*Journal of Fluorescence, Vol. 15, No. 3, May 2005 (© 2005)*
DOI: 10.1007/s10895-005-2631-2

# Performance Validation for Microplate Fluorimeters

**Robert Giebeler,[1,3] Evelyn McGown,[1] Todd French,[1] and John C. Owicki[2]**

*Received July 1, 2004; accepted August 17, 2004*

As the sophistication of instruments that make fluorimetric measurements on samples in microplates has increased, so has the need for methods to validate instrumental performance. This paper describes a solid-state validation microplate that tests multiple aspects of fluorescence performance, including signal linearity, gain, noise, sensitivity, wavelength accuracy, and polarization stability. Both the operating principles and the validation of the validation microplate are discussed.

**KEY WORDS:** Validation; microplate; fluorescence; intensity; wavelength.

## INTRODUCTION

Validation and calibration are two complementary aspects of quality control for analytical instruments. Validation involves periodic measurements that determine whether an instrument is operating within its specifications; calibration involves adjustment of an instrument to operate within its specifications. This paper describes a solid-state microplate that incorporates standards for fluorescence intensity, polarization, and wavelength. The microplate can be used to validate a variety of aspects of optical performance. It can also be used for instrument calibration by service and instrument-manufacturing personnel.

### Background on Intensity and Polarization Validation

Unlike spectrophotometric measurements of optical density, measurements of fluorescence intensity on the same sample notoriously vary from instrument to instrument and even from day to day on the same instrument. The principal reason for the difference is that fluorimetry lacks the ratiometric character of spectrophotometry, and it thus does not cancel out many instrument-dependent factors. Attempts to remedy this problem, effectively replacing "Arbitrary Units" and "Relative Fluorescence Units" by "Calibrated Fluorescence Units," have involved normalizing experimental intensities to the intensities of reference samples [1].

Because instrumental response varies with wavelength, intensity validation typically must be done at the same excitation and emission wavelengths that will be used for experimental samples. Components that contribute to wavelength dependence of instrumental response include lamps, monochromators, filters, and detectors. Intensity variations on the excitation side of the optical path can be compensated by using a beam splitter to send some of the excitation light to a reference detector.

It would be very useful for fluorescence-intensity validation to have fluorescence-intensity standards that are traceable to international standards, analogous to National Institute of Standards and Technology (NIST) certified absorption standards available in the USA. NIST does provide standard reference materials (SRMs) such as fluorescein, but these are not certified as fluorescence intensity standards (only the concentration is certified for SRM 1932). SRM 1932 is a component of the molecules of equivalent soluble fluorophore (MESF) approach in flow cytometry, to convert the signal from a fluorescent particle to the number of fluorophores in solution that would have given the same signal [2,3]. This

[1] Molecular Devices Corporation, 1311 Orleans Dr., Sunnyvale, California 94089.
[2] Owicki Consulting, 956 N. California Avenue, Palo Alto, California 94303.
[3] To whom correspondence should be addressed. E-mail: Bob_Giebeler@moldev.com

approach is based upon liquid samples, with attendant concerns about sample handling and reproducibility in various laboratory settings. A NIST program exists to prepare a solid-state SRM as an intensity standard for cuvette, but not microplate, fluorimetry (P. DeRose, talk at 2004 Pittcon). Since there is no directly NIST-traceable solid-state fluorescence intensity standard for microplate fluorimeters, we have chosen to base our standard on a material having a stable fluorescence intensity that we can modify at will using NIST-traceable absorbance standards.

In developing a fluorescence intensity standard for microplate fluorimeters, the choice between liquid- and solid-state materials is a difficult one. Liquid standards are physically more similar to experimental samples than are solid standards. For example, refractive effects, such as those caused by the meniscus, are similar between standard and sample. In addition, liquid standards tend to be more broadly applicable across different instrumental platforms. The geometry of the interaction between excitation and emission light and sample differs considerably from instrument to instrument. For example, solid samples that scatter light, are opaque, or contain opaque masks may work well in one instrument but give very different results in another.

However, a liquid standard also has significant drawbacks: difficulties obtaining high precision of concentrations, photobleaching, environmental stability and the inconvenience and labor cost of preparing fresh validation standards for each day's test. Even with the careful MESF approach, reproducibility may be worse than 10% [2]. These disadvantages are significant enough that all commercial validation microplates employ solid-state standards. Our validation microplate differs from other commercial varieties principally in that we provide NIST-traceable intensity control and NIST-traceable wavelength validation.

The development of fluorescence intensity standards enables multiple types of instrumental validation. For example, aside from inner-filter effects [4], fluorescence intensity should be a linear function of fluorophore concentration. Instrumental artifacts, however, may limit linearity. Limitations in detector technology that can cause deviations from linearity include pulse overlap at high intensities in photon-counting mode with photomultiplier tubes and saturation effects in analog detection circuits. Hence, there is need for methods that validate linearity for signal intensities that span orders of magnitude—the operation range of a microplate reader.

Fluorescence intensity standards may also enable the validation and calibration of fluorescence polarization and anisotropy measurements. Both techniques require two intensity measurements per data point, made with parallel and perpendicular polarizers. The ratiometric nature of the measurements automatically corrects most of the effects that are problematic for standardizing fluorescence-intensity measurements. The effect that is *not* automatically corrected is the instrumental bias in intensities between the two polarization orientations used for measurements [4].

Instrumental components that may show wavelength-dependent polarization bias include the light source, diffraction gratings, reflective surfaces, and detectors. The standard compensation procedure is to multiply one of the two intensity measurements by a (wavelength-dependent) correction called the G factor. For cuvette instruments, the right angle between excitation and emission optical paths permits determination of the G factor on any experimental sample, without recourse to polarization standards. The epifluorescence optical system of typical microplate instruments, however, does not permit this internal calibration. Unless the optical design of such an instrument is inherently free of polarization bias, the instrument must be calibrated with a reference material of known polarization that has fluorescence spectra similar to those of the sample [5]. A solution of a fluorescent label is widely used to calibrate a biochemical assay employing the same label, but the fluorescence polarization of the label is typically rather temperature dependent. A photochemically stable solid-state polarization standard with small temperature dependence would be a useful validation tool for microplate fluorimeters.

## Background on Wavelength Calibration

Miller [6] enumerated four main strategies for calibrating the monochromators of spectrofluorimeters, all based on the use of sharp spectral features at known wavelengths:

1. Sharp peaks in the spectrum of the instrument's lamp, typically a xenon arc lamp;
2. Sharp peaks in the spectrum of a lamp inserted into the instrument's optical path expressly for calibration, typically a mercury arc lamp;
3. Sharp absorption minima in the excitation light due to the insertion of a suitable filter into the instrument's optical path; and
4. Sharp fluorescence emission peaks of certain fluorophores such as aromatic hydrocarbons or lanthanides, either in solution or in polymeric glasses.

Variations on these methods have included using inflection points [7] and isosbestic points [8] rather than spectral extrema as the calibration points. The relevant ASTM Standard Test Method [9] describes calibrating the emission monochromator with the lines of a mercury lamp and then calibrating the excitation monochromator against the already-calibrated emission monochromator by using a light-scattering sample. Solutions of substances with sharp absorbance features, such as holmium oxide, have been used as reference samples to calibrate the monochromators of spectrophotometers [10], but this strategy cannot be used directly in spectrofluorimeters. Of particular interest for the present paper, Paladini and Erijman [11] interposed a holmium oxide filter between the detector and a cuvette containing a fluorophore, thus using the emission of the fluorophore as the light source with which the sharp absorption bands of the holmium oxide could be analyzed.

For methods that employ sharp absorbance (more precisely, transmittance) spectra to validate monochromators in spectrofluorimeters, reference spectra of the absorbing materials are generally obtained by spectrophotometry. The larger bandwidths typical of monochromators in spectrofluorimeters lead to spectral distortions as the reference transmittance spectrum is multiplied by wavelength-dependent instrumental factors such as the lamp spectrum and then convolved with the bandpass function of the monochromator [9]. The results may be (a) loss of transmittance extrema; (b) shifts in the wavelength of the remaining extrema; and (c) overall broadening of features, which reduces the sharpness of the extrema and decreases the precision of their measured locations.

Venable and Eckerle [7] studied the effects of monochromator bandwidth on the measured transmittance spectrum of didymium glass in the spectral region 400–750 nm. The positions of transmittance minima shifted by 0–3.8 nm for bandwidths in the range 1.5–10.5 nm, and the shifts were not always monotonic with bandwidth. The authors also found that only six of the 15 minima found with a 1.5 nm bandwidth survived at a 10.5 nm bandwidth.

## EXPERIMENT AND RESULTS

### Process for Validating the Validation Microplates

A validation microplate must itself be validated before it can reliably serve its purpose. In this section we describe that validation process, which is termed the Gold Standard process and is depicted in Fig. 1. The process was based on qualifying a FlexStation® microplate spectrofluorimeter (Molecular Devices) as a "Gold Standard" instrument.

The accuracy of the excitation monochromator of the Gold Standard instrument was determined with a NIST-traceable spectrum analyzer. The accuracy of the emission monochromator was determined using the calibrated excitation signal as an input by reflecting the excitation output directly to the emission optics. Due to detector saturation limitations, we attenuated the excitation signal by scattering some of the excitation light out of the emission path.

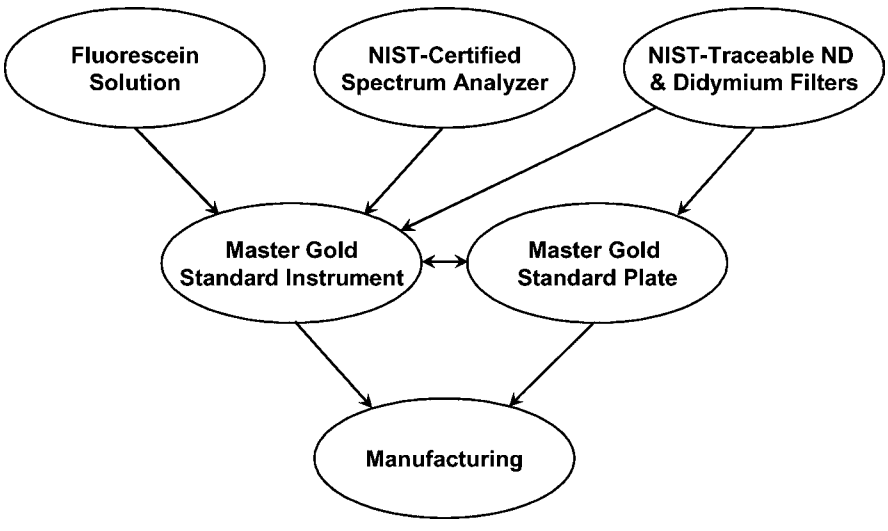


**Fig. 1.** Flow chart for the Gold Standard validation process. ND means neutral density. See text for details.

We used a scatterer instead of a conventional attenuating filter to minimize the introduction of wavelength artifacts into the emission light.

A stable fluorescence-intensity response of the Gold Standard instrument was validated as follows. Fluctuations in the intensity of excitation light were eliminated by ratioing the fluorescence signal to a built-in reference signal in the instrument. A beam splitter was used to direct a small portion of the excitation light to the highly linear solid-state reference detector. Fluctuations in the sensitivity of the emission detection system were compensated for by an internal fluorescence standard. Linearity of the response of the emission side of the instrument was verified by determining that the ratiometric signal was independent of lamp intensity and that neutral-density filters gave attenuations that were consistent with their optical densities as measured on a NIST-traceable spectrophotometer.

The temporal stability of the intensities reported by the Gold Standard instrument was determined by measuring the fluorescence intensity of 200 $\mu$L of 100 nM sodium fluorescein, pH 9.0, in a 96-well microplate. The solution was freshly prepared from a standard reference lot of solid sodium fluorescein for each measurement. The precision of measurements with the sodium fluorescein solution was not as good as that with the solid-state reference material in the validation plate, but we used sodium fluorescein because we believed that the drift in its intensity would be minimal over periods for which the temporal stability of the fluorescence intensity of the solid-state material had not yet been verified. Over 12 months of tests, the variations in intensity measurements of sodium fluorescein on the Gold Standard instrument were indistinguishable from the imprecision of preparing the test solutions, about 3%. In other words, any instability of the instrument was smaller than 3%.

A "Gold Standard" validation microplate was then validated on the Gold Standard instrument. This microplate was used to validate other instruments used in the general manufacturing of validation microplates.

In summary, the chain of validation was as follows. The Gold Standard instrument was validated with respect to monochromator wavelength by a NIST-traceable spectrum analyzer and with respect to intensity response by a standard solution of sodium fluorescein. A Gold Standard validation microplate was validated by the Gold Standard instrument. Other instruments were validated with the Gold Standard validation microplate, and these were used to validate validation microplates in the general manufacturing process. Unless otherwise noted, all data in this paper were obtained with the Gold Standard validation microplate.

## Solid-State Fluorescence Reference Material

The fluorescence intensity of a good reference material should be stable with respect to time (years), photochemical degradation, and temperature. One type of reference material is lanthanide glass. We did not choose to use this material in the validation plates for two reasons:

- Its fluorescence intensity suffers from significant temperature dependence, limiting its usefulness when the temperature is not strictly controlled or measured for compensation.
- Its fluorescence is characterized by long (hundreds of $\mu$s) lifetime, which can be problematic for use in microplate readers that use flash lamps and integrate only during the flash.

After studying a variety of fluorescent materials, including commercially available products, we found a promising material based on a fluorescent plastic and tested three different formulations (denoted A, B, and C) that seemed likely to meet the stability requirements and had suitable spectral properties and positional uniformity. Figure 2 shows excitation and emission spectra for the three materials. The three polymeric materials are spectroscopically distinct from one another. Type A material was ultimately selected for the validation microplate.

Figure 3 compares the absorbance and excitation spectra for the material chosen for the validation plate. The

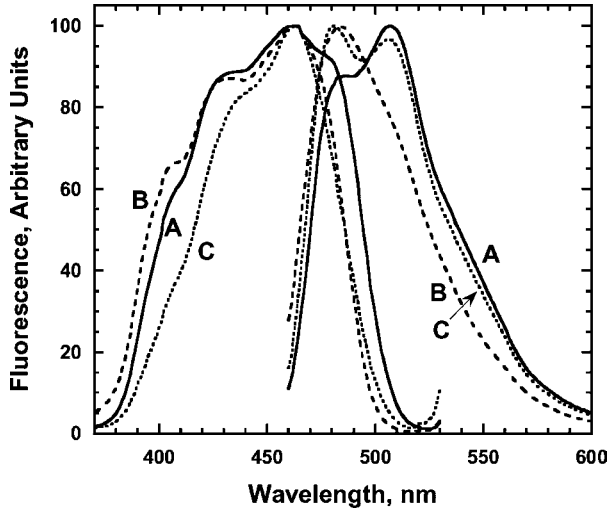


**Fig. 2.** Excitation (left) and emission (right) spectra of three fluorescent plastic materials. Spectra from fluorescent plastics type A, B, and C were obtained on a SpectraMax® Gemini EM microplate fluorimeter (Molecular Devices). Excitation spectra were taken with emission at 550 nm in the presence of a 530 nm longpass filter. Emission spectra were taken with excitation at 440 nm in the presence of a 455 nm longpass filter.

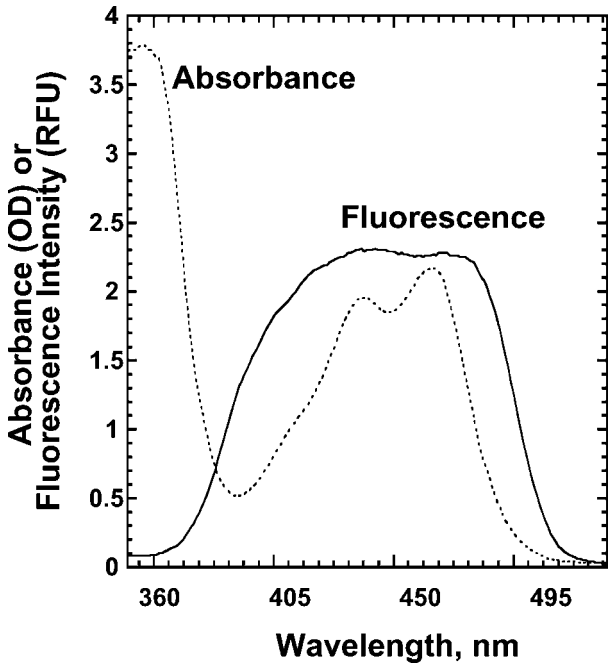


**Fig. 3.** The absorbance and fluorescence excitation spectra of the plastic used in the validation plate differ. The short-wavelength absorbance peak presumably arises from the plastic matrix. Part, but not all, of the difference between the two spectra at longer wavelengths is due to the narrower bandwidth of the monochromator in the spectrophotometer (1.5 nm in a SpectraMax Plus[384], Molecular Devices) compared to the fluorimeter (7 nm in a SpectraMax Gemini EM, Molecular Devices). The emission wavelength was 550 nm, with a 530 nm longpass filter.

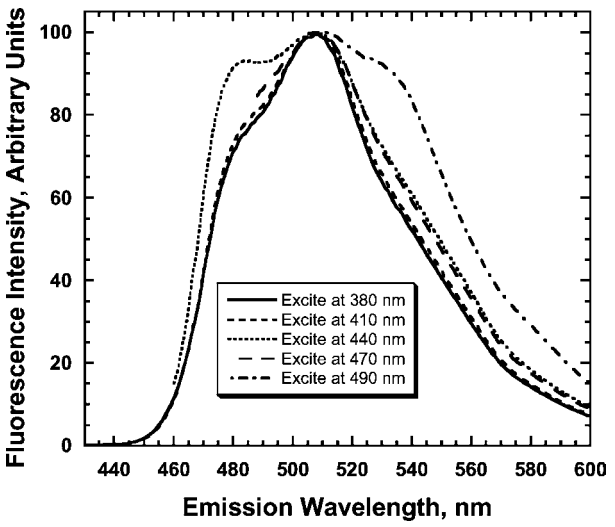


**Fig. 4.** The emission spectrum of the fluorescent plastic in the validation plate depends significantly on the excitation wavelength. This implies the existence of multiple spectroscopically distinct fluorescent species. Data were obtained on a SpectraMax Gemini EM microplate fluorimeter.

strong ultraviolet absorbance presumably arises from the plastic matrix. Otherwise, the difference in shape between the two spectra suggests that the fluorescence arises from multiple spectroscopically distinct species. The presence of multiple fluorescent species is further suggested by the fact that the shape of the emission spectrum depends on the excitation wavelength, as is shown in Fig. 4.

Figure 5 shows the temperature dependence of the fluorescence intensities of the fluorescent plastic in the validation plate and the lanthanide glass internal fluorescence standard, both plotted as a function of wavelength. To observe the temperature dependence of the lanthanide glass intensity, the instrument's automatic intensity-temperature compensation was disabled. The stability of a fluorescence-intensity measurement in this configuration is 2%, which corresponds to about $0.1\%C^{-1}$ in the measured temperature coefficient.

The figure shows that the temperature coefficients of both the fluorescent plastic and the lanthanide glass depend on the emission wavelength. They also depend somewhat on the excitation wavelength (data not shown). Such behavior is not unexpected in materials that contain multiple spectroscopically distinct species. The temperature dependence was no more severe than $+0.3\%C^{-1}$ for the fluorescent plastic and $-0.8\%C^{-1}$ for the lanthanide glass. The plastic showed more stability than the lanthanide in the mid-visual (500–650 nm) range.

Figure 6 shows the temporal stability of fluorescence intensity, measured over about 13 months. The plastics

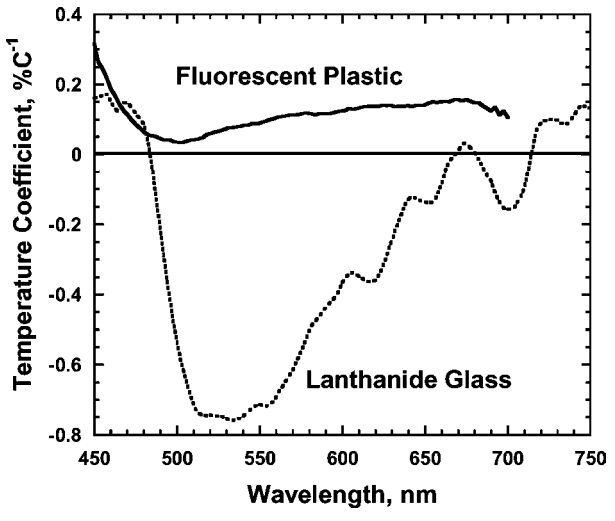


**Fig. 5.** Temperature dependence of the fluorescence intensity of the fluorescent plastic and lanthanide glass. The temperature coefficient is defined as the average change in intensity per degree between 23°C and 45°C for the fluorescent plastic, and between 27°C and 45°C for the lanthanide glass. The plastic was excited at 400 nm, the lanthanide at 423 nm in the Gold Standard instrument. The curves have been smoothed.

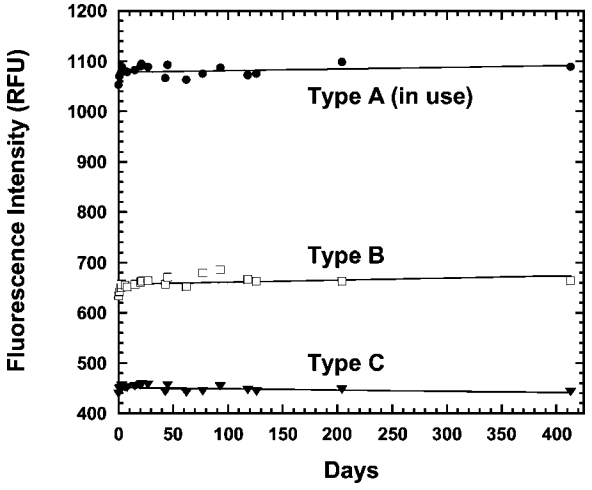


**Fig. 6.** Temporal stability of three fluorescent plastics. Each data point is based on measurements of two samples (eight for Type A), eight measurements per sample. Lines are linear regressions. Standard errors of data points are 0.2% to 0.8%. The excitation wavelength was 485 nm, the emission wavelength 525 nm, in the Gold Standard instrument.

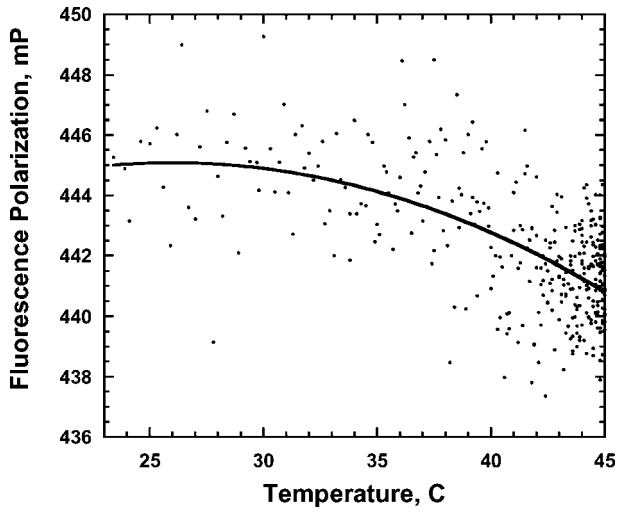


**Fig. 7.** Effect of temperature on the fluorescence polarization of the fluorescent plastic. Data were taken as the temperature of the sample was raised from 23 to 45°C over 45 minutes in a prototype instrument (excitation at 485 nm, emission at 525 nm). The curve is a quadratic regression.

were stored on a laboratory benchtop with ambient fluorescent light and were periodically assayed in the Gold Standard instrument. The slopes of the linear-regression lines in the figure are (in units of % per year) $1.1 \pm 0.9$ for Type A (used in the validation plate), $2.3 \pm 1.5$ for Type B, and $-1.8 \pm 1.1$ for Type C. None of these slopes is significantly different from zero according to an F test ($p > 0.10$). The fact that fluctuations in intensity of the three materials are positively correlated suggests that they are due in large part to variations in instrument response rather than in the materials themselves.

Given that fluorescence polarization depends on the ratio of two intensity measurements, it is not surprising that the fluorescence polarization of the fluorescent plastic used in the validation plate is stable over time. Fluorescence-polarization measurements were made in a prototype instrument every eight seconds for 16 hours (excitation at 485 nm, emission at 525 nm). The standard deviation of the measurements over the entire period was 1.8 mP, against a mean fluorescence polarization of 445.1 mP. Linear regression gave a slope of $0.038 \pm 0.004$ mP/hour, which is small but significantly different from zero ($p < 0.001$).

The fluorescence polarization is relatively insensitive to temperature in the range 23 to 45°C. As is shown in Fig. 7, the fluorescence polarization decreases only by about 4 mP over this temperature range. This decrease is barely distinguishable from the noise in the measurements, which is best quantified by the 1.7 mP standard deviation of the residuals from a quadratic regression curve.

The material chosen for the validation plate was also tested for photochemical stability. One column in a validation plate was exposed to 3,000,000 flashes of the xenon arc lamp in a life-test fixture with a decrease in fluorescence intensity of less than 1%. Exposure to daily sun on a windowsill for three days decreased the fluorescence by less than 1%.

The final stability tests determined to what extent the fluorescence intensity of the plastics depended exposure to high temperature and high humidity. A sample of the plastic was held at 49°C and 80% relative humidity for 3 days and then held at 60°C with low humidity for two weeks. These manipulations changed the fluorescence intensity by less than 2%.

## Optical Design of Fluorescence-intensity Standards

Four different fluorescence intensities were obtained with a single fluorescent plastic by placing neutral-density filters on top of the plastic, as is shown in Fig. 8A. The single-pass attenuations of the filters were 0.5, 1.0, 1.5, and 2.0 OD. Since both excitation and emission light must pass through the filter, this achieves attenuations of approximately 1, 2, 3, and 4 OD, a $10^3$ range. Using glass instead of fluorescent plastic and without a neutral-density filter provides a fifth intensity that approximates background.

The optical properties of the fluorescent plastic and neutral-density filters vary somewhat from sample to sample. Nevertheless, it is possible to select materials so that

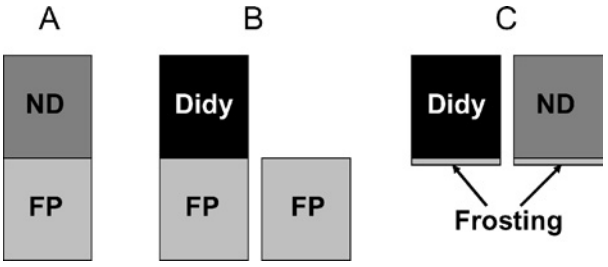


**Fig. 8.** Schematic cross-sectional views of materials in validation plate. A. Fluorescein intensity standard, comprising a neutral-density filter (ND) over the fluorescent plastic (FP). B. Wavelength standard for fluorescence method, comprising a didymium filter (Didy) over fluorescent plastic and (for ratiometry) fluorescent plastic. C. Wavelength standard for reflectance method, comprising a didymium filter frosted on the bottom side and (for ratiometry) a neutral-density filter frosted on the bottom side. A neutral-density filter is used in the reflectance method as a solid support for the frosted surface and because of the high intensity of light reflected by the frosting. If light were not attenuated, different photomultiplier tube settings might be necessary for the two spectra, complicating the ratiometry. Illumination is from above in all cases.

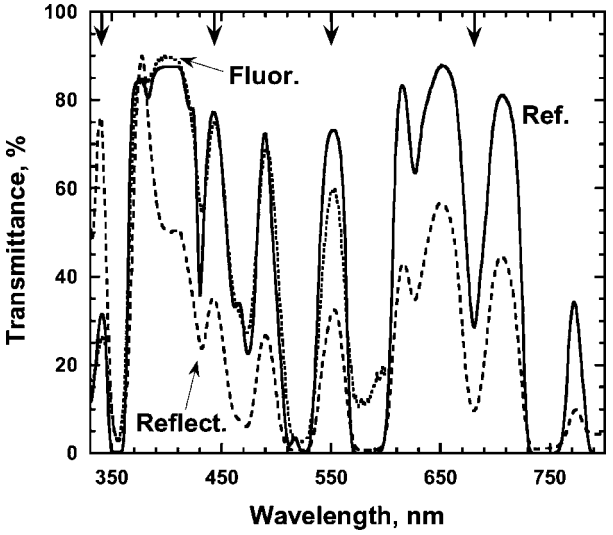


**Fig. 9.** Wavelength validation of the excitation monochromator. The ratios of spectra with and without the didymium filter are shown for the fluorescence (Fluor.) and reflection (Reflect.) methods; the spectra were taken with a SpectraMax Gemini EM, 7 nm excitation monochromator bandwidth. Also shown is the reference transmittance spectrum of the didymium filter (Ref.), taken with a SpectraMax Plus$^{384}$ instrument, 1.5 nm monochromator bandwidth. The ratio spectra were normalized for convenience of presentation, which does not affect the wavelengths of the extrema. The four extrema that were selected for wavelength validation are indicated by arrows pendant from the upper axis.

the well-to-well variations in fluorescence intensity are small within a microplate. For example, the coefficient of variation for the fluorescence intensity measured on a set of four combinations of plastic and 1-OD neutral density filters (32 wells) is typically no more than 0.5%.

## Optical Design of Monochromator Wavelength Standards

Our goal was to validate the wavelength accuracy of the monochromators to within ±2 nm. This is challenging, because it is much smaller than the minimum width of observable spectral features, which is governed by the bandpass of monochromators. Compared to spectrophotometers, spectrofluorimeters usually have monochromators with a large bandpass (7 nm for excitation and 9 or 18 nm for emission in Molecular Devices instruments) to improve light throughput.

A didymium glass filter in the optical path creates spectra with sharp minima and maxima (extrema); see Figure 9 for a transmission spectrum. The positions of extrema for each lot of filters were determined with a NIST-traceable spectrophotometer. Two complementary strategies were developed to use the didymium filters in the validation plate to validate the wavelength response of monochromators.

In the first strategy ("fluorescence method," Fig. 8B) a layer of fluorescent plastic is placed beneath the didymium filter to serve as a light source. To validate the excitation monochromator, an excitation scan is performed at fixed emission wavelength (Fig. 9). This scan contains the didymium transmittance spectrum modified by other factors such as the excitation spectrum of the fluorescent plastic. A second scan is performed on fluorescent plastic without the intervening didymium filter. The ratio of the two scans approximates the transmission spectrum of the didymium filter. The emission monochromator is validated by a similar procedure, except that the emission monochromator is scanned at fixed excitation wavelength (results in Fig. 10).

The second strategy ("reflectance method," Fig. 8C) differs from the first in the source of light that is detected by the emission optics. Instead of a layer of fluorescent plastic, the side of the didymium filter away from the optic system (the lower side) is frosted to produce a source of diffusively reflected, or scattered, light. To validate the excitation monochromator, that monochromator is scanned while the emission monochromator is set for zero-order diffraction (i.e., reflection) to direct all wavelengths to the detector. The result is the transmittance spectrum of didymium, modified by instrumental factors such as the wavelength-dependent response of the detector. A second scan is performed on neutral-density glass, again frosted on the lower side. The ratio of the two scans approximates the transmittance spectrum of didymium (Fig. 9). The emission monochromator is validated by essentially

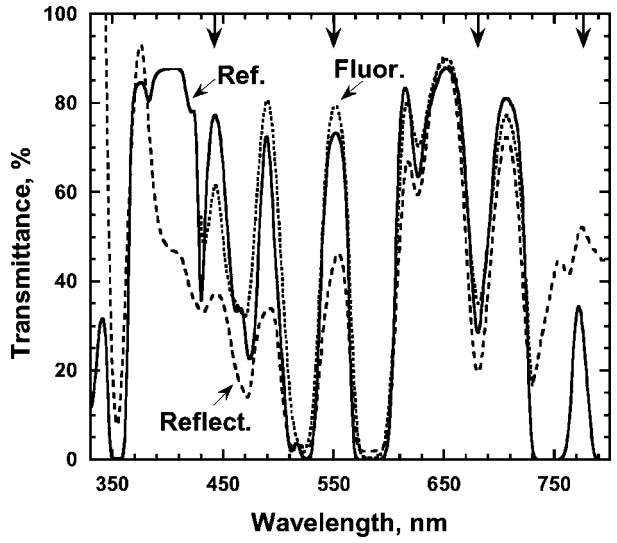


**Fig. 10.** Wavelength validation of the emission monochromator. The ratios of spectra with and without the didymium filter are shown for the fluorescence (Fluor.) and reflection (Reflect.) methods; the spectra were taken with a SpectraMax Gemini EM, 9 nm emission monochromator bandwidth. Also shown is the reference transmittance spectrum of the didymium filter (Ref.), taken with a SpectraMax Plus$^{384}$ instrument, 1.5 nm monochromator bandwidth. The ratio spectra were normalized for convenience of presentation, which does not affect the wavelengths of the extrema. The four extrema that were selected for wavelength validation are indicated by arrows pendant from the upper axis.

the same method, except instead scanning the emission monochromator and fixing the excitation monochromator in the zero-order diffraction configuration (results in Fig. 10).

Once the ratiometric didymium spectrum is obtained, the measured positions of extrema (maxima and minima) can be checked against standard values obtained by spectrophotometry. The wavelength shifts due to the relatively broad bandwidths of the fluorimeters monochromators are, for most of the extrema, less than the tolerance of the validation procedure.

A subset of the extrema from the didymium spectrum was chosen for use with the validation microplate, based on factors such as sharpness, relative insensitivity to bandwidth effects, and coverage of the wavelength range of interest. Table I lists the wavelengths from a representative validation microplate, both the spectrophotometrically determined reference wavelengths and the wavelengths determined by the four ratiometric procedures described here. All deviations of validation wavelengths from reference values are within the 2 nm tolerance.

An advantage of the fluorescence method is that it more closely approximates fluorescence from a solution,

**Table I.** Wavelengths of Extrema in Didymium Spectra

| Monochromator | Reference | Observed | Error | Method |
|---|---|---|---|---|
| Excitation | 340.6 | 339.9 | −0.7 | Fluorescence |
| Excitation | 442.9 | 444.3 | +1.4 | Fluorescence |
| Excitation | 551.1 | 552.9 | +1.8 | Reflectance |
| Excitation | 681.4 | 682.3 | +0.9 | Reflectance |
| Emission | 442.9 | 443.9 | +0.1 | Reflectance |
| Emission | 551.1 | 551.9 | +0.8 | Fluorescence |
| Emission | 681.4 | 682.1 | +0.7 | Reflectance |
| Emission | 773.7 | 774.7 | +1.0 | Reflectance |

*Note*. All wavelengths are in nm. The reference wavelengths were obtained by the manufacturer of the didymium filter using a spectrophotometer with a 3 nm bandwidth. The Gold Standard instrument (7 nm excitation bandwidth, 18 nm emission bandwidth) was used for the validation. Each validation result is based on 20 repeated measurements of eight didymium wells and four reference wells on the microplate (see Fig. 11 and Table II). The standard error of the mean (SEM) for a single measurement across eight wells averaged from 0.1 to 0.3 nm. The SEM for the 20 repeated measurements of the average across the plate ranged from 0.0 to 0.1 nm.

more resembling the normal mode of instrumental operation. The two methods are useful over somewhat different wavelength ranges: the fluorescence method where the fluorescent plastic excites or emits appreciably, and the reflection method for wavelengths that are efficiently passed by both the excitation and emission optical systems. Both methods were implemented in the final validation microplate.

## Layout of Manufactured Validation Microplate

Figure 11 shows the final layout of the validation plate (now sold by Molecular Devices as the SpectraTest$^{TM}$ FL1), which mimics a 96-well microplate.

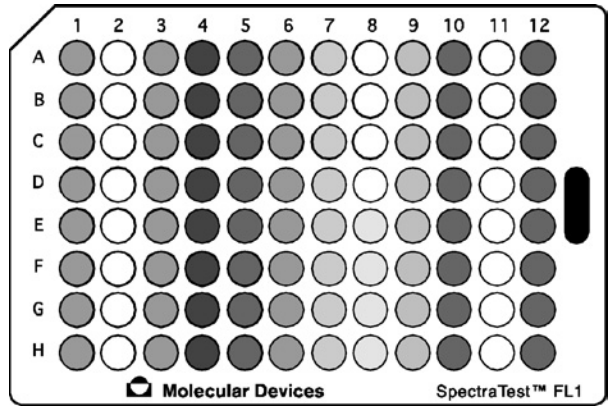


**Fig. 11.** Layout of SpectraTest FL1 validation microplate. See Table II and text for details.

**Table II.** Key to Layout of Validation Microplate in Fig. 11

| Column | Rows | Material (Purpose) |
|---|---|---|
| 1 | All | Attenuator + fluorescent plastic (CFU Magnitude 3) |
| 2 | All | Glass (background, CFU Magnitude 0) |
| 3 | All | Attenuator + fluorescent plastic (CFU Magnitude 3) |
| 4 | All | Attenuator + fluorescent plastic (CFU Magnitude 1) |
| 5 | All | Attenuator + fluorescent plastic (CFU Magnitude 2) |
| 6 | All | Attenuator + fluorescent plastic (CFU Magnitude 4) |
| 7 | All | Frosted didymium (reflectance method for wavelength) |
| 8 | A–D | Frosted attenuator (reflectance method for wavelength) |
| 8 | E–H | Fluorescent plastic (fluorescence method for wavelength) |
| 9 | All | Didymium + fluorescent plastic (fluorescence method for wavelength) |
| 10 | All | Attenuator + fluorescent plastic (CFU Magnitude 3) |
| 11 | All | Glass (background, CFU Magnitude 0) |
| 12 | All | Attenuator + fluorescent plastic (CFU Magnitude 3) |

*Note*. CFU refers to calibrated fluorescence units. Magnitude 0 is the background intensity obtained from glass. Magnitudes 1, 2, 3, and 4 are intensity standards based on the fluorescent plastic with neutral-density filters that are successively weaker, creating approximately a 10-fold intensity ratio in the adjacent magnitudes.

Each column contains a different configuration, and all row positions within a column are nominally identical, except for column 8, which contains the materials for ratiometric wavelength calibration. See Table II for a more detailed description of the layout.

## DISCUSSION

### Validation of Fluorescence Intensity and Polarization

The basis of all the fluorescence-intensity standards in the validation microplate is a fluorescent plastic whose fluorescence intensity is quite stable with respect to the parameters that are likely to be operationally important: time, light exposure, temperature, and humidity. A series of fluorescence-intensity standards spanning a thousand-fold range of intensity is constructed by overlaying this single fluorescent plastic with NIST-traceable neutral-density filters. Thus, the unavailability of NIST traceability for fluorescence-intensity is largely overcome by combining a bright, stable source of fluorescence with NIST-traceable absorbance material.

As we noted in the Introduction, solid-state standards have many advantages, primarily precision, convenience, and economy of labor, which are all exhibited in the validation microplate of this paper. The microplate also has the limitations of the solid-state approach. These limitations arise from the fact that the geometry of the solid-state material differs from that of the liquid samples for which microplate fluorimeters are designed. The solid-state material has no meniscus. It is not confined to the well of a typical microplate. Its fluorescence is not necessarily uniformly distributed within the structure.

In consequence, the relationship between fluorescence intensities determined on the validation plate and on liquid samples may depend on the optical design of the instrument. For example, the ratio of intensities measured for the validation microplate and for 100 $\mu$L of 100 nM sodium fluorescein in a microplate well may differ from one instrument design to another. The sensitivity of measured intensity to sample geometry in microplate fluorometry is a well-known phenomenon: in contrast to cuvette-based analysis, measured fluorescence intensity normally depends on the volume of the liquid sample and on the shape of the meniscus.

Sample geometry can also affect sensitivity to problems with the positioning of the microplate. If each well in a microplate is identically filled, each should yield the same fluorescence intensity. Mechanical mis-adjustments can create biases, for example across the plate along the row or column directions. We have observed that some causes of bias such as excitation beam position or focus may affect solid state and liquid samples differently.

The stability of the fluorescence intensity in the validation microplate is mirrored by the stability of the fluorescence polarization, which depends on a ratio of intensity measurements. Although this renders the validation plate a useful tool for monitoring the consistency of fluorescence-polarization measurements, we urge caution in using it to calibrate the G factor for an instrument. It has been our experience that G factors can differ among fluorophores with roughly similar spectroscopic properties even when the instrument settings are identical.

The G factor depends on wavelength, and fluorimeters typically employ rather broad bandwidths. For different fluorophores the distribution of absorption and

emission presumably can differ enough within the instrumental bandwidths that wavelength-dependent instrumental polarization biases average differently. Hence, at a particular set of excitation and emission wavelengths and bandpasses, the G factor for the fluorescent plastic in a validation microplate may not be the same as the G factor for the fluorophore used in an experimental sample.

### Validation of Wavelength

Validation of wavelength settings is more complex in spectrofluorimeters than spectrophotometers because of broader bandwidths and added complexity of the optical system. We have implemented a strategy to calibrate wavelengths to within 2 nm, a fraction of the monochromator bandwidths. This depends on the wavelengths of sharp features in NIST-traceable didymium filters coupled with the use of fluorescent plastic or diffusively reflective glass as a secondary light source at the sample position. The broad bandwidths of the optical systems in fluorimeters erase some extrema of the didymium transmission spectrum and significantly shift others. It was, however, possible to choose the extrema listed in Table I that were not seriously affected. For a more detailed discussion of these issues, see the Appendix. The use of the wavelengths in Table I enables validation of excitation wavelengths from 340 to 680 nm and emission wavelengths from 440 to 770 nm.

### Types of Validations that can be Performed with the Validation Plate

In this paper we have emphasized the validation of the accuracy of calibrated fluorescence intensity units, stable fluorescence polarization, and wavelength response of the monochromators. A variety of additional instrumental functions can also be validated:

- *Detector linearity*. The linearity of the response of the detector (often a photomultiplier tube) can be validated over three orders of magnitude of signal intensity with the fluorescence-intensity standards on the validation microplate.
- *Calibration of detector output at different gain settings*. The dynamic range of instruments is often extended by adjusting the gain setting of the detection system inversely with the brightness of the sample, in one or more steps. Changing the voltage on photomultiplier tubes is a common example. The fluorescence intensity standards on the validation microplate permit calibration of the detector to enable comparison of fluorescence-intensity measurements taken at different gain settings.
- *Accuracy of well-to-well measurements*. Fluorescence-intensity measurements on otherwise identical wells should not depend on factors such as the positions of the wells on the microplate. The distribution of replicate fluorescence-intensity standards on the validation microplate enables this validation, within the capability of the solid-state format as discussed above.
- *Precision of repeated intensity measurements*. The precision of repeated measurements on the same well will depend on the instrument's excitation and detection (optical, electrical) systems and, if the microplate is repositioned between measurements, also on the instrument's mechanical systems. The fluorescence-intensity standards on the validation plate enable analysis of this precision at a wide range of intensities.
- *Precision of wavelength response of monochromators*. When a monochromator is repeatedly slewed to some wavelength, the actual wavelengths that are reached will be a distribution about the intended wavelength. The wells on the validation plate that contain didymium and fluorescent plastic can be used to determine this precision as follows. To test the excitation monochromator, it is repeatedly set to a wavelength at which the didymium transmittance is rapidly varying, ensuring large signal changes due to wavelength errors. The emission monochromator is positioned at a broad extremum on the didymium transmission spectrum to optimize signal stability. To test the emission monochromator, the instructions for the excitation and emission monochromators are interchanged.

## CONCLUSIONS

We have described a solid-state microplate that can be used to validate the major functions of a microplate spectrofluorimeter, including fluorescence intensity and wavelength responses. In addition, it provides a stable reference for fluorescence polarization and can be used to validate a variety of other functions, such as detector function, the precision and well-to-well accuracy of intensity measurements, and the precision of wavelength settings.

With the aid of the validation microplate, fluorescence intensities can be reported in "Calibrated Fluorescence Units" (CFUs) rather than "Arbitrary Units" or "Relative Fluorescence Units." These CFUs allow quantitative comparisons between intensity measurements made at different times on the same instrument or made on different instruments of the same model.

## ACKNOWLEDGMENTS

This work was supported by Molecular Devices' internal research into fluorescence standards. We would like to thank Joe Machamer for review and Jan Hummel for assistance preparing this paper.

## APPENDIX A: EFFECTS OF FINITE MONOCHROMATOR BANDWIDTH ON APPARENT TRANSMITTANCE SPECTRA

We have already mentioned that the wavelengths of extrema in a sharp transmission spectrum can be shifted appreciably due to wavelength dependence of instrumental response and finite bandwidth of excitation and emission monochromators. In this Appendix we describe these effects more formally. We also discuss approximate computational methods for analyzing them in the context of the wavelength-validation methods described in this paper. The mathematical description given below can be visualized by aid of Fig. A1.

We begin by defining the optical properties of the components of the validation plate. The reference transmittance spectrum of didymium is designated $T(\lambda)$; it would be measured on a spectrophotometer whose monochromator has very narrow bandwidth. The fluorescent plastic has excitation spectrum $E(\lambda)$ and emission spectrum $F(\lambda)$. For a multi-component fluorescent material such as we describe, the shapes of $E(\lambda)$ and $F(\lambda)$ depend somewhat on the emission and excitation wavelengths, respectively. In the interest of brevity, we will not include that dependence in our treatment here. The frosted glass reflects light impinging on it with a wavelength-dependent efficiency $R(\lambda)$.

The optical components of the fluorimeter are:

$L(\lambda) \equiv$ spectrum of lamp in spectrofluorimeters
$M_x(\lambda) \equiv$ efficiency spectrum of excitation monochromator
$B_x(\lambda - \lambda_x) \equiv$ bandpass function of excitation monochromator (the transmission efficiency at wavelength $\lambda$ for a monochromator set at $\lambda_x$)
$M_m(\lambda) \equiv$ efficiency spectrum of emission monochromator
$B_m(\lambda - \lambda_m) \equiv$ bandpass function of emission monochromator (the transmission efficiency at wavelength $\lambda$ for a monochromator set at $\lambda_m$)
$D(\lambda) \equiv$ efficiency spectrum of detector in spectrofluorimeter
$D_{ref}(\lambda) \equiv$ efficiency spectrum of reference detector in spectrofluorimeter

Wavelength validation requires various kinds of intensity measurements, which can be expressed as convolutions of products of optical functions with a monochromator bandpass function. We will use the notation $\otimes$ to indicate convolution that is performed physically by the instrument, at absorption and detection events, and give the following examples to define the notation:

$$(LM_xTE) \otimes B_x \equiv \int_{-\infty}^{\infty} L(\lambda)\, M_x(\lambda)\, T(\lambda)\, E(\lambda)\, B_x(\lambda - \lambda_x)\, d\lambda \qquad \text{(A1)}$$

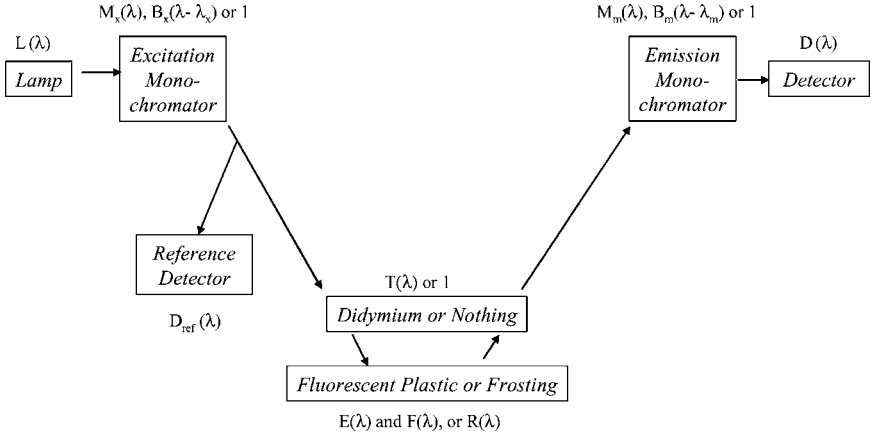


**Fig. A1.** Optic layout of fluorimeter for wavelength calibration. The wavelength-dependent functions are defined in the text. When a monochromator is set for reflectance, it does not substantially modify the light in a wavelength-dependent way, and its function is set to 1. Likewise, when light passes through nothing or a neutral-density filter rather than didymium in the validation microplate, the function is set to 1 to indicate the approximate wavelength independence of the effect.

$$(FTM_mD) \otimes B_m \equiv \int_{-\infty}^{\infty} F(\lambda)\, T(\lambda)\, M_m(\lambda)\, D(\lambda)\, B_m(\lambda - \lambda_m)\, d\lambda \quad \text{(A2)}$$

Equation (A1) describes the total light absorbed by fluorescent plastic through the didymium filter when the excitation monochromator is set at $\lambda_x$. Equation (A2) describes the signal from the detector due to emission from fluorescent plastic that is attenuated by the didymium filter when the emission monochromator is set at $\lambda_m$. More precisely, Equation (A2) represents the signal relative to the amount of light absorbed by the fluorescent plastic, as given in Equation (A1).

Suppressing wavelength-independent factors, we can write equations for the apparent didymium transmittance spectra obtained by the four techniques described in this paper. Excitation and emission scans are indicated by the subscripts "x" and "m," respectively, and the fluorescence and reflectance methods are indicated by the subscripts "fluor" and "refl." In all cases, the apparent transmission spectra are obtained by ratioing spectra taken with and without the didymium filter.

$$T^{app}_{fluor,x}(\lambda_x) = \frac{(LM_xTE) \otimes B_x/(LM_xD_{ref}) \otimes B_x}{(LM_xE) \otimes B_x/(LM_xD_{ref}) \otimes B_x} \quad \text{(A3)}$$

$$T^{app}_{refl,x}(\lambda_x) = \frac{(LM_xTRTD) \otimes B_x/(LM_xD_{ref}) \otimes B_x}{(LM_xRD) \otimes B_x/(LM_xD_{ref}) \otimes B_x} \quad \text{(A4)}$$

$$T^{app}_{fluor,m}(\lambda_m) = \frac{(FTM_mD) \otimes B_m/(LM_xD_{ref}) \otimes B_x}{(FM_mD) \otimes B_m/(LM_xD_{ref}) \otimes B_x} \quad \text{(A5)}$$

$$T^{app}_{refl,m}(\lambda_m) = \frac{(LTRTM_mD) \otimes B_m/(LD_{ref}) \otimes 1}{(LRM_mD) \otimes B_m/(LD_{ref}) \otimes 1} \quad \text{(A6)}$$

Note the normalization of the numerators and denominators in the equations A3–A6 by the signal from the reference detector, $(LM_xD_{ref}) \otimes B_x$ or $(LD_{ref}) \otimes 1$. These factors would appear to cancel; in reality they do not, to the extent that there are fluctuations in lamp output between the measurements with and without the didymium filter. Parenthetically, the reference detector factors only depend on excitation wavelength and not the emission wavelength, an important consideration for the computational approximations below. For the excitation methods, there are factors depending on the (constant) emission wavelength that are not shown in the equations because they cancel rigorously in the ratio if the detector response is stable. Finally, note that in the reflectance method the light of the scanned wavelength passes through the didymium filter twice. T is therefore squared in the convolution; this does not in itself distort the positions of extrema in the apparent transmittance spectra.

The apparent transmittance spectra in Equations (A3–A6) approach $T(\lambda)$ (or $T^2(\lambda)$) if the monochromator bandwidths are infinitely narrow. The effects of finite bandwidth could be studied computationally by numerical convolution if the various functions in the equations were known. It is possible–though laborious–to measure them all.

We have instead performed some approximate calculations to correlate observed shifts in the wavelengths of some of the extrema with those predicted by the convolution equations. For example, Equation (A7) represents a computational approximation of the experimental apparent transmittance spectrum described by Equation (A4). The "*" notation indicates a convolution carried out numerically rather than physically within the instrument. Three types of experimental data were input to Equation (A7). The first two were $T(\lambda)$, obtained with a narrowband spectrophotometer, and $B_m(\lambda - \lambda_m)$, obtained by scanning the emission monochromator past the excitation monochromator, for which $B_x(\lambda - \lambda_x)$ had been measured with a spectrum analyzer. The third was an emission scan of the fluorescent plastic, $(FM_mD) \otimes B_m$, which was used to approximate the unconvolved product $F(\lambda)M_m(\lambda)D(\lambda)$ in the numerator of Equation (A4).

$$T^{calc}_{fluor,m}(\lambda_m) = \{[T(FM_mD) \otimes B_m]^* B_m\}/[(FM_mD) \otimes B_m] \quad \text{(A7)}$$

When applied to the transmittance extrema reported by the fluorescence technique for the emission monochromator using the method of Table I, the calculation agrees with the experimental observation that the shifts are within tolerance (1.7 nm calculated, 0.8 nm observed at 551.1 nm; 0.4 nm calculated, 1.8 nm observed at 681.4 nm).

Analogous approximations were made to compute the shifts in peaks expected for the other three methods (corresponding to Equations A3, A5, and A6) to verify that the calculated shifts were all within tolerance.

## REFERENCES


1. A. K. Gaigalas *et al.* (2001). *J. Res. Natl. Inst. Stand. Technol.* **106**, 381–389.
2. L. Wang *et al.* (2002). *J. Res. Natl. Inst. Stand. Technol.* **107**, 339–353.
3. A. Schwartz *et al.* (2002). *J. Res. Natl. Inst. Stand. Technol.* **107**, 83–91.
4. J. Lakowicz (1999). *Principles of Fluorescence Spectroscopy*, 2nd Ed., Kluwer Academic/Plenum, New York.
5. J. Owicki (2000). *J. Biomol. Screening* **5**, 297–306.

6. J. Miller (1981). In J. Miller (Ed.), *Standards in Fluorescence Spectroscopy*, Chapman & Hall, London, pp. 8–14.
7. W. Venable, Jr. and K. Eckerle (1979). Standard Reference Materials: Didymium Glass Filters for Calibrating the Wavelength Scale of Spectrophotometers—SRM 2009, 2010, 2013, and 2014, National Bureau of Standards, Washington, DC.
8. W. J. Korzun and W. G. Miller (1986). *Clin. Chem.* **32**, 162–165.
9. ASTM International (1972). Standard Test Method for Spectral Bandwidth and Wavelength Accuracy of Fluorescence Spectrometers (Reapproved 1998), ASTM International, West Conshohocken, PA.
10. J. C. Travis *et al*. (2002). *Anal. Chem.* **74**, 3408–3415.
11. A. Paladini and L. Erijman (1988). *J. Biochem. Biophys. Methods* **17**, 61–66.