Spectrofluorometry

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Spectrofluorometers

- Spectrofluorometers (or fluorescence spectrophotometers) measure the fluorescence signature of an analyte in a sample based on its specific excitation and emission wavelengths.
- The fluorescence signature can be correlated to the concentration level of the analyte in the sample.
- A spectrofluorometer can be used in basic and applied research:
  - biofuels analysis,
  - biotechnology applications,
  - quality control,
  - medical diagnostics,
  - plasma monitoring
  - polymer analysis
  - as a tool in teaching laboratories

Principles

- Fluorescence is an emission phenomenon where an energy transition from a higher to a lower state is accompanied by radiation.
- Only molecules in their excited forms are able to emit fluorescence; thus, they have to be brought into a state of higher energy prior to the emission phenomenon.

...Principle

- A molecule in its electronic and vibrational ground state (S0v0) can absorb photons matching the energy difference of its various discrete states.
- The required photon energy has to be higher than that required to reach the vibrational ground state of the first electronic excited state (S1v0).
- The excess energy is absorbed as vibrational energy (v>0), and quickly dissipated as heat by collision with solvent molecules.
- The molecule thus returns to the vibrational ground state (S1v0).
- These relaxation processes are non-radiating transitions from one energetic state to another with lower energy, and are called internal conversion (IC).
- From the lowest level of the first electronic excited state, the molecule returns to the ground state (S0) either by emitting light (fluorescence) or by a non-radiative transition.
- Upon radiative transition, the molecule can end up in any of the vibrational states of the electronic ground state (as per quantum mechanical rules).

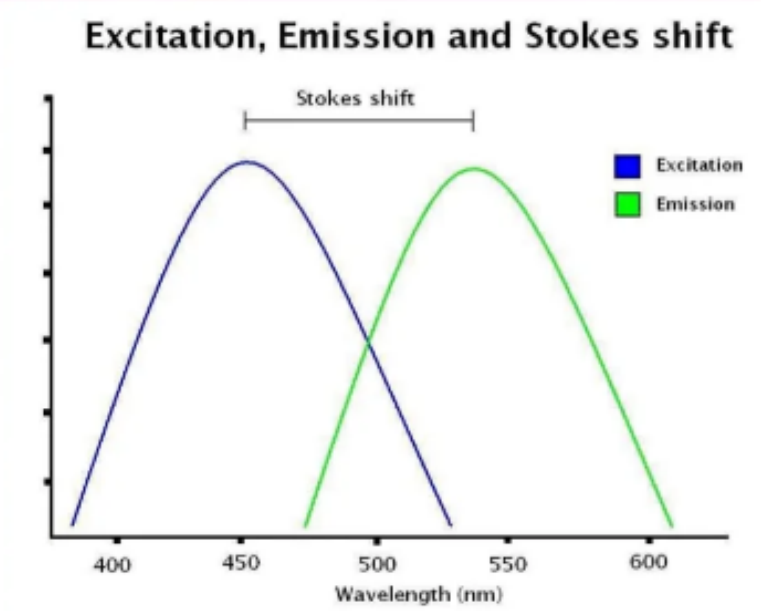
- Since radiative energy is lost in fluorescence as compared to the absorption, the fluorescent light is always at a longer wavelength than the exciting light (Stokes shift).
- The emitted radiation appears as band spectrum, because there are many closely related wavelength values dependent on the vibrational and rotational energy levels attained.
- An associated phenomenon in this context is phosphorescence which arises from a transition from a triplet state (T1) to the electronic (singlet) ground state (S0).
- The molecule gets into the triplet state from an electronic excited singlet state by a process called **intersystem crossing (ISC).**
- The transition from singlet to triplet is quantum-mechanically not allowed and thus only happens with low probability in certain molecules where the electronic structure is favourable.
- Such molecules usually contain heavy atoms.
- The rate constants for phosphorescence are much longer and phosphorescence thus happens with a long delay and persists even when the exciting energy is no longer applied.

Excitation and Emission of Fluorophores

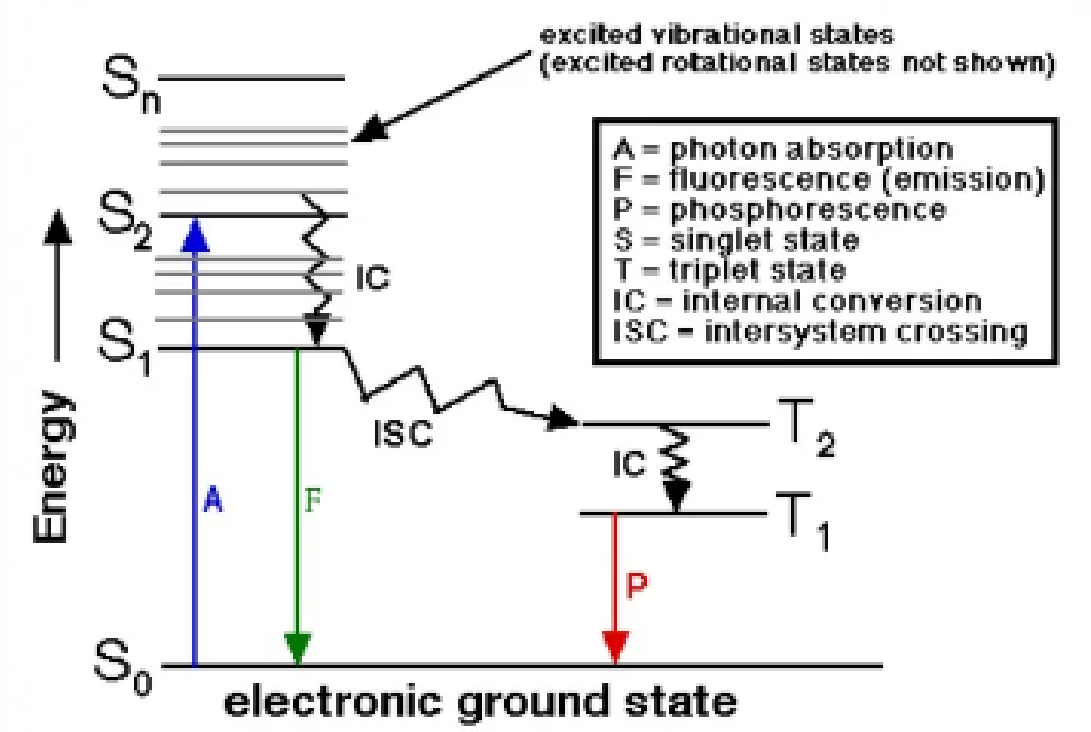
- The physics behind fluorescence involves the different electronic and vibrational states that fluorophores can exist in.
- An electronic state is divided into multiple vibrational states.
- Photons, that have energies in the ultraviolet to blue-green range of the spectrum can trigger an electronic transition from a lowest vibration in the ground state to one of the vibrational levels in a higher electronic excited state.
- As soon as the energy input from the photon (in other words the excitation) stops, the fluorophore molecule relaxes into the lowest vibrational level of the excited electronic state.
- The fluorophore remains in this state for some time (around 10 nanoseconds, known as the fluorescence lifetime) and then returns to the electronic ground state.
- This return to the ground state is associated with a release of energy, known as fluorescence emission.

Quantum yield

- The number of photons emitted by a fluorophore, relative to the number of photons absorbed, is called the quantum yield. A fluorophore with a large quantum yield, like rhodamine, will display a bright emission.
- The difference between excitation and emission wavelengths is called Stokes shift.
- Stokes' studies of fluorescent substances led to the formulation of Stokes' Law, which states that the wavelength of fluorescent light is always greater than that of the exciting radiation.



- The process that happens between excitation and emission is illustrated using Jablonski diagrams (named after the father of fluorescence spectroscopy, Alexander Jablonski).



- **Triplet excited state: An excited state in which unpaired electron spins occur.(when the analyte returns from a higher- energy state to a lower- energy state with the opposite spin)**
- **Singlet excited state: An excited state in which all electron spins are paired. (When the analyte returns from a higher- energy state to a lower- energy state with the same spin)**

A molecule's fluorescence quantum yield is influenced by external Variables such as:

- Temperature
- viscosity of solvent
- pH
- Increasing temperature generally decreases Φf because more frequent collisions between the molecule and the solvent increases external conversion.
- Decreasing the solvent's viscosity decreases Φf for similar reasons.
- For an analyte with acidic or basic functional groups, a change in pH may change the analyte's structure and, therefore, its fluorescent properties.

- Intrinsic fluorophores are molecules with a natural fluorescence, such as chlorophyll and the aromatic amino acids.
- Extrinsic fluorophores are those that are added to a sample to provide fluorescence, or to change the spectral properties of the sample.
- Examples of extrinsic fluorophores are fluorescein and rhodamine, but there are many more.
- Each fluorophore has its own characteristic properties, such as fluorescence lifetime, intensity, and position of the emission wavelength, thus, each fluorophore will yield a unique fluorescence spectrum.
- A fluorescence spectrum is a plot of the fluorescence intensity as a function of wavelength.
- Fluorescent intensity F is dependent on both intrinsic properties of the compound (fluorescence quantum yield Φf), and on readily controlled experimental parameters including:
  - intensity of the absorbed light I_0
  - molar absorption coefficient Ɛ
  - path length of the cell b
  - concentration of the fluorophor in solution c

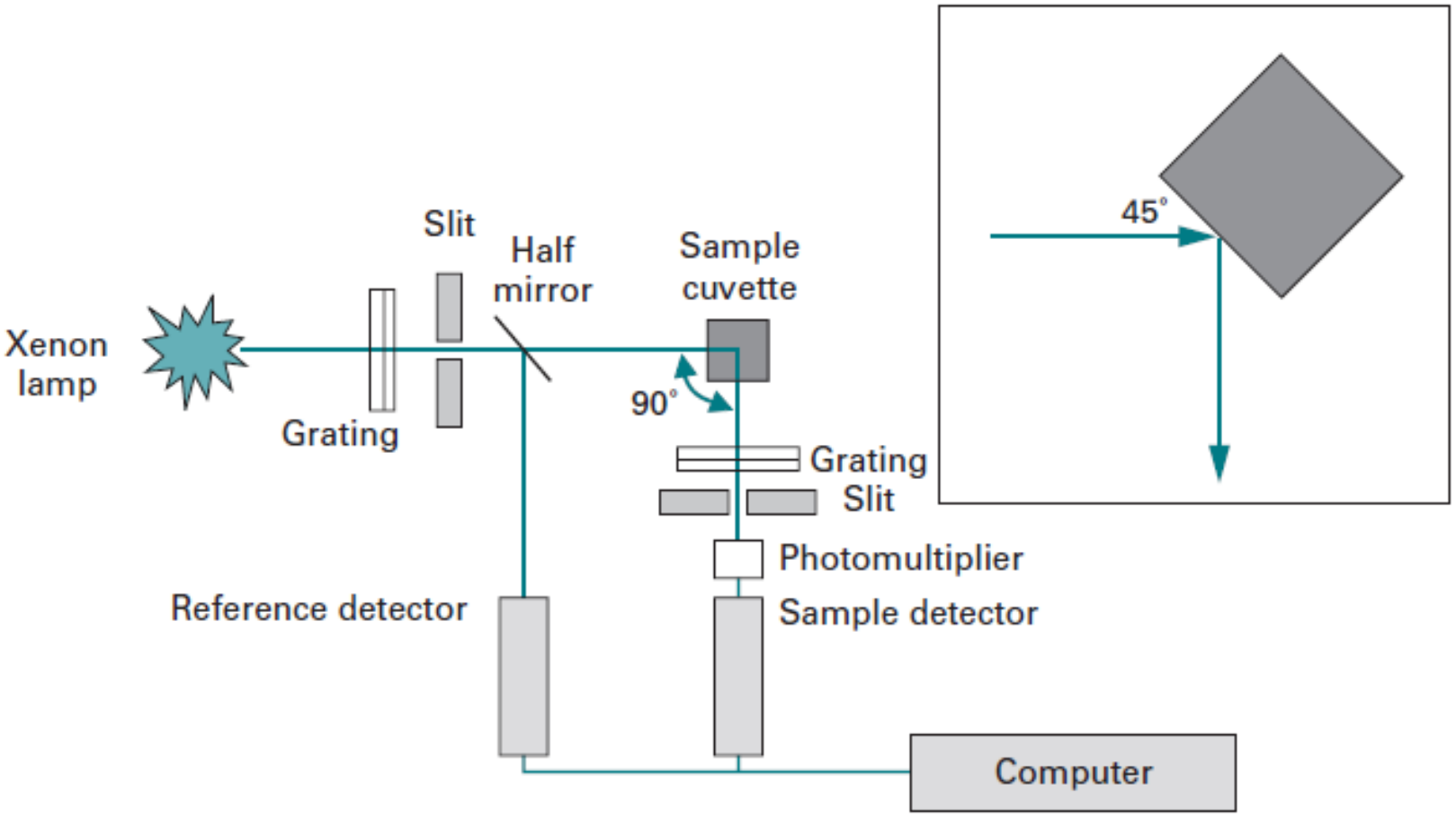
$$F = \phi I_0 (1 - e^{-\varepsilon b c})$$

How a spectrofluorometer works?

- The essential components of a spectrofluorometer are a light source, an excitation monochromator, a sample cell/cuvette, an emission monochromator and a detector.
- Fluorometers quantify biological analytes as a function of fluorescence.
- This requires the sample to be bound to a specific fluorescent agent and loaded into the instrument in a cell/cuvette holder.
- The light source sends out light at the excitation wavelength of an analyte in a sample.
- Before it reaches the sample, the light passes through the excitation monochromator, which transmits a wavelength specific to the excitation spectrum of the analyte while blocking other wavelengths.
- The light from the excitation monochromator passes through the sample contained in the sample cell/cuvette holder and excites the analyte (fluorophore).
- Fluorophores absorb light of a distinct excitation wavelength and emit, or fluoresce, light of reduced energy thus a longer wavelength.

...How a spectrofluorometer works?

- The emitted light passes through the emission monochromator positioned at a right angle to the excitation light.
- The emission monochromator minimizes light scatter and screens the emission light before it reaches the detector.
- The detector measures the emitted light, displays the fluorescence value and produces the fluorescence signature of the analyte.
- The fluorescence value is proportional to the concentration level of the analyte in the sample.


Slit
Half
mirror
Sample
cuvette
Xenon
lamp
Grating
90°
Grating
Slit
Photomultiplier
Reference detector
Sample detector
Computer
45°

What can Spectrofluorometer Do?

- It has been used for the direct or indirect quantitative and qualitative analysis by measuring the fluorescent intensity F.
- It is relatively inexpensive and sensitive (the sensitivity of fluorescence is approximately 1,000 times greater than absorption spectrophotometric methods).

Applications of fluorescence spectroscopy

Bioscience

- In the biosciences, one of the most frequent applications of fluorescence spectroscopy is the high precision quantification of DNA and RNA using ethidium bromide as an extrinsic fluorophore.
- Another modern application is SMRT (single molecule real-time) DNA sequencing. In its ability to produce long read single molecules with high accuracy, it is predicted to be central to the next genetic diagnostic revolution.
- Proteins possess three intrinsic fluorophores: tryptophan, tyrosine and phenylalanine, although the latter has a very low quantum yield and its contribution to protein fluorescence emission is thus negligible. The main application for intrinsic protein fluorescence aims at conformational monitoring.

Industrial

- Fluorescence spectroscopy is used in several industrial settings as a fast, noninvasive technique in the assessment of contamination.
- For example, it has been used to detect contaminating organic compounds in groundwater, after hydraulic fracturing for gas exploration.

... Applications of fluorescence spectroscopy

Chemical

- An important chemical application of fluorescence spectroscopy can be found in the field of nanoparticle synthesis for potential medical uses, such as drug delivery.

Environmental

- In environmental monitoring, the technique also has wide application.
- High-resolution fluorescence spectroscopy and 3D-excitation emission matrix fluorescence spectroscopy are used to characterize dissolved organic matter in these samples and then based on that, optimize treatment processes for landfill.

... Applications of fluorescence spectroscopy

Pharmaceutical

- Spectrofluorometric techniques are also used in the pharmaceutical field to analyze drugs.
- An example is the analysis of co-formulated tablets prescribed as cholesterol medication.

Agricultural

- In agriculture, spectroscopic techniques are also widely applied for instance in the identification of different crop varieties.
- Likewise, total luminescence spectroscopy can be used by tea manufacturers as a quick, affordable and objective alternative to employing trained tea tasters, to discriminate between similar types of tea.

THANKS