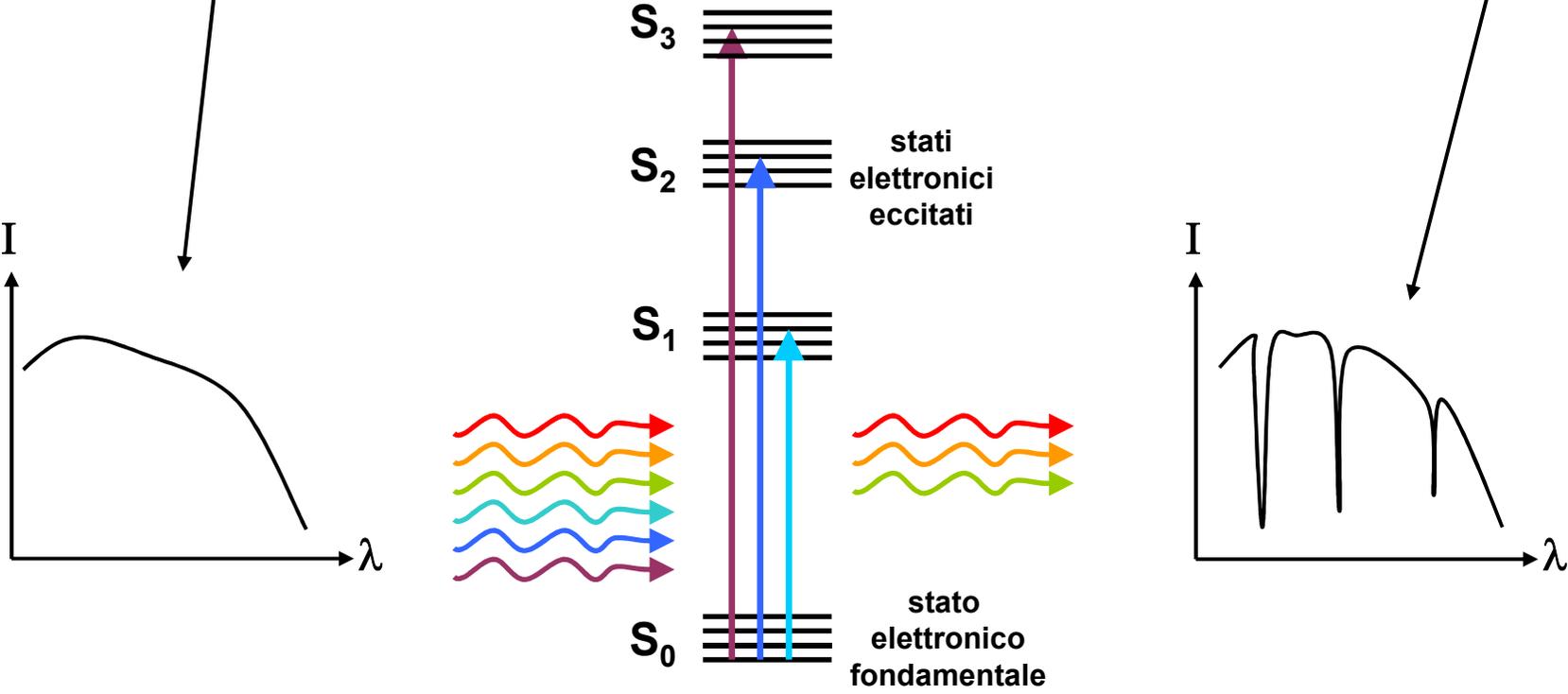
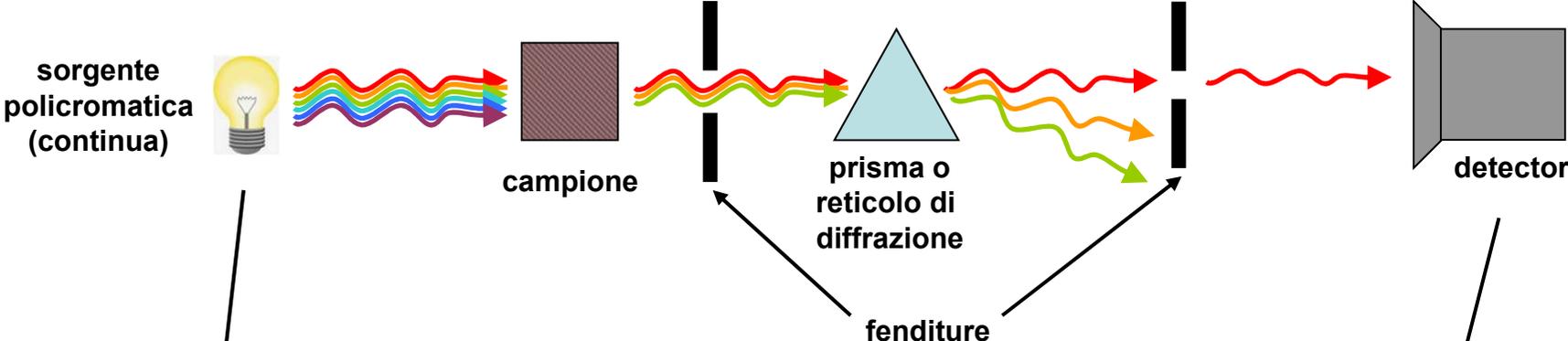


PARTE II

Assorbimento UV-visibile

La Spettroscopia di Assorbimento nell'UV-visibile



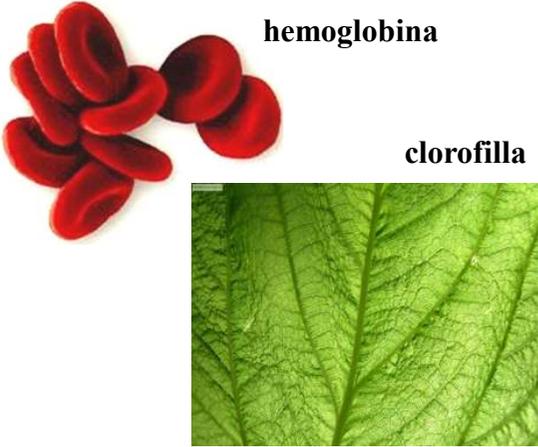
La Spettroscopia di Assorbimento nell'UV-visibile

MOLECOLE CHE ASSORBONO NEL VISIBILE

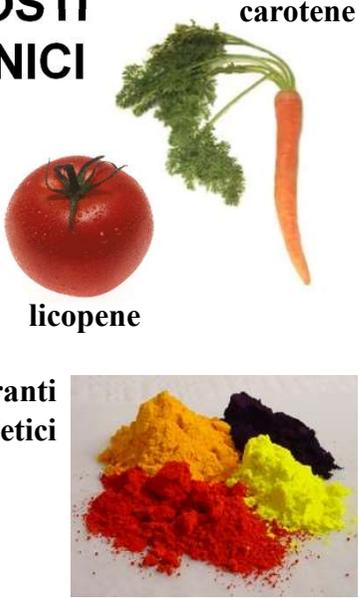
COMPOSTI INORGANICI



COMPOSTI "METALLORGANICI"

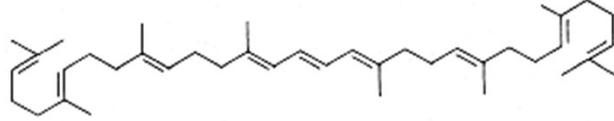


COMPOSTI ORGANICI

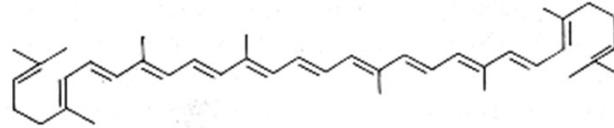


La Spettroscopia di Assorbimento nell'UV-visibile

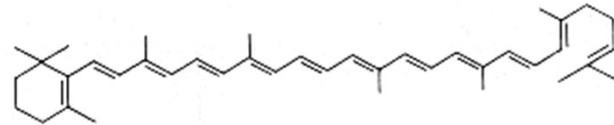
phytoene (C₄₀H₆₄; colorless; λ_{\max} , 285 nm)



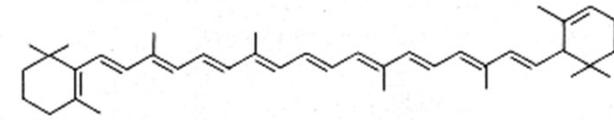
lycopene (C₄₀H₅₆; red; λ_{\max} , 476 nm)



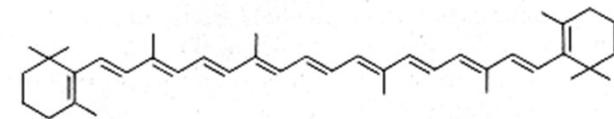
γ -*carotene* (C₄₀H₅₆; orange; λ_{\max} , 460 nm)



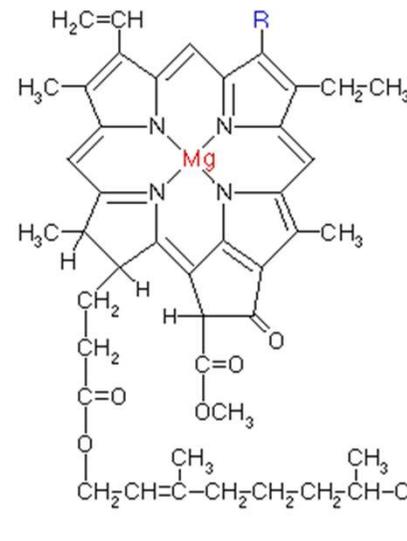
α -*carotene* (C₄₀H₅₆; orange; λ_{\max} , 456 nm)



β -*carotene* (C₄₀H₅₆; orange; λ_{\max} , 463 nm)

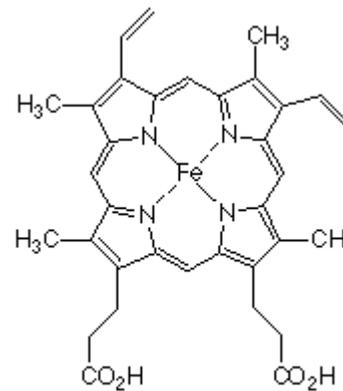
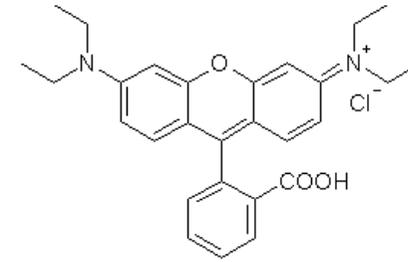


“SISTEMI π ” delocalizzati

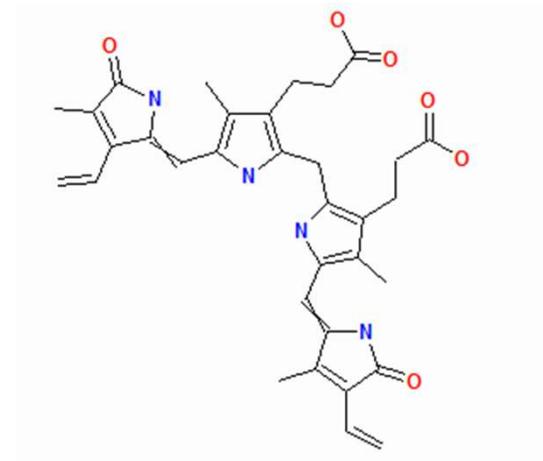


R-CH₃ in chlorophyll *a*
CHO in chlorophyll *b*

The structure of chlorophyll

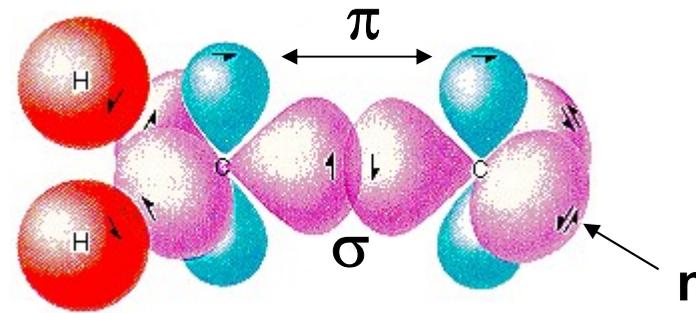
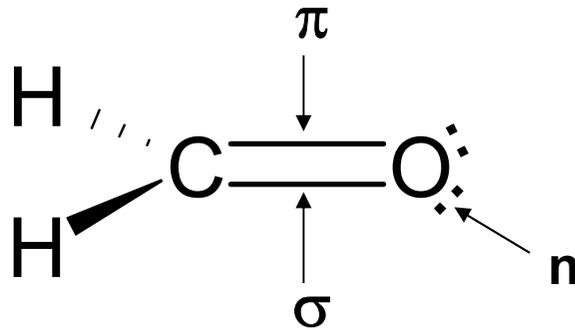


Heme

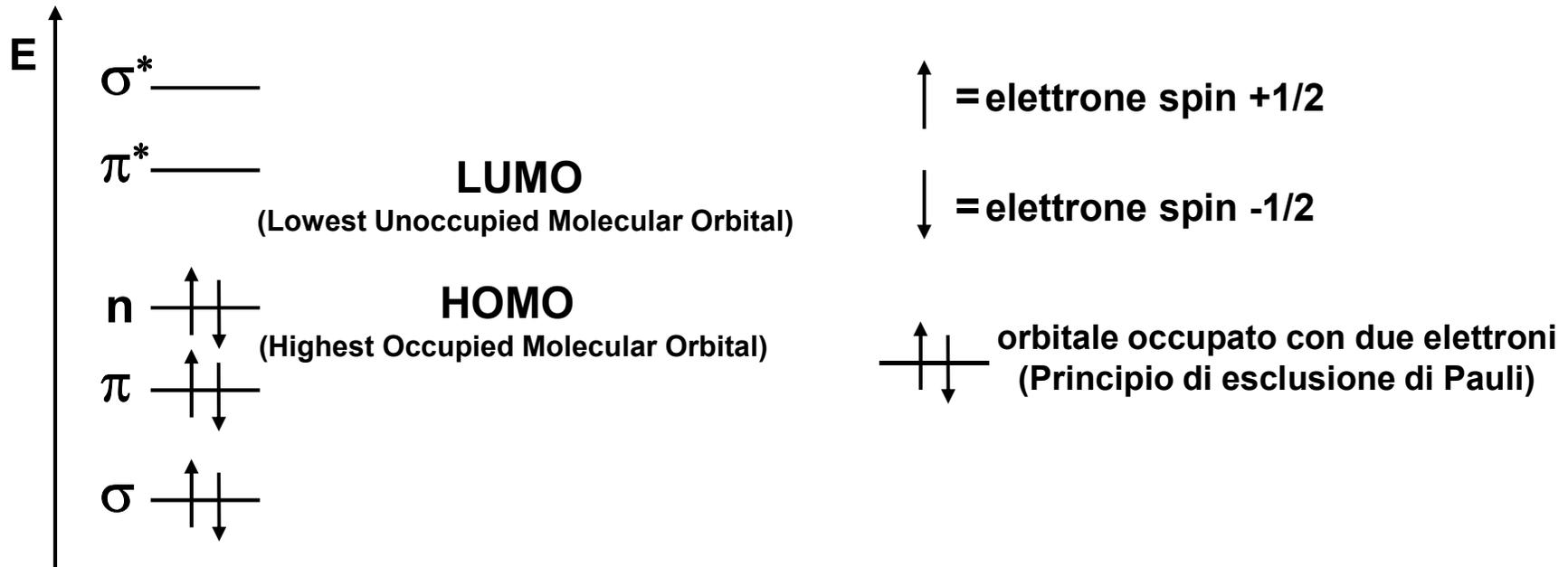


La Spettroscopia di Assorbimento nell'UV-visibile

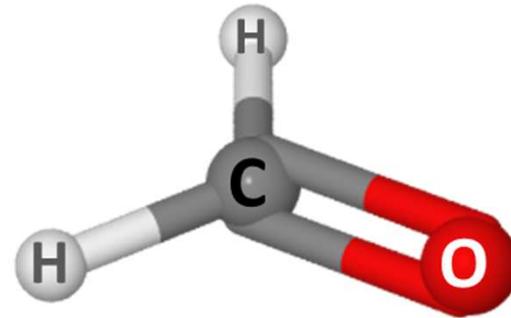
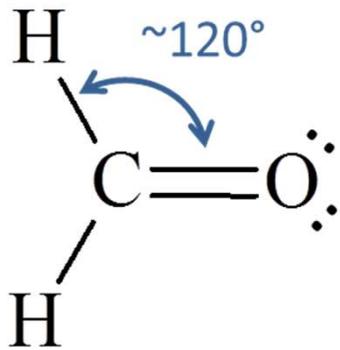
orbitali σ , π e n



formaldeide

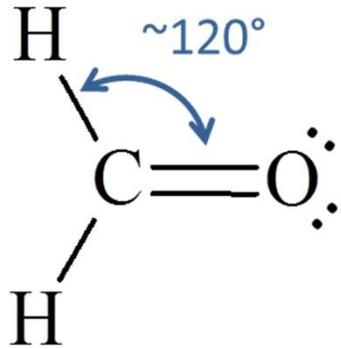


formaldeide

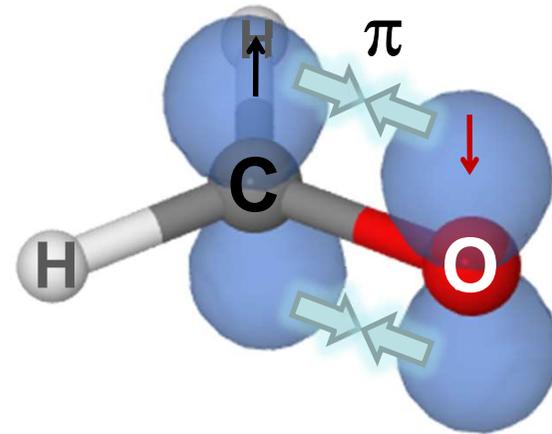
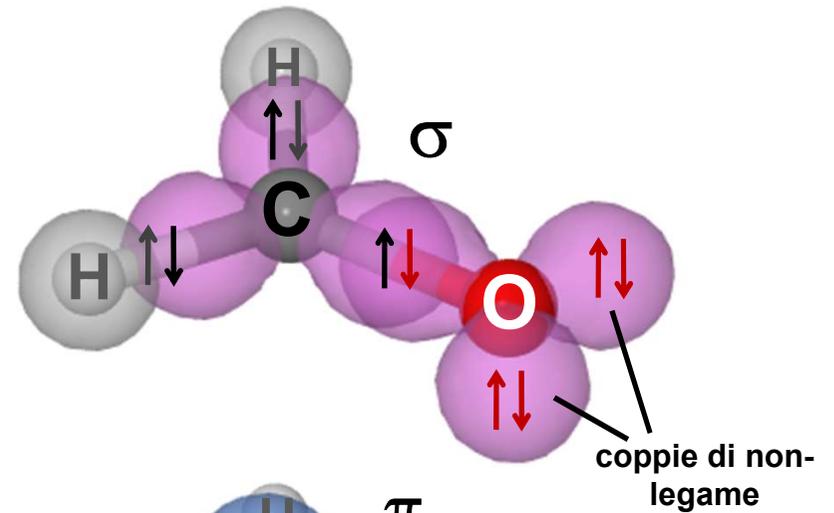
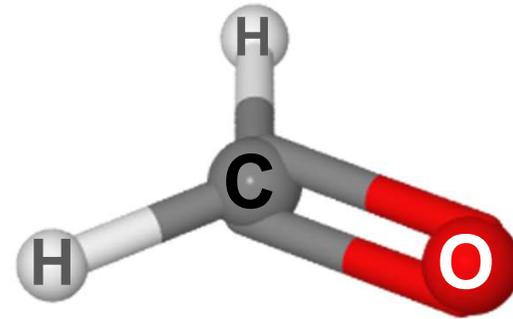
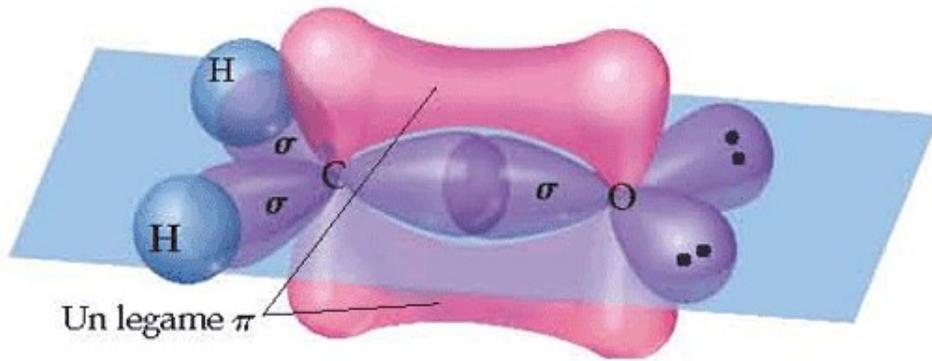


*Come sono gli stati
elettronici?*

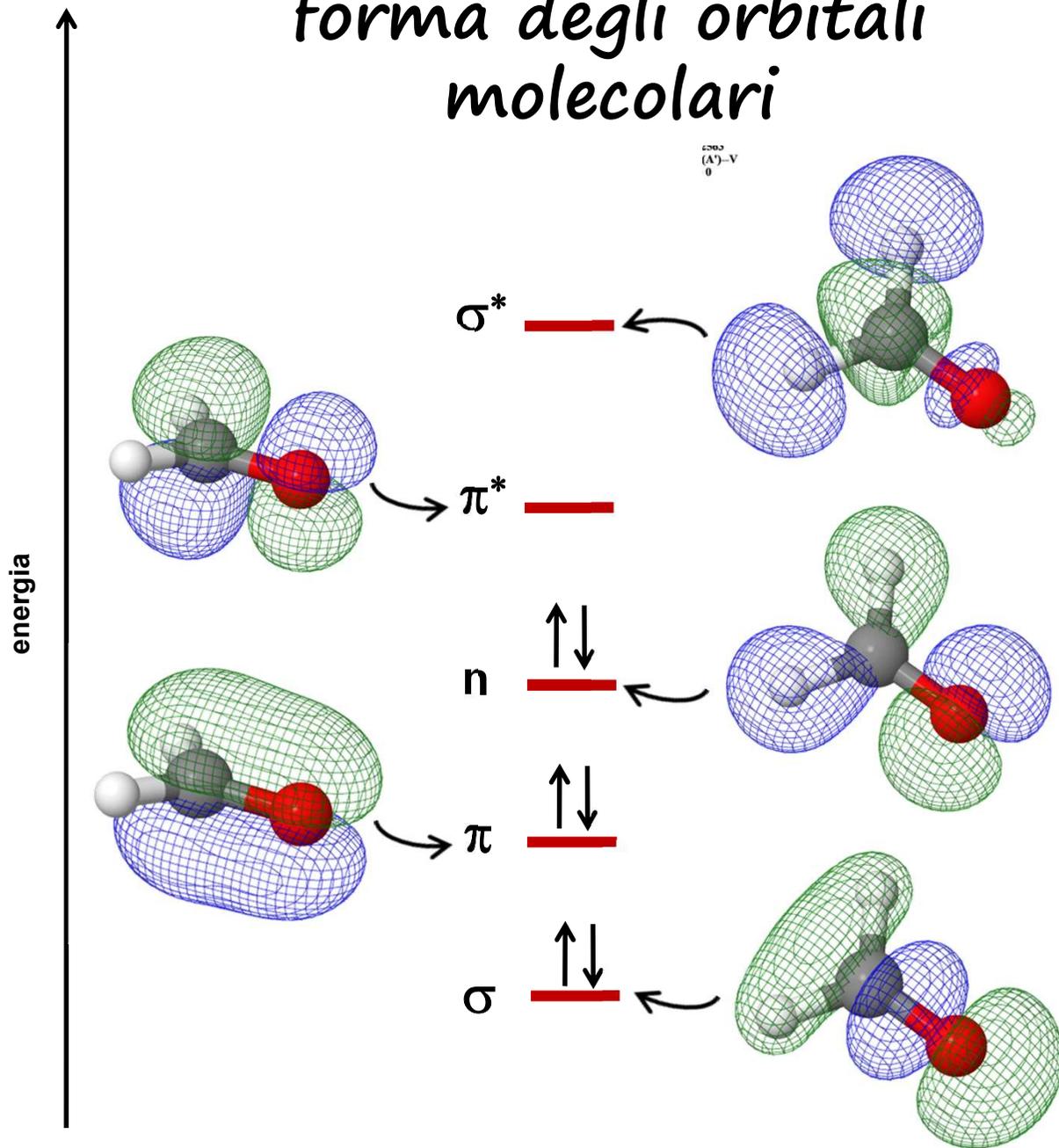
descrizione legame VBT



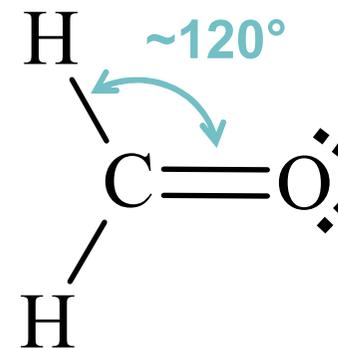
orbitali σ , π e n



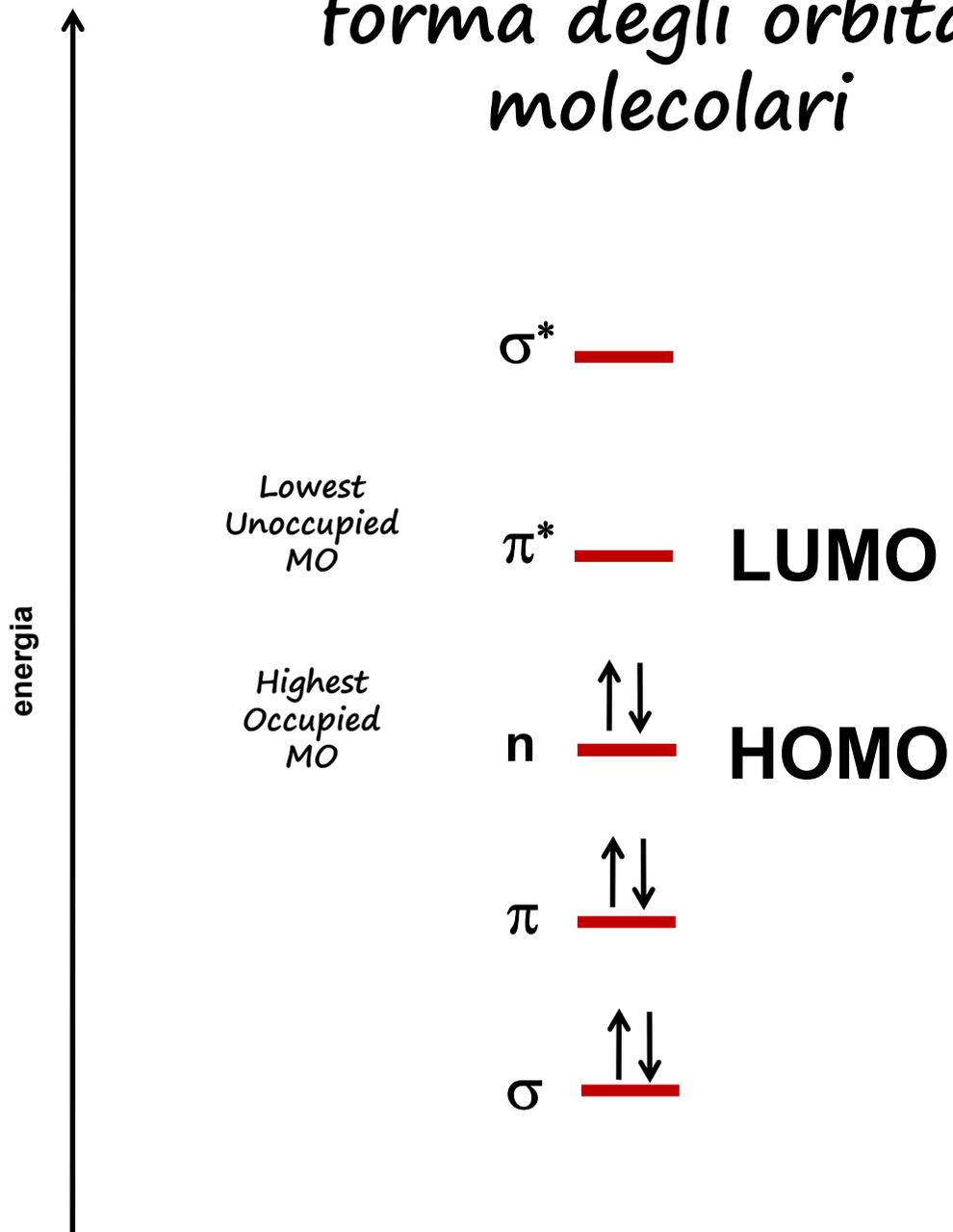
forma degli orbitali molecolari



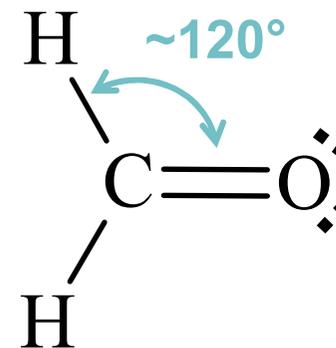
orbitali
delocalizzati su
tutta la
molecola, non
solo tra due
nuclei



forma degli orbitali molecolari

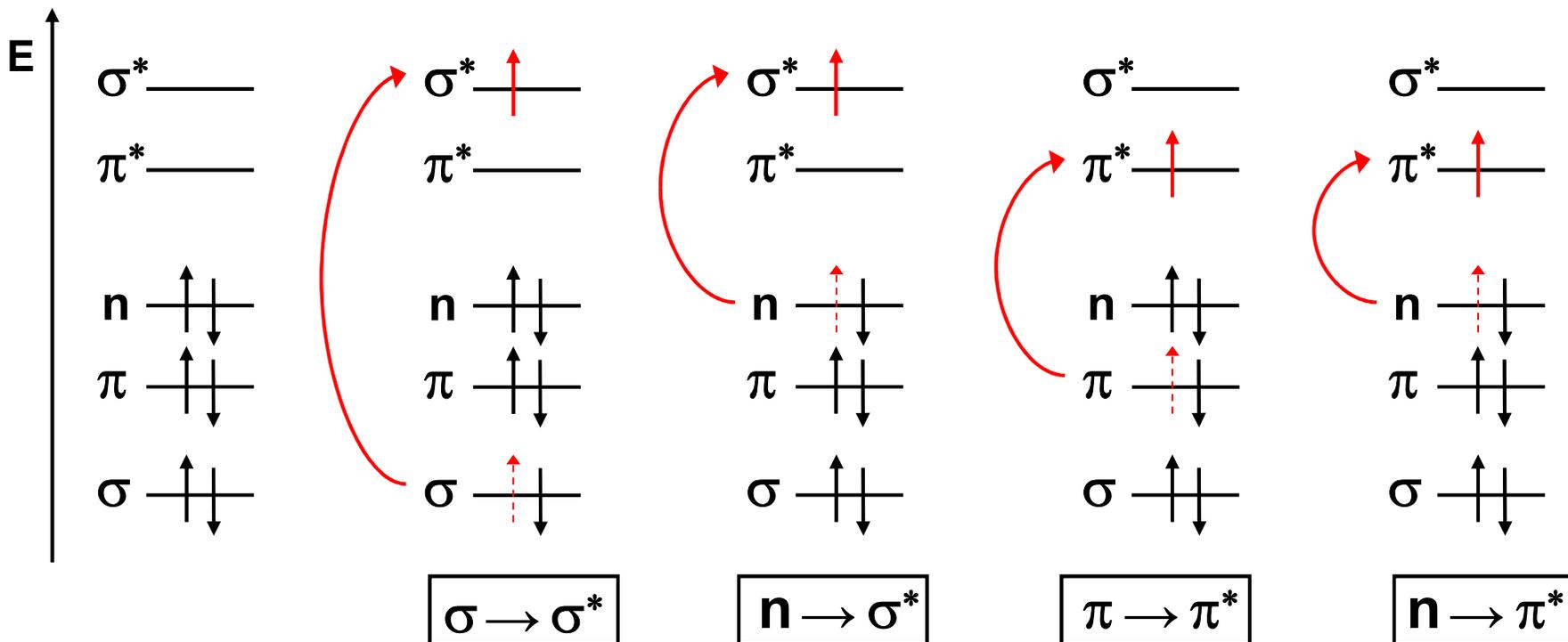


orbitali
delocalizzati su
tutta la
molecola, non
solo tra due
nuclei

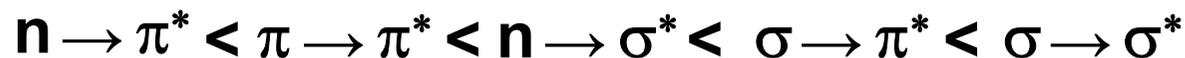


La Spettroscopia di Assorbimento nell'UV-visibile

Possibili transizioni elettroniche



In generale la scala di Energia delle transizioni è la seguente:



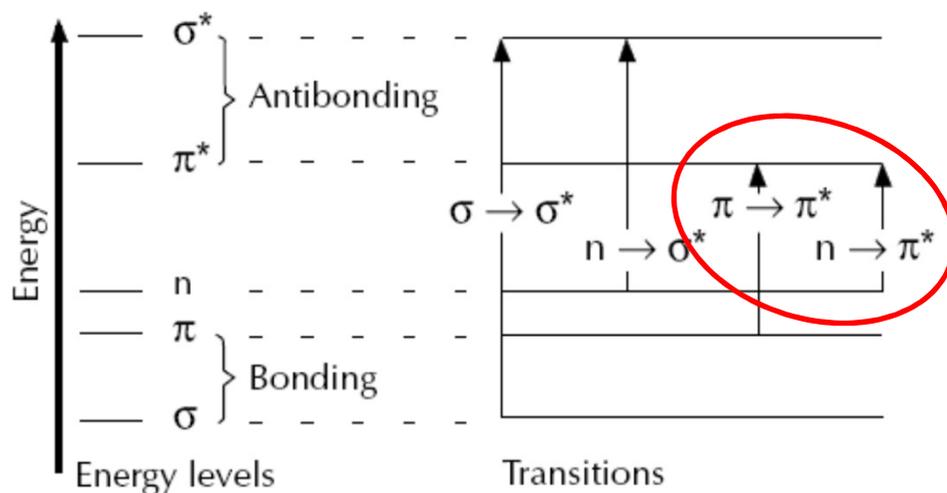
La Spettroscopia di Assorbimento nell'UV-visibile

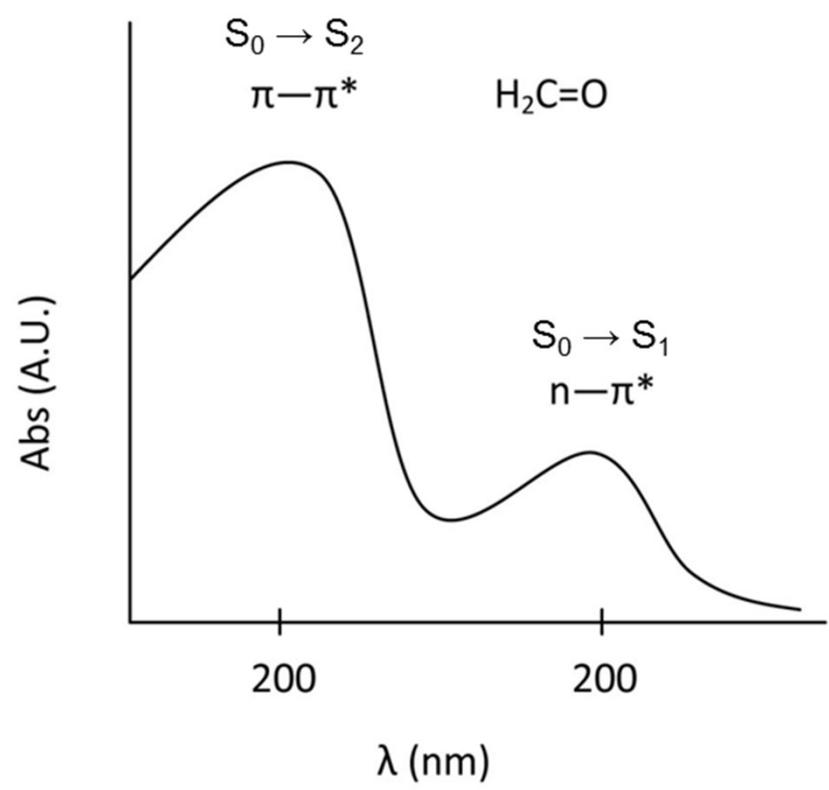
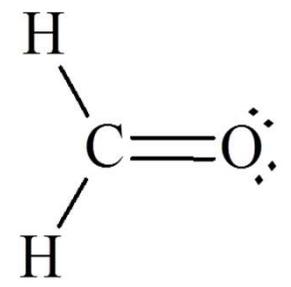
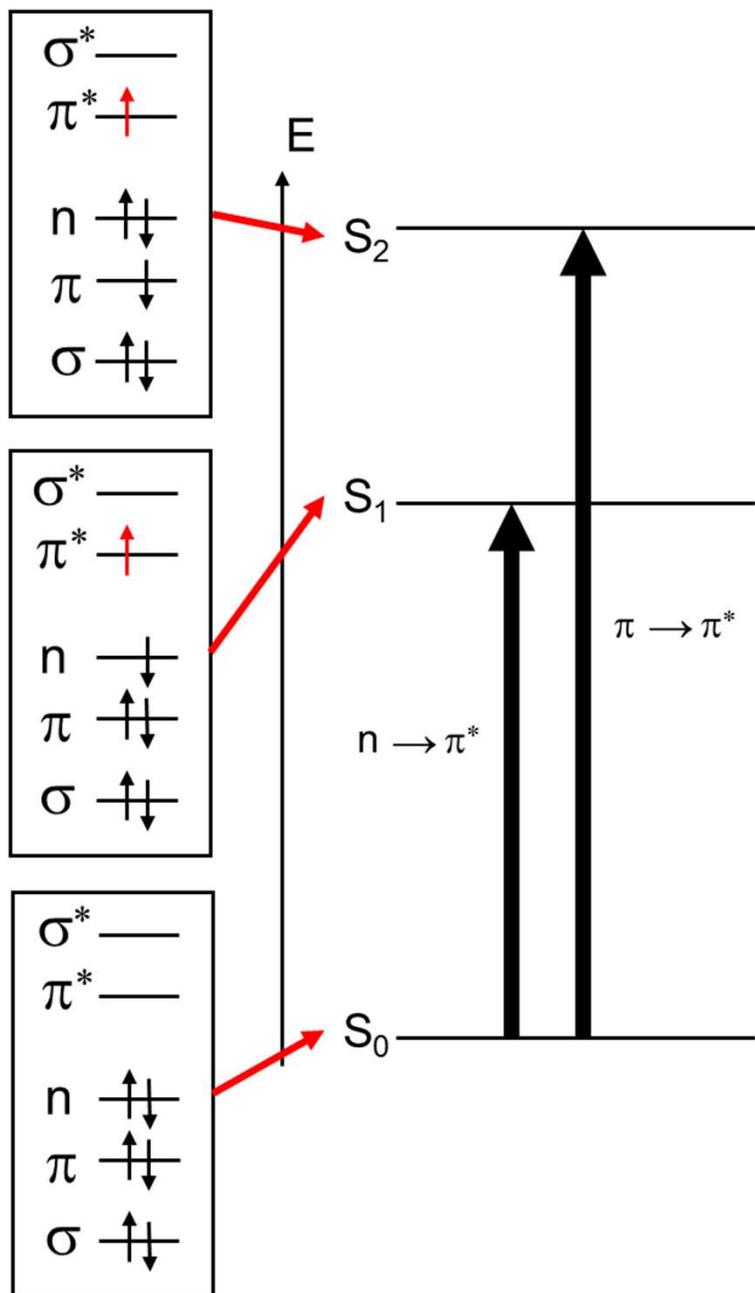
Table 10.5 Electronic Transitions Involving n , σ , and π Molecular Orbitals

Transition	Wavelength Range (nm)	Examples
$\sigma \rightarrow \sigma^*$	< 200	C—C, C—H
$n \rightarrow \sigma^*$	160–260	H ₂ O, CH ₃ OH, CH ₃ Cl
$\pi \rightarrow \pi^*$	200–500	C=C, C=O, C=N, C≡C
$n \rightarrow \pi^*$	250–600	C=O, C=N, N=N, N=O

le transizioni $\sigma \rightarrow \sigma^*$ hanno un'energia elevata

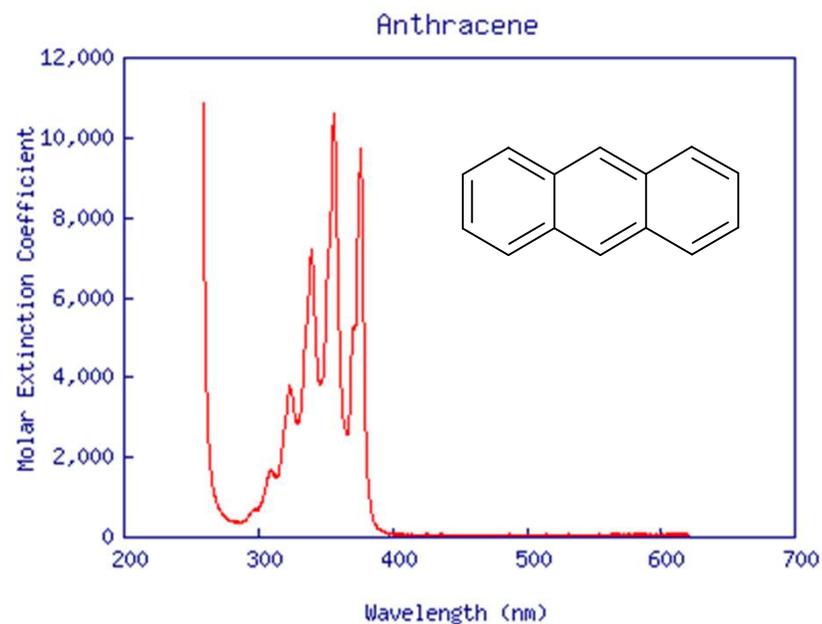
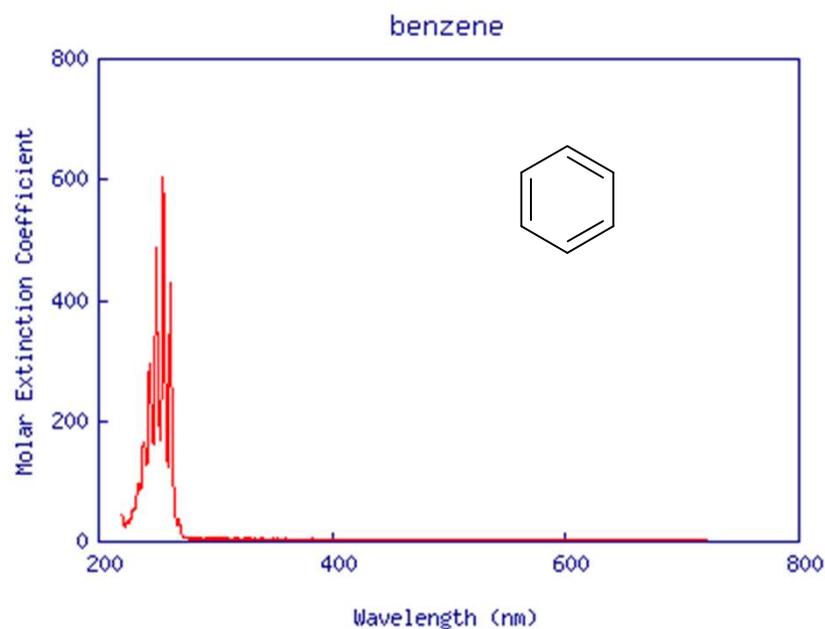
nell'UV-visibile sono comuni le transizioni $\pi \rightarrow \pi^*$ e $n \rightarrow \pi^*$





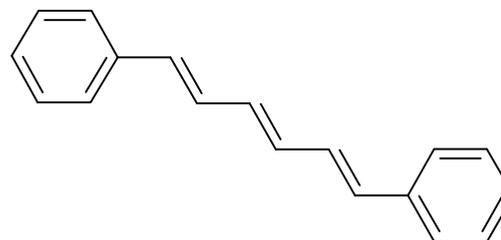
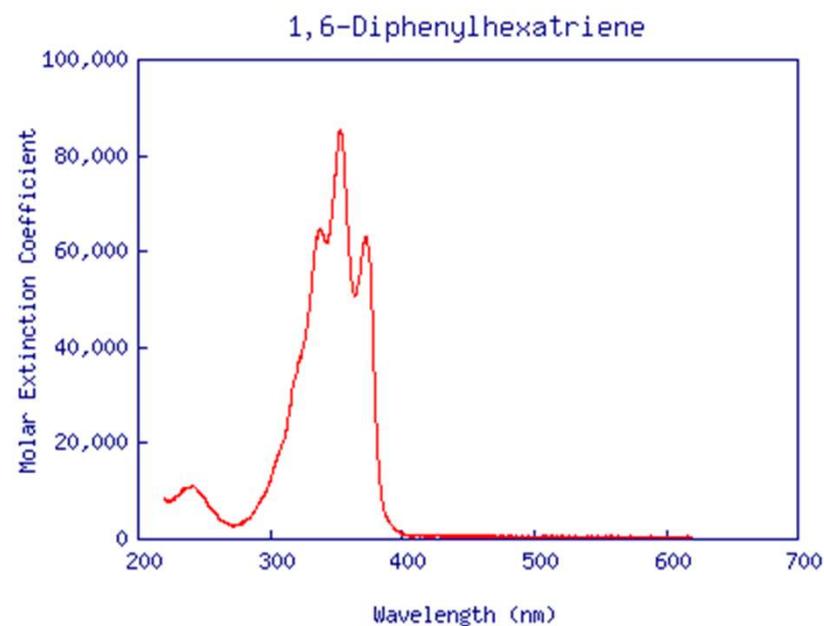
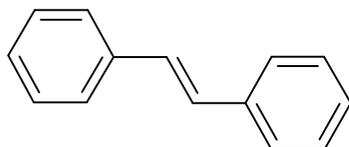
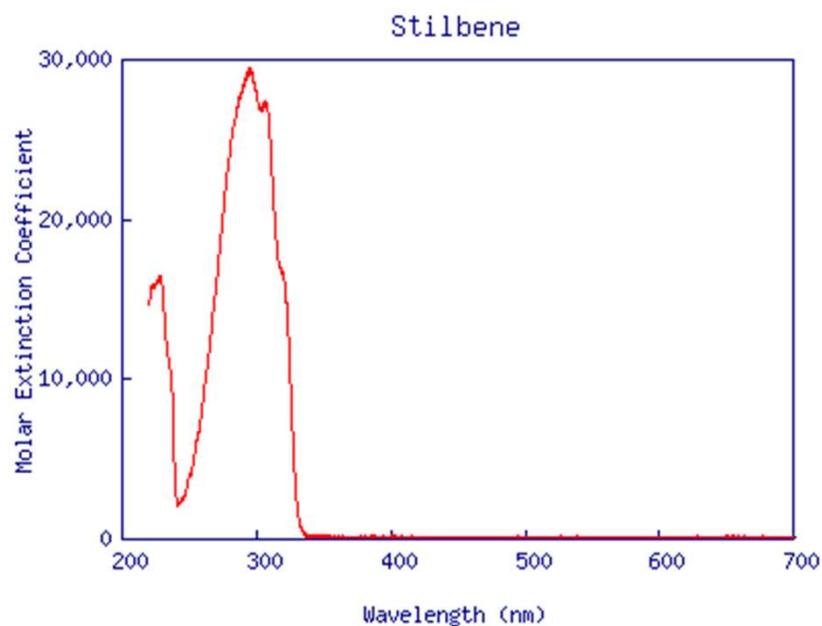
La Spettroscopia di Assorbimento nell'UV-visibile

Regola "generica": più è esteso un sistema π , è più bassa e l'energia corrispondente alla transizione $\pi-\pi^*$



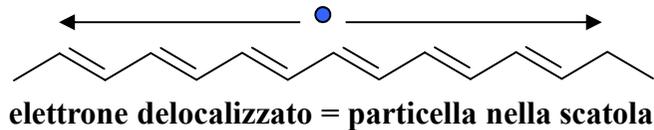
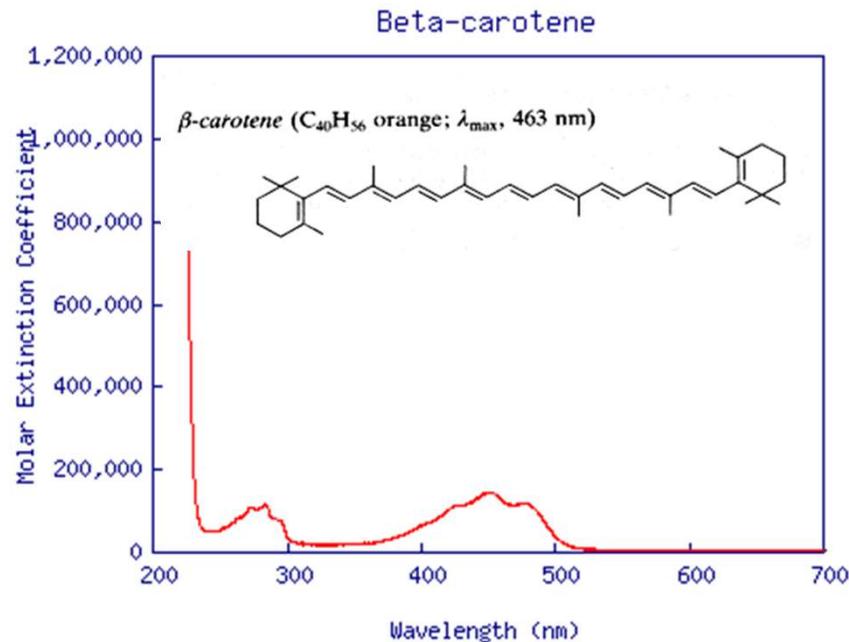
La Spettroscopia di Assorbimento nell'UV-visibile

Regola "generica": più è esteso un sistema π , è più bassa e l'energia corrispondente alla transizione $\pi-\pi^*$



La Spettroscopia di Assorbimento nell'UV-visibile

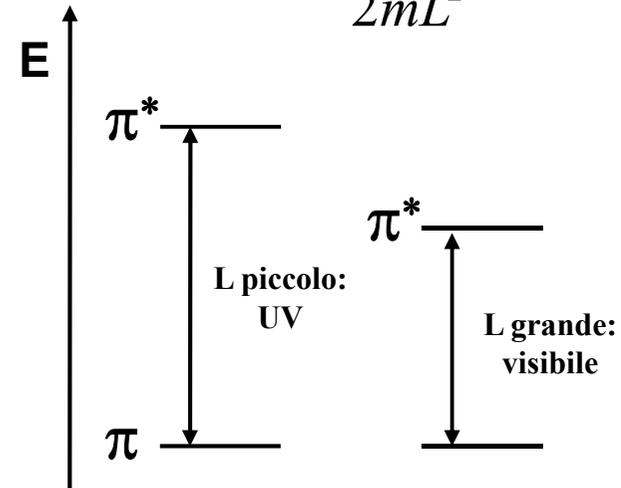
Regola "generica": più è esteso un sistema π , è più bassa e l'energia corrispondente alla transizione $\pi-\pi^*$



Energia particella nella scatola!

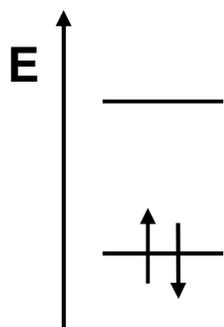
$$E_n = \frac{\hbar^2 \pi^2}{2mL^2} n^2$$

$$\Delta E = \frac{\hbar^2 \pi^2}{2mL^2} (2n + 1)$$



La Spettroscopia di Assorbimento nell'UV-visibile

“molteplicità” di spin = $2S+1$ “S” singoletto ($S=0$)
 “T” tripletto ($S=1$)

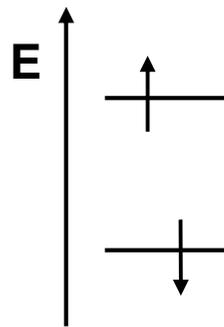


“S”

stato fondamentale
SINGOLETTO

$$S = \uparrow(+1/2) + \downarrow(-1/2) = 0$$

$$2S + 1 = 1$$

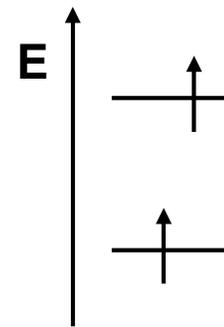


“S”

stato eccitato
SINGOLETTO

$$S = \uparrow(+1/2) + \downarrow(-1/2) = 0$$

$$2S + 1 = 1$$



“T”

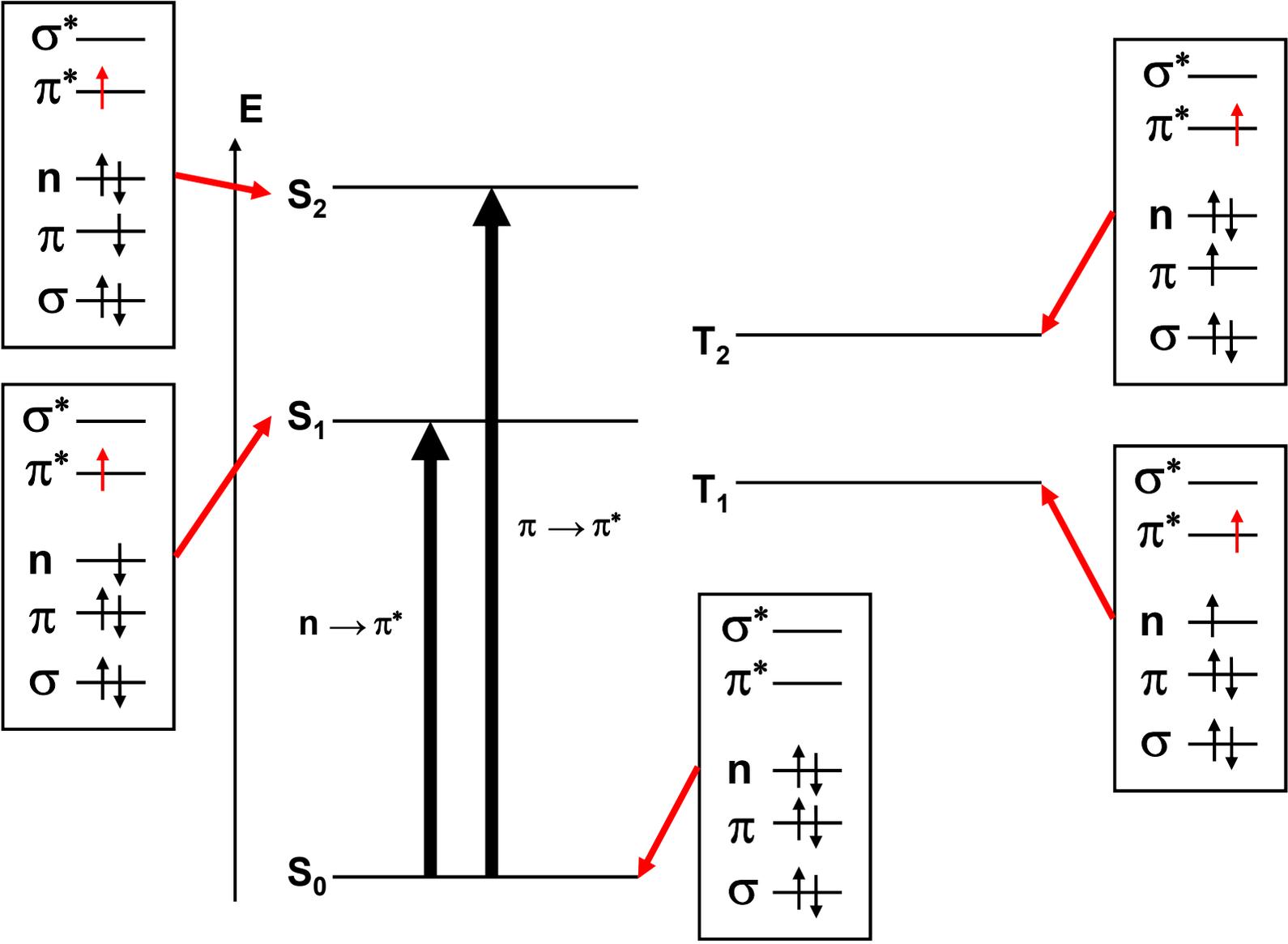
stato eccitato
TRIPLETTO

$$S = \uparrow(+1/2) + \uparrow(+1/2) = 1$$

$$2S + 1 = 3$$

↓ = elettrone spin -1/2 ↑ = elettrone spin +1/2

La Spettroscopia di Assorbimento nell'UV-visibile



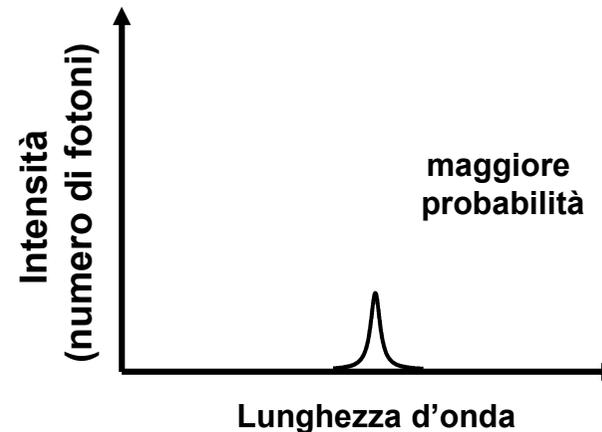
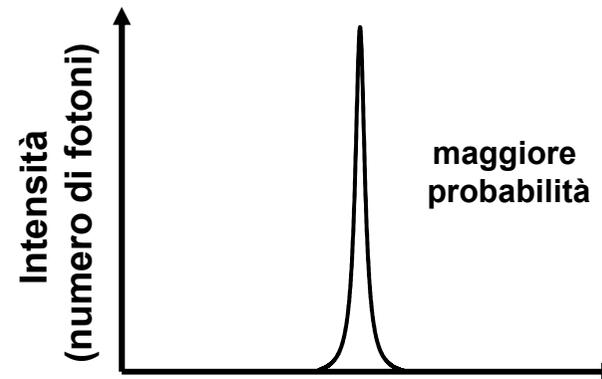
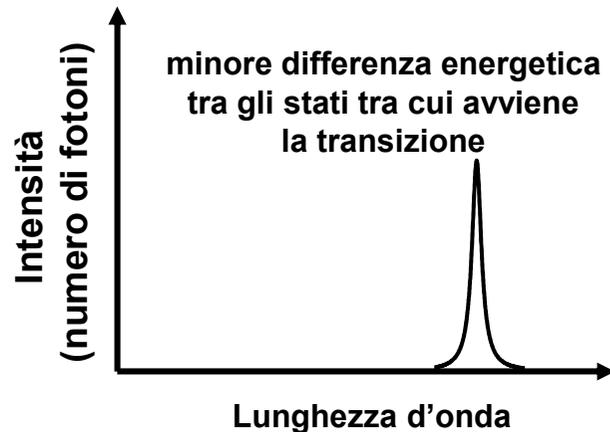
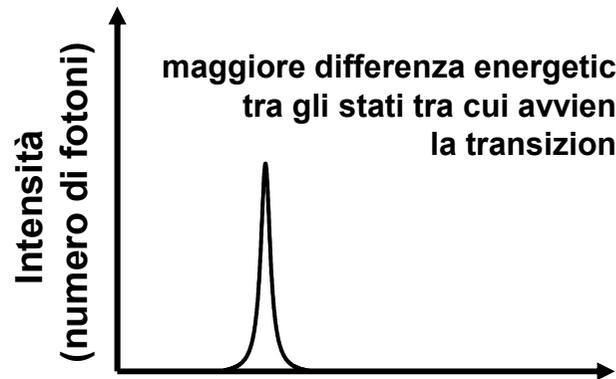
La Spettroscopia di Assorbimento nell'UV-visibile

lunghezza d'onda/frequenza legata alla
differenza energetica (ΔE) di una transizione

...e l'intensità?

Molto difficile da prevedere/calcolare
Legata a molti fattori

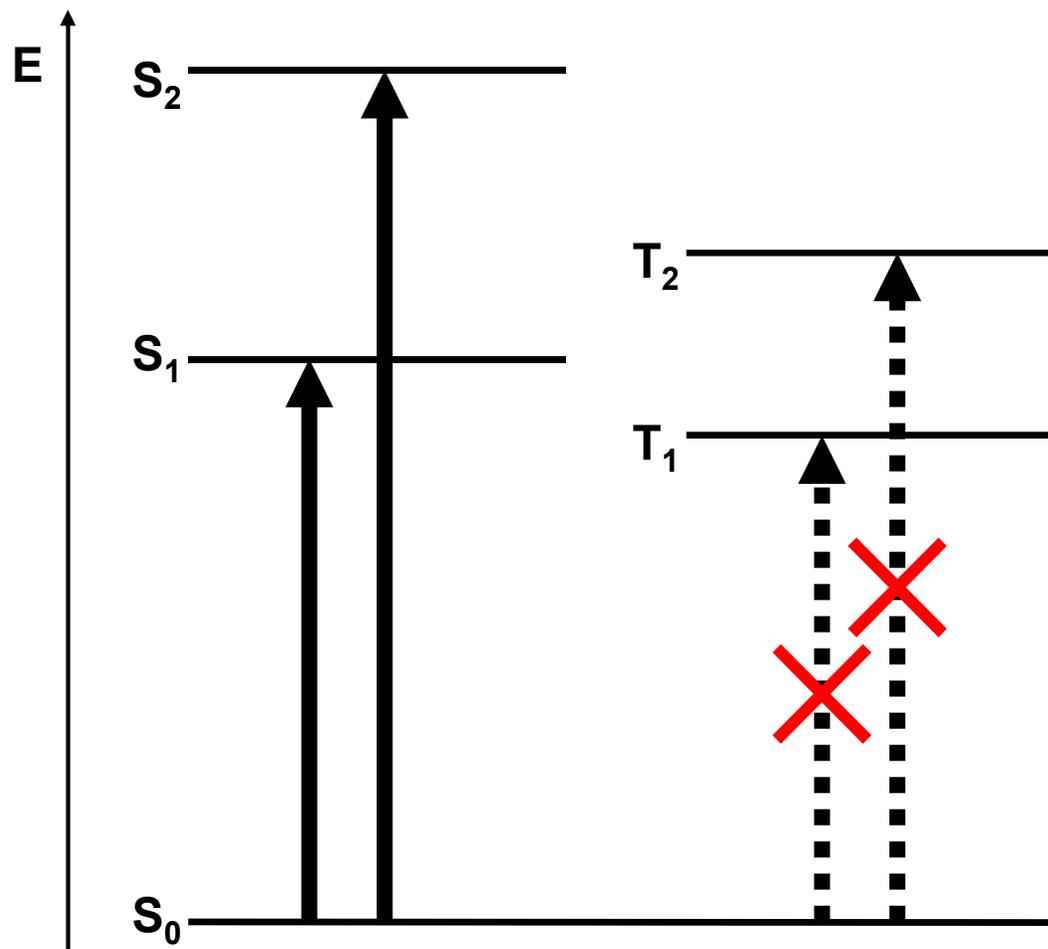
È legata alla probabilità che una transizione avvenga



La Spettroscopia di Assorbimento nell'UV-visibile

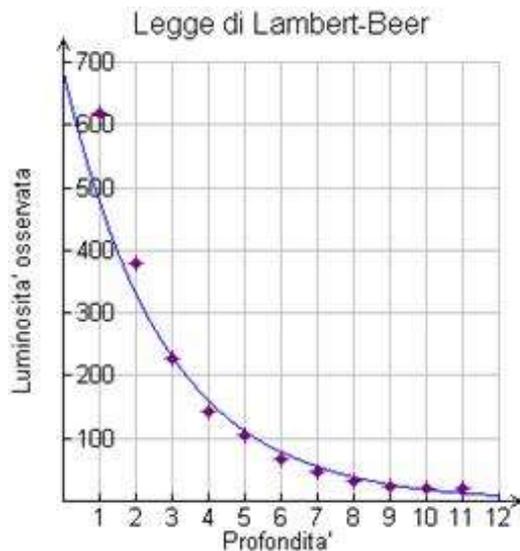
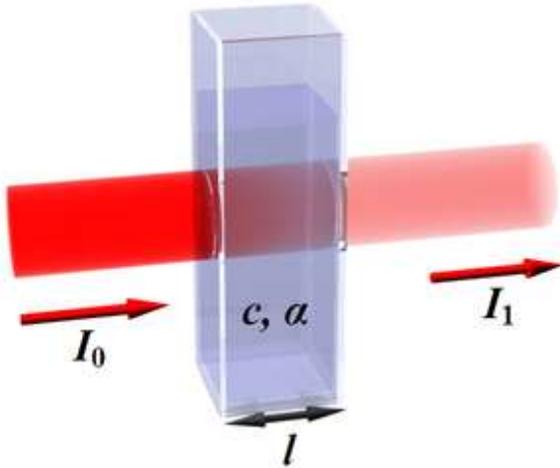
REGOLE DI SELEZIONE: ci dicono quali transizioni sono “permesse”

Per l'assorbimento nell'UV-Visibile: $\Delta S=0$



La Spettroscopia di Assorbimento nell'UV-visibile

Legge di Beer-Lambert



ASSORBENZA

$$A(\lambda) = \log \frac{I_{\lambda}^0}{I_{\lambda}^1} = -\log T(\lambda)$$

TRASMITTANZA $T(\lambda) = \frac{I_{\lambda}^1}{I_{\lambda}^0}$

$$A(\lambda) = \log \frac{I_{\lambda}^0}{I_{\lambda}^1} = \alpha(\lambda)lc$$

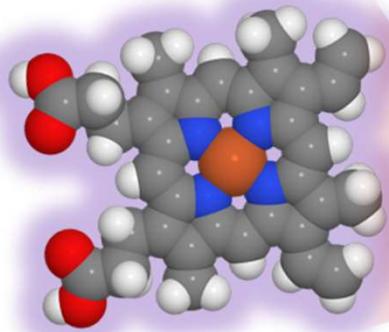
↑
COEFFICIENTE DI ESTINZIONE MOLARE

se conosciamo il coeff.di est. mol.
possiamo calcolare la concentrazione

**UV-Vis = METODO ANALITICO
QUANTITATIVO**

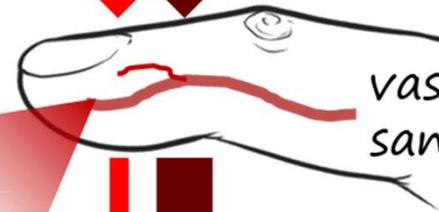
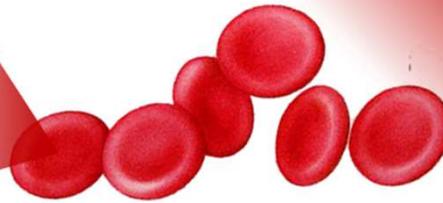
Applicazioni

L'esempio dell'ossimetro



emoglobina

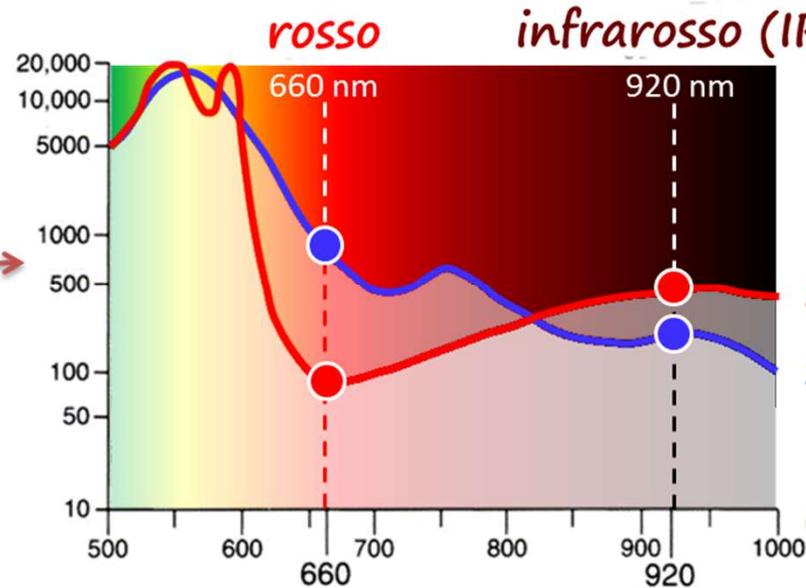
globuli rossi



"ossimetro"



quantità di luce "assorbita" dalla materia



sanguine con ossigeno

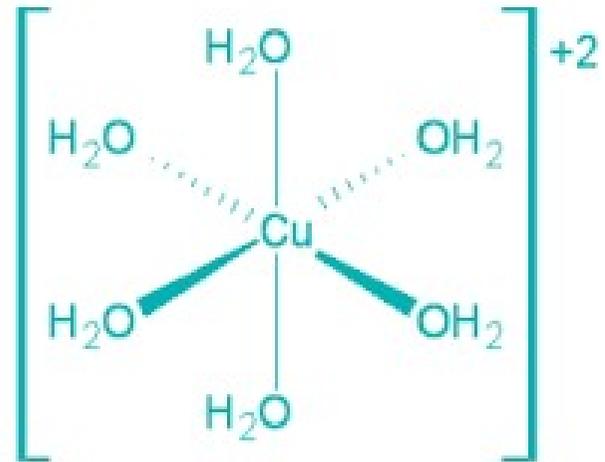
sanguine senza ossigeno

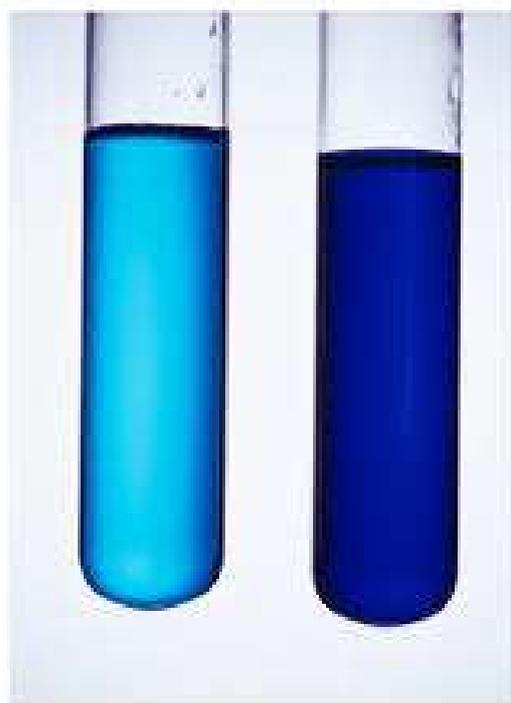
colore (lunghezza d'onda espressa in nm)

La Spettroscopia di Assorbimento nell'UV-visibile

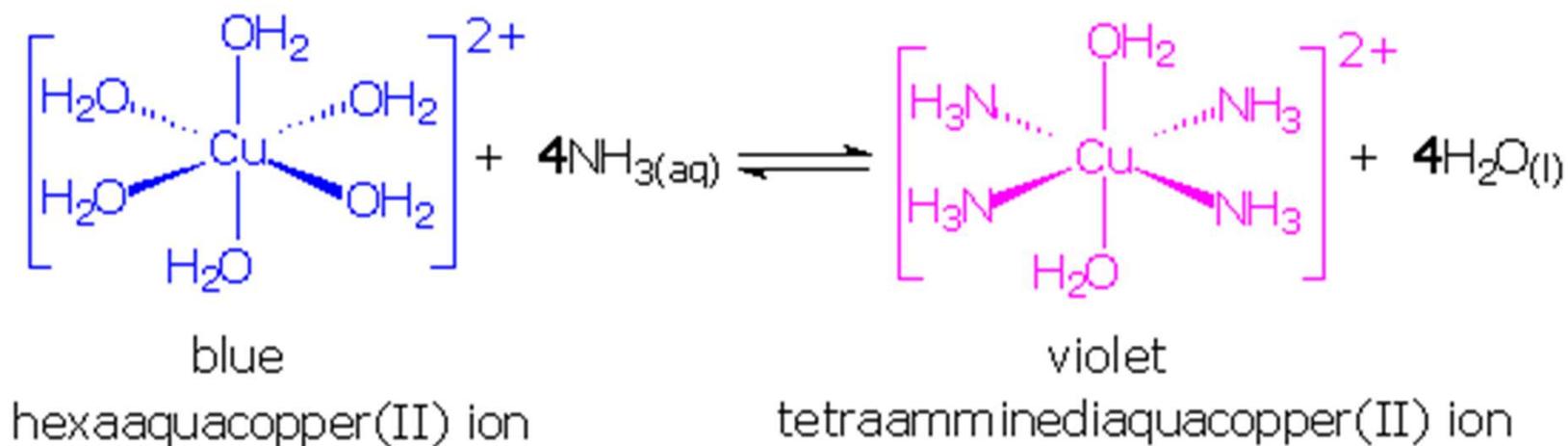
COMPOSTI INORGANICI: complessi METALLI di Transizione

soluzione aq. Cu (II)
(solfato di Rame)

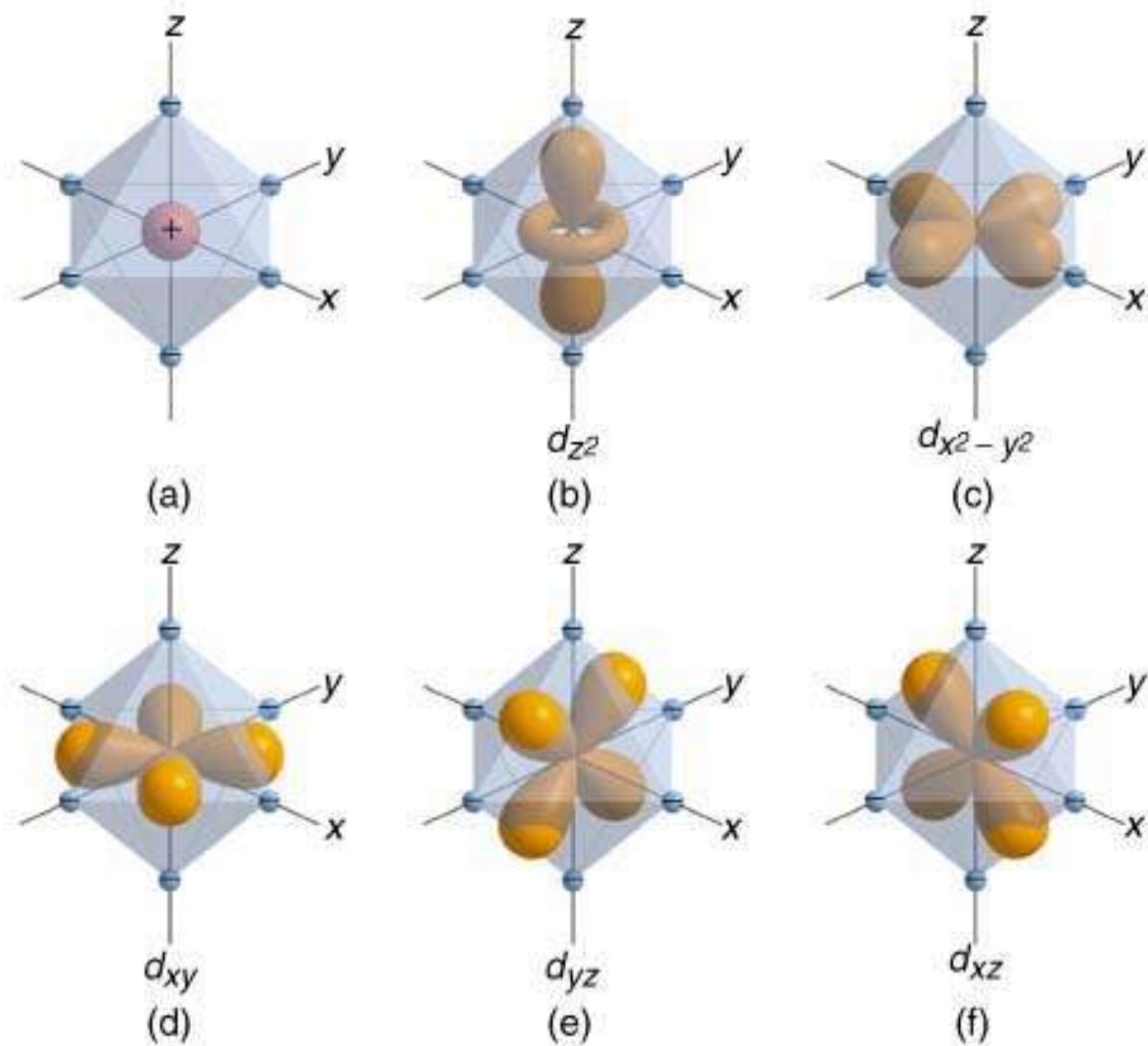




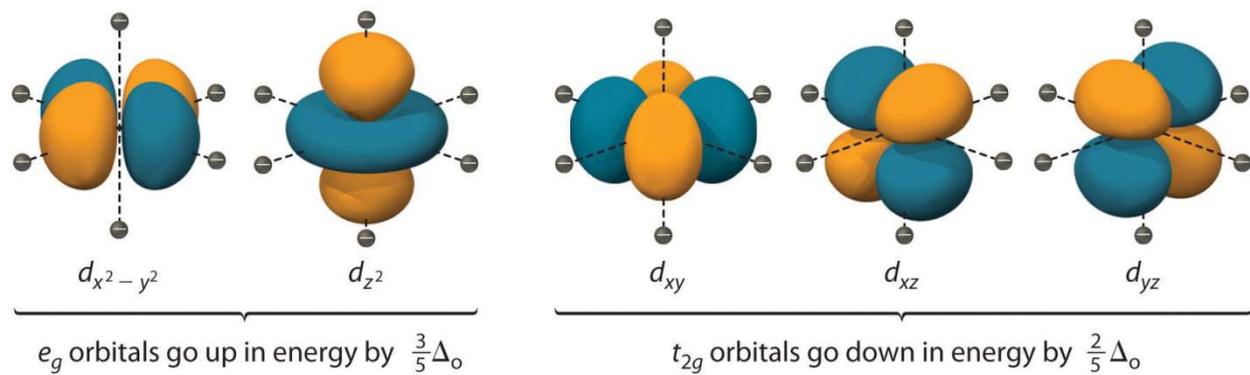
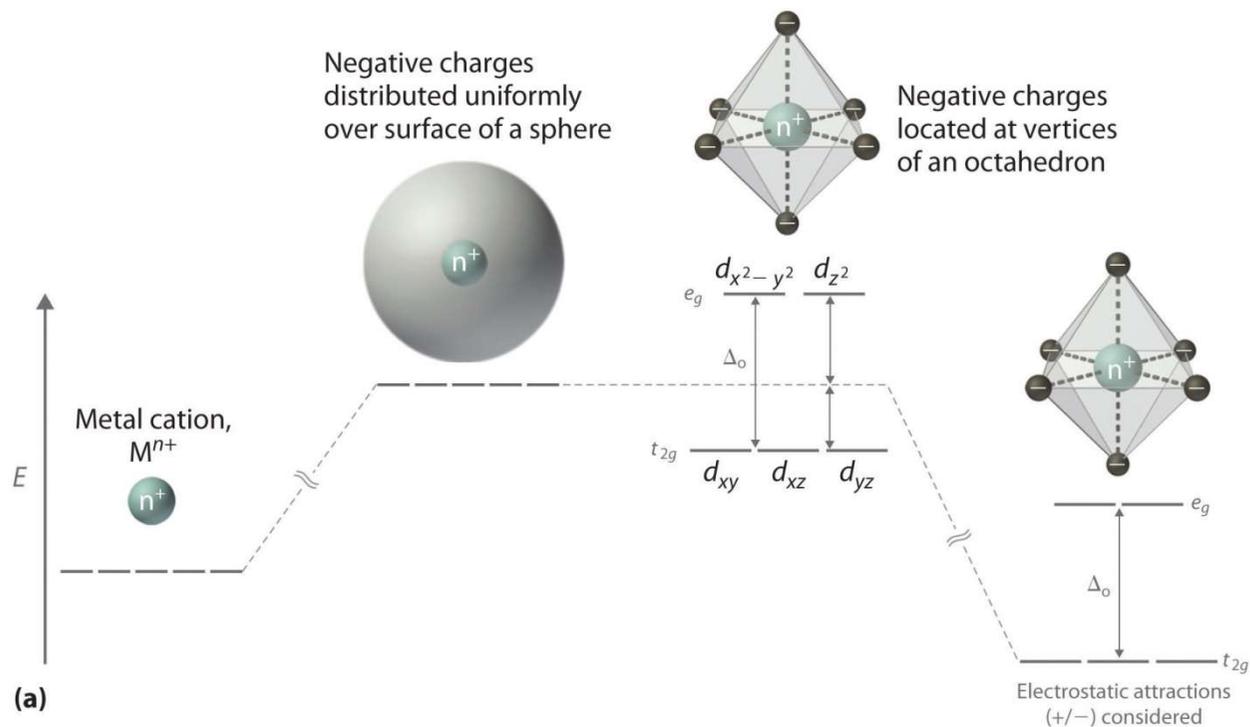
**colore
dipende
dai leganti
del
metallo**



Crystal Field Theory



Crystal Field Theory



(b)

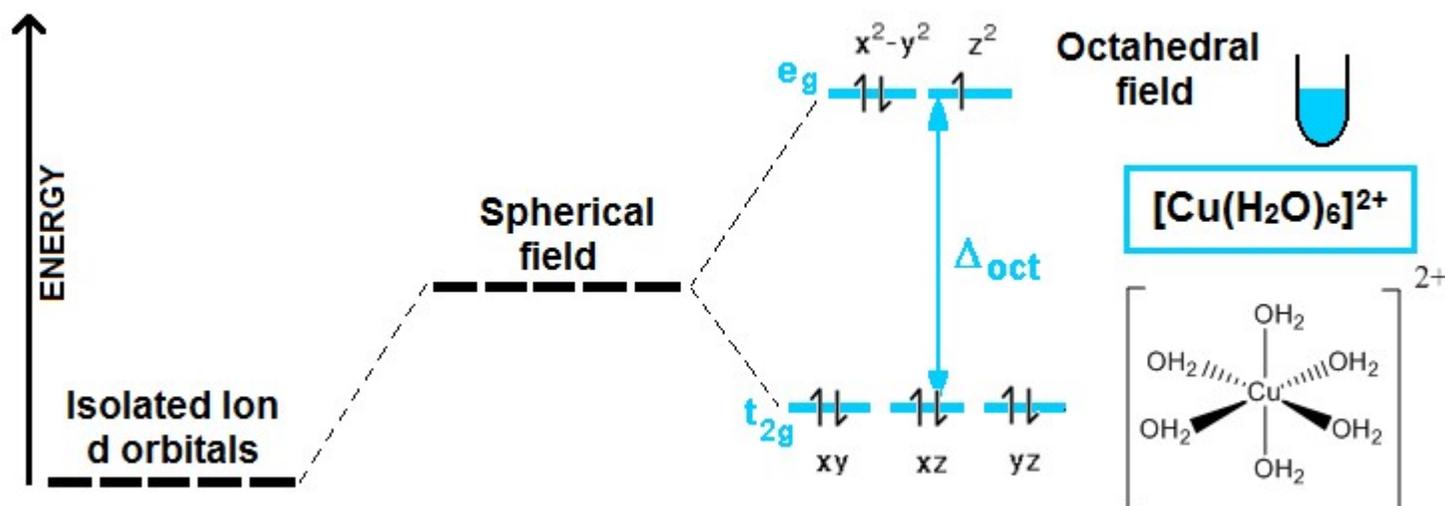
Crystal Field Theory



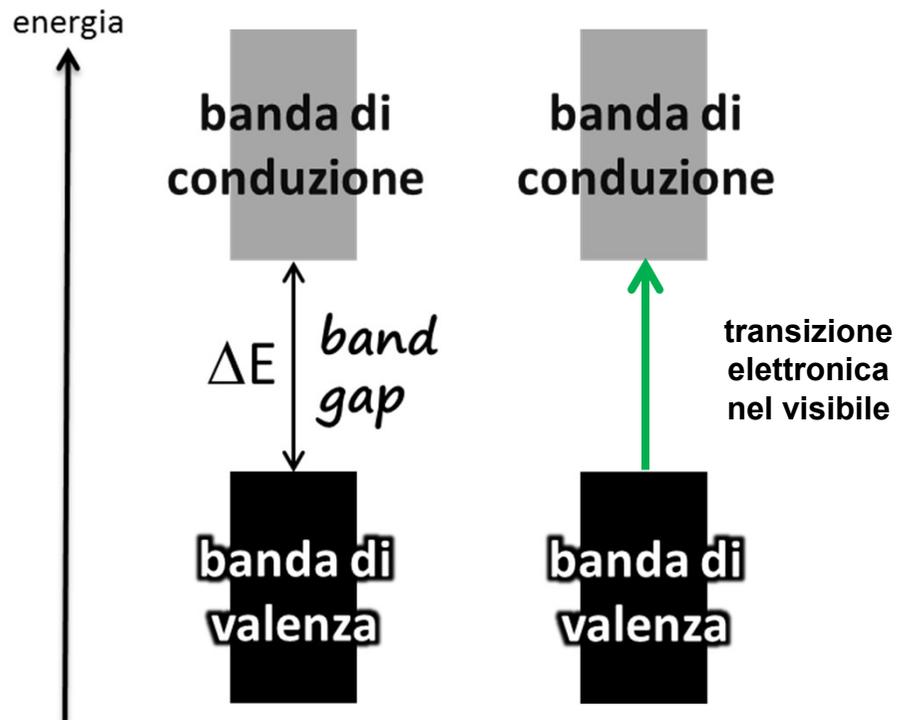
turquoise



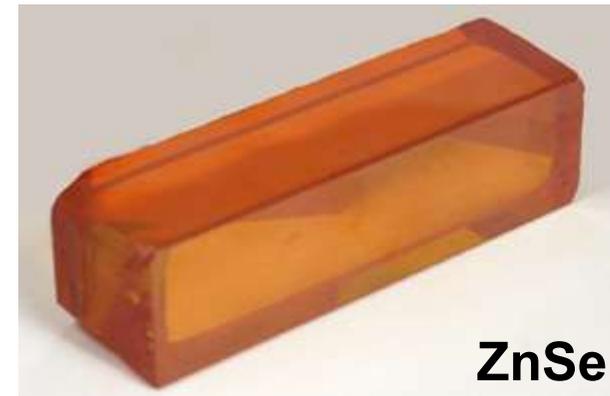
color caused by the presence of Cu^{2+} ions coordinated by OH^- and H_2O



assorbimento nei solidi inorganici covalenti

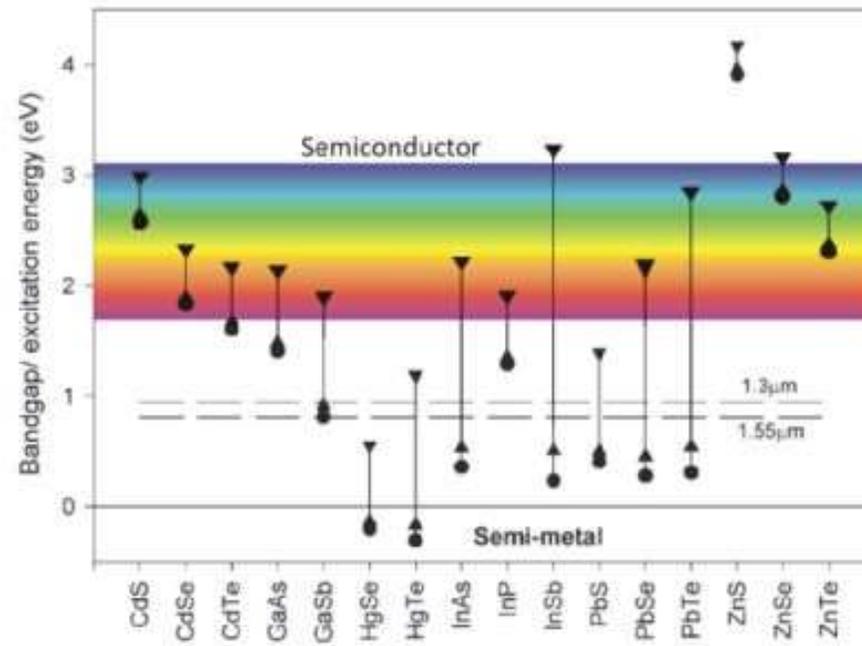


CdS

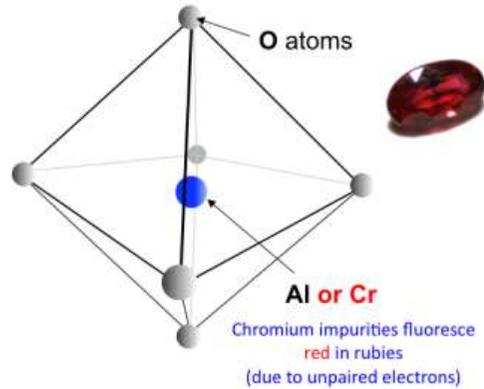


ZnSe

assorbimento nei solidi inorganici covalenti



Al_2O_3 : Corundum Crystal Structure



Ruby

~1% Cr^{3+} doped Al_2O_3

Absorbs yellow-green region

Emits red

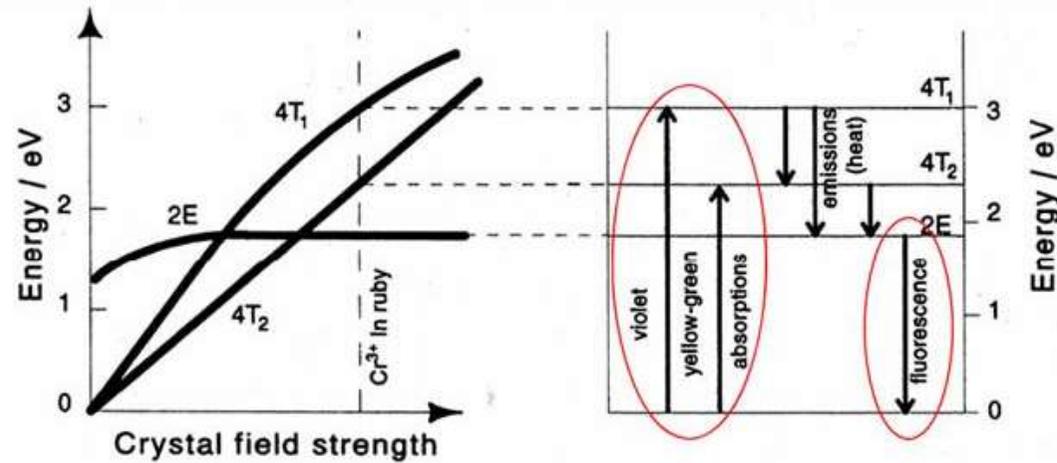
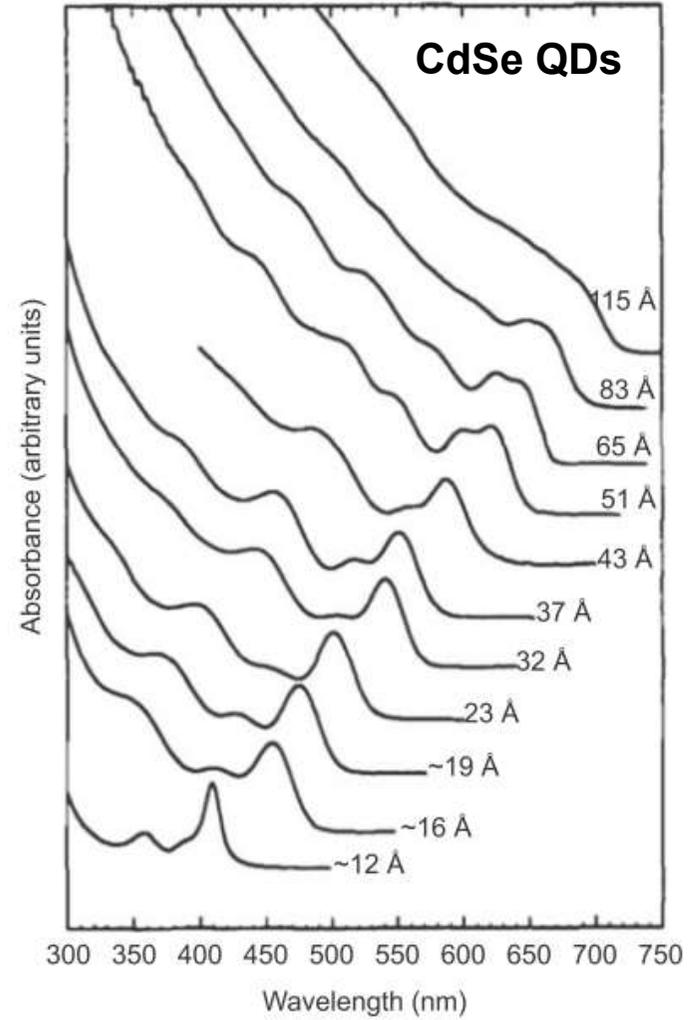
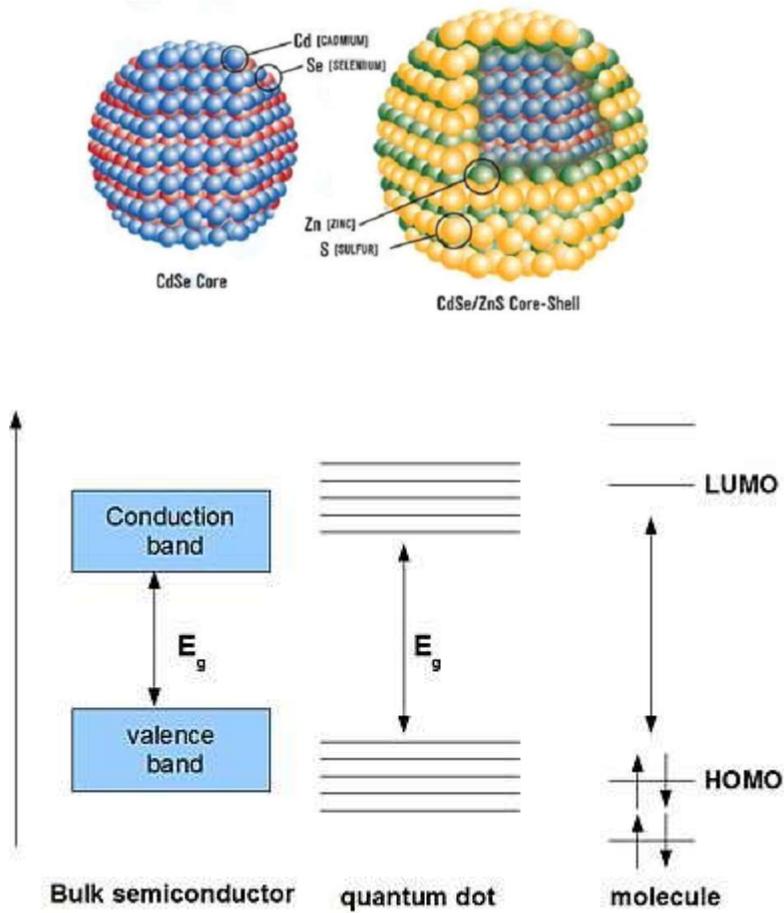
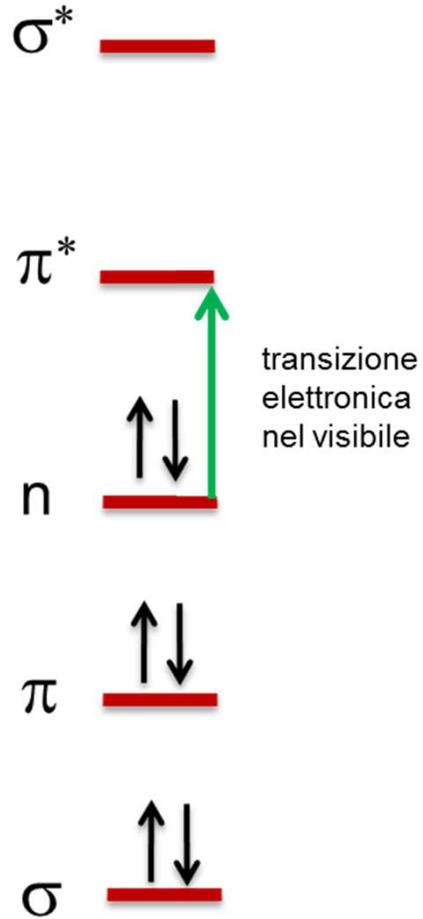


Figure 2.9. The effect of crystal field strength on the splitting of the energy levels of Cr^{3+} ions in a lattice. Styled after K. Nassau (1980), *Scientific American*, October 1980, 134.

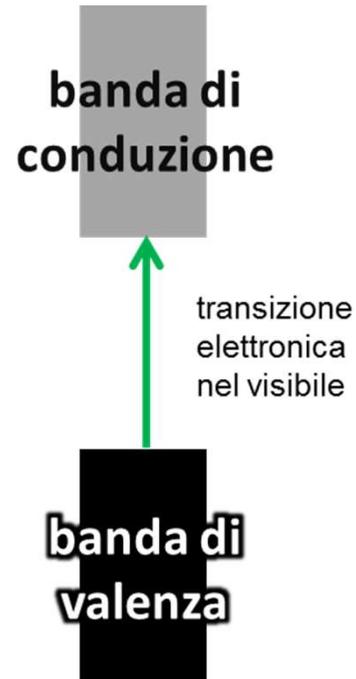
quantum dots



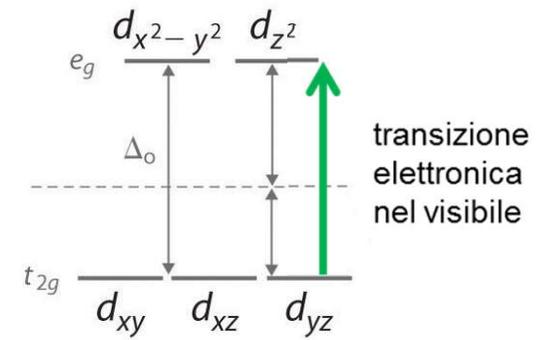
**molecules/
molecular solids**



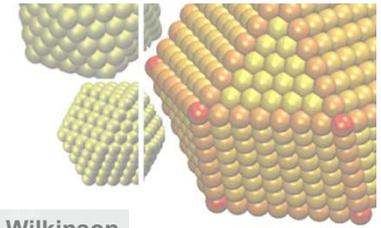
**non-molecular solids
(semiconductors)**



**transition metals
ions**



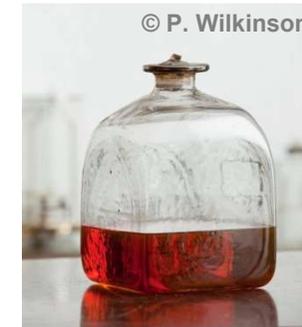
metal nanoparticles



The Lycurgus Cup (British Museum)



Stained glass (Chartres cathedral)



“Ruby gold” (Faraday Museum)

4th century

12th century

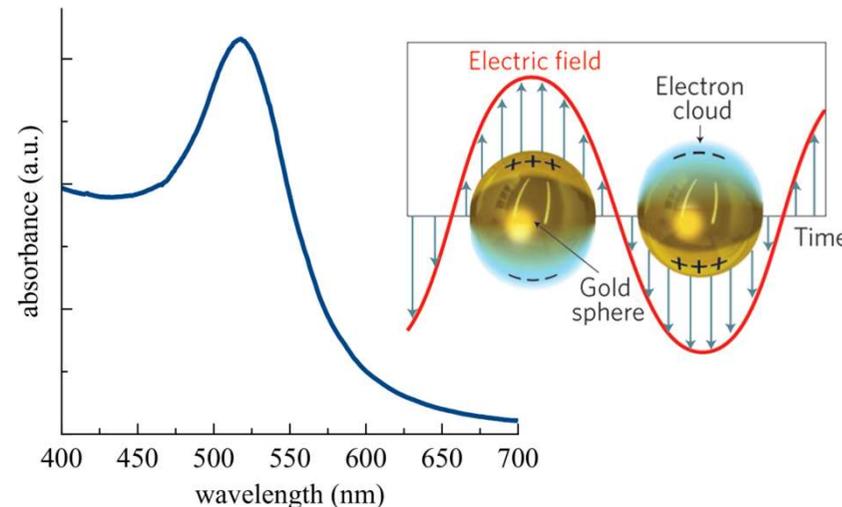
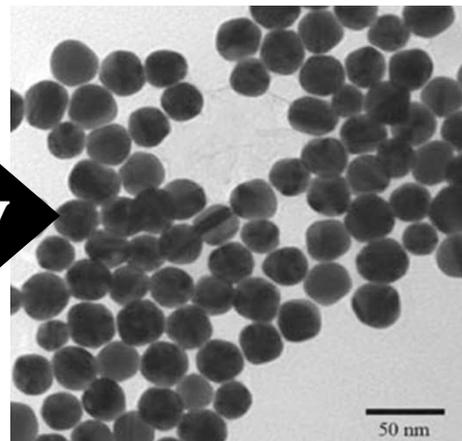
19th century



M. Faraday (1791-1869)
Phil. Trans. R. Soc. 147 (1857), 145-181

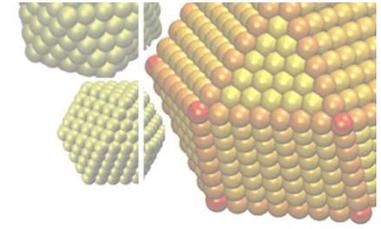
nanotechnology
plasmonics

21st century

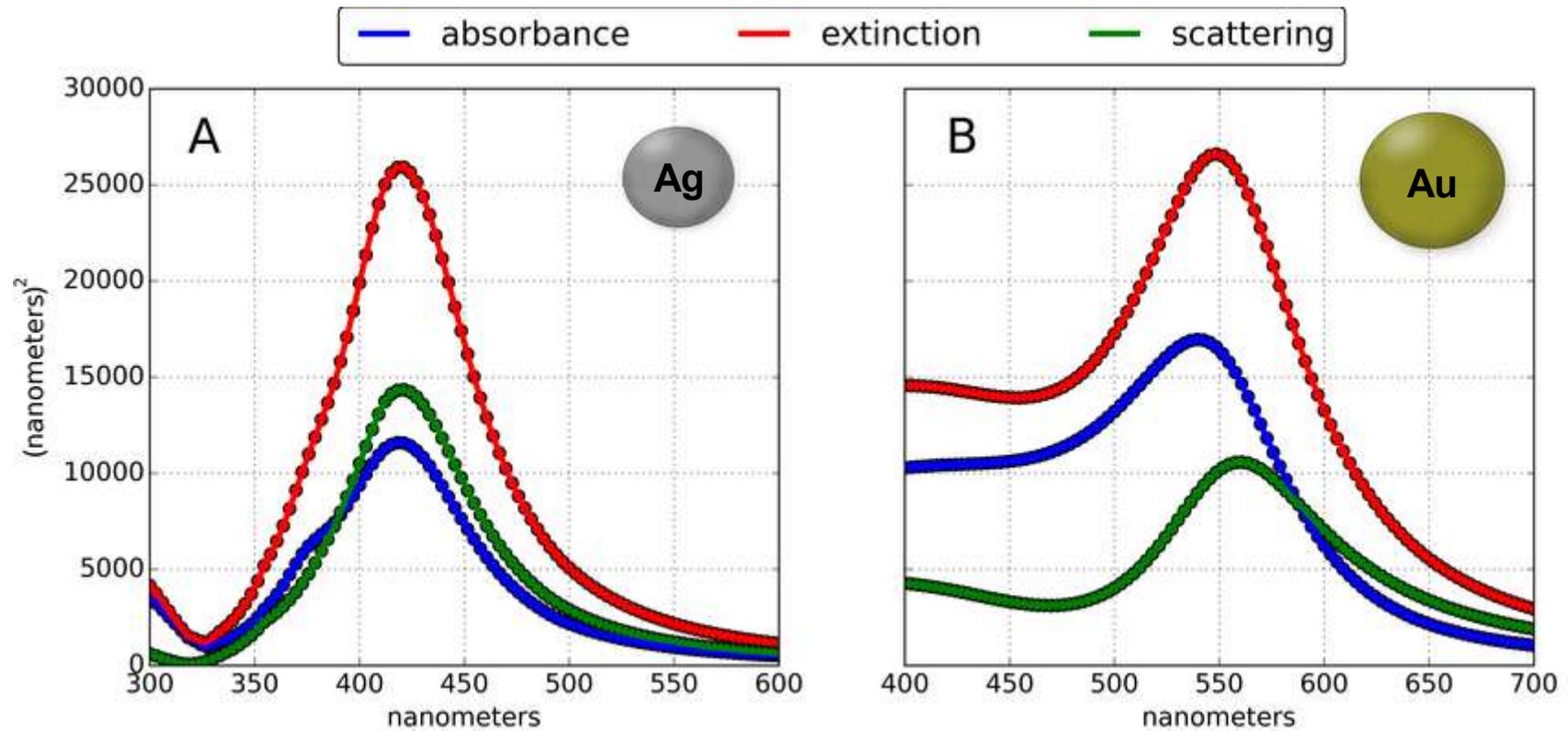


ML Juan et al. Nat. Photon. 5, 349–356 (2011)
Willems and RP Van Duyne Annu. Rev. Phys. Chem 58, 267–97 (2007)

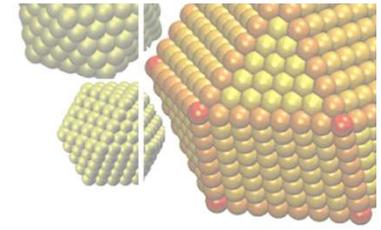
metal nanoparticles



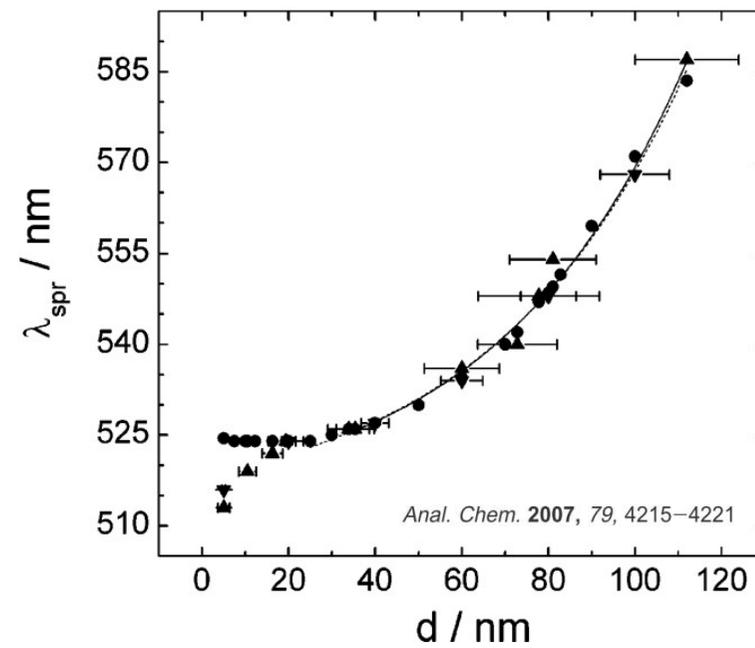
estinzione = assorbimento + diffusione



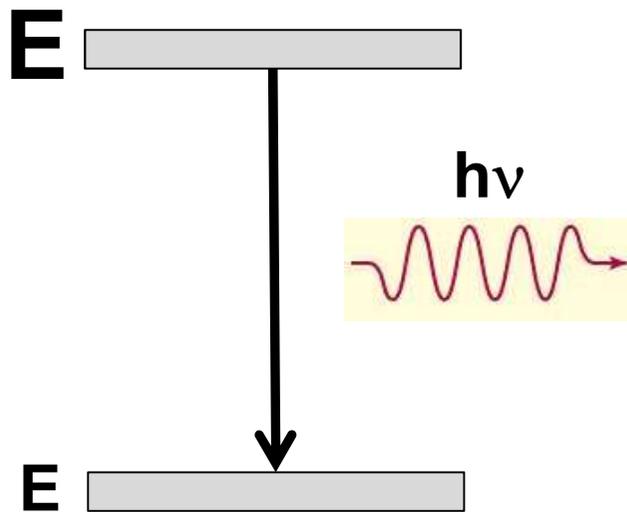
metal nanoparticles



dimensione – massimo di estinzione

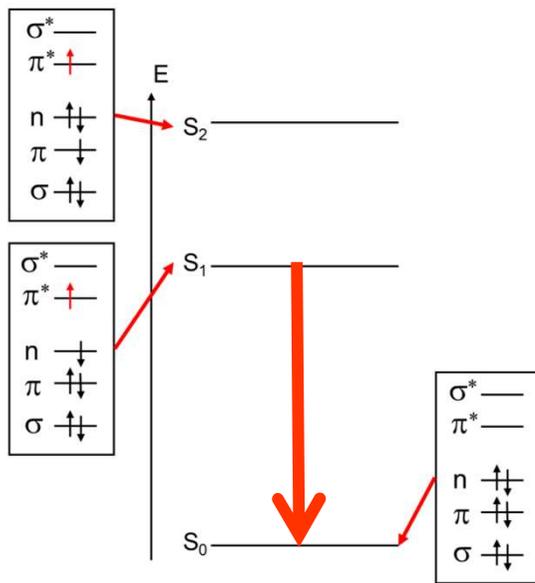


Fluorescenza

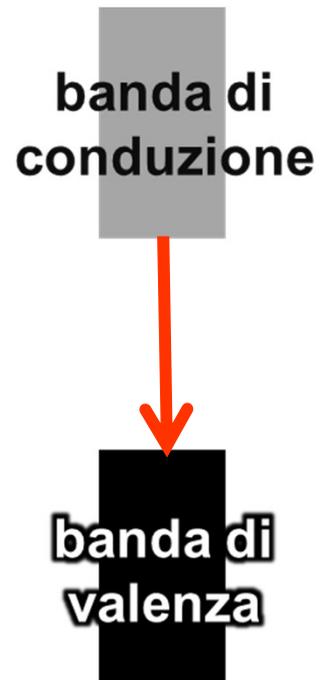


**emissione di radiazione in
conseguenza di una
transizione elettronica da
uno stato elettronico ad
energia maggiore ad uno ad
energia minore**

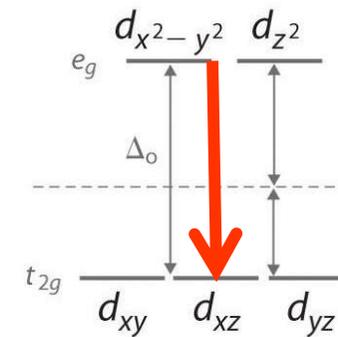
**molecules/
molecular solids**



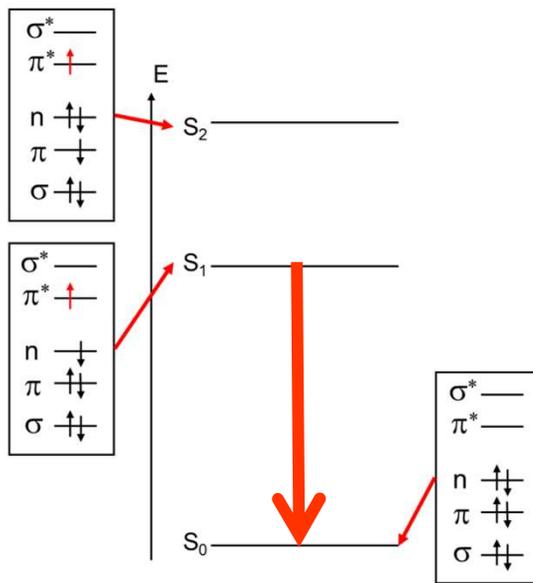
**non-molecular solids
(semiconductors)**



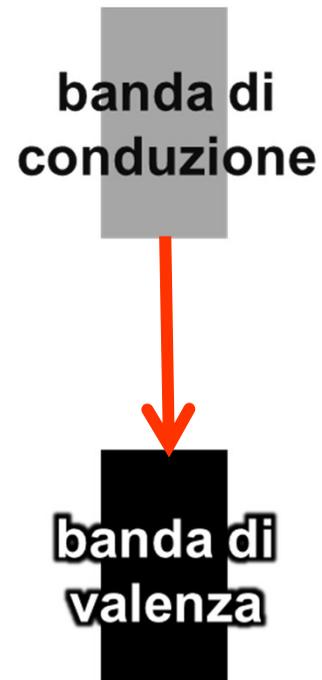
**transition metals
ions**



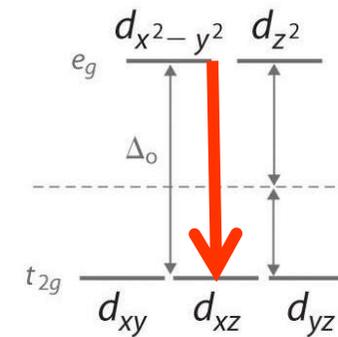
molecules/ molecular solids



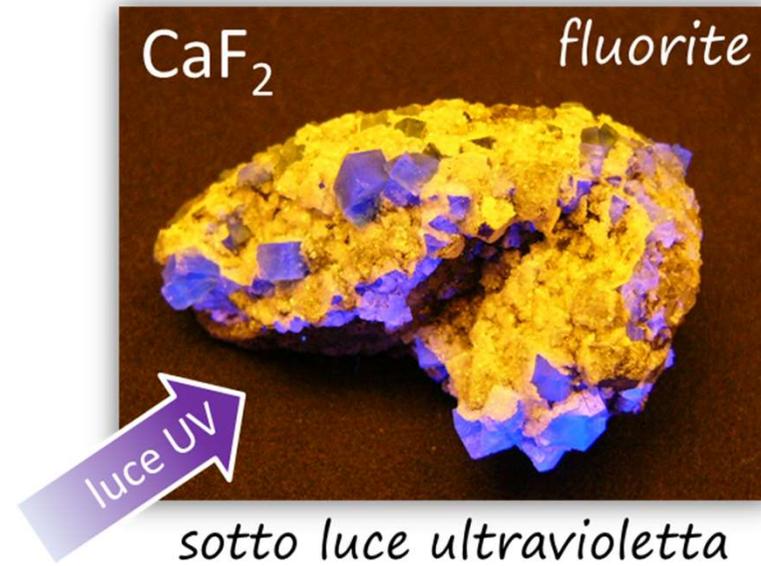
non-molecular solids (semiconductors)

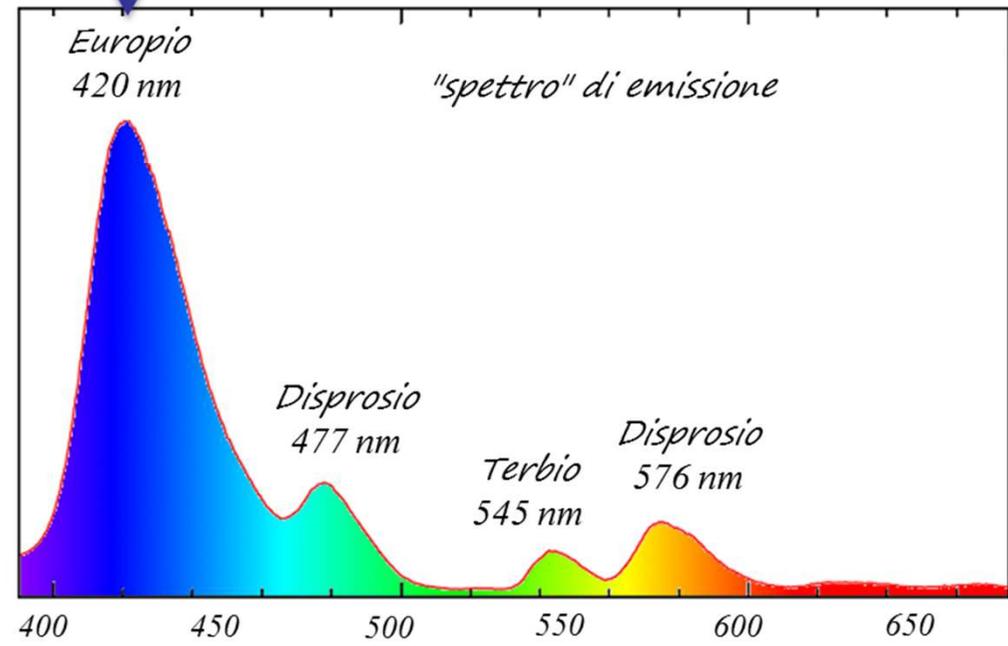
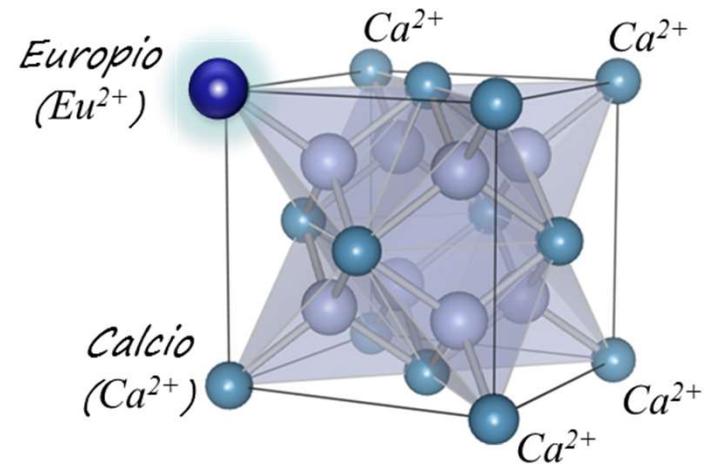


transition metals ions



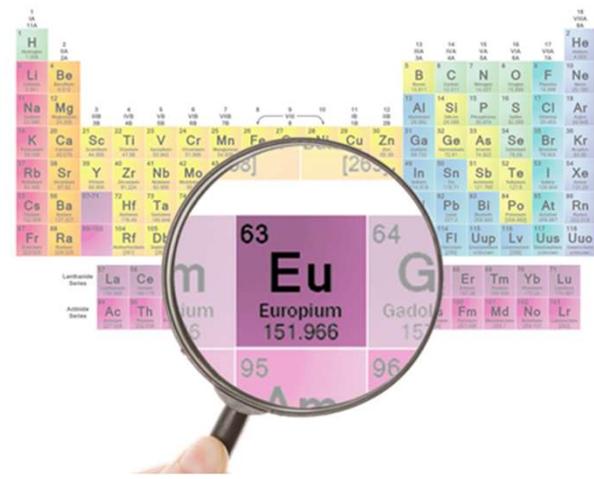
Fluorescenza (fotoluminescenza) di minerali non utilizzata per identificazione, ma per localizzazione



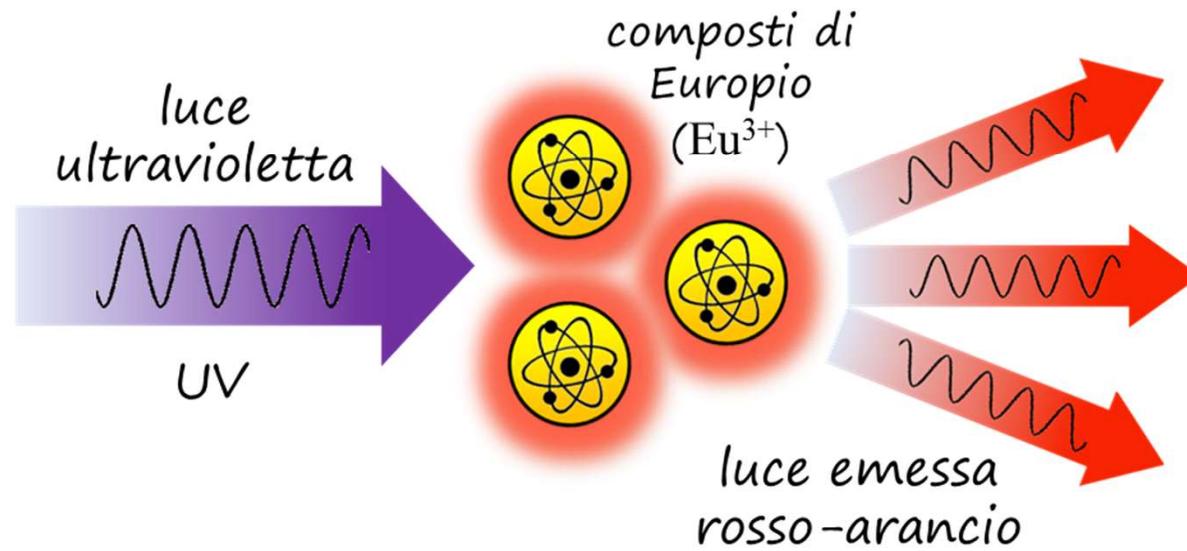


quantità di luce "emessa" dalla materia

colore (lunghezza d'onda espressa in nm)



metodo
anti-contraffazione



Fluorescenza (fotoluminescenza) di minerali

non utilizzata per identificazione, ma per localizzazione



Piezospettroscopia Al_2O_3

Al_2O_3 :Corundum Crystal Structure

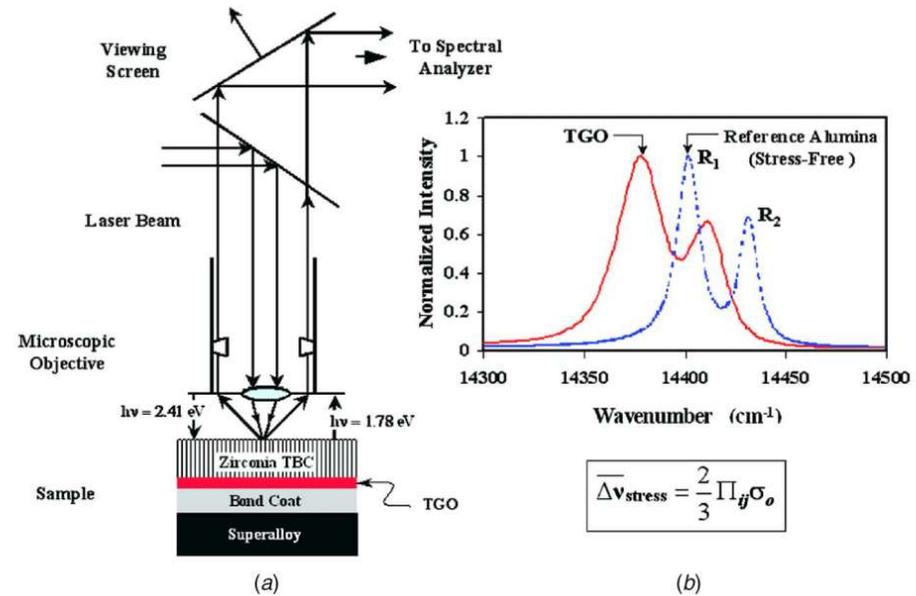
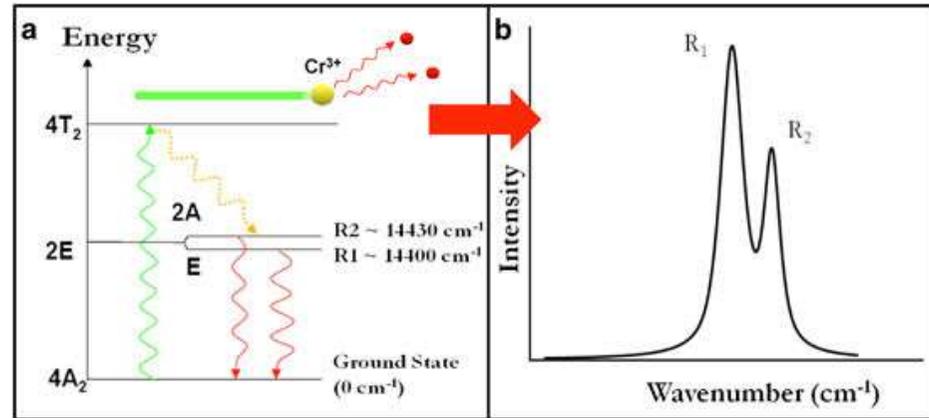
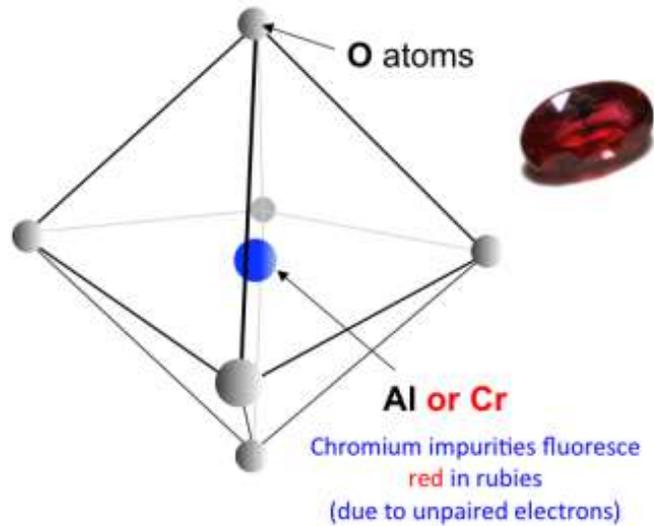
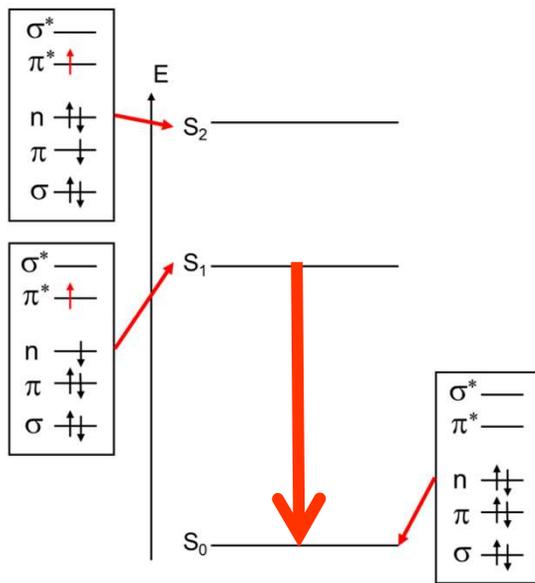
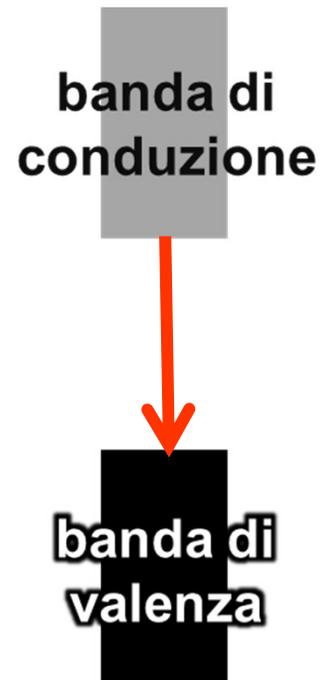


Fig. 1 (a) Schematic illustration of the photostimulated luminescence piezospectroscopy technique, and (b) typical R_1/R_2 fluorescence spectra for chromium-containing stress-free and stressed $\alpha\text{-Al}_2\text{O}_3$

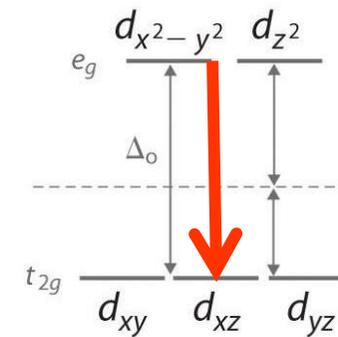
molecules/ molecular solids



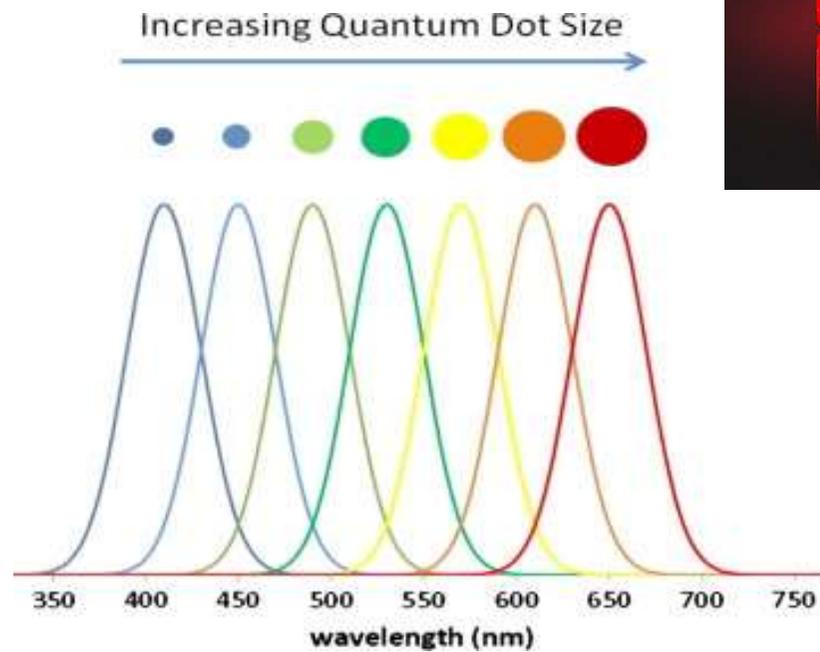
non-molecular solids (semiconductors)



transition metals ions

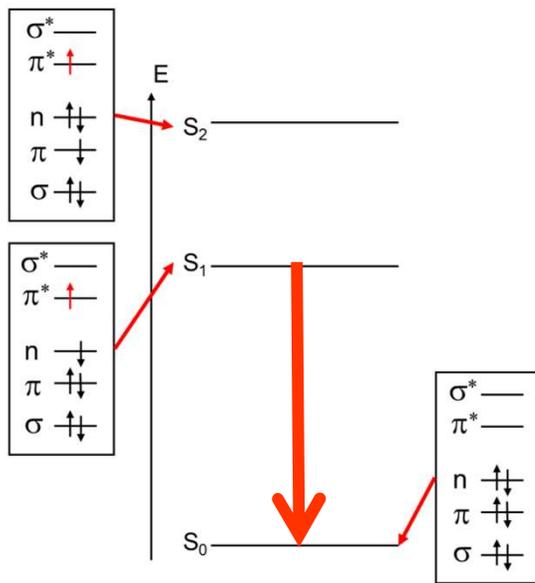


Fluorescenza quantum dots

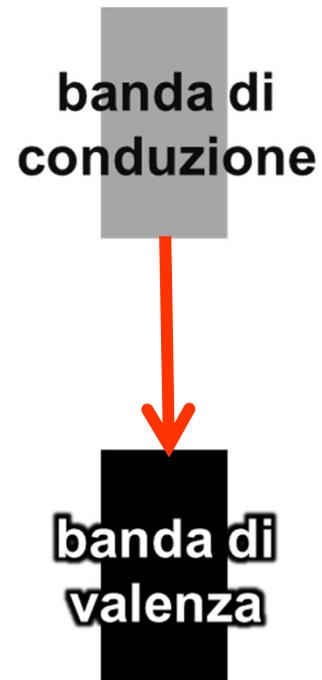


utilizzata, insieme con
l'assorbimento UV-vis, per
misurare le dimensioni

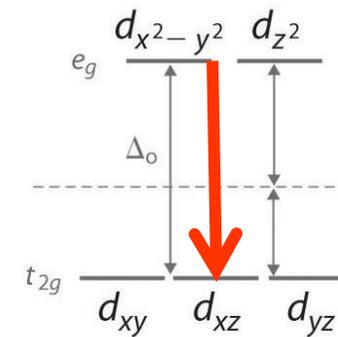
molecules/ molecular solids



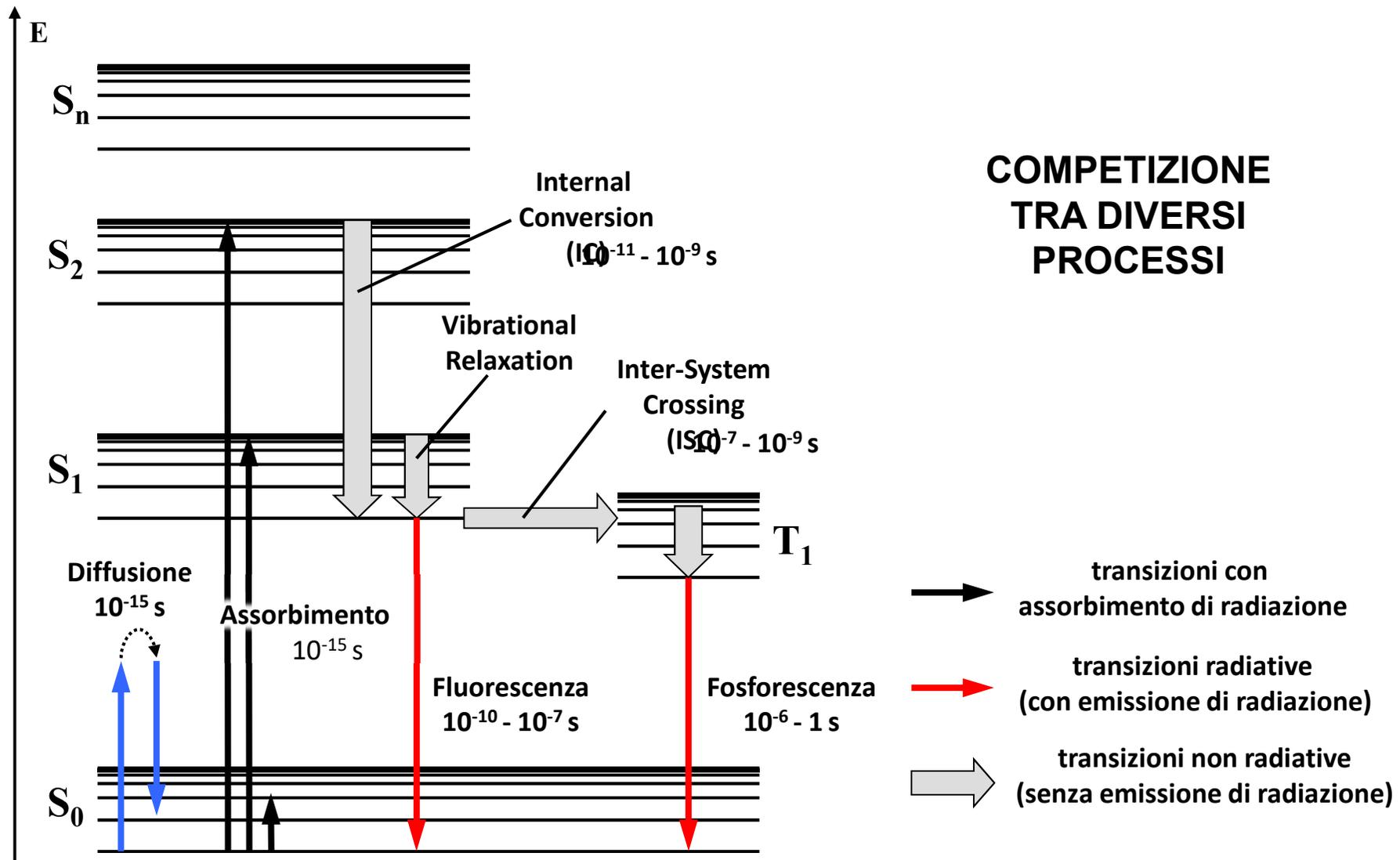
non-molecular solids (semiconductors)



transition metals ions



Interazione tra Radiazione e Materia (diagramma di Jablonski)

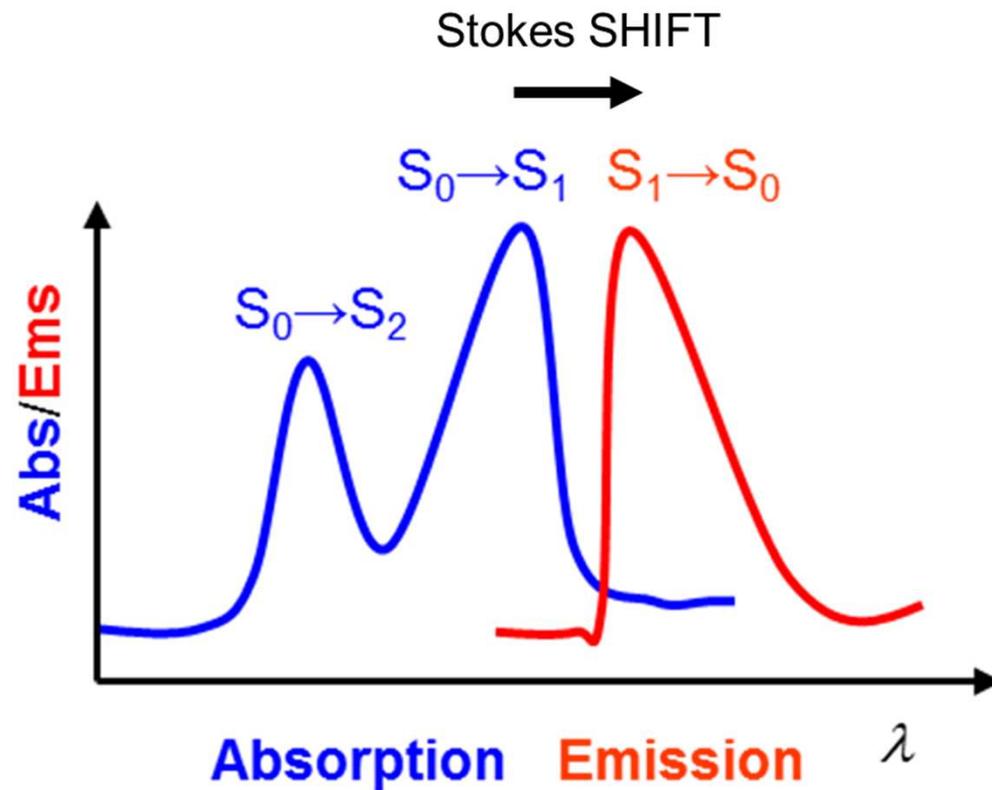
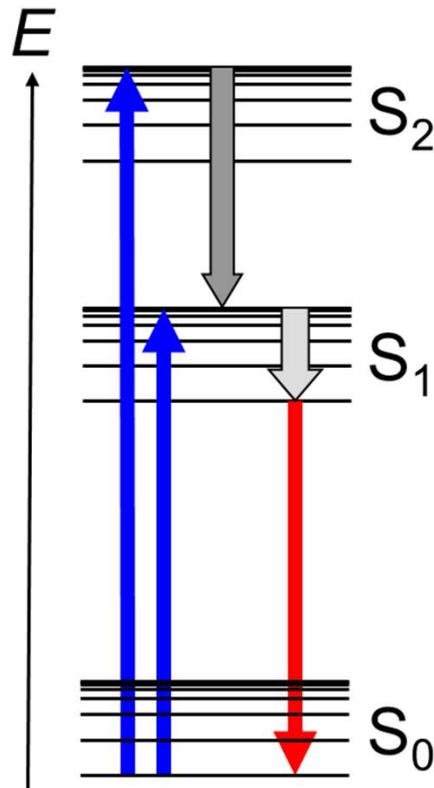


Caratteristiche della Fluorescenza

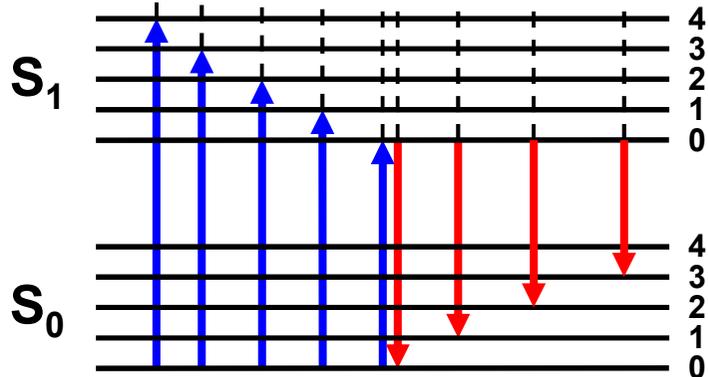
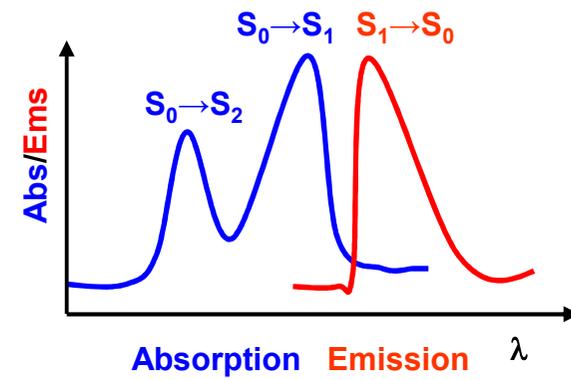
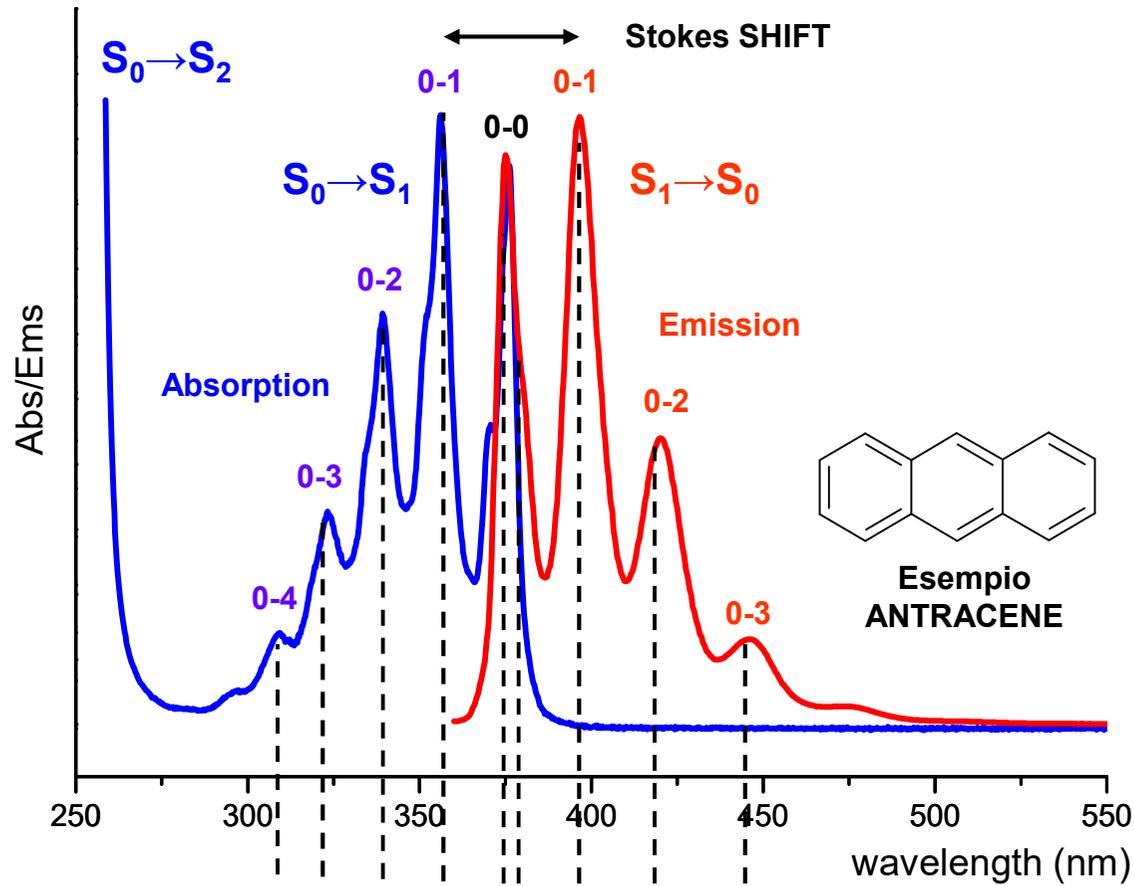
1 Lo STOKES SHIFT è la differenza tra il massimo dell'assorbimento e della fluorescenza

2 L'emissione di fluorescenza è indipendente dalla lunghezza d'onda utilizzata per l'eccitazione

3 L'emissione di fluorescenza ricorda specularmente lo spettro di assorbimento (MIRROR IMAGE RULE)

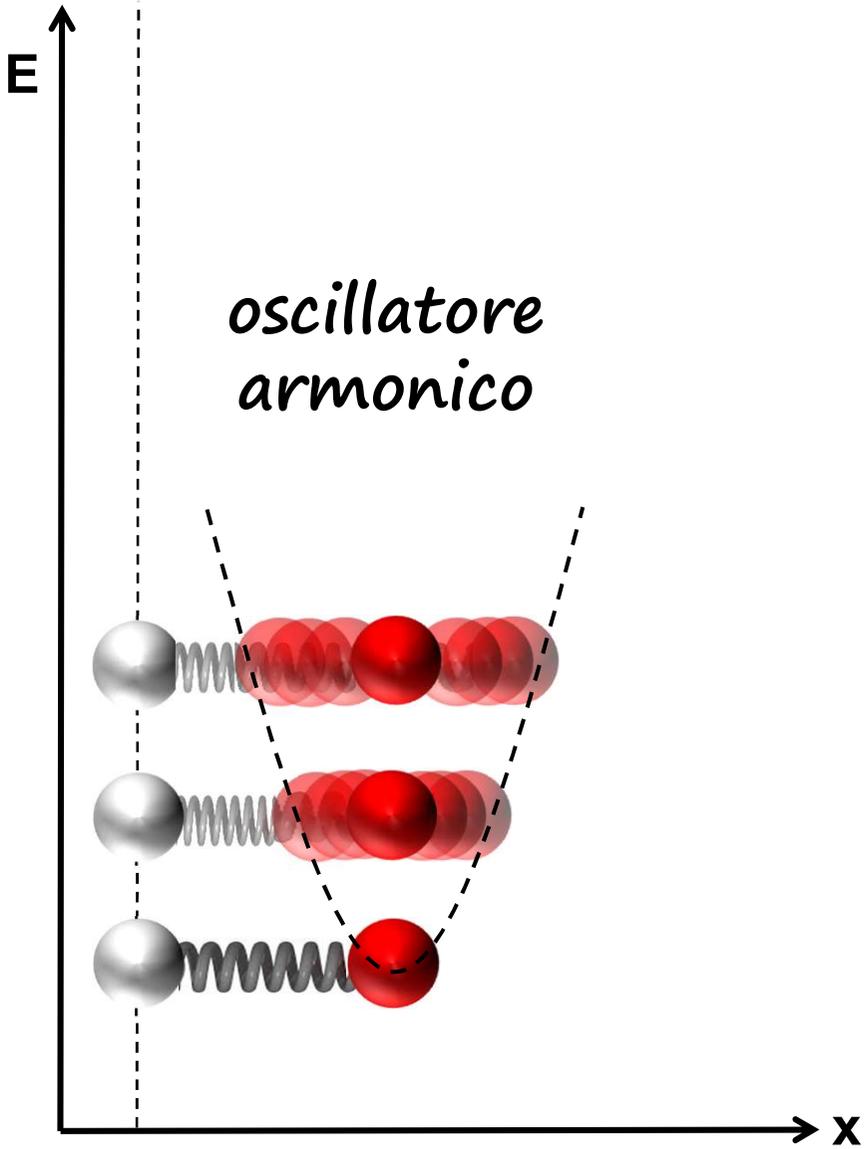


Caratteristiche della Fluorescenza

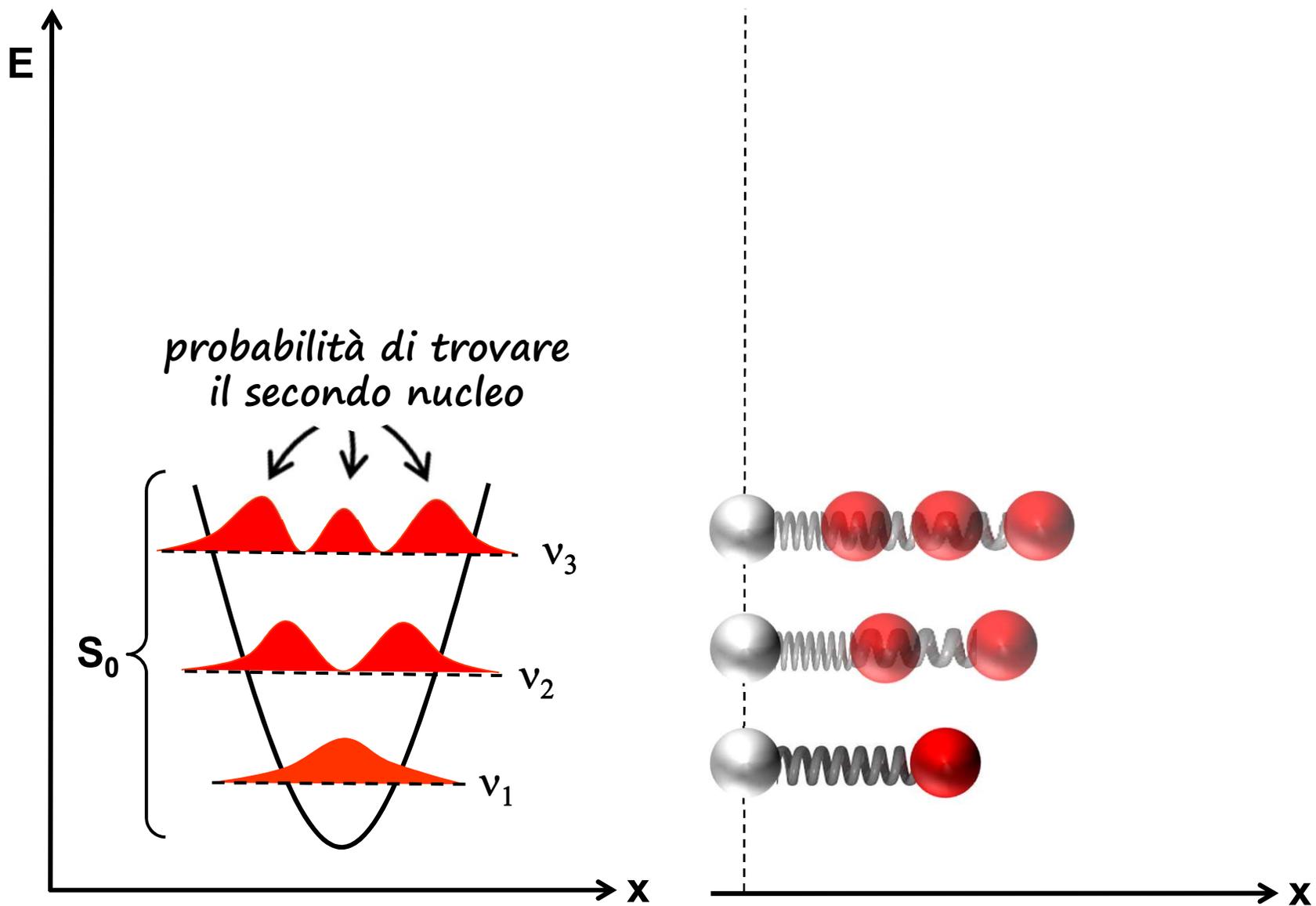


DIVERSA INTENSITA' DOVUTA A FATTORE DI FRANCK-CONDON

Franck-Condon

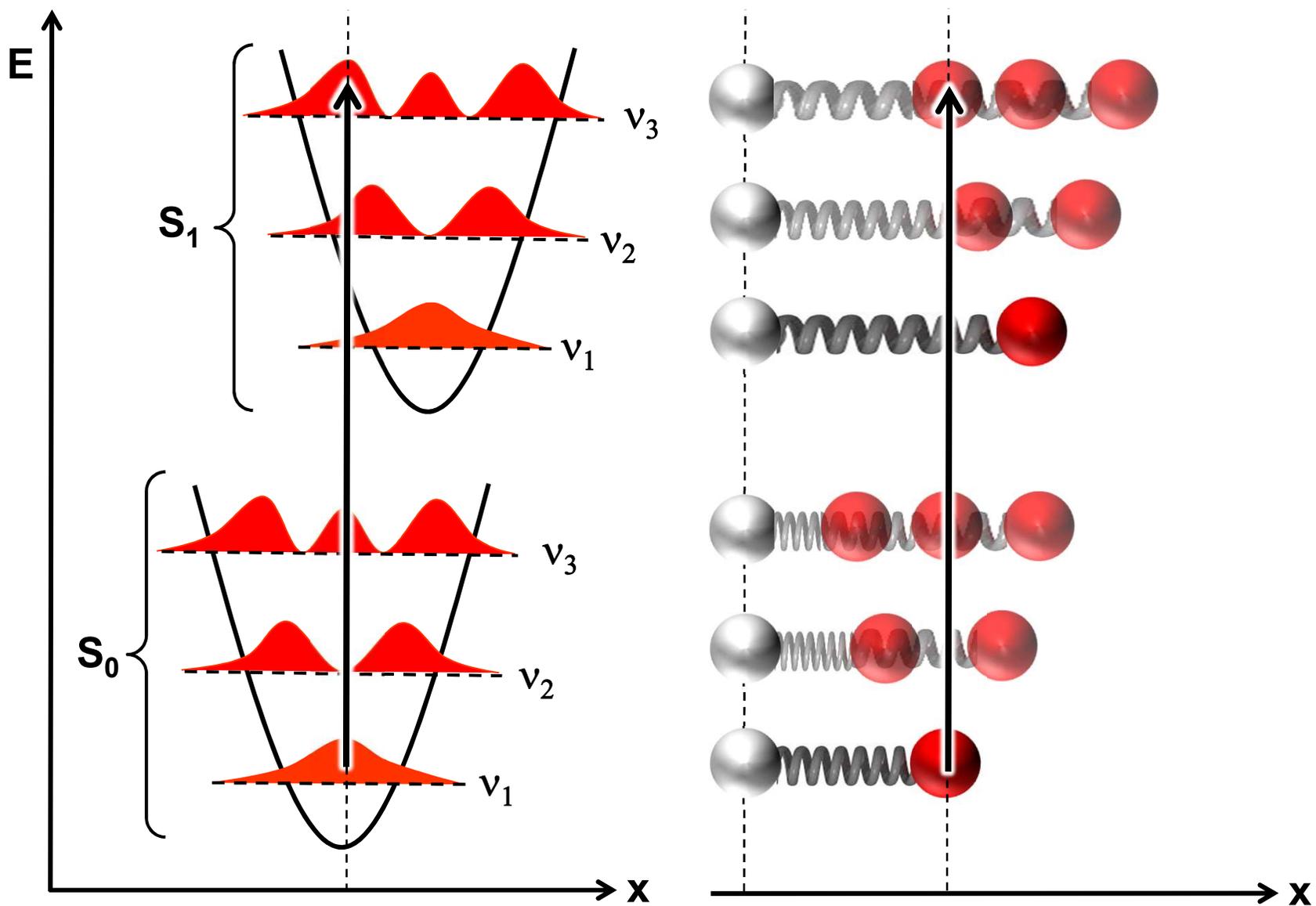


Franck-Condon



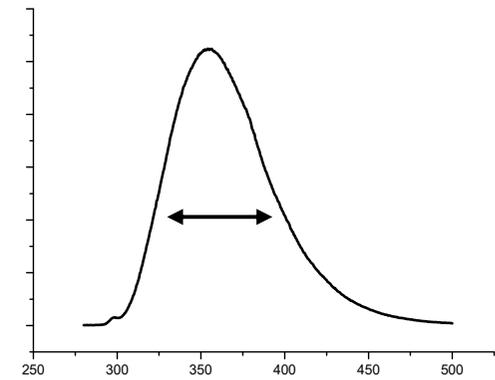
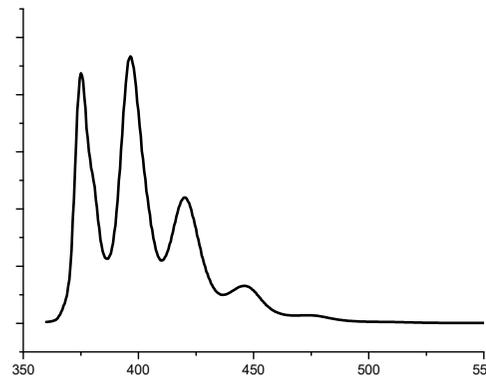
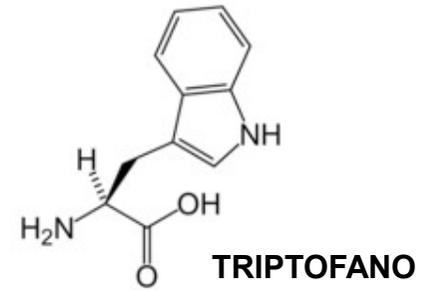
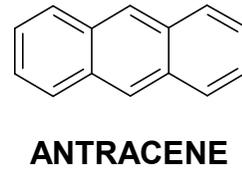
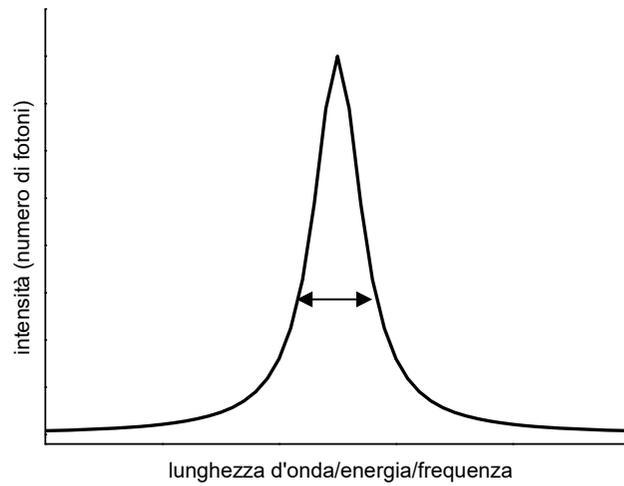
Franck-Condon

(gli elettroni sono più veloci dei nuclei)

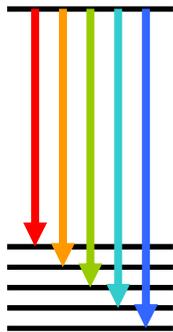


Caratteristiche della Fluorescenza

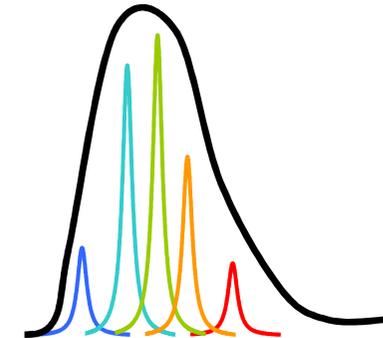
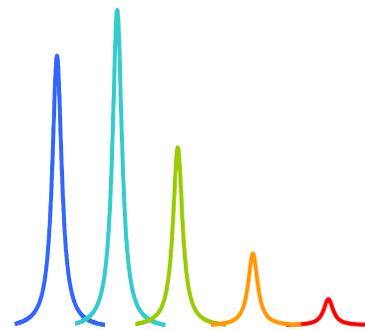
“ALLARGAMENTO” DI BANDA



ALLARGAMENTO “OMOGENEO”



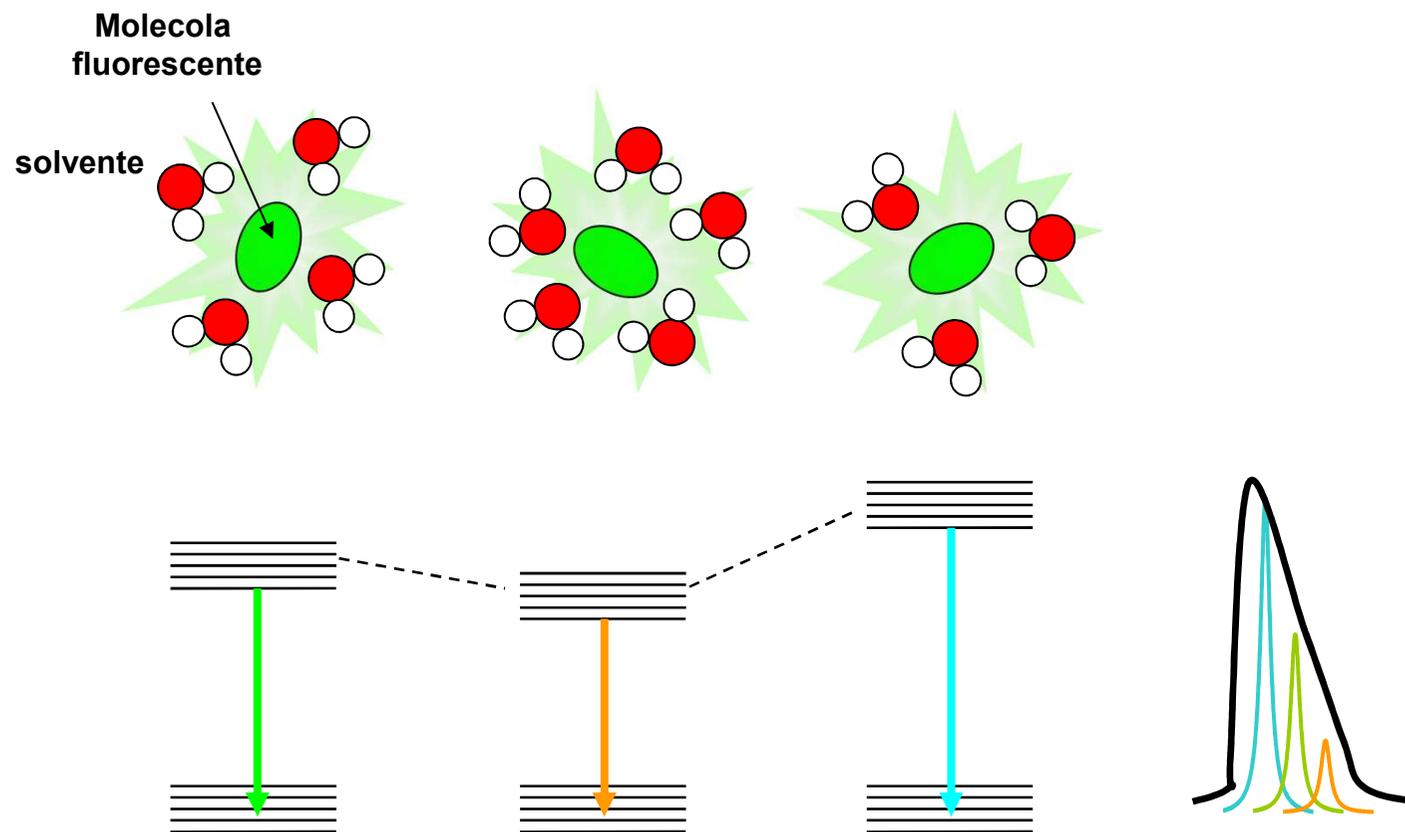
Dovuto al fatto che ci sono
sotto-livelli vibrazionali



Caratteristiche della Fluorescenza

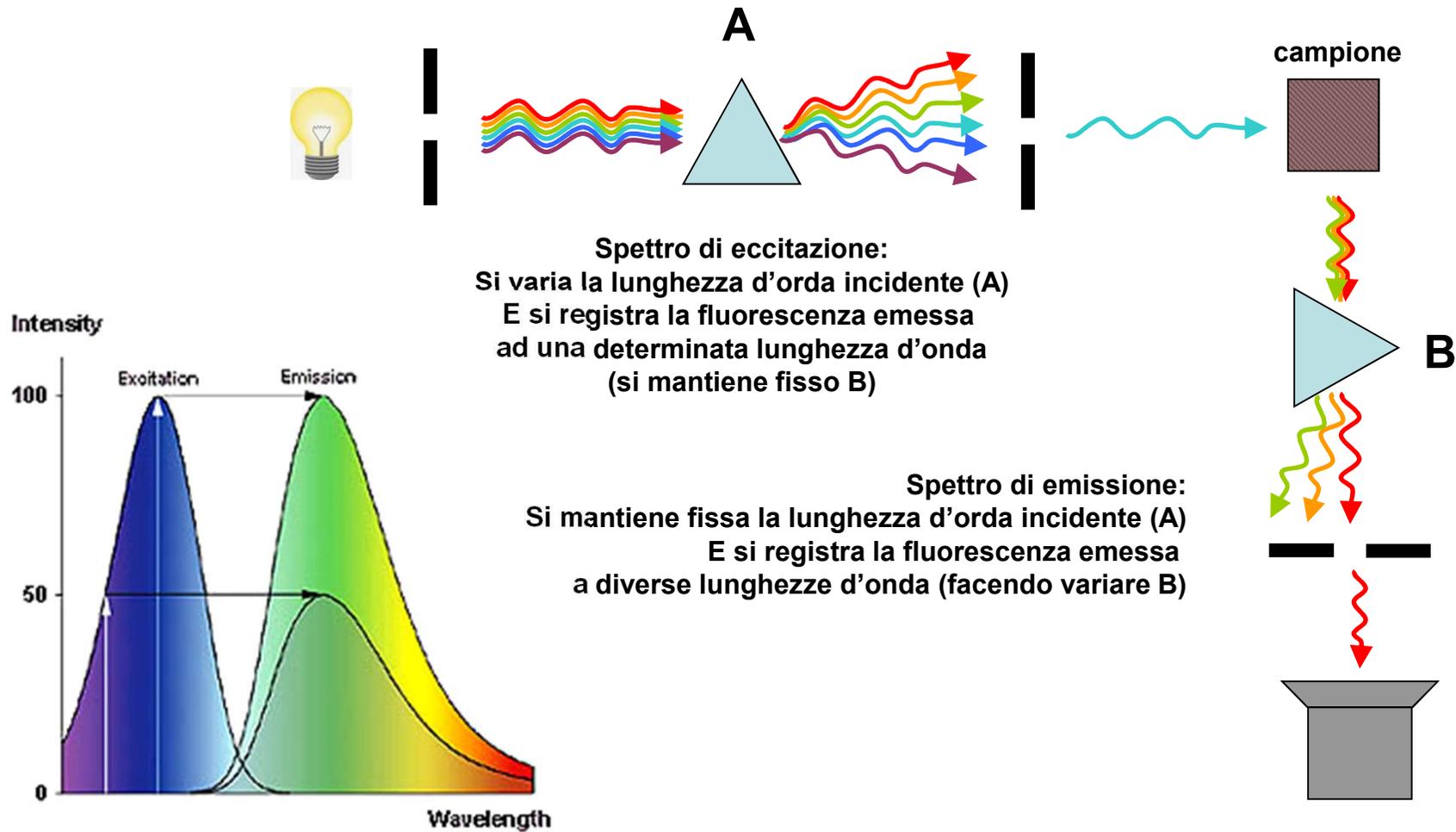
EFFETTI FATTORI AMBIENTALI

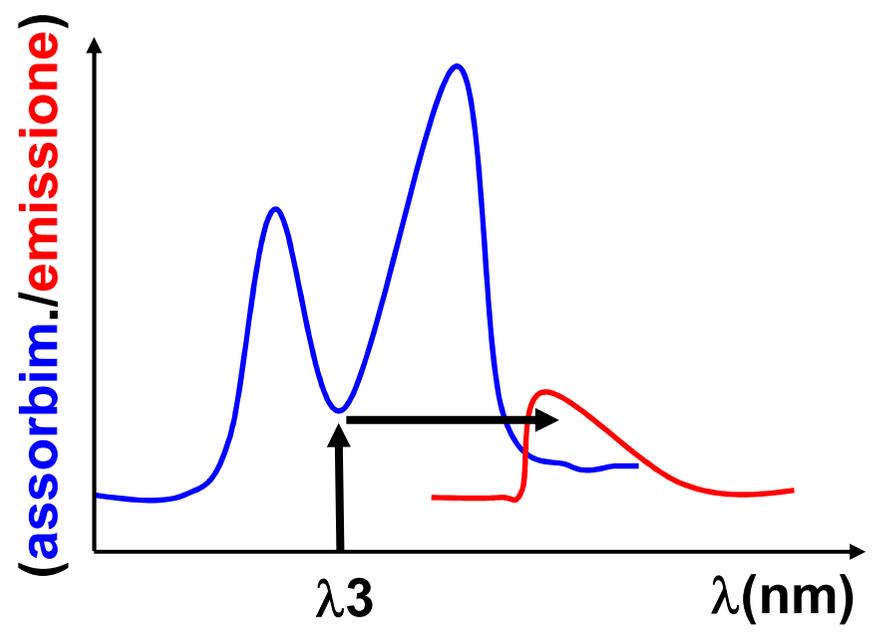
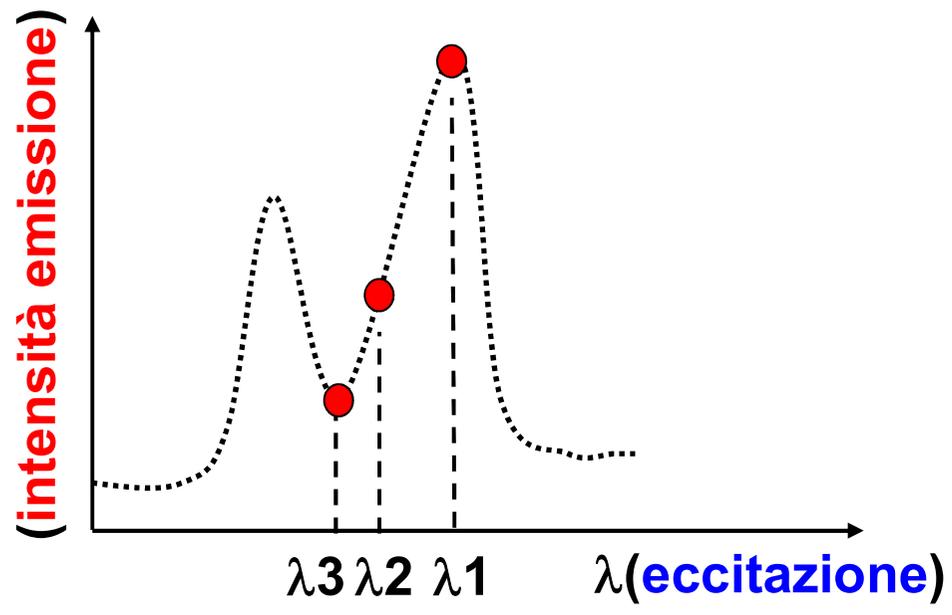
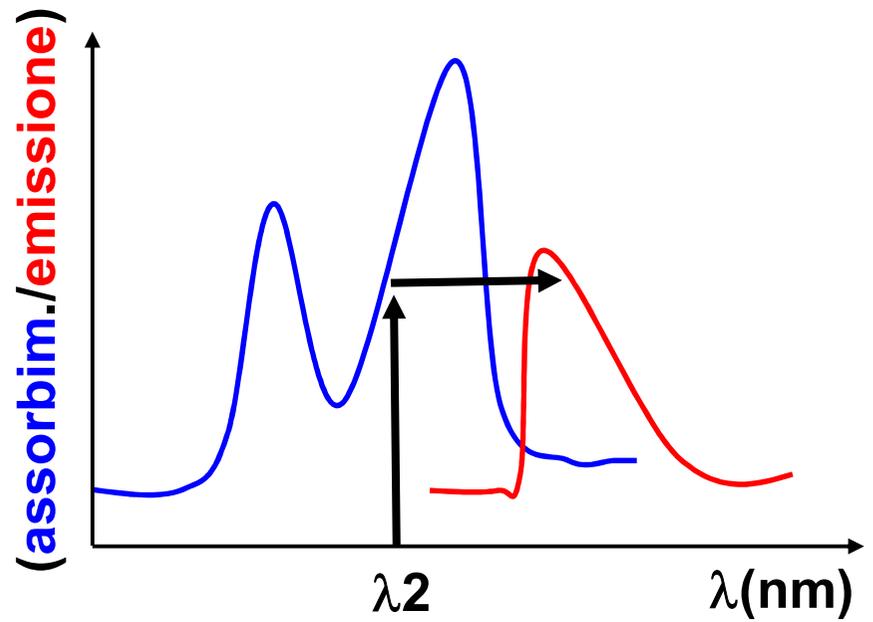
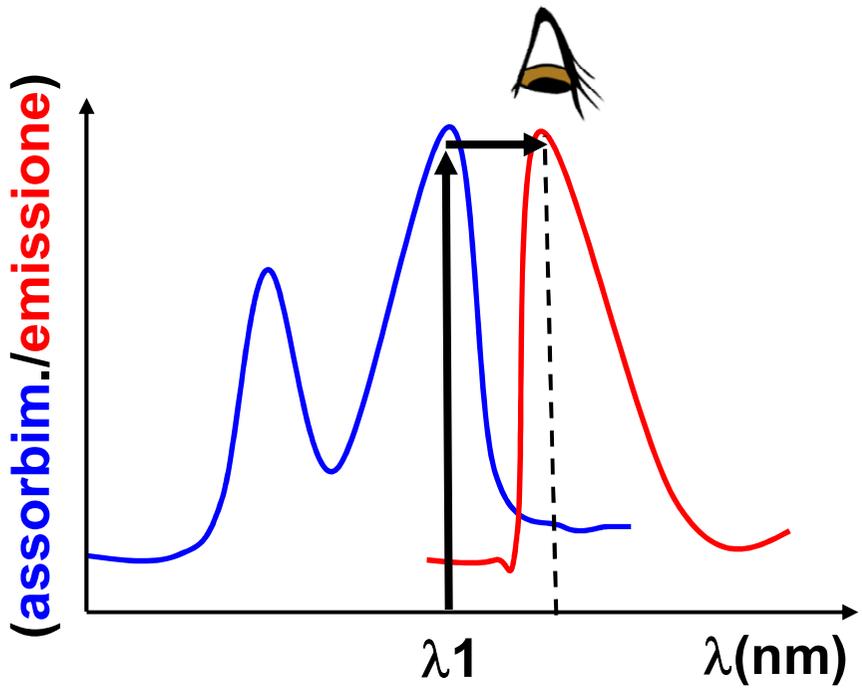
ALLARGAMENTO DI BANDA "IN-OMOGENEO" DOVUTO ALLE FLUTTUAZIONI DEL SOLENTE ATTORNO AL FLUOROFORO



Caratteristiche della Fluorescenza

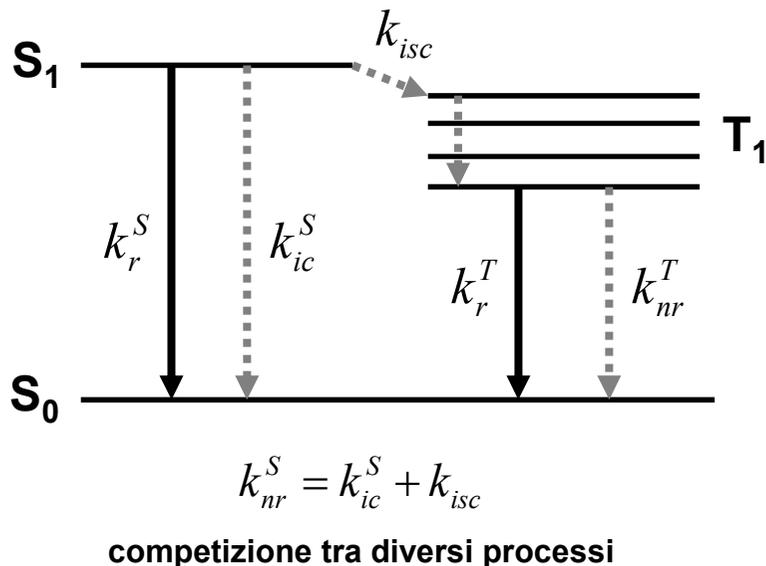
SPETTRI DI EMISSIONE ED ECCITAZIONE





Caratteristiche della Fluorescenza

Lifetime e Quantum Yield



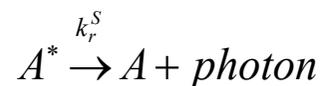
numero molecole A nello stato eccitato

$$-\frac{d[{}^1A^*]}{dt} = (k_r^S + k_{nr}^S)[{}^1A^*]$$

$$[{}^1A^*] = [{}^1A^*]_0 \exp\left(-\frac{t}{\tau_S}\right)$$

fluorescence lifetime

$$\tau_S = \frac{1}{k_r^S + k_{nr}^S}$$



RESA QUANTICA
(quantum yield)

$$\Phi = \frac{k_r^S}{k_r^S + k_{nr}^S} = k_r^S \tau_S$$

efficienza processo radiativo

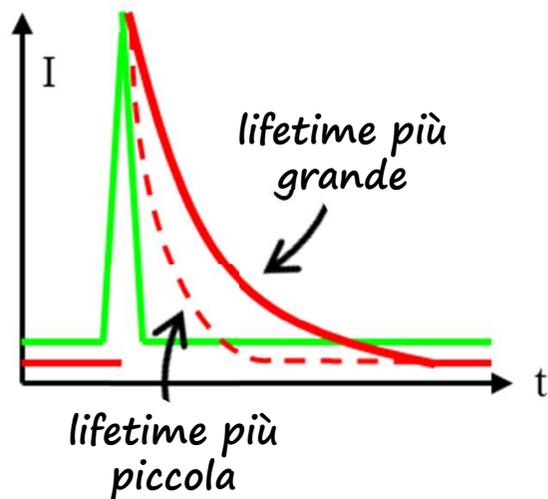
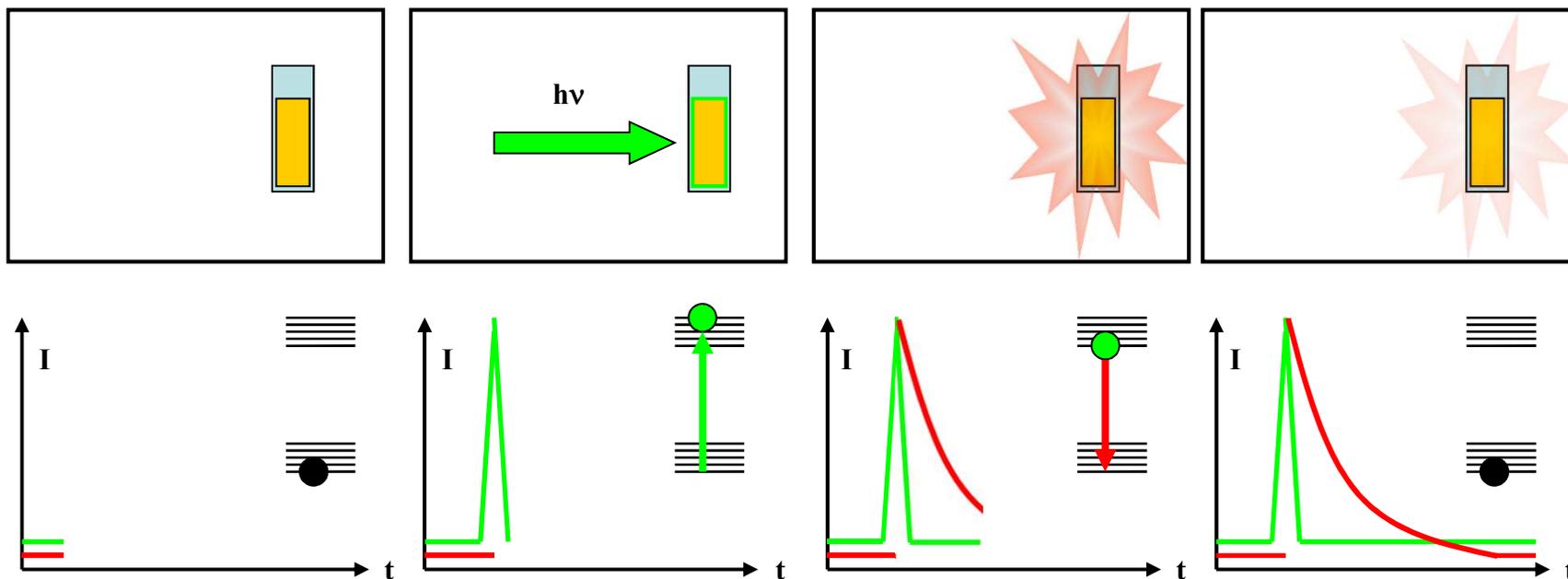
$$\Phi = \frac{\text{photons emitted}}{\text{photons absorbed}}$$

$$i_F(t) = k_r^S [{}^1A^*] = k_r^S [{}^1A^*]_0 \exp\left(-\frac{t}{\tau_S}\right)$$

$$I_F(t) = I_0 \exp\left(-\frac{t}{\tau_S}\right)$$

fluorescence intensity

Caratteristiche della Fluorescenza



COME SI MISURA?
TIME-RESOLVED SPECTROSCOPY

$$I_F(t) = I_0 \exp\left(-\frac{t}{\tau_S}\right)$$

fluorescence intensity

Caratteristiche della Fluorescenza

Lifetime e Quantum Yield

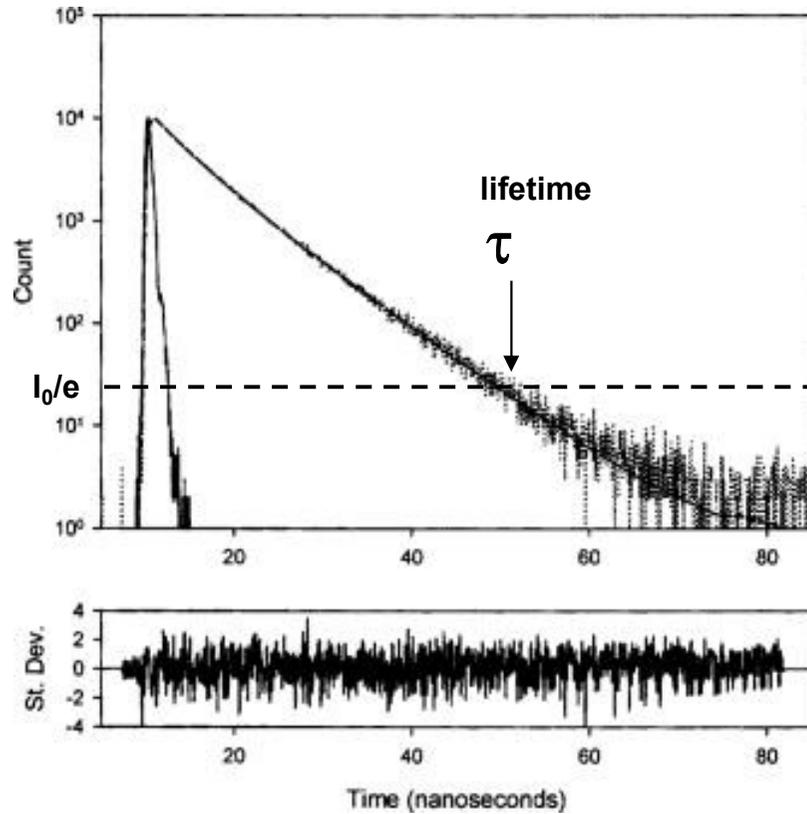


Table 2. Fluorescence Lifetimes of Some Dyes and Biological Materials Commonly Used in LSM

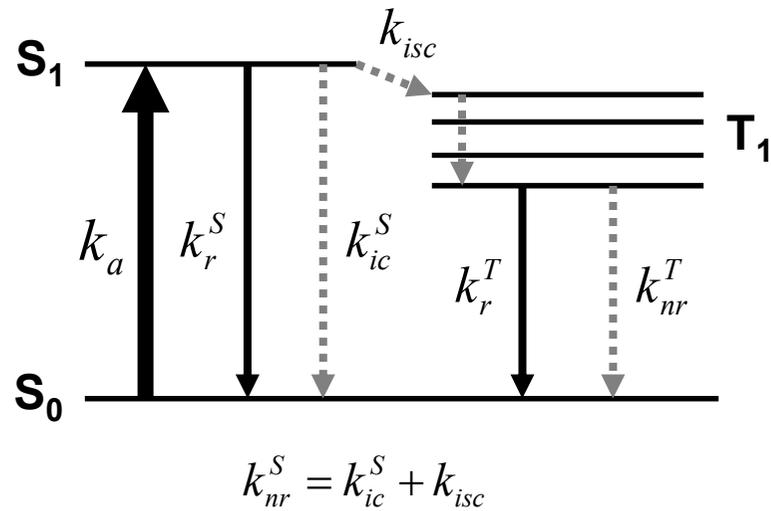
Compound	Fluorescence lifetime
Acridine orange	9.2 ns ^a
Eosin B	6.3 ns ^a
Ethidium bromide	1.7 ns ^b
FITC-BSA	0.5 ns ^c
Green fluorescent protein	3 ns ^d
Indocyanine green	0.6 ns ^c
NADH	1.7 ns ^f
Rhodamine 6G	3.9 ns ^a
Rhodamine B	3.2 ns ^a
Rose bengal	5.8 ns ^a
Tryptophan	2.5–3.7 ns ^g
Elastin	2.3 ns ^h
Collagen	5.3 ns ^h

$$I_F(t) = I_0 \exp\left(-\frac{t}{\tau_S}\right)$$

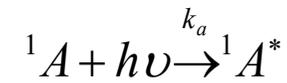
fluorescence intensity

Caratteristiche della Fluorescenza

Lifetime e Quantum Yield



Steady-state fluorescence (stato stazionario)



$$\frac{d[{}^1A^*]}{dt} = 0 = k_a \alpha N_0 - (k_r^S + k_{nr}^S)[{}^1A^*]$$

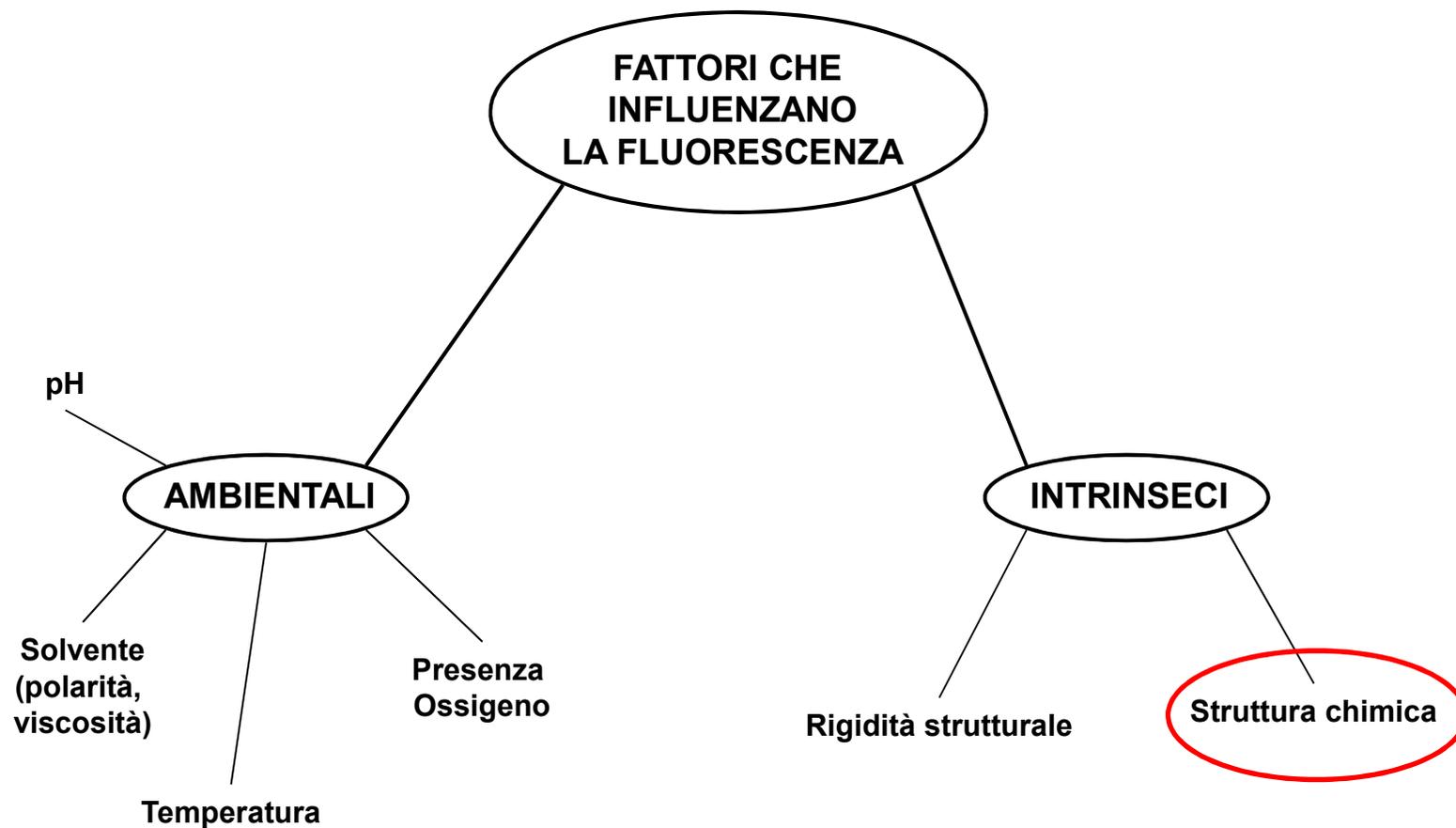
amount of absorbed photons
per unit volume

$$i_F = k_r^S [{}^1A^*] = \alpha I_0 \frac{k_r^S}{k_r^S + k_{nr}^S} = \alpha I_0 \Phi$$

intensity of
incident light

$$[{}^1A^*] = \frac{\alpha I_0}{k_r^S + k_{nr}^S}$$

Caratteristiche della Fluorescenza



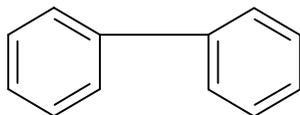
Caratteristiche della Fluorescenza

EFFETTI FATTORI INTRINSECI

ESEMPIO RIGIDITA'

Quantum Yield 0.2

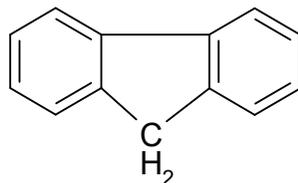
bifenile



meno rigido

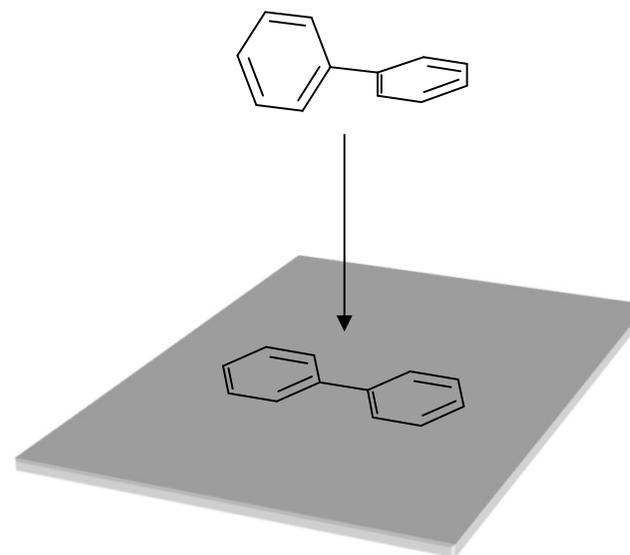
Quantum Yield 0.9

fluorene



più rigido

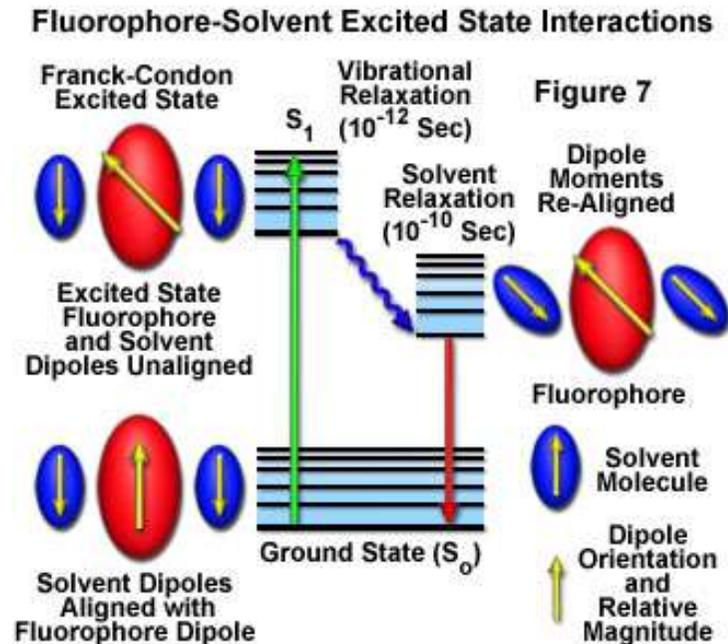
**Più flessibilità = più stati energetici
accessibili alla molecola
= più modi per “smaltire”
energia con conversione
interna**



Resa quantica più alta su superfici

Caratteristiche della Fluorescenza

EFFETTI FATTORI AMBIENTALI



EFFETTO SOLVENTE (SOLVENT RELAXATION)

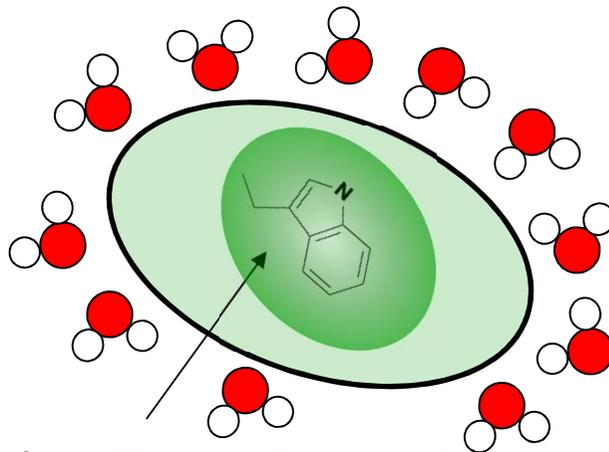
NELLO STATO ECCITATO
LA MOLECOLA é DIVERSA
RISPETTO ALLO STATO FONDAMENTALE

IL SOLVENTE SI ADATTA ALLA
NUOVA SITUAZIONE, SI ABBASSA
L'ENERGIA COMPLESSIVA
(FLUOROFORO+SOLVENTE)
DELLO STATO ECCITATO

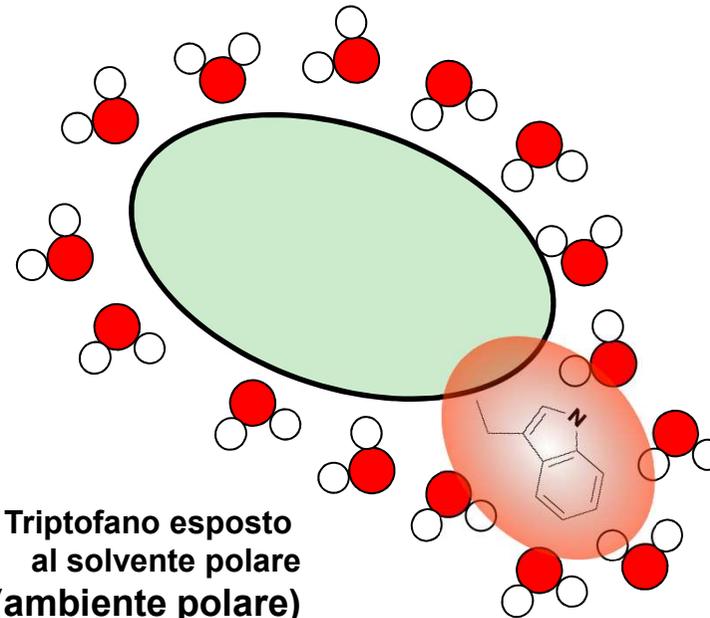
L'EFFETTO CAMBIA DA
SOLVENTE A SOLVENTE
(AD ESEMPIO CON LA POLARITA')

Caratteristiche della Fluorescenza

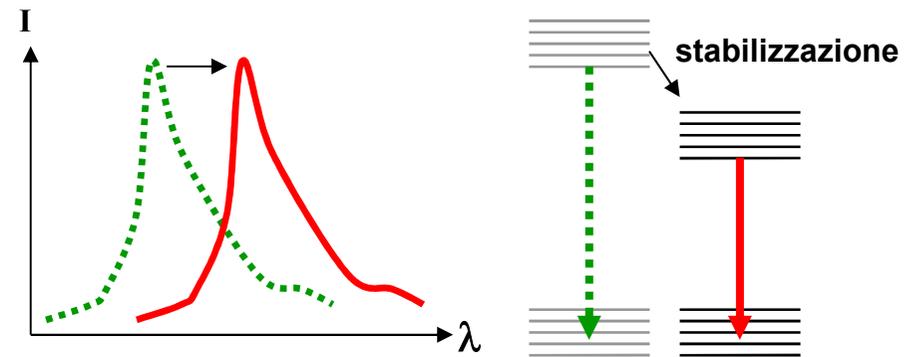
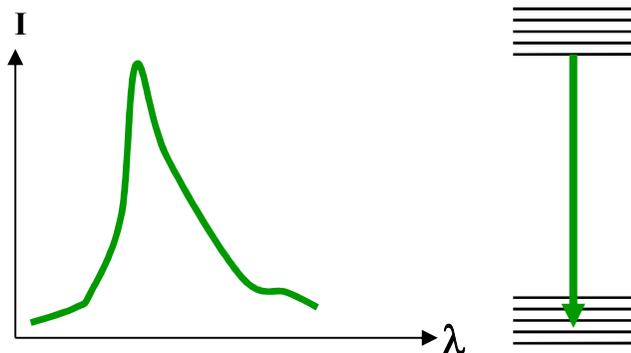
EFFETTI FATTORI AMBIENTALI



Triptofano all'interno di una proteina
(ambiente apolare)



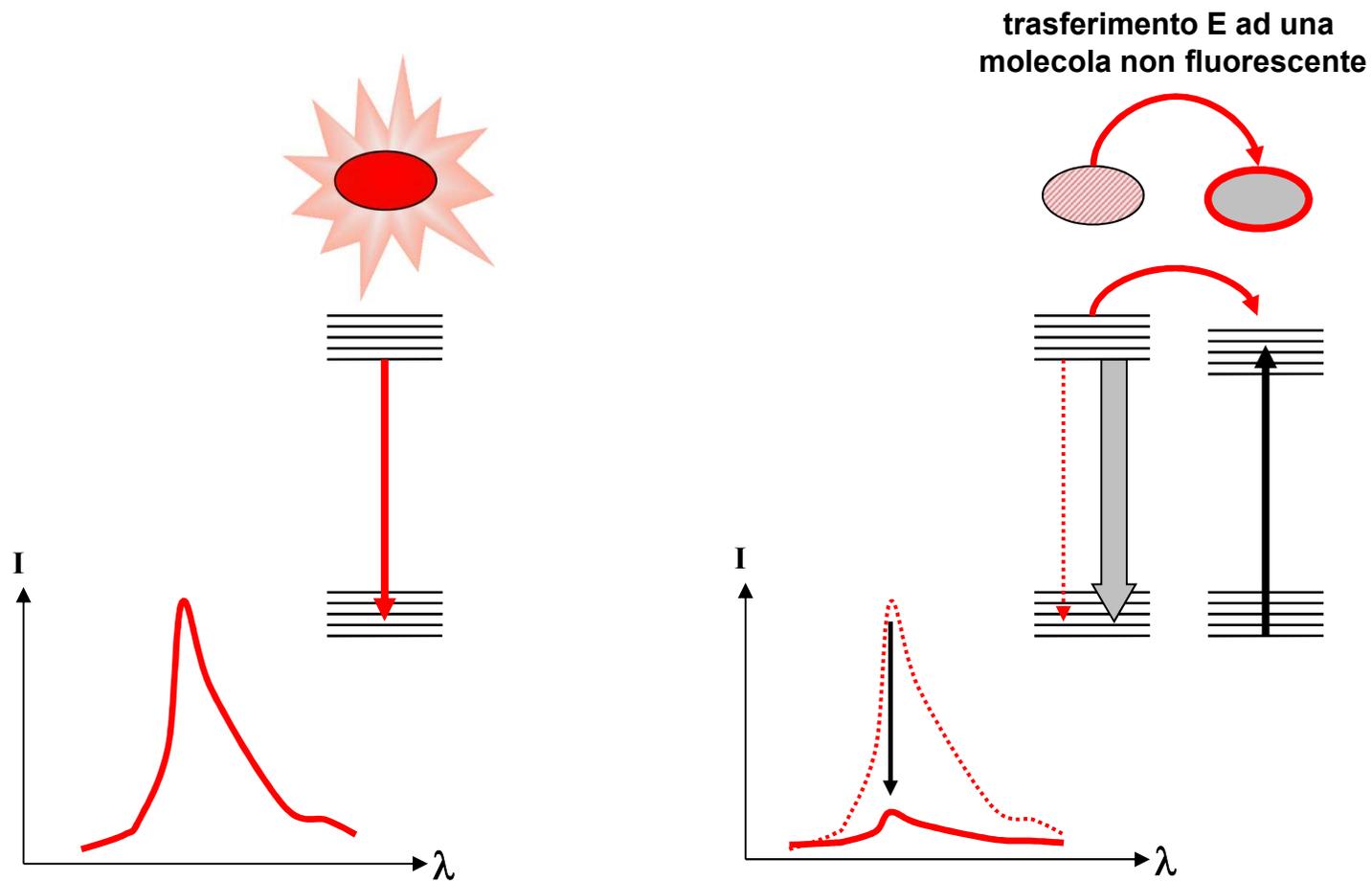
Triptofano esposto
al solvente polare
(ambiente polare)



Caratteristiche della Fluorescenza

EFFETTI FATTORI AMBIENTALI

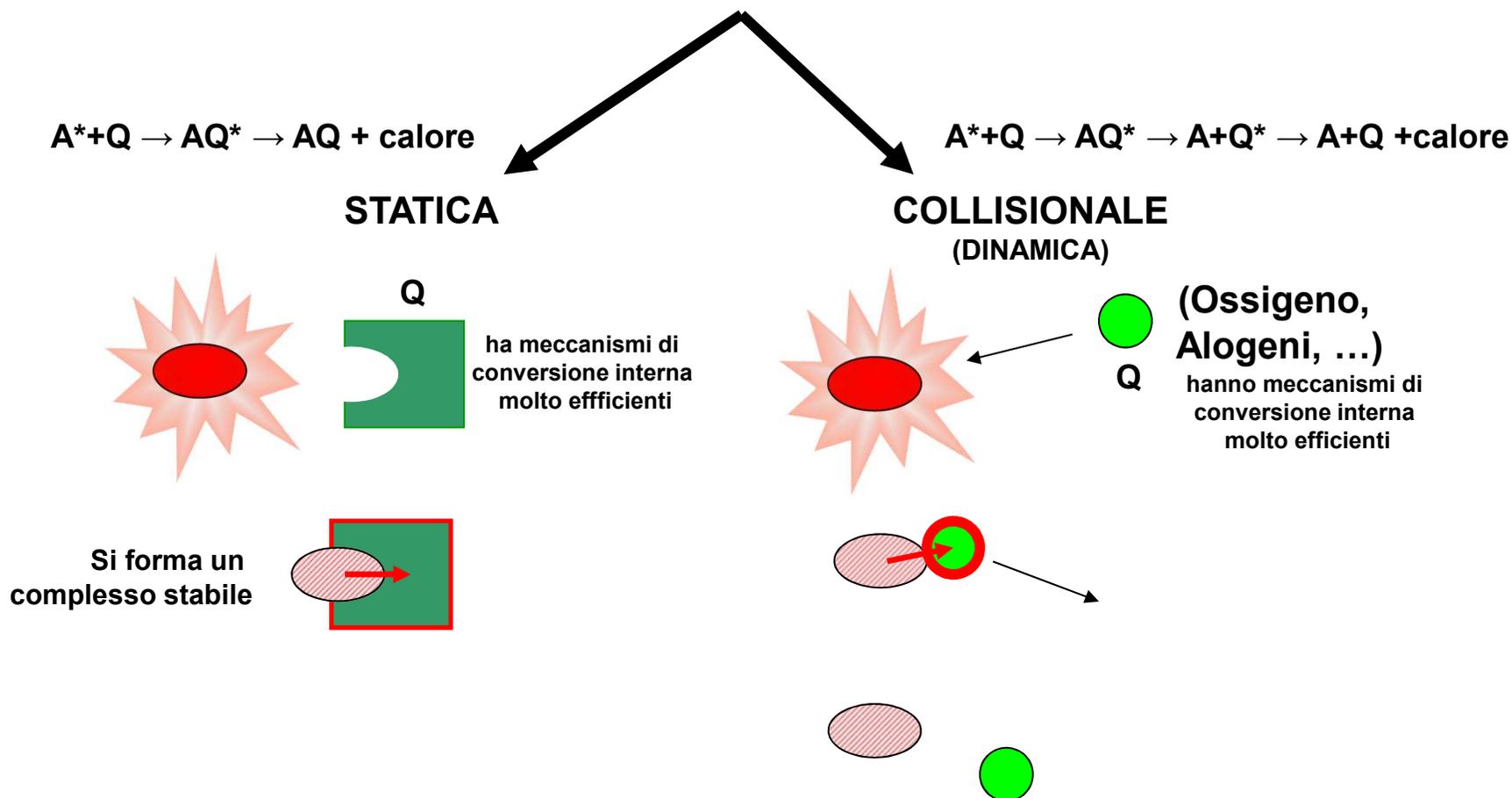
QUENCHING: Diminuzione della intensità di Fluorescenza



Caratteristiche della Fluorescenza

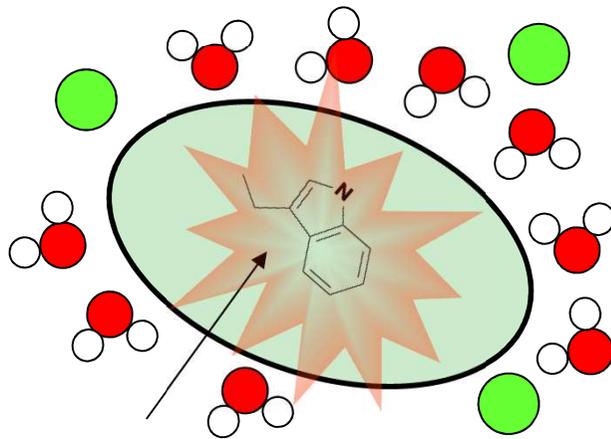
EFFETTI FATTORI AMBIENTALI

QUENCHING: Diminuzione della intensità di Fluorescenza

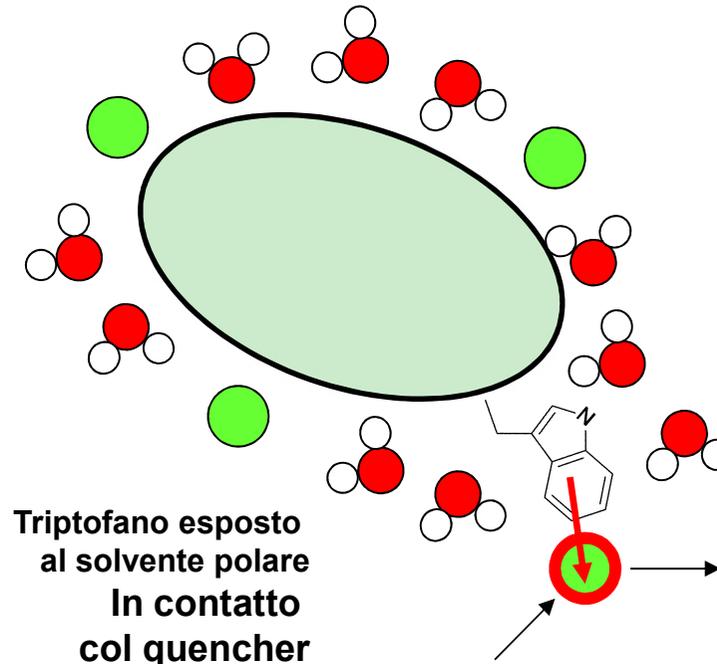


Caratteristiche della Fluorescenza

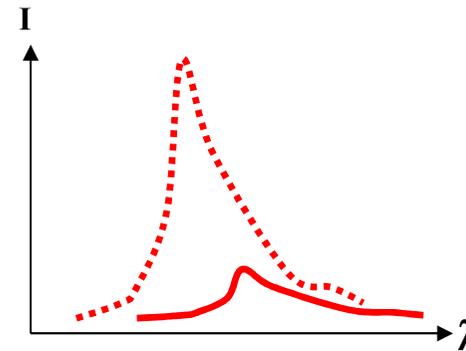
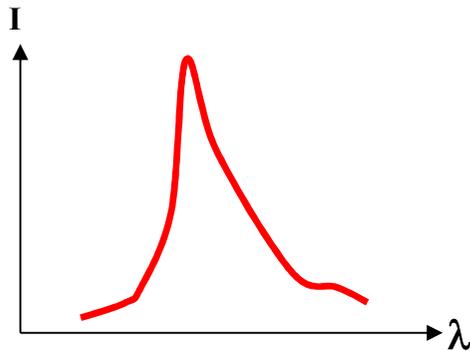
QUENCHING COLLISIONALE



Tryptofano all'interno di una proteina
No contatto con quencher



Tryptofano esposto al solvente polare
In contatto col quencher



Caratteristiche della Fluorescenza

QUENCHING COLLISIONALE

Equazione di STERN-VOLMER

$$I_0/I = 1 + k\tau [Q]$$

Intensità Fluorescenza In assenza di Q → I_0
 Intensità Fluorescenza In presenza di Q → I
 Lifetime fluoroforo → $k\tau$
 Concentrazione Quencher → $[Q]$

$$k\tau = K_{SV}$$

(costante di Stern Volmer)

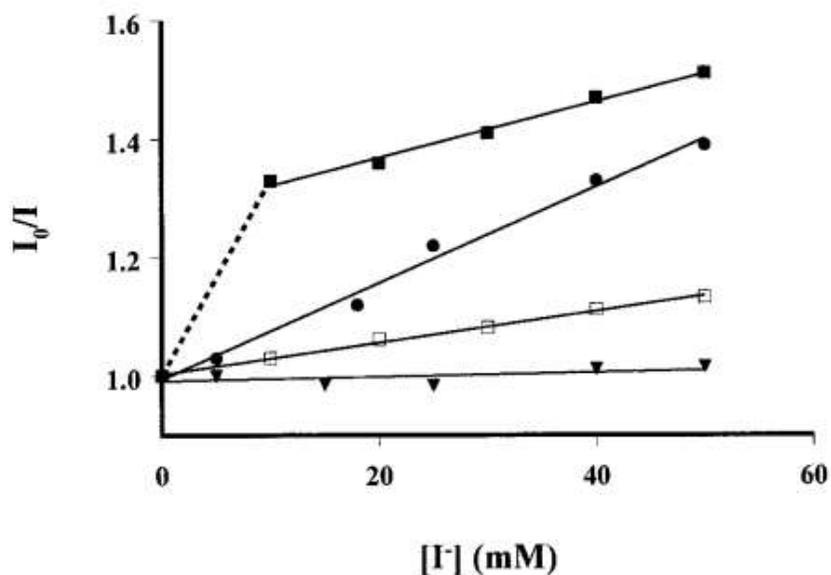


FIG. 2. Stern-Volmer plots for the quenching by iodide of tryptophan in solution (●), OVA (▼), and BSA at low ionic strength (■) and BSA at ionic strength = 2 M (□).

TABLE I
Quenching by iodide

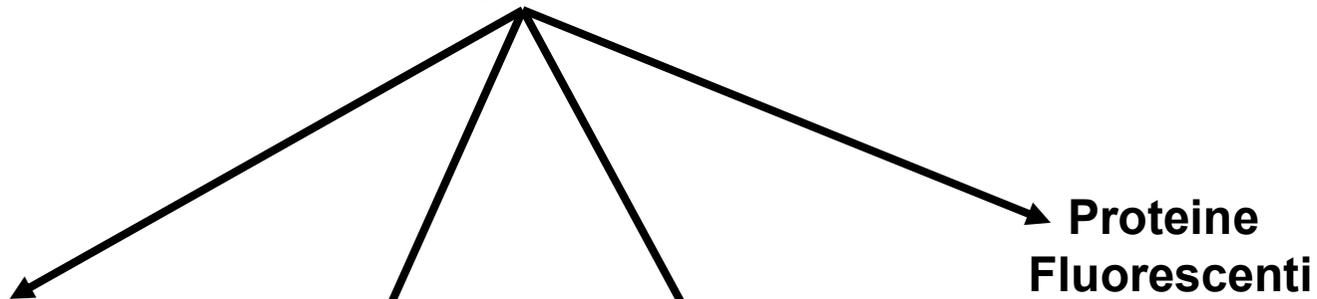
	K_{SV} (M^{-1})
Trp (solution)	8.1 ± 0.4
OVA	0.33 ± 0.02
BSA ₁ ^a	26 ± 0.6
BSA ₂ ^a	4.7 ± 0.3
BSA ^b	2.1 ± 0.2

^a K_{SV} determined at low ionic strength.

^b K_{SV} determined at ionic strength = 2 M.

Caratteristiche della Fluorescenza

Fluorofori Estrinseci (fluorescent Dyes/Labels/Probes)



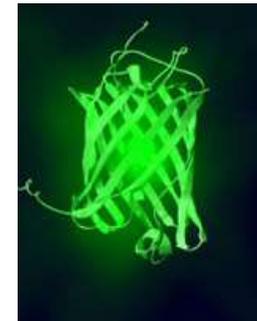
Lantanidi

Periodic Table of the Elements

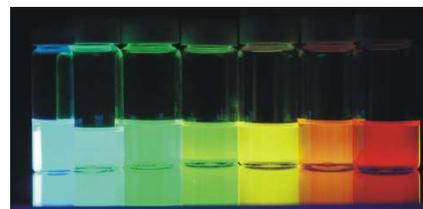
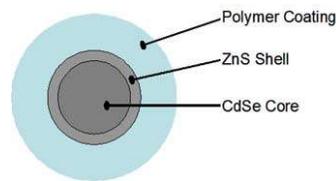
1	H	2	He																																																												
3	Li	4	Be	5	B	6	C	7	N	8	O	9	F	10	Ne																																																
11	Na	12	Mg	13	Al	14	Si	15	P	16	S	17	Cl	18	Ar																																																
19	K	20	Ca	21	Sc	22	Ti	23	V	24	Cr	25	Mn	26	Fe	27	Co	28	Ni	29	Cu	30	Zn	31	Ga	32	Ge	33	As	34	Se	35	Br	36	Kr																												
37	Rb	38	Sr	39	Y	40	Zr	41	Nb	42	Mo	43	Tc	44	Ru	45	Rh	46	Pd	47	Ag	48	Cd	49	In	50	Sn	51	Sb	52	Te	53	I	54	Xe																												
55	Cs	56	Ba	57	La	58	Ce	59	Pr	60	Nd	61	Pm	62	Sm	63	Eu	64	Gd	65	Tb	66	Dy	67	Ho	68	Er	69	Tm	70	Yb	71	Lu	72	Hf	73	Ta	74	W	75	Re	76	Os	77	Ir	78	Pt	79	Au	80	Hg	81	Tl	82	Pb	83	Bi	84	Po	85	At	86	Rn
87	Fr	88	Ra	89	Ac	90	Th	91	Pa	92	U	93	Np	94	Pu	95	Am	96	Cm	97	Bk	98	Cf	99	Es	100	Fm	101	Md	102	No	103	Lr																														

* Lanthanide Series: Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu
 * Actinide Series: Th, Pa, U, Np, Pu, Am, Cm, Bk, Cf, Es, Fm, Md, No, Lr

Proteine Fluorescenti



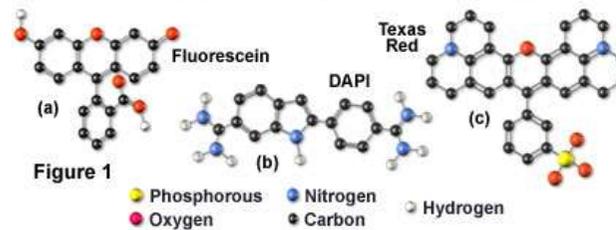
Quantum Dots (nanocristalli di semiconduttore)



2.3 → 5.5
Size (nanometers)

Sintetici (molecole organiche)

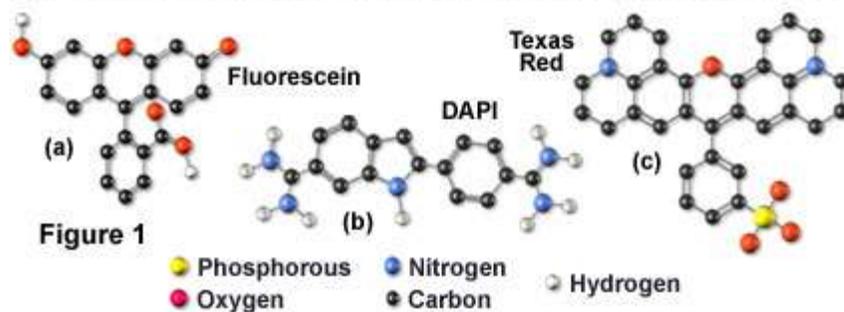
Common Fluorophores in Widefield and Confocal Microscopy



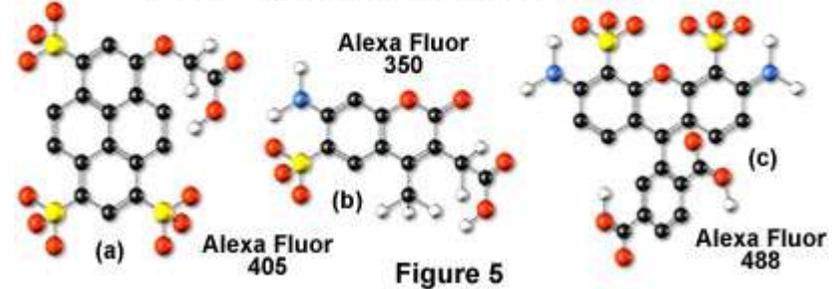
Caratteristiche della Fluorescenza

FLUOROFORI SINTENTICI

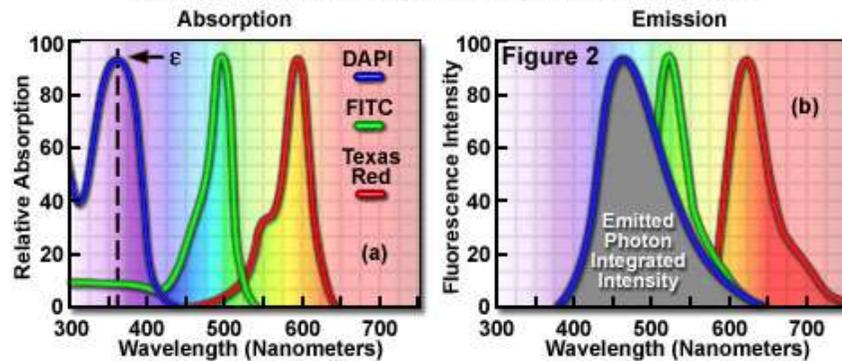
Common Fluorophores in Widefield and Confocal Microscopy



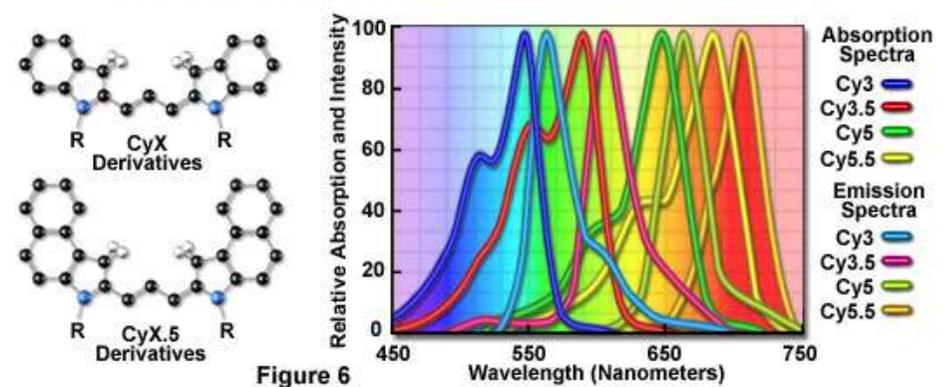
Alexa Fluor Synthetic Fluorochromes



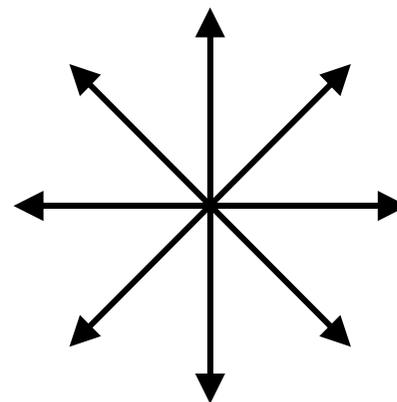
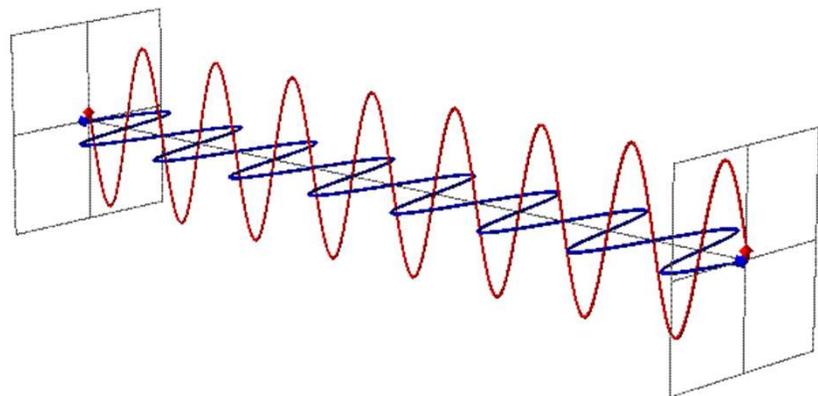
Spectral Profiles of Popular Traditional Fluorophores



Structure and Spectral Profiles of Cyanine Fluorochromes

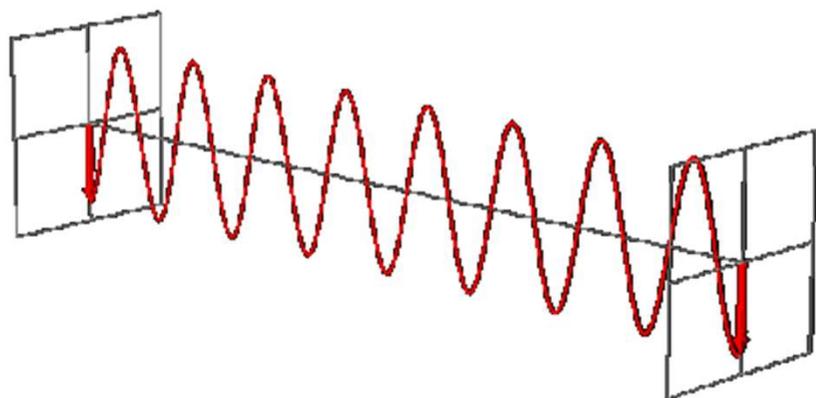


polarizzazione della luce



piani in cui vibra il campo elettrico di una radiazione incidente

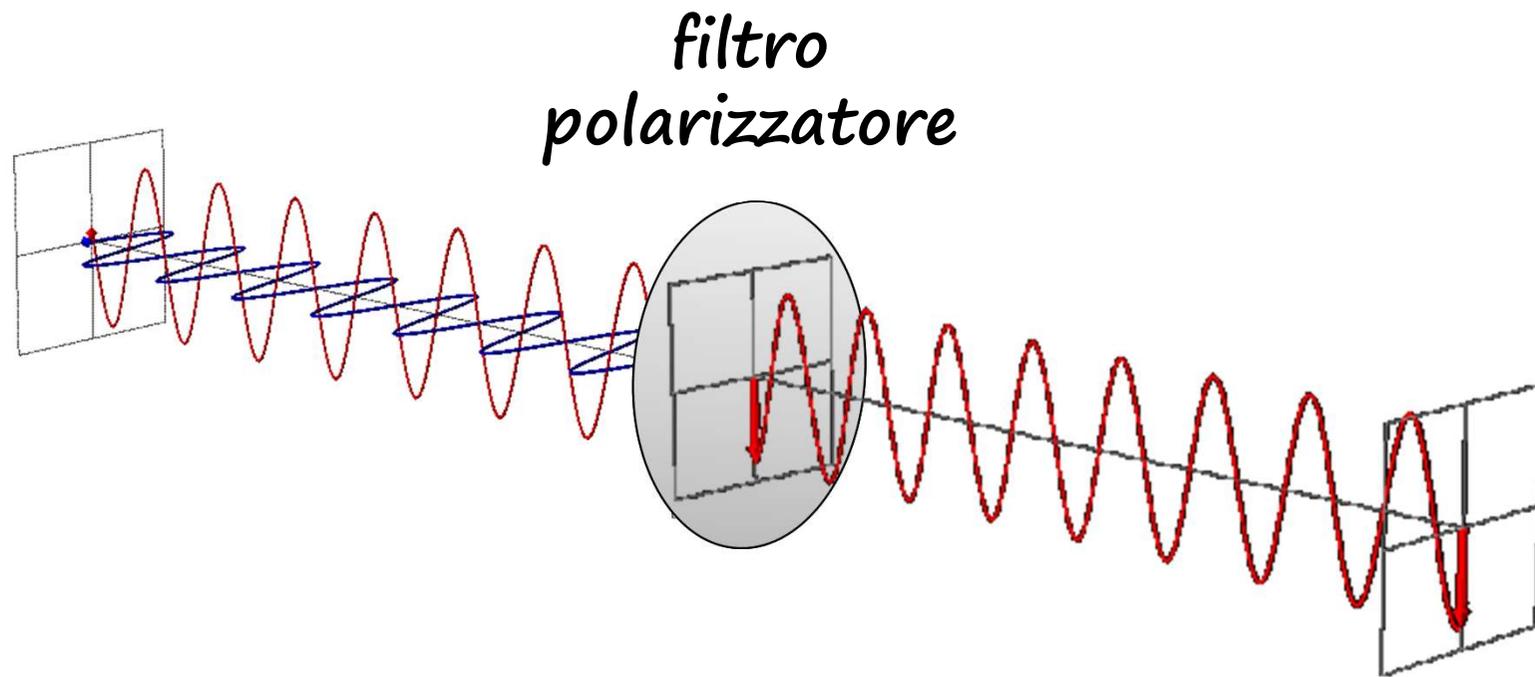
radiazione
NON polarizzata



vibra in un solo piano

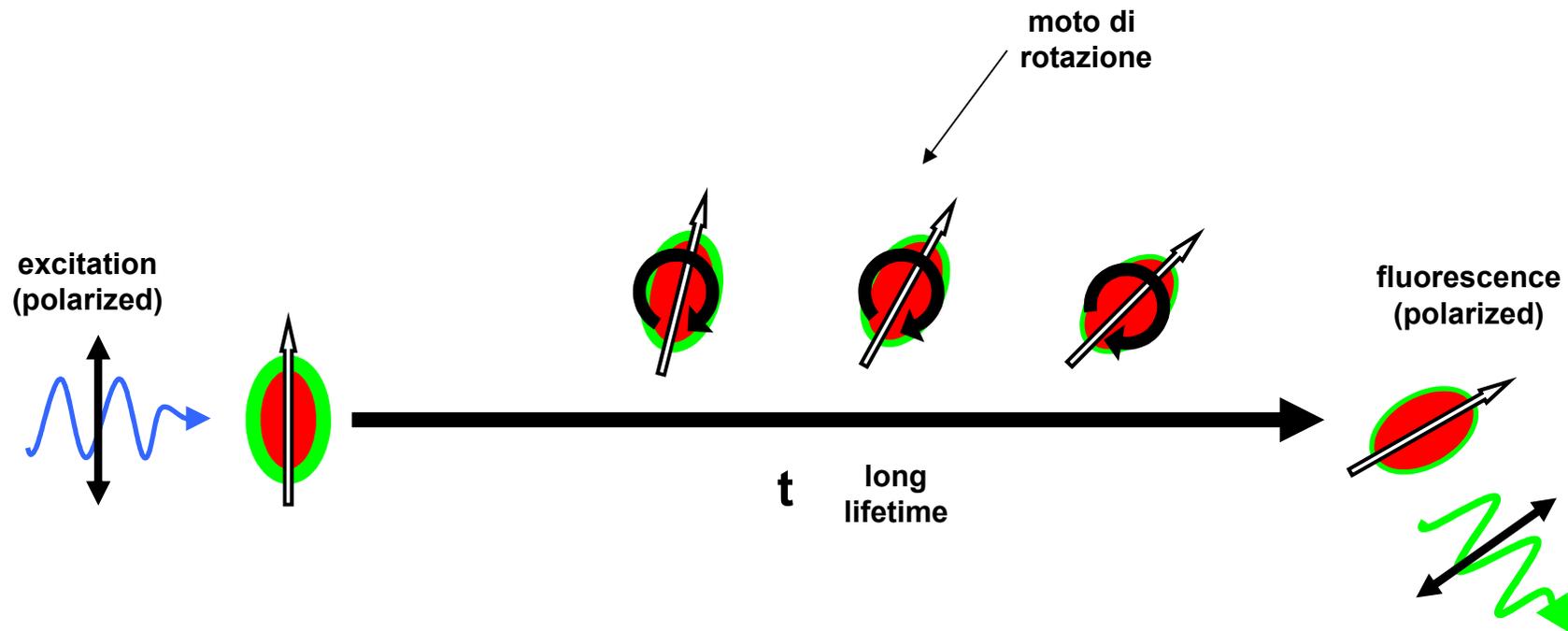
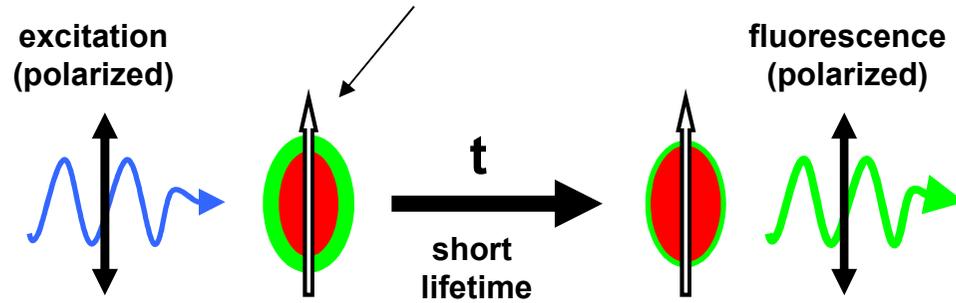
radiazione
polarizzata

polarizzazione della luce



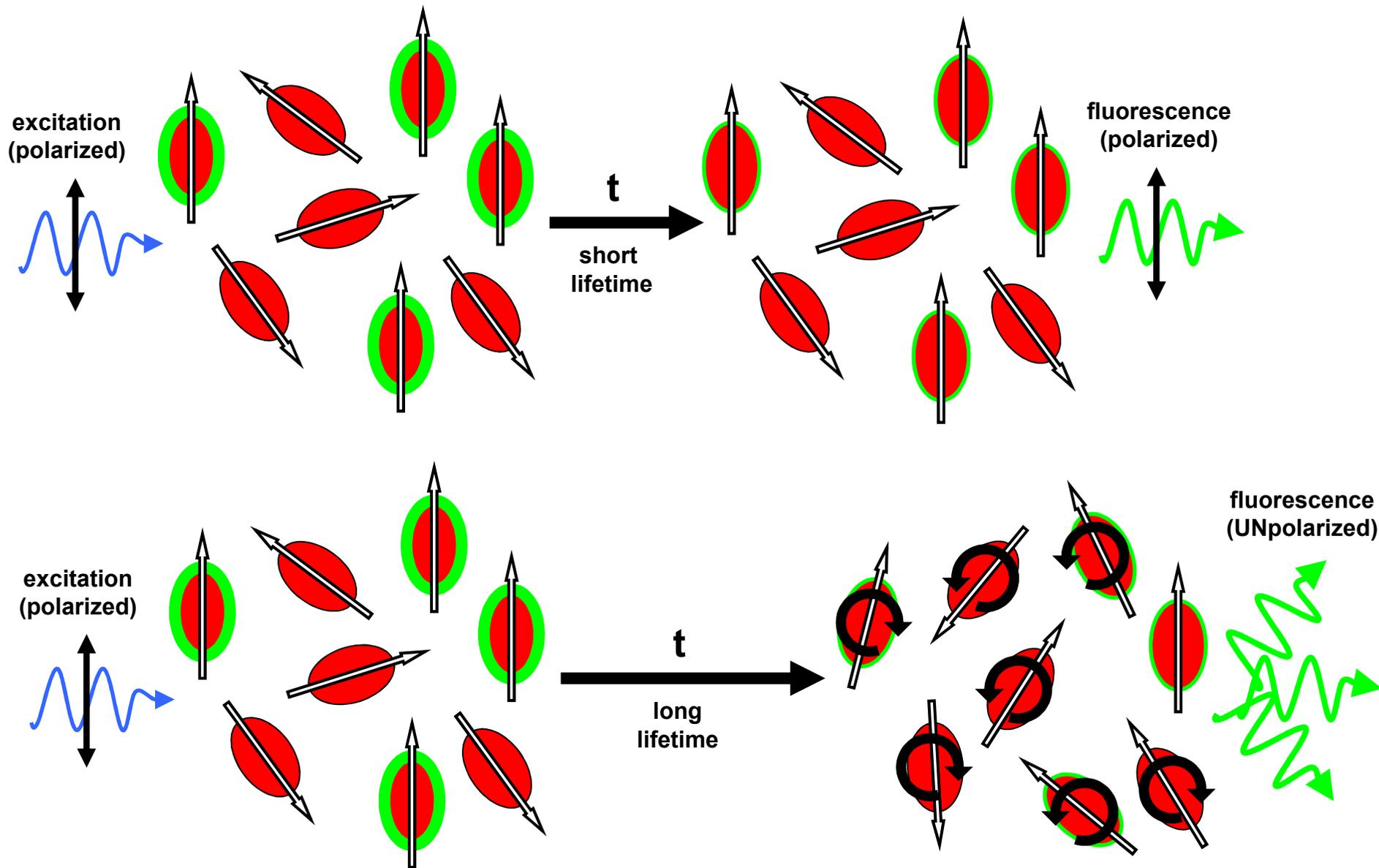
Caratteristiche della Fluorescenza

I fluorofori assorbono (ed emettono) preferenzialmente la luce polarizzata parallelamente alla direzione del momento di dipolo (transition dipole moment)

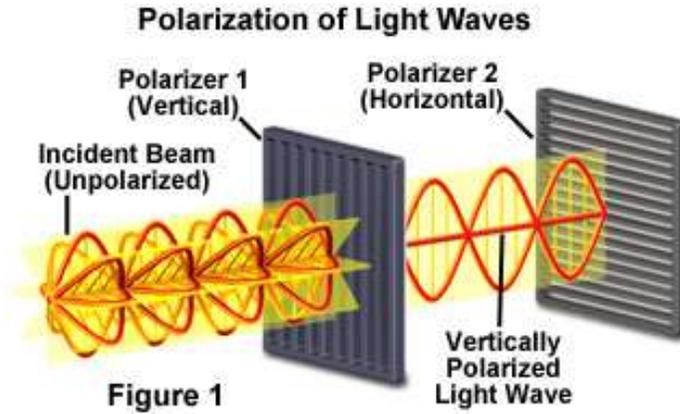
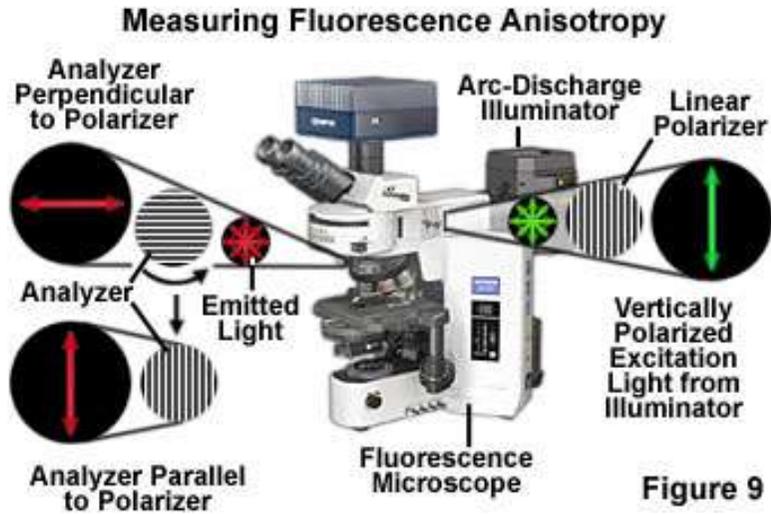


Caratteristiche della Fluorescenza

ANISOTROPIA

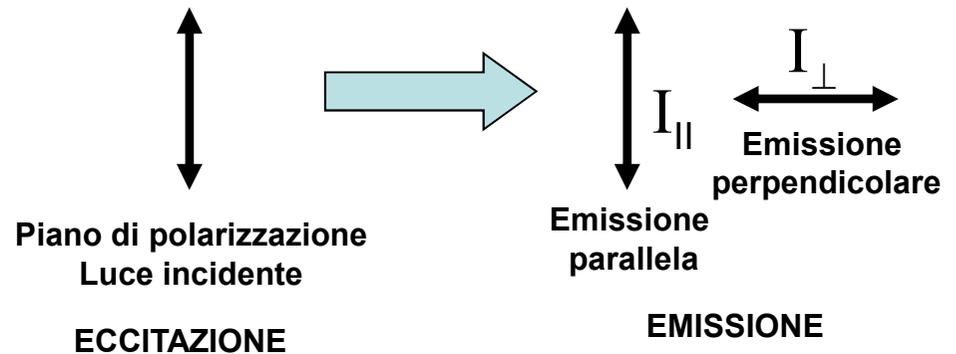


Caratteristiche della Fluorescenza

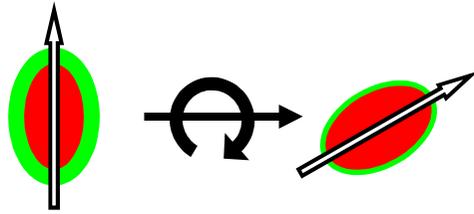


polarization	$p = \frac{(I_{ } - I_{\perp})}{(I_{ } + I_{\perp})}$
anisotropy	$r = \frac{(I_{ } - I_{\perp})}{(I_{ } + 2I_{\perp})}$

Polarizzazione ed anisotropia esprimono entrambi lo stesso fenomeno e possono essere facilmente interconvertite



Caratteristiche della Fluorescenza



Relazione
ANISOTROPIA – MOBILITA' ROTAZIONALE

(Perrin equation)

anisotropia "limite"
(in assenza di rotazione)

fluorescence lifetime

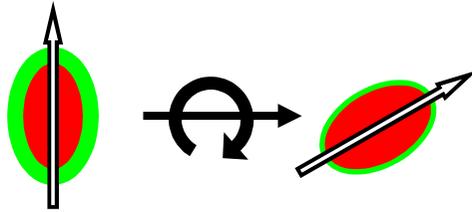
$$\frac{r_0}{r} = 1 + \frac{RT\tau}{\eta V}$$

viscosità *volume molecolare*

tempo di correlazione rotazionale

$$\frac{\eta V}{RT} = \phi$$
$$\frac{r_0}{r} = 1 + \frac{\tau}{\phi}$$

Caratteristiche della Fluorescenza



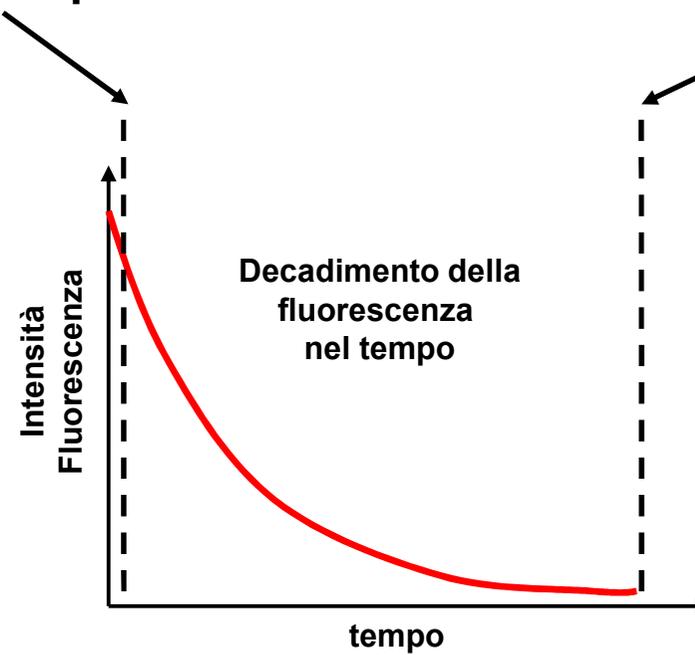
**Relazione
ANISOTROPIA – MOBILITA' ROTAZIONALE**

Tempi di rotazione caratteristici per piccoli
Fluorofori in soluzione

50-100 ps

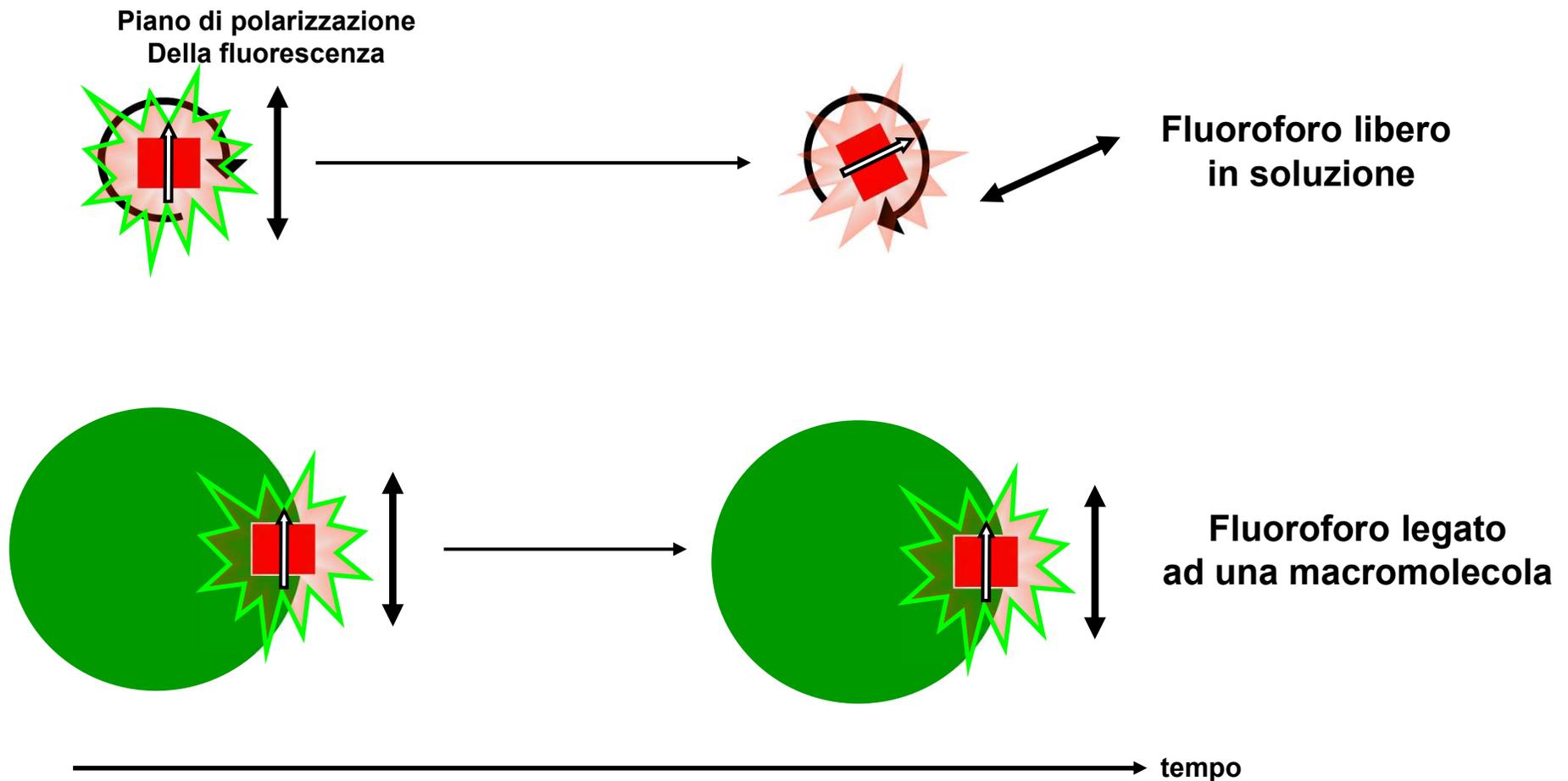
Tempi di rotazione caratteristici per
macromolecole (es. proteine) in soluzione

10-50 ns



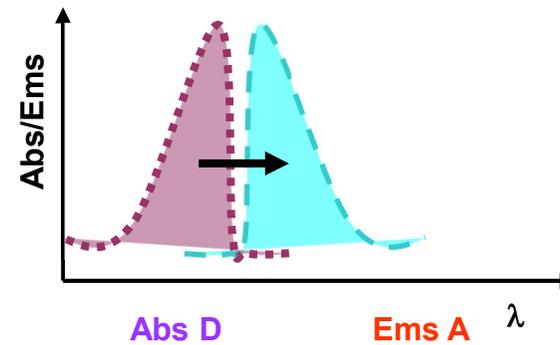
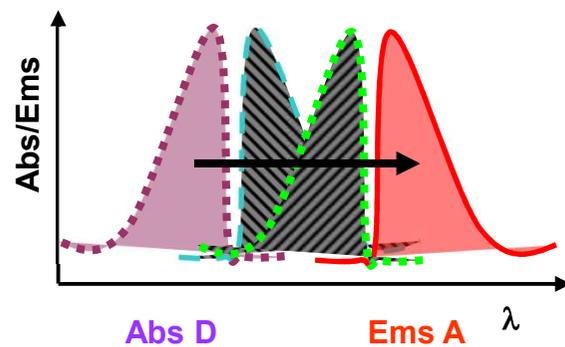
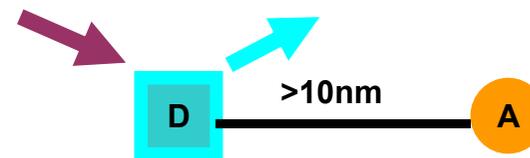
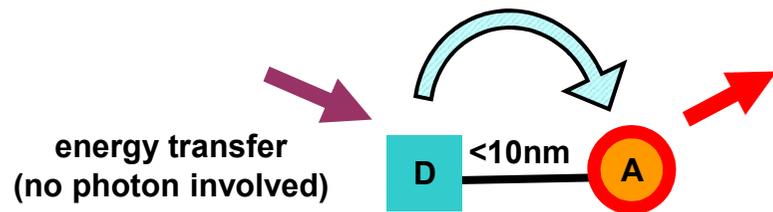
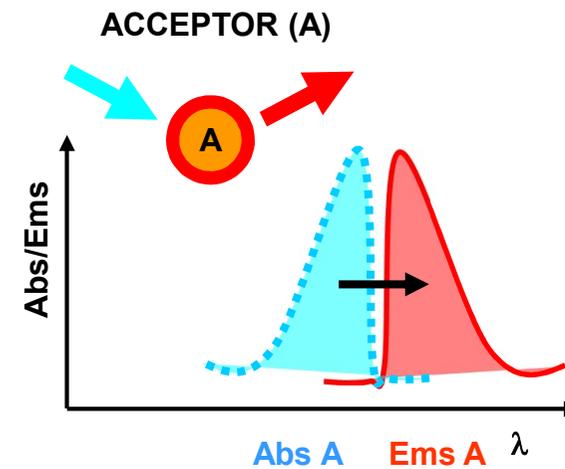
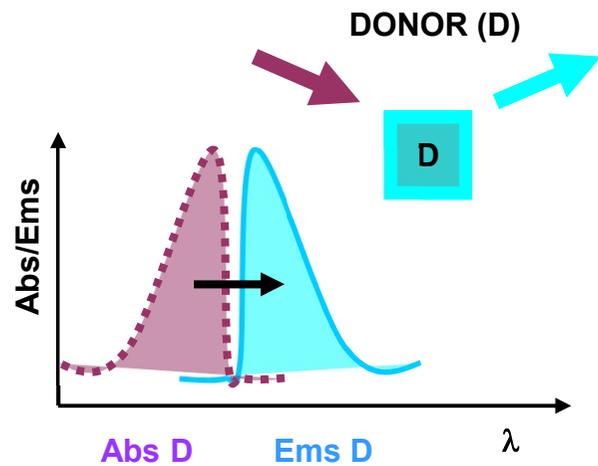
Fluorescenza: applicazioni anisotropia

L'associazione di un fluoroforo ad una molecola più grande
aumenta il tempo medio di rotazione e
conseguentemente la polarizzazione/anisotropia



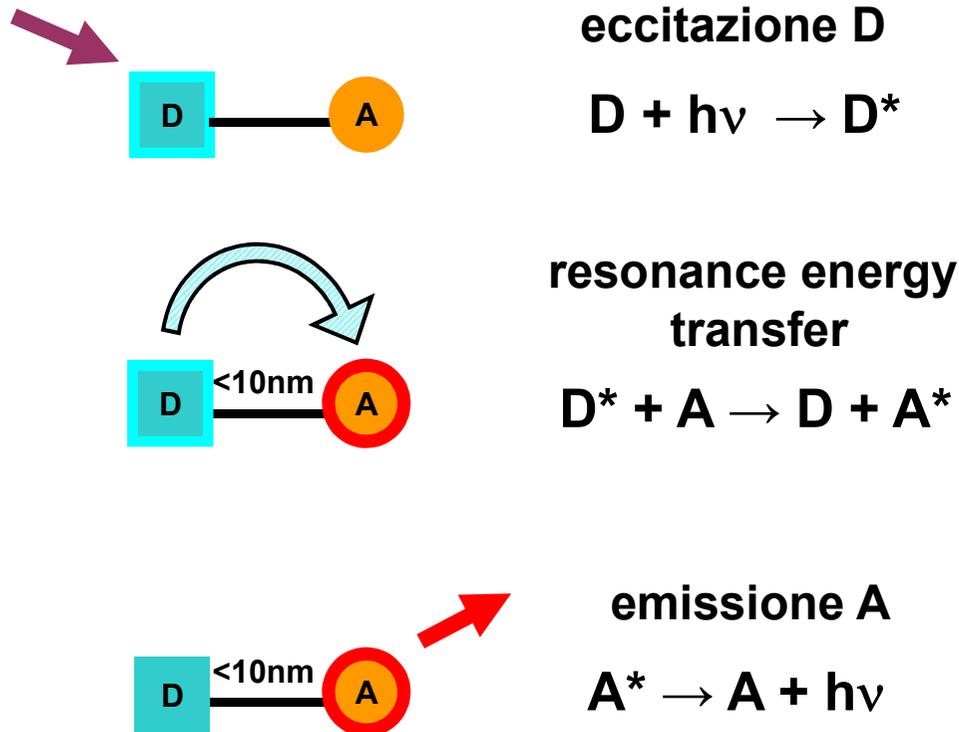
Fluorescenza: applicazioni

Förster Resonance Energy Transfer - FRET



Fluorescenza: applicazioni

Förster Resonance Energy Transfer - FRET



relazione efficienza FRET (E) – distanza (r)

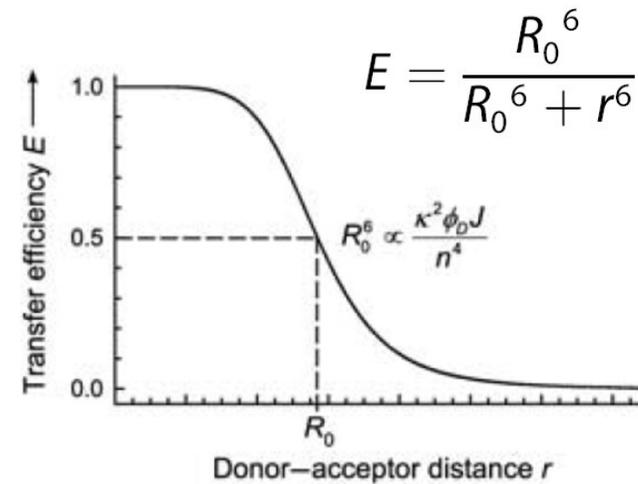
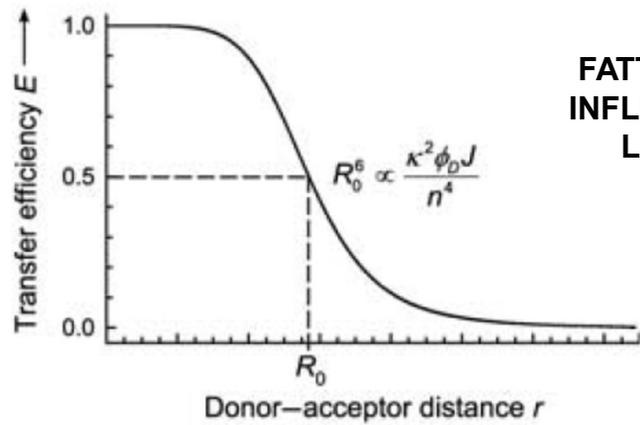


Figure 1. Distance dependence of the Förster resonance energy transfer efficiency between a suitable pair of chromophores, calculated according to Equation (1). The characteristic Förster distance R_0 is calculated from the orientational factor κ^2 , the donor quantum yield ϕ_D , the overlap integral J , and the refractive index of the medium n .

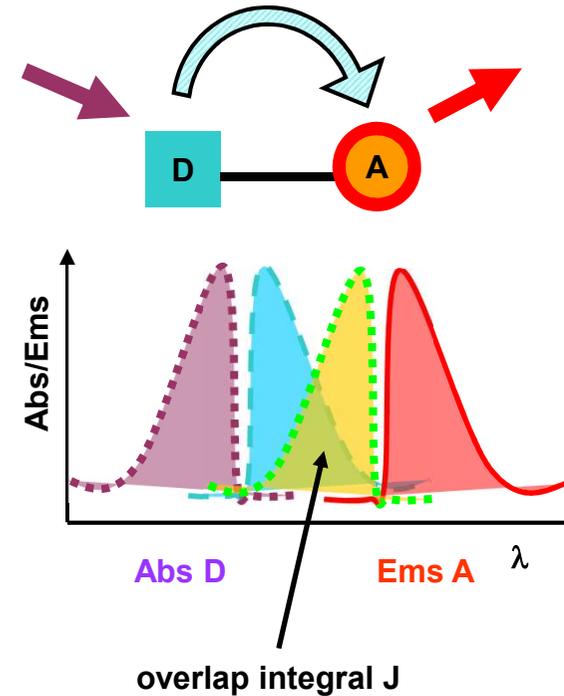
Fluorescenza: applicazioni

relazione efficienza FRET (E) – distanza (r)

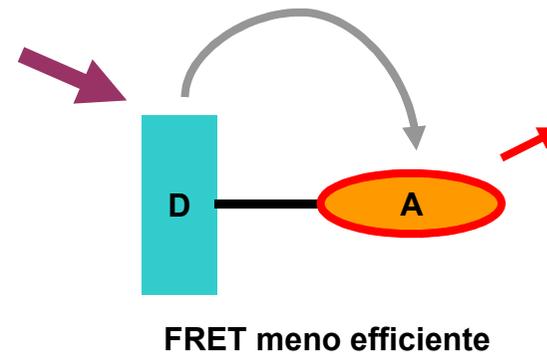
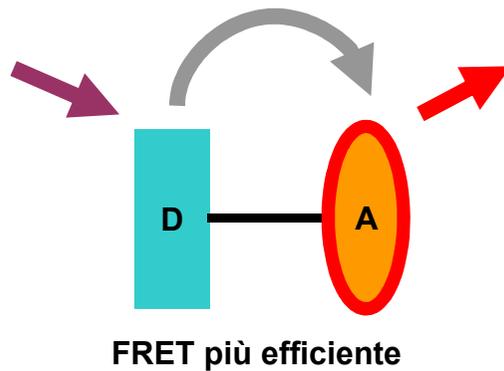


FATTORI CHE INFLUENZANO LA FRET

Figure 1. Distance dependence of the Förster resonance energy transfer efficiency between a suitable pair of chromophores, calculated according to Equation (1). The characteristic Förster distance R_0 is calculated from the orientational factor κ^2 , the donor quantum yield ϕ_D , the overlap integral J , and the refractive index of the medium n .



orientational factor κ



Fluorescenza: applicazioni

*ENERGY TRANSFER: A SPECTROSCOPIC RULER**

BY LUBERT STRYER AND RICHARD P. HAUGLAND†

DEPARTMENT OF BIOCHEMISTRY, STANFORD UNIVERSITY SCHOOL OF MEDICINE, PALO ALTO,
AND THE DEPARTMENT OF CHEMISTRY, STANFORD UNIVERSITY

Communicated by Arthur Kornberg, May 29, 1967

PNAS 1967;58;719-726

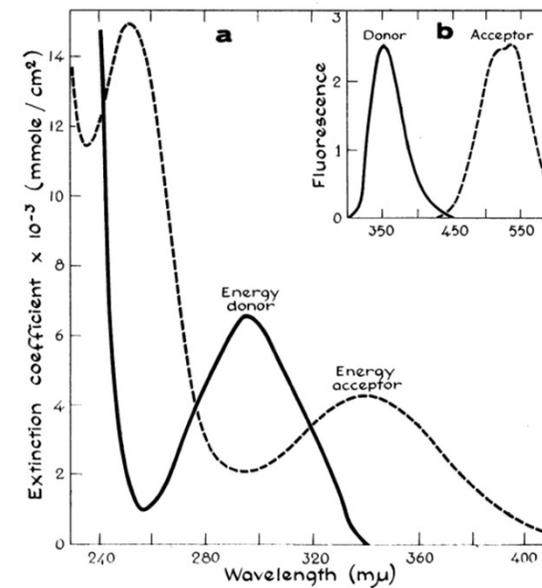
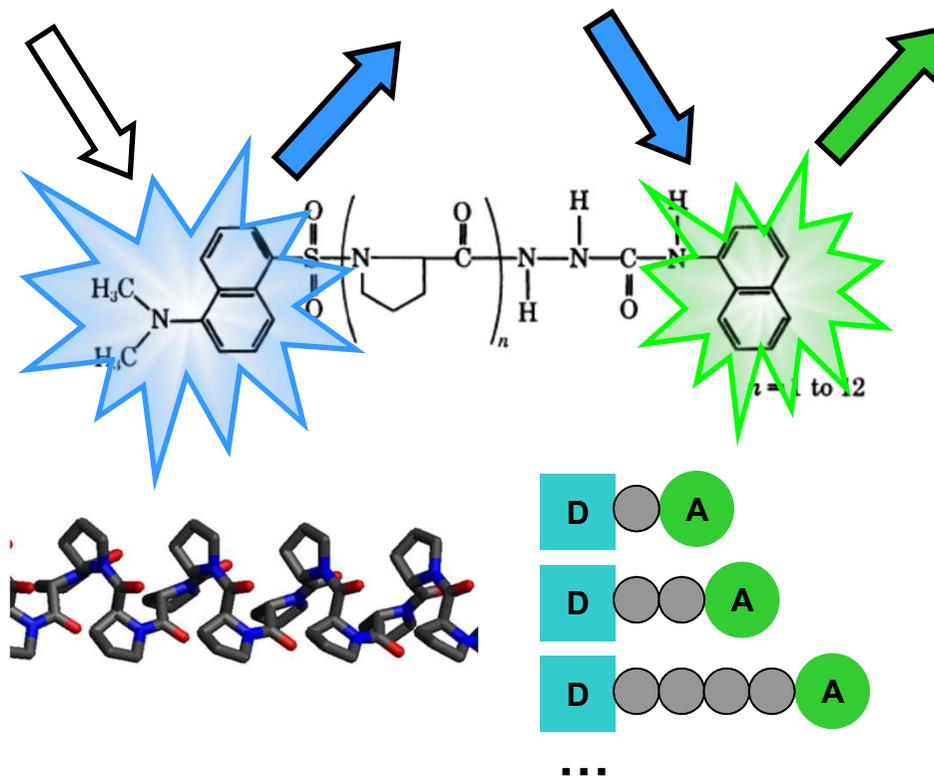


FIG. 2.—(a) Absorption spectrum of the energy donor (1-acetyl-4-(1-naphthyl) semicarbazide, —) and the energy acceptor (dansyl-L-prolyl-hydrazide, ----) in ethanol; (b) emission spectrum of the energy donor (—) and the energy acceptor (----) in ethanol.

Fluorescenza: applicazioni

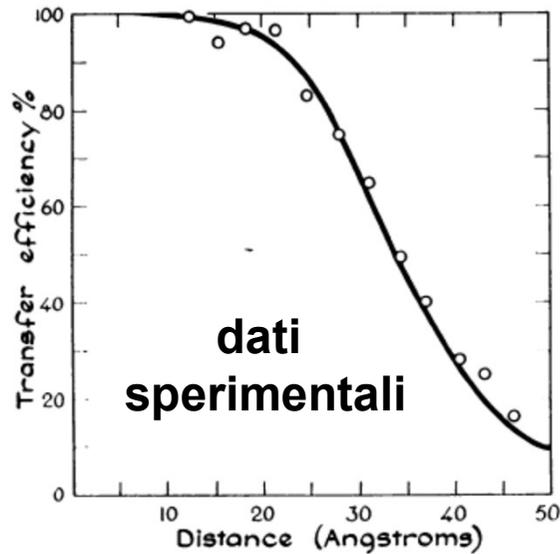
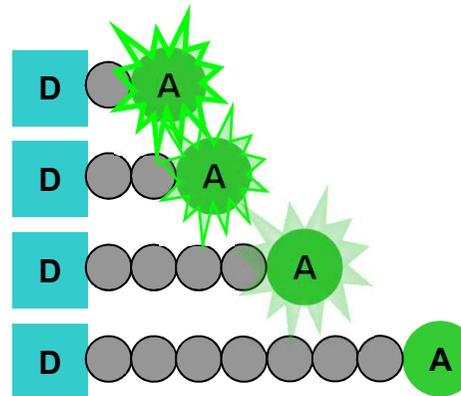
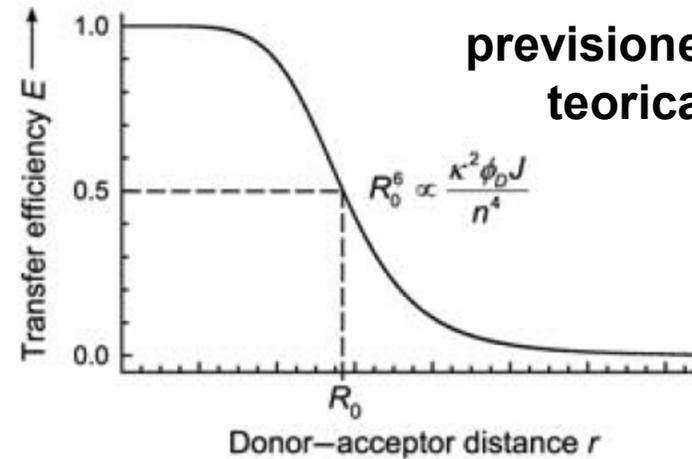


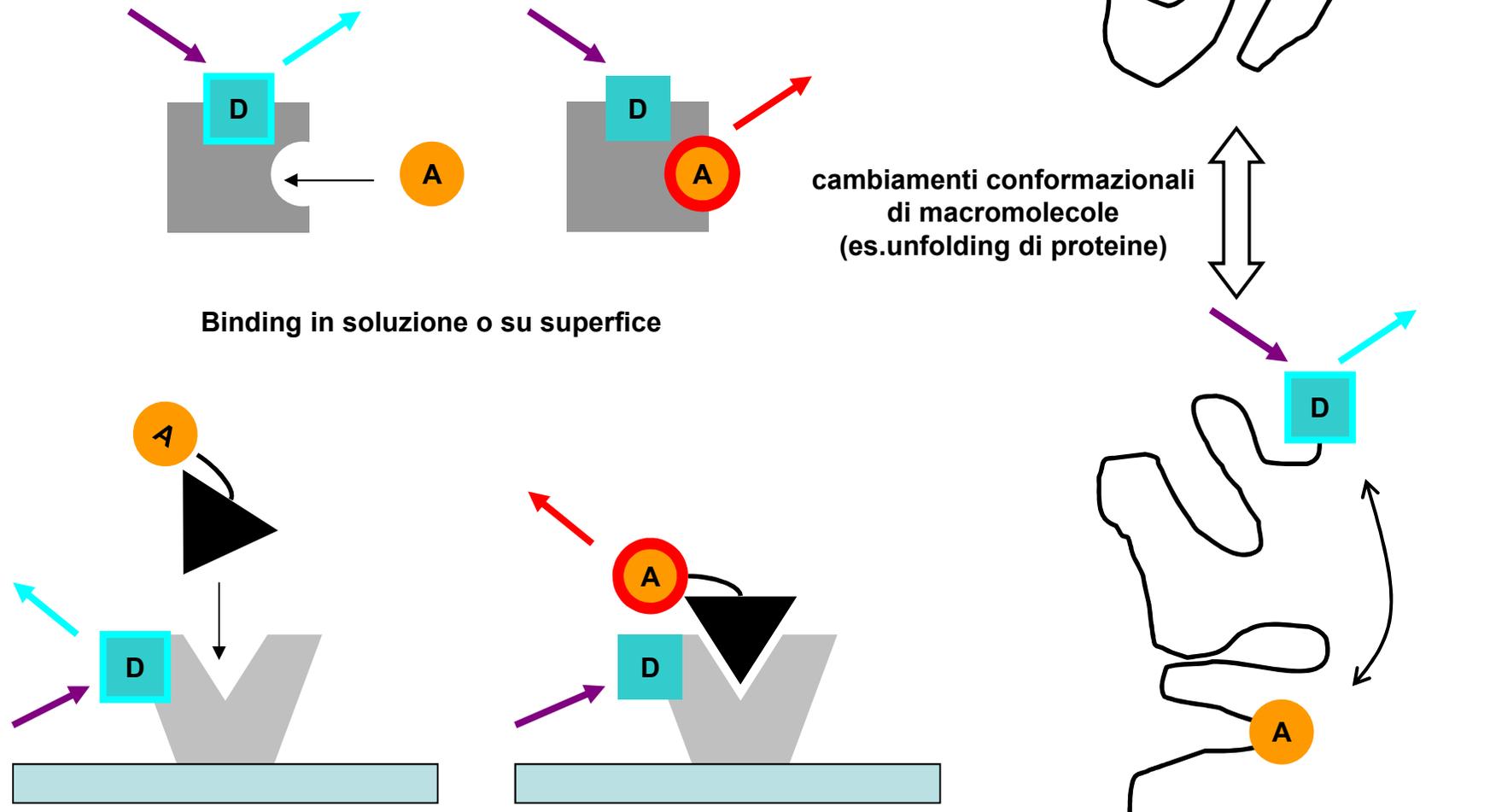
FIG. 4.—Efficiency of energy transfer as a function of distance in dansyl-(L-prolyl)_n-α-naphthyl, *n* = 1 to 12. The α-naphthyl and dansyl groups were separated by defined distances ranging from 12 to 46 Å. The energy transfer is 50% efficient at 34.6 Å. The solid line corresponds to an r^{-6} distance dependence.

Conferma (validazione) della teoria:
misurando l'efficienza della FRET si può risalire alla distanza (“metro” spettroscopico)



Fluorescenza: applicazioni

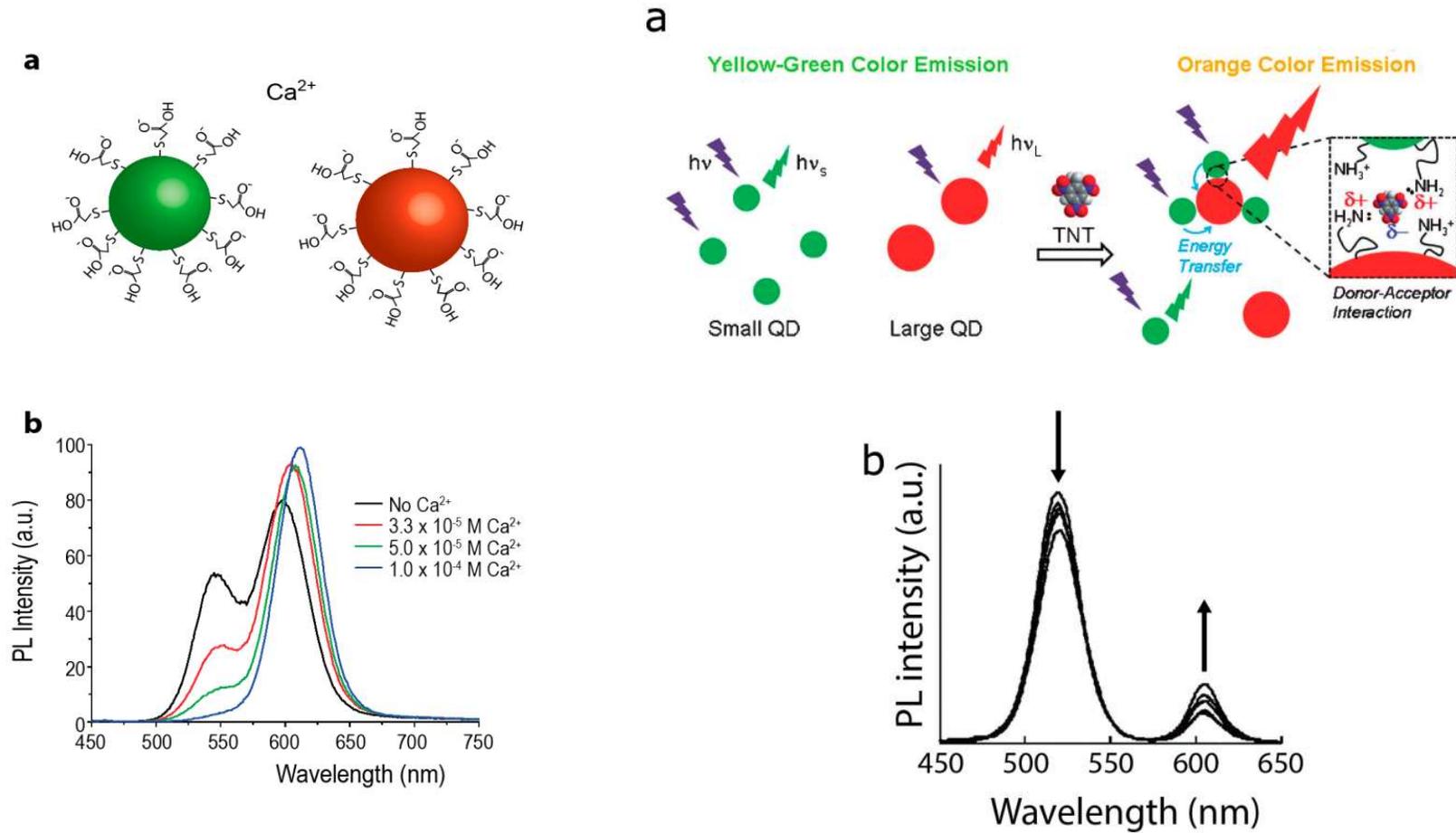
APPLICAZIONI FRET: VASTISSIME



Förster Resonance Energy Transfer between Quantum Dot Donors and Quantum Dot Acceptors

Kenny F. Chou¹ and Allison M. Dennis^{2,*}

Sensors **2015**, *15*, 13288-13325; doi:10.3390/s150613288

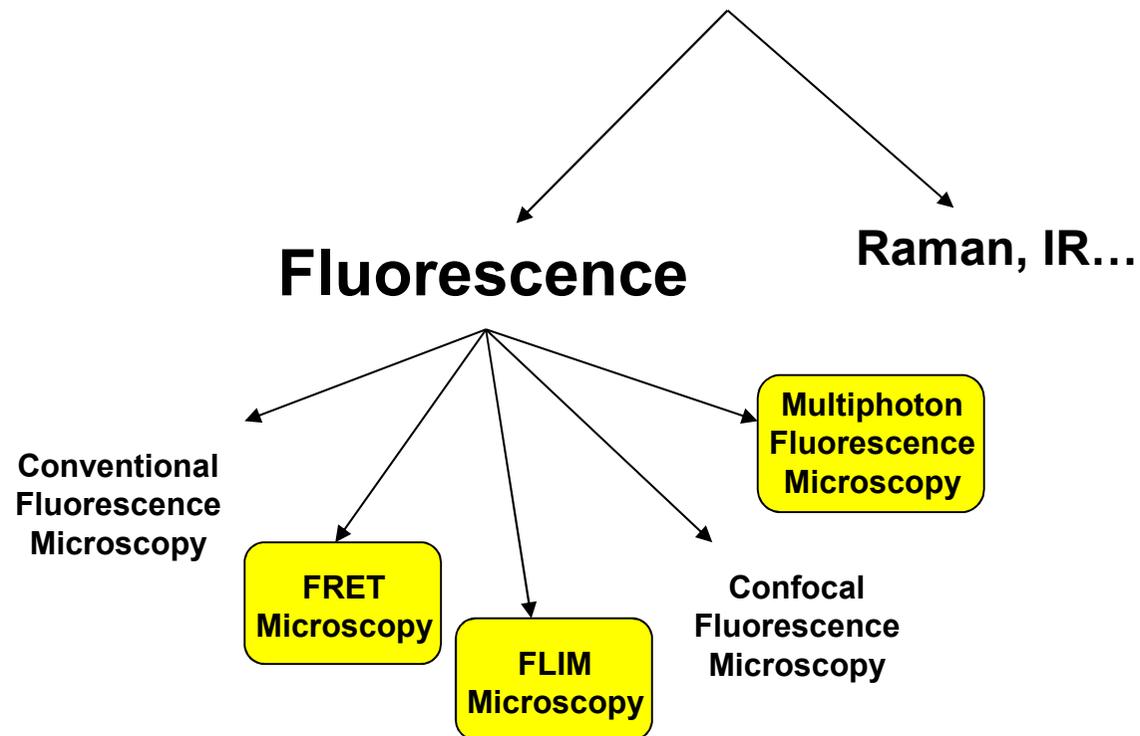


Fluorescenza: applicazioni

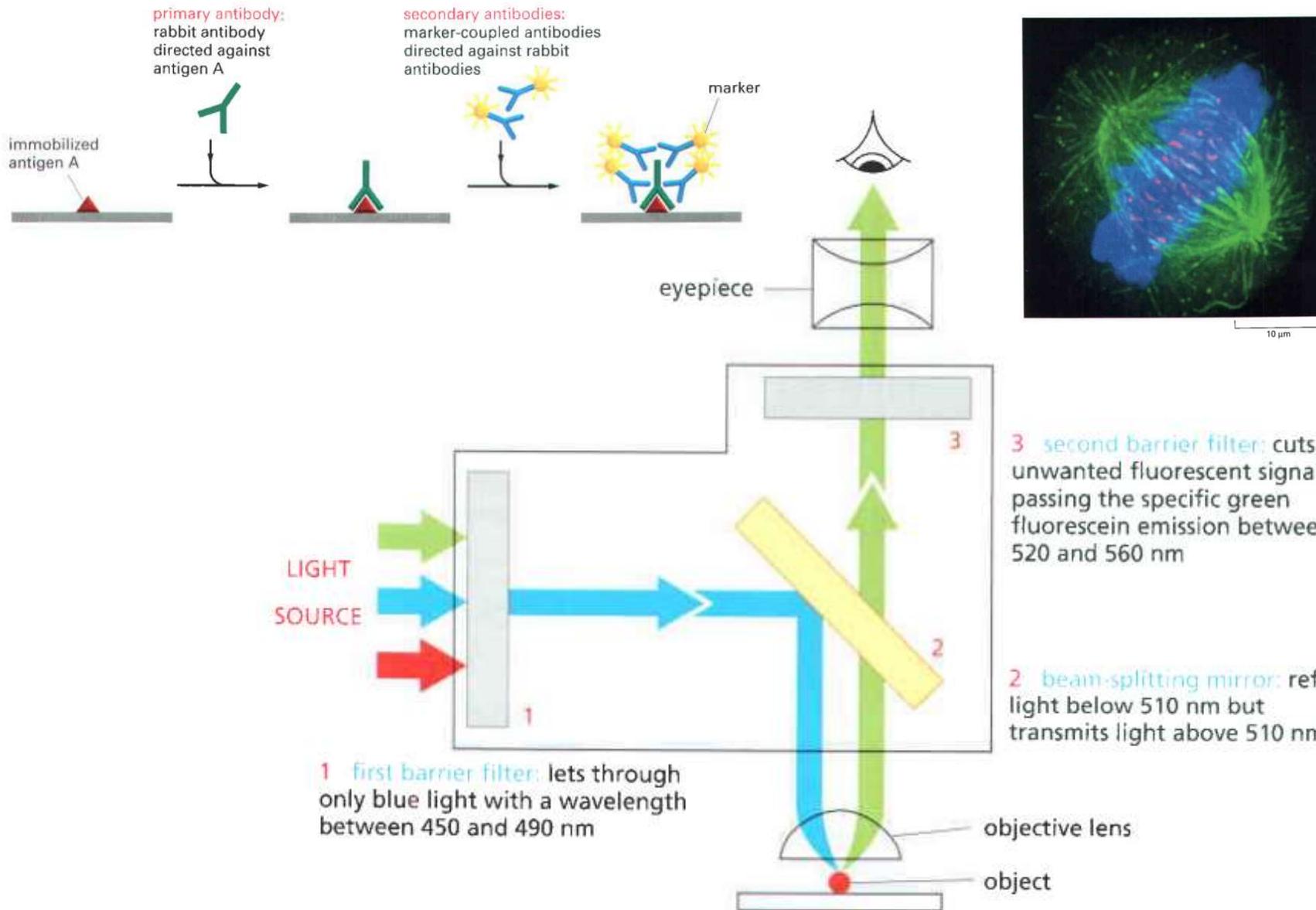
“Chemical” (optical) Microscopy / Imaging

i.e. spatial representation
of chemical information

(p2p mapping, wf imaging)

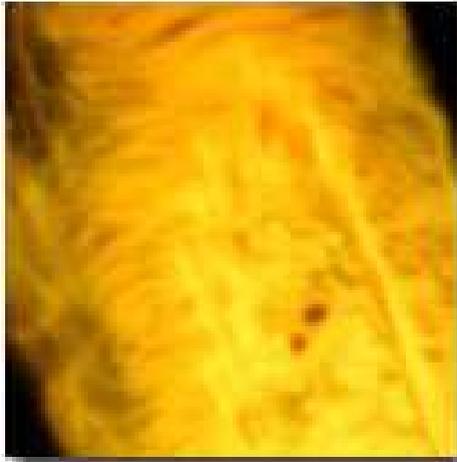


Microscopia a (di) fluorescenza

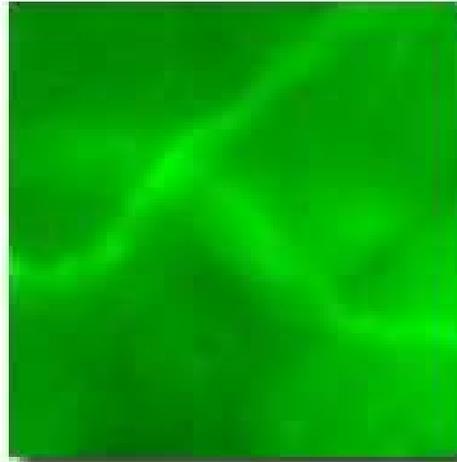


Microscopia CONFOCALE

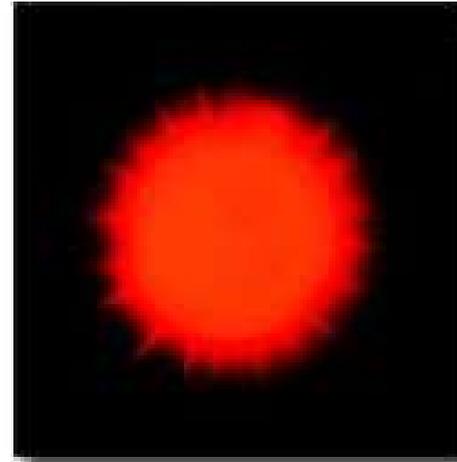
Confocal and Widefield Fluorescence Microscopy



(a)

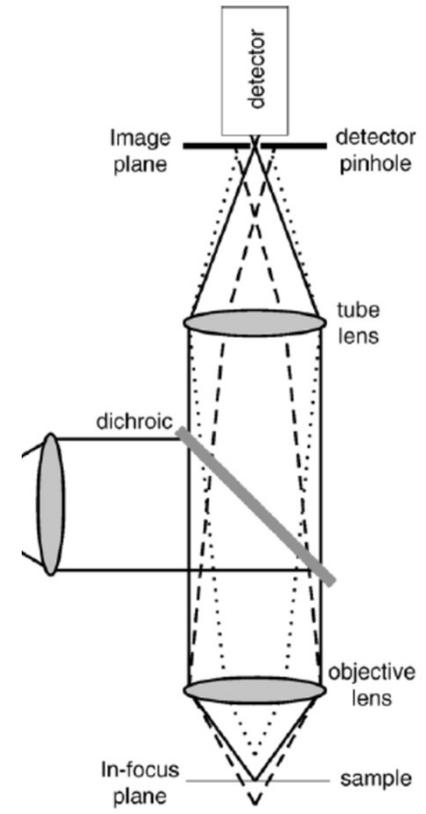
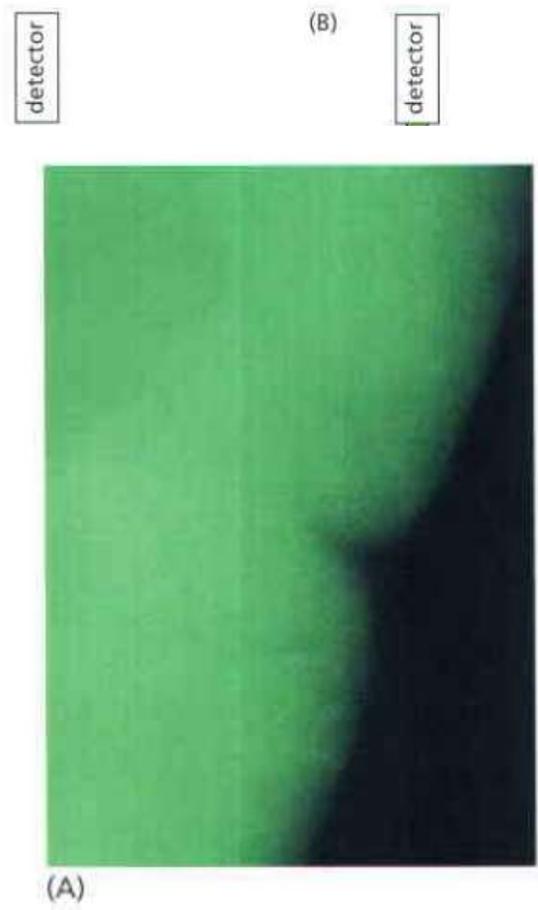
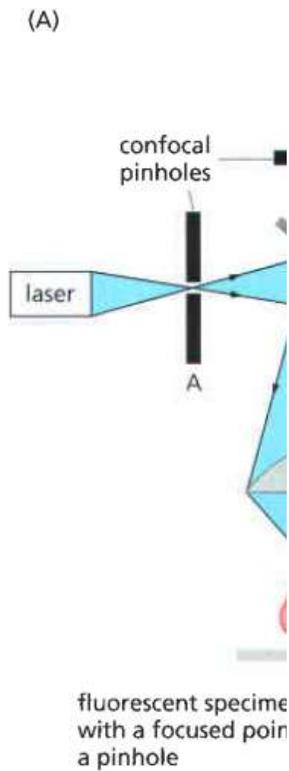


(b)



(c)

Microscopia CONFOCALE



Widefield versus

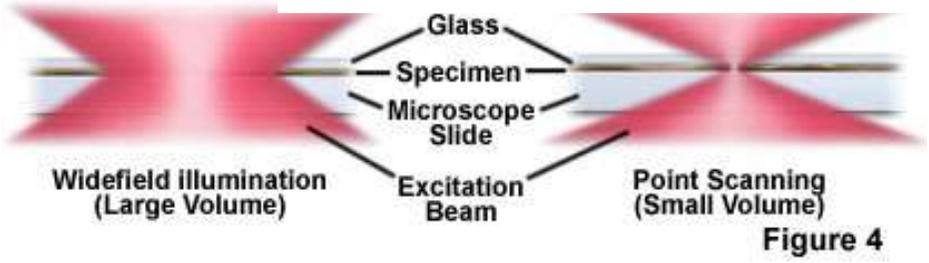
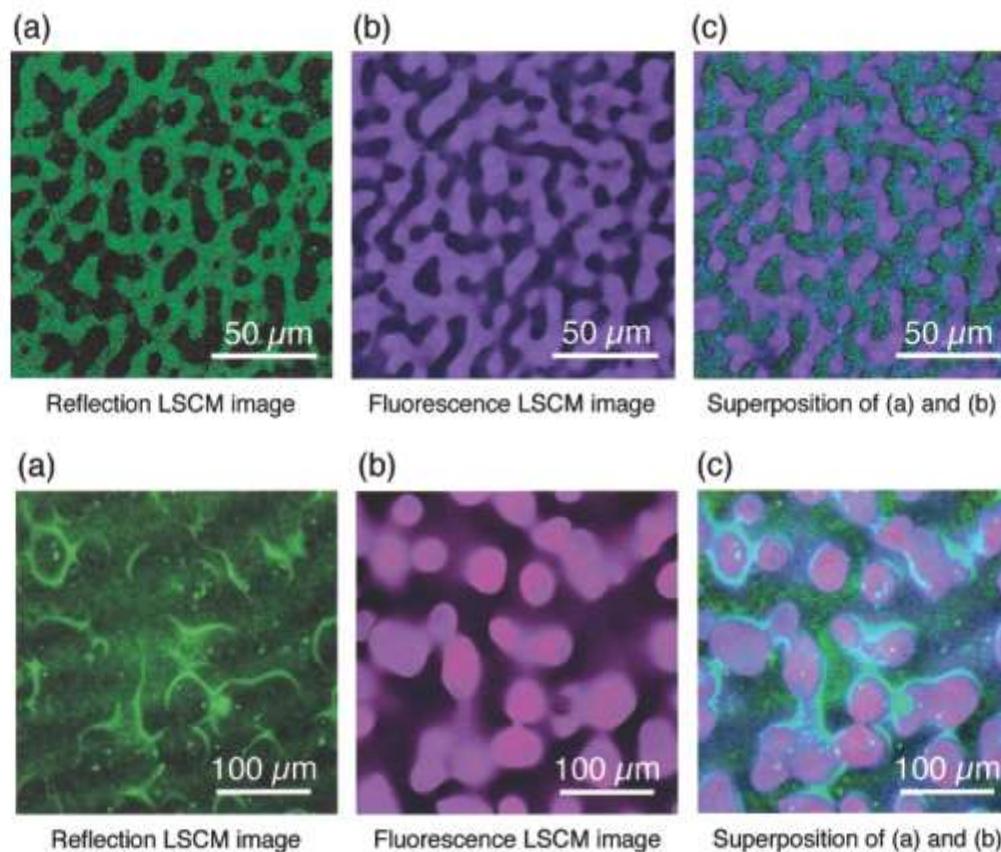


Figure 4

NON confocale,
anche la luce proveniente da punti
diversi dal fuoco dell'obiettivo vengono
riportati al rivelatore

Observation of Fine Structure in Bicontinuous Phase-Separated Domains of a Polymer Blend by Laser Scanning Confocal Microscopy

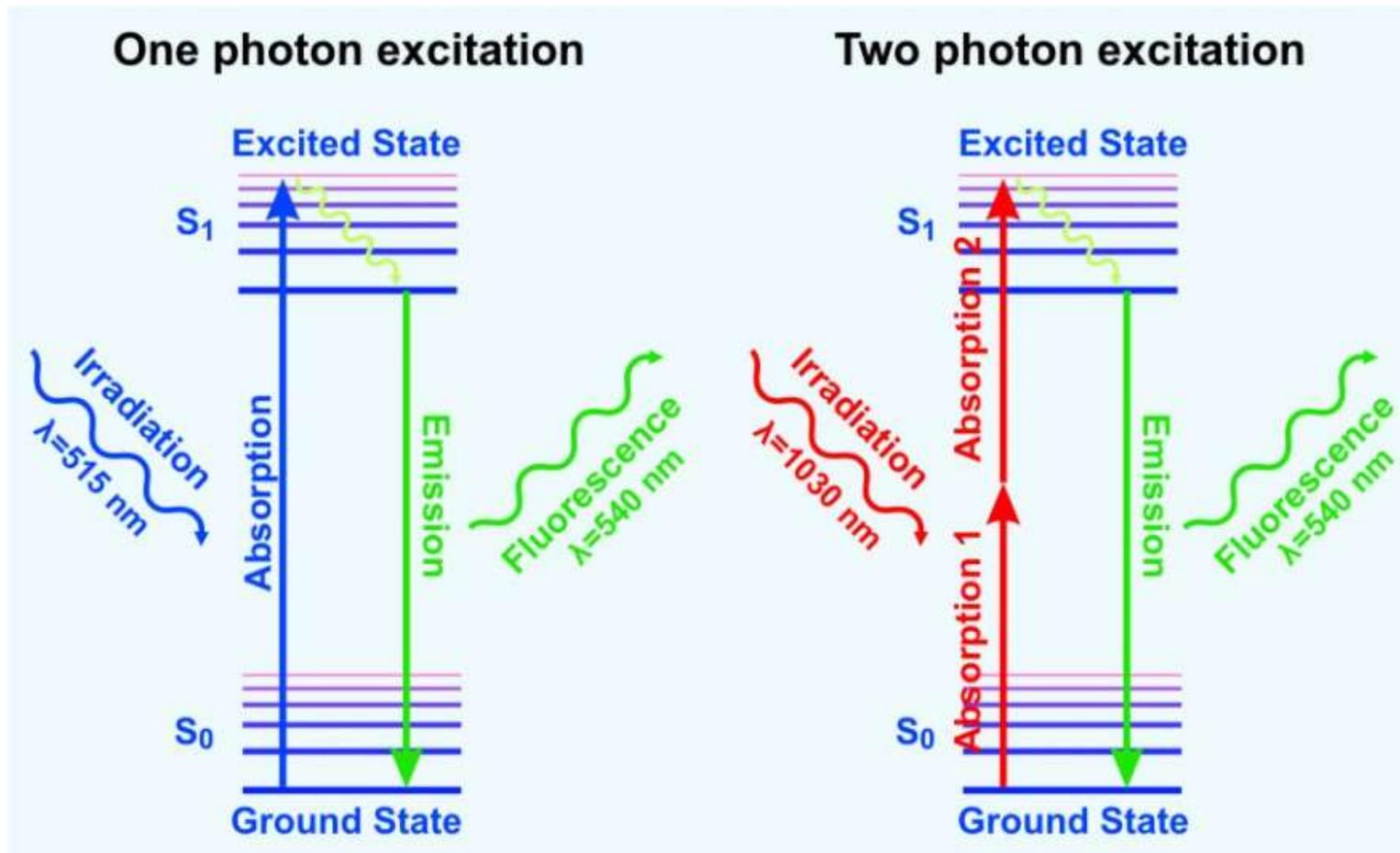
Hiroshi Jinnai,^{*,†,‡} Hiroshi Yoshida,^{†,§} Kohtaro Kimishima,^{†,||} Yoshinori Funaki,^{†,⊥}
Yoshitsugu Hirokawa,^{†,¶} Alexander E. Ribbe,^{†,⊗} and Takeji Hashimoto^{*,†,○}



Fluorescenza: applicazioni

2 Photons Excitation Fluorescence (2PEF) microscopy

Si usano due fotoni invece di uno



Fluorescenza: applicazioni

Advanced Drug Delivery Reviews 58 (2006) 788–808

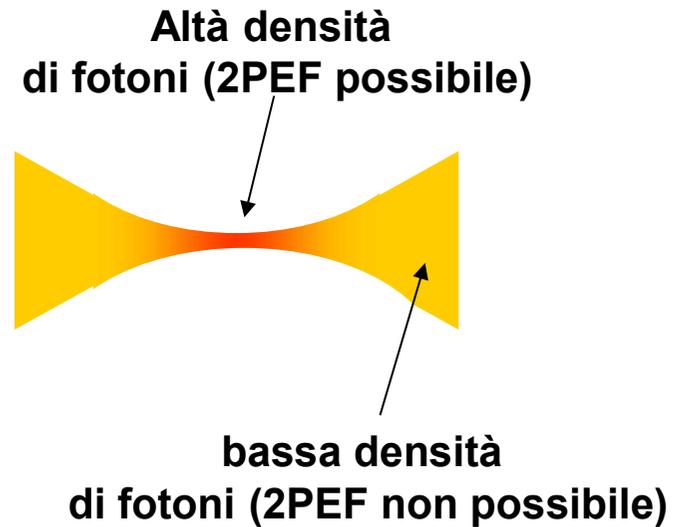
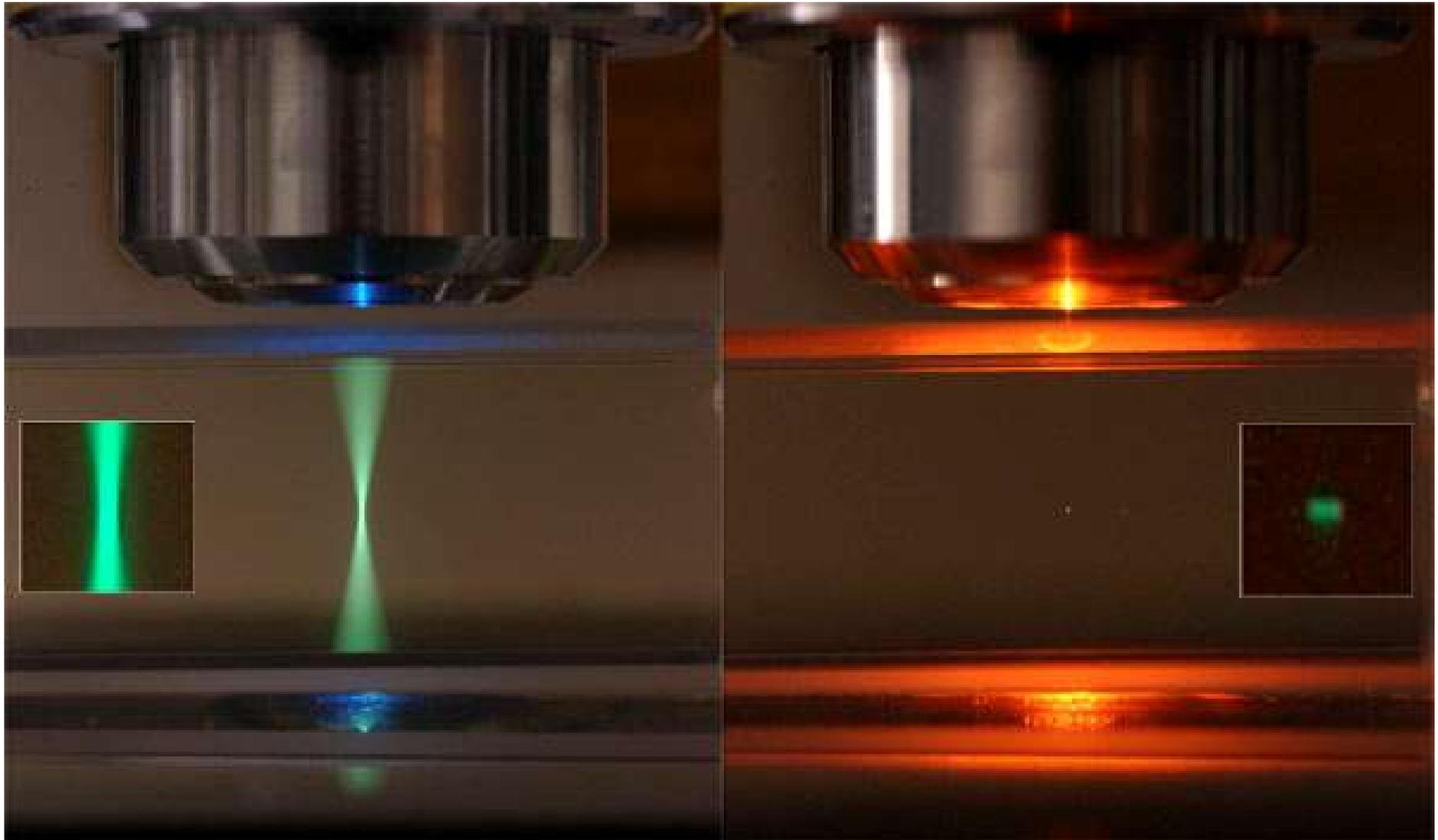
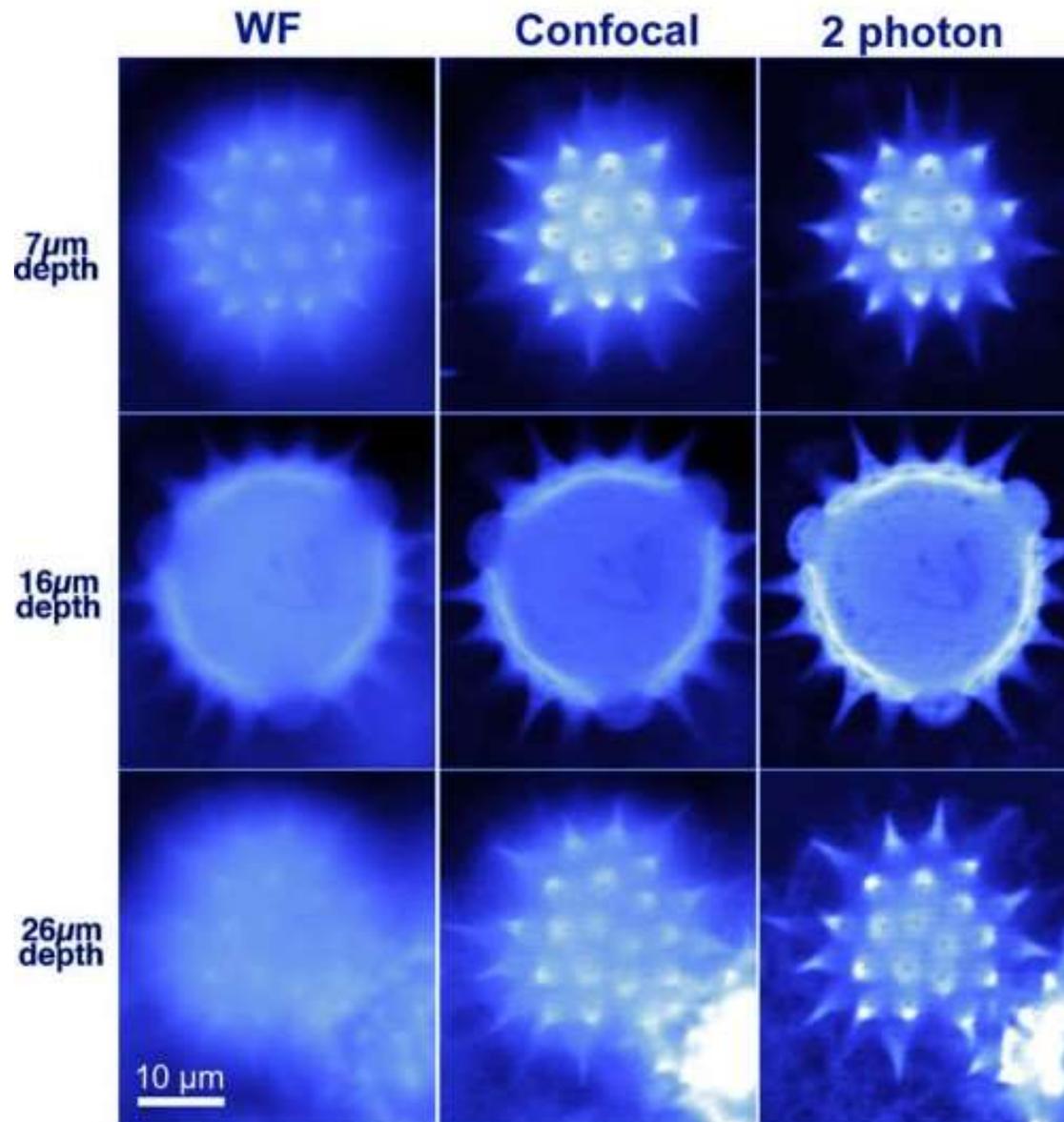


Fig. 3. An experiment to illustrate the difference between ordinary (single-photon) excitation of fluorescence and two-photon excitation. The cuvette contains a solution of the dye safranin O, which normally emits yellow light when excited by green. The upper lens focuses green (543 nm) light from a continuous-wave (CW) helium-neon laser into the cuvette and produces the expected conical pattern of excitation, fading to the left due to the self-absorption in the concentrated dye solution. The lower objective lens focuses a pulsed infrared (1046 nm) beam from a neodymium-YLF laser. One infrared photon alone is not sufficiently energetic to excite fluorescence, so that two photons need to team up and join their energies. Two-photon fluorescence excitation is proportional to the intensity squared, confining fluorescence generation to a small spot in the focal plane where the density of photons is high. This focal volume (arrowed) can be raster-scanned to anywhere in the cuvette thereby creating a point-wise, sequential 3-D representation of fluorescence intensity. (Image credit: Brad Amos/Science Photo Library, London).

CONFOCALITA' ottenuta senza pinhole (intrinseca)

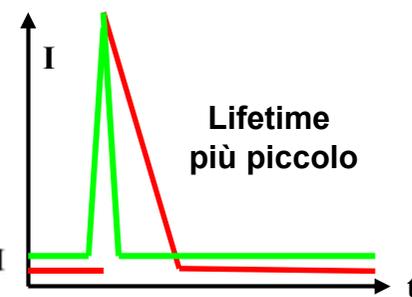
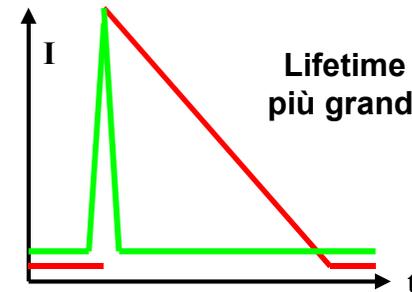
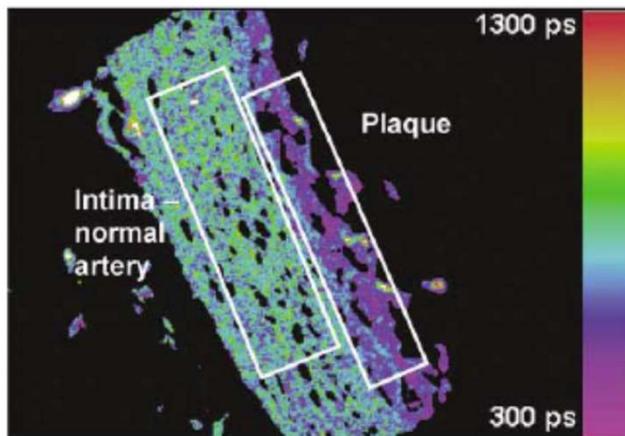
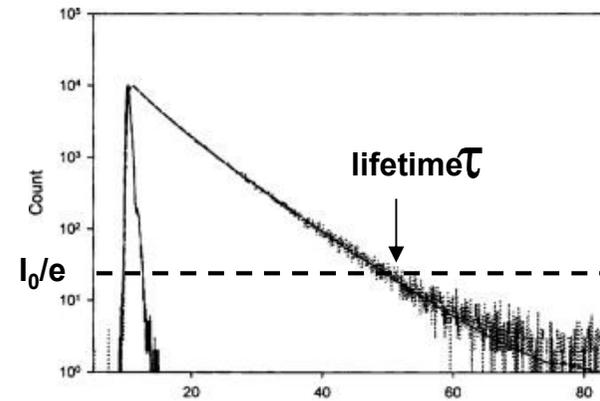
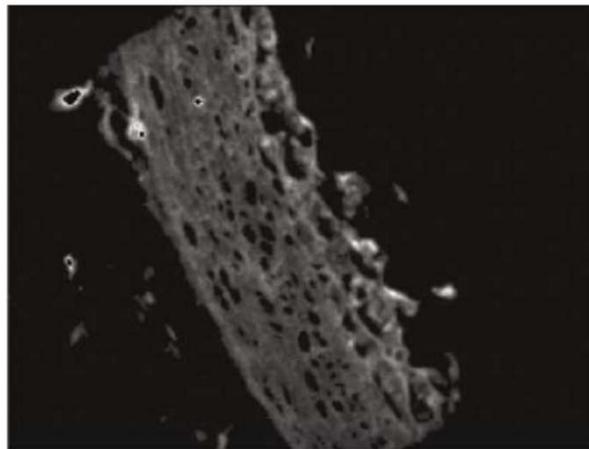




Fluorescent pollen grain was imaged at three depths below its surface (7, 16, and 26 μ m) by widefield, confocal and 2 photon technique. (<http://candle.am/microscopy/>)

Fluorescenza: applicazioni

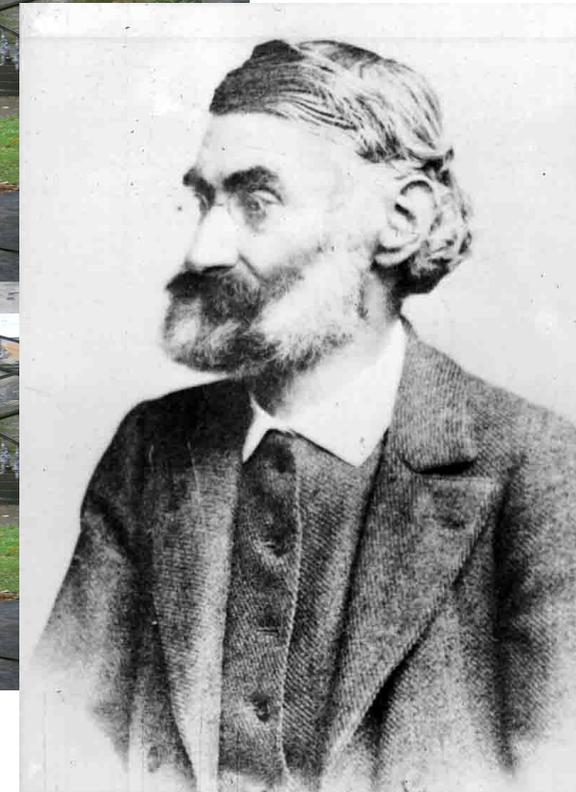
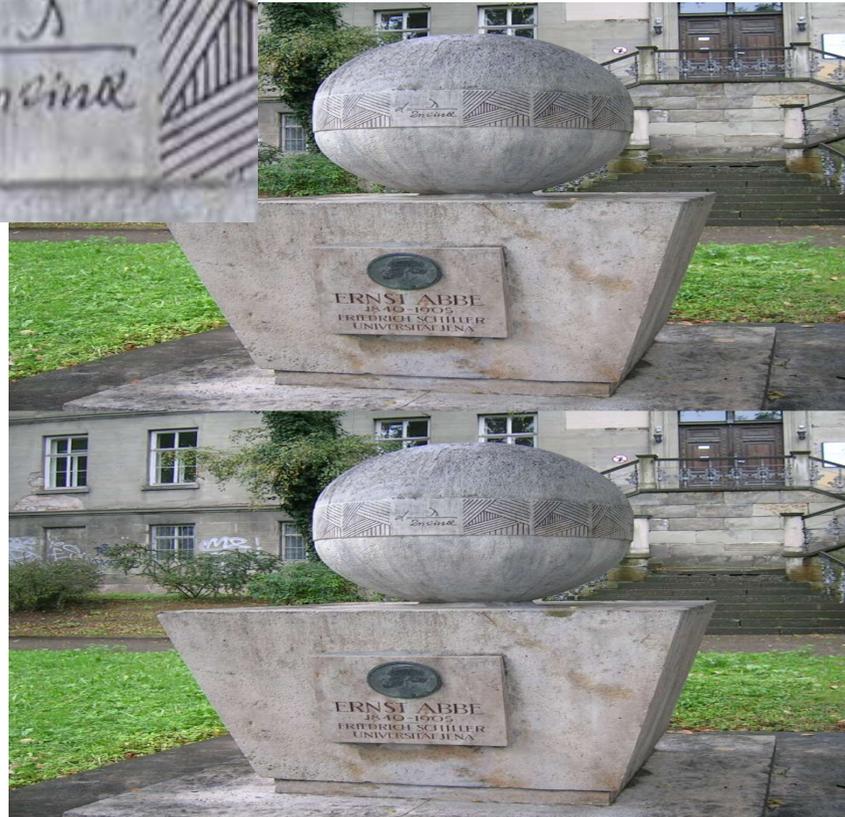
Fluorescence Lifetime Imaging (FLIM)



Differenze Lifetime
Usate per discriminare
Intorno chimico
IMAGING

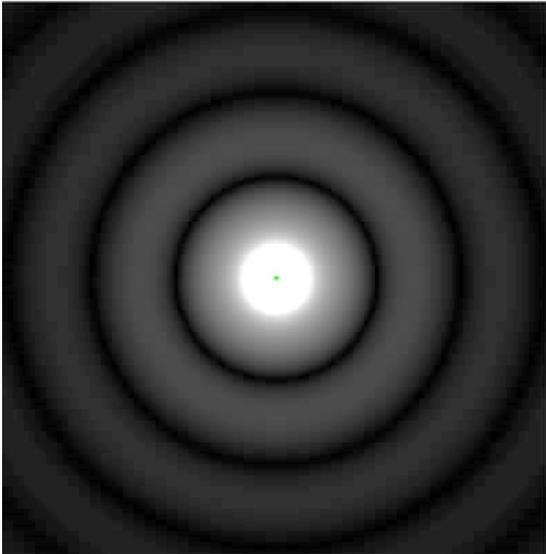
Fig. 7 (a) Wide-field fluorescence image and (b) wide-field FLIM image of unstained section of rabbit artery with atherosclerotic plaque.

limite di diffrazione (criterio di Abbe)



Ernst Abbe (1840-1905)

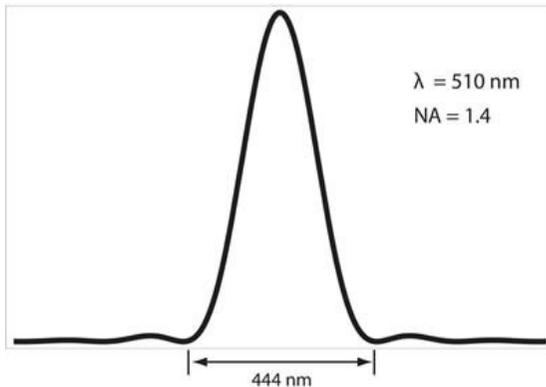
limite di diffrazione (criterio di Abbe)



Intensity profile of a single fluorophore emitting light onto a CCD camera; this is known as the Airy pattern.

The fluorophore is at the center of the image, and can be considered a point source.

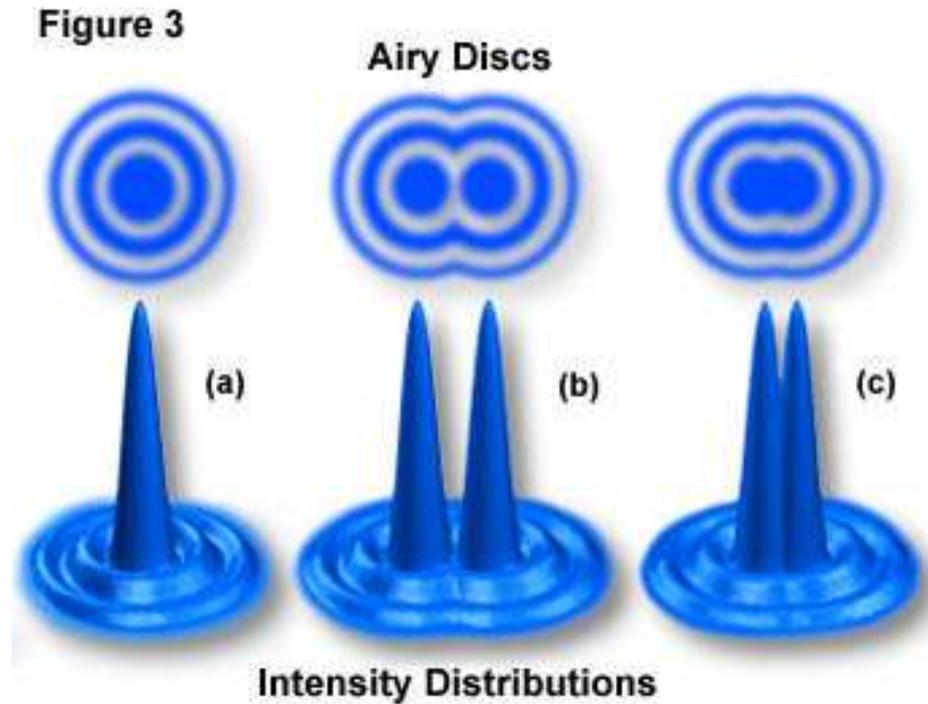
This profile is a result of the wave nature of light as the photons from the fluorescent protein diffract (scatter) off of the aperture of the objective and interfere with each other.



Thus, a point source (the fluorescent protein) is no longer viewed as a point source, but rather as a diffuse, delocalized intensity pattern.

Microscopia CONFOCALE

Problema risoluzione spaziale:



$$R = \lambda / 2 NA$$
$$(NA = n \sin \theta)$$

$$r \equiv \frac{0.5 \lambda}{NA} \equiv \frac{0.5 \lambda}{n \sin(\theta)}$$

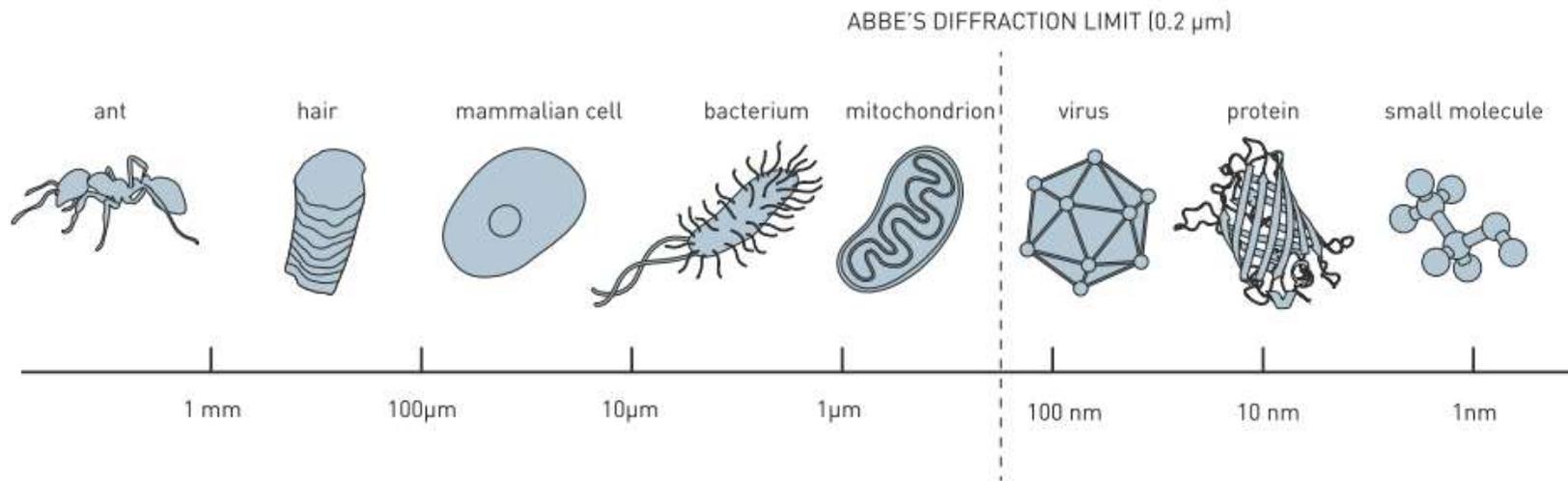


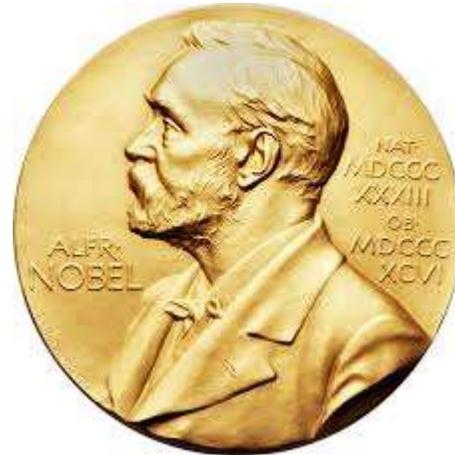
Figure 1. At the end of the 19th century, Ernst Abbe defined the limit for optical microscope resolution to roughly half the wavelength of light, about 0.2 micrometre. This meant that scientists could distinguish whole cells, as well as some parts of the cell called organelles. However, they would never be able to discern something as small as a normal-sized virus or single proteins.



Stefan Hell

**Max Planck Institute for
Biophysical Chemistry,
Göttingen,**

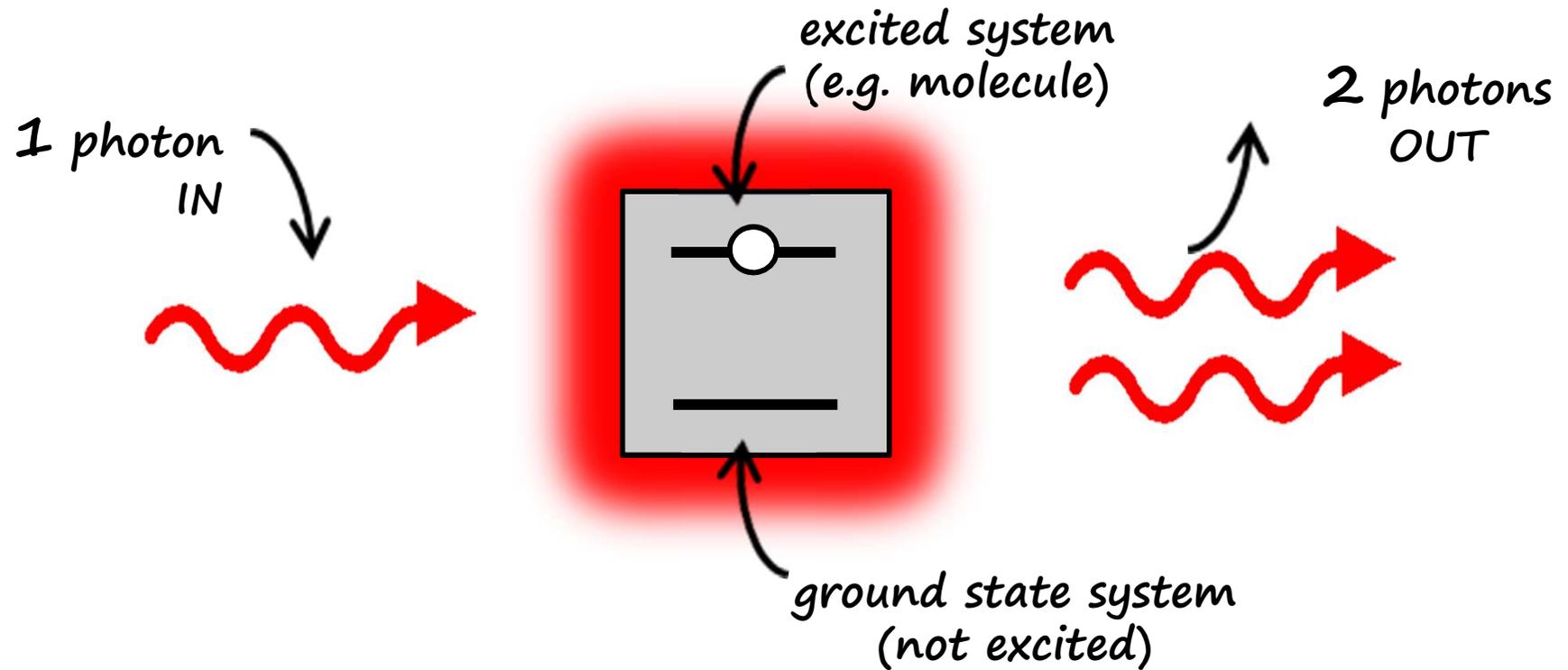
The Nobel Prize in Chemistry 2014 Eric Betzig, Stefan W. Hell, William E. Moerner

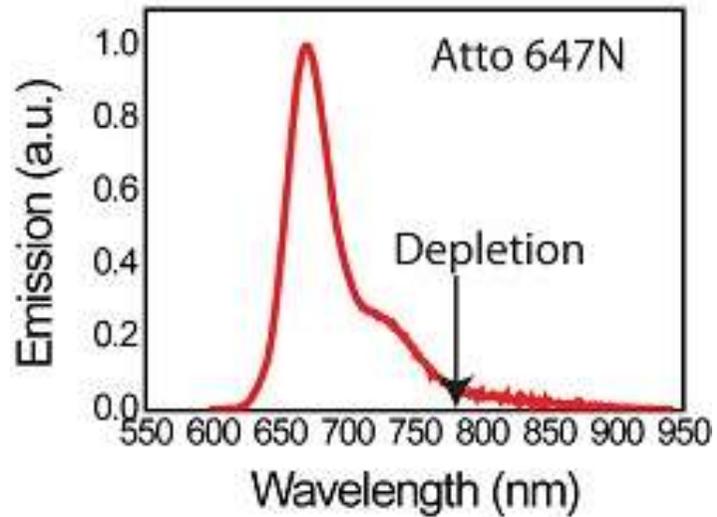
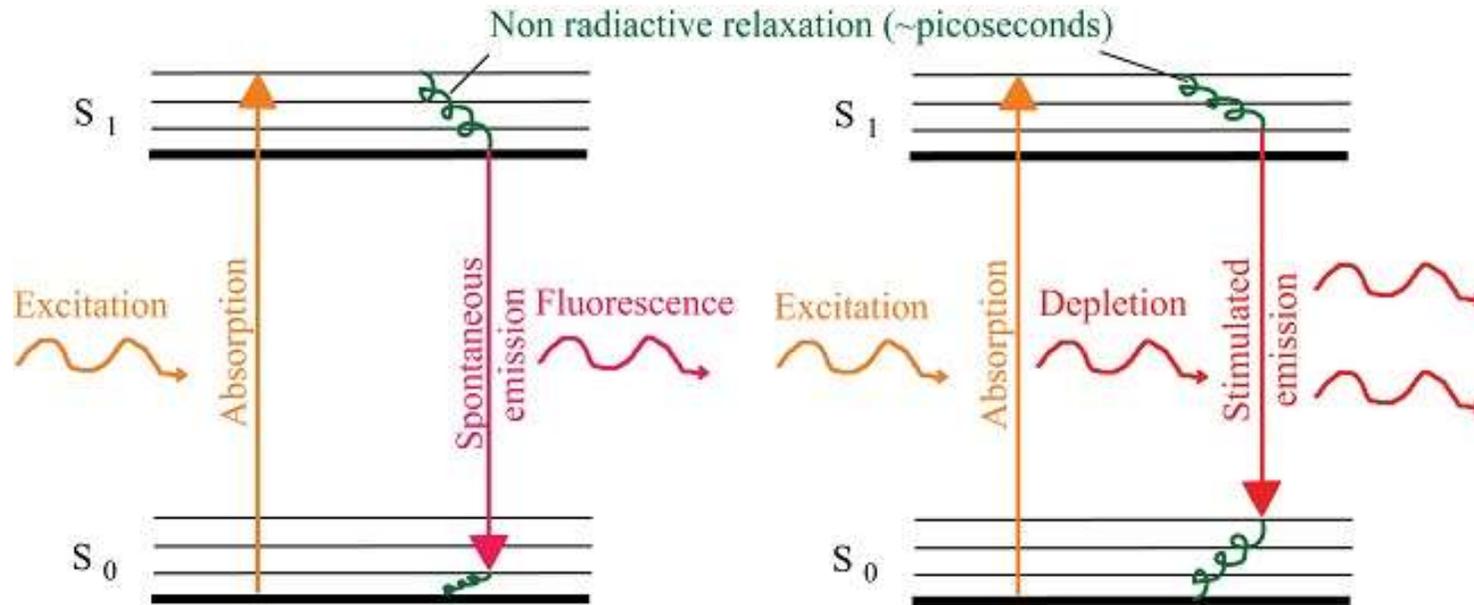


**"for the development of super-
resolved fluorescence
microscopy"**

**MICROSCOPIA STED
(Stimulated Emission Depletion)**

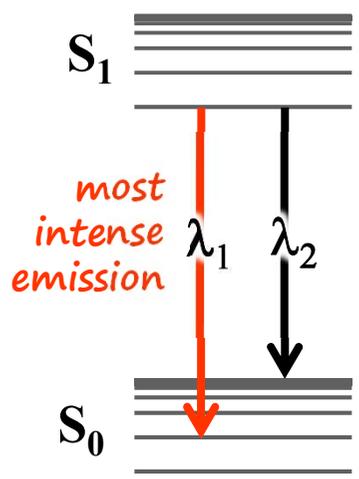
STIMULATED EMISSION





The key issue is that we can precisely control the wavelength of stimulated emission output, which can be significantly different from the typical wavelength of fluorescence.

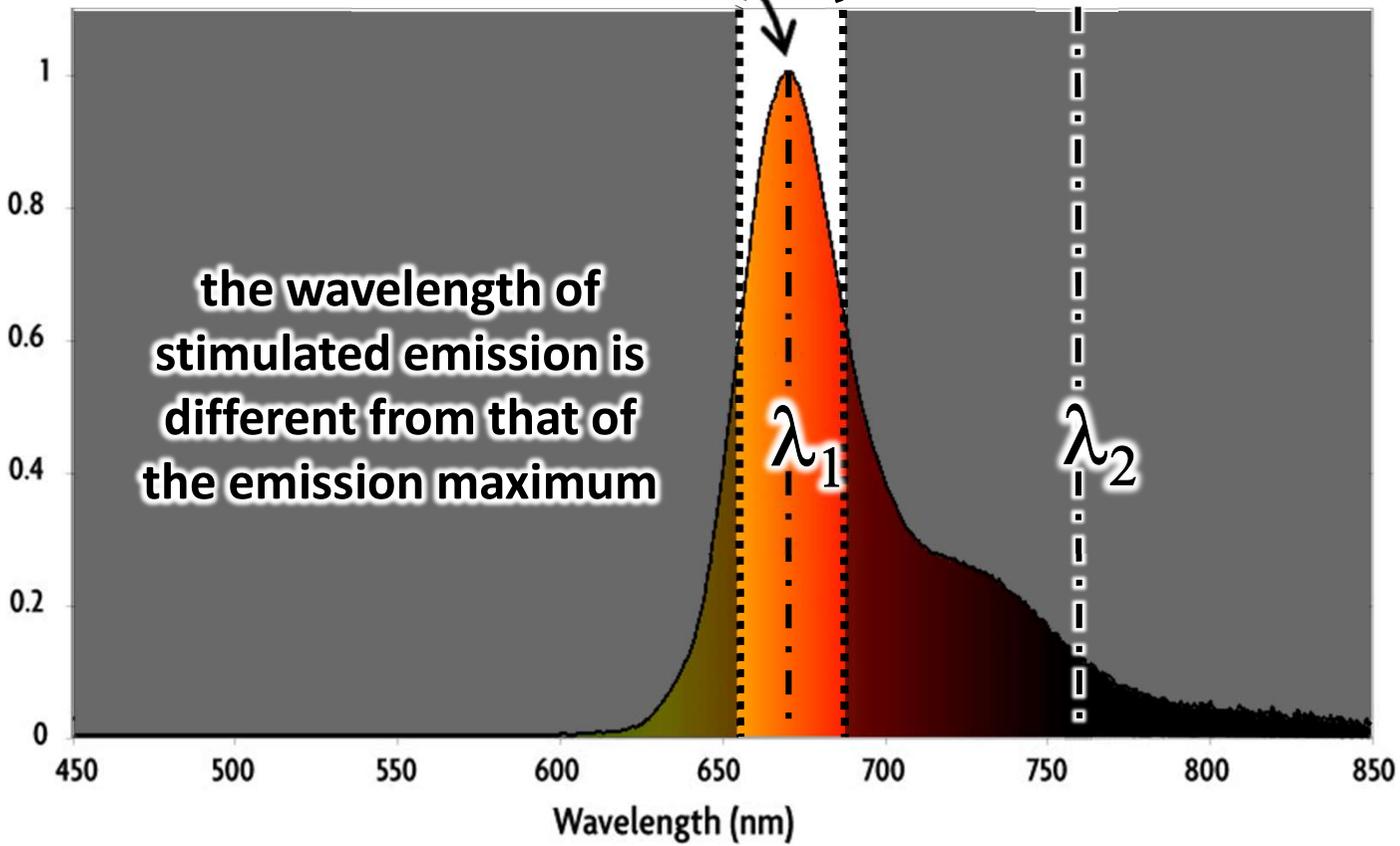
stimulated emission is NOT observed (depleted)



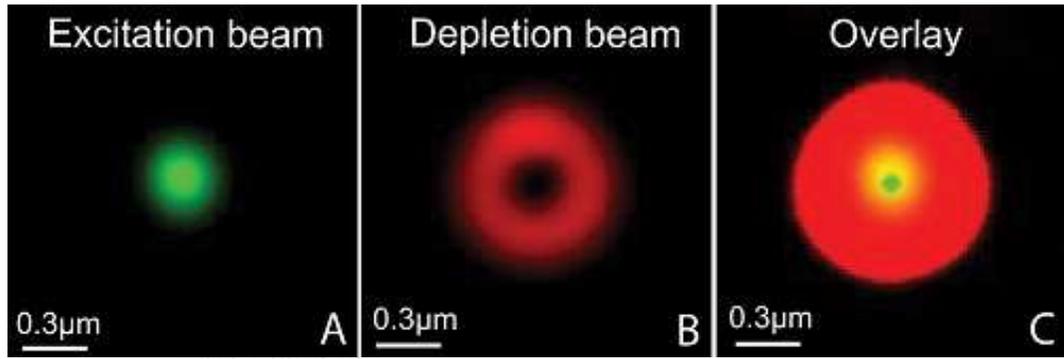
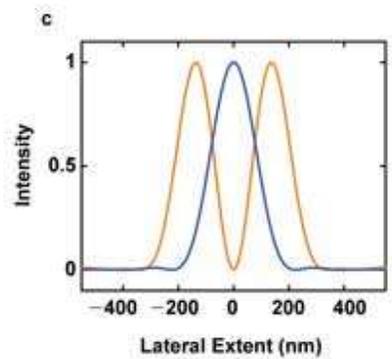
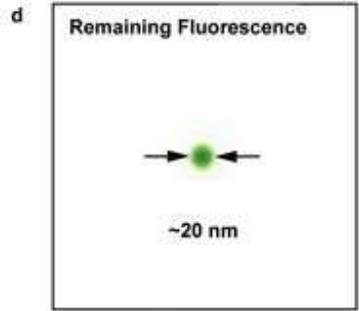
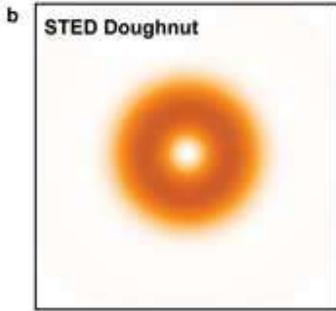
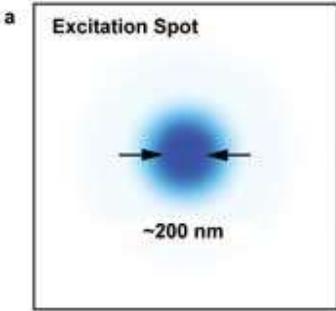
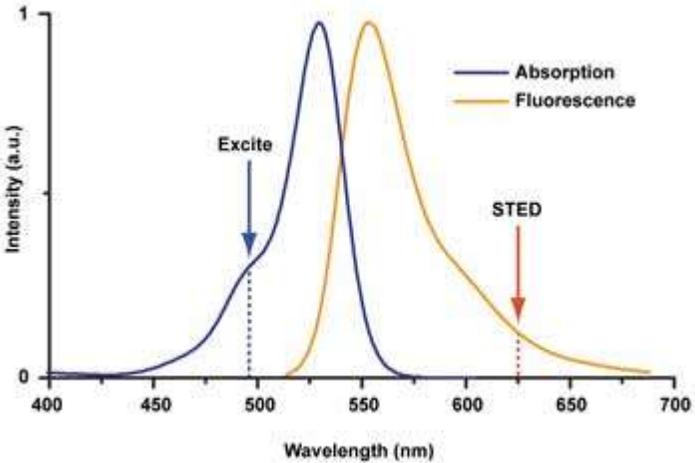
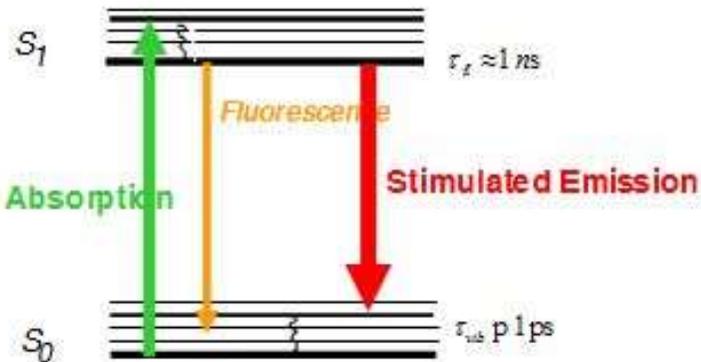
stimulated emission

most intense emission

stimulated emission



MICROSCOPIA STED (Stimulated Emission Depletion)



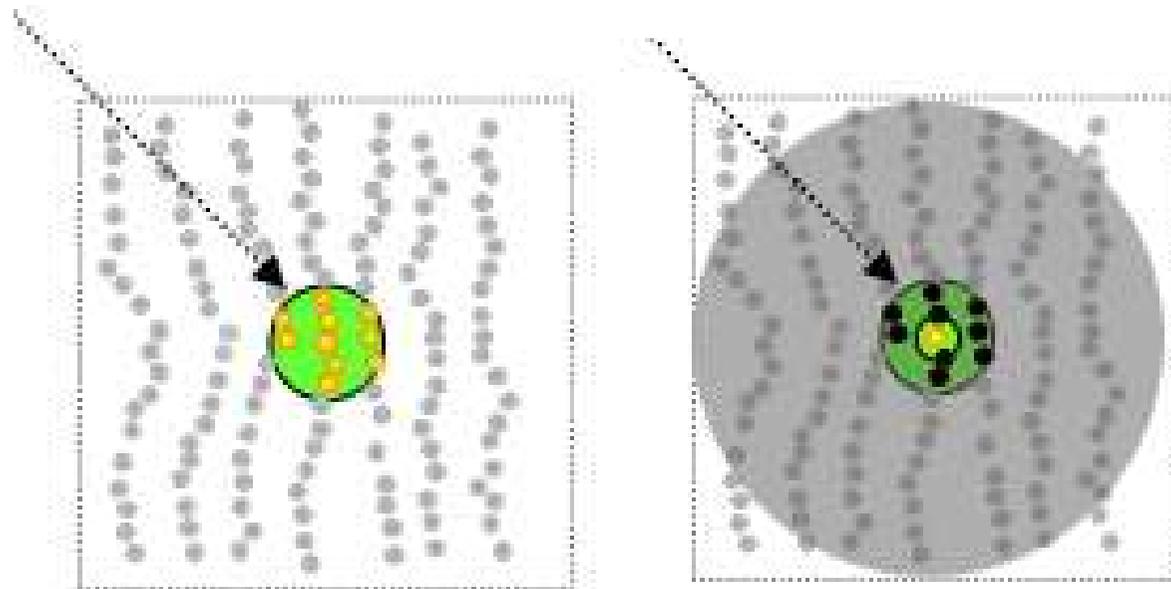
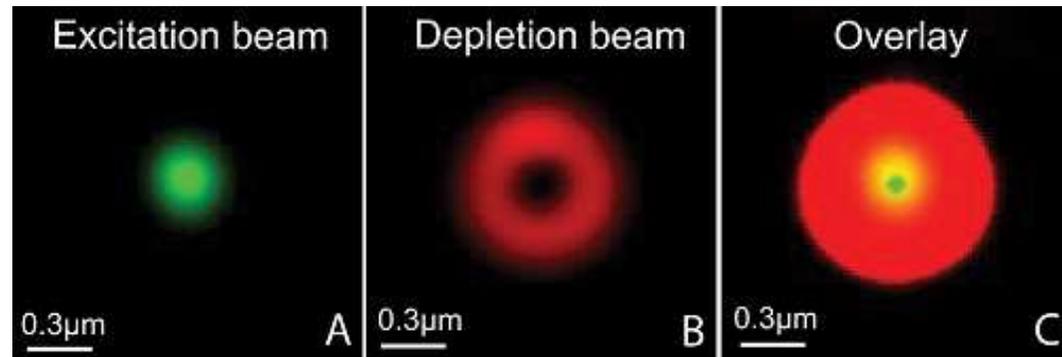
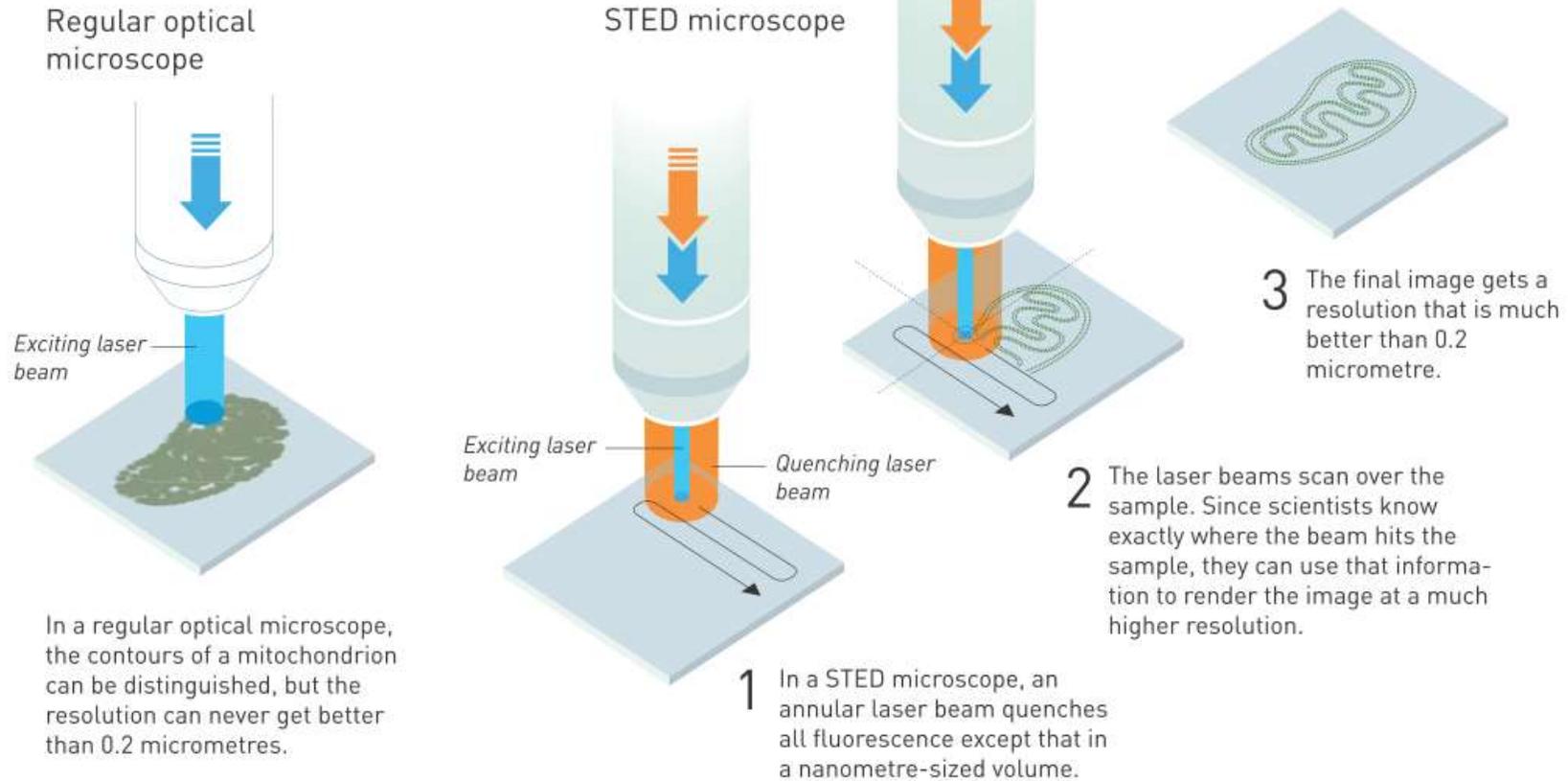


Figure 2

The principle of STED microscopy



Regular optical microscope

Exciting laser beam

In a regular optical microscope, the contours of a mitochondrion can be distinguished, but the resolution can never get better than 0.2 micrometres.

STED microscope

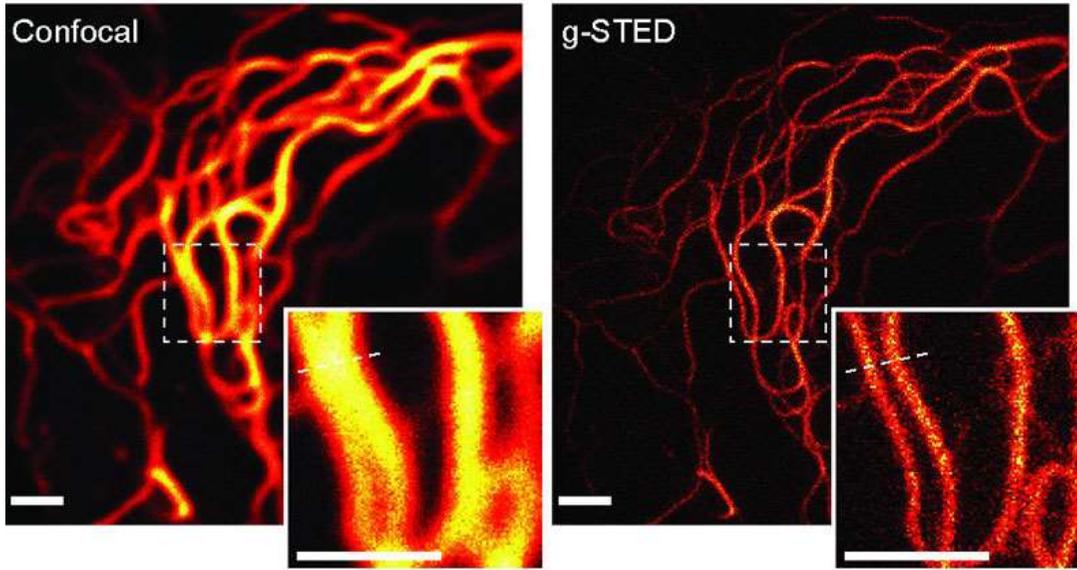
Exciting laser beam

Quenching laser beam

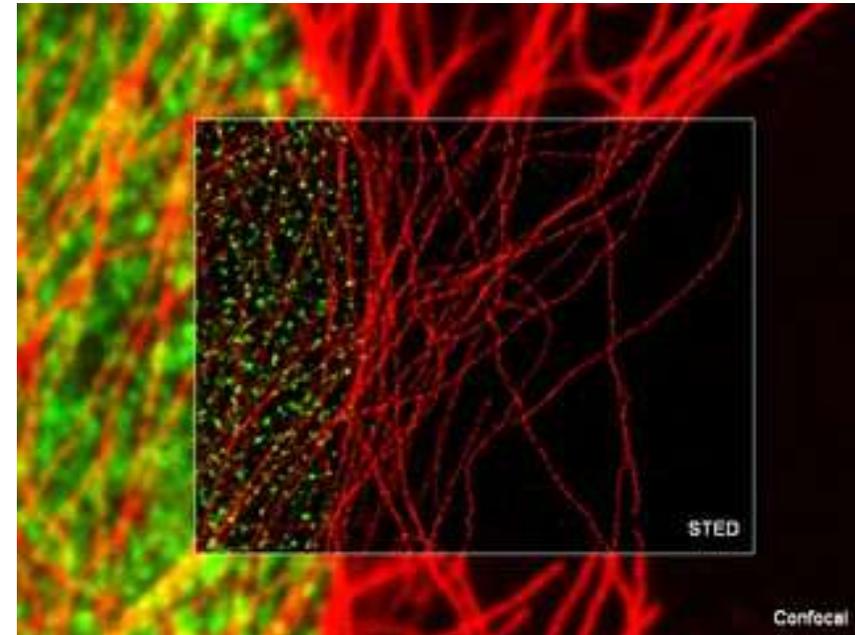
1 In a STED microscope, an annular laser beam quenches all fluorescence except that in a nanometre-sized volume.

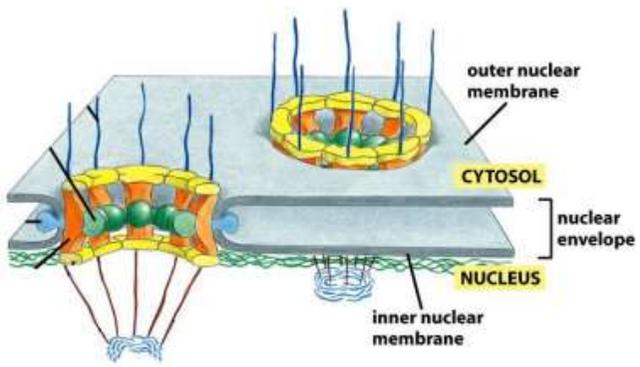
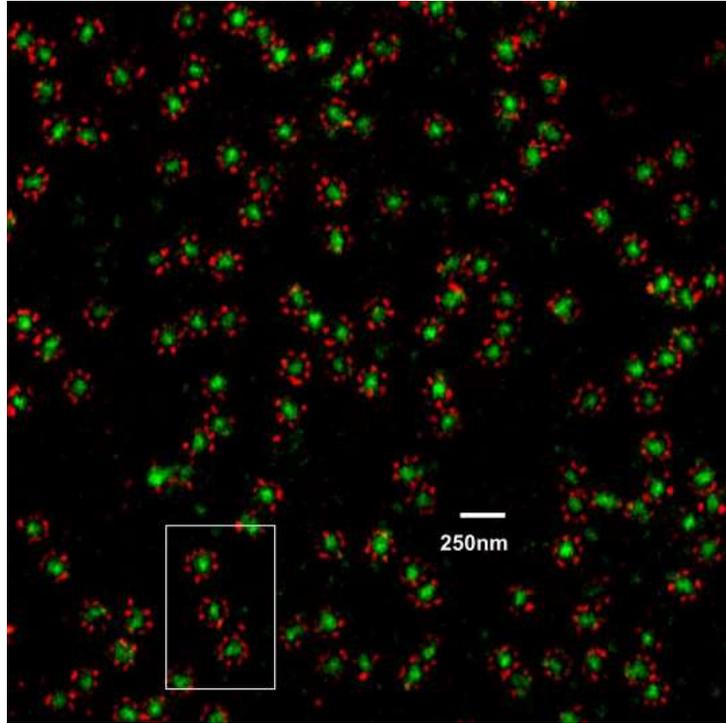
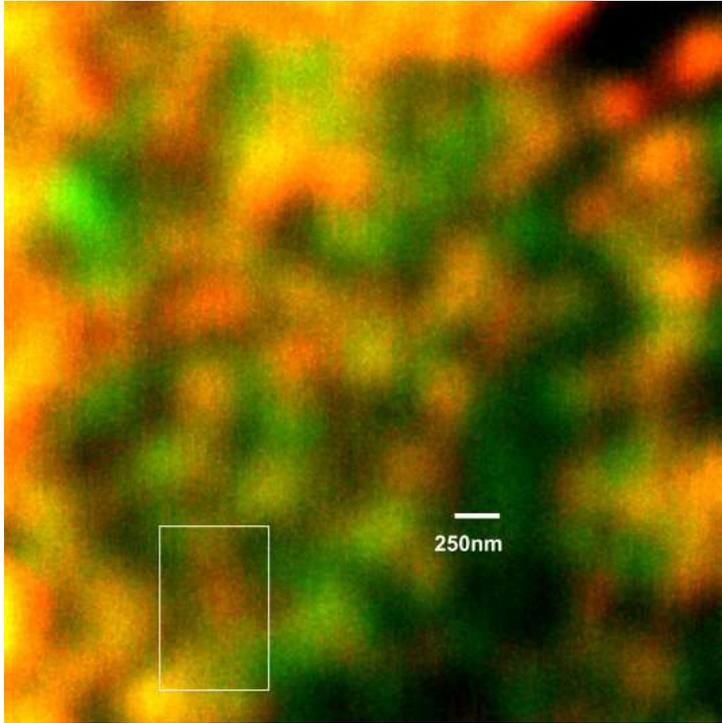
2 The laser beams scan over the sample. Since scientists know exactly where the beam hits the sample, they can use that information to render the image at a much higher resolution.

3 The final image gets a resolution that is much better than 0.2 micrometre.

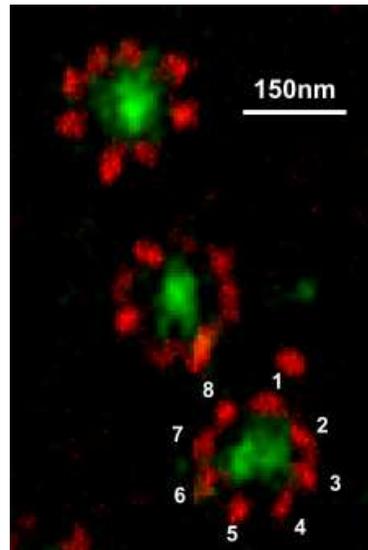


**Fluorophore-labeled keratin filaments
in a living cell, bar = 1 micron**





Nuclear pore complex



Super-resolution Fluorescence Imaging for Materials Science

Dominik Wöll* and Cristina Flors*

Small Methods 2017, 1, 1700191

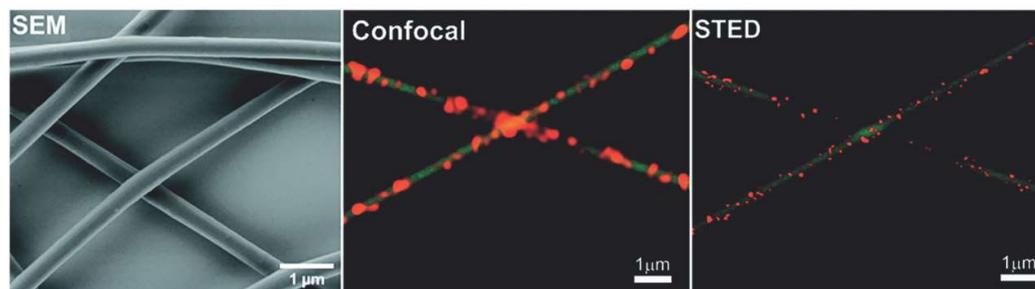


Figure 4. SEM, confocal microscopy and STED microscopy images of electrospun nanofibers labeled with fluorescent nanoparticles. Reproduced with permission.^[55a] Copyright 2011, American Chemical Society.

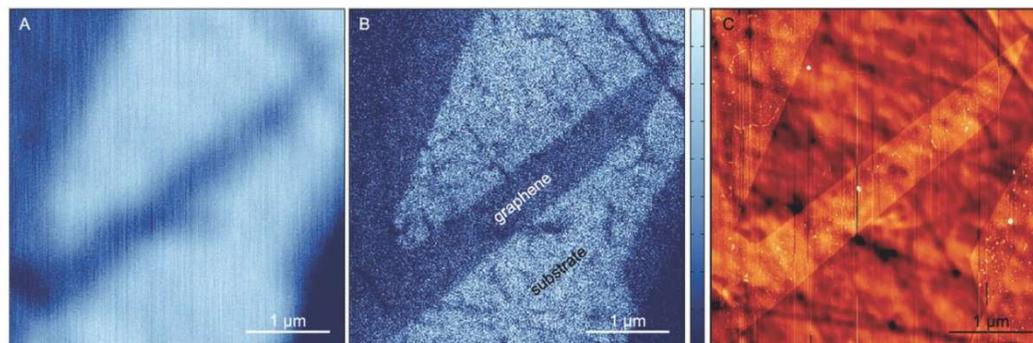


Figure 6. Super-resolution imaging of graphene by "FQM + STED". A) Diffraction-limited FQM image. B) With an additional 532 nm donut-shaped depletion beam, super-resolution imaging of graphene is obtained. C) AFM image of the same region. Reproduced with permission.^[80] Copyright 2012, American Chemical Society.