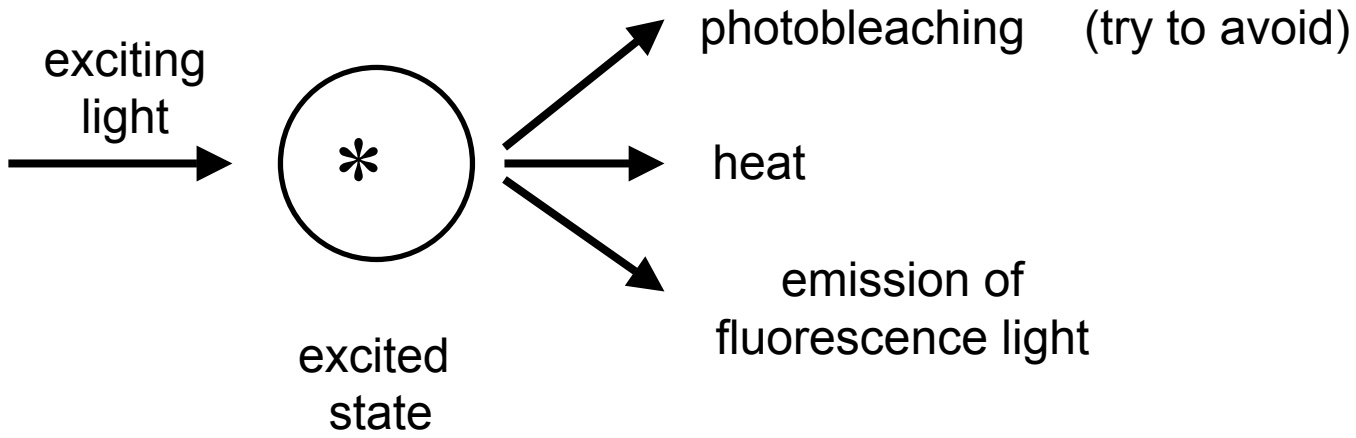
Fluorescence

1. What is fluorescence
2. Technical issues
3. Uses of Fluorescence:
   - Quantitation
   - Ligand binding
   - Conformational changes
4. Measuring distances
5. Fluorescence quenching
6. Fluorescence lifetimes

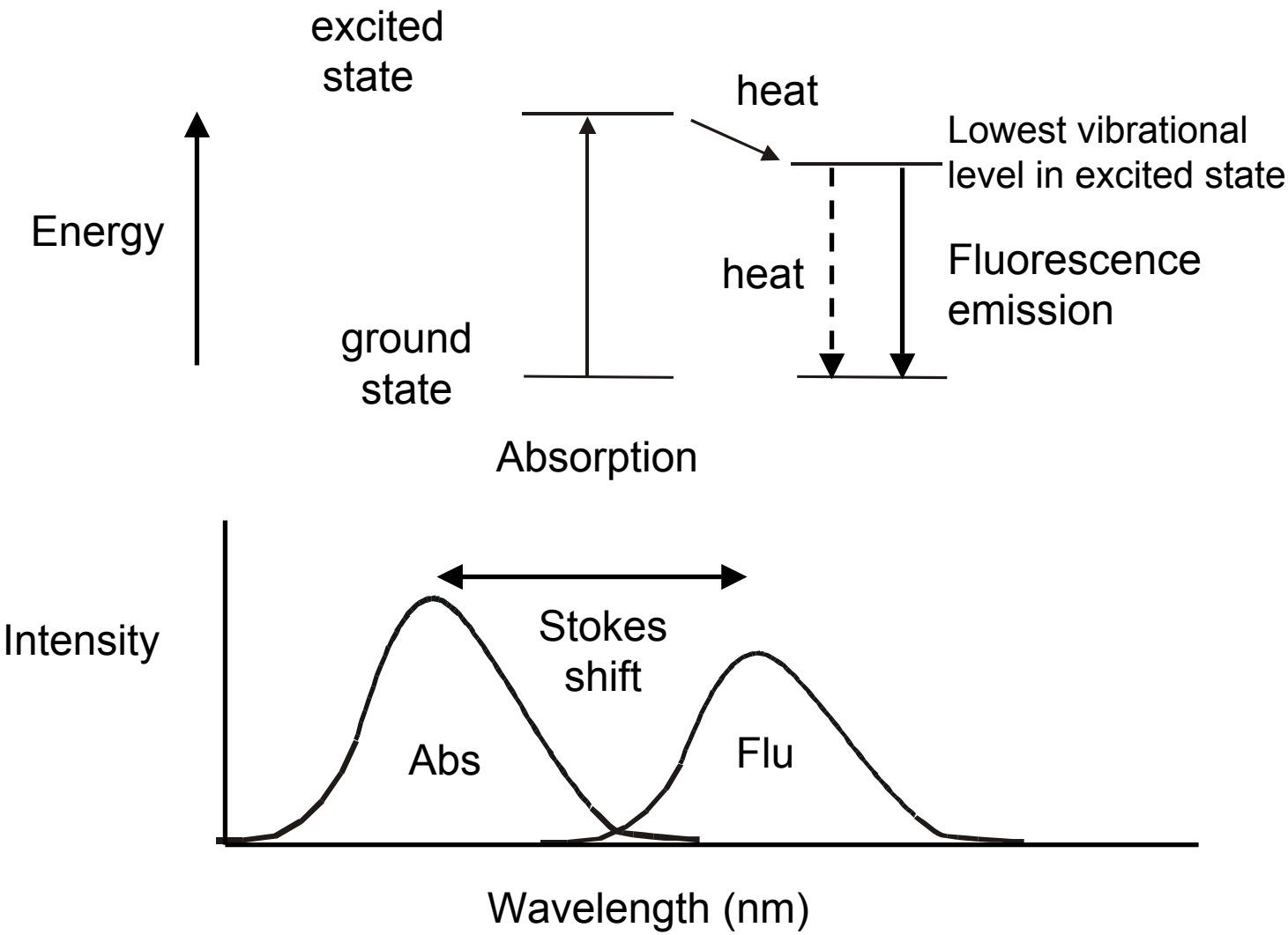
Fluorescence

What happens after a molecule has adsorbed light

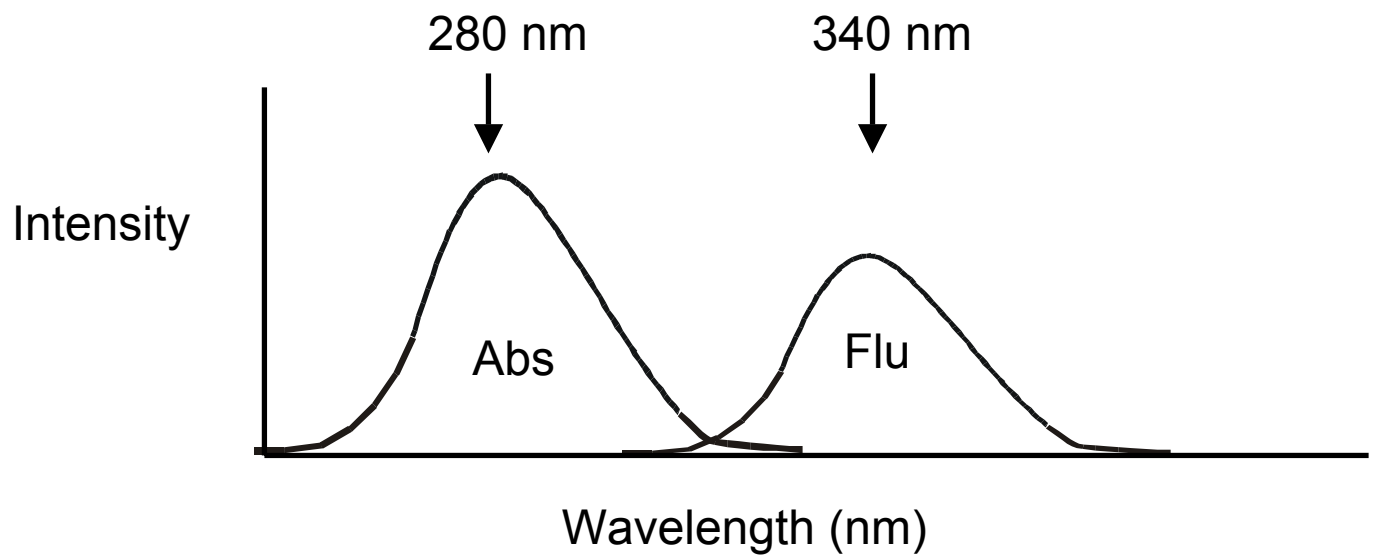


Lifetime 1 –10 10^{-9} secs
(1-10 nsecs)

Fluorescence



Fluorescence of Trp



Fluorescent amino acids: Trp, Phe, Tyr
Fluorescence of proteins is dominated by Trp

Fluorescence

1. What is fluorescence
2. Technical issues
3. Uses of Fluorescence:
   - Quantitation
   - Ligand binding
   - Conformational changes
4. Measuring distances
5. Fluorescence quenching
6. Fluorescence lifetimes

Fluorescence Instrumentation

Spectrofluorimeter

Looks at samples in solution
(Typically 3 mls)
High sensitivity (conc 0.1 μM)

Fluorescence microscope

Looks at spatial distribution in 2D or
3D (confocal)

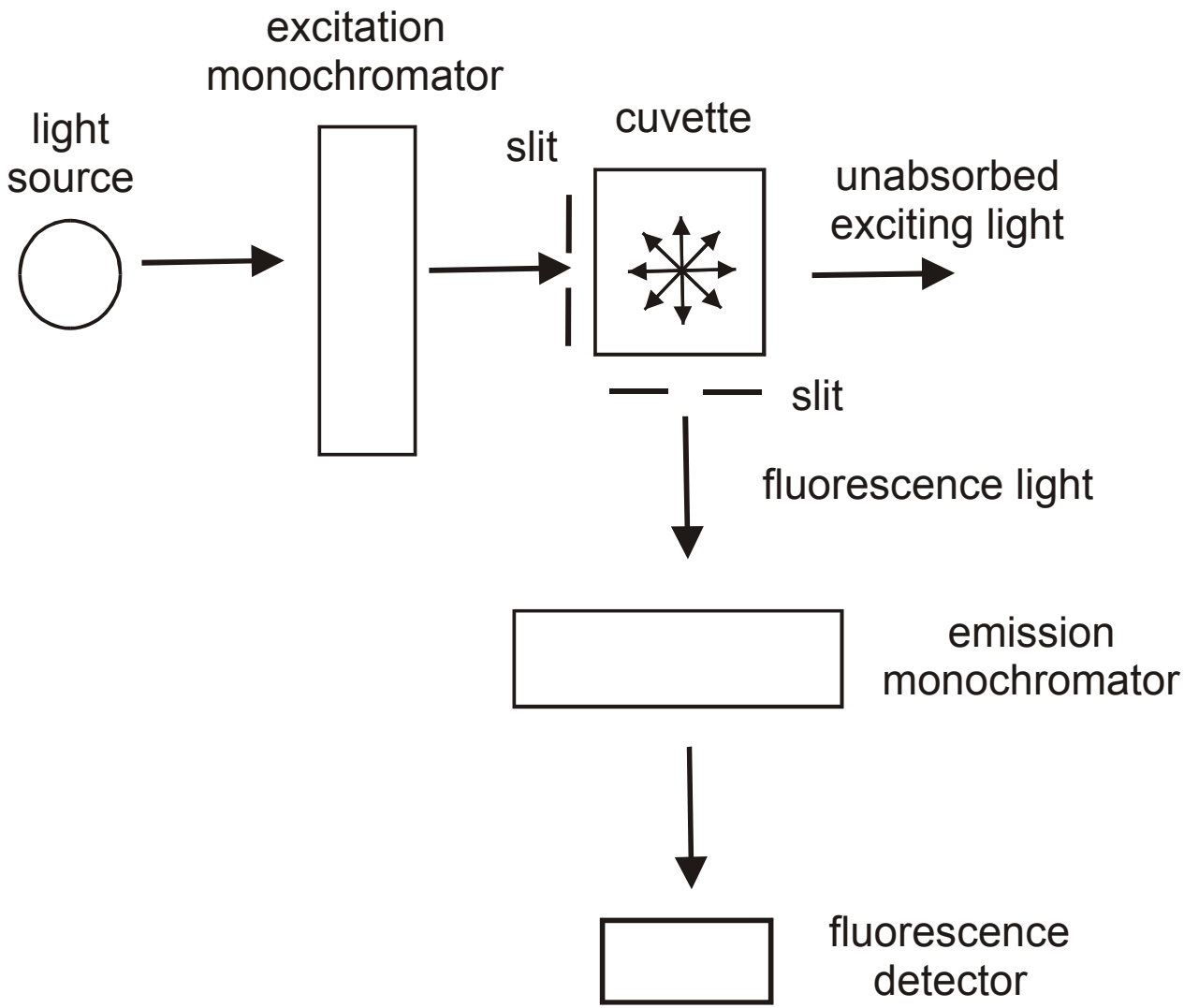
Source of Radiation

Arc lamp (450 watt Xenon) + monochromator
Laser (generally fixed wavelength
eg Argon-ion laser 488 nm

Detector

Phototube (can be electrically cooled)
CCD camera on fluorescence microscopes

Fluorimeter (right angle geometry)



Sensitivity

Down to ca 0.01 μM (ca 100 times more sensitive than absorption spectroscopy

Limited by:

- Scattered light (problem with cloudy samples)
- Background fluorescence
  - Impurities in buffers (use Ultra-pure reagents)
  - Autofluorescence in cells

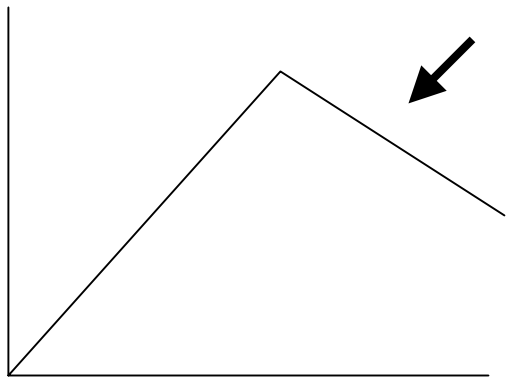
Ideal fluorescence molecule:

- High extinction coefficient ε (Absorbs light well)
- High quantum yield
- Large Stoke's Shift
- High wavelength of emission

Usually contains multiple –C=C-

Sensitivity

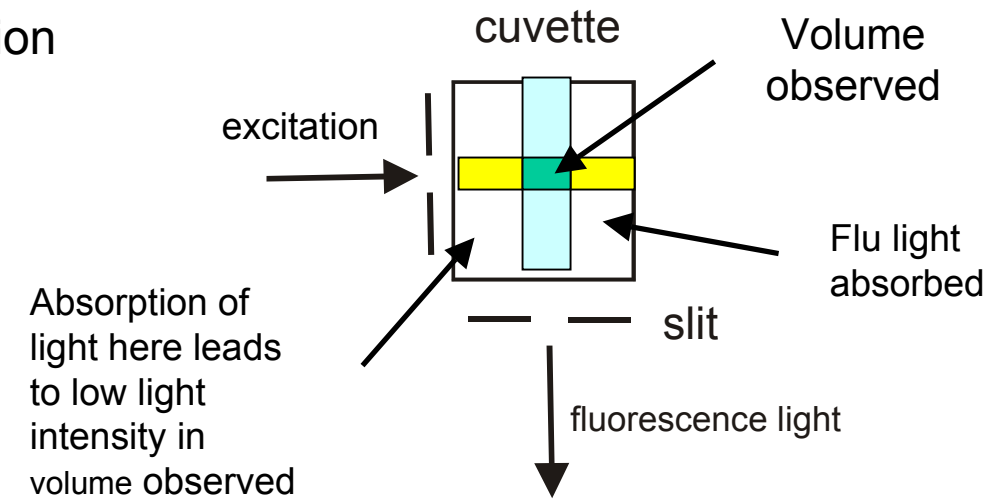
Common problem : concentration too high



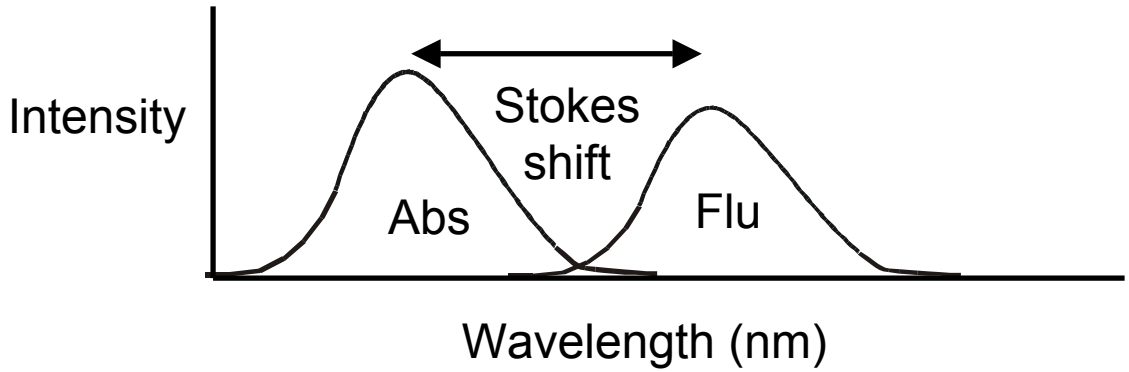
Inner filter effect

Optical Density less than 0.05 to avoid inner filter effect

Inner Filter Effect



Fluorescence Parameters



Wavelength of maximum absorption (equiv. to abs. Spectrum)
intensity of absorption – extinction coefficient ε
Wavelength of maximum fluorescence emission
environmentally sensitive
Fluorescence quantum yield Q (related to intensity of emission)
environmentally sensitive

(cont.)

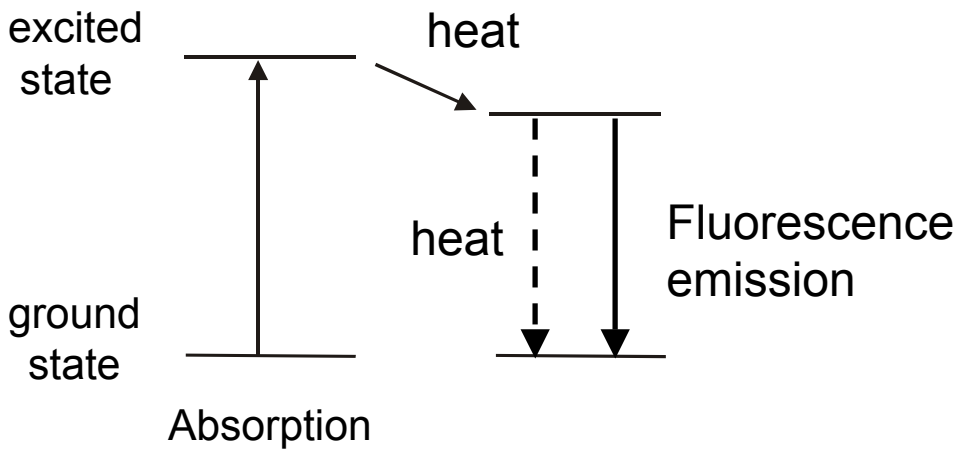
Fluorescence Parameters (cont.)

Fluorescence quantum yield Q (related to intensity of emission)

$$Q = \frac{\text{no. of photons emitted}}{\text{no. of photons absorbed}}$$

Q between 0 and 1

(Trp Q ca 0.2)

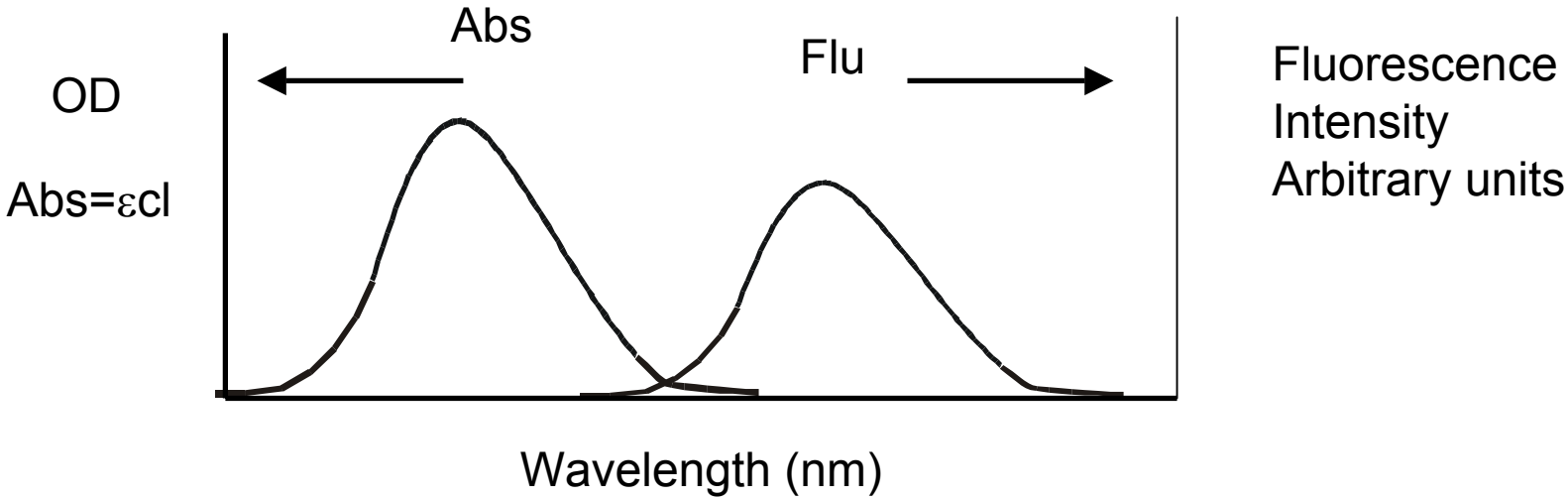


Fluorescence lifetime

Time molecules remains in the excited state

typically 1-10 nsecs

Fluorescence Intensity Measurements



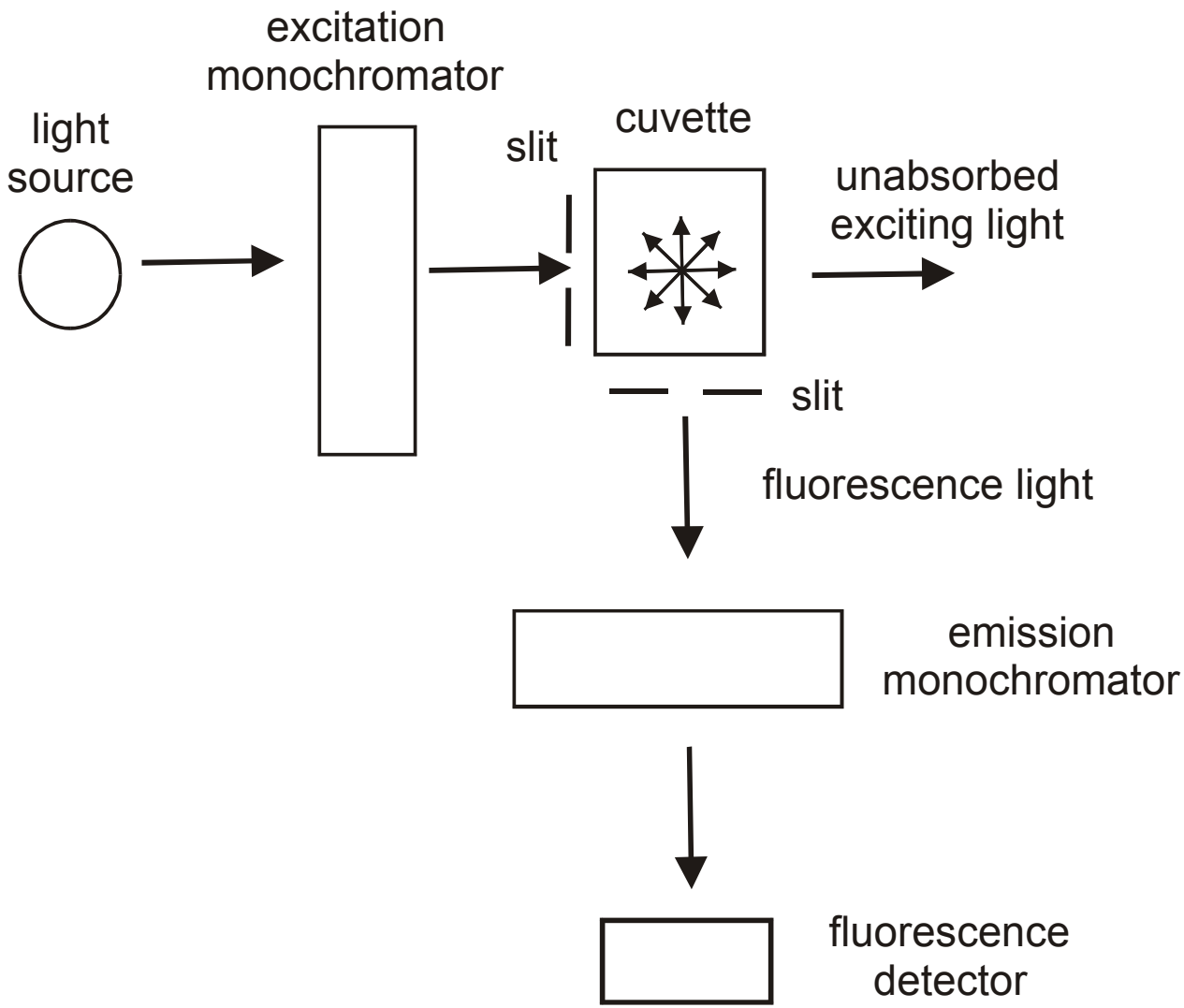
Fluorescence intensity is measured in ‘arbitrary units’

Size of signal depends on sensitivity of the fluorimeter

Thus need to standardize:

measure relative to some convenient standard solution

Fluorimeter (right angle geometry)



Fluorescence

1. What is fluorescence
2. Technical issues
3. Uses of Fluorescence:
   - Quantitation
   - Ligand binding
   - Conformational changes
4. Measuring distances
5. Fluorescence quenching
6. Fluorescence lifetimes

Uses of Fluorescence

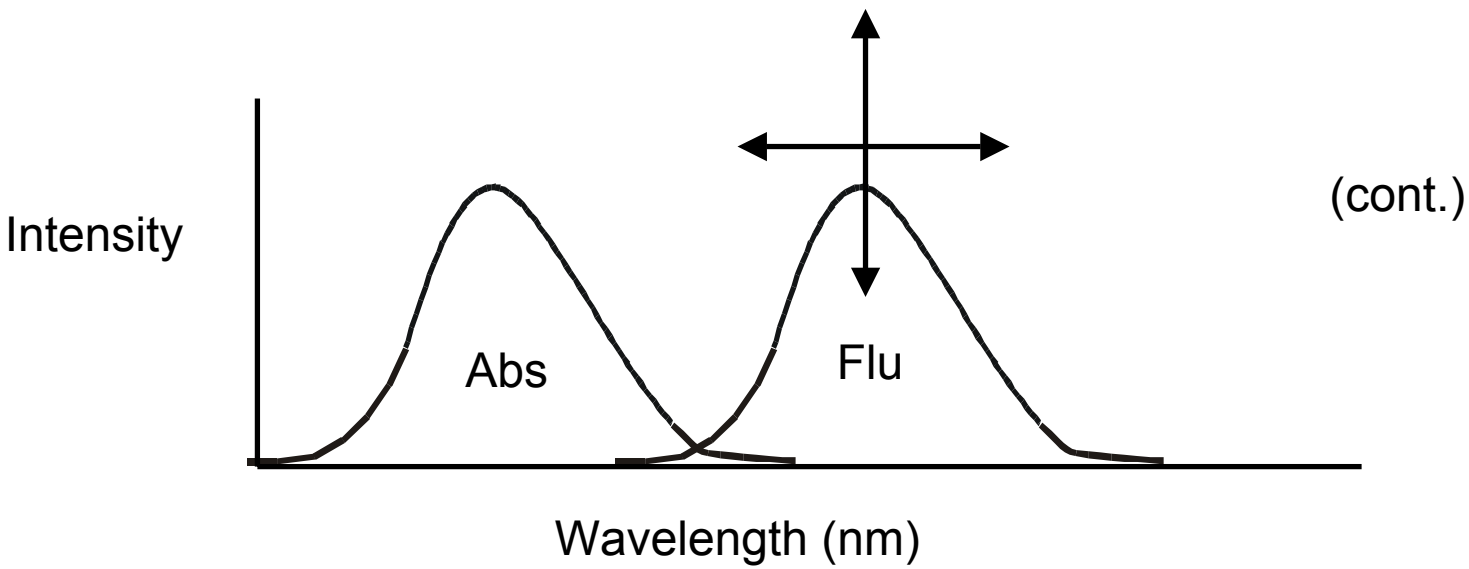
Quantitate material:

Flu. Int. proportional to conc.

Environmental change:

Flu maximum can shift (spectral shift)

Q can change (intensity change)



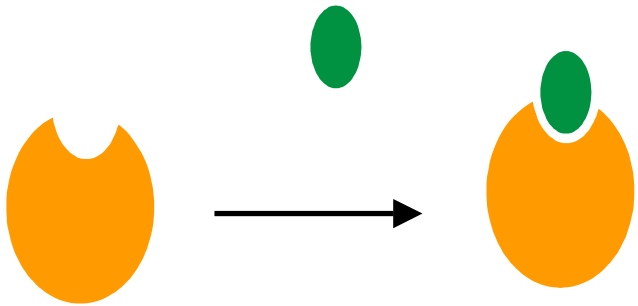
Uses of Fluorescence (cont.)

Conformational changes on a protein

- Trp fluorescence
- Labelled protein eg at a Cys residue with a fluorescence probe

Ligand binding

- Changes in protein fluorescence
- Changes in ligand fluorescence if ligand is fluorescent



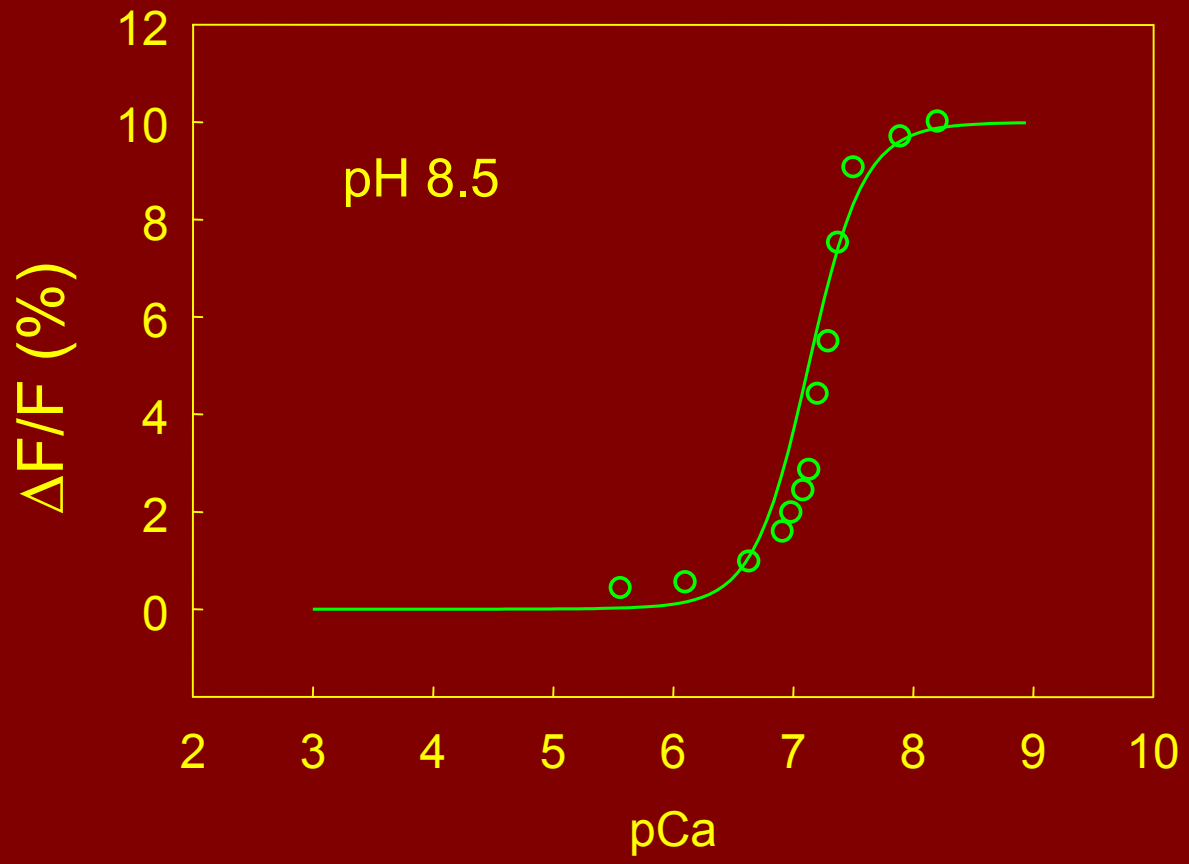
Examples: Ca^{2+} binding to Ca^{2+} -ATPase

Trp residues

Ca^{2+} binding sites

Ca^{2+}-ATPase + Ca^{2+}

Change in Trp fluorescence intensity



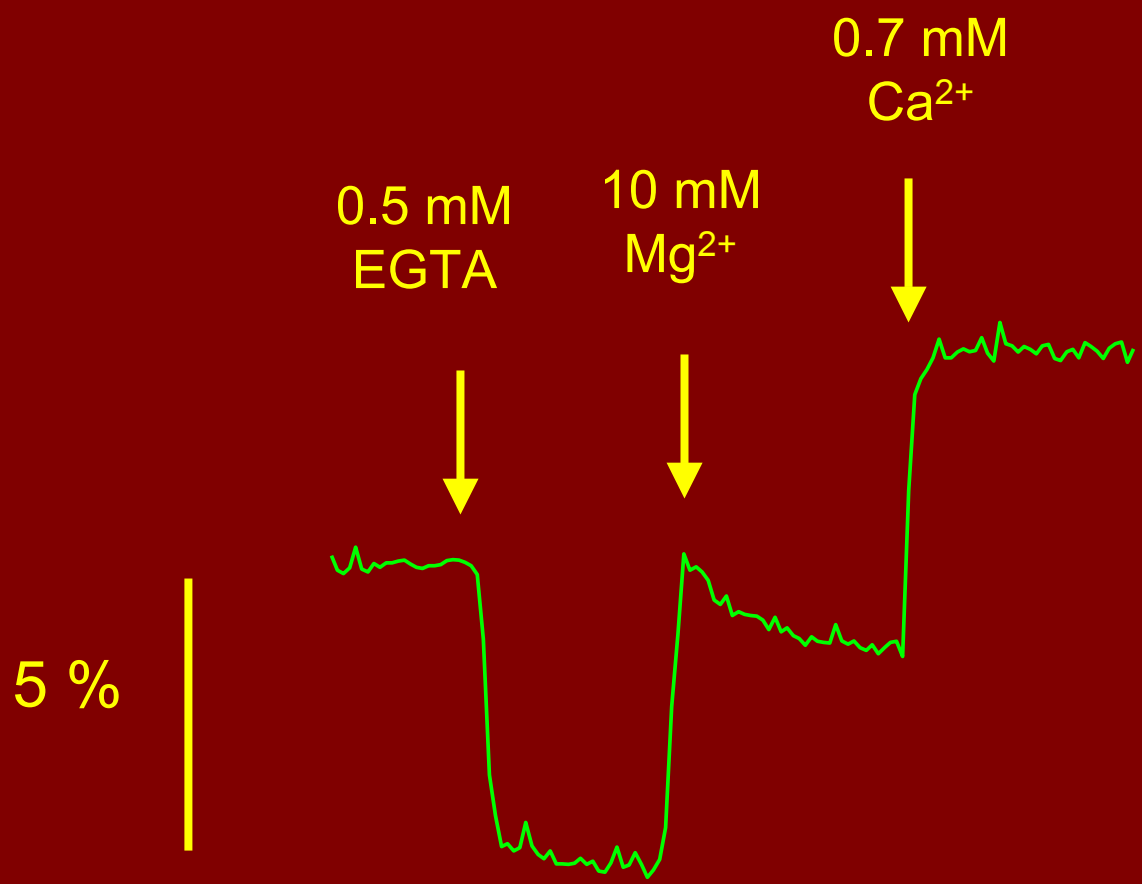
Cys residues

NBD-Cl

Bromomethyl-coumarin

Cys-344

NBD-labelled Ca^{2+}-ATPase



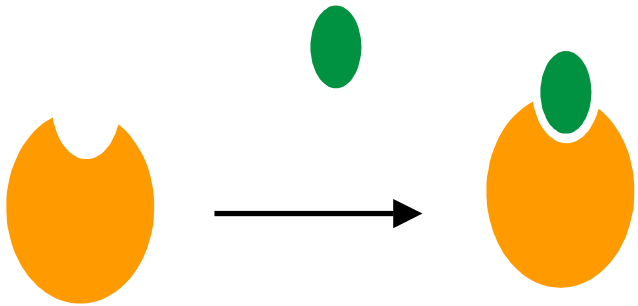
Uses of Fluorescence (cont.)

Conformational changes on a protein

- Trp fluorescence
- Labelled protein eg at a Cys residue with a fluorescence probe

Ligand binding

- Changes in protein fluorescence
- Changes in ligand fluorescence if ligand is fluorescent



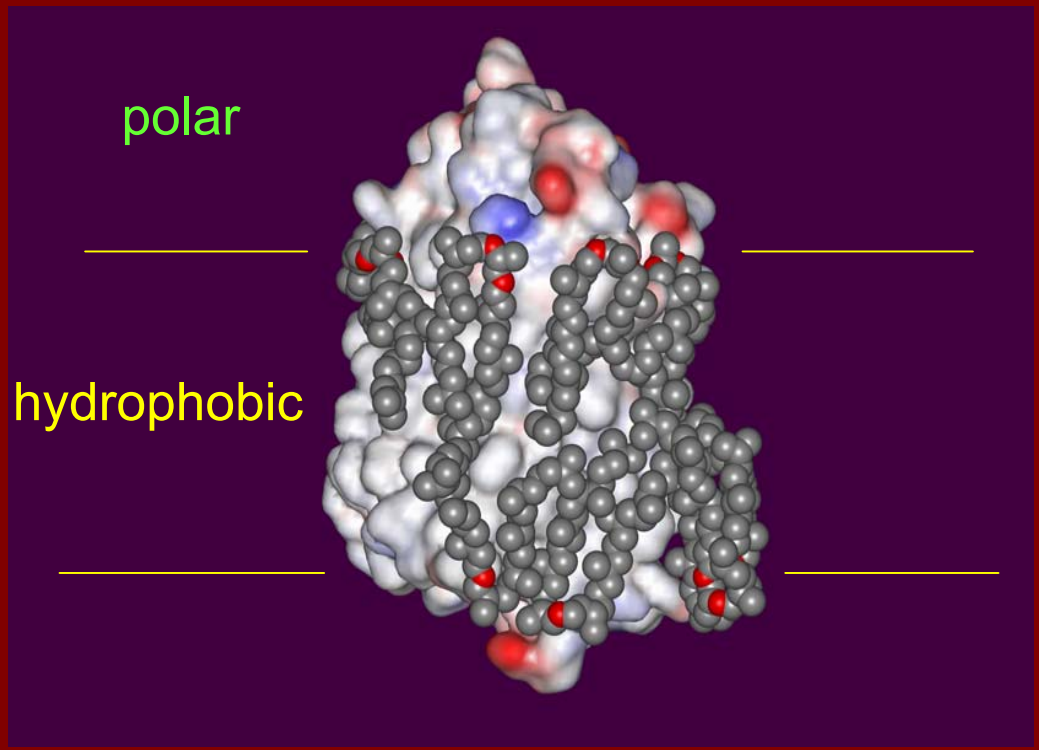
Examples: Ca^{2+} binding to Ca^{2+} -ATPase

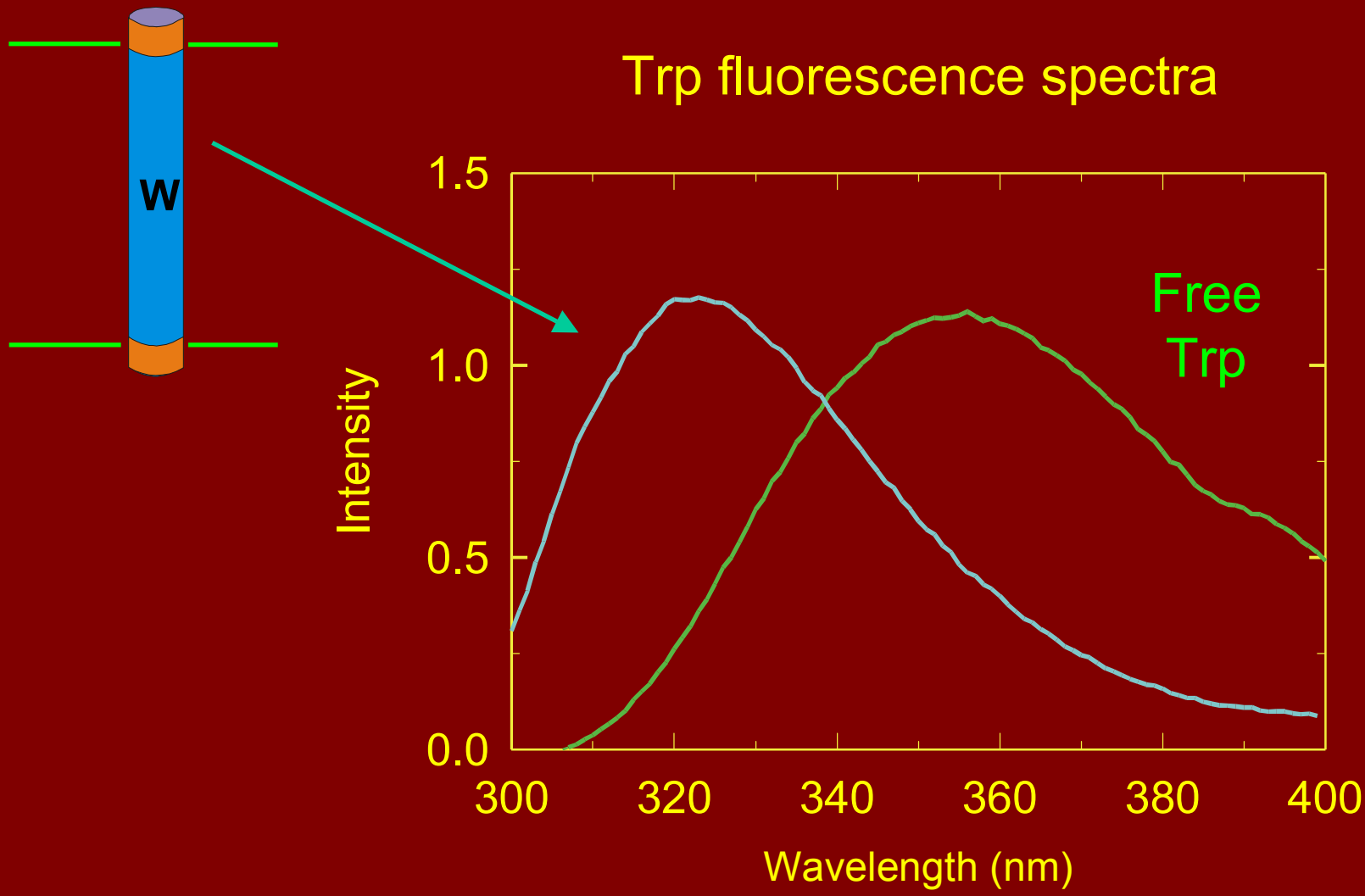
Location of Trp residues in a membrane protein

Uses of fluorescence
To report on environment

different λ_{max} for Trp

Bacteriorhodopsin



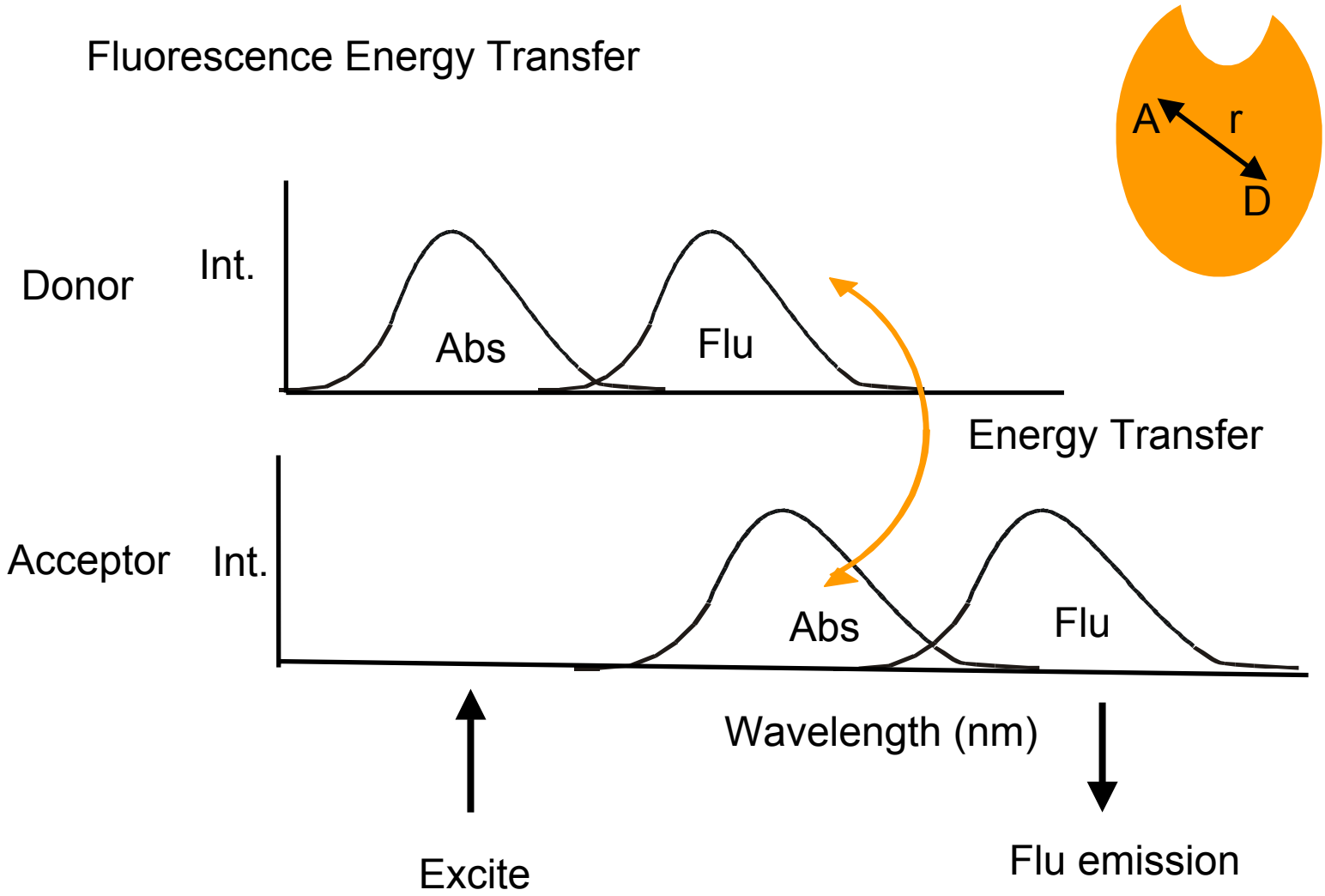

W
Trp fluorescence spectra
Intensity
1.5
1.0
0.5
0.0
300
320
340
360
380
400
Wavelength (nm)
Free
Trp

Fluorescence

1. What is fluorescence
2. Technical issues
3. Uses of Fluorescence:
   - Quantitation
   - Ligand binding
   - Conformational changes
4. Measuring distances
5. Fluorescence quenching
6. Fluorescence lifetimes

Uses of Fluorescence – Distance Measurements

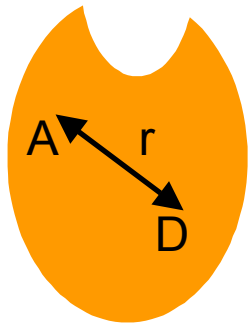
Fluorescence Energy Transfer



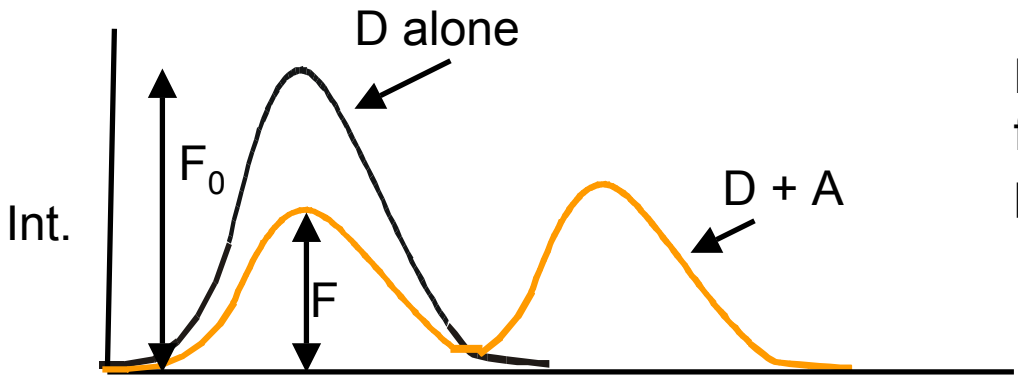
(cont.)

Uses of Fluorescence – Distance Measurements (cont.)

Efficiency of Energy Transfer proportional to r^6



Fluorescence emission spectra



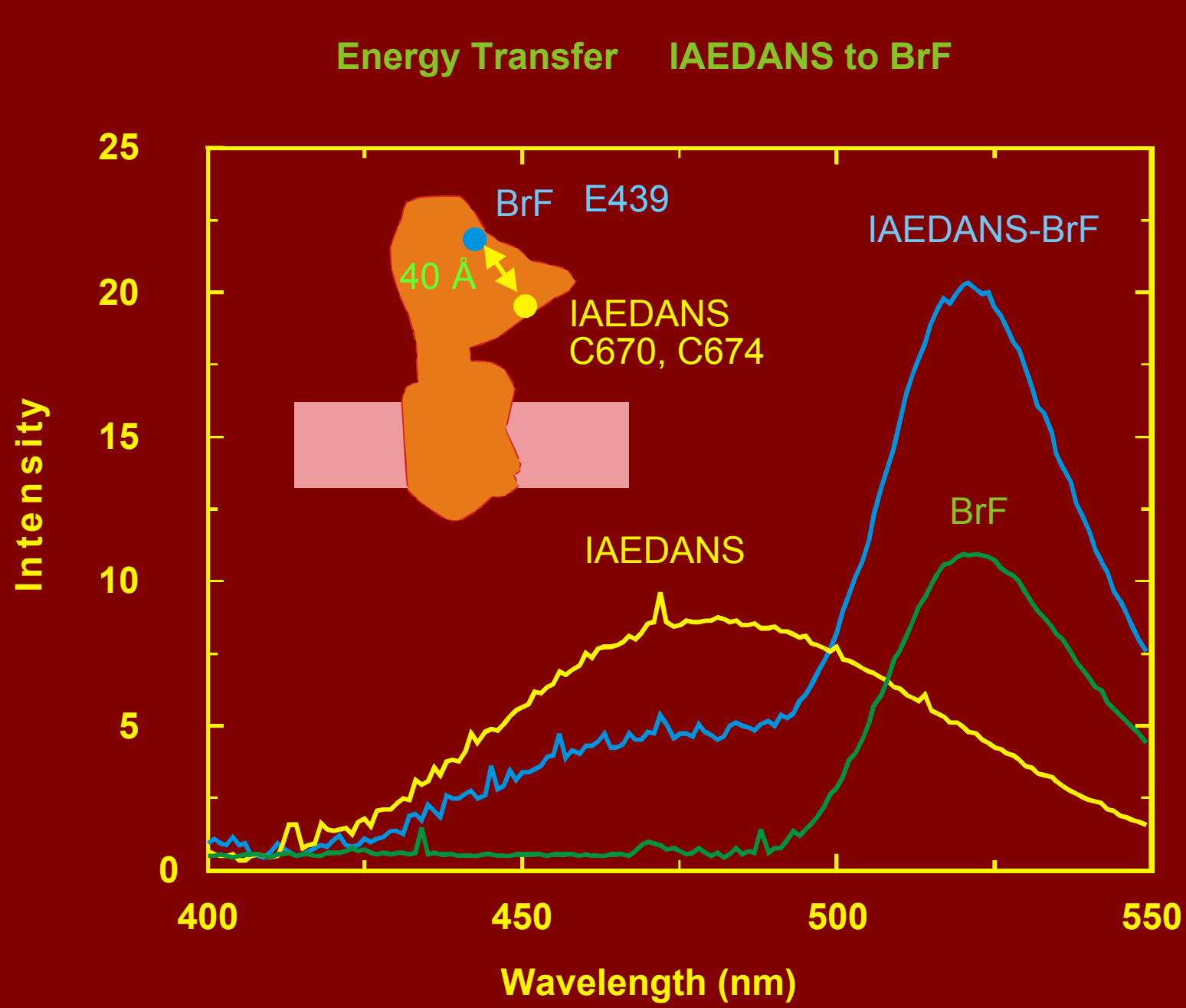
Measure decrease in F for donor caused by the presence of the acceptor

Efficiency of transfer $E = 1 - F/F_0$

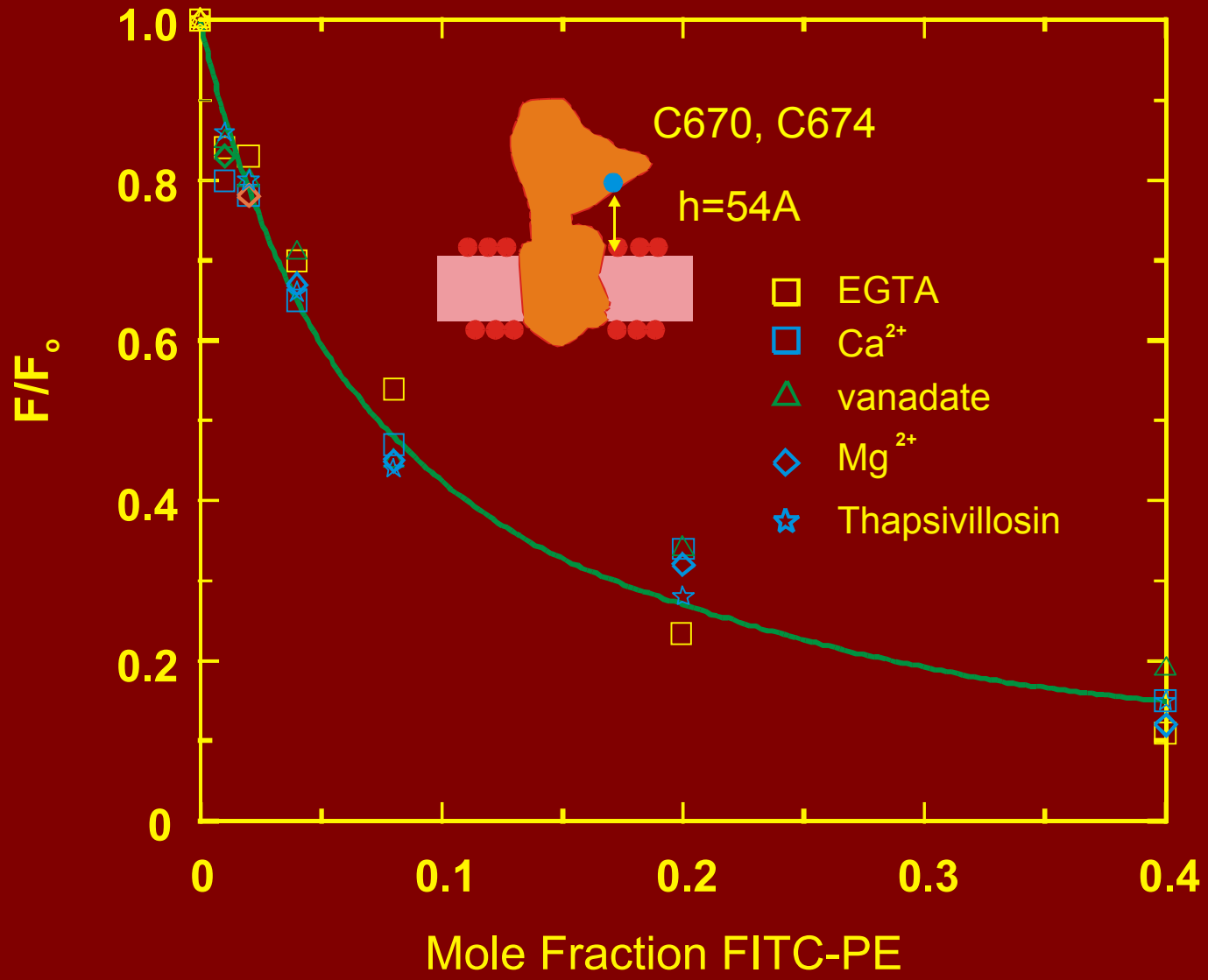
Forster theory: $E = R_0^6/(r^6 + R_o^6)$

R_0 = distance of separation at which E = 50%

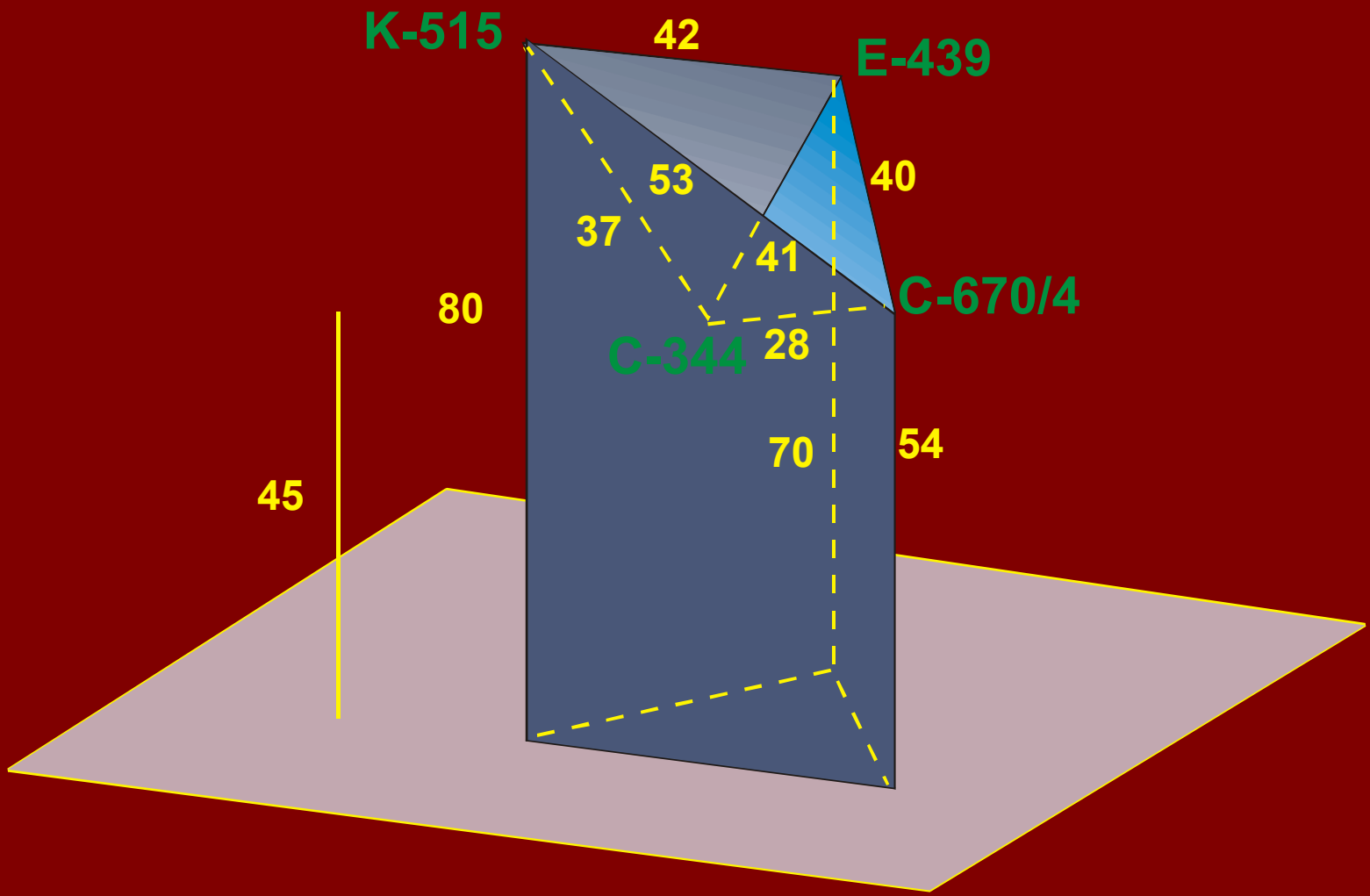
typically 50 Å


Energy Transfer IAEDANS to BrF
25
20
15
10
5
0
Intensity
400
450
500
550
Wavelength (nm)
BrF E439
40 Å
IAEDANS
C670, C674
IAEDANS-BrF
BrF
IAEDANS

IAEDANS-ATPase to FITC-PE

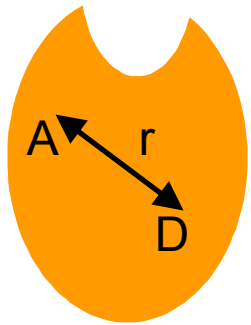


Fluorescence Energy Transfer

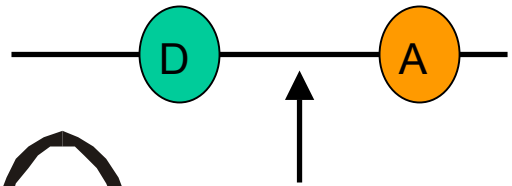


Uses of Fluorescence – Distance Measurements (cont.)

Measure distances on a protein

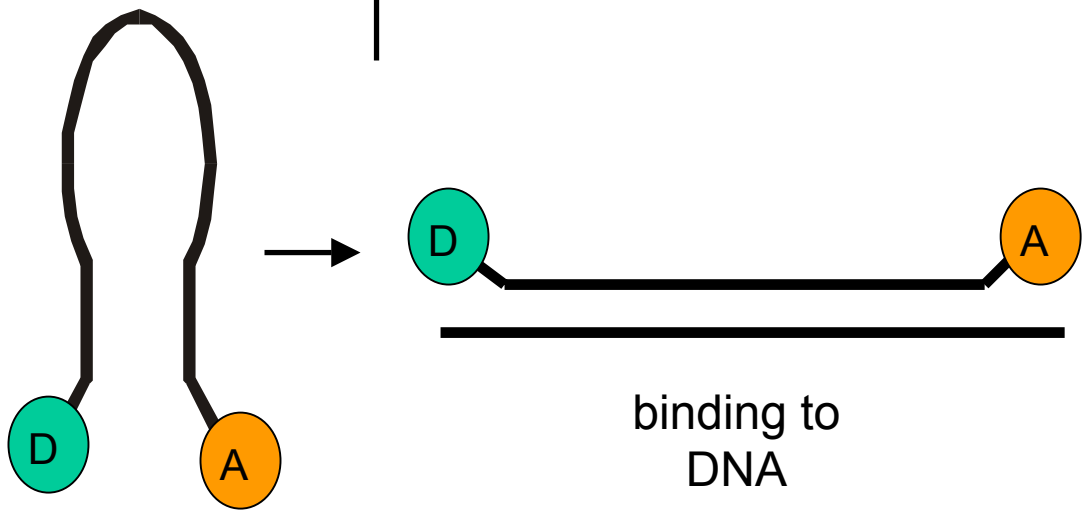


Peptide Hydrolysis



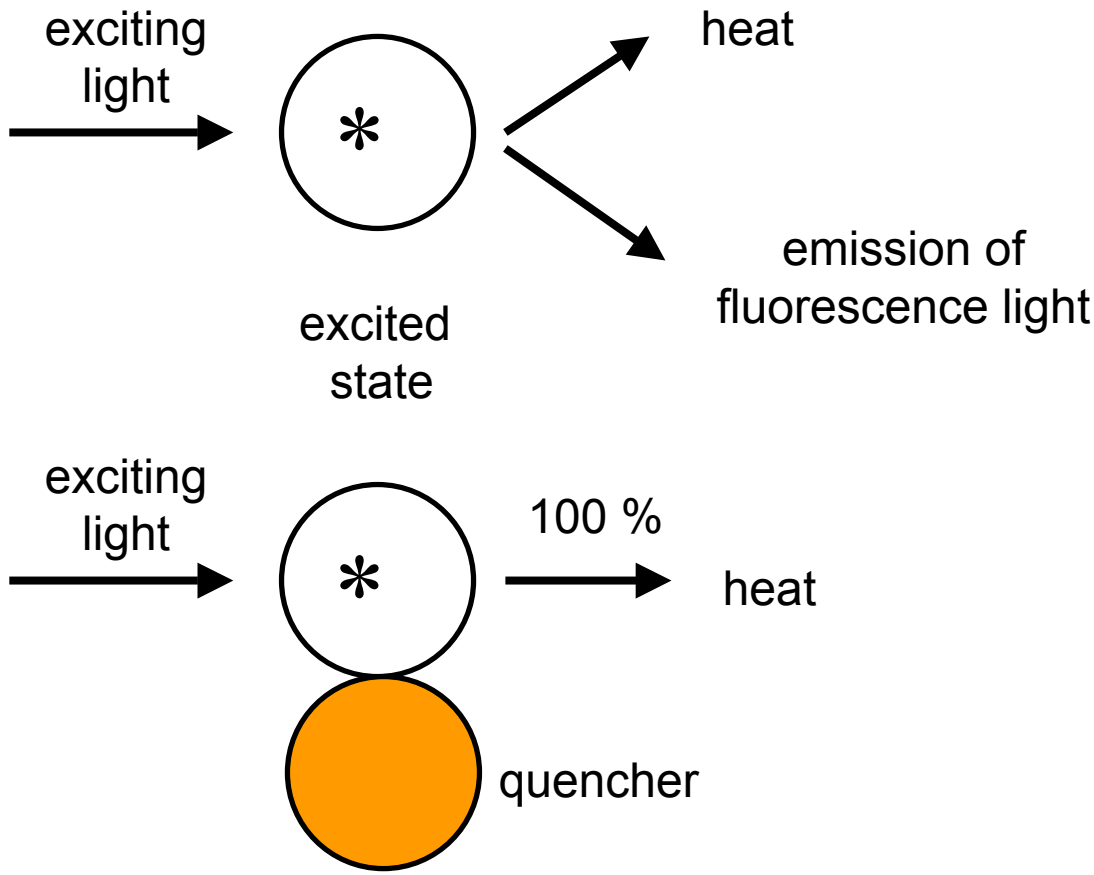
Molecular beacons

(DNA or RNA)



Uses of Fluorescence – Fluorescence quenching

Short range – requires contact between fluorophore and quencher



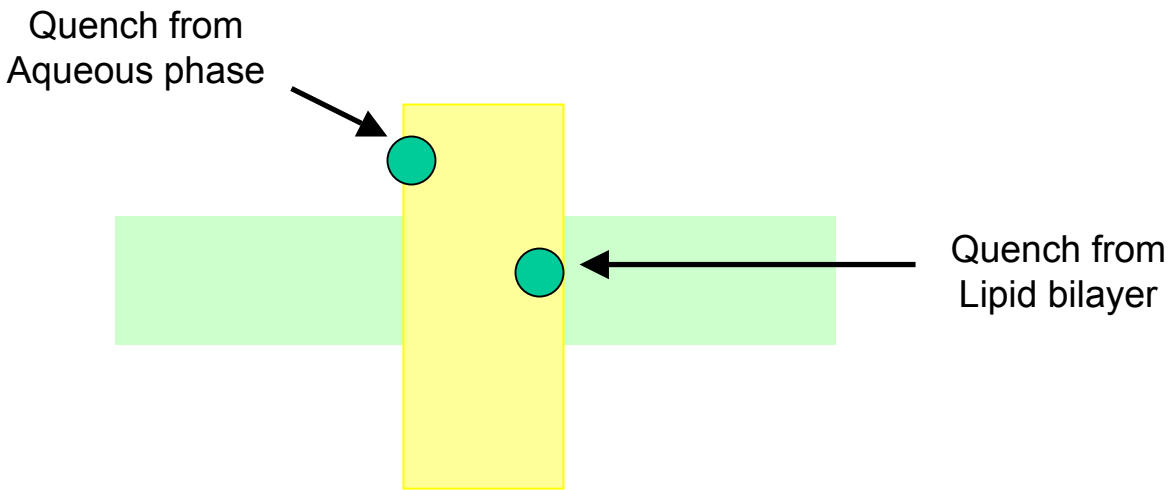
Uses of Fluorescence – Fluorescence quenching

Short range – requires contact between fluorophore and quencher

Water soluble – iodide (I^-), acrylamide, O_2

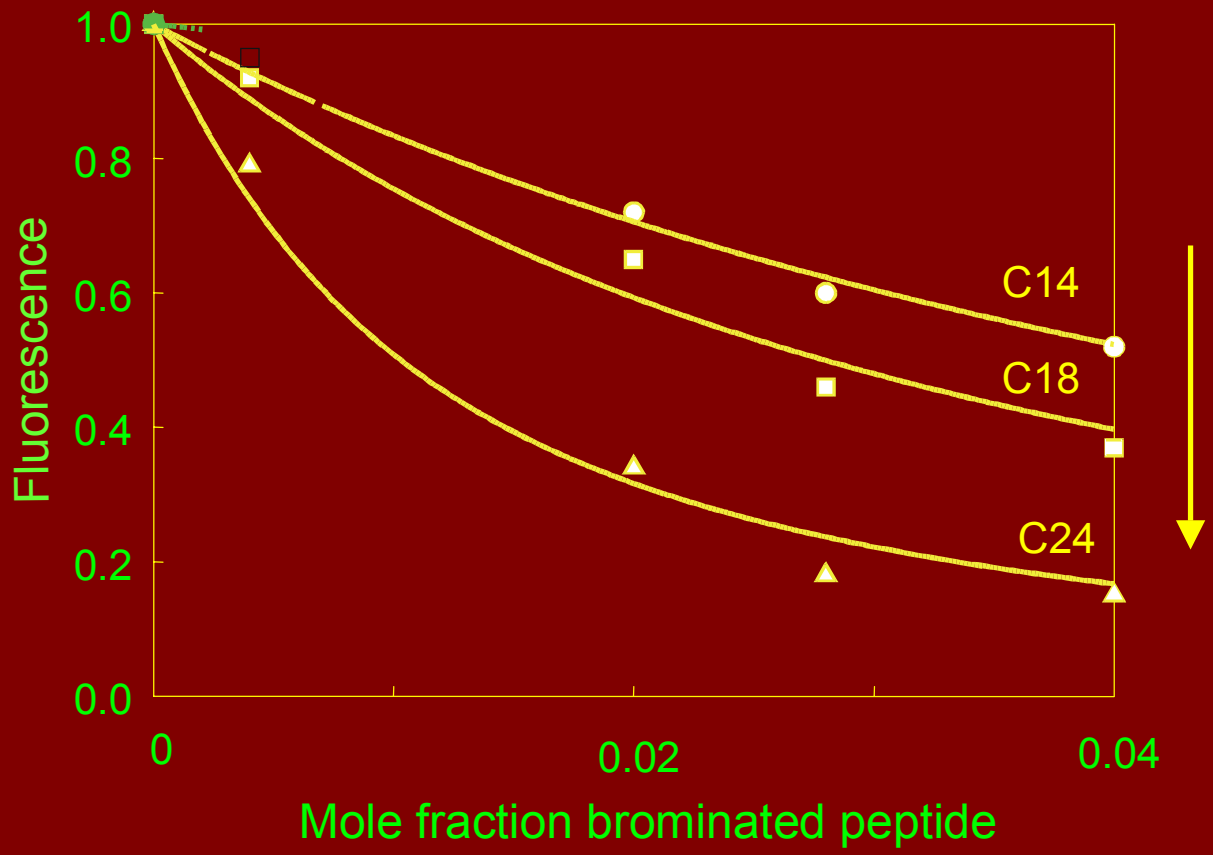
Hydrophobic – aliphatic bromides

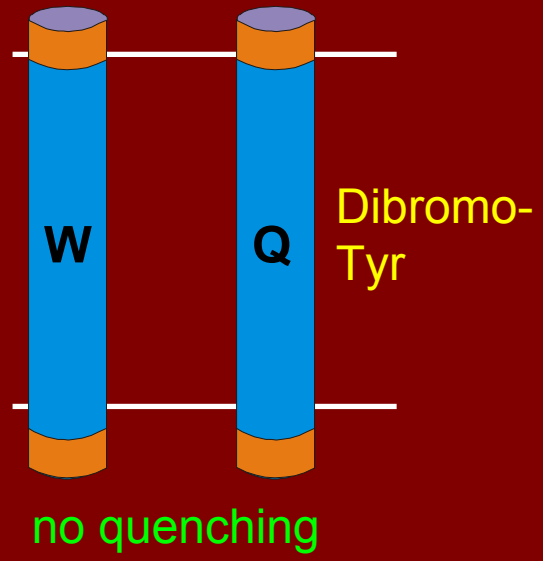
Use to test accessibility



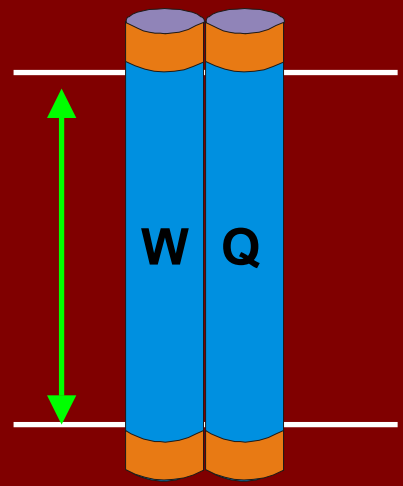
Trp containing peptide

Dibromo-tyrosine containing peptide





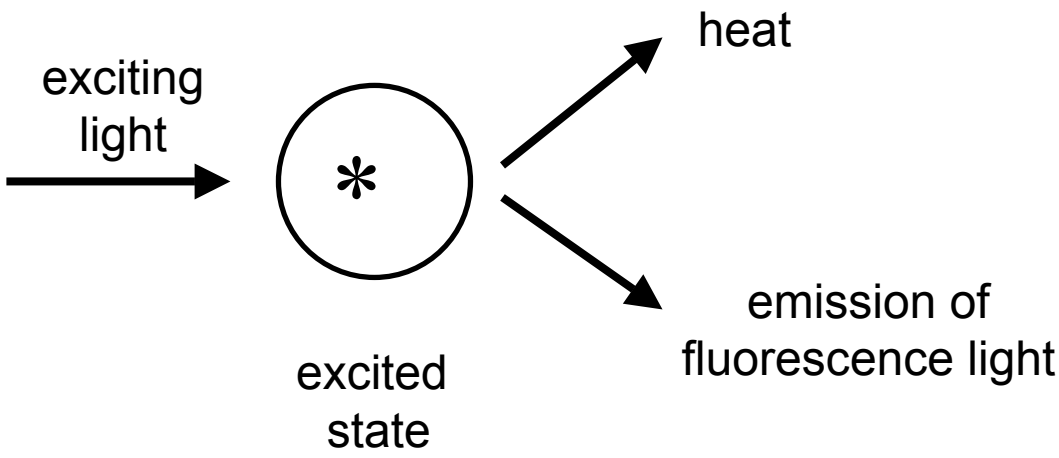
Thickness



Fluorescence

1. What is fluorescence
2. Technical issues
3. Uses of Fluorescence:
   - Quantitation
   - Ligand binding
   - Conformational changes
4. Measuring distances
5. Fluorescence quenching
6. Fluorescence lifetimes

Fluorescence lifetimes



Lifetime 1 –10 10^{-9} secs
(1-10 nsecs)

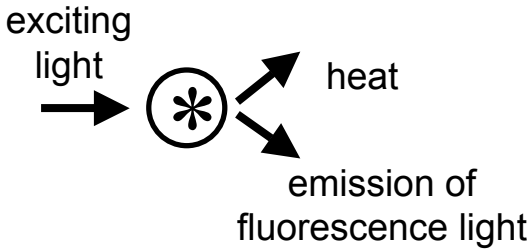
Measure using pulse methods

Excite fluorescence with a short pulse of light

Measure time between excitation and emission of a photon of light

(cont)

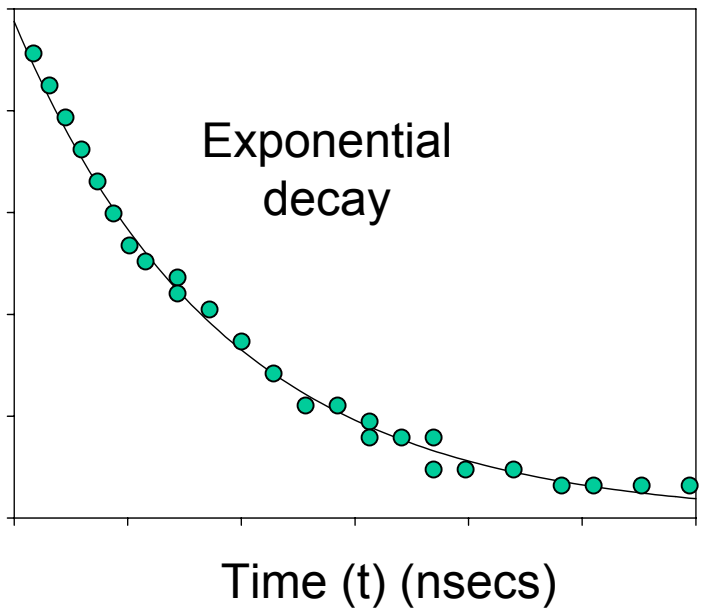
Fluorescence lifetimes (cont)



No. of photons emitted at time t N_t

$N_t = N_o \exp(-t/\tau)$

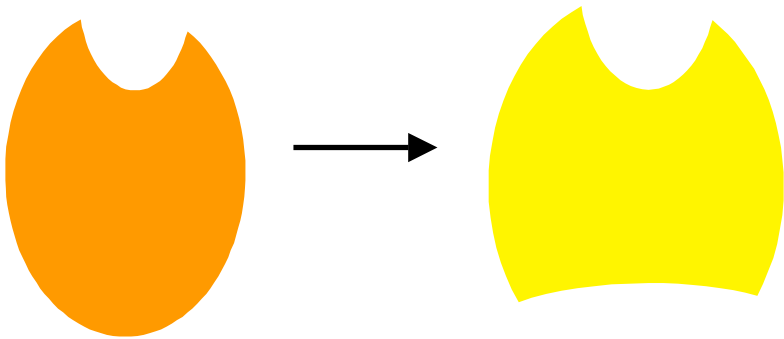
τ = fluorescence lifetime



Uses of Fluorescence lifetimes

Environmentally sensitive
protein changes etc.

Protein change



Intensity	F_1	F_2
Lifetime	τ_1	τ_2

Often:

$$\frac{F_1}{F_2} = \frac{\tau_1}{\tau_2}$$

Advantage of τ : this is an absolute measurement
Disadvantage of F ; this is in arbitrary units, and varies with amount of protein