FLUORESCENCE SPECTROSCOPY AND IMAGING

Spettroscopia

- Tutte le spettroscopie consistono nella misura delle interazione energia-materia.
- L'assorbimento di energia da parte di una molecola può provocare delle variazioni chimiche o fisiche (proprietà degli elettroni o del nucleo) della specie chimica.
- L'assorbimento o l'emissione possono fornire informazioni sulla struttura della molecola e/o le variazioni che una essa subisce.

Interazione tra energia e materia

λ (nm)	**ν (Hz)**	**Regione dello spettro**	**Interazioni (Spettroscopia)**
10^{6} - 10^{10}	$3 \cdot 10^{11}$ - $3 \cdot 10^{7}$	Radio	Spin nucleare, spin elettronico (NMR – EPR)
10^{3} - 10^{5}	$3 \cdot 10^{14}$ - $3 \cdot 10^{12}$	Radiazioni InfraRosse	Vibrazioni, rotazioni (IR)
$4 \cdot 10^{2}$ - $8 \cdot 10^{2}$ (400-800)	$7.5 \cdot 10^{14}$ - $3 \cdot 10^{14}$	Luce Visibile	Transizioni elettroniche (Spettroscopie ottiche)
$2 \cdot 10^{2}$ - $3 \cdot 10^{2}$ (200-300)	$1.5 \cdot 10^{15}$ - $1 \cdot 10^{15}$	Luce UltraVioletta	
10^{-3} - 10^{0}	$3 \cdot 10^{20}$ - $3 \cdot 10^{17}$	Raggi X	Gusci interni (Spettroscopie X)

Assorbimento e fluorescenza

- La luminescenza è un processo di **emissione di radiazioni** a seguito di un **assorbimento di energia**.
- Si parla di fluorescenza quando si osserva un processo di **emissione di luce** a seguito del rilassamento di uno stato elettronico eccitato generato a da un **assorbimento di luce**.
- La scala dei tempi con la quale avviene l'emissione è diversa da quella con la quale avviene l'assorbimento.
- Come conseguenza sono diverse le proprietà della materia che influiscono sui due fenomeni e, quindi, diverse le informazioni che se ne possono ricavare.

Luminescence is the emission of light from any substance, and occurs from electronically excited states. Luminescence is formally divided into two categories—fluorescence and phosphorescence—depending on the nature of the excited state.

Luminescence: emission of photons from electronically excited states of atoms, molecules, and ions.

- **Fluorescence**: average lifetime **from $<10^{-10}$ to 10^{-7} sec** from singlet states.
- **Phosphorescence**: Average lifetime **from 10^{-5} to $>10^{+3}$ sec** from triplet excited states.

Fluorescence occurs at much smaller scales then **absorption** (**10^{-15} sec**):

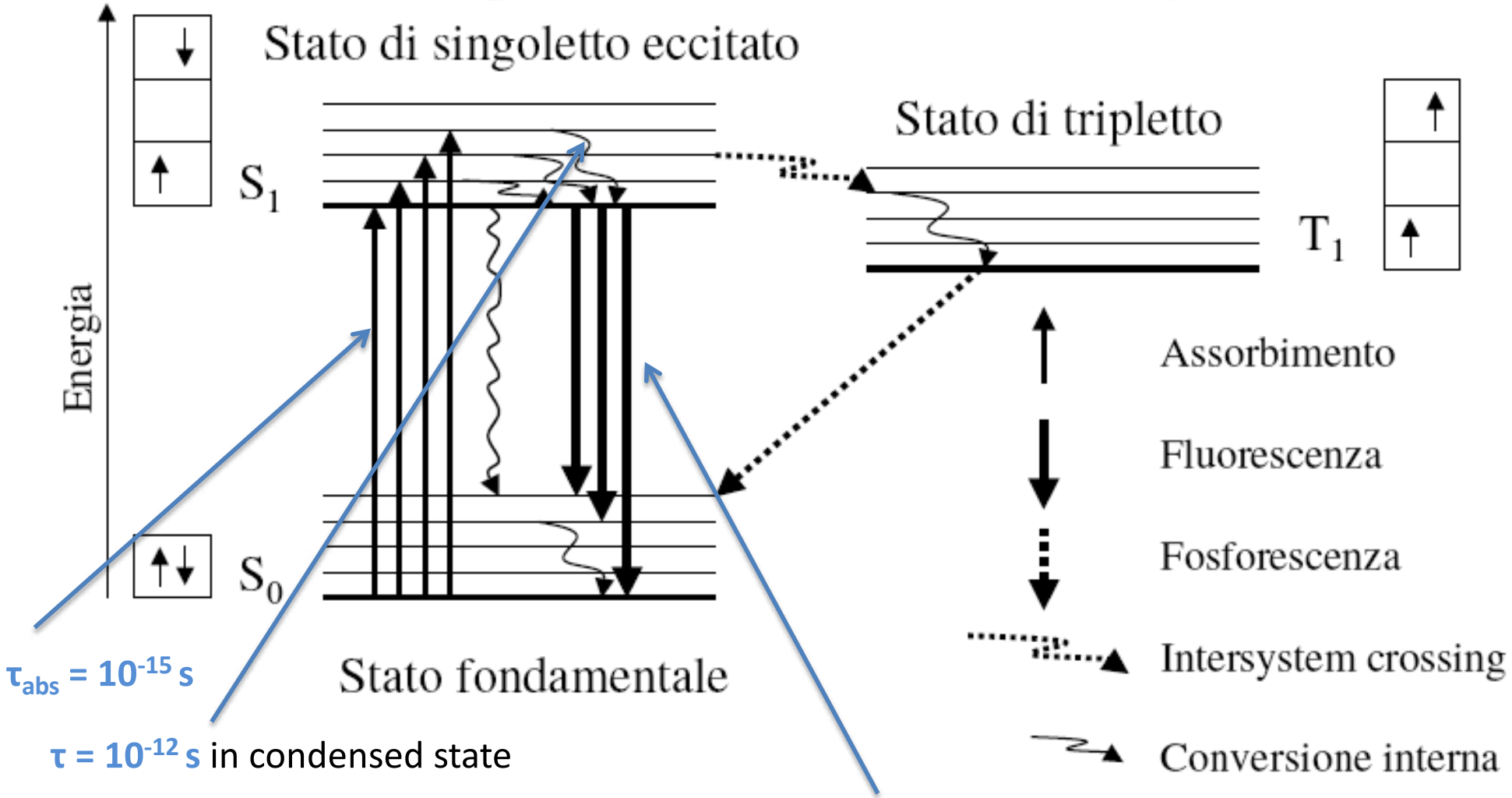
this allows the spectrum to carry information about the perturbation or changes occurring within this time scale.

Its existence was predicted by Einstein

N.B.: **The lifetime of an excited state is the inverse of the transition rate from that excited state.**

Le transizioni elettroniche

(Diagramma di Jablonsky)

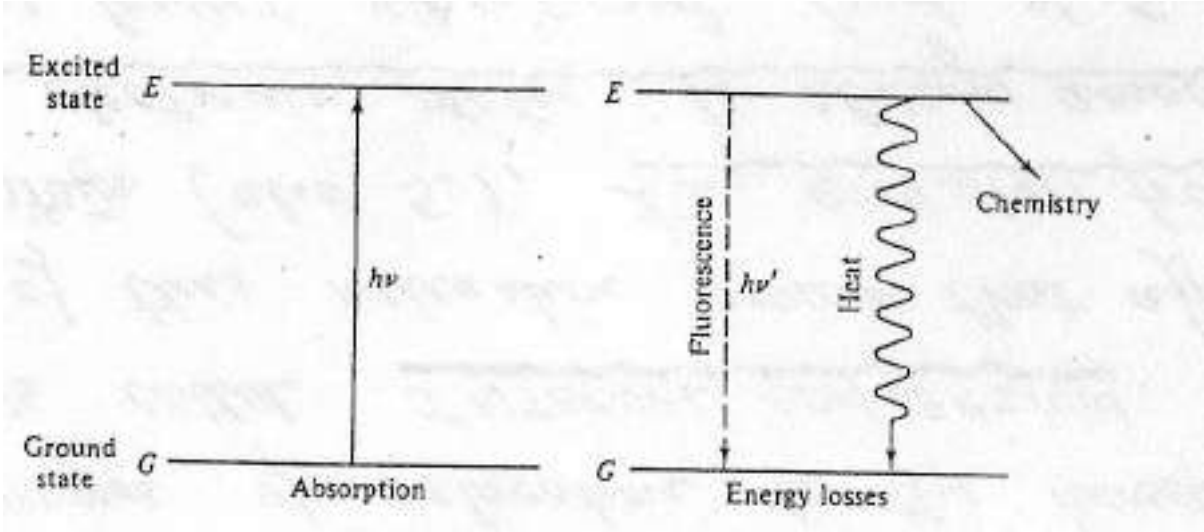


$\tau_{abs} = 10^{-15}$ s

$\tau = 10^{-12}$ s in condensed state

$\tau = 10^{-8}$- 10^{-9} s fluorescence decay (5% of total emission)

- Most of the energy absorbed by a molecule is released back into the surroundings in the form of heat. The collisions experienced by the molecules from the surrounding solvent constitute the most effective mechanism of de-excitation of molecules. However, a small faction of the absorbed energy can be re-emitted in the form of radiation of smaller frequency. This phenomenon is called LUMINESCENCE. Here we will concentrate in particular on its most important form: FLUORESCENCE.



Photophysics: Jablonski Diagram

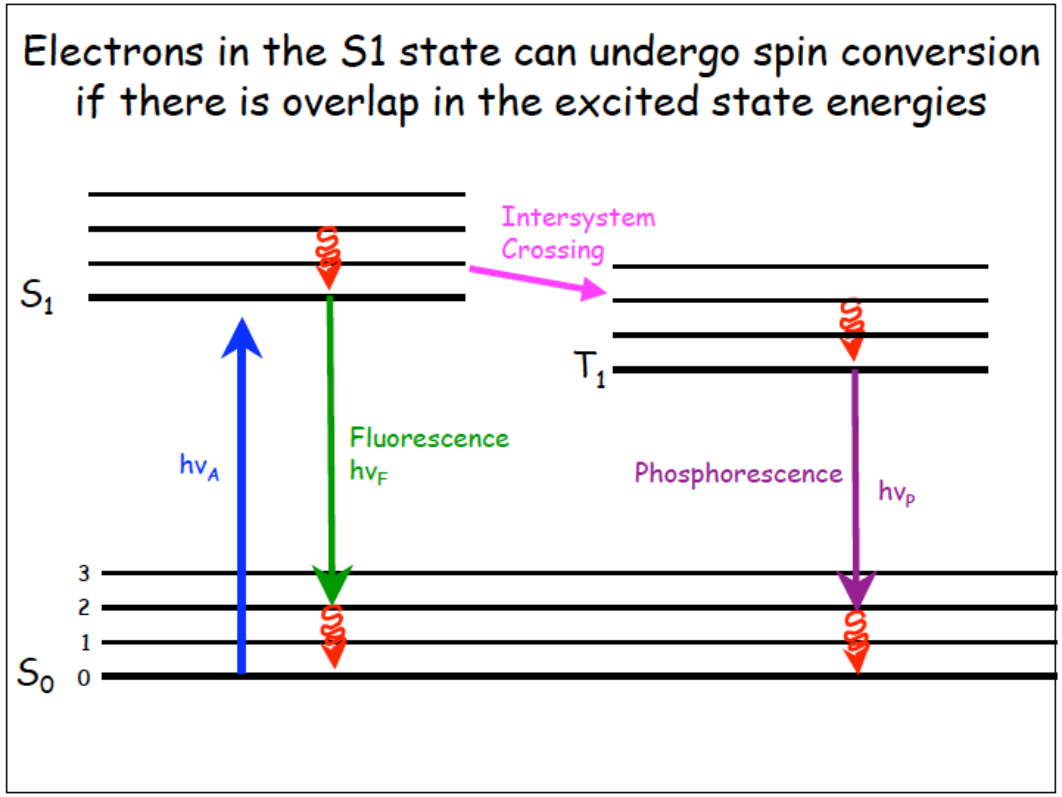
- Photoexcitation from the ground electronic state S_0 creates excited states S_1, (S_2, …, S_n)of the molecule
- At each of these energy level the fluorophore can exist in different vibrational levels (0,1,2)
- Kasha's rule: Rapid relaxation from excited electronic and vibrational states precedes nearly all fluorescence emission.
  - (track these processes using femtosecond spectroscopy)

Internal Conversion: Molecules rapidly (10^{-14} to 10^{-11} s) relax to the lowest vibrational level of S_1.
The emission from the excited level is therefore always associated to the lowest vibrational level of S_1

At RT the ratio of the vibrational population between the excited and the ground state is very small, about 0:

$$R = e^{-\Delta E / kT}$$

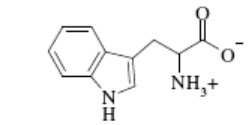
Photophysics: Jablonski Diagram



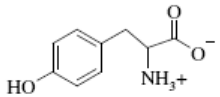
Intersystem crossing: Molecules in S_1 state can also convert to first triplet state T_1. Emission from T_1 is termed **phosphorescence**, shifting to longer wavelengths (lower energy) than fluorescence. Transition from S_1 to T_1 is called intersystem crossing. These transition are not allowed and therefore are slow. Heavy atoms such as Br, I, and metals promote ISC.

- The small fraction of molecules which decay emitting radiation from the lowest vibrational level of the first excited singlet state to the ground singlet state conserve spin (are allowed) and therefore have a shorter lifetime (the inverse of the transition rate), which is of the order of 1-10 nseconds (transition rates of about 10^8 sec^{-1})

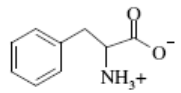
- From this is clear that all molecules fluoresce. But some particularly strong fluorescent molecules are those that posses delocalized electrons through conjugated double bonds. For example, aromatic molecules are often fluorescent



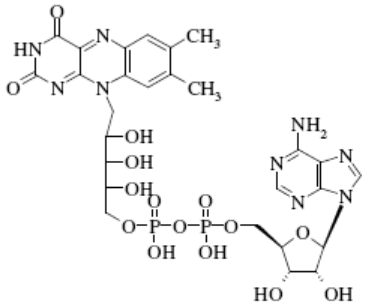
Triptofano (W)



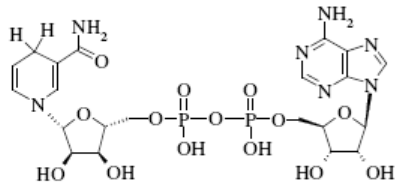
Tyrosina (Y)



Fenilalanina (P)

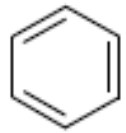
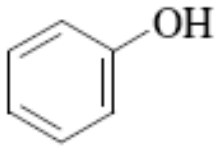
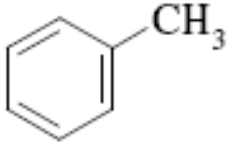
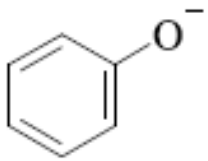
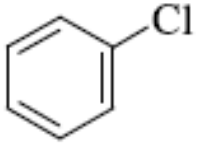
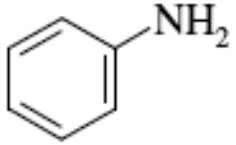
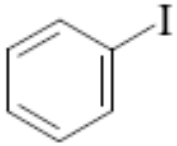
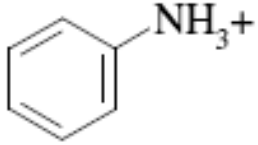


FAD

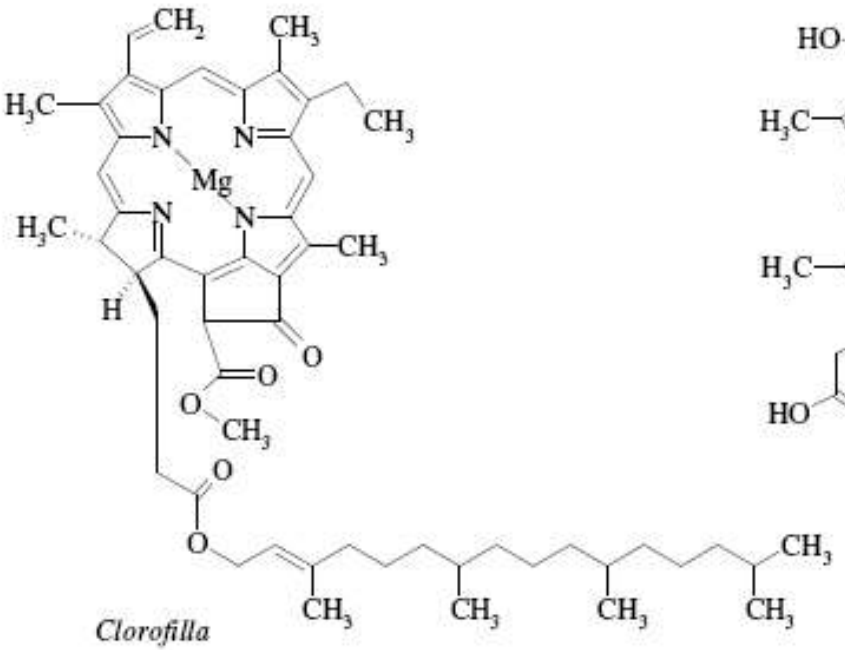


NADH

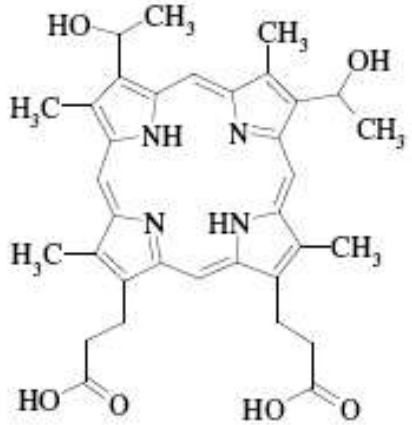
Effetto dei sostituenti sulla fluorescenza del benzene

Molecola	λ_{ex} - λ_{em} (nm)	Intensità	Molecola	λ_{ex} - λ_{em} (nm)	Intensità
	270-310	10		285-265	18
	270-320	17		310-400	10
	275-345	7		310-405	20
	-	0		-	0

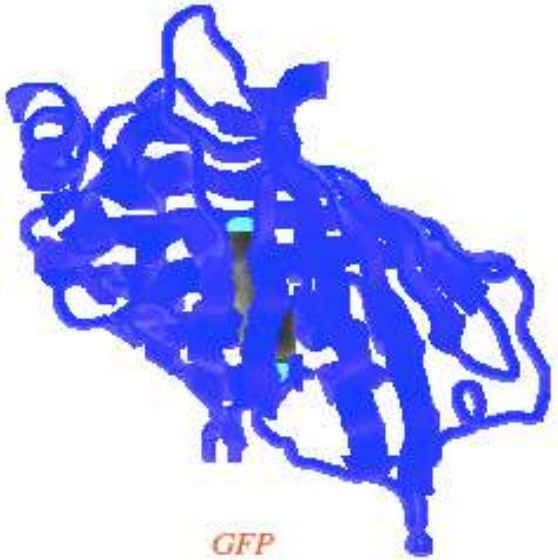
…altre biomolecole fluorescenti…



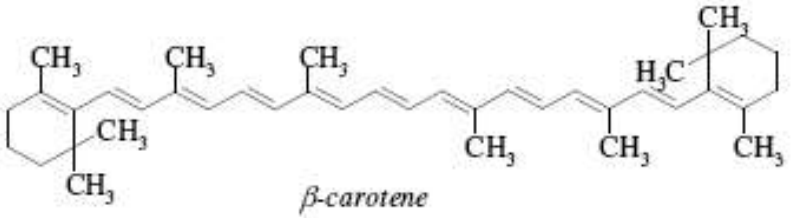
Clorofilla



Ematoporfirina

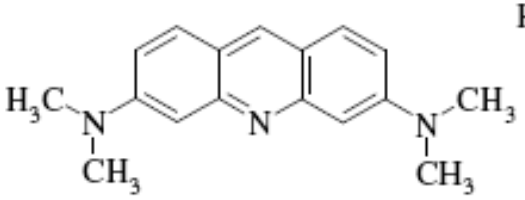


GFP

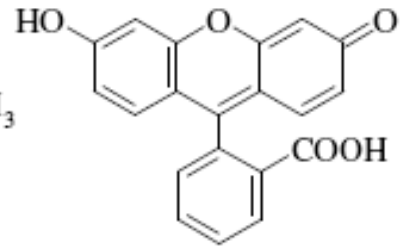


β-carotene

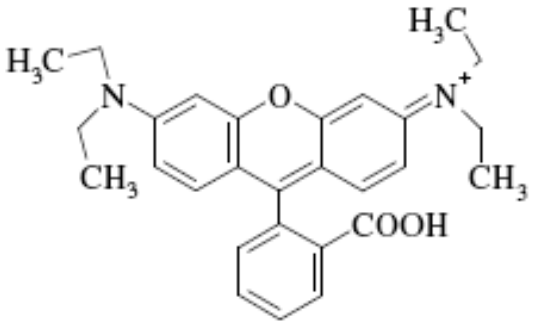
…e molecole fluorescenti usate in biologia



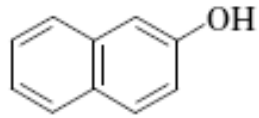
Arancio di acridina



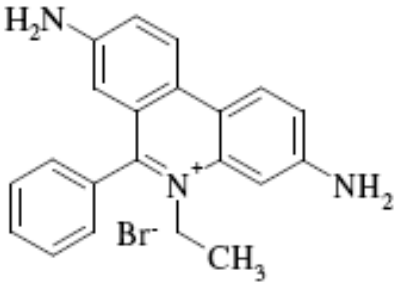
Fluoresceina



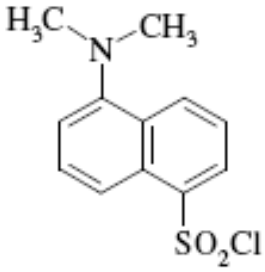
Rodamina B



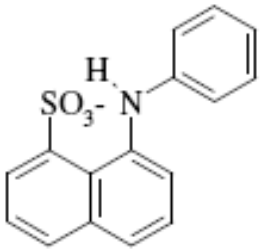
Naftolo



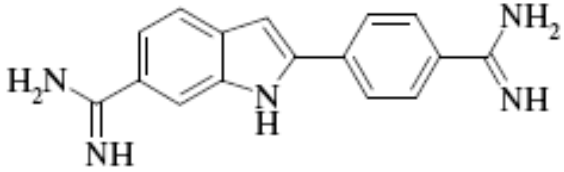
Bromuro di etidio



Dansile



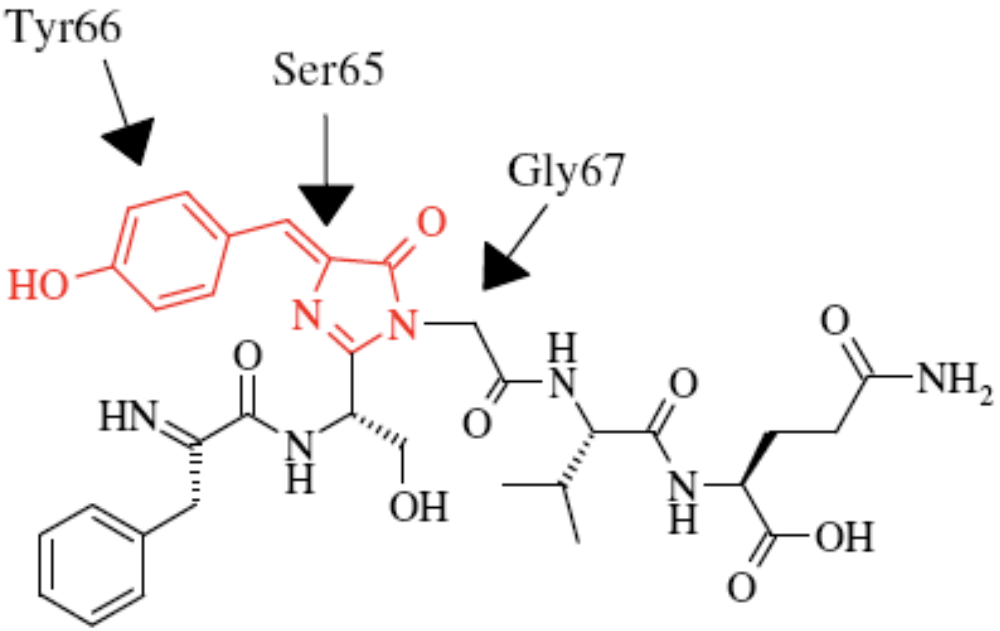
ANS

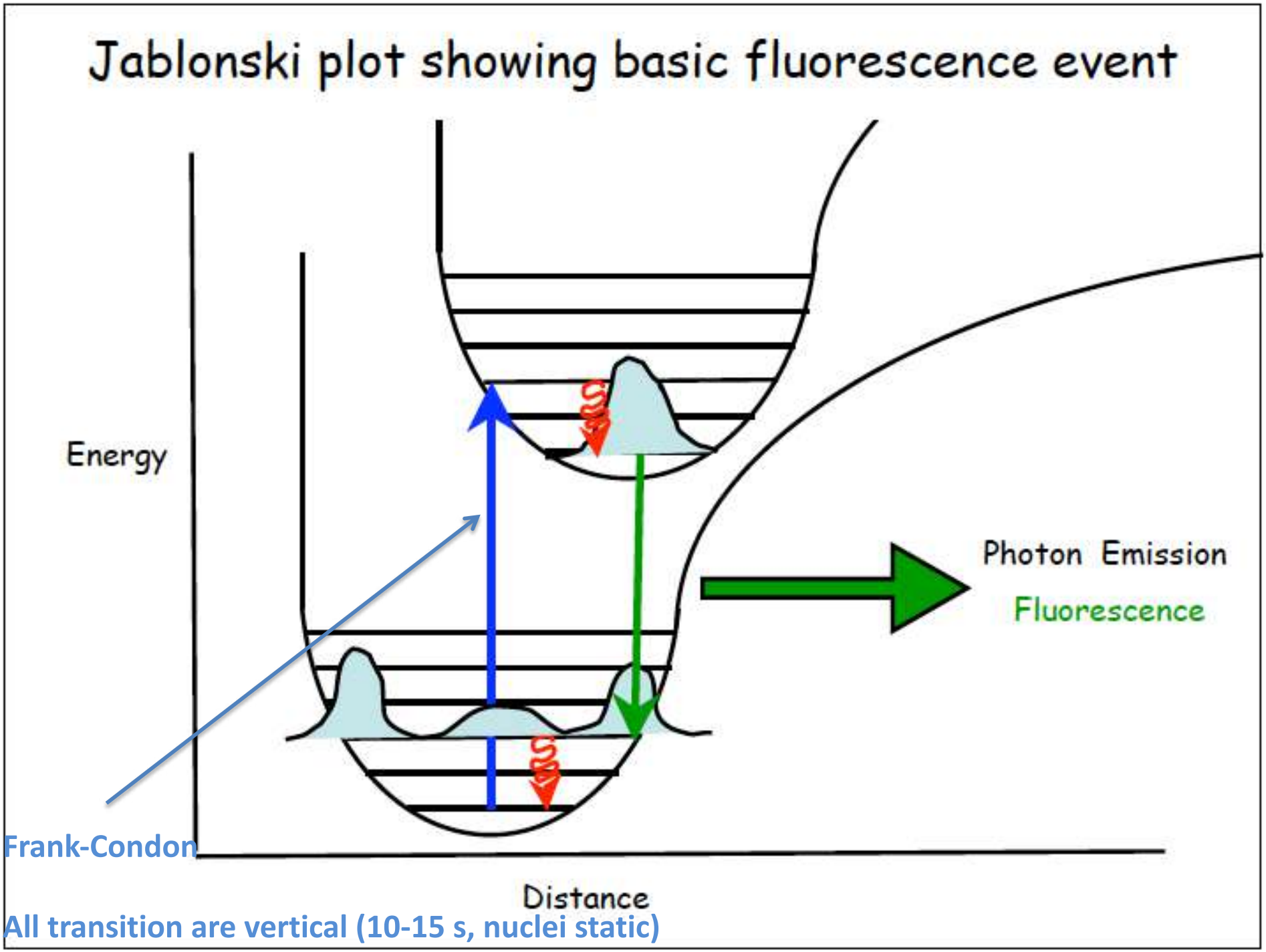


DAPI

Fluoroforo della GFP

- Il fluoroforo della GFP proviene da una modificazione postrascrizionale della proteina con condensazione di tre AA (Ser65, Tyr66, Gly67)



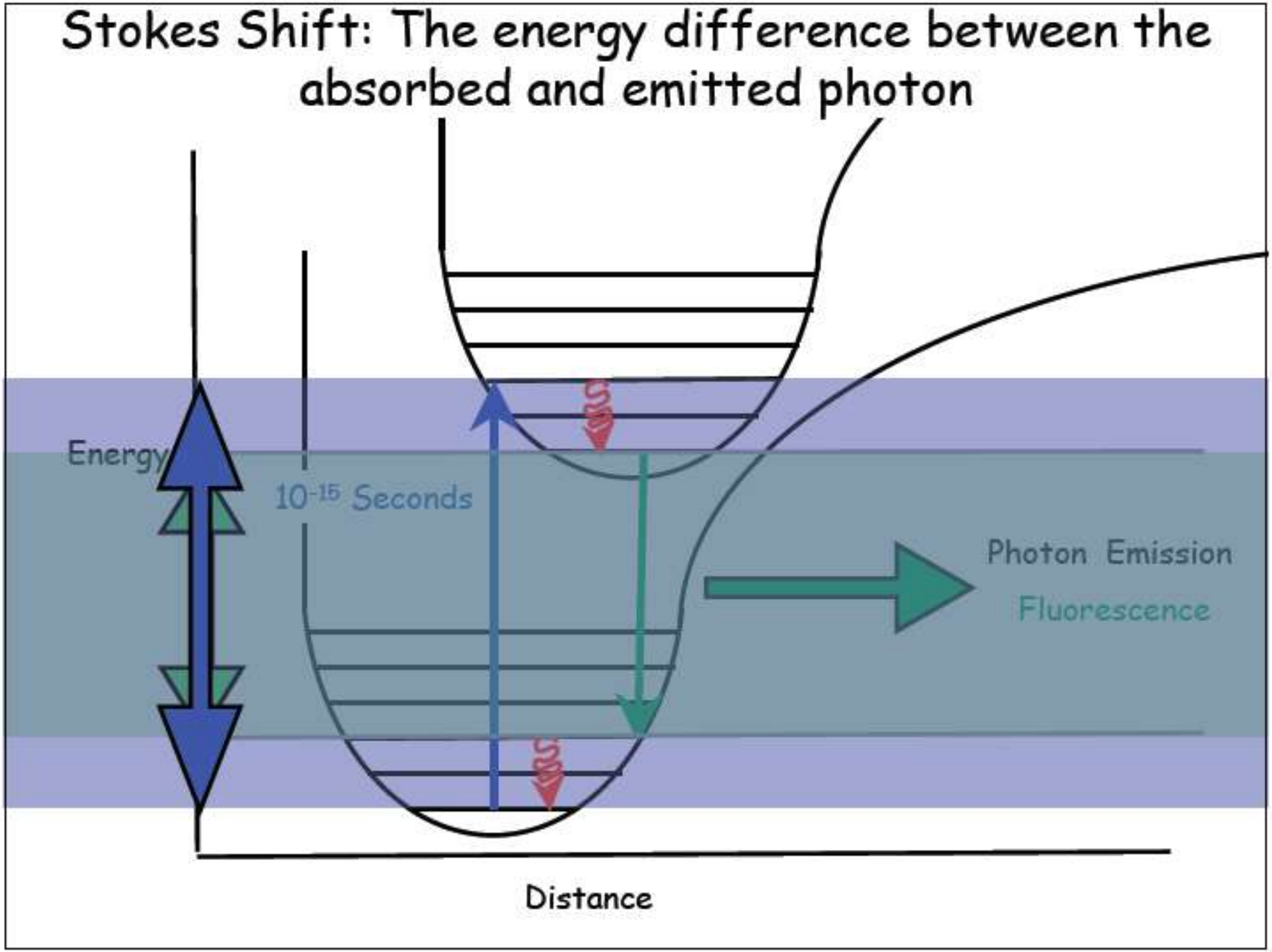

Jablonski plot showing basic fluorescence event
Energy
Photon Emission
Fluorescence
Frank-Condon
Distance
All transition are vertical (10-15 s, nuclei static)

Because **of internal conversion**:

absorption spectra reflect the vibrational levels of the excited states

fluorescent spectra reflect vibrational states of the ground level

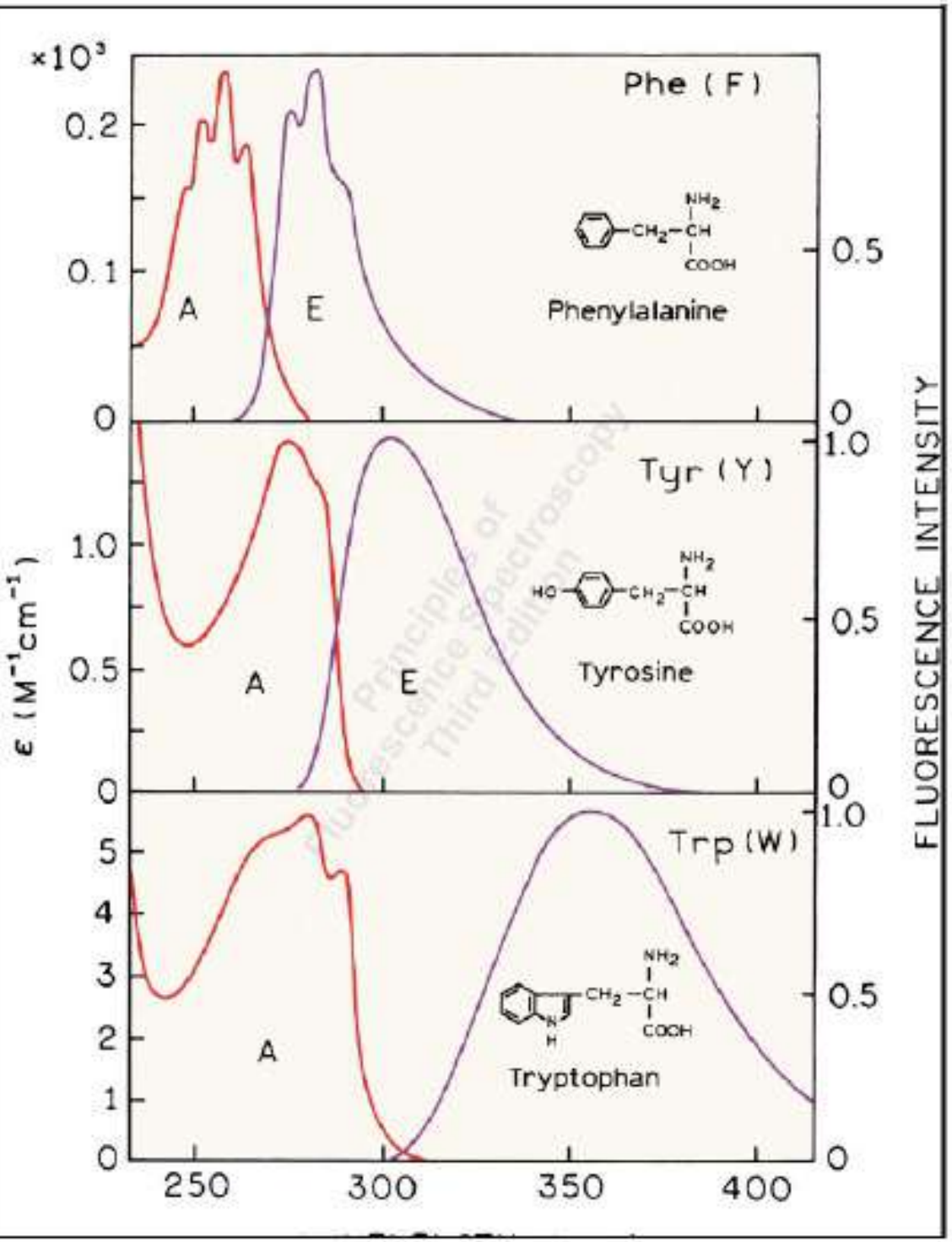
The spectrum of the emitted light is invariant with the excitation WL.
Only exception is azulene, which can emit from S1 and S2


Stokes Shift: The energy difference between the absorbed and emitted photon
Energy
10^{-15} Seconds
Photon Emission
Fluorescence
Distance

Stoke's Shift

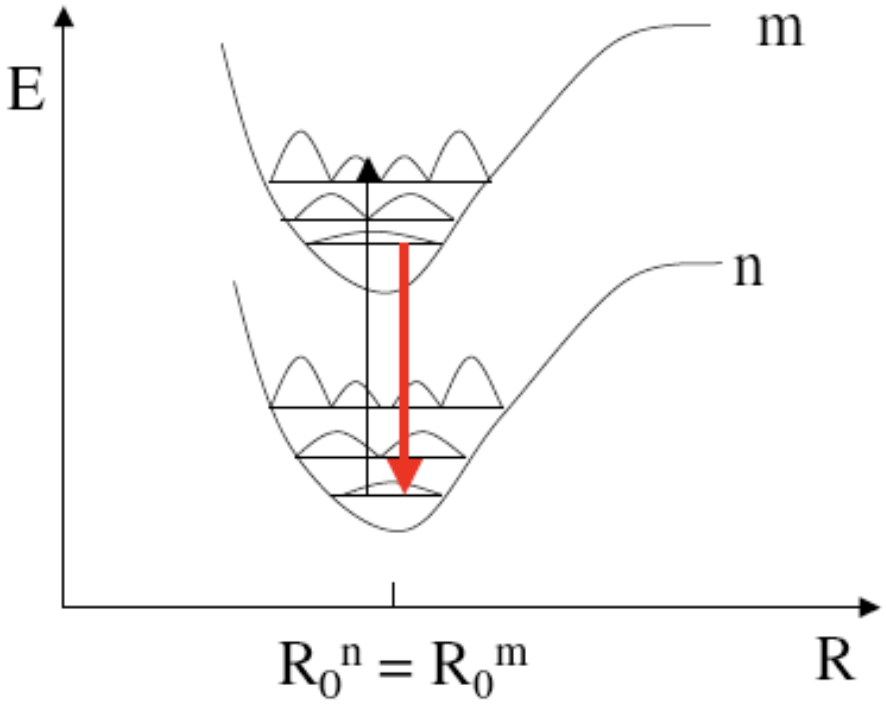
Fluorescence will always occur at longer wavelengths

(lower energies) than absorbance.



The mirror image rule:

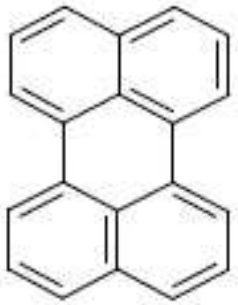
The fluorescence emission often appears as the mirror image of the absorption spectrum (specially when associated to S0-S1 transition)

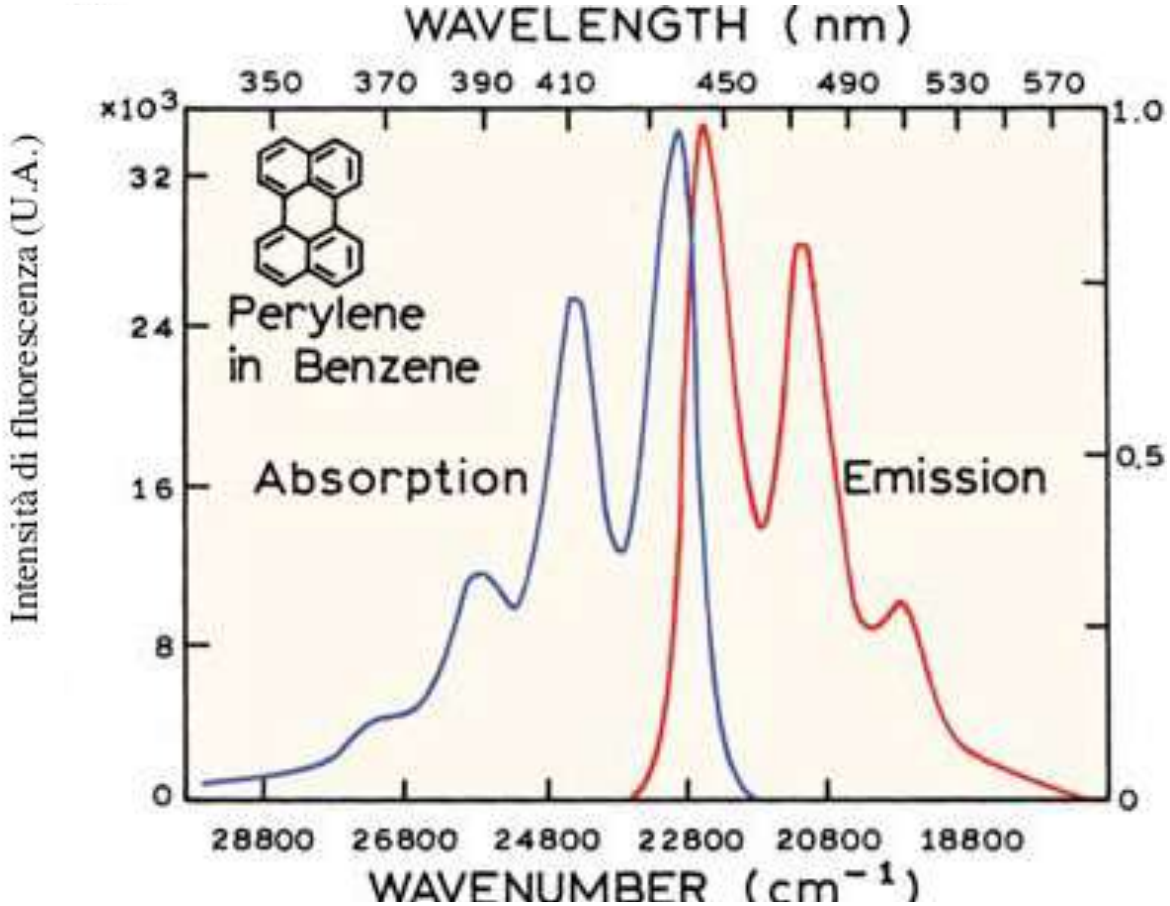


The generally symmetric nature of these spectra is a result of the same transitions being involved in both absorption and emission, and the similar vibrational energy levels of S0 and S1. In most fluorophores these energy levels are not significantly altered by the different electronic distributions of S0 and S1.

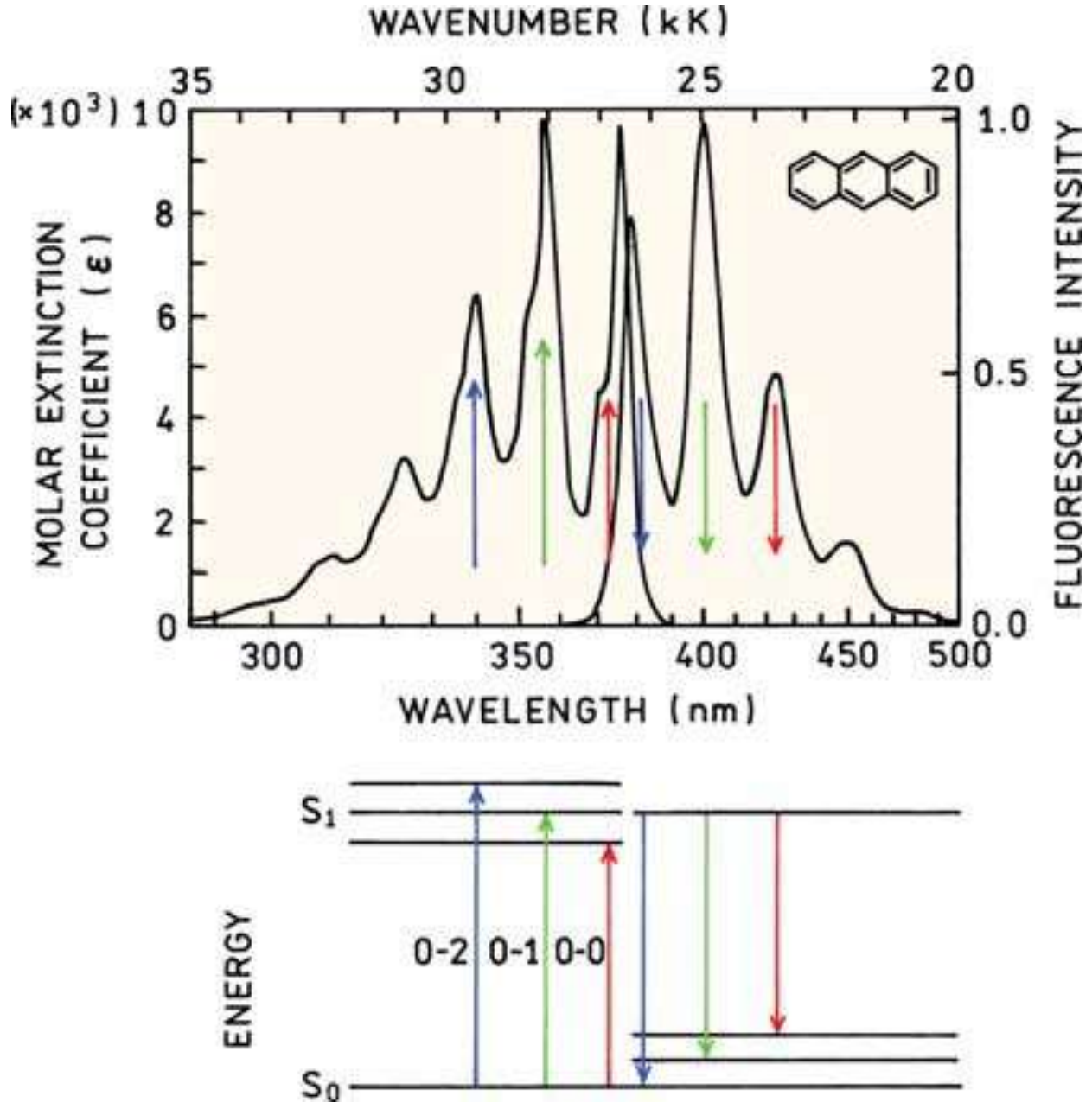
Perilene

- Il perilene ha una struttura compatta, non ci sono variazioni di struttura allo stato eccitato, si ha simmetria speculare.



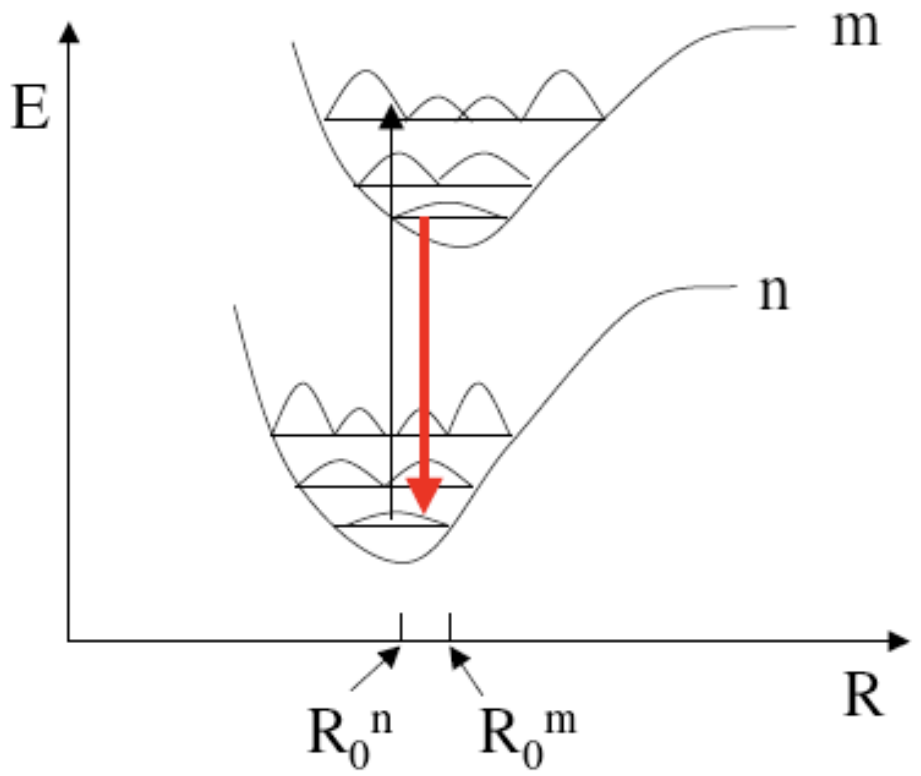


Anthracene


WAVENUMBER (kK)
35
30
25
20
(×10³) 10
8
6
4
2
0
MOLAR EXTINCTION COEFFICIENT (ε)
1.0
0.5
0.0
FLUORESCENCE INTENSITY
300
350
400
450
500
WAVELENGTH (nm)
S₁
S₀
ENERGY
0-2
0-1
0-0

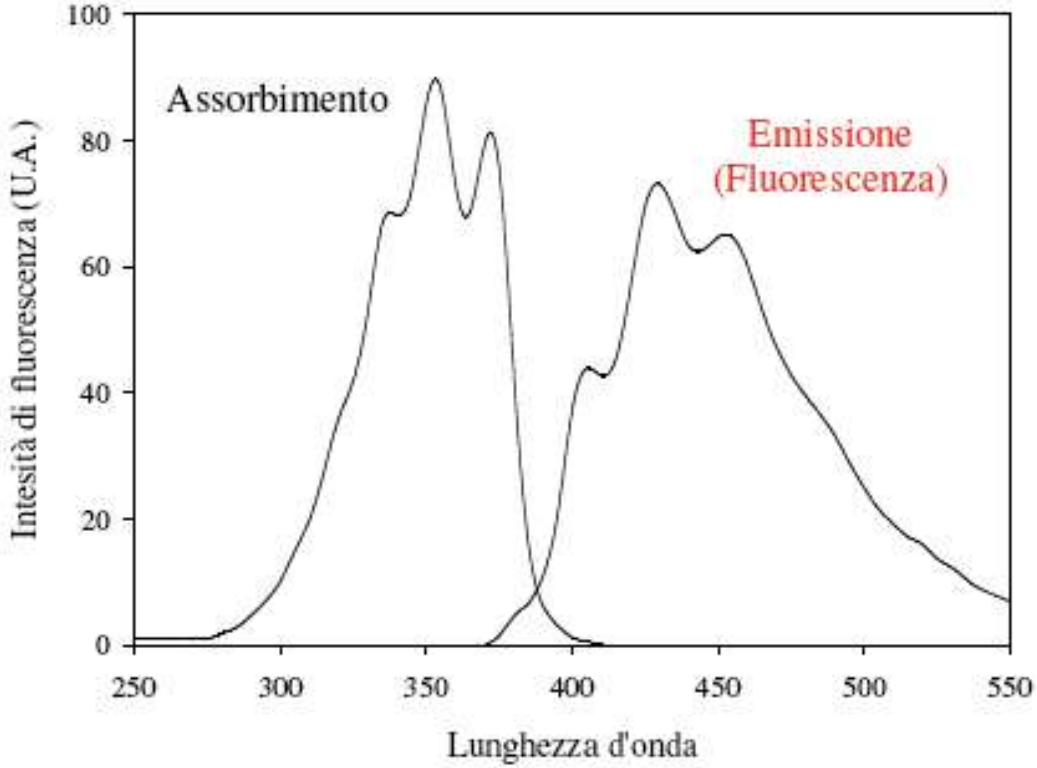
Transizione asimmetrica

- Se lo stato eccitato subisce una perturbazione (cambia la struttura della molecola, avvengono reazioni allo stato eccitato, vi è energy transfer o charge transfer, ecc.) si perde la simmetria speculare tra lo spettro di eccitazione e lo spettro di emissione.

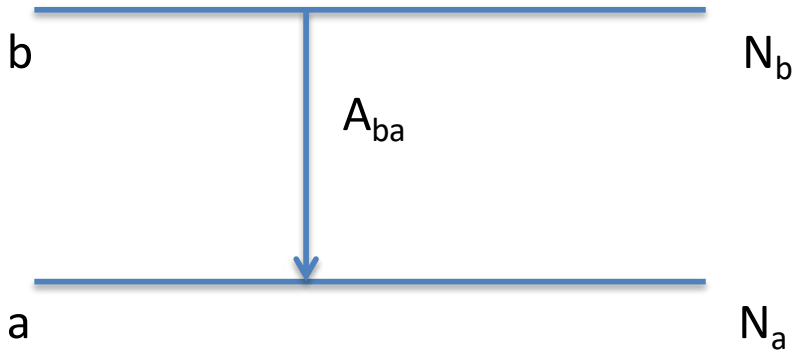


Difenilesatriene (DPH)

- Il DPH può subire una isomerizzazione *cis-trans* allo stato eccitato. Lo spettro di emissione non è speculare a quello di eccitazione.



Fluorescence lifetime and quantum yield



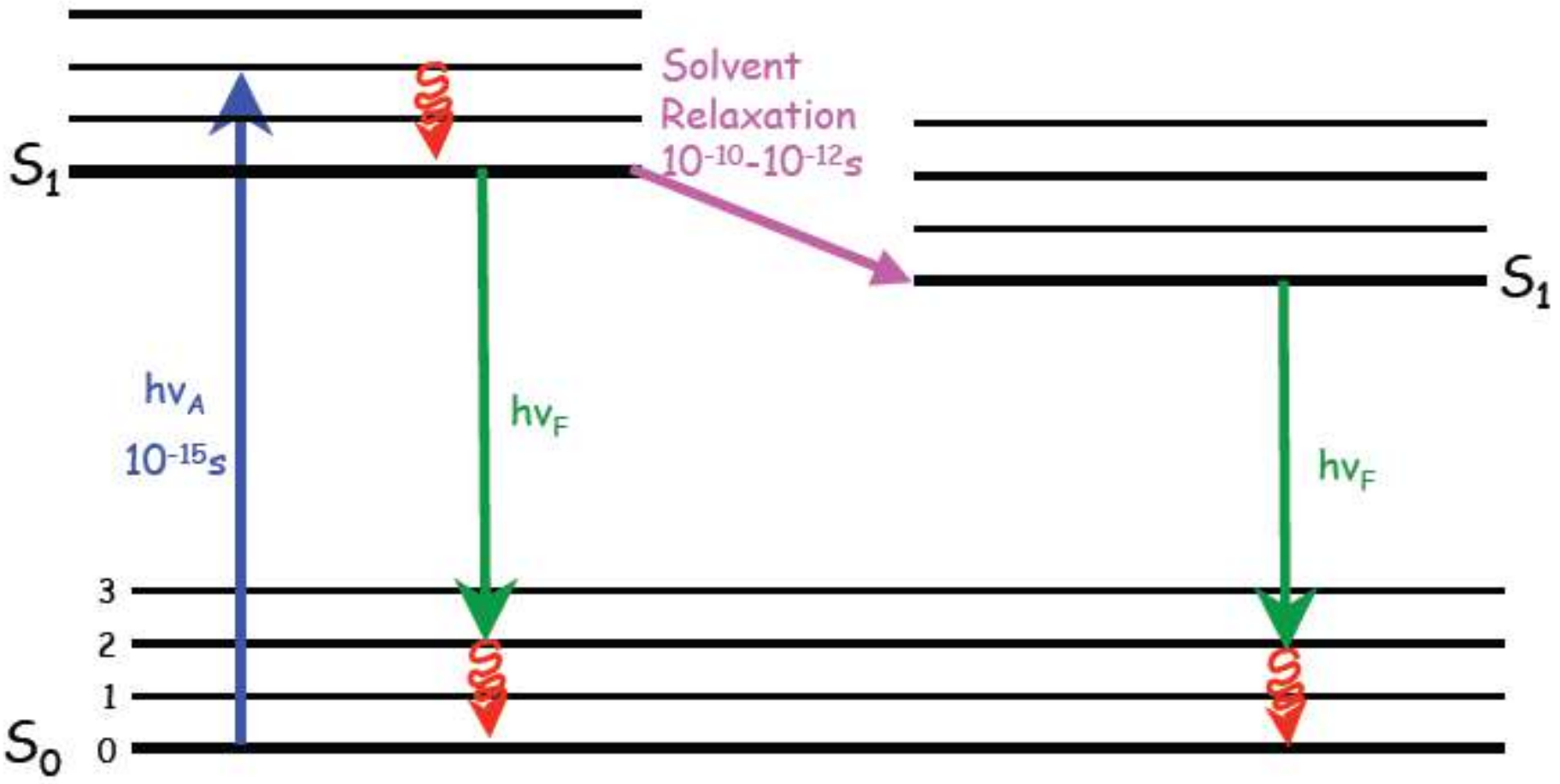
De-excitation rate in the absence of radiation:

$$N_b(t) = N_b(0) \exp(-t/\tau_R)$$

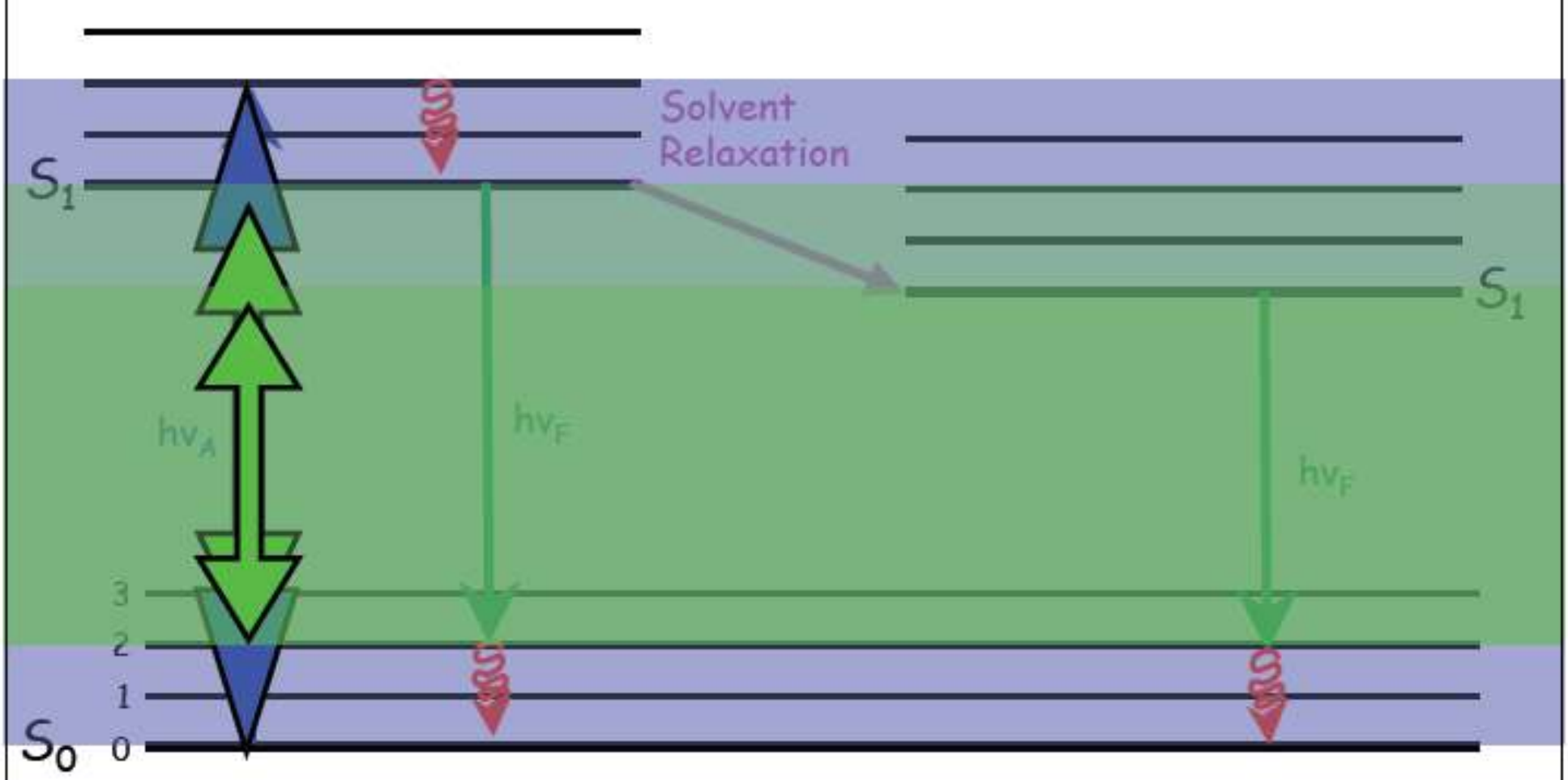
$\tau_R = 1/A_{ba}$ Radiative life-time

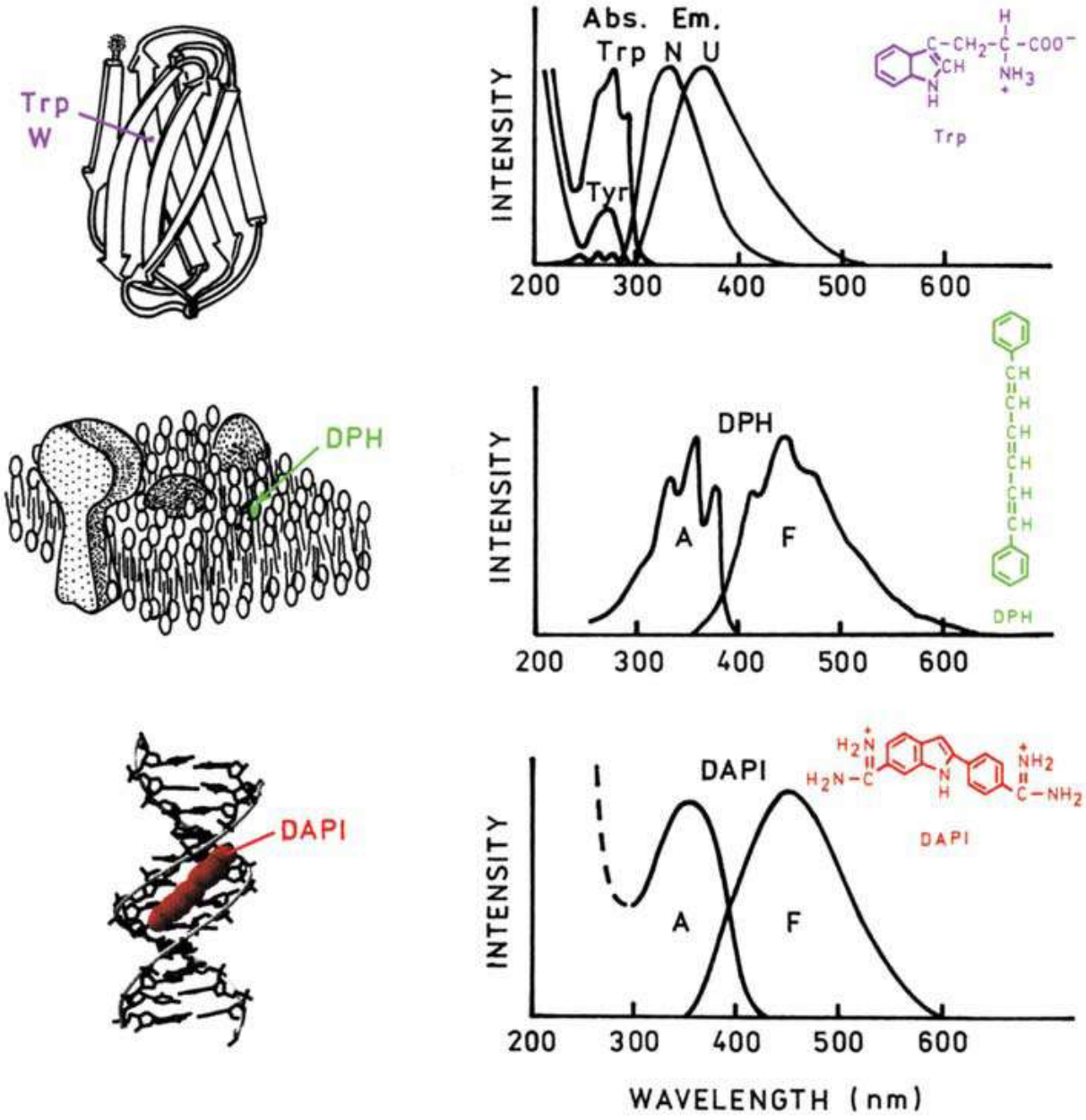
Indeed the excited state can decay in many ways.
The fraction of excited singlets that become de-excited due to fluorescence is called **Fluorescence Quantum Yield**
Collision decay by: collision with solvent; dissipation through internal vibrational modes;

The excited state lifetime allows enough time for solvent to re-organize around it.



Relaxation of a polar solvent increases the Stokes Shift

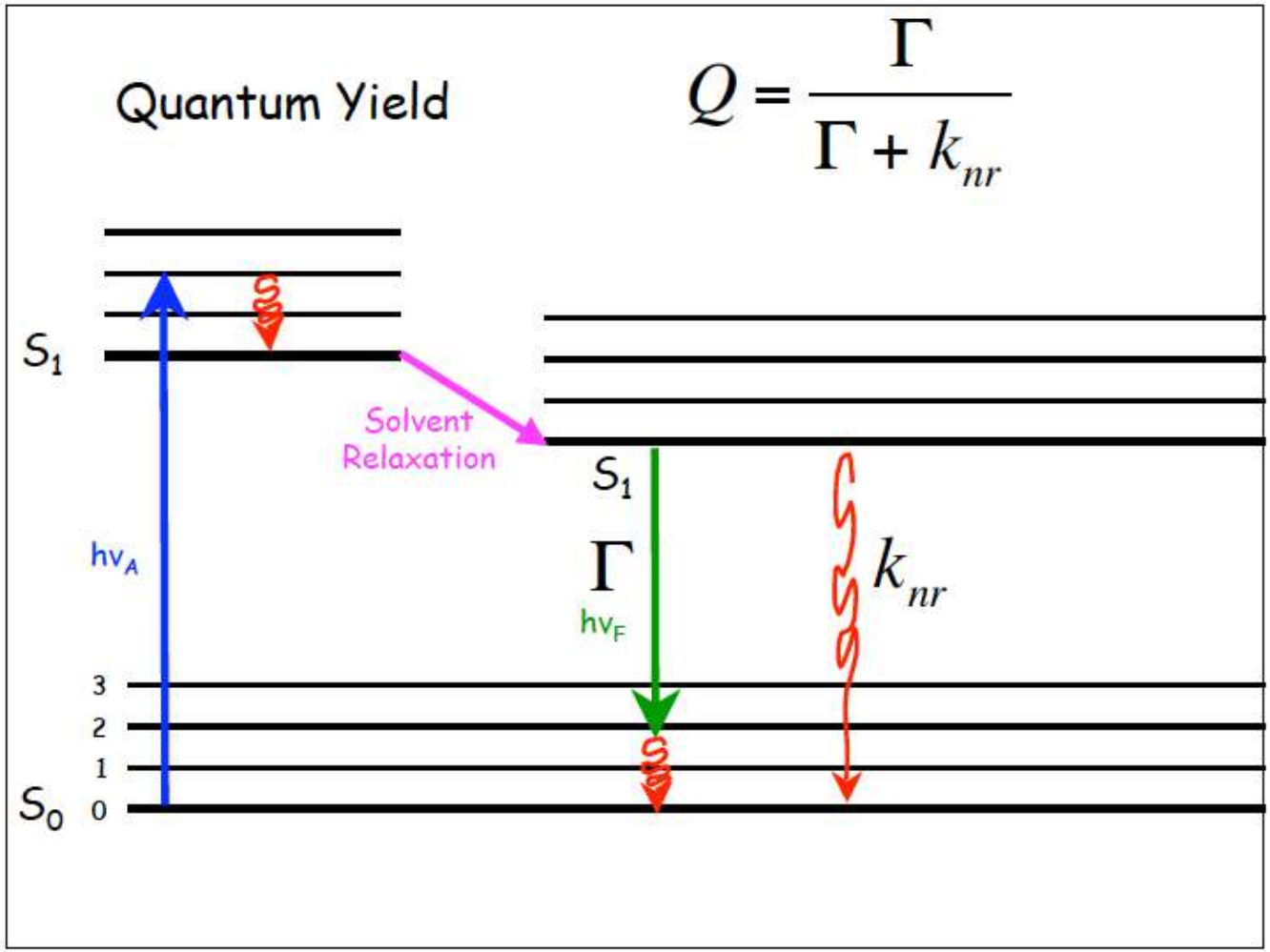



Trp
W
Abs. Em.
Trp N U
Tyr
INTENSITY
200
300
400
500
600
Trp
DPH
DPH
A
F
INTENSITY
200
300
400
500
600
DPH
DAPI
DAPI
A
F
INTENSITY
200
300
400
500
600
DAPI
WAVELENGTH (nm)

FLUORESCENCE LIFETIMES AND QUANTUM YIELDS

Quantum yield is the number of emitted photons relative to the number of absorbed photons.

The lifetime is the time available for the fluorophore to interact with or diffuse in its environment, and hence the information available from its emission.

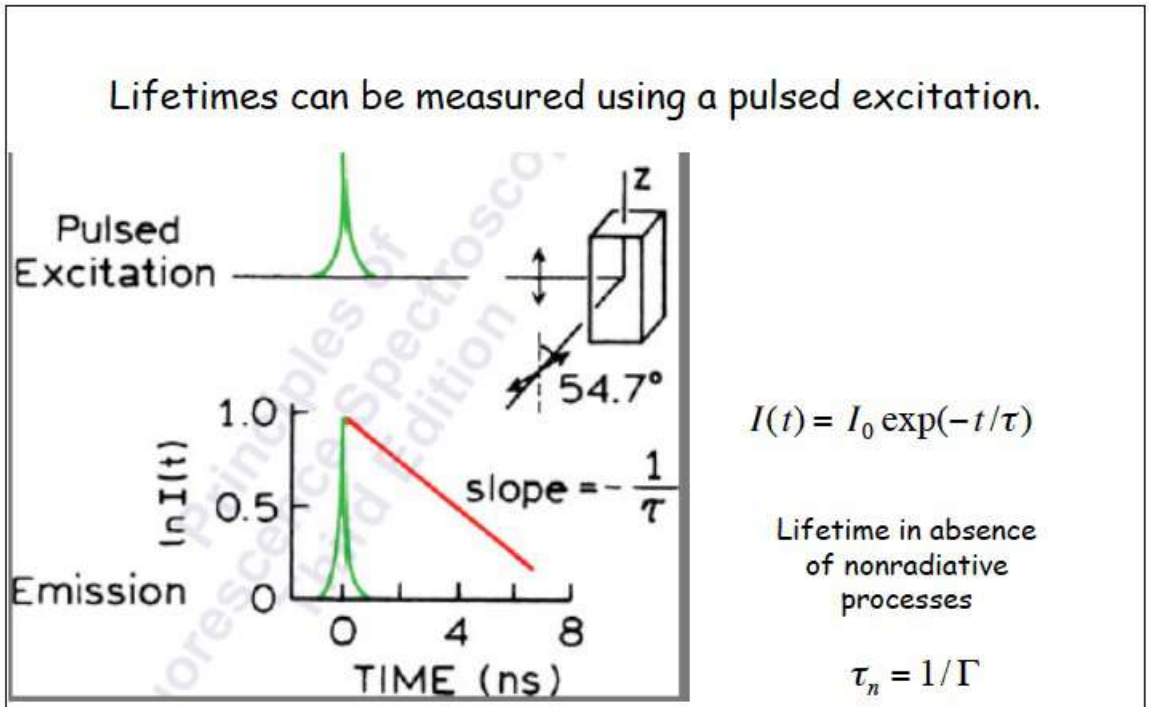


A main route of non-radiative decay is via coupling to vibrations of water.

FLUORESCENCE LIFETIMES AND QUANTUM YIELDS

The **lifetime** of the excited state is defined by the average time the molecule spends in the excited state prior to return to the ground state. Generally, fluorescence lifetimes are near 10 ns.

$$\tau = \frac{1}{\Gamma + k_{nr}}$$



$$I(t) = I_0 \exp(-t/\tau)$$

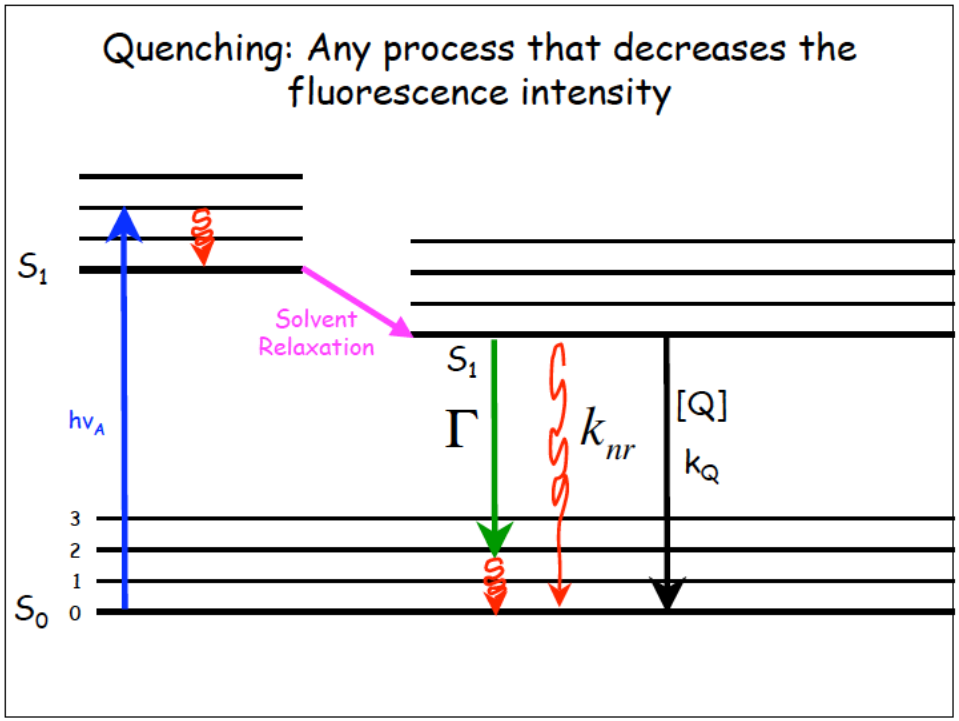
Lifetime in absence of nonradiative processes

$$\tau_n = 1/\Gamma$$

$$\tau_n = \tau / Q$$

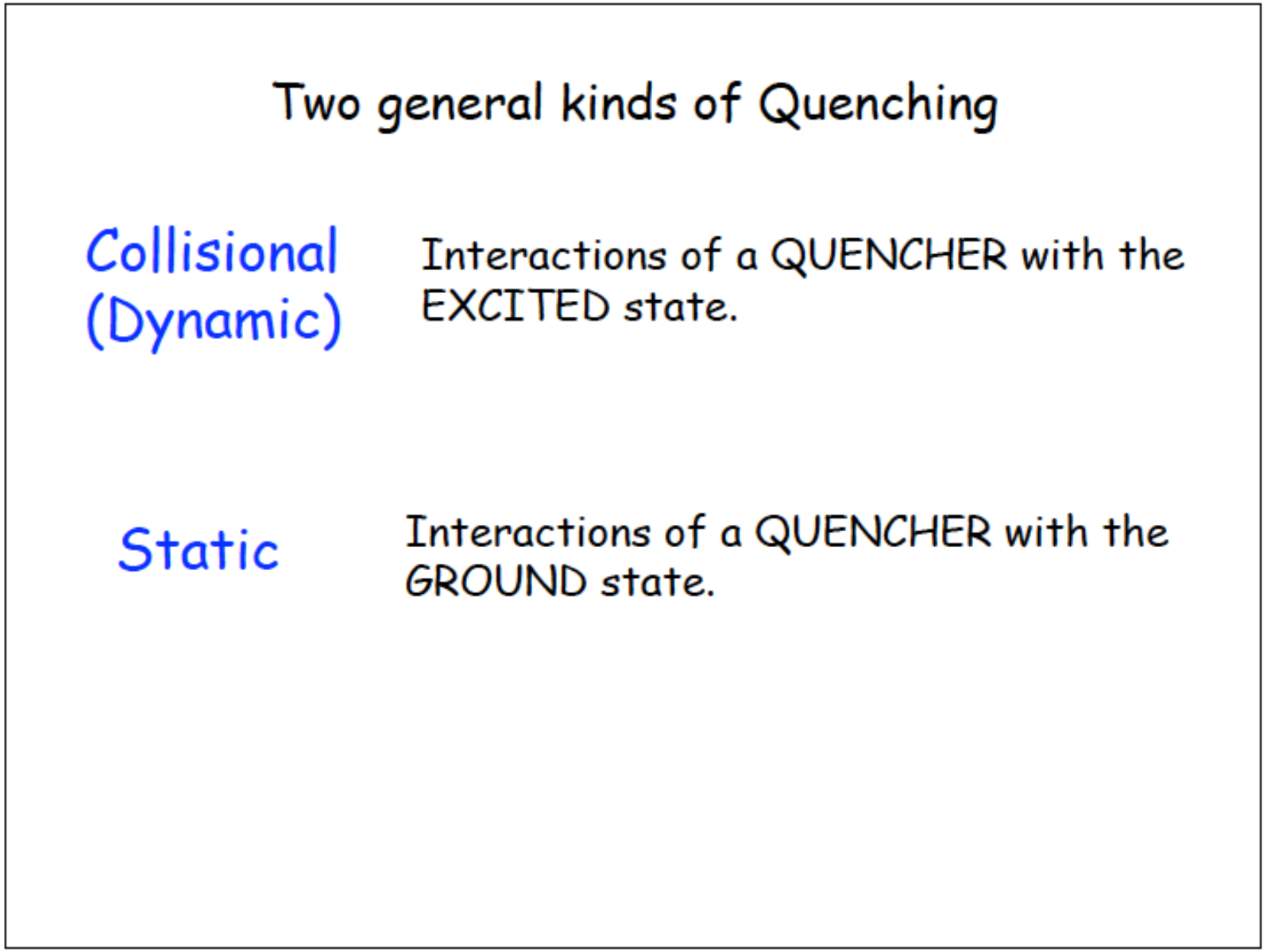
Fluorescence emission is a random process, and few molecules emit their photons at precisely t = τ. The lifetime is an average value of the time spent in the excited state.
For a single exponential decay 63% of the molecules have decayed prior to t = τ and 37% decay at t > τ.

The lifetime of the fluorophore in the absence of nonradiative processes is called the **intrinsic or natural lifetime**.



Indeed, many biochemical fluorophores do not behave as predictably as unsubstituted aromatic compounds.

These discrepancies occur for a variety of unknown and known reasons, such as a fraction of the fluorophores located next to quenching groups, which sometimes occurs for tryptophan residues in proteins.



Fluorophores can form nonfluorescent complexes with quenchers. This process is referred to as static quenching since it occurs in the ground state and does not rely on diffusion or molecular collisions.

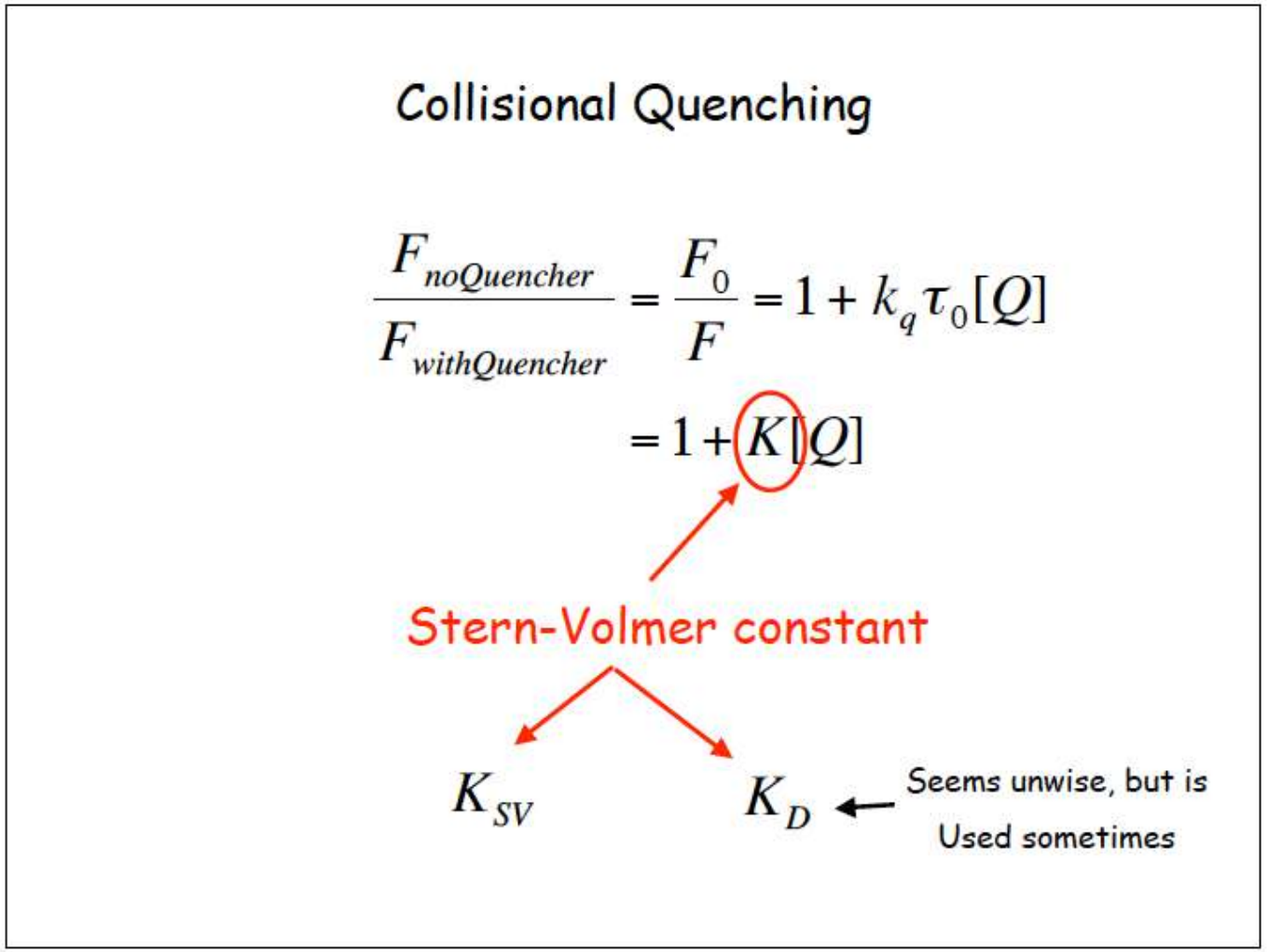
Collisional Quenching

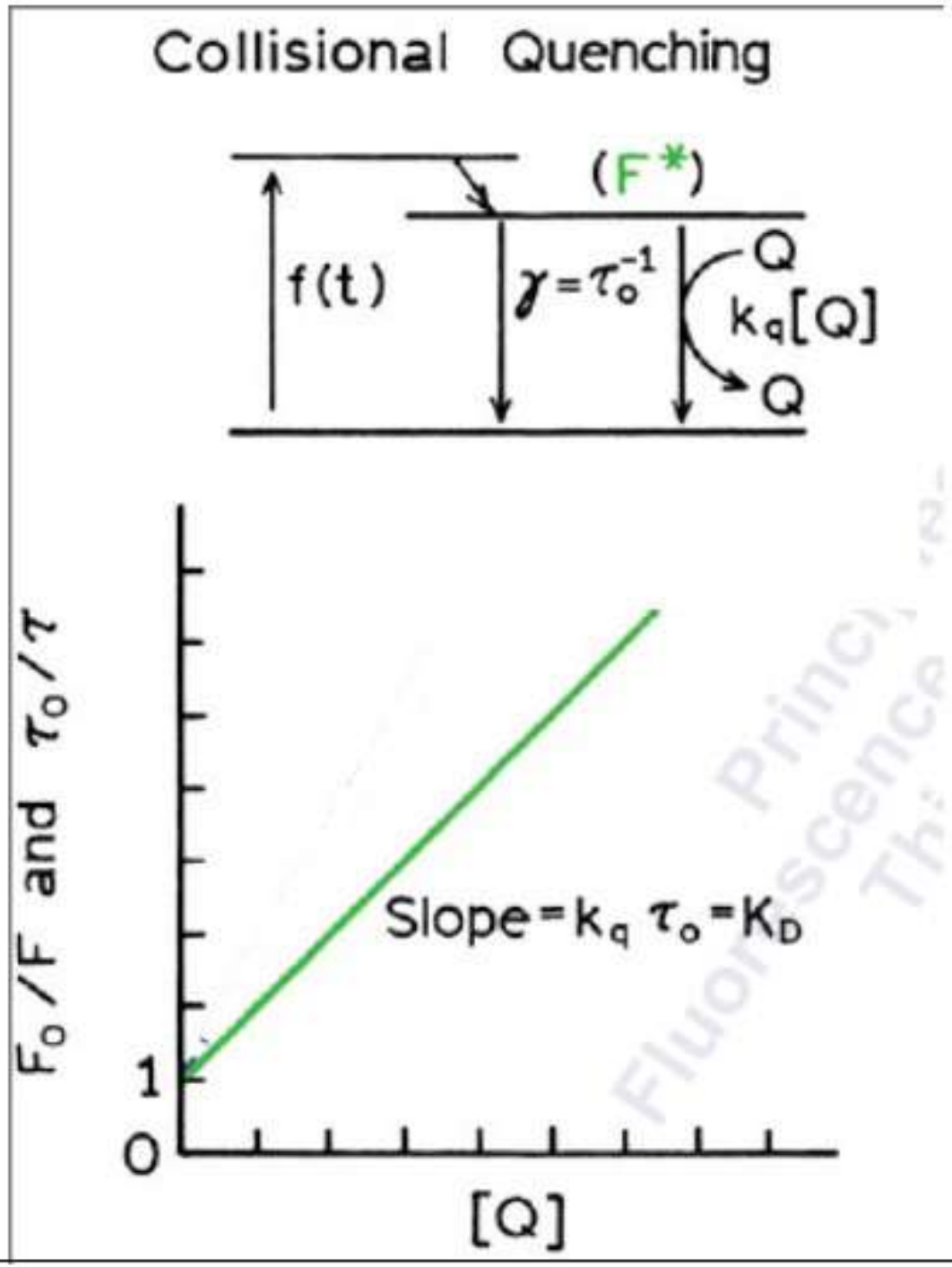
The excited-state fluorophore is deactivated upon contact with some other molecule in solution: the Quencher.

The Quencher must diffuse to the fluorophore during the lifetime of the excited state.

Upon contact, the fluorophore returns to the ground state without emission of a photon.

In this expression K is the Stern-Volmer quenching constant, k_q is the bimolecular quenching constant, τ_0 is the unquenched lifetime, and [Q] is the quencher concentration.




Collisional Quenching
(F*)
f(t)
γ = τo-1
Q
kq[Q]
Q
Fo/F and τo/τ
Slope = kq τo = KD
1
0
[Q]

A fluorophore buried in a macromolecule is usually inaccessible to water soluble quenchers, so that the value of K is low. Larger values of K are found if the fluorophore is free in solution oron the surface of a biomolecule.

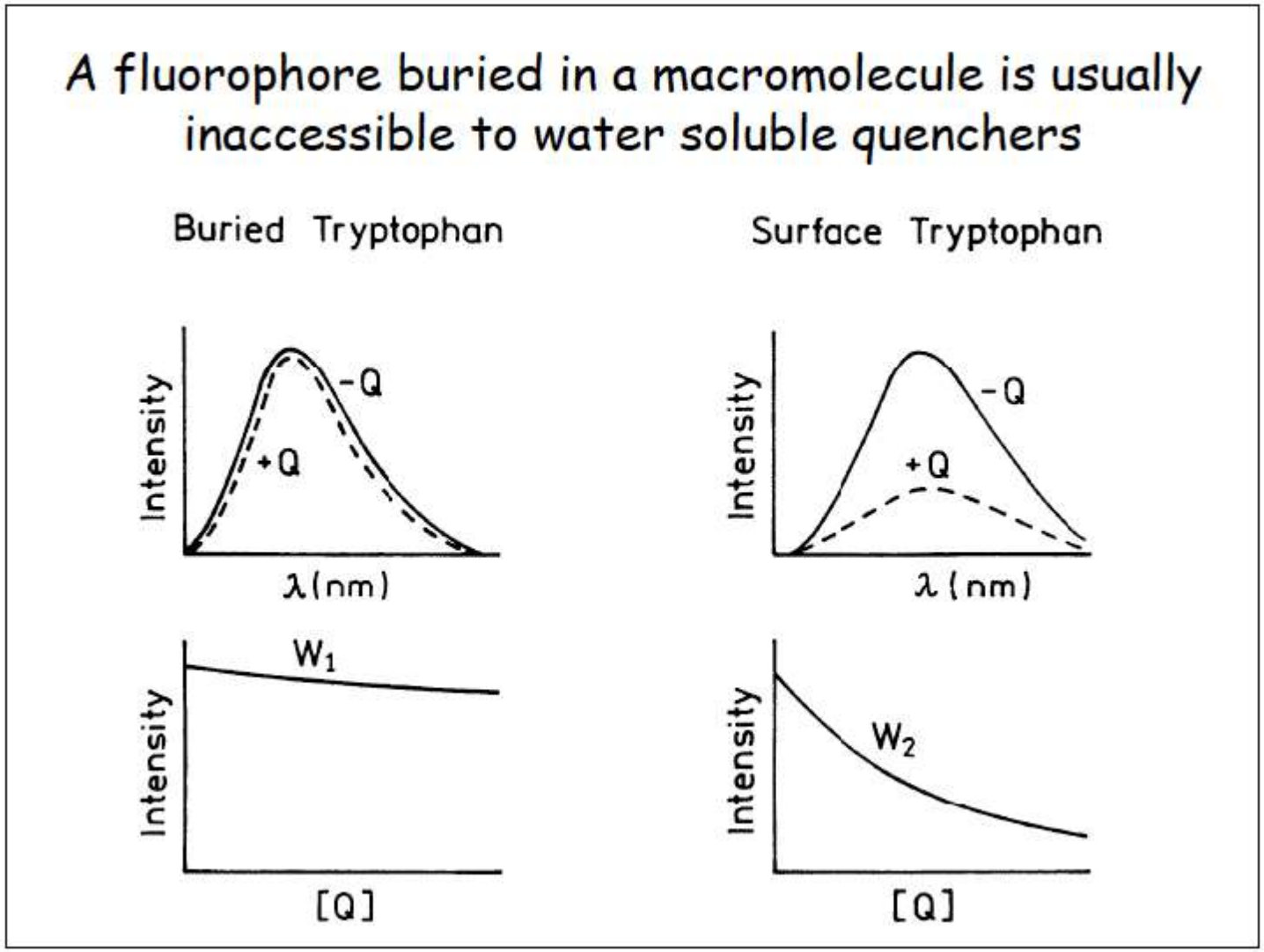


Table 8.1. Quenchers of Fluorescence

Quenchers	Typical fluorophore	References
Acrylamide	Tryptophan, pyrene, and other fluorophores	5–7, 176–180
Amines	Anthracene, perylene	2, 124, 181–186
Amines	Carbazole	187
Amine anesthetics	Perylene, anthroyloxy probes	188–190
Bromate	–	191
Bromobenzene	Many fluorophores	192
Carbon disulfide	Laser dyes, perylene	193
Carboxy groups	Indole	194
Cesium (Cs^+)	Indole	195
Chlorinated compounds	Indoles and carbazoles	196–199
Chloride	Quinolinium, SPQ	200–203
Cobalt (Co^{2+})	NBD, PPO, Perylene (Energy transfer for some probes)	204–210
Dimethylformamide	Indole	211
Disulfides	Tyrosine	212
Ethers	9-Arylxanthyl cations	213
Halogens	Anthracene, naphthalene, carbazole	214–229
Halogen anesthetics	Pyrene, tryptophan	230–232
Hydrogen peroxide	Tryptophan	233
Iodide	Anthracene	234–237
Imidazole, histidine	Tryptophan	238
Indole	Anthracene, pyrene, cyanoanthracene	239–241
Methylmercuric chloride	Carbazole, pyrene	242
Nickel (Ni^{2+})	Perylene	243–244
Nitromethane and nitro compounds	Polycyclic aromatic hydrocarbon	245–256
Nitroxides	Naphthalene, PAH, Tb^{3+}, anthroyloxy probes	257–266
NO (nitric oxide)	Naphthalene, pyrene	267–270
Olefins	Cyanonaphthalene, 2,3-dimethylnaphthalene, pyrene	271–273
Oxygen	Most fluorophores	274–290
Peroxides	Dimethylnaphthalene	291
Picolinium nicotinamide	Tryptophan, PAH	292–296
Pyridine	Carbazole	297
Silver (Ag^+)	Perylene	298
Succinimide	Tryptophan	299–300
Sulfur dioxide	Rhodamine B	301
Thallium (Tl^+)	Naphthylamine sulfonic acid	302
Thiocyanate	Anthracene, 5,6-benzoquinoline	303–304
Xenon		305

Oxygen, Halides (Iodide, Bromide)

Thought to cause fluorophore to undergo intersystem crossing to triplet state.

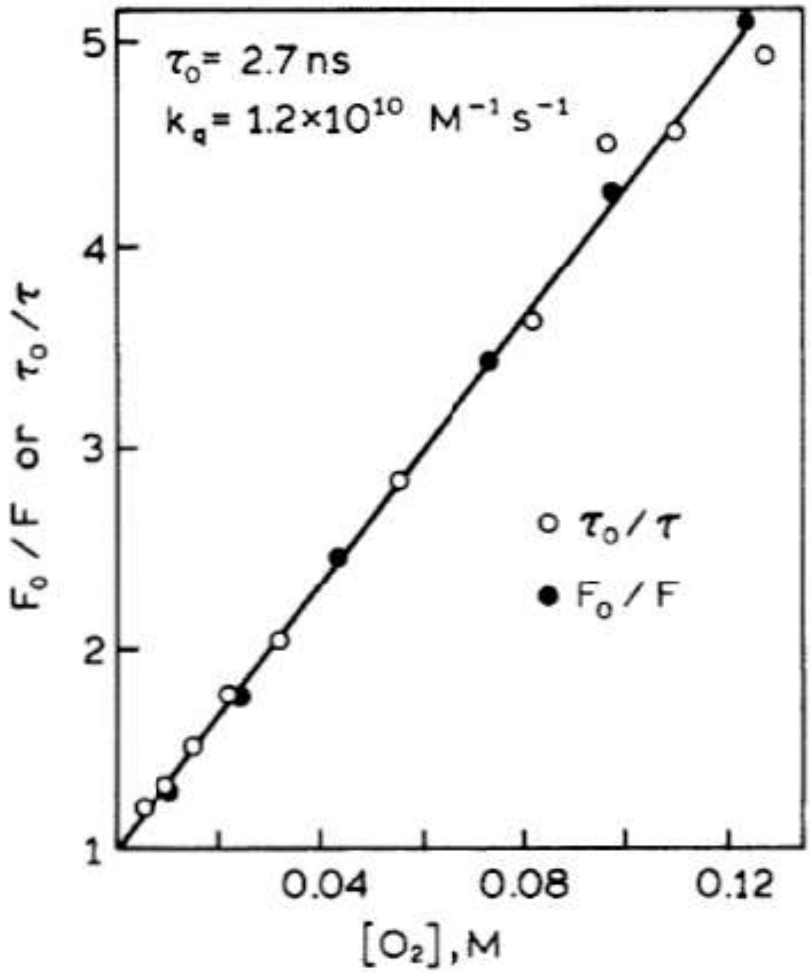
Cu^{2+}, PB^{2+}, Cd^{2+}

Thought to cause donation of an electron from the fluorphore in the excited state.

Acrylamide

Quenchers: oxygen, halogens, amines, and electron-deficient molecules like acrylamide.

Oxygen quenching of Tryptophan fluorescence



CAN THERE BE ENOUGH DIFFUSION OF QUENCHER DURING THE EXCITED STATE LIFETIME?

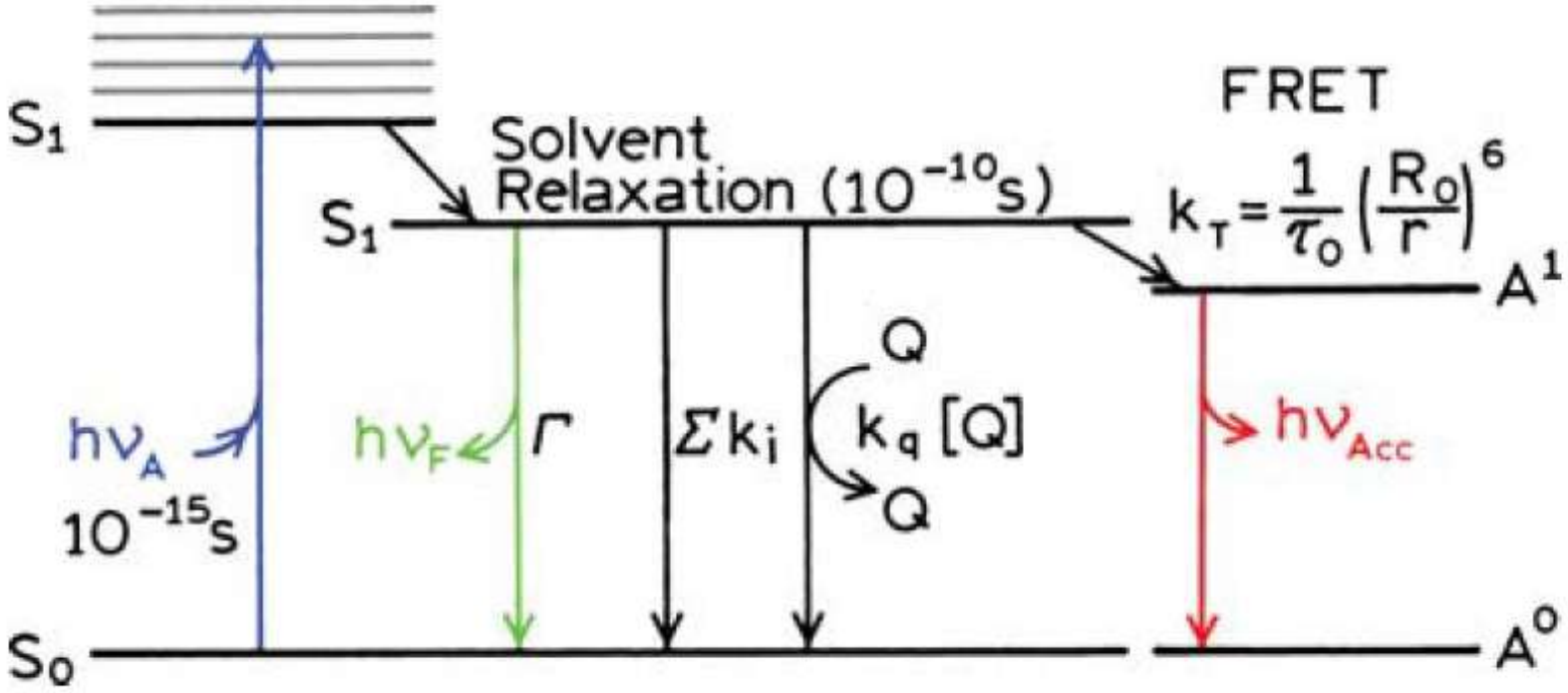
If a fluorophore in the excited state collides with an oxygen molecule, then the fluorophore returns to the ground state without emission of a photon.
The diffusion coefficient (D) of oxygen in water at 25C is 2.5×10^{-5} cm^2/s.

Suppose a fluorophore has a lifetime of 10 ns. Although 10 ns may appear to be a brief time span, it is in fact quite long relative to the motions of small molecules in fluid solution.
The average distance $(\Delta x^2)^{1/2}$ an oxygen molecule can diffuse in 10^{-8} s or 10 ns is given by the Einstein equation:

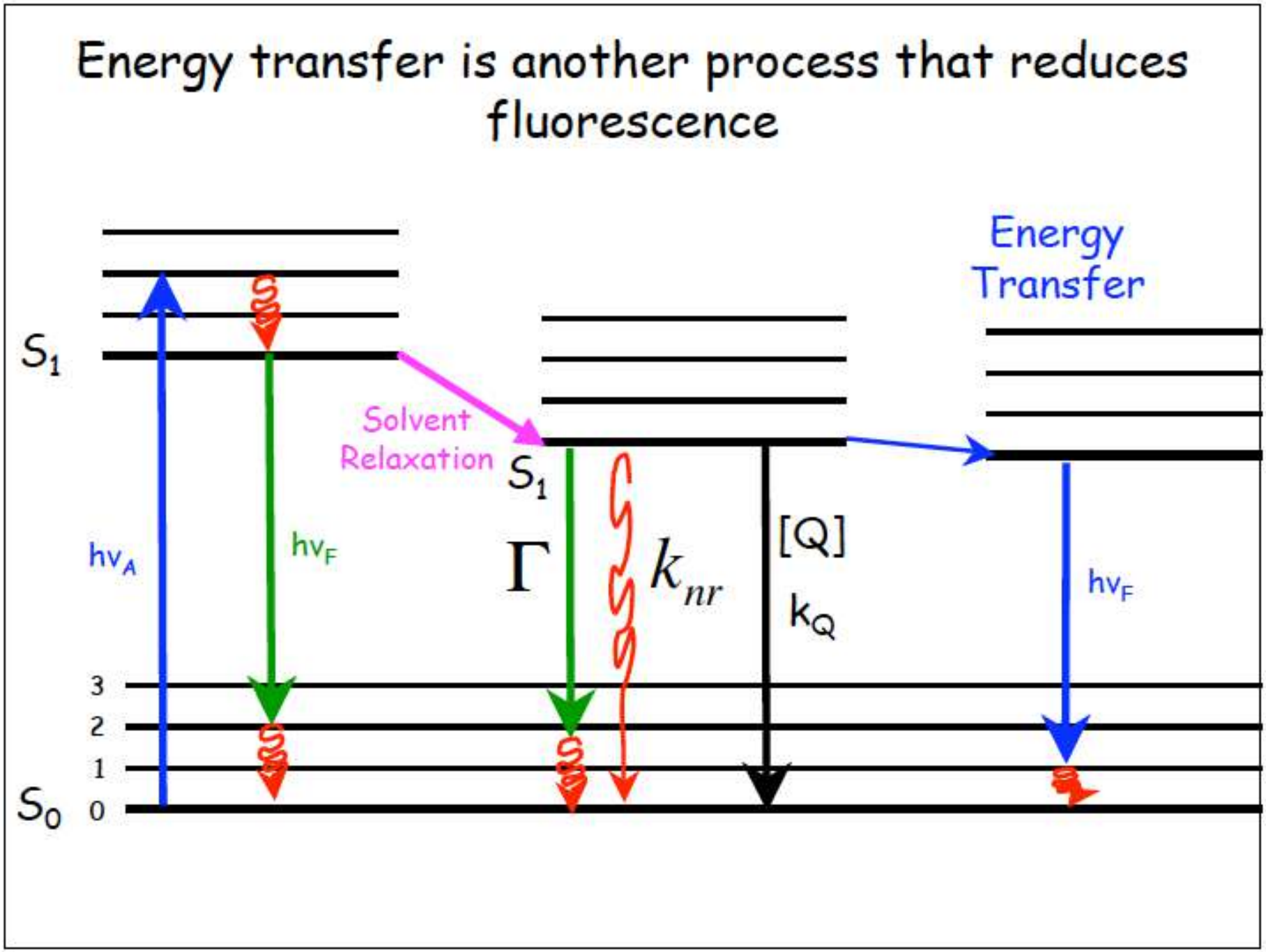
$$\sqrt{\overline{\Delta x^2}} = \sqrt{2 D_Q \tau}$$

O_2 $\quad D = 2.5 \times 10^{-8}\ cm^2\ s^{-1}$

Use Trp Lifetime of 4 ns ⟶ 44Å


S1
Solvent
Relaxation (10^-10 s)
S1
FRET
k_T = 1/τ_0 (R_0/r)^6
A^1
hν_A
10^-15 s
hν_F
Γ
Σk_i
Q
k_q [Q]
Q
hν_Acc
S0
A^0

Energy transfer is another process that reduces fluorescence



Energy Transfer: commonly called FRET: Fluorescence Resonance Energy Transfer

Occurs when an "Acceptor" molecule takes the excited state energy from a "Donor" fluorophore.

No photon is emitted from the donor
-->Donor fluorescence is reduced.

No photon is involved in the transfer.

The energy transfer occurs by electronic dipolar coupling between the Donor and Acceptor molecules when they are in close proximity to each other.

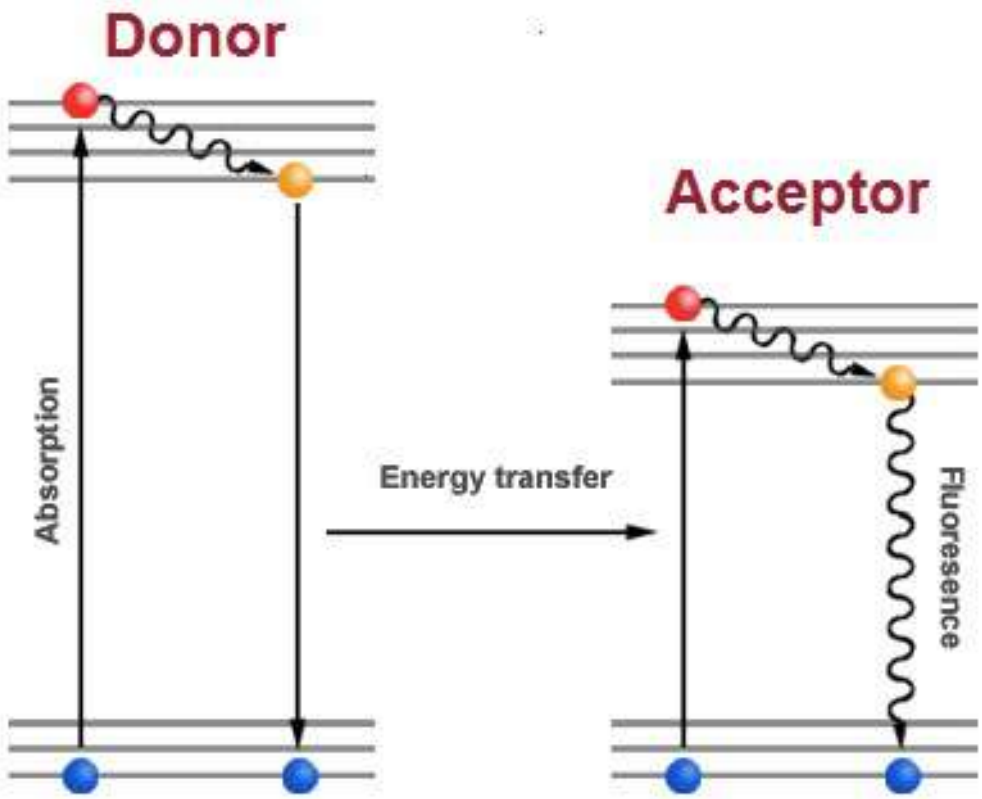
Advanced Optical Microscopies-5

Förster (Fluorescence) Resonance Energy Transfer (FRET)

- The technique of FRET, when applied to optical microscopy, permits to determine the approach between two molecules within several nanometers

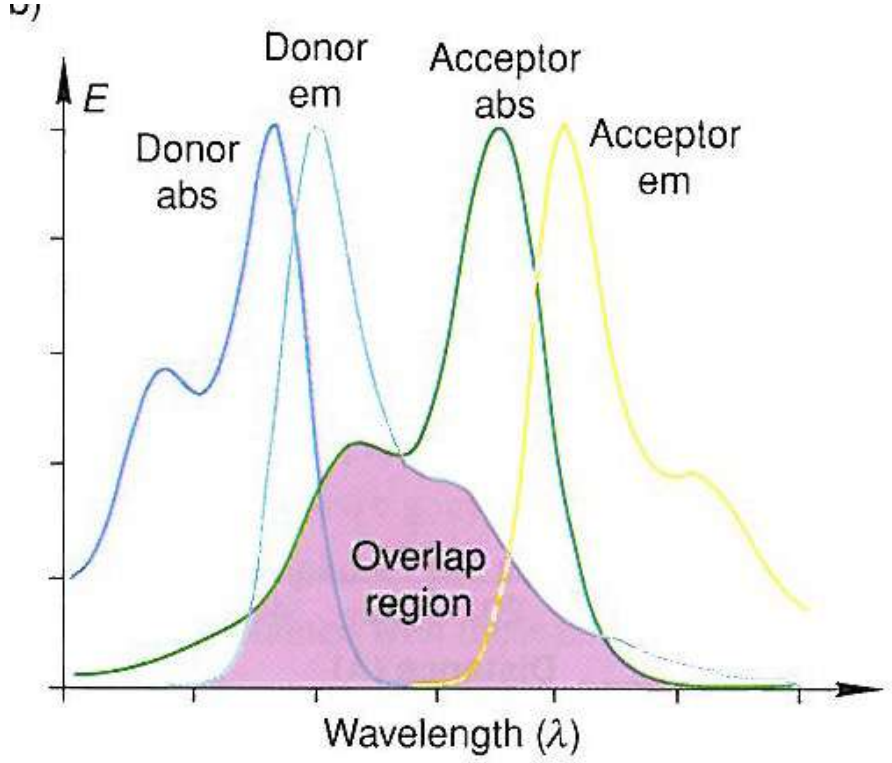
- FRET is a distance dependant radiationless transfer of energy from an excited donor fluorophore to a suitable acceptor fluorophore, is one of few tools available for measuring nanometer scale distances and the changes in distances, both in vitro and in vivo.
- It can be classified as a super-resolution techniques (i.e. STED)

Fluorescence Resonance Energy Transfer (FRET)



The mechanism of FRET involves a donor fluorophore in an excited electronic state, which may transfer its excitation energy to a nearby acceptor chromophore in a non-radiative fashion through long-range dipole-dipole interactions.

Fluorescence Resonance Energy Transfer (FRET)



In presence of suitable acceptor, the donor fluorophore can transfer its excited state energy directly to the acceptor without emitting a photon.

Fluorescence Resonance Energy Transfer (FRET)

- The theory supporting energy transfer is based on the concept of treating an excited fluorophore as an oscillating dipole that can undergo an energy exchange with a second dipole having a similar resonance frequency. In this regard, **resonance energy transfer is analogous to the behavior of coupled oscillators, vibrating at the same frequency.**
- In contrast, radiative energy transfer requires emission and reabsorption of a photon and depends on the physical dimensions and optical properties of the specimen, as well as the geometry of the container and the wavefront pathways.
- Unlike radiative mechanisms, resonance energy transfer can yield a significant amount of structural information concerning the donor-acceptor pair.

Fluorescence Resonance Energy Transfer (FRET)

- In the process of FRET, initially a donor fluorophore absorbs the energy due to the excitation of incident light and transfer the excitation energy to a nearby chromophore, the acceptor.

$$D + h\upsilon \rightarrow D^{*}$$

$$D^{*} + A \rightarrow D + A^{*} \quad [\mathrm{D} \rightarrow \text{donor}, \mathrm{A} \rightarrow \text{Acceptor}]$$

$$A^{*} \rightarrow A + h\upsilon'$$

- Energy transfer manifests itself through decrease or quenching of the donor fluorescence and a reduction of excited state lifetime accompanied also by an increase in acceptor fluorescence intensity.

Fluorescence Resonance Energy Transfer (FRET)

- There are few criteria that must be satisfied in order for FRET to occur. These are:

(i) the fluorescence emission spectrum of the donor molecule must overlap the absorption or excitation spectrum of the acceptor chromophore. The degree of overlap is referred to as spectral overlap integral (J).

(ii) The two fluorophore (donor and acceptor) must be in the close proximity to one another (typically 1 to 10 nanometer).

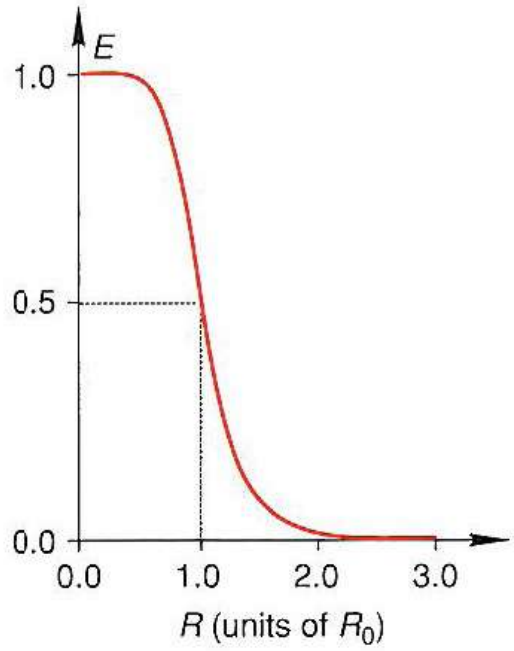
(iii) The transition dipole moments of the donor and acceptor must be approximately parallel to each other.

(iv) The fluorescence lifetime of the donor molecule must be of sufficient duration to allow the FRET to occur.

Fluorescence Resonance Energy Transfer (FRET)

Förster showed that the efficiency of the FRET process (E_{FRET}) *depends on the inverse* sixth power of the distance between the donor and acceptor pair (r) and is given by:

$$E_{FRET} = R_0^6 / (R_0^6 + r^6)$$



where R_0 *is the Förster radius at which half of the excitation energy of donor is transferred to* the acceptor chromophore. Therefore Förster radius (R_0) *is referred to as the distance at which the* efficiency of energy transfer is 50% and is about 2-6 nm

Fluorescence Resonance Energy Transfer (FRET)

- The Förster radius (R_0) *depends on the fluorescence quantum yield of the donor in the* absence of acceptor (f_d)*, the refractive index of the solution* (η) *, the dipole angular orientation of* each molecule (K^2) *and the spectral overlap integral of the donor-acceptor pair (J) and is given by*

$$R_0 = 9.78 \times 10^3 (\eta^{-4} . f_d . J)^{1/6} A^0$$

Fluorescence Resonance Energy Transfer (FRET)

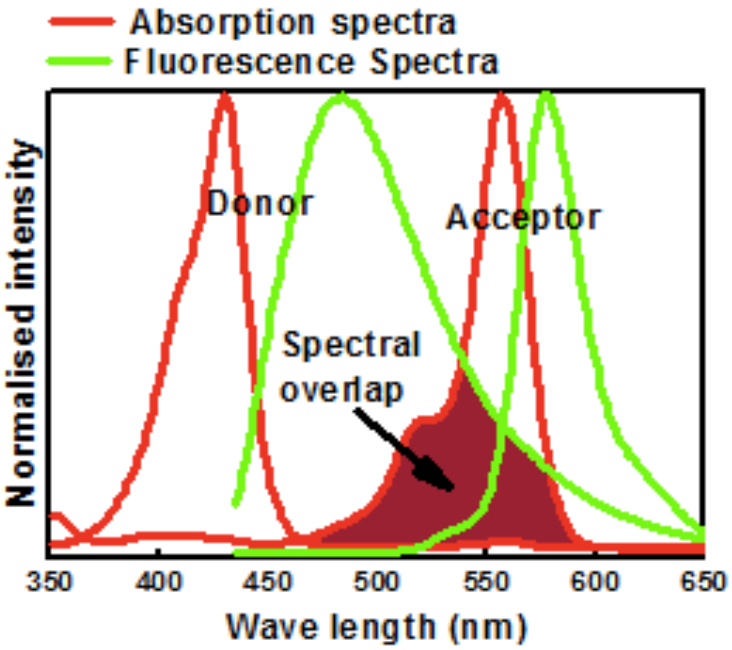
- In summary, the rate of FRET depends upon the extent of spectral overlap between the donor acceptor pair, the quantum yield of the donor, the relative orientation of the donor-acceptor transition dipole moments and the distance separating the donor-acceptor chromophore.

  Any event or process that affects the distance between the donor-acceptor pair will affect the FRET rate, consequently allowing the phenomenon to be quantified, provided that the artifacts can be controlled or eliminated. As a result, FRET is often referred to as a `**spectroscopic/molecular ruler**´, for example to measure the distance between two active sites on a protein that have been labelled with suitable donor-acceptor chromophore, and therefore monitoring the conformational changes through the amount of FRET between the fluorophores.

Fluorescence Resonance Energy Transfer (FRET)

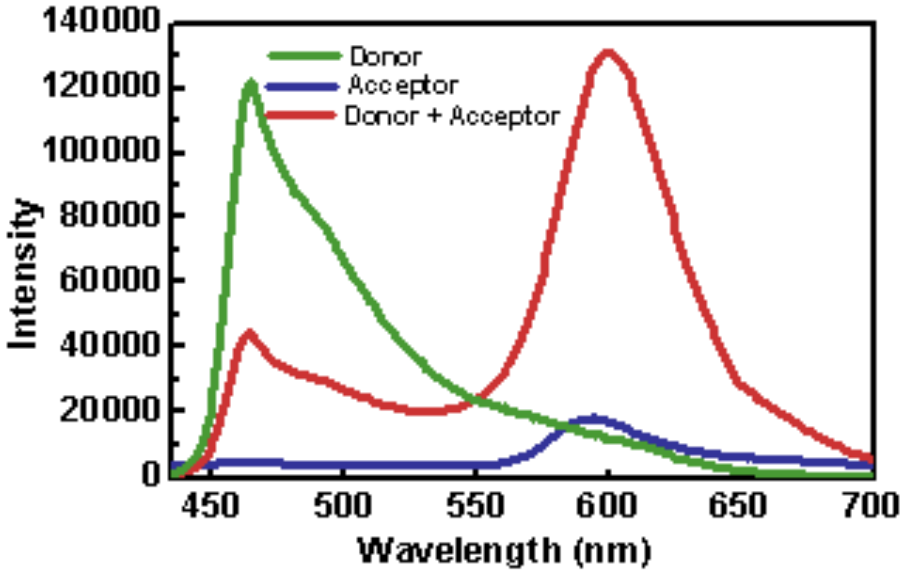
The detection and quantitation of FRET can be made in a number of different ways. Simply the phenomenon can be observed by exciting a specimen containing both the donor and acceptor molecules with light emitted at wavelengths centered near the emission maximum of the acceptor.

Because FRET can result in both a decrease in fluorescence of the donor molecule as well as an increase in fluorescence of the acceptor, a ratio metric determination of the two signals can be made. The advantage of this method is that a measure of interaction can be made that is independent of the absolute concentration of the sensor.



Fluorescence Resonance Energy Transfer (FRET)

- Because not all acceptor moieties are fluorescent, they can be used as a means to quench fluorescence. In these instances, those interactions that result in a fluorescent donor molecule coming in close proximity to such a molecule would result in a loss of signal. Inversely, reactions that remove the proximity of a fluorescent donor and a quencher would result in an increase in fluorescence. Figure 3 illustrates the detection of FRET by observing the fluorescence spectra of the donor-acceptor pair.



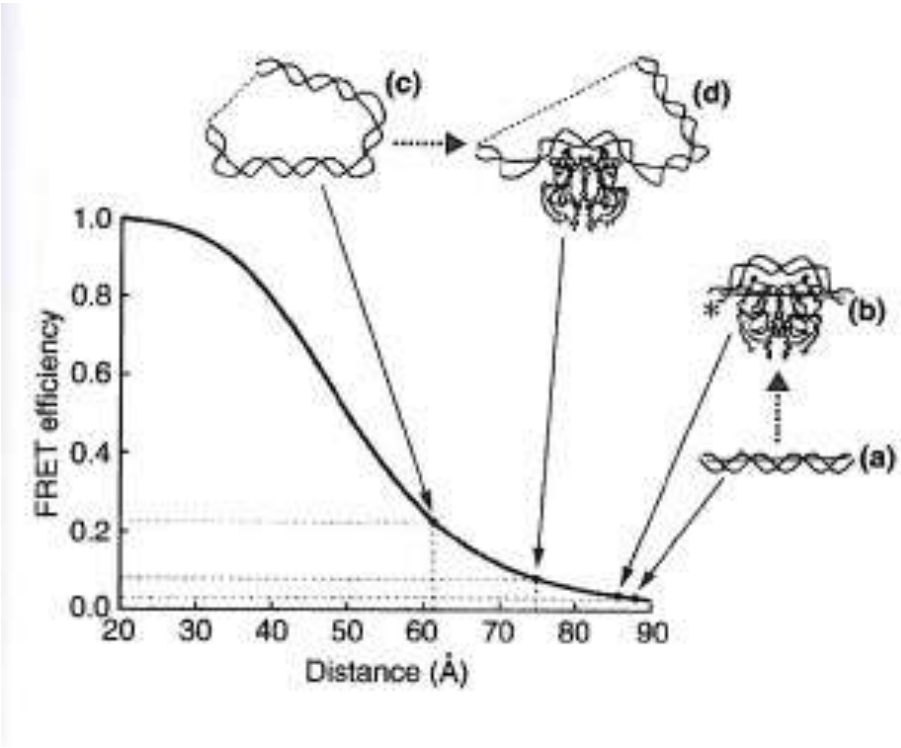
Another alternative method, is to measure the fluorescence lifetime of the donor fluorophore in the presence and absence of the acceptor chromophore. FRET will cause a decrease in excited lifetime of the donor fluorophore.

Fluorescence Resonance Energy Transfer (FRET)

- The strong distance-dependence of the FRET efficiency has been widely utilized in studying the structure and dynamics of proteins and nucleic acids, in the detection and visualization of intermolecular association and in the development of intermolecular binding assays.
- FRET is a particularly useful tool in molecular biology as the fraction, or efficiency, of energy that is transferred can be measured, and depends on the distance between the two fluorophores. The distance over which energy can be transferred is dependent on the spectral characteristics of the fluorophores, but is generally in the range 10–100A°.

Fluorescence Resonance Energy Transfer (FRET)

- Thus, if fluorophores can be attached to known sites within molecules, measurement of the efficiency of energy transfer provides an ideal probe of inter- or intramolecular distances over macromolecular length scales. Indeed, fluorophores used for this purpose are often called ‘‘probes’’.



Fluorescence (Förster) Resonance Energy Transfer (FRET)

- Techniques for measuring FRET are becoming more sophisticated and accurate, making them suitable for a range of applications. FRET has been used for measuring:
- the structure
- conformational changes
- interactions between molecules
- as a powerful indicator of biochemical events.

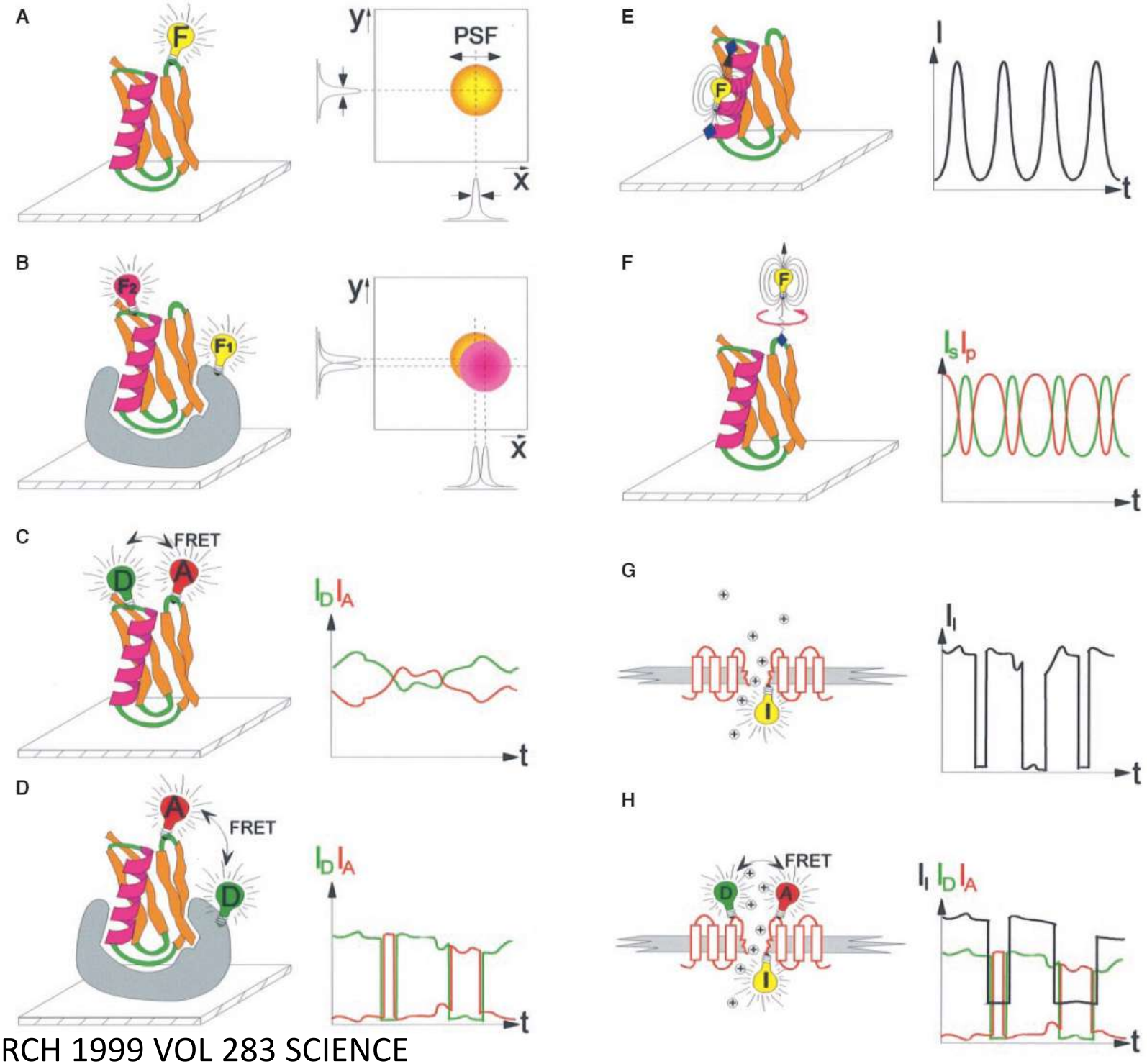

A
F
y
PSF
x
B
F2
F1
y
x
C
FRET
D
A
IDIA
t
D
A
FRET
D
IDIA
t
E
F
I
t
F
F
IsIp
t
G
I
II
t
H
FRET
D
A
I
II IDIA
t

Fig. 1. Labeling schemes (left) and physical observables (right). (**A**) Localization of a macromolecule labeled with a single fluorophore F with nanometer accuracy. The point-spread-function (PSF) can be localized within a few tenths of a nanometer. (**B**) Colocalization of two macromolecules labeled with two noninteracting fluorophores, F_1 and F_2. Their distance can be measured by subtracting the center positions of the two PSFs. (**C**) Intramolecular detection of conformational changes by spFRET. D and A are donor and acceptor; I_D and I_A are donor and acceptor emission intensities; t is time. (**D**) Dynamic colocalization and detection of association or dissociation by intermolecular spFRET. Donor and acceptor intensities are anticorrelated both in (C) and (D). (**E**) The orientation of a single immobilized dipole can be determined by modulating the excitation polarization. The fluorescence emission follows the angle modulation. (**F**) The orientational freedom of motion of a tethered fluorophore can be measured by modulating the excitation polarization and analyzing the emission at orthogonal **s** and **p** polarization detectors. I_S and I_p are emission intensities of **s** and **p** detectors. (**G**) Ion channel labeled with a fluorescence indicator I. Fluctuations in its intensity I_I report on local ion concentration changes. (**H**) Combination of (C) and (G). D and A report on conformational changes whereas I reports on ion flux.

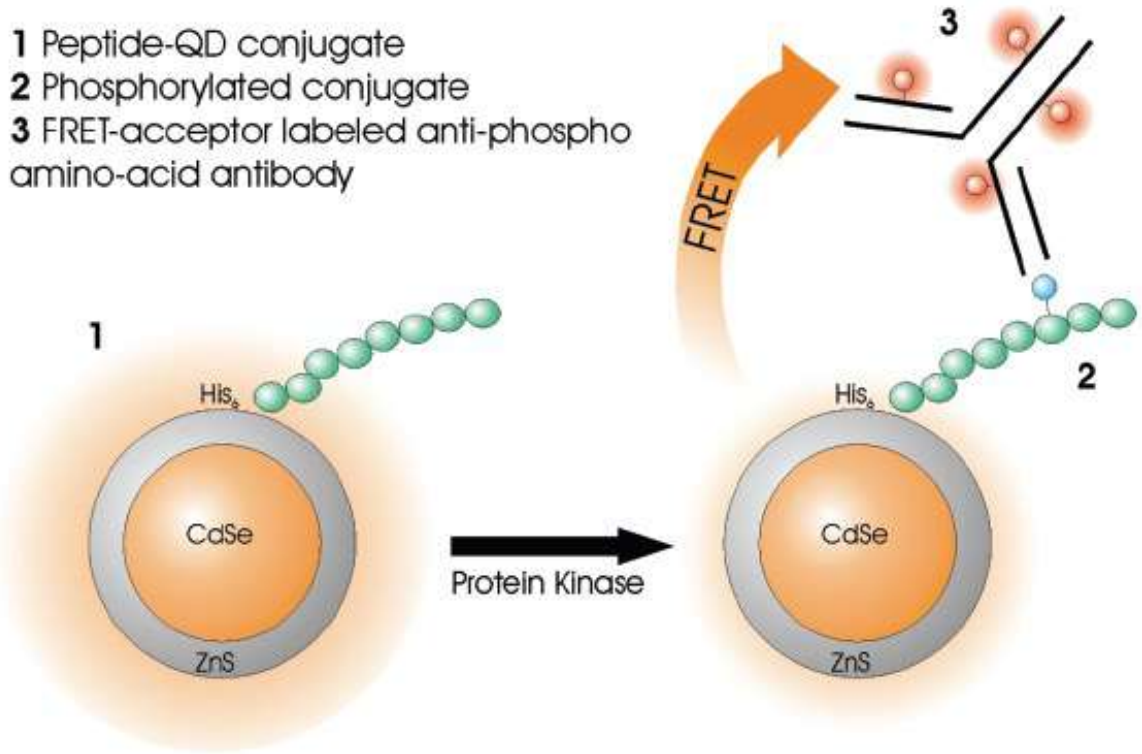


Figure 1. Schematic representation of kinase-mediated phosphorylation of peptide–QD conjugates, antibody recognition of phosphopeptide, and FRET detection.

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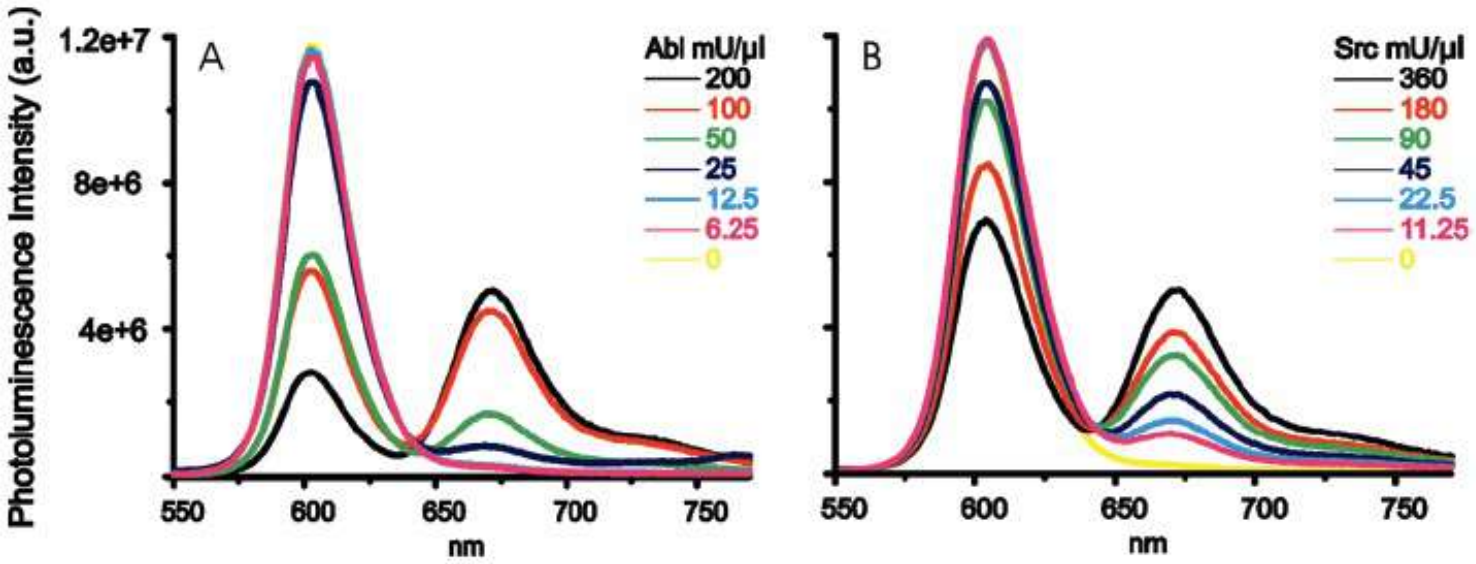

Figure 3. Steady-state emission spectra (λ_{ex} = 400 nm) of Abl (A) and Src (B) kinase reactions using peptide−QD conjugate substrates following 1 h enzyme reactions and addition of FRET-acceptor labeled antibody.

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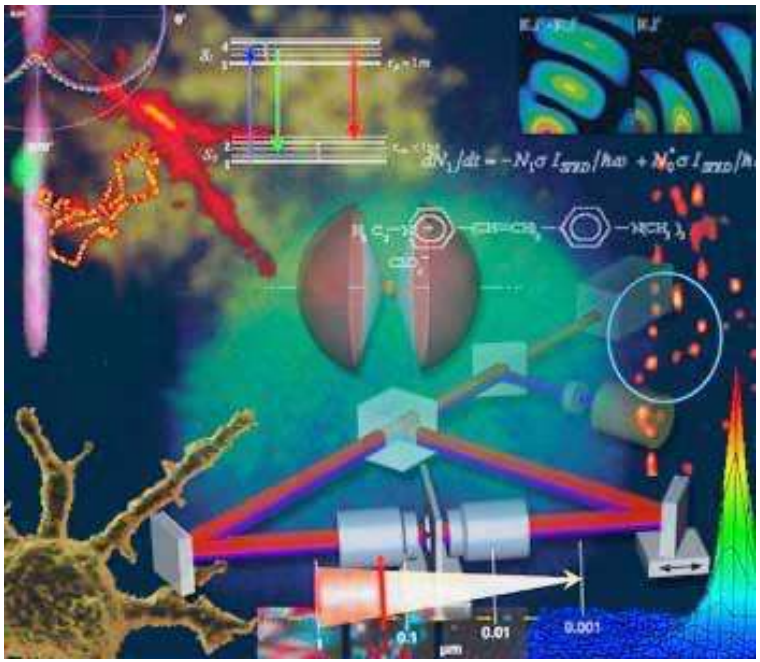
Max Planck Society

Fluorescence Nanoscopy

4Pi / STED / RESOLFT



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19

Far-Field Optical Nanoscopy

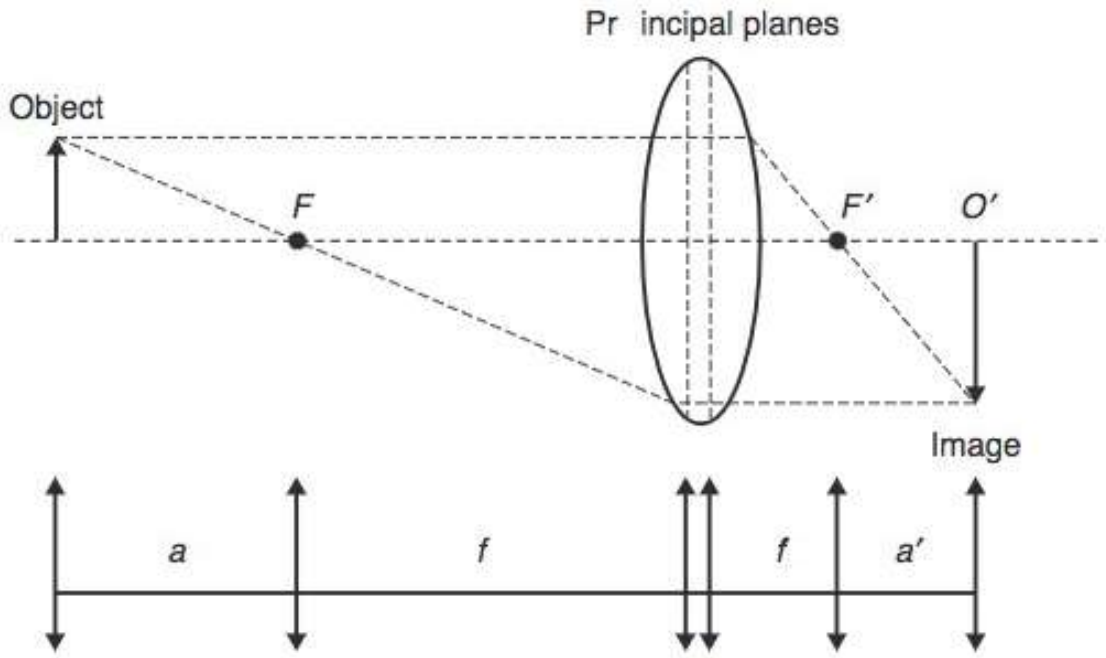
Stefan W. Hell

By providing a spatial resolution down to the atomic scale, electron and scanning probe microscopy have revolutionized our understanding of life and matter. Nonetheless, optical microscopy has maintained its key role in many fields, in particular in the life sciences. This stems from a number of rather exclusive advantages, such as the noninvasive access to the interior of (living) cells and the specific and highly sensitive detection of cellular constituents through fluorescence tagging. As a matter of fact, lens-based fluorescence microscopy would be almost ideal for investigating the three-dimensional (3D) cellular interior if it could resolve details far below the wavelength of light. However, until not very long ago, obtaining a spatial resolution on the nanometer scale with an optical microscope that uses lenses and focused visible light was considered unfeasible [1, 2].

S. Hell, “Far field optical nanoscopy” Springer

PRINCIPLES OF OPTICAL MICROSCOPY

One lens microscope



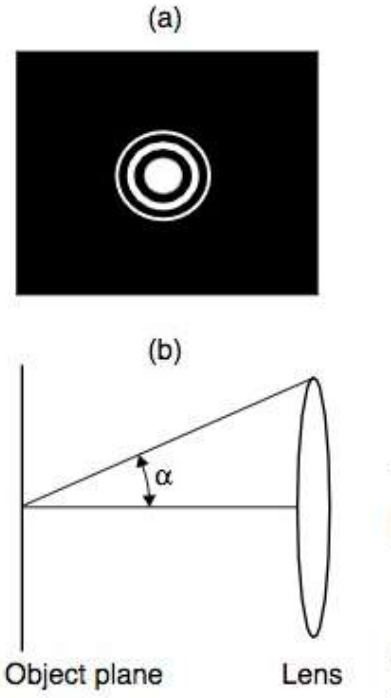
From geometrical optics:

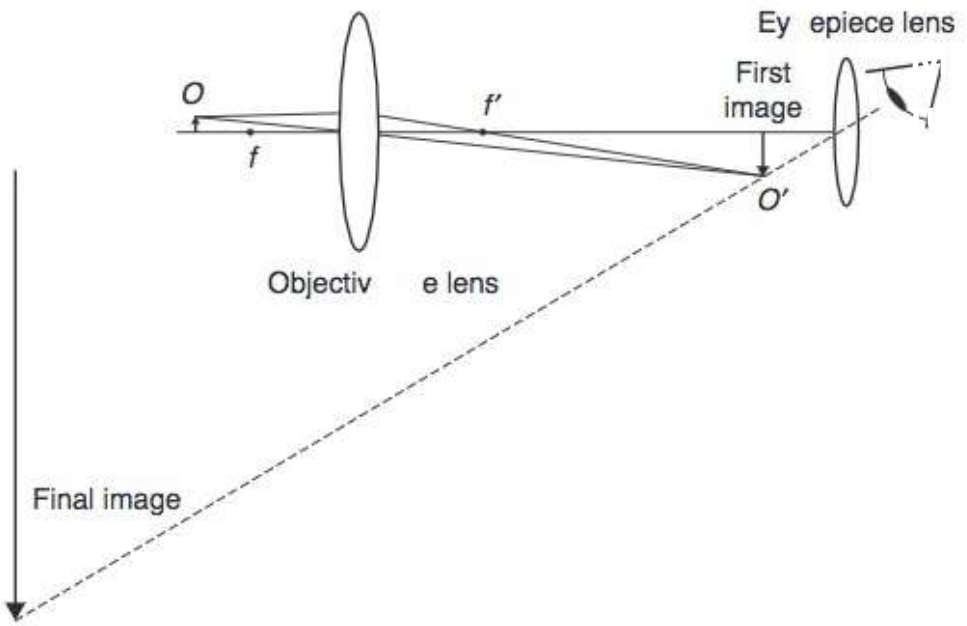
aa’ = ff’
magnification = -f/a

Requires a lens with very short focal length and observer eye very close to the lens!

Compound lens microscope

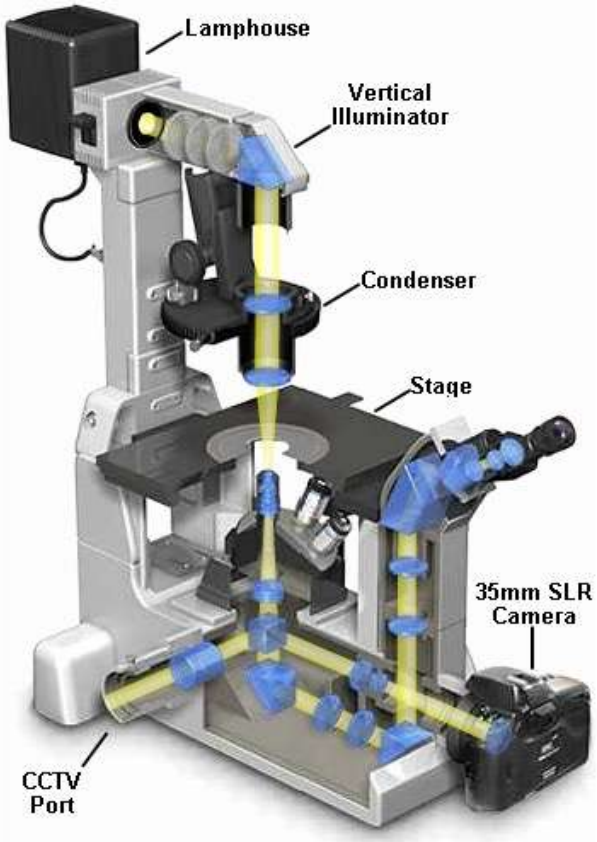
Requires two lenses: an objective plus an eyepiece

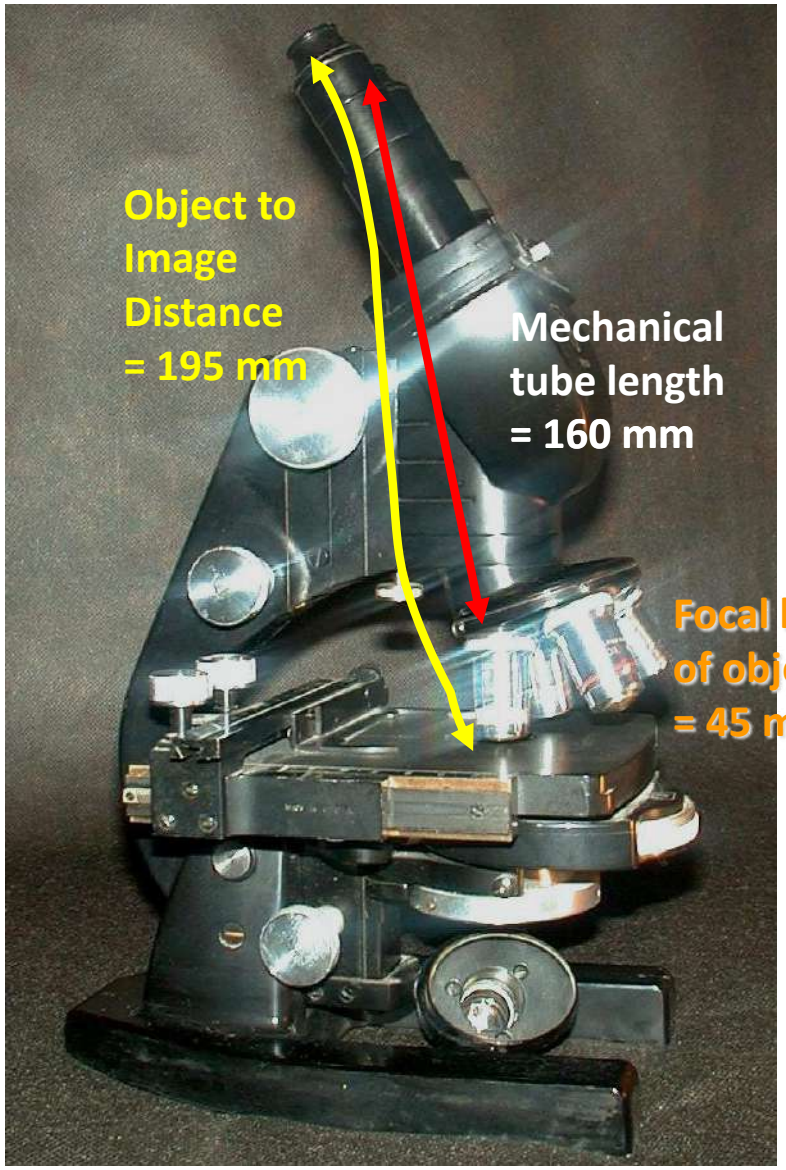




The object is positioned beyond the objective. The object-magnified image is further magnified by the eyepiece and focused onto the retina of the eye

Compound lens microscope

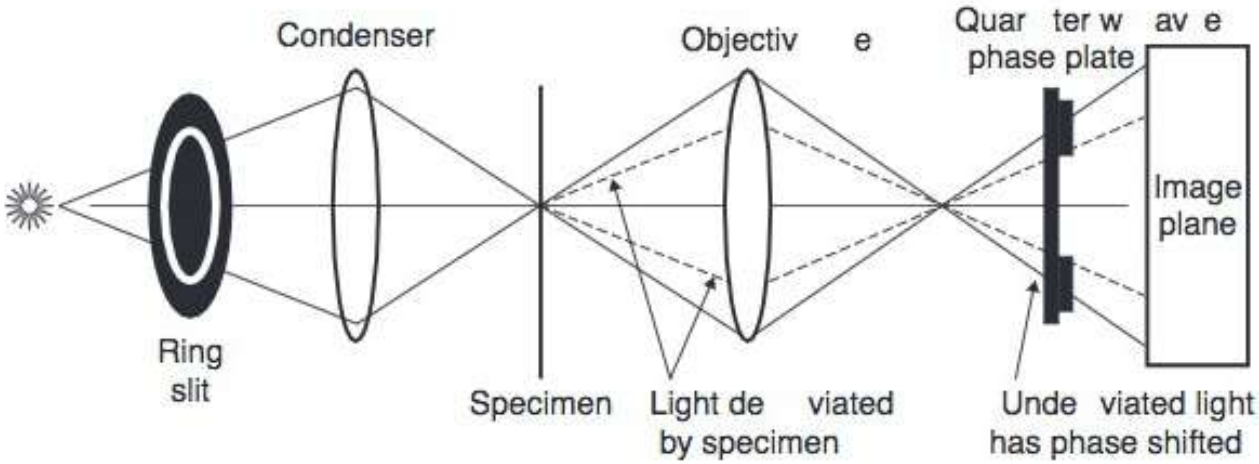




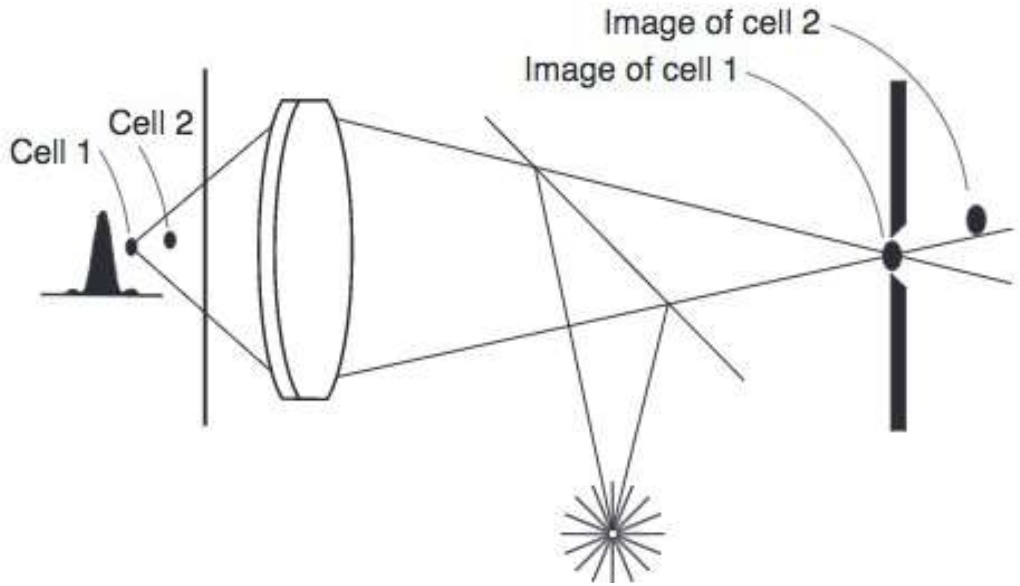
Key ideas in optical microscopy

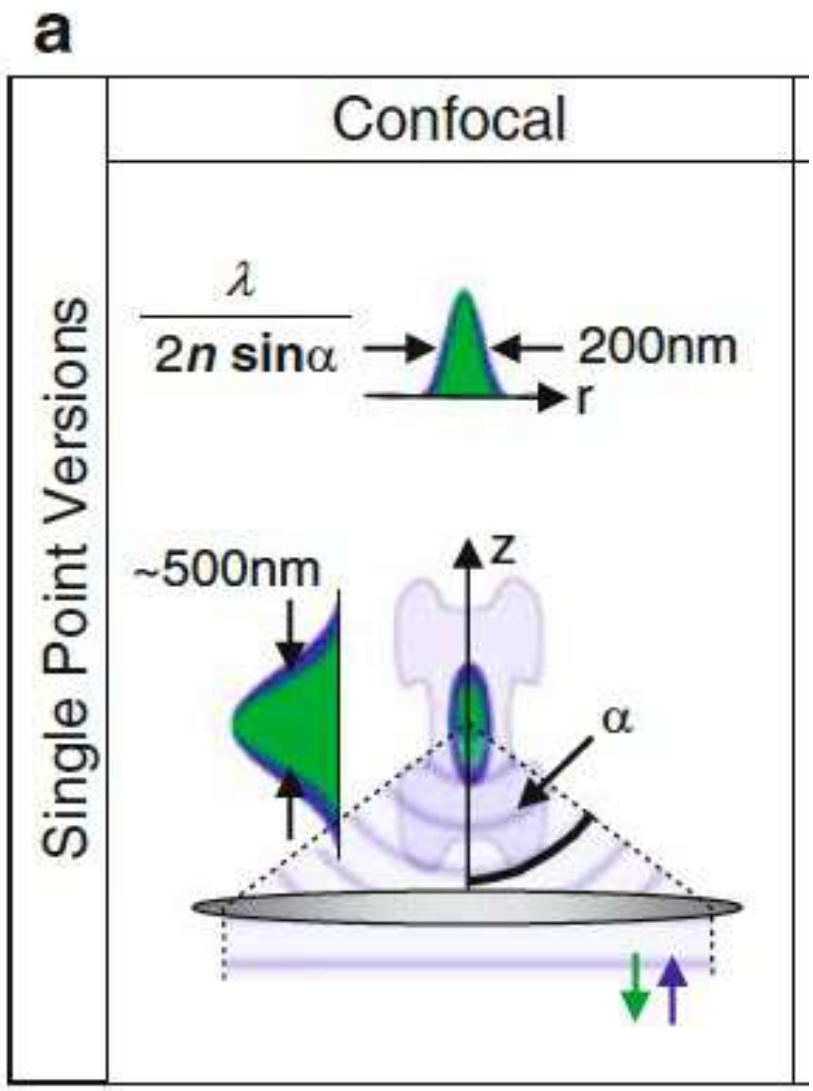
- The light microscope is an instrument for producing enlarged images of objects that are too small to be seen unaided; such images may be viewed directly with a viewing screen or photographic apparatus or special electronic device.
- Because biological macromolecules are generally transparent to light numerous contrast-enhancing techniques have been developed including dark-field, phase-contrast, polarization and interference microscopy.
- The diffraction limit of resolution power of a microscope is about half of the wavelength of the illuminating light; an increase of the resolving power of the microscope is possible only by increasing the numerical aperture of the optical system or by using a shorter wavelength.
- In contrast to the ordinary light microscope, the confocal microscope employs a point-like illumination and detection arrangement; by restricting the observed volume, the technique keeps nearby scatterers from contributing to the detected signal.
- The NSOM technique of imaging is based on the use of an 'optical stethoscope', which places a small near-field light source a very short distance from the sample (less than the wavelength of light).
- Images in NSOM are obtained by scanning the light source over the sample, and the resolution is limited only by the diameter of the light source.
- NSOM links the world of conventional optical microscopy with scanned probe imaging techniques, the most common of which is atomic force microscopy.

Phase contrast microscopy

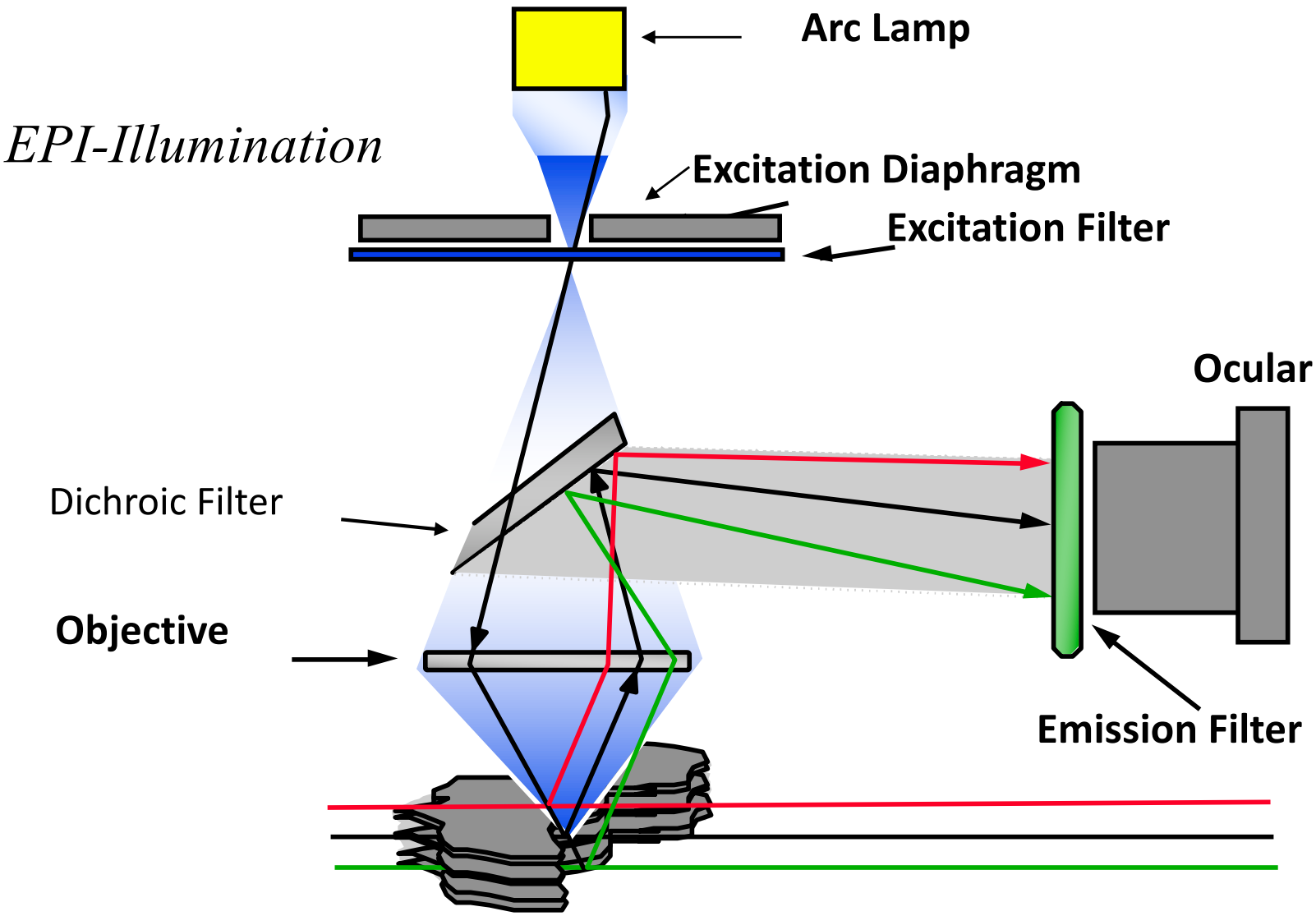


Confocal microscopy




a
Confocal
Single Point Versions
λ
2n sinα
200nm
r
~500nm
z
α

Fluorescent Microscope



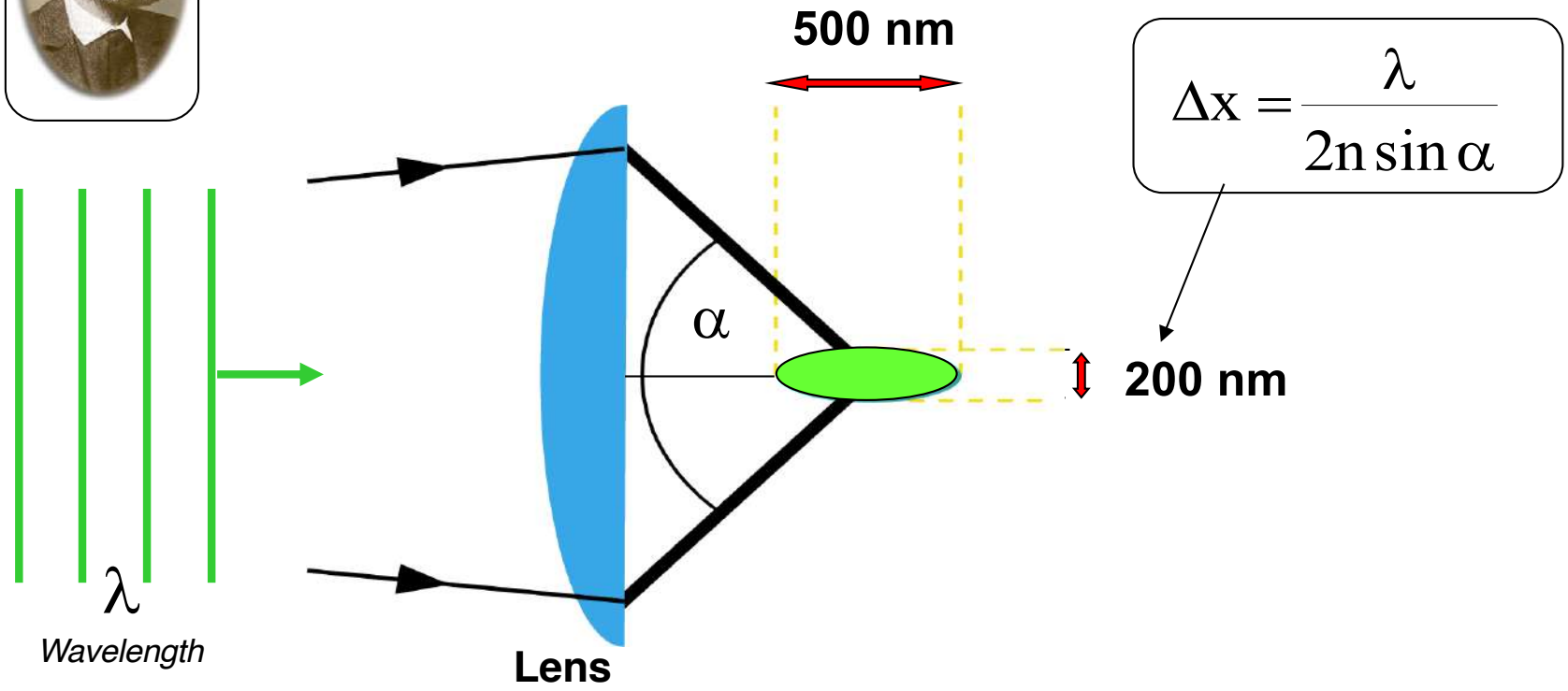
Fluorescence Microscope

upright

inverted

Diffraction limit

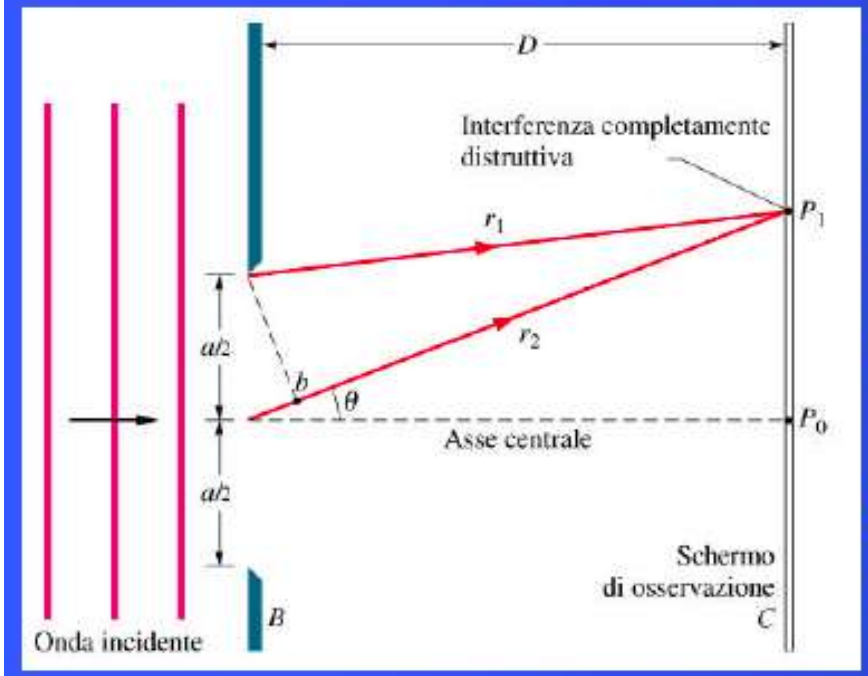
Focusing a propagating light wave means causing it to interfere constructively at a certain point in space, called the geometrical focal point (0,0,0). Due to diffraction a focal intensity pattern $I(x, y, z)$ emerges around (0,0,0), which is also referred to as the intensity point-spread-function (PSF) of the lens. $I(x, y, z)$ features a central maximum called the focal spot (Fig. 19.1a) whose full-width-half-maximum (FWHM) is $\Delta r \approx \lambda/(2n \sin \alpha)$ in the focal plane and $\Delta z \approx \lambda/(n \sin^2 \alpha)$ along the optic axis [3]. λ is the wavelength of light, α denotes the semi-aperture angle of the lens, and n is the refractive index of the object medium (Fig. 19.1a). Discerning similar objects lying within this spot is usually precluded because they are illuminated in parallel and hence give off (fluorescence) photons in parallel. Likewise, the propagation of the emitted (fluorescence) light that is collected by a lens and focused to an image plane is governed by a similar function $I_{\mathrm{em}}(x, y, z)$, describing the blur of the coordinate from where the photons originated.



E. Abbe (1873), *Arch. Mikroskop. Anat.* 9, 413.

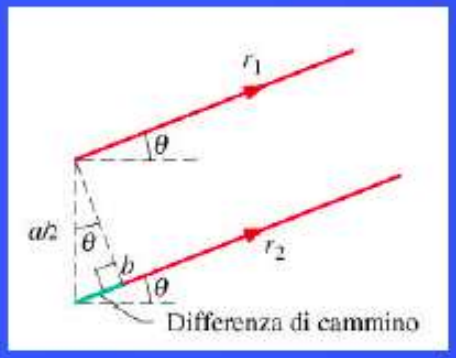
Diffrazione da singola fenditura

Consideriamo un'onda piana di **lunghezza d'onda λ** che viene difratta da una sottile **fenditura di lunghezza a**.



Per individuare la posizione dei massimi e dei minimi nella figura di diffrazione, consideriamo la fenditura a suddivisa in tanti punti, ognuno dei quali sarà sorgente di onde sferiche secondarie, e calcoliamo la **differenza di cammino ottico tra due raggi originati da punti a distanza a/2** l'uno dall'altro. Innanzitutto calcoliamo la **posizione della prima frangia scura**

Le onde originate nella fenditura sono in fase ed interferiscono distruttivamente in P_1, quindi in P_1 arrivano con uno sfasamento di $\lambda/2$. Facciamo anche l'ipotesi che **D >> a**.



$$\Delta L = \frac{a}{2}\sin\theta$$

Vale per ogni coppia di raggi che arriva in P_1

Per avere interferenza distruttiva deve essere

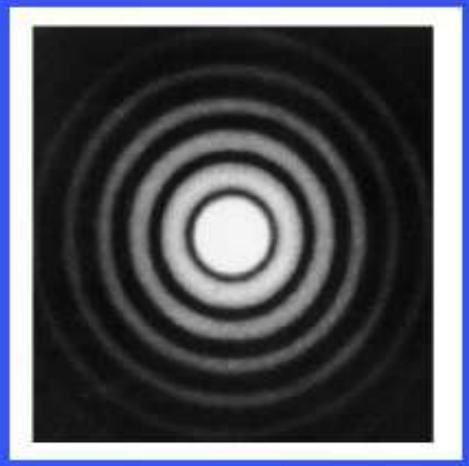
$$\frac{a}{2}\sin\theta = \frac{\lambda}{2} \Rightarrow a\sin\theta = \lambda$$

primo minimo

Se ora **diminuiamo a**, l'effetto di **diffrazione aumenta**, ovvero aumenta l'angolo **θ** a cui si trova il primo minimo, se **a = λ**, allora **$\theta_1 = 90^o$** e il **massimo centrale copre tutto lo schermo**.

Per trovare i **minimi successivi** si procede in modo analogo, ma questa volta si divide la **fenditura in quattro parti** ciascuna di ampiezza **a/4**.

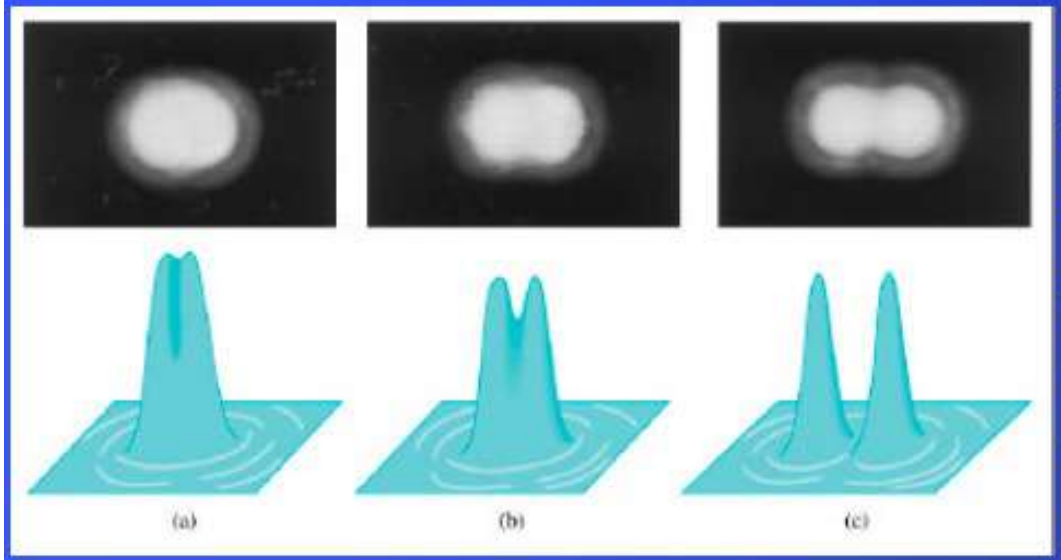
Diffrazione attraverso un foro circolare



Prendiamo ora un foro circolare di diametro d, la figura di diffrazione che si ottiene è formata da cerchi lumiosi e scuri alternati. Per la posizione del primo minimo si trova

$$\sin\theta = 1{,}22\frac{\lambda}{d}$$

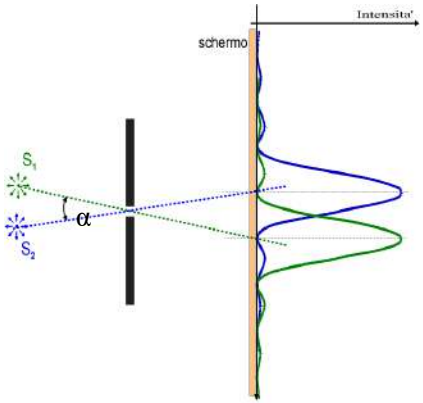
Potere risolvente

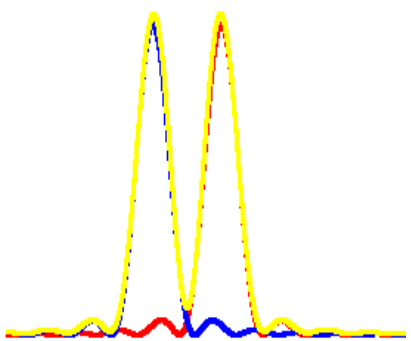


Criterio di Rayleigh

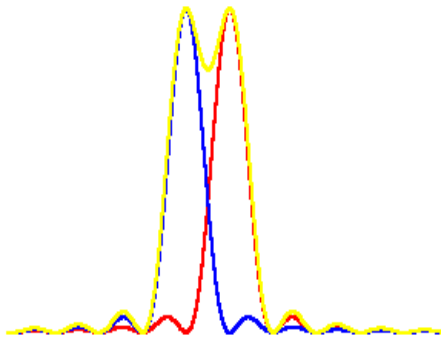
Due sorgenti luminose puntiformi sono risolubili se la loro distanza angolare è tale che il max. centrale della figura di diffrazione di una coincide con il primo minimo della figura di diffrazione dell'altra

Risoluzione: criterio di Reyleigh

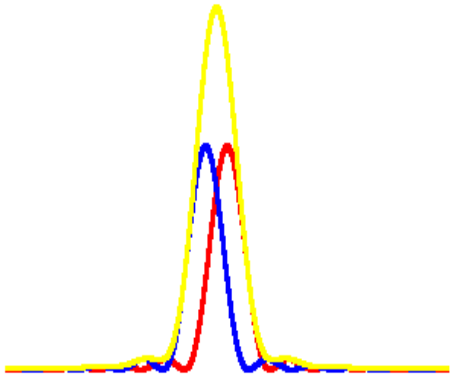




$\alpha = 2\theta_1$



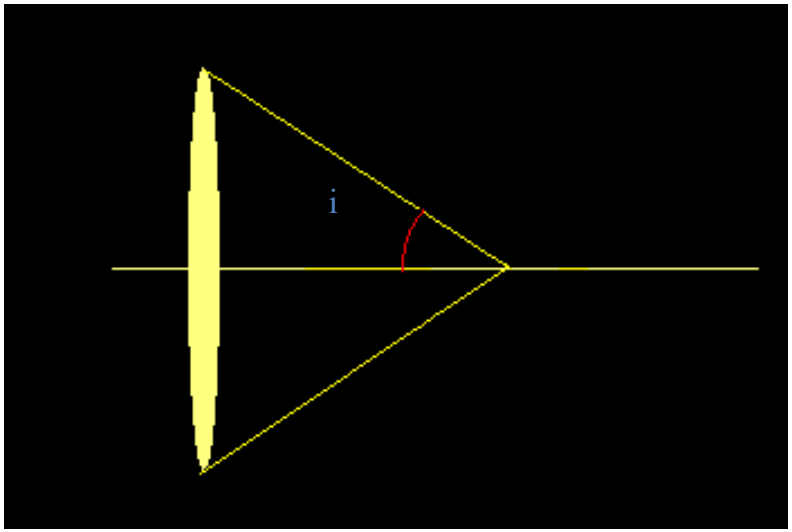
$\alpha = \theta_1$



$\alpha = \theta_1/2$

$$\alpha \geq \theta_1 = 1.22 \frac{\lambda}{D}$$

telescopio

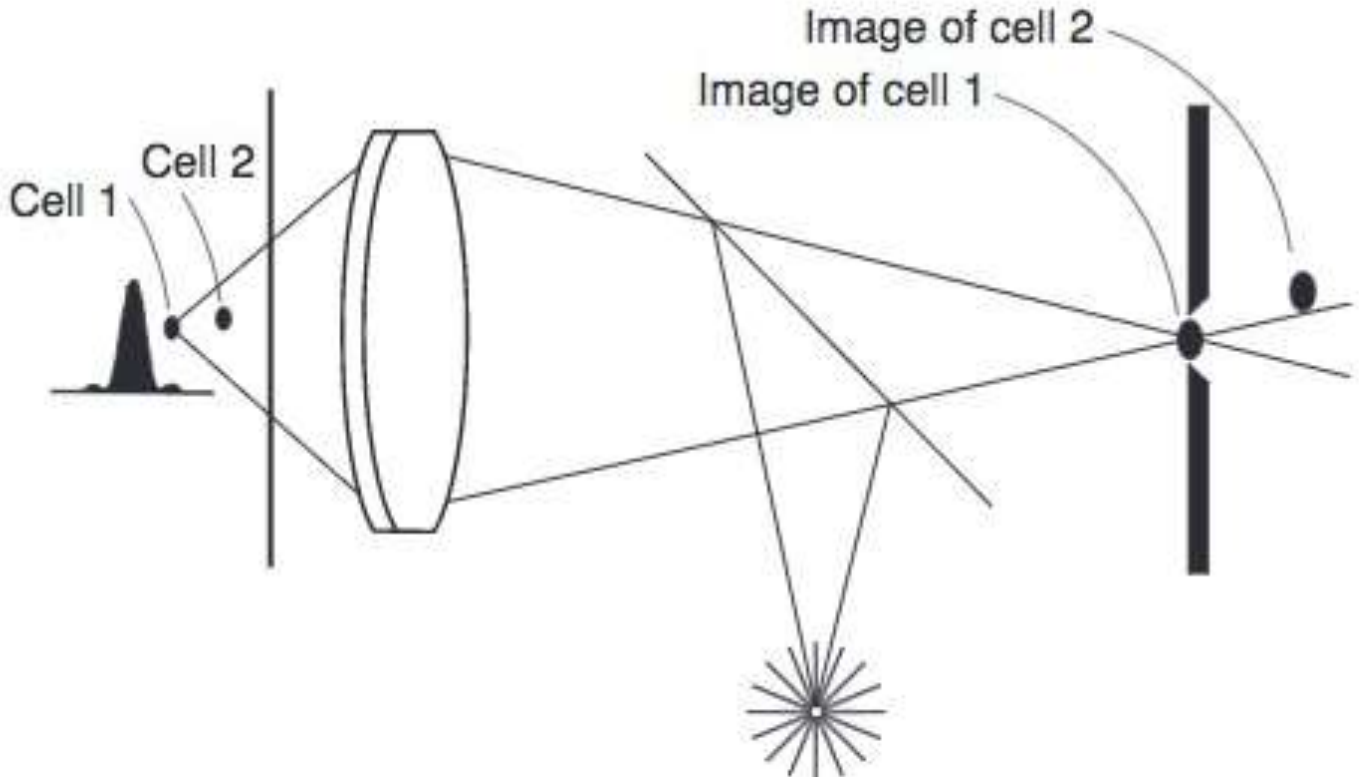


$$s \geq \frac{\lambda}{2n \cdot \mathrm{sen}(i)}$$

microscopio

Apertura numerica

Confocal optical microscopy

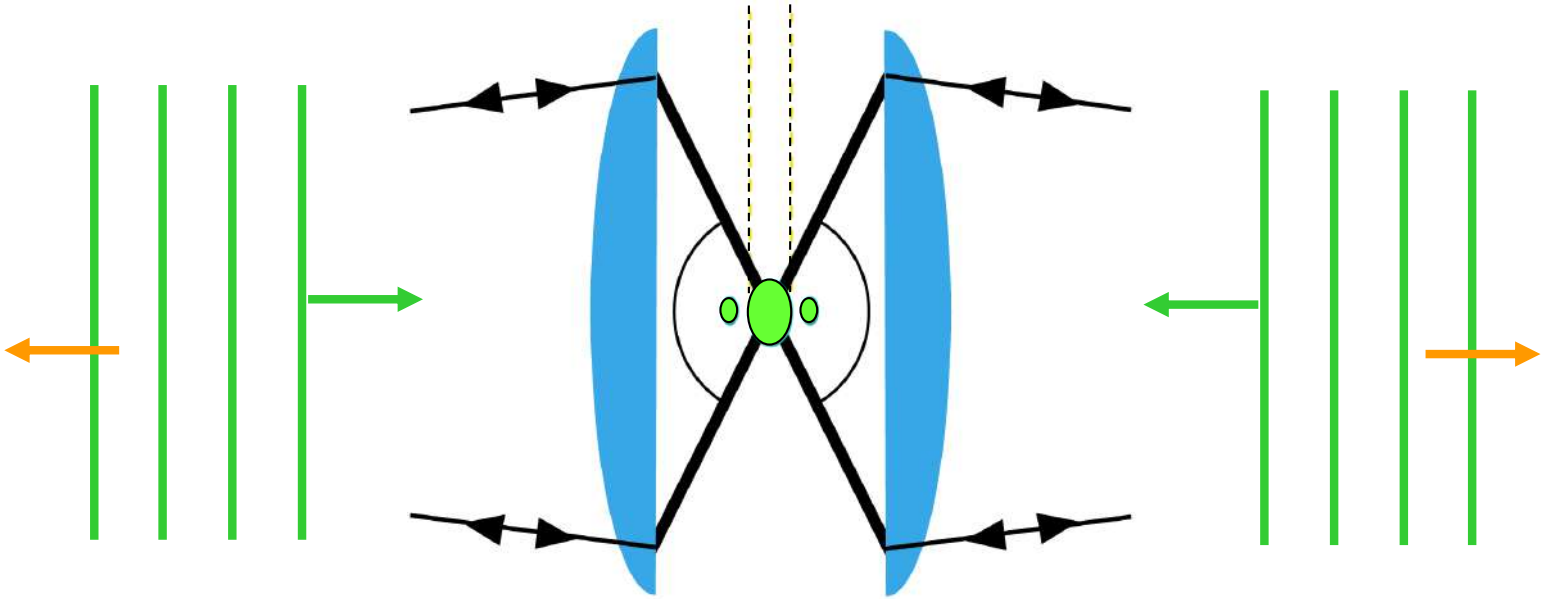


Max Planck Society

4Pi- Microscopy: resolution improvement in **z**

4Pi- Microscopy:

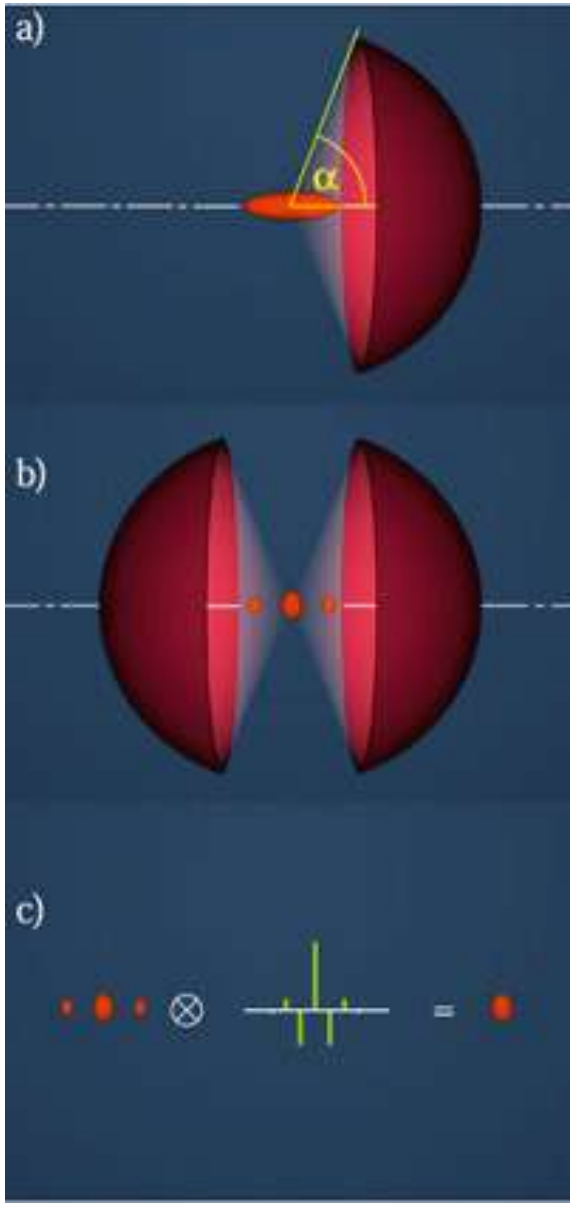
70 - 140 nm



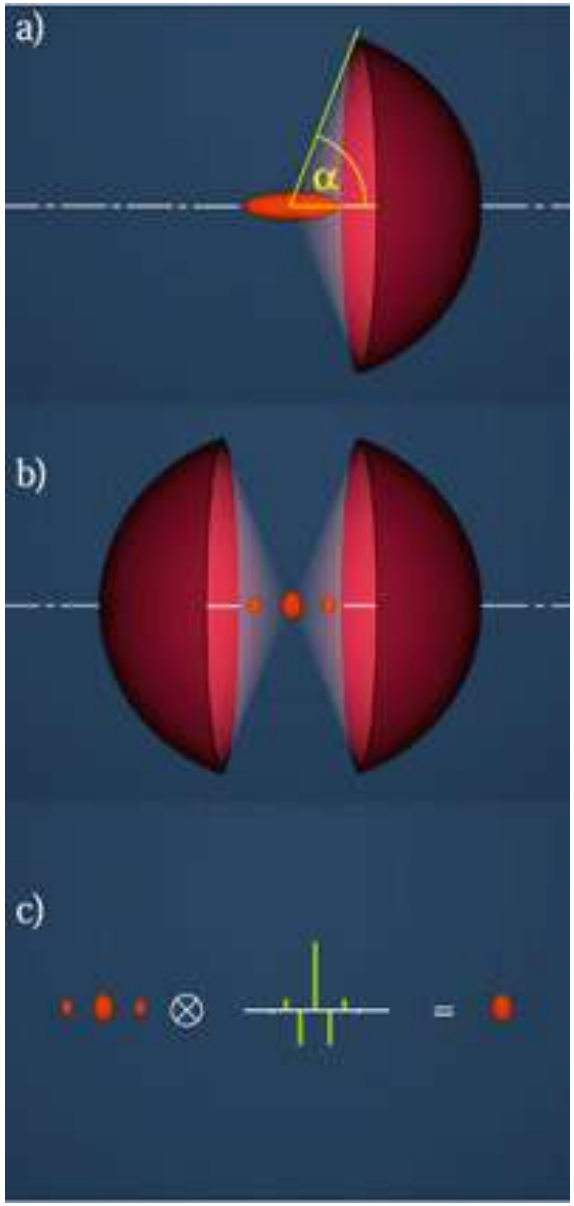
$$\vec{E}^{4Pi}(r,z,\varphi) = \vec{E}_1(r,z,\varphi) + \vec{E}_2(r,-z,\varphi)$$

Coherent illumination and/or fluorescence detection

S.W. Hell (1990), *Europ. Patent* OS 0491289.
S.W. Hell, et al. (1992), *Opt. Commun.* **93**, 277.
M. Schrader, et al. (1998), *Biophys. J.* **75**, 1659.
H. Gugel, et al. (2004), *Biophys. J.* **87**, 4146.

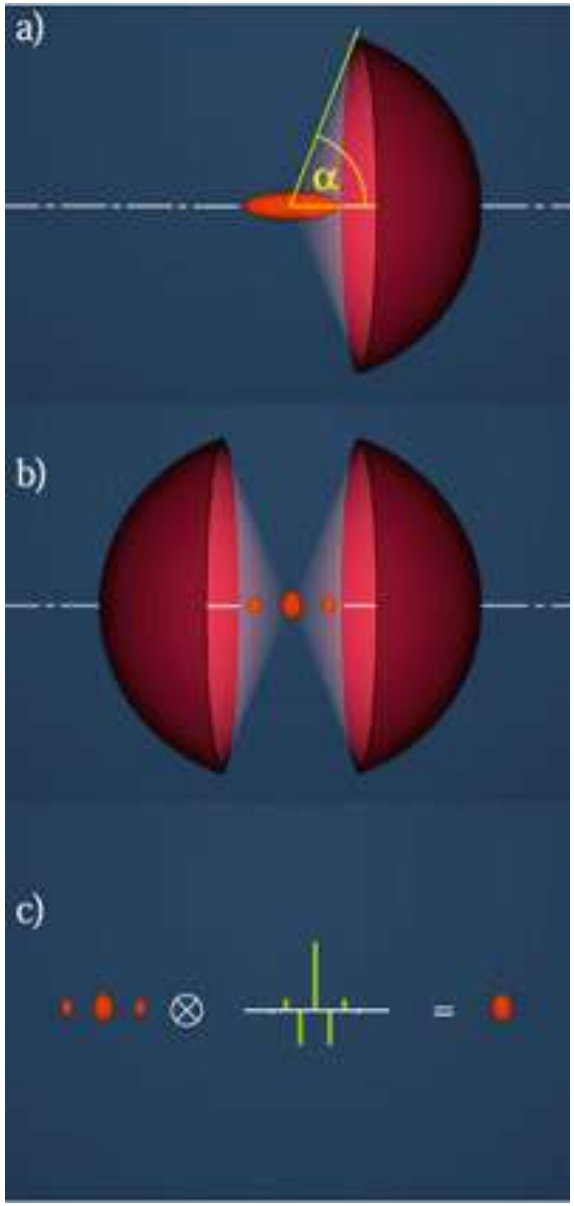


Ernst Abbe discovered that the focal spot size decreases with the microscope's aperture angle i.e. with the size of the spherical wavefront that is produced by the objective lens. But a regular objective lens, even of the largest aperture, produces just a segment of a spherical wavefront coming from a single direction. As a result the focal spot is longer (z) than wide (x,y) [Fig. 1a]. By contrast, a full spherical wavefront of a solid angle of 4π would lead to a spherical spot and hence to an improvement of spatial resolution in the z-direction.



The idea: Since there are no lenses or mirrors that could provide such a wavefront across a significantly large field of view, the idea behind our 4Pi-microscope is to mimic the 'close to ideal' situation by **using two opposing objective lenses coherently**, so that the two wavefronts add up and join forces.

Allowing the illumination wavefronts to constructively interfere in the sample produces a main focal spot that is sharper in the z-direction by about 3-4 times (4Pi of type A). A similar improvement is obtained if the lenses add their collected fluorescence wavefronts in a common point detector (4Pi of type B). Doing both together is best, of course, and leads to a 5-7-fold improvement of resolution along z (4Pi of type C).



The sidelobe challenge: If the two segments were full spherical halves, the focal spot would be a (nearly) spherical spot, too. But since a considerable solid angle is not provided by the lenses, interference typically spawns off 2 axial side-lobes which, if not taken into account, lead to artefactual images. We deal with this challenge by an appropriate mathematical filter. This filter does not require any information about the object, apart from the height and location of the lobes. Linear filtering is possible if the lobes are significantly less than 50% of the main sharp maximum. This can be reliably fulfilled if multiphoton excitation of the dye is applied. Linear mathematical filtering is fast and a single effective spot is readily achieved.

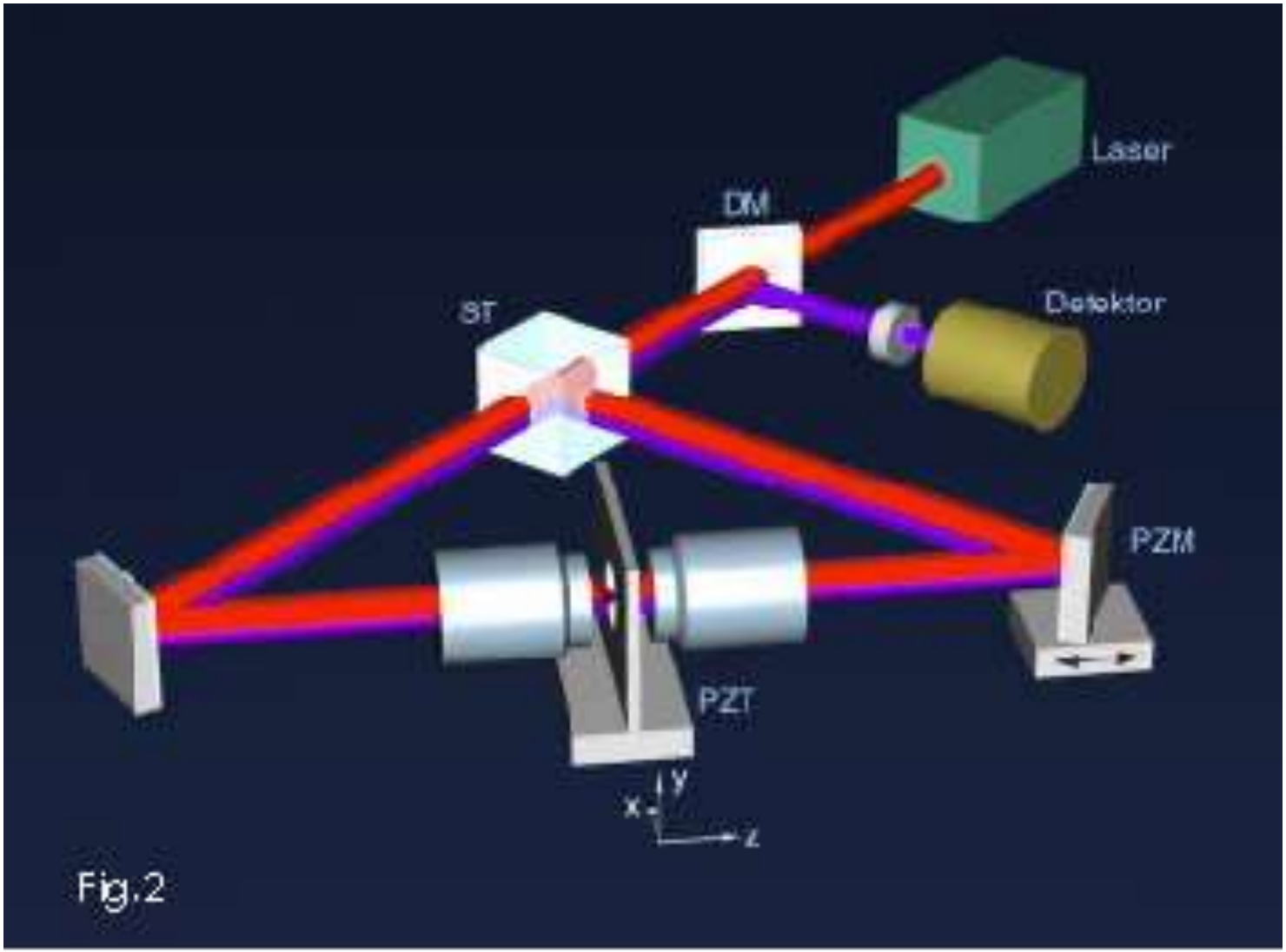
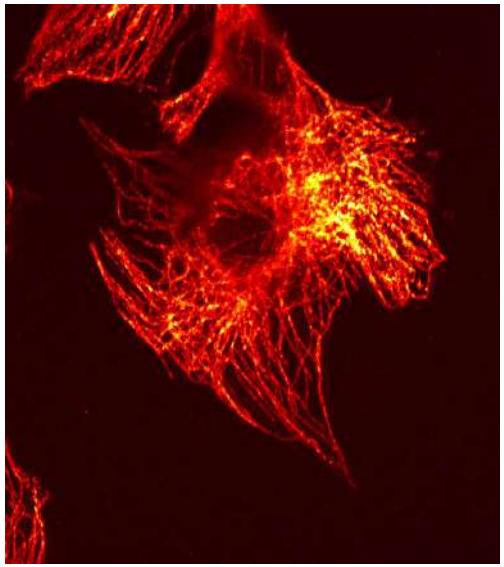
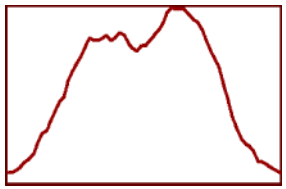


Fig.2



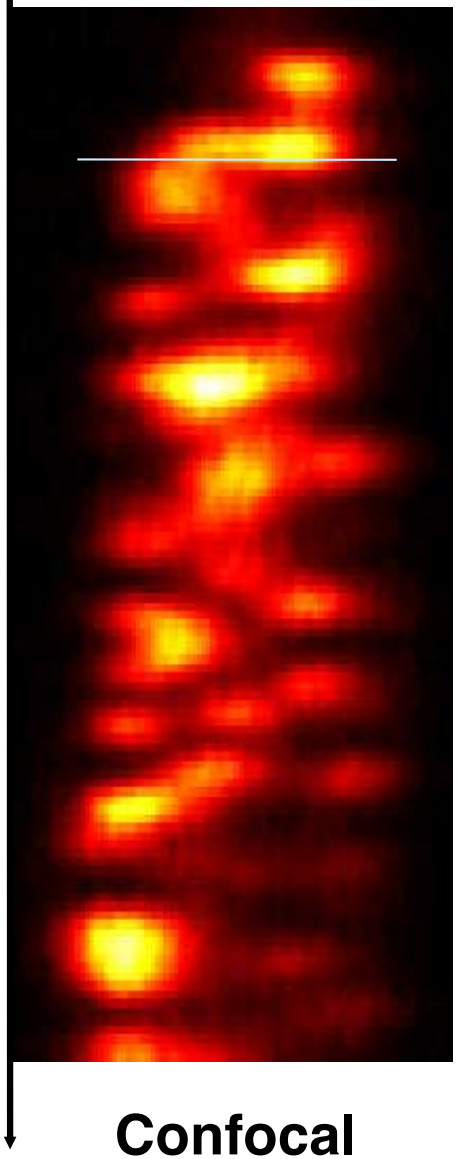
Microtubules, mouse fibroblast
Immunofluor, Oregon Green



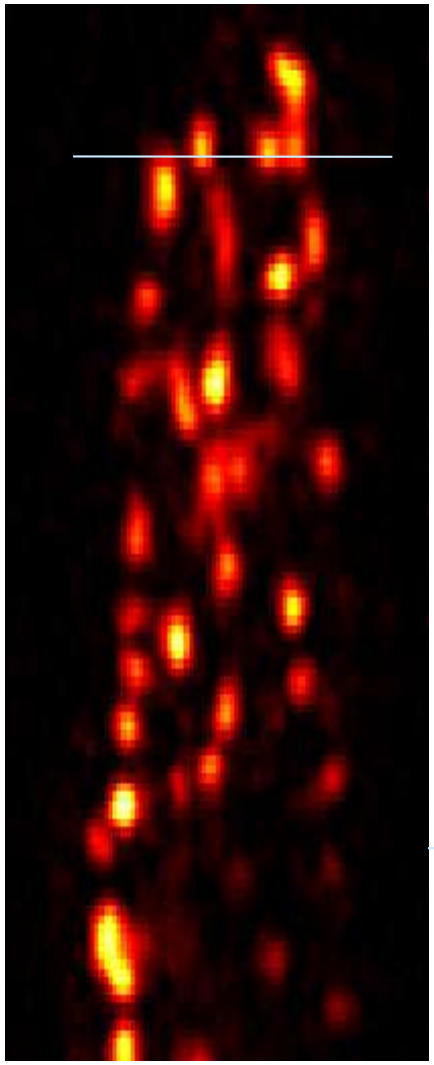
2 µm

Z

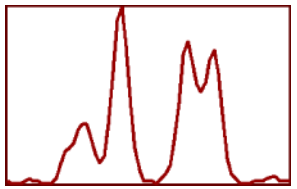
X



Confocal



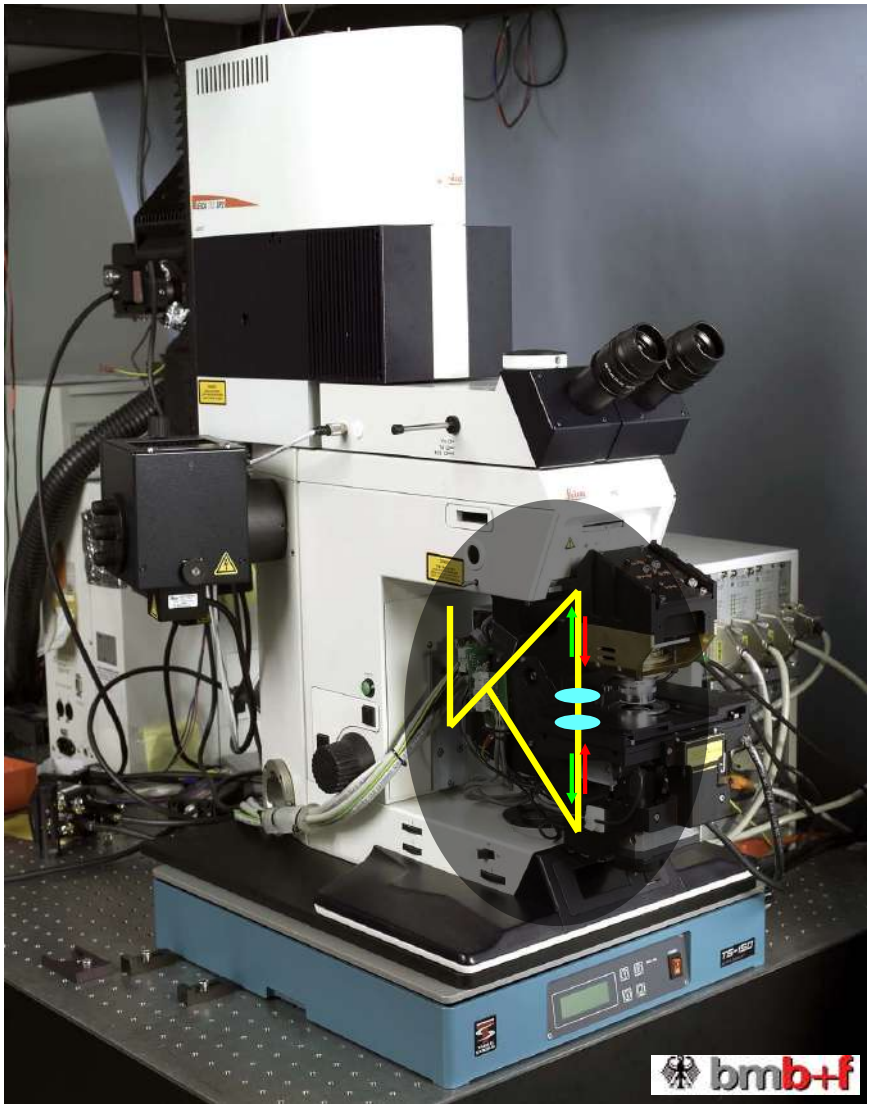
4Pi

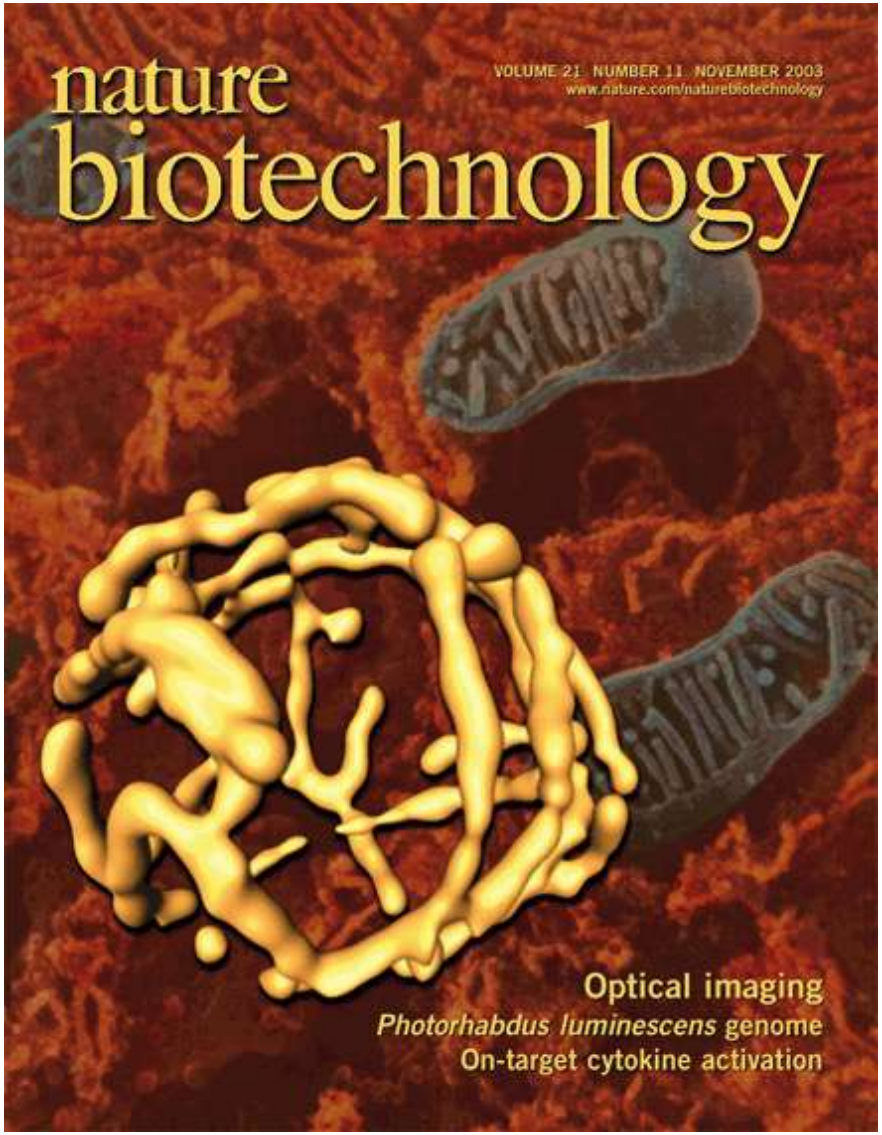


2 µm

S.W. Hell, et al. (1992), *Opt. Commun.* **93**, 277.
M. Schrader, et al. (1998), *Biophys. J.* **75**, 1659.
H. Gugel, et al. (2004), *Biophys. J.* **87**, 4146.

Commercial 4Pi-microscope





Z- resol < 90 nm (Live cells /aqueous cond.)

H. Gugel, et al. (2004), *Biophys J* **87**, 4146.

Max Planck Society

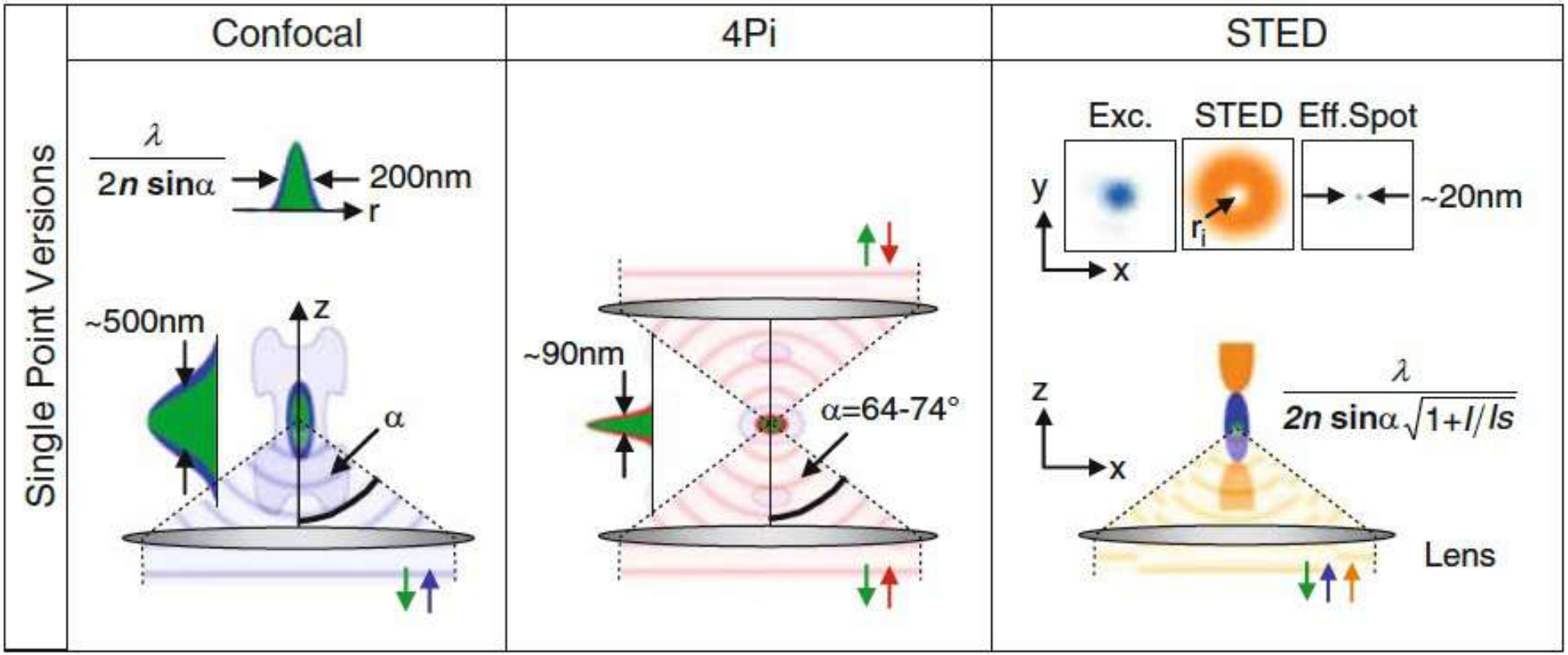
STED microscopy

1st physical concept to break the diffraction barrier in *far-field* fluorescence microscopy

S.W. Hell & J. Wichmann (1994), *Opt. Lett.* **19**, 780.

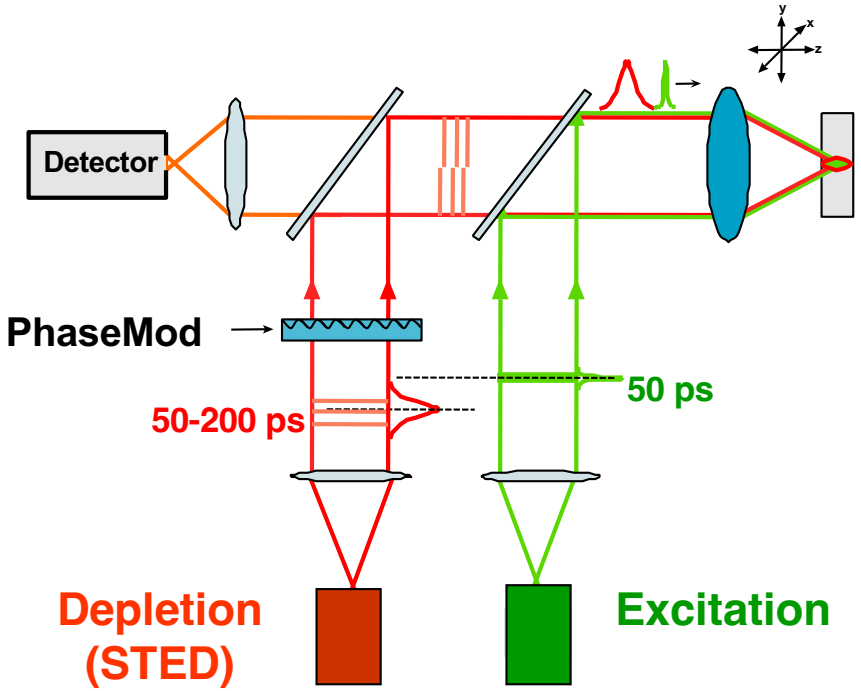
In the targeted read-out mode, the bright state A is established at coordinate r_i by driving an optical transition $A \rightarrow B$ with a light intensity distribution $I = I(r)$ featuring an intensity minimum, ideally a zero, at coordinate r_i (Figs. 19.1d and 19.2a). Applying $I(r)$ transfers the markers virtually everywhere to B, except at the zero-intensity point r_i where the molecules can still remain in A. The rate of the transition $A \rightarrow B$ is given by $k_{AB} = \sigma I$, with σ denoting the optical cross-section for $A \rightarrow B$. In order to effectively switch the molecule to B, the optically induced rate k_{AB} must outperform any competing spontaneous transitions between A and B. Since these spontaneous rates are given by the inverse lifetimes $\tau_{A,B}$ of the states A and B, we obtain: $k_{AB} = \sigma I \gg (\tau_{A,B})^{-1}$. Therefore, applying an intensity I that is much larger than the "saturation intensity" $I_s = (\sigma\, \tau_{A,B})^{-1}$ shifts the molecule everywhere to B except in the proximity of the zero-intensity point r_i of $I(r)$. Thus, we obtain a narrowly confined region $r_i \pm \Delta r/2$ in which the molecule can still be in A. The width Δr of this region or spot is readily calculated as

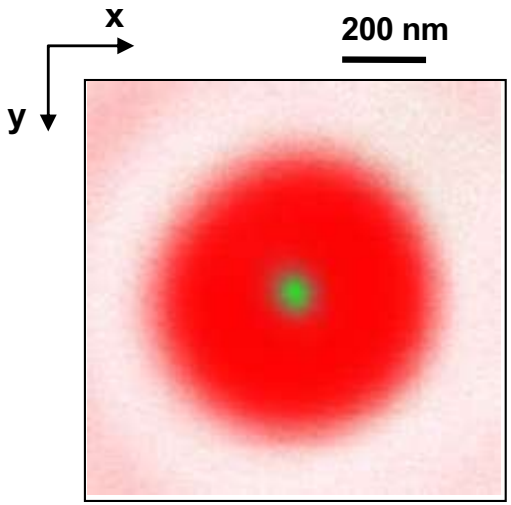
$$\Delta r \approx \frac{\lambda}{2\, n\, \sin\alpha \sqrt{1 + a I_{\max}/I_s}}. \tag{19.1}$$


Single Point Versions
Confocal
4Pi
STED
λ
2n sinα
200nm
r
~500nm
z
α
~90nm
α=64-74°
Exc.
STED
Eff.Spot
y
x
~20nm
z
x
λ
2n sinα√1+I/Is
Lens

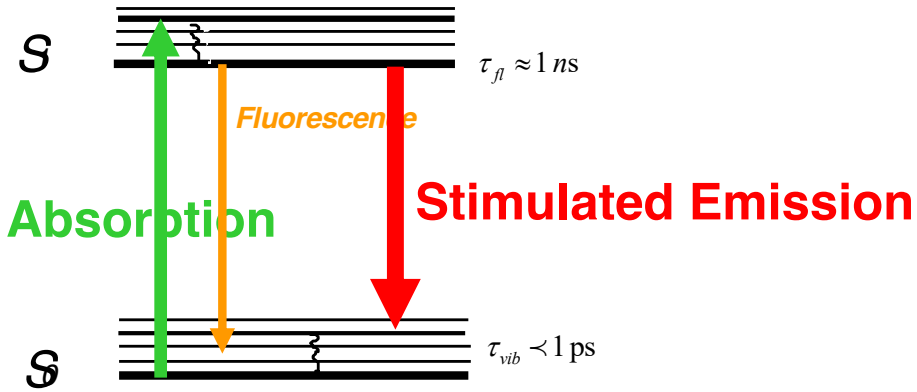
STED Microscopy

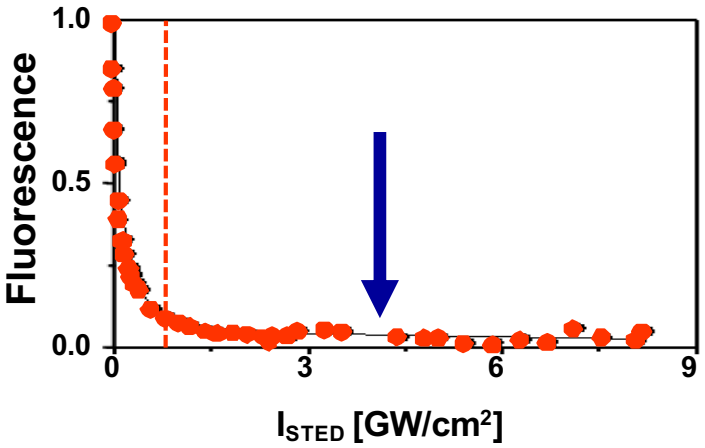
S.W. Hell & J. Wichmann (1994), *Opt. Lett.* **19**, 780.



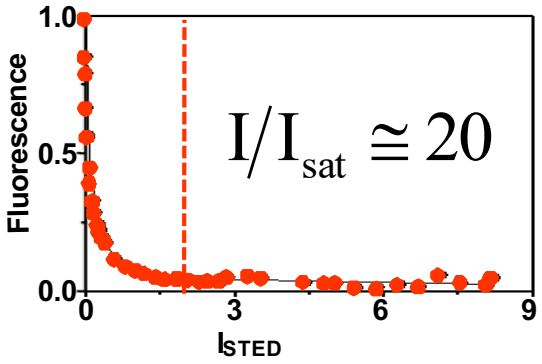


The stronger the STED beam the narrower the fluorescent spot!

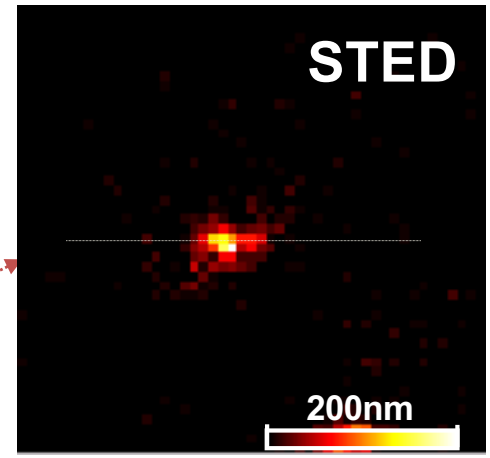


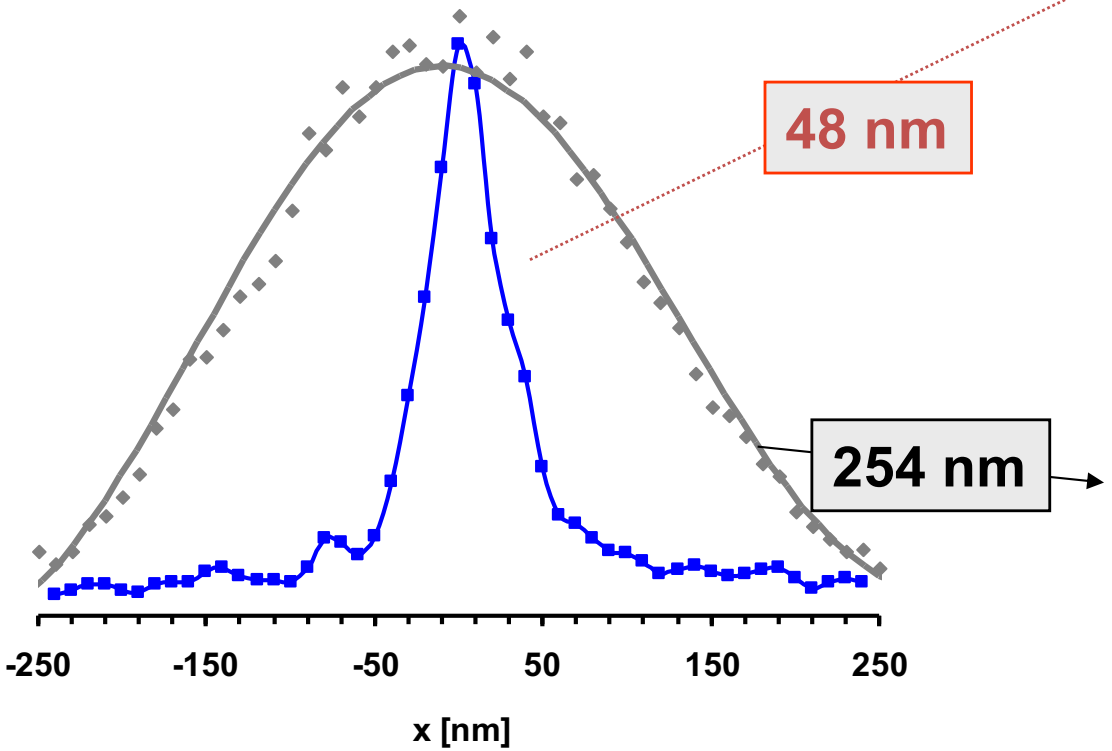


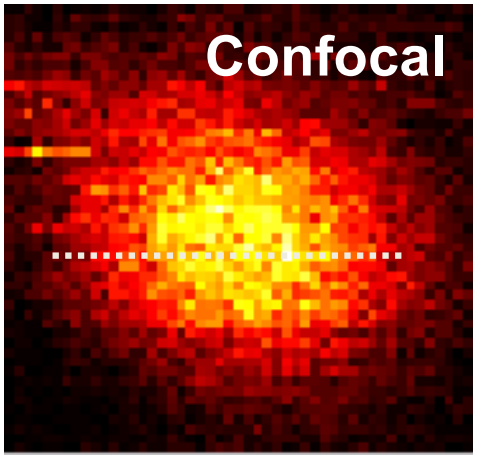
Focal spot *... probed with 1 molecule*



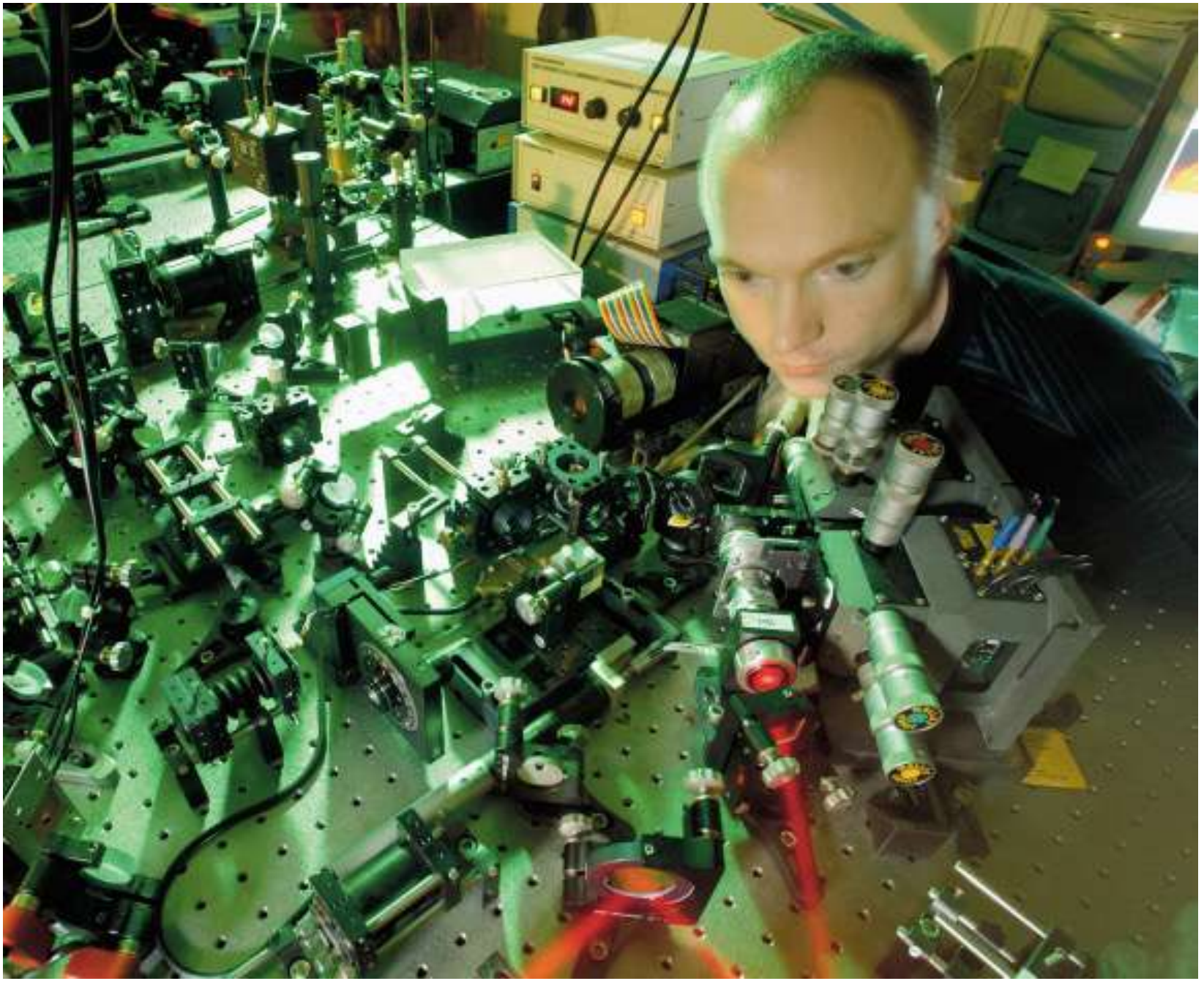
λ_{STED}= 770 nm





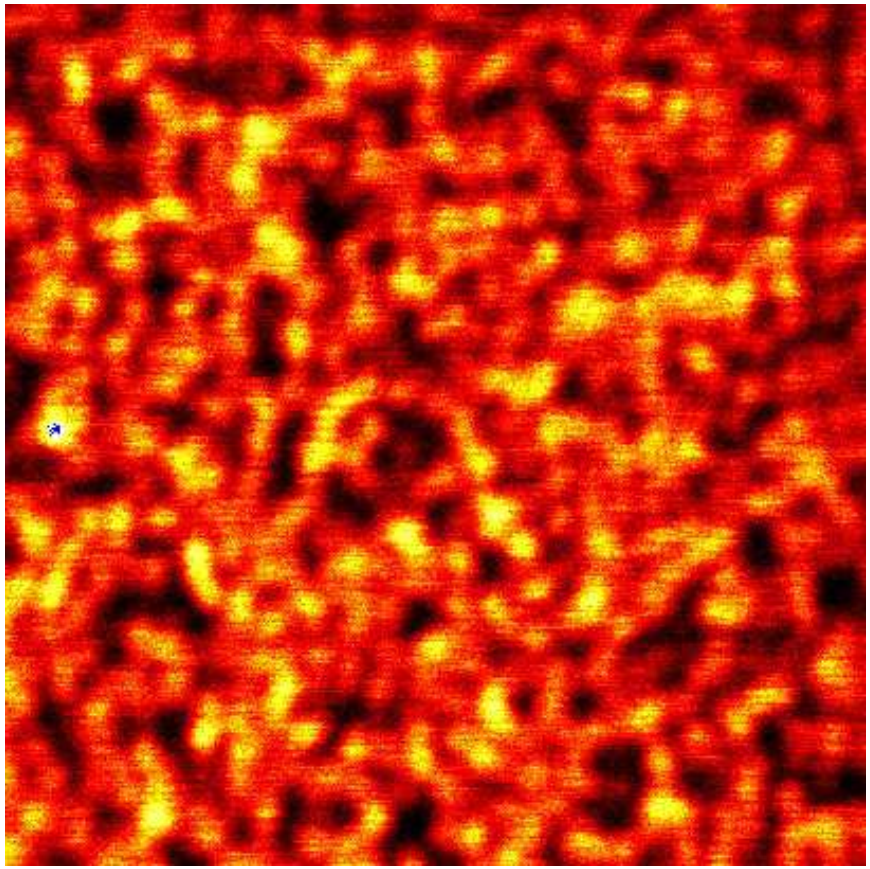


V. Westphal & S.W. Hell (2005), Phys. Rev. L



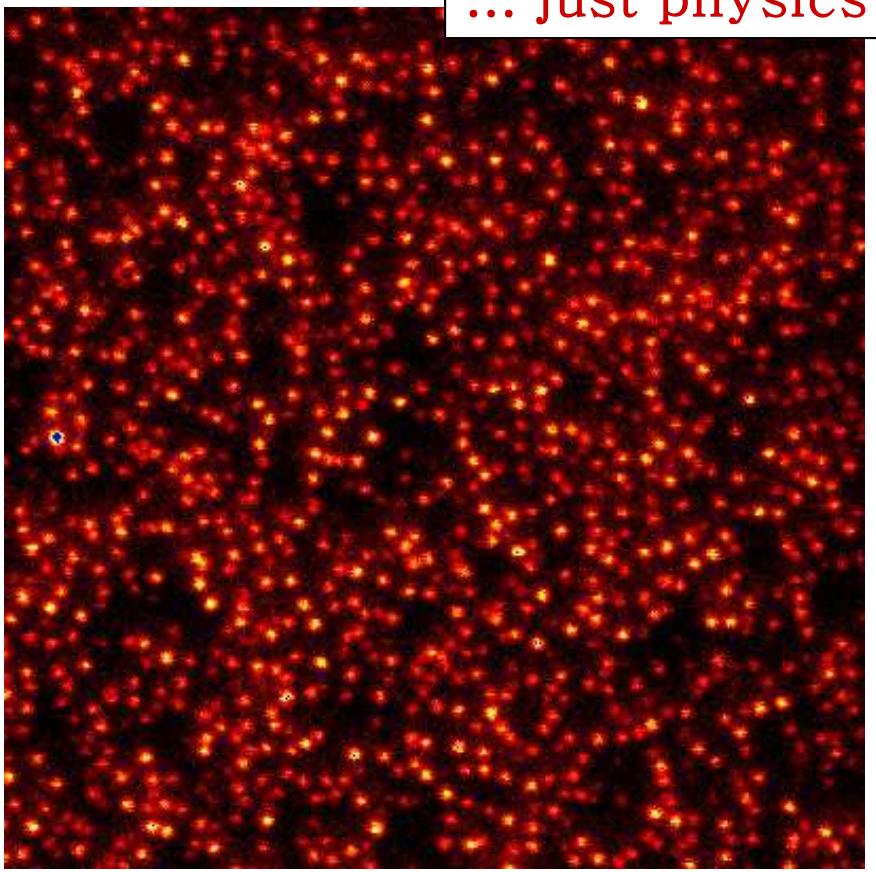
Imaging 40 nm fluorescence beads:

Confocal



10 counts/0,3ms 204

STED



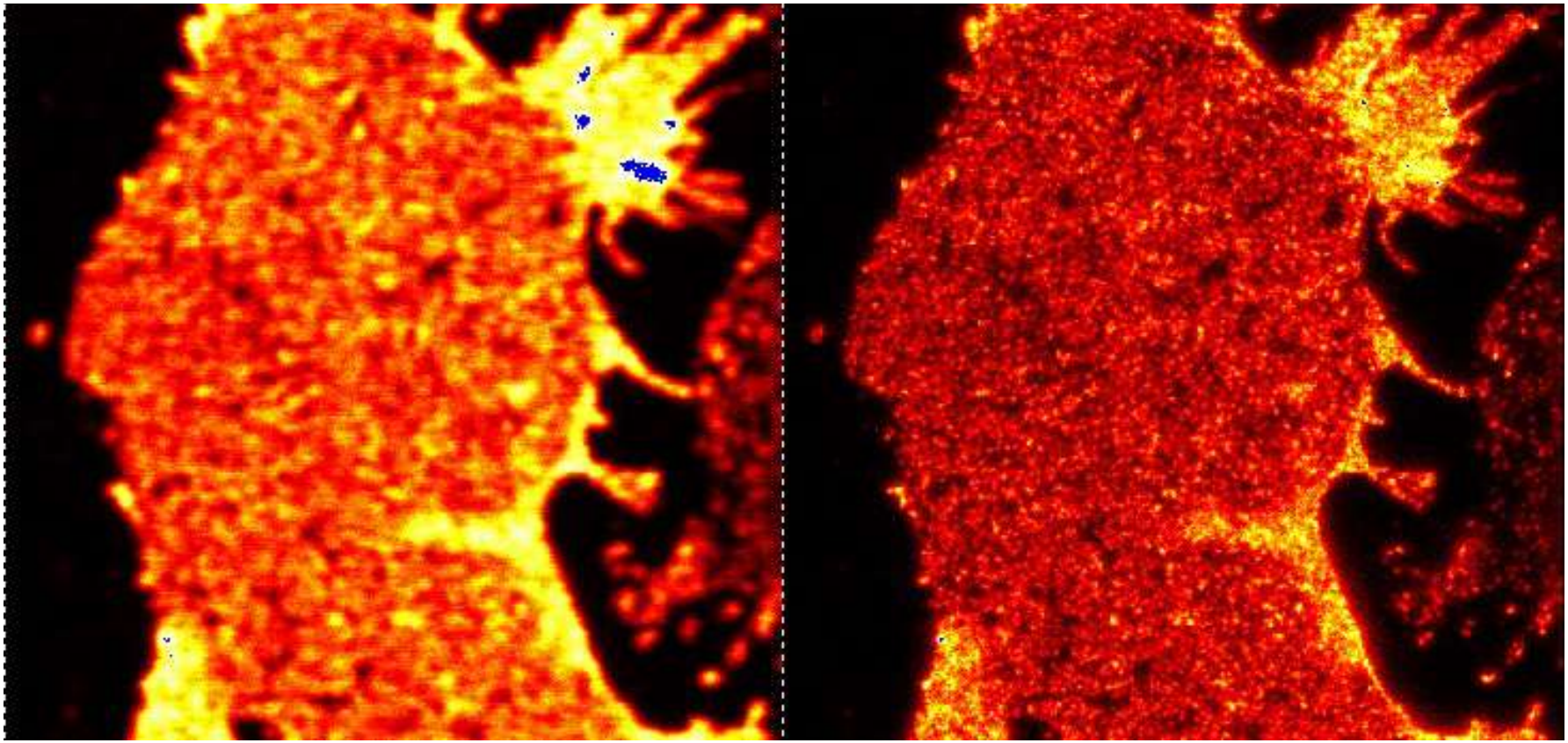
5 counts/0,3ms 89

... just physics !

Imaging protein distribution on cell membrane: SNAP 25

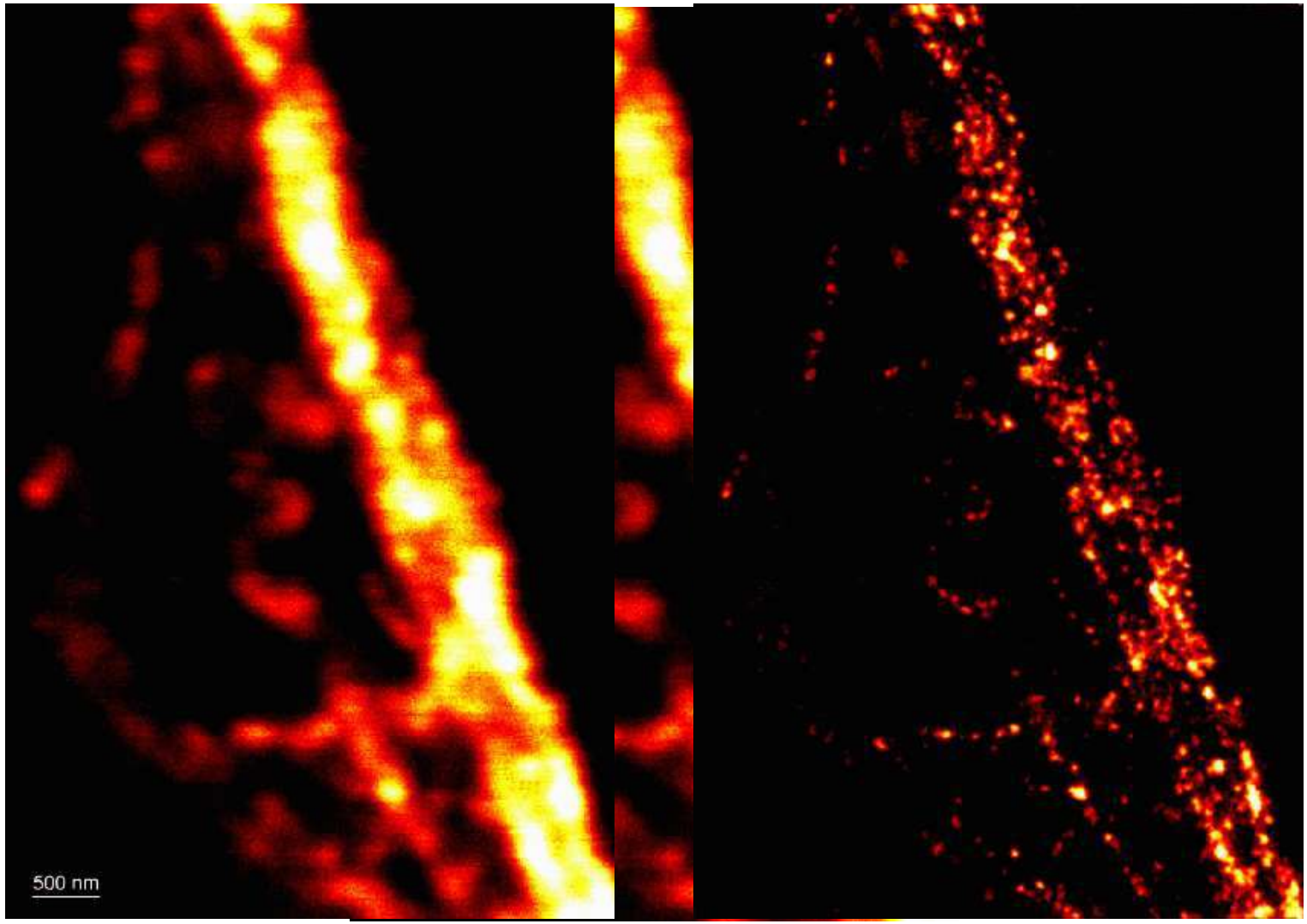
...just physics !

Confocal: **STED:**



Heavy subunit of neurofilaments in neuroblastoma

Confocal

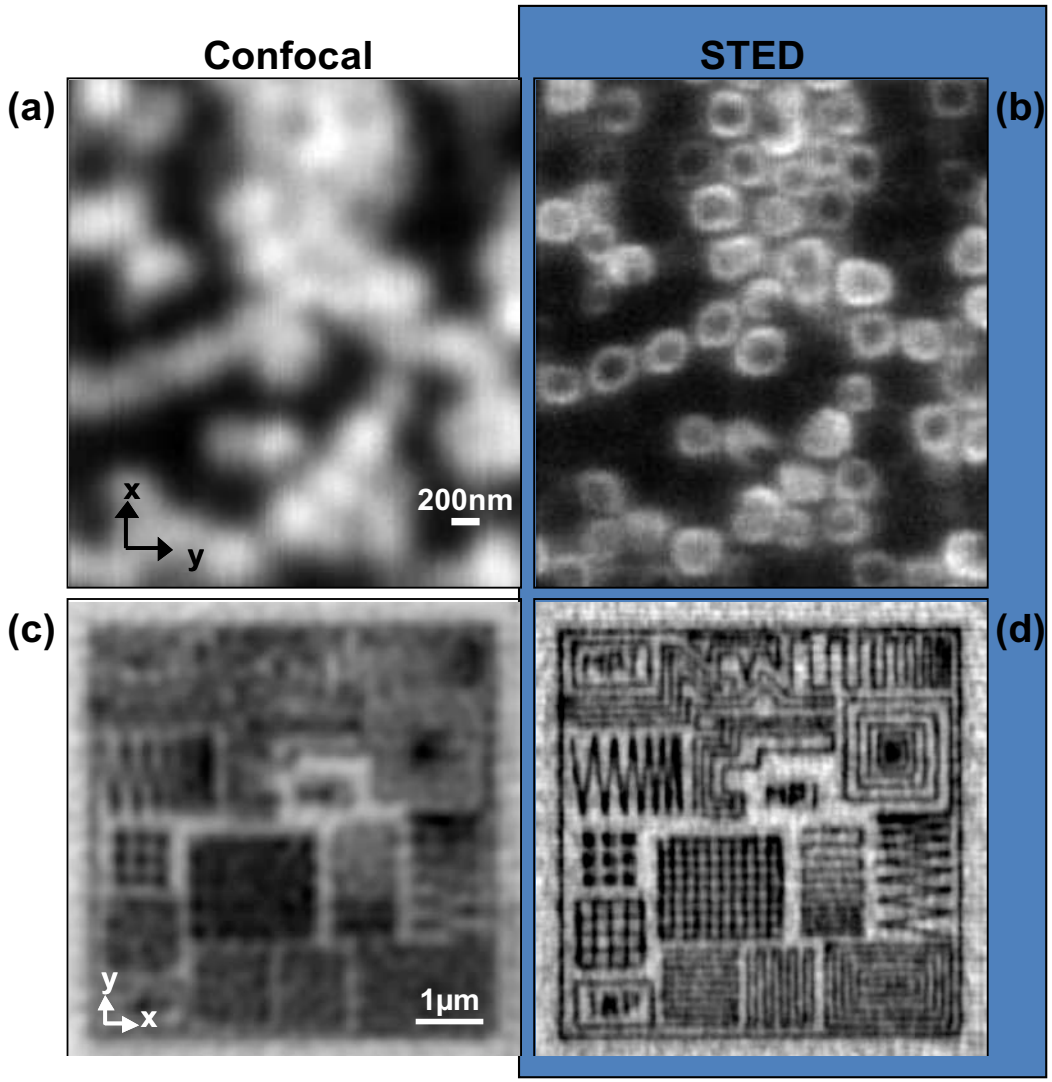


STED

G. Donnert, et al. (2006), *PNAS*

Max Planck Society

STED Microscopy: Sometimes only *resolution*…



Pores in a porous membrane marked with a fluorescent dye

Fluorescence dye marked nanostructures produced by electron beam lithography in a polymer

…makes subdiffraction images !

V. Westphal, S.W. Hell (2005), *Phys. Rev. Lett.* **94**, 143903.
V. Westphal, J. Seeger, T. Salditt, S. W. Hell (2005) , *J. Phys. B* **38**, S695.

STED microscopy

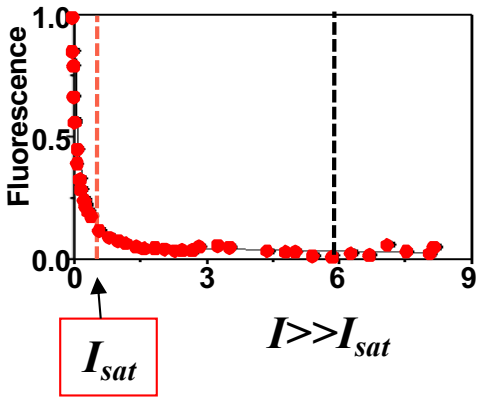
- Resolution is not limited by the wavelength of light!

- Resolution just depends on the level of fluorescence depletion.

- Resolution at the molecular scale is possible with visible light and regular lenses!

- Resolution follows a new law; a modification of Abbe's law:

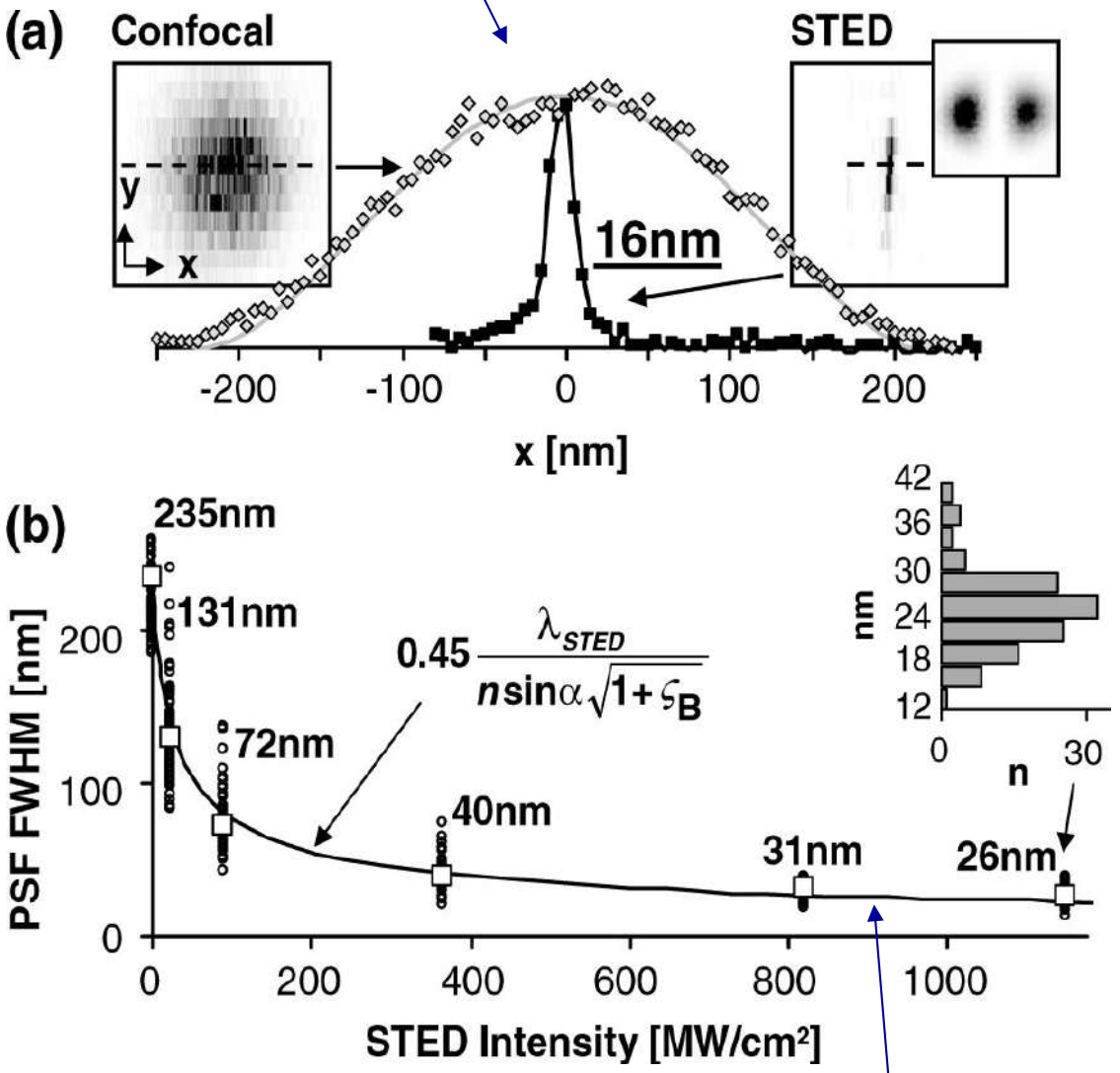
$$\Delta x \approx \frac{\lambda}{2n \sin\alpha \sqrt{1 + I/I_{sat}}}$$



S.W. Hell (2003), *Nature Biotech.* **21**, 1347.
S.W. Hell (2004), *Phys. Lett. A* **326**, 140.
V. Westphal & S.W. Hell (2005), *Phys. Rev. Lett.* **94**, 143903.

Max Planck Society

Sharpest focal spot



Validation of square-root resolution law

V. Westphal, S.W. Hell (2005), *Phys. Rev. Lett.* **94**, 143903.

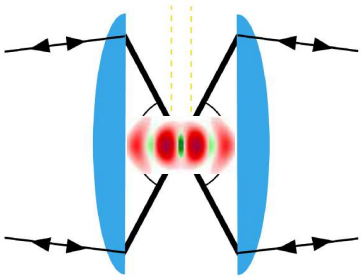
Max Planck Society

4Pi- STED Microscopy

Axial (z) resolution 30-50 nm and beyond …

Max Planck Society

The combination: STED-4Pi-Microscopy

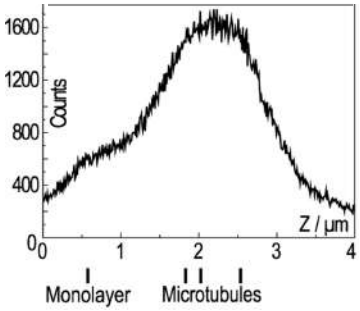


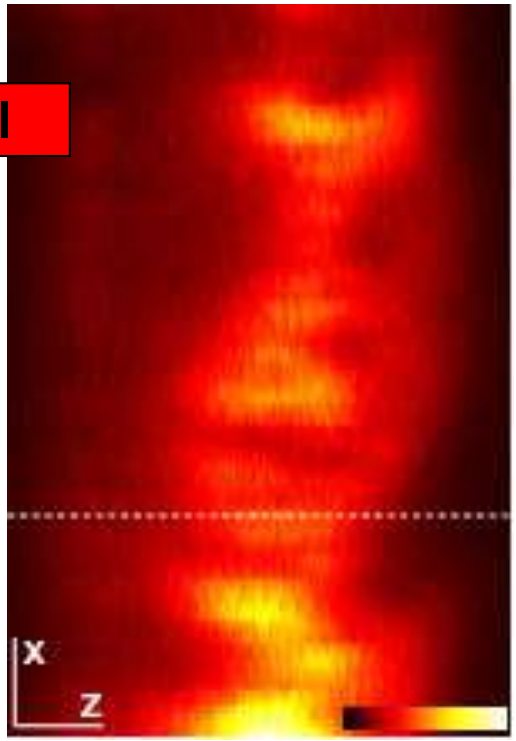
Monolayer

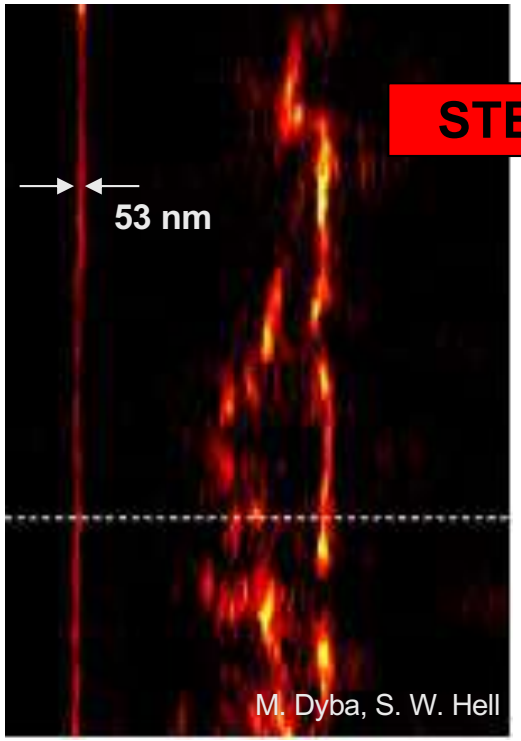
Monolayer

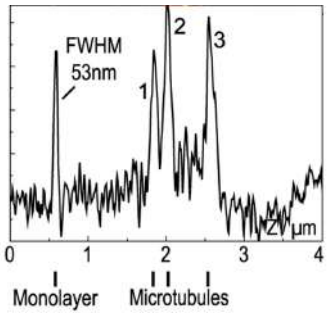
confocal

STED-4Pi









Fluorescently tagged microtubuli
with an axial resolution of 50-70 nm

M. Dyba, S. Jakobs, S.W. Hell (2003), *Nature Biotechnol.* **21**, 1303.

Acknowledgements / References:

Physics:

G. Donnert et al *PNAS, 103 (2006)*

K. Willig, J. Keller, M. Bossi, S.W. Hell *New J Phys, 8 (2006)*

V. Westphal, S.W. Hell *Phys Rev Lett, 94 (2005)*

L. Kastrup, H. Blom, C. Eggeling, S.W. Hell *Phys Rev Lett, 94 (2005)*

M. Hofmann, C. Eggeling, S. Jakobs, S.W. Hell *PNAS, 102 (2005)*

Applications:

K. Willig, S. Rizzoli, R. Jahn, S.W. Hell *Nature, April 13, (2006)*

R. Kittel, et al *Science, May 19, (2006)*

Pictures/Movies

www.nanoscopy.de