Fluorescence: Basic Instrumentation

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Principles

A typical modern spectrofluorimeter, PC1 from ISS, is shown in Figure 1. We have adopted the nomenclature of "fluorimeter" for steady-state instrumentation and "fluorometer" for lifetime instrumentation – a custom, which

originated with Gaviola[1] who termed his lifetime instrument a "fluorometer". Clearly the starting point for any

fluorescence observation is the light source. Fortunately, we no longer need to rely on sunlight as the principle

excitation source for fluorescence. A comprehensive discussion of the development of light sources is beyond the

scope of this chapter, but a good index of a wide range of light sources (and many other relevant topics such as

filters, monochromators and photodetectors) can be found in the book by Moore et al. [2].
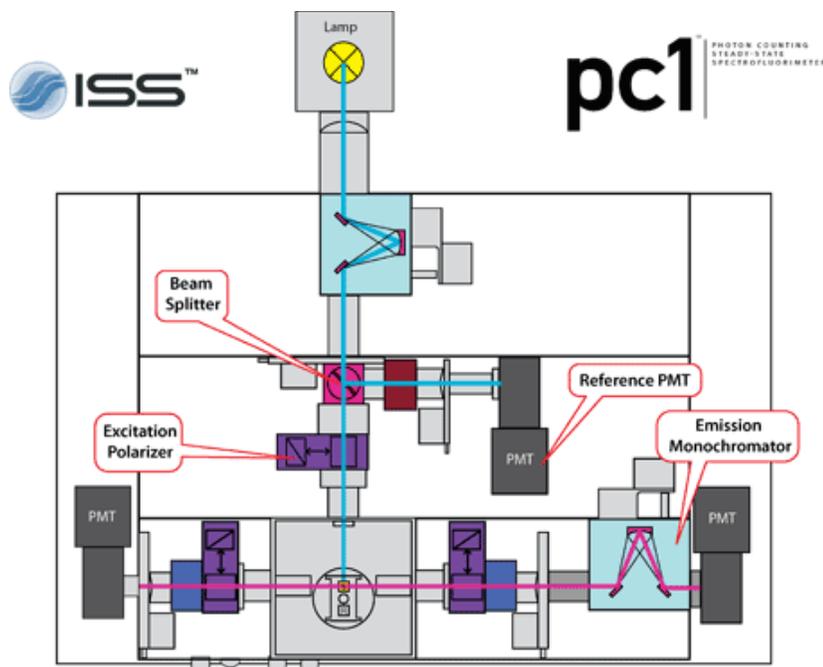
Figure 1 Schematic drawing of PC1, the photon-counting spectrofluorimeter from ISS.

We shall restrict ourselves to consideration of the light sources most commonly employed in commercial fluorescence

instrumentation, namely the xenon arc lamp, the xenon-mercury arc lamp, lasers, light-emitting diodes (LEDs) and

laser diodes. We should note that the most common light sources used in absorption spectrophotometry, the

deuterium and tungsten lamp, are rarely used in fluorescence since they are relatively weak photon sources.

Light Sources

The most relevant aspect of light sources for our present discussion is the useful wavelength range. From this point of view the xenon arc lamp is by far the common light source in commercial instruments since it produces usable light from the ultraviolet to the infrared. This range is quite adequate for most fluorescence studies on biological samples since such studies are usually limited by the absorption characteristics of water at either end of this spectral range, and by photodamage in the deep ultraviolet.

An example of the light distribution from a Cermax xenon arc lamp, from 200 nm to 1100 nm, is shown in Figure 2. Xenon arc lamps provide significant illumination out to around 1300 nm. Clearly the intensity of this light source depends dramatically on the wavelength – a fact, which has a significant impact on the excitation spectrum. The line radiation from 800—1000 nm is the result of bound-bound transitions in the xenon atoms and ions. The continuum is made up primarily of recombination radiation from gas ions capturing electrons into bound states (free- bound transitions) and from Bremsstrahlung radiation (free-free transitions).

The xenon-mercury arc has the characteristics of the xenon arc source but is dominated by very prominent lines due to the mercury transitions. The more prominent mercury lines are near 254 nm, 297 nm, 302 nm, 313 nm, 365 nm, 405 nm, 436 nm, 546 nm and 578 nm [3]. Much of the early work on proteins carried out in the former Soviet Union utilized 296.7 nm as the excitation wavelength since mercury lamps were a common light source at that time; the choice of this wavelength was appropriate since tyrosine absorption is negligible above 295 nm and hence tryptophan residues could be preferentially excited.

The use of lasers in fluorescence has primarily been restricted to time-resolved instrumentation, due to the intensity and spatial characteristics of laser sources (with the notable exception of fluorescence activated cell sorters – FACS). Of course laser sources have distinct emission lines characteristic of the atomic processes involved. The most commonly used lasers in modern biological fluorescence spectroscopy are the argon ion laser, the helium cadmium laser, the neodymium YAG (Nd:YAG) laser and, more recently, the titanium sapphire laser. The most commonly used argon ion laser lines are near 488 nm and 514 nm. But large frame, high power argon ion lasers (for example the Spectra-Physics Model 2045 laser in the authors' lab) produce lines in the deep UV at 275 nm and between 300-305 nm as well as the mid-UV near 334 nm, 351 nm and 364 nm and in the visible near 457 nm, 476 nm, 488 nm, 497 nm, 501 nm, 514 nm and 528 nm.

Helium-cadmium lasers produce lines near 325 nm and 442 nm. Nd:YAG lasers emit at 1064 nm and are typically doubled or quadrupled to 532 nm and 266 nm, respectively (frequency-doubled Nd:YAG lasers are now readily available as green emitting laser pointers!).

Often, the doubled Nd:YAG output at 532 nm is used to pump a dye laser (typically using a rhodamine-based dye) whose output in a range around 600 nm can then be doubled to produce UV light over a range around 300 nm. The titanium-sapphire laser, which emits over a range of around 700 – 1000 nm, is presently the light source of choice for

multi-photon excitation. A relevant characteristic of laser sources is the time-profile of their output – for example typical argon ion or helium cadmium lasers are operated as continuous sources (termed CW; intensity essentially time-invariant) whereas Nd:YAG and titanium-sapphire lasers are usually operated as pulsed sources.
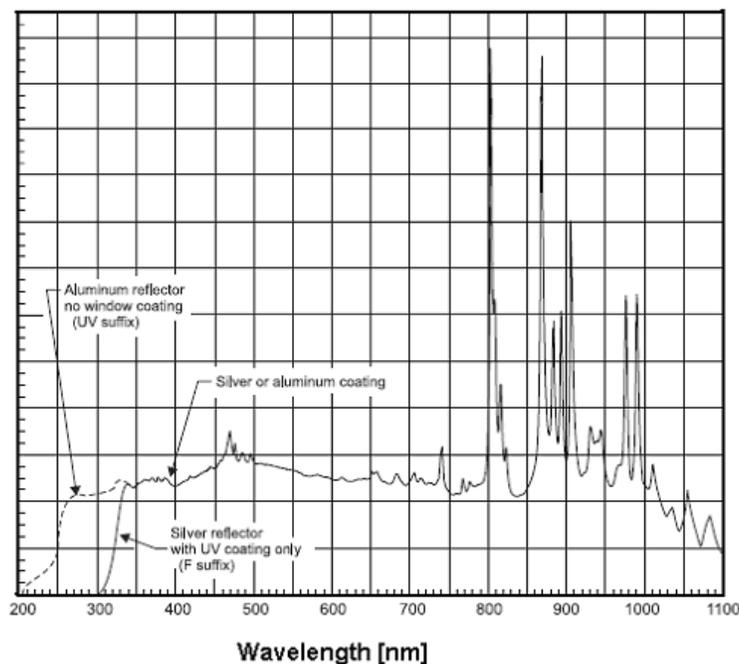
Figure 2. Spectral output of a Cermax Xenon Lamp used in ISS spectrofluorometers.

LEDs are becoming more popular since the list of available wavelengths is growing and, more importantly, extending deeper into the UV. LED sources down to 265 nm are available commercially. LEDs are much less intense than lasers (and not collimated) but have the advantages that they are relatively inexpensive, low power, solid-state devices that generate very little heat and which provide usable intensities over a narrow (but not discrete) spectral range. Their energy output can also be directly modulated which suggests time-resolved applications (see ISS Application Note: Frequency-Domain Spectroscopy Using 280-nm and 2300-nm LEDs). Another solid-state device, the laser diode, provides monochromatic radiation; near-UV laser diodes have become available which are much more intense than LED's but which for now only extend down to about 370 nm. If the excitation light source is not at a discrete wavelength, i.e., a laser or laser diode, then a device capable of wavelength selectivity is required – typically either an optical filter or a monochromator. For excitation purposes, the most useful optical filter is usually an interference-type filter, which typically can isolate wavelengths with a few nanometers resolution. The figure of merit for these types of filters is their FWHM – or full-width at half- maximum. For example, an interference filter centered at 400 nm with a FWHM of 5 nm transmits 50% of the intensity at 395 nm and 405 nm that it transmits at 400 nm. Such filters are not always very efficient, especially as the wavelength decreases. For example, the peak light transmission efficiency of a typical UV interference filter may be ~20% compared to ~70 to 80% for an interference filter centered in the visible wavelength region.

Monochromators

Monochromators are the most common and versatile devices used to isolate specific wavelengths of light from broad-band sources such as xenon-arc lamps. Monochromators operate by dispersing the incident light – most people are very aware of the light dispersing qualities of a prism (most people have observed the excellent light dispersion qualities of raindrops which results in rainbows, but far fewer notice that the weaker of the double rainbows, which can occur under particularly sunny conditions, has the order of the colors reversed due to the additional raindrop-interior reflection). The spectral region selected by a monochromator depends on the design of the monochromator and, ultimately, on the physical size of the monochromator slits; the key consideration here is the dispersion of the monochromator, which allows one to convert the physical width of a slit (e.g., in millimeters) to the FWHM of the spectral region passed. For example, the monochromators in the instrument shown in **Figure 1** utilize fixed-slits (as opposed to infinitely variable slits) and have dispersions of 8 nm per millimeter.

Slits ranging from 0.025 mm to 4 mm are commonly available, which thus supply spectral resolutions ranging from 0.2 nm to 32 nm. Different monochromators of course have different dispersion factors but the common feature is that the smaller the slit, the higher will be the spectral resolution. This resolution comes with a cost, namely, a reduction in the light intensity – a two-fold reduction in each slit width (entrance or exit) results in an approximate 4-fold decrease in light intensity. In commercial spectrofluorimeters, prism-based monochromators are not commonly used, one reason being that a linear scan of the prism assembly will not result in a linear dispersion of wavelengths.

For many years, commercially available monochromators (such as those from Bausch and Lomb or Jarrell Ash) used planar ruled diffraction gratings as the dispersive element. These devices worked well but exhibited parasitic light levels, due to imperfections in the ruling process, which could seriously hamper measurements of turbid samples. This source of stray light was dramatically reduced when concave holographic gratings became available. These types of diffraction gratings are made using interference patterns generated onto photoresist substrates using laser sources. Regardless of the type of grating utilized, the efficiency with which a monochromator transmits light will show both wavelength and polarization dependence.

Polarizers

After the excitation wavelength is isolated it is sometimes passed through a polarizing device to select one plane of polarization (see also ISS Technical Note: Fluorescence Polarization). A variety of devices, both naturally occurring and manmade, have been used to polarize light. The Vikings, in fact, used a "sunstone" to observe the location of the sun on foggy or overcast days (magnetic compasses were unreliable at higher latitudes), which lasted a long time at the high northern latitudes! This stone is now thought to have been composed of the mineral cordierite, a natural polarizing material.

By taking advantage of the fact (even if they didn't know the reasons behind it) that scattered sunlight was highly polarized while light coming along the direction of the sun was not, Vikings could observe the distribution of the sky's brightness through the sunstone and localize the sun's position and, if the time of day were known, the compass directions.

Nowadays, the most common polarizers are either dichroic devices, which operate by effectively absorbing one plane of polarization (e.g., Polaroid type-H sheets based on stretched polyvinyl alcohol impregnated with iodine, which were invented in 1926 by E.H. Land while he was a freshman at Harvard) or double refracting calcite (CaCO3) crystal polarizers – discovered independently by Erasmus Bartholin and Christiaan Huygens in the 17th century - which differentially disperse the two planes of polarization (examples of this class of polarizers are Nicol polarizers, Wollaston prisms and Glan-type polarizers such as the Glan-Foucault, Glan-Thompson and Glan-Taylor polarizers).

Typical film polarizers are inexpensive but do not transmit efficiently in the ultraviolet. (Quiz: the interested reader should work out which direction of polarized light polarizing sunglasses pass; hint – glare or light reflected from surfaces is largely polarized in a horizontal direction) Calcite prism polarizers, on the other hand, transmit well into the ultraviolet (<240 nm) but are expensive (in the range of US $1000) and have small apertures and angular acceptance tolerances, which means that incident light cannot be tightly focused (a comparison of the wavelength dependent polarizing efficiencies of several types of polarizing devices was given in [4].

In the ISS PC1, the exciting light impinges upon the sample and the emission is viewed at right angles to the direction of excitation (Figure 1). This 90° observation angle is primarily intended to reduce the extent of exciting light that passes to the detection side. Only when samples are relatively turbid, e.g., in the case of lipid suspensions or membrane samples, will significant levels of exciting light be scattered and potentially reach the photodetector (since optical filters and monochromators are not perfect). We should also note that the focal length of the lenses just before and after the sample have a small influence on the measured polarization. Specifically, the larger the numerical aperture of the lenses focusing the excitation and collecting the emitted light (i.e., the shorter the focal length and hence the larger the cone of collected light) the lower will be the measured polarization compared to the true polarization [5]. This effect is most serious in microscope optics and will have only a small influence on measurements taken with normal spectrofluorimeters.

A typical instrument such as the ISS PC1, shown in Figure 1, may yield a polarization lower by only a few percents from the true value. After excitation by the polarized light, the emitted light can (if desired) also be passed through a polarizing device, and the spectral region of interest can be isolated by an optical filter or a second monochromator. Typical filters used in the emission side are either bandpass filters (which transmit a broader spectral region than interference filters – FWHMs can be several tens of nanometers or greater) or cut-on filters. This latter filter is often referred to as a cut-off filter depending on the viewpoint of whether the transmission commences sharply at a given wavelength (cuts-on) or equivalently if the optical density decreases sharply at that wavelength (cuts-off).

Regardless, the operational principle of these types of filters, which are also known as longpass filters, is that they can be used to block any excitation light scattered towards the emission direction and then collect a large percentage of the total emission. Web sites, which contain transmission data for many types of filters, include *www.mellesgriot.com*, *www.omegafilters.com*, and *www.corion.com* - a useful handbook containing information about filters for fluorescence microscopy can be found at *www.chroma.com.*

Light Detectors

Most modern instruments use photomultiplier tubes (PMTs) for detection and quantification of the emitted light. These devices are, of course, based on the photoelectric effect, i.e., the ejection of electrons from metallic surfaces as a consequence of incident light – the theory for this effect was developed by Albert Einstein who received the Nobel Prize for this work and not his more famous theory of relativity. The original phototubes were based on a simple arrangement to collect the emitted photoelectrons and produce an electric current, which could then be quantified.
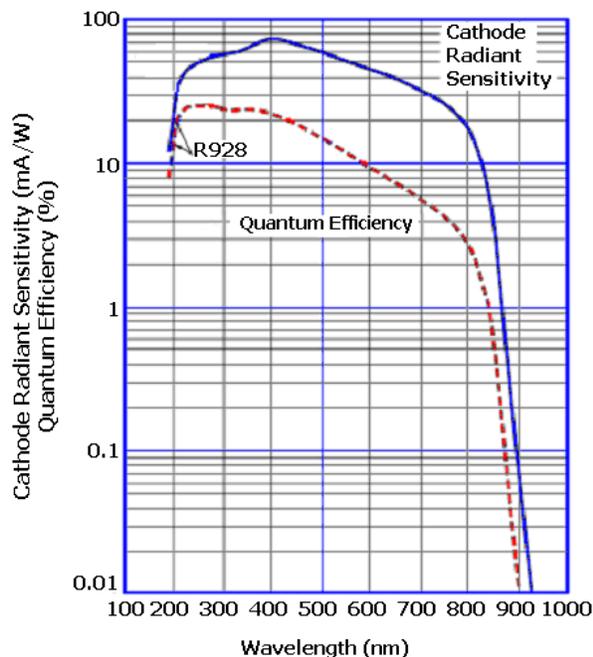
Figure 3. Sensitivity and Quantum Efficiency for the PMT Model R928 by Hamamatsu

These early devices were not much of an improvement over the human eye, although they did offer the considerable advantage of protecting the observer from the deleterious effect of UV or IR radiation on the visual system. PMT devices were soon developed, however, that had multiple plates after the photosensitive cathode, called dynodes, held at progressively more positive voltages, which acted as secondary electron emitting surfaces and which would eject several electrons for each incident electron and hence multiply the effect many-fold (practical gains above 10^9 anode electrons per photoelectron can be achieved for short light pulses though continuous gains of around 10^7 are typical due to thermal loading in the final dynodes). In the last few decades, significant progress had been made on the commercialization of PMTs with "extended red-response", which essentially means PMTs that can efficiently detect light out to above 800 nm. The model R928 by Hamamatsu features extremely high quantum efficiency, high current amplification, good S/N ratio and wide spectral response from UV to near infrared (Figure 3) and is therefore a commonly used PMT in today's spectrofluorimeters. *For detailed information on a variety of PMTs and other type of detectors see http://usa.hamamatsu.com.*

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