



Fluorescence from Highly Diluted and Sub-Picomolar Samples

Introduction

The Fluorolog[®] modular spectrofluorometer and the benchtop FluoroMax[®] spectrofluorometer are able to detect sub-picomolar ($<10^{-12}$ M) fluorescein according to the conventions established by the ASTM Committee E-13 on Molecular Spectroscopy (ANSI/ASTM E579-76). Other commercial manufacturers of spectrofluorometers specify their Minimum Detectable Concentration (MDC) somewhat differently. This *Technical Note* demonstrates how HORIBA Jobin Yvon achieves such a low MDC.

A further demonstration of the FluoroMax[®]'s sensitivity is shown by detection of up to 10^8 -fold dilutions of fluorescent microspheres, a concentration of 2.3×10^4 mL⁻¹.

Sub-picomolar fluorescein on a Fluorolog[®]

The measurements were taken on a Fluorolog[®] spectrofluorometer with double-grating monochromators, a 450-W xenon lamp, and an R928 photomultiplier tube operated at 950 V in photon-counting mode. The bandpass was set to 4.0 nm on both excitation and emission monochromators. Integration time was 10 s, with a single scan and no smoothing. Excitation of the sample was at 480 nm; emission was scanned in 1 nm steps and corrected for dark counts. Scans were taken under ambient room conditions.

Figure 1 shows spectra of 50 fM (0.05 pM or 5×10^{-14} M) fluorescein in 0.01 N NaOH(aq), a blank of 0.01 N

aqueous NaOH, and the subtracted spectrum of fluorescein from the solvent. The large peak near 515 nm clearly shows the presence of fluorescein at a 0.05 pM concentration.

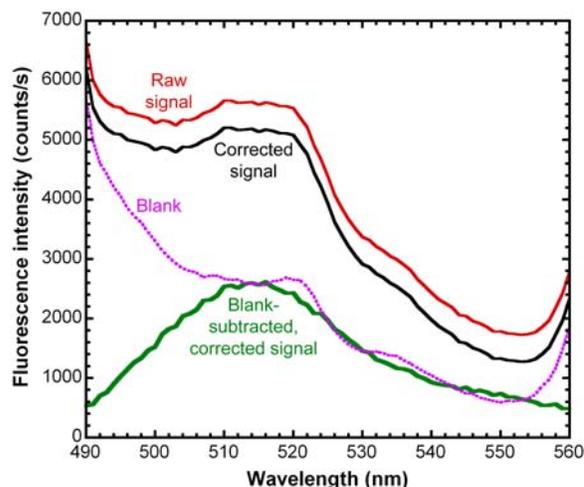


Fig. 1. Emission from 50 fM fluorescein in 0.01 N NaOH: Raw signal, corrected signal, blank (0.01 N NaOH), and blank-subtracted signal on a Fluorolog[®].

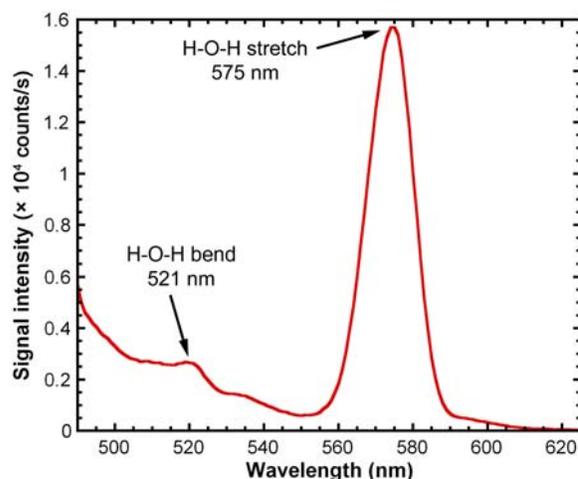


Fig. 2. Raman spectrum of water. $\lambda_{exc} = 480$ nm on a Fluorolog[®].

At the highest wavelengths, at the extreme right of Fig. 1, the edge of the water Raman O-H stretch is visible. A clearer graph of this region is shown in Fig. 2, with 480 nm excitation. The Raman peak is inherently broad, centered at 575 nm, along with a weaker H-O-H bending mode near 521 nm. The bending mode is also visible in the solvent-containing spectra in Fig. 1. This weak bending mode demonstrates the excellent sensitivity of the Fluorolog[®]. In Figs. 1 and 3, spectral subtraction completely eliminates the O-H and H-O-H modes.

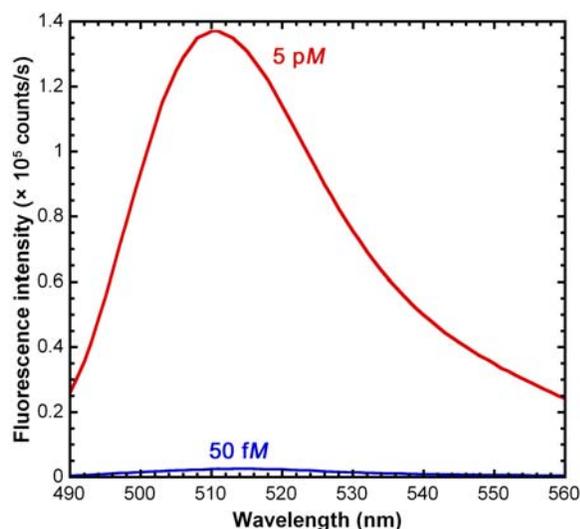


Fig. 3. Comparison of emission spectra from two concentrations of fluorescein on a Fluorolog[®]: (a) 5 pM, and (b) 50 fM. Both spectra are blank-subtracted and corrected for dark counts.

The Standard Test Method according to the ASTM was intended to establish the minimum detectable fluorescence of quinine sulfate. We applied this method to fluorescein because a number of customers specifically requested detection limits for that compound. The MDC is determined by the limiting signal-to-noise ratio according to

the equation

$$\text{MDC} = \frac{C}{S_{\text{sample}} - S_{\text{blank}}} \times \frac{\text{Noise}_{\text{p-p}}}{5}$$

where C is the concentration of the test solution, S is the signal from the sample or blank, and the $\text{Noise}_{\text{p-p}}$ is the total rms noise from all sources. For the subtracted spectrum in Fig. 1, we calculate an MDC of ~ 10 fM ($\sim 10^{-14}$ M) fluorescein.

Measurements at the femtomolar level are plagued with technical problems originating primarily in the sample. Because of contamination or improper cleaning, detection of fluorescein at “zero” concentration is not uncommon. This is one reason why ASTM resorted to the above method of extrapolation. By running scans at higher concentrations, freedom from background contamination can be better assured.

Our spectrum of fluorescein results from a subtraction of a blank, guaranteeing that our signal is greater than the background. As further insurance against impurities and contamination, the cuvettes were soaked in alcoholic KOH, rinsed, soaked in chromic acid, rinsed, and then soaked in nitric acid. Aliquots of all solutions were scanned before and after the sample to eliminate any effects from leaching of contaminants from the glassware.

Sub-picomolar fluorescein on a FluoroMax[®]

These measurements were taken using the standard 150-W xenon lamp and R928P photomultiplier tube. The bandpass was 5 nm on excitation and emission slits. Integration time was only 0.5 s, with a single scan and no smooth-

ing. Excitation of the sample occurred at 485 nm, and fluorescence was recorded in 1 nm steps under ambient room conditions. The data were blank-subtracted and corrected for dark counts.

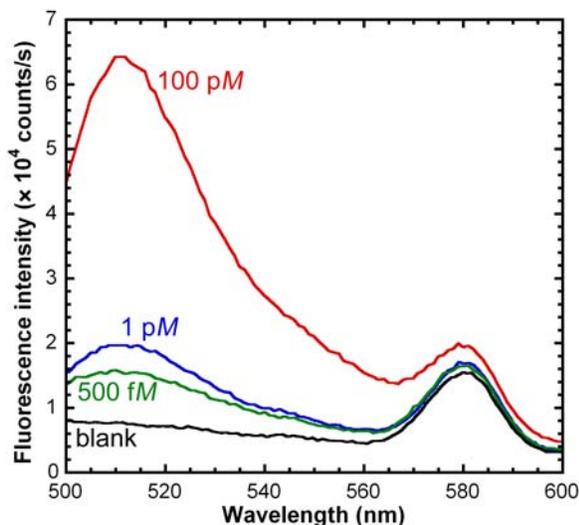


Fig. 4. Comparison of emission spectra from fluorescein on a FluoroMax[®]. Spectra are corrected for dark counts.

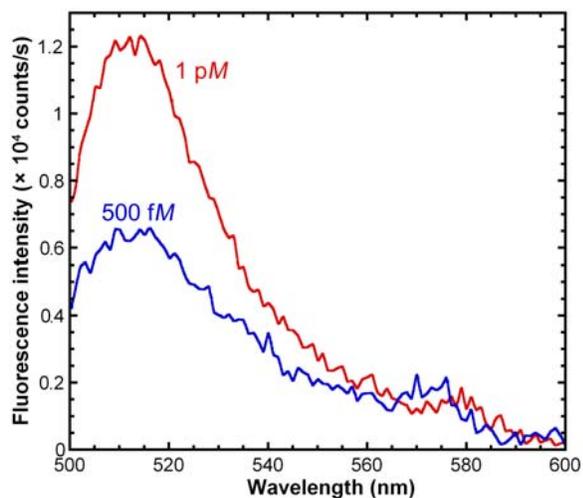


Fig. 5. Comparison of emission spectra from two concentrations of fluorescein on a FluoroMax[®]: (a) 1 pM, and (b) 500 fM. Both spectra are blank-subtracted and corrected for dark counts.

Fig. 4 shows the uncorrected spectra of several concentrations of

fluorescein in 0.01 N NaOH(aq). Note the Raman O–H stretch at 580 nm. Corrected spectra for 1 pM and 500 fM (0.5 pM) fluorescein are shown in Fig. 5. Using Fig. 5, a calculation of the MDC on the FluoroMax[®] performed via ASTM's method gives an MDC of ~15 fM.

High dilutions of microspheres

Three samples of internally-dyed fluorescent polystyrene microspheres (Duke Scientific, Palo Alto, CA) suspended in water were examined using a FluoroMax[®] spectrofluorometer:

- 0.20 ± 0.01 μm dia. blue polystyrene beads ($2.3 \times 10^{12} \text{ mL}^{-1}$); $\lambda_{em} = 447$ and 473 nm
- 0.20 ± 0.02 μm dia. green polystyrene beads ($2.3 \times 10^{12} \text{ mL}^{-1}$), $\lambda_{em} = 508$ nm
- 0.30 ± 0.02 μm dia. red polystyrene beads ($6.7 \times 10^{11} \text{ mL}^{-1}$); $\lambda_{em} = 612$ nm

As with the fluorescein experiment above, blank-subtraction from a sample of pure water, a step size = 1 nm, and 3 nm bandpass for excitation and emission monochromators were employed.

Data for the three suspensions of microspheres are shown in Figs. 6–8. Blue microspheres were detectable down to a dilution of 10^6 , or a concentration of 2.3×10^6 beads mL^{-1} . With blank-subtraction, they provided fluorescence peaks at 444 nm and 471 nm (Fig. 6). The red microspheres could be detected when diluted 10^7 times, or a concentration of 6.7×10^4 beads mL^{-1} (Fig. 7), giving a peak at 571 nm. Green microspheres were detectable at even lower concentrations, down to a dilution of 10^8 times, or a concentration of 2.3×10^4 beads mL^{-1} , with a fluorescence peak at 509 nm (Fig. 8). Reasons for the

difference in detectability among the dyes may be differing quantum yields of the dyes or inhomogeneities in dye-molecule dispersion through each microsphere.

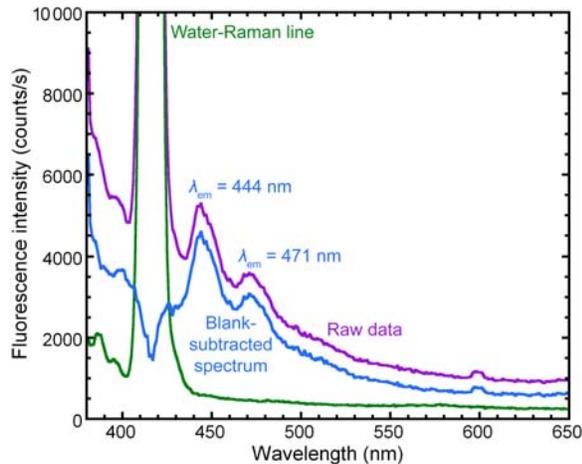


Fig. 6. Blank-subtracted emission spectrum (light blue) of blue microspheres diluted 10^6 -fold, a concentration of 2.3×10^6 beads mL^{-1} . $\lambda_{\text{exc}} = 365$ nm. Raw data are in purple; the blank (pure water) is green. Integration time = 3 s.

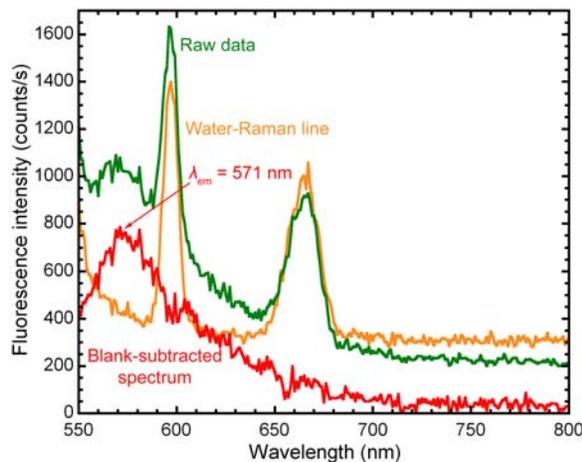


Fig. 7. Blank-subtracted emission spectrum (red) of red microspheres at a 10^7 -fold dilution, a concentration of 6.7×10^4 beads mL^{-1} . $\lambda_{\text{exc}} = 542$ nm. Raw data are in green; the blank (pure water) is orange. Integration time = 1 s.

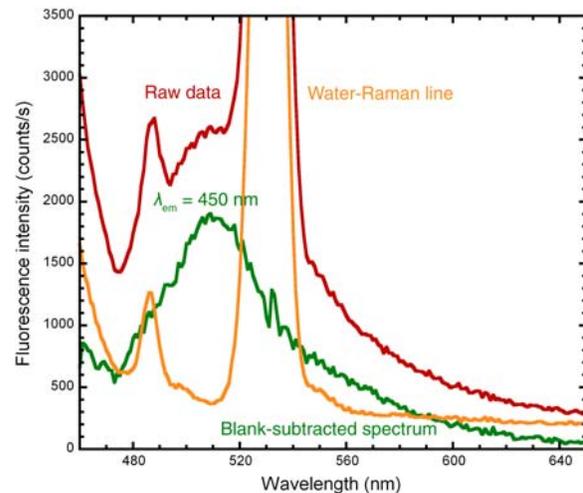


Fig. 8. Blank-subtracted emission spectrum (green) of green microspheres at a 10^8 -fold dilution, a concentration of 2.3×10^4 beads mL^{-1} . $\lambda_{\text{exc}} = 450$ nm. Raw data are in red; the blank (pure water) is orange. Integration time = 3 s.

Conclusions

We conclude from these results that the ASTM method reliably predicted the actual MDC (scanned) of the Fluorolog[®] system, and that the Fluorolog[®] is a spectrofluorometer capable of measuring some of the weakest possible fluorescence from nearly any sample. We also find that the FluoroMax[®] is a highly sensitive instrument, capable of detecting fluorescein nearly as well as the Fluorolog[®], and can measure fluorescent microsphere dilutions of up to 100 million, corresponding to concentrations as low as 2.3×10^4 particles mL^{-1} .

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