A Luminescence Tutorial

Introduction

The use of fluorescence spectroscopy in the life sciences is expanding rapidly.
The reasons are obvious: no other technique can offer as much in terms of sensitivity and selectivity with such moderate operational costs.
Furthermore, the technique can be used to dynamically follow phenomena of physiological importance in intact living systems using probe concentrations low enough not to overly perturb the system.

The technique is used more and more as a 'black box' technique, and rightly so:
the majority of practitioners of fluorescence have little interest in the highly theoretical aspects of the technique since it offers only a means to an end.
This standpoint is completely understandable, although in order to fully appreciate and optimise the technique, understanding the theory is essential.

The theoretical and practical aspects of fluorescence analysis are not too complicated, although most written work on the subject either approaches the basics with little attempt at conceptualisation (or at explaining practical aspects); at the other end of the scale some of the more thorough work assumes an in-depth knowledge of the theory.

The truth is that many modern users are using the technique in very applied research environments where the *application* of the technique is more important than the theory of the measurement technique itself.

This tutorial is an attempt to cut a middle line between theory and practicality, also attempting to provide understandable images to relate the theory.

At times the analogies and graphical images displayed may stray from the path of absolute truth in order to attempt clarification: despite this it is hoped that
the ideas are genuinely useful in understanding the fascinating and powerful subject of luminescence spectroscopy.

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Chapter 1. Theory of luminescence

The nature of light

Light is composed of a continous electromagnetic spectrum.
The region of most interest in luminescence spectroscopy is the near Ultra Violet
To the near Infra Red. The term electromagnetic is used to describe the fact that light is composed of electrical and magnetic functions.

The absorption of light - creation of the excited state

The absorption of light by a molecule involves the transition of an electron from a low energy (ground, S_0) state to a higher energy (excited, S_1) state. These states are illustrated using the Jablonski diagram, which shows the ground and excited states as discrete energy domains, each of which has separate vibrational electronic levels. The large energy difference between the levels means that thermal energy absorbed by the molecule cannot cause electrons to be promoted to higher states, only to higher vibrational levels within a state.

The process of light absorption is quantised, this means that the incident photon must have at least enough energy to promote the electron to a higher state in order for absorption to occur. Light of insufficient energy will not be absorbed by the molecule. This absorption energy requirement depends on the difference in energy between ground and excited states, this is in turn directly dependent on the structure of the molecule and how it interacts with it's environment. The relationship between the energy of the ground and excited states is given by:

$$E = E_1 - E_0 = h\nu$$

where h is Planck's constant and n is the frequency.
The ground state must be highly populated and the excited state must be unpopulated
for this to occur, that means that there must be a significant number of molecules with orbital electrons in the ground state available for light absorption, also the energy state which the orbital electron will be promoted to must be empty.
(This is just common sense - if the ground state is not highly populated then there won't be any ground state electrons to be excited. Similarly, the target excited state orbital must be able to accept the promoted electron).

When absorption occurs, an electron is promoted from an orbital containing two electrons with opposite spin to an orbital containing only one electron. The spin state of the two electrons remains paired, that is they have opposite spin. This is the singlet state. The exception is in phosphorescence, where external factors cause the two electrons to have equal spin (the triplet state).

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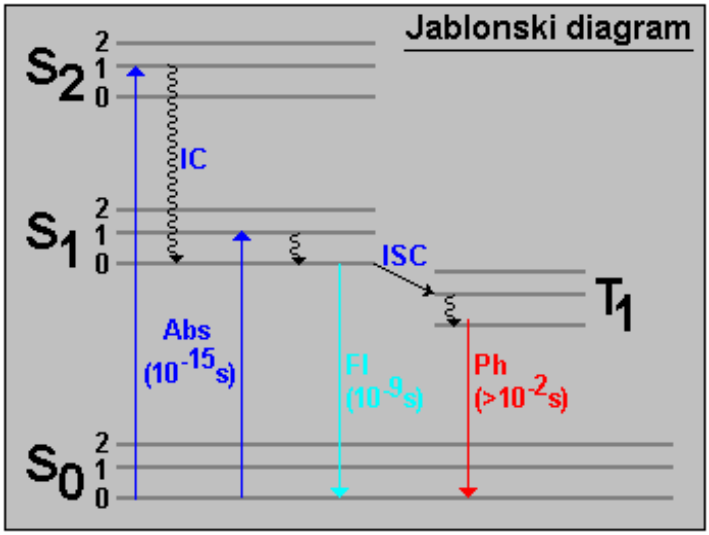
Absorption is a very fast process, typically occuring within 10^{-15} seconds. The Franck-Condon principle states that because of the rapidity of the transition, displacement of atoms in the molecule does not occur during absorption of light. The fact that the arrows showing energy transitions are vertical is intended to show this.



Abs is the absorption process, and takes place over 10^{-15} to 10^{-12} seconds.
IC is internal conversion, and is a relaxation process whereby energy is lost from higher vibronic levels as the electron stabilises to the v=0 vibrational level.
ISC is intrasystem crossing, this involves the transition from a singlet state to a triplet, so is spin forbidden.
S0, S1 and S2 are all singlet states, being the ground state and two excited states respsectively. T1 is a triplet state. Fl is a radiative transition from the S1 excited singlet state to ground which results in the emission of a photon (fluorescence). Ph is the radiative transition from the triplet state to ground resulting in phosphorescence.

Absorption of light is further selective due to the existence of an absorption dipole moment. Only that portion of light having an electric dipole equivalent to the transition dipole of the absorbing molecule will be absorbed. This principle is of critical importance in polarisation analysis: this will be discussed in a later chapter.

Photobleaching - incident-light induced

Under normal conditions, most molecules will have >99% of bonding electrons in the ground state. Absorption of light can then lead to the phenomenon of luminescence emission. Under extreme incident light intensities this situation can change, leading to a significant number of electrons in an excited state.

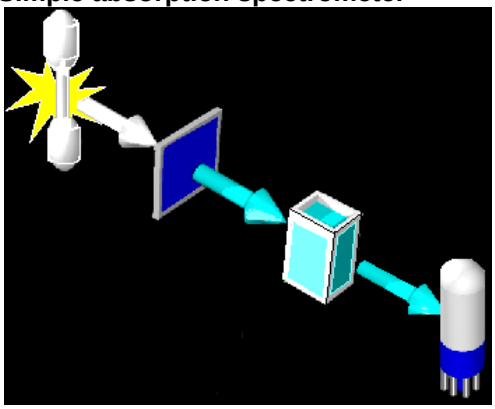
Further transition of these electrons to a higher excited state does not lead to further emission of luminescence, since emission always occurs during the transition from the lowest excited state to the ground state and not between higher excited states.
In other words, one photon is absorbed for each jump to a higher excitation state, although only one will be emitted from the transition back to the ground state.

This causes a type of photobleaching and loss of linearity: normally, however, the light intensities required would be caused only by the use of a powerful laser.

Chapter 2. -How does the instrument work?

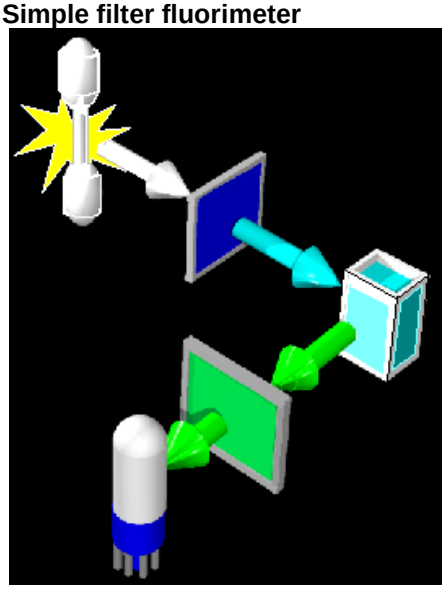
Absorption spectrometers and fluorimeters are fundamentally different in their construction.
In absorption spectrometry, a relatively large amount of light passes through the sample . The sample absorbs some of this light: the residual light is detected and the degree of absorption is presented as an absolute figure.
The detector measures a small change in light intensity on top of a large background light signal.

Simple absorption spectrometer



In a fluorimeter, the light emitted by the sample is measured at right angles in order to avoid the excitation light reaching the detector. The background signal is very low, fluorescence from the sample is a very small signal on top of this.

Simple filter fluorimeter



Practical problems arise in the use of such a design. Firstly, many modern fluorescence assays require the selection of more than one wavelength, or the scanning of spectra.

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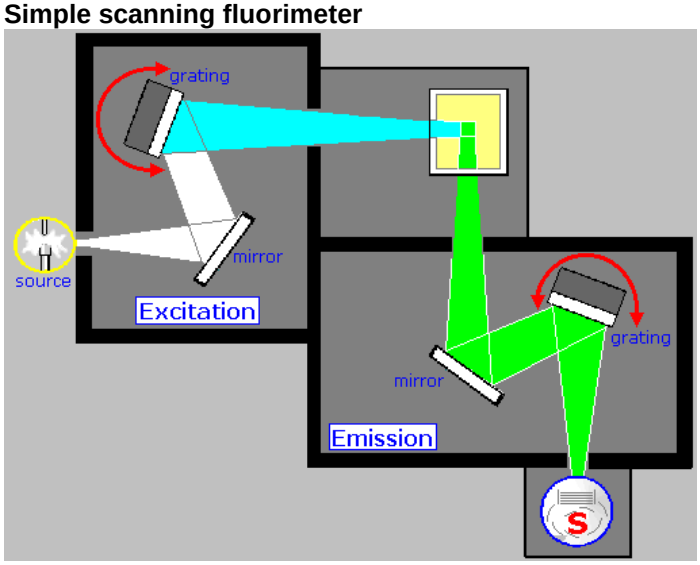
Most modern fluorimeters therefore contain monochromators based on gratings, which allow the selection of a narrow band of light. Light emerges from the source and enters the excitation monochromator which selects a narrow band of light. This is absorbed by the sample, which emits fluorescence. A small amount of this fluorescence passes into the emission monochromator, where the desired emission wavelength is selected and measured by the detector (photomultiplier).

Simple scanning fluorimeter



As the grating is rotated, the wavelength of light changes. By detecting the effect of this on the measured signal, fluorescence spectra can be scanned. If the excitation monochromator is moved, then the excitation spectrum is scanned. This is the equivalent of the absorption spectrum, in that it relates to light absorbed, but it differs in that it is actually a measure of the efficiency of the range of excitation wavelengths at producing fluorescence. Even though the excitation spectrum is being scanned, it is of course fluorescence emission which is being measured. If the fluorescence signal is measured while the emission grating is rotated, then the emission spectrum is scanned.

Whatever the type of measurement made using the LS-50B, the signal comes from the sample photomultiplier.
There is no ´Excitation´ detector, only Reference and Sample detectors.

The reference detector is used for removing instrumental artefacts from excitation spectra, and does not ´see´ light from the sample!

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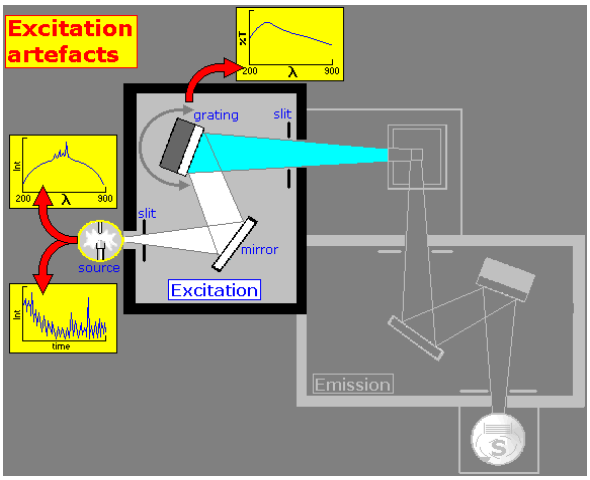
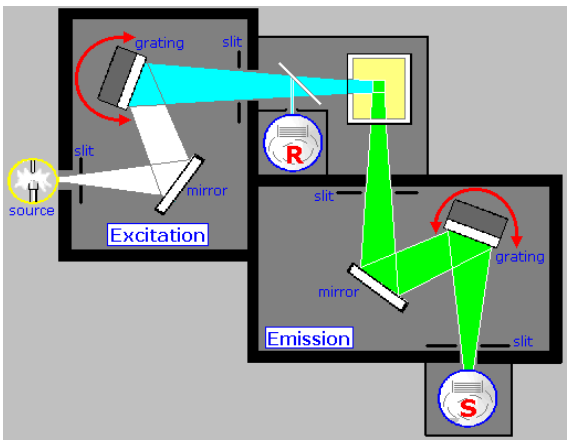
This picture is not complete, however. Components of the excitation system have distinct characteristics which affect the light which falls on the sample:

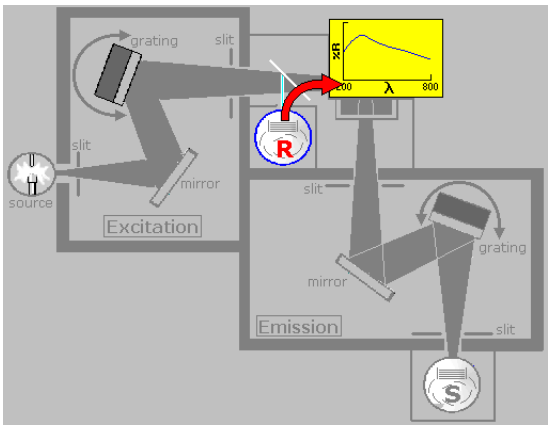


The source has wavelength-dependent and time-dependent fluctuations: the excitation grating has a wavelength-dependent transmission.
For this reason, a small amount of light is sampled immediately before it hits the sample. This is the reference channel, used for excitation correction. The signal measured is always calculated as:

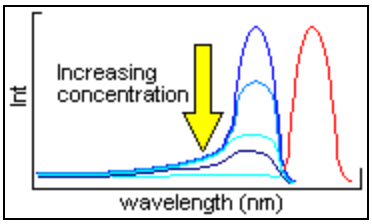
Reported **Int**ensity = (**S**ample signal)**/**(**R**eference signal)

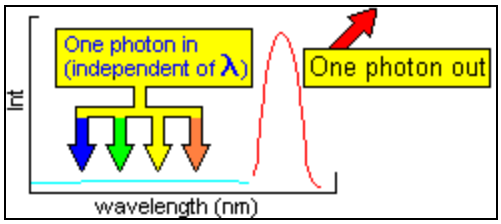


Unfortunately, the addition of the reference detector to remove excitation artefacts adds another-the reference detector itself has a distinct wavelength-dependent sensitivity spectrum:



For this reason, the reference detector itself is corrected using a quantum counter.
This is a concentrated solution of a fluorescent dye which has special properties: the concentration is so high that the excitation spectrum is completely distorted to flatness. The effect of this is that when a photon is absorbed, independent of the wavelength, one photon is emitted:



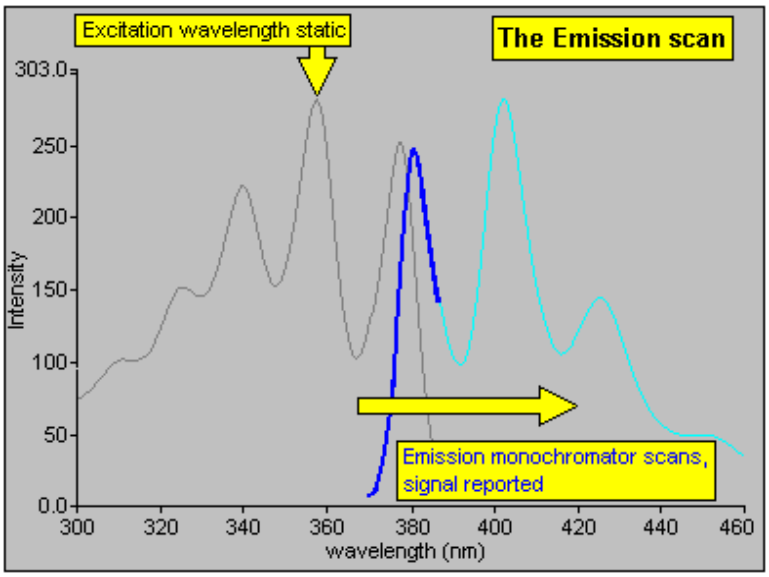


This system is therefore wavelength independent, so should produce a flat excitation spectrum. The measured spectrum of this sample therefore contains information concerning the response curve of the reference detector. All subsequent excitation data is divided by the stored response curve: excitation data is then fully corrected for instrument artefacts.

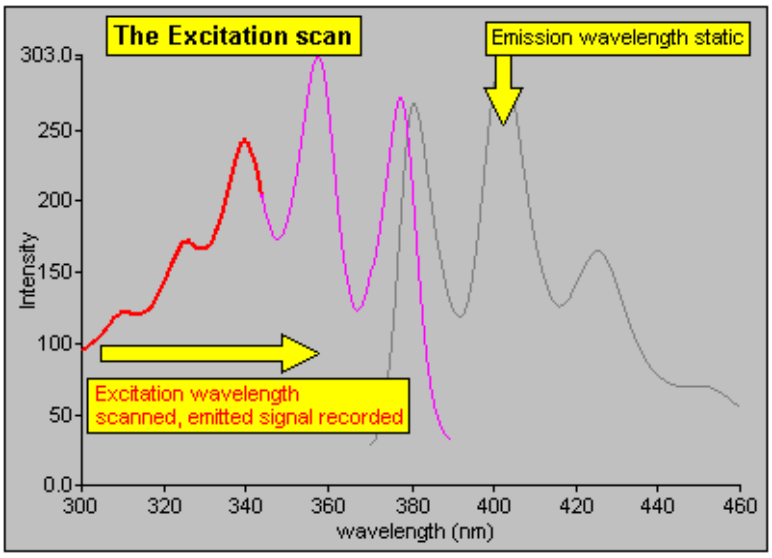
Chapter 3. Qualitative Practical considerations

How are excitation and emission spectra recorded?

This is a commonly misunderstood principle, mainly due to familiarity with absorption spectroscopy. Whether the user collects an excitation or emission spectrum, the signal which is measured is fluorescence emission.
For an emission spectrum, the excitation monochromator is sent to a fixed wavelength, the emission monochromator is then scanned to determine the distribution of light emitted by the sample:



For an excitation scan, the emission monochromator is sent to a fixed wavelength, and the excitation wavelength is scanned. During this scan, the signal displayed represents how much fluorescence emission is produced by each excitation wavelength. This signal is measured on the **emission** photomultiplier:



Whatever the type of measurement made using the LS-50B, the signal comes from the sample photomultiplier. There is no ´Excitation´ detector, only Reference and Sample detectors.
The reference detector is used for removing instrumental artefacts from excitation spectra, and does not ´see´ light from the sample!

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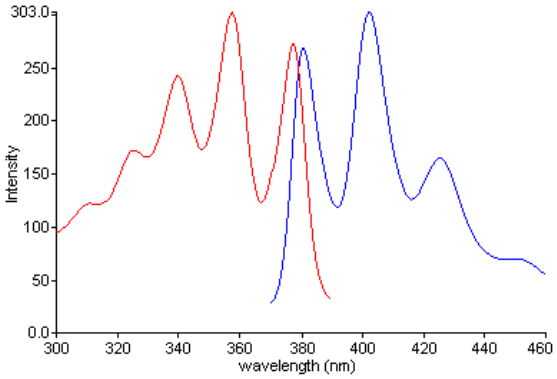
Scanning spectra

Luminescence analysis involves the use of two spectra to select the species under analysis: these are the excitation spectrum and the emission spectrum.
When scanning an excitation spectrum, the emission wavelength is fixed. When scanning the emission spectrum, the excitation wavelength if fixed. Normally, one would choose the intensity maxima for these fixed wavelengths:



Of course, the selection of the optimal wavelengths is rather a paradox: in order to scan the emission we must know the excitation wavelength, but in order to scan the excitation spectrum to obtain this, we need to know the emission wavelength.....but normally one would have at least a general idea of the wavelengths for analysis. Many modern fluorimeters have 'Pre-scan´ functions built in to help if the spectral properties are completely unknown.
Equally important is the correct choice of scan range, since if this is wider than it needs to be, then there is a strong likelihood that scatter peaks will appear, sometimes confusing the user as to the correct analytical wavelengths.
Also, different instruments have their own characteristics which lead to spectral artefacts, and unless these are corrected for, then the excitation spectra and/or the emission spectra from two fluorimeters will not be comparable. For this reason it is always worthwhile taking a little time to fully establish the optimal excitation and emission wavelengths for an assay: assuming that those published can be used directly is dangerous, and could lead to the loss of many months of work.
The suggested steps taken to correctly identify the analytical wavelengths are:

- Scan the emission spectrum
- Scan the excitation spectrum
- Re-scan the emission spectrum with the confirmed excitation wavelength
- Re-scan the emission spectrum with lower excitation wavelength to check for scatter

The Emission spectrum

To scan the emission spectrum given the spectra shown above, we would choose 360nm as the excitation wavelength. Selection of the emission scan range follows some simple rules:

<u>The scan start wavelength</u>

The emission maximum is almost always at a longer wavelength than the excitation wavelength, since energy is lost within the excited state. For this reason, it makes no sense to start the emission scan at a wavelength below or at the excitation wavelength. This leads to the situation where both monochromators are at the same wavelength, which allows Rayleigh scatter to pass directly through to the sample detector. This produces a large, symmetrical peak which can confuse the user into thinking it is an analytically useful peak.
The width of this Rayleigh scatter peak depends on the slit widths used: the wider these are, then the wider the peak will be. For this reason, choose a start wavelength equal to:

Excitation wavelength + (excitation slit width + emission slit width)/2

so for the example given, this would be:

350 + (5 + 5)/2 = 355nm

The scan end wavelength

In most cases, the width of the emission spectrum is not very large, being typically between 100 and 200nm. It is highly unlikely that the emission band would extend much further than this, since this would require a very large energy loss within the excited state. There is no danger in setting a very large emission scan range, but this does lengthen the scan time and can also show second order scatter peaks which can be confusing. If we set the end of the scan to avoid the second order peak, then we obtain a scan end wavelength of approximately 690nm.
In practice, this is found to be far too large, but having scanned too far it is easy to then reduce the scan range. It is too easy to scan first with a short scan range and therefore miss important analytical peaks.

The Excitation Spectrum

To scan the excitation spectrum, we would choose 400nm as the emission wavelength. Selection of the excitation range to scan also follows some simple rules:

The scan end wavelength

The emission maximum is almost always at a longer wavelength than the excitation wavelength, since energy is lost within the excited state. For this reason, it makes no sense to end the excitation scan at a wavelength above or at the emission wavelength.
This leads to the situation where both monochromators are at the same wavelength, which allows Rayleigh scatter to pass directly through to the sample detector. This produces a large, symmetrical peak which can confuse the user into thinking it is an analytically useful peak.
The width of this Rayleigh scatter peak depends on the slit widths used: the wider these are, then the wider the peak will be. For this reason, choose an end wavelength equal to:

Emission wavelength - (excitation slit width + emission slit width)/2

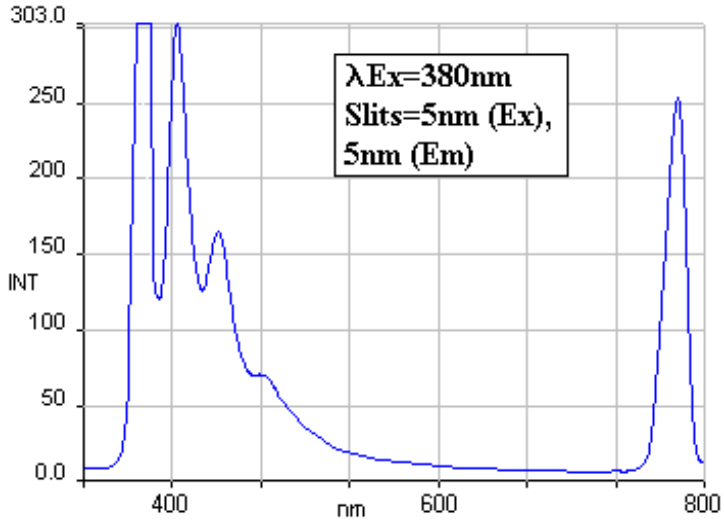
so for the example given, this would be:

400 - (5 + 5)/2 = 395nm

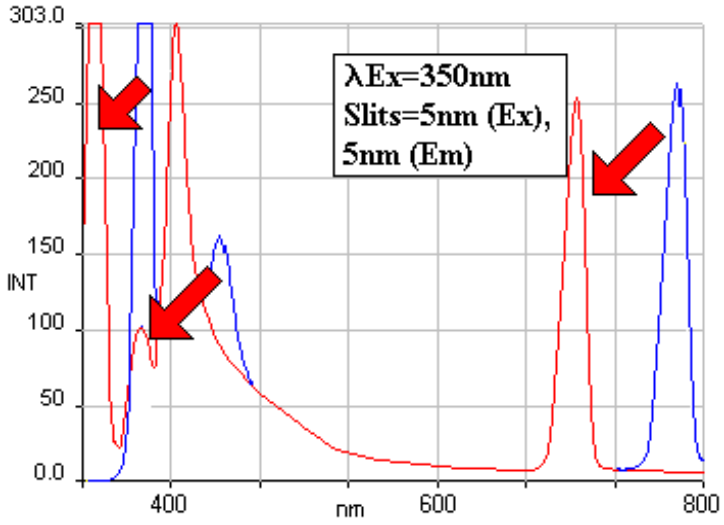
Identifying and removing scatter

In normal practice, spectra obtained in fluorescence spectroscopy do not show a high degree of structure. A further complication is brought about by the existence of various types of scatter, these are Rayleigh, Raman and Second order scatter respectively. A more detailed description of the scatter types is included later.

One simple test can be employed to positively and immediately identify scatter. The user simply carries out an emission scan using a suitable excitation wavelength, then repeats the emission scan using a lower excitation wavelength, for example 20nm lower.
Any peaks which remain in a similar position are probably due to some form of luminescence, but any peaks which move towards lower wavelength are due to scatter.
In the first example, an emission spectrum has been scanned at an excitation wavelength of 380nm:



As can be seen, many peaks were observed. When the emission spectrum is re-scanned at a different excitation wavelength (350nm), then all scatter-related peaks shift along
the wavelength axis, the new spectrum is shown in red, peaks from the previous spectrum in blue:



Only one clear fluorescence peak can be identified in the above spectrum (that at around 404nm).

Predicting the position of scatter peaks

How much the scatter peaks will change when the excitation wavelength is changed is predictable. For example, if the following instrument parameters are used:

Excitation wavelength	350nm
Slits	10/10nm
Emission scan start	330nm
Emission scan end	720nm
Sample	10^{-10} M quinine sulphate in 0.1N sulphuric acid

Then the following peaks would be observed:

Peak Wavelength	Source
350	Rayleigh scatter
397	Raman scatter
460	fluorescence
700	Second order scatter

If the emission spectrum is re-scanned using the same conditions except for excitation at 366nm, then the following peaks would be observed:

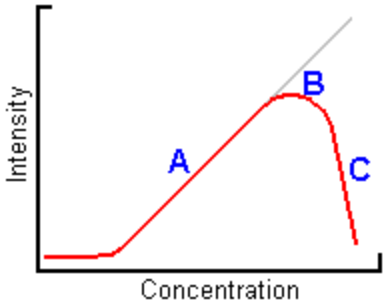
Peak Wavelength	Source
366	Rayleigh scatter
414	Raman scatter
460	fluorescence
732	Second order scatter

Note also that in some cases, further harmonics can be observed, leading to further confusion. For example, if the excitation wavelength is set to 400nm, then a small peak can be observed at 600nm. The excitation light beam produces a harmonic at 200nm, this passes through the emission monochromator at 3 times the wavelength of the light.
Secondary Raman peaks may also be observed, so that a Raman peak at 397nm produces a second order peak at 796nm.
Whatever the source of the scatter peak, changing the excitation wavelength always produces a wavelength shift in the scatter peak position.

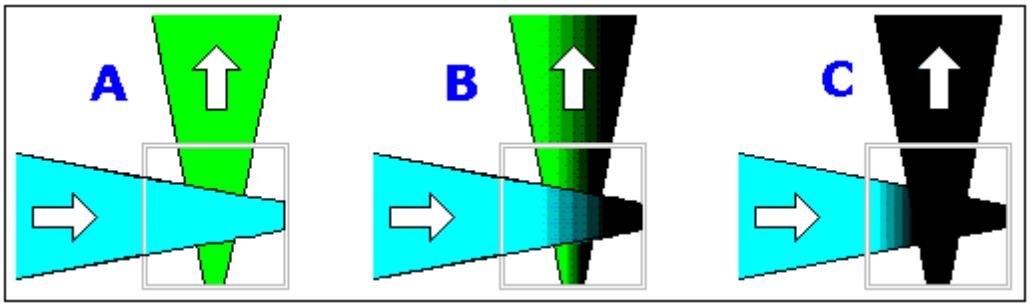
Chapter 4. Quantitative Practical issues

Non-linearity due to high concentration (inner filtering)

The calibration graph of any cuvette-based assay, over a broad concentration range, would appear as follows:



Above a linear range (A), the measured intensity flattens (B) until the measured intensity is almost totally lost (C).
This is an absorption phenomenon named Inner Filtering, and prevents the excitation beam from reaching the centre of the cuvette where the sample is measured.
In the diagram below, a view from above the cuvette is shown for each of the three graph regions A, B and C.



Example A shows the case for a linear sample concentration. The blue excitation beam is focussed into the sample cuvette, and most passes through without being absorbed. The emission beam is also focussed, so the instrument measures the part of the sample where the excitation and emission beams overlap.

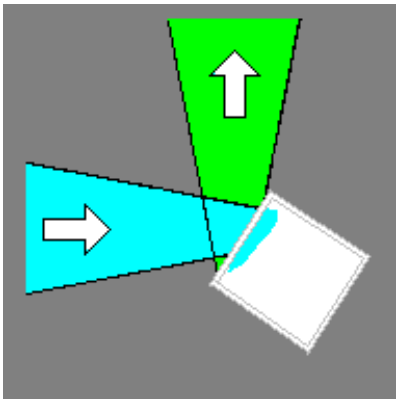
In example B, the excitation light is totally absorbed within the pathlength of the cuvette. Some light falls on the central, measured part of the cuvette, but this is much reduced compared to the diagram above.
In this case, some of the sample molecules located in the darker region of the measured volume are not measured-they do not fluoresce since there is no light to be absorbed.

In severe cases (case C), the excitation light is absorbed within the first millimetre or so of the light path. Virtually no light reaches the measured part of the cuvette, so the measured fluorescence intensity is very low or even zero.

There are several ways to avoid this inner filtering, including:

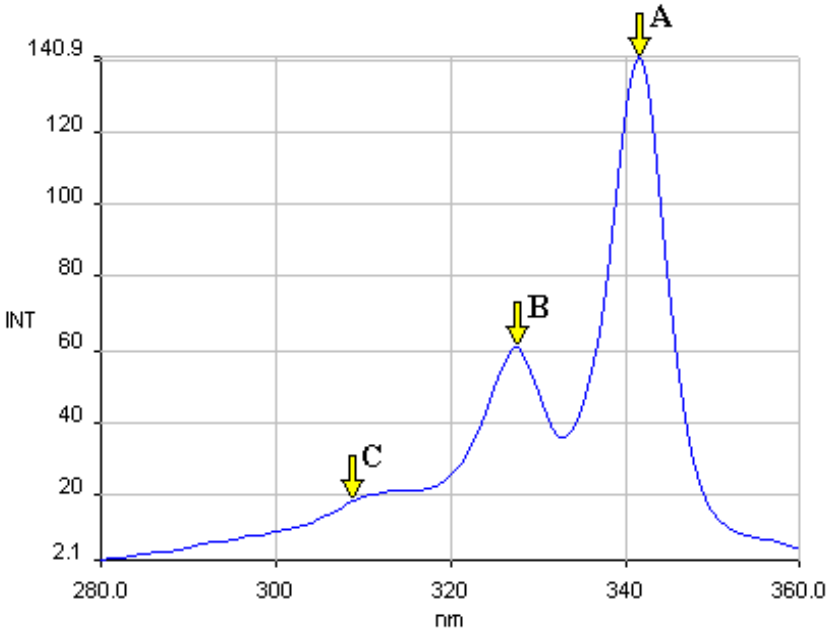
- Ensuring that the absorption of the sample at the excitation wavelength is low (O.D. < 0.1).
- Using a semi-micro cuvette which has a smaller light path (or of course a flow-cell), minimising the pathlength and hence absorption effect.

- Measuring the sample using front surface illumination. The front surface accessory holds the sample surface (in this case a wall of the cuvette) at 60 degrees to the incident light beam, so avoiding scatter. In this case, the emission from the sample is measured at 90 degrees to the excitation beam:



The surface fluorescence is measured, so there is little effective path length through the sample, making the measurement less susceptible to absorption effects.

- Since the inner filter effect is due to the absorbance of light, choose a different excitation wavelength. Since the excitation and absorbance spectra are similar, then at a wavelength different to the maximum, the sample will absorb less light and the inner filter effect will be lower.
In the following example, using the excitation wavelength at ´A´ has led to inner filtering.
Using the wavelength at ´B´, the inner filtering will be reduced by around 2.5-fold.
Using the wavelength at ´C´, the inner filtering should be ten fold lower, giving ten fold greater dynamic range and better linearity.
Note that from principles of fluorescence, the shape of the emission spectrum, and hence the position of the emission maximum, MUST be independent of the excitation wavelength.
Note also that when choosing a different excitation wavelength than the maximum, then chossing a LOWER wavelength will usually give benefits over a HIGHER wavelength, since there will be less Rayleigh scatter and probably less Raman scatter.



Solvents & buffers-absorption effects

An often overlooked fact is that the solvent/buffer can also corrupt assay linearity, even if it has no background fluorescence. Inner filtering caused by excessive sample concentration is relatively easy to identify, because the preparation of a calibration graph will immediately show lack of linearity over a certain domain.
When the solvent/buffer causes inner filtering because of absorption at the excitation wavelength, this is harder to identify since the solvent/buffer may have no fluorescence and hence give no measurable signal on a fluorimeter. Furthermore, changing the sample concentration may not bring the assay into a linear region, however much the sample is diluted.
In any case, the simple expedient of checking the absorption of the sample at the excitation wavelength will clearly show inner filtering, regardless of the cause. If attempts to dilute an over-concentrated sample to below 0.06 O.D., then the solvent/buffer is probably at fault. Measure the solvent/buffer alone (as a blank) to verify this. Ensure that the blank contains ALL components of the sample apart from the analyte, so that for a PicoGreen-based DNA assay, for example, the blank would contain buffer and PicoGreen but NO DNA

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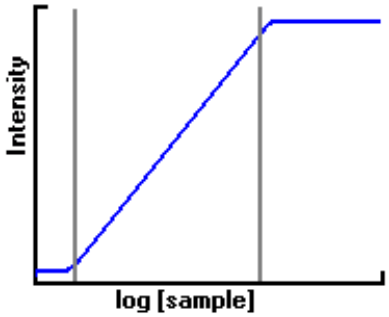
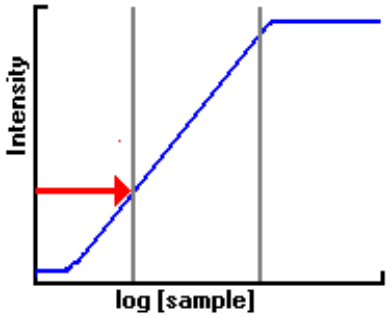
Background luminescence

This can be the luminescence from the buffer/solvent, from living cells, from the solvent used to introduce an agonist/fluorescent dye, or even from the cuvette or sample holder.
The most significant effect of background luminescence is that the dynamic range of the instrument is severely shortened.
This dynamic range is the usable portion of the response curve of the instrument:



below a certain concentration, the sample cannot be discriminated from the background.
Above the instrument´s detection range, response is flattened due to detector saturation.
The usable range in the graph above lies between the two grey lines.

In the graph below, the presence of background intensity compresses the lower end range of the dynamic range. Since fluorescence instruments normally have dynamic range of between 4 and 6 decades, this effect can cause 2 - 3 orders of magnitude of the limit of sensitivity.



The practice of automatically subtracting a single background value from reference samples and unknown samples is rather dangerous, since one may not be aware of the effect that the background signal is having on the dynamic range. Also, background signals have relatively low signal to noise ratios, so the measurement of a single blank can lead to erratic background level subtraction.

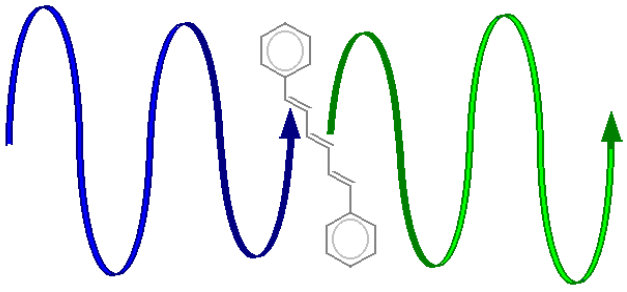
Chapter 5. Application-specific issues

Polarisation measurements

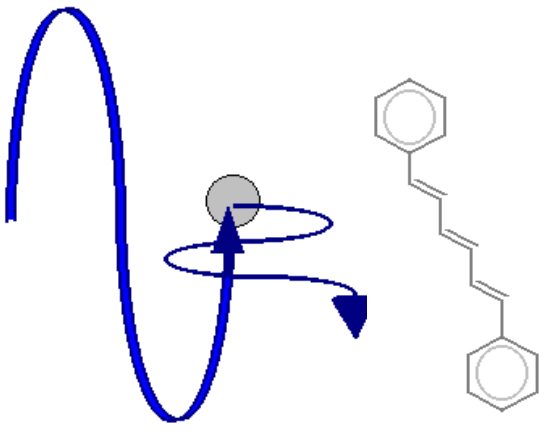
Polarisation measurements are also susceptible to non-linearity problems. These are caused either by inner filtering due to high concentration, or by light scattering, which causes depolarisation and corruption of the measured polarisation value.

Inner filtering due to probe concentration causes one or more of the components of the polarisation measurement to be non-linear. (Polarisation measurements consist of two intensities which are recorded with the polarisers in Vertical/Vertical and Vertical/Horizontal (Ex/Em).
The polarisers decrease light by around 66% each, and when the polarisers are crossed (V/H), the intensity is lower than when both are parallel (V/V).
If inner filtering occurs, it will probably affect the two intensities differently, so one component may be non-linear but the other linear. It may also be that one intensity is off-scale, the other on-scale. This is not so obvious to diagnose since the use sees the result of the processed data.

More problematic is depolarisation. Here the sample itself scatters light at the excitation and/or the emission wavelength. This scattering is dependent on particulates in the sample: many polarisation samples contain lipid vesicles or cells which scatter light very effectively.
For example, a highly polarised molecule absorbs light in the plane of the polarised excitation beam and emits light in the same orientation:

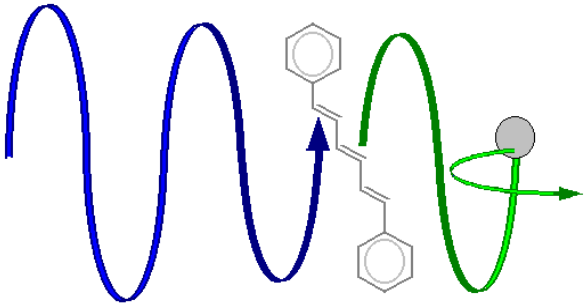


If particulates in the sample scatter the excitation light before it hits the moecule, then the excitation light is no longer vertically polarised. The molecule in the example above would not absorb light:



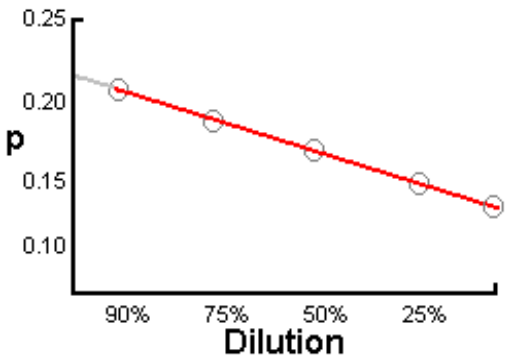
Instead, molecules in a random orientation would be excited.

If the polarised emission from the molecule is scattered by particulates, then the emission signal would be randomised with respect to its polarisation:



The effect of both of these scattering phenomena is that the observed polarisation will be lowered. There are two ways to determine that the interference does not occur. Measuring the absorbance at the excitation and the emission wavelengths is useful as a first approximation. As with inner filtering, the absorbance should be less than or equal to 0.06 absorbance units.

A more accurate way to correct for the problem is to create a dilution curve. The polarisation value should be largely independent of concentration, but the effect of the scattering by micelles and particulates is strongly concentration dependent. Simply dilute the micelle-labelled probe suspension with buffer, starting with 1:1, 2:1, 4:1 and 8:1 dilution and measure the polarisation values. Plot polarisation vs. dilution:



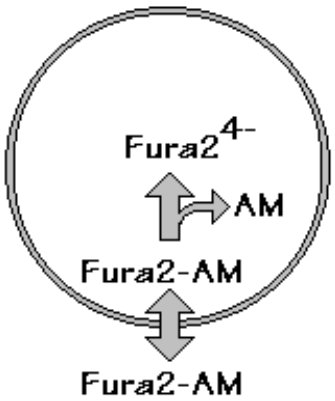
Extrapolate the dilution to 0% micelles. At this point, the true polarisation/anisotropy value (independent of micelle-based scattering) will be reported. If necessary, dilute the sample further until the polarisation/anisotropy remains constant.

Further justification for dilution to reduce scatter depolarisation is that the G-Factor strongly depends on scattering: if the micelle concentration is high, reducing measured p/a values, then on aggregation and settling of micelles, this intereference is reduced, changing values for GF, p and a during an experiment.

A Luminescence Tutorial

Using fluorescence probes in cells -Loading cells using intracellular ion probes such as FURA-2, BCECF etc.

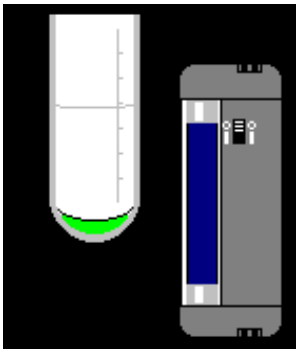
One of the most critical steps in working with intracellular ion-binding dyes is the loading of the dye into the cell. The dye is introduced as an ester which can freely diffuse through the cell membrane. Once inside the cell, the ester link is broken by the action of enzymes occuring naturally inside the cell.
The dye can then bind to its target ion and is also trapped within the cell since it can no longer permeate the membrane due to its charge.



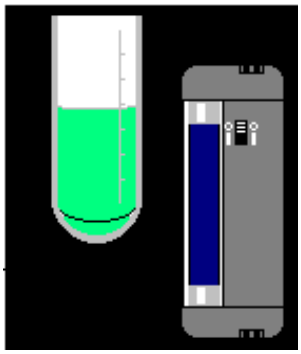
The permeation and hydrolysis are fundamental design concepts of the dye, and are well described in the literature. Even at this early stage in the preparation of the experiment, however, problems can occur. The structure of the cell membrane (particularly with plants) may prevent the permeation of the dye.
The cell may eject the dye once it passes through the membrane. More common is the failure of the cells to hydrolyse the dye.

Checking that the dye is loaded into the cells

To check the loading of dye into the cells, a hand-held UV lamp can be used.
After the first centrifugation step to remove excess dye, observe the centrifuge tube in a darkened room or glove box with the hand-held lamp. If loading has been successful, then an intense fluorescence from the pellet should be seen, with little fluorescence in the supernatant:

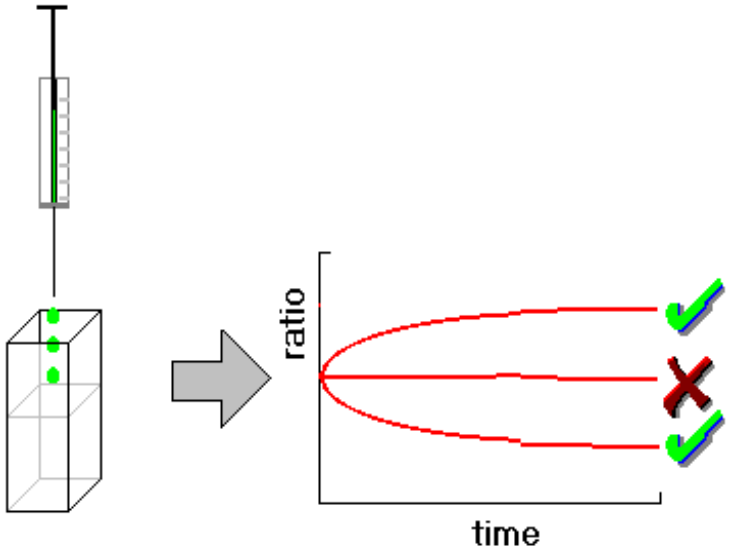


If loading was not successful for any reason, then the fluorescence intensity in the supernatant and pellet will be approximately equal:



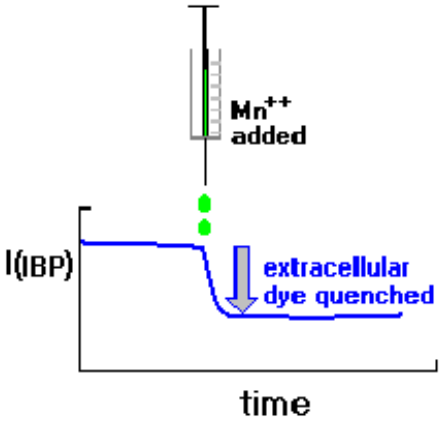
Checking that the cells are correctly hydrolysing the probe

If the cells are not in good condition, then the respiratory enzymes which would normally cause hydrolysis of the dye-ester will not be active enough. The dye will not be hydrolysed and will therefore not be able to report intracellular ion concentration.
The unhydrolysed dye displays spectral characteristics similar to the completely bound dye.
This corresponds to complete saturation by target ion, a condition highly unlikely to occur in physiological conditions. When the dye is hydrolysed it is then responsive to ion concentration and shows a wavelength shift accordingly. In a control experiment, this can be used to check the hydrolysis during loading of dye in the cuvette.
Here, 2ml of cell suspension (approximately 200,000 to 2 million cells depending on the cell type) in buffer are inserted into a quartz cuvette and this is inserted into the cuvette holder and thermostatted to the same temperature as that used in dye loading.
The cell suspension is then treated with an aliquot of dye (to a final dye concentration of for example 1 micromolar) and the ratio of bound/unbound probe is measured for 1 hour.
As dye is successively hydrolysed, a change in the ratio would be expected. If the ratio remains virtually static, then one must suspect that the cells are not healthy and dye hydrolysis is not taking place. Correct loading and hydrolysis would lead to an increase or decrease in the ratio depending on the dye:



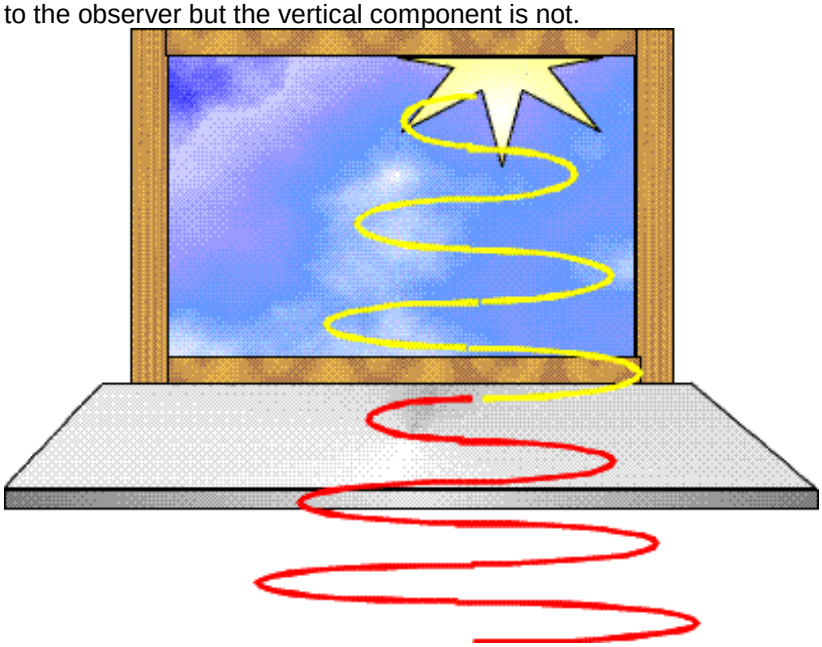
Checking for leakage of the dye

Even if the dye enters the cell and is then hydrolysed, it may be that it is then actively expelled by the cell, or the cell is leaky and allows the dye to emerge into the extracellular matrix.
In either case, extracellular hydrolysed dye is sensitive to the addition of reagent.
In the case of FURA-2, complexing with manganese ions causes very efficient quenching of fluorescence.



Polarisation measurements - Explanation

Light consists of vertical and horizontal components. This effect can be seen by observing how a flat surface reflects sunlight. This is because flat surfaces reflect only the light which lies in the plane of the surface. Sunlight is thus ´selected´ by the flat surface: the horizontally polarised component of the sunlight is reflected to the observer but the vertical component is not.



When the reflected light is observed through a polariser (or pair of sunglasses), then as the polariser is rotated, the surface appears alternately darker then lighter.
The polariser selectively passes light in one plane, so as this plane is in agreement with the reflected light, the reflected light (or ´glare´)can be seen. When the planes of the reflected light and the polariser are perpendicular to each other, the reflected light is filtered out and the surface appears dark.

Fluorescent molecules also selectively absorb light. This is because the electronic orbital which is responsible for the light absorption has absorption and emission dipoles.
These dipoles determine the orientation of light which the molecule absorbs, and how strong this selectivity is.
Because the absorption process is very rapid (~10^{-15} seconds), then the absorption process is like a flash in photography when used to freeze objects: even with continuous light sources, and in a sample containing many molecules which are randomly oriented, only those which are correctly aligned are excited.

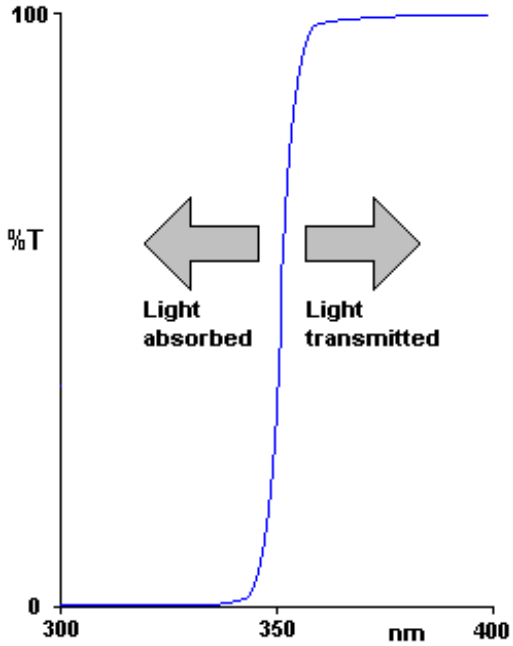
These excited molecules will then emit a photon (as fluorescence) within perhaps 10^{-9} to 10^{-8} seconds. When this occurs, the emitted light is also polarised in the same plane as the molecule´s electric dipole.
This phenomenon provides very useful information about the molecule´s interaction with its environment.
If the molecule is held completely rigid throughout the lifetime of the fluorescence emission, then the emitted light will be polarised in the same plane as the exciting light. If the molecule can freely rotate and tumble, then the emitted light will consist of randomly polarised components.

A Luminescence Tutorial

Chapter 6. Glossary

Cut-off filter

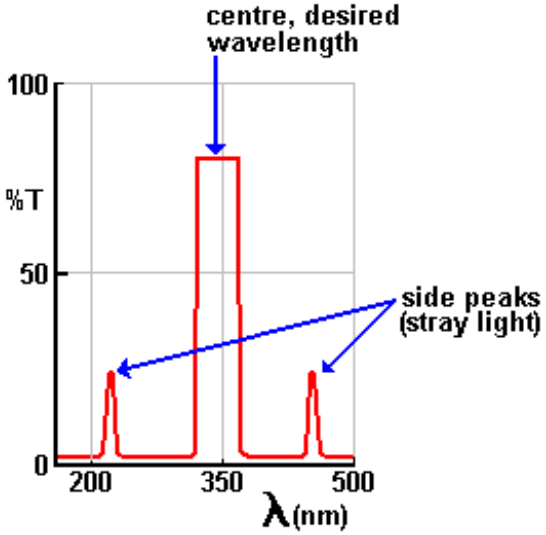
Also called a high-pass filter: this transmits light above the quoted cut-off wavelength but strongly absorbs light below it. It´s transmission spectrum looks similar to the following example, for a 350nm cut-off filter:



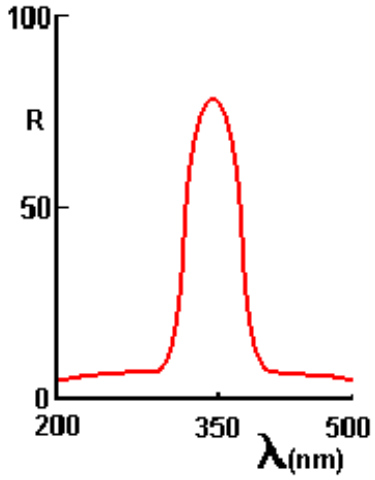
Cut-off filters are normally used to remove Second order scatter but are also useful in enhancing sensitivity by measuring a broad band of light rather than a relatively narrow band selected using a monochromator. In this mode, Cut-Off filters are best used in conjunction with a Total Emission mirror.

Stray light

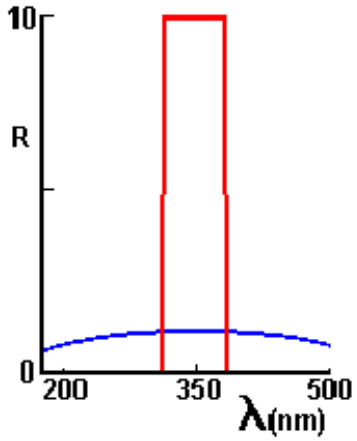
Every type of optical monochromator has stray light characteristics, although gratings normally suffer more from this than filters. Stray light is the band of wavelengths of light outside the nominally selected wavelength band which escape through the monochromator system. For a filter, this may be the presence of side bands outside the desired transmission range:



For a grating, the stray light is caused by the inability of the grating to supply completely ´pure´ light. A typical curve of monochromator reflectivity against wavelength would appear as follows:



This may not appear too alarming, but by zooming in around the desired transmission wavelength we see the problem:



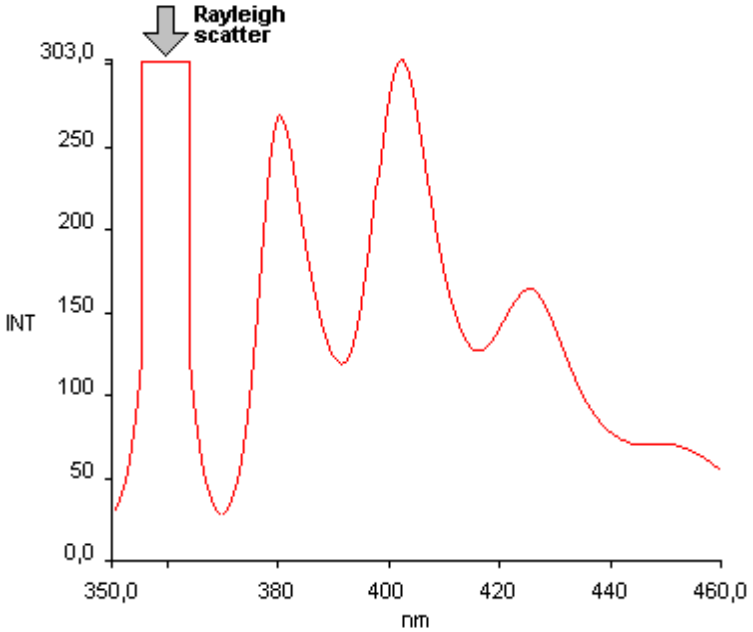
The desired band (shown in red) is superimposed upon broad band stray light (shown in blue).
This broad band may only have a relative intensity equal to 0.1% to 1% of the transmission maximum, but in fluorescence spectrometry, 1% of the excitation light reflected through to the sample detector represents a very high signal.
In cuvette or flowcell-based assays, this is not a particular problem, since the sample does not scatter much light into the sample detector. When the sample can scatter light, however, this becomes a major problem. It means that in addition to delivering the desired light to excite the sample, we also deliver light across a broad spectrum, including the emission wavelength, which can then produce huge background signals. It can also increase noise dramatically, because:

- the broad band stray light will be measured by the emission detector, but may not induce fluorescence in the sample: this produces a ´decoupled´ source of noise

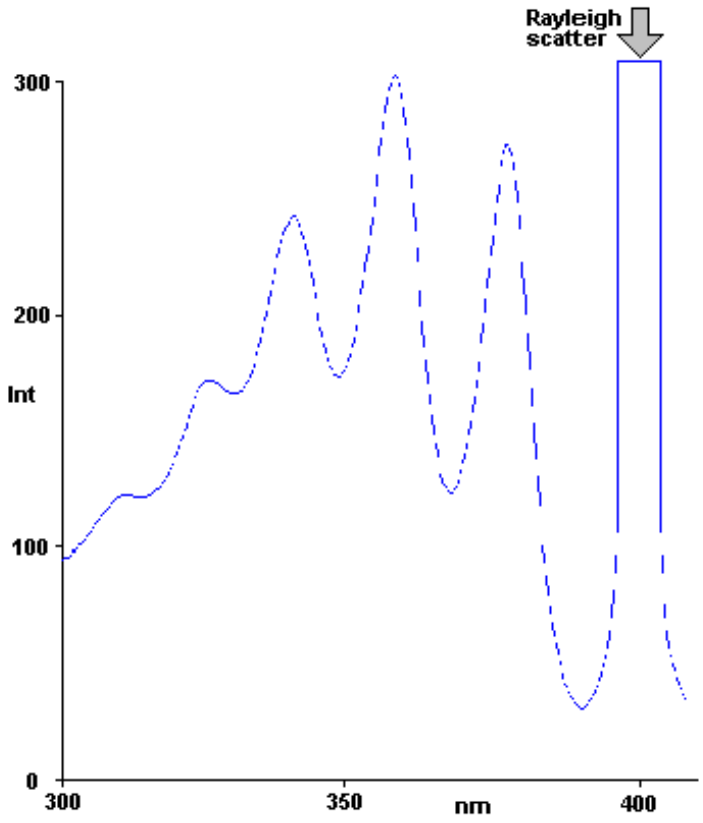
- any changes in the light scattering properties of the sample can have large effects on the background signal: when these changes occur between blank samples, then the standard deviation of error of the blank noise will increase sharply although the actual fluorescence level of the blank may not have changed significantly.

Rayleigh scatter

Rayleigh scatter is caused by the scattering of excitation light directly through the emission monochromator onto the sample detector. It can be observed when scanning either excitation or emission spectra. When scanning emission spectra, the Rayleigh scatter is observed near to the start of the spectrum:



When scanning excitation spectra, the Rayleigh scatter is observed near to the end of the spectrum:

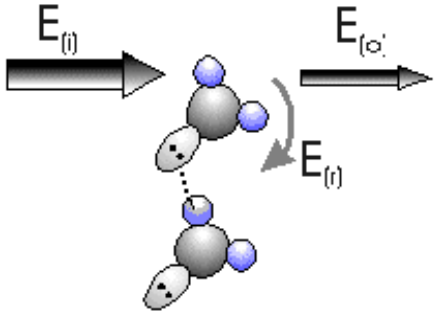


The intensity of the observed Rayleigh peak depends on the sample: where there is much light scatter, then

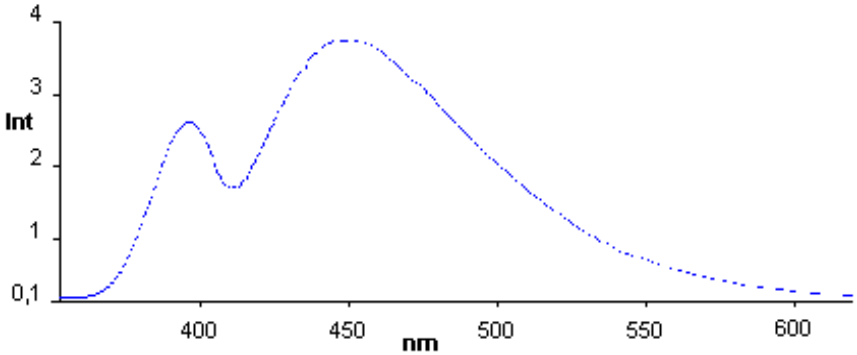
the peak can be very large. Where the scattering is low, the peak can be almost invisible. Scattering of light depends on the amount of particulate matter, or refractive species such as micelles or cells in the sample. Scattering can also be much increased by the nature of the sampling accessory: when there are reflective objects within the measured sample area, then this can cause back-reflection of light into the emission monochromator.
In some cases, where the sample detector of the instrument has no overload protection, allowing Rayleigh scatter directly thorugh the optical system could lead to permanent damage, or instability for several minutes while the detector ‘settles down´.

Raman scatter

Raman scatter is a solvent-based phenomenon. It is caused by collision of the incident light photon with the solvent molecule, imparting rotational motion on it (in other words, the incident light collides with the solvent molecule and sets it tumbling).



The energy absorbed by the solvent (in this case water) depends upon the degree of solvent-solvent interaction and the temperature. The higher the degree of hydrogen bonding and Van der Waal´s forces present, then the more tightly bound the solvent molecules and the higher the energy absorbed. At any given temperature for any given solvent, however, this energy is constant, and leads to the appearance of a small, symmetrical peak at longer wavelength than the excitation wavelength. This can lead to the incorrect identification of a Raman bands as an analytical peak, particularly at low concentrations where the Raman band becomes increasingly significant. The following figure shows an emission spectrum of a common fluorophore at concentration 10^{-8} M.

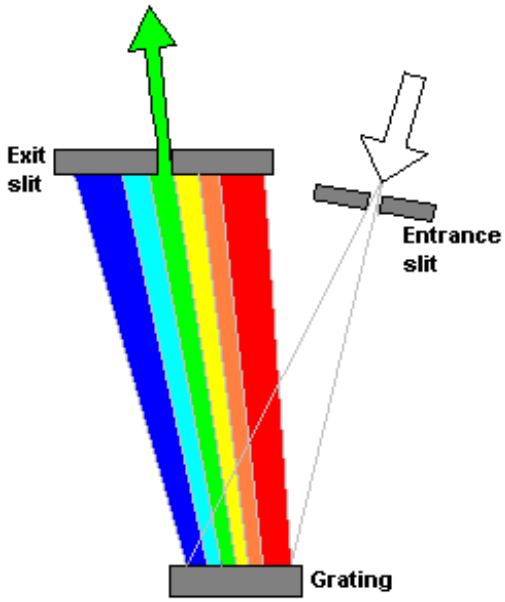


The emission peak of the sample at around 450nm is only a factor of two higher in intensity than the Raman band at around 397nm. As the concentration decreases, then the Raman band becomes even more significant. At a concentration of 10^{-10} M or less,
the Raman band can be the highest observed peak.

The difference between the excitation wavelength and the Raman peak is given by an energy value E(o), so the difference will not be constant with changing excitation wavelength. For example, if the excitation wavelength is set to 350nm, then the Raman peak would be seen at 397nm. The following table shows some Raman band wavelengths for water.

Second order scatter

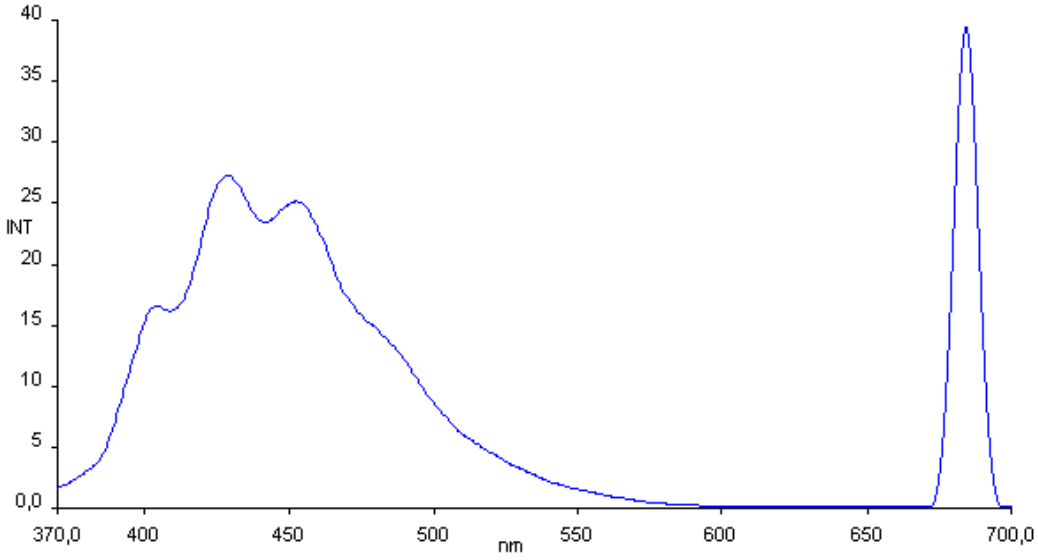
(which is seen when the emission monochromator is driven through a wavelength equal to twice the excitation wavelength)
Second order scatter is an instrument artefact, and is caused by a property of the grating in the monochromator. The grating disperses light in a predictable manner, so that it can be rotated to pass a known wavelength of light to the sample or to the sample detector.
This is done using slits to select bands of light at definite locations to separate out the desired wavelength:



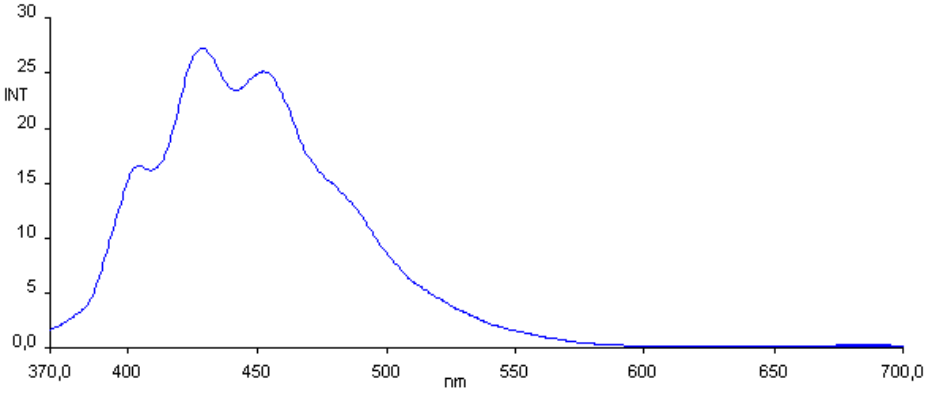
Unfortunately, the grating also passes other wavelengths at this selected position. These other wavelengths are harmonics of the fundamental wavelength, so for example if the emission grating is set to 500nm, then it will pass 500nm light to the detector but also 250nm light, 1000nm light and so on.
If the emission monochromator scans through a wavelength equal to twice the excitation wavelength, then the emission monochromator allows through the light at the excitation wavelength as one of its harmonics. Similarly, as the excitation monochromator is scanned, it produces harmonics of it´s currently selected wavelength. These harmonics complicate the observed spectrum and can confuse the user as to the correct analytical wavelength to use.

A Luminescence Tutorial

In the following example, the emission spectrum of DPH (diphenylhexatriene) has been scanned between 370nm and 700nm. The excitation wavelength was 340nm, the excitation and emission slits were both set to 5nm. As the emission monochromator scanned through 680nm, a symmetrical peak was observed.



It is critical to note that second order scatter is caused by light at the excitation wavelength and not at emitted light at 680nm. This is often obvious because the user observes a strong peak at a wavelength above the limit of the detector, since ´standard´photomultipliers have an upper limit in their response of around 650nm. Fluorescence emission peaks above the detector´s response range would be recorded with very low sensitivity, if at all.
Second order peaks can therefore be removed using a suitable cut-off filter in the emission beam between the sample and the monochromator. This filter allows any wavelength above it´s cut-off wavelength through to the monochromator, but cuts off the second order scatter because this is below the cut-off wavelength.
When the emission spectrum of DPH is re-scanned with a 350nm cut-off filter between the sample and the emission monochromator, the result is as follows:



The position of the second order scatter peak can be particularly confusing when the emission peak lies around twice the typical excitation wavelength. Examples include ethidium bromide and uranium. Although one would expect the scatter peak to be independent of sample concentration and therefore simple to identify, this is not always so.
If the sample concentration is high enough to cause significant absorption of the excitation light, then not as much light reaches the measured, central part of the cuvette, so the amount of observed scattered light decreases in a sample concentration-dependent manner.