UV-Visible Fluorescence Spectrometers

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Abbreviations

CCD	charge-coupled devices
PMT	photomultiplier tube
UV	ultraviolet

Fluorescence (and phosphorescence) spectrometers essentially illuminate a sample with light (the 'excitation') of a chosen wavelength and detect the resultant 'emission' of light deriving from fluorescence, luminescence, or phosphorescence phenomena induced in the sample. The excitation would typically be of light within the wavelength region extending from the far-ultraviolet (UV) (175 nm) to the red end of visible (*c.* 600 nm), with the corresponding emission being detected in the UV–visible or near-infrared wavelengths (*c.* 200–900 nm) as appropriate.

Fluorescence Spectrometers

Fluorescence spectrometers can be divided into either lifetime or steady-state instruments, depending on whether they resolve the temporal behavior of the emission (or more correctly the excited state) or not, respectively.

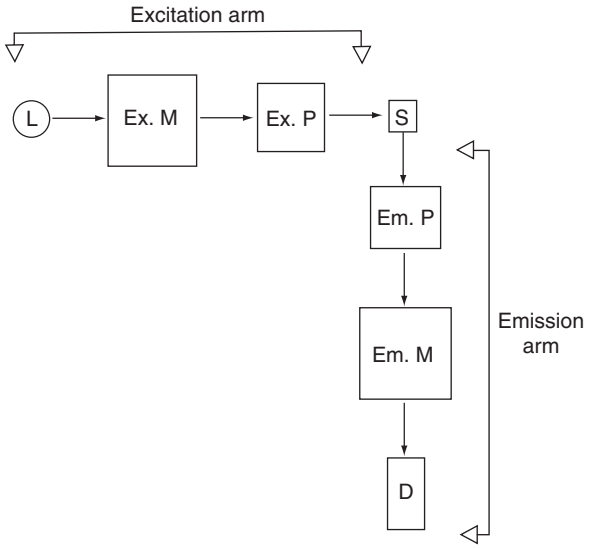

Figure 1 Conceptual diagram of a fluorescence spectrometer. L = light source, Ex. M = excitation monochromator(s), Ex. P = excitation polarizer, S = sample, Em. P = emission polarizer, Em. M = emission monochromator(s), and D = detector. Copyright of Chiralabs Ltd (2009) with permission.

In both cases, there are strong similarities with single-beam (ultraviolet) UV–visible absorption instruments and the reader is referred to the UV–visible absorption spectrometers section of the encyclopedia for further relevant details, especially regarding stray light.

However, crucially, the levels of photons detected in fluorescence (or equally phosphorescence) are typically much lower than those in absorbance: in the former one is detecting the few photons that are emitted by the sample, in the latter one is detecting those of the light source attenuated by the number absorbed by the sample. As a consequence, certain features are optimized differently for fluorescence. In addition, due to the low levels of photons to be detected, it is extremely important to exclude all sources of ambient light from the instrument.

Fluorescence is typically detected orthogonal to the direction of the excitation beam incident on the sample (**Figure 1**), so as to spatially delineate the emission photons from those of the excitation beam and minimize those from Rayleigh and Raman scattering, although these always provide a residual level. However, this is not an absolute requirement and instruments to measure the fluorescence in, for example, a well-plate format have nonorthogonal geometries dictated by the location of the sample and the nature of the vessel containing it.

Light Sources

As the fluorescence is directly proportional to the number of photons absorbed by the sample (in the absence of inner-filter/self-shadowing effects of excessive absorption), it is advantageous to employ very high-intensity light sources; xenon arcs are highly suitable. With the higher levels of light impinging on a sample, unwanted photoreactions can be a problem, as can heat generation in the sample, which accelerates all reactions. Alternatives are pulsed sources, which provide broadband radiation over short periods of time and thus may minimize some of the problems of steady-state arcs. Furthermore, in lifetime instruments they provide a means of determining the time lag between absorption and subsequent emission of the photons by the sample – the emission lifetime. In this respect pulsed lasers and flash lamps such as hydrogen arcs are popular. For very portable, low-powered, or small devices, high-intensity, light-emitting diodes have been employed successfully as light sources.

Monochromators

To distinguish the wavelength dependencies of a sample's excitation and emission spectra, monochromators are placed in both the excitation and emission optical paths. In very basic instruments, filters may be substituted for monochromators. Similarly, for instruments operating at a single excitation wavelength, laser sources can be used to good effect and negate the need for an excitation monochromator. The emission-side monochromator and detector may also be replaced with a fixed dispersive element (e.g., a diffraction grating) and a solid-state array detector.

The selection of excitation wavelength and detected emission wavelength may be independently controlled. Thus, the excitation wavelength may be fixed and the emission wavelength scanned to give the emission spectrum, or vice versa to give the excitation spectrum. On many of the higher specification instruments, it is possible to automatically scan both the emission and excitation wavelengths to give an excitation–emission 2-D maps or fixed offset maps.

Additional excitation intensity may be achieved by greater spectral bandwidths employed on the excitation side, although this may compromise the spectral resolution of the results. Similarly, the number of photons detected can be increased by modestly increasing the spectral bandwidth on the emission side. However, again this will have a corresponding effect on spectral resolution.

Sample Compartment

Solution studies are typically carried out using fused quartz rectangular cuvettes (cells) that have orthogonal faces optically transparent and flat; for historical reasons, these are typically of 1 cm pathlength. However, many alternative variants are available, including thermostated flow cells, micro cells, and a plethora of specialist types. Whatever the cell construction, for reliable results cells must be located in a fixed, reproducible orientation and position in the sample compartment. Many instrument manufacturers provide a wide range of attachments for controlling the sample, such as thermostated cell holders, stirrers, sippers, and cell autochangers.

Likewise, there are numerous attachments for solid samples. As an alternative, optical fibres allow the study of samples remote from the spectrometer. Essentially, a fibre optic redirects the light from the sample compartment to the external sample, with another returning the resultant beam back to the detector. By these means, fluorescence can be monitored using remote cells, surface and submersible probes. Adaptations to fluorescence spectrometers such that they can measure fluorescence from samples in a well-plate format, or vials and tubes are also available.

Detectors

The detector in a standard fluorescence spectrometer is most frequently a photomultiplier tube (PMT) or a solid-state semiconductor-based device; PMTs generally have a greater sensitivity and are employed in many of the most demanding research-grade instruments. More recent detectors include charge-coupled devices (CCD) and photomultipliers arrays, which will no doubt become more commonplace in the future. In the most sensitive of instruments, the detector is cooled to reduce noise and thus improve the signal-to-noise levels.

Calibration

The two axes of a fluorescence spectrum, namely, the wavelength (or correspondingly the energy, frequency, or wave number) and the fluorescence intensity (or photon counts), dictate that these two scales of an instrument ideally be calibrated.

The wavelength scale calibration, that is, the adjustment of the monochromators to ensure the correct wavelength, can be accomplished by the use of either a series of line emissions from discharge lamps, the precise wavelengths of which have been tabulated, or through standard filters with known absorption spectra. For general convenience, the filter method is the one of choice. The most common filters used are those of holmium oxide or didymium (a mixture of neodymium and praseodymium) oxide in glass. However, these can be difficult to produce consistently and can show variations of some ± 4 nm in peak positions at the long wavelength end of their useful range (240–685 nm). Therefore, for accurate calibration, it is necessary to use filters provided with a table of determined peak positions from a reputable source such as a national laboratory for standards. As an alternative to glasses, solutions containing lanthanide ions have proved useful, with less variation but a corresponding decrease in convenience. As a further check, the spectral characteristics of many common fluorophores are known and can be employed as secondary calibrants of wavelength.

In contrast, many fluorescence studies are carried out without recourse to calibration of the fluorescence intensity or the correction for instrumental response variations with wavelength, or even with time. For some investigations this is adequate, but the reasons for not calibrating stem primarily from its difficulty rather than its irrelevance; for absolute measurements, intensity calibration is essential.

To correct excitation spectra, it is necessary to determine the wavelength dependence of the light intensity of the excitation side of the instrument, with the emission side fixed. A common method is to employ a 'quantum counter'; essentially a compound whose absorption spectrum (at an appropriate concentration) is such that more than 99% of all the exciting photons are absorbed over a sufficiently wide wavelength range and whose emission spectrum and quantum yield are independent of the excitation wavelength over this range. The most frequently used quantum counter is rhodamine B in glycerol or ethylene glycol (at 3–8 $g\,l^{-1}$). This solution exhibits constant (to within 2%) fluorescence efficiency at 610–620 nm when excited in the range 350–600 nm, and only $\pm 5\%$ variation for excitation between 250 and 350 nm. Measurement of the apparent excitation spectrum of such a sample, monitored at an emission wavelength between 610 and 620 nm, allows direct determination of the wavelength dependence of the excitation side of the instrument.

The effectiveness of this method has led to instruments in which a quantum counter is incorporated into the design by diverting a portion of the excitation light to a separate quantum counter and detector. Although such a system importantly allows for correction of any temporal variations while measurement on samples is ongoing, it does introduce a difference in the optical path from that of the true excitation beam, with a potential inaccuracy.

Determination of the wavelength dependence of the emission side of an instrument is more problematical. Ideally, light from a 'standard lamp,' that is, one whose calibrated spectral distribution is known, is directly introduced into the emission optical path from the sample compartment. Measurement of the apparent 'emission' spectrum and comparison with the known true distribution of the lamp give the wavelength characteristics of the emission side.

One practical variation of this method is to employ the light from the excitation side of the instrument, for which the wavelength characteristics have already been determined via, say a quantum counter. To direct this light into the emission optical path, it is necessary to place a reference scatterer into the sample compartment. Such scatterers must have no appreciable wavelength dependence over the wavelength range of interest (mirrors, while achieving the redirection, have wavelength dependencies that make them poor choices). Common choices are flat cakes of magnesium oxide (MgO) or barium sulfate with potassium sulfate binder ($BaSO_4$ in K_2SO_4), which can be mounted in the sample position at an angle of 45° to both the excitation and emission optical paths. When using such devices, it is important to take care that the light intensity reaching the detector is not excessive, as this would lead to saturation, inaccurate measurement, and possible damage to the detector; the use of narrow spectral bandwidths or attenuators may be required in these circumstances.

As an alternative to standard lamps and their derivatives, there are numerous compounds whose absolute fluorescence spectra have been documented and may be employed to deduce the emission wavelength characteristics of the instrument. Nonetheless, the calibration of the excitation arm is still an essential procedure before the emission arm can be likewise calibrated.

Stray Light and the Inner Filter Effect

One of the main reasons for an apparent deviation from a linear response of a sample with concentration is the effect of stray light. In an ideal spectrometer, only light of the correct wavelength (within the spectral bandwidth window) would illuminate the sample and thence only fluorescence of the correct wavelength would reach the detector and be monitored. Any additional sources of light in a real spectrometer may be thought of as 'stray light.' Broadly there are six potential sources of stray light in a fluorescence instrument:

1. scattering from the sample;
2. fluorescence/scattering of the cell, etc.;
3. ambient light leakage into the instrument;
4. scattering/transmission/reflection of light by routes other than via the sample;
5. imperfections in the monochromators and light source optics;
6. imperfections in the detector optics.

The first of these, scattering by the sample, can be a serious issue when working with emission wavelengths close to the excitation wavelength. As a phenomenon specific to the sample, it is not within the realms of 'instrumental' stray light and must be considered on a case-by-case basis. However, it also provides a mechanism for additional 'second-order' anomalous signals, which may appear at distant wavelengths, due to the characteristics of diffraction-grating monochromators (see below), the scattered light masquerading as fluorescence.

The sources (2), (3), and (4) are manifestations of poor instrumental design; instruments should be light-tight and the sample should be sufficiently masked in a blackened compartment to ensure that only light impinging on the sample reaches the detector. This latter condition is sometimes unfortunately overlooked by instrument manufacturers, who may, for example, introduce reflective components in the sample compartment, or cell holders that do not fully mask the cell to within its useable aperture and beyond the dimensions of the light beam.

Finally, the last two sources are, to some degree, unavoidable instrumental stray light. Nonetheless, they can be minimized through careful design and maintenance. Imperfections in the optical surfaces and compromises in the positioning of components in the monochromators, and elsewhere, give rise to unwanted reflections or dispersion. Thus, the choice of optimal component configurations, light baffles, and component quality is crucial to the stray light performance. Diode array instruments may suffer through internal reflections in the optical surface covering the array, leading to apparent illumination of the incorrect array elements.

In particular, diffraction gratings are not perfect and furthermore, even in ideal circumstances, they generate repetitions of the wavelength range – the 'higher-order' diffractions alongside the first-order (principal) diffraction, albeit of typically weaker intensity. The most important of these, the second-order diffraction, will give light of a wavelength half that of the first-order diffraction but along essentially the same trajectory. Thus, the second order transmission of the emission monochromator of the principal excitation wavelength, if scattered by particles in a sample, can masquerade as fluorescence at twice the wavelength of the principal excitation wavelength. Consequently, care must be taken in interpreting fluorescence signals observed at multiples of the excitation wavelength. As a corollary, this anomaly can be used to advantage in allowing the monitoring of scattering alongside fluorescence without excessive photon counts for some samples.

Excessive absorption by the sample can also provide a means for both stray light to have an exaggerated effect and for the appearance of anomalous fluorescence intensities. For example, if the absorption of a sample in a fluorescence cell is *c.* 1 AU (along the direction of excitation), then the intensity of the excitation light at the center of the cell will be only *c.* 32% of that at the front face (intensity is logarithmically related to absorption). Clearly, if the fluorescence is measured from this central portion it will be of much lower intensity than that at the front face, potentially giving the impression that the fluorescence is quenched or diminished. This 'inner-filter' effect can be easily avoided by ensuring that concentration of the sample, together with the dimensions and geometry of the cell, is such that the absorption at the excitation wavelength is kept below 0.1 AU.

Fluorescence Anisotropy and Polarization

As in absorption spectroscopy, instrumental polarization effects can yield unwanted artifacts and therefore it is appropriate to introduce depolarizers into the optical path before and after the sample if the aim is to monitor the true unpolarized fluorescence spectrum. Unfortunately, this will reduce the light levels commensurately and is therefore frequently not pursued. Other methods, involving the use of polarizers set at 'magic angles' to minimize some unwanted polarization effects, have been devised but are even less frequently employed.

However, deliberate polarization can be used to great advantage in probing the environment and motion of fluorescent molecules and groups in larger macromolecules. In this case, rotatable plane polarizers are inserted in the optical path just before and after the sample; spectra are acquired in the four possible combinations of the polarizers, each in the horizontal or vertical orientation relative to the 'horizontal' plane defined by the excitation and emission optical paths. By comparison of the four spectra, the mobility of the fluorescent group during the lifetime of the excited state can be deduced.

Fluorescence Lifetime Instruments

Lifetime instruments share most of the optical arrangement of steady-state instruments. Indeed, there are commercial instruments that combine both into one versatile spectrometer. The essential optical difference is in the use of intense pulsed light sources, with an emission pulse width typically of the order of 1 ns or less. By coupling the detector and light source trigger with sophisticated electronics and postacquisition processing, it is possible to correlate the time between the absorption and subsequent emission of photons by the sample. Essentially, the excitation pulse corresponds to the absorption profile in time. In *time-correlated single photon counting methods*, the delay for the first photon to be subsequently detected is then recorded. This is repeated many thousands of times to give a statistical distribution from which the emission time profile can be deconvoluted.

Alternatively, if the lifetime is sufficiently long, as in phosphorescence, then the complete decay curve of emitted photons from a single excitation pulse can be directly monitored – the *pulse excitation method*. Finally, rather than employing a flash lamp to provide a pulse of excitation, the intensity of continuous excitation can be modulated and the phase lag of resulting oscillations on emission intensity observed – the *phase-resolved method*. Whichever method is adopted, in turn the data can be analyzed in terms of the fluorescence or phosphorescence lifetimes of the molecular species involved.

Imaging Instruments

The advent of imaging detectors, such as CCD cameras and more recently photomultiplier arrays, has prompted

the development of monochromators that are able to spectrally disperse the individual pixels of an image, while preserving the spatial integrity of the image. Consequently, absorption and fluorescence instruments have been developed that are able to produce a spectroscopic image of a sample, each pixel of the image being a complete spectrum. Such instruments have great utility in the investigation of inhomogeneous material for which traditional methods are only able to give spatially averaged results, especially if the imaging system is a microscope.

See also: Biochemical Applications of Fluorescence Spectroscopy, Fluorescence Polarization and Anisotropy, Fluorescence Theory, Organic Chemistry Applications of Fluorescence Spectroscopy, UV-Visible Absorption Spectrometers.

Further Reading

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