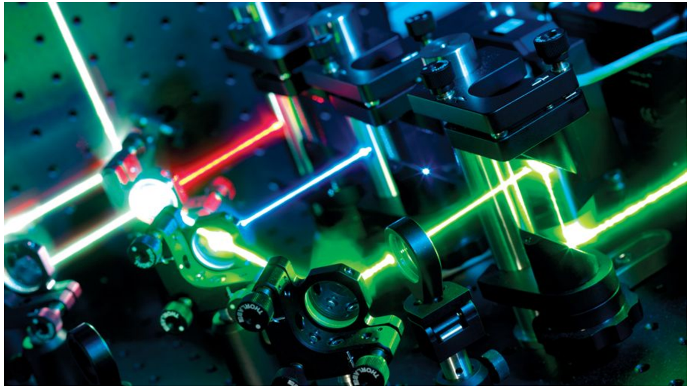
The physics behind

Single Molecule Spectroscopy

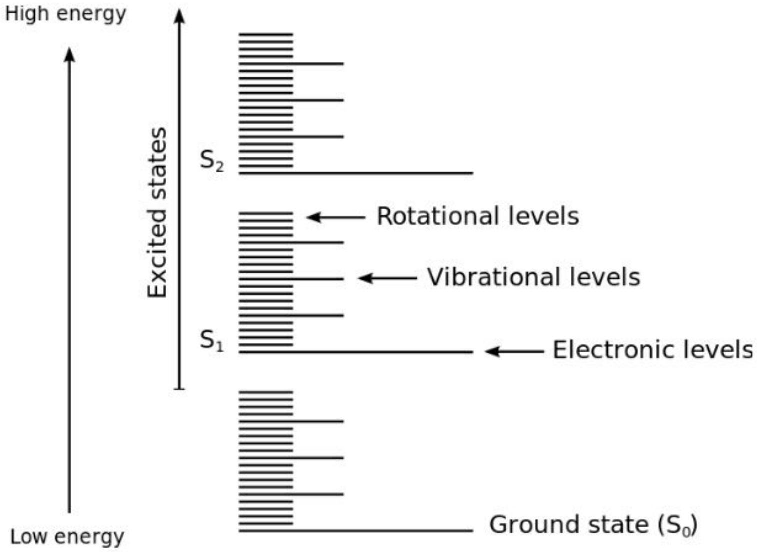
Adapted from Lakowicz *Principles of Fluorescence Spectroscopy*

Kif Lim

Workshop 3/2/23



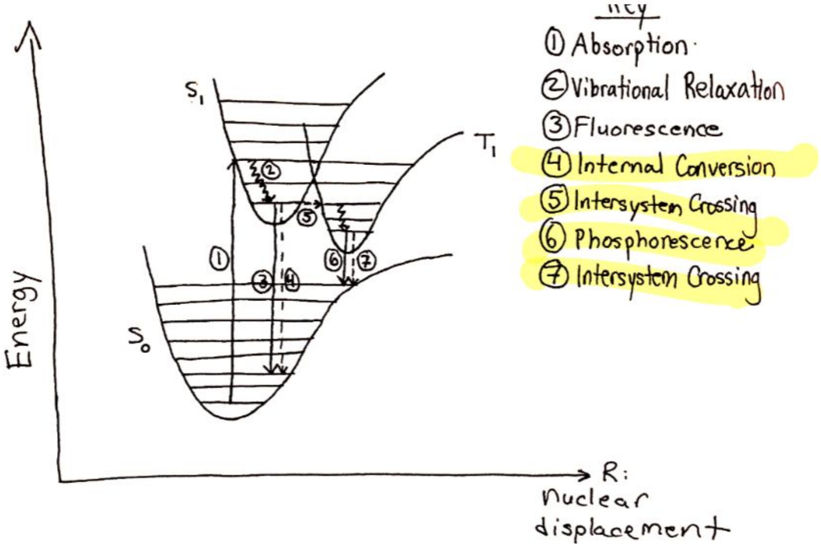
Review of Fluorescence Workshop



Divisions of energy transitions:

1. Electronic Transition occur when electrons move from one energy level to another.
2. Vibrational Transitions are due to the elastic movement of chemical bonds. These transitions occur between different vibrational levels of the same electronic state.
3. Rotational motions involve changes in the molecule’s angular momentum and occur within the same vibrational state.

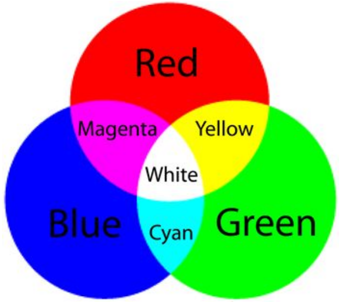
Review of Fluorescence Workshop

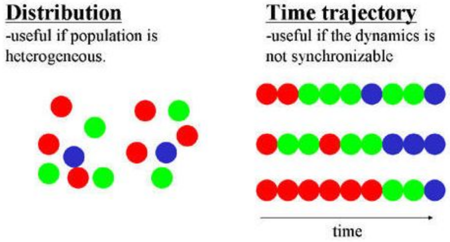


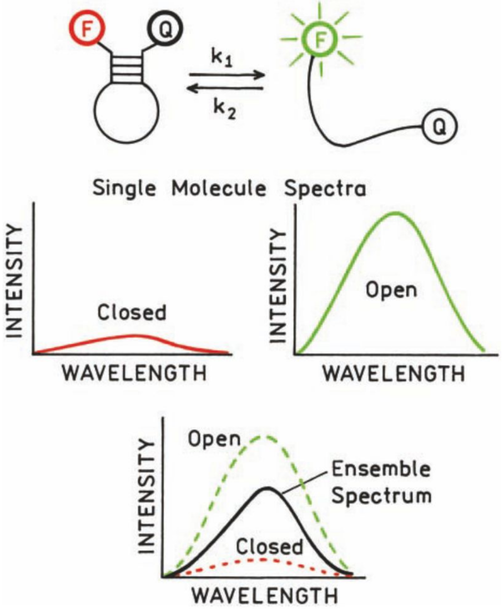
The Jablonski diagram summarizes all the possible transitions that can occur after a molecule is photoexcited.

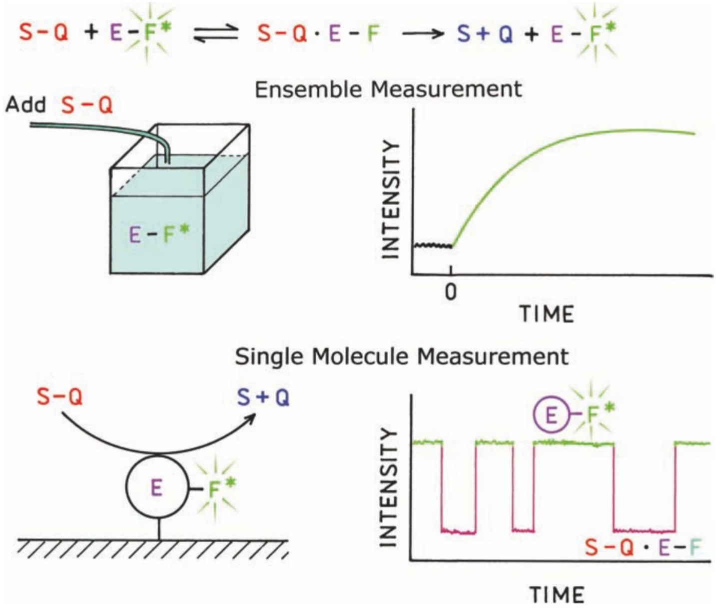
Absorption	Electron excited to a higher energy level	10^{-18} s
Vibrational Relaxation	Non-radiative transition to lowest vibrational state that involves transferring kinetic energy to other molecules.	10^{-15} s
Fluorescence	Radiative emission of light from electronically excited state to ground electronic state.	10^{-9} s
Internal Conversion	Non-radiative emission from electronically excited state to ground electronic state.	10^{-7} s
Intersystem Crossing	Transition from excited singlet state to triplet state requiring change in electron spin. No energy change.	10^{-6} s
Phosphoresc ence	Radiative relaxation from excited triplet state to singlet ground state.	10^{-5} s

Why single-molecule?







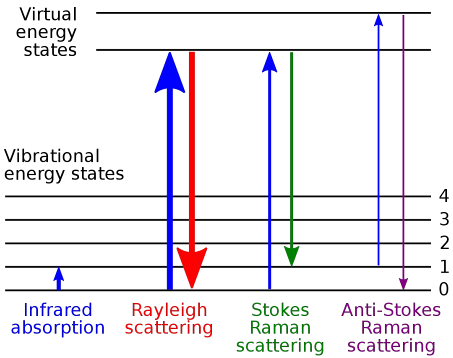


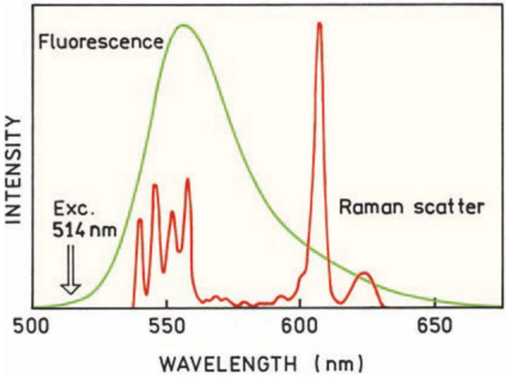
- Single-molecule imaging eliminates ensemble averaging, allow us observe heterogeneity in a population.
- For instance, single-molecule measurements can capture intermediate states of a process.
- Single-molecule detection is also useful to study reaction kinetics without synchronizing the reaction.

Challenges with imaging single-molecules

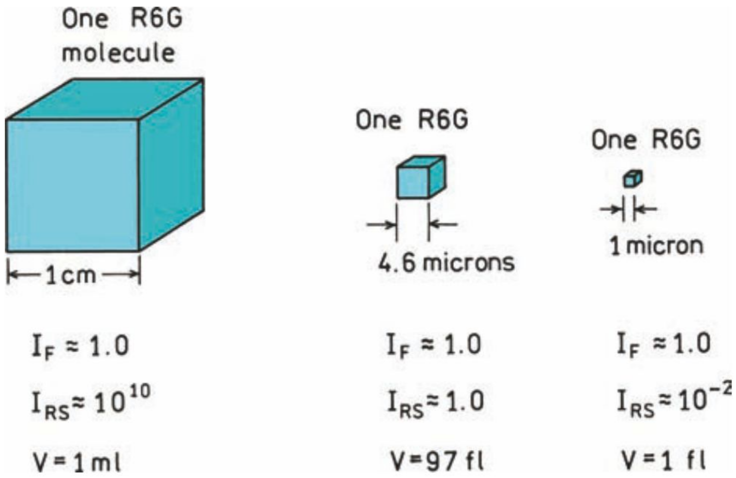
- Impurities in the sample
- Single-fluorophores can bleach or blink easily.
    - Blinking occurs when molecules undergo intersystem crossing to the triplet state. They remain dark until they undergo phosphorescence to the ground singlet state.
- Emission by optical components
- Scattered light
- Non-specific binding
- **Collective Raman Scattering of solvent molecules**

The detectability of single-molecules

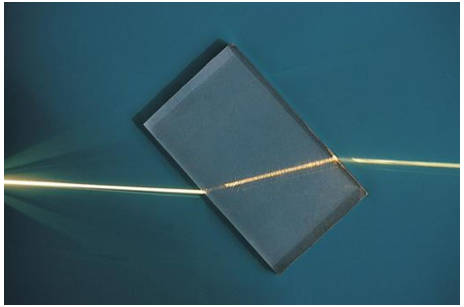


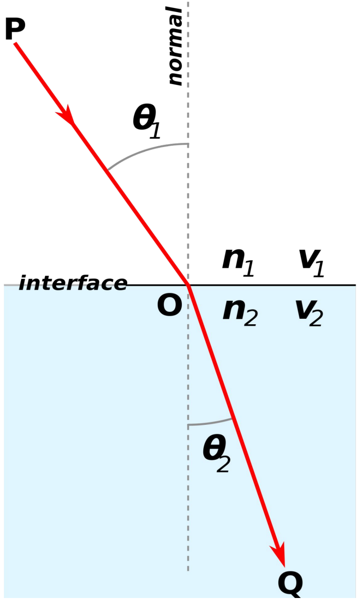


- The collective Raman scattering of solvent molecules can outcompete the fluorescence of a single-molecule.
- This requires is to limit the observed volume when imaging fluorophores. Typical single-molecule experiments are designed so that the volume observed is less than 1 femtoliter or 1 μm^3.
- TIRF and confocal scopes provide a way to limit the observed volumes.



Refraction is the physical basis of TIRF

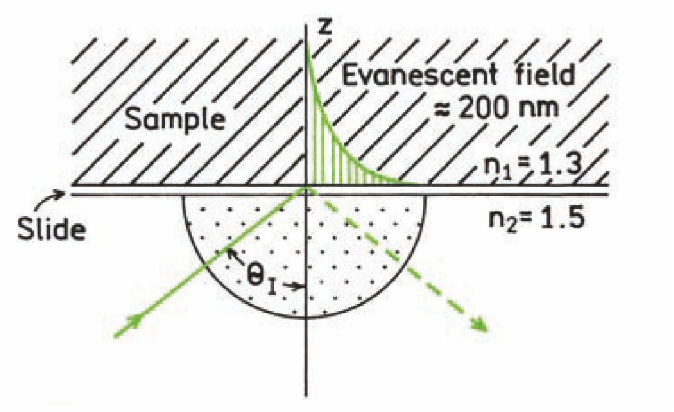




$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

- Refraction is the bending of light as it passes from one medium to another.
- If $n_2 > n_1$, then $\theta_2 < \theta_1$ (the refracted angle decreases).
- If $n_2 < n_1$, then $\theta_2 > \theta_1$ (the refracted angle increases).
- The incident angle when $\theta_2 = 90^o$ is known as the critical angle.
- TIR occurs when the incident angle exceeds the critical angle.

Total Internal Reflection (TIRF) Optics

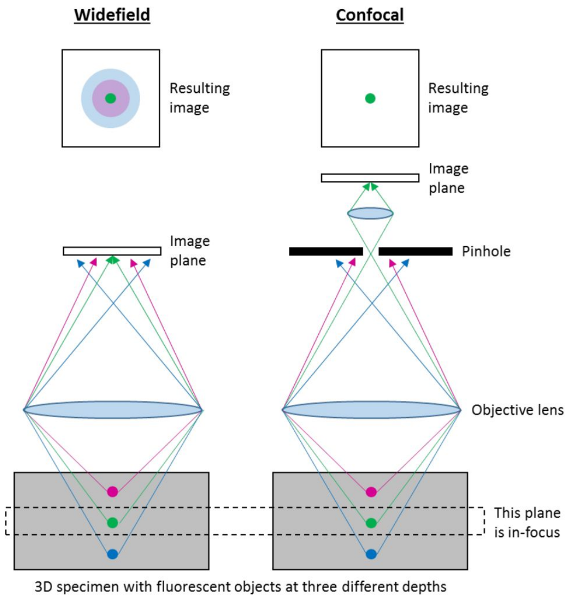


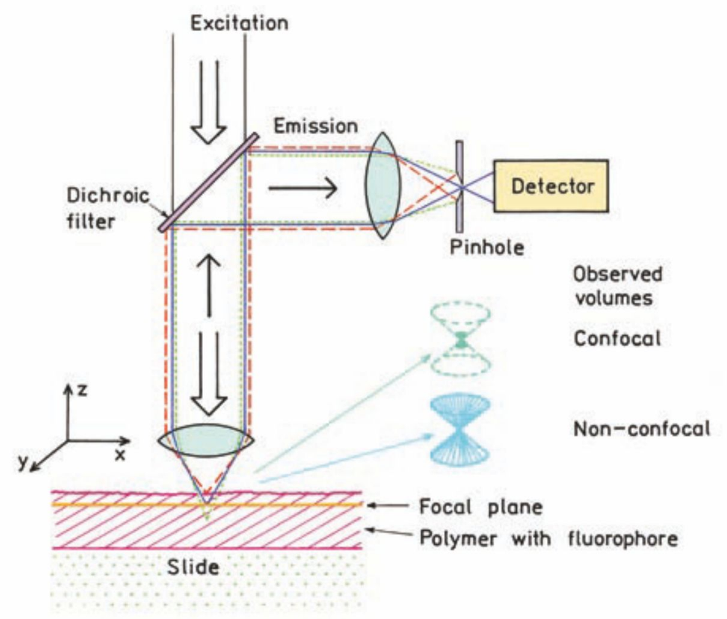
$$\theta_C = \sin^{-1}\left(\frac{n_1}{n_2}\right)$$

$$I(z) = I(0)\exp(-z/d)$$

- Reflection occurs at the interface between the glass coverslip and the small film of aqueous medium.
- While the incident light is reflected off the interface, the intensity of light can penetrate a short distance into the sample.
- TIRF reduces the illuminated distance in the z-axis, thereby reducing the effective observed volume.

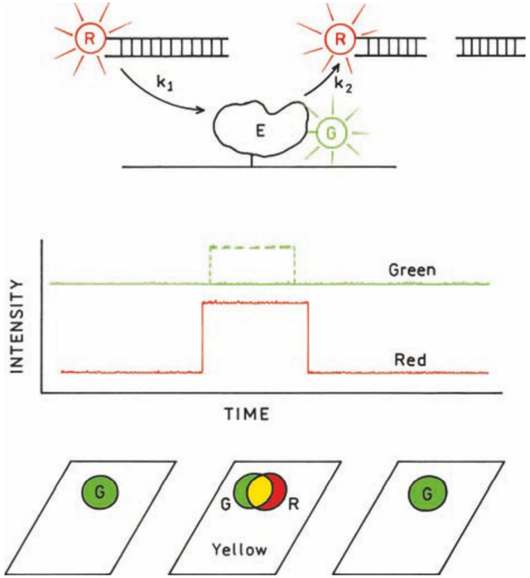
Confocal Optics

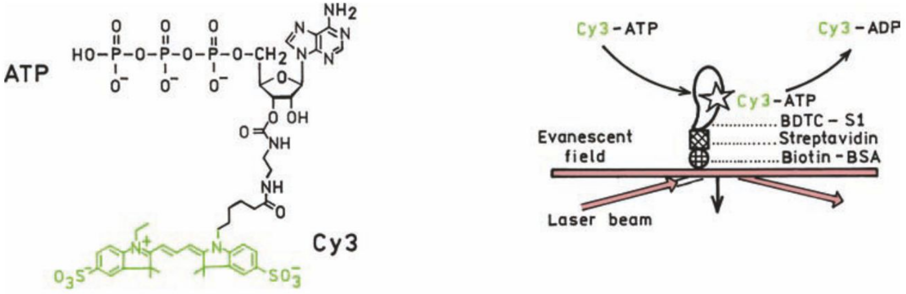


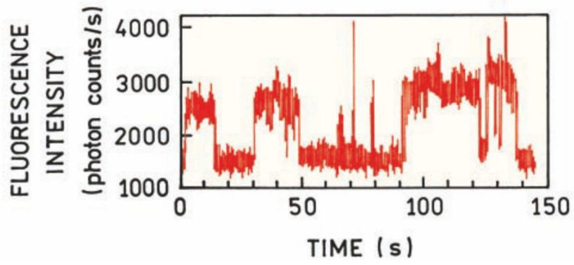


- In confocal optics, a pinhole placed at the focal point of light blocks out-of-focus light. Any light above or below the focal plane is blocked because it either converges too early or too late.
- Therefore, confocal optics also reduce the observed volume. The signal from a single fluorophore can be 500X the Raman scatter of solvent molecules.

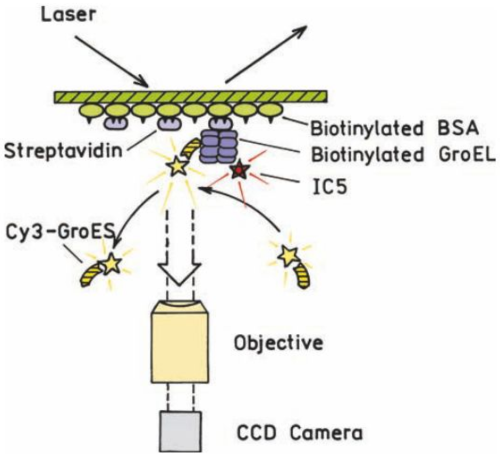
Example 1: Single-Molecule Enzyme Kinetics

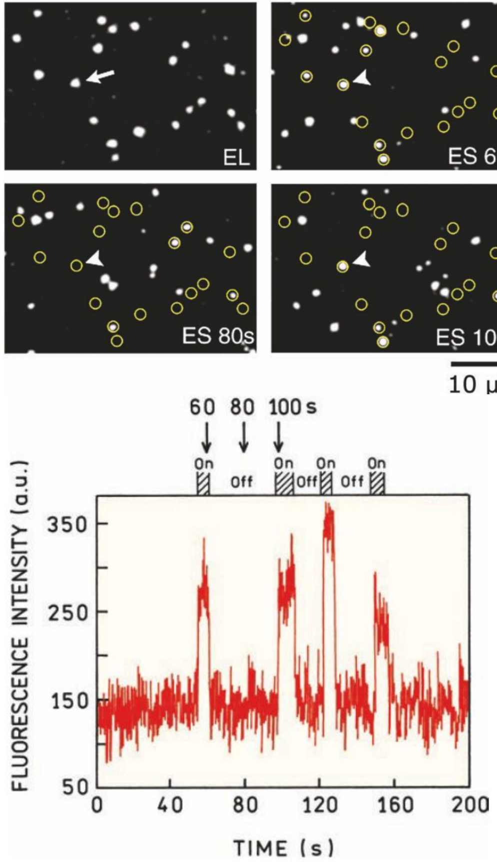


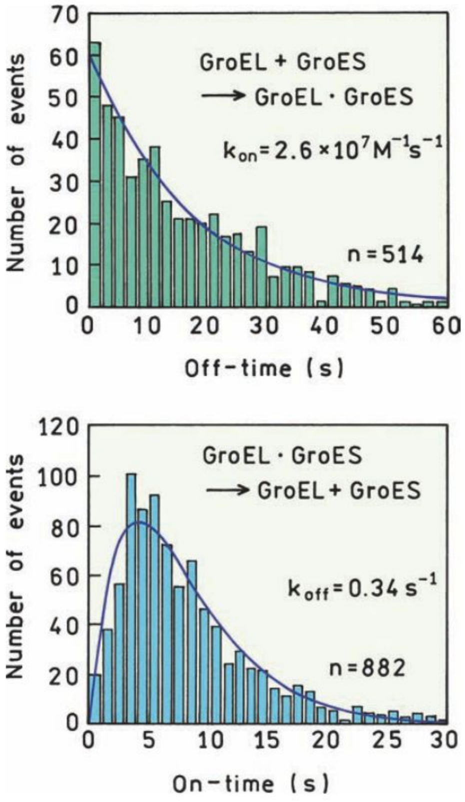




Example 2: Single-Molecule Studies of a Chaperonin Protein

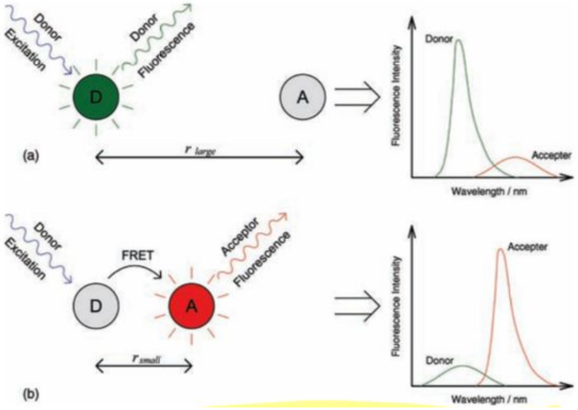


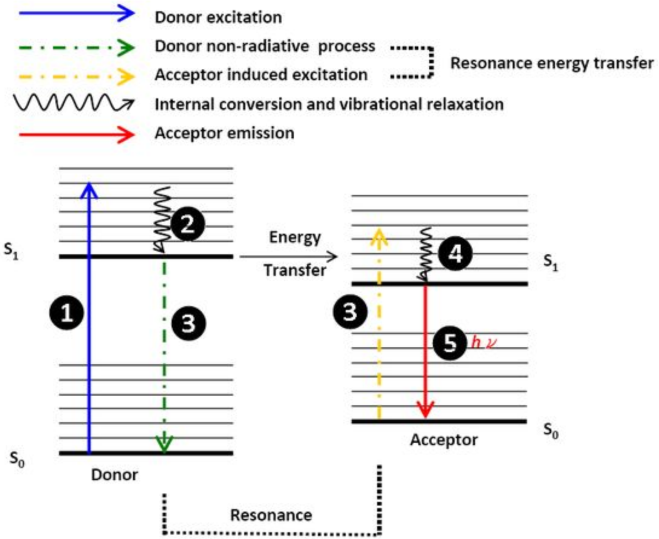


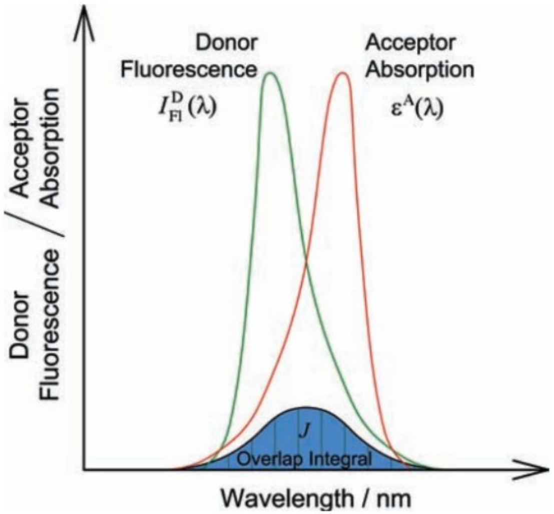


Forster Resonance Energy Transfer (FRET)

$$k_{ET} = \frac{1}{r^6}\kappa^2$$

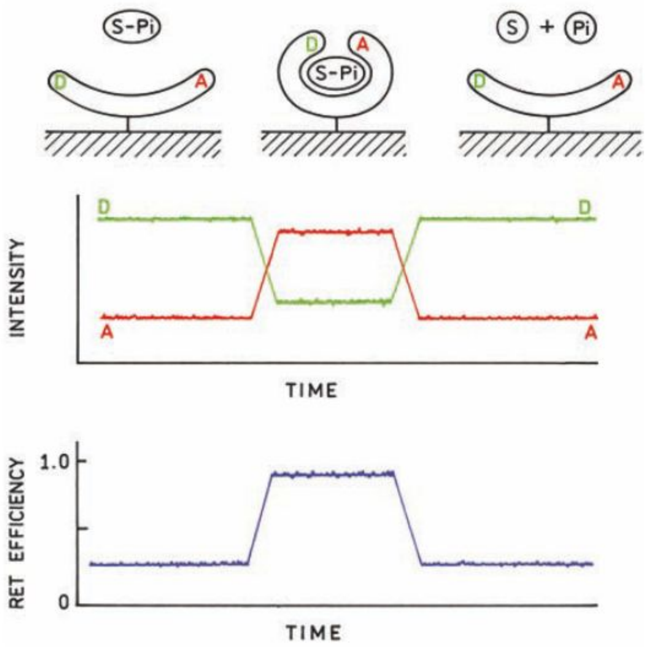


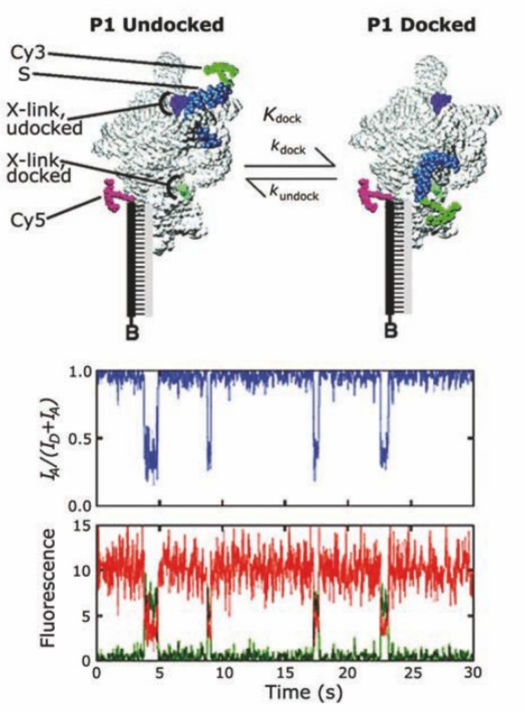




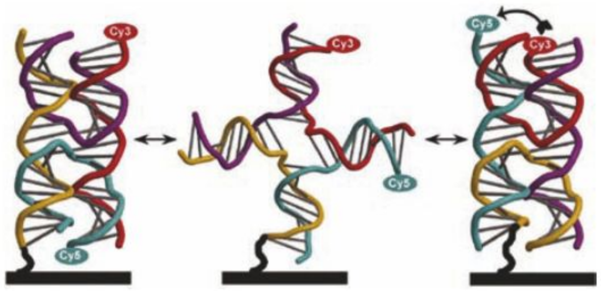
- FRET involves the transfer of excitation energy from a donor to acceptor fluorophore.
- FRET requires that the donor and acceptor are in close proximity (<80 Å), with a distance dependence of $1/r^6$.
- The rate of energy transfer is directly proportional between the spectral overlap between donor fluorescence and acceptor absorption.

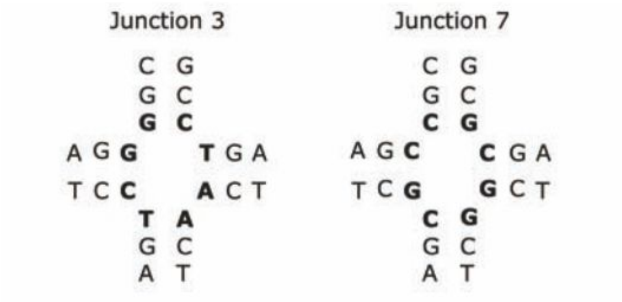
Single-Molecule FRET

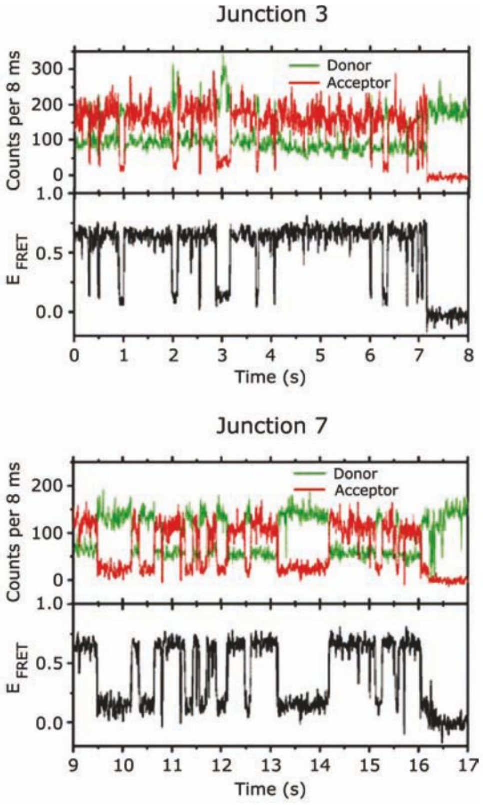




Example 3: Conformational Dynamics of a Holliday Junction







The single-molecule toolkit

- **Single-molecule imaging**
- **Single-molecule FRET**
- Optical/Magnetic Tweezers
    - C-traps
- DNA Curtains
- Single-molecule sequencing
- Single-molecule polarization assay
- Among many other variations… the question drives the tools used.