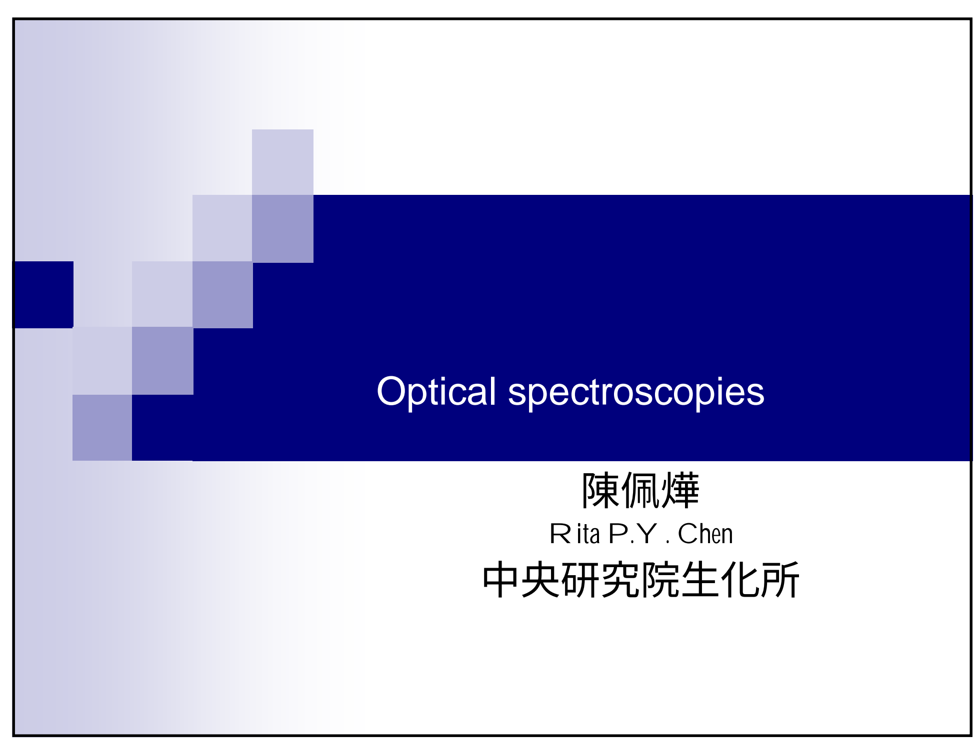
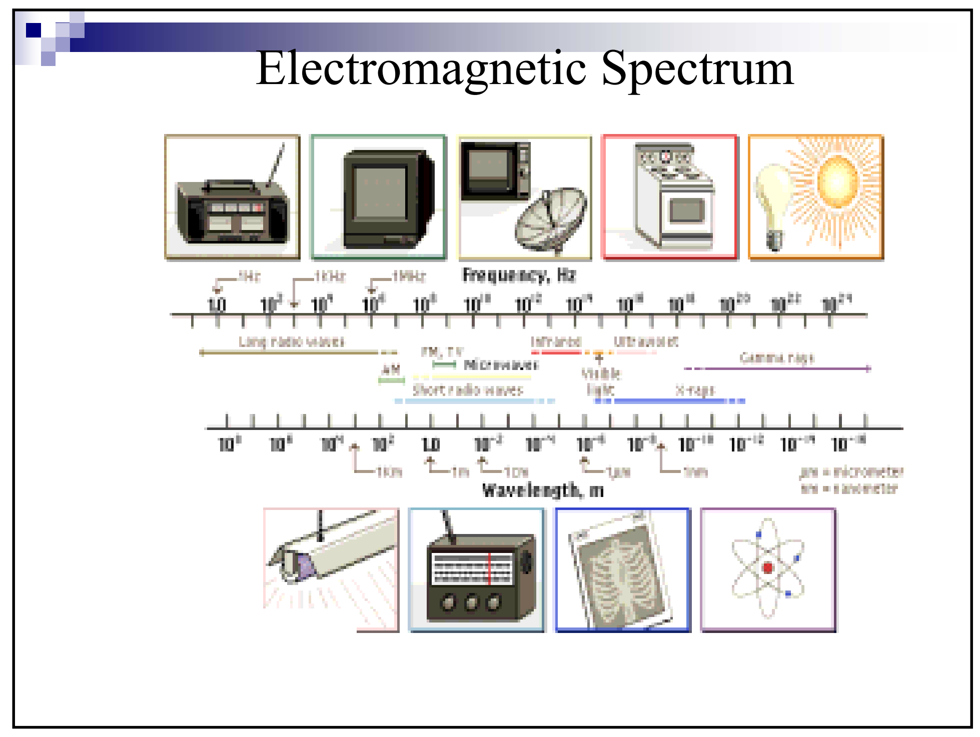
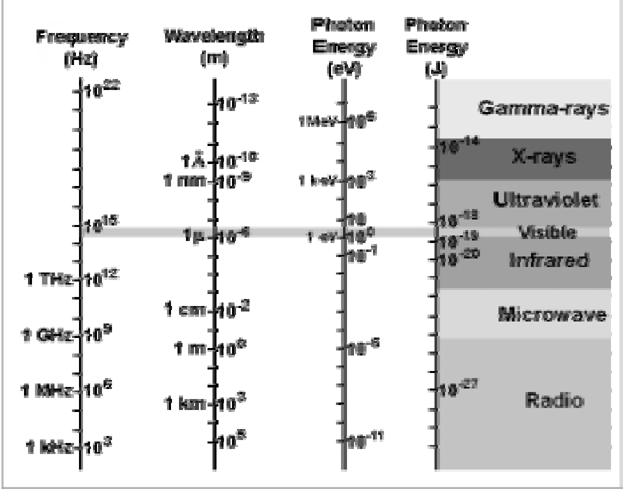

Optical spectroscopies
陳佩燁
Rita P.Y. Chen
中央研究院生化所

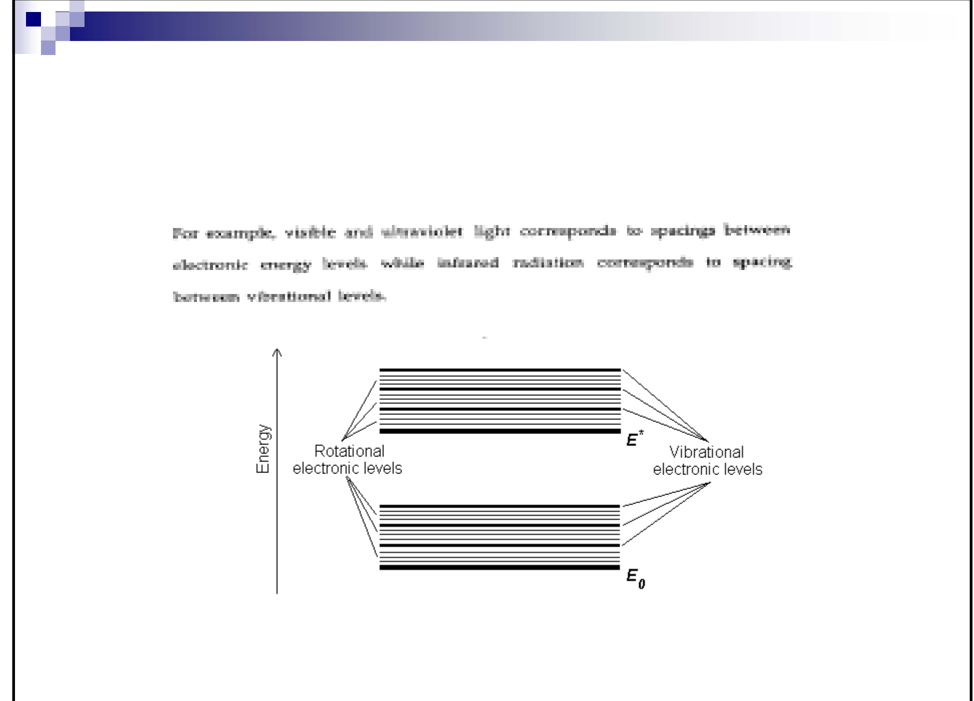

Electromagnetic Spectrum
Frequency, Hz
Long radio waves
FM, TV
AM
Microwaves
Short radio waves
Infrared
Ultraviolet
Visible light
Gamma rays
X-rays
Wavelength, m

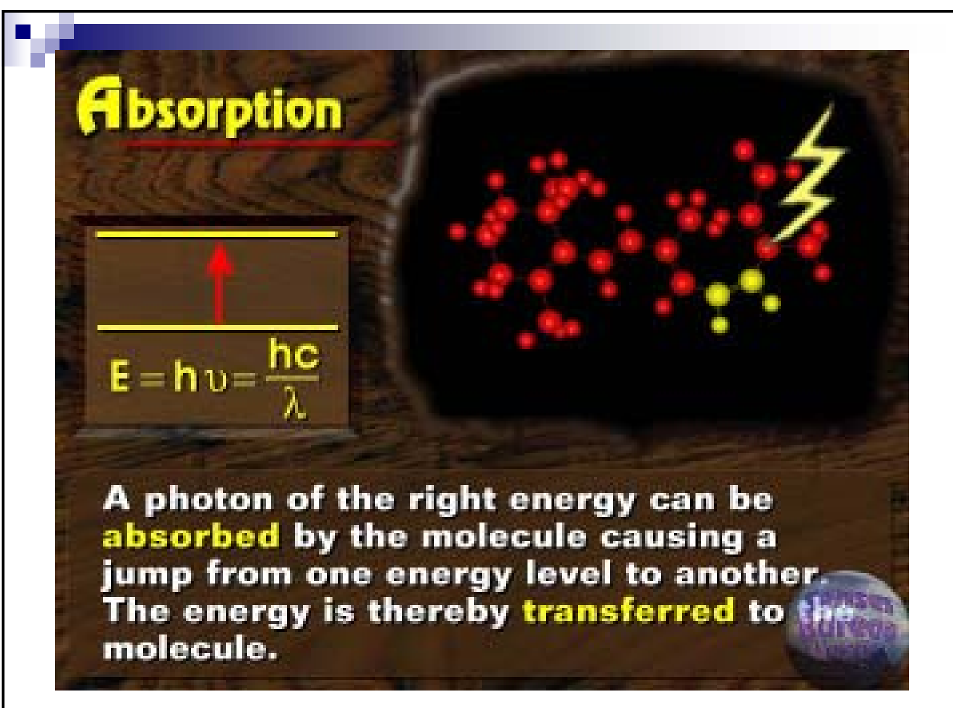


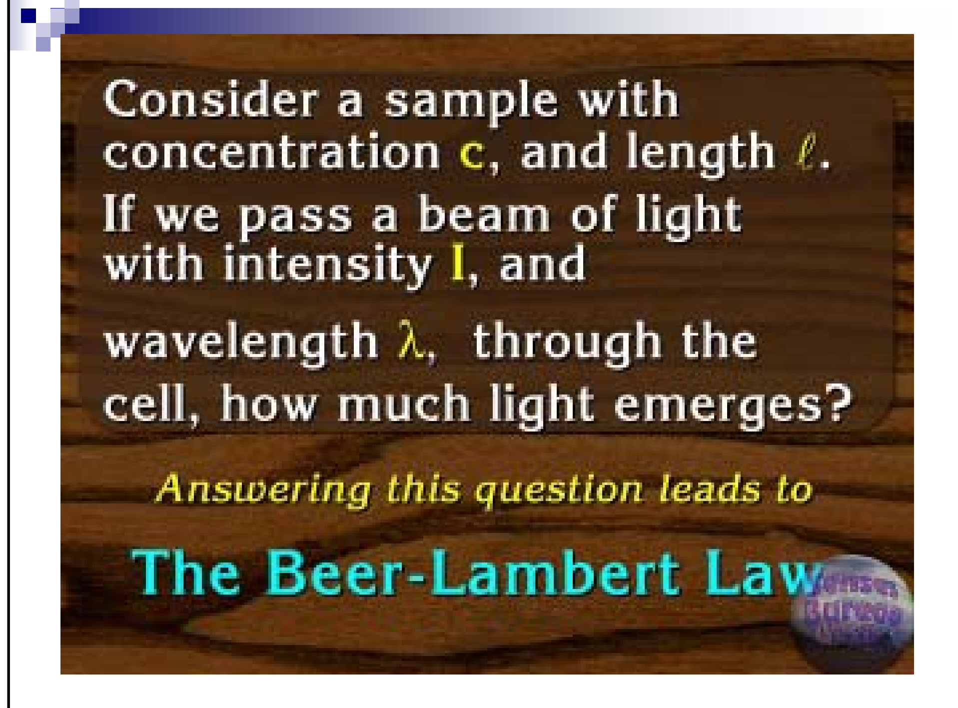
$$f = \frac{c}{\lambda}\ ; E = hf = \frac{hc}{\lambda}$$

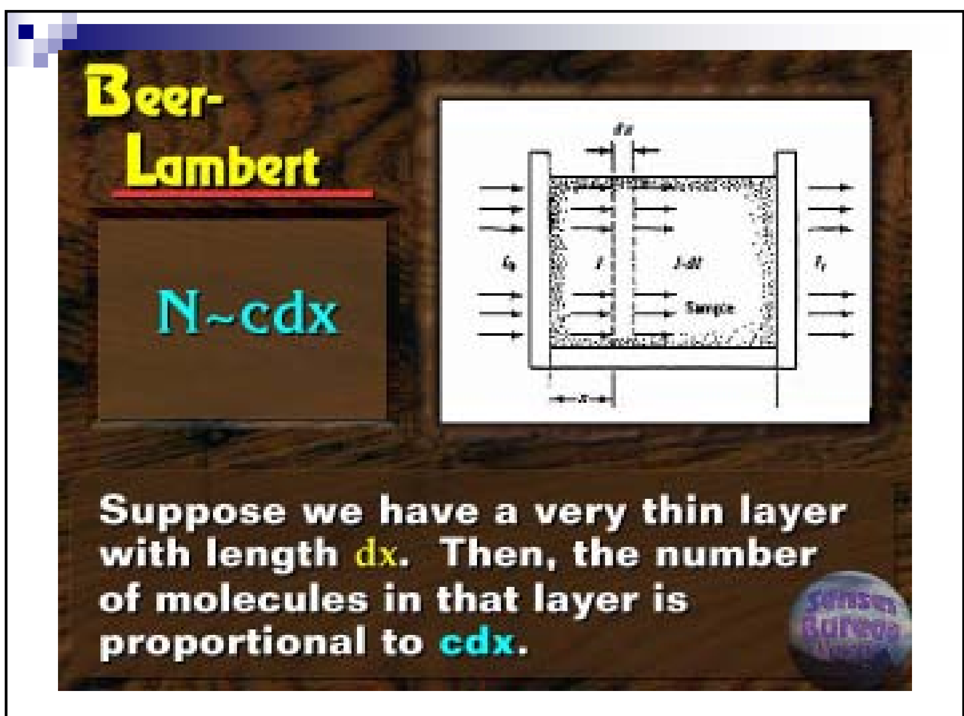
$h = 6.63 \times 10^{-34}$ J s , c= 3×10^{8} m

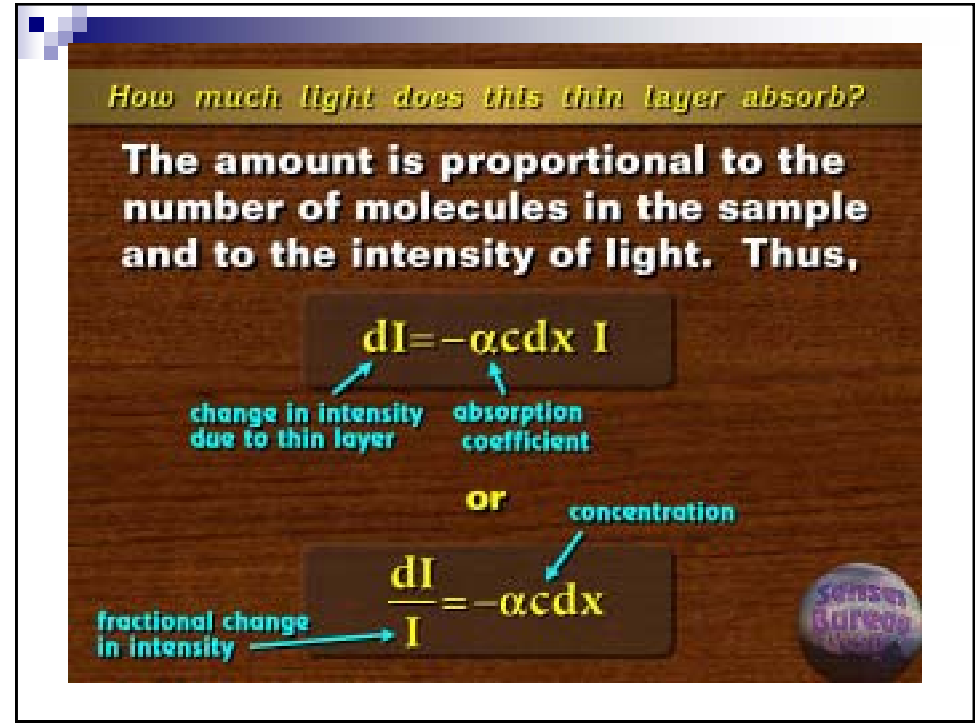
1 eV = 1.6×10^{-19} J




Absorption
E = hυ = hc/λ
A photon of the right energy can be absorbed by the molecule causing a jump from one energy level to another. The energy is thereby transferred to the molecule.


Consider a sample with concentration c, and length ℓ.
If we pass a beam of light with intensity I, and wavelength λ, through the cell, how much light emerges?
Answering this question leads to
The Beer-Lambert Law


Beer-Lambert
N~cdx
dx
I_0
I
I-dI
I_t
Sample
Suppose we have a very thin layer with length dx. Then, the number of molecules in that layer is proportional to cdx.


How much light does this thin layer absorb?
The amount is proportional to the number of molecules in the sample and to the intensity of light. Thus,
$dI = -\alpha c dx\ I$
change in intensity due to thin layer
absorption coefficient
or
concentration
$\frac{dI}{I} = -\alpha c dx$
fractional change in intensity

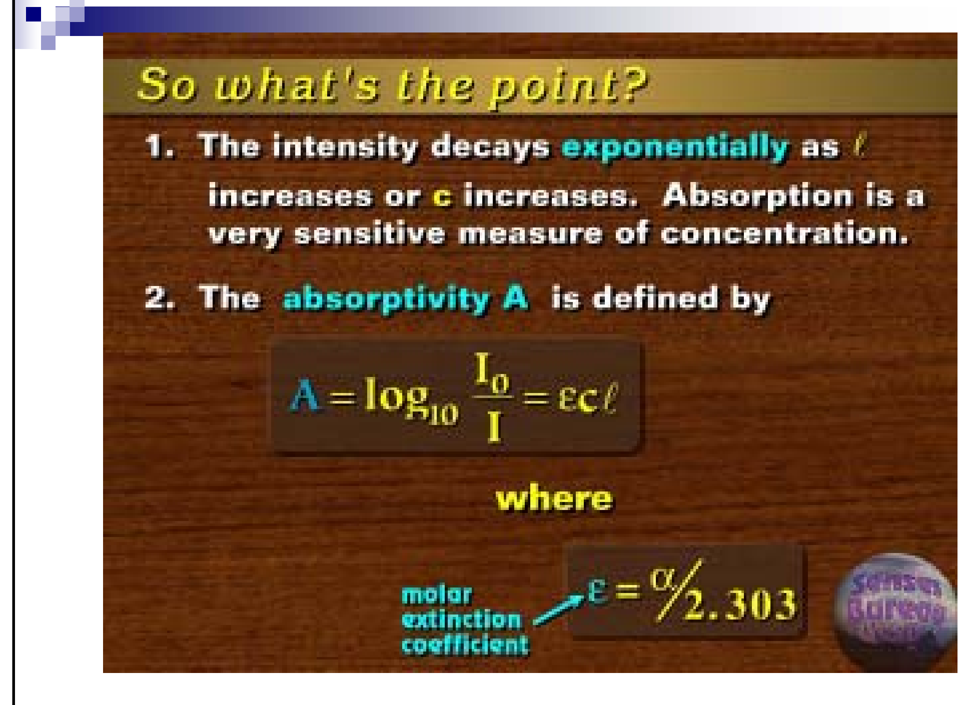
What is the total change in intensity due to the entire sample ? (I_0 = initial intensity I_f = final intensity)

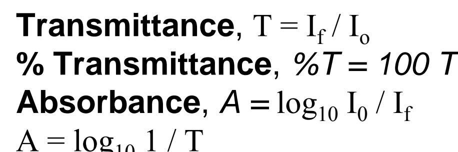
$$\int_{I_0}^{I_f} \frac{dI}{I} = -\int_0^{\ell} \alpha c\, dx$$

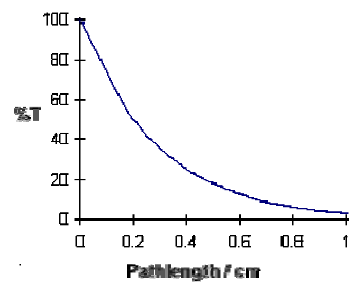
$$\ell n I_f - \ell n I_0 = -\alpha c \ell$$

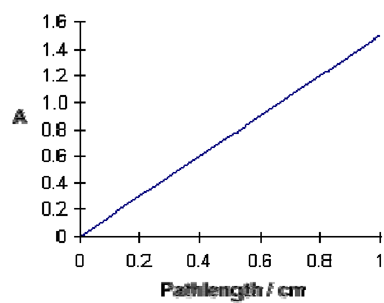
$$\Rightarrow \ell n \frac{I_f}{I_0} = -\alpha c \ell \qquad \Rightarrow \frac{I_f}{I_0} = e^{-\alpha c \ell}$$

$$\Rightarrow I_f = I_0 e^{-\alpha c \ell} \Rightarrow \text{Beer–Lambert law}$$









Absorbance

- O.D. units or absorbance is expressed in logarithmic terms so they are additive.
- An object of O.D. of 1.0 absorbs 90% of the light. Only ? % of the light reaches detector. When another object of O.D. 1.0 placed in the path, further 90% of this light is absorbed and only ? % of the original light is transmitted by the second object.
- It is possible to express the absorbance of a mixture of substances at a particular wavelength as the sum of the absorbances of the components.
- The extinction coefficient indicates how efficient the molecule will absorb photons.

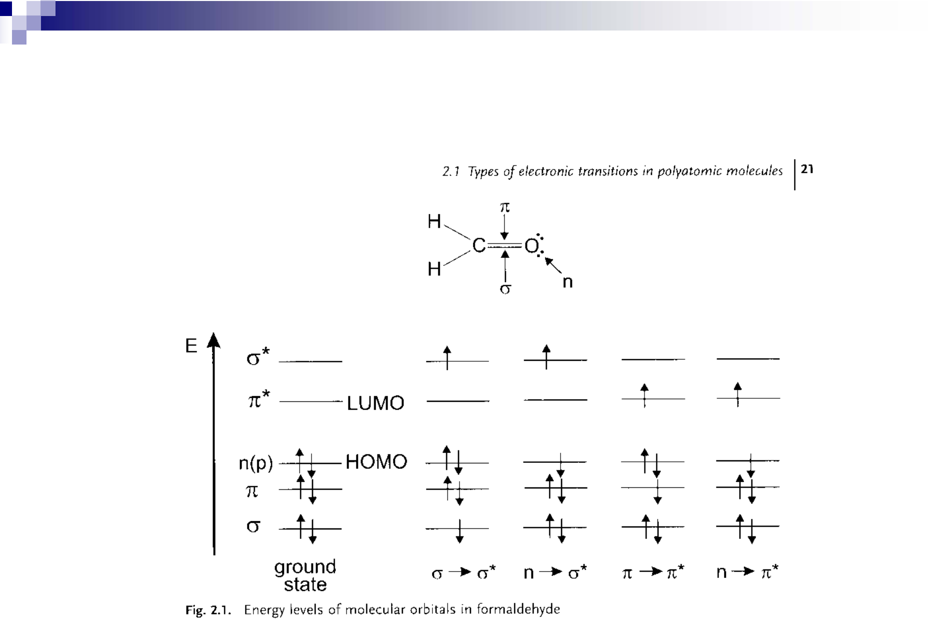
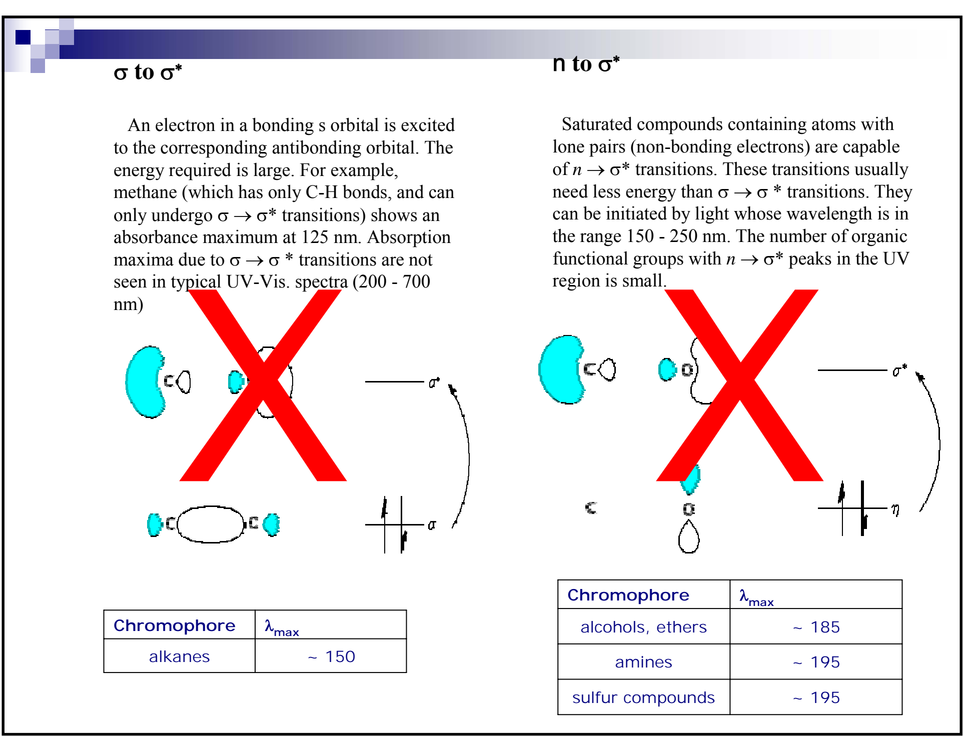


Fig. 2.1. Energy levels of molecular orbitals in formaldehyde (HOMO: Highest Occupied Molecular Orbitals; LUMO: Lowest Unoccupied Molecular Orbitals) and possible electronic transitions.



σ to σ*

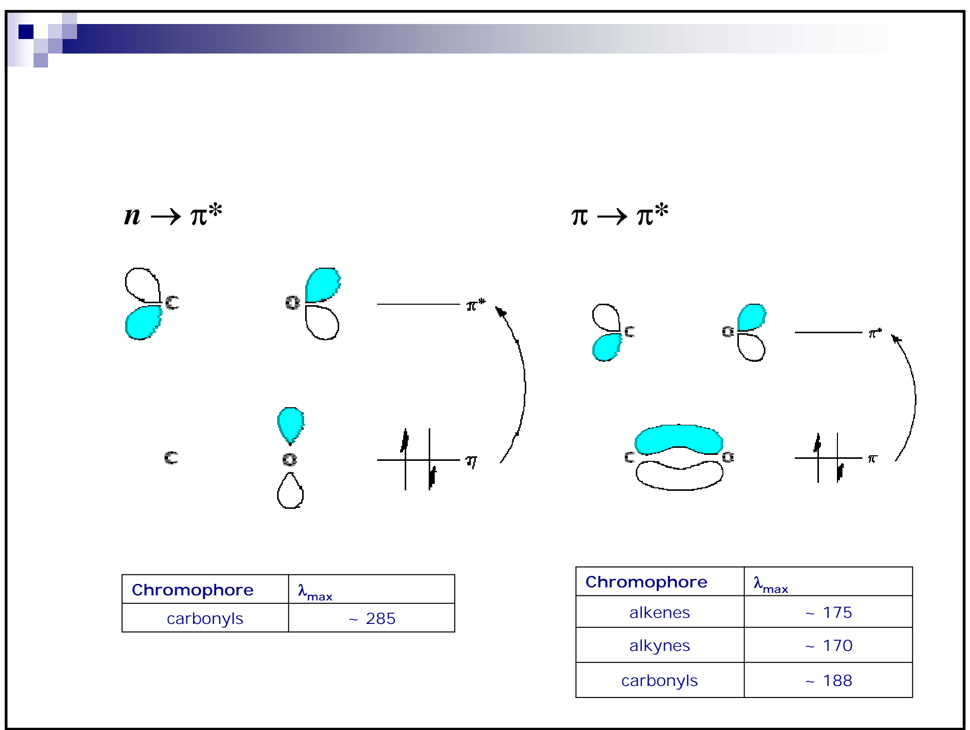
An electron in a bonding s orbital is excited to the corresponding antibonding orbital. The energy required is large. For example, methane (which has only C-H bonds, and can only undergo $\sigma \rightarrow \sigma^*$ transitions) shows an absorbance maximum at 125 nm. Absorption maxima due to $\sigma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

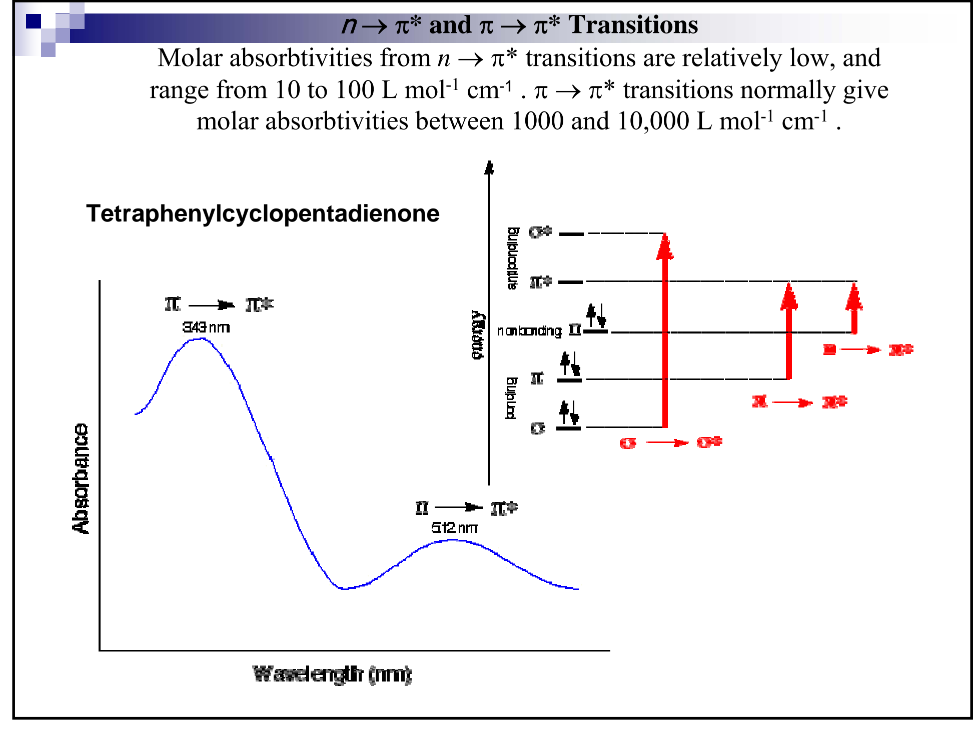
Chromophore	λ_{max}
alkanes	~ 150

n to σ*

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.

Chromophore	λ_{max}
alcohols, ethers	~ 185
amines	~ 195
sulfur compounds	~ 195


n → π*
π → π*
π*
n
π*
π
Chromophore
λmax
carbonyls
~ 285
Chromophore
λmax
alkenes
~ 175
alkynes
~ 170
carbonyls
~ 188

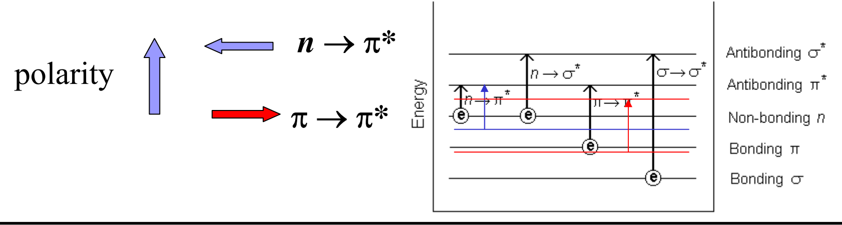

n → π* and π → π* Transitions
Molar absorbtivities from n → π* transitions are relatively low, and range from 10 to 100 L mol-1 cm-1 . π → π* transitions normally give molar absorbtivities between 1000 and 10,000 L mol-1 cm-1 .
Tetraphenylcyclopentadienone
π → π*
343 nm
n → π*
512 nm
Absorbance
Wavelength (nm)
energy
antibonding
σ*
π*
nonbonding
n
bonding
π
σ
n → π*
π → π*
σ → σ*

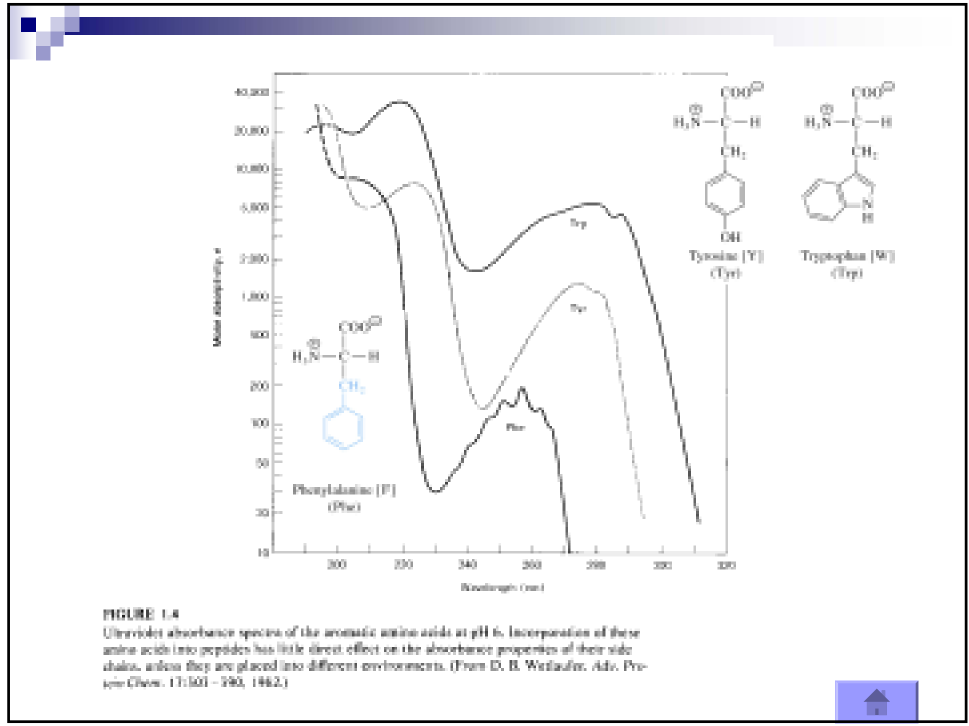
The table below gives a list of common solvents and the minimum wavelength from which they may be used in a 1 cm cell.

Solvent	Minimum Wavelength (nm)
acetonitrile	190
water	191
cyclohexane	195
hexane	195
methanol	201
ethanol	204
ether	215
methylene chloride	220
chloroform	237
carbon tetrachloride	257

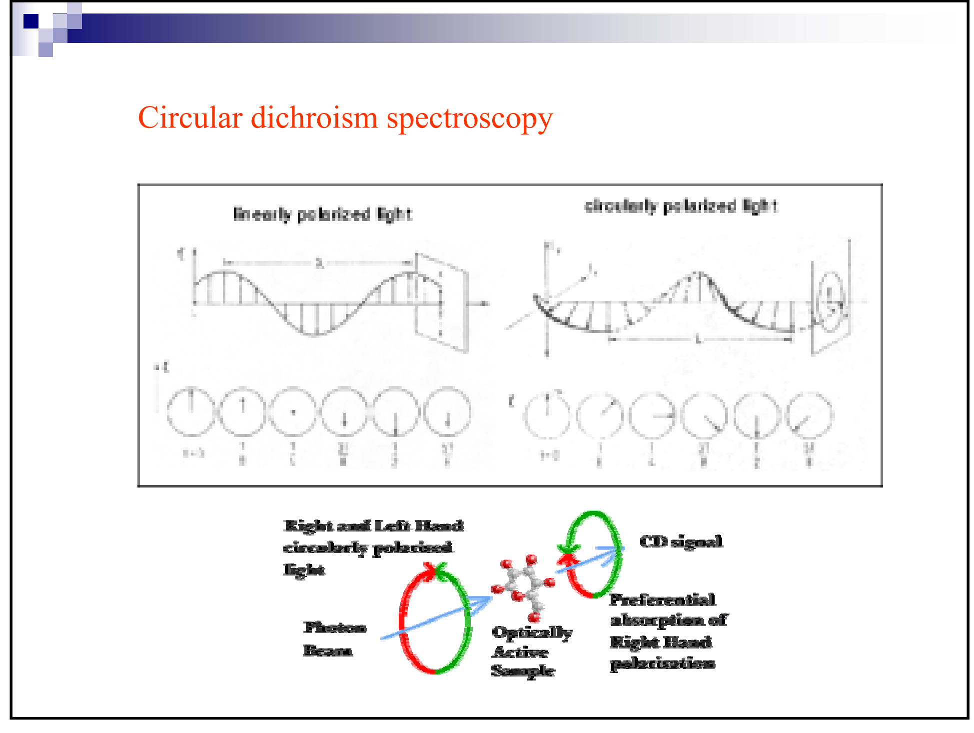
Solvent effect in absorption spectrum

The solvent in which the absorbing species is dissolved also has an effect on the spectrum of the species. Peaks resulting from $n \rightarrow \pi^*$ transitions are shifted to shorter wavelengths (*blue shift*) with increasing solvent polarity. This arises from increased solvation of the lone pair, which lowers the energy of the *n* orbital. Often (but *not* always), the reverse (i.e. *red shift*) is seen for $\pi \rightarrow \pi^*$ transitions. This is caused by attractive polarisation forces between the solvent and the absorber, which lower the energy levels of both the excited and unexcited states. This effect is greater for the excited state, and so the energy difference between the excited and unexcited states is slightly reduced - resulting in a small red shift. This effect also influences $n \rightarrow \pi^*$ transitions but is overshadowed by the blue shift resulting from solvation of lone pairs.

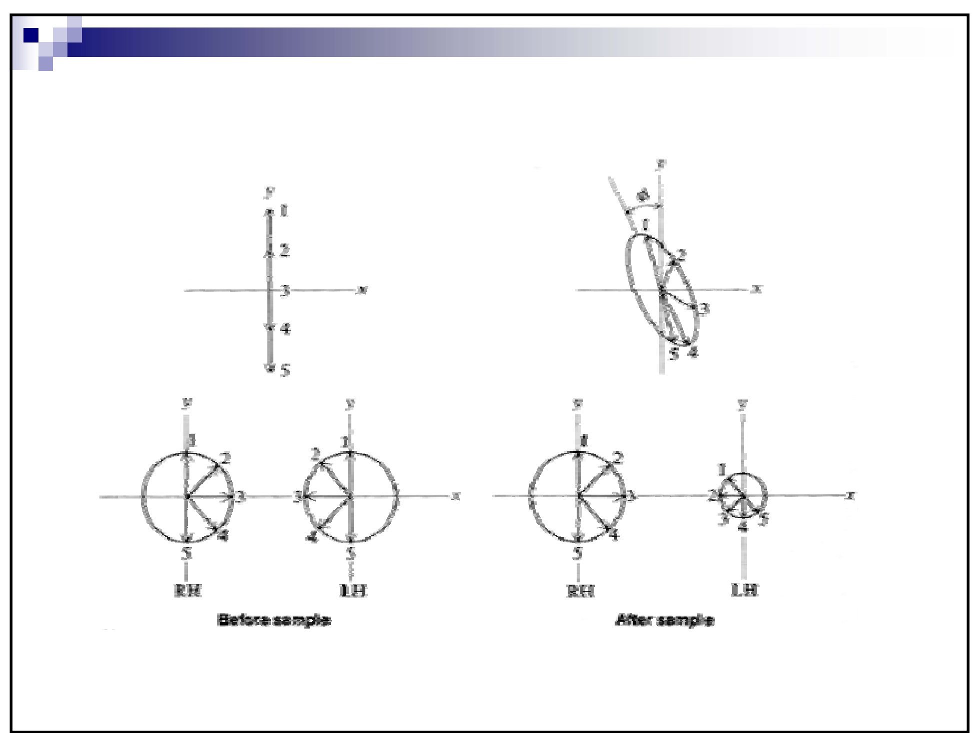


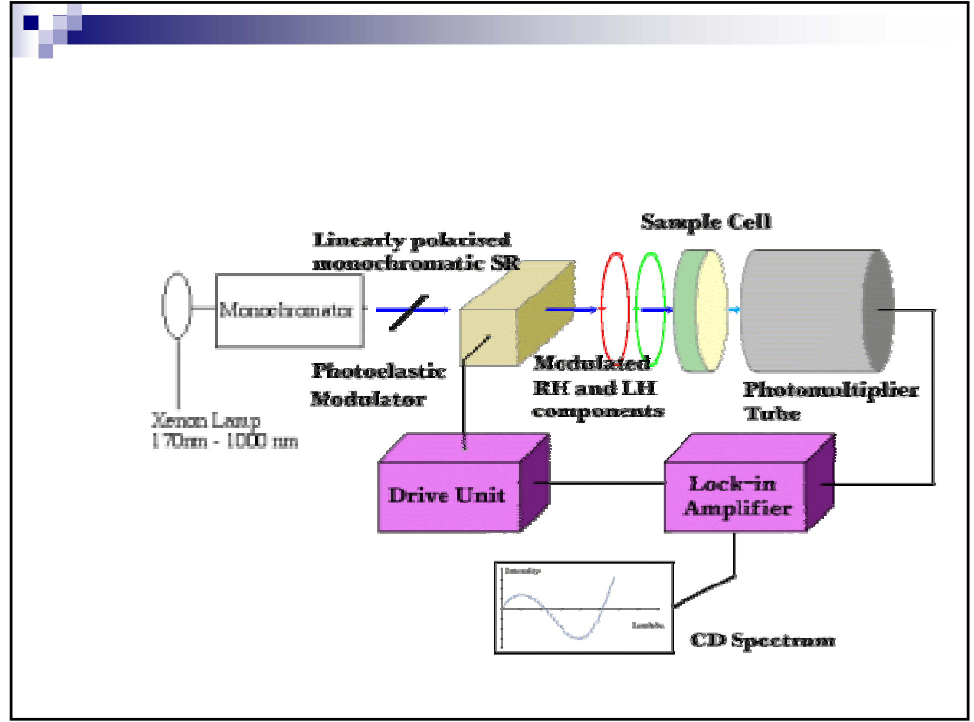


Tyrosine [Y]
(Tyr)
Tryptophan [W]
(Trp)
Phenylalanine [F]
(Phe)
FIGURE 1.4
Ultraviolet absorbance spectra of the aromatic amino acids at pH 6. Incorporation of these amino acids into peptides has little direct effect on the absorbance properties of their side chains, unless they are placed into different environments. (From D. B. Wetlaufer, Adv. Protein Chem. 17:303 - 390, 1962.)



Circular dichroism spectroscopy
linearly polarized light
circularly polarized light
Right and Left Hand circularly polarised light
Photon Beam
Optically Active Sample
CD signal
Preferential absorption of Right Hand polarization


Before sample
After sample
RH
LH

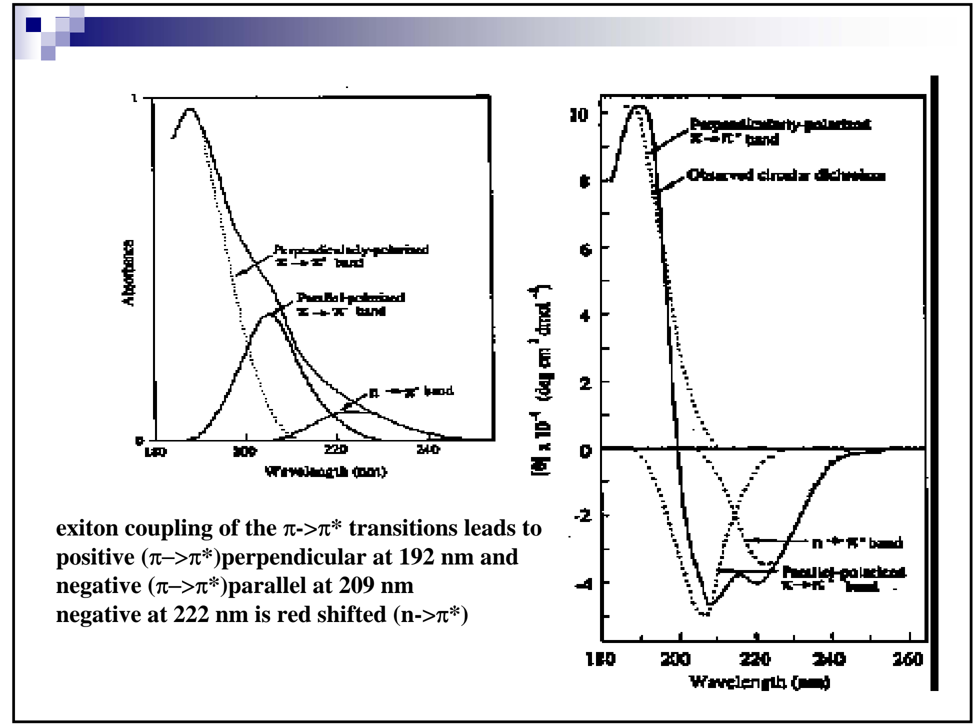

Sample Cell
Linearly polarised monochromatic SR
Monochromator
Photoelastic Modulator
Modulated RH and LH components
Photomultiplier Tube
Xenon Lamp
170nm - 1000 nm
Drive Unit
Lock-in Amplifier
CD Spectrum

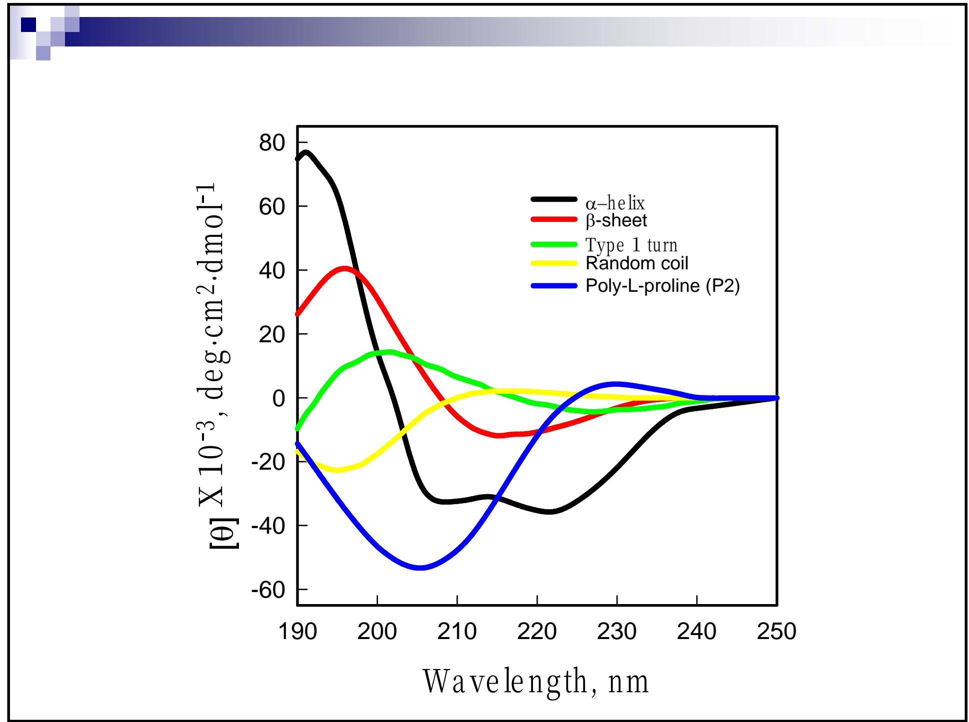
Far UV CD

n -> π* (210-230 nm) centered around 220 nm,
ε = ~100 mol^{-1} dm^3 cm^{-1},
involves non-bonding electrons of O of the carbonyl

π -> π* centered around 190 nm
ε = ~7000 mol^{-1} dm^3 cm^{-1},
π -> π*dominated by the carbonyl π-bond, and also affected by the involvement of the nitrogen in the π-orbitals

The intensity and energy of these transitions depends on ϕ and ψ (i.e., secondary structure)





Beer-Lambert law

$A = -\log_{10}(I_{in}/I_{out})$

$A = \varepsilon \times b \times c$

$\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ differential absorbance of a 1 mol/l solution in a 1 cm cell

Measured θ , ellipticity, is the rotation in degrees of a 1dmol/cm3 solution and a pathlength of 1 cm

Molar ellipticity: $[\theta] = \dfrac{\theta \times 100 \times Mr}{c \times l}$ c: mg/ml l: cm

degrees cm^2 $dmol^{-1}$

Mean residue ellipiticity: $[\theta]_{MRW} = [\theta]$ /residue number

degrees cm^2 $dmol^{-1}$ $residue^{-1}$

$\Delta\varepsilon = [\theta] / 3298$

Litre mol^{-1} cm^{-1} or Litre $(mol\ residue)^{-1}$ cm^{-1}

Use far-UV CD to determine amounts of secondary structure in proteins

generate basis sets by determining spectra of pure α-helix, β-sheet, etc. of synthetic peptides

or deconvoluting CD spectra of proteins with know structures to generate basis sets of each of secondary structure

poly-L-lysine {(Lys)n} can adopt 3 different conformations merely by varying the pH and temperature

random coil at pH 7.0
α-helix at pH 10.8
β-form at pH 11.1 after heating to 52°C and recooling

CD spectrum of unknown protein = *f*αSa(l) + *f*βSb(l) + *f*RCSRC(l), where Sa(l), Sb(l), and SRC(l) are derived from poly-L-lysine basis spectra.

21.4.4 Computer Programs

Some of the computer programs, available in the public domain, for estimating secondary structure fractions from protein CD are given below. They can be downloaded from the Internet or requested from the authors.

1. SELCON3 [76] (self-consistent method, version 3).
   Contact: sreeram@lamar.colostate.edu or rww@lamar.colostate.edu
   Internet: http://lamar.colostate.edu/~sreeram/SELCON3
2. CDsstr [72] (uses an extremely flexible basis set).
   Contact: johnsowc@ucs.orst.edu
3. CONTIN [55] (ridge regression method).
   Internet: http://www.provencher.de/contin-cd.html
4. CDNN [62] (backpropagation neural network method).
   Internet: http://bioinformatik.biochemtech.uni-halle.de/cdnn/index.html
5. K2D [64] (Kohonen neural network method).
   Internet: http://www.embl-heidelberg.de/~andrade/k2d.html
6. Varselec [57] (variable selection method).
   Contact: johnsowc@ucs.orst.edu
7. CCA [60] (convex constraint analysis).
   Internet: http://www2.chem.elte.hu/protein/programs/cca/
8. Prot CD [77] (DEFCLASS, tertiary class determination, and CONTIN, Varselec, CDEstima).
   Contact: venyamin@mayo.edu
9. HELOK [58] (two-step ridge regression method).
   Contact: inbio@gbs.apc.org

The disadvantage of this method is that although these basis sets are easily determined by direct measurement, **they do not always agree from one lab to another**. In addition, chain length and aggregation effect the basis set spectra. However, this method is usually accurate to within 10% for α-helix content.

Technique	Secondary Structure	carboxypeptidase	α-chymotrypsin	myoglobin
	α	23%	8%	68%
X-ray	β	18%	22%	0%
	RC + other	59%	70%	32%
	α	13%	12%	68%
CD using $(Lys)_n$ Basis Sets	β	31%	23%	5%
	RC + other	56%	65%	27%

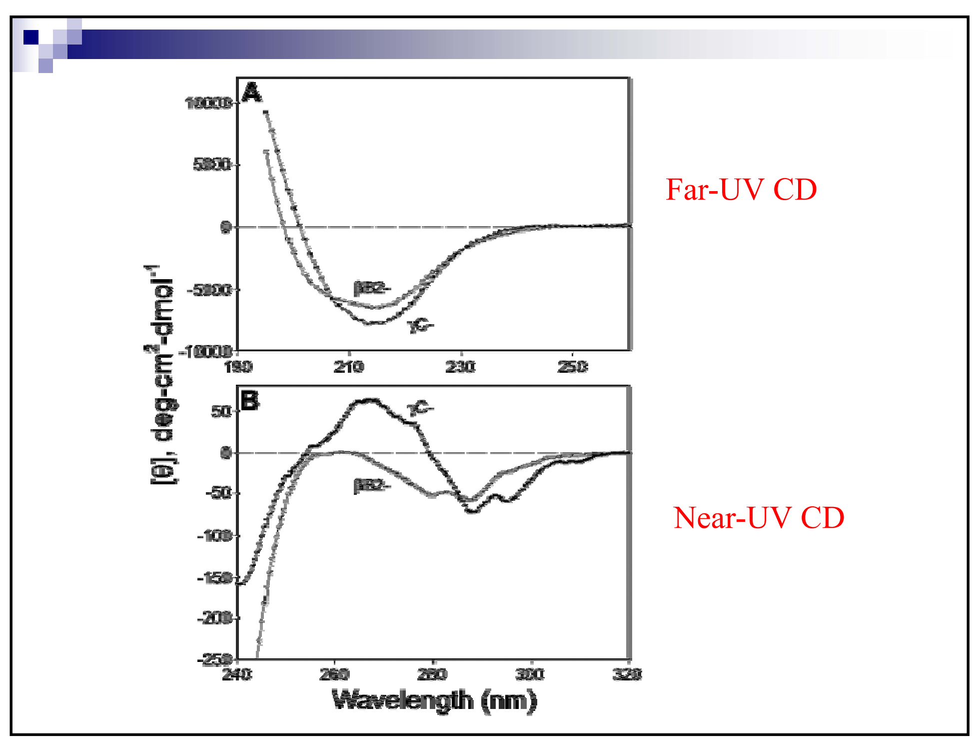
11: Optical spectroscopy to characterize protein conformation

Table 3. Absorbance of various salt and buffer substances in the far-UV region[a]

Compound	No absorbance above:		Absorbance of a 0.01 M solution in a 0.1 cm cell at: 210nm	200nm	190nm	180nm
$NaClO_4$		170 nm	0	0	0	0
NaF, KF		170 nm	0	0	0	0
Boric acid		180 nm	0	0	0	0.5
NaCl		205 nm	0	0.02	> 0.5	0.5
Na_2HPO_4		210 nm	0	0.05	0.3	> 0.5
NaH_2PO_4		195 nm	0	0	0.01	0.15
Na acetate		220 nm	0.03	0.17	> 0.5	> 0.5
Glycine		220 nm	0.03	0.1	> 0.5	> 0.5
Diethylamine		240 nm	0.4	> 0.5	> 0.5	> 0.5
NaOH	pH 12	230 nm	≥ 0.5	> 2	> 2	> 2
Boric acid, NaOH	pH 9.1	200 nm	0	0	0.09	0.3
Tricine	pH 8.5	230 nm	0.22	0.44	> 0.5	> 0.5
Tris	pH 8.0	220 nm	0.02	0.13	0.24	> 0.5
Hepes	pH 7.5	230 nm	0.37	0.5	> 0.5	> 0.5
Pipes	pH 7.0	230 nm	0.20	0.49	0.29	> 0.5
Mops	pH 7.0	230 nm	0.10	0.34	0.28	> 0.5
Mes	pH 6.0	230 nm	0.07	0.29	0.29	> 0.5
Cacodylate	pH 6.0	210 nm	0.01	0.20	0.22	> 0.5

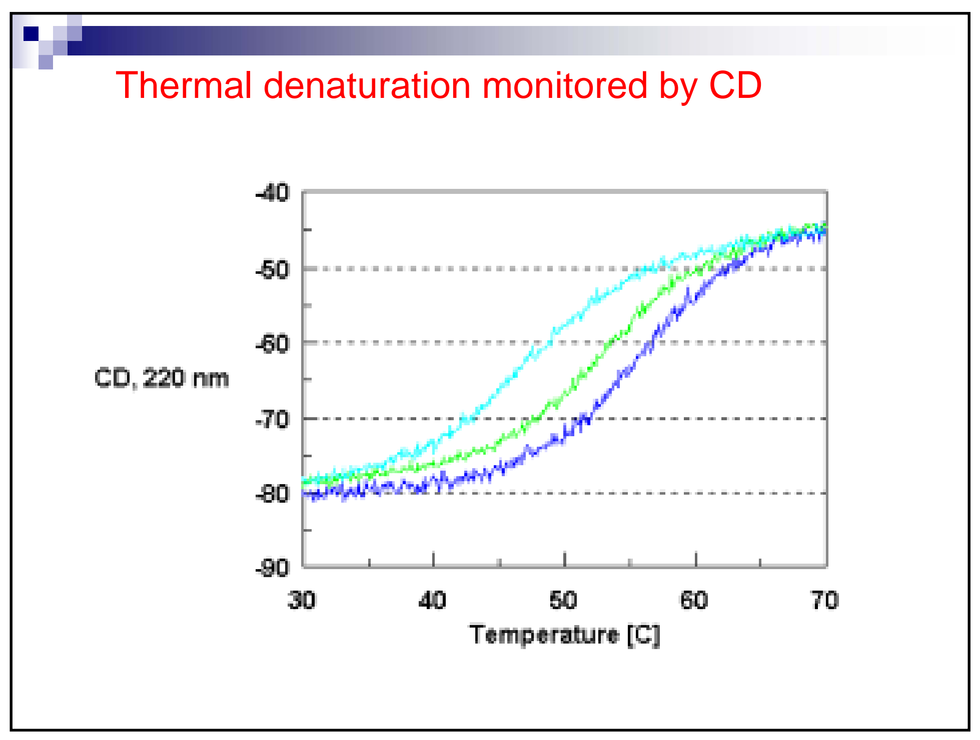
[a] Buffers were titrated with 1 M NaOH or 0.5 M H_2SO_4 to the indicated pH values.

Protein structure IRL press

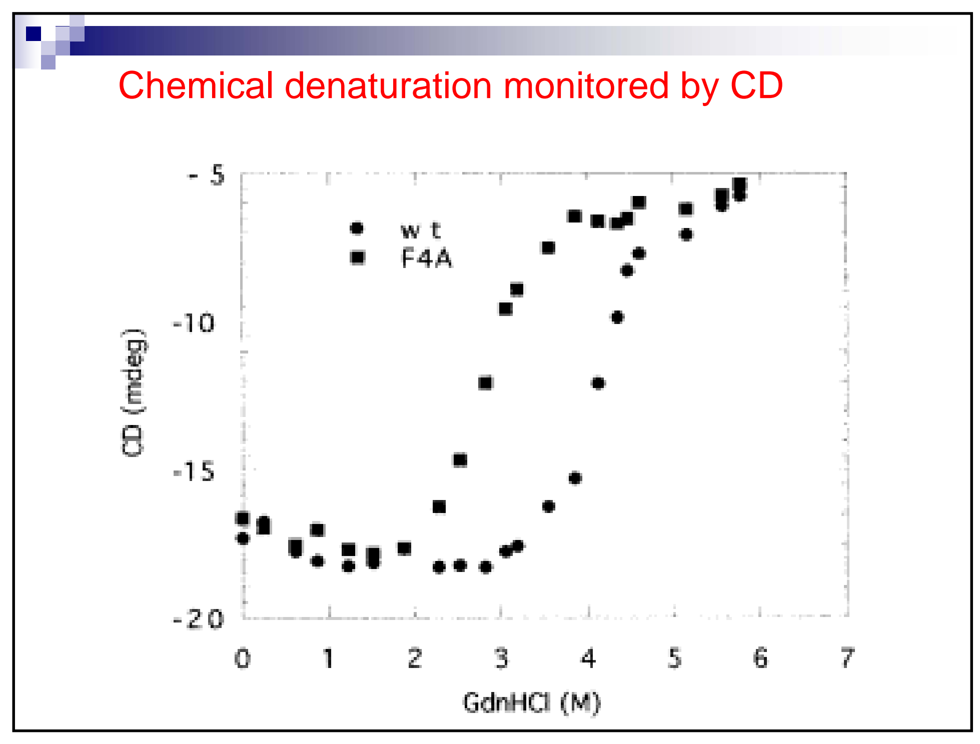


Near UV CD

Wavelength Range	*Chromophore Contributions*
250-270nm	*side chain Phe*
270-290nm	*side chain Tyr*
280-300nm	*side chain Trp*
250-350nm	*disulphide bond*



Thermal denaturation monitored by CD
CD, 220 nm
-40
-50
-60
-70
-80
-90
30
40
50
60
70
Temperature [C]



Chemical denaturation monitored by CD
CD (mdeg)
- 5
-10
-15
-20
w t
F4A
0
1
2
3
4
5
6
7
GdnHCl (M)

$$U \underset{}{\overset{K_{fold}}{\rightleftharpoons}} F$$

intensity at 303 nm

GdnHCl (M)

yF=1189+80.6x[GdnHCl]

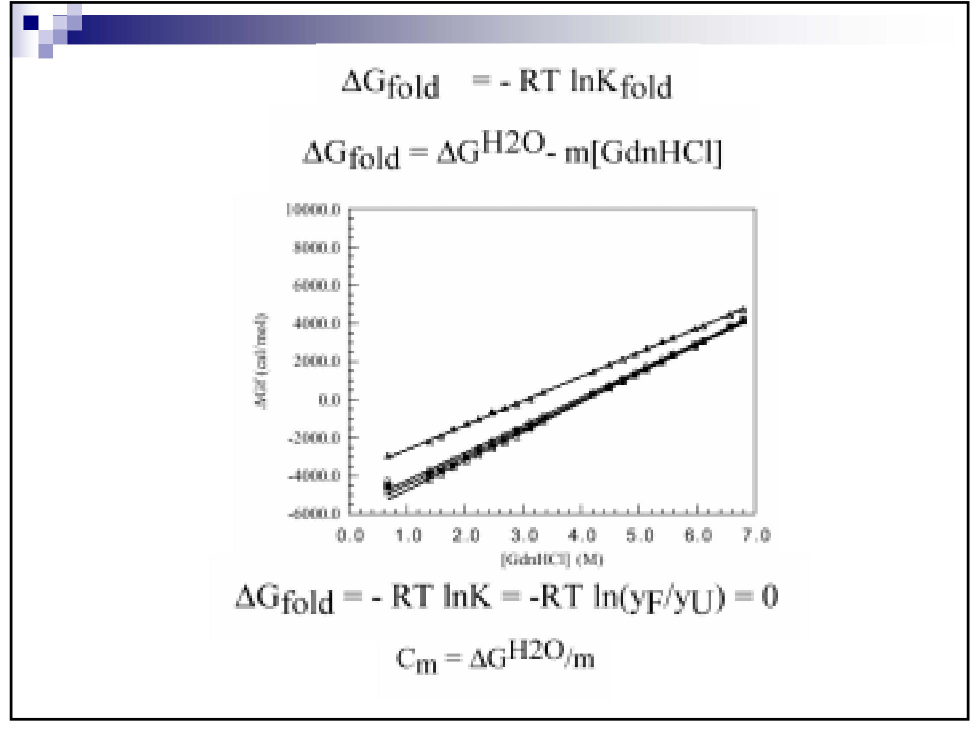
yU=319+40.3x[GdnHCl]

$$f_F + f_U = 1$$

$$y = y_F f_F + y_U f_U$$

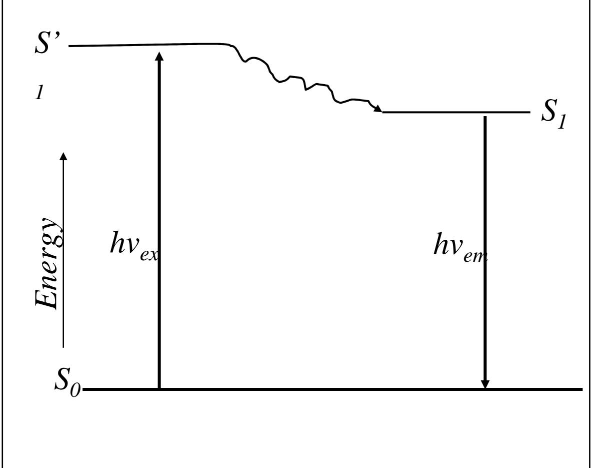
$$f_F = \frac{y_U - y}{y_U - y_F}$$

$$K_{fold} = \frac{f_F}{f_U} = \frac{f_F}{1 - f_F} = \frac{y_U - y}{y - y_F}$$



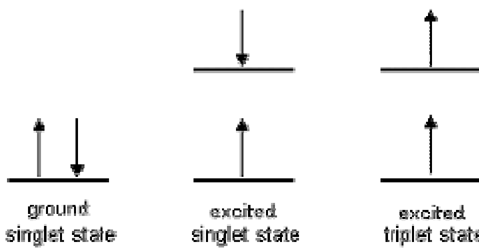
Fluorescence

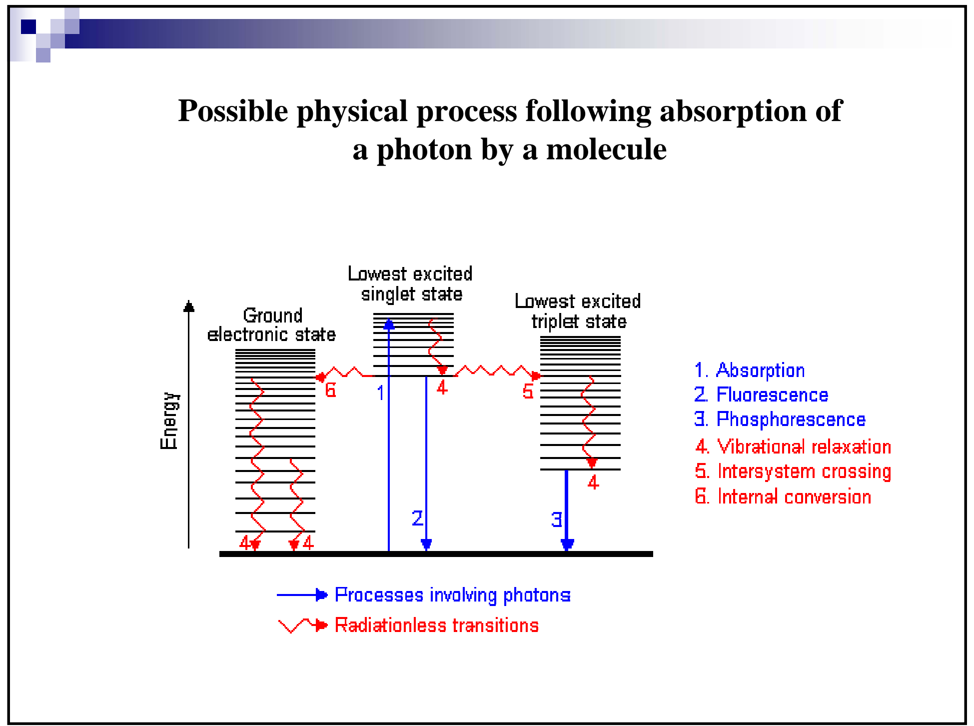
Photon emission as an electron returns from an excited state to ground state



The electronic states of most organic molecules can be divided into *singlet* states and *triplet* states;

Singlet state: All electrons in the molecule are spin-paired
Triplet state: One set of electron spins is unpaired





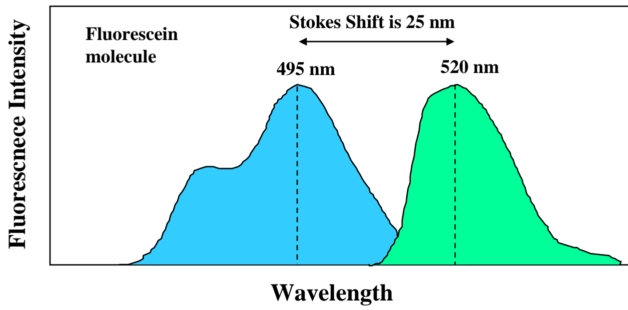
Fluorescence

- Fluorescing molecules (fluorophores) are usually aromatic
- Resolution of fluorescence is usually much better than absorption because you are measuring at right angles to the direction of exciting light (better signal to noise) so can work with much lower concentrations
- The wavelength of absorption is related to the size of the chromophores
- Smaller chromophores, higher energy (shorter wavelength)

Fluorescence

Stokes Shift:

The difference in wavelength between absorbed and emitted quanta. The emitted wavelength is always longer (if single photons are absorbed) or equal to the incident wavelength, due to energy conservation; the difference is absorbed as heat in the atomic lattice of the material.



Stokes shift increases with solvent polarity.

Fluorescence intensity is proportional to the product of ε and Q_f

- **Quantum Yield**

$$Q_f = \frac{\text{photons emitted}}{\text{photons absorbed}} = \frac{k_r}{k_r + k_{nr}}$$

Fluorescence Lifetime (τ)

- is the time delay between the absorbance and the emission

$$\tau = \frac{1}{k_r + k_{nr}}$$

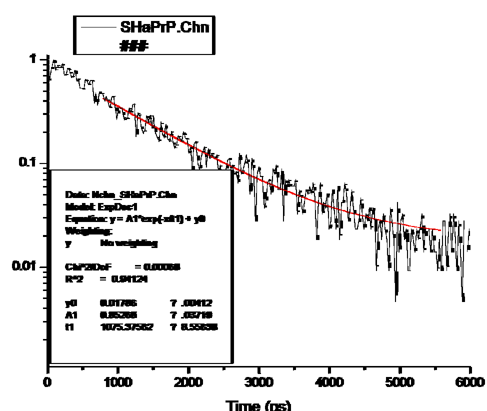
- Many of these measurements are made possible by the fluorescence lifetime, the average time that a molecule spends in the excited state before emitting a photon and returning to the ground state. It is an important and unique feature of an excited state.
- Fluorescence lifetimes are very short. Most fluorescence lifetimes fall within the range of hundreds of picoseconds to hundreds of nanoseconds. The fluorescence lifetime can function as a molecular stopwatch to observe a variety of interesting molecular events. An antibody may rotate slightly within its molecular environment. A protein can change orientation. A critical binding reaction may occur. Because the time-scale of these events is similar to the fluorescence lifetime, the measurement of the fluorescence lifetime allows the researcher to peer into the molecule and observe these phenomena

Fluorescence lifetime measurement

♣ Fluorescence lifetime can be measured by measuring the decay of the total fluorescence intensity following pulsed excitation which reflects the average time that a molecule remains in the singlet excited state. The fluorescence decay spectra were collected using time-correlated single-photon counting.

➢Many dynamic events can deactivate the excited state and hence influence the lifetime, including solvent relaxation, fluctuation in macromolecular conformation, rotations of side-chains, interactions with neighboring residues, and quenching by exogeneous agents.

➢Fluorescence lifetime of a fluorophore in a protein or nucleic acid is highly dependent upon its local environment and can vary from a few picoseconds to tens of nanoseconds.



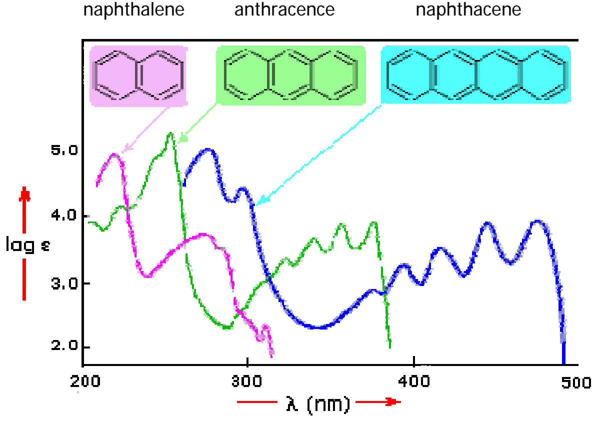
Tab. [illegible] Quantum yields and lifetimes of some aromatic hydrocarbons

Compound	Formula	Solvent (temp.)	Φ_F	τ_0 (ns)	Φ_{ISC}	Φ_P	τ_T (s)
Benzene		Ethanol (293 K)	0.04	31			
		EPA[a] (77 K)				0.17	7.0
Naphthalene		Ethanol (293 K)	0.21	2.7	0.79		
		Cyclohexane (293 K)	0.19	96			
		EPA (77 K)				0.06	2.6
Anthracene		Ethanol (293 K)	0.27	5.1	0.72		
		Cyclohexane (293 K)	0.30	5.24			0.09
		EPA (77 K)					
Perylene		n-Hexane	0.98		0.02		
		Cyclohexane (293 K)	0.78	6			
Pyrene		Ethanol (293 K)	0.65	410	0.35		
		Cyclohexane (293 K)	0.65	450			
Phenanthrene		Ethanol (293 K)	0.13		0.85		
		n-Heptane (293 K)	0.16	0.60			
		EPA (77 K)				0.31	3.3
		Polymer film	0.12		0.88		0.11

a) EPA: mixture of ethanol, isopentane, diethyl ether 2:5:5 v/v/v

Effects of molecular structure on fluorescence

➢ **Extent of p-electron system :** An increase in the extent of the π-electron system (i.e. degree of conjugation) leads to a shift of the absorption and fluorescence spectra to longer wavelengths and to an increase in the fluorescence quantum yield.



➢ **Substituted aromatic hydrocarbons:**

1. Internal heavy atom effect: the presence of heavy atom are substitutents of aromatic molecules (e.g. Br, I) results in fluorescence quenching because of the increased probability of intersystem crossing
2. Electron-donating substituents (-OH, -OR, -NH2, -NHR, -NR2) induce an increase in the molar absorption coefficient and a shift in both absorption and fluorescence spectra. They become broad and structureless.
3. Sulfonate: The solubility of many fluorophores is achieved by grafting sulfonate groups. It causes small red-shift in fluorescence spectrum and a slight decrease in fluorescence quantum yield.

Tab. 3.3. Heavy atom effect on emissive properties of naphthalene (from Wehry 1990)

	Φ_F	k_{isc}/s^{-1}	Φ_P	τ_P/s
Naphthalene	0.55	1.6×10^6	0.051	2.3
1-Fluoronaphthalene	0.84	5.7×10^5	0.056	1.5
1-Chloronaphthalene	0.058	4.9×10^8	0.30	0.29
1-Bromonaphthalene	0.0016	1.9×10^9	0.27	0.02
1-Iodonaphthalene	< 0.0005	$> 6 \times 10^9$	0.38	0.002

Bernard Valeur, Molecular Fluorescence , Wiley-VCH

❖**Fluorescence is very dependent on the environment.**

•Small molecules and other compounds can absorb energy and quench (lower) fluoresence.

•Temperature has a big effect.

•Dielectric constant e.g. aqueous vs hydrophobic/polar

Effect of temperature

- Generally, an increase in temperature results in a decrease in the fluorescence quantum yield and the life time because the non-radiative processes related to thermal agitation (collisions with solvent molecules, intramolecular vibrations and rotations…) are more efficient at higher temperature.

Effect of local environment

♣ **Aromatic residues (especially Tryptophan) are naturally occurring fluorophores which are good probes of folding.**

A tryptophan side-chain in a hydrophobic core has a high fluorescence intensity (not always)and a blue-shifted (shorter wavelength) emission maximum.

Exposed tryptophans (or tryptophans from unfolded proteins) have low fluorescence intensity (not always)and a red-shifted longer wavelength) emission maximum.

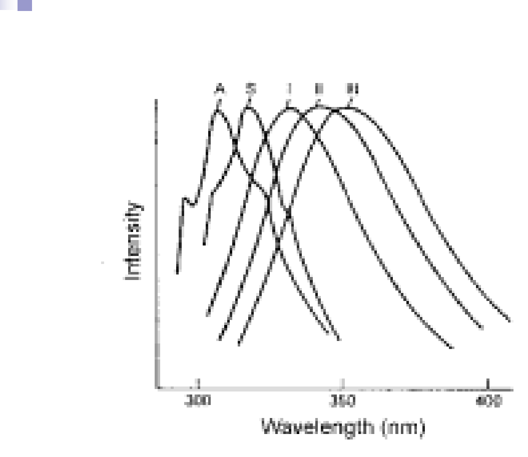
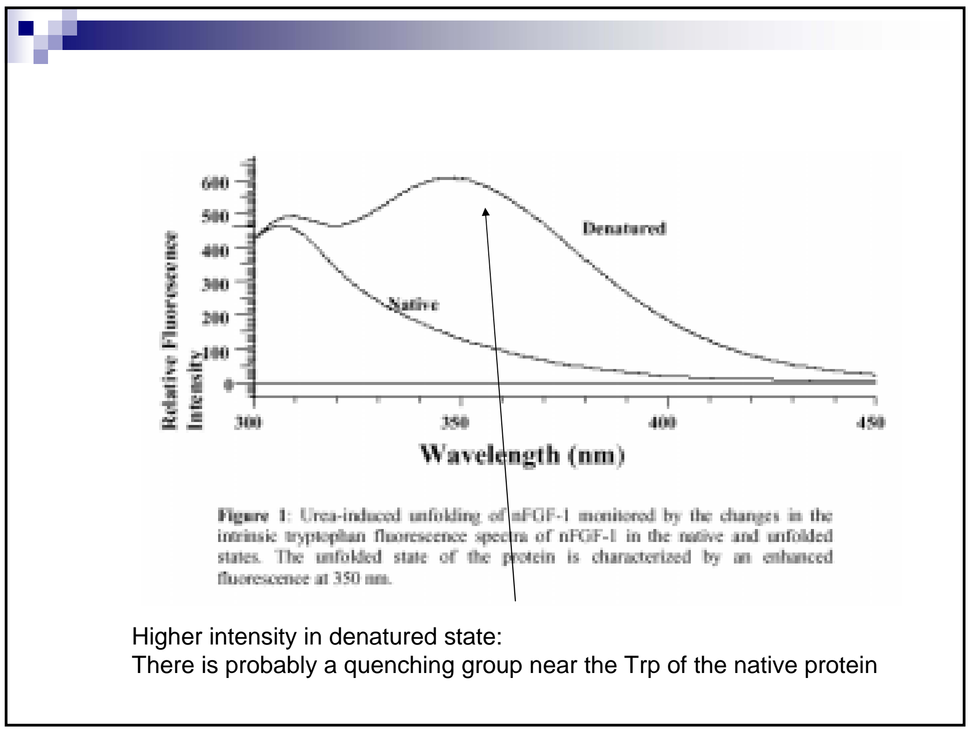
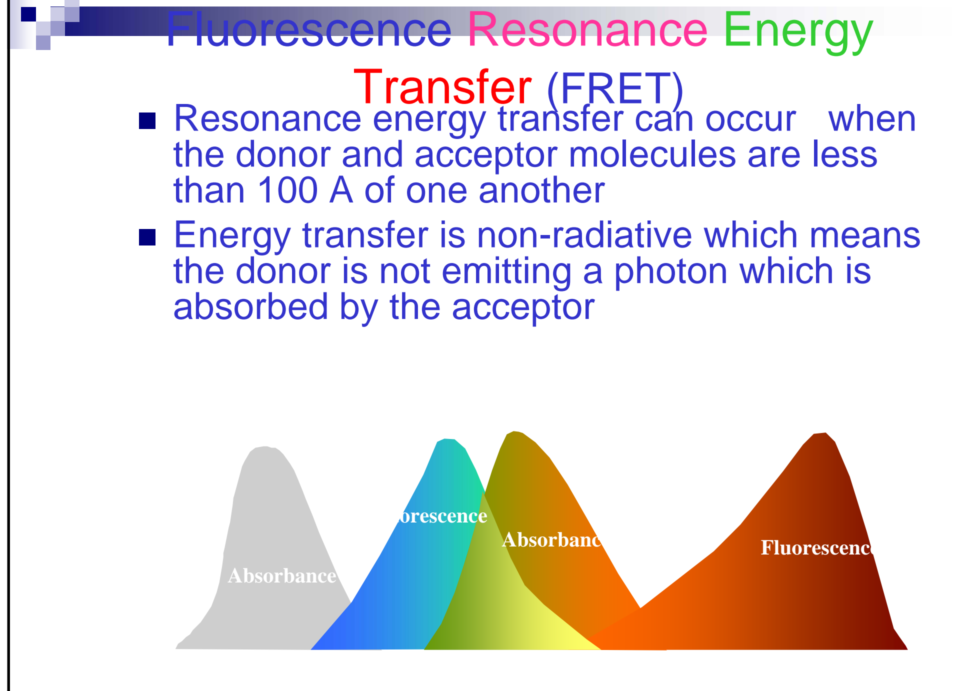
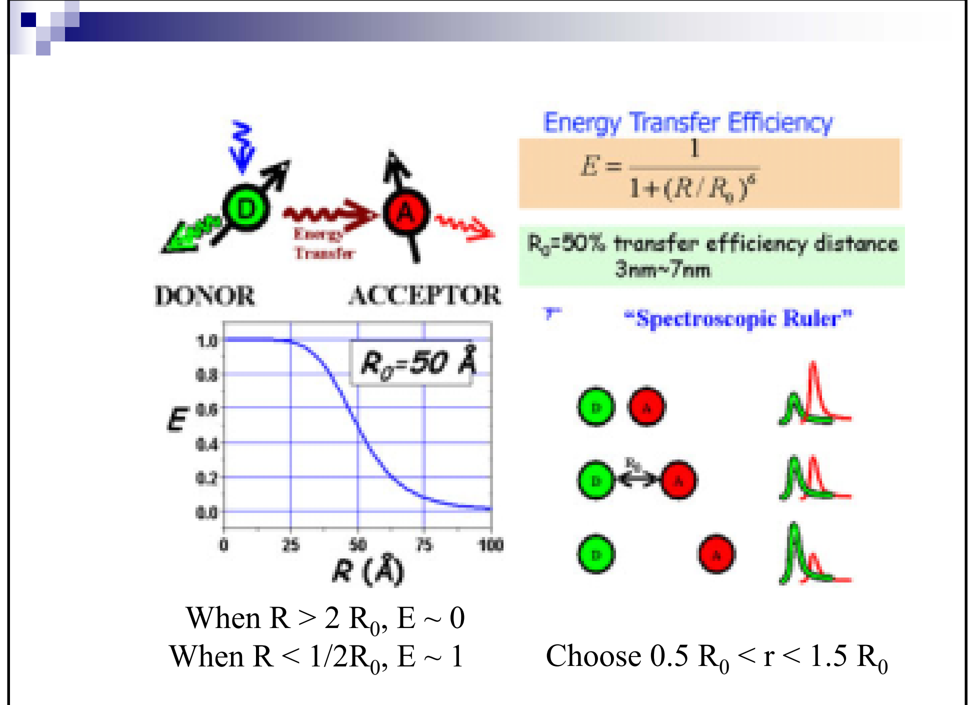


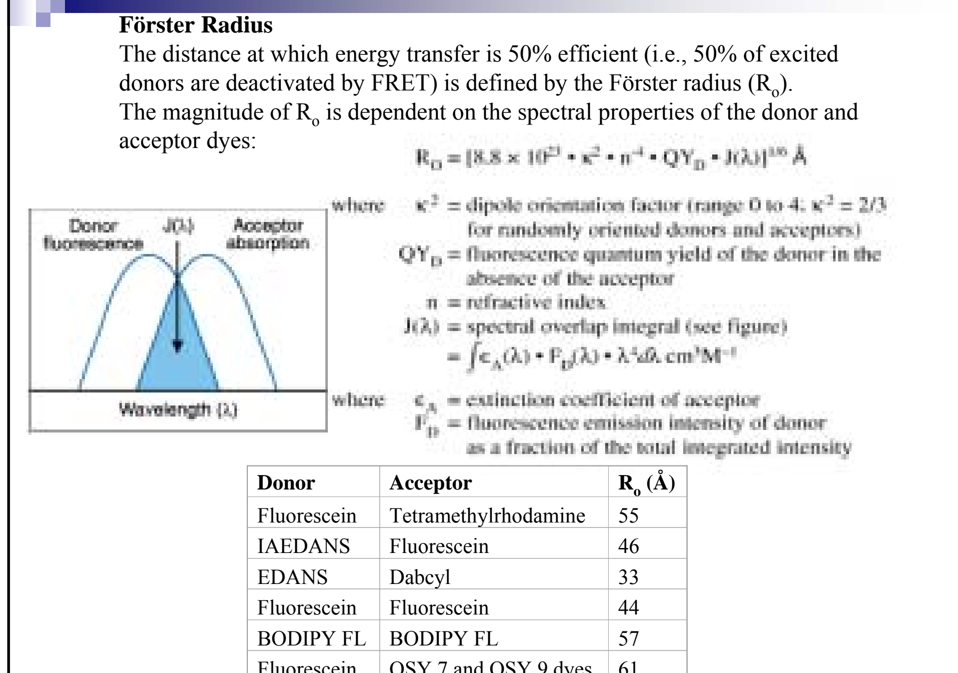
Figure 2 Normalised fluorescence spectra of tryptophan residues belonging to five spectral classes A, S, I, II and III according to Burstein.[illegible] Extreme variability of the emission of the indole fluorophore, depending on polarity and mobility of its environment, makes tryptophan fluorescence a sensitive tool in protein analysis. (Reproduced by permission of ONTI NCBI.)

A: extremely nonpolar environment inside the protein globule
S: relatively nonpolar environment inside the protein globule, interacting with neighboring polar group
I: polar but rigid environment inside the protein globule, interacting with neighboring polar group
II: at protein surface, in contact with bound water or other polar group
III: at protein surface, in contact with free water, typical for unfolded protein

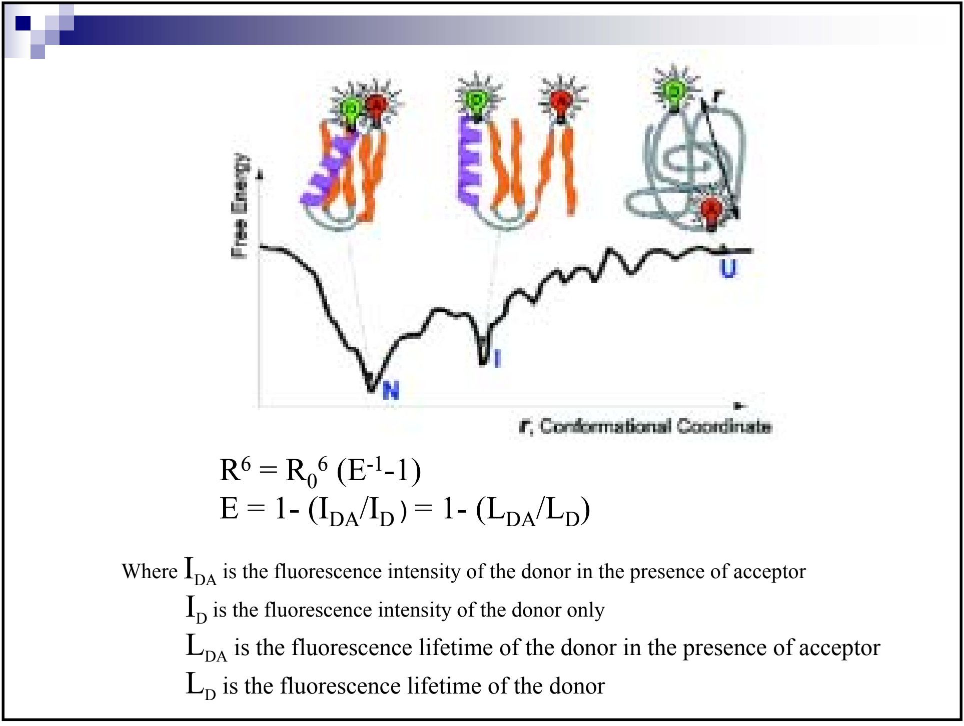

Relative Fluorescence Intensity
600
500
400
300
200
100
0
300
350
400
450
Wavelength (nm)
Denatured
Native
Figure 1: Urea-induced unfolding of nFGF-1 monitored by the changes in the intrinsic tryptophan fluorescence spectra of nFGF-1 in the native and unfolded states. The unfolded state of the protein is characterized by an enhanced fluorescence at 350 nm.
Higher intensity in denatured state:
There is probably a quenching group near the Trp of the native protein


Fluorescence Resonance Energy Transfer (FRET)
Resonance energy transfer can occur when the donor and acceptor molecules are less than 100 A of one another
Energy transfer is non-radiative which means the donor is not emitting a photon which is absorbed by the acceptor
Absorbance
Absorbanc
Fluorescenc

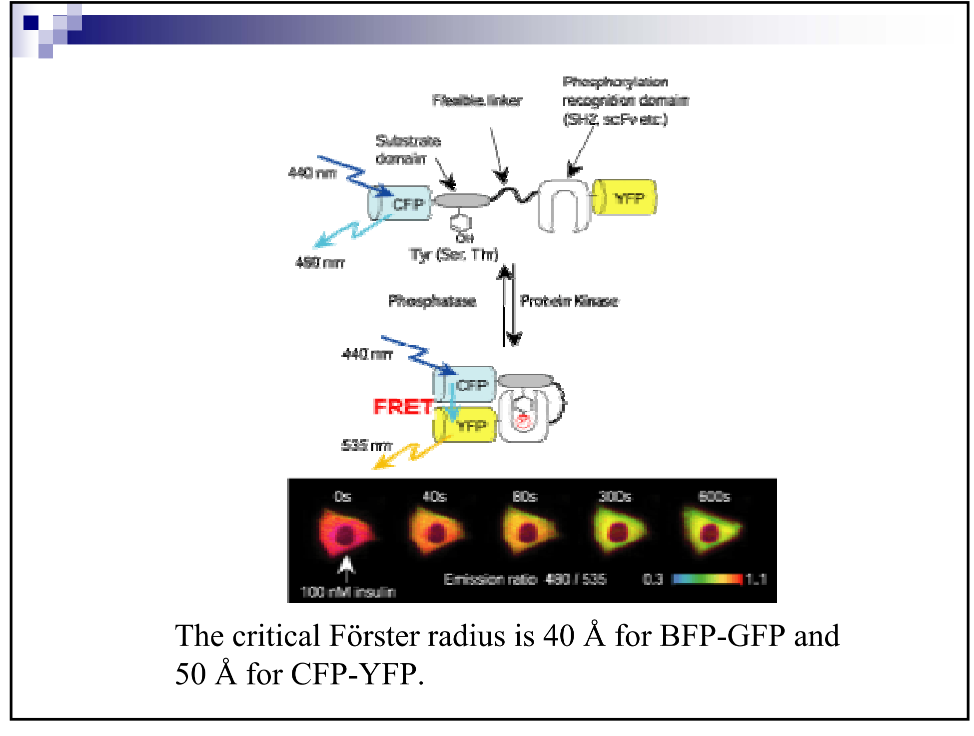




Donor	Acceptor	R_o (Å)
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46
EDANS	Dabcyl	33
Fluorescein	Fluorescein	44
BODIPY FL	BODIPY FL	57
Fluorescein	QSY 7 and QSY 9 dyes	61



Free Energy
U
I
N
r, Conformational Coordinate
$R^6 = R_0^6 (E^{-1}-1)$
$E = 1- (I_{DA}/I_D) = 1- (L_{DA}/L_D)$
Where I_{DA} is the fluorescence intensity of the donor in the presence of acceptor
I_D is the fluorescence intensity of the donor only
L_{DA} is the fluorescence lifetime of the donor in the presence of acceptor
L_D is the fluorescence lifetime of the donor



Phosphorylation recognition domain (SH2, scFv etc.)
Flexible linker
Substrate domain
440 nm
CFP
YFP
480 nm
Tyr (Ser, Thr)
Phosphatase
Protein Kinase
440 nm
FRET
CFP
YFP
535 nm
0s
40s
80s
300s
600s
100 nM insulin
Emission ratio 480 / 535
0.3
1.1
The critical Förster radius is 40 Å for BFP-GFP and 50 Å for CFP-YFP.

Fluorescence Polarization (FP)

Fluorescence polarization measurements provide information on molecular orientation and mobility and processes that modulate them, including receptor–ligand interactions, proteolysis, protein–DNA interactions, membrane fluidity and muscle contraction

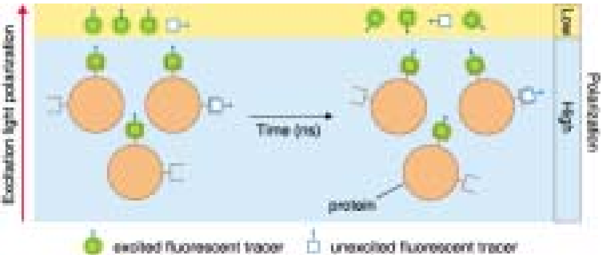
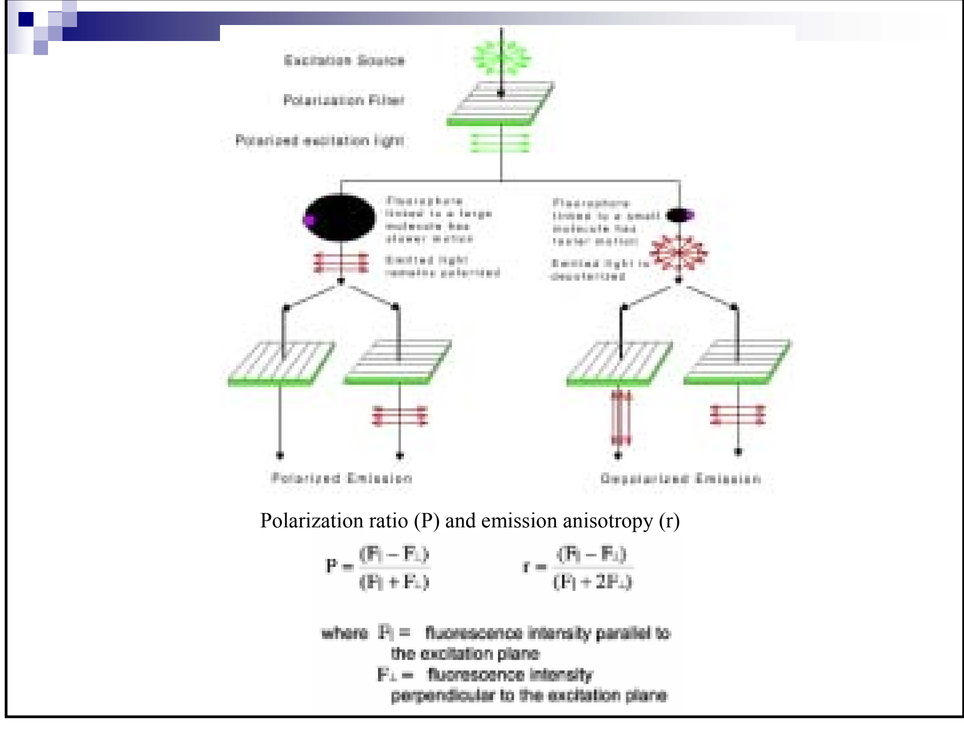


Figure 1. Physical basis of fluorescence polarization assays. Dye molecules with their absorption transition vectors (arrows) aligned parallel to the electric vector of linearly polarized light (along the vertical page axis) are selectively excited. For dyes attached to small, rapidly rotating molecules, the initially photoselected orientational distribution becomes randomized prior to emission, resulting in low fluorescence polarization. Conversely, binding of the low molecular weight tracer to a large, slowly rotating molecule results in high fluorescence polarization. Fluorescence polarization therefore provides a direct readout of the extent of tracer binding to proteins, nucleic acids and other biopolymers.

http://www.probes.com/handbook/boxes/1572.html



Polarization ratio (P) and emission anisotropy (r)

$$P = \frac{(F_{\parallel} - F_{\perp})}{(F_{\parallel} + F_{\perp})} \qquad r = \frac{(F_{\parallel} - F_{\perp})}{(F_{\parallel} + 2F_{\perp})}$$

where $F_{\parallel}$ = fluorescence intensity parallel to the excitation plane

$F_{\perp}$ = fluorescence intensity perpendicular to the excitation plane

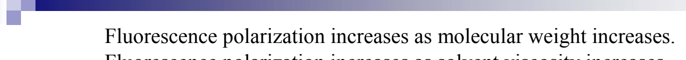
Fluorescence anisotropy measurement

$$P = \frac{(F_{\parallel} - F_{\perp})}{(F_{\parallel} + F_{\perp})} \qquad r = \frac{(F_{\parallel} - F_{\perp})}{(F_{\parallel} + 2F_{\perp})}$$

where $F_{\parallel}$ = fluorescence intensity parallel to the excitation plane
$F_{\perp}$ = fluorescence intensity perpendicular to the excitation plane

$$r = (I_{VV} - G \cdot I_{VH}) / (I_{VV} + 2G \cdot I_{VH})$$

where I_{VV} is the fluorescence intensity recorded with excitation and emission polarization in vertical position, and I_{VH} is the fluorescence intensity recorded with the emission polarization aligned in horizontal position. The G factor is the ratio of sensitivities of detection system for vertically and horizontally polarized light $G = I_{HV}/I_{HH}$.



Fluorescence polarization increases as molecular weight increases.
Fluorescence polarization increases as solvent viscosity increases.
Fluorescence polarization decreases as the excited state lifetime of the dye increases.

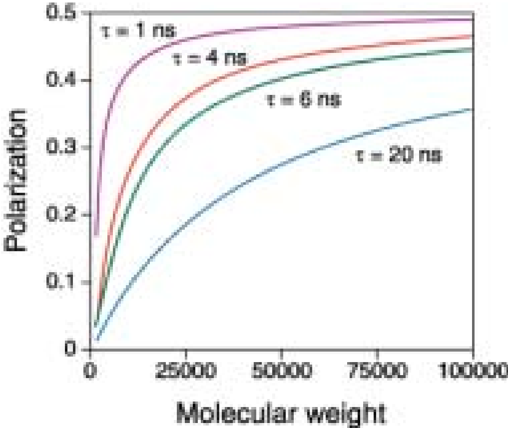


Figure 2. Simulation of the relationship between molecular weight (MW) and fluorescence polarization (P). Simulations are shown for dyes with various fluorescence lifetimes ( ): 1 ns (cyanine dyes) in purple, 4 ns (fluorescein and Alexa Fluor 488 dyes) in red, 6 ns (some BODIPY dyes) in green and 20 ns (dansyl dyes) in blue. At MW = 1000, P = 0.167 for = 1 ns, P = 0.056 for = 4 ns, P = 0.039 for = 6 ns and P = 0.012 for = 20 ns. Simulations assume Po (the fundamental polarization) = 0.5 and rigid attachment of dyes to spherical carriers.

How does the FluoroTect™ System Work?

The FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System uses a charged lysine tRNA molecule labeled with the fluorophore BODIPY®-FL at the ε -amino acid position of lysine (Figure 1). The BODIPY®-FL fluorophore, with an absorbance at 502nm and an emission at 510nm, was developed to be compatible with widely used excitation sources and common optical filter sets. The labeled FluoroTect™ tRNA is added directly to the translation reaction, and the labeled lysine is incorporated into the synthesized protein.

Recently, a method using BODIPY®-FL-labeled methionine was shown to be able to detect nanogram levels of protein using laser-based fluorescent gel scanning. For the FluoroTect™ System, lysine was chosen as the labeled amino acid because it is one of the more frequently used amino acids, comprising, on average, 6.6% of protein's amino acids. This compares to 1.7% for methionine.

FTIR signal

- Transitions in Vibrational levels.
- Signals arise from transition that are localized.
  - C=O stretch, C-H, N-H stretch, bending modes, etc.
- Signals result from asymmetric stretch← O=C=O → not active in FTIR
- Lower energy than electronic
- Less used: less intense (ε often 10-x less).
- Higher concentrations needed.
- Interference from H_2O.

FTIR as a tool to study protein structure

- In Vibrational transitions, bond type determines vibrational frequency of transition, (cm^{-1}).
- Diagnostic absorption bands:
  - Amide I absorption; also has contributions from C=O stretch. (1630 – 1660 cm^{-1}) (1660 cm^{-1} = 6024 nm)
  - Amide I Depends on strength of H-bonds involving C=O and N-H
  - Also N-H stretch (3300), N-H bend (1520-1550)
- Differences in H-bond geometry, and pattern of 2° structures gives characteristic amide I (& II) absorptions

Sensitivity of FTIR to 2° structure

- Stronger H-bonds yield changes in vibrational frequencies.
- H-bond makes it easier to stretch along axis of C=O or N-H, but harder to bend, vs. Non-H-bond
- Lowest frequency is for long aggregates
  - β-sheets over long stretches of extended structure.

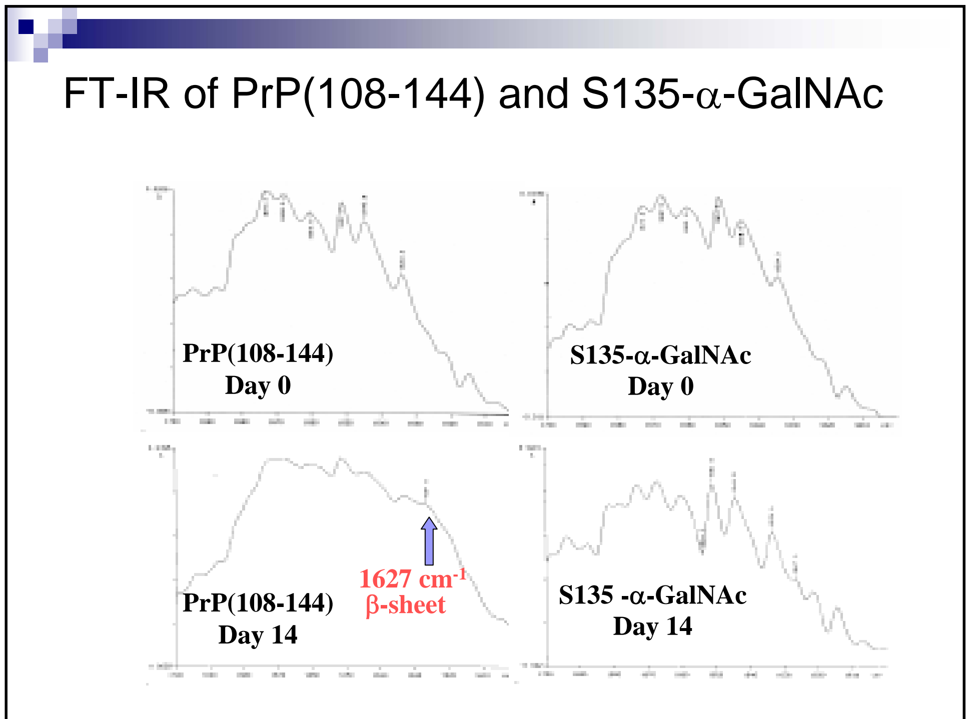
Characteristic FTIR bands

Antiparallel β-sheet and aggregates	1675-95 cm^{-1}
3_{10}-helix	1660-70
α-helix	1648-60
unordered	1640-48
β-sheet	1625-40
Aggregates	1610-28

- These ranges are only guidelines, general rules.
- Other factors like solvent effects, distortion of 2° elements, prosthetic groups can alter frequencies

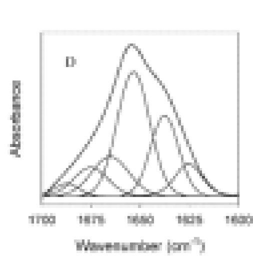
Advantages of using FTIR

- Identify presence of protein 2° structures
- Detect changes in protein 2° structures
- Fast kinetic events (stopped flow)
- Good for rapidly interconverting samples (can resolve both components instead of averages).
- No size limit (compared to NMR).
- Good for membranes (even vesicles, bilayers, solids, films. 2D crystals, gels.
- Light scattering isn't a big problem.
  - Samples can be suspensions or inhomogeneous dispersions.
- Excellent tool for studying protein aggregation, prions, amyloids, etc. (kinetics, progression).
- Organic solvents are good
  - TFE, etc. for hydrophobic peptides



Limitations of FTIR

- Width and separation of peaks gives overlap: mixtures of 2° elements can be hard to distinguish.
- Deconvolution is needed. Changes in spectra can be due to shift of one type of 2° structure to another, or to an increase of one type.
  - Hard to distinguish.
  - Deconvolution methods are not always clean.
  - Can be difficult to quantify.
  - Better for β-sheet than α-helix, so good complement to CD.
- Water interference. So FTIR works best with solids, or films.



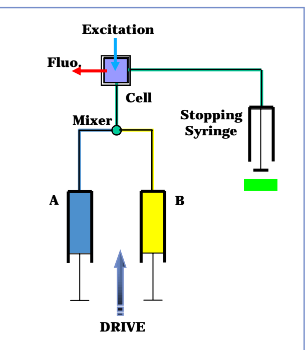
Water effects on FTIR spectra

- Water is BIG problem:
  - O-H bending mode at 1644 cm^{-1}
  - Obscures amide I band
- Can subtract water signal (blank), if a small amount of water is in sample (e.g. bound to protein).
- But if water is solvent, O-H absorbs so strongly that you can't subtract it and still retain sample signal.
- Solutions - use D_2O instead
  - O-D stretch is lower than O-H by 400 cm^{-1}

Slow and fast kinetics:

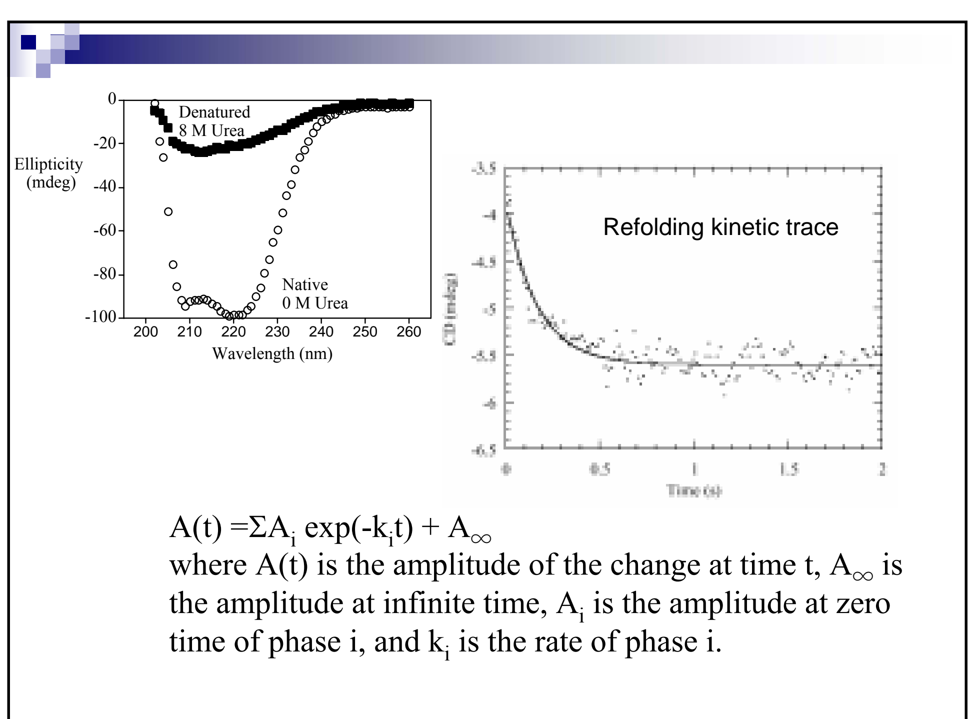
UV-VIS, CD, FTIR, Fluorescence

Stopped Flow: how does it work?



Standard stopped flow schematic

- Under pneumatic drive activation, the two small volumes of solutions are driven from high performance syringes through a high efficiency mixer.
- The resultant mixture passes through a measurement flow cell and into a stopping syringe.
- Just prior to stopping, a steady state flow is achieved.
- As the solution fills the stopping syringe, the plunger hits a block, causing the flow to be stopped instantaneously.
- Using appropriate techniques, the kinetics of the reaction can be measured in the cell.



$A(t) = \Sigma A_i \exp(-k_i t) + A_\infty$

where $A(t)$ is the amplitude of the change at time t, A_∞ is the amplitude at infinite time, A_i is the amplitude at zero time of phase i, and k_i is the rate of phase i.

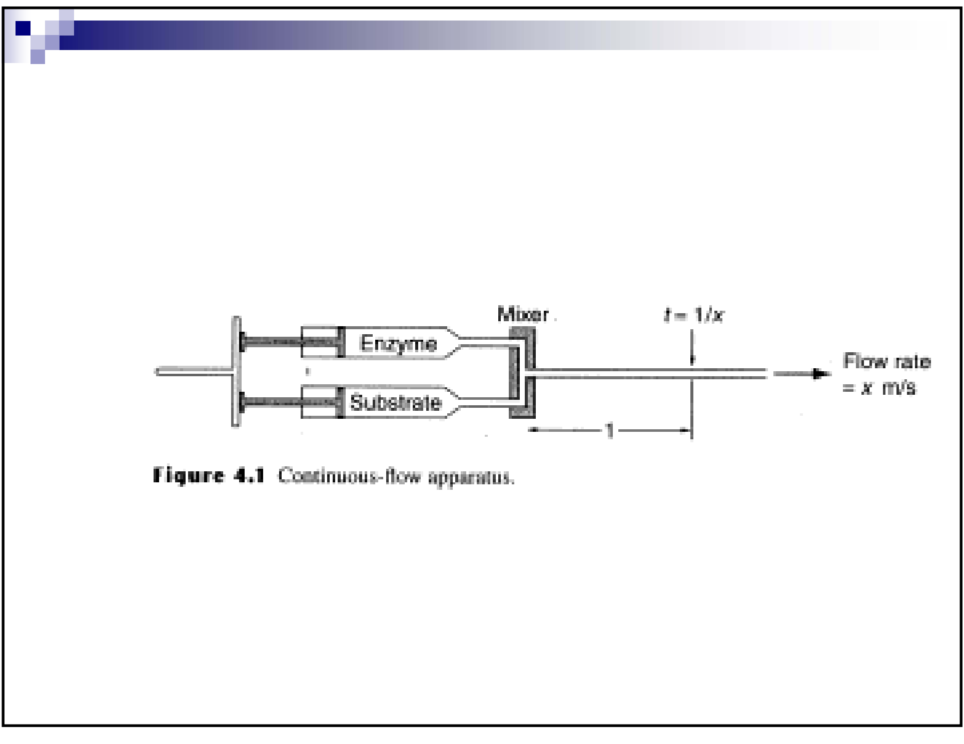


Figure 4.1 Continuous-flow apparatus.

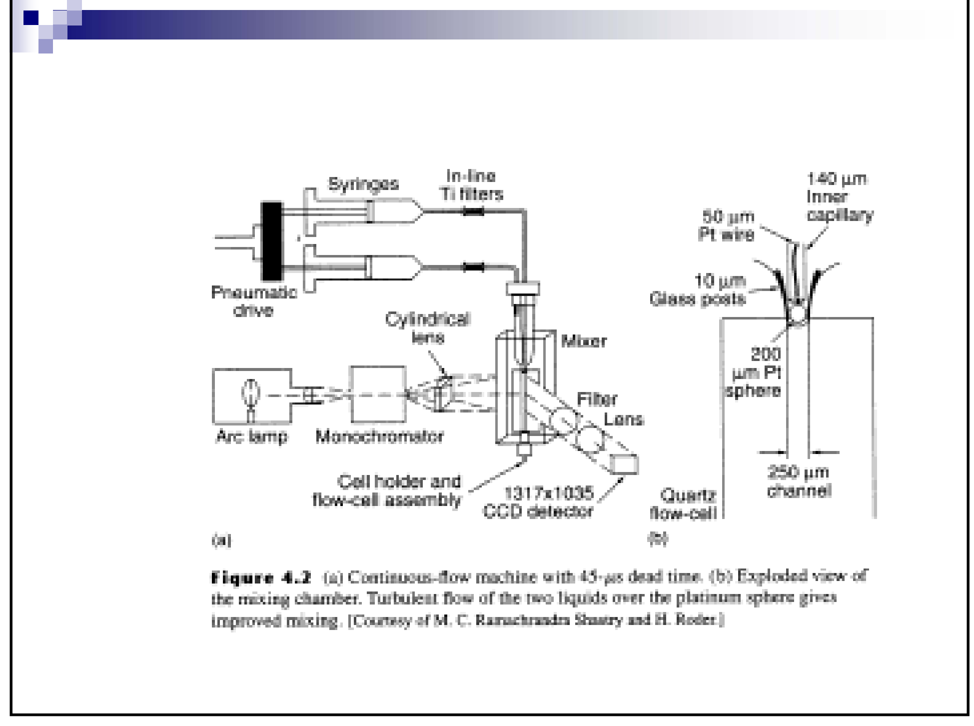


Figure 4.2 (a) Continuous-flow machine with 45-µs dead time. (b) Exploded view of the mixing chamber. Turbulent flow of the two liquids over the platinum sphere gives improved mixing. [Courtesy of M. C. Ramachandra Shastry and H. Roder.]

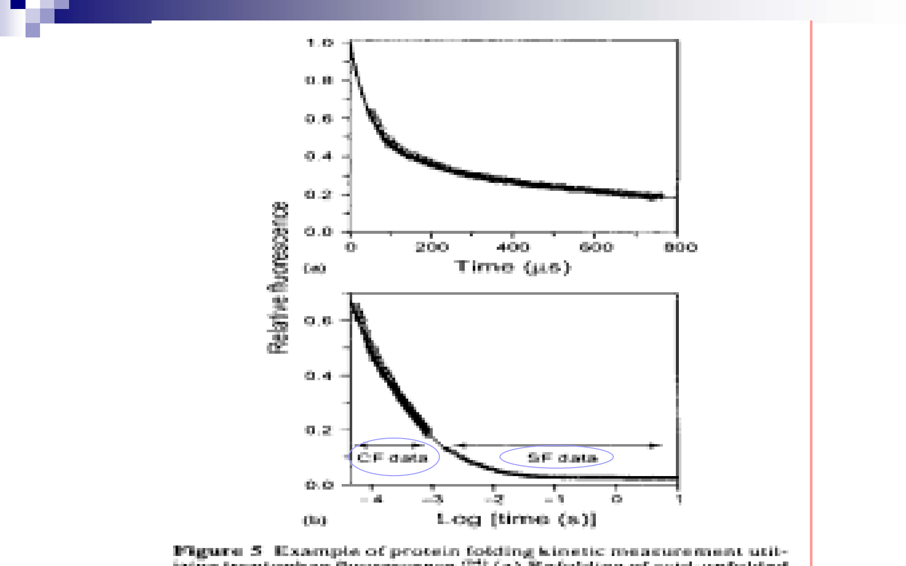


Figure 5 Example of protein folding kinetic measurement utilising tryptophan fluorescence. (a) Refolding of acid-unfolded horse cytochrome *c* was followed on the submillisecond timescale in a continuous flow capillary mixing apparatus. (b) Combination of continuous flow (CF) and conventional stopped-flow (SF) experiments allows collection of data over a wide range of time (symbols) and determination of the five kinetic phases with correlation times ranging from tens of microseconds to tens of milliseconds (solid line). (Reproduced by permission of the Biophysical Society.)