Today’s class:

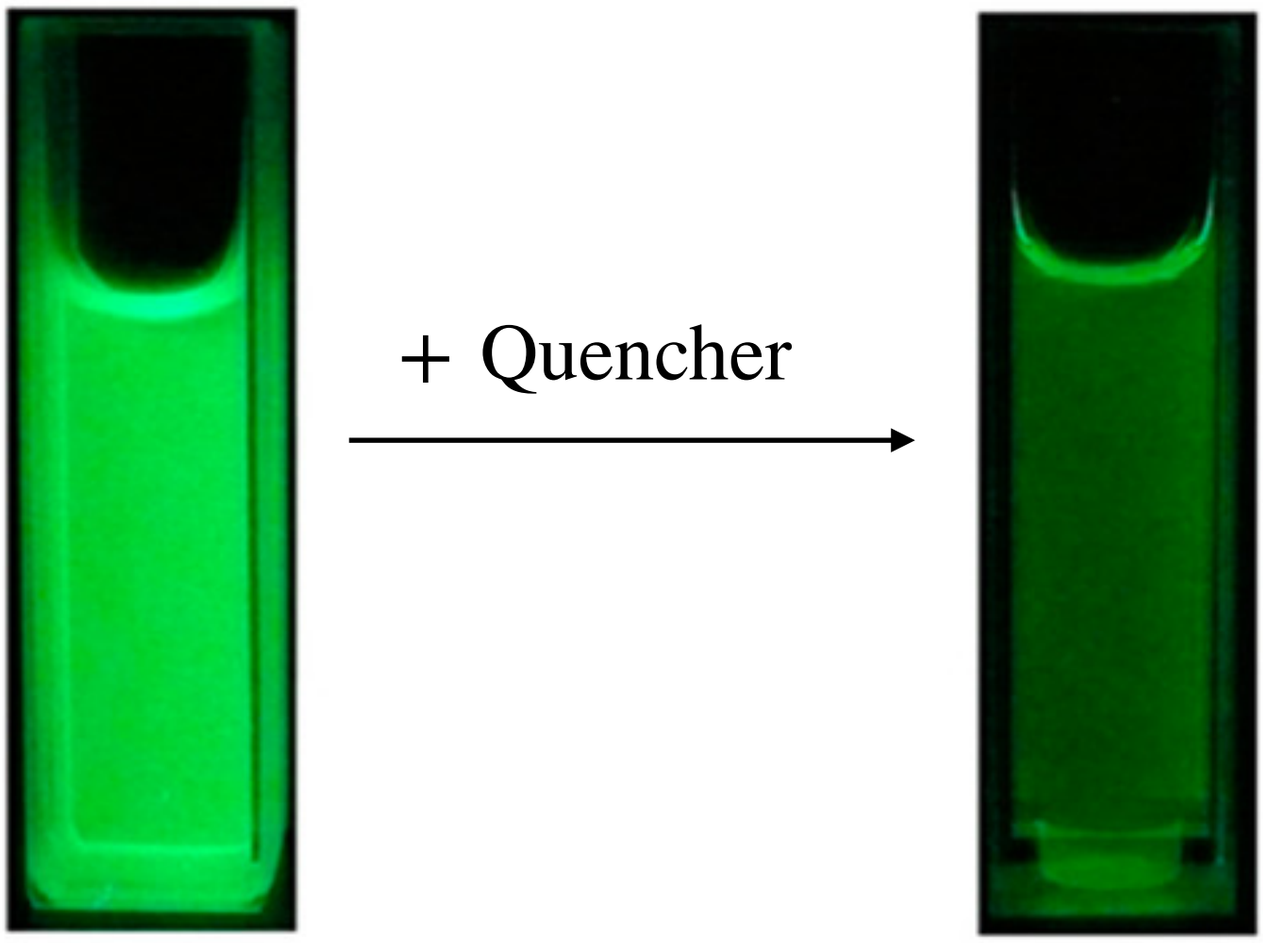
Bioluminescence part 3

This lecture follows the materials from the following books

- *Physical Chemistry for Life Sciences, by PW Atkins and JD Paula, Oxford, 2006*

Quenching of fluorescence

Fluorescence quenching is the non-radiative removal of the excitation energy from a fluorescent molecule and the elimination of its fluorescence.



Quenching can be a desired process if focusing on energy transfer or undesired side reaction decreasing the quantum yield of fluorescence

Quencher opens up a new channel of deactivation

Regular deactivation channels for fluorescence

Absorption: $S + h\nu_i \longrightarrow S^*$ $\quad v_{abs} = I_{abs}$

Fluorescence: $S^* \longrightarrow S + h\nu_f$ $\quad v_f = k_f[S^*]$

Intersystem crossing: $S^* \longrightarrow T^*$ $\quad v_{ISC} = k_{ISC}[S^*]$

Internal conversion: $S^* \longrightarrow S$ $\quad v_{IC} = k_{IC}[S^*]$

The addition of a quencher, Q, opens an additional channel for deactivation of S*:

Quenching: $S^* + Q \longrightarrow S + Q$ $\quad v_Q = k_Q[Q][S^*]$

Kinetics of quenching

The steady-state approximation for [S*] now gives

$$\frac{\mathrm{d}[\mathrm{S}^*]}{\mathrm{d}t} = I_{\mathrm{abs}} - (k_{\mathrm{f}} + k_{\mathrm{IC}} + k_{\mathrm{ISC}} + k_{\mathrm{IC}} + k_{\mathrm{Q}}[\mathrm{Q}])[\mathrm{S}^*] = 0$$

and the fluorescence quantum yield in the presence of the quencher is

$$\phi_{\mathrm{f}} = \frac{k_{\mathrm{f}}}{k_{\mathrm{f}} + k_{\mathrm{ISC}} + k_{\mathrm{IC}} + k_{\mathrm{Q}}[\mathrm{Q}]}$$

We can identify the fluorescence lifetime in the presence of quencher as

$$\tau = 1/(k_{\mathrm{f}} + k_{\mathrm{ISC}} + k_{\mathrm{IC}} + k_{\mathrm{Q}}[\mathrm{Q}])$$

When [Q] = 0, the quantum yield is

$$\phi_{\mathrm{f},0} = \frac{k_{\mathrm{f}}}{k_{\mathrm{f}} + k_{\mathrm{ISC}} + k_{\mathrm{IC}}}$$

← Quantum yield without the quencher

Kinetics of quenching …*contd*

It follows that

$$\frac{\phi_{f,0}}{\phi_f} = \left(\frac{k_f}{k_f + k_{ISC} + k_{IC}}\right) \times \left(\frac{k_f + k_{ISC} + k_{IC} + k_Q[Q]}{k_f}\right)$$

$$= \frac{k_f + k_{ISC} + k_{IC} + k_Q[Q]}{k_f + k_{ISC} + k_{IC}}$$

$$= 1 + \frac{k_Q}{k_f + k_{ISC} + k_{IC}}[Q]$$

Using definition of τ_0,

$$\tau_0 = \frac{1}{k_f + k_{ISC} + k_{IC}}$$

$$\frac{\phi_{f,0}}{\phi_f} = 1 + \tau_0 k_Q[Q]$$

Stern-Volmer equation

Lifetime of fluorescence without the quencher

Rate of quenching

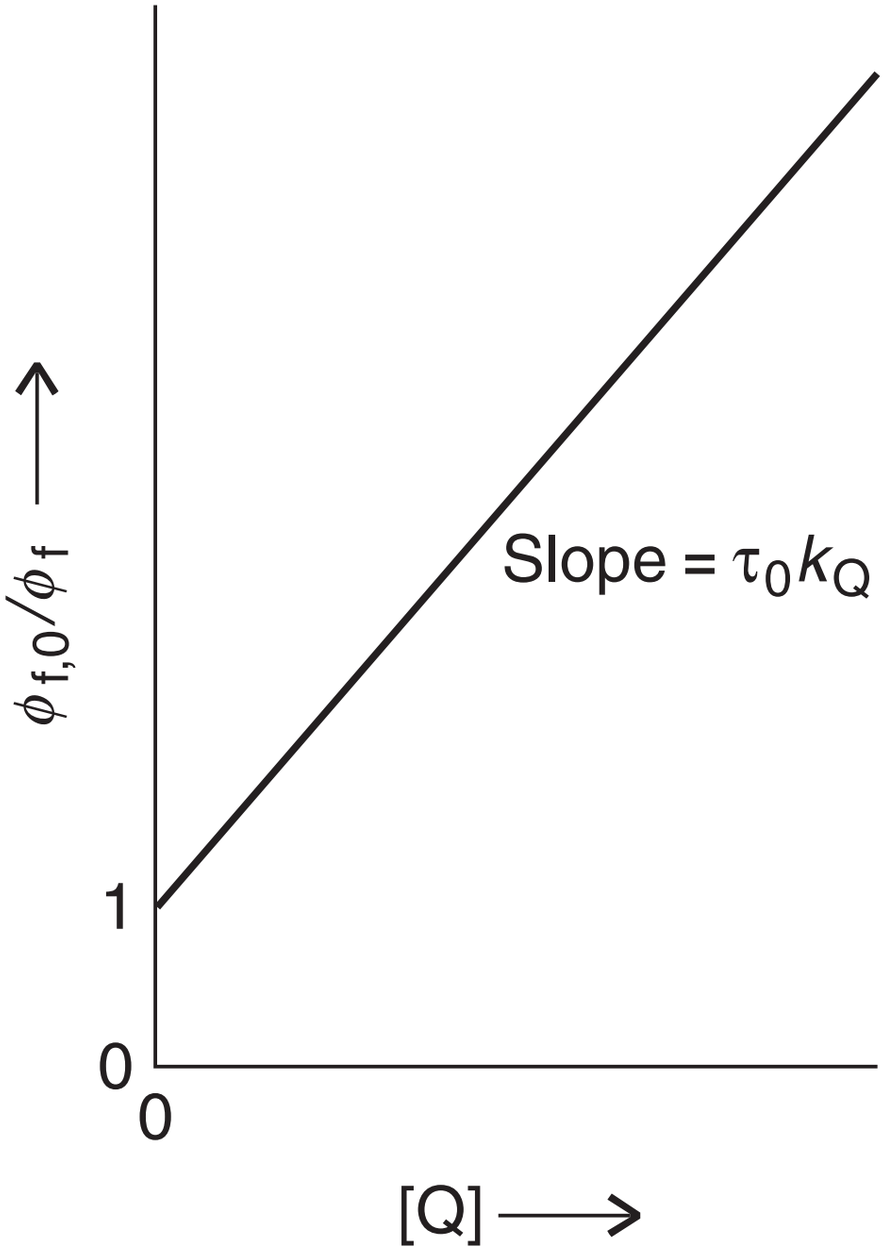
Molar conc of quencher

Stern-Volmer plot

Stern-Volmer equation

$$\frac{\phi_{f,0}}{\phi_f} = 1 + \tau_0 k_Q[Q]$$

Can also be applied for phosphorescence quenching



The SV formula sometimes written as

$$\frac{I_{F,0}}{I_F} = 1 + K_{SV}[Q]$$

Fluorescence intensity

where $K_{SV} = \tau_0 k_Q$

Called the Stern-Volmer constant, unit = M^{-1}

Determination of rate constant of quenching from fluorescence lifetime

The quenching of tryptophan fluorescence by dissolved O_2 gas was monitored by measuring emission lifetimes at 348 nm in aqueous solutions. Determine the quenching rate constant for this process from the following data:

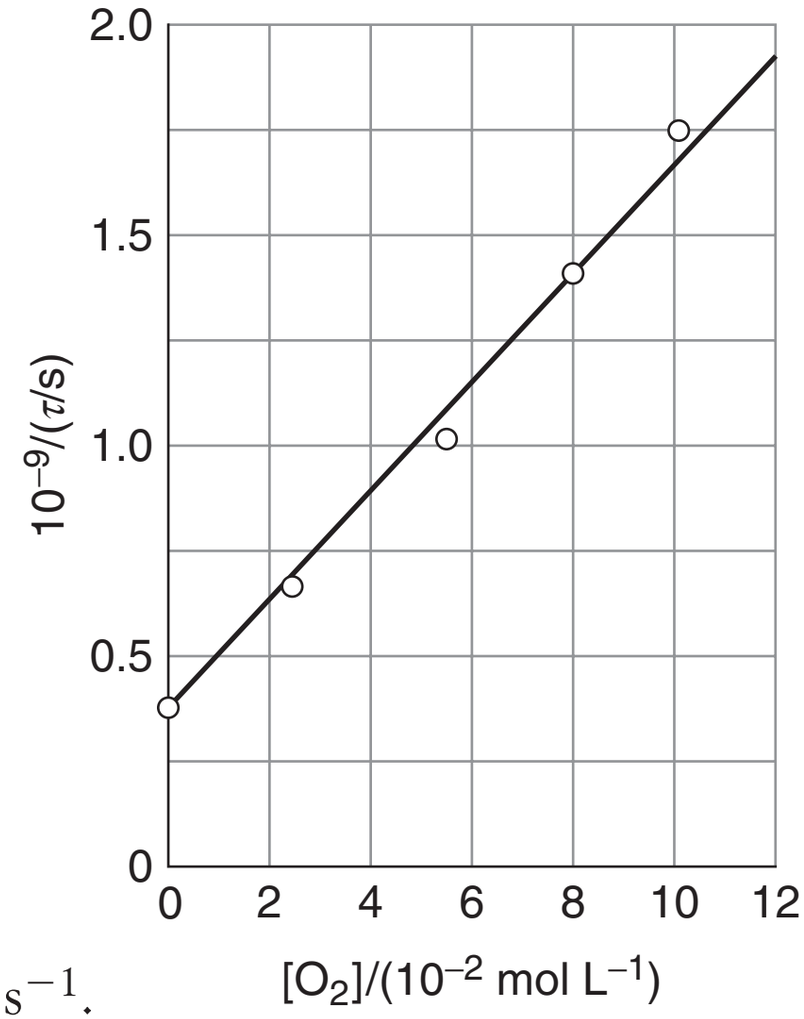
$[O_2]/(10^{-2}\ \text{mol L}^{-1})$	0	2.3	5.5	8	10.8
$\tau/(10^{-9}\ \text{s})$	2.6	1.5	0.92	0.71	0.57

From previous results, we know

$$\tau_0 = \frac{\phi_{f,0}}{k_f} \qquad \tau = \frac{\phi_f}{k_f}$$

$$\Longrightarrow \frac{\phi_{f,0}}{\phi_f} = \frac{\tau}{\tau_0}$$

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_Q[Q]$$



The slope of the line is 1.3×10^{10}, so $k_Q = 1.3 \times 10^{10}\ \text{L mol}^{-1}\ \text{s}^{-1}$.

Mechanisms of quenching

Three common mechanisms of deactivation of singlet or triplet state

Collisional deactivation

$$S^* + Q \rightarrow S + Q$$

Electron transfer

$$S^* + Q \rightarrow S^+ + Q^- \text{ or } S^- + Q^+$$

Resonance energy transfer

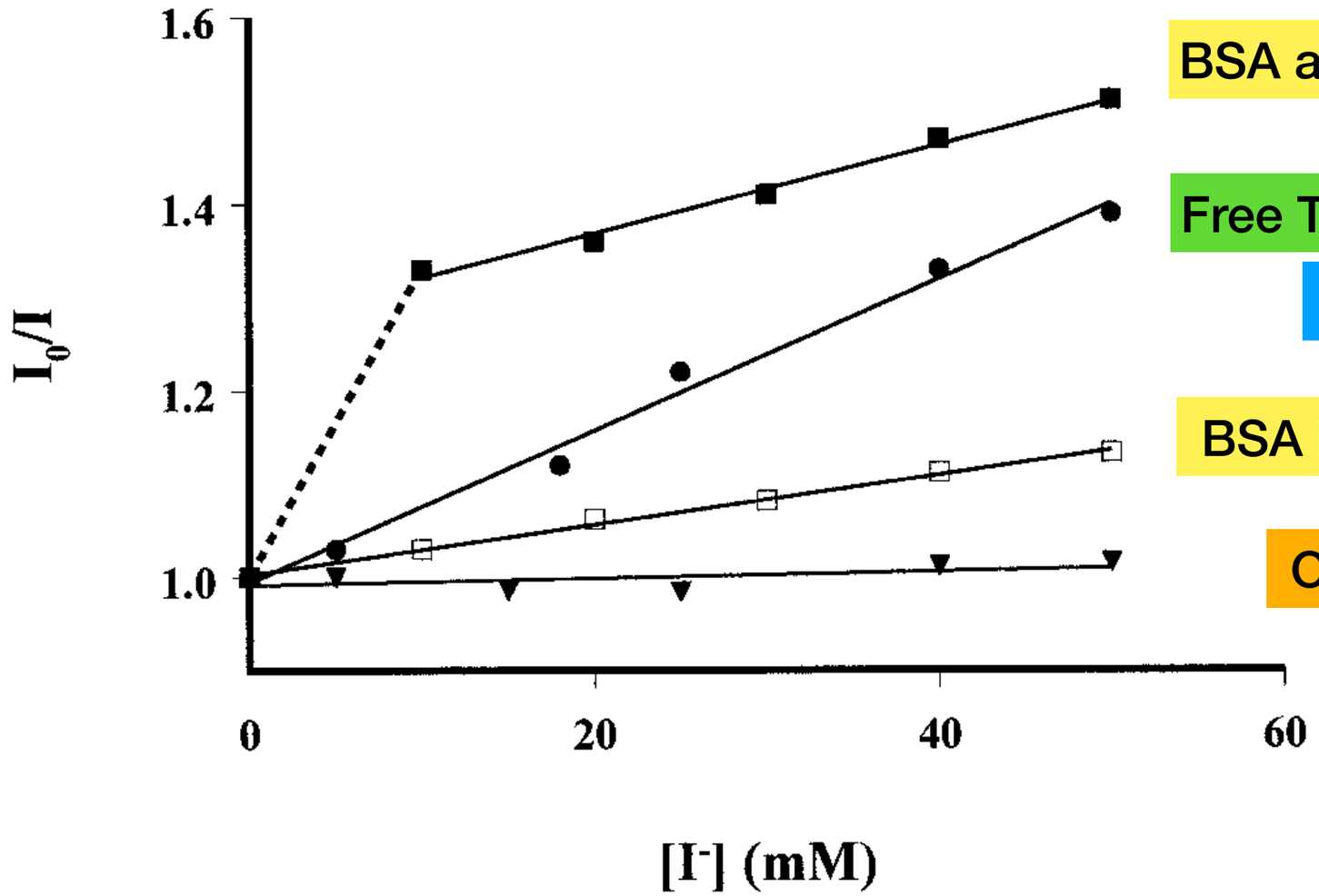
$$S^* + Q \rightarrow S + Q^*$$

Quenching of fluorescence by collision

$$S^* + Q \rightarrow S + Q$$

- Collisional quenching is particularly efficient when the quencher is a heavy species, such as iodide ion
- Here the quencher receives energy from the fluorescing species and then decays non-radiatively to the ground state.
- Used to determine the accessibility of specific locations on protein

Quantification of accessibility of amino acids through fl. quenching by iodide



BSA at low ionic strength

Free TRP in solution

Strong quenching

BSA in high ionic strength

Ovalbumin

All TRP are buried within the protein

Moeller et al BIOCHEMISTRY AND BIMOLECULAR BIOLOGY EDUCATION, 2002

The biphasic behavior of BSA-TRP fluorescence quenching shows there are two TRP populations with different accessibility

Modified Stern-Volmer equation for biphasic quenching

BSA has two TRP residues which are non-identical

We can write $\phi_f = \phi_f^1 + \phi_f^2$ for two residues

$$\implies \phi_f = \frac{\phi_{f,0}^1}{1 + K_{SV}^1[Q]} + \frac{\phi_{f,0}^2}{1 + K_{SV}^2[Q]}$$

If residue 2 is not accessible $\implies \phi_f = \frac{\phi_{f,0}^1}{1 + K_{SV}^1[Q]} + \phi_{f,0}^2$

In absence of quencher, $\phi_{f,0} = \phi_{f,0}^1 + \phi_{f,0}^2$

$$\implies \phi_f = \frac{\phi_{f,0}^1}{1 + K_{SV}^1[Q]} + \phi_{f,0} - \phi_{f,0}^1$$

Modified Stern-Volmer equation for biphasic quenching …*contd*

$$\phi_f = \frac{\phi_{f,0}^1}{1 + K_{SV}^1[Q]} + \phi_{f,0} - \phi_{f,0}^1$$

Reorganization gives:

$$\frac{\phi_{f,0}}{\Delta\phi_f} = \frac{1}{f_a}\frac{1}{K_{SV}^1[Q]} + \frac{1}{f_a} \qquad \text{where } \Delta\phi_f = \phi_{f,0} - \phi_f$$

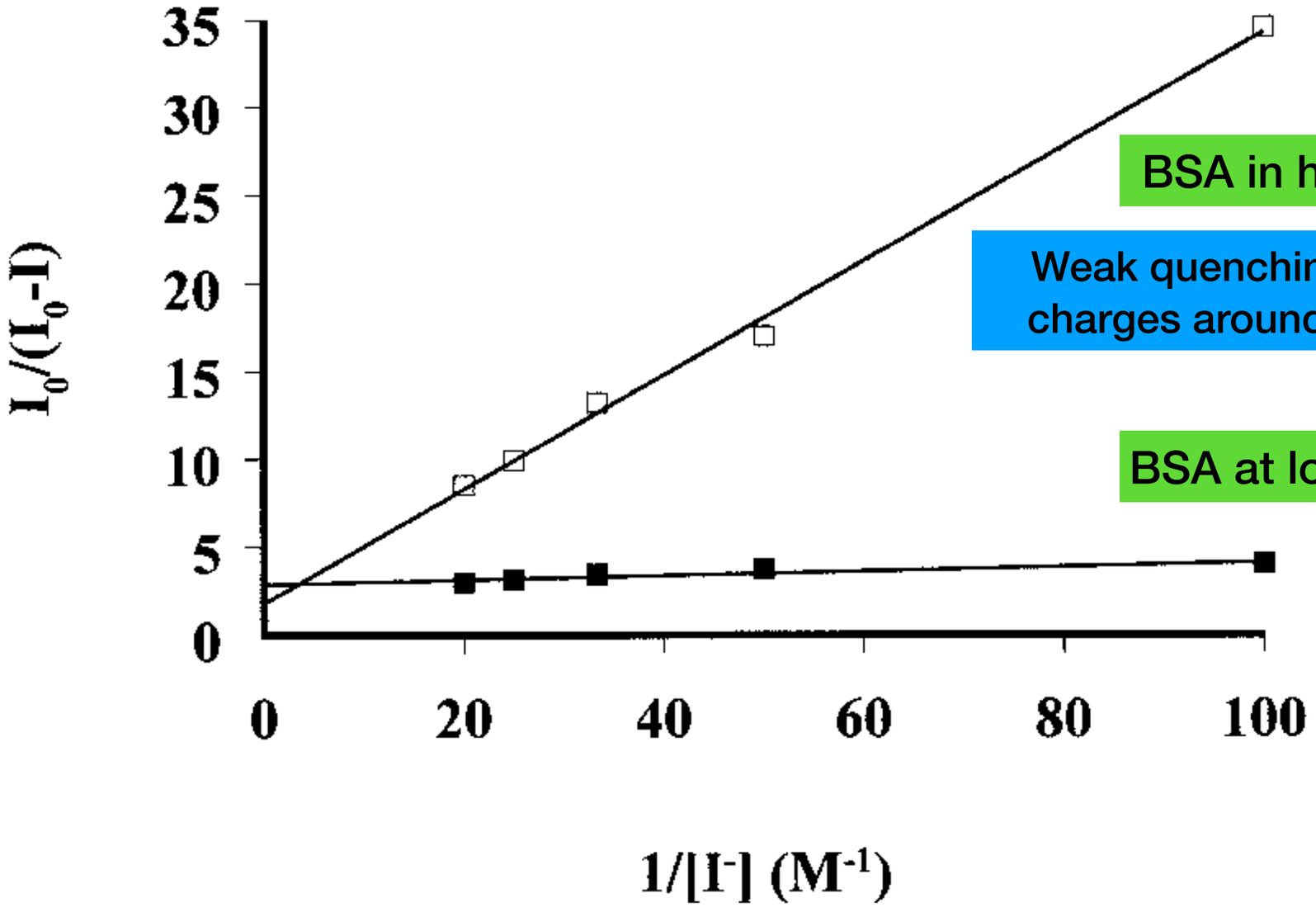
where $f_a = \dfrac{\phi_{f,0}^1}{\phi_{f,0}}$ the accessible fraction

the inaccessible fraction $f_b = 1 - f_a = \dfrac{\phi_{f,0} - \phi_{f,0}^1}{\phi_{f,0}} = \dfrac{\phi_{f,0}^2}{\phi_{f,0}}$

Thus a plot of $\phi_{f,0}/\Delta\phi_f$ vs $1/[Q]$ should be a straight line, providing the SV constant K_{SV}^1 for accessible TRP from slope and the accessible fraction from intercept

Modified Stern-Volmer equation for biphasic quenching …*contd*

Modified Stern-Volmer plot provides quenching information about just the accessible fraction



BSA in high ionic strength

Weak quenching because of other charges around TRP screen iodide

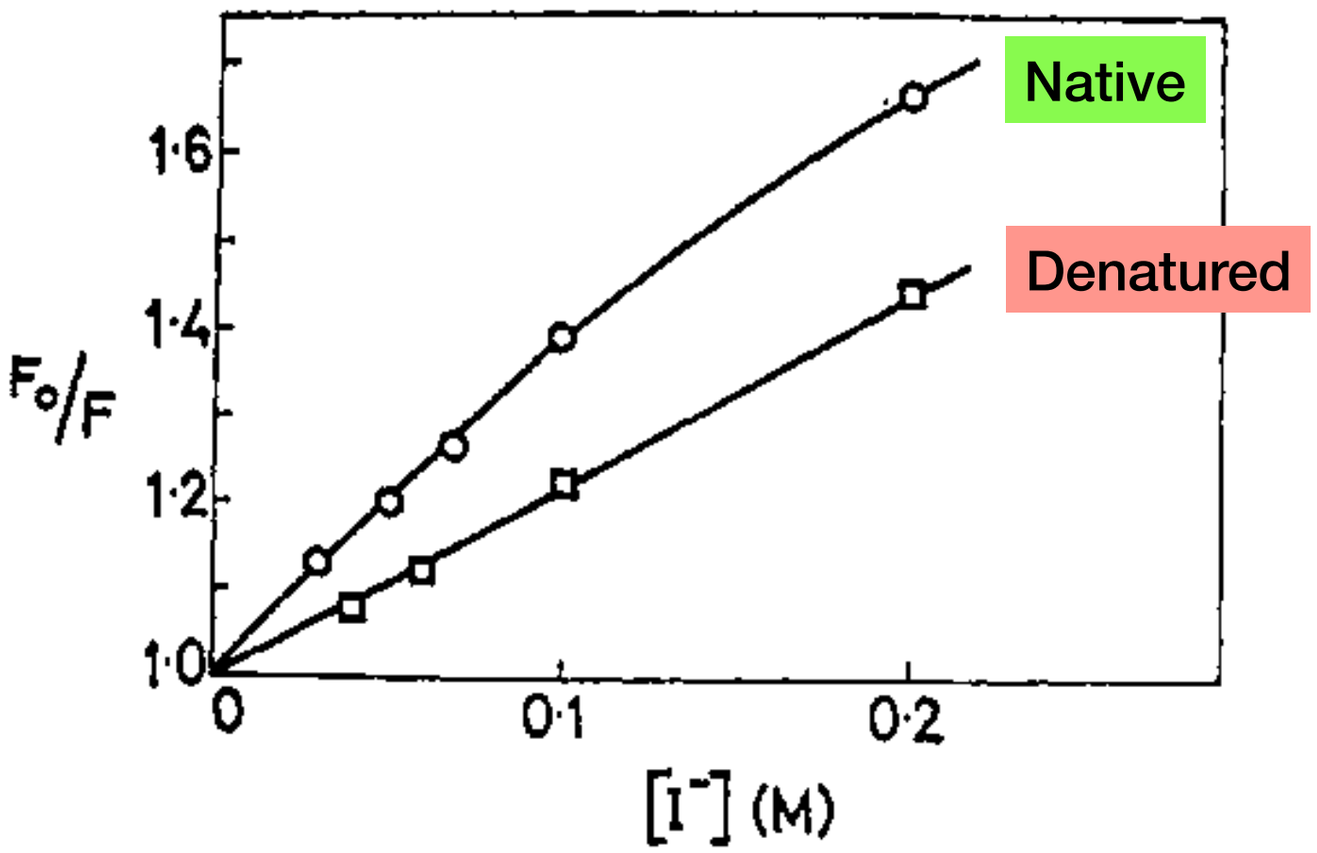
BSA at low ionic strength

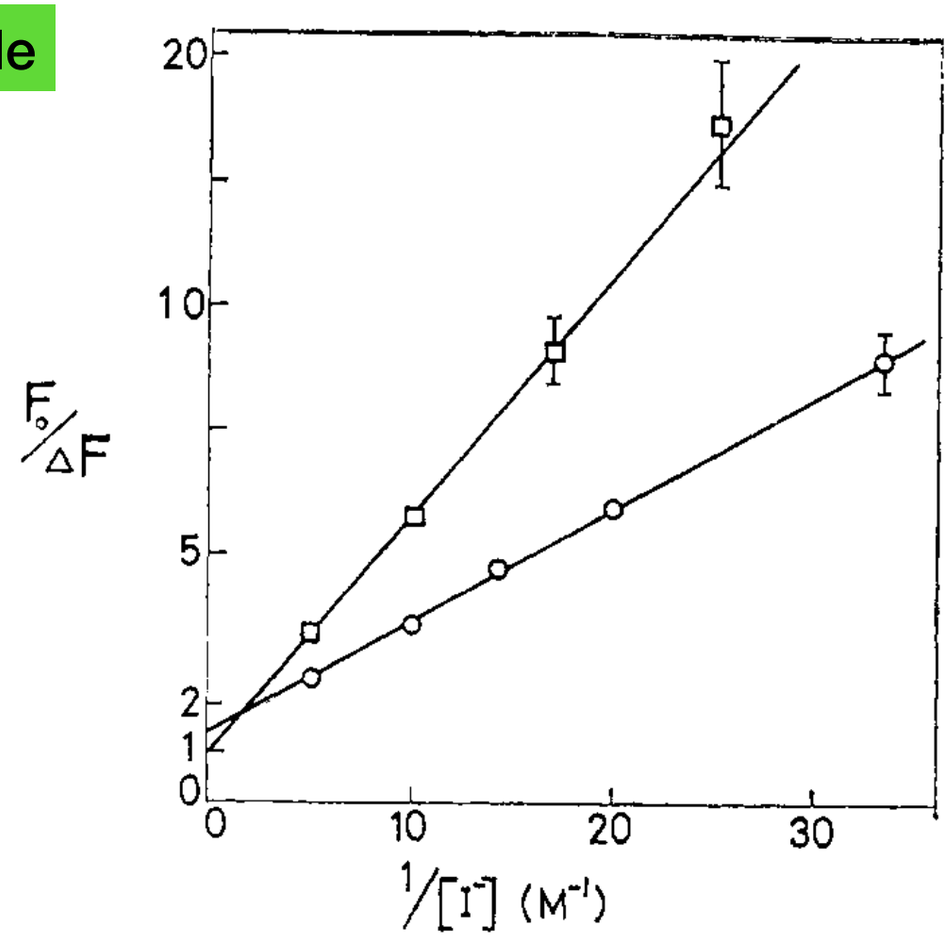
Strong quenching

Moeller et al BIOCHEMISTRY AND BIMOLECULAR BIOLOGY EDUCATION, 2002

Modified Stern-Volmer was first proposed for denaturation of Lysozyme

TRP fluorescence quenching of Lysozyme by iodide





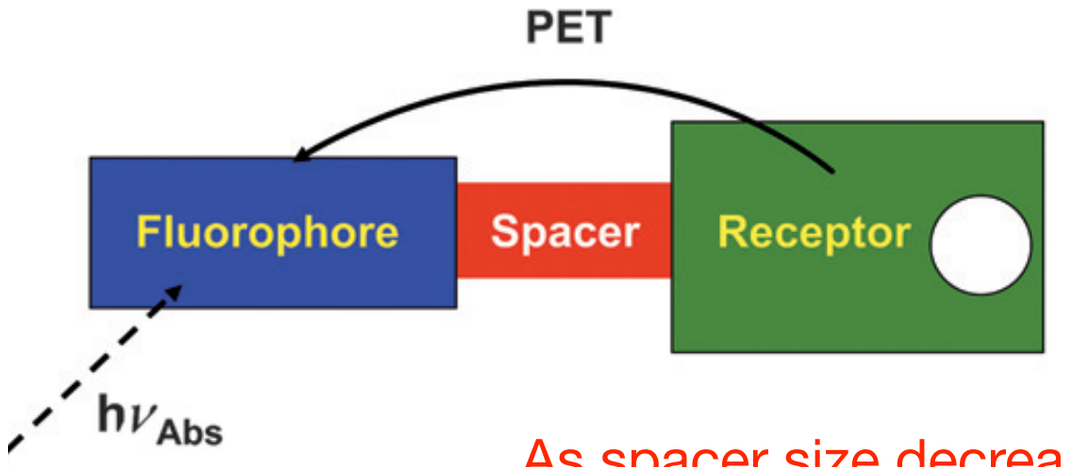
Lehrer et al Biochemistry 1971

- Here the non-linearity of the native case indicates accessible and inaccessible TRP
- Upon unfolding, all the TRP are exposed to quenching so regular SV plot is observed

Quenching of fluorescence by photo induced electron transfer

$$S^* + Q \rightarrow S^+ + Q^- \text{ or } S^- + Q^+$$

Quenching of fluorescence due to PET between the fluorophore and a receptor/donor in vicinity

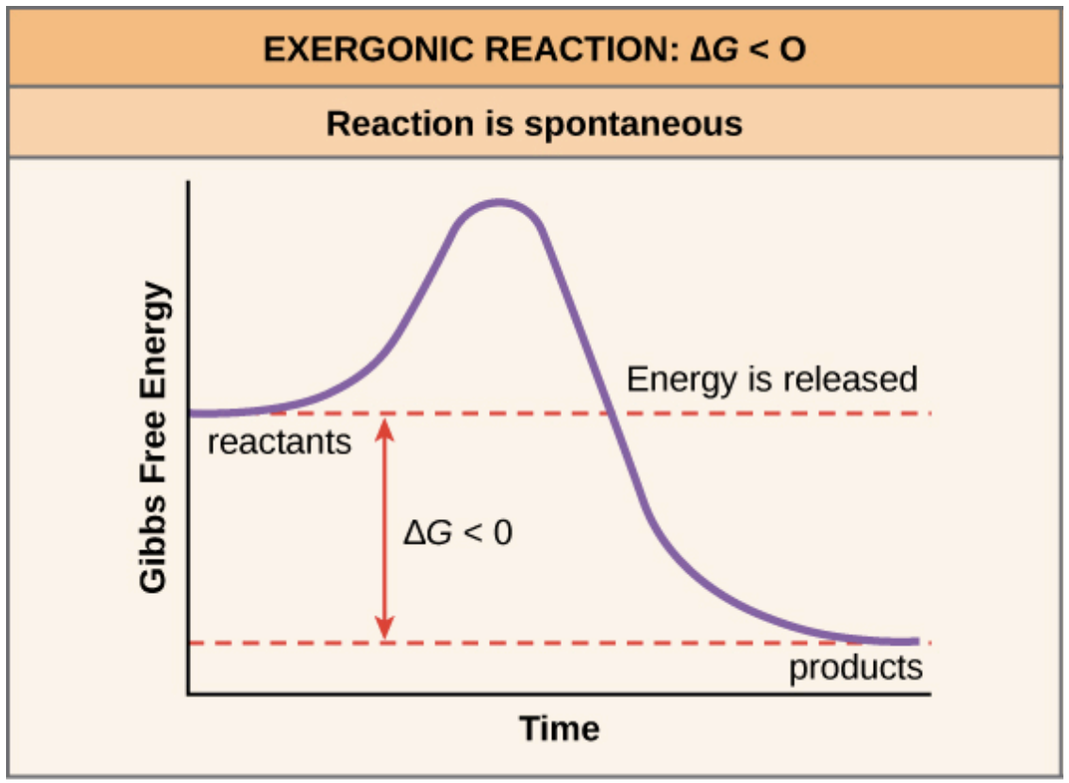


As spacer size decreases PET become more efficient and quenching increases

- **Distance between the donor and acceptor**

- **Free energy for reaction**

- ΔG for PET has to be $-\text{ve}$
- As magnitude of ΔG increases PET become more efficient
- Rate of PET also increases if the energy cost for molecular rearrangement of the donor fluorophore and the receptor is matched by ΔG

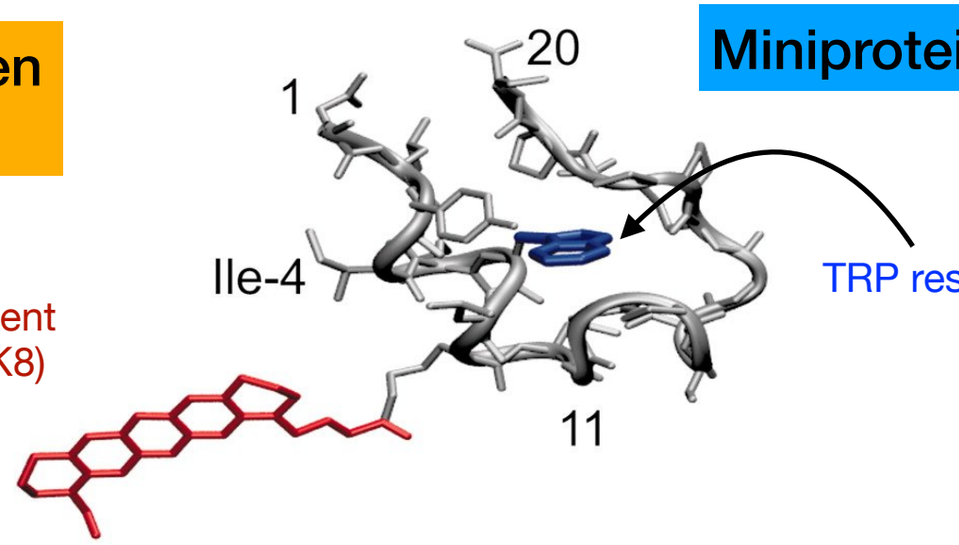


Quenching by PET example

Here PET happens when the protein unfolds

Miniprotein Trp-cage

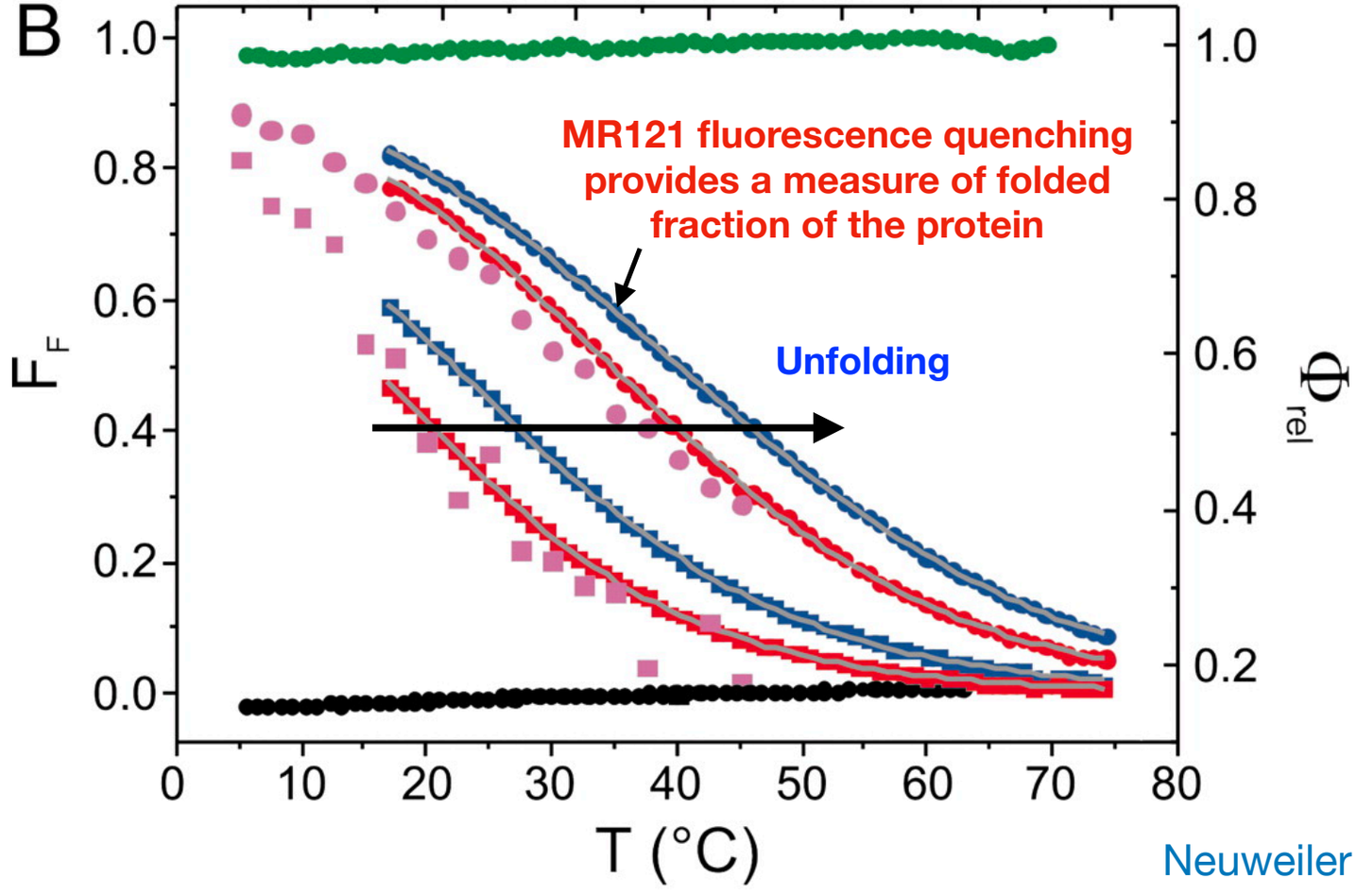
Smallest protein that can fold



e^- acceptor

e^- donor

Folded fraction

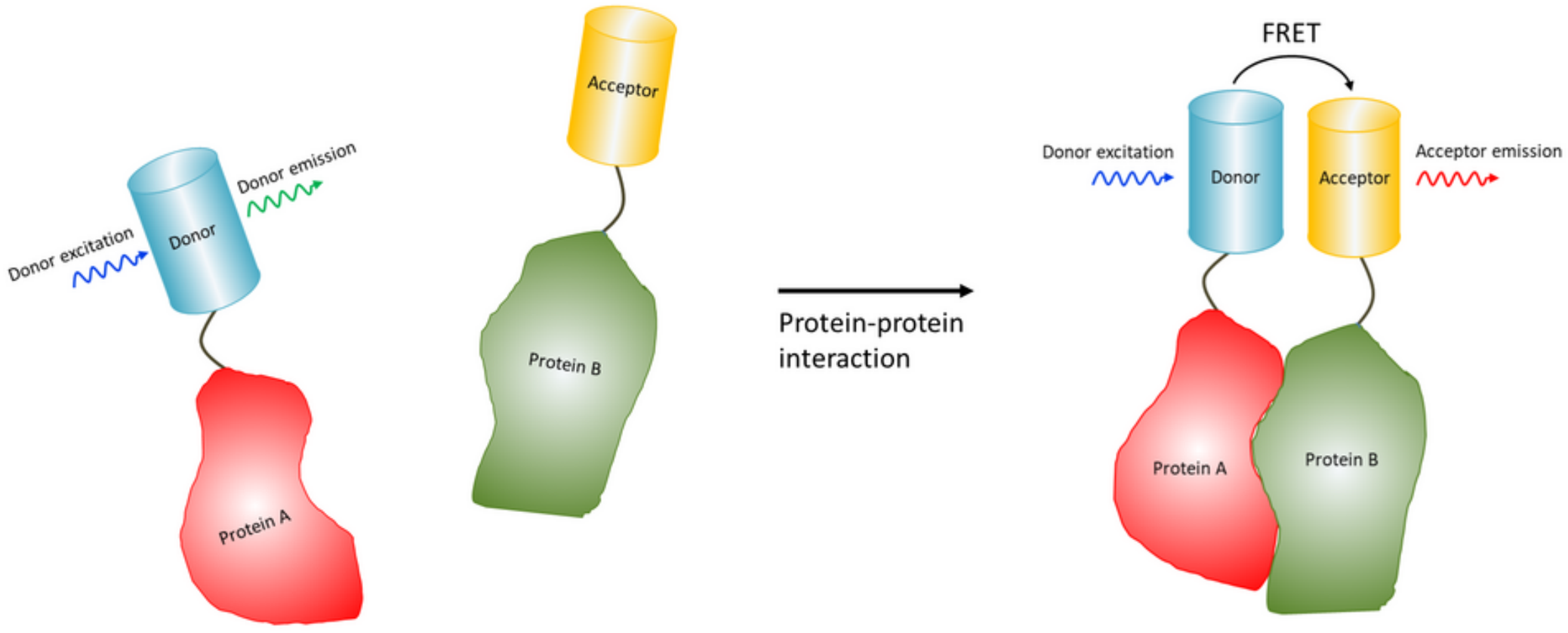


Neuweiler et al PNAS 2005

Quenching of fluorescence by FRET

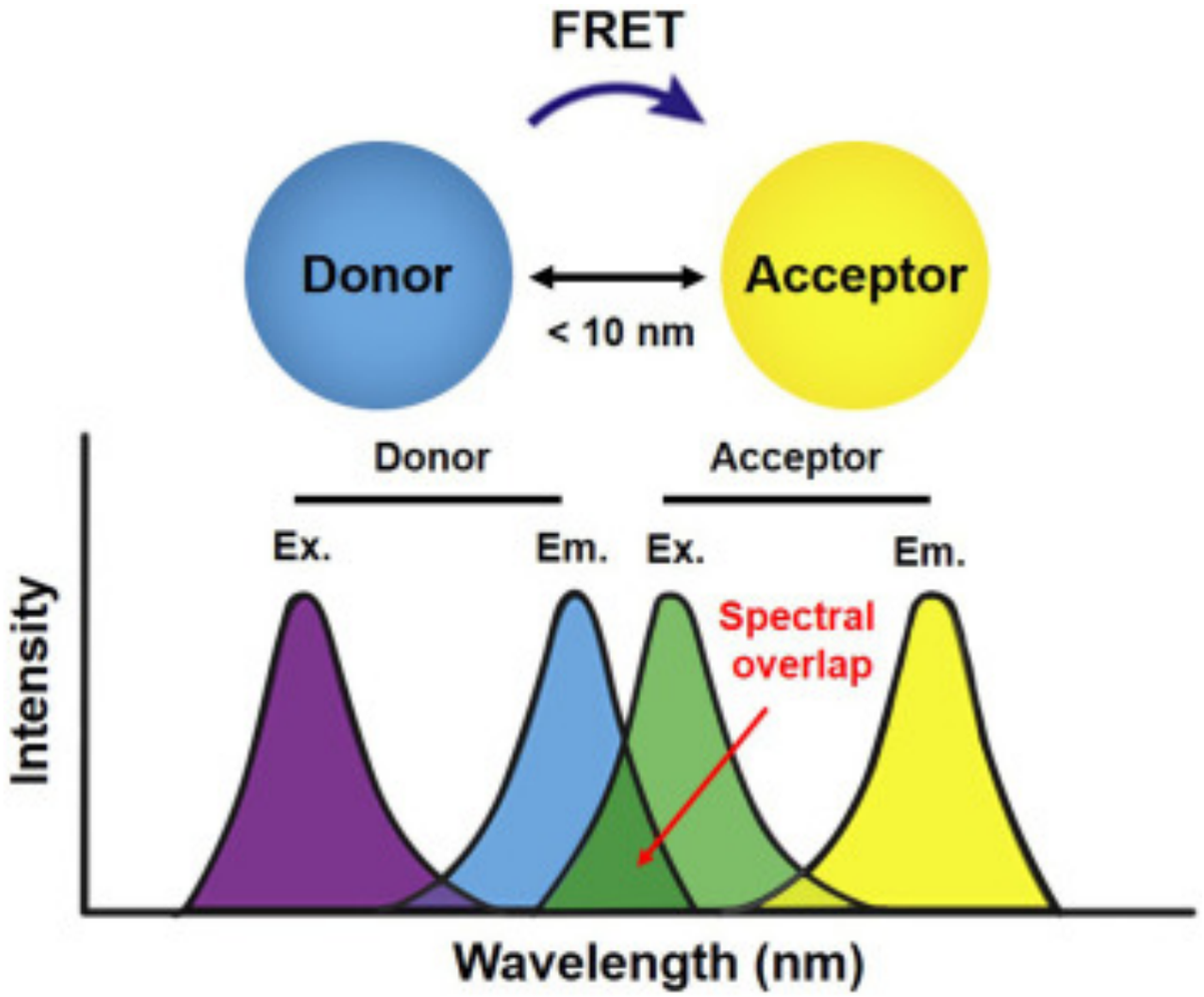
FRET = Fluorescence Resonance Energy Transfer

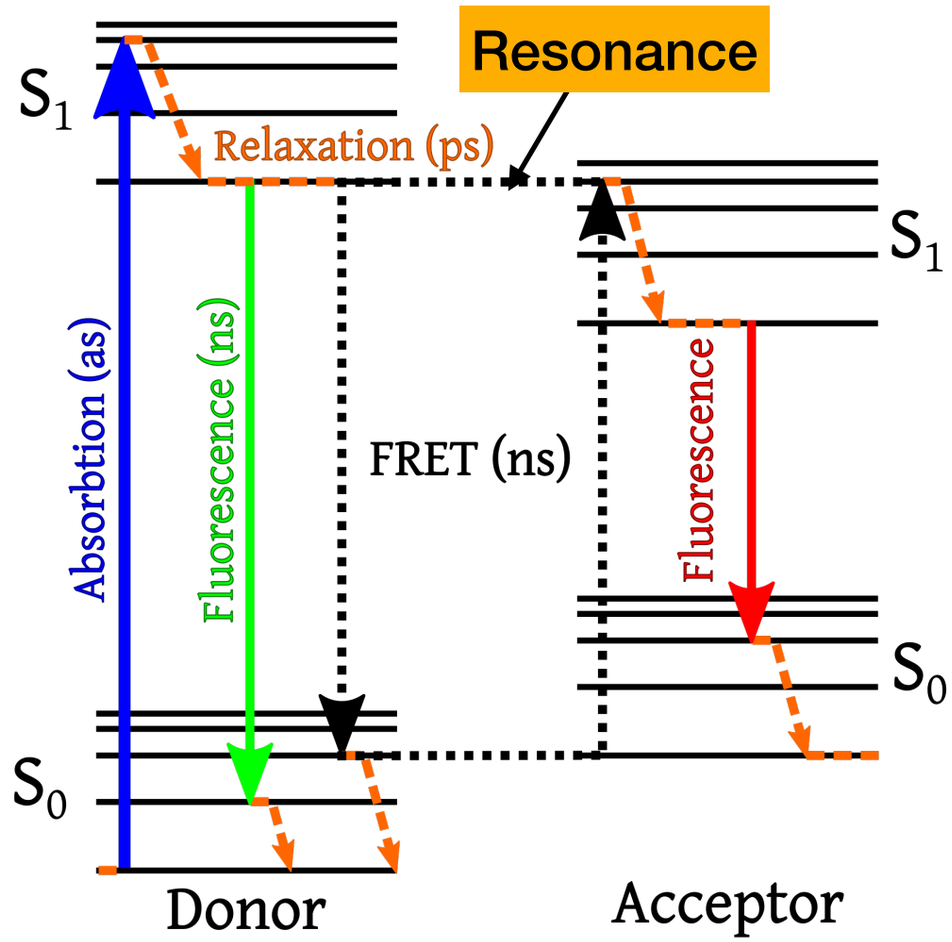
Excited donor to ground-state acceptor energy transfer when they are close



FRET is an intrinsic probe for protein-protein interactions

Förster's theory of FRET





Success of FRET is high if

- The energy donor and acceptor are separated by a short distance (of the order of few nm)
- Photons emitted by the excited state of the donor can be absorbed directly by the acceptor.

Förster's theory of FRET

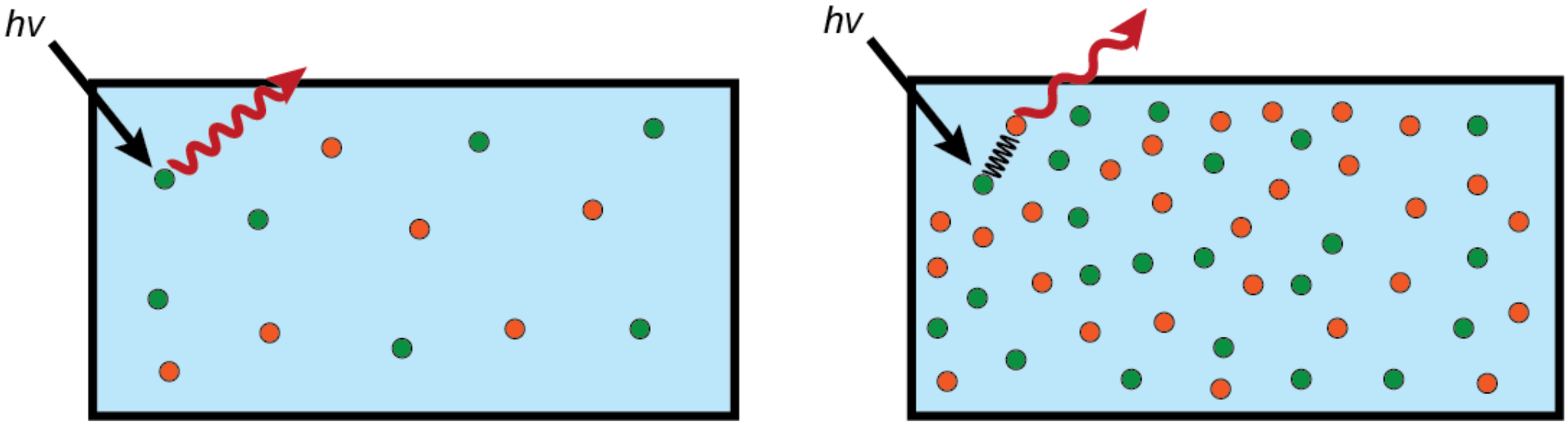
Efficiency of FRET is given by

$$\epsilon_{fret} = \frac{R_0^6}{R_0^6 + R^6}$$

Where R_0 = Förster's distance or radius specific to donor-acceptor pairs, obtained from quantum mechanical calculations

Significance of R_0 = donor-acceptor distance for which the FRET efficiency is 50%

Concentration dependence of FRET

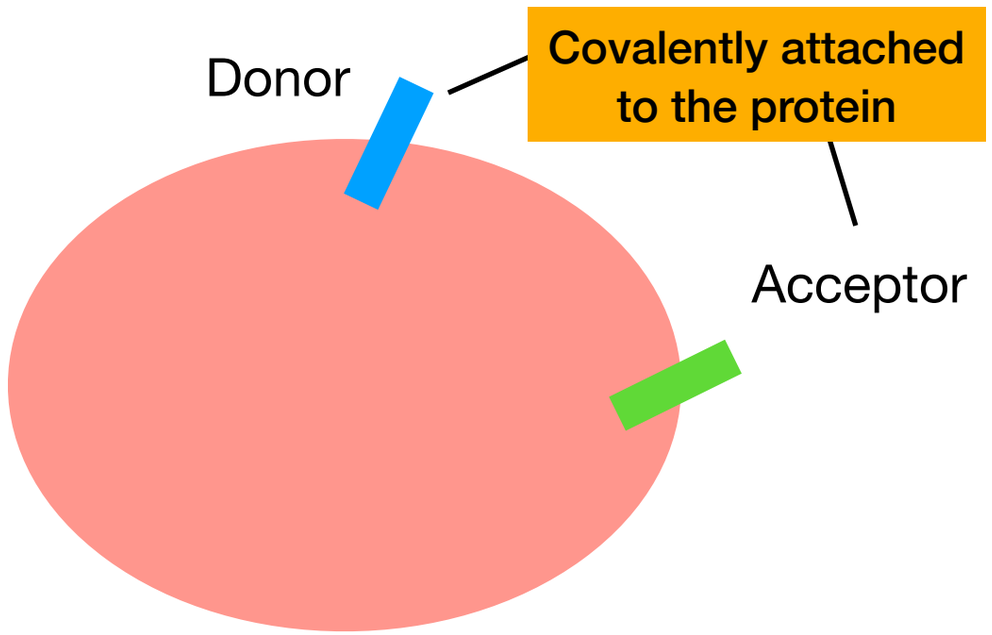


Low concentration, large *R,* no FRET

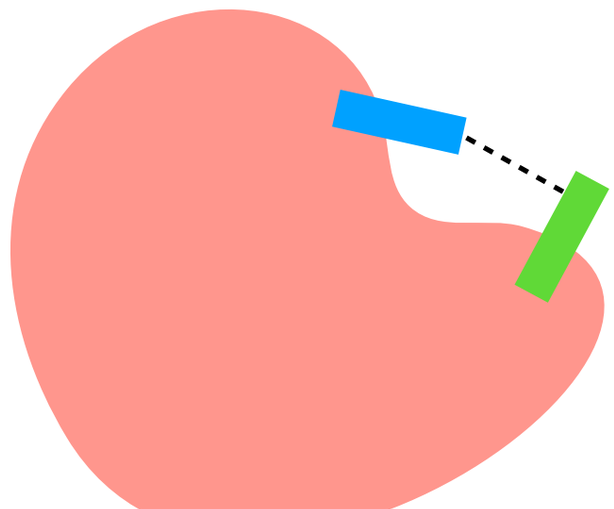
High concentration, small *R,* FRET

With increase in concentrations of the donor and acceptor FRET may increase as their mutual distances become smaller

Applications of FRET in biology



No FRET



FRET

- From the FRET efficiency we can measure the distance and hence study structural changes of the protein
- Even dynamic conformational changes can be tracked through FRET
- CFP-YFP FRET pairs have been used to study cell apoptosis also as cell death reduces the FRET efficiency
- Cell surface receptor clustering has been studied through FRET