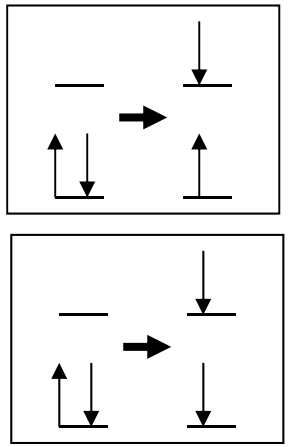
Fluorescence Spectroscopy

Principles of Fluorescence

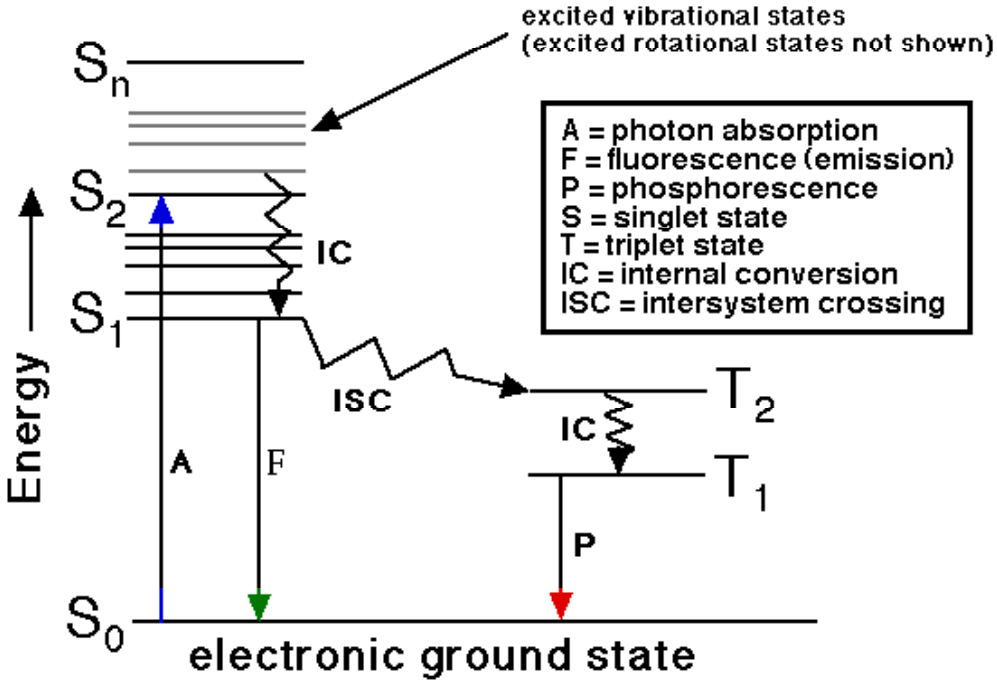
- **Luminescence**
  - Emission of photons from electronically excited states
  - Two types of luminescence:
    - Relaxation from singlet excited state
    - Relaxation from triplet excited state
  - Singlet and triplet states
    - Ground state – two electrons per orbital; electrons have opposite spin 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



Principles of Fluorescence

- **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

- **Energy level diagram (Jablonski diagram)**

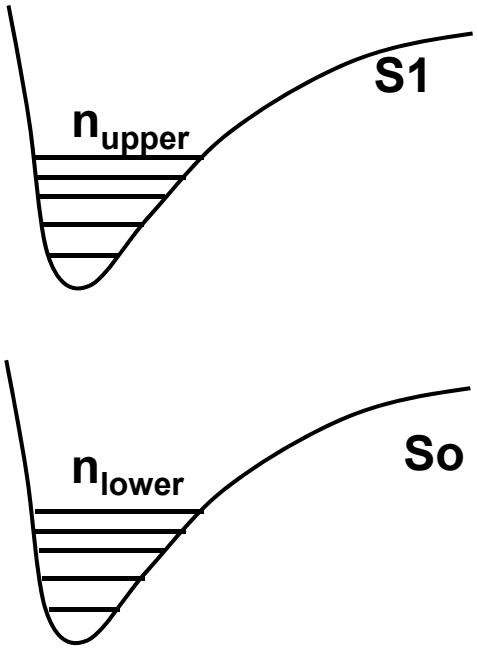


Principles of Fluorescence

- **Population of energy levels**
  - 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



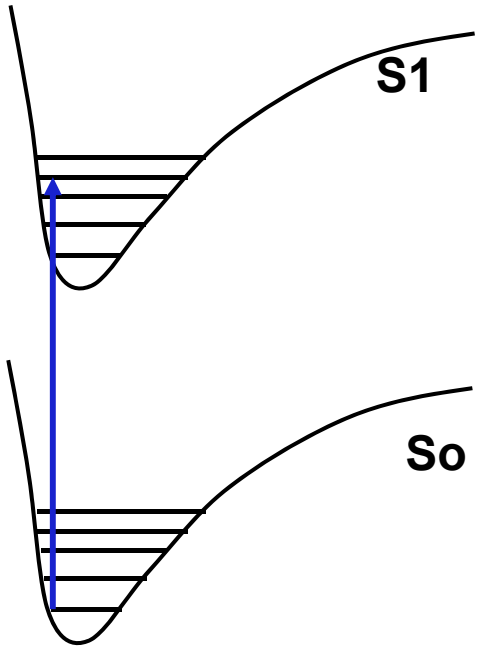
Principles of Fluorescence

- **Excitation**
  - Light is absorbed; for dilute sample, Beer-Lambert law applies
    - Magnitude of ε reflects probability of absorption
    - Wavelength of ε dependence corresponds to absorption spectrum

$$A = \varepsilon(\lambda)Cl$$

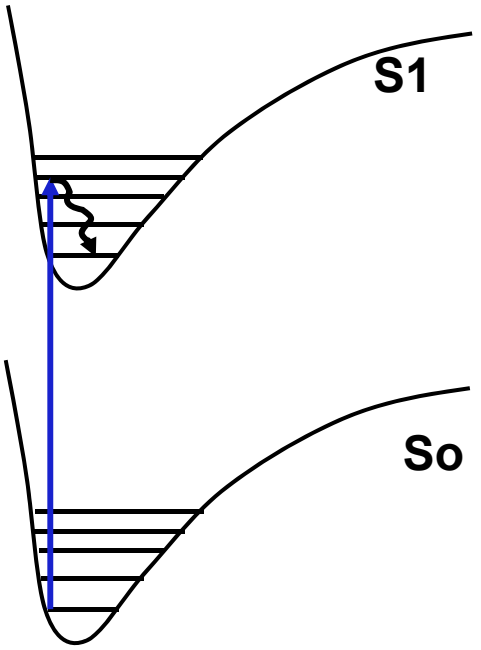
ε = molar absorption coefficient (M^{-1} cm^{-1})
C = concentration, l = pathlength

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



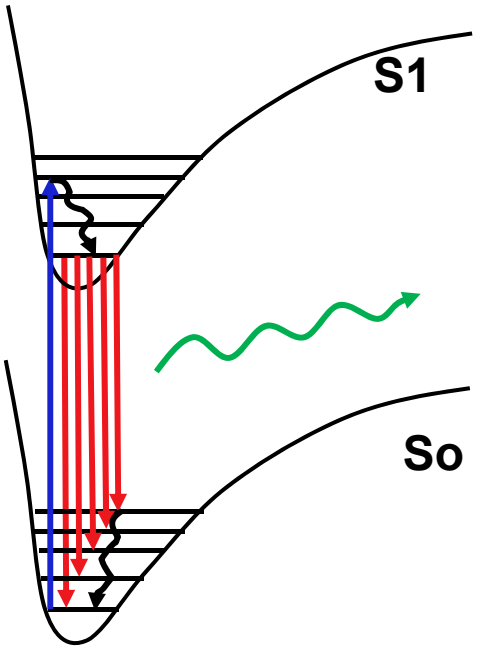
Principles of Fluorescence

- **Non-radiative relaxation**
  - The electron is promoted to higher vibrational level in S1 state than the vibrational level it was in at the ground state
  - Vibrational deactivation takes place through intermolecular collisions
    - A time scale of10^{-12} s (faster than that of fluorescence process)



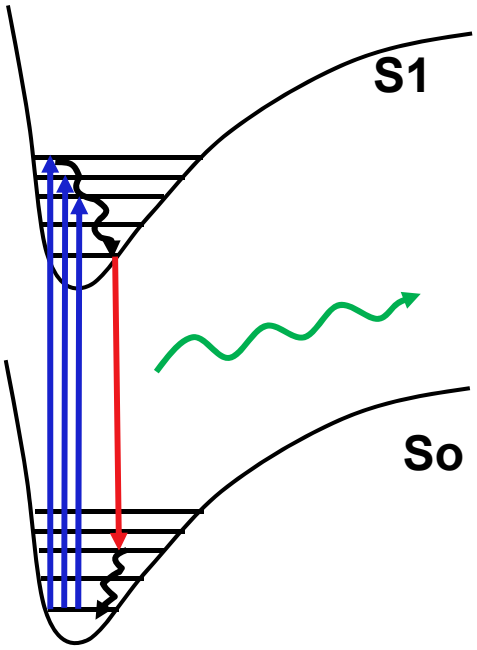
Principles of Fluorescence

- **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)
  - The energy of the emitted photon is lower than that of the incident photons
  - Emission Spectrum
    - 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



Principles of Fluorescence

- **Emission**
  - Stokes shift
    - Fluorescence light is red-shifted (longer wavelength than the excitation light) relative to the absorbed light
    - Internal conversion can affect Stokes shift
    - Solvent effects and excited state reactions can also affect the magnitude of the Stokes shift
  - Invariance of emission wavelength with excitation wavelength
    - Emission wavelength only depends on relaxation back to lowest vibrational level of S1
    - For a molecule, the same fluorescence emission wavelength is observed irrespective of the excitation wavelength



Principles of Fluorescence

- **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

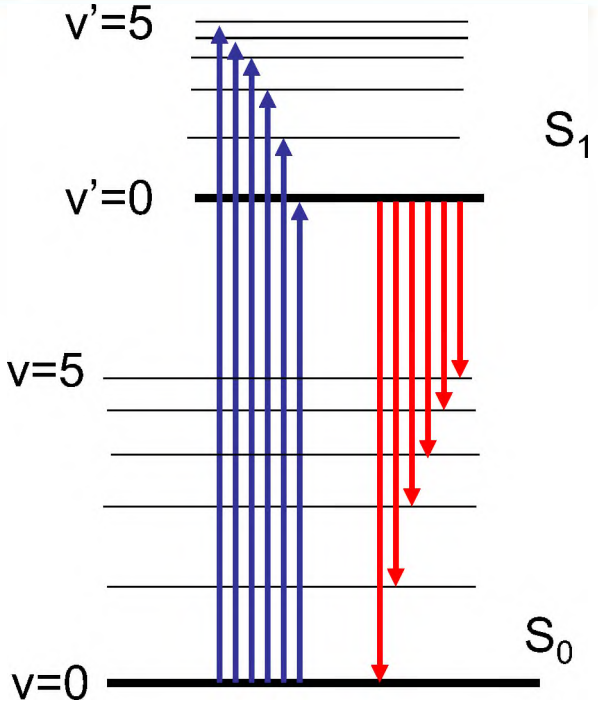
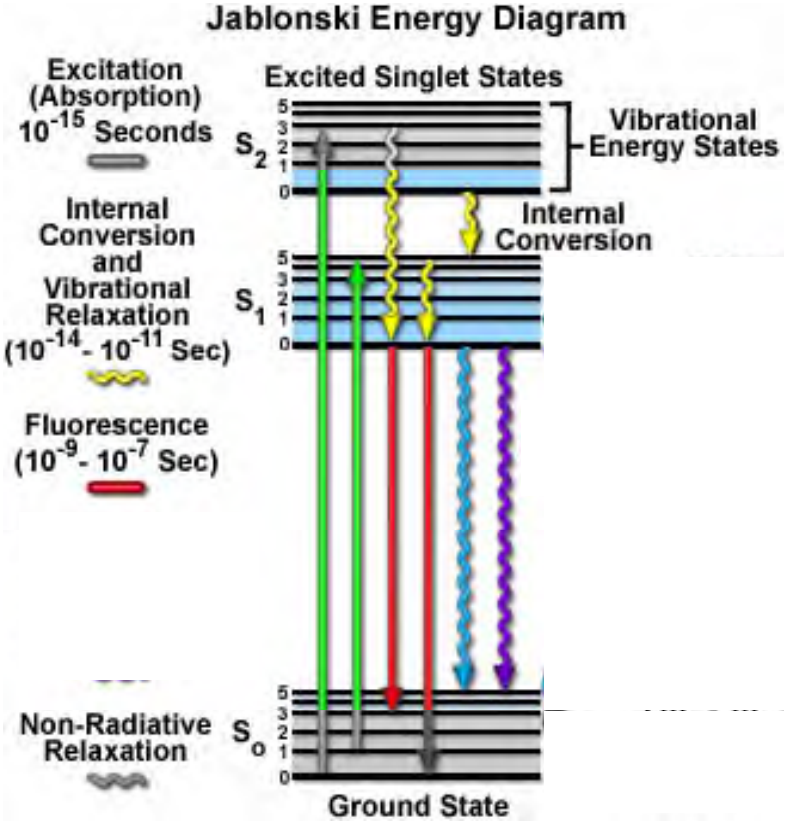


Figure 3

Principles of Fluorescence

- **Internal conversion vs. fluorescence emission**
  - Electronic energy increases --> the energy levels grow more closely spaced
    - Overlap between the high vibrational energy levels of S_{n-1} and low vibrational energy levels of S_n more likely
    - This overlap makes transition between states highly probable
  - Internal conversion: a transition between states of the same multiplicity
    - Time scale of 10^{-12} s (faster than that of fluorescence process)
  - Significantly large energy gap between S_1 and S_0
    - S_1 lifetime is longer → radiative emission can compete effectively with non-radiative emission



Principles of Fluorescence

- **Internal conversion vs. fluorescence emission**
  - 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

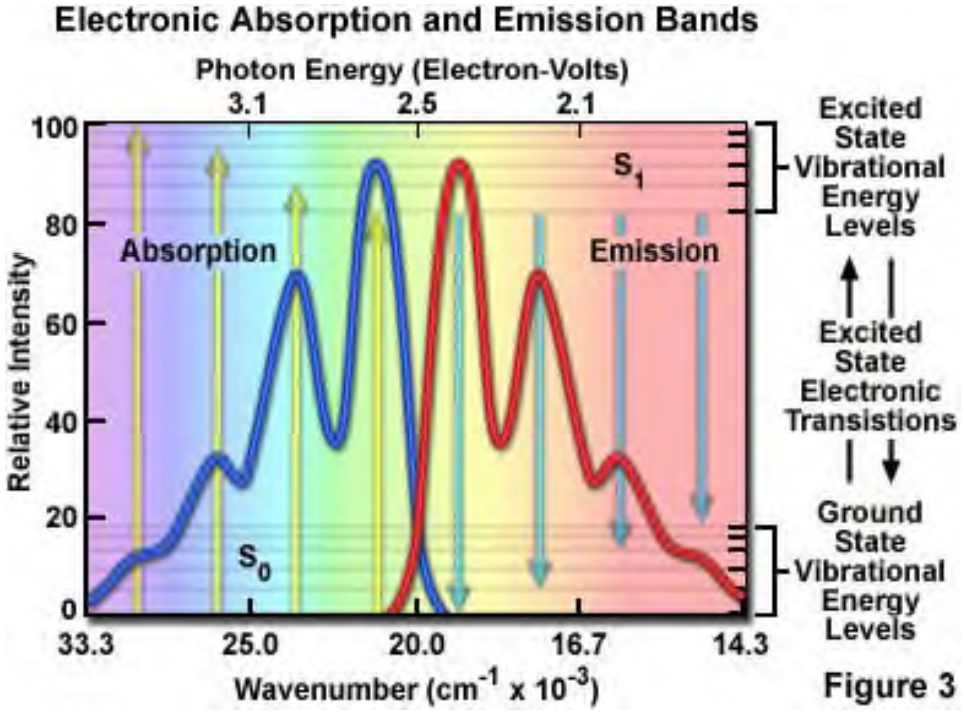
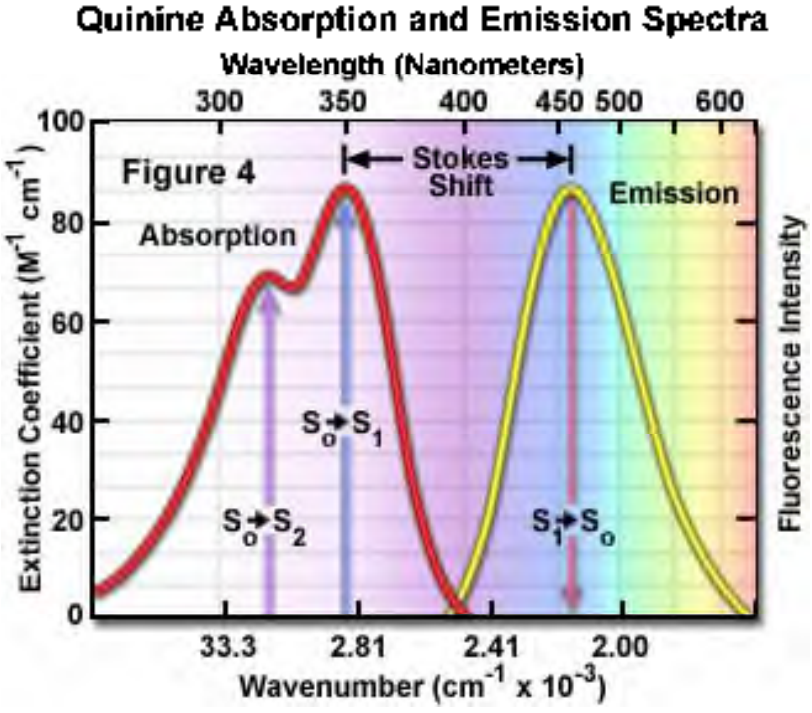


Figure 3



Principles of fluorescence

- **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 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
  - T1 → S0 transition is also forbidden → T1 lifetime significantly larger than S1 lifetime (10-3-102 s)

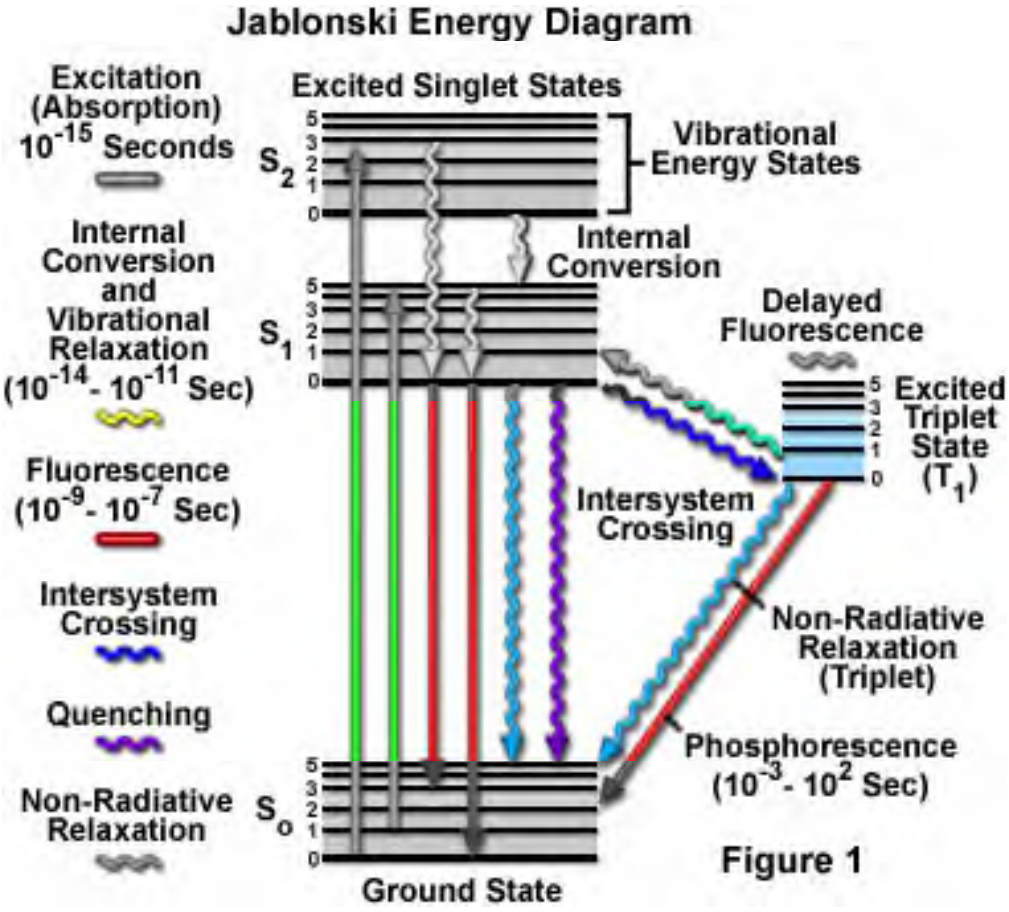


Figure 1

Quantum yield and life time

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

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

- **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 Φ_f=1**
- **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+}

Quantum yield and life time

- **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**

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

- **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 varjations in excitation intensity, concentration or sources of optical loss
  - Compatible with clinical measurements in vivo

Quantum yield and life time

- **Fluorescence life-time methods**
  - Short pulse excitation followed by an interval during which the resulting fluorescence is measured as a function of 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

Fluorescence Intensity

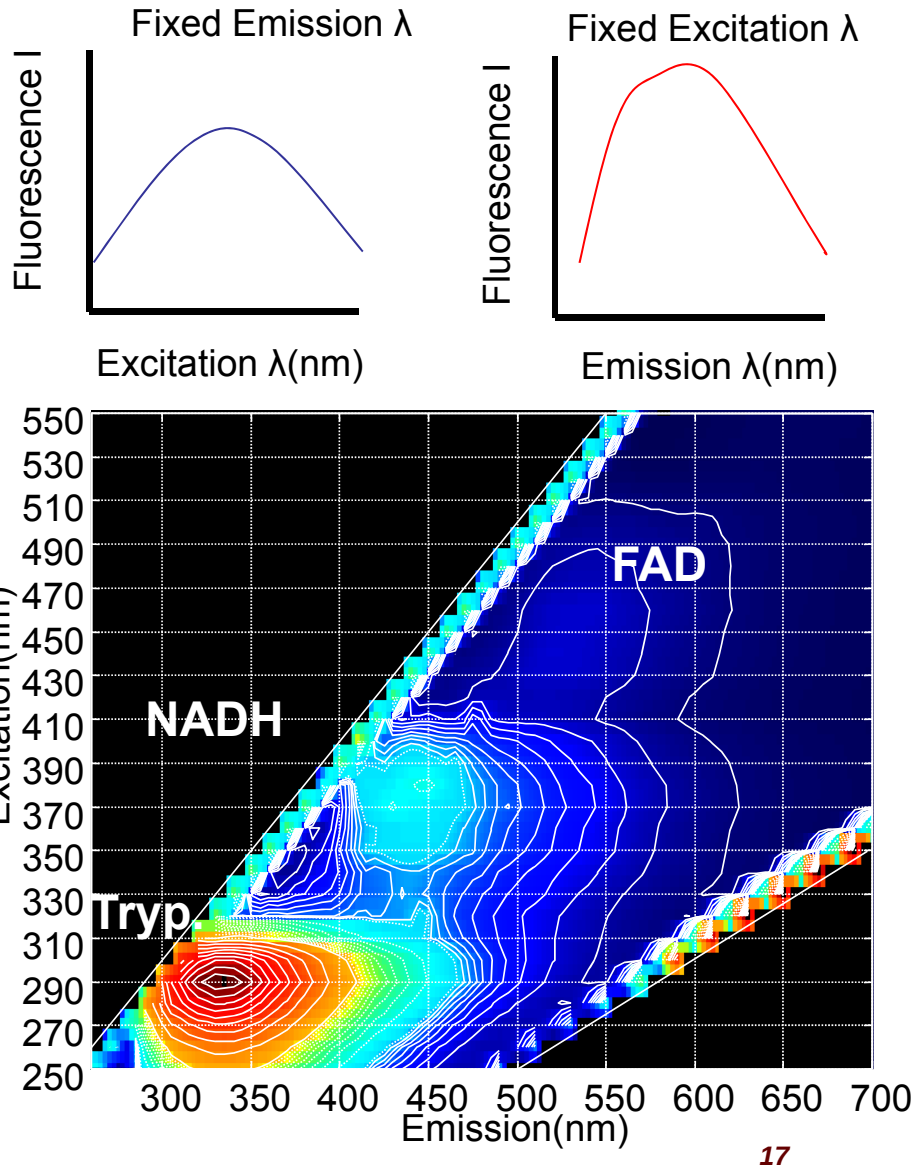
- **Absorbed intensity for a dilute solution**
  - Very small absorbance
  - From the Beer-Lambert law

$$F\left(\lambda_x, \lambda_m\right) = I_A \Phi(\lambda_m) Z = \left[2.303 I_o \varepsilon\left(\lambda_x\right) CL\right] \Phi\left(\lambda_m\right) Z$$

  - where,
    - Z – instrumental factor (collection angle)
    - I_o – incident light intensity
    - ε – molar extinction coefficient
    - Φ – quantum yield
    - C – concentration
    - L – path length

Fluorescence Intensity

- **Emission spectrum**
  - Hold excitation wavelength fixed, scan emission
  - Reports on the fluorescence spectral profile
  - reflects fluorescence quantum yield, $\Phi_k(l_m)$
- **Excitation spectrum**
  - Hold emission wavelength fixed, scan excitation
  - Reports on absorption structure
  - reflects molar extinction coefficient, $\varepsilon(l_x)$
- **Excitation-Emission Matrix (EEM)**
  - Composite
  - Good representation of multi-fluorophore solution



Quenching, Bleaching & Saturation

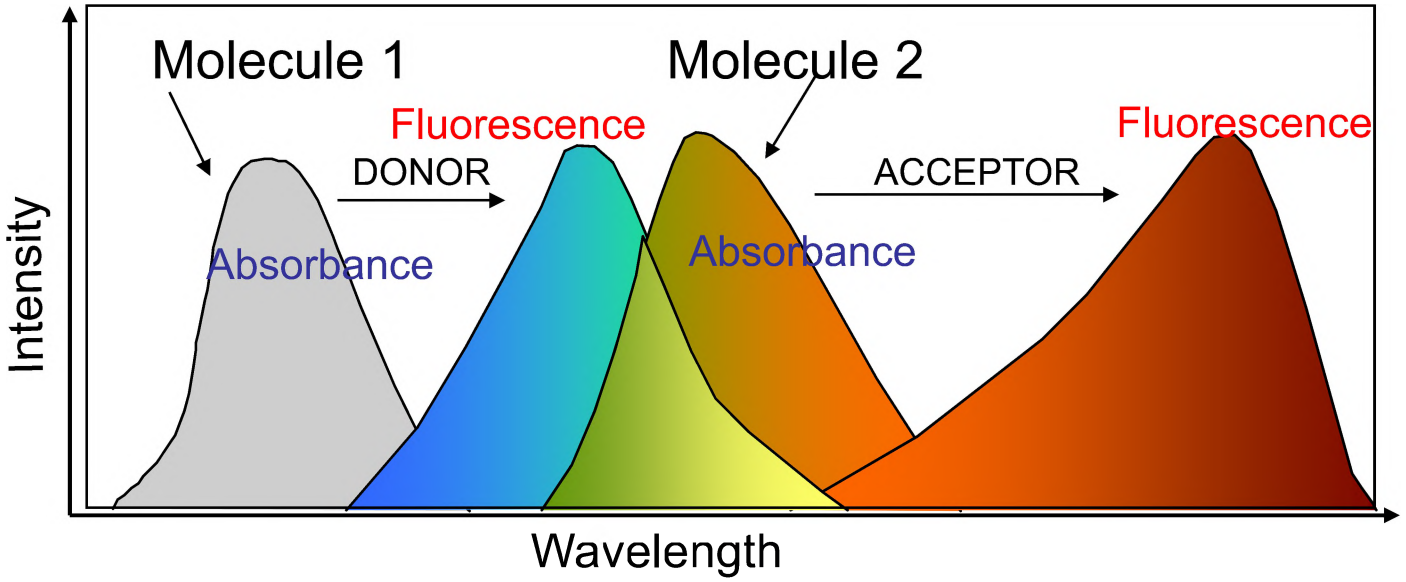
- **Quenching**
  - Excited molecules relax to ground states via nonradiative pathways avoiding fluorescence emission (vibration, collision, intersystem crossing)
  - Molecular oxygen quenches by increasing the probability of intersystem crossing
  - Polar solvents such as water generally quench fluorescence by orienting around the exited state dipoles

Quenching, Bleaching & Saturation

- **Photobleaching**
  - Defined as the irreversible destruction of an excited fluorophore
  - Photobleaching is not a big problem as long as the time window for excitation is very short (a few hundred microseconds)
- **Excitation Saturation**
  - The rate of emission is dependent upon the time the molecule remains within the excitation state (the excited state lifetime □f)
  - Optical saturation occurs when the rate of excitation exceeds the reciprocal of □f
  - Molecules that remain in the excitation beam for extended periods have higher probability of interstate crossings and thus phosphorescence

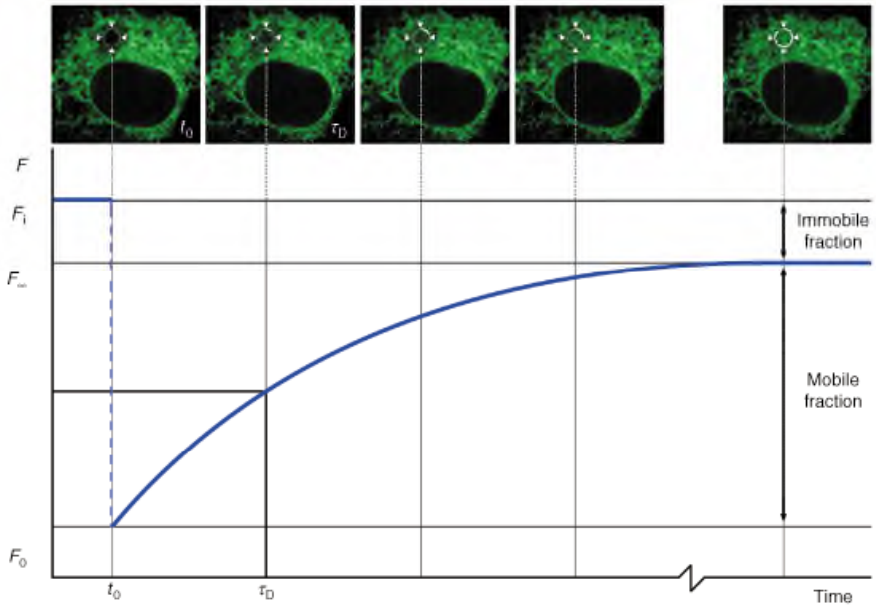
Fluorescence Resonance Energy Transfer (FRET)

- **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
  - FRET is very sensitive to the distance between donor an acceptor and is therefore an extremely useful tool for studying molecular dynamics



Fluorescence Recovery after Photo-bleaching (FRAP)

- **Useful technique for studying transport properties within a cell, especially transmembrane protein diffusion**
  - FRAP can be used to estimate the rate of diffusion, and the fraction of molecules that are mobile/immobile
  - Can also be used to distinguish between active transport and diffusion
- **Procedure**
  - Label the molecule with a fluorophore
  - Bleach (destroy) the fluorophore is a well defined area with a high intensity laser
  - Use a weaker beam to examine the recovery of fluorescence as a function of time



Nature Cell Biology **3**, E145 - E147 (2001)

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.

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				
	Lifetime (ns)		Area		Lifetime (ns)		Area		
	τ_1	τ_2	A_1	A_2	τ_1	τ_2	A_1	A_2	References
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

Fluorescence Instrumentation

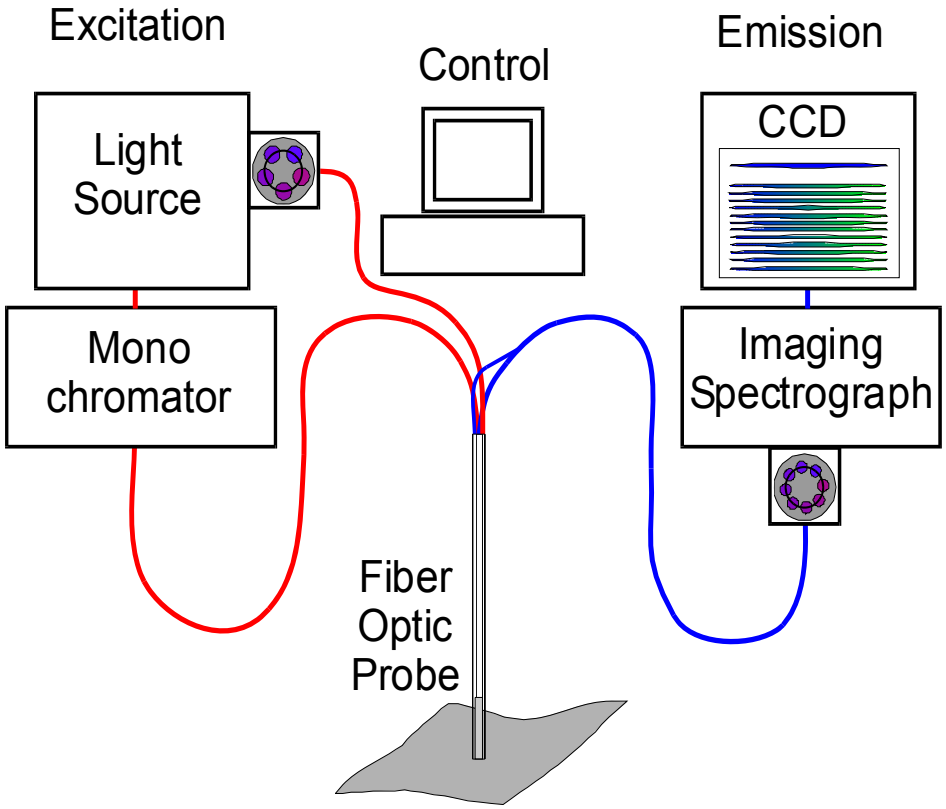
- **Fluorescence is a highly sensitive method (can measure analyte concentration of 10^{-8} M)**

- **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

Fluorescence Instrumentation

- **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)
    - Filters
    - Monochromator
    - Spectrograph
  - Detector
    - PMT
    - CCD camera



Applications

- **Test definitions**

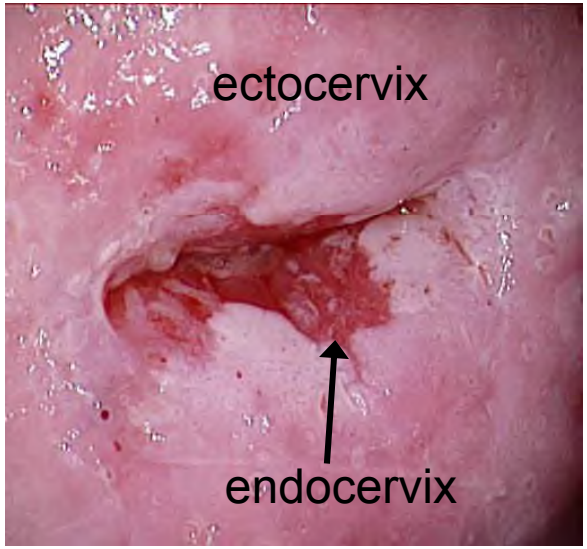
	Has disease	Does not have disease	
Tests positive	**(A)** **True positive**	**(B)** **False positive**	**(A+B)** **Total # who test positive**
Tests negative	**(C)** **False negative**	**(D)** **True negative**	**(C+D)** **Total # who test negative**
	(A+C) **Total # who have disease**	**(B+D)** **Total # who do not have disease**	

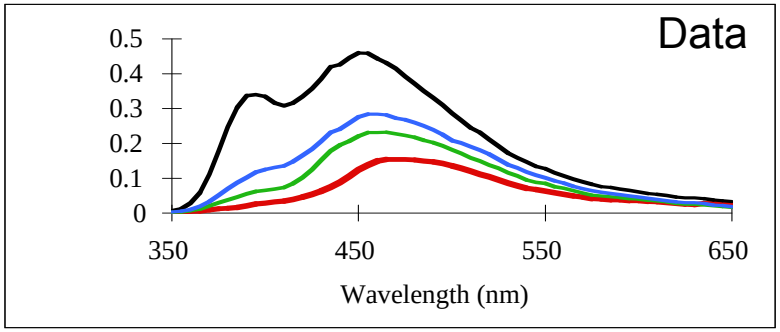
Sensitivity=A/(A+C)
Specificity=D/(B+D)

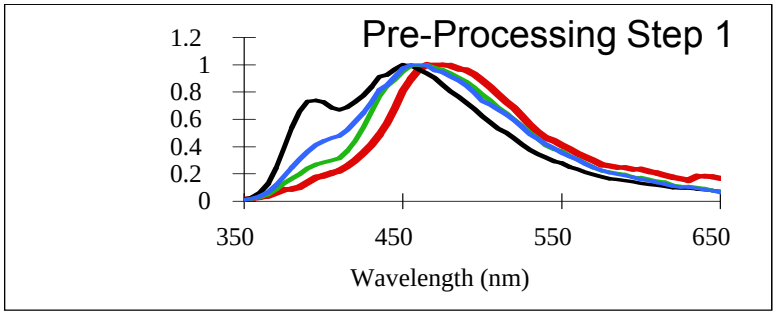
Positive predictive value=A/(A+B)
Negative predictive value=D/(C+D)

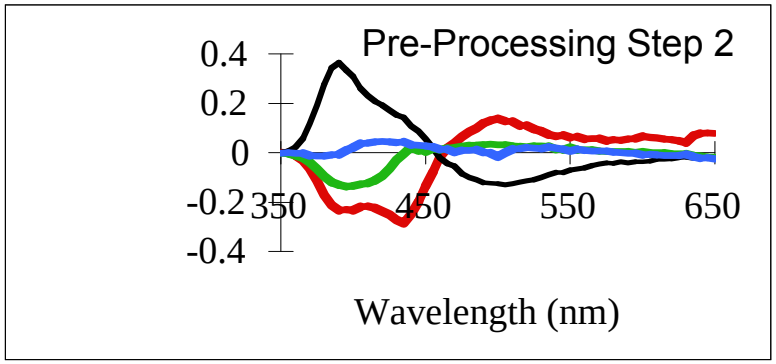
Applications

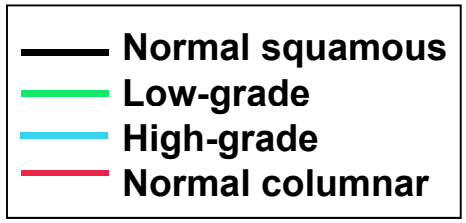
- **Detection of cervical pre-cancerous lesions**











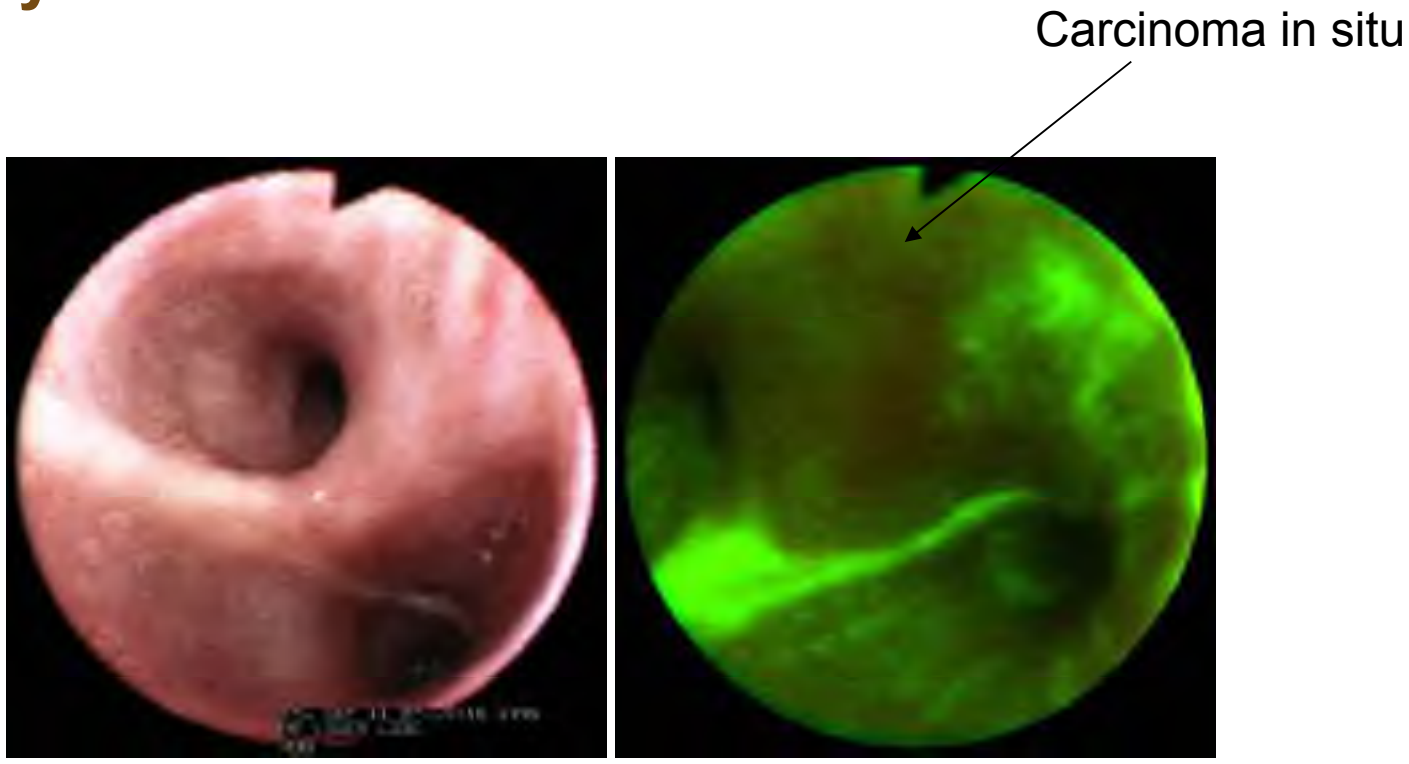
Applications

- **Detection of cervical pre-cancerous lesions**

	SILs vs. NON SILs		HG SIL vs. Non HG SIL	
Classification	Sensitivity	Specificity	Sensitivity	Specificity
Pap Smear Screening	62% ±23	68%±21	N/A	N/A
Colposcopy in Expert Hands	94%±6	48%±23	79%±23	76%±13
Full Parameter Composite Algorithm	82%±1.4	68%±0.0	79%±2	78%±6
Reduced-Parameter Composite Algorithm	84%±1.5	65%±2	78%±0.7	74%±2

Applications

- **Detection of lung carcinoma in situ using the LIFE imaging system**



White light bronchoscopy

Autofluorescence ratio image

Courtesy of Xillix Technologies (www.xillix.com)

Applications

- **Detection of lung carcinoma in situ using the LIFE imaging system**
  - Autofluorescence enhances ability to localize small neoplastic lesions

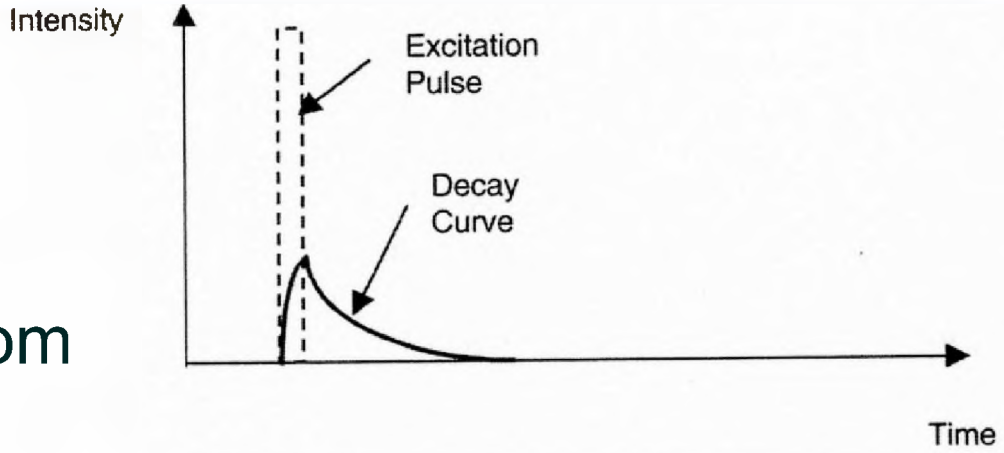
	Severe dysplasia/Worse		Intraepithelial Neoplasia	
	WLB	WLB+LIFE	WLB	WLB+LIFE
Sensitivity	0.25	0.67	0.09	0.56
Positive predictive value	0.39	0.33	0.14	0.23
Negative predictive value	0.83	0.89	0.84	0.89
False positive rate	0.10	0.34	0.10	0.34
Relative sensitivity	2.71		6.3	

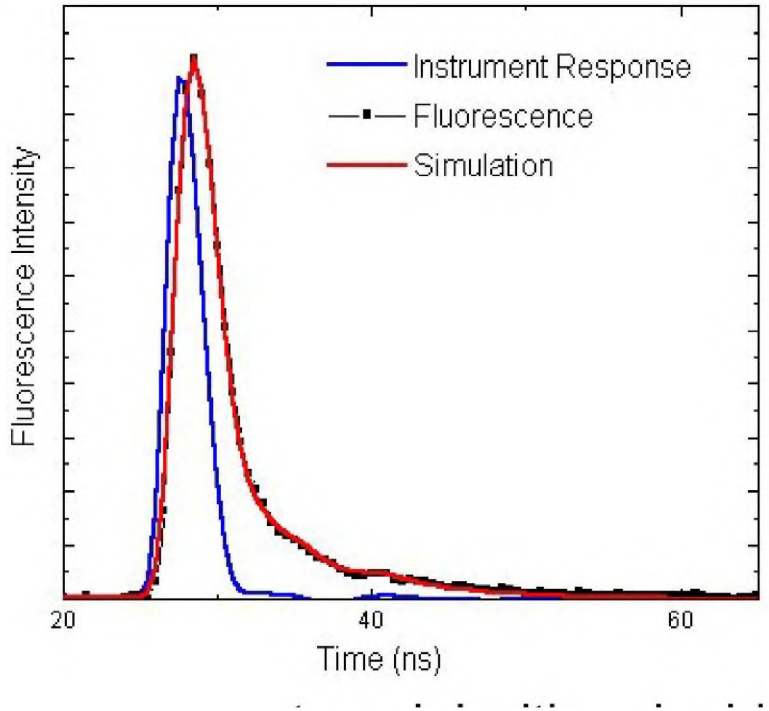
S Lam et al. Chest 113: 696-702, 1998

Applications

- **Fluorescence lifetime measurements**
  - Autofluorescence lifetimes measured from colon tissue in vivo
  - Analysis via iterative re-convolution
    - Two component model with double exponential decay

$$F(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$$





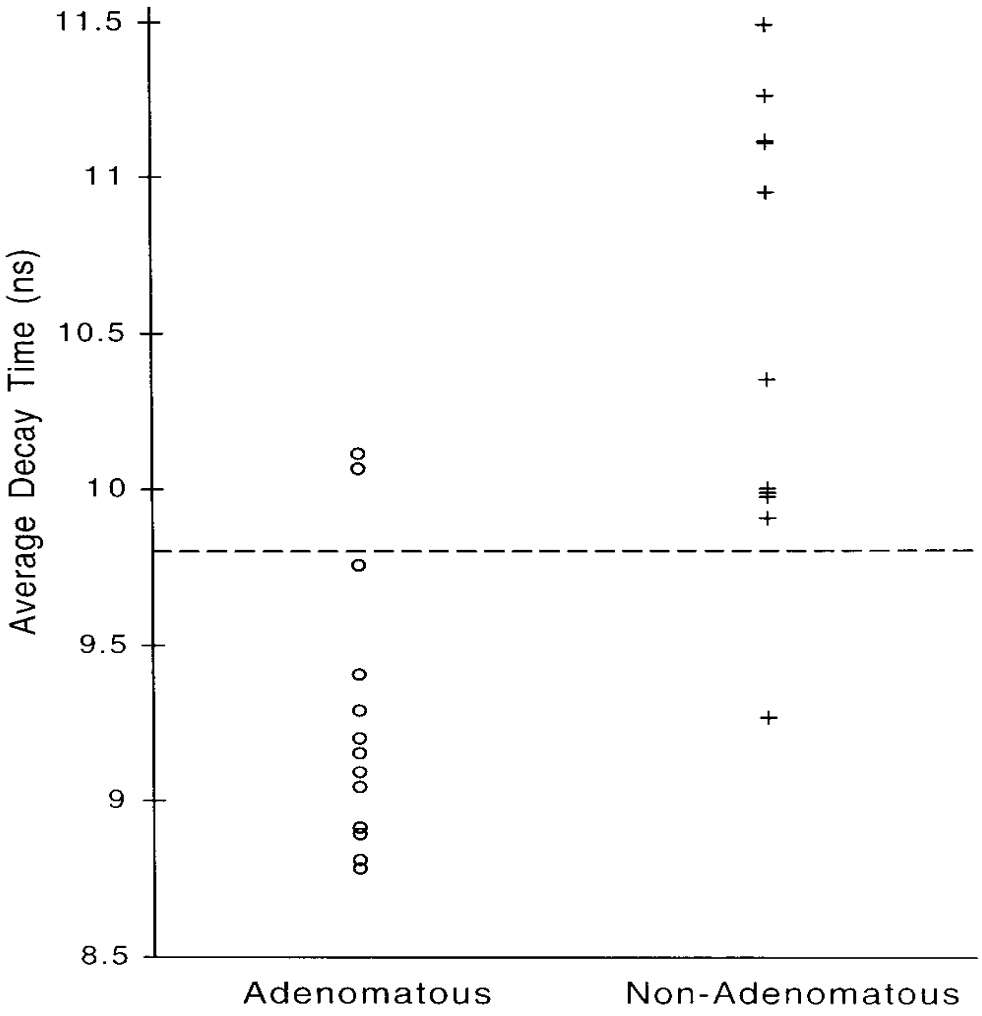
M.-A. Mycek et al. GI Endoscopy 48:390-4, 1998

Applications

- **Fluorescence lifetime measurements**
  - Autofluorescence lifetimes used to distinguish adenomatous from non-adenomatous polyps in vivo

$\tau_1 = 6.03$ ns $\tau_2 = 0.72$ ns

Area1 = 0.42 Area2 = 0.58



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