THE JOURNEY OF THE PHOTON: PHOTO-PHYSICS AND FLUORESCENCE INSTRUMENTATION

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Spectrochemical analysis is one of the most sensitive and powerful tools for us to use in understanding the environment around us. One of the techniques is optical spectroscopy which involves the interaction of optical electromagnetic radiation with matter and often this requires the measurement of absorption or emission of light. The instrumentation for such measurement systems involves many skills including, optics mechanism, electronics, signal processing as well as software

Il viaggio del fotone: foto-fisica e strumentazione per fluorescenza
L'analisi spettrochimica è uno degli strumenti più sensibili e potenti da utilizzare nella comprensione dell'ambiente che ci circonda. Una delle tecniche impiegate è la spettroscopia ottica, che comporta l'interazione della radiazione elettromagnetica ottica con la materia; spesso questo richiede la misurazione di assorbimento o di emissione di luce. La strumentazione per tali sistemi di misura coinvolge molti aspetti tra cui meccanismi ottici, elettronica, elaborazione del segnale così come un software opportuno.

Even in the 1850s, as optical measurements and instrumentation were beginning to expand dramatically in capability and acceptance, it was realised that a thorough knowledge of the instrument operation and its characteristics was required in order to interpret the measurements correctly. Robert Bunsen famously said: "A chemist who is not also a physicist is nothing at all". His meaning was that a physical knowledge is required to understand many processes and measurements and by understanding these a chemist can ensure he is making "true" measurements, of course this statement applies to all scientists and not just chemists!
In thinking about optical spectroscopy measurements there are essentially four types as described in Tab. 1.

Tab.1
Types of optical spectroscopy

Method	Measured Quantity	Examples
Absorption	Absorbance or the ratio of transmitted to incident radiant power, $A = -\log(\mathbf{P}/\mathbf{P_O})$	Atomic absorption UV-VIS molecular absorption IR absorption
Emission	Radiant power of emission, $\mathbf{P_E}$	ICP and DCP emission, spark emission, laser-induced breakdown emission flame emission, DC arc emission
Luminescence	Radiant power of luminescence, $\mathbf{P_L}$	Molecular fluorescence and phosphorescence, chemi- and bio-luminescence, atomic fluorescence
Scattering	Radiant power of scattering, $\mathbf{P_S}$	Raman scattering, Mie scattering, turbidity

Luminescence spectroscopy is the optical spectroscopy technique described further along with the instrumentation requirements and errors associated with it. Luminescence can be divided into many forms determined by the mode from which the light is generated, see Tab. 2.

Tab. 2
Types of luminescence

External influence	Luminescence type
Chemical reaction	chemi-luminescence
Biochemical reaction	bio-luminescence
Electrical discharge (recombination of ions and electrons at an electrode)	electro-luminescence
Interaction with accelerated electrons	cathodo-luminescence
High temperature	thermo-luminescence
Absorption of radiation	photo-luminescence • *fluorescence* • *phosphorescence*

Tab. 3
A brief summary of fluorescence before "quantum theory"

Year	Scientist	Observation/Achievement
1565	N. Monardes	Emission of light by infusion of wood (*Lignum nephriticum*), first observation for fluorescence
1600's	Cascariolo/ Licetus	Studies of Bolognese Stone, first detailed observation of phosphorescence and non-thermal light emission
1833	D. Brewster	Emission for chlorophyll and fluorspar crystals.
1833	J. Herschel	Quinine bisulphate (epipolic dispersion)
1842	E. Becquerel	First statement of λem>λex (calcium sulphate studies by UV exc.
1852/3	G.G. Stokes	Emission of quinine sulfate solution by UV light (irrefrangibility of light), then the introduction of the term "*fluorescence*"
1888	E. Weidemann	Introduction of the term "*luminescence*"
1905/10	E.L. Nicols, E. Merrit	First fluorescence excitation spectrum of a dye. Mirror symmetry between ABS and EM spectra
1919	O. Stern & M. Volmer	Relationship of fluorescence quenching
1922/24	S.I. Vavilov	Exc wavelength independence of fluorescence quantum yield First determination of fluorescence yield of dye solution

One of the most popular techniques is photo-luminescence, of which fluorescence and phosphorescence are special cases that are very commonly used for analytical purposes. Both derive from absorption of radiation to generate a luminescence from:

- fluorescence: "spontaneous emission of radiations (luminescence) from an excited molecular entity with retention of spin multiplicity";
- phosphorescence: "phenomenological, term used to describe long-lived luminescence. In mechanistic photochemistry, the term designates luminescence involving a change in spin multiplicity, typically from triplet to singlet or vice versa. (Note: e.g. the luminescence from a quartet state to a doublet state is also phosphorescence)".

Luminescence observation has been reported for several centuries (Tab. 3) but it was not until 1852 when Sir George Gabriel Stokes made his now famous experiment using quinine water and observed a shift in colour from the excitation to the emission light that the phenomena of fluorescence was first termed.

About eighty years later Perrin and Jabłonski explained the phenomenon using quantum theory and presented the now universally used Perrin-Jabłonski energy level diagram to explain the process of fluorescence and phosphorescence, Fig. 1.

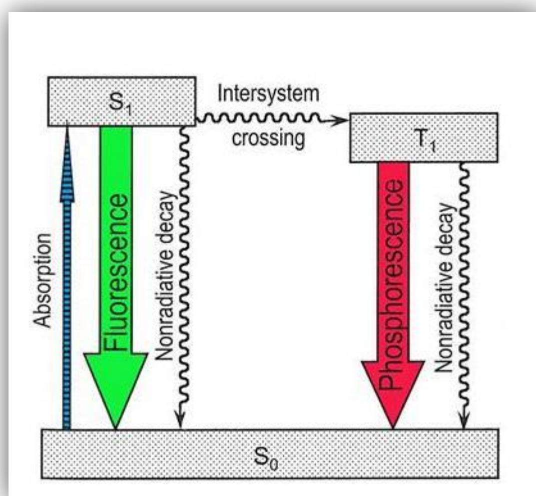

Fig. 1
Perrin-Jabłonski Diagram, 1935, from Valour and Bereran-Santos, 2013

The importance of luminescence measurements over absorption measurement is in its intrinsic sensitivity. The reason for this is that a luminescence signal emanates from a low background signal. Where - as an absorption the signal is often a small change - a large signal offset. An example of this is the dilution experiment of Alexa 488 a commonly used fluorescence probe for biological studies. In Fig. 2 this experiment demonstrates that a 40,000 times dilution sample still yields a concentration measurement of skill and a signal-to-noise ratio better than 15:1. The corresponding absorption measurement is unmeasurable after a five times dilution from stock!

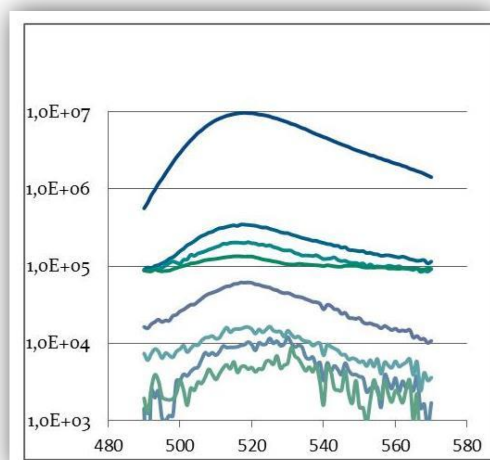

Fig. 2
Alexa 488 dilution study. 40,000 time dilution and still a fluorescence s/n>15:1

Luminescence measurement offers a range of measurements possibilities that allows measurements to be made that can sensitivity probe molecular behaviour and the associated micro-environments. As a result such measurements have become common place in many labs ranging from chemistry, physics, electronics, biochemists, environmental as well as quality control application. All of these application

areas and measurements need careful measurement practice as well as detailed evaluation of correction factors and instrumentation artefacts.
All luminescence steady state measurements make a measure of the emission intensity from a sample. There are four main types of scan commonly used (Tab. 4), and these form the basis of other more complex measurements. Getting these correct as well as ensuring suitable correction files in the key to success.

Tab. 4

Type	Method
Excitation Scan	Select emission wavelength and pass band, λ_{em}, $\Delta\lambda_{em}$, and hold fixed Select excitation pass band, $\Delta\lambda_{exc}$ Scan excitation monochromator across excitation spectral region of interest
Emission Scan	Select excitation wavelength and pass band, λ_{exc}, $\Delta\lambda_{exc}$, and hold fixed Select emission pass band, $\Delta\lambda_{em}$ Scan emission monochromator across emission spectral region of interest
Synchronous Scan	Select excitation and emission pass bands, $\Delta\lambda_{exc}$ and $\Delta\lambda_{em}$ Select wavelength offset between excitation and emission wavelengths, $\Delta\lambda$ Scan excitation and emission monochromators in synchronization with the offset $\Delta\lambda$
Excitation-Emission Matrices	Select excitation and emission pass bands, $\Delta\lambda_{exc}$ and $\Delta\lambda_{em}$ Scan emission spectra (over a spectral range) as a function of excitation wavelength

All fluorimeters comprise key components, such as: an excitation source, wavelength selection devices for both excitation and emission channels as well as a beam directing optics and detectors for monitoring signals. A typical layout is presented in Fig. 3.

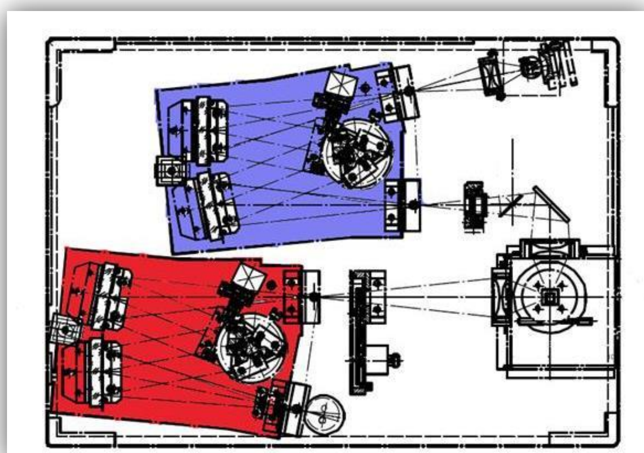

Fig. 3
Typical fluorimeter components layout

In a ideal fluorimeter one would like to have:
- extremely sensitive, and no noise signals;
- measures excitation and emission spectra, i.e. the photon flux emitted at each wavelength;
- not sensitive to interfering signals e.g. Raman, Rayleigh scattering, straylight, fluorescence from solvents, etc.;
- measures the “true spectra” of the sample that is corrected for the non-uniform spectral output of light sources and wavelength dependent efficiency of monochromators and detectors;

- the light source must yield a constant photon output at all wavelengths;
- the monochromator must pass photons of all wavelengths with equal efficiency;
- the monochromator must be independent of polarisation;
- the detectors must detect photons of all wavelengths with equal efficiency.

The reality of any fluorimeter system is in fact quite different! Lamp sources such as Xenon bulbs have their own characteristics spectral output which is non-uniform with wavelength and often has sharp emission spectral features, see for example in Fig. 4. At the same time, the emission signal intensity from the sample is related to the incident excitation power level. As a result it is important to be able to measure the lamp spectral emission in real time and to correct for this as well as possible lamp temporal stability.

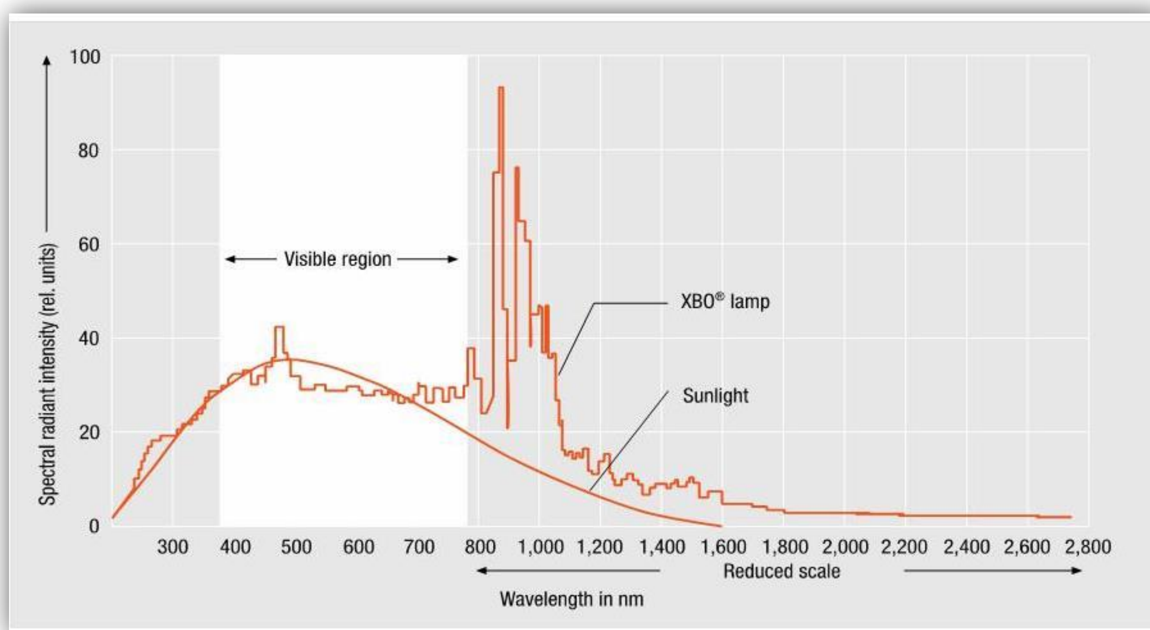

Fig. 4
Typical Xenon lamp emission spectrum, from Osram, New Technology and Applications, 2008

Not only do lamps have a wavelength dependent output but the wavelength selection devices like monochromators with diffraction grating also do. Even worse is that the wavelength diffraction efficiency is in fact also polarisation dependent and in some cases caused by Wood's anomalies can fall close to zero response. Thus the combined effect of lamp emission and monochromator response must be very carefully combined instrument design and also method in real-time monitoring (Fig. 5).

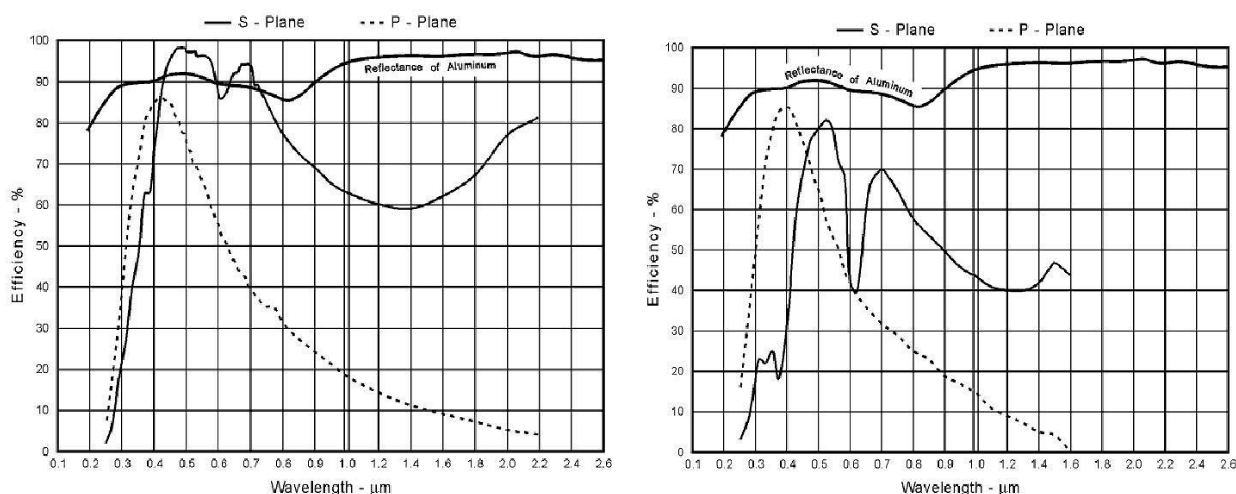

Fig. 5
Typical diffraction grating efficiency curves for 1200 g/mm, 500nm blaze diffraction gratings from different masters and measured under Littrow conditions. Reproduced with kind permission from Richardson Grating Lab, Newport Corporation

The normal method of meaning the relative excitation intensity is to pick-off a small amount of light from the excitation cloud and then to measure this using a high-stability silicon UV photodiode.

The analysing or emission channel has a similar yet complimentary spectral transmission problem such that the emission from sample is 'distorted' in measurement by the transmission efficiency of the analysing monochromator and the spectral responsivity of the measurement detector.

As a result corrected emission spectra should be a standard function in a fluorimeter. Such corrections are usually made using a highly stabilised tungsten lamp under constant current conditions such that emission spectrum is well known and stable. Once this is applied the measurement can be corrected for the monochromator and detector spectral efficiencies.

The application fields of luminescence spectroscopy are very wide indeed. In all cases a detailed understanding of instrument performances and units are essential for ensuring good measurement practice. With this in mind one should also consider what the often-quoted fluorescence units are? In principle they describe the intensity axis of the spectral plot. There is, in fact, no standard definition of what these units are and they are therefore varying from instrument-to-instrument and laboratory-to-laboratory. At the same time it is not common for instruments to be characterised in an absolute and strict radiometric meaning, although relative spectral corrections can be applied. In photon counting type systems it is also necessary to describe the signal level correctly: different user request often the signal in counts (as measured in the defined integration time), as counts per second (as corrected signal for the integration time) and as photon numbers in which either of the former is corrected for photon energy. This is important as by the conversion from counts to photons spectral shifts can occur. As a result it is important to ensure that a correct understanding of the signal meaning and corrections is available to the user. The measured signal intensities is therefore only relative unless a very full and strict radiometric calibration is made.

There are many non-instrument effects that can affect the quality of fluorescence measurements and these should be controlled as part of the experimental methods and implementation used. Errors can be attributed to all sorts of sources caused by, for example:

- errors in sample concentration
- inner-filter effects
- temperature changes
- lack of pH control
- non-linearity
- wrong or contaminated solvents
- adsorption of sample
- aggregation effects
- overlapping spectral bands
- and so on.

Careful instrument set-up and sample preparations are key to successful, reliable, and repeatable fluorescence measurements and care should be taken at each stage[1].

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