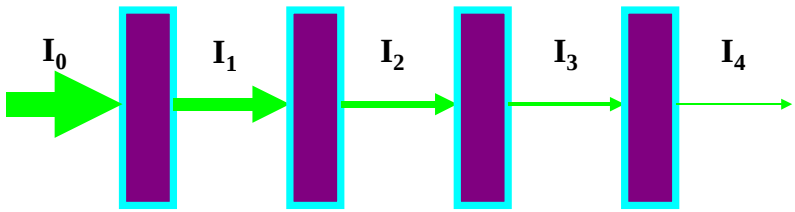
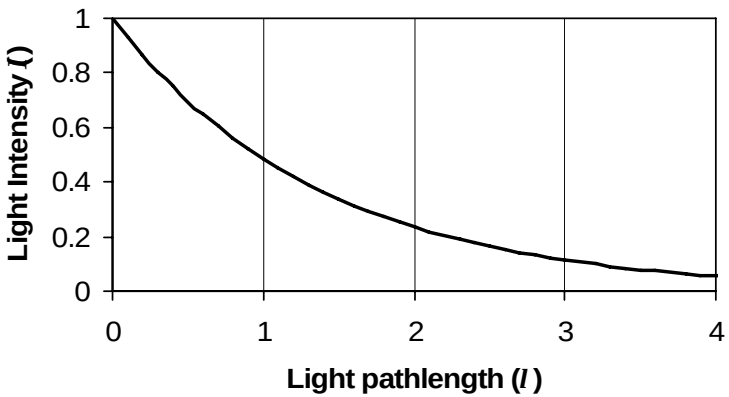
Light Absorption – Lambert-Beer Law

Light goes through a number of cuvettes.



$$\frac{I_0}{I_1} = \frac{I_1}{I_2} = \frac{I_2}{I_3} = \frac{I_3}{I_4} = n$$



This qualitative relationship is described by the following function for light intensity:

$$I_i = I_o n^{-i} \quad \Rightarrow \quad I_i = I_o 10^{-i \log_n 10}$$

where ***i*** is the number of cuvettes. The last expression may be generalized for any cuvette with light path-length ***l***: $I = I_o 10^{-kl}$

where ***k*** is a coefficient which depends on concentration, ***c***, and molar absorptivity, ***ε***:

$$k = \varepsilon \times c$$

Therefore, $I = I_o 10^{-\varepsilon cl} \quad \Rightarrow \quad \frac{I}{I_0} = 10^{-\varepsilon cl} \quad \Rightarrow \quad \frac{I_0}{I} = 10^{\varepsilon cl} \quad \Rightarrow \quad \log \frac{I_0}{I} = \varepsilon cl$

$$Absorbance = A = \log \frac{I_o}{I} = \varepsilon cl$$

$$Transmittance = T = \frac{I}{I_0} = 10^{-A}$$

Lambert-Beer Law Contd.

Dimensions:

A	dimensionless
l	cm
c	M
ε	$M^{-1}cm^{-1}$ (molar absorptivity or extinction coefficient)

Connection between absorbance and transmittance:

A	**T**
0	**1**
1	0.1
2	0.01
3	0
∞	**0**

Major application of Beers' law - determination of unknown concentration by measuring absorbance:

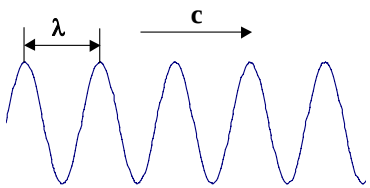
$$c = \frac{A}{\varepsilon l}$$

Absorption Spectra

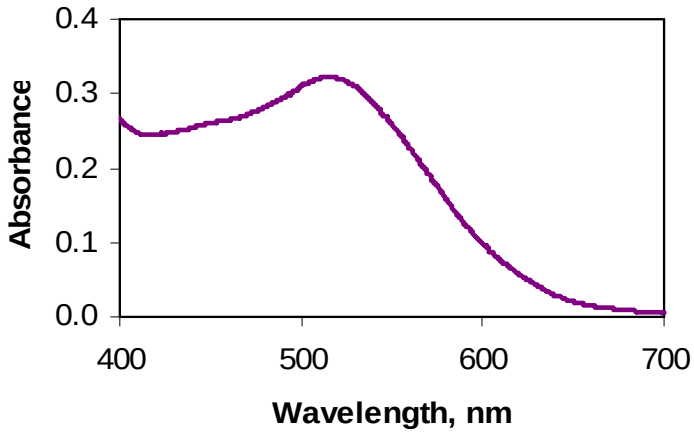
Light absorption depends on wavelength of light $A_\lambda = \varepsilon_\lambda c l$

Light has dual nature

1. Wave



λ – wavelength
c – speed of light wave propagation
ν = c/λ – frequency



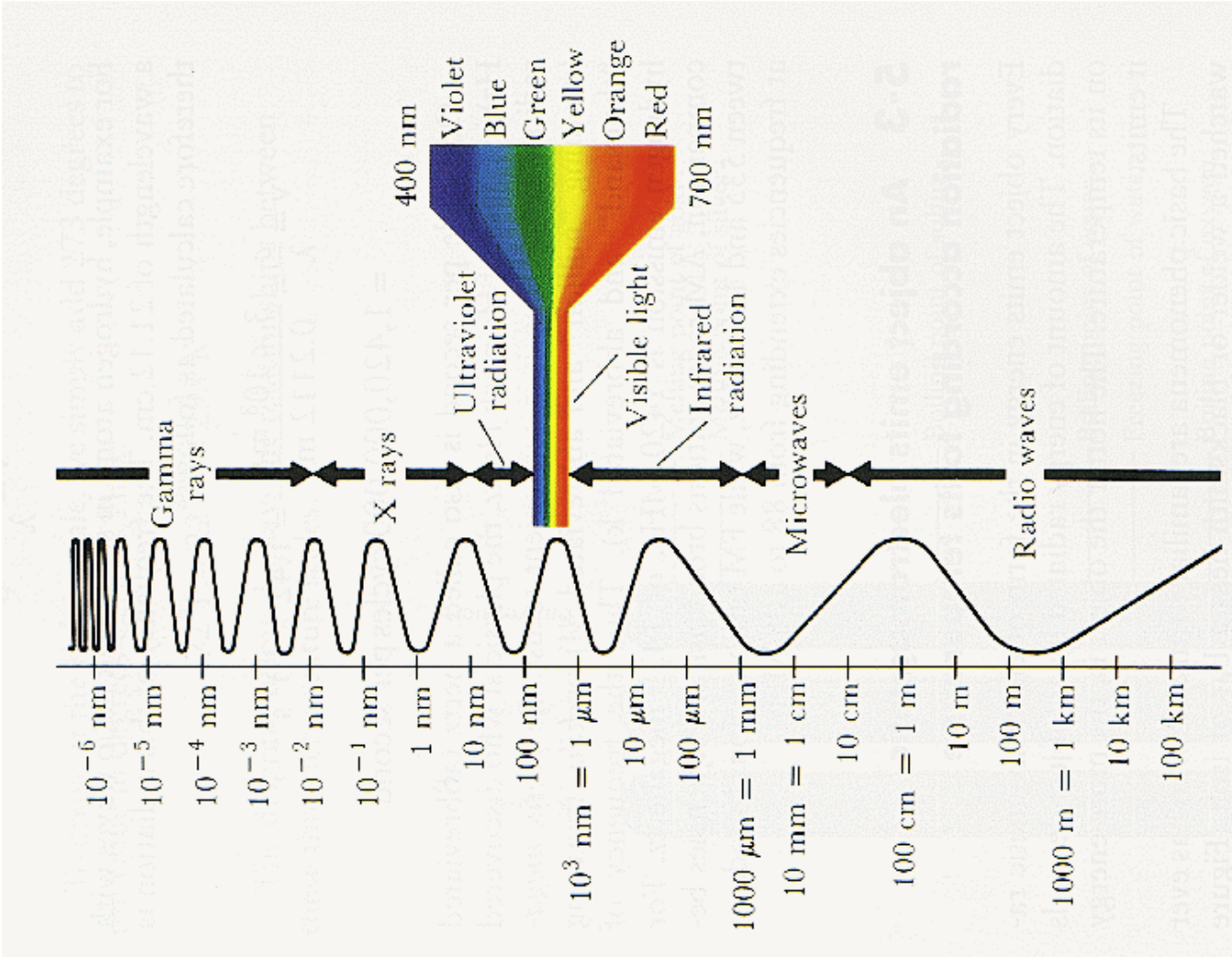
Absorption spectrum of red wine (Canadian merlot, 1998)

2. Particle

Energy of light particle: $E = h\nu = \frac{hc}{\lambda}$

λ (or ν) determines the color of light

Spectrum of Electromagnetic Waves



Spectrophotometry deals with light within ~200–1000 nm

Mechanism of light absorption

Electronic transition is the transition between different electronic states

Ground state is the state with lowest electronic energy

Excited state is the state with higher electronic energy.

Electronic transition to the excited state is caused by absorption of a photon

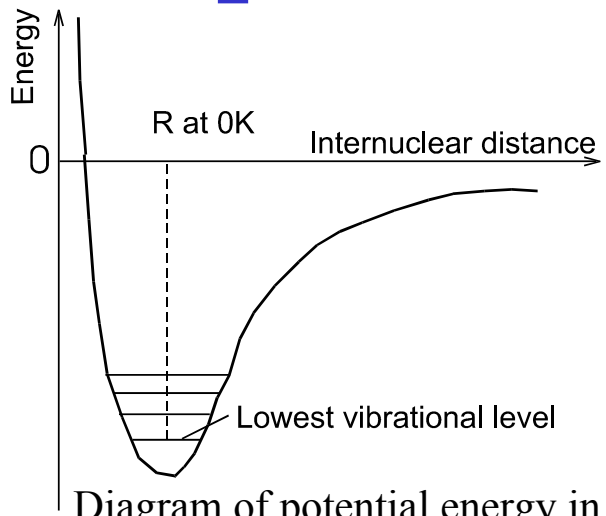
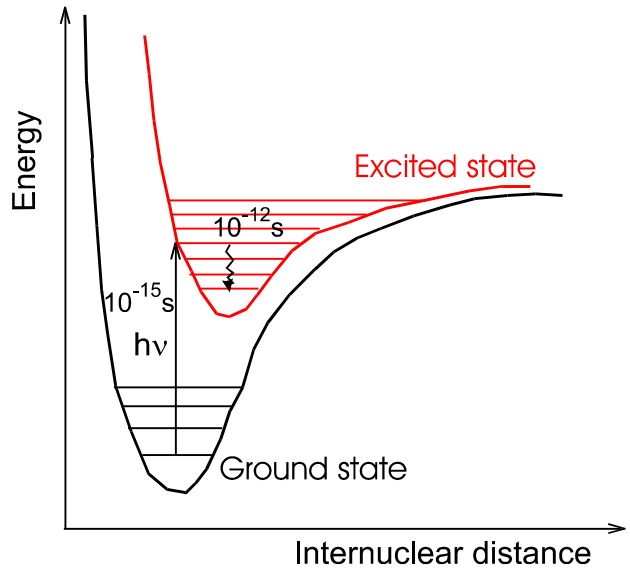


Diagram of potential energy in diatomic molecule

1) Inter-nuclear distance is greater in the excited state than in the ground state

2) **Franck-Condon principle**: electronic transition is much faster than rearrangement of nuclei → the distance between nuclei is not able to change during electronic transition

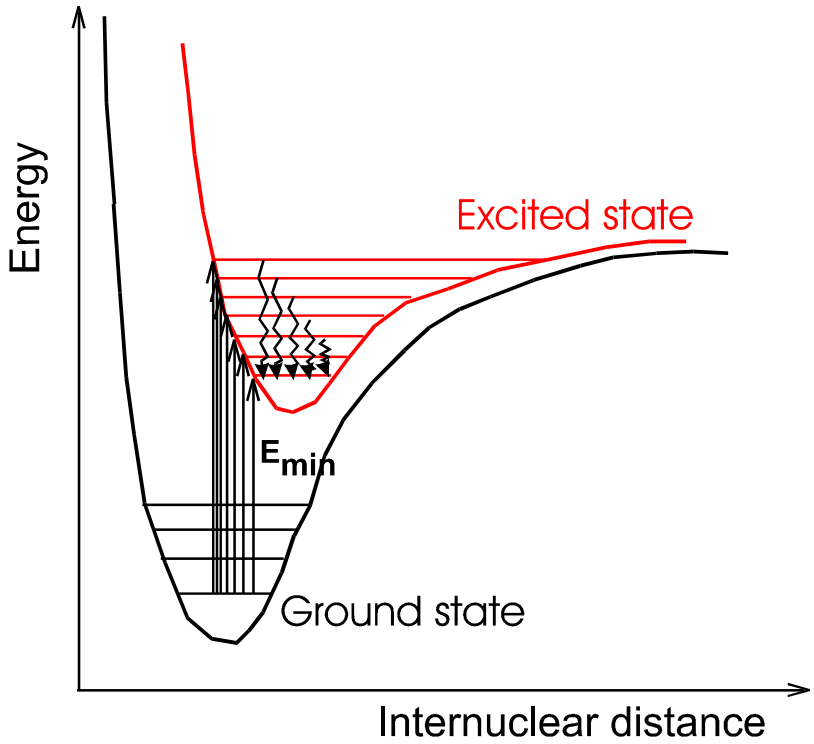
} Vertical transitions are most probable



Vertical transition results in occupation of higher vibrational energy levels ⇒ Vibrational energy dissipates producing heat ⇒ **Light absorption is always accompanied by sample heating**

Small molecules at low temperature

Even at 0K the molecule has vibration. Therefore, a number of vertical transitions can occur to different vibrational levels of the excited state.



This transitions will require photons of different energy, resulting in an absorption spectrum.
At low temperatures for small molecules transitions to different vibrational levels can be resolved

⇓

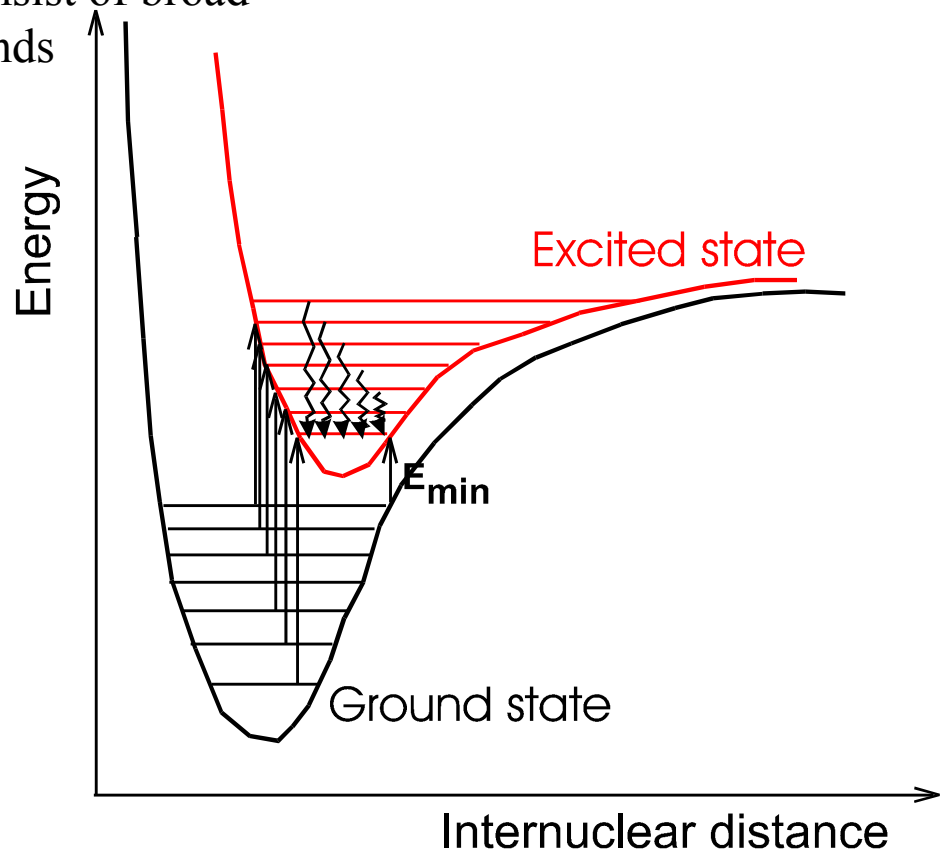
The spectrum consists of very narrow bands.

Multi-atomic molecules at high temperatures

1) Multiatomic molecules have many possible electronic transitions

2) At high temperatures excitation can also occur from different vibrational levels of the ground state

Absorption spectra consist of broad bands



Using absorption spectra to study biochemical reactions

Absorption spectrum is sensitive to minor changes in chemical structure of a molecule. Therefore, chemical reactions and changes in biopolymer conformation can be followed by monitoring absorption spectra.

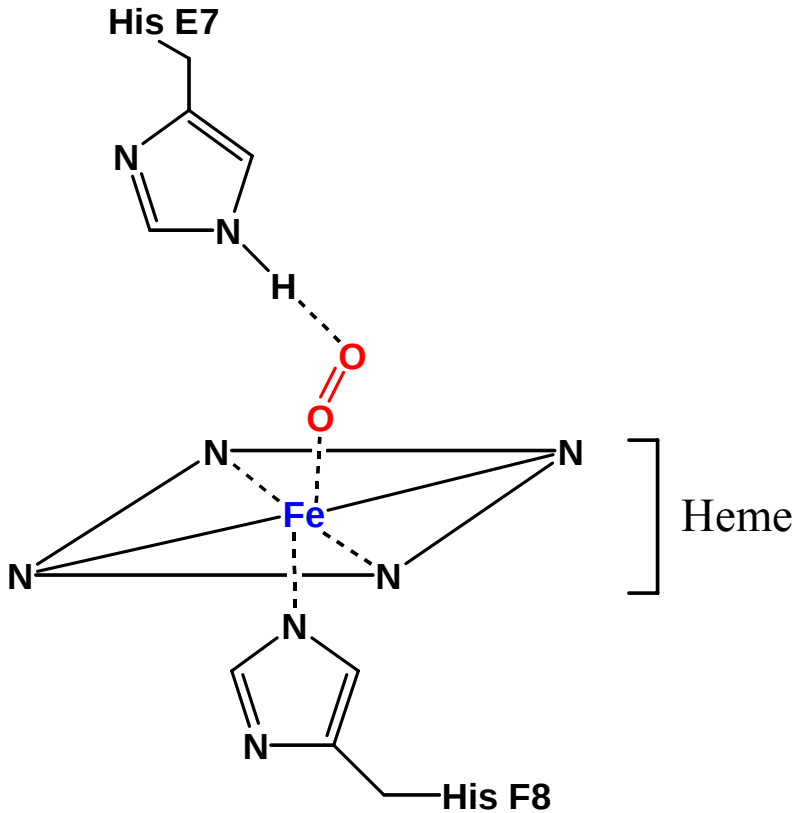
Example: oxygenation of hemoglobin

Hemoglobin is a protein with MW of 70 kDa

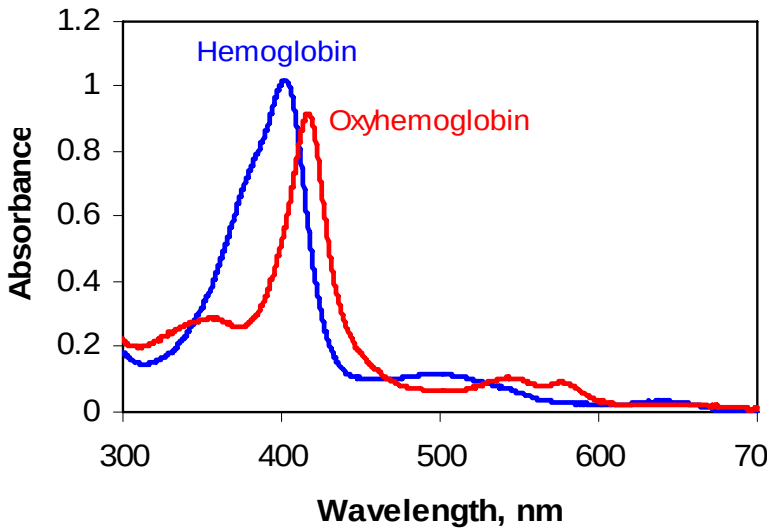
Contains 4 heme groups

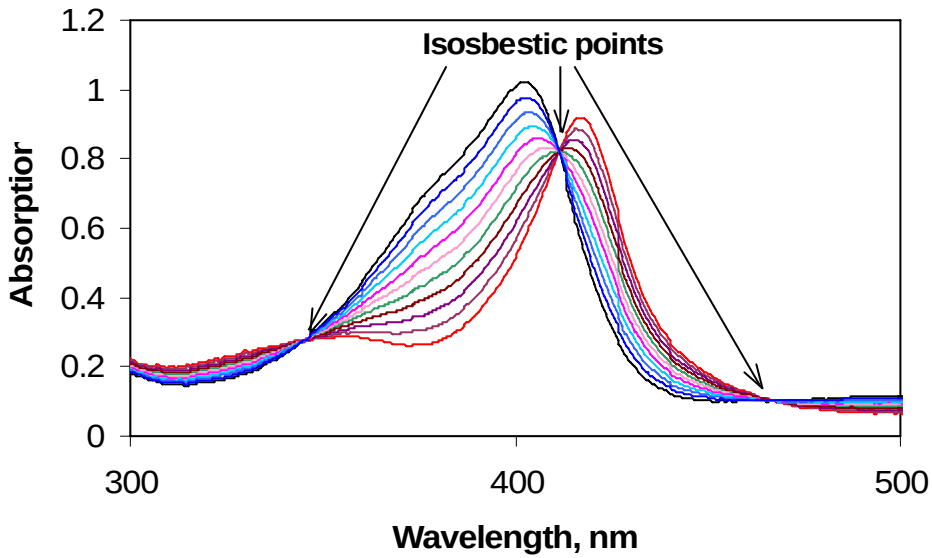
Every heme can bind a molecule of oxygen non-covalently (oxygenation)

Oxygenation drastically changes the absorption spectrum



Oxygenation of Hemoglobin





- Isosbestic points are wavelengths at which the molar absorptivities of the substances are equal

- The presence of isosbestic points is a good indication that only one product forms from a substrate

Analysis of a Mixture

Absorbance is an additive function → Absorbance of a mixture is a sum of the absorbances of the components:

$$A = \varepsilon_X [X] l + \varepsilon_Y [Y] l + \varepsilon_Z [Z] l + \ldots$$

- It is a linear equation. [X], [Y], [Z] are unknowns.
- To find the unknowns the number of unknowns must be equal to the number of equations → to determine the unknown concentrations of N substances in a mixture absorbances of the mixture must be measured at N wavelengths

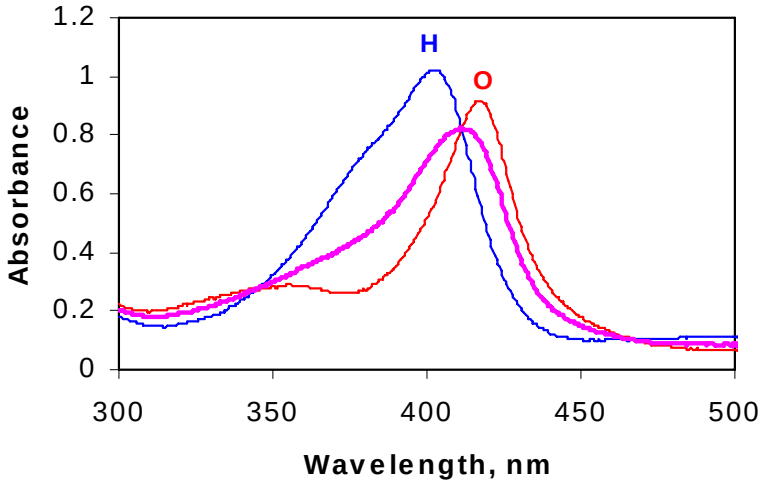
The ***molar absorptivities*** of all the pure components at N wavelengths must be measured a priory using pure substances.

Analysis of two-component mixture (hemoglobin and oxyhemoglobin)

Two components require two wavelengths. The wavelength are chosen to maximize the differential molar absorptivity:

For wavelength λ_1: $\left|\varepsilon_1^H - \varepsilon_1^O\right| = \max$

For wavelength λ_2: $\left|\varepsilon_2^H - \varepsilon_2^O\right| = \max$



Absorption of the mixture at wavelength λ_1:

$$A_1 = \varepsilon_1^H [H] l + \varepsilon_1^O [O] l$$

Absorption of the mixture at wavelength λ_2:

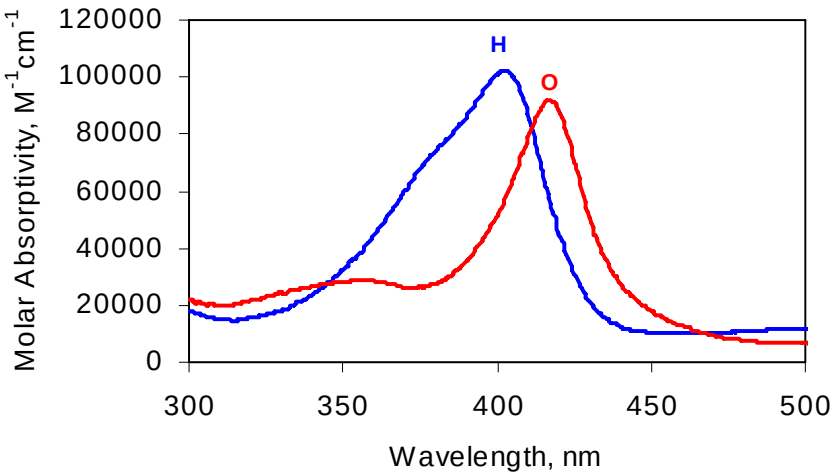
$$A_2 = \varepsilon_2^H [H] l + \varepsilon_2^O [O] l$$

To solve this system of equation with regards to [H] and [O] we must:

1) measure the molar absorptivities of pure hemoglobin and oxyhemoglobin at two wavelengths: ε_1^H, ε_2^H, ε_1^O, and ε_1^O

Hemoglobin and Oxyhemoglobin Contd.

2) Measure absorbances, A_1 and A_2, of the mixture at two wavelengths.
3) Solve the system:
From equation 1:



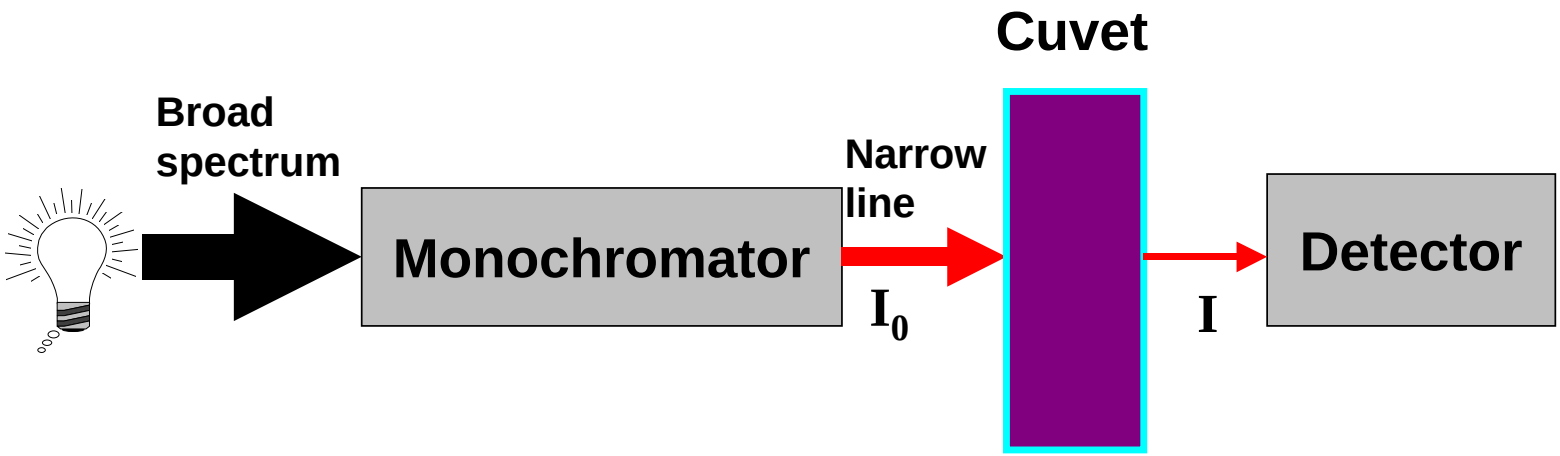
$$[H] = \frac{A_1 - \varepsilon_1^O [O] l}{\varepsilon_1^H l}$$

Substitute [H] in equation 2: $$A_2 = \varepsilon_2^H \frac{A_1 - \varepsilon_1^O [O] l}{\varepsilon_1^H l} l + \varepsilon_2^O [O] l$$

Solve last expression with regards to [O]: $$[O] = \frac{A_2 \varepsilon_1^H - A_1 \varepsilon_2^H}{(\varepsilon_2^O \varepsilon_1^H - \varepsilon_2^H \varepsilon_1^O) l}$$

Using expressions 3 and 4: $$[H] = \frac{A_1 \varepsilon_2^O - A_2 \varepsilon_1^O}{(\varepsilon_2^O \varepsilon_1^H - \varepsilon_2^H \varepsilon_1^O) l}$$

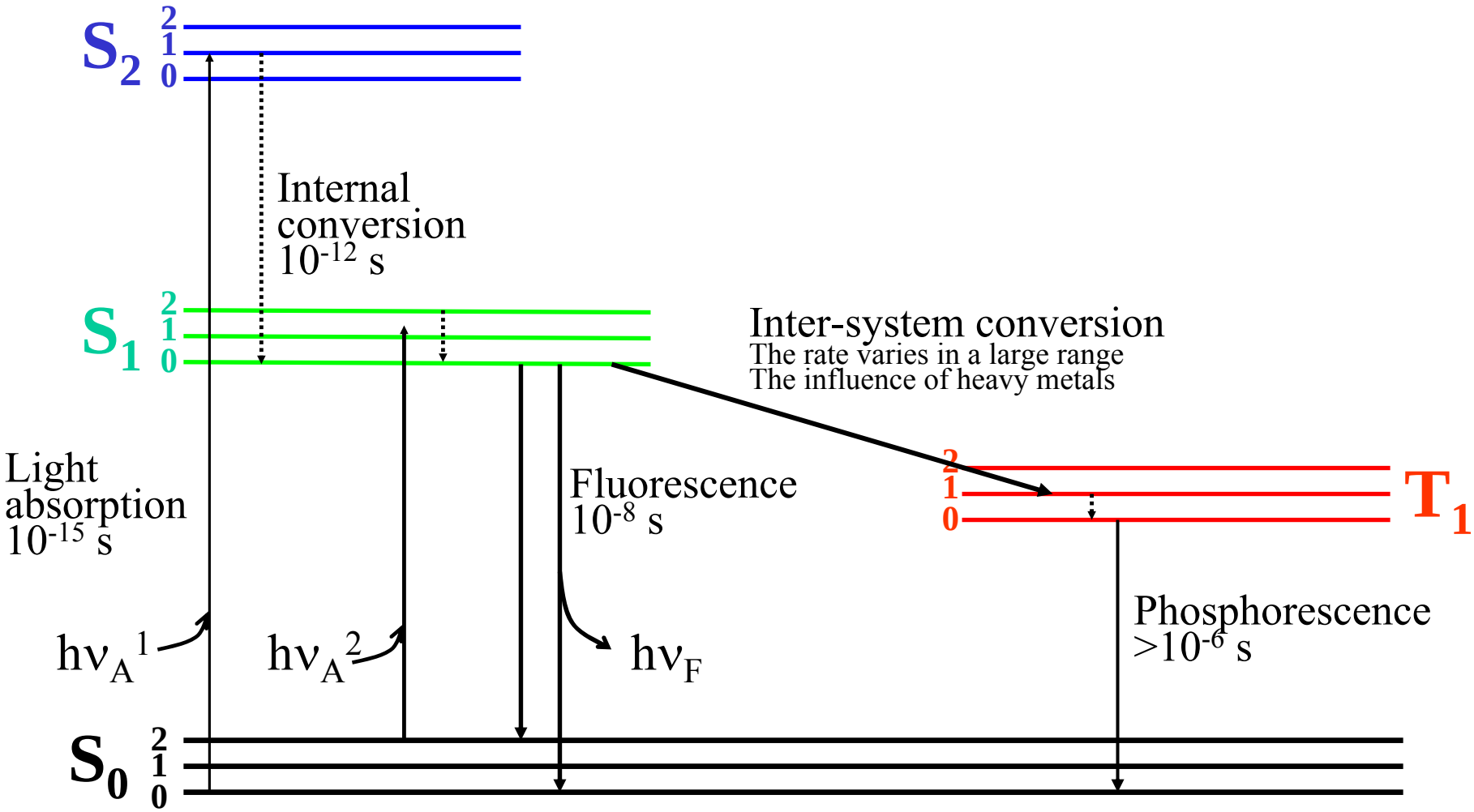
Spectrophotometers



1. Baseline is the measurement of $\mathbf{I_0}$ as a function of λ using a cuvet with only a solvent.
2. Sample reading is the measurement of **I** as a function of λ using the cuvet with a sample.
3. Calculation of absorbance: $\mathbf{A = \log(I_0/I)}$

Conventional spectrophotometers
Price range CA$ 15-20K

Jablonski Diagram - Fluorescence vs. Phosphorescence



Fluorescence Lifetime

Fluorescence intensity decays exponentially:

$$I = I_0 e^{-kt}$$

where k is the monomolecular rate constant of fluorescence decay, which can be determined experimentally from the kinetics of decay

Definition: lifetime of fluorescence:

$$\tau = 1/k$$

do not confuse with half-life time, $t_{1/2} = \ln 2/k$

The rate constant of fluorescence decay is in general a function of two components, radiative rate, k_r and non-radiative rate, k_{nr}:

$$k = k_r + k_{nr}$$

The lifetime accordingly is $\tau = 1/(k_r + k_{nr})$

Natural lifetime, τ_n, is that for $k_{nr} = 0$:

$$\tau_n = 1/k_r$$

$$\Rightarrow \tau/\tau_n = k_r/(k_r + k_{nr})$$

Quantum Yield

Quantum yield: $$Q = \frac{\text{number of emitted photons}}{\text{number of absorbed photons}}$$

Substances with the highest Q (~1), such as rhodamines are the brightest emitters

Quantum yield relates with the lifetime:

$$Q = k_r/(k_r + k_{nr})$$

$$\tau/\tau_n = k_r/(k_r + k_{nr}) = Q$$

Thus, τ_n can be found as: $\tau_n = \tau/Q$

Note: that both τ and Q are experimentally measurable parameters

Problem:

k_r typical for fluorescence is 10^8 s^{-1}

k_{nr} typical is ~ 10^9 s^{-1}

Calculate Q typical for fluorescence

Solution: $Q = k_r/(k_r + k_{nr}) = 10^8/(10^8 + 10^9) \approx 0.1$

Quenching of Fluorescence

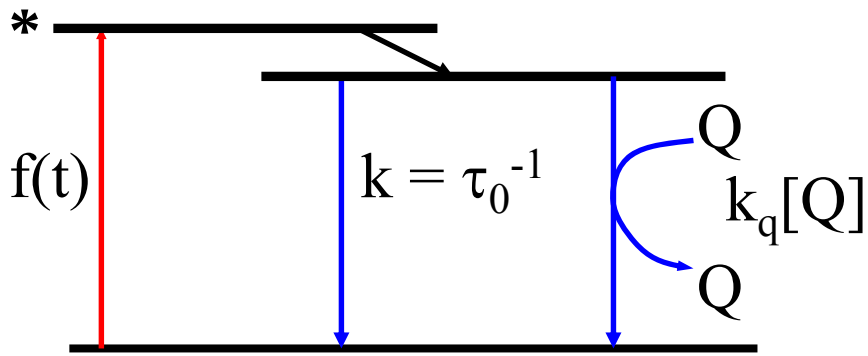
Two major mechanisms of quenching exist:
- **Dynamic** or **collisional** (depends on the diffusion of fluorophore and quencher)
- **Static** (the formation of non-radiative complexes with quenchers that does not depend on the diffusion of fluorophore and quencher)

Dynamic quenching is characterized by the **Stern-Volmer eqn.**:

$$\mathbf{I_0/I = 1 + k_q \tau_0 [Q]}$$

Where $\mathbf{I_0}$ and **I** are fluorescence intensities with and without quencher, $\mathbf{k_q}$ is a bimolecular rate constant of quenching ($M^{-1}s^{-1}$), $\boldsymbol{\tau_0}$ is the lifetime of the fluorophore in the absence of quenchers, **[Q]** is the concentration of quencher.

Stern-Volmer Equation



Simplified Jablonski diagram:
f(t) is the rate of excitation which depends on:
(i) incident light intensity,
(ii) concentration of fluorophore,
(iii) absorptivity of fluorophore

$k = \tau_0^{-1}$ is the decay rate of fluorophore in the absence of the quencher
τ_0 is the lifetime of fluorophore in the absence of the quencher
[F*] is the concentration of fluorophore in the excited (*) state

In the steady state, when f(t) = const, the concentration of the fluorophore in the excited state does not change:

$$[F^*] = \text{const}$$

Thus:

$$d[F^*]/dt = 0$$

Note: d[F*]/dt = the rate of formation *minus* the rate of decay

Stern-Volmer Equation contd.

In the absence of the quencher: $\mathbf{d[F^*]/dt = f(t) - k[F^*]_0 = 0}$
Where $[F^*]_0$ is the concentration of F* in the the absence of quencher

In the presence of the quencher: $\mathbf{d[F^*]/dt = f(t) - k[F^*] - k_q[F^*][Q] = 0}$

$f(t) - k[F^*]_0 = f(t) - k[F^*] - k_q[F^*][Q]$
$k[F^*]_0 = (k + k_q[Q])[F^*]$
$[F^*]_0 /[F^*] = (k + k_q[Q]) /k = 1 + k_q[Q]/k = 1 + k_q\tau_0[Q]$
$\mathbf{[F^*]_0 /[F^*] = 1 + k_q\tau_0[Q]}$

The intensities of irradiation in the absence and the presence of quencher, $\mathbf{I_0}$ **and** $\mathbf{I}$, are proportional to the concentration of fluorophore in the excited state: $\mathbf{I_0 = k[F^*]_0}$, and $\mathbf{I = k[F^*]}$, then:

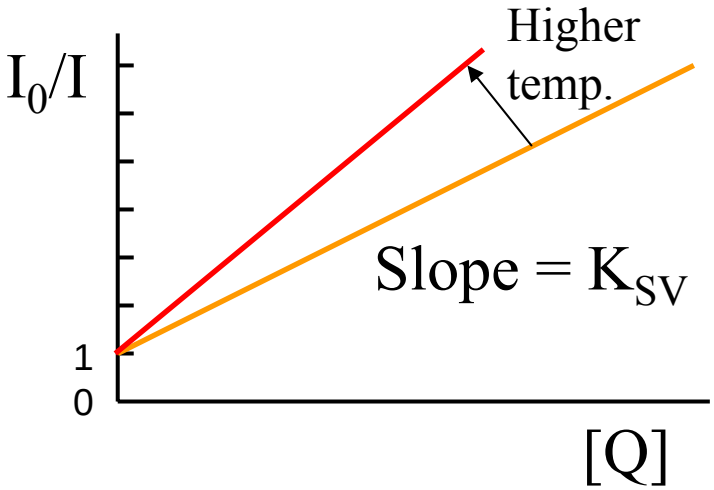
$$\mathbf{I_0 /I = 1 + k_q\tau_0[Q]}$$

Prove that: $\tau_0/\tau = 1 + k_q\tau_0[Q]$

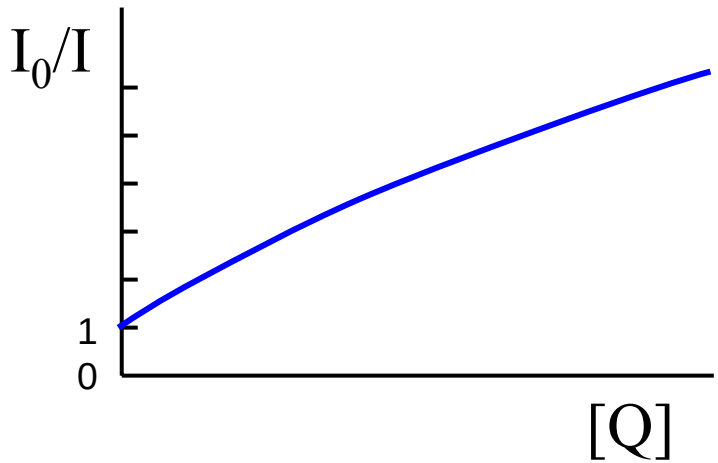
Stern-Volmer Constant, $K_{SV} = k_q \tau_0$

$I_0 / I = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q]$

K_{SV} can be experimentally measured from the Stern-Volmer plot:



- The linearity of the SV plot in general indicates that there is a single class of fluorophore
- The increase of the slope with T indicates that quenching is dynamic (collision frequency increases with T)



More than 1 fluorophore with at least one of them less accessible to the quencher leads to SV plot deviation toward the [Q]-axis

Efficiency of Quenching

The bimolecular rate constant of quenching, $\mathbf{k_q}$, is proportional to the diffusion controlled bimolecular rate constant, $\mathbf{k_d}$. The coefficient of proportionality is the efficiency of quenching φ:

$$\mathbf{k_q} = \varphi\, \mathbf{k_d} \qquad \varphi \leq 1$$

Determination of φ: $\varphi = \mathbf{k_q} / \mathbf{k_d}$

k_q can be calculated from $k_q = K_{SV}/\tau_0$

K_{SV} can be determined from the SV plots

τ_0 can be determined from the kinetics of fluorescence decay

k_d can be calculated using the Smoluchovski equation:

$$k_d = \frac{4\pi N_A}{1000}(R_f + R_q)(D_f + D_q)$$

where $\mathbf{N_A}$ is Avogadro number, $\mathbf{R_f}$ and $\mathbf{R_q}$ are molecular radii of the fluorophore and quencher, respectively, $\mathbf{D_f}$ and $\mathbf{D_q}$ are diffusion coefficients of the fluorophore and quencher, respectively

Efficiency of Quenching contd.

The radii can be estimated from the molecular structures

Diffusion coefficients can be calculated from the Stokes-Einstein eqn.:

$$D = kT/6\pi\eta R$$

where $k = 1.38 \times 10^{-23}$ JK^{-1} is the Boltzman constant

η is the solvent viscosity ($\sim 9 \times 10^{-4}$ kg m^{-1} s^{-1} for water)

Example: Find φ for quenching of tryptophan by oxygen

Step 1: finding k_q

The experimental value of $K_{SV} = 32.5$ M^{-1}

The experimental value of $\tau_0 = 2.7$ ns

$$k_q = K_{SV}/\tau_0 = 1.2 \times 10^{10}\ M^{-1}s^{-1}$$

Step 2: finding k_d

At 25°C in water: $D_{O_2} = 2.5 \times 10^{-5}$ cm^2/s, $D_{trypt} = 0.66 \times 10^{-5}$ cm^2/s

Assume that $R_{O_2} + R_{tryp} = 5$ Å

$$k_d = \frac{4\pi N_A}{1000}(R_{O_2} + R_{tryp})(D_{O_2} + D_{tryp}) = 1.2 \times 10^{10}\ M^{-1}s^{-1}$$

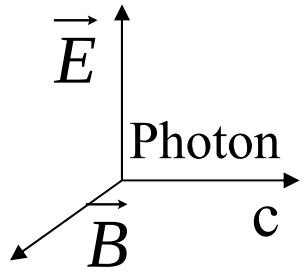
Step 2: finding φ

$$\varphi = k_q / k_d = 1$$

Fluorescence Polarization or Anisotropy

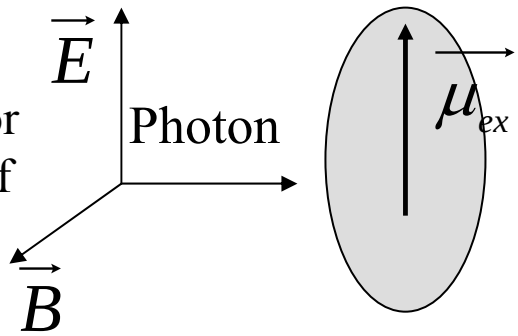
1. Absorption of Polarized Light

- Polarization is the direction of the electric vector of the electromagnetic wave. Light is polarized if all photons have the same direction of $\vec{E}$ (lasers are most convenient sources of polarized light)

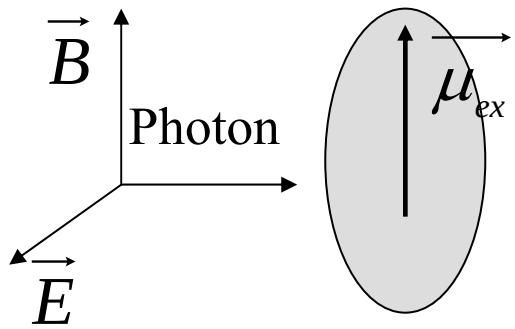


- Fluorophores ***preferably*** absorb photons whose electric vector is aligned with the transition dipole moment, $\vec{\mu}_{ex}$, of fluorophore.
- The $\vec{\mu}_{ex}$ has a defined orientation with respect to molecular axes.
- Thus, if a fluorophore solution is illuminated by polarized light then only a fraction of randomly oriented molecules will absorb light (those with a favorable orientation).
- Thus, the population of molecules excited with polarized light will have the same orientation.

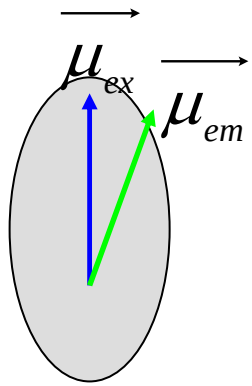
Polarization **favorable** for absorption of photon



Polarization **unfavorable** for absorption of photon



2. Emission of Polarized Light



- The emission dipole moment, μ_{em}, is not the same as the excitation dipole moment, μ_{em}. It is associated with the change of molecule geometry in the excited state.

- Therefore, the polarization of emitted light will be different from that of excitation light.
- The degree of differences in the polarization of excitation and emission lights is described by **fluorescence anisotropy**:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{I_{\parallel} - I_{\perp}}{I_{Total}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of fluorescence in the direction parallel and perpendicular to $\overrightarrow{\mu_{ex}}$ respectively.

Note: 1. ($I_{\parallel} + 2I_{\perp}$) is the total intensity of fluorescence, so that fluorescence anisotropy does not depend on fluorescence intensity. "2" in the denominator shows that in a 3-D space there are 2 indistinguishable directions perpendicular to $\overrightarrow{\mu_{ex}}$

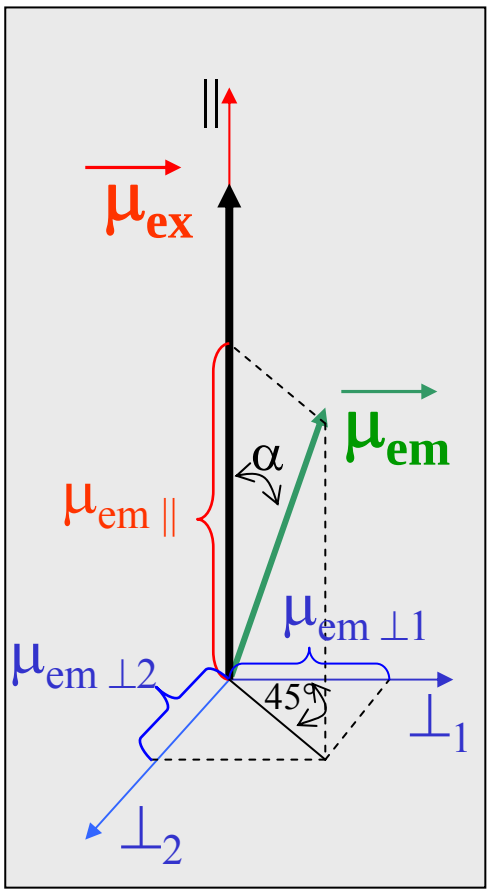
2. Maximum possible anisotropy of 1 (when $I_{\perp} = 0$) would be observed if: (i) the fluorophore had $\overrightarrow{\mu_{ex}} = \overrightarrow{\mu_{em}}$, (ii) all fluorophore molecules were oriented favorably and (ii) all fluorophore molecules were **immobile**

3. Maximum fluorescence anisotropy of randomly oriented solution of fluorophore is 0.4 due to excitation photoselection

4. Light scattering and reflection can produce r = 1

Calculation of Fluorescence Anisotropy

Problem: Calculate fluorescence anisotropy for a solid solution of fluorophore which is oriented so that μ_{ex} of all molecules are aligned with the direction of polarization of excitation light. The angle between $\vec{\mu}_{ex}$ and $\vec{\mu}_{em}$ is α



Solution:

- To calculate the anisotropy we need to know $I_{||}$ and $I_{\perp}$.
- $I_{||}$ and $I_{\perp}$ are dependent on the projections of μ_{em} on the || ($\mu_{em\,||}$) and ⊥ axes ($\mu_{em\,\perp1}$ and $\mu_{em\,\perp2}$):

$$I_{||} = a\,\mu_{em\,||} \qquad I_{\perp1} = a\,\mu_{em\,\perp1} \qquad I_{\perp2} = a\,\mu_{em\,\perp2}$$

where a is a constant

- The projections $\mu_{em\,\perp1}$ and $\mu_{em\,\perp2}$ are identical due to the two ⊥ axes being indistinguishable from each another, thus:

$$I_{||} = a\,\mu_{em\,||} = a\,\mu_{em}\cos\alpha$$

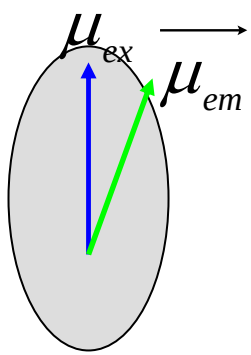
$$I_{\perp} = I_{\perp1} = I_{\perp2} = a\,\mu_{em}\sin\alpha\cos 45^{\circ} = 0.5\times 2^{1/2}\,a\,\mu_{em}\sin\alpha$$

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} = \frac{a\,\mu_{em}\cos\alpha - 0.5\times 2^{1/2}\,a\,\mu_{em}\sin\alpha}{a\,\mu_{em}\cos\alpha + 2\times 0.5\times 2^{1/2}\,a\,\mu_{em}\sin\alpha} = \frac{\cos\alpha - 0.5\times 2^{1/2}\sin\alpha}{\cos\alpha + 2^{1/2}\sin\alpha}$$

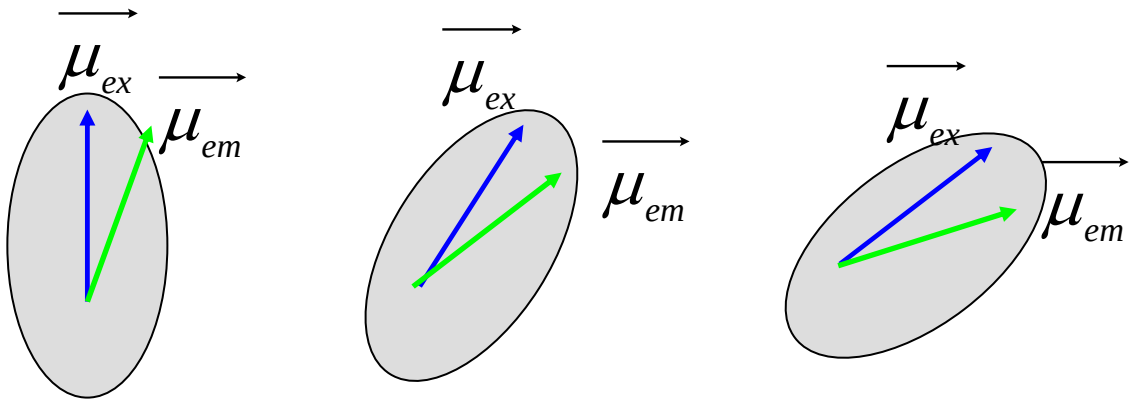
2. Rotational Depolarization of Fluorescence

Reasons for fluorescence anisotropy of a random solution of fluorophore to be less than **0.4**:

1. $\vec{\mu}_{ex} \neq \vec{\mu}_{em}$



2. Rotation of a fluorophore molecule during the lifetime of fluorescence



t = 0 t = 5 ns t = 10 ns

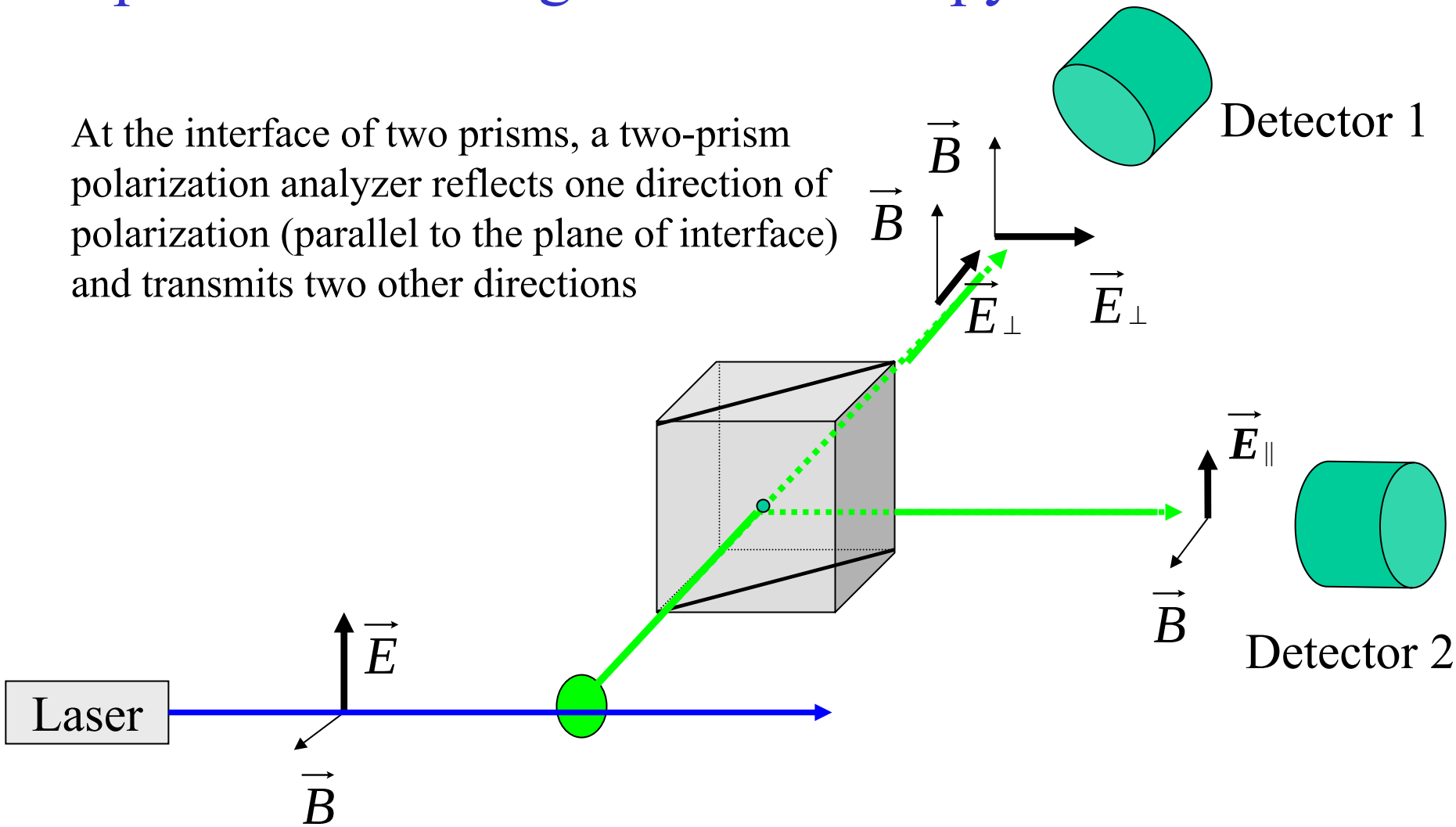
Anisotropy Increases with Increasing Size of Fluorophore Molecule

- The rate of rotational depolarization increases with an increasing rate of molecular rotation (rotational diffusion)
- The rate of rotational diffusion increases with decreasing the size of fluorophore.
- This property of fluorescence anisotropy is used to sense interactions between small fluorophores and large biomolecules: anisotropy increases upon binding of fluorophore to a large molecule.
- The equilibrium dissociation constant of the protein-fluorophore complex, $K_d = k_{off}/k_{on}$, can be calculated based on the anisotropy measurements.

$$\text{Protein} + \text{Fluorophore} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{Protein•Fluorophore}$$

Experimental Design for Anisotropy Measurements

At the interface of two prisms, a two-prism polarization analyzer reflects one direction of polarization (parallel to the plane of interface) and transmits two other directions



Determination of K_d Using Anisotropy

Assume that fluorophore F forms a dynamic complex with protein P:

$$P + F \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} P\bullet F$$

The equilibrium binding constant is defined as: $$K_d = \frac{[P]_{eq}[F]_{eq}}{[P \bullet F]_{eq}}$$

where $[P]_{eq}$, $[F]_{eq}$, and $[P\bullet F]_{eq}$ are equilibrium concentrations of free protein, free fluorophore, and the complex, respectively. We will express $[P]_{eq}[F]_{eq}$ $[P\bullet F]_{eq}$ through fluorescence anisotropy and total concentrations of protein and fluorophore, $[P]_0$ and $[F]_0$ using two principles:

1) Anisotropy is an additive function, that is

$$r = r_F \frac{[F]_{eq}}{[F]_0} + r_{P\bullet F} \frac{[P \bullet F]_{eq}}{[F]_0}$$

2) Mass balance requires that:

$$[P]_0 = [P\bullet F]_{eq} + [P]_{eq}$$

and

$$[F]_0 = [P\bullet F]_{eq} + [F]_{eq}$$

Determination of K_d Using Anisotropy contd.

$$r = r_F \frac{[F]_{eq}}{[F]_0} + r_{P\bullet F} \frac{[P\bullet F]_{eq}}{[F]_0} = r_F \frac{[F]_{eq}}{[F]_0} + r_{P\bullet F} \frac{[F]_0 - [F]_{eq}}{[F]_0} = r_F \frac{[F]_{eq}}{[F]_0} + r_{P\bullet F}\left(1 - \frac{[F]_{eq}}{[F]_0}\right) =$$

$$r_F \frac{[F]_{eq}}{[F]_0} + r_{P\bullet F} - r_{P\bullet F} \frac{[F]_{eq}}{[F]_0} = r_{P\bullet F} + \frac{[F]_{eq}}{[F]_0}\left(r_F - r_{P\bullet F}\right) \Rightarrow \boxed{\frac{[F]_{eq}}{[F]_0} = \frac{r - r_{P\bullet F}}{r_F - r_{P\bullet F}} = R}$$

Experimentally measurable parameter

Thus:

1. $[F]_{eq} = [F]_0 R$
2. $[P\bullet F]_{eq} = [F]_0 - [F]_{eq} = [F]_0 - [F]_0 R = [F]_0 (1 - R)$
3. $[P]_{eq} = [P]_0 - [P\bullet F]_{eq} = [P]_0 - [F]_0 (1 - R)$

By substituting the expressions 1-3 into $K_d = \frac{[P]_{eq}[F]_{eq}}{[P\bullet F]_{eq}}$ we get:

$$K_d = \frac{\{[P]_0 - [F]_0(1-R)\}[F]_0 R}{[F]_0(1-R)} = \frac{[P]_0 - (1-R)}{(1/R - 1)} \quad \text{where } R = \frac{r - r_{P\bullet F}}{r_F - r_{P\bullet F}}$$

$$K_d = \frac{[P]_0 - (1-R)}{(1/R - 1)} \quad \text{where } R = \frac{r - r_{P\bullet F}}{r_F - r_{P\bullet F}}, \quad 0 < R < 1$$

Determination of r, r_F and $r_{P\bullet F}$

- $\mathbf{r_F}$ is anisotropy of free fluorophore. It is measured in the absence of the protein
- **r** is the anisotropy of fluorophore at a finite concentration of protein so that only part of fluorophore is bound to a protein:

$$r = r_F \frac{[F]_{eq}}{[F]_0} + r_{P\bullet F} \frac{[P \bullet F]_{eq}}{[F]_0} \quad \textit{and} \quad 0 < \frac{[F]_{eq}}{[F]_0} < 1, \text{ preferably } \frac{[F]_{eq}}{[F]_0} \approx 0.5$$

- $\mathbf{r_{P\bullet F}}$ is the anisotropy of the P•F complex, that can be *experimentally* found by using high enough concentration of protein to ensure that $[P\bullet F]_{eq}/[F]_0 \rightarrow 1$ and $[F]_{eq}/[F]_0 \rightarrow 0$ and thus

$$r = r_F \frac{[F]_{eq}}{[F]_0} + r_{P\bullet F} \frac{[P \bullet F]_{eq}}{[F]_0} = r_F \times 0 + r_{P\bullet F} \times 1 = r_{P\bullet F}$$

- Practically, r is measured as a function of $[P]_0$ (Scotchard plot) to find the saturating value of r which is assumed to be equal to $r_{P\bullet F}$

Other Parameters that Can be Used for the Determination of K_d

- Anisotropy can be used for the determination of K_d because it is an **additive parameter** (anisotropy of a mixture is equal to the sum of anisotropies of its components)
- Any additive parameter that has a different value for F and P•F can be used to find K_d
- Can fluorescence lifetime or intensity be used?
- Can fluorescence resonance energy transfer (FRET) be used?
- Mobility in capillary electrophoresis can be used for calculation of K_d, because:

Home assignment

$$\mu = \mu_F \frac{[F]_{eq}}{[F]_0} + \mu_{P\bullet F} \frac{[P\bullet F]_{eq}}{[F]_0}, \qquad \mu = \frac{v}{E} = \frac{L}{tE}$$

where t is migration time

$$\frac{1}{t} = \frac{1}{t_F}\frac{[F]_{eq}}{[F]_0} + \frac{1}{t_{P\bullet F}}\frac{[P\bullet F]_{eq}}{[F]_0}$$

Fluorescence Energy Transfer

Reaction involved in ET between donor (D) and acceptor (A):

$D + h\nu \rightarrow D^*$

$D^* + A \xrightarrow{k_{ET}} D + A^* + \text{heat}$

Two types of processes are distinguished:

- Non-Resonance Energy Transfer requires collision of D and A:

$$d[A^*]/dt = k_{ET}[D^*][A]$$

where $k_{ET} = \varphi \times k_d$, φ is the efficiency of ET and k_d is a diffusion-controlled rate constant. The process is similar to quenching! It is rare (e.g. spin-forbidden T→S or S→T) and is not widely used as an analytical tool.

2. Fluorescence Resonance Energy Transfer (FRET) by the Förster mechanism is widely used to study the dynamics of molecular interactions. The rate constant of FRET is conventionally expressed as a monomolecular one:

$$k_{FRET}(r) = \frac{1}{\tau_0^D}\left(\frac{R_0}{r}\right)^6$$

where

τ_0^D is the lifetime of D in the absence of A,

R_0 is the Förster radius, and

r is the distance between the D and A.

FRET – the Meaning of R_0

$$k_{FRET}(r) = \frac{1}{\tau_0^D}\left(\frac{R_0}{r}\right)^6$$

What is the physical meaning of R_0?

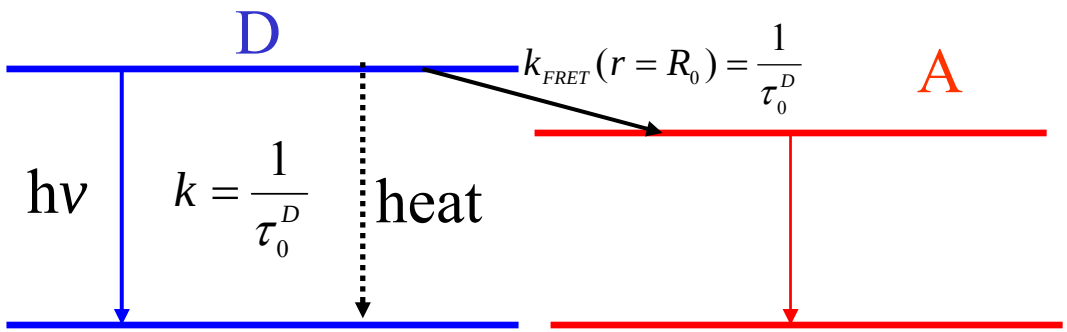
R_0 is the distance between D and A at which the efficiency of energy transfer is equal to 1/2 (half of energy is transferred from D to A). Indeed, when $r = R_0$, then:

$$k_{FRET}(r = R_0) = \frac{1}{\tau_0^D}\left(\frac{1}{1}\right)^6 = \frac{1}{\tau_0^D}$$

Typical R_0 are 20 – 90 Å

Q: What is the efficiency of ET for $r = R_0$?

Recall that the rate constant, k, of radiative+non-radiative transitions in D is the same as that of FRET: $k = k_{FRET} = 1/\tau_0^D$



The efficiency of ET: $\varphi = k_{FRET}/(k_{FRET} + k)$ (=1/2 for $r = R_0$)

Calculation of R_0

$$R_0 = \sqrt[6]{\frac{9000(\ln 10)\kappa^2 Q_D}{128\pi^5 N_A n^4} \int_0^\infty F_D(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}$$

where

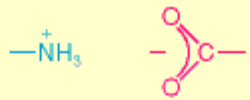
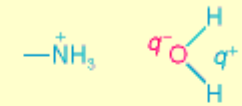
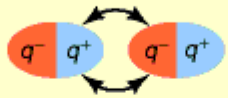
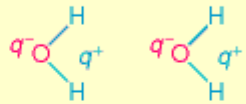
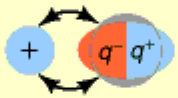
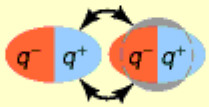
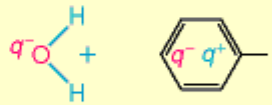
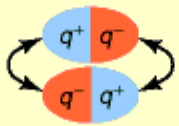
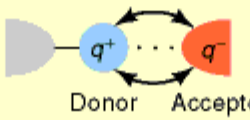
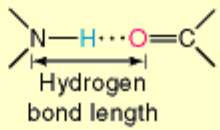
- Q_D is the quantum yield of D in the absence of A
- n is the refractive index of the medium (1.4 for biomolecules in aqueous solns.)
- N_A is Avogadro's number
- κ^2 is the orientation factor (2/3 for randomly oriented molecules)
- $F_D(\lambda)$ is the normalized spectrum of fluorescence of D and $\varepsilon(\lambda)$ is the extinction coefficient of A

$\int_0^\infty F_D(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda$ is called the integral of spectral overlap

What is the reason of 6 in $(R_0/r)^6$?

Dipole-dipole coupling mechanism makes the distance dependence so strong

Types of intermolecular interactions in solution

Type of Interaction	Model	Example	Dependence of Energy on Distance
(a) Charge–charge Longest-range force; nondirectional			$1/r$
(b) Charge–dipole Depends on orientation of dipole			$1/r^2$
(c) Dipole–dipole Depends on mutual orientation of dipoles			$1/r^3$
(d) Charge–induced dipole Depends on polarizability of molecule in which dipole is induced			$1/r^4$
(e) Dipole–induced dipole Depends on polarizability of molecule in which dipole is induced			$1/r^5$
(f) Dispersion Involves mutual synchronization of fluctuating charges			$1/r^6$
(g) van der Waals repulsion Occurs when outer electron orbitals overlap			$1/r^{12}$
(h) Hydrogen bond Charge attraction + partial covalent bond			Length of bond fixed

FRET – The Bioanalytical Use

1. Calculation of the distance between two fluorophores:

- The distance between tyrosine and tryptophan in a protein
- R_0 is calculated using the formula $R_0 = \sqrt[6]{\frac{9000(\ln 10)\kappa^2 Q_D}{128\pi^5 N_A n^4}\int_0^\infty F_D(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}$ and, then, ***r*** is calculated as follows:

$$r = \sqrt[6]{\frac{R_0^6}{k_{FRET}(r)\tau_0^D}} = \sqrt[6]{\frac{R_0^6}{\frac{k}{1/\varphi - 1}\tau_0^D}} = \sqrt[6]{\frac{R_0^6}{\frac{1}{1/\varphi - 1}}} = \sqrt[6]{(1/\varphi - 1)R_0^6}$$

$\varphi = k_{FRET}/(k_{FRET} + k)$ $\quad$ $k = 1/\tau_0^D$

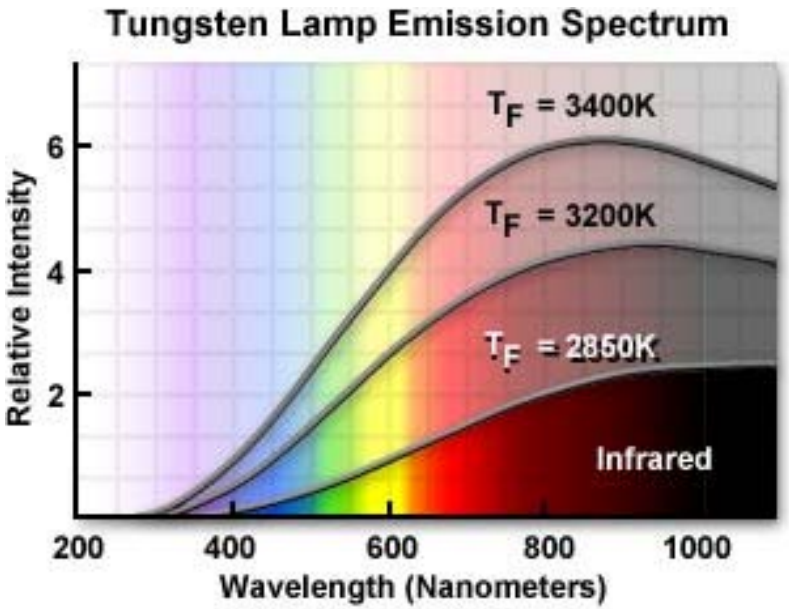
The efficiency of FT, φ is determined experimentally

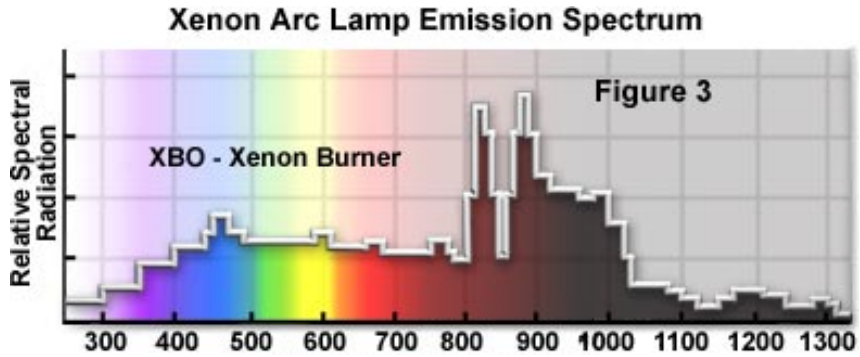
2. FRET-based reporters such as molecular beacons

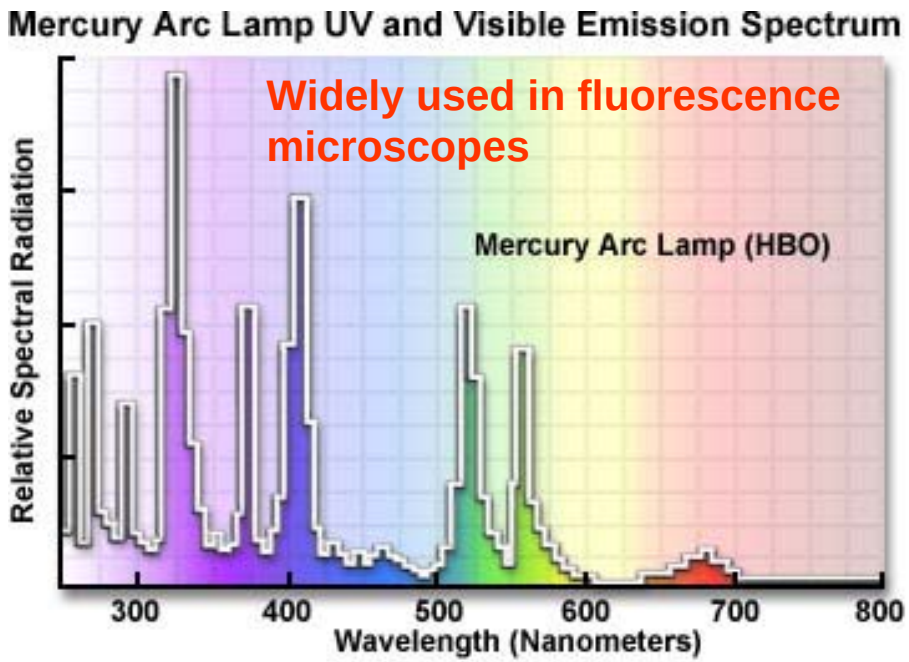
Light Sources. Lamps

Never touch optical surfaces with bare fingers

Characteristics of lamps as light sources: Tunable λ (requires monochromator), Low cost, Low power at any fixed λ, No polarization



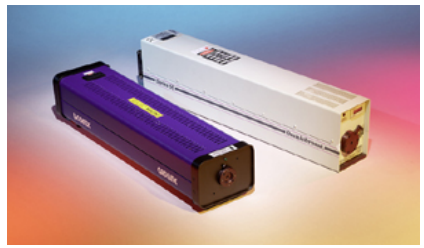




Mercury and Xenon arc lamps require caution during operation because of the danger of explosion due to very high internal gas pressures and extreme heat generated during use.

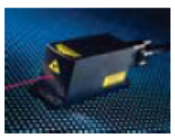
Light Sources. Lasers

Advantages: monochromatic light, high power, high level of polarization
Disadvantages: high cost, impossible to tune λ



Examples
- Helium-cadmium laser, 325nm, 442 nm
- Helium-neon laser, 543 nm, 594 nm, 632.8 nm
- Diode-pumped solid-state lasers, 457 nm, 640-685 nm (temp-dependant), 1064 nm.
- Argon-ion, 488 nm, 514 nm

Lasers deliver 1-200 mW in light. Eye damage is possible during direct exposure

	Model	Wavelength	Output Power
	Cyan OEM (Cyan™ OEM 488 nm CW Laser)	488 nm	10–50 mW
	Cyan Scientific (Cyan™ Scientific 488 nm CW Laser)	488 nm	10–50 mW
	Excelsior OEM (Excelsior® OEM CW Lasers)	473 nm 488 nm 532 nm 561 nm 1064 nm	5–500 mW
	Excelsior Direct Diode (Excelsior® Direct Diode CW Lasers)	375 nm 405 nm 440 nm 635 nm 785 nm	8–50 mW
	Excelsior Scientific (Excelsior® Scientific CW Lasers)	473 nm 532 nm 561 nm 1064 nm	10–800 mW

Objectives

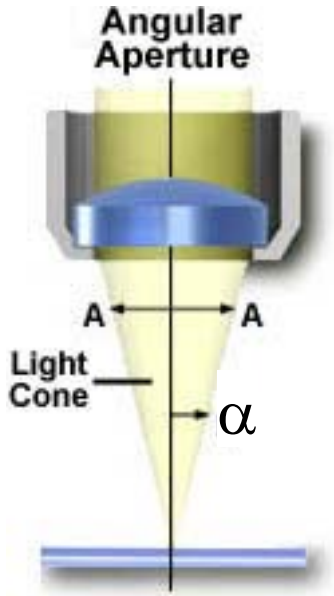
Magnification: The ratio between the sizes of the image and object when object is at the working distance.

Working distance: Distance from the objective to the object at which the image will be in focus

Angular Aperture: AA = sin α

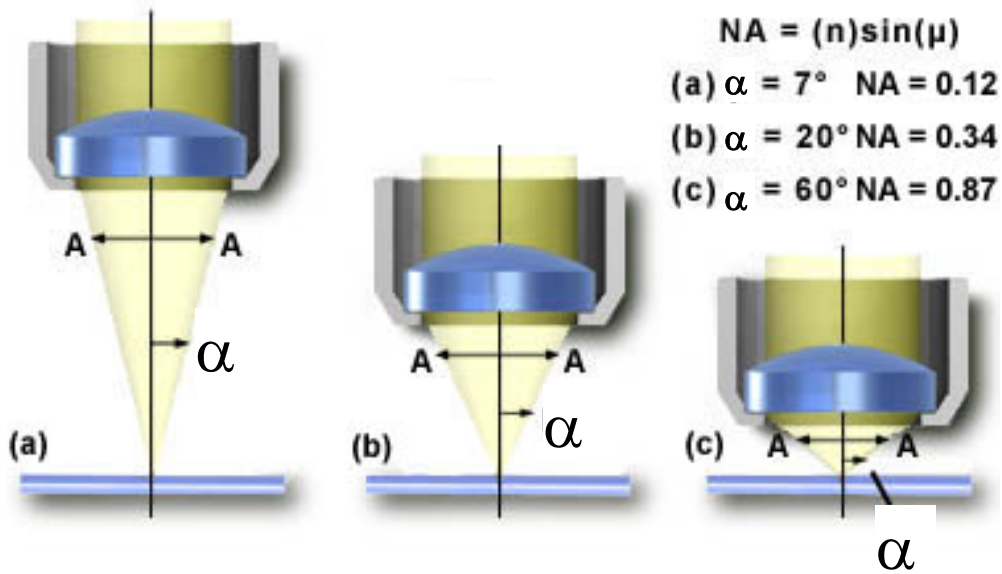
Numerical Aperture: NA = *n*(sin α)

where *n* is the refractive index of the imaging medium between the front lens of the objective and the specimen cover glass, a value that ranges from 1.00 for air to 1.51 for specialized immersion oils.

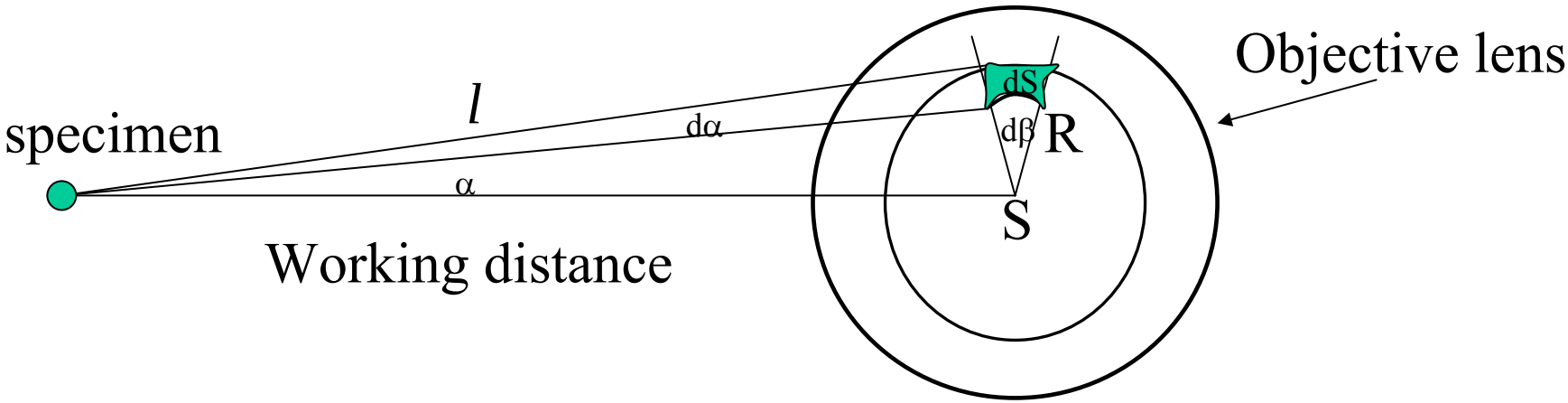


Most objectives in the magnification range between 60x and 100x (and higher) are designed for use with immersion oils.

Manufacturers: Melles Griot, Newport, Leica, Olympus, etc.



How Much of Isotropic Fluorescence Light Does the Air Lens of Known **Angular Aperture** Collect?



The part of light collected by a lens is the ratio between the solid body **S** that defines the aperture and the solid body S_{sph} of the sphere:

$S/S_{sph} = S/4\pi l^2$

$$dS = l\, d\alpha\, R d\beta = l\, d\alpha (l \sin\alpha) d\beta = l^2 \sin\alpha\, d\alpha\, d\beta$$

$$S = l^2 \int_0^{2\pi}\int_0^{\alpha} (\sin\alpha\, d\alpha) d\beta = -l^2 \int_0^{2\pi} (\cos\alpha\,|_0^{\alpha}) d\beta =$$

$$-l^2 \int_0^{2\pi} (\cos\alpha - 1) d\beta = l^2 \int_0^{2\pi} (1-\cos\alpha) d\beta = 2\pi\, l^2 (1-\cos\alpha)$$

$S/S_{sph} = S/4\pi l^2 = 2\,\pi l^2\,(1-\cos\alpha)/4\pi l^2 = (1-\cos\alpha)/2$

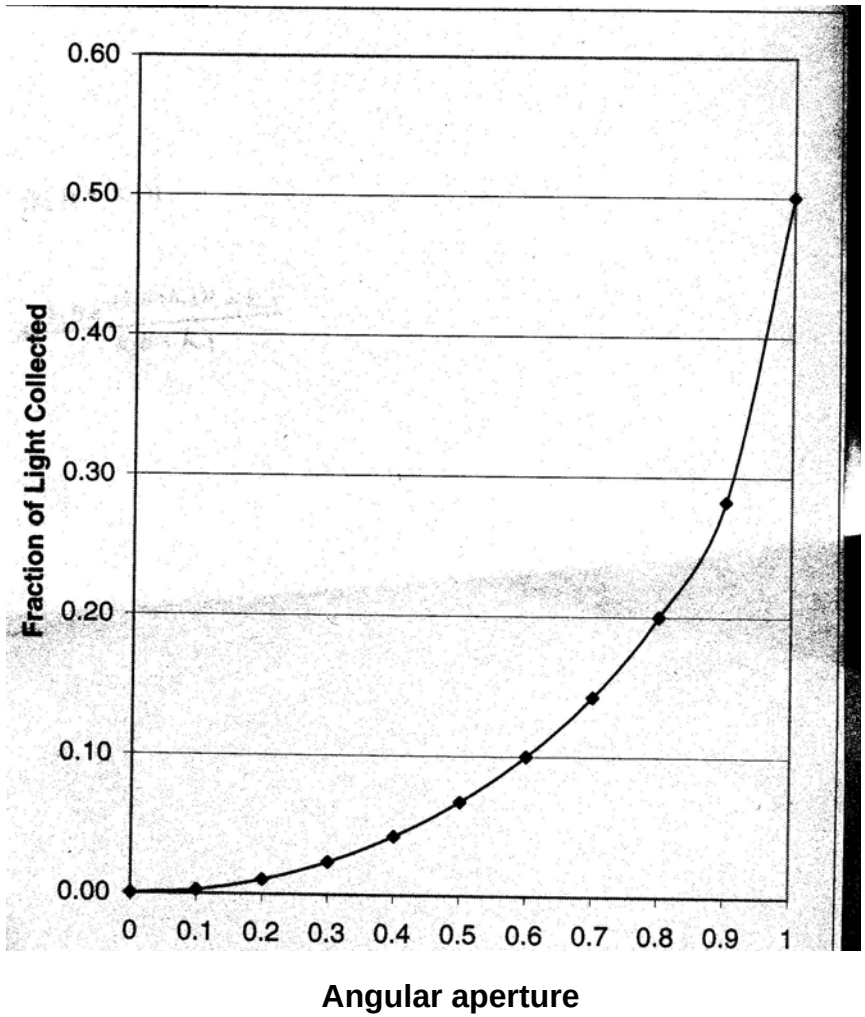
How Much of Isotropic Fluorescence Light Does an Air Lens of Known **AA** Collect? Contd.

$AA = \sin \alpha$

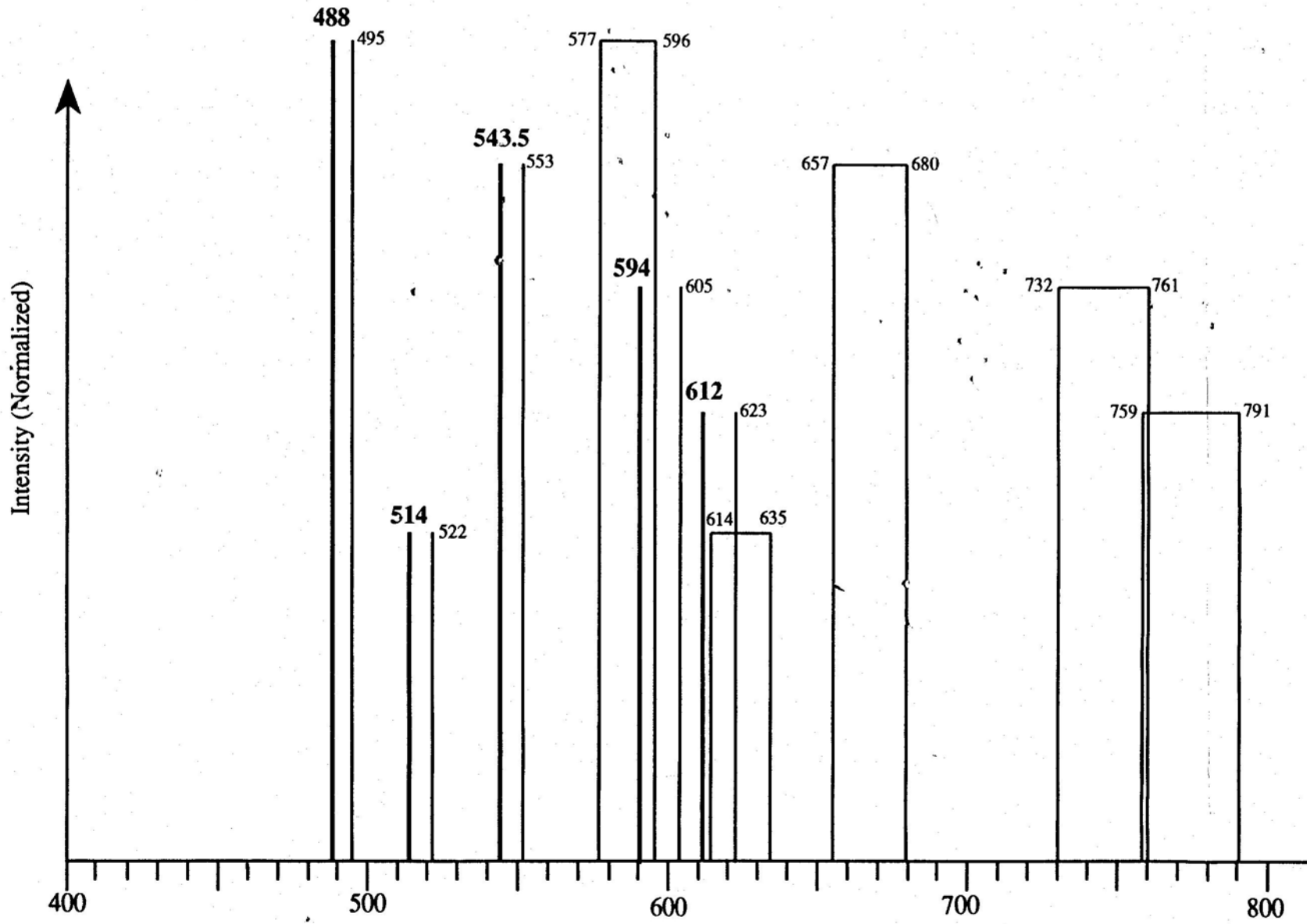
$\alpha = \arcsin (AA)$

$S/S_{sph} = (1 - \cos\alpha)/2 =$

$= \{1 - \cos(\arcsin(AA))\}/2$

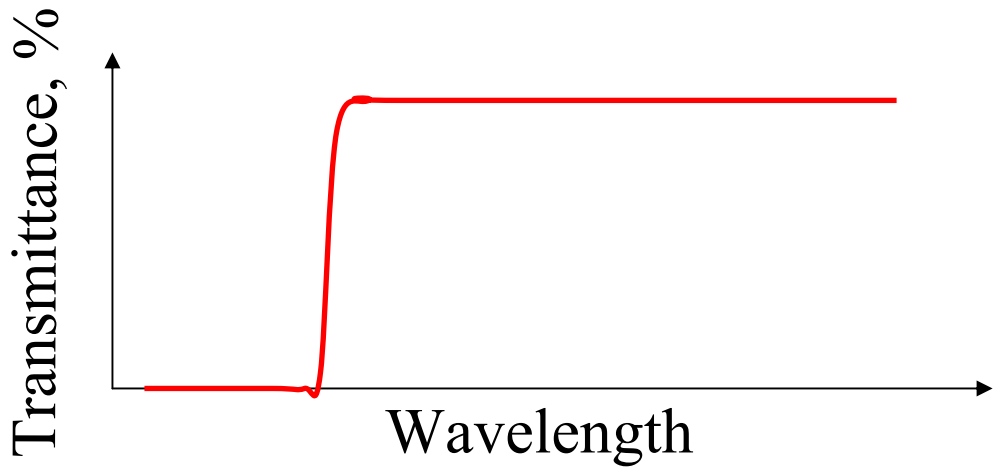


Raman Scattering on H_2O

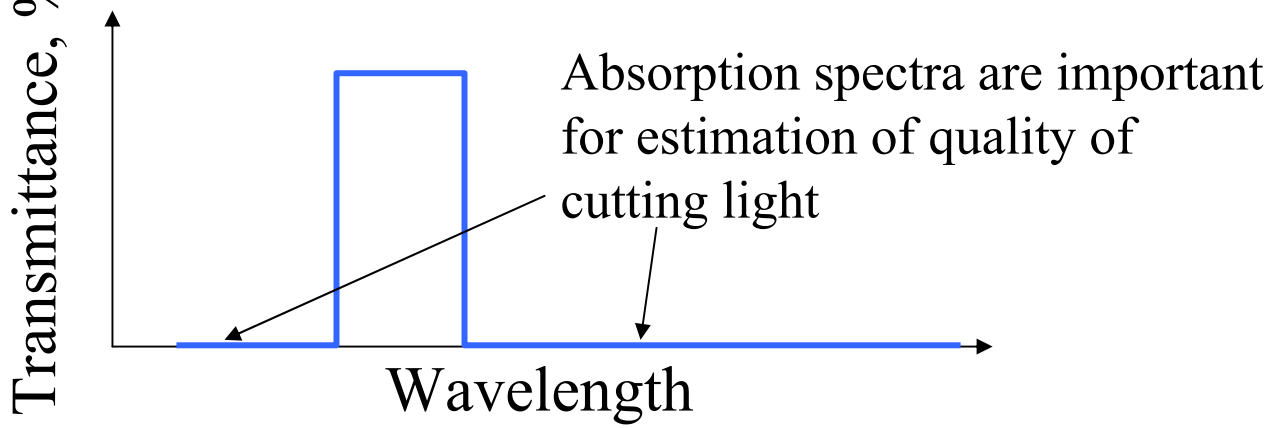


Filters

Cut-off filters:



Band-pass filters:

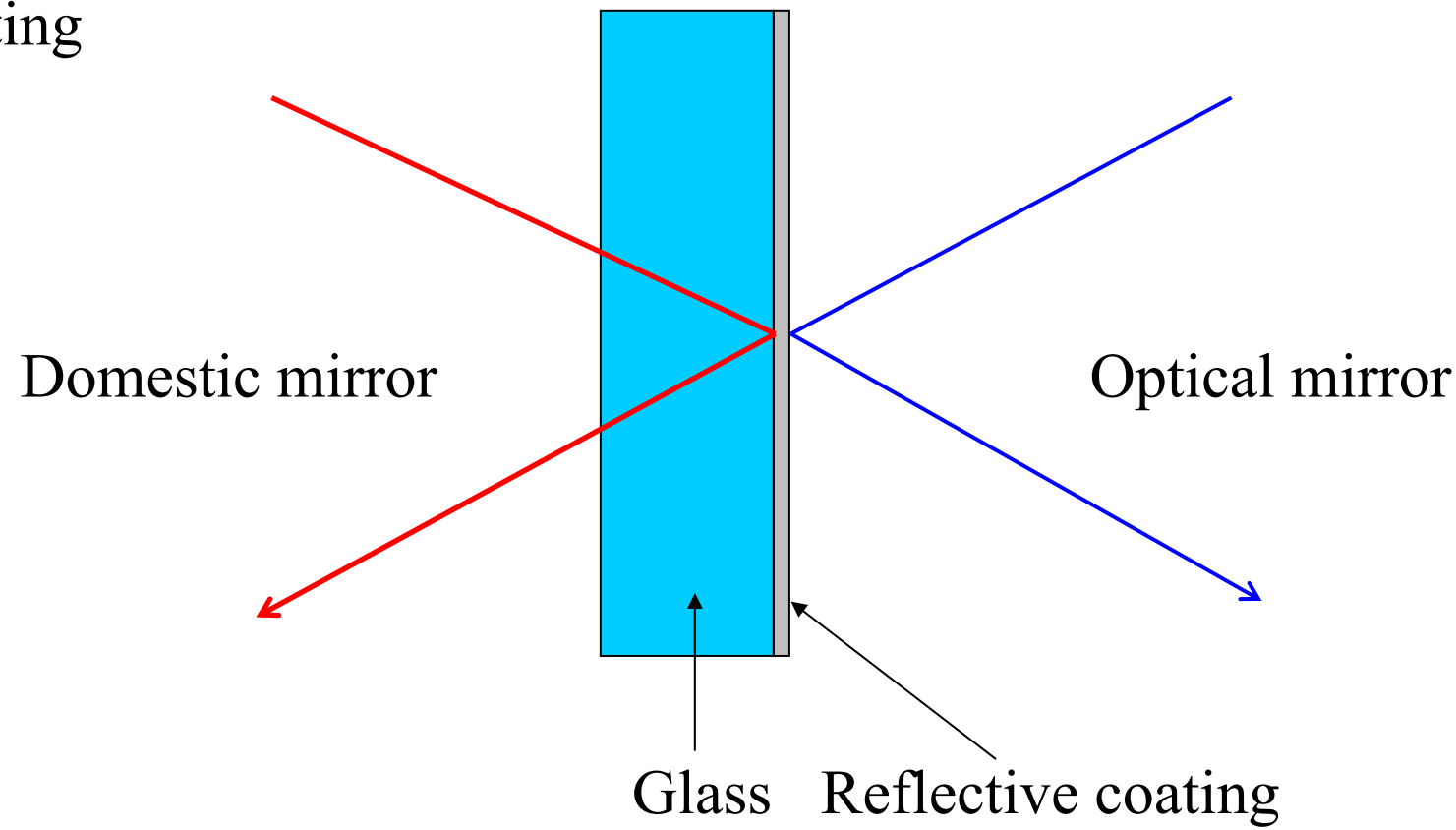


Nomenclature: *530DF30* means that the transmittance band is centered at 530 nm and has a **width** of 30 nm (530 ± 15 nm).

Manufacturers: Omega Opticals (www.omegafilters.com), Chroma

Mirrors

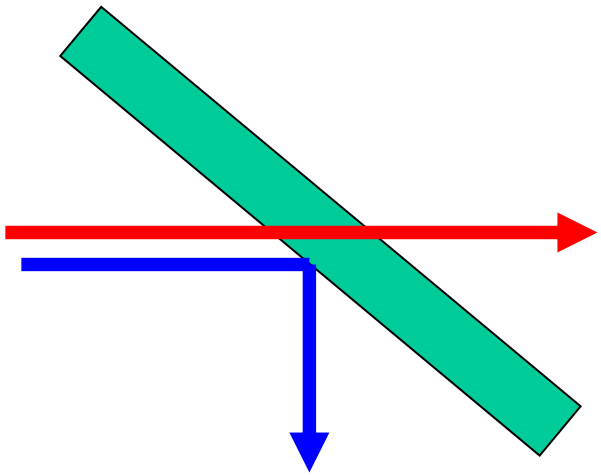
- In household mirrors, the reflecting surface is protected by glass, we look through glass.
- In optical mirrors the reflective surface is exposed; therefore it should be protected from oxidation by a layer of dielectric coating



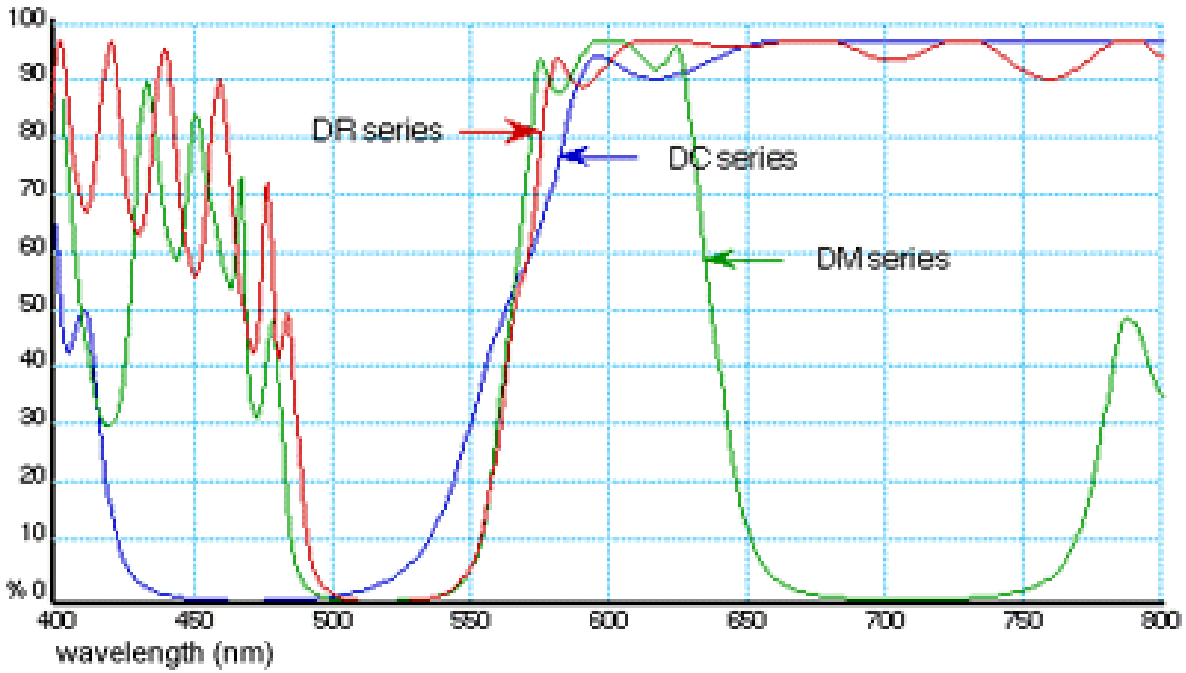
Manufacturers: Omega Opticals (www.omegafilters.com), Chroma

Dichroic Mirrors (Beamsplitters)

Dichroic beamsplitters typically operate at 45° to incident light. They are designed to separate light of short and long wavelengths into two separate channels. Dichroic mirrors reflect low-wavelength light and transmit high-wavelength light



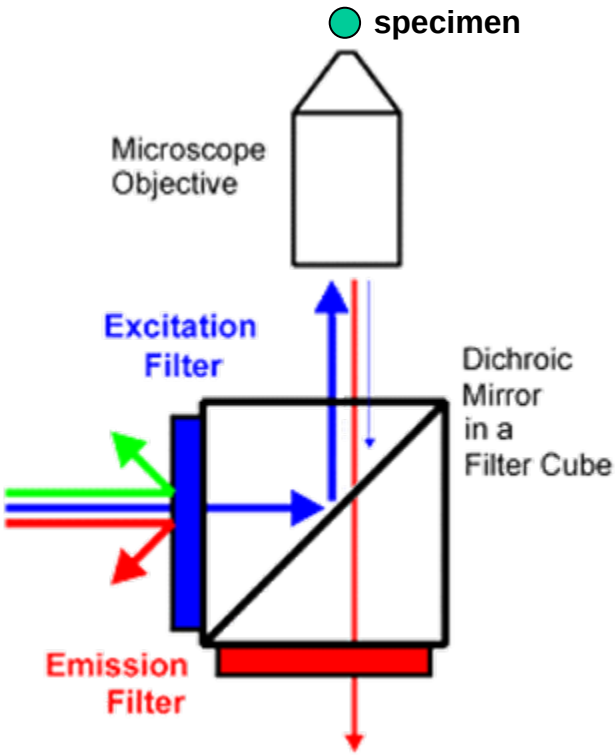
Nomenclature: for example 490DCLP means DiChroic Long Pass. 490 nm corresponds to 80%/20% transmittance/refraction



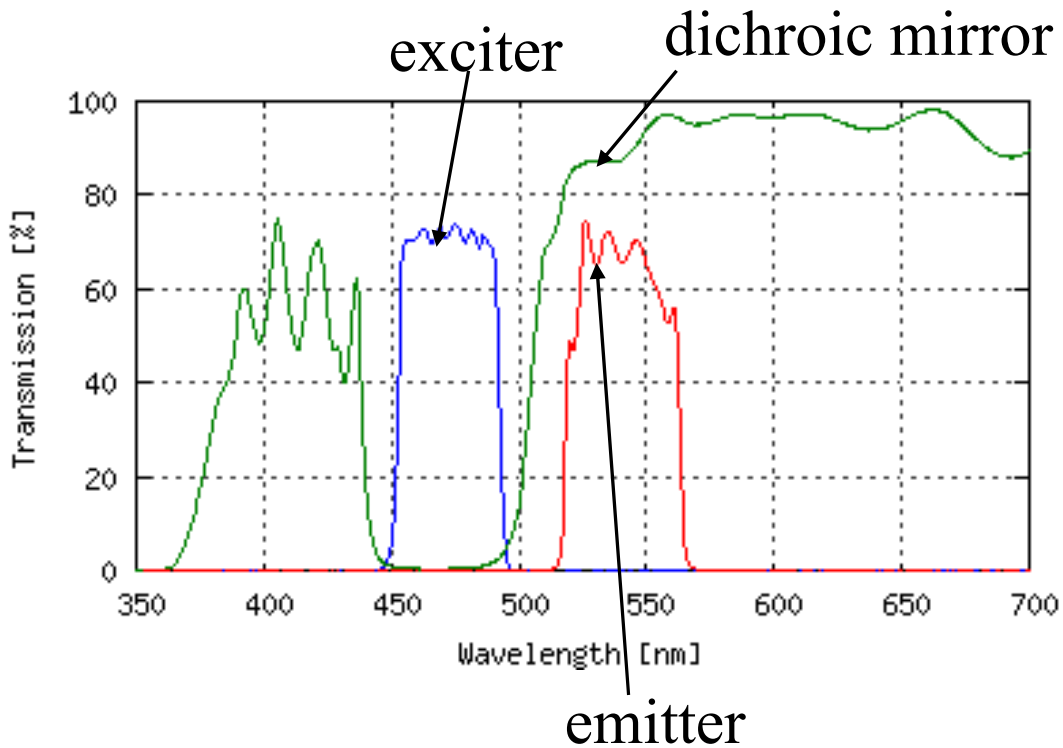
Manufacturers: Omega Opticals (www.omegafilters.com), Chroma

Filter Cubes

Filter cubes are used in fluorescence microscopes and fluorescence detection systems to allow excitation of fluorescence and collection of fluorescence light with the same objective lens

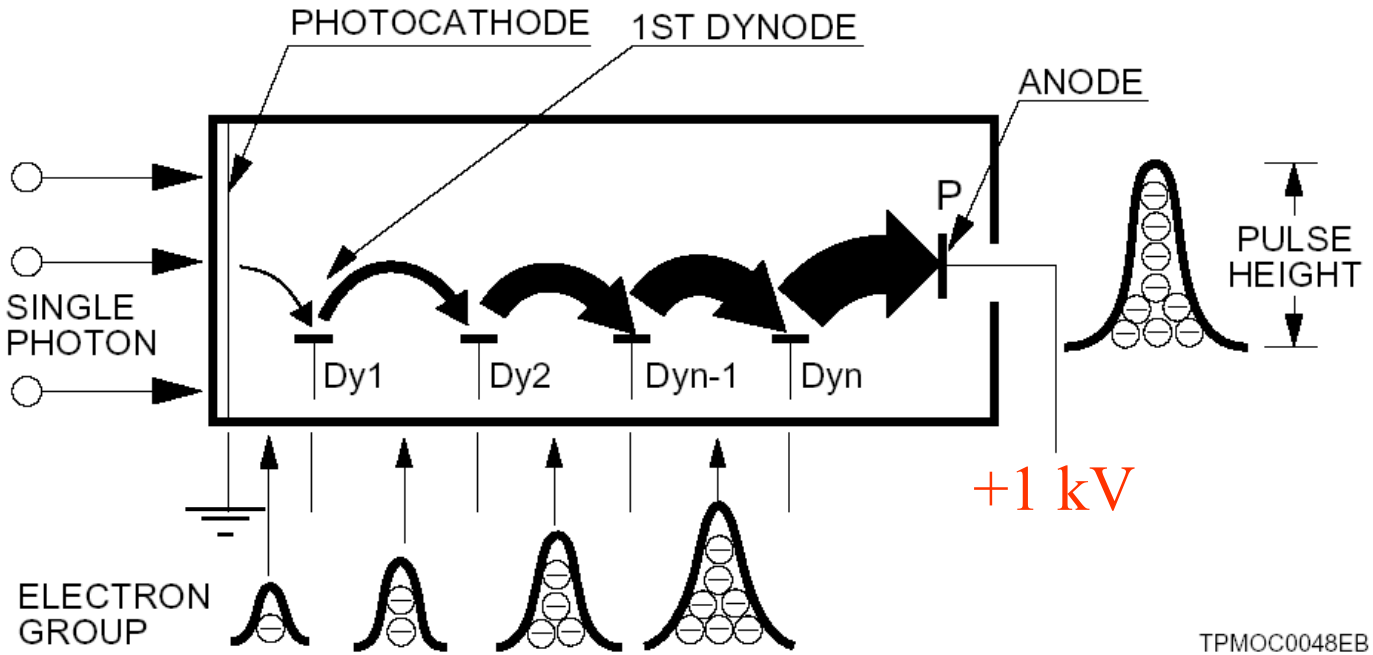


EYE or detector



Manufacturers: Omega Opticals (www.omegafilters.com), Chroma

Light Detectors - PMTs



Modes of operation:

1) Current (higher noise, higher dynamic range)
2) Photon counting (noise reduction due to the discrimination of pulses that correspond to single electrons born on the photocathode. Lower dynamic range due to the need to resolve between single photons. The pulse width is 50 ns (5×10^{-8} s). The dynamic range is 0 – 10^5 counts/s.

Manufacturers: Hamamatsu (www.hamamatsu.com)