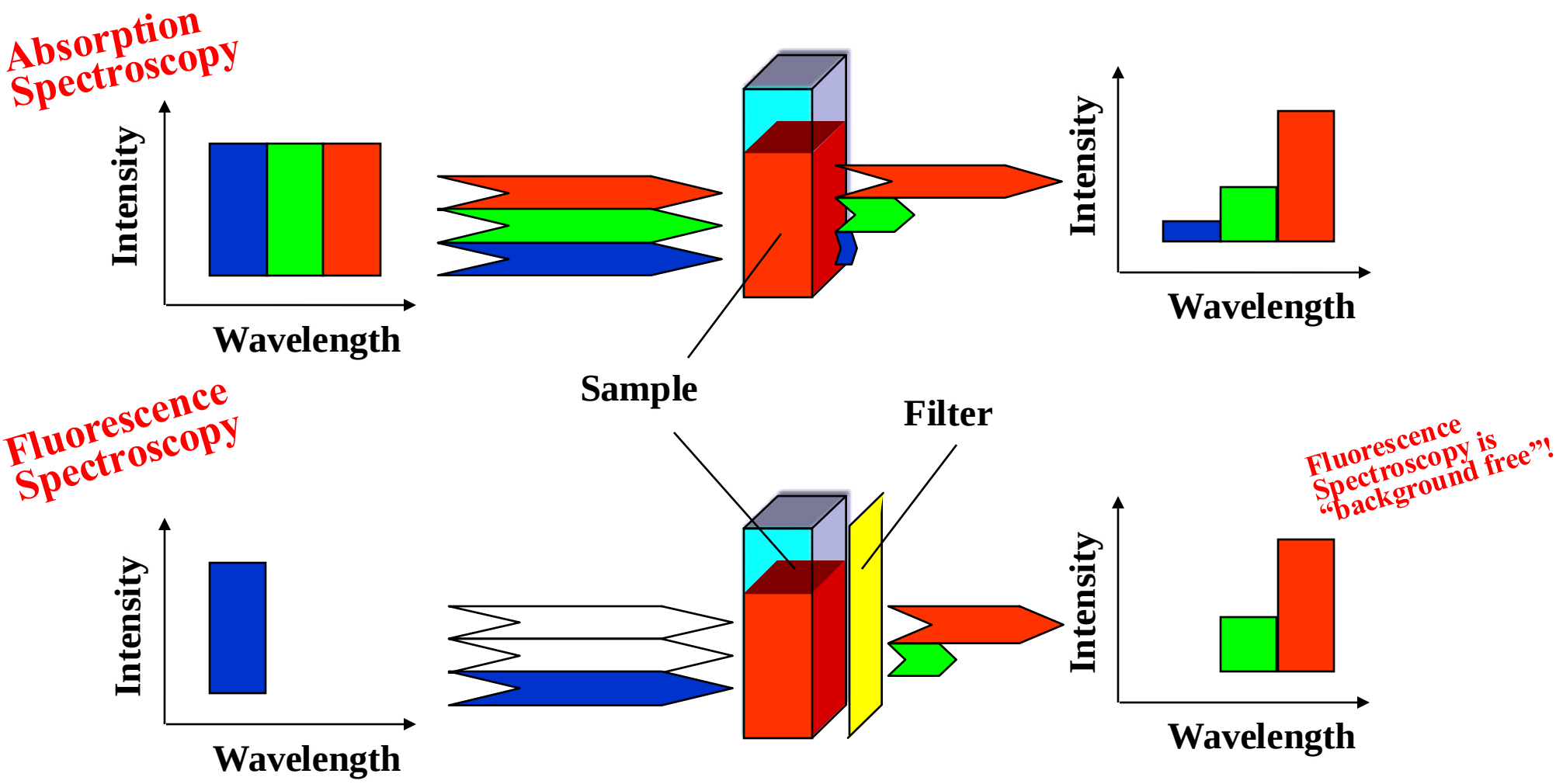
7.2 Fluorescence Spectroscopy

I. Luminescence
II. Principles of Fluorescence
III. Quantum yield and lifetime
IV. Fluorescence Intensity / Spectroscopy
V. Biological Fluorophores
VI. Fluorochromes used in vivo for oncologic Applications

Absorption and Fluorescence Spectroscopy



I. Luminescence

Luminescence

- Emission of photons from electronically excited states

- Two types of luminescence:

  Relaxation from singlet excited state

  Relaxation from triplet excited state

I. Luminescence

Singlet and triplet states

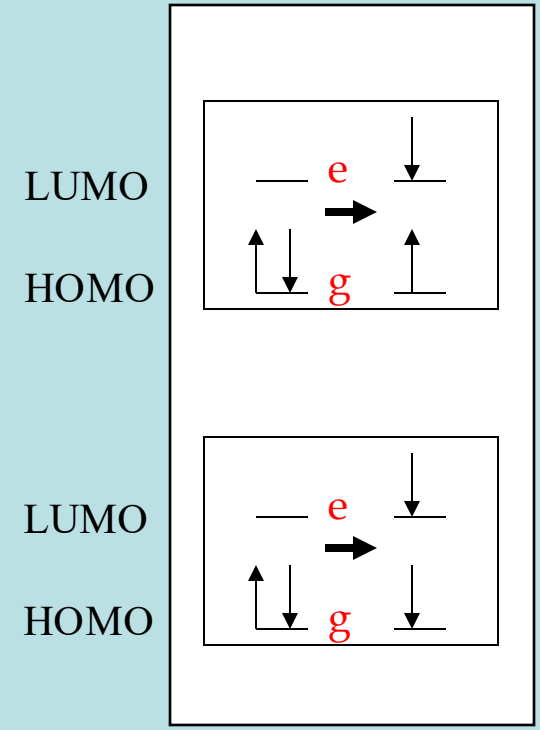
Ground state – two electrons per orbital;
electrons have opposite spin and are paired

Singlet excited state

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

Triplet excited state

Electrons in both orbitals have same spin orientation; spin flip may occurs when there is interaction of spin with a magnetic field



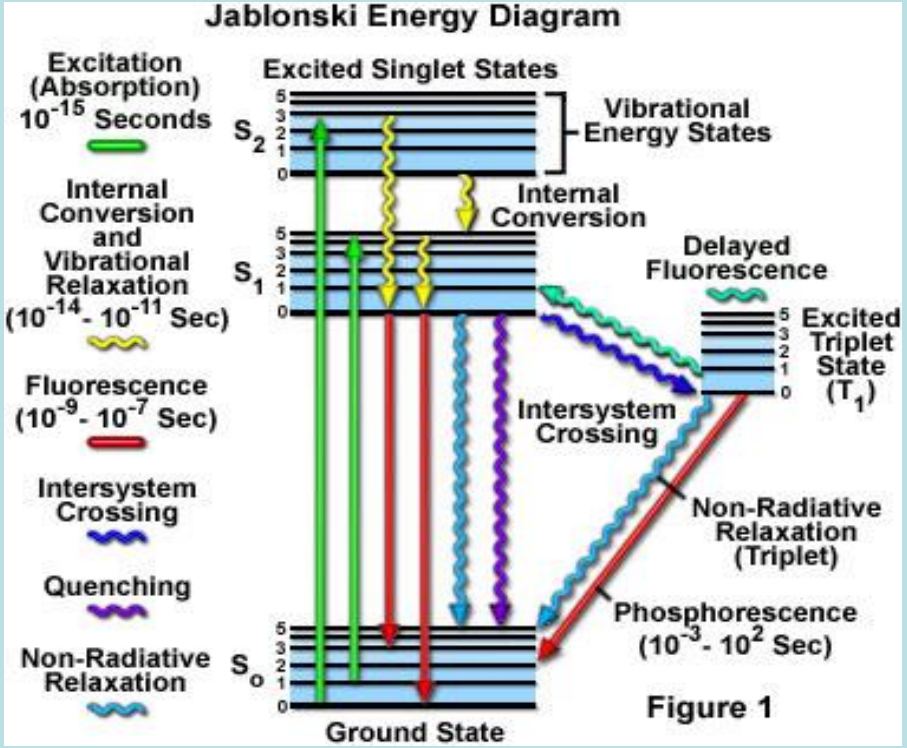
I. Luminescence

Types of emission

- **Fluorescence** – return from excited singlet state to ground state; does not require change in spin orientation
- **Phosphorescence** – 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. Luminescence

Energy level diagram (Jablonski diagram)




Alexander Jablonski (1898-1980)

Alexander Jablonski, who would one day come to be known as the father of fluorescence spectroscopy, was born in Ukraine and educated in the University of Warsaw, Poland. His doctoral dissertation, entitled "***On the influence of the change of wavelengths of excitation light on the fluorescence spectra***", concerned what would become the primary focus of his professional career. His work resulted in his introduction of what is now known as a **Jablonski Energy Diagram**, a tool that can be used to explain the kinetics and spectra of fluorescence, phosphorescence, and delayed fluorescence.

Jablonski diagrams (see Figure 1) are often used as a starting point for discussions regarding the absorption and emission of light.

II. Principles of Fluorescence

II. Principles of fluorescence

1. Absorption
2. Fluorescence
3. Stokes shift
4. Invariance of emission wavelength
5. Mirror image rule

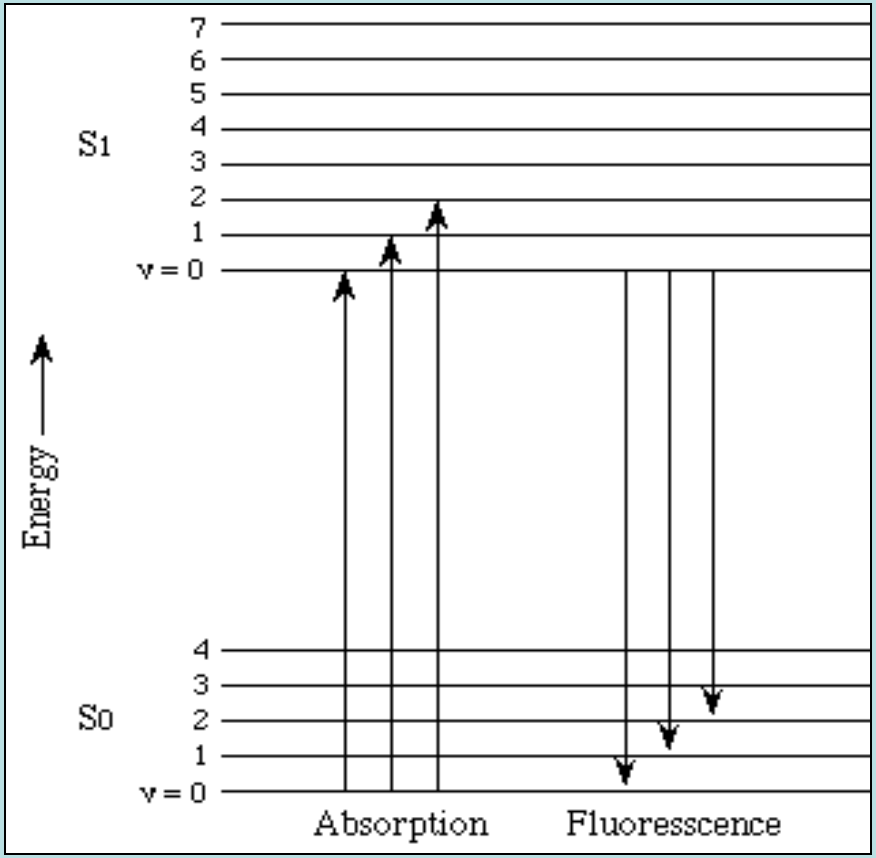
II. Principles of Fluorescence

1a. Absorption

- At room temperature (300 K), and for typical electronic and vibration energy levels, one can calculate the ratio of molecules in upper and lower states
- At room temperature, everything starts out at the lowest vibrational energy levels of the ground state
- When a molecule is illuminated with light at a resonance frequency, it is promoted to a vibrational energy level of the excited state

II. Principles of Fluorescence

1b. Absorption



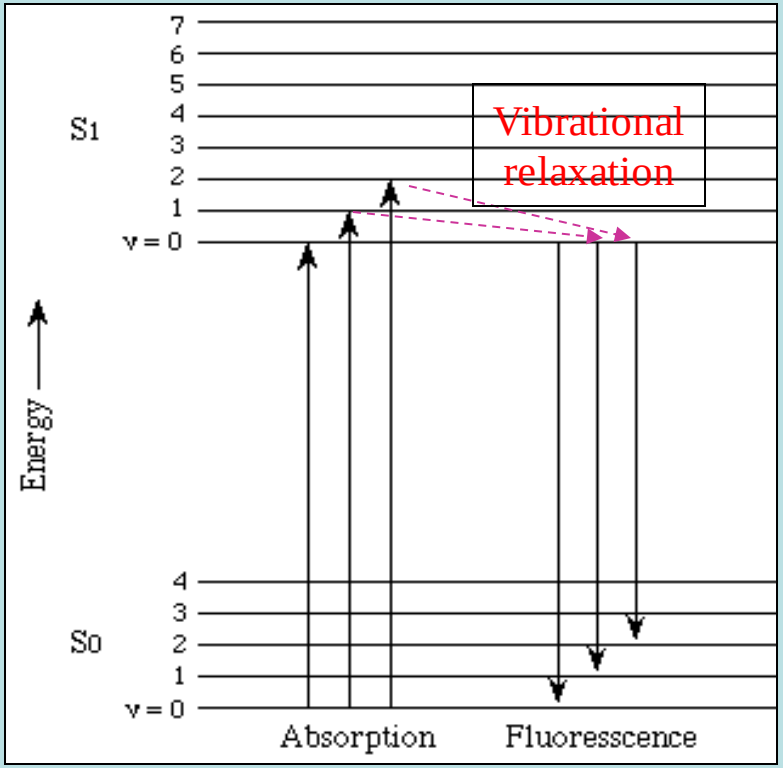
II. Principles of Fluorescence

2a. Fluorescence

- Excitation - following light absorption, a fluorophore is excited to some higher vibrational energy level of S_1 or S_2 (10^{-15} s).
- Internal convertion + Vibrational relaxation - molecule relaxes back to lowest vibrational energy level of S_1 (10^{-12} s)
- Emission – relaxation back to a vibrational energy level of the ground electronic state

II. Principles of Fluorescence

2b. Fluorescence

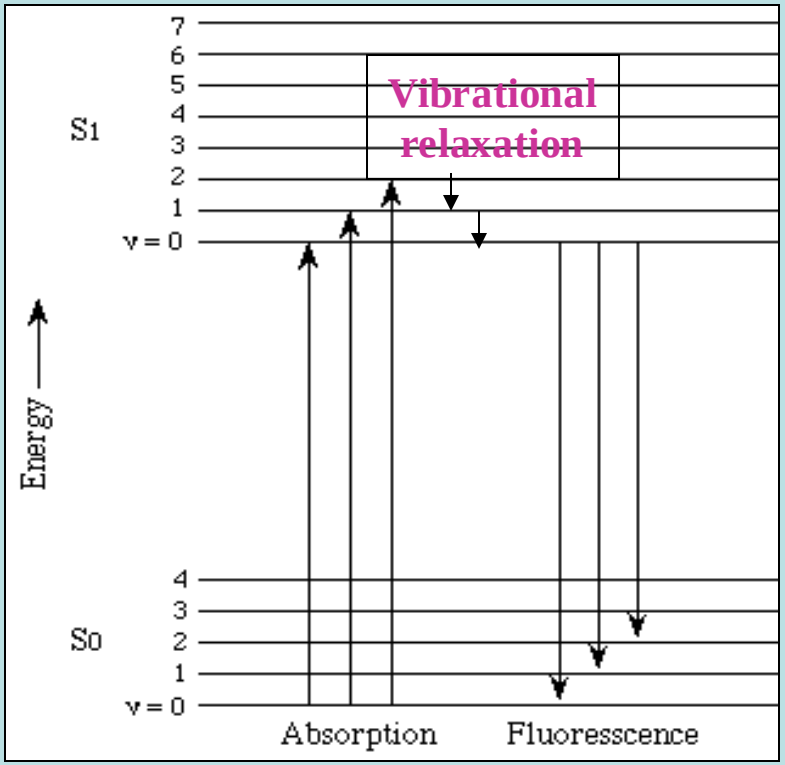


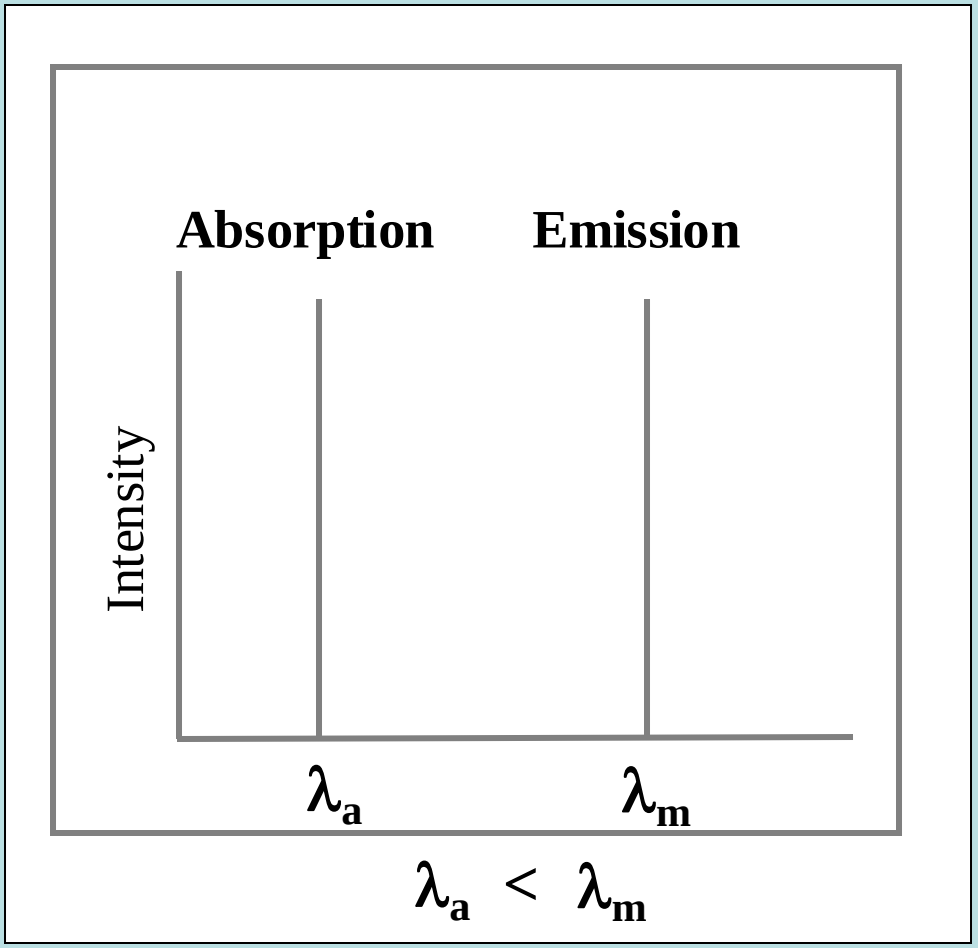
II. Principles of Fluorescence

3a. Stokes shift

- The fluorescence light is red-shifted (longer wavelength than the excitation light) relative to the absorbed light ("Stokes shift").
- Internal conversion + vibrational relaxation can cause Stokes shift
- Solvent effects (charge redistribution in the molecule environment induced by the change of dipole moment between the ground and excited states) and excited state reactions (due to a change of reactivity) can also cause a Stokes shift

II. Principles of Fluorescence





II. Principles of Fluorescence

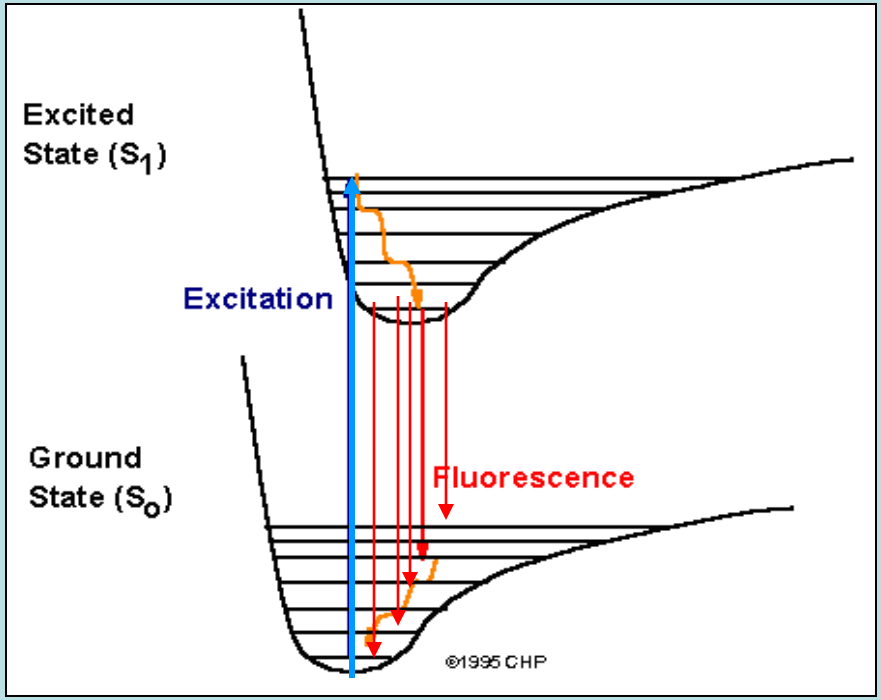
4a. Invariance of emission spectrum with excitation wavelength

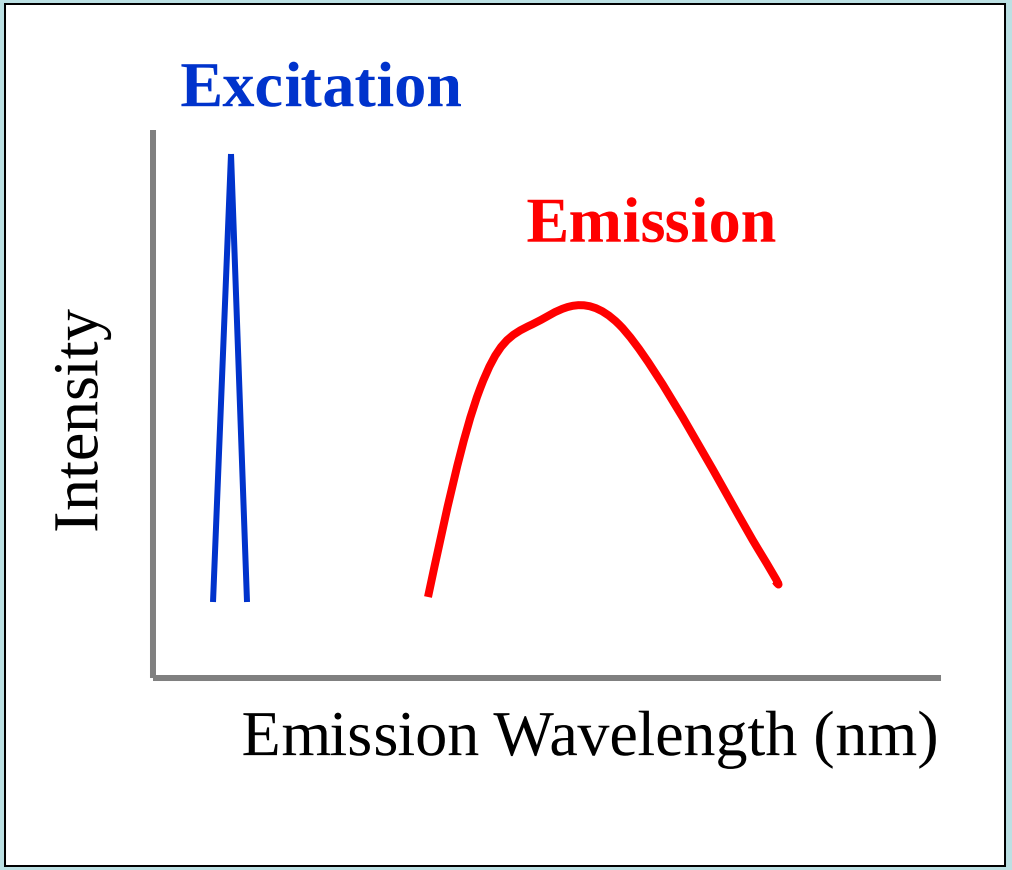
- For a molecule, the same fluorescence emission wavelength is observed irrespective of the excitation wavelength

- Emission is only due to relaxations from the lowest vibrational level of S_1

II. Principles of Fluorescence

4b. Invariance of emission spectrum with excitation wavelength





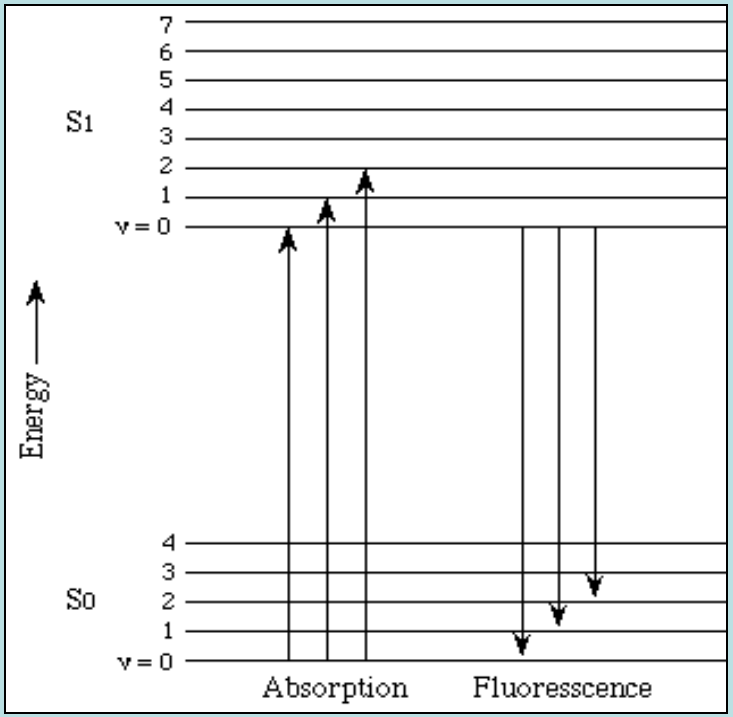
II. Principles of Fluorescence

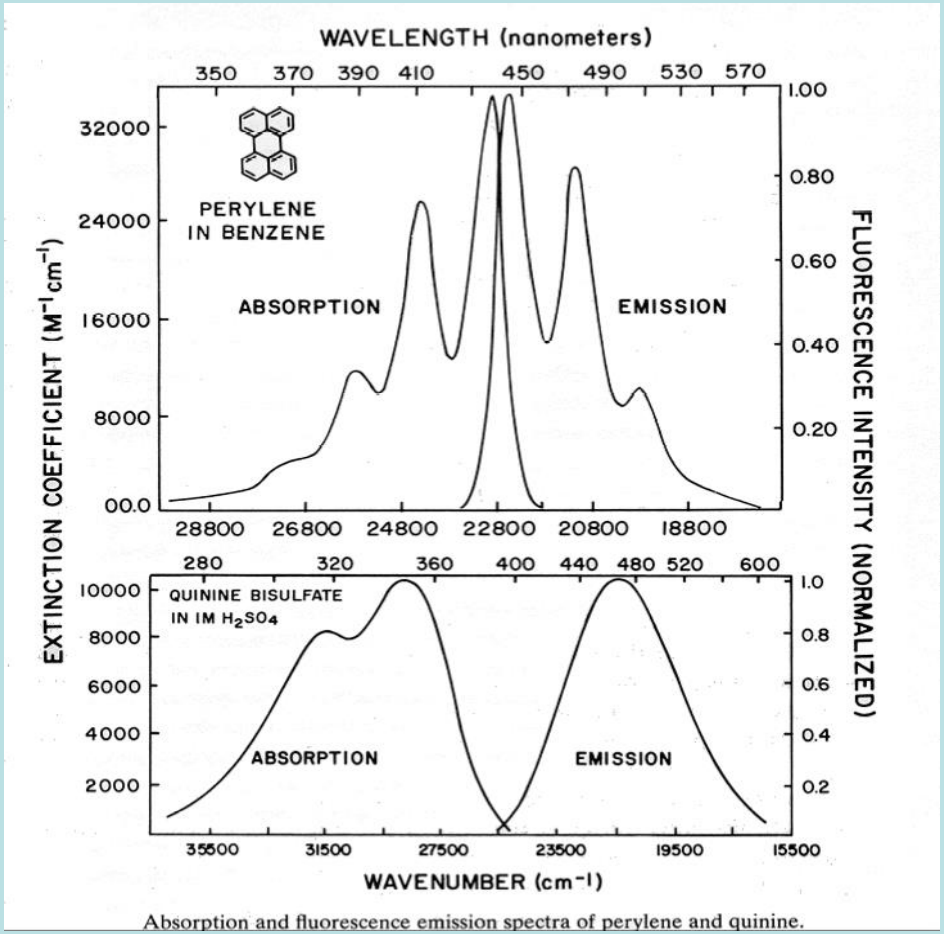
5a. Mirror image rule

- An absorption spectrum reflects the vibrational levels of the electronically excited state
- An emission spectrum reflects the vibrational levels of the electronic ground state
- Vibrational levels in absorption and emission are frequently similar !
- Fluorescence emission spectrum is a mirror image of the absorption spectrum (S_o to S_1 transition)

II. Principles of Fluorescence

5b. Mirror image rule





Absorption and fluorescence emission spectra of perylene and quinine.

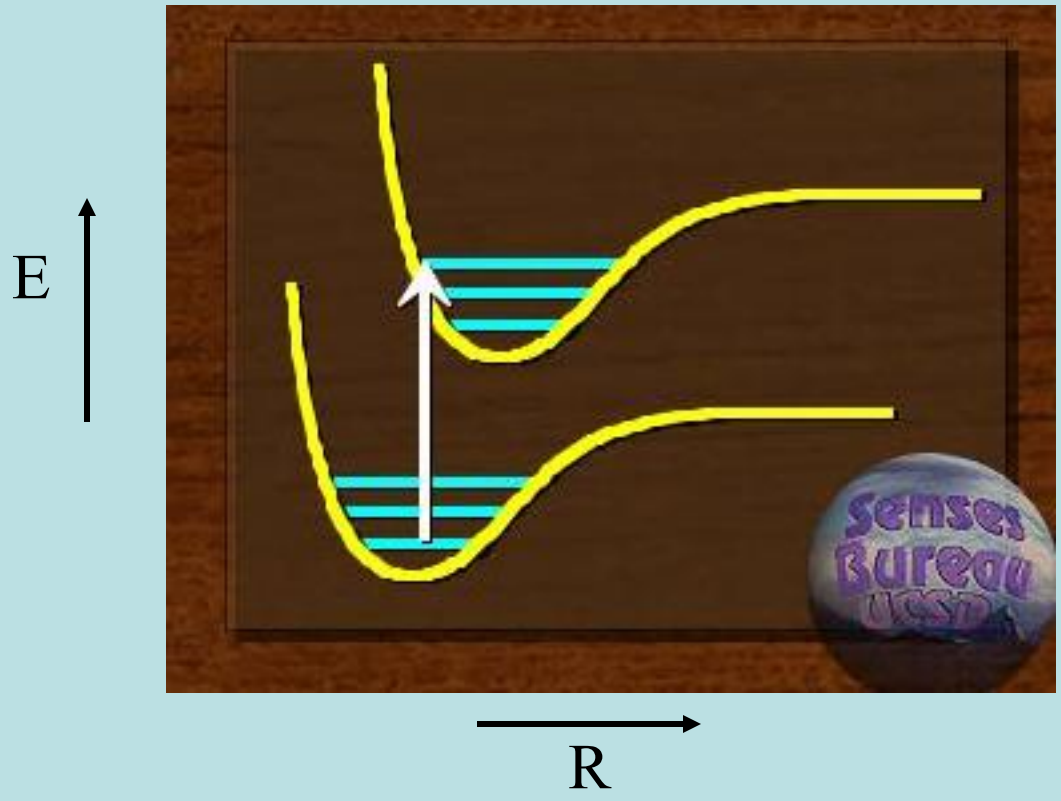
II. Principles of Fluorescence

Franck Condon principle

- The time for an electronic transition is:
  $f = E/h$; ~ 10^{-15} s (at 420 nm)
- Franck Condon principle: electronic transitions occur so rapidly that during the transition the nuclei are static
- Thus, all electronic transitions are vertical

II. Principles of Fluorescence

Franck Condon principle

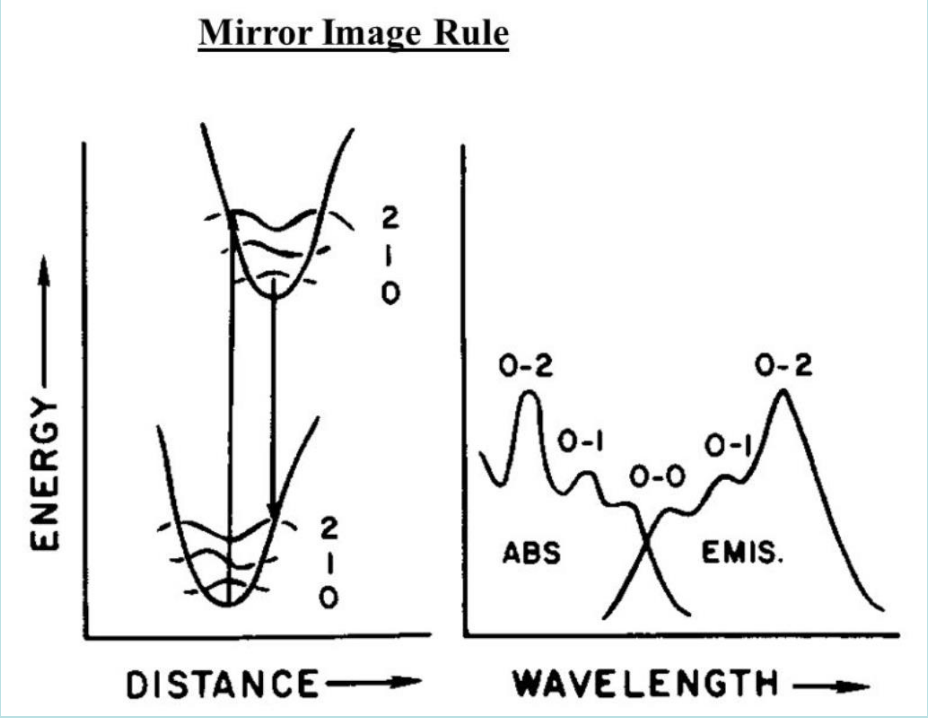


All electronic transitions are vertical !

II. Principles of Fluorescence

5b. Mirror image rule and Franck-Condon principle

(If a particular transition probability is large in absorption, the reciprocal transition is also the most probable emission)



III. Quantum Yield and Lifetime

III. Quantum yield and lifetime

1. Modified Jablonski diagram
2. Lifetime
3. Quantum yield

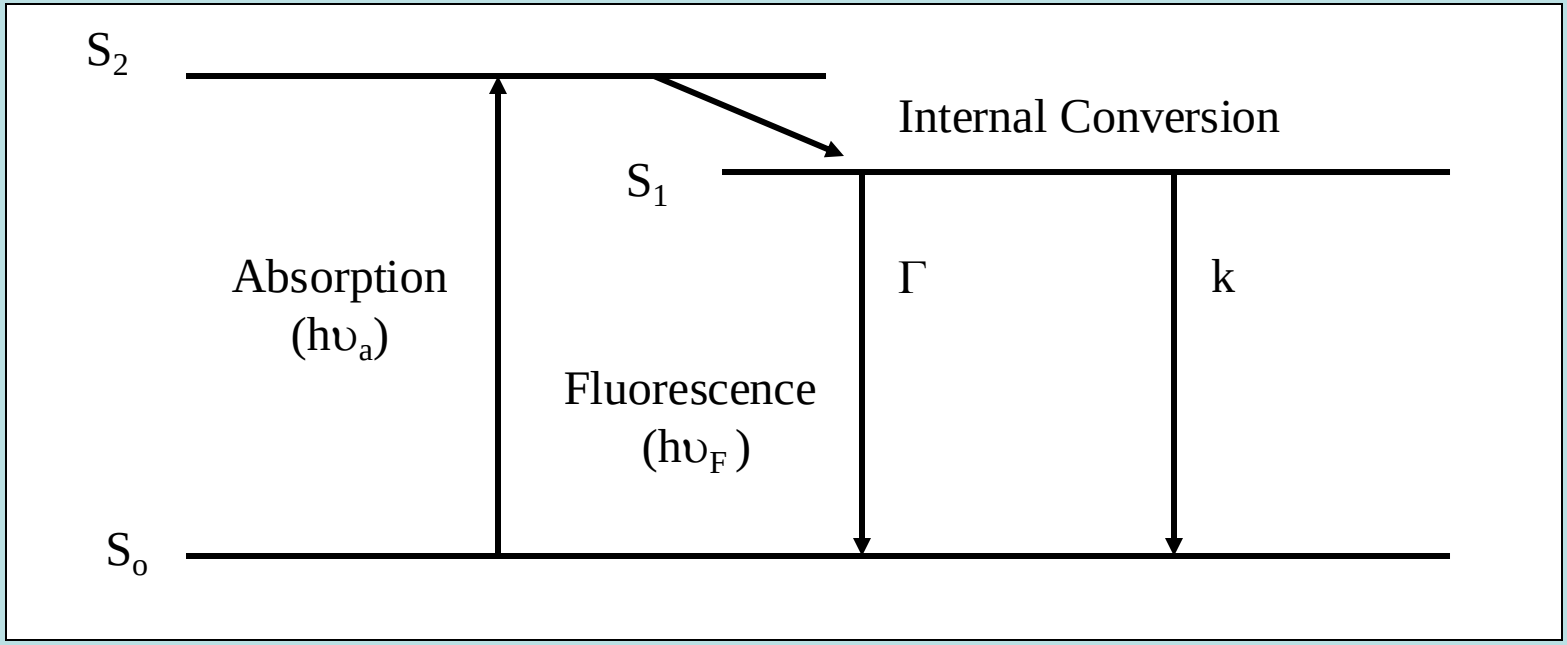
III. Quantum Yield and Lifetime

1. Modified Jablonski diagram

k - rate of radiationless decay [s^{-1}]

Γ - rate of radiative decay (fluorescence) [s^{-1}]

Both depopulate the excited state



III. Quantum Yield and Lifetime

2. Lifetime

Lifetime of the excited state is the average time a molecule spends in the excited state before returning to ground state (~10 ns)

$$t = \frac{1}{G + K}$$

Note: τ is the average time spent in the excited state

III. Quantum Yield and Lifetime

2b. Characteristics of life time

- Looks at how excited states depopulate over time
- Can reflect properties of environment
- Can reflect molecular dynamics of molecule
- Concentration independent

III. Quantum Yield and Lifetime

3a. Fluorescence quantum yield

Fluorescence quantum yield: ratio of the number of photons emitted as fluorescence to the total number of de-excitations:

$$Q = \frac{G}{G + K}$$

III. Quantum Yield and Lifetime

3b. Characteristics of quantum yield

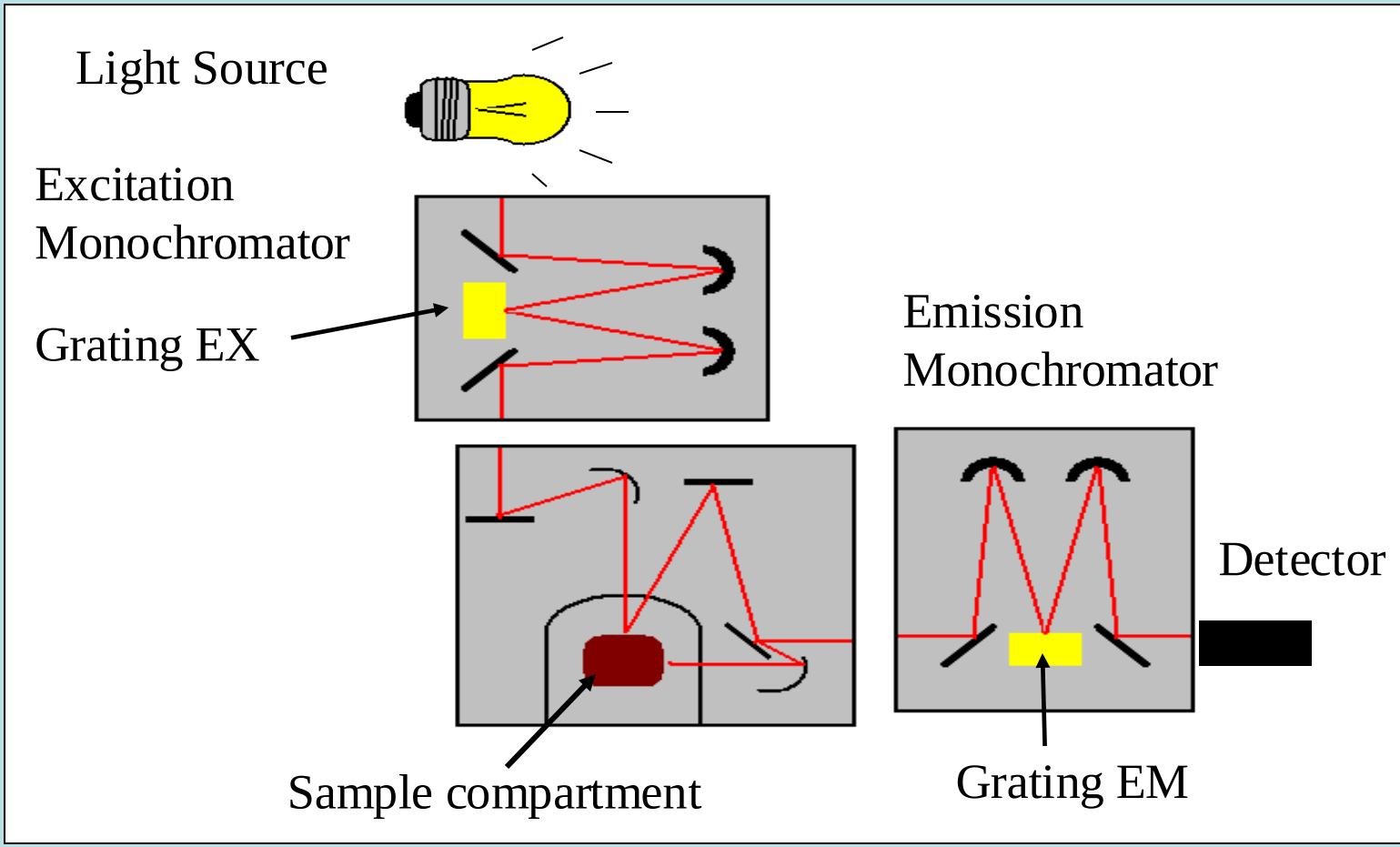
- A molecule may be non-fluorescent if it has a large rate of radiationless decay or a slow rate of emission
- Rate constants sensitive to the fluorophore environment
- Quantum yield is difficult to measure; generally compared with standard sample quantum yield
- Concentration dependent!

IV. Fluorescence Spectroscopy

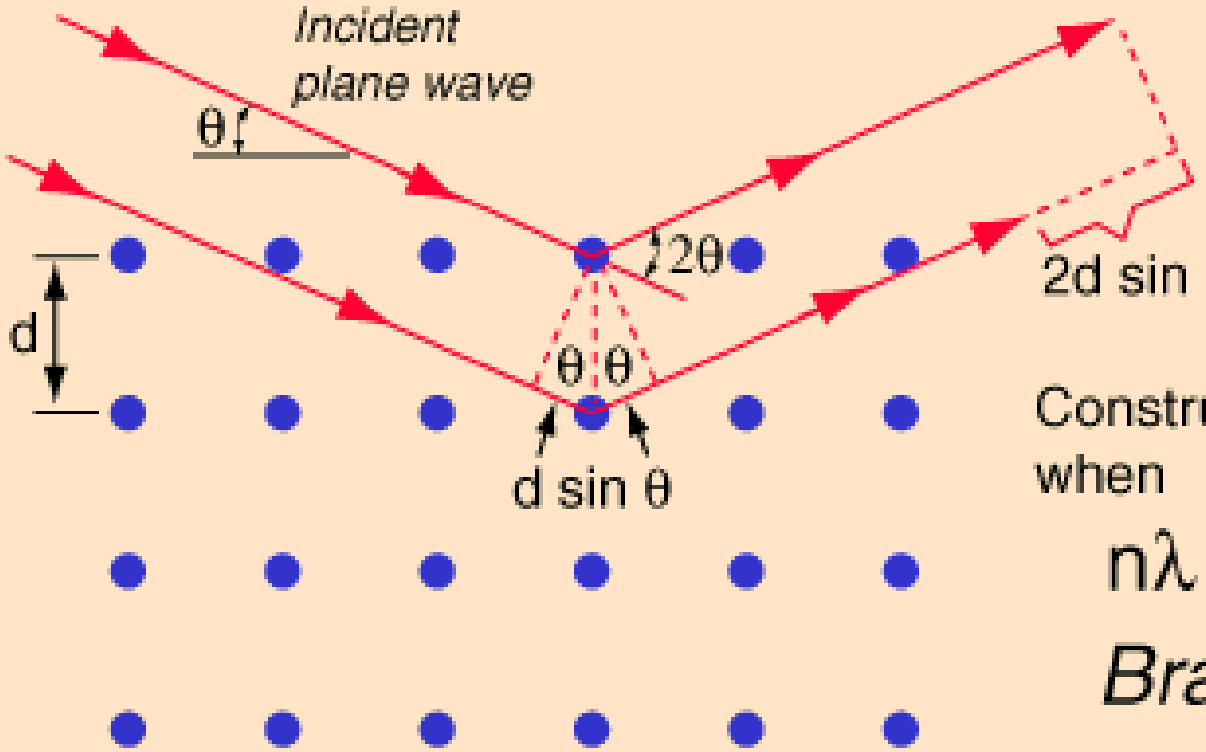
IV. Fluorescence intensity

1. Fluorescence intensity expression
2. Fluorescence spectra

Schematic of a spectrofluorometer



Bragg's Law



Constructive interference when

$$n\lambda = 2d \sin\theta$$

Bragg's Law

IV. 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:

$$F(\lambda_x, \lambda_m) = I_A(\lambda_x) f(\lambda_m)$$

where,

I_A – light absorbed to promote electronic transition

ϕ – quantum yield

IV. Fluorescence Intensities

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

$$I_A(I_x) = 2.303 I_o \epsilon C L$$

where,

I_o – Initial intensity

ε – molar extinction coefficient

C – concentration

L – path length

IV. 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:

$$F(\lambda_x, \lambda_m) = I_o 2.303 \epsilon(\lambda_x) C L f(\lambda_m)$$

where,

I_o – incident light intensity

C – concentration

L – path length

ϕ – quantum yield

ε – molar extinction coeff.

IV. Fluorescence Intensities

1d. Measured fluorescence intensity

If we include instrument collection angle:

$$F(I_x, I_m) = I_o 2.303 \epsilon(I_x) C L f(I_m) Z$$

where,

Z – instrumental factor

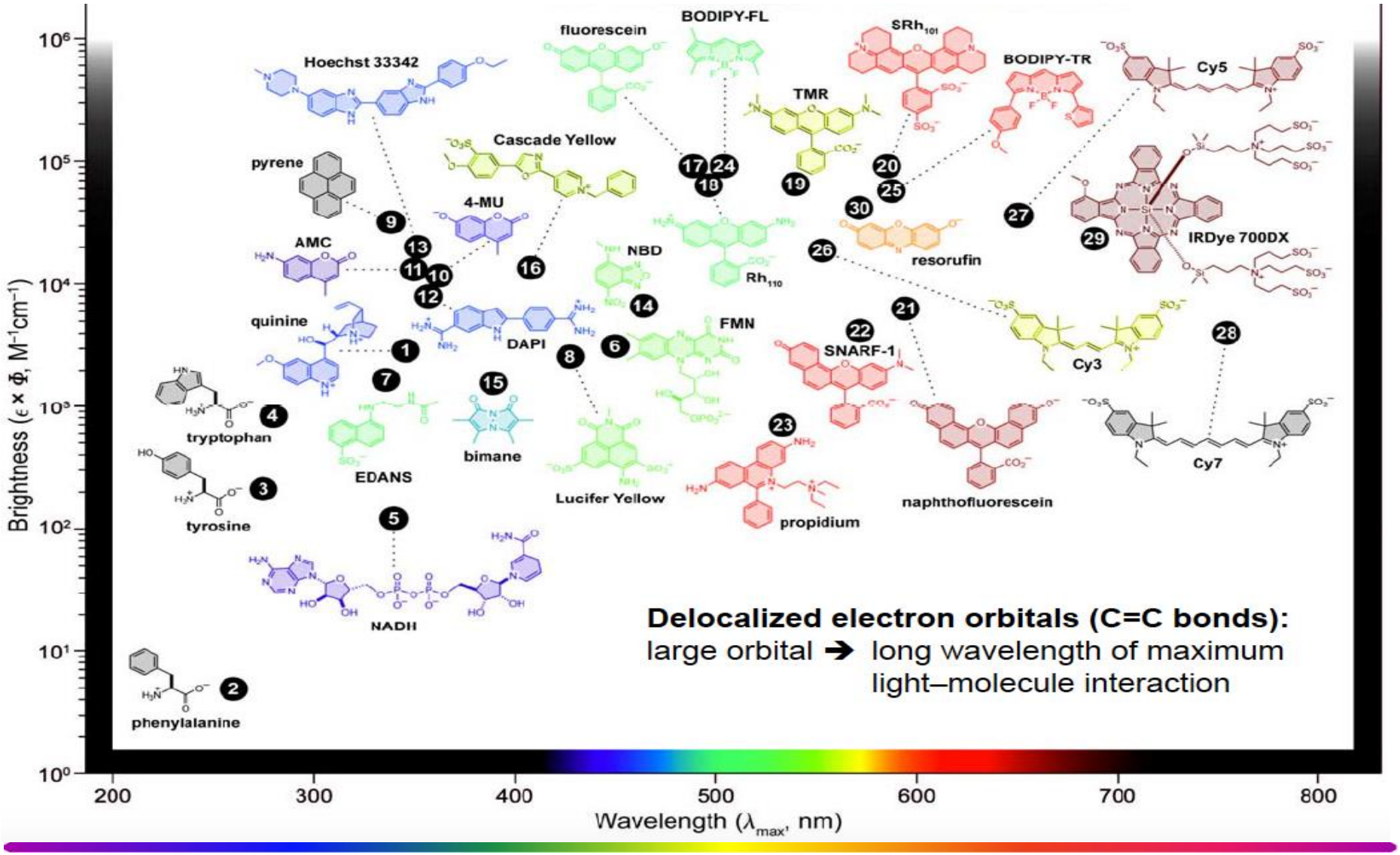
I_o – incident light intensity

ε – molar extinction coefficient

C – concentration

L – path length

IV. Fluorescence Intensities



IV. Fluorescence Intensities

2a. Fluorescence spectra

- **Excitation spectrum**
  - Hold emission wavelength fixed, scan excitation
  - Reports on absorption structure

    Reflects molar extinction coefficient, $\varepsilon(\lambda_x)$

$$F(I_x, I_m) = I_o 2.303 \epsilon(I_x) C L f(I_m) Z$$

IV. Fluorescence Intensities

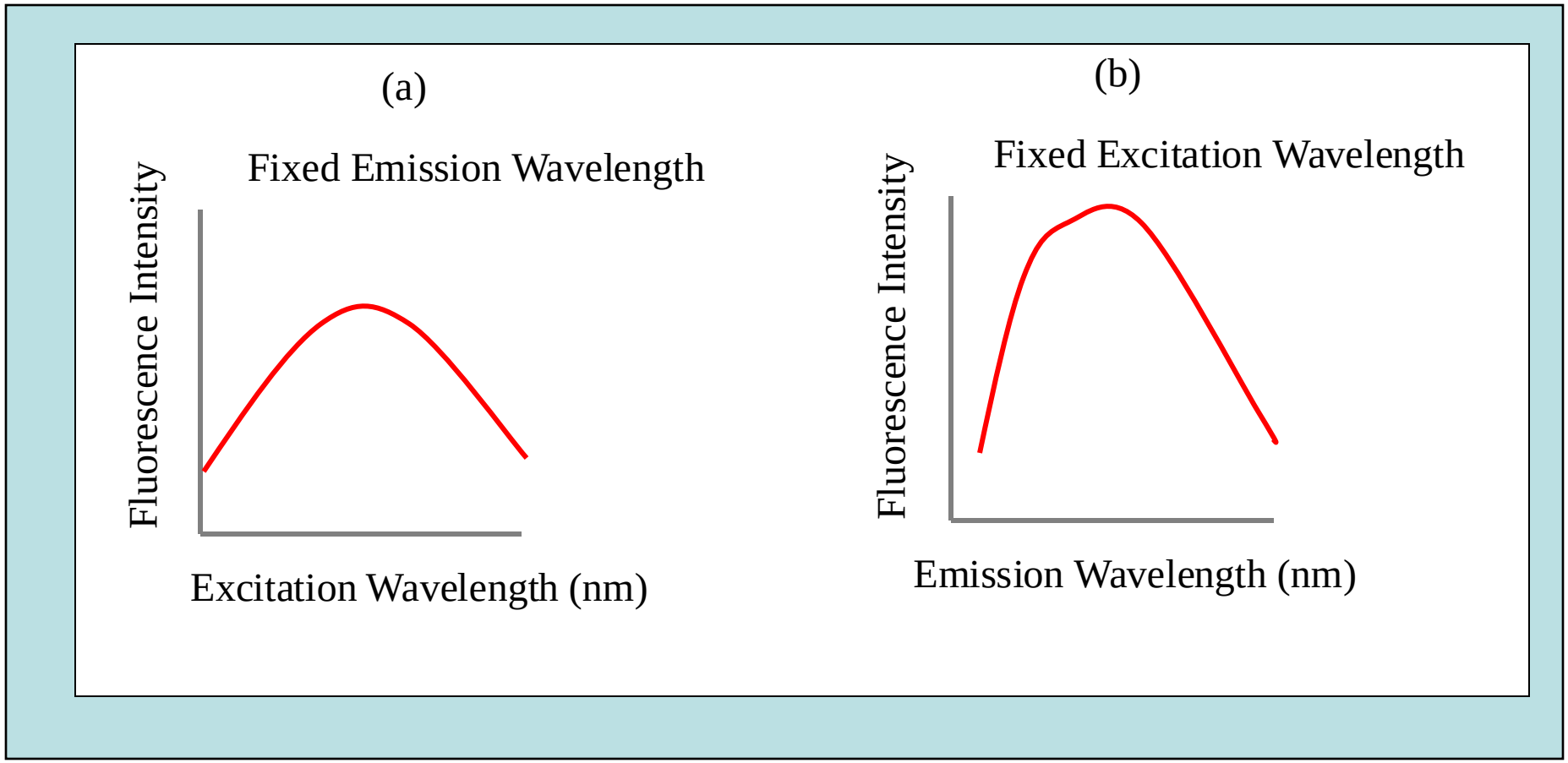
2b. Fluorescence spectra

- Emission spectrum
  - Hold excitation wavelength fixed, scan emission
  - Reports on the fluorescence spectral profile

    Reflects fluorescence quantum yield, $\phi(\lambda_m)$

$$F(\lambda_x, \lambda_m) = I_o 2.303 \epsilon(\lambda_x) C L f(\lambda_m) Z$$

IV. Fluorescence Intensities

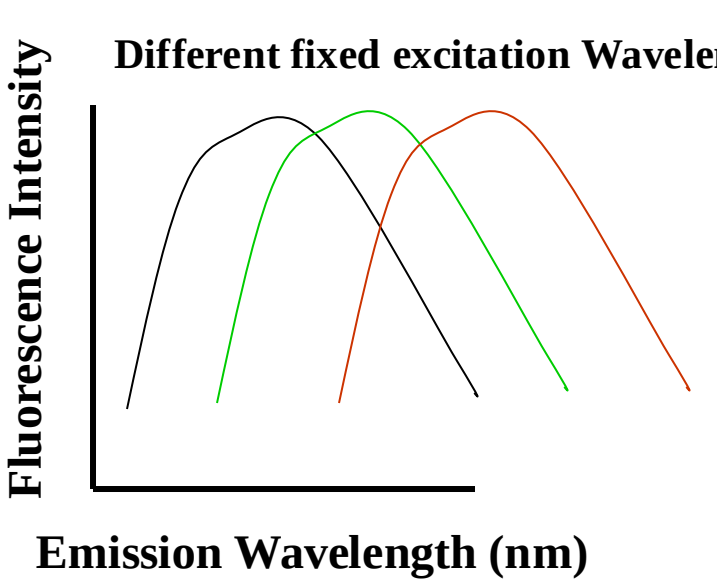


IV. Fluorescence Intensities

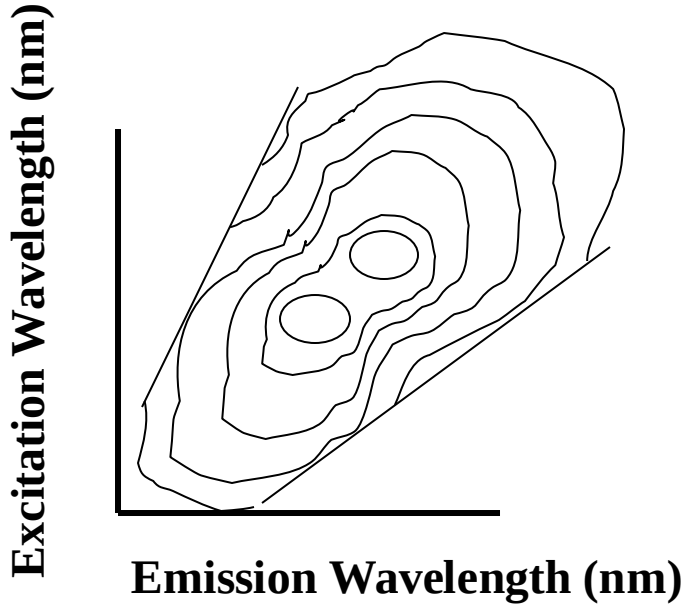
2c. Fluorescence spectra

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

IV. Fluorescence Intensities



Emission spectrum



Excitation-emission matrix

V. Biological Fluorophores

V. Biological fluorophores

1. Tables + spectra
2. EEM map
3. Epithelial cell suspension
4. Collagen

V. Biological Fluorophores

–Endogenous Fluorophores

amino acids

structural proteins

enzymes and co-enzymes

vitamins

lipids

porphyrins

–Exogenous Fluorophores

Fluorescein

Cyanine dyes

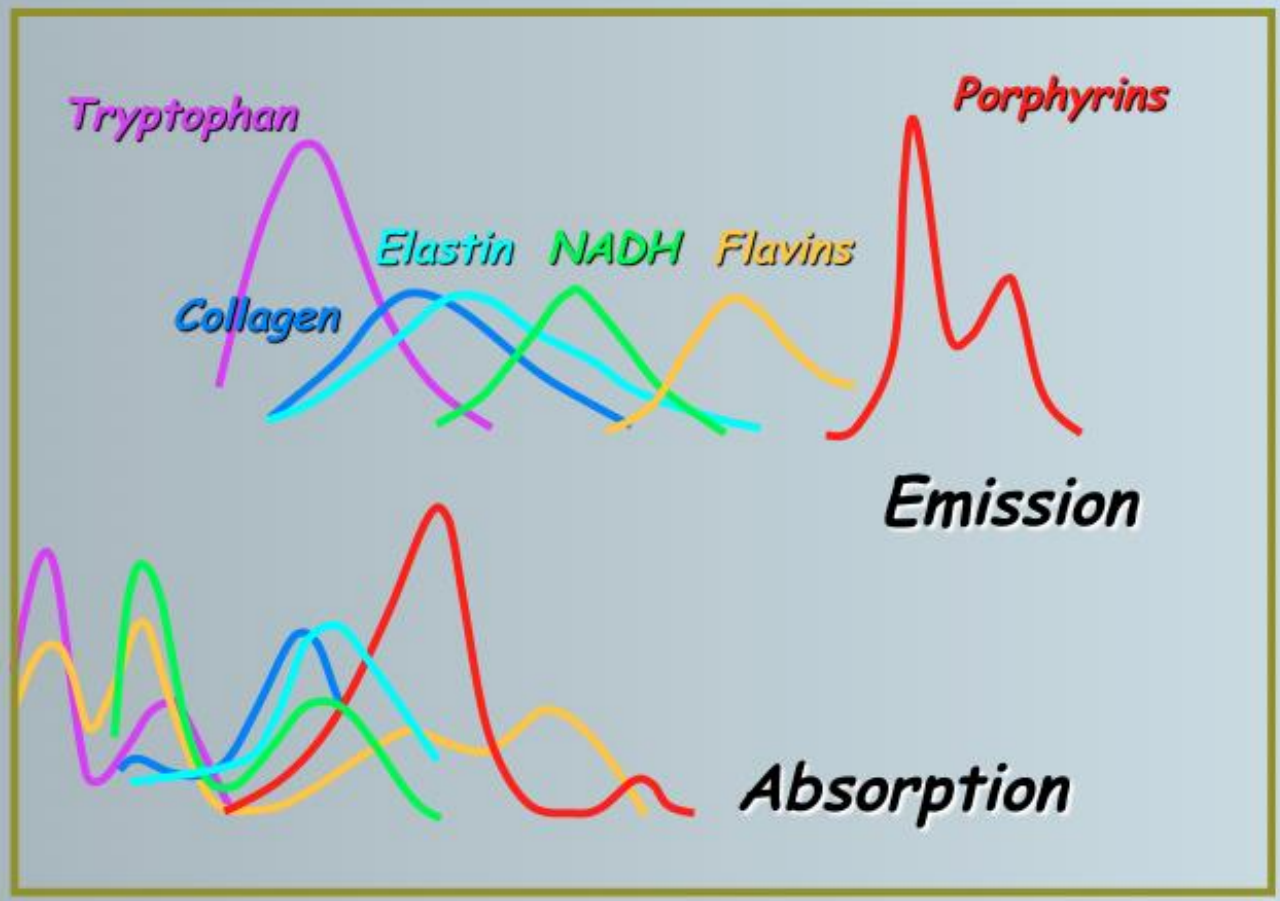
Photosensitizers

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.

Spectroscopy of Endogenous Fluorophores

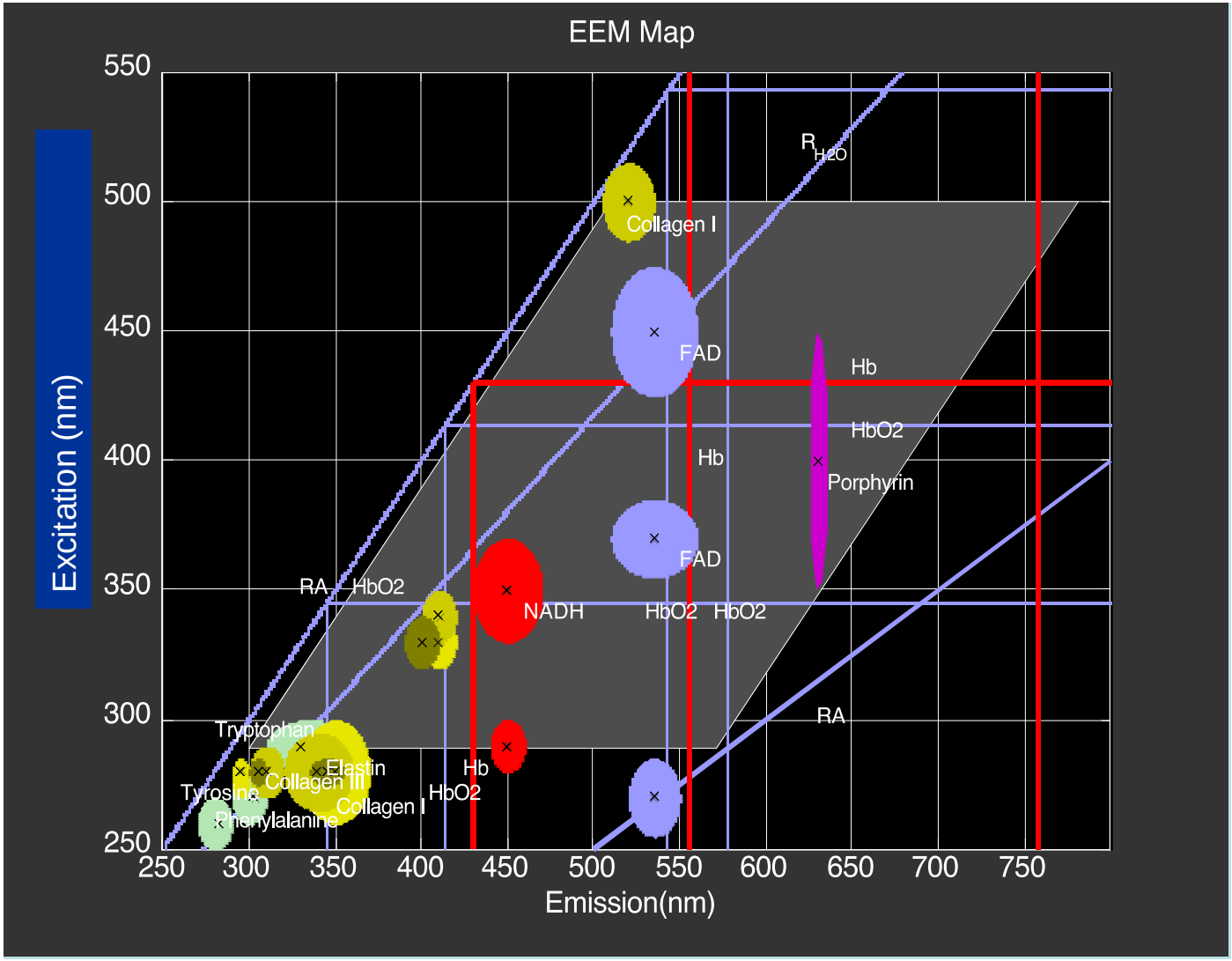


Yield of the tissue Autofluorescence :

- 10^{-3} - 10^{-4} under violet (400 nm) excitation
- 10^{-2} - 10^{-3} under UV (340 nm) excitation

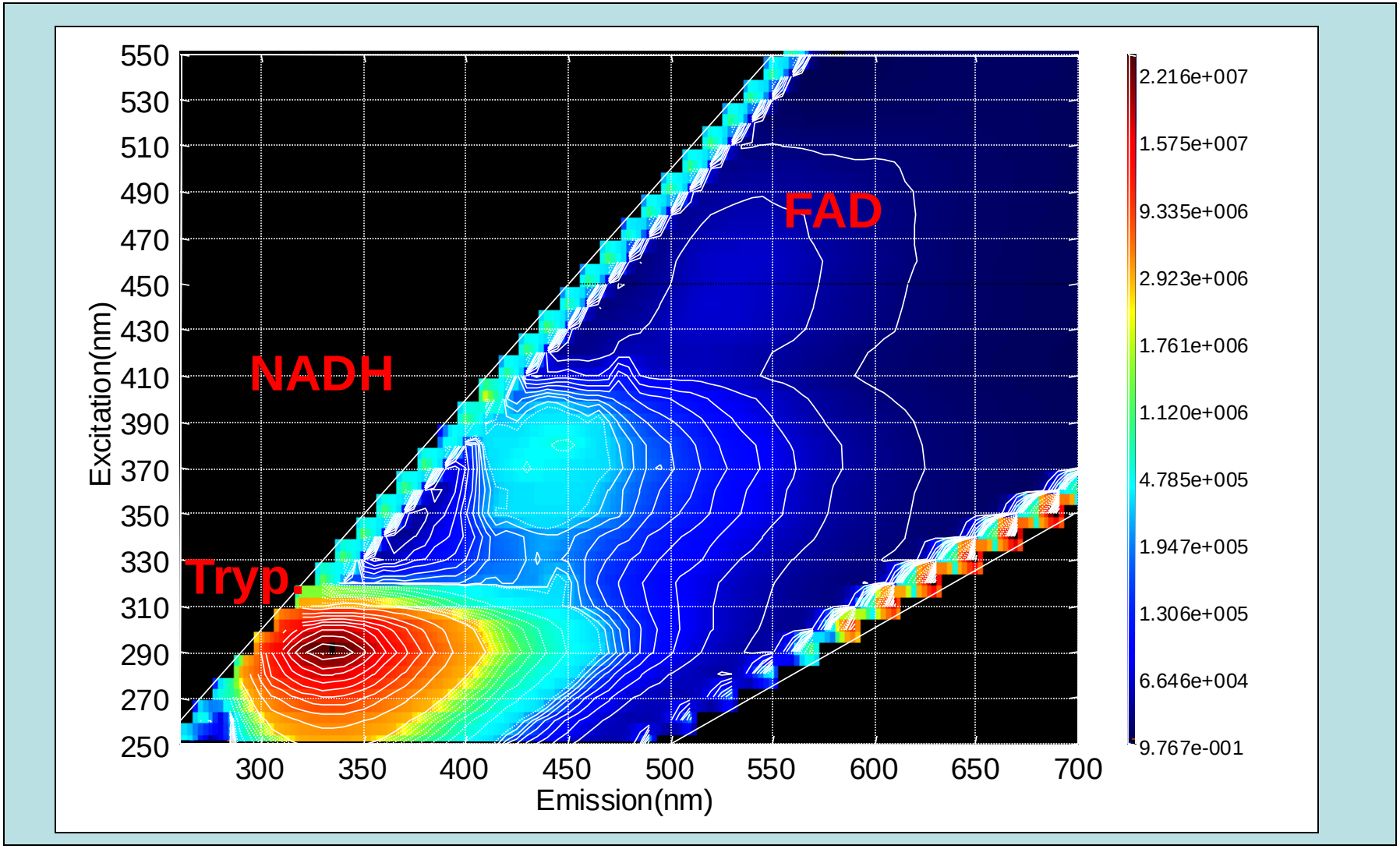
Wagnières et al.
Photochem. Photobiol.
68(5), 1998.

V. Biological Fluorophores: Ecitation – Emission Matrix (EEM)

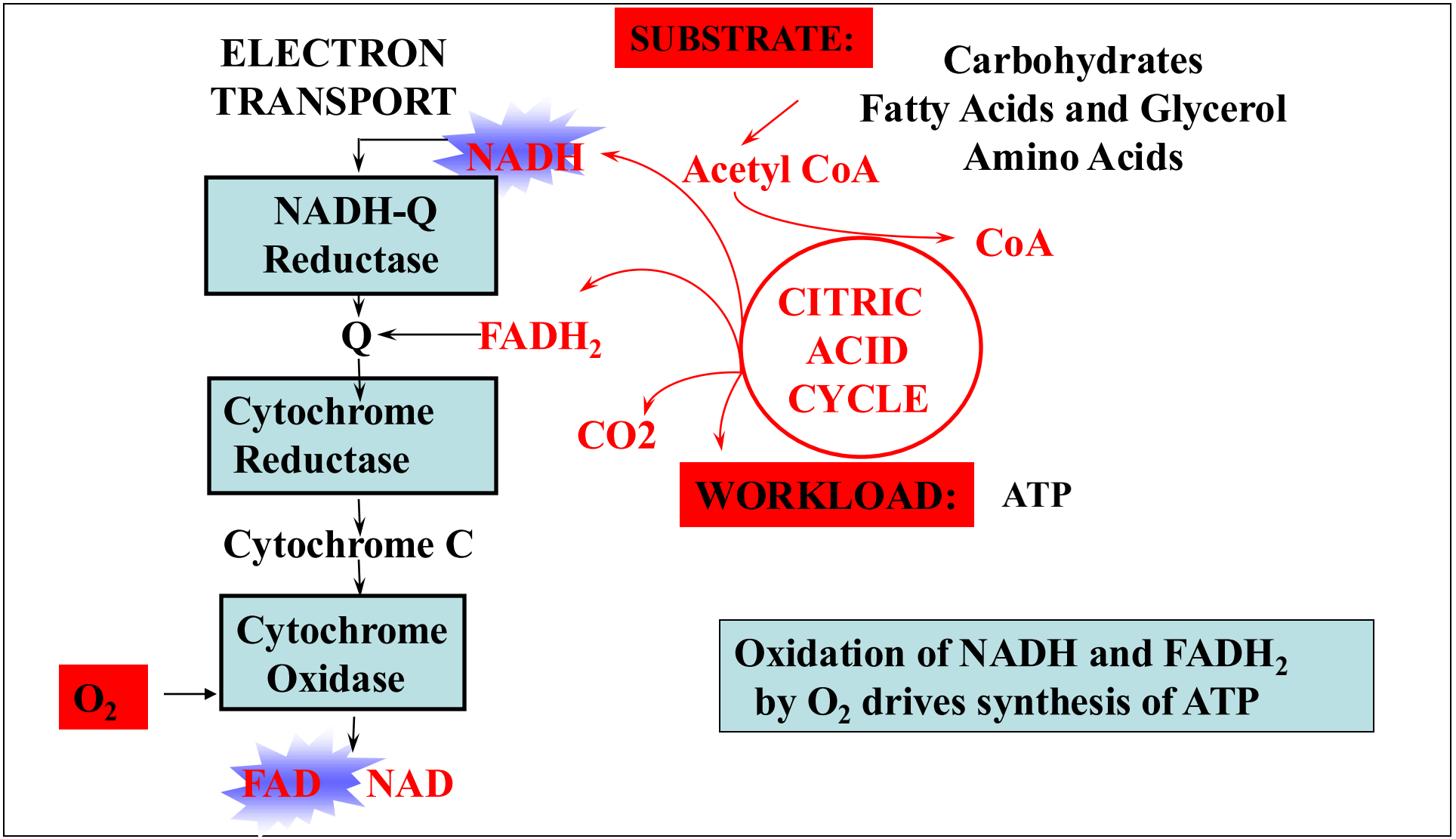


Epithelial Cell Suspension

NADH: Nicotinamide-Adenine Dinucleotide
FAD: Flavin adenine dinucleotide
Tryp.: Tryptofan



Mitochondrial Energy Generation



Metabolic Indicators

Metabolism

Redox Ratio: FAD / NADH

↓ **Redox ratio** ~ **Metabolic Rate** ↑

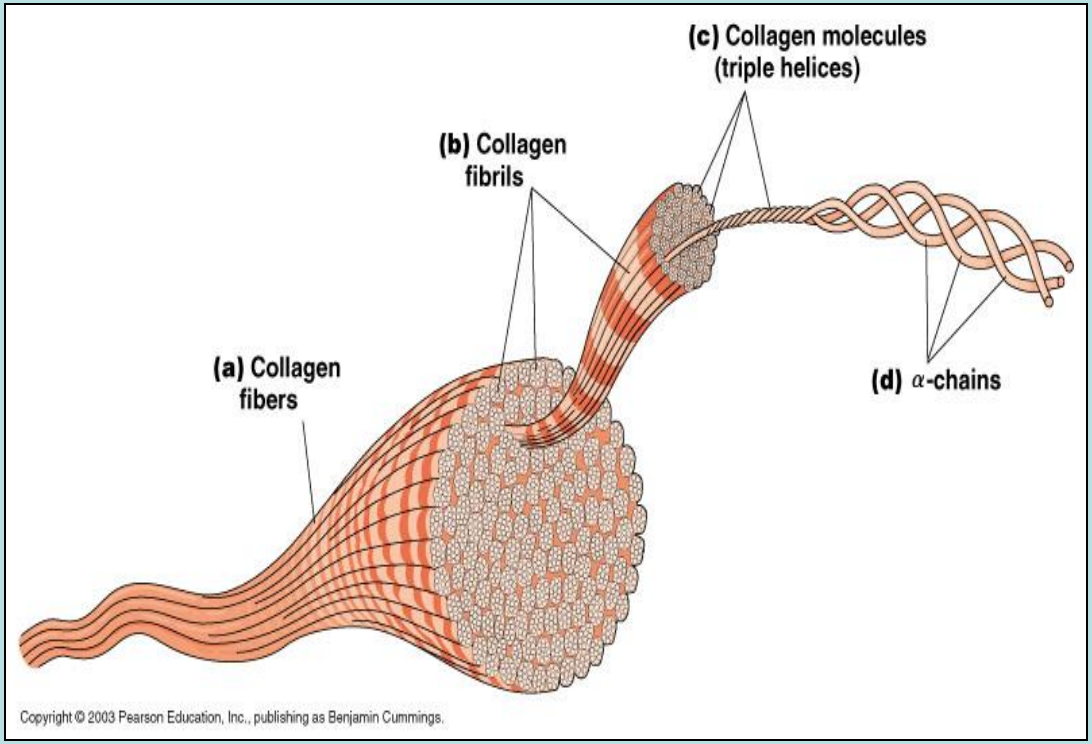
V. Biological Fluorophores

Collagen

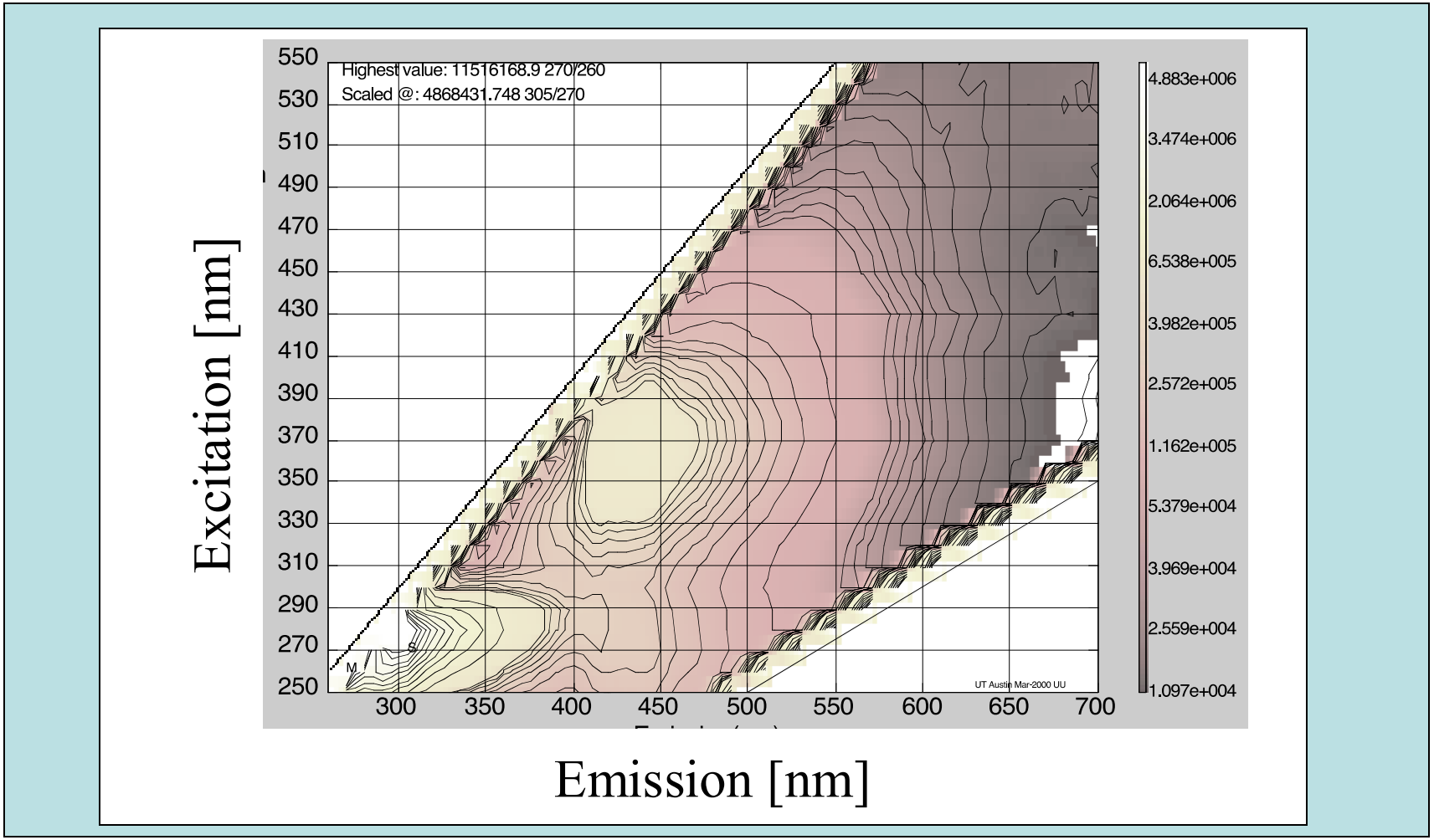
- It is the major extracellular matrix component, which is present to some extent in nearly all organs and serves to hold cells together in discrete units
- Collagen fluorescence in load-bearing tissues is associated with cross-links, hydroxylysyl pyridoline (HP) and lysyl pyridinoline (LP).
- Collagen crosslinks are altered with age and with invasion of cancer into the extracellular matrix

V. Biological Fluorophores

Collagen



Collagen I (gel)



VI. Fluorochromes used *in vivo* for oncologic Applications

Endogenous

Synthesized in the Body

- Elastin
- Flavins
- NADH
- Collagen
- …

PD in the bronchi

Endogenously induced

Induced by exogenous Precursors

- ALA±deriv. induced PPIX

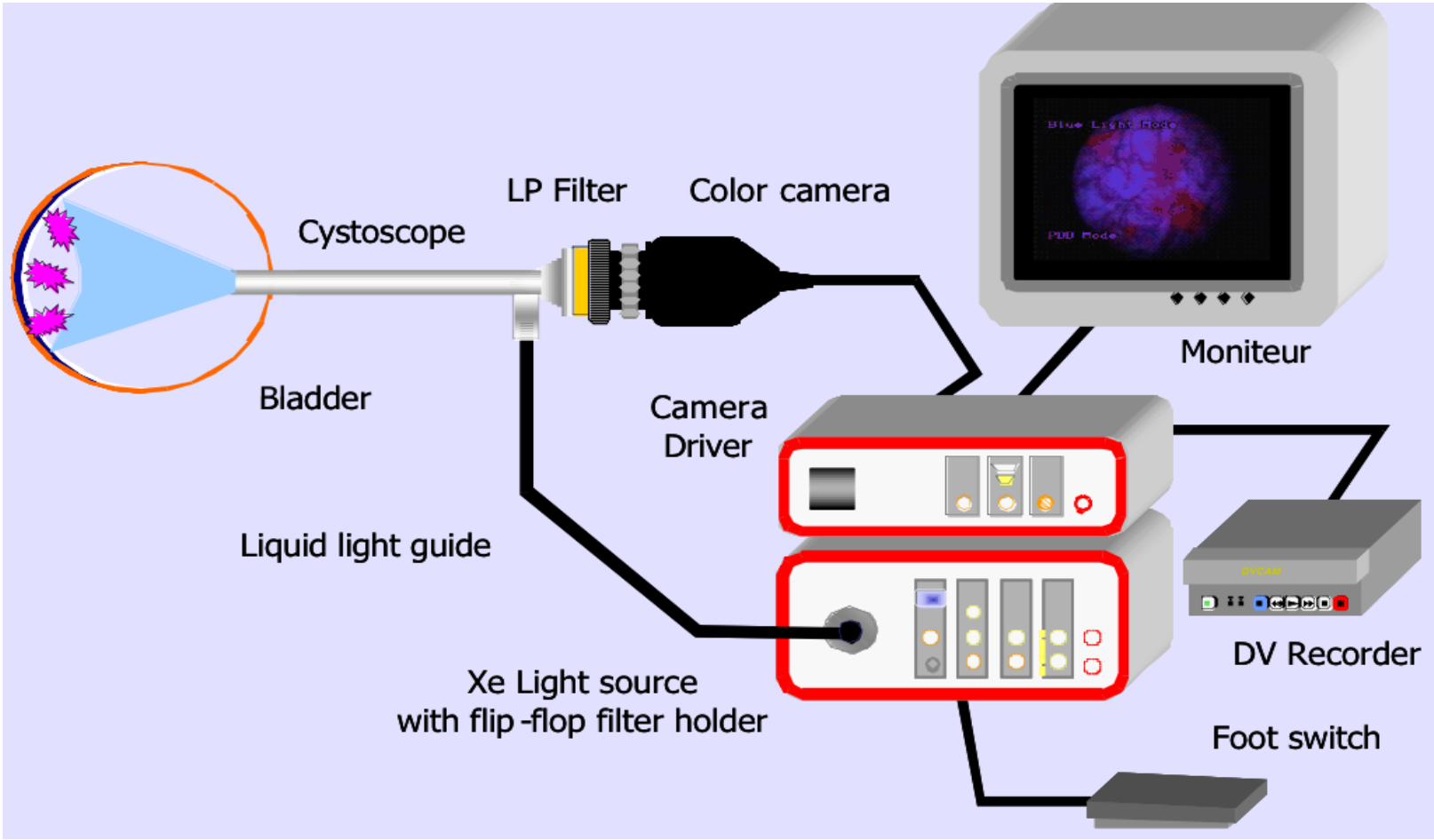
PD in the bladder

Exogenous

Synthesized before Administration

- FITC
- ICG

Principle of Diagnosis by Endoscopic Fluorescence Imaging

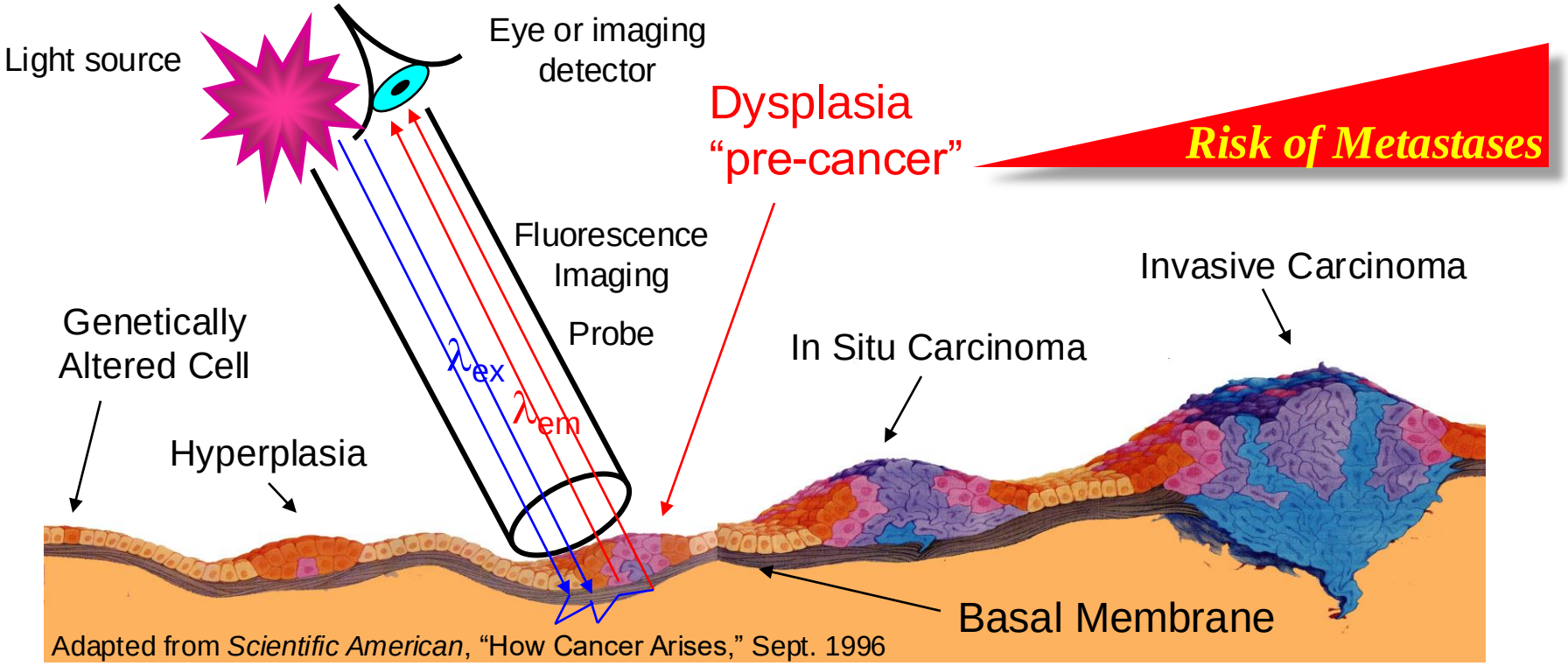


Advantages of Diagnosis by Endoscopic Fluorescence Imaging

- « High » resolution (10 - 100 μm)
- Localisation of already detected lesions
- Optical contrast agents/factors available
- Cost effective (less than 1000 CHF/procedure)
- High « physical sensitivity » (10 - 100 ng/g)
- Ideal for superficial lesions

➔ Well suited for the detection of early cancer !

Localization of early carcinoma by fluorescence imaging: Principle



Stages detectable by conventional methods →

Fluorescence imaging →

Spectral and Spatial Information

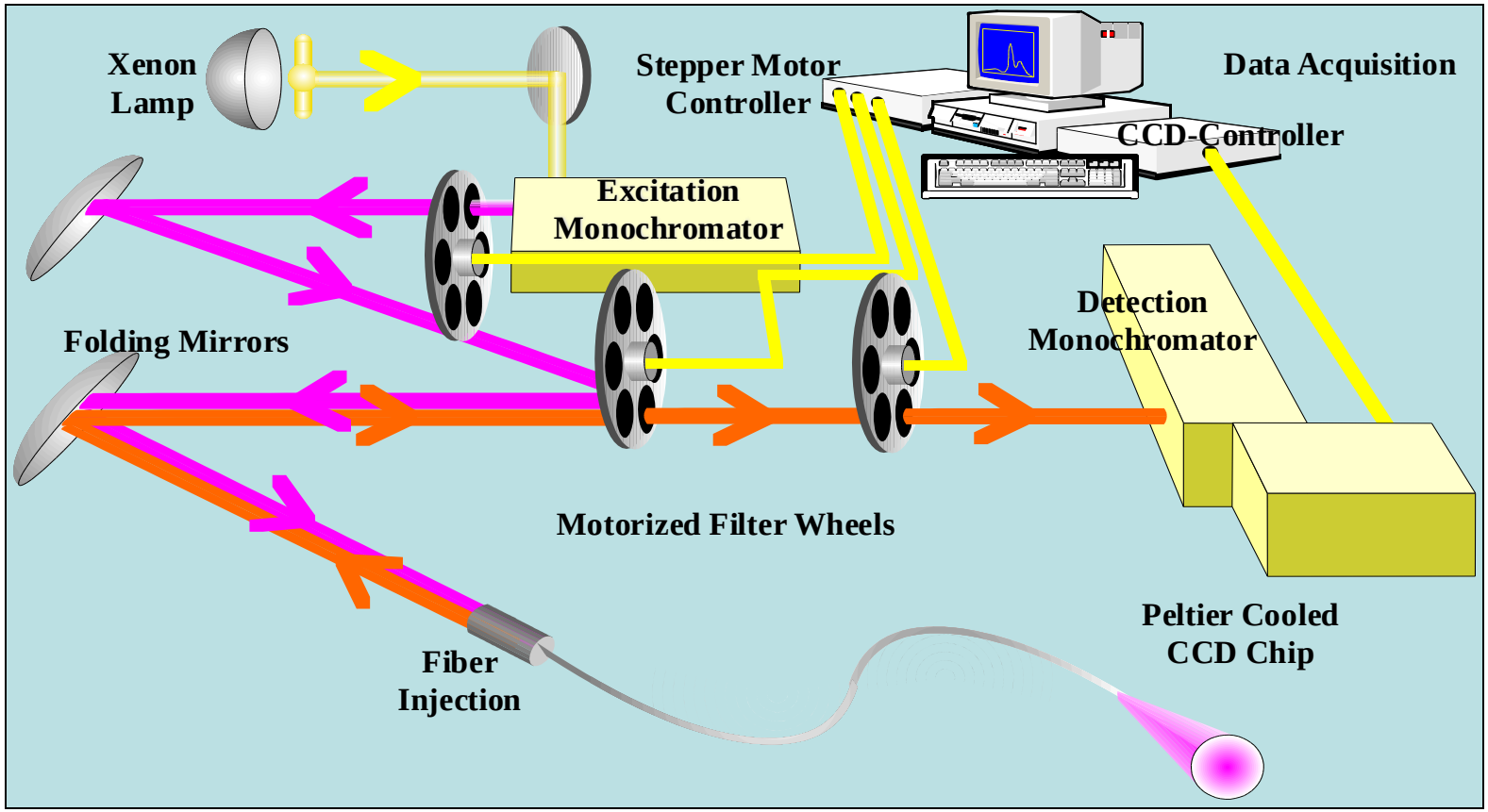
Point systems
Spectral information in
a small spatial domain

Imaging systems
Spatial information in
selected spectral domain(s)

Applications:
- Tissue characterization
- Tissular drug level

Applications:
- Detection
- Demarcation
- Biopsy guidance

Optical fiber-based spectrofluorometer for clinical endoscopic measurements

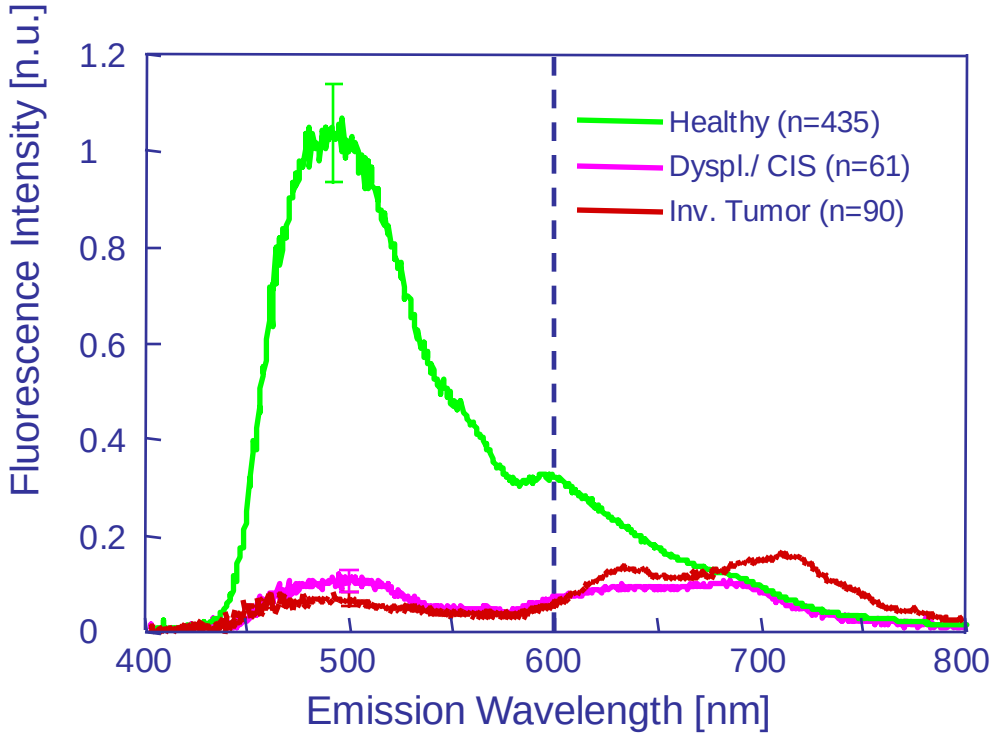


In vivo Spectrofluorometry of the bronchial wall

M&M

- Endoscopic measurements performed on 34 patients
- Excitation at 405 nm
- Non contact measurements (tissue-fiber distance: 3.5 mm)

Zellweger et al., JBO, 6(1), 41-52, 2001



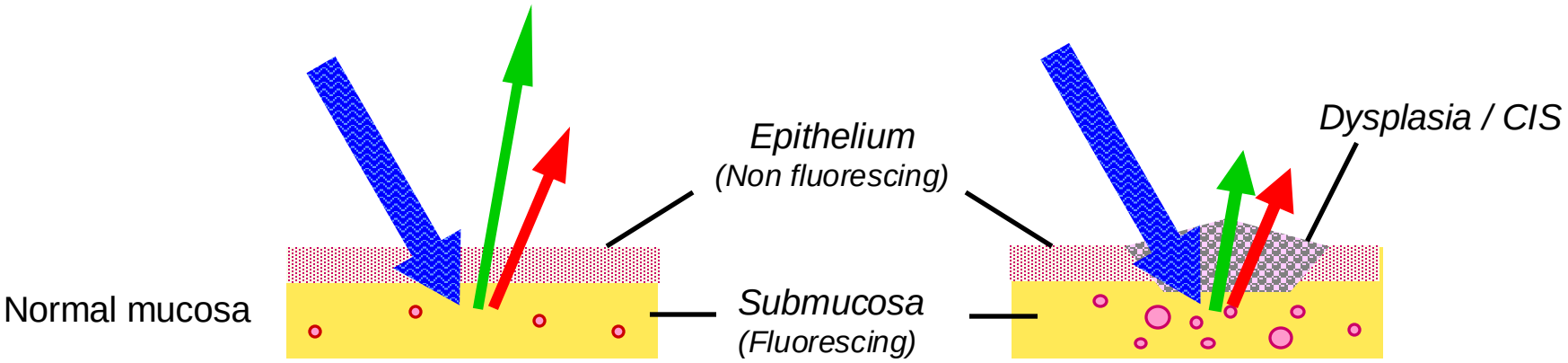
Increasing Malignancy:

"Green" Intensity ↘ Red/Green Ratio ↗

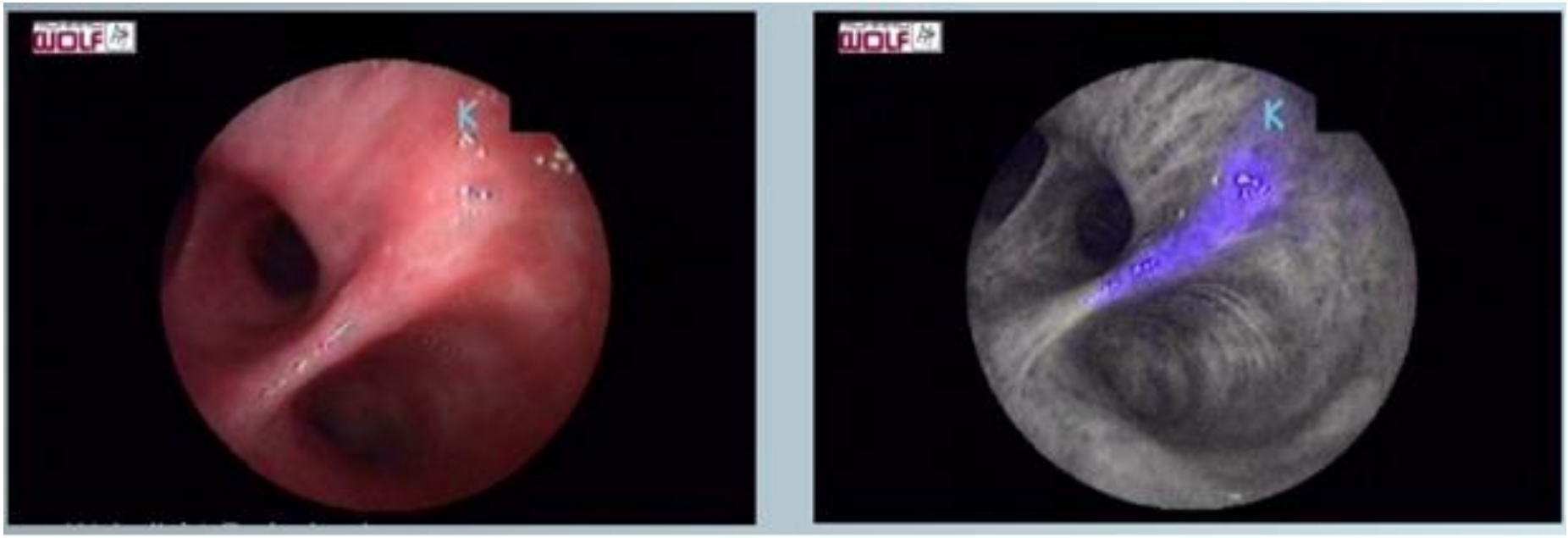
Origin of the T/N autofluorescence intensity and spectral contrast

- **The contrast is probably due to a thickening of the epithelium combined with an increased hemoglobin concentration in the submucosa (neo-vascularisation)**

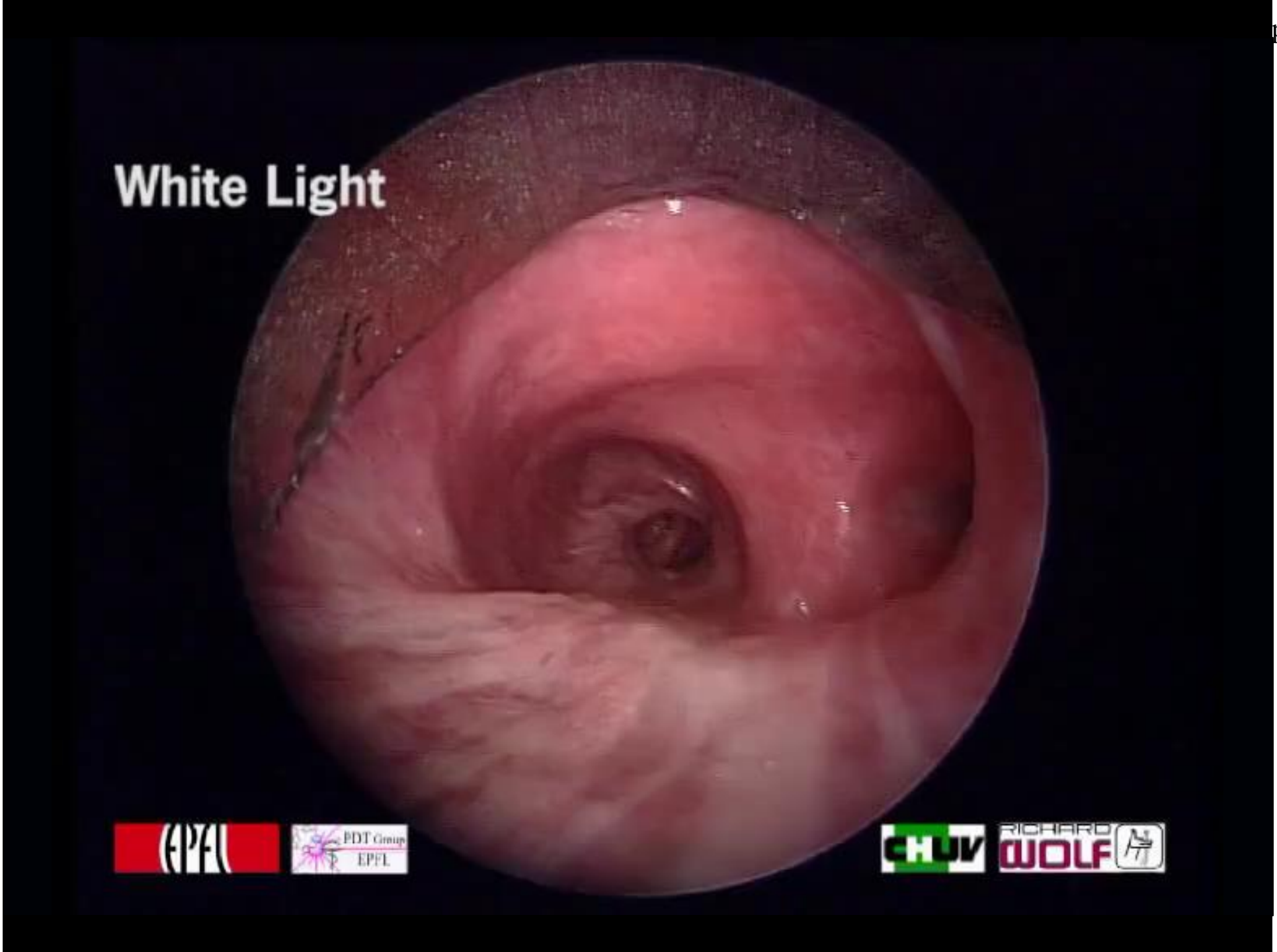
- **Alterations of the fluorescing molecules and their quenching probably play only a secondary role, if any.**



Carcinoma in situ (CIS)



Detection of bronchial carcinoma in situ (CIS) by autofluorescence bronchoscopy


White Light
EPFL
PDT Group
EPFL
CHUV
RICHARD WOLF