Lo studio delle singole molecole, che bisogno c'è?

Le molecole possono 'fare' cose complicate, o inattese. Se una popolazione è sincronizzata, una tecnica di studio di popolazione può caratterizzarle

Ma se le molecole fanno cose complicate e non sono sincronizzate, o diverse molecole fanno cose diverse … è meglio caratterizzarle una alla volta.

Bulk Methods vs. Single Molecule Methods

Single Molecule Methods:

- Molecules display a fast, instantaneous dynamics
- Behavior appear random and stochastic
- Fluctuations are predominant
- Molecules are seen to co-exist in various states. Populations are multimodal
- Molecules can be found in states far from the mean of the population (extreme states)

Single molecule fluorescence

• Can it be done? How? Why?

– Yes. In many different ways.

• Reasons for single molecule fluorescence:

– To examine molecular heterogeneity and diversity

– To observe intermediates or structural changes in processes that cannot be synchronized

- monitor time-course of fluorescence

- kinetic trajectories of single molecules

- conformational changes of single molecules

- spectral fluctuations of single molecules

- excited-state dynamics

- catalytic turnover of single molecules

- macromolecular associations of single molecules

- translational and rotational motion

Why single Molecule Methods?

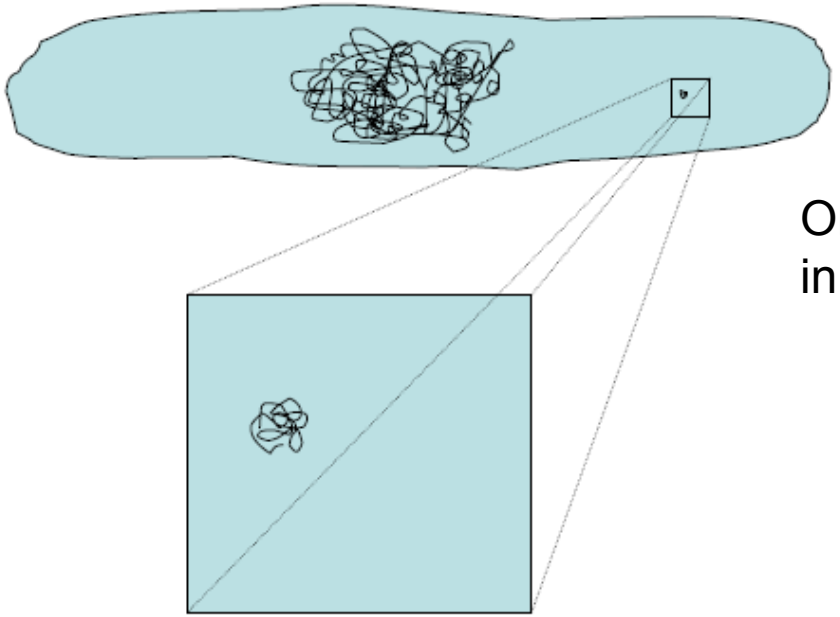
The microscopic view matters in describing the cell interior:

Many cellular processes, such as:

Chromosome replication and segregation

DNA transcription, recombination, and RNA translation

Are often carried out by a few molecules. Far from displaying smooth dynamics these process are stochastic in nature.



One molecule in an *E. coli* cell (about 1 μm^3) in volume is at a concentration of ~ 1.6 nM.

Why single Molecule Methods?

The advent of methods of single molecule manipulation has made it possible, for the first time to:

Measure the forces that maintain the 3D structure of macromolecules

Characterize the stress-strain relationships of molecules

Measure the forces generated in chemical & biochemical reactions

Investigate time-averaged and time-dependent fluctuations

Characterize the dynamics of molecular motors

Exert External forces and torques to alter the extent and fate of chem. Rxns.

Single molecule fluorescence: Experimental approaches

- Solution measurements:
  - Fluctuation Correlation Spectroscopy
    - Confocal
    - Multiphoton
  - Photon Counting Histogram / FIDA
- Immobilized molecules:
  - TIRF
  - Scanning confocal
  - Scanning 2-photon
- Imaging:
  - Fluorescence microscopy
  - FLIM (FLIM-FRET)

Single molecule fluorescence: theoretical principles

- Require the ability to generate and detect sufficient number of photons to discriminate the single molecule from background and from other molecules
- Probability of observation of a molecule < 1
- A single fluorophore typically emits 105 photons before bleaching

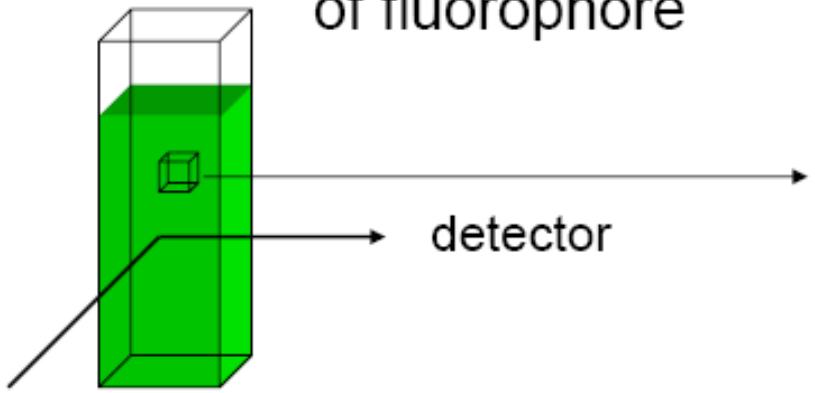
Single molecule fluorescence: experimental considerations

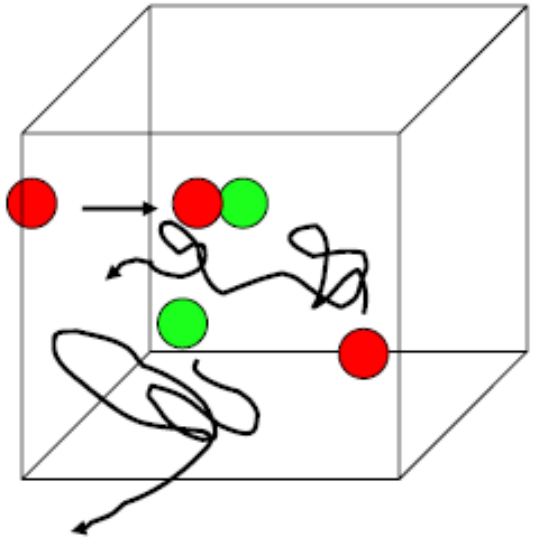
- **Excitation**
  - – High NA objective lens
  - – “Bright” fluorophores
    - High extinction coefficient
    - High quantum yield
  - – Exclude quenchers
    - particularly molecular oxygen!
    - O2 scavengers include β-mercaptoethanol (BME), catalase

- **Emission**
  - – Wavelength dependence of detectors
  - – Spectral separation from excitation
  - – Efficient detection optics
  - – Autofluorescence and contaminant fluorescence
  - – Photobleaching and ISC
  - – Scatter:
    - elastic (Rayleigh)
    - inelastic (Raman)

Fluctuation Correlation Spectroscopy: theoretical principle

- equilibrium solution of fluorophore





At low concentrations and/or in VERY small observation volume

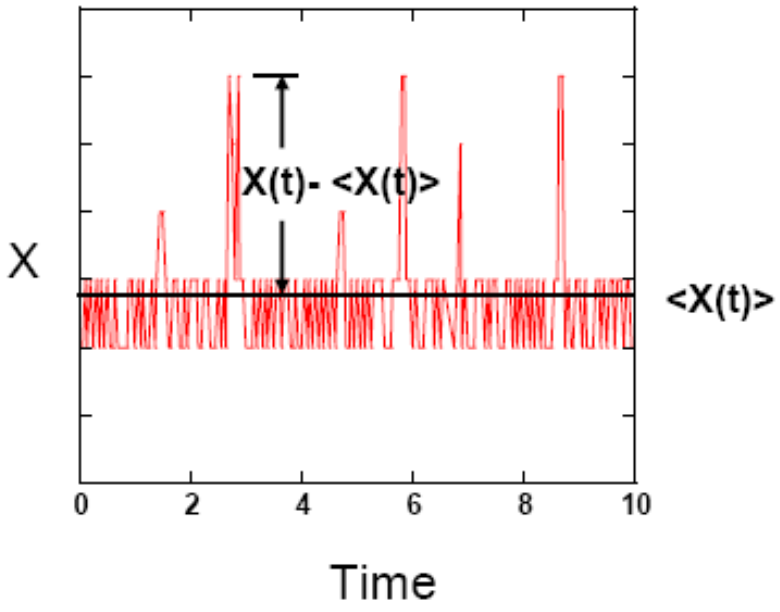
non-equilibrium behavior
fluctuations in particle number

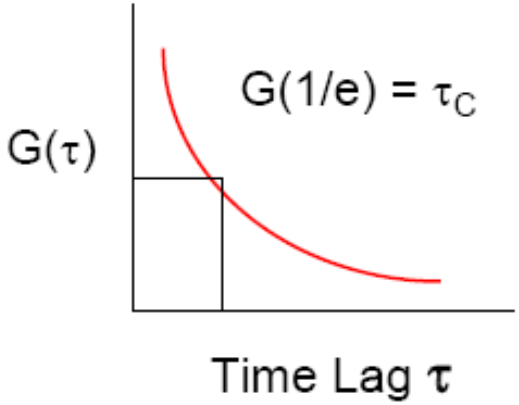
Correlation Analysis

Autocorrelation: Statistical method for determination of randomness in time-derived data sets
- commonly used in electronics and signal-processing

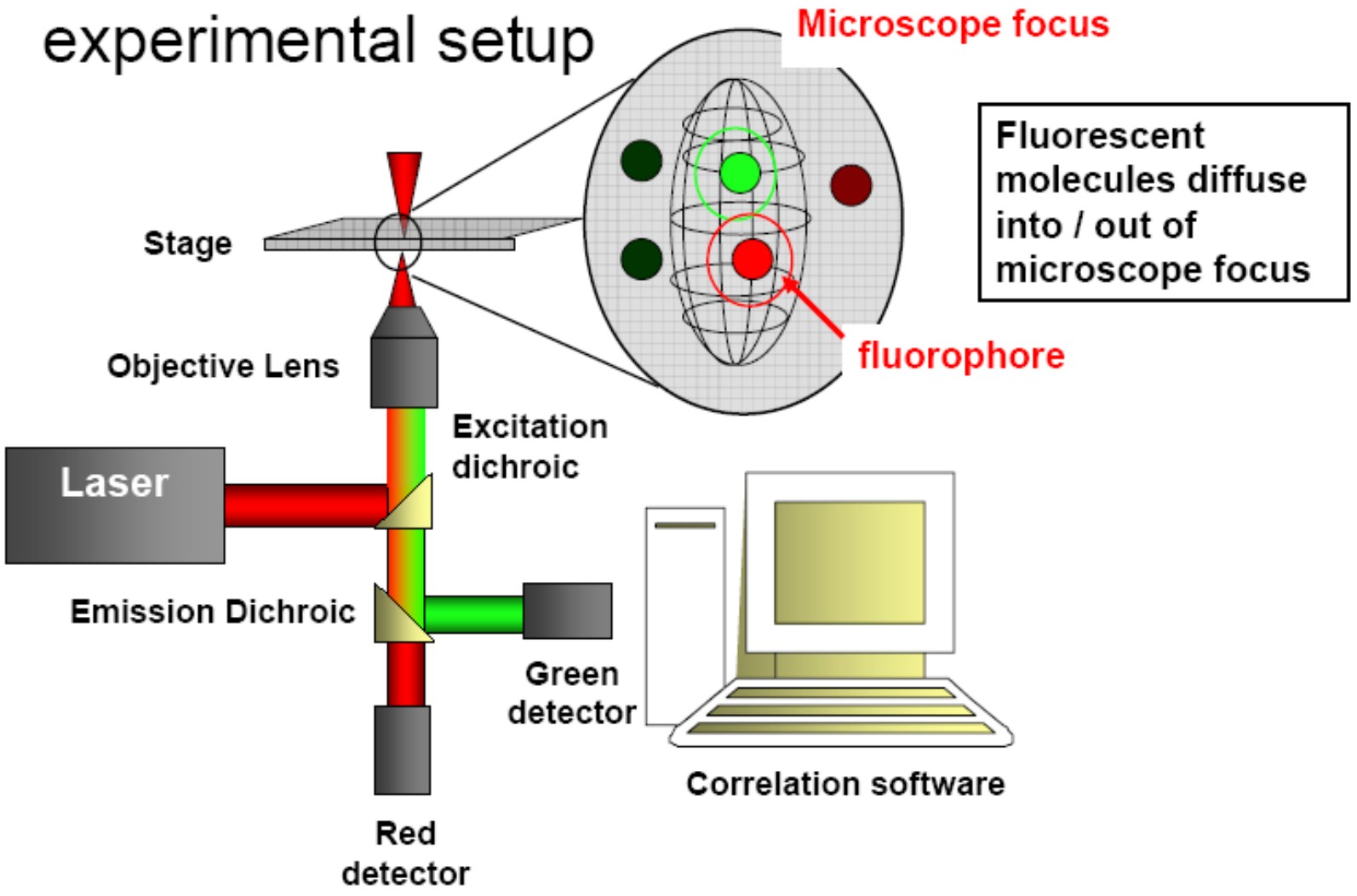
$$G(\tau) = \langle X(t_1)X(t_2)\rangle$$
$$= \langle X(t)X(t+\tau)\rangle$$

$$= \langle (X(t) - \langle X(t)\rangle)(X(t+\tau) - \langle X(t+\tau)\rangle)$$

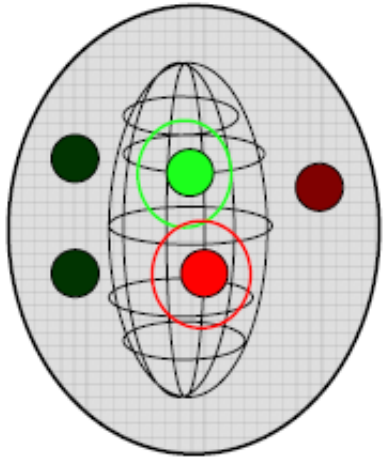




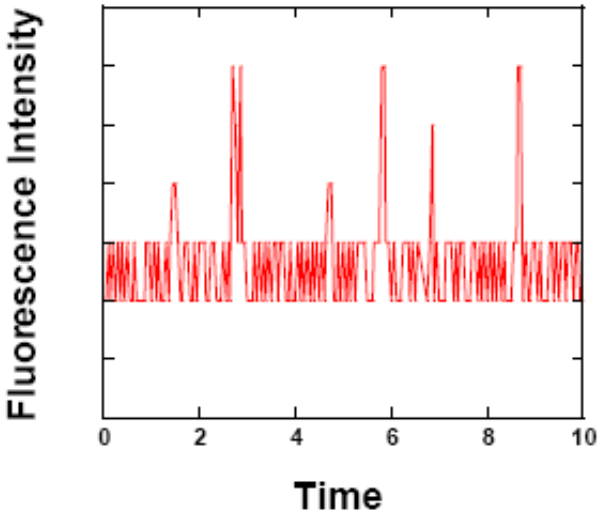
Correlation Spectroscopy: experimental setup



Fluctuation Analysis



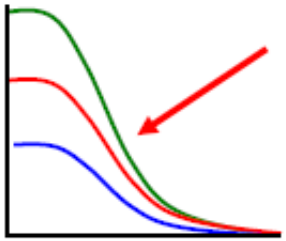
Measure fluorescence as a function of time



Data analysis

Static data - conc

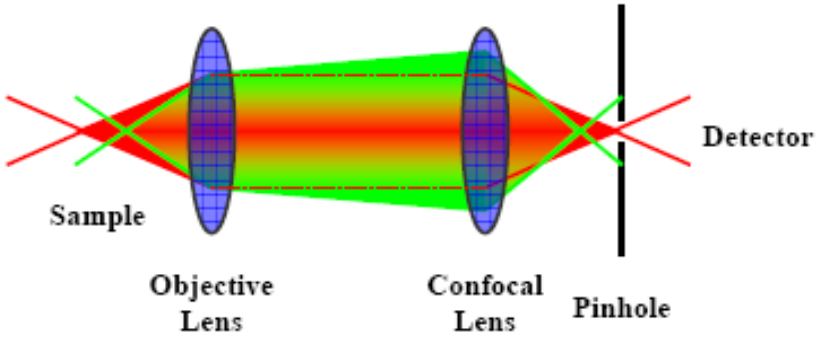
Dynamic data:
- diffusion
- quenching
- kcat
- blinking
- photophysics



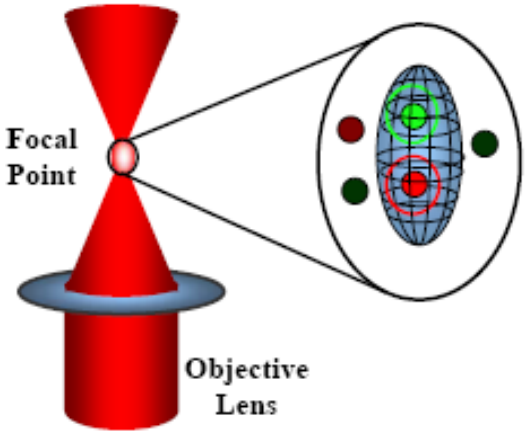
Y-intercept = 1/conc
Decay = diffusion rate

$G(0) \alpha 1/N$
$G(\infty) = 0$
$G(1/e) = \tau_D$
= average residence time

Confocal vs. multiphoton



Detector aperture is at the image focal plane of objective
CONFOCAL
Out of focus excitation light is screened by aperture



Excitation α (laser intensity)^2

$\therefore$ excitation ONLY occurs at the focal point

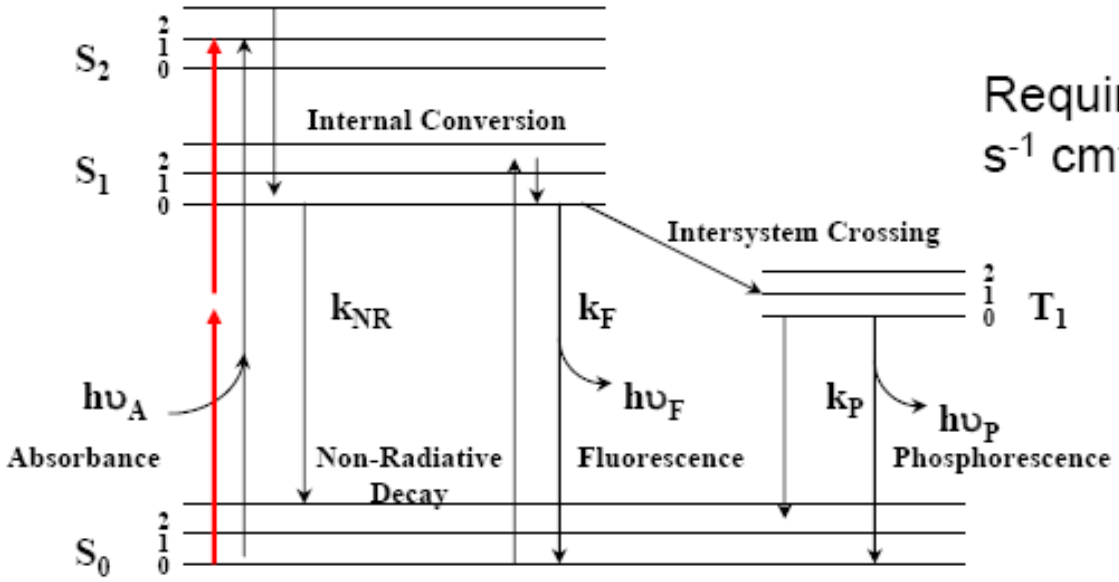
- mode-locked Ti:Sapph laser
- average power ~1W

2-photon excitation

2 photons must interact with the same fluorophore at the same time

Maria Göppert-Mayer (1931)

Requires photon flux ~10^{18} s^{-1} cm^{-2}



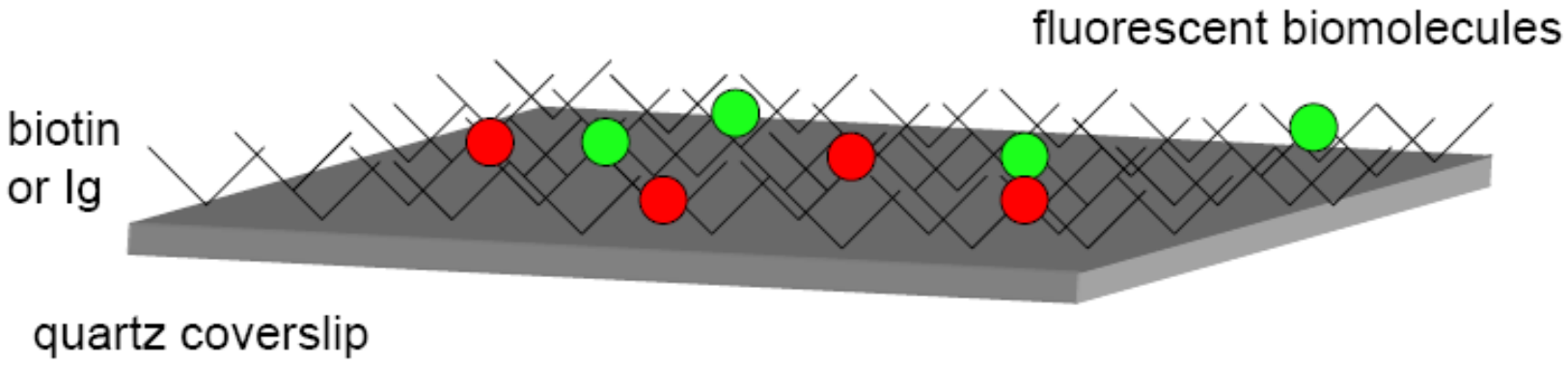
E α 1/ λ ∴ 2 x 900nm photons ~ 1 x 450nm

Solution measurements: Strengths and weaknesses

- Pros:
  - Solution measurement!
  - Experimentally easy
  - Can assay many many events quickly
  - Can access very fast processes, very short-lived species
  - Wide time-variation
  - In vivo measurements are possible!
- Cons:
  - Requires low concentration of fluorophores
  - Data stream is I vs t
  - Diffusion rate α with $MW^{1/3}$
  - Small numbers of very fluorescent particles can kill analysis (aggregates)
  - Contaminant fluorescence

Single molecule fluorescence: immobilized molecules

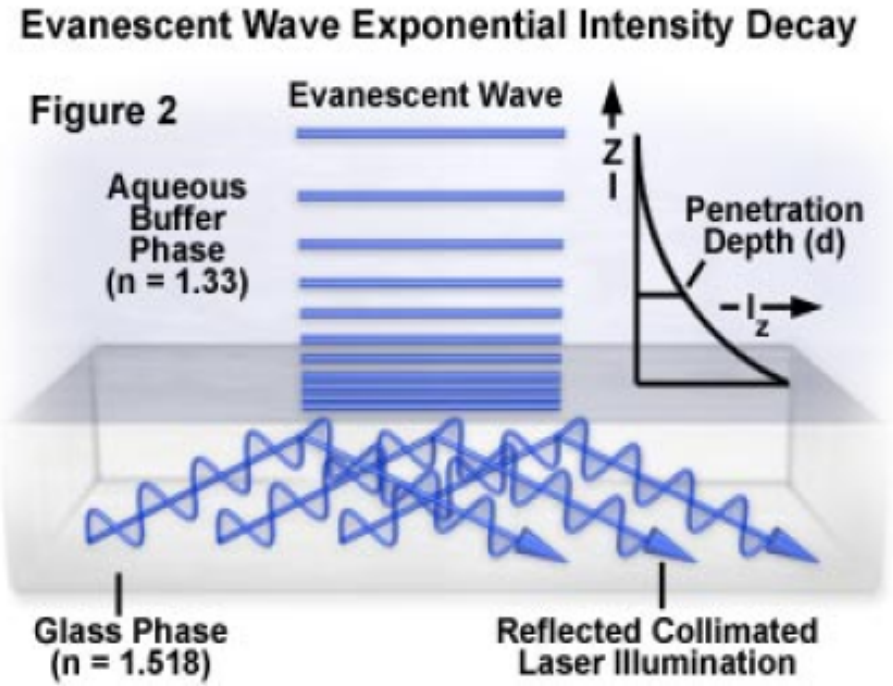
- true “single molecule” fluorescence
- functionalize a quartz microscope slide
  - Biotin/strepatividin
  - Ni-NTA / His-tagged proteins
  - antibody / antigen
  - agarose gel
- attach fluorescent labeled molecules
- monitor time-course of fluorescence



Immobilized molecules: Strengths and weaknesses

- Pros:
  - Experimental artifacts from solution measurements are easily detected
  - Inhomogeneity of labeling is apparent
  - Eliminates any uncertainty in the nature of the observations
  - timescales longer than diffusion time accessible
- Cons:
  - Not a solution measurement!
  - Artifacts from immobilization
  - Orientation wrt surface may be a factor
  - Immobilization may make buffer exchange or addition of substrate difficult
  - Non-specific binding / aggregation
  - Contaminant fluorescence

Total Internal Reflection Fluorescence (TIRF): principles

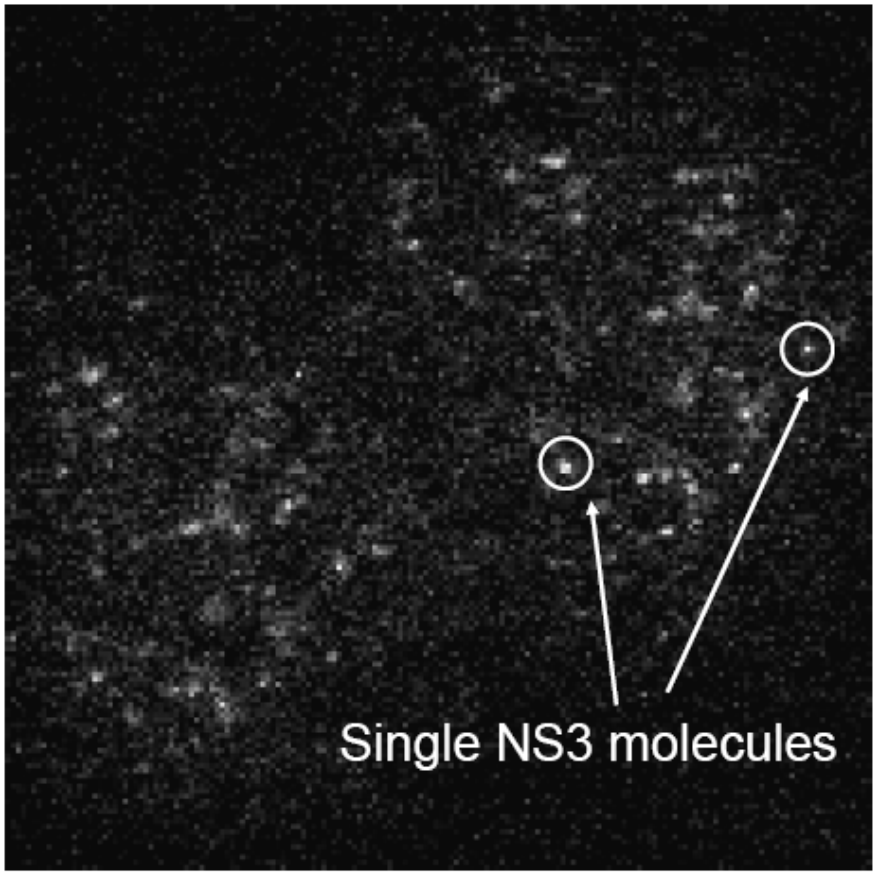


$$\sin \theta_c = \frac{n_1}{n_2}$$

$$I_z = I_0 e^{-z/d}$$

- incident laser is totally internally reflected
- generates an evanescent field propagating along interface
- field extends $\leq$ 100nm
- excitation only at interface, minimizes background
- widely used in lipid biophysics

NS3h: single molecule fluorescence



- TIRF
- immobilized NS3 (or immobilized RNA with labeled NS3 bound)

~30pM labeled NS3

- follow time-course of fluorescence from single molecules

Scanning Confocal Microscopy (LSCM)

- Rather than excite the entire field, use a laser to "scan" the field
- Detect emission using photomultiplier or photodiode
- Reconstruct a picture using software

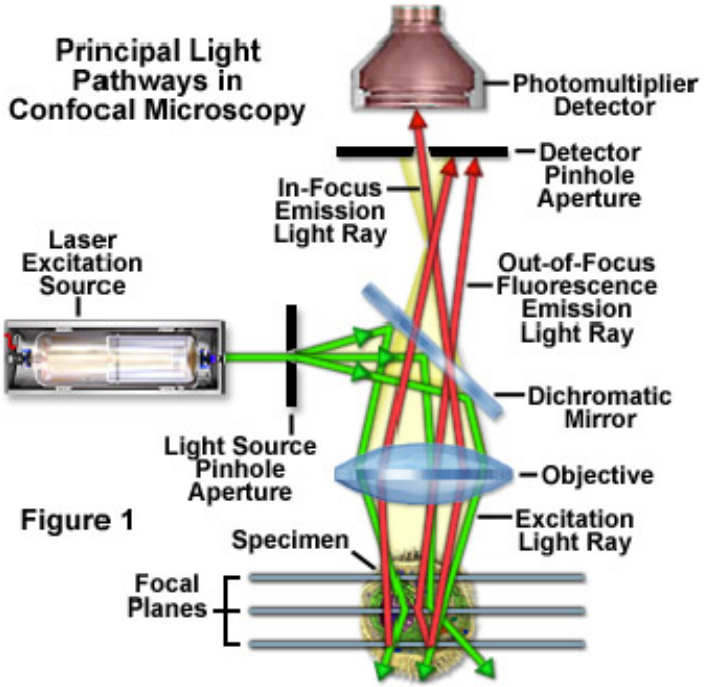
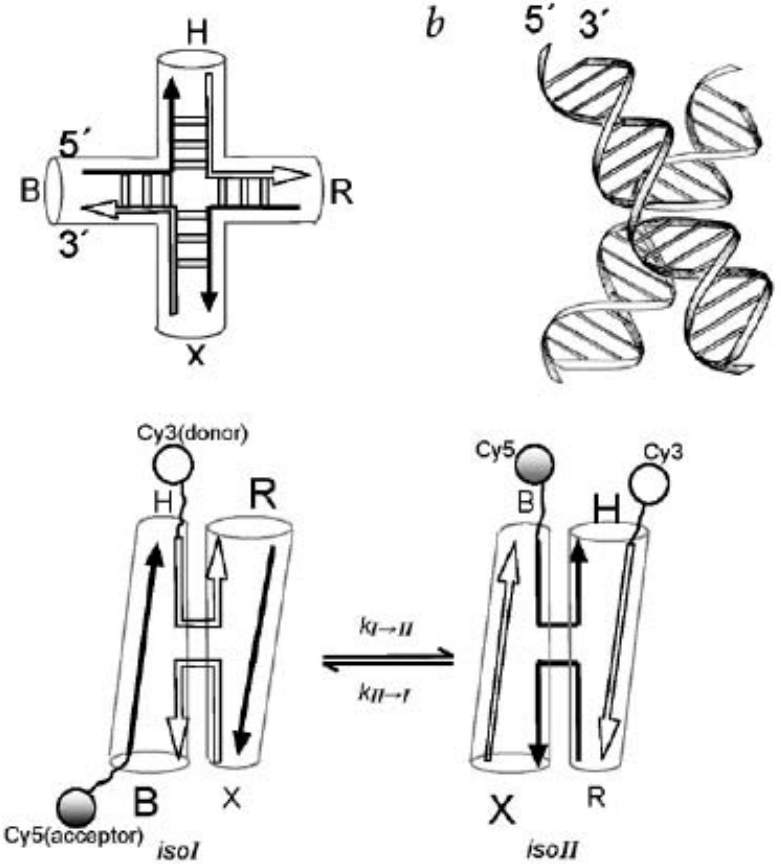
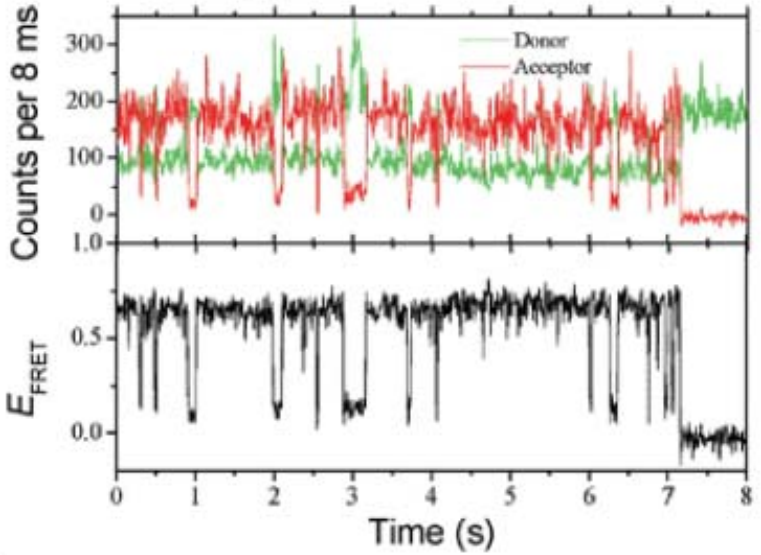


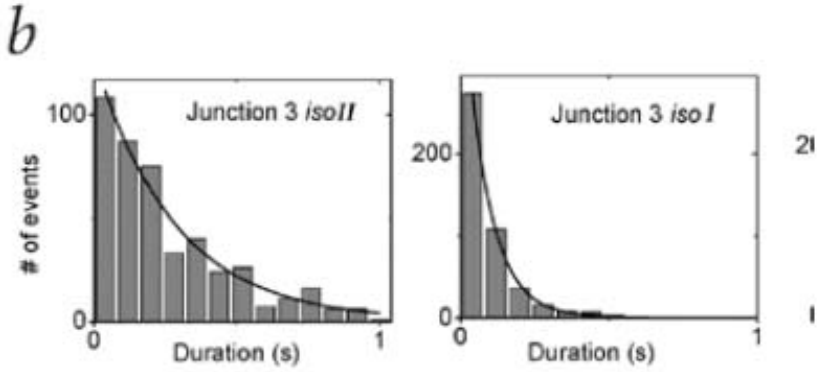
Figure 1

- Image "sectioning" = z-axis slices
  allows 3-D image to be rendered
  or 2-D image can be examined
- 2 types of scanning:
  move sample (early LSCM)
  move excitation (modern)
    - galvanometer
    - Nipkow disc
- emission must be "descanned"
  must be colinear with excitation in order to pass through the pinhole

Single molecule FRET: conformational changes







McKinney et al. NSB, 2002

TIRF and scanning comparison

- TIRF:
  - Acquisition rate limited by camera
  - Massively parallel data acquisition
- Scanning
  - Fast acquisition by PMT
  - Scanning rate limited by photon acquisition