

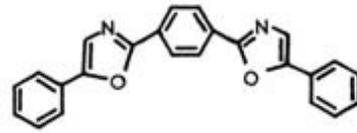
FLUORESCENCE SPECTROSCOPY AND IMAGING

Principles of Fluorescence Spectroscopy

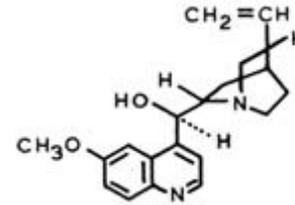
Third Edition

Joseph R. Lakowicz

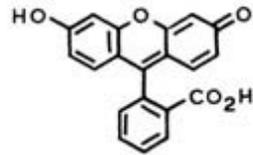
*University of Maryland School of Medicine
Baltimore, Maryland, USA*



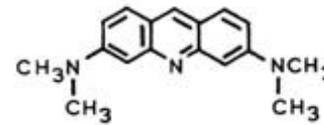
POPOP



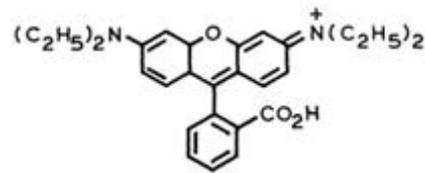
Quinine



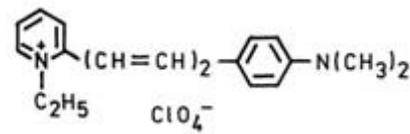
Fluorescein



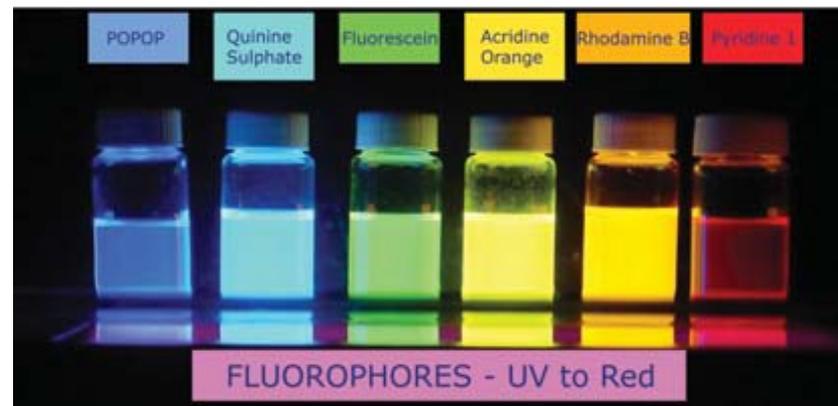
Acridine Orange



Rhodamine B



Pyridine 1



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)

LUMINESCENCE

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.

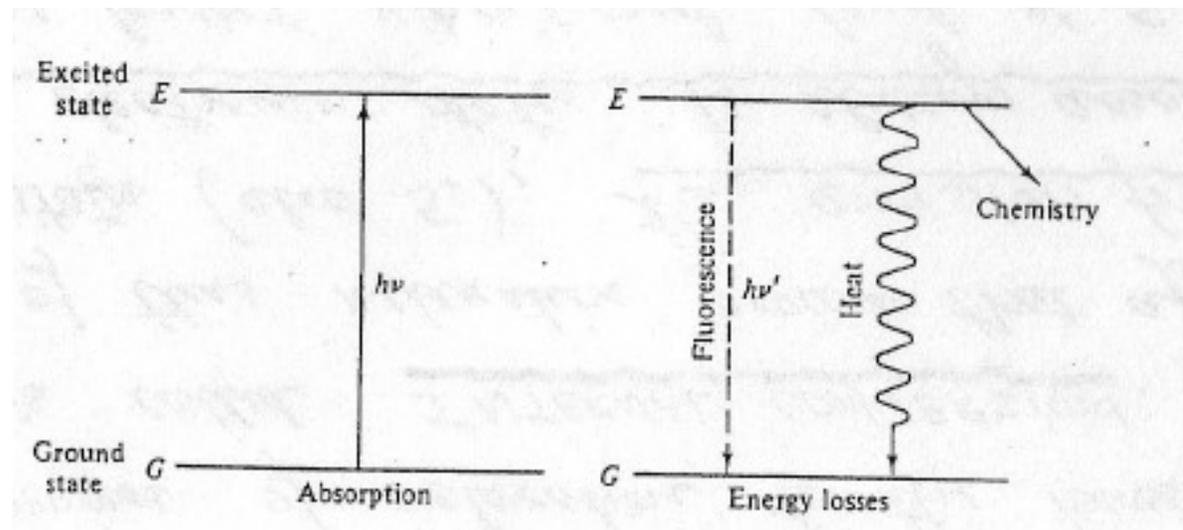
- **Fluorescence:** average lifetime **from $<10^{-10}$ to 10^{-7} sec** from singlet states.
- **Phosphorescence:** Average lifetime **from 10^{-5} to $>10^3$ sec** from triplet excited states.

Fluorescence occurs at much smaller scales than **absorption (10^{-15} sec)**: this allows the spectrum to carry information about the perturbation or changes occurring within this time scale.

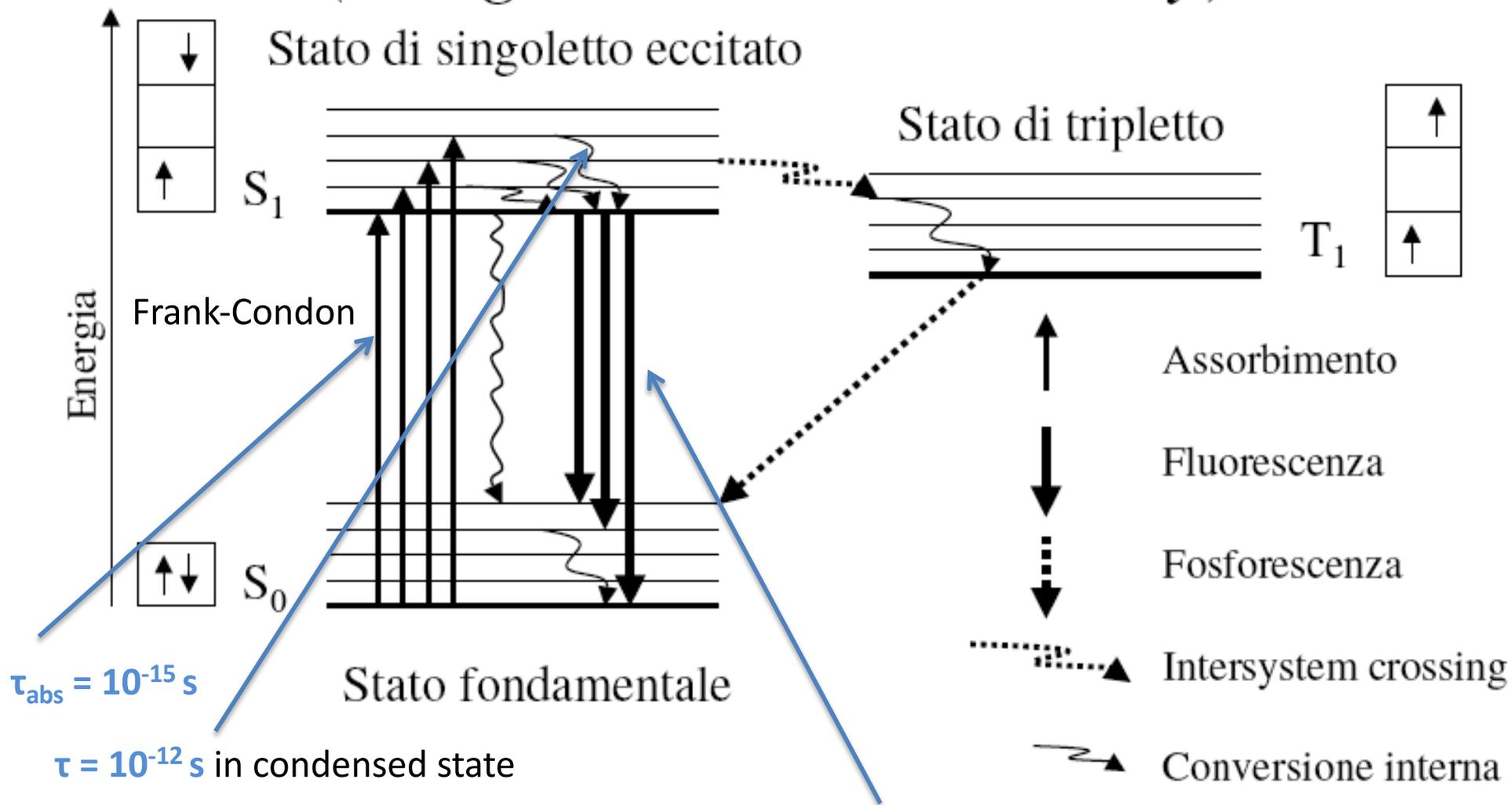
Its existence was predicted by Einstein

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

- 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 fraction 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.

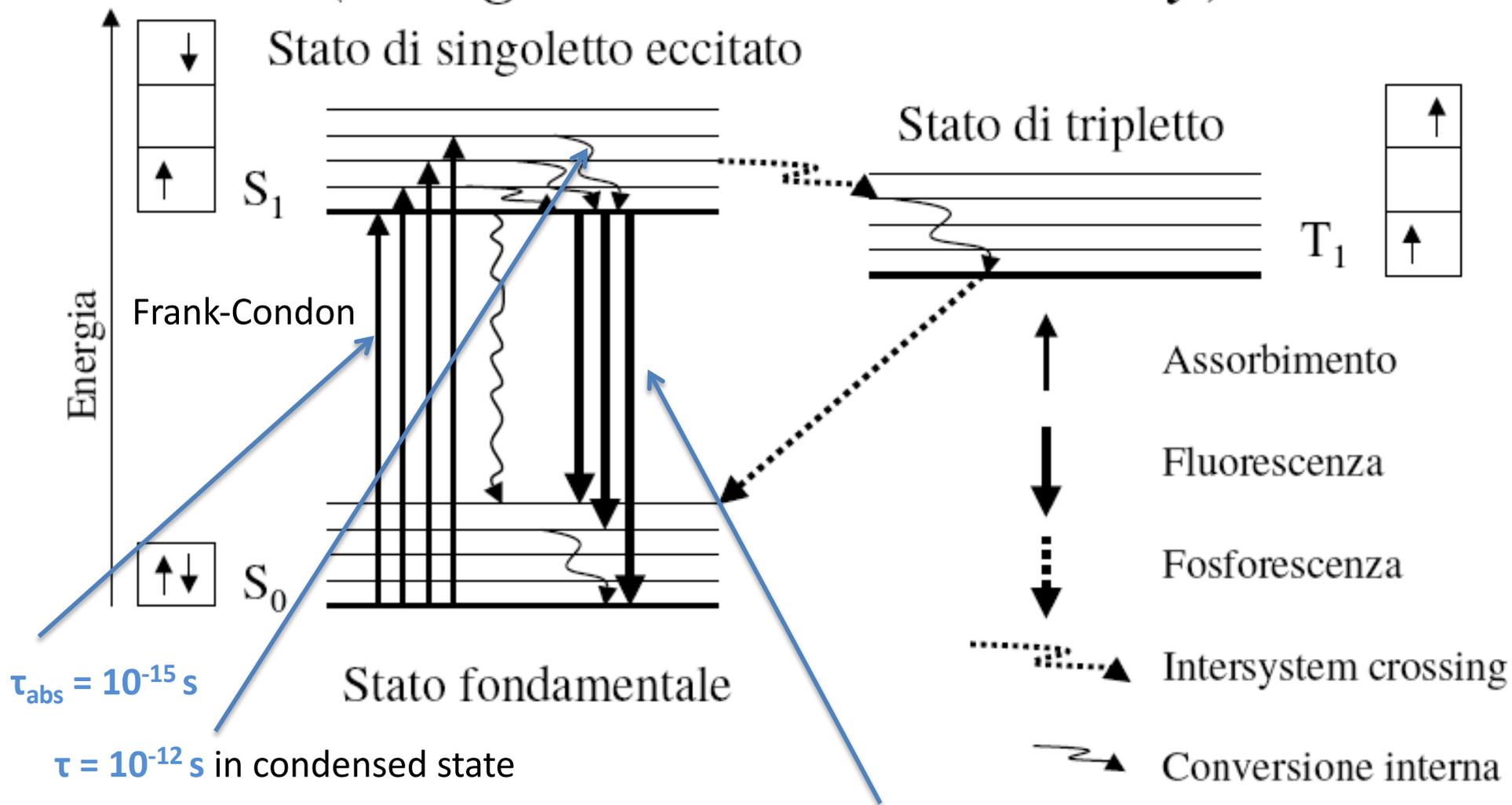


Le transizioni elettroniche (*Diagramma di Jablonsky*)

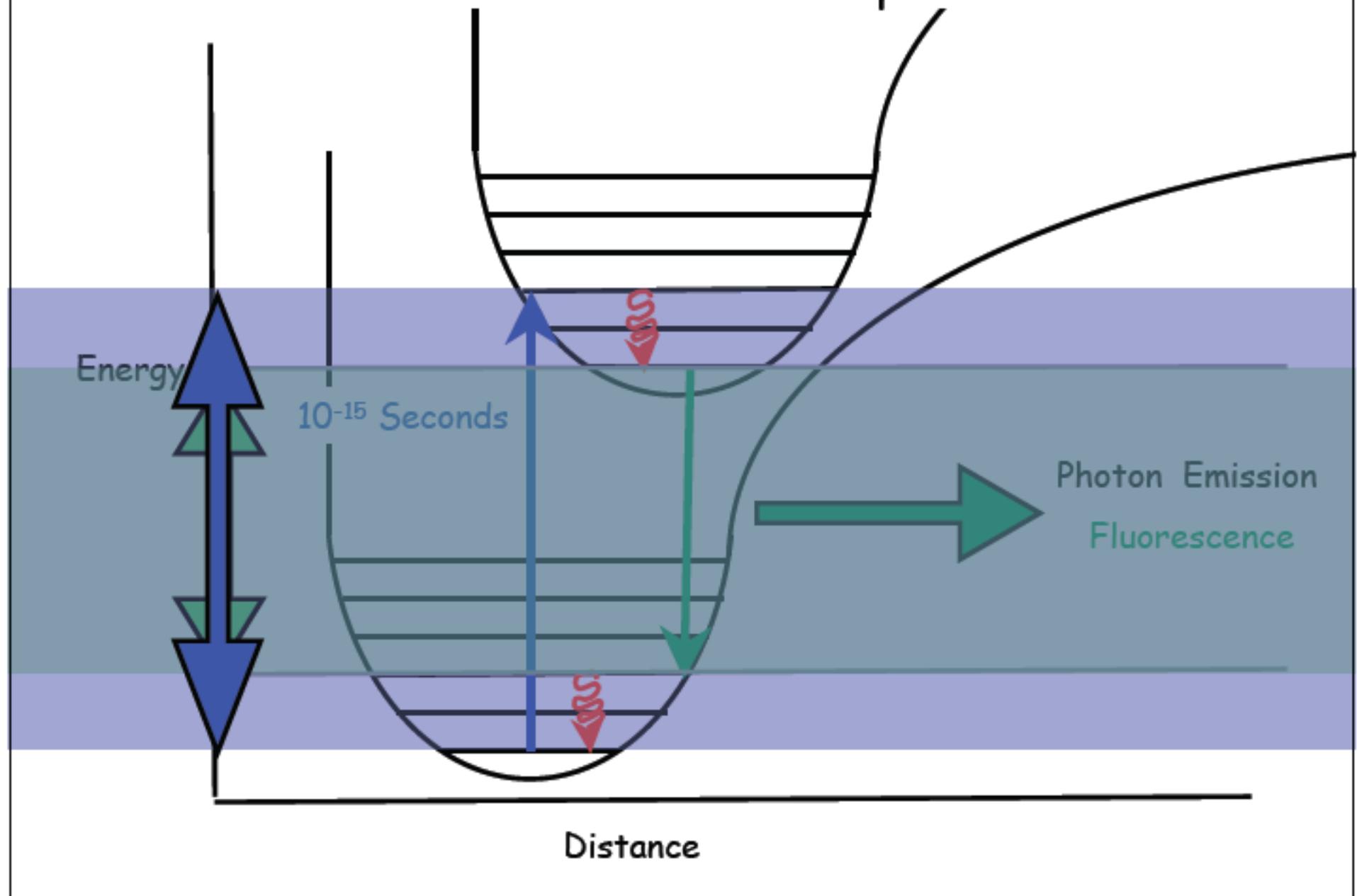


$\tau = 10^{-8} - 10^{-9} \text{ s}$ fluorescence decay (5% of total emission)
average fluorescent lifetime: 1 ns!

Le transizioni elettroniche (*Diagramma di Jablonsky*)

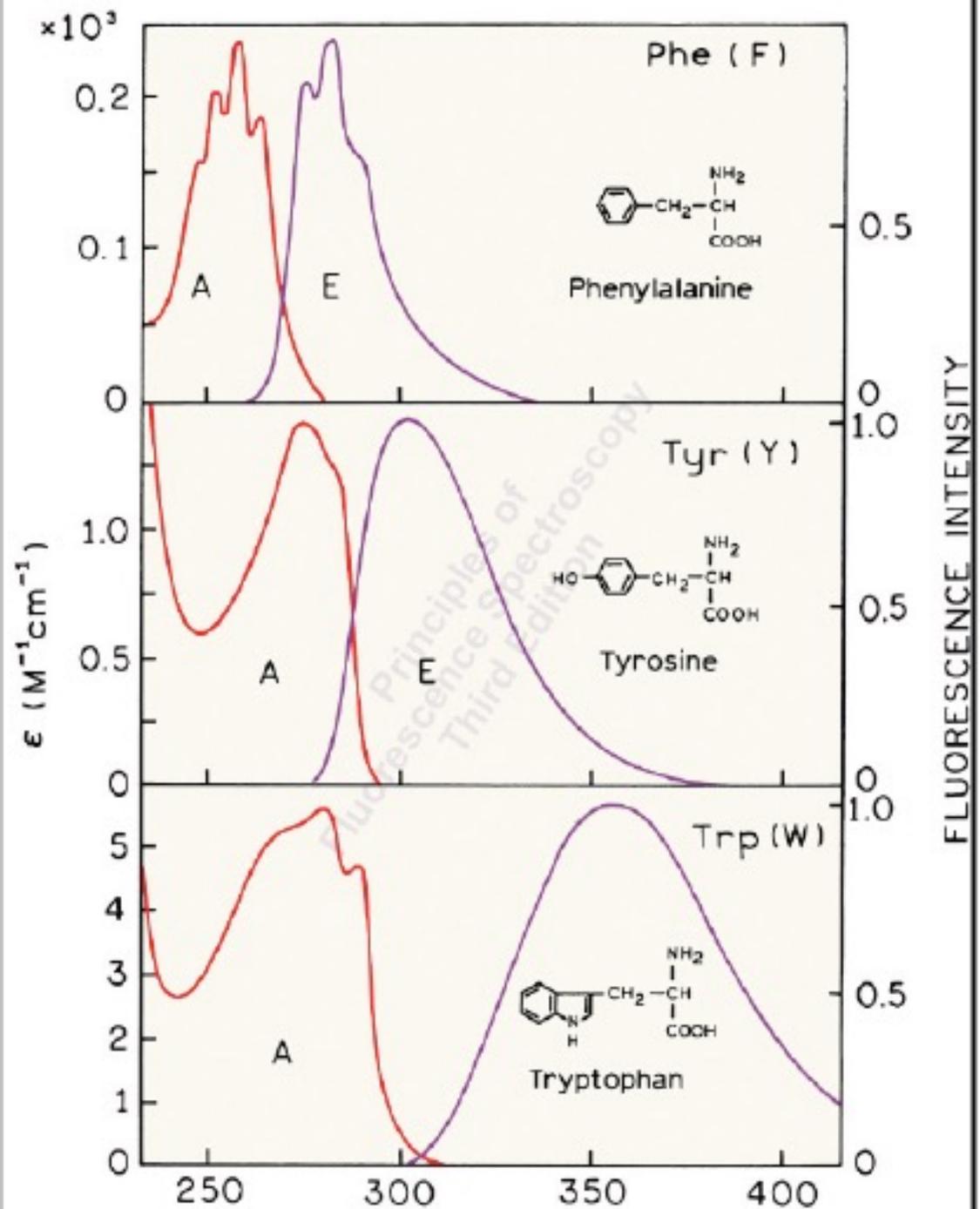


Stokes Shift: The energy difference between the absorbed and emitted photon



Stoke's Shift

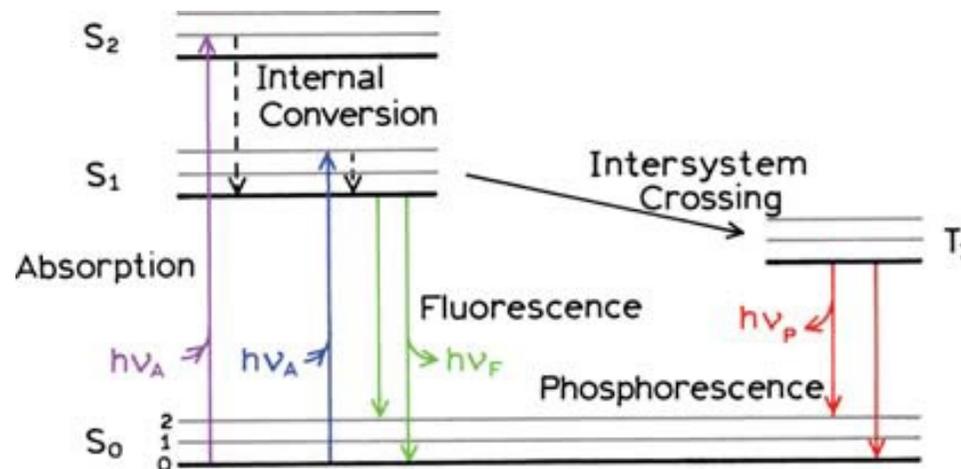
Fluorescence
will always
occur at longer
wavelengths
(lower energies)
than
absorbance.



Another general property of fluorescence is that the **same fluorescence emission spectrum is generally observed irrespective of the excitation wavelength**. This is known as Kasha's rule.

Upon excitation into higher electronic and vibrational levels, the excess energy is quickly dissipated, leaving the fluorophore in the lowest vibrational level of S_1 .

This relaxation occurs in about 10^{-12} s, and is presumably a result of a strong overlap among numerous states of nearly equal energy. Because of this rapid relaxation, emission spectra are usually independent of the excitation wavelength.



Photophysics: Jablonski Diagram

- Photoexcitation from the ground electronic state S_0 creates excited states $S_1, (S_2, \dots, S_n)$ 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)

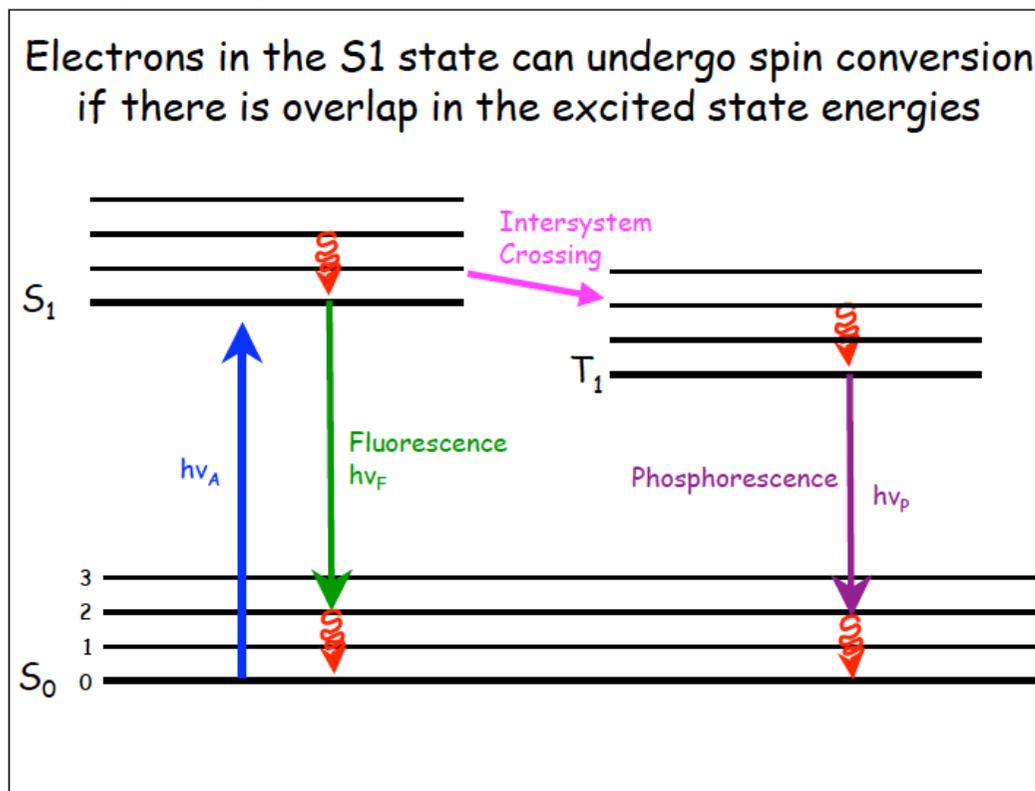
Internal Conversion: Molecules rapidly (10^{-14} to 10^{-11} s) relax to the lowest vibrational level of S_1 .

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:

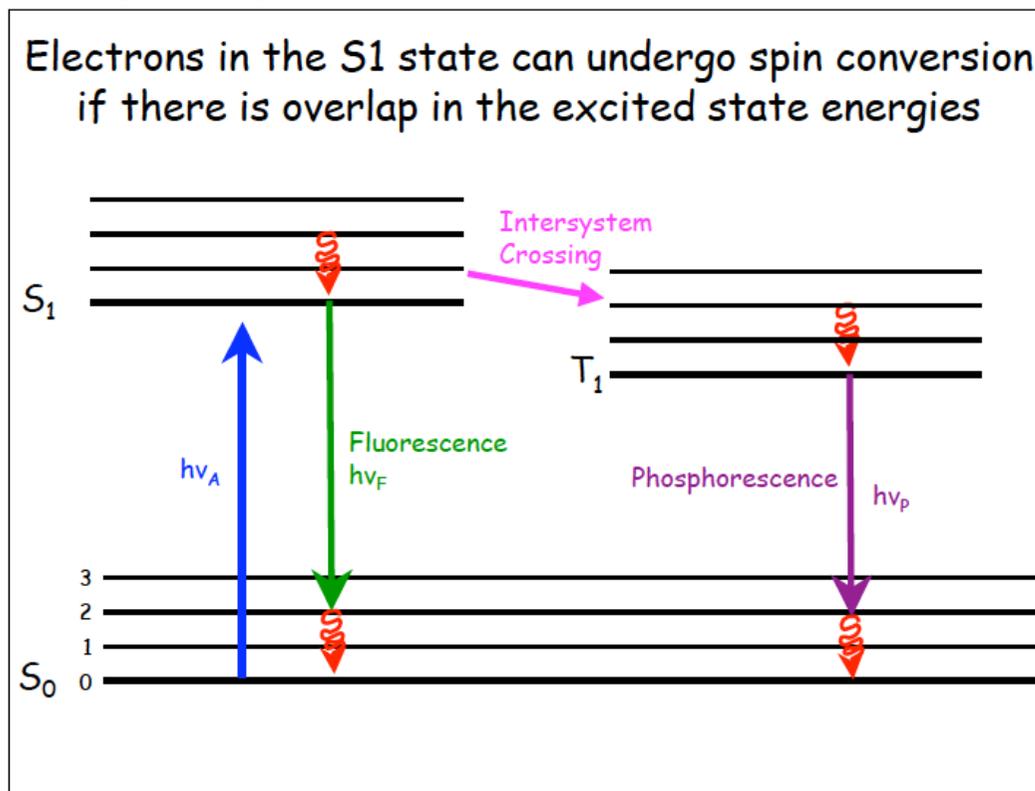
$$R = e^{-\Delta E/kT}$$

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.

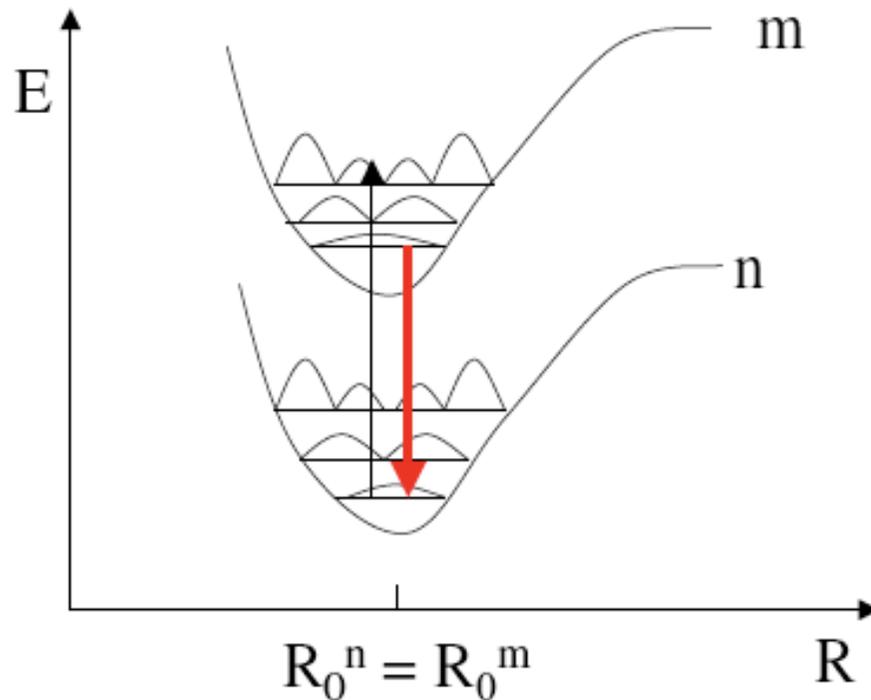
Photophysics: Jablonski Diagram



Phosphorescence is usually not seen in fluid solutions at room temperature. This is because there exist many deactivation processes that compete with emission, such as non-radiative decay and quenching processes.

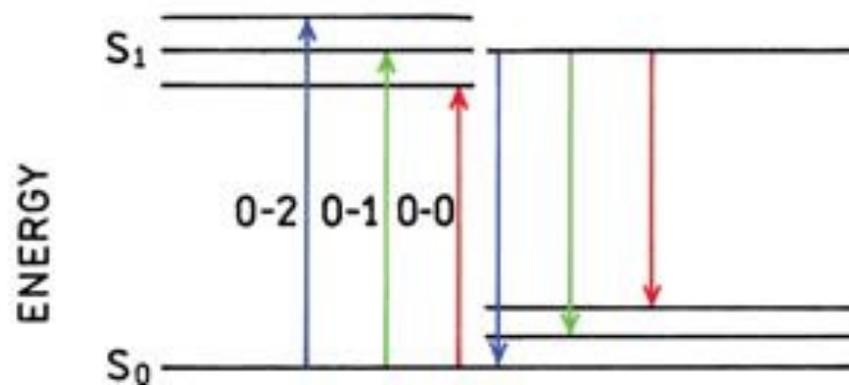
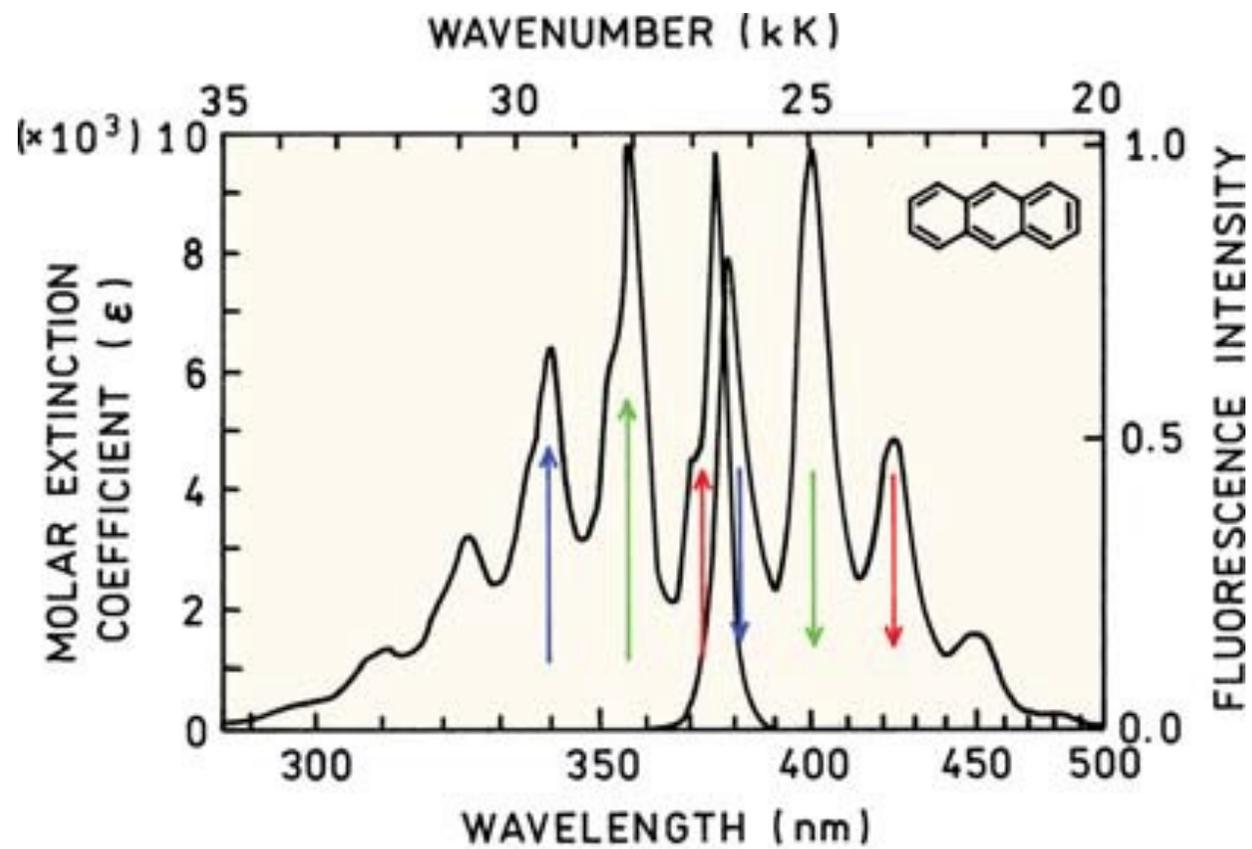
The mirror image rule:

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

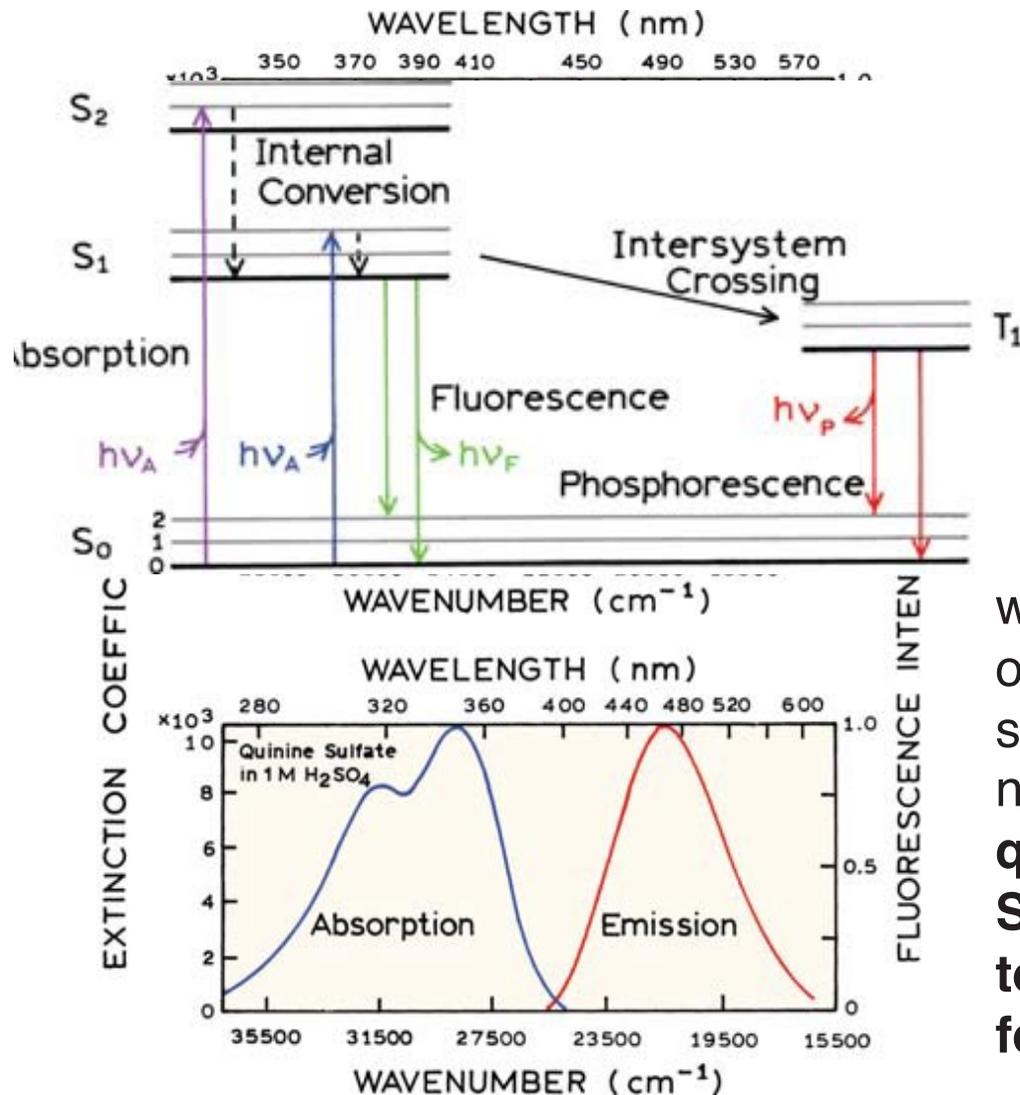


The generally symmetric nature of these spectra is a result of the same transitions being involved in both absorption and emission, and the similar vibrational energy levels of S₀ and S₁. In most fluorophores these energy levels are not significantly altered by the different electronic distributions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

Anthracene



Photophysics: Jablonski Diagram

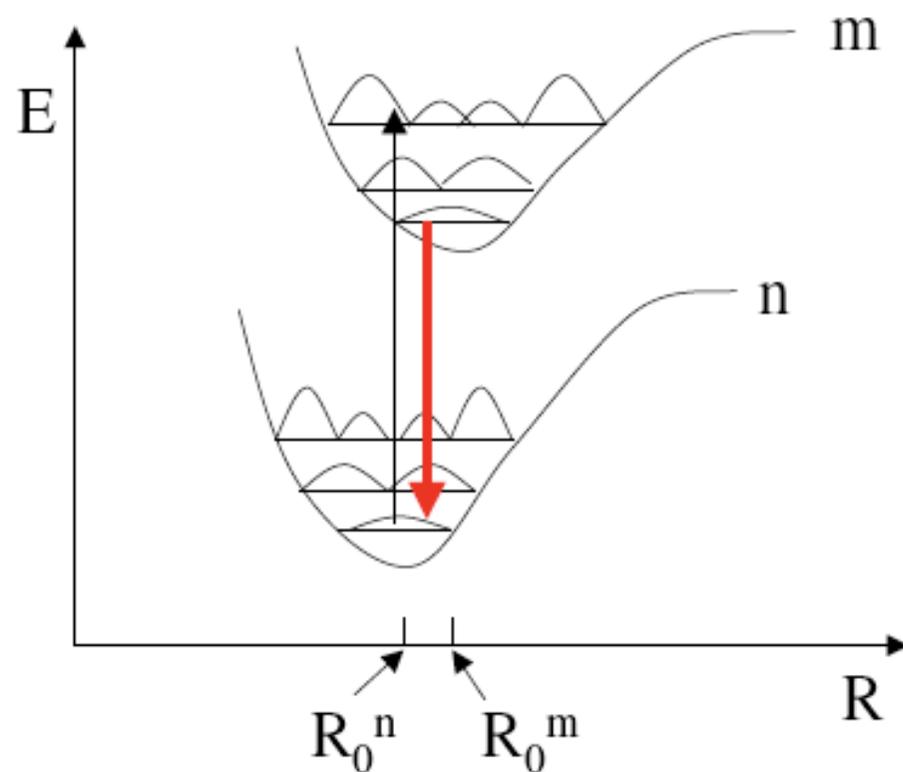


It is interesting to ask why perylene follows the mirror image rule, but quinine emission lacks the two peaks seen in its excitation spectrum at 315 and 340 nm.

In the case of quinine, the shorter wavelength absorption peak is due to excitation to the second excited state (S_2), which relaxes rapidly to S_1 . Emission occurs predominantly from the lowest singlet state (S_1), so emission from S_2 is not observed. **The emission spectrum of quinine is the mirror image of the S_0 to S_1 absorption of quinine, not of its total absorption spectrum. This is true for most fluorophores.**

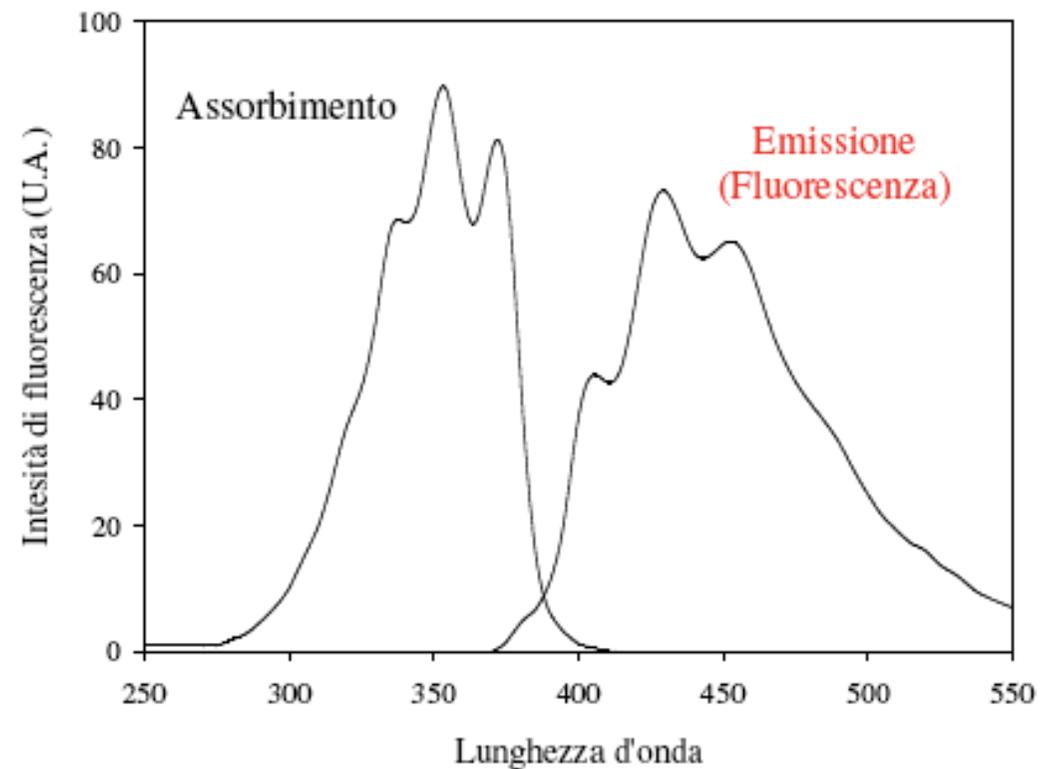
Transizione asimmetrica

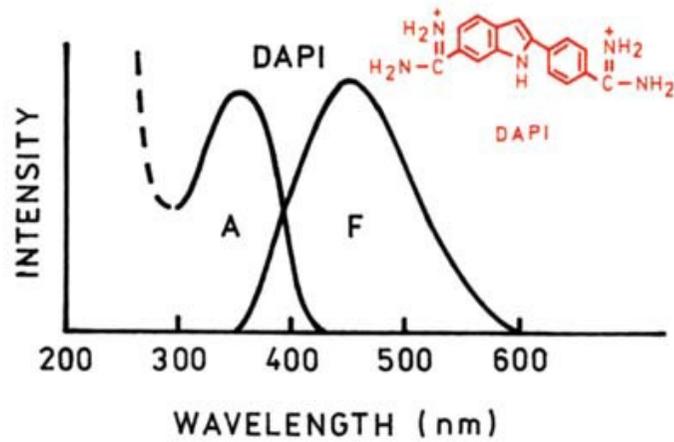
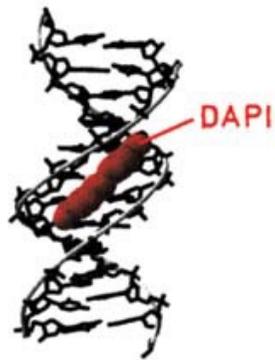
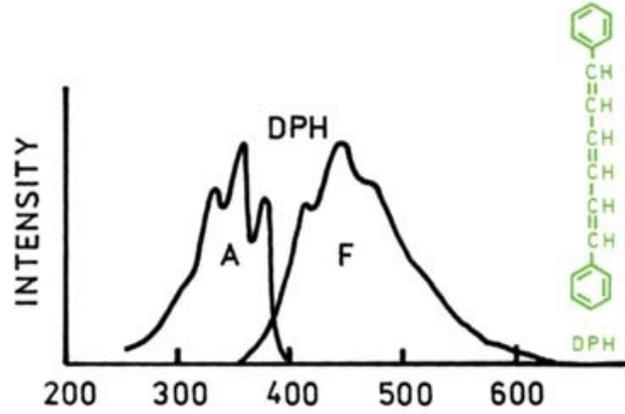
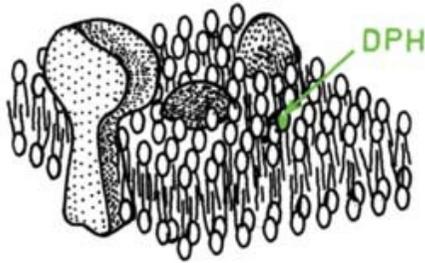
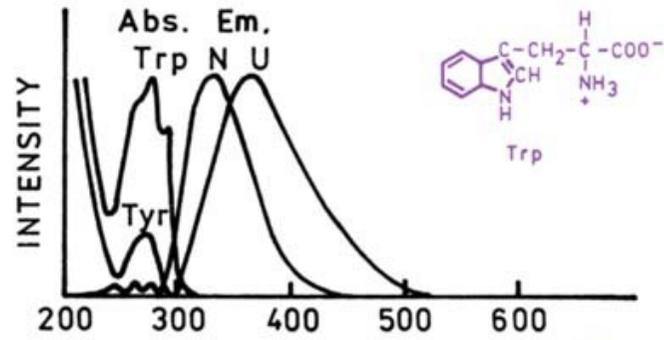
- Se lo stato eccitato subisce una perturbazione (cambia la struttura della molecola, avvengono reazioni allo stato eccitato, vi è energy transfer o charge transfer, ecc.) si perde la simmetria speculare tra lo spettro di eccitazione e lo spettro di emissione.



Difenilesatriene (DPH)

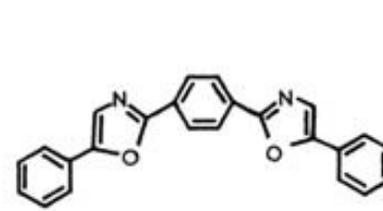
- Il DPH può subire una isomerizzazione *cis-trans* allo stato eccitato. Lo spettro di emissione non è speculare a quello di eccitazione.



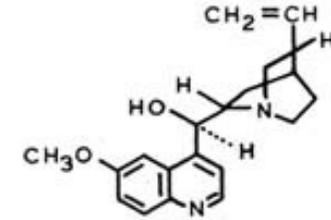


WAVELENGTH (nm)

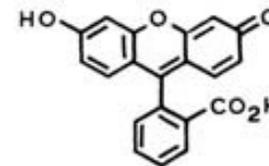
- The small fraction of molecules which decay emitting radiation from the lowest vibrational level of the first excited singlet state to the ground singlet state conserve spin (are allowed) and therefore have a shorter lifetime (the inverse of the transition rate), which is of the order of 1-10 nseconds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



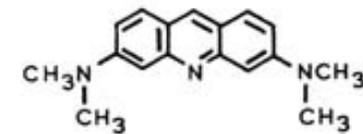
POPOP



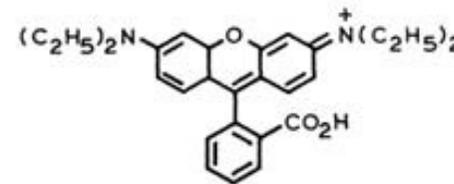
Quinine



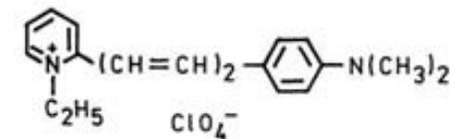
Fluorescein



Acridine Orange

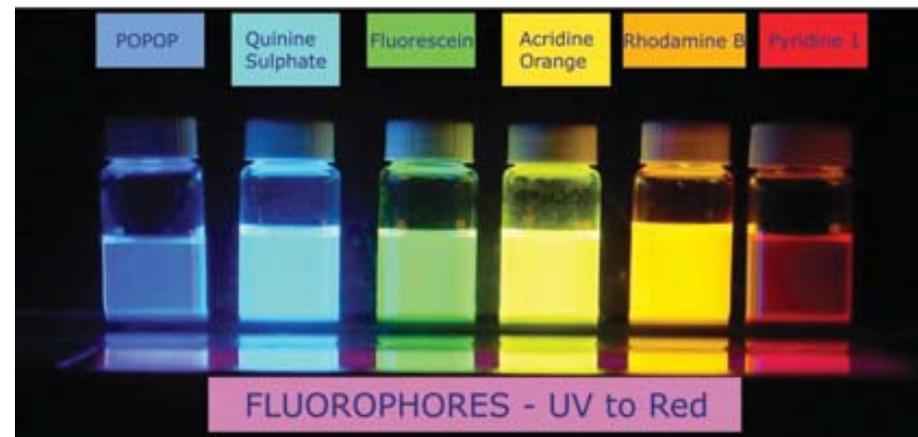


Rhodamine B

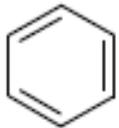
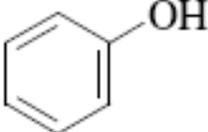
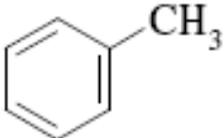
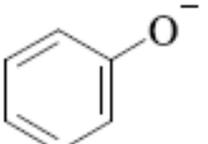
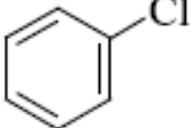
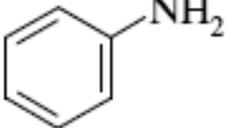
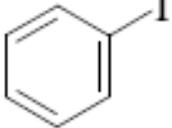
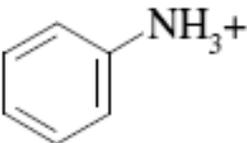


Pyridine 1

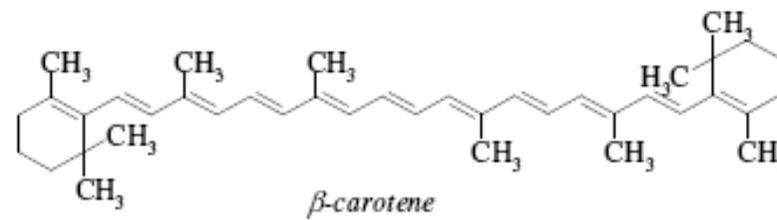
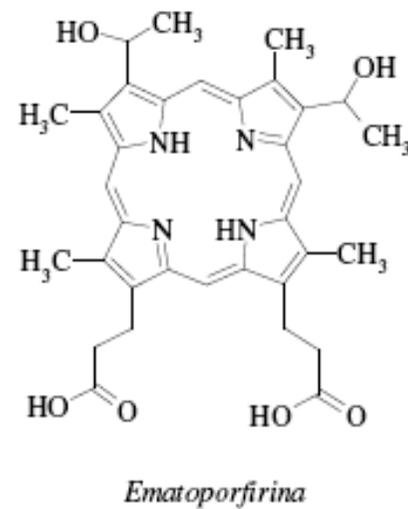
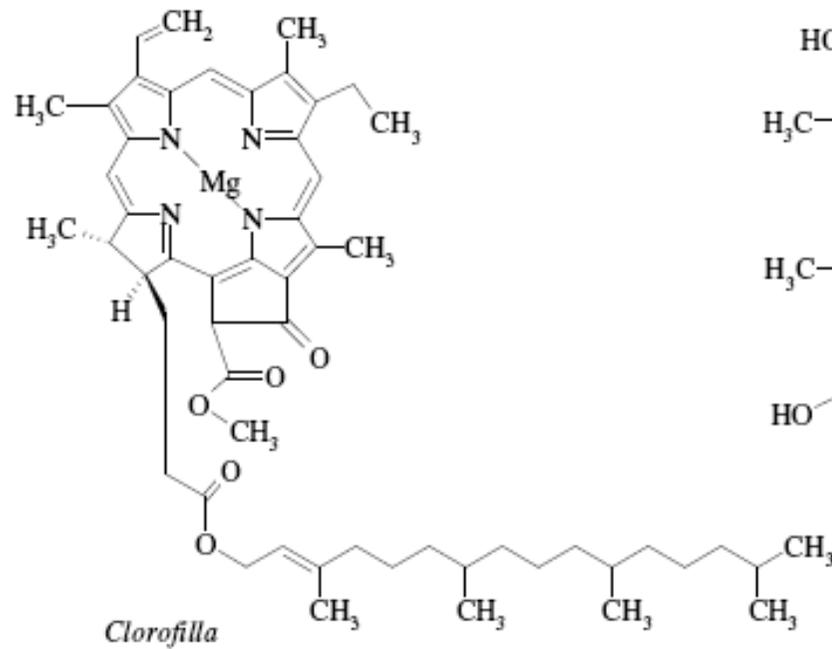
- From this is clear that all molecules fluoresce. But some particularly strong fluorescent molecules are those that possess delocalized electrons through conjugated double bonds. For example, **aromatic molecules are often fluorescent**



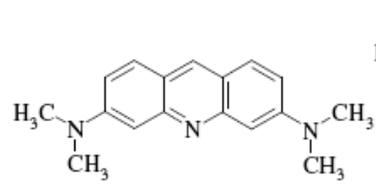
Effetto dei sostituenti sulla fluorescenza del benzene

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

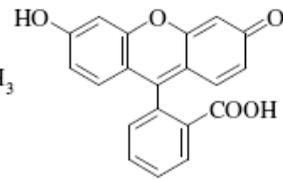
...altre biomolecole fluorescenti...



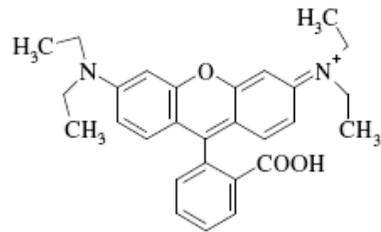
...e molecole fluorescenti usate in biologia



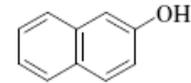
Arancio di acridina



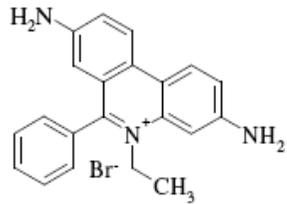
Fluoresceina



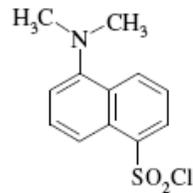
Rodamina B



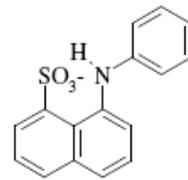
Naftolo



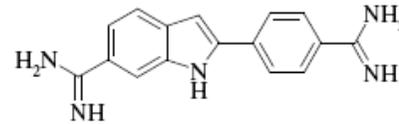
Bromuro di etidio



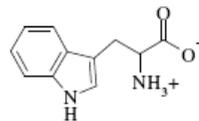
Dansile



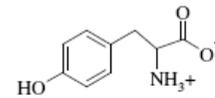
ANS



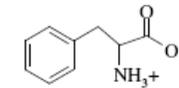
DAPI



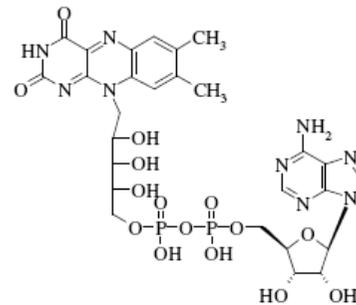
Triptofano (W)



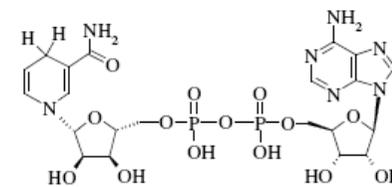
Tirosina (Y)



Fenilalanina (P)



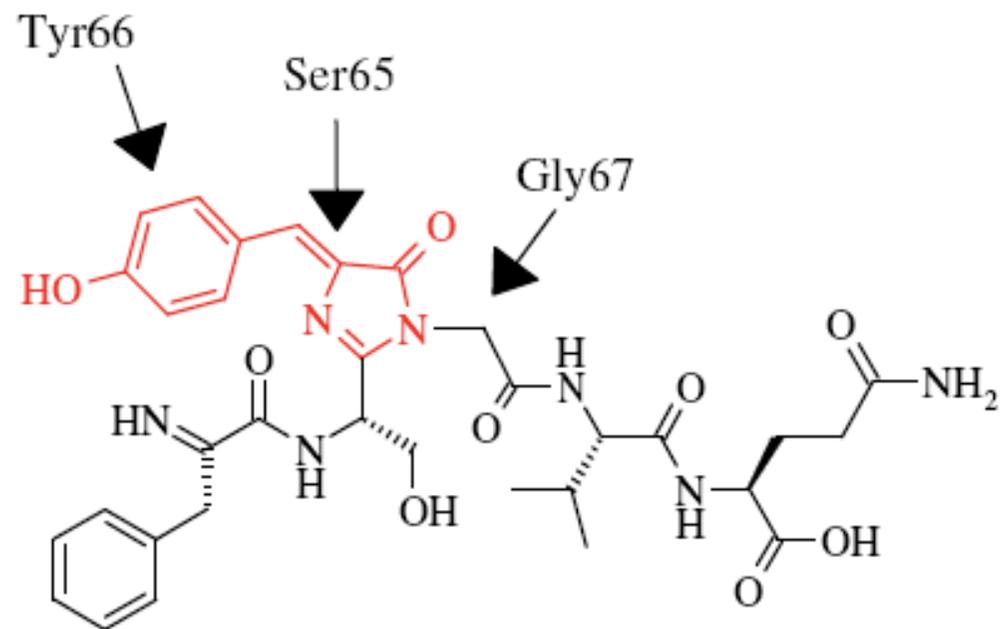
FAD



NADH

Fluoroforo della GFP

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



Fluorescence lifetime and quantum yield

The fluorescence lifetime and quantum yield are perhaps the most important characteristics of a fluorophore.

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

Substances with the largest quantum yields, approaching unity, such as rhodamines, display the brightest emissions.

$$Q = \frac{\Gamma}{\Gamma + k_{nr}} \quad K_{nr} = \text{rate of non-radiative decay}$$

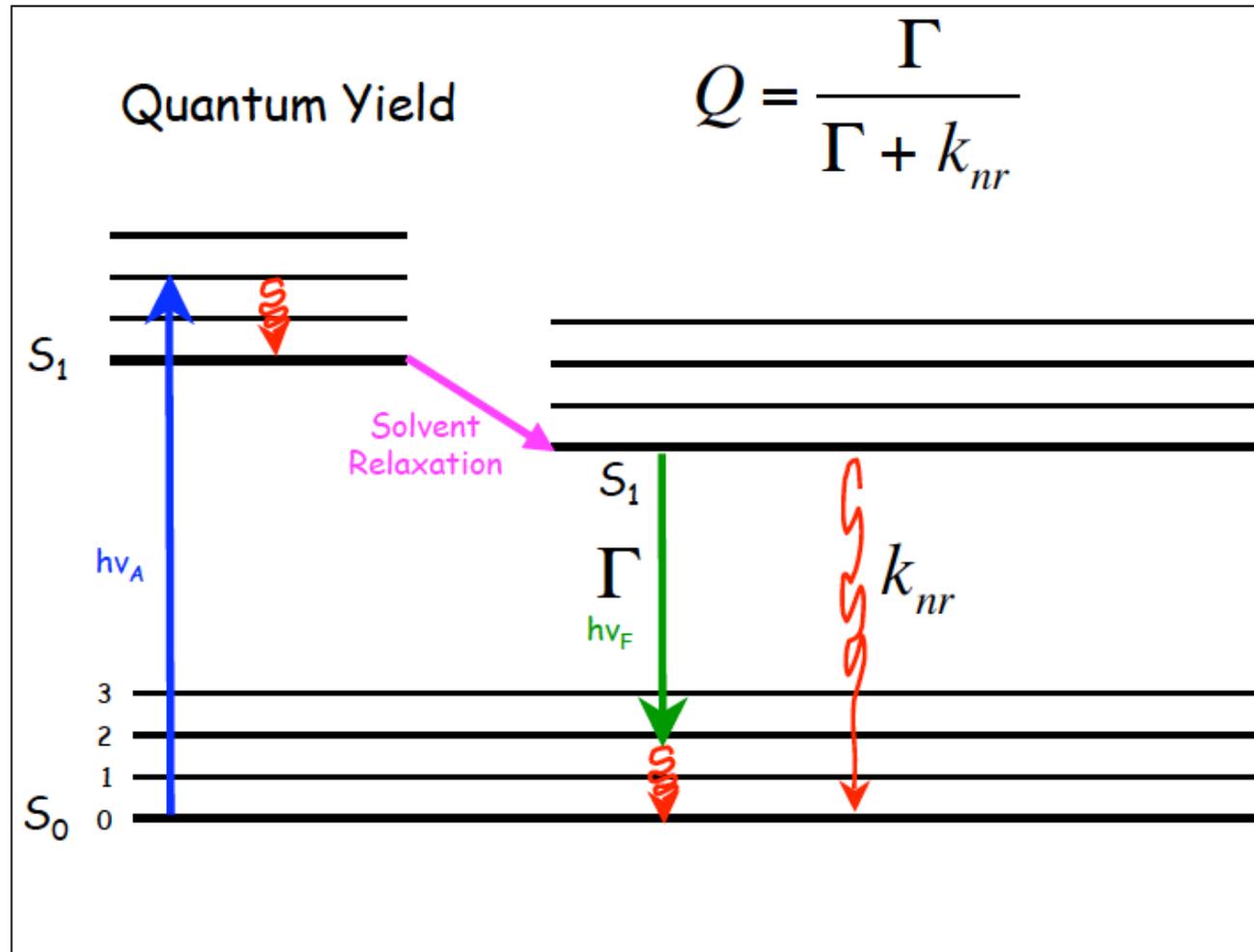
Lifetime: time available for the fluorophore to interact with or diffuse in its environment (time spent in excited state), and hence the information available from its emission

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

FLUORESCENCE LIFETIMES AND QUANTUM YIELDS

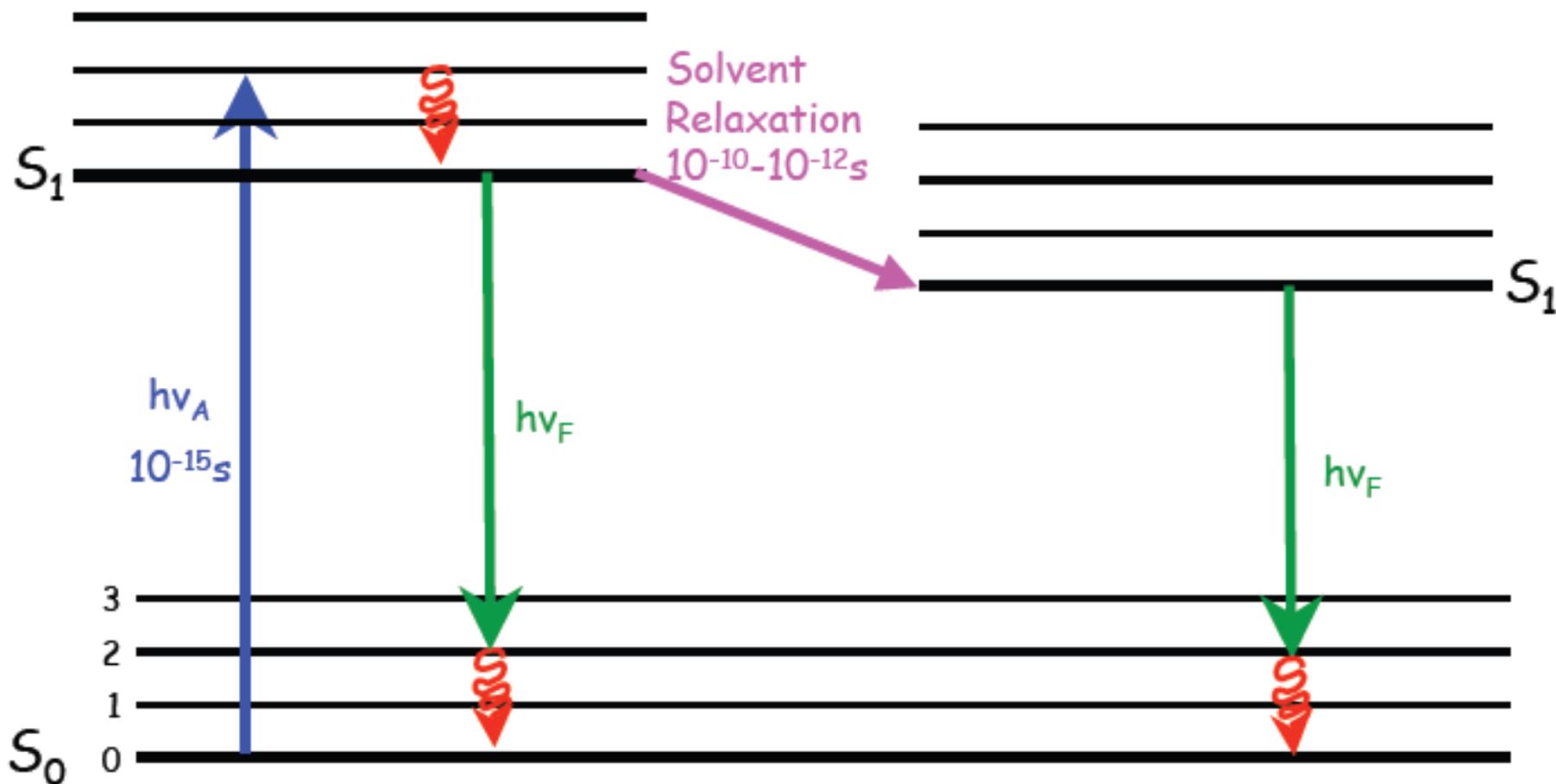
Quantum yield is the number of emitted photons relative to the number of absorbed photons.

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. (also: dissipation through internal vibrational modes)

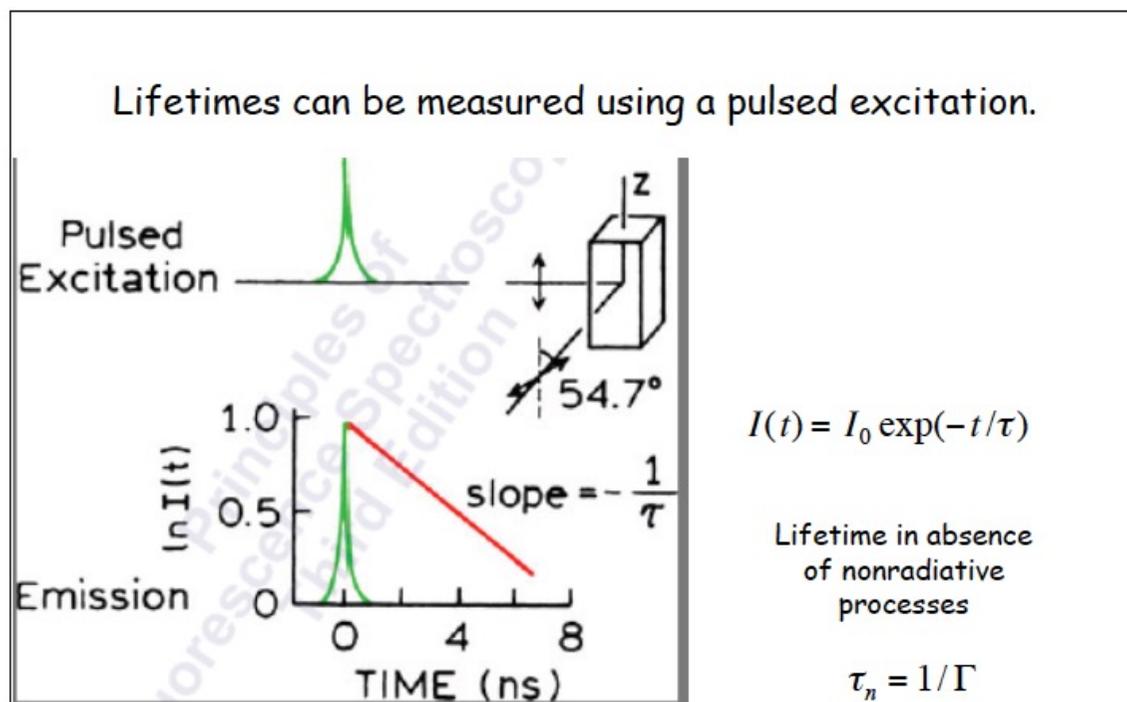
The excited state lifetime allows enough time for solvent to re-organize around it.



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.

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



$$\tau_n = \tau / Q$$

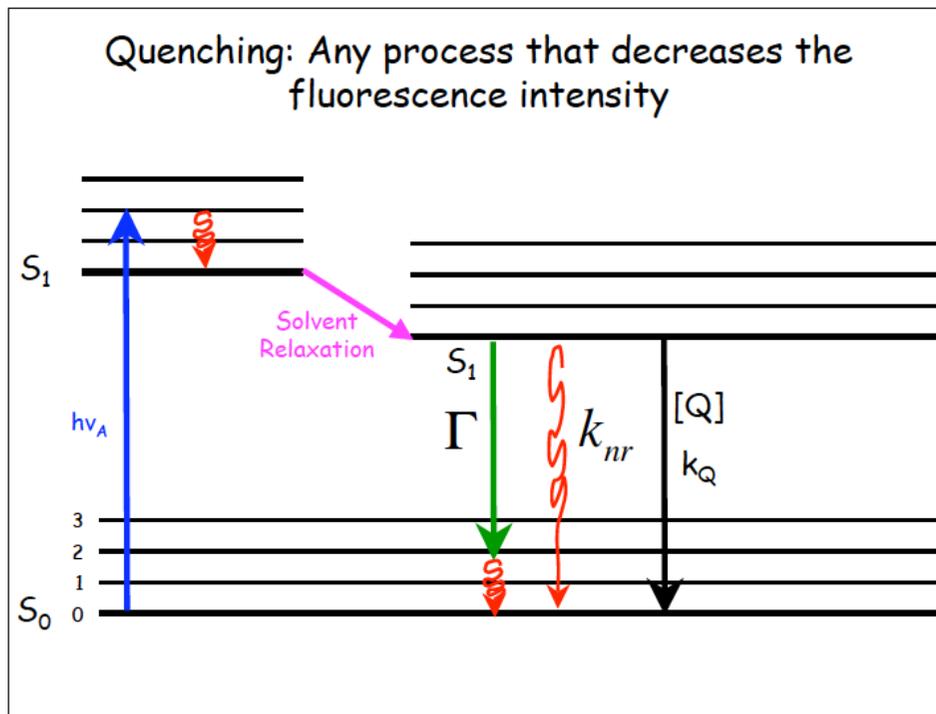
Fluorescence emission is a random process, and few molecules emit their photons at precisely $t = \tau$. The lifetime is an average value of the time spent in the excited state.

For a single exponential decay 63% of the molecules have decayed prior to $t = \tau$ and 37% decay at $t > \tau$.

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

Fluorescence quenching

The intensity of fluorescence can be decreased by a wide variety of processes. Such decreases in intensity are called **quenching**. Quenching can occur by different mechanisms.



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.

One of best known quencher is molecular oxygen: remove dissolved oxygen from solution!
(paramagnetic ox. Push fluorophore via intersystem crossing to triplet state)

Two general kinds of Quenching

Collisional
(Dynamic)

Interactions of a QUENCHER with the EXCITED state.

Static

Interactions of a QUENCHER with the GROUND state.

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

$$\frac{F_{noQuencher}}{F_{withQuencher}} = \frac{F_0}{F} = 1 + k_q \tau_0 [Q]$$
$$= 1 + K[Q]$$

Stern-Volmer constant

K_{SV}

K_D

Seems unwise, but is
Used sometimes

K_D^{-1} = quencher concentration at which 50% of the intensity is quenched

The Stern-Volmer equation can be derived by consideration of the fluorescence intensities observed in the absence and presence of quencher. The fluorescence intensity observed for a fluorophore is proportional to its concentration in the excited state, $[F^*]$. Under continuous illumination a constant population of excited fluorophores is established, and therefore $d[F^*]/dt = 0$. In the absence and presence of quencher the differential equations describing $[F^*]$ are

$$\frac{d[F^*]}{dt} = f(t) - \gamma[F^*]_0 = 0 \quad (8.2)$$

$$\frac{d[F^*]}{dt} = f(t) - (\gamma + k_q[Q])[F^*] = 0 \quad (8.3)$$

where $f(t)$ is the constant excitation function, and $\tilde{a} = \tau_0^{-1}$ is the decay rate of the fluorophore in the absence of quencher. In the absence of quenching the excited-state population decays with a rate $\tilde{a} = (\Gamma + k_{nr})$, where Γ is the radiative rate and k_{nr} is the non-radiative decay rate. In the presence of quencher there is an additional decay rate $k_q[Q]$. With continuous excitation the excited-state population is constant, so the derivatives in these equations can be set to zero. Division of eq. 8.3 by 8.2 yields

$$\frac{F_0}{F} = \frac{\gamma + k_q[Q]}{\gamma} = 1 + k_q\tau_0[Q] \quad (8.4)$$

which is the Stern-Volmer equation.

The Stern-Volmer equation may also be obtained by considering the fraction of excited fluorophores, relative to the total, which decay by emission. This fraction (F/F_0) is given by the ratio of the decay rate in the absence of quencher (γ) to the total decay rate in the presence of quencher ($\gamma + k_q[Q]$):

$$\frac{F}{F_0} = \frac{\gamma}{\gamma + k_q[Q]} = \frac{1}{1 + K_D[Q]} \quad (8.5)$$

which is again the Stern-Volmer equation. Since collisional quenching is a rate process that depopulates the excited state, the lifetimes in the absence (τ_0) and presence (τ) of quencher are given by

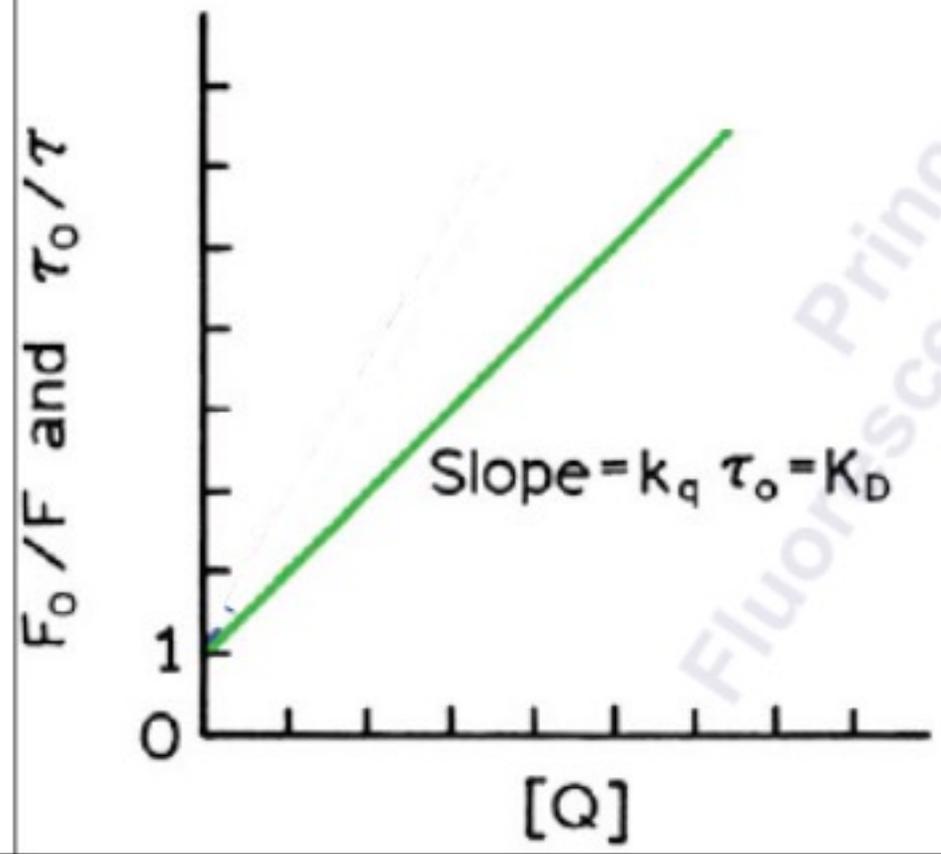
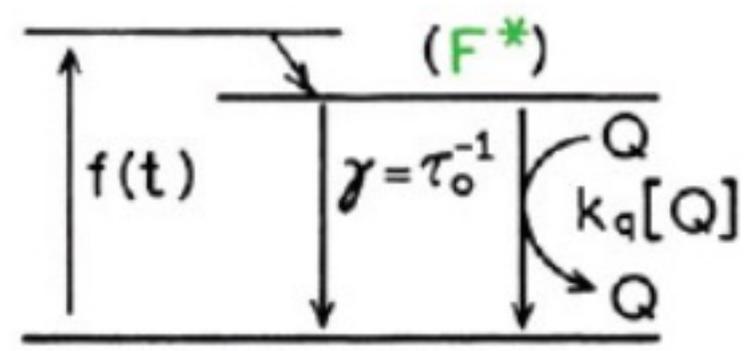
$$\tau_0 = \gamma^{-1} \quad (8.6)$$

$$\tau = (\gamma + k_q[Q])^{-1} \quad (8.7)$$

and therefore

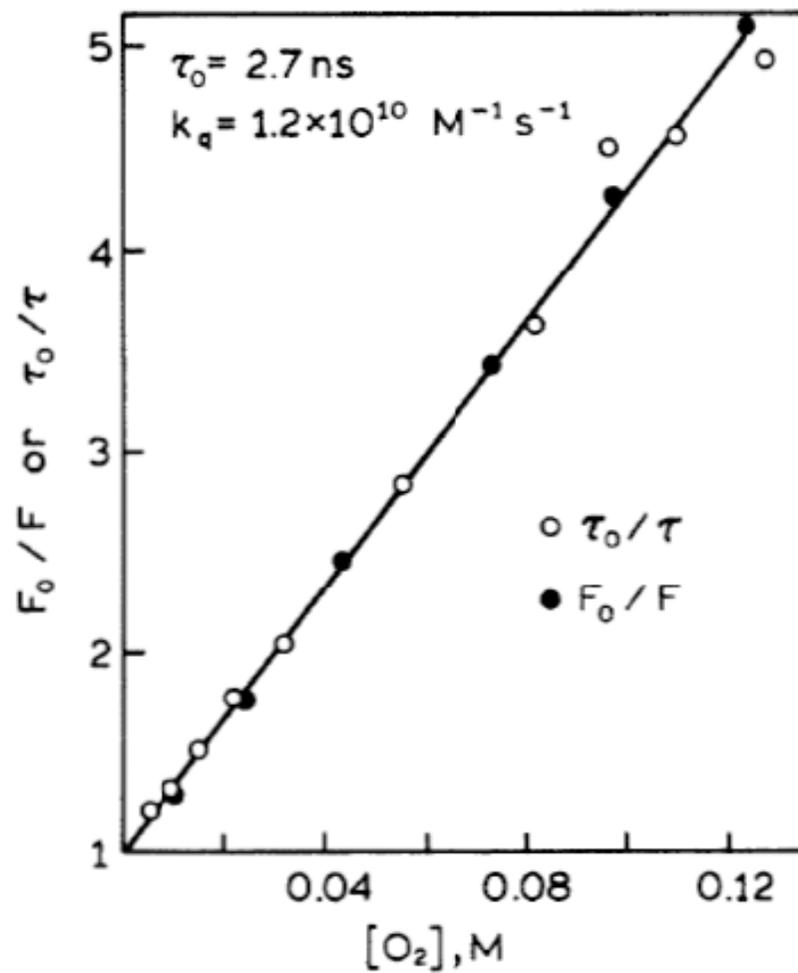
$$\frac{\tau_0}{\tau} = 1 + k_q\tau_0[Q] \quad (8.8)$$

Collisional Quenching



Principles of Fluorescence Spectroscopy
Th...

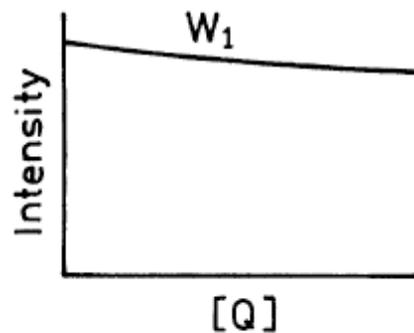
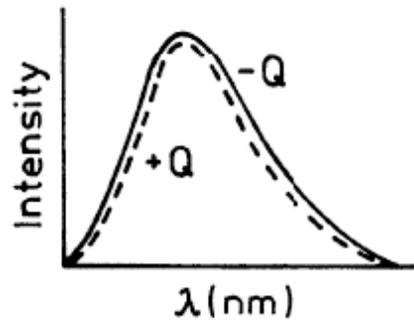
Oxygen quenching of Tryptophan fluorescence



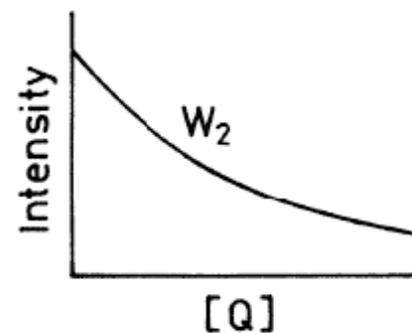
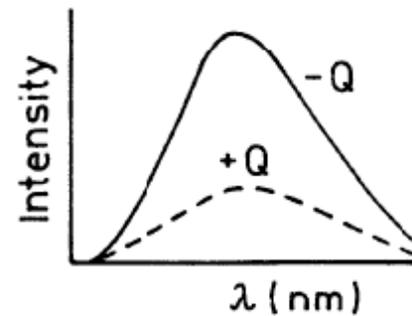
A fluorophore buried in a macromolecule is usually inaccessible to water soluble quenchers, so that the value of K is low. Larger values of K are found if the fluorophore is free in solution or on the surface of a biomolecule.

A fluorophore buried in a macromolecule is usually inaccessible to water soluble quenchers

Buried Tryptophan



Surface Tryptophan



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 25 C is $2.5 \times 10^{-5} \text{ cm}^2/\text{s}$.

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:

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

$$\text{O}_2 \quad D = 2.5 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$$

Use Trp Lifetime of 4 ns \longrightarrow 44 Å

Some fluorophores have lifetimes as long as 400 ns, hence diffusion of oxygen molecules may be observed over distances of 450 Å

Static Quenching

Formation of a complex between the **Quencher** and the fluorophore that **does not** rely on diffusion of the excited state.

Formation of a complex between **Quencher** and **Ground** state

$$K_s = \frac{[FQ]}{[F][Q]} \quad \text{Association (binding) constant}$$

$$[F_T] = [F] + [FQ] \quad K_s = \frac{[F]_T - [F]}{[F][Q]} = \frac{[F]_T}{[F][Q]} - \frac{1}{[Q]}$$

Static Quenching

$$K_S = \frac{[F]_T}{[F][Q]} - \frac{1}{[Q]}$$

$$K_S = \frac{[I_T]}{[I_{Obs}][Q]} - \frac{1}{[Q]}$$

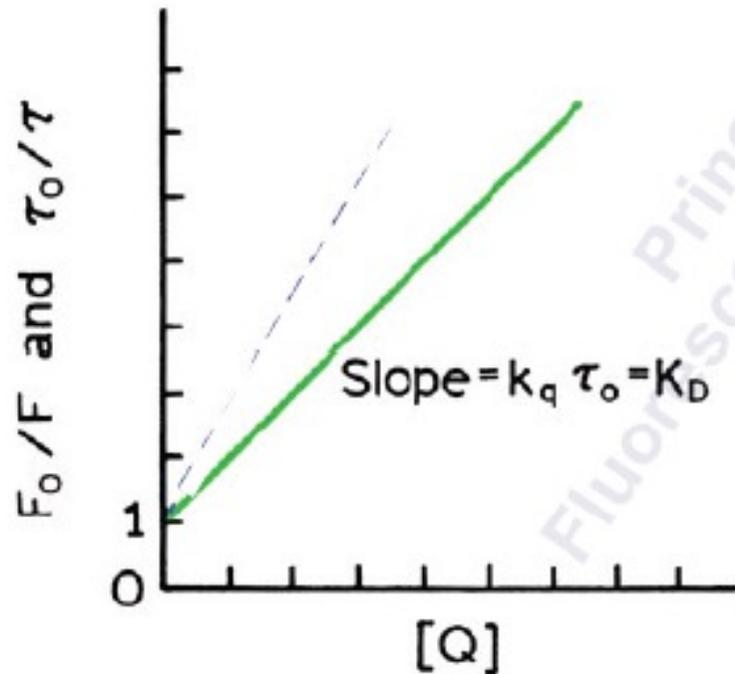
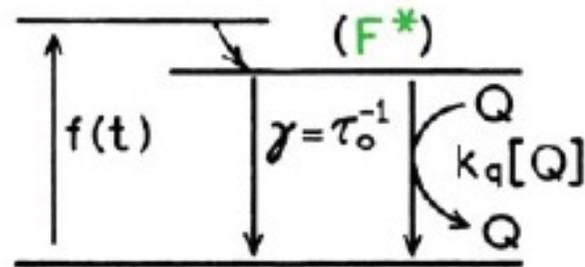
$$\frac{F_{noQuencher}}{F_{withQuencher}} = \frac{F_0}{F} = 1 + [Q]K_S$$

$$[Q]K_S = \frac{[I_T]}{[I_{Obs}]} - 1$$

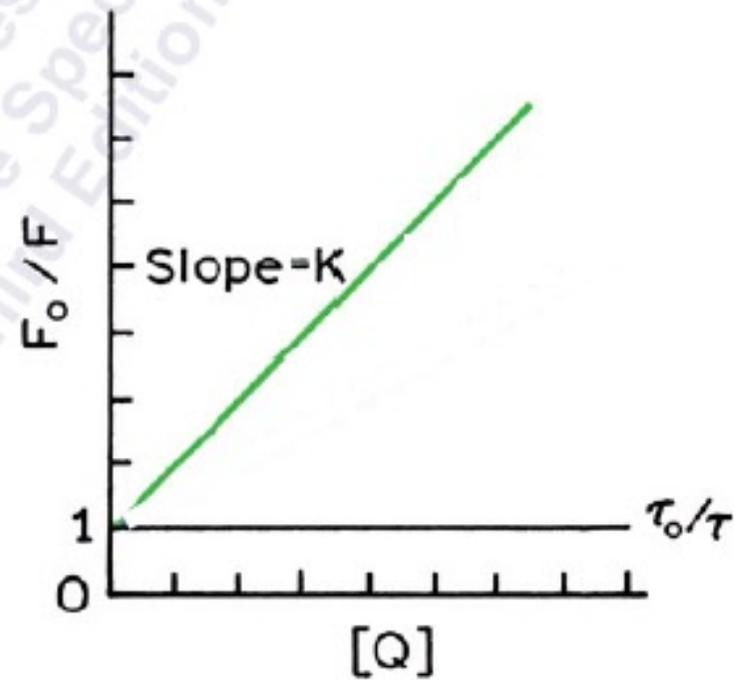
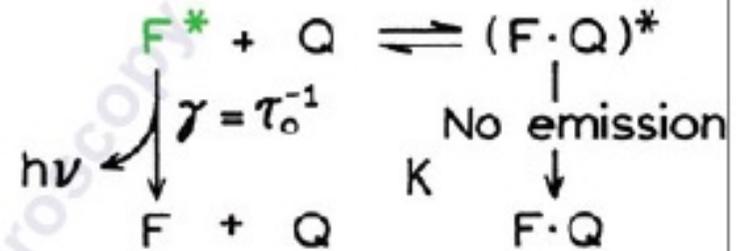
$$[Q]K_S + 1 = \frac{[I_T]}{[I_{Obs}]}$$

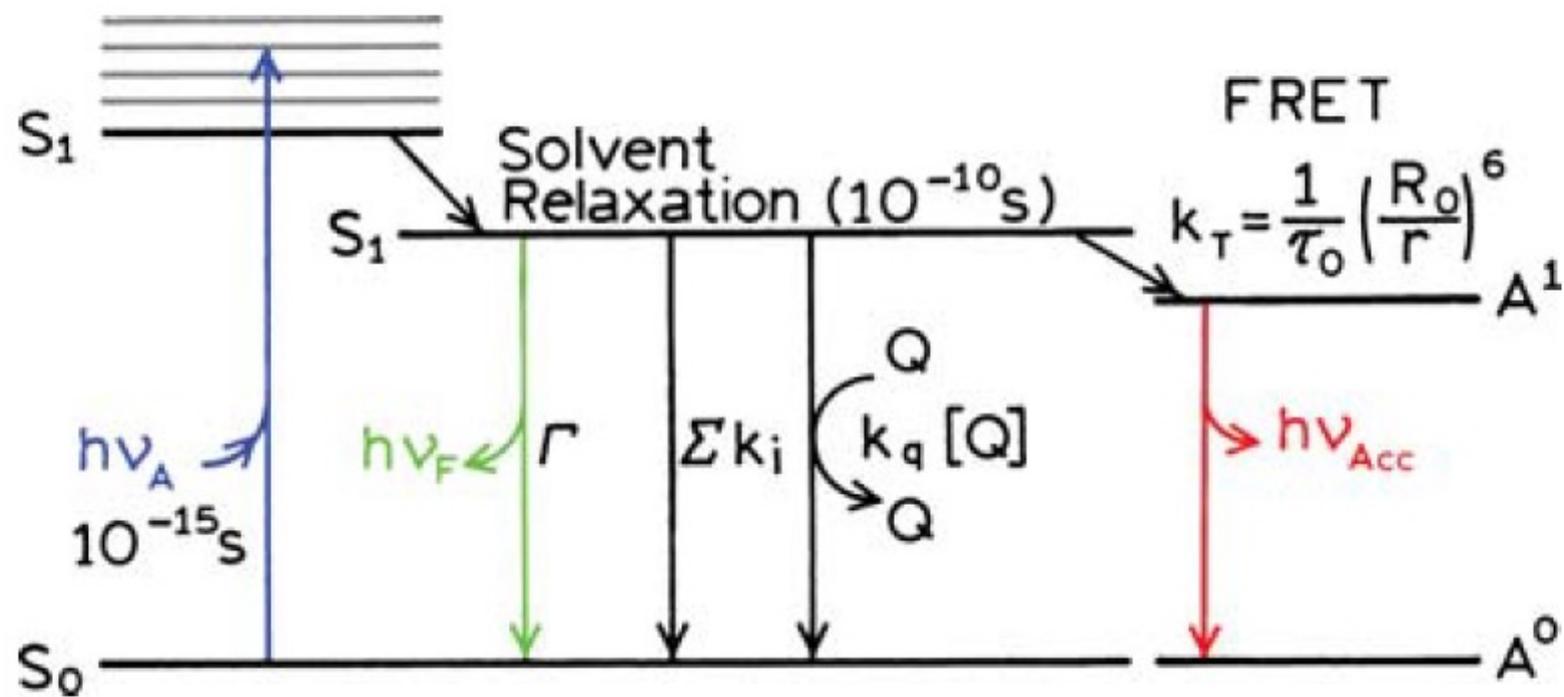
Fluorescence quenching

Collisional Quenching



Static Quenching





Gold surface as a quencher

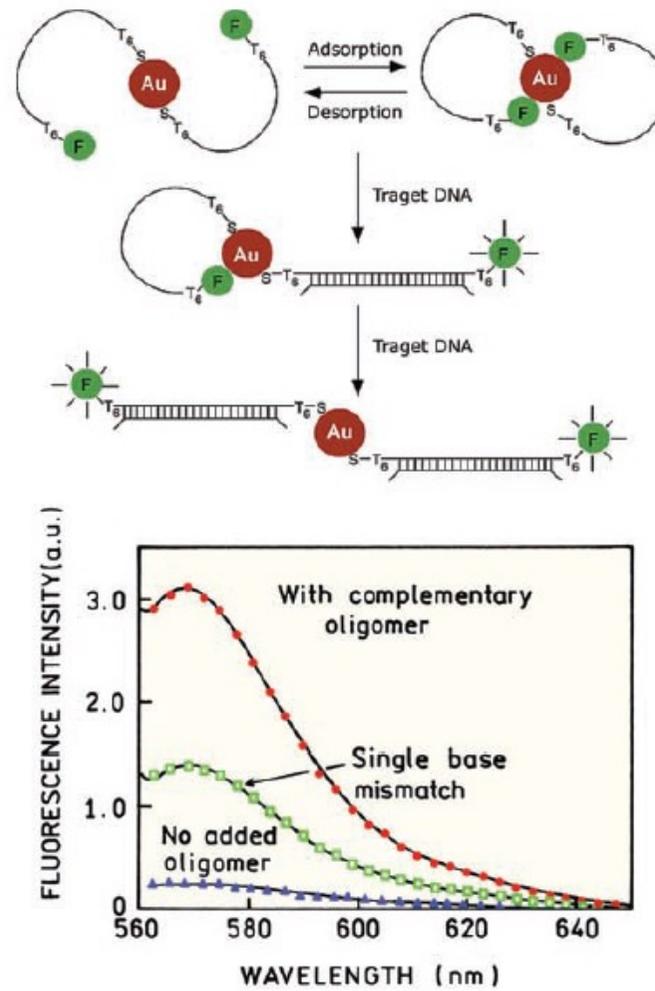
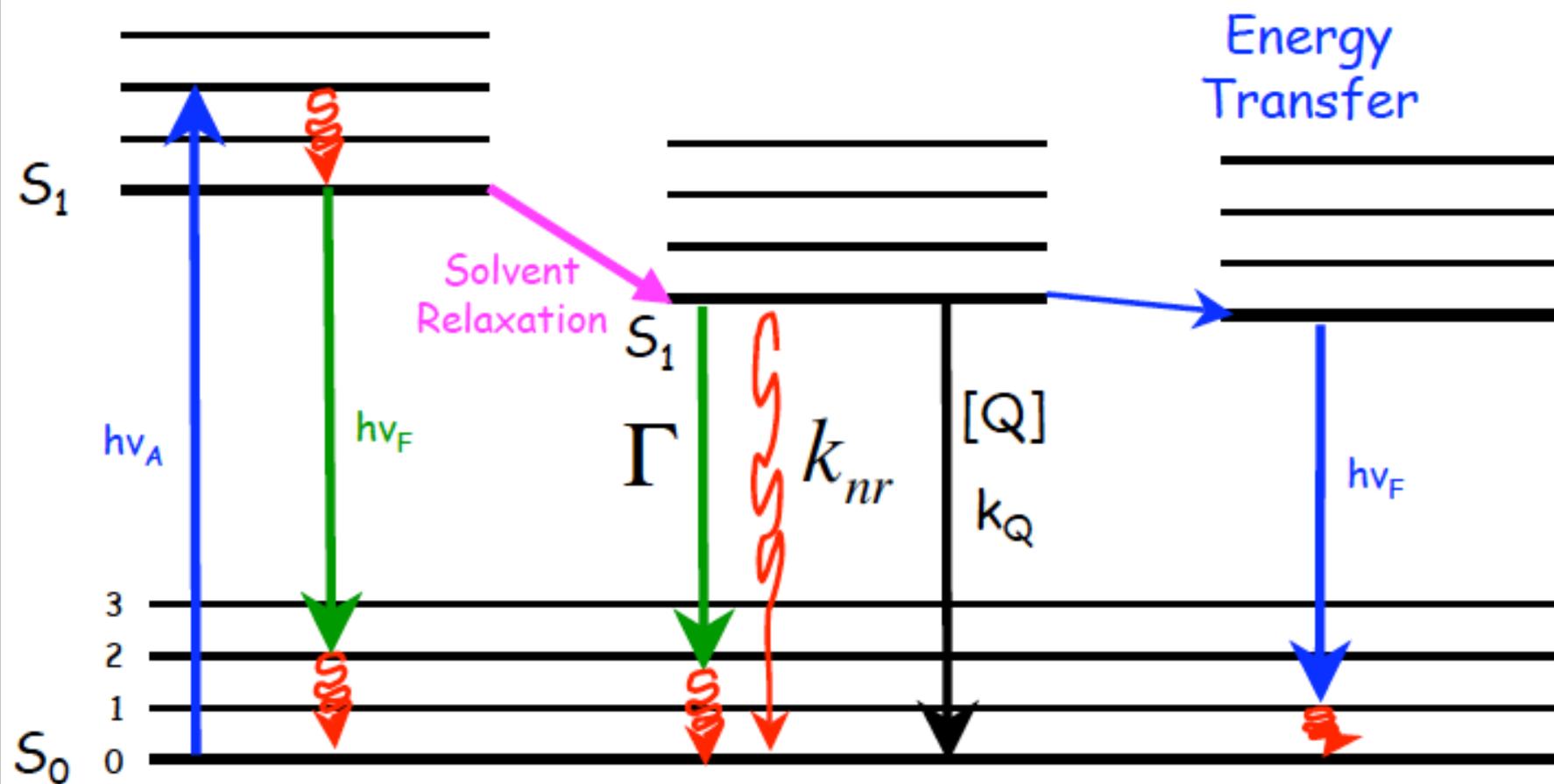


Figure 8.64. Schematic and emission spectra for a TMR-labeled oligo bound to gold colloids. Revised and reprinted with permission from [152]. Copyright © 2002, American Chemical Society.

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.

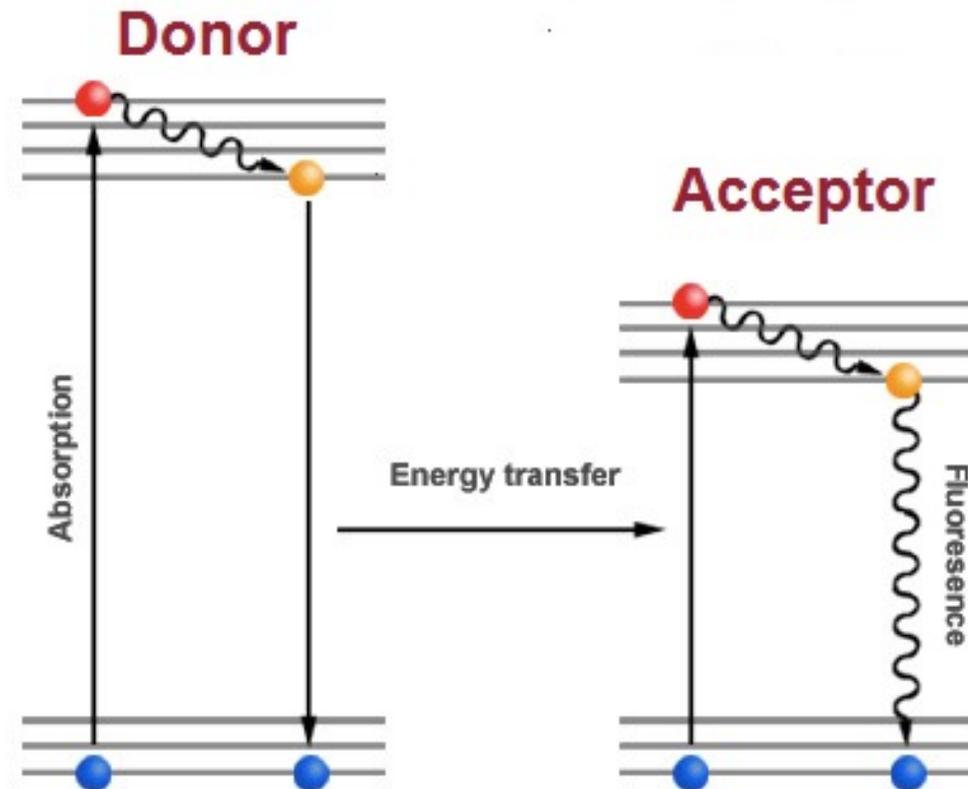
No photon is involved in the transfer.

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

Förster (Fluorescence) Resonance Energy Transfer (FRET)

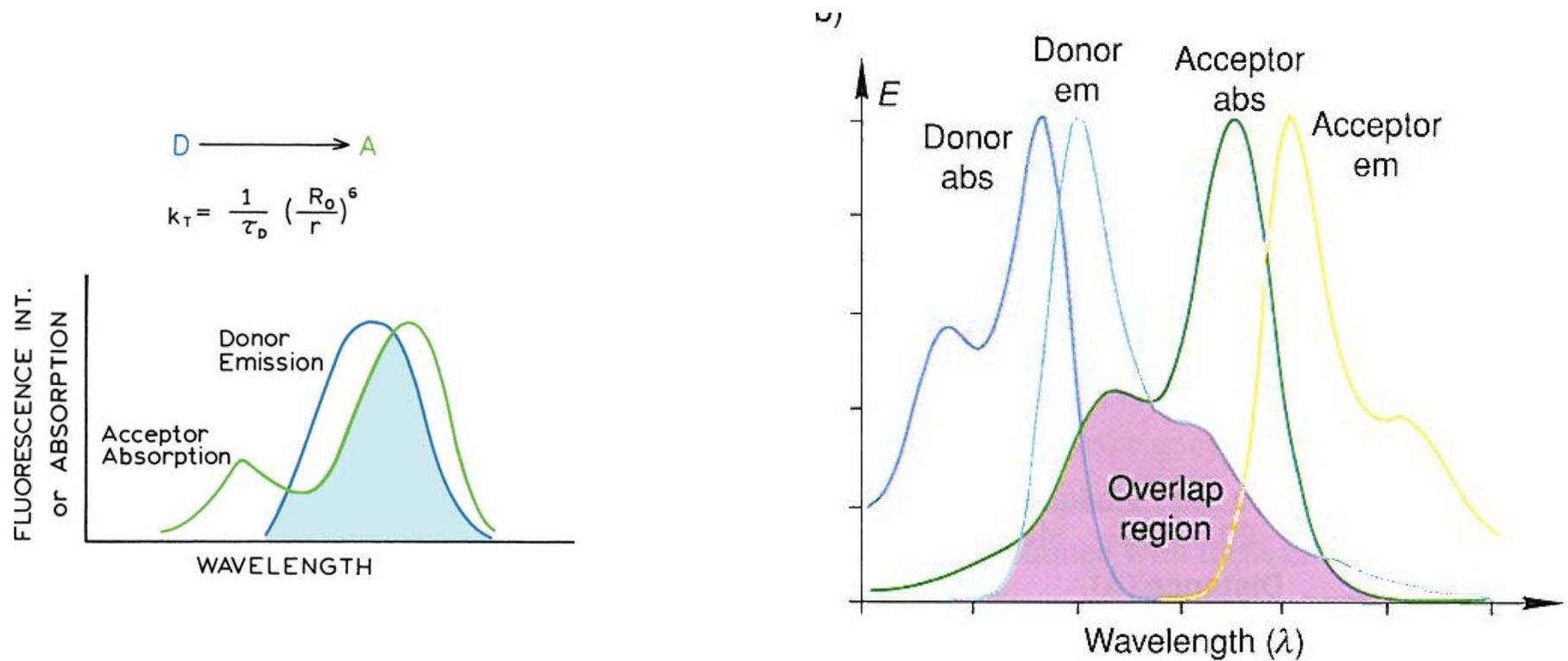
- The technique of FRET, when applied to optical microscopy, permits to determine the approach between two molecules within several nanometers
- FRET is a distance dependant radiationless transfer of energy from an excited donor fluorophore to a suitable acceptor fluorophore, is one of few tools available 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)



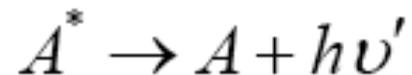
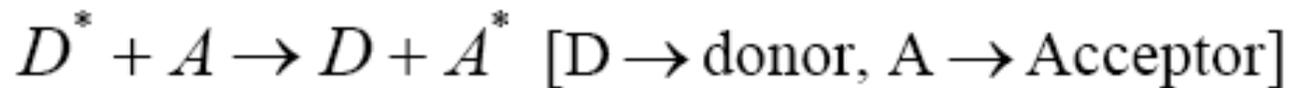
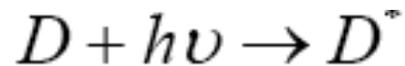
In presence of suitable acceptor, the donor fluorophore can transfer its excited state energy directly to the acceptor without emitting a photon.

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.



- 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)

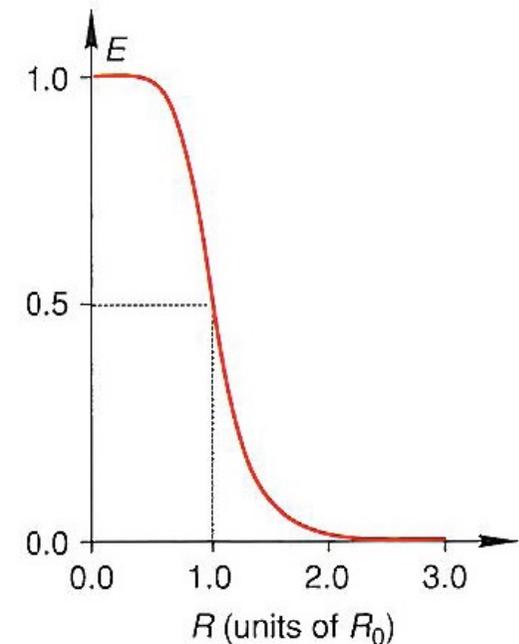
- There are few criteria that must be satisfied in order for FRET to occur. These are:
 - (i) the fluorescence emission spectrum of the donor molecule must overlap the absorption or excitation spectrum of the acceptor chromophore. The degree of overlap is referred to as spectral overlap integral (J).
 - (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

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

Fluorescence Resonance Energy Transfer (FRET)

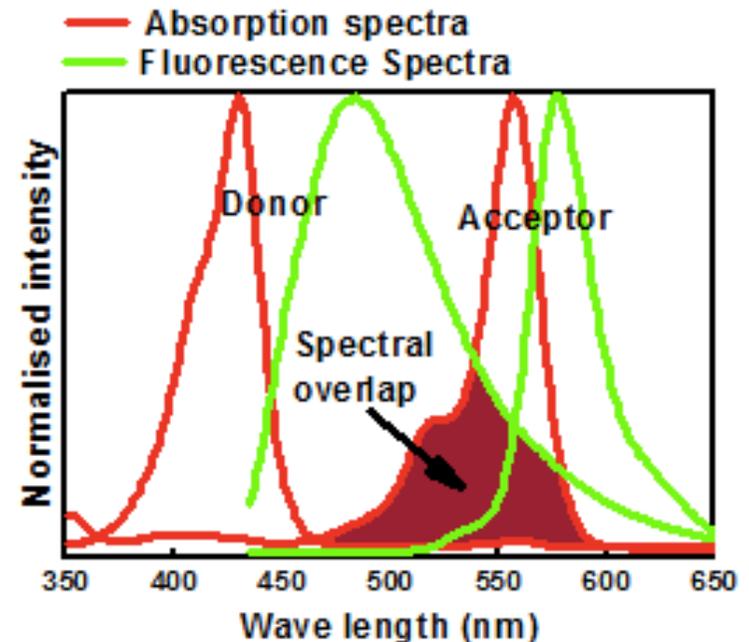
- In summary, the rate of FRET depends upon the extent of spectral overlap between the donor acceptor pair, the quantum yield of the donor, the relative orientation of the donor-acceptor transition dipole moments and the distance separating the donor-acceptor chromophore.

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)

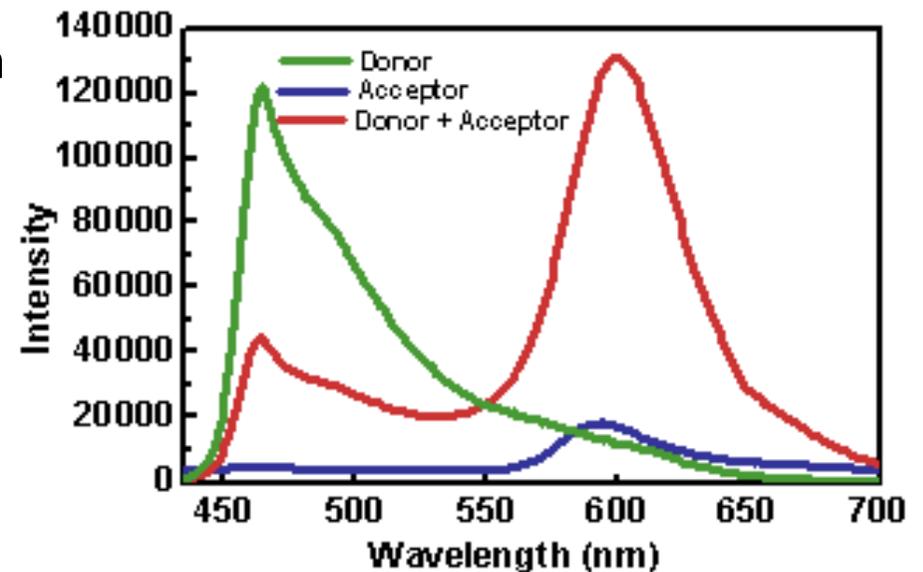
The detection and quantitation of FRET can be made in a number of different ways. Simply the phenomenon can be observed by exciting a specimen containing both the donor and acceptor molecules with light emitted at wavelengths centered near the emission maximum of the acceptor.

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.



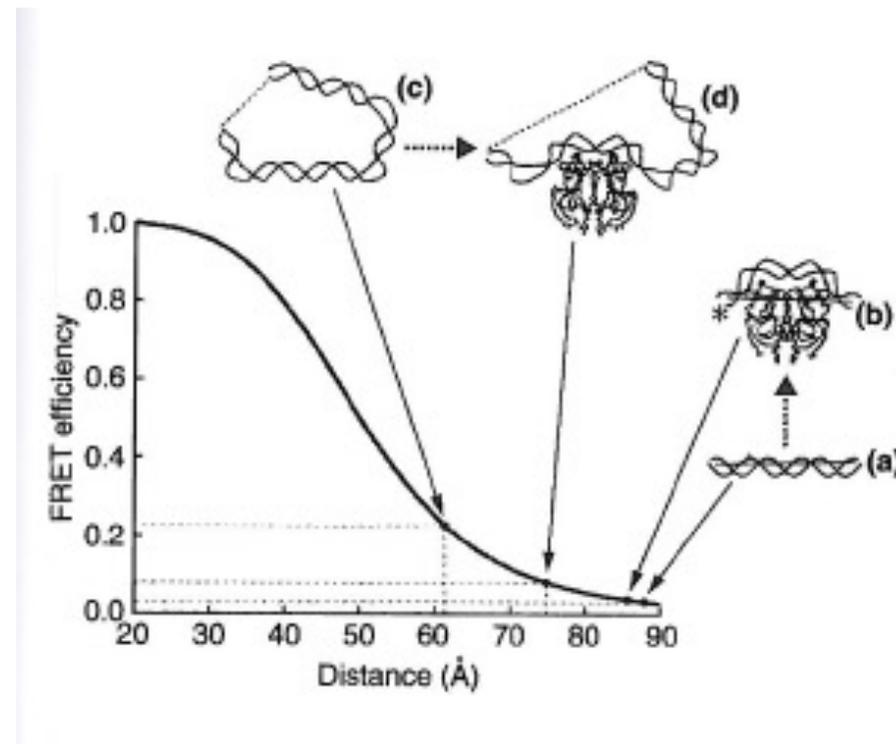
Another alternative method, is to measure the fluorescence lifetime of the donor fluorophore in the presence and absence of the acceptor chromophore. FRET will cause a decrease in excited lifetime of the donor fluorophore.

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 10–100Å°.

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.

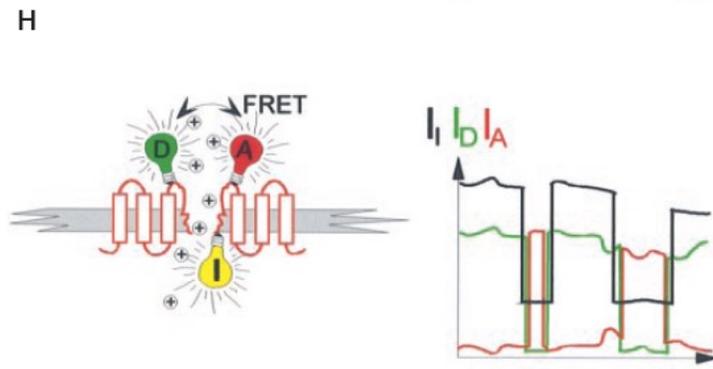
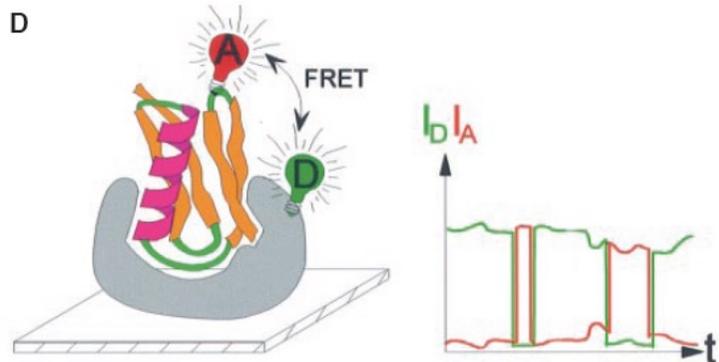
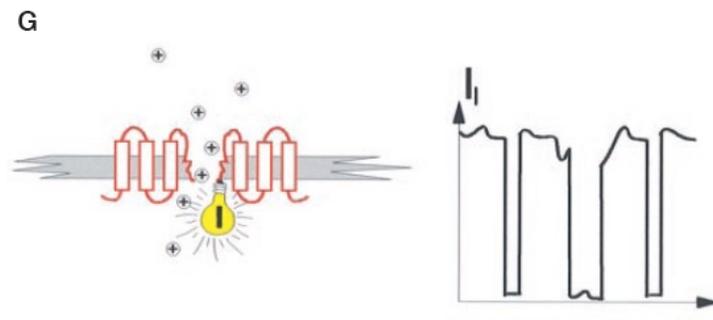
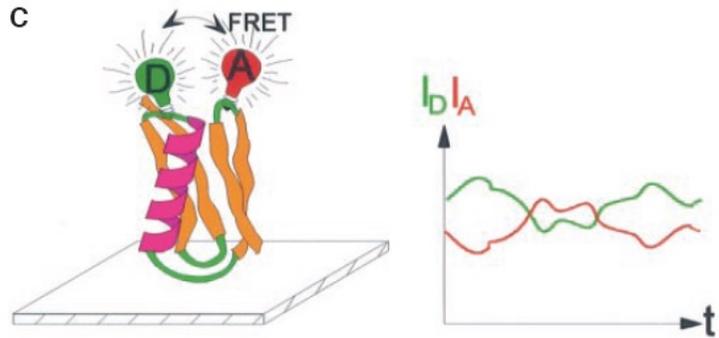
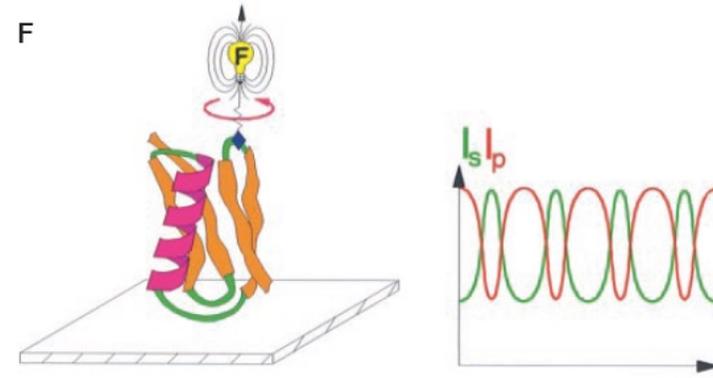
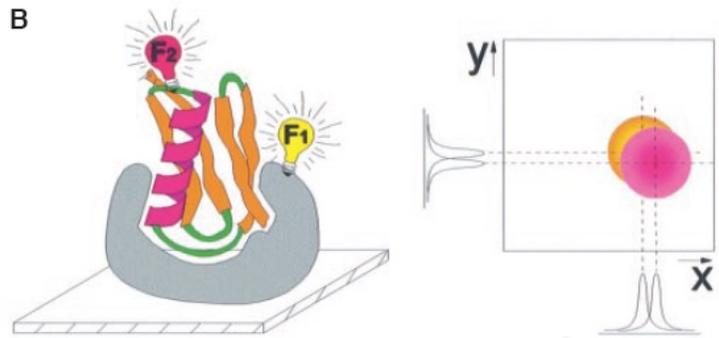
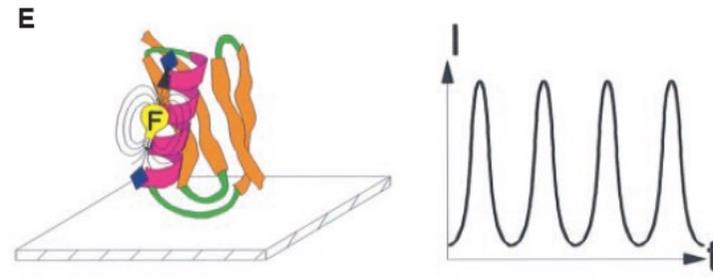
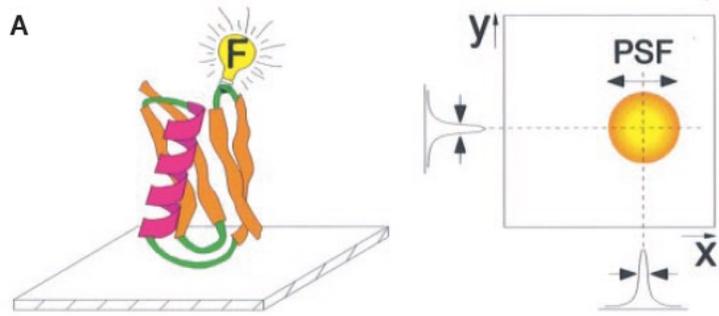


Fig. 1. Labeling schemes (left) and physical observables (right). **(A)** Localization of a macromolecule labeled with a single fluorophore F with nanometer accuracy. The point-spread-function (PSF) can be localized within a few tenths of a nanometer. **(B)** Colocalization of two macromolecules labeled 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.

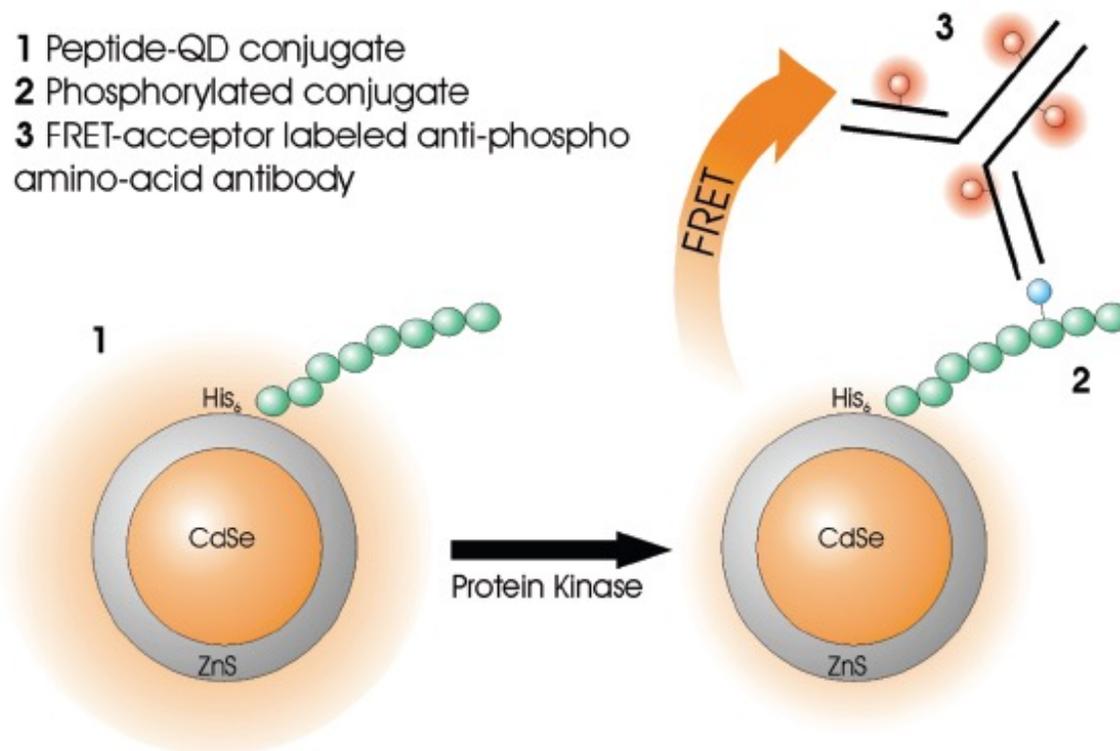


Figure 1. Schematic representation of kinase-mediated phosphorylation of peptide–QD conjugates, antibody recognition of phosphopeptide, and FRET detection.

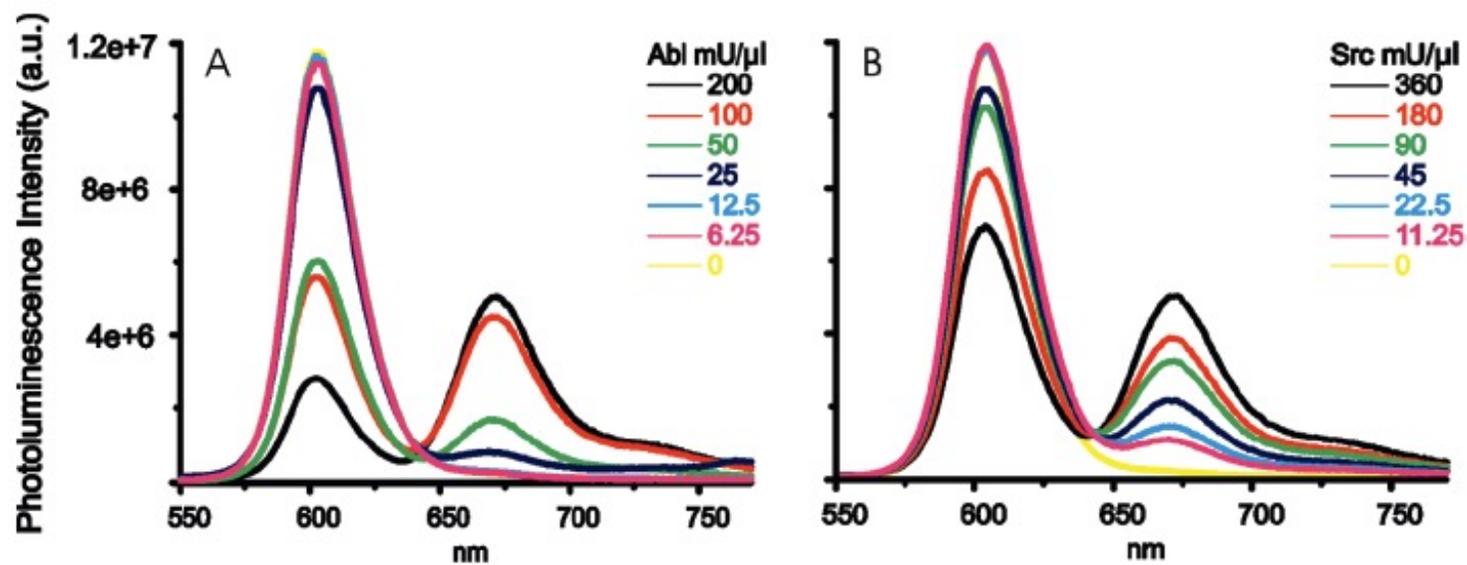


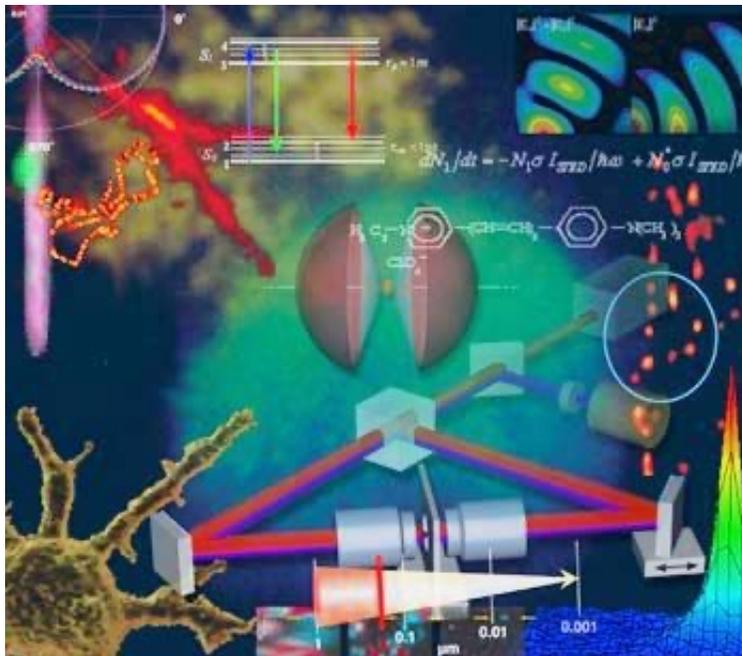
Figure 3. Steady-state emission spectra ($\lambda_{\text{ex}} = 400 \text{ nm}$) of Abl (A) and Src (B) kinase reactions using peptide–QD conjugate substrates following 1 h enzyme reactions and addition of FRET-acceptor labeled antibody.



Max Planck
Society

Fluorescence Nanoscopy

4Pi / STED / RESOLFT



Stefan W. Hell

[Max Planck Institute for Biophysical Chemistry](#)

[Department of NanoBiophotonics](#)

Göttingen, Germany

hell@nanoscopy.de



Far-Field Optical Nanoscopy

Stefan W. Hell

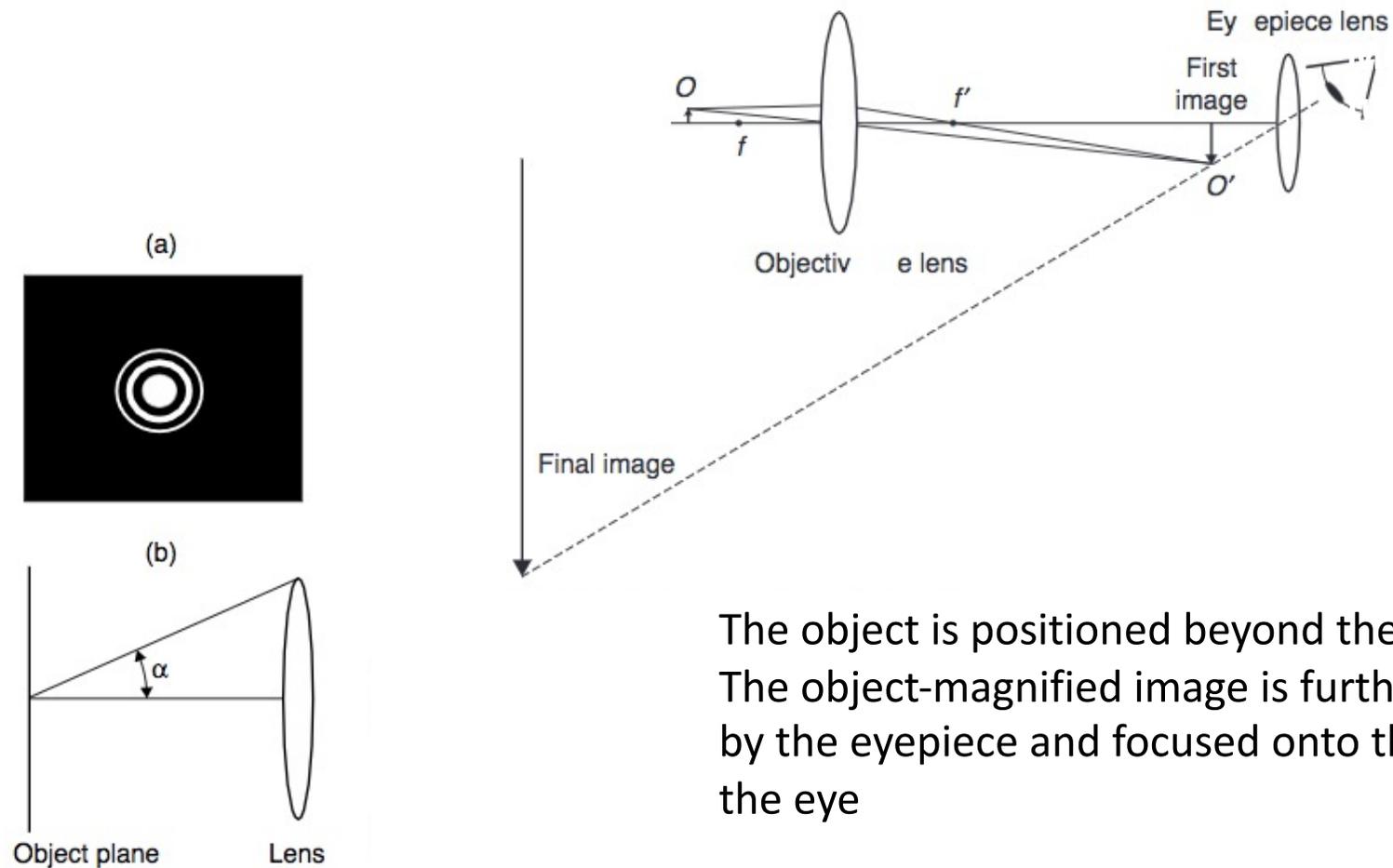
By providing a spatial resolution down to the atomic scale, electron and scanning probe microscopy have revolutionized our understanding of life and matter. Nonetheless, optical microscopy has maintained its key role in many fields, in particular in the life sciences. This stems from a number of rather 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

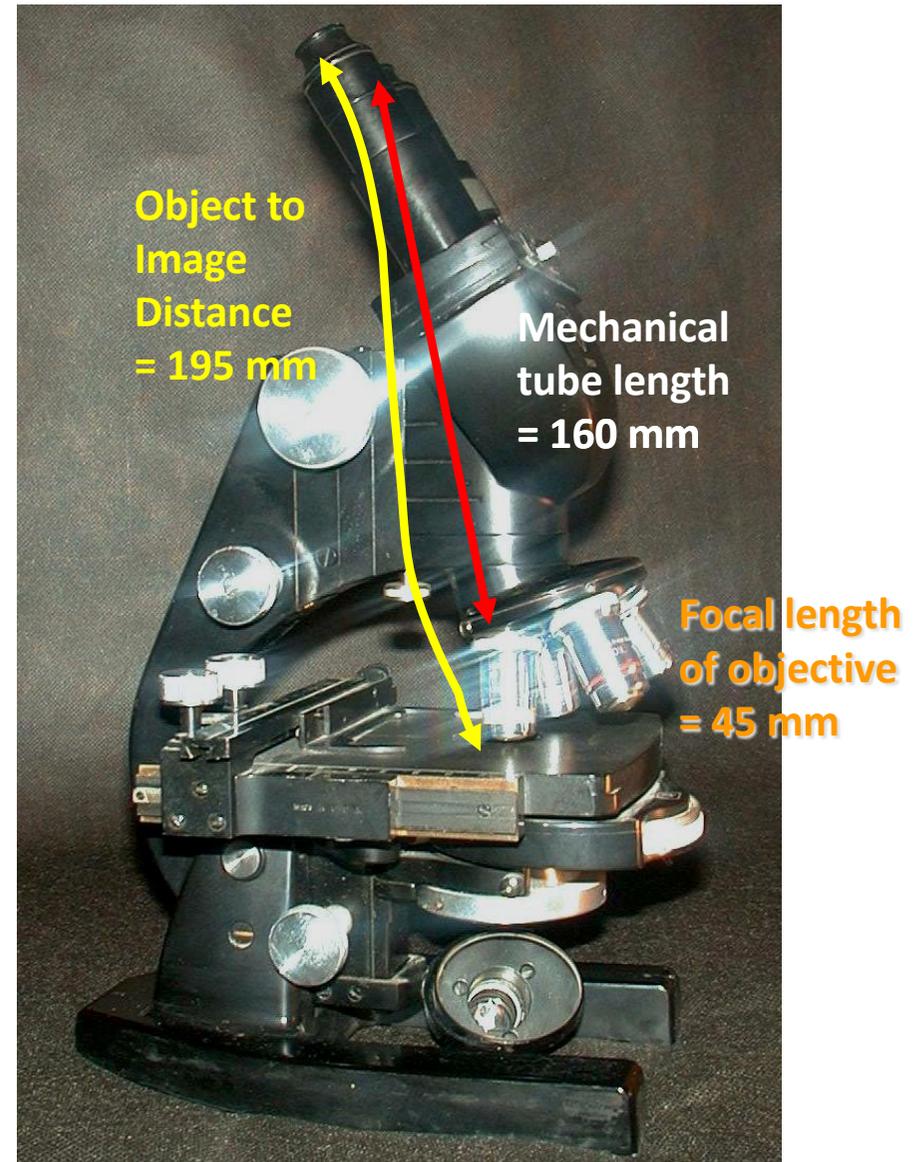
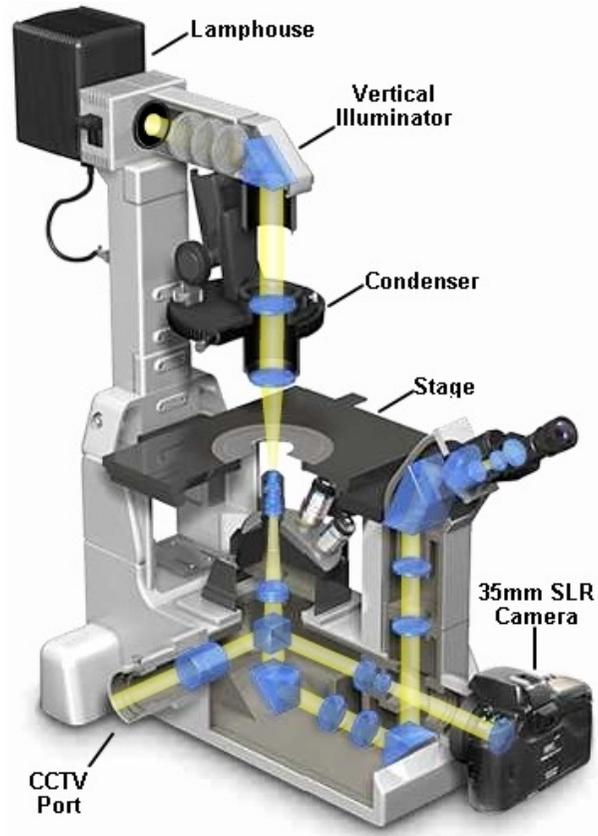
Compound lens microscope

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

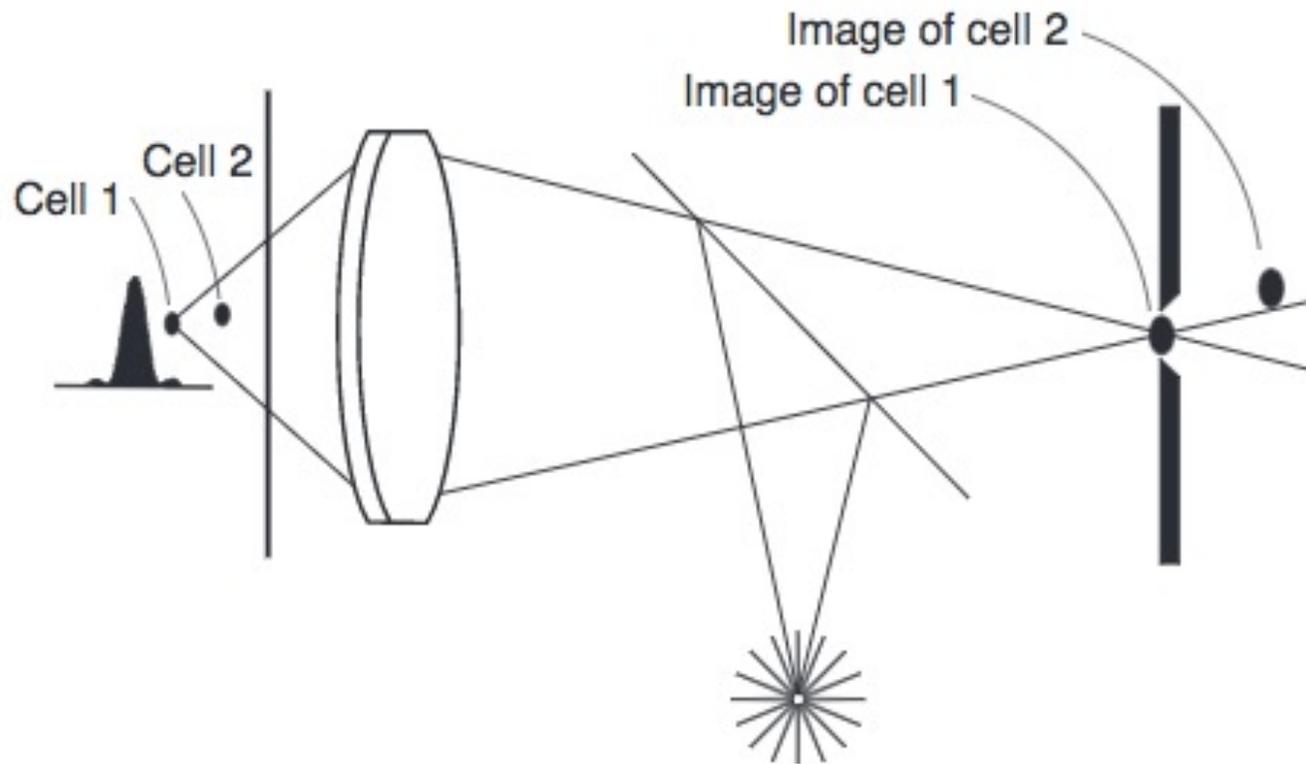
Compound lens microscope



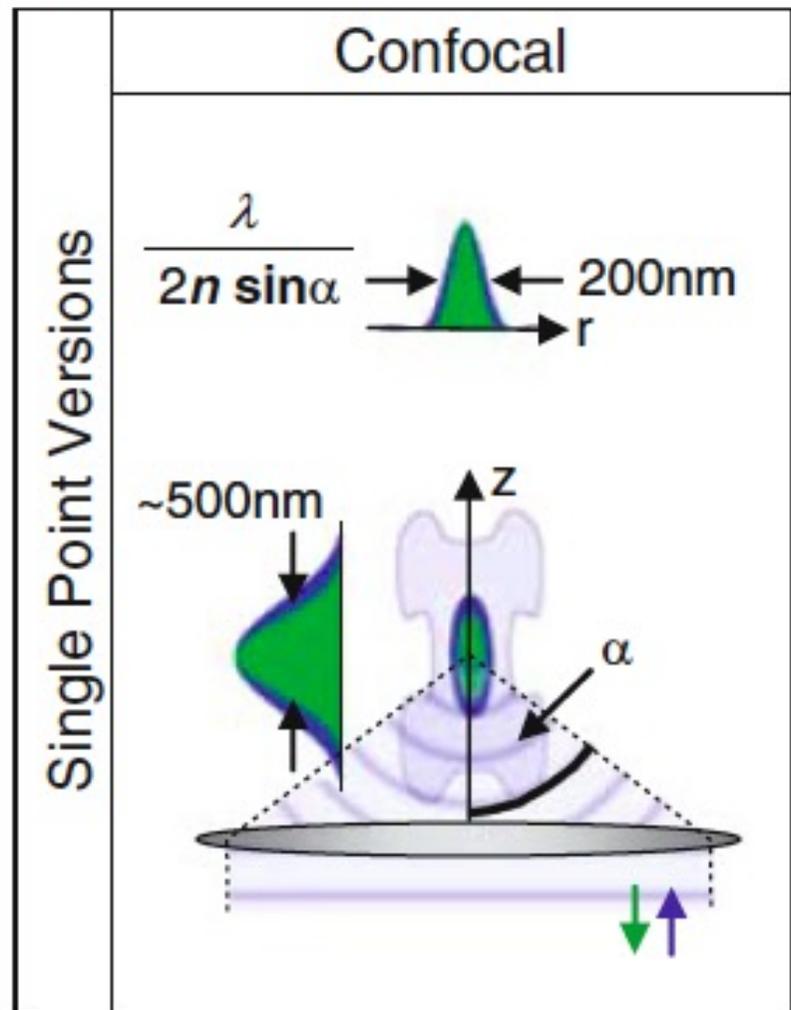
Key ideas in optical microscopy

- 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.

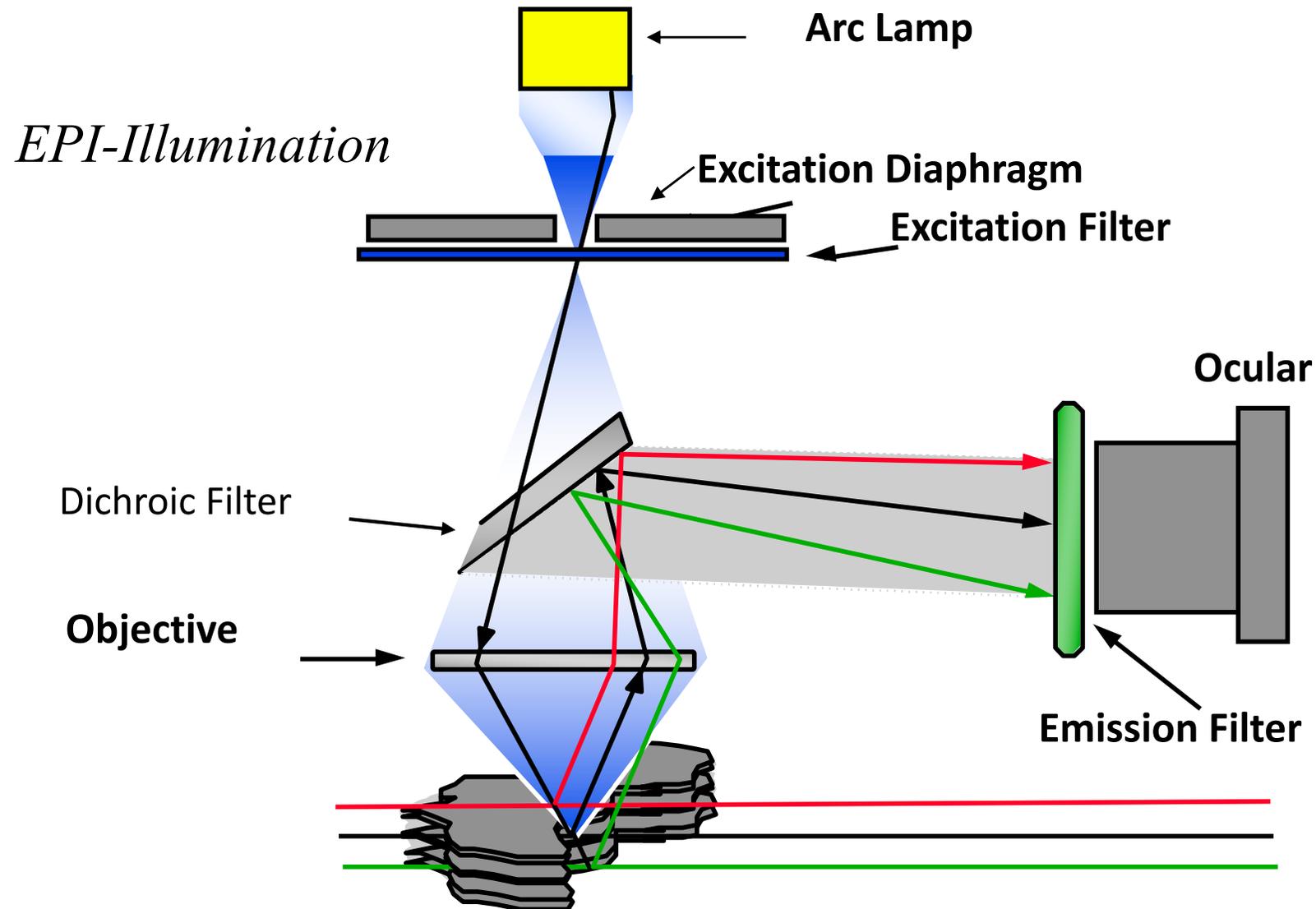
Confocal microscopy



a



Fluorescent Microscope



Fluorescence Microscope

upright

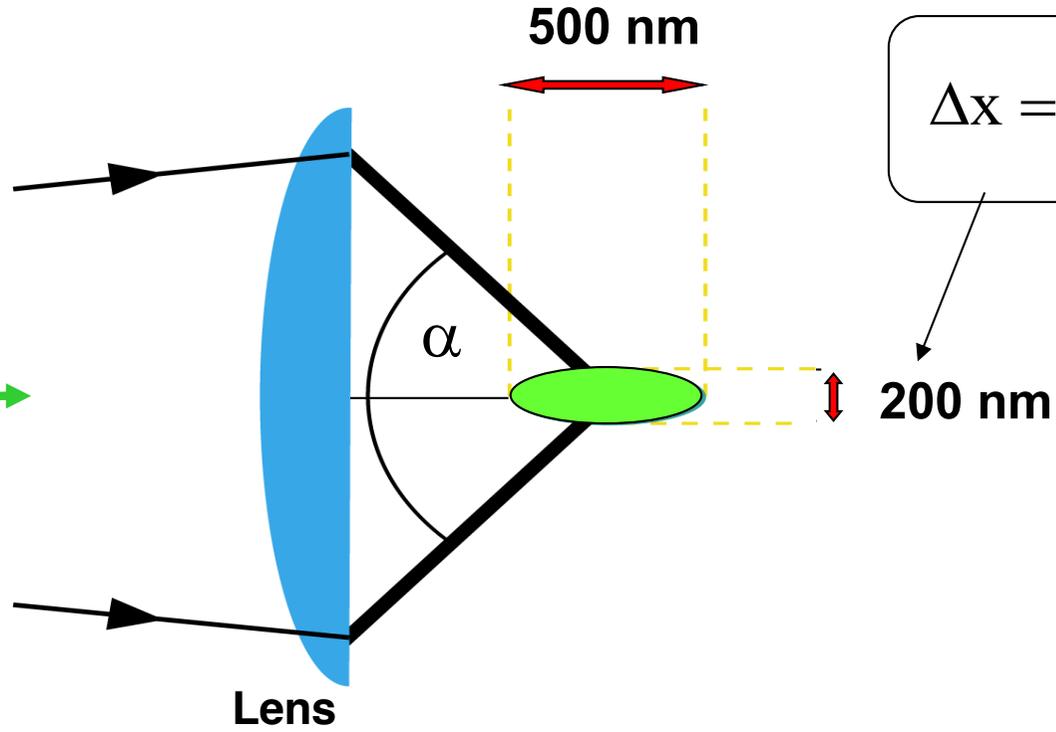
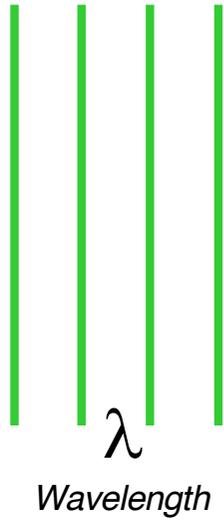
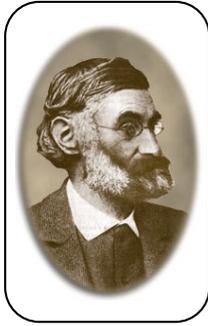


inverted



Diffraction limit

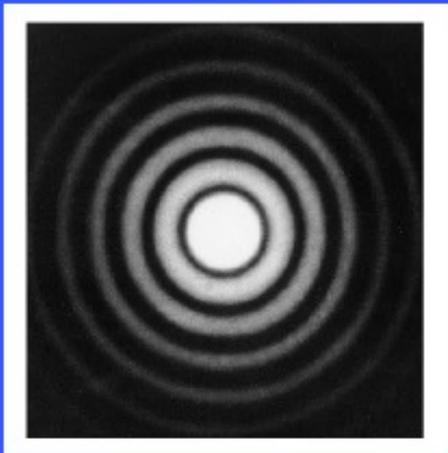
Focusing a propagating light wave means causing it to interfere constructively at a certain point in space, called the geometrical focal point $(0,0,0)$. Due to diffraction a focal intensity pattern $I(x, y, z)$ emerges around $(0,0,0)$, which is also referred to as the intensity point-spread-function (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 $I_{\text{em}}(x, y, z)$, describing the blur of the coordinate from where the photons originated.



$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$



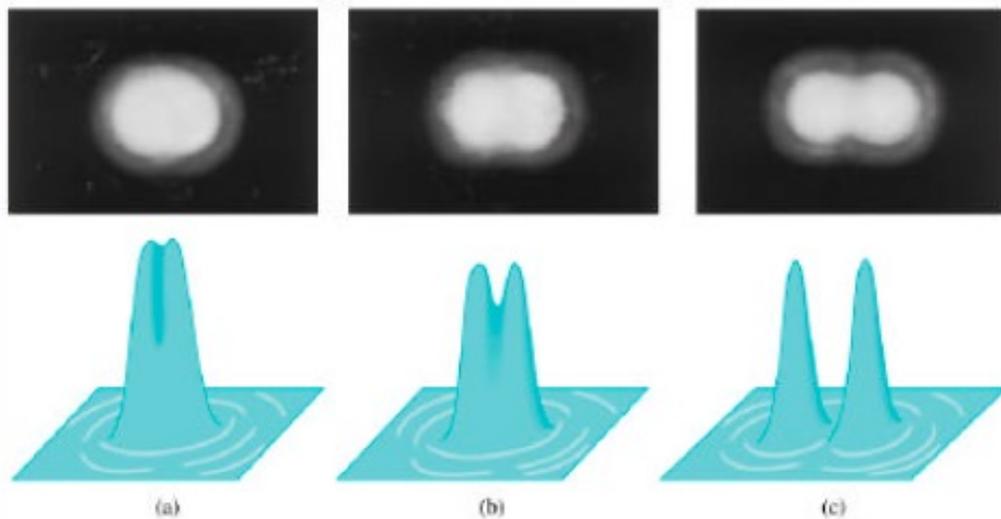
Diffrazione attraverso un foro circolare



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

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

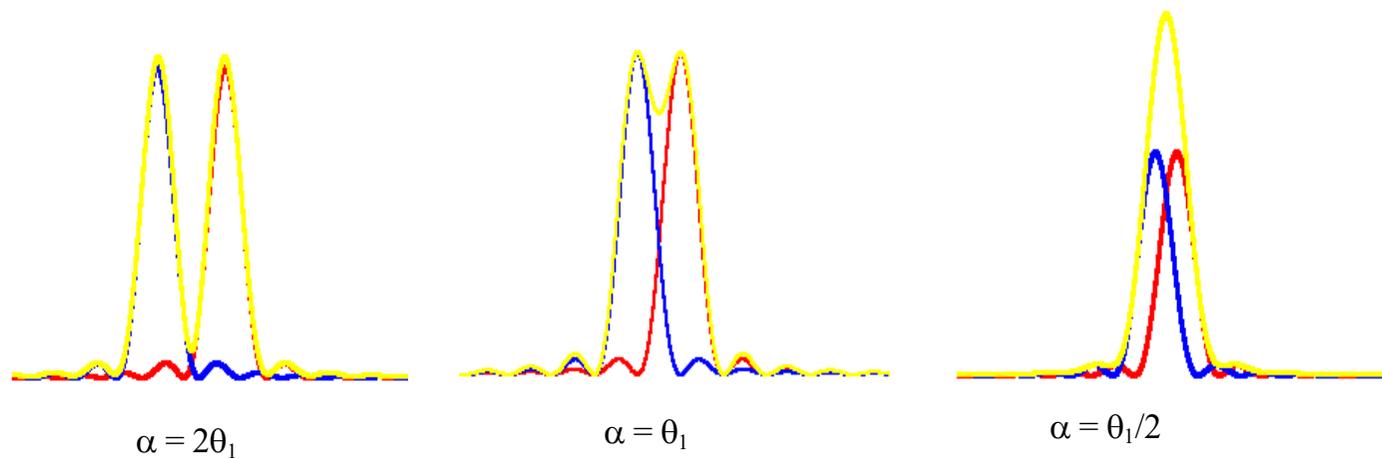
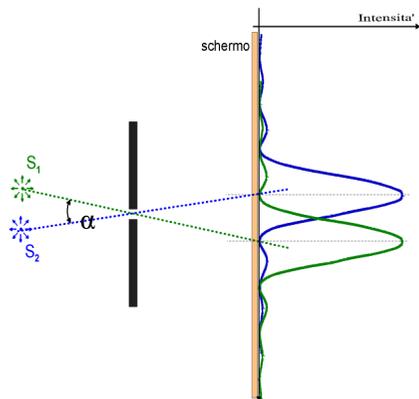
Potere risolvete



Criterio di Rayleigh

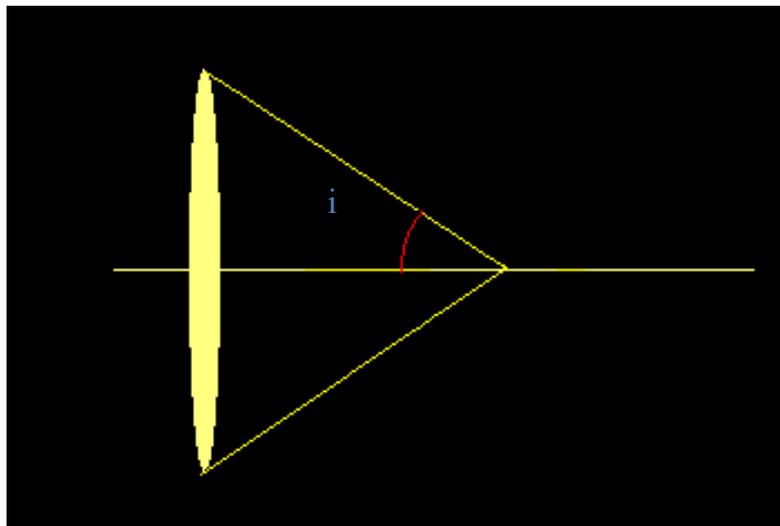
Due sorgenti luminose puntiformi sono risolubili se la loro distanza angolare è tale che il max. centrale della figura di diffrazione di una coincide con il primo minimo della figura di diffrazione dell'altra

Risoluzione: criterio di Reyleigh



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

telescopio



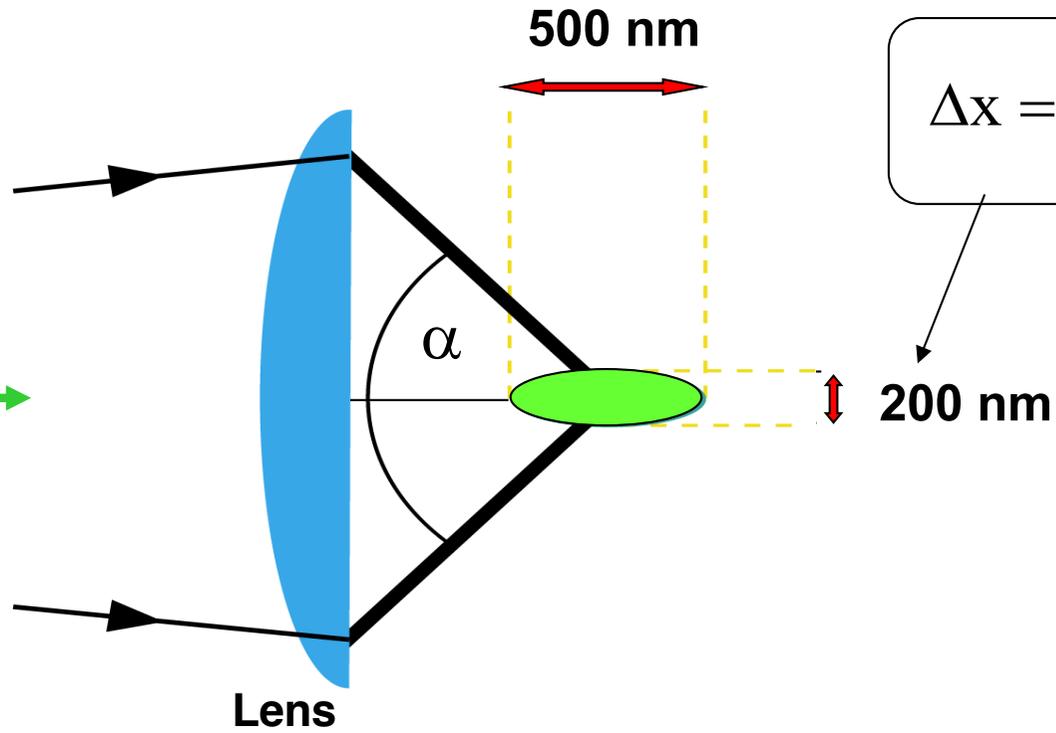
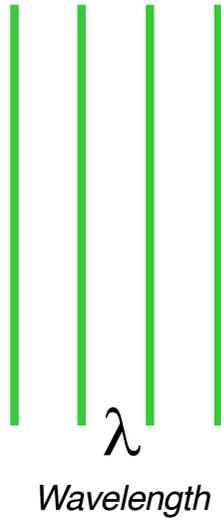
$$s \geq \frac{\lambda}{2n \cdot \text{sen}(i)}$$

microscopio

Apertura numerica

Lens less microscope: scanning near field optical microscopy

A logical consequence of the fact that light cannot be focused more sharply than the diffraction limit was to give up propagating waves and lenses and **confine the light by means of a subdiffraction-sized aperture or tip**. Placing this aperture in sub- $\lambda/2$ proximity to the object and scanning it across the object renders optical images with subdiffraction resolution. Proposed by Synge [4] in 1928 and again by Ash and Nicholls in 1972 [5], this concept was invigorated in the wake of the invention of the scanning tunneling microscope as near-field optical microscopy [6, 7]. A fundamental difference of near-field optical microscopy to its lens-based (far-field) counterpart is that it relies on non-propagating, evanescent light fields fading out exponentially within distance $\sim\lambda/2$ from the object. Giving up focusing therefore comes at a high price: one is bound to imaging surfaces.



$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$





[Max Planck
Society](#)

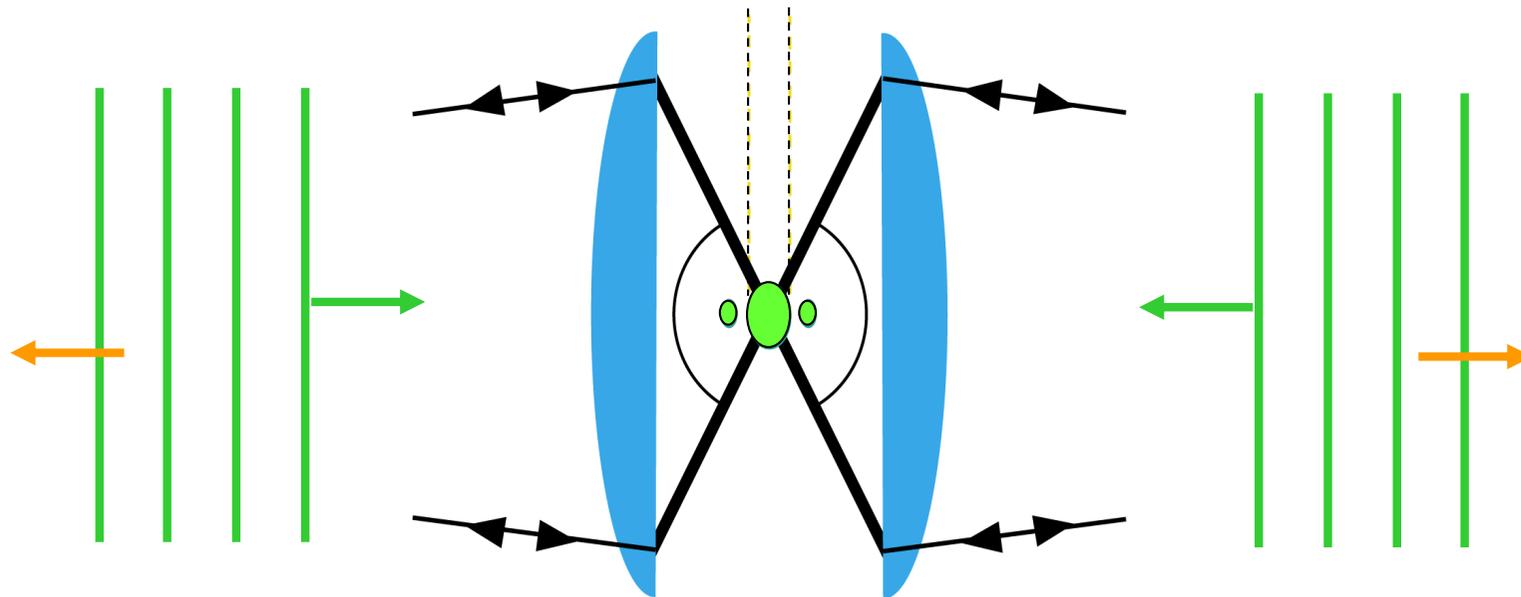
4Pi- Microscopy: resolution improvement in **Z**





4Pi- Microscopy:

70 - 140 nm

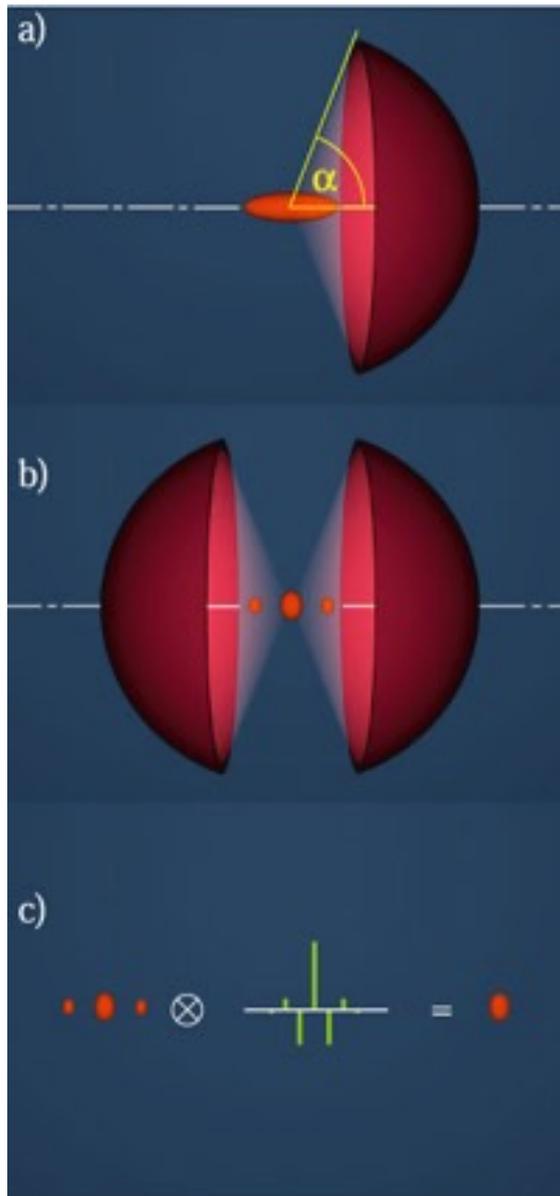


$$\vec{E}^{4Pi}(r, z, \varphi) = \vec{E}_1(r, z, \varphi) + \vec{E}_2(r, -z, \varphi)$$

Coherent illumination and/or fluorescence detection

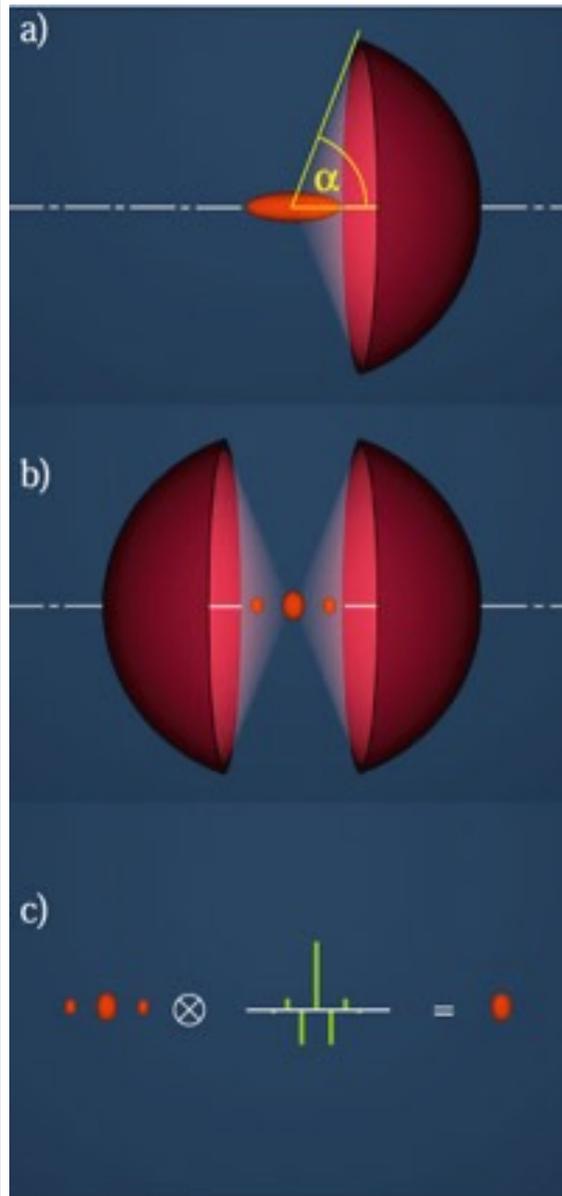
[S.W. Hell \(1990\), *Europ. Patent* OS 0491289.](#)
[S.W. Hell, et al. \(1992\), *Opt. Commun.* **93**, 277.](#)
[M. Schrader, et al. \(1998\), *Biophys. J.* **75**, 1659.](#)
[H. Gugel, et al. \(2004\), *Biophys. J.* **87**, 4146.](#)





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.

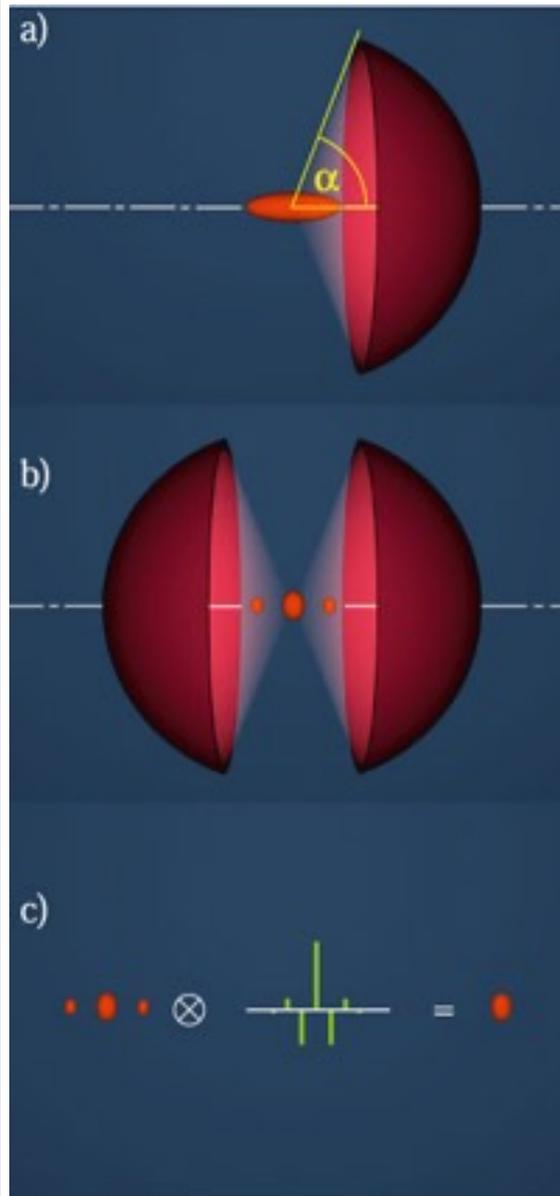




The idea: Since there are no lenses or mirrors that could provide such a wavefront across a significantly large field of view, the idea behind our 4Pi-microscope is to mimic the 'close to ideal' situation by **using two opposing objective lenses coherently**, so that the two wavefronts add up and join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

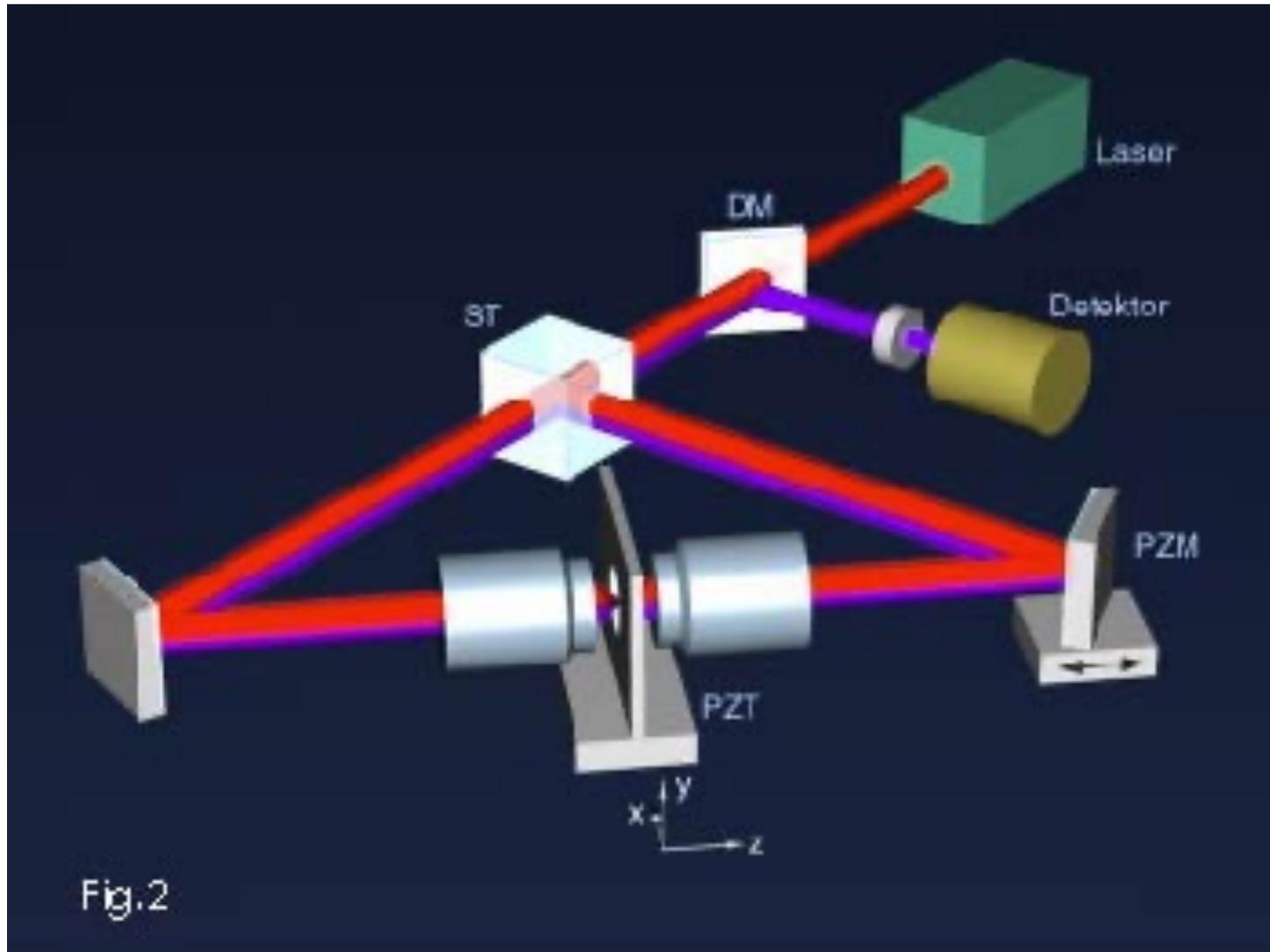
Allowing the illumination wavefronts to constructively interfere in the sample produces a main focal spot that is sharper in the z-direction by about 3-4 times (4Pi of type A). A similar improvement is obtained if the lenses add their collected fluorescence wavefronts in a common point detector (4Pi 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).

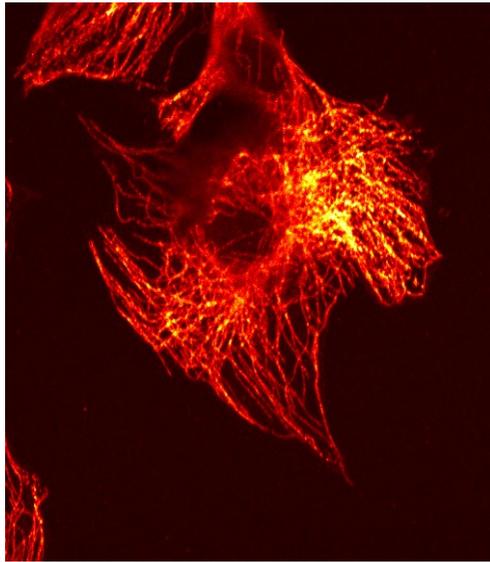




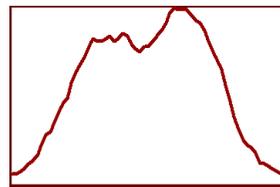
The sidelobe challenge: If the two segments were full spherical halves, the focal spot would be a (nearly) spherical spot, too. But since a considerable solid angle is not provided by the lenses, interference typically spawns off 2 axial side-lobes which, if not taken into account, lead to artefactual 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.







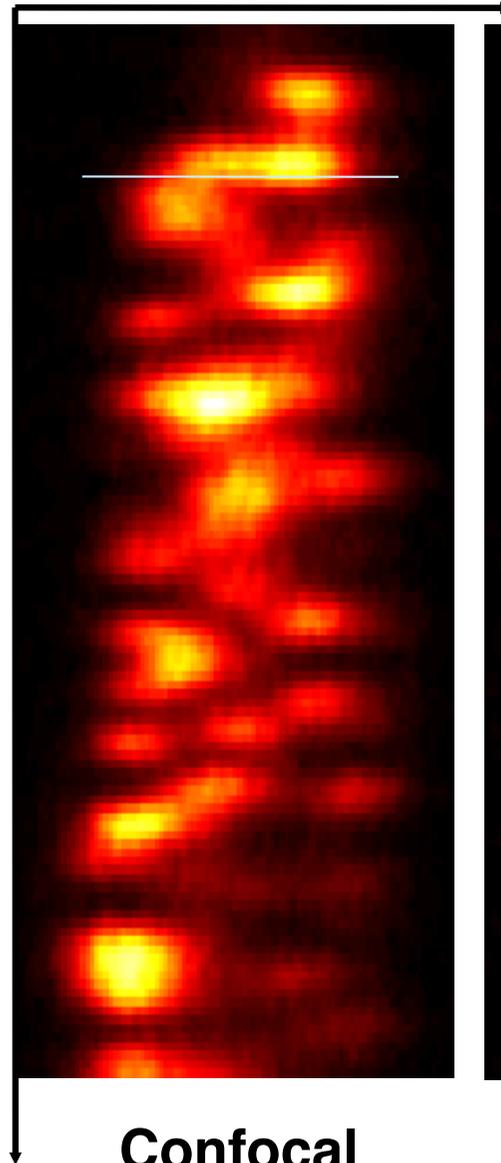
Microtubules, mouse fibroblast
Immunofluor, Oregon Green



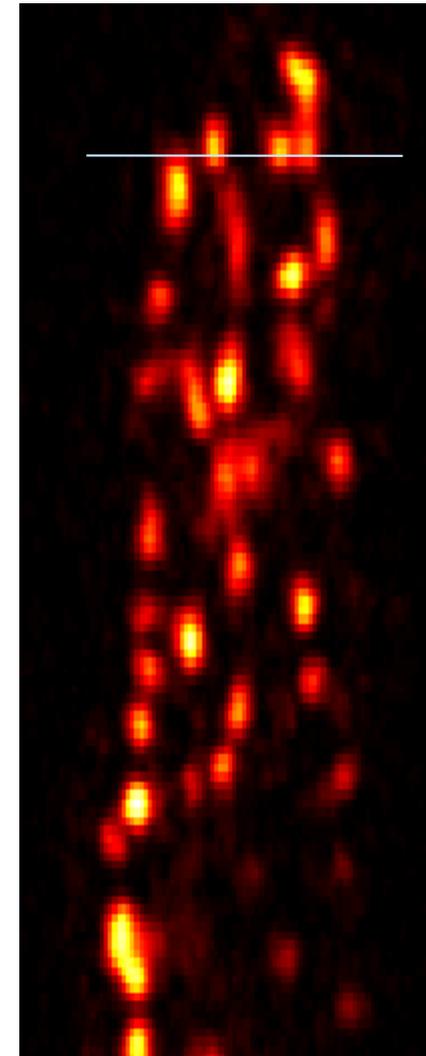
2 μm

X

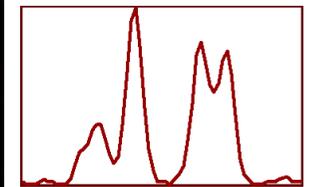
Z



Confocal



4Pi



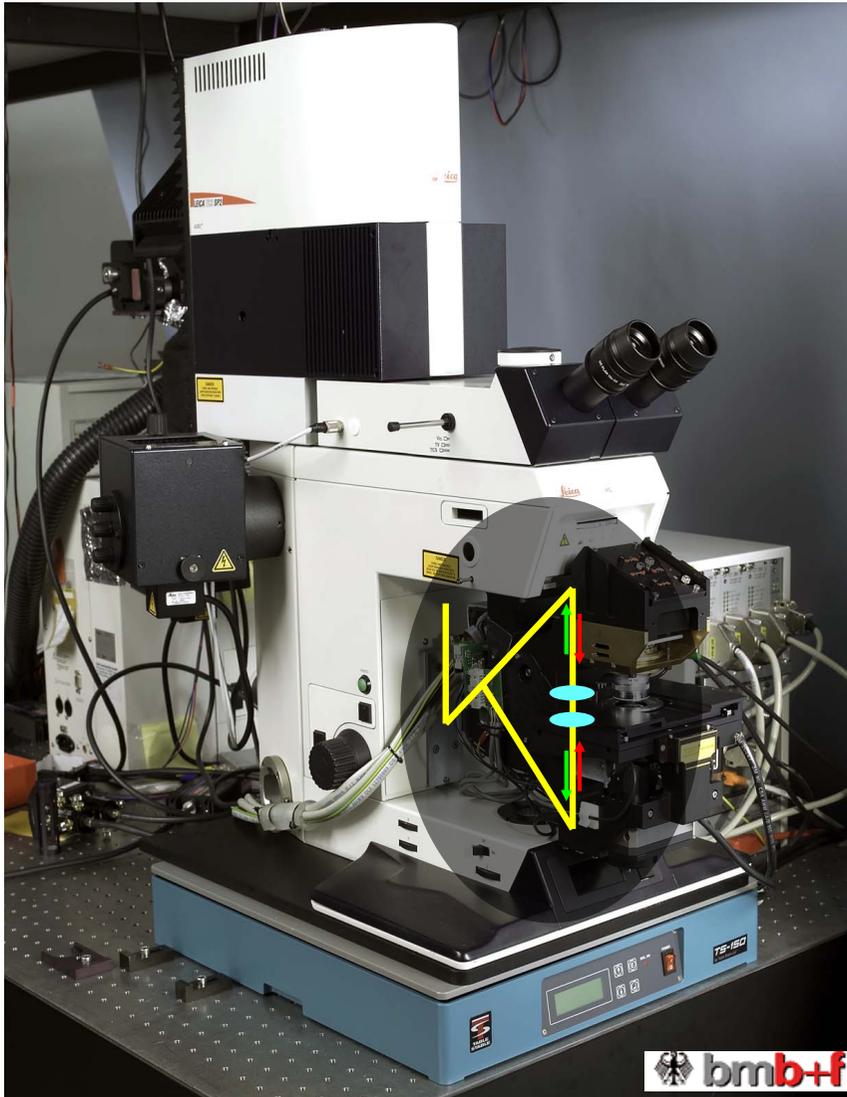
2 μm



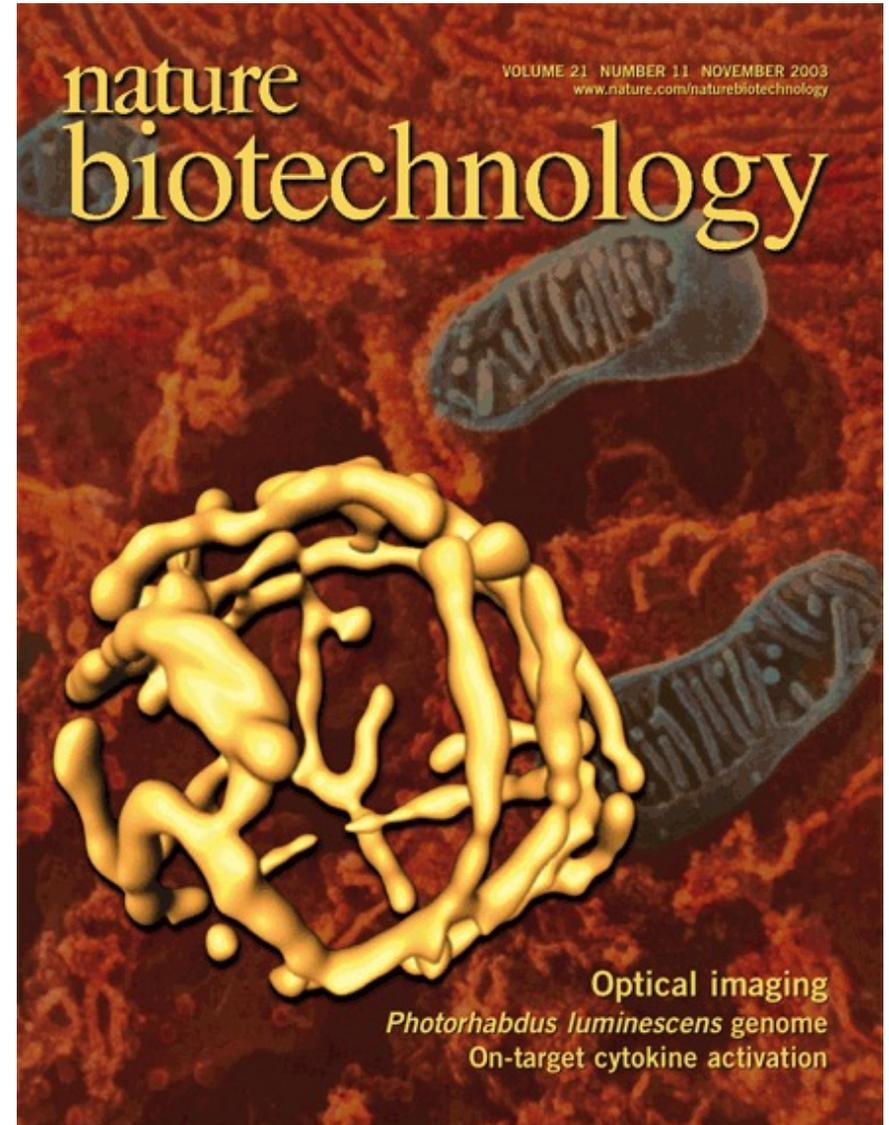
[S.W. Hell, et al. \(1992\), *Opt. Commun.* **93**, 277.](#)
[M. Schrader, et al. \(1998\), *Biophys. J.* **75**, 1659.](#)
[H. Gugel, et al. \(2004\), *Biophys. J.* **87**, 4146.](#)



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

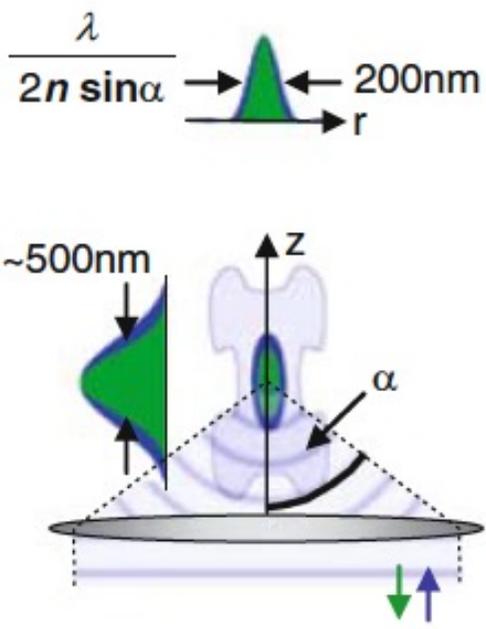


In the targeted read-out mode, the bright state A is established at coordinate r_i by driving an optical transition $A \rightarrow B$ with a light intensity distribution $I = I(r)$ featuring an intensity minimum, ideally a zero, at coordinate r_i (Figs. 19.1d and 19.2a). Applying $I(r)$ transfers the 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

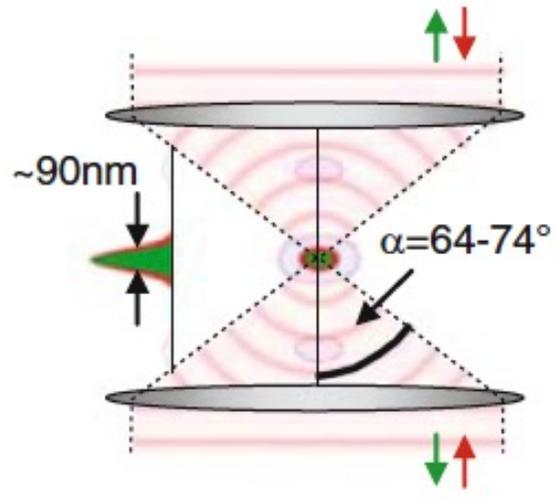
$$\Delta r \approx \frac{\lambda}{2 n \sin \alpha \sqrt{1 + a I_{\max} / I_s}}. \quad (19.1)$$

Single Point Versions

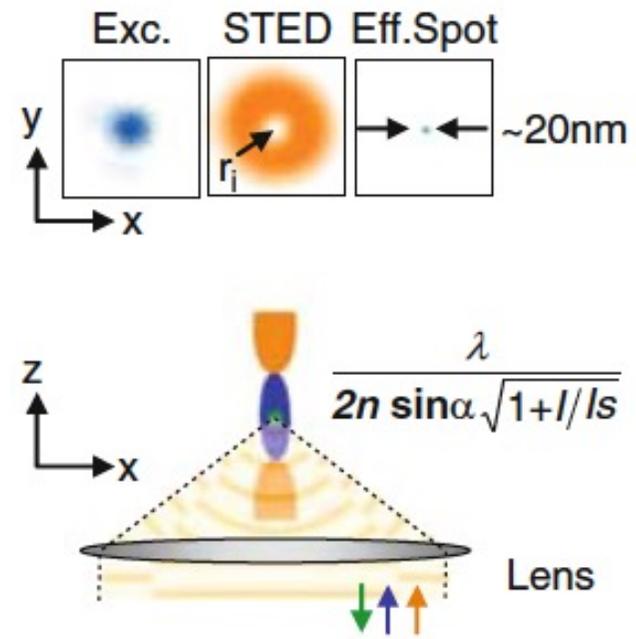
Confocal



4Pi



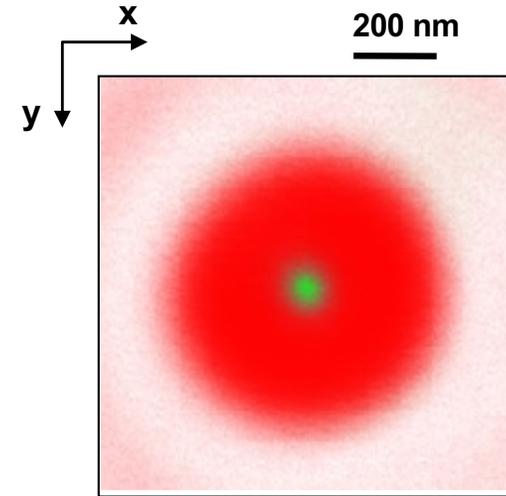
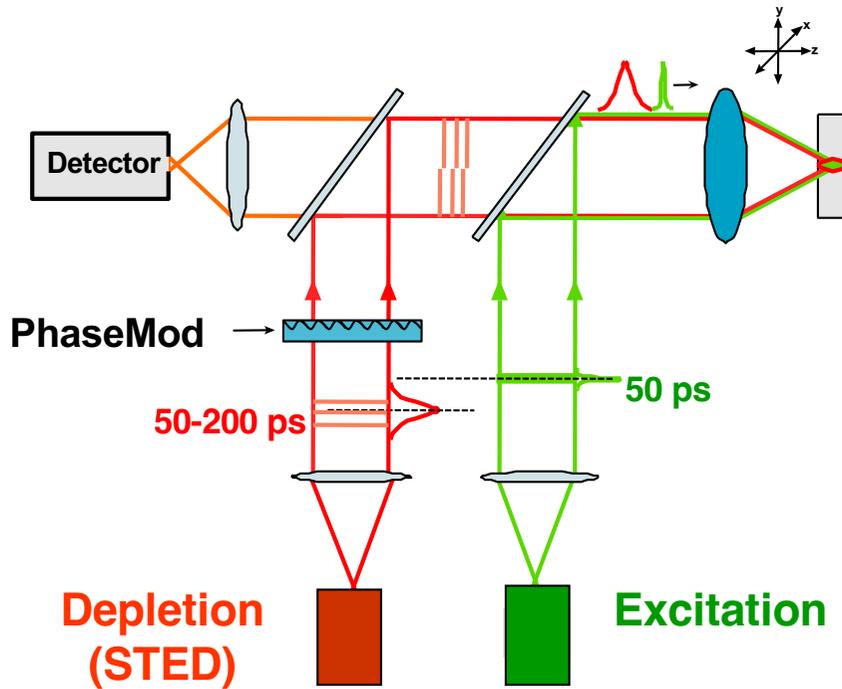
STED



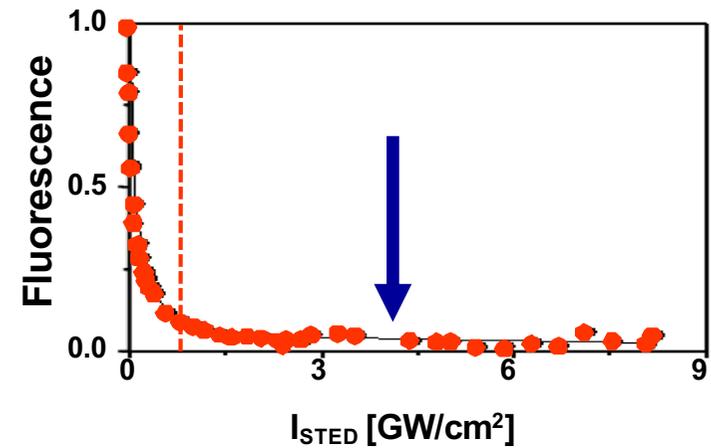
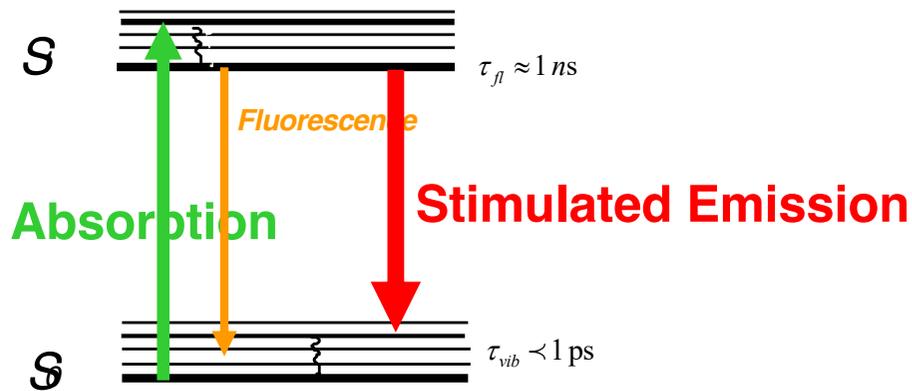


STED Microscopy

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

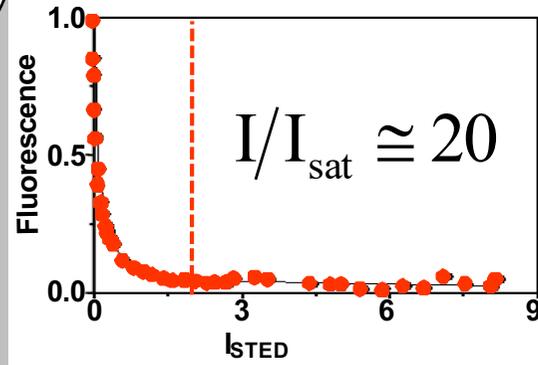


The stronger the STED beam the narrower the fluorescent spot!

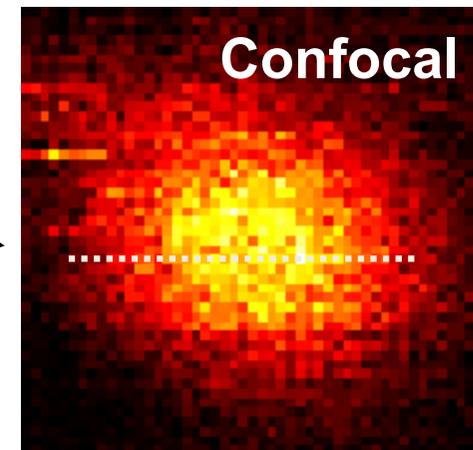
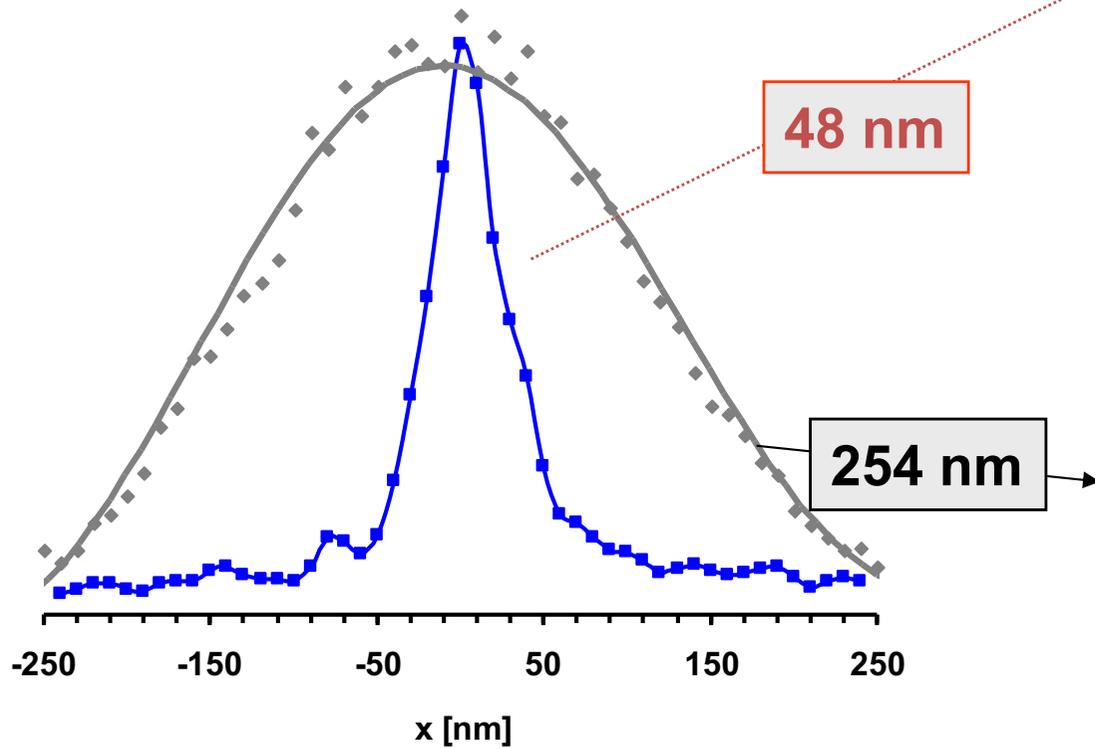
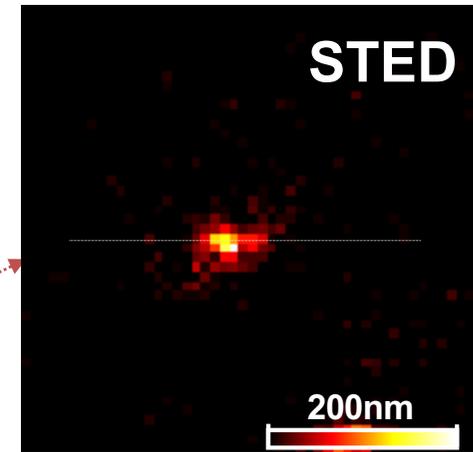


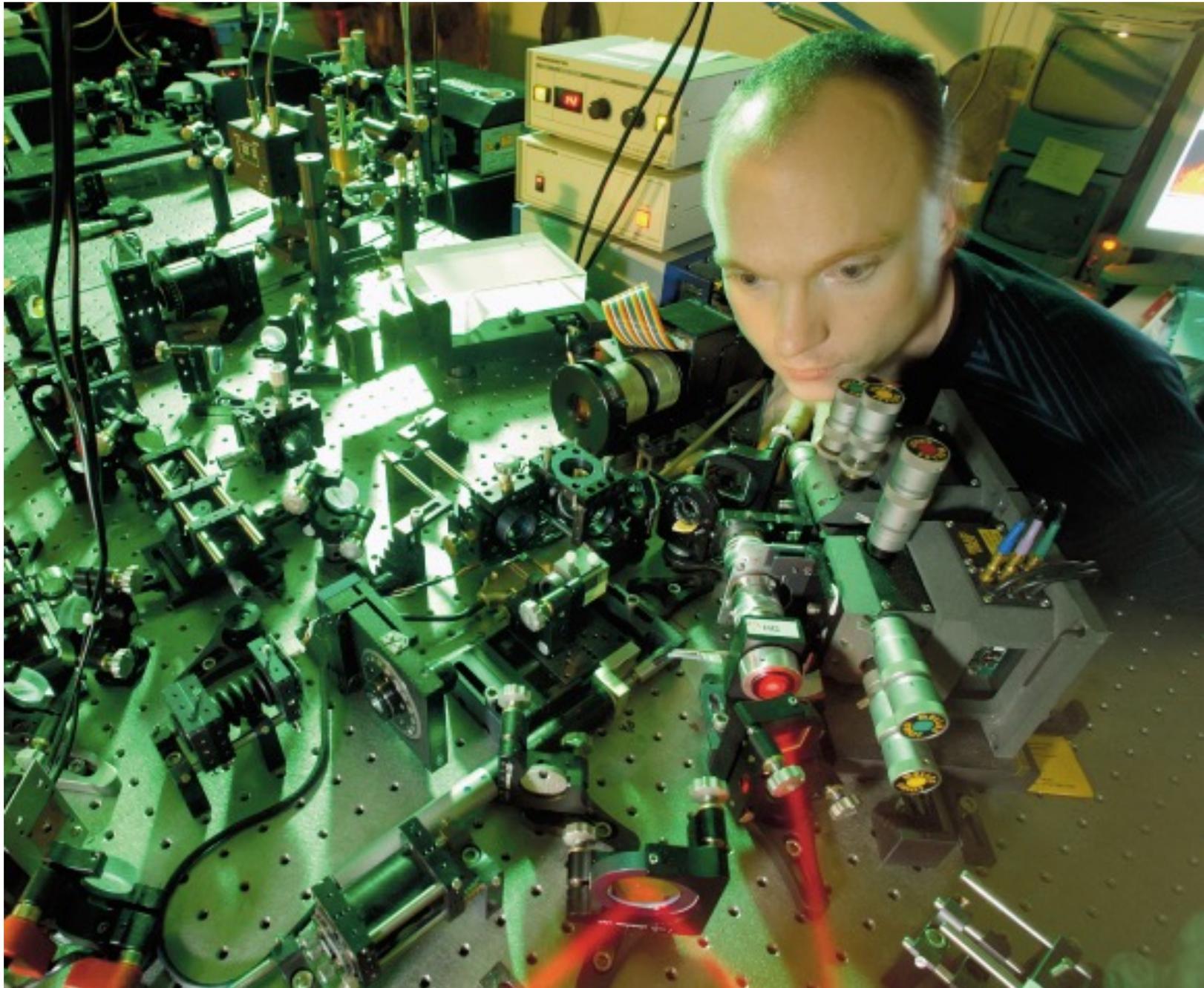


Focal spot ... probed with 1 molecule



$\lambda_{\text{STED}} = 770 \text{ nm}$

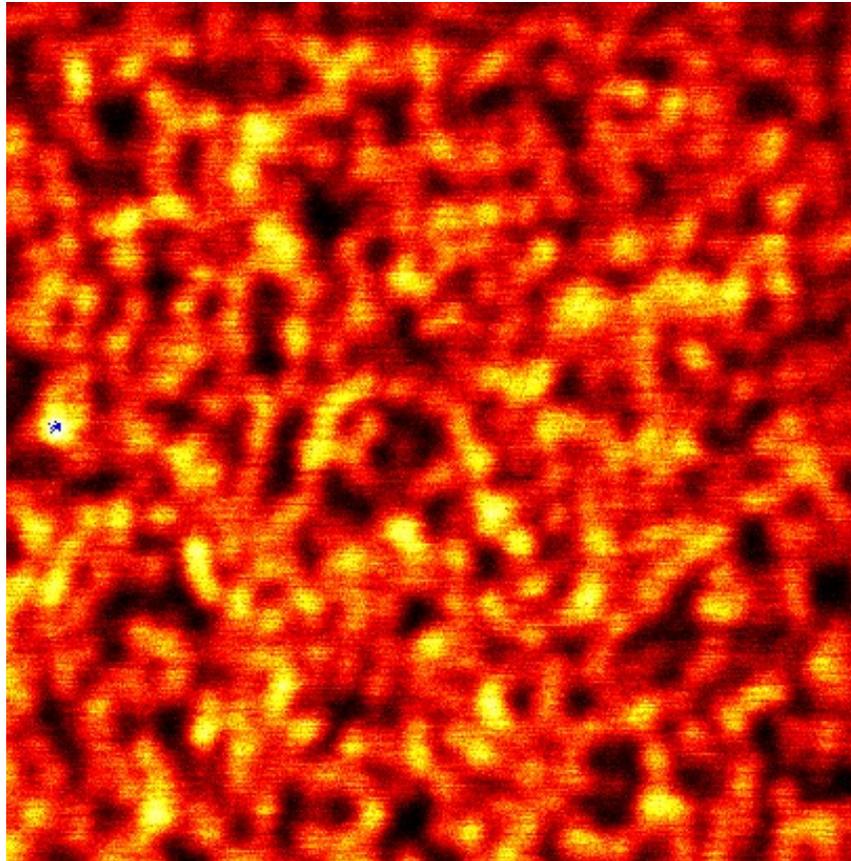






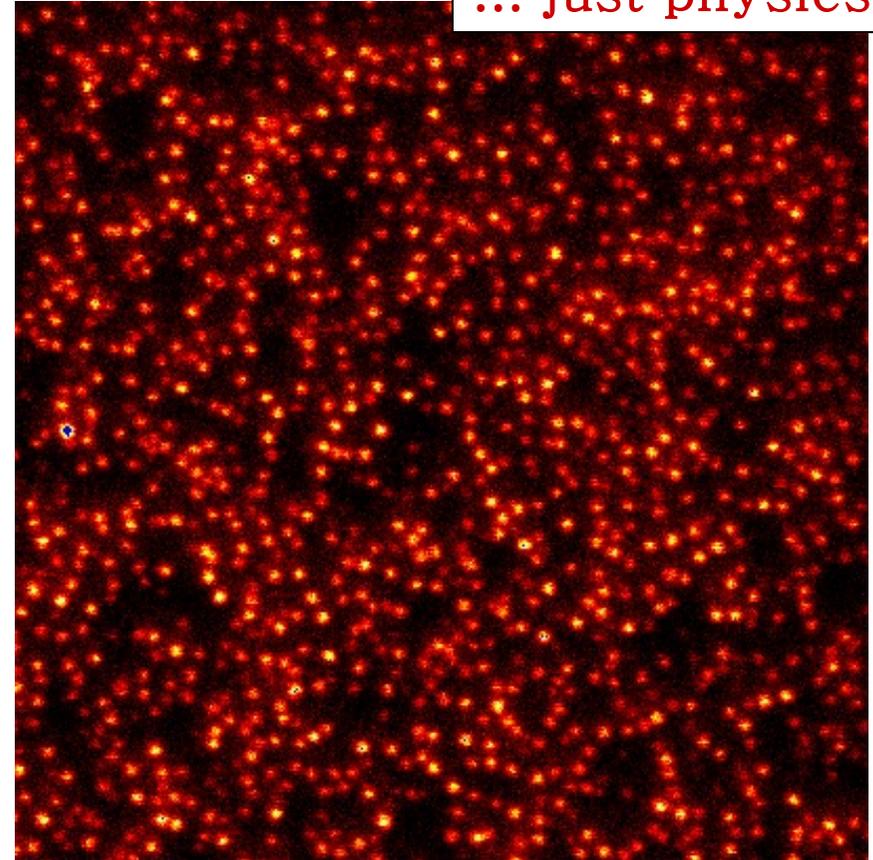
Imaging 40 nm fluorescence beads:

Confocal



STED

... just physics !



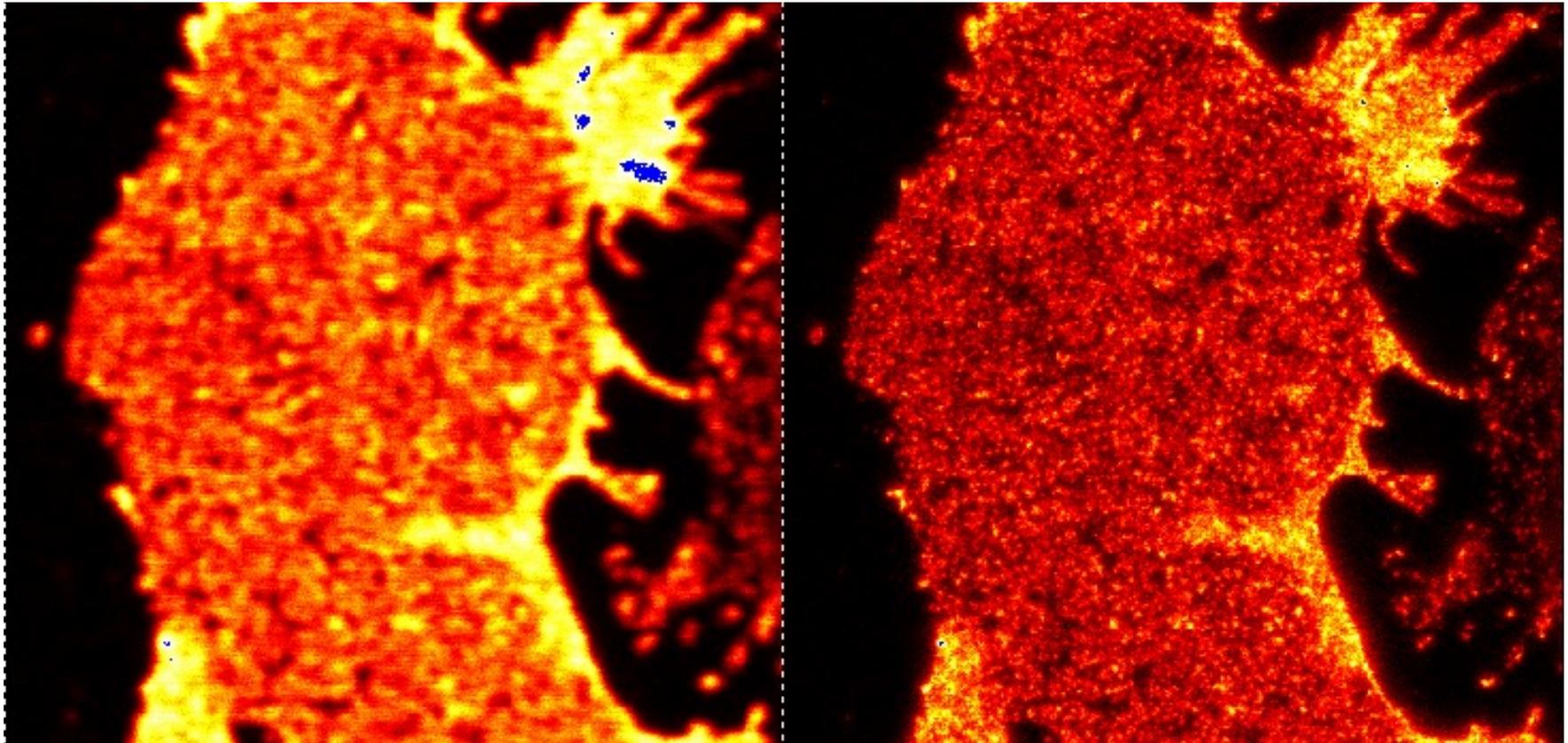


Imaging protein distribution on cell membrane: SNAP 25

...just physics !

Confocal:

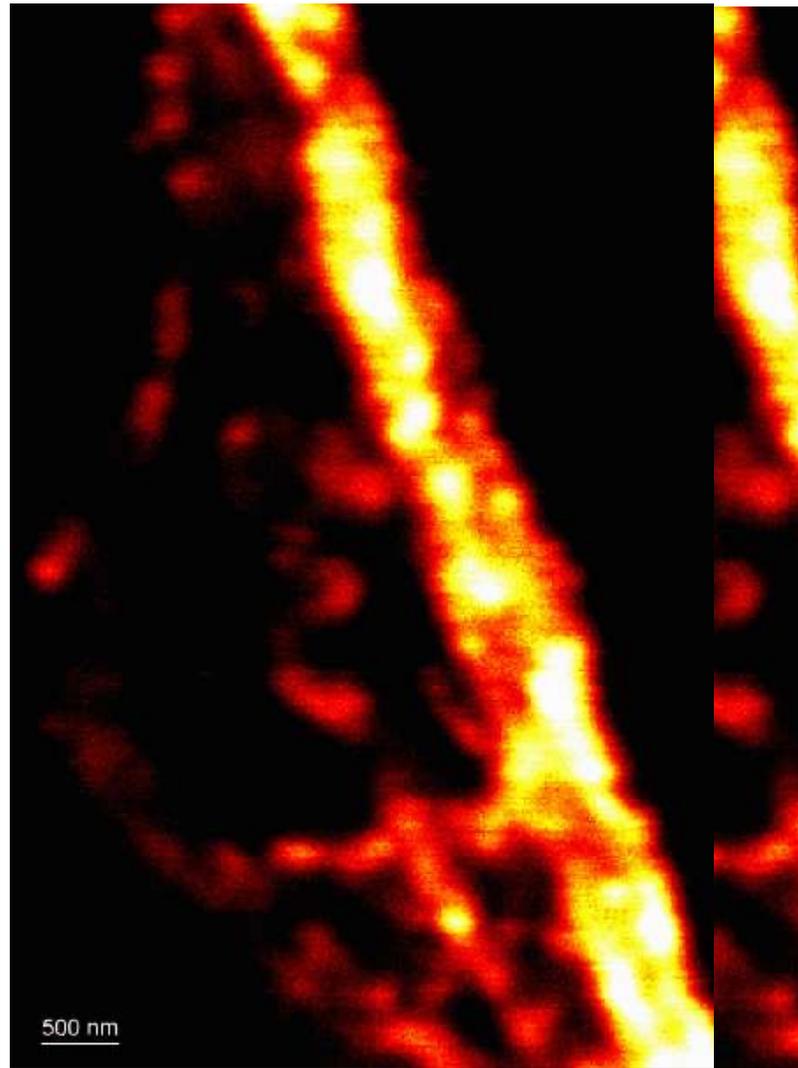
STED:



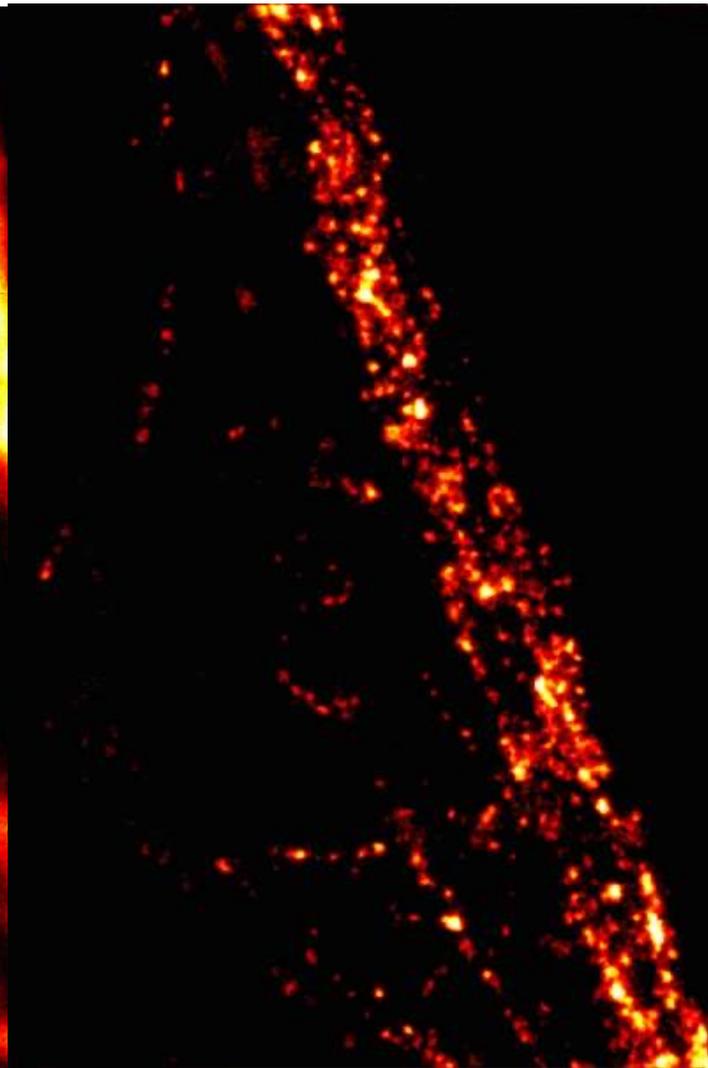


Heavy subunit of neurofilaments in neuroblastoma

Confocal

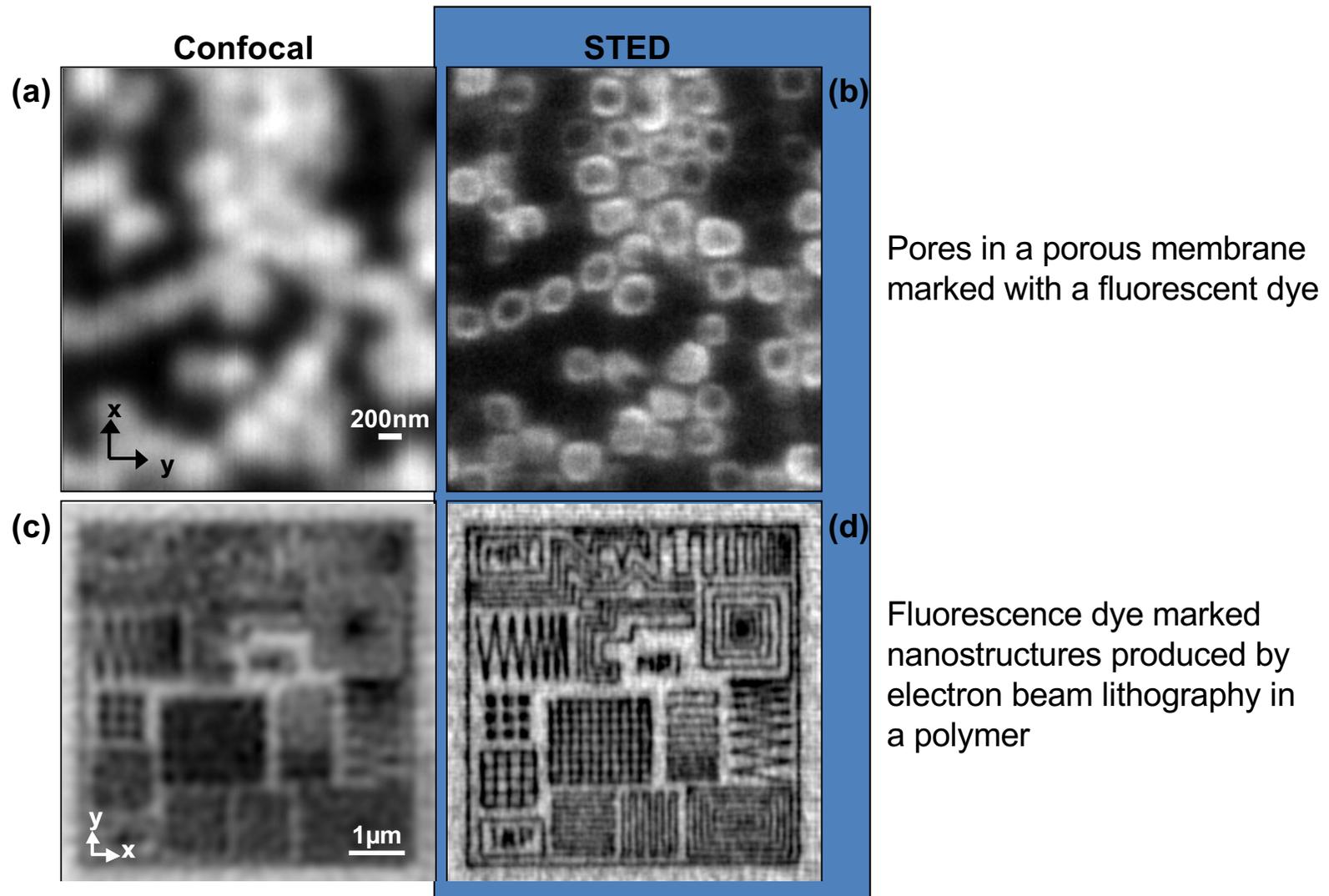


STED





STED Microscopy: Sometimes only *resolution*...



...makes subdiffraction images !

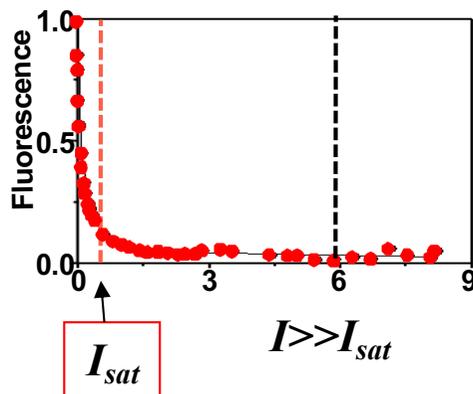




STED microscopy

- 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.](#)

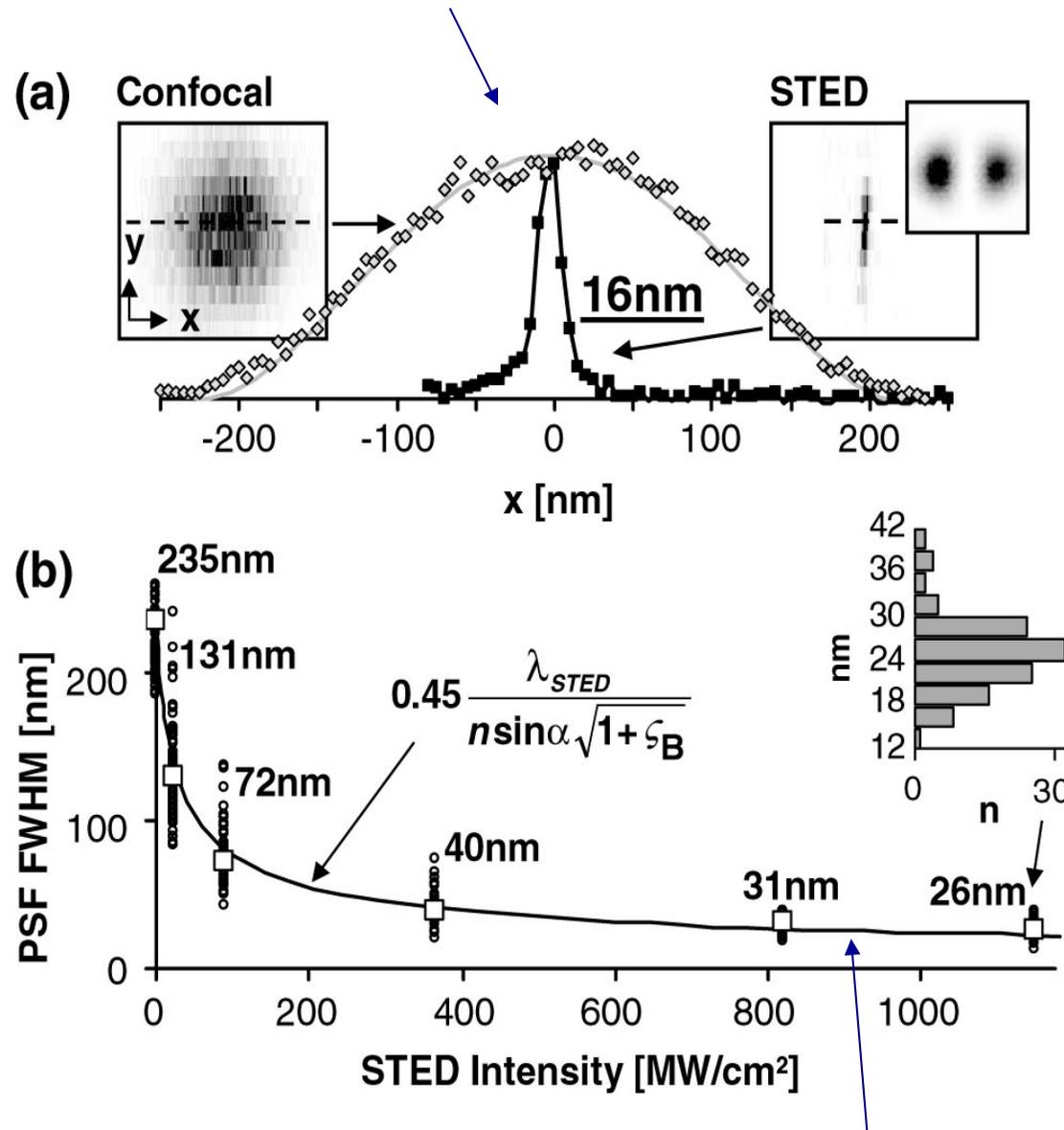
[S.W. Hell \(2004\), *Phys. Lett. A* **326**, 140.](#)

[V. Westphal & S.W. Hell \(2005\), *Phys. Rev. Lett.* **94**, 143903.](#)





Sharpest focal spot



Validation of square-root resolution law





[Max Planck
Society](#)

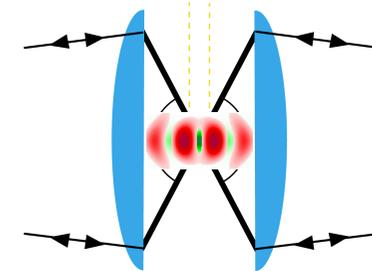
4Pi- STED Microscopy

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



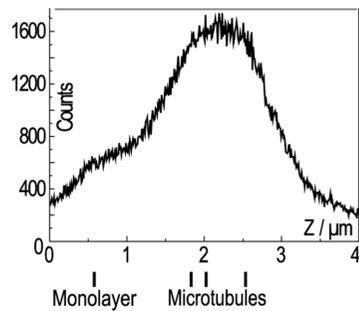
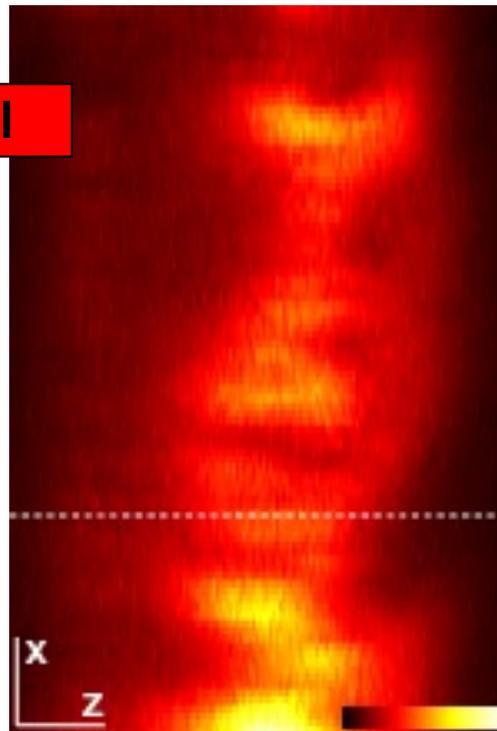


The combination: STED-4Pi-Microscopy



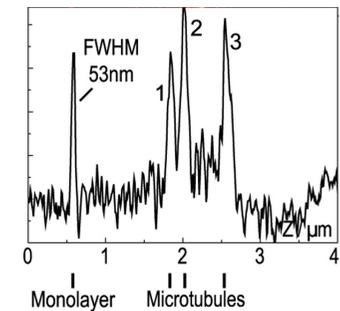
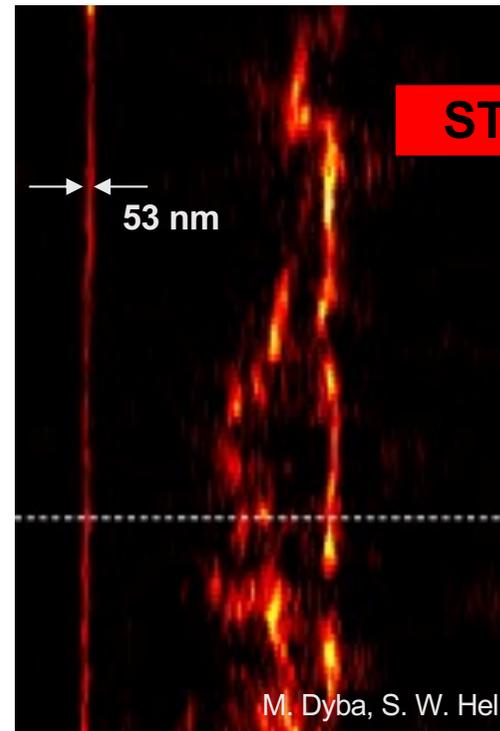
↓ Monolayer

confocal



↓ Monolayer

STED-4Pi



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





[Max Planck
Society](#)

RESOLFT

Reversible Saturable (Switchable) Linear Fluorescence Transitions

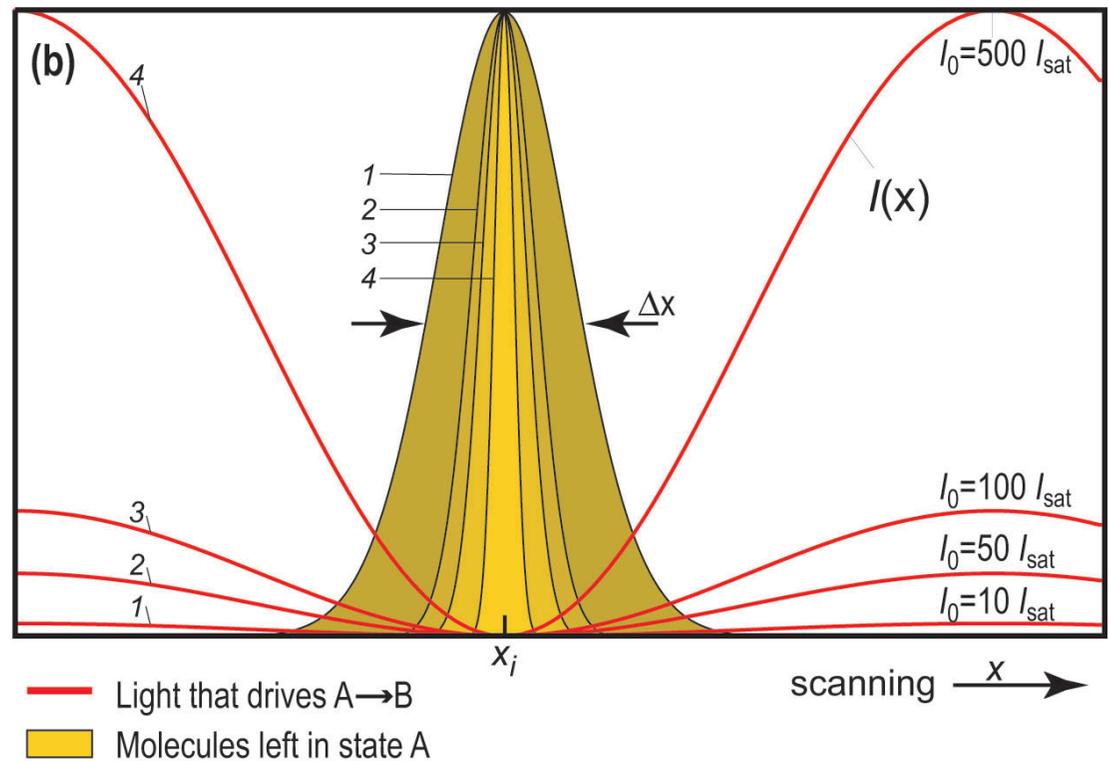
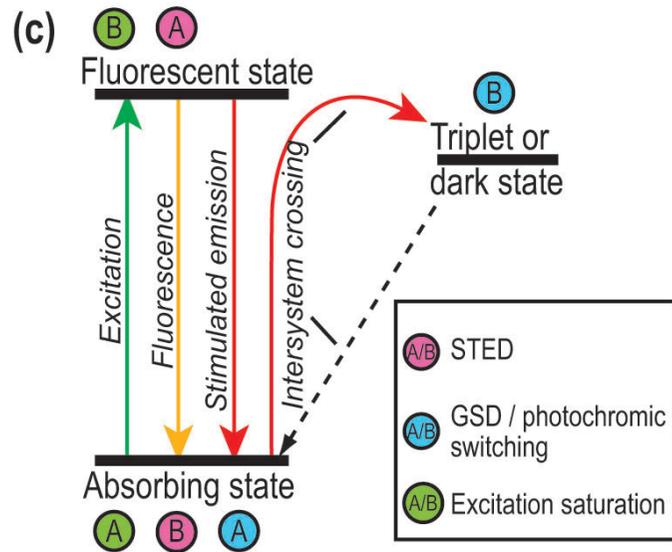
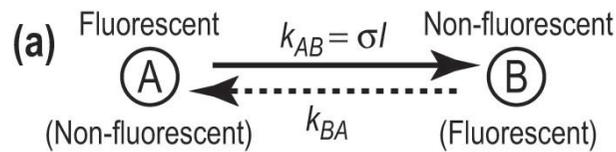
is

the generalized principle of STED microscopy





RESOLFT: Reversible Saturable Optical (Fluorescent) Transition





Acknowledgements / References:

Physics:

G. Donnert et al

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L. Kastrup, H. Blom, C. Eggeling, S.W. Hell

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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

