

# Characterization of Standard Reference Material 2944, Bi-Ion-Doped Glass, Spectral Correction Standard for Red Fluorescence

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**Abstract:** Standard Reference Material (SRM) 2944 is a cuvette-shaped, Bi-ion-doped glass, recommended for optimal use for relative spectral correction of emission from 590 nm to 805 nm and day-to-day performance verification of steady-state fluorescence spectrometers. Properties of this standard that influence its effective use or contribute to the uncertainty in its certified emission spectrum were explored here. These properties include its photostability, absorbance, dissolution rate in water, anisotropy and temperature coefficient of fluorescence intensity. The expanded uncertainties ( $k=2$ ) in the certified spectrum are about 4 % around the nominal peak maximum at 704 nm and increase to about 6% at the wings, using an excitation wavelength of 515 nm.

**Keywords:** fluorescence; instrument qualification; Bi glass; spectral correction; SRM; standards

## Introduction

The increasing use of quantitative fluorescence-based assays in clinical, biotechnological, pharmaceutical and other health-related areas has fueled demand for fluorescence standards. [1] A variety of certified reference materials [2,3,4,5,6,7,8] and related standardization documents [9,10,11,12,13,14] have recently become available in response to this demand. Standard Reference Material (SRM) 2944 is the fifth fluorescence SRM in a series of recently released NIST standards certified as spectral correction standards for emission. Many of its fluorescence properties have been characterized and are described here to understand better the uncertainties and limitations of its use as a standard. Similar characterizations of SRMs 2940,[15] 2941 [16], 2942 [17] and 2943 [18] have been reported previously.

SRM 2944 is a ready-to-use, cuvette-shaped, Bi-ion-doped, solid glass standard whose certified values can be used to correct fluorescence emission spectra for distortions in the measured spectral shape, i.e., relative intensity correction, due to the changing responsivity with wavelength of the detection system of a steady-state fluorescence spectrometer. SRM 2944 can be used in combination with SRMs 2940, 2941, 2942 and 2943 to calibrate fluorescence instruments through the near UV and visible regions and into the near infrared (NIR) from 320 nm to 830 nm. An algorithm to seamlessly integrate data from multiple SRMs in this series is presently being developed by the authors. These SRMs can also be used as day-to-day intensity standards for performance verification, due to their resistance to photodegradation.

The certified values of SRM 2944 are to be used as follows to obtain correction factors as a function of emission wavelength. The SRM is excited at a fixed wavelength of 515 nm while the emission is collected from 530 nm to 830 nm, preferably using the instrument parameters given in the certificate. [6] Due to decreasing signal to noise levels away from the peak maximum at the edges of the certified range, the emission range from  $\lambda_{EM} = 590$  nm to 805 nm is recommended as optimal for most instruments and applications. The measured spectrum is then normalized to a peak intensity of one at the peak maximum, nominally 704 nm, i.e., divide all measured intensity values by the corresponding value at the peak maximum. Each certified value is then divided by its corresponding normalized, measured value to obtain correction factors. The measured emission spectrum of an unknown sample that falls in the effective emission range of the SRM can then be corrected by multiplying its measured intensities by the correction factors at the corresponding emission wavelengths. Even though the correction factors must be determined using the SRM at a 515 nm excitation wavelength, they may be applied to the spectral correction of emission independently of the excitation wavelength of a sample. This assumes the responsivity of the detection system is independent of the excitation wavelength, which is true for most instruments since the optical path of detected emission typically does not change with excitation wavelength.

Due to glass fabrication limitations, SRM 2944 was produced from three separate glass melts. Although the same “recipe” was used for each melt, the optical properties of each are statistically discernible. Accordingly, SRM 2944 was produced and certified in three batches, labeled Series A, B, and C, and will be released in succession. All batches display very similar, but not identical, behavior. The results shown here are representative of all three batches, except where noted, so the suffix A, B or C will only be used when a difference between batches was observed.

## Experimental

A more detailed experimental description of many of these procedures has already been reported. [16] All uncertainties given here are  $2\sigma$  uncertainties, i.e., at the 95 % confidence level, unless specified otherwise.

*Samples:* The glass was melted at 1300 °C in a high purity alumina crucible, using a base glass composition with mass fractions of  $P_2O_5 = 70$  % ( $Ca(H_2PO_4)_2H_2O$  and  $NH_4 H_2PO_4$  used),  $CaO = 26$  % ( $Ca(H_2PO_4)_2H_2O$  used),  $Al_2O_3 = 3.0$  % ( $Al_2O_3$  used), and a dopant mass fraction of  $Bi_2O_3 = 0.51$  %. Non-oxidizing conditions were maintained during melting by flowing 100 % Ar gas into the crucible.

The glass was cut into cuvette-shaped pieces (12.5 mm x 12.5 mm x 45.0 mm) with three long sides polished, to be used with a 90° transmitting detection geometry, and one long side frosted, to be used with a front-face detection geometry [19] for minimizing excitation beam penetration and as a surface on which to focus for microscope applications. One glass batch had a final composition with mass fractions of  $P_2O_5 = 79$  %  $\pm 16$  %,  $CaO = 13$  %  $\pm 3$  %,  $Al_2O_3 = 7$  %  $\pm 1$  %,  $Bi_2O_3 = 0.17\%$   $\pm 0.04$  % and other trace

oxides = 0.5 %  $\pm$  0.1 %, as determined using X-ray fluorescence. Similar results were found for the other two batches.

*Fluorescence Measurements:* All steady-state fluorescence spectra were taken on a SPEX Fluorolog 3 [20] (Jobin Yvon, Edison, NJ) spectrofluorometer using a continuous 450 W Xe lamp excitation source, except where noted. A small fraction of the excitation beam was reflected, using a fused silica window, to a “reference” photodiode just before the sample to monitor the relative excitation intensity as a function of time and wavelength. The wavelength accuracy achieved over the entire wavelength range of the instrument was  $\pm$  0.2 nm for both emission and excitation, as determined using atomic lamps. The relative radiometric accuracy as a function of wavelength of the reference (excitation) and signal (emission) detection systems was corrected using a calibrated detector and a calibrated light source with calibrated diffuse reflector, respectively, traceable to the NIST realization of the International System of Units (SI). [21,22,23,24,25] All fluorescence measurements were taken at 25 °C using a 90° transmitting geometry with the excitation beam incident on and normal to one of the polished glass surfaces. The excitation wavelength was 515 nm, and the typical scanning range for emission spectra was from 530 nm to 830 nm, using excitation and emission bandwidths of 3 nm. The ratio of signal to reference intensities is given as the “fluorescence intensity” in what follows to correct for signal intensity fluctuations due to changes in the excitation intensity with time, and all emission spectra are corrected for the responsivity of the detection system. A more detailed description of the qualification of the fluorescence spectrometer, related uncertainties and experimental conditions for certification and the determination of spectral correction factors is given elsewhere. [26]

A fluorescence spectrometer with pulsed excitation (Varian Eclipse) was used with 5 nm bandwidths for both excitation and emission, pulse duration = 2  $\mu$ s, PMT voltage = 800 V. In “fluorescence mode,” a PMT gate of 40  $\mu$ s was used with no delay time between the excitation pulse and the gate. In “phosphorescence mode,” a PMT gate of 10 ms was used with a delay time of 10 ms. Correction factors for relative spectral correction were determined for this instrument to emission wavelengths up to 760 nm using Federal Institute for Materials Research and Testing - Germany (BAM) certified reference materials (CRMs) [6], so corrected spectra could be compared between instruments using pulsed and continuous excitation. CRMs were used here to save the time needed to set up physical transfer standards, such as a calibrated light source. This emphasizes the ease-of-use of NIST SRMs and other CRMs, which can be measured in the same way as typical samples.

*Polarizers:* Glan Thompson polarizers were used just after the excitation monochromator and just before the emission monochromator to measure the fluorescence intensities  $I_{VV}$ ,  $I_{VH}$ ,  $I_{HV}$  and  $I_{HH}$ , which were then used to determine fluorescence anisotropy ( $r$ ), where the first and second subscripts indicate the polarization setting of the excitation and emission polarizers, respectively, using V to indicate vertical or 0° polarization and H to indicate horizontal or 90° polarization. These measurements were taken at a fixed emission wavelength, corresponding to the peak maximum for SRM 2944. F and G values [27] were determined as described previously. [16,26]

*Photostability Testing Methods:* The fluorescence intensity of SRM 2944 was measured periodically after 4 to 6 hours of continuous irradiation. These measurements were taken on the Fluorolog 3, after the sample was removed from the irradiation chamber and its temperature was allowed to equilibrate in the sample compartment of the fluorometer. The irradiation chamber used a 150 W Xe arc lamp with an NG-11 neutral density filter put between the lamp and the chamber to block wavelengths less than 400 nm. Infrared radiation from the lamp was not blocked.

A fiber optic with a 400  $\mu\text{m}$  diameter aperture attached to an Ocean Optics S2000 spectrometer with an 8 nm bandwidth was used to measure the irradiance of the light incident on the samples as a function of wavelength. The relative spectral responsivity of the spectrometer was calibrated using a calibrated tungsten halogen lamp. The excitation irradiation incident on the samples, when they were excited in our fluorometer at an excitation bandwidth and wavelength of 3 nm and 515 nm, respectively, was measured using both a calibrated Si detector and the fiber optic spectrometer. The comparison of the two measurements was used to calibrate the absolute responsivity of the fiber optic spectrometer.

*Lifetime:* Fluorescence lifetimes were measured on an ISS K2 fluorometer with a K2LF accessory. A monochromator was used to set the excitation wavelength at 515 nm. An LP-630 long pass filter was placed before the emission PMT to block light with wavelengths less than 630 nm when measuring SRM 2944. A Ludox suspension (excitation light scatterer) was used as a lifetime reference with a lifetime of 0.0 ns. Note that Ludox scatters light, but does not fluoresce, making its scattering lifetime on the order of femtoseconds. No emission wavelength selector was used with the lifetime reference. The K2 was scanned over 10 frequencies in the range from 5 kHz to 1500 kHz.

## **Results and discussion**

### *Corrected Fluorescence Spectra and Uncertainties*

The corrected emission spectrum is a single broad peak with a nominal maximum at 704 nm and a full-width at half the maximum intensity (FWHM) of 118 nm (see Fig. 1). This spectral shape is consistent with Bi-ion-doped glasses reported previously, although the positions and broadness of peaks have been found to change with the base glass composition. [28,29,30,31] Homogeneity of the glass was measured on a centimeter scale by collecting the spectrum for each SRM 2944 sample in both a normal and a raised (0.5 cm) position and comparing them. Both spectra were found to be statistically identical for all samples, implying that they are spatially homogeneous.

The combined standard uncertainty in the relative fluorescence intensity was calculated for each certified intensity value in the fluorescence spectrum by adding in quadrature the  $1\sigma$  standard uncertainties due to 1) spatial uncertainty of the excitation beam's position on the sample (causing secondary inner filter effect uncertainties), 2) variation of F and G polarization ratios between instruments, 3) temperature uncertainty, 4) excitation and emission wavelength and bandwidth uncertainty, 5) uncertainty in the spectral shape

correction (due to uncertainty in the radiance and reflectance values of the calibrated light source and reflector) and 6) standard deviation of the certification data, and taking the square root of the sum. The combined standard uncertainty was then multiplied by an expansion factor  $k = 2$  to obtain the total expanded uncertainty ( $U_{95}$ ). The spectrum of SRM 2944 and the associated uncertainties in the certified values are shown in Fig. 1 and reported in the certificate. [6] The values for  $U_{95}$  are about 4 % near the peak maximum and 6% at the wings. The sides or wings of the peak refer to the regions of the spectrum to either side of the peak maximum where the intensities are 10 % to 20 % of the peak maximum.

The excitation spectrum of SRM 2944, spectrally corrected for excitation intensity, has a broad peak maximum in the visible region at 515 nm with a FWHM of 190 nm (see Fig. 2). This excitation peak corresponds to an absorbance band around 463 nm with a FWHM of 120 nm (see Fig. 3). An excitation peak is also observed in the UV from 360 nm to 300 nm with a luminescence intensity more than twice as strong as the visible peak. The SRM was not certified using UV excitation for several reasons. Firstly, UV irradiation may cause solarization of the glass, which would result in an apparent decrease in luminescence with irradiation time. In addition, absorption of the excitation beam by the glass matrix at wavelengths less than 310 nm causes large inner filter effects. For these reasons, we chose an excitation wavelength of 515 nm.

The certified values will only yield effective spectral correction factors when the SRM is excited at 515 nm, because the shape of the emission spectrum is excitation wavelength dependent. A 1.0 nm shift of the excitation wavelength in either direction causes the resulting emission spectrum to deviate from the certified values by 2% or less in the optimal region from 590 nm to 805 nm. Deviations due to a 1.0 nm change in the excitation bandwidth are less than 0.7 % in the optimal region, and those due to a 1.0 nm change in the emission bandwidth are insignificant, being 0.1 % or less in the optimal region and less than 0.5 % across the entire certified emission spectrum.

#### *Corrosion Study*

The mass of a Bi-ion-doped glass sample immersed in deionized water was measured over a period of 30 days. The rate of dissolution was  $10.1 \text{ ng}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ , which is equal to a log dissolution rate of  $-8.0 \text{ g}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ . For comparison, a similar study found in the literature on a calcium aluminum phosphate base glass (47 mol % CaO, 3 mol %  $\text{Al}_2\text{O}_3$ , 50 mol %  $\text{P}_2\text{O}_5$ ) showed a log dissolution rate from  $-7.51 \text{ g}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$  to  $-7.73 \text{ g}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ . [32] Window glass is reported to have a log dissolution rate in the range of  $-8.0 \text{ g}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$  to  $-8.5 \text{ g}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ . [32] The log dissolution rate of SRMs 2942 and 2943 were found to be  $-8.0 \text{ g}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$  [17] and  $-8.1 \text{ g}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$  [18], respectively. The composition of these glasses is nearly identical to that of SRM 2944, except for the dopant.

#### *Absorbance and Inner Filter Effects*

Inner filter effects (IFE) are due to absorption by the sample of either the excitation beam before it reaches the detection region, known as the primary IFE, or the emission

before it leaves the sample, known as the secondary IFE. Both cause the measured fluorescence intensity ( $F$ ) to decrease, the extent of which can be easily calculated using the measured absorbances  $A(\lambda_{EX})$  and  $A(\lambda_{EM})$  of the sample at the excitation and emission wavelengths, respectively. [33, 34] Samples with  $A(\lambda_{EX})$  and  $A(\lambda_{EM})$  values less than 0.04 ( $T = 91\%$ ), corresponding to intensity changes of less than 5%, are generally considered to be small enough to ignore, as is the case here.

SRM 2944 has a primary IFE at its excitation wavelength of 22%, 25% and 17% for batches A, B and C, respectively. In spite of this, all IFEs will be observed with the same magnitude whenever the SRMs are measured under the same conditions, so they should not matter when the conditions specified on the SRM certificate are followed. On the other hand, the positions of the excitation beam and detection path on the sample can change over time or between instruments, resulting in a corresponding change in IFE values. Ideally, the detection region, where the excitation beam and emission detection path overlap, should always be at the center of the cuvette. In reality, this position can change due to misalignment of the excitation source, optics and sample over time or due to differences in optical alignment between samples. A 1 mm change in the position of the excitation beam or detection path would cause a change in the measured fluorescence intensity of 4% or less at the peak maximum for all three batches. These absolute intensity differences due to IFEs can affect the SRM when used for day-to-day intensity verification of instrument performance.

When these SRMs are being used with their certified values for relative spectral correction, only changes in relative intensity versus  $\lambda_{EM}$  are significant. This means that the primary IFE, which is independent of  $\lambda_{EM}$ , will not affect SRM performance. Only changes in the secondary IFE with  $\lambda_{EM}$  can affect the spectral correction when the position of the detection region changes. The percent error in the measured relative emission spectrum due to IFEs was calculated with the same 1 mm change in position. As might be expected, the relative IFE errors are smaller than the corresponding absolute errors, given above, with those for SRM 2944 being less than 2% for all three batches (see Fig. 4).

#### *Photostability*

Photostability is likely the most important characteristic of a solid, robust fluorescent reference material that is meant to be used repetitively in the lab or field. The SRM was irradiated with visible light from a 150 W Xe lamp to test the glass under intensity conditions similar to those expected under normal use. After irradiation of SRM 2944 for 30 hours, its fluorescence emission spectra showed no changes in spectral shape or fluorescence intensity, within our uncertainties ( $\pm 0.5\%$  for fluorescence intensity at the peak). The irradiance incident on the SRMs was equal to about  $3.0 \text{ mW cm}^{-2}$  ( $0.37 \text{ mW cm}^{-2} \text{ nm}^{-1}$ ) at 515 nm, the excitation wavelength. The incident irradiance at other wavelengths from 400 nm to 680 nm was comparable to this. The excitation irradiance incident on the samples, when they were excited in our fluorometer at an excitation bandwidth of 3 nm, was measured to be about equal to the irradiance in the irradiation chamber. If the intensity in the irradiation chamber at other excitation wavelengths, where sample absorption produces fluorescence, is considered (this was done using the

excitation fluorescence spectrum), then the effective excitation intensity in the irradiation chamber was about 18 times greater than that of our fluorometer at 515 nm. This was calculated using the intensity values of the excitation fluorescence spectrum at each wavelength to weight the corresponding excitation intensity values in the irradiation chamber. Therefore, 30 hours of exposure in the irradiation chamber is estimated to correspond to about 540 hours (22.5 days) of continuous excitation in our fluorometer. Ultraviolet (UV) light from the Xe lamp was blocked, using a filter, to prevent UV solarization of the glass, which is known to change the absorption of metal-ion-containing glasses. [35, 36] SRM 2944 is not recommended as a performance verification standard at excitation wavelengths below 400 nm due to this effect.

#### *Anisotropy and Polarization Effects*

Samples with non-zero values for fluorescence anisotropy ( $r$ ) will show different fluorescence intensities and spectral shapes on different instruments, since each fluorometer has its own polarization ratios or factors, where  $I_{V,EX} / I_{H,EX}$ , referred to as the  $F$  factor, is the ratio of the vertically and horizontally polarized components of the excitation intensity and  $R_V / R_H$ , referred to as the  $G$  factor, is the ratio of the responsivities of the detection system to vertically and horizontally polarized light. The values of these polarization factors are dependent on the unique components of individual instruments, such as gratings, other optics, lamps and detectors.

The  $r$  value for SRM 2944 was measured to be  $0.10 \pm 0.01$  at its fluorescence peak maximum. The uncertainty is the standard deviation of 5 measurements. The anisotropy of SRM 2944 changed significantly with emission wavelength ( $\lambda_{EM}$ ), as shown in Fig. 5. Y error bars representing  $1\sigma$  standard deviations for the average  $r$  values, a trendline and its corresponding equation are also given in the figure. The measured  $r$  values for the Bi glass from 580 nm to 800 nm are fitted to a linear equation. With EX and EM polarizers in place, the intensity of detected fluorescence becomes too weak at emission wavelengths greater than 800 nm and less than 580 nm to measure accurate  $r$  values, so the fitted equation must be used to extrapolate the  $r$  values in these regions, when necessary.

The  $F$  factor at 515 nm is 0.24. The range of  $G$  factors for our instrument is from 0.55 to 0.15 over the emission wavelength range of the SRM. These  $F$  and  $G$  values are typical for monochromator-based systems. [37] We estimated  $\pm 25\%$  to be a typically expected instrument-to-instrument difference between the  $F$  and  $G$  values of our instrument and those of other users for conventional fluorometers designed to cover the visible emission region with greatest sensitivity. With this assumption, differences in the absolute intensity at the peak maximum and in the relative intensities across the emission spectrum that can be expected due to variations in  $F$  and  $G$  values between instruments were calculated. [38] The absolute intensity difference at the peak maximum was calculated to be 0.4 % for SRM 2944. The differences in the relative intensity across the emission spectrum normalized to one at the peak maximum were calculated to be less than 1 % across the entire certified spectrum, see Fig. 6.

#### *Temperature Dependence*

The fluorescence peak intensity as a function of temperature was measured between 10 °C and 40 °C (see Fig. 7). The slope of the linear least-squares fitted straight line to the plotted points was taken to be the temperature coefficient. This value corresponds to  $-0.25 \% / ^\circ\text{C} \pm 0.01 \% / ^\circ\text{C}$  for SRM 2944 at 25 °C.

Emission spectra taken at several temperatures from 40 °C to 10 °C were compared to that at 25 °C by percent difference. Since the temperature dependence of the percent difference in this temperature range was found to be linear with changes in temperature, the percent difference fit at 10 °C (having the largest percent difference curve), is used to calculate the uncertainty in the certified values corresponding to the uncertainty in temperature, by taking the percent difference values as a function of wavelength and dividing each by 30 ( $15 ^\circ\text{C} / 0.5 ^\circ\text{C} = 30$ ). Spectral differences due to a  $\pm 0.5 ^\circ\text{C}$  change in temperature were found to be less than 0.8 %, for SRM 2944 across its emission wavelength range (see Fig. 8). It should also be noted that no significant spectral differences with temperature were measurable above the noise level from 665nm to 830 nm. The differences in this region shown in Fig. 8 only reflect the observed noise level.

#### *Fluorescence Lifetimes and Pulsed Excitation*

The time decay of fluorescence for SRM 2944 was found to be single exponential, when excited at 515 nm. The fitted lifetime for SRM 2944 was  $\tau = 3.6 \mu\text{s} \pm 0.5 \mu\text{s}$  with  $\chi^2 = 20$ . This lifetime is similar to those of Bi-doped phosphate glasses reported in the literature. [39]

The corrected emission spectrum for SRM 2944 was also determined on an instrument with pulsed excitation in fluorescence mode and compared to the certified spectrum taken on the Fluorolog 3. The fluorescence spectra look very similar using either pulsed or continuous excitation (see Fig. 9) with the relative intensity values from the pulsed instrument differing from the certified values by less than 6 % in the peak region and by 10% or less in the region from 590 nm to 760nm. These differences are within the combined uncertainties of the certified values and the uncertainties related with the pulsed instrument measurements. These results imply that the fluorescence emitted within 40  $\mu\text{s}$  of the excitation pulse has the same spectral profile as the longer, time-averaged fluorescence. The 40  $\mu\text{s}$  PMT gate duration was chosen as a typical value for conventional pulsed fluorimeters, suggesting that SRM 2944 can also be used as a spectral correction standard for instruments with pulsed excitation.

SRM 2940 was also used to determine correction factors for the pulsed fluorometer to emission wavelengths up to 800 nm, the longest wavelength for which SRM 2940 has been certified. This was done, in spite of the fact that SRM 2940 is not recommended for use with instruments using pulsed excitation, to determine if it could be used as a spectral correction standard with such instruments under certain conditions. Unlike SRM 2944, SRM 2940 does show a significant spectral difference with pulsed versus continuous excitation. An emission spectrum of SRM 2940 was collected on the pulsed fluorometer in phosphorescence mode with a delay time of 10 ms between the excitation pulse and the PMT gate and a PMT gate of 10 ms. Under these conditions, only the longer lifetime

component of the fluorescence is detected. This component has been shown to be similar to the fluorescence detected under continuous excitation, unlike the shorter lifetime component. Correction factors were determined using the measured emission spectrum under these conditions and the certified values for SRM 2940, according to the SRM certificate. These correction factors, when applied to the emission spectrum of SRM 2944 taken with pulsed excitation, gave a corrected spectrum that was in good agreement with the certified spectrum (See Fig. 9). In this case, the relative intensity values from the pulsed instrument differed from the certified values by less than 6 % in the peak region and by 20% or less in the region from 590 nm to 800nm. Note that the corrected spectrum in Fig. 9 (pulsed-SRM2940) does become increasingly noisy due to the decreasing fluorescence signal of SRM 2940 at wavelengths greater than 680 nm.

## **Conclusion**

SRM 2944, a Bi-ion-doped glass in the shape of a standard cuvette, has been certified as a relative spectral correction standard for fluorescence emission from 530 nm to 830 nm. The expanded uncertainties in the certified values are about 4 % near the peak maximum at 704 nm. Errors in the measured emission spectrum due to inner filter effects in conjunction with excitation beam misalignment were found to be less than 2 % across the entire wavelength range, resulting from a 1 mm displacement of the detection region from the center of the cuvette. Corresponding errors due to varying polarization ratios (F and G factors) between instruments were found to be less than 1 % across the same wavelength range, assuming a 25 % difference between the F and G values of our instrument and those of other conventional fluorometers. The fluorescence anisotropy and temperature coefficient of fluorescence intensity for the SRM were measured to be  $0.10 \pm 0.01$  and  $-0.25 \% / ^\circ\text{C} \pm 0.01 \% / ^\circ\text{C}$ , respectively, at the peak maximum at 25 °C. SRM 2944 possesses good photostability with no photodegradation observed under common lamp-based excitation conditions at wavelengths greater than 400 nm.

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## Figure captions

Fig. 1 : The certified fluorescence spectrum of SRM 2944 with intensity in relative power units (E) and the corresponding uncertainty envelope obtained by adding and subtracting the total expanded uncertainty (U95) to the certified values. The relative percent uncertainty is labeled in the peak and wing regions of the spectrum. The certified spectrum is normalized to one at the peak maximum at 704 nm.

Fig. 2: The fluorescence excitation spectrum of SRM 2944 with emission collected at 704 nm. The spectrum is normalized to one at the peak maximum at 515 nm.

Fig. 3: Absorbance spectra of the three batches of SRM 2944, corrected for the Fresnel reflections at the air-glass interfaces.

Fig. 4: Percent error in the measured fluorescence emission spectrum of SRM 2944 due to a secondary inner filter effect resulting from a 1 mm displacement of the detection region/excitation beam from the center of the cuvette.

Fig. 5: The dependence of the fluorescence anisotropy ( $r$ ) of SRM 2944 on emission wavelength. The error bars for anisotropy represent  $1\sigma$  standard deviations. The error bars for wavelength are smaller than the point size used.

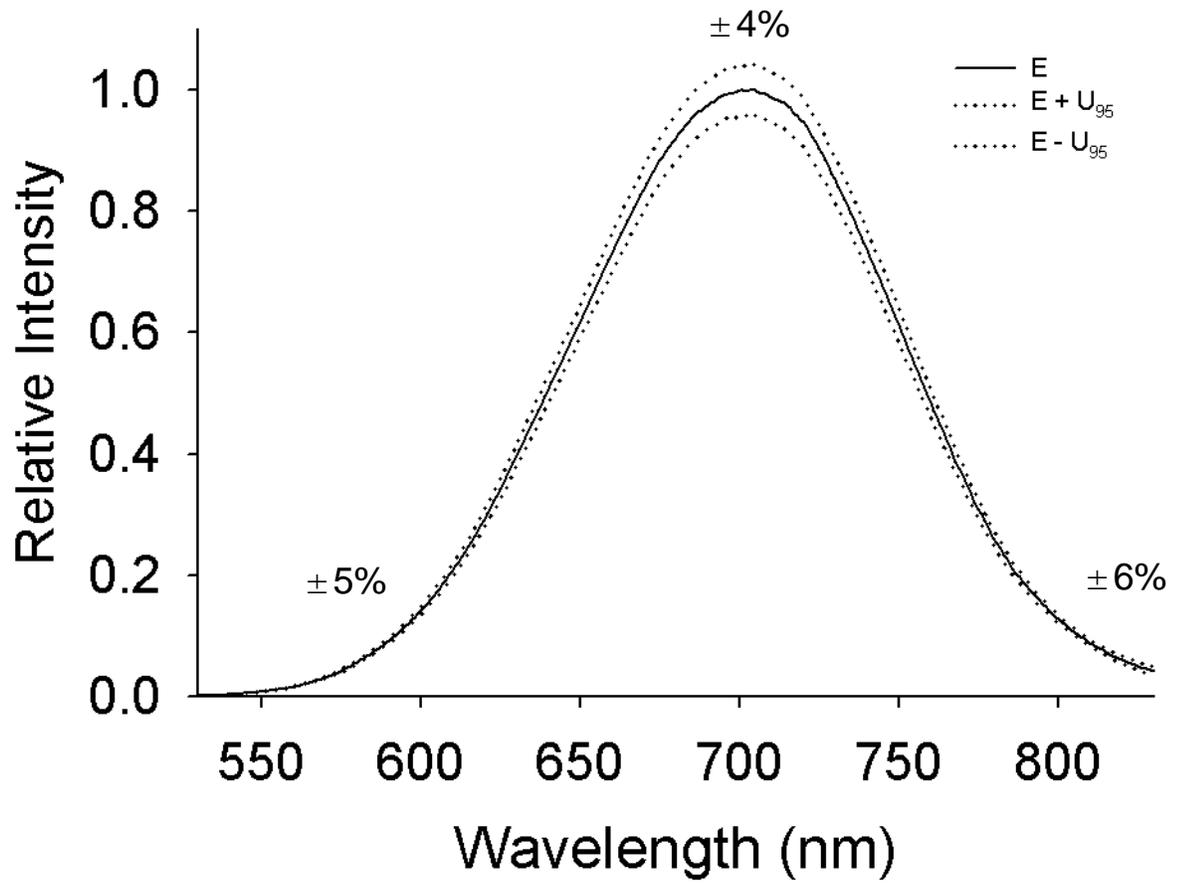
Fig. 6: Percent error in the measured fluorescence emission spectrum of SRM 2944 due to a  $\pm 25\%$  change in the polarization ratios (F and G factors) of a fluorescence spectrometer from those of the instrument used to certify the standard.

Fig. 7: The temperature dependence of the fluorescence intensity of SRM 2944 at the peak maximum. The error bars represent  $1\sigma$  standard deviations.

Fig. 8: The percent difference in the fluorescence spectrum of SRM 2944 caused by a  $\pm 0.5\text{ }^\circ\text{C}$  change in temperature from  $25\text{ }^\circ\text{C}$  is shown as fitted trendlines.

Fig. 9: Spectrally corrected fluorescence spectra of SRM 2944 taken on instruments with pulsed and continuous excitation. The pulsed spectra were corrected using both BAM CRMs and SRM 2940. SRM 2940 was used in phosphorescence mode under selective instrumental conditions.

Figure 1



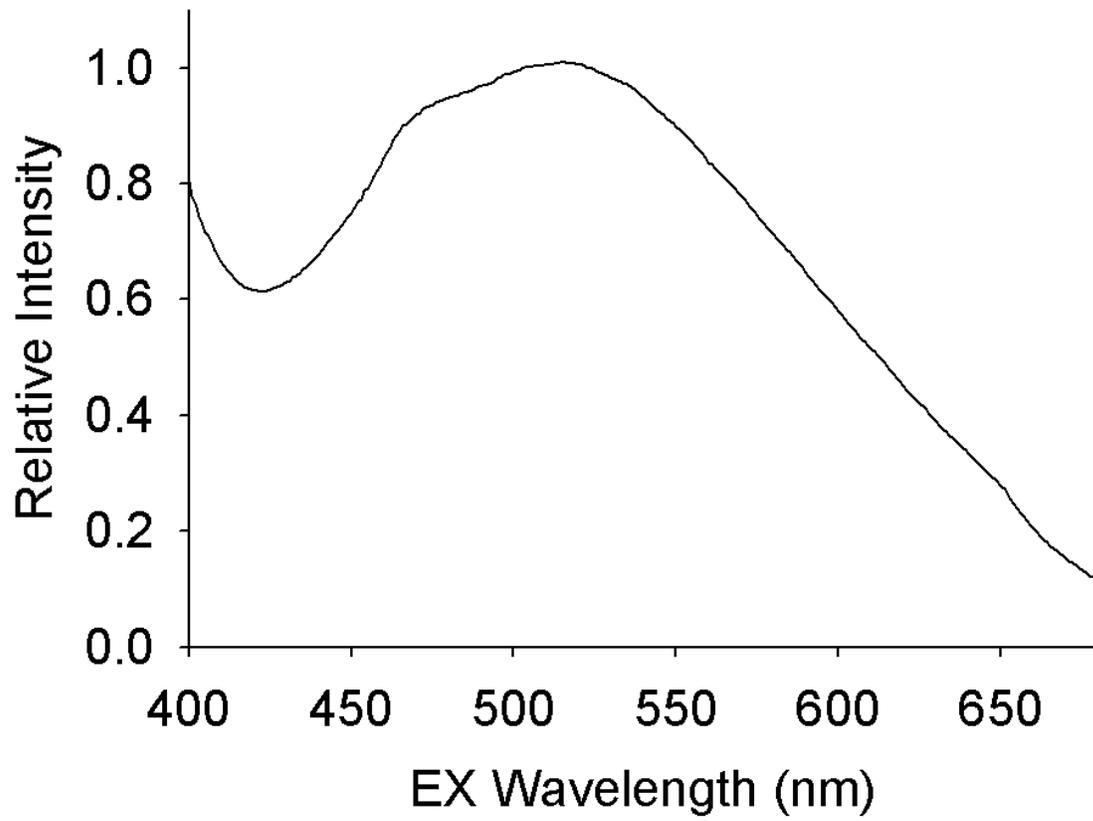


Figure 2

Figure 3

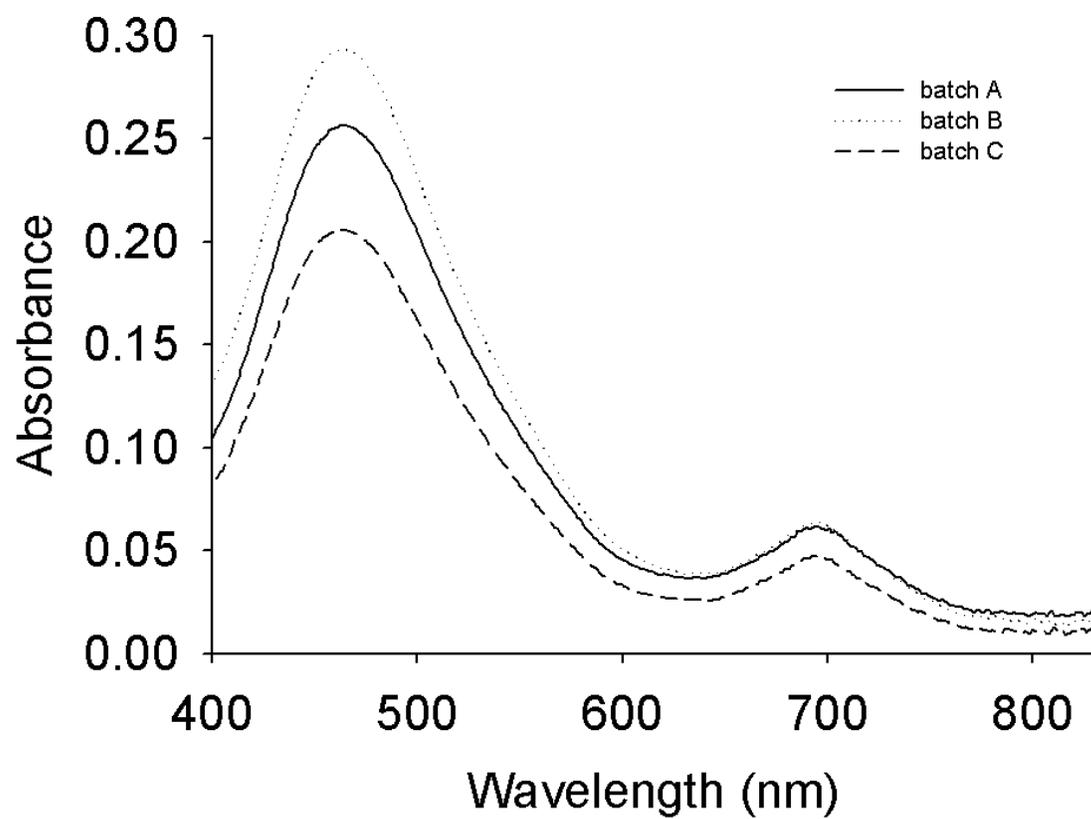


Figure 4

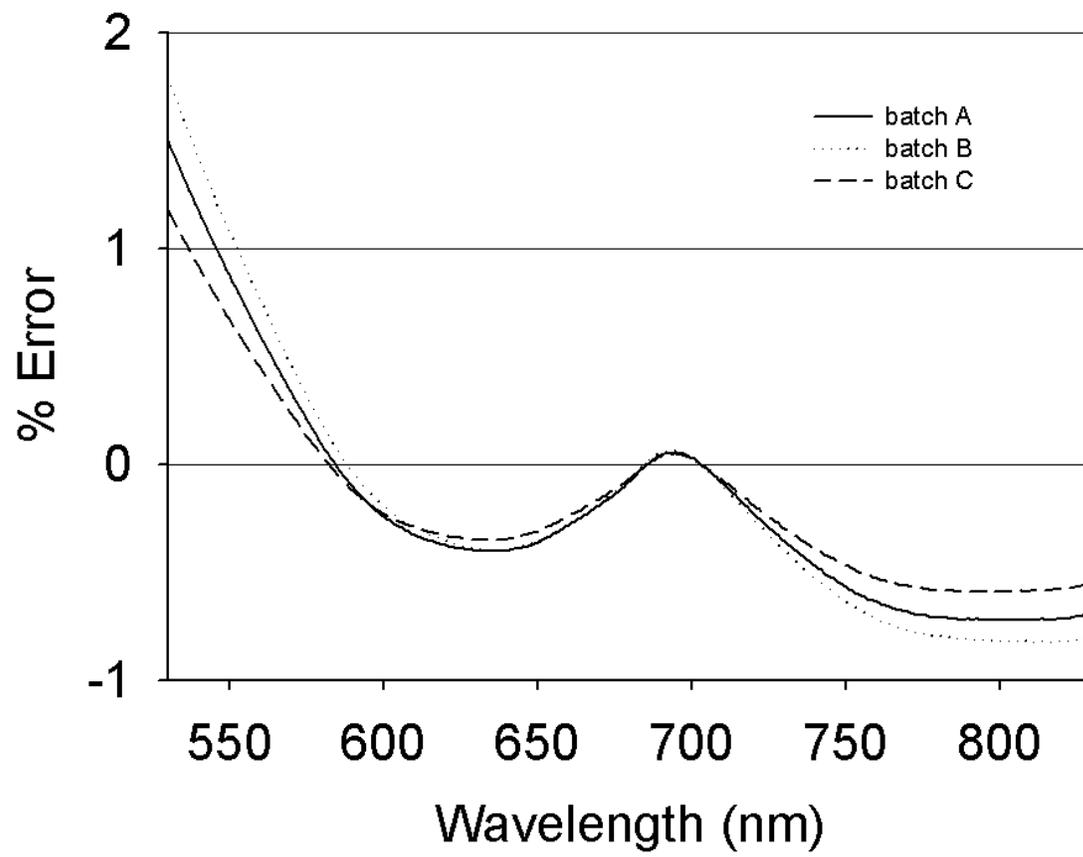


Figure 5

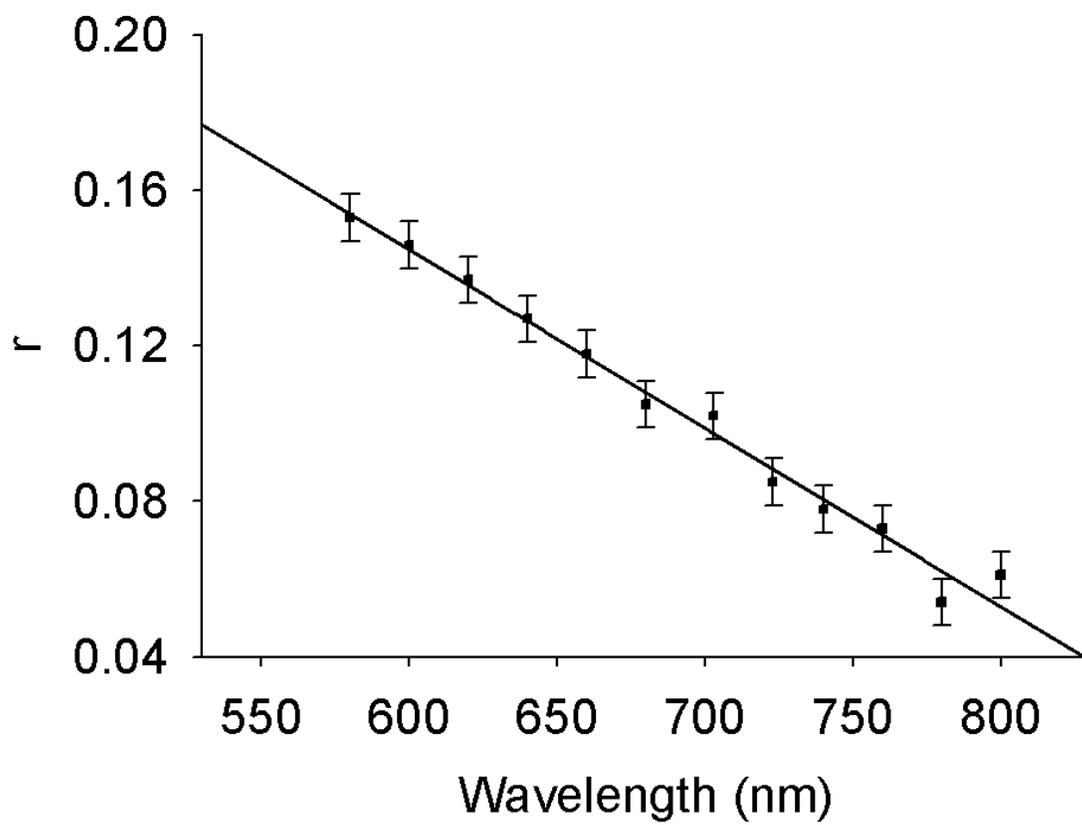


Figure 6

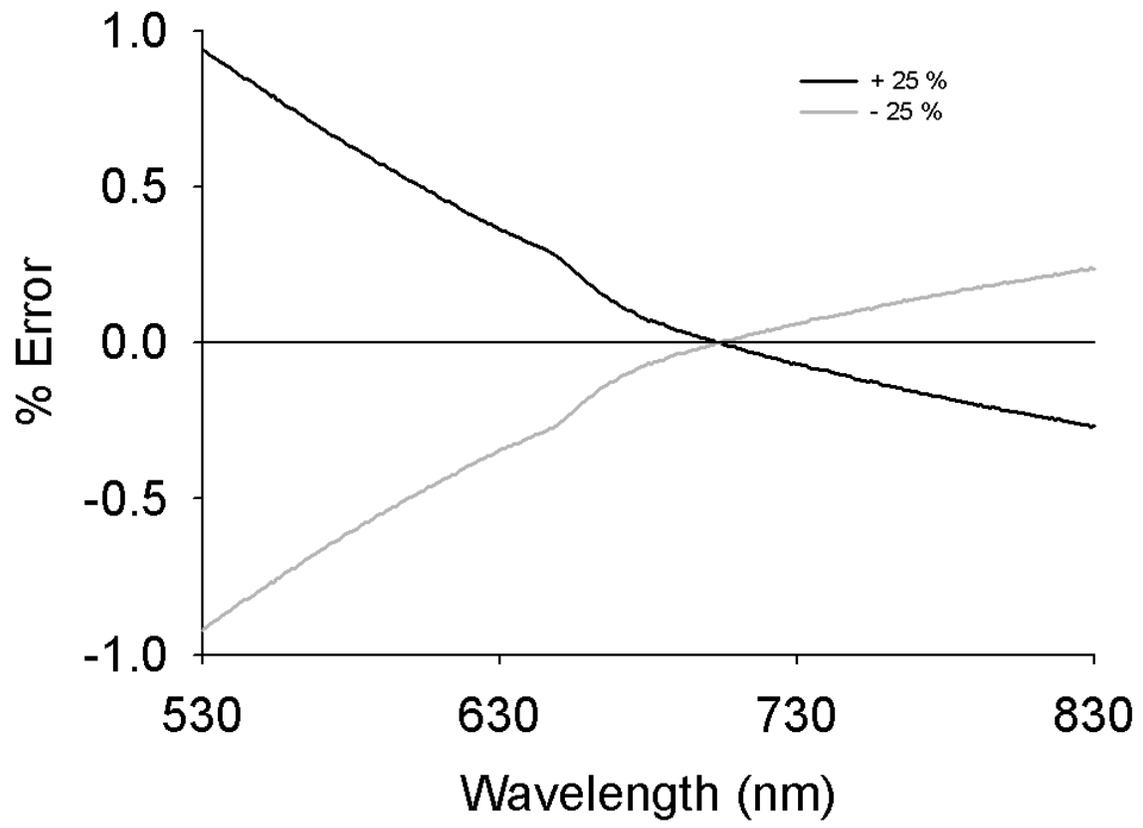


Figure 7

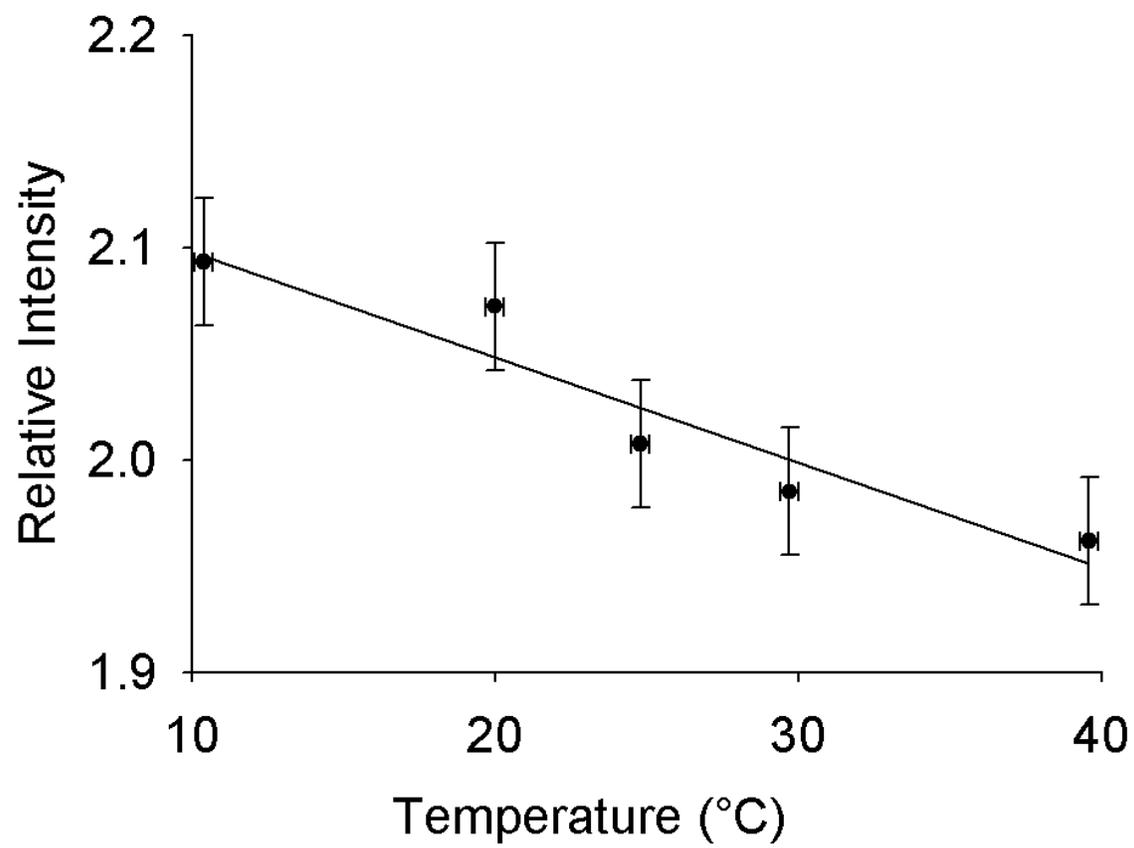


Figure 8

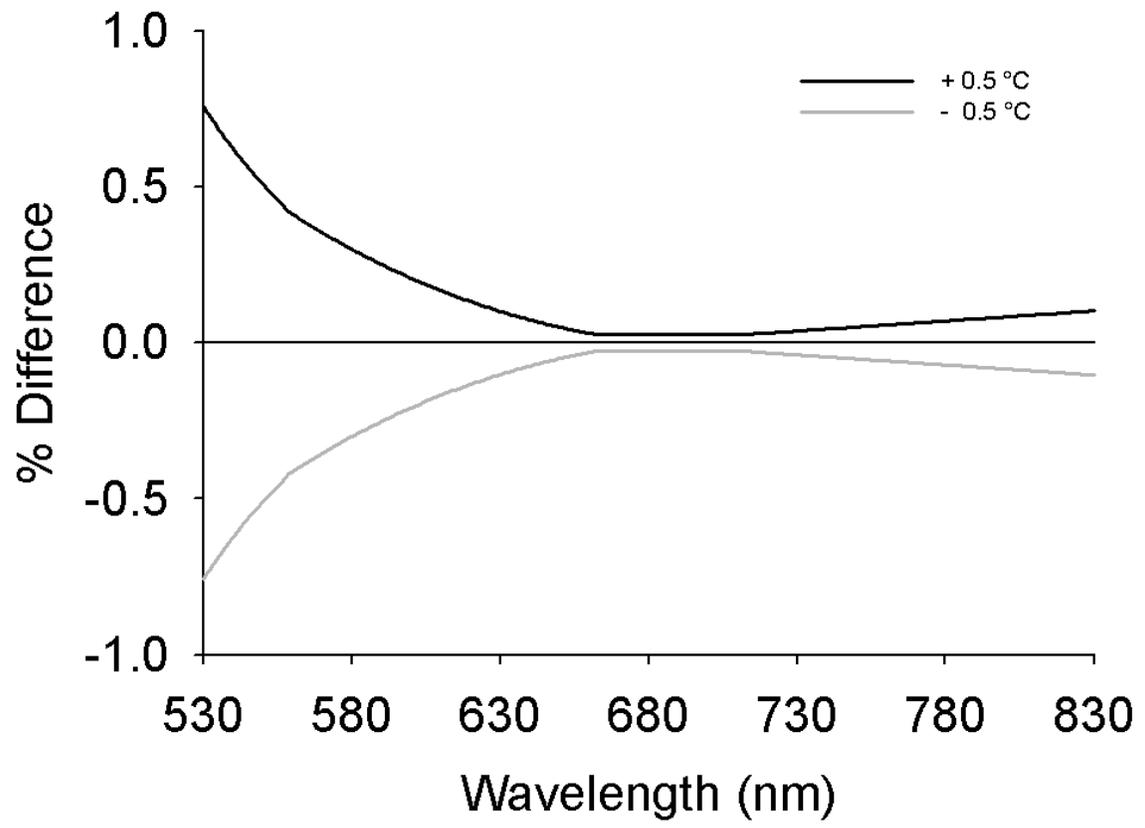
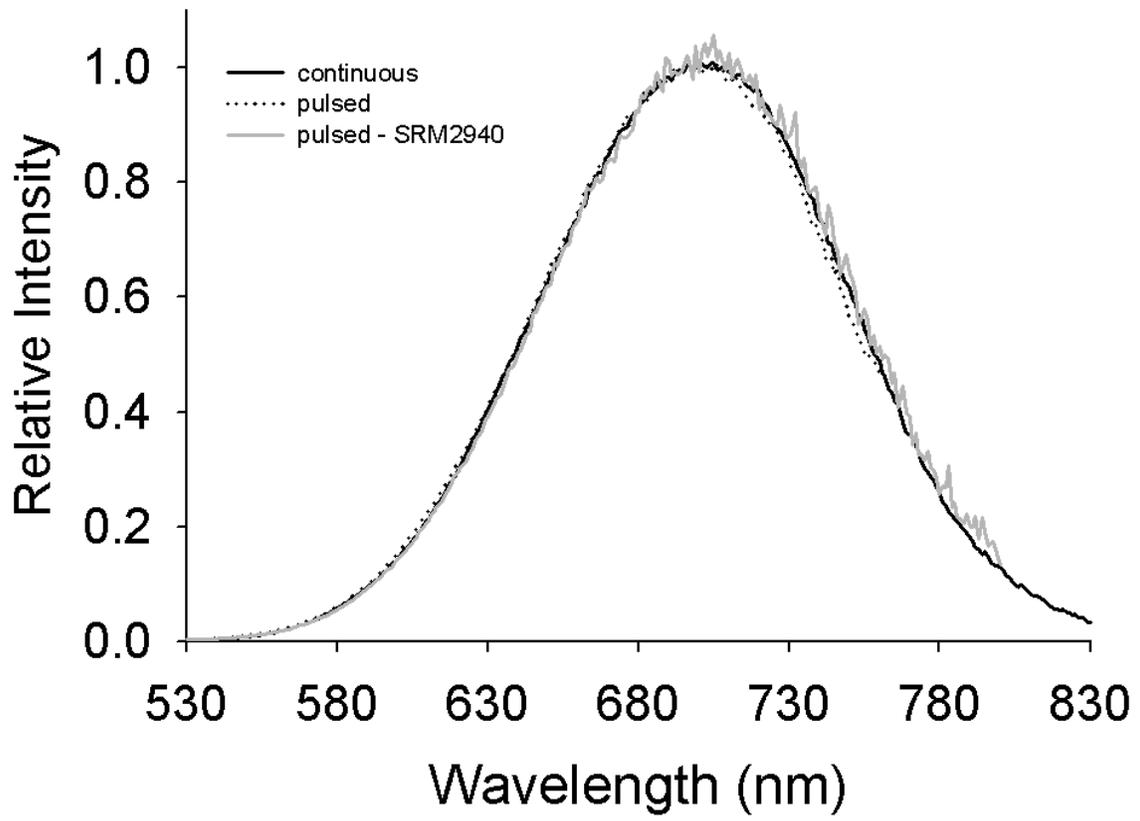


Figure 9



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