Instrumentation for Fluorescence Spectroscopy

The success of fluorescence experiments requires attention to experimental details and an understanding of the instrumentation. There are also many potential artifacts that can distort the data. Light can be detected with high sensitivity. As a result, the gain or amplification of instruments can usually be increased to obtain observable signals, even if the sample is nearly nonfluorescent. These signals seen at high amplification may not originate with the fluorophore of interest. Instead, the interference can be due to background fluorescence from the solvents, light leaks in the instrumentation, emission from the optical components, stray light passing through the optics, light scattered by turbid solutions, and Rayleigh and/or Raman scatter, to name a few interference sources.

An additional complication is that there is no ideal spectrofluorometer. The available instruments do not yield true excitation or emission spectra. This is because of the nonuniform spectral output of the light sources and the wavelength-dependent efficiency of the monochromators and detector tubes. The polarization or anisotropy of the emitted light can also affect the measured fluorescence intensities because the efficiency of gratings depends on polarization. It is important to understand and control these numerous factors. In this chapter we will discuss the properties of the individual components in a spectrofluorometer, and how these properties affect the observed spectral data. These instrumental factors can affect the excitation and emission spectra, as well as the measurement of fluorescence lifetimes and anisotropies. Additionally, the optical properties of the samples—such as optical density and turbidity—can also affect the spectral data. Specific examples are given to clarify these effects and the means to avoid them.

2.1. SPECTROFLUOROMETERS

2.1.1. Spectrofluorometers for Spectroscopy Research

With most spectrofluorometers it is possible to record both excitation and emission spectra. An emission spectrum is the wavelength distribution of an emission measured at a single constant excitation wavelength. Conversely, an excitation spectrum is the dependence of emission intensity, measured at a single emission wavelength, upon scanning the excitation wavelength. Such spectra can be presented on either a wavelength scale or a wavenumber scale. Light of a given energy can be described in terms of its wavelength λ, frequency ν, or wavenumber. The usual units for wavelength are nanometers, and wavenumbers are given in units of cm^{-1}. Wavelengths and wavenumbers are easily interconverted by taking the reciprocal of each value. For example, 400 nm corresponds to $(400 \times 10^{-7} \text{ cm})^{-1} = 25{,}000 \text{ cm}^{-1}$. The presentation of fluorescence spectra on the wavelength or wavenumber scale has been a subject of debate. Admittedly, the wavenumber scale is linear in energy. However, most commercially available instrumentation yields spectra on the wavelength scale, and such spectra are more familiar and thus easier to interpret visually. Since corrected spectra are not needed on a routine basis, and since accurately corrected spectra are difficult to obtain, we prefer to use the directly recorded technical or uncorrected spectra on the wavelength scale.

For an ideal instrument, the directly recorded emission spectra would represent the photon emission rate or power emitted at each wavelength, over a wavelength interval determined by the slit widths and dispersion of the emission

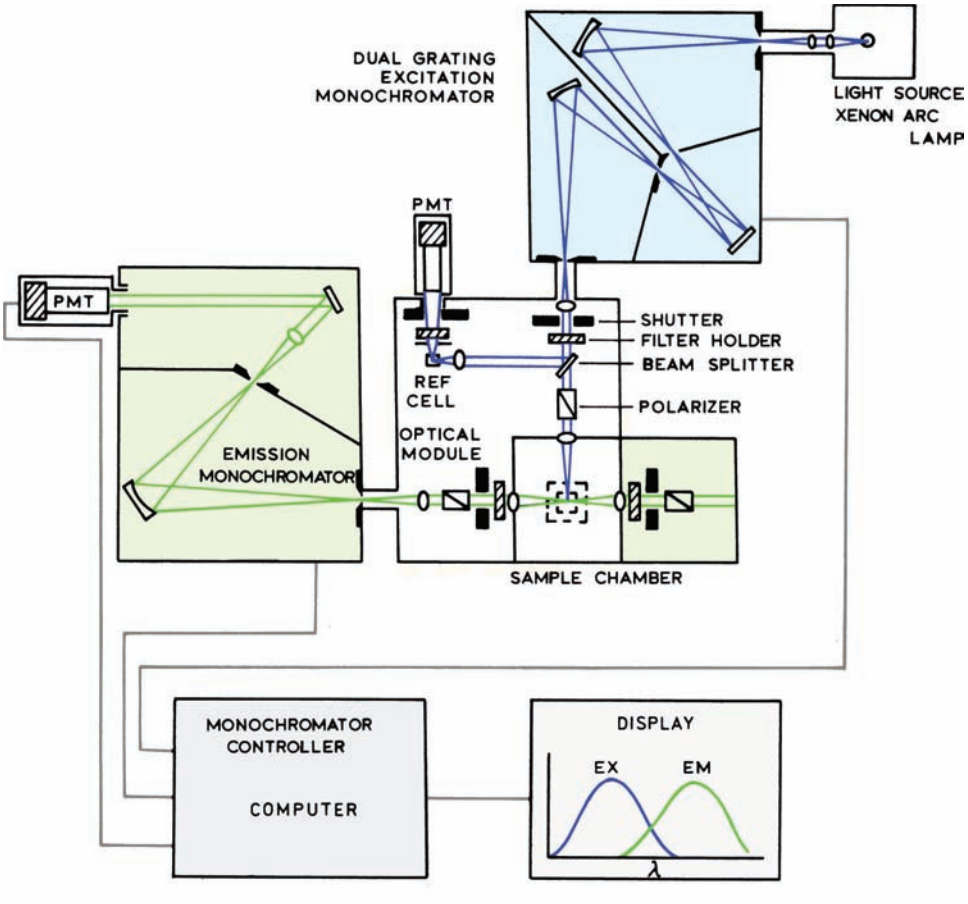

Figure 2.1. Schematic diagram of a spectrofluorometer [1].

monochromator. Similarly, the excitation spectrum would represent the relative emission of the fluorophore at each excitation wavelength. For most fluorophores the quantum yields and emission spectra are independent of excitation wavelength. As a result, the excitation spectrum of a fluorophore can be superimposable on its absorption spectrum. However, such identical absorption and excitation spectra are rarely observed because the excitation intensity is different at each wavelength. Even under ideal circumstances such correspondence of the excitation and absorption spectra requires the presence of only a single type of fluorophore, and the absence of other complicating factors, such as a nonlinear response resulting from a high optical density of the sample or the presence of other chromophores in the sample. Emission spectra recorded on different instruments can be different because of the wavelength-dependent sensitivities of the instruments.

Figure 2.1 shows a schematic diagram of a general-purpose spectrofluorometer: this instrument has a xenon lamp as a source of exciting light. Such lamps are generally useful because of their high intensity at all wavelengths ranging upward from 250 nm. The instrument shown is equipped with monochromators to select both the excitation and emission wavelengths. The excitation monochromator in this schematic contains two gratings, which decreases stray light, that is, light with wavelengths different from the chosen one. In addition, these monochromators use concave gratings, produced by holographic means to further decrease stray light. In subsequent sections of this chapter we will discuss light sources, detectors and the importance of spectral purity to minimize interference due to stray light. Both monochromators are motorized to allow automatic scanning of wavelength. The fluorescence is detected with photomultiplier tubes and quantified with the appropriate electronic devices. The output is usually presented in graphical form and stored digitally.

The instrument schematic also shows the components of the optical module that surrounds the sample holder. Ver-