Supporting Information

State-of-the Art Comparability of Corrected Emission Spectra – Part I: Spectral Correction with Physical Transfer Standards and Spectral Fluorescence Standards by Expert Laboratories

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1. Instrumentation

Table 1S provides an overview of the fluorescence measuring instruments used by the four expert laboratories that participated in this study.

⟨Insert Table 1S, here⟩

S2 Calibration Procedures and Physical Standards

Each participating laboratory performed an instrument qualification according to the PTS-based calibration procedures they had developed and routinely followed for their fluorescence spectrometers. This qualification included the following steps, typically in the given order: determination of 1.) the wavelength accuracy and spectral resolution of both the excitation and emission monochromator, 2.) the linear range of the emission detection system, and 3.) the relative spectral responsivity of the detection system ($s(\lambda)$). An overview of these instrument qualification procedures and the transfer standards used as well as the relative uncertainties is given in Table 2S.

⟨Insert Table 2S, here⟩

The measured uncorrected data obtained from these state of the art fluorescence instruments were also provided ($I_u(\lambda_{em})$): i.e., instrument-specific relative spectral radiance or emission spectrum of the spectral radiance transfer standard or the previously calibrated emission channel, dependent on the chosen calibration procedure used, along with the certified values of the physical transfer standards ($I_{cert}(\lambda_{em})$; in the case of PTS, no blank or background subtraction was performed for $I_u(\lambda_{em})$ in contrast to the measurements with RMs, see equation 3S). These data enabled the calculation of a wavelength-dependent spectral correction factor $C_{PTS}(\lambda_{em})$, see equation 1S, that equals the relative spectral responsivity $s(\lambda_{em})$ of the emission channel.. [1]

$$C_{PTS}(\lambda_{em}) = I_u(\lambda_{em}) / I_{cert}(\lambda_{em}). \quad \text{(eq. 1S)}$$

A spectrally corrected, instrument-independent emission spectrum $I_c(\lambda_{em})$ using $C_{PTS}(\lambda_{em})$ was then calculated for each dye from the measured, spectrally uncorrected dye spectrum after blank correction according to equations 2S and 3S.

$$I_c(\lambda_{em}) = I_u(\lambda_{em}) / C_{PTS}(\lambda_{em}) \quad \text{(eq. 2S)}$$

$I_u(\lambda_{em})$ in equation 2S equals the spectrally uncorrected emission spectrum of the dye, corrected for scattering and fluorescence from the solvent and dark counts from the detector by subtraction of the spectrally uncorrected emission spectrum of the solvent (termed blank spectrum; $I_b(\lambda_{em})$) obtained under identical measurement conditions, see equation 3S.

$$I_u(\lambda_{em}) = I_m(\lambda_{em}) - I_b(\lambda_{em}) \quad \text{(eq. 3S)}$$

The calibration procedures including standards used that are detailed in the following section and the resulting uncertainties are summarized in Table 2S.

NIST. The details of the calibration procedures used have been reported elsewhere. [2] 1) Wavelength accuracy: The atomic lines of Hg, Xe, Ne, and Kr low pressure atomic discharge pen-type lamps (Oriel Inc.) placed at the sample position were used to determine the wavelength accuracy of the emission monochromator from 295 nm to 795 nm ($\Delta\lambda_{EM}$ = 0.1 nm, integration time t_i = 0.05 s, scanning interval s_i = 0.05 nm). The wavelength accuracy of the excitation monochromator from 220 nm to 550 nm was determined using a calibrated diffuse reflector at the sample position to scatter light at wavelengths selected by the excitation monochromator into the emission monochromator. The emission monochromator, after calibration of its wavelength scale, was then used to find the wavelength of the fixed position of the excitation monochromator by scanning over the position ($\Delta\lambda_{EX}$ / $\Delta\lambda_{EM}$ = 1.0 / 0.1 nm t_i = 0.5 s, s_i = 0.1 nm). 2) Linearity of the emission detection system: A scattering solution was placed at the sample position to scatter the excitation light into the detection system. The intensity of the scattered light was controlled by means of calibrated neutral density filters with absorbances (A) A = 0.1 to A = 4.0, placed before the sample that attenuated the excitation and thus the scattered light. The signal measured by the detection system was used to determine its linearity. 3) Relative spectral responsivity of the emission channel: $s(\lambda)$ was obtained with two methods (method 1: 370 nm to 800 nm; method 2: 300 nm to 370 nm) that were combined to cover the wavelength region of 300 nm to 800 nm. For method 1, a calibrated diffuse reflector (sintered polytetrafluoroethylene, PTFE) was placed at

the sample position to reflect the spectral radiance of a calibrated source or spectral radiance transfer standard into the detection system. The emission monochromator was scanned and the PMT signal was collected. The measured spectral shape of the lamp spectrum was compared with the known shape of the standard´s spectral radiance or emission spectrum to determine spectral correction factors from 370 nm to 800 nm. For method 2, a calibrated detector at the sample position was used to collect the spectral radiance of the excitation channel as a function of wavelength (step 1). Then, the excitation and emission monochromators were scanned synchronously with the calibrated reflector at the sample position and the PMT signal was collected as a function of wavelength (step 2). The known radiance of the excitation channel or excitation beam, determined in step 1, was then compared to that measured by the detection system in step 2 to determine spectral correction factors below 370 nm.

NRC. The details of the NRC fluorescence instrumentation, standards and calibration procedures are described in detail elsewhere. [3, 4] Briefly, the calibration of the NRC reference spectrofluorometer involved both conventional spectrophotometric and radiometric calibration procedures. The spectrophotometric procedures included wavelength and photometric scale, stray light, and degree of polarization. The radiometric calibration procedures included spectral irradiance of the excitation unit, spectral responsivity of the emission unit and characterization of the instrument´s slit scattering function for a given spectral bandpass. 1) Wavelength accuracy: The wavelength scale of the excitation and emission units of the instrument were calibrated for both sets of holographic gratings used to cover the wavelength range of operation of the instrument. These two grating conditions were calibrated independently using 22 spectral lines of Hg, Cs, He, and Cd low pressure atomic discharge lamps (Oriel Inc., pencil-style spectral calibration lamps). For calibration of the excitation unit, the monitor detector (Hamamatsu S-1227 silicon diode) was used to record the data, whereas for the calibration of the emission unit, the calibration lamp was mounted on the sample stage and the PMT was used as the analyzing detector. The wavelength calibration functions are the combination of a first order linear fit to the raw data and a second order polynomial fit to the residual differences. 2) The linearity of the analyzing detector, a thermoelectrically-cooled InGaAs PMT (Hamamatsu R6872) situated behind a

ground Suprasil diffuser, was characterized using the NRC-designed high precision variable aperture device, which is based on the double aperture method. [5] 3) Relative spectral responsivity of the emission channel: $s(\lambda)$ of the emission unit was characterized for both gratings A and B by recording the analyzing detector´s signal for a source of known spectral radiance at the sample position. This source consisted of a Lambertian reflecting diffuser of known 45°/0° spectral radiance factor, which was illuminated at 45° incidence by a standard spectral irradiance lamp/plane mirror combination. The spectral diffuse reflectance standard was a pressed tablet of polytetrafluoroethylene powder, 1.0 gm/cm^3) traceable to a master pressed PTFE spectral radiance factor standard calibrated by NIST (NIST certificate: 844256382-96, calibration uncertainty (k = 1): 0.2%). The standard spectral irradiance lamp was a 200 W quartz-halogen lamp, which was mounted in a housing with a baffle tube limiting the source aperture. The incident light was reflected by the plane mirror mounted at 22.5°. This lamp/mirror arrangement was fixed on a kinematic mount and has been calibrated by NRC as a unit for absolute spectral irradiance, see Table 2S. This calibrated source unit was mounted in the sample compartment of the spectrofluorometer and aligned to give 45° incidence at the sample position. For this study, the emission unit was calibrated for spectral conditions corresponding to this intercomparison, i.e., a 5 nm bandpass and 2 nm measurement interval.

PTB. 1) The calibration procedures generally follow the procedures reported above. [1] Wavelength accuracy: Prior to check and correction of the wavelength accuracy of the fluorometer´s monochromators, the optical path was aligned with the aid of a red and a green HeNe laser. The wavelength accuracy of the emission monochromator was determined in the spectral range between 350 nm and 800 nm using a scattering cuvette with a small hole in the center, in which a fiber was inserted guiding light from a low pressure mercury/argon source HG-1 (Ocean Optics Inc.) into the hole. The homemade scattering cuvette was filled with non-fluorescing glass spheres acting as scatterers embedded in a polymer matrix. The wavelength accuracy of the excitation channel was determined by a series of synchronous scans within the relevant wavelength region of the emission monochromator. The bandwidth used was 0.2 nm, the step size 0.1 nm. 2) The linearity and 3) the relative spectral

responsivity of the emission channel were both determined with a calibrated integrating sphere-type spectral radiance transfer standard UK2891 (10 W quartz halogen lamp, Gigahertz-Optik GmbH; operated at 500 mA in a current-stabilized mode) and a calibrated non-fluorescent diffuse reflectance standard (Labsphere SRS99, diameter of *ca*. 40 mm and thickness of *ca*. 8 mm; calibrated spectral radiance factor). The spectral irradiance reaching the emission detector was controlled via the distance between the reflectance standard and the lamp, as detailed in the BAM section below, or by neutral density filters.

BAM. All calibrations and subsequent fluorescence measurements were performed at a room temperature of (25±1) °C. The instrument and the transfer standards were thermally equilibrated to minimize drift. 1) Wavelength accuracy: The wavelength accuracy of the emission monochromator was determined in the spectral range of 300 nm to 810 nm with a cuvette-shaped, low pressure mercury/argon discharge lamp CAL-2000 (Ocean Optics Inc; pen-type lamp inside a metal cuvette with a small reflector in the center, model HR4000CG-UV-NIR), placed at sample position exactly at the intersection point of the foci of the excitation and emission monochromator lenses. The bandwidth of the emission monochromator was 0.25 nm, the scan step width 0.1 nm (uncertainty +/- 0.05 nm), the integration time 1.11 s/nm, and the scanning interval 10 steps/nm. The spectral resolution of the emission monochromator was determined with this Hg-Ar lamp using the FWHM (full width at half height of the maximum) of the mercury doublet at 577 nm and 579 nm and a monochromator slit width of 1 nm. The wavelength accuracy of the excitation channel was determined by a series of scans over fixed wavelength intervals of the previously calibrated emission monochromator with a white standard at the sample position. Also, the spectral position of selected mercury lines.was recorded using a mercury lamp placed in the lamp housing in front of the excitation monochromator with a calibrated detector at the sample position. 2) Linearity of the emission detection system: The linear range of the detection system was determined using a ratioing method developed by BAM [1] under routine operating conditions prior to spectral calibration with particular attention to slit widths/spectral bandpass, detector voltage, and detection mode used. This method measures the spectral irradiance of the instrument´s

excitation source, scattered into the detector with the aid of a calibrated non-fluorescent diffuse reflectance standard, at two different settings of the emission polarizer (P_{em}) at a selected wavelength; in this case, 400 nm (P_{em} = 0° and 90°) and 620 nm (P_{em} = 0° and 20°). The reproducibility of the polarizer alignment was previously determined to 1000:1. Deviations from a constant value, that exceed the estimated uncertainty of these fluorescence measurements, give the upper limit of the linearity of the emission detection system. Controlled variation of the light intensity was achieved by placing several attenuators, here neutral density filters, in the filter holder in the excitation channel. The upper limit of the linear range of the UV/vis detection system was determined to 10,000 cts/s for 400 nm and 620 nm. All subsequent calibrations and fluorescence measurements were performed with photon counting rates ≤ 10,000 cts/s. 3) Relative spectral responsivity of the emission channel: $s(\lambda)$ of the detection system was determined with a calibrated integration sphere-type spectral radiance transfer standard BN9701 (10 W-quartz halogen lamp placed inside an OP.DI.MA integrating sphere, Gigahertz-Optik GmbH; inner diameter of 50 mm, size of radiating area = 8 mm×10 mm; operated at 800 mA in the current-stabilized mode; certificate 1912-PTB-05) and a calibrated non-fluorescent diffuse reflectance standard (type BN-R98-S01, Gigahertz Optik GmbH, OP.DI.MA, diameter of *ca*. 50 mm and thickness of *ca*. 8 mm; spectral radiance factor calibrated for 45°/0° measurement geometry; certificate PTB 4.52-001068/05). The emission correction curves were measured in relative intensities for the measurement conditions used, i.e., for the spectral bandpasses of the emission monochromator (1 nm; 4 nm) and emission polarizer settings (0°), see Table 3S. To minimize the uncertainties of the corrected emission spectra, the source-based calibration of the two emission channels was performed not only at similar instrument settings as used for the measurements of the dye solutions, but also at spectral radiances comparable to those emitted by typical fluorescent samples. [1, 6, 7] To achieve the necessary reduction of the spectral radiance of the integrating sphere-type spectral radiance transfer standard, without affecting its emission characteristics and without interrupting the traceability chain, we used the quadratic distance dependence of diffuse illumination. [1] To realize this, the white standard was placed at the sample position normal to the direction of detection of the emission monochromator and illuminated with the

lamp at an angle of 45°. The lamp was mounted on an optical bench attached to the fluorometer´s sample compartment at an angle of 45° and the scattered light was detected at an angle of 0° with the emission detection system. With this set up, the spectral irradiance at the white standard and, thus the spectral radiance reaching the emission detection system, could be controlled simply by variation of the distance between lamp and white standard. To minimize the influence of scattered light, the measurement was performed in the dark (dark room and/or black tube-type coverage equipped with ring apertures for the beam emitted from the lamp and special housing for the sample compartment).

S3 Samples

Eight dyes were measured: seven dyes provided by BAM and one dye supplied by NIST; all provided as ready-to-use solutions. The BAM dyes consisted of: five RMs: F001 to F005 (ethanol, solvent) [7] and two test dyes: BAM dye X (ethanol, solvent) and BAM dye X (acetonitrile, solvent). The one dye supplied by NIST was NIST Standard Reference Material® (SRM®) 936a quinine sulfate dihydrate (dye QS; 0.1 mol/L perchloric acid, solvent). [8, 9] These dyes were chosen to cover the spectral region used for calibration, i.e., 300 nm to 800 nm. BAM dye X was also selected for its slightly structured emission spectrum to check on effects of spectral resolution and the accuracy of the emission wavelength scale. Two concentrations were chosen for each dye such that the resulting absorbances at the main absorption maximum were 0.04 and 0.08. This guaranteed that all participants, especially NRC using a less sensitive colorimetric set up, were able to obtain adequate signals. Prior to this comparison, BAM and NIST measured the emission spectra of the BAM dyes and SRM 936a, respectively, to ensure that these spectra did not depend on dye concentration within the concentration range used for this laboratory intercomparison. For each dye, the respective solvent was supplied for measurement of the corresponding blank spectrum. [1]

S4 Protocols, Measurement Conditions, and Requested Data

The eight dyes F001 to F005, X, QS, and Y were measured according to detailed SOPs evaluated and provided by BAM and NIST to minimize sample-related measurement uncertainties. In the case of F001 to F005, these SOPs closely resemble the SOPs supplied by BAM with these RMs available from Sigma Aldrich and BAM. The following excitation wavelengths and emission ranges were recommended: F001 (280 nm; 300–450 nm), F002 (315 nm; 325–530 nm), F003 (380 nm; 390–600 nm), F005 (420 nm; 440–710 nm), F005 (550 nm; 560–780 nm), dye X (281 nm; 295–470 nm), dye QS (347.5 nm; 375-675 nm), and dye Y (462 nm; 490–780 nm). The excitation wavelengths of the BAM fluorophores corresponded to the dye´s longest wavelength absorption maximum. The spectra had to be measured with a spectral resolution of 1 to 2 nm, preferably at 25°C. To account for temperature-induced spectral changes, BAM had previously measured the temperature dependence of the emission spectra within the room temperature range of 20°C to 30°C. The following measurement cycle was recommended with the date and time for each measurement to be provided: 3 scans dye F001, 3 scans solvent ethanol using conditions dye F001 (i.e. “blank dye F001”) etc. in the order of dye F001 to F005, dye X, SRM 936a (dye QS), and dye Y. If two concentrations, i.e. A = 0.04 and A = 0.08 (F001 to F005; dye X, dye Y) were used for a dye, for instance dye F001, 3 scans dye F001 should be substituted by 3 scans dye F001 (A = 0.04) and 3 scans dye F001 (A = 0.08). For each dye-solvent pair, use of the same cuvette was recommended. If for any reason different cuvettes were employed, this had to be indicated. If the instrument calibration and the measurement of the dye samples were not performed on the same day, two samples differing in their spectral emission range had to be measured on the day of the instrument calibration to account for temporal changes of the instrument. As the dyes are nearly isotropic emitters, the use of polarizers was not mandatory. [7]

The measurement parameters used by the study participants are summarized in Table 3S. The measurement protocol employed by NRC was not in strict conformance to that specified for this study since the NRC instrumentation and procedures have been optimized for colorimetric rather than analytical applications. Firstly, the instrument has a measurement geometry of 45° illumination and normal viewing compared to the other participants that used a 0°/90°-geometry. In order to approximate

a normal illumination and 90° viewing geometry on the NRC instrument, a tubular-shaped sample cuvette was used that was mounted in a V-shaped blackened holder. The cuvette was positioned in the measurement beam to give a maximum detector signal. By using the diode laser alignment procedure routinely employed with this instrument for aligning the sample mounts, it was possible to reproduce this optimum sample position to within 0.04 mm. Secondly, standard colorimetric methods recommend that the measurement interval should be equal to the spectral bandpass. In the case of the NRC instrument which has been optimized for 5-nm band-pass conditions, the detection system had been calibrated for a 5-nm measurement interval. Since the comparison protocol for this study specified a measurement interval of 1 or 2 nm, the NRC instrument had to be re-calibrated just prior to this study using a 2 nm-measurement interval, see *Calibration Procedures and Physical Standards* and Table 2S. Finally, the signals measured on the NRC instrument for these fluorescent dye samples were much weaker than typically encountered when measuring fluorescent surface colors. For this reason, the NRC experimental conditions were not kept constant during this study, but were optimized to enhance the measurement sensitivity for each sample e.g., by the use of different gratings for the excitation and emission monochromators depending upon the excitation and emission ranges of the samples, see sections on *Participants* and *Calibration Procedures and Physical Standards* and Table 3S.

⟨Insert Table 3S, here⟩

Requested Data. The following data were provided by each participant: 1) Description of fluorescence measuring system and routine calibration procedures, see previous section and Tables 1S and 2S. 2) Raw spectra of dyes and solvent (wavelength and intensity values with no corrections or averaging, date, and time of measurement indicated as well as all the measurement parameters used), see previous section and Table 3S. 3) Averaged spectra (wavelength and average intensity values) of the three raw spectra obtained for each dye including the corresponding solvent or blank spectra. 4)

Background-corrected spectra.* 5) Wavelength accuracy data tables, see e.g. Table 2S. 6) Emission correction file(s): PTS-based relative spectral responsivity (including wavelength-dependent uncertainties) and RM-based emission correction curve. 7) Spectrally corrected emission spectra of all eight dyes using the routine PTS-based emission correction including the respective wavelength-dependent uncertainties (see also Table 2S), and 8) Spectrally corrected emission spectra of the three test dyes X, QS, and Y using the RM-based standardized emission correction. 9) All raw data (raw: no background correction, no spectral correction, single scans, i.e., no averaging; separate data for each dye and each blank or background spectrum) provided as ASCII files.

*Data Analysis by Participants***.** The following steps were performed by each participant: 1) Averaging of the three spectra for each dye/sample or blank/solvent spectrum. 2) Consideration of the instrument´s linear detection range: the intensity values of each averaged spectrum had to be either corrected for non-linearity errors or the signal intensities had to be recorded within the (previously determined) linear range. The "linearity-related error" had to be included into the calibration uncertainty budget (see Table 2S). 3) Background correction, i.e., subtraction of the average background intensity from the average sample intensity for each sample/blank pair at each wavelength. 4) Wavelength accuracy correction: Correction of the wavelength values of each background-corrected spectrum using the wavelength scale calibration function, if necessary, or inclusion of the wavelength accuracy error into the uncertainty. 5) Spectral Correction of the emission spectra with the previously determined routine PTS-based emission correction $C_{PTS}(\lambda_{em})$. The correction function should be expressed in spectral radiance units, i.e., on a wavelength scale, not in spectral photon radiance units, i.e., on an energy scale. [1, 2, 8, 10] Determination of a standardized RM-based emission correction with the BAM-certified spectral fluorescence standards F001 to F005 and the BAM software *LINKCORR* following a BAM SOP as a basis for a common

* For each dye, the average (spectrally uncorrected) blank spectrum $I_b(\lambda_{em})$ was subtracted from the measured average (spectrally uncorrected) dye spectrum $I_m(\lambda_{em})$ to obtain the average, (spectrally uncorrected) blank-corrected spectrum $I_u(\lambda_{em})$, see equation 3S, which is referred to here also as the raw spectrum (S_{raw}): $I_u(\lambda_{em}) = I_m(\lambda_{em}) - I_b(\lambda_{em})$.

method for the characterization of the spectral characteristics of the emission channel and common data evaluation. *LINKCORR* calculates the ratios $Q^{F001\text{-}F005}(\lambda_{em})$ of the BAM-corrected emission spectra $I_{corr,BAM}(\lambda_{em})$ and the measured spectrally uncorrected, blank-corrected emission spectra $I_u(\lambda_{em})$, see equation 3S, for each spectral fluorescence standard, [1, 7] see equation 4S

$$Q^{F001\text{-}F005}(\lambda_{em}) = I_{corr,BAM}(\lambda_{em}) / I_u(\lambda_{em}) \quad \text{(eq. 4S)}$$

Weighted combination of $Q^{A\text{-}E}(\lambda_{em})$ by *LINKCORR* yields an overall emission correction curve that corresponds to the inverse relative spectral responsivity of the instrument $1/s(\lambda_{em})$ equaling $1/C_{PTS}(\lambda_{em})$. Corrected (instrument-independent) emission spectra are then obtained upon multiplication of measured blank-corrected spectra with this correction curve $I_c(\lambda_{em}) = I_u(\lambda_{em}) \times 1/C_{PTS}(\lambda_{em})$, see also equation 2S.

7) RM-based correction of the emission spectra of dyes X, QS, and Y.

Data pre-treatment: Complete datasets (i.e., containing measurement results from all participants) were available for an absorbance of 0.08 for all eight dyes. This is related to the lower spectral sensitivity of the colorimetric setup. Data assessment thus focuses on measurements with dye solutions with A = 0.08. The spectral format of the corrected non-normalized spectra of the study participants varied due to different start and end points and different spacing/step widths. The emission spectra were truncated to give common start and end points by simply cutting possible edges/tails either below a 5 % relative signal level or at the smallest and largest wavelength position at which data points were available for all participants depending on which criterion applied first. Subsequently, the truncated data were projected onto a common sampling grid. A sampling interval of 2 nm was chosen to accommodate the results of all laboratories. For the preliminary inspection of the data, they were normalized by the maximum value in the measured spectra. As the detector signals at maximum emission can show a non-negligible variability, the maximum emission intensity may not necessarily coincide with the actual maximum of the emission spectrum.

Determination of the intercomparison reference function (ICRF). Although there have been a great number of analyses originating from different fields of measurement and testing, where measurands are

not single values, but functional relationships between two or even more variables, the problem of how to define a good ICRF still remains open. Albeit some attempts have been made at the level of the Bureau International des Poids et Mesures (BIPM) for defining key comparison reference values (KCRVs), [11] a generally accepted procedure is still lacking.

An ICRF is normally found by an appropriate fit procedure which minimizes the residual scatter, i.e. the residual deviations of the measured points from the ICRF. Besides the most commonly used minimization of the (absolute) sum of squared deviations (SSD) shown in equation 5S

$$SSD = \sum_{j,k}^{J,K} [f_k \cdot y_{jk}(\varphi(x_{jk})) - ICRF(\varphi(x_{jk}))]^2 = \min \qquad \text{(eq. 5S)}$$

(see also equation 1 in the manuscript), other approaches are feasible, e.g., the minimization of the sum of relative deviations according to equation 6S

$$SSD_{rel} = \sum_{j,k}^{J,K} [\frac{f_k \cdot y_{jk}(\varphi(x_{jk})) - ICRF(\varphi(x_{jk}))}{ICRF(\varphi(x_{jk}))}]^2 = \min \qquad \text{(eq. 6S)}$$

Equations 5S and 6S lead to different results since they emphasize different parts of the emission spectra: equation 5S optimizes the peak region while equation 5S focuses mainly on the tails where the relative contributions to the SSD are large. Equation 5S includes the trivial solution, namely $\forall\ f_k = 0$, ICRF ≡ 0, and therefore SSD = 0. Care must be taken to prevent the optimization algorithm from yielding the trivial solution. In the most general case, the minimization problem does not have a unique solution. Allowing for a shift in the independent variable causes an ambiguity, namely a common constant which cannot be defined by optimization. This is because results may be highly comparable, but still not necessarily true. To avoid ambiguity, additional constraints and assumptions on f_k and $\varphi(x_k)$ are needed.

The ICRFs were constructed on the grounds of statistical reasoning and in a principles-based approach. For the former, an appropriate statistical estimate like the mean, the median, or any other directly calculated estimate from the available data forms the ICRF point- or sector-wise. Interpolating functions like splines, kernels, and polygons for pre-defined ranges of the emission spectra are also

feasible. In a principles-based approach, physically reasonable model functions are assumed to describe the actual emission spectrum with parameters to be defined by regression. This approach was tested but failed to describe the fine structure of the observed spectra, in particular slight shoulders in the vicinity of the emission maximum which are characteristic for some of the dyes.

Models and adjustments. Finding appropriate f_k is mandatory in the process of defining the ICRFs. If one allows for adjustments in the independent variable, the problem of ambiguity arises. A unique solution can only be obtained when additional constraints are used, e.g. by allocating the minimum-variance solution at a certain point on the x scale. This corresponds to the mean value of the original maximum positions of the emission spectra. Besides a possible shift of the spectra, one might also assume a distortion of the individual wavelength scales rendering the minimization problem even more ambiguous. However, distortions introduced by improper calibration of the wavelength scale are not very plausible when considering the established procedures used in this study for the characterization of the wavelength accuracy of the emission monochromators, the expertise of the participants, and the uncertainties of the wavelength accuracy of the participating laboratories summarized in Table 2S.

2D averaging. 2D averaging consisted of the following steps: 1) Each spectrum included in the optimization procedure was made "continuous" by an interpolation rule which retained the experimentally determined data points in space, and allowed continuous interpolation between them. For this study, frequency polygons, i.e., straight-line interpolations between data points were used. 2) Emission spectra were subjected to scaling (y-axis) and shifting (x-axis). 3) At any of the iteration steps, each intensity value of the measured emission spectra was attributed to the closest actual sampling position (normally the sampling points of the original, non-shifted grid) in the independent variable. Here, the average was taken over k, i.e., the data points in the close vicinity of the actual sampling point of all the spectra measured. Note, that the average of the shifted positions is normally different from the sampling points on the original grid. The ICRFs are then represented by a frequency polygon interpolating all data points calculated. 4) Individual deviations of each laboratory from the ICRFs and

the total SSDs were estimated as derived in the previously discussed interpolation rule. 5) The total SSDs were minimized by adjusting the parameters f_k and δ_k in an iterative procedure. 6) After reaching convergence, i.e., finding best-fit estimates for f_k and δ_k, the joint confidence region (JCR) for each of the data points, which make up the ICRFs, was determined. Upper and lower confidence intervals (CIs) of the point on the ICRFs were then estimated as the points where the bisecting line of the ICRF frequency polygon passed through the JCR.

S5 Results for PTS-based spectral correction

⟨Insert Figure 1S, here⟩

The relative differences between the ICRF and the reference value for the PTS-based corrected emission spectrum of QS (CI: confidence interval; k = 2) are summarized in Figure 1S. [8] For most parts of the spectral region covered by the emission of QS, these deviations are within the NIST-stated uncertainties. However, for wavelengths ≥ 555 nm, the deviations reach the very edge of the expanded uncertainties.

S6 Results for RM-based spectral correction

The BAM RMs display broad and unstructured emission spectra to minimize the dependence of the shape of the spectra on instrument resolution/spectral band-pass, a very small overlap between absorption and emission for a minimum influence of dye concentration and measurement geometry on spectral shape, and moderate to high fluorescence quantum yields for optimal signal-to-noise ratios and minimum influence of stray light, solvent emission, and fluorescent impurities on spectral shape. [1, 12] Due to their small emission anisotropy (r), e.g., r ≤ 0.05 within the analytically relevant room temperature range, F001 to F005 can be used without polarizers, see also Table 3S. [1, 7] Due to their

spectral radiances and radiating volumes closely matching those of routinely measured dye solutions, these dyes enable the straightforward determination of the relative spectral responsivity of fluorescence instruments under routine measurement conditions. To eliminate potential sources of uncertainty, prior to this interlaboratory comparison, the emission spectra of these dyes were evaluated by BAM under the measurement conditions (dye concentration, temperature etc.) to be used by the participating laboratories if these conditions deviated somewhat from the certifying conditions. The wavelength-dependent uncertainties of the RM-based corrected emission spectra of the participants were calculated by BAM and NIST as described in the section *Data assessment* in the manuscript.

S7 Sources of calibration uncertainty

During the course of this study and additional studies with non-expert or field laboratories, we identified two major sources of uncertainty for spectral correction: 1) Instrument characterization and measurement of the emission spectra under non-identical experimental conditions and 2) non-linearities of the emission detection system that could result in distorted emission spectra. Uncertainties of the wavelength scale or wavelength accuracy are typically not problematic for monochromator/PMT-based detection systems.

Different instrument settings for instrument calibration and fluorescence measurements. The influence of different emission monochromator gratings was demonstrated by one participant with additional data (Figure 2S). For wavelengths ≤ 425 nm, a different grating was used for measuring dye QS than was employed for the measurement of the emission spectra of F001 to F005 and other data submitted by this laboratory. As follows from Figure 2S, the resulting corrected dye spectrum strongly deviates, by as much as 25 % from the corrected emission spectra of the other participants and the ICRF. This clearly demonstrates the considerable impact of changes in the instrument operation conditions for instrument characterization and subsequent fluorescence measurements on the quality of the corrected emission spectra. Similar effects are observed for a PTS-based calibration.

⟨Insert Figure 2S, here⟩

Detector non-linearities. Figure 3S shows the influence of detector non-linearities on emission spectra in the case of dye F003 by exploiting the proportionality between fluorescence intensity and absorption factor $f(\lambda_{ex})$. [13] For the illustration of these effects, different measurement parameters were used than for the interlaboratory comparison; in particular, larger spectral bandpasses of the excitation and emission monochromators. Also, background corrected, spectrally uncorrected data were compared. The curves shown in Figure 3S (top) present the ratio between the normalized emission spectra of F003 measured at different dye concentrations or absorption factors $f(\lambda_{ex})$, see equation 7S, and the emission spectrum obtained at the lowest concentration used as reference. In equation 7S, T equals the transmission, A the absorbance, ε the molar (decadic) absorption coefficient and l the optical pathlength.

$$f(\lambda_{ex}) = 1 - T(\lambda_{ex}) = 1 - 10^{-A(\lambda_{ex})} = 1 - 10^{-\varepsilon(\lambda_{ex}) c\, l} \qquad \text{(eq. 7S)}$$

For the data evaluation procedure chosen, two emission spectra of identical spectral shape and ideal proportionality between emission intensity and absorption factor (full detector linearity) yield a straight line parallel to the *x*-axis at a value of unity. Surprisingly very small deviations from the linear range of the detector result in a slight downwards shift of the straight line (still parallel to the *x*-axis) and even slight distortions of the spectrum´s spectral shape are clearly seen by deviations from the straight line. This underlines the considerable influence of detector non-linearities on the absolute value of the signal and on the spectral shape of emission spectra. Also, this simple and straightforward procedure presents a very sensitive tool for the determination of the linear range of a spectrofluorometer´s detection system. Prerequisites are here fluorophores revealing concentration-independent emission spectra (no dimer formation or aggregation; minimum overlap between absorption and emission bands to avoid inner filter effects and reabsorption, and only use of comparatively small absorbances). [1, 14]

⟨Insert Figure 3S, here⟩

In the lower panel of Figure 3S, typical spectral distortions due to detector non-linearities are compared with the corresponding non-linearity-corrrected data obtained from the measured data corrected with a non-linearity calibration procedure commonly used in spectroscopy. This correction included also a constant term for residual background noise. Parameters for the correction function were determined by minimizing the sum of the squared deviations of all ratios from the theoretical value unity. Here, a single coefficient (quadratic term) for describing the non-linearity effects proved to be sufficient. With the aid of this correction, the resulting gain in the sum of squared deviations reached a value of more than 32. As can be clearly seen in the lower panel of Figure 3S, all ratios of the non-linearity corrected spectra center around the theoretical line, but still display a slight waviness. This waviness, however, does not exceed 3 % relative for all wavelengths and spectra except in the wings) and is thus still at the level of the method reproducibility. Hence, this procedure presents an effective method for the subsequent analysis of the effects of detector non-linearities.

Another elegant method for the determination of the linear range of detection systems is highlighted in Figure 4S. [1] Figure 4S displays the ratio of the emission intensities measured at a single wavelength with the emission polarizer (P_{em}) set to 90° (maximum transmission efficiency of the emission monochromator) and 0° (minimum transmission efficiency of the emission monochromator), respectively, as a function of the spectral irradiance reaching the detector. Controlled variation of the spectral radiance of the instrument´s excitation source, scattered with a non-fluorescent reflection standard at the sample position into the emission channel of the fluorometer, was achieved with the aid of several calibrated neutral density filters placed in the filter holder in the excitation channel (after the excitation monochromator and in front of the white standard). The excitation monochromator and the emission monochromator were both set to 400 nm. For detector non-linearity to have a negligible influence, the response ratio of the signals at 90° and 0° should be constant. At larger spectral radiances, the response ratio decreases as a consequence of the onset of detector saturation for the more intense signal resulting for P_{em} = 90°. At higher spectral radiances, considerable deviations from linearity result

in a downward bending of both signals (see Figure 4S). The onset of the upper limit of the linear range of the detector depends on instrument design, especially on the type of the detector used, on detection mode (photon counting; analog), and e.g. in the case of photomultiplier tubes, the applied voltage.

⟨Insert Figure 4S, here⟩

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