Light can be absorbed and re-emitted by matter – luminescence (photo-luminescence). There are two types of luminescence, in this discussion: fluorescence and phosphorescence.

A Fraction of the light energy (radiation) absorbed by molecules is channeled into other energy regimes like vibrational energy, phonons, rotational energy, etc. The remaining energy is then re-emitted as photons at a longer wavelength (lower photon energy) than the absorbed photon.

A phonon is a quantized mode of vibration occurring in a rigid crystal lattice of a solid.

**Fluorescence Spectroscopy**

![Fluorescence Diagram](image)

**Jablonski Diagram:** Shows the fate of excited state molecules.

**Excited vibrational and rotational levels of T<sub>1</sub> electronic state**

**Luminescent molecules absorb in UV-VIS region.**

![Absorption Spectrum](image)

**Absorption:** S<sub>0</sub> → S<sub>1</sub> very fast; 10<sup>-15</sup> - 10<sup>-13</sup> s

**External Conversion:** radiation-less transitions to lower state by collisional deactivation

**Internal Conversion - IC:** radiation-less transitions to lowest vibrational meta stable excited electronic state. (Kasha’s Rule)

**Intersystem Crossing - ISC:** transition with spin change (S ↔ T).

**Fluorescence:** emission not involving spin change (S<sub>1</sub> → S<sub>0</sub>) efficient, short lived S<sub>1</sub>; 10<sup>-9</sup> - 10<sup>-5</sup> s.

**Phosphorescence:** emission involving spin change (T<sub>1</sub> → S<sub>0</sub>) long lived T<sub>1</sub> 10<sup>-3</sup> - 10 s; less probable transition; “forbidden”.

**Dissociation:** excitation energy transfer to v. high energy, v, vibrational state breaks bond – photolysis.
**Fluorescence** is the process of absorbing and re-emitting light on a time scale of about $10^{-8}$ seconds (rapid relaxation/shorter excited state lifetime) while phosphorescence processes are much slower, taking about $10^{-3}$ to $10$ s to occur (slower relaxation/longer excited state lifetime).

The spectroscopy of the emission radiation from relatively short lived excited states - fluorescence spectroscopy.

Experiment: Molecules capable of fluorescing (fluorophore) are placed in a cuvette and is exposed relatively high-energy (UV-Vis), monochromatic radiation.

Subsequent to the absorption of energy, some molecules, will relax by emitting radiation of lower photon energy.

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The light emitted by the fluorophore is made up of a range of different wavelengths (continuous ‘radiation’, practically).

IC puts the fluorophore into the lowest energy state $S_0$. The fluorophore then returns to the ground state by emission of photons with energy $\approx h\nu$.

For every photon that is absorbed, the frequency (and energy) of the emitted photon will vary since the emitted photon’s energy (and frequency) depends on which vibrational level of $S_0$ (e.g. 0, 1, 2, 3, etc.) the fluorophore relaxes/returns to.

Fluorescence

$\lambda_{\text{max}}$ of excitation (absorption) $\leq$ that of fluorescence (emission).

T1 can ISC to $S_0$, thus phosphorescence intensity is generally very low.
Often a Stokes shift is observed.

\[ \lambda_0 \text{ for absorption} \]

\[
\lambda_{\text{ex}} = \lambda_{\text{max of UV}}^c
\]

\[
P_b = P_0 10^{-\varepsilon_{\text{bc}}} \]

\[
\lambda_{\text{em}} = \lambda_{\text{max of UV}}^c
\]

\[
P_0 = P_1 10^{-\varepsilon_{\text{em}}} \]

\[
P_{b1} = P_1 10^{-\varepsilon_{\text{bc}}}
\]

\[
P_{b2} = P_1 10^{-\varepsilon_{\text{bc}}}
\]

\[
P_{b3} = P_1 10^{-\varepsilon_{\text{bc}}}
\]

\[
F = k' P_0 10^{-\varepsilon_{\text{bc}}} \left(1 - 10^{-\varepsilon_{\text{bc}}} \right) 10^{-\varepsilon_{\text{em}} b_3 c}
\]

\[
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\]

**Pre-filter and Post-filter Effects**

- Beam power entering plane @ \( b_1 \): \( P'_b = P_0 10^{-\varepsilon_{\text{bc}}} \)
- Beam power leaving plane @ \( b_2 \): \( P' = P_1 10^{-\varepsilon_{\text{bc}}} \)
- Power absorbed: \( \left( P'_b - P' \right) \)
- Emission intensity: \( P_{\text{em}} \propto \left( P'_b - P' \right) = k \left( P'_b - P' \right) \)
- Emission intensity from the cell, \( I \), after post filter effects; \( F \)
- Fluorescence, \( F \) : \( F = I \propto P_0 10^{-\varepsilon_{\text{bc}}} \)
  \[
  F = k' \left[ P'_b - P' \right] 10^{-\varepsilon_{\text{bc}}} \\
  F = k' P_0 10^{-\varepsilon_{\text{bc}}} - P_1 10^{-\varepsilon_{\text{bc}}} \left(1 - 10^{-\varepsilon_{\text{bc}}} \right) 10^{-\varepsilon_{\text{bc}}} \\
  F = k' P_0 10^{-\varepsilon_{\text{bc}}} \left(1 - 10^{-\varepsilon_{\text{bc}}} \right) 10^{-\varepsilon_{\text{bc}}}
  \]

Loss of power in distance \( b_1 \), pre-filter

Loss in distance \( b_3 \), post-filter, self absorption reduces the signal, can change the shape of true emission spectrum
At low concentrations, the exponential terms are close to zero simplifying:

\[ F = k'P_0(1 - 10^{-x \ln 10})^{10^{-x \ln 10}} \]

using the identity; \( 10^x = 1 - x \ln 10 + \frac{(x \ln 10)^2}{2!} - \frac{(x \ln 10)^3}{3!} + \ldots \)

and at \( x << 1 \)

\[ 10^x = 1 - x \ln 10; \quad 1 - 10^x = x \ln 10 \]

\[ F = k'P_0(1 - 10^{-x \ln 10}) = k'\Phi \epsilon_{ex} b c \ln 10 = k'\Phi \epsilon_{ex} b c \ln 10 \]

defining \( k = k'\Phi \epsilon_{ex} b c \ln 10 \)

\[ F = kP_0 c \]

At low concentrations emission intensity is directly proportional to the analyte concentration.

\[ F = k'P_0(1 - 10^{-x \ln 10})^{10^{-x \ln 10}} \]

Part of irradiation gets lost in, the \textit{pre-filter} region. Part of fluorescence may get lost in the \textit{post-filter} region: \textit{is significant at high concentrations}. Radiation damping; color quenching due to overlap (self absorbance).

Result – measured fluorescence is less than absolute emittance.

\[ \Phi = \text{quantum yield} < 100\% \]

\[ \Phi = \text{quantum yield} < 1 \]

Often this is the type observed

Quantitative Aspect:

The relationship between fluorescence intensity and analyte concentration is:

\[ F = k\Phi P_0(1 - 10^{-x \ln 10}) \]

where \( F \) = fluorescence intensity, \( k \) = geometric instrumental factor, \( \Phi \) = quantum yield (photons emitted/photons absorbed),

\( P_0 \) is the radiant power of the excitation source, \( \epsilon_{ex} \) = molar absorptivity coefficient, \( b \) is the path length of observed region, and \( c \) is the analyte concentration (\( \epsilon_{ex} \), \( b \), and \( c \) are the same as in the Beer’s Law).
Expanding the previous equation in a series and neglecting the higher terms gives:

\[ F = 2.303k\Phi_e bP_0c = kc \]

where \( k = 2.303k\Phi_e bP_0 \)

Valid at concentrations <10^{-5} M.

Note: fluorescence intensity \( F \) is linearly proportional to analyte concentration.

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**Generation of Excitation and Emission spectra**

**Procedure 1**

Excitation spectra are very similar to the UV spectra. Thus as a first step one can take the UV spectrum; determine the wavelength of maximum absorption wavelength, \( \lambda_{ex} \).

Set the excitation monochromator at \( \lambda_{ex} \) and scan the emission monochromator, record the detector output, which is the emission spectrum. Determine the emission maximum wavelength \( \lambda_{em} \).

Set the emission monochromator at \( \lambda_{em} \) and scan the excitation monochromator, record the detector output, which is the true excitation spectrum.

**Procedure 2**

Set the excitation monochromator at a low wavelength and scan the emission monochromator, record the detector output, which is the emission spectrum. Determine the emission maximum wavelength \( \lambda_{em} \).

Set the emission monochromator at \( \lambda_{em} \) and scan the excitation monochromator, record the detector output, which is the true excitation spectrum. The maximum value of \( \lambda \) in this spectrum is \( \lambda_{ex} \).
Detection limit: superior, (1/1000) that of UV-VIS.

Limitations:
Limitations of the Beer’s Law also affect quantitative Fluorimetry. Fluorescence measurements are susceptible to inner-filter effects. These effects include excessive absorption of the excitation radiation (pre-filter effect) and self-absorption of fluorescence emission (post-filter effect).

Raleigh Scatter:
Excitation radiation being scattered by solvent, appears in the fluorescence spectrum as a ‘pseudo’ peak. Avoid if possible.

Raman Scatter:
Part of excitation radiation being transferred to solvent molecules, appears in the emission spectrum shifted way toward red. 1/1000 times weaker than Raleigh scatter. Easily correctable.

In luminescence spectroscopy, the superior detection limits come about due to two reasons.

1. Emission spectroscopy – measures absolute emissions (as opposed to absorption spectroscopies which measures small differences of P and P₀ at low concentrations).

2. The emission is monitored with the minimal of background signals – by virtue of the geometry of observation of the emission beam at right angle.

In samples containing macromolecules or unfiltered particles, scatter is an inevitable contributor to the background signal.

Phosphorescence Spectroscopy

A quasi-stable electron excited state involving a changed spin state (triplet) via intersystem crossing would decay slowly because transition to the initial state singlet is forbidden; Selection rule (ΔS=0).

In phosphorescence, light emitted by an atom or molecule persists after the exciting source is removed because of the inherently longer lifetimes of the triplet state.

Thermal energy would also raise the electron to the triplet state thus phosphorescence is temperature-dependent.
Energy dissipation after the excitation of a sample with a pulsed xenon source in phosphorescence mode.

Pulsed mode - further eliminates any background emissions detected by the detector because the source is turned off during emission signal detection.

Note: in samples containing macromolecules or unfiltered particles, scatter is an inevitable contributor to the background signal.

Absorption: Highest when $10^{-\varepsilon_{\text{em}}b_c}$ is at its lowest, i.e. the irradiating beam (excitation monochromator) is set at $\lambda_{\text{max.ex}}$.

Detector signal is at its highest when the emission monochromator is set at $\lambda_{\text{max.em}}$.

$$F = k' P_0 10^{-\varepsilon_{\text{ex}}b_c} (1 - 10^{-\varepsilon_{\text{ex}}b_2c}) 10^{-\varepsilon_{\text{em}}b_2c}$$
Synchronous Fluorescence Spectroscopy

Synchronous fluorescence spectroscopy (SFS) introduced by Lloyd (1971), is another fluorometric technique where the fluorescence signal is recorded when excitation and emission wavelengths are simultaneously scanned keeping in between them, a fixed wavelength interval (offset value, $\Delta \lambda$; $|\Delta \lambda| = \lambda_{\text{max,em}} - \lambda_{\text{max,ex}}$) throughout the spectrum acquisition.

When both monochromators are changed simultaneously at the offset there comes a point when excitation monochromator is at $\lambda_{\text{max,ex}}$, emission monochromator will be exactly at $\lambda_{\text{max,em}}$ thereby produces a large signal in the detector.

The main purpose of synchronous scanning is to generate spectra having decreased bandwidths.

With a mixtures of fluorescent components, synchronous scanning with judiciously selected offsets would greatly simplify the spectrum by decreasing the extent of spectral overlaps.

SFS at optimized offset values offers a very simple and effective means of obtaining data from several compounds in a single scan with improved peak resolution (Deyl et al., 1999).
ELISA – Enzyme-linked Immuno-Sorbent Assay.

Technique based on the principle of antigen-antibody interaction. In response to ‘foreign molecules (antigen)’, living systems produce a protein (antibodies).

\[ \text{antigen} + \text{antibody 1} = \text{adduct (complex)} \quad K_{eq} >> 0 \]

The adduct formation is due to the antibody specifically recognizing a part (moiety) of the antigen (molecular recognition).

A **second antibody (with a labeled enzyme)** which can recognize another moiety of the analyte (antigen) is allowed to interact with the ‘adduct’.

Introduce a reactant that could be converted to a chromophore or a fluorophore by reaction with the enzyme.

Depending on the time allowed for the enzyme catalyzed reaction to take place, the concentration chromophoric/fluorophoric product would vary.

The enhancement of chromophoric/fluorophoric signal is significant. Each adduct would produce many many detectable chromophores or fluorophores.

Product amplification increases the spectroscopic signal, proportional to the analyte/antigen concentration thus enhancing the sensitivity.