

A High-Accuracy Fluorescence Spectrometer and Standard Reference Materials for True Fluorescence Spectra to Enable Quantitative Fluorometry

NIST scientists are developing critical resources to enable scientists to measure the true spectral shape and absolute intensity of fluorescence, as opposed to uncorrected spectral shape and relative fluorescence. Fluorometers, such as high-throughput plate and chip readers, are used extensively in medical research and clinical laboratory diagnostics. The new NIST methods and Standard Reference Materials (SRMs) are expected to enable innovation in drug discovery, diagnostic medicine, and homeland security

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To qualify a research-grade fluorescence spectrometer for measuring true fluorescence spectra, enabling the certification of fluorescence Standard Reference Materials.

Luminescence measurements have become the detection methods of choice for new clinical and biochemical assays, and related high-throughput techniques, due to their extraordinary selectivity and sensitivity. These new analytical methods are becoming increasingly more quantitative, requiring standards to calibrate the luminescence measuring instruments that they utilize and aid in the validation of the methods. Reported here is the first step in this process, which is to qualify a research-grade fluorescence spectrometer for measuring true fluorescence spectra of reference material candidates. “True” spectra are defined here as those with fluorescence intensity, relative or absolute as required, and wavelength both being reported with high accuracy and known precision, after wavelength has been calibrated and corrections for excitation intensity and detection system responsivity have been applied.

SRMs for relative spectral correction of emission and intensity validation will enable measurements of fluorescence spectra to be compared over time and between instruments with relative ease by users, even non-experts.

Qualifying a fluorometer for measuring true spectra requires 1) excitation wavelength and bandwidth calibration, 2) emission wavelength and bandwidth calibration, 3) radiometric calibration of the excitation source as a function of wavelength (i.e., excitation correction) for excitation

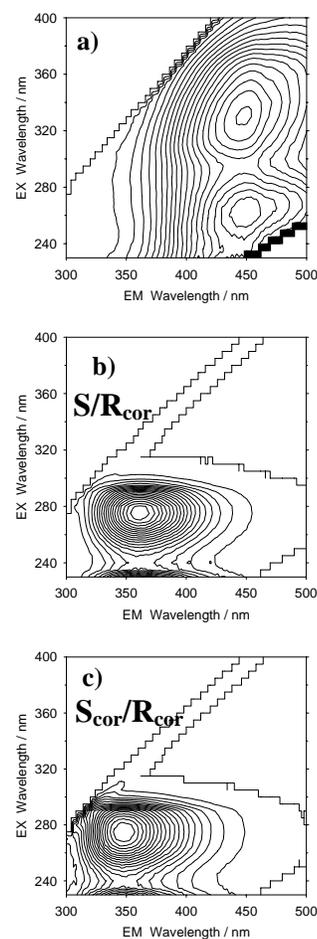
spectra and 4) radiometric calibration of the emission detection system as a function of wavelength (i.e., emission correction) for emission spectra. We have utilized conventional methods for calibrating intensity, using physical transfer standards (i.e., a calibrated light source, a calibrated detector and a calibrated diffuse reflector), and wavelength, using atomic lamps. Correction curves for fluorescence intensity as a function of wavelength were calculated.

Figure: Comparison of contour-plotted, fluorescence 3-D spectrum for tryptophan, where the fluorescence intensity is:

a) S the uncorrected signal measured at the detector, maxima at $(\lambda_{EX}, \lambda_{EM}) = (265 \text{ nm}, 452 \text{ nm})$ and $(330 \text{ nm}, 446 \text{ nm})$,

b) S/R_{cor} the signal corrected for the excitation intensity or flux at the sample position, maximum at $(\lambda_{EX}, \lambda_{EM}) = (275 \text{ nm}, 358 \text{ nm})$, and

c) S_{cor}/R_{cor} instrument-independent signal, corrected for both the instrument's excitation flux and its spectral responsivity, maximum at $(\lambda_{EX}, \lambda_{EM}) = (275 \text{ nm}, 346 \text{ nm})$.



Our high-accuracy fluorescence spectrometer has been qualified to measure both relative and absolute intensity-corrected fluorescence spectra throughout the visible region and beyond (310 nm to 800 nm). Both calibrated source (CS)- and calibrated detector (CD)-based methods for spectral correction of fluorescence were developed and compared. The CS-based method gave uncertainties, typically about $\pm 5\%$ for relative spectral correction, that were

about half that of the CD-based method for determining both relative and absolute spectral correction factors. We demonstrated that absolute spectral correction factors can be determined using either method without knowing the optical geometry of the instrument.

Currently, fluorescence intensity is nearly always expressed on a relative scale, making the comparison of intensity between different fluorometers difficult or impossible. Fluorescence spectra are often not corrected for the detection system's responsivity as a function of wavelength and in some cases are not even corrected for the intensity of the excitation source as a function of wavelength, resulting in a quantitatively incorrect and perhaps even qualitatively misleading spectral shape. This includes most of the fluorescence spectra found in the literature. The availability of SRMs for relative spectral correction of emission and intensity validation will enable the shape and absolute intensity, respectively, of fluorescence spectra to be compared over time and between instruments with relative ease by users, even non-experts. SRMs 2940 and 2941 have already been certified for spectral correction and intensity validation using our high-accuracy instrument and others are soon to follow.

Future Plans:

We are presently working to extend the wavelength region, where true fluorescence spectra can be measured, of our fluorescence spectrometer into the NIR region from 800 nm to 1000 nm, a region into which many new biological and clinical assays are beginning to extend. We are also adding accessories to our instrument to enable the routine measurement of absolute fluorescence quantum yields, an area where the need for standards is also increasing.