NISTIR 7458

Standard Guide to Fluorescence - Instrument Calibration and Validation

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Technology Administration, U.S. Department of Commerce

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1. Scope

To list the available materials and methods for each type of calibration or correction for fluorescence instruments (spectral emission correction, wavelength accuracy, etc.) with a general description, the level of quality, precision and accuracy attainable, caveats, and useful references given for each entry. The listed materials and methods are intended for the qualification of fluorometers as part of complying with regulatory and other QA/QC requirements. Precision and accuracy or uncertainty are given at a 1 σ confidence level and are approximated in cases where these values have not been well established.[1]

2. Wavelength Accuracy

Methods for determining the accuracy of the emission (EM) or excitation (EX) wavelength for a fluorescence instrument are given here with an emphasis on monochromator (mono) based wavelength selection. An example spectrum, which has been spectrally corrected, is given for each method.

Table 1 : Summary of Methods for Determining Wavelength Accuracy

Sample	λ region	Drop-In	Off-Shelf	Uncertainty	Caveats	Established Values	Ref.
Pen Lamp	UV-NIR (EM)	Maybe	Y	± 0.1 nm or better	alignment	Y	1, 2
Dy-YAG crystal	470nm-760nm (EM) 255nm-480nm (EX)	Y	Y	± 0.1 nm		Y	3
Eu glass	570nm-700nm (EM) 360nm-540nm (EX)	Y	Y	± 0.2 nm		N	5, 6
Anthracene in PMMA	380nm-450nm (EM) 310nm-380nm (EX)	Y	Y	± 0.2 nm	limited range	N	
Ho_2O_3 + DR	330nm-800nm (EM or EX)	Maybe	Y	± 0.4 nm	need blank	Y	9-11
Xe Source	400nm-500nm (EX)	Y	Y	± 0.2 nm	limited range, calibration	N	12
Xe Source + DR	UV-NIR	Maybe	Y	± 0.2 nm	EX mono must be calibrated	Y	12
Water Raman	UV-blue	Y	Y	± 0.2 nm	EX mono must be calibrated	N	13

[1] Certain commercial equipment, instruments, or materials are identified in this paper 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.

2.1 Low Pressure Atomic Lamps [1, 2]

These low pressure atomic lamps, often referred to as pen lamps due to their size and shape, should be placed at the sample position and pointed toward the detection system for emission wavelength accuracy determination. The emission wavelength selector (λ_{EM}-selector) is then scanned over the wavelength range of interest. (see Fig. 1) High accuracy is only achieved when the lamp is aligned properly into the wavelength selector, e.g., the light fills the entrance slit of the monochromator. Atomic lines that are too close to other atomic lines to be resolved by the instrument should not be used, i.e., may not be appropriate for low resolution instruments. Although these lamps can be placed at the excitation source position for excitation wavelength accuracy determination, weaker signals are typically observed, e.g., by a reference detector, and alignment is more difficult than for the emission wavelength accuracy determination.

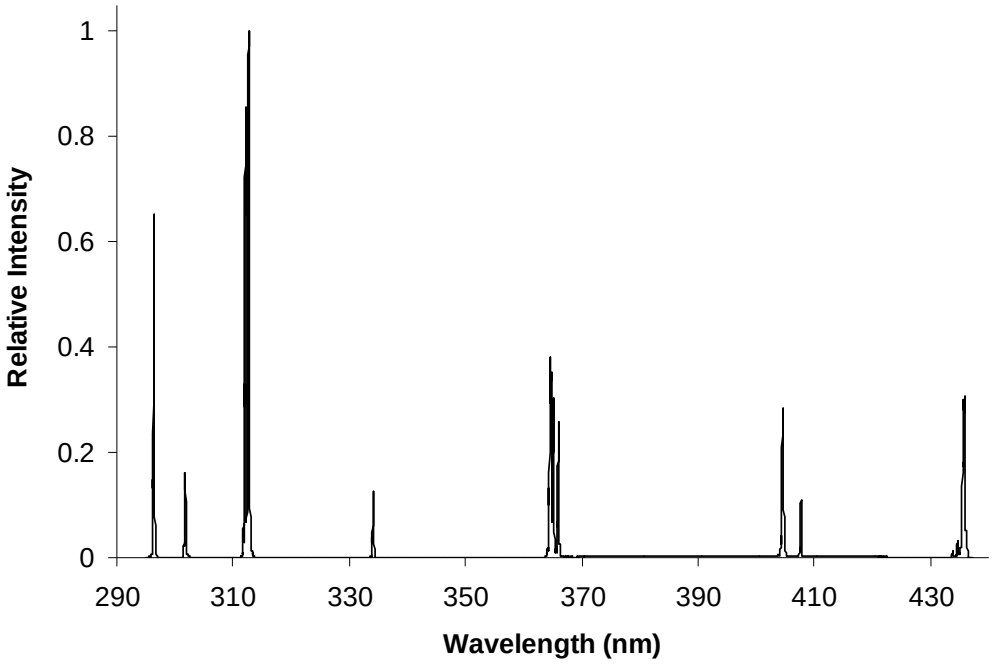

Fig. 1: Hg pen lamp spectrum

2.2 Dy-YAG crystal [3]

This sample is available in standard cuvette format, so it can simply be dropped into a cuvette holder. An excitation or emission spectrum is then collected for an excitation or emission wavelength accuracy determination, respectively. (see Fig. 2) Peaks that are too close to other peaks to be resolved by the instrument should not be used, i.e., may not be appropriate for low resolution instruments.

2.3 Eu-doped glass [4] or PMMA [5, 6]

This sample is available in standard cuvette format, so it can simply be dropped into a cuvette holder. An excitation or emission spectrum is then collected for an excitation or emission wavelength accuracy determination, respectively. (see Fig. 3) Accurate peak

positions for this glass have not been well established and the positions of peaks can change somewhat depending on the particular glass matrix used. For these reasons, a one-time per sample determination of these peak positions using another wavelength calibration method is recommended.

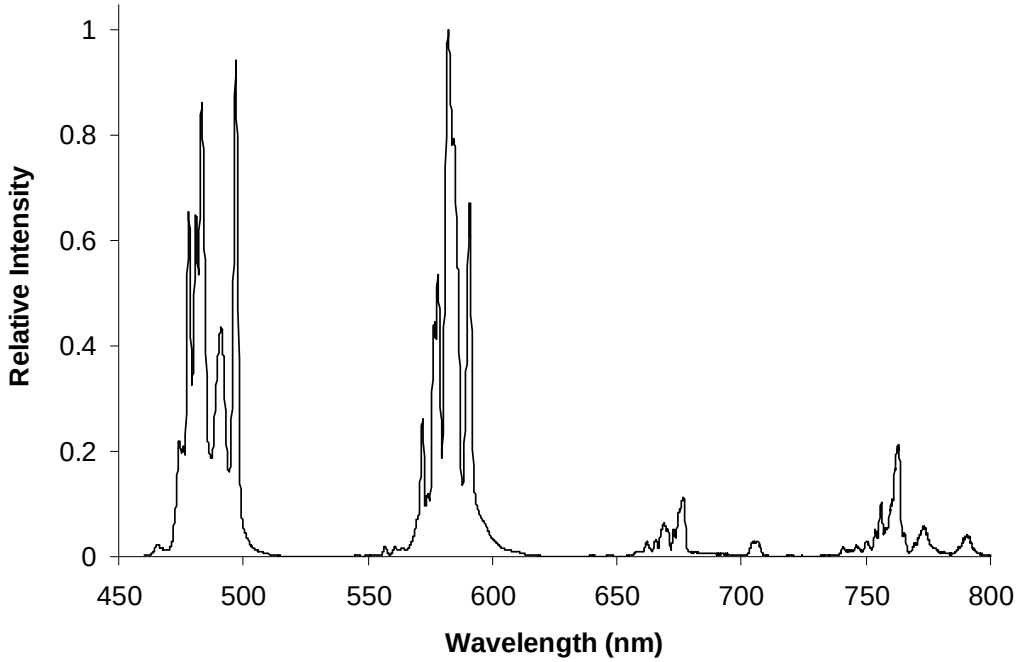

Fig. 2: Emission spectrum of a Dy-YAG crystal excited at 352.7 nm.

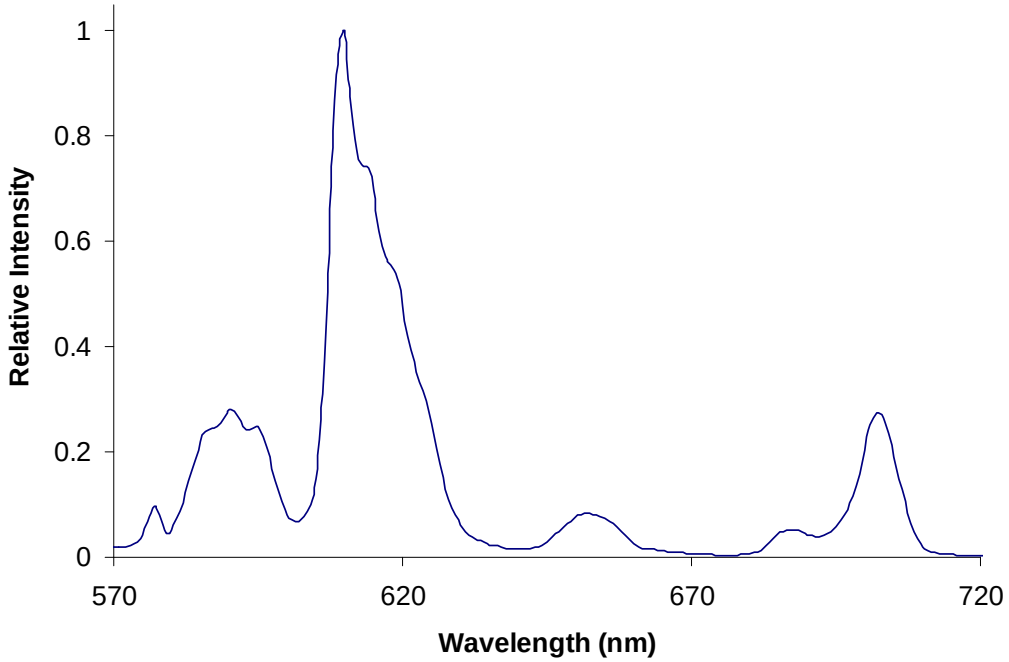

Fig. 3: Emission spectrum of a Eu-ion-doped glass excited at 392 nm.

2.4 Anthracene-doped PMMA [7, 8]

This sample is available in standard cuvette format, so it can simply be dropped into a cuvette holder. An excitation or emission spectrum is then collected for an excitation or emission wavelength accuracy determination, respectively. (see Fig. 4)

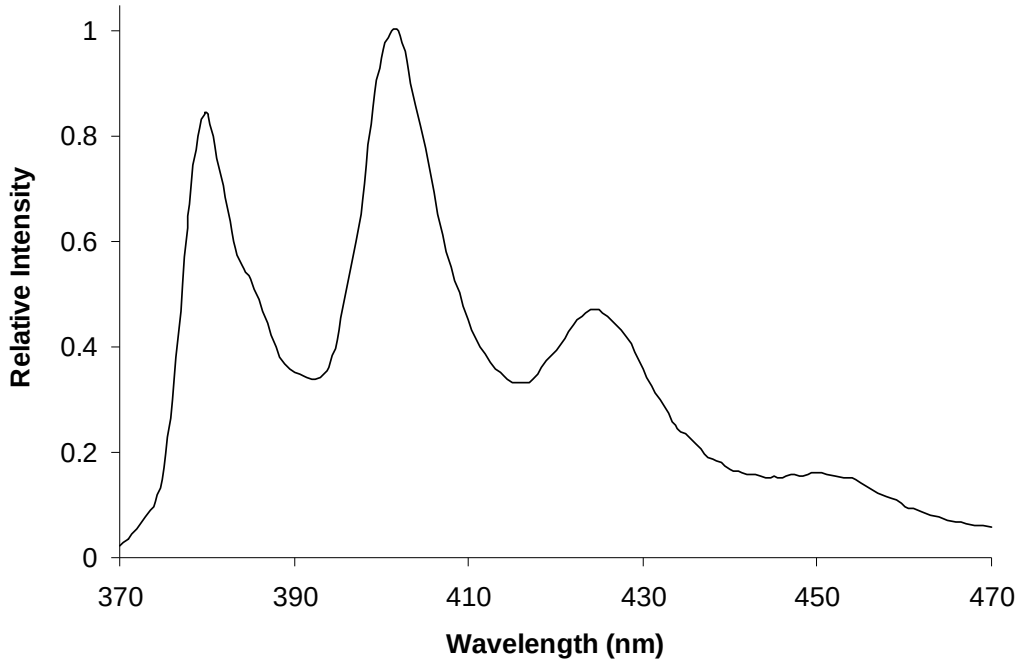

Fig. 4: Emission spectrum of anthracene-doped PMMA excited at 360 nm.

2.5 Ho_2O_3 solution or doped glass with diffuse reflector, scatterer or fluorescent dye [9-11]

This sample is available in standard cuvette format, so it can simply be dropped into a cuvette holder. An excitation or emission spectrum is then collected for an excitation or emission wavelength accuracy determination, respectively. The wavelength selector not being scanned must be removed or set to zero order. The diffuse reflector, scatterer or fluorescent dye is scanned with and without the Ho_2O_3 sample in place and the ratio of the two intensities is calculated to obtain an effective transmittance spectrum with dips in the intensity ratio corresponding to absorption peaks of the sample. (see Fig. 5)

2.6 Xe Source Lamp [12]

This method is for fluorometers that use a high pressure Xe arc lamp as an excitation source. A few peaks between 400 and 500 nm can be used, but most of these are due to multiple lines, so their positions are not well established. (see Fig. 6) For this reason, a determination of these peak positions (one-time per lamp) using another wavelength calibration method is recommended. For EX wavelength calibration the excitation wavelength selector (λ_{EX}-selector) is scanned while collecting the reference detector

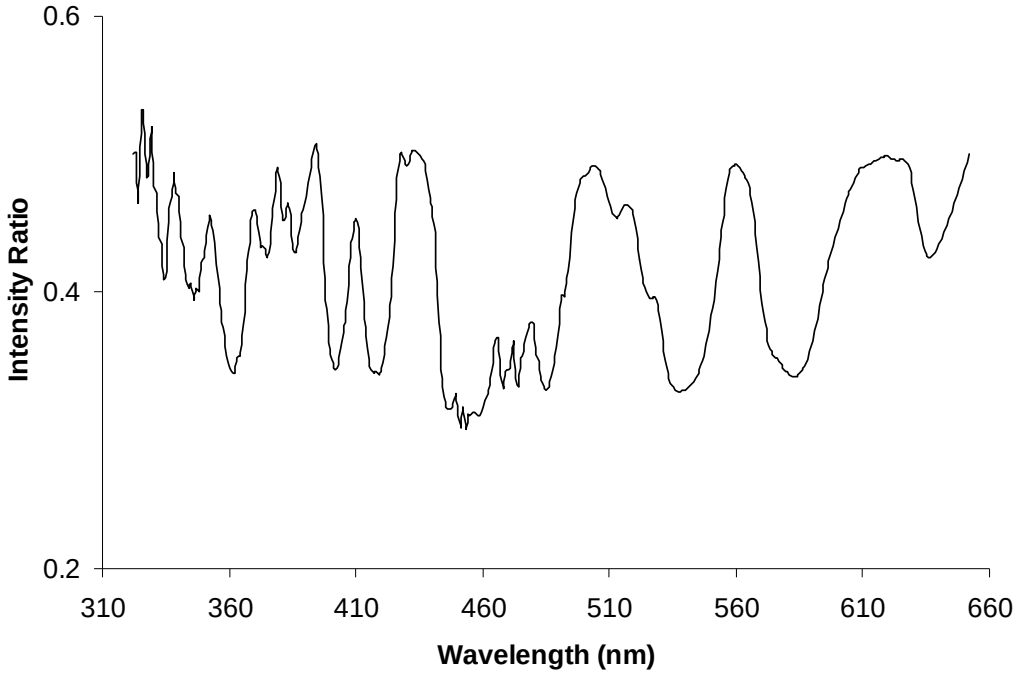

Fig. 5: Effective transmittance spectrum of a Ho_2O_3 doped glass with diffuse reflector.

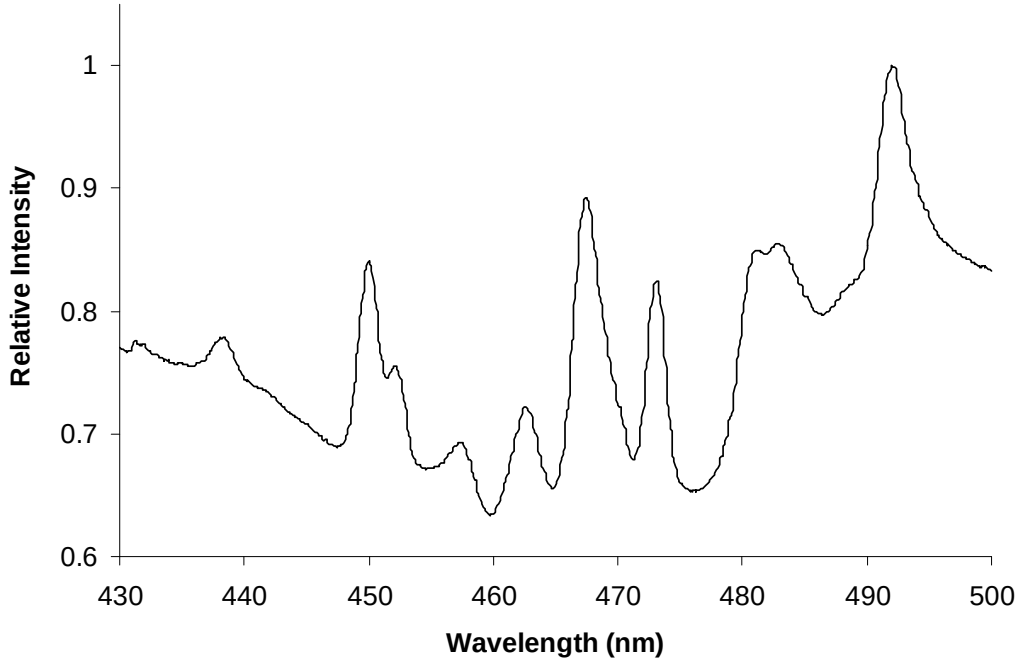

Fig. 6: Xe source lamp (high pressure, 450 W) spectrum in a spectral region containing peak structure.

signal. If this is used for EM wavelength calibration, a diffuse reflector or scatterer must be placed at the sample position and the λ_{EX}-selector must be removed or set to zero order.

2.7 Instrument Source with diffuse reflector or scatterer [12]

A dilute scattering solution in a standard cuvette or a solid diffuse reflector at 45 degrees relative to the excitation beam can be used to scatter the excitation beam into the detection system. One wavelength selector is fixed at a wavelength of interest and the other scans over the fixed wavelength. (see Fig. 7) The difference between the fixed wavelength and the observed peak position is the wavelength bias between the two wavelength selectors at that wavelength. Either the excitation or the emission wavelength selector must have a known accuracy at the desired wavelengths in order to use this method to calibrate the unknown side.

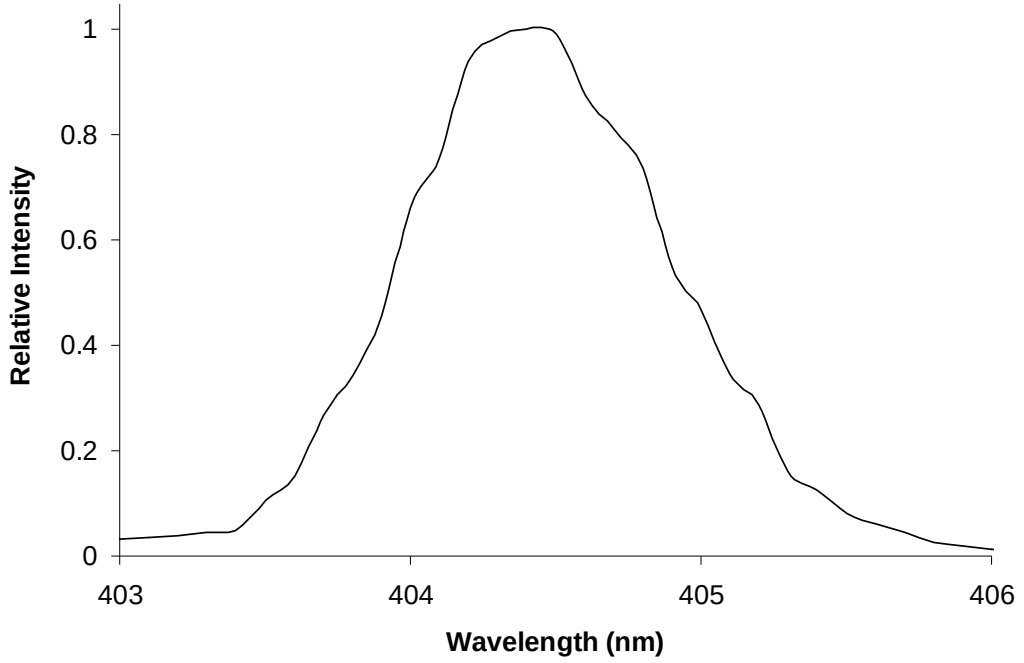

Fig. 7: Excitation source profile with excitation wavelength fixed at 404.3 nm (excitation bandwidth of 1.0 nm) and emission monochromator scanned (emission bandwidth of 0.1 nm)

2.8 Water Raman [13]

Deionized water is used. One wavelength selector is fixed at a wavelength of interest and the other is scanned. (see Fig. 8) The water Raman peak appears at a wavelength that is about 3400 cm^{-1} lower in energy than the excitation wavelength. [14] The Raman scattering intensity is proportional to λ^{-4}, so the Raman intensity quickly becomes too weak to use this method when going into the visible region. Either the excitation beam or the emission wavelength selector must have a known accuracy at the desired wavelengths in order to use this method to calibrate the unknown side.

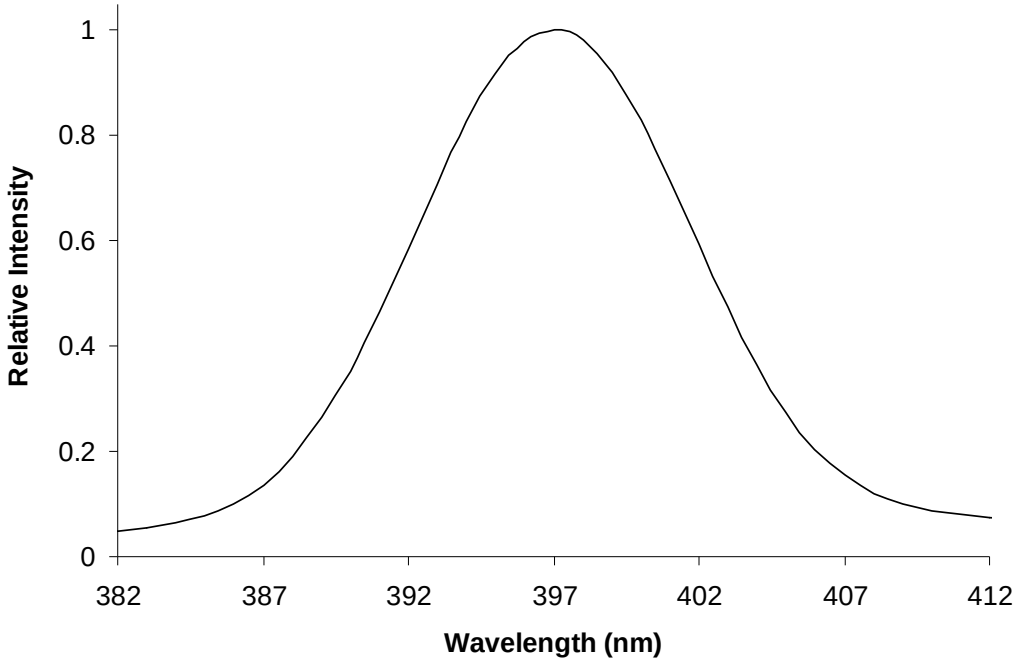

Fig. 8: Water Raman spectrum with excitation wavelength set at 350 nm and excitation and emission bandwidths at 5 nm.

3. Spectral Slit Width Accuracy

Spectral slit width accuracy of the emission or excitation wavelength selector can be determined by measuring the spectral bandwidth, taken to be the full width at half the peak maximum (FWHM), of a single line of a pen lamp, using the same setup and with the same caveats described in section 2.1. [1] For fluorescence spectrometers with both excitation and emission monochromators, an alternative method may be used where one monochromator is scanned over the position of the other, using the setup described in section 2.7 [12] The uncertainties involved in both methods have not been well established, but ± 0.5 nm or better is estimated here based on what has been reported.

4. Linearity of the Detection System

Several methods can be used to determine the linear intensity range of the detection system. They can be separated into three types, based on the tools used to vary the intensity of light reaching the detector: 1) double aperture, 2) optical filters and/or polarizers, 3) fluorophore concentrations. The double aperture method is the most well established and probably the most accurate when done correctly, but is also the most difficult to perform. [15, 16] A variety of methods using optical filters, polarizers or a combination of the two have been reported. [12, 17] These methods require high quality, often costly, components and some expertise on the part of the user. The third method is the most popular and easiest to implement. It uses a set of solutions obtained by serial

dilution of a fluorescent stock solution, similar to that used for obtaining calibration curves for analyte concentration, as described in section 5.1. In this case, solutions with low concentration (A<0.05 at 1cm pathlength) should be used and fluorophore adsorption to cuvette walls may affect measurements at vey low concentrations. [18] Users must insure that the fluorescence intensities of samples are reproducible and do not decrease over the time period that they are being excited and measured, because the organic dyes typically used can be prone to photobleaching.

5. Spectral Correction of Detection System Responsivity

Calibration of the relative responsivity of the emission detection system with emission wavelength, also referred to as spectral correction of emission, is necessary for successful quantification when intensity ratios at different emission wavelengths are being compared or when the true shape or peak maximum position of an emission spectrum needs to be known. Such a calibration is necessary because the relative spectral responsivity of a detection system can change significantly over its wavelength range (see Fig. 9). It is

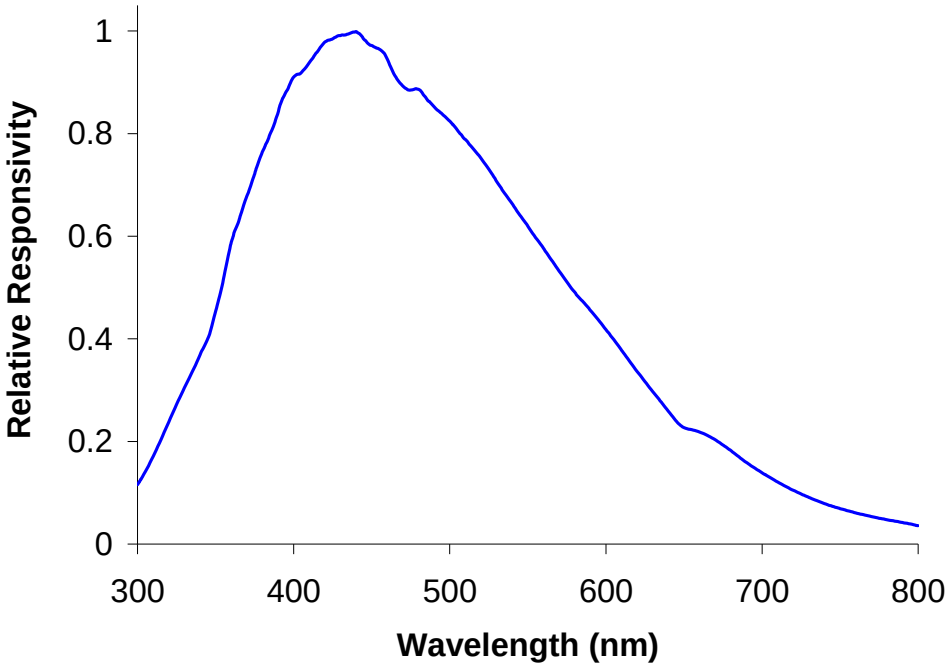

Fig. 9: Example of the relative spectral responsivity of an emission detection system (grating monochromator-PMT based), [12] for which a correction needs to be applied to a measured emission spectrum to obtain its true spectral shape (relative intensities).

highly recommended that the wavelength accuracy (see section 2) and linear range of the detection system be determined (see section 4) before this calibration is performed and that appropriate steps are taken (e.g., the use of attenuators) to insure that all measured intensities during this calibration are within the linear range. Also note that when using an emission polarizer, the spectral correction for emission is dependent on the polarizer setting. In addition, users must be careful to insure that signal intensities and correction

factors are expressed consistently, either in power units or in photon units. Some detectors, such as those with photon-counting capabilities and quantum counter detectors, give a signal that is proportional to the number of incident photons, while other detectors give a signal that is proportional to the incident power. Energy and photon signals can be interconverted easily (see *Planck's constant* in section 12).

Table 2 : Summary of Methods for Determining Spectral Correction of Detection System Responsivity

Sample	λ region	Drop-In	Off-Shelf	Uncertainty	Caveats	Certified Values	Ref.
CS	UV-NIR	N	Y	< ± 5 %	difficult setup	Y	12, 17 20-22
CD + CR	UV-NIR	N	Maybe	± 10 %	difficult setup	Y	12, 20, 21
CRMs	UV-NIR	Y	Y	± 5 %		Y	23-25

5.1 Calibrated Light Source (CS) – Tungsten [19] Lamp [12, 20, 21, 17, 22]

The light from a CS is directed into the emission detection system by placing the CS at the sample position. If the CS is too large to be placed at the sample position, a calibrated diffuse reflector (CR) may be placed at the sample position to reflect the light from the CS into the emission detection system. The λ_{EM}-selector is scanned over the emission region of interest, using the same instrument settings as that used with the sample, and the signal channel output (S″) is collected. The known radiance of the CS incident on the detection system (L) can be used to calculate the relative correction factor (C_{CS}), such that $C_{CS} = L / S''$. The corrected emission intensity is equal to the product of the signal output of the sample (S) and C_{CS}.

5.2 Calibrated Detector (CD)[2] with Calibrated Diffuse Reflector (CR) [12, 20, 21]

This is a two-step method. The first step uses a CD to measure the flux of the excitation beam as a function of excitation wavelength, as described in section 3.1. The second step uses a CR with reflectance R_{CR} to reflect a known fraction of the flux of the excitation beam into the detection system. This is done by placing the CD at the sample position at a 45° angle, assuming a 0°/90° instrument geometry, and synchronously scanning both the λ_{EX}- and λ_{EM}-selectors over the emission region of interest while collecting both the signal output (S′) and the reference output (Rf′). This method enables the relative correction factor (C_{CD}) to be calculated using the equation $C_{CD} = (C_R R_{CR} Rf') / S'$, see section 3.1 for definition of terms.

[2] It is assumed in what follows that a calibrated detector is either a photodiode mounted inside an integrating sphere or a photodiode alone, whose spectral responsivity is known. The former is typically the more accurate of the two, because the integrating sphere insures spatially uniform illumination of the photodiode.

5.3 Certified Reference Materials (CRMs) [23-25]

The CRMs presently available are either organic dye solutions or solid, inorganic glasses released by national metrology institutes with certified relative fluorescence spectra, i.e., relative intensity and uncertainty values are given as a function of emission wavelength at a fixed excitation wavelength. They have been designed to closely resemble typical samples. A CRM is placed at the sample position and its spectrum is collected and compared to the certified spectrum according to the instructions given on the accompanying certificate, yielding spectral correction factors for the instrument. The corrected emission spectra of some commonly used dyes have also been reported recently in the literature. [26, 27]

6. Spectral Correction of Excitation Beam Intensity

Calibration of the excitation intensity with excitation wavelength is necessary for successful quantification when intensity ratios at different excitation wavelengths are being compared or when the true shape or peak maximum position of an excitation spectrum needs to be known. Such a calibration is necessary because the relative spectral flux of an excitation beam at the sample can change significantly over its wavelength range (see Fig. 10). The neglect of excitation intensity correction factors can cause even greater errors than that of emission correction factors. [12, 28] Fortunately, many fluorescence instruments have a built-in reference detection system to monitor the intensity of the excitation beam. This is usually done using a photodiode or a PMT, or a quantum counter detector to measure a fraction of the excitation beam that is split off from the rest of the beam. The collected reference signal can be used to correct the fluorescence signal for fluctuations due to changes in the excitation beam intensity. Reference detectors are often not calibrated with excitation wavelength, introducing errors, which can be particularly large over longer excitation wavelength ranges (e.g., greater than 50 nm) or in a wavelength region where the excitation intensity changes rapidly with excitation wavelength, such as the UV. It is highly recommended that the wavelength accuracy of the excitation wavelength selector (see section 2) and linear range of the detection system used to measure the excitation beam be determined (see section 4) before a spectral correction of the excitation beam is performed and that appropriate steps are taken (e.g., the use of attenuators) to insure that all measured intensities during this calibration are within the linear range. Signals and correction factors must be expressed consistently in either power or photon units (see section 5). Also note that when using an excitation polarizer, the spectral correction for excitation intensity is dependent on the polarizer setting.

Table 3 : Summary of Methods for Determining Spectral Correction of Excitation Beam Intensity

Sample	λ region	Drop-In	Off-Shelf	Uncertainty	Caveats	Certified Values	Ref.
CD - Si	UV-NIR	N	Y	± 2 %	difficult setup	Y	12, 17
Quantum Counter	UV-NIR	Y	Y	± 5 %	limited range	N	22, 29
Photodiode - Si	UV-NIR	N	Y	≤ ± 50 %		N	12

6.1 Calibrated Detector - Si Photodiode (CD – Si) [12, 17]

A CD is put at the sample position with the excitation beam incident on it. The output of the CD (S_{CD}) is measured as a function of emission wavelength by scanning the λ_{EX}-selector over the excitation region of interest using the same instrument settings as that used with the sample. The known responsivity of the CD (R_{CD}) is used to calculate the flux of the excitation beam (ϕ_x), such that $\phi_x = S_{CD} / R_{CD}$. The instrument's reference detector can also be used to measure the intensity of the excitation beam by measuring its output (Rf_{CD}) simultaneously with S_{CD}. Then the correction factor for the responsivity of the reference detector $C_R = \phi_x / Rf_{CD}$.

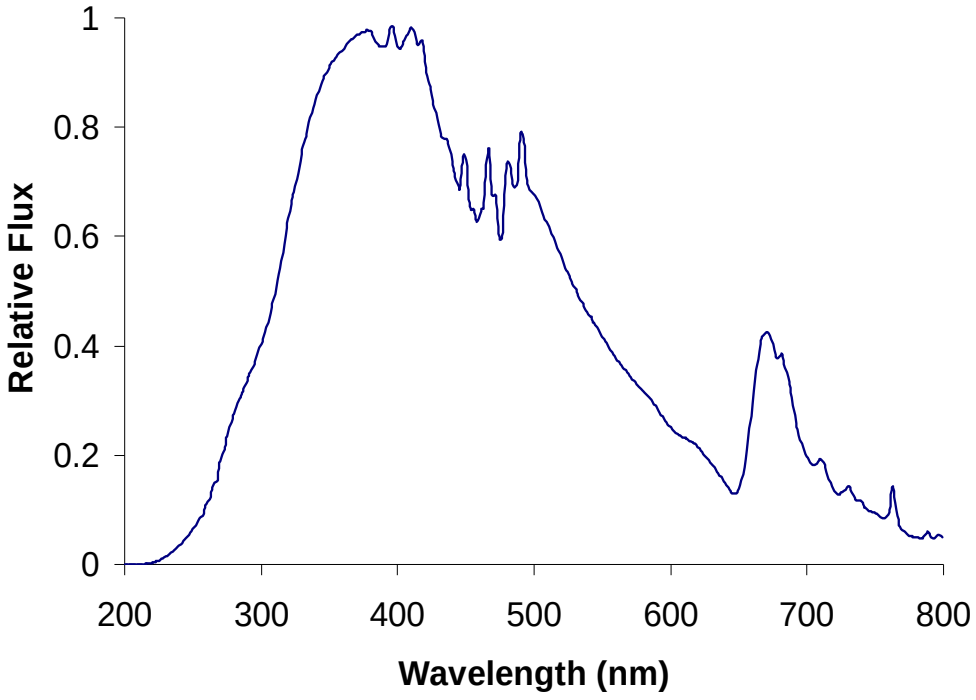

Fig. 10: Example of the relative flux of an excitation beam (Xe lamp-grating monochromator based), [12] for which a correction needs to be applied to a measured excitation spectrum to obtain its true spectral shape (relative intensities).

6.2 Quantum Counters [22, 29]

A quantum counter solution is a concentrated dye solution that absorbs all of the photons incident on it and has an emission spectrum whose shape and intensity do not change with excitation wavelength. The quantum counter solution is placed at the sample position in a quartz cuvette. If front face detection is possible, then a standard cuvette can be used with the excitation beam at normal incidence. If 90-degree detection is only possible, then a right-triangular cuvette can be used with the excitation beam at 45-degree incidence to the hypotenuse side and one of the other sides facing the detector. Scan the excitation wavelength over the region of interest with the emission wavelength fixed at a position corresponding to the long-wavelength tail of the emission band and collect the signal intensity (S_{QC}). The instrument's reference detector can also be used to measure the intensity of the excitation beam by measuring its output (Rf_{QC}) simultaneously with S_{QC}. Then the correction factor for the responsivity of the reference detector $C_R = S_{QC} / Rf_{CD}$ is calculated. Note that each quantum counter has a limited range. For instance, Rhodamine B can achieve the specified uncertainty from 250 nm to 600 nm. Beyond this range the intensity falls off and uncertainties increase. Also note that S_{QC} will be proportional to the quantum flux at the sample, not the flux in power units.

6.3 Si Photodiode (uncalibrated) [12]

This is used in the same way as a calibrated Si photodiode (see section 3.1), except its spectral responsivity is not known. A Si photodiode is sometimes erroneously assumed to have a responsivity that is qualitatively flat over its effective range. In fact, using its output to correct an excitation spectrum can lead to quantitatively significant errors, particularly over a large excitation range and in the UV region. Although, using an uncalibrated Si photodiode for such a correction will almost always yield a more accurate spectrum than when using no correction.

7. Calibration Curves for Concentration

Calibration curves of fluorescence intensity, i.e., instrument responsivity, as a function of fluorophore concentration can be determined for a particular instrument and fluorophore. Reference materials composed of the fluorophore of interest must be used. The highest accuracy is obtained when the fluorophore in both the standard and the sample experience the same microenvironment. For example, they are dissolved in the same solvent or attached to the same biomolecules. This type of calibration enables concentrations and amounts of fluorophores to be compared over time and between instruments without determining the absolute responsivity of the instrument (see section V).

7.1 Fluorophores with specified purity & uncertainty [30]

If the purity of a fluorophore (e.g., a high-purity, organic dye powder) is known, then it can be put in the same microenvironment (e.g., solvent) as an unknown sample to

produce a standard sample. Several standard samples should be produced to cover the concentration range of interest. These standard samples are measured under the same conditions as that of any unknowns and the fluorescence intensities are recorded. Fluorescence intensity versus standard sample concentration is plotted and the points are fitted to a polynomial, typically a straight line. The concentration of an unknown is determined by using the fitted polynomial along with the measured intensity of the unknown to find the corresponding concentration.

7.2 Fluorophore Solutions with specified concentration & uncertainty [31, 32]

Standard solutions with known concentrations can be used in the same way as a standard fluorophore (see section 4.1). In this case the fluorophores are in solution, so they are ready to use or they can be diluted to produce standard solutions of lower concentration. In both cases, the solvent used in the standard and unknown solutions should be the same.

7.3 Molecules of Equivalent Soluble Fluorophore (MESF) [33-35]

The matching of microenvironments between sample and standard solutions, as emphasized in the last two sections, cannot always be achieved. This is of particular concern when the sample contains immobilized fluorophores, e.g., those attached to a cell. In many such cases, it is very difficult or impossible to determine the concentration of fluorophores in a candidate standard solution. MESF units are used, particularly in flow cytometry, to quantify such complex systems. These units express the fluorescence intensity of a fluorescent analyte, e.g., several immobilized fluorophores bound to a microbead or cell, as the corresponding number of free fluorophores of the same type in a standard solution with the same intensity. The MESF scale for a particular fluorophore is determined using the same procedure as that given in section 4.2. This scale is transferred from a conventional fluorometer to a flow cytometer, using fluorophore-labeled microbead suspensions with pre-determined MESF values.

8. Day-to-Day and Instrument-to-Instrument Intensity

The stability of an instrument over time and comparability between instruments of fluorescence intensity is made possible by performance validation standards. The fluorescence intensity of such standards can be monitored over time and between instruments, enabling an absolute intensity scale to be established without performing absolute fluorescence measurements. These standards must emit a fluorescence intensity that does not change with time or irradiation. Another possibility is that they be single-use standards that can be made with a highly reproducible fluorescence intensity. It is not necessary for these standards to reproduce the exact spectrum of analyte samples, but they should be measurable with routine instrument settings, e.g., typical excitation intensity, bandwidths and emission wavelengths.

8.1 Cuvette Format [25, 5 , 6 , 8 , 24 , 23]

This is the most commonly used format in conventional, benchtop fluorometers, as well as in many portable instruments. Both solid and liquid standards are available for this format and most can be used in both 0°/90° and front-face geometries. Standards of this type have been released by NMIs and industry, but the most well known of these is high purity water, where its Raman line is used as a pseudo-fluorescence signal.[36, 37, 13] Unfortunately, the "Water Raman" method is effectively limited to the UV to violet region of the spectrum. Inorganic solid standards are the most robust, most photostable, longest lasting and easiest-to-use of fluorescent samples available in a cuvette format, although organic dyes may more closely resemble the behavior of fluorescent probes.

8.2 Microwell Plate Format [38, 6 , 39]

Some solid materials, similar to those used to make some of the cuvette standards mentioned in section 5.1, have been used to make microwell plate reference materials. These have typically been made by taking a microplate-sized piece of the material and putting a mask over it that mimics the well boundaries. Organic dye solutions, such as those used in section 5.1, can be put into the empty microwells of any plate and used as a standard. Of course, a fresh dye solution of known concentration has to be dispensed each time such a standard is used. The reference materials described here can be used as day-to-day intensity standards for filter-based instruments if the same filter is always used. For instrument-to-instrument comparisons or when filters are changed, the spectral differences between filters must be considered. No microwell plate standards have been recommended by NMIs or generally accepted by the community at-large to this point.

8.3 Microarray Format [40-43]

Standard slides containing arrays of fluorescent dye samples with a morphology and intensity that is more consistent than that of a typical microarray sample are commercially available. No microarray standards have been recommended by NMIs or generally accepted by the community at-large to this point.

9. Limit of Detection and Sensitivity [44, 13]

Methods and reference materials used in section 8 can also be used for determining limit of detection and sensitivity by employing samples that approach the limit of detection of the instrument, i.e., within two orders of magnitude of the background intensity of a blank or of the noise of the detection system, and comparing the sample intensity to the background intensity or noise.

10. Lifetimes [45, 46]

Time-domain and frequency-domain measurements are the two types of fluorescence measurements used to determine fluorescence lifetimes. Conventional instruments include those based on time-correlated single-photon counting (time-domain) and

multifrequency phase and modulation (frequency-domain) techniques, which are typically used to measure lifetimes from picoseconds to microseconds. More simple, time-domain instruments are commonly used to measure lifetimes on the order of milliseconds or longer. Criteria for fluorescence lifetime standards include 1) high purity, 2) a single exponential decay component, and 3) a lifetime independent of excitation and emission wavelengths. Possibly, the most thorough comparison of fluorescence lifetime candidates was recently performed by nine expert laboratories. [45] Almost all of the candidates mentioned in the literature for use as lifetime standards have been liquid, organic dye solutions, probably due to the more complex, excited-state kinetics that exist in most solid fluorescent samples. Lifetime standards are measured in the same way as typical unknown samples. A bias in the measured lifetime or an observed multi-exponential decay of the standard indicates the presence of systematic errors in the instrument.

11. Fluorescence Quantum Yield [47]

Defined as the ratio of the number of molecules that fluoresce a photon to the number of molecules that absorb a photon from the excitation source, this quantity is an intrinsic property of a particular molecular species. Sample effects, such as inner filter effects, often introduce errors into measured quantum yield values. Absolute quantum yields can be particularly difficult to measure accurately, due to instrumental errors that need to be avoided or corrected and experimental setups requiring some expertise on the part of the investigator. Because of this, relative quantum yields are much more commonly measured than absolute by employing a species with a known quantum yield as a reference. This makes the accuracy of the unknown, relative quantum yield dependent on the accuracy of the known, reference value. Unfortunately, the fluorescence quantum yields of very few species have been well established. Absolute spectroscopic methods also use a reference, e.g., a diffuse scatterer, but no reference value needs to be known in advance, as the reference has an effective quantum yield of one. Only methods that can use a conventional fluorescence spectrometer as a detector are summarized below. All of these methods are capable of yielding quantum yield values with an accuracy of ± 10 %, when systematic uncertainties are minimized.

11.1 Absolute methods

11.1.1 Optically Dilute Samples (A < 0.05)

This most commonly used absolute method was developed by Weber and Teale.[48] The variation of this method described here, using a spectrometer as a detector, was first done by Eastman.[49] An optically dilute scattering solution is used as a reference, such as colloidal silica or glycogen. A right angle geometry and identical instrument settings (e.g., excitation wavelength, emission range, bandwidths, etc.) should be used for both sample and reference measurements. In addition, the response of the detector should not be polarization dependent and the excitation light should be unpolarized, or correction factors should be determined to compensate for these. If all of these conditions are not met, then large systematic errors may result.

Solutions at several optical densities, preferable all with A < 0.05, of both the fluorescent sample (f subscript) and the scatterer (s subscript) are made and their absorbances (A) and emission spectra with fluorescence signals (S) are measured at the excitation wavelength. The values of S_f/A_f and S_s/A_s extrapolated to zero optical density, m_f and m_s, respectively, are calculated. [50] The response of the detection system (R_d) as a function of emission wavelength is determined. The polarization (p) and the refractive index (n) of the sample and the reference are measured. The quantum yield (ϕ) is equal to a summation over the emission wavelength range of the product R_d (m_f / m_s) (n_f / n_s)^2 ($3+p_f$)/($3+p_s$). Alternatively, the emission monochromator can be set to zero-order, so all wavelengths reach the detector at once, thereby, removing the summation from the equation. This is less tedious and closer to Weber and Teale's original method where no emission monochromator was used, although stray light is more likely to introduce error in this case.

11.1.2 Optically Dense Samples

This method, first described by Vavilov,[51] is similar to the Weber and Teale method, but a solid, diffuse scatterer, e.g., barium sulfate or sintered polytetrafluoroethylene, with a known diffuse reflectance is used as a reference. Front face detection with the sample parallel to the detector is the best optical geometry. [52] This method requires a detailed knowledge of the optical geometry of the instrument used, among other complications. Therefore, this method is only recommended when optically dense samples must be used.

11.1.3 Integrating Sphere at Sample [53]

An integrating sphere can be placed at the sample position of a fluorescence spectrometer with the sample placed inside the sphere. This eliminates the need for refractive index, polarization, and spatial anisotropy corrections. Excitation and emission ports, facing the excitation beam and detection system (right angle geometry), respectively, are open on the integrating sphere. The light coming out of the exit port is collected by the detection system. If the sample holder in the sphere is able to rotate the sample in and out of the direct path of the excitation beam, this enables secondary excitation (reabsorption) and emission (reemission) to be subtracted out, and the absorptance (α) of a sample to be measured accurately with the same system. The response of the sample-integrating sphere-detection system must first be determined as a function of wavelength. The quantum yield and absorptance can then be calculated using the following equations:

$$\phi_f = \frac{E_{in} - (1-\alpha)E_{out}}{X_{empty}\alpha} \qquad \alpha = \frac{X_{out} - X_{in}}{X_{out}} \qquad (1)$$

where E_{in} and E_{out} are the integrated emission spectra of the sample after direct (sample in the beam path) and secondary (sample out of the beam path) excitation, respectively. X is the integrated profile of the excitation, obtained with the integrating sphere-detection system. Both the emission spectra and the excitation profile are collected with the excitation beam set at a fixed wavelength and the appropriate spectral range being chosen

or scanned by the detection system. The subscripts empty, in and out refer to an empty sphere (no sample), the sample directly in the path of the beam, and the sample in the sphere but out of the direct path of the beam, respectively.

11.2 Relative methods

A relative method using an optically dilute quantum yield standard (reference) with a quantum yield that is known with high accuracy is the most commonly used method for quantum yield determination. Absorbance values (A) and emission spectra for the unknown sample and standard reference are measured at their corresponding excitation wavelengths. A reference detector is used to measure the relative intensity of the excitation beam simultaneously with the collection of the emission spectra. The response of the detection system (R_d) as a function of emission wavelength is determined and used to correct the emission spectra. The following equation is used to calculate the quantum yield:

$$\phi_f = \phi_r \left[\frac{A_r(\lambda_r)}{A_f(\lambda_f)} \right] \left[\frac{I_r(\lambda_r)}{I_f(\lambda_f)} \right] \left[\frac{n_f}{n_r} \right] \left[\frac{E_f}{E_r} \right], \qquad (2)$$

where n is refractive index at the peak emission wavelength, I(λ) is the relative excitation intensity at excitation wavelength λ, E is the integrated area under the corrected emission spectrum and subscripts f and r refer to the unknown and reference samples, respectively. The fluorescence quantum yields of only a short list of compounds have been characterized to the extent necessary to be used as standards [54], with quinine sulfate being the most thoroughly established. [30]

This same procedure can be used to measure the relative quantum yield of optically dense samples. In this case, the absorbance ratio in eq. (2) is equal to one, since the excitation beam is absorbed completely, thereby simplifying the equation. Optically dense samples are used for both the unknown and the reference. Unfortunately there are many complications associated with measuring optically dense samples, making this method less accurate than its optically dilute counterpart, in most cases.

12. DEFINITION OF TERMS [55, 56]

absorption coefficient (α) – a measure of absorption of radiation from an incident beam as it traverses a sample according to Bouguer's law, $I/I_o = e^{-\alpha b}$, where I and I_0 are the transmitted and incident intensities, respectively, and b is the pathlength of the beam through the sample. Note that transmittance $T = I/I_o$ and absorbance $A = -\log T$

Beer-Lambert law (or **Beer's law** or **Beer-Lambert-Bouquer law**) – relates the dependence of the absorbance (A) of a sample on its pathlength (see *absorption coefficient*, α) and concentration (c), such that $A = \alpha bc$

calibrated detector (CD) - a light detector whose responsivity as a function of wavelength has been determined along with corresponding uncertainties. [57]

calibrated light source (CS) – a light source whose radiance as a function of wavelength has been determined along with corresponding uncertainties. [58, 59]

calibrated diffuse reflector (CR) - a Lambertian reflector whose reflectance as a function of wavelength has been determined along with corresponding uncertainties. [60]

certified reference material (CRM) – a material with properties of interest, whose values and corresponding uncertainties have been certified by a standardizing group or organization.

diffuse scatterer – a material that scatters light in multiple directions; this includes diffuse reflectors, which are often Lambertian, and scattering solutions, which are not Lambertian.

fluorescence anisotropy (r) – a measure of the degree of polarization of fluorescence, defined as $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the observed fluorescence intensities when the fluorometer's emission polarizer is oriented parallel and perpendicular, respectively, to the direction of the polarized excitation.

fluorescence band – a region of a fluorescence spectrum where the intensity passes through a maximum, usually corresponding to a discrete electronic transition

fluorescence lifetime – parameter describing the time decay of the fluorescence intensity of a sample component; if a sample decays by first-order kinetics, this is the time required for its fluorescence intensity and corresponding excited state population to decrease to 1/e of its initial value.

fluorescence quantum efficiency – the ratio of the number of fluorescence photons leaving an emitter versus the number of photons absorbed.

fluorescence quantum yield (Φ) – the probability that a molecule or species will fluoresce once it has absorbed a photon. This quantity is an innate property of the species and is typically calculated for a sample as the ratio of the number of molecules that fluoresce versus the number of molecules that absorbed.

flux (or **radiant flux**) – rate of propagation of radiant energy, typically expressed in watts (W); *spectral flux* is the flux per unit spectral bandwidth, typically expressed in W / nm.

grating equation – describes the relationship between the angle of diffraction and wavelength of radiation incident on a grating, i.e., $m\lambda = d (\sin\alpha + \sin\beta)$, where d is the groove spacing on the grating, α and β are the angles of the incident and diffracted

wavefronts, respectively, relative to the grating normal and m is the diffraction order, which is an integer.[61]

inner filter effects – a decrease in the measured quantum efficiency of a sample due to significant absorption of the excitation beam and /or reabsorption of the emission of the sample by itself. This causes the measured quantum efficiency to be dependent on the absorbance, concentration, and excitation and emission pathlengths of the sample. [62, 63]

intensity – a measure of the amount of electromagnetic energy present. This general definition, which is used here, is synonymous with or directly proportional to the signal output of a photodetector or the flux of a sample or light source. A more specific definition, often used in radiometry, is "the radiant flux per unit solid angle from a point source," which is typically expressed in W/sr.

Lambertian reflector – a surface that reflects light according to Lambert's law, i.e., the light is unpolarized and has a radiance that is isotropic or independent of viewing angle.

limit of detection – an estimate of the lowest concentration of an analyte that can be measured with a given technique, often taken to be the analyte concentration with a measured signal to noise ratio of three.

noise level – the peak-to-peak noise of a blank

photobleaching – a loss of emission or absorption intensity by a sample due to exposure to light. This loss can be reversible or irreversible with the latter typically referred to as *photodegradation* or *photodecomposition*.

Planck's constant (h) - relates the energy (E) of a photon to its frequency (ν), such that $E = h\nu$.

quantum counter – a photoluminescent emitter with a quantum efficiency that is independent of excitation wavelength over a defined spectral range. When a quantum counter is combined with a detector to give a response proportional to the number of incident photons, the pair is called a *quantum counter detector*.

quasi-absolute fluorescence intensity scale – a fluorescence intensity scale that has been normalized to the intensity of a fluorescent reference sample or artifact under a fixed set of instrumental and experimental conditions. This artifact should be known to yield a fluorescence intensity that is reproducible with time and between instruments under the fixed set of conditions.

Raman scattering – inelastic scattering of radiation (the wavelengths of the scattered and incident radiation are not equal) by a sample that occurs because of changes in the polarizability of the relevant bonds of a sample during a molecular vibration. The

radiation being scattered does not have to be in resonance with electronic transitions in the sample, unlike fluorescence. [64]

Rayleigh scattering – elastic scattering of radiation by a sample, i.e., the scattered radiation has the same energy (same wavelength) as the incident radiation.

responsivity (spectral) – ratio of the photocurrent output and the radiant power collected by a light detection system. *Spectral responsivity* is the responsivity per unit spectral bandwidth.

sensitivity – a measure of an instrument's ability to detect an analyte under a particular set of conditions

spectral bandwidth (or **spectral bandpass** or **resolution**) – a measure of the capability of a spectrometer to separate radiation or resolve spectral peaks of similar wavelengths.

spectral slit width – the mechanical width of the exit slit of a spectrometer divided by the linear dispersion in the exit slit plane.

transition dipole moment – an oscillating dipole moment induced in a molecular species by an electromagnetic wave that is resonant with an energy transition of the species, e.g., an electronic transition. Its direction defines that of the transition polarization, and its square determines the intensity of the transition.

13. Other Guideline/Recommendation Documents [40, 65, 66]

References

1. ASTM E 388-04, "Standard Test Method for Spectral Bandwidth and Wavelength Accuracy of Fluorescence Spectrometers," In: Annual book of ASTM standards, vol 03.06 (2004, original version 1972).
2. keywords: standard pen lamp
3. Lifshits, I.T. and Meilman, M.L., "Standard Sample for Calibrating Wavelength Scales of Spectral Fluorimeters," *Sov.J.Opt.Technol.*, **55**, 487 (1988).
4. Other rare earth doped glasses have narrow excitation and emission transitions, but Eu-doped glass is the only one listed because it is one of the most commonly used and most readily available. See reference 4 for a list of other doped glasses and their effective wavelength ranges.
5. Velapoldi, R.A., Epstein, M.S., "Luminescence Standards for Macro- and Microspectrofluorimetry," Ch. 7 in ACS Symposium Series 383 *Luminescence Applications in Biological, Chemical, Environmental and Hydrological Sciences*, Goldberg, M.C., Ed., American Chemical Society, Washington, DC (1989) p. 98.
6. keywords: fluorescent reference standards

7. Other polyaromatic hydrocarbon doped PMMAs have narrow excitation and emission transitions, including those with ovalene, p-terphenyl and naphthalene.
8. keywords: fluorescence standards, reference materials, PMMA
9. Certificate of Analysis, Standard Reference Material® 2065 Ultraviolet-Visible-Near-Infrared Transmission Wavelength Standard, National Institute of Standards and Technology (2002).
10. Certificate of Analysis, Standard Reference Material 2034 Holmium Oxide Solution, National Institute of Standards and Technology (1985).
11. Paladini, A.A., Erijman, L., "New Method for Spectrofluorometer Monochromator Wavelength Calibration," *J.Biochem.Biophys.Methods*, **17**, 61 (1988).
12. DeRose, P.C., Early, E.A., Kramer, G.W., "Qualification of a Fluorescence Spectrometer for Measuring True Fluorescence Spectra," *Rev.Sci.Instru.*, **78**, 033107 (2007).
13. "Technical Note: The Measurement of Sensitivity in Fluorescence Spectroscopy," Photon Technology International (1997-1999). http://www.pti-nj.com/tech_3.html
14. Lakowicz, J.R., "Principles of Fluorescence Spectroscopy," second edition, Kluwer Academic/Plenum Publishers, New York (1999) p.39.
15. Mielenz, K.D., Eckerle, K.L., "Spectrophotometer Linearity Testing Using the Double-Aperture Method," *Appl.Opt.*, **11**, 2294 (1972).
16. Zwinkels, J.C., Gignac, D.S. "Automated High Precision Variable Aperture for Spectrophotometer Linearity Testing," *Appl. Opt.*, **30**, 1678 (1991).
17. a) Hollandt, J., Taubert,R.D., Seidel, J., Resch-Genger, U., Gugg-Helminger, A., Pfeifer, D., Monte, C. and Pilz, W., "Traceability in Fluorometry-Part I: Physical Standards," *J.Fluoresc.*, **15**, 301 (2005). b) Resch-Genger, U., Pfeifer, D., Monte, C., Pilz, W., Hoffmann, A., Spieles, M., Rurack, K., Hollandt, J., Taubert,D., Schonenberger, B. and Nording, P., "Traceability in Fluorometry-Part II: Spectral Fluorescence Standards," *J.Fluoresc.*, **15**, 315 (2005).
18. ASTM E 578 -07, "Standard Test Method for Linearity of Fluorescence Measuring Systems," In: Annual book of ASTM standards, vol 03.06 (2007, original version 1983).
19. Other types of calibrated lamps can be used, but tungsten is ideal in the visible range due to its broader, featureless spectral structure and high intensity.
20. Roberts, G.C.K., Chapter 7, "Correction of Excitation and Emission Spectra," in *Techniques in Visible and Ultraviolet Spectrometry*, Vol. 2, *Standards in Fluorescence Spectrometry*, J.N.Miller, Ed. (Chapman and Hall, New York, 1981), pp. 54-61.
21. Costa, L.F., Mielenz, K.D., Grum, F., Chapter 4, "Correction of Emission Spectra," in *Optical Radiation Measurements*, Vol. 3, *Measurement of Photoluminescence*, K.D.Mielenz, Ed., Academic Press, New York (1982), pp. 139-174.
22. Hofstraat, J.W., Latuhihin, M.J., "Correction of Fluorescence Spectra,"*Applied Spec.*, **48**, 436 (1994).
23. Certificate of analysis, Standard Reference Material 936a, quinine sulfate dihydrate. National Institute of Standards and Technology (1994). (https://srmors.nist.gov/view_detail.cfm? srm=936A).
24. Certificate of analysis, Certified reference materials BAM-F001 - BAM-F005, Calibration Kit,Spectral Fluorescence Standards Federal Institute for Materials Research and Testing (BAM) (2006). These are available from BAM or from Sigma-Aldrich.

25. a) Certificate of Analysis, Standard Reference Material® 2940: Relative Intensity Correction Standard for Fluorescence Spectroscopy: Orange Emission, National Institute of Standards and Technology (2007). (https://srmors.nist.gov/view_detail.cfm?srm=2940) b) Certificate of Analysis, Standard Reference Material® 2941: Relative Intensity Correction Standard for Fluorescence Spectroscopy: Green Emission, National Institute of Standards and Technology (2007). (https://srmors.nist.gov/view_detail.cfm?srm=2941)
26. Gardecki, J.A., Maroncelli, M., "Set of Secondary Emission Standards for Calibration of the Spectral Responsivity in Emission Spectroscopy," *Appl. Spectr.*, **52**, 1179 (1998).
27. Velapoldi, R.A., Tonnesen, H.H., "Corrected Emission Spectra and Quantum Yields for a Series of Fluorescent Compounds in the Visible Spectral Region, *J. Fluoresc.*, **14,** 465 (2004).
28. Holbrook, R.D., DeRose, P.C., Leigh, S.D., Rukhin, A.L., Heckert, N.A., "Excitation-Emission Matrix Fluorescence Spectroscopy for Natural Organic Matter Characterization: A Quantitative Evaluation of Calibration and Spectral Correction Procedures", *Appl.Spec.*, **60**, 791 (2006).
29. Demas, J.N. "Measurement of Photon Yields," in *Optical Radiation Measurements*, Volume 3, *Measurement of Photoluminescence*, K.D.Mielenz, Ed., Academic Press, New York (1982) pp 228-233.
30. Velapoldi, R.A., Mielenz, K.D. "A Fluorescence Standard Reference Material: Quinine Sulfate Dihydrate", NBS Special Publication 260-64, U.S. Government Printing Office, Washington, DC
(1980) p. 27.
31. Certificate of Analysis, Standard Reference Material® 1932: Fluorescein Solution, National Institute of Standards and Technology (2003). (https://srmors.nist.gov/view_detail.cfm?srm= 1932)
32. ASTM E 578 -07, "Standard Test Method for Linearity of Fluorescence Measuring Systems," In: Annual book of ASTM standards, vol 03.06 (2007, original version 1983).
33. Wang, L., Gaigalas, A.K., Abbasi, F., Marti, G.E., Vogt, R.F., Schwartz, A., "Quantitating Fluorescence Intensity from Fluorophores: Practical Use of MESF Values," *J. Res. Natl. Inst. Stand. Technol.*, **107**, 339 (2002).
34. Schwartz, A., Wang, L., Early, E., Gaigalas, A.K., Zhang, Y-z., Marti, G.E., Vogt, R.F., "Quantitating Fluorescence Intensity from Fluorophores: The Definition of MESF Assignment," *J. Res. Natl. Inst. Stand. Technol.*, **107**, 83 (2002).
35. Schwartz, A., Gaigalas, A.K., Wang, L., Marti, G.E., Vogt, R.F., Fernandez-Repollet, E., "Formalization of the MESF Unit of Fluorescence Intensity," *Cytometry*, **57B**, 1 (2004).
36. Kovach, R.J., Peterson, W.M., "The Measurement of Sensitivity in Fluorescence Spectroscopy," *Am. Lab.*, **32G** (1994).
37. Froehlich, P., "Under the sensitivity specification for a fluorescence spectrophotometer," *Int. Lab.*, **42** (1989).
38. Giebeler, R., McGown, E., French, T., Owicki, J.C., "Performance Validation for Microplate Fluorimeters," *J.Fluoresc.*, **15**, 363 (2005).
39. Hoffmann, K., Resch-Genger, U., Nitschke, R., "Simple Tool for the Standardization of Confocal Spectral Imaging Systems", *G.I.T. Imaging & Microscopy*, Issue 3 (2005) p. 18.

40. DeRose, P.C., Resch-Genger, U., Wang, L., Gaigalas, A.K., Kramer, G.W., Panne, U., "Need for and Metrological Approaches towards Standardization of Fluorescence Measurements from the View of National Metrology Institutes," in Resch-Genger, U. (ed) *Standardization in Fluorometry: State-of-the Art and Future Challenges*, Vol. 5 of *Springer Series on Fluorescence* (Springer-Verlag GmbH, Berlin Heidelberg) 2007, in press.
41. Zou, S., He, H-J., Zong, Y., Shi, L., Wang, L. "DNA Microarrays: Application, Future Trends and Need for Standardization", in Resch-Genger, U. (ed) *Standardization in Fluorometry: State-of-the Art and Future Challenges*, Vol. 5 of *Springer Series on Fluorescence*, Springer-Verlag GmbH, Berlin Heidelberg (2007) in press.
42. Zong, Y., Wang, L., Zhang, S., Shi, L., "How to Evaluate a Microarray Scanner",in Hardiman, G. (ed) *Microarrays Methods and Applications-Nuts & Bolts*, DNA Press (2003) p. 99.
43. Wang, L., Gaigalas, A.K., Satterfield, M.B., Salit, M., Noble, J., "Microarray Preparation Parameters and Their Effects on the Fluorescence Properties of Spotted Oligonucleotide DNA Microarrays", in Rampal, J.B. (ed) *Microarrays: Methods and Protocols*, 2nd edn., Humana Press Inc. (2007).
44. ASTM E 579 -04, "Standard Test Method for Limit of Detection of Fluorescence of Quinine Sulfate," In: Annual book of ASTM standards, vol 03.06 (2004, original version 1984).
45. Boens, N., Ameloot, M., et.al., "Fluorescence Lifetime Standards for Time and Frequency Domain Fluorescence Spectroscopy," *Anal.Chem.*, **79**, 2137 (2007).
46. Lakowicz, J.R., "Principles of Fluorescence Spectroscopy," second edition, Kluwer Academic/Plenum Publishers, New York (1999) pp.645, 95, 141.
47. a) Demas, J.N. "Measurement of Photon Yields," in *Optical Radiation Measurements*, Volume 3, *Measurement of Photoluminescence*, Mielenz, K.D., Ed., pp 195-248, Academic Press, New York (1982). b) Demas, J.N., Crosby, G.A. "The Measurement of Photoluminescence Quantum Yields. A Review," *J.Phys.Chem.*, **75**, 991 (1971).
48. Weber, G., Teale, F.W.J., "Determination of the Absolute Quantum Yield of Fluorescent Solutions," *Trans.Faraday Soc.*, **53**, 646 (1957).
49. Eastman, J.W., *Photochem.Photobiol.*, **6**, 55 (1967).
50. Dawson, W.R., Kropp, J.L., *J.Opt.Soc.Amer.*, **55**, 822 (1965).
51. Vavilov, S.I., *Z.Phys.*, **42**, 311 (1927).
52. a) Melhuish, W.H., *J.Opt.Soc.Amer.*, **54**, 183 (1964). b) Melhuish, W.H., *New Zealand J.Sci.Tech.*, **37**, 142 (1955).
53. Porres, L., Holland, A., Palsson, L-O, Monkman, A.P., Kemp, C., Beeby, A., "Absolute Measurements of Photoluminescence Quantum Yields of Solutions Using an Integrating Sphere," *J.Fluoresc.*, **16**, 267 (2006).
54. Lakowicz, J.R., "Principles of Fluorescence Spectroscopy," second edition, Kluwer Academic/Plenum Publishers, New York (1999) p.53.
55. ASTM E 131-02, "Standard Terminology Relating to Molecular Spectroscopy," In: Annual book of ASTM standards, vol 03.06 (2002).
56. Braslavsky, S.E., "Glossary of Terms Used in Photochemistry, 3rd Edition, (IUPAC Recommendations 2006)," *Pure Appl.Chem.*, **79**, 293 (2007).

57. Larason, T.C., Bruce, S.S., Parr, A.C., Natl. Bur. Stand. (U.S.) Spec. Publ. 250-41, U.S. GPO, Washington, D.C. (1998).
58. Walker, J.H., Saunders, R.D., Jackson, J.K., McSparron, D.A., Natl. Bur. Stand. (U.S.) Spec. Publ. 250-20, U.S. GPO, Washington, D.C. (1987).
59. Walker, J.H., Saunders, R.D., Hattenburg, A.T., Natl. Bur. Stand. (U.S.) Spec. Publ. 250-1, U.S. GPO, Washington, D.C. (1987).
60. Weidner, V.R., Hsia, J.J., Natl. Bur. Stand. (U.S.) Spec. Publ. 250-6, U.S. GPO, Washington, D.C. (1987).
61. Palmer, C., "Diffraction Grating Handbook," fourth edition, Richardson Grating Laboratory, Rochester, N.Y. (2000).
62. Yappert, M.C., Ingle, Jr., J.D., *Appl.Spec.* **43**, 759 (1989).
63. Parker, C.A., Barnes, W.J., *Analyst* **82**, 606 (1957).
64. Chalmers, J., Griffiths, P., Eds., "Handbook of Vibrational Spectroscopy," John Wiley & Sons, Ltd, New York (2002).
65. DeRose, P.C., "Recommendations and Guidelines for Standardization of Fluorescence Spectroscopy," NISTIR 7457, National Institute of Standards and Technology (2007).
66. Marti, G.E., Vogt, R.F., Gaigalas, A.K., Hixson, C.S., Hoffman, R.A., Lenkei, R., Magruder, L.E., Purvis, N.B., Schwartz, A., Shapiro, H.M., Waggoner, A., "Fluorescence Calibration and Quantitative Measurements of Fluorescence Intensity, Approved Guideline," NCCLS, I/LA24-A, Vol. 24 No. 26 (2004).