Lecture 15

Fluorescence spectroscopy and imaging:

Basic principles and sources of contrast

Outline for Fluorescence

I. Principles of Fluorescence
II. Quantum Yield and Lifetime
III. Fluorescence Spectroscopy
IV. Biological Fluorophores
V. Fluorescence Instrumentation
VI. Fluorescence Measurements

I. Principles of Fluorescence

1. Luminescence

- Emission of photons from electronically excited states

- Two types of luminescence:

  Relaxation from singlet excited state
  Relaxation from triplet excited state

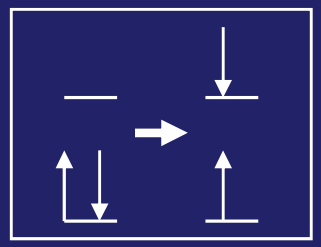
I. Principles of Fluorescence

2. Singlet and triplet states

- Ground state – two electrons per orbital; electrons have opposite spin and are paired

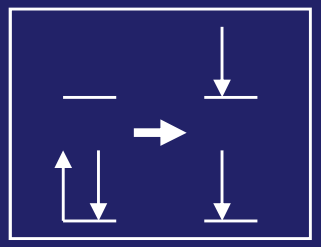
- Singlet excited state

  Electron in higher energy orbital has the opposite spin orientation relative to electron in the lower orbital



- Triplet excited state

  The excited valence electron may spontaneously reverse its spin (spin flip). This process is called intersystem crossing. Electrons in both orbitals now have same spin orientation



I. Principles of Fluorescence

3. Types of emission

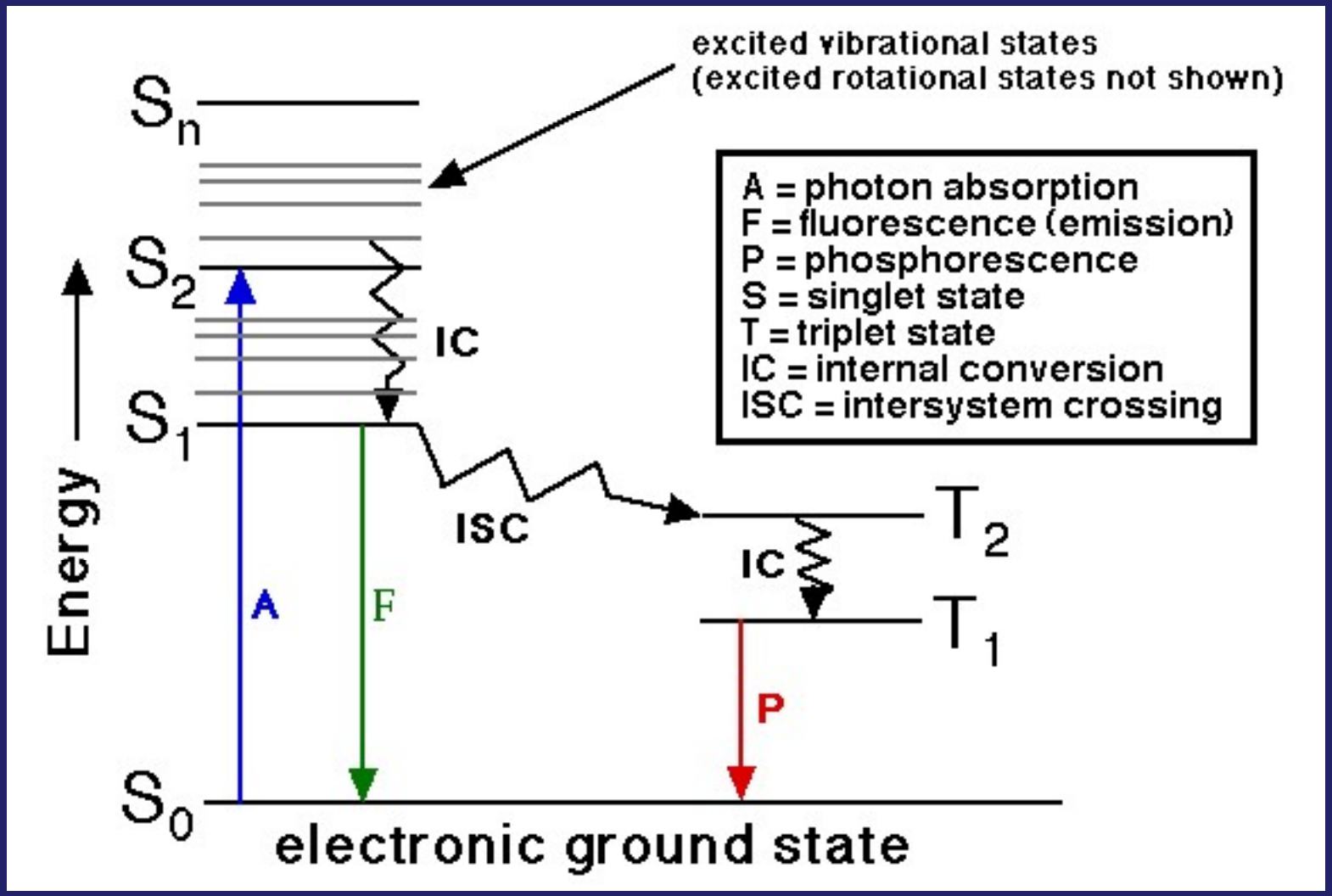
- Fluorescence – return from excited singlet state to ground state; does not require change in spin orientation (more common of relaxation)

- Phosphoresence – return from a triplet excited state to a ground state; electron requires change in spin orientation

- Emissive rates of fluorescence are several orders of magnitude faster than that of phosphorescence

I. Principles of Fluorescence

4. Energy level diagram (Jablonski diagram)



I. Principles of Fluorescence

5a. Fluorescence process: Population of energy levels

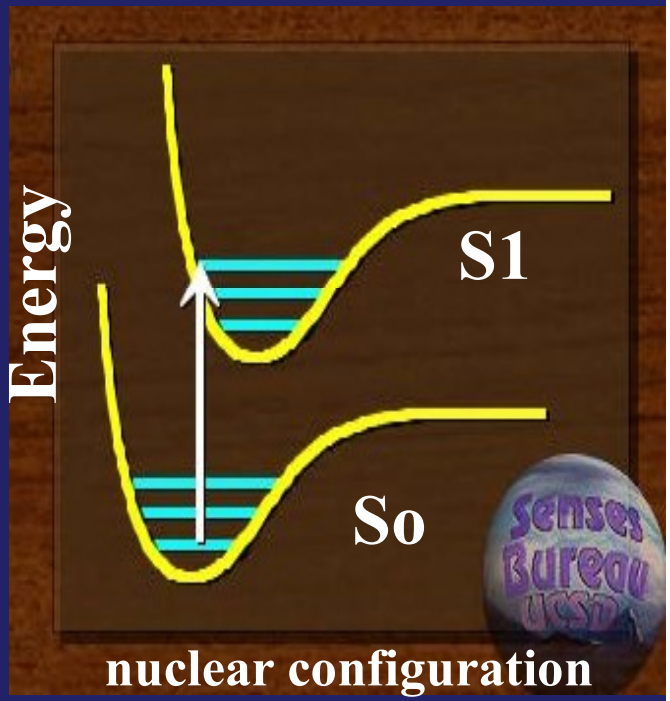
- At room temperature (300 K), and for typical electronic and vibration energy levels, can calculate the ratio of molecules in upper and lower states

$$\frac{n_{upper}}{n_{lower}} = \exp\left(-\Delta E / kT\right)$$

k=1.38*10^{-23} JK^{-1} (Boltzmann's constant)
ΔE = separation in energy level

I. Principles of Fluorescence

5b. Fluorescence process: Excitation



- At room temperature, everything starts out at the lowest vibrational energy level of the ground state

- Suppose a molecule is illuminated with light at a resonance frequency

- Light is absorbed; for dilute sample, Beer-Lambert law applies $A = \varepsilon c l$
  where ε is molar absorption (extinction) coefficient (M^{-1} cm^{-1}); its magnitude reflects probability of absorption and its wavelength dependence corresponds to absorption spectrum

- Excitation - following light absorption, a chromophore is excited to some *higher* vibrational energy level of S_1 or S_2

- The absorption process takes place on a time scale (10^{-15} s) much faster than that of molecular vibration → "vertical" transition (Franck-Condon principle).

I. Principles of Fluorescence

5c. Fluorescence process: Non-radiative relaxation

- In the excited state, the electron is promoted to an anti-bonding orbital→ atoms in the bond are less tightly held → shift to the right for S_1 potential energy curve →electron is promoted to higher vibrational level in S_1 state than the vibrational level it was in at the ground state

- Vibrational deactivation takes place through intermolecular collisions at a time scale of 10^{-12} s (faster than that of fluorescence process)

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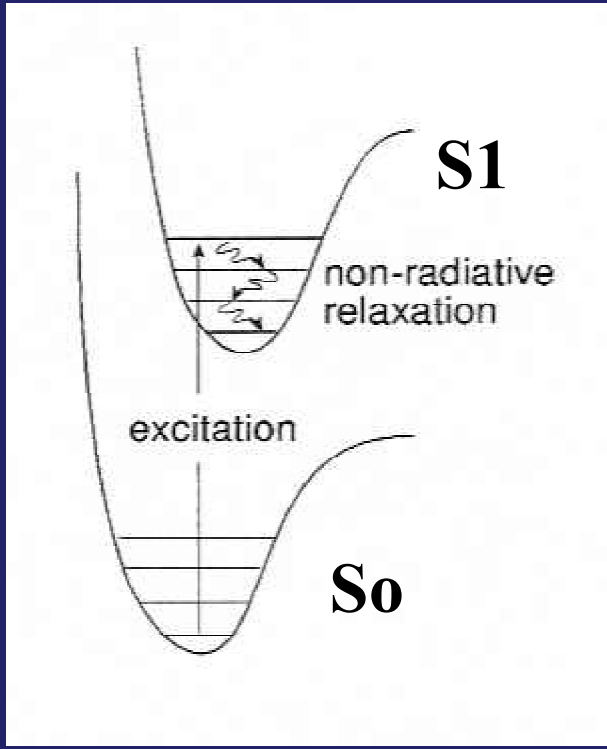
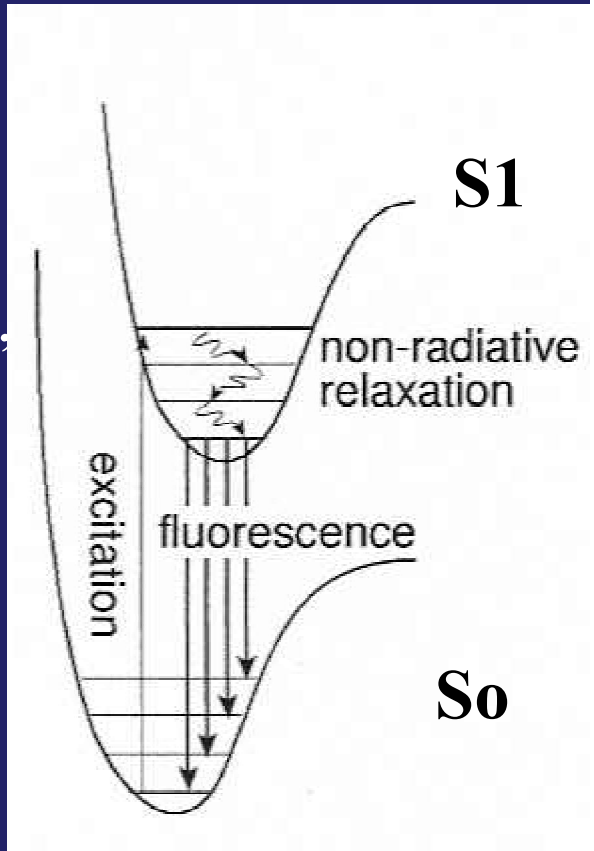


I. Principles of Fluorescence

5d. Fluorescence process: Emission

- The molecule relaxes from the lowest vibrational energy level of the excited state to a vibrational energy level of the ground state (10^{-9} s)
- Relaxation to ground state occurs faster than time scale of molecular vibration → "vertical" transition
- The energy of the emitted photon is lower than that of the incident photons



I. Principles of Fluorescence

6a. Stokes shift

- The fluorescence light is red-shifted (longer wavelength than the excitation light) relative to the absorbed light ("Stokes shift").

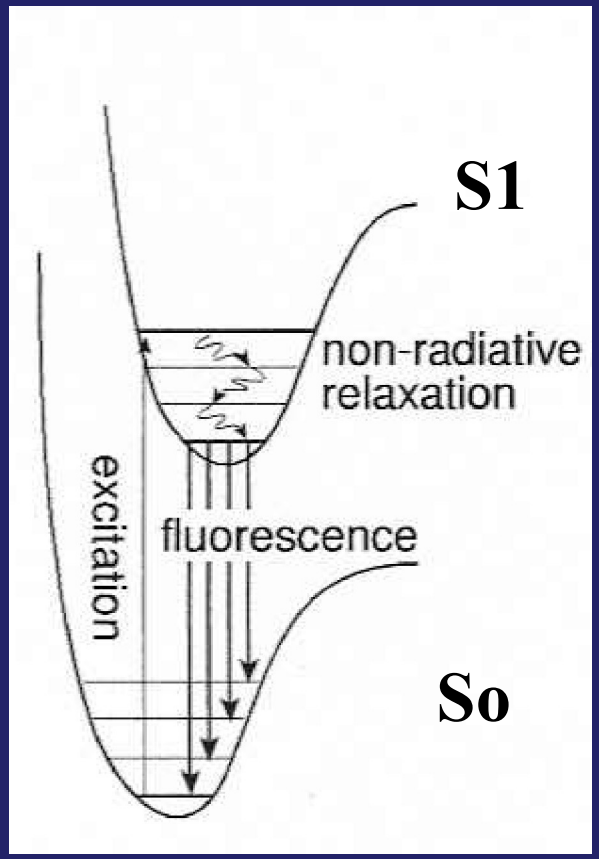
- Internal conversion (see slide 13) can affect Stokes shift

- Solvent effects and excited state reactions can also affect the magnitude of the Stoke's shift

I. Principles of Fluorescence

6b. Invariance of emission wavelength with excitation wavelength

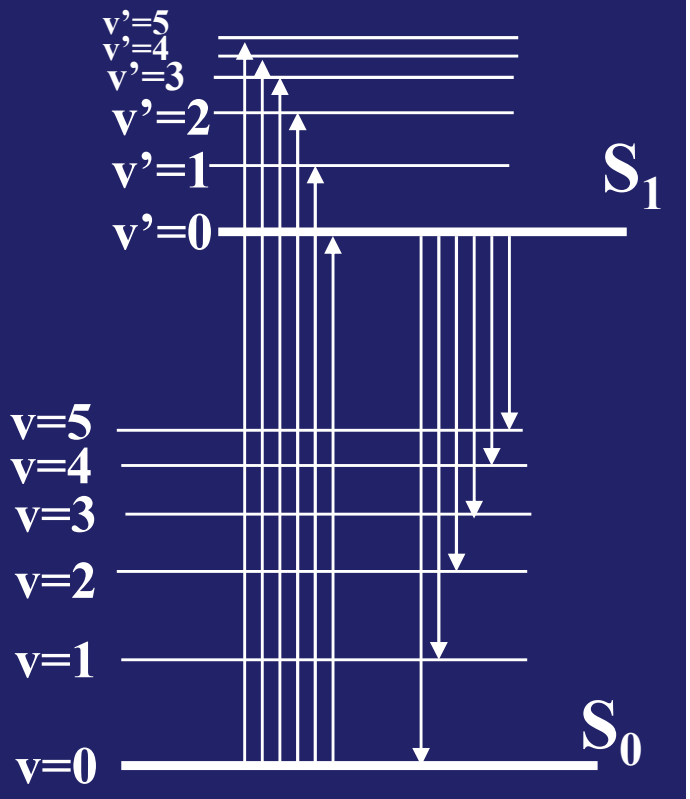
- Emission wavelength only depends on relaxation back to lowest vibrational level of S_1
- For a molecule, the same fluorescence emission wavelength is observed irrespective of the excitation wavelength



I. Principles of Fluorescence

6c. Mirror image rule

- Vibrational levels in the excited states and ground states are similar
- An absorption spectrum reflects the vibrational levels of the electronically excited state
- An emission spectrum reflects the vibrational levels of the electronic ground state
- Fluorescence emission spectrum is mirror image of absorption spectrum



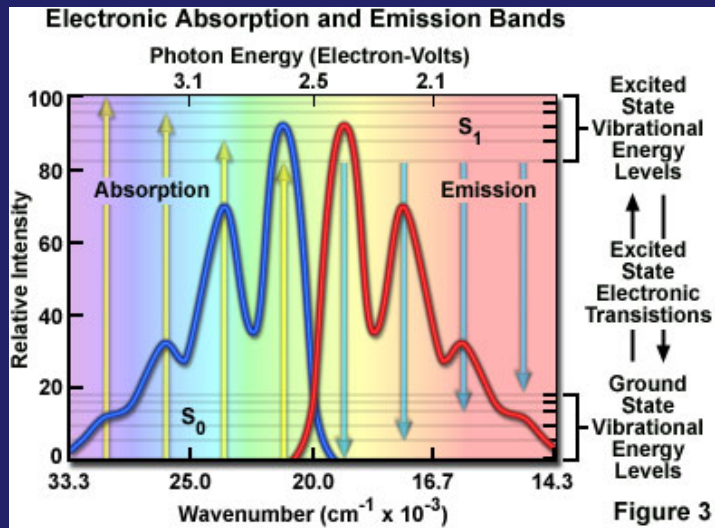
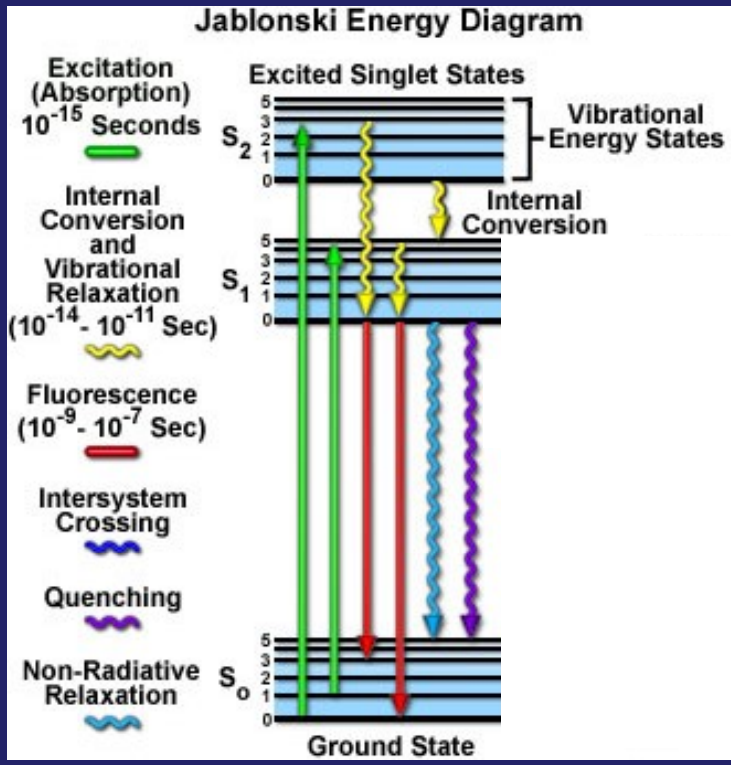


Figure 3

I. Principles of Fluorescence

6d. Internal conversion vs. fluorescence emission

- As electronic energy increases, the energy levels grow more closely spaced
- It is more likely that there will be overlap between the high vibrational energy levels of S_{n-1} and low vibrational energy levels of S_n
- This overlap makes transition between states highly probable
- **Internal conversion** is a transition occurring between states of the same multiplicity and it takes place at a time scale of 10^{-12} s (faster than that of fluorescence process)
- The energy gap between S_1 and S_0 is significantly larger than that between other adjacent states → S_1 lifetime is longer → radiative emission can compete effectively with non-radiative emission



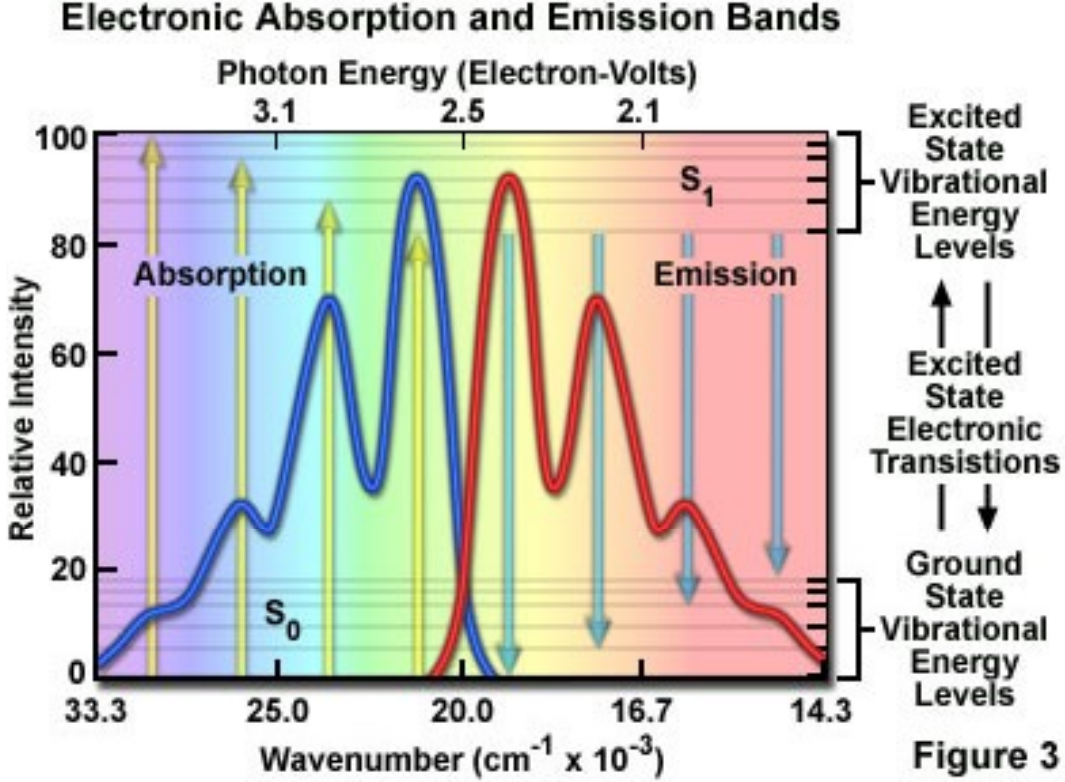
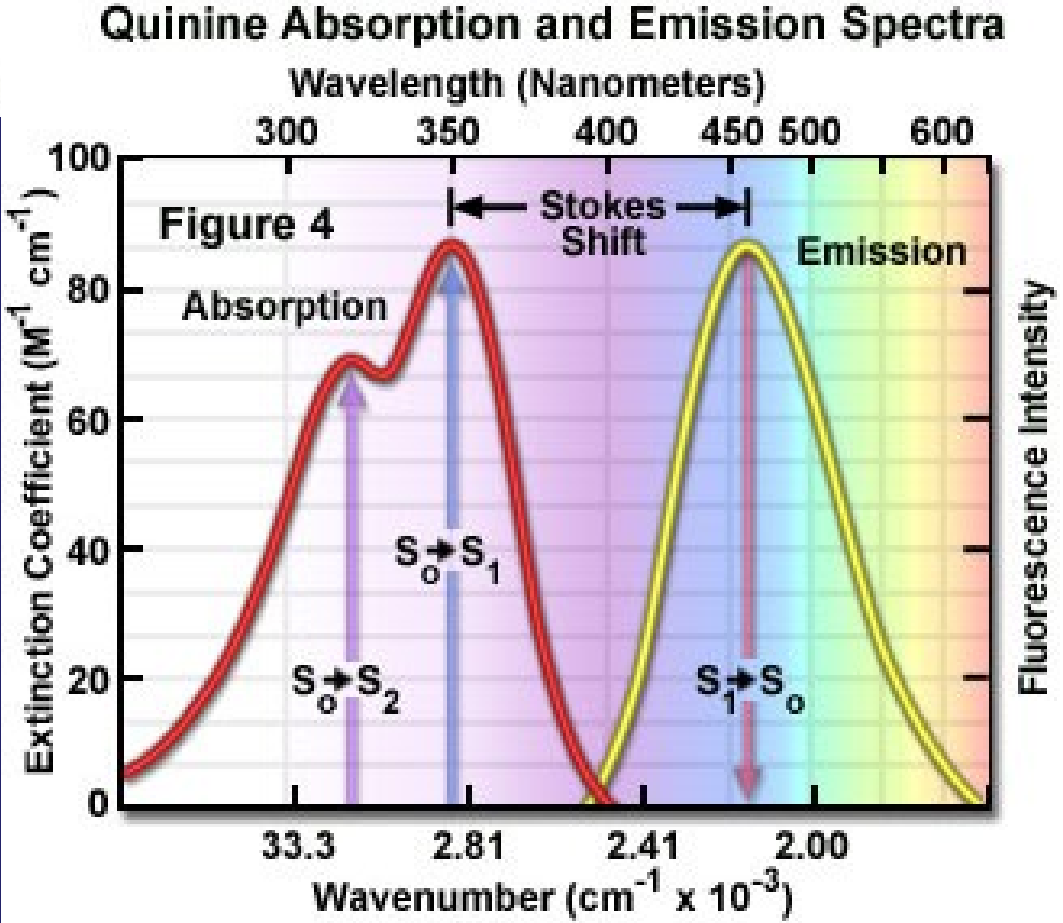


Figure 3

Mirror-image rule typically applies when only $S_0 \rightarrow S_1$ excitation takes place

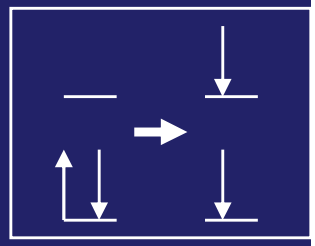
Deviations from the mirror-image rule are observed when $S_0 \rightarrow S_2$ or transitions to even higher excited states also take place



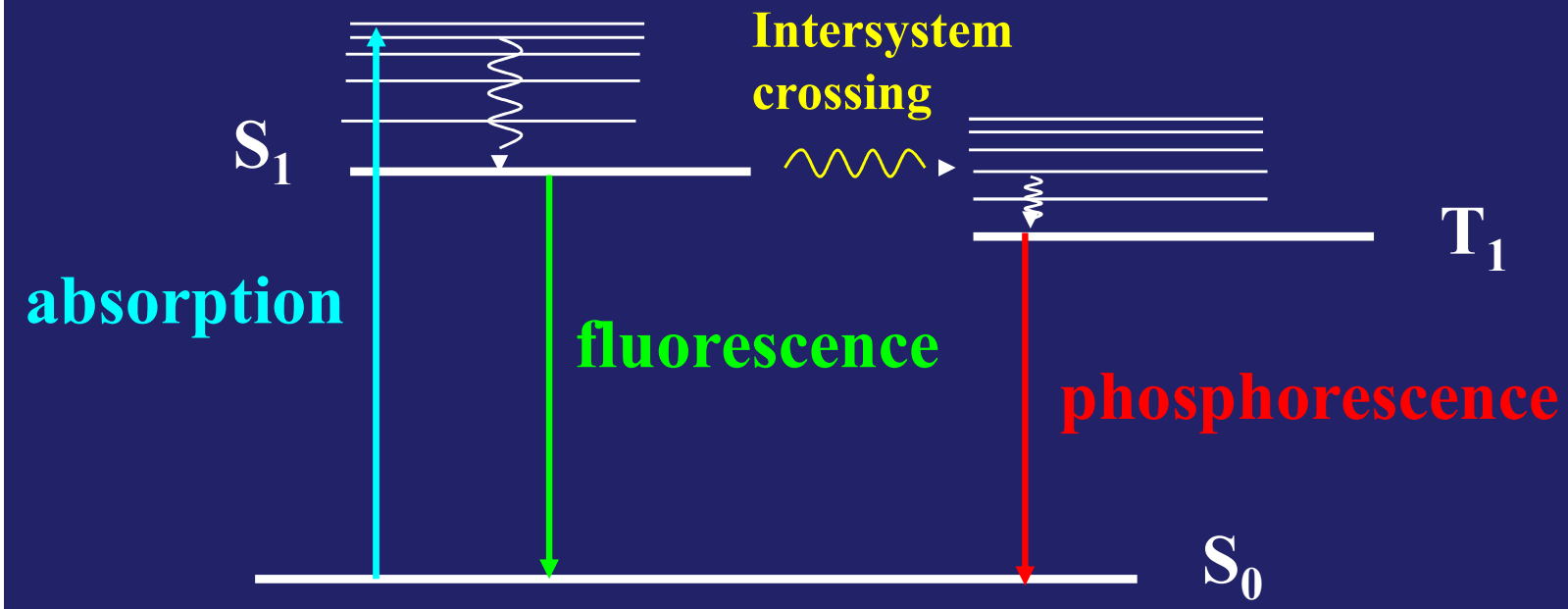
I. Principles of fluorescence

6e. Intersystem crossing

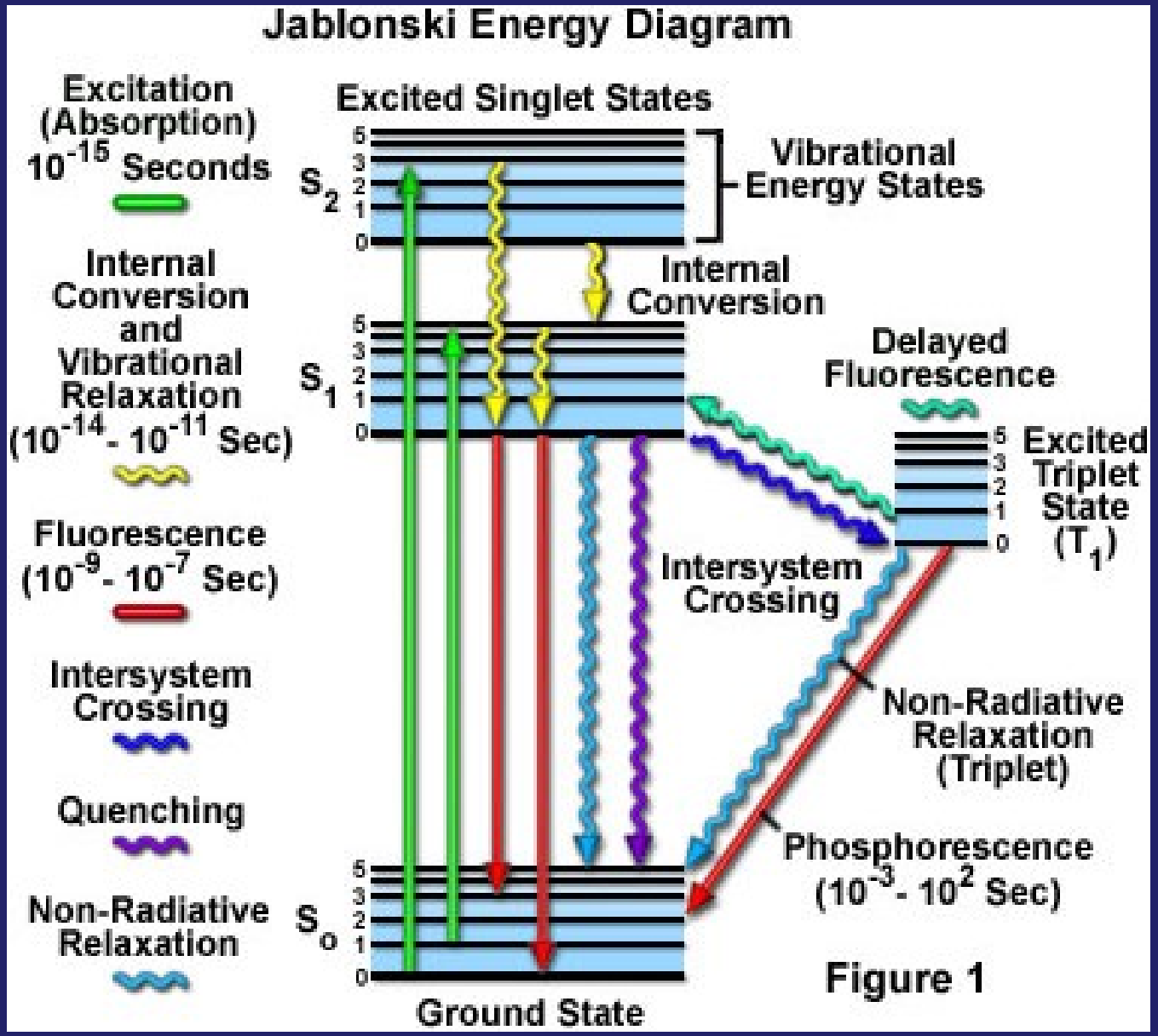
- Intersystem crossing refers to non-radiative transition between states of different multiplicity



- It occurs via inversion of the spin of the excited electron resulting in two unpaired electrons with the same spin orientation, resulting in a state with Spin=1 and multiplicity of 3 (triplet state)
- Transitions between states of different multiplicity are formally forbidden
- Spin-orbit and vibronic coupling mechanisms decrease the "pure" character of the initial and final states, making intersystem crossing probable
- $T_1 \rightarrow S_0$ transition is also forbidden $\rightarrow$ T_1 lifetime significantly larger than S_1 lifetime (10^{-3}-10^{2} s)

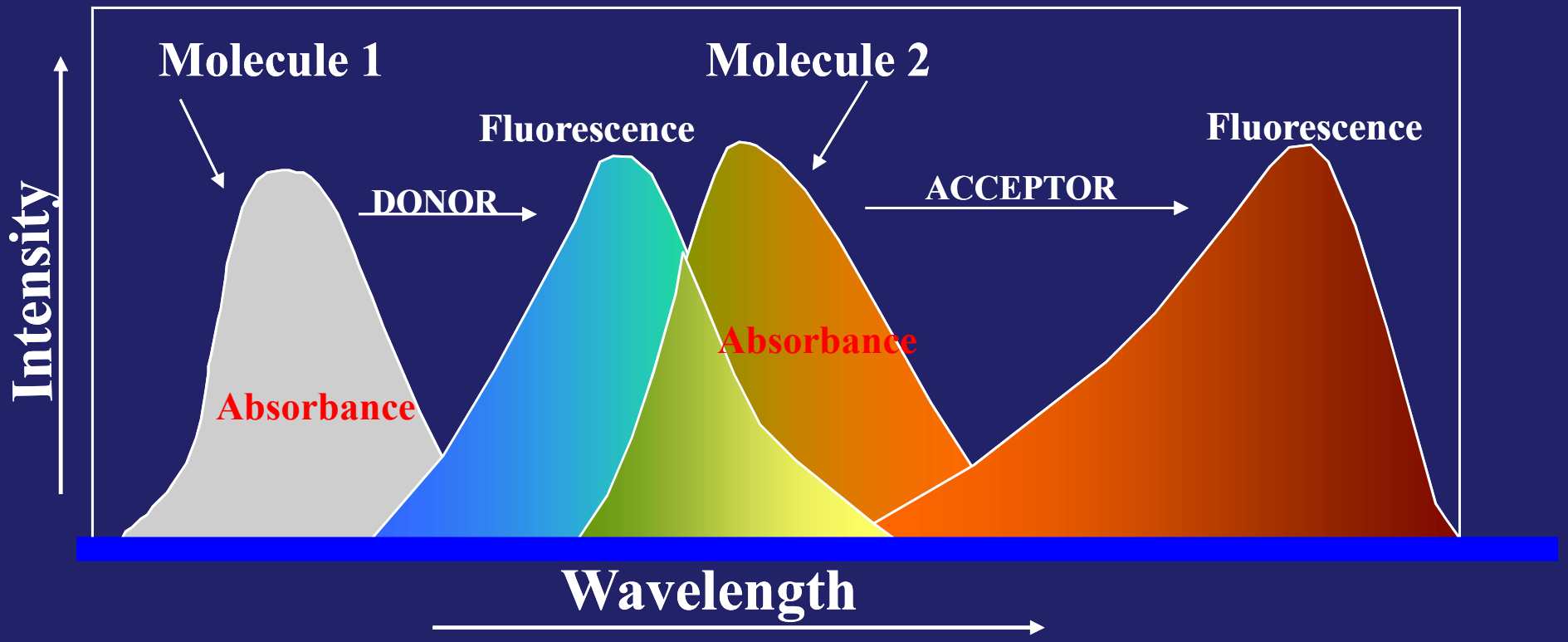


I. Principles of fluorescence



I. Principles of fluorescence

- Fluorescence energy transfer (FRET)



Non radiative energy transfer – a quantum mechanical process of resonance between transition dipoles

Effective between 10-100 Å only

Emission and excitation spectrum must significantly overlap

Donor transfers non-radiatively to the acceptor

II. Quantum yield and lifetime

1a. Quantum yield of fluorescence

- Quantum yield of fluorescence, Φ_f, is defined as:

$$\Phi_f = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}}$$

- In practice, is measured by comparative measurements with reference compound for which has been determined with high degree of accuracy.

- Ideally, reference compound should have
  - the same absorbance as the compound of interest at given excitation wavelength
  - similar excitation-emission characteristics to compound of interest (otherwise, instrument wavelength response should be taken into account)
  - Same solvent, because intensity of emitted light is dependent on refractive index (otherwise, apply correction

$$\frac{\Phi_f^u}{\Phi_f^s} = \frac{I_f^u}{I_f^s} \times \frac{n^2(u)}{n^2(s)}$$

  - Yields similar fluorescence intensity to ensure measurements are taken within the range of linear instrument response

II. Quantum yield and life time

1b. Fluorescence lifetime

- Another definition for Φ_f is

$$\Phi_f = \frac{k_r}{\sum k}$$

where k_r is the radiative rate constant and Σk is the sum of the rate constants for all processes that depopulate the S_1 state.

- In the absence of competing pathways $\Phi_f = 1$
- Radiative lifetime, τ_r, is related to k_r

$$\tau_r = \frac{1}{k_r}$$

- The observed fluorescence lifetime, is the average time the molecule spends in the excited state, and it is

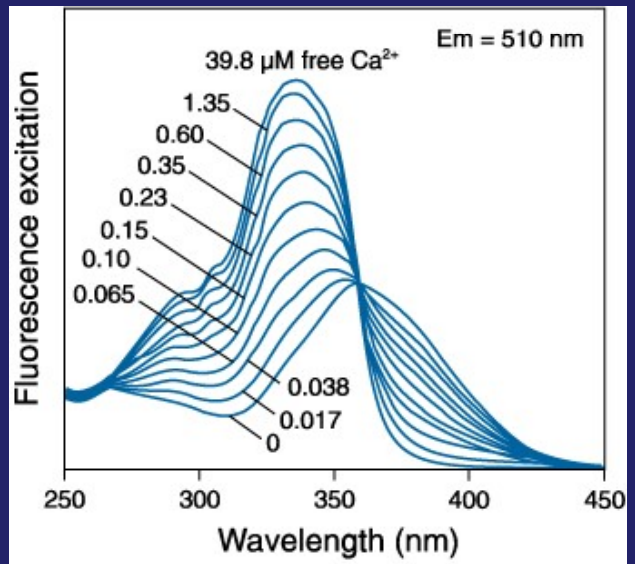
$$\tau_f = \frac{1}{\sum k}$$

II. Quantum Yield and Lifetime

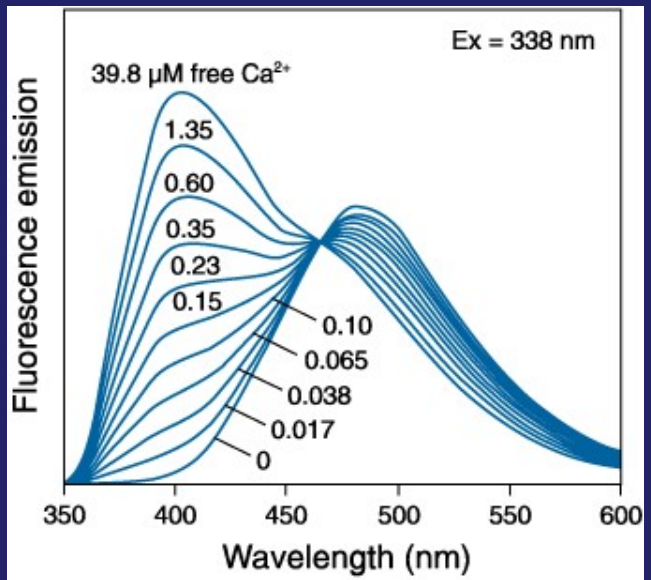
2a. Characteristics of quantum yield

- Quantum yield of fluorescence depends on biological environment
- Example: Fura 2 excitation spectrum and Indo-1 emission spectrum and quantum yield change when bound to Ca^{2+}

Fura-2 changes in response to varying [Ca2+]



Indo-1 changes in response to varying [Ca2+]



II. Quantum yield and lifetime

2b. Characteristics of life-time

- Provide an additional dimension of information missing in time-integrated steady-state spectral measurements
- Sensitive to biochemical microenvironment, including local pH, oxygenation and binding
- Lifetimes unaffected by variations in excitation intensity, concentration or sources of optical loss
- Compatible with clinical measurements in vivo

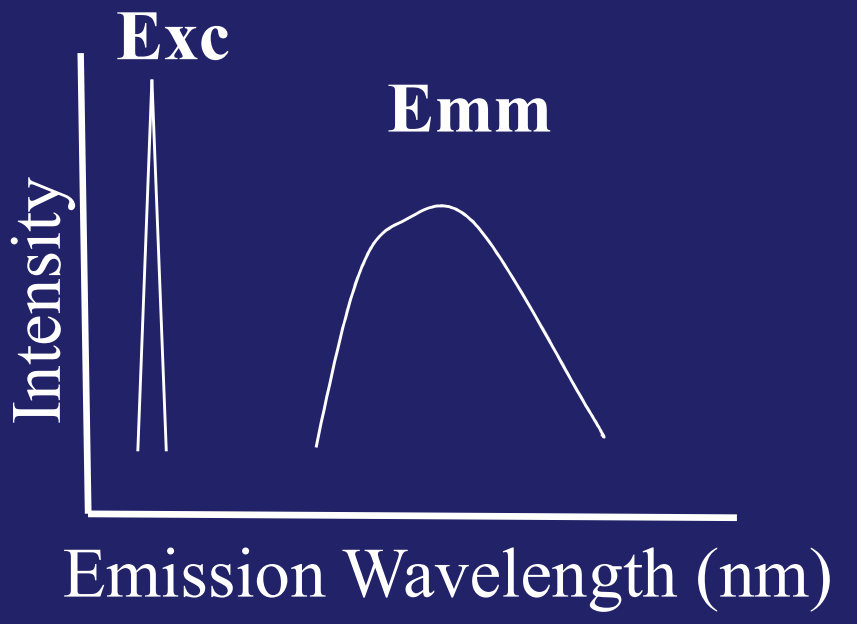
Courtesy of M.-A. Mycek, U Michigan

II. Quantum Yield and Lifetime

3a. Fluorescence emission distribution

- For a given excitation wavelength, the emission transition is distributed among different vibrational energy levels

- For a single excitation wavelength, can measure a fluorescence emission spectrum



II. Quantum Yield and Lifetime

3b. Heisenberg's uncertainty principle

- Values of particular pairs of observables cannot be determined simultaneously with high precision in quantum mechanics

- Example of pairs of observables that are restricted in this way are:

  - Momentum and position
  - Energy and time

II. Quantum Yield and Lifetime

3c. Heisenberg's uncertainty principle

- Momentum and position : $$\Delta p_x \, \Delta x \geq \frac{\hbar}{2}$$

- Energy and time: $$\Delta E \, \Delta t \geq \frac{\hbar}{2}$$

II. Quantum Yield and Lifetime

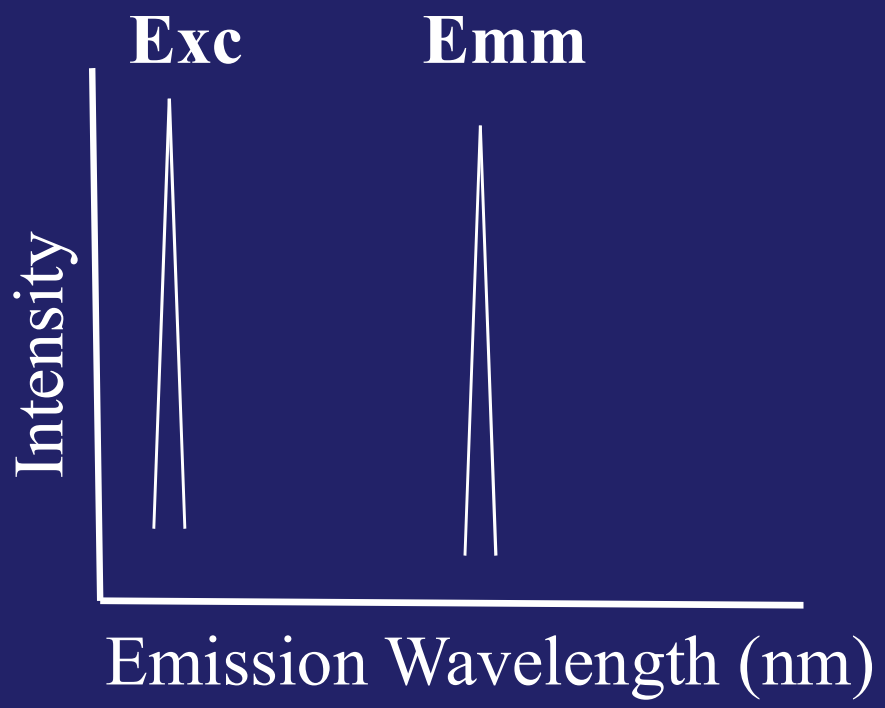
3d. Effect on fluorescence emission

- Suppose an excited molecule emits fluorescence in relaxing back to the ground state

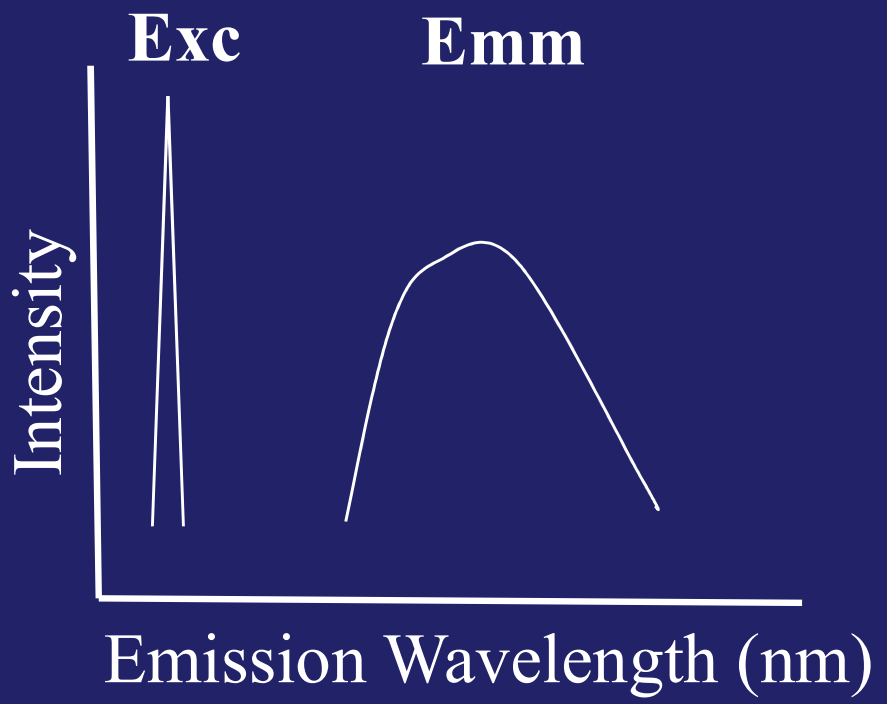
- If the excited state lifetime, τ is long, then emission will be monochromatic (single line)

- If the excited state lifetime, τ is short, then emission will have a wider range of frequencies (multiple lines)

Large $\Delta\tau$ – small ΔE



Small $\Delta\tau$ – large ΔE



III. Fluorescence Intensity

1. Fluorescence intensity expression
2. Fluorescence spectra

III. Fluorescence Intensities

1a. Fluorescence intensity

The fluorescence intensity (F) at a particular excitation (λ_x) and emission wavelength (λ_m) will depend on the absorption and the quantum yield:

$$\mathbf{F}(\lambda_x, \lambda_m) = \mathbf{I}_A(\lambda_x)\phi(\lambda_m)$$

where,

I_A – light absorbed to promote electronic transition

ϕ – quantum yield

III. Fluorescence Intensities

1b. From the Beer-Lambert law, the absorbed intensity for a dilute solution (very small absorbance)

$$I_A(\lambda_x) = 2.303 I_o \varepsilon(\lambda_x) CL$$

$$\text{for } \varepsilon(\lambda_x) CL << 1$$

where,

I_o – Initial intensity

ε – molar extinction coefficient

C – concentration

L – path length

III. Fluorescence Intensities

1c. Fluorescence intensity expression

The fluorescence intensity (F) at a particular excitation (λ_x) and emission wavelength (λ_m) for a dilute solution containing a fluorophore is:

$$\mathbf{F}(\lambda_x, \lambda_m) = \mathbf{I_o}\, 2.303\, \varepsilon(\lambda_x)\mathbf{CL}\,\phi(\lambda_m)$$

where,

I_o – incident light intensity

C – concentration

L – path length

ϕ – quantum yield

ε – molar extinction coefficient

III. Fluorescence Intensities

1d. Measured fluorescence intensity

If we include instrument collection angle:

$$\mathbf{F}(\lambda_x, \lambda_m) = \mathbf{I_o}\, 2.303\, \varepsilon(\lambda_x)\mathbf{CL}\,\phi(\lambda_m)\mathbf{Z}$$

where,

Z – instrumental factor

I_o – incident light intensity

ε – molar extinction coefficient

C = concentration

L – path length

III. Fluorescence Intensities

2a. Fluorescence spectra

- Emission spectrum
  - Hold excitation wavelength fixed, scan emission
  - Reports on the fluorescence spectral profile
    - » reflects fluorescence quantum yield, $\phi_k(\lambda_m)$

$$\mathbf{F}(\lambda_x, \lambda_m) = \mathbf{I_o}\, 2.303\, \varepsilon(\lambda_x)\mathbf{CL}\,\phi(\lambda_m)\mathbf{Z}$$

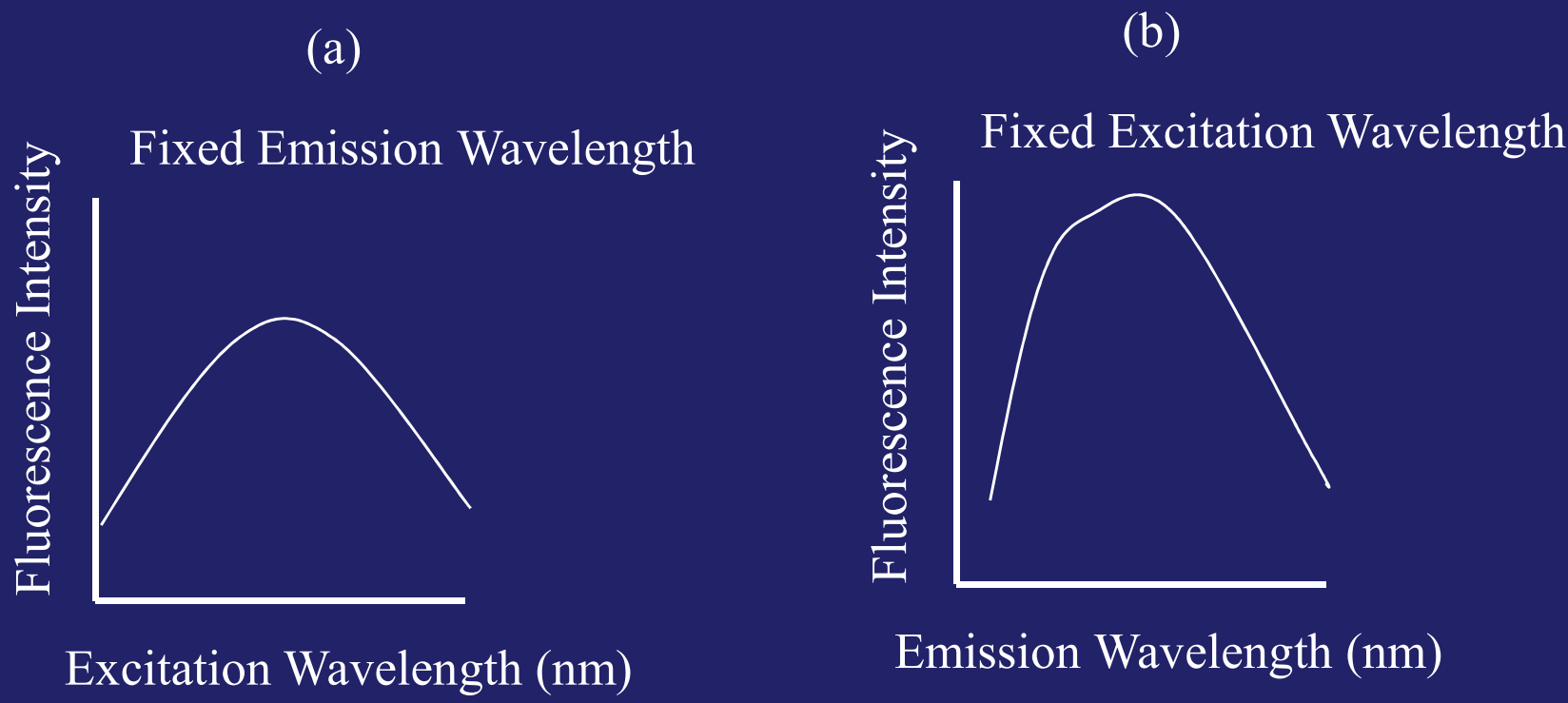
III. Fluorescence Intensities

2b. Fluorescence spectra

- Excitation spectrum
  - Hold emission wavelength fixed, scan excitation
  - Reports on absorption structure
    - » reflects molar extinction coefficient, $\varepsilon(\lambda_x)$

$$\mathbf{F}(\lambda_x, \lambda_m) = \mathbf{I_o}\, 2.303\, \varepsilon(\lambda_x)\mathbf{CL}\,\phi(\lambda_m)\mathbf{Z}$$

III. Fluorescence Intensities

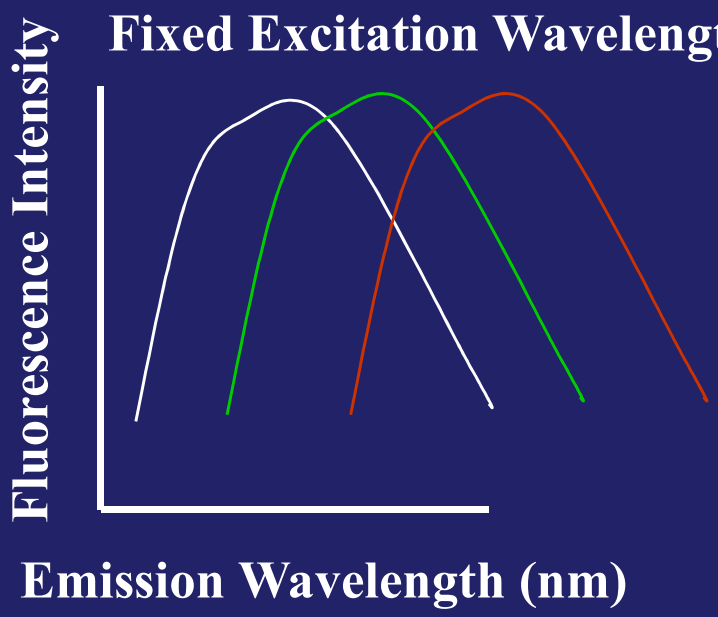


III. Fluorescence Intensities

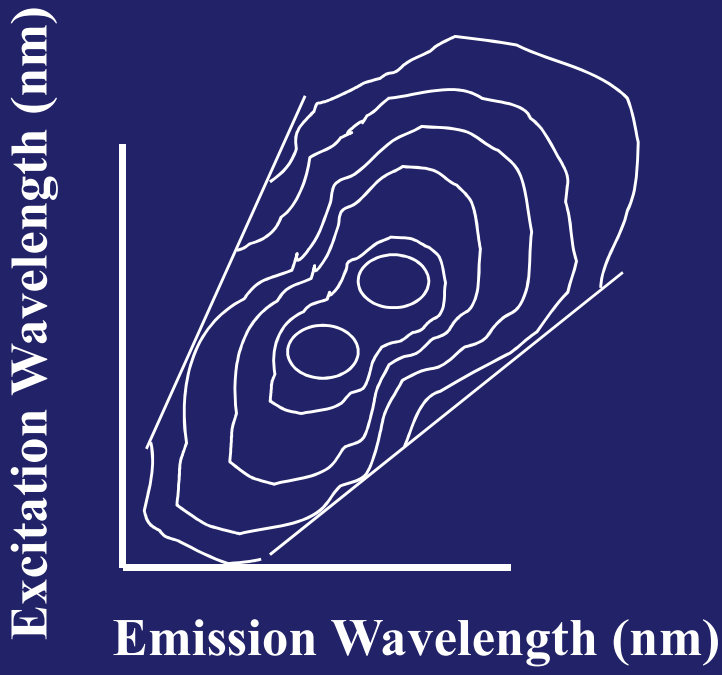
2c. Fluorescence spectra

- Composite: Excitation-Emission Matrix
  - » Good representation of multi-fluorophore solution

III. Fluorescence Intensities



Emission spectrum



Excitation-emission matrix

IV. Biological Fluorophores

1. Table
2. EEMs of Epithelial cell suspension
3. EEMs of Collagen

IV. Biological Fluorophores

–Endogenous Fluorophores

- amino acids
- structural proteins
- enzymes and co-enzymes
- vitamins
- lipids
- porphyrins

–Exogenous Fluorophores

- Cyanine dyes
- Photosensitizers
- Molecular markers – GFP, etc.

Endogenous fluorophores	Excitation maxima (nm)	Emission maxima (nm)
Amino acids		
Tryptophan	280	350
Tyrosine	275	300
Phenylalanine	260	280
Structural proteins		
Collagen	325	400, 405
Elastin	290, 325	340, 400
Enzymes and coenzymes		
FAD, flavins	450	535
NADH	290, 351	440, 460
NADPH	336	464
Vitamins		
Vitamin A	327	510
Vitamin K	335	480
Vitamin D	390	480
Vitamin B_6 compounds		
Pyridoxine	332, 340	400
Pyridoxamine	335	400
Pyridoxal	330	385
Pyridoxic acid	315	425
Pyridoxal 5′-phosphate	330	400
Vitamin B_{12}	275	305
Lipids		
Phospholipids	436	540, 560
Lipofuscin	340–395	540, 430–460
Ceroid	340–395	430–460, 540
Porphyrins	400–450	630, 690

FAD, flavin adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; AND(P)H, reduced nicotinamide adenine dinucleotide phosphate.

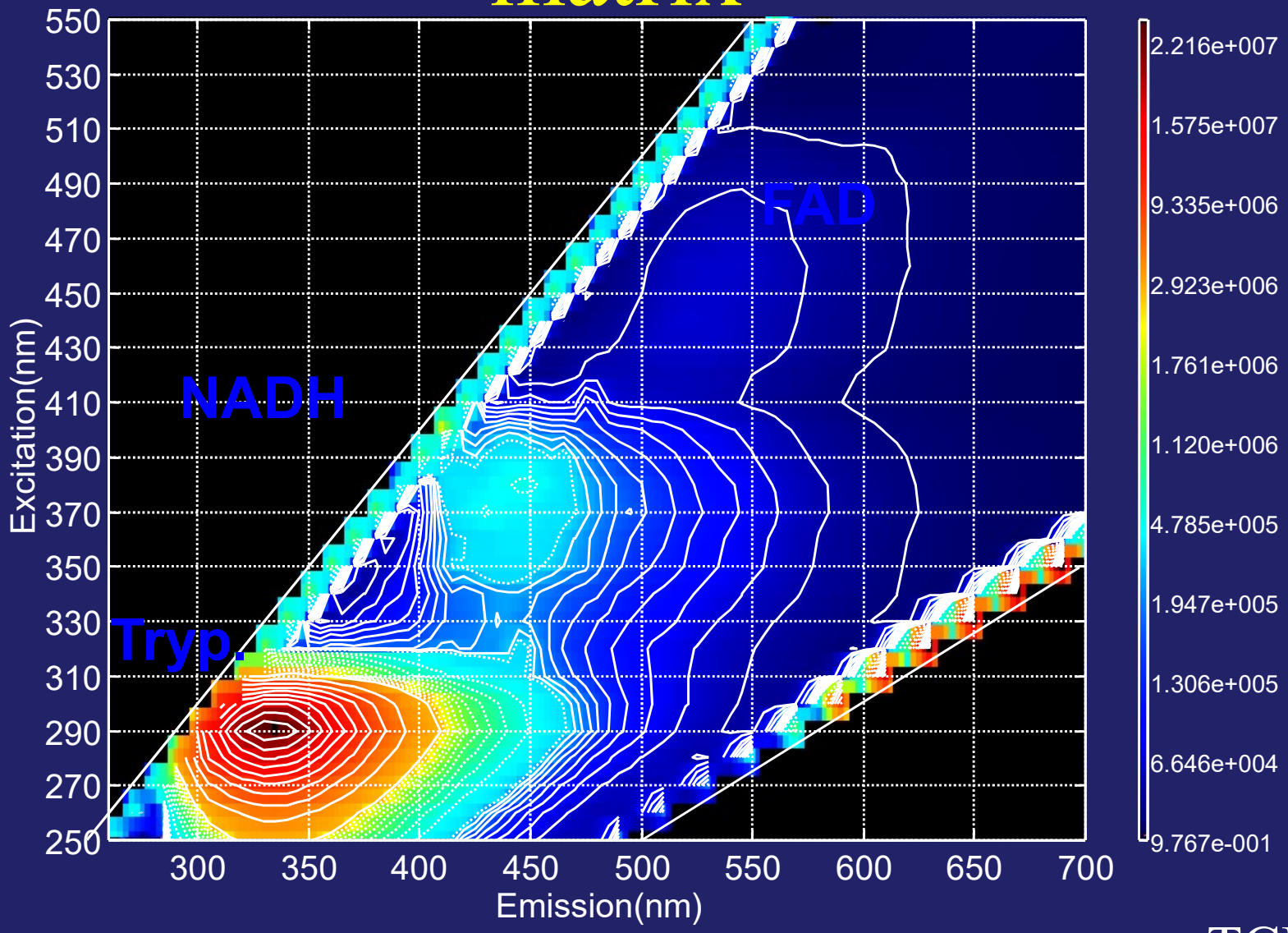
IV. Biological Fluorophores

TABLE III. Measured fluorescence lifetimes and amplitudes for endogenous biological fluorophores *in vitro* (337.1 nm excitation), with comparison to literature values.

Fluorophore $<5\times10^{-6}$ M in PBS	Measured				Literature				References
	Lifetime (ns)		Area		Lifetime (ns)		Area		
	τ_1	τ_2	A_1	A_2	τ_1	τ_2	A_1	A_2	
Tryptophan	0.54	4.45	0.33	0.66	0.62	3.20	0.22	0.78	58
					0.53	3.10	0.33	0.67	37
					1.47	2.78	0.72	0.28	55
Elastin	1.63	7.36	0.36	0.64	1.7	8.3			57
					1.3	5.8			57
					1.4	6.7	0.58	0.42	55
					1.12	6.57	0.27	0.73	28
FAD	2.63				2.3				33
					2.85				28
NADH (free)	0.36				0.3				36
					0.26	0.66			59
					0.24	0.58	0.77	0.23	55
					0.4				33
					0.38	1.38	0.97	0.03	28

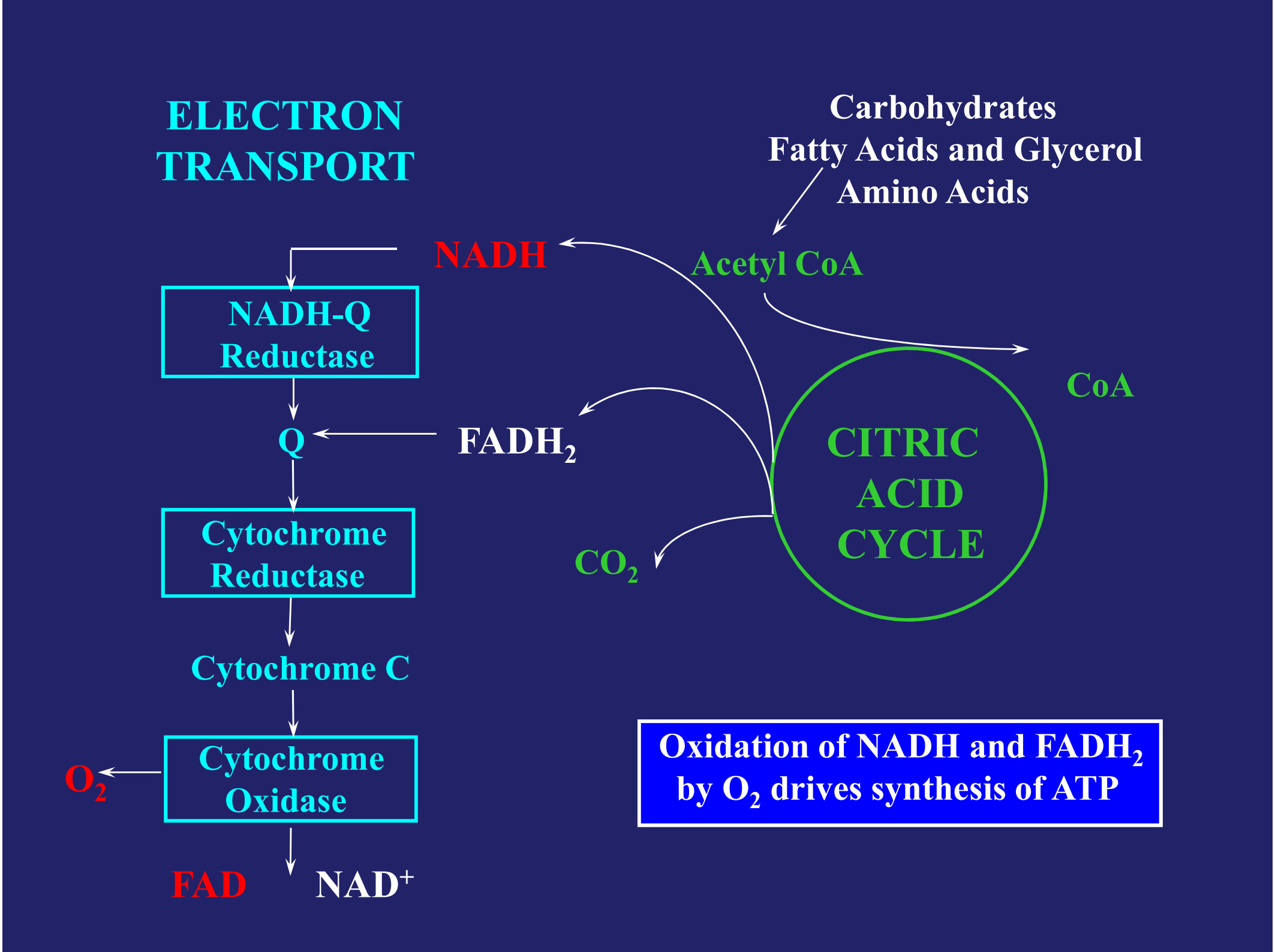
Ref. J.D. Pitts and M.-A. Mycek: Review of Scientific Instruments, Vol.72, No.7, pp.3061-3072 (2001).

Epithelial Cell Suspension Fluorescence intensity excitation-emission matrix



Courtesy of N. Ramanujam

TCL-1


ELECTRON TRANSPORT
Carbohydrates
Fatty Acids and Glycerol
Amino Acids
NADH
Acetyl CoA
NADH-Q Reductase
CoA
Q
FADH2
CITRIC ACID CYCLE
Cytochrome Reductase
CO2
Cytochrome C
Cytochrome Oxidase
O2
FAD
NAD+
Oxidation of NADH and FADH2 by O2 drives synthesis of ATP

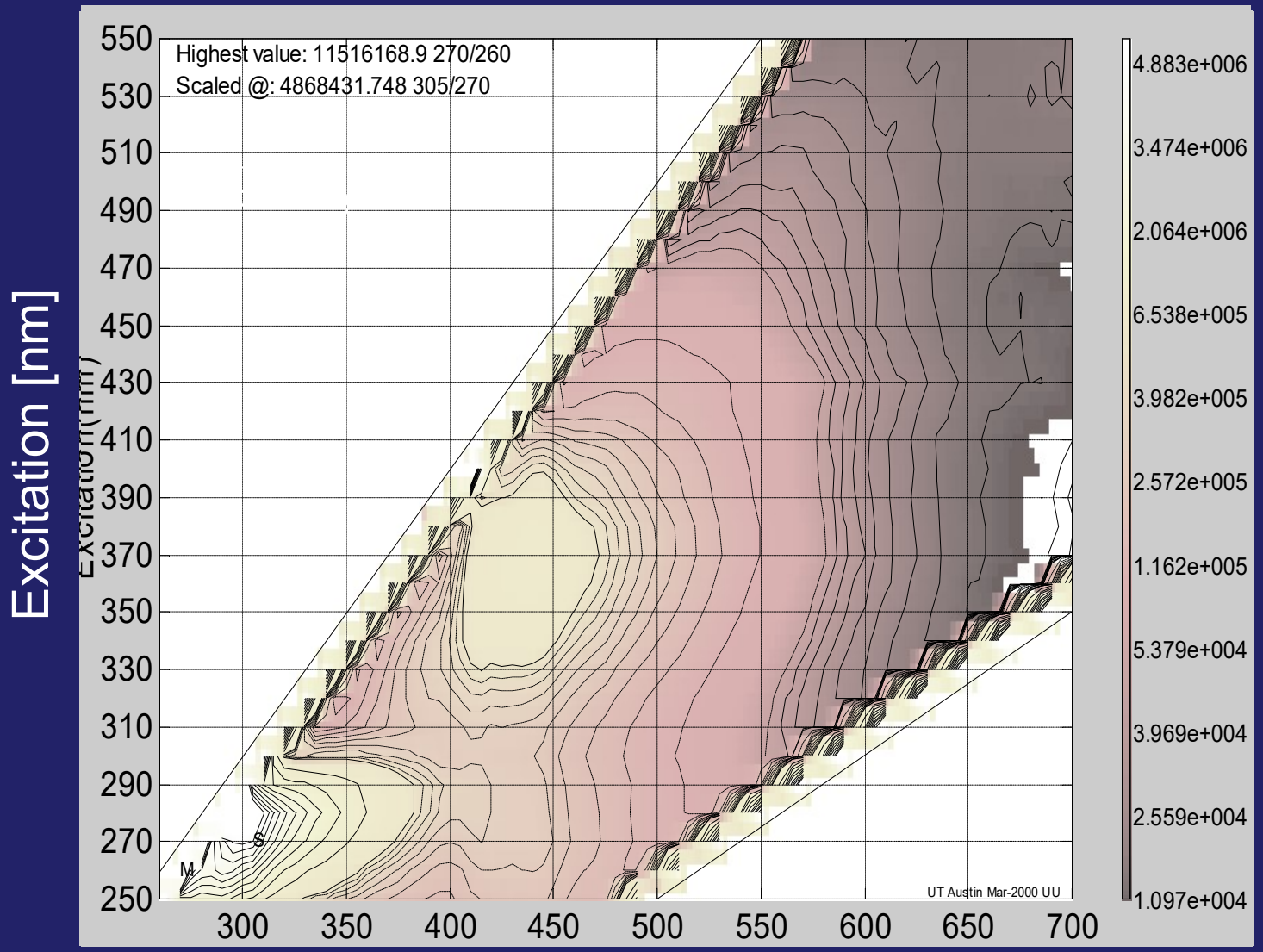
Metabolic Indicators

Metabolism

Redox Ratio: FAD / (FAD+NADH)

↓ Redox ratio ~ ↑ Metabolic Rate

Collagen I (gel)



K. Sokolov

IV. Biological Fluorophores

Collagen

- It is the major extracellular matrix component, which is present to some extent in nearly all organs and serves to hold cells together in discrete units

- Collagen fluorescence in load-bearing tissues is associated with cross-links, hydroxylysyl pyridoline (HP) and lysyl pyridinoline (LP).

- Collagen crosslinks are altered with age and with invasion of cancer into the extracellular matrix

V. Fluorescence Instrumentation

1. Introduction
2. Components of a spectrofluorometer
3. Description of key components

V. Fluorescence Instrumentation

1. Introduction

- Fluorescence is a highly sensitive method (can measure analyte concentration of 10^{-8} M)

- Important to minimize interference from:
  - Background fluorescence from solvents
  - Light leaks in the instrument
  - Stray light scattered by turbid solutions

- Instruments do not yield ideal spectra:
  - Non-uniform spectral output of light source
  - Wavelength dependent efficiency of detector and optical elemens

V. Fluorescence Instrumentation

2. Major components for fluorescence instrument

- Illumination source
  - Broadband (Xe lamp)
  - Monochromatic (LED, laser)
- Light delivery to sample
  - Lenses/mirrors
  - Optical fibers
- Wavelength separation (potentially for both excitation and emission)
  - Monochromator
  - Spectrograph
- Detector
  - PMT
  - CCD camera

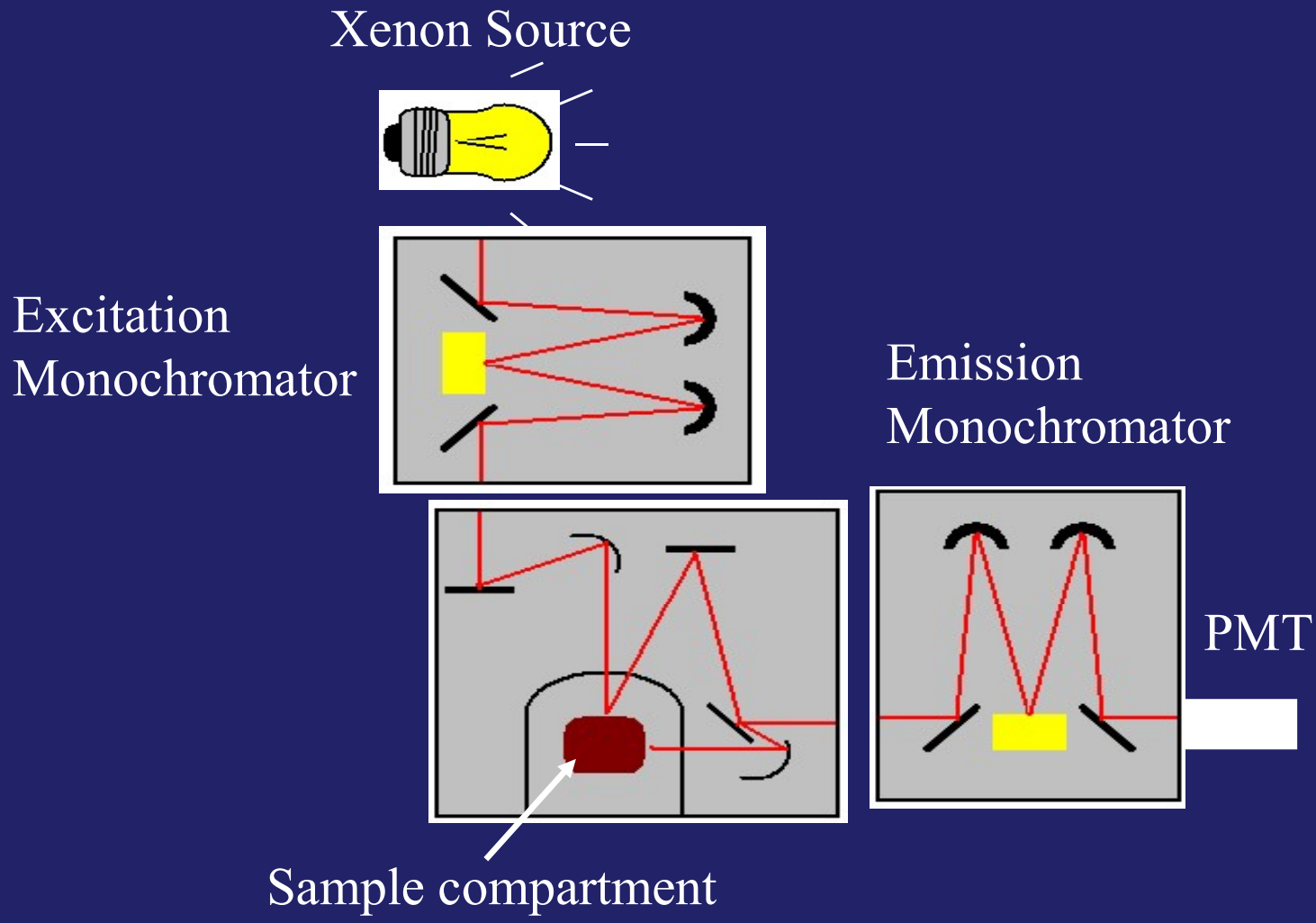
V. Fluorescence Instrumentation

Components of the spectrofluorometer (standard fluorescence lab instrument for in vitro samples)

- Xenon lamp (> 250 nm)
- Excitation and emission monochromator
  - Each contains two gratings to increase purity of the light
  - Automatic scanning of wavelength through motorized gratings
- Sample compartment
- Photo multiplier tube

V. Fluorescence Instrumentation

2. Spectrofluorometer schematic



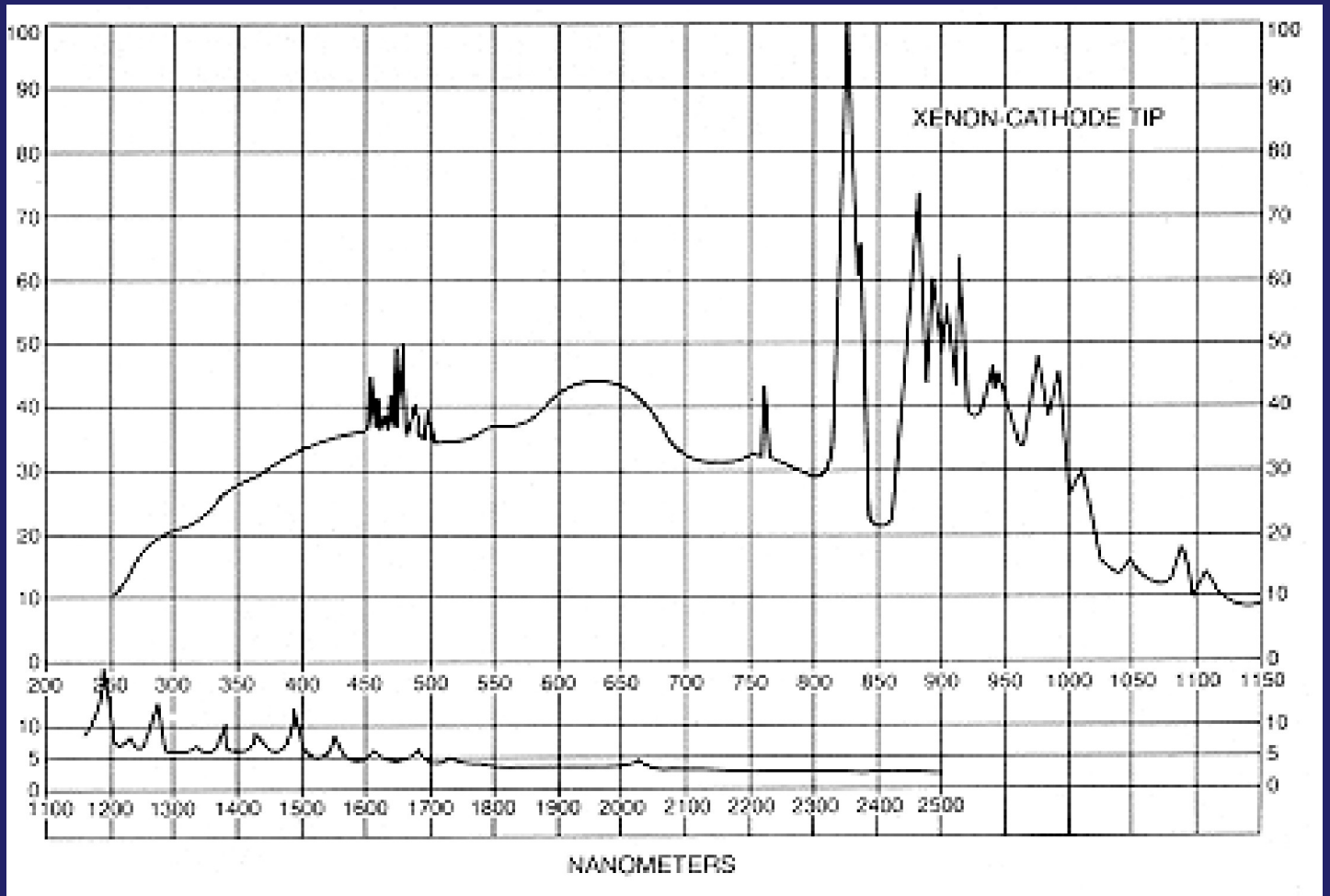
V. Fluorescence Instrumentation

3a. Xenon light source

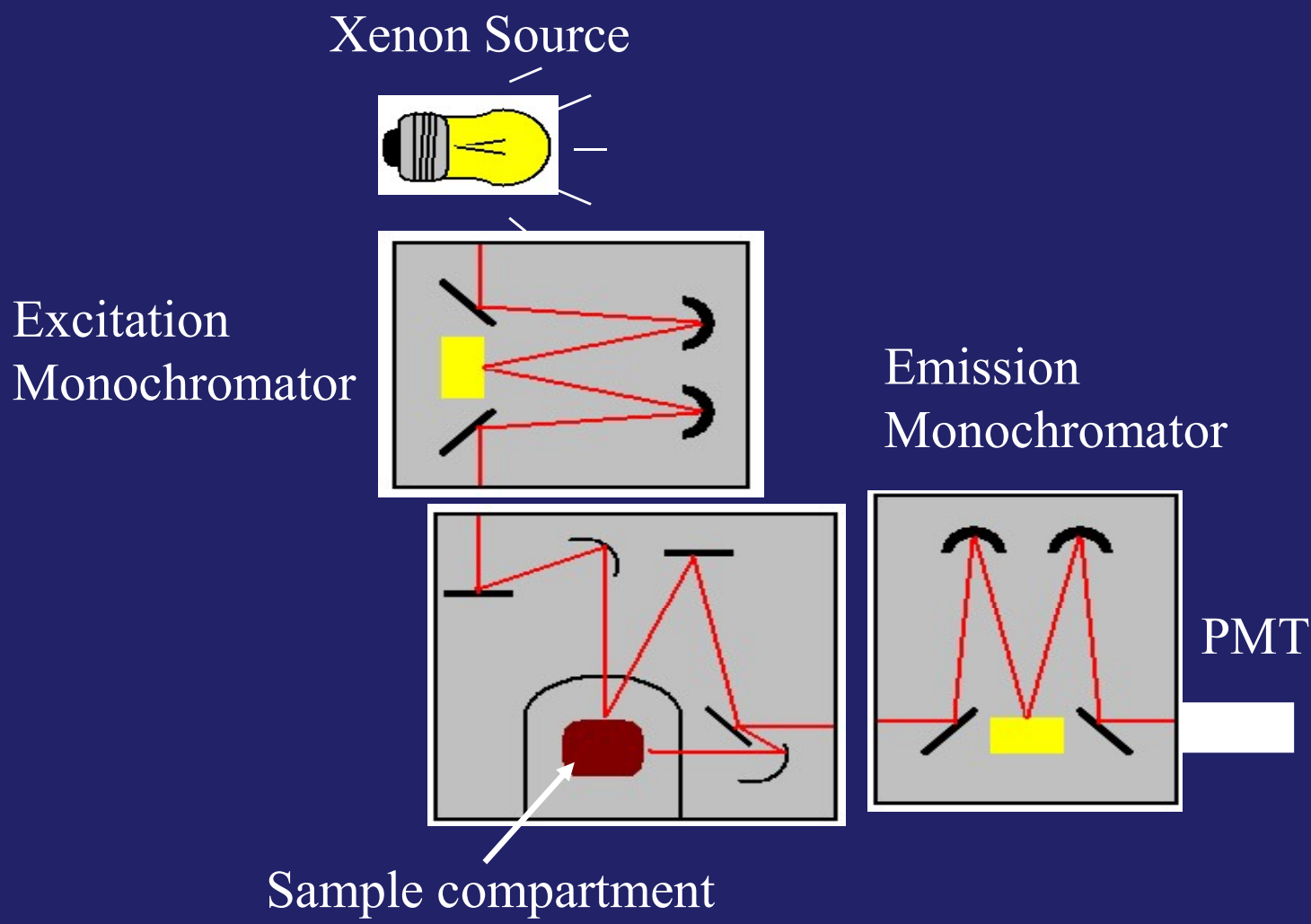
- Continuous output from Xenon: 270-1100 nm
- Power – typically 200-450 W
- Lifetime of ~2000 hours
- Strong dependence on wavelength

V. Fluorescence Instrumentation

3a. Xenon light source: broad illumination in the near UV-visible range

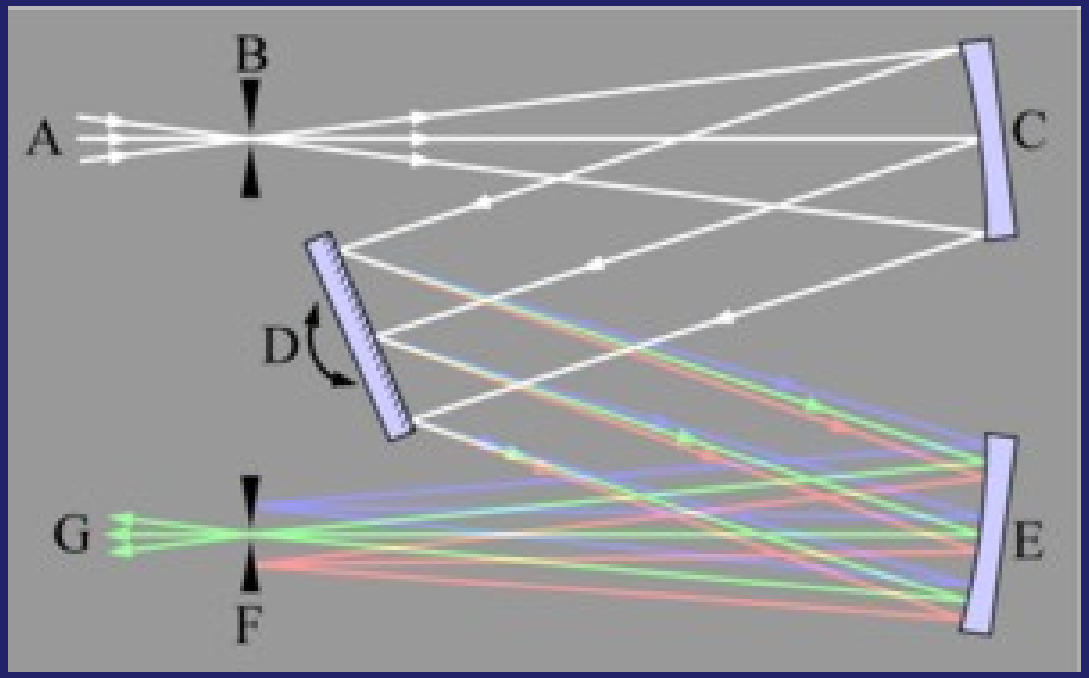


V. Fluorescence Instrumentation

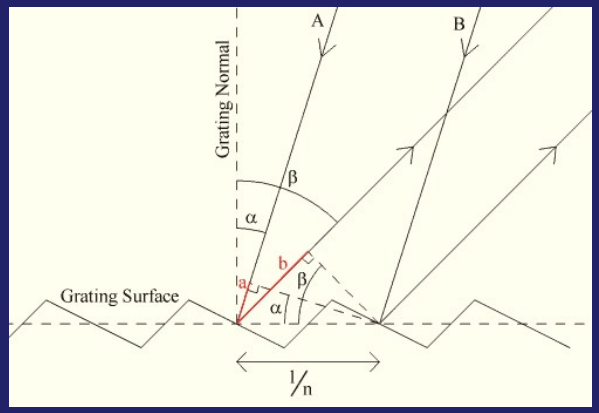


V. Fluorescence Instrumentation

3b. Monochromator: only a small range of wavelengths are focused at the exit slit determined by angle of light incident on the diffraction grating



Principle of diffraction grating operation



$$\sin\alpha + \sin\beta = 10^{-6} Kn\lambda,$$

where K = diffraction order

n = # of lines per mm

λ = wavelength

$D = \beta - \alpha$ = constant

V. Fluorescence Instrumentation

3b. Monochromator – Spectral Resolution

Inversely proportional to product of dispersion (nm/mm) of grating and the slit width (mm)

~ 5 nm sufficient for fluorescence measurements of biological media

Signal increases with the slit width

V. Fluorescence Instrumentation

3b. Monochromator – Stray light

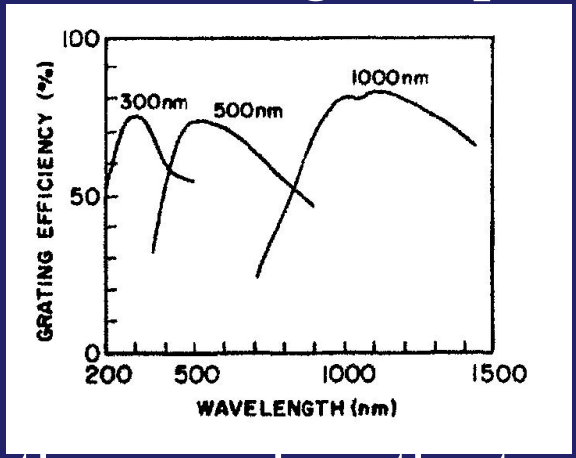
- Light which passes through monochromator besides that of desired wavelength

- Double grating monochromator (stray light rejection is 10^{-8} – 10^{-12}) but signal is decreased

V. Fluorescence Instrumentation

3b. Monochromator – Signal efficiency

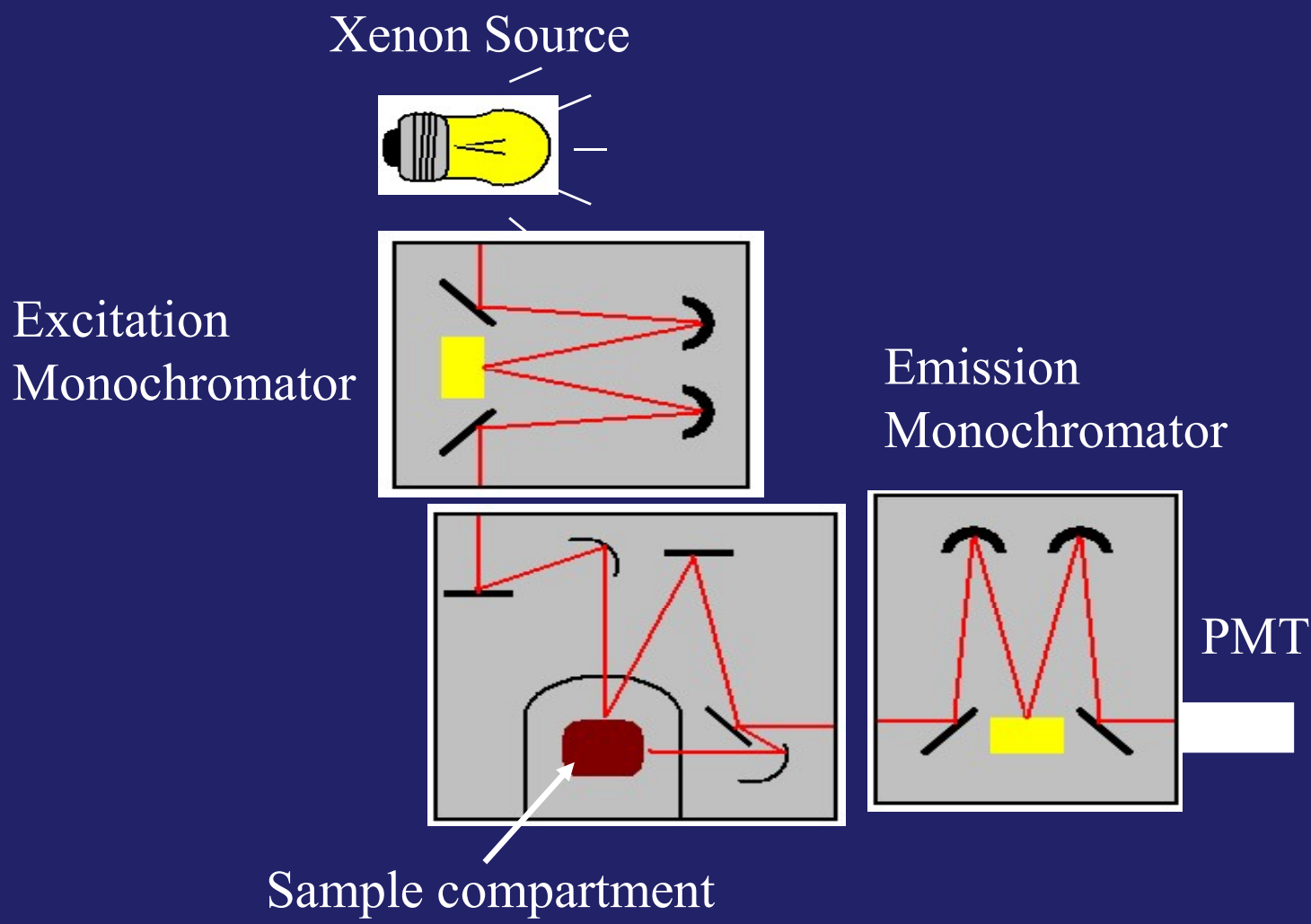
- Grating has a wavelength dependent efficiency



- Can choose the wavelength at which grating is blazed (maximal efficiency)

- Excitation monochromator should have high efficiency in the UV; emission monochromator should have high efficiency in the visible

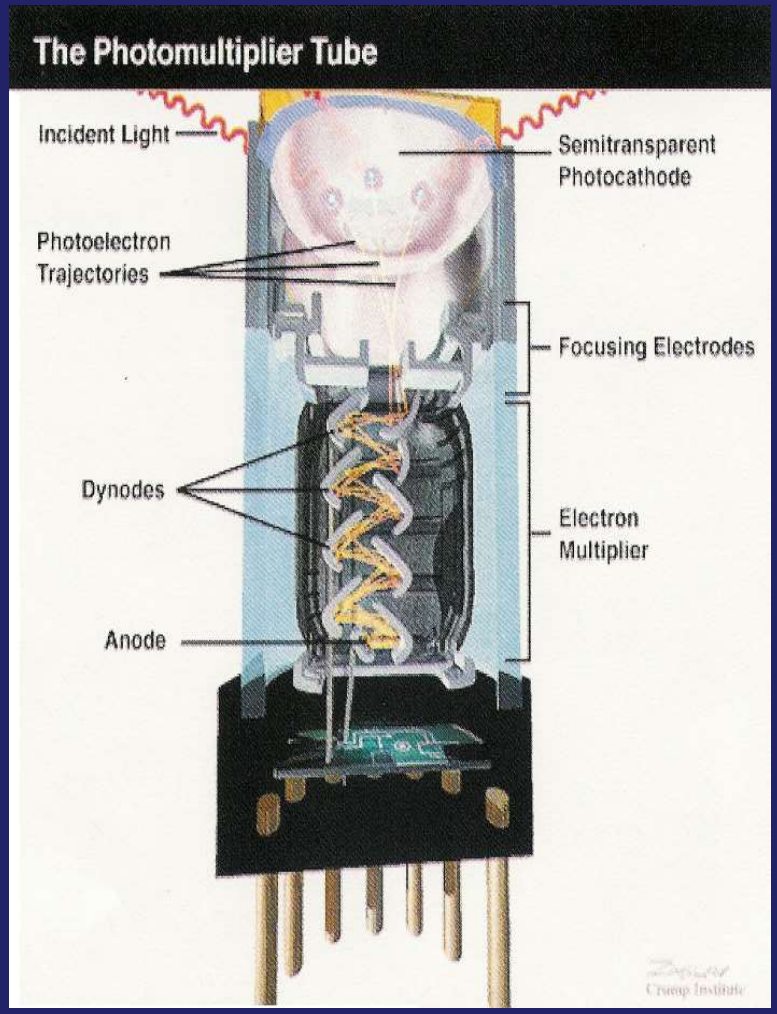
V. Fluorescence Instrumentation



V. Fluorescence Instrumentation

3c. Photomultiplier tube

- Contains a photocathode:
  light sensitive material, which yields electrons upon interaction with photons based on photoelectric effect.
- Electrons are multiplied by a series of dynodes
- Provides current output proportional
  to light intensity



V. Fluorescence Instrumentation

3c. PMT – Linearity response

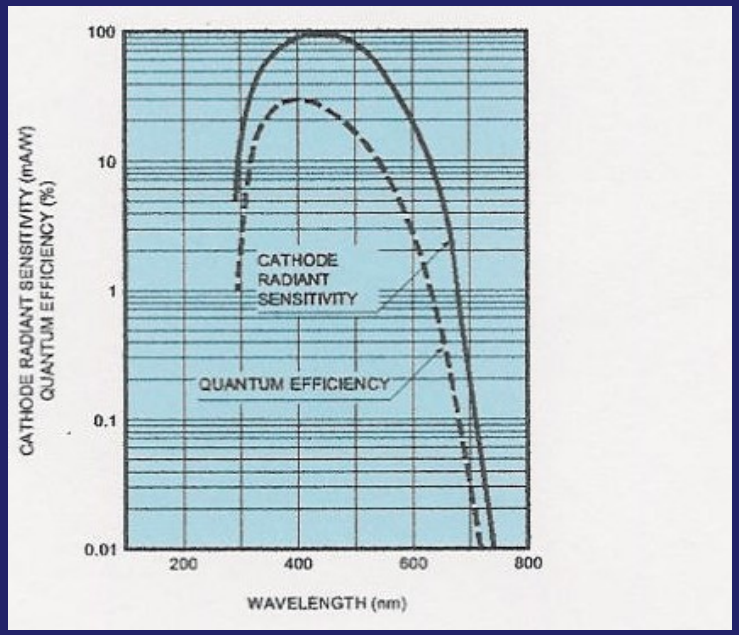
- Current from PMT is proportional to light intensity
- Under high intensity illumination, PMT will saturate (dynamic range); at low intensity, limited by dark noise
- Excessive light can damage photocathode, resulting in loss of gain and increased dark noise (thermal noise)

V. Fluorescence Instrumentation

3c. PMT – Quantum efficiency

- Quantum efficiency gives the photon to electron conversion efficiency

- Highly dependent on wavelength

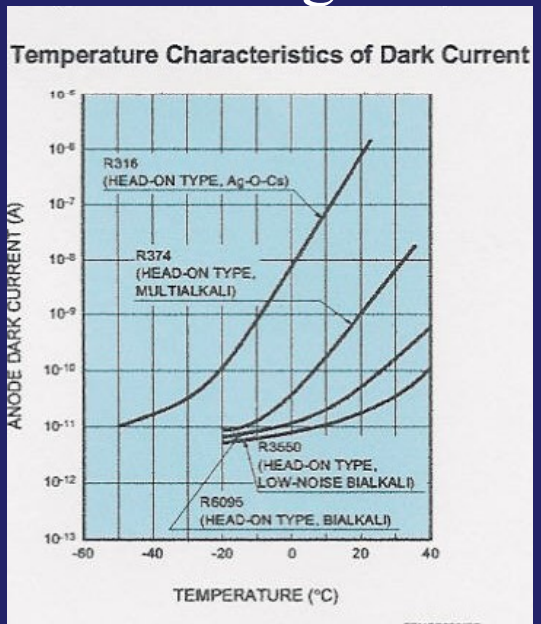


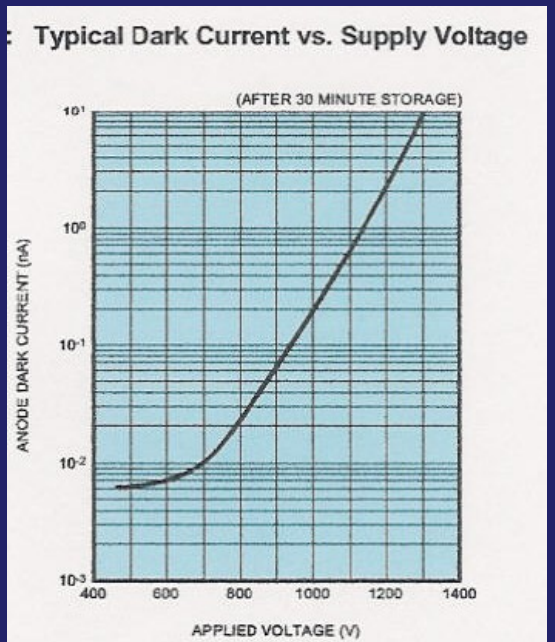
V. Fluorescence Instrumentation

3c. Key components – Noise

- Dark current – Noise due to thermal generation; increases with temperature and high voltage

- Shot noise – proportional to the square root of the signal





VI. Fluorescence Measurements

VI. Fluorescence measurements

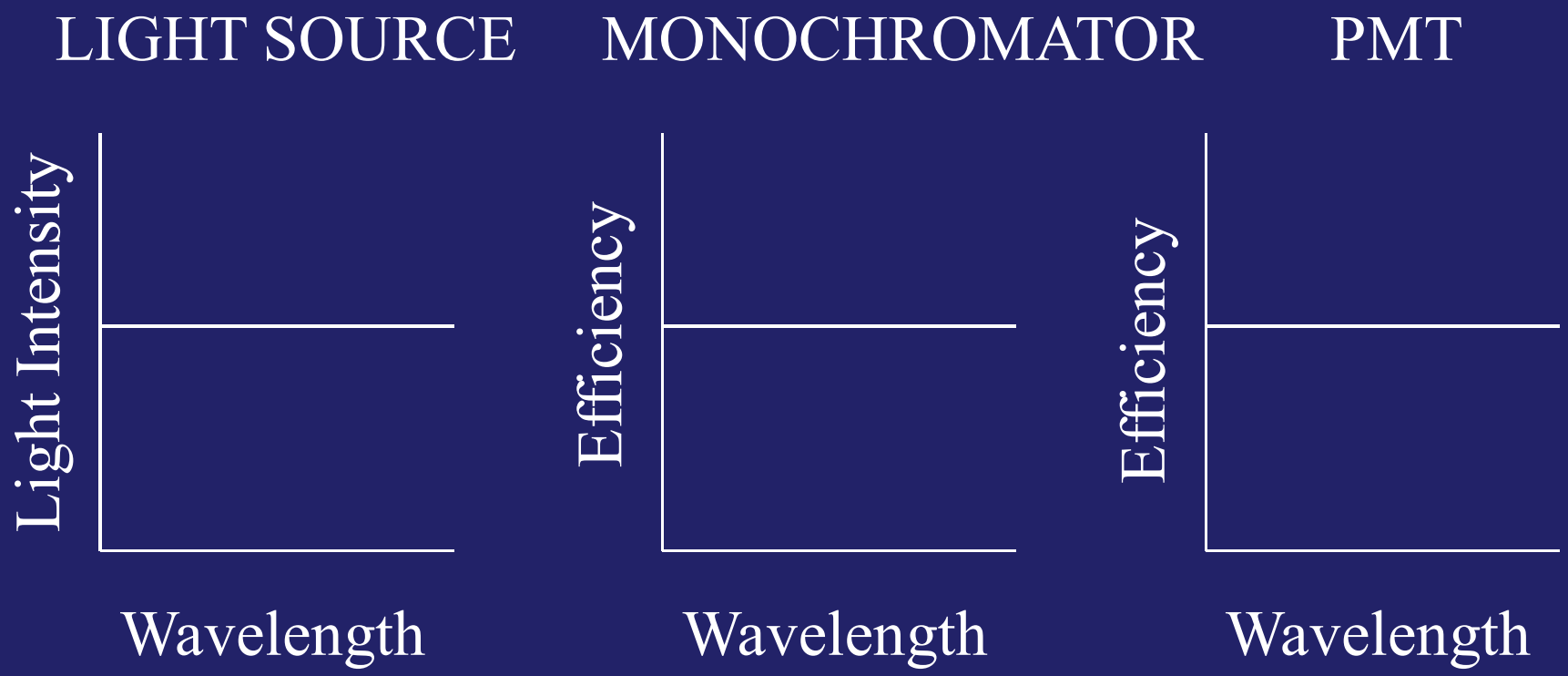
1. Instrument non-uniformities
2. Excitation wavelength calibration
3. Emission wavelength calibration
4. Setup parameters for emission spectrum
5. Routine experimental procedure
6. Collection geometry
7. Blank scans
8. Typical fluorescence spectrum

VI. Fluorescence Measurements

1a. Ideal spectrofluorometer

- Light source must yield constant photon output at all wavelengths
- Monochromator must pass photons of all wavelengths with equal efficiency
- The PMT must detect photons of all wavelengths with equal efficiency

VI. Fluorescence Measurements



VI. Fluorescence Measurements

1b. Distortions in excitation and emission spectra

- Light intensity from light source is a function of wavelength
- Monochromator efficiency is a function of wavelength
- The PMT does not have equal efficiency at all wavelengths

VI. Fluorescence Measurements

1c. Calibration

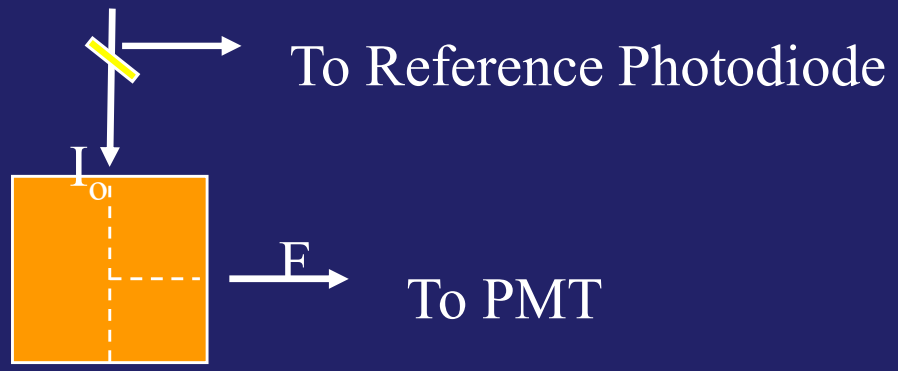
- Correction of variations in wavelength of Xenon lamp and excitation monochromator
  - Need to do when measuring excitation spectra or emission spectra at multiple excitation wavelengths

- Correction of emission monochromator and PMT
  - Need to do when measuring emission spectra

VI. Fluorescence Measurements

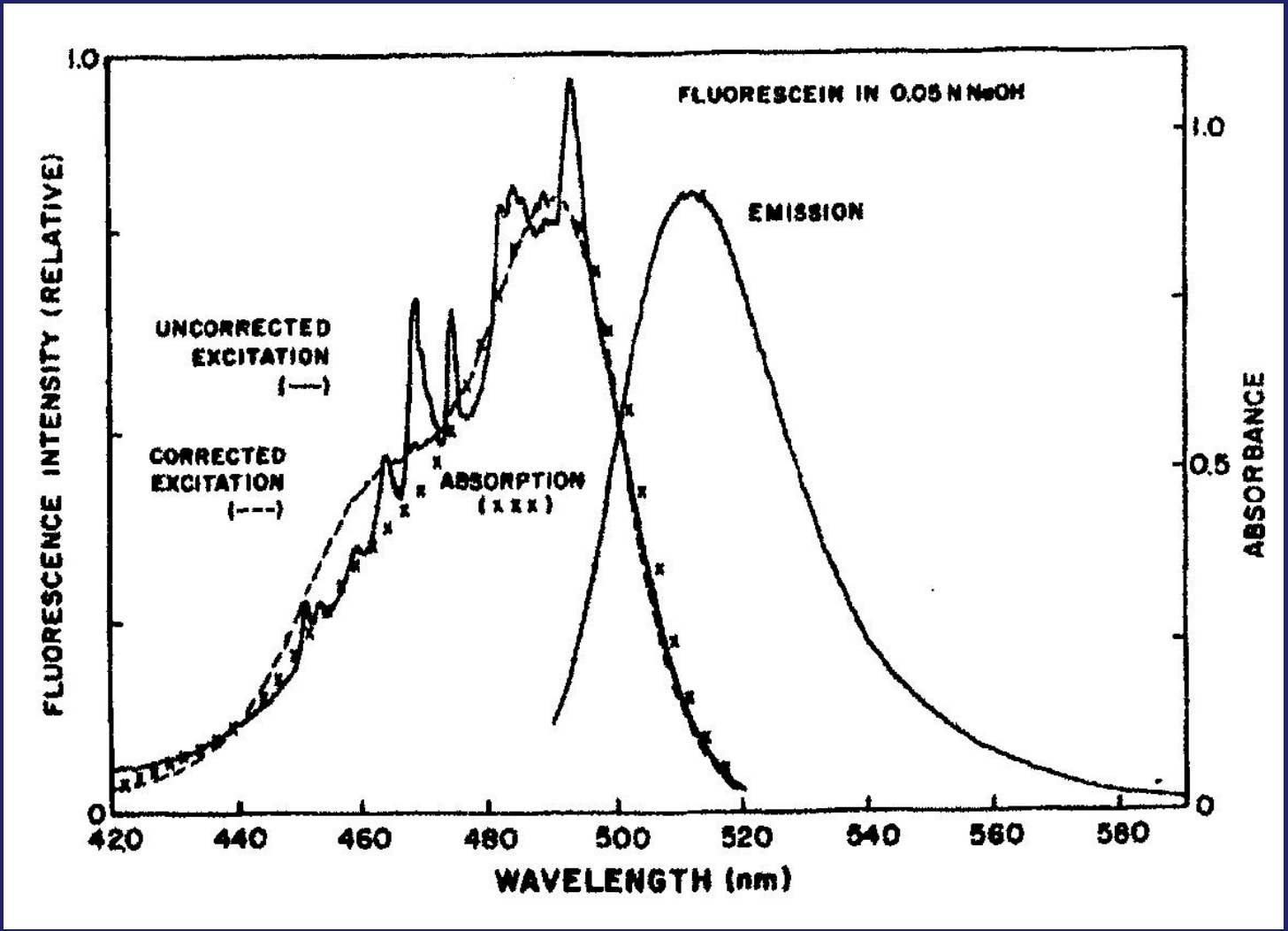
2a. Excitation wavelength calibration

- Excitation spectra are distorted primarily by the wavelength dependent intensity of the light source
- Can use reference photodetector (calibrated) next to sample compartment to measure fraction of excitation light
- The measured intensity of the reference channel is proportional to the intensity of the exciting light



VI. Fluorescence Measurements

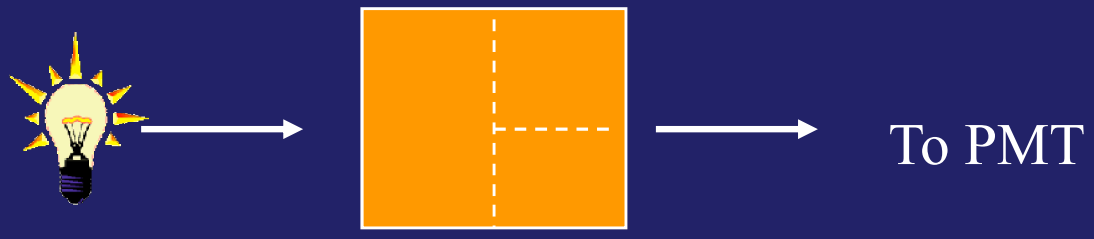
2b. Effect of excitation wavelength calibration



VI. Fluorescence Measurements

3a. Emission wavelength calibration

- Need correction factors
- Measure wavelength dependent output from a calibrated light source
- Standard lamps of known and calibrated spectral outputs are available from the National Institute of Standards and Testing (NIST)
- This measurement is typically done by factory; it is difficult to perform properly with commercial fluorimeter



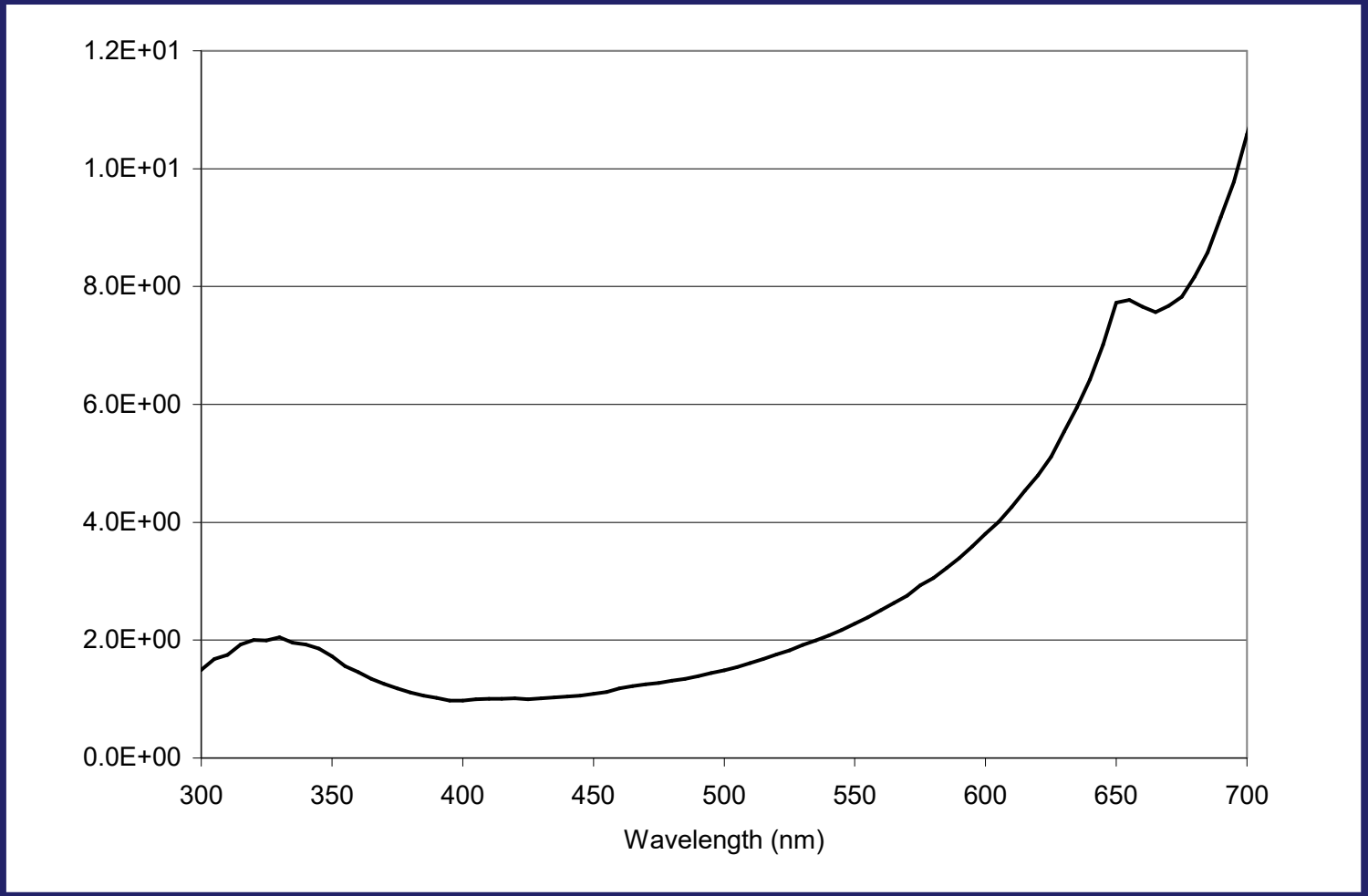
VI. Fluorescence Measurements

3b. Emission wavelength calibration procedure

- Measure intensity versus wavelength ($I(\lambda)$) of standard lamp with spectrofluorometer
- Obtain the spectral output data ($L(\lambda)$) provided for the lamp
- Correction factor: $S(\lambda) = L(\lambda)/ I(\lambda)$
- Multiply emission spectrum with correction factor

VI. Fluorescence Measurements

3c. Emission wavelength calibration curve

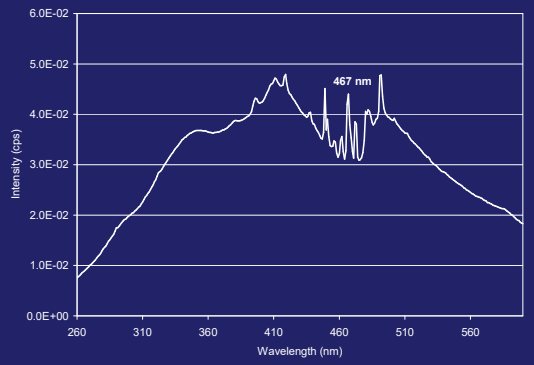


VI. Fluorescence Measurements

5. Routine experimental procedures

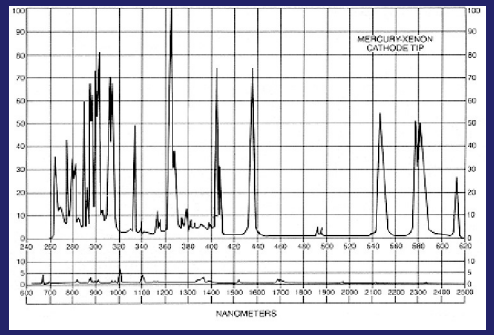
- Check wavelength calibration of excitation monochromator

Xe lamp scan



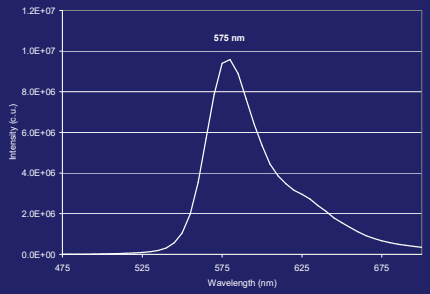
- Check wavelength calibration of emission monochromator

Hg lamp spectrum scan



- Check throughput of spectrofluorometer

Rhodamine standard scan

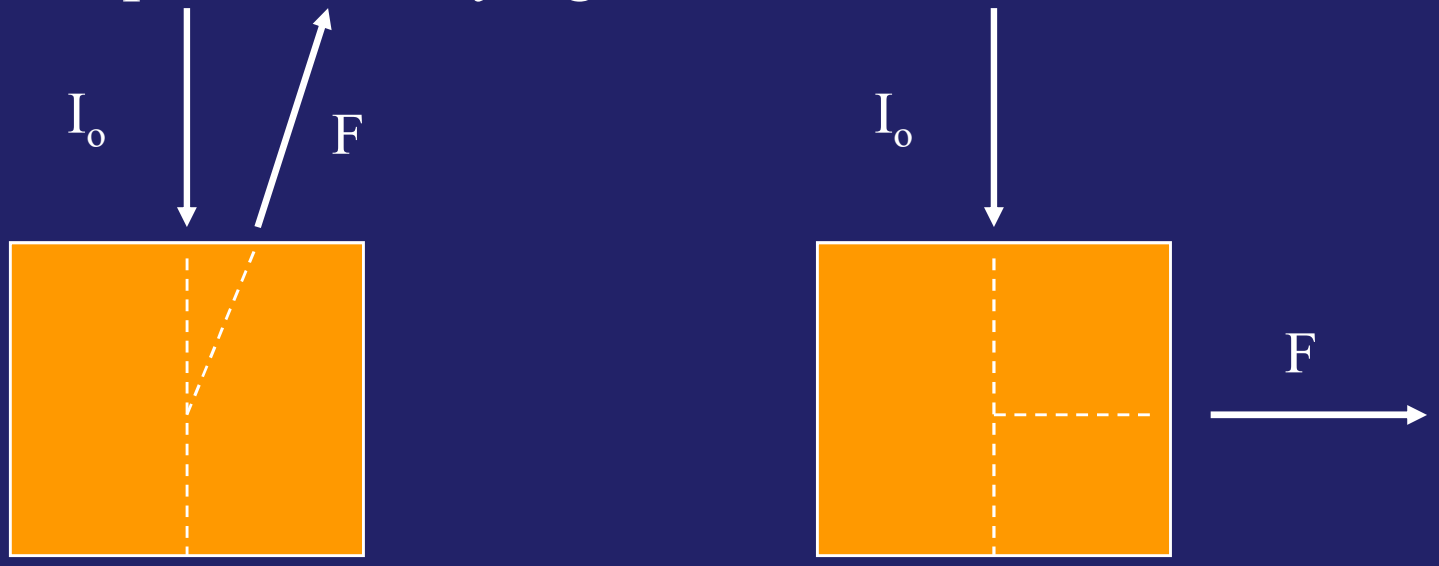


VI. Fluorescence Measurements

6a. Collection geometry in sample compartment

- Front face – collection is at a 22 degree angle relative to the incident beam; appropriate for an optically absorbing / scattering sample; more stray light

- Right angle – collection is at a right angle to the incident light; appropriate for optically transparent sample; less stray light



Front Face Right Angle

VI. Fluorescence Measurements

6c. Features of right angle illumination

- Appropriate for optically transparent sample

- At high optical densities, signal reaching detector will be significantly diminished

VI. Fluorescence Measurements

6d. Features of front face illumination

- Appropriate for an optically absorbing / scattering sample
- At high optical densities, light is absorbed near the surface of the cuvette containing the absorber; therefore fluorescence is detectable
- Fluorescence independent of concentration at high optical densities

VI. Fluorescence Measurements

7. Blank scan

- Blank is identical to sample except it does not contain fluorophore
- Measuring the fluorescence of these samples allows the scattering (Rayleigh and Raman) to be assessed
- In addition, such samples can reveal the presence of fluorescence impurities, which can be subtracted

VI. Fluorescence Measurements

8. Typical fluorescence emission spectrum at 340 nm excitation (the different components)