Fluorescence Resonance Energy Transfer (FRET)

Actually *seeing* molecular proximity

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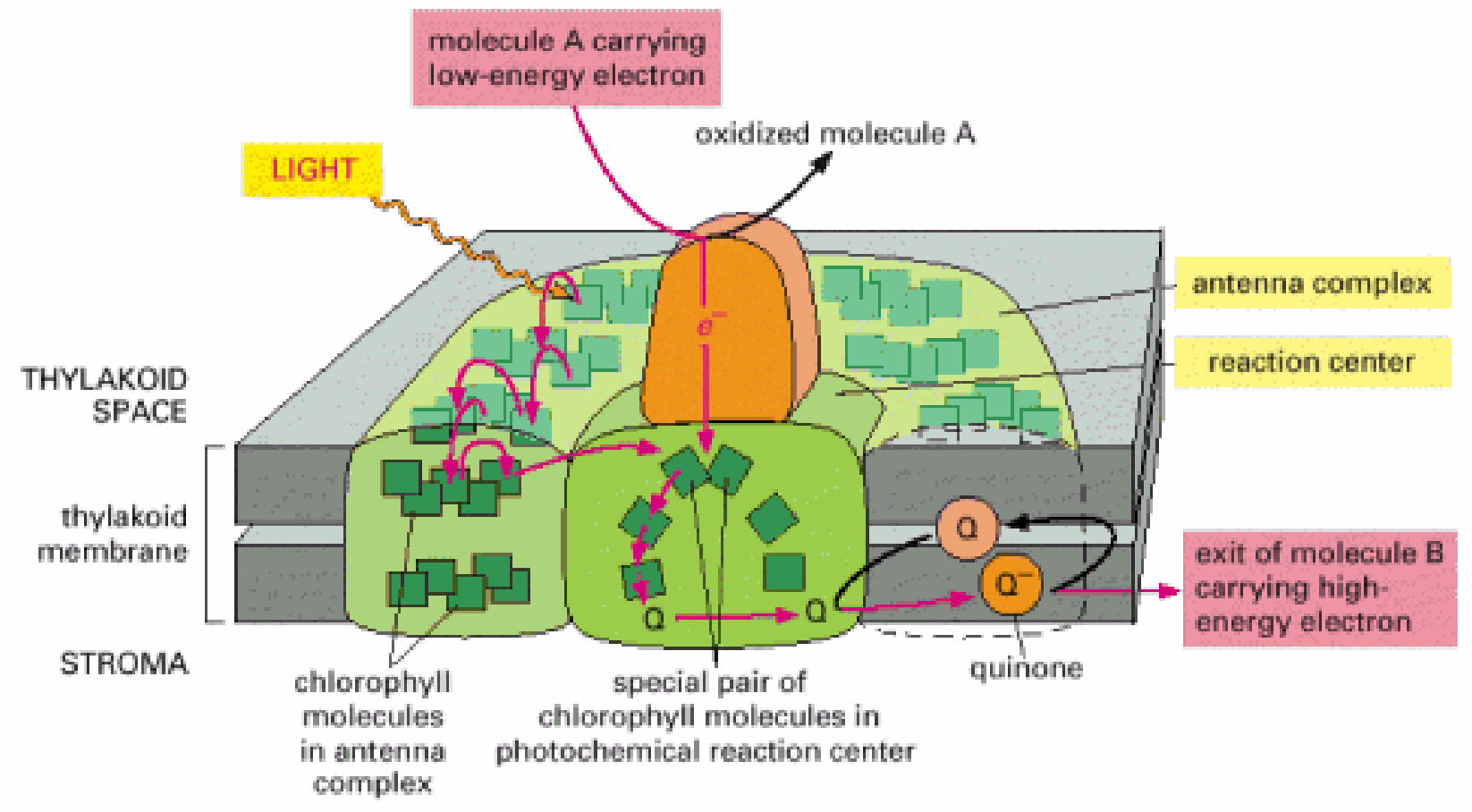
Outline

- What is FRET?
  - Introduction
- How does it work?
  - Physical principles of FRET
- How is it measured?
  - Experimental strategies
- What is it used for?
  - Applications in biology

Quick facts

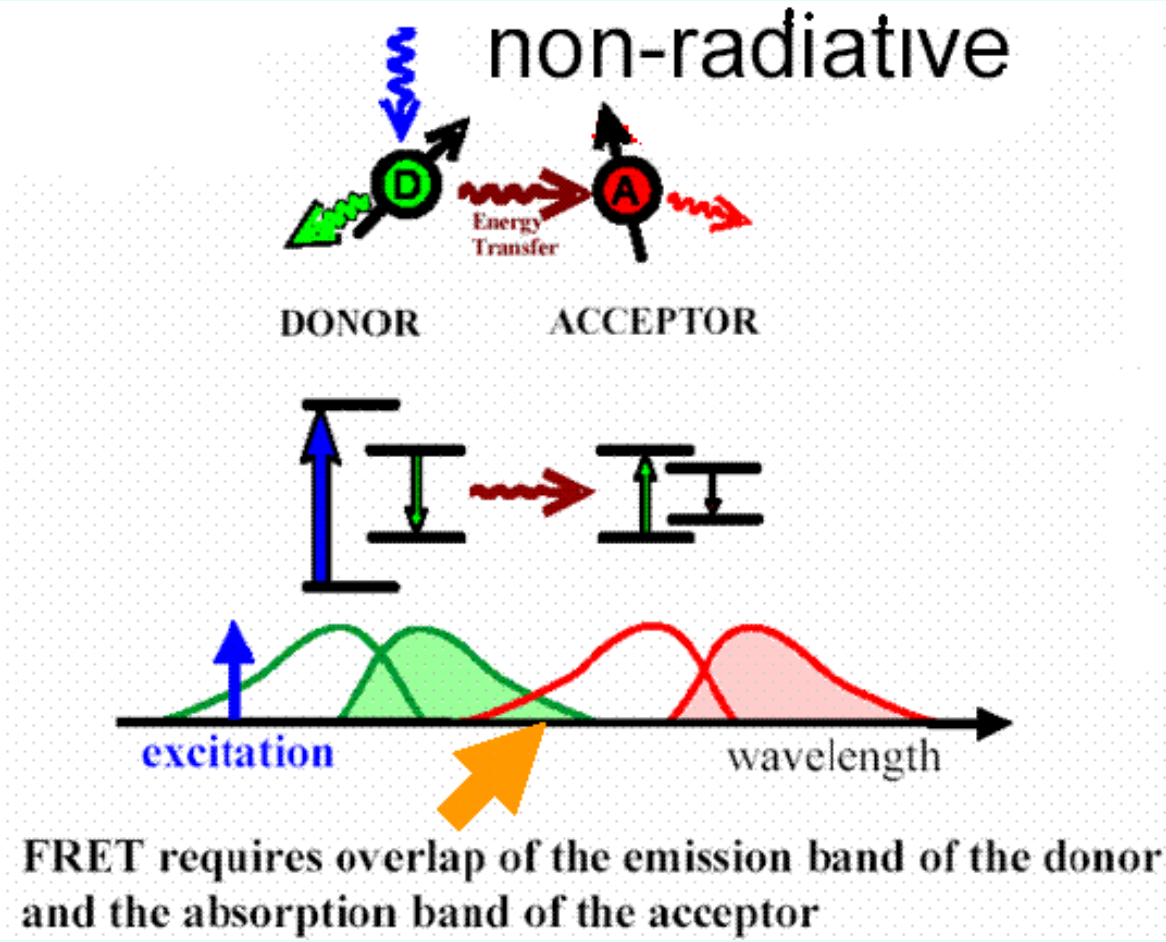
- Powerful tool in biophysics, biochemistry, and cell biology
- Relies on fluorescence detection, hence:
  - high sensitivity
  - modalities: spectroscopy (steady-state, time-resolved, stopped-flow), microscopy, flow cytometry, high-throughput tech.
  - works well both *in vitro* and *in vivo*
- Requires two: a donor and an acceptor
- Reports proximity of molecules or moieties within a molecule
- Works in the range of approx. 1-10 nm

Where it occurs in nature



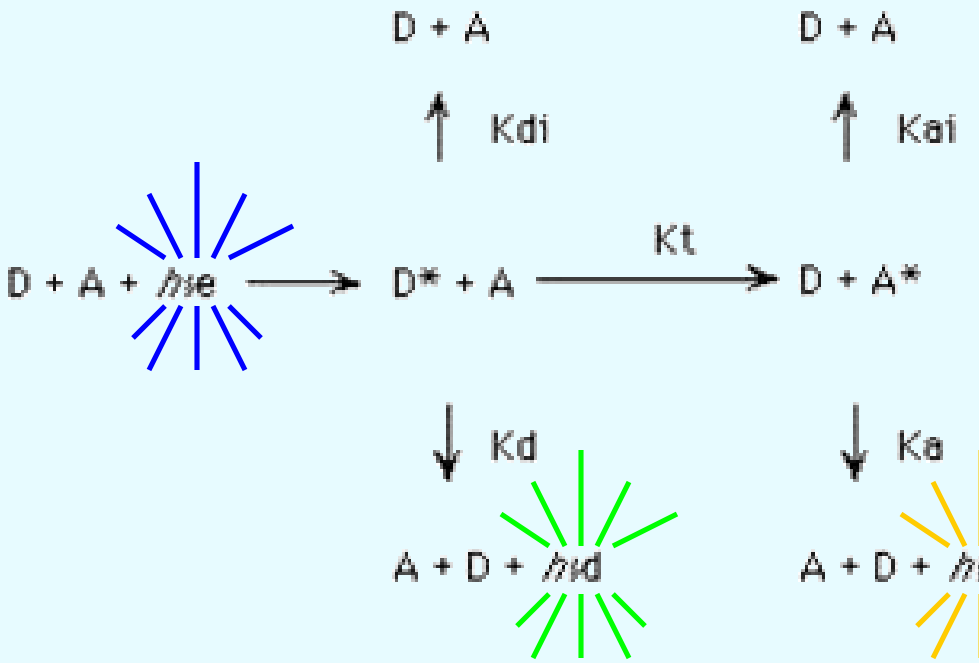
The antenna complex and photochemical reaction center in a photosystem.

Physical principles of FRET



- nonradiative
- weak dipole-dipole interaction
- decays as $1/R^6$
- no overlap of electron clouds
- spectral shape unchanged

Kinetics of fluorescence and FRET



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

Förster radius

excited donor lifetime

distance between D and A

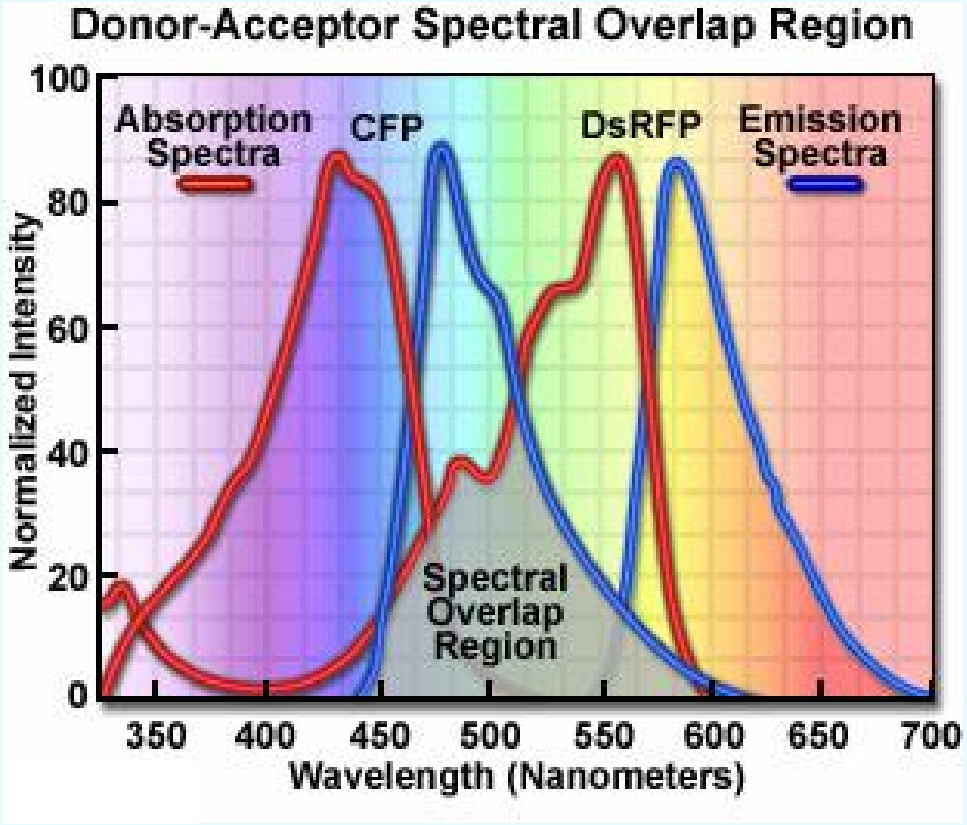
Förster radius (R_0)

- the distance between D & A at which the rate of fluorescence emission equals the rate of FRET
- 50% of excited donors will emit light, and 50% will pass energy to acceptors
- R_0 is a function of the donor quant. yield, spectral overlap, and orientation
- Can be calculated for a given donor-acceptor pair from spectroscopic data

$$R_0 \propto (\kappa^2 Q_D J)^{1/6}$$

Förster radius

depends on the overlap of acceptor absorbance spectrum and donor emission spectrum



$$R_0 \propto (\kappa^2 Q_D J)^{1/6}$$

$$J = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$

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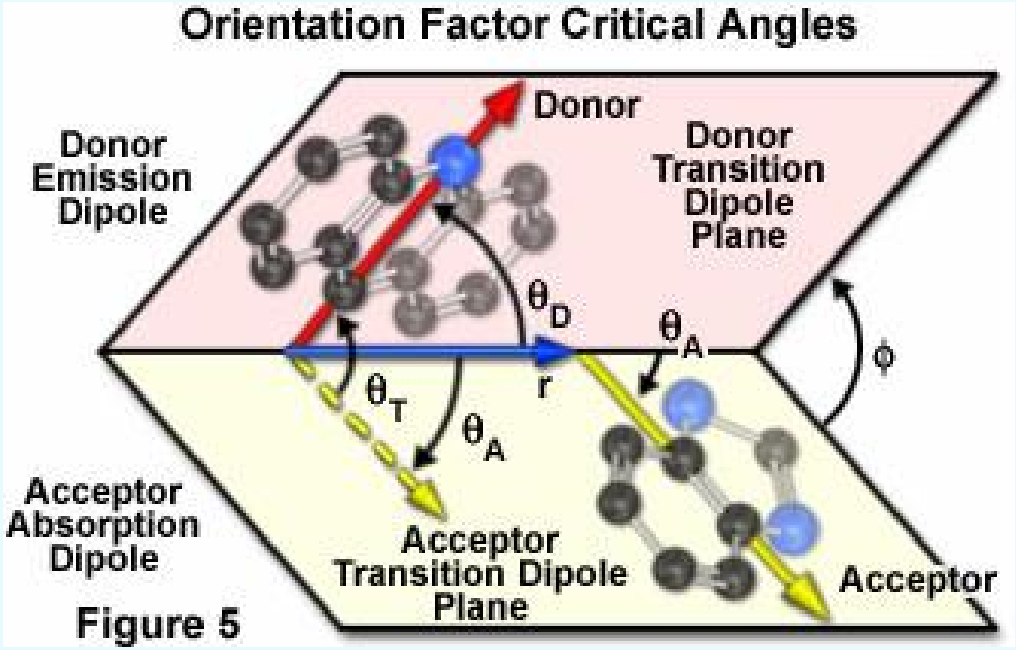
Förster radius

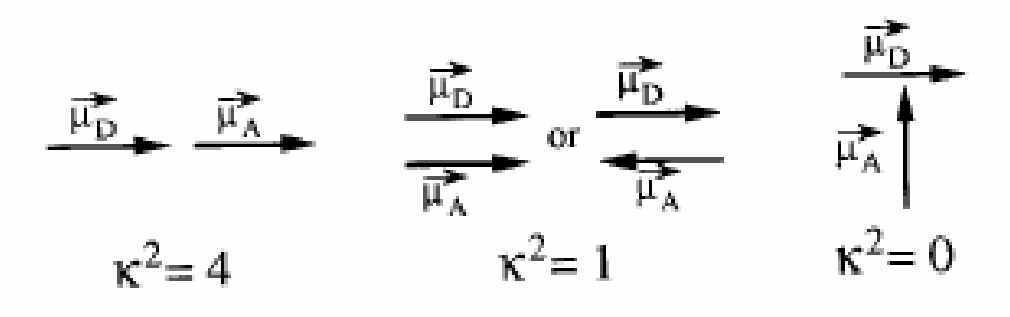
depends on the mutual orientation of donor and acceptor transition moments



$$R_0 \propto (\kappa^2 Q_D J)^{1/6}$$

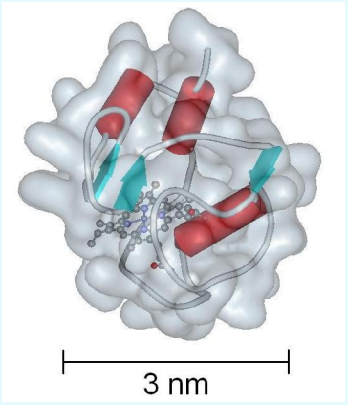
dynamically averaged value:

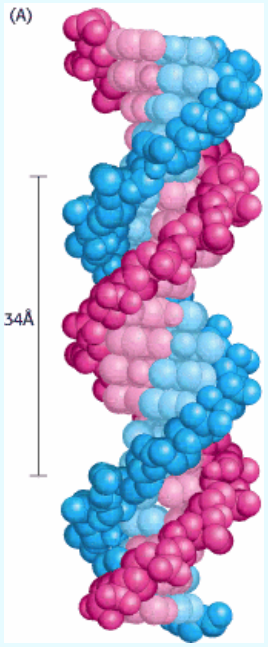
$$\kappa^2 = \frac{2}{3}$$

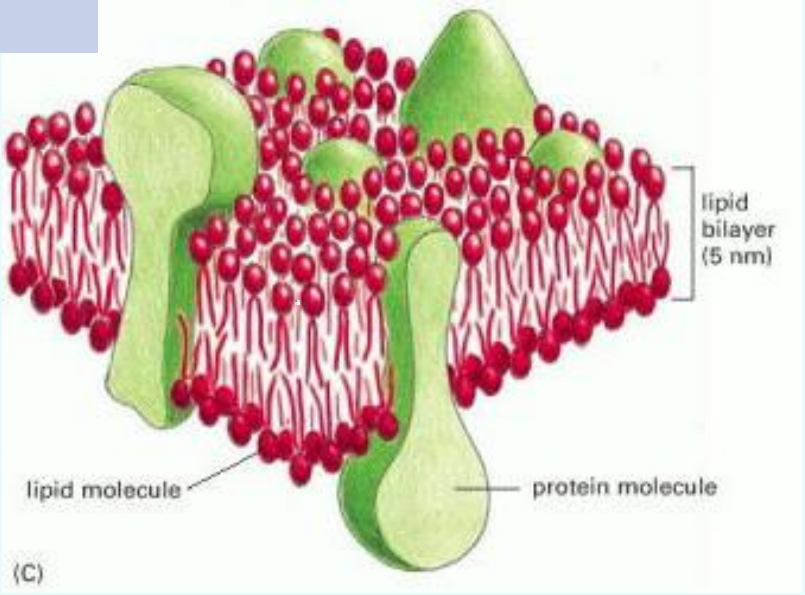


Typical values of R_0

Donor	Acceptor	Förster Distance (nanometers)
Tryptophan	Dansyl	2.1
Dansyl	FITC	3.3 - 4.1
CF	Texas Red	5.1
Fluorescein	Tetramethylrhodamine	4.9 - 5.5
Cy3	Cy5	>5.0
GFP	YFP	5.5 - 5.7
BODIPY FL	BODIPY FL	5.7
Rhodamine 6G	Malachite Green	6.1
Cy5	Cy5.5	>8.0

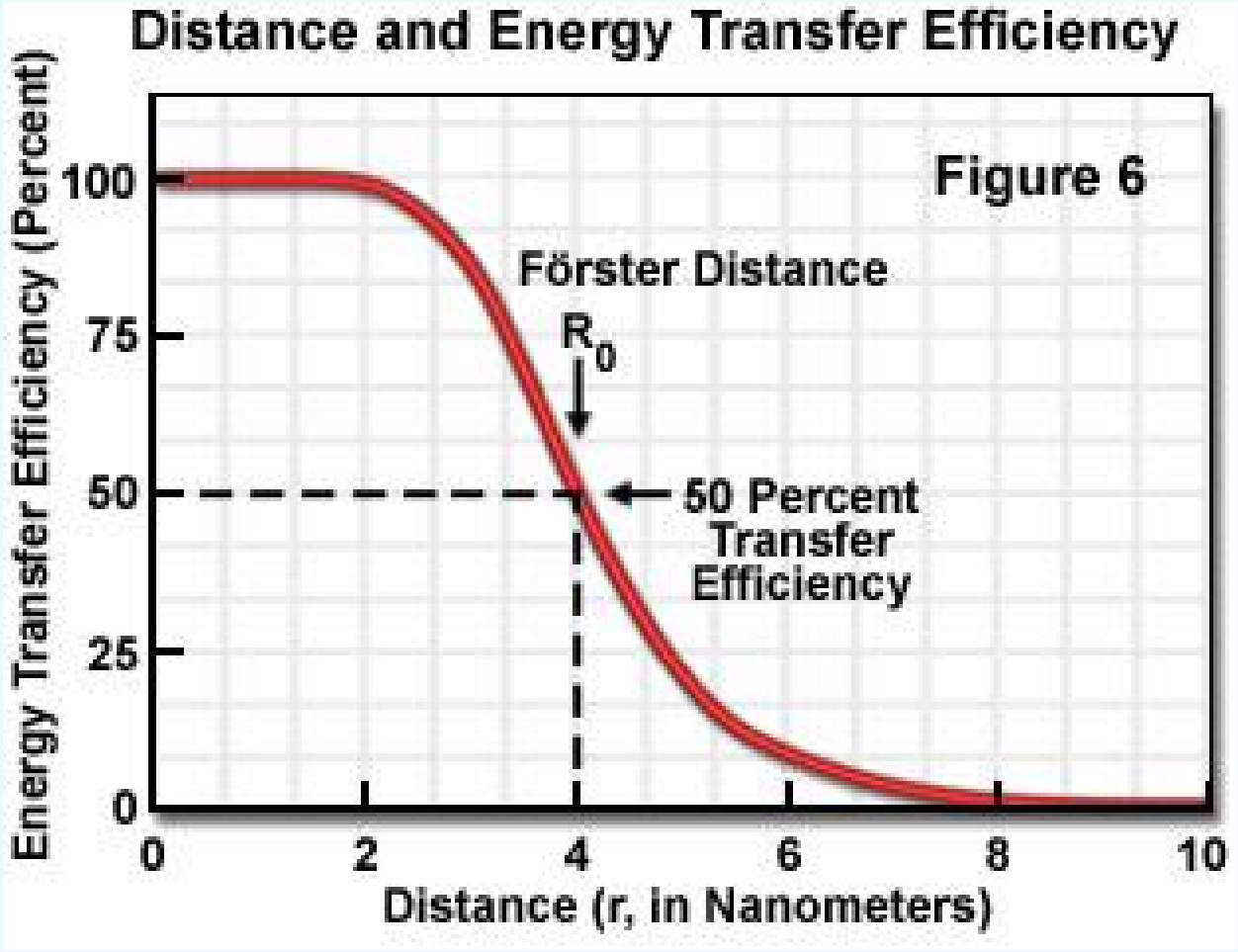




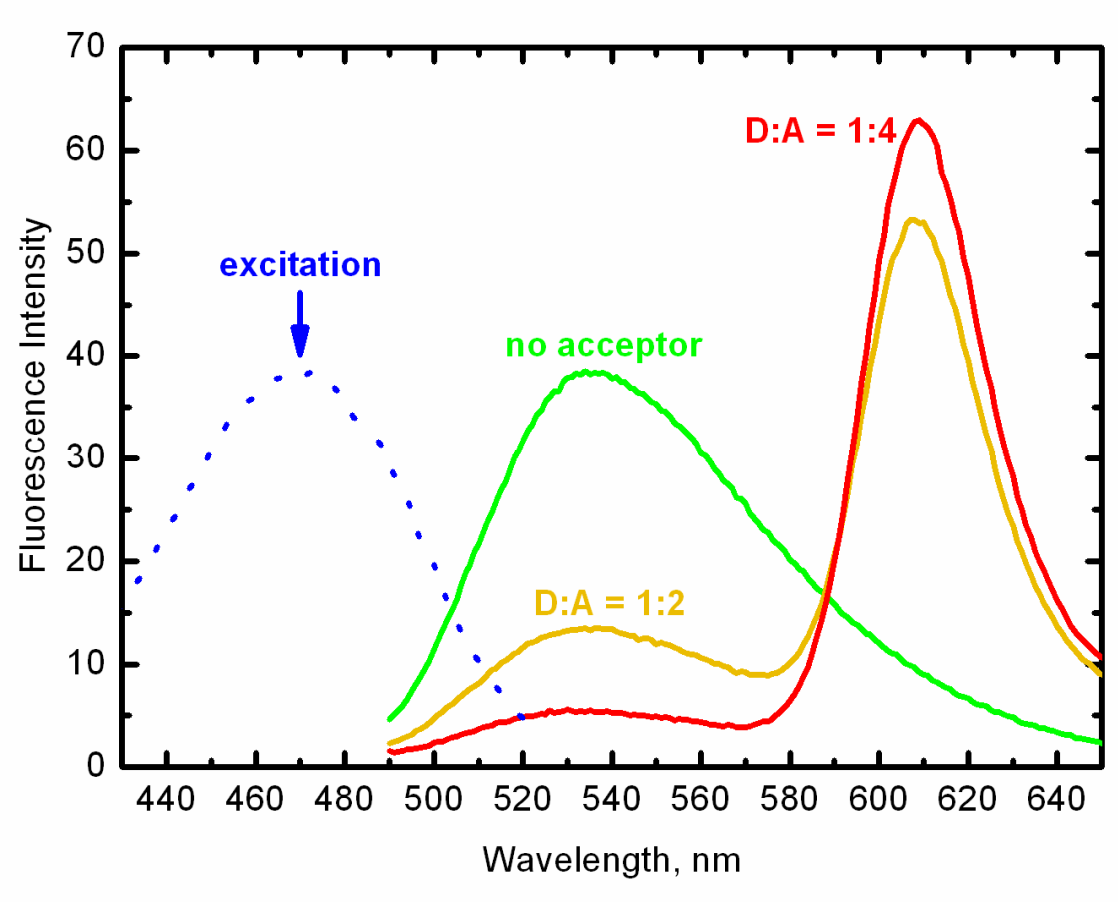


Efficiency of energy transfer

$$E = \frac{k_T}{k_T + \tau_D^{-1}} = \frac{R_0^6}{R_0^6 + R^6}$$



Determining the efficiency of FRET



NBD-PC liposomes à TexasRed-temporin B

- by donor intensity:

$$E = 1 - \frac{I_{DA}}{I_D}$$

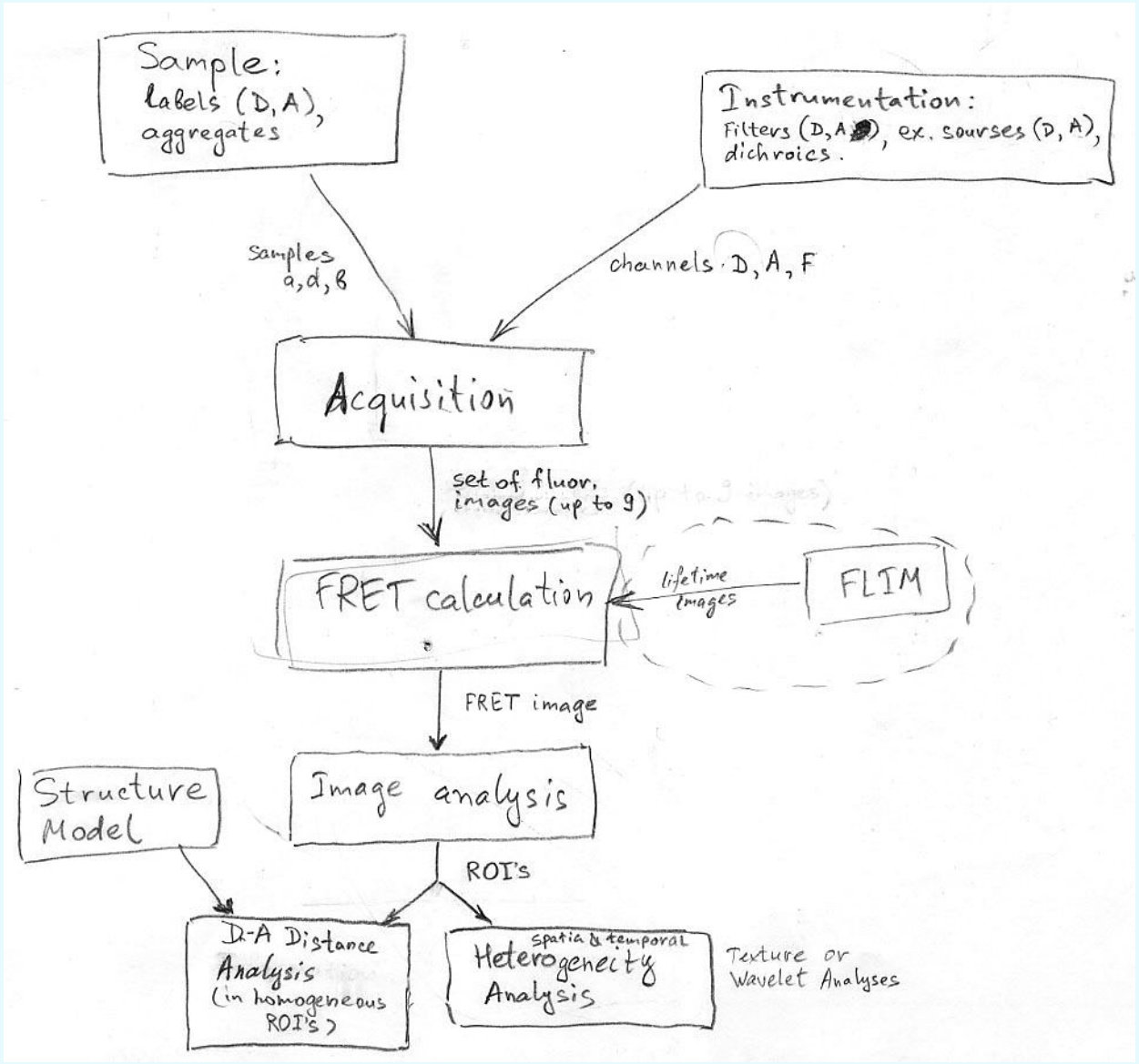
- by donor fluorescence lifetime:

$$E = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_D \rangle}$$

- by acceptor intensity:

$$E = \frac{\varepsilon_A C_A}{\varepsilon_D C_D} \left(\frac{I_{AD}}{I_A} - 1 \right)$$

FRET microscopy studies: workflow

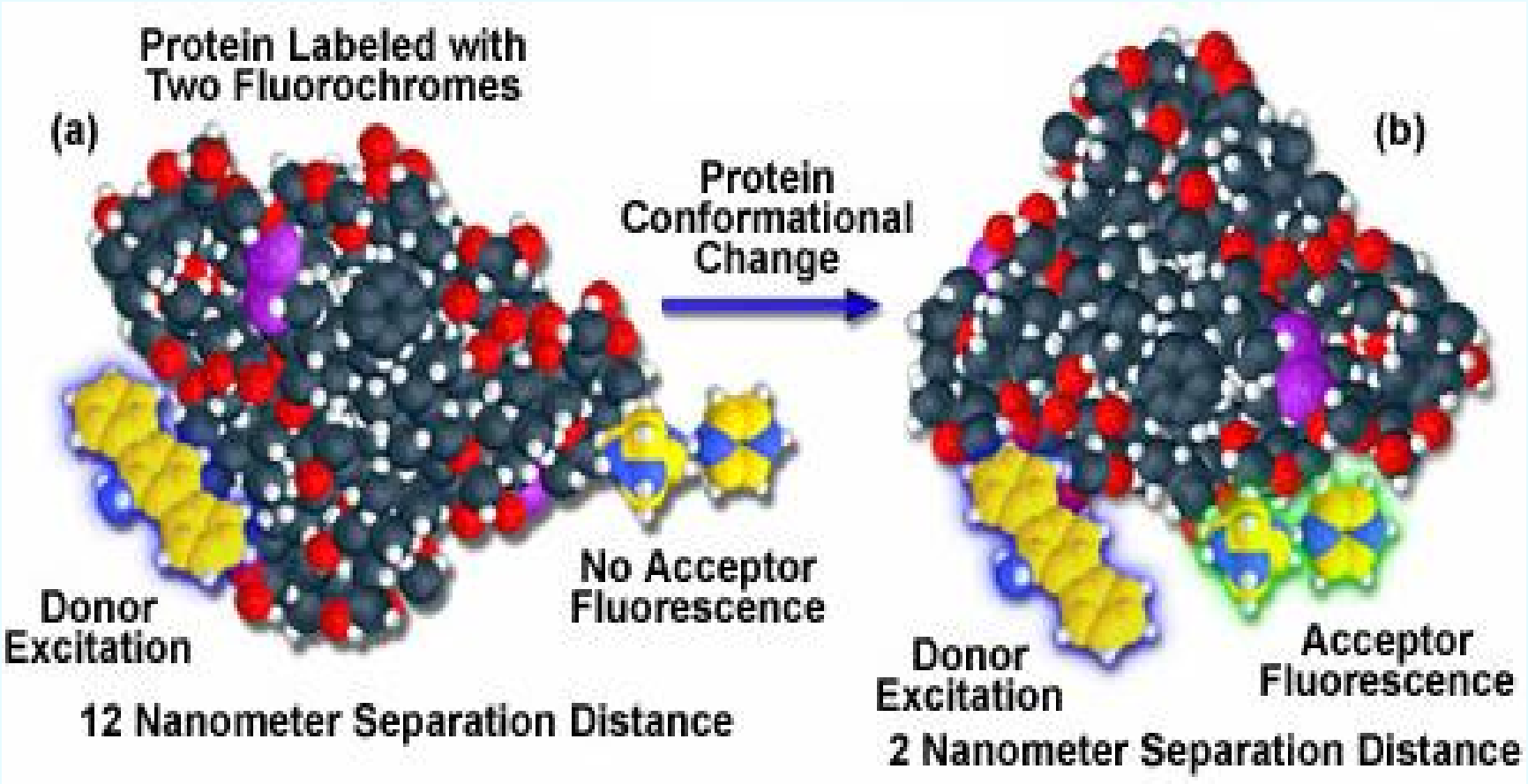


Applications of FRET

- Changes: Association, aggregation, conformational changes, enzymatic activity
- Absolute distances: Structural studies complementary to NMR, EPR, X-ray crystallography, SAS, CryoEM

- Objects/techniques:
  - macromolecules (structure, dynamics, biochemical reactions)
  - supramolecular complexes (multisubunit proteins, polymers, aggregates, prot.-nucl. acid, protein-lipid interactions, membrane fusion, lipid rafts)
  - cellular structures/processes (signalling, transport)
  - whole cells (expression, viability)

Intramolecular FRET:
detecting conformational changes



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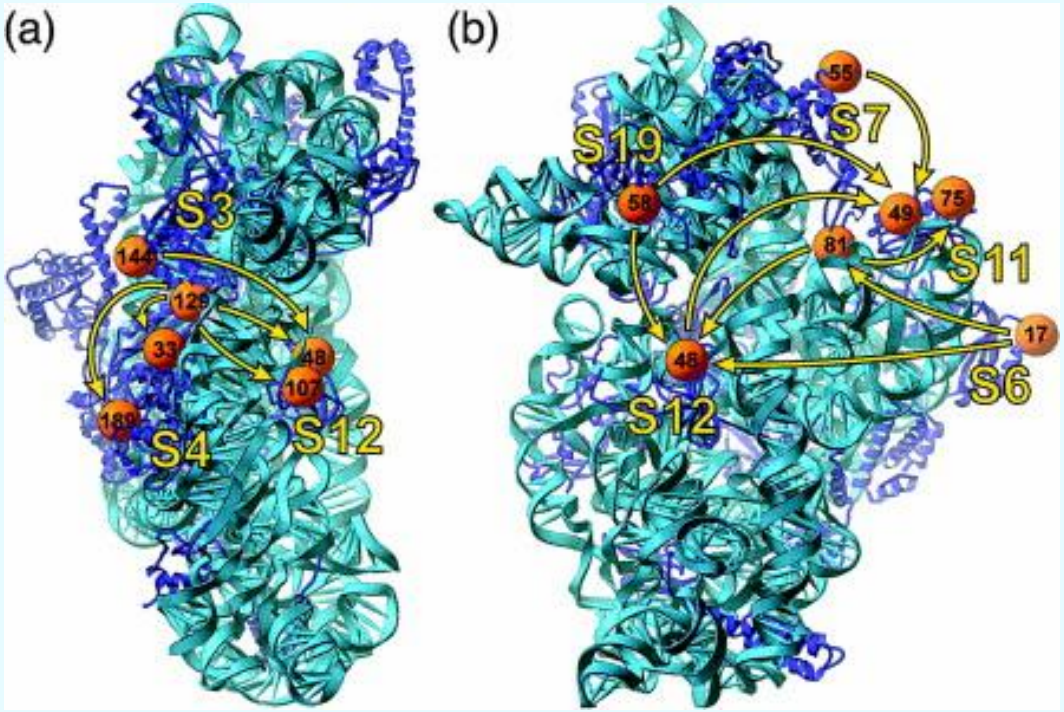
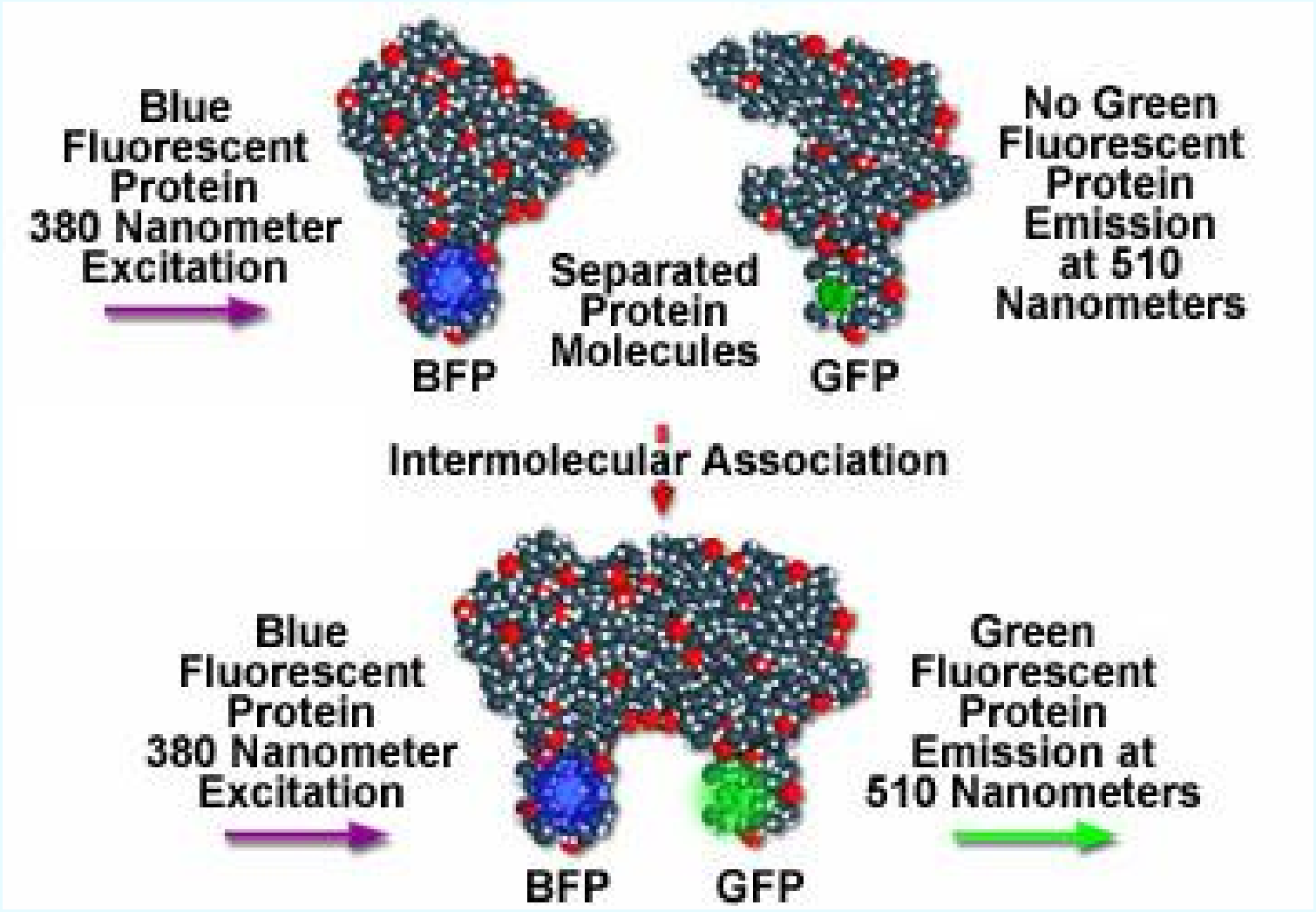
Example: Internal Movements within the 30 S Ribosomal Subunit



- Single-Cys mutants
- 13 D-A pairs
- Alexa 488 à Alexa 568
- Association with 50 S subunit
- Agree with X-ray & cryoEM
- New information unavailable from X-ray & cryoEM

Hickerson et al. (2005) *J Mol Biol* 354:459

Intermolecular FRET:
detecting interactions of biomolecules



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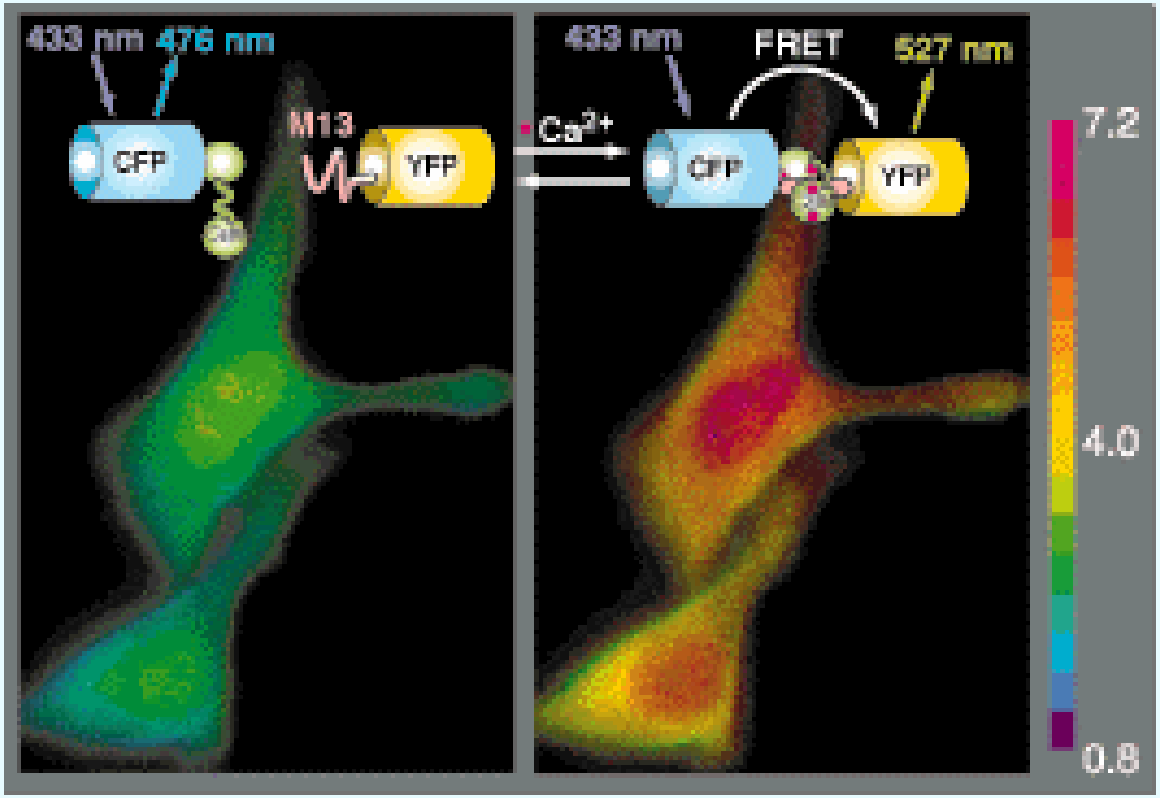
Gene-encoded two-component Ca^{2+} indicator



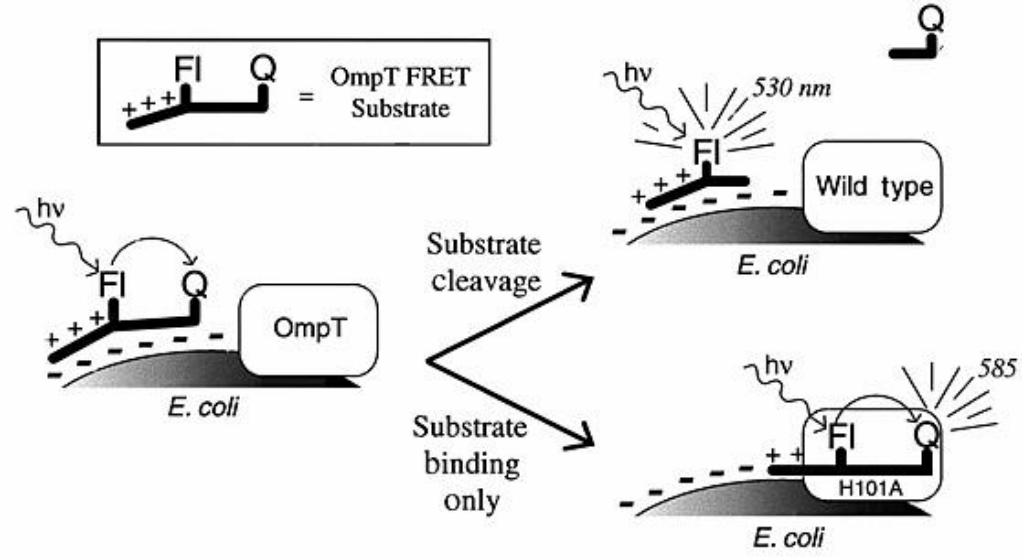
FRET constructs for measuring intracellular calcium. CFP-labeled calmodulin and YFP-labeled calmodulin binding peptide (M13-YFP) were coexpressed. High Ca^{2+} levels (right) lead to binding and FRET emission of YFP (pseudo color red); low Ca^{2+} levels (left) lead to little FRET and mostly blue emission (pseudocolor green).

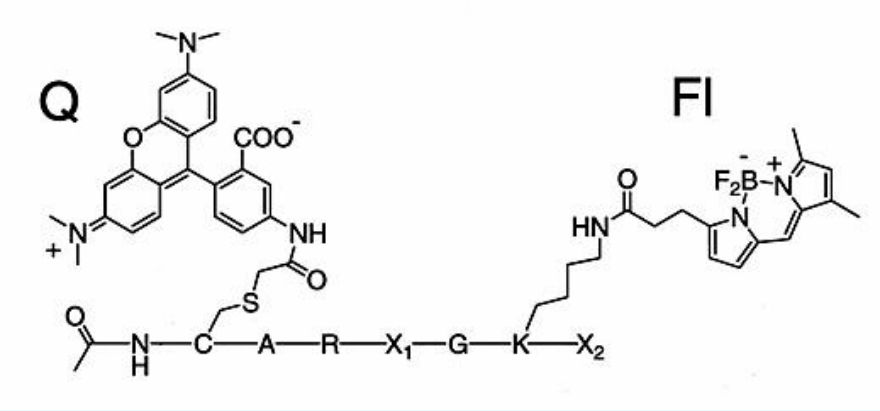
The left panel shows two cells before stimulation, while the right panel shows the same cells after elevation of cytosolic Ca^{2+} by 0.1 mM histamine.

Tsien & Miyawaki (1998) *Science* 280:1954-1955.

FRET in Flow Cytomery

Function-based isolation of novel enzymes from a large library



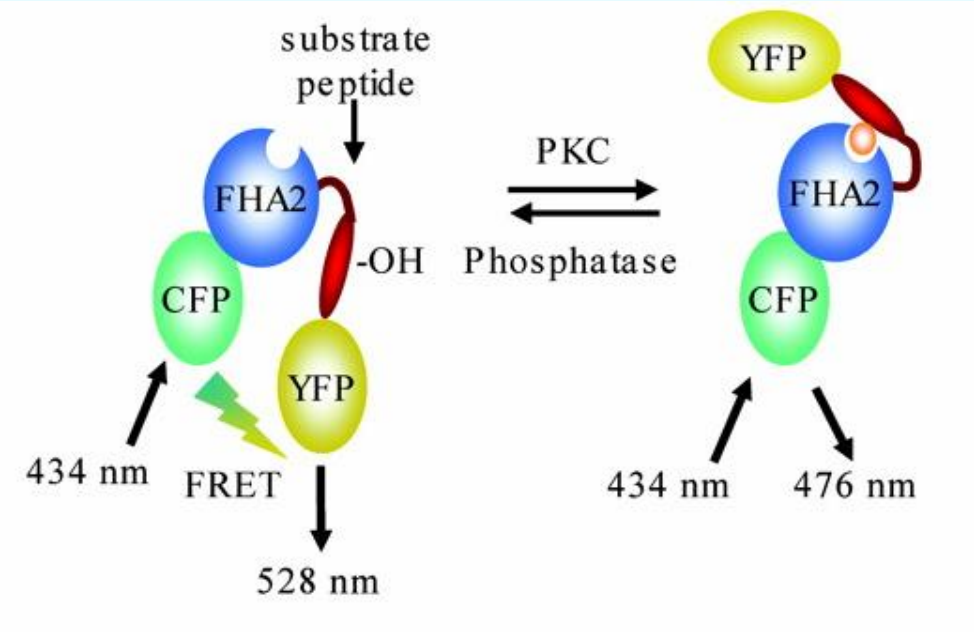


Protein variants are displayed on the surface of microorganisms and incubated with a synthetic substrate consisting of (1) a fluorescent dye (2) a positively charged moiety (3) the target scissile bond, and (4) a fluorescence resonance energy transfer (FRET) quenching partner. Enzymatic cleavage of the scissile bond results in release of the FRET quenching partner while the fluorescent product is retained on the cell surface, allowing isolation of catalytically active clones by fluorescence-activated cell sorting (FACS).

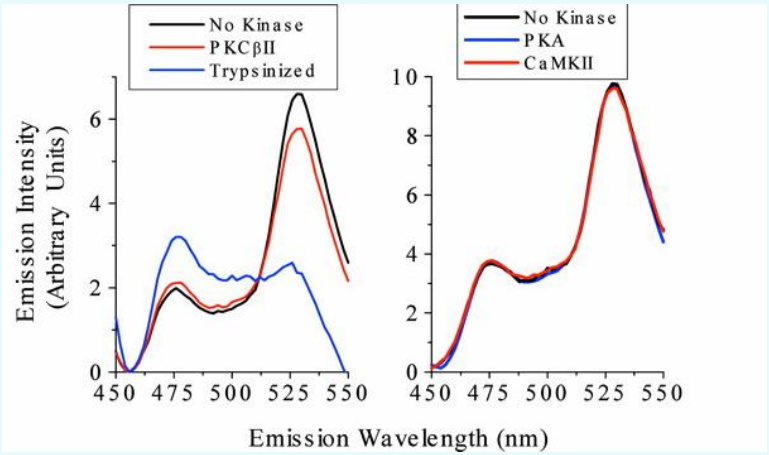
NB: Cys/Lys doble labelling

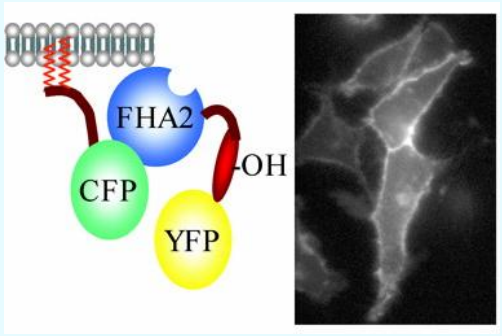
Olsen et al. (2000) *Nat Biotechnol* 18:1071

Genetically encoded FRET reporter of PKC phosphorylation



CKAR is comprised of mCFP, the FHA2 domain of Rad53p, a PKC substrate sequence, and mYFP. The substrate sequence, when phosphorylated, binds the FHA2 phospho-peptide–binding domain. This conformational change results in a change in FRET, reversible by phosphatases.





Violin *et al.* (2003) *J Cell Biol* **161**:899-909

Advantages and disadvantages

The upside...

- FRET is relatively cheap!!!
- It is very efficient in measuring changes in distances.
- You measure distances in molecules in solution.
- You only need a few µM of labeled proteins.
- Once you have labeled your molecule, you can have a measurement rapidly.
- You can measure distances or changes in distances in complex of molecules

...and the downside

- The precision of the measure is impaired by the uncertainty of the orientation factor and by the size of the probes
- When measuring a change in distance between two probes, the result is a scalar and give no indications of which probe (donor and/or acceptor) moves.
- The presence of free labels in solution could mask a change in energy transfer.
- These measurements give the average distance between the two probes.

Suggested reading

- P.R. Selvin (2000) The renaissance of fluorescence resonance energy transfer. *Nat Struct Biol.* 7:730-4.
- P.R. Selvin (1995) Fluorescence resonance energy transfer. *Meth Enzymol* 246:300-334.
- J.R. Lakowicz (2006) Principles of Fluorescence Spectroscopy, 3rd edn. Springer.
- Olympus Resource Center: Fluorescence resonance energy transfer (FRET) microscopy. http://www.olympusfluoview.com/applications/fretintro.html
- J. Matko, M. Edidin (1997) Energy transfer methods for detecting molecular clusters on cell surfaces. *Meth Enzymol* 278:444-462.