UNITE

SPECTROSCOPIC TECHNIQUES

I.C. **LABORATORY OF BIOCHEMICAL METHODOLOGIES AND EXPERIMENTAL MODELS**

- MODULE: **BIOCHEMICAL METHODOLOGIES**

BIOS-07/A

5 ECTS (CFU)



Spectroscopic techniques

BY THE END OF THE LESSON, THE STUDENT WILL BE ABLE TO:

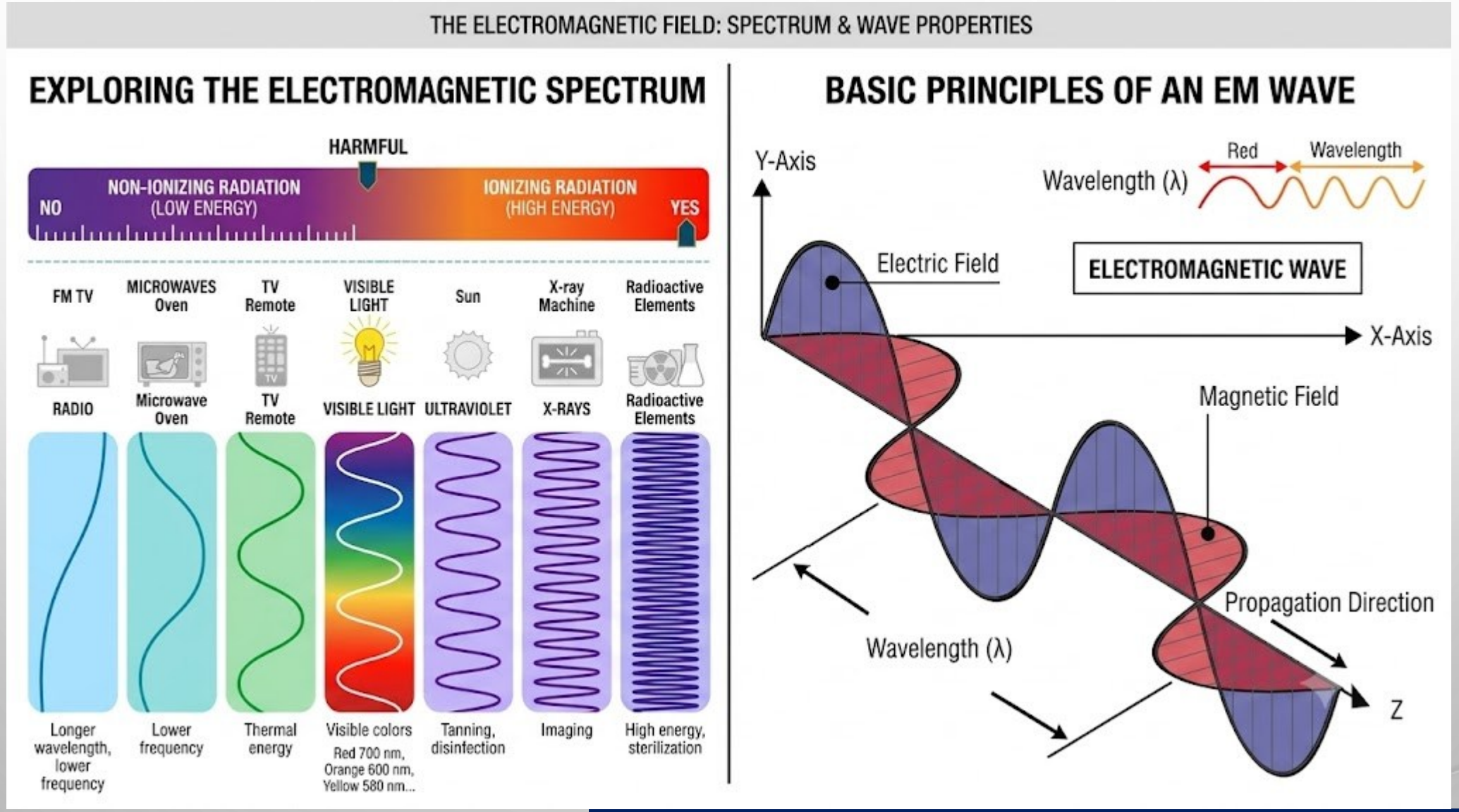
- Describe the fundamental properties of electromagnetic radiation (wavelength, frequency, energy) and the relationships between them.
- State and apply the Beer-Lambert Law to calculate the concentration of a solution based on measured absorbance.
- Distinguish between the three main spectroscopic techniques in biochemistry: UV-Vis, fluorescence, and infrared spectroscopy.
- Identify the main biological chromophores (aromatic amino acids, nitrogenous bases, prosthetic groups) and their respective absorption wavelengths.
- Explain the physical mechanism of fluorescence, the Franck-Condon principle, and the Stokes shift.
- Illustrate the principle and applications of FRET (Fluorescence Resonance Energy Transfer) and BRET in molecular biology.
- Recognize the factors influencing fluorescence intensity (solvent, pH, temperature, quenching).

Spectroscopic techniques

- **Spectroscopic techniques** are based on the interaction of light with matter and probe certain features of a sample to learn about its consistency or structure.
- **Light is electromagnetic radiation,** a phenomenon exhibiting **different energies.**
- When light interacts with matter, it can emerge with other characteristics and properties, and therefore different molecular features of matter can be probed.
- Conceptually, matter can either **annihilate (absorb)** or **create (emit) light ,** or it can **scatter light.**

An understanding of the properties of electromagnetic radiation and its interaction with matter leads to an appreciation of the variety of types of spectrum and different spectroscopic techniques, and their applications to the solution of biological problems.

Spectroscopic techniques: Electromagnetic Radiation Properties



Wilson and Walker's - Principle and Techniques of Biochemistry and Molecular Biology - 8th edition 2018

Spectroscopic techniques: Electromagnetic Radiation Properties

Electromagnetic radiation is characterized by:

- A wavelength (λ), which is the distance between two crests and is measured in nanometres (nm);
- A frequency (ν), which is related to λ via the propagation speed (c);

$$\nu = c/\lambda$$

- An energy (E), which is derived using:

$$E = h\nu$$

h = Planck's constant ($h = 6.63 \times 10^{-34}$ J s)

Spectroscopy is the study of the interactions of electromagnetic radiation with matter.

Which is a quantum phenomenon

For biological samples, depending on the radiation energy and the type of interaction, different types of spectra can be obtained that provide various physical and/or chemical insights into the sample being analyzed:

- Information on the structure of matter, the surrounding microenvironment, and molecular dynamics:
- Molecules undergo Brownian motion, enabling their rotation and diffusion in solution;
- Thermal energy (which drives vibrational motion) is closely linked to the dynamic properties of molecules.

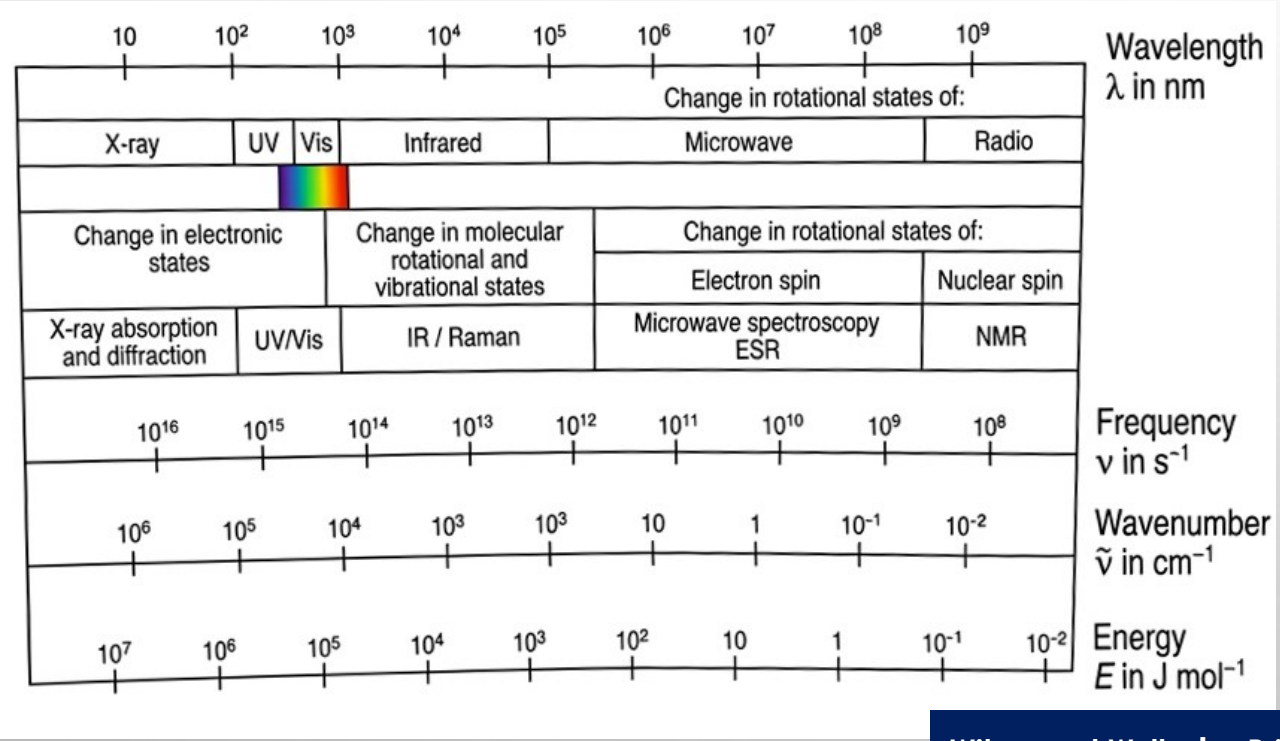
Interaction with matter

The origin of the electromagnetic radiation is due to energy changes within matter itself.

Molecules energy absorption induces different types of transition; these transitions are **quantum phenomena** and correspond to either the **absorption** or **emission** of an **energy quantum** (a **photon**).

Depending on the order of magnitude of the energy of the radiation, given by the frequency or the wavelength, different states of the molecules are affected:

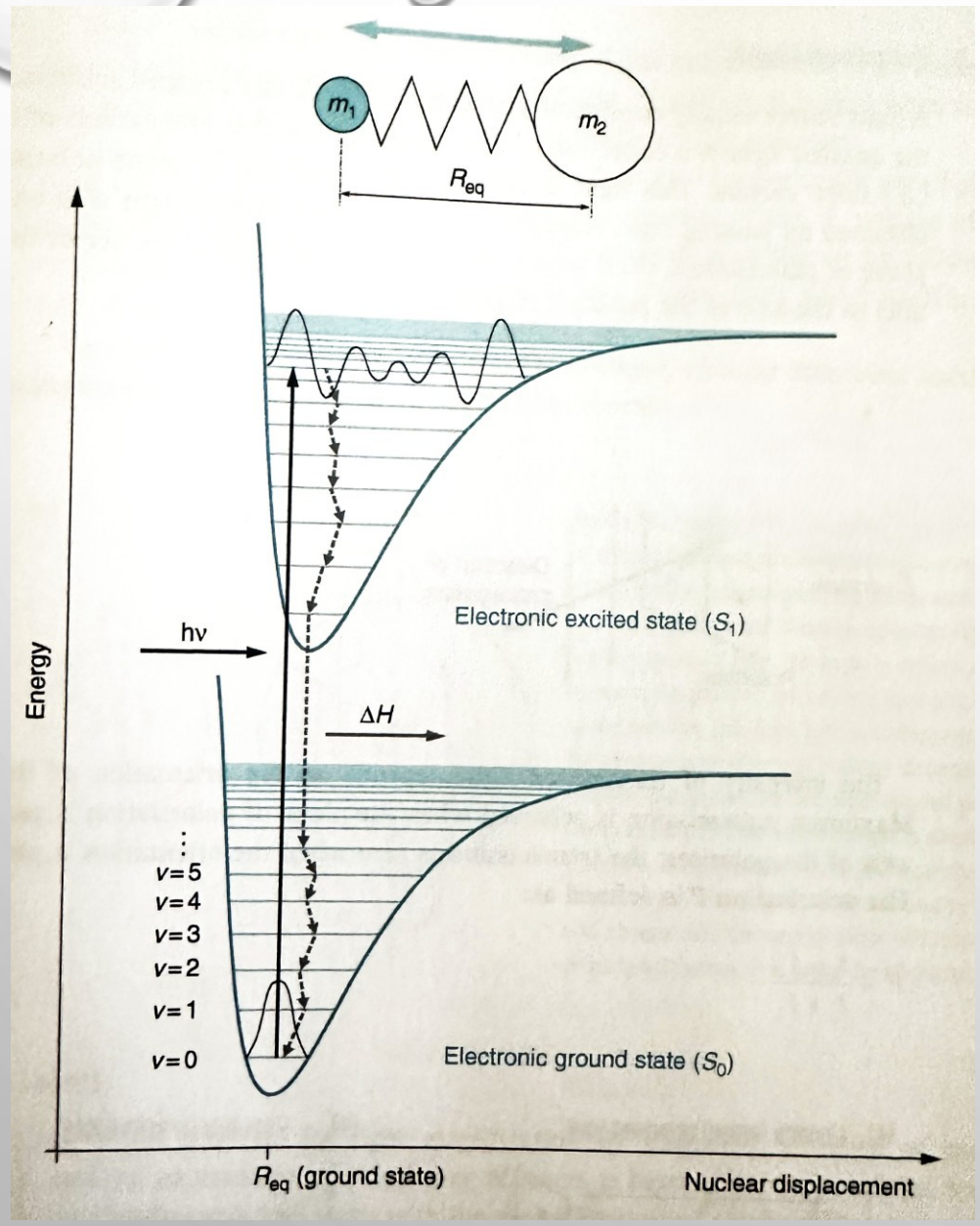
Molecular (sub)structures responsible for interaction with electromagnetic radiation are called **CHROMOPHORES**



Spectrum of magnetic radiation organized by increasing wavelength, and thus decreasing energy, various radiation and thus interaction with matter and the resulting spectroscopic application

Wilson and Walker's - Principle and Techniques of Biochemistry and Molecular Biology - 8th edition 2018

Interaction with matter



Diatomic molecules have **rotation** or **vibrational** transitions → discrete energies that only merge into continuum at very high energy

Each electronic state of a molecule posseses its own set of rotational and vibrational levels →represented in the **Jablonsky Diagram(figure) :**

- **horizontal lines** represent **rotational and vibrational levels**
- **vertical lines** represent **possible transitions**

For a transition to occur energy must be absorbed

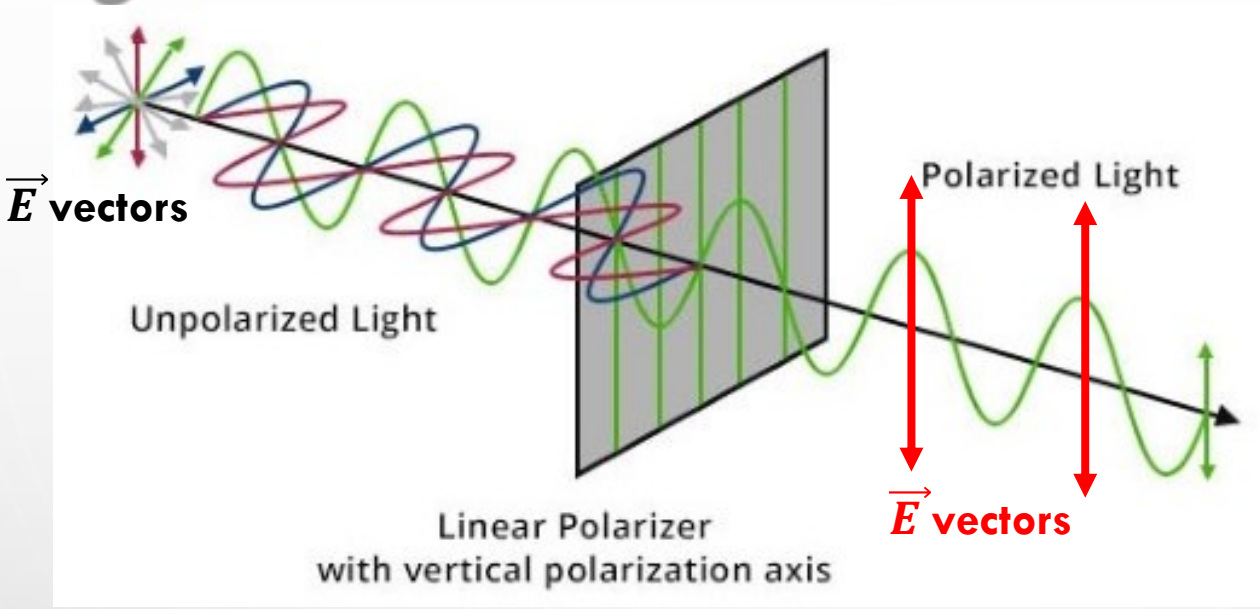
$$\Delta E = E_{final} - E_{start} = h \cdot v$$

Generally electrons reside in the **ground state (S_0)** and if energy is putted into the system they can reach the **excited state (S_1)**

If the energy comes from **electromagnetic radiation** there will be an **absorption spectrum** and the electron go from ground state to excited state, the molecule will also be in excited vibrational and rotational state; subsequent **relaxation** will revert the electron to ground state. For **non-fluorescent molecules → emission of heat (ΔH).**

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Interaction with matter: polarized light



Generation of linearly polarised light

A light source (emitted light) is generally composed by waves with multiple orientation of the electric field ($\vec{E}$) vectors = **non-polarised light**

⇓

Polariser that transmits light with a single plane of polarization can transform the unpolarized light into **linearly of plane polarised light**

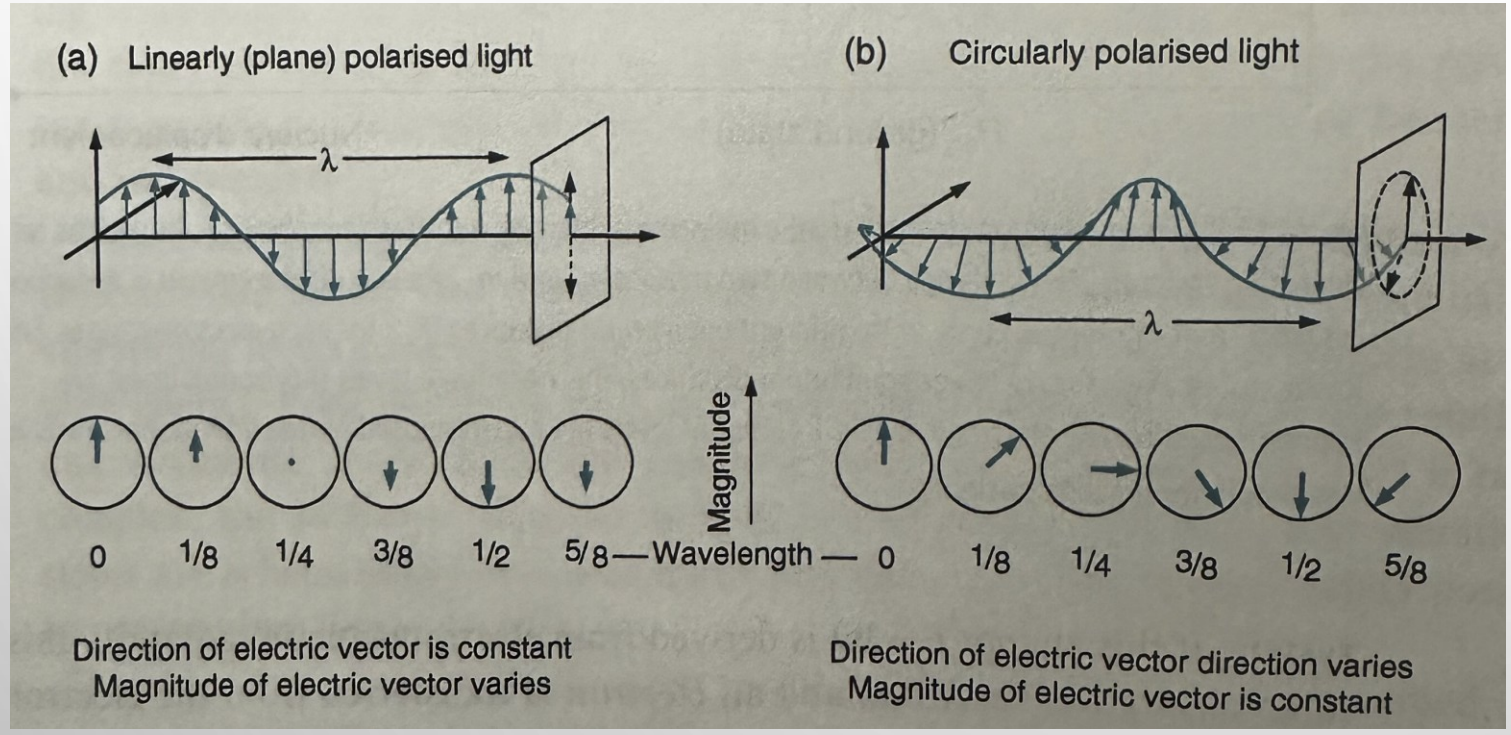
The intensity of the transmitted light depend on the orientation of the polariser → higher if parallel; 0 if perpendicular:
Polarization (P), can vary between +1 and -1:

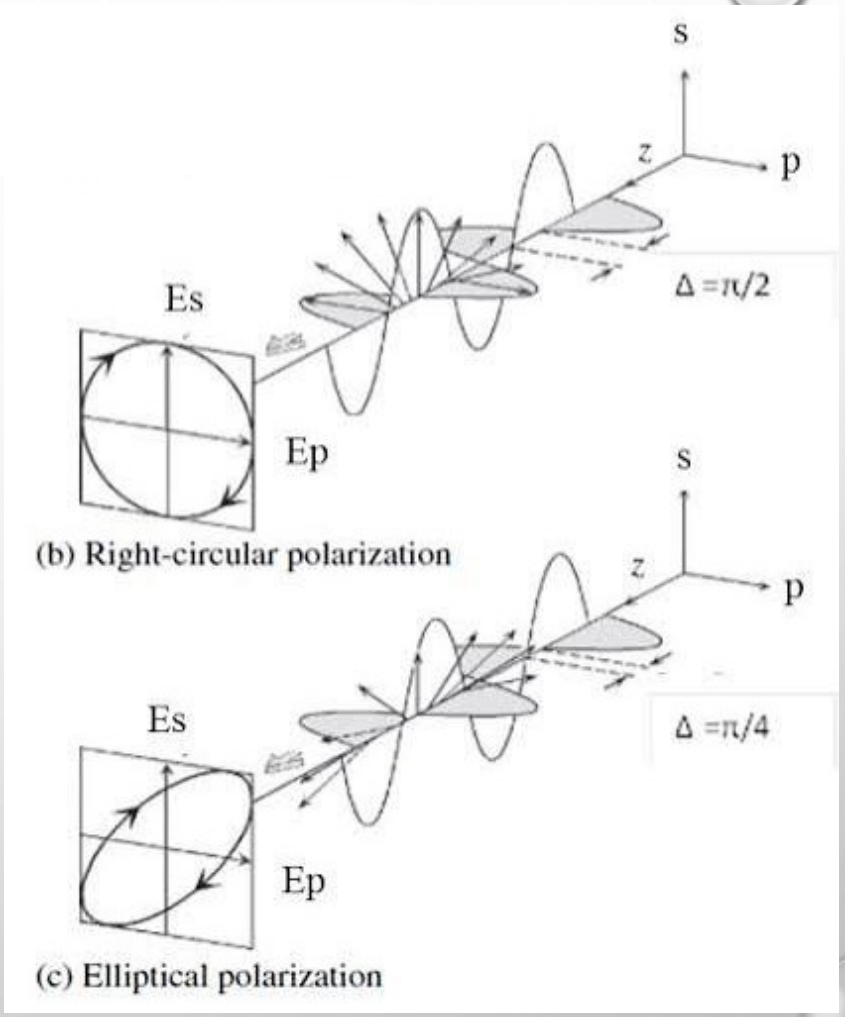
$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$ where I are intensities

Interaction with matter: polarized light

When two perpendicular light waves are ¼ wavelength out of phase, their combined $\vec{E}$ vector rotates as it moves forward — its tip tracing a helix:

- **Circularly polarized light**
- **Elliptically polarized light**





Light sources: lasers

LASER = Light Amplification by Stimulated Emission of Radiation.

Stimulated emission





One photon in → two identical, in-phase photons out.

HOW IT WORKS

1. **Pump** Intense flashing light (pumping) lifts many electrons to the excited state.
2. **Stimulate** A photon of exactly ΔE makes an excited electron fall back to the ground state.
3. **Amplify** It releases a 2nd identical, in-phase photon — repeated, this builds a cascade of coherent light.

LASER LIGHT IS

Coherent	Monochromatic	Intense
in phase	narrow bandwidth	high power

In the life sciences: high intensity + precisely defined wavelengths make lasers ideal for modern spectroscopy — laser-read fluorescent labels in DNA sequencing enabled whole-genome sequencing.

Spectroscopic techniques: UV/Vis spectroscopy

Measuring light absorption to identify and quantify biomolecules

Absorbing UV/visible light probes a molecule's electronic structure — and, through it, its size, form and flexibility.

Why it matters: UV/Vis is the most widely used analytical technique in the life sciences — and the basis of most colorimetric assays

Chromophores: are the groups that absorb UV/Vis light — typically molecules with several conjugated double bonds (more conjugation → longer wavelength).

THREE TRANSITION TYPES CAN ABSORB THIS ENERGY

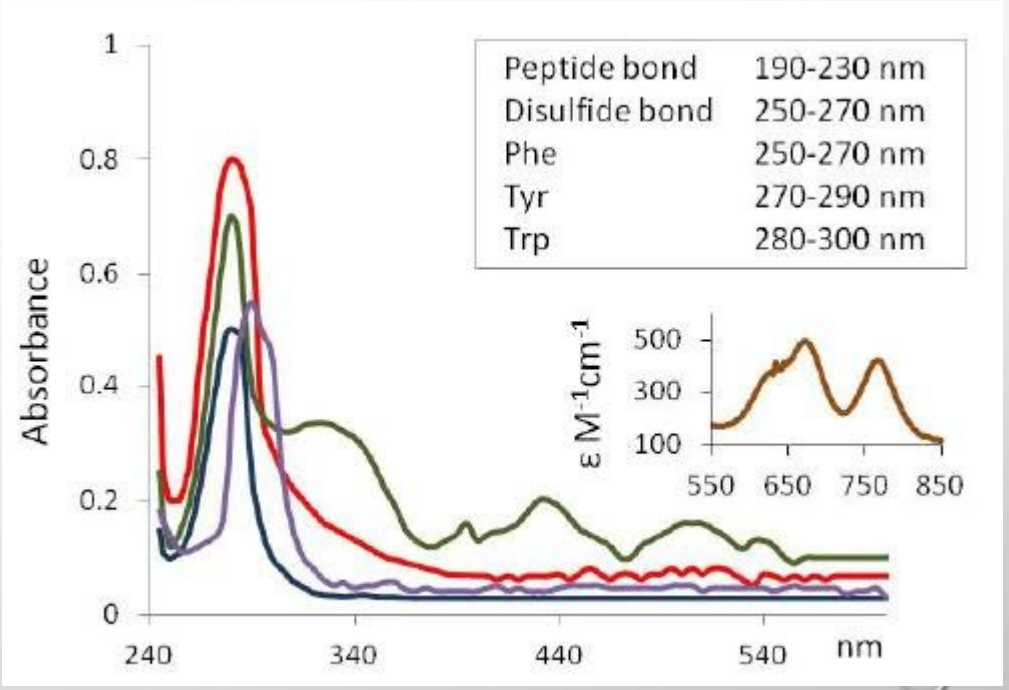
1. **Electronic:** promote an electron to a higher level — the dominant UV/Vis effect.
2. **Vibrational:** stretch & bend of bonds; finer energy steps.
3. **Rotational:** whole-molecule rotation; the smallest steps.

Spectroscopic techniques: UV/Vis spectroscopy

Chromophores in proteins

Three intrinsic absorbers — the peptide backbone plus two aromatic side chains dominate.

Chromophore	λmax	Notes
Peptide bond (amide)	190 nm	far-UV, intense (+210–220 nm)
Tryptophan	280 nm	dominates the protein band
Tyrosine	274 nm	Tyr + Trp → band at ~278 nm
Phenylalanine	257 nm	weak
Cystine (S–S)	250 nm	weak



Spectroscopic techniques: UV/Vis spectroscopy

Beyond the backbone: pigments, coenzymes & nucleic acids

Prosthetic groups and coenzymes add strong, environment-sensitive bands — handy reporters of function.

Species	λmax
Carotenoids	420, 450, 480 nm
Porphyrins / haem (Soret)	~400 nm
FAD	438 nm
NADH	340 nm
NAD^+	260 nm
Nucleic-acid bases	260–275 nm

Environment-sensitive: haem bands shift with the iron-bound ligand → structure–function studies (ex. Haemoglobin).

Redox reporters: FAD, NADH & NAD^+ track electron-transfer reactions

Nucleic acids: bases absorb at 260 nm — the basis of DNA/RNA quantitation.

Spectroscopic techniques: UV/Vis spectroscopy

The Beer-Lambert law

Absorbance (A) is proportional to concentration and path length — the foundation of quantitation.

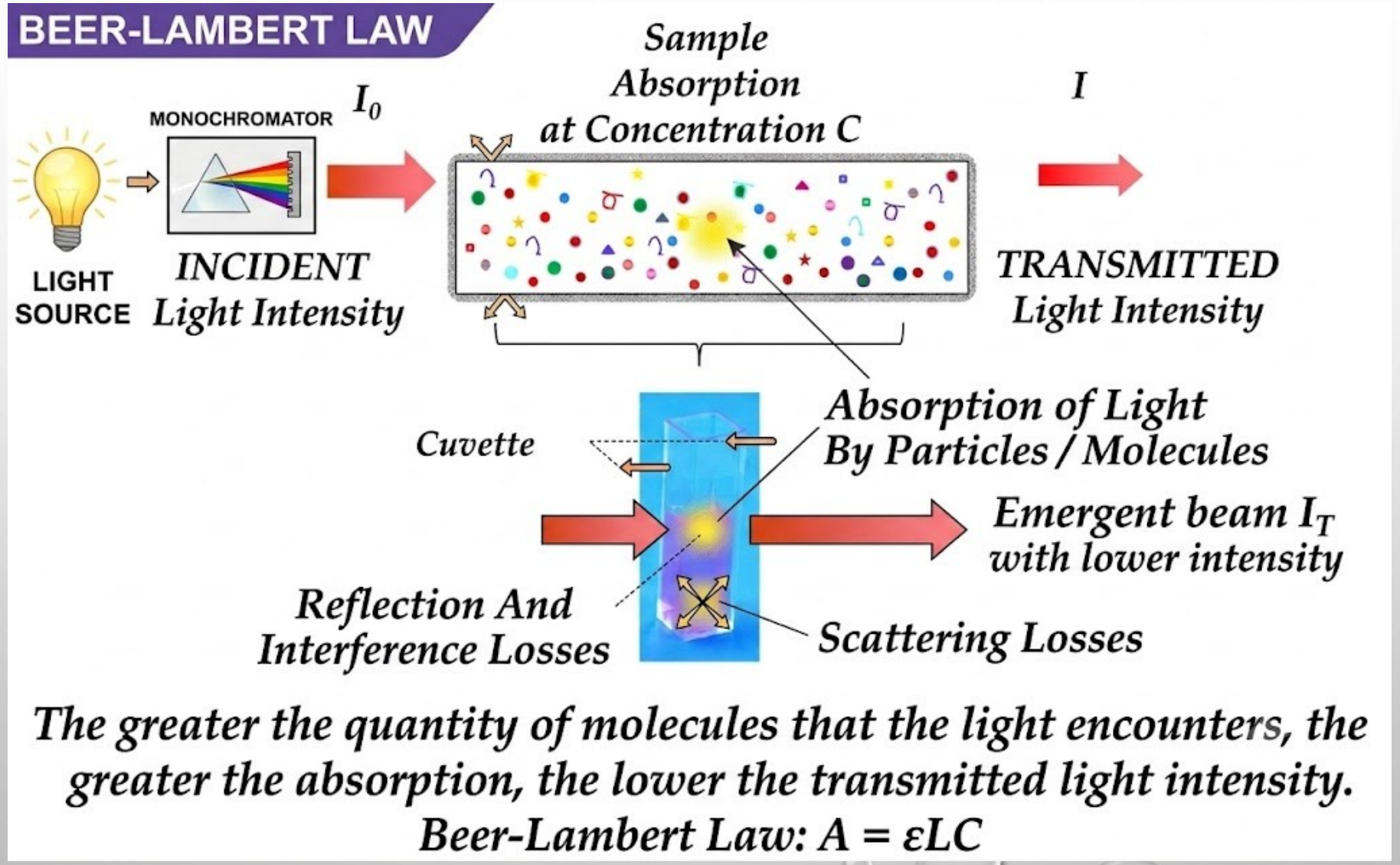
$$A = \varepsilon \cdot c \cdot d \qquad = \log(I_0/I) = \log(1/T)$$

A absorbance (shown by the spectrophotometer); **T** = I/I_0 transmission; **ε** molar absorption coefficient or **extinction coefficient** ($dm^3\ mol^{-1}\ cm^{-1}$) = the chance of a photon being absorbed by matter; **c** concentration ($mol\ dm^{-3}$); **d** path length (cm); and the raw extinction coefficient $a = 2.303 \cdot \varepsilon$.

Valid at low concentration only (aggregation causes deviations). Absorbances are additive across species, and light scattering makes A read too high.

Spectroscopic techniques: UV/Vis spectroscopy

The Beer-Lambert law



Spectroscopic techniques: UV/Vis spectroscopy

When absorbance misleads: deviations & optical density

Linearity breaks down at high absorbance — and turbid samples scatter light rather than absorb it.

1)Deviations from Beer–Lambert

- **High absorbance** the A-vs-c line bends; keep max A around 0.5.
- **Stray light** for good S/N, signal should be ≥10× the stray light.
- **Molecular events** dimerisation at high c changes the spectrum.

2) Optical density (scattering)

- **Turbid samples** bacterial cultures scatter light instead of absorbing it.
- **Apparent absorbance** recorded as optical density (OD) — a turbidity readout.
- **Use** biomass / cell-density measurements; nephelometers or a normal UV/Vis.

3) Environmental effects on absorption: the spectrum depends on the chromophore's surroundings — so it can be used to probe them.

↑ **Bathochromic (red shift)** λmax moves to longer wavelength;

↑ **Hyperchromic** absorbance increases ("more colour");

↓ **Hypsochromic (blue shift)** λmax moves to shorter wavelength

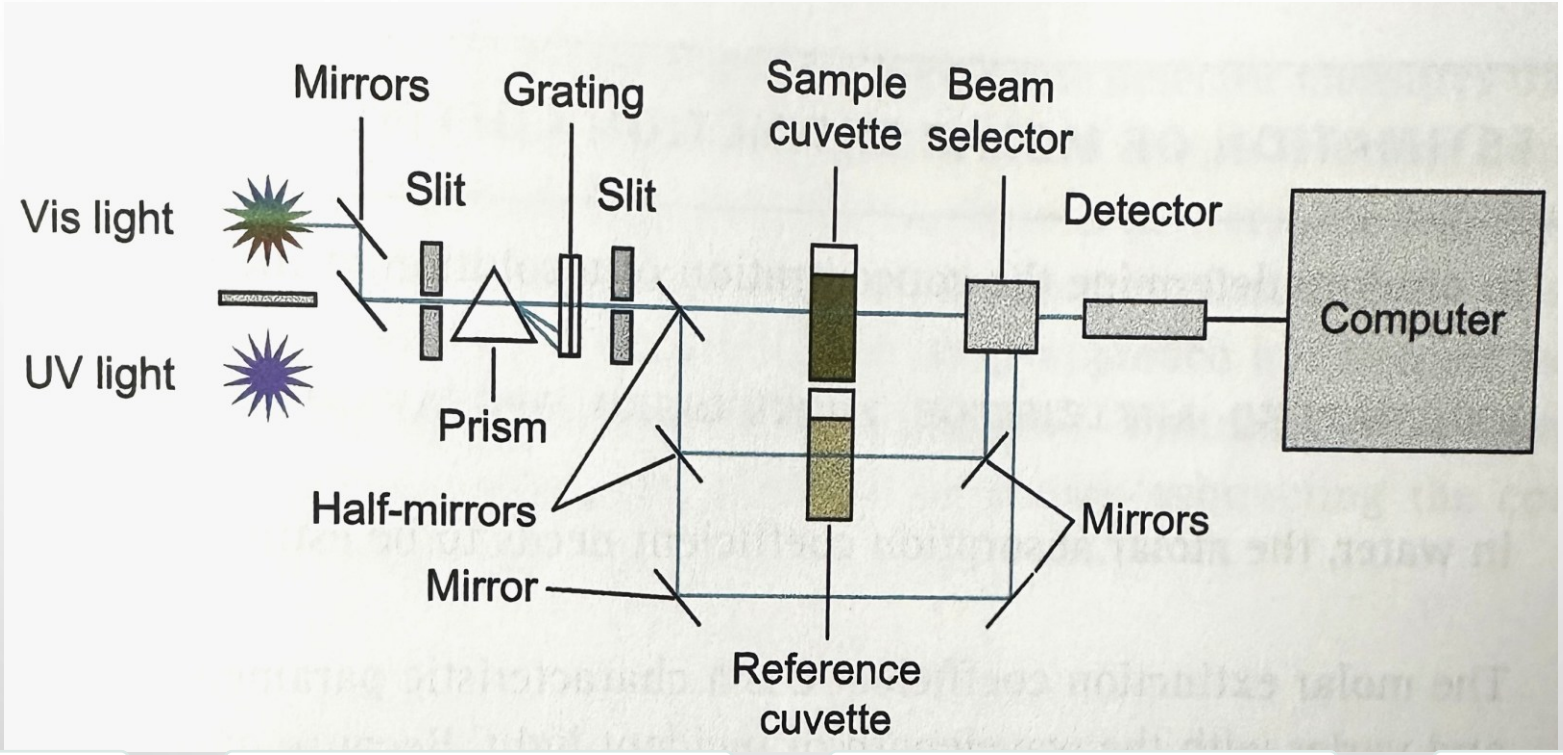
↓ **Hypochromic** absorbance decreases ("less colour").

Driven by three factors:
1) protonation/deprotonation (pH, redox); 2) solvent polarity; 3) orientation & order.

Spectroscopic techniques: UV/Vis spectroscopy

Inside the spectrophotometer

Four core components in series — plus a reference channel to cancel source drift.



Light source	→	**Monochromator**	→	**Sample cuvette**	→	**Detector**
tungsten (visible) + deuterium (UV)		prism or grating (grating = higher res.)		glass/plastic = visible quartz = UV		reads the transmitted light

Dual-beam design: a half-mirror splits the beam through sample and reference (blank), so source fluctuations cancel. Many instruments instead subtract a stored baseline in software.

Colorimetry vs spectrophotometry: colored filters isolate a broad band (colorimetry, plate readers); prisms/gratings isolate a narrow band (spectrophotometry). Narrower slit = more reproducible but less sensitive.

Spectroscopic techniques: UV/Vis spectroscopy

Applications

- METALLOPROTEIN STUDIES: Some proteins contain chromophore groups (e.g., the heme group in hemoglobin). In such cases, absorption in the visible region can be exploited to analyze structural changes in the molecule surrounding the chromophore—such as the oxygenation state in the case of hemoglobin.

- ENZYME KINETICS: If the substrate or product of an enzymatic reaction absorbs light in a spectral region where other substrates or products do not, reaction kinetics can be monitored via spectrophotometry.

- COLORIMETRY: Colorless biochemical compounds can be converted into colored compounds through chromogenic reactions (Colorimetric methods for protein concentration determination: Biuret, Lowry, Bradford)

Spectroscopic techniques: UV/Vis spectroscopy

Applications Example

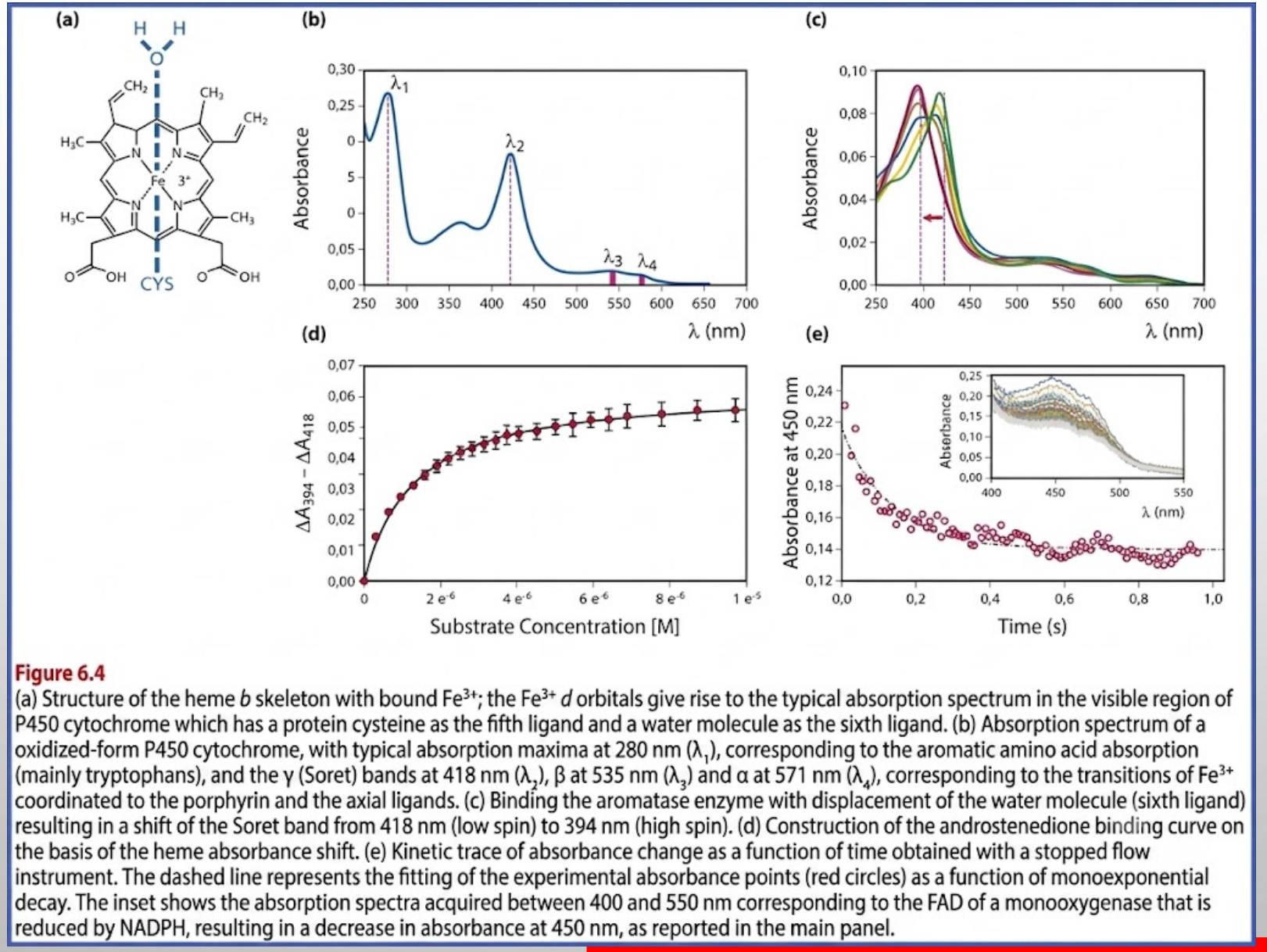


Figure 6.4

(a) Structure of the heme *b* skeleton with bound Fe^{3+}; the Fe^{3+} *d* orbitals give rise to the typical absorption spectrum in the visible region of P450 cytochrome which has a protein cysteine as the fifth ligand and a water molecule as the sixth ligand. (b) Absorption spectrum of a oxidized-form P450 cytochrome, with typical absorption maxima at 280 nm (λ_1), corresponding to the aromatic amino acid absorption (mainly tryptophans), and the γ (Soret) bands at 418 nm (λ_2), β at 535 nm (λ_3) and α at 571 nm (λ_4), corresponding to the transitions of Fe^{3+} coordinated to the porphyrin and the axial ligands. (c) Binding the aromatase enzyme with displacement of the water molecule (sixth ligand) resulting in a shift of the Soret band from 418 nm (low spin) to 394 nm (high spin). (d) Construction of the androstenedione binding curve on the basis of the heme absorbance shift. (e) Kinetic trace of absorbance change as a function of time obtained with a stopped flow instrument. The dashed line represents the fitting of the experimental absorbance points (red circles) as a function of monoexponential decay. The inset shows the absorption spectra acquired between 400 and 550 nm corresponding to the FAD of a monooxygenase that is reduced by NADPH, resulting in a decrease in absorbance at 450 nm, as reported in the main panel.

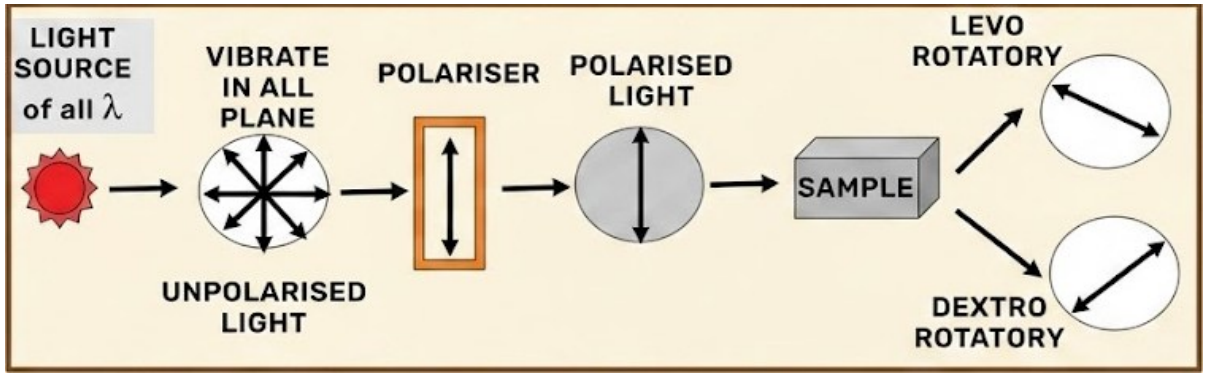
Spectroscopic techniques: Circular Dichroism (CD) Spectroscopy

Optically active isomers:

- allow the rotation of plane-polarised light
- Have identical chemical composition and topology
- mirror images cannot be superimposed (chiral)

Polarimetry measures the angle through which the plane of polarisation is changed after linearly polarised light is passed through a solution containing a chiral substance

Optical rotatory dispersion (ORD) spectroscopy is an analytical technique that measures how the angle of rotation of plane-polarized light by a chiral substance changes as the wavelength of the light varies. The angle α between the plane of the resulting linearly polarised light against that of the incident light is dependent on the refractive index for left and right circularly polarised light.



Spectroscopic techniques: Circular Dichroism (CD) Spectroscopy

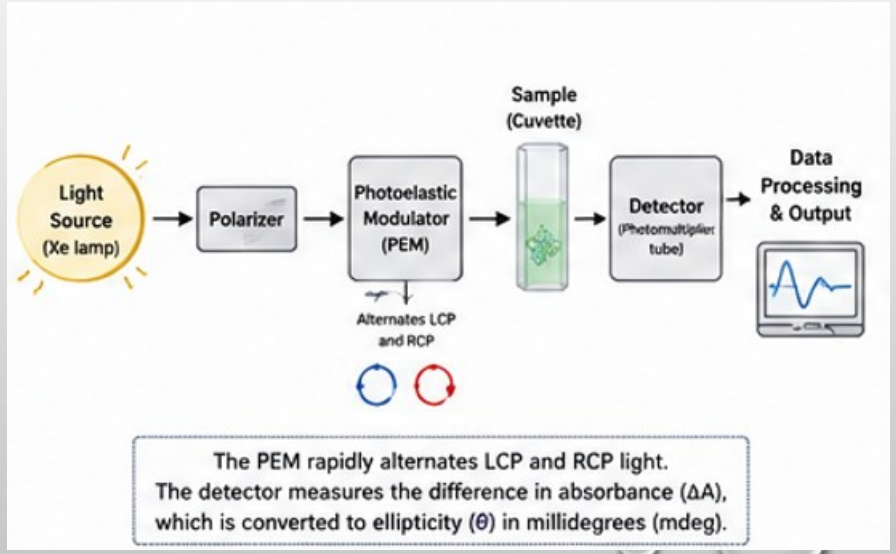
Depending on the sample the left- and right- handed polarised components are absorbed differently and the difference in absorption coefficient is called circular dichroism (CD)

$$\Delta\varepsilon = \varepsilon_{left} - \varepsilon_{right}$$

Results from protein CD experiment are reported as ellipticity (ϑ), which normalization yields the molar ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$)

$$[\theta] = \frac{\theta_{\text{mdeg}} \times 0.1}{c \cdot l}$$

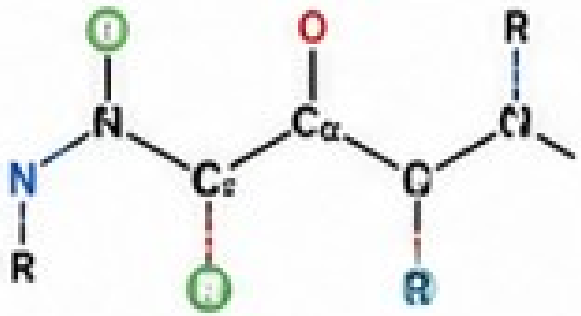
l = path length of the cuvette in centimeters (cm); c = molar concentration (M)



Spectroscopic techniques: Circular Dichroism (CD) Spectroscopy

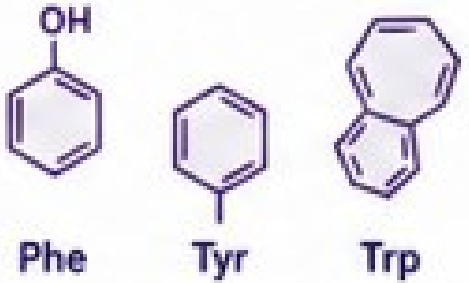
Far-UV CD vs Near-UV CD spectra

FAR-UV CD (190–250 nm)	NEAR-UV CD (250–320 nm)
Probes the peptide backbone and is sensitive to secondary structure.	Probes the environment of aromatic amino acids and disulfide bonds.

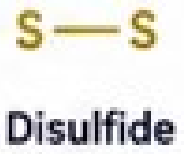


Measures:

- α-Helix
- β-Sheet
- Turns
- Random coil



Phe Tyr Trp



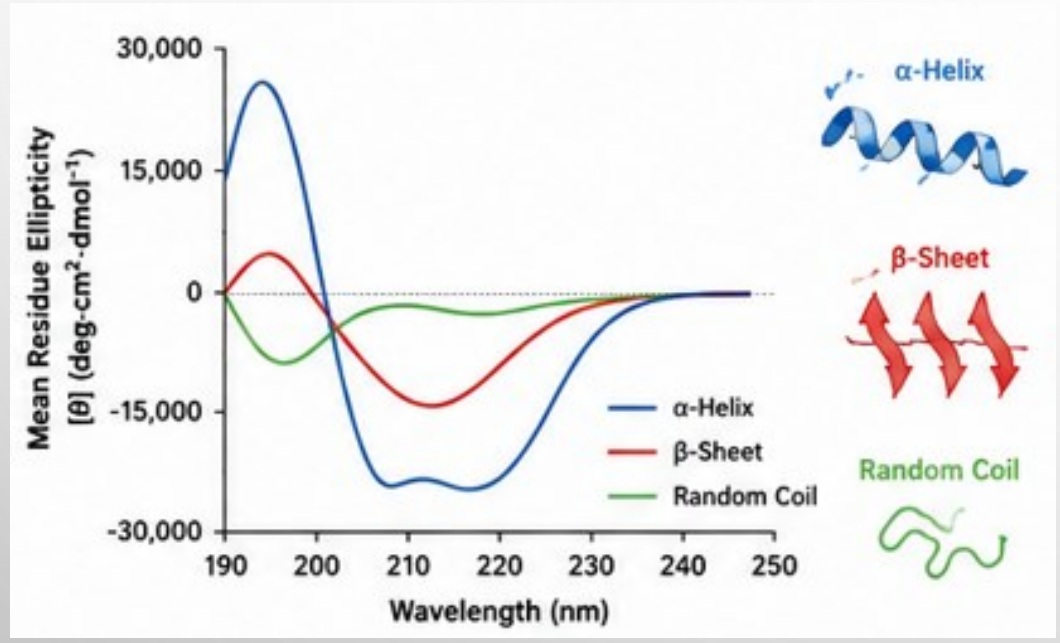
Disulfide bond

Measures:

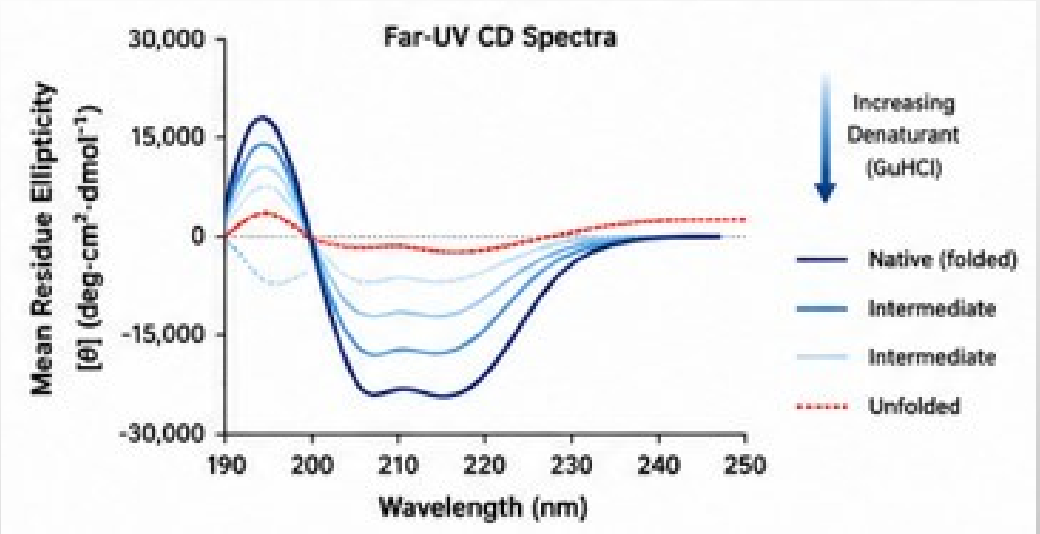
- Tertiary structure
- Side-chain packing
- Disulfide bonds

Spectroscopic techniques: Circular Dichroism (CD) Spectroscopy

Far-UV CD allow secondary structure determination and thus to follow the thermal folding-unfolding of the protein







Loss of a-helical/b-sheet content leads to:

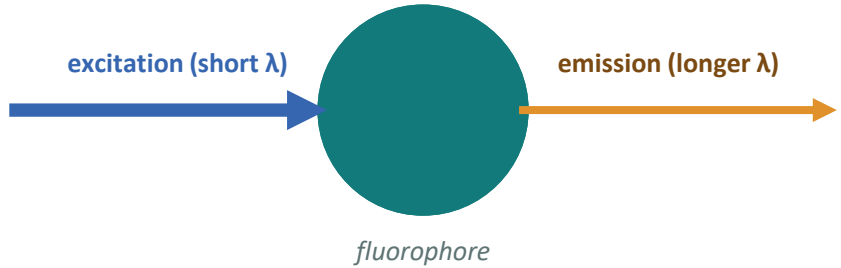
- Decrease in negative ellipticity at 208 and 222nm
- Spectrum approach to the random coil one

Spectroscopic techniques: Fluorescence Spectroscopy

What is fluorescence?

Light emitted when an excited molecule relaxes to the ground state — always at a longer wavelength than the exciting light.

- **Emission phenomenon (accompanied by radiation)** only excited molecules emit — they must be excited first.
- **Stokes shift** energy lost non-radiatively first → emission λ > excitation λ.
- **Band spectrum** many close vibrational/rotational levels give a broad band.
- **The fluorophore** the fluorescent group; usually a rigid aromatic ring system.



Energy is lost as heat before emission — so the photon comes out "redder."

Spectroscopic techniques: Fluorescence Spectroscopy

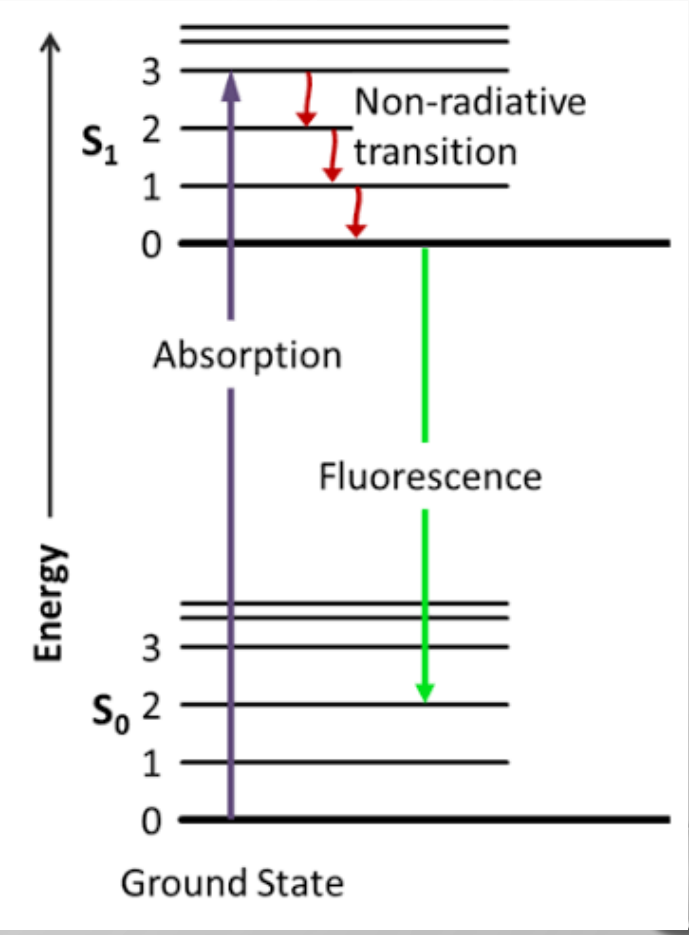
What is fluorescence?

The Jablonski diagram: absorb → relax → emit

Absorption, then non-radiative relaxation, then emission from the lowest excited level.

- **Absorption** a photon lifts the molecule to a higher vibrational level of S_1.
- **Internal conversion** excess vibrational energy is lost as heat down to S_1, v=0.
- **Fluorescence** emission from S_1 (v=0) to a vibrational level of S_0.

Consequences: the emitted photon is lower-energy (Stokes shift), landing on many S_0 levels → a band that mirrors the absorption spectrum.



Spectroscopic techniques: Fluorescence Spectroscopy

Why so few molecules fluoresce?

Non-radiative decay usually wins the race — so good fluorophores are rigid, aromatic ring systems.

Flexible molecules have high ground-state vibrational levels that overlap the excited state → energy leaks away as heat (internal conversion). Rigid aromatics block this route and fluoresce.

Quantum yield Φ

Φ = photons emitted / photons absorbed

$= k_f / (k_f + k_{IC} + k_{ISC} + \ldots)$. Dimensionless — the only absolute measure of fluorescence (usually compared relatively).

Lifetime τ

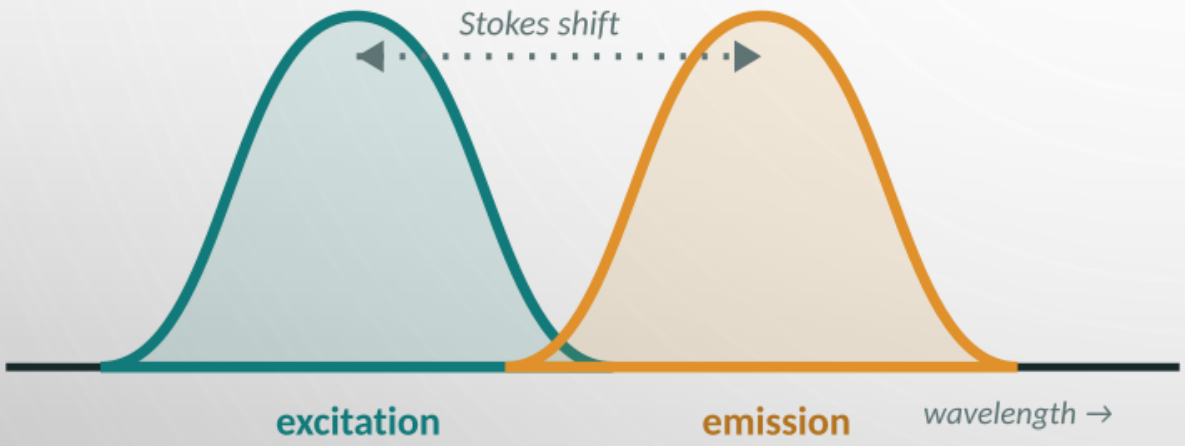
The excited state decays exponentially (first-order). τ is the time for the excited population to fall to 1/e.

The effective lifetime is shortened by every non-radiative route: internal conversion, quenching, FRET, inter-system crossing and excited-state reactions.

Spectroscopic techniques: Fluorescence Spectroscopy

Excitation and emission spectra

The emission spectrum is independent of the excitation wavelength and mirrors the absorption spectrum.

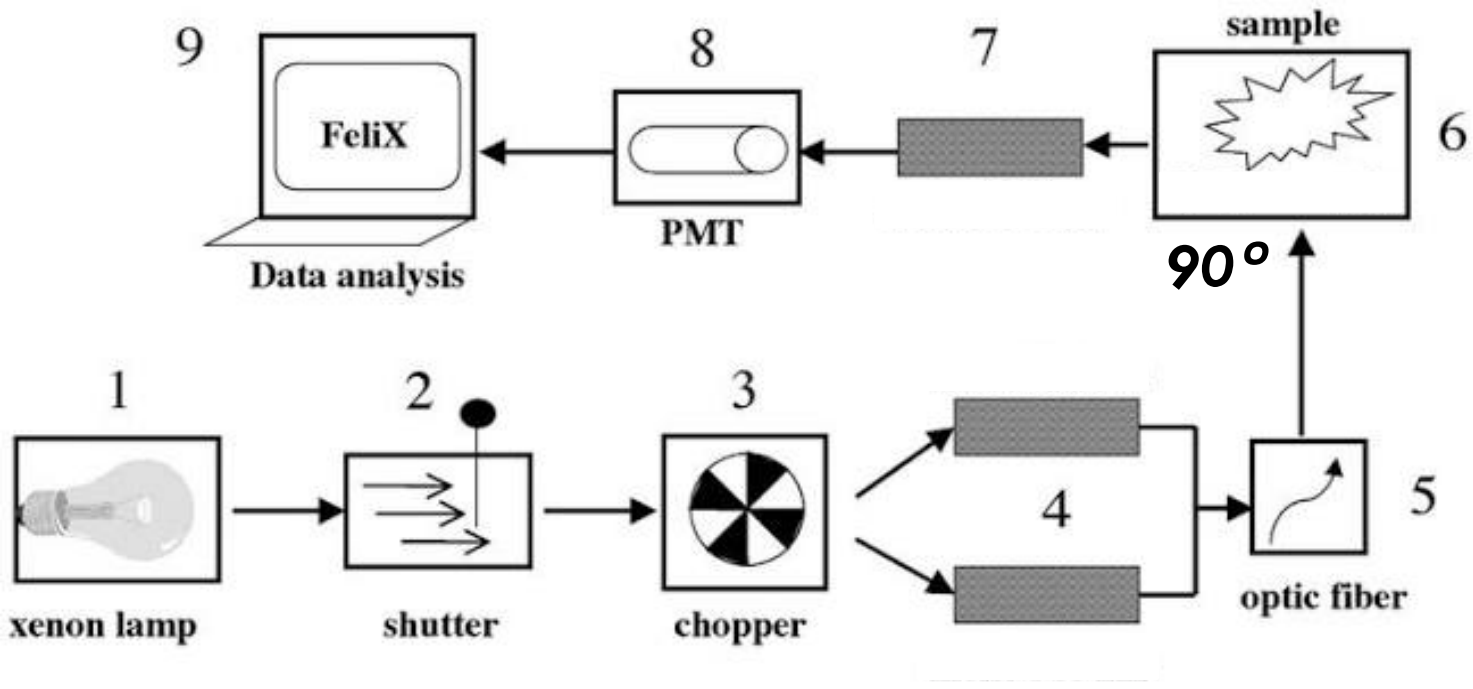


- **Excitation spectrum** fix λ_{em}, scan the excitation wavelength.
- **Emission spectrum** fix $\lambda_{ex}{}^{c}$, scan the emission wavelength.
- **Mirror image** emission mirrors absorption, shifted to longer λ.
- **Stokes shift** the gap between the two peaks.

Spectroscopic techniques: Fluorescence Spectroscopy

The spectrofluorometer

Two monochromators, with the detector set at 90° to read emission against a dark background.



- **Two monochromators** excitation set to a shorter λ than emission.
- **90° geometry** perpendicular detection → very low background.
- **High sensitivity** best at low fluorophore concentration.

Inner-filter effect: at high absorbance, light is absorbed before/after the fluorophore and readings distort. Keep A < 0.05 (use micro-cuvettes, or front-face illumination). Temperature control matters — emission intensity is temperature-dependent.

Spectrofluorometer hardware system with dual excitation wavelengths. The illumination produced by the xenon or other arc lamp (1) first encounters a rotating chopper disk (3), which alternates the direction of the incident beam to one of one/two monochromators (4), via a focusing mirror. A shutter (2) is included to protect the sensitive PMT detector (8) from excessive light. The monochromator outputs are collected by a quartz fibreoptic bundle that focuses the excitation light onto the sample (5). The sample holder is designed to accommodate a cuvette and can have a provision for sample stirring and for variable temperature (6). The sample compartment is attached to the emission monochromator and the photon counts are measured using a PMT (8). The fluorescence spectrometer is linked to a computer equipped with an acquisition system and appropriate software (9), for example FeliX (Photon Technology International Inc. Princeton, NJ).

Spectroscopic techniques: Fluorescence Spectroscopy

The spectrofluorometer light sourses

The source must be bright and broad: fluorescence intensity is proportional to the excitation intensity.

Xenon arc (Xe)
Continuous & intense, ~200–700+ nm. The standard for steady-state work — any excitation wavelength.

Pulsed xenon (flash)
High peak power, low average. Reduces photobleaching; used for time-resolved (lifetime) measurements.

Mercury (Hg)
A line source: a few intense lines (254–578 nm). Ideal when one matches the fluorophore; common in fluorescence microscopy.

Mercury–xenon (Hg–Xe)
Xenon continuum plus sharp Hg lines — a compromise between broad coverage and brightness.

Tungsten–halogen (quartz)
Strong in the visible / near-IR but weak in the UV; a secondary source for excitation in the visible.

Lasers & LEDs
Monochromatic and very intense (lasers: LIF, confocal, single-molecule) or compact & stable (LEDs, incl. pulsed for lifetimes).

Why not a deuterium lamp? The UV source of absorption spectrophotometers is too weak here — fluorescence needs the high brightness of xenon.

Spectroscopic techniques: Fluorescence Spectroscopy

Intrinsic protein fluorescence: Trp & Tyr

Two built-in fluorophores report on protein conformation and local environment.

- **Tryptophan dominates** selectively excited at 295–305 nm.
- **280 nm excites both** Tyr + Trp contribute to the spectrum.
- **Tyrosine is weaker** quenched when ionised or near $-NH_3^+$/$-COO^-$ or a Trp.
- **Reports conformation** binding or folding changes the Trp signal.

EMPIRICAL RULES

Less-polar environment
blue (hypsochromic) shift + higher intensity at λmax.

Polar environment
quantum yield falls as temperature rises.

Protonated acidic neighbour
quenches tryptophan fluorescence.

Spectroscopic techniques: Fluorescence Spectroscopy

Intrinsic protein fluorescence: Trp & Tyr

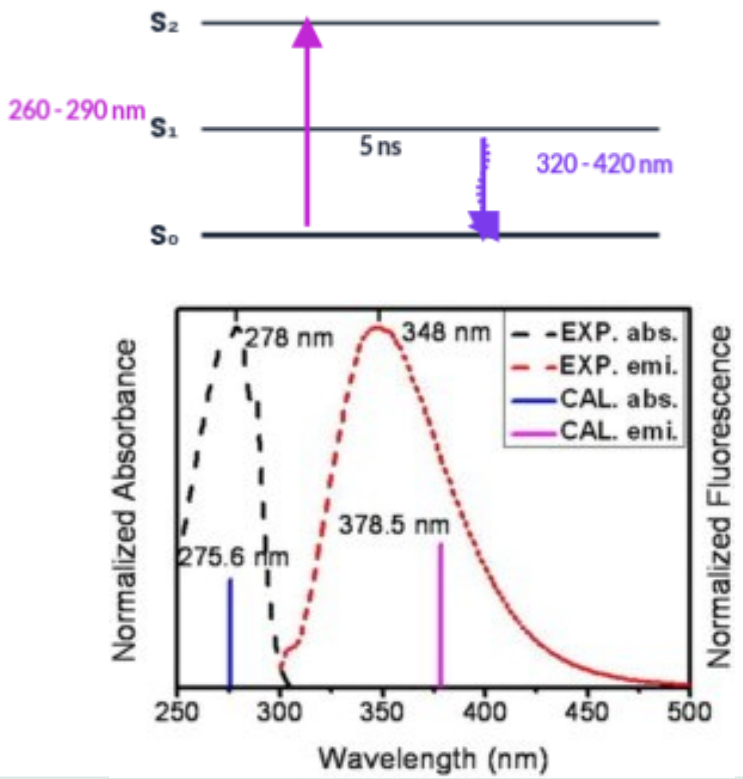
Intrinsic Fluorophores

Aromatic amino acids (phenylalanine, histidine, tryptophan, and tyrosine) emit fluorescent light. In particular, **tryptophan** exhibits a significantly more intense spectrum than the others.

Microenvironment Sensitivity

This fluorescence emission depends on the surrounding local environment and provides key structural information about a protein's state (e.g., whether a **Trp residue** is buried inside the hydrophobic core or exposed to water).

Tryptophan Energy Transitions & Spectra



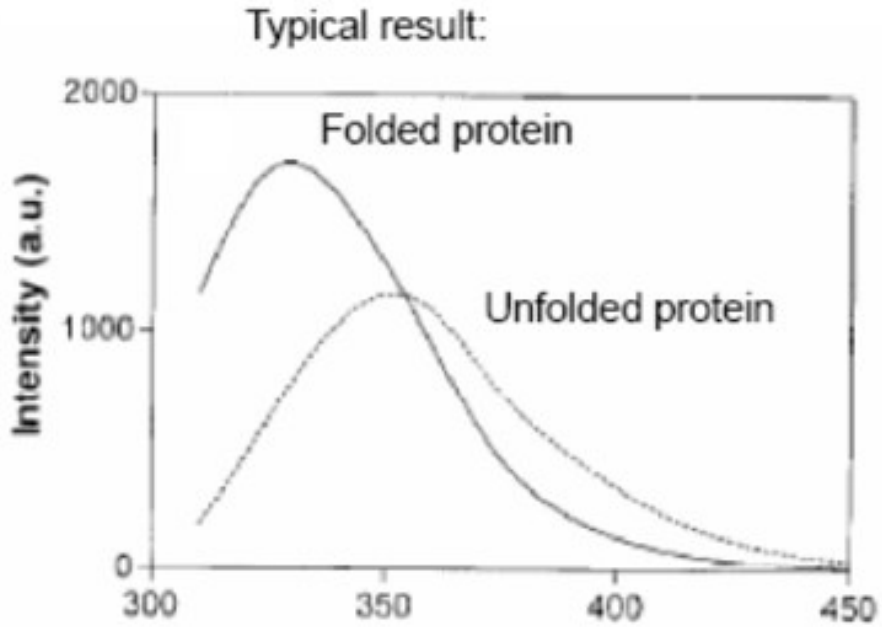
Spectroscopic techniques: Fluorescence Spectroscopy

Intrinsic protein fluorescence: Trp & Tyr

Because aromatic amino acids (phenylalanine, histidine, tryptophan, and tyrosine) emit fluorescence light, in particular, tryptophan has a more intense spectrum than the others.This light depends on the surrounding environment and allows for obtaining information on the state of a protein (e.g., if a TRP is buried or exposed to water).

The emission maximum is very sensitive to the polarity of the environment.
For example, a tryptophan in water emits at 350 nm, while a tryptophan well-protected by the protein emits at 330 nm.

Protein folding and unfolding



Wilson and Walker's - Principle and Techniques of Biochemistry and Molecular Biology - 8th edition 2018

Spectroscopic techniques: Fluorescence Spectroscopy

Extrinsic fluorophores & probes

When the target isn't fluorescent, attach a probe: non-perturbing, environment-sensitive, and tightly bound at one site.

ANS / SYPRO Orange

Amphiphilic dyes: weak in water, bright on hydrophobic patches (475→450 nm). Basis of differential scanning fluorimetry (DSF) for ligand binding & stability.

Fluorescamine / OPA

Conjugating reagents that derivatise primary amines for amino-acid analysis; OPA is non-fluorescent until it reacts.

Fura-2 / Indo-1 / Quin-2

Ca^{2+}-chelating probes for metal homeostasis in vitro and in cells (watch for cross-reactivity).

SYBR Green (→ replaced EtBr)

Intercalates dsDNA; emission rises ~30× on binding; safer, non-teratogenic vs ethidium bromide.

Spectroscopic techniques: Fluorescence Spectroscopy

Labelling with fluorescent proteins

Genetically fuse GFP (or a colour variant) to a target protein to turn it into a fluorescent reporter.

- **Genetic fusion** GFP joined N- or C-terminally to the target sequence.
- **Visualise & track** fluorescence microscopy; monitor expression (esp. membrane proteins).
- **"Living colour"** variants RFP, BFP, CFP, YFP enable real-time, multi-colour imaging.

Photo-activatable GFP: An engineered GFP that changes structure under a 413 nm pulse. Same colours as native GFP ($\lambda_{ex}{}^{c}$ **488 nm,** λ_{em} **509 nm**) — but emission jumps **~100×** after the activating flash, so a chosen pool of molecules can be switched on and followed.

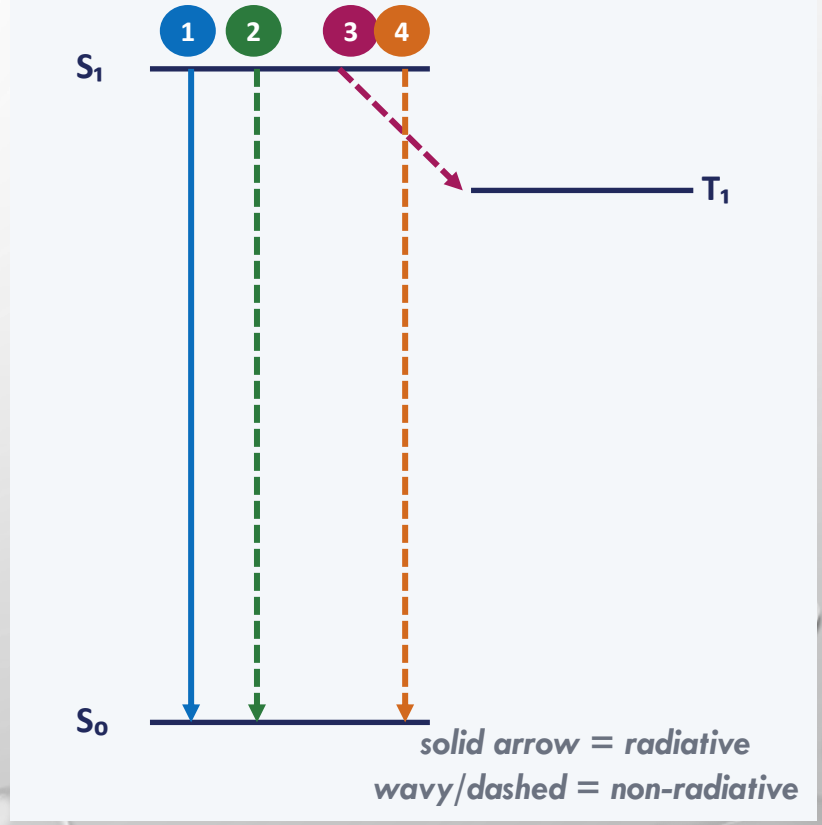
Spectroscopic techniques: Fluorescence Spectroscopy

Factors influencing the fluorescence phenomenon

All molecules that absorb in the visible/UV range are potentially fluorescent, but the phenomenon is actually significant only for some of them, because of non-radiative dissipative processes that compete with the emission of radiation.

Four deactivation pathways of the S_1 state at room temperature

1. **FLUORESCENCE EMISSION**
   $S_1 \longrightarrow S_0 + h\nu$
2. **INTERNAL CONVERSION**
   $S_1 \rightsquigarrow S_0 + \text{heat}$
3. **INTERSYSTEM CROSSING**
   $S_1 \rightsquigarrow T_1$
4. **COLLISIONAL QUENCHING**
   $S_1 + Q \rightsquigarrow S_0 + \text{heat}$



Spectroscopic techniques: Fluorescence Spectroscopy

Factors influencing the fluorescence phenomenon

There are four deactivation processes of the S_1 state at room temperature that allow the molecule to lose its excess electronic energy and return to the S_0 state.

An example of Q is oxygen (O_2), which quenches a significant fraction of the fluorescence of aromatic hydrocarbons.

4 **COLLISIONAL QUENCHING**

$$S_1 + Q \rightsquigarrow S_0 + \text{heat}$$

O_2

77K

** O_2 also inhibits phosphorescence. This explains why, although the phosphorescence of solids can easily be observed at room temperature, it is often impossible to observe the phosphorescence of solutions: O_2 molecules, absorbing energy through collisions with excited species, quench their phosphorescence. To avoid this, solutions are cooled in liquid nitrogen (77 K) and frozen.*

Spectroscopic techniques: Fluorescence Spectroscopy

Variables influencing the fluorescence phenomenon

1 **SOLVENT**

The solvent can interact with the ground state, but interacts even more strongly with the excited state.

2 **pH**

Can affect the electronic structure of the molecule, especially if it bears acidic hydrogens; e.g. many phenols are fluorescent at neutral or acidic pH, but not at alkaline pH.

3 **TEMPERATURE**

Affects the viscosity of the matrix and therefore the frequency of collisions with surrounding particles; measurements are generally taken at room temperature, protecting the sample from the heat generated by the lamps.

4 **MATRIX**

May contain substances that absorb part of the exciting or emitted radiation.

5 **QUENCHING**

Chemical interactions with particular species present in the matrix can attenuate the signal.

6 **CONCENTRATION**

Fluorescence emission increases linearly with concentration only at low concentration values.

M. Maccarrone, Biochemical and Biomolecular Methodologies, Zanichelli, 2019 — p. 22

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Spectroscopic techniques: Fluorescence Spectroscopy

Variables influencing the fluorescence phenomenon

When a molecule emits light, it has always absorbed energy beforehand. When a molecule absorbs energy, it does not always emit light.

Type of transition

Fluorescence is linked to the absorption of ultraviolet radiation with $\lambda > 250$ nm. Radiation of shorter wavelength carries enough energy that excited states are deactivated through different mechanisms, so fluorescence from $\sigma \rightarrow \sigma^*$ transitions is rarely observed. Emission is essentially limited to the lower-energy $\pi \longrightarrow \pi^*$ or $n \longrightarrow \pi^*$ process, whichever has the lower energy.

Empirically, fluorescence is most common in compounds whose lowest-energy transition is $\pi \longrightarrow \pi^*$: its quantum yield is higher, both because the transition is more probable and because the processes competing with fluorescence occur less readily.

Comparing the two transition types

	$\pi \longrightarrow \pi^*$	$n \longrightarrow \pi^*$
Excited-state lifetime	10^{-9}–10^{-7} s	10^{-7}–10^{-5} s
Relative quantum yield	Higher	Lower
Relative molar absorptivity	100–1000 × higher	reference
Fluorescence observed	Commonly	Rarely

M. Maccarrone, Biochemical and Biomolecular Methodologies, Zanichelli, 2019 — p. 23

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Spectroscopic techniques: Fluorescence Spectroscopy

Applications

Qualitative analysis

- Identification of the structure of a substance (comparison of absorption and emission spectra; analysis of the effects of pH, solvent, and decay time).
- Analysis of protein structure (fluorescence of aromatic residues).
- Energy-transfer studies: resonance energy transfer between a donor and an acceptor, occurring when they are at the appropriate distance and the emission spectrum of the donor overlaps the absorption spectrum of the acceptor.
- Cell separation by fluorescent antibody labelling.
- Enzyme assays and measurements of enzyme kinetics.

Spectroscopic techniques: Fluorescence Spectroscopy

Applications

Quantitative analysis

- Measurement of the concentration of fluorescent molecules (intrinsic fluorescence) or of non-fluorescent molecules after derivatization with fluorophores or by using fluorescent probes (extrinsic fluorescence).

- The fluorescence of some fluorophores is quenched when they bind to a specific molecule or macromolecule (e.g., ethidium bromide–DNA). This phenomenon can be exploited to quantify materials present at very low concentrations.

Spectroscopic techniques: Fluorescence Spectroscopy

FRET: a spectroscopic ruler

Resonance energy transfer due to fluorescence

- A phenomenon in which energy is transferred between two fluorophores.

- A widely used technique for visualizing biological molecules (such as proteins, lipids, and nucleic acids) in relation to one another.

- It allows the distance between two molecules to be determined and characterized with high precision. The mechanism uses two fluorescent molecules, called the donor and the acceptor.

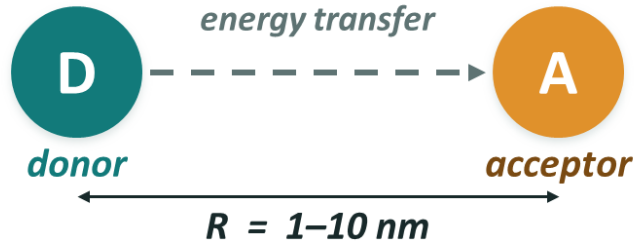
Spectroscopic techniques: Fluorescence Spectroscopy

FRET: a spectroscopic ruler

Non-radiative energy transfer from an excited donor to a nearby acceptor — steeply distance-dependent ($\propto 1/R^6$).

REQUIREMENTS

- **Spectral overlap** donor emission ↔ acceptor excitation ("resonance").
- **Close proximity** 10–100 Å between the two chromophores.
- **Parallel dipoles** near-parallel transition dipoles.



The spectroscopic ruler

Because efficiency $\propto 1/R^6$, FRET is exquisitely sensitive to 1–10 nm distances — ideal for watching **molecular interactions and conformational changes** (protein–protein, protein–DNA, protein–ligand), even in single molecules and living cells, in real time. Common pairs like CFP/YFP drive enzyme, protease and **high-throughput drug-screening** assays. A non-fluorescent acceptor can be used to cut background.

Spectroscopic techniques: Fluorescence Spectroscopy

FRET: spectra overlap

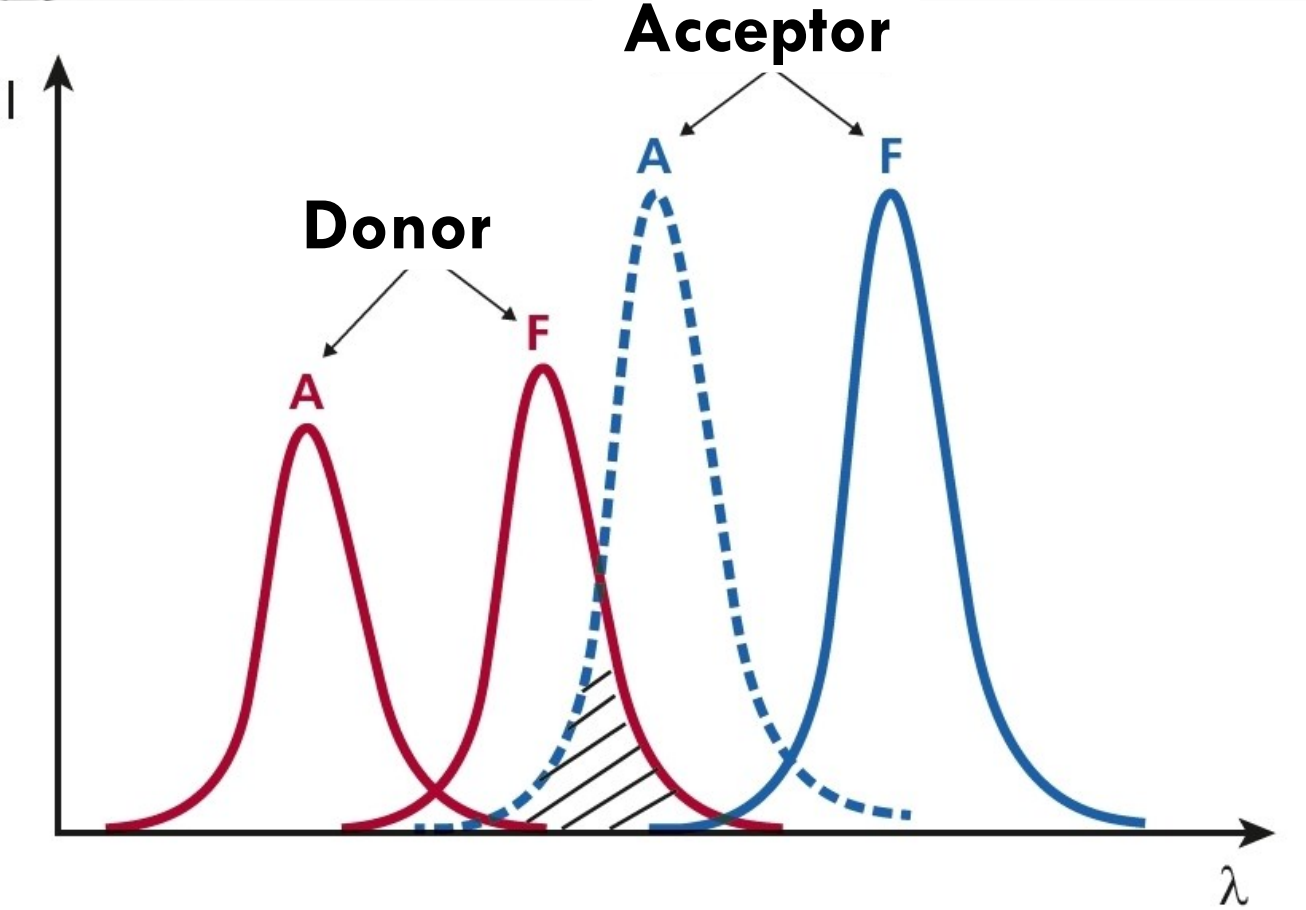


Figure 6.9 — Resonance energy transfer between donor and acceptor.

The hatched region represents the overlap between the donor's fluorescence emission and the acceptor's absorption spectrum.

A = absorption; F = fluorescence.

For the process to occur, the donor emission energy must correspond to the acceptor absorption energy — the two must be in resonance.

If the acceptor is not fluorescent, the energy is dissipated through non-radiative processes.

Spectroscopic techniques: Fluorescence Spectroscopy

Fluorescent proteins as FRET probes

- The most widely used fluorophores in FRET belong to the GFP (Green Fluorescent Protein) family.
- These protein molecules are much easier to handle than classical organic fluorophores, which can present substantial problems with purification, chemical modification, and intracellular delivery.
- GFP and its derivatives — RFP (red), BFP and CFP (blue), and YFP (yellow) — can be fused to the protein of interest using genetic-engineering technologies.
- This makes fluorescent proteins powerful genetically encoded reporters for monitoring protein behavior and molecular proximity.

Spectroscopic techniques: Fluorescence Spectroscopy

GFP: structure and chromophore formation

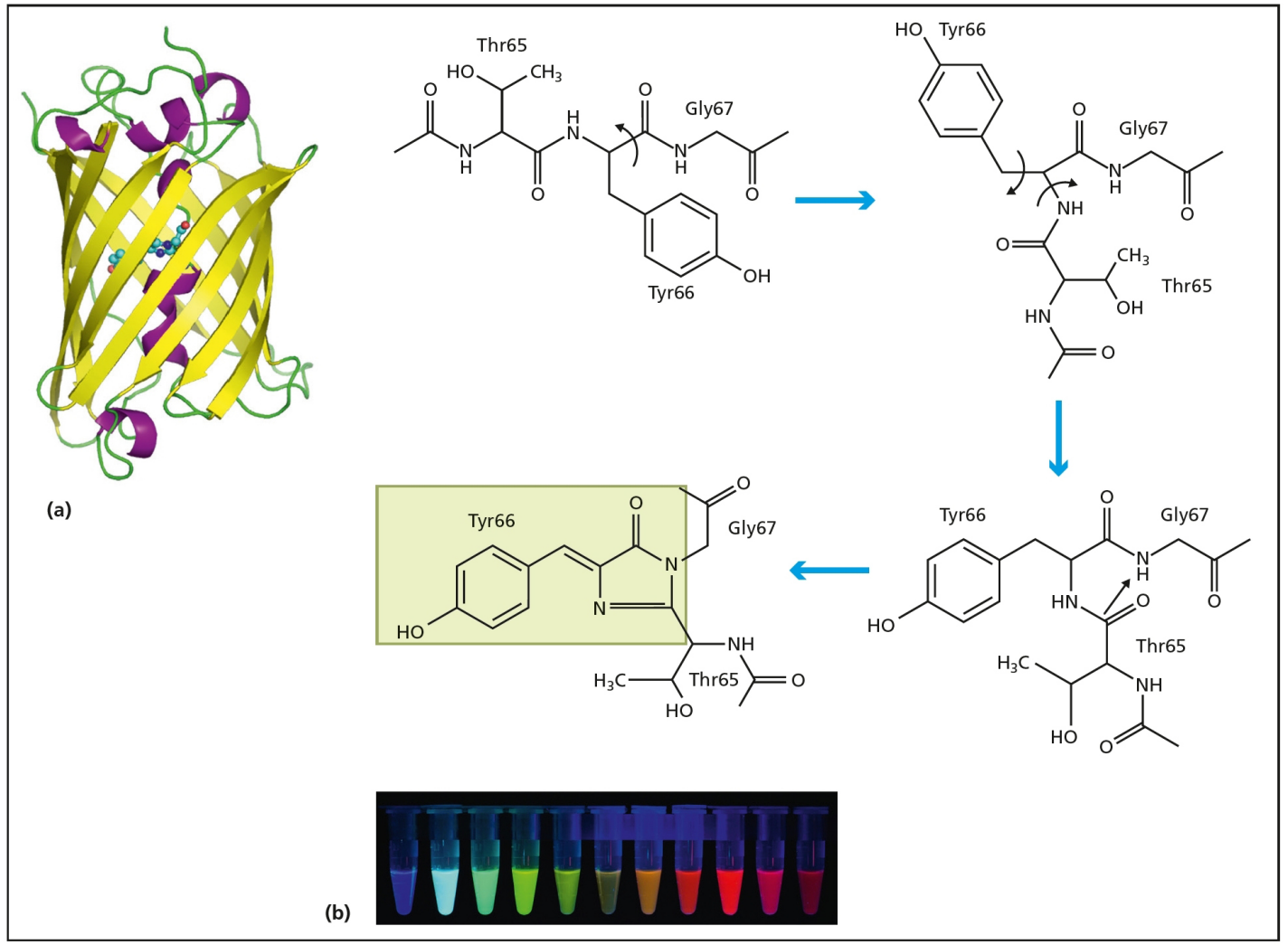
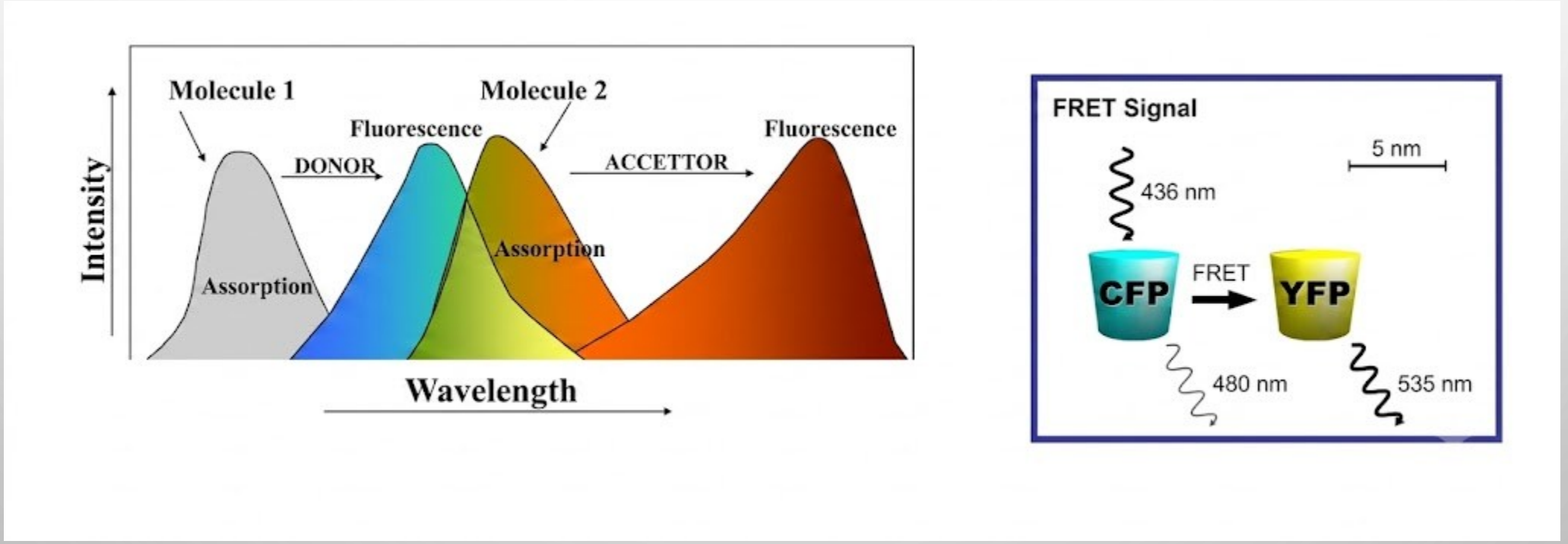


Figure 6.10
(a) Crystal structure of green fluorescent protein (GFP) from the jellyfish Aequorea victoria, showing the fluorophore inside the β-barrel. (b) Chromophore formation from Thr65, Tyr66, and Gly67 through torsion, cyclization, dehydration, and oxidation. Different mutations have generated a wide range of fluorescent proteins with distinct absorption and emission spectra.

Spectroscopic techniques: Fluorescence Spectroscopy

CFP–YFP as a FRET pair

In the figure, the donor is CFP (Cyan Fluorescent Protein) and the acceptor is YFP (a variant of Yellow Fluorescent Protein). The CFP emission spectrum and the YFP absorption spectrum overlap extensively between 450 and 550 nm (gray area), making them a suitable pair of molecules for FRET.

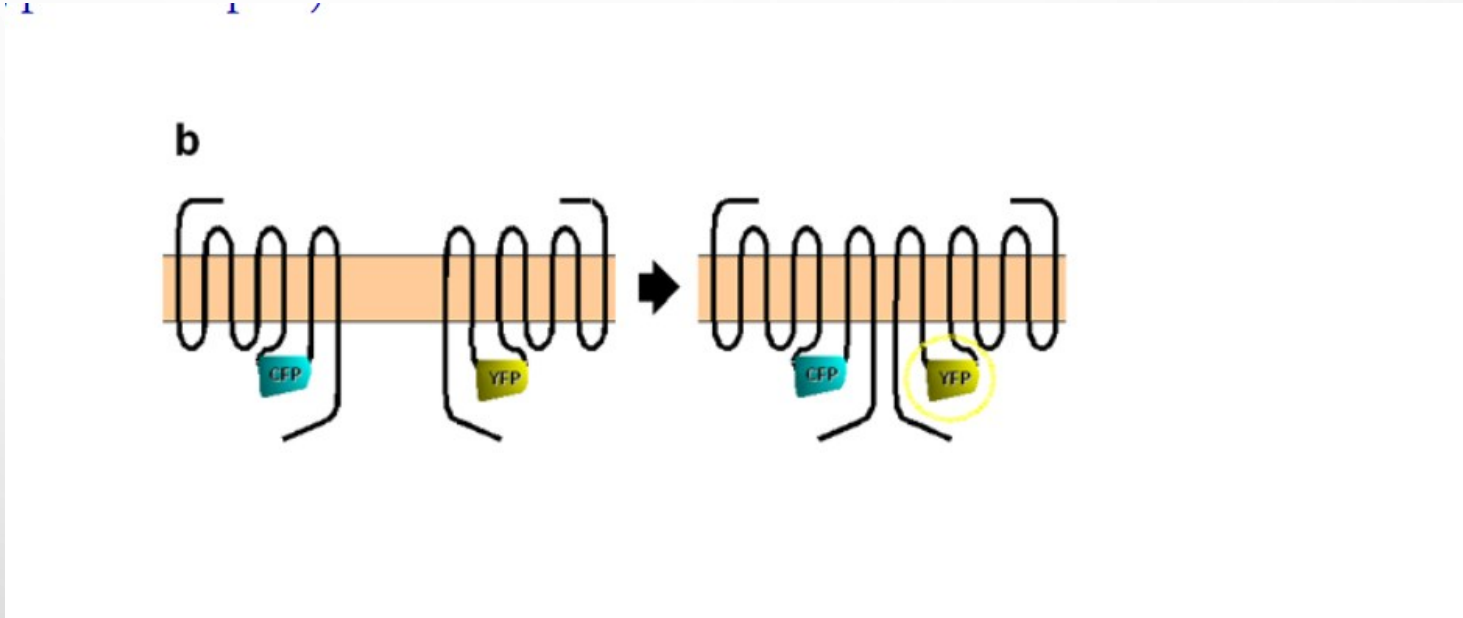


Spectroscopic techniques: Fluorescence Spectroscopy

Applications to macromolecular interactions

FRET is a useful tool for quantifying interactions between biological macromolecules, including:

- **protein–protein**
- **protein–nucleic acid**
- **protein–lipid**



Spectroscopic techniques:

Fluorescence Recovery After Photobleaching (FRAP)

Measuring molecular mobility and diffusion

Principle

- A high-intensity light pulse irreversibly bleaches fluorophores in a selected region.
- The bleached region shows a sharp drop in fluorescence.
- Unbleached molecules diffuse into the observation area.
- Fluorescence recovery is monitored over time.
- The recovery rate allows calculation of diffusion coefficients.

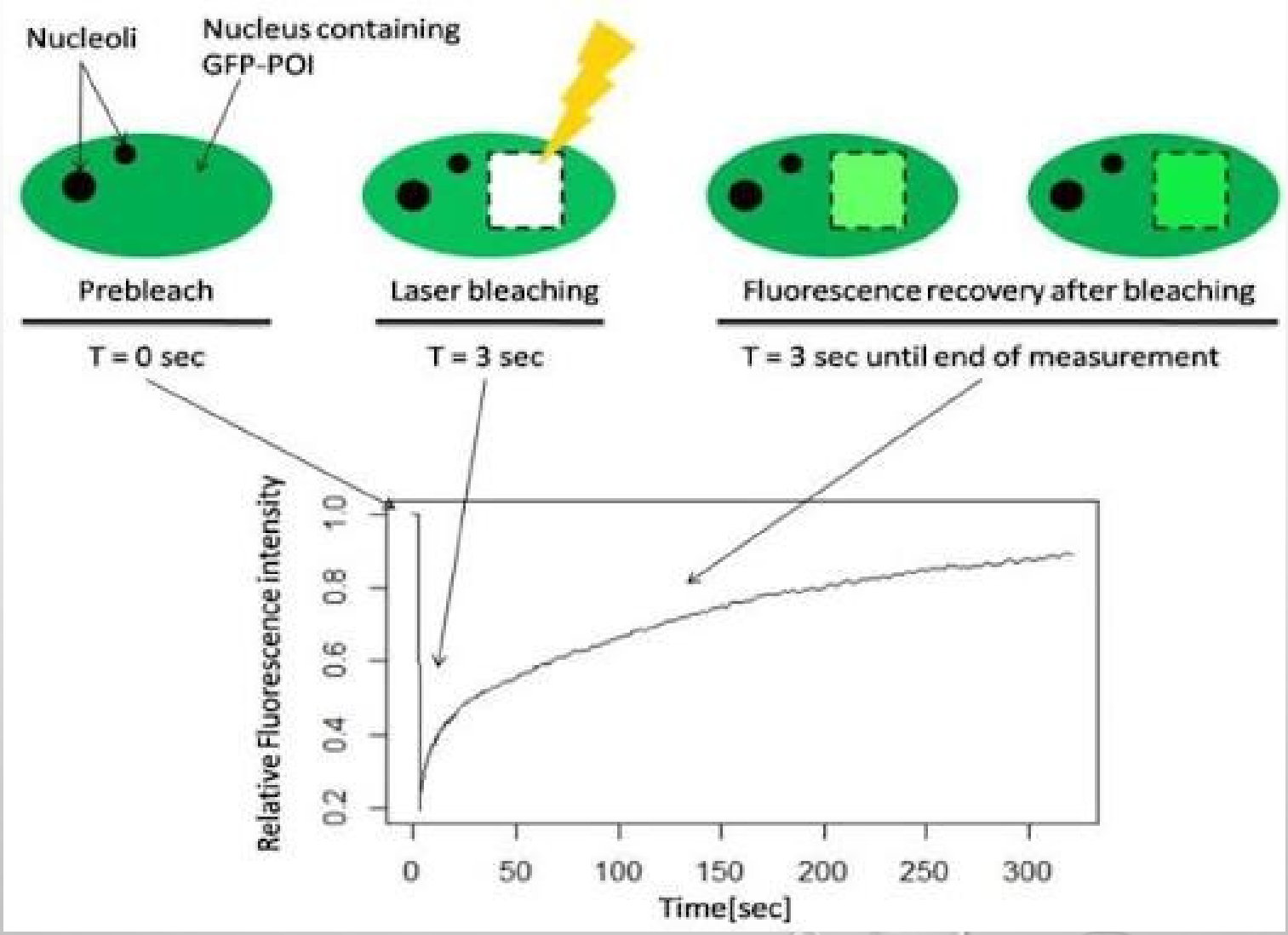
Typical applications

- NBD-labelled phospholipids incorporated into biological or artificial membranes.
- Membrane proteins such as receptors.
- Fluorescently labelled proteins inside cells.

Bleach → diffusion → fluorescence recovery

Spectroscopic techniques:

Fluorescence Recovery After Photobleaching (FRAP)



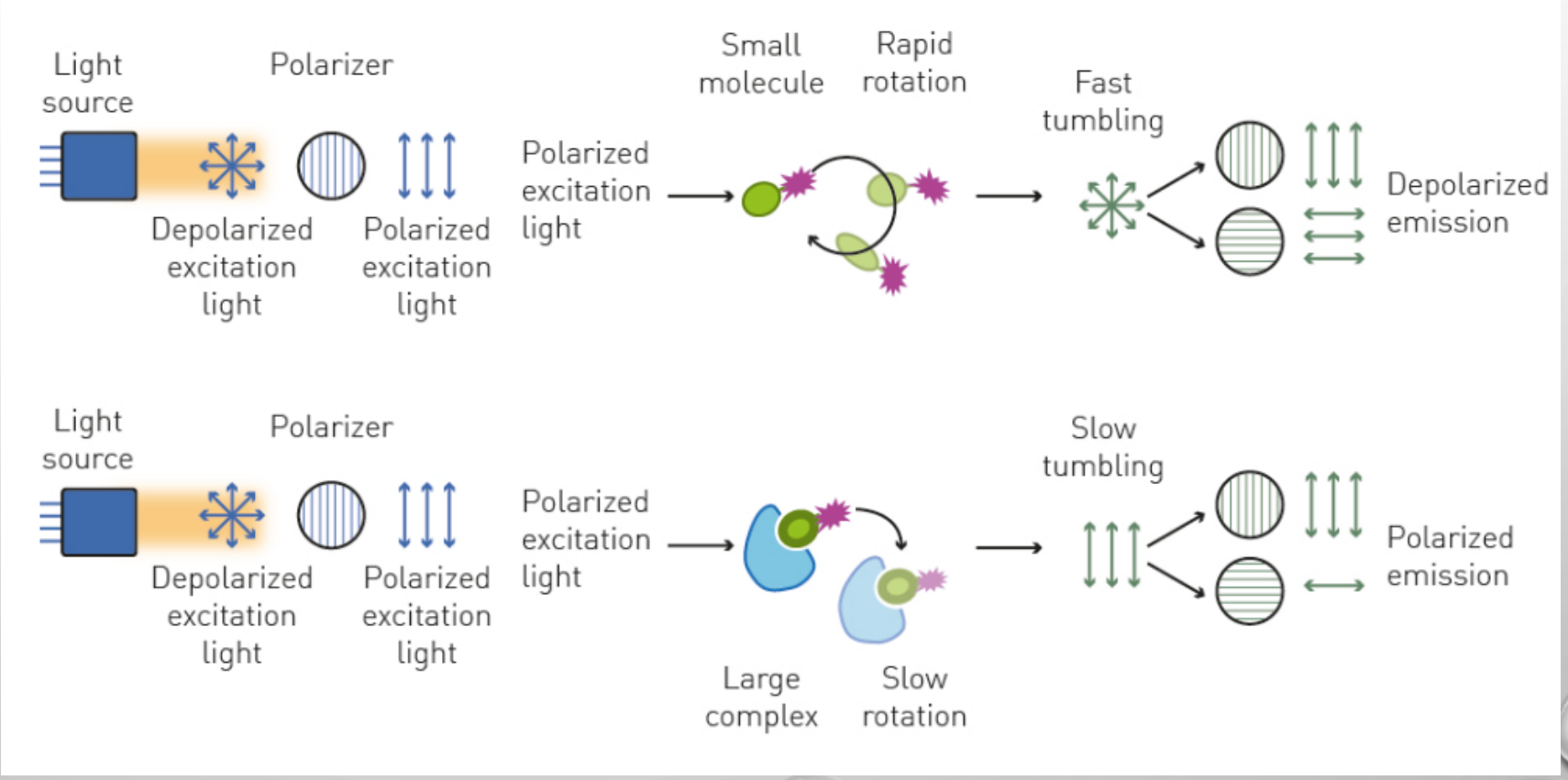
Spectroscopic techniques:

Fluorescence Polarisation

Information on molecular orientation and mobility

- Fluorescence polarisation provides information on the orientation and mobility of fluorescent molecules.
- Absorption of polarised excitation light is highest when the polarisation plane is parallel to the absorption dipole moment.

- For randomly oriented chromophores in solution, polarisation P is below 0.5.
- Reduced rotational freedom, for example after binding to a larger molecule, changes the measured polarisation.
- Temperature and solvent viscosity affect Brownian motion and therefore polarisation.
- Experimentally, a polariser is placed in the excitation path and a second polariser is placed before the detector, parallel or perpendicular to the first.



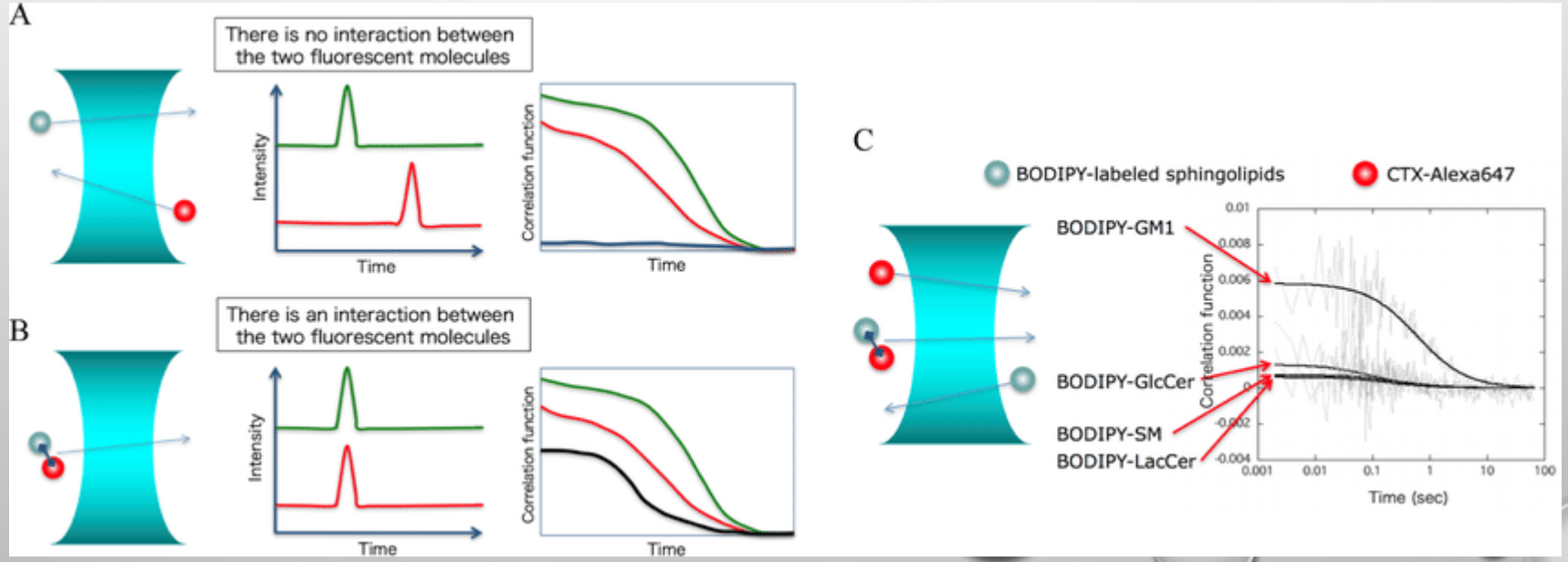
Spectroscopic techniques:

Fluorescence Cross-Correlation Spectroscopy (FCCS)

Detecting correlated molecular fluctuations

- FCCS measures temporal fluorescence fluctuations between two differently labelled molecules as they diffuse through a small sample volume.
- Separate detection channels record the two fluorescence signals.
- Cross-correlation analysis extracts information on the dynamics of dual-labelled molecules.
- The technique can characterize diffusion coefficients.
- It provides binding constants and kinetic rates of binding.
- It can determine molecular interactions in solutions and cells.

Channel 1 + Channel 2 → cross-correlation → molecular interaction / dynamics

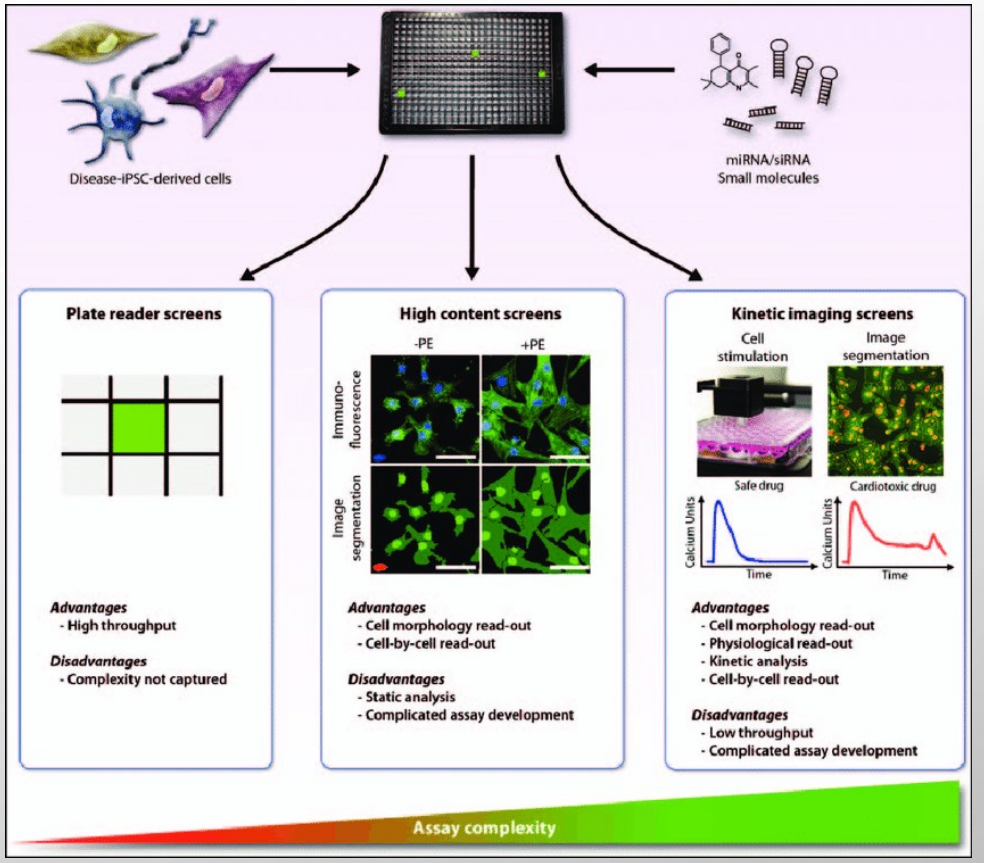


Spectroscopic techniques:

Fluorescence Microscopy & High-Throughput Assays

Spatial and temporal tracking of labelled biomolecules

- Fluorescence emission is a valuable monitoring tool in biological and biochemical applications.
- Proteins or other macromolecules can be tagged with GFP or RFP.
- Fluorescent proteins enable spatial and temporal tracking of labelled species.
- CFP can act as a fluorescence donor and YFP as an acceptor for FRET-based mechanistic studies.
- Cells expressing labelled proteins are illuminated at the excitation wavelength and observed through a filter that excludes the exciting light.
- Fluorescence images can be overlaid computationally with visual images for localisation; distinct labels can also enable colocalisation studies.

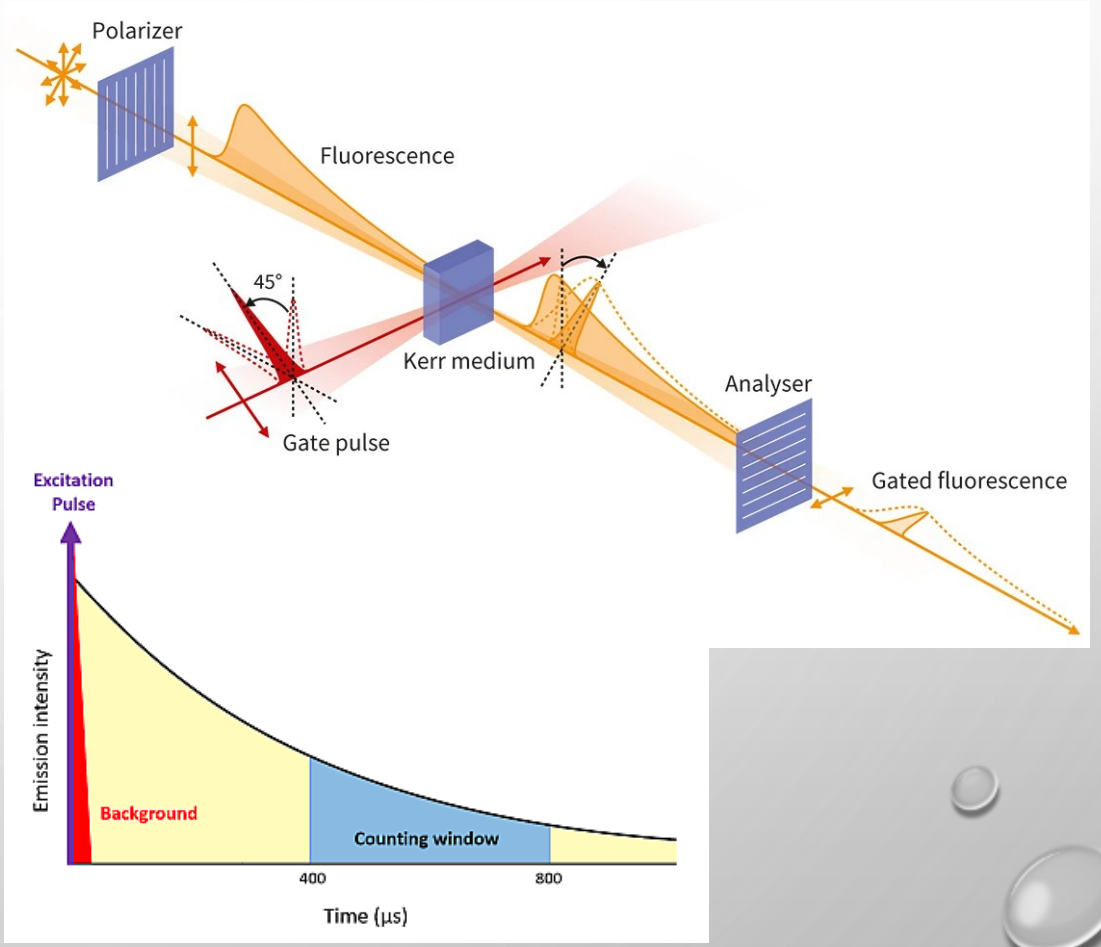


Spectroscopic techniques:

Time-Resolved Fluorescence Spectroscopy

Measuring fluorophore lifetimes

- Emission of a single photon follows a probability distribution.
- Time-correlated single-photon counting records emitted photons after pulsed excitation.
- Sampling many excitation cycles reconstructs the probability distribution.
- Fluorescence decay follows an exponential distribution; its time constant is the fluorophore lifetime.
- Curve fitting can identify the number of fluorescent species, within certain limits, and determine their lifetimes.
- Different species may correspond to different fluorophores or distinct conformations of the same fluorophore.



Pulsed excitation → photon counting vs. time → decay curve → fluorescence lifetime

Spectroscopic techniques:

Phosphorescence

- Phosphorescence arises from a transition from a triplet excited state (T) to the singlet ground state (S_0).
- The molecule reaches the triplet state from an excited singlet state through intersystem crossing (ISC).
- The singlet-to-triplet transition is quantum-mechanically forbidden and occurs with low probability in favourable molecular structures.
- Such molecules often contain heavy atoms.
- Phosphorescence is much slower than fluorescence, so emission is delayed and can persist after excitation stops.
- Instrumentation exploits this property by introducing a delay between excitation and measurement.
- Phosphorescence is observed at low temperature or in highly viscous media.

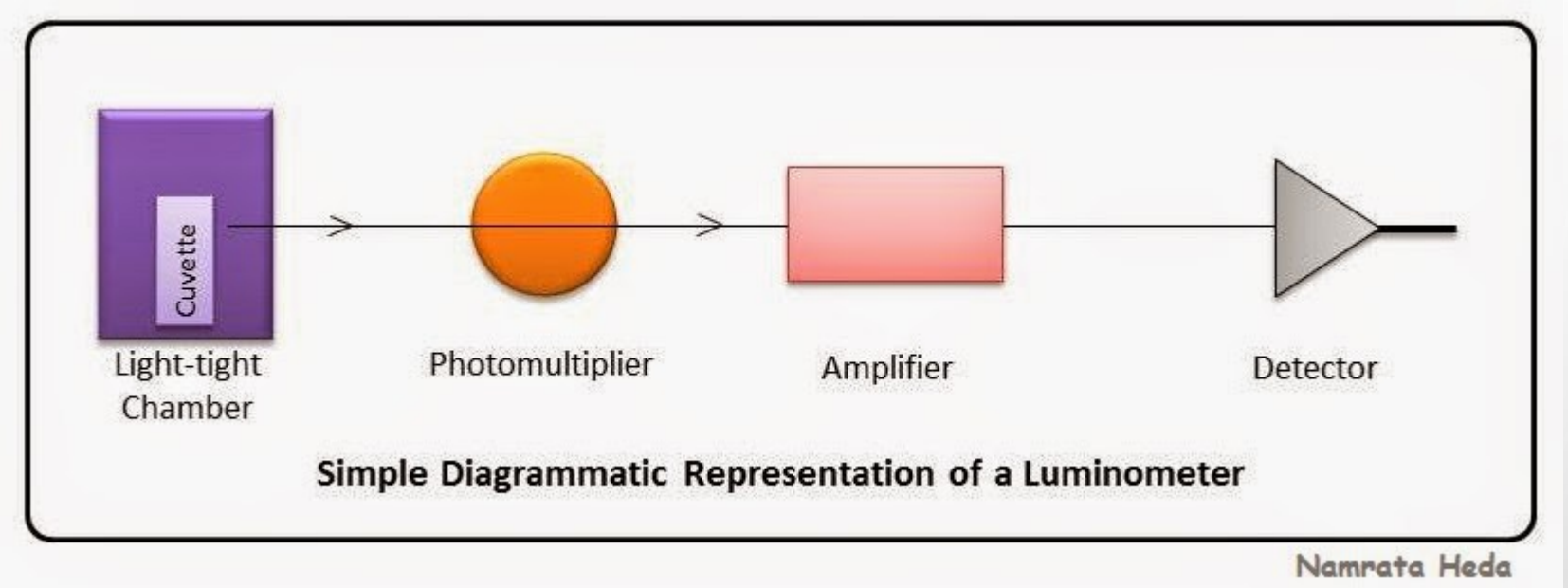
$$S_1 \xrightarrow{ISC} T_1 \xrightarrow{\text{phosphorescence}} S_0$$

Luminometry

Measuring luminescence produced by chemical or enzymatic reactions

- Luminometry measures luminescence: emission of visible electromagnetic radiation generated by a reaction.
- Unlike fluorescence, luminescence does not require external optical excitation.
- Chemiluminescence results from chemical excitation; for example, luminol + oxygen produces 3-aminophthalate, whose fluorescence is observed as chemiluminescence.
- Bioluminescence is the analogous phenomenon in which the reaction leading to the emitting product is enzymatic.
- Luciferase is the most commonly used enzyme in bioluminescence.
- Bioluminescence is highly sensitive because the underlying reactions can have very high quantum yields.
- Because there is no optical excitation, autofluorescence problems in assays are eliminated.

Luminometry: Instrumentation



- No light source or monochromator is required.
- A photomultiplier tube is commonly used to amplify the output signal.
- Strict temperature control is important because chemical and especially enzymatic reactions are temperature-sensitive.

Luminometry: Applications: chemiluminescence

- Luminol and its derivatives can undergo highly efficient chemiluminescent reactions.
- Enzymatically generated H_2O_2 can be detected through light emission at about 430 nm in the presence of luminol and microperoxidase.
- Competitive binding assays can determine low concentrations of hormones, drugs and metabolites in biological fluids.
- These assays rely on competition between labelled and unlabelled ligands for high-affinity binding sites on proteins such as antibodies or cell receptors.
- If the concentration of binding protein is known, the concentration of unlabelled ligand can be determined under saturation conditions.
- Using only labelled ligand allows determination of protein concentration and therefore the number of available binding sites.
- Chemiluminescence can also monitor oxidative processes in leukocytes during phagocytosis and the effects of pharmacological or toxicological agents.

Spectroscopic techniques:

A FRET-based approach using native fluorescent or luminescent proteins

Principle:

- BRET uses the FRET effect with native fluorescent or luminescent proteins as chromophores.
- A natural example occurs in the sea pansy Renilla reniformis.
- Renilla luciferase oxidises coelenterazine and emits light at about 480 nm.
- This light directly excites GFP, which then emits fluorescence at about 509 nm.

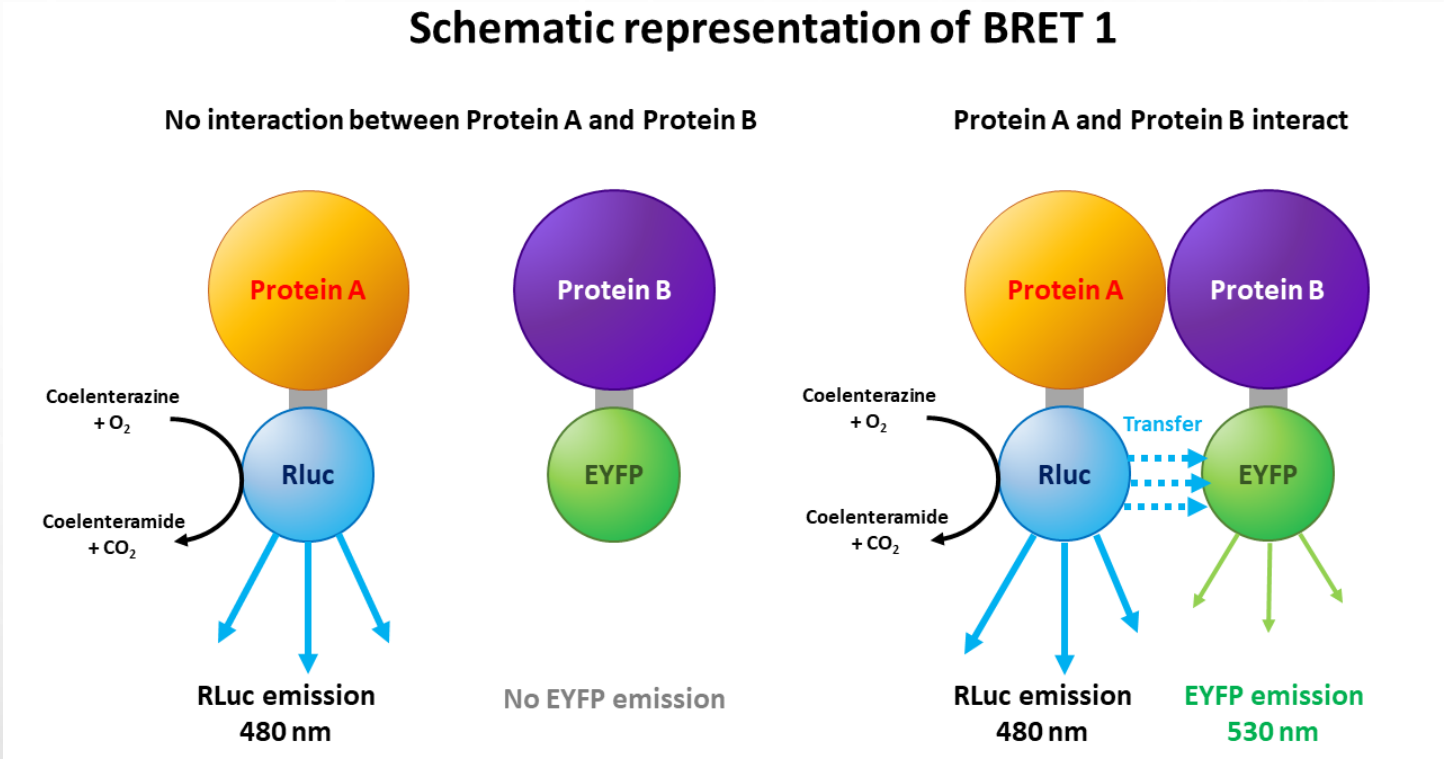
Conceptual energy-transfer scheme

Renilla luciferase → GFP

480 nm → 509 nm

Luciferase emission → GFP excitation → GFP fluorescence

Schematic representation of BRET 1



Spectroscopic techniques: BRET

Application: quantitative monitoring of protease activity

A protease substrate is engineered as a fusion protein containing a GFP variant at the N-terminal side and dRED at the C-terminal side.

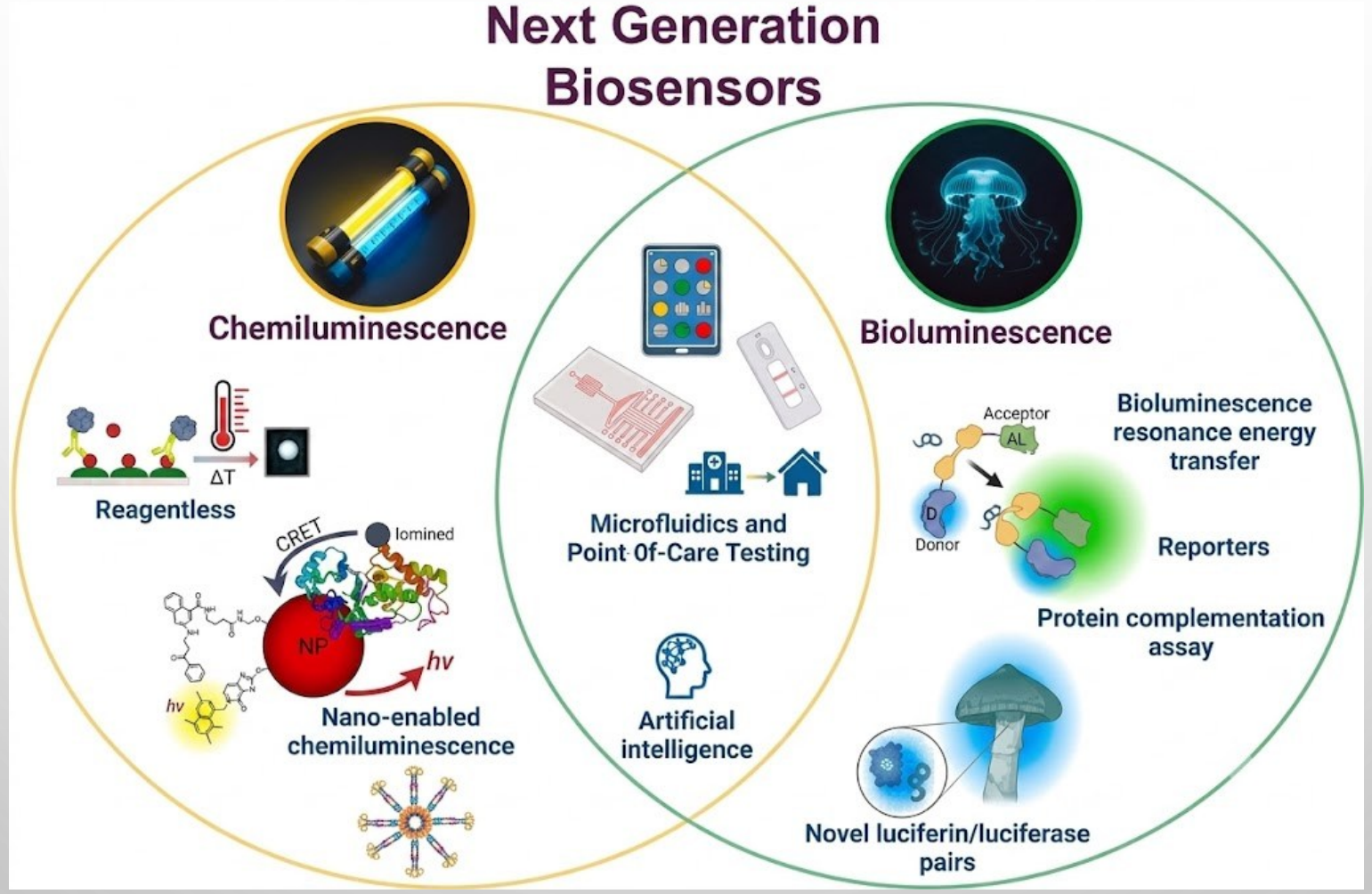
- GFP acts as the FRET donor.
- dRED acts as the red-fluorescent FRET acceptor.
- Protease cleavage separates donor and acceptor, abolishing the FRET effect.
- The system can therefore directly monitor protease activity.
- Combined with two-photon excitation spectroscopy, FRET analysis can also support kinetic analysis.

GFP — protease substrate — dRED

Luminometry:
Applications: bioluminescence

- Firefly luciferase is widely used to measure ATP concentrations.
- Bioluminescence assays can be substantially more sensitive than spectrophotometric and fluorimetric assays.
- The method can be applied to ATP-interconversion systems and to ADP, AMP and cyclic AMP.
- Enzymes that can be assayed include pyruvate kinase, adenylate kinase, phosphodiesterase, creatine kinase, hexokinase and ATP sulfurylase.
- Other substrates include creatine phosphate, glucose, GTP, phosphoenolpyruvate and 1,3-diphosphoglycerate.
- Bacterial luciferase can determine reduced electron-transfer cofactors such as NADH, NADPH and reduced flavin mononucleotides.
- Luciferase-coupled assays can be applied to a broad range of redox enzyme reaction systems.

Luminometry: Applications



Shining light on biosensors: Chemiluminescence and bioluminescence in enabling technologies; Roda B., et al. (2024), TrAC Trends in Analytical Chemistry Volume 180, 117975