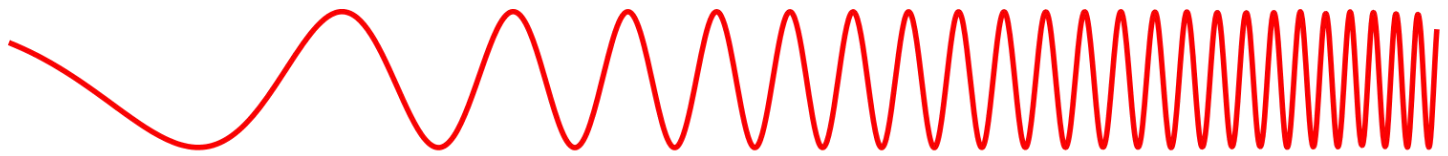
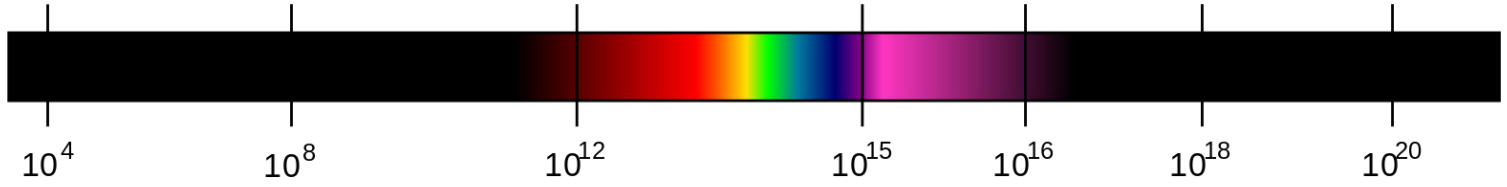
UV-Visible absorbance spectroscope

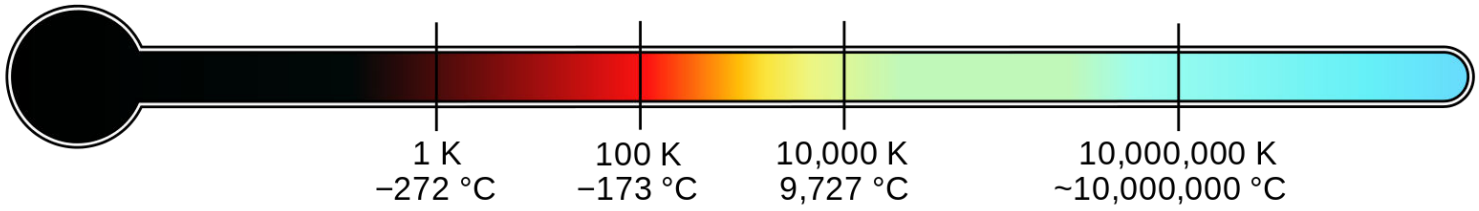
Electromagnetic spectrum



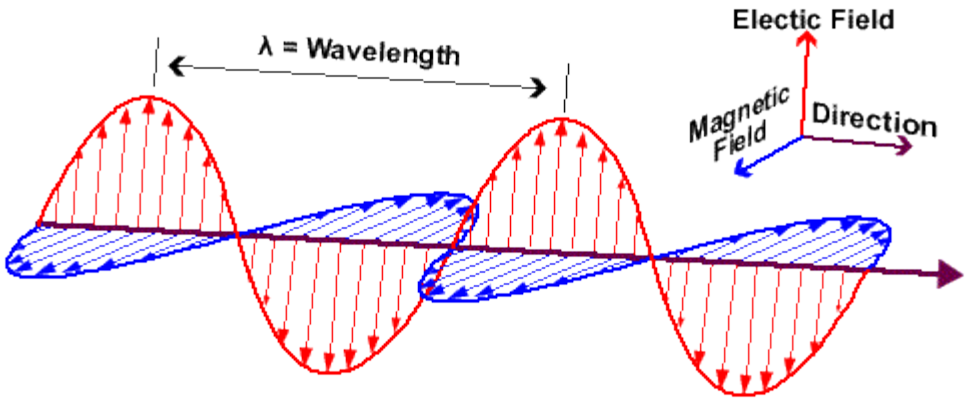
Radiation Type	**Radio**		**Microwave**	**Infrared**	**Visible**	**Ultraviolet**	**X-ray**	**Gamma ray**
Wavelength (m)	10^{3}	10^{-2}		10^{-5}	0.5×10^{-6}	10^{-8}	10^{-10}	10^{-12}
Approximate Scale of Wavelength	Buildings	Humans	Butterflies	Needle Point	Protozoans	Molecules	Atoms	Atomic Nuclei

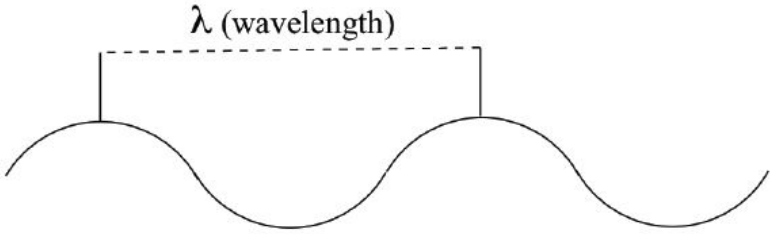


Temperature of objects at which this radiation is the most intense wavelength emitted



The electromagnetic radiation

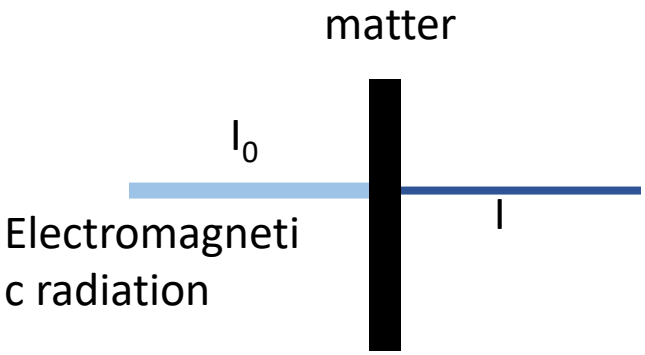




$E = hc/\lambda$ or $E = h\nu$

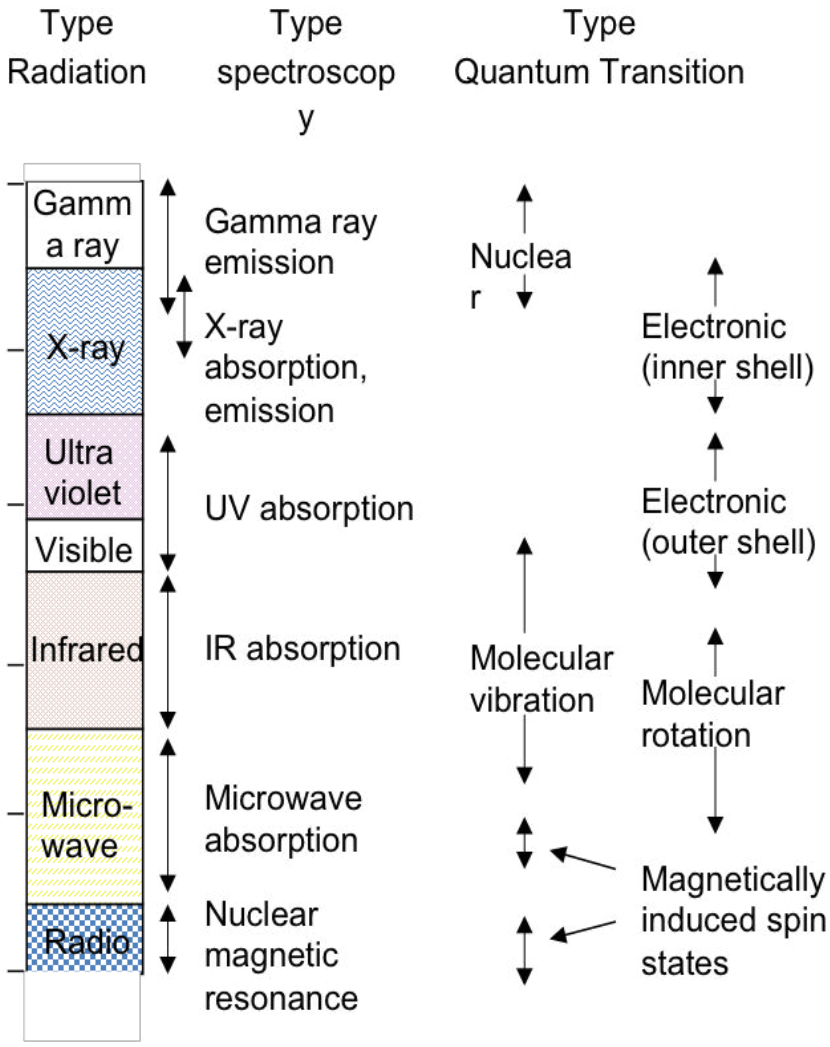
where E is energy in kJ/mol, λ (the Greek letter lambda) is wavelength in meters, c is 3.00×10^{8} m/s (the speed of light), and h is 3.99×10^{-13} kJ·s·mol^{-1} , a number known as Planck's constant.

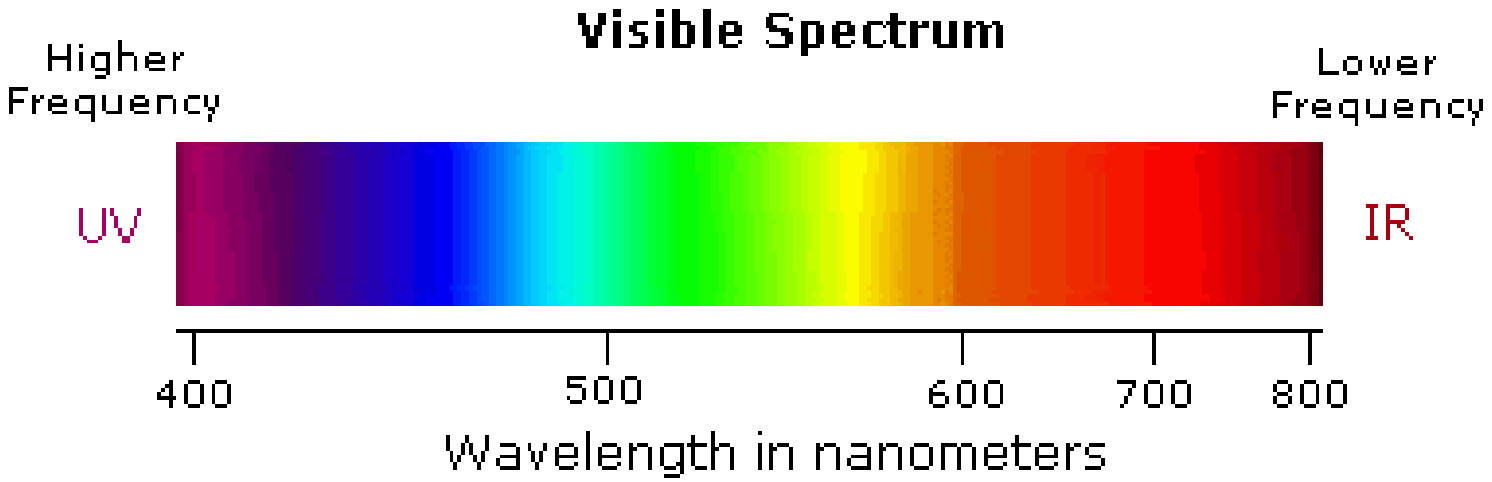
Molecular Spectroscopy



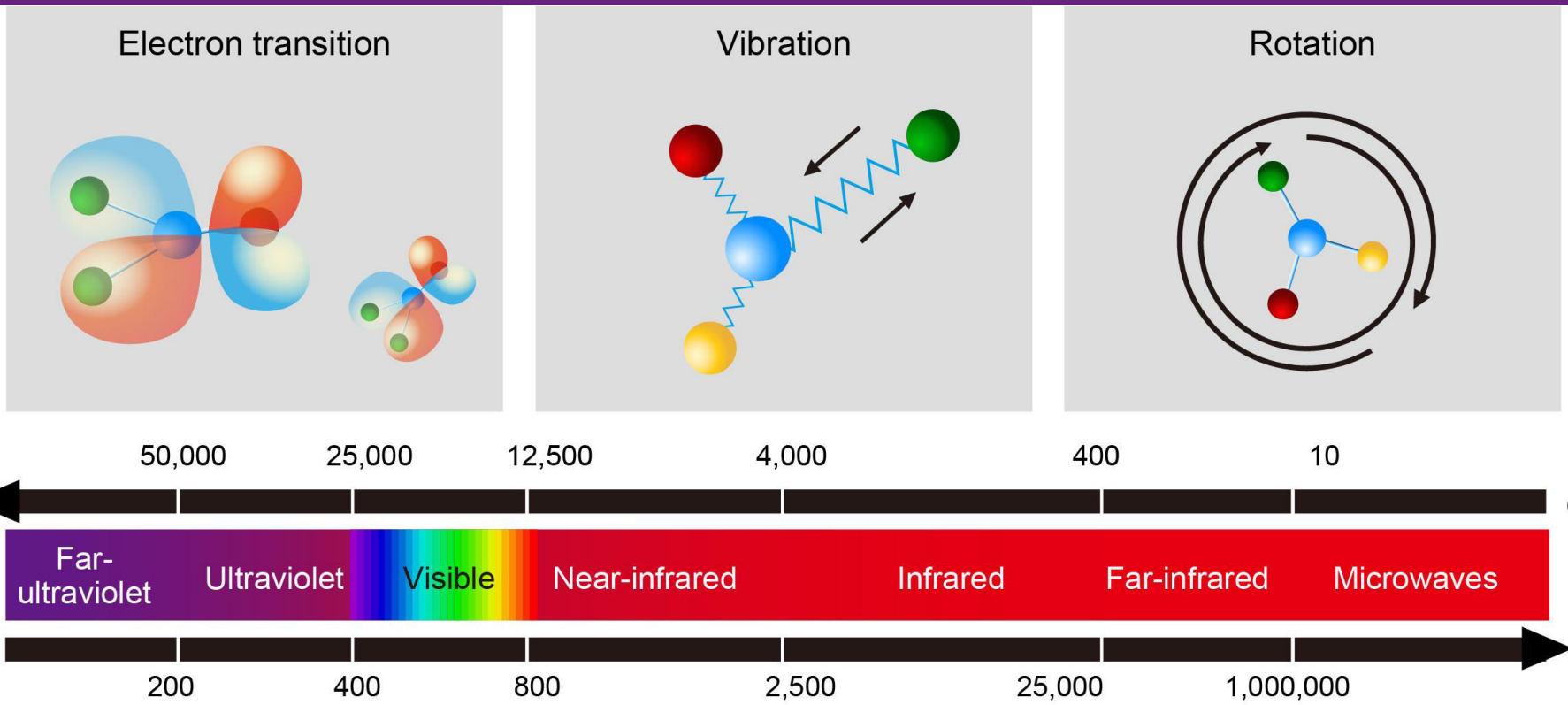
Interaction of EM radiation with any matter leads to the change in its

- Intensity: (Absorption spectroscopy, NMR, Elastic scattering techniques (SAXS, SANS)
- Emission: Fluorescence, Phosphorescence, Raman scattering
- Change in polarization: ORD, CD, FP



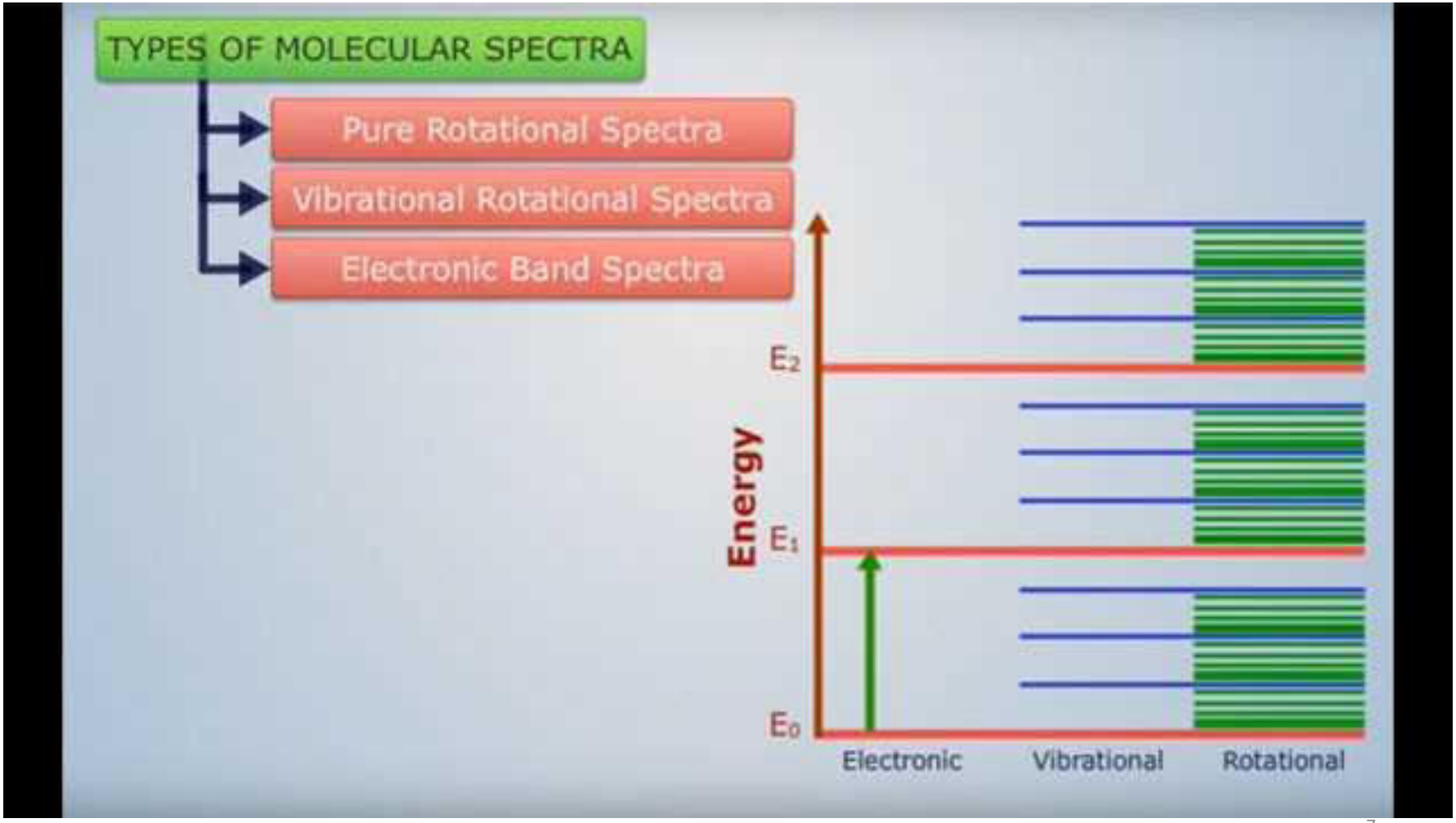


- Ultraviolet: 100 ~ 400nm
- Violet: 400 - 420 nm
- Indigo: 420 - 440 nm
- Blue: 440 - 490 nm
- Green: 490 - 570 nm
- Yellow: 570 - 585 nm
- Orange: 585 - 620 nm
- Red: 620 - 780 nm



Molecular transitions

Types of Molecular Spectra



Origin of electronic spectra

Absorptions of UV-vis photons by molecule results in electronic excitation of molecule with chromophore.

The electronic transition involves promotion of electron from an electronic ground state to higher energy state, usually from a molecular orbital called HOMO to LUMO.

BIOLOGICAL CHROMOPHORES

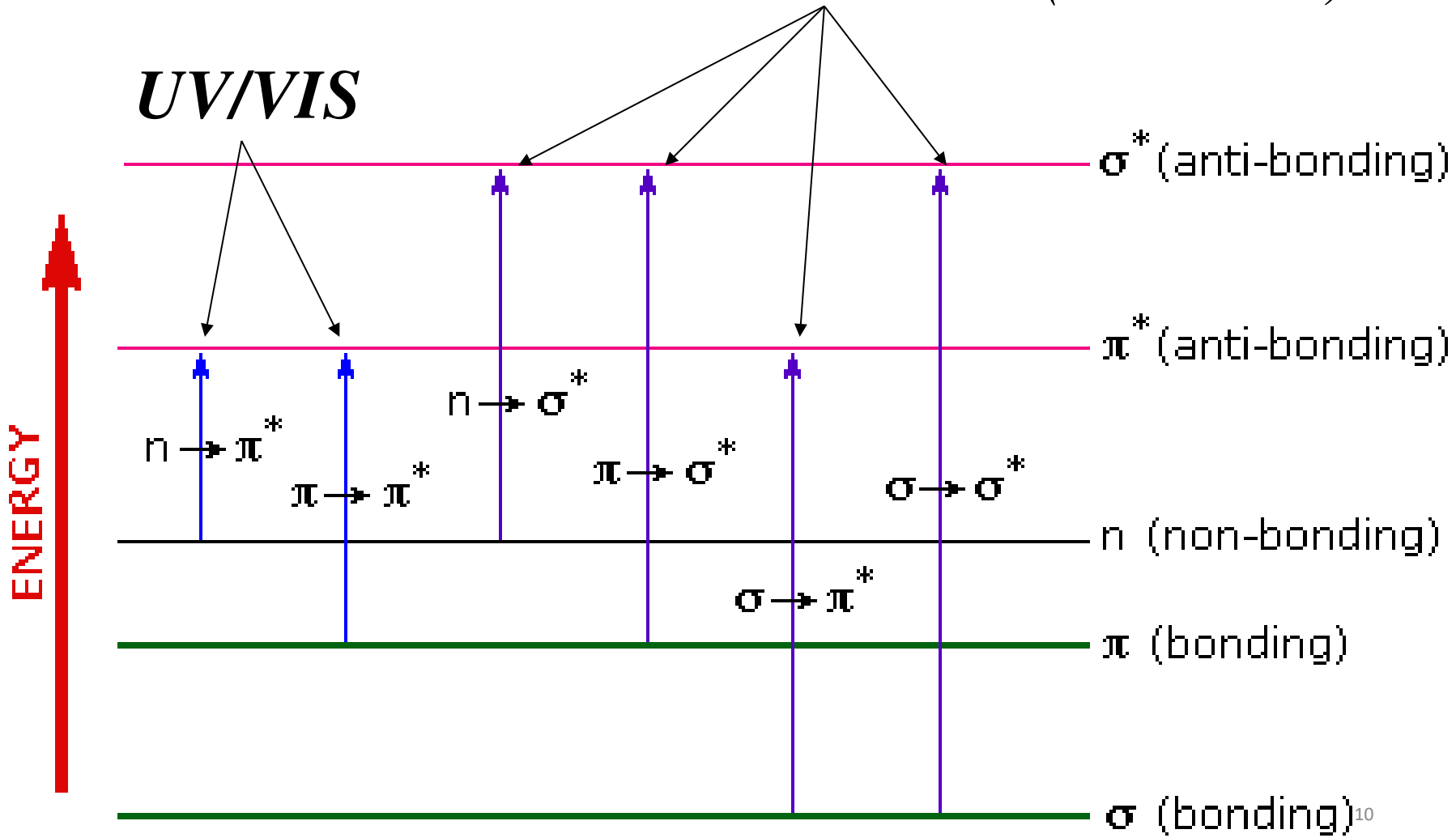
1. The peptide bonds and amino acids in proteins
   - The pi (π) bond electrons of the peptide group are delocalized over the carbon, nitrogen, and oxygen atoms. The n-π^* transition is typically observed at 210-220 nm, while the main π-π^* transition occurs at ~190 nm.
   - Aromatic side chains contribute to absorption at λ> 230 nm

2. Purine and pyrimidine bases in nucleic acids and their derivatives

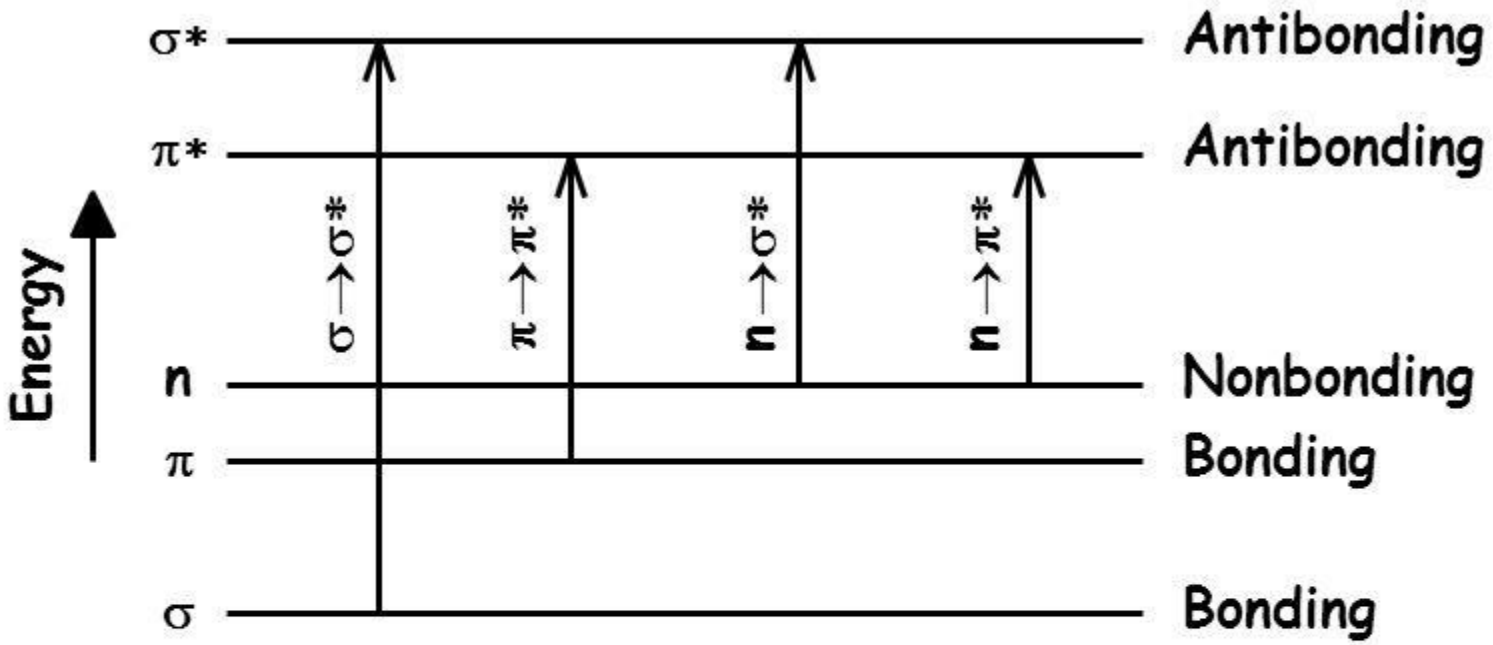
3. Highly conjugated double bond systems

ABSORPTION OF ULTRAVIOLET AND VISIBLE RADIATION

Vacuum UV or Far UV (λ<190 nm)



Allowed Electronic transition



Electronic transitions can occur between various states.
The energy of the transitions increases in the following order:

$$(n \rightarrow \pi^*) < (\pi \rightarrow \pi^*) < (n \rightarrow \sigma^*) < (\sigma \rightarrow \sigma^*)$$

$\sigma \rightarrow \sigma^*$ Transitions

- An electron in a bonding **s** orbital is excited to the corresponding antibonding orbital. The energy required is large. For example, methane (which has only C-H bonds, and can only undergo $\sigma \rightarrow \sigma^*$ transitions) shows an absorbance maximum at 125 nm. Absorption maxima due to $\sigma \rightarrow \sigma^*$ transitions are not seen in typical UV-VIS spectra (200 - 700 nm)

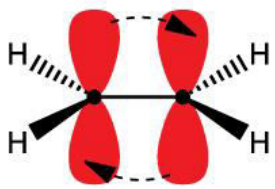
$n \rightarrow \sigma^*$ Transitions

- Saturated compounds containing atoms with lone pairs (non-bonding electrons) are capable of $n \rightarrow \sigma^*$ transitions. These transitions usually need less energy than $\sigma \rightarrow \sigma^*$ transitions. They can be initiated by light whose wavelength is in the range 150 - 250 nm. The number of organic functional groups with $n \rightarrow \sigma^*$ peaks in the UV region is small.

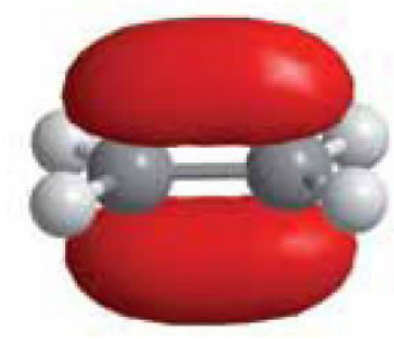
$n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ Transitions

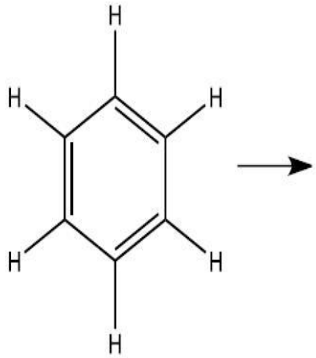
- Most absorption spectroscopy of organic compounds is based on transitions of *n* or π electrons to the π^* excited state.
- These transitions fall in an experimentally convenient region of the spectrum (200 - 700 nm). These transitions need an unsaturated group in the molecule to provide the π electrons.

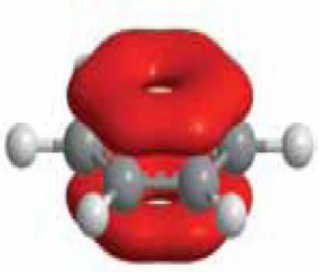
Conjugated systems



sideways overlap of half-filled p orbitals



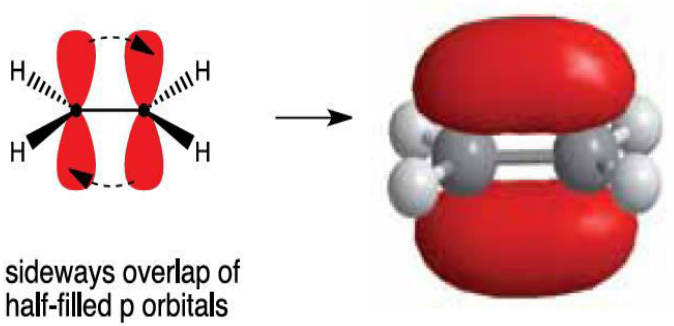




π bonds are formed by sideways overlap of the half-filled p orbitals on the two carbon atoms of a double bond. The two red shapes shown in the diagram below for ethene are part of the same π bonding orbital. Both of the electrons are found in the resulting π bonding orbital in the ground state.

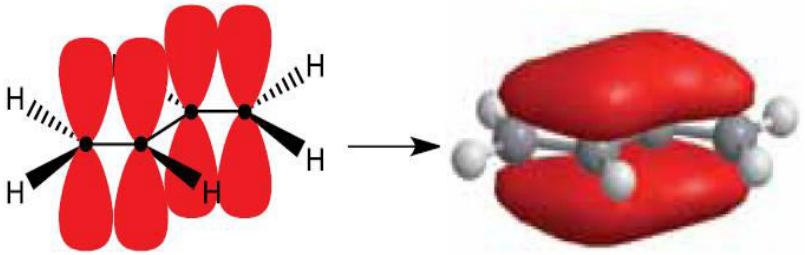
Molecules that contain **conjugated systems**,i.e. **alternating single and double bonds**, will have their electrons delocalised due to overlap of the p orbitals in thedouble bonds.

Electronic Delocalization

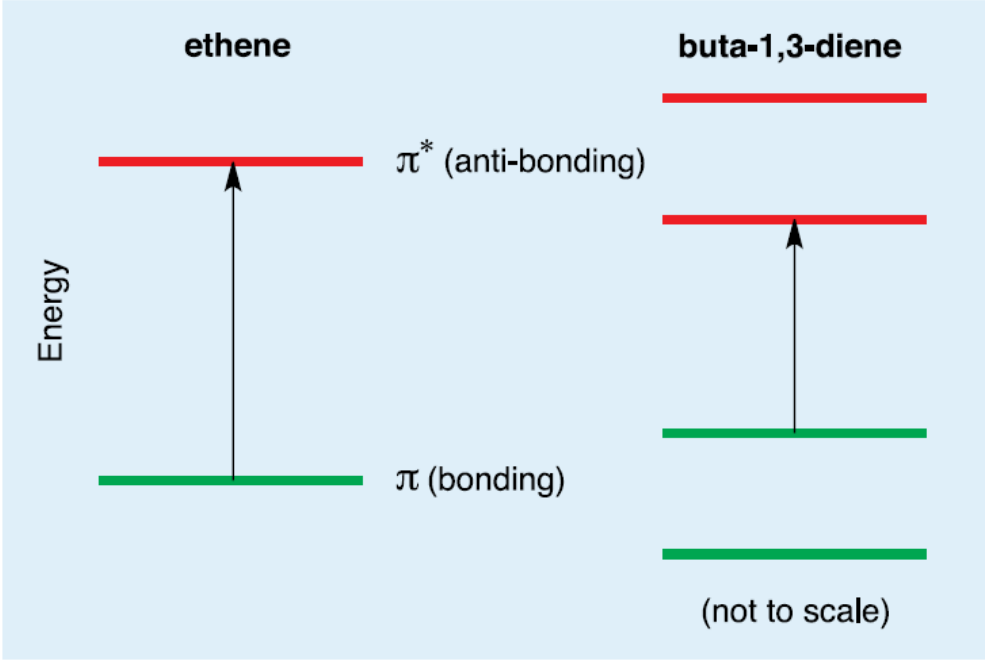


sideways overlap of half-filled p orbitals

Ethene

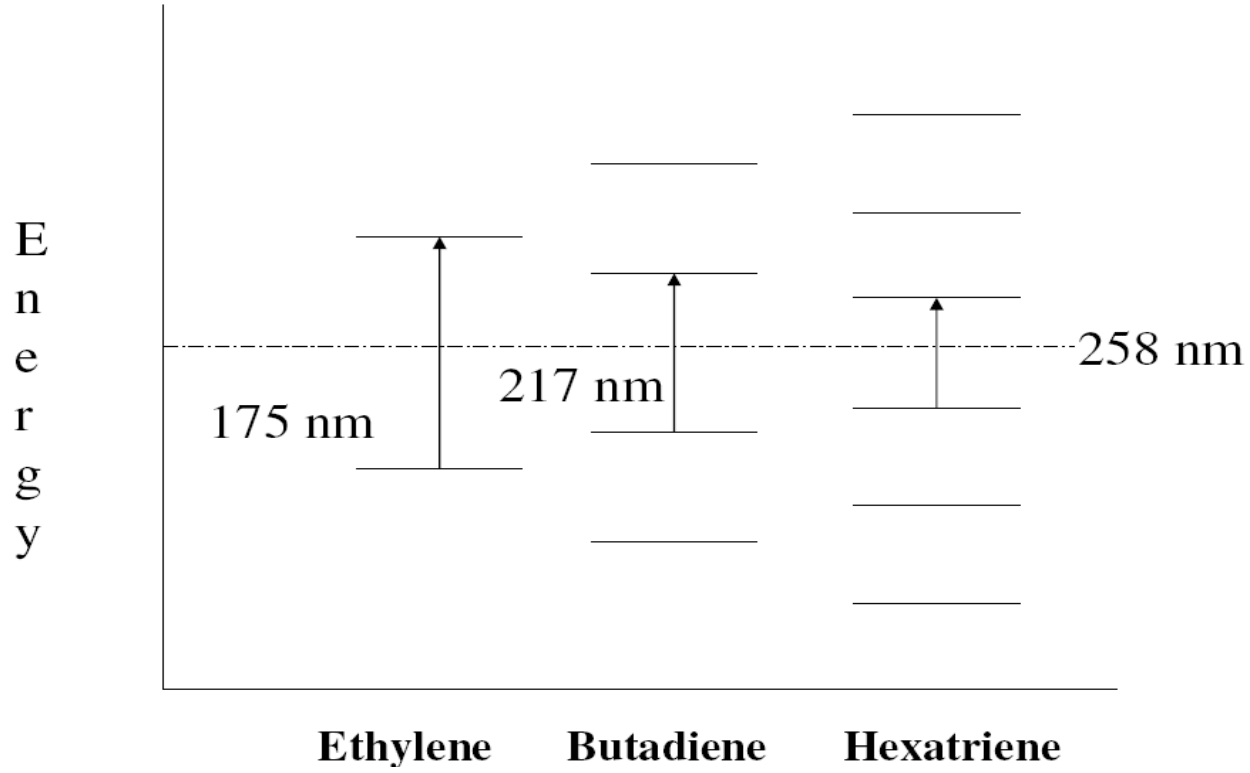


buta-1,3-diene



As the amount of delocalisation in the molecule increases the energy gap between the π bonding orbitals and π anti-bonding orbitals gets smaller and therefore light of lower energy, and longer wavelength, is absorbed.

The effect of conjugation of alkene



Absorbing species containing p, s, and *n* electrons

- Absorption of ultraviolet and visible radiation in organic molecules is restricted to certain functional groups (*chromophores*) that contain valence electrons of low excitation energy.
- **Chromophore:** an atom or group whose presence is responsible for the colour of a compound.
- Common examples include retinal (used in the eye to detect light), various food colorings, fabric dyes (azo compounds), pH indicators, lycopene, β-carotene, and anthocyanins.

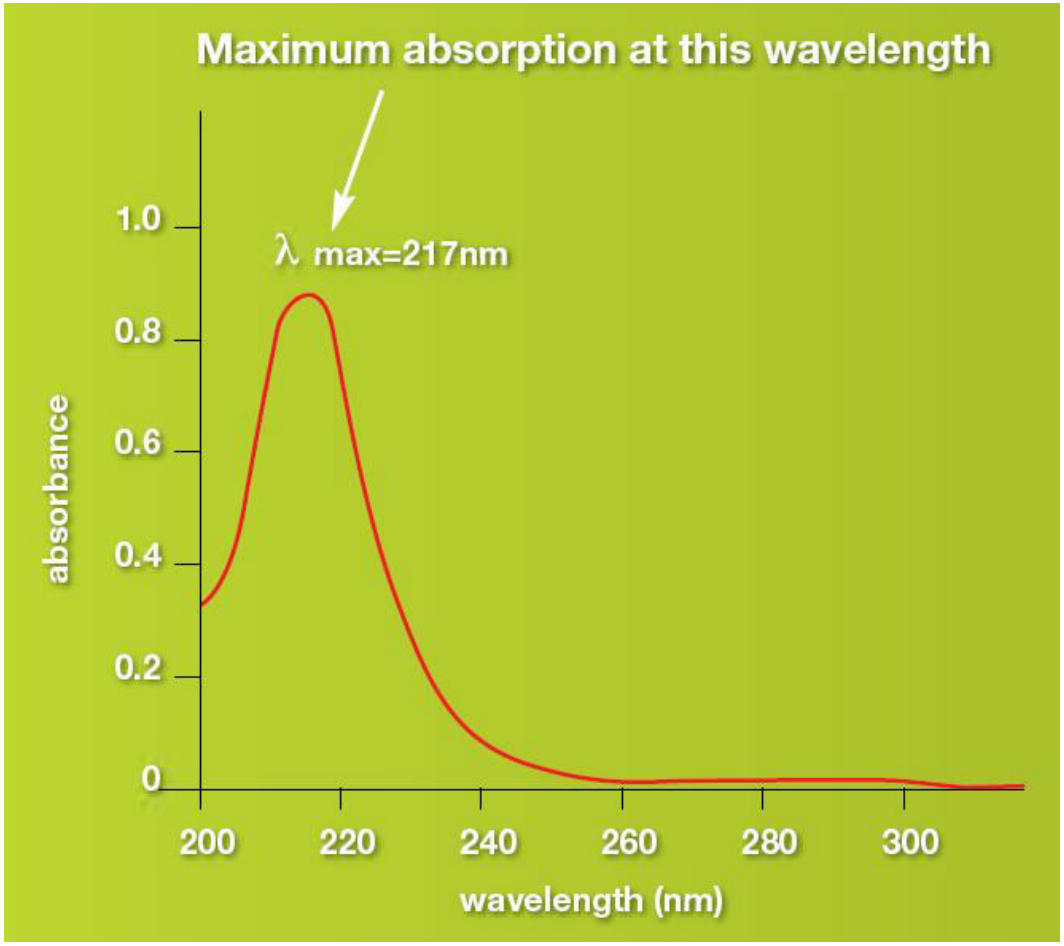
- An **auxochrome** is a group of atoms attached to a chromophore which modifies the ability of that chromophore to absorb light.
- Some commonly known auxochromic groups are: -OH, -NH2, -OR, -NHR, and –NR2

Chemical Structure & UV Absorption

Chromophoric Group:- The group in molecules which contain the electronic system that give rise to absorption in the ultra-violet region.

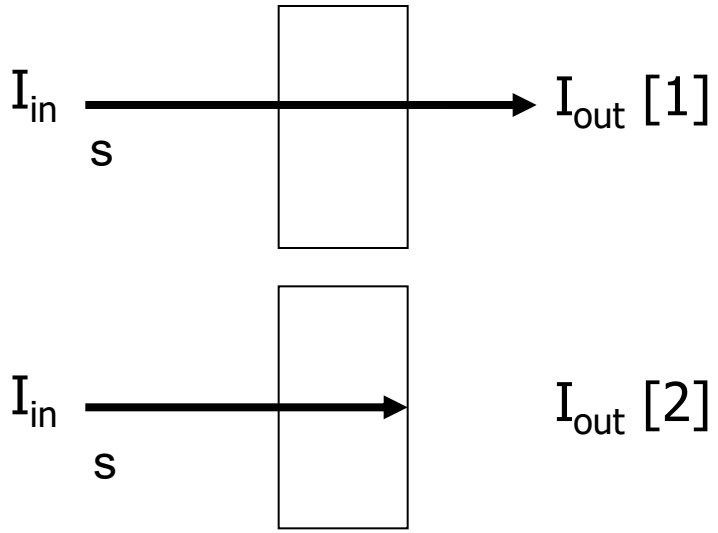
Group	**Structure**	**λ_{max}, nm**
Peptide	- c(=O –N(H)-	214
Carbonyl	> C=O	280
Azo	-N=N-	262
Nitro	-N=O	270
Thioketone	-C=S	330
Nitrite	-NO2	230
Conjugated Diene	-C=C-C=C-	233
Conjugated Triene	-C=C-C=C-C=C-	268
Conjugated Tetraene	-C=C-C=C-C=C-C=C-	315
Benzene		261
Phenolate ion		230

UV-visible absorption spectrum for buta-1,3-diene



Beer-Lambert's law

Absorbance Vs. Concentration (c)

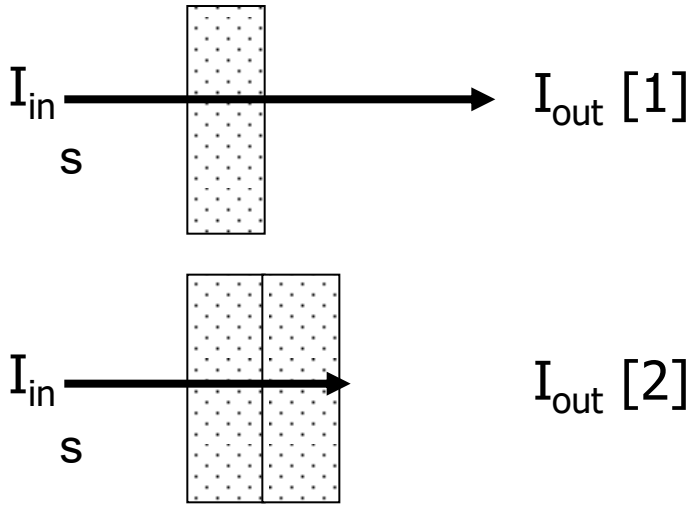


$$A \propto c$$

This is not true at high concentrations

Unit of concentration = M

Absorbance Vs. Path length (b)



$$A \propto b$$

Path length- the distance that light travels in the sample

Unit of path length = cm

Beer-Lambert's law

$$A \propto c \rightarrow A \propto bc \leftarrow A \propto b$$

$$\boxed{A = abc}$$

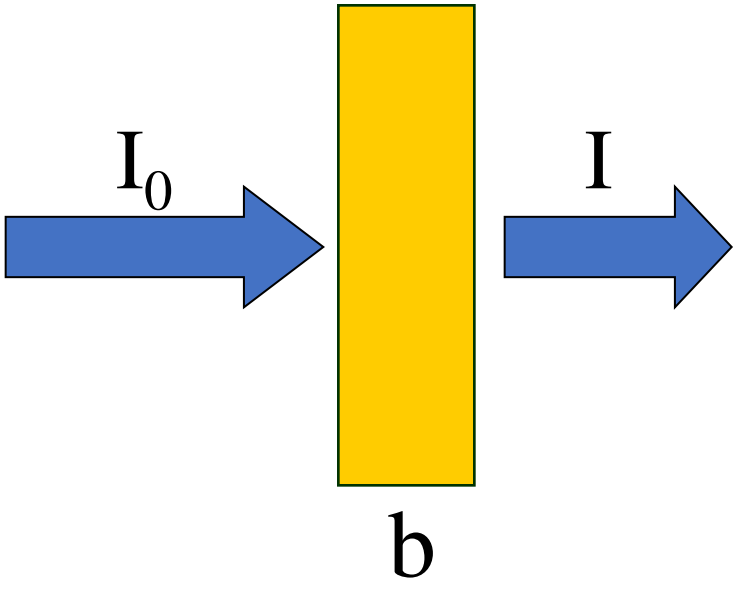
a – Molar absorptivity

Units of molar absorptivity:

$$unit\ of\ A = (unit\ of\ a)(unit\ of\ b)(unit\ of\ c)$$

$$unit\ of\ a = \frac{unit\ of\ A}{(unit\ of\ b)(unit\ of\ c)}$$

Transmittance



$$T = \frac{I}{I_0} \Rightarrow \frac{dI}{I_0} = kcdb$$

$$\int_{I_0}^{I} \frac{dI}{I_0} = -kc\int_0^b db$$

$$\Rightarrow \ln(\frac{I}{I_0}) = -kbc = 2.303\log(\frac{I}{I_0})$$

$$\Rightarrow -\log(\frac{I}{I_0}) = -\log T = A = \varepsilon bc$$

$$\varepsilon = \frac{k}{2.303}$$

$$unit\ of\ a = \frac{unit\ of\ A}{(unit\ of\ b)(unit\ of\ c)}$$

$$unit\ of\ a = \frac{unitless}{(cm)(M)} = \frac{1}{(cm)(M)} = cm^{-1}M^{-1} = M^{-1}cm^{-1}$$

"*A*" is unitless but "*a*" has units $M^{-1}cm^{-1}$

Definition of molar absorptivity

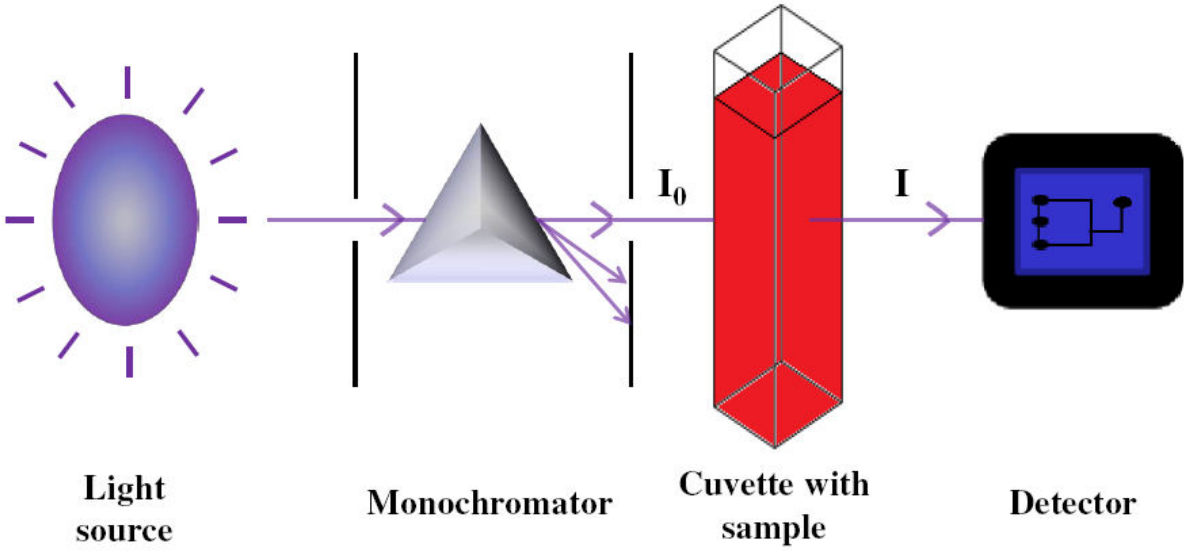
$$A = abc$$

$$a = \frac{A}{(b)(c)}$$

a - is a measure of the amount of light absorbed per unit concentration and pathlength

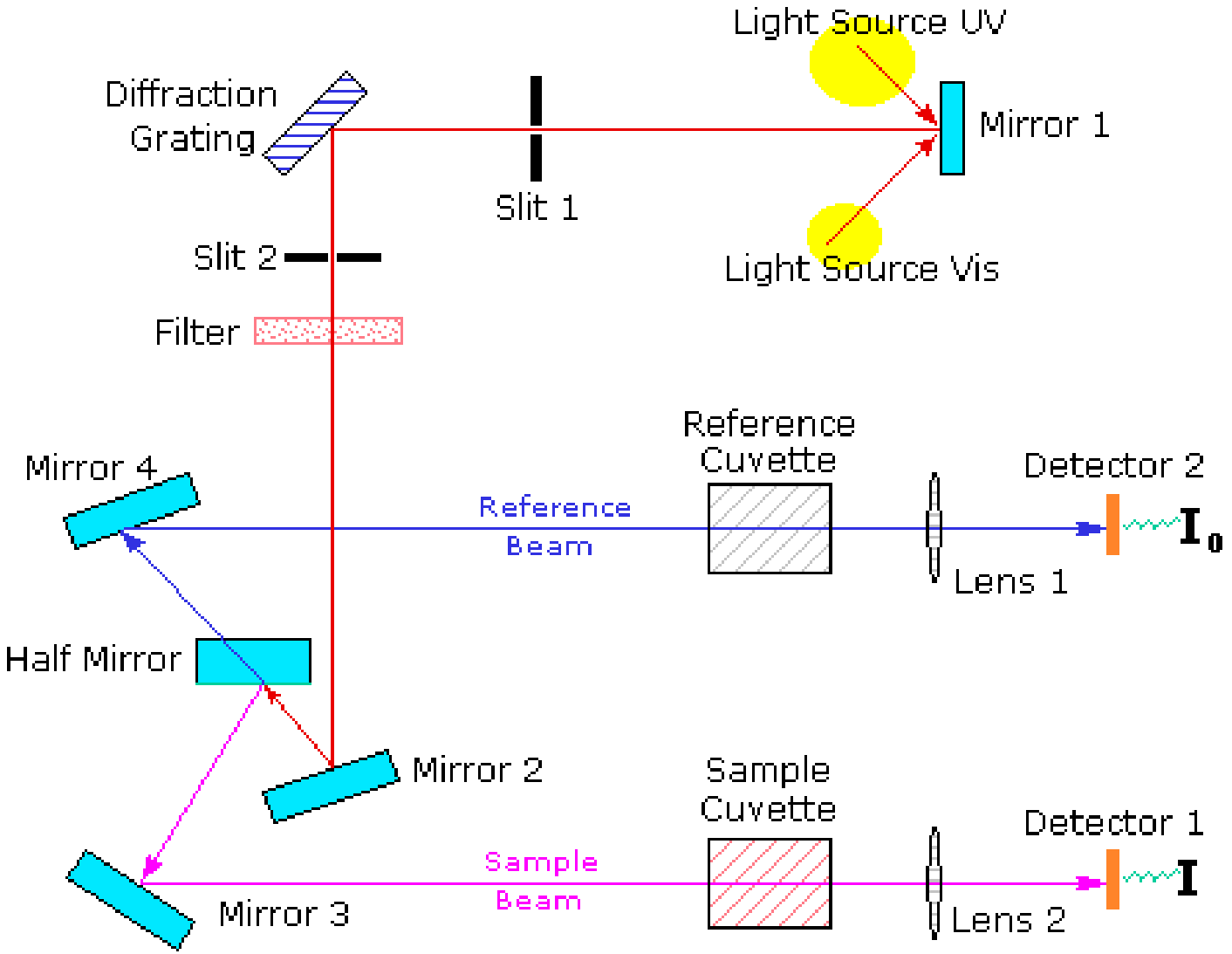
A compound with a high molar absorptivity is very effective at absorbing light (of the appropriate wavelength)

Spectrophotometer Experimental set up



- Various designs of spectrophotometers : Fixed and variable wavelength; Single, split and double beam
- Cuvettes: Glass or plastic – visible
  Quartz – visible, UV
  Acrylic – visible, UV

Instrumentation

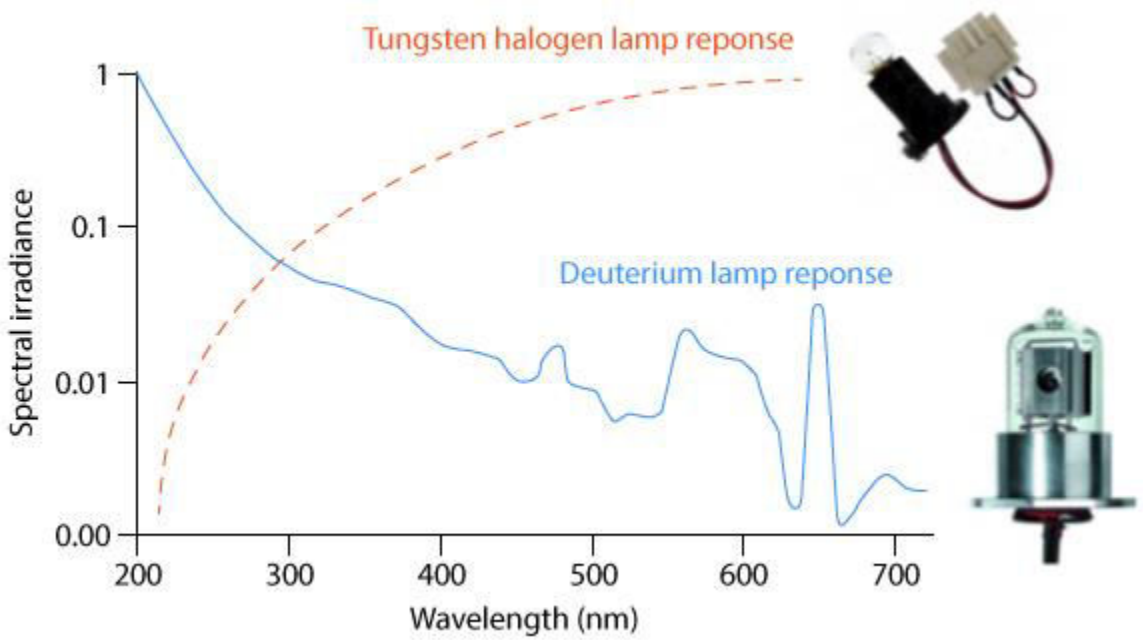


Components of a Spectrophotometer

Light Source

- Deuterium Lamps - a truly continuous spectrum in the ultraviolet region is produced by electrical excitation of deuterium at low pressure (160 ~ 375 nm).
- Tungsten Filament Lamps - the most common source of visible and near infrared radiation (350~2500 nm)
- Xenon lamp:
- Wavelength range

(190-800 nm)



Components of a Spectrophotometer
Wavelength selector

- Wavelength selection can be done two ways.

1. Using band pass filters (useful in colorimetry)

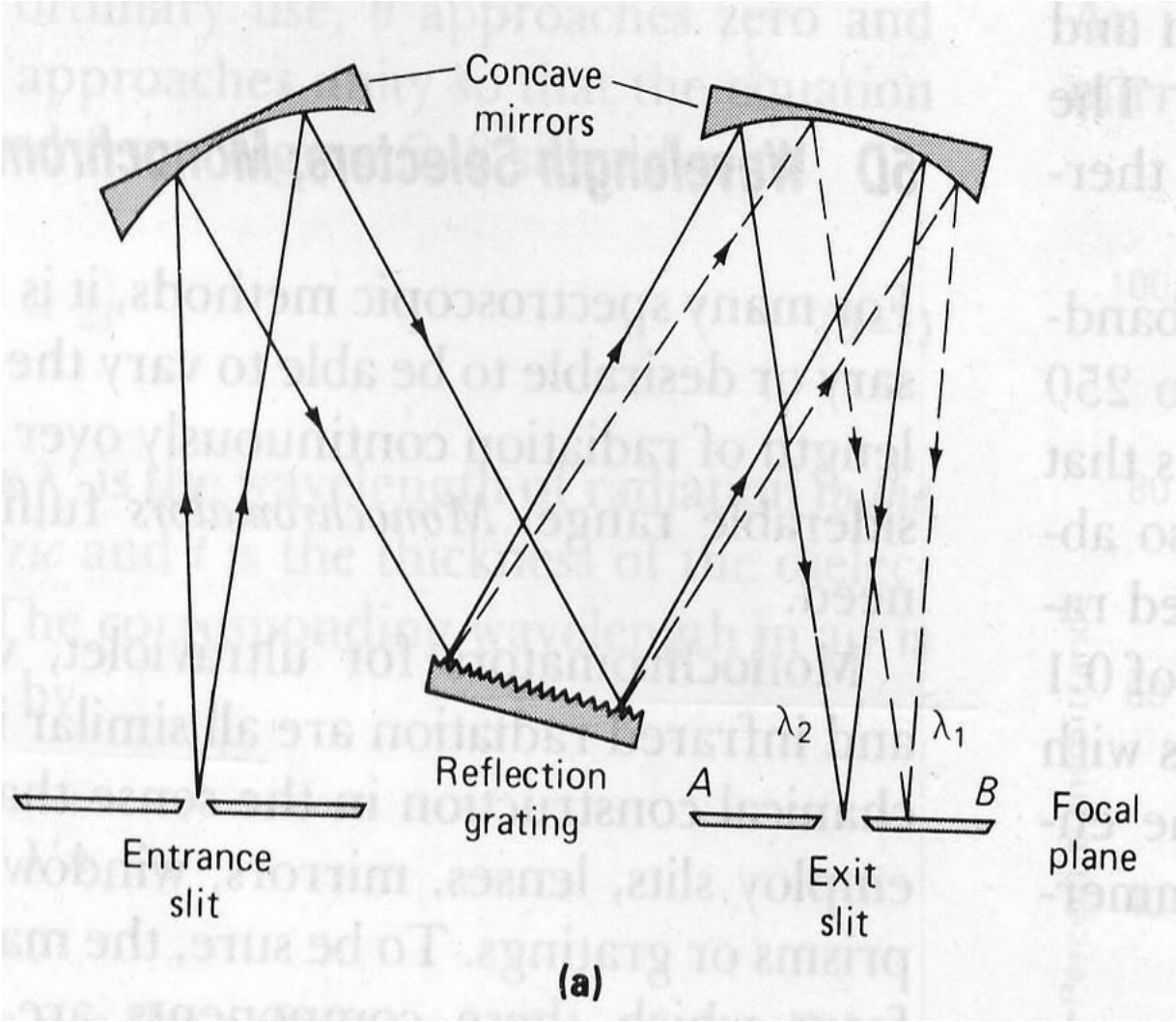
2. Prism

3. Monochromators (broad range use): A monochromator is an optical device that transmits a mechanically selectable narrow band of wavelengths of light or other radiation chosen from a wider range of wavelengths available at the input.

- Used in analysis: the monochromator will sequentially select for the detector to record the different components (spectrum) of any source or sample emitting light.

Monochromator
Czerny-Turner design

- Broad-band illumination source is aimed at an entrance slit.
- Light from the slit is focused at a curved mirror (the collimator) such that the light reflected from the mirror is collimated (focused at infinity).
- The collimated light is diffracted from the grating and then is collected by another mirror
- Mirror refocuses the dispersed light on the exit slit.



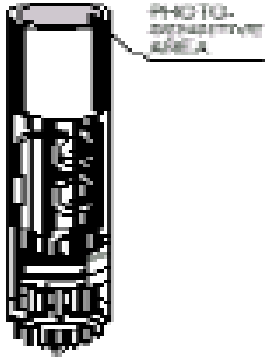
Absorption cell

- Receptacle for Sample.
- Material
  - Quartz, Fused Silica(Using over 190nm)
  - Glass (Using Over Visible Region)

Detector

PMT

- Phototube



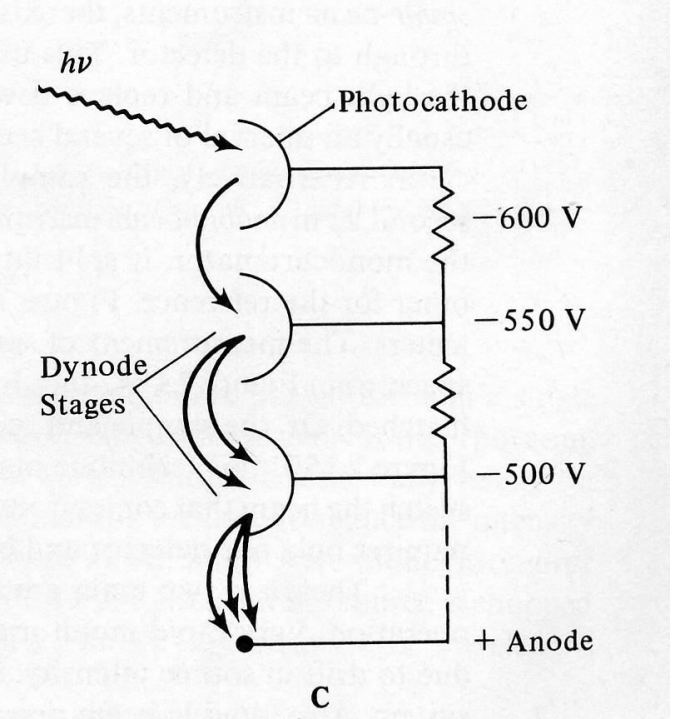
APD & CCD
(Avalanche photo diode and Charged couple diode)

- Photodiode & CCD

Detector

Photomultiplier

- THIS DETECTOR IS A VACUUM TUBE WITH A CESIUM-COATED PHOTOCATHODE AND SEQUENTIALLY PLACED DYNODES.
- PHOTONS OF SUFFICIENTLY HIGH ENERGY HITTING THE CATHODE CAN DISLODGE ELECTRONS, WHICH ARE COLLECTED AT THE ANODE.
- PHOTON FLUX IS MEASURED BY THE CURRENT FLOW IN THE SYSTEM.

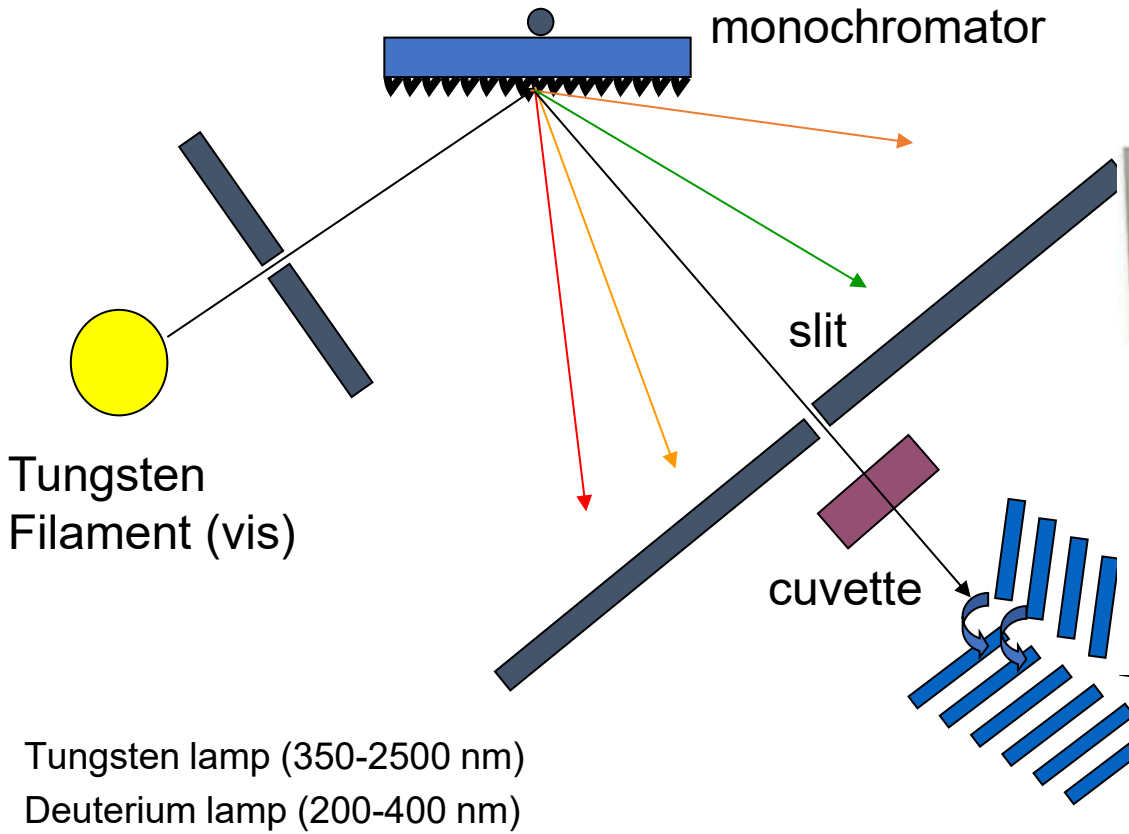


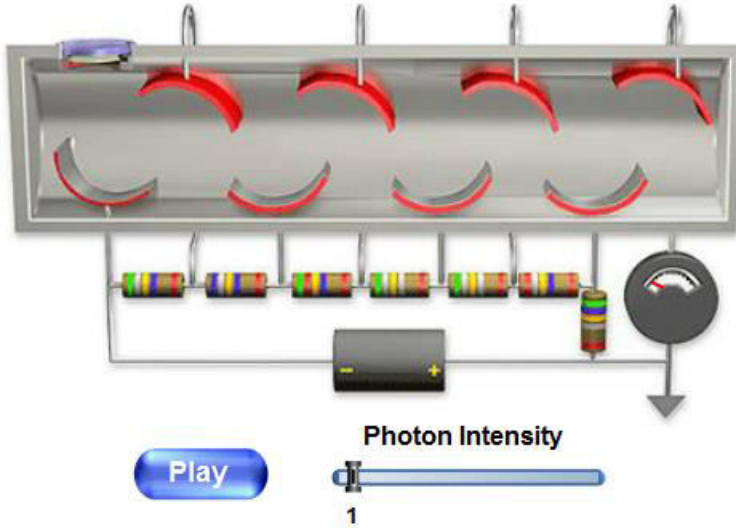
Detector

Principle of Photomultiplier

- The type is commonly used.
- The detector consists of a photo-emissive cathode coupled with a series of electron-multiplying dynode stages, and usually called a photomultiplier.
- The primary electrons ejected from the photo-cathode are accelerated by an electric field, so as to strike a small area on the first dynode.
- The impinging electrons strike with enough energy to eject two to five secondary electrons, which are accelerated to the second dynode to eject still more electrons.
- A photomultiplier may have 9 to 16 stages, and overall gain of 10^6~10^9 electrons per incident photon.

Types of spectrophotometer
Scanning Instrument





- Tungsten lamp (350-2500 nm)
- Deuterium lamp (200-400 nm)
- Xenon Arc lamps (200-1000 nm)

The grating can either be movable or fixed. If a single detector, such as a photomultiplier tube or photodiode is used, the grating can be **scanned** stepwise (**scanning spectrophotometer**) so that the detector can measure the light intensity at each wavelength (which will correspond to each "step"

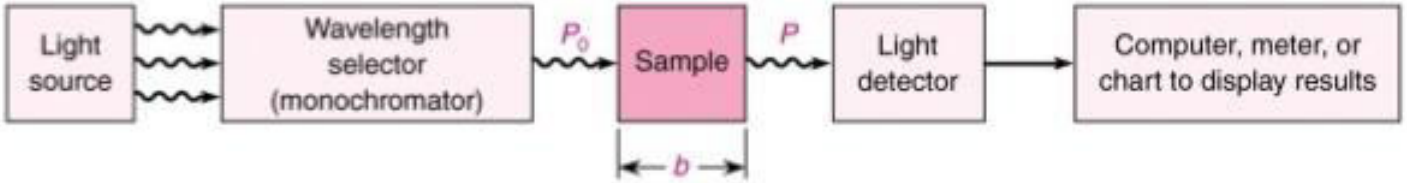
Single and Double Beam Spectrometer

- Single-Beam: There is only one light beam or optical path from the source through to the detector.
- Double-Beam: The light from the source, after passing through the monochromator, is split into two separate beams-one for the sample and the other for the reference.

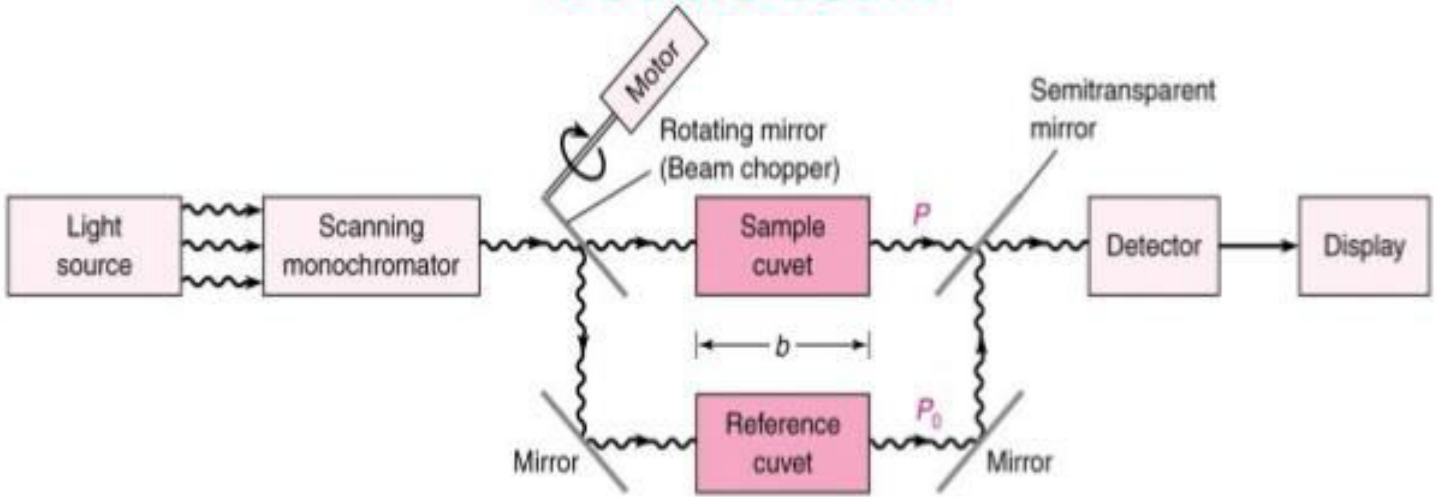
SINGLE AND DOUBLE BEAM SPECTROMETER

The Spectrophotometer

Single-beam



Double-beam



Limits to Beer's Law

- Chemical Deviations
  - absorbing of light undergoes association, dissociation or reaction with the solvent
- Instrumental Deviations
  - non-monochromatic radiation
  - stray light

Limits to Beer's Law

Chemical Deviations

- High concentration - particles too close.
- Average distance between ions and molecules are diminished to the point.
- Affect the charge distribution and extent of absorption.
- Cause deviations from linear relationship.

Limits to Beer's Law

Chemical Deviations

- Chemical interactions - monomer-dimer equilibria, metal complexation equilibria, acid/base equilibria and solvent-analyte association equilibria.

  The extent of such departure can be predicted from molar absorptivity and equilibrium constant.

Terms describing UV absorptions

1. **Bathochromic shift**: shift to longer λ, also called red shift.
2. **Hypsochromic shift**: shift to shorter λ, also called blue shift.
3. **Hyperchromism**: increase in ε of a band.
4. **Hypochromism**: decrease in ε of a band.

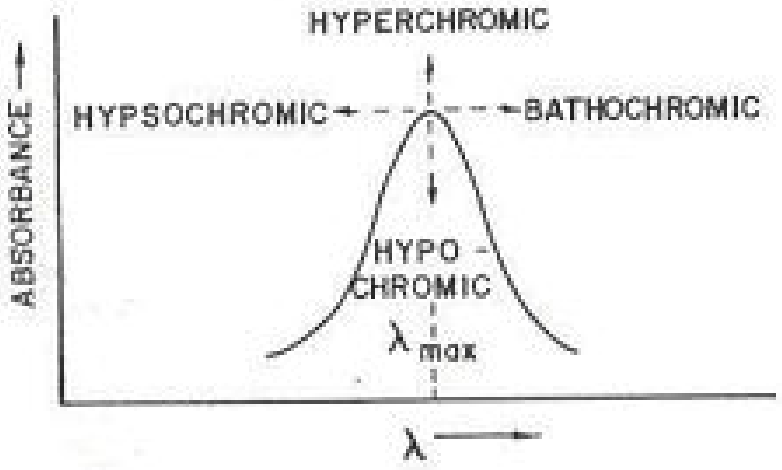
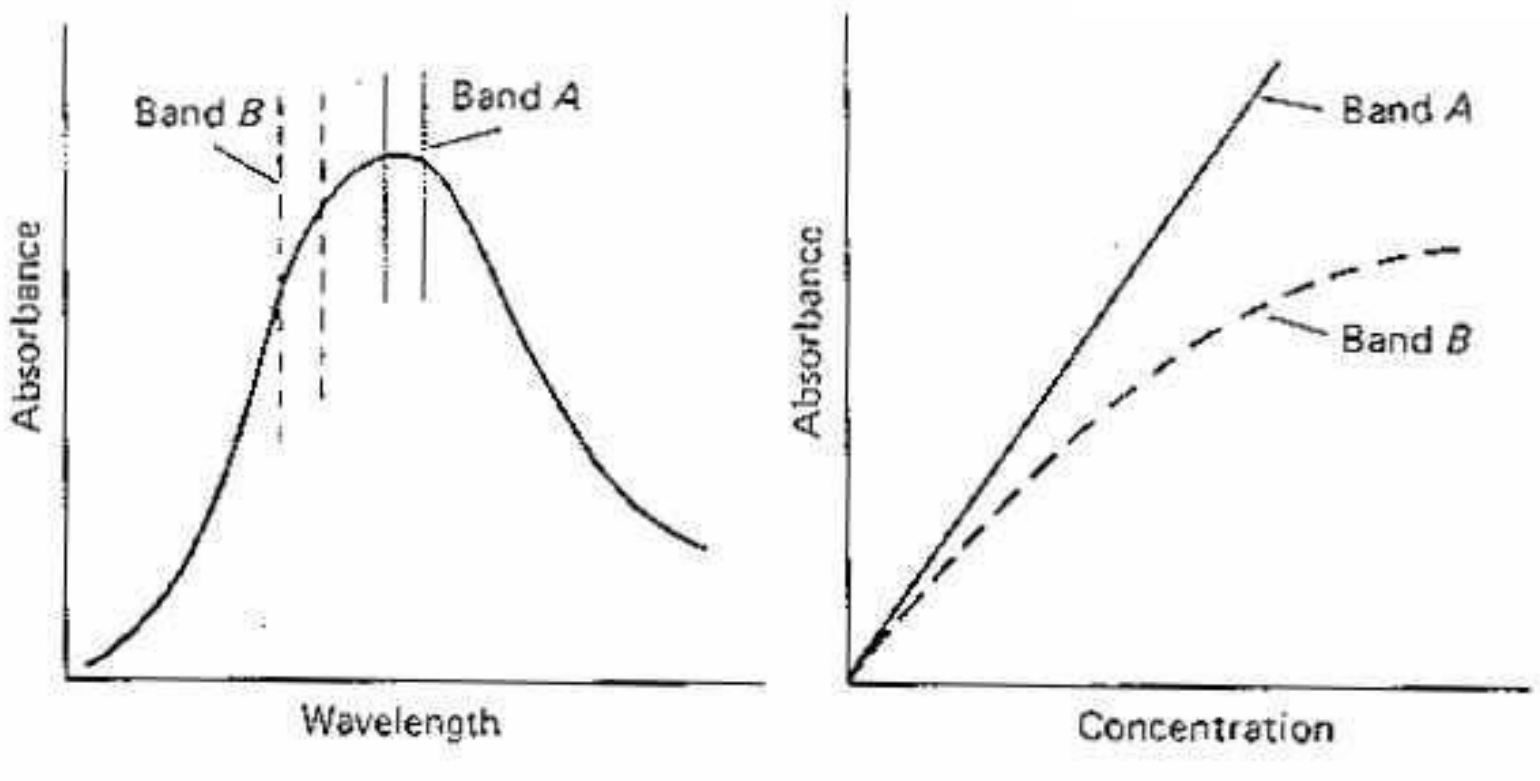


Figure 11-3 Terminology of shifts in the position of an absorption band.

Limits to Beer's Law

Instrumental Deviations

- non-monochromatic radiation

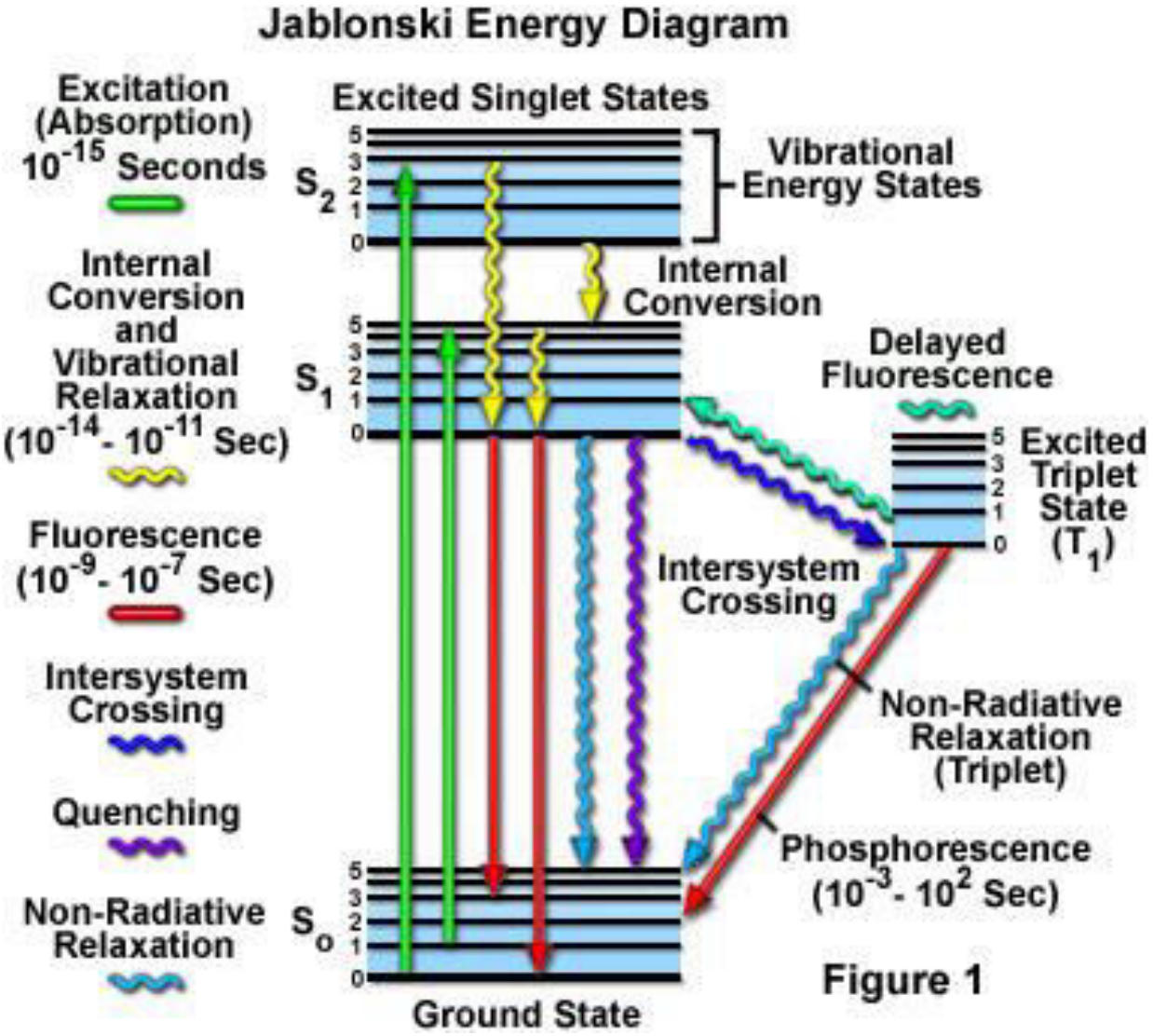


Fluorescence Spectroscopy

Fluorescence Spectroscopy

1. High sensitivity
2. Selectivity
3. Wide time scale

Jablonski diagram



Introduction to Fluorescence

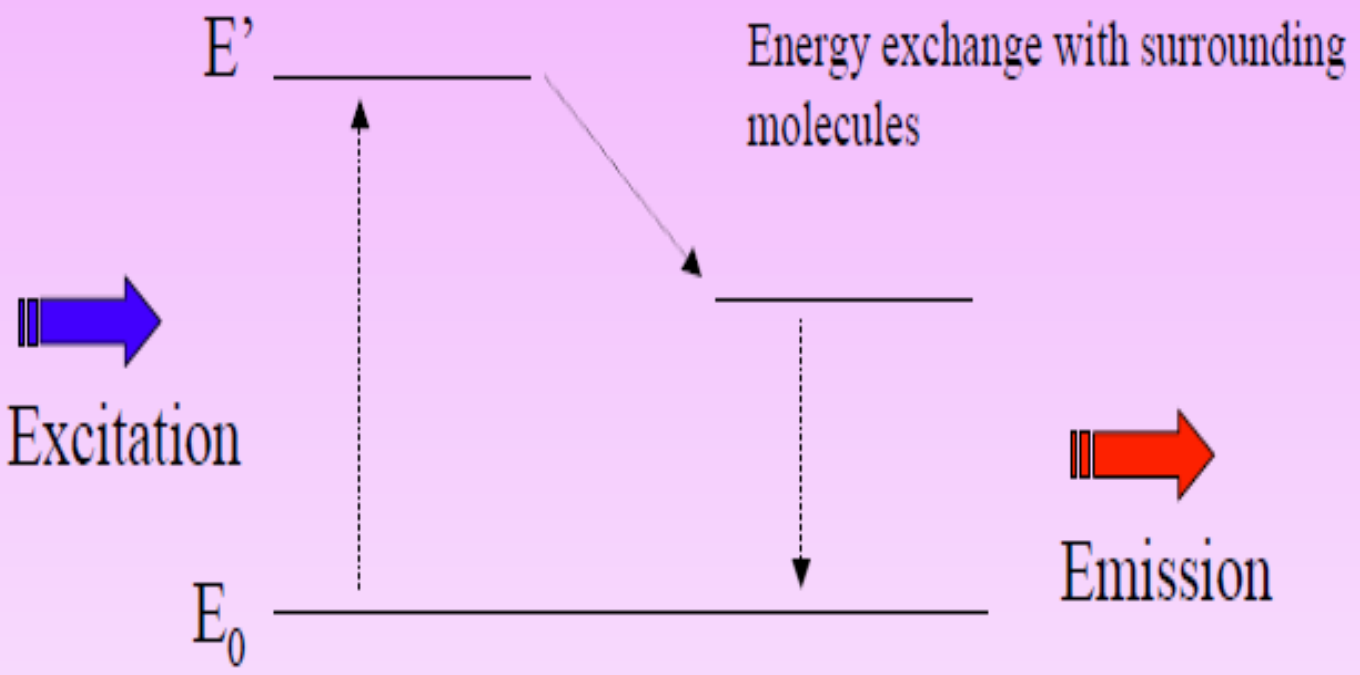
- Luminescence: emission of photons from electronically excited states of atoms, molecules, and ions.
- Fluorescence: Average lifetime from $<10^{-10}$ to 10^{-7} sec from singlet states.
- Phosphorescence: Average lifetime from 10^{-5} to $>10^{+3}$ sec from triplet excited states.

Principles

- Interaction of photons with molecules results in promotion of valence electrons from ground state orbitals to high energy levels.
- The molecules are said to be in excited state.
- Molecules in excited state do not remain there long but spontaneously relax to more stable ground state ($<10^{-10}$ to 10^{-7} sec).

- The relaxation process is brought about by collisional energy transfer to solvent or other molecules in the solution.
- Some excited molecules, however, return to the ground state by emitting the excess energy as light.
- This process is called fluorescence.

Fluorescence spectroscopy – The process (1)



The emitted light & its characteristics

1. It is usually of longer wavelength (lower energy) than the excited light. This is because part of the energy associated with S state is lost as heat energy.
2. The emitted light is composed of many wavelengths which results in fluorescence spectrum.

3. 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 favorable. 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.

Factors governing fluorescence intensity

1. Internal conversion k_{ic}: De-excitation by collision with solvent or by internal vibrational modes. Increases with increase in temperature.
2. K_q De-excitation from collision with solute molecule complexes (q)

$$S1 + Q \xrightarrow[k_q]{} S0 + Q$$

Aromatic chromophore τ = 1 ns to 100 ns

Common quenchers O_2, i^- Ions de-excite essentially every time they collide with excited singlet. At mM CONCENTRATION OF QUENCHER COLLISION CAN APPROACH RATES OF 10^{-8} S^{-1}. Therefore appreciable quenching is observed.

3. Intersystem crossing (k_{isc}): In this process nominally forbidden spin exchange converts an excited singlet to an excited triplet. This can then convert to ground state singlet either by phosphorescence or internal conversion.

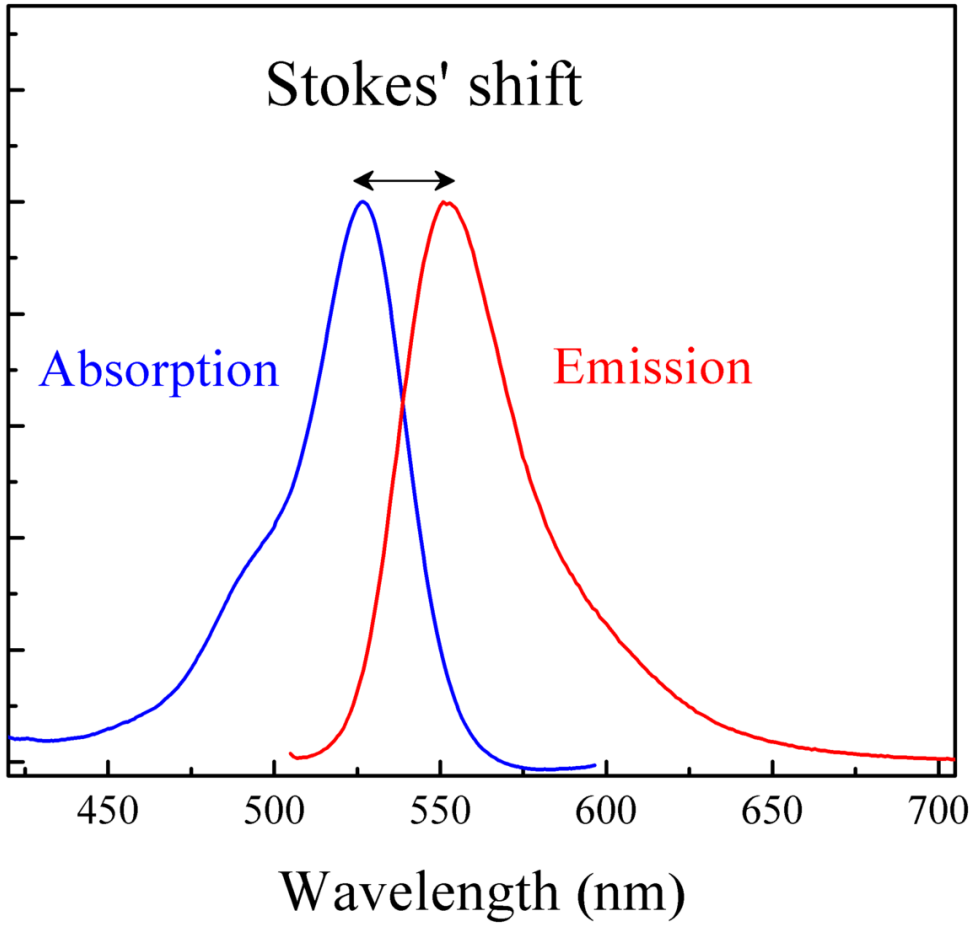
Fluorescence lifetime

$$1/\tau = k_r + \Sigma k_{nr}$$

The **fluorescence lifetime** is a measure of the time a fluorophore spends in the excited state before returning to the ground state by emitting a photon [1]. The **lifetimes** of fluorophores can range from picoseconds to hundreds of nanoseconds.

Stoke's shift

- 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.



Quantum yield, Q

- The fluorescence intensity is described in terms of quantum yield.
- The quantum **yield Q** is the *ratio of the number of photons emitted to the number of photons absorbed.*

$$\Phi = \frac{N(\text{em})}{N(\text{abs})} = \frac{k_r}{k} = \frac{k_r}{k_r + k_{IC} + k_{ISC} + k_{reaction} + k_Q c(Q) + k_{FRET}} = \frac{\tau}{\tau_r}$$

The quantum yield is a dimensionless quantity, and, most importantly, the only absolute measure of fluorescence of a molecule. Measuring the quantum yield is a difficult process and requires comparison with a fluorophore of known quantum yield. In biochemical applications, this measurement is rarely done. Most commonly, the fluorescence emissions of two or more related samples are compared and their relative differences analyzed.

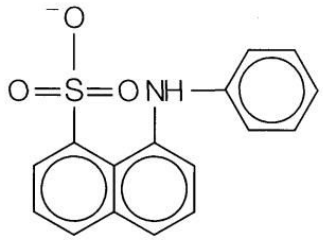
Intrinsic Fluorescence

- Some biomolecules are having intrinsic fluorescence i.e., they fluoresce themselves.
- The amino acids with aromatic groups, examples: phenylalamine, tyrosine, tryptophan are fluorescent. Hence, proteins containing these amino acids have intrinsic fluorescence.
- The purine and pyrimidine bases and some coenzymes e.g. NAD and FAD are also intrinsic fluorescent molecules.
- Intrinsic fluorescence is used to study protein conformation changes and to probe the location of active site and coenzymes in enzymes.

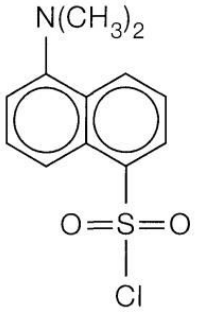
Extrinsic Fluorescence

- These are fluorescent molecules that are added in biochemical system under observation.
- Extrinsic fluorescence has been used to study the binding of fatty acids to serum albumin, to characterize the binding sites for cofactors and substrates in enzyme molecules and to study the intercalation of small molecules into the DNA double helix.

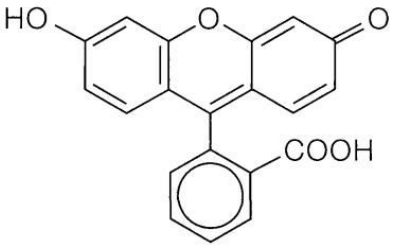
Fluorescent molecules useful in biochemical studies.



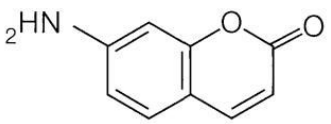
1-Anilino-8-naphthalene sulfonate (ANS)



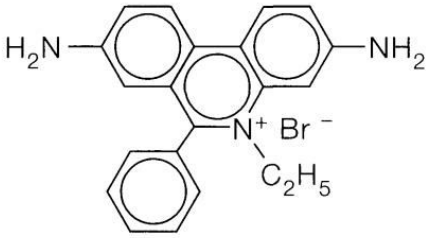
Dansyl chloride



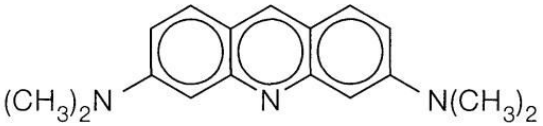
Fluorescein



Aminomethyl coumarin (AMC)



Ethidium bromide

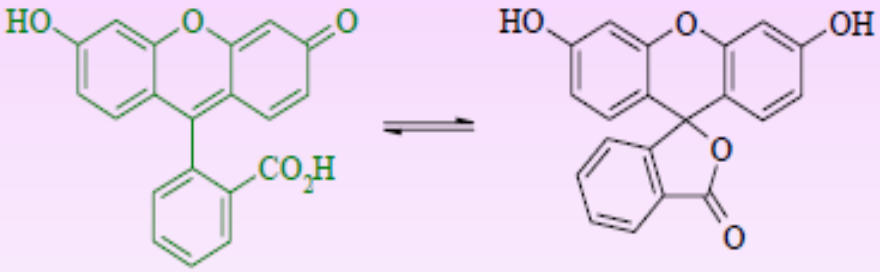


Acridine orange

- ANS, dansyl chloride, fluorescein are used for protein studies.
- Ethidium, proflavine and acridines are used for nucleic acid characterization.
- Ethidium bromide has enhanced fluorescence when bound to double stranded DNA, but not with single stranded DNA.

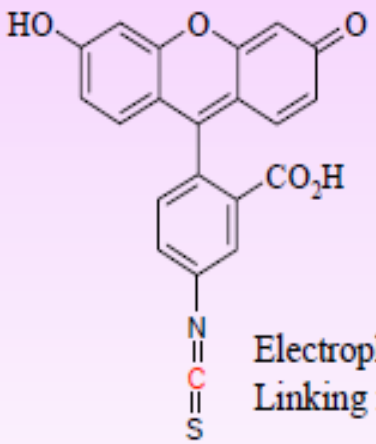
Not all compounds fluoresce. Fluorophores require

- Aromatic ring
- The potential for increased extended conjugation
- At least one electron donating group –OH, -NH_2 attached to the aromatic ring (note : electron withdrawing groups can diminish or often destroy fluorescence



Fluorescent

Fluoroscein



Electrophilic
Linking functional group

Fluoroscein isothiocyanate (FITC)

Instrumentation

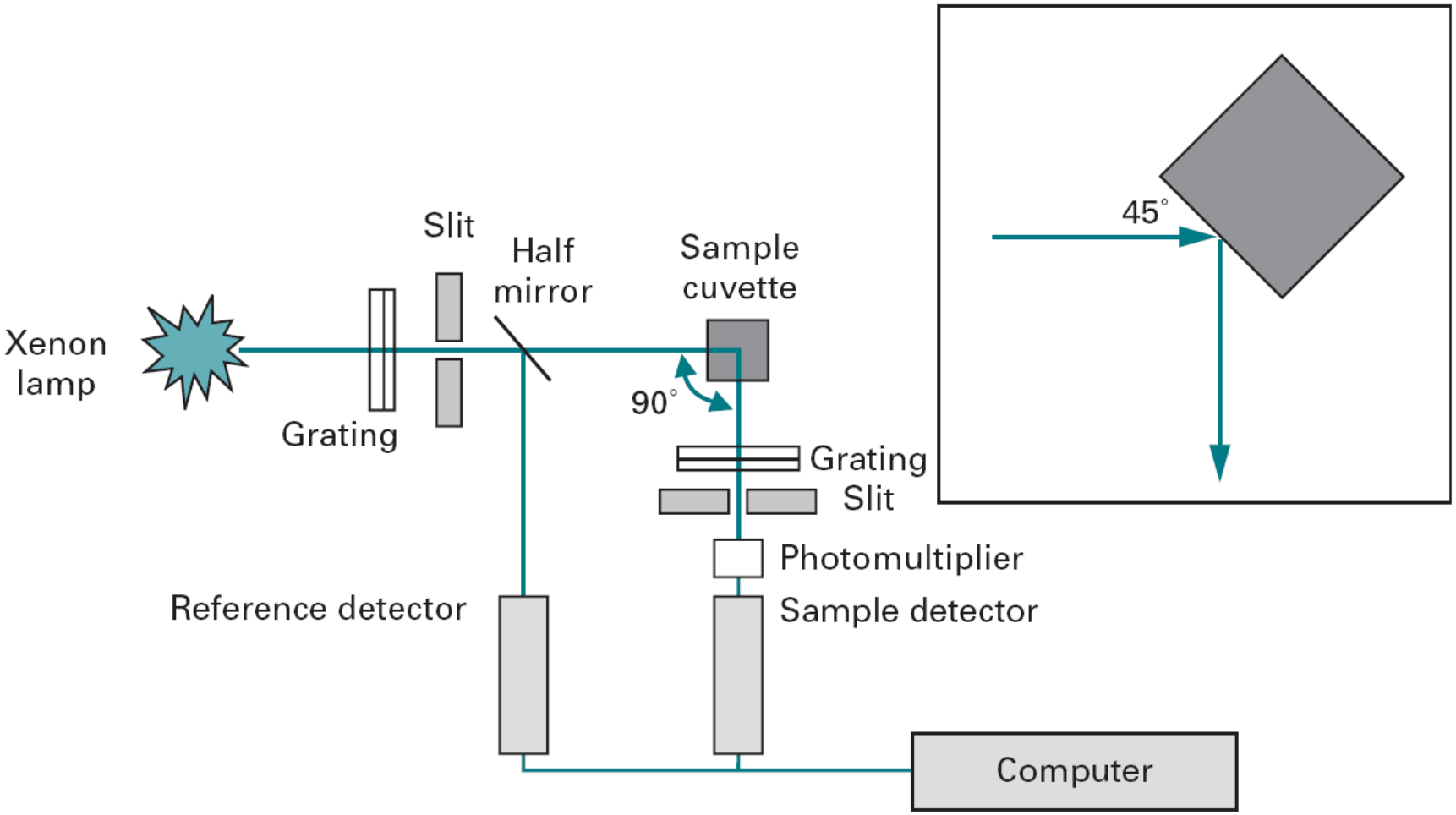


Fig. 12.9 Schematics of a spectrofluorimeter with 'T' geometry (90°). Optical paths are shown as green lines. Inset: Geometry of front-face illumination.

- THE BASIC INSTRUMENT IS A SPECTROFLUOROMETER.
- IT CONTAINS A LIGHT SOURCE, TWO MONOCHROMATORS, A SAMPLE HOLDER AND A DETECTOR.
- THERE ARE TWO MONOCHROMATORS, ONE FOR SELECTION OF THE EXCITATION WAVELENGTH, ANOTHER FOR ANALYSIS OF THE EMITTED LIGHT.
- THE DETECTOR IS AT 90 DEGREES TO THE EXCITATION BEAM.
- UPON EXCITATION OF THE SAMPLE MOLECULES, THE FLUORESCENCE IS EMITTED IN ALL DIRECTIONS AND IS DETECTED BY PHOTOCELL AT RIGHT ANGLES TO THE EXCITATION LIGHT BEAM.

- THE LAMP SOURCE USED IS A XENON ARC LAMP THAT EMITS RADIATION IN THE UV, VISIBLE AND NEAR-INFRARED REGIONS.
- THE LIGHT IS DIRECTED BY AN OPTICAL SYSTEM TO THE EXCITATION MONOCHROMATOR, WHICH ALLOWS EITHER PRESELECTION OF WAVELENGTH OR SCANNING OF CERTAIN WAVELENGTH RANGE.

- The exciting light then passes into the sample chamber which contains fluorescence cuvette
- A special fluorescent cuvette with four translucent quartz or glass sides is used.
- When the excited light impinges on the sample cell, molecules in the solution are excited and some will emit light.
- Light emitted at right angles to the incoming beam is analyzed by the emission monochromator.
- The wavelength analysis of emitted light is carried out by measuring the intensity of fluorescence at preselected wavelength.
- The analyzer monochromator directs emitted light of the preselected wavelength to the detector.
- A photomultiplier tube serves as the detector to measure the intensity of the light.
- The output current from the photomultiplier is fed to some measuring device that indicates the extent of fluorescence.