2   Tutorial on Fluorescence and Fluorescent Instrumentation

Brief Tutorial on Fluorescence

Fluorescence occurs when a molecule absorbs light photons from the u.v.-visible light spectrum, known as excitation, and then rapidly emits light photons as it returns to it's ground state. Fluorimetry characterizes the relationship between absorbed and emitted photons at specified wavelengths. It is a precise quantitative analytical technique that is inexpensive and easily mastered. This chapter outlines the basic concepts and theories on instrument setup and fluorescent dyes in solution.

All chemical compounds absorb energy which causes excitation of electrons bound in the molecule, such as increased vibrational energy or, under appropriate conditions, transitions between discrete electronic energy states. For a transition to occur, the absorbed energy must be equivalent to the difference between the initial electronic state and a high-energy state. This value is constant and characteristic of the molecular structure. This is termed the excitation wavelength. If conditions permit, an excited molecule will return to ground state by emission of energy through heat and/or emission of energy quanta such as photons. The emission energy or wavelength of these quanta are also equivalent to the difference between two discrete energy states and are characteristic of the molecular structure.

Fluorescence occurs when a molecule absorbs photons from the u.v.-visible light spectrum (200-900 nm), causing transition to a high-energy electronic state and then emits photons as it returns to its initial state, in less than 10^{-9} sec. Some energy, within the molecule, is lost through heat or vibration so that emitted energy is less than the exciting energy; i.e., the emission wavelength is always longer than the excitation wavelength. The difference between the excitation and emission wavelengths is called the Stokes shift.

Fluorescent compounds or fluorophors can be identified and quantified on the basis of their excitation and emission properties. Figure 2-1 shows the excitation and emission spectra of a yellow-green fluorescent dye (Molecular Probes, Inc.). The excitation spectra is determined by measuring the emission intensity at a fixed wavelength, in this case 506 nm, while varying the excitation wavelength. The emission spectra is determined by measuring the variation in emission intensity wavelength for a fixed excitation wavelength, in this case 495 nm.

As shown in Figure 2-1, this dye has a detectable emission intensity for a broad excitation range (440-492 nm). Maximum emission occurs at a unique excitation wavelength of 495 nm. Emitted light is detected for a broad wavelength range (492–600 nm), however, when excited at 495 nm, maximum emission occurs at 506 nm. The excitation and emission properties of a compound are fixed, for a given instrument and environmental condition, and can be used for identification and quantification.

The principal advantage of fluorescence over radioactivity and absorption spectroscopy is the ability to separate compounds on the basis of either their excitation or emission spectra, as opposed to a single spectra. This advantage is further enhanced by commercial fluorescent dyes that have narrow and

distinctly separated excitation and emission spectra. Table 2-1 lists excitation and emission wavelength pairs which cause maximum emission for 10 common fluorescent dyes.
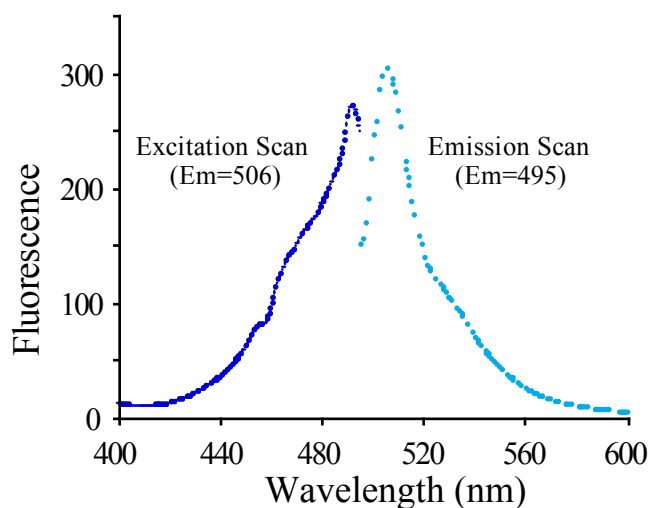

Figure 2-1

Excitation and emission spectra of a yellow-green fluorescent dye (Molecular Probes, Inc.). The Stokes shift of this dye is relatively small (11 nm). In theory, peak intensity of the excitation and emission scans should be equivalent.

Although, maximum emission occur only for specific excitation and emission wavelength pairs, the magnitude of fluorescent intensity is dependent on both intrinsic properties of the compound and on readily controlled experimental parameters, including intensity of the absorbed light and concentration of the fluorophor in solution.

The intensity of emitted light, F, is described by the relationship

$$F = \phi I_0 (1 - e^{-\varepsilon bc}) \tag{1}$$

where ϕ is the quantum efficiency, I_0 is the incident radiant power, ε is the molar absorptivity, b is the path length of the cell, and c is the molar concentration of the fluorescent dye (Guilbault, 1990) .

The quantum efficiency is the percentage of molecules in an excited electronic state that decay to ground state by fluorescent emission; i.e., rapid emission of a light photon in the range of 200-900 nm. This value is always less than or equal to unity and is characteristic of the molecular structure. The quantum efficiency for some fluorescent dyes is presented in Table 2-1. A high efficiency is desirable to produce a higher relative emission intensity. All non-fluorescent compounds have a quantum efficiency of zero.

The intensity of the excitation light, which impinges on the sample, depends of the source type, wavelength and other instrument factors. The light source, usually mercury or xenon, has a characteristic spectrum for emission intensity relative to wavelength. The properties and advantages of sources are discussed below in the Instrumentation Section.

For dilute concentrations, where $\varepsilon bc < 0.05$, equation (1) reduces to the form:

$$F = k\phi I_0 \varepsilon bc \tag{2}$$

Table 2-1
Excitation/Emission Wavelengths and Quantum Efficiencies of Fluorescent Dyes in 2-ethoxyethyl acetate

Color	Excitation Wavelength (l_{ex}, nm)	Emission Wavelength (l_{em}, nm)	Quantum Efficiency
Blue	360	423	0.7
Blue-Green	430	467	0.7
Green	445	492	0.7
Yellow-Green	485	506	0.9
Yellow	517	524	0.9
Orange	534	552	0.9
Orange-Red	553	569	0.9
Red	566	598	0.9
Crimson	610	635	0.4
Scarlet	646	680	0.1

If ϕ, I_0, ε and b remain constant, the relationship between the fluorescence intensity and dye concentration is linear (Guilbault, 1990).

At high dye concentrations or short path lengths, fluorescence intensity relative to dye concentration decreases as a result of "quenching". As the concentration of molecules in a solution increases, probability increases that excited molecules will interact with each other and lose energy through processes other than fluorescent emission. Any process that reduces the probability of fluorescent emission is known as quenching. Other parameters that can cause quenching include presence of impurities, increased temperature, or reduced viscosity of the solution media (Guilbault, 1990).

Introduction to Experimentation

A schematic representation of a fluorimeter is shown in Figure 2-2. The light source produces light photons over a broad energy spectrum, typically ranging from 200 to 900 nm. Photons impinge on the excitation monochromator, which selectively transmits light in a narrow range centered about the specified excitation wavelength. The transmitted light passes through adjustable slits that control magnitude and resolution by further limiting the range of transmitted light. The filtered light passes into the sample cell causing fluorescent emission by fluorphors within the sample. Emitted light enters the emission monochromator, which is positioned at a 90º angle from the excitation light path to eliminate background signal and minimize noise due to stray light. Again, emitted light is transmitted in a narrow range centered about the specified emission wavelength and exits through adjustable slits, finally entering the photomultiplier tube (PMT). The signal is amplified and creates a voltage that is proportional to the measured emitted intensity. Noise in the counting process arises primarily in the PMT. Therefore, spectral resolution and signal to noise is directly related to the selected slit widths.

Since source intensity may vary over time, most research grade fluorimeters are equipped with an additional "reference PMT" which measures a fraction of the source output just before it enters the excitation monochromator, and used to ratio the signal from the sample PMT.

Not all fluorimeters are configured as described above. Some instruments employ sets of fixed band-pass filters rather than variable monochromators. Each filter can transmit only a select range of wavelengths. Units are usually limited to 5 to 8 filters and are therefore less flexible. Fiber optics are also employed for "surface readers", to transmit light from the excitation monochromators to the sample surface and then transport emitted light to the emission monochromators. This setup has the advantage of speed, but has the disadvantages of increased signal to noise, due to the inline geometry, and smaller path length which increase the probability of quenching.
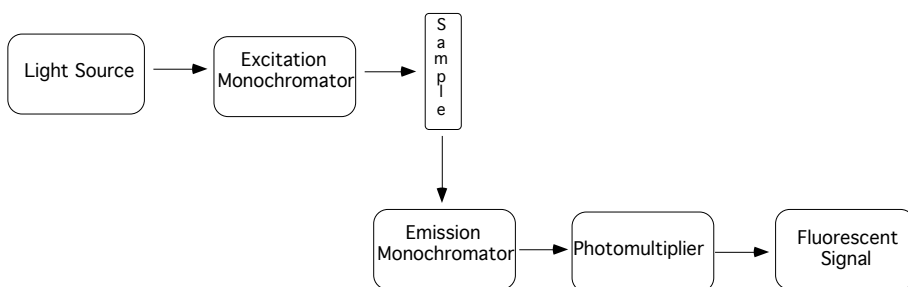

Figure 2-2 Top:

Schematic representation of a fluorescence spectrophotometer. The excitation and emission monochromators are variable band-pass filters.
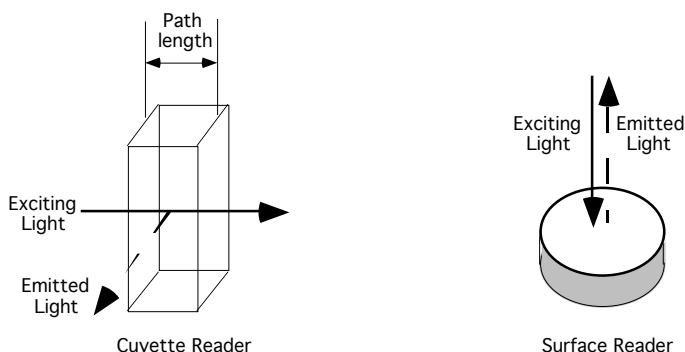
Figure 2-2 Bottom:

Two methods of measuring fluorescence. The cuvette reader excites the sample over the entire path length and reads the emitted light at right angles. The surface reader excites the sample from the top and reads the emitted light returning along the same path direction.

Fluorescent methods have three significant advantages over absorption spectroscopy. First, two wavelengths are used in fluorimetry, but only one in absorption spectroscopy. Emitted light from each fluorescent color can be easily separated because each color has unique and narrow excitation spectra. This selectivity can be further enhanced by narrowing the slit width of the emission monochromator so that only emitted light within a narrow spectral range is measured. Multiple fluorescent colors within a single sample can quantified by sequential measurement of emitted intensity using a set of excitation and emission wavelength pairs specific for each color. The second advantage of fluorescence over absorption spectroscopy is low signal to noise, since emitted light is read at right angles to the exciting light. For absorption spectrophotometry, the excitation source, sample and transmitted light are configured in line, so that the absorption signal is the small difference between the exciting light and the transmitted light, both of which are quite intense. The third advantage is that fluorescent methods have a greater range of linearity. Because of these differences, the sensitivity of fluorescence is approximately 1,000 times greater than absorption spectrophotometric methods (Guilbault, 1990).

A major disadvantage of fluorescence is the sensitivity of fluorescence intensity to fluctuations in pH and temperature. However, pH effects can be eliminated by using nonaqueous solvents, and normal room temperature fluctuations do not significantly affect the fluorescence intensities of commercial dye solutions.

It is important to consider interactions between different types of compounds in a given solution. For instance, one potential problem with neighboring fluorescent colors is that the emitted photons from one compound may cause excitation of a compound that fluoresces at a longer excitation wavelength, causing a reduction in the observed emitted intensity. This would be greatest when the emission wavelength overlaps the excitation wavelengths of two colors. An example is shown in Figure 2-3, where the emission light from the red dye is measured in a solution containing increasing concentrations of crimson dye. It was found that the decrease in red emission intensity was less than 3% for crimson concentrations that were almost three-fold greater than the red microspheres. Potential interactions should be considered when choosing combinations of colors for use in experiments requiring multiple fluorescent microspheres. Future quantification of absorption and emission effects caused by companion dyes will enable us to apply a mathematical correction matrix to reduce a source of error and allow greater flexibility in choice of color combinations.

Emission Scan
Excitation = 580
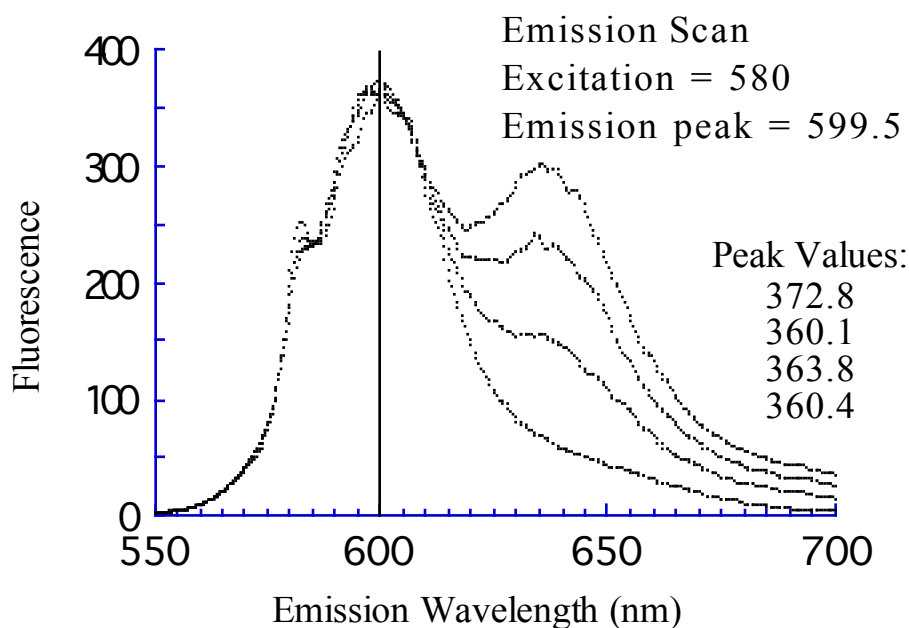Emission peak = 599.5

Peak Values:
372.8
360.1
363.8
360.4

Figure 2-3

Emission spectra of mixtures of red and crimson fluorescent dye (at an excitation wavelength of 580 nm). Per ml of solvent, 2,500 red microspheres were present together with 0, 2,000, 4,000 or 6,000 crimson microspheres. With increasing crimson concentration the reading for red decreases (<3%) due to absorption of the emitted red fluorescence by the crimson (see text). Note that the emission signal at 640 nm (peak of crimson emission) is not read when the sample is excited at 580 nm.

A significant problem can arise when absorption of excited or emitted light from a fluorescent dye occurs in the presence of non-fluorescent substances, significantly decreasing the fluorescence signal and causing erroneous results. Nonfluorescent dyes commonly used for vital stains in tissue samples absorb light from 400- to 680-nm wavelengths.   Absorption curves of five commonly used nonfluorescent dyes are shown in Figure 2-4. These dyes significantly interfere with excited and/or emitted light from the dyes in the fluorescent microspheres. Users should check vital dyes to see if they fluoresce and when they do not, assume they can be used in conjunction with fluorescent dyes without considering the absorption characteristics of vital dyes. A simple method is used to determine if a substance interferes with your fluorescence signal. Make a fluorescent sample containing the fluorescent dyes of interest and read them at the appropriate excitation and emission wavelengths. Add the vital dye or other substance to the solution and reread the fluorescence, taking into account any dilution that may have occurred. If the fluorescent signals are the same, your vital dye probably does

not affect your fluorescence signal. **Note:** It is best to test this with fluorescence signals and vital dye concentrations in the ranges commonly encountered.

Many fluorescent compounds interact with the excitation light to decompose or otherwise change their structure. To minimize this possibility, an excitation energy of the longest wavelength that results in a detectable fluorescent emission may be more suitable than employing peak excitation energy (Guilbault, 1990).
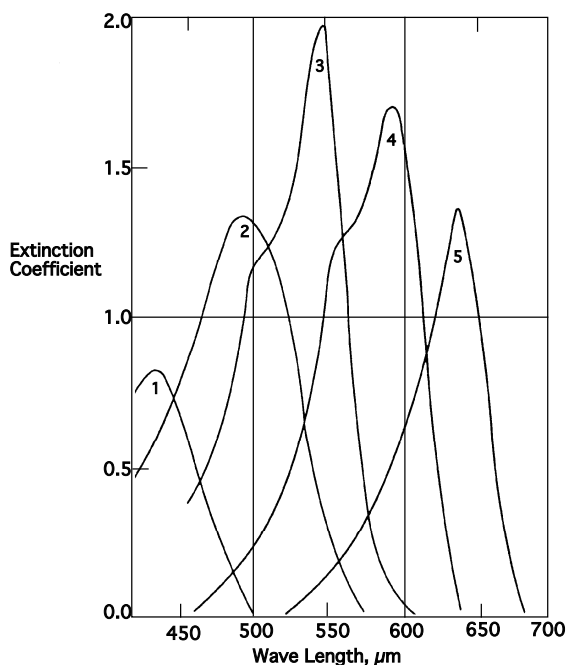

Figure 2-4:

Absorption spectra of five different dyes: 1) Tartrazine (yellow); 2) orange G; 3) fuchsin (red); 4) crystal violet; and 5) Neptune blue BG (patent blue V).

Fluorescence Instrumentation

Perkin-Elmer LS-50B

To obtain accurate data, it is necessary to understand several key aspects of fluorescence spectrophotometry and how these pertain to use of the LS-50B.

All fluorescence instruments contain three basic elements: a source of light, a sample holder, and a detector (Figure 2-2). To be of analytical utility, a system must be equipped with adjustable monochromators that can accurately select excitation and emission wavelengths. It is also essential to monitor and correct any fluctuations in source intensity. The Perkin-Elmer Models LS-50 and LS-50B Luminescence Spectrometer measure the fluorescence intensity of samples in either a continuous scan over a range of wavelengths or at select excitation and emission wavelength pairs. Blood flow analysis data require accurate detection of fluorescence intensities for a sample containing multiple fluorophores. This can be accomplished accurately and rapidly by using a wavelength program that performs serial intensity measurements on a single sample at predefined excitation and emission wavelength pairs.

The Xenon Source

The LS-50B employs a pulsed xenon source that produces a high output using a low voltage, 9.9 watts, resulting in longer lamp life with minimal ozone and heat production. Equally important, the pulsed source reduces potential photobleaching of the sample, during analysis, by several orders of magnitude over continuous sources. The xenon flash lamp produces a 10-µsec pulse of radiation in 16 msec. In fluorescence mode, the photomultiplier tube detector is gated for an 80-msec period in synchronization with the lifetime of the lamp pulse.

Photomultiplier Tube

A photomultiplier dark current is acquired prior to the onset of each lamp pulse and is subtracted from that pulse for correction of phototube dark current. The instrument measures and corrects every flash of the lamp to improve sensitivity at low levels of fluorescence, making it possible to measure samples in room light, thus freeing the user from working through septa in light-tight compartments.

Determining Fluorescence Ratios (LS-50B)

Intensity of emitted light depends on a number of factors, including intensity of incident exciting light (the more powerful the exciting light, the stronger the emitted fluorescence intensity). All light sources currently used in fluorescence instrumentation lack stability over long periods. This causes the output of a xenon lamp to fluctuate as a function of time, which affects measured fluorescence intensity of a given sample, all other conditions being equal. To perform accurate quantitative analyses, these fluctuations must be monitored and corrected to the measured fluorescence intensity. The LS-50B automatically makes this correction by determining the ratios of real-time lamp intensity to sample intensity. The lamp intensity is monitored continuously with a beam splitter to divert a portion of the exciting light to a reference photomultiplier tube (just after it exits the excitation monochromator and prior to entering the sample compartment). The output signal ratio is then scaled and multiplied by a rhodamine correction spectrum stored within the instrument. Comparison with the rhodamine spectrum corrects the wavelength-dependent response of the photomultiplier tubes and the transmission characteristics of the beam splitter. Determining source ratios permits sample fluorescence to be measured free of lamp-related artifacts.

Signal Processing (LS-50B)

When operated in wavelength programming mode, the instrument automatically sets the excitation and emission wavelengths for each dye and dwell time for the specified integration time. The instrument then averages the appropriate number of lamp pulse cycles for the specified integration time. Longer integration time reduces the signal-to-noise ratio for the sample fluorescence intensity. When calculating the optimal sample integration, consider that 1) there are 60 lamp pulses per sec, and 2) the noise in a sample measurement is reduced by the square root of the number of lamp pulses used. Because of the square root relationship between noise reduction and pulse number, there is a point of diminishing return for long integration times. An integration time exceeding 2 sec is necessary only when fluorescence

intensities being measured are very small. **Note:** Long integration times (>5 sec) for multi-component samples can result in prohibitively long total analysis time.

Slit Settings (LS-50B)

An important feature of the LS-50B is the availability of continuous variable slit adjustment (0.1-nm increments) on both the excitation and emission monochromators. This flexibility allows the user to fine-tune the instrument for both selectivity and sensitivity in dye discrimination and measurement. The slits can best be described as volume controls for the fluorescence intensity. For optimal instrumental performance, the excitation slit width automatically controls the sample photomultiplier tube voltage. This control provides an optimum signal-to-noise ratio as a function of sample intensity.

In general, a wider slit setting causes higher fluorescence signal measurements. However, because of the fluorescence ratioing system used in the LS-50B (see above), widening the excitation slit width will not increase the reported fluorescent signal ratio, but does increase sample fluorescence signal, resulting in an improved signal-to-noise ratio.

The spectral overlap of dyes used for blood flow analysis can be significant when all available dyes are employed in one experiment; wide slit settings should be avoided in these experiments as they prevent accurate separation of colors. For assays that require five or fewer dyes, the judicious selection of dyes that have minimal or no spectral overlap can be selected (see recommended color combinations in Section 4). As a result, slit settings up to 10-15 nm can be employed for maximum sensitivity. For assays using more than five dyes, care must be taken to insure that a wide slit setting will not result in significant cross-talk between adjacent dyes. With these types of assays there will always be a trade-off between sensitivity and selectivity of dye discrimination.

Solvent and tissue background fluorescent signals can become significant as slit widths are increased. High background signals in the Blue region are frequently seen when using the extraction method with lung tissues. We have no experience with other tissue, but recommend testing all tissue for background fluorescence (see Section 3).

Preparation of Sample

Fluorescence is a very sensitive technique. This is the one criterion that makes it a viable replacement to many radioisotope-labeling procedures. However, it is extremely susceptible to interference by contamination of trace levels of organic chemicals. Potential sources of contamination are ubiquitous since any aromatic organic compound can be a possible source of fluorescence signal. For example, the researcher is a possible source of this type of contamination since oils secreted by the skin are fluorescent. Good laboratory procedure is essential in preventing solvents and chemicals from becoming contaminated with high background fluorescence that could hinder low-level measurements. Solvents should be of the highest level purity obtainable commercially. In addition, care must be taken to eliminate all forms of solid interference (suspended particulates such as dust and fibers). These will float in and out of the sampling area of the cuvette via convection currents, and cause false signals due to light scattering while they remain in the instrument's beam.

Sample Temperature

All fluorophores are subject to intensity variations as a function of temperature. In general fluorescence intensity decreases with increasing temperature due to increased molecular collisions that occur more frequently at higher temperatures. These collisions bleed energy from the excited state that produces fluorescence. The degree of response of an individual compound to temperature variations is unique to each compound. While many commercially available dyes are selected for their temperature stability, care should be taken to eliminate exposure of samples to drastic temperature changes during measurement. If possible, the temperature of instrument's sample compartment should be regulated via a circulating water bath. At lower assay temperatures, higher fluorescence signal will be generated. We have found a 50% decrease in the fluorescence signal of yellow-green microspheres when exposed to 160°C for 15 minutes.

Sample pH

Fluorescence variations due to pH changes are caused by the different ionizable chemical species formed by these changes. The results from these pH variations can be quite drastic since new ionization forms of the compound are produced. For blood flow analysis, the amount of pH variation is dependent on the tissue processing technique. A buffer step has been added to the final rinse for negative pressure filtration technique to minimize pH variations (see Section 5).

Sample Exposure to Light

The fluorescent dyes within microspheres are very stable, losing less then 1% of their fluorescent signal in six months. Once the microspheres are dissolved in solvent, stability decreases. Exposure to sunlight has been shown to significantly degrade dye in less than one week. Therefore we recommend that the samples be stored in the dark both prior to and after extraction.

Fluorimeter-to-Fluorimeter Variability

There is substantial machine-to-machine variability between fluorimeters, even from the same manufacturer. When the same sample is read on two different fluorimeters the fluorescence signals will not necessarily be equivalent. It may be possible to correct for this variability using the internal controls run prior to and during a fluorimeter session (see Section 3). All samples for a given experiment should be read on the same fluorimeter, using identical experimental conditions.

Other Instruments

Hitachi F-2000

The Hitachi F-2000 is another fluorescent spectrophotometer that has been used successfully to separate six different colors of microspheres (Chein et al., 1995). Although its spillover matrix is somewhat greater than the Perkin Elmer machine (Section 4), all colors can be easily separated if the spillover is corrected using a matrix inversion method (solving for a system of linear equations).

ISA-Spex FluoroMax-2

The FluoroMax-2 is a commercially available spectrofluorometer that offers high sensitivity, fast-scanning capability, and selectivity for research and routine fluorescence measurements. The basic components include the source, slits, excitation monochromator, sampling compartment, reference detector, emission monochromator and detector.

The Source

The xenon source that supplies UV performance, is mounted vertically, thereby eliminating sagging of the arc and increasing stability and useful life. The lamp lifetime extends to 1200 hours in the FluoroMax-2 system.

The xenon source is focused onto the entrance slit of the excitation monochromator with an elliptical mirror. Light collection is maximized throughout the system to provide high sensitivity. Besides insuring efficient collection, the reflective surface keeps all wavelengths focused on the slit, unlike lenses that have chromatic aberrations that make them most efficient only at one wavelength.

The Slits

The slits themselves are bilaterally, continuously adjustable from the computer in units of bandpass (wavelength) or millimeters. This preserves maximum resolution and instant reproducibility. The bandpass can range from 0-30 nm depending on the signal strength. For weakly fluorescing samples it is advantageous to increase the bandpass and collect more light. For highly fluorescent samples the narrow bandpass is recommended to avoid exposing the detector to too high signal levels.

The Monochromators

The excitation monochromator is an aspheric design that insures that the image of the light diffracted by the grating fits through the slit. This is an important feature when wanting to measure fluorescence from extremely small sample volumes. The FluoroMax-2 measures high sensitivity regardless of sample volume. The gratings themselves are plane, ruled gratings that avoid the two major disadvantages of the more common concave holographic gratings: poor polarization performance and inadequate imaging during scans that throws away light. The unique wavelength drive scans the grating at speeds as high as 200 nm/s. The grating grooves are blazed to provide maximum light in the UV and visible region.

The Reference Detector

Before the excitation light reaches the sample, a photodiode reference detector monitors the intensity as a function of time and wavelength to correct for any change in output due to age or wavelength.. The photodiode detector has a wider wavelength response range than the older, traditional rhodamine-B quantum counter, and requires no maintenance.

The Sample Chamber

A spacious sample chamber is provided to allow the use of a long list of accessories for special samples, and encourage the user to utilize a variety of sample schemes.

Detector

Emission detector electronics employ photon- counting for low-light-level detection. Photon counting concentrates on signals that originate from fluorescence photons, ignoring the smaller pulses originating in the pmt electronics. The more common method of analog detection adds noise and signal together hiding low signals in the noise.

The emission detector housing also contains and integral high voltage supply that is factory set to provide the maximum count rate, while eliminating most of the dark noise. An optional detector extends the useful range of the system further into the IR.

The Emission Monochromator

All features of the excitation monochromator are also incorporated into the emission monochromator. Gratings are blazed to provide maximum efficiency in the visible.

Computer Control

The FluoroMax-2 is controlled by a PC via a serial link. On start up, the system automatically calibrates and presents itself for either new experiments or stored routines.

The Fluorescence Measurements

The type of scans automatically defined in the sophisticated DataMax software allow the following types of fluorescence measurements:

Excitation	Constant Wavelength Analysis
Emission	Multiwavelength Scanning
Synchronous	Single Point Analysis
Time Base Scans	Recall Experiment

References

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