Application of UV-Visible spectroscopy in biological science

(With protein and nucleic acids approach)

By:

Mehdi Imani

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“Knowledge is power”

Summary of workshop

1. *Importance of spectroscopy in biological studies*
2. *Nature of light*
3. *Spectrophotometer configuration*
4. *Types of spectrophotometer cuvettes*
5. *Protein and DNA chromophores*
6. *Absorbtion and emission spectra of some molecules*
7. *Basics of protein and DNA assays*

Importance of spectroscopy

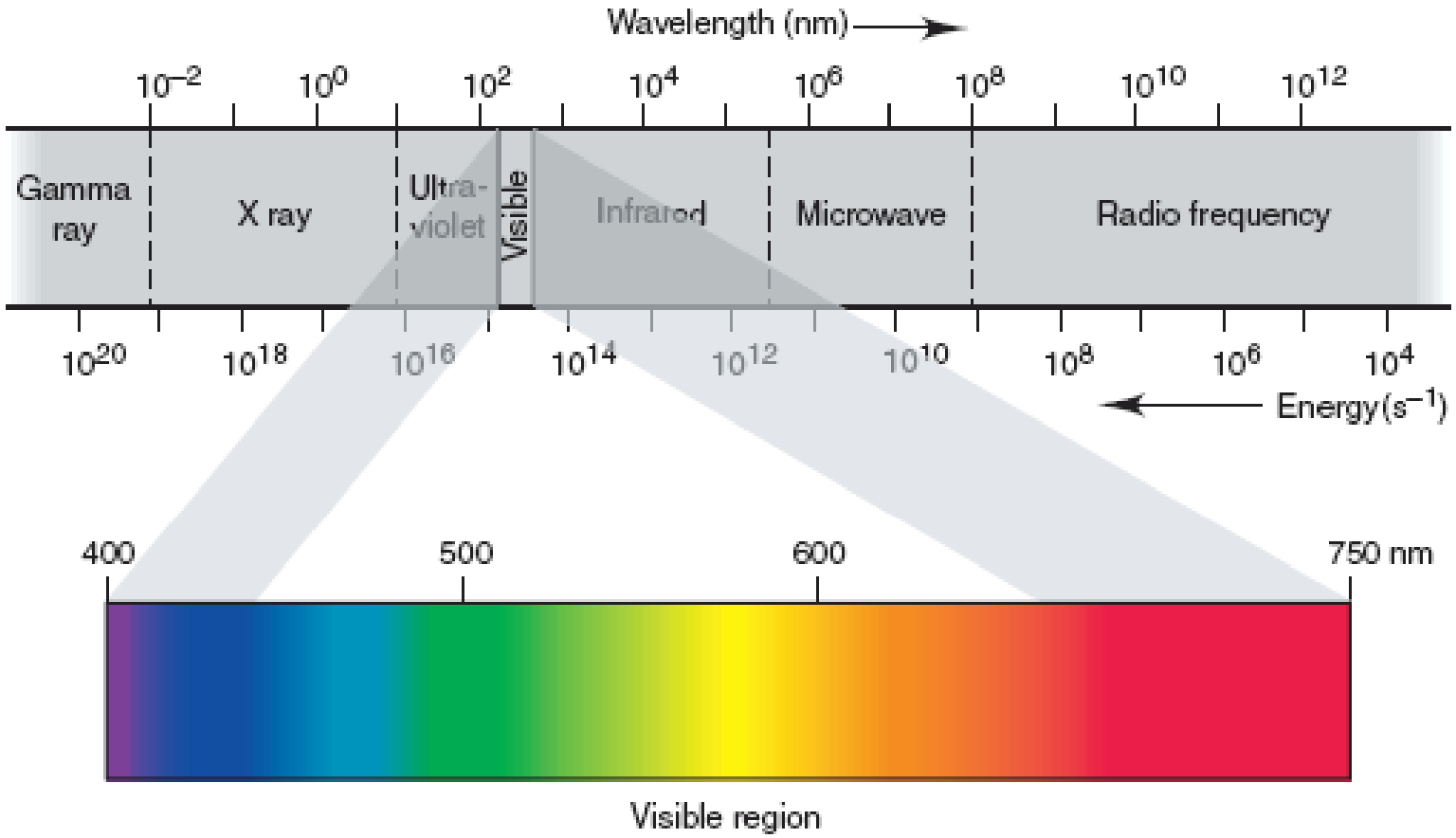
1. Spectroscopy is a powerful tool for studying biological systems.

2. It often provides a convenient method for analysis of individual components in a biological system **such as proteins, nucleic acids, and metabolites.**

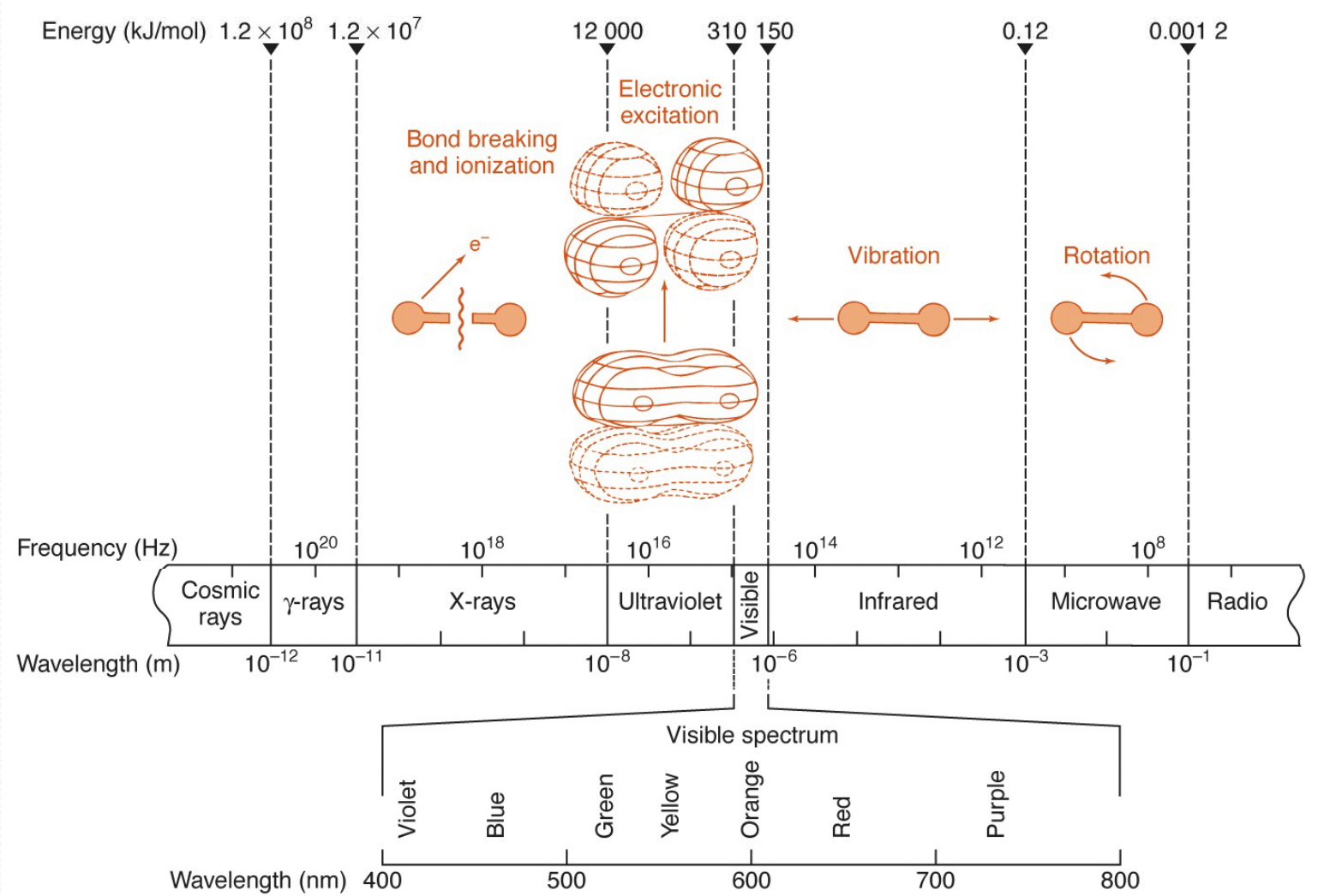
The aims of this workshop

1. *Grasp the basic concepts of spectroscopic methods.*
2. *Understand and differentiate various and confusing form of spectroscopic methods.*
3. *Be able to understand and determine* ***λmax*** *in case of necessity.*
4. ***Be able to estimate the protein concentration using UV and Colorimetric methods.***
5. ***Be able to determine the concentration of nucleic acids DNA or RNA accurately.***

Electromagnetic Spectrum (Light)



Regions of Electromagnetic Spectrum-the "colour" of light



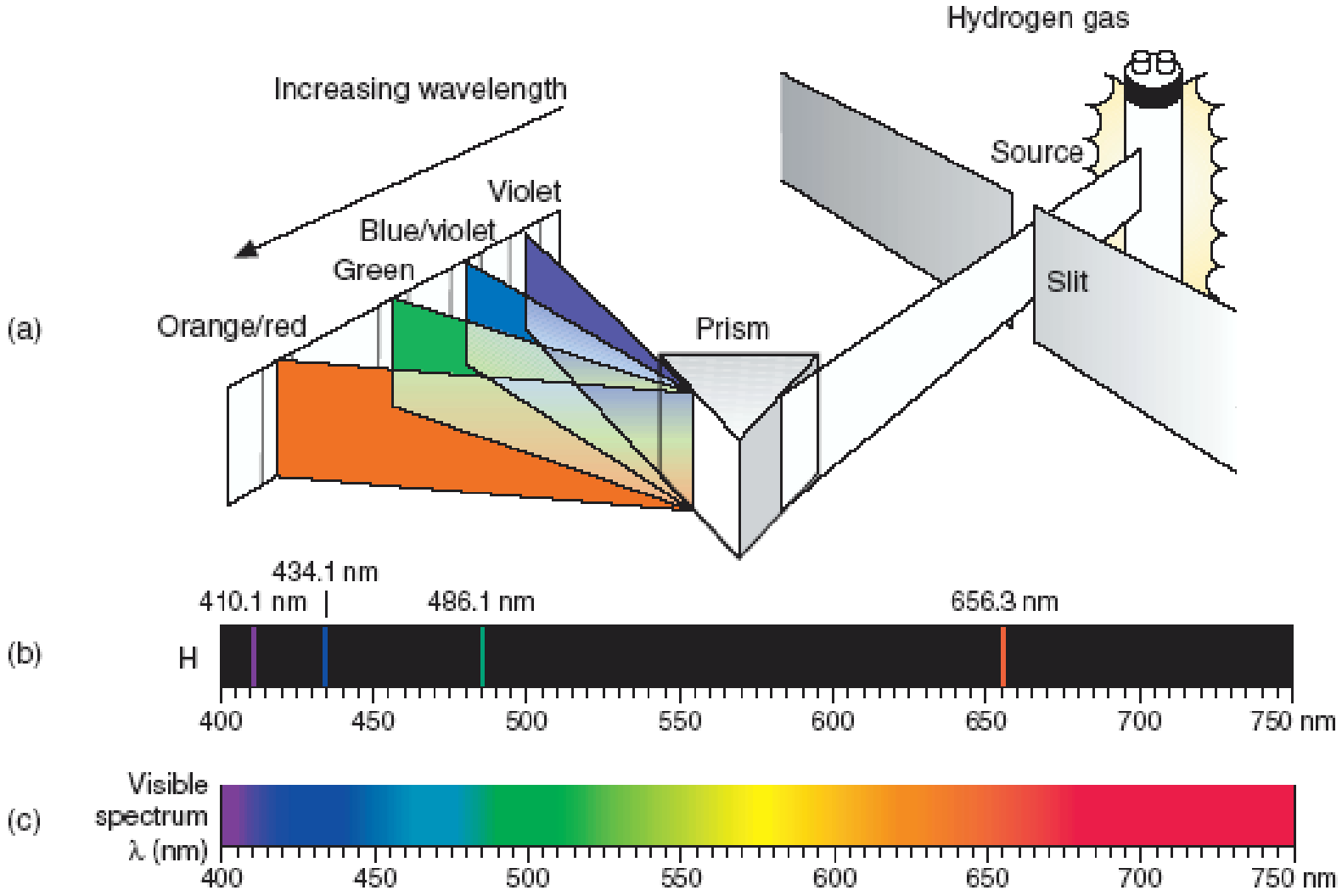
Some type of spectroscopy

- ✓ *Infrared Spectroscopy*
- ✓ *Raman Spectroscopy*
- ✓ *NMR Spectrometers*
- ✓ *MASS Spectrometry*
- ✓ *Circular Dichroism*
- ✓ *Fluorescence*
- ✓ ***UV-Visible***

Energy content of the wave

$$E = \frac{h \cdot c}{\lambda}$$

$$E = h \cdot \nu$$


Hydrogen gas
Increasing wavelength
Source
Violet
Blue/violet
Green
Slit
(a)
Orange/red
Prism
434.1 nm
410.1 nm
486.1 nm
656.3 nm
(b)
H
400
450
500
550
600
650
700
750 nm
Visible
spectrum
λ (nm)
(c)
400
450
500
550
600
650
700
750 nm

Light excitation and absorption

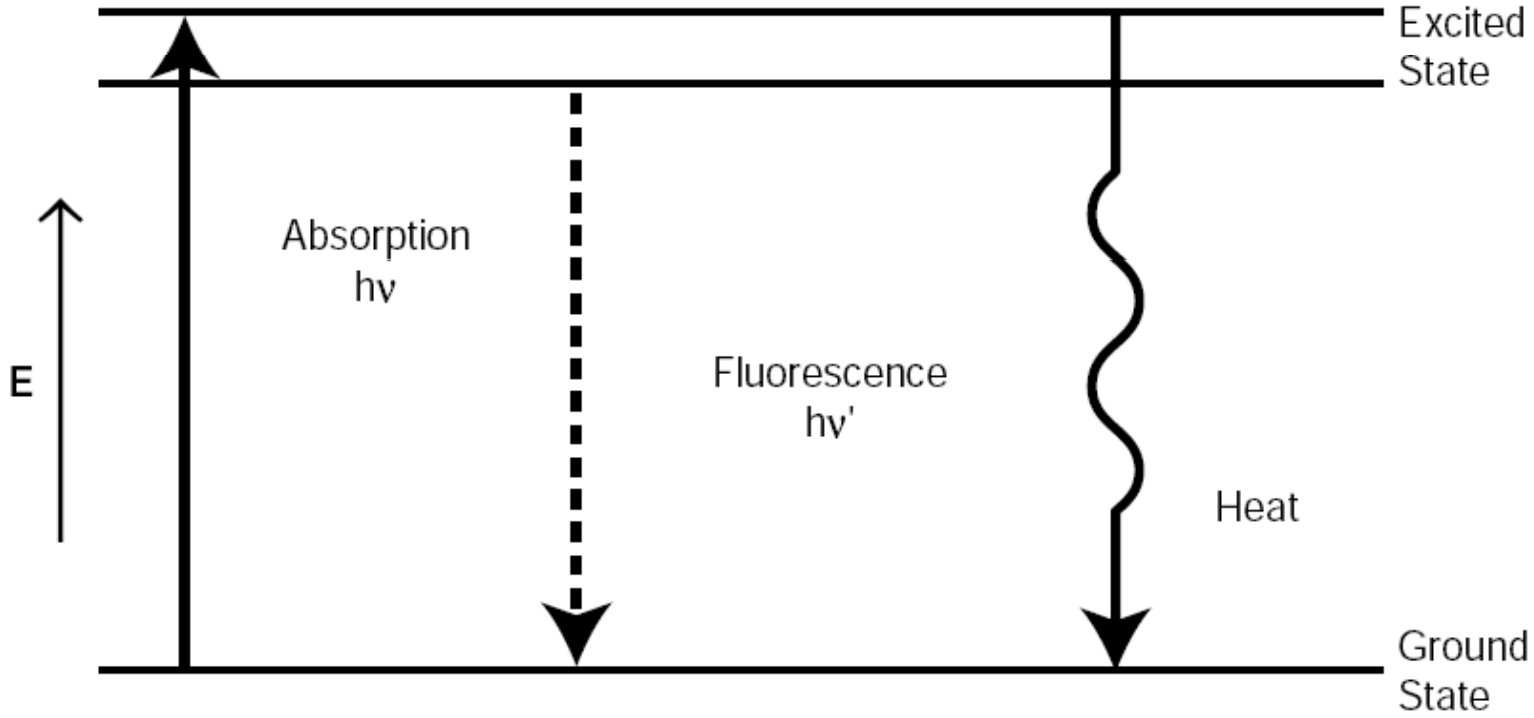
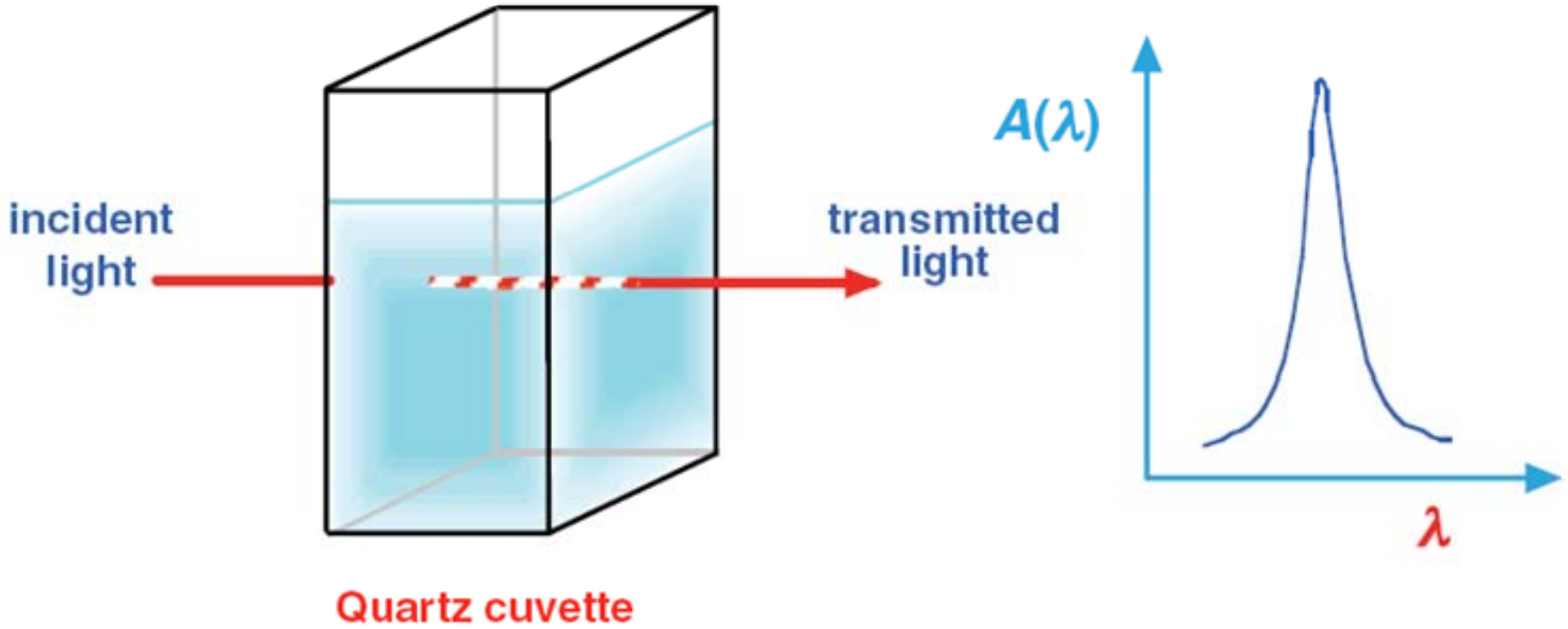
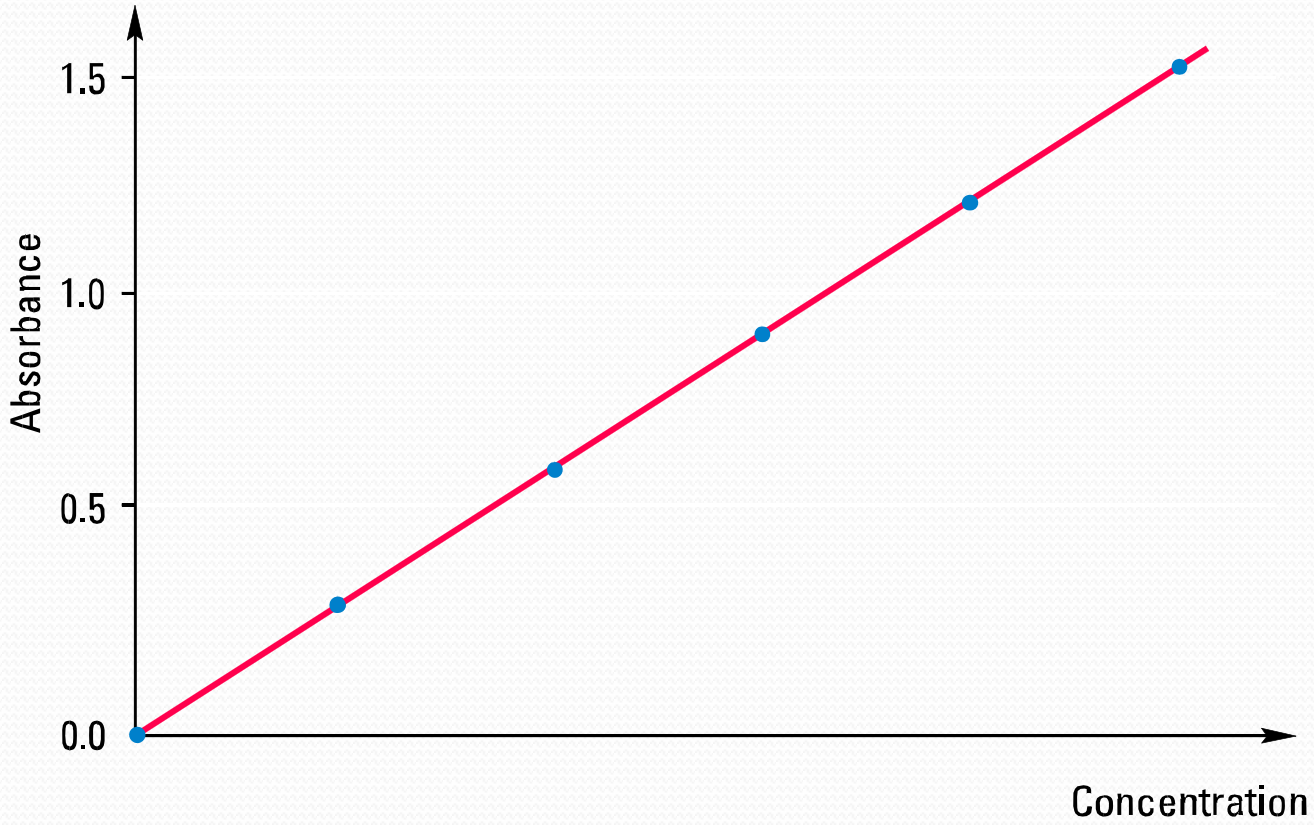


Figure 3-1. Simplified schematic diagram of electron excitation by absorbance of radiation. An electron is excited to a higher energy level by radiation of frequency υ. It returns to its ground state through fluorescence at a frequency υ′ and/or by dissipation of heat. A more complete diagram is presented in Figure 3-11.

Illustration of light absorbtion and transmission



The Beer-Lambert Law



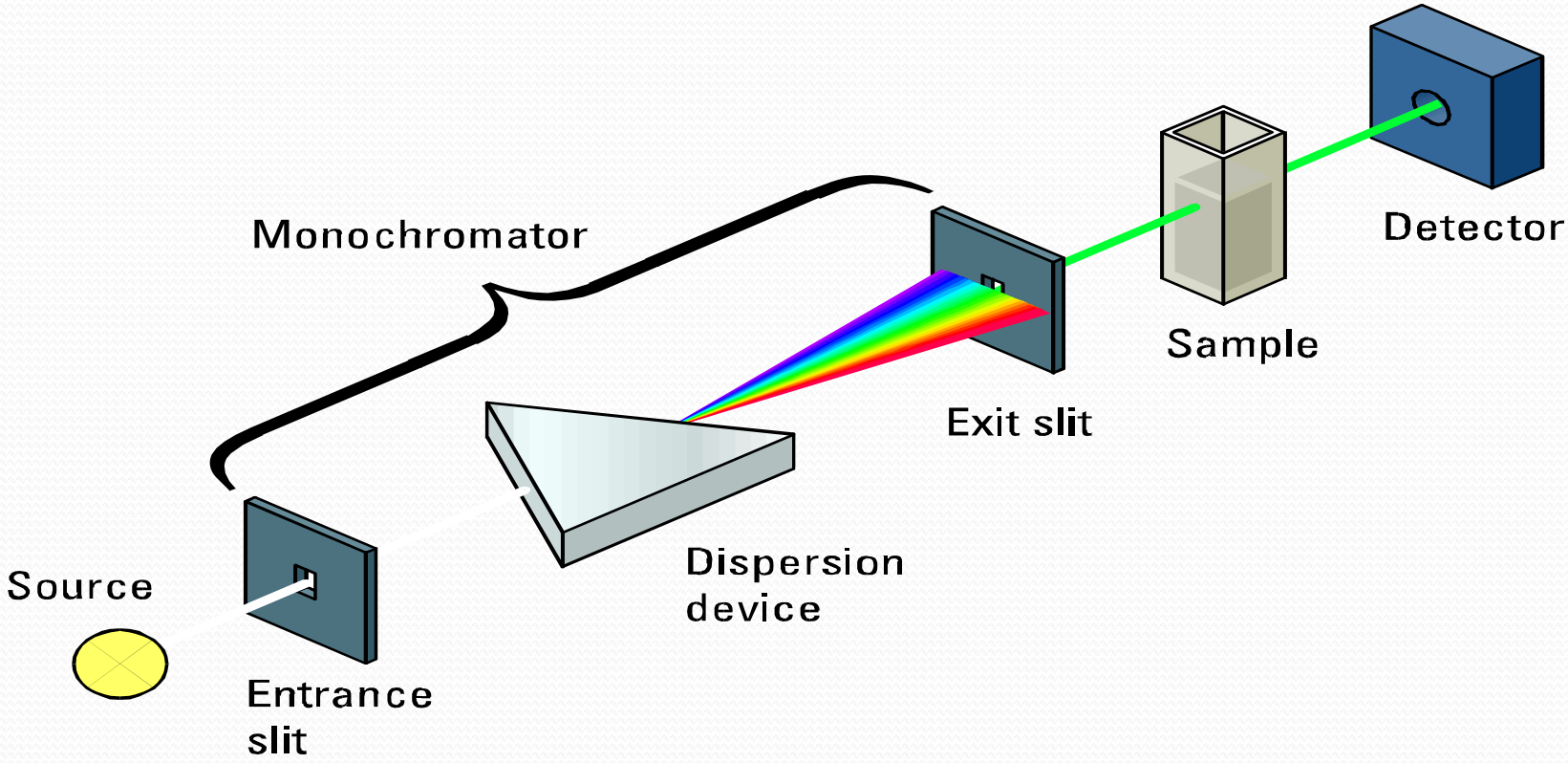
$$A = -\log T = -\log\left(I / I_0\right) = \log\left(I_0 / I\right) = \varepsilon \cdot b \cdot c$$

Spectrophotometer configurations

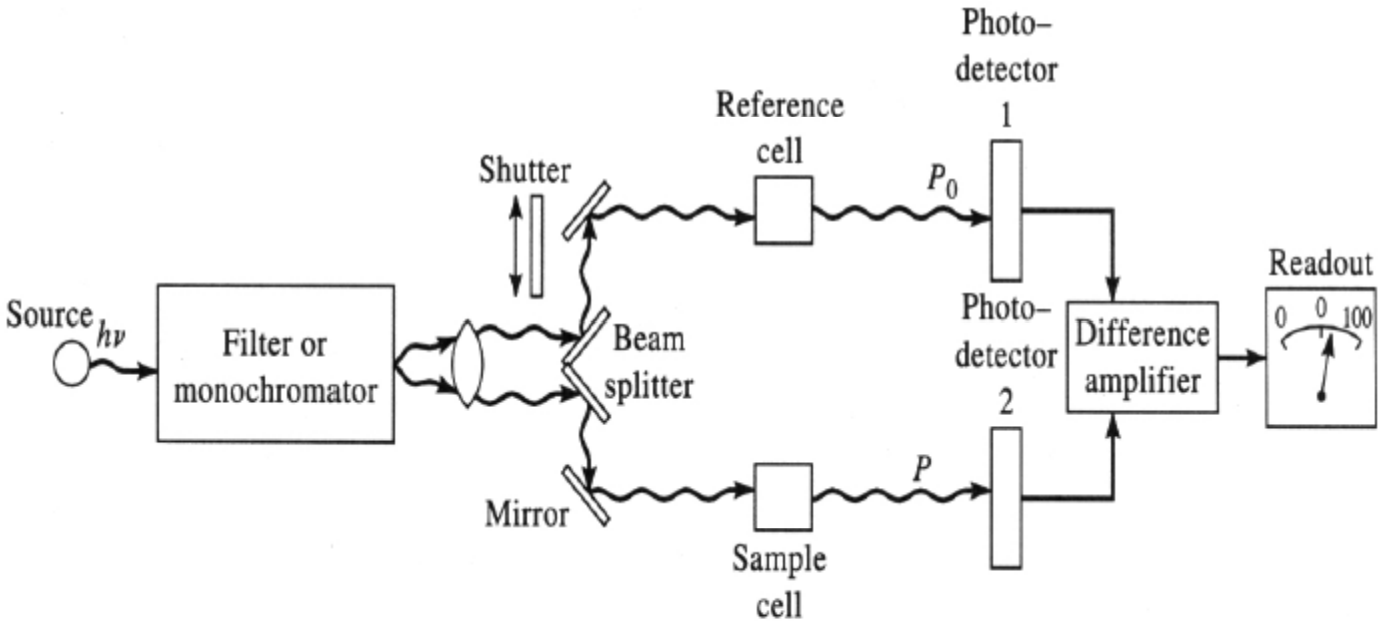
- ✓ **Single beam spectrophotometers**

- ✓ **Split beam or 'double beam' spectrophotometers**

Single beam spectrophotometers

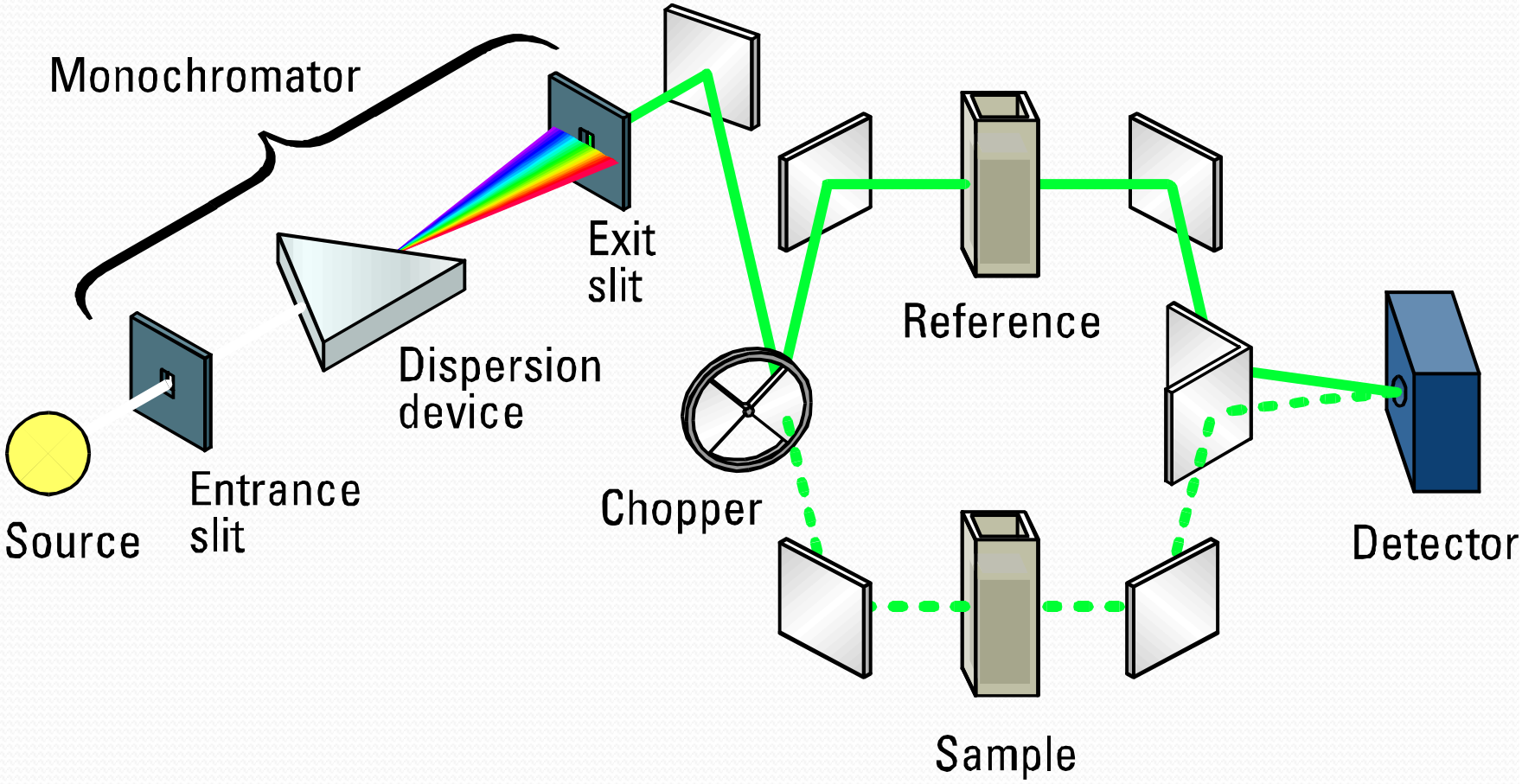


Double beam spectrophotometers



- Sample and reference are measured simultaneously and the signal from the reference is subtracted from the sample signal.
- A major drawback of this type of instrument is the requirement of two detectors, which makes the instrument more expensive.

Double beam spectrophotometers



light Source

- **Tungsten lamp**

Used for the visible region of the spectrum (350 –800 nm).

Usually constructed of a quartz or glass bulb containing a tungsten filament.

- **H2 or D2 lamp**

Used for the ultraviolet region of the spectrum (160 –350 nm).

The excited D2will dissociate to give a continuous band of radiation.

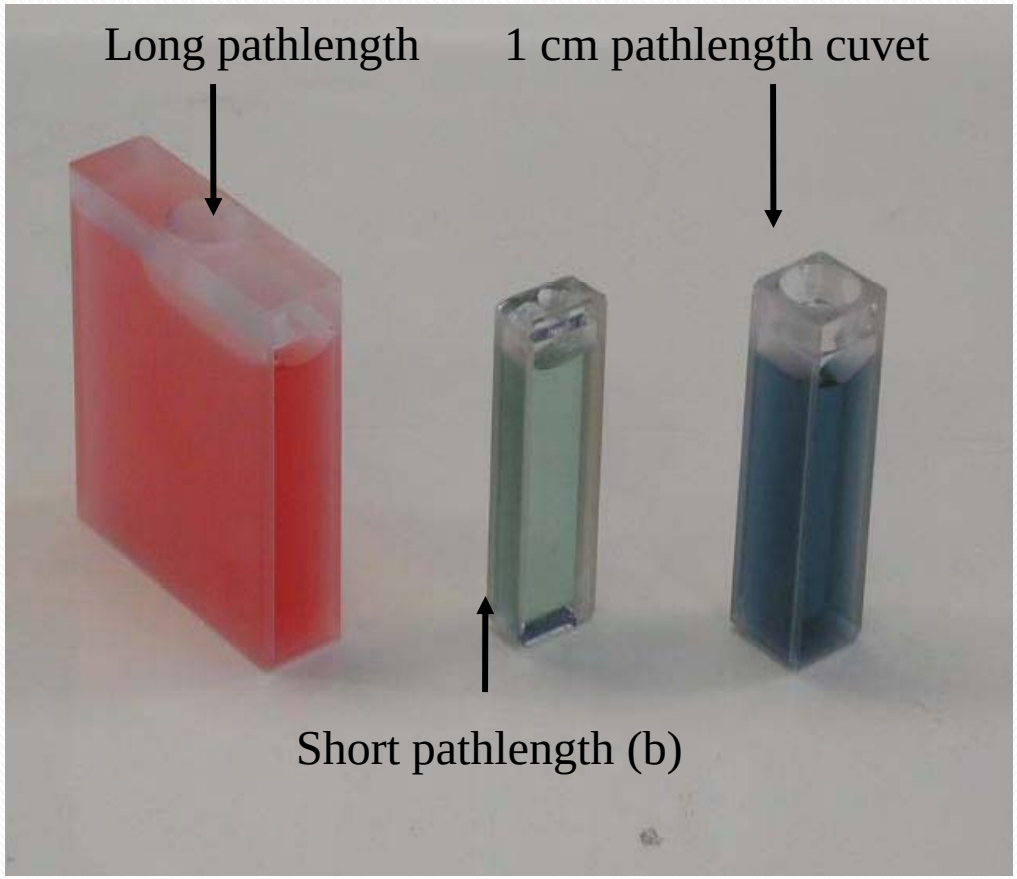
Constructed of a quartz bulb filled with deuterium and a pair of electrodes.

H2 or D2 lamp

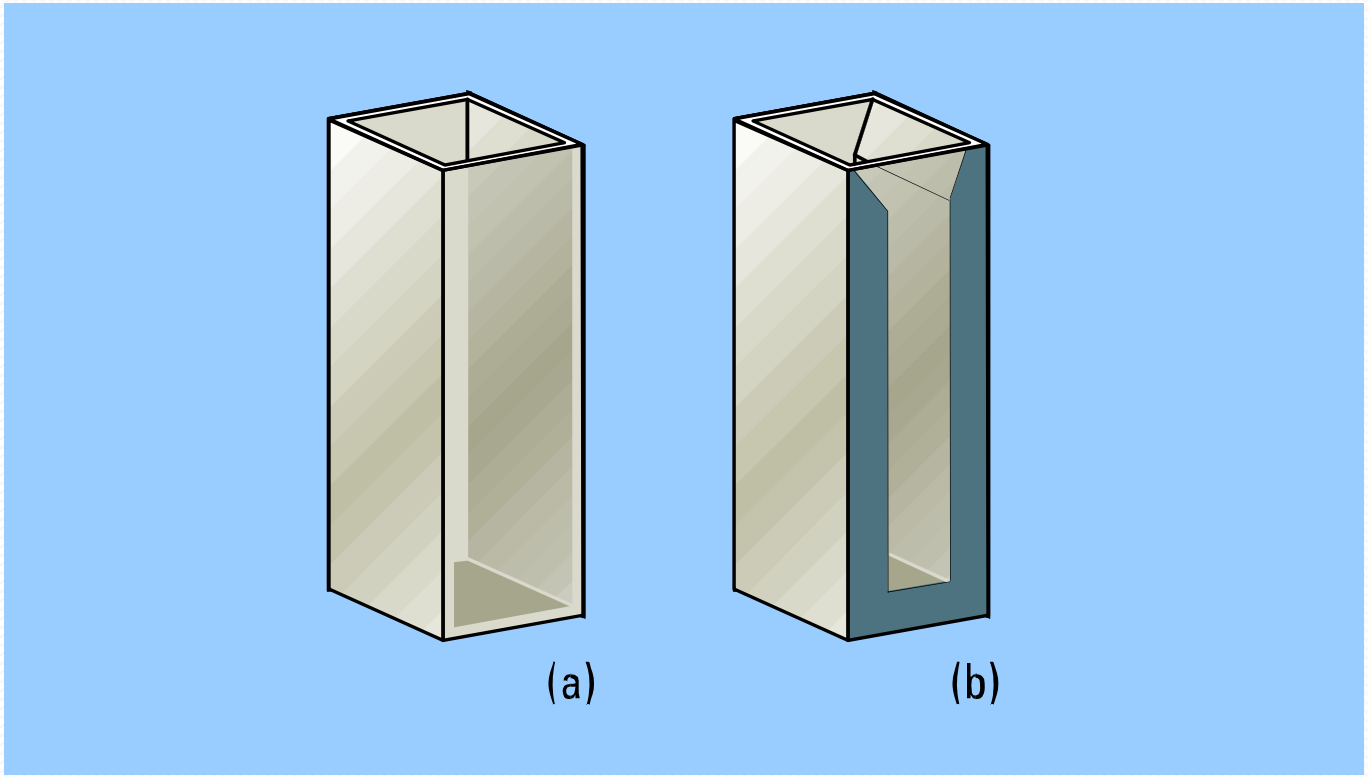
Overview of carry-Varian spectrophotometer

Sample containers (cuvettes)

- **For Visible and UV spectroscopy, a liquid sample is usually contained in a cell called a cuvette.**
- **Glass is suitable for visible but not for UV spectroscopy because it absorbs UV radiation. Quartz can be used in UV as well as in visible spectroscopy**

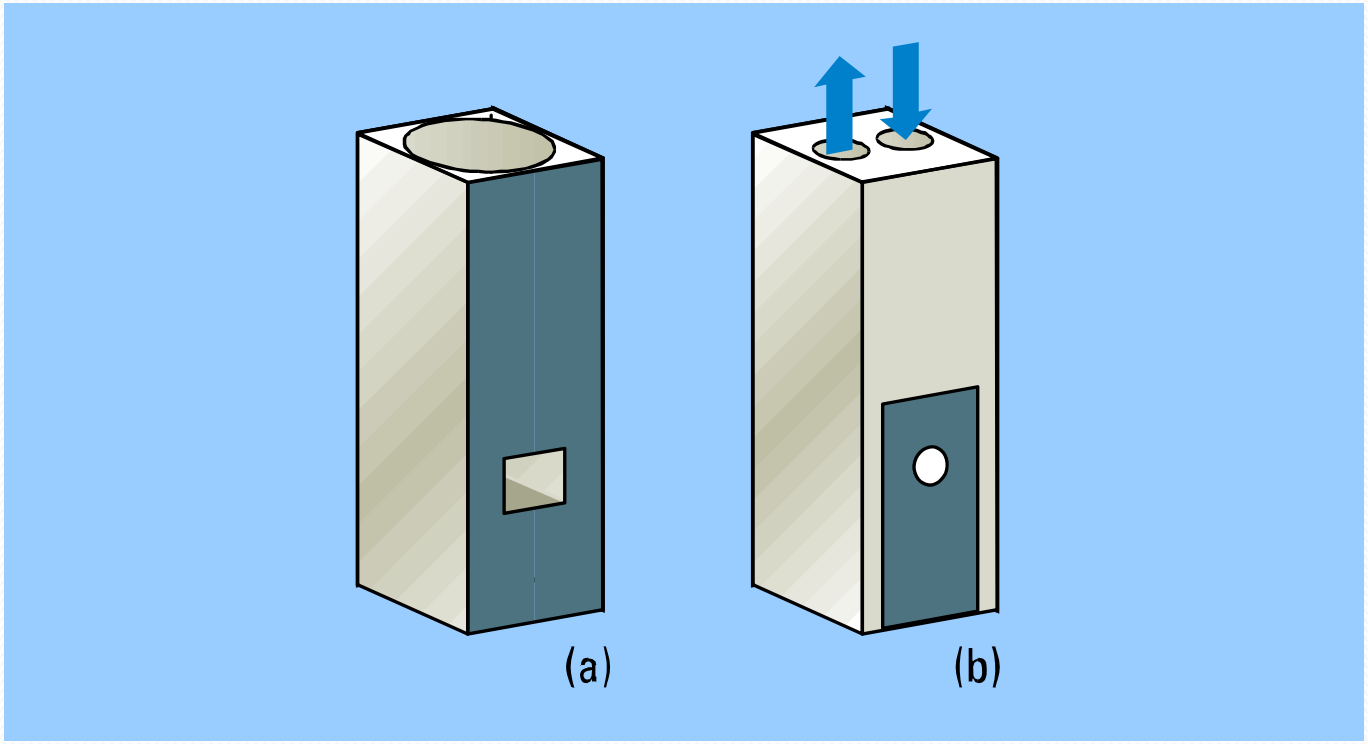


Cell Types I



Open-topped rectangular standard cell (a)
and apertured cell (b) for limited sample volume

Cell Types II

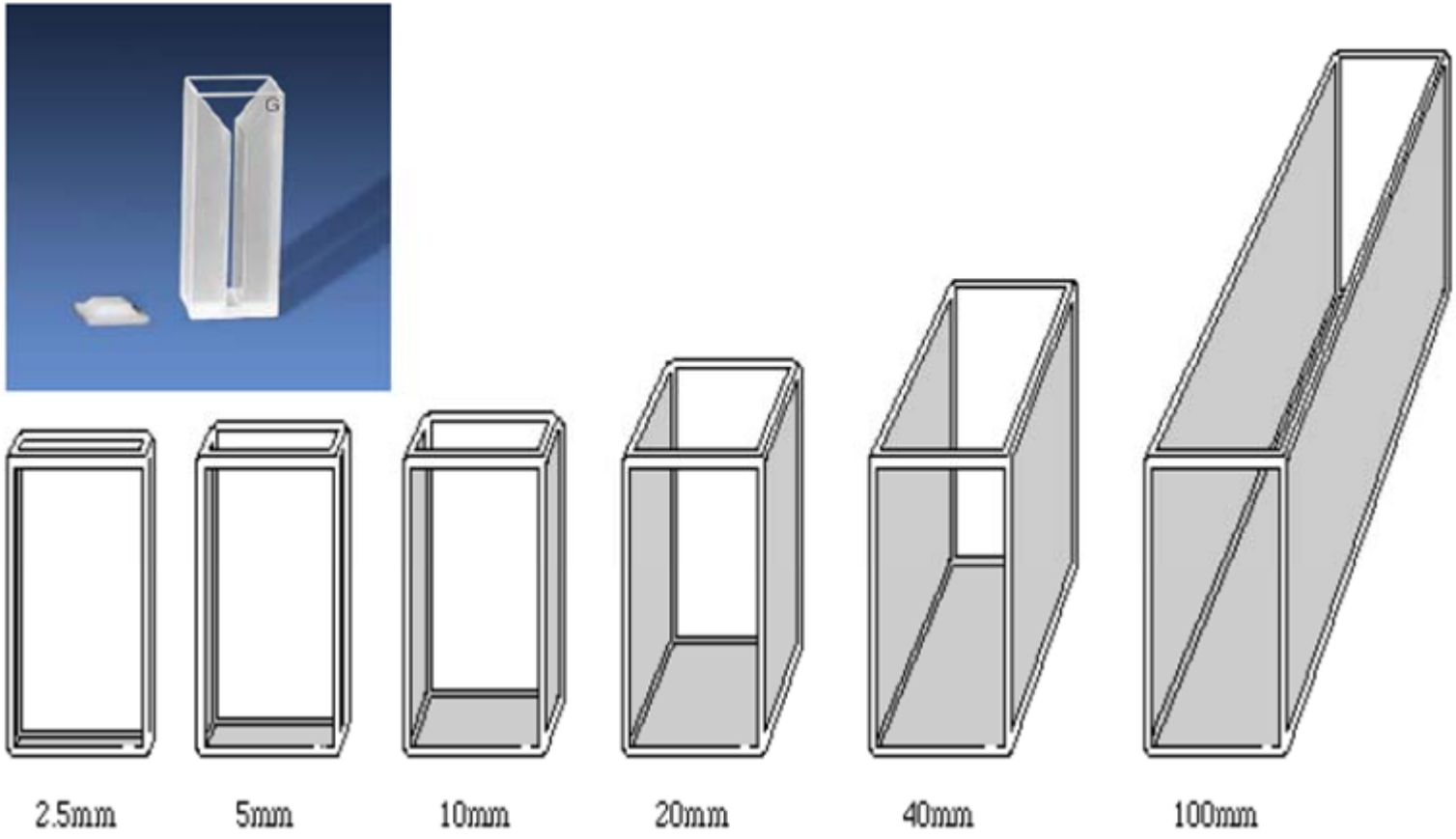


Micro cell (a) for very small volumes and flow-through cell (b) for automated applications


G

Silica glasses

- Much cheaper.



Disposablle Cuvettes & Caps

- Plastic (disposable) cuvettes can also be used in the visible region and transmit light from 350-900 nm
- **You must make sure the solvent won't dissolve the cuvette!**

Nanodrop spectrophotometer

Ultraviolet spectra of proteins and nucleic acids.

Why do proteins and DNA absorb light?

Chromophores

Table 4.1 Summary of the main **Chromophore/Fluorophore residues** in proteins and nucleic acids with absorption and fluorescence characteristics given A_{max} is wavelength of maximum absorbance, ε_{max} is maximum extinction coefficient value, I_{max} is wavelength of maximum fluorescence intensity, and τ_R is radiative lifetime.

Fluorophore	Conditions	A_{max}/nm	$10^{-3} \times \varepsilon_{max}$/ M^{-1} cm^{-1}	I_{max}/nm	ϕ_F	τ_R/nsec
Tryptophan	aqueous, pH7	280	5.7	348	0.2	2.6
Tyrosine	aqueous, pH7	274	1.3	303	0.14	3.6
Phenylalanine	aqueous, pH7	257	0.2	282	0.04	6.4
Adenine	aqueous, pH7	260	13.4	321	2.6×10^{-4}	<0.02
Guanine	aqueous, pH7	275	8.1	329	3.0×10^{-4}	<0.02
Cytosine	aqueous, pH7	267	6.1	313	0.8×10^{-4}	<0.02
Uracil	aqueous, pH7	260	9.5	308	0.4×10^{-4}	<0.02

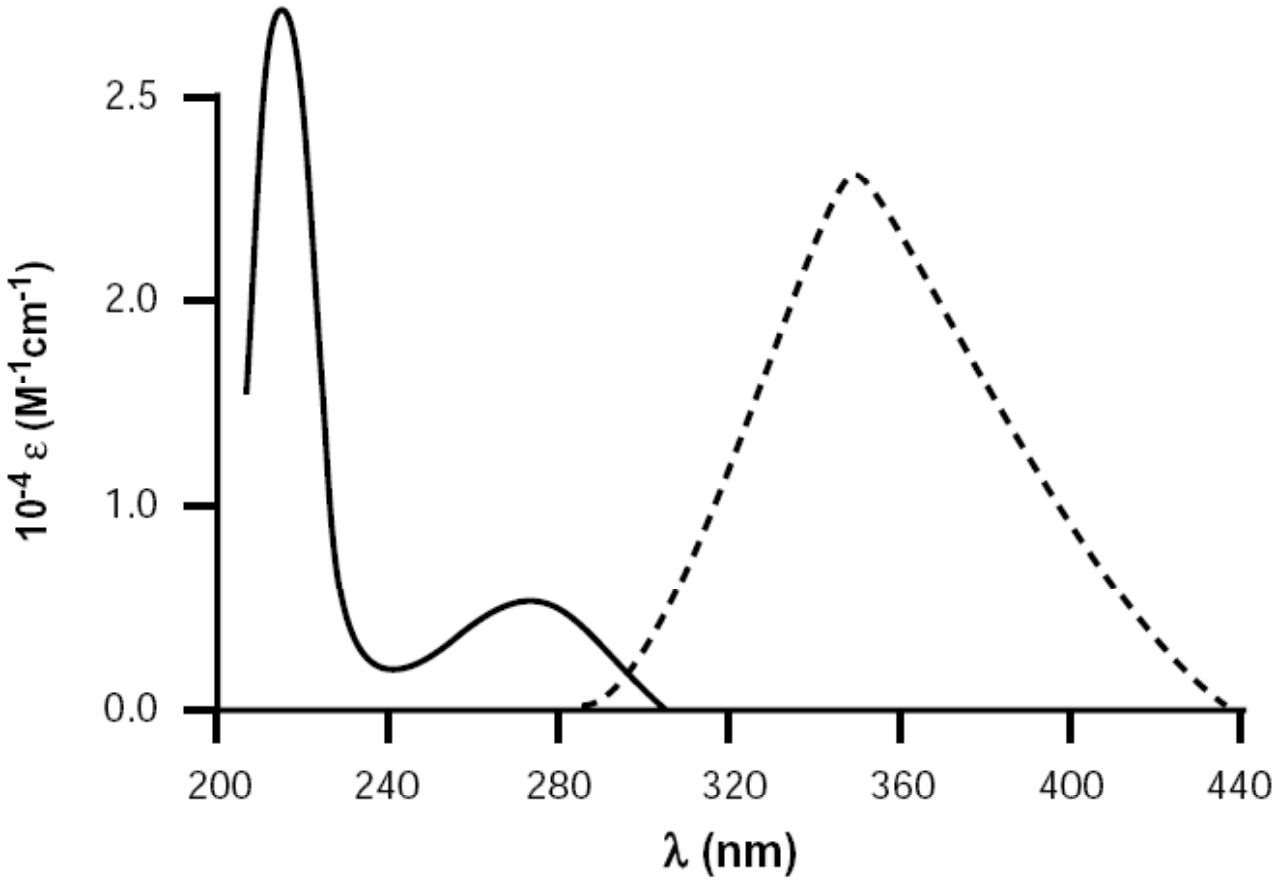


Figure 3-12. Absorption and fluorescence spectrum of tryptophan. The solid line is the extinction coefficient, and the dashed line is the fluorescence emission in arbitrary units with excitation at approximately 275 nm. D. Freifelder, *Physical Biochemistry,* 2nd edition, W. H. Freeman, New York, 1982, p. 539. © 1976, 1982 by W. H. Freeman and Company. Used with permission.

Importance and Determination of λmax

Alkaline Phosphatase Assay Kit (Colorimetric)

The kit uses *p-nitrophenyl phosphate (pNPP) as a phosphatase* substrate which turns yellow (**λmax= 405 nm**) when dephosphorylated by ALP.

Some Natural Chromophores

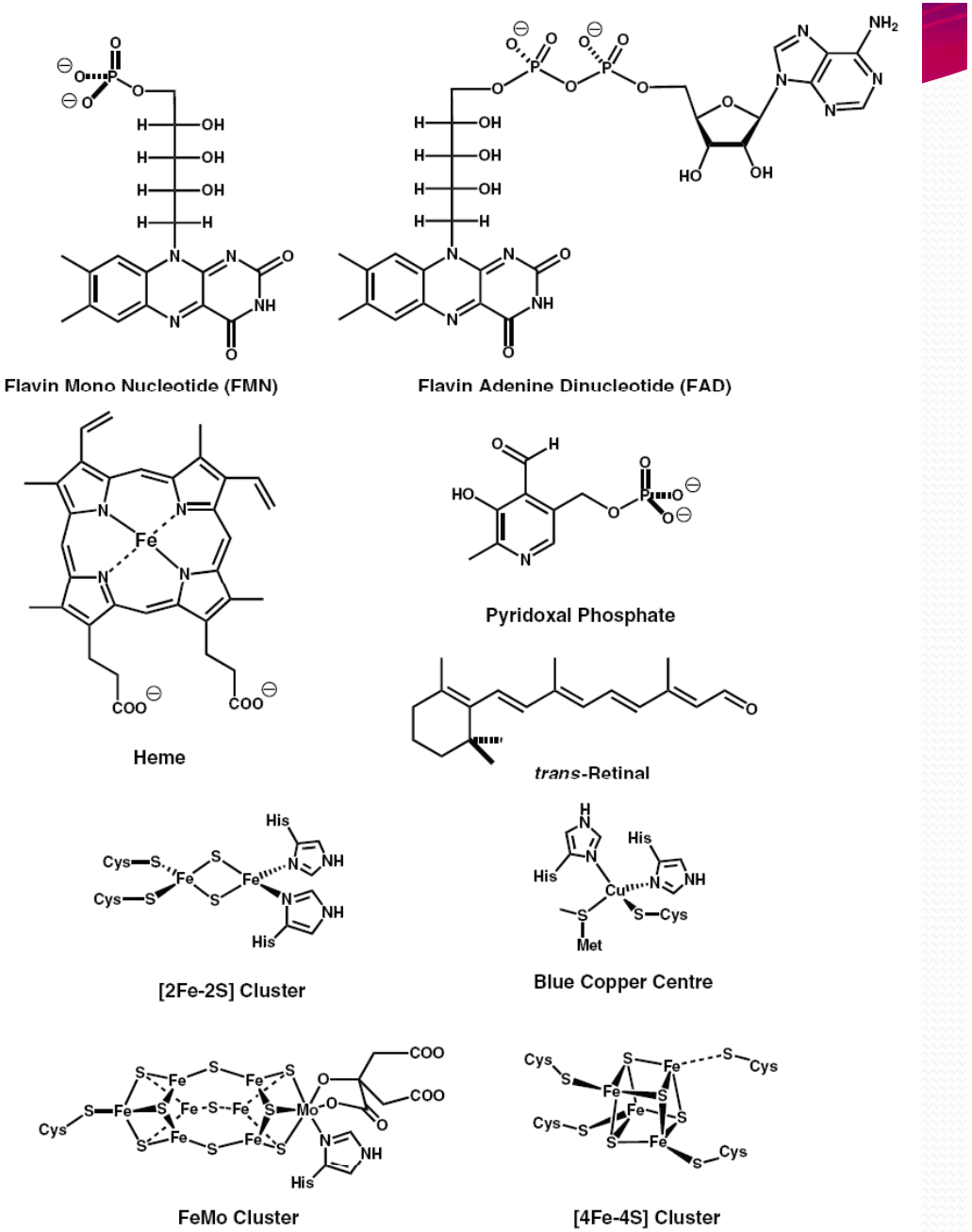


Figure 4.2 Structures of main **prosthetic groups** that contribute significantly to the UV-visible spectroscopy of proteins. Prosthetic groups are non amino acid-based moieties that are covalently attached to the proteins concerned and play an integral part of the structure and function of proteins to which they are covalently attached.

Absorption spectra of some molecules

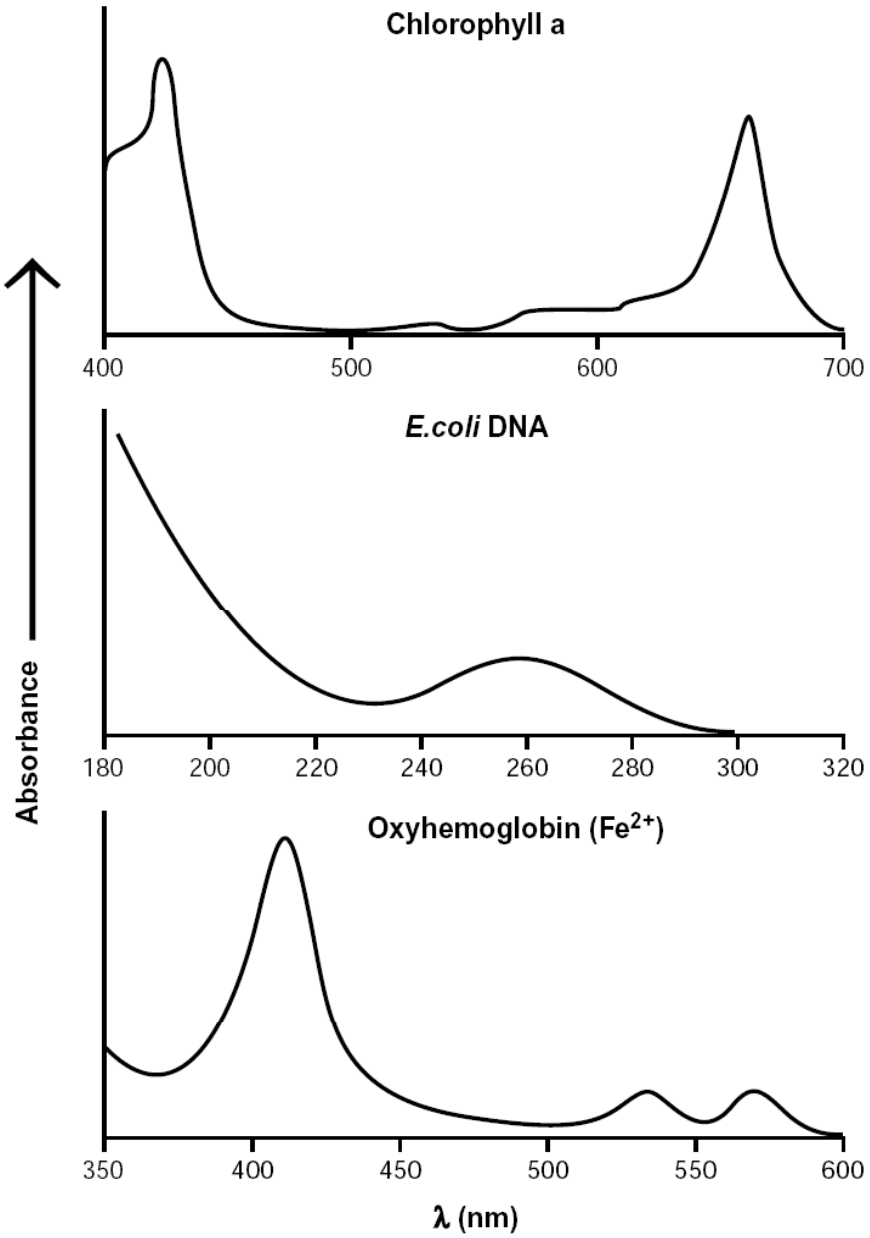
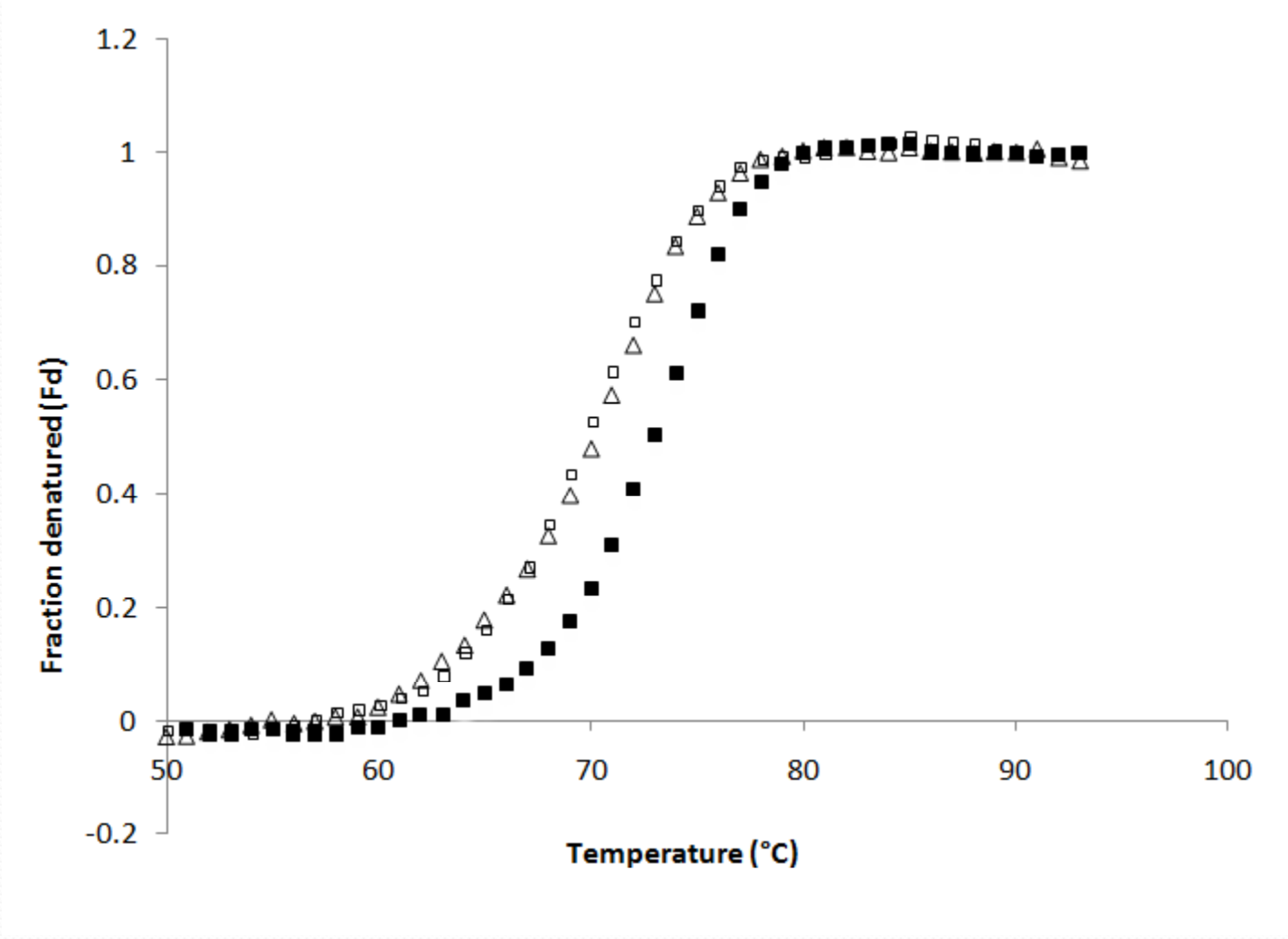


Figure 1-8. Absorption of light by biological molecules. The absorbance scale is arbitrary and the wavelength, λ, is in nanometers. Chlorophyll *a* solutions absorb blue and red light and are green in color. DNA solutions absorb light in the ultraviolet and are colorless. Oxyhemoglobin solutions absorb blue light and are red in color.

Spectra of thermal denaturation of protein



Imani et al. submitted to *Cell Biochemistry & Function*

Spectra of Melting DNA

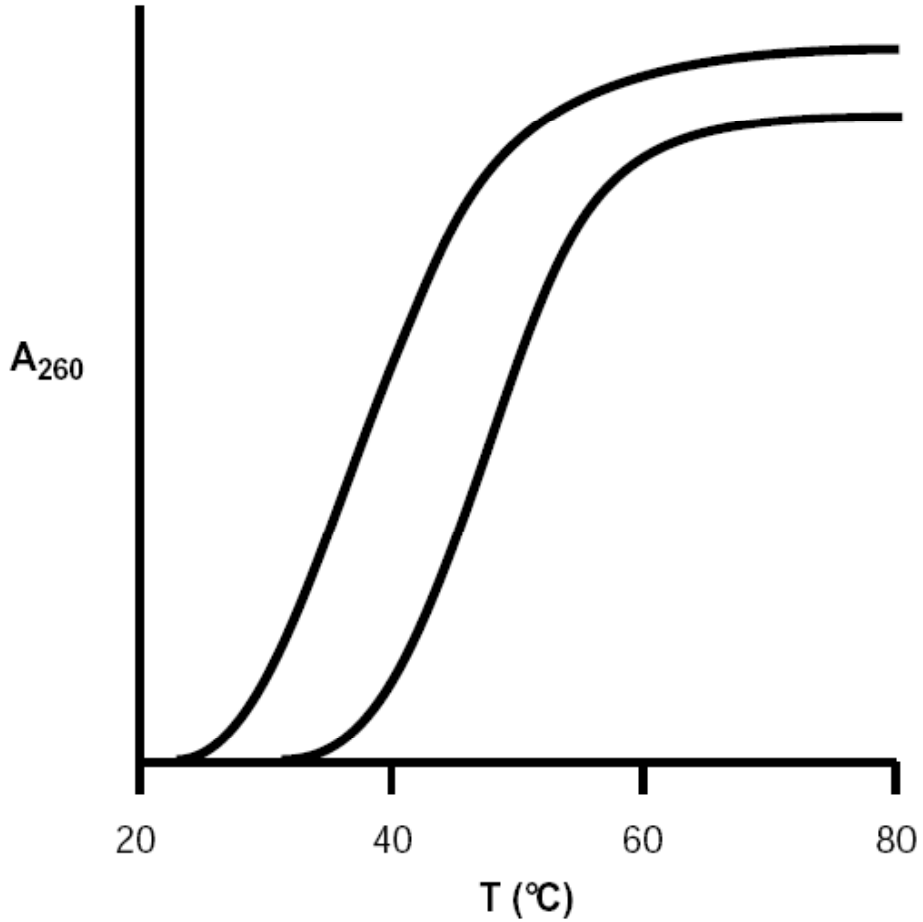


Figure 3-6. Schematic representation of DNA melting for two different DNA. The absorbance at 260 nm increases as the temperature is raised and the double-stranded structure breaks down. The temperature at which the melting occurs increases as the amount of G-C base pairs increases.

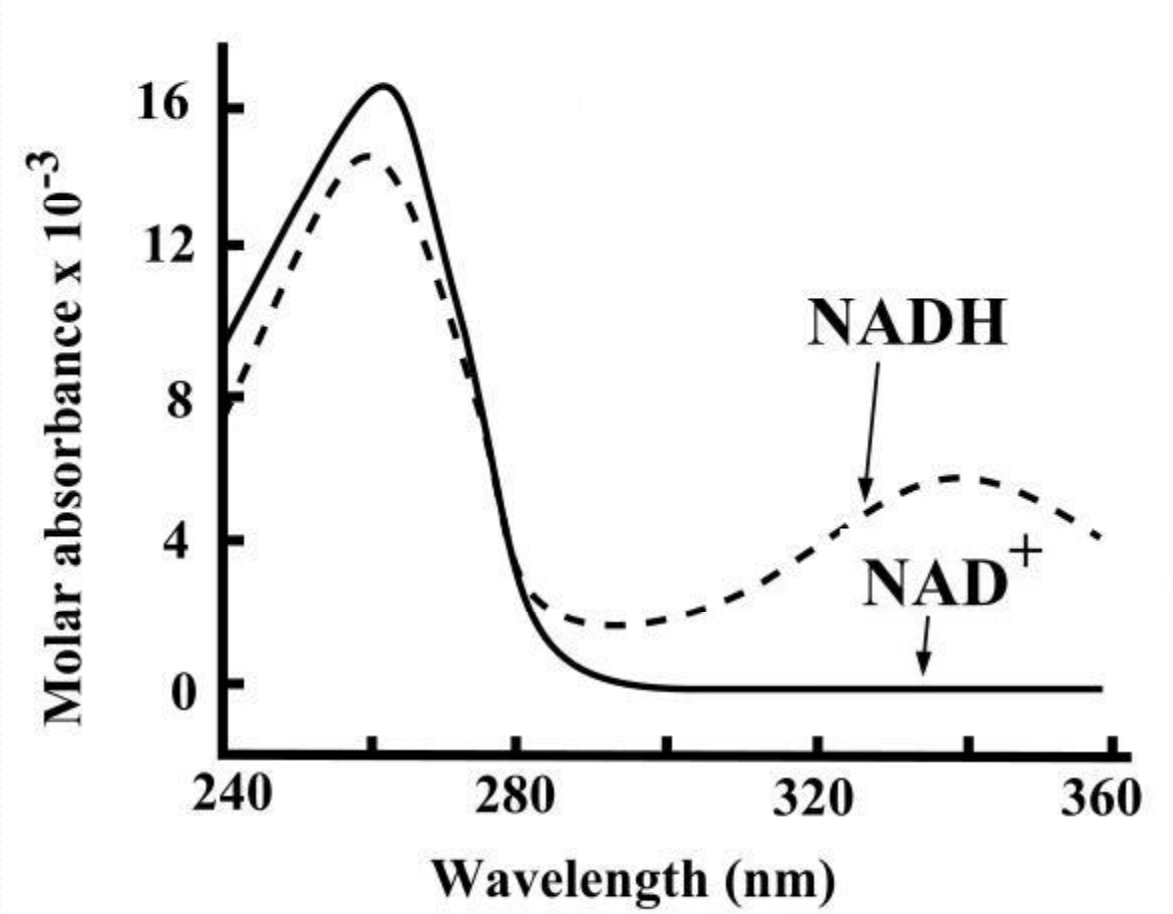
Absorbance of various buffer components

Compound	No absorbance above	Absorbance of a 0.01 M solution in a 0.1-cm cell			
		210 nm	200 nm	190 nm	180 nm
$NaClO_4$	170 nm	0	0	0	0
NaF, KF	170 nm	0	0	0	0
Boric acid	180 nm	0	0	0	0
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
Sodium 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

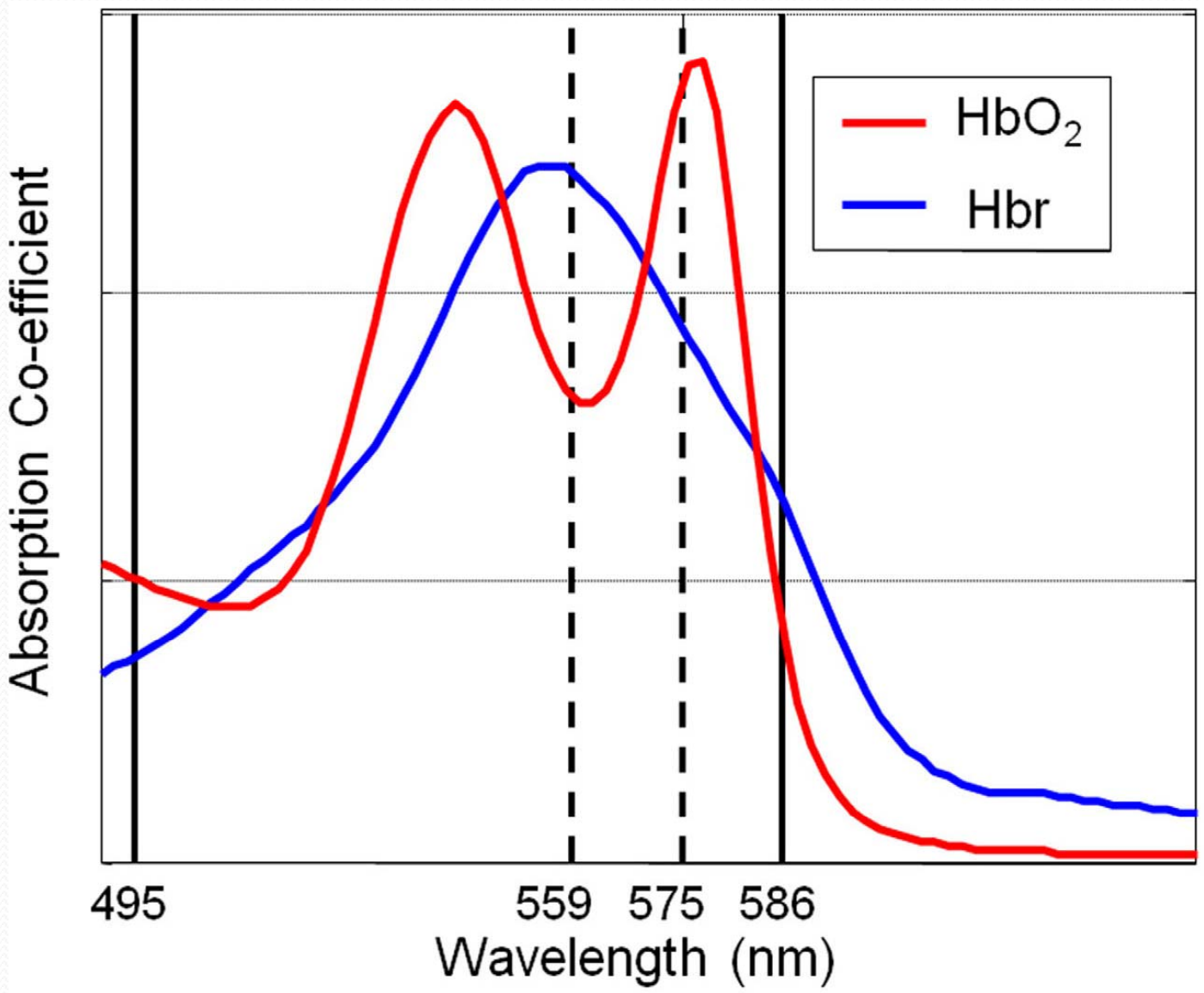
Absorbtion And Emission Of Some other Chromophores

Absorbtion Spectra of NAD+ and NADH

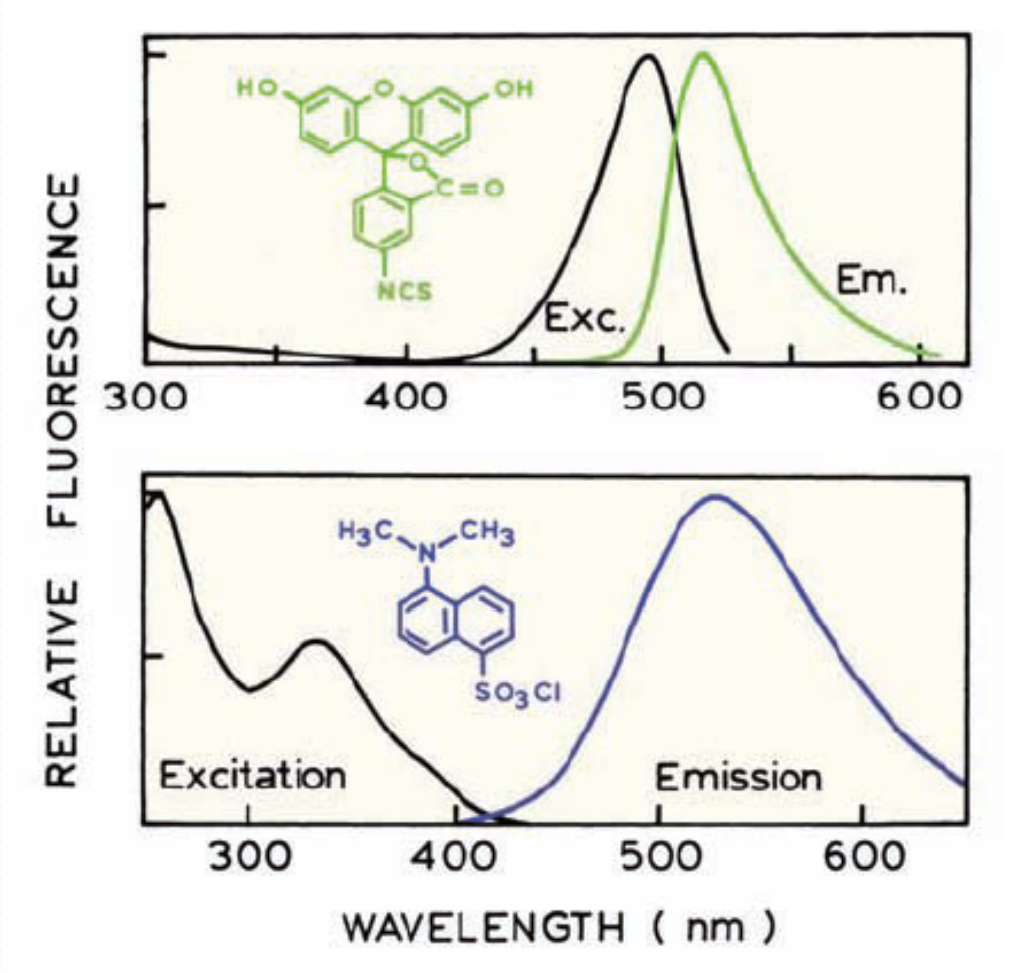
λmax of NADH: 340 nm



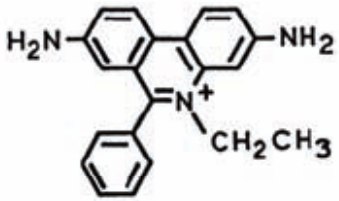
Absorbtion of OxyHB and Deoxy HB



Excitation and emission spectra of FITC and DNS-Cl labeled antibodies.

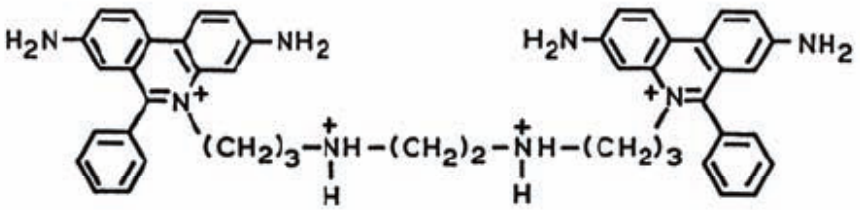


DNA Probes



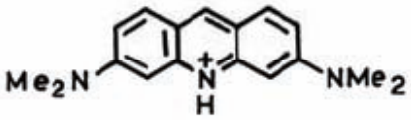
Ethidium Bromide

518/605 nm



Ethidium Homodimer

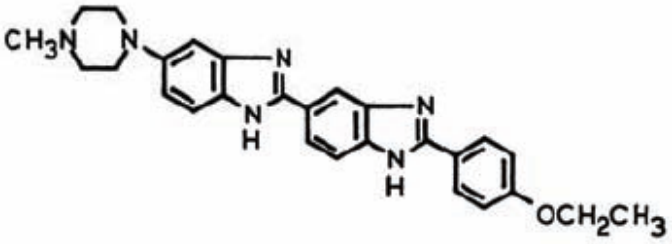
528/617 nm



Acridine Orange

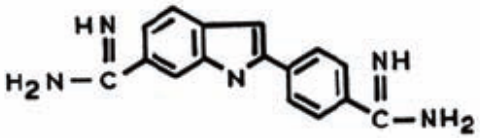
500/526 nm DNA

460/650 nm RNA



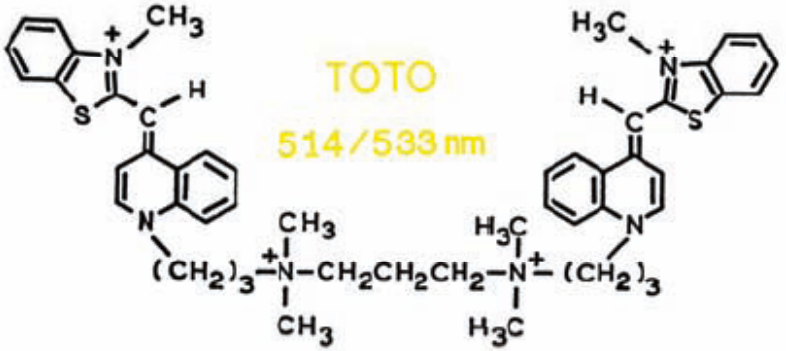
Hoechst 33342

350/460 nm



DAPI

355/461 nm



TOTO

514/533 nm

Figure 3.23. Representative DNA probes. Excitation and emission wavelengths refer to DNA-bound dye.

Some Non-natural Chromophores

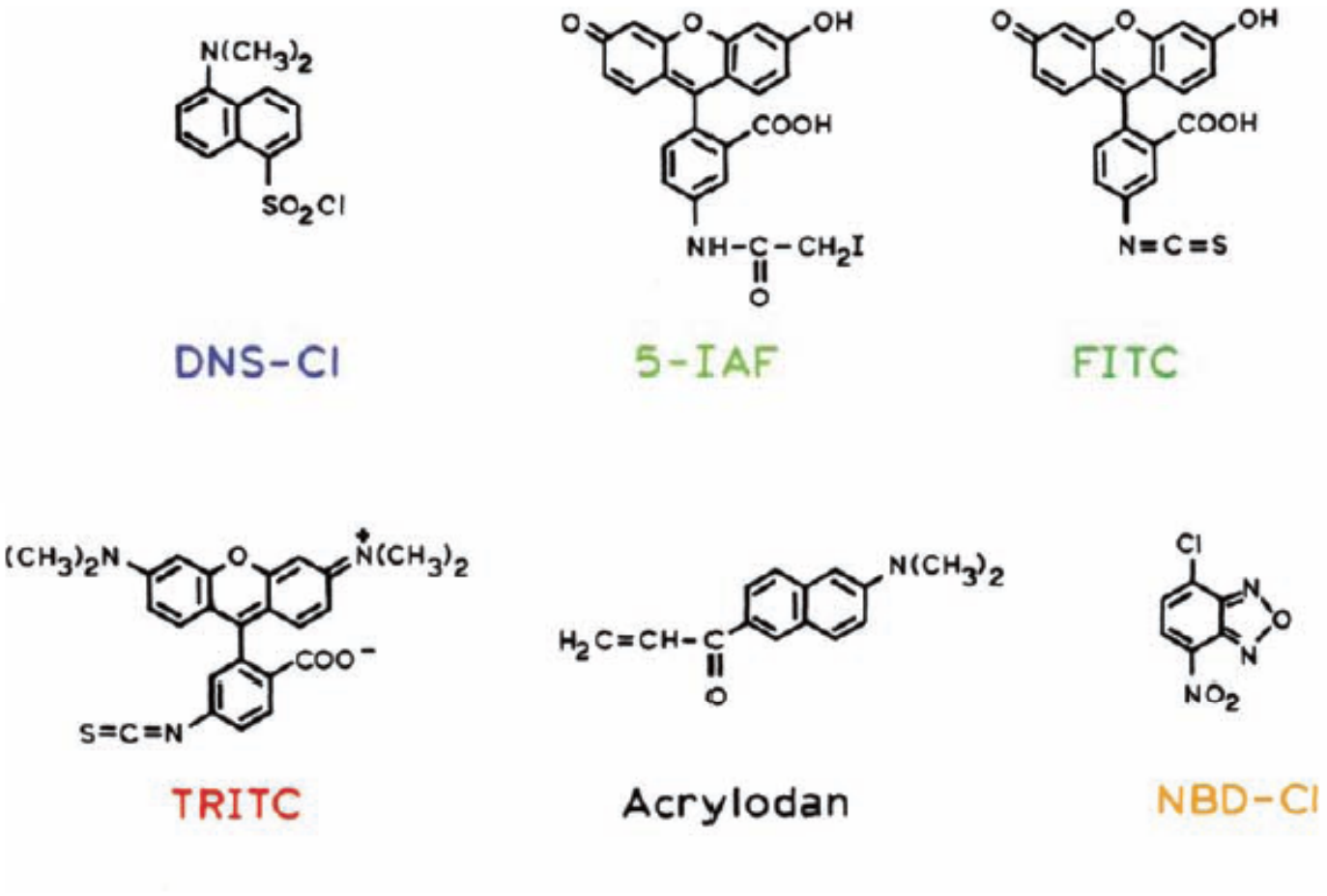
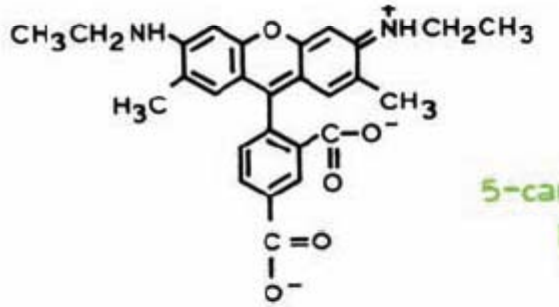
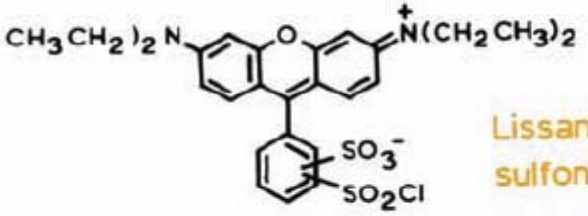


Figure 3.8. Reactive probes for conjugation with macromolecules.

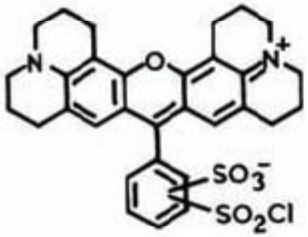
Cont.



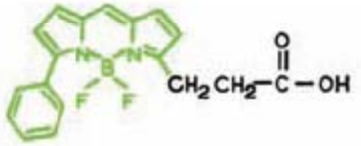
5-carboxyrhodamine 6G hydrochloride



Lissamine rhodamine B sulfonyl chloride

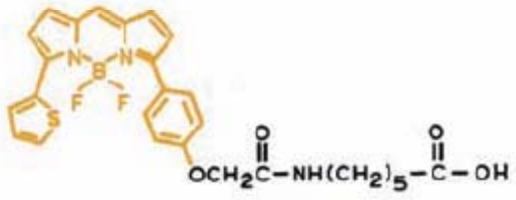


Texas Red sulfonyl chloride



BODIPY-R6G

BODIPY- 581/591



BODIPY- Texas Red

GFP (from *Aequorea victoria*)

a ?? kDa protein

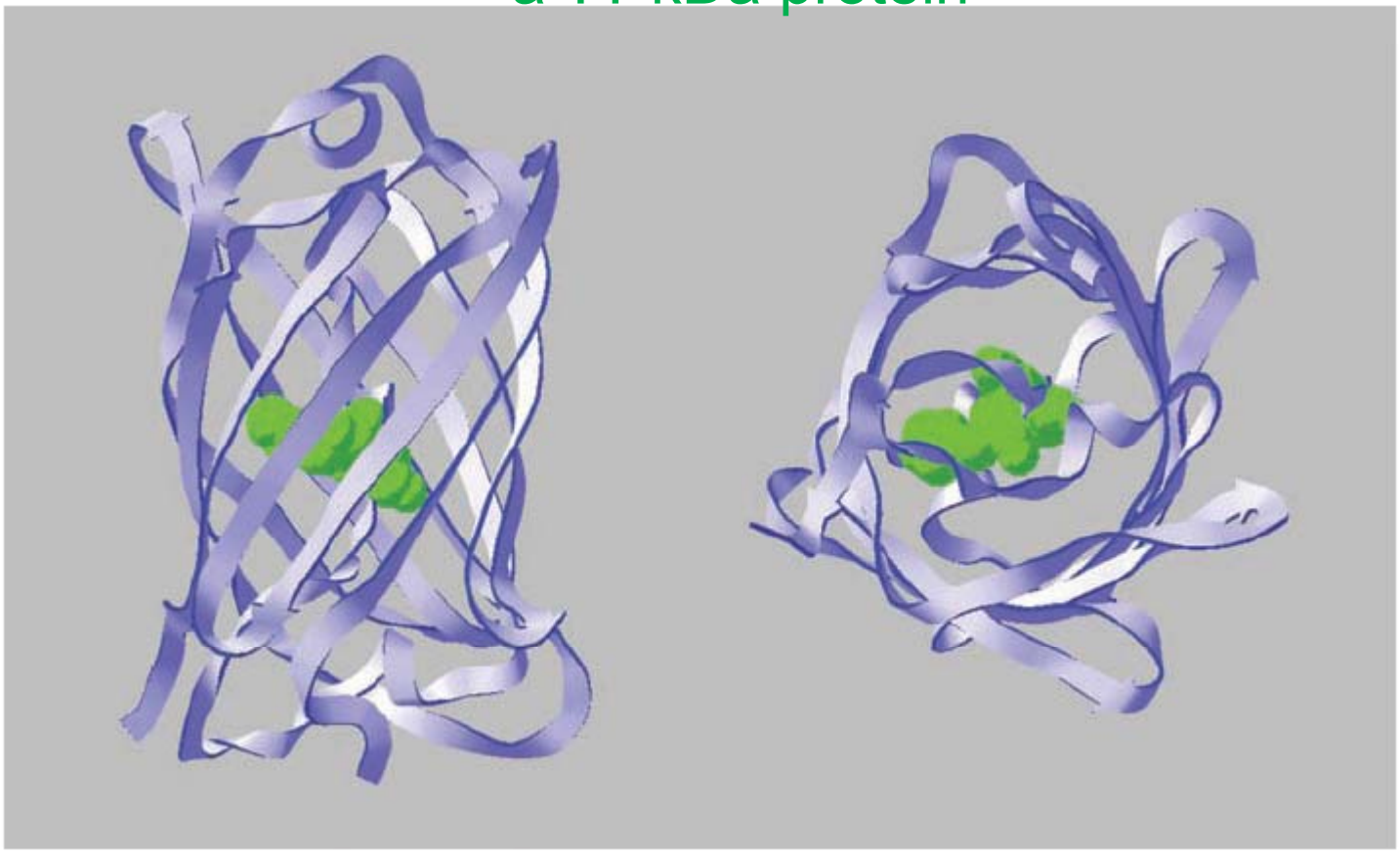


Figure 3.33. β-Barrel structure of GFP. Side and top view. The chromophore is linked covalently to the protein.

Excitation (Top) and Emission (bottom) wavelength of GFP mutants

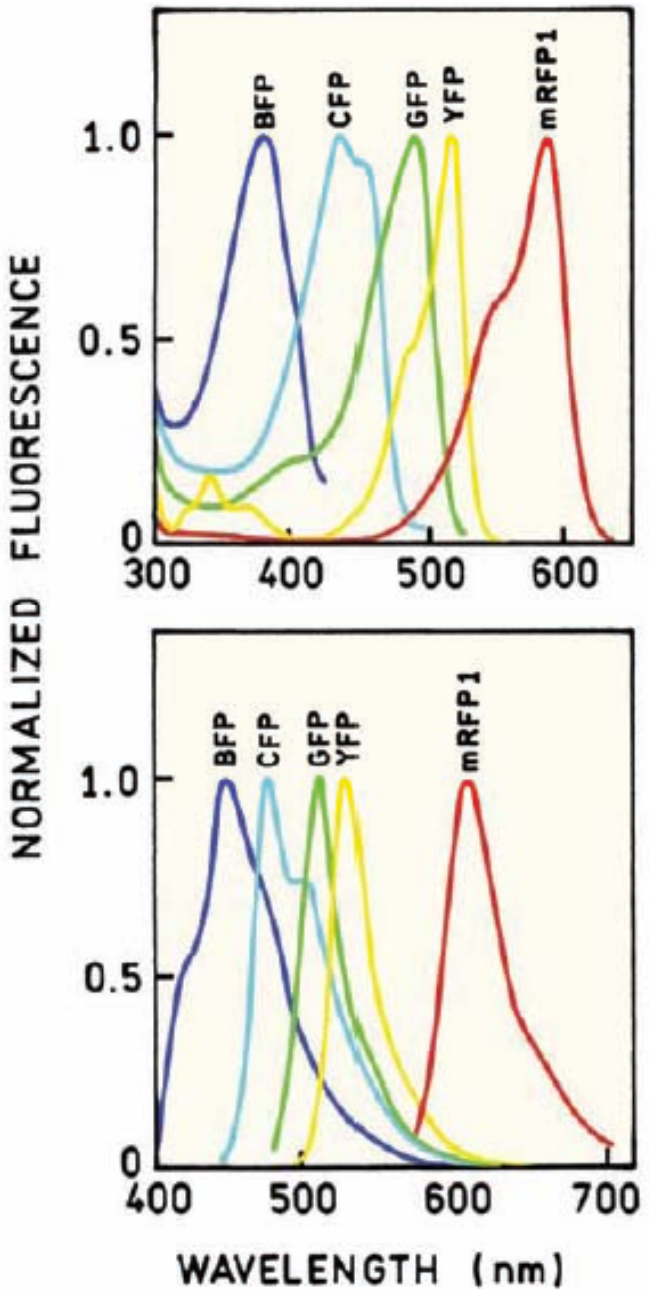
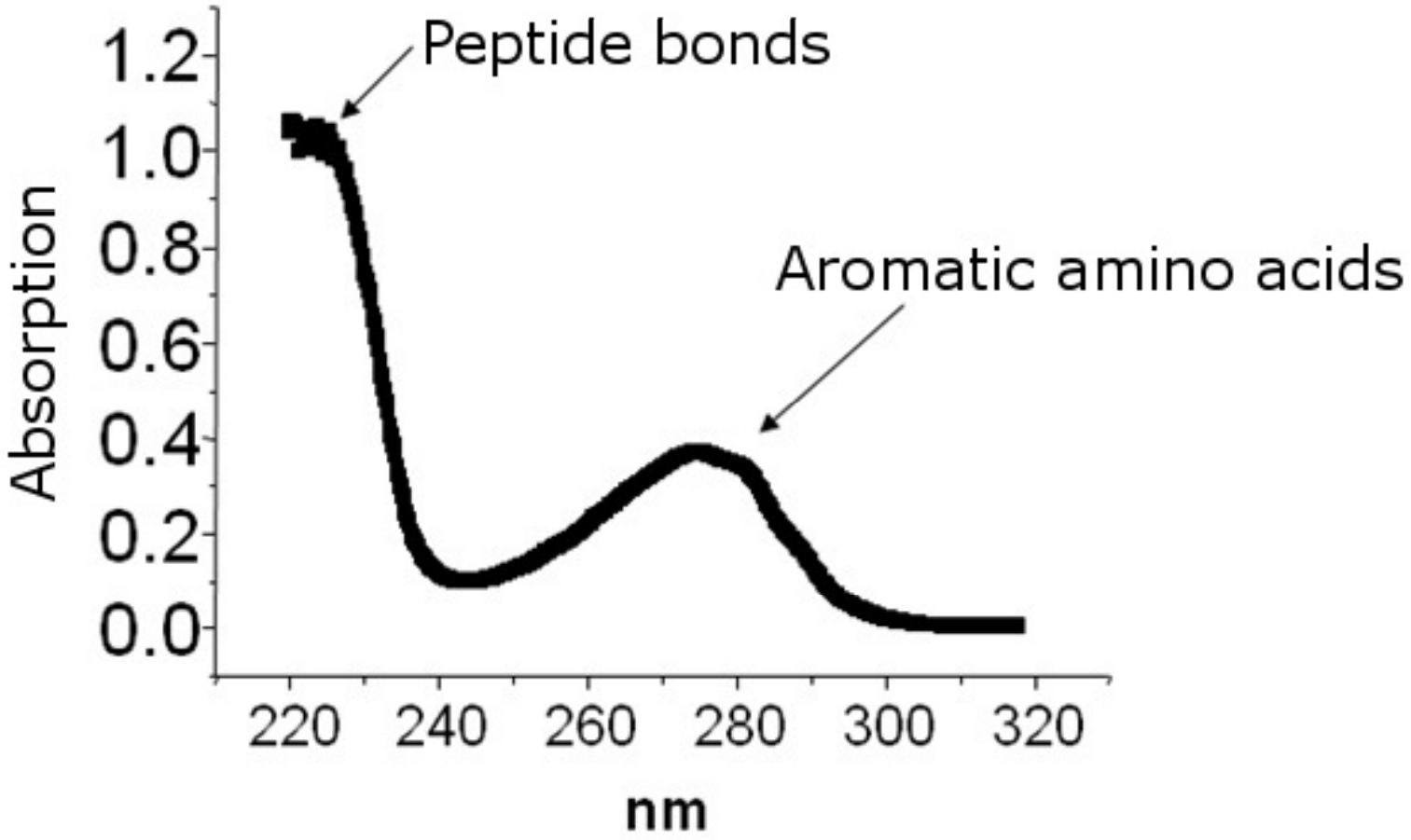


Figure 3.35. Excitation (top) and emission spectra (bottom) of Gl mutants. Revised from [129].

Quantification of proteins and Nucleic acids

Why do we need to determine protein concentration?

Absorbtion spectrum of protein



Working with Proteins

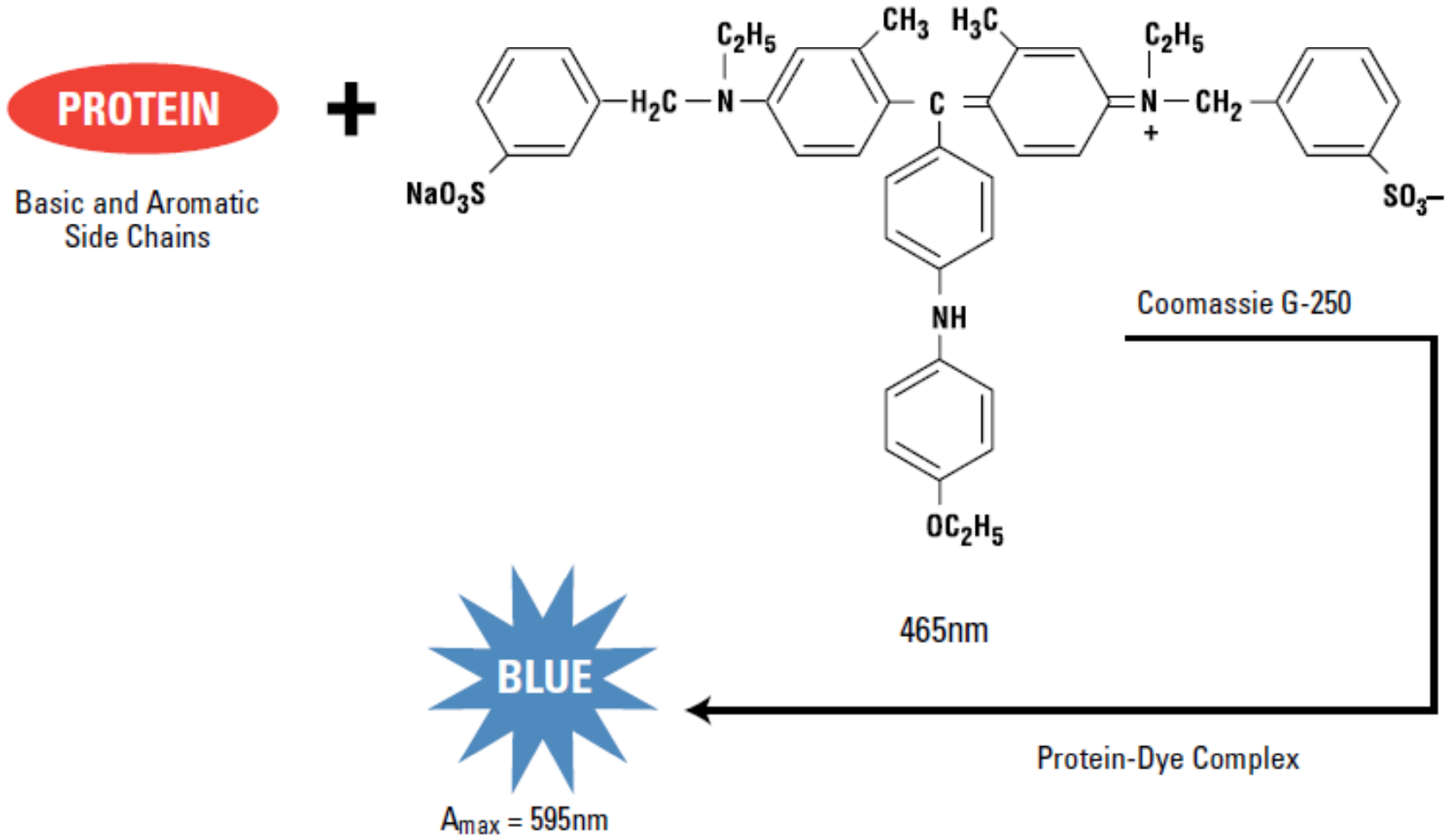
1. Cell lysates for immunobloting.
2. Protein excretion in urine.
3. Serume protein
4. Enzyme specific activity of any organism (bacteria, plants, and mammals).
5. Protein structural stability in various condition.

Different methods of protein assay

- Copper-based Protein Assay Chemistries
  - ✓Peptides and the Biuret Reaction
  - ✓Bicinchoninic Acid (BCA) Protein Assays
  - ✓Lowry Protein Assays
  - ✓Biuret protein assay
- Dye-based Protein Assay Chemistries
  - ✓Coomassie Dye (Bradford) Protein Assays
- UV absorbtion assay

Pierce Protein Assay Product	Detection (Amax)	Compatibilities (Note 1)
BCA	562nm	Yes: Detergents No: Reducing agents; Chelators
BCA - Reducing Agent Compatible	562nm	Yes: Detergents; Reducing agents No: Chelators
Micro BCA	562nm	Yes: Detergents No: Reducing agents; Chelators
660 nm	660nm	Yes: Detergents; Reducing agents; SDS sample buffer (Note 2) No: Ionic detergents (unless IDCR is used, Note 2)
Coomassie Plus	595nm	Yes: Most reducing agents; Chelators No: Detergents
Coomassie (Bradford)	595nm	Yes: Most reducing agents; Chelators No: Detergents
Modified Lowry	750nm	Yes: SDS No: Most detergents; Reducing agents; Chelators

Chemistry of Bradford assay



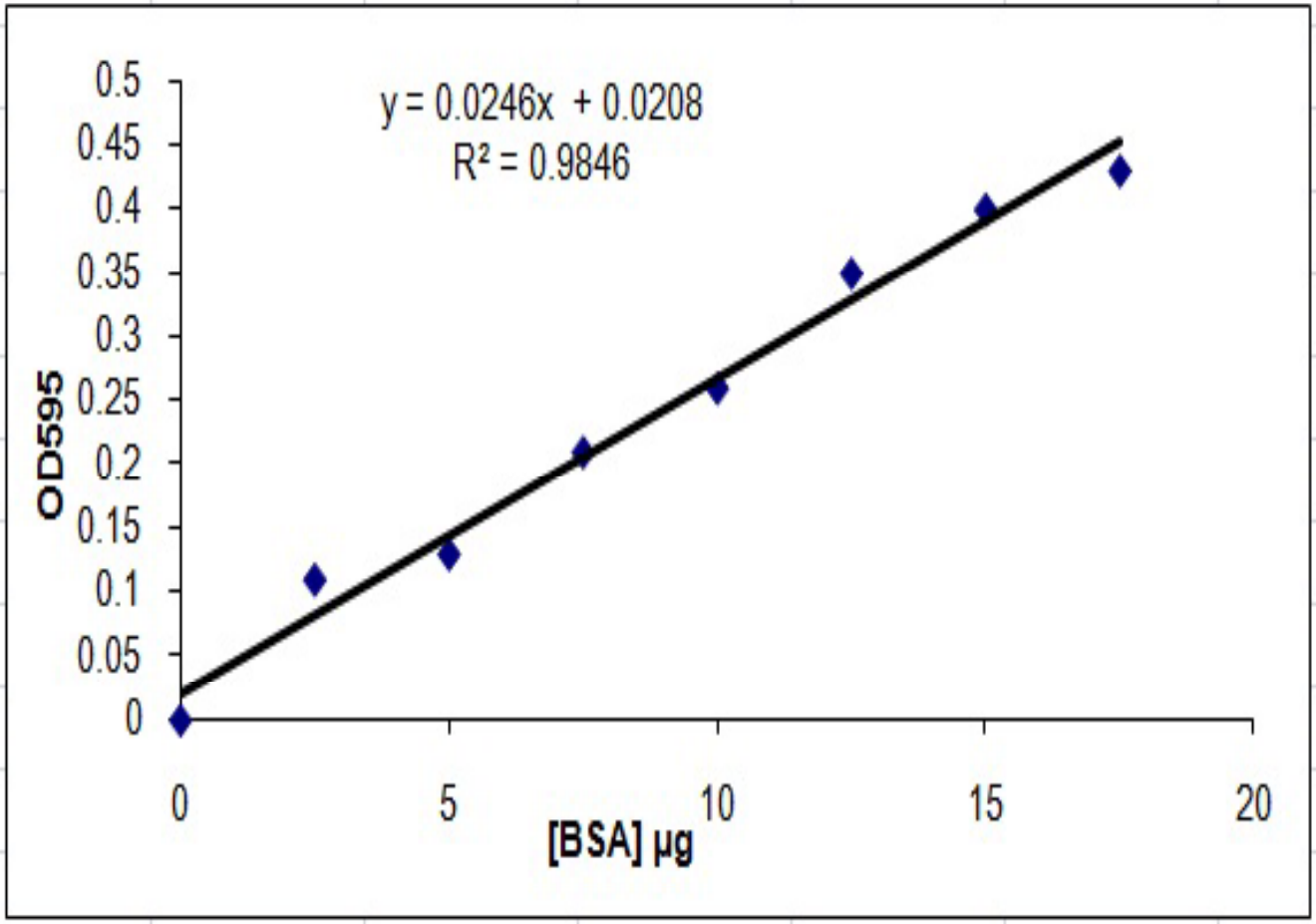
Protein standard curve

- **Standards** (solutions of known concentration) of the compound of interest are made, treated, and their absorbances (ABS) and concentration values are used to create a **Standardization Graph**.

BSA standard curve

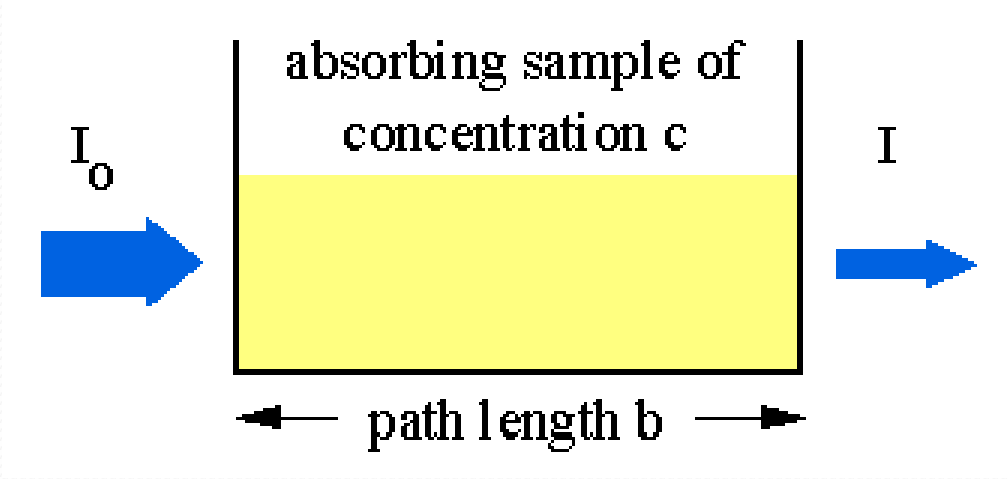
Protein concentration standard curve

BSA (ug)	OD595
0	0
2.5	0.11
5	0.13
7.5	0.21
10	0.26
12.5	0.35
15	0.4
17.5	0.43



Absorbance at 280 nm

Beer-Lambert law (or Beer's law)



Absorbance at 280 nm (cont.)

- OD280 = 1 equals 1mg/ml
- Using absorbtion coefficient or molar extinction coefficient *(for pure proteins)*

absorbtion coefficient or molar extinction coefficient

Definition: absorbtion of certain amount of protein at a distinct
wavelength in a 1 cm cuvette.

For example: A^1% of acetyl cholin esterase is 22.9 and Em of it is 5.27x10^5

چند سوال درباره محاسبه غلظت پروتئین

1) 0.2 ml از 10 ml محلول پروتئینی را در حجم کلی ۱ سی سی رقیق می کنیم. جذب 280 nm آن معادل 0.75 می باشد. غلظت محلول پروتئینی را محاسبه کنید.

2) ضریب جذب (A^1%) استیل کولین استراز در 0.1 M NaCl و فسفات سدیم با pH 7 در طول موج 280 nm در کوتی به طول 1 cm معادل 0.34 می باشد. غلظت این پروتئین چند درصد است.

3) ضریب خاموشی مولی (Em) استیل کولین استراز 5.27x10^5 می باشد. جذب 280 nm محلولی از آن در کوتی به طول 1 cm معادل 0.22 می باشد. غلظت مولی این پروتئین چقدر است.

رابطه بین غلظت پروتئین با مولاریته

$$mg/ml = M \times Mw \text{ of protein}$$

What about protein concentration in cells or plasma?!!!

The reference range for *albumin concentrations in serum* is approximately 35 - 50 g/L (3.5 - 5.0 g/dL).

Mw: 67 kDa

Calculate its molarity ???!!!

Measurement of DNA concentration

Why do we need DNA concentration?

1. *After genomic DNA isolation.*
2. *Molecular biology works.*
3. *DNA laddering for apoptosis*
4. *Conventional PCR*
5. *RNA isolation*
6. *RT-PCR*
7. *Structural conformation in various concentration*

Working with DNA

1. Measure DNA concentration using the **NanoDrop** program. Record the absorbance values at 260 nm and 280 nm.

2. OD260 nm **1 = 50 ng/µl dsDNA**, **or 50 µg/ml**

3. OD260 nm **1 = 40ng/µl ssDNA**, **or 40µg/ml**

4. For **RNA OD 1 = 40 µg/ml**

5. **A260/A280**. A value of **greater than 1.7** indicates essentially pure DNA, while lower values indicate phenol or protein contamination.

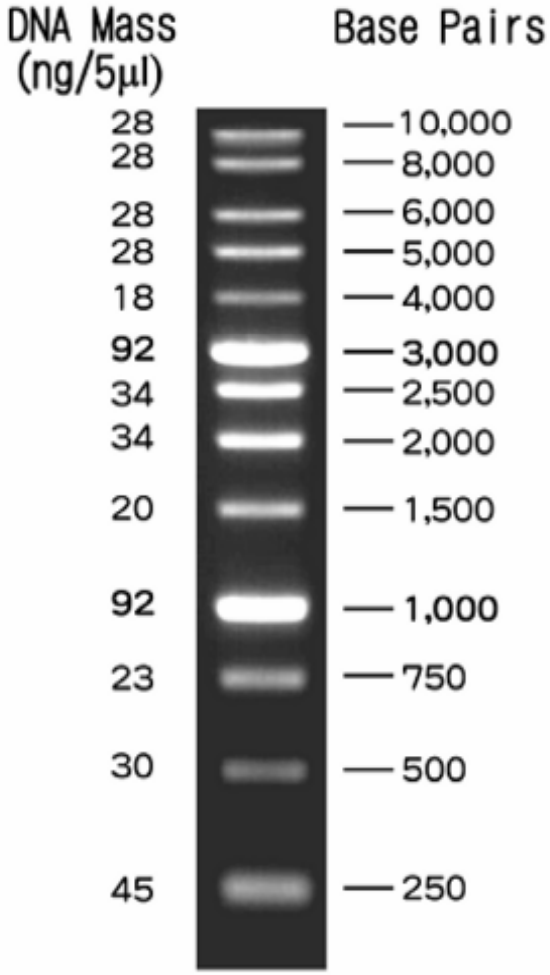
6. For **RNA OD 1 = 40 µg/ml**

رابطه بین غلظت DNA با مولاریته

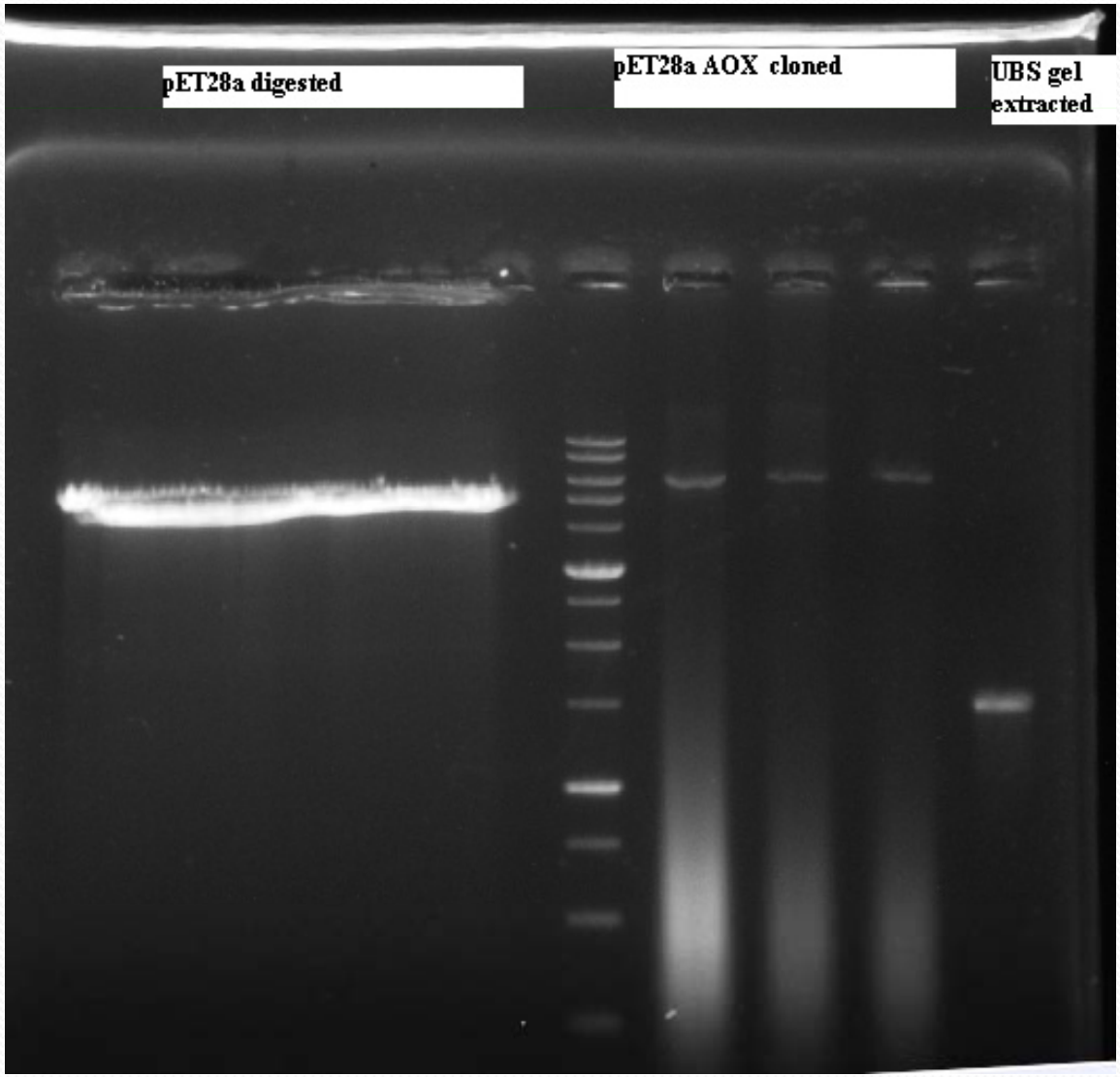
Mw of a base pair = 660 g/mole

Mw of a single base = 330 g/mole

Estimation DNA concentration (Cont.)

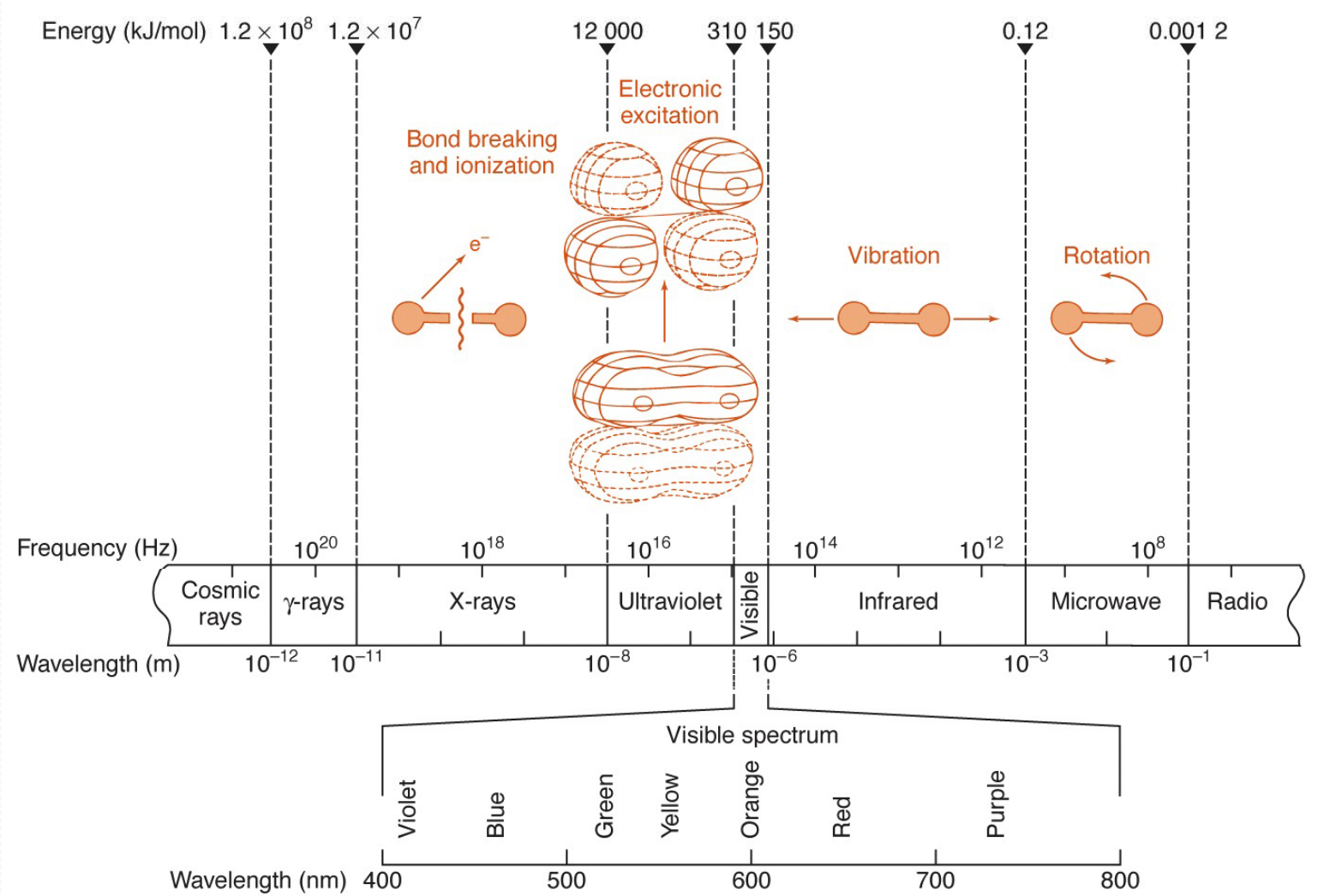


1 % TAE agarose gel



Thanks for participation

Regions of Electromagnetic Spectrum-the "colour" of light



Chemical Sensing Probes

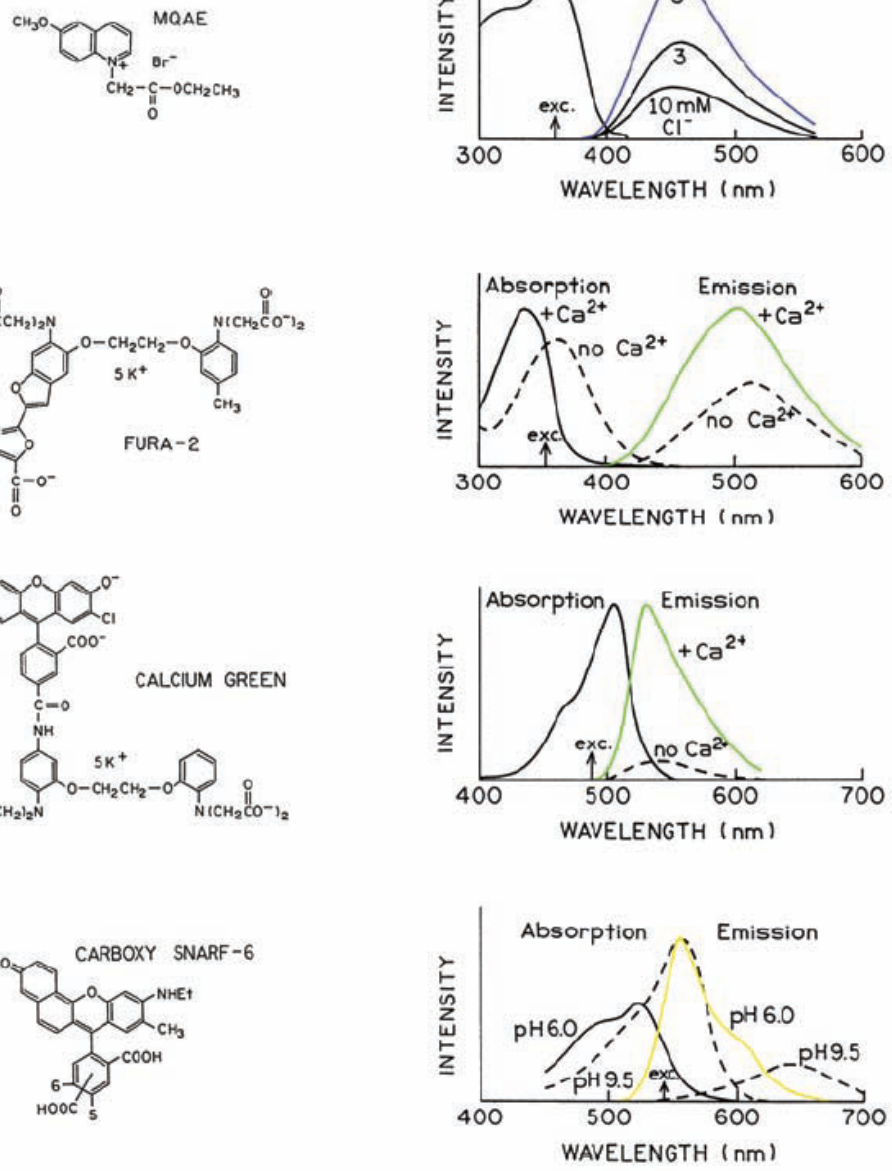


Figure 3.27. Chemical sensing probes (left) and thus spectra (right).

Fluorogenic Probes

7-Umbelliferyl phosphate (7-UmP) ELISA

galactoside of Umbelliferone or 7-hydroxy-4-methylcoumarin

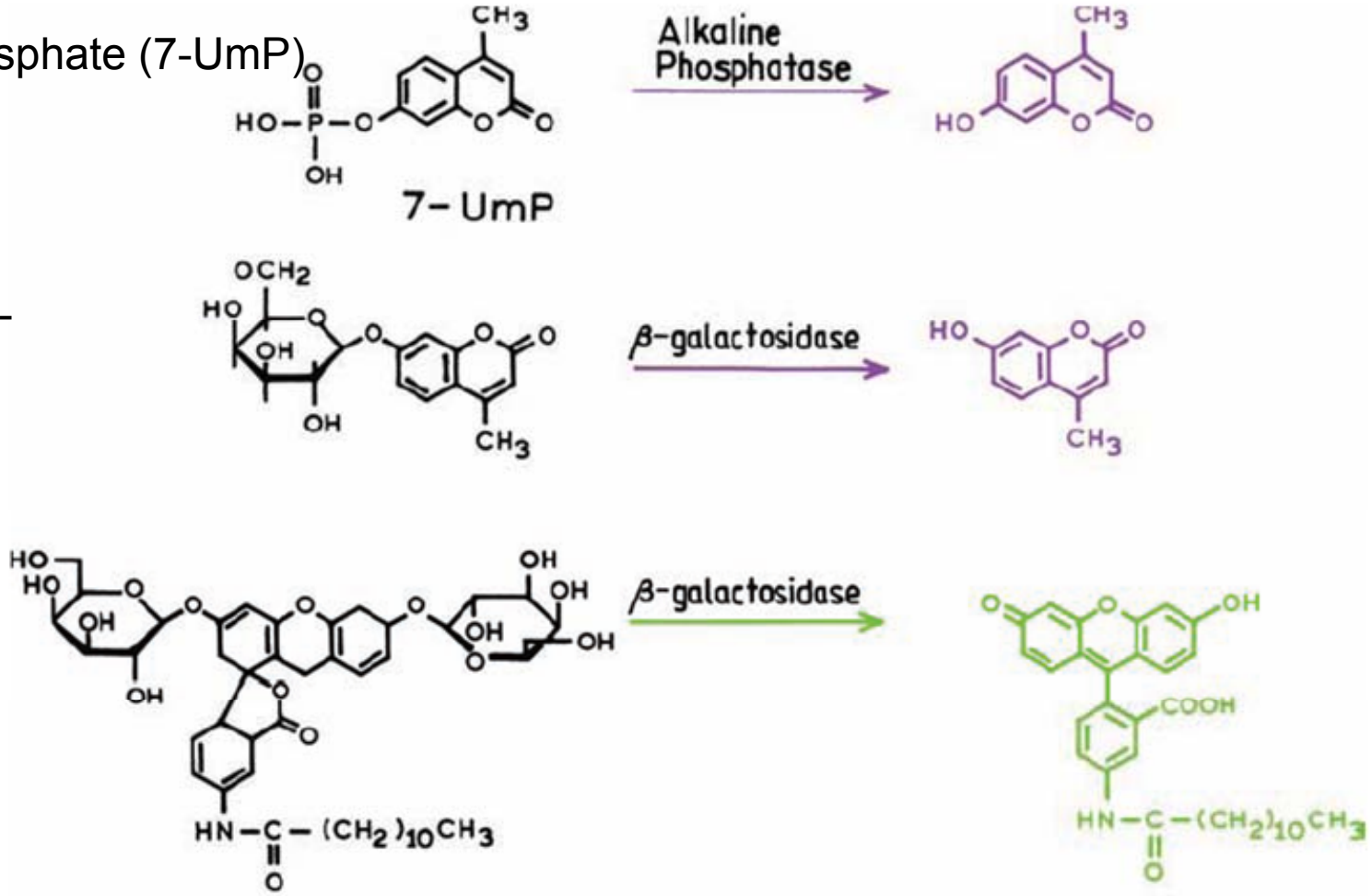


Figure 3.28. Fluorogenic probes.

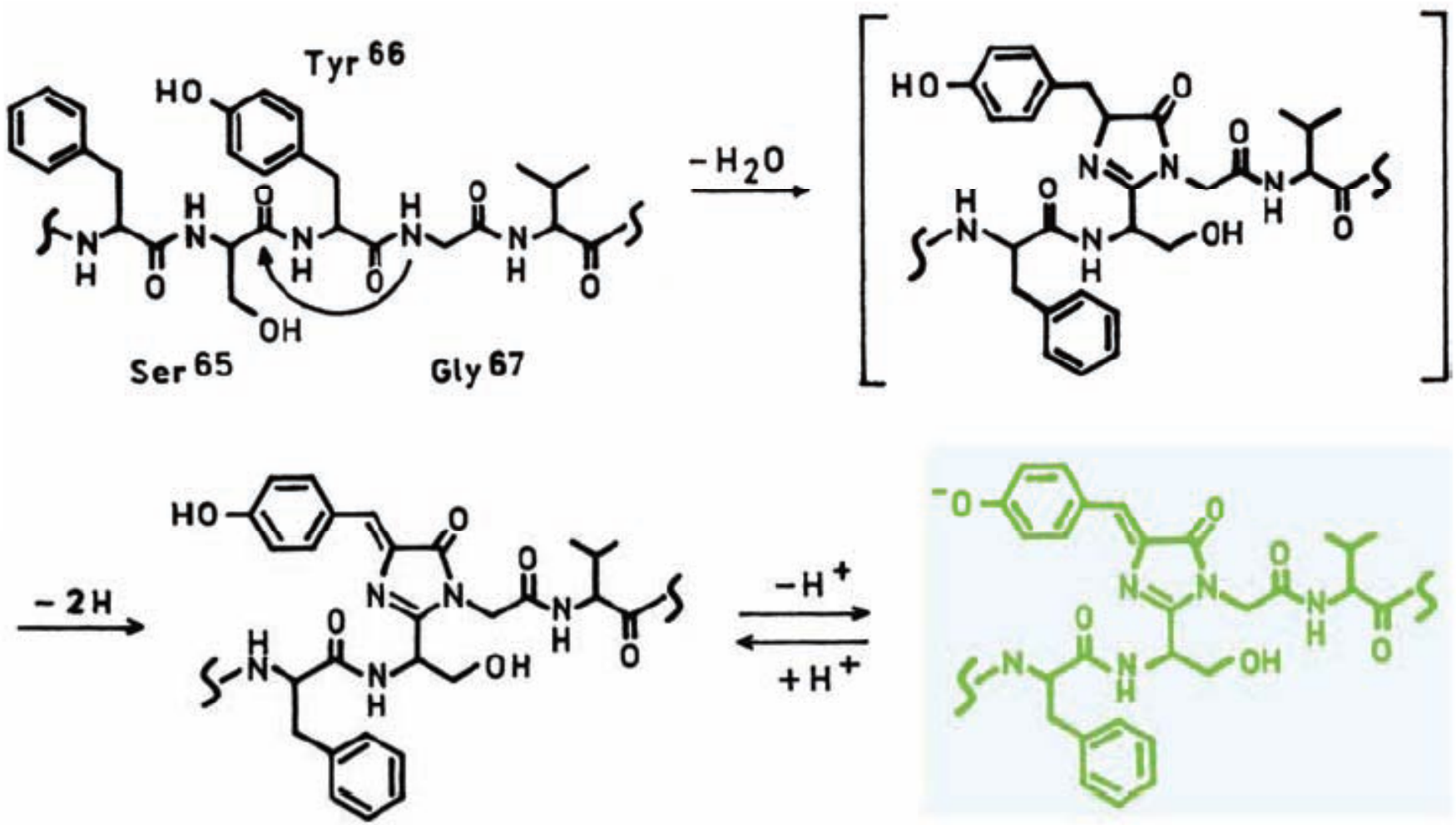


Figure 3.34. Spontaneous formation of the fluorophore in GFP by the serine–tyrosine–glycine residues. From [115].

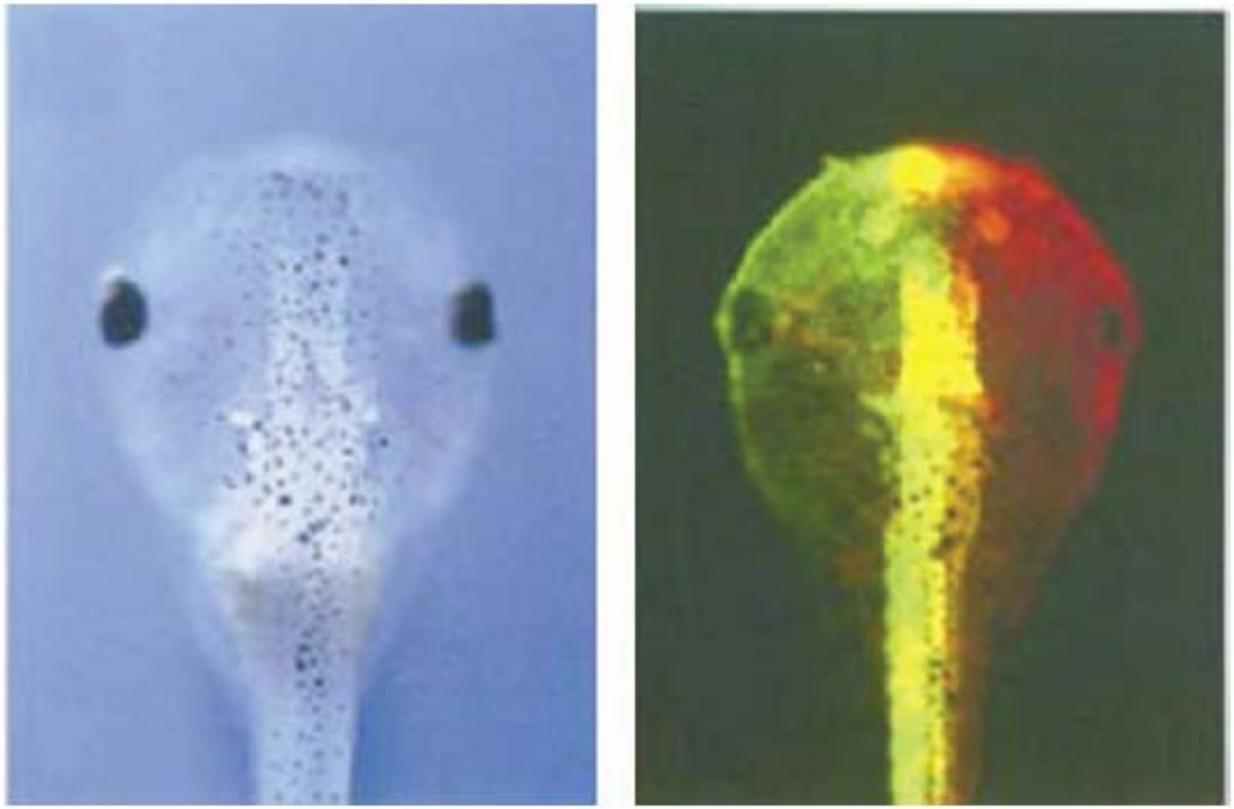


Figure 3.36. Color photograph (right) of a *Xenopus* embryo injected with the mRNA from a green fluorescent coral protein (right side) and a red fluorescent coral protein (left side). The left side shows a white light photograph. Reprinted from [125].


Unknown Xenopus sp.

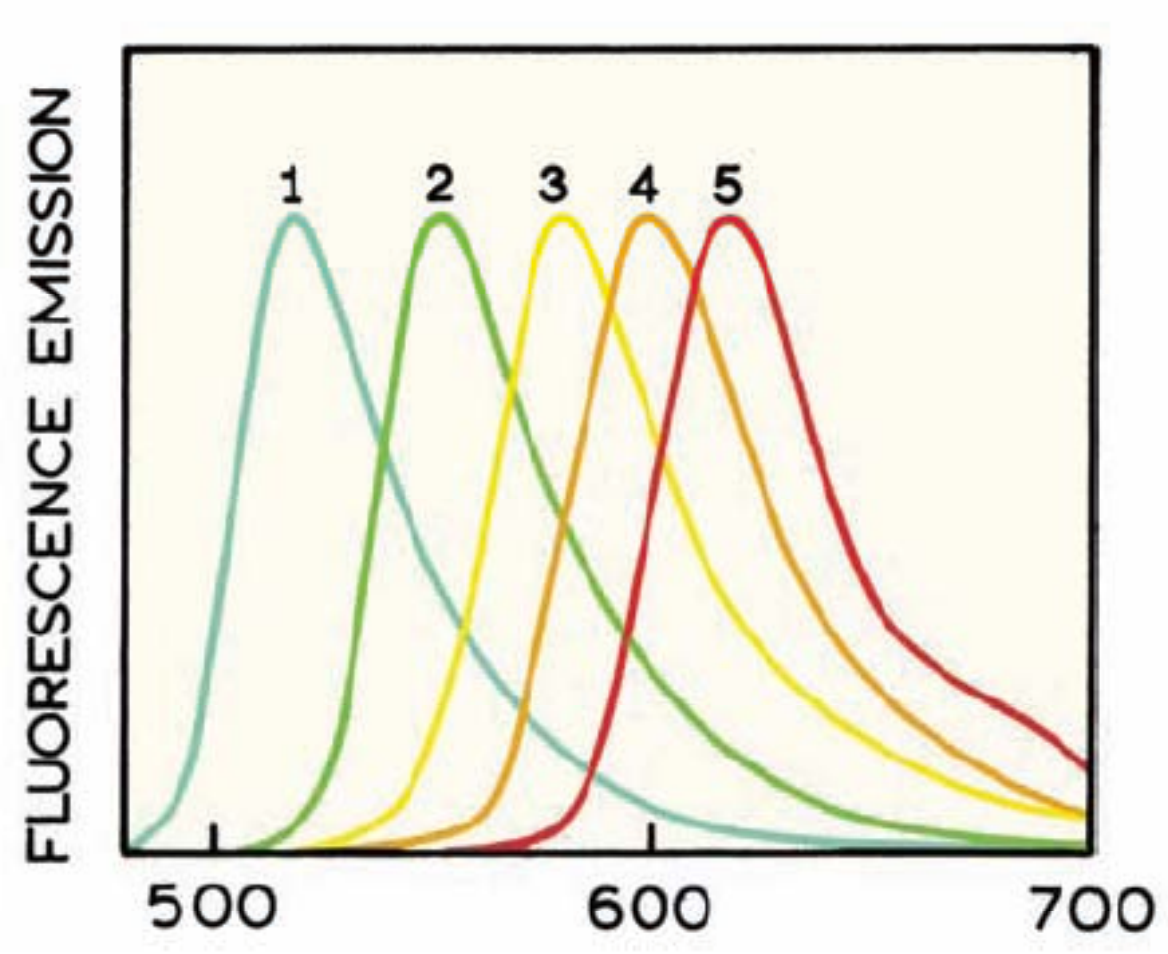
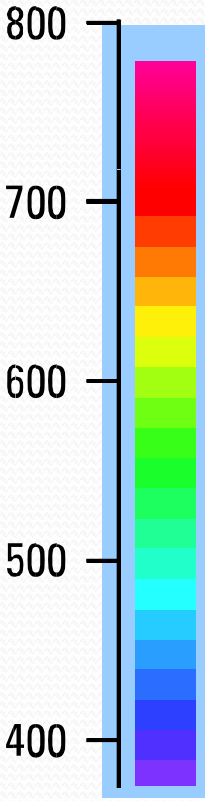


Figure 3.11. Structures and normalized fluorescence emission spectra of goat anti-mouse IgG conjugates of (1) fluorescein, (2) rhodamine 6G, (3) tetramethylrhodamine, (4) Lissamine rhodamine B, and (5) Texas Red dyes. Revised from [30].

Absorbance and Complementary Colors



Wavelength [nm]	Absorbed color	Complementary color
650-780	red	blue-green
595-650	orange	greenish blue
560-595	yellow-green	purple
500-560	green	red-purple
490-500	bluish green	red
480-490	greenish blue	orange
435-480	blue	yellow
380-435	violet	yellow-green

Other Fluorescent Proteins

Phytofluors: A New Class of Fluorescent Probes
Phycobiliproteins

Table 3.2. Properties of Some Major Phycobiliproteinsa

Protein	Subunit composition	Approx. mol. wt.	$\varepsilon(M^{-1}\ cm^{-})$	Total bilins per protein	λ_{ab}^{max} (nm)	λ_{ab}^{max} (nm)	Quantum yield
Allophycocyanin	$(\alpha\beta)_3$	100,000	700,000	6	650	660	0.68
B-Phycoerythrin	$(\alpha\beta)_6\gamma$	240,000	2,400,000	34	543,562	576	0.98
R-Phycoerythrin	$(\alpha\beta)_6\gamma$	240,000	2,200,000	34	495,536,565	576	0.84

[a]From [143] and [144]. C-phycocyanine (620/642 nm) and C-phycoerythrin (562/576 nm) have a subunit structure $(\alpha\beta)_n$, n = 1–6, with molecular weights from 36,500 to 240,000.

Beer law deviations

- ***Chemical Deviations***

- ***Instrument Deviations***

❑ *Chemical Deviations*

*Absorbance usually becomes nonlinear with concentration when **cis** greater than 0.10 M.*

- Above concentrations of 0.10 M, the distance between analyte molecules decreases to the extent that they change each others charge distribution, effectively changing the way they absorb radiation (i.e. ε changes).

*Absorbance becomes nonlinear when **chemical reactions** occur.*

- *If the associates, dissociates, or reacts with the solvent or other components in the solution deviations from Beer's Law will occur.*

Instrument Deviations

- Effect of polychromatic radiation
- Ideally, a monochromator will pass radiation of a single wavelength, but in reality the monochromator passes a band of radiation. The bandwidth of the spectrometer will affect the linearity of Beer's Law.