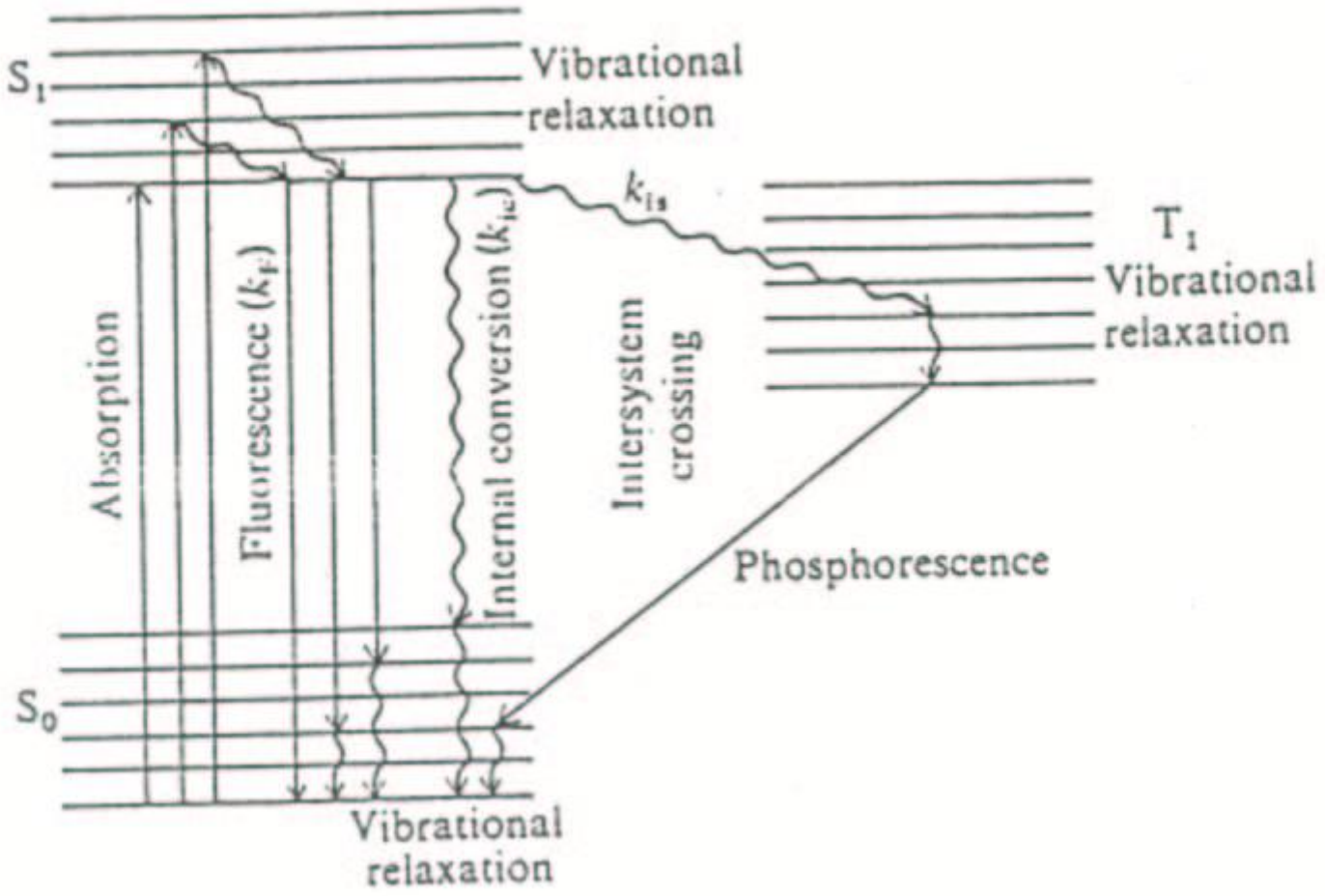
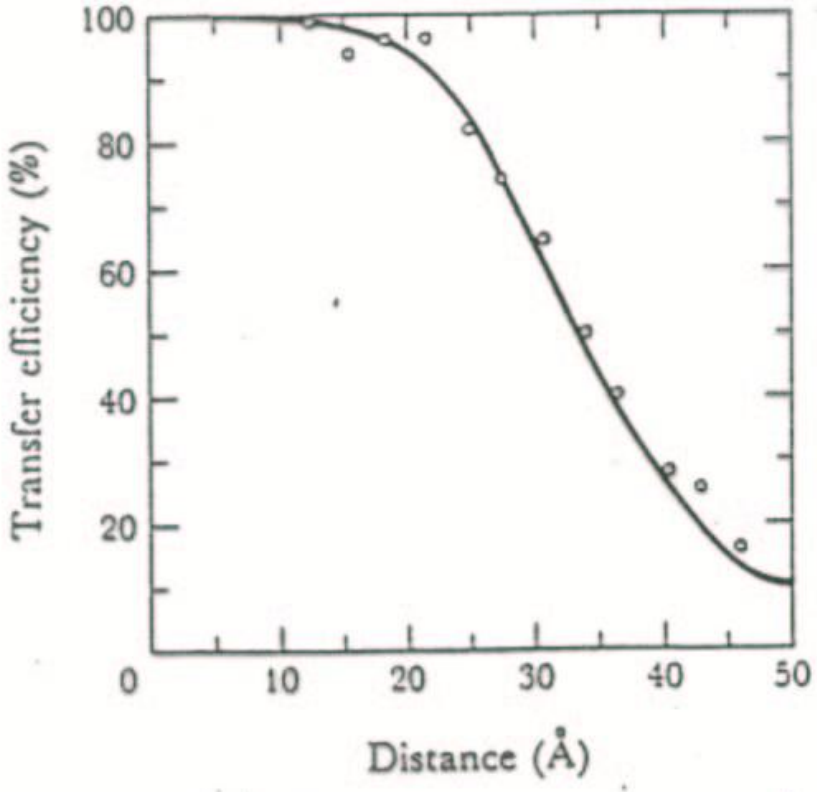
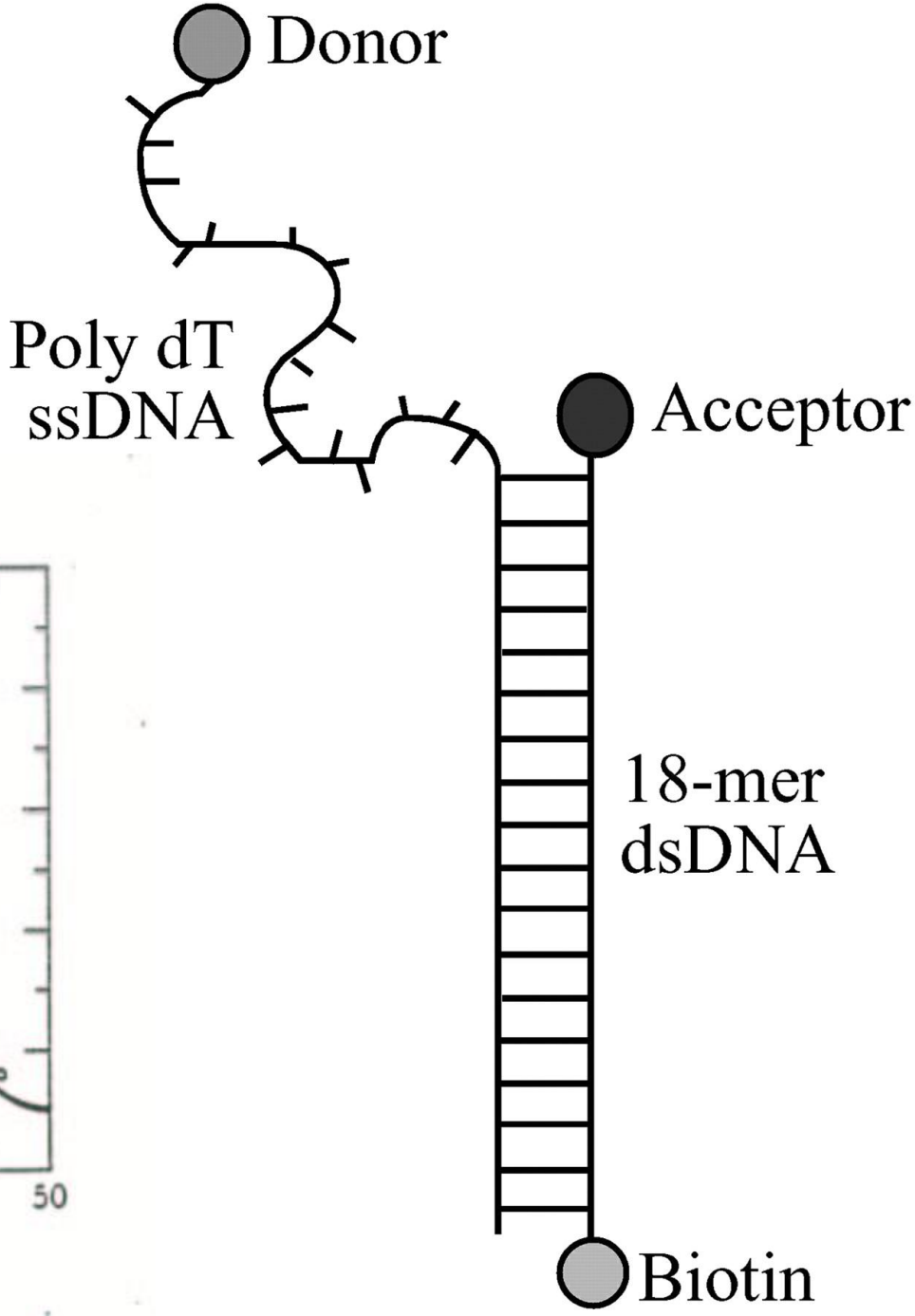
Fluorescence

- Absorption of light occurs within ~10^{-15} seconds, leaving a molecule in an excited state
- What happens next?
  - If no photon is re-emitted, the molecule probably loses the energy via a collision with solvent molecules
  - If a photon is emitted then it can be of several types:
    - Scattered at the same frequency/energy
    - Fluorescent at a longer wavelength (takes ~ ns)
    - Phosphorescent – similar to fluorescence but transition is from a triplet state (with electrons parallel ↑↑ ; fluorescence is from a singlet state with paired e^-↑↓) (takes > msec)
    - Resonant energy transfer (FRET) – donor and acceptor groups have a common vibrational energy level: A + hf $\rightarrow$A*; A* + B $\rightleftharpoons$ A + B* ; B* $\rightarrow$ B + hf ; A & B must lie close to one another – technique can be used as a “yardstick”

Energy Levels




Transfer efficiency (%)
100
80
60
40
20
0
10
20
30
40
50
Distance (Å)


Donor
Poly dT
ssDNA
Acceptor
18-mer
dsDNA
Biotin

Quantum Yield

- All of these processes compete with one another
- The quantum yield for fluorescence

$$Q_{fluorescence} = \frac{\#\ fluorescent\ \ photons}{\#\ absorbed\ \ photons}$$

Each other process has a Q and all must add up to 1:

$$\Sigma Q_i = 1$$

Two types of factors affecting $Q_{fluorescence}$:

– internal – with more vibrational levels closely spaced (more flexible bonds), fluorescence is more easily quenched, losing energy to heat

best fluors are stiff ring structures: Tryp, Tyr

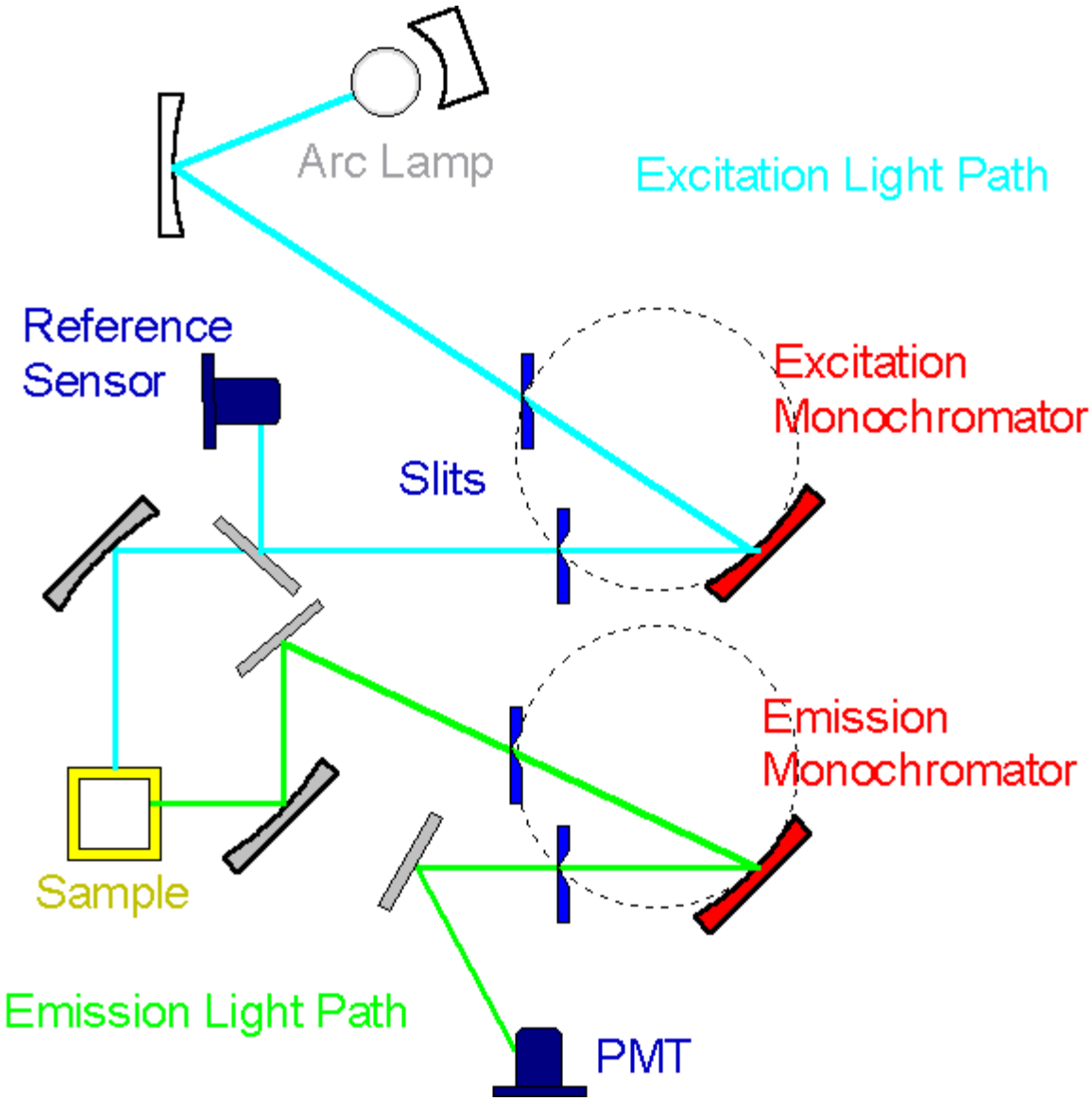
– environmental factors such as T, pH, neighboring chemical groups, concentration of fluors; generally more interesting

Instrumentation

1. 90^o measurement to avoid scattering or direct transmitted beam
2. Very low concentration can be used to keep I_{fluor} linear in concentration

$$I = I_o Q(1 - e^{-\varepsilon c \ell}) = (\,for\ small\ c) I_o Q \varepsilon c \ell = Kc$$

3. Sensitivity is very high since no bkgd signal – no difference measurement (blank) needed as in absorption
4. Measure either I vs $\lambda_{emitted}$ for a given λ_{inc} = emission spectrum OR measure I vs $\lambda_{exciting}$ at fixed $\lambda_{emitted}$ = excitation spectrum
5. Simple fluorometer uses interference filters for incident & 90^o emission – better machines use gratings and scan to get a spectrum


Arc Lamp
Excitation Light Path
Reference
Sensor
Excitation
Monochromator
Slits
Emission
Monochromator
Sample
Emission Light Path
PMT

Spectra

Record uncorrected spectra directly –

3 types of corrections needed:

a. Output I_o of light source varies with λ_{inc}

b. Variable losses in monochromators with λ_{inc} or emitted

c. Variable response of PMT with $\lambda_{emitted}$

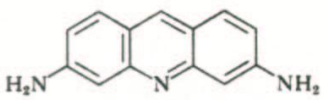
Typically absolute measurements are not done and so no corrections are made – only comparisons

Fluors

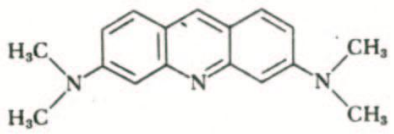
- Intrinsic: "chromophore" = e.g. Try, Tyr, Phe – best is Try; I_{fluor} depends strongly on environment
- Extrinsic: attach fluor to molecule of interest; must:
  - Be tightly bound at unique location
  - Have fluorescence that is sensitive to local environment
  - Not perturb molecules being studied

  Examples: ANS & dansyl chloride fluoresce weakly in water, but strongly in non-polar solvents;

  Acridine O used with DNA – green on d-s, red-orange on s-s

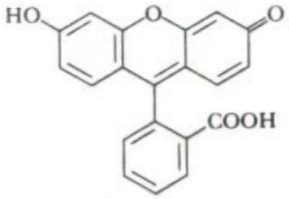


Proflavin

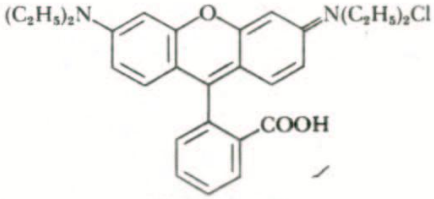


Acridine orange

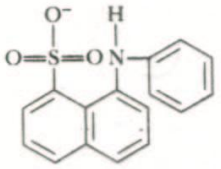
Green on d-s DNA; red-orange on s-s DNA



Fluorescein

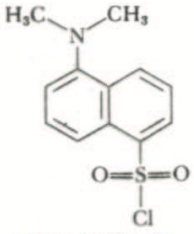


Rhodamine B



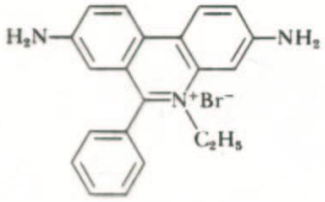
1-Anilino-8-naphthalene sulfonate (ANS)

Weak in water; strong in non-polar solvents

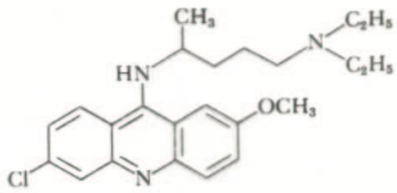


Dansyl chloride

Used with DNA



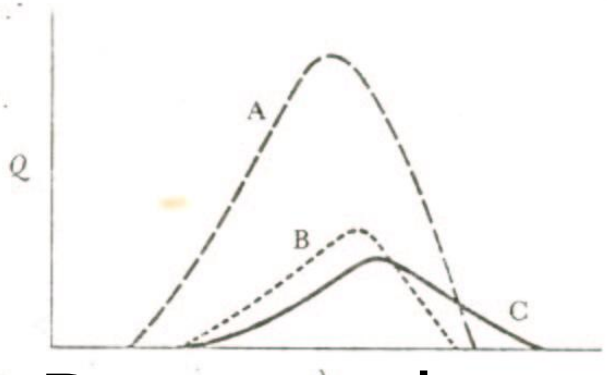
Ethidium bromide



Quinacrine chloride

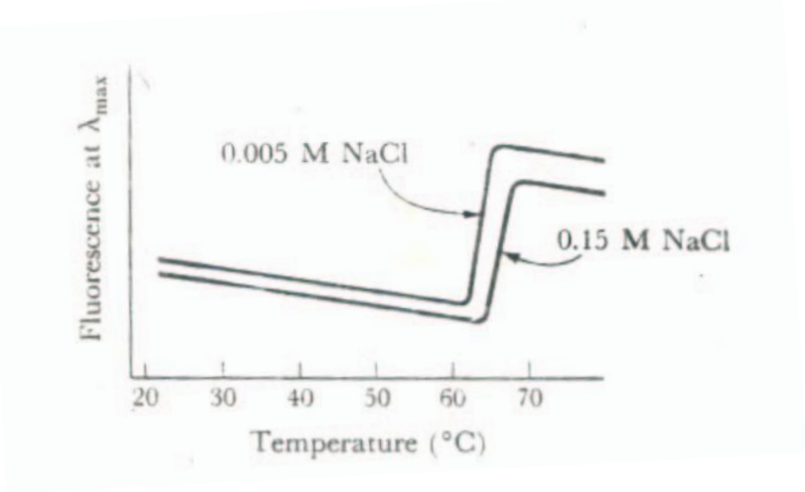
Two Application Examples

1. Detect conformational changes in an enzyme when a co-factor binds



A w/o added co-factor; B with added co-factor; C = free Tryptophan

2. Denaturation of a protein

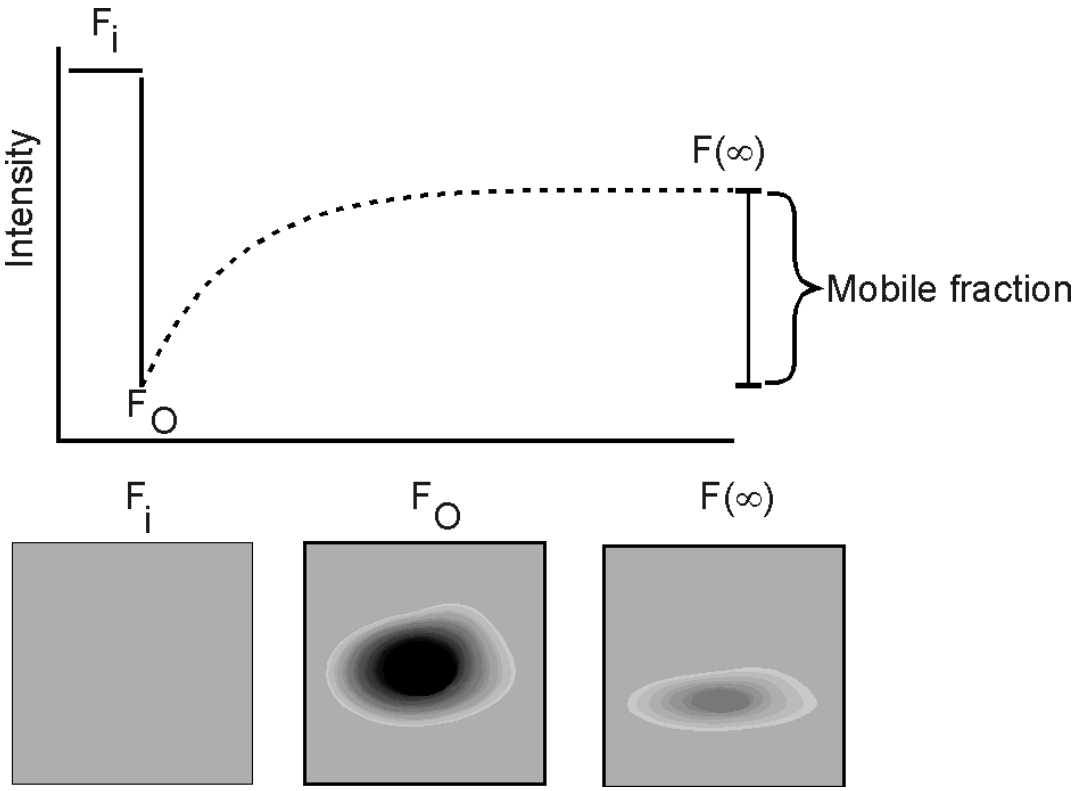


Helix-coil transition of a protein; in 0.15 M NaCl the protein is more stable – higher T needed for transition

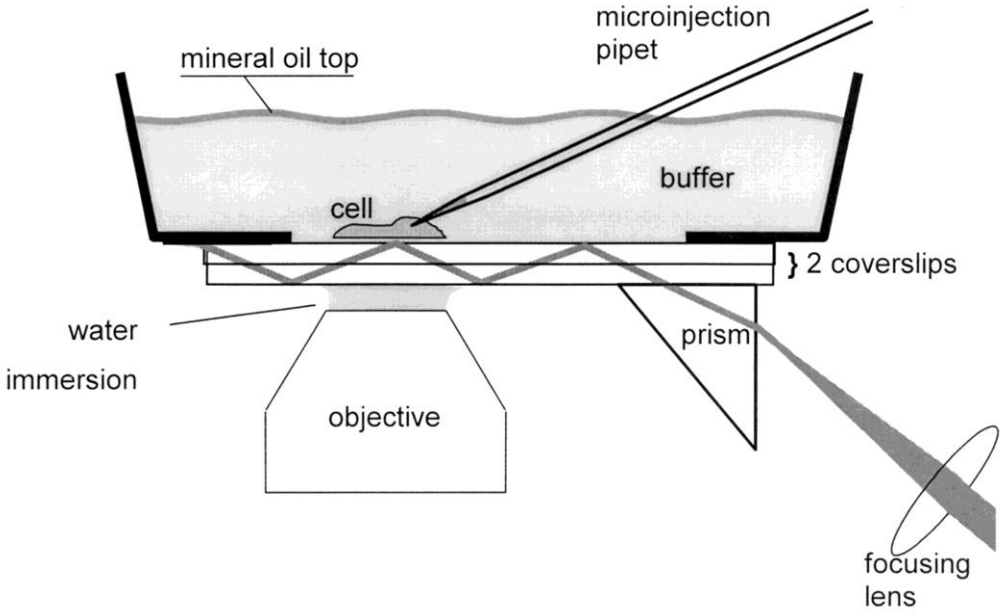
FRAP

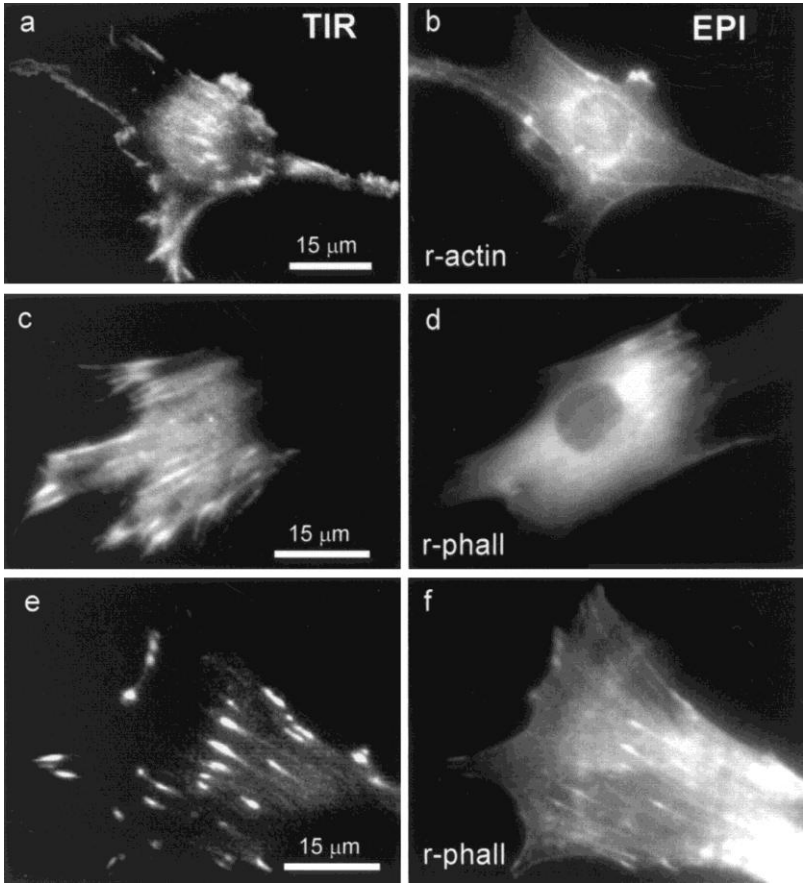
- High power bleach pulse
- Low power probe
- Look at 2-D diffusion

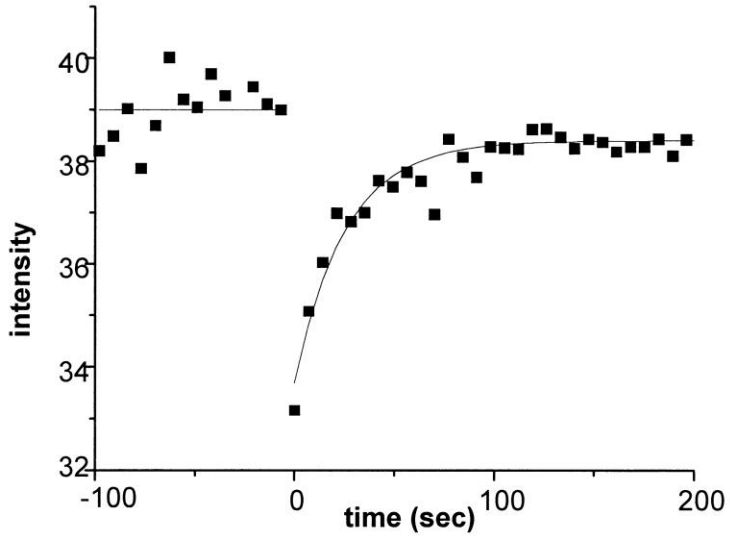
  $\langle r^2 \rangle = 4Dt \sim \text{size}^2$ beam focus



TIR-FRAP







Rhodamine labeled actin/phalloidin