single-molecule fluorescence resonance energy transfer

(2) determing the Förster radius: quantum yield, donor lifetime, spectral overlap, anisotropy

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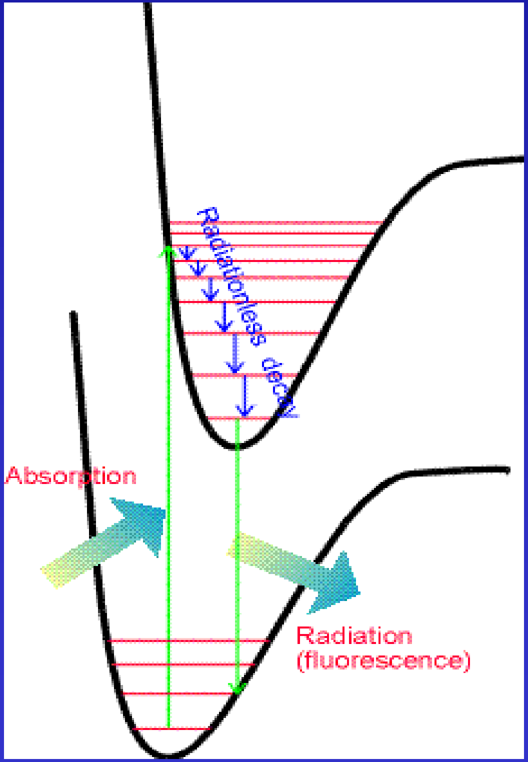
fluorescence

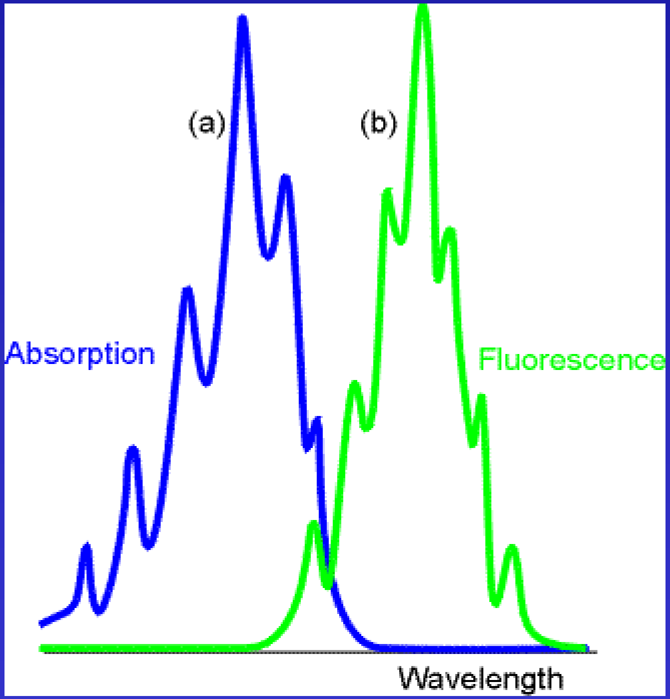
(1) absorbance of one photon (fs)

(2) vibrational relaxation (ps)

(3) fluorescence parameters:



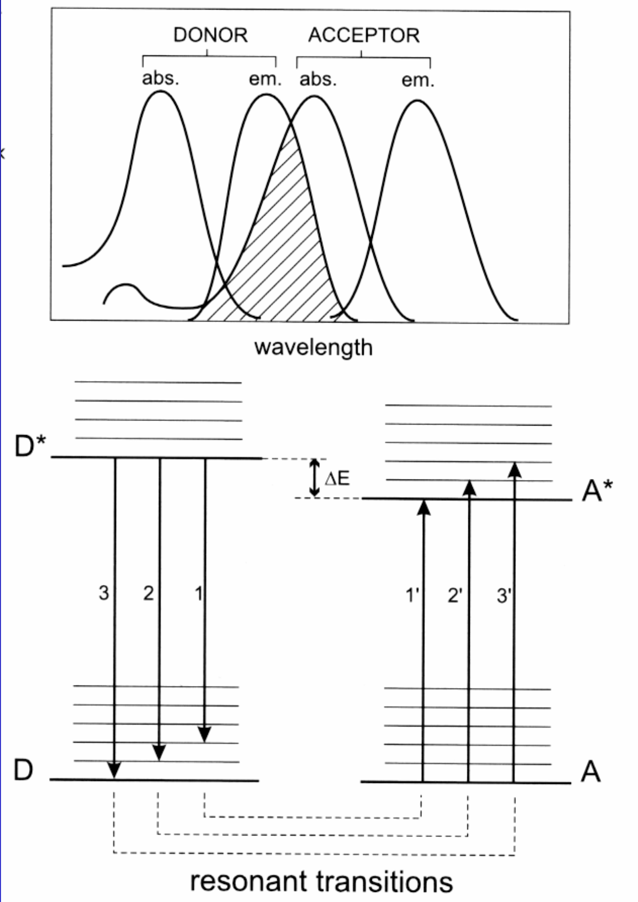
spectrum
quantum yield
lifetime
anisotropy



relaxation modes

- internal conversion (IC)
- intersystem crossing (ISC)
- fluorescence
- phosphorescence
- delayed fluorescence (after reverse ISC)
- triplet-triplet annihilation
- triplet-triplet transition
- photoinduced intramolecular charge transfer (TICT)
- intermolecular de-excitation (quenching or **FRET**)
- photochemical transformation
- excimer or exciplex formation

fluorescence resonance energy transfer : FRET (1)



- non-radiative energy transfer
- electronic energy transfer
- coulomb interaction:
  long-range dipole-dipole IA (Förster)

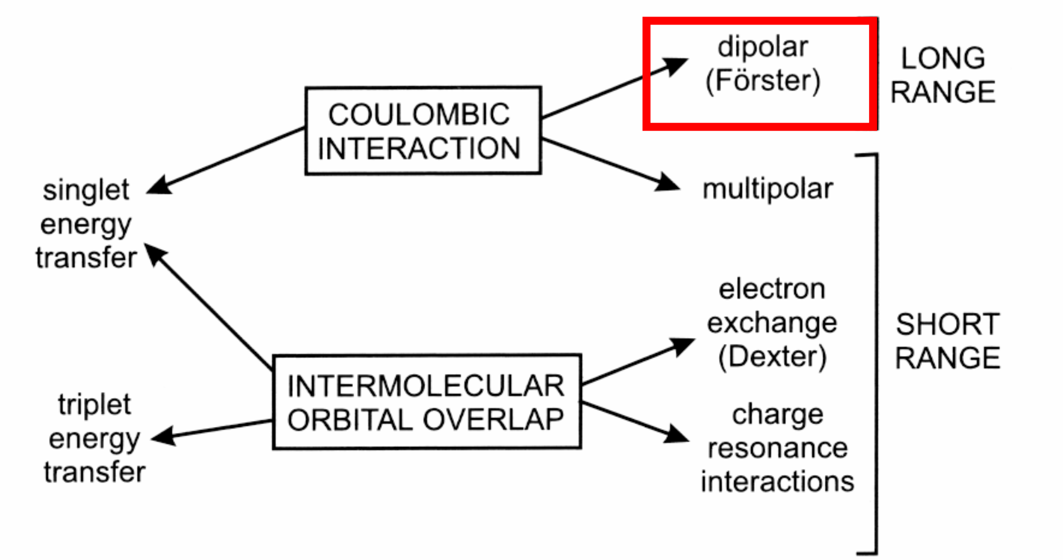


Fig. 4.13. Types of interactions involved in non-radiative transfer mechanisms.

fluorescence resonance energy transfer : FRET (2)

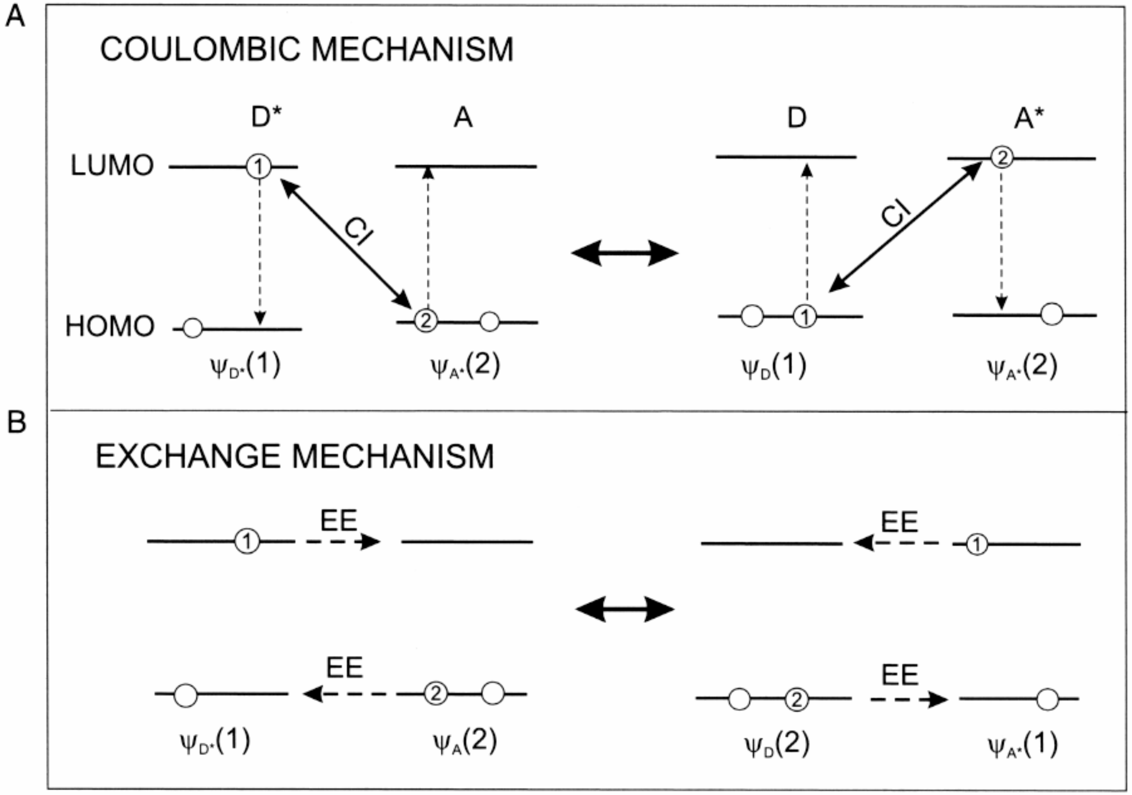
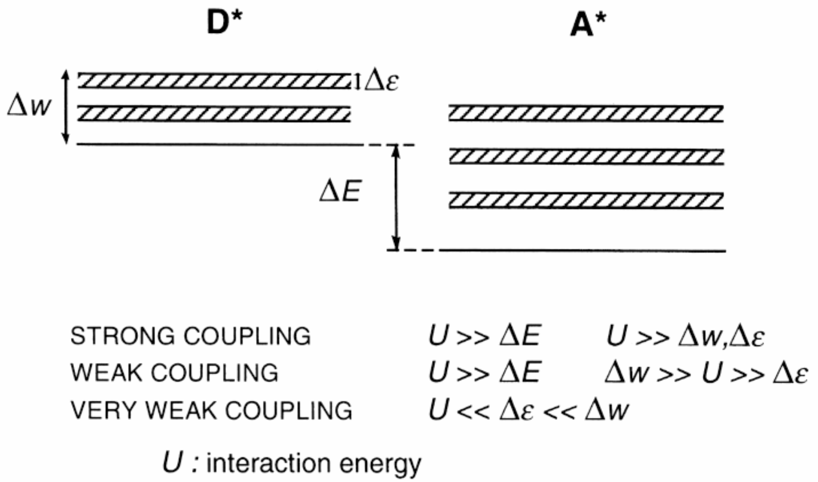


Fig. 4.14. Schematic representation of the (A) Coulombic and (B) exchange mechanisms of excitation energy transfer. CI: Coulombic interaction; EE: electron exchange.

intermolecular energy transfer:

- RET
- reabsoprtion
- complex formation
- collisional quenching

fluorescence resonance energy transfer : FRET (3)



STRONG COUPLING	$U >> \Delta E$	$U >> \Delta w, \Delta\varepsilon$
WEAK COUPLING	$U >> \Delta E$	$\Delta w >> U >> \Delta\varepsilon$
VERY WEAK COUPLING	$U << \Delta\varepsilon << \Delta w$	

U : interaction energy

Fig. 4.15. Distinction between strong, weak and very weak coupling.

dipole interaction energy:

$$U_{dd} = 5.04 \frac{M_D \bullet M_A}{r^3} \underbrace{(\cos\theta_{DA} - 3\cos\theta_D \cos\theta_A)}_{\kappa}$$

U_{dd} in cm^{-1}

Δw: absorption bandwidth
$\Delta\varepsilon$: width of an isolated vibronic level

strong coupling: two new absorption bands, energy transfer faster than vibrational relaxation, delocalized ex.energy, coherent transfer → exciton theory
distance dependende is r^{-3}
weak coupling: transfer rate faster than vibrational relaxation but slower than nuclear motions, distance dependende is r^{-3}
very weak coupling: interaction energy is lower than the vibrational bandwidth,
stringent conditions for resonance, thermal and solvent broadening of vibronic bandwidths
transfer rate depends on the square of U, and distance dependende is r^{-6} .

fluorescence resonance energy transfer : FRET (4)

Förster long-range dipole-dipole interaction (very weak coupling)

$$k_T^{dd} = k_D\left[\frac{R_0}{r}\right]^6 = \frac{1}{\tau_D^0}\left[\frac{R_0}{r}\right]^6$$

k_T : rate constant (r.c.)
for energy transfer
k_D : donor emission r.c.
r : distance between
donor and acceptor
τ^0_D : donor lifetime in
the absence of acceptor

R_0 : Förster radius for
$k_T = k_D + k_{Di}$

Förster singlet-singlet radiationless resonance eletronic excitation energy transfer FSSRREEET

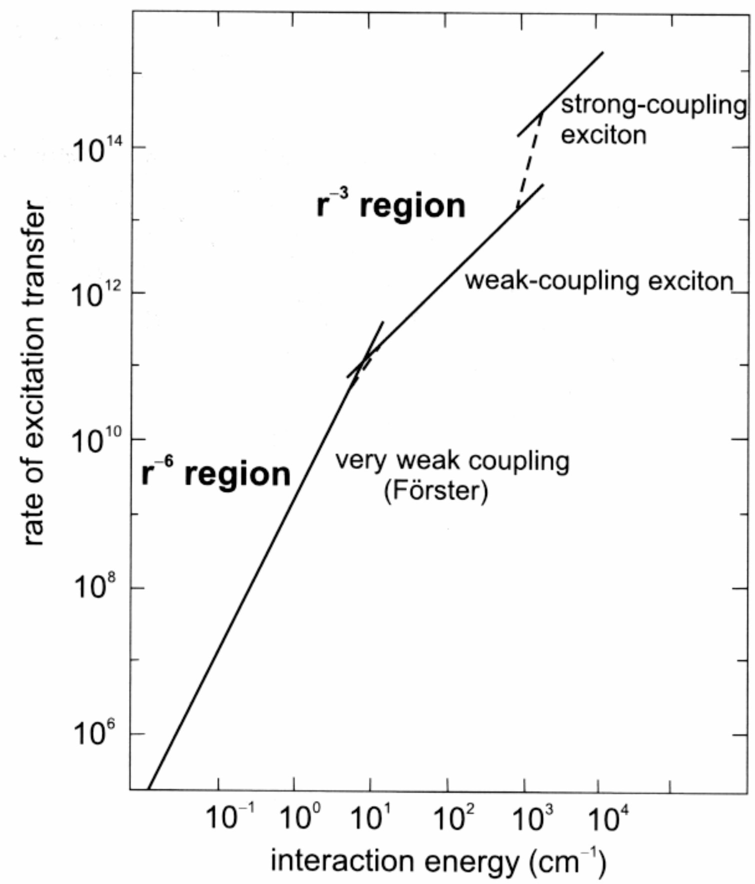


Fig. 4.16. Transfer rates predicted by Förster for strong, weak and very weak coupling.

time dependence of FRET

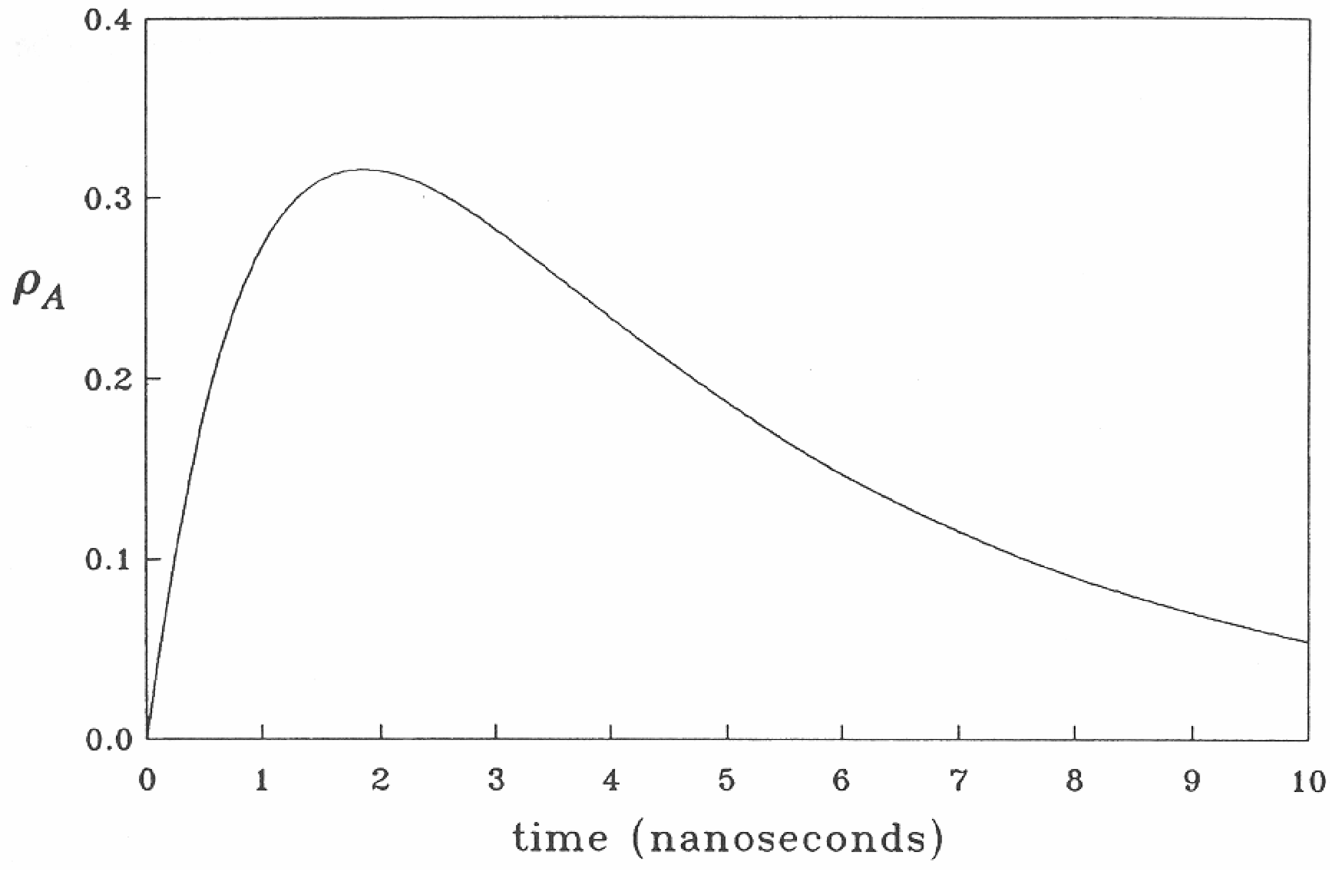


Figure 2.11. The probability ρ_A that a fluorescent acceptor is excited versus time according to Eq. (2.34), with the following parameter values: $\rho_{A0}^{dir} = 0$, $\rho_{D0} = 1$, $k_T = 0.50\ \text{ns}^{-1}$, $k_D + k_{Di} = 0.50\ \text{ns}^{-1}$, $k_A + k_{Ai} = 0.25\ \text{ns}^{-1}$. The acceptor emission is proportional to ρ_A.

fluorescence resonance energy transfer : FRET (5)

$$k_T = \frac{1}{\tau_d}\left(\frac{R_0}{r}\right)^6$$

distance dependence and Förster radius R_0

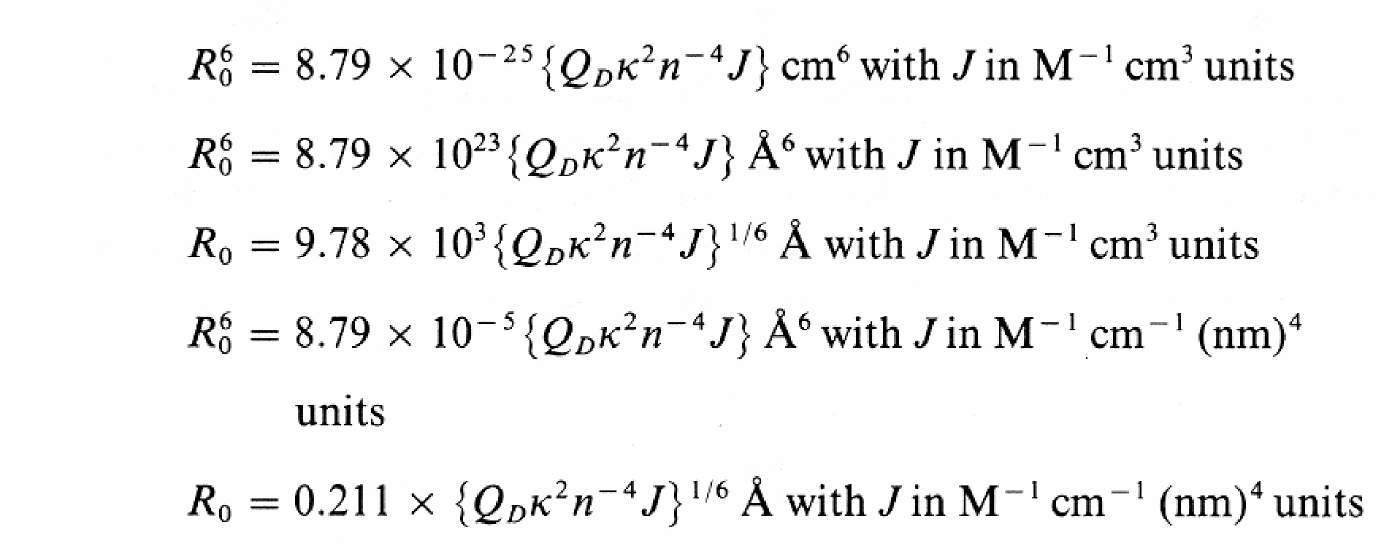
$$k_T = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 n^4 N r^6 \tau_d}\int_0^\infty \frac{F_d(\bar{\nu})\varepsilon_a(\bar{\nu})}{\bar{\nu}^4}\,d\bar{\nu}$$

$$k_T = (r^{-6} J \kappa^2 n^{-4} \lambda_d) \times 8.71 \times 10^{23}\ \mathrm{sec}^{-1}$$

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 N n^4}\int_0^\infty \frac{F_d(\bar{\nu})\varepsilon_a(\bar{\nu})\,d\bar{\nu}}{\bar{\nu}^4}\,d\bar{\nu}$$

units of R_0

- units follow the definition of the overlap integral



$R_0^6 = 8.79 \times 10^{-25}\{Q_D \kappa^2 n^{-4} J\}$ cm^6 with J in $\text{M}^{-1}\,\text{cm}^3$ units

$R_0^6 = 8.79 \times 10^{23}\{Q_D \kappa^2 n^{-4} J\}$ Å^6 with J in $\text{M}^{-1}\,\text{cm}^3$ units

$R_0 = 9.78 \times 10^{3}\{Q_D \kappa^2 n^{-4} J\}^{1/6}$ Å with J in $\text{M}^{-1}\,\text{cm}^3$ units

$R_0^6 = 8.79 \times 10^{-5}\{Q_D \kappa^2 n^{-4} J\}$ Å^6 with J in $\text{M}^{-1}\,\text{cm}^{-1}\,(\text{nm})^4$ units

$R_0 = 0.211 \times \{Q_D \kappa^2 n^{-4} J\}^{1/6}$ Å with J in $\text{M}^{-1}\,\text{cm}^{-1}\,(\text{nm})^4$ units

- *apparent R_0'* : with donor quantum yield excluded

fluorescence resonance energy transfer : FRET (6)

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 N n^4} J$$

Förster radius R_0 depends on

ϕ_d^0 : the quantum yield of donor fluorophore in the absence of FRET acceptor

κ^2 : orientational factor of transition dipoles

J : spectral overlap (λ or $\tilde{\nu}$)

n : refractive index

quantum yield (for fluorescence)

- ratio of the number of emitted photons to the number of absorbed photons
- ratio of the rate constant for radiative deactivation ($S_1 \rightarrow S_0$) to the sum of rate constants for all deactivation processes
- ratio of the lifetime of S_1 (fluorescence lifetime) to the radiative lifetime (in absence of any non-radiative deactivation)

quantum yield measurements (1)

determing $\phi_d{}^0$: the quantum yield of donor fluorophore
in the absence of FRET acceptor

methods: (i) integration of the fluorescence spectrum and subsequent normalization using a reference (known quantum yield)

$$\phi_{sample} = \frac{OD_{ref} \cdot F_{sample} \cdot n^2_{sample}}{OD_{sample} \cdot F_{ref} \cdot n^2_{ref}} \bullet \phi_{ref}$$

ϕ : fluorescence quantum yield

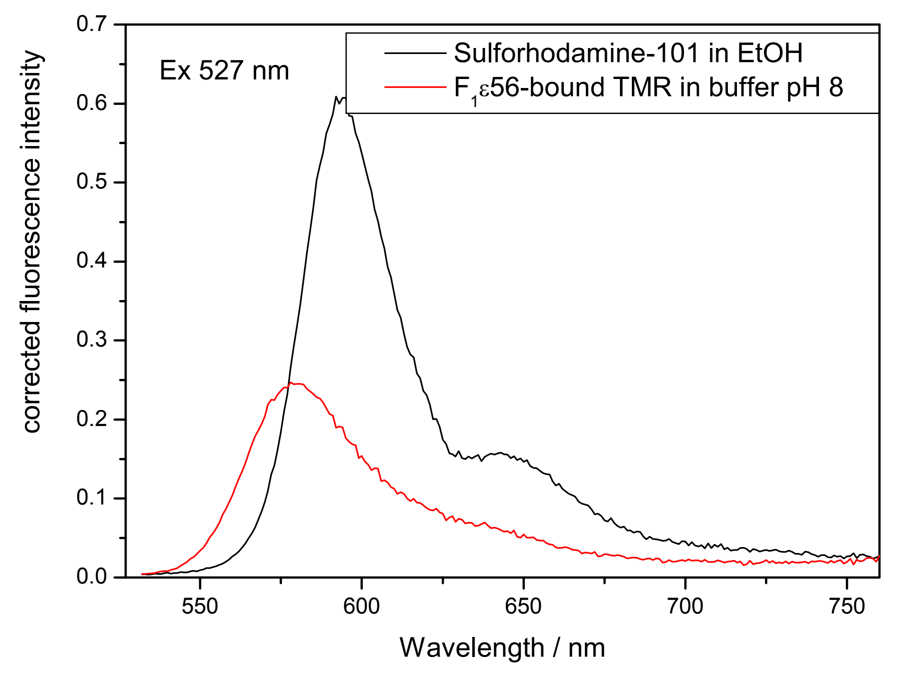
F : integrated fluorescence intensity, excitation at λ_{EX}

OD: optical density of the solution at λ_{EX}

n: index of refraction (H_2O: 1.33)

quantum yield measurements (2)

determing ϕ @ λ_{EX} (527nm)



$\phi_{ref,\ rh101}$	1.00
OD_{ref}	0.332
F_{ref}	31.582
n^2 (EtOH)	1.8496
OD_{sample} TMR@F_1-ATPase	0.488
F_{sample}	16.043
n^2 (buffer)	1.7689
ϕ_{sample}	0.361

quantum yield measurements (3)

determing ϕ_d^0 : wavenumbers $\tilde{\nu}$ or wavelengths λ?

depending on the monochromator type

- prism based: use wavenumbers
- grating mono: use wavelengths

conversion problem for finite bandwidths ?

$$F_\lambda(\lambda_F)\Delta\lambda_F = F_{\tilde{\nu}}(\tilde{\nu}_F)\Delta\tilde{\nu}_F$$

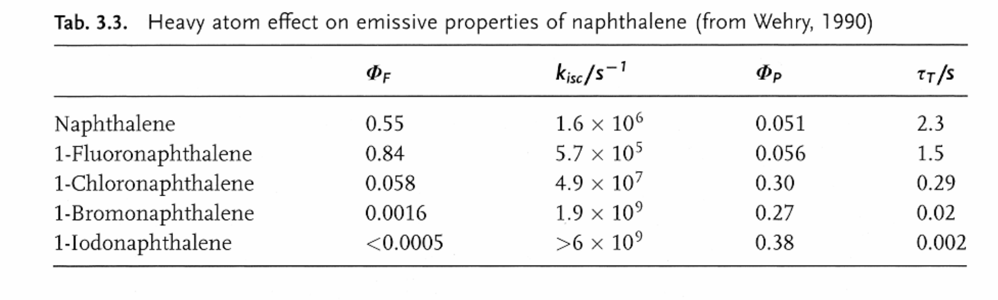
$$\Delta\tilde{\nu}_F = \frac{1}{\lambda_F} - \frac{1}{(\lambda_F + \Delta\lambda_F)} \geq 0,$$

$$F_\lambda(\lambda_F)\Delta\lambda_F = F_{\tilde{\nu}}(\tilde{\nu}_F)\frac{1}{\lambda_F(\lambda_F + \Delta\lambda_F)}$$

with $\Delta\lambda_F \ll \lambda_F$ *follows*

$$F_\lambda(\lambda_F)\boxed{\lambda_F^2} = F_{\tilde{\nu}}(\tilde{\nu}_F)$$

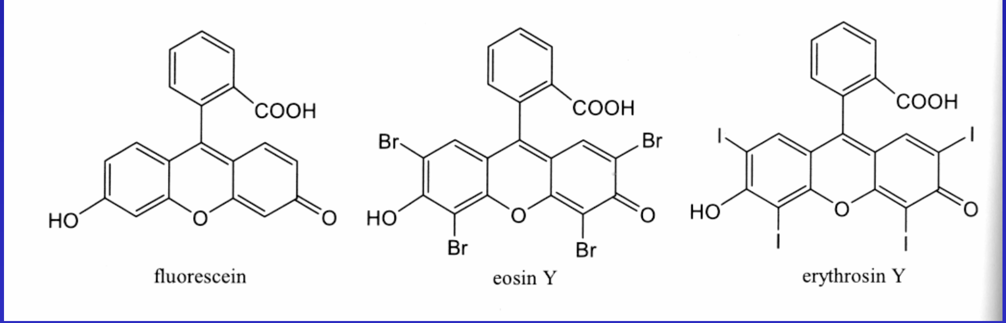
structure of fluorophores: quantum yields of rhodamines



Tab. 3.3. Heavy atom effect on emissive properties of naphthalene (from Wehry, 1990)

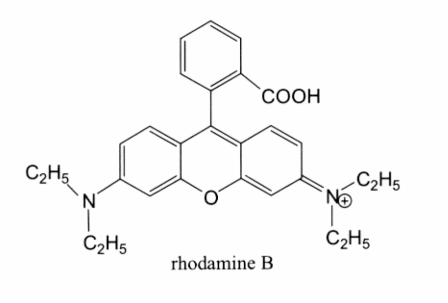
	Φ_F	k_{isc}/s^{-1}	Φ_P	τ_T/s
Naphthalene	0.55	1.6×10^6	0.051	2.3
1-Fluoronaphthalene	0.84	5.7×10^5	0.056	1.5
1-Chloronaphthalene	0.058	4.9×10^7	0.30	0.29
1-Bromonaphthalene	0.0016	1.9×10^9	0.27	0.02
1-Iodonaphthalene	<0.0005	$>6 \times 10^9$	0.38	0.002

internal quenching
by 'heavy atom effect'
(ISC, triplett formation)
due to spin-orbit-coupling,
depends on Z^4

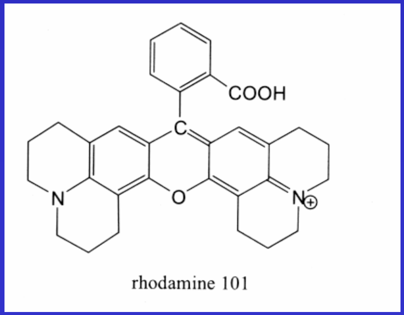


fluorescein eosin Y erythrosin Y

decreasing ϕ_d →



rhodamine B

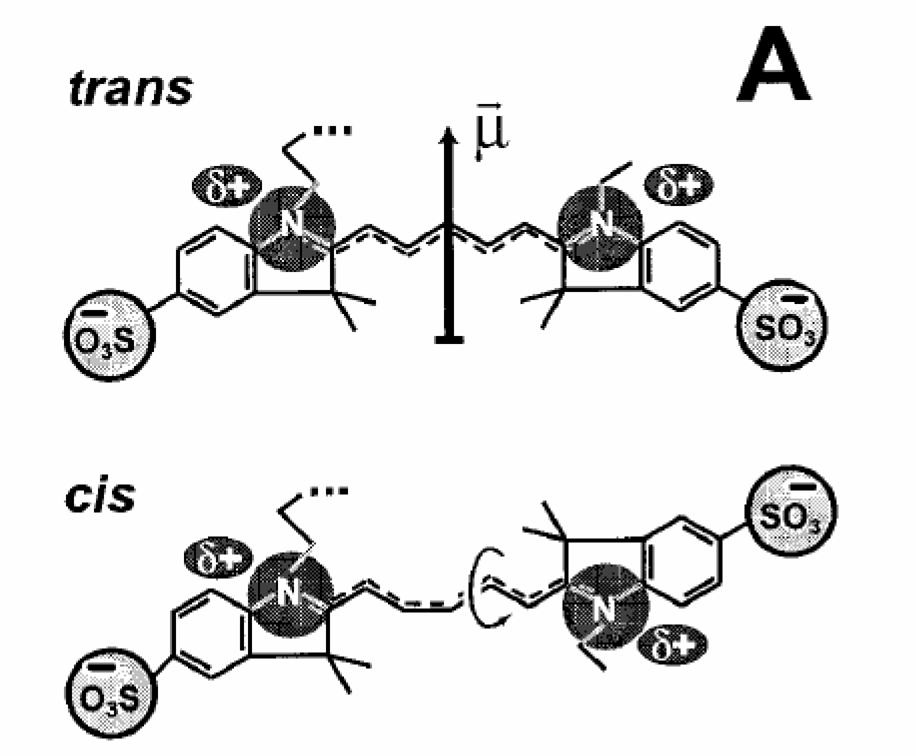


rhodamine 101

increasing ϕ_d →

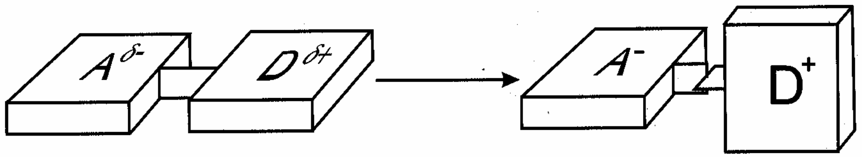
rigid structure reduces
internal conversion
(vibronic relaxation)

photoisomerization of Cy5

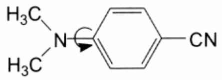


only *trans*-form is fluorescent, rate of photo-isomerization is EX power dependend
(CAM Seidel et al. J.Phys.Chem A 105, 6851-66)

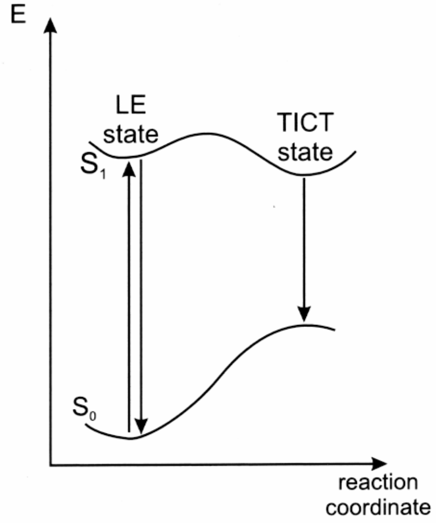
twisted intramolecular charge transfer (TICT)

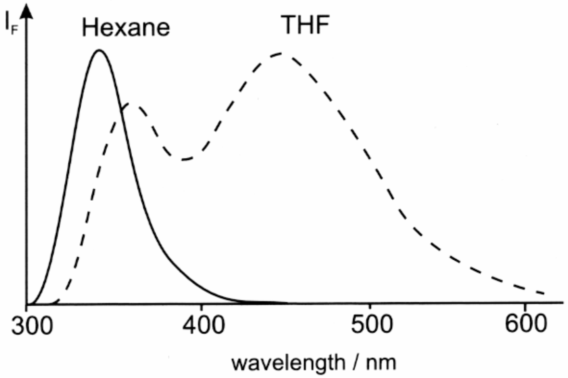


Scheme 3.3

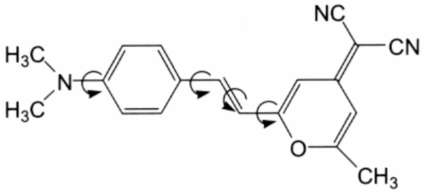


DMABN

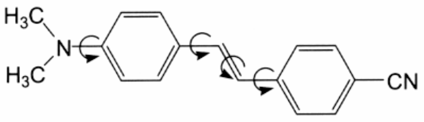




electron-donor and acceptor groups within the fluorophore, two emitting states result in dual fluorescence



DCM



DCS

intermolecular or collisional quenching (1)

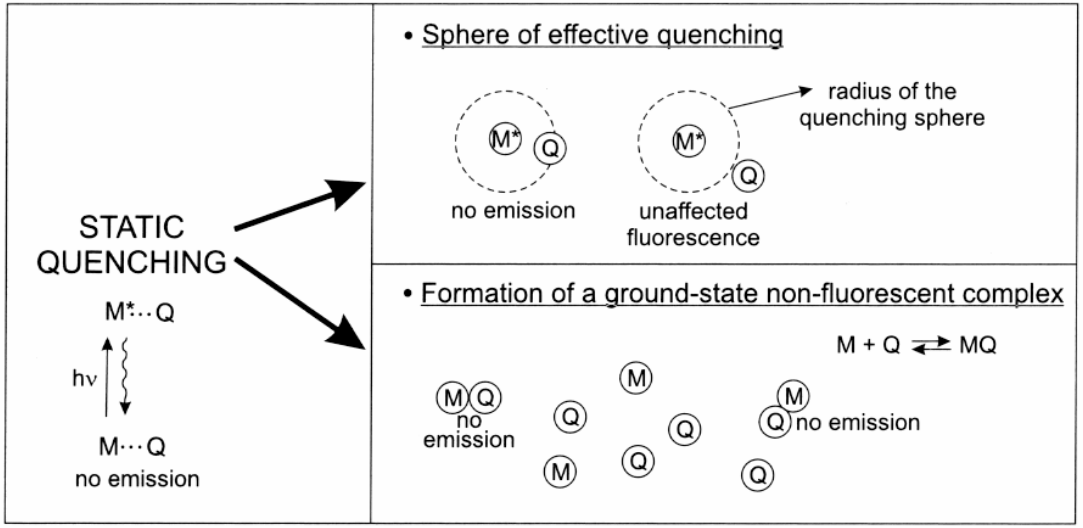
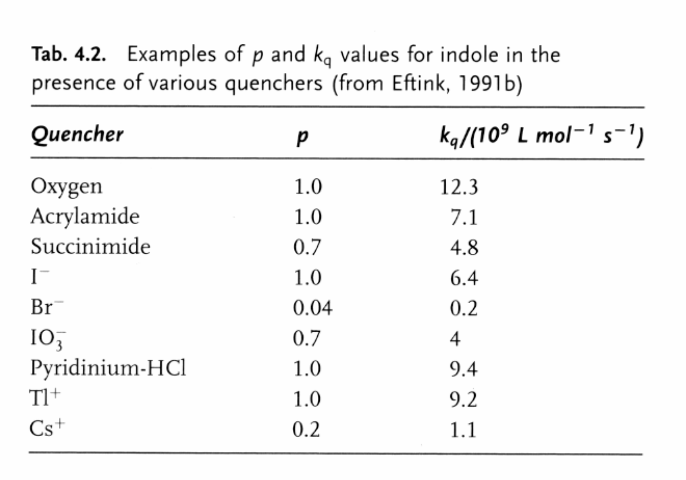


Fig. 4.1. Illustration of static quenching.



Tab. 4.2. Examples of p and k_q values for indole in the presence of various quenchers (from Eftink, 1991b)

Quencher	*p*	$k_q/(10^9\ \mathrm{L\ mol^{-1}\ s^{-1}})$
Oxygen	1.0	12.3
Acrylamide	1.0	7.1
Succinimide	0.7	4.8
I^-	1.0	6.4
Br^-	0.04	0.2
IO_3^-	0.7	4
Pyridinium-HCl	1.0	9.4
Tl^+	1.0	9.2
Cs^+	0.2	1.1

- collision with a heavy atom
- electron transfer
- proton transfer
- self-quenching (excimer = excited dimer)

collisional quenching (2)

static quenching:

at high conc. of Q,
Q is always adjecent to the fluorophore,
pseudo-first-order kinetics

dynamic quenching:

diffusion controlled,
mutual approach of Q,
second-order kinetics,

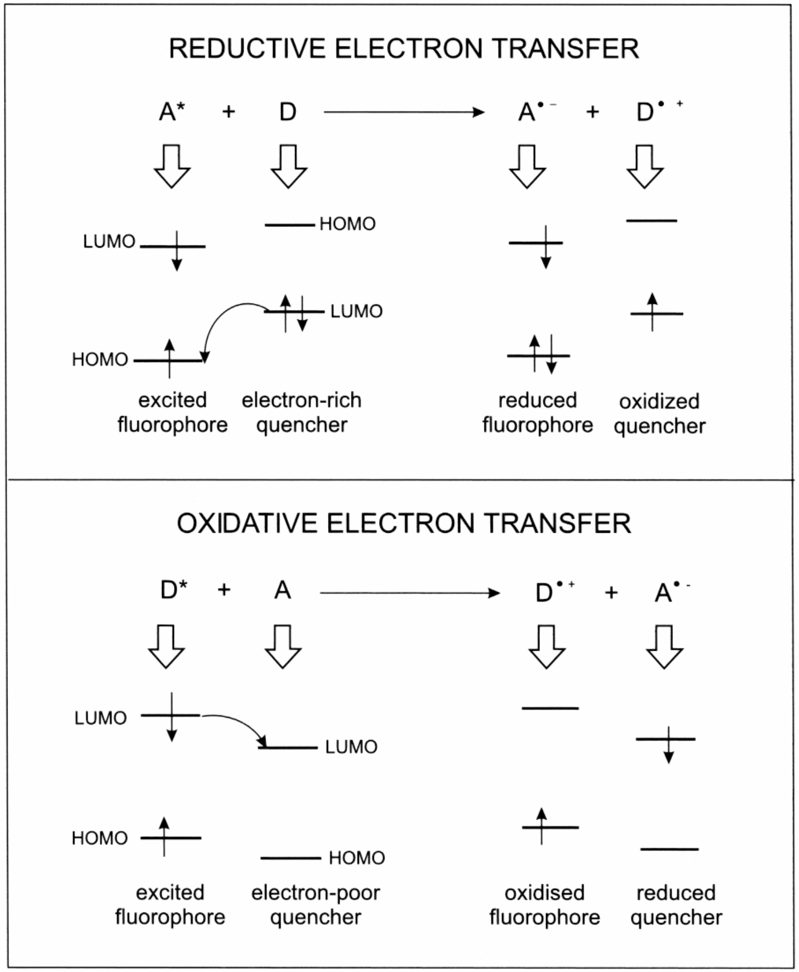
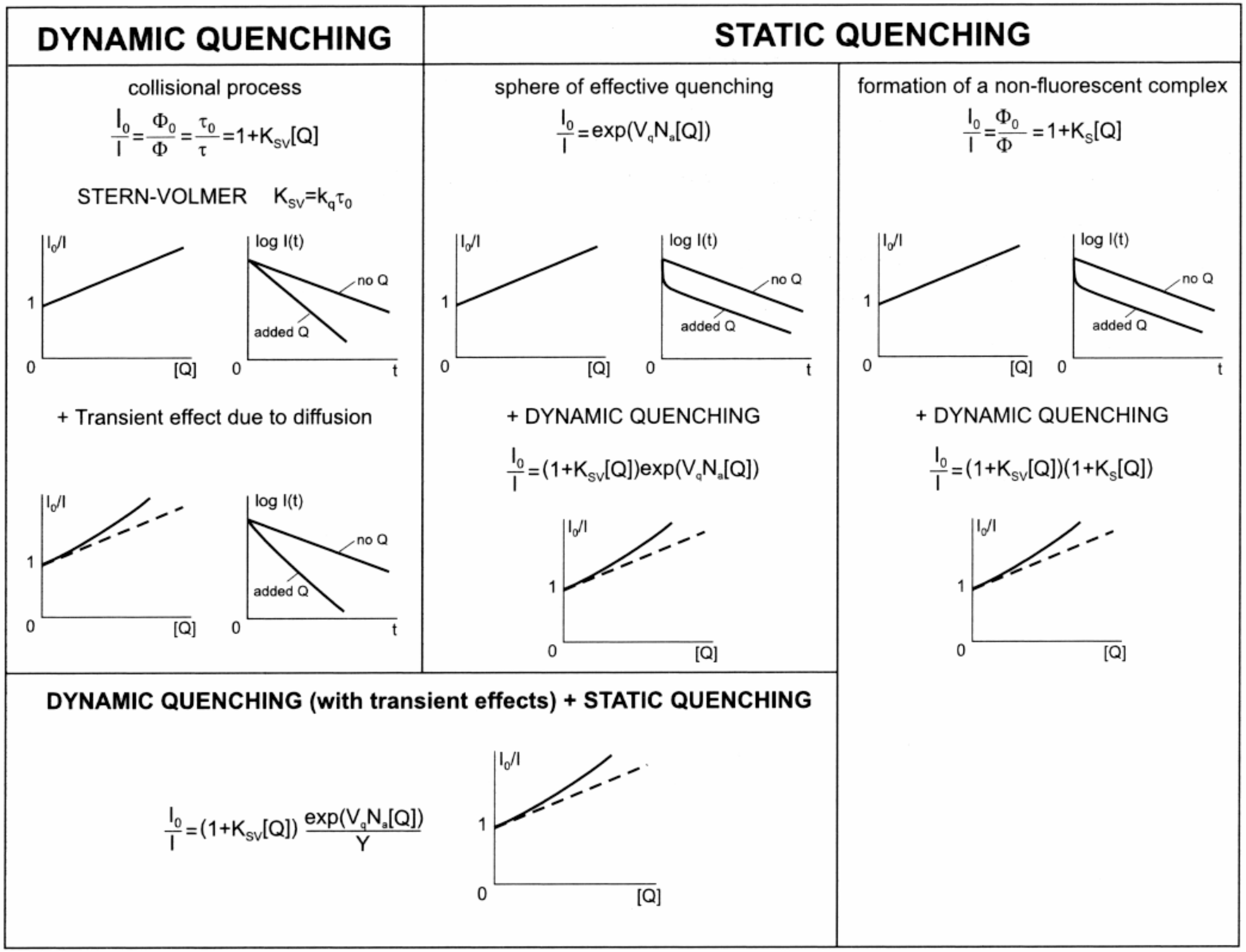


Fig. 4.3. Reductive and oxidative electron transfers.

quenching processes



DYNAMIC QUENCHING

collisional process

$$\frac{I_0}{I}=\frac{\Phi_0}{\Phi}=\frac{\tau_0}{\tau}=1+K_{SV}[Q]$$

STERN-VOLMER $K_{SV}=k_q\tau_0$

+ Transient effect due to diffusion

STATIC QUENCHING

sphere of effective quenching

$$\frac{I_0}{I}=\exp(V_qN_a[Q])$$

+ DYNAMIC QUENCHING

$$\frac{I_0}{I}=(1+K_{SV}[Q])\exp(V_qN_a[Q])$$

formation of a non-fluorescent complex

$$\frac{I_0}{I}=\frac{\Phi_0}{\Phi}=1+K_S[Q]$$

+ DYNAMIC QUENCHING

$$\frac{I_0}{I}=(1+K_{SV}[Q])(1+K_S[Q])$$

DYNAMIC QUENCHING (with transient effects) + STATIC QUENCHING

$$\frac{I_0}{I}=(1+K_{SV}[Q])\,\frac{\exp(V_qN_a[Q])}{Y}$$

Fig. 4.2. Distinction between dynamic and static quenching.

quantum yield measurements (4)

determing ϕ_d^0 : the quantum yield of donor fluorophore in the absence of FRET acceptor

methods: 1) integration of the fluorescence spectrum and subsequent normalization using a standard

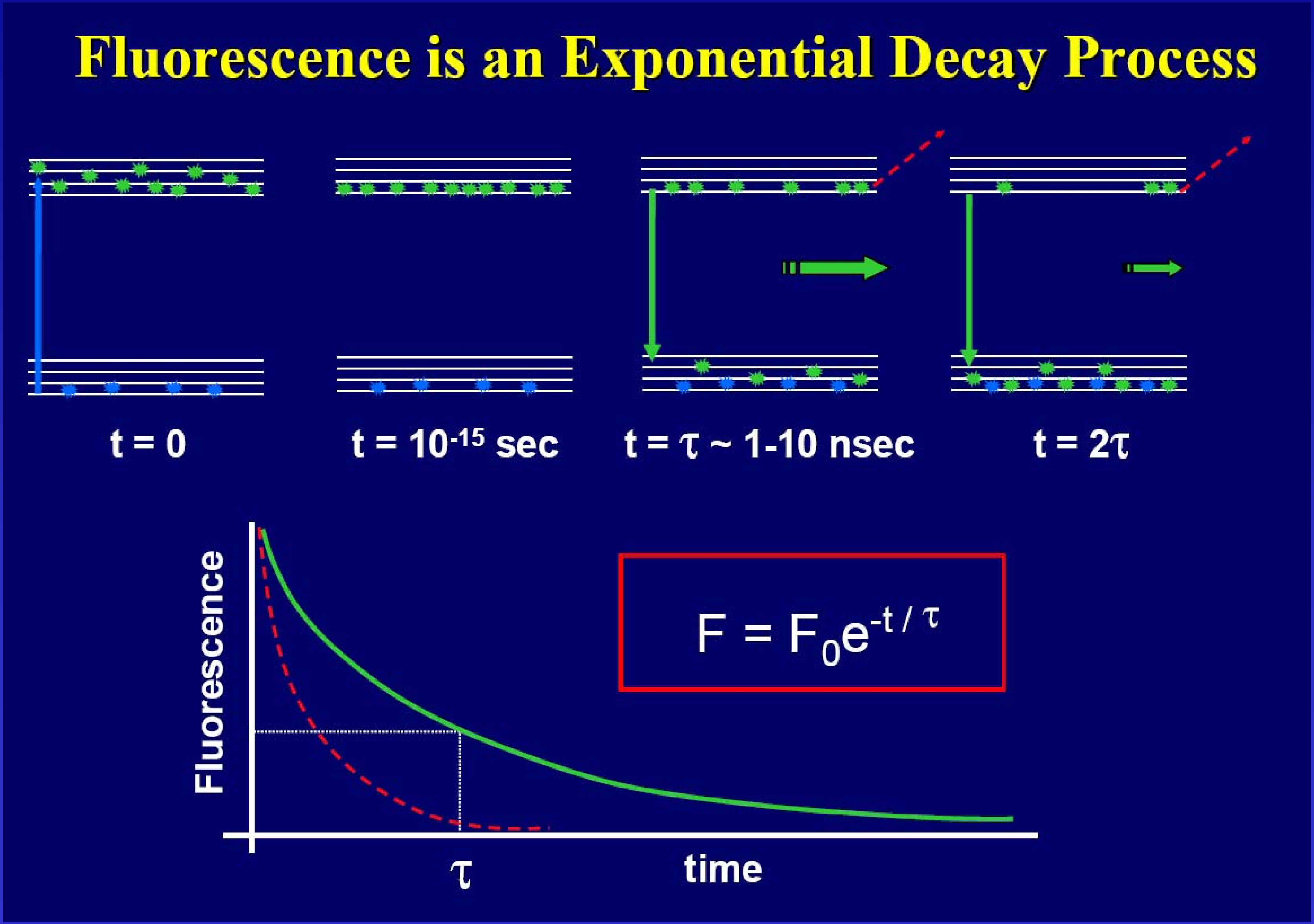
2) lifetime measurements

τ_S : lifetime of fluorescence

τ_0 : radiative lifetime in the absence of non-radiative de-excitation or calculated, Strickler-Berg relation J. Chem. Phys. 37, 814 (1962)

$$\phi = \frac{\tau_S}{\tau_0}$$

fluorescence lifetime



Fluorescence is an Exponential Decay Process

t = 0

t = 10^{-15} sec

t = τ ~ 1-10 nsec

t = 2τ

$$F = F_0 e^{-t/\tau}$$

lifetime measurements

- pulsed excitation: ps-/ fs laser with MHz repetition rate
- fluorophores with ns lifetime
- SPAD (or fast PMT for single photon counting)
- TCSPC : time correlated single photon counting
  electronic with ps time resolution / MHz count rates
- programs for online analysis

SMD: time resolution of detection << lifetime <<
overall detection time of a single molecule (burst)

TCSPC with PC card

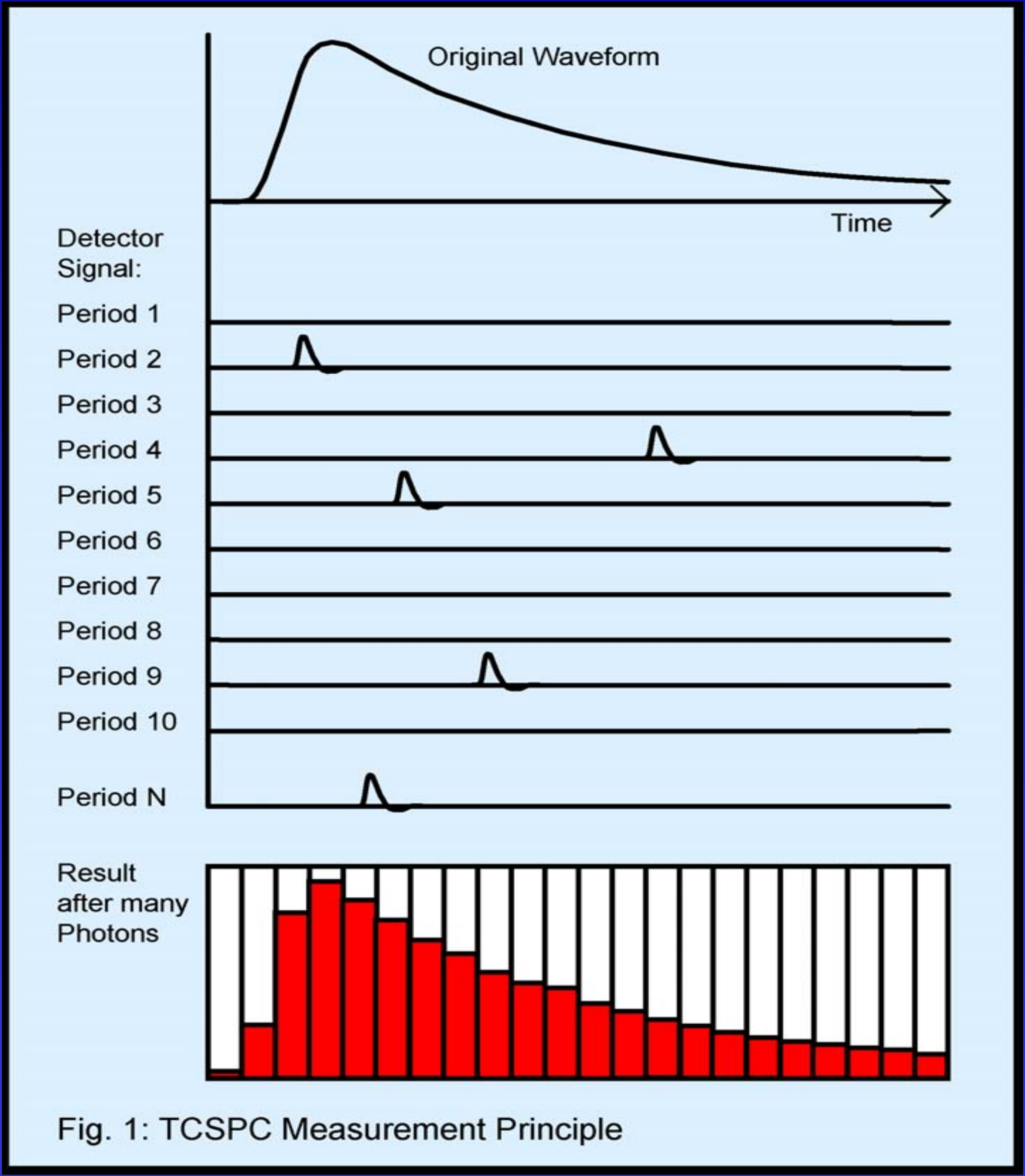


Fig. 1: TCSPC Measurement Principle

limits using lifetimes

- measurements of τ_0
- static quenching of fluorophores result in decreases quantum yield, but not always measurable in fluorescence lifetime

Förster radius R_0 and donor quantum yield

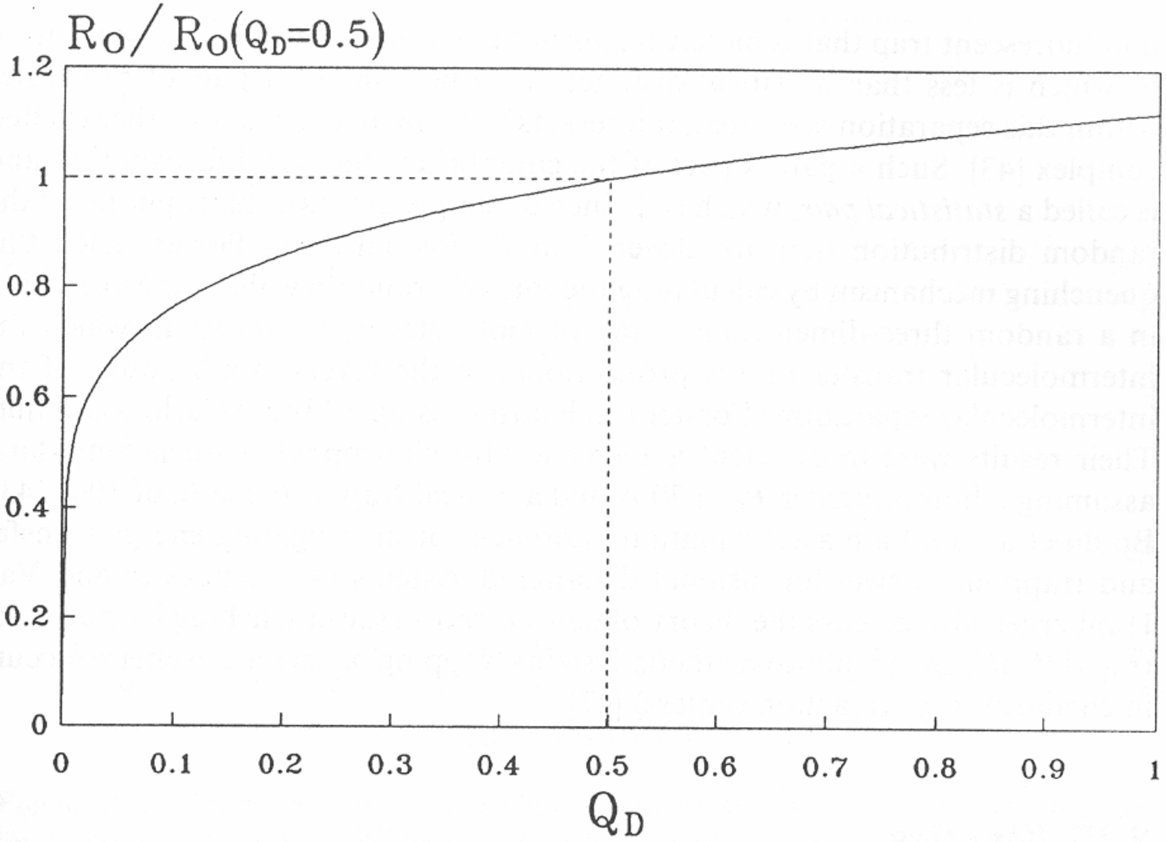


Figure 2.10. The relative Förster distance versus Q_D, the quantum yield of the donor in the absence of acceptor. R_0 denotes the Förster distance and $R_0(Q_D = 0.5)$ is the Förster distance at $Q_D = 0.5$. Q_D varies between 0 and 1 (see Chapter 7 for data).

fluorescence resonance energy transfer : FRET (7)

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 N n^4} J$$

Förster radius R_0 depends on

ϕ_d^0 : the quantum yield of donor fluorophore
in the absence of FRET acceptor

κ^2 : orientational factor of transition dipoles

J : spectral overlap

n : refractive index

fluorescence resonance energy transfer : FRET (8)

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 N n^4} J$$

spectral overlap (depends on λ):

→

$$J = \int_0^\infty F_d(\lambda)\varepsilon_a(\lambda)\lambda^4\, d\lambda$$

Spectral overlap

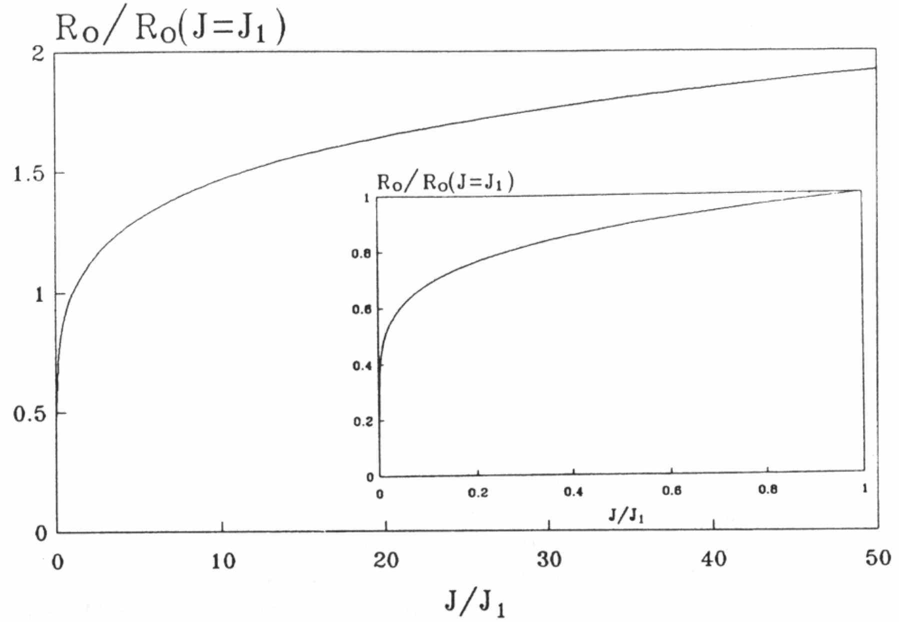


Figure 2.7. The relative Förster distance versus the relative overlap integral, J/J_1, where J is the overlap, and J_1 represents a typical value of this integral—say, 10^{14} M^{-1} cm^{-1} nm^4. R_0 denotes the Förster distance and $R_0(J/J_1 = 1)$ is the Förster distance if $J = J_1$. J-values vary over a wide range (see Chapter 7).

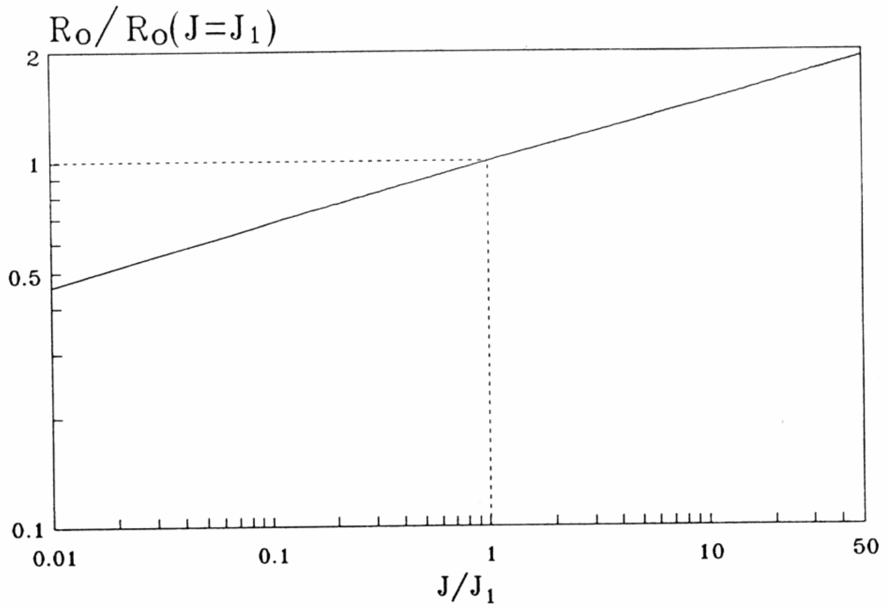


Figure 2.8. The relative Förster distance versus the relative overlap integral on a log–log scale. The symbols have the same meaning as in Figure 2.7.

Alexa dyes: R_0 depends on J

ε_A →

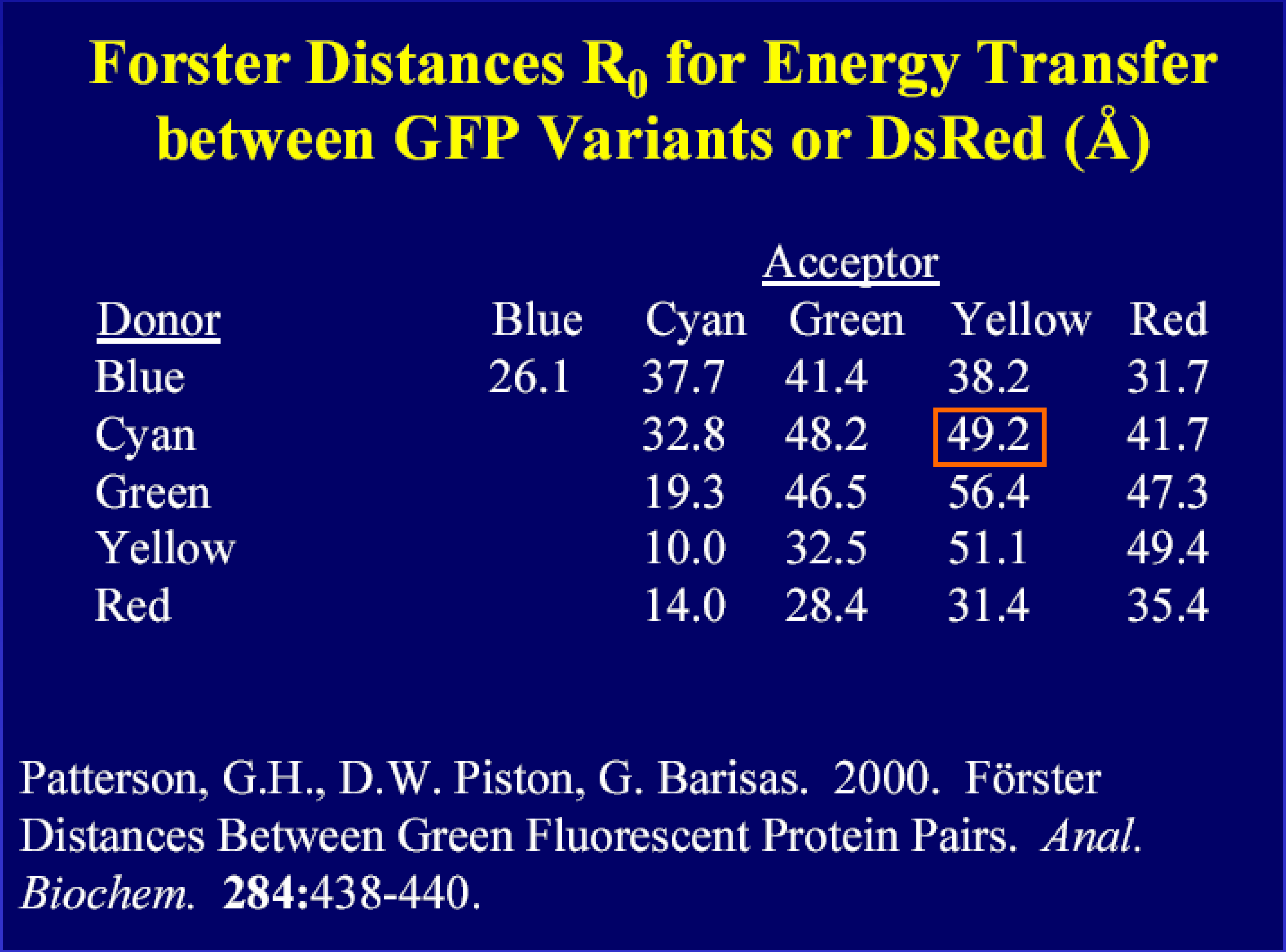
J ↓

Donor	**Acceptor**					
	Alexa Fluor 488	**Alexa Fluor 546**	**Alexa Fluor 555**	**Alexa Fluor 568**	**Alexa Fluor 594**	**Alexa Fluor 647**
Alexa Fluor 350	50					
Alexa Fluor 488	NA	64	70	62	60	56
Alexa Fluor 546		NA		70	71	74
Alexa Fluor 555			NA		47	51
Alexa Fluor 568				NA		82
Alexa Fluor 594					NA	85
Alexa Fluor 647						NA

* R_0 values in angstroms (Å) represent the distance at which fluorescence resonance energy transfer from the donor dye to the acceptor dye is 50% efficient. Values were calculated from spectroscopic data.

... and the donor quantum yield

R_0 of autofluorescent proteins



Forster Distances R_0 for Energy Transfer between GFP Variants or DsRed (Å)

	Acceptor				
Donor	Blue	Cyan	Green	Yellow	Red
Blue	26.1	37.7	41.4	38.2	31.7
Cyan		32.8	48.2	49.2	41.7
Green		19.3	46.5	56.4	47.3
Yellow		10.0	32.5	51.1	49.4
Red		14.0	28.4	31.4	35.4

Patterson, G.H., D.W. Piston, G. Barisas. 2000. Förster Distances Between Green Fluorescent Protein Pairs. *Anal. Biochem.* **284:**438-440.

fluorescence resonance energy transfer : FRET (9)

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^4 N n^4} J$$

Förster radius R_0 depends on

ϕ_d^0 : the quantum yield of donor fluorophore in the absence of FRET acceptor

κ^2 : orientational factor of transition dipoles

J : spectral overlap

n : refractive index

Förster radius R_0 and index of refraction n

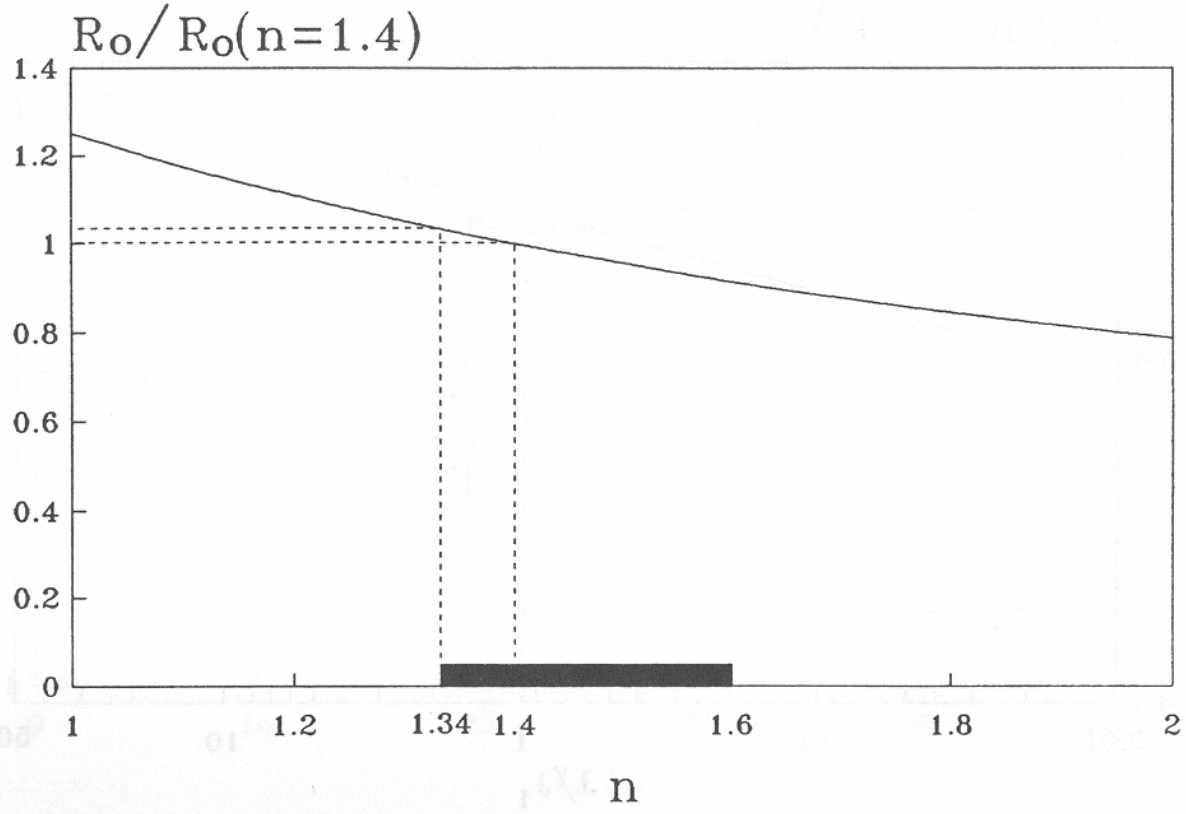


Figure 2.9. The relative Förster distance versus *n*, the refractive index of the medium in which the donor and acceptor are embedded. R_0 denotes the Förster distance and $R_0(n = 1.4)$ is the Förster distance at $n = 1.4$. All n-values in the literature are in the 1.34–1.6 range. The values 1.34 and 1.4 are the ones that are most frequently used.

correction for protein-embedded fluorophores ?

or:

spectral properties (J) already include protein environment.

fluorescence resonance energy transfer : FRET (10)

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 N n^4} J$$

Förster radius R_0 depends on

ϕ_d^0 : the quantum yield of donor fluorophore
in the absence of FRET acceptor

κ^2 : orientational factor of transition dipoles

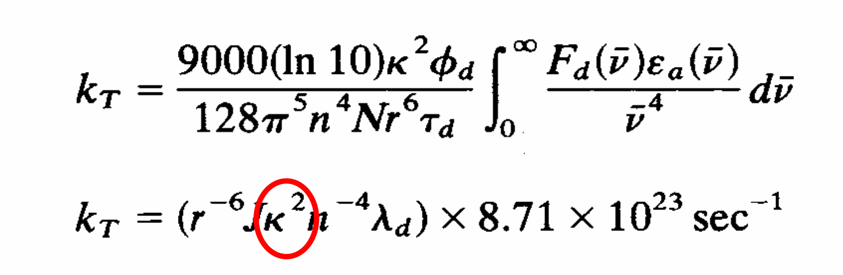
J : spectral overlap

n : refractive index

fluorescence resonance energy transfer : FRET (11)

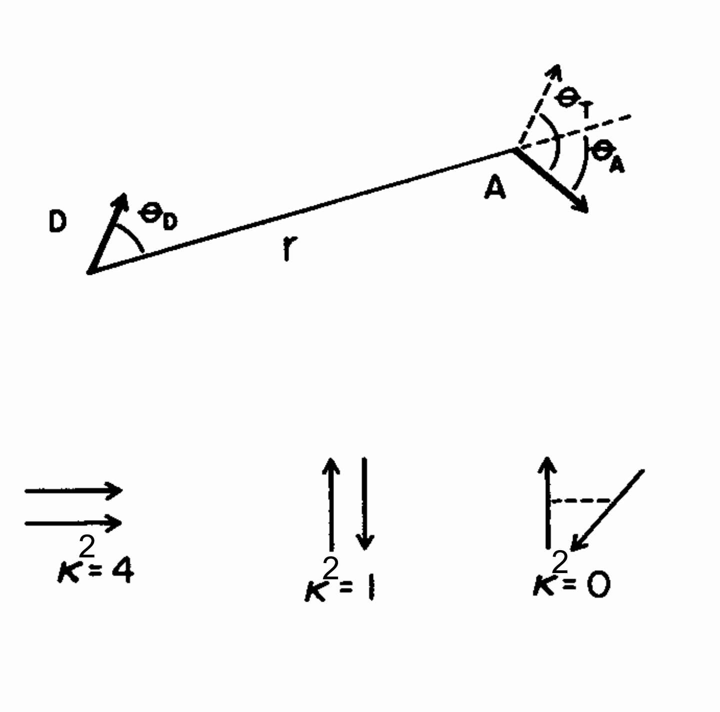
orientational factor κ^2

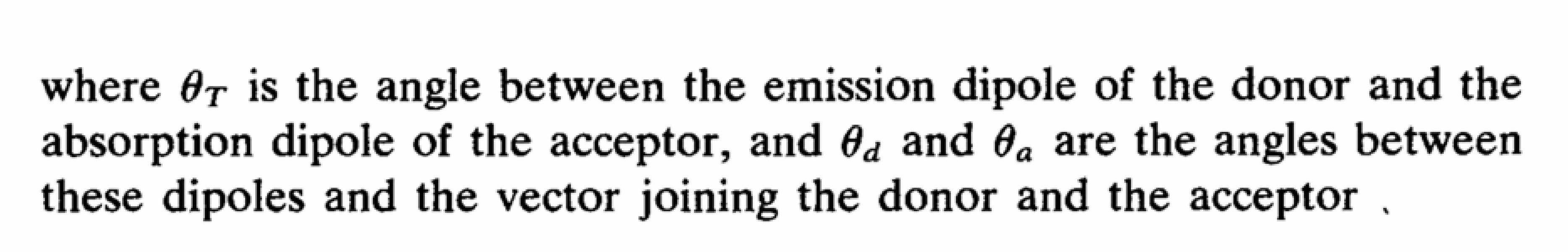
freely rotating: $\kappa^2 = 2/3$



$$k_T = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 n^4 N r^6 \tau_d}\int_0^\infty \frac{F_d(\bar{\nu})\varepsilon_a(\bar{\nu})}{\bar{\nu}^4}\,d\bar{\nu}$$

$$k_T = (r^{-6}J\boxed{\kappa^2}n^{-4}\lambda_d) \times 8.71 \times 10^{23}\ \mathrm{sec}^{-1}$$





where θ_T is the angle between the emission dipole of the donor and the absorption dipole of the acceptor, and θ_d and θ_a are the angles between these dipoles and the vector joining the donor and the acceptor .

measuring FRET efficiency

steady state:

- decrease in donor fluorescence
- comparison between absorption spectrum and the excitation spectrum (through observation of acceptor fluorescence)
- enhancement of acceptor fluorescence

time-resolved:

- decay of donor fluorescence
- increase in acceptor fluorescence

$$E = 1 - (\tau_{da}/\tau_d)$$

$$E = 1 - (F_{da}/F_d)$$

measuring FRET dependencies

J : spectral overlap

- G. Weber, F.J.W.Teale (1959) Discuss Faraday Soc. 27, 134-151, with fluorescently labelled heme proteins in ferri- and ferroheme forms;
- R.P. Haugland, J. Yguerabide, L. Stryer (1969) PNAS 63, 23-30

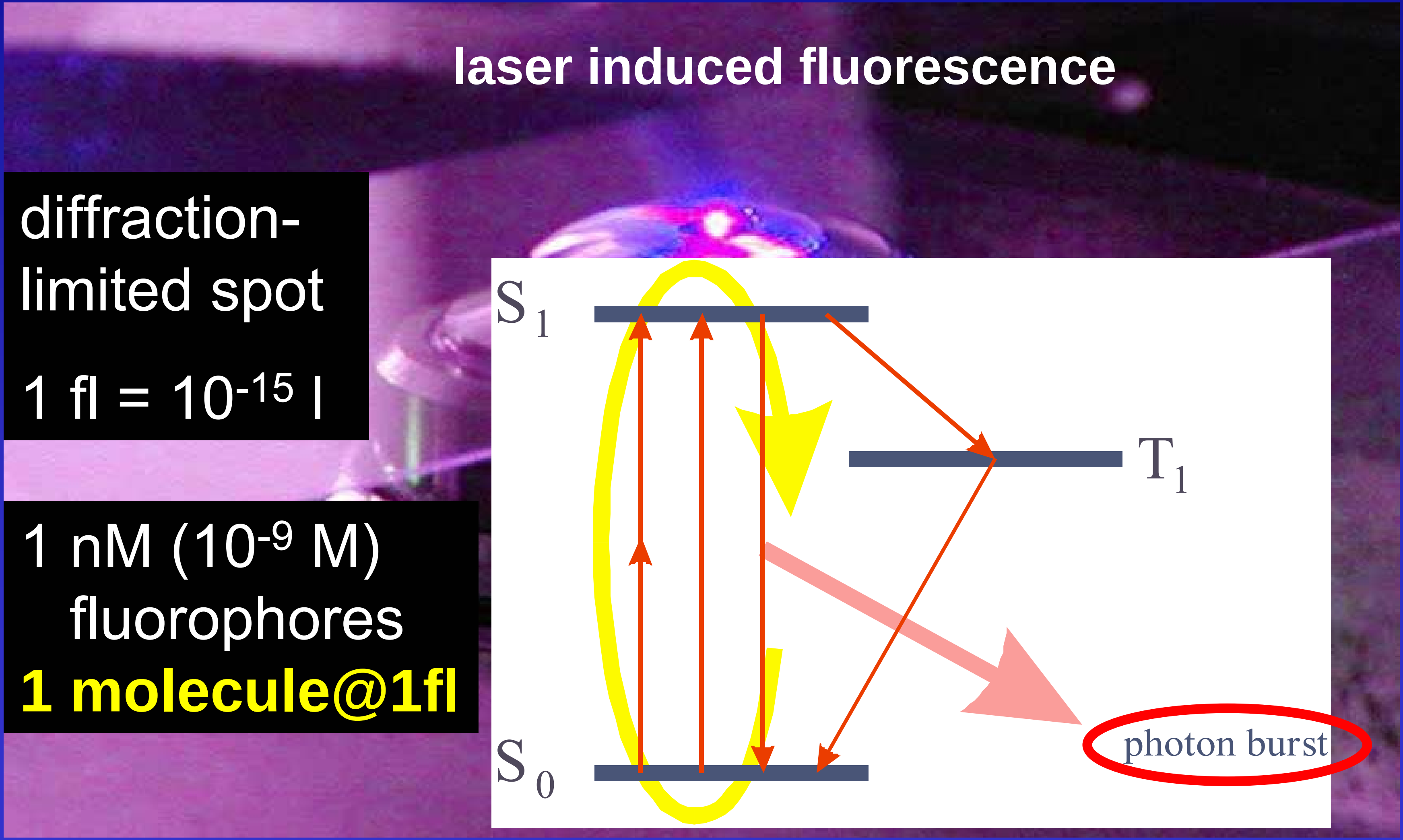
R^{-6} dependence

L.Stryer, R.P. Haugland, (1967) PNAS 58, 719-726

κ^2 : orientational factor of transition dipoles

- R.M. Hochstrasser, D.K. Negus (1984) PNAS 81, 4399-4403

principle of
single-molecule fluorescence detection



laser induced fluorescence

diffraction-limited spot

1 fl = 10^{-15} l

1 nM (10^{-9} M) fluorophores

1 molecule@1fl

single-molecule spectroscopy

- quantum yield = molecular brightness,
  number of photons per fluorophore
- triplett state population measurable by FCS
- collisional quenching = dark state / FCS
- spectral overlap: problem of spectral shifts, spectral diffusion = photon detection in two spectral regions (→ M. Sauer)

literature

- http://www.biophys.leidenuniv.nl/Teaching/Spectroscopy/Fluorescence.pdf (T.Schmidt)
- Bernard Valeur, Molecular Fluorescence, Wiley-VCH 2002
- B. Wieb van der Meer, George Coker III, S.-Y. Simon Chen, Resonance Energy Transfer; VCH 1994
- Joseph Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press

next 09/06/2003

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