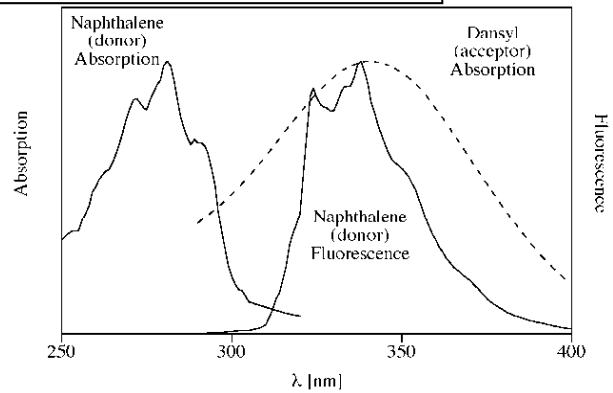
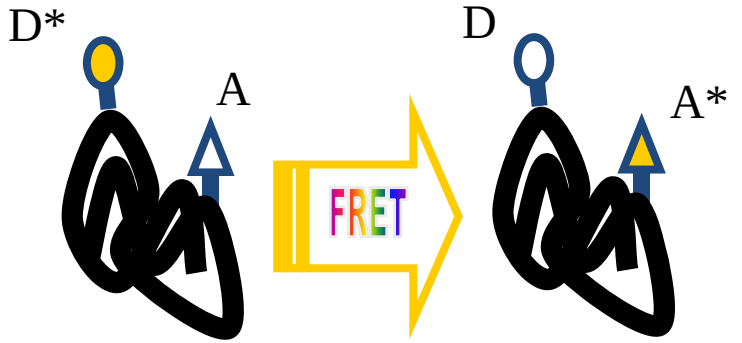
Trasferimento di energia in risonanza alla Förster (FRET)

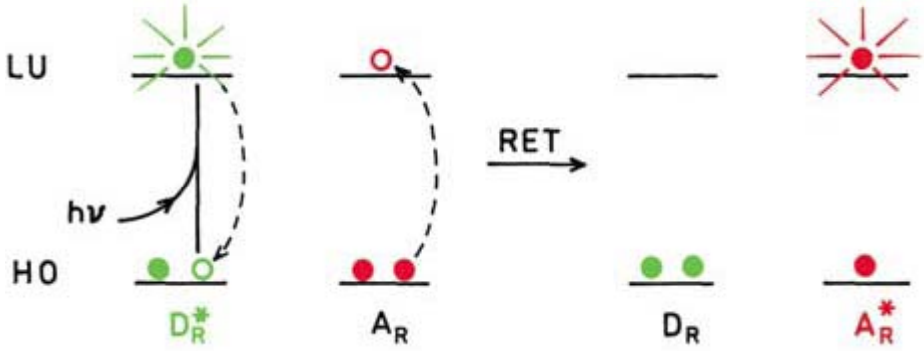
FRET: introduzione

È una tecnica per misurare distanze inter- o intra-molecolari nell'intervallo 1-10 nm

- I due punti di interesse devono essere marcati con due cromofori diversi: il "donatore" (che deve essere fluorescente) e "l'accettore".
- Lo spettro di emissione del donatore deve sovrapporsi (almeno parzialmente) allo spettro di assorbimento dell'accettore.
- In queste condizioni un donatore eccitato, anziché emettere fluorescenza, può trasferire la propria energia di eccitazione all'accettore, attraverso un fenomeno di risonanza.
- La probabilità di questo fenomeno dipende dalla distanza ed orientazione tra le due sonde, che quindi possono essere misurate.







FRET: vantaggi

(rispetto ad altre tecniche strutturali, più potenti, come la cristallografia a raggi X o l'NMR)

- Strumentazione semplice ed economica.
- Elevata sensibilità (è richiesto poco campione).
- Esperimenti in soluzione, in condizioni fisiologiche.
- Possibilità di effettuare misure in vivo.
- Ampio intervallo di distanze misurabili.
- Risoluzione temporale nell'ordine dei nanosecondi.

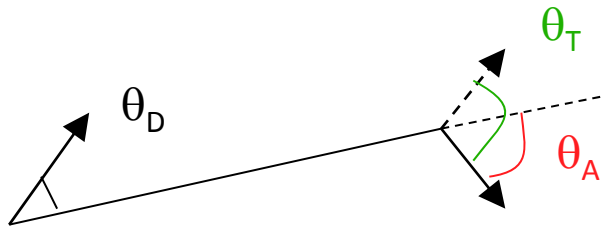
FRET: teoria

- Teoria perturbativa di ordine 0
- Funzione d'onda imperturbate
- Potenziale d'interazione dipolo-dipolo

$$V = \frac{\vec{\mu_D} \cdot \vec{\mu_A}}{R^3} - 3\frac{\left(\vec{\mu_D} \cdot \vec{R}\right)\left(\vec{\mu_A} \cdot \vec{R}\right)}{R^5} =$$

$$= k\frac{\mu_D \mu_A}{R^3}$$

$$k = \cos\theta_T - 3\cos\theta_D \cos\theta_A$$



$$k_{FRET} \propto \left|\left\langle \psi_D \psi_{A^*} \middle| V \middle| \psi_{D^*} \psi_A \right\rangle\right|^2 = \frac{k^2}{R^6}\left|\left\langle \psi_D \psi_{A^*} \middle| \mu_D \mu_A \middle| \psi_{D^*} \psi_A \right\rangle\right|^2 =$$

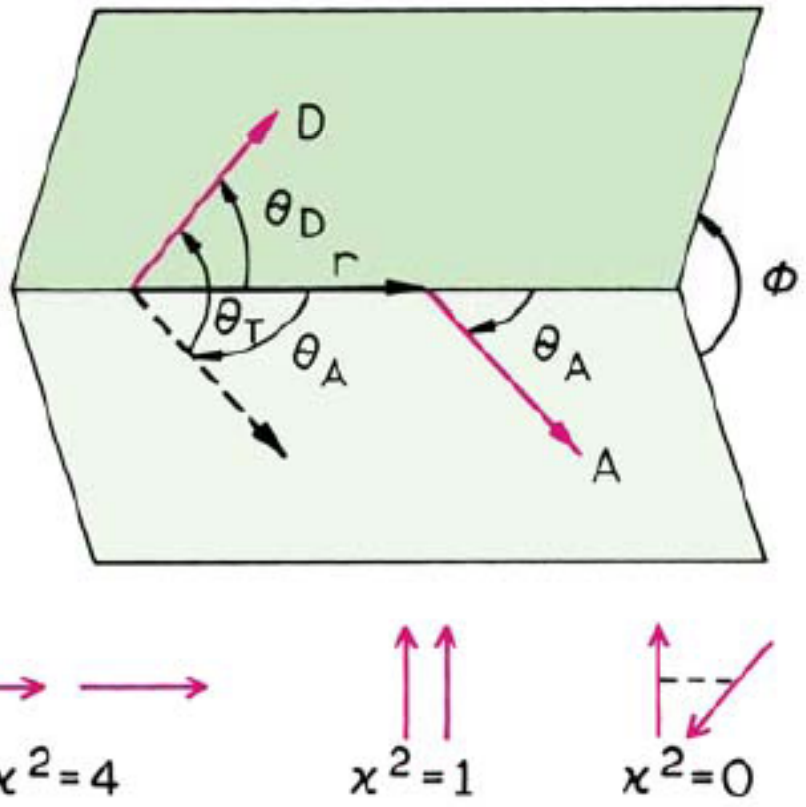
$$= \frac{k^2}{R^6}\left|\left\langle \psi_D \middle| \mu_D \middle| \psi_{D^*} \right\rangle\right|^2 \left|\left\langle \psi_{A^*} \middle| \mu_A \middle| \psi_A \right\rangle\right|^2 \propto \frac{k^2}{R^6}\frac{k_r^D}{\nu^3}\frac{\varepsilon^A}{\nu} = \frac{k^2}{R^3}\frac{\phi_0^D}{\tau_0^D \nu^3}\frac{\varepsilon^A}{\nu}$$

FRET: teoria

$$k_{FRET} = \frac{1}{\tau_D^0}\left(\frac{R_0^{\,6}}{R^6}\right)\left(\frac{3}{2}k^2\right)$$

$$R_0^{\,6} = \frac{2}{3}\alpha\, n^{-4}\, \Phi_D^0\, J$$

$$J = \frac{\int_0^\infty f_D(\nu)\, \varepsilon_A(\nu)\, \nu^{-4} d\nu}{\int_0^\infty f_D(\nu)\, d\nu}$$



$$x^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$

$$x^2 = (\sin\theta_D \sin\theta_A \cos\Phi - 2\cos\theta_D \cos\theta_A)^2$$

Figure 13.5. Dependence of the orientation factor κ^2 on the direction of the emission dipole of the donor and the absorption dipole of the acceptor.

Per rotazione libera di donatore ed accettore durante il tempo di vita del donatore:

$$\left\langle k^2 \right\rangle = \frac{2}{3} \quad \text{media dinamica}$$

Per fluorofori orientati casualmente, ma immobili durante il tempo di vita (matrice solida o viscosa):

$$\left\langle k^2 \right\rangle = 0.476 \quad \text{media statica}$$

$$\sqrt[6]{2/3} = 0.93 \qquad \sqrt[6]{0.476} = 0.87$$

FRET: efficienza

E= probabilità che un donatore eccitato trasferisca la sua energia.

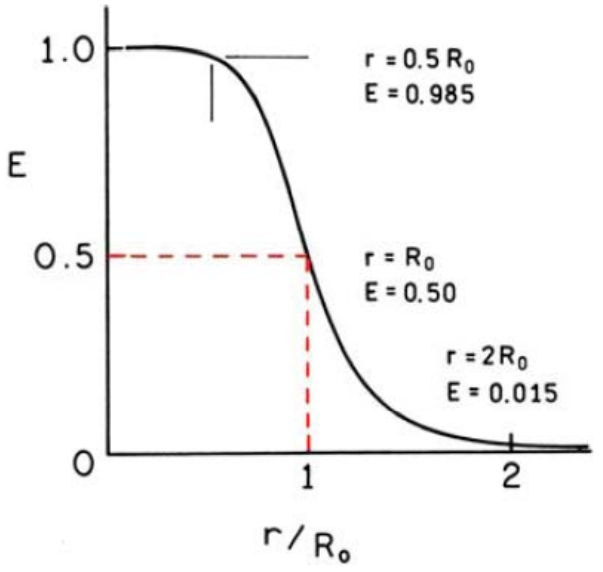
$$E = \frac{k_{FRET}}{k_r^D + k_{nr}^D + k_{FRET}}$$

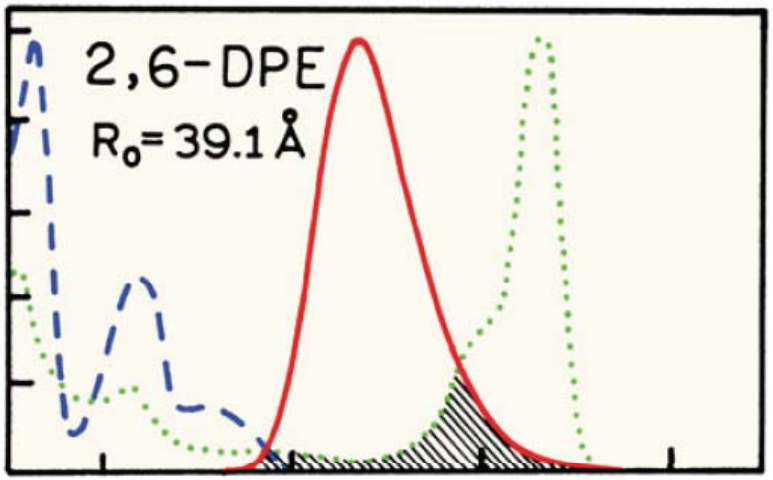
$$E = \frac{1}{1 + \frac{k_r^D + k_{nr}^D}{k_{FRET}}} = \frac{1}{1 + \frac{1}{\tau_0^D k_{FRET}}} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6 \left(\frac{3}{2}k^2\right)^{-1}}$$

- In generale, l'efficienza dipende dall'orientazione.
  - Se i cromofori ruotano liberamente sulla scala dei tempi della fluorescenza, allora $k^2=2/3$.

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

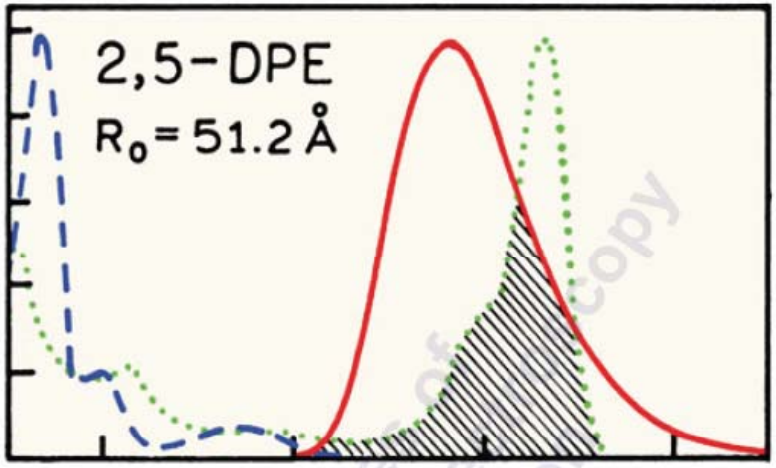
- L'efficienza dipende dalla sesta potenza della distanza
  - Elevata sensibilità
  - Si misurano bene solo distanze confrontabili con R_0 (da $R_0/2$a $2R_0$).
- Si può avere un R_0 ottimale scegliendo opportunamente i cromofori (ossia la loro sovrapposizione spettrale).



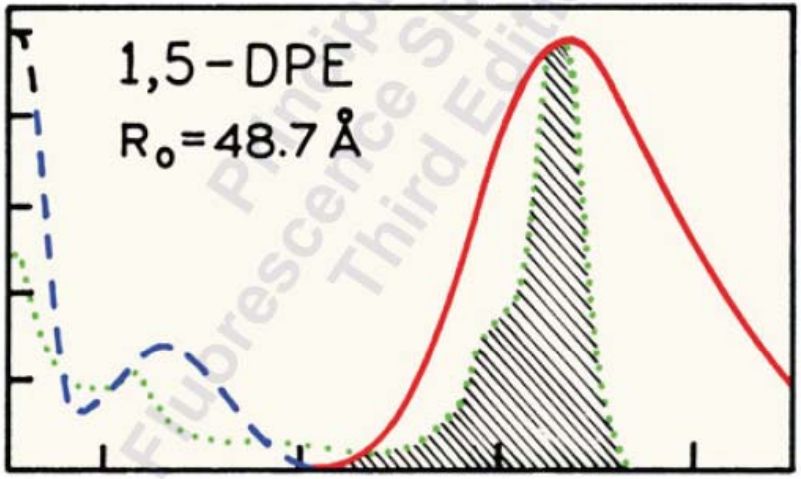


Red = donor emission

Dotted green = acceptor absorption

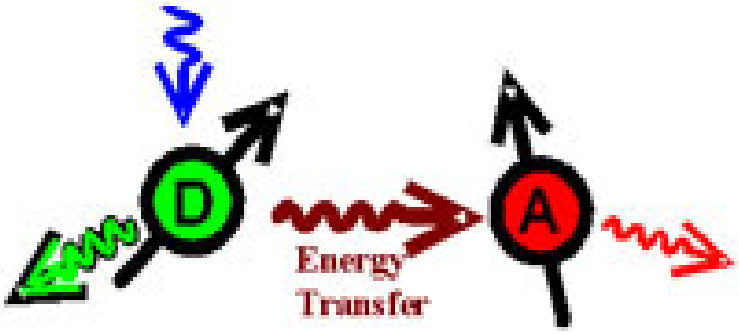


Dansyl-labeled phospatidylethanolamine

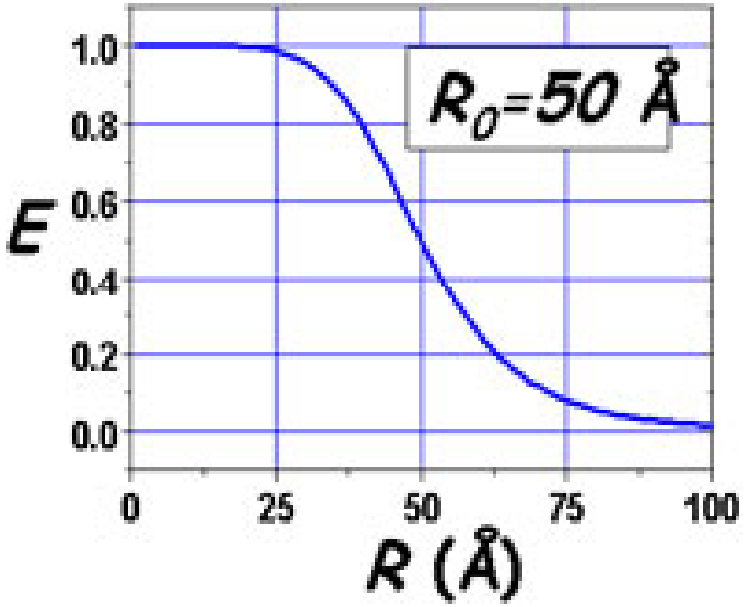


Donor	***Acceptor***	***R_0(Å)***
Naphthalene	Dansyl	22
Pyrene	Coumarin	39
Terbium	Rhodamine	65
Tryptophan	Dansyl	21-24
Tryptophan	Heme	29
Tryptophan	Pyrene	28
Bodipy	Bodipy	57
Tryptophan	DPH	40
Tryptophan	Tyrosine-NO_2	26

A spectroscopic ruler



DONOR ACCEPTOR

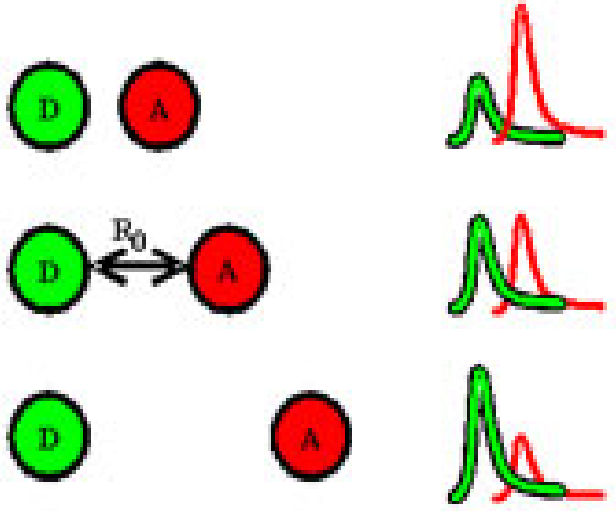


Energy Transfer Efficiency

$$E = \frac{1}{1+(R/R_0)^6}$$

R_0=50% transfer efficiency distance
3nm~7nm

"Spectroscopic Ruler"



FRET: misura dell'efficienza

$$E = \frac{k_{FRET}}{k_r^D + k_{nr}^D + k_{FRET}}$$

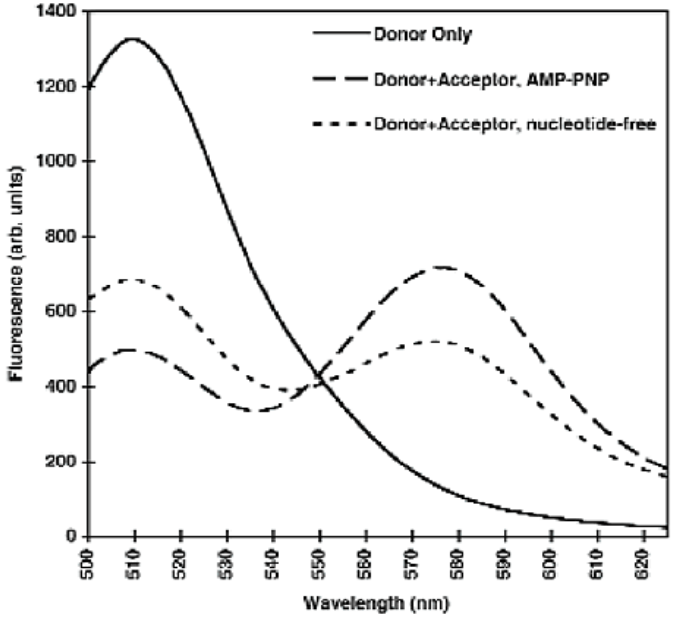
L'efficienza si può misurare dalla diminuzione della fluorescenza del donatore

$$\Phi_0^D = \frac{k_r^D}{k_r^D + k_{nr}^D} \qquad \Phi_A^D = \frac{k_r^D}{k_r^D + k_{nr}^D + k_{FRET}}$$

$$\frac{1}{\Phi_A^D} = \frac{k_r^D + k_{nr}^D + k_{FRET}}{k_r^D} \qquad \frac{1}{\Phi_A^D} - \frac{1}{\Phi_0^D} = \frac{k_r^D + k_{nr}^D + k_{FRET}}{k_r^D} - \frac{k_r^D + k_{nr}^D}{k_r^D} = \frac{k_{FRET}}{k_r^D}$$

$$E = \frac{\frac{1}{\Phi_A^D} - \frac{1}{\Phi_0^D}}{\frac{1}{\Phi_A^D}} = 1 - \frac{\Phi_A^D}{\Phi_0^D}$$

$$E = 1 - \frac{F_A^D}{F_0^D}$$



Oppure dalla diminuzione del tempo di vita del donatore

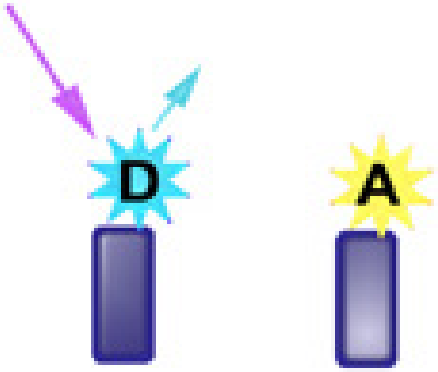
$$E_{ET} = 1 - \frac{\tau}{\tau_D^0}$$

$$\tau_D = \frac{1}{k_r + k_{nr} + k_{ET}} \qquad \tau_0^D = \frac{1}{k_r + k_{nr}}$$

$$1 - \frac{\tau}{\tau_D^0} = 1 - \frac{k_r + k_{nr}}{k_r + k_{nr} + k_{ET}} = \frac{k_{ET}}{k_r + k_{nr} + k_{ET}} = E_{ET}$$

Oppure dall'aumento della fluorescenza dell'accettore
(se è fluorescente!)

$$F_A(\lambda_A^{em}) \propto \varepsilon_A(\lambda^{ex})\phi_A$$

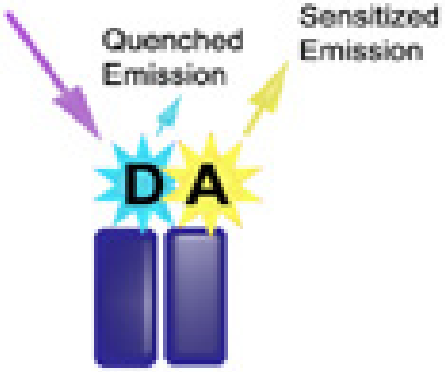


No Energy Transfer

$$F_{AD}(\lambda_A^{em}) \propto \varepsilon_A(\lambda_{ex})\phi_A + \varepsilon_D(\lambda_{ex})\phi_A E$$

$$\frac{F_{AD}(\lambda_A^{em})}{F_A(\lambda_A^{em})} = \frac{\varepsilon_A(\lambda_{ex})\phi_A + \varepsilon_D(\lambda_{ex})\phi_A E}{\varepsilon_A(\lambda_{ex})\phi_A} =$$

$$= 1 + \frac{\varepsilon_D(\lambda_{ex})E}{\varepsilon_A(\lambda_{ex})}$$



FRET - Energy Transfer

$$E = \frac{\varepsilon_A(\lambda_{ex})}{\varepsilon_D(\lambda_{ex})}\left[\frac{F_{AD}(\lambda_A^{em})}{F_A(\lambda_A^{em})} - 1\right]$$

FRET: applicazioni

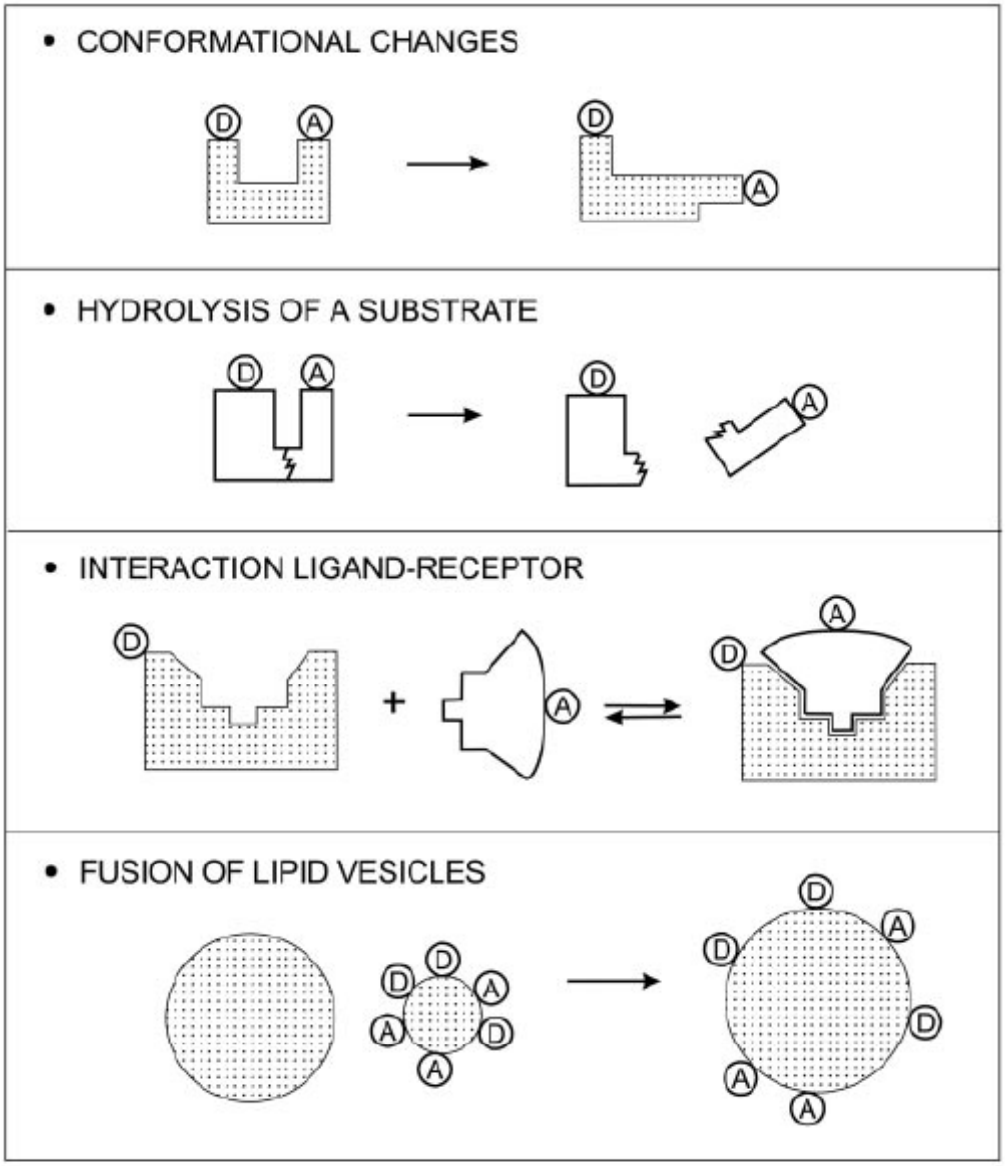
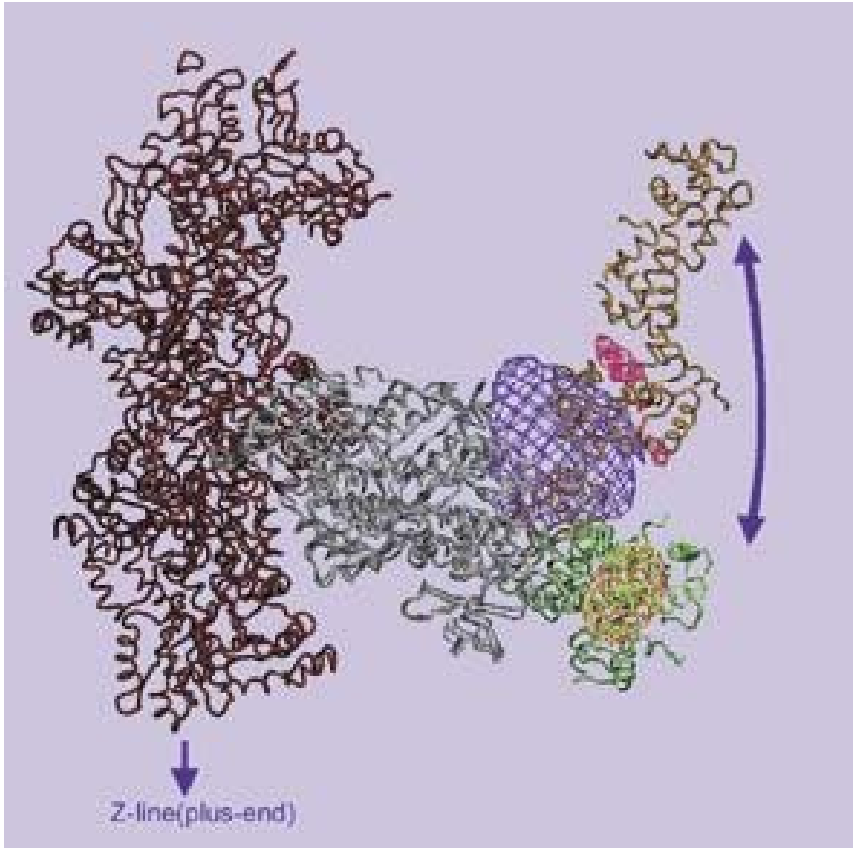
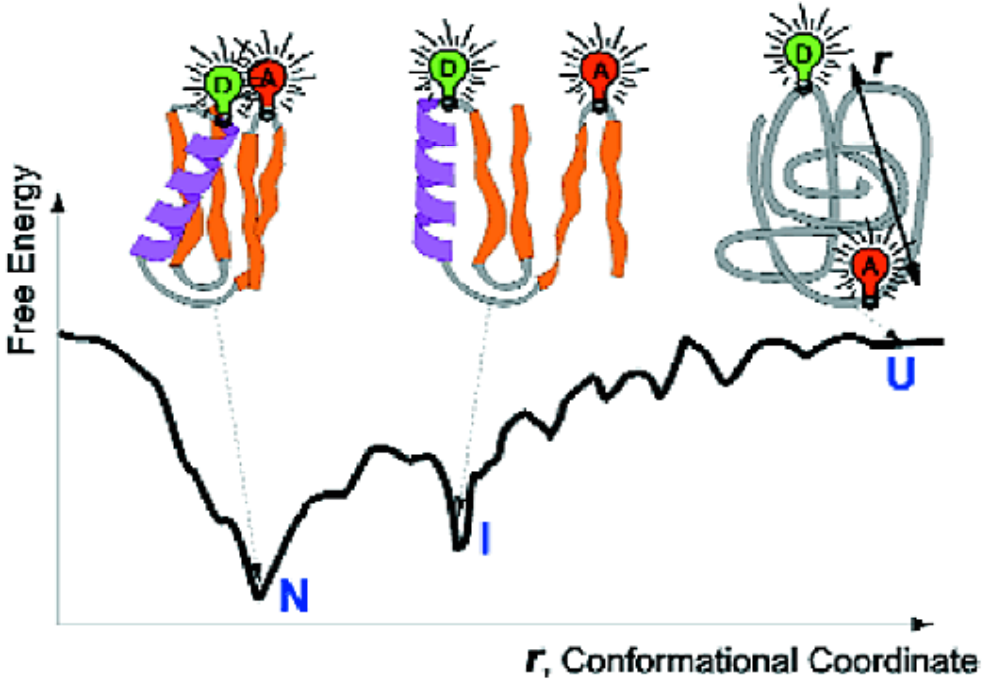


Fig. 9.5. Some applications of RET.

FRET: applicazioni

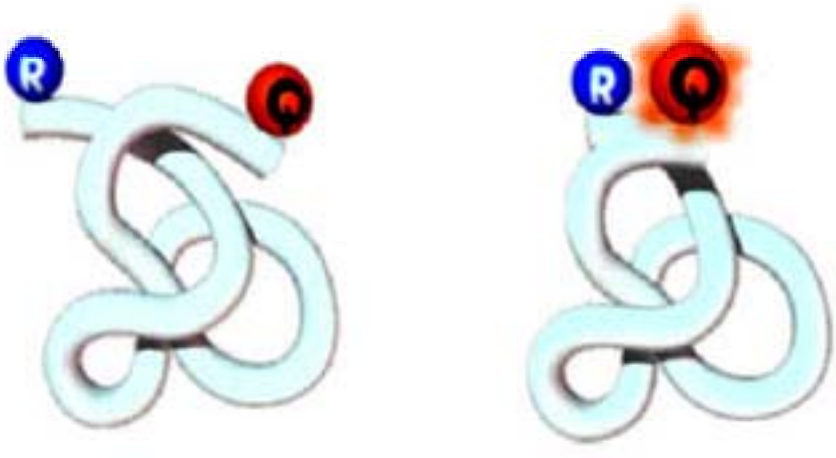


Struttura di macromolecole



Time-resolved energy transfer

Measuring conformational dynamics:



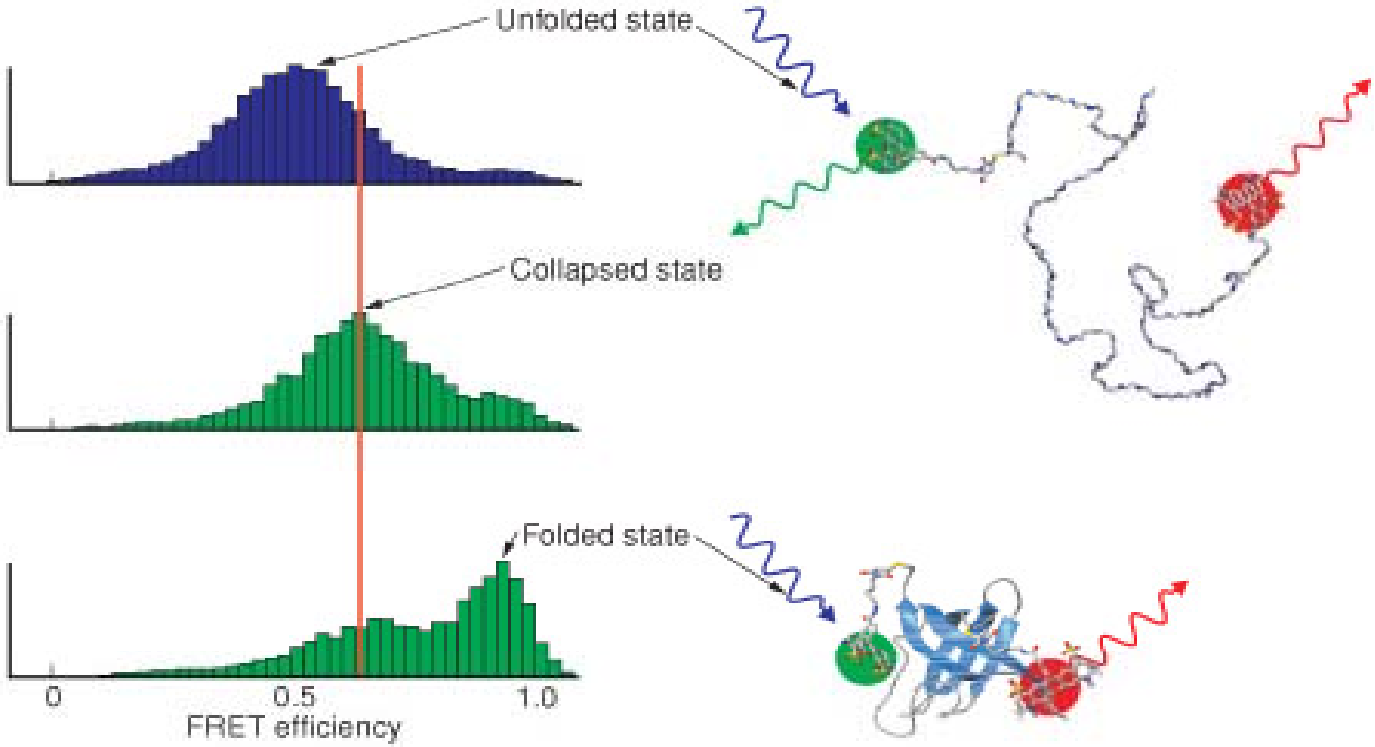
$$k_{ET} = \frac{1}{\tau_D^0}\left(\frac{R_0}{R}\right)^6$$

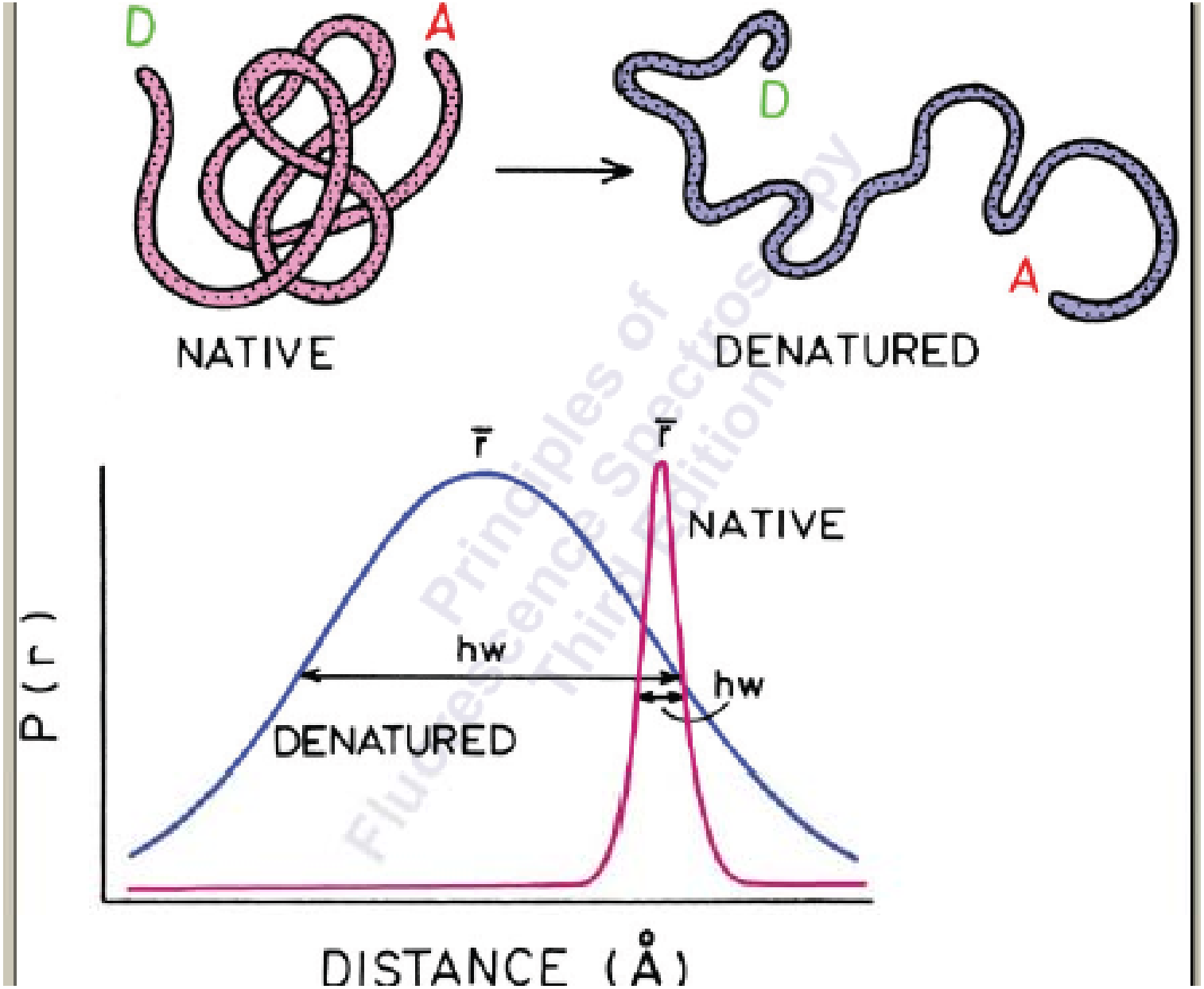
$$I_{DA}(t) = I_{DA}^0 e^{-(k_{ET} + \frac{1}{\tau_D})t}$$

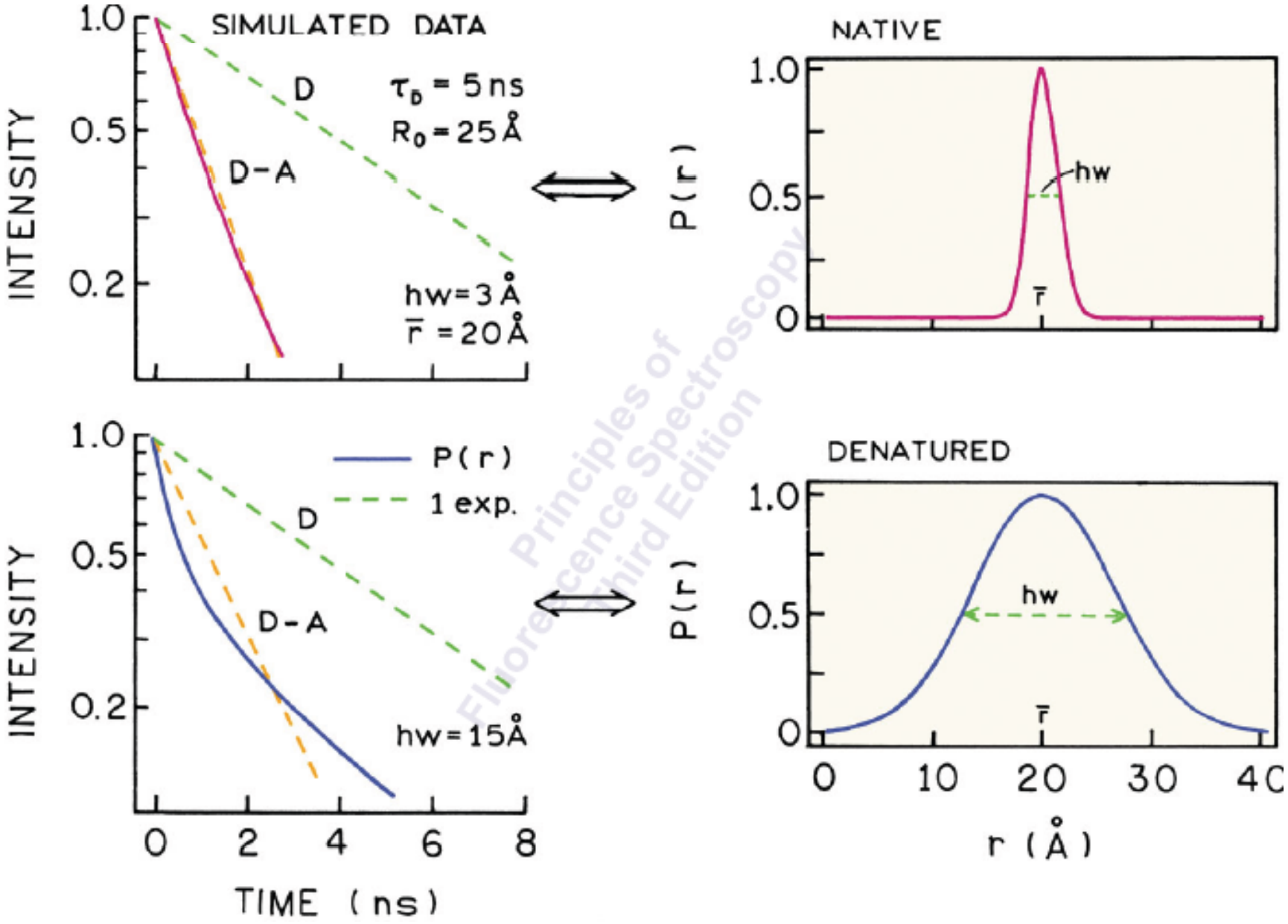
For a gaussian distribution of distances:

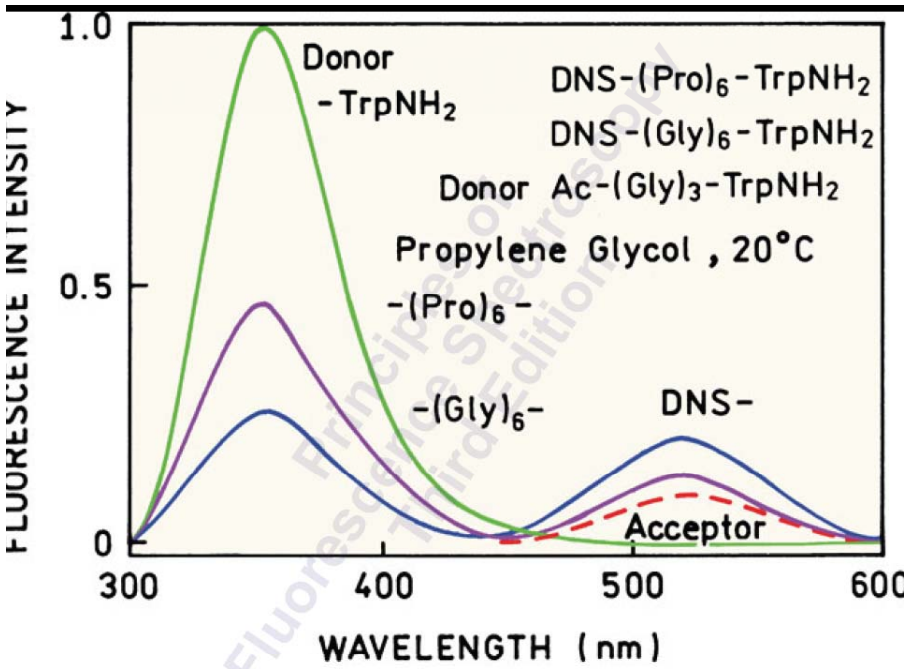
$$P(r) = \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{1}{2}\left(\frac{R-\overline{R}}{\sigma}\right)^2}$$

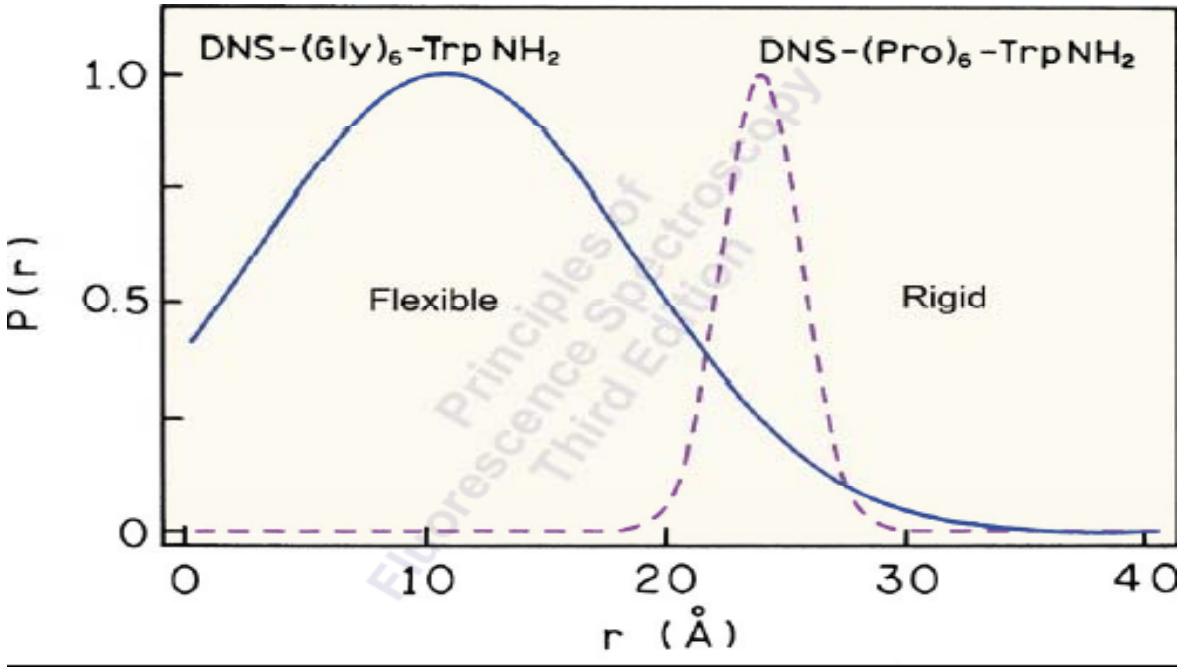
$$I_{DA}(t) = I^{0}_{DA} \int_{0}^{\infty} P(R)\, e^{-(k_{ET} + \frac{1}{\tau_D})t}\, dR$$

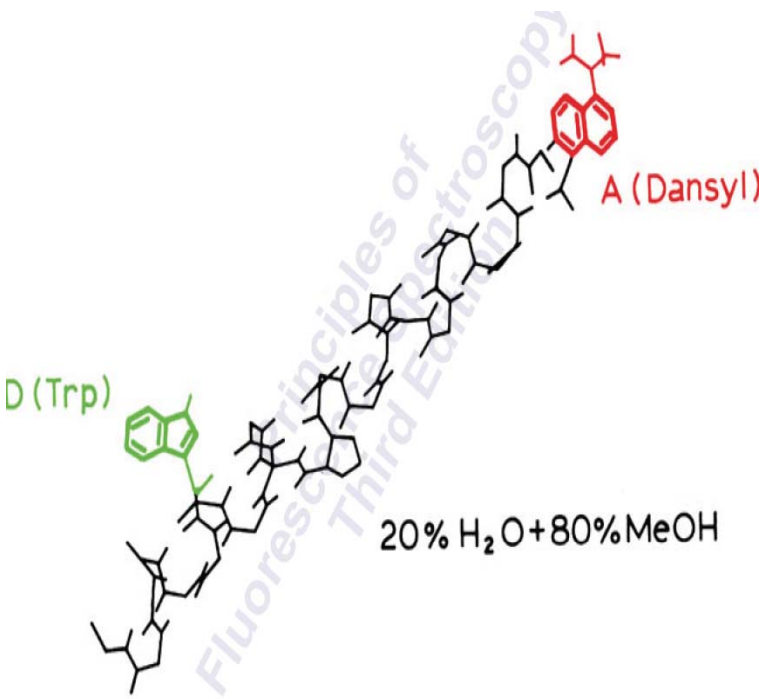


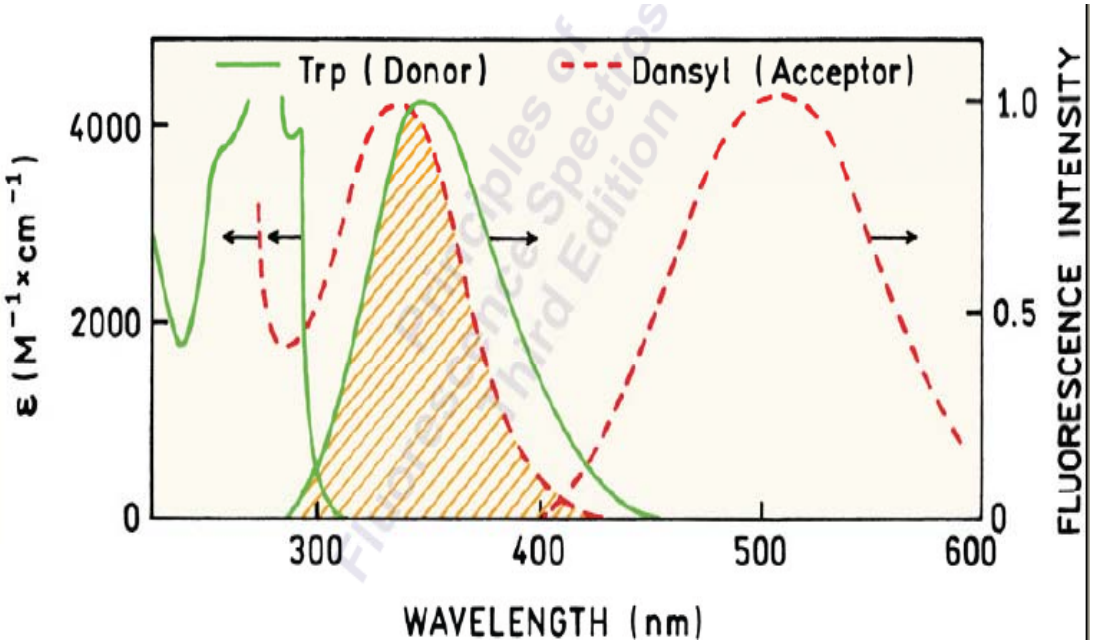

D
A
NATIVE
D
A
DENATURED
P(r)
r̄
r̄
NATIVE
hw
hw
DENATURED
DISTANCE (Å)


SIMULATED DATA
INTENSITY
1.0
0.5
0.2
D
D-A
τ_D = 5 ns
R_0 = 25 Å
hw = 3 Å
$\bar{r}$ = 20 Å
P(r)
NATIVE
hw
$\bar{r}$
0
P(r)
1 exp.
hw = 15 Å
TIME (ns)
0
2
4
6
8
DENATURED
10
20
30
40
r (Å)

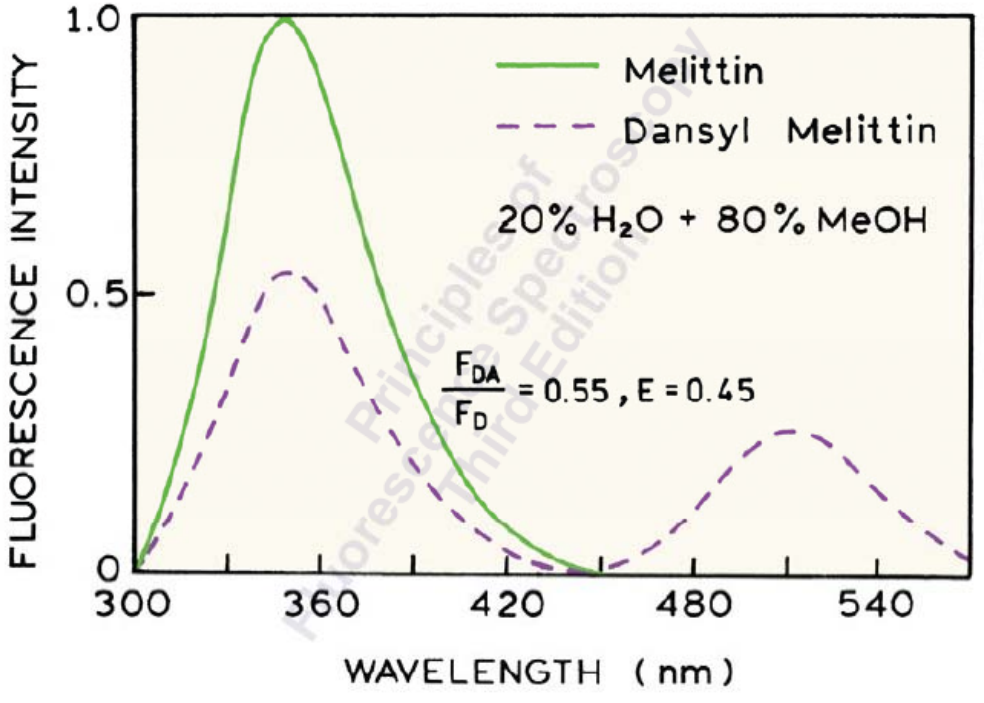

FLUORESCENCE INTENSITY
1.0
0.5
0
300
400
500
600
WAVELENGTH (nm)
Donor
-TrpNH2
DNS-(Pro)6-TrpNH2
DNS-(Gly)6-TrpNH2
Donor Ac-(Gly)3-TrpNH2
Propylene Glycol , 20°C
-(Pro)6-
-(Gly)6-
DNS-
Acceptor


DNS-(Gly)6-Trp NH2
DNS-(Pro)6-TrpNH2
P(r)
1.0
0.5
0
Flexible
Rigid
0
10
20
30
40
r (Å)

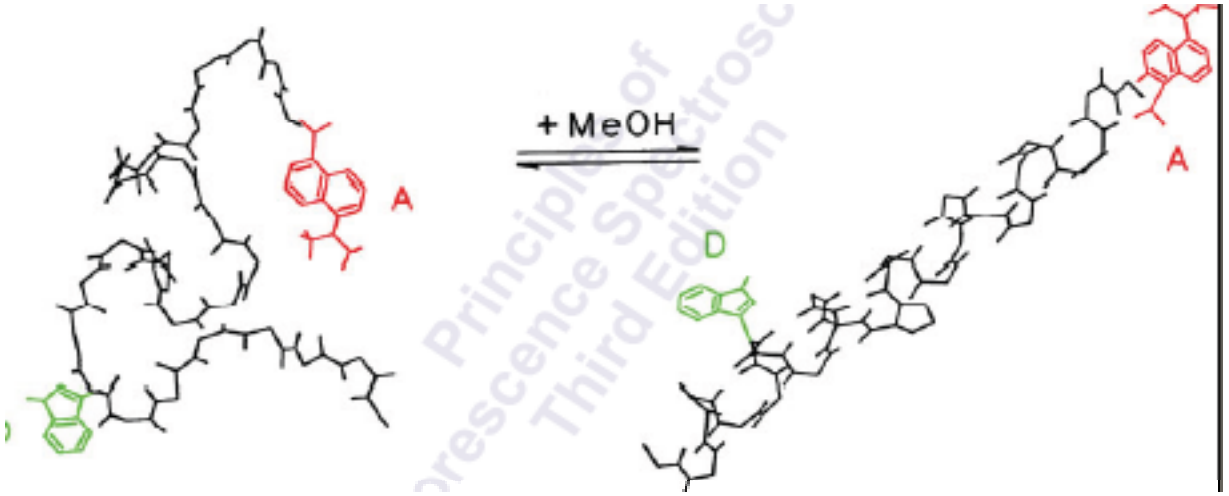




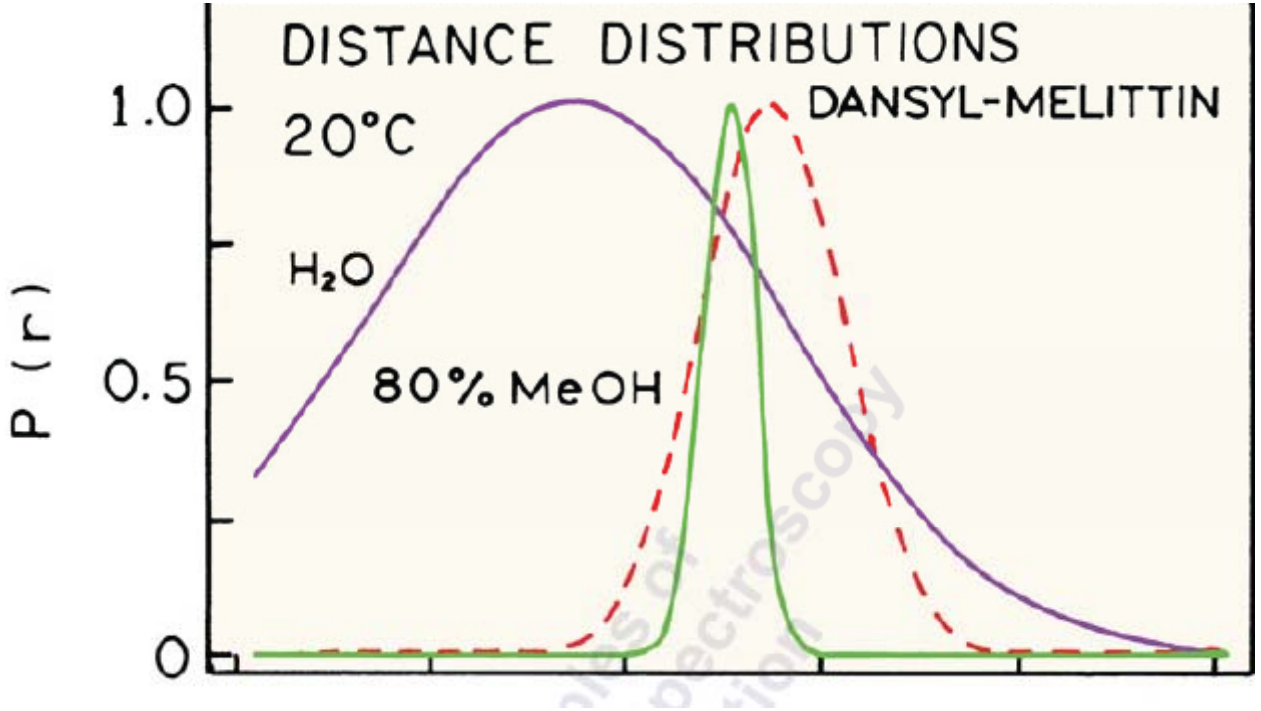
Dansyl-labeled melittin



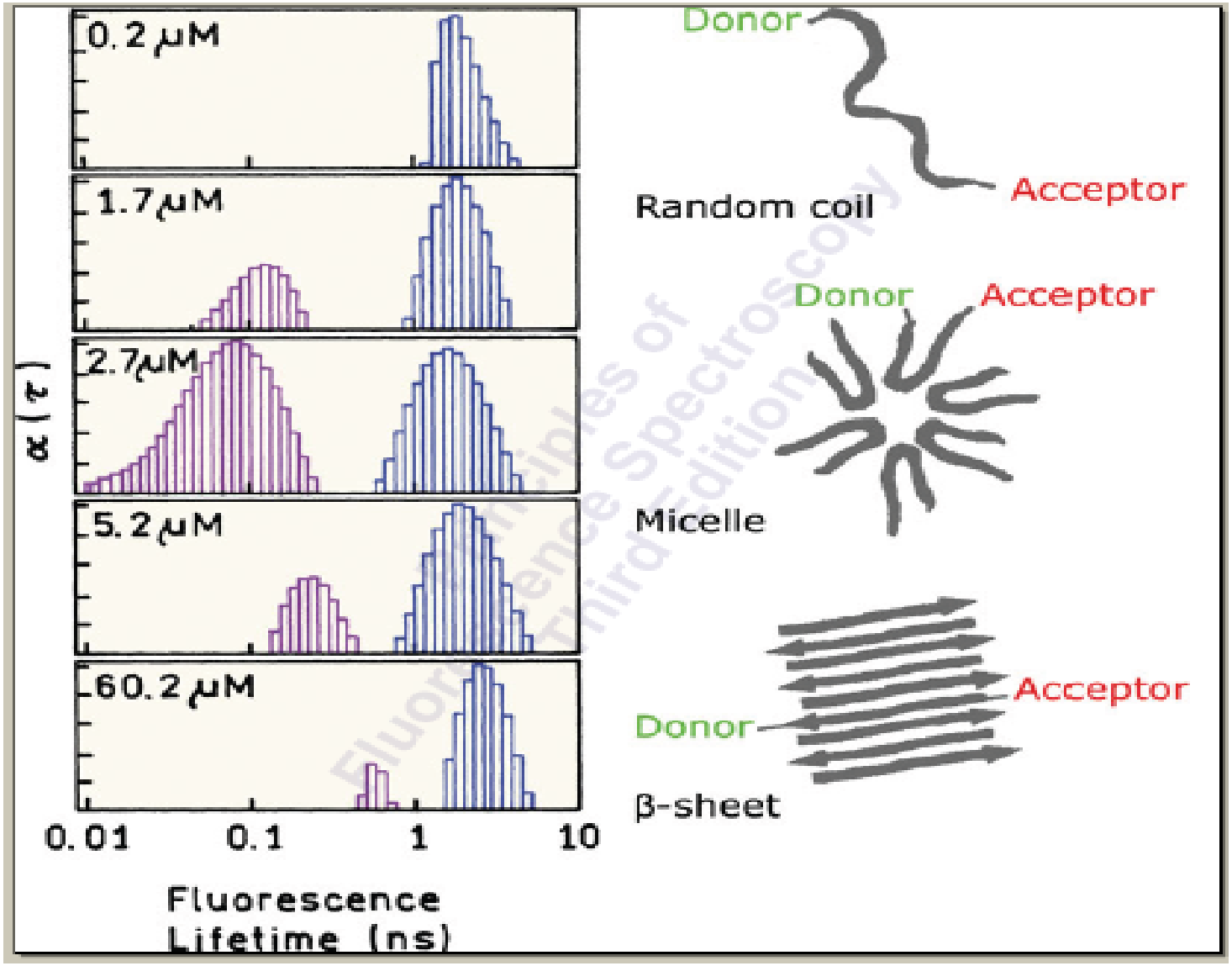
Melittin



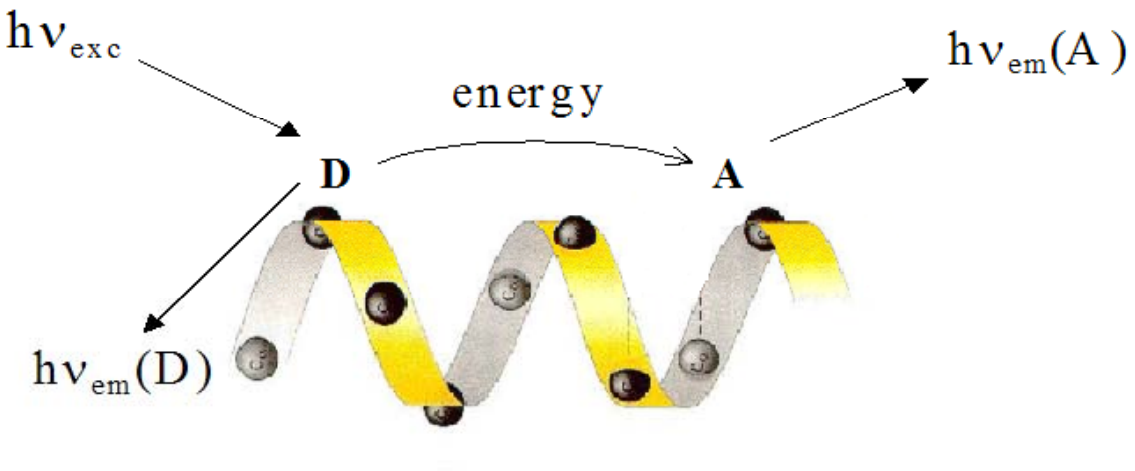
Coil-helix transition



Aggregation of Amyloid peptides



Peptides Foldamers for Energy Transfer



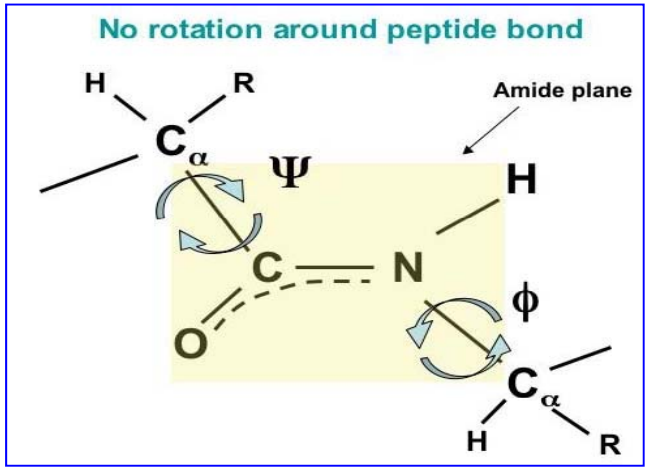
Förster Energy Transfer
dipole-dipole interaction

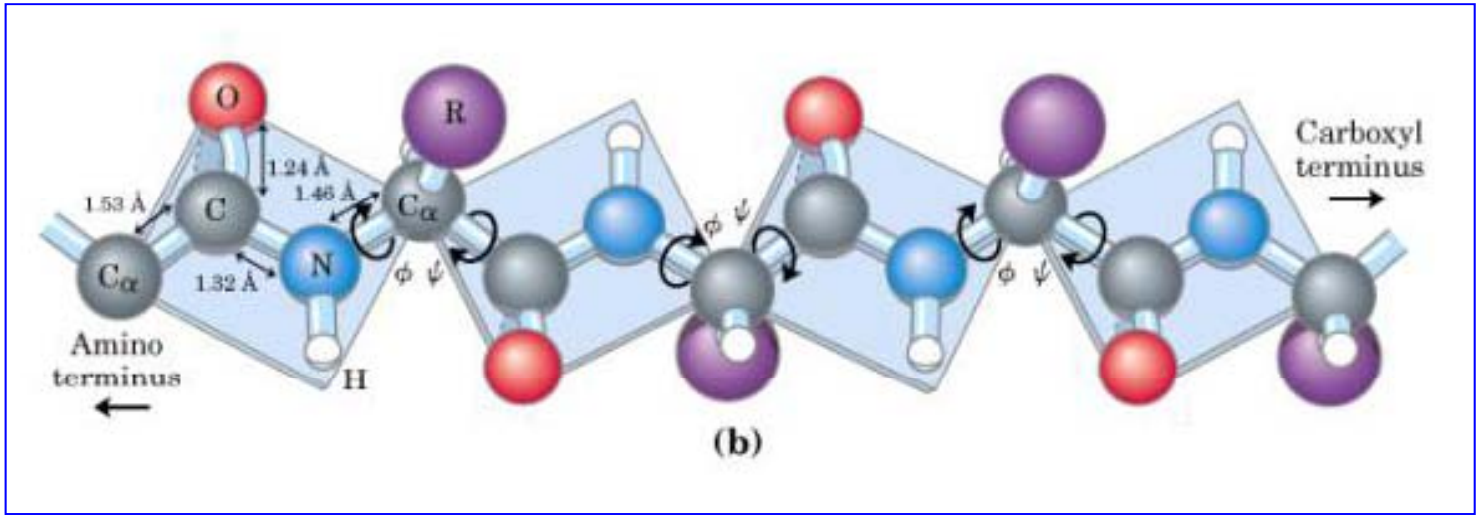
$$E = \frac{1}{1 + \frac{2}{3k^2}\left(\frac{R}{R_0}\right)^6}$$

Peptide foldamers

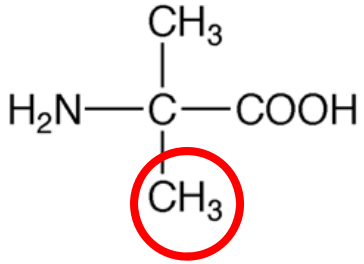
'Foldamers are molecules that have well-defined and predictable folding properties in solution.'

S. H. Gellman,
Accounts of Chemical Research 1998, 31, 173.

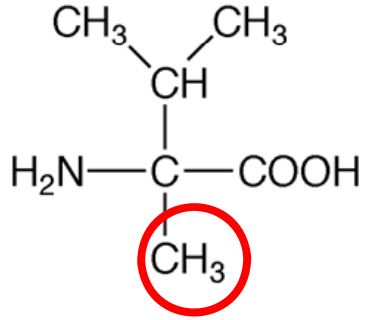




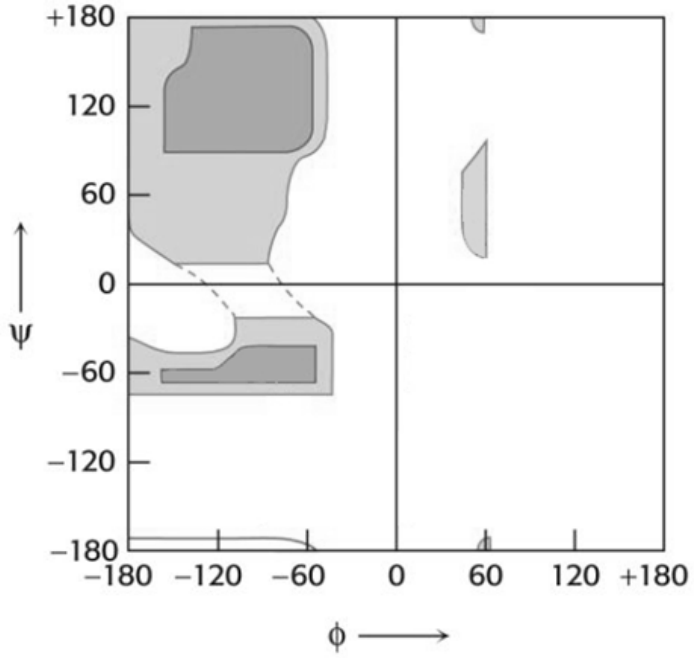
$C^{\alpha,\alpha}$ disubstituted aminoacids

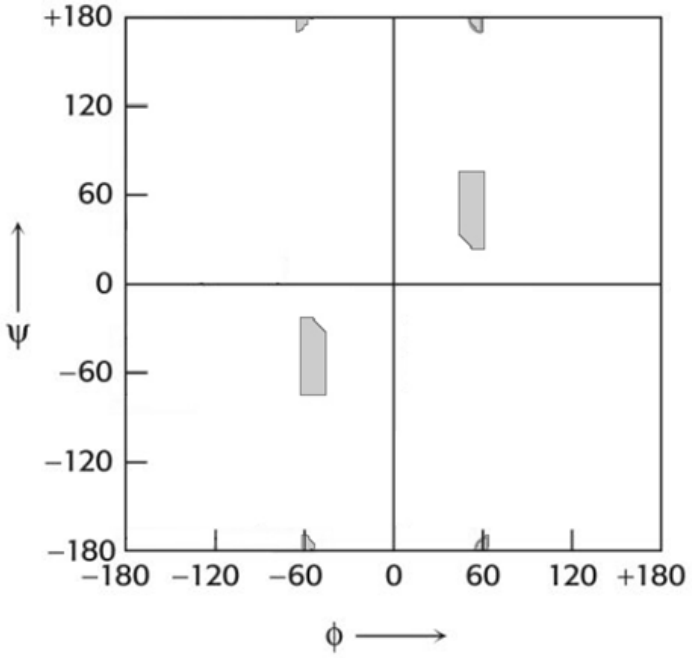


Aib



(αMe)Val





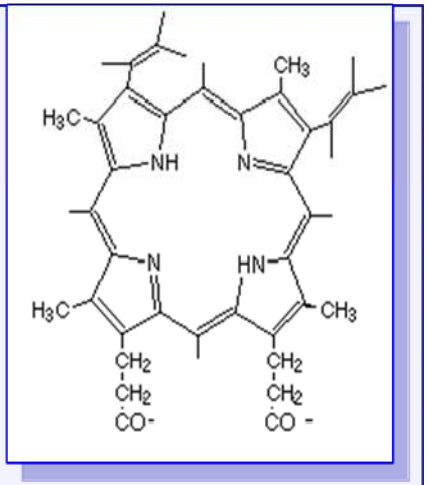
P-Aib-Aib-Aib-N

P-Aib-Aib-Aib-Aib-Aib-Aib-N

P-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib - N

P-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-N

P-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-Aib-N



P

Fmoc-Toac-(αMe)Val- (αMe)Val- NH*t*Bu

Fmoc-(αMe)Val-Toac-(αMe)Val- (αMe)Val- NH*t*Bu

Fmoc-(αMe)Val-(αMe)Val-Toac-(αMe)Val- (αMe)Val- NH*t*Bu

Fmoc-(αMe)Val-(αMe)Val- (αMe)Val-Toac-(αMe)Val- (αMe)Val- NH*t*Bu

Ac-Toac-Trp-Aib-Aib-Aib-Aib-O*t*Bu

Ac-Toac-Aib-Trp-Aib-Aib-Aib-O*t*Bu

Ac-Toac-Aib-Aib-Trp-Aib-Aib-O*t*Bu

Ac-Toac-Aib-Aib-Aib-Trp-Aib-O*t*Bu

Boc-(R)Bin-Toac-Ala-Aib-Aib-Ala-O*t*Bu

Boc-(R)Bin-Aib-Toac-Ala-Aib-Ala-O*t*Bu

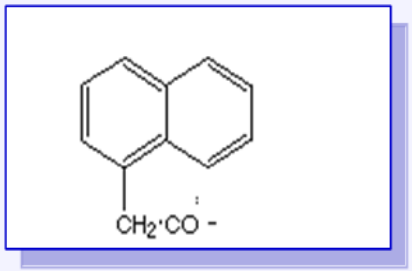
Boc-(R)Bin-Aib-Ala-Toac-Aib-Ala-O*t*Bu

Boc-(R)Bin-Aib-Aib-Ala-Toac-Ala-O*t*Bu

Boc-Aal- Aib-Toac-Ala-Aib-Ala-O*t*Bu

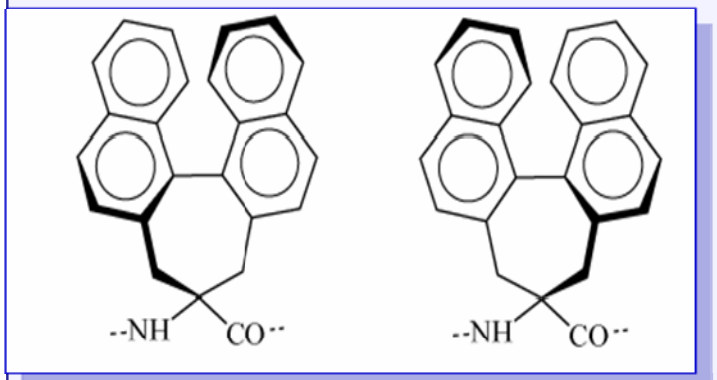
Boc-(S)Bin-Ala-Aib-Toac-Ala-Ala-O*t*Bu

Boc-Aal- Aib-Ala-Toac-Aib-Ala-O*t*Bu

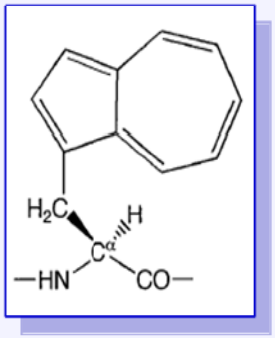


N

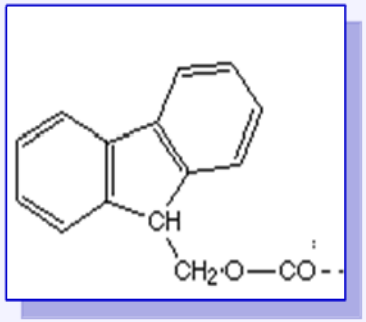
Ciclo{Orn-[Aib-Aib-Trp-Aib-Aib-Z]-Asp-[Aib-Aib-Toac-Aib-Aib-OtBu]}



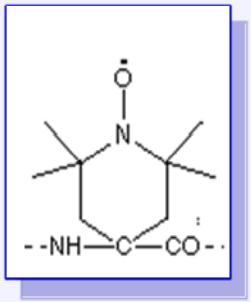
(S)-Bin (R)-Bin



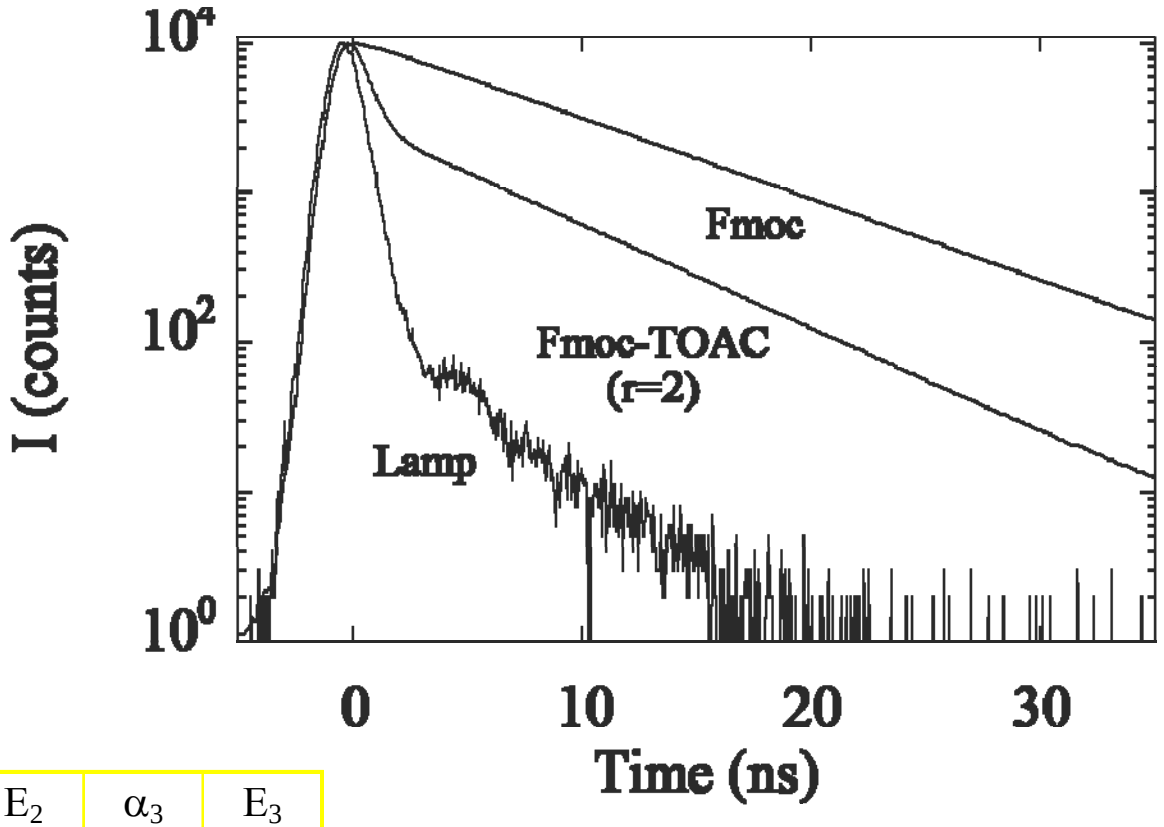
L-Aal



Fmoc

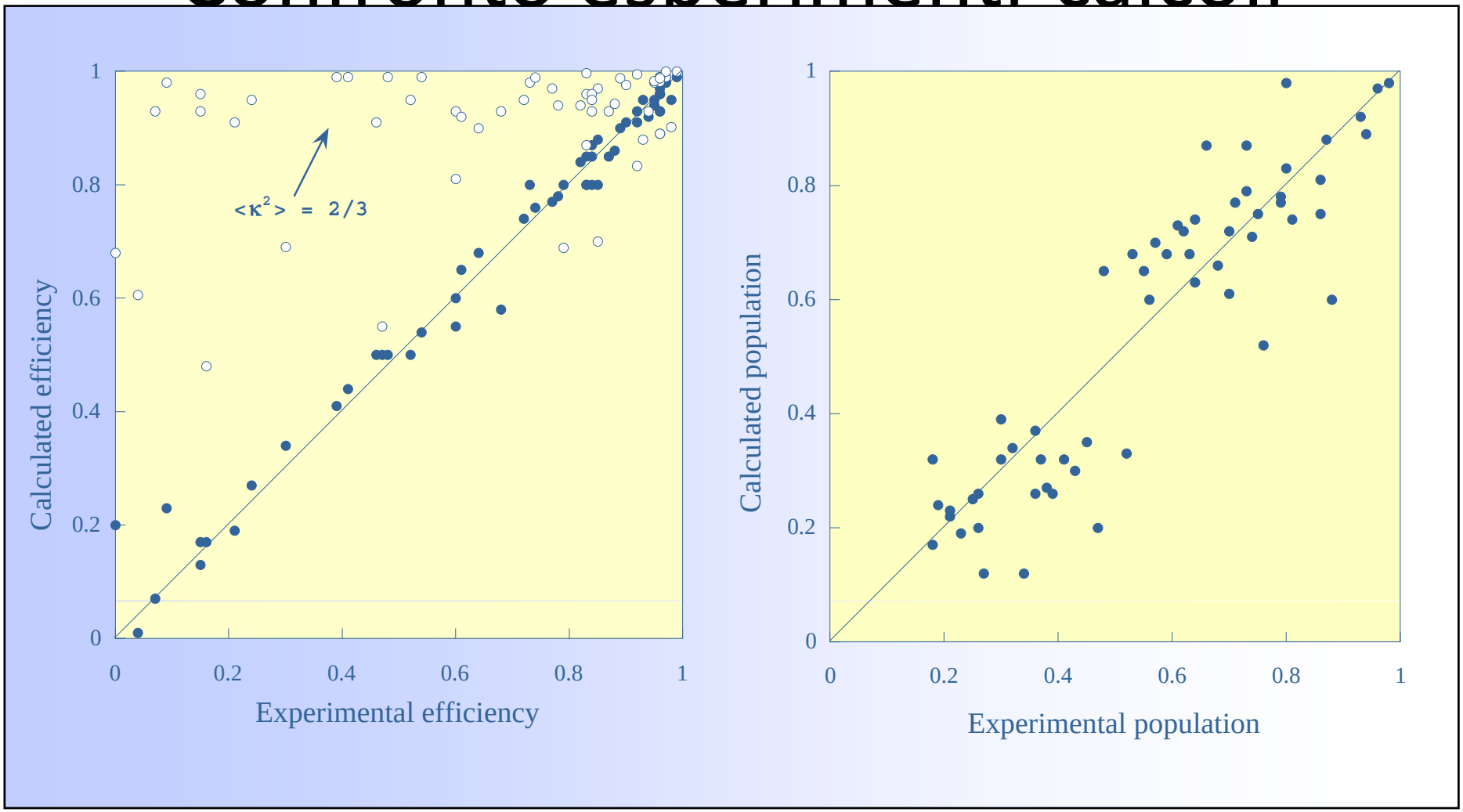


Toac

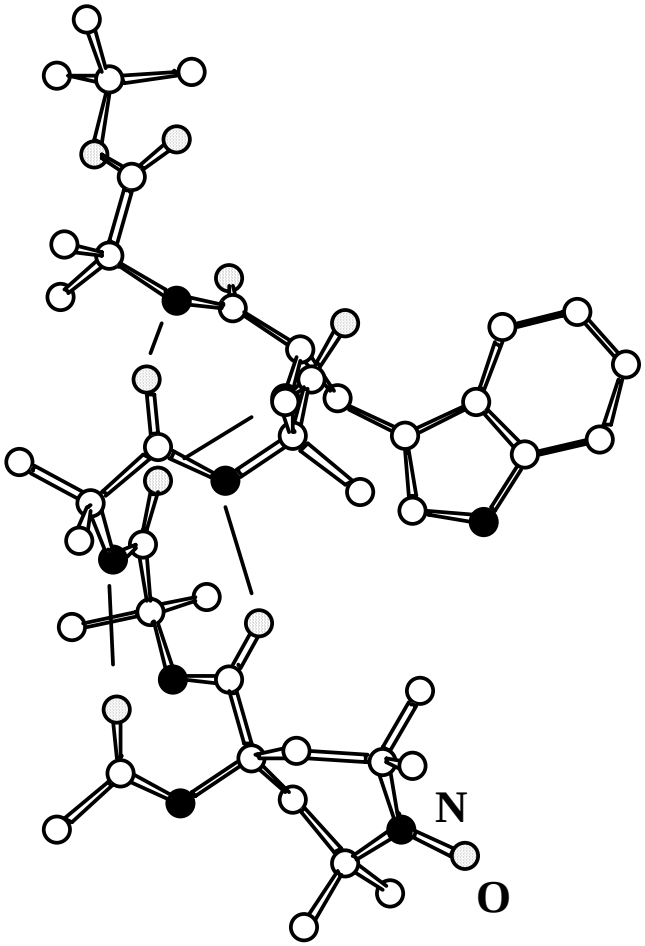


Peptide	r	α_1	E_1	α_2	E_2	α_3	E_3
Trp-TOAC	0	0.86	0.96	0.11	0.86	0.04	0.14
	1	0.86	0.93	0.12	0.83	0.02	0.10
	2	0.56	0.95	0.30	0.68	0.14	0.06
	3	0.80	0.96	0.16	0.71	0.04	0.21
Fmoc-TOAC	0	0.94	0.96	0.06	0.28	-	-
	1	0.98	0.90	0.02	0.24	-	-
	2	0.88	0.98	0.02	0.63	0.10	0.00
	3	0.76	0.92	0.18	0.79	0.06	0.00

Confronto esperimenti-calcoli

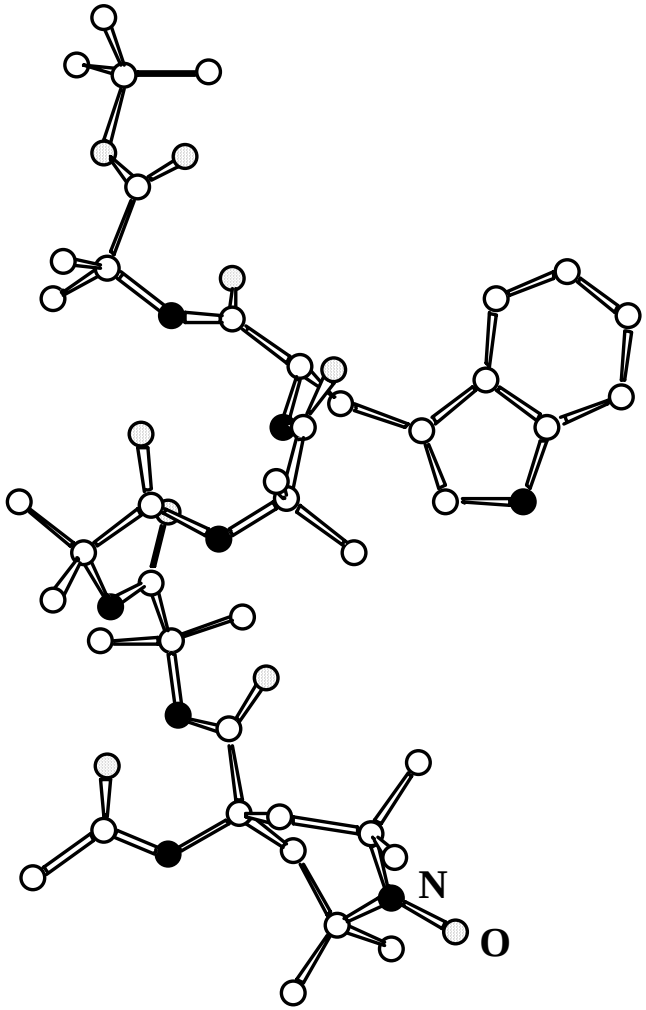


Soluzione

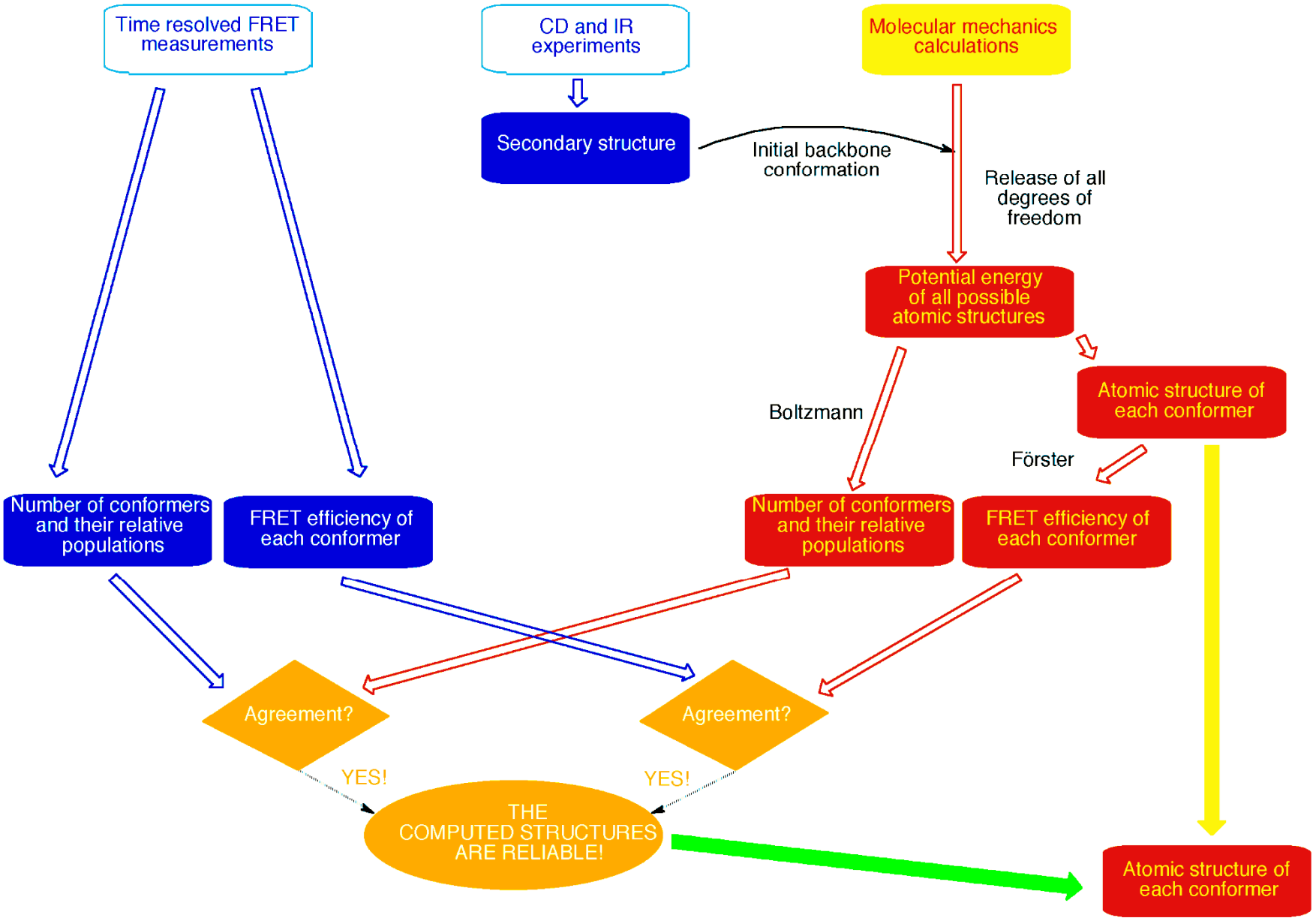


Meccanica molecolare
(confermata dai dati di fluorescenza)

Cristallo

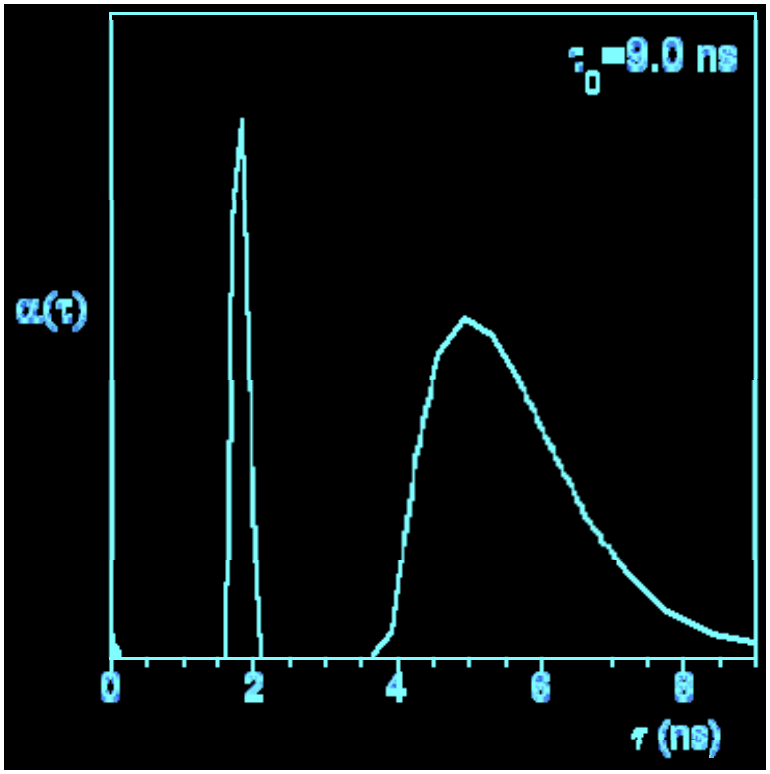


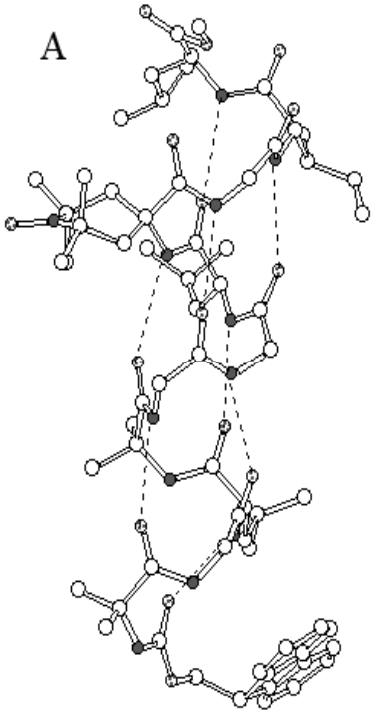
Raggi X

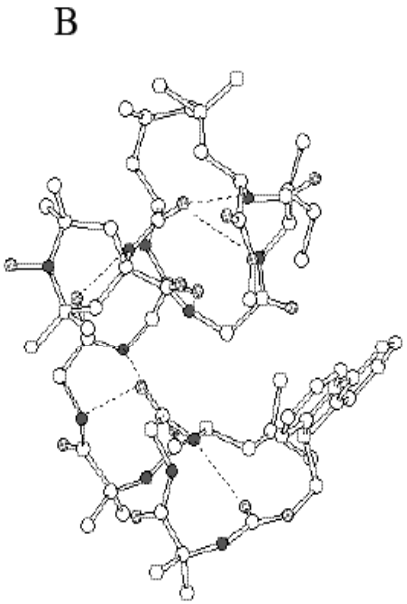

Time resolved FRET measurements
CD and IR experiments
Molecular mechanics calculations
Secondary structure
Initial backbone conformation
Release of all degrees of freedom
Potential energy of all possible atomic structures
Boltzmann
Förster
Atomic structure of each conformer
Number of conformers and their relative populations
FRET efficiency of each conformer
Number of conformers and their relative populations
FRET efficiency of each conformer
Agreement?
Agreement?
YES!
YES!
THE COMPUTED STRUCTURES ARE RELIABLE!
Atomic structure of each conformer

Fluorescence Energy Transfer

Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol

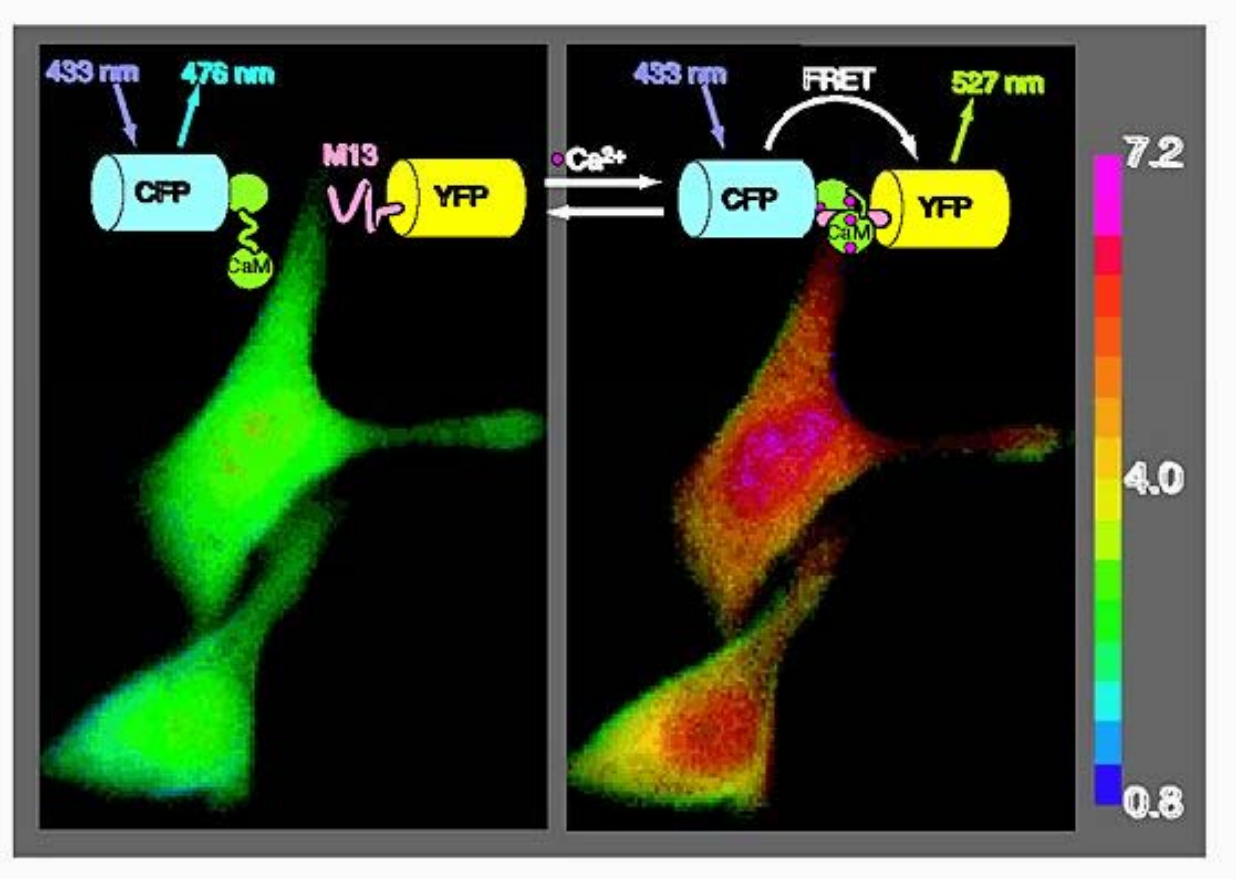






FRET: applicazioni

Processi di associazione

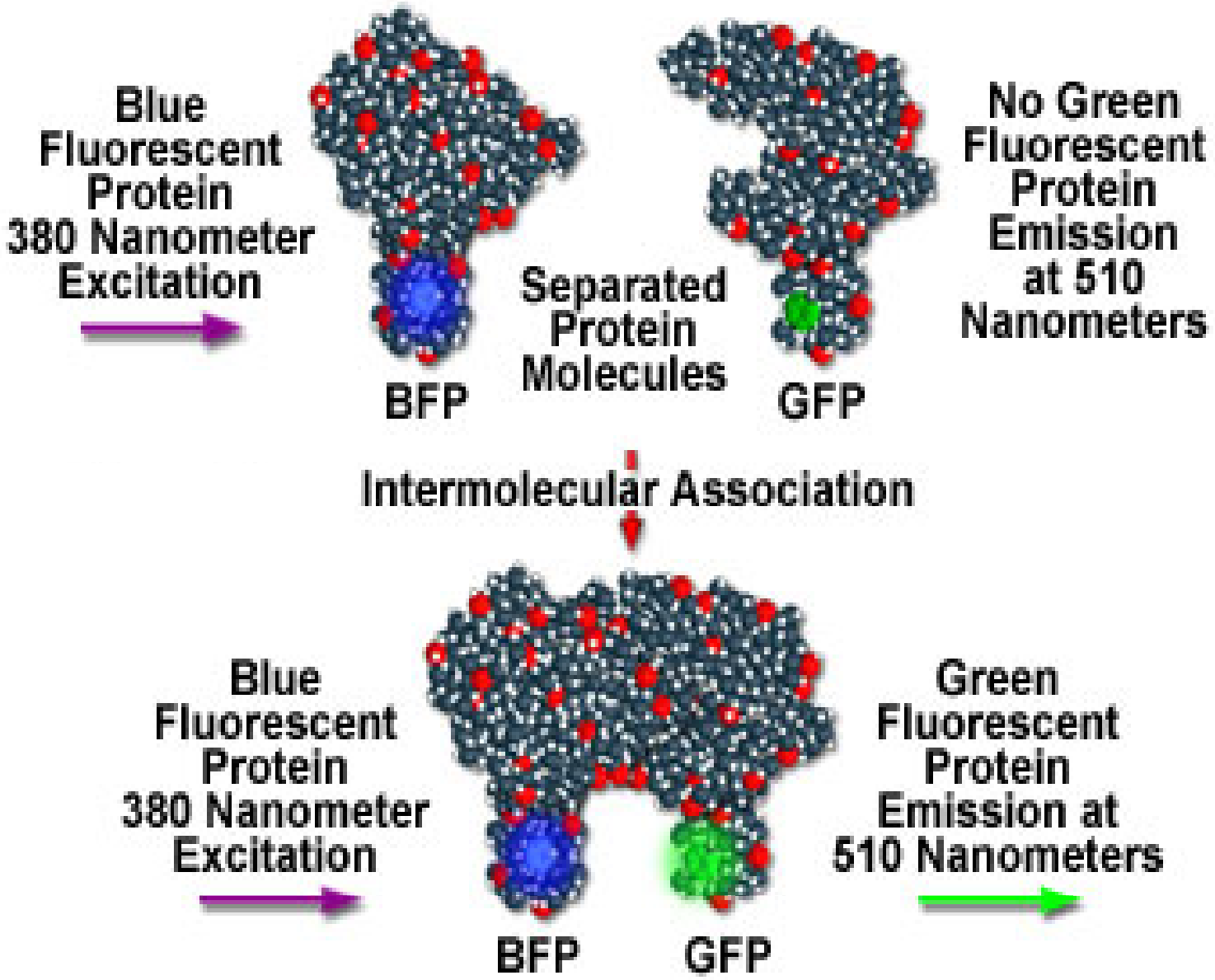


In vivo detection of calcium binding

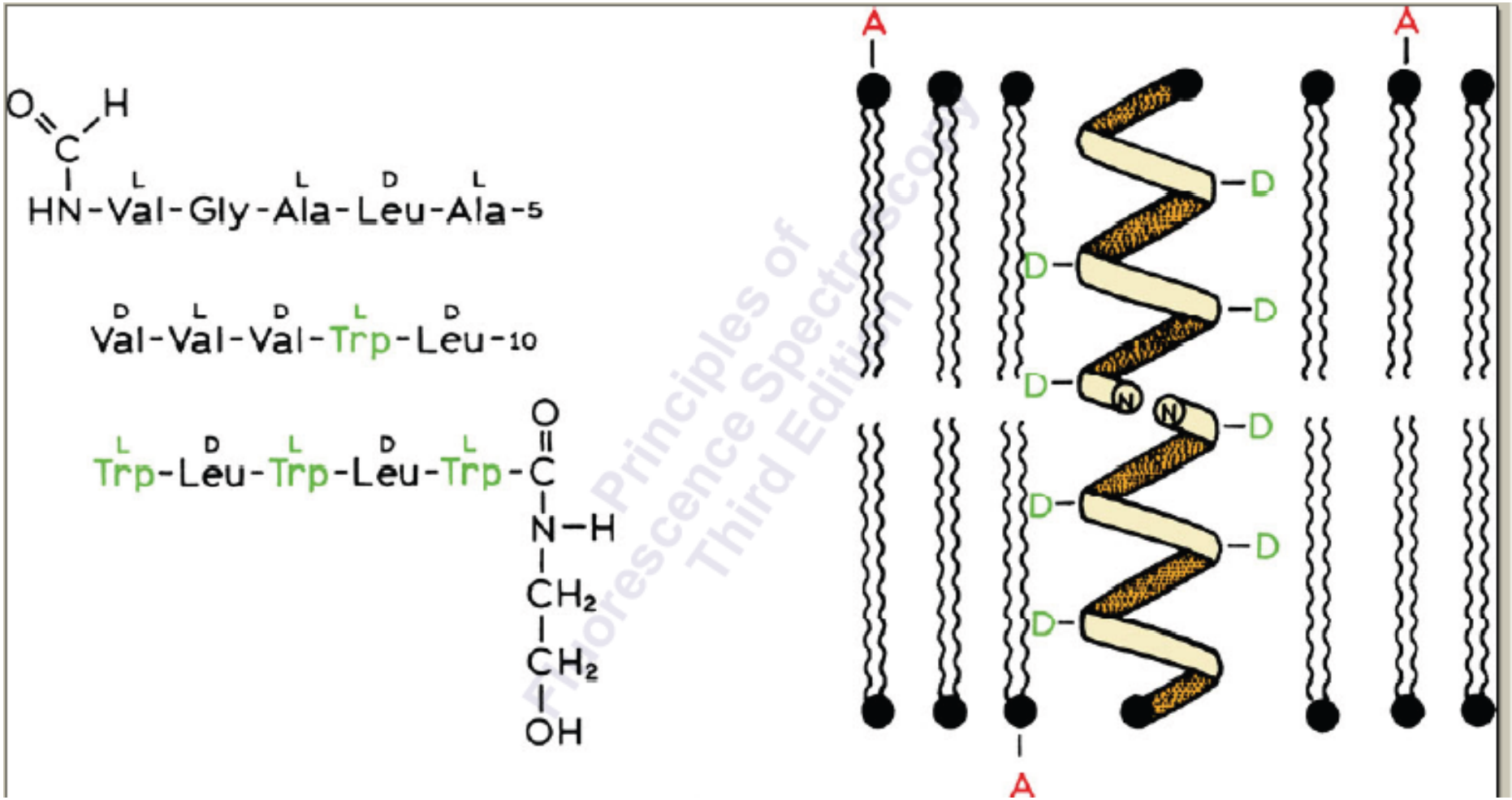
CFP= cyan fluorescent protein YFP=yellow fluorescent protein CaM=Calmodulin
M13=Calmodulin Binding peptide

(Tsien et al. 1998, Science 280:1954)

FRET Detection of *in vivo* Protein-Protein Interactions

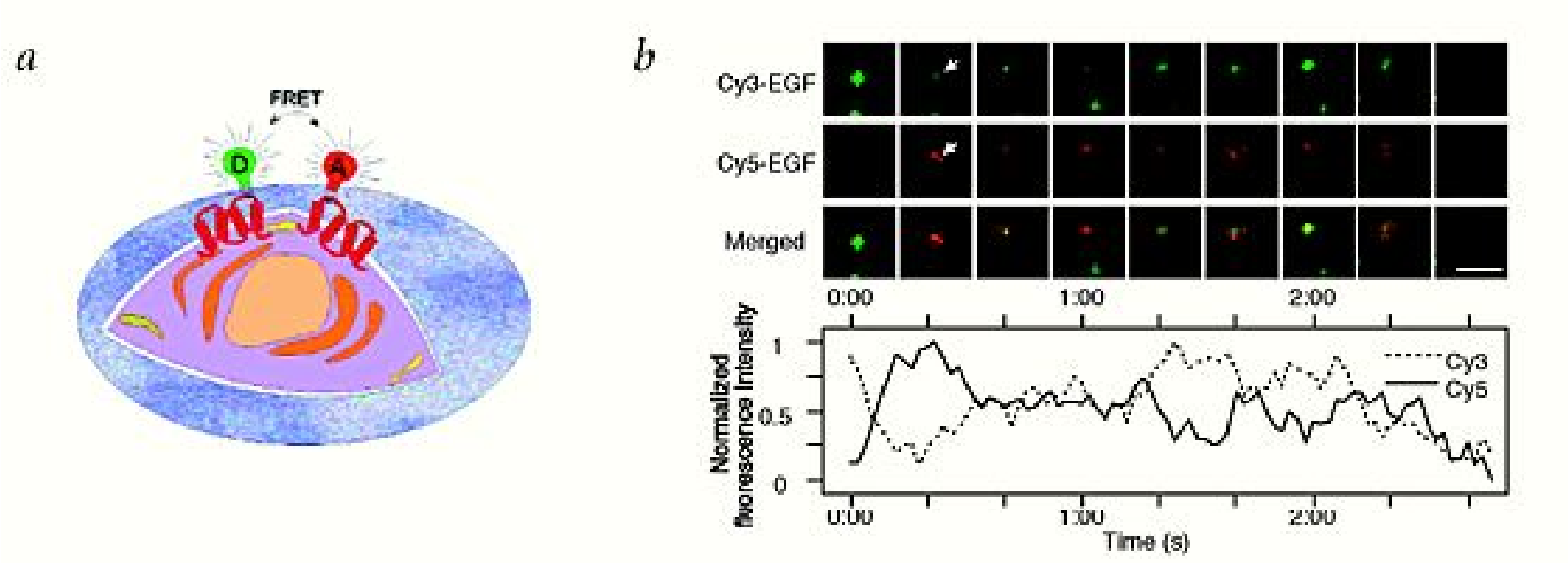


Membrane permeabilization



FRET: applicazioni

Processi di associazione

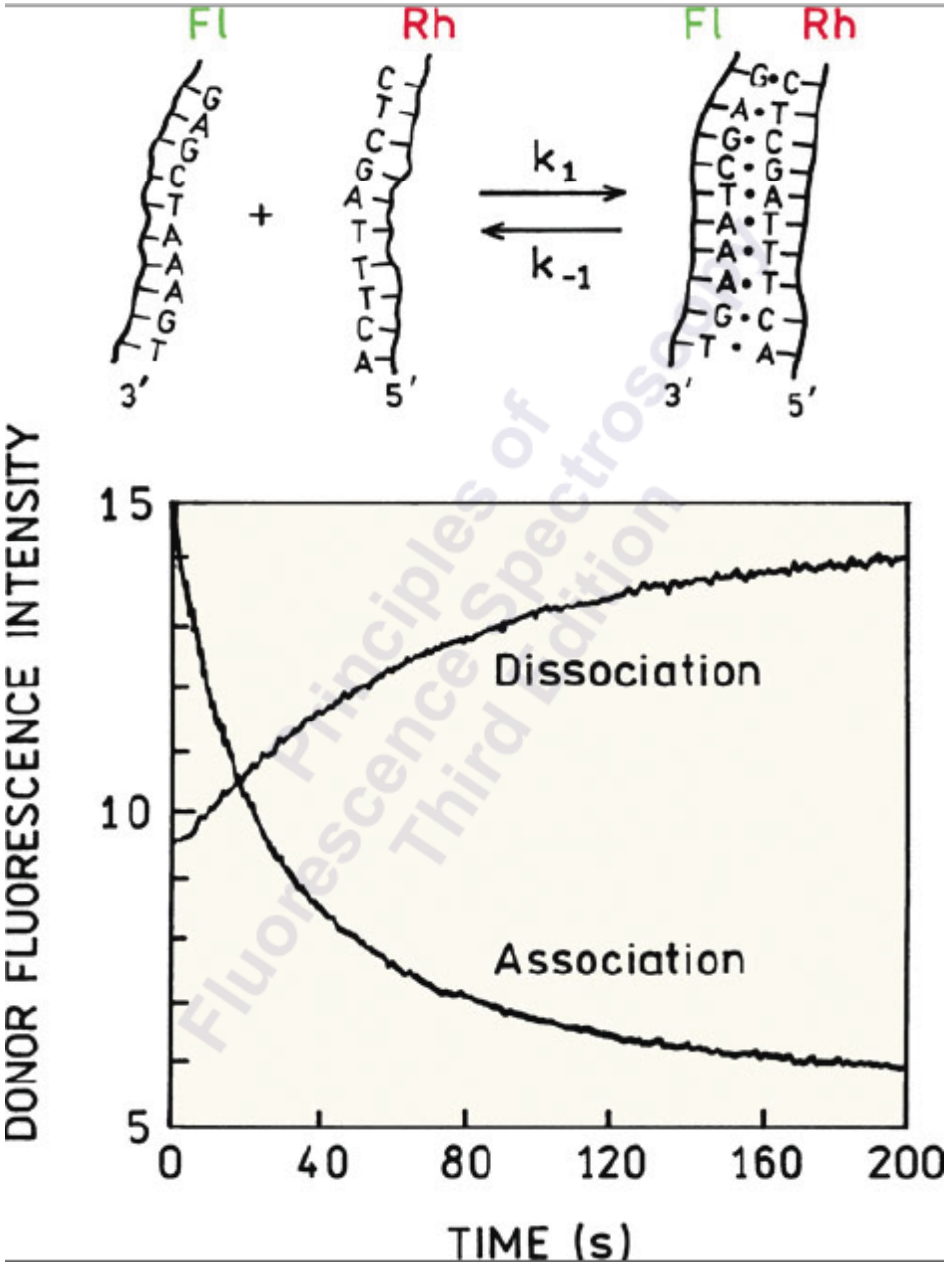


Single molecule detection of association between two epidermal growth factor receptors in the membrane of a living cell

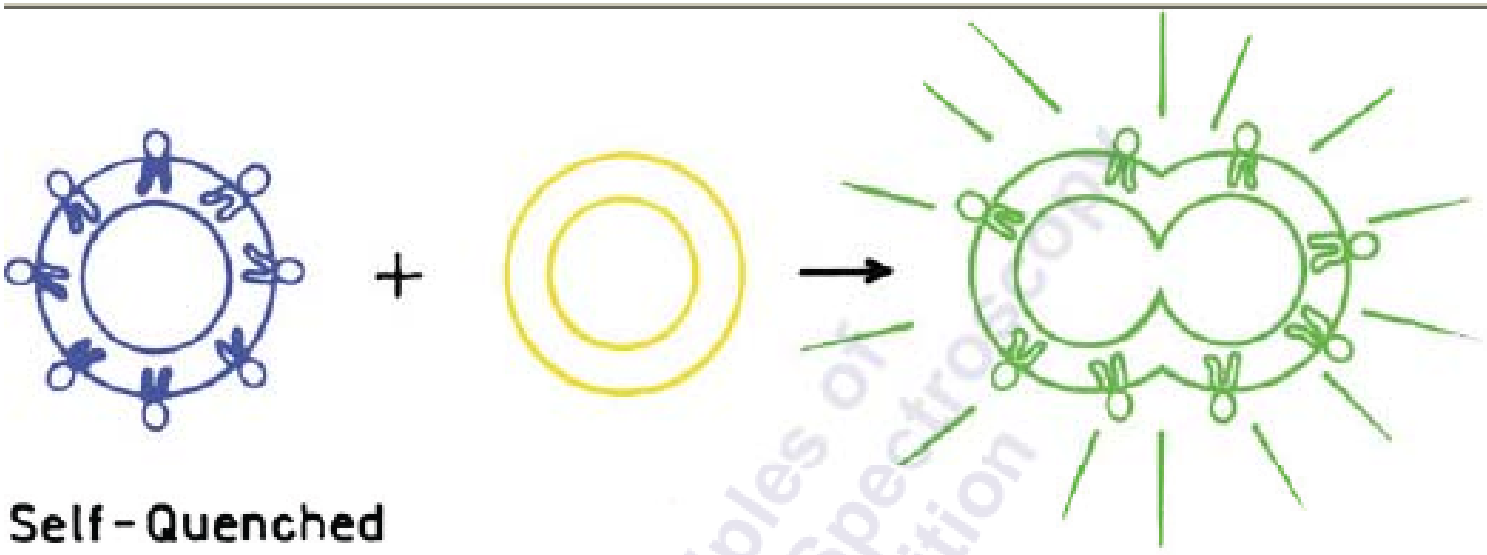
(Sako et al. 2000, Nature Cell Biol. 2:168)

FRET: applicazioni

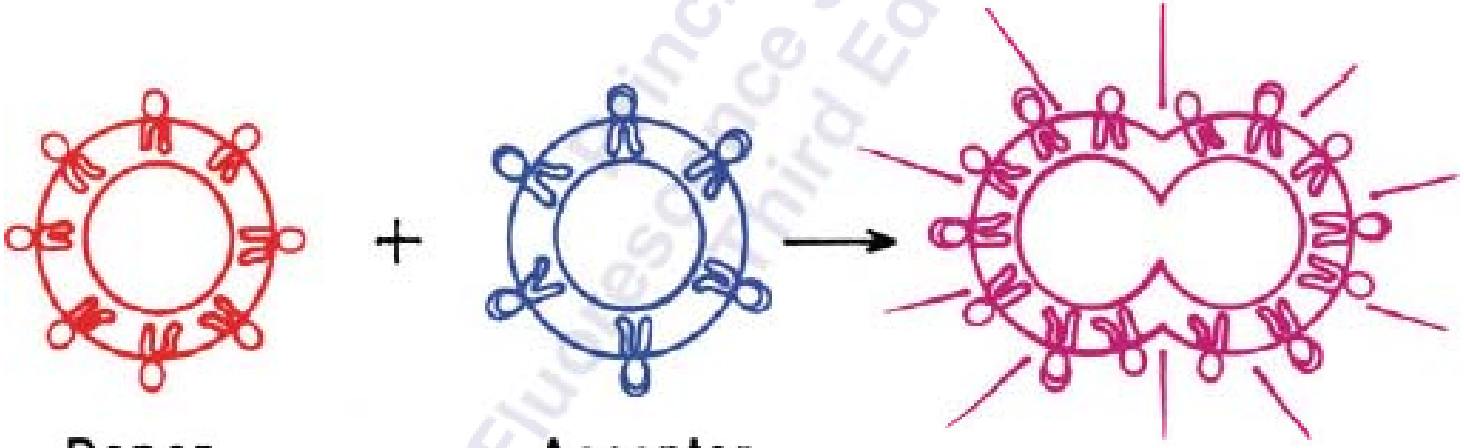
Ibridizzazione



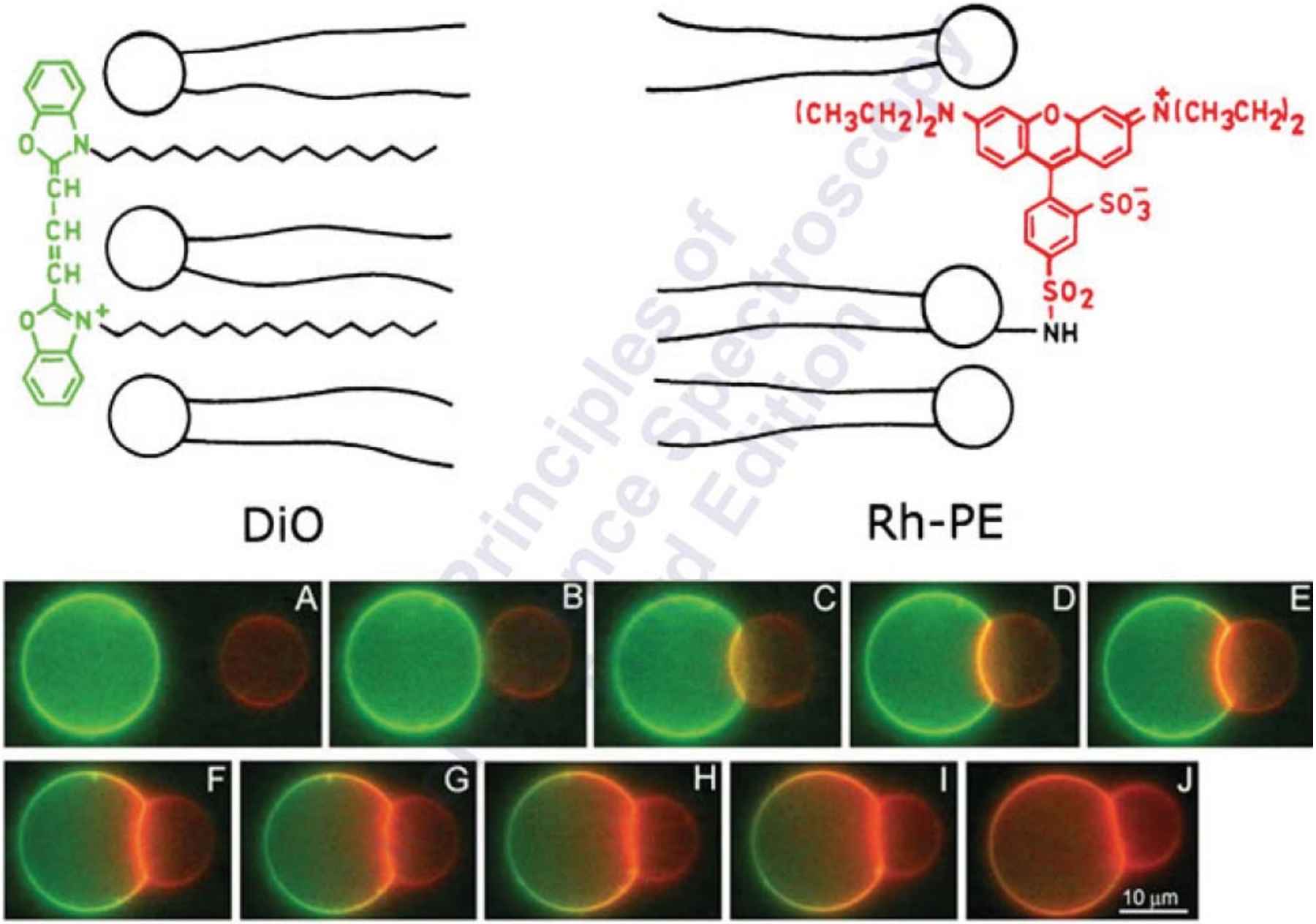
Membrane fusion



Self-Quenched

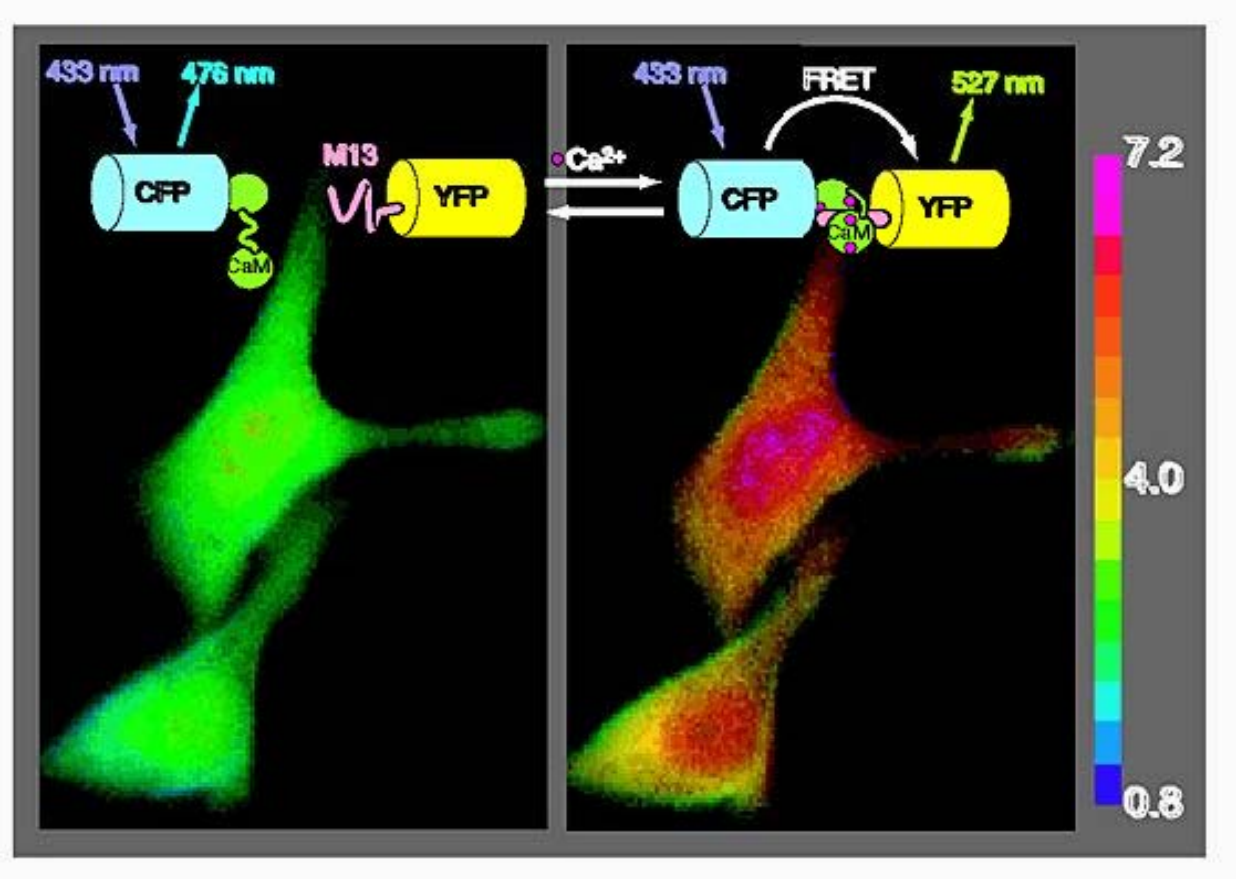


Donor Acceptor


DiO
Rh-PE
A
B
C
D
E
F
G
H
I
J
10 µm

FRET: applicazioni

Processi di associazione



In vivo detection of calcium binding

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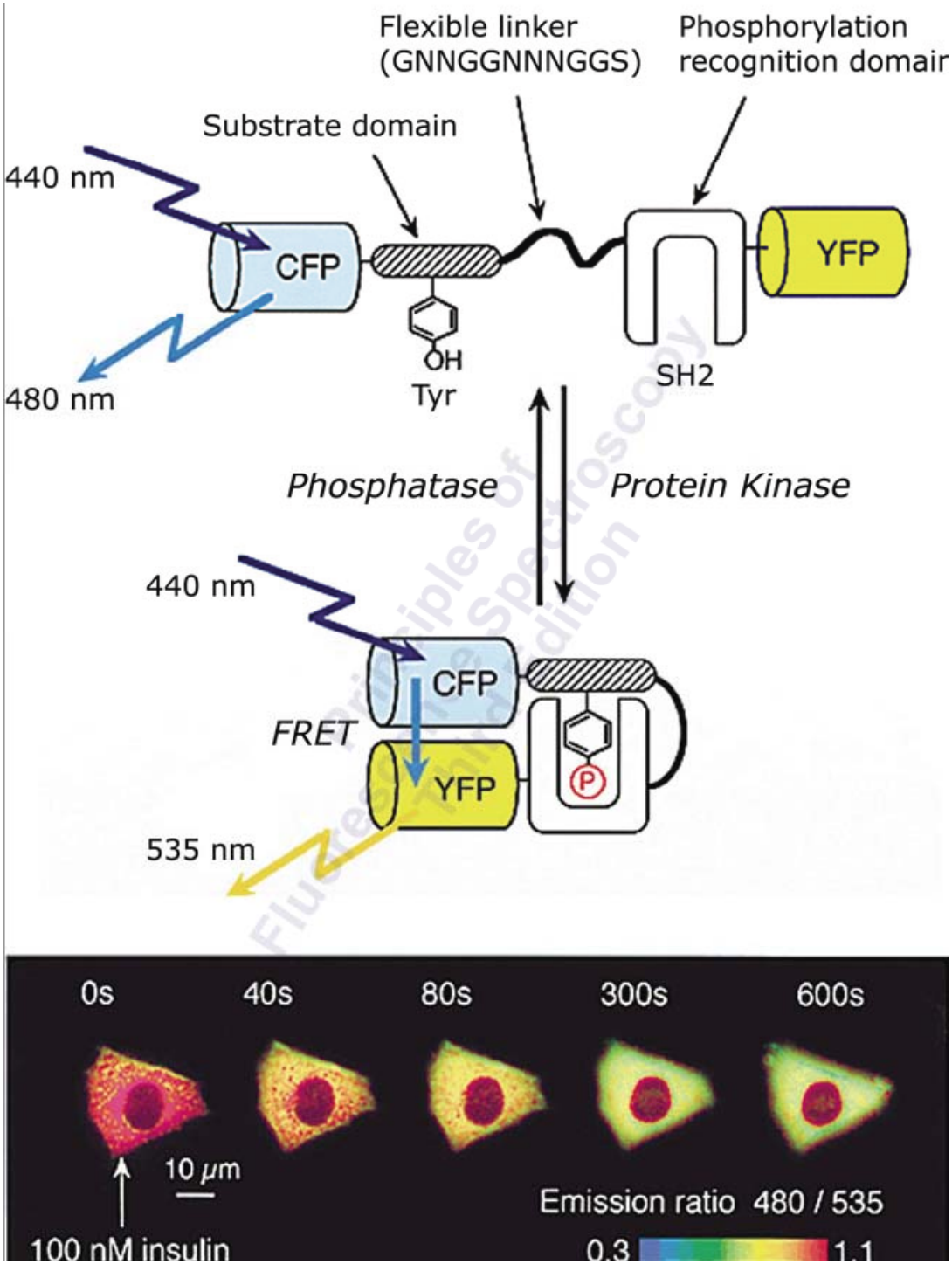
(Tsien et al. 1998, Science 280:1954)

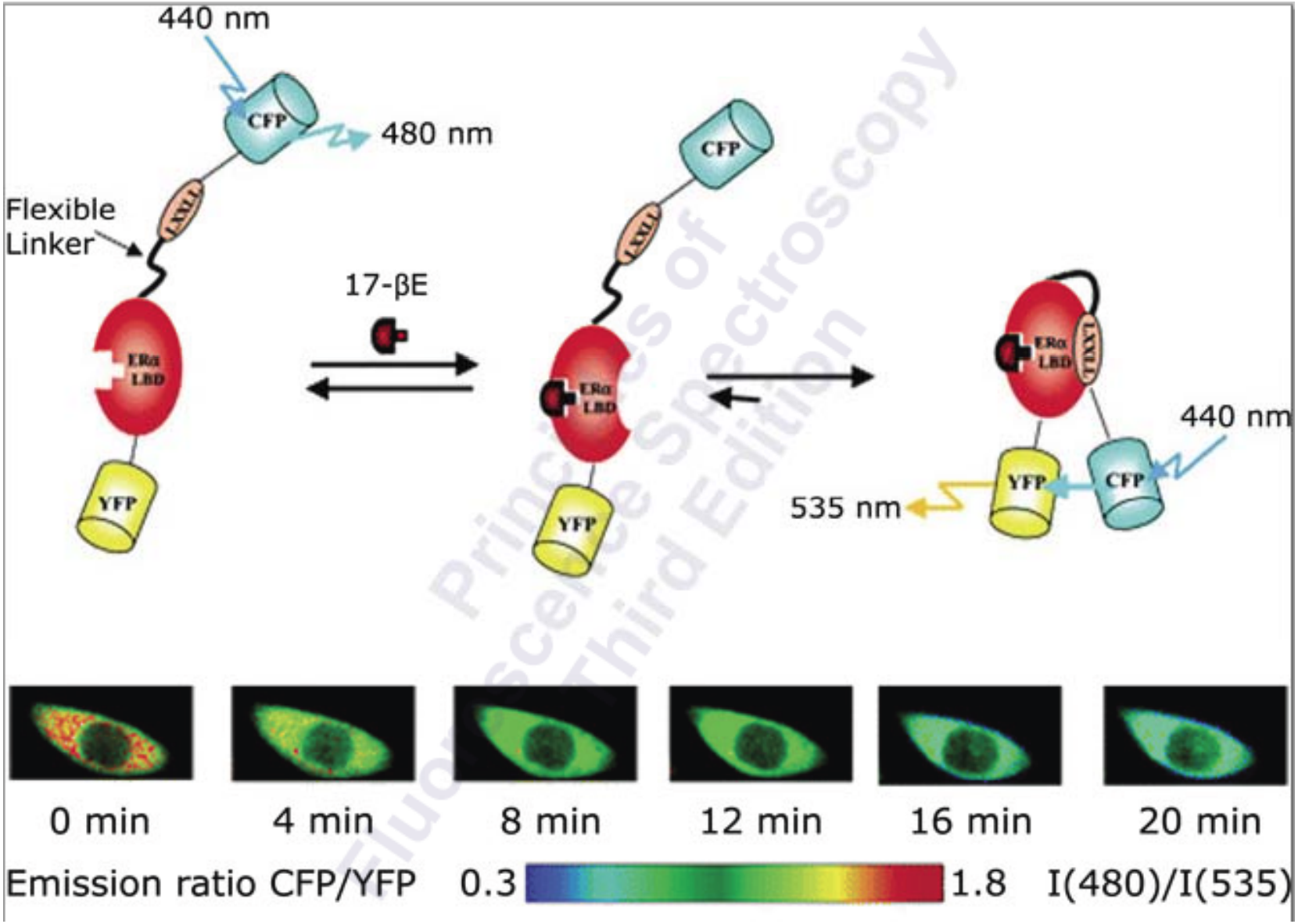
FRET: applicazioni

Sensore di fosforilazione



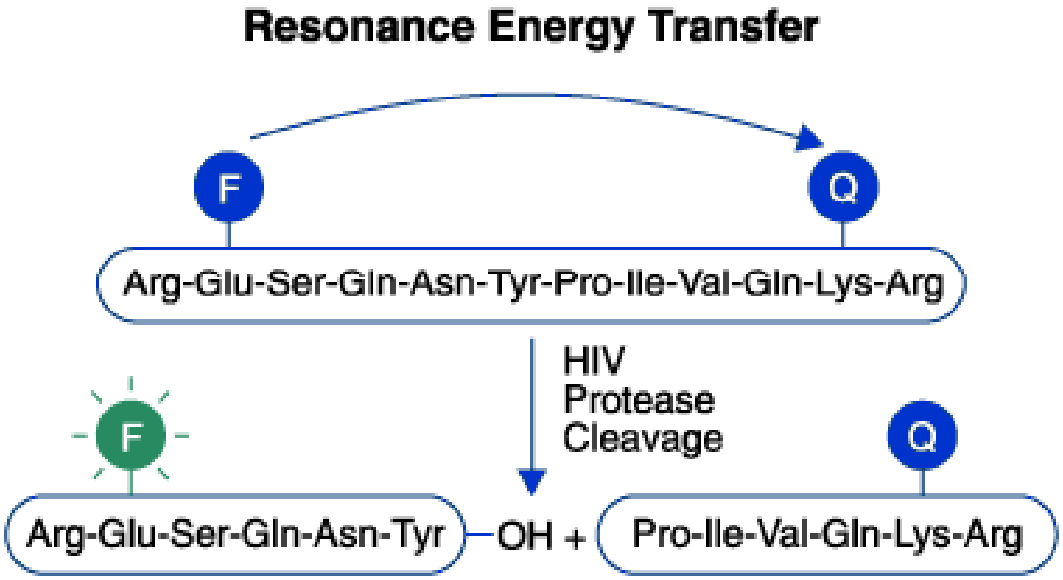
FRET: applicazioni

Sensore di estrogeni



FRET: applicazioni

Attività enzimatica



Nota: il metodo FRET permette la misura di attività enzimatica in vivo, utilizzando la microscopia di fluorescenza.