*a brief introduction*

# spFRET
# –
# Single molecule spectroscopy

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SE Elements of Nanophotonics

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# Outline of the talk …

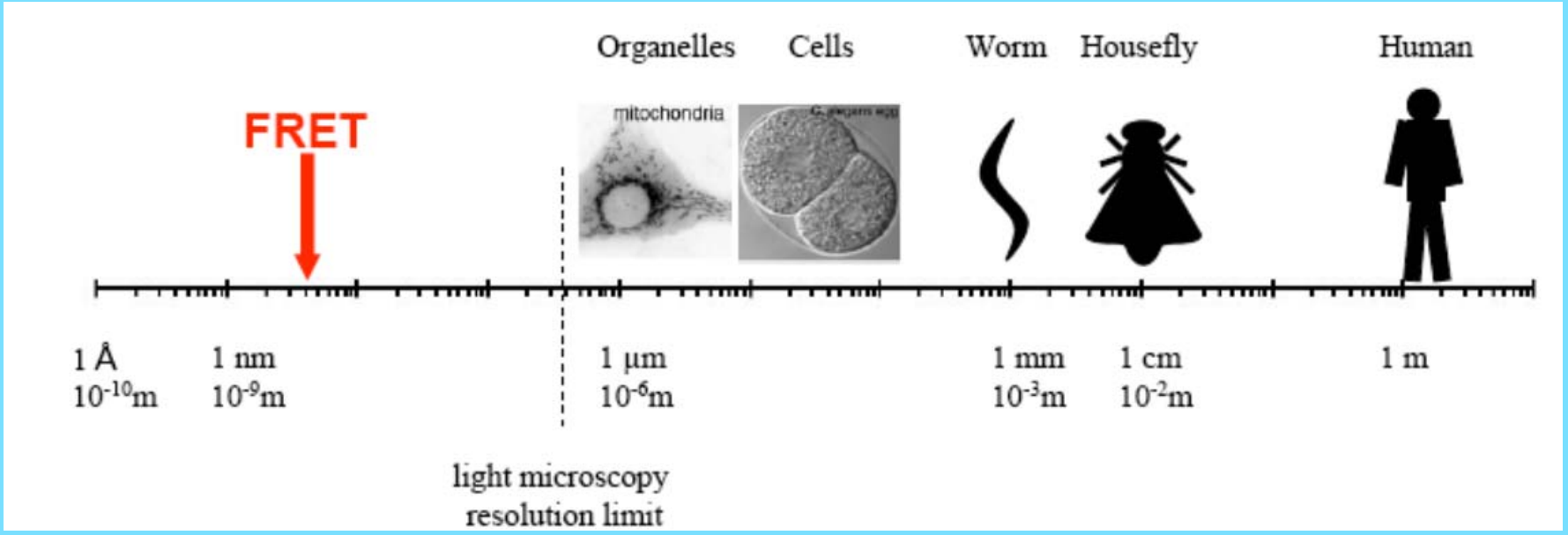
- Introduction
- FRET overview
- few examples
- Conclusions

# Single molecule spectroscopy …

Main (biological) motivation:

- Molecular interactions
- Enzymatic activity
- Reaction kinetics
- Conformational dynamics
- Molecular degrees of freedom
- Alterations in changing chemical background
- Probing the biological function of macromolecules
- Allows identification, sorting & quantitatively comparision of subpopulations in ensembles

# The dimensions …




FRET allows:

- the observation in the nm regime
- studying the molecular structure (e.g. protein folding)

# FRET …

**F**örster **R**esonance **E**nergy **T**ransfer

Identified by Theodor Förster in 1946.

→ FRET relies on the distance-dependent energy transfer between a donor flourophore and acceptor flourophore.

→ FRET is sensitive to distance information and radiometric nature of measurement as well.

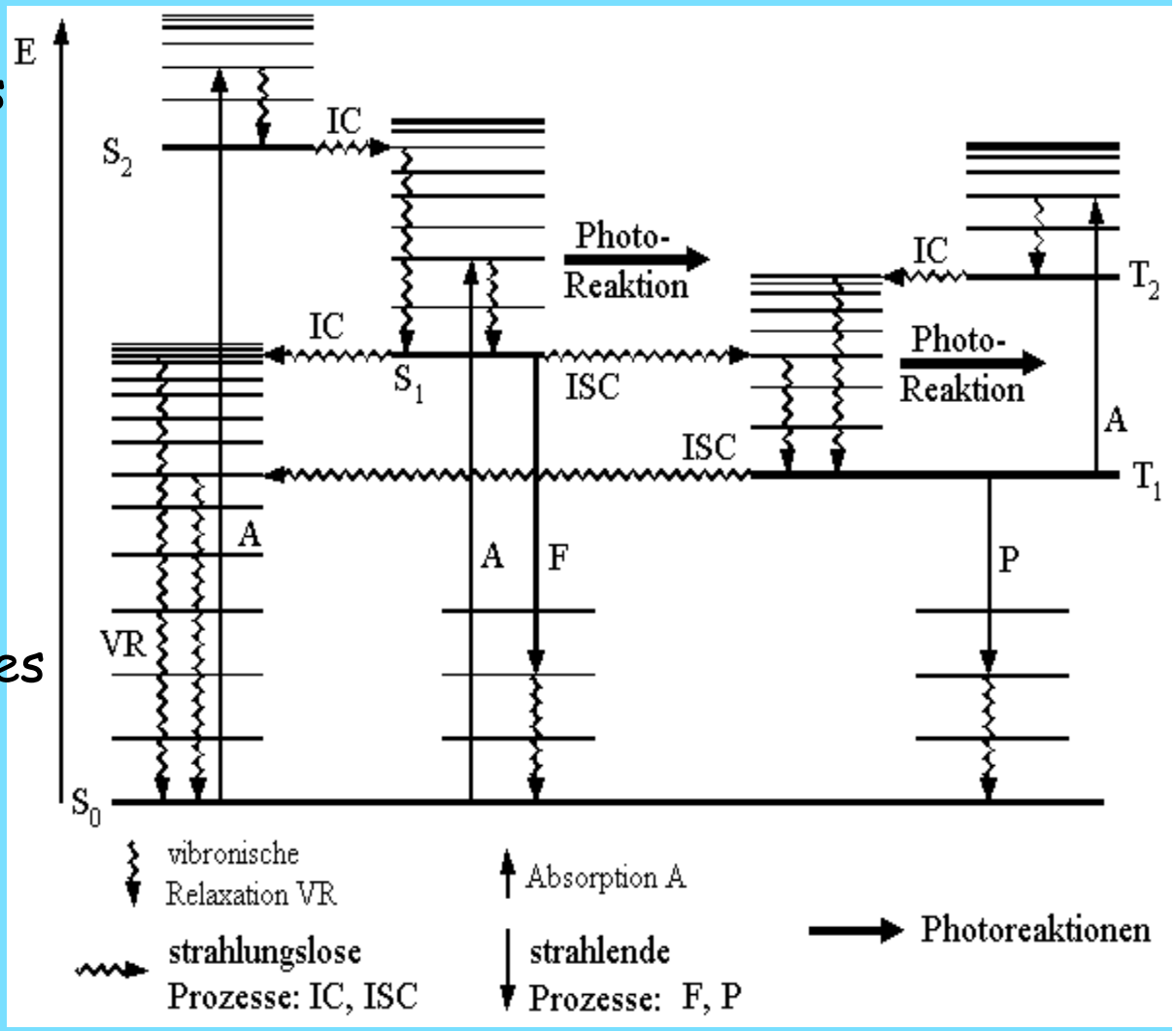
→ Complex statistical description of the data not necessary, as the two instantaneous signals can be compared directly.

# Jablonski Diagram …

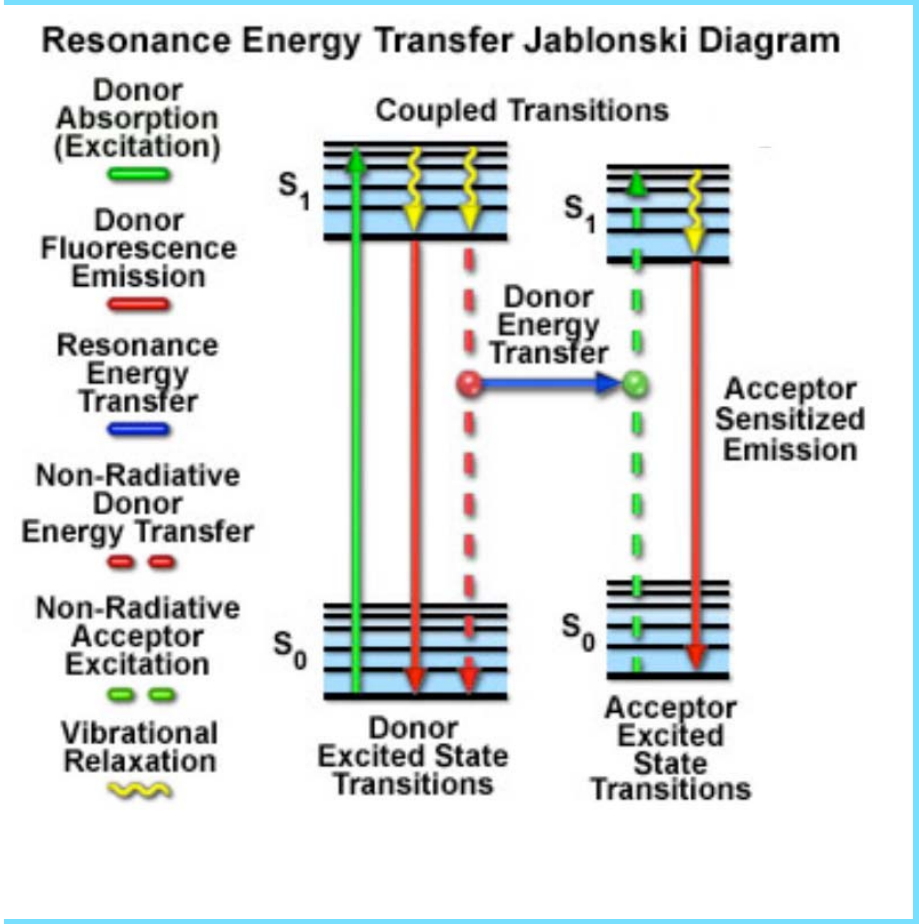
"Term scheme" of molecules

More complex de-excitation mechanisms need to be taken into account:

- Non-radiative processes
- Radiative processes
- Photoreactions



# FRET's Jablonski Diagram …




Precondition:

- Donor – Acceptor must be closely together, e.g. separation in 20 – 100 Å
- Acceptor must absorb in the flourescence emission spectrum of the donor
- Donor emission dipole must be parallel to the acceptor excitation dipole

→ *FRET is dipole-dipole interaction.*

# FRET mechanism …

Energy is transferred non-radiatively from an excited flourophore (donor) to another chromophore (acceptor).

→ to the typical excited lifetime a new term is introduced:

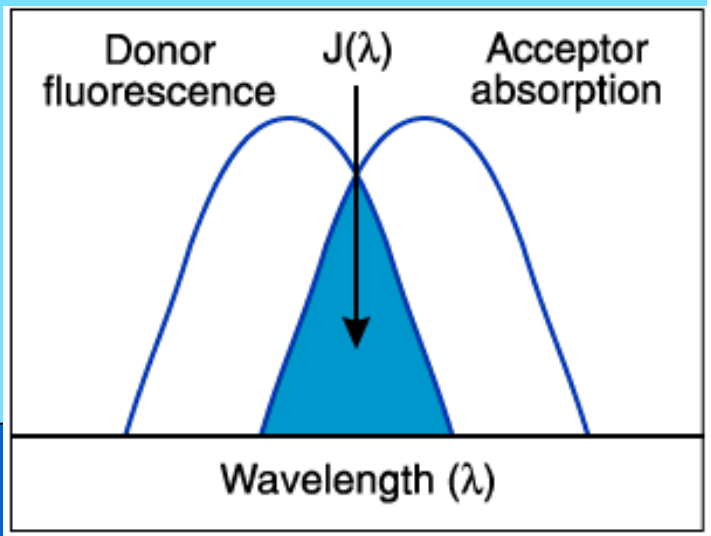
$$\tau_{donor}^{-1} = k_{int.conv.} + k_{int.systm.cross.} + k_{quench} + k_{Flour.} + k_{FRET}$$

Transfer rate $k_{FRET}$ dependent on the different properties in the donor / acceptor / donor+acceptor system:

Orientation factor — Quantum efficiency

$$k_{FRET} = \frac{9000(ln10)\cdot\kappa^2 Q_D\cdot J}{128\pi^5 n^4 N_A \tau_D R^6} = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6$$

index of refraction — distance



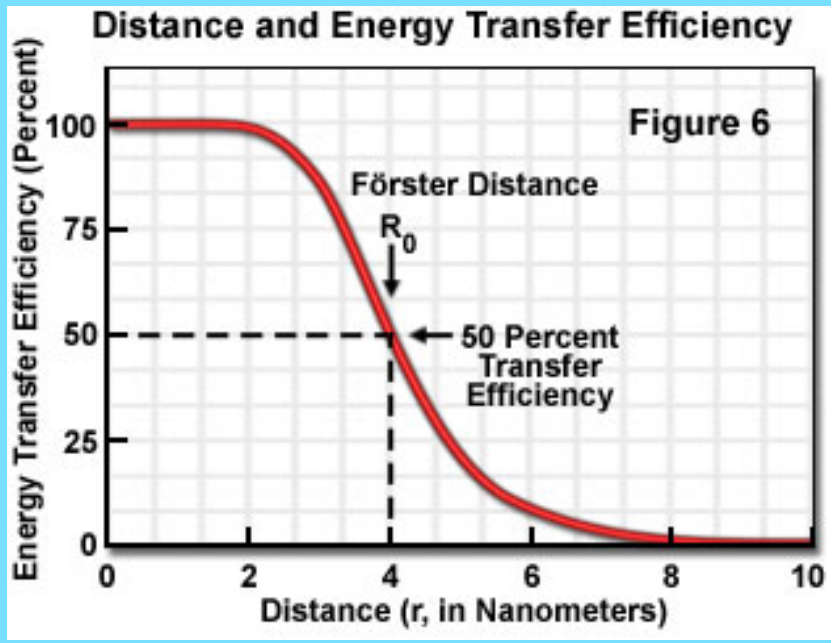
# FRET mechanism …

Key aspect for FRET is the dependence of the energy transfer rate on the spatial distance between donor and acceptor.

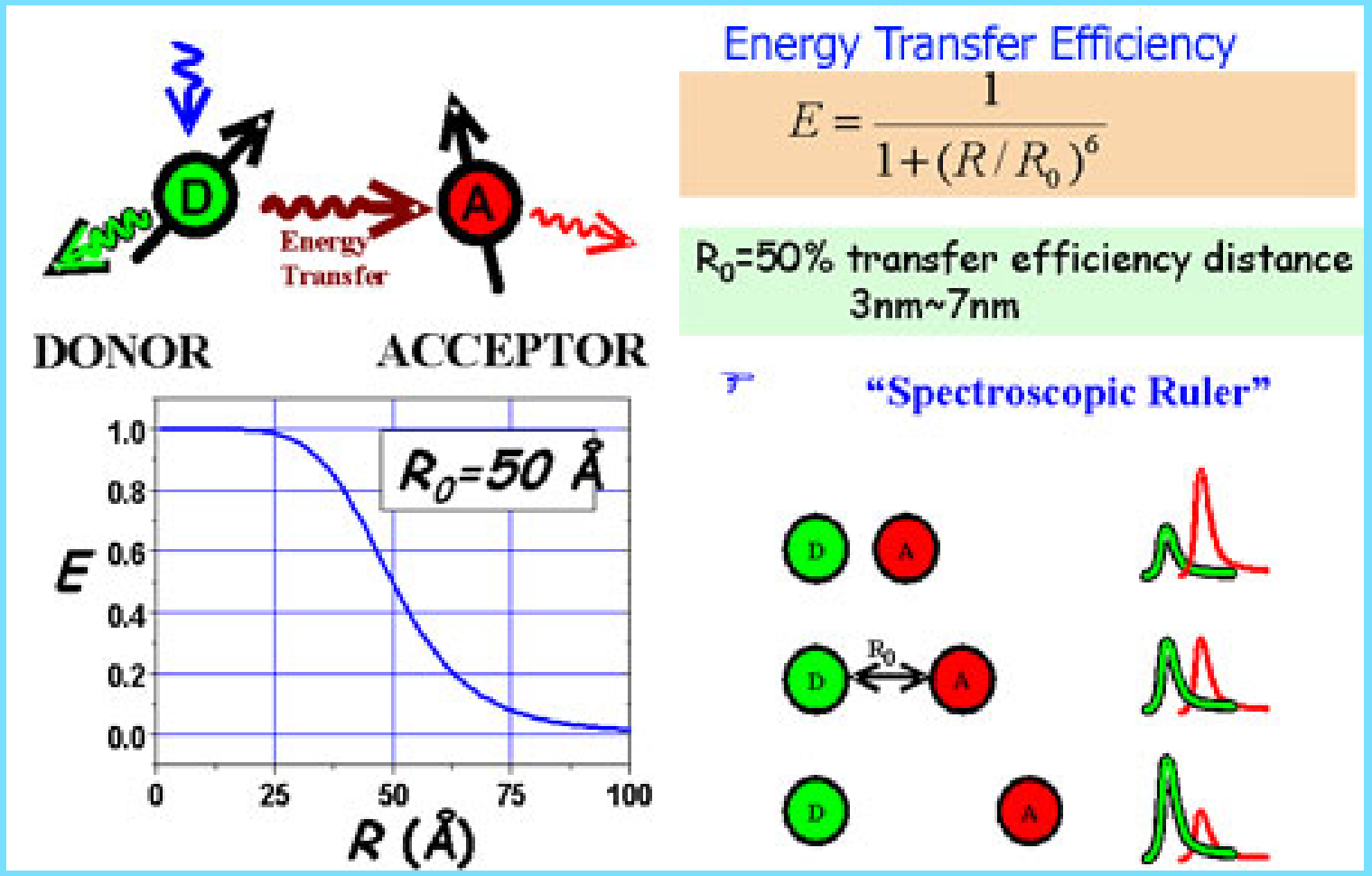
$$k_{FRET} = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6$$

Experimental determination of the *transfer efficiency* in the FRET system:

$$E_{FRET} = 1 - \frac{\Phi_D^A}{\Phi_D} = 1 - \frac{I_D^A}{I_D} = 1 - \frac{\tau_D^A}{\tau_D}$$



# Schematic FRET …



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# Problems using FRET …

Reference value of unperturbed lifetime mostly not known

**R** is of great interest, but some parameters in R might change during experiment (e.g. translocations) making data interpretation difficult.

→ Central factor is the orientation factor κ, but tabulated values not always correct.

Donor – acceptor stocihiometry needs to be taken into account as well.

Using FRET two challenges arise:

- the formalism must be appropriate for quantifying FRET under conditions of abitrary, unknown, intermolecular + intramolecular stoichiometries, distributions and environments.
- continuous methods of observation are desirable.

# Molecule detection schemes …

Intramolecular FRET

Intermolecular FRET

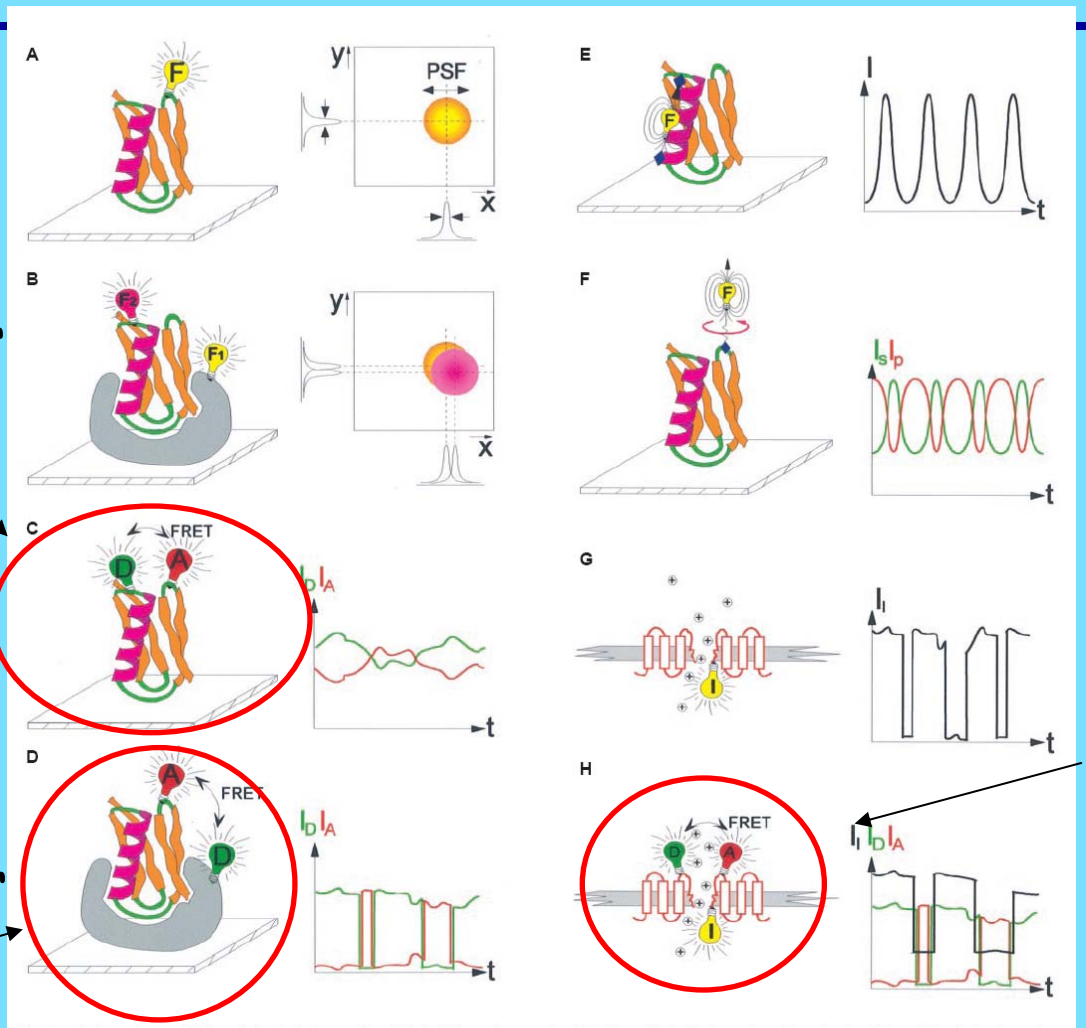


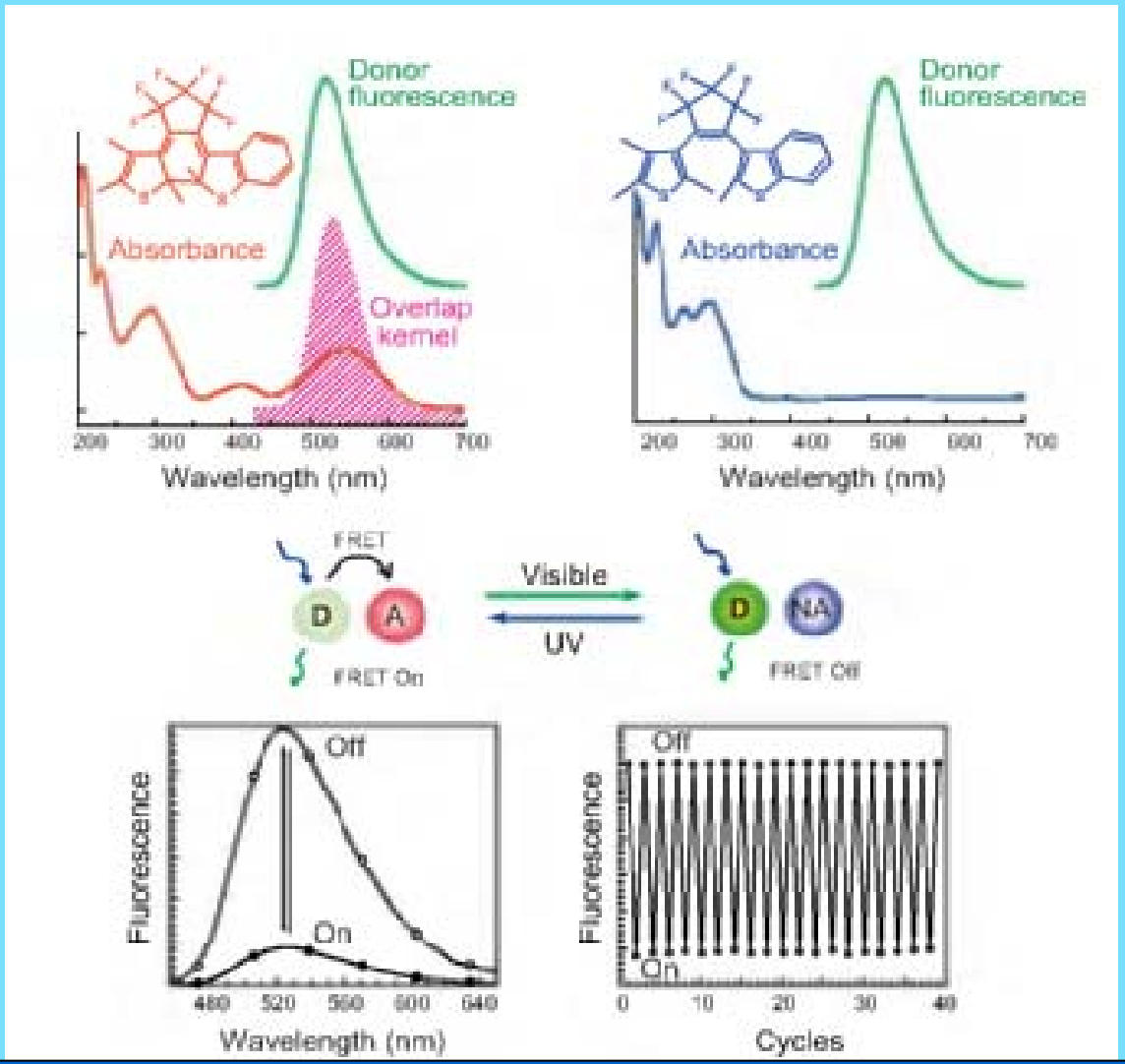

**Fig. 1.** Labeling schemes (left) and physical observables (right). **(A)** Localization of a macromolecule labeled with a single fluorophore F with nanometer accuracy. The point-spread-function (PSF) can be localized within a few tenths of a nanometer. **(B)** Colocalization of two macromolecules labeled with two noninteracting fluorophores, $F_1$ and $F_2$. Their distance can be measured by subtracting the center positions of the two PSFs. **(C)** Intramolecular detection of conformational changes by spFRET. D and A are donor and acceptor; $I_D$ and $I_A$ are donor and acceptor emission intensities; $t$ is time. **(D)** Dynamic colocalization and detection of association or dissociation by intermolecular spFRET. Donor and acceptor intensities are anticorrelated both in (C) and (D). **(E)** The orientation of a single immobilized dipole can be determined by modulating the excitation polarization. The fluorescence emission follows the angle modulation. **(F)** The orientational freedom of motion of a tethered fluorophore can be measured by modulating the excitation polarization and analyzing the emission at orthogonal **s** and **p** polarization detectors. $I_S$ and $I_P$ are emission intensities of **s** and **p** detectors. **(G)** Ion channel labeled with a fluorescence indicator I. Fluctuations in its intensity $I_I$ report on local ion concentration changes. **(H)** Combination of (C) and (G). D and A report on conformational changes whereas I reports on ion flux.

Ion channel measurement + FRET

# Intramolecular FRET …

Allows the study of intramolecular dynamics:

- Folding of protein structures
- Conformational dynamics



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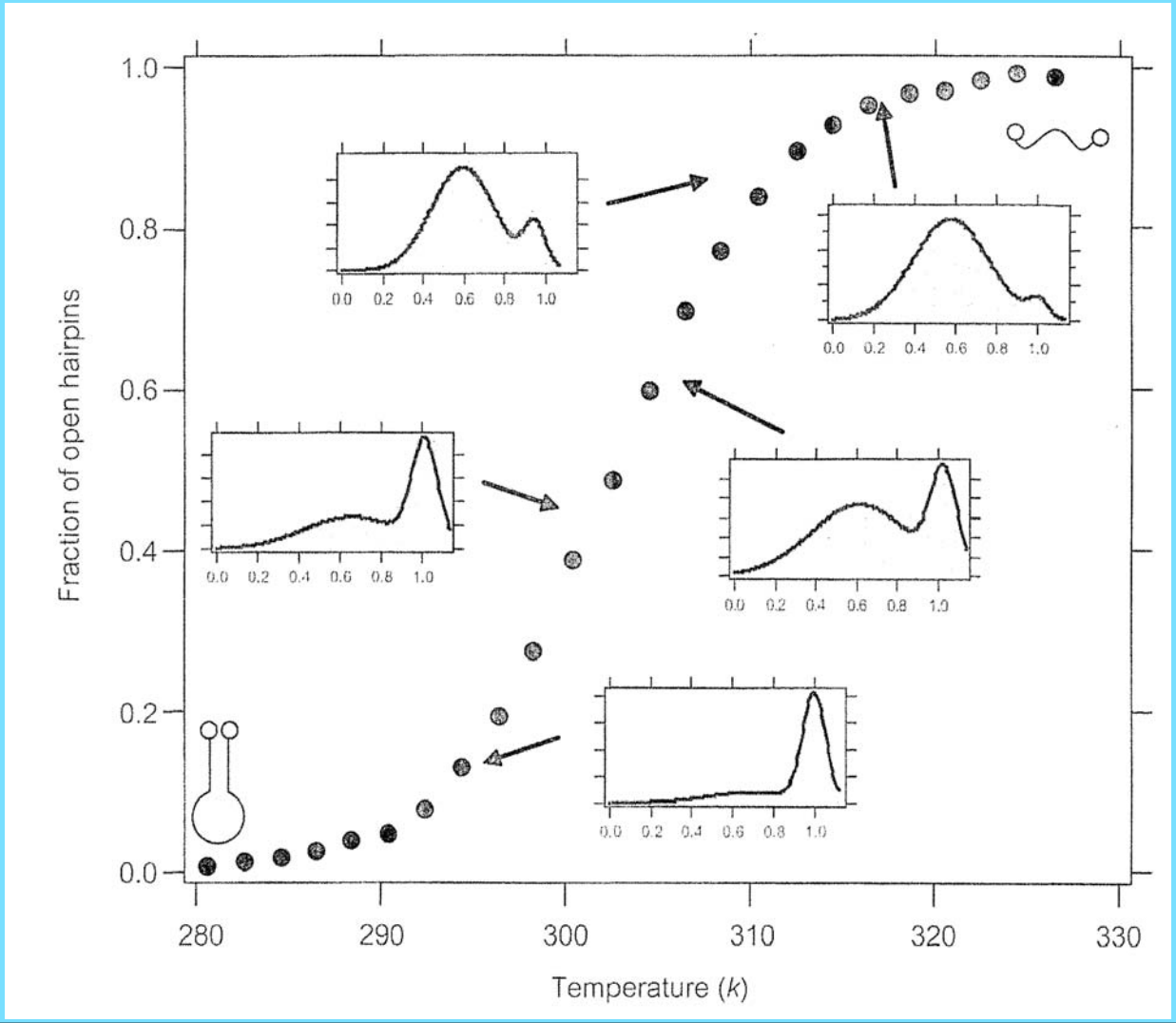
# Intramolecular FRET …

Observation of single molecule reaction dynamics in time dependent chemical & physical environment.



# Intermolecular FRET …

Precise measurement of interaction between the molecule and the environment.

→ (independent) of probe preparation

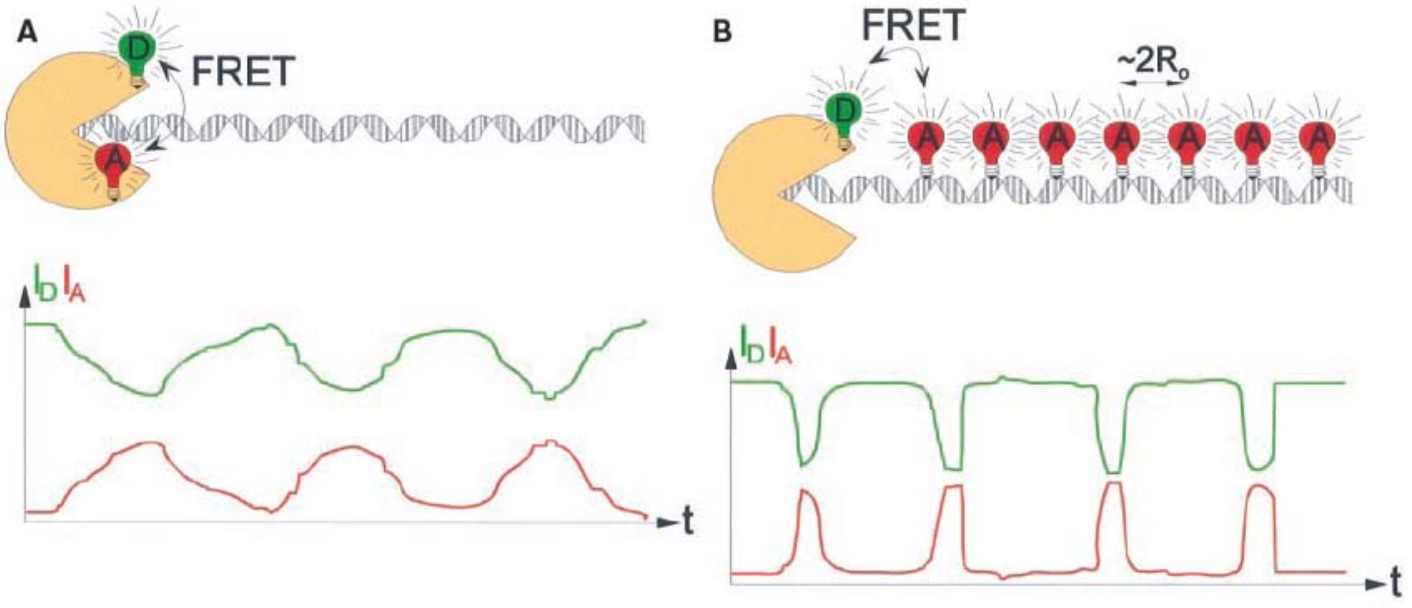



**Fig. 5.** A cartoon illustrating (**A**) intramolecular and (**B**) intermolecular spFRET nuclease-DNA interactions. Intramolecular spFRET measures conformational dynamics of the enzyme during catalysis. Intermolecular spFRET measures association, catalysis, and dissociation of substrate molecules. Multiple acceptors at equal distances on the DNA act as a "ruler." $R_0$ is the Förster radius (distance at which 50% of the energy is transferred). This scheme can be generalized to many other protein-DNA interactions.

# Stochiometry …

Measurement of conformational distributions in freely diffusing single molecules.

→ Usage of two DNA constructs with varying intramolecular fluorophores showed the possibility to distinguish sub-ensembles.

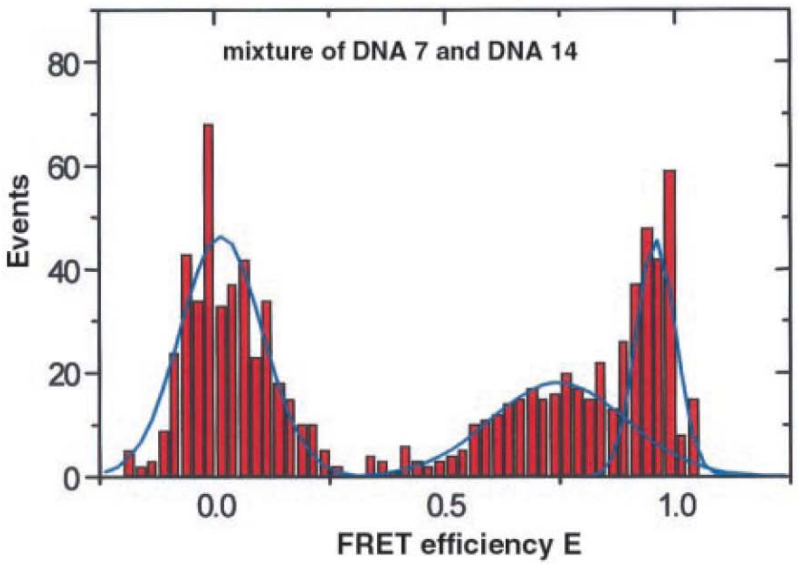
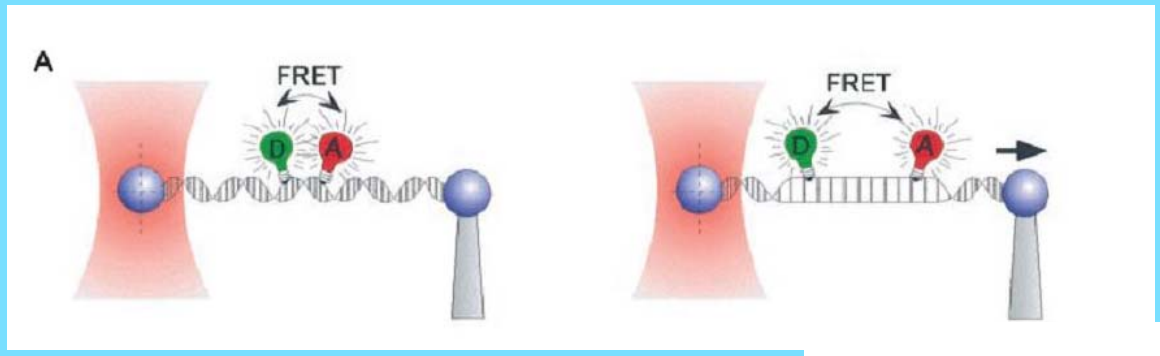


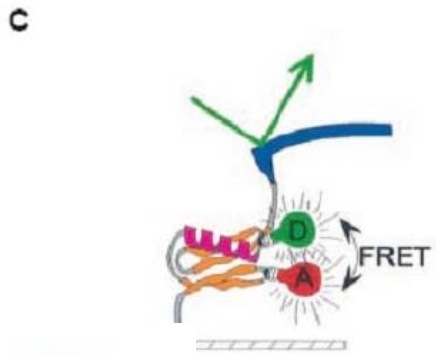

**Fig. 2.** FRET Histogram of a sample containing a 1:1 mixture of two different double-stranded DNA molecules with 7– and 14–base pair (bp) separation between donor and acceptor. The peak around zero results from faster photobleaching of acceptors (compared with that of donors), leaving donor-only–labeled molecules; the two peaks at energy transfer efficiency $E \sim 0.7$ (14-bp separation) and $E \sim 1$ (7-bp separation) demonstrate the ability to identify subpopulations according to their conformational states.

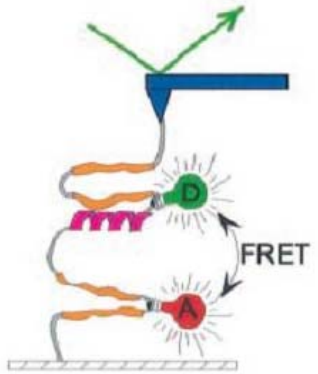
# Possible applications of FRET …

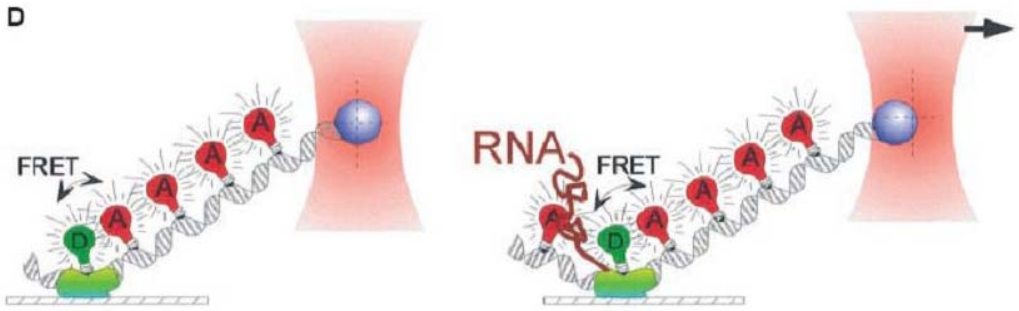



Observing conformational dynamics with optical tweezers

"Mechanical" (e.g. AFM tip) forces on a macromolecule




Protein dynamics

# Thank you for your attention!

*Selected references:*

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