, many of the newer fluorescence-based assays are employed in the highly regulated areas of clinical diagnostics, drug production, and environmental monitoring. In addition, the desire for analyte quantification from fluorescence measurements is ever-increasing in both conventional areas, e.g., spectrofluorometry, flow cytometry, and fluorescence assay technologies and newer areas with more complex instrumentation, e.g., fluorescence imaging techniques such as (confocal) fluorescence microscopy and fluorescence-based microarray technology. These developments demanded a reassessment of effective instrument calibration and qualification procedures and encouraged several NMIs, including the Federal Institute for Materials Research and Testing (BAM, Germany), NIST (USA), National Physical Laboratory (NPL, UK), National Research Council (NRC, Canada), and the Physikalisch-Technische Bundesanstalt (PTB, Germany), to become more active in the past several years in fluorescence standardization. In combination with the output from recent fluorescence workshops and questionnaires, organized by NIST [29–34] and BAM [35], this situation motivated BAM and NIST to dedicate research activities to the development of reliable instrument-type and application-specific fluorescence standards for various fluorescence techniques. In this chapter, recent, ongoing, and future standard activities at NIST and BAM are presented and directions still needing to be explored and addressed by NMIs in the fluorescence area are highlighted.

## 2

### **Fluorescence Standards: Types, Requirements and Quality Criteria for Choice**

#### 2.1

##### **Classification of Fluorescence Standards**

Fluorescence standards can be divided into three general types: (i) instrument calibration standards, both physical and chemical, i.e., chromophore-

based, for the determination and correction of instrument bias; (ii) application-specific fluorescence standards, based on routinely measured fluorophores or closely related<sup>3</sup> chromophores that take into account the fluorescence properties of typically measured samples and are thus particularly useful for the validation of methods involving such samples; and (iii) instrument validation standards, both physical and chemical, for periodic checks of instrument performance [9]. Standards of the first type include spectral fluorescence standards with known corrected, i.e., instrument-independent, spectra [9]. A classic example of application-specific fluorescence standards are fluorescence intensity standards that relate chemical concentration to instrument response. Such materials are fluorophore-specific. Fluorescence quantum yield standards [9], which belong to this class of standards as well, can be used to link fluorescence intensity to an absolute scale. Examples of instrument validation standards are day-to-day intensity standards, which test the instrument's day-to-day performance and long-term stability and may even enable correction for these variabilities. Such standards can also be applied as instrument-to-instrument intensity standards for the comparison of measured fluorescence intensities between instruments when measurement parameters are fixed. Requirements on different types of fluorescence standards, similar to those given here, have recently been defined by BAM [9].

## 2.2

### Quality Criteria for Fluorescence Standards

The value of a physical or chemical fluorescence standard depends on its suitability and reliability for the respective task, e.g., instrument characterization or quantification. Criteria for the production of reference materials – and thus also of fluorescence standards – are stated in *ISO Guide 34* [36] and *ISO Guide 35* [37], the calculation of uncertainties is described in the *Guide of the Measurement of Uncertainty* (GUM) [38], and traceability is defined in *EN ISO/IEC 17025* [28].

The reliability of a fluorescence standard is determined by the characterization of its calibration-relevant properties [9]. This includes the characterization of the instrument and the measurement conditions used for the determination of the calibration-relevant quantity or quantities, the standard's stability under application-relevant conditions that determine its shelf life and storage conditions, and the uncertainty of the certified/characterized quantity or quantities. Moreover, additional information relevant for proper use of the standard, a well-defined scope, limitations for use, and a tested standard operation procedure (SOP) should be mandatory. In principle, fit-for-purpose

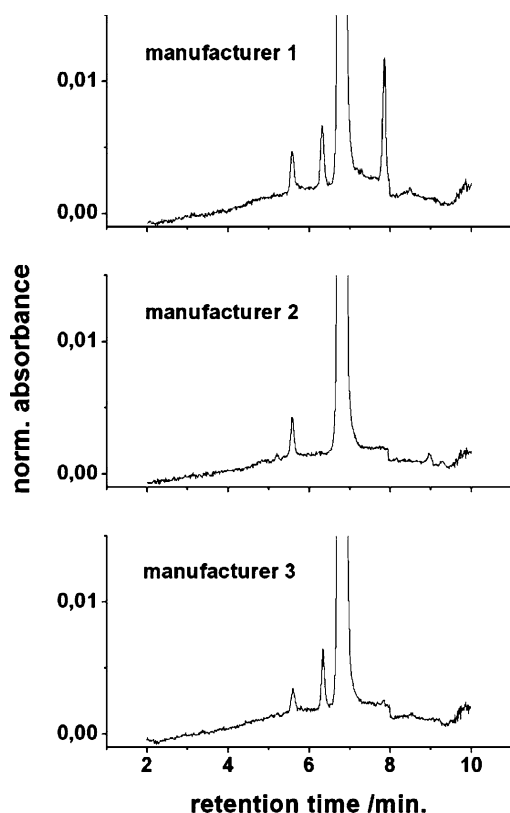
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<sup>3</sup> “Closely related” refers to spectral behavior, i.e., matching or similar absorption and emission spectra.

fluorescence standards, designed for a broad community of users of fluorescence techniques, need to consider typical samples to be encountered and measurement conditions commonly employed. These include measurement geometry, format of the sample, and common instrument settings. Also, the radiated/emitted intensity and the size and shape of the radiating volume of standard and sample should be comparable. This allows instrument calibration and fluorescence measurements to be made under similar conditions while the instrument's detection system is operated within its linear range.

To minimize standard-related uncertainties for the three types of fluorescence standards, information on the wavelength and temperature dependence of the application-relevant fluorescence properties is mandatory as well as on the concentration dependence of these features where applicable. Values of the fluorescence anisotropy should be provided as this quantity determines the need for the use of polarizers and the size of polarization-related contributions to the overall uncertainty. Also the determination of the standard's purity including method of analysis and respective uncertainty is important as dye purity can affect the standard's spectroscopic properties, photochemical and thermal stability, and reproducibility. Dye purity can vary substantially between different dye manufacturers and lots (see Fig. 1) and has historically contributed to errors in reported spectroscopic quantities, such as molar absorption coefficient [39] and fluorescence quantum yield [40]. For liquid standards, the solvent to be employed also needs to be characterized with respect to properties potentially affecting fluorescence, such as water content for hygroscopic solvents and pH and ionic strength for aqueous solvents. Alternatively, the solvent should be provided with the standard at a constant quality level. Solid standards require additional characterization of the homogeneity of the dye's distribution in the matrix to guarantee a uniform fluorescence. Additional requirements inherent to the respective application and type of fluorescence standard can be found in the corresponding sections.

The majority of fluorescence standards recommended in the literature, commercially available, or in-house prepared [9] do not meet these criteria. This results in an enhanced calibration or measurement uncertainty or, at worst, an instrument characterization that is not reliable. Also, these standards typically do not yield traceability to the spectral radiance or spectral responsivity scale relevant for fluorometry; see also Chap. 3 on the traceability of fluorometry in this book [41]. In contrast, reference materials from NMIs are supplied with certified values and uncertainties that apply to the individual or batch material and follow the *GUM*, are produced according to *ISO Guides 34* and *35*, and are traceable to SI units whenever possible. In addition, NMIs are typically required to have the highest confidence possible in the measured values for reference materials before these materials can be released as CRMs [42]. Measurement integrity of (certified) values is assured by the determination of these values with multiple, independent techniques, the investigation of all known or suspected sources of error, and the performance



**Fig. 1** HPLC diagram of an exemplary organic dye revealing typical deviations in dye purity between batches from different dye manufacturers. Depending on the chemical nature and spectroscopic properties of the impurities, this can affect the spectroscopic properties of a dye, such as molar absorption coefficient (at a selected wavelength) and fluorescence quantum yield, as well as its photochemical and thermal long-term stability

of interlaboratory comparisons with other NMIs. At present, NIST and BAM are the only NMIs releasing CRMs for the characterization of fluorescence instruments [43–50].

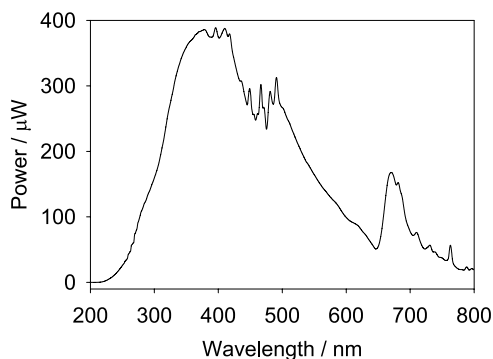
### 2.3

#### Fluorescence Calibration Standards

Outcomes from recent workshops organized by NIST and BAM and results from distributed questionnaires have highlighted the importance of fluorescence calibration standards and certified, corrected fluorescence spectra to the fluorescence community [29–35]. This includes wavelength standards with multiple narrow emission bands spanning the UV/Vis/NIR spectral region to validate the wavelength accuracy of wavelength-selecting optical

components [51] and so-called emission and excitation standards having broad unstructured spectra for the determination of the spectral responsivity and intensity characteristics of fluorescence instruments [9, 52–54]. A number of spectral fluorescence standards have been reported in the literature and several are available from commercial (non-NMI) sources. However, none of these materials are supplied with traceable and certified, corrected fluorescence data and corresponding wavelength-dependent uncertainties [9, 55].<sup>4</sup> In particular, the reliability of the corrected emission spectra of emission standards is often questionable.

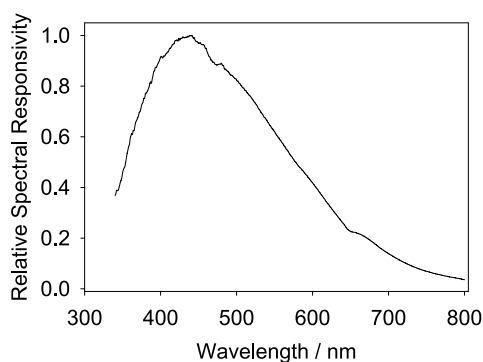
The importance of such fluorescence calibration standards, particularly of emission standards, is obvious as measured fluorescence signals are not only determined by the analyte's absorptance at the excitation wavelength and its spectral fluorescence yield, but also contain contributions from the instrument-specific spectral irradiance at the sample position and the instrument-specific spectral responsivity of the emission channel or detection system [9, 14, 56–58]. These two quantities are wavelength dependent (see Figs. 2 and 3 [58, 59]). This is a result of the combination of the wavelength dependence of the spectral radiance of the excitation light source, the transmittance of components like lenses, mirrors, filters, monochromator gratings and polarizers in the optical paths of the excitation and emission channels, and the spectral responsivity of the detector of the fluorescence instrument. In addition, they are polarization dependent, as discussed in Chaps. 3 and 6 in [41, 60], and time dependent, due to aging of instrument components.



**Fig. 2** Excitation intensity or flux at the sample position of a typical spectrofluorometer measured with a calibrated detector

<sup>4</sup> See for instance Invitrogen or former Molecular Probes, Starna GmbH, Matech Precision Dynamics Corp., Labsphere Inc., LambdaChem GmbH and Photon Technology International Inc. (DYAG,) FA-2036. Certain commercial equipment, instruments, or materials are identified in this chapter to foster understanding. Such identification does not imply recommendation or endorsement by 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.

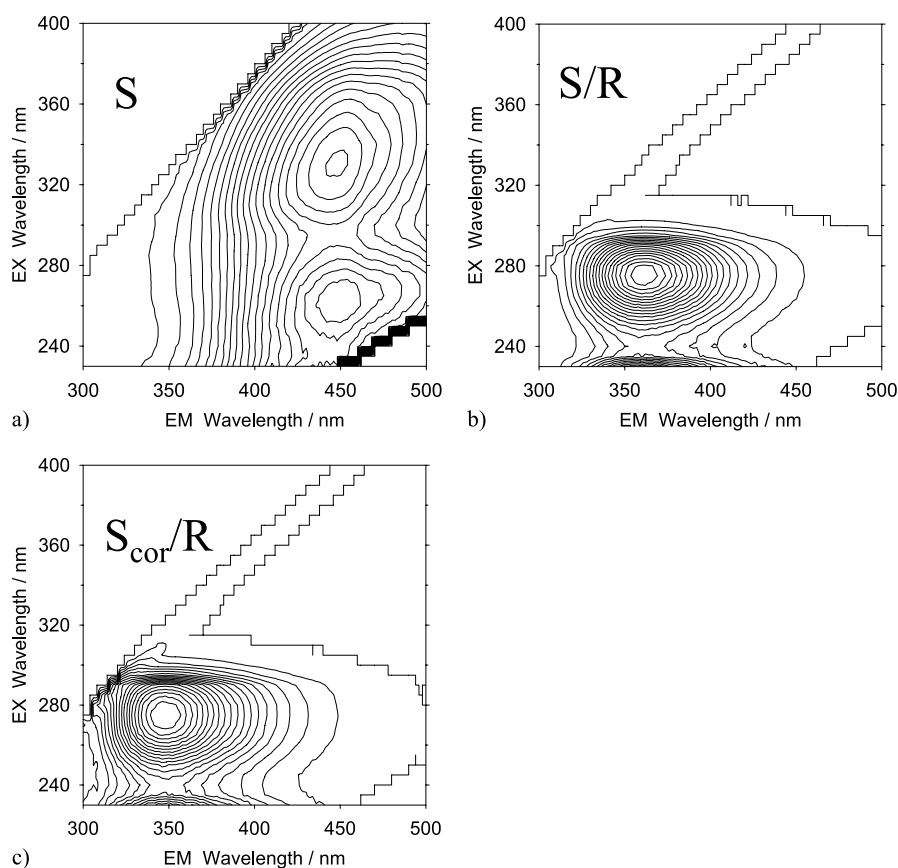




**Fig. 3** Relative spectral responsivity of the emission channel of a typical spectrofluorometer measured with a calibrated lamp

The influence of these instrument-specific quantities on fluorescence data is illustrated in Fig. 4 [58, 59] for an excitation-emission matrix (EEM) or so-called total fluorescence spectrum of tryptophan. This figure compares the measured uncorrected signal  $S$  (Fig. 4a), the excitation intensity-corrected signal  $S/R$  (Fig. 4b) that takes into account the wavelength dependence of the spectral irradiance of the instrument's excitation channel shown in Fig. 2, and the excitation- and emission corrected, i.e., instrument-independent, signal  $S_{\text{cor}}/R$  (Fig. 4c). The last considers both instrument-specific quantities. As follows from a comparison of these signals, without any spectral correction, two peaks are observed instead of one with both dramatically shifted by 100 nm or more (see Fig. 4a). When only the excitation intensity correction is applied, a single, qualitatively correct spectrum is obtained, but the peak is still shifted by about 12 nm (see Fig. 4b). The obvious differences between these data clearly demonstrate the need for and importance of the spectral correction of excitation and emission spectra and EEMs for comparable fluorescence data.

As only relative fluorescence intensities are typically measured, correction of measured data for the relative spectral irradiance of the excitation channel (termed excitation correction) and/or the relative spectral responsivity of the emission channel (termed emission correction) are sufficient [9, 56–58], as detailed in Chap. 3 in [41]. In most cases, there is no need to link fluorescence intensity to an absolute scale, i.e., to absolute radiometric units. When quantification from or longterm comparability of measured relative fluorescence intensities is desired, the requirements of most users can be satisfied using either a calibration curve for concentration or a (relative) day-to-day intensity standard, as described in a forthcoming section, in addition to the correction for the instrument's spectral characteristics.



**Fig. 4** Comparison of contour-plotted EEM spectra for tryptophan, where the fluorescence intensity is **a**  $S$  the measured uncorrected signal, maxima at  $(\lambda_{EX}, \lambda_{EM}) = (265 \text{ nm}, 452 \text{ nm})$  and  $(330 \text{ nm}, 446 \text{ nm})$ , **b**  $S/R$  the signal corrected for the spectral irradiance at the sample position, shown in Fig. 2, maximum at  $(\lambda_{EX}, \lambda_{EM}) = (275 \text{ nm}, 358 \text{ nm})$ , and **c**  $S/R_c$  the instrument-independent signal, corrected for both the instrument's spectral irradiance and its spectral responsivity, displayed in Figs. 2 and 3, maximum at  $(\lambda_{EX}, \lambda_{EM}) = (275 \text{ nm}, 346 \text{ nm})$

### 2.3.1

#### Emission Standards with Certified Corrected Emission Spectra

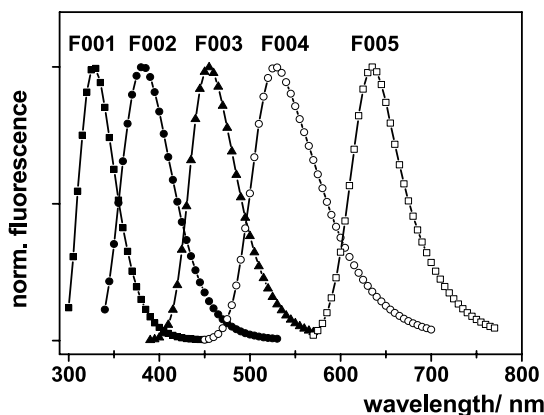
The relative spectral responsivity of the emission channel of a fluorescence instrument can be most easily determined with chromophore-based emission standards, whose corrected, i.e., instrument-independent emission spectra are known and preferably certified. Accordingly, both BAM and NIST have responded to the increasing need for quality assurance in fluorescence in the past several years by qualifying fluorescence spectrometers for measuring

corrected fluorescence spectra [56, 58, 59]. These instruments have been and will be used for the certification of corrected fluorescence spectra with known uncertainties [44, 45, 61]. As a first step towards standardization of the spectral characteristics of fluorescence instruments on a broad level, BAM and NIST have independently developed new sets of standards for the determination of the relative spectral responsivity of fluorescence instruments and the spectral correction of emission data.

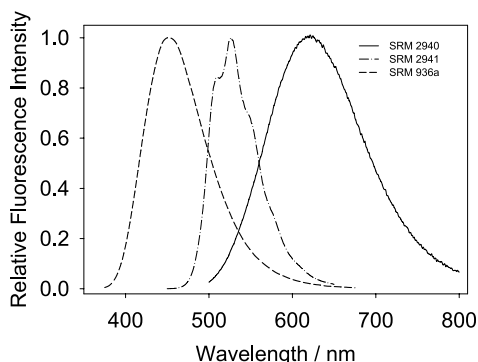
The BAM Calibration Kit *Spectral Fluorescence Standards* released in January 2006 [62, 63] consists of the five spectral fluorescence standards BAM-F001 through BAM-F005 (equaling the organic dyes A–E in earlier publications) with certified, normalized, corrected emission spectra covering the spectral range from 300 to 770 nm as a set (see Fig. 5). These dyes are provided as ready-made bottled solids, either as a kit [61–63] or individually [46–50]. They are readily transformed into dye solutions that can be measured without any additional dilution steps with routinely used settings of the fluorescence instrument to be calibrated. To minimize standard-related uncertainties, BAM developed the software *LINKCORR* for data evaluation and spectra linking [9, 61, 62].<sup>5</sup> The wavelength-dependent, expanded relative uncertainties supplied include uncertainties related to the calibration of the BAM fluorometer and to the measurement of fluorescence spectra with this instrument, as well as material-related uncertainties, such as batch-to-batch dye homogeneity and thermal stability [61–63]. The influence of other parameters, such as the bandwidth of the emission monochromator, temperature, and the excitation wavelength, was minimized by careful choice of standard materials [46–50, 61–63]. Due to their liquid nature and the minimum spectral overlap between their absorption and emission spectra, BAM-F001 through BAM-F005 offer a unique flexibility with respect to measurement geometry, format, and type of fluorescence instrument to be calibrated [63] and have been successfully used for the characterization of microplate readers and for the characterization of the spectral characteristics of spectral imaging systems [64]. Moreover, the BAM spectral fluorescence standards can be used in conjunction with all types of light sources (continuous and pulsed-lamps, lasers etc.) due to their short fluorescence lifetimes of a few nanoseconds.

The two new NIST standards, SRMs 2940 (*Orange Emission*) and 2941 (*Green Emission*) *Relative Intensity Correction Standards for Fluorescence Spectroscopy*, cover the spectral region from 395 to 780 nm, when combined with SRM 936a *Quinine Sulfate Dihydrate* as a set (see Fig. 6 [59]) [65]. SRMs 2940 and 2941 [44, 45] are ready-to-use, cuvette-shaped glasses with three long sides polished and one side frosted, allowing measurements using instruments with 0°/90°, front-face or epifluorescence geometries. Similar to

<sup>5</sup> BAM-F001–BAM-F005 ready-made from Sigma-Aldrich Production GmbH (former Fluka Production GmbH) are available from BAM or from Sigma-Aldrich. The corresponding product numbers from Sigma-Aldrich are 97003-1KT-F for the Calibration Kit and 72594, 23923, 96158, 74245, and 94053 for BAM-F001–BAM-F005, respectively.



**Fig. 5** Normalized corrected emission spectra of the Calibration Kit *Spectral Fluorescence Standards* BAM-F001–BAM-F005



**Fig. 6** Normalized corrected emission spectra of the spectral correction standards SRMs 2940, 2941, and 936a

BAM-F001 through BAM-F005, the supplied uncertainties of the certified corrected emission spectra of the NIST SRMs arise from uncertainties related to the measurements by and calibration of the spectrofluorometer used for certification [58], as well as from material-related uncertainties. In the case of the new NIST SRMs, the latter includes the dependence of the emission spectra on excitation wavelength, spectral bandpass, concentration (inner filter effects), polarization of the excitation beam, responsivity of the detection system to fluorescence with different polarizations, and sample temperature. Information provided with each standard will include the temperature coefficient for fluorescence intensity and the anisotropy of the fluorescence at the peak maximum along with instructions for use. Software for calculating values and uncertainties of the certified spectra for any emission wavelength or wavelength interval will also be supplied. The certified values of these

SRMs are valid for ten years from the date of issue (April 2007). Due to the comparably long luminescence lifetimes of the dopants, inorganic metal ions, in the  $\mu\text{s}$  to  $\text{ms}$  region, use of these materials is recommended for instruments equipped with continuous light sources. In the case of pulsed light sources, the emission properties of these dopants can be affected by measurement parameters, such as pulse duration, delay, and gate [9], see also Sect. 2.5. For instance, the certified values for SRM 2941 can, but those for SRM 2940 cannot, be used, with pulsed light sources.

### 2.3.2

#### **Interlaboratory Comparison on Corrected Emission Spectra**

Along with the certification of their new spectral fluorescence standards, NIST and BAM organized a comparison amongst the NMIs (NRC, PTB, NIST, and BAM) active in the area of high precision spectrofluorometry to evaluate the comparability of their emission spectra measurements. This study, which will provide the basis for future steps towards instrument standardization and will be jointly published, focused on the determination of the relative spectral responsivity of spectrofluorometers in a  $0^\circ/90^\circ$  (PTB, BAM, and NIST) and  $45^\circ/0^\circ$  (NRC) measurement geometry using both physical transfer standards, i.e., calibrated lamps and detectors, and spectral fluorescence standards, here BAM-F001 through BAM-F005.

### 2.3.3

#### **Chromophore-Based Wavelength Standards**

Current research activities at NIST and BAM include the development of wavelength standards for the control of the wavelength accuracy and spectral resolution of fluorescence measuring systems in the UV/Vis/NIR spectral region [9, 51]. These materials are not intended as substitutes for atomic discharge lamps that, having extremely narrow emission lines and well known spectral band positions (including uncertainties) [65], are commonly used for the calibration of the wavelength scale of high precision spectrofluorometers to an accuracy of typically  $\pm 0.5 \text{ nm}$  [11, 67, 68]. However, chromophore-based wavelength standards present an elegant, easy-to-use alternative to such lamps, particularly for compact fluorescence instruments with a reduced spectral resolution, such as portable fluorometers, spectral imaging systems, or certain microplate readers, which are becoming increasingly more prevalent [9, 51]. Suitable materials should emit a multitude of narrow emission bands or lines in the UV/Vis/NIR region properly separated by at least 20 nm. Structured or very narrow bands may be used for the determination of the instrument's spectral resolution.

A variety of materials are potentially suited as chromophore-based wavelength standards, including inorganic crystals and glasses and organic-doped

polymers [9, 55, 69]. Both BAM and NIST are developing glass-based wavelength standards, such as cuvette-shaped glasses doped with a variety of different inorganic metal ions [9, 51]. Since the narrow spectra of these candidate wavelength standards are emitted as a consequence of absorption of the instrument's excitation light, and the intensity of the luminescence can be controlled via dopant concentration, the shape and size of the radiating volume and the spectral radiance/fluorescence intensity of such a solid standard can be made comparable to that of commonly measured samples. Accordingly, they can be used under routine measurement conditions. This may not necessarily be true for a comparatively bright atomic discharge lamp which must be attenuated to avoid detector saturation and where improper alignment of the lamp can lead to wavelength biases. BAM's candidate wavelength standards, which are currently tested with spectrofluorometers at a  $0^\circ/90^\circ$  measurement geometry, may be eventually provided in different formats for different fluorescence techniques including, for instance, fluorescence microscopy. As spectral bandpass can vary amongst instrument manufacturers, e.g., portable fluorometers or spectral imaging systems, and potential candidates for wavelength standards do not typically have symmetric emission bands, these wavelength standards will be certified at multiple bandwidths or spectral bandpasses, some narrow and some broad similar to the certification of the Calibration Kit Spectral Fluorescence Standards BAM-F001–BAM-F005 [46–50, 61, 62] and SRM 2034 *Holmium Oxide Solution* or SRM 2065 *UV/Vis/NIR Transmission Wavelength / Vacuum Wavenumber Standard*, wavelength standards for UV/Vis absorbance.

## 2.4

### Application-Specific Fluorescence Intensity Standards

Quantification of fluorophores from measurements of fluorescence intensities is hampered by two facts: the very challenging determination of absolute fluorescence intensities and the sensitivity of the molar absorption coefficients and fluorescence quantum yields of most chromophores to their microenvironment. Measurement of absolute fluorescence intensities imposes very strong requirements on instrument calibration because the spectral characteristics of the fluorescence instrument used need to be considered, as detailed in the previous section, as well as the collection efficiency of the instrument's detection system, a similarly instrument-specific property that is even more difficult to measure. Tedious measurements of absolute fluorescence intensities can be elegantly circumvented by the use of fluorescence intensity standards that link the measured fluorescence intensity of a sample to that of a standard, thereby defining a relative intensity scale comparable across instruments and laboratories. In the following, approaches to and examples of fluorescence intensity standards [9] from NIST and BAM, along with strategies to minimize the influence of dye microenvironment on quantification are

presented. Day-to-day and instrument-to-instrument intensity standards that have a similar scope are detailed in a later section.

#### 2.4.1

##### Fluorescence Quantum Yield Standards

Fluorescence quantum yield standards, which are employed as a reference for the determination of the (relative) fluorescence quantum yield of an analyte, are typically not based on the same fluorophore as the sample, contrary to the fluorescence intensity standards detailed in the next section that relate chemical concentration to instrument response [9]. They do not require matching of the spectra of the standard and the sample, but both systems should absorb and emit within comparable spectral regions [9]. However, spectrally corrected emission spectra for both the standard and the sample are mandatory in the vast majority of cases [1, 4, 5, 14]. The most stringent requirements on these standards are a reliable fluorescence quantum yield with a stated uncertainty and properly defined and reported measurement conditions, such as matrix, oxygen concentration, temperature, and excitation wavelength, as well as dye purity and applicable concentration range. These requirements are typically not fulfilled for most fluorescence quantum yield standards described and recommended in the literature [9] (see also Chap. 4 on fluorescence quantum yield standards) [70]. This encouraged BAM to investigate different strategies for the reliable determination of fluorescence quantum yields and to characterize existing and develop new fluorescence quantum yield standards. As a first step towards standards with certified fluorescence quantum yields, BAM is testing a set of fluorophores emitting in the UV/Vis/NIR region and characterizing a new, recently built reference fluorometer designed for the determination of absolute fluorescence quantum yields [57, 58]. Such standards and the measurement of absolute fluorescence quantum yields are likely to gain in importance due to an increasing desire for reliable relative and absolute fluorescence quantum yields of a variety of materials, including those used in LEDs and OLEDs [71],<sup>6</sup> as well as NIR fluorophores.

#### 2.4.2

##### Standards that Relate Chemical Concentration to Instrument Response

Standards that relate chemical concentration to instrument response compare the fluorescence intensity of a sample to that of a standard of known fluorophore content under identical measurement conditions to quantify the concentration or number of fluorophores. Accordingly, the same fluorophore as that to be quantified in the sample is used for the production of this type

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<sup>6</sup> LED: light emitting diode; OLED: organic light emitting diode.

of intensity standard. To eliminate errors in quantification, the dye purity in the reference material needs to be determined (see Sect. 2.2 and Fig. 1) [39], aggregation should be avoided, and the chromophore(s) to be detected in the sample and in the standard have to be in the same microenvironment to guarantee identical molar absorption coefficients (at the excitation wavelength), fluorescence spectra, and fluorescence quantum yields [72]. In December 2004, NIST issued SRM 1932 *Fluorescein Solution*, a solution of high and well characterized purity of the most widely used fluorescent label, fluorescein, dissolved in a borate buffer at pH 9.5, and certified this solution for concentration. This included the identification of the chemical nature of impurities and the determination of their relative percentages, using at least seven different analytical techniques including  $^1\text{H}$ -NMR, HPLC, and a variety of elemental analyses [43], following the NIST guidelines for the traceable assessment of chemical purity [73].

### 2.4.3

#### The MESF Concept

For the majority of fluorescence techniques, the criterion of matching microenvironments between sample and standard can only be met to a certain degree. This is particularly true for the comparison of free and immobilized fluorophores, i.e., dyes attached to beads, particles or macro- and biomolecules, e.g., polymers, proteins, antibodies, and DNA. Accordingly, different concepts for fluorescence intensity standards have been developed that all aim at the consideration and minimization of the effect of dye microenvironment on fluorophore quantification. For flow cytometry, which has become a very prominent technique in clinical diagnostics for acquiring cell counts, such as the fraction of cells infected with a particular antigen in a blood sample, the concept of molecules of equivalent soluble fluorophore (MESF) has been introduced [74, 75] as an exemplary scheme for reliable quantification in complex microenvironments.

In flow cytometry, the antibody binding capacity (ABC) or absolute number of antibodies bound to a single cell is the most important quantity to be determined [76, 77]. However, the molar absorption coefficient at the excitation wavelength and especially the fluorescence quantum yield can change dramatically upon attachment to an antibody [78]. Moreover, the number of fluorophores bound to a particular antibody is both difficult to control and to determine, and the label density can vary within a batch of labelled antibodies and between different batches. In addition, flow cytometers can only measure fluorescence intensities of micrometer-sized structures such as cells suspended in a liquid. As fluorescently labelled cells are perishable, the calibration of the fluorescence channel number of flow cytometers is commonly performed with sets of microbeads with different amounts of surface-bound fluorophores and assigned MESF units. These units express



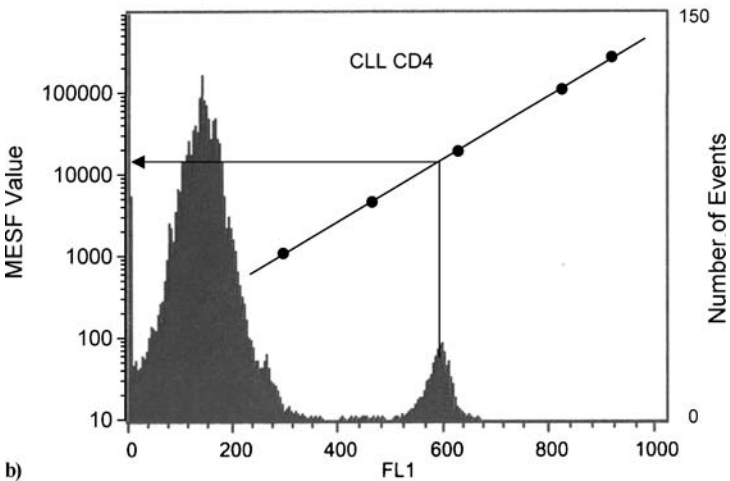
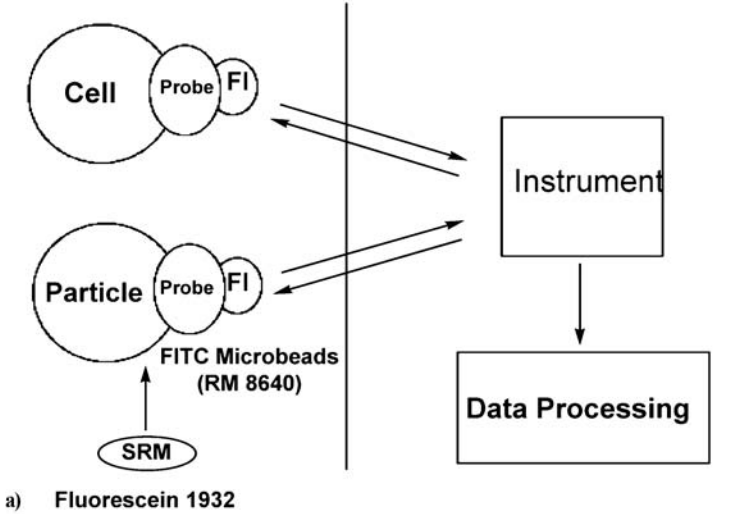
the nominal fluorescence intensity of each type of microbead in terms of the number of free fluorophores in a standard solution giving an equivalent fluorescence intensity in the same matrix under similar measurement conditions [9, 79–81] (see also chapters on flow cytometry in this book). The application and reliability of the MESF concept requires well characterized bead-type MESF standards for typical fluorescent labels [82–84], but few standards of this type and required quality are available. In response to this, the flow cytometry community has strongly expressed the need for better standards of this type in recent years to assign MESF values [29–33, 85]. As a first step to meet these demands, NIST developed SRM 1932 mentioned in the previous section [43],<sup>7</sup> a concentration standard for fluorescein, the first fluorescence channel, FL1, in flow cytometers. SRM 1932 enables the determination of concentration curves and assignment of MESF values of suspensions of fluorescein-labeled beads, which can then be used to calibrate the fluorescence intensity scale of FL1 in terms of MESF values, as illustrated in Fig. 7, with the number density of the microbeads being determined with a Coulter counter. Based on this intensity calibration, the fluorescence intensity of a single cell stained with fluorescein-labeled antibodies is then determined in terms of MESF values.

Even though the MESF concept provides a straightforward intensity scale comparable across instruments, laboratories, and over time, it is not designed to derive the absolute number of fluorophores in a sample. In addition, the instability of many organic dyes, such as fluorescein, which are known to degrade when exposed to oxygen or light, requires fresh fluorophore solutions for each calibration. Accordingly, results over time depend on the reproducibility of these solutions. When applied to integral (broad-band) fluorescence intensities, such as those typically detected in flow cytometry, the MESF approach relies on matching fluorescence spectra of the fluorophore in the standard and in the sample or at least knowledge of the spectral deviations. Here, the availability of corrected emission spectra of fluorophore-labeled microbeads and representative samples in typically used solvents, for instance from NMIs, such as BAM and NIST, may be helpful to estimate the size of these contributions to the overall uncertainty of quantification. Moreover, the MESF methodology needs to be extended to the simultaneous measurement of multiple fluorophores to enable the development of assay kits that calibrate each one of the multiple fluorescence channels in terms of MESF units. One way to approach these challenges with the limited resources of NMIs could involve collaborations with manufacturers of MESF standards and the organization of regular interlaboratory comparisons.

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<sup>7</sup> A standard fluorescein solution similar to SRM 1932 is sold by Invitrogen. See the on-line publication by Molecular Probes at [http://probes.invitrogen.com/lit/bioprobe45/bp45\\_16.pdf](http://probes.invitrogen.com/lit/bioprobe45/bp45_16.pdf).

# MESF Assignment Process



**Fig. 7** Illustration of the MESF assignment process. **a** MESF assignment of sets of microbeads with different amounts of surface-labeled fluorescein (RM 8640) with fluorescein solution (SRM 1932). These microbeads are used to calibrate the intensity axis of the fluorescence channel FL1 of flow cytometers thereby enabling the determination of the MESF values of fluorescein labeled cells. **b** Representative single-parameter histogram for CD4 T cells from a B-cell chronic lymphocytic leukemia (CLL) patient and a calibration curve generated with RM 8640 microbeads. The vertical and horizontal lines with arrow display the conversion of the channel number to the interpolated MESF values based on the calibration curve

## 2.5

### Standards for Day-to-Day and Instrument-to-Instrument Intensity

#### 2.5.1

##### General Applications

Most users of fluorescence techniques are not particularly interested in the measurement of absolute fluorescence intensities, but in the determination and consideration of the long-term stability of their fluorescence instrument and aging-induced changes in its absolute (spectral) sensitivity, as well as the comparability of fluorescence intensities across instruments. Accordingly, standards for day-to-day and instrument-to-instrument intensity are highly requested by many users of fluorescence techniques. Moreover, the availability of suitable standards of this type will strongly contribute to an improved quality assurance in fluorometry and eventually to the standardization of instrument qualification and method validation procedures for particular fluorescence techniques. Of equal interest for many users and especially for instrument manufacturers is the instrument's spectral sensitivity under a particular set of conditions or limit of detection for a particular analyte. Standard methods for measuring the range of linearity of fluorescence instruments, their dynamic range, and the limit of detection for particular analytes have been introduced by ASTM [12, 13], but the development of novel fit-for-purpose methods—preferably in combination with the supply of suited and well characterized materials to provide the necessary basis for the reliability and reproducibility of the respective procedure—along with their international acceptance is needed.

The significance of day-to-day and instrument-to-instrument standards is related not only to the ever-increasing use of fluorescence measurements and according need for the qualification of fluorescence instruments but also to the use of portable fluorometers, microplate and microarray readers, as well as fluorescence microscopes in drug discovery and clinical diagnostics. For these highly regulated areas, day-to-day and instrument-to-instrument standards are elegant tools to establish a comparable fit-for-purpose intensity scale as well as control charts [9, 10, 74, 79] for the comparison of fluorescence data over time and between laboratories. For the majority of these instruments, which are filter-based and accordingly perform integral and not spectrally resolved fluorescence measurements, spectral correction is not yet an issue.

Day-to-day and instrument-to-instrument intensity standards do not necessarily need to closely match routinely measured samples. However, they should be measurable with typical instrument settings to guarantee the reliability of the instrument performance and the determination of the spectral sensitivity under applicable conditions, e.g., at typical spectral irradiances, within a relevant spectral region [9]. The most stringent requirement is

either a sufficient, well-characterized stability under applicable conditions, or, for single-use standards, an excellent reproducibility, preferably in combination with an assigned uncertainty. Further prerequisites are known corrected spectra, if their intensities need to be compared with those of other fluorophores or between instruments with different spectral bandpasses.

The best-known day-to-day and instrument-to-instrument intensity standard of excellent stability is water (typically provided in a sealed cell to prevent the uptake of impurities) in combination with the so-called Raman test incorporated into the software of many spectrofluorometers [9, 54, 86].<sup>8</sup> Although very convenient, this method is effectively limited to excitation wavelengths below 400 nm due to the  $\lambda^{-4}$ -dependence of the intensity of scattered light. Fluorophores dissolved in a solvent or embedded into a solid matrix offer a greater flexibility with respect to the spectral region of excitation and emission. In the case of solutions of organic or inorganic dyes, their suitability is closely linked to the known purity of not only the dye, but also of the solvent. Depending on the chosen fluorophore, the close spectral match to typical fluorescent labels can be advantageous. Because many users of fluorescence techniques favor solid materials with an excellent long-term stability (desired shelf lives of two or more years) over liquid standards to be regularly replaced, inorganic fluorophores in a glass or polymer matrix are particularly attractive candidate materials [9, 51, 55, 69, 87]. As prerequisites for ease-of-use, such solid materials should be transparent, measurable without the use of polarizers, tested for the homogeneity of the fluorophore content and the long-term stability under application-specific conditions [36, 37],<sup>9</sup> and should come with an SOP for proper use. Ideally, they should also be usable for many different techniques and thus provided in different formats or shapes. Some materials are commercially available, but their corrected fluorescence spectra and other relevant information are typically not supplied or reported [9].

The significance of day-to-day and instrument-to-instrument intensity standards has motivated NIST and BAM to develop such materials in different formats for different fluorescence techniques ranging from steady-state fluorescence spectroscopy to fluorescence microscopy to fluorescence-based microarray technology. Attractive candidates currently tested by both institutes are inorganic glasses doped with inorganic fluorophores displaying either broad emission spectra, such as SRMs 2940 and 2941 [44, 45], or narrow line-shaped spectra covering the UV/Vis/NIR spectral regions [9, 51, 69]. These metal ion-doped glasses are very robust and do not photodegrade when excited by a fluorometer's conventional light source, even over long periods of

<sup>8</sup> This test, usually performed at an excitation wavelength of 350 nm and a detection wavelength of 397 nm, employs the Raman line of water to compare the long-term spectral sensitivity of a single fluorescence instrument or spectral sensitivities between instruments.

<sup>9</sup> The homogeneity of the dopant also needs to be considered as part of the photochemical stability studies to determine if local photo degradation effects are significant.

time. For instance, due to their excellent long-term stability, projected to be ten years, SRMs 2940 and 2941 are prescribed also for use as day-to-day intensity standards. Since many metal-ion-based dopants have comparably long emission lifetimes, on the order of milliseconds in many cases [88], when pulsed light sources are used, the emission properties of these dopants can be affected by measurement parameters, such as pulse duration, delay, and gate [9]. Accordingly, reference materials containing such dopants should be used as day-to-day intensity standards only for a constant set of measurement parameters. Their suitability as instrument-to-instrument intensity standards is linked to the application of comparable measurement conditions for the fluorescence instruments to be compared. As this is often difficult to guarantee, especially since many fluorescence users are not aware of the influence and control of the relevant instrument parameters, it is recommended to use materials containing such long lived emitters, as instrument-to-instrument intensity standards only with instruments using a continuous (non-pulsed) excitation source [9, 44, 45]. In addition to solid materials, BAM is investigating the potential of the liquid spectral fluorescence standards BAM-F001 to BAM-F005 for this application not only in a cuvette format, but also, e.g., in a slide-shaped microchannel device for fluorescence microscopy [9, 51, 64].

### 2.5.2

#### **Selected Applications: Filter-Based Instruments**

The bright future of fluorescence-based assays and microarray experiments in medical diagnostics and drug discovery encouraged NIST and BAM to dedicate resources to the development of day-to-day intensity and instrument-to-instrument intensity standards for these areas. Moreover, instrument qualification is an important part of assay validation as required by regulatory agencies, such as the United States Food and Drug Administration (FDA). The majority of microplate readers, certain types of fluorescence microscopes, microarray readers and scanners, as well as portable fluorometers are filter-based instruments that use bandpass filters for wavelength and bandwidth selection of both excitation and emission. These filters typically have a maximum transmittance near the wavelength corresponding to the excitation or emission maximum of the dye of interest. However, the spectral transmittance of these filters often varies between manufacturers and on a batch-to-batch basis. This, combined with the instrument-specific, spectral responsivity of the detection system, makes fluorophore-specific intensity standards, based on the same fluorescent labels as used in assays, very attractive. When the standard and label are spectrally matched, neither a correction for filter transmittance nor for the spectral responsivity of the detection system has to be performed. Due to the ever-increasing number of fluorescent dyes, however, the production of a standard for every type of fluorescent label is not practical or straightforward and is thus not currently followed either by NIST

or BAM. Commercial fluorophore-specific standards are available, e.g., for the labels Cy3 and Cy5.

Day-to-day intensity standards for microplate readers can be made from solid materials, such as the chromophore-doped glasses already being studied at NIST and BAM. In principle, such standards can be produced by putting a mask over a glass slide, thereby mimicking, e.g., a 96-well microplate, or by filling the wells of a validation microplate with solid materials [87]. In addition, a microfluidic approach, which is generally gaining in importance in the assay area, may be suitable for the adaptation of liquid fluorescence standards to the characterization of microplate readers. Both NIST and BAM have tested existing liquid CRMs for the characterization of microplate readers. For instance, SRM 1932 fluorescein solution has been used in an interlaboratory comparison of NMIs to evaluate non-biological variations associated with 96-well microplate readers, i.e., for the determination of the limit of detection, dynamic range, and instrument variables [89].<sup>10</sup> BAM successfully employed BAM-F001 through BAM-F005 for the determination of the relative spectral responsivity of monochromator-based fluorescence microplate readers and for the determination of the range of linearity and limit of detection of both filter- and monochromator-based instruments [51, 64].

The variability in the spectral characteristics of filters does not affect the use of day-to-day intensity standards as long as the same filter is always employed. This variability, however, can affect the signals obtained with instrument-to-instrument intensity standards. To account for the influence of the wavelength dependence of the transmittance of such filters on measured fluorescence intensities, the transmittance spectra of individual filters, or batches of filters need to be known. One approach for producing an effective intensity standard currently discussed at NIST is the supply of a suitable reference material for a particular spectral region, similar to a spectral correction standard, along with software that can link the measured fluorescence intensity onto an absolute scale by considering the transmittance spectrum of the filters used, along with the corrected fluorescence spectrum of the standard. This is one possible way to compare spectral sensitivity and absolute intensity between instruments that use the same type of filter set, without relying on standards that must spectrally match specific fluorescent labels.

Generally, the quality assurance in the assay area could benefit from a closer collaboration of standard manufacturers with NMIs, in addition to the development of standards by NMIs. For instance, NMIs could aid in the determination of desirable characteristics of existing commercial standards and validation microplates, such as photostability, homogeneity of the fluorescence intensity across the plate, corrected emission spectra, and NMI

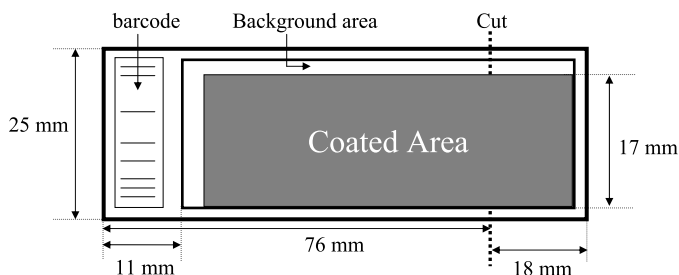
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<sup>10</sup> CCQM P58 "Fluorescence measurement for the life sciences" with CCQM referring to the committee consultative de la matiere and mutual recognition agreement.

traceability. NMIs could also contribute to the development of guidelines for instrument performance validation, together with instrument and standard manufacturers, regulatory agencies, technical and scientific organizations, and users from industry and academics.

The obvious future importance of microarray technology in strongly regulated areas, such as medical diagnostics and homeland security, motivated NIST to get involved with the development of instrument-type and biological standards for this research area [9]. The logistics of developing, producing, and certifying day-to-day and instrument-to-instrument intensity standards for fluorescence-based microarray scanners are further complicated, beyond that of standards for microplate readers, by the use of a focused laser for the excitation of small blotted samples, on the order of 10 to 100  $\mu\text{m}$ , which are scanned similarly to a confocal microscope. Accordingly, a standard suitable for the characterization of a microarray scanner needs to be several orders of magnitude more photostable compared to a corresponding intensity standard for spectrofluorometry. In addition, the fluorescence intensity from the standard has to be independent of the instrument geometry at a constant laser spot volume and power, as some microarray readers use an epifluorescence and others a transmitting measurement geometry. Ideally, a fit-for-purpose standard should be made in a blotted pattern similar to a typically measured biochip. However, it is almost impossible to reach the necessary homogeneity and photostability with such a design. A fluorescent film is likely to be a better candidate for achieving these characteristics. Such a film should be comparable to or thinner than the size of the focused laser spot, typically having a 5  $\mu\text{m}$  to 10  $\mu\text{m}$  diameter, to render the fluorescence intensity independent of instrument geometry.

Only recently, a microarray standards interest group, which includes representatives from most of the major microarray reader manufacturers and from NIST, agreed on specifications for two standards for day-to-day and instrument-to-instrument intensity comparison and calibration. One standard should possess a typical medium intensity, corresponding to about 500 fluorophores per square micrometer, and the other a low intensity, corresponding to about 0.5 fluorophores per square micrometer (see Fig. 8). Both standards will be based on a continuous fluorescent thin film on a 1 mm thick glass slide with a coating thickness of less than 10  $\mu\text{m}$ . These standards will be certified as possessing an intensity uniformity of 99% or better averaged over areas of 250  $\mu\text{m}^2$ , when excited at 532 and 633 nm and supplied with the certified corrected emission spectrum. The same pair of standards can be used for both excitation wavelengths or one pair can be made for each wavelength, depending on the characteristics of the potential candidates. NIST is presently characterizing candidate materials in collaboration with commercial material manufacturers to determine if materials that appropriately fit the desired standards criteria can be found.



**Fig. 8** Design of consensus material standards for microarrays. Comprised of a glass slide (thickness of 0.7 to 1.0 mm) coated with a fluorescent thin film (thickness of 10  $\mu\text{m}$  or less) with a fluorescence intensity uniformity of 99% over 250  $\mu\text{m}$  scanning subareas. An uncoated background area and a barcode will be used to subtract substrate fluorescence and to identify the sample. To produce standards at the two most commonly used chip sizes the standard will be cut along the *dotted line*

Meanwhile, NIST is also testing a commercially available validation slide from Full Moon BioSystems<sup>11</sup> to assess the performance of an array scanner (see also Chap 26 [90]). This slide is composed of a series of known concentrations of either Cy3 or Cy5 embedded in a polymer matrix and, in principle, can be used to validate the scanners in terms of dynamic range, detection limit, and scanning uniformity [91, 92]. The emission spectrum of Cy5 fluorophores on oligonucleotide microarray slides matches that on the commercial validation slide and the spectrum of the Cy3 fluorophores on this slide is red-shifted by 5 nm in comparison to that on the oligonucleotide arrays [93]. This slide does photodegrade, but preliminary results suggest that fluorescence intensity changes with time and between instruments are detectable using this slide.

### 3 Conclusion

The urgent need for fluorescence calibration standards and day-to-day intensity standards for the UV/Vis/NIR spectral region motivated NIST and BAM to develop certified reference materials to meet these demands and to perform a first interlaboratory comparison on spectral correction. The ultimate goal here is to make fluorescence measurements comparable across instruments, laboratories and over time. This comparison marks the beginning of a fruitful collaboration of NMIs in this area.

<sup>11</sup> Certain commercial equipment, instruments, or materials are identified in this chapter to foster understanding. Such identification does not imply recommendation or endorsement by 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.



To improve the quality assurance in fluorometry and the comparability of fluorescence data on a broad level, reliable, yet fit-for-purpose, fluorescence standards with certified, calibration-relevant properties are mandatory, along with internationally accepted recommendations and guidelines for instrument calibration and performance validation. The fulfillment of these requirements encourages a close interaction and collaboration between NMIs, instrument manufacturers, and regulatory agencies. To help users of fluorescence techniques with the choice of fit-for-purpose standards, quality criteria for the different classes of fluorescence standards in need have been defined by NIST and BAM. Moreover, the determination of uncertainties for representative fluorescence measurements for different fluorescence techniques seems to be helpful, including interlaboratory comparisons between NMIs, expert laboratories, and routine users. These collaborations help to establish the achievable repeatability and accuracy of analyte determinations and assay results that can be eventually considered in recommendations and guidelines. Additionally, workshops and special training courses on drawbacks and sources of uncertainties of fluorescence techniques and instrument characterization—jointly organized by NMIs—may help to broaden the understanding for the need of an improved quality assurance in fluorometry within the fluorescence community.

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## References

1. Lakowicz JR (ed) (1999) Principles of fluorescence spectroscopy, 2nd edn. Kluwer Academic/Plenum Press, New York
2. Lakowicz JR (ed) (1992–2004) Topics in fluorescence spectroscopy series, Vol. 1–8. Plenum Press, New York
3. Valeur B (ed) (2002) Molecular Fluorescence, Principles and Applications. Wiley-VCH, Weinheim
4. Wolfbeis OS (ed) (2001–2004) Springer series on fluorescence, methods and applications, Vol. 1–3. Springer, Berlin Heidelberg New York
5. Schulman SG (ed) (1985–1993) Molecular luminescence spectroscopy, Parts 1–3. Wiley Interscience, New York
6. Mason WT (1999) Fluorescent and luminescent probes for biological activity, 2nd edn. Academic Press, San Diego
7. Stokes GG (1852) On the change of refrangibility of light. Phil Trans R Soc Lond 142:463
8. Udenfriend S (1995) Development of the spectrophotofluorometer and its commercialization. Protein Sci 4:542

9. Resch-Genger U, Hoffmann K, Nietfeld W, Engel A, Ebert B, Macdonald R, Neukammer J, Pfeifer D, Hoffmann A (2005) How to improve quality assurance in fluorometry: fluorescence-inherent sources of error and suited fluorescence standards. *J Fluoresc* 15:337
10. Gaigalas AK, Li L, Henderson O, Vogt R, Barr J, Marti G, Weaver J, Schwartz A (2001) The development of fluorescence intensity standards. *J Res Nat Inst Stand Technol* 106:381
11. ASTM E 388-04 (2004) Spectral bandwidth and wavelength accuracy of fluorescence spectrometers. In: Annual book of ASTM standards, vol 03.06 (original version 1972)
12. ASTM E 578-01 (2001) Linearity of fluorescence measuring system. In: Annual book of ASTM standards, vol 03.06 (original version 1983)
13. ASTM E 579-04 (2004) Limit of detection of fluorescence of quinine sulfate. In: Annual book of ASTM standards, vol 03.06 (original version 1984)
14. Miller JN (ed) (1981) Techniques in visible and ultraviolet spectrometry, Vol. 2, Standards in fluorescence spectrometry. Chapman and Hall, New York
15. Eaton DF (1988) Reference compounds for fluorescent measurements. *Pure Appl Chem* 60:1107
16. Eaton DF (1990) Recommended methods for fluorescence decay analysis. *Pure Appl Chem* 62:1631
17. Shelton CF (1968) NBS Tech Note 417, Spectral emission properties of NBS standard phosphor samples under photo-excitation. US Government Printing Office, Washington, DC
18. Mavrodineanu R, Shultz JI, Menis O (eds) (1973) NBS Spec Pub 378, Accuracy in spectrophotometry and luminescence measurements. US Government Printing Office, Washington, DC (first appeared in (1972) *J Res Nat Bur Stand* 76A)
19. Mielenz KD, Velapoldi RA, Mavrodineanu R (eds) (1977) NBS Spec Pub 466, Standardization in spectrophotometry and luminescence measurements. US Government Printing Office, Washington, DC (first appeared in (1976) *J Res Nat Bur Stand* 80A)
20. Cehelnik ED, Mielenz KD, Velapoldi RA (1975) Polarization effects on fluorescence measurements. *J Res Nat Bur Stand* 79A:1
21. Mielenz KD, Cehelnik ED, McKenzie RL (1976) Elimination of polarization bias in fluorescence intensity measurements. *J Chem Phys* 64:370
22. Velapoldi RA, Mielenz KD (1980) NBS Spec Pub 260-64, A fluorescence standard reference material: quinine sulfate dihydrate. US Government Printing Office, Washington, DC
23. (1979) Certificate of analysis, Standard Reference Material 936, quinine sulfate dihydrate. National Bureau of Standards (This certificate is expired, replaced by [25])
24. (1994) Certificate of analysis, Standard Reference Material 936a, quinine sulfate dihydrate. National Institute of Standards and Technology (<http://ts.nist.gov/ts/htdocs/230/232/232.htm>)
25. (1989) Certificate of analysis, Standard Reference Material 1931, fluorescence emission standards for the visible region. National Institute of Standards and Technology (This SRM is no longer available.)
26. Thompson A, Eckerle KL (1989) Standards for corrected fluorescence spectra. *Proc SPIE-Int Soc Opt Eng* 1054:20
27. Saunders G, Parkes H (1999) Analytical molecular biology: quality and validation. RSC, Cambridge
28. (2005) ISO/IEC 17025, 2nd edn. International Organization for Standardization, Geneva

29. DeRose PC (2000) NIST workshop on luminescence standards for chemical analysis, Sept 1999. *J Res Nat Inst Stand Technol* 105:631 (<http://nvl.nist.gov/pub/nistpubs/jres/105/4/j54ce-der.pdf>)
30. Workshop (Jan 1998) Fluorescence intensity standards. NIST
31. Workshop (June 2000) New directions in fluorescence intensity standards. NIST
32. Workshop (March 2005) Towards national traceability in fluorescence intensity measurements. NIST
33. Workshop (Feb 2006) Improved antibody-based metrology in flow cytometry, NIST (comment: a ref. article should be available soon, the other 3 workshops, refs 31–33 were the precursors to this one)
34. Workshop (Dec 2002) Fluorescence standards for microarray assays. NIST (<http://www.cstl.nist.gov/biotech/fluormicroarray/FluorMicroarrayWkshp12-10-2002.html>)
35. Workshop (June 2003) Bioanalytical and biomedical applications of fluorescence techniques: instrument characterization and validation, traceability and need for reference materials. Resch-Genger U (BAM), Macdonald R (PTB), BERM-9
36. (2000) ISO; General Requirements for the Competence of Reference Material Producers, Second edition
37. (2003, draft) ISO; Reference materials — General and statistical principles for certification.
38. (1993) ISO; Guide to the expression of uncertainty in measurement; ISBN 92-67-10188-9, 1st edn. International Organization for Standardization, Geneva
39. DeRose PC, Kramer GW (2005) Bias in the absorption coefficient determination of a fluorescent dye, Standard Reference Material 1932 fluorescein solution. *J Luminesc* 113:314
40. Benson RC, Kues HA (1977) Absorption and fluorescence properties of cyanine dyes. *J Chem Eng Data* 22:379
41. Resch-Genger U, Pfeifer D, Hoffmann K, Flachenecker G, Hoffmann A, Monte C (2008) Linking fluorometry to radiometry: traceability and physical and fluorescence Standards. In: Resch-Genger U (ed) *Standardization in Fluorometry: State-of-the Art and Future Challenges*. Springer, Berlin Heidelberg
42. May W, Parris R, Beck C, Fassett J, Greenberg R, Guenther F, Kramer G, Wise S, Gills T, Colbert J, Gettings R, MacDonald B (2000) Definitions of terms and modes used at NIST for value-assignment of reference materials for chemical measurements, NIST Special Publication 260-136. US Government Printing Office, Washington, DC
43. (2004) Certificate of analysis, Standard Reference Material 1932, Fluorescein solution. National Institute of Standards and Technology (<http://ts.nist.gov/ts/htdocs/230/232/232.htm>)
44. (2007) Certificate of analysis, Standard Reference Material 2940, Relative intensity correction standard for fluorescence spectroscopy: Orange emission. National Institute of Standards and Technology. Certification of emission spectra in 1 nm-intervals. (<http://ts.nist.gov/ts/htdocs/230/232/232.htm>)
45. (2007) Certificate of analysis, Standard Reference Material 2941, Relative intensity correction standard for fluorescence spectroscopy: Green emission. National Institute of Standards and Technology. Certification of emission spectra in 1 nm-intervals. (<http://ts.nist.gov/ts/htdocs/230/232/232.htm>)
46. (2006) Certificate of analysis, Certified Reference Material BAM-F001, Spectral fluorescence standard for the determination of the relative spectral responsivity of fluorescence instruments within its emission range. Federal Institute for Materials Research and Testing (BAM)

47. (2006) Certificate of analysis, Certified Reference Material BAM-F002, Spectral fluorescence standard for the determination of the relative spectral responsivity of fluorescence instruments within its emission range. Federal Institute for Materials Research and Testing (BAM)
48. (2006) Certificate of analysis, Certified Reference Material BAM-F003, Spectral fluorescence standard for the determination of the relative spectral responsivity of fluorescence instruments within its emission range. Federal Institute for Materials Research and Testing (BAM)
49. (2006) Certificate of analysis, Certified Reference Material BAM-F004, Spectral fluorescence standard for the determination of the relative spectral responsivity of fluorescence instruments within its emission range. Federal Institute for Materials Research and Testing (BAM)
50. (2006) Certificate of analysis, Certified Reference Material BAM-F005, Spectral fluorescence standard for the determination of the relative spectral responsivity of fluorescence instruments within its emission range. Federal Institute for Materials Research and Testing (BAM)
51. Hoffmann K, Monte C, Pfeifer D, Resch-Genger U (2005) Standards in fluorescence spectroscopy: Simple tool for the characterization of fluorescence instruments, *GIT Lab J Eur* 9:29
52. Hofstraat JW, Latuhihin MJ (1994) Correction of fluorescence spectra. *Appl Spectrosc* 48:436
53. Gardecki JA, Maroncelli M (1998) Set of secondary emission standards for calibration of the spectral responsivity in emission spectroscopy. *Appl Spectrosc* 52:1179
54. Kovach RJ, Peterson WM (1994) The measurement of sensitivity in fluorescence spectroscopy. *Am Lab* 26:G32
55. Lifshitz IT, Meilman ML (1989) Standard sample for calibrating wavelength scales of spectral fluorometers. *Sov J Opt Technol* 55:487
56. Hollandt J, Taubert DR, Seidel J, Resch-Genger U, Gugg-Helminger A, Pfeifer D, Monte C (2005) Traceability in fluorometry: Part I, Physical standards. *J Fluoresc* 15:301
57. Monte C, Resch-Genger U, Pfeifer D, Taubert RD, Hollandt J (2006) Linking fluorescence measurement to radiometric units. *Metrologia* 43:S89
58. DeRose PC, Early EA, Kramer GW (2007) Qualification of a fluorescence spectrometer for measuring true fluorescence spectra. *Rev Sci Instrum* 78:033107
59. DeRose PC, Early EA, Kramer GW (2008) Measuring and certifying true fluorescence spectra with a qualified fluorescence spectrometer. In: *Proc 5th Oxford Conf on spectrometry*. Crown, UK
60. Zwinkels J (2008) Surface fluorescence: the only standardized method of measuring luminescence. In: Resch-Genger U (ed) *Standardization in Fluorometry: State-of-the Art and Future Challenges*, Springer, Berlin Heidelberg
61. Resch-Genger U, Pfeifer D (2006) Certification report, Calibration kit Spectral fluorescence standards BAM-F001–BAM-F005, BAM, Berlin
62. (2006) Certificate of analysis, Certified reference materials BAM-F001–BAM-F005, Calibration Kit, Spectral Fluorescence Standards for the determination of the relative spectral responsivity of fluorescence instruments. Federal Institute for Materials Research and Testing (BAM). Certification according to ISO guides 34 and 35 in 1 nm-steps for three different spectral bandpasses of the BAM fluorometer.
63. Pfeifer D, Hoffmann K, Hoffmann A, Monte C, Resch-Genger U (2006) The calibration kit, Spectral fluorescence standards: A simple tool for the standardization of the spectral characteristics of fluorescence instruments. *J Fluoresc* 16:581

64. Hoffmann K, Resch-Genger U, Nitschke R (2005) Simple tool for the standardization of confocal spectral imaging systems. *GIT Imaging Microsc* 3:18
65. DeRose PC, Smith MV, Blackburn DH, Kramer GW (2008) Characterization of Standard Reference Material 2941, uranyl-ion-doped glass, spectral correction standard for fluorescence. *J Luminesc* 128:257
66. [www.physics.nist.gov/PhysRefData/Handbook/index.html](http://www.physics.nist.gov/PhysRefData/Handbook/index.html)
67. Harrison GR (1982) MIT wavelength tables, Vol. 2, Wavelengths by element. MIT Press, Cambridge, MA
68. Zaidel AN, Prokofev VK, Raikii SM, Slavnyi VA, Shreider EY (1970) Tables of spectral lines. Plenum Press, New York
69. Velapoldi RA, Epstein MS (1989) Luminescence standards for macro- and microspectrofluorimetry. In: Goldberg MC (ed) ACS symposium series 383, Luminescence applications in biological, chemical, environmental and hydrological sciences. American Chemical Society, Washington, DC, p 98
70. Rurack K (2008) Fluorescence quantum yields: traceability, methods of determination and standards. In: Resch-Genger U (ed) *Standardization in Fluorometry: State-of-the Art and Future Challenges*, Springer, Berlin Heidelberg
71. de Mello JC, Wittmann HF, Friend RH (1997) An improved experimental determination of external photoluminescence quantum efficiency. *Adv Mater* 9:230
72. Wise SA, Sander LC, May WE (1993) Determination of polycyclic aromatic hydrocarbons by liquid chromatography. *J Chromatogr* 642:329
73. Duewer DL, Parris RM, White V E, May WE, Elbaum H (2004) NIST Spec Pub 1012, An approach to the metrologically sound traceable assessment of the chemical purity of organic reference materials. US Government Printing Office, Washington, DC
74. Schwartz A, Gaigalas AK, Wang L, Marti GE, Vogt RF, Fernandez-Repollet E (2004) Formalization of the MESF unit of fluorescence intensity. *Cytometry* 57B:1
75. (2004) Report of Investigation, Reference Material 8640, Microspheres with immobilized fluorescein isothiocyanate. National Institute of Standards and Technology
76. Hultin LE, Matud JL, Giorgi JV (1998) Quantitation of CD38 activation antigen expression on CD8+ T cells in HIV-1 infection using CD4 expression on CD4+ T lymphocytes as a biological calibrator. *Cytometry* 33:123
77. Iyer SB, Hultin LE, Zawadzki JA, Davis KA, Giorgi JV (1998) Quantitation of CD38 expression using QuantiBRITE™ beads. *Cytometry* 33:206
78. Gruber HJ, Hahn CD, Kada C, Riener CK, Harms GS, Ahrer W (2000) Anomalous fluorescence enhancement of Cy3 and Cy3.5 versus anomalous fluorescence loss of Cy5 and Cy7 upon covalently linking to IgG and noncovalent binding to avidin. *Bioconj Chem* 11:696
79. Wang L, Gaigalas AK, Abbasi F, Marti GE, Vogt RF, Schwartz A (2002) Quantitating fluorescence intensity from fluorophores: Practical use of MESF values. *J Res Nat Inst Stand Technol* 107:339
80. Lenkei R, Mandy F, Marti G, Vogt R (eds) (1998) Special issue on quantitative fluorescence cytometry: An emerging consensus. *Cytometry* 33
81. Schwartz A, Wang L, Early E, Gaigalas AK, Zhang Y-Z, Marti GE, Vogt RF (2002) Quantitating fluorescence intensity from fluorophores: The definition of MESF assignment. *J Res Nat Inst Stand Technol* 107:83
82. Schwartz A, Marti GE, Gratama JW, Fernandez-Repollet E (1998) Standardizing flow cytometry: A classification system of fluorescence standards used for flow cytometry. *Cytometry* 33:106
83. Schwartz A, Mendez M, Santiago G, Diaz L, Fernandez-Repollet E (1997) Applications of common quantitative fluorescent standards to multiple platforms: Com-

- parison of commercial fluorescent calibration standards used in quantitative flow cytometry immunophenotyping analysis as a function of pH environment. *Clin Immunol* 17:14
84. Zenger VE, Vogt R, Mandy F, Schwartz A, Marti GE (1998), Quantitative flow cytometry: interlaboratory-variation. *Cytometry* 33:138
  85. Marti GE, Vogt RF, Gaigalas AK, Hixson CS, Hoffman RA, Lenkei R, Magruder LE, Purvis NB, Schwartz A, Shapiro HM, Waggoner A (2004) Fluorescence calibration and quantitative measurements of fluorescence intensity, Approved guideline, NCCLS, I/LA24-A, vol 24 No. 26
  86. Froehlich P (1989) Under the sensitivity specification for a fluorescence spectrophotometer. *Int Lab* 19:42
  87. Gibeler R, McGown E, French T, Owicki JC (2005) Performance validation of microplate fluorimeters. *J Fluoresc* 15:363
  88. Parke S, Watson AI, Webb RS (1970) Fluorescence decay times of divalent manganese in inorganic glasses. *J Phys D Appl Phys* 3:763
  89. Howarth P, Redgrave F (2003) *Metrology in short*, 2nd edn. MKom Aps, Denmark
  90. Sige Z, He H-J, Zong Y, Shi L, Wang L (2008) DNA microarrays: applications, future trends and need for standardization. In: Resch-Genger U (ed) *Standardization in Fluorometry: State-of-the Art and Future Challenges*. Springer, Berlin Heidelberg
  91. Zong Y, Wang Y, Zhang S, Shi Y (2003) How to evaluate a microarray scanner. In: Hardiman G (ed) *Microarrays methods and applications-nuts & bolts*. DNA Press, USA
  92. Shi L, Tong W, Su Z, Han T, Han J, Puri RK, Fang H, Frueh FW, Goodsaid FM, Guo L, Branham WS, Chen JJ, Xu ZA, Harris SC, Hong H, Xie Q, Perkins RG, Fuscoe JC (2005) Microarray scanner calibration curves: characteristics and implications. *BMC Bioinformatics* 6:S11
  93. Wang L, Gaigalas AK, Satterfield MB, Salit M, Noble J (2007) Evaluating the quality of data from microarray measurements. *Methods Mol Biol* 381:121

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