Fundamentals in Biophotonics

Single molecule Localization Microscopy (SMLM)

Single Molecule FRET with Total Internal Reflection Microscopy

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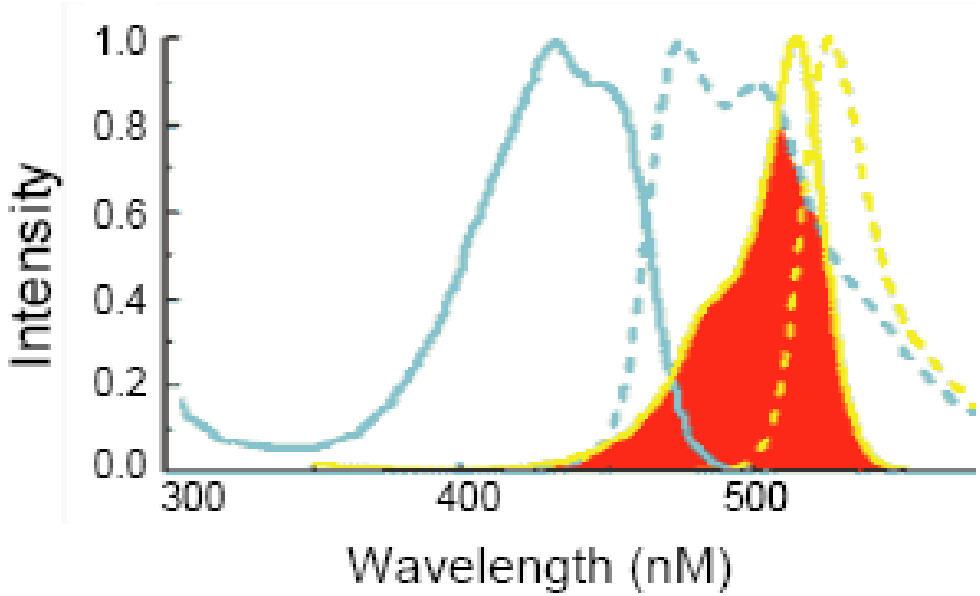
Single Molecule FRET with Total Internal Reflection Microscopy

- **Outline**
- **Single molecule Forster resonance energy transfer physics background**
- **TIRF basic principle**
- **Dye choice labeling of nucleic acids and proteins**
- **Surface preparation and data acquisition**

FRET-Förster Resonance Energy Transfer

- First Identified in 1946 by Theodor Förster
- Non-radiative energy transfer between molecules with **overlapping emission-excitation** spectra Excitation energy of a donor molecule **is nonradiatively transferred** to an acceptor via interaction of two induced dipoles .

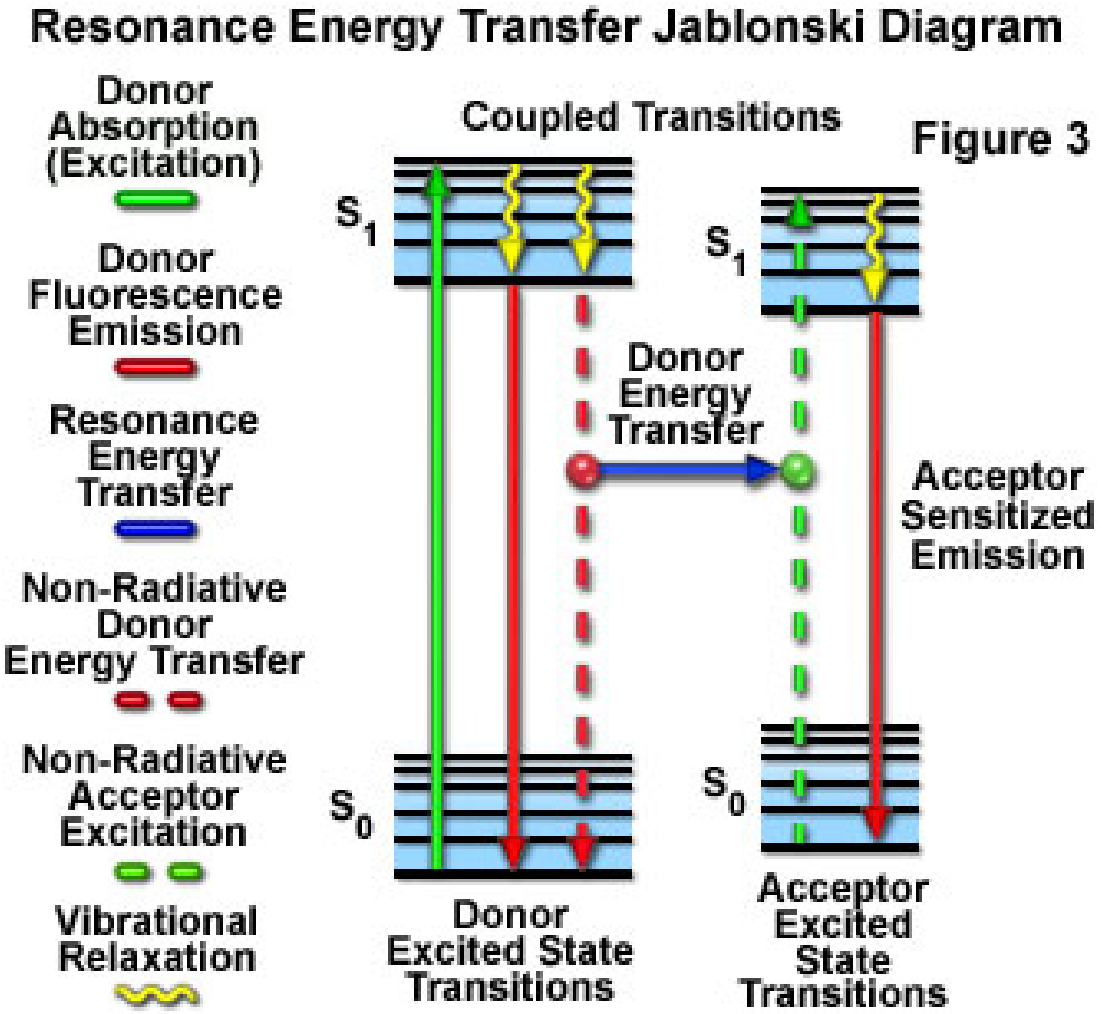


$$E_t = \frac{1}{1 + (r / R_o)^6}$$

http://www.bphys.uni-linz.ac.at/bioph/res/icg/fret.htm

FRET- The Quantum Theory

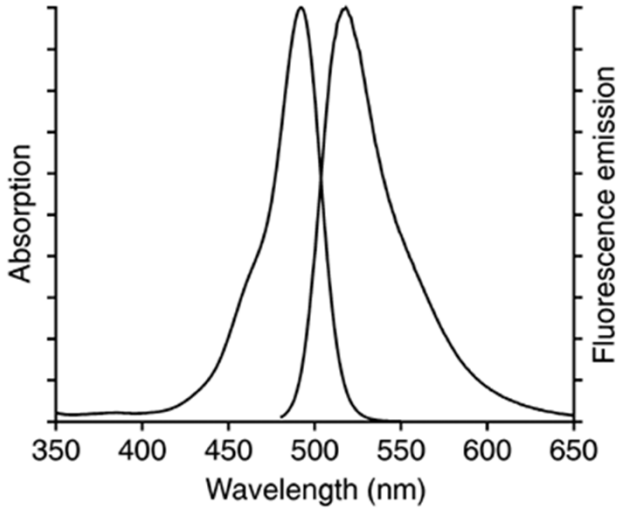
- Energy is transferred through the resonant coupling of the dipole moments of donor and acceptor
- Energy transition between a donor and acceptor with a finite probability based on proximity

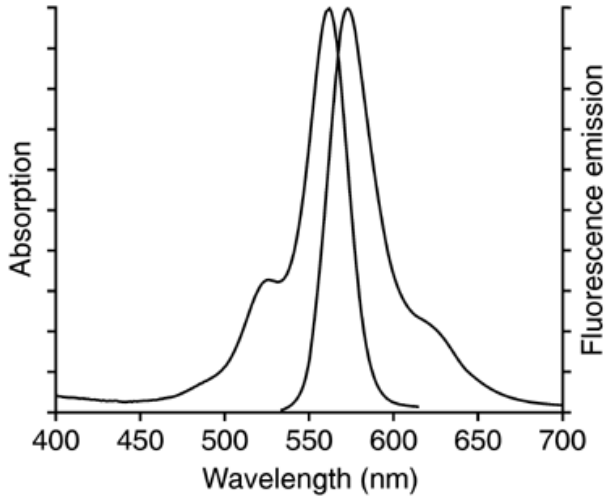


FRET

- When two fluorophores are close together it is possible that one of them absorbs the light (donor), then transfers the energy to the neighboring fluorophore (acceptor), which then emits the light.
- The two conditions for this to happen are:
- Transition dipole interaction between the two fluorophores (i.e., they need to be close together and aligned).
- Significant overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor.

Example: **Fluorescein** (donor) and **Alexa-546** (acceptor):





FRET efficiency and distance Example of pairs of fluorophores

Donor	Acceptor	Ro (Å)
Fluorescein	Tetramethylrhodamine	49-55
Cy-3	Cy-5	53
Alexa 488	Alexa 568	62
Alexa 546	Alexa 594	71
Alexa 594	Alexa 647	85
CFP	YFP	49
GFP	DsRed	47

Ro values (in Å) represent the distance at which fluorescence resonance energy transfer from the donor dye to the acceptor dye is 50% efficient.

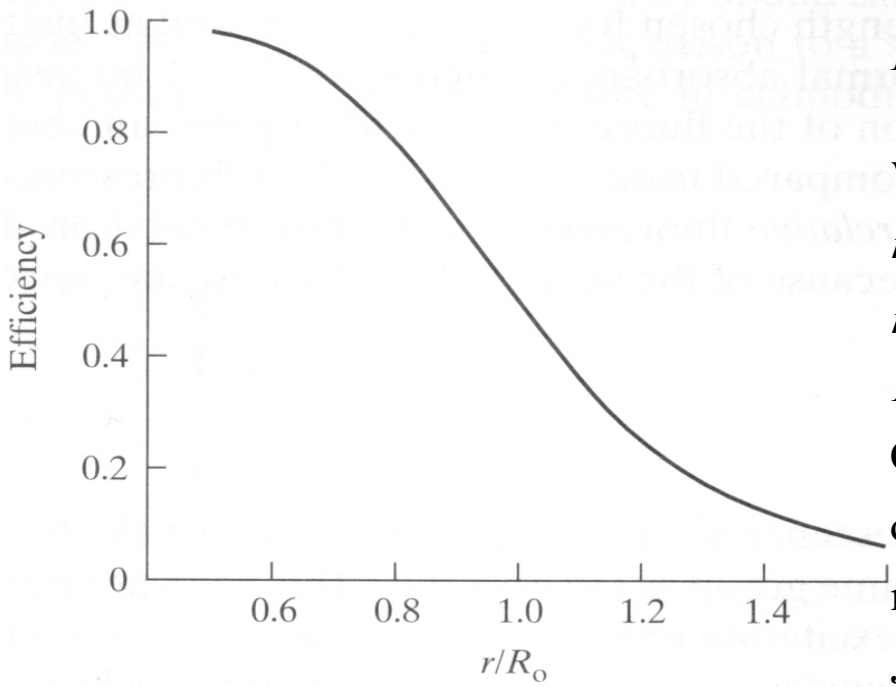
Choosing a Fluorophore

- 1. Fluorophore pair with significant spectral overlap
- 2. Pair should be compatible by chosen instrumentation
  - -Ability to be excited
  - -Proper filters for detection
  - -Lifetime
- 3. Can I label the molecule accurately?
- 4. Does fluorescent label interfere with molecule's function?

- Dyes must be
  - photostable-so that they emit millions of photons before photobleaching
  - Bright high extinction coefficient and quantum yield
  - Showing little intensity fluctuations (on biological timescales)
  - Excitable and emitting in visible wavelengths
  - Relatively small so that perturbation is minimal to the system
  - Commercially available in a form that can be conjugated to biomolecules

FRET

Basically, FRET is a great method to determine the distance between two fluorophores (molecules) in the range of ~1-10 nm.



Efficiency of transfer:

$$E_{transfer} = \frac{1}{1+\left(\frac{r}{R_0}\right)^6}$$

$$R_0 = \left[\frac{9000\ln(10)\cdot\kappa^2\cdot QY_D\cdot J(\lambda)}{128\pi^5\cdot N_A\cdot n^4}\right]^{1/6} \quad \text{Å}$$

where

κ^2 = dipole orientation factor range form $0-4$

$\kappa^2 = 2/3$ for randomly oriented donors and acceptors

$N_A = Avogadro's\ number$

QY_D = fluoresence quantum yield of donor in the absence of the acceptor

n = refractive index

$J(\lambda)$ = spectral overlap integral

$$J(\lambda) = \int \varepsilon_\lambda(\lambda)\cdot F_D(\lambda)\cdot\lambda^4 d\lambda \quad cm^3M^{-1}$$

where

$\varepsilon_\lambda = extinction\ coefficient\ of\ acceptor$

F_D = fluresence emission intensity of donor as a fraction of the totoal integrated intensity

Primary Conditions for FRET

- Free software for calculating R0 is available from
- http://www.photochemcad.com/

- **DISTANCE** : Donor and acceptor molecules must be in close proximity (10-100 Å)
- **SPECTRUM OVERLAP:** The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor
- **DIPOLE-DIPOLE ORIENTATION**: Donor emission dipole and acceptor excitation dipole must be approximately parallel

FRET: What is it Good For

Applications:

Structure and conformation of proteins

Spatial distribution and assembly of protein complexes

Receptor/ligand interactions

Immunoassays

Probing interactions of single molecules

Structure and conformation of nucleic acids

Real-time PCR assays and SNP detection

Detection of nucleic acid hybridization

Primer-extension assays for detecting mutations

Automated DNA sequencing

Distribution and transport of lipids

Membrane fusion assays

Membrane potential sensing

Fluorogenic protease substrates

Indicators for cyclic AMP and zinc

QUIZ TIME!

Ready to review some material?

Then open kahoot!

GOOD FRET pairs

Appreciable overlap between **donor emission** and **acceptor absorption**

Large separation between **donor and acceptor emission**

Comparable emission quantum yield , which guarantees clearly anticorelated intensity changes

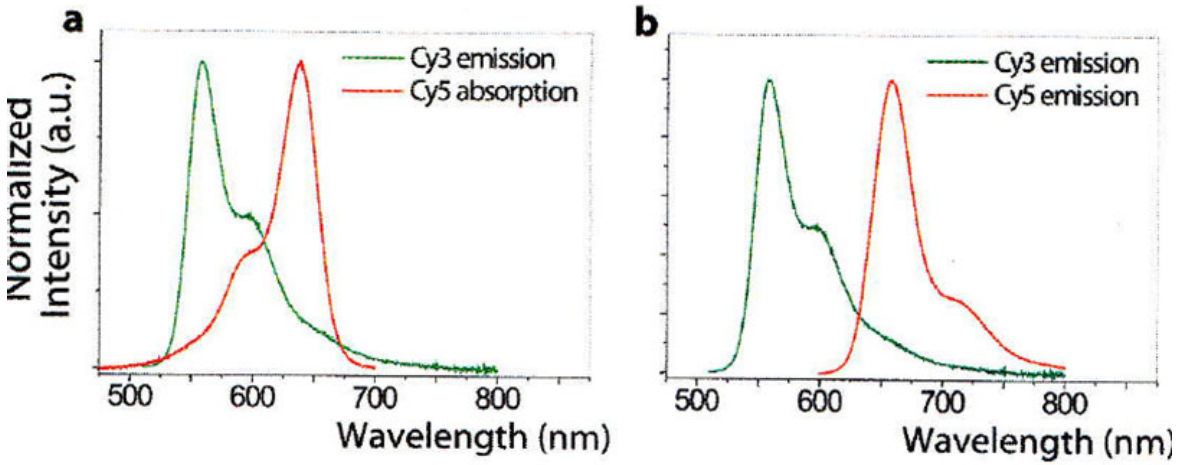
Cy 3 and Cy5 extremely popular

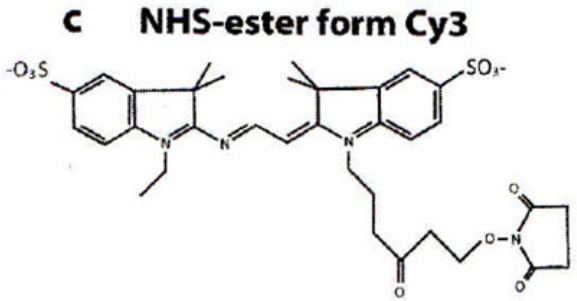
Spectral separation 100 nm

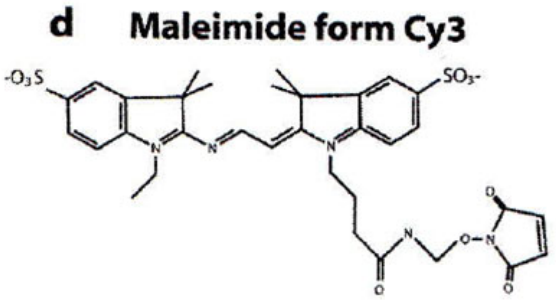
Photostable

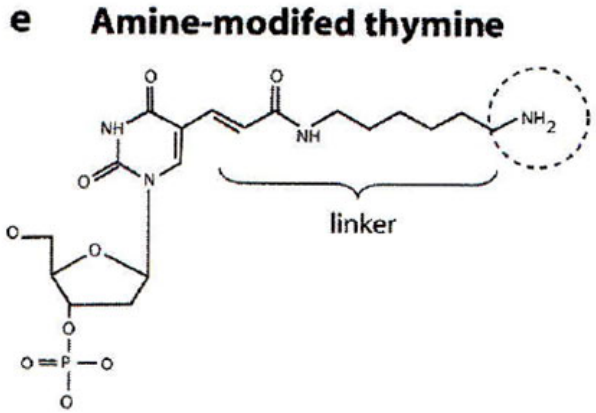
Comparable quantum yield 0.25

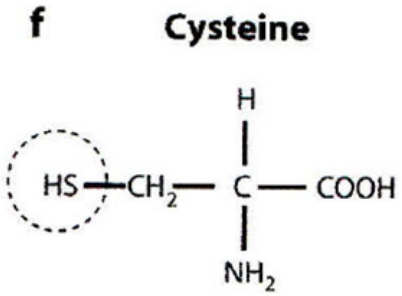
Commercially available in thiol, amine and other reactive forms











Labeling

- FRET is insensitive to incomplete labeling of host molecules
- **Labeling RNA DNA**
- Companies that provide
- IDT – 5’ end offers labeling 5-100 nucleotides

  3’ end - 5 to 50 nucleotides

Dyes are inserted through phosphoramidite chemistry / **Nucleoside phosphoramidites** are used to synthesise short nucleic acid chains

- **Labeling proteins**

  thiol groups (SH) show very high specificity and Cystein is only amino acid that has the thiol group

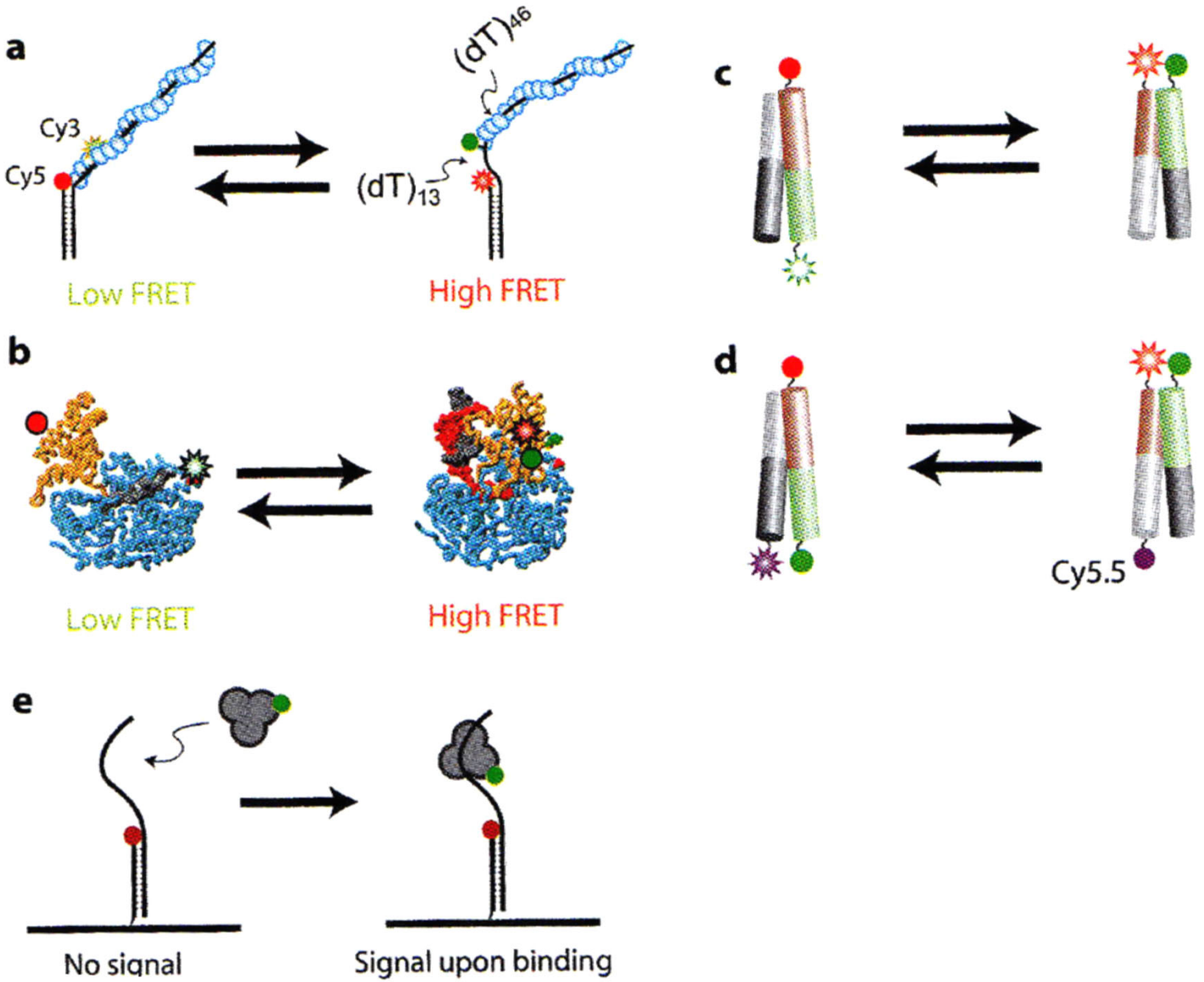
  thiol group reacts with melanmide form of a dye

  very simple mixing yields good labeling

  preferably only one FRET pair per protein, unfortunately many proteins have more than one Cystein – fto remove few Cystein solution

  site directed mutagenesis

Sample labeling



Sample labeling general design RULES

- If the protein recognizes a certain chemical feature of DNA the fluorophore should be placed elsewhere
- For internal labeling keep the backbone continuity of DNA
- In the DNA the part that interacts with a protein may be placed away from the glass surface to avoid potential physical constraints
- If using 3 color FRET design pairs so that 2 acceptors are very well separated
- For studying DNA protein binding events attach donor to the protein and acceptor to the DNA –binding produces sudden increase in fluorescence
- Avoid high protein concentration - to suppress background fluorescence

Surface immobilization strategies

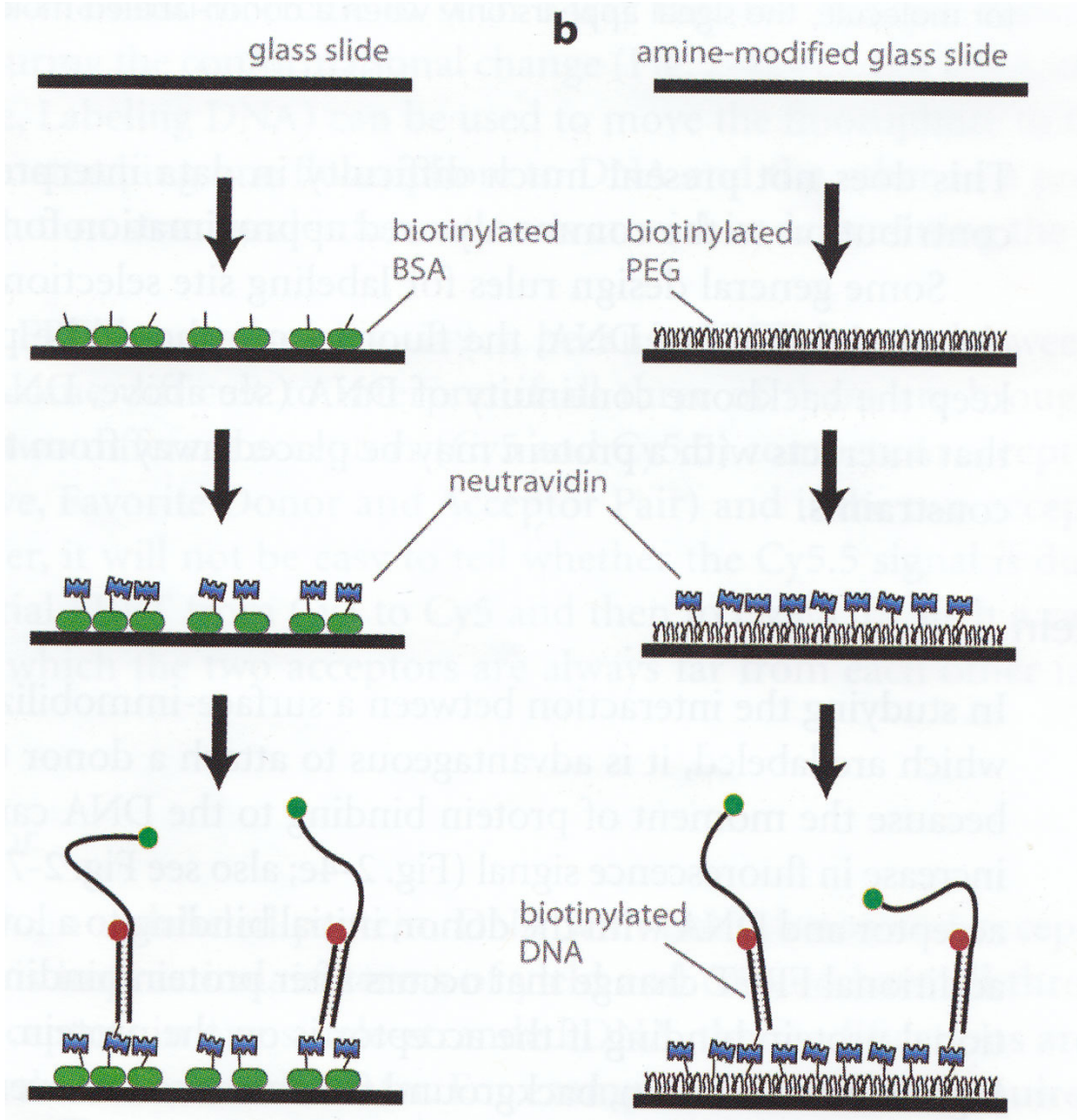
- Two strategies
- By using biotin neutravidin glass or quartz

-negative charge good for DNA

RNA at neutral pH repels DNA/RNA

- Or amine modifies glass surface with biotinylated PEG

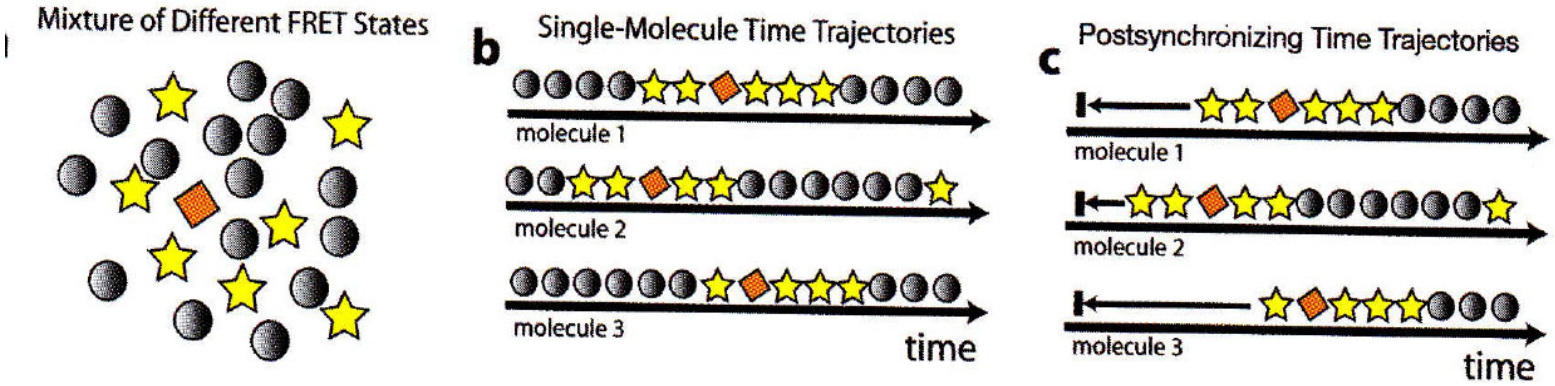
- Better suited for proteins since BSA coated surfaces are to adhesive



smFRET advantages

- In comparison to other sm techniques smFREt is less prone to environmental noise

1. It is inherently ratiometric technique where we measure the ratio between two colors
2. It reports on the internal movements of molecules in their center of mass frame thus variations in excitation and detection efficiencies between molecules are mostly tolerable
3. it is relatively easy to acquire data for several thousands of molecules and therefore kinetic rates could be determined with the highest accuracy screening out intrinsic heterogeneity between single molecules
4. However photo physical properties of organic dyes limit its resolution to several milliseconds



Single molecule fluorescence **FRET**

- Like bulk fluorescence, except on a single fluorophores
- **Challenges:**
  - Photobleaching: Most fluorophores photobleach, i.e. after absorbing many photons (usually a few million), they chemically rearrange and stop fluorescing (end of experiment).
  - Signal to noise ratio (need to reduce noise and have sensitive detection)
  - Must have very, very clean sample.
  - Detecting weak signals requires very sensitive instrumentation
- **Needed:**
  - A strong fluorophore (high absorbance and Q-yield).
  - Intense light source (often laser).
  - Very sensitive camera/detector
  - Eliminate all background light.
  - Illuminate a very small region only
  - Confocal microscopy (images only slices (stacks) of a sample)
  - Use TIR (total internal reflection).

QUIZ TIME!

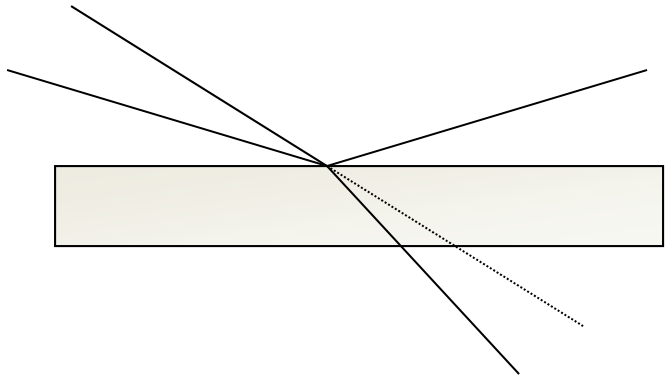
Ready to review some material?

Then open kahoot!

Total Internal Reflection Fluoresence **TIRF**

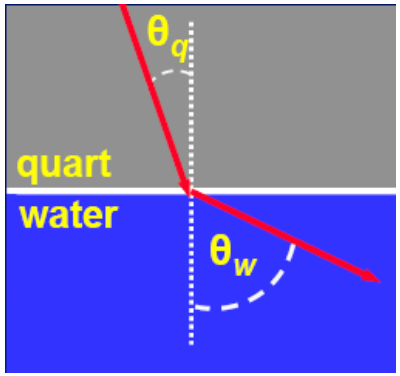
- What is TIRF?
- Why do we constantly use acronyms to describe everything?
- Microscope Configurations
    - -Prism vs Prismless
- Biological Applications
    - -Brief Aside
- Unique attributes to our system
    - -Calibrated TIRF planes
    - -TIRF-FRET
- TIRF-photoactivation

Index of refraction “bends” light



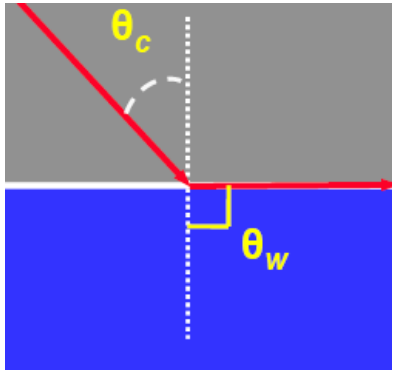
water	1.33
air	1.0003
glass	1.517
coverglass	1.523
immersion oil	1.516
cell cytosol	1.38
mount	variable

Propagation of Light at a Solution-Surface Interface



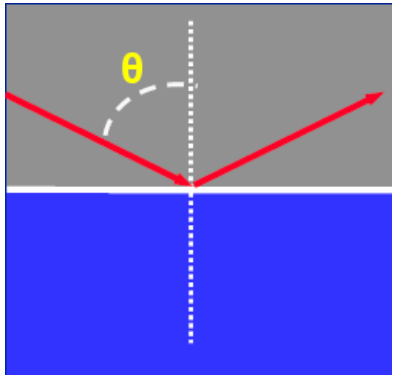
1. Refraction

$$n_{quartz} \sin\theta_q = n_{water} \sin\theta_w$$



2. Propagation along interface

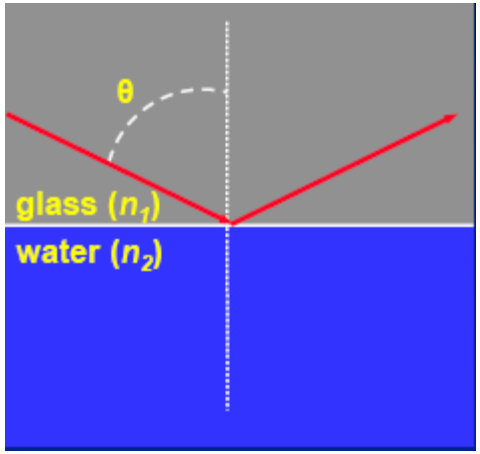
-occurs at the "critical angle" θc



3. Total Internal reflection

-occurs when θ> θc

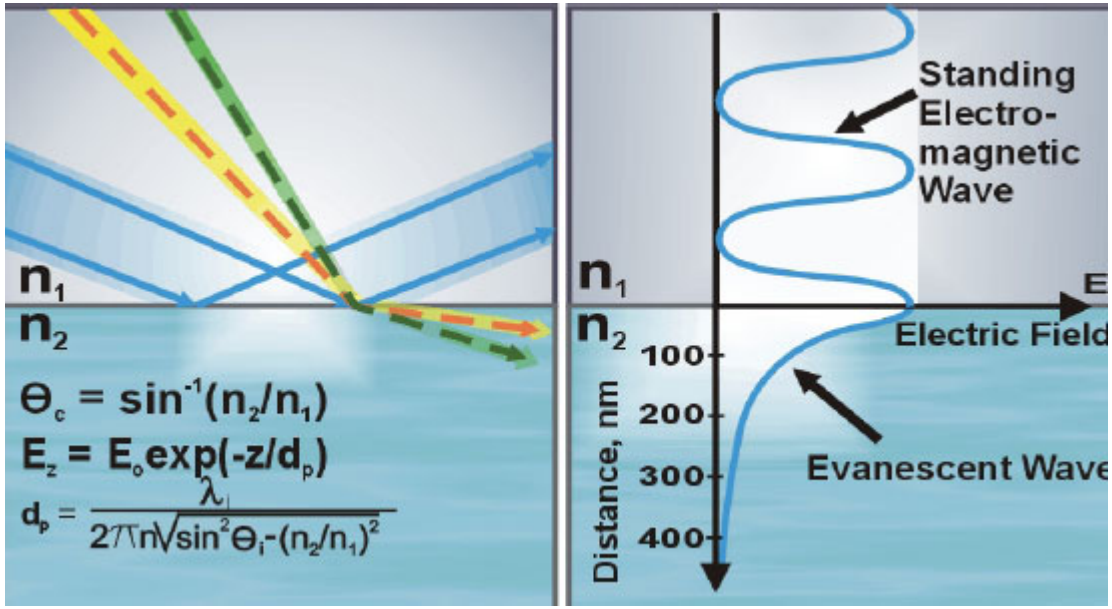
Total Internal Reflection Fluoresence **TIRF**



1. TIR occurs under two conditions

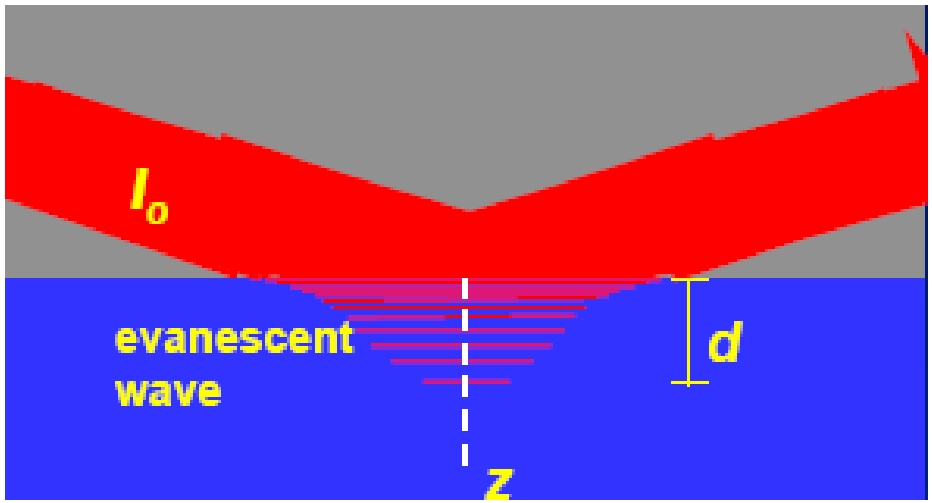
$$n_1 > n_2$$

$$\theta > \theta_C$$



$$d_P = \frac{\lambda_i}{2\pi n\sqrt{\sin^2 \vartheta_i - \left(\frac{n_2}{n_1}\right)^2}}$$

The Evanescent Wave



- **An electromagnetic field extends into the medium of lower refractive index**
- **The evanescent depth *d extends* ~ *λ***
- **The intensity decreases exponentially**

$$I(z) = I_0 e^{-z/d}$$

$$d_P = \frac{\lambda_i}{2\pi n \sqrt{\sin^2 \vartheta_i - \left(\frac{n_2}{n_1}\right)^2}}$$

note: d is only the depth at which the intensity of the evanescent wave is 37% of the initial intensity. Thus, can empirically determine the experimental depth at which fluorophores are visible using fluorescent beads (Keyel, Watkins, and Traub 2004 JBC)

$\lambda_0 = 488$; $n_2 = 1.52$; $n_1 = 1.38$ $d_{empirical} = 190$ nm
$\lambda_0 = 647$; $n_2 = 1.52$; $n_1 = 1.38$ $d_{empirical} = 238$ nm
$\lambda_0 = 488$; $n_2 = 1.78$; $n_1 = 1.38$ $d_{empirical} = 142$ nm

Total Internal Reflection Fluoresence **TIRF**

- An evanescent field is generated into the solution phase
- Fluorescent molecules that enter the field fluoresce
- Maximum fluorescence at the surface

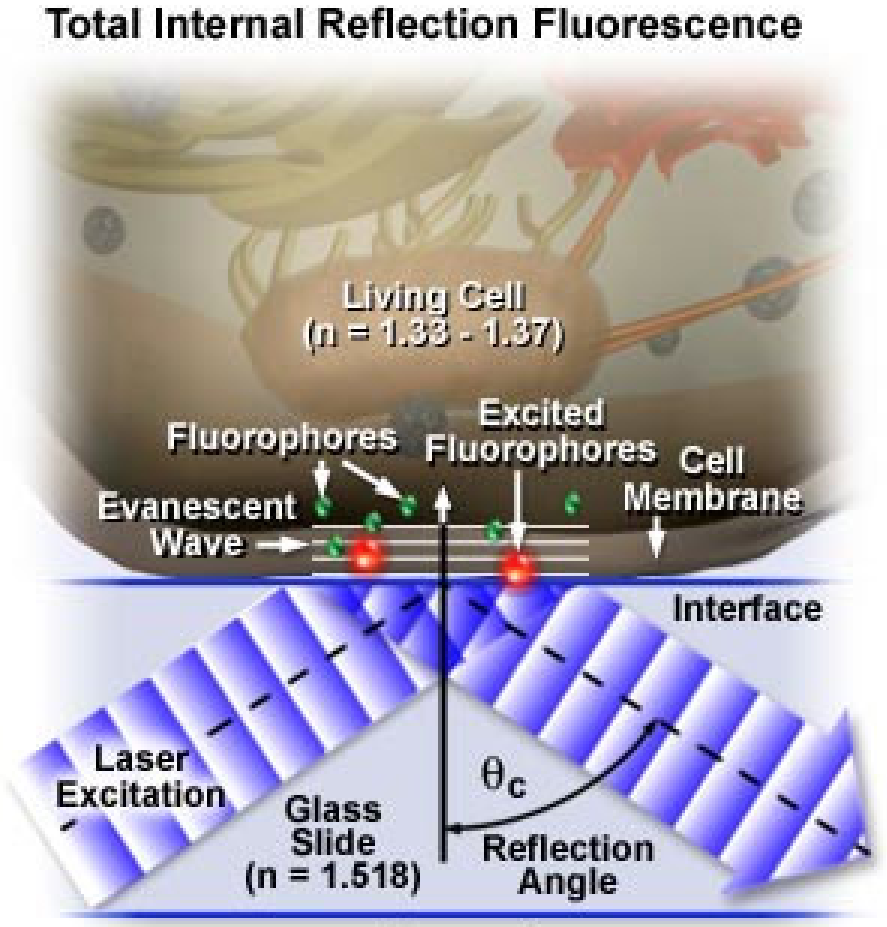
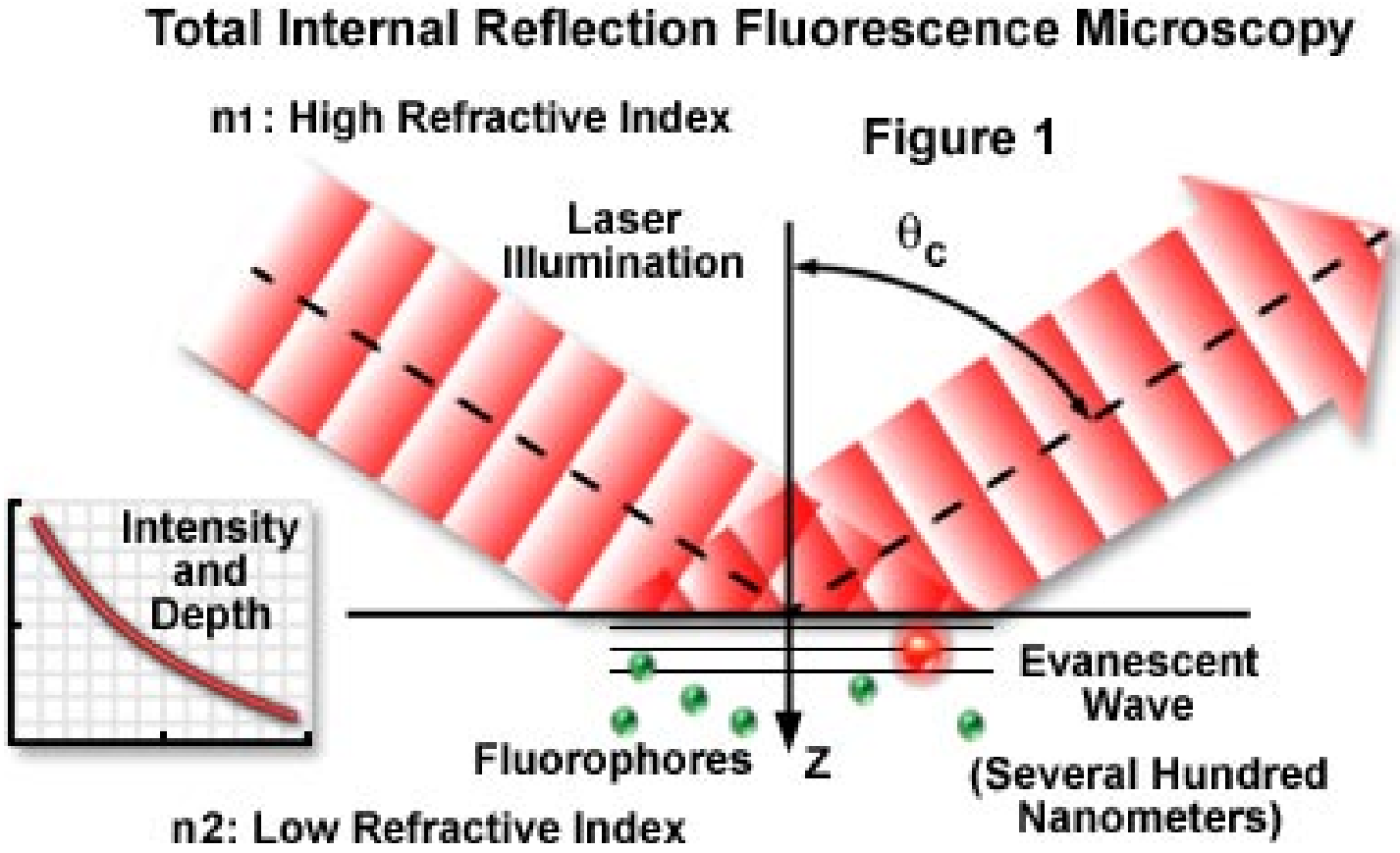


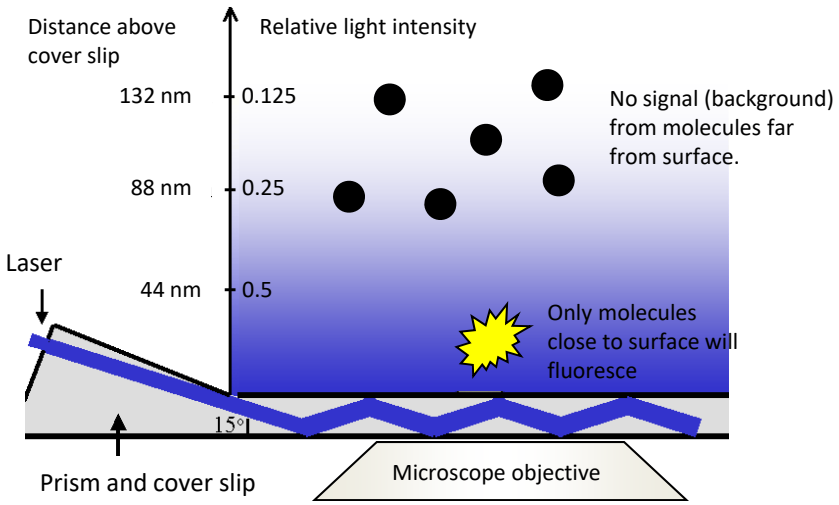
Figure 1

The basics of imaging cells by TIRF microscopy

- At a specific critical angle [θcritical = sin-1(n1/n2)] light is totally reflected from the glass/water interface. The reflection generates a very thin electromagnetic field that has an identical frequency to that of the incident light, providing a means to selectively excite fluorophores within ≤ 100 nm of the coverslip.



Prism based TIRF



$$I(z) = I_0 e^{-\frac{z}{d}}$$

penetration depth:

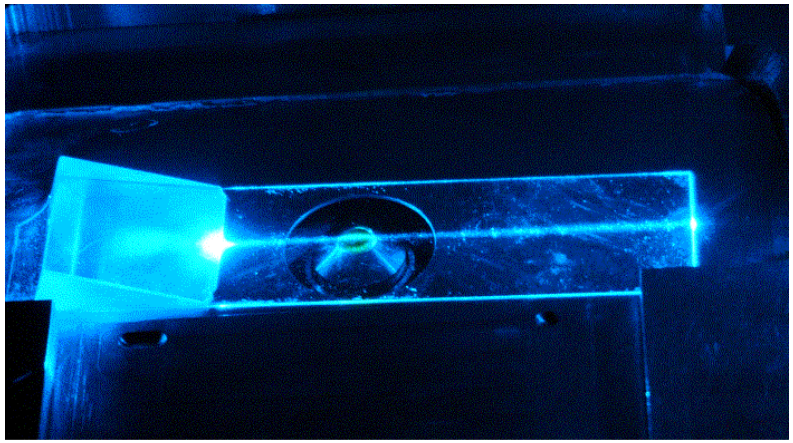
$$d = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2\theta_1 - n_2^2}}$$

n_1 …index of refraction of glass slide

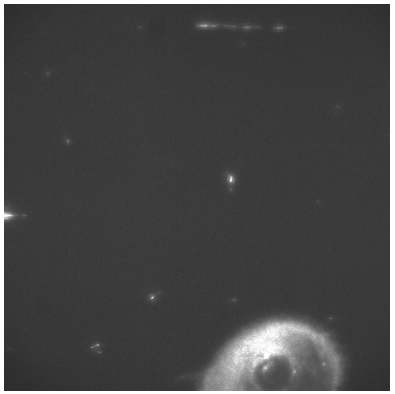
n_2 …index of refraction of water

θ … incident angle

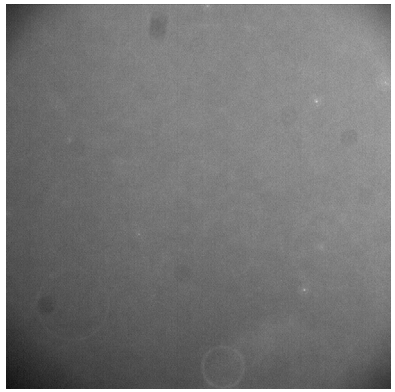
λ … wavelength of light



TIRF

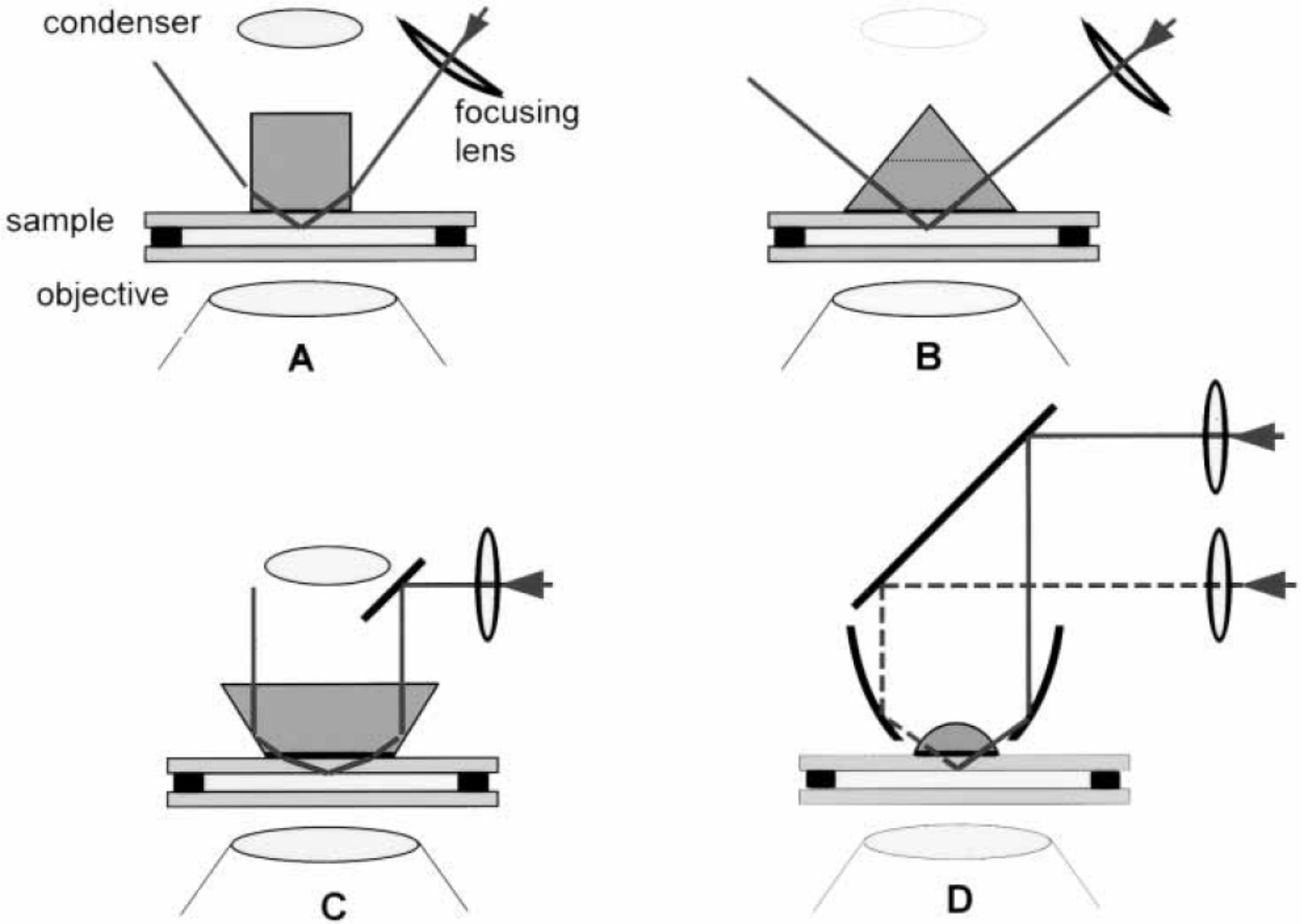


No TIRF



Prism based TIRF

- Prism-based TIRF limit access to sample



TIRF Comparison

Prism Method

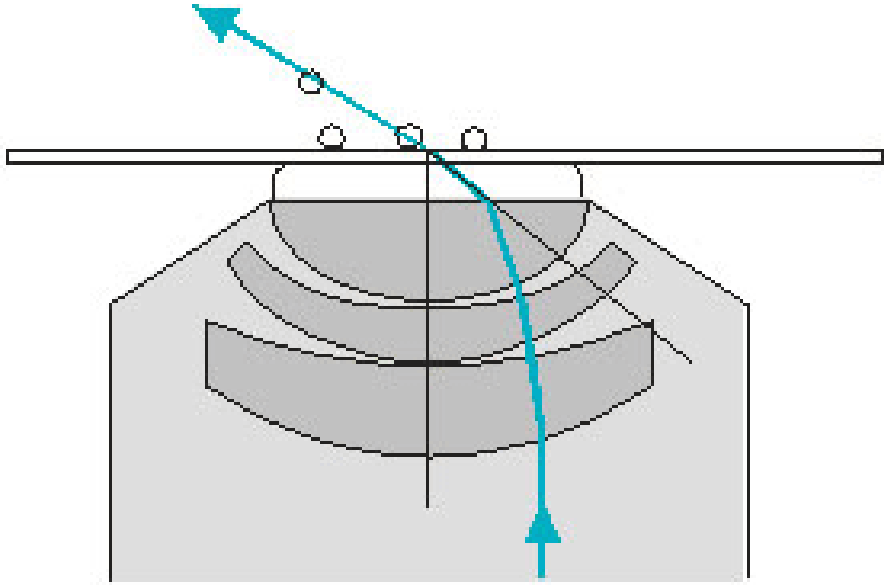
1. “Purer” evanescent wave
2. Limited access to sample
3. Few commercial manufactures
4. Open laser systems
5. Typically lower NA objectives

Prism-less Method

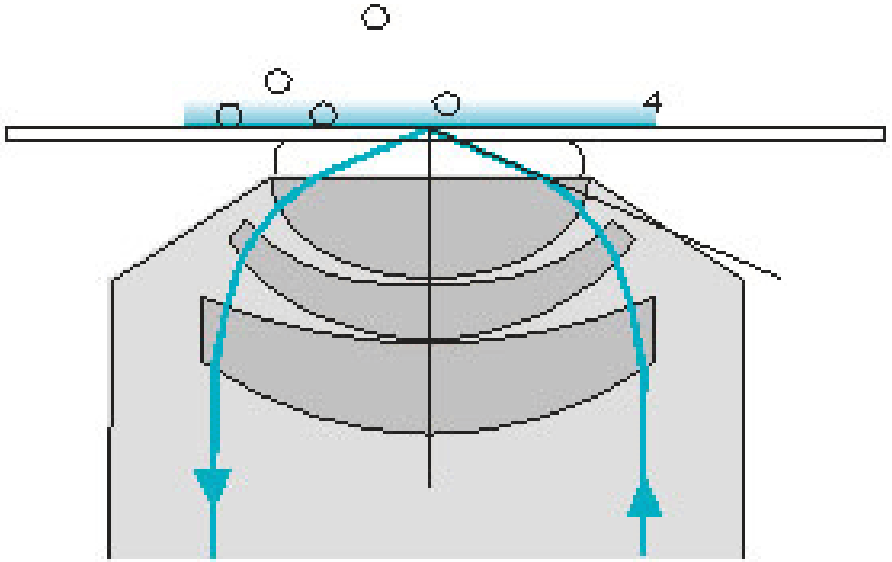
1. Higher NA will allow confinement closer to surface
2. Not as pure an evanescent wave as prism
3. Commercial system readily available

Objective-based TIRF illumination

Ray paths (schematic):



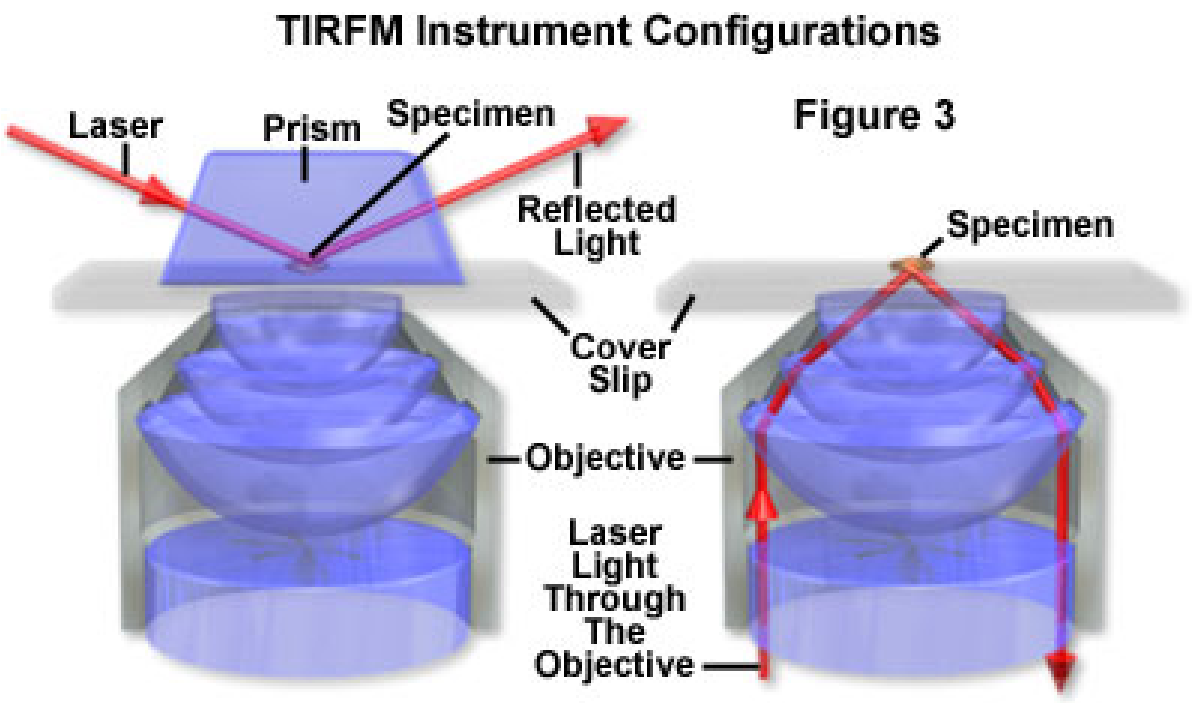
Angle of incidence smaller than the critical angle.



Total reflection
1: Objective,
2: Immersion oil n = 1.518,
3: Cover slip n = 1.518,
4: Evanescent field,
5: Mountant n = 1.33…1.38

TIRF is commonly done inside the objective

- TIRF objectives are now starting to come with compensation collars for varying temperature and cover slip thickness



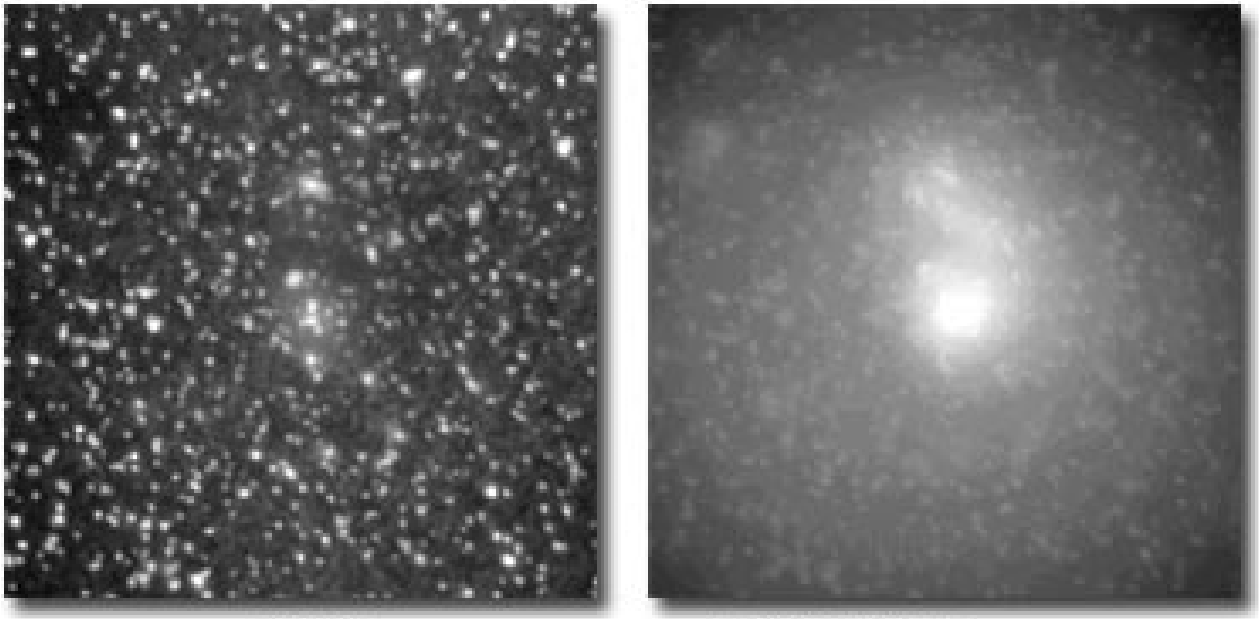
100X 1.65 NA objective:
$\theta_c = \sin^{-1}(n_1/n_2) = 50.83^o$
[calculated using $n_2 = 1.78$ (RI coverglass and immersion liquid) and $n_1 = 1.38$]

100X 1.45 NA objective:
$\theta_c = \sin^{-1}(n_1/n_2) = 65.22^o$
[calculated using $n_2 = 1.52$ (RI coverglass and immersion liquid) and $n_1 = 1.38$]

Maximum Angle θ_m from the optical axis that TIR will occur is:
$NA = n_2 \sin \theta_m$
60X 1.45 NA $\theta_m = 72.54^o$
100X 1.65 NA $\theta_m = 67.97^o$

TIRF

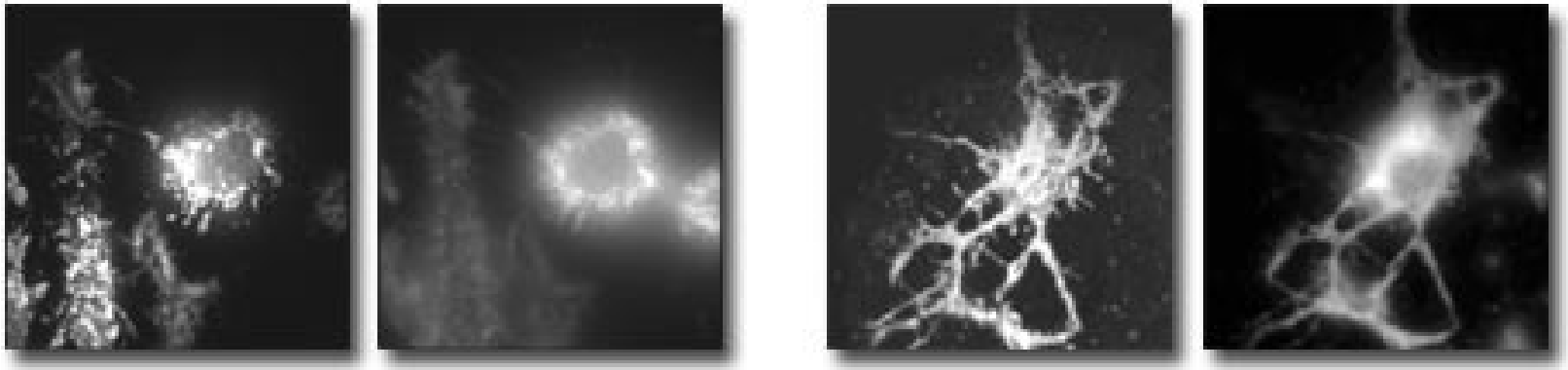
Fluorescent Beads and Cheek Cell



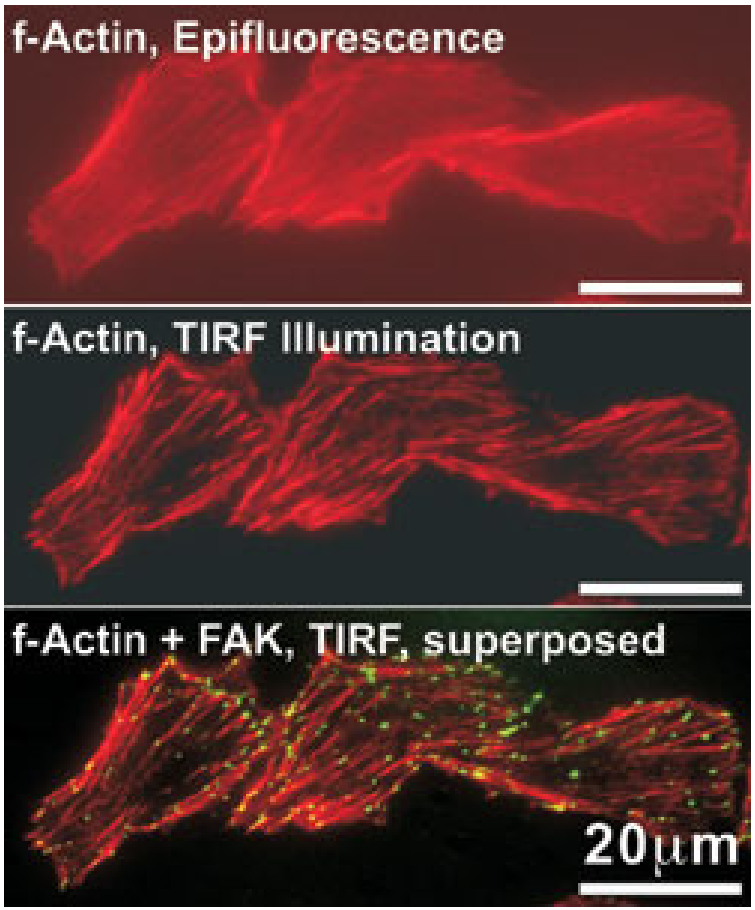
TIRFM Widefield Fluorescence

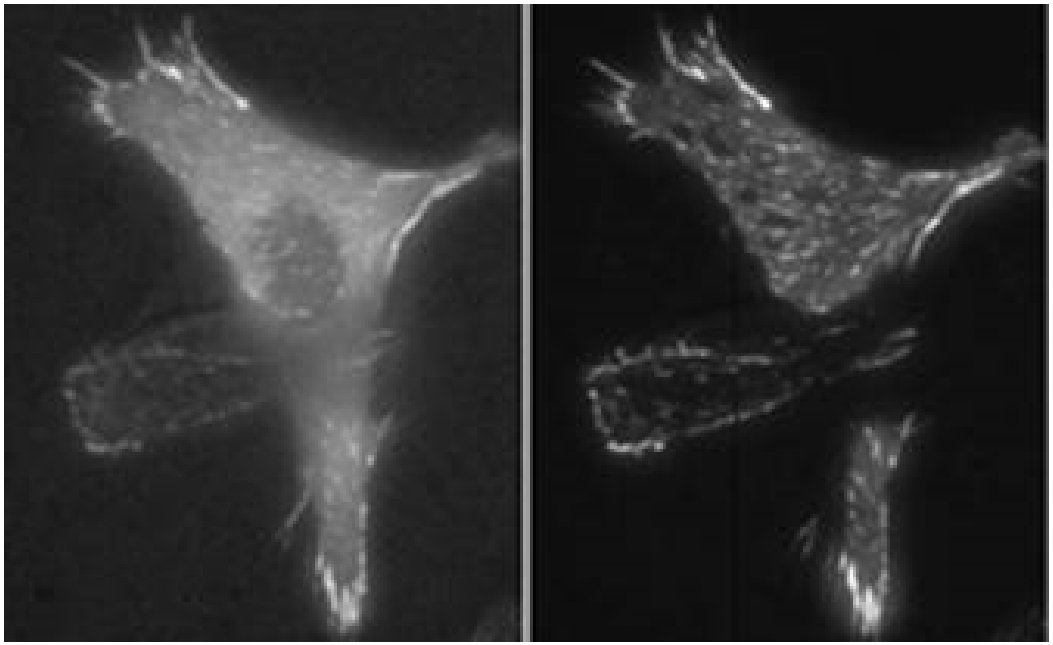
Figure 7

DiI-Stained HeLa Cell (Figure 8) **DiI-Stained Neuron (Figure 9)**

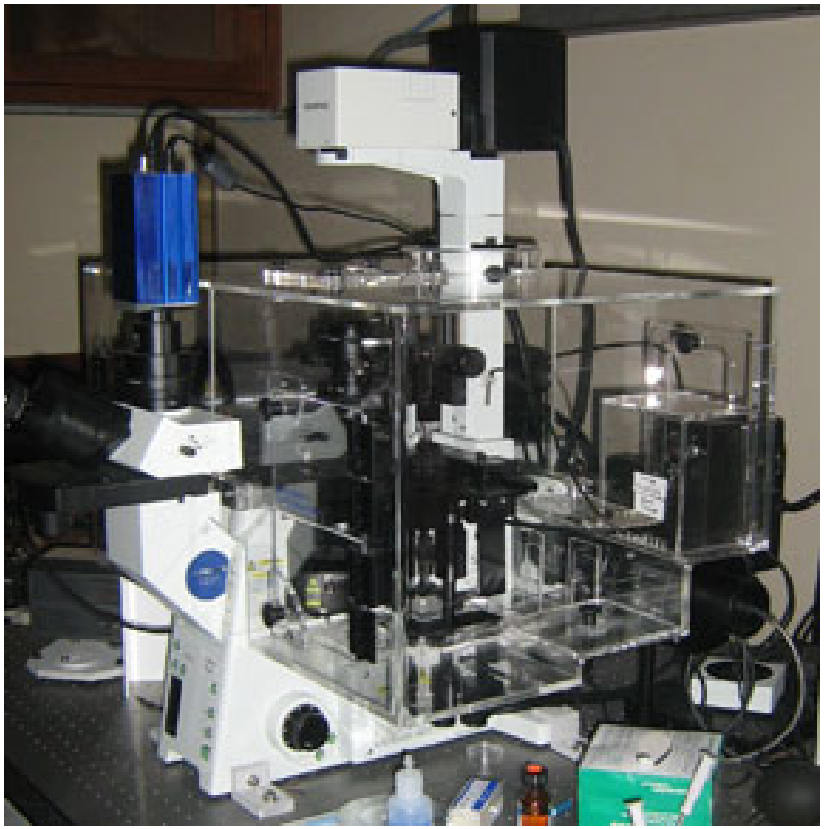


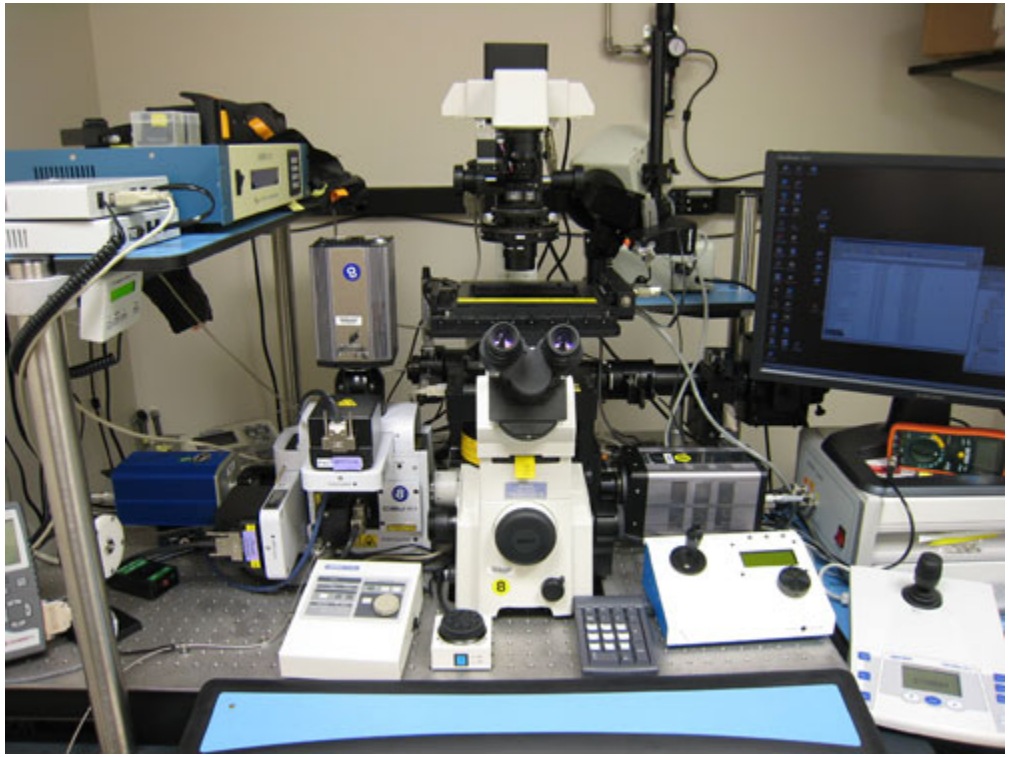
TIRFM FLM TIRFM FLM


f-Actin, Epifluorescence
f-Actin, TIRF Illumination
f-Actin + FAK, TIRF, superposed
20μm



TIRF setup





QUIZ TIME!

Ready to review some material?

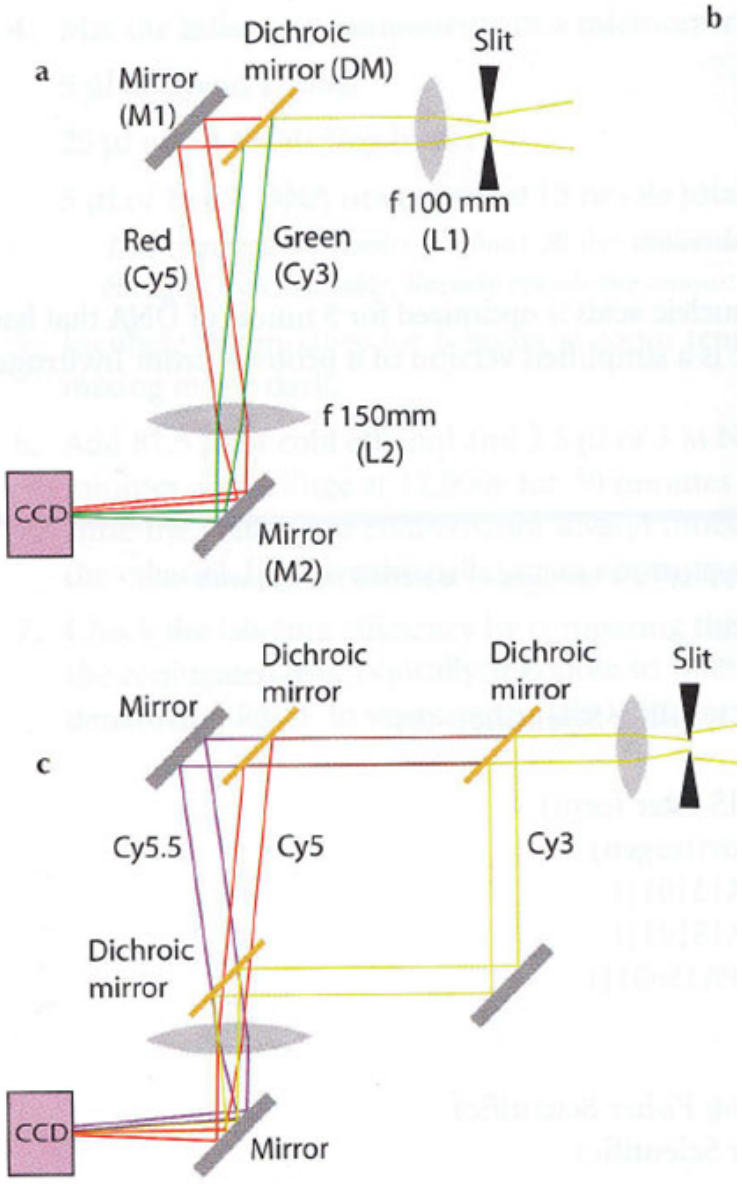
Then open kahoot!

smFRET TIRF setup multicolor detection

- Build around Olymous IX 71 microscope
- Two color emission optics
  Collimated beam goes through a dichroic mirror and the donr and acceptor images are projected on one-half of the CCD camera each

- Three color setup

  donor color is separated first , same trick is employed to split acceptors signals



Typical DATA from smFRET

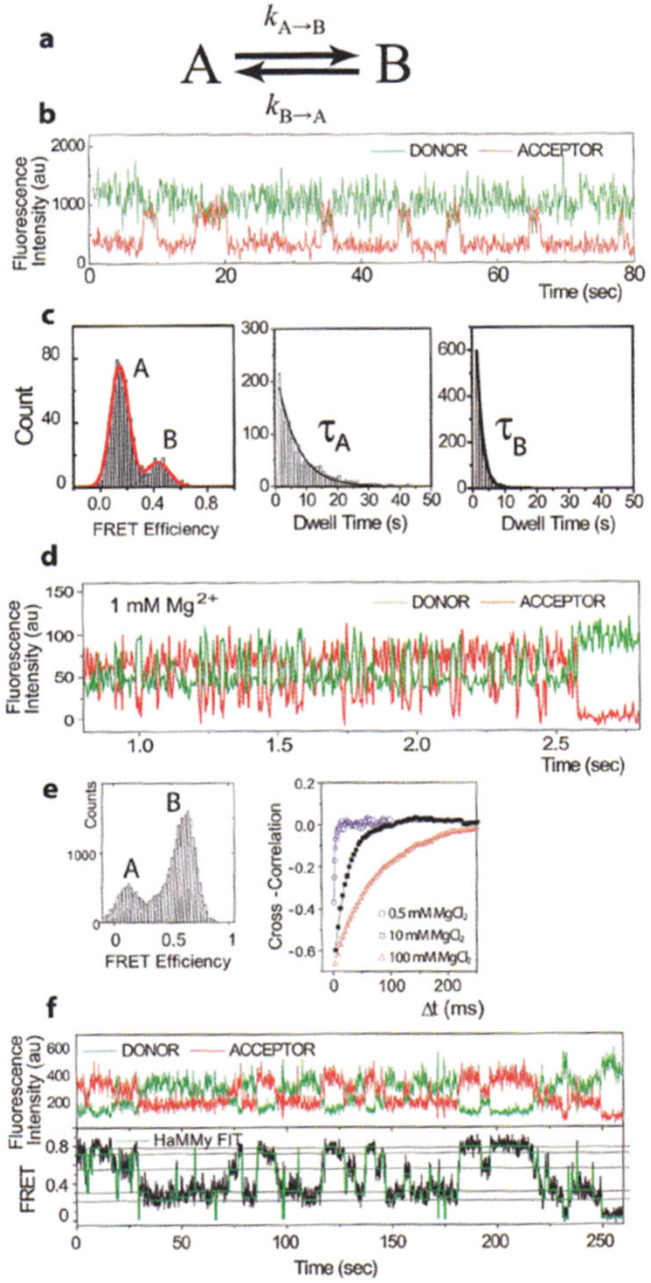
- FIRST find **FRET efficiency**

  apparent FRET efficiency through

$$E_{app} = \frac{I_A}{\left(I_A + I_D\right)}$$

What we actually measure are the raw intensities of the donor and acceptor channels I_{A0} and I_{D0} we need than to correct for leakage of donor signal to the acceptor channel

$$E_{app} = \frac{I_A^0 - \alpha \cdot I_D^0}{\left(I_A^0 + I_D^0 - \alpha \cdot I_D^0\right)}$$

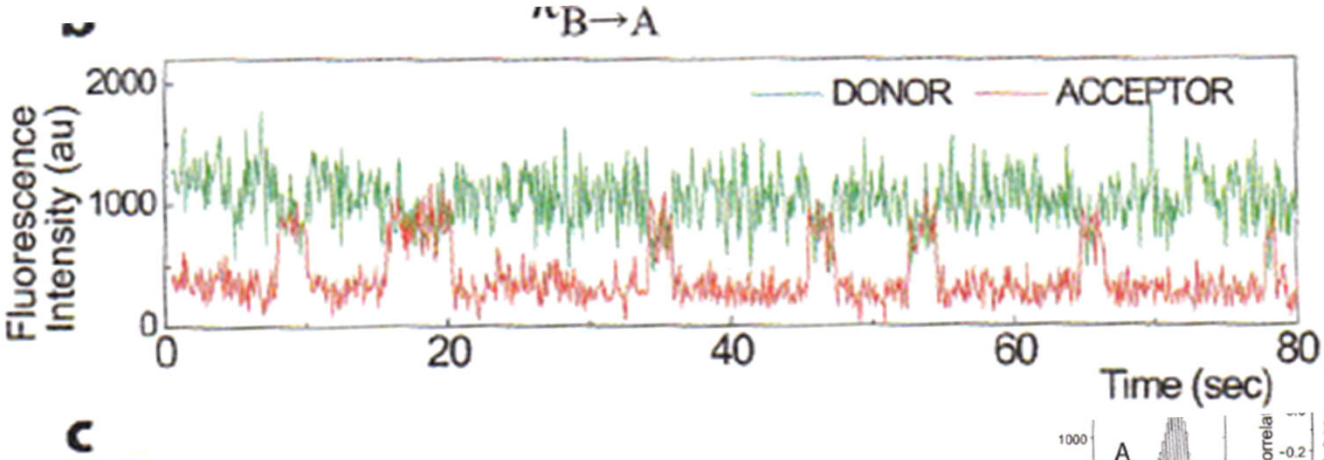


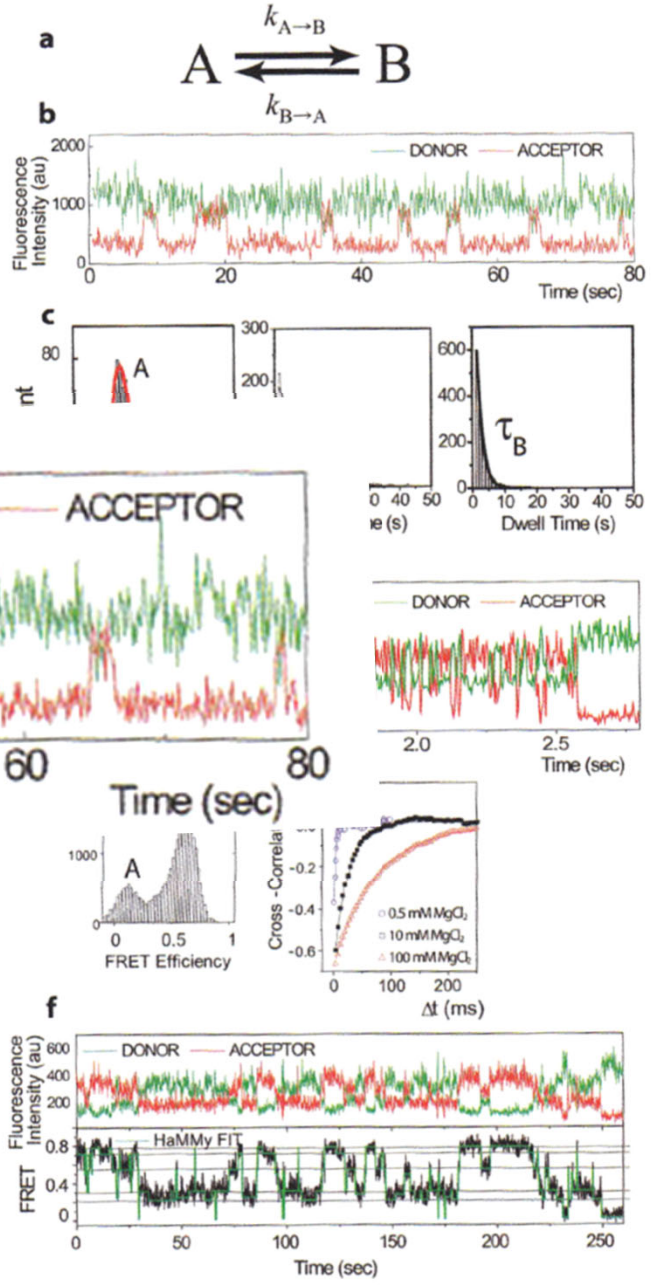
Typical DATA from smFRET

FRET efficiency

$$E = \frac{I_A}{\left(I_D + \gamma \cdot I_D\right)}$$

Where γ is correction factor





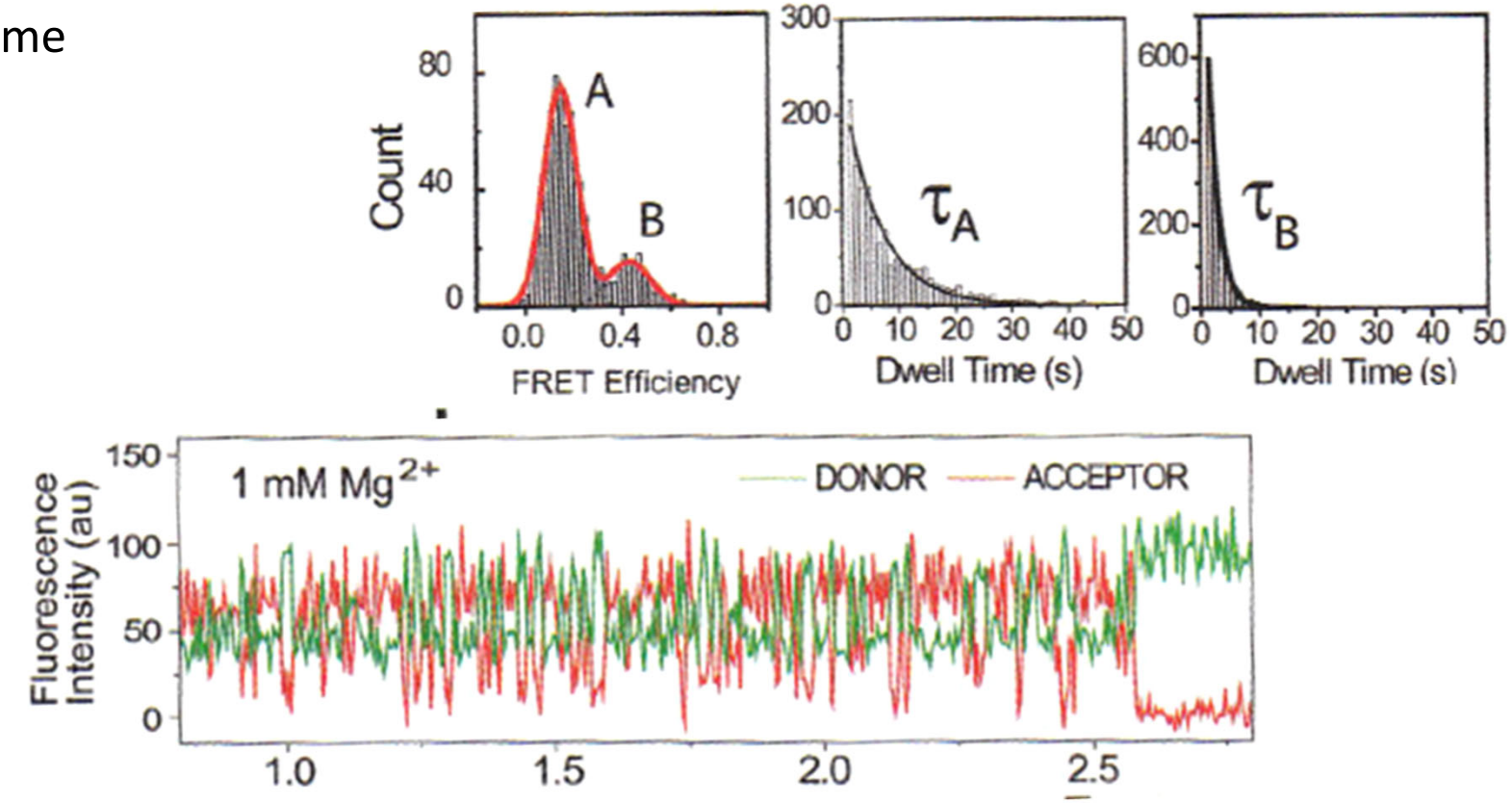
Easy to notice two states

Apply automated threshold analysis

We fit the dwell time histogram to of each state to obtain lifetimes τ_A and τ_B

Typical DATA from smFRET

- Dwell time

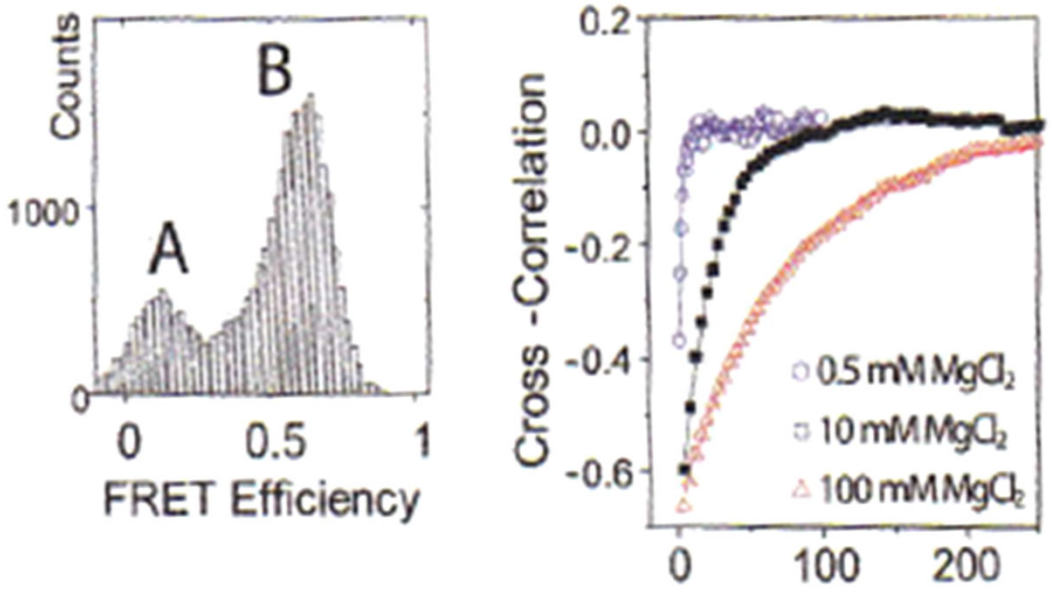


If the timescale of the fluctuations is **very fast** use **cross correlations** this type of analysis can tell us if donor and acceptor are fluctuating in anticorrelated manner

$$CC(\tau) = \int I_D(t) I_A(t-\tau) dt$$

$$-Ae^{-(k_{A\to B}+k_{B\to A})\tau}$$

Typical DATA from smFRET



$$-Ae^{-(k_{A\to B}+k_{B\to A})\tau}$$

From the cross correlation curve fit we can obtain the sum of two rates and if FRET efficiency histogram is clean enough in combination we can determine both

$$k_{A\to B} \quad \text{and} \quad k_{B\to A}$$

For more than one state more evolved algorithms are need than just simple treshholding
And cross correlation analysis – **USE HIDDEN MARKOV MODELING**

Hidden Markov Modeling tools

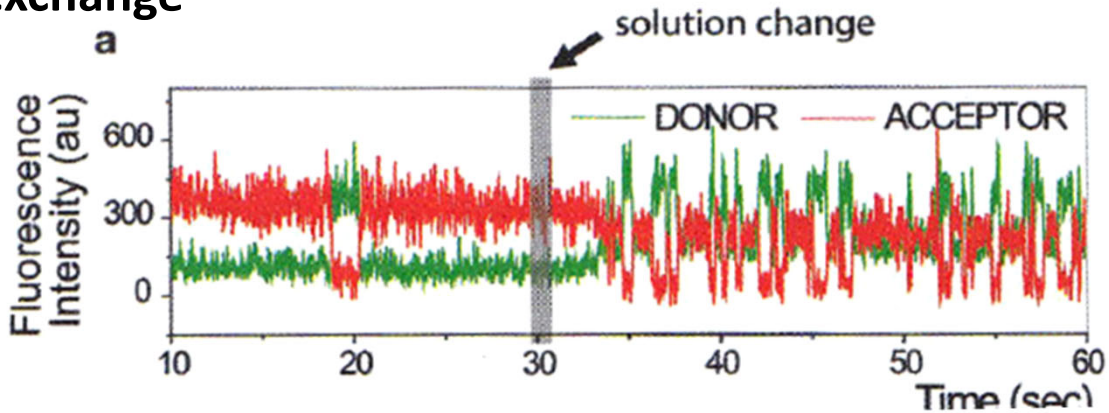
- Rule inspect your traces if states are well separted no need for Hidden Markov modeling

- For multiple states or unclean data use it ! HaMMy is tool developed in Ha lab

- http://bio.physics.illinois.edu/HaMMy.html

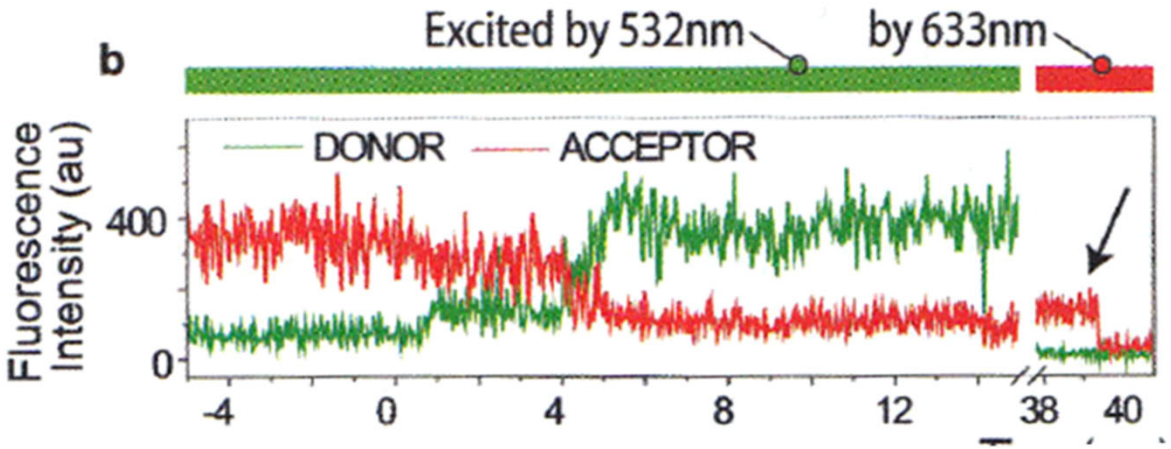
Other types of traces

- **Solution exchange**



- **Disappearance of signal**

When FRET signal drops to zero to be sure that this comes form the relative dye positioning and not simply form photo bleaching of the donor direct acceptor excitation uses to confirm the data



Other types of traces

- **FRET histograms**