Syllabus - Chem 728 - Physical Biochemistry

Tu	**2/2**	Overview - EM Radiation, Energies, Quantum nature of everything - Lec Notes - Video
Th	2/4	Electronic Spectra II - Transition dipole moments; symmetry; Einstein coeff; Boltzmann distrib - Lec Notes - Video - extra tutorial
Tu	2/9	Circular Dichroism (& Linear) - demo of polarization - Lec Notes - Video
Th	2/11	Fluorescence I - Jablonski Diagram, fundamentals - Lec Notes - Video - Quenching supplement
Tu	2/16	Fluorescence II - Lifetimes, quenching of all sorts - Lec Notes - Video
Th	2/18	Fluorescence III - Lifetimes, FRET (and dyes, beacons), anisotropy - Lec Notes - Video
Tu	2/23	Fluorescence IV - Fluorimeters, Anisotropy, Intro to single molecule - Lec Notes - Video
Th	2/25	**In-class Quiz**; Fluorescence V - TIRF, Laser traps - Lec Notes - Video
Tu	3/2	Single molecule (cont), AFM, Scattering - Lec Notes - Video
Th	3/4	Scattering, FLIM-FRET, Aniso imaging, Bit of R - Lec Notes - Video - DLS Long Video
Tu	3/9	Intro to **R** - be sure that your computer has **R** installed - Lec Notes (No video)
Th	3/11	Curve fitting - Lec Notes - Video - (big ugly R script)
Tu	3/16	R wrap up, Error, Sig Figs, Intro thermodynamics - Lec Notes - Video (partial)
Th	3/18	Ligand Binding - Lec Notes - Video - R script

Tu	3/23	Kinetics - 21st Century approaches - Lec Notes - Video - R script - Adv R script
Th	3/25	Hands on with R - simulating kinetics - Lec Notes - Video - R script - Inhib script
Tu	3/30	Cooperativity - Lec Notes - Video - Coop Review Article - Adv Coop @ Wikipedia!
Th	4/1	Isothermal Titration Calorimetry - Lec Notes - Video
Tu	4/6	Adv ITC & UV-Vis practicals - Lec Notes - Video - R script
Th	4/8	Surface Plasmon Resonance & Spectral Deconvolution - Lec Notes - Video - R script
Tu	4/13	Bio-Layer Interferometry / Thermophoresis - Lec Notes - Video
Th	4/15	SEC-MALS - ZetaSizer - Doppler DLS - Lec Notes - Video
Tu	4/20	Wednesday class schedule - No class
Th	4/22	Kinetics Workshop: [Transcr Term], [Transcr Inhib], [2ColorReporter] video
Tu	4/27	Guest Lecture - heliX dynamic biosensors - Video -
Th	4/29	**In-class Quiz**; extras
Tu	5/4	Summary Wrap Up - Lec Notes - Video

CHEM728 Physical Biochemistry Spring 2021

- Tu/Th 8:30a-9:45a Moodle-provided Zoom session
- Understanding chemical, physical, and biological properties of proteins and nucleic acids.
- 1) Thermodynamic and Kinetic behavior and experiment.
- 2) Spectroscopic tools for understanding structure (static and dynamic) and activity (binding, catalysis, etc) of biopolymers.
- The course will not (this year) include hands-on activities with, but will discuss in depth, the tools available in the Institute for Applied Life Sciences (IALS) Biophysical Characterization Facility.
- Instructor: Craig Martin (message through Moodle)
- Prerequisites: BIOCHEM 423, 523, or CHEM 423, and CHEM 471 or 475.
  ★ Or see Prof Martin

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Textbooks / Lecture Notes

Modern Biophysical Chemistry, 2nd edition
- by Walla, Peter Jomo
- ISBN: 9783527337736

Binding and Kinetics for Molecular Biologists
- by Goodrich, James A.; Kugel, Jennifer F. - Cold Spring Harbor Lab Press
- ISBN: 9781621820796
- Sadly, this book is out of print. Search eBay, perhaps?

Full lecture notes are available here in Moodle and should be sufficient for most of you.
Last year's notes are available here. This year's notes will be posted below as they become available (see Syllabus, below)

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R Statistical Package

Exams and homework/projects will require the use of the open source R statistical package, freely available for Windows and Macs. As you learn and explore, please post your experience to the R Moodle Forum for this course. Questions? Tips? Anything…

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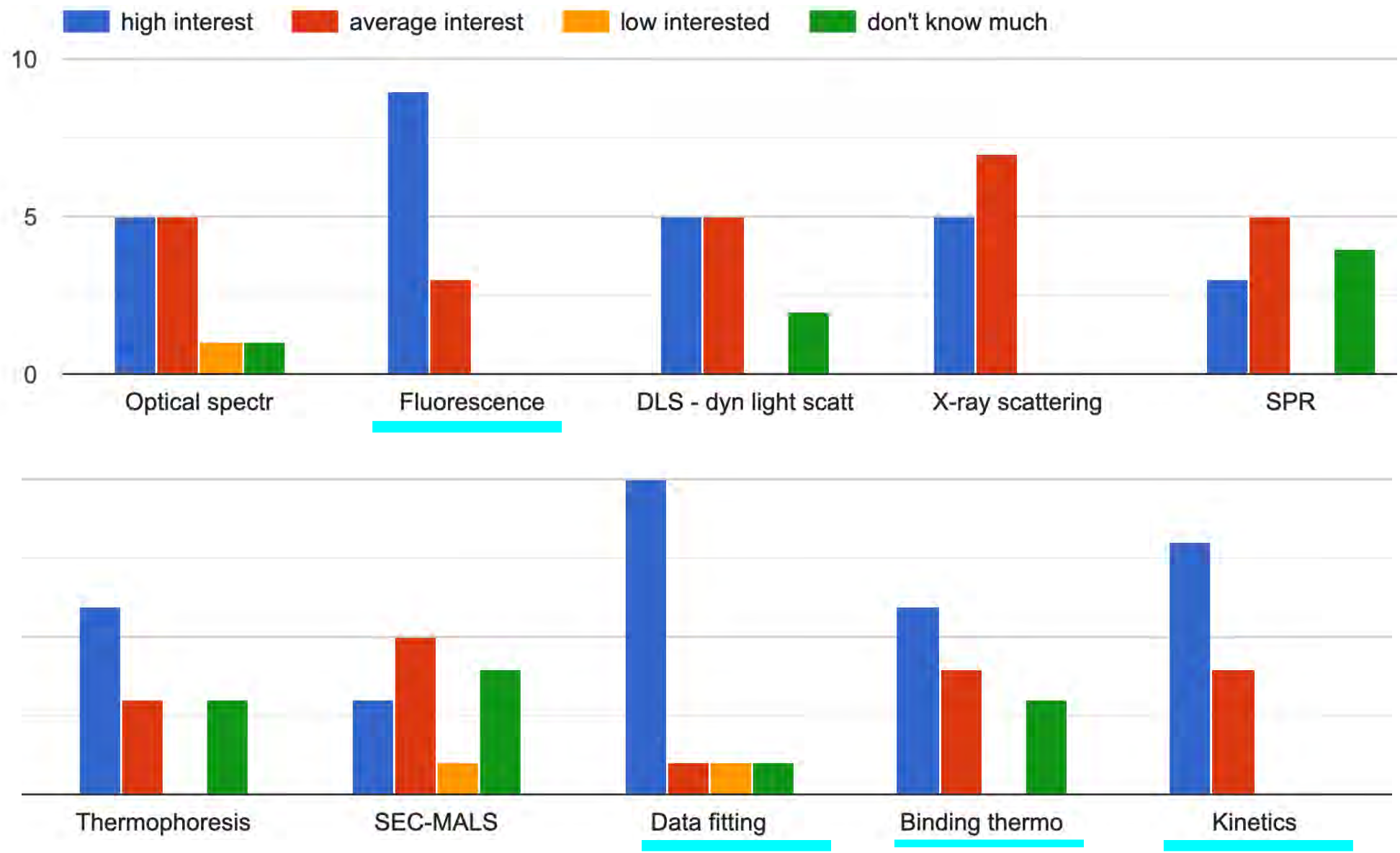
Grading

- 30% Take Home Exam 1
- 30% Take Home Exam 2
- 10% In-class Quizzes
- 20% Homework / Projects
- 10% Class participation, including in Moodle Forums (required)

Survey Results

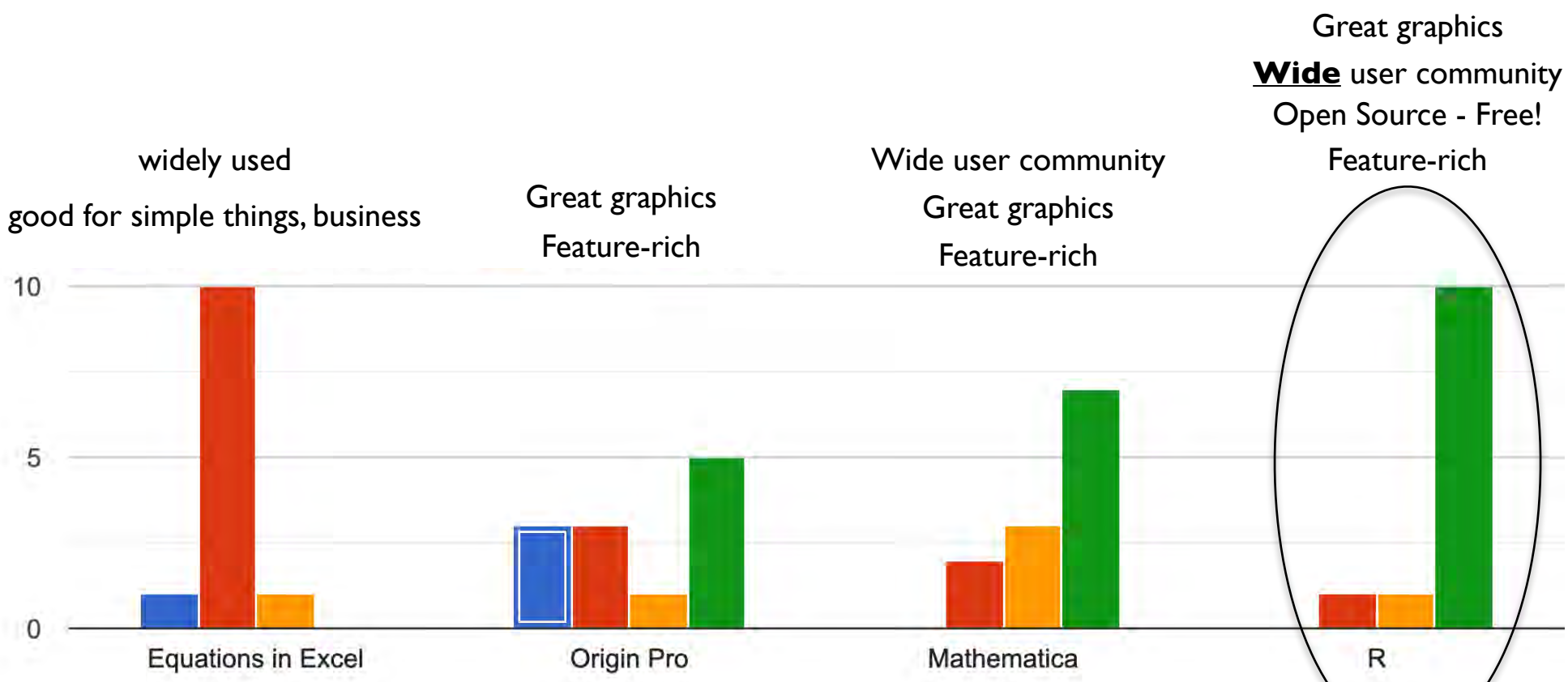
Spring 2021



Survey Results

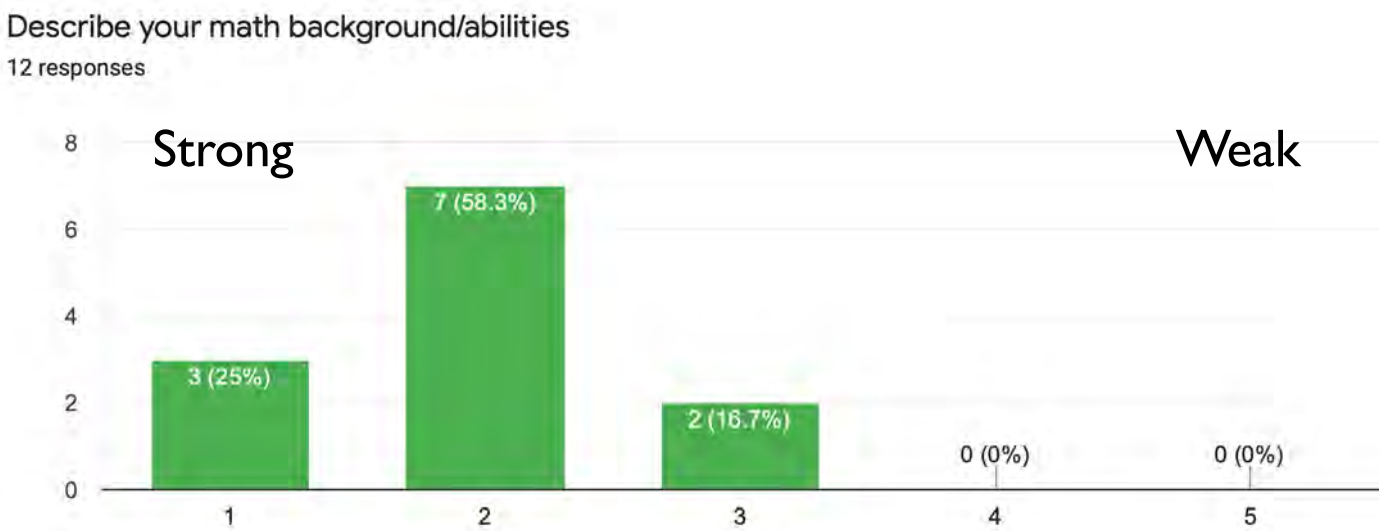
Spring 2021

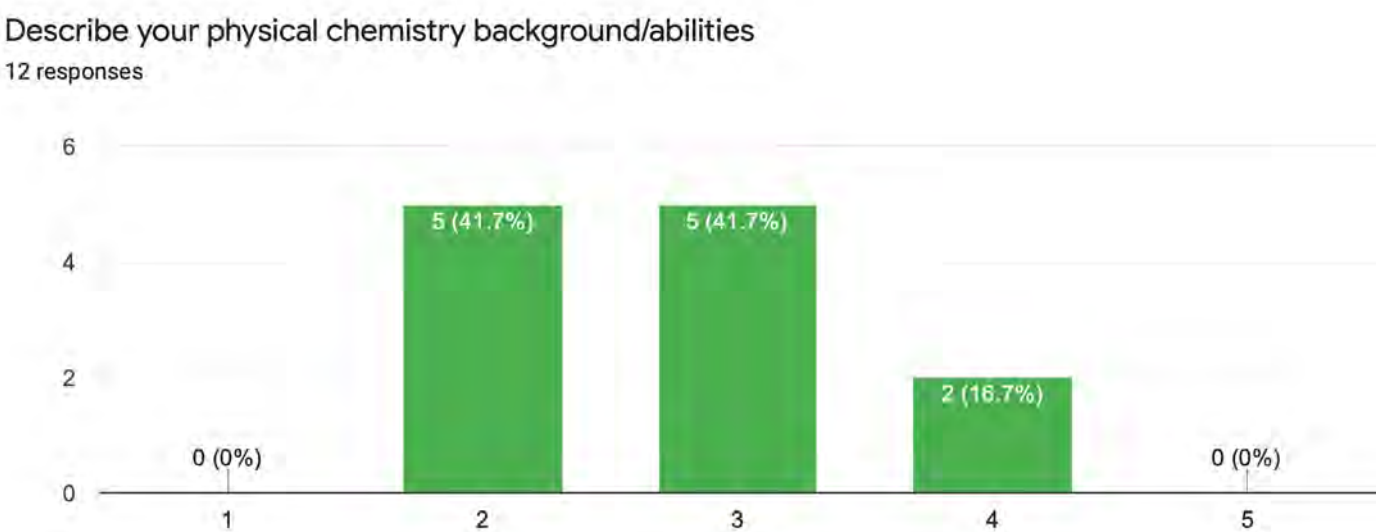
Describe your knowledge of the following software tools



Survey Results

Spring 2021

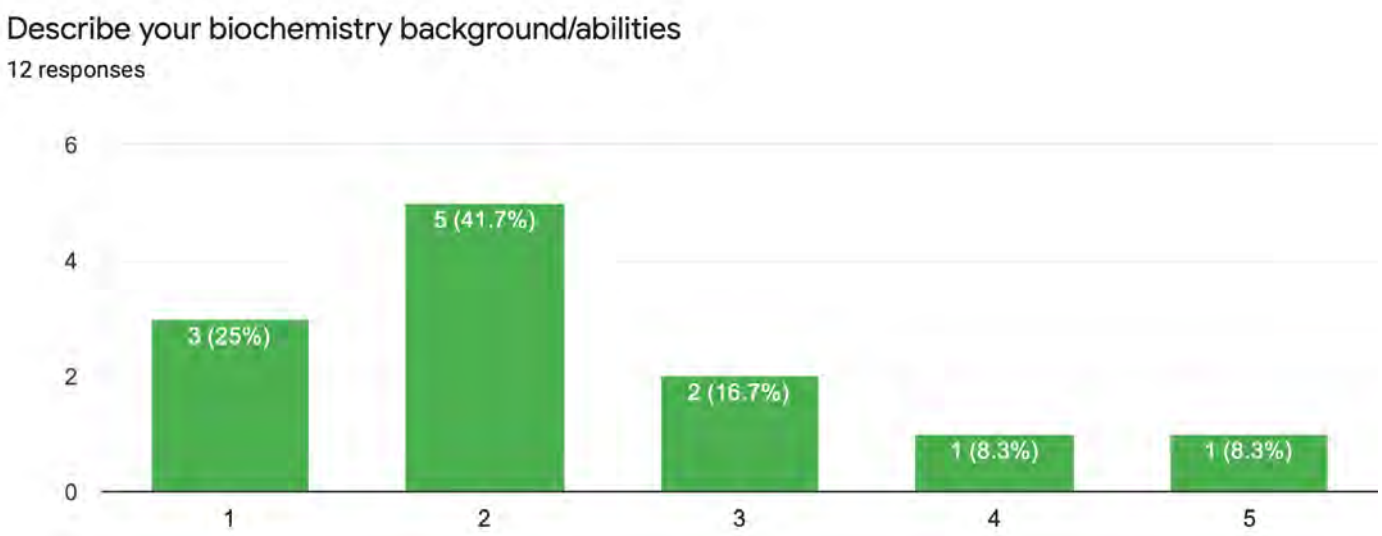




Survey Results

Spring 2021

Strong Weak



Survey Results

What else would you like to learn?

I wouldn't mind knowing more about crystallography and Cryo-EM

- Differential Scanning Calorimeter

- Basic ideas about curve fitting, Fourier transform, and spectral deconvolution

- Allosteric and cooperative binding

Some examples for real-life applications of the listed techniques would be nice.

Common mathematical methods to model molecular behavior.

N/A my main goal was to learn theory behind techniques I plan to use in my research.

Survey Results

Characteristics of a Great Course

Good slides and videos posted on the website to allow for looking back if technical difficulties occur

Access of relearning from available recordings

Creating a comfortable space for discussions

Discussion

Encouraging/opportunities for participation

Engaging

Lecture engagement

Interactive Clear presentation

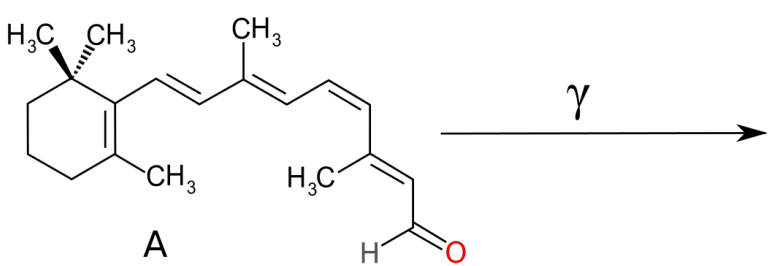
Quantum Nature of Matter and Energy

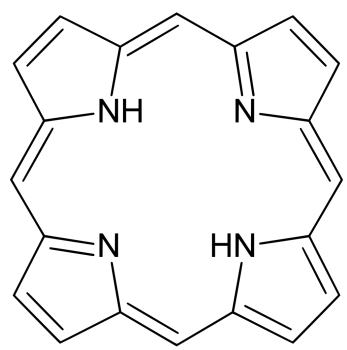
Feb 1, 2021

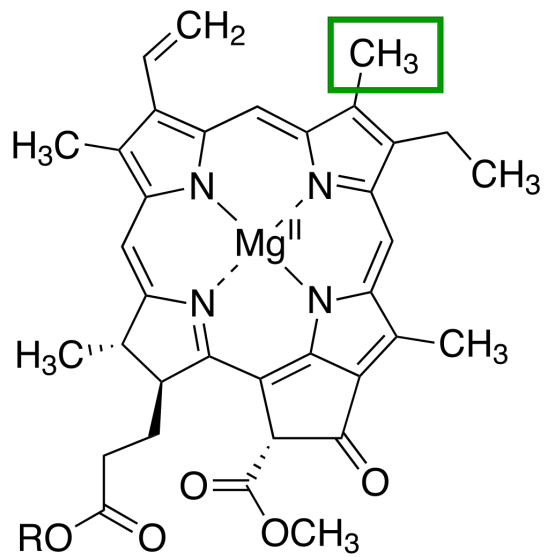
What is electromagnetic radiation?

Basics

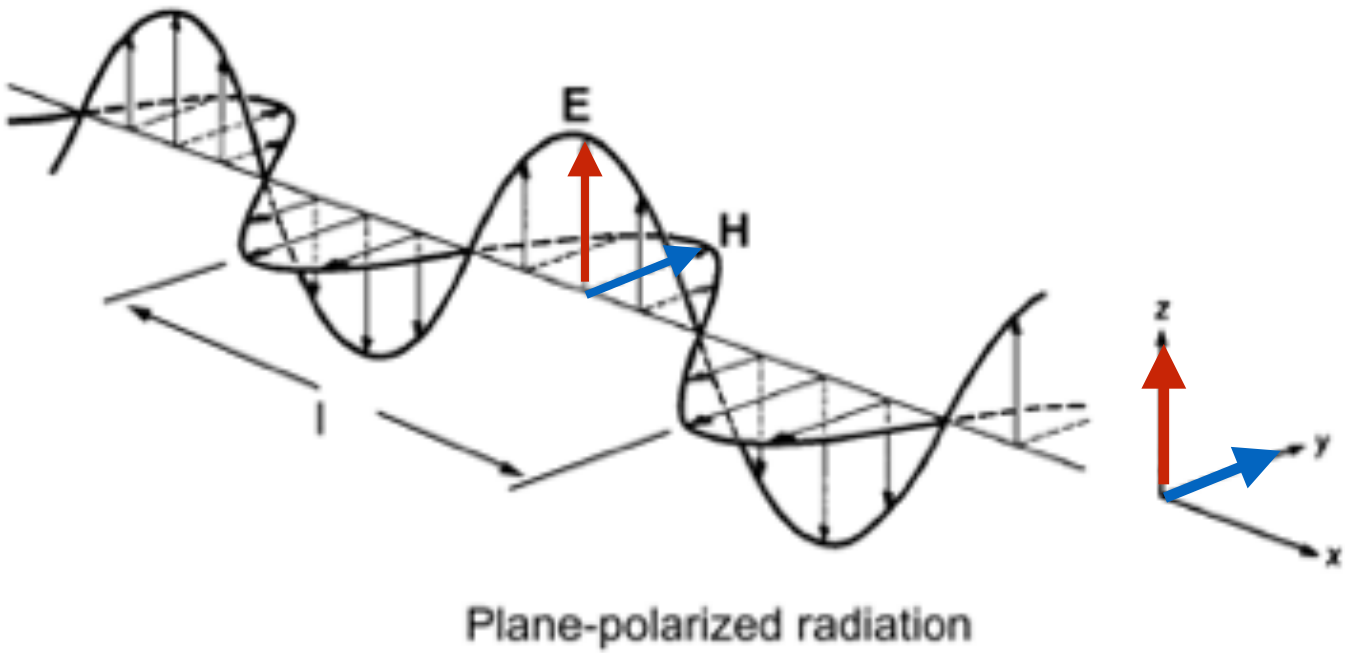
What spectroscopic difference(s) can you predict about these molecules?





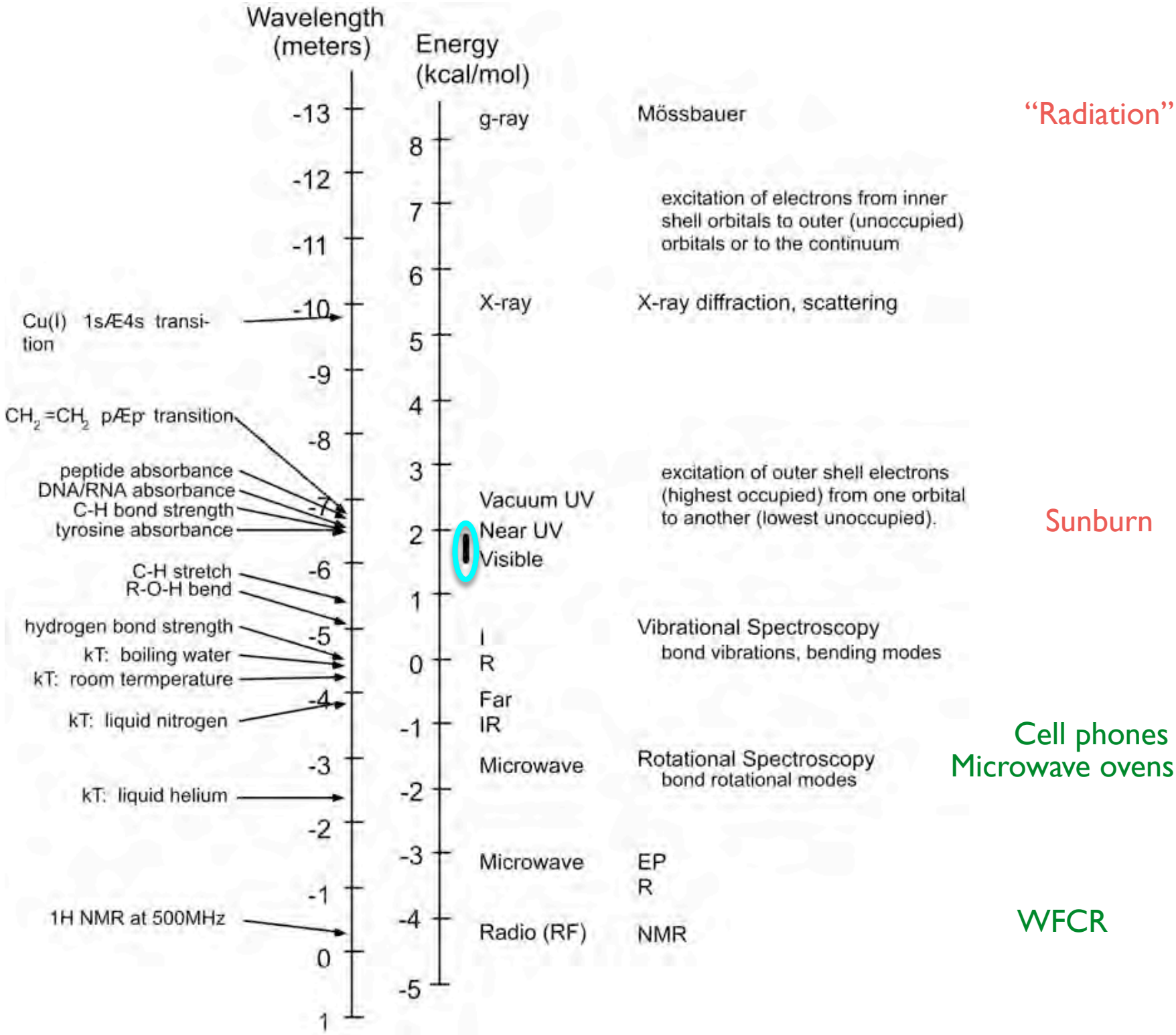


Electromagnetic Radiation



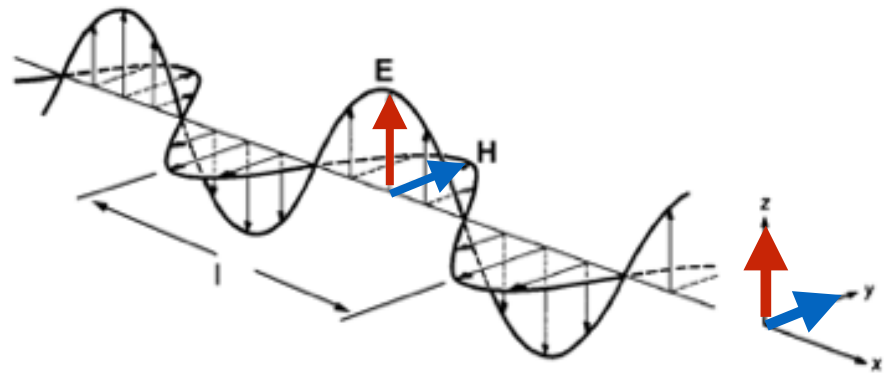
Plane-polarized radiation

$$E = h\nu = \frac{hc}{\lambda}$$

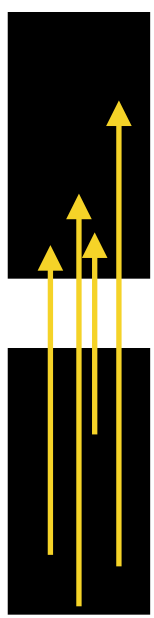

Wavelength (meters)
Energy (kcal/mol)
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
0
1
8
7
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
g-ray
X-ray
Vacuum UV
Near UV
Visible
I
R
Far
IR
Microwave
Microwave
Radio (RF)
Mössbauer
excitation of electrons from inner shell orbitals to outer (unoccupied) orbitals or to the continuum
X-ray diffraction, scattering
excitation of outer shell electrons (highest occupied) from one orbital to another (lowest unoccupied).
Vibrational Spectroscopy
bond vibrations, bending modes
Rotational Spectroscopy
bond rotational modes
EP
R
NMR
Cu(I) 1s/E4s transition
CH2=CH2 pÆp* transition
peptide absorbance
DNA/RNA absorbance
C-H bond strength
tyrosine absorbance
C-H stretch
R-O-H bend
hydrogen bond strength
kT: boiling water
kT: room termperature
kT: liquid nitrogen
kT: liquid helium
1H NMR at 500MHz
"Radiation"
Sunburn
Cell phones
Microwave ovens
WFCR

Electromagnetic Radiation

$$E = h\nu = \frac{hc}{\lambda}$$

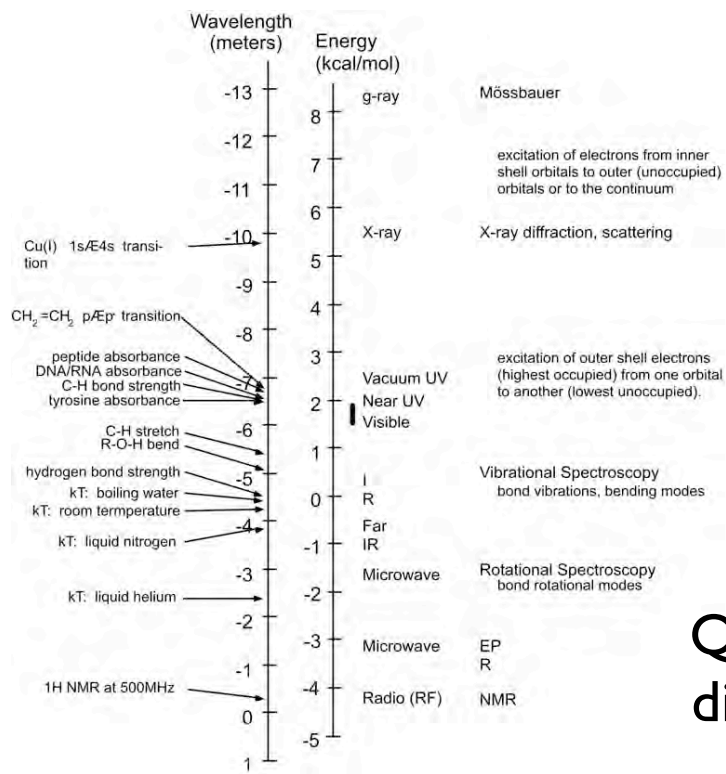


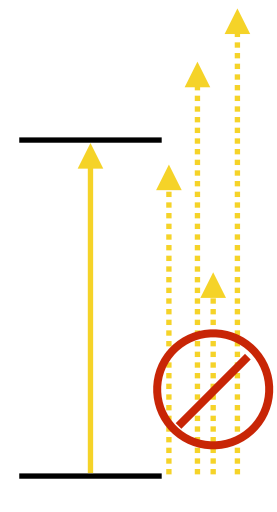
Plane-polarized radiation



$E_{excited}$

E_{ground}





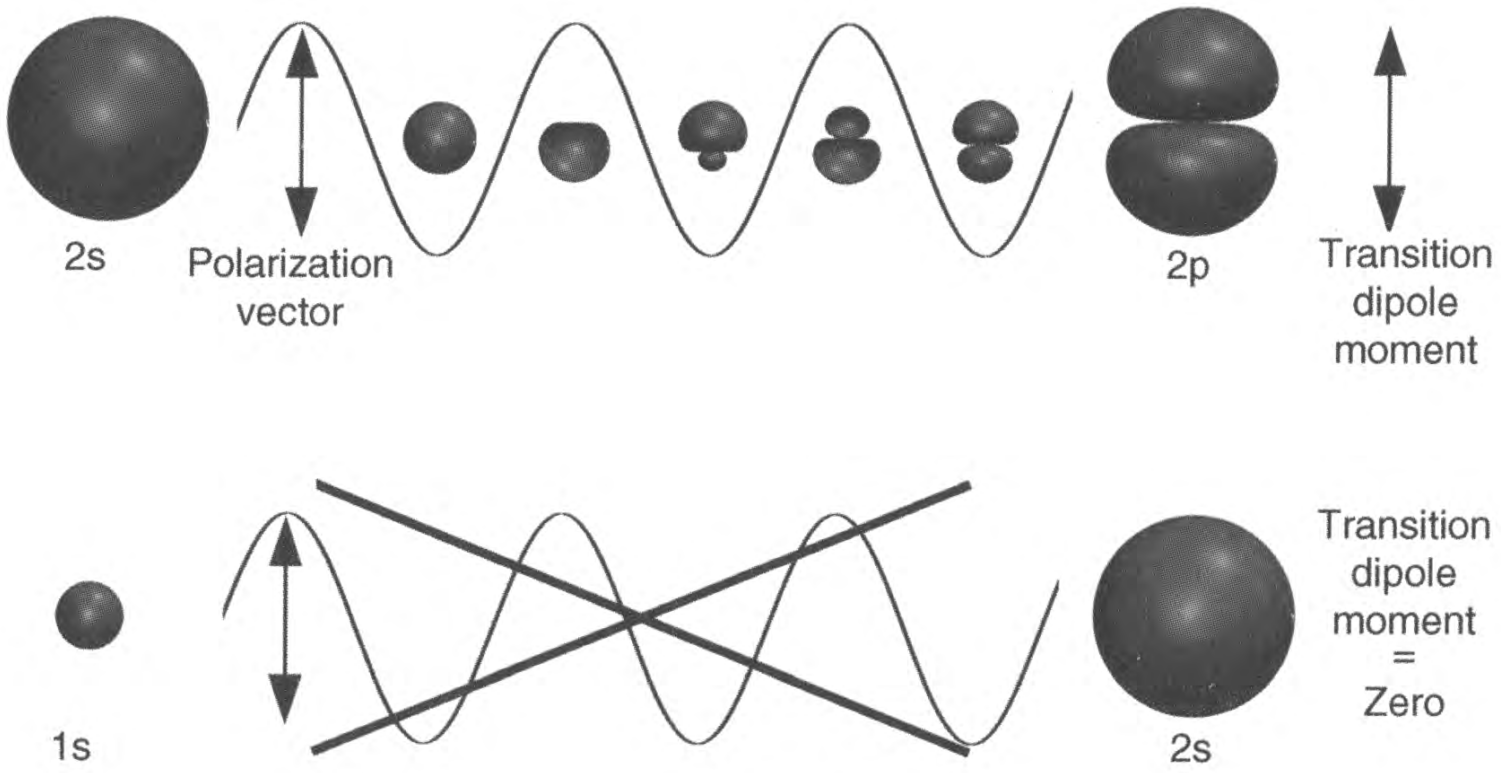
$E_{excited}$

E_{ground}

Classical: electrons can have any energy

QM: electrons can have only discrete (quantized) energies

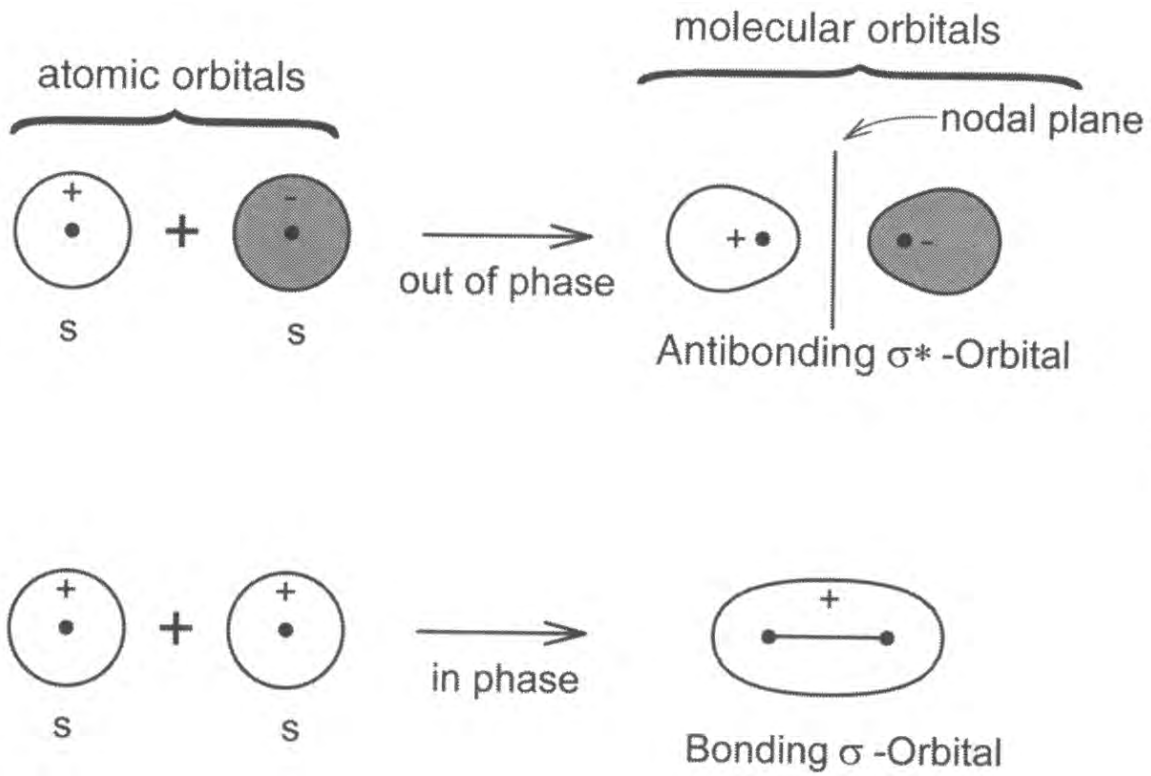
Transition Dipole Moment

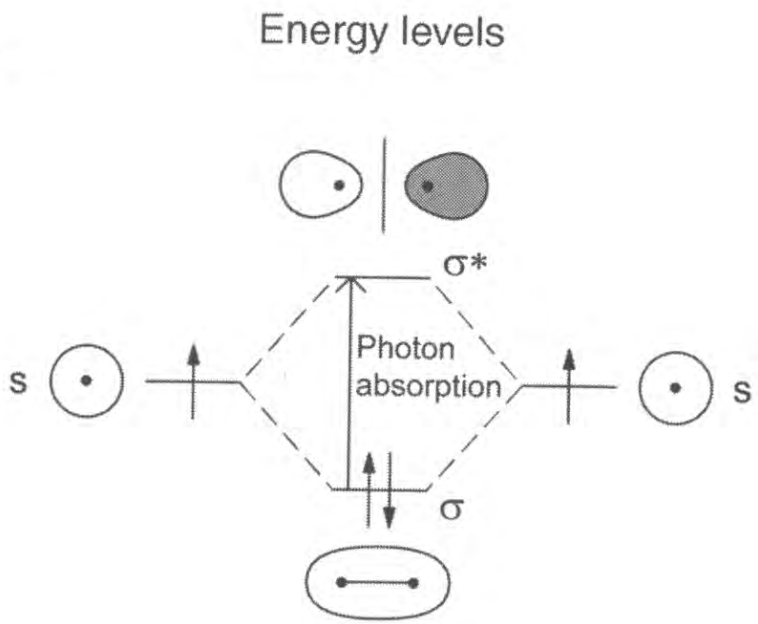


Why can't a photon excite 1s to 2s?

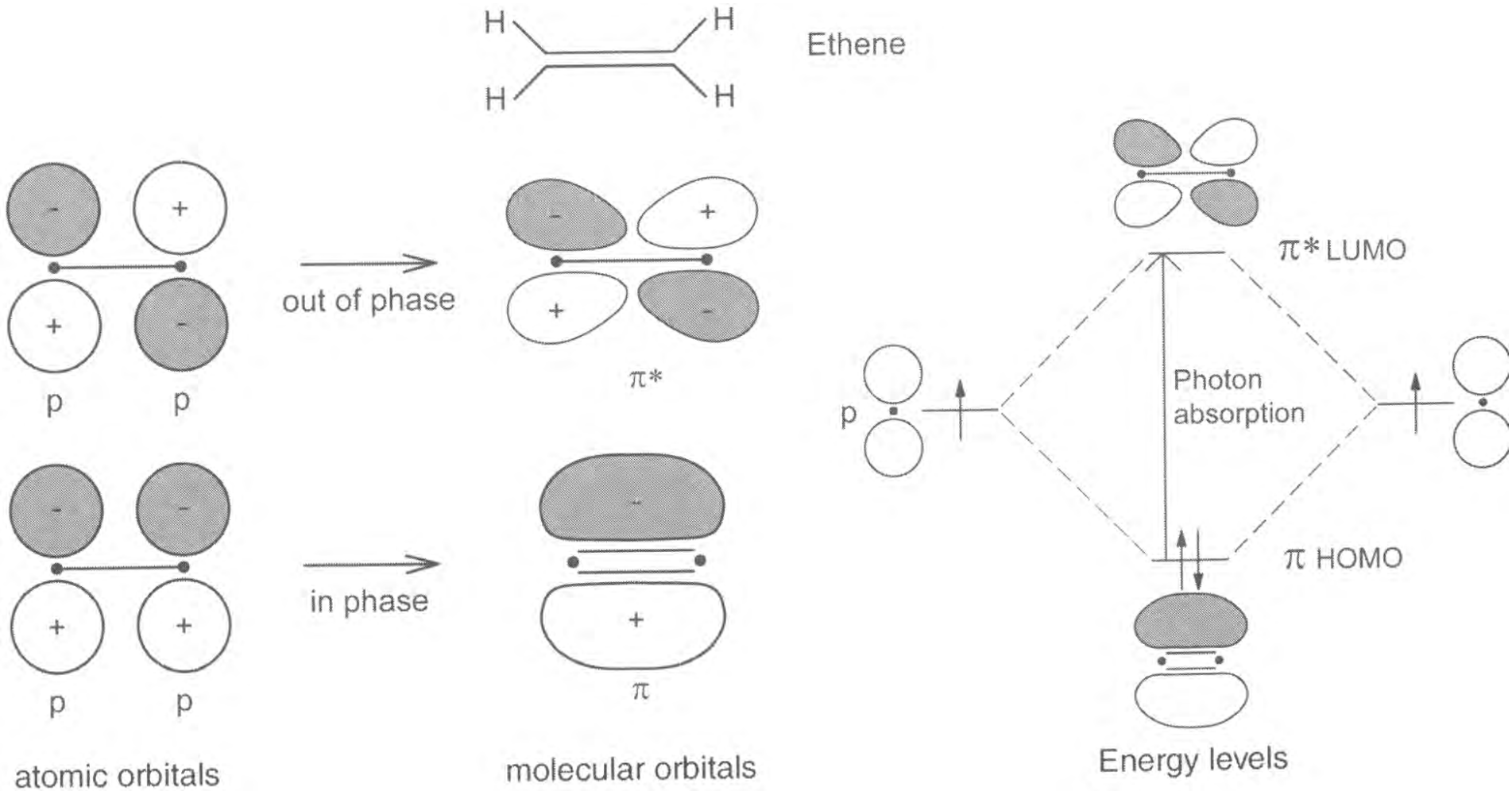
Can three perpendicular photons excite 1s to 2s? Why not?

Transition Dipole Moment

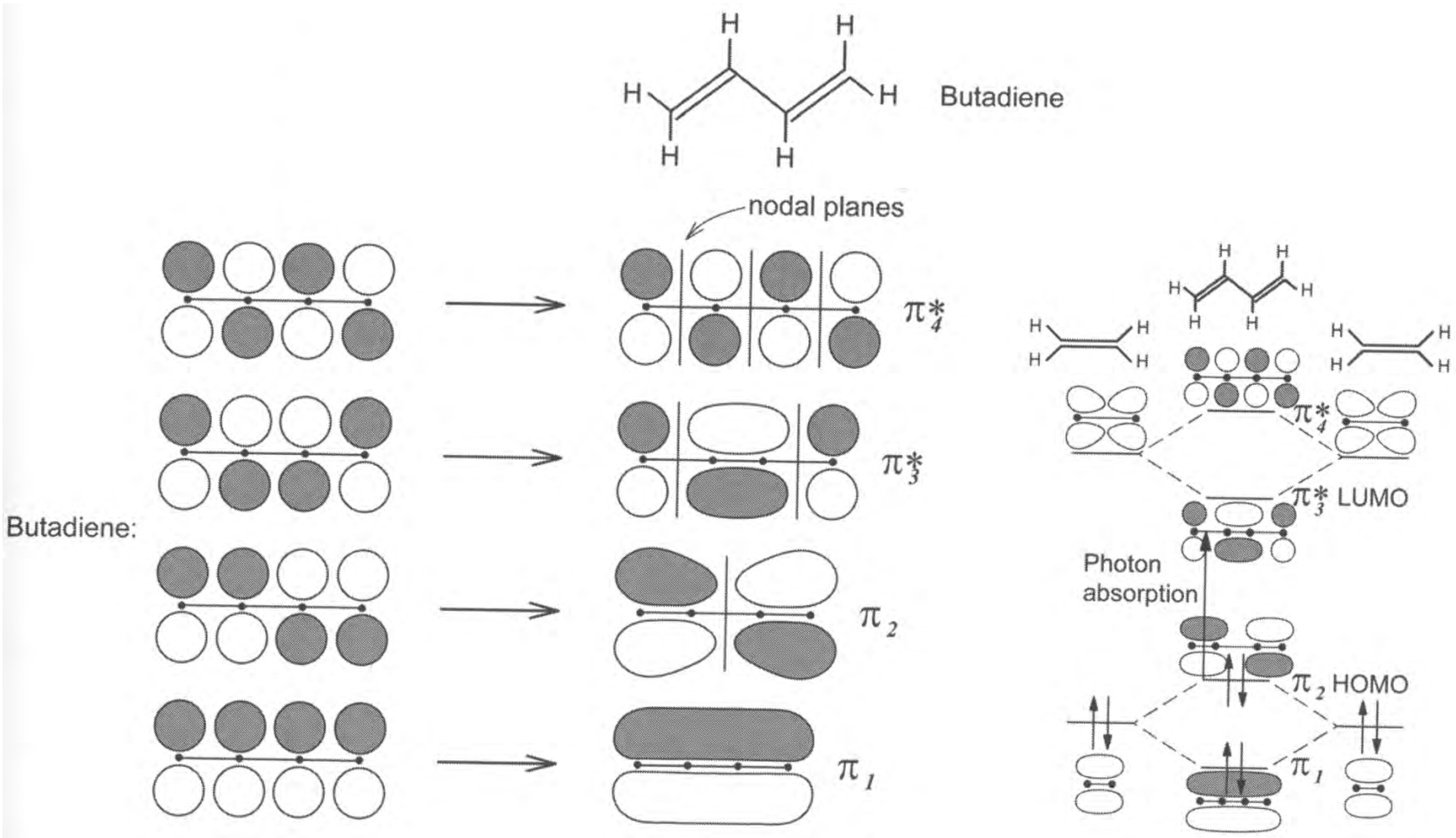




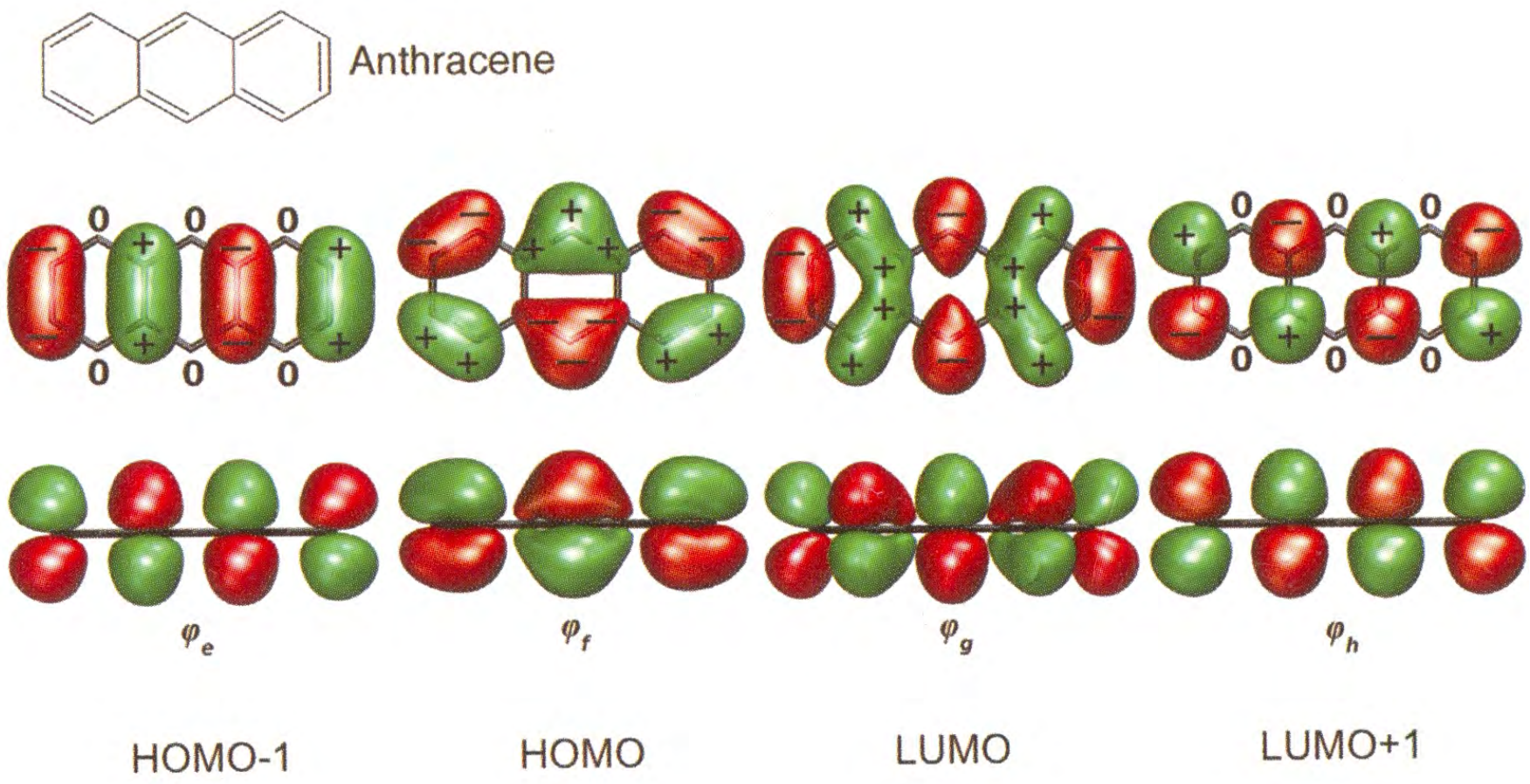
Transition Dipole Moment



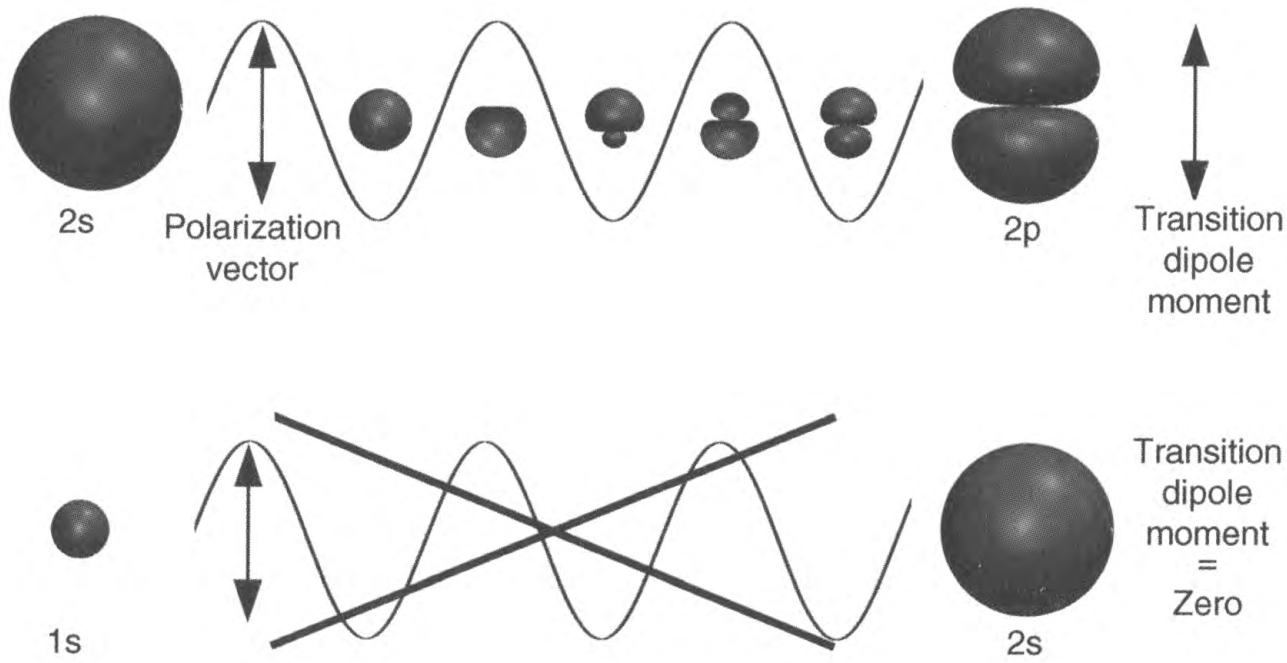
Transition Dipole Moment



Transition Dipole Moment



Transition Dipole Moment



$$\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\psi^2\,\partial x\,\partial y\,\partial z$$

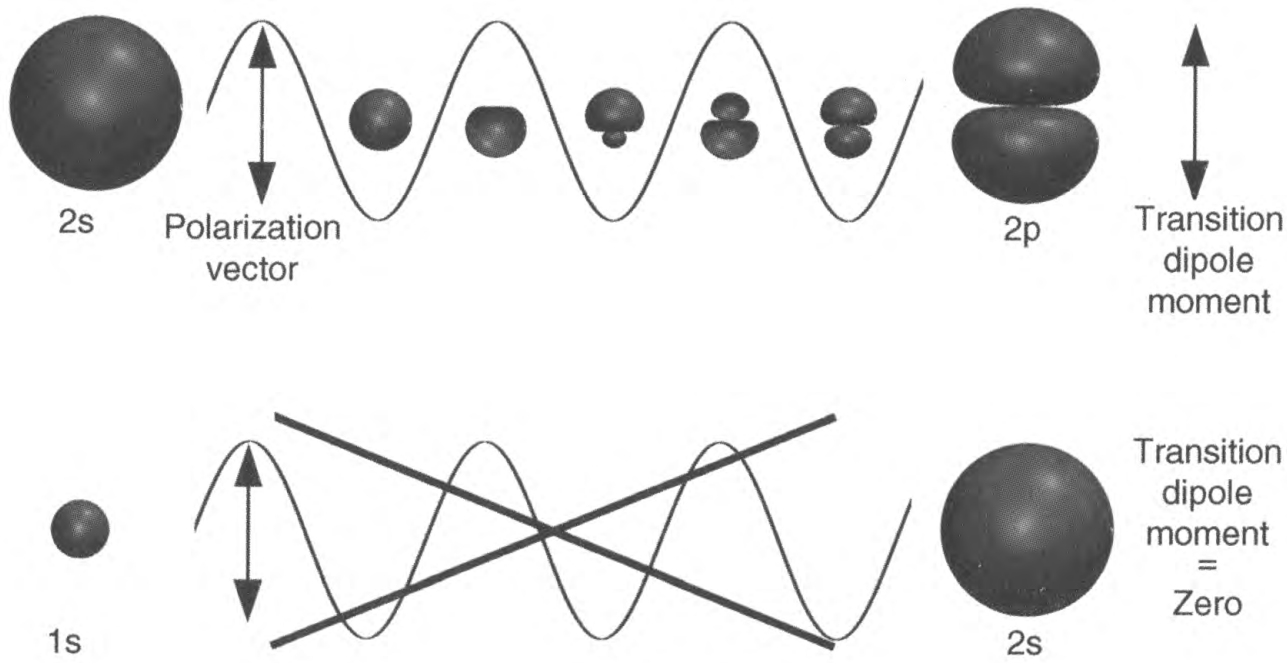
Operator

Final orbital

Initial orbital

$$\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\psi_2\bar{\mu}\psi_1\,\partial x\,\partial y\,\partial z$$

Transition Dipole Moment



$$\left(\int_{-\infty}^{+\infty}\psi_2\bar{\mu}_x\psi_1\,\partial x\right)\left(\int_{-\infty}^{+\infty}\psi_2\bar{\mu}_y\psi_1\,\partial x\right)\left(\int_{-\infty}^{+\infty}\psi_2\bar{\mu}_z\psi_1\,\partial x\right)$$

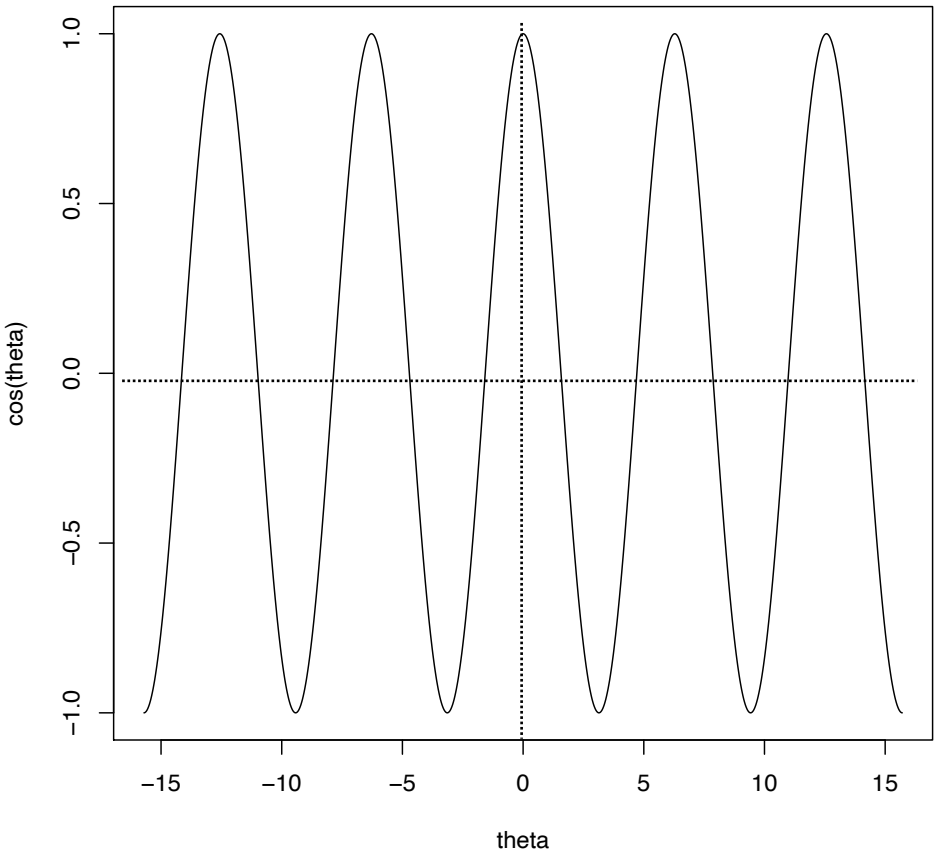
$$\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\psi_2\bar{\mu}\psi_1\,\partial x\,\partial y\,\partial z$$

Easy Calculus

Integration is Area Under the Curve

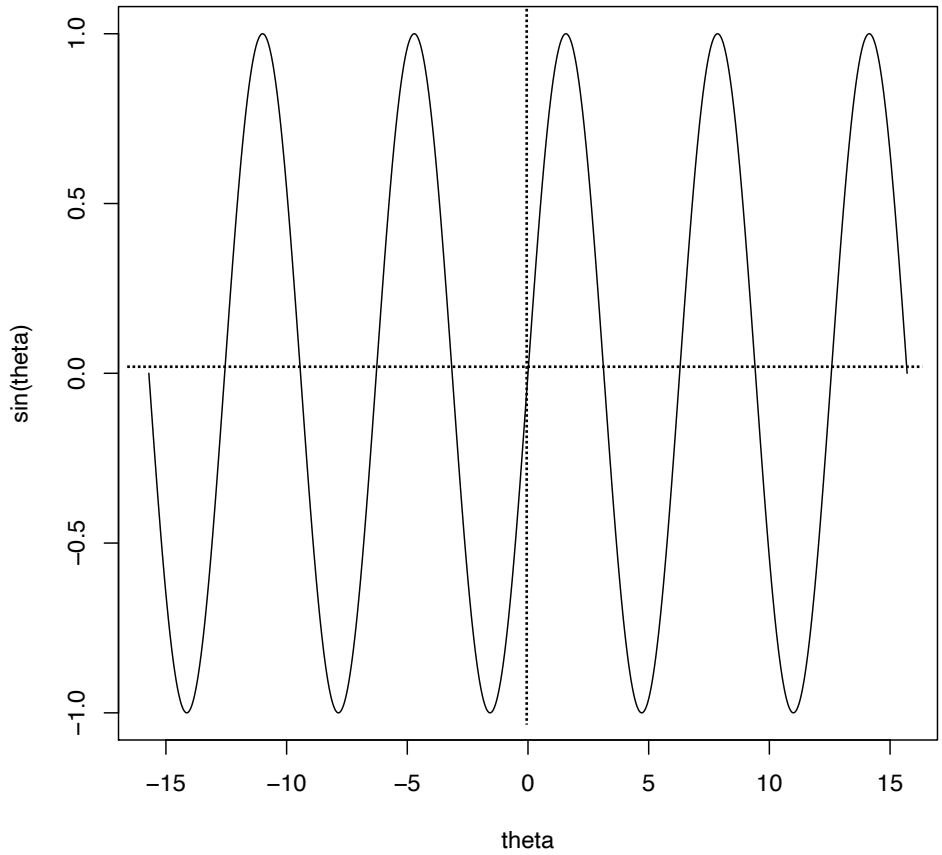
Cos

Even



Sin

Odd



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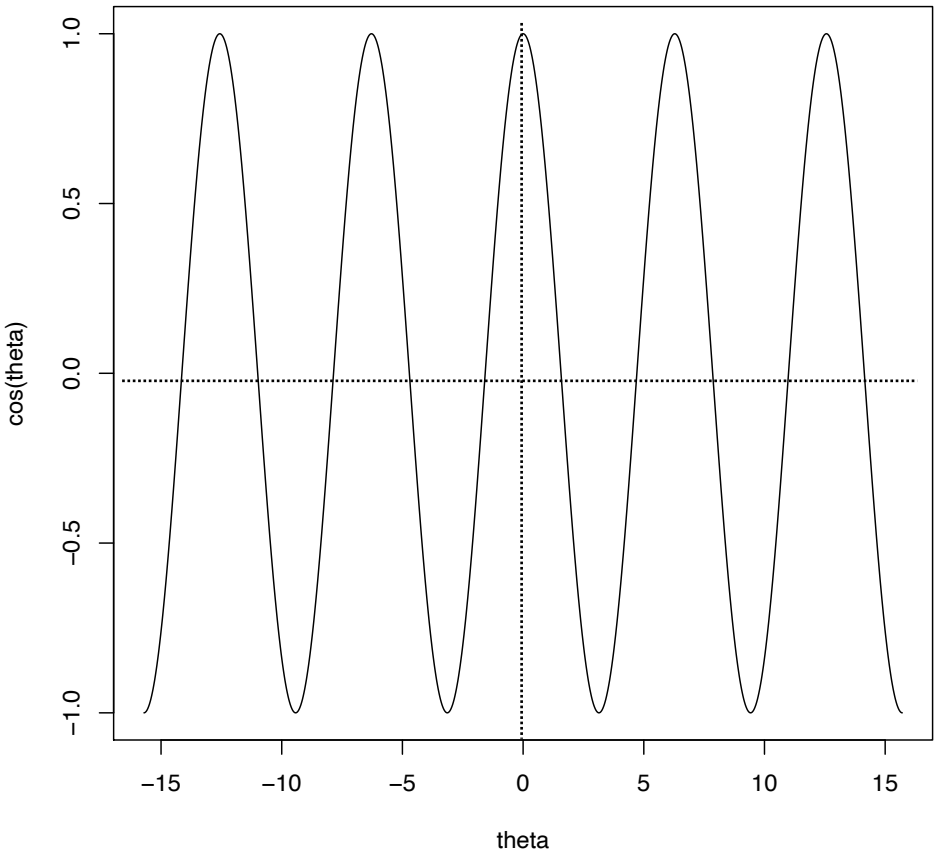

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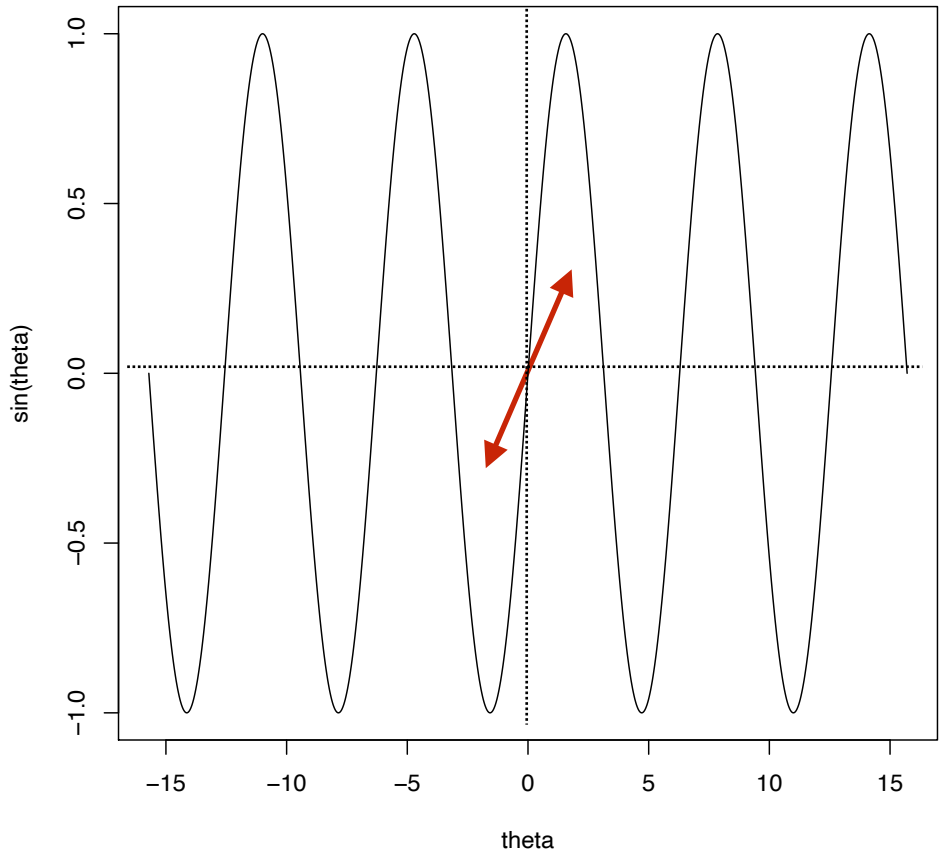

Easy Calculus

Integration is Area Under the Curve

Cos

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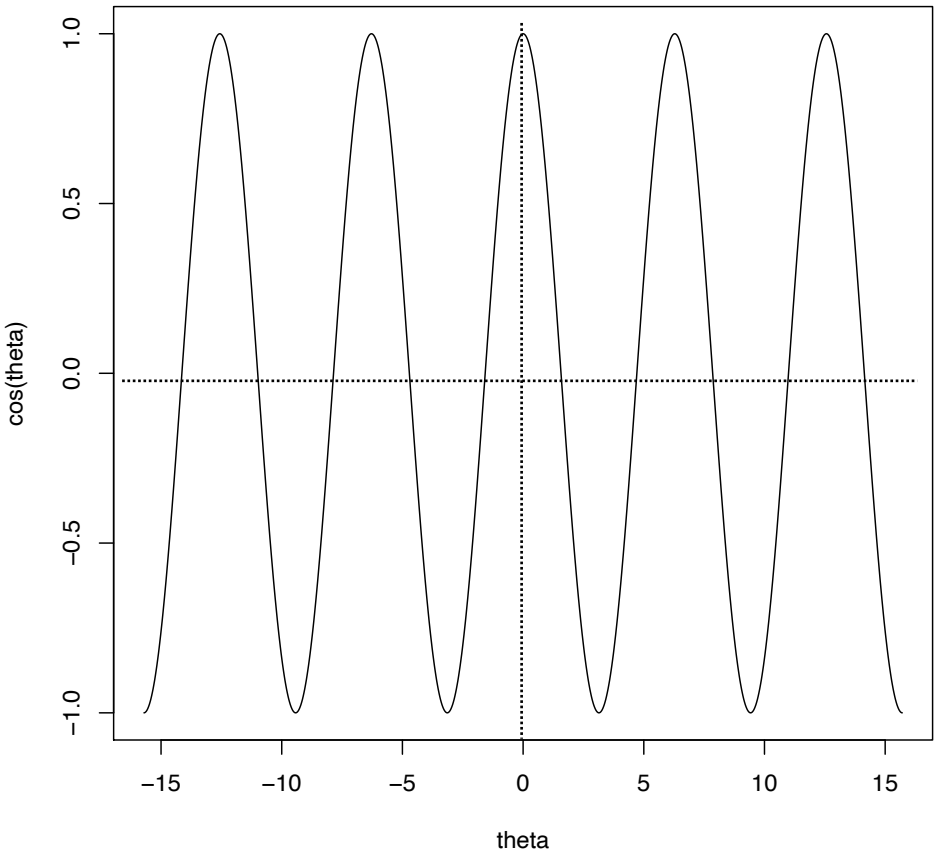

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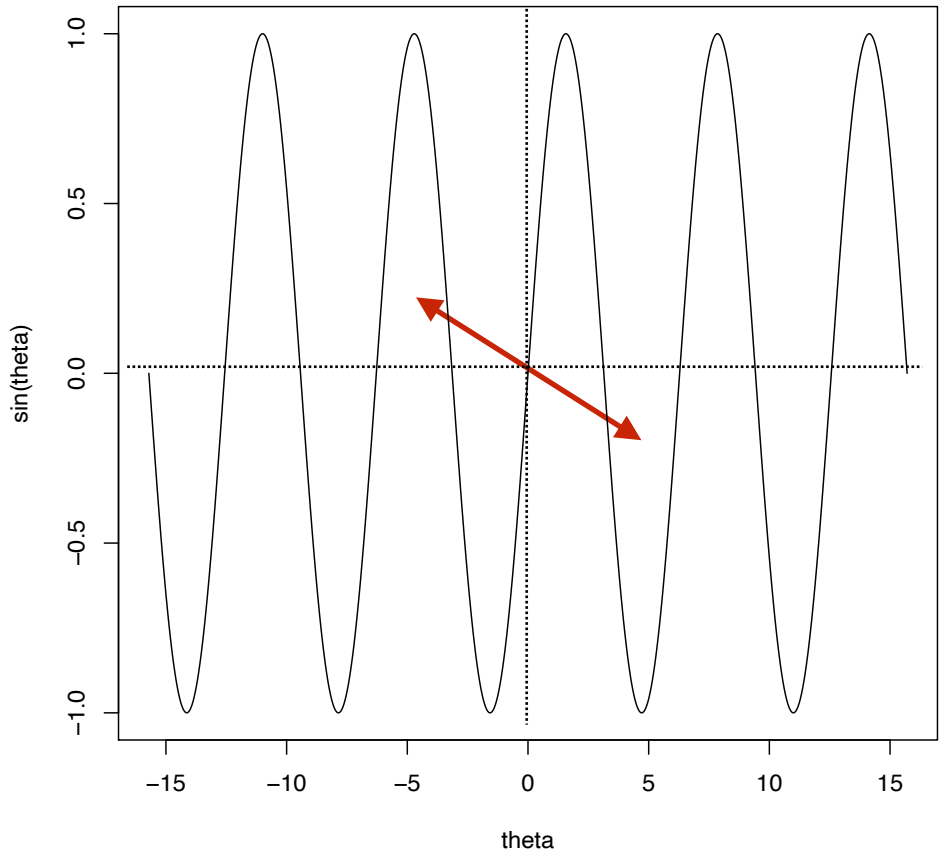

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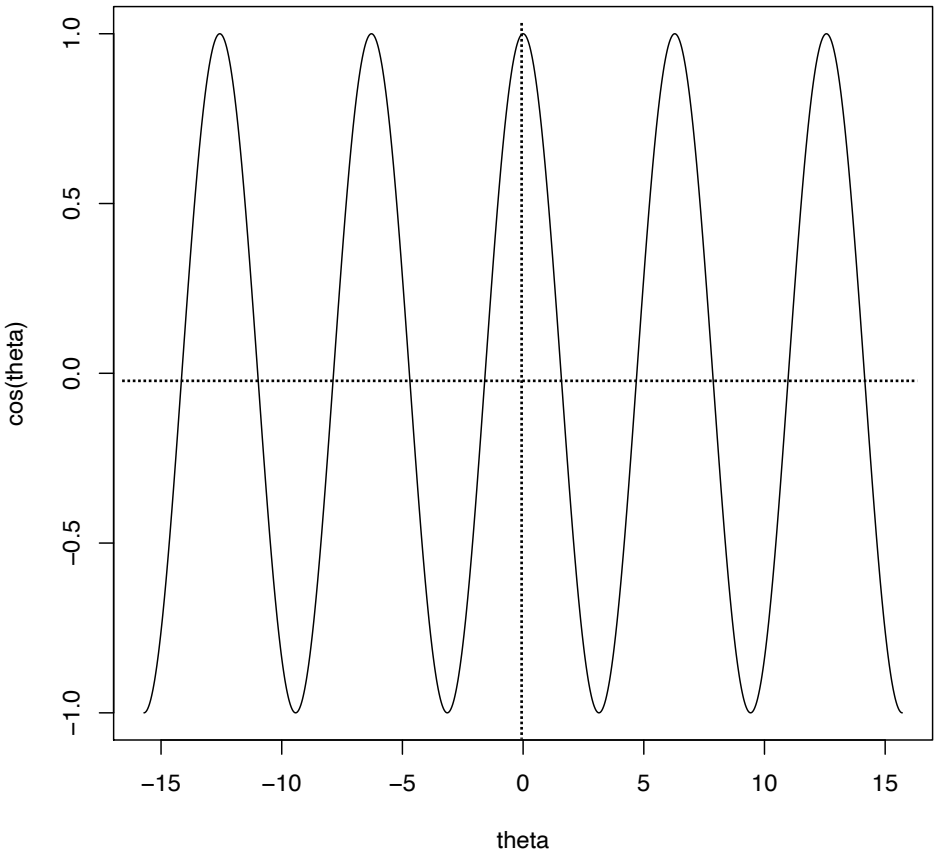

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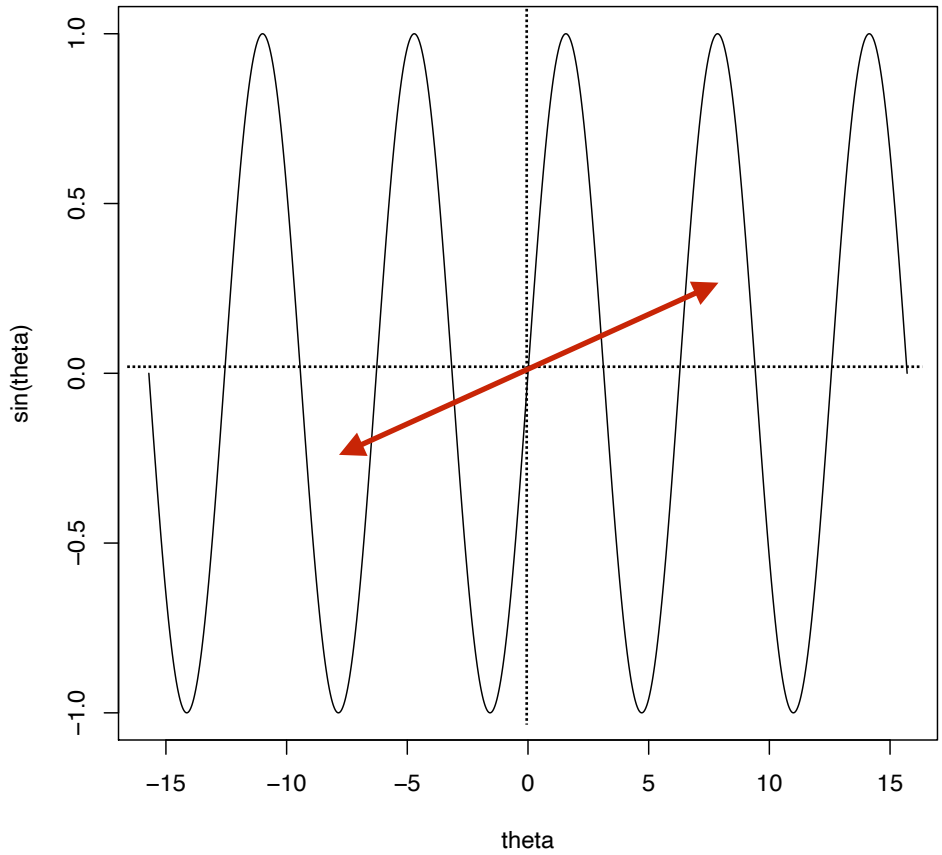

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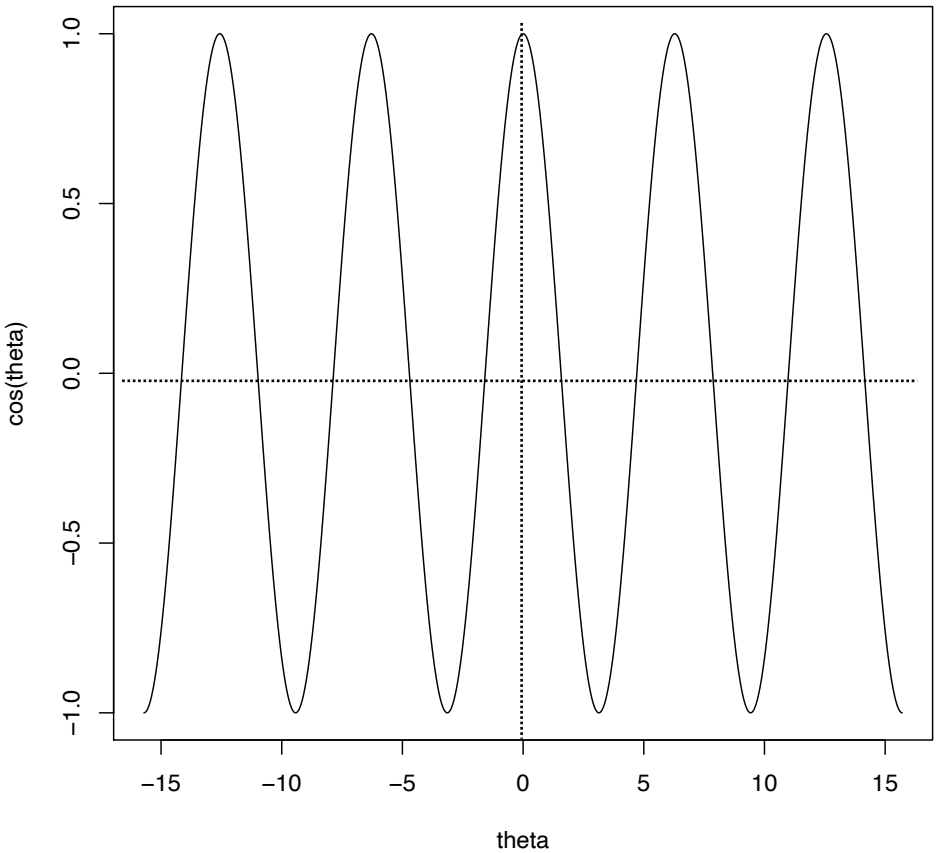

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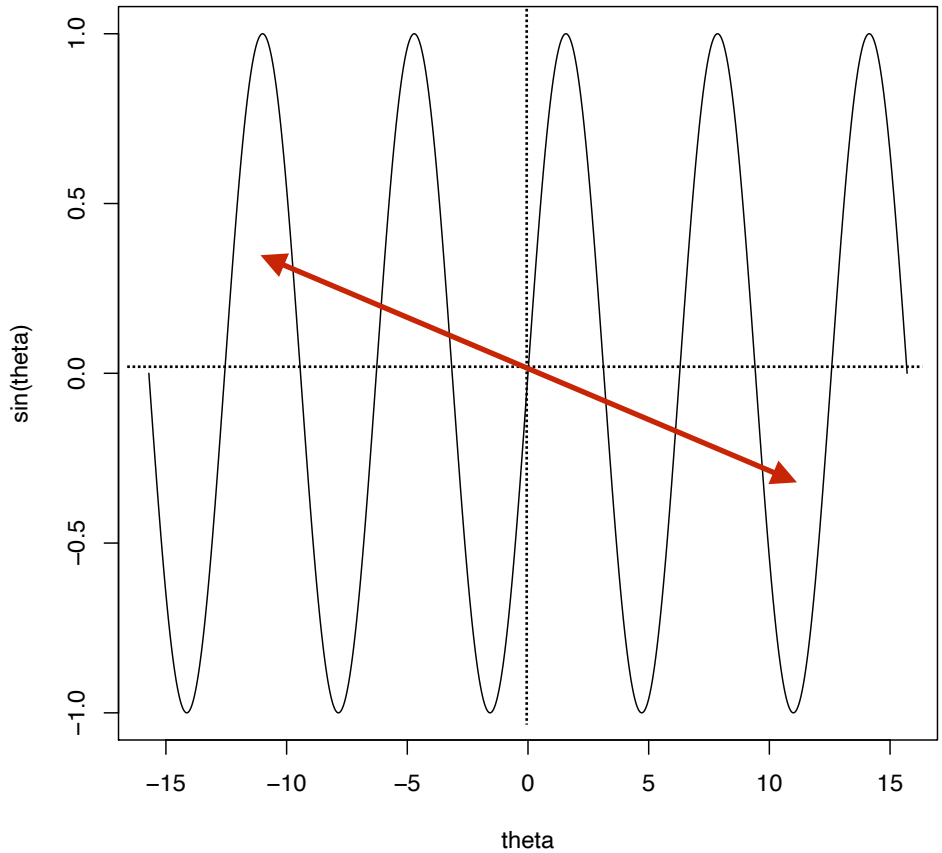

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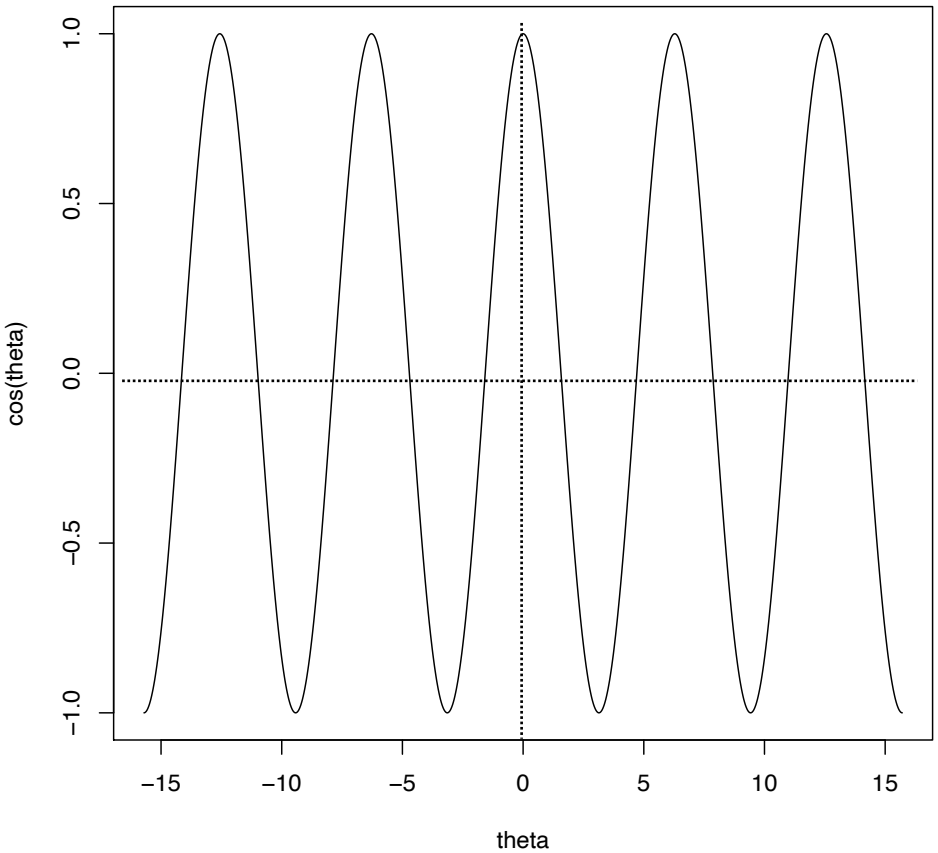
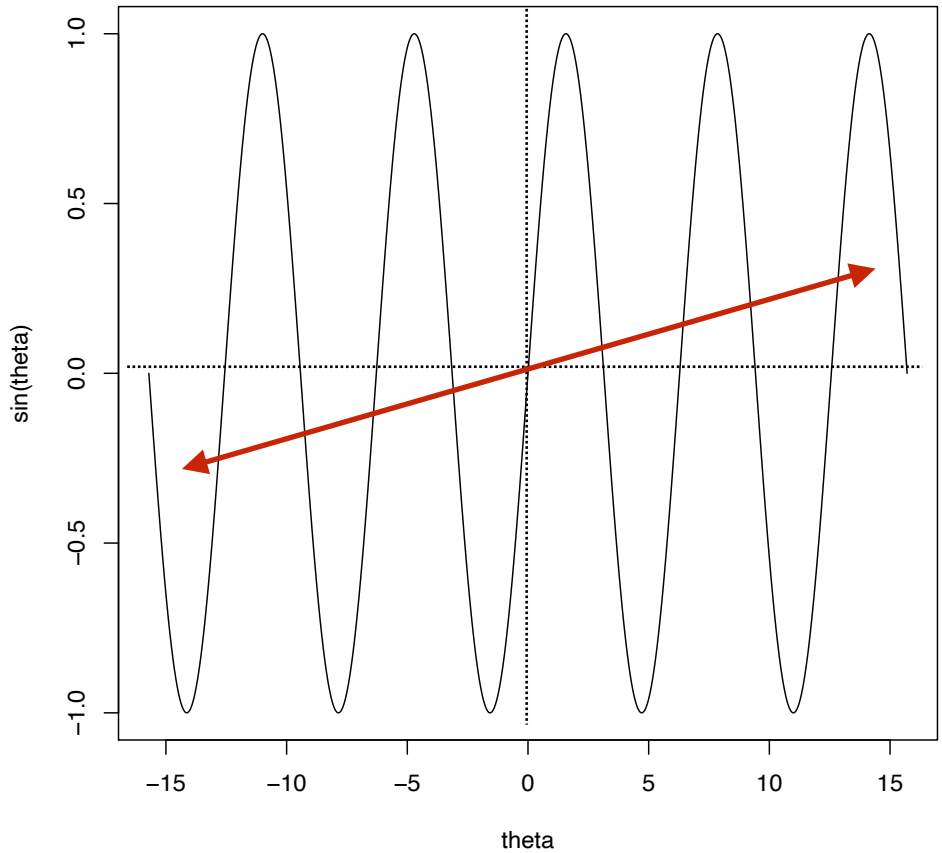


Sin

Odd

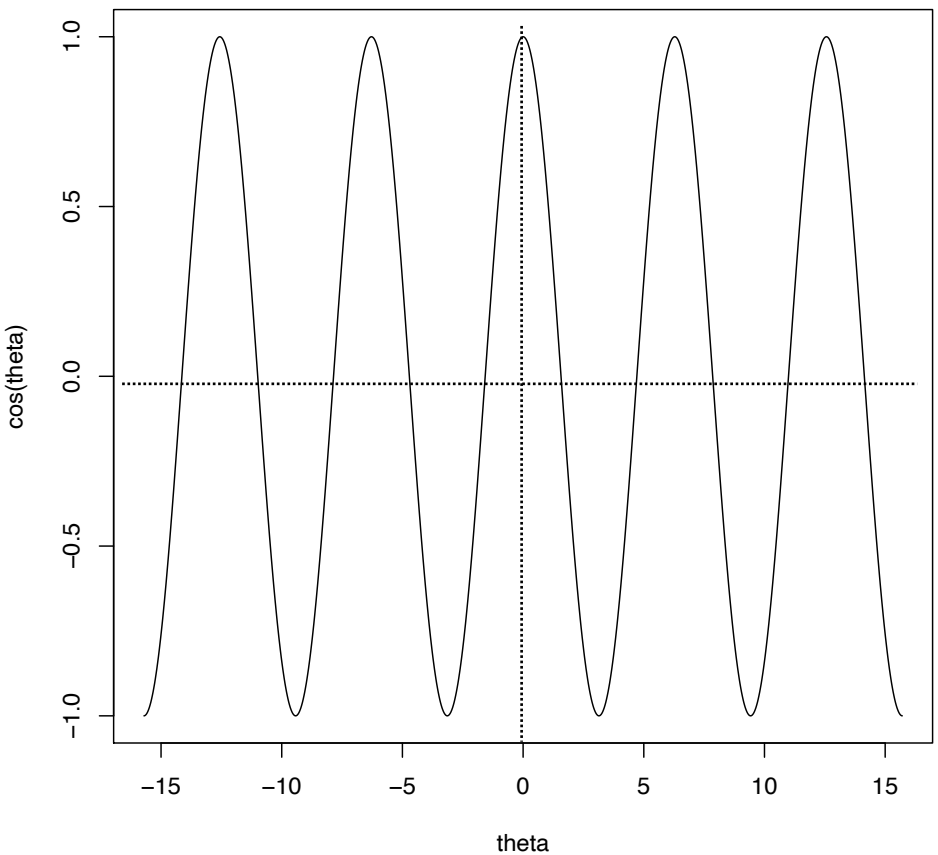


Easy Calculus

Integration is Area Under the Curve

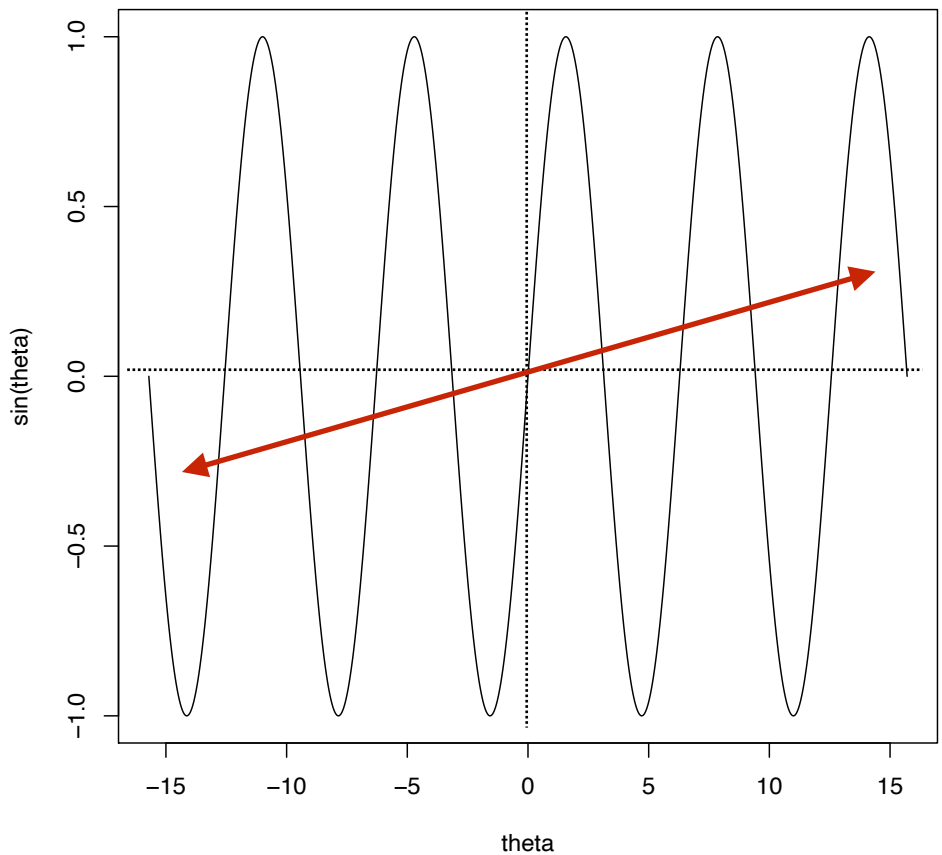
Cos

Even



Sin

Odd



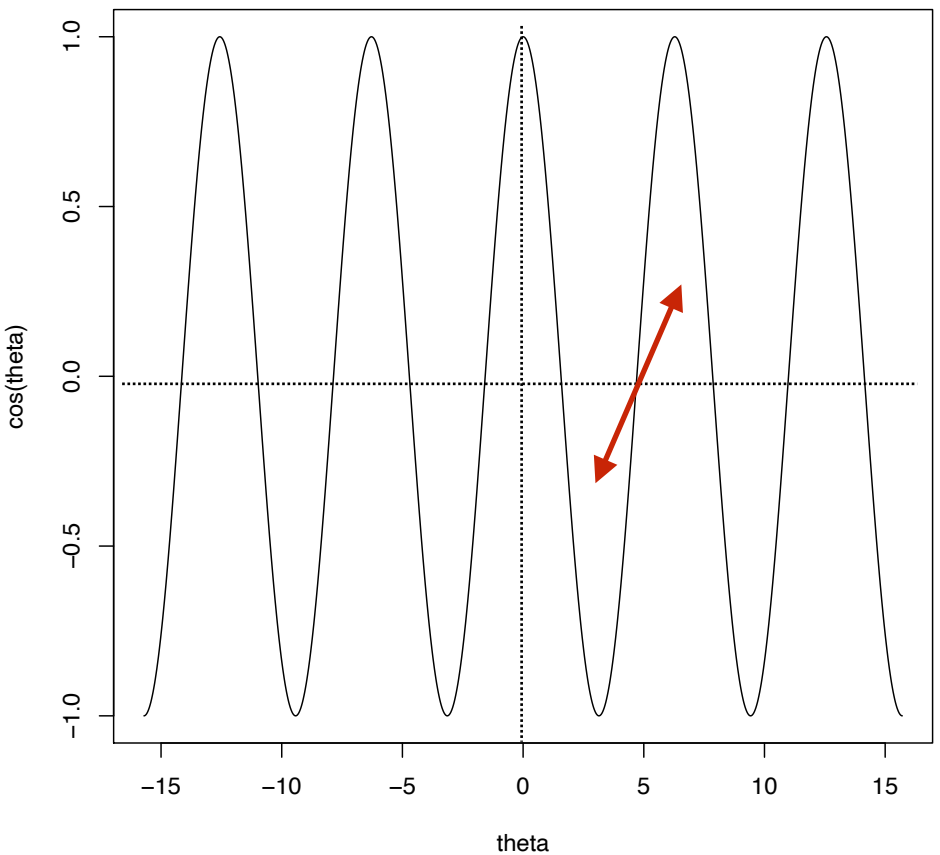
Zero

Easy Calculus

Integration is Area Under the Curve

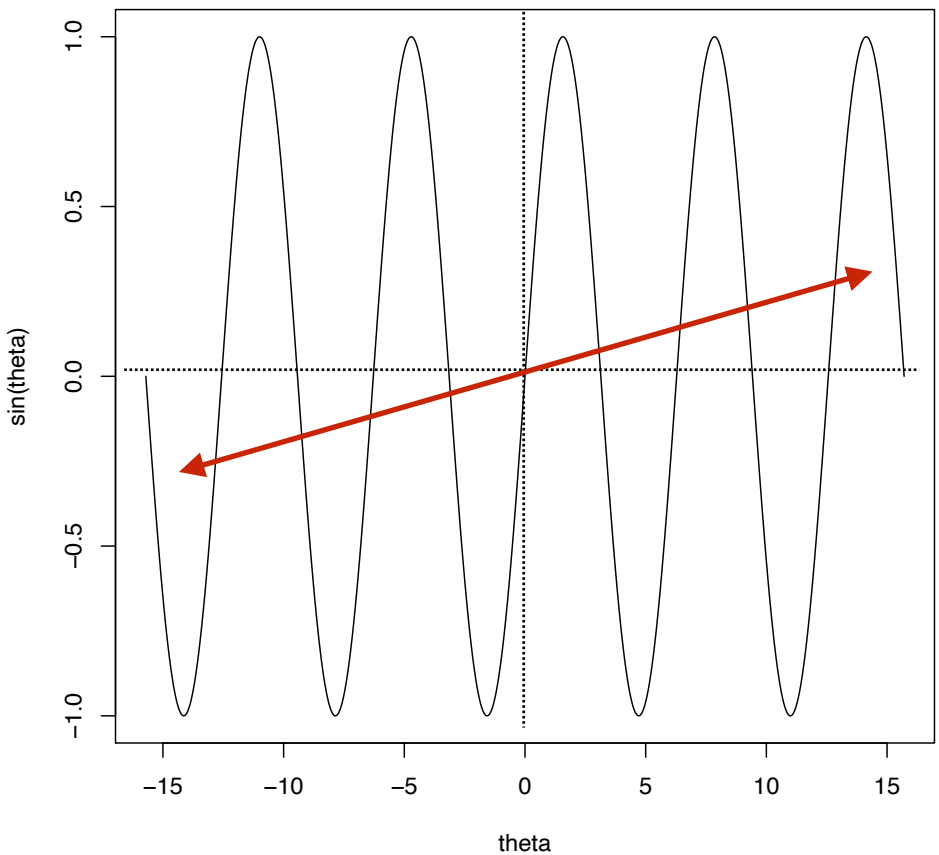
Cos

Even



Sin

Odd



Zero

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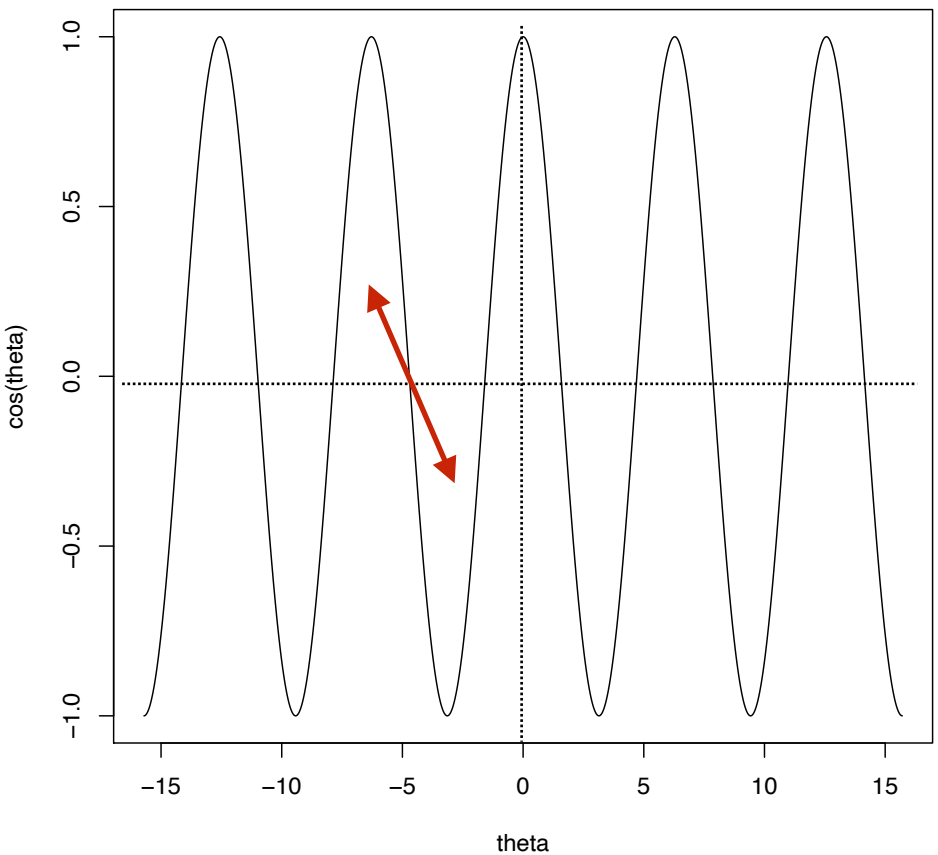
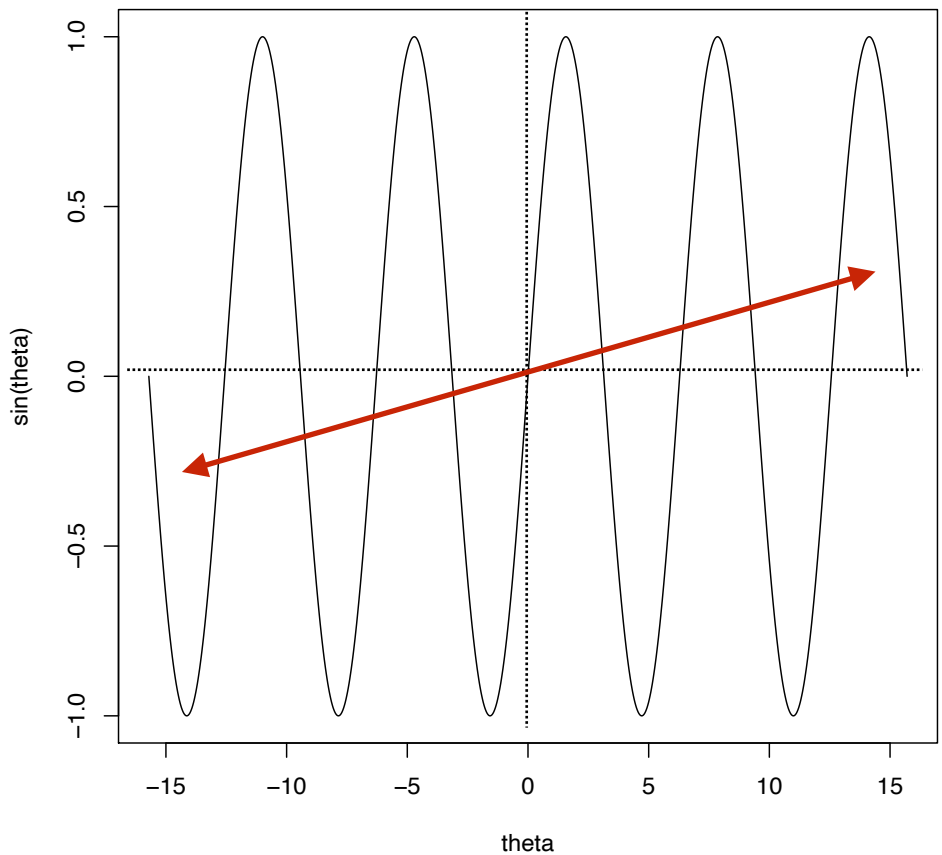
Cos

Even



Sin

Odd



Zero

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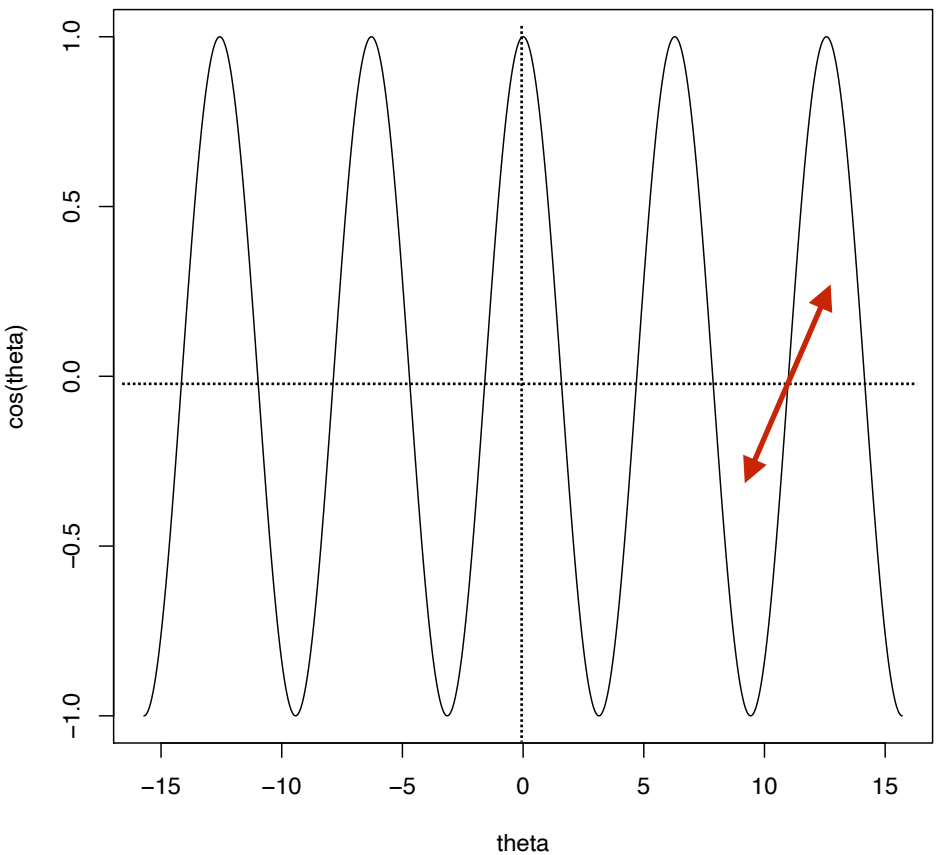
Integration is Area Under the Curve

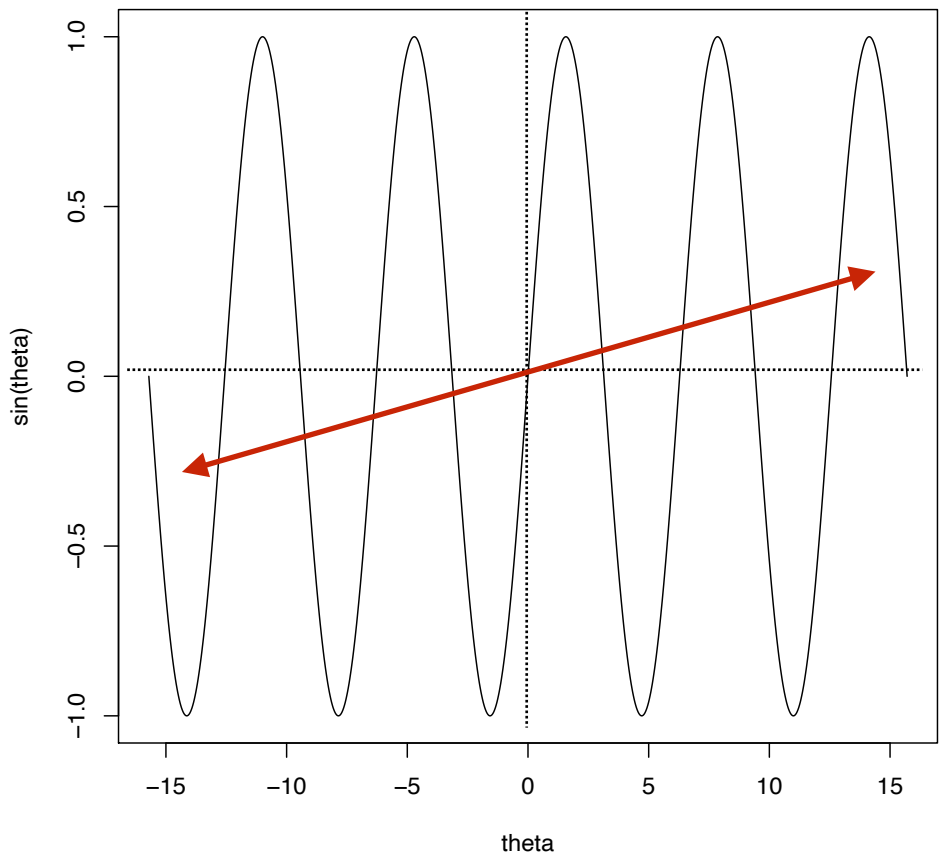
Cos

Even



Sin

Odd



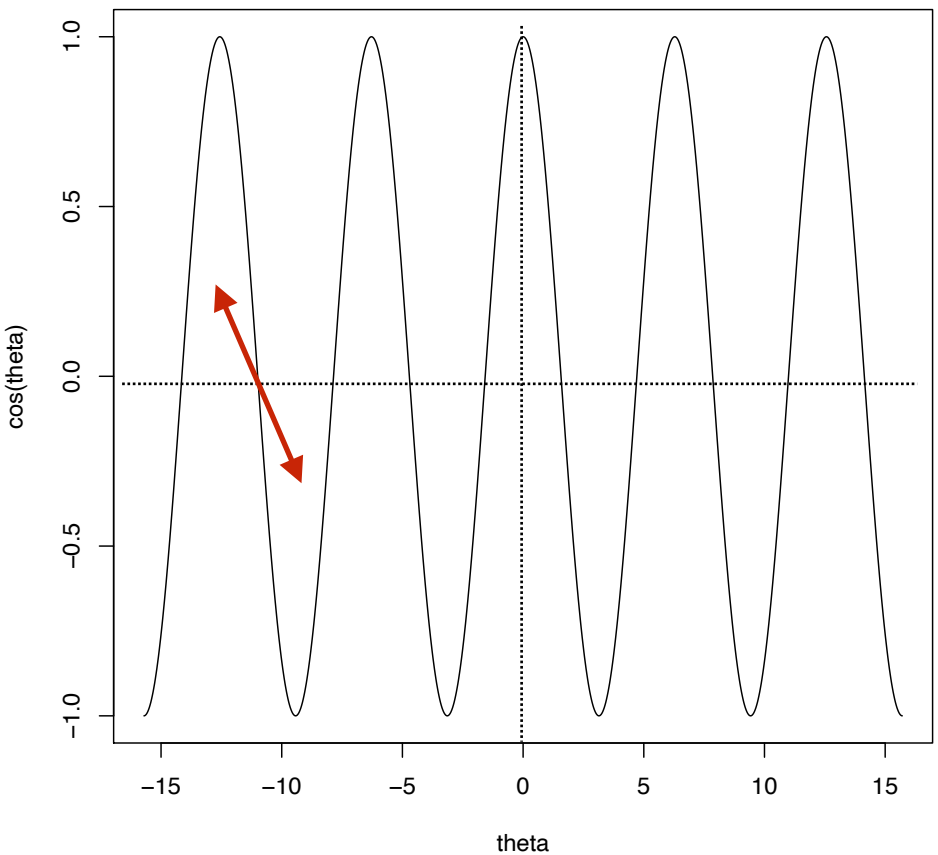
Zero

Easy Calculus

Integration is Area Under the Curve

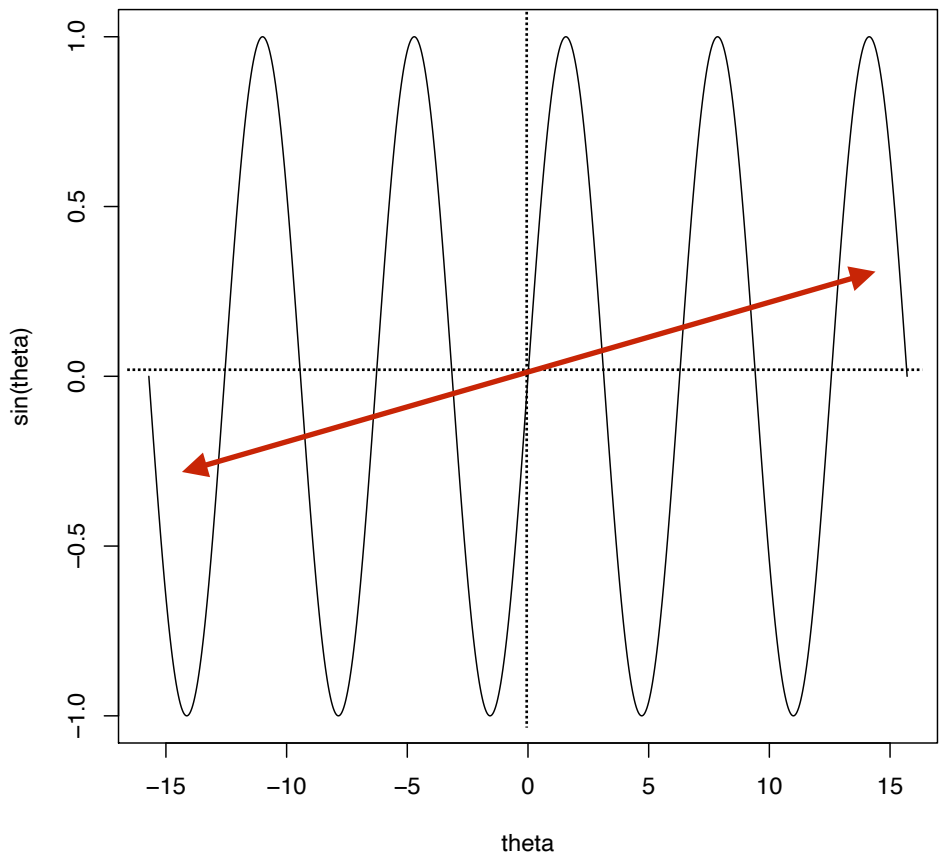
Cos

Even



Sin

Odd



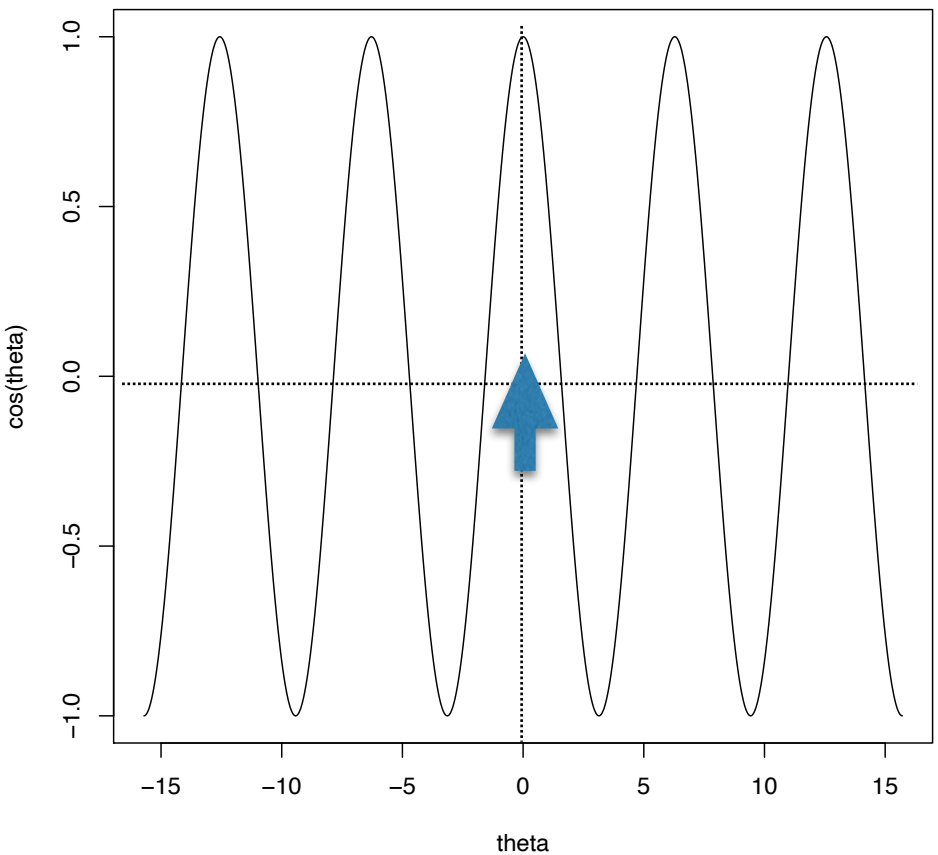
Zero

Easy Calculus

Integration is Area Under the Curve

Cos

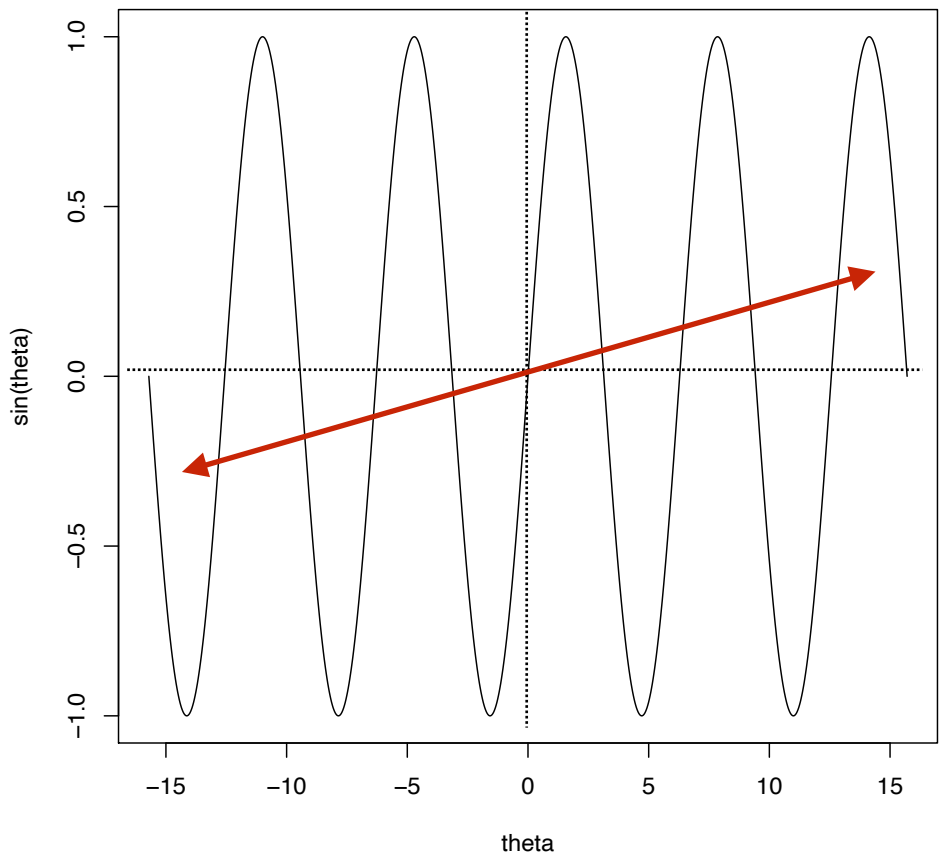
Even



Not Zero

Sin

Odd



Zero

Transition Dipole Moment

Integrals the easy way

$$\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_x \psi_1 \, \partial x$$

even

↓

Non-zero

$$\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_x \psi_1 \, \partial x$$

odd

↓

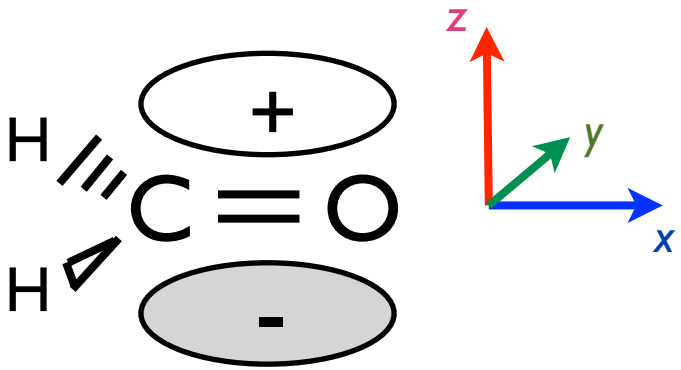
Zero

$$\left(\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_x \psi_1 \, \partial x\right)\left(\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_y \psi_1 \, \partial x\right)\left(\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_z \psi_1 \, \partial x\right)$$

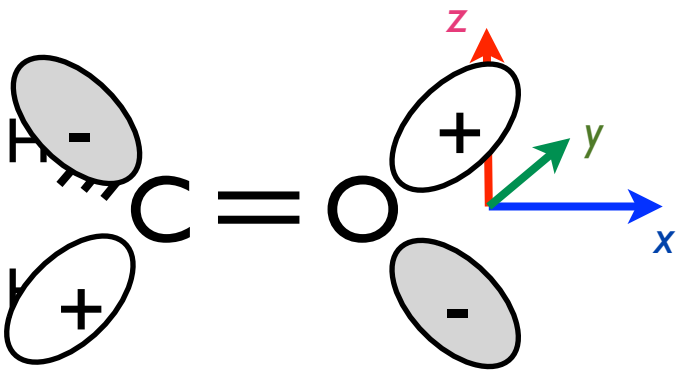
≠0 (even) ≠0 (even) 0 (odd)

Formaldehyde

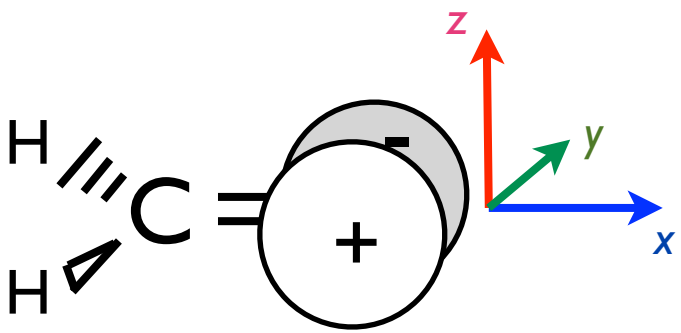
$\langle\Psi_2|\mu_x|\Psi_1\rangle\langle\Psi_2|\mu_y|\Psi_1\rangle\langle\Psi_2|\mu_z|\Psi_1\rangle$



x	y	z
even	even	odd



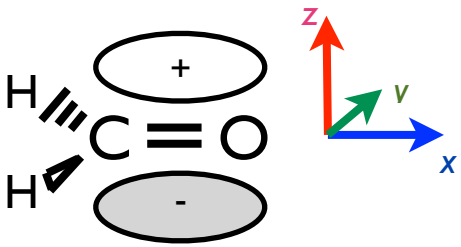
odd	even	odd



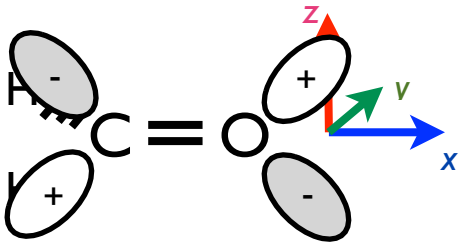
even	odd	even

→	μ_x	odd	even	even
↗	μ_y	even	odd	even
↑	μ_z	even	even	odd

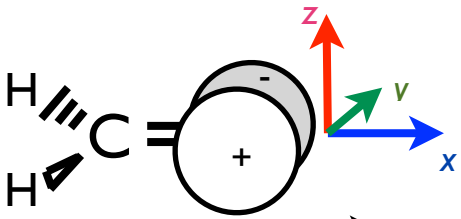
Formaldehyde: π to π*



	x	y	z
	even	even	odd



	x	y	z
	odd	even	odd



	x	y	z
	even	odd	even
μ_x	odd	even	even
μ_y	even	odd	even
μ_z	even	even	odd

$\int \pi \mu_x \pi^* dx$ e o o → *even*

$\int \pi \mu_x \pi^* dy$ e e e → *even*

$\int \pi \mu_x \pi^* dz$ o e o → *even*

$\int \pi \mu_y \pi^* dx$ e e o → *even*

$\int \pi \mu_y \pi^* dy$ e o e → *odd*

$\int \pi \mu_y \pi^* dz$ o e o → *even*

$\int \pi \mu_z \pi^* dx$ e o → e_{even}

$\int \pi \mu_z \pi^* dy$ e e e → *even*

$\int \pi \mu_z \pi^* dz$ o o o → *odd*

$\langle \pi|\mu|\pi^* \rangle = \langle \pi|\mu_x|\pi^* \rangle + \langle \pi|\mu_y|\pi^* \rangle + \langle \pi|\mu_z|\pi^* \rangle$

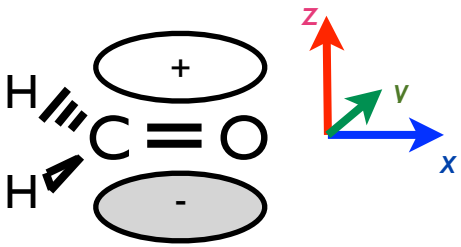
allowed (eoo)(eee)(oeo) + (eeo)(eoe)(oeo) + (eeo)(eee)(ooo)

(eoe) **zero** (ooo) **zero**

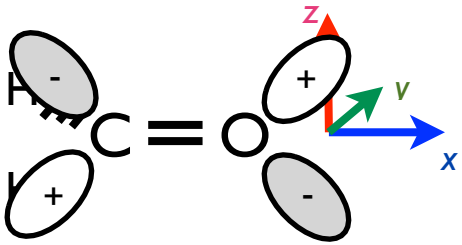
dx dy dz dx dy dz dx dy dz

non-zero + **zero** + **zero**

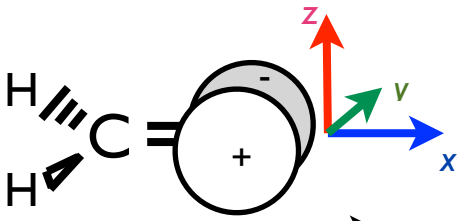
Formaldehyde: n to π*



	x	y	z
	even	even	odd



	x	y	z
	odd	even	odd



	x	y	z
	even	odd	odd
μ_x	odd	even	even
μ_y	even	odd	even
μ_z	even	even	odd

$\int n\mu_x\pi^* dx$ e o o — *even*

$\int n\mu_x\pi^* dy$ o e e — *odd*

$\int n\mu_x\pi^* dz$ e e o — *odd*

$\int n\mu_y\pi^* dx$ e e o — *odd*

$\int n\mu_y\pi^* dy$ o o e — *even*

$\int n\mu_y\pi^* dz$ e e o — *odd*

$\int n\mu_z\pi^* dx$ e e o — *even*

$\int n\mu_z\pi^* dy$ o e e — *odd*

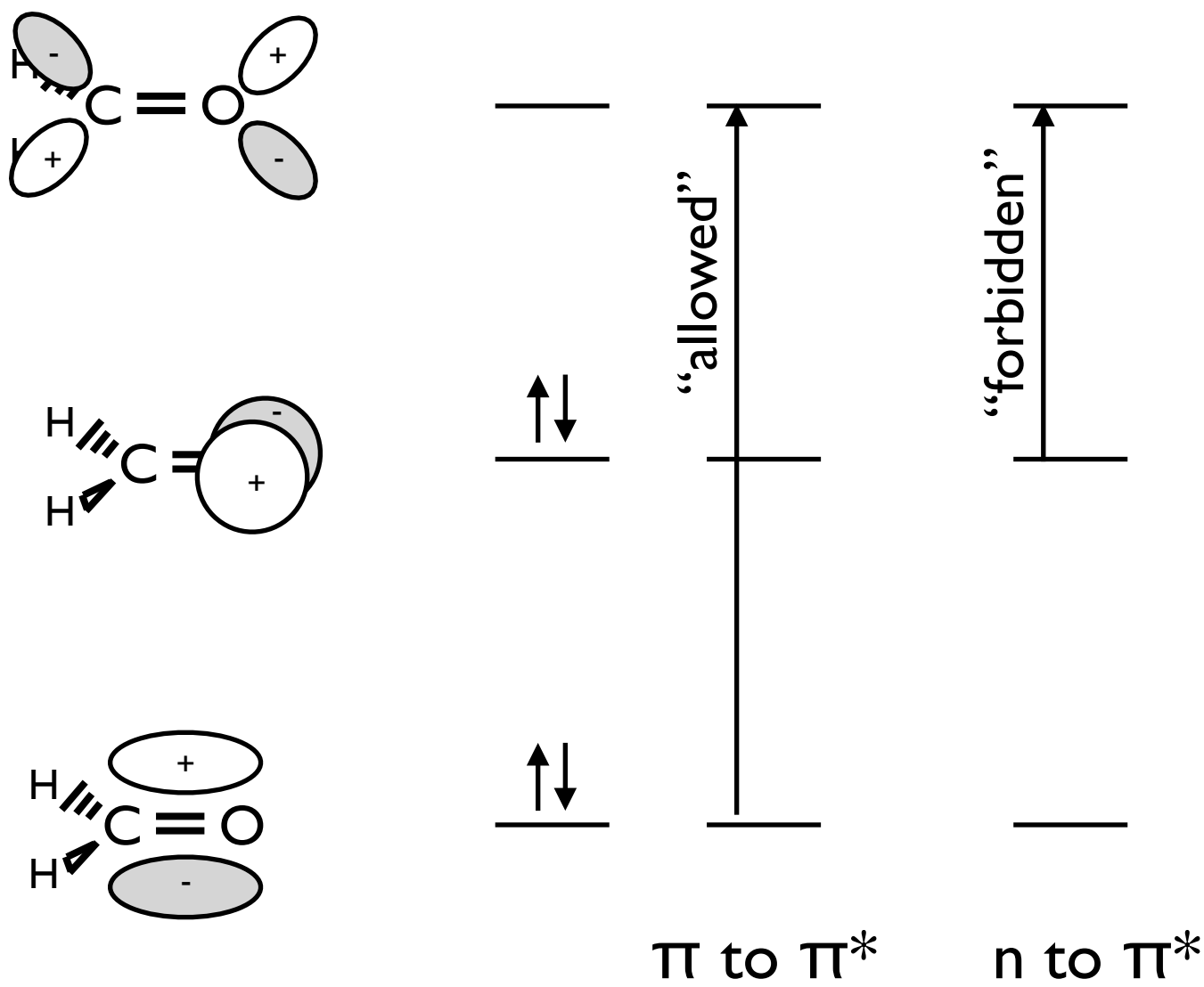
$\int n\mu_z\pi^* dz$ e o o — *even*

$\langle n|\mu|\pi^* \rangle = \langle n|\mu_x|\pi^* \rangle + \langle n|\mu_y|\pi^* \rangle + \langle n|\mu_z|\pi^* \rangle$

disallowed (eoo)(oee)$_{\text{zero}}$(eeo)$_{\text{zero}}$ + (eeo)$_{\text{zero}}$(ooe)(oeo)$_{\text{zero}}$ + (eeo)(oee)$_{\text{zero}}$(ooo)

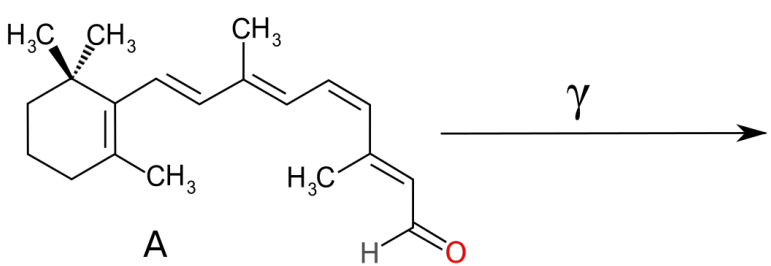
zero + **zero** + **zero**

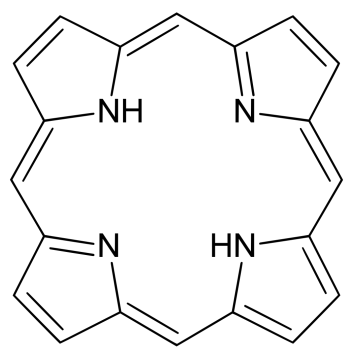
Formaldehyde

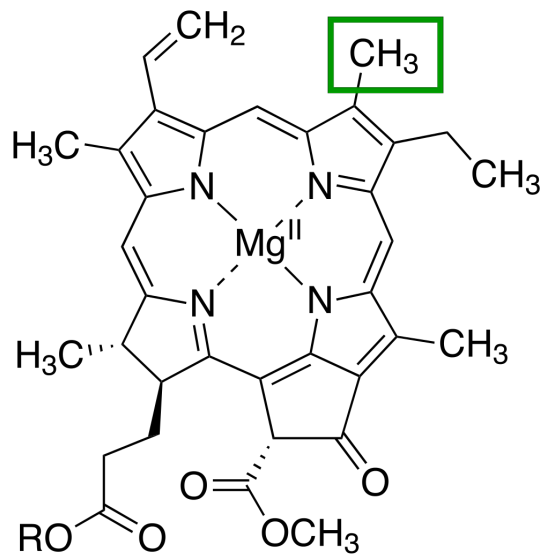


Basics

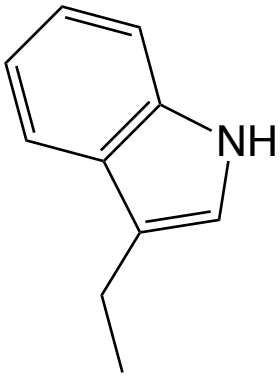
What spectroscopic difference(s) can you predict about these molecules?



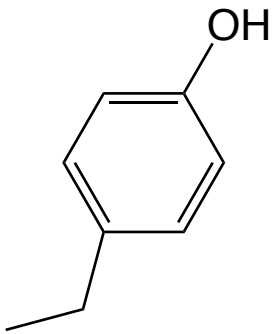




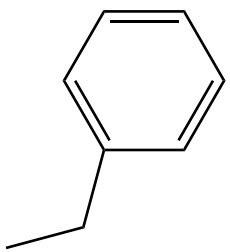
Theory and Experiment



Trp 280 nm 5050 M^{-1} cm^{-1}



Tyr 274 nm 1440 M^{-1} cm^{-1}



Phe 257 nm 220 M^{-1} cm^{-1}

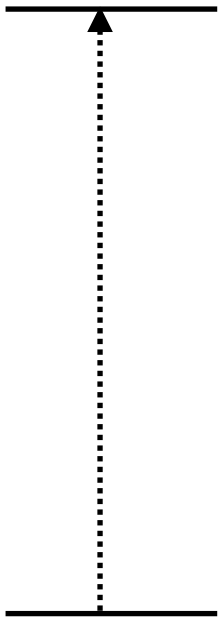
(Electronic) environment also matters!

Take home lesson

- Symmetry in a molecule (more correctly, in an electronic orbital) allows the *possibility* that transitions will be *forbidden*
- Even changes in the molecule's (electronic) environment can influence this.
    - Hyperchromic effect

Time Dependent Schrödinger Equation (polarized light)

$$P_b = IC_b(t)^2 = \frac{\left|\langle \vec{\psi}_b | \vec{\mu} | \vec{\psi}_a \rangle \bullet \vec{\mathbf{E}}_0\right|^2}{\hbar^2} \frac{t^2 \sin^2\left(\left(\frac{(E_b - E_a)}{\hbar} - \omega\right)t\right)}{2\left[\left(\frac{(E_b - E_a)}{\hbar} - \omega\right)t\right]}$$



Transition probability maximal when the energy of the photon exactly matches the difference in energy between the ground and excited states

But that is not enough!

Electronic Spectra II-III

Feb 4, 2021

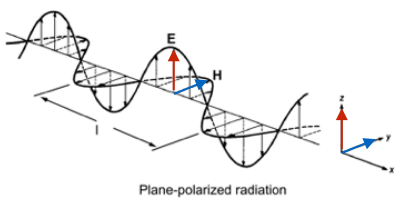
Time Dependent Schrödinger Equation (polarized light)

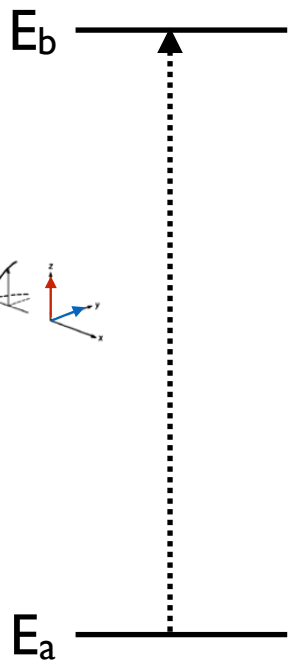
$$P_b = IC_b(t)^2 = \frac{\left|\langle \vec{\psi}_b | \vec{\mu} | \vec{\psi}_a \rangle \bullet \vec{\mathbf{E}}_0\right|^2}{\hbar^2} \frac{t^2 \sin^2\left(\left(\frac{(E_b - E_a)}{\hbar} - \omega\right)t\right)}{2\left[\left(\frac{(E_b - E_a)}{\hbar} - \omega\right)t\right]}$$

transition dipole moment

Light intensity

Energy Match





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For energy exactly matching the transition energy:

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = IC_b^{\omega=\Delta E}(t)^2 = \frac{\left|\langle \psi_b | \mu | \psi_a \rangle \bullet E_0\right|^2}{2\hbar^2}$$

For non-polarized light, integrate over all space:

$$\left|\langle \psi_b | \mu | \psi_a \rangle \bullet E_0\right|^2 = \frac{1}{3}\left|\langle \psi_b | \mu | \psi_a \rangle\right|^2 \left|E_0\right|^2$$

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = \frac{\left|\langle \psi_b | \mu | \psi_a \rangle\right|^2 \left|E_0\right|^2}{6\hbar^2}$$

Time Dependent Schrödinger Equation

For energy exactly matching the transition energy:

For non-polarized light, integrate over all space:

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = \frac{\left|\langle \psi_b | \mu | \psi_a \rangle\right|^2 \left| E_0 \right|^2}{6\hbar^2}$$

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = \frac{\left|\langle \psi_b | \mu | \psi_a \rangle\right|^2 \left| E_0 \right|^2}{6\hbar^2} = \frac{4\pi \left|\langle \psi_b | \mu | \psi_a \rangle\right|^2}{6\hbar^2} \frac{\left| E_0 \right|^2}{4\pi} = \frac{2\left|\langle \psi_b | \mu | \psi_a \rangle\right|^2}{3\hbar^2} I(\upsilon) = B_{AB} I(\upsilon)$$

"Probability of transition"

"concentration of light"

What does this look like?

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = B_{AB} I(\upsilon)$$

Time Dependent Schrödinger Equation

For energy exactly matching the transition energy:

For non-polarized light, integrate over all space:

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = \frac{\left|\langle \psi_b | \mu | \psi_a \rangle\right|^2 \left| E_0 \right|^2}{6\hbar^2}$$

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = \frac{\left|\langle \psi_b | \mu | \psi_a \rangle\right|^2 \left| E_0 \right|^2}{6\hbar^2} = \frac{4\pi \left|\langle \psi_b | \mu | \psi_a \rangle\right|^2}{6\hbar^2} \frac{\left| E_0 \right|^2}{4\pi} = \frac{2\left|\langle \psi_b | \mu | \psi_a \rangle\right|^2}{3\hbar^2} I(\upsilon) = B_{AB} I(\upsilon)$$

"Probability of transition"

"concentration of light"

What does this look like?

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = B_{AB} I(\upsilon)$$

$$-\frac{\partial A}{\partial t} = k[A]$$

Time Dependent Schrödinger Equation

For energy exactly matching the transition energy:

For non-polarized light, integrate over all space:

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = \frac{|\langle \psi_b | \mu | \psi_a \rangle|^2 |E_0|^2}{6\hbar^2}$$

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = \frac{|\langle \psi_b | \mu | \psi_a \rangle|^2 |E_0|^2}{6\hbar^2} = \frac{4\pi |\langle \psi_b | \mu | \psi_a \rangle|^2}{6\hbar^2} \frac{|E_0|^2}{4\pi} = \frac{2|\langle \psi_b | \mu | \psi_a \rangle|^2}{3\hbar^2} I(\upsilon) = B_{AB} I(\upsilon)$$

"Probability of transition"

"concentration of light"

What does this look like?

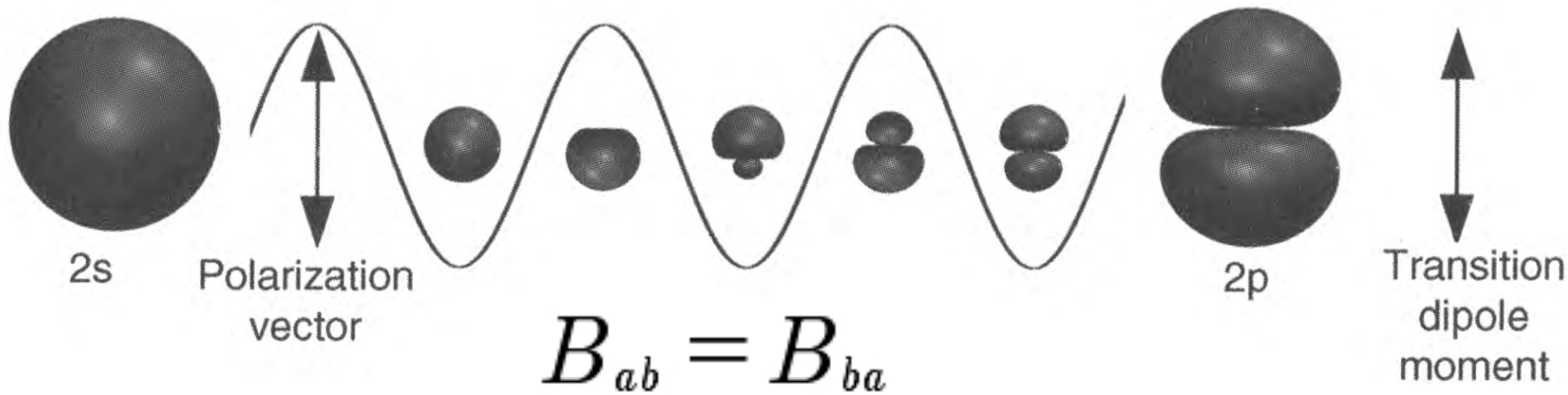
$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = B_{AB} I(\upsilon)$$

$$-\frac{\partial A}{\partial t} = k[A]$$

"Probability of reaction"

"concentration of reagent"

Time Dependent Schrödinger Equation



$$B_{ab} = B_{ba}$$

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = \frac{|\langle \psi_b | \mu | \psi_a \rangle|^2 |E_0|^2}{6\hbar^2} = \frac{4\pi |\langle \psi_b | \mu | \psi_a \rangle|^2}{6\hbar^2} \frac{|E_0|^2}{4\pi} = \frac{2|\langle \psi_b | \mu | \psi_a \rangle|^2}{3\hbar^2} I(\upsilon) = B_{AB} I(\upsilon)$$

"Probability of transition"

"concentration of light"

What does this look like?

$$\frac{\partial P_b^{\omega=\Delta E}}{\partial t} = B_{AB} I(\upsilon)$$

$$-\frac{\partial A}{\partial t} = k[A]$$

"Probability of reaction"

"concentration of reagent"

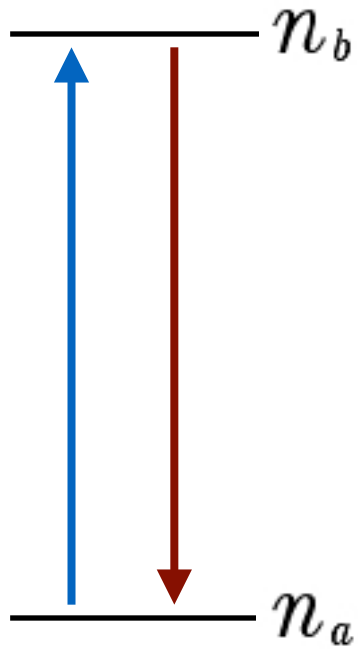
Absorption of Light Inducing a Transition

$$-\frac{\partial A}{\partial t} = k[A] \qquad \frac{\partial P_b^{\omega=\Delta E}}{\partial t} = B_{AB} I(\upsilon)$$

$$-\frac{\partial I(\upsilon)}{\partial t} = h\upsilon(n_a B_{ab} - n_b B_{ba}) I(\upsilon)$$

$$B_{ab} = B_{ba}$$

$$-\frac{\partial I(\upsilon)}{\partial t} = h\upsilon(n_a - n_b) B_{ab} I(\upsilon)$$



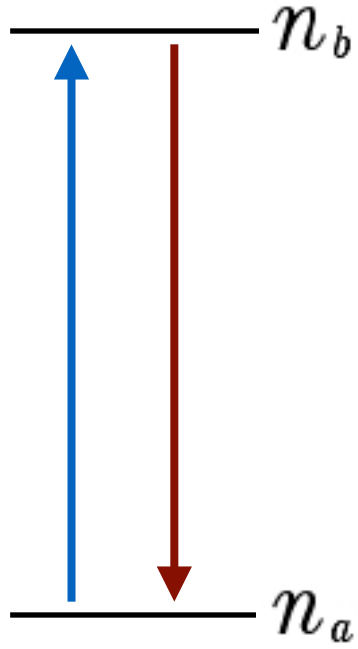
Absorption of Light Inducing a Transition

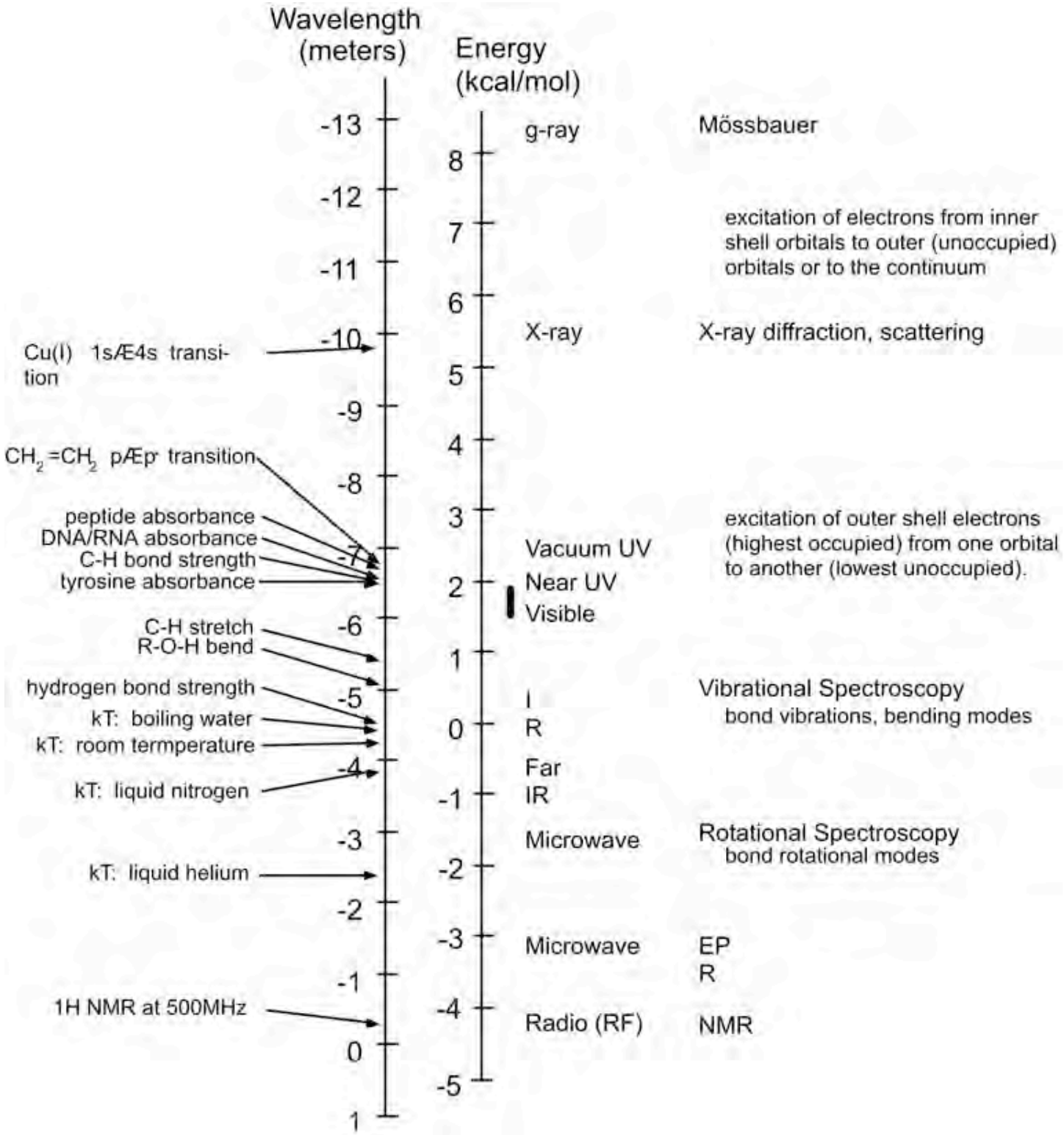
$$-\frac{\partial I(\upsilon)}{\partial t} = h\upsilon(n_a - n_b)B_{ab}I(\upsilon)$$

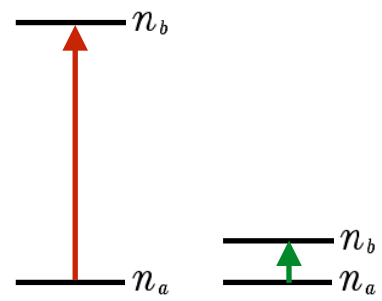
Boltzmann Equilibrium

$$\frac{n_b}{n_a} = e^{\frac{-(E_b - E_a)}{kT}} = e^{\frac{-\Delta E}{kT}}$$

$$-\frac{\partial I(\upsilon)}{\partial t} = h\upsilon(1 - e^{\frac{-\Delta E}{kT}})n_a B_{ab}I(\upsilon)$$







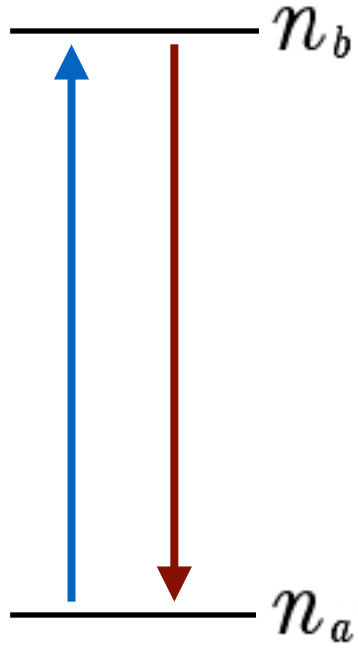
log ΔG	ΔG	n_b/n_a
2	100	5.7964E-74
1	→ 10	4.7459E-08
0	1	1.852E-01
-1	0.1	8.4482E-01
-2	0.01	9.8328E-01
-3	→ 0.001	9.9832E-01
-4	0.0001	9.9983E-01
-5	0.00001	9.9998E-01

Absorption of Light Inducing a Transition

$$-\frac{\partial I(\upsilon)}{\partial t} = h\upsilon\left(1 - e^{\frac{-\Delta E}{kT}}\right) n_a B_{ab} I(\upsilon)$$

ΔE large 0

ΔE small infinity



But remember that as more systems go up than down, the system will no longer be at Boltzmann equilibrium

The populations will equalize

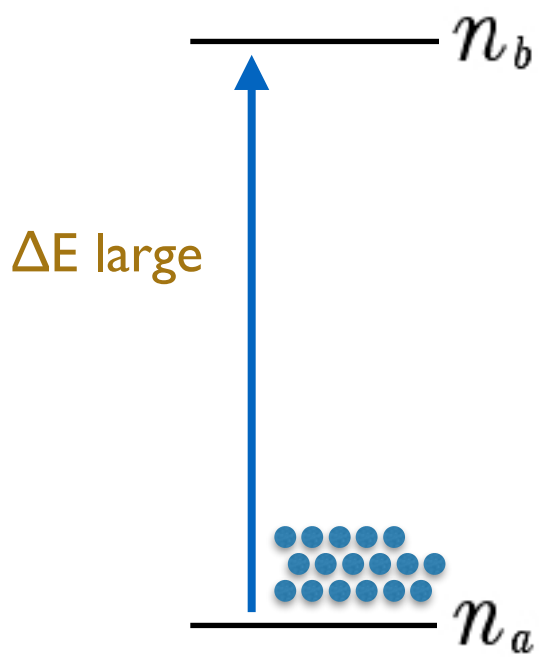
$$-\frac{\partial I(\upsilon)}{\partial t} = h\upsilon(n_a - n_b) B_{ab} I(\upsilon)$$

0

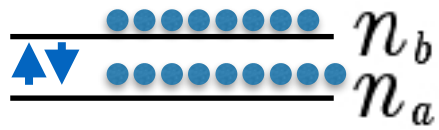
SATURATION

Absorption of Light Inducing a Transition

$$-\frac{\partial I(\upsilon)}{\partial t} = h\upsilon\left(1 - e^{\frac{-\Delta E}{kT}}\right) n_a B_{ab} I(\upsilon)$$



Optical Abs



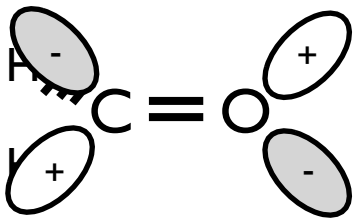
NMR

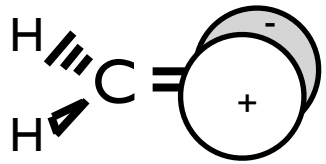
Formaldehyde

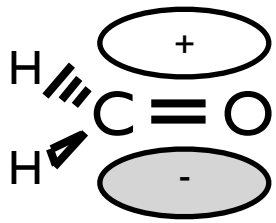
polarizability - interaction with solvent

more extended

more polarizable

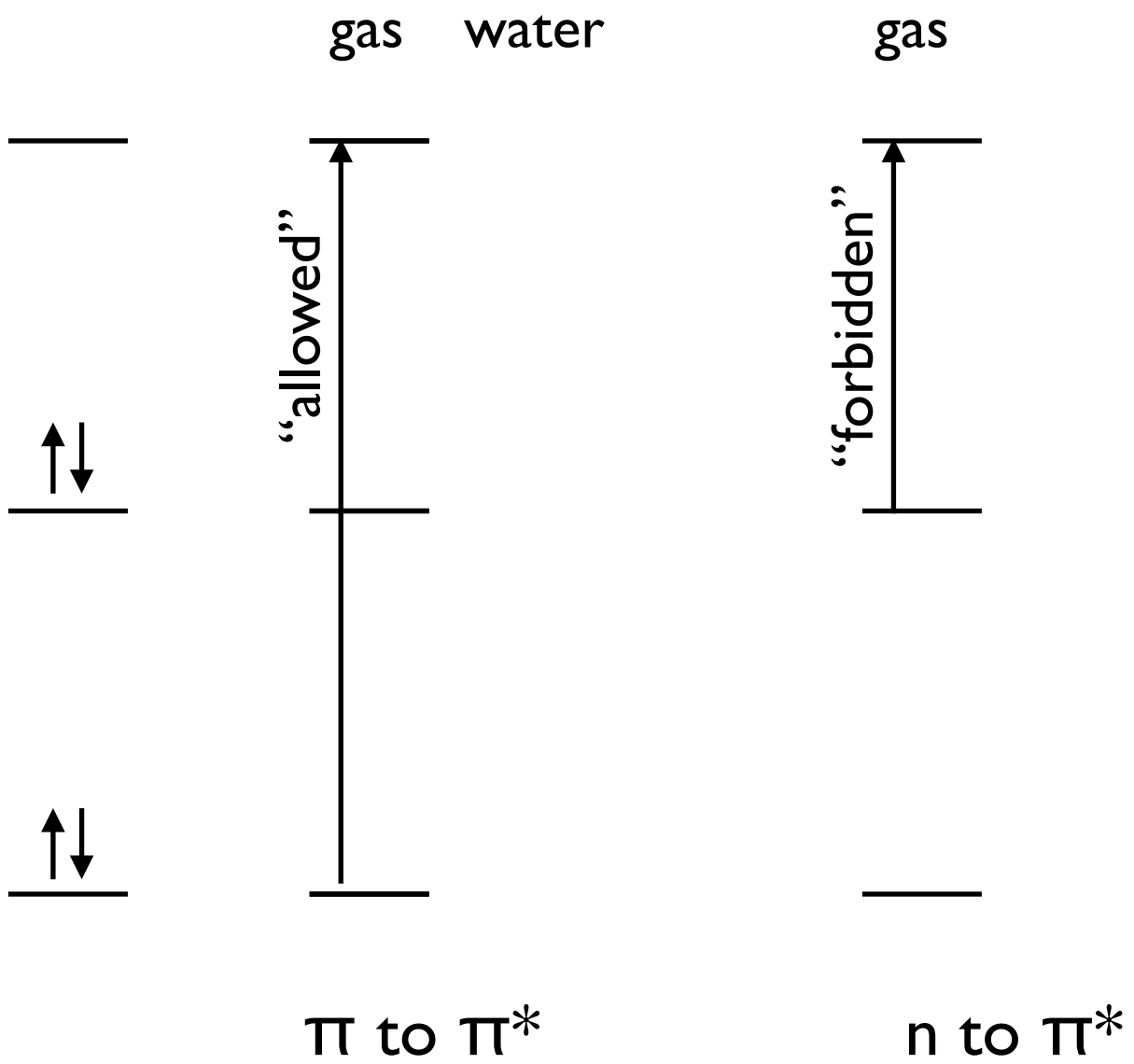






more compact

less polarizable

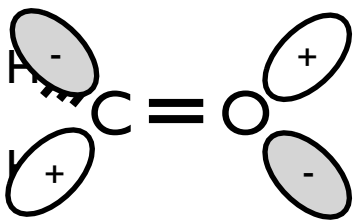


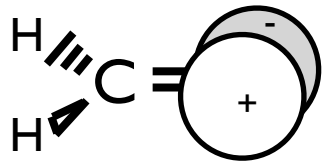
Formaldehyde

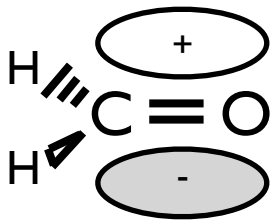
polarizability - interaction with solvent

more extended

more polarizable

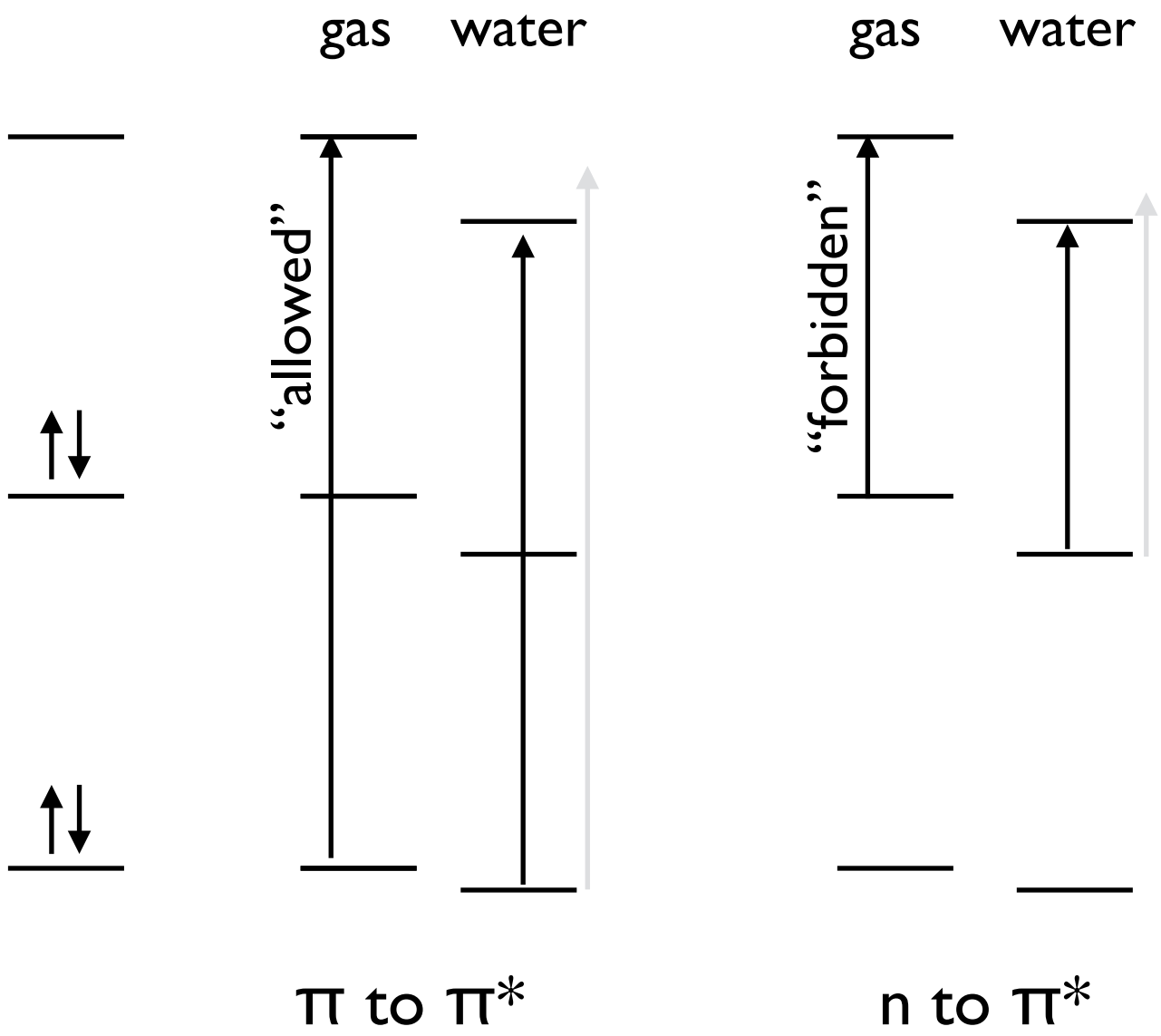




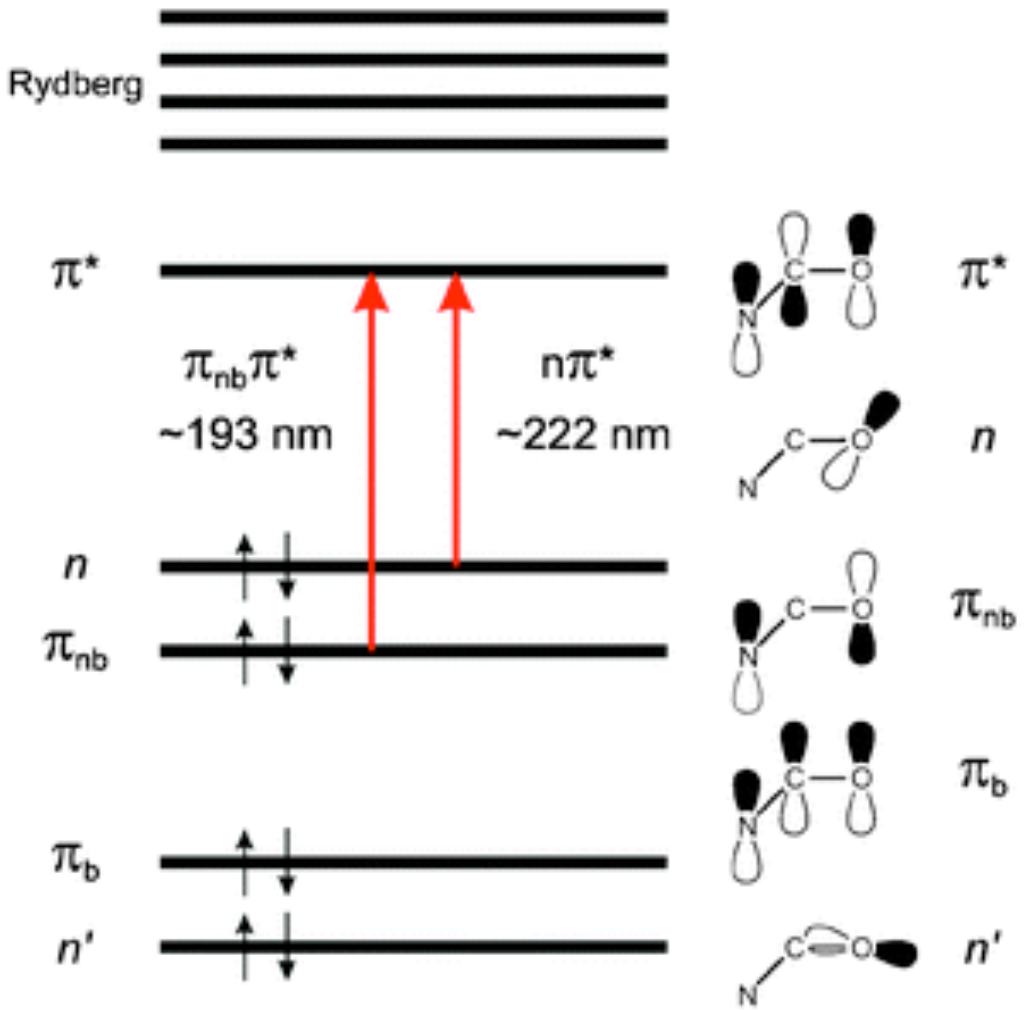


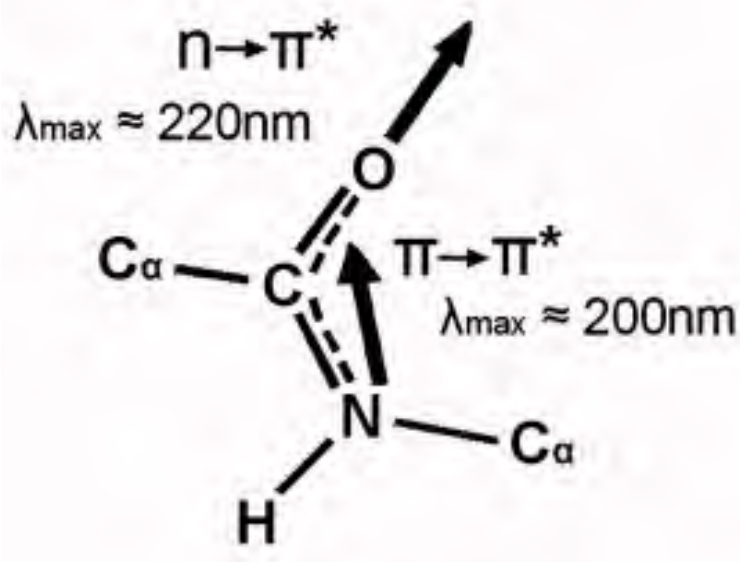
more compact

less polarizable



Peptide bond

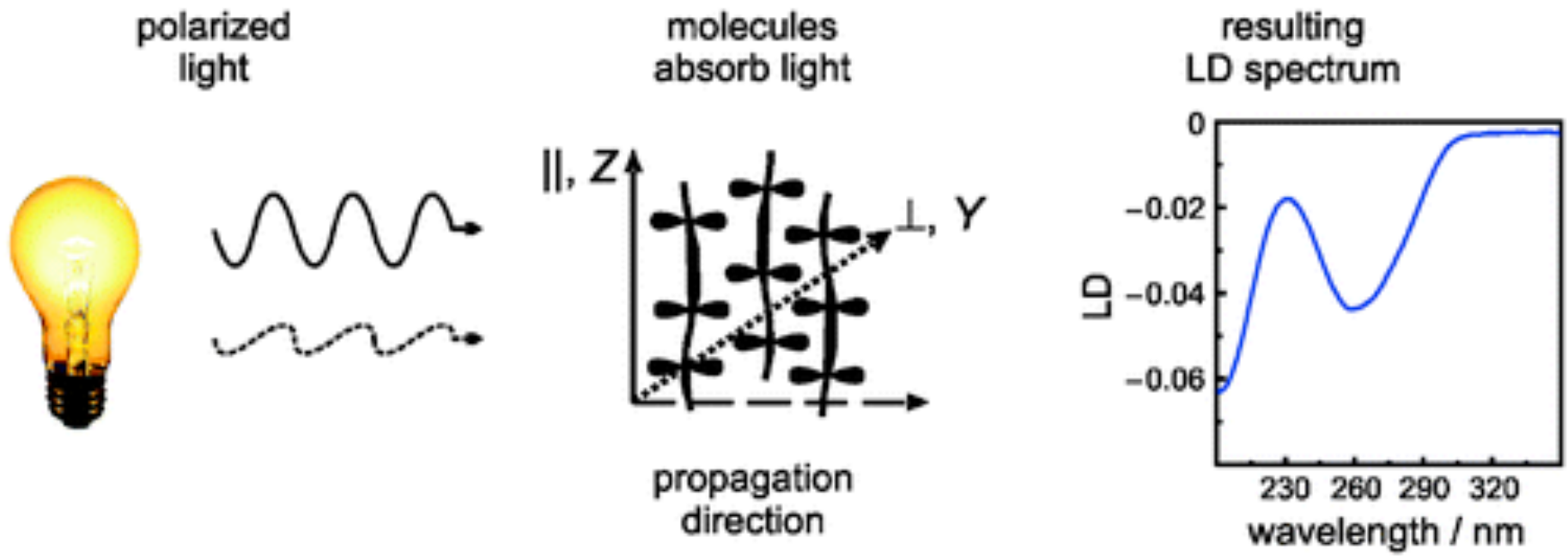




Whitmore & Wallace (2007)
Biopolymers 89, 392-400

Bullheller, Rodger, & Hirst (2007) Phys Chem Chem Phys 9, 2020-2035

Linear dichroism



$$\langle \pi|\mu|\pi^* \rangle = \langle \pi|\mu_x|\pi^* \rangle + \langle \pi|\mu_y|\pi^* \rangle + \langle \pi|\mu_z|\pi^* \rangle$$

allowed (eoo)(eee)(oeo) + (eeo)(eoe)(oeo) + (eeo)(eee)(ooo)

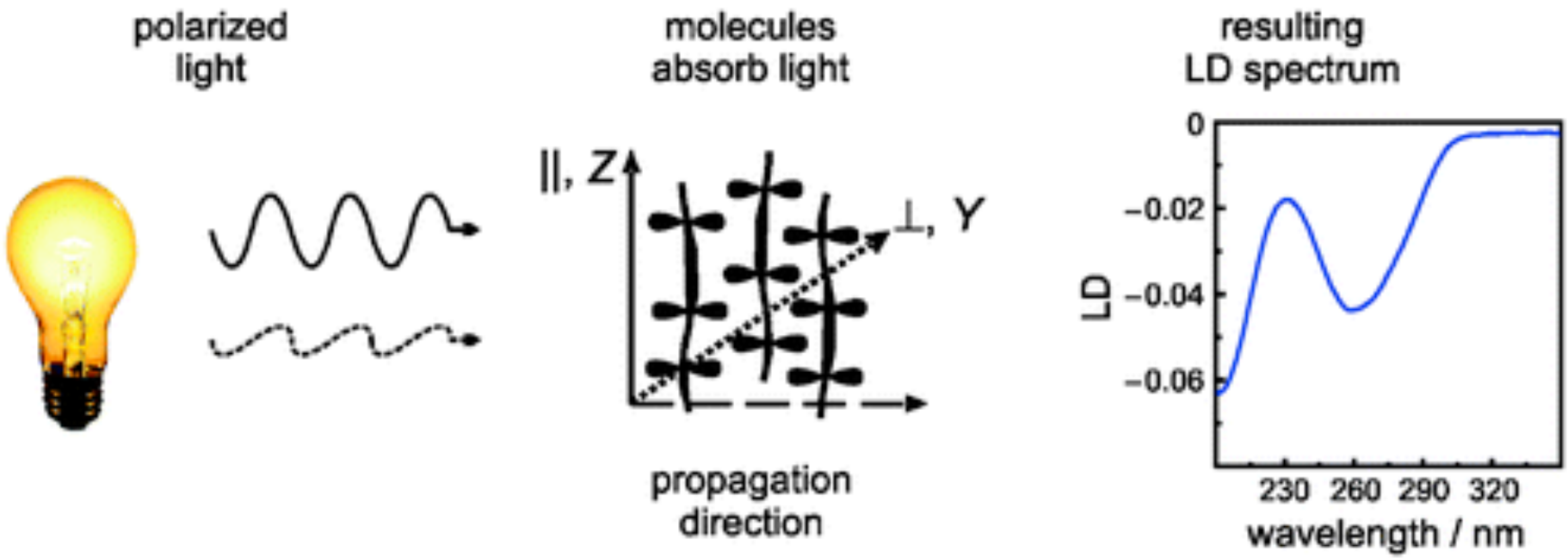
(eoe) zero; (ooo) zero

dx dy dz | dx dy dz | dx dy dz

non-zero + **zero** + **zero**

Bullheller, Rodger, & Hirst (2007) Phys Chem Chem Phys 9, 2020-2035

Linear dichroism



$\langle \pi | \mu_x | \pi^* \rangle$

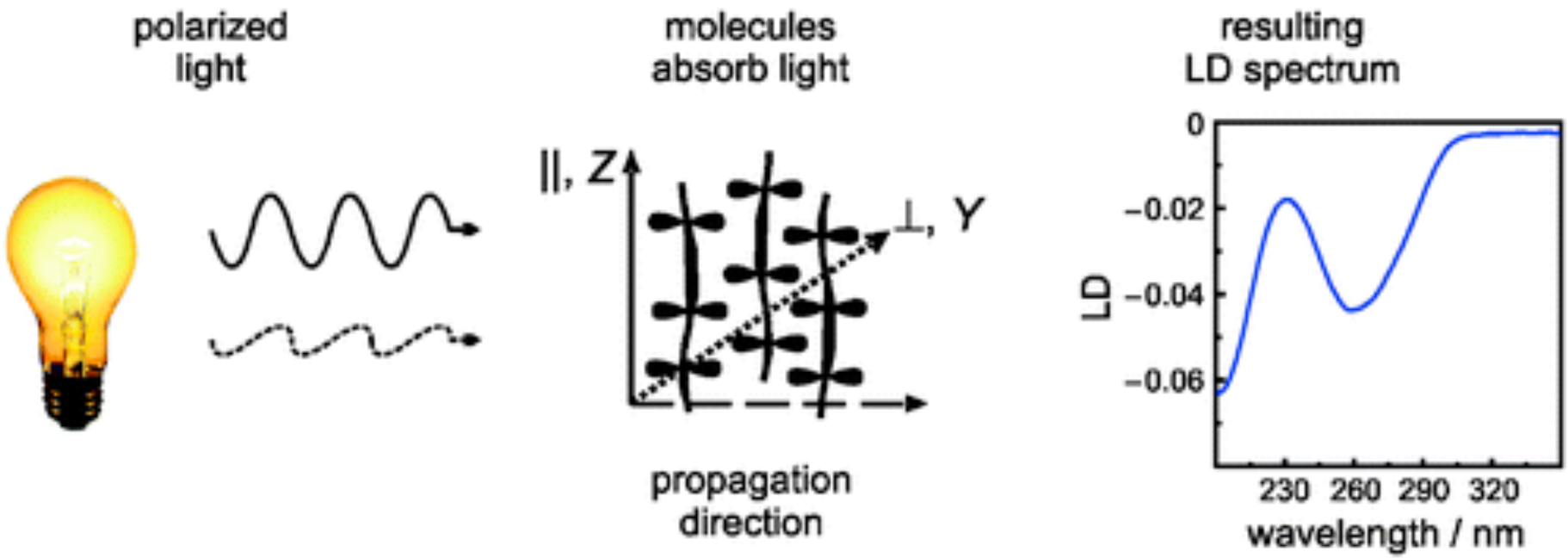
(eoo)(eee)(oeo)

dx dy dz

non-zero

allowed

Linear dichroism



$\langle \pi | \mu_y | \pi^* \rangle$

(eeo)(eoe)(oeo)

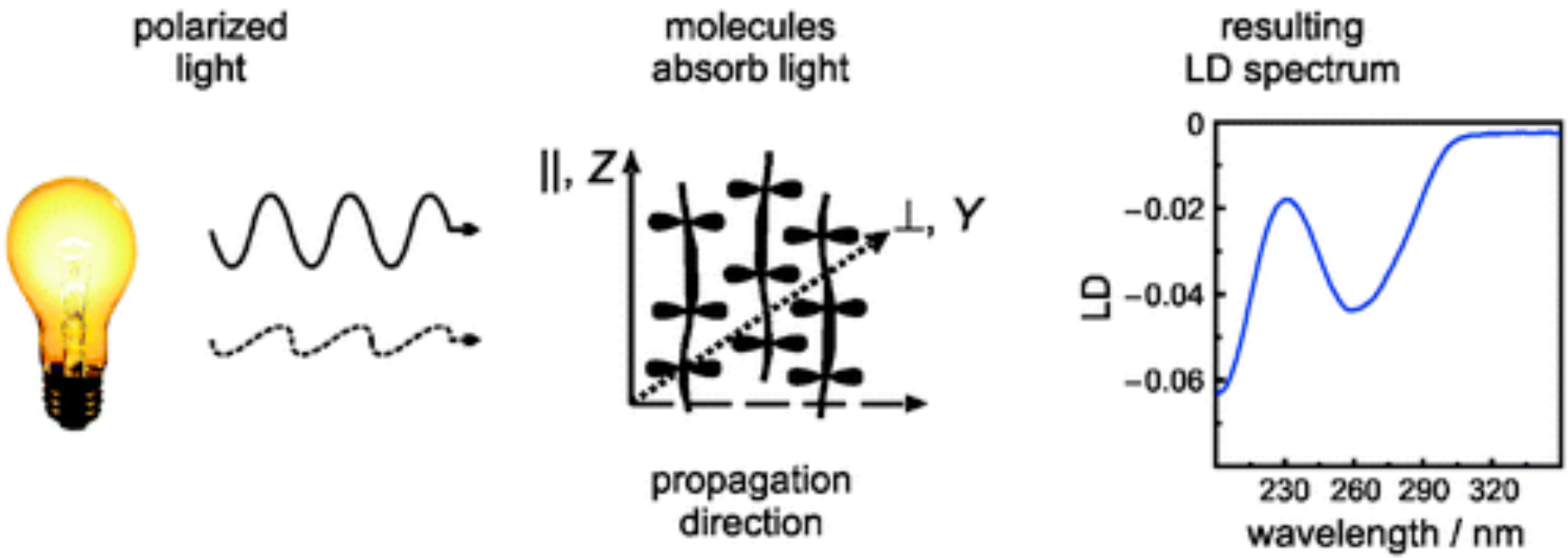
zero

dx dy dz

zero

forbidden

Linear dichroism



$\langle \pi | \mu_z | \pi^* \rangle$

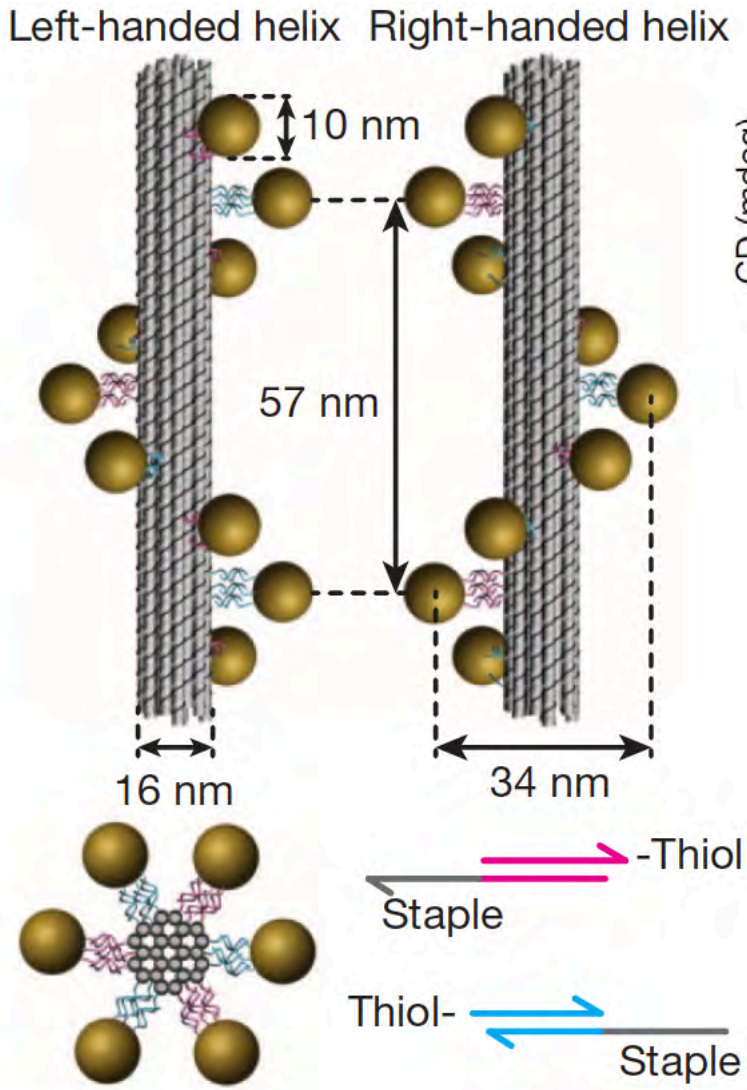
(eeo)(eee)(ooo)
zero

dx dy dz

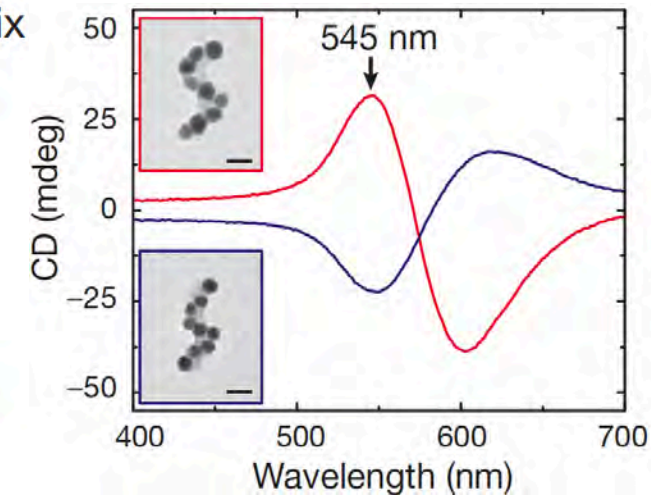
zero

forbidden

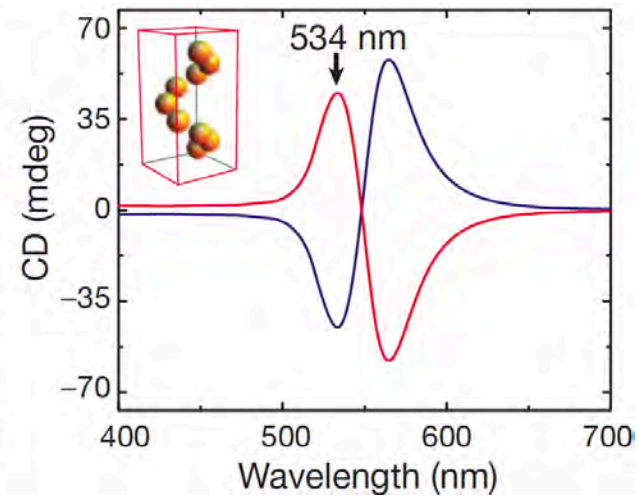
Circularly polarized light - preferential absorption



Experiment



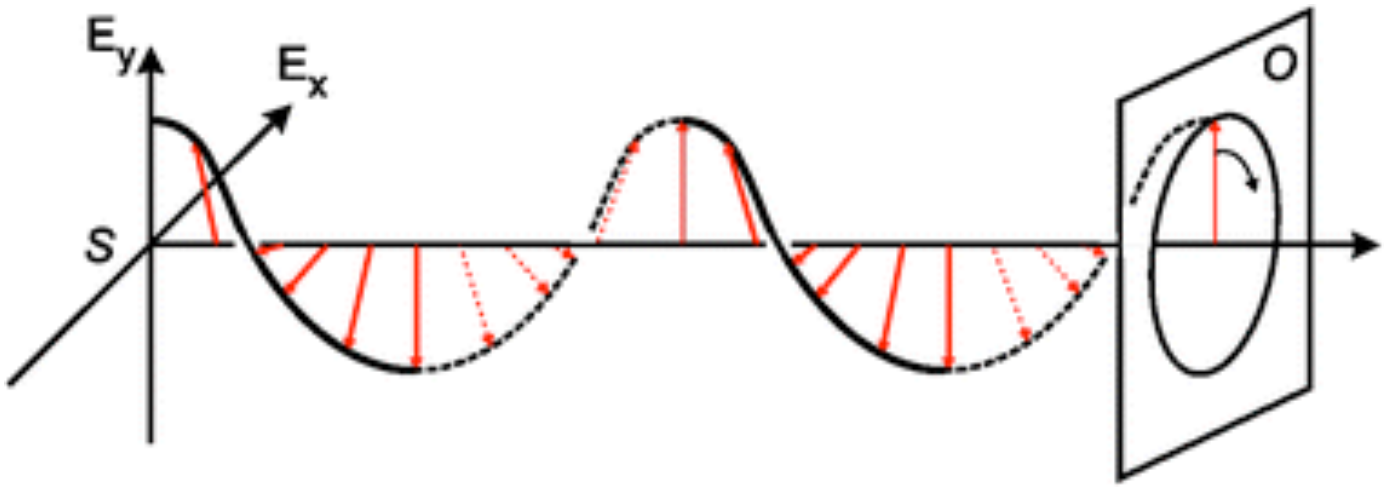
Theory

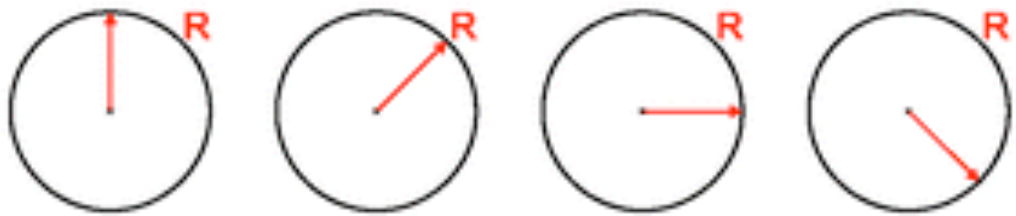


Circular dichroism of self-assembled gold nanohelices. Experimental (a and c) and theoretical (b and d) CD spectra of **left-handed (red lines)** and **right-handed (blue lines)** helices of nine gold nanoparticles show characteristic bisignate signatures in the visible. … c, The CD signal increases owing to collective plasmonic enhancement by a factor of 400 for assemblies of nanoparticles with 16-nm diameter, rendering the noise in the spectra invisible (as in a). The peak position for left-handed helices exhibits a red-shift from 524 nm to 545 nm. d, The corresponding theoretical calculation predicts a 500-fold enhancement of the signal and a peak shift from 523 nm to 534 nm. The CD spectra were recorded at concentrations of nanohelices of 1.5 nM in a and 0.4 nM in c. The insets in a and c show TEM images of left-handed (red frame) and right-handed (blue frame) nanohelices

DNA-based self-assembly of chiral plasmonic nanostructures with tailored optical response, (2012) Nature 483, 311-314
Anton Kuzyk1, Robert Schreiber, Zhiyuan Fan ,Günther Pardatscher1, Eva-Maria Roller, Alexander Högele, Friedrich C. Simmel1, Alexander O. Govorov & Tim Liedl

Circularly polarized light

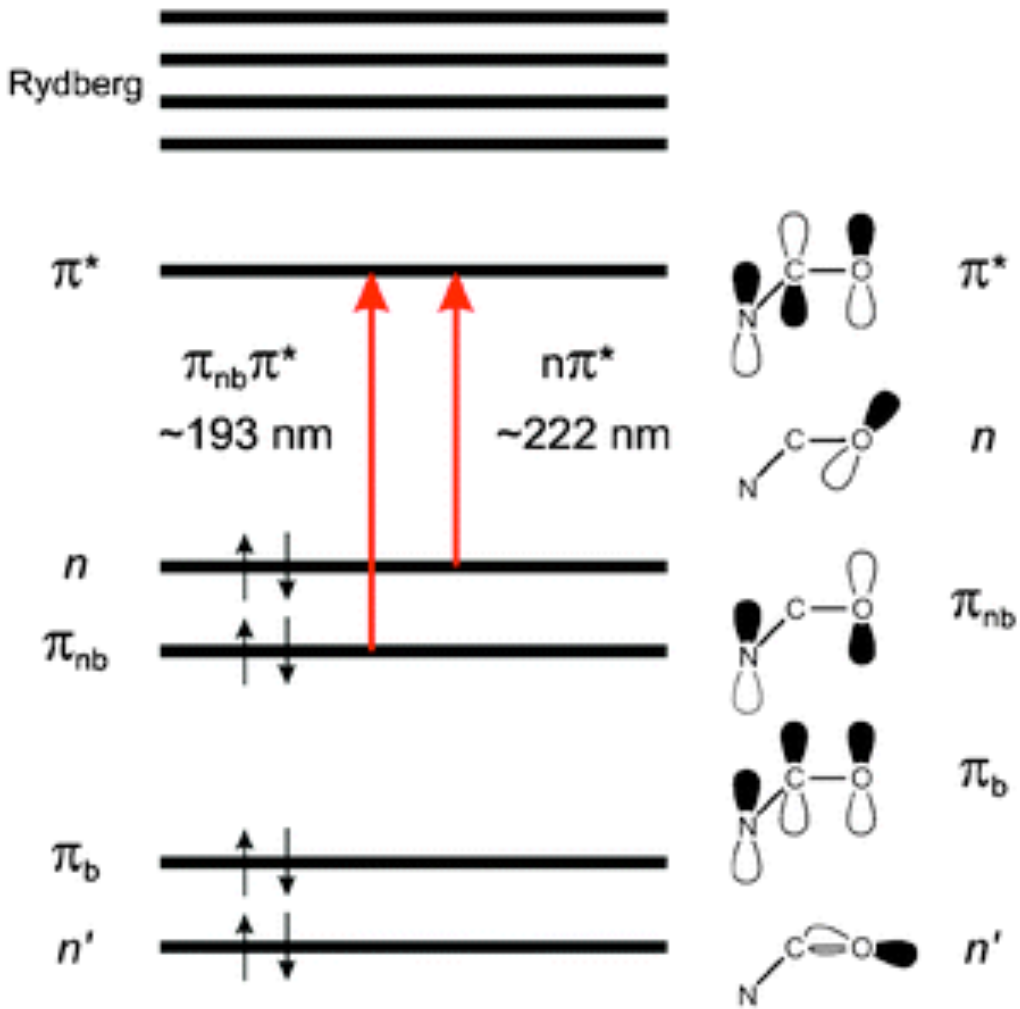


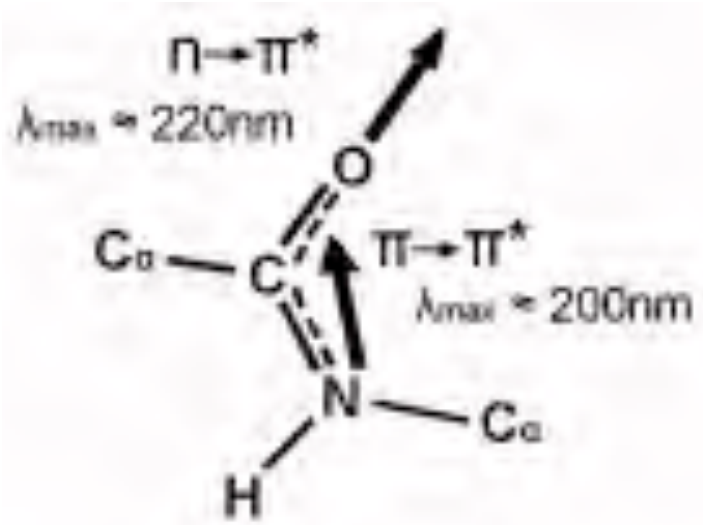


$$CD = \Delta A_\lambda = A_\lambda^{LeftCP} - A_\lambda^{RightCP}$$

Bullheller, Rodger, & Hirst (2007) Phys Chem Chem Phys 9, 2020-2035

Peptide bond

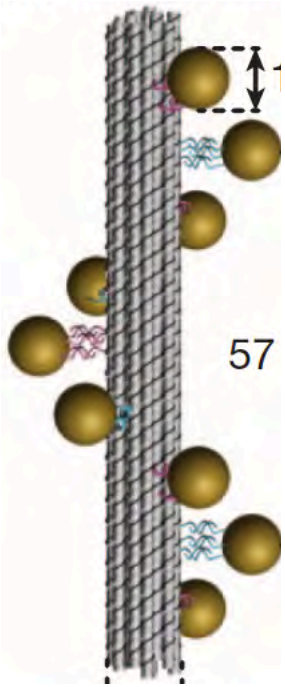


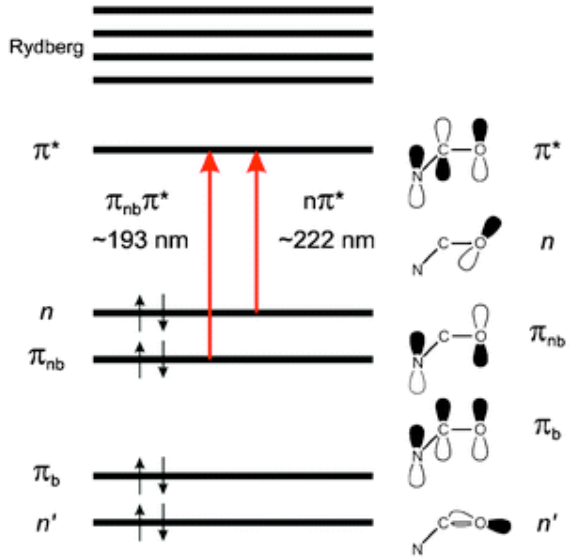


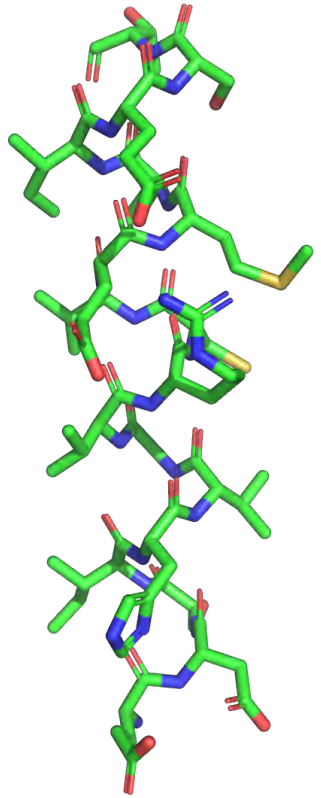
Whitmore & Wallace (2007)
Biopolymers 89, 392-400

Bullheller, Rodger, & Hirst (2007) Phys Chem Chem Phys 9, 2020-2035

Circular Dichroism





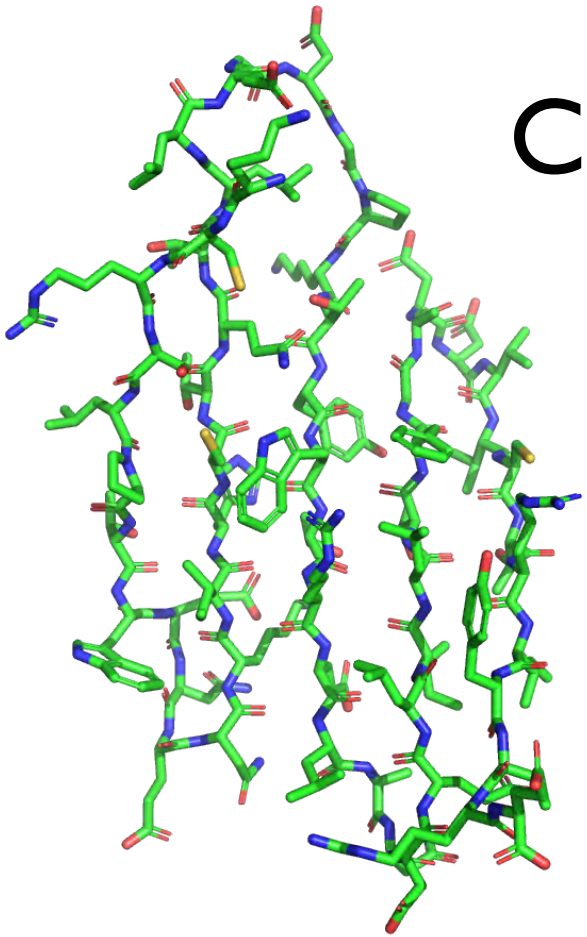


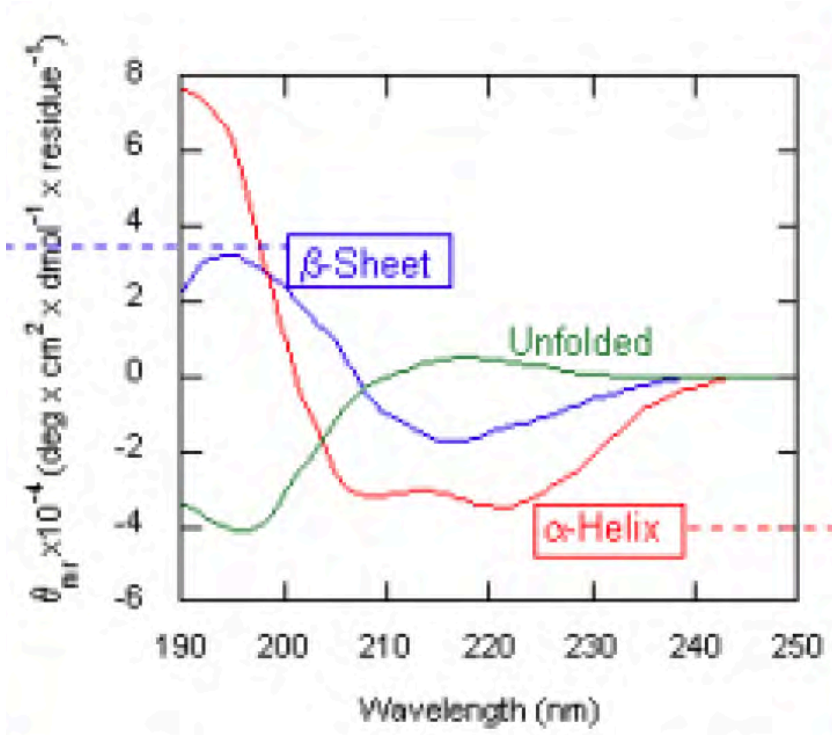
The origin of protein CD is the chiral nature of the polypeptide backbone, whose electronic transitions give rise to distinctive bands in the far UV. phenomenon of CD

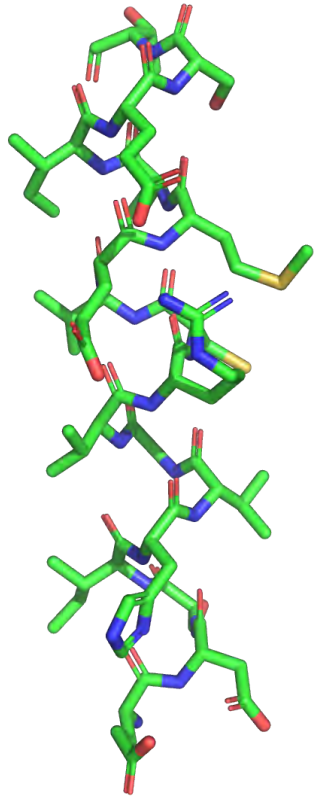
The coupling of electric dipoles ($\pi \rightarrow \pi^*$ electronic transition) in a repeating helical structure leads to one in-phase combination with a net polarization parallel to the helix axis and two out-of-phase combinations with a net polarization perpendicular to the axis.

The electric field of circularly polarized light causes a linear displacement of charge (along the bond) in the $\pi \rightarrow \pi^*$ transition. The magnetic field on the other hand induces a circulation of charge. These two interactions occur at the same time, and the combination of linear and circular displacement leads to a helical movement of electrons with which left and right circularly polarized light interact differently, leading to the phenomenon of CD

Circularly polarized light







$$CD = \Delta A_\lambda = A_\lambda^{LeftCP} - A_\lambda^{RightCP}$$

unitless

$$\varepsilon = \frac{A_\lambda}{Cl}$$ *M-1 cm-1*

$$\Delta\varepsilon_\lambda = \varepsilon_{LeftCP} - \varepsilon_{RightCP} = \frac{\Delta A_\lambda}{C\,l}$$

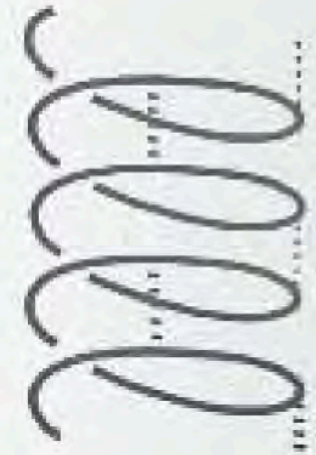
M-1 cm-1

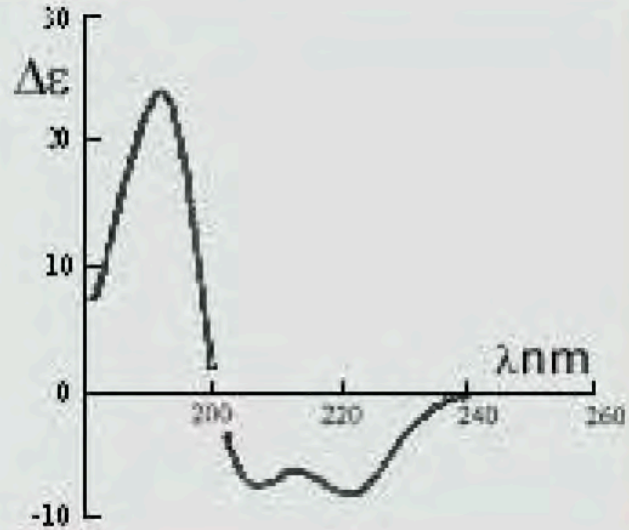
$$\Delta\varepsilon_\lambda^{CMR} = \varepsilon_\lambda^{LeftCP} - \varepsilon_\lambda^{RightCP} = \frac{\Delta A_\lambda}{NC\,l}$$

(M aa)-1 cm-1

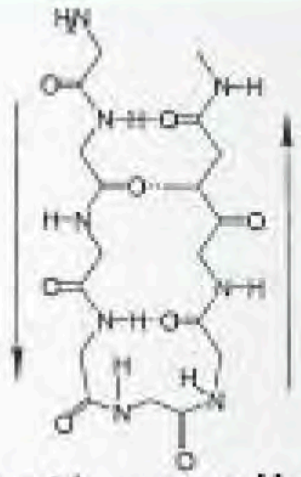
$$CD = \Delta A_\lambda = A_\lambda^{LeftCP} - A_\lambda^{RightCP}$$

α-helix
(H-bonded)

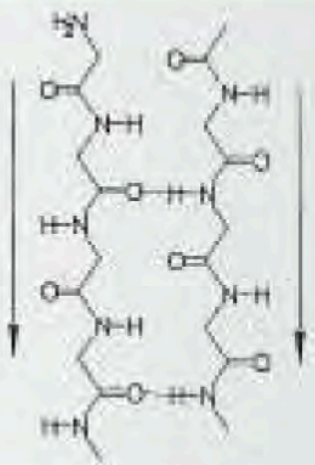




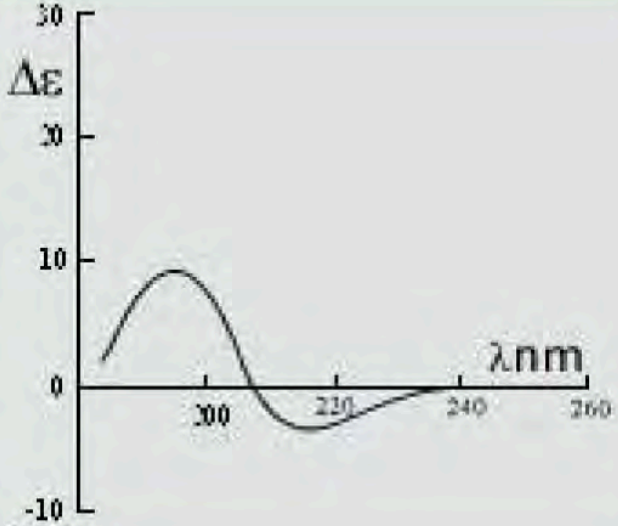
β–sheet
parallel and
anti-parallel
(H-bonded)



anti-parallel



parallel



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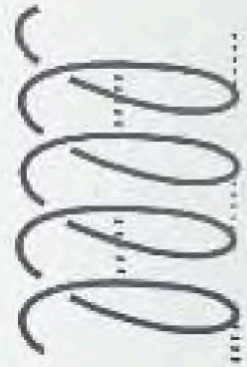
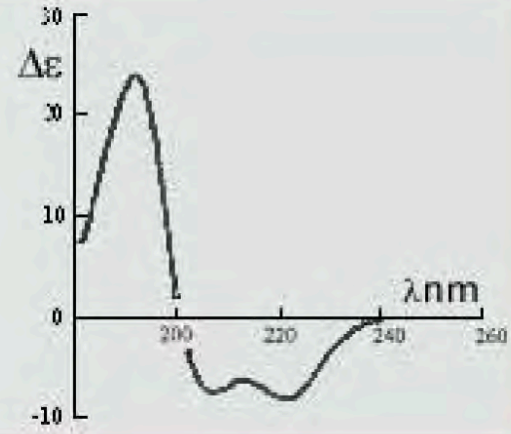
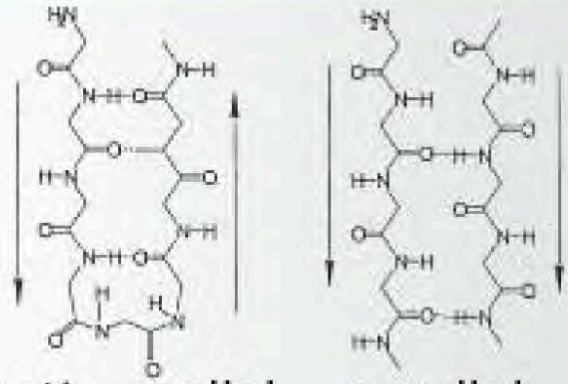
α-helix
(H-bonded)

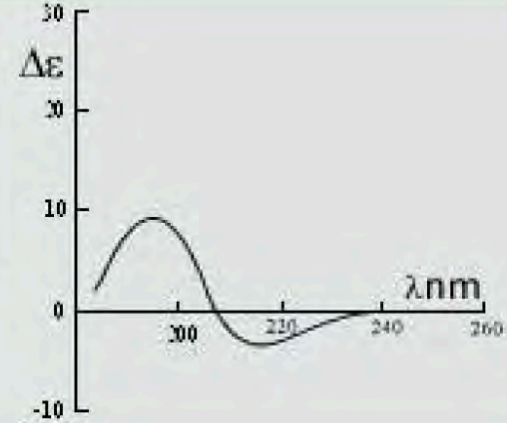




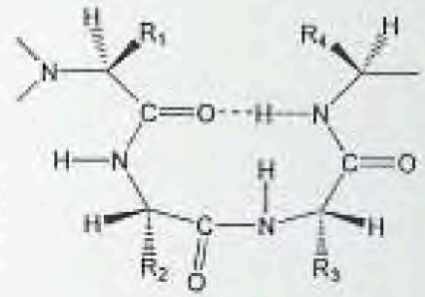
β–sheet
parallel and
anti-parallel
(H-bonded)

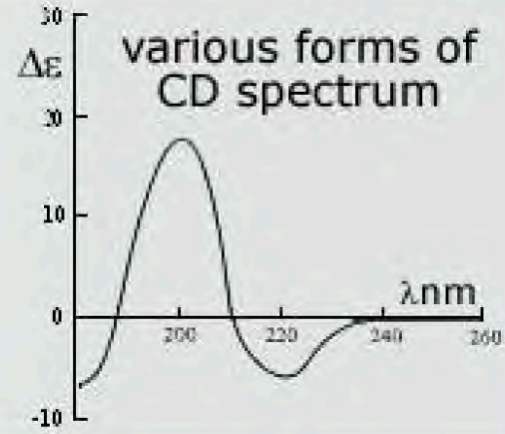


anti-parallel parallel



β-turn
(Type I,II,III.....)
γ–turns
(some turns not
H-bonded in proteins)





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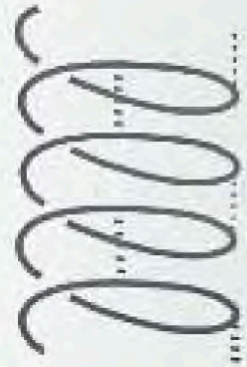
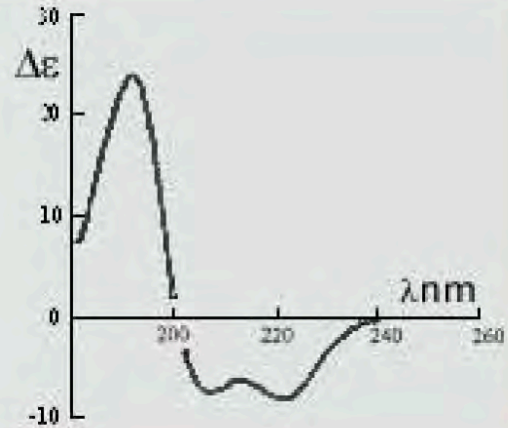
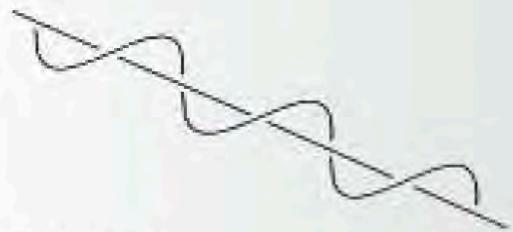
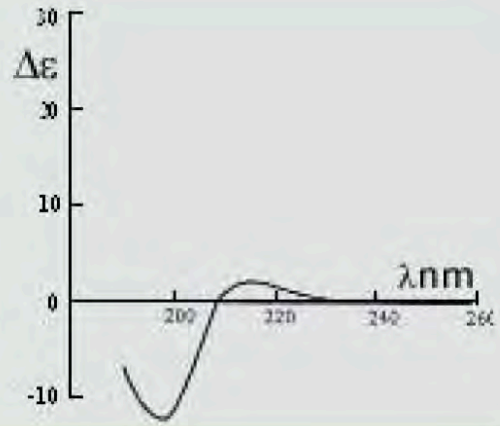


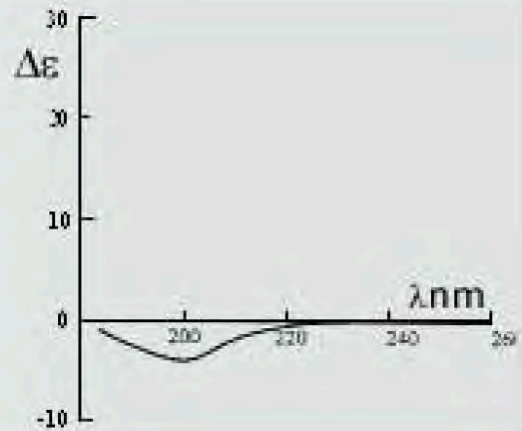


Polyproline
PII helix
31-helix
(H-bonded)
(left hand
extended helix,
no intramolecular H-bonds)

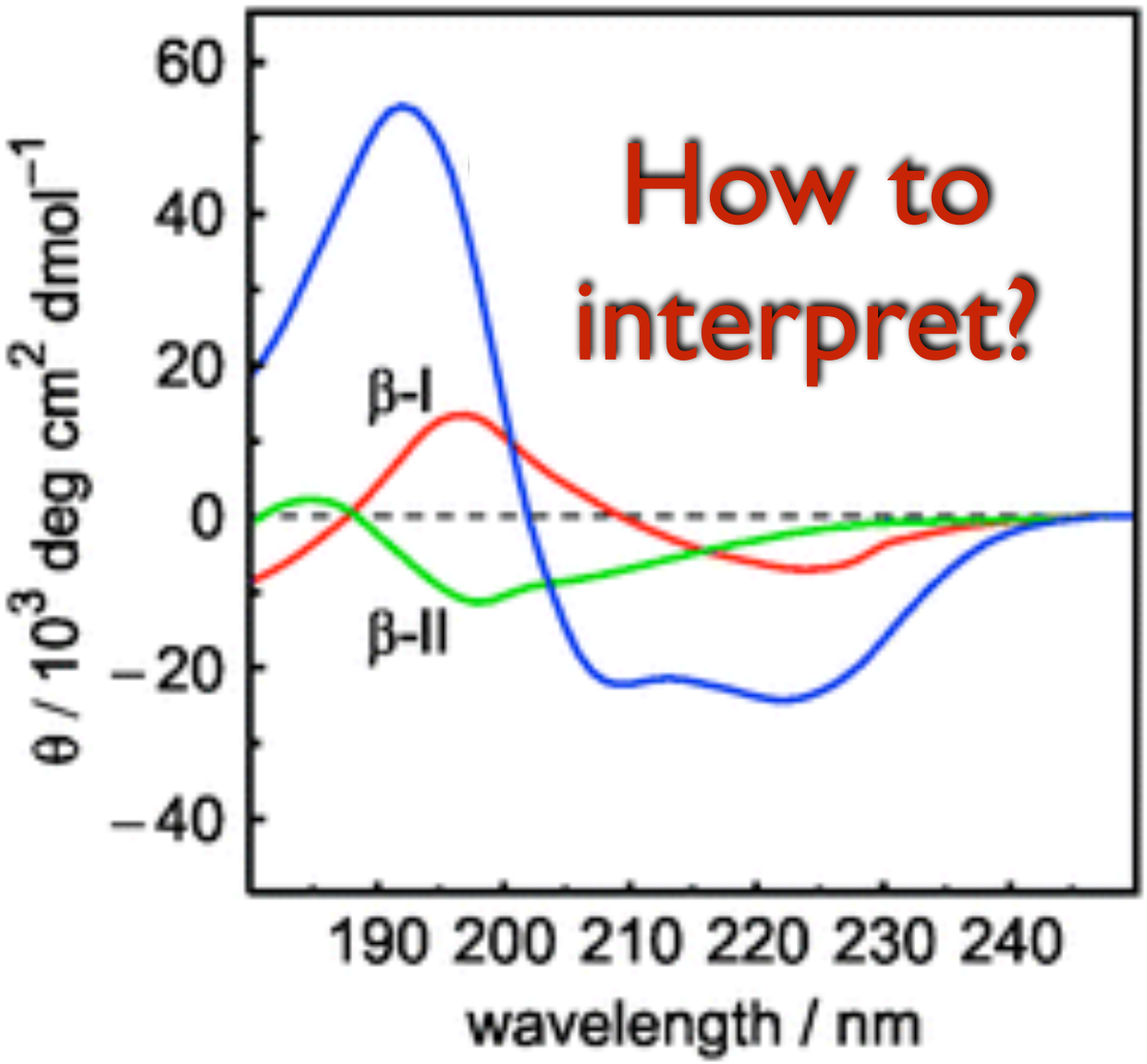


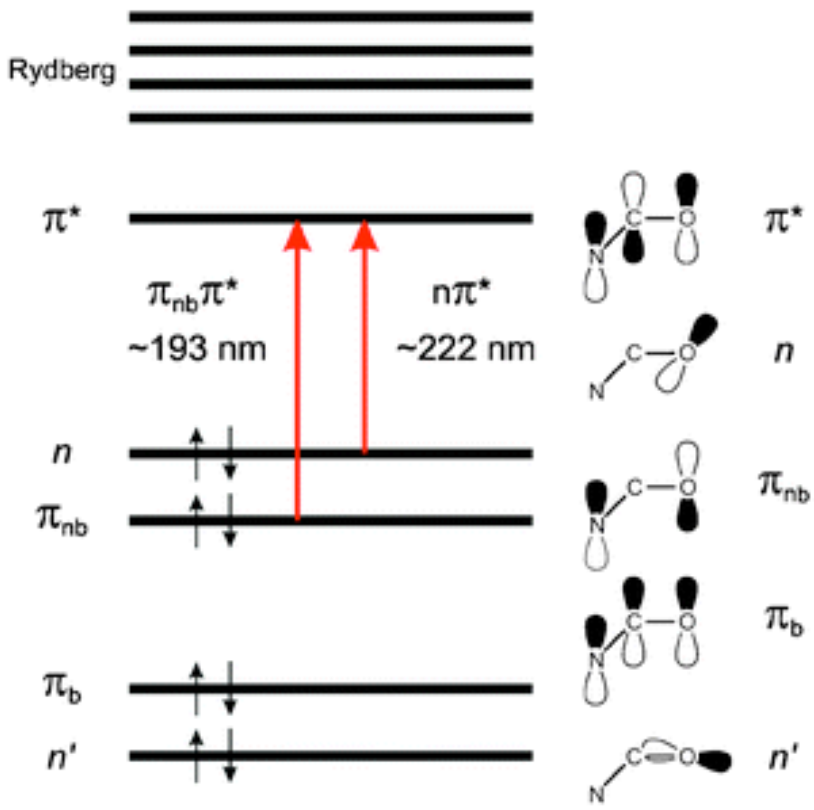


Irregular
(Disordered)



Circular Dichroism - peptide bonds





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Bullheller, Rodger, & Hirst (2007) Phys Chem Chem Phys 9, 2020-2035

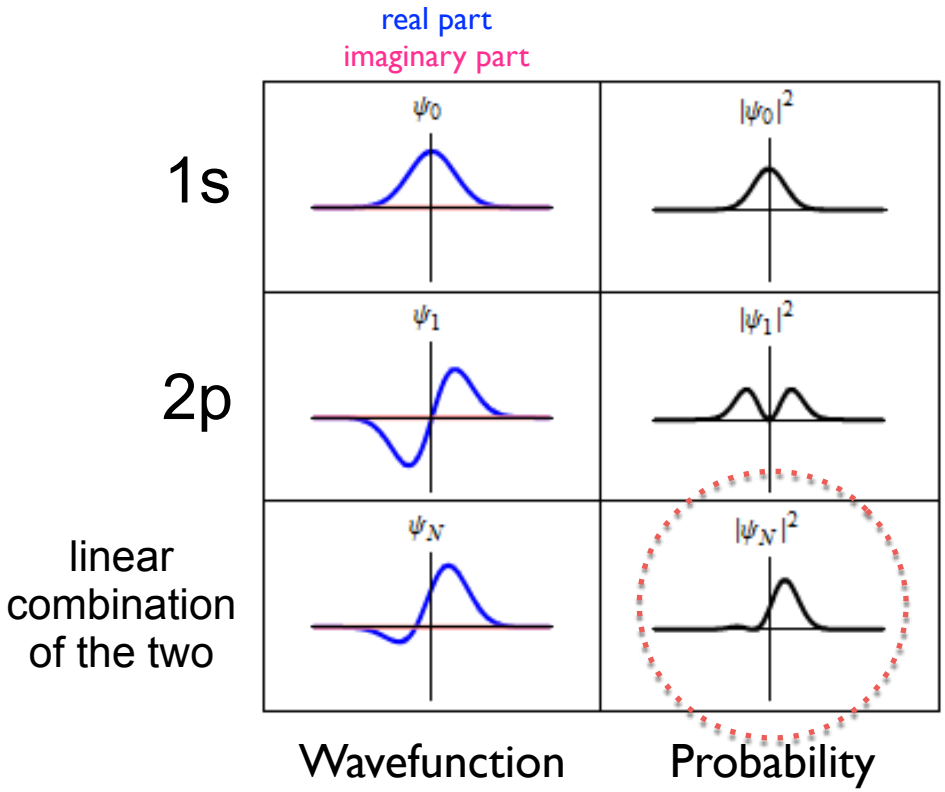
But first…

Zhaolin and Jian have a discussion going in our Moodle Forum on the nature of the transition dipole moment

According to Schroedinger, the electron gets from state 2 to state 1 by a continuous transition through intermediate superposition states. So halfway through our example, the electron is in a state that is a superposition of the 2s and 2p states.

The transition dipole moment is then the actual dipole moment evaluated for the superposition of states.

Note that the “position” of the electron is moving (oscillating) in time - an oscillating dipole!



https://en.wikipedia.org/wiki/Transition_dipole_moment

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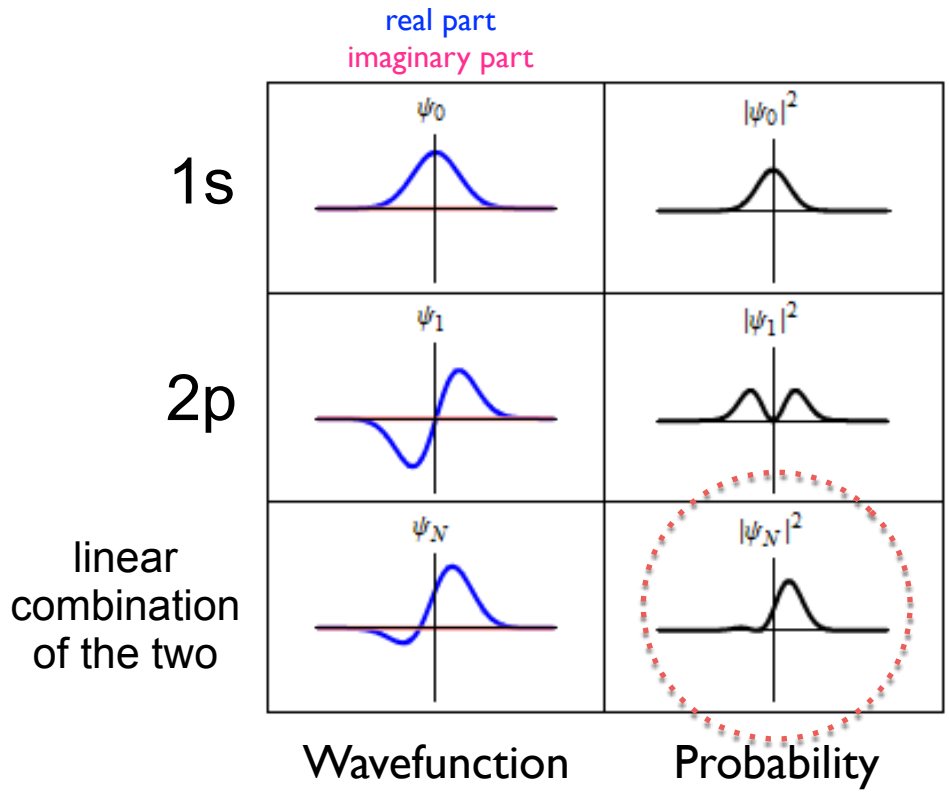
Note that 1s -> 2p transition is “forbidden” - why?



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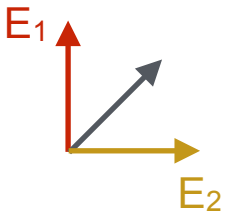

From last time

Nathanael asked about circularly polarized light and linearly polarized light. In fact, you can make one with combinations of the other. See this Kahn Academy video
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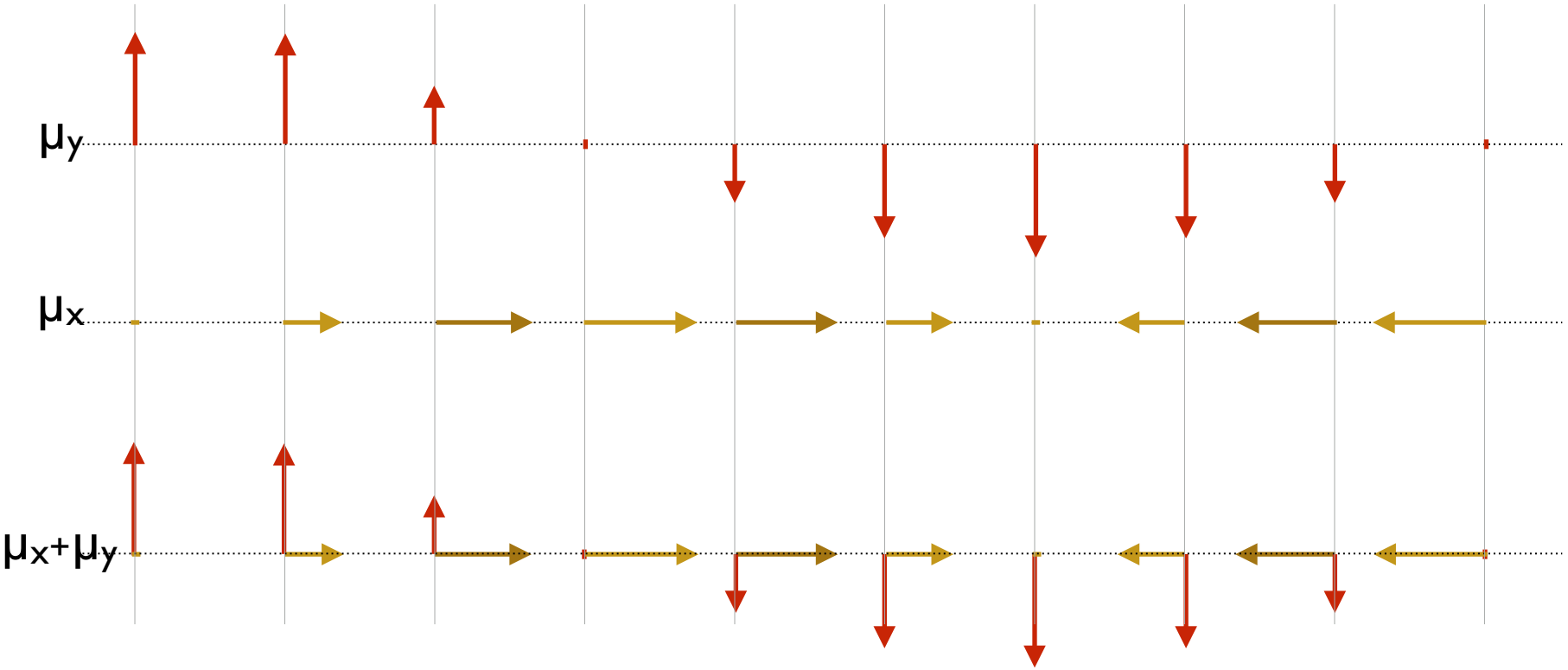

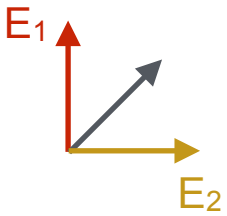

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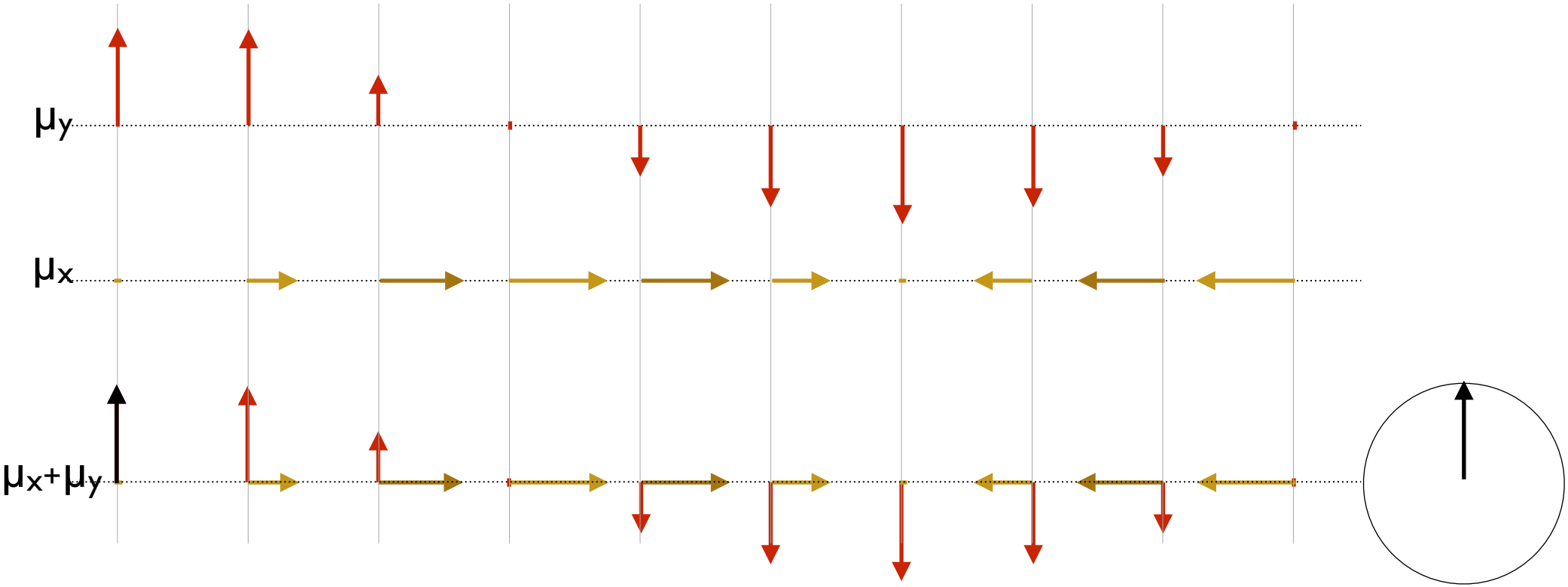

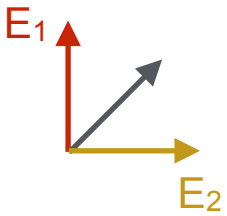

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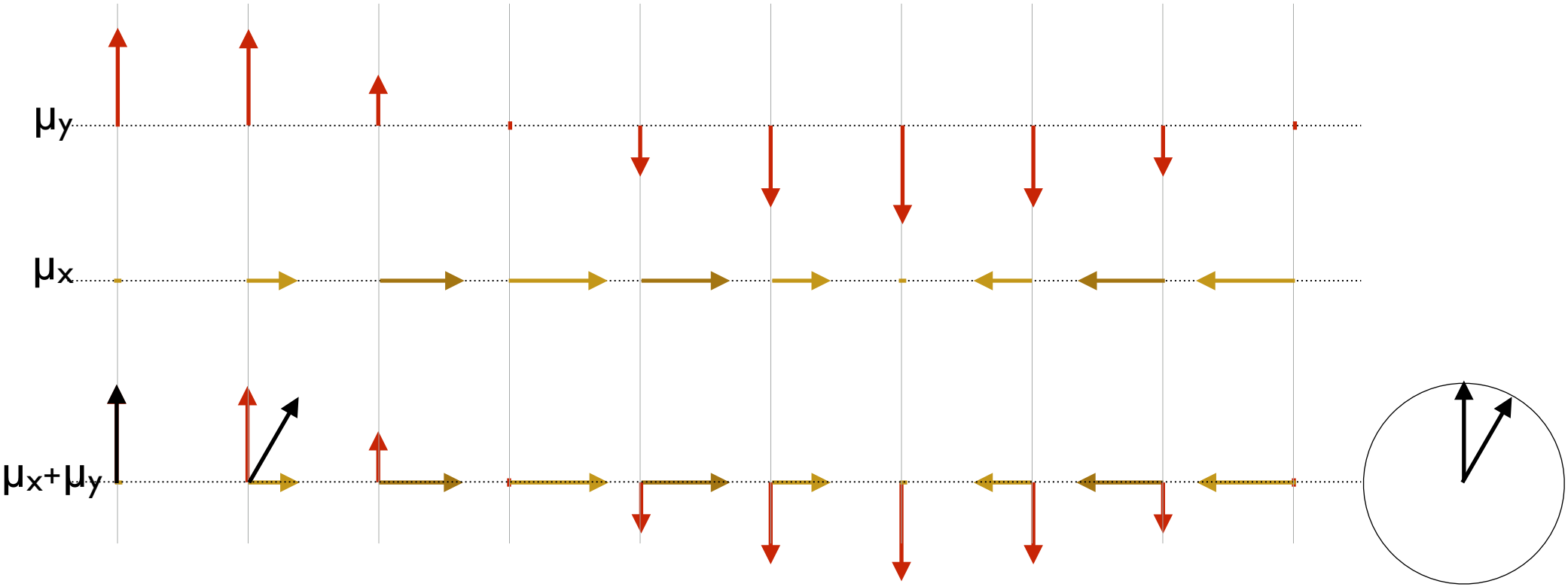

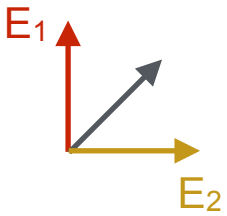

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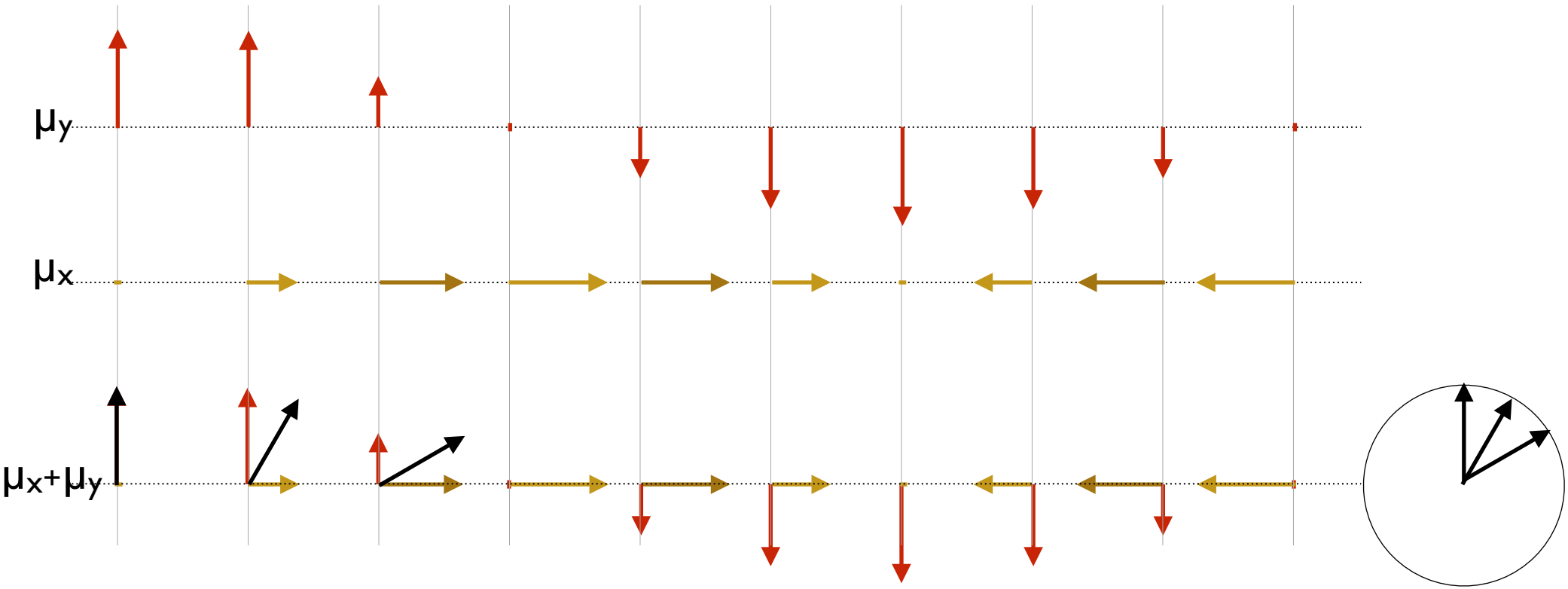

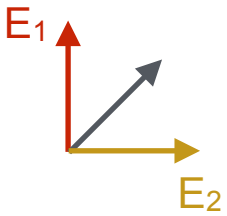

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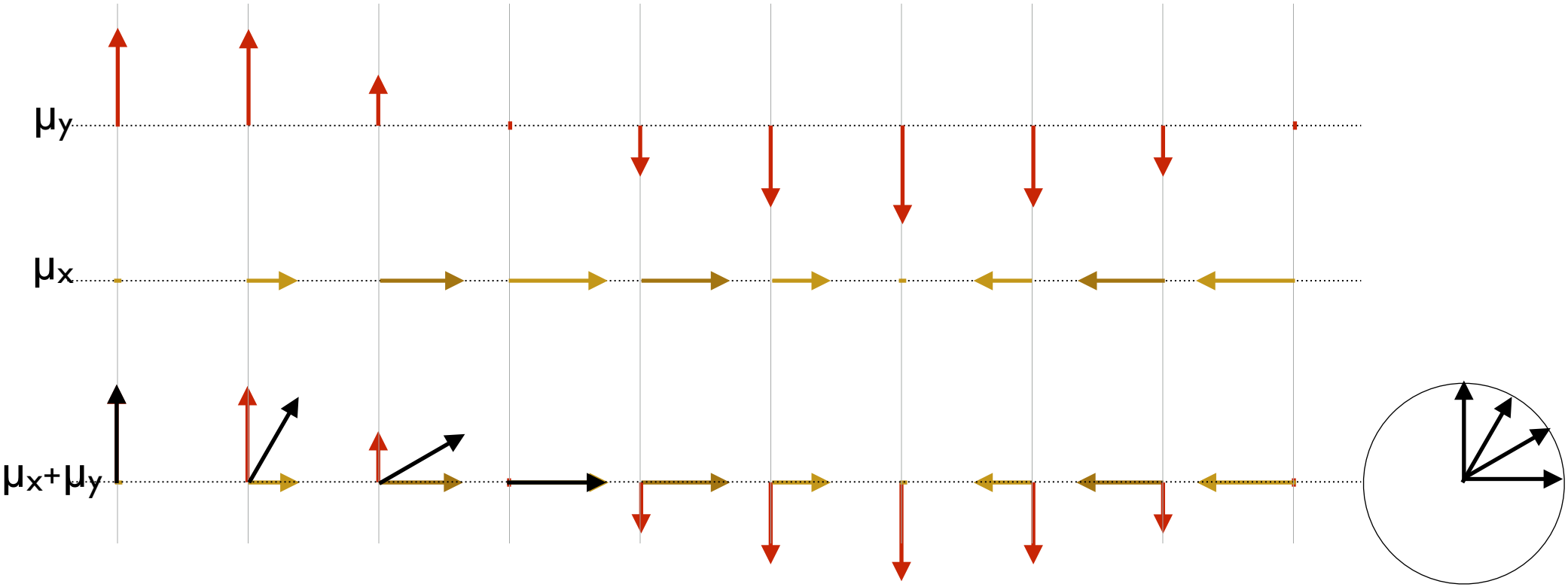

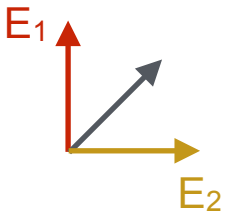

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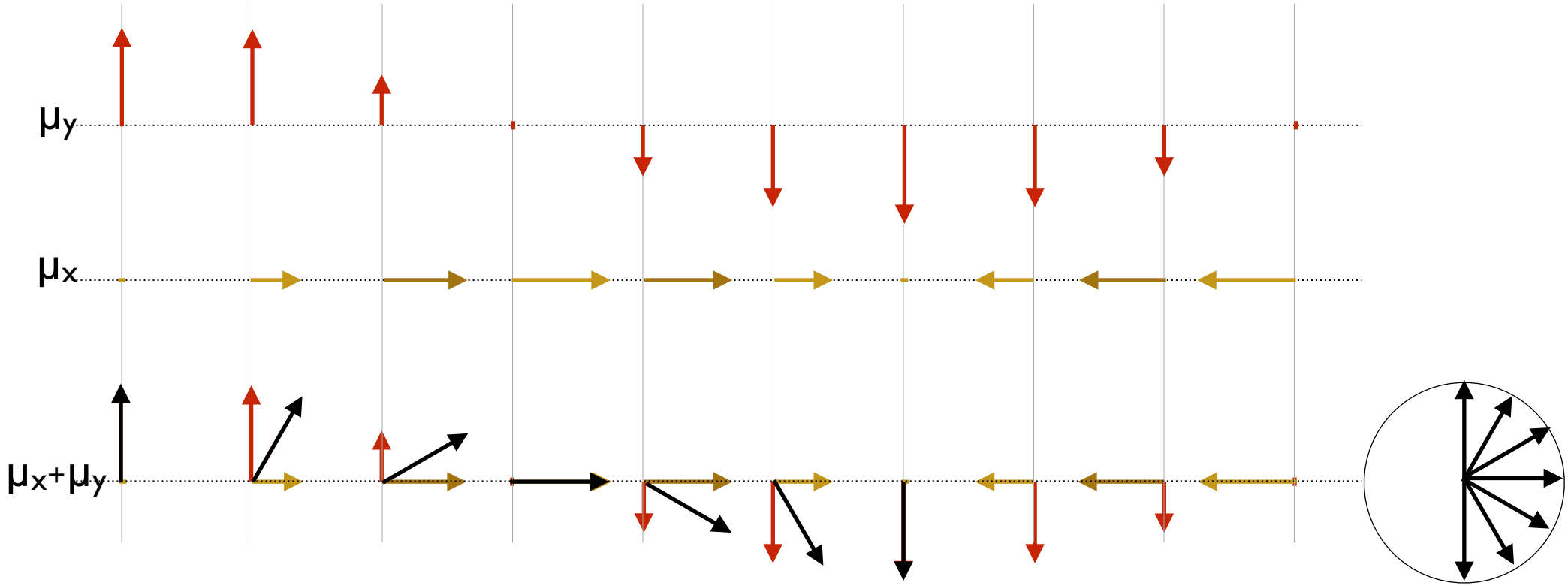

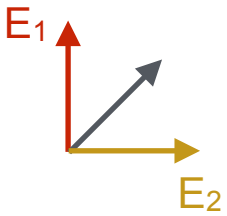

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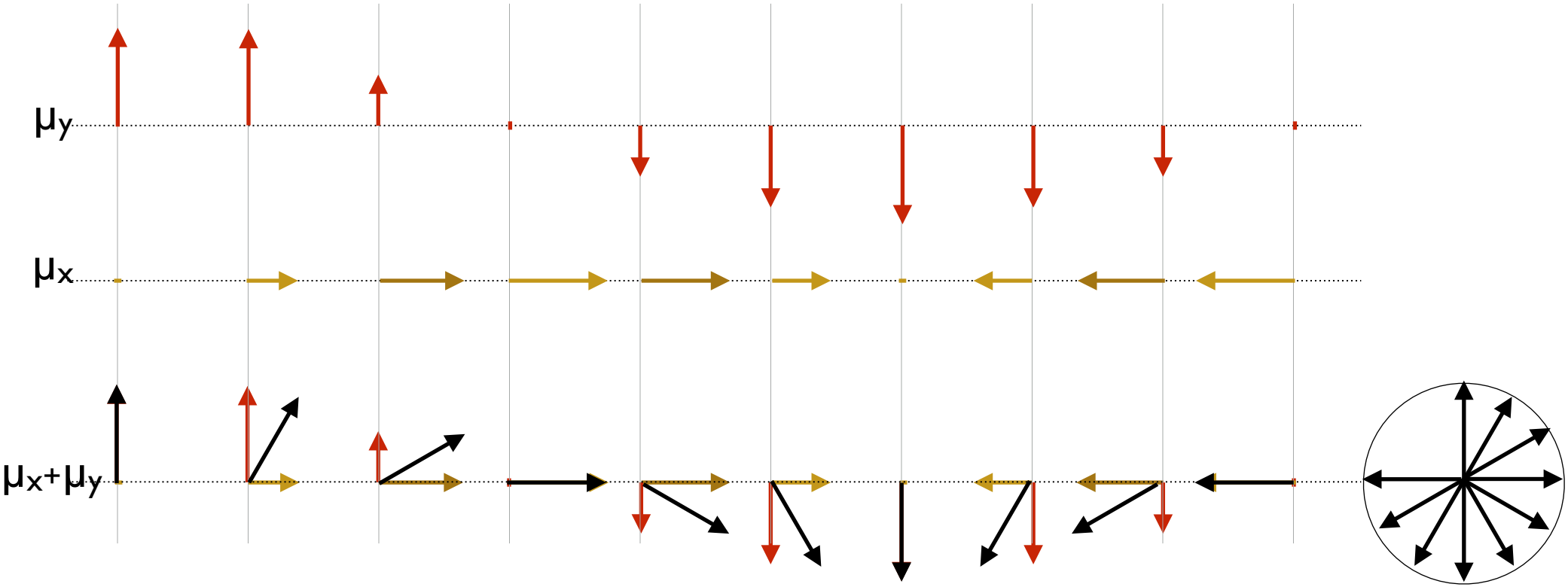

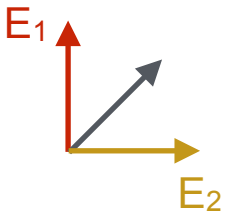

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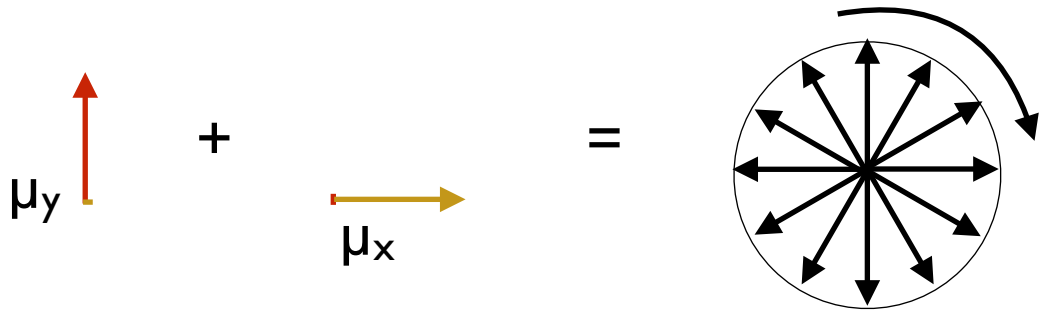
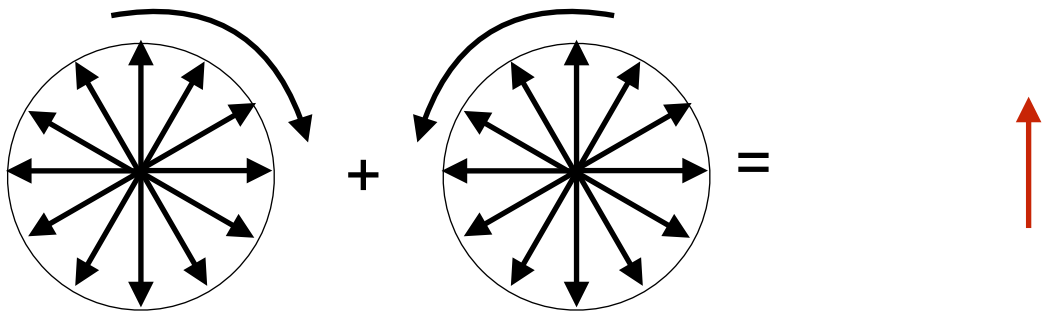
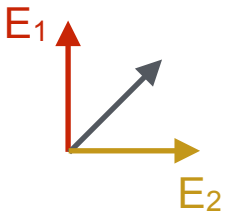




equal parts



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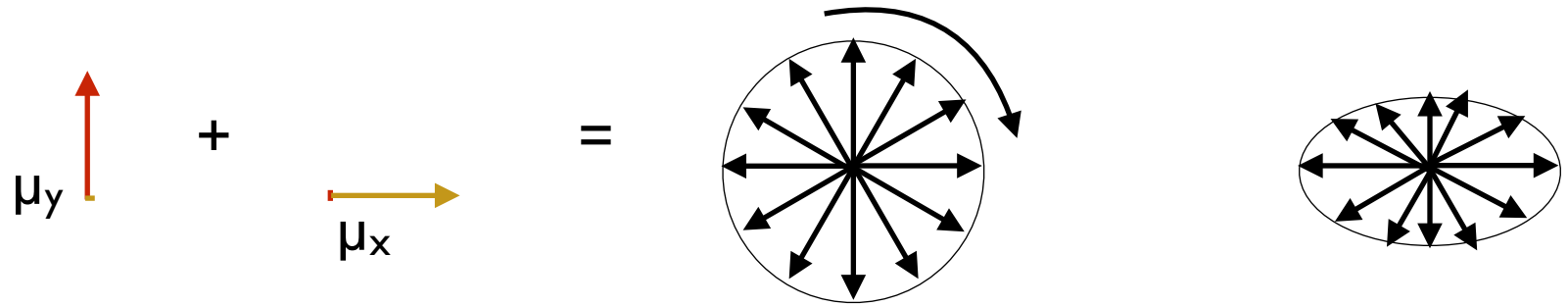
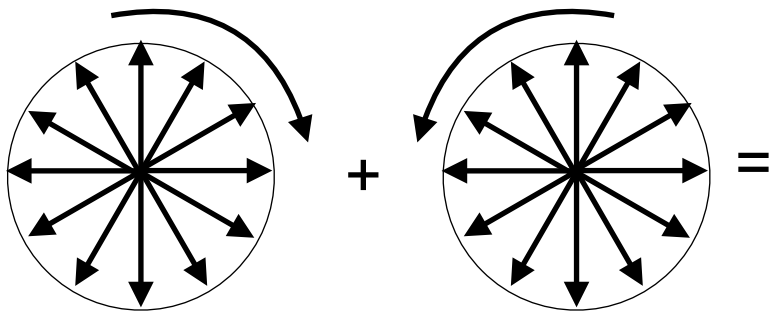


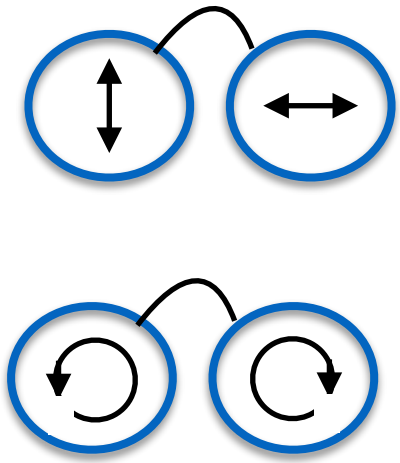


equal parts

unequal parts

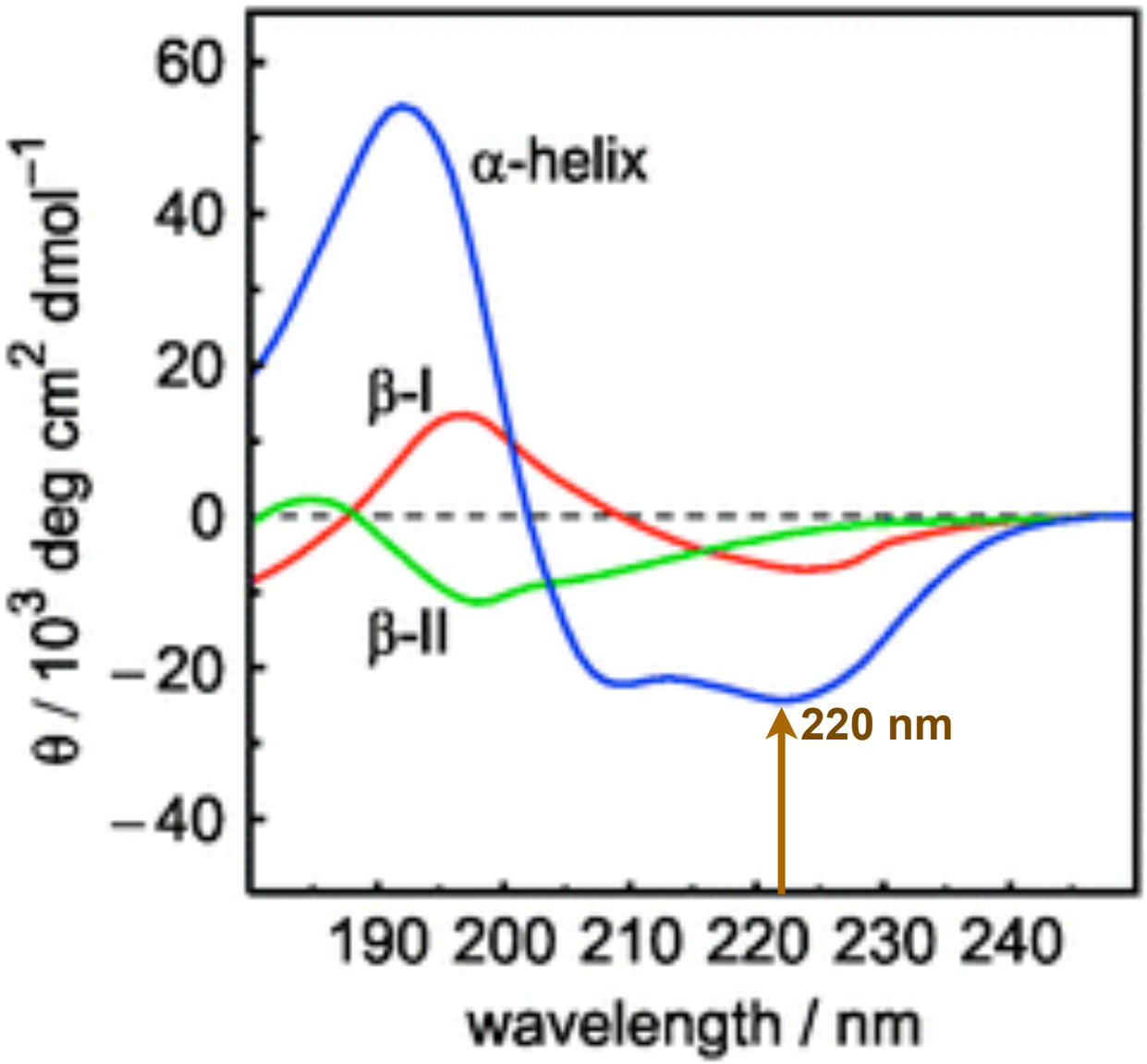
In the reel world

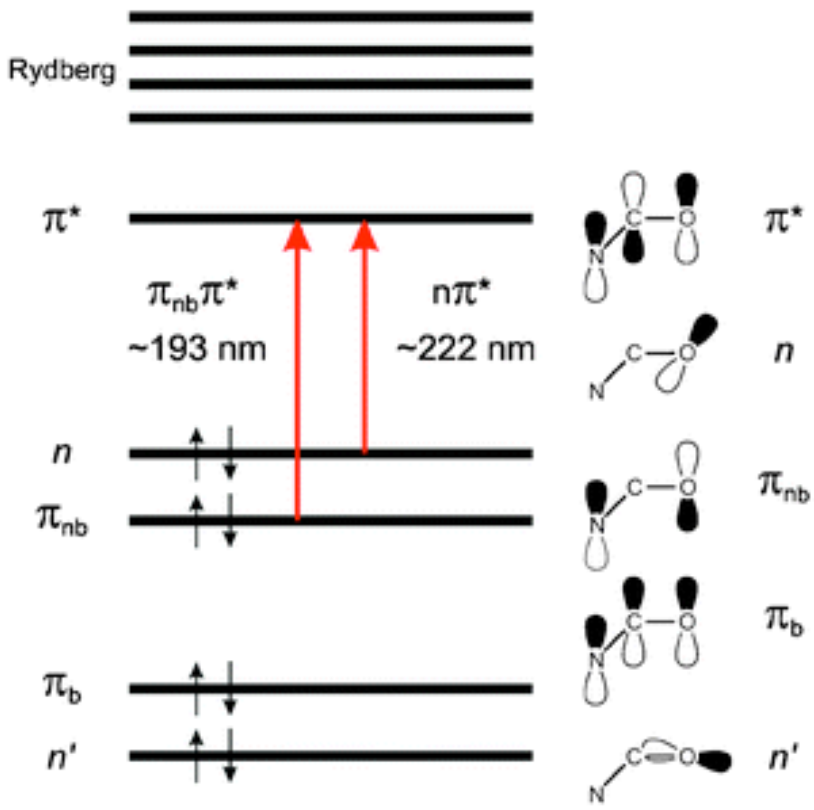
Where have you used
circular dichroism
outside of science?



3D movies use circular polarization and left vs right absorbing chromophores!!

Circular Dichroism - peptide bonds





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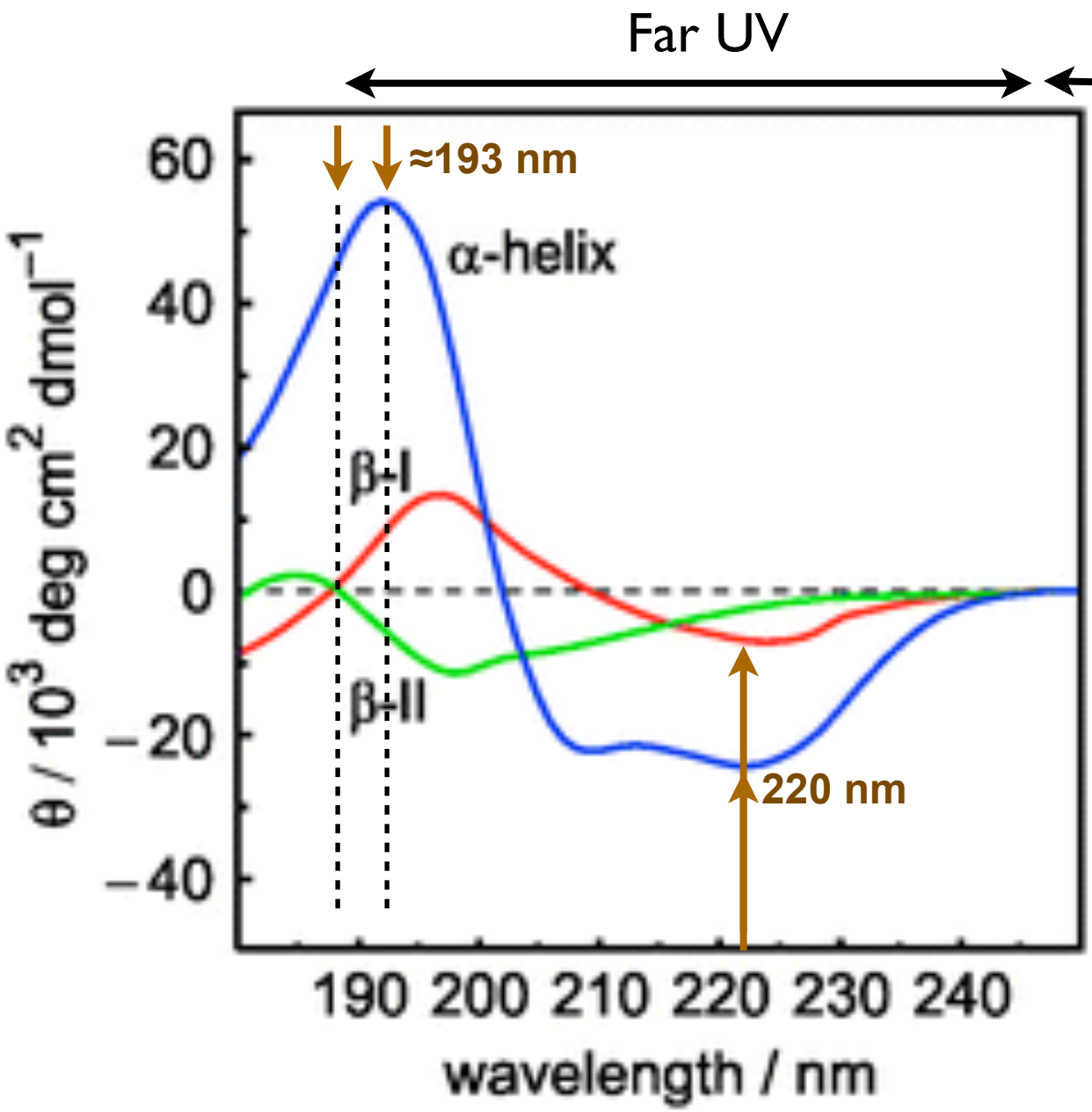
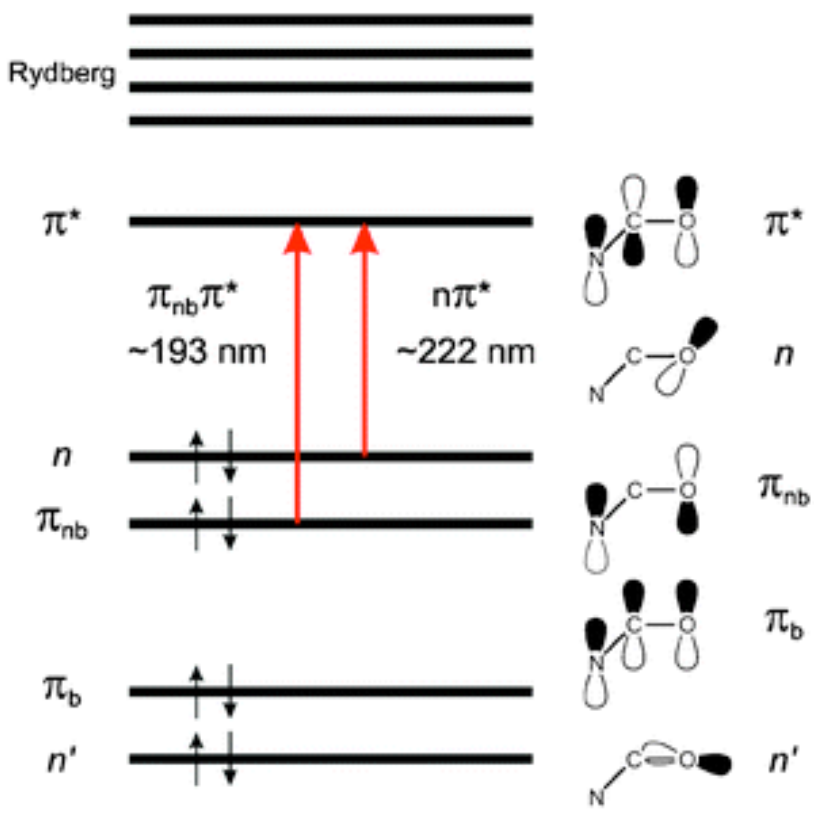
Bullheller, Rodger, & Hirst (2007) Phys Chem Chem Phys 9, 2020-2035

Circular Dichroism - peptide bonds





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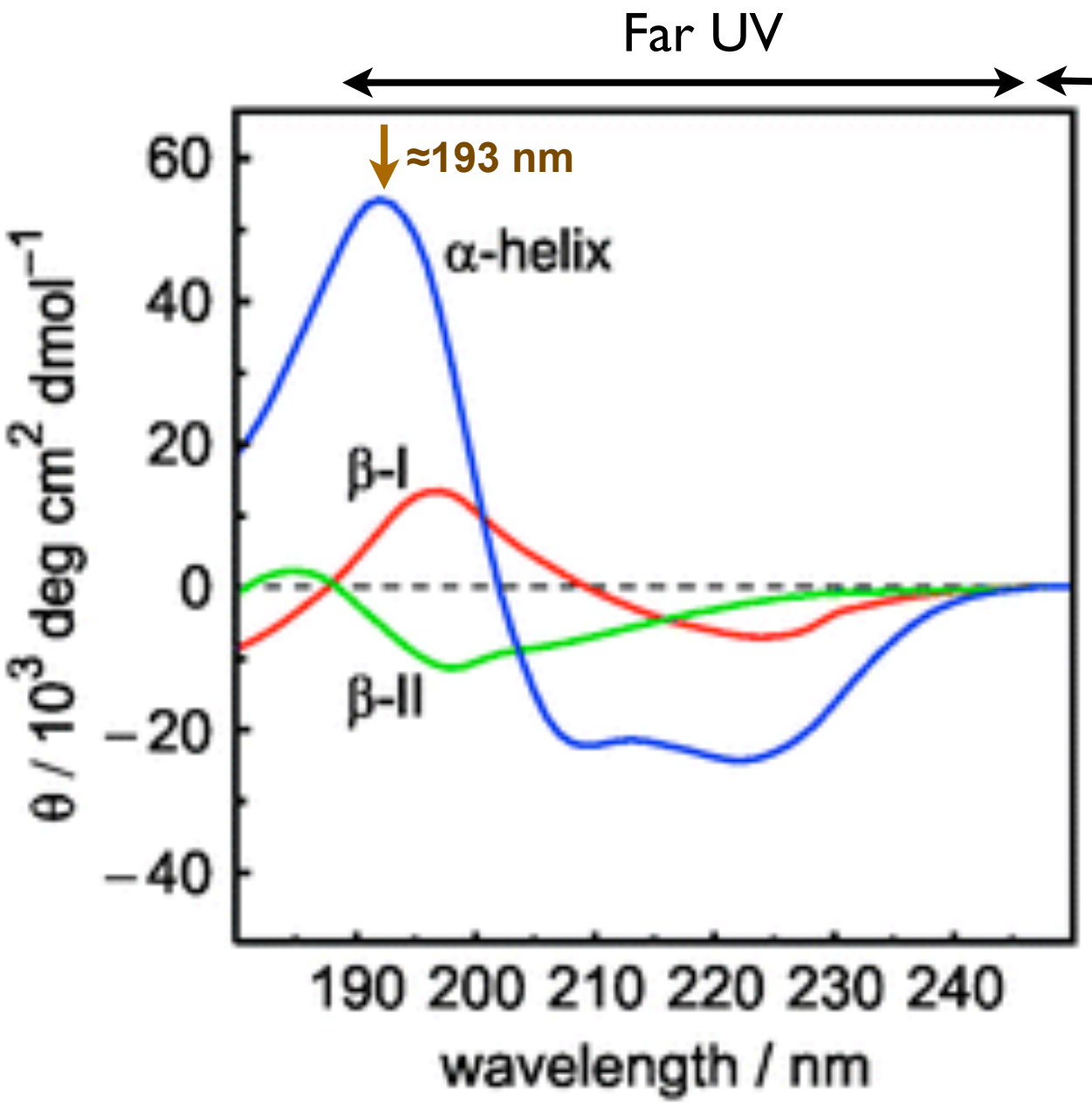
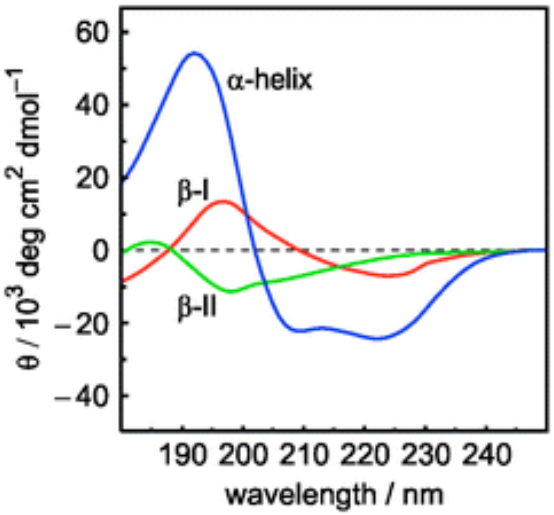


Traditional approaches for discerning structure from CD include using spectral "basis sets." The fit involves adding together varying ratios of each contributor until the summed spectrum best fits the experimental spectrum.

Simple fits might include only 2-3 contributing spectra, others might use more complex basis sets (but see warning to come about doing so!).

$$CD = \Delta A_\lambda = A_\lambda^{LeftCP} - A_\lambda^{RightCP}$$



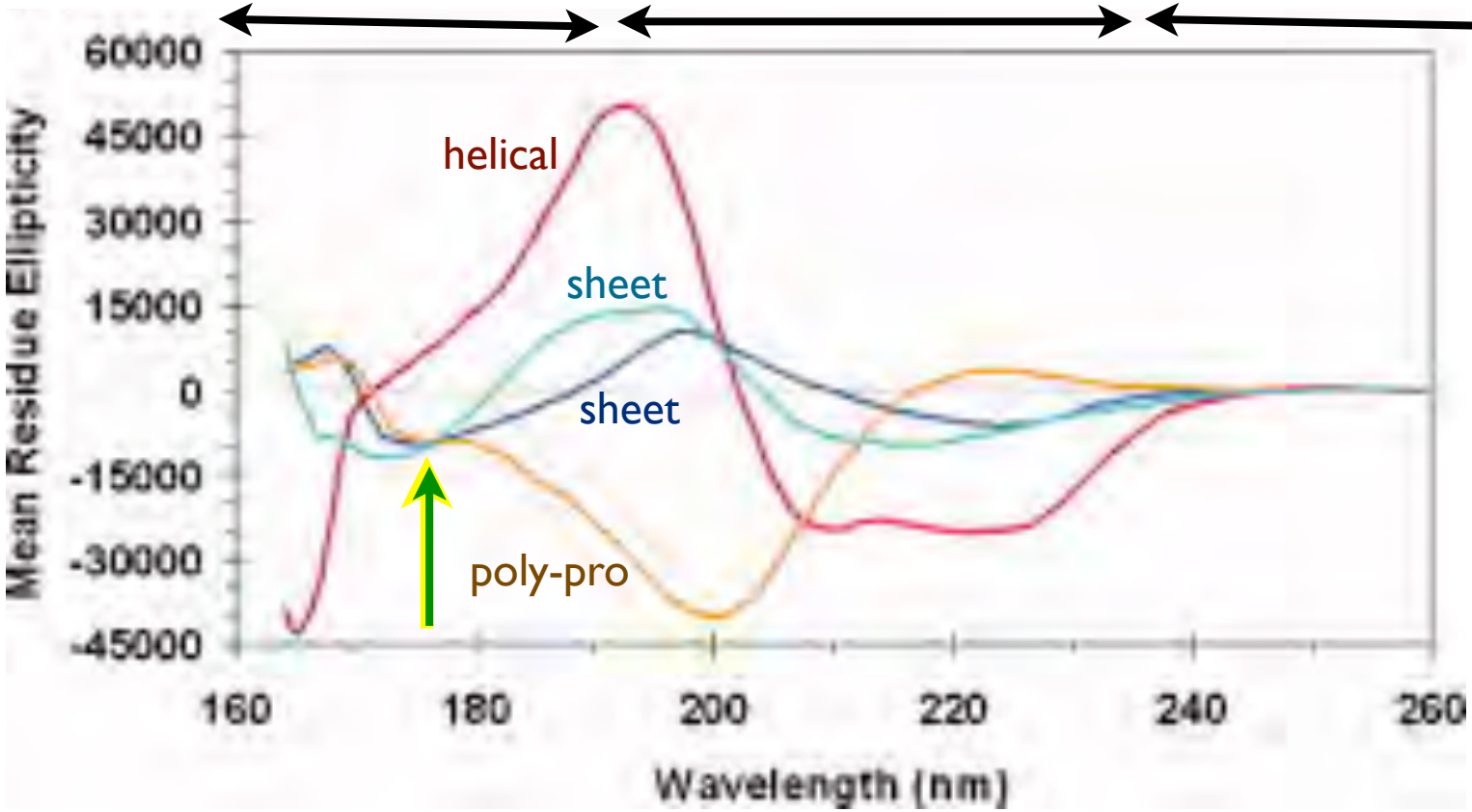
Absorption by H_2O

SRCD
(synchrotron radiation CD)

VUV
(very low wavelength)

Far UV

Near UV



Whitmore & Wallace (2007) Biopolymers 89, 392-400

On-line Tools

Predict 2° structure from spectra

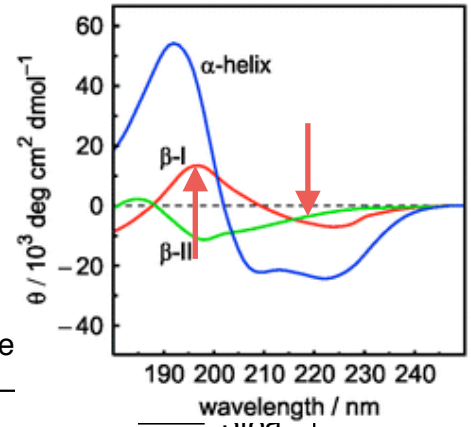
DICHROWEB http://www.cryst.bbk.ac.uk/cdweb

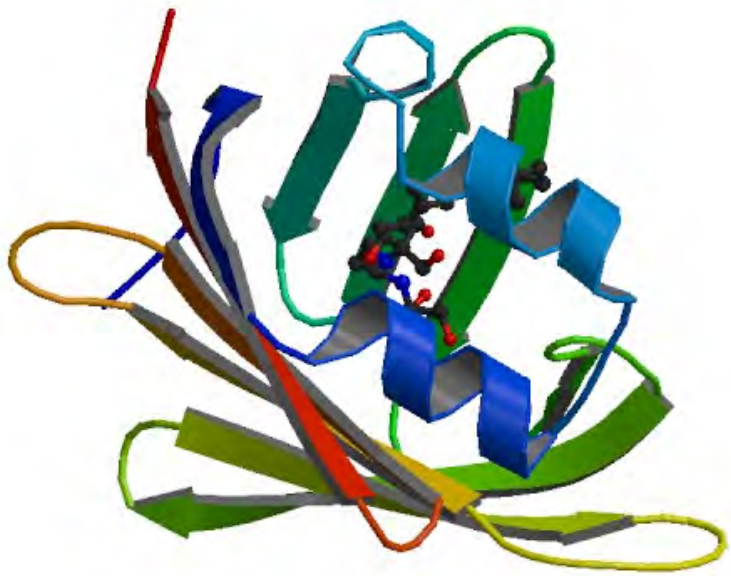
Predict spectra from 3D structure

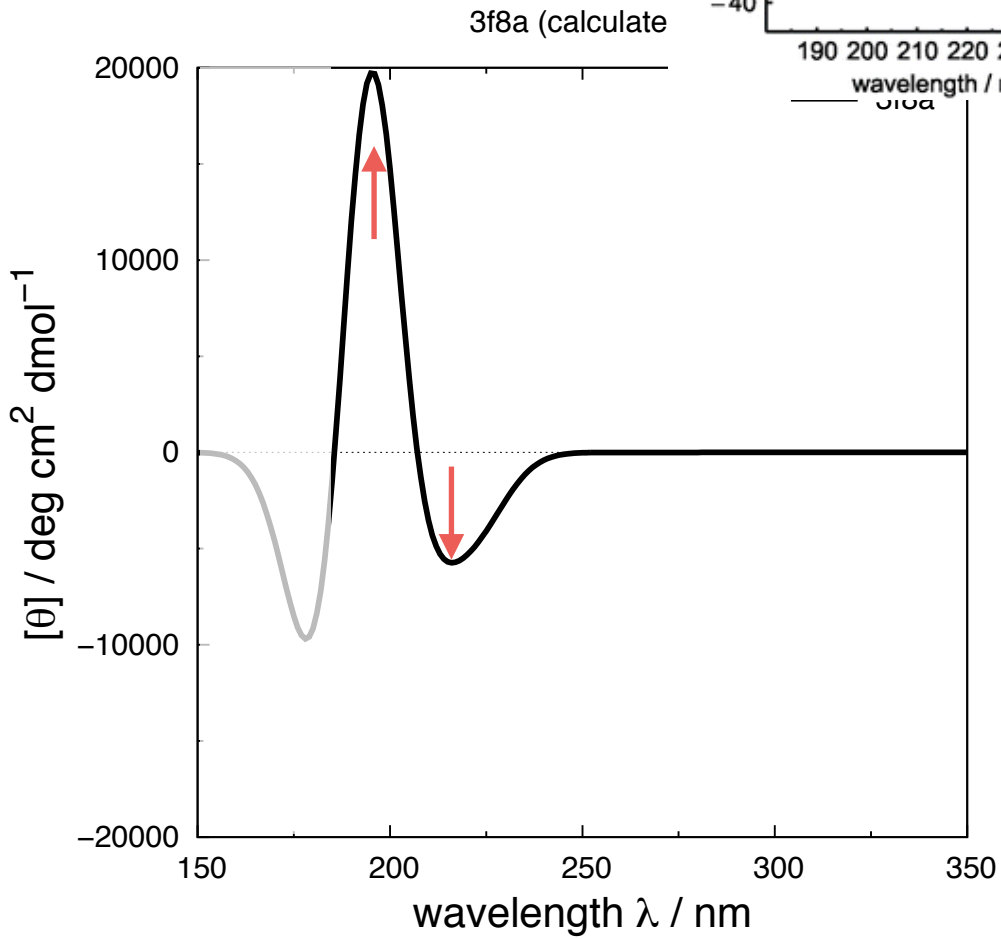
DICHROCalc http://comp.chem.nottingham.ac.uk/dichrocalc/

CD Spectroscopy

Cellular Retinoic Acid Binding Protein





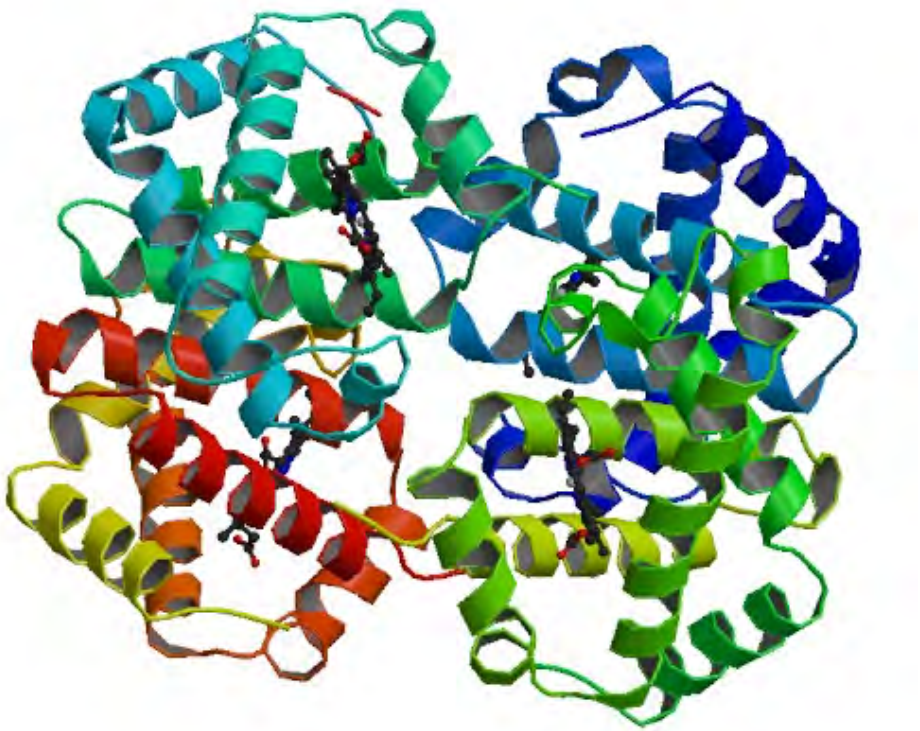


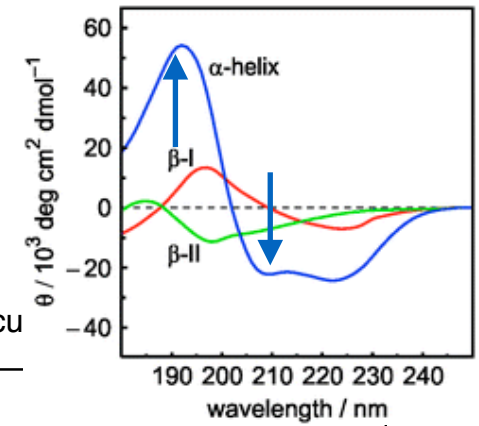
Predict spectra from 3D structure

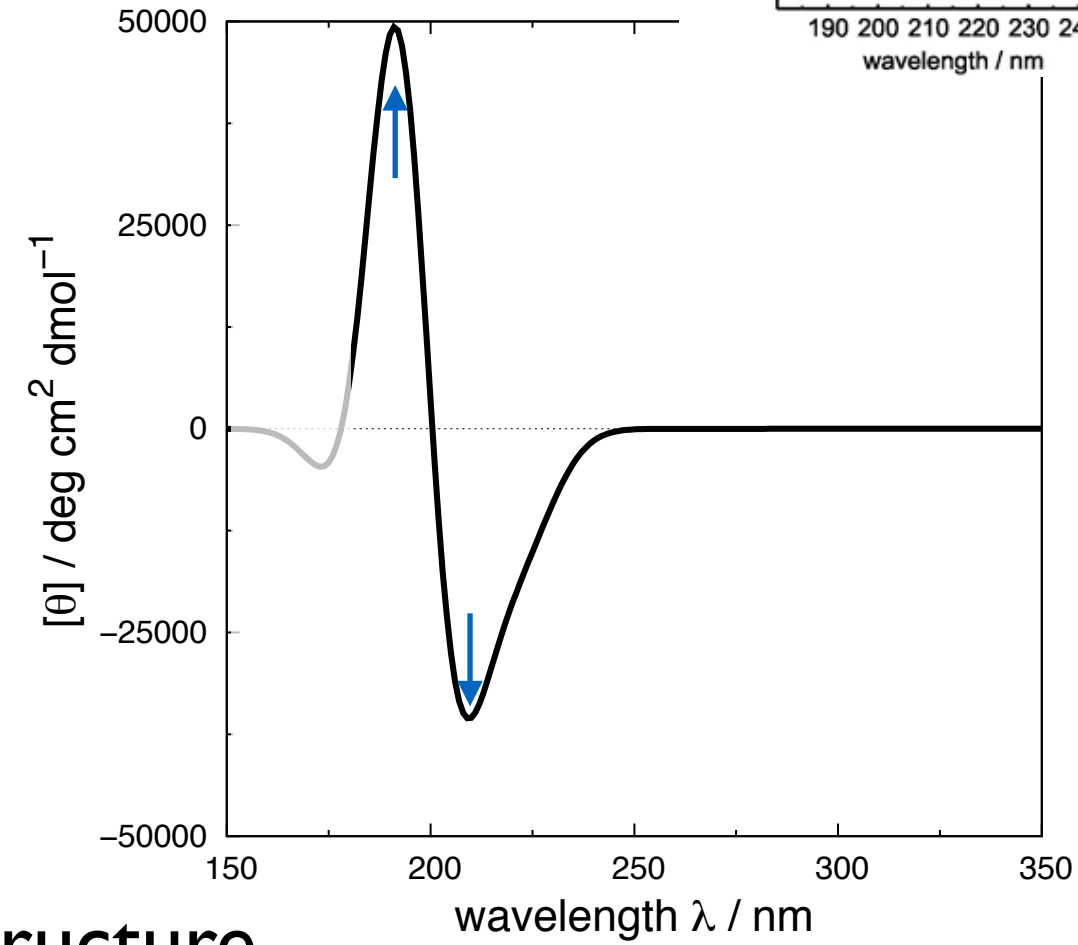
DICHROCalc http://comp.chem.nottingham.ac.uk/dichrocalc/

CD Spectroscopy

Hemoglobin



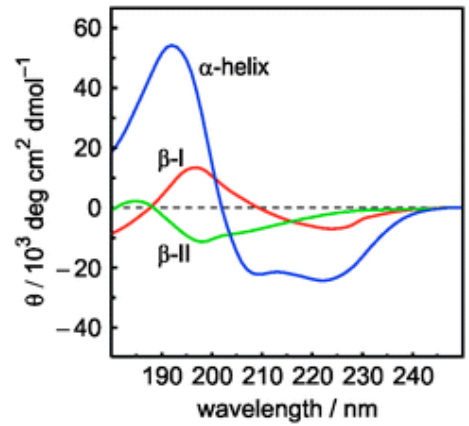




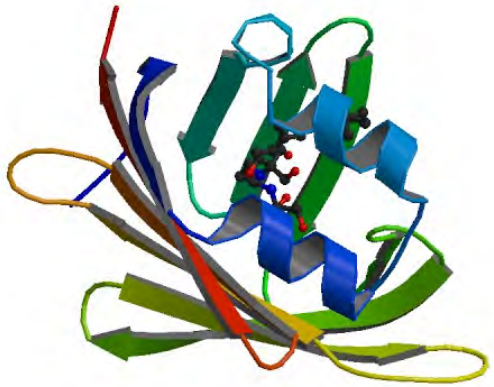
Predict spectra from 3D structure

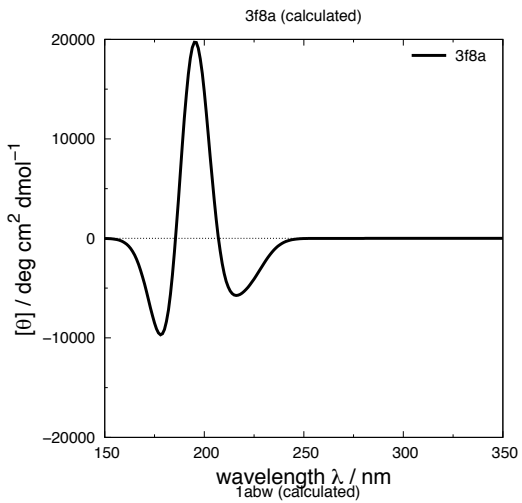
DICHROCalc http://comp.chem.nottingham.ac.uk/dichrocalc/

CD Spectroscopy



Cellular Retinoic Acid Binding Protein

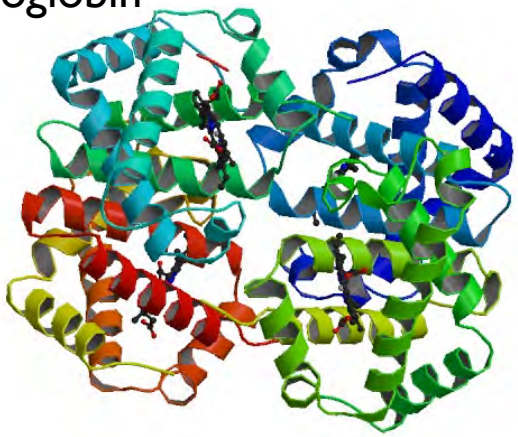


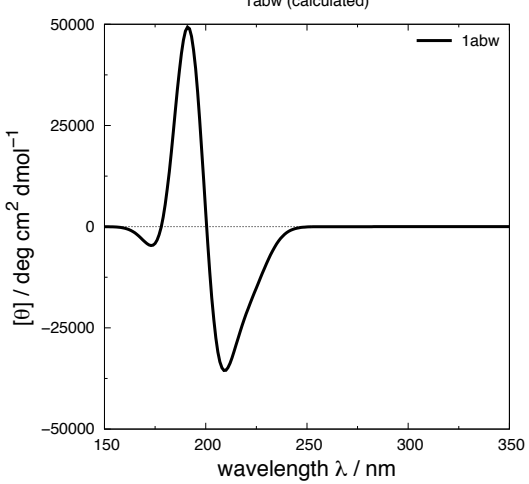


+20,000

-20,000

Hemoglobin





+50,000

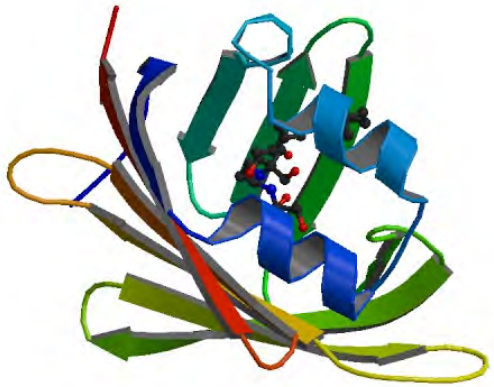
-50,000

Predict spectra from 3D structure

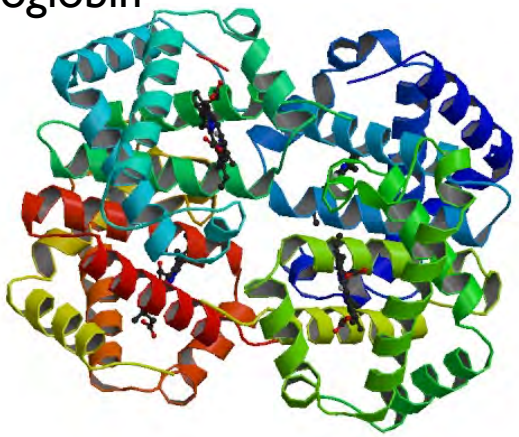
DICHROCalc http://comp.chem.nottingham.ac.uk/dichrocalc/

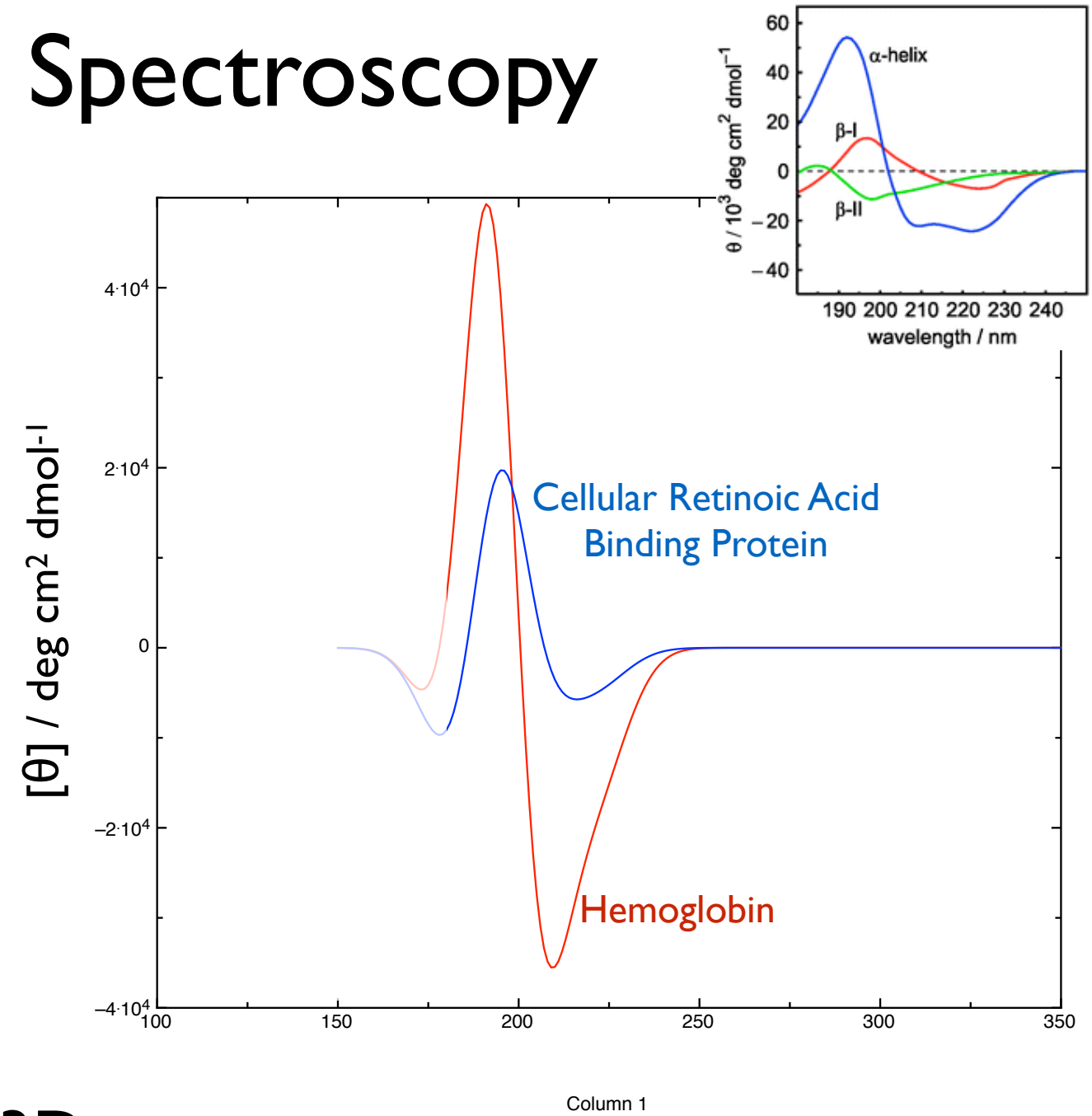
CD Spectroscopy

Cellular Retinoic Acid Binding Protein



Hemoglobin



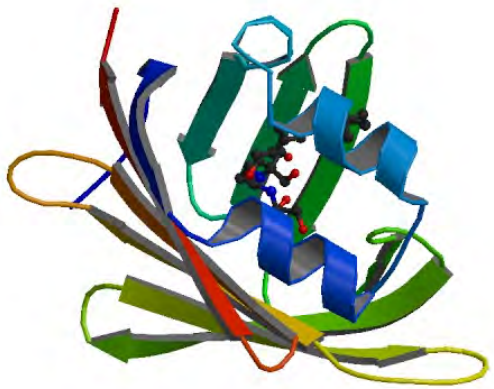


Predict spectra from 3D structure

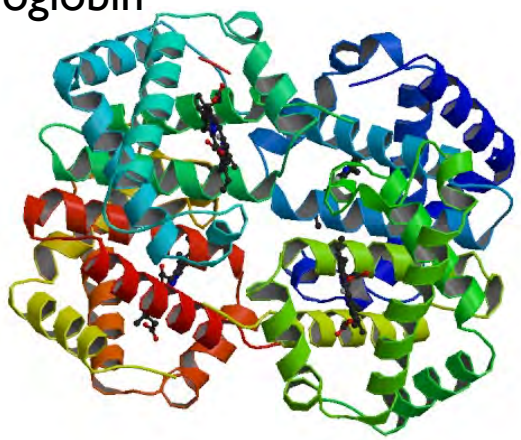
DICHROCalc http://comp.chem.nottingham.ac.uk/dichrocalc/

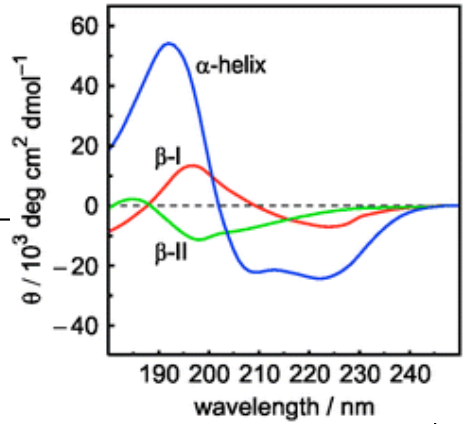
CD Spectroscopy

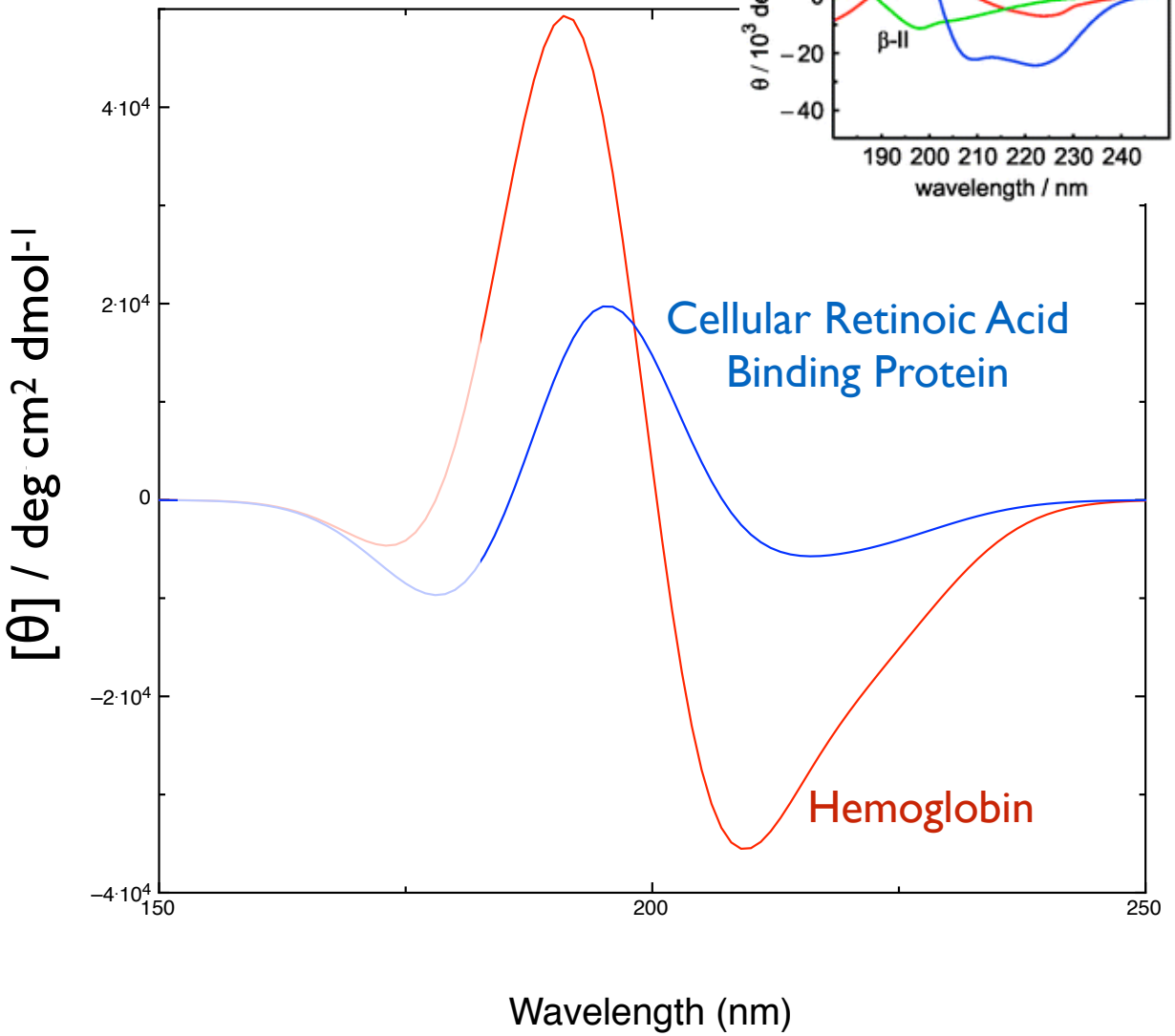
Cellular Retinoic Acid Binding Protein



Hemoglobin





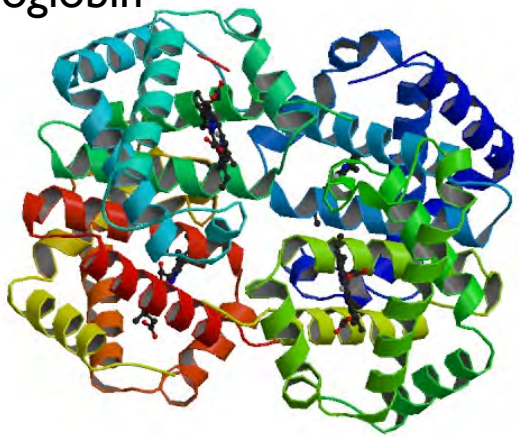


Predict spectra from 3D structure

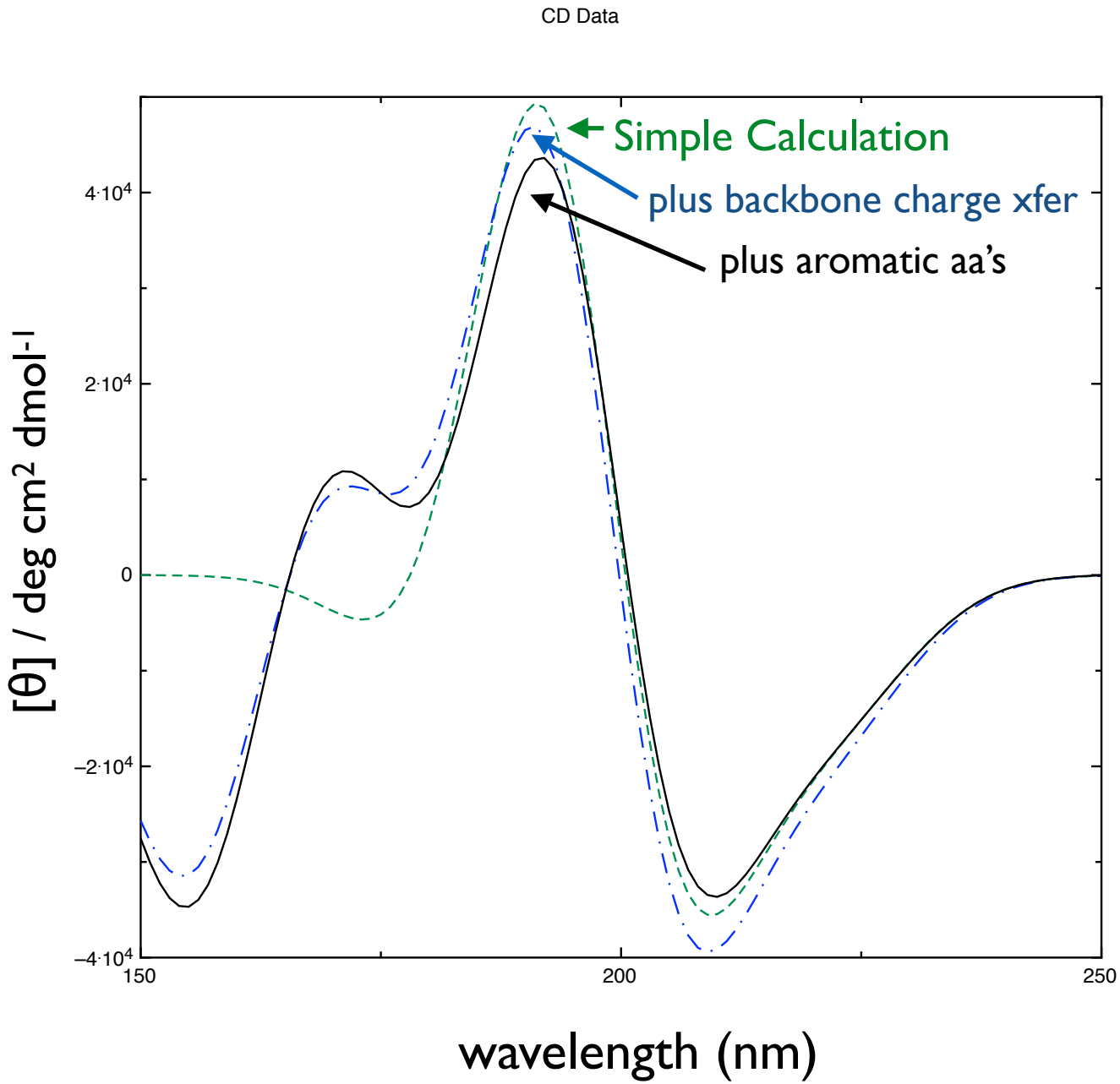
DICHROCalc http://comp.chem.nottingham.ac.uk/dichrocalc/

Not so simple

Hemoglobin

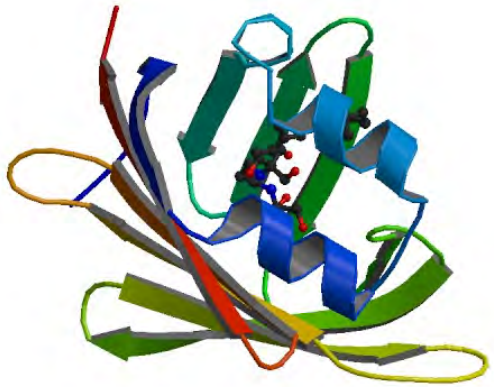


Ab initio and other computational approaches are trying to go beyond using simple basis sets. As they do, they try to include more and more sophisticated interactions, including backbone-backbone charge transfer transitions, and aromatic aa-backbone CT transitions. Most of the changes are toward the far UV, as shown at right.

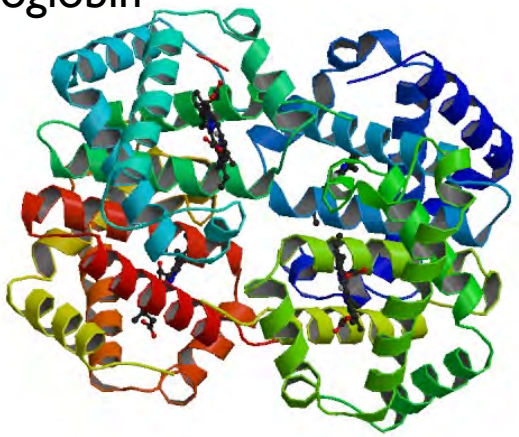


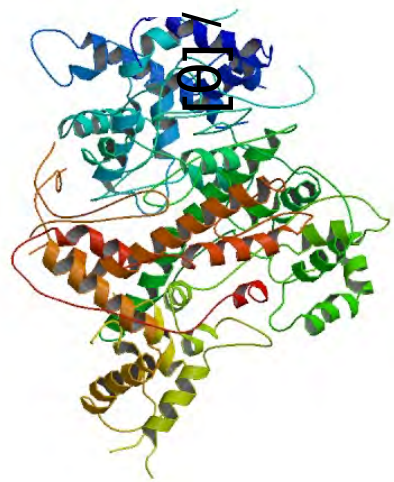
CD Spectroscopy

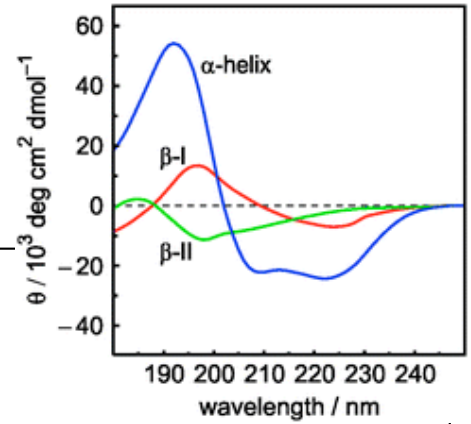
Cellular Retinoic Acid Binding Protein

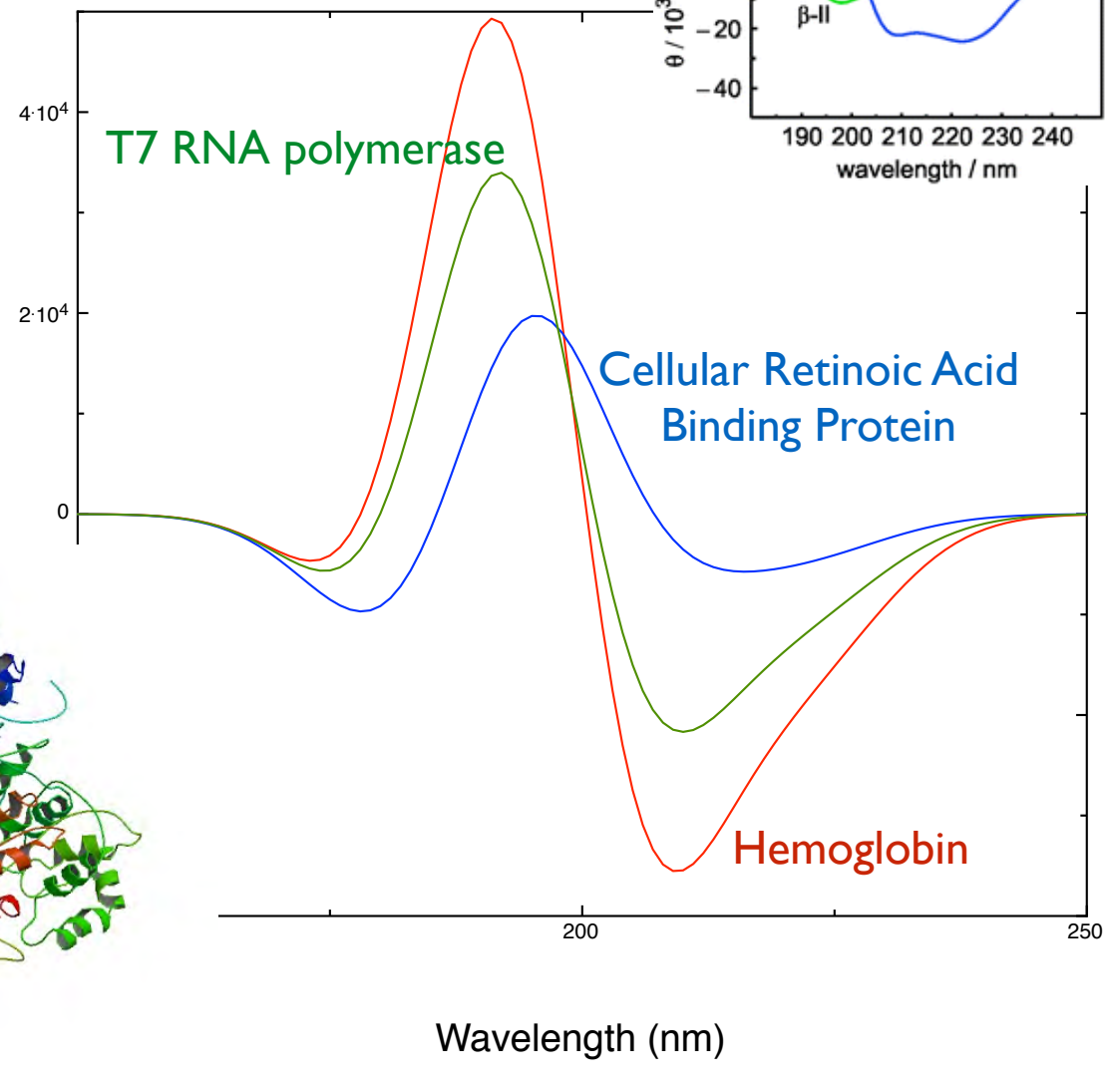


Hemoglobin









Predict spectra from 3D structure

DICHROCalc http://comp.chem.nottingham.ac.uk/dichrocalc/

CD - things to be careful of

There is the possibility that if used in a blackbox manner, users can produce less than ideal (or even erroneous) conclusions from CD. Thus a number of precautions need to be considered in the use and interpretation of the results.

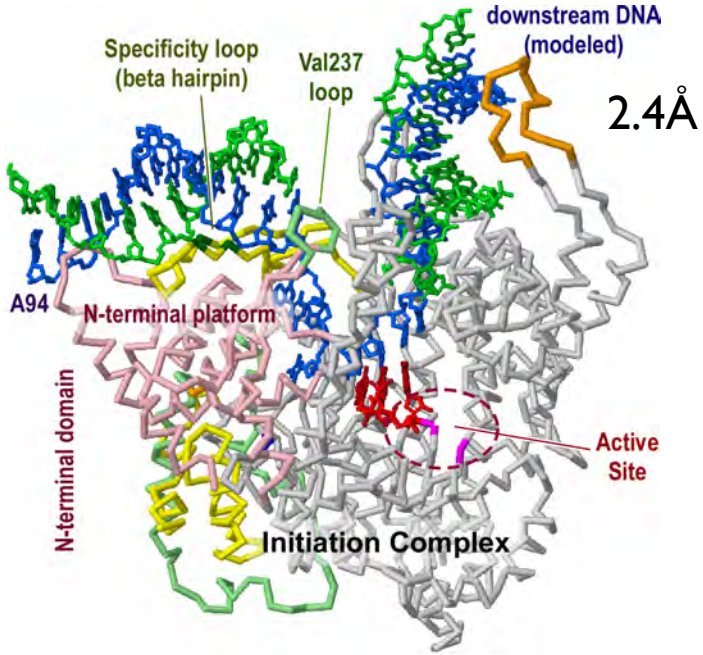
1. The amount of data must be sufficient to solve for the desired number of secondary structure components. Data that only extends to 200 nm contains at most two eigenvectors, and hence the results should only be interpreted in terms of two components (i.e., how much is helix and how much is not helix). Any interpretation of such data that attempts to deconvolute into more components than these will be an over-interpretation of the data.

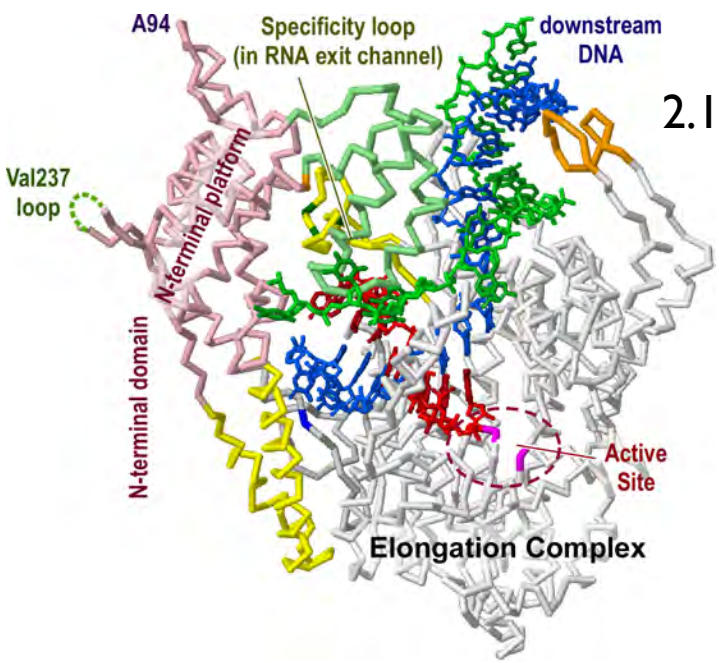
2. A low value for the NRMSD or any other goodness-of-fit parameter does not always indicate it represents a correct solution. A low NRMSD value (0.1) is a necessary but not sufficient condition for accuracy in secondary structure determination. However a high value is a good indication that either the analysis has gone wrong (often because the magnitude of the spectrum is incorrect) or the reference database is inappropriate for the characteristics of the protein being analysed. It is also important to note that some algorithms, notably CDSSTR, nearly always produce the lowest NRMSD due to the way they fit the data, but they very often are not the most correct solution.[36]

3. Reference databases derived from globular soluble proteins are not appropriate for the analysis of proteins (or peptides) in nonaqueous solutions.[38] (4) It is absolutely essential to have precisely correct concentration measurements (not just estimates from colorimetric assays) and an accurate measurement of the cell pathlength (the values cited by the manufacturers, especially for very short pathlengths, can err by 30% or more).[42] The consequence of concentration and pathlength errors is that the magnitude of the spectrum produced will err by a corresponding amount and result in incorrect analyses.

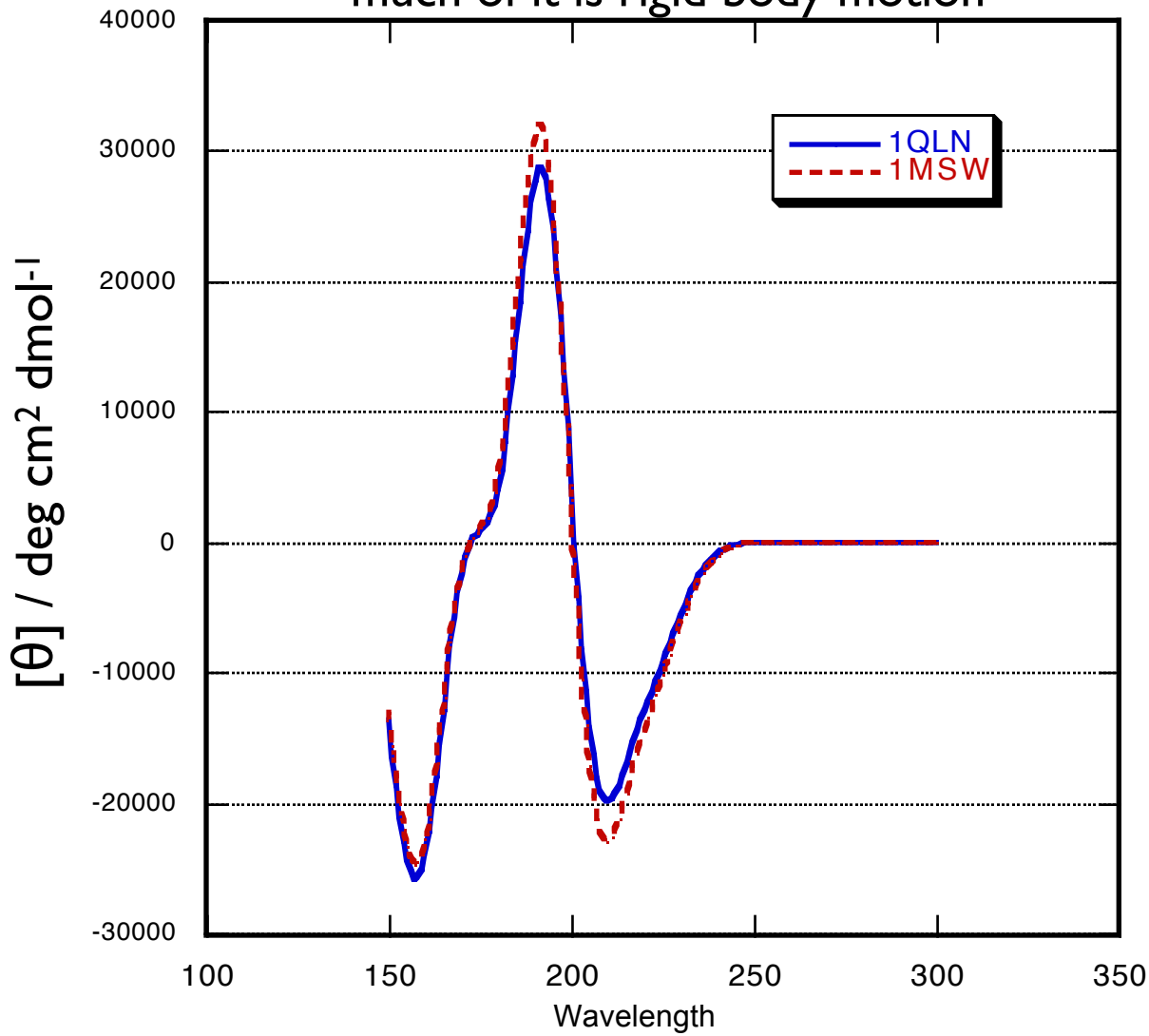
Whitmore & Wallace (2007) Biopolymers 89, 392-400

Detect conformational change?





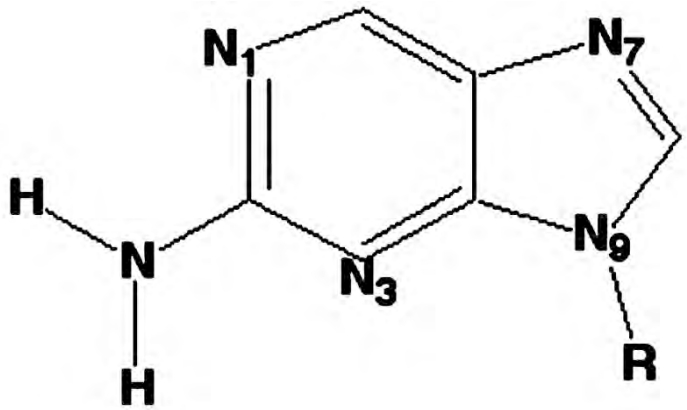
Large structural change, but much of it is rigid body motion



Much better for local helix to coil transition in a relatively small protein

Structures and abbreviations

a



$Abs_{max} \approx 300$ nm

b

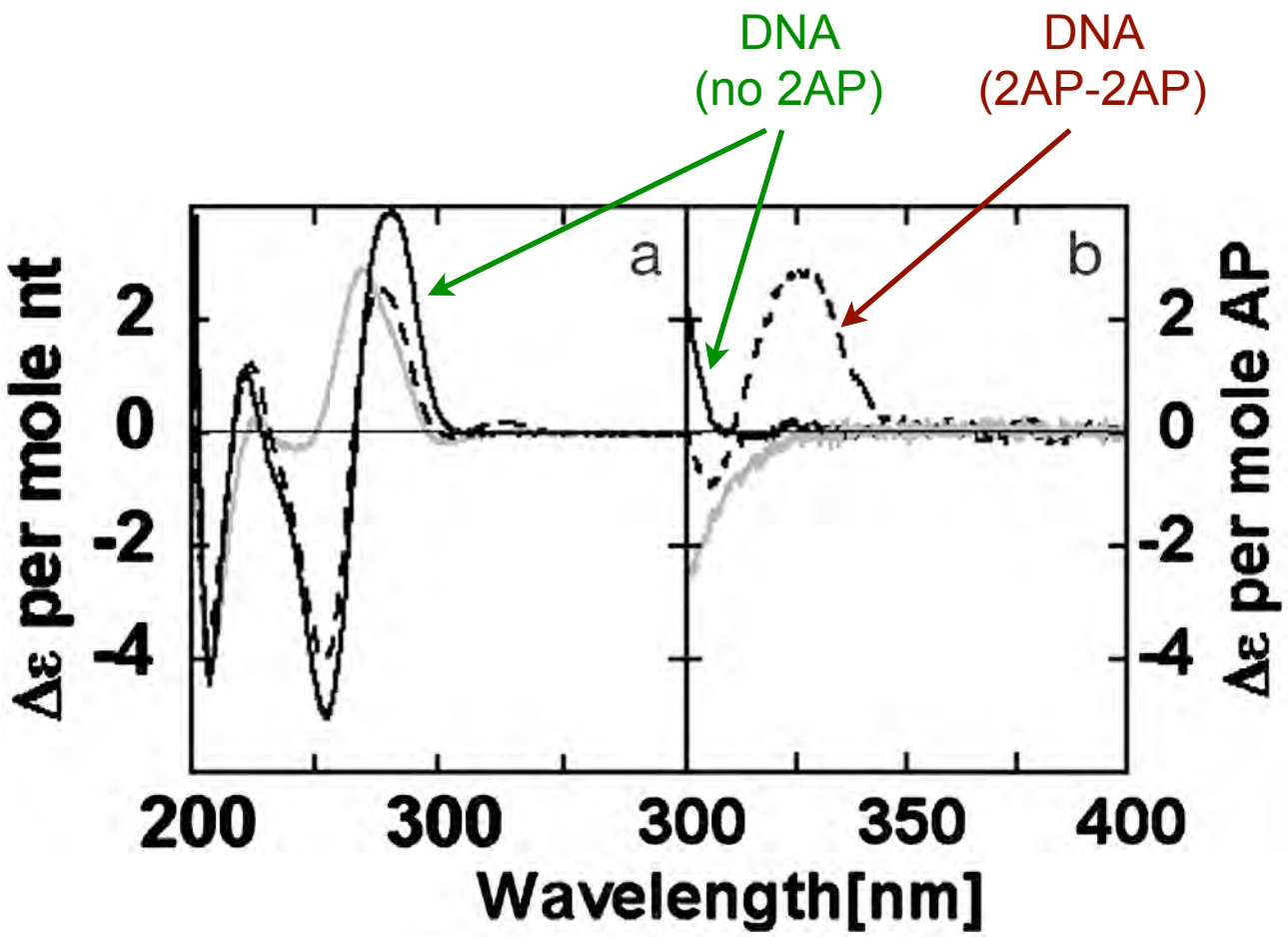
—	5'-CGA AGT GCG AAG GCT
–X–	5'-CGA AGT GCG APAC GCT
–XX–	5'-CGA AGT GCG APAPC GCT
–XGX–	5'-CGA AGT GCAPGAPC GCT

═	5'-CGA AGT GCG AAG GCT 3'-GCT TCA CGC TTG CGA
--X--	5'-CGA AGT GCG APAC GCT 3'-GCT TCA CGC T TG CGA
--XX--	5'-CGA AGT GCG APAPC GCT 3'-GCT TCA CGC T T G CGA
--XGX--	5'-CGA AGT GCAPGAPC GCT 3'-GCT TCA CG T C TG CGA
--XX-R	5'-CGA AGT GCG APAPC GCT 3'-GCU UCA CGC U U G CGA

2-aminopurine dimer

Johnson N. P. et.al. PNAS 2004;101:3426-3431

CD Spectra of ds oligonucleotides containing AP dimer

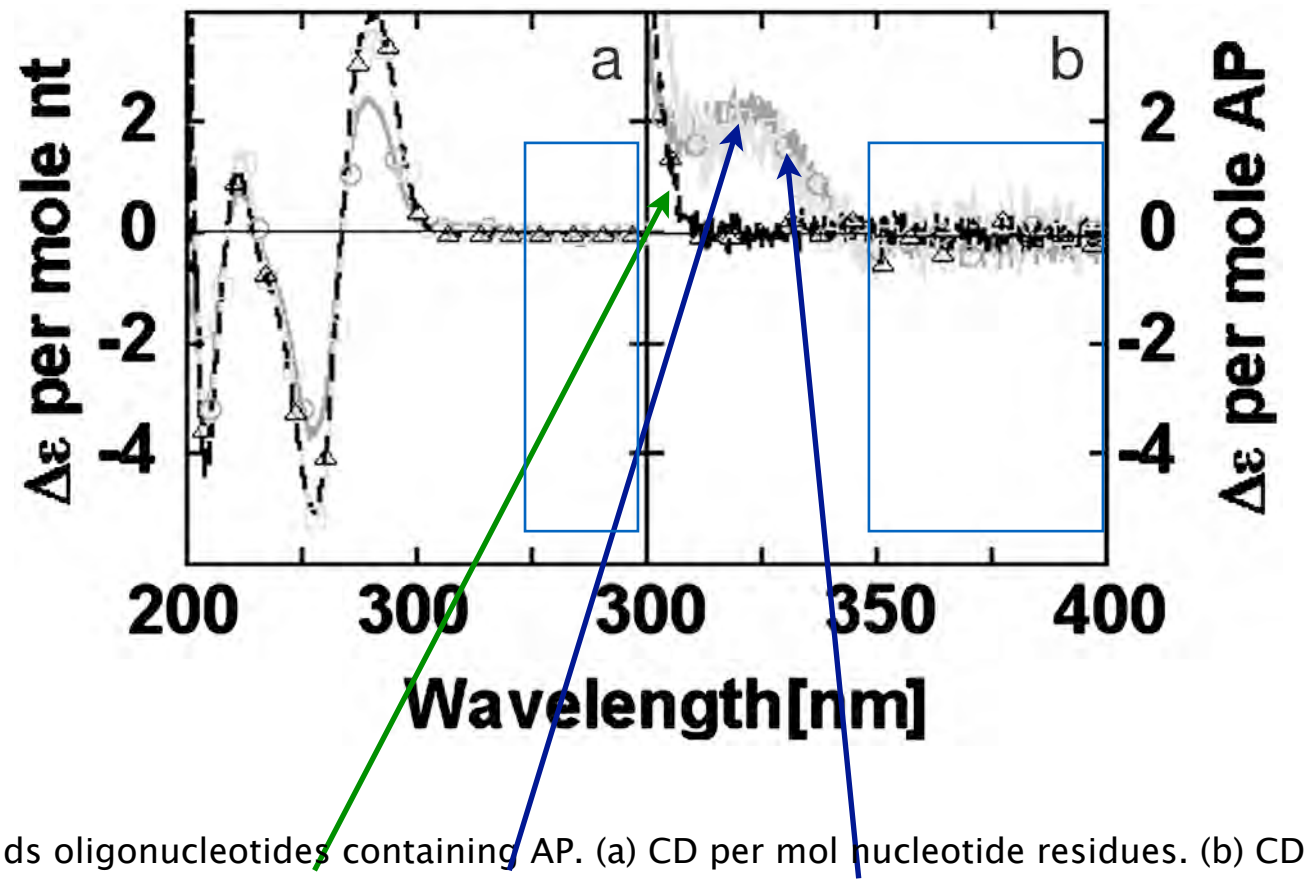


CD Spectra of ds oligonucleotides containing AP dimer. (a) CD per mol nucleotide residues. (b) CD per mol AP residues. Oligonucleotides: ----- (dark solid line), --XX-- (dashed line), and --XX--$_R$ (light solid line).

Johnson N. P. et.al. PNAS 2004;101:3426-3431

CD Spectra of ds oligonucleotides containing AP

A number of experiments were carried out to further characterize the 328-nm peak of the CD spectra. Double-stranded oligonucleotides containing a single AP residue (--X--) or two AP residues separated by an intervening base (--XGX--), showed similar CD spectra above 300 nm (Fig. 4). These spectra had a single peak with maximum intensity at 320 nm, differing markedly from the CD spectrum of the --XX-- species (Fig. 3).

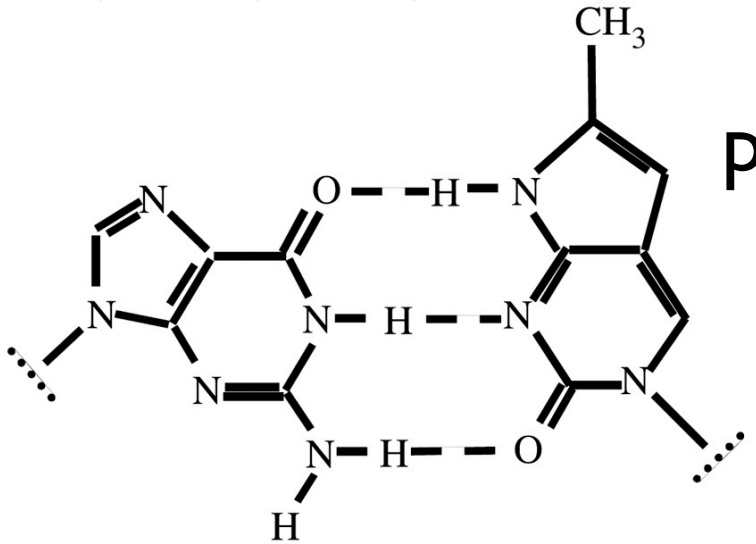


CD Spectra of ds oligonucleotides containing AP. (a) CD per mol nucleotide residues. (b) CD per mol AP residues. Oligonucleotides: --------- (triangles), --X-- (squares), and --XGX-- (circles).

Johnson N. P. et.al. PNAS 2004;101:3426-3431

Structure of a guanine-PC base pair (a), and sequences and nomenclature of ss oligonucleotides used in this study (b). ssPCC is the complementary strand used to form the ds oligonucleotides dsPC0, dsPC1, dsPC2, dsPC1A, and dsPC1B.

a



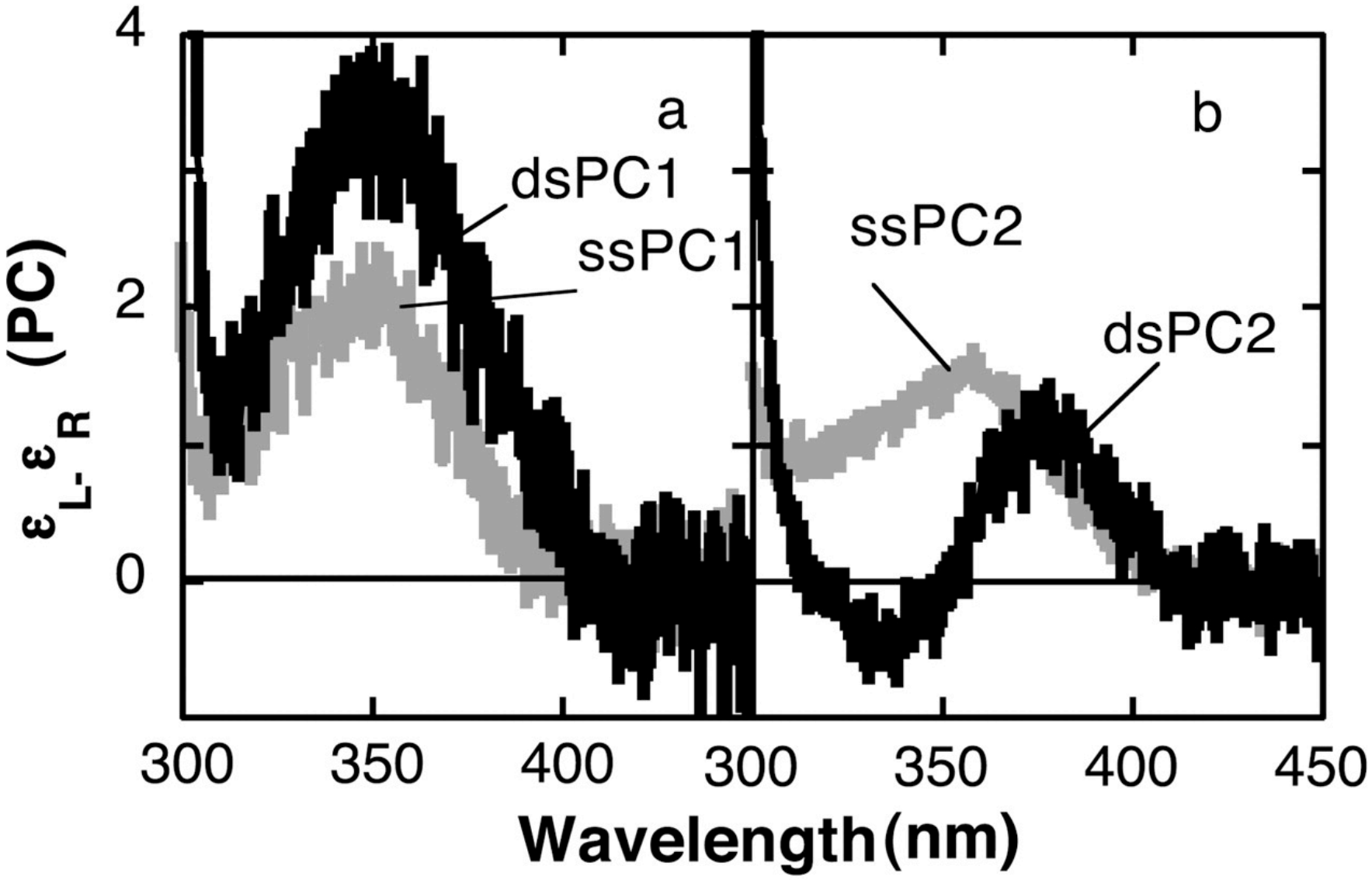
how is this different from dC?

b

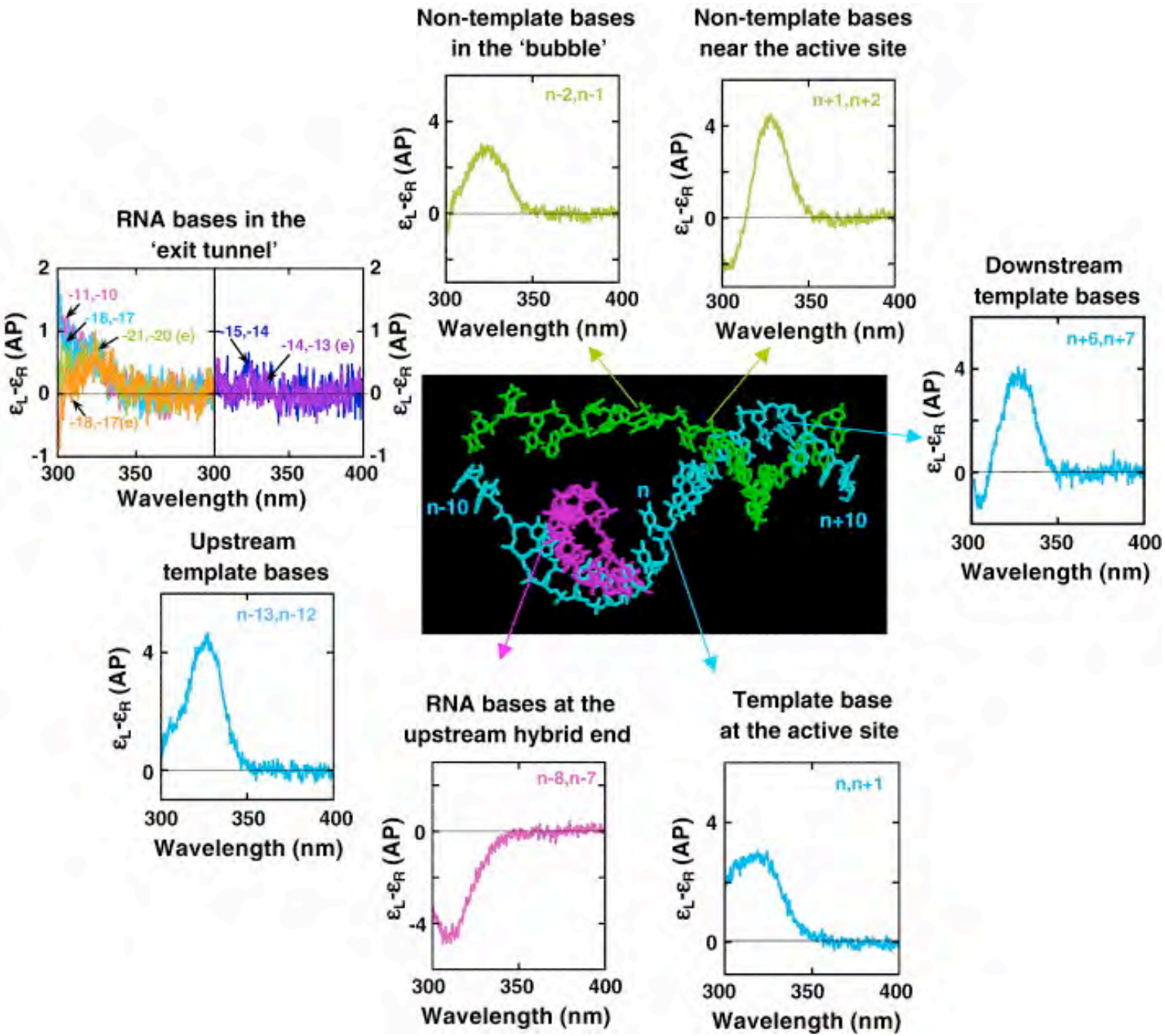
Name	Sequence
ssPC0	5’-GCC CAA CCA TAC CCG-3’
ssPC1	5’-GCC CAA (PC)CA TAC CCG-3’
ssPC2	5’-GCC CAA (PC)(PC)A TAC CCG-3’
ssPC1A	5’-GCC (PC)AA C(PC)A TAC CCG-3’
ssPC1B	5’-GCC CAA C(PC)A TA(PC) CCG-3’
ssPCC	3’-CGG GTT GGT ATG GGC-5’

Johnson N. P. et.al. PNAS 2005;102:7169-7173

Low-energy CD spectra of 7 μM ss oligonucleotide and 3.5 μM ds oligonucleotides



Johnson N. P. et.al. PNAS 2005;102:7169-7173



Datta et al, J Mol Biol 360, 800-813 (2006)

Circular Dichroism Recap - Practical Stuff

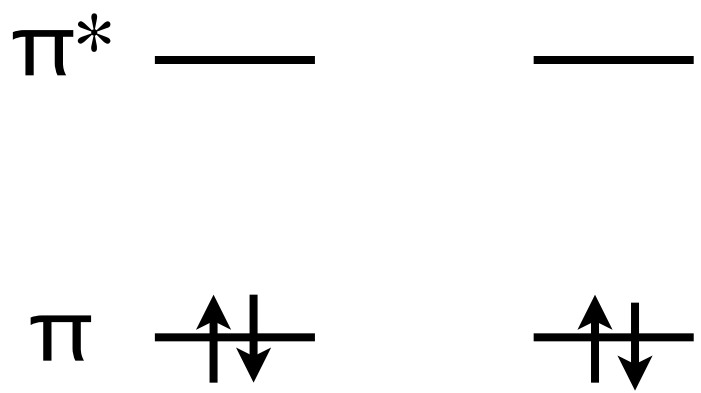
- Can try to estimate 2° structure of protein whose structure is not known.
  - Two approaches
    - Fit to 2 or more basis sets (*be careful*!)
      - number of sets fit
      - using appropriate basis sets
    - Fit to *ab initio* calculations (*be careful*!)
  - Not done so much anymore…

- Can measure conformational changes
  - Mostly empirical (*be careful*!)

Electronic Energy Levels

Use ethylene (ethene) as a simple model

$CH_2=CH_2$



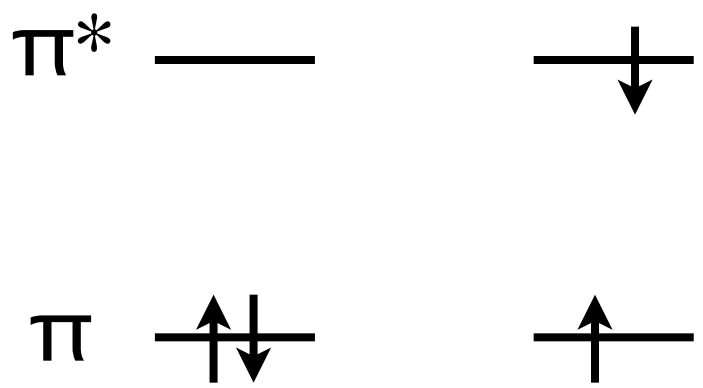
S_0

(singlet)

Electronic Energy Levels

Use ethylene (ethene) as a simple model

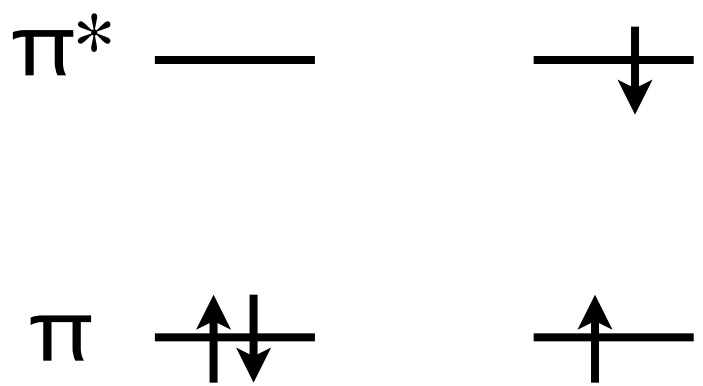
$CH_2=CH_2$



Electronic Energy Levels

Use ethylene (ethene) as a simple model

$CH_2=CH_2$



Electronic Energy Levels

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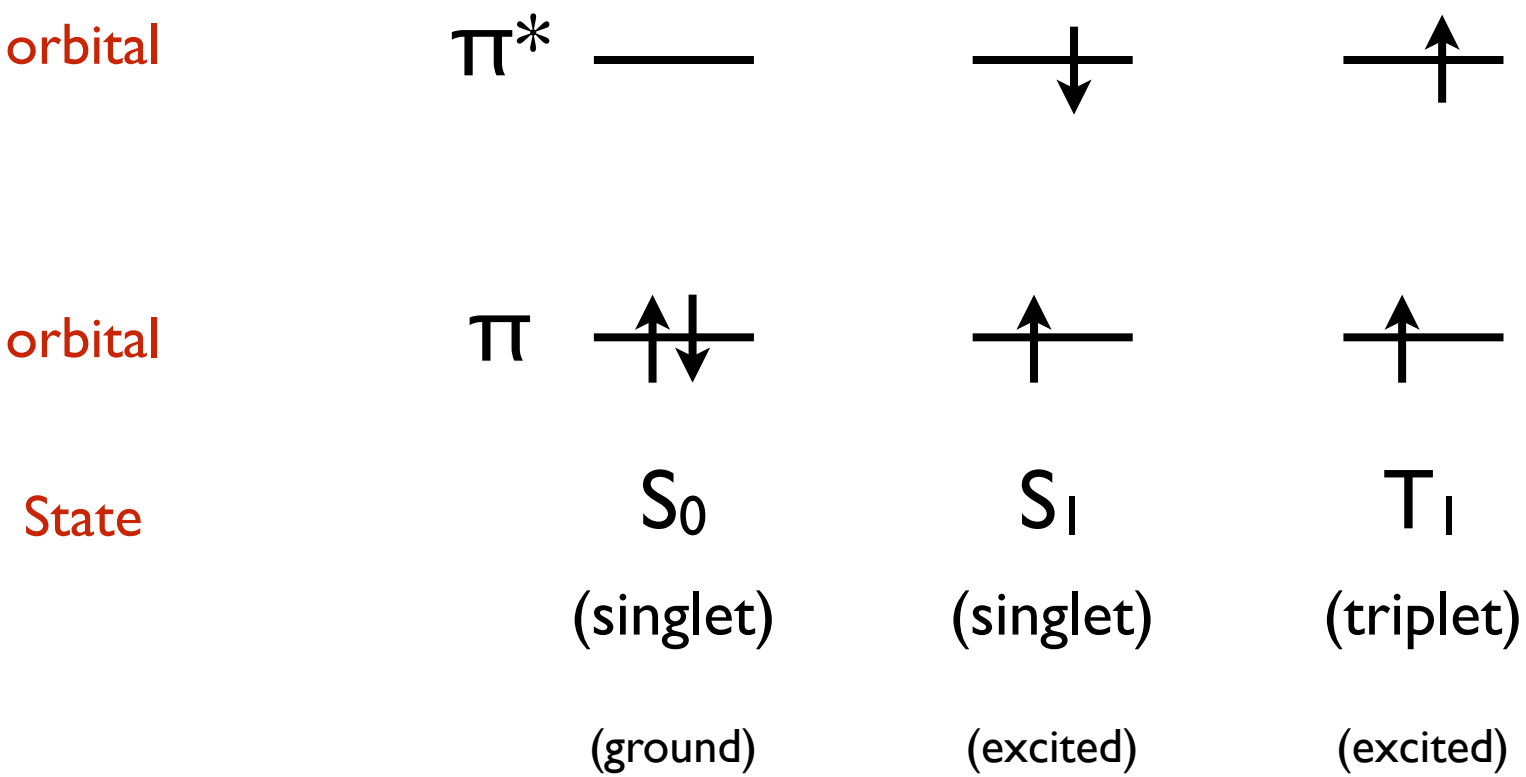

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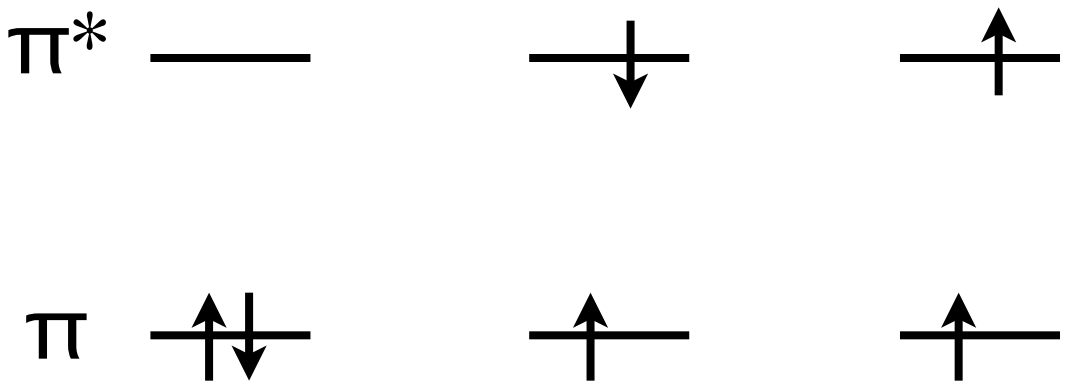


S_0 (singlet) (ground)

S_1 (singlet) (excited)

T_1 (triplet) (excited)

$S_0 \rightarrow S_1$

Allowed, subject to

$$\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\psi_2\bar{\mu}\psi_1\,\partial x\,\partial y\,\partial z$$

$S_0 \rightarrow T_1$

Forbidden - why?

Requires TWO transitions: change in orbital AND spin flip

Remember this for later

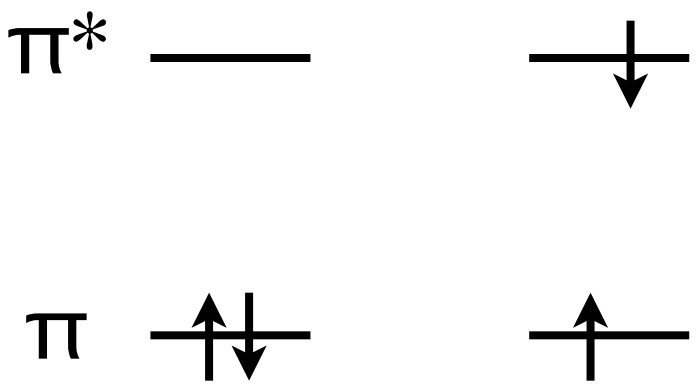
Why is fluorescence emission *always* at longer wavelength than the exciting absorbance?

(Stokes shift)

Electronic Energy Levels

Use ethylene (ethene) as a simple model

$CH_2=CH_2$



S_0 (singlet) (ground)

S_1 (singlet) (excited)

$CH_2=CH_2$ CH_2-CH_2

$CH_2=CH_2$

Bond order = 2

Bond length = 1.3 Å

CH_2-CH_2

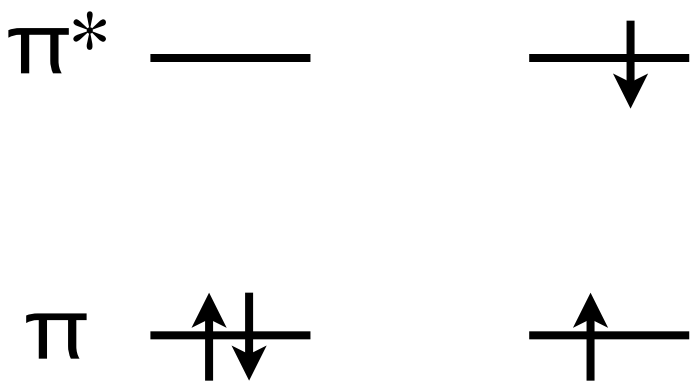
Bond order = 1

Bond length = 1.5 Å

Electronic Energy Levels

Use ethylene (ethene) as a simple model

$CH_2=CH_2$



S_0 (singlet) (ground)

S_1 (singlet) (excited)

$CH_2=CH_2$ CH_2-CH_2

$CH_2=CH_2$

Bond order = 2

Bond length = 1.3 Å

CH_2-CH_2

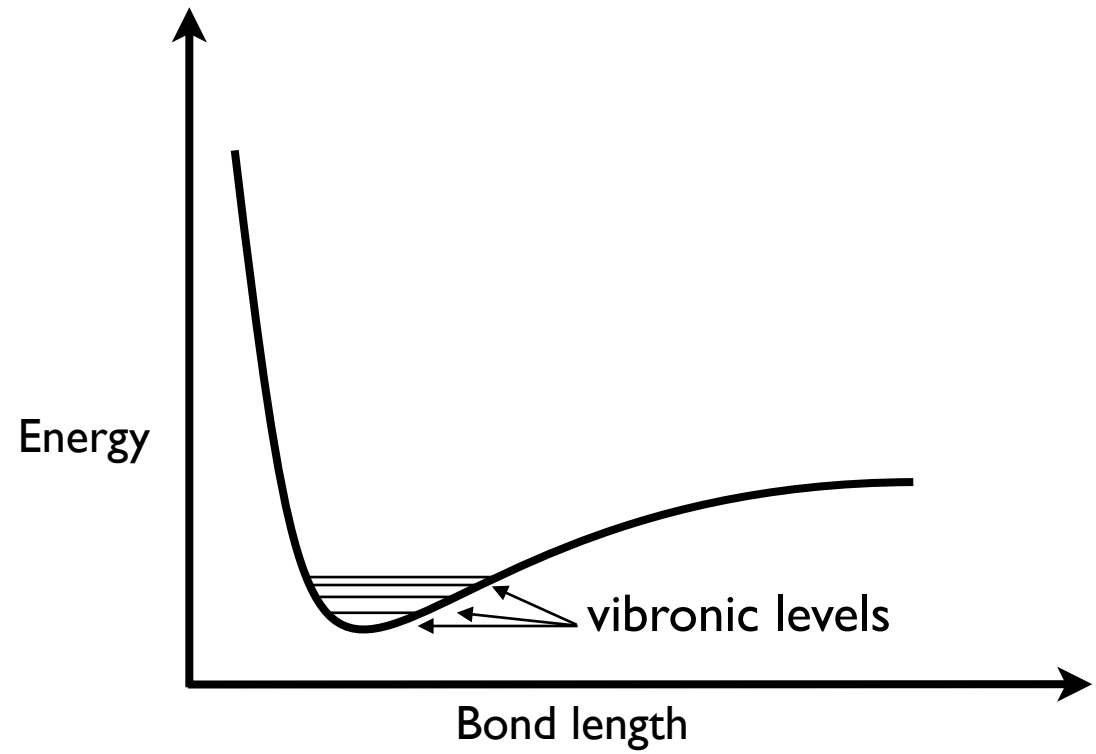
Bond order = 1

Bond length = 1.5 Å

But at T > 0K, bonds vibrate!

Bonds *lengths* oscillate in length!

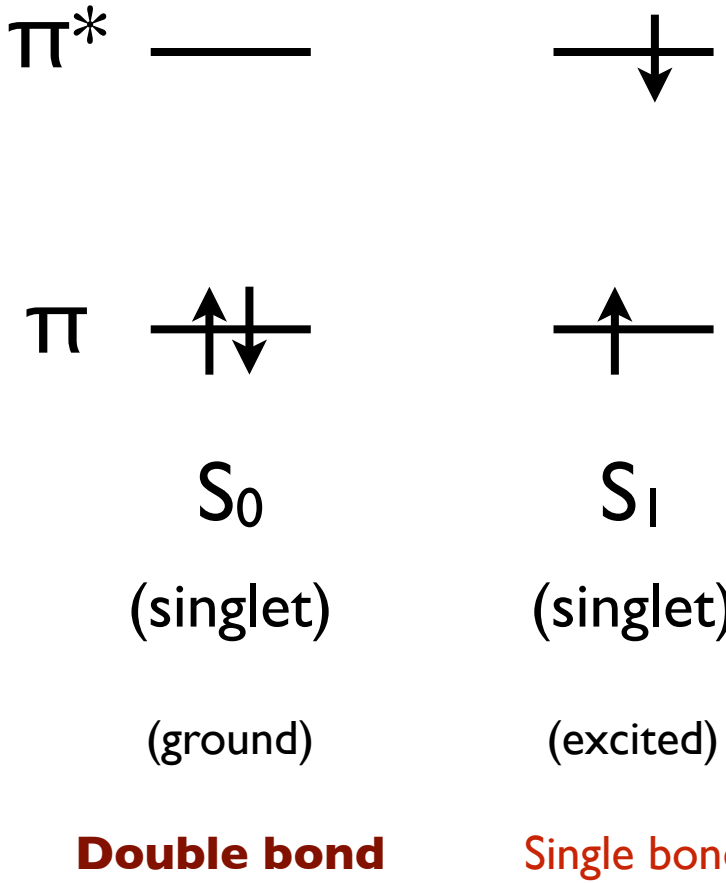
Higher excited vibrational states
vibrate with *bigger amplitude*



Electronic Energy Levels

Use ethylene (ethene) as a simple model

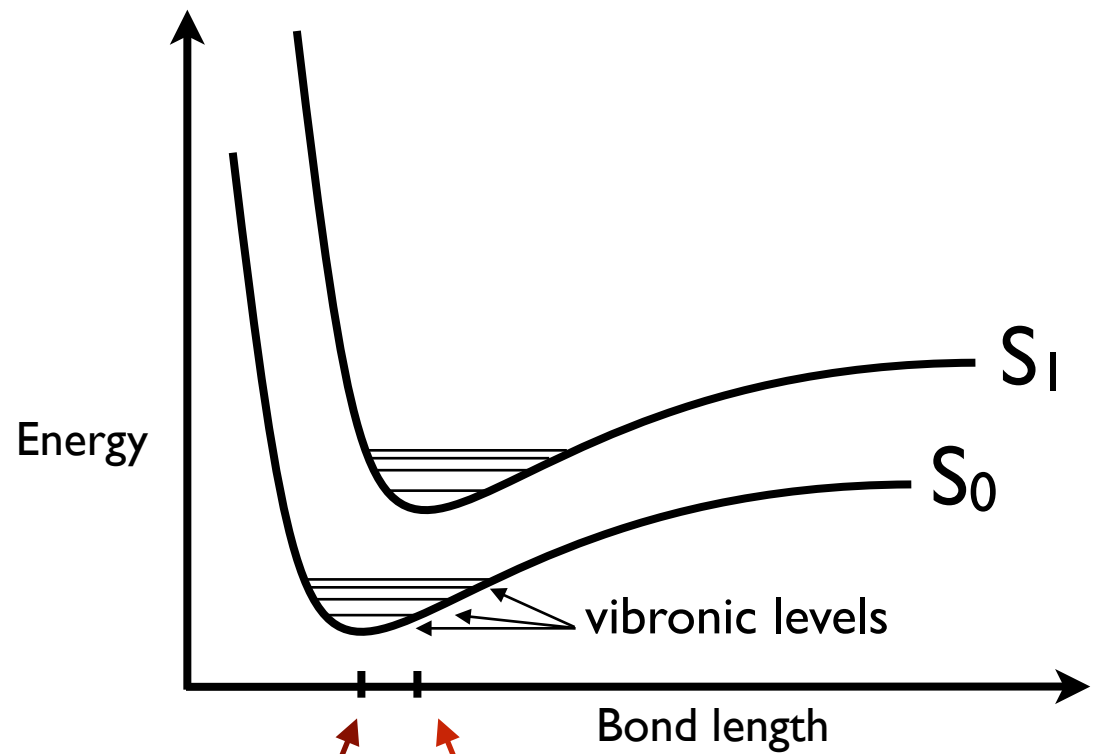
$CH_2=CH_2$



Double bond

Single bond

What can we say about bond lengths?



Bond order = 2
Bond length = 1.3 Å

Bond order = 1
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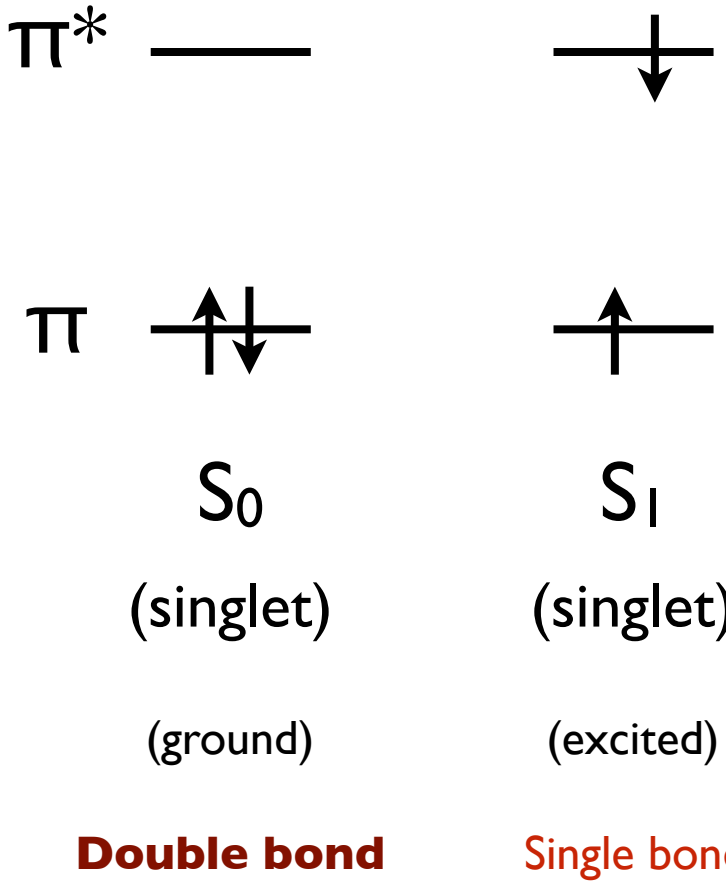
Electronic Energy Levels

Use ethylene (ethene) as a simple model

$CH_2=CH_2$

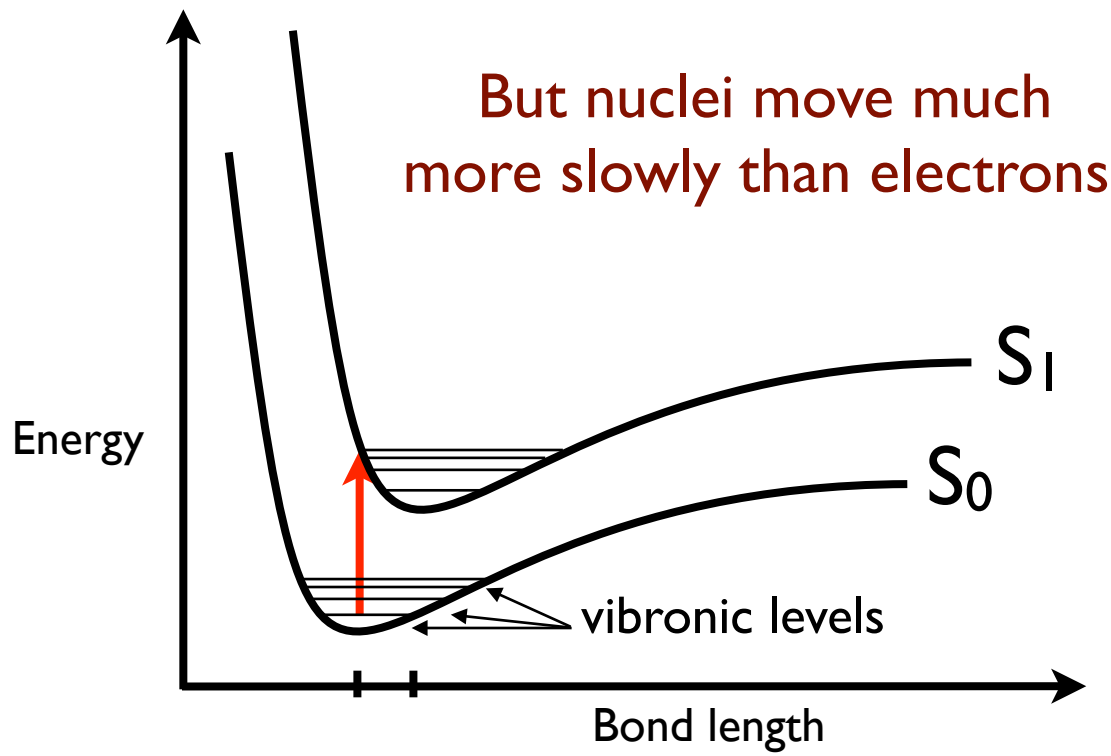


What can we say about bond lengths?

S_0 - short bond

S_1 - long bond

But nuclei move much more slowly than electrons!



Electronic Energy Levels

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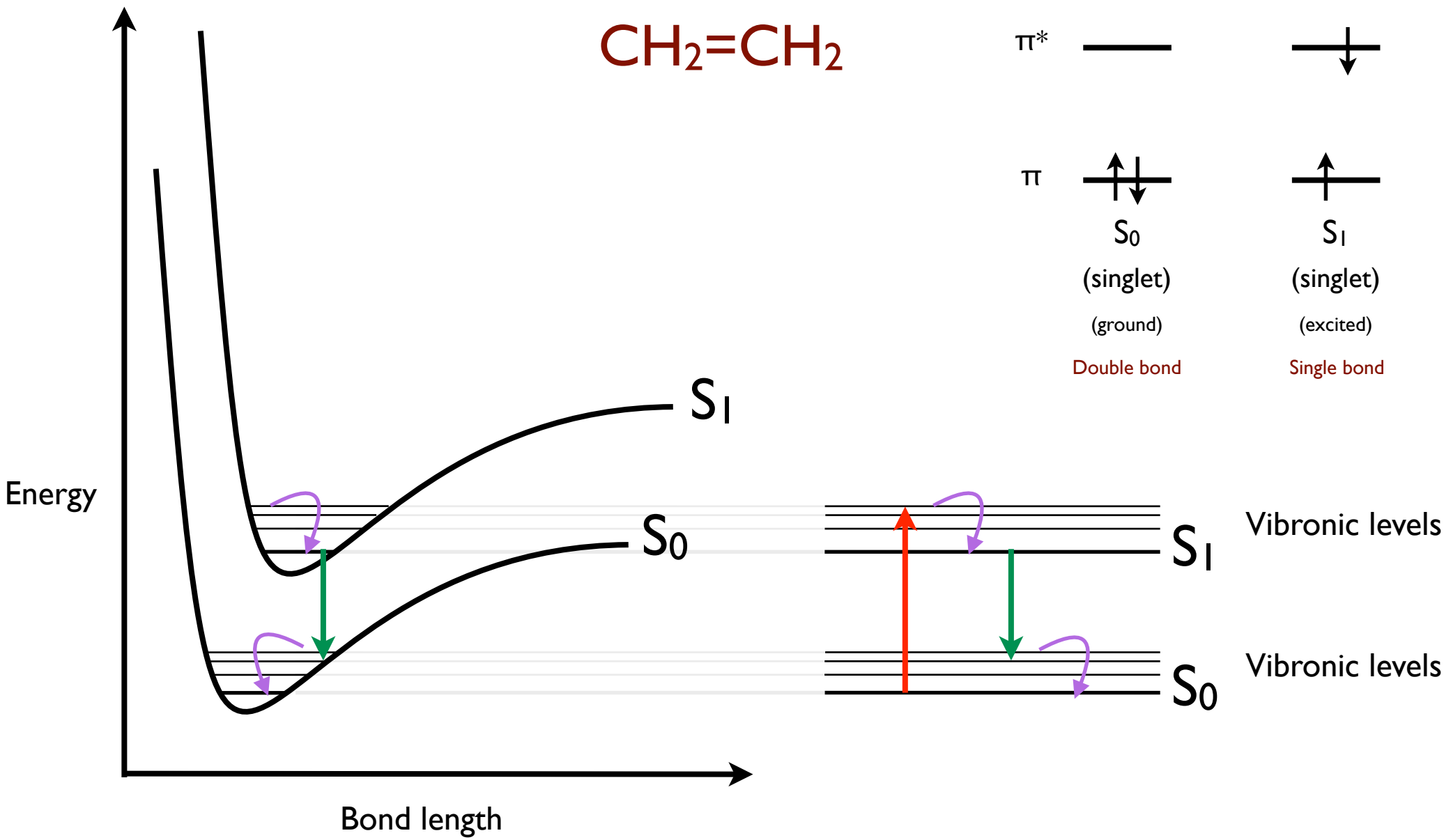

Electronic Energy Levels

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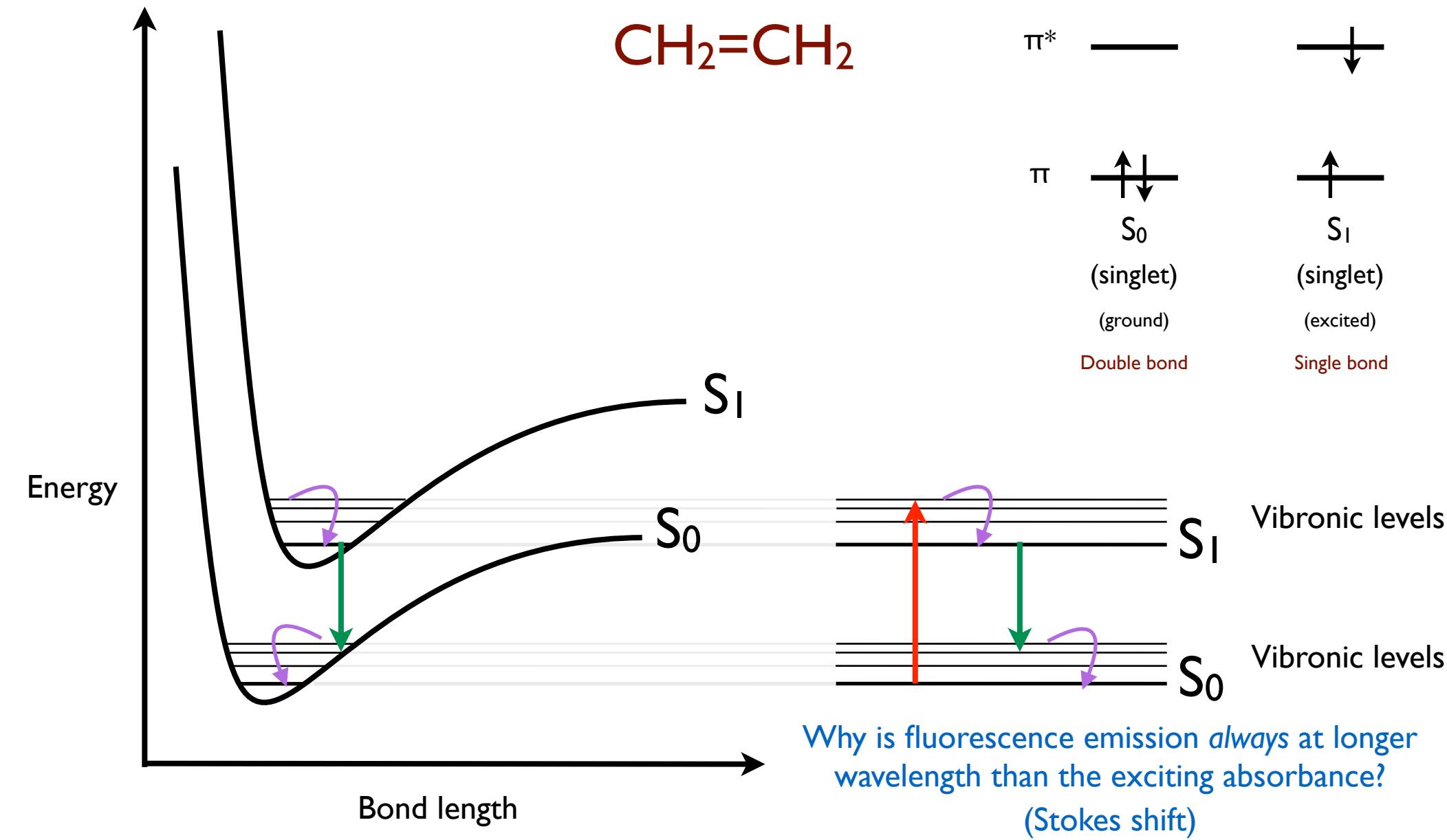

Why is fluorescence emission *always* at longer wavelength than the exciting absorbance?

(Stokes shift)

Electronic Energy Levels

Use ethylene (ethene) as a simple model

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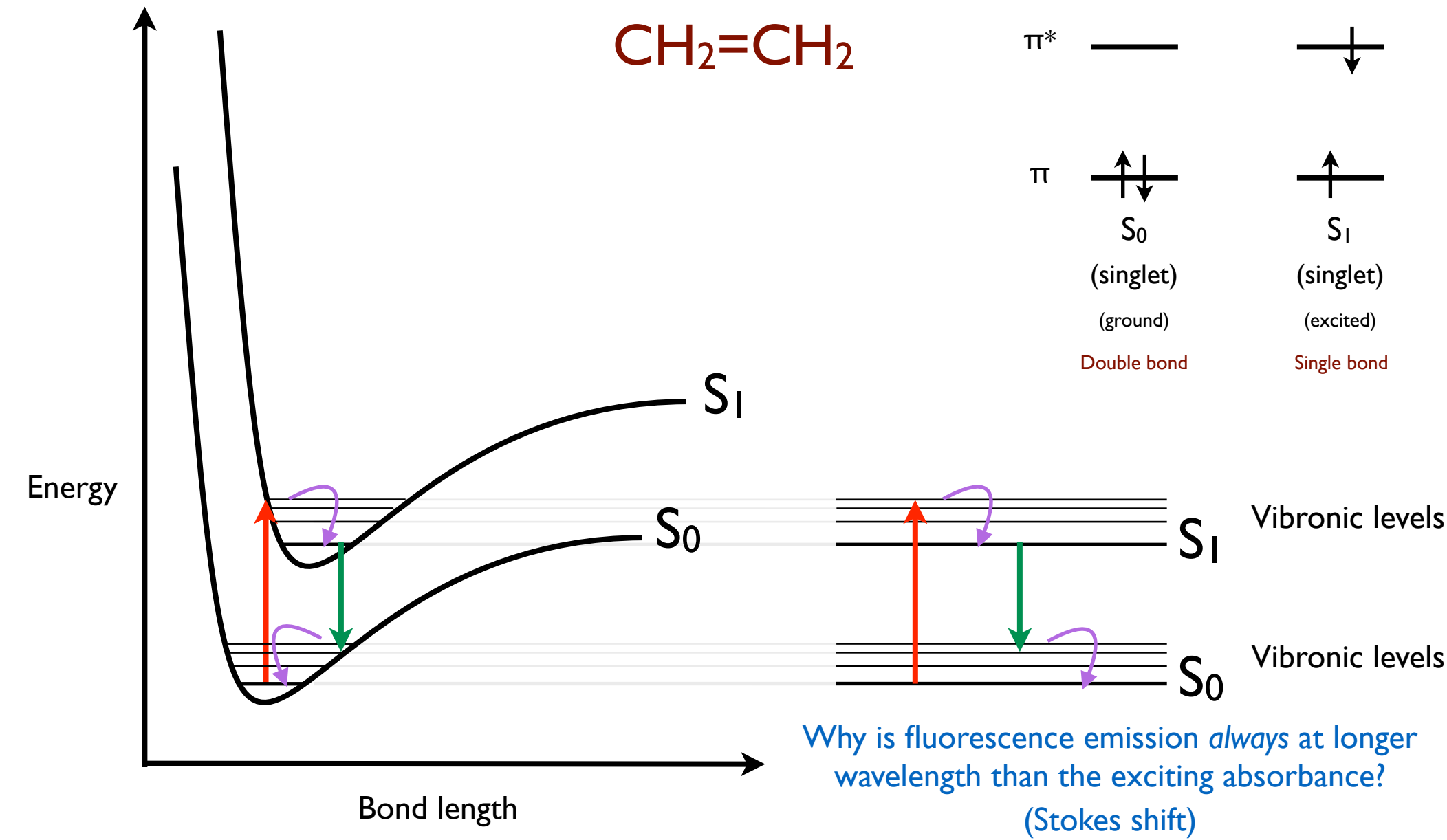
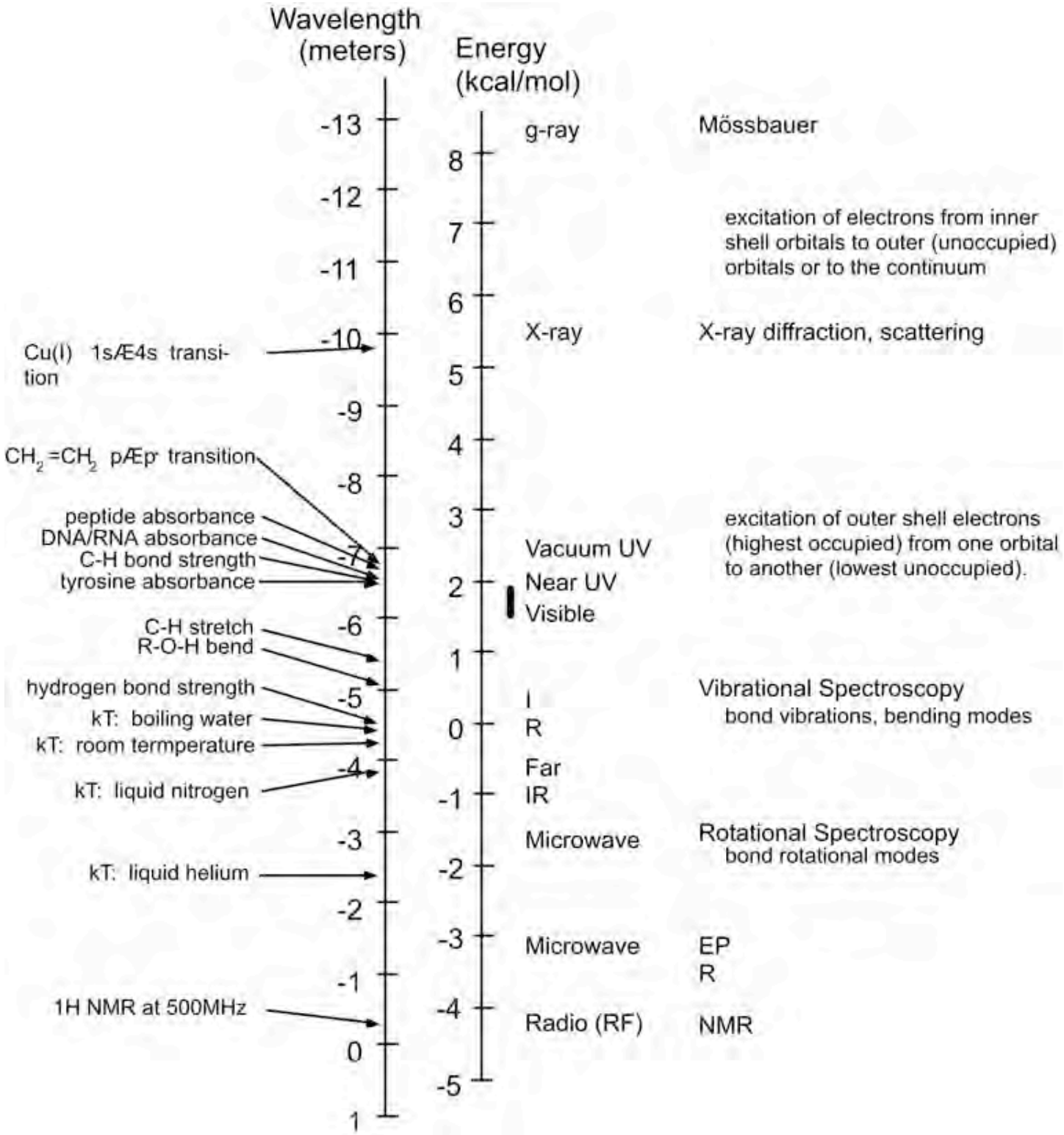
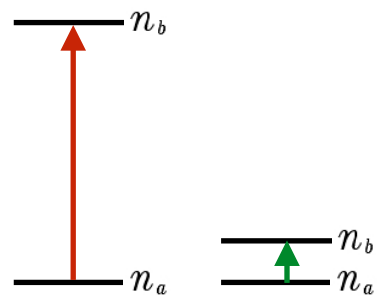


Why is fluorescence emission *always* at longer wavelength than the exciting absorbance?

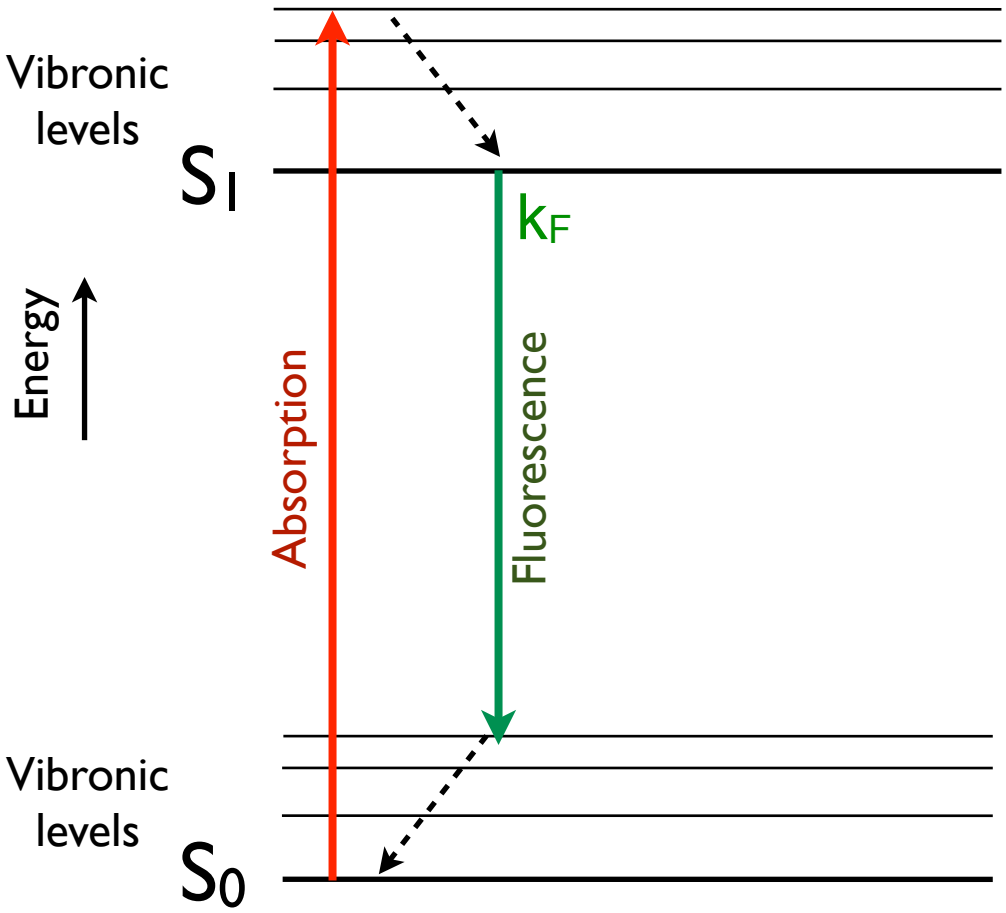
(Stokes shift)



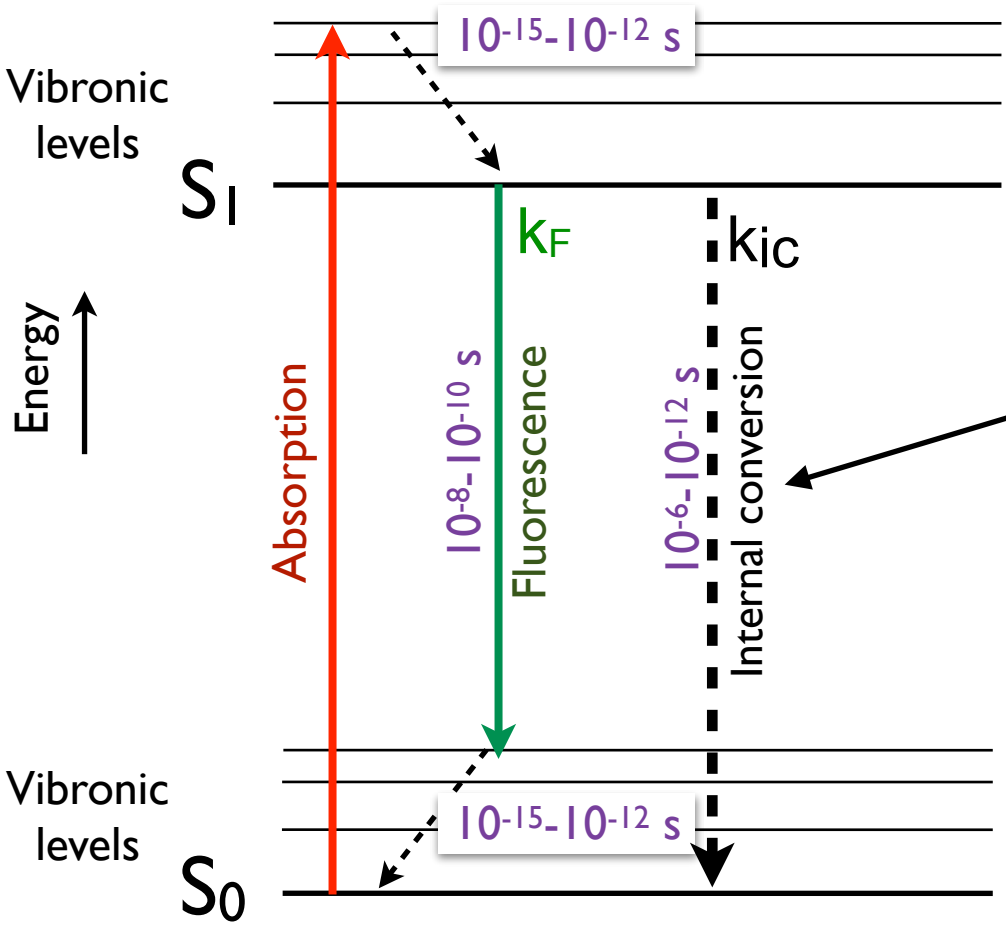


log ΔG	ΔG	n_b/n_a
2	100	5.7964E-74
1	→ 10	4.7459E-08
0	1	1.852E-01
-1	→ 0.1	8.4482E-01
-2	0.01	9.8328E-01
-3	0.001	9.9832E-01
-4	0.0001	9.9983E-01
-5	0.00001	9.9998E-01

Electronic Energy Levels

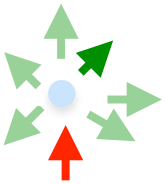


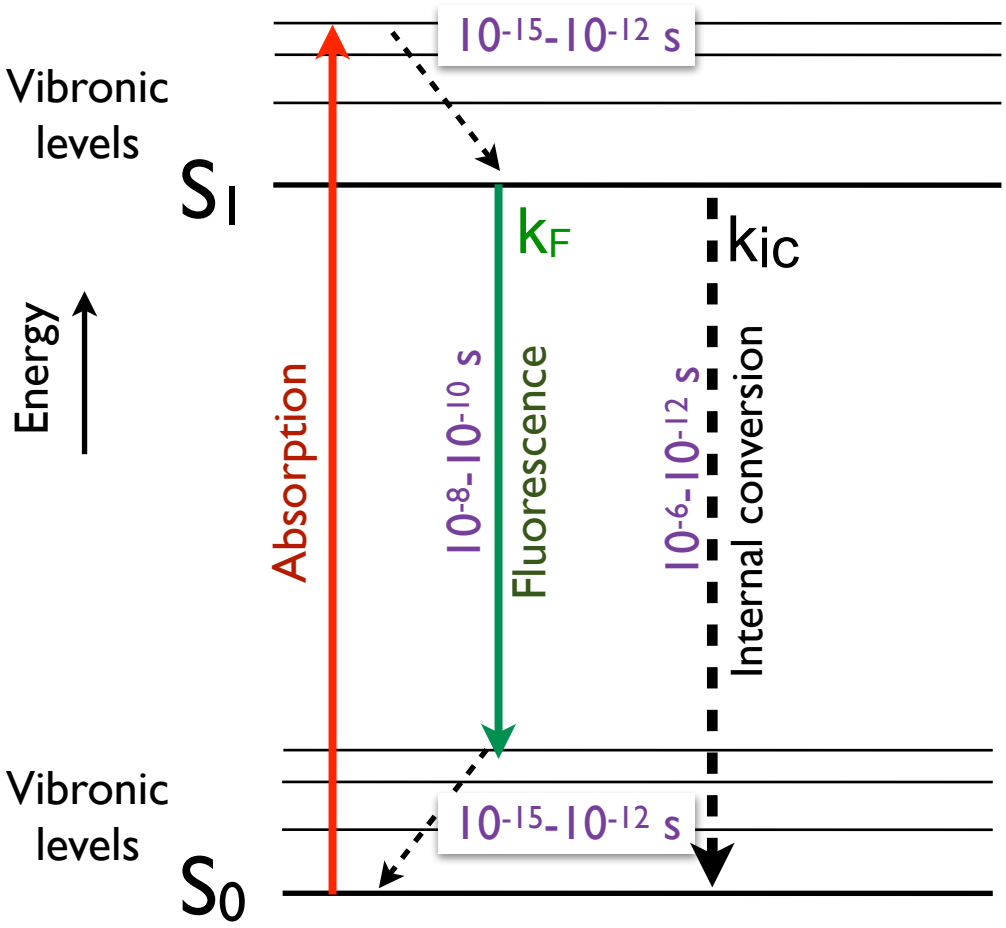
Electronic Energy Levels

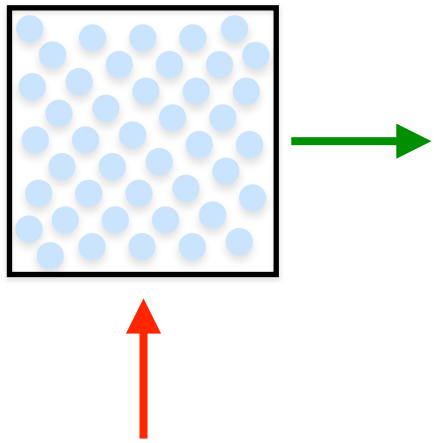


there are generally many mechanisms for internal conversion - the environment is complex!

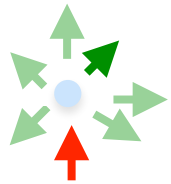
Electronic Energy Levels

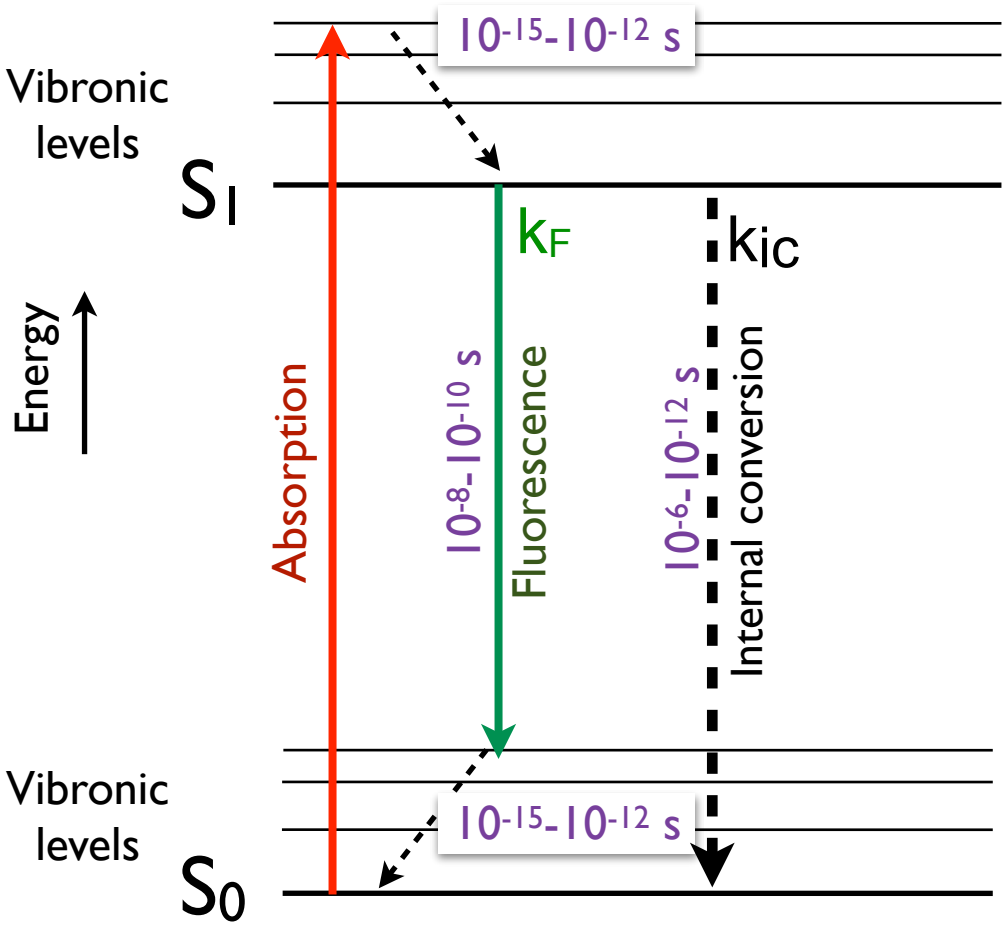


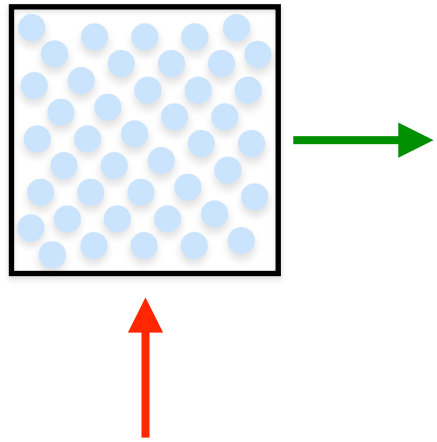




Electronic Energy Levels

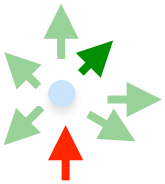


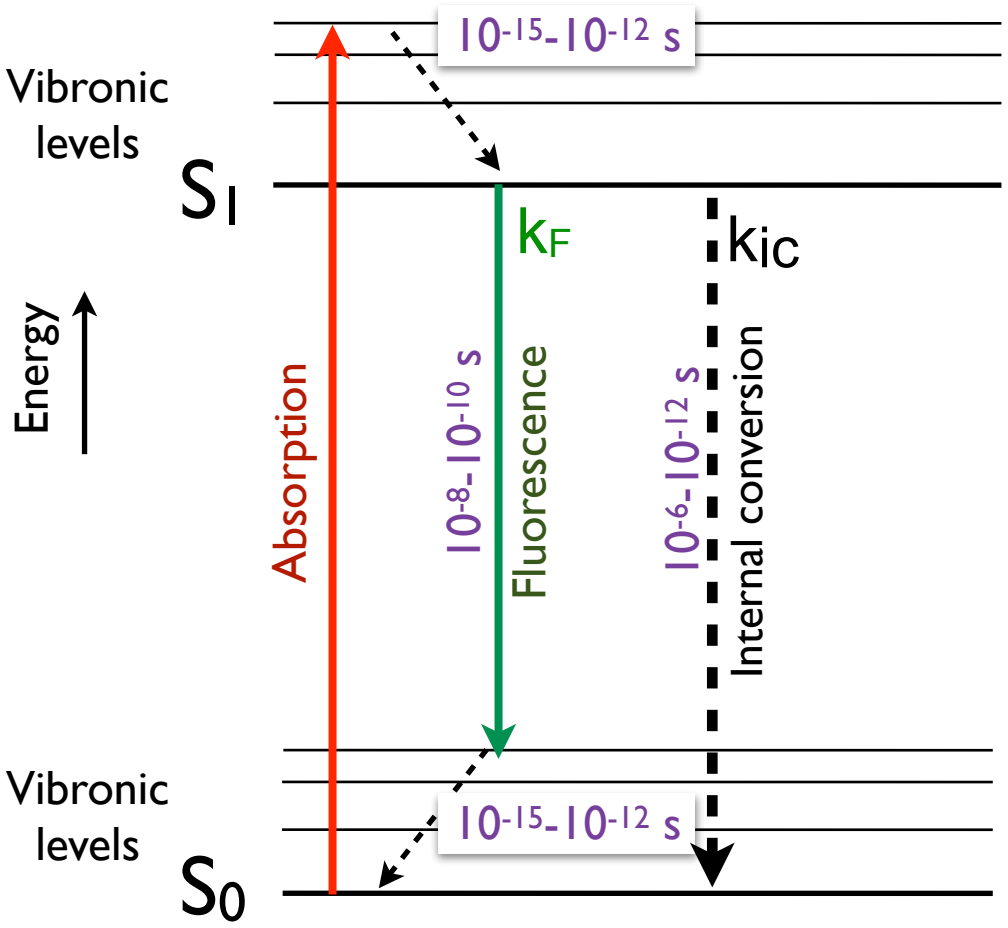


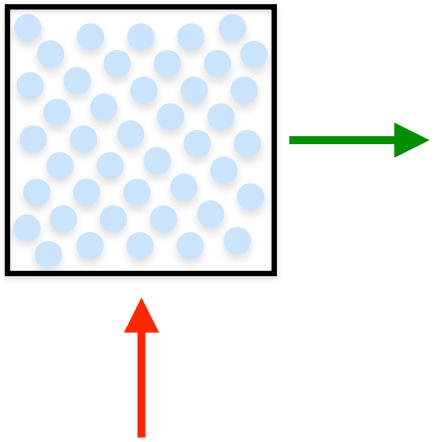


What goes up, must come down

Electronic Energy Levels



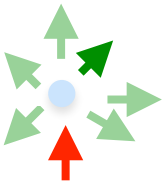


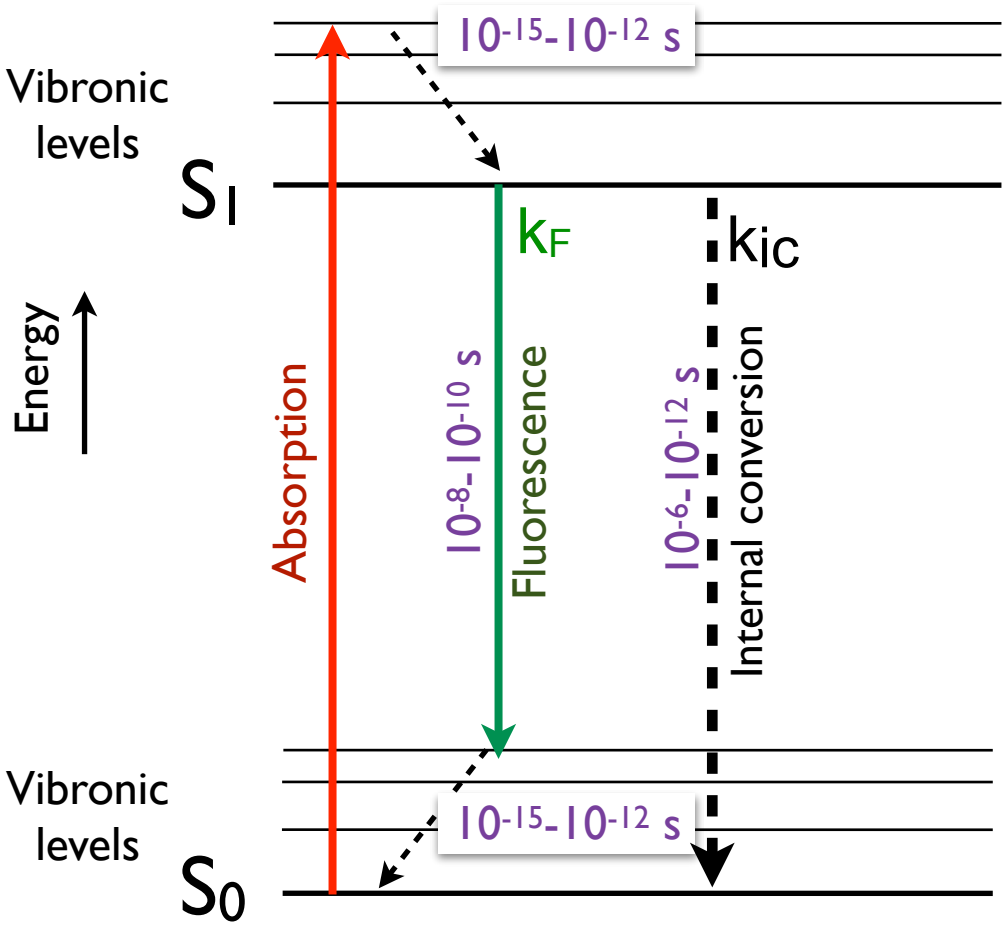


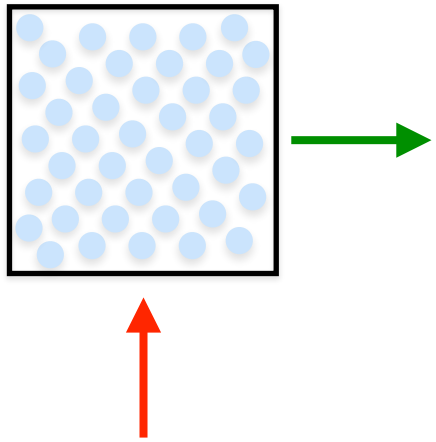
What goes up, must come down

$$k_{abs}[S_0] = \{k_F + k_{ic}\}[S_1]$$

Electronic Energy Levels





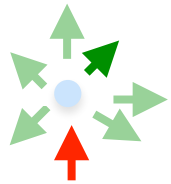


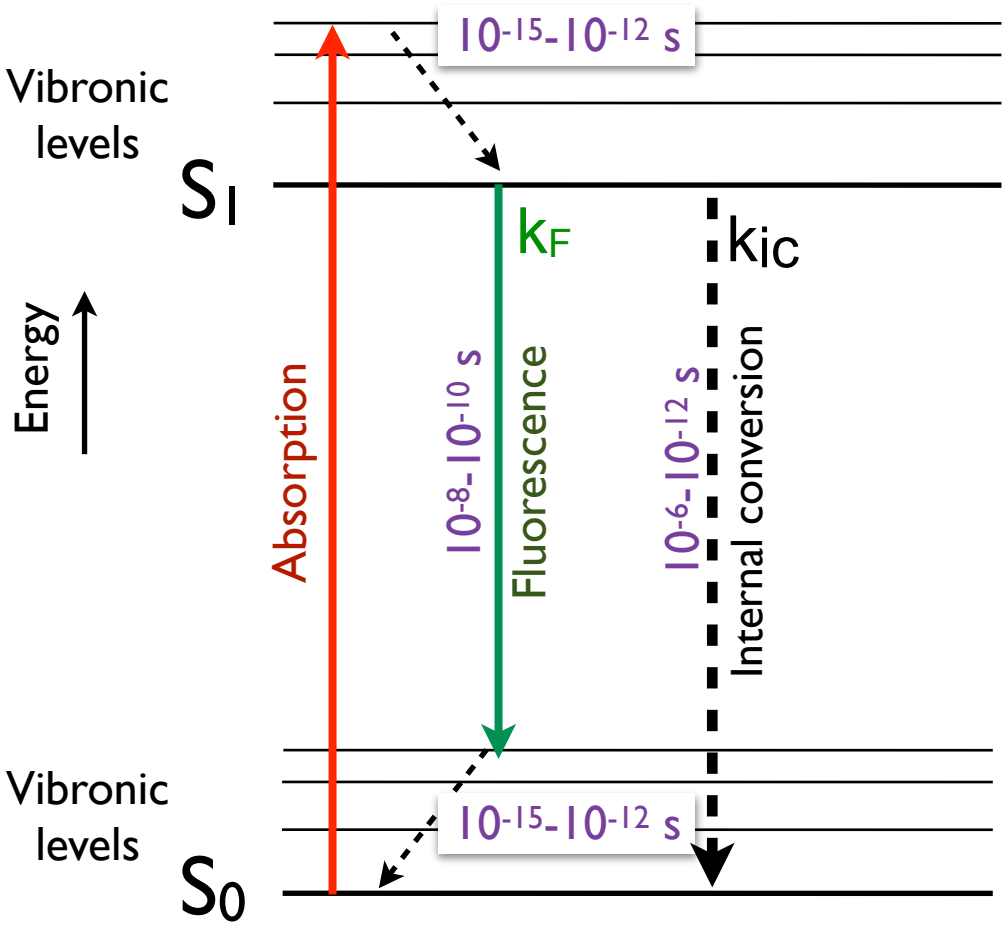
What goes up, must come down

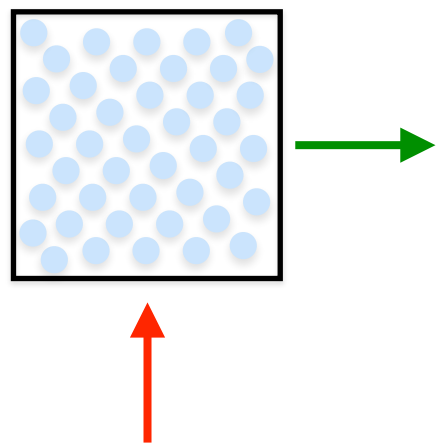
$$k_{abs}[S_0] = \{k_F + k_{ic}\}[S_1]$$

 # molecs excited

Electronic Energy Levels







note that we don't use or need to know k_{abs}, $[S_0]$, or $[S_1]$

What goes up, must come down

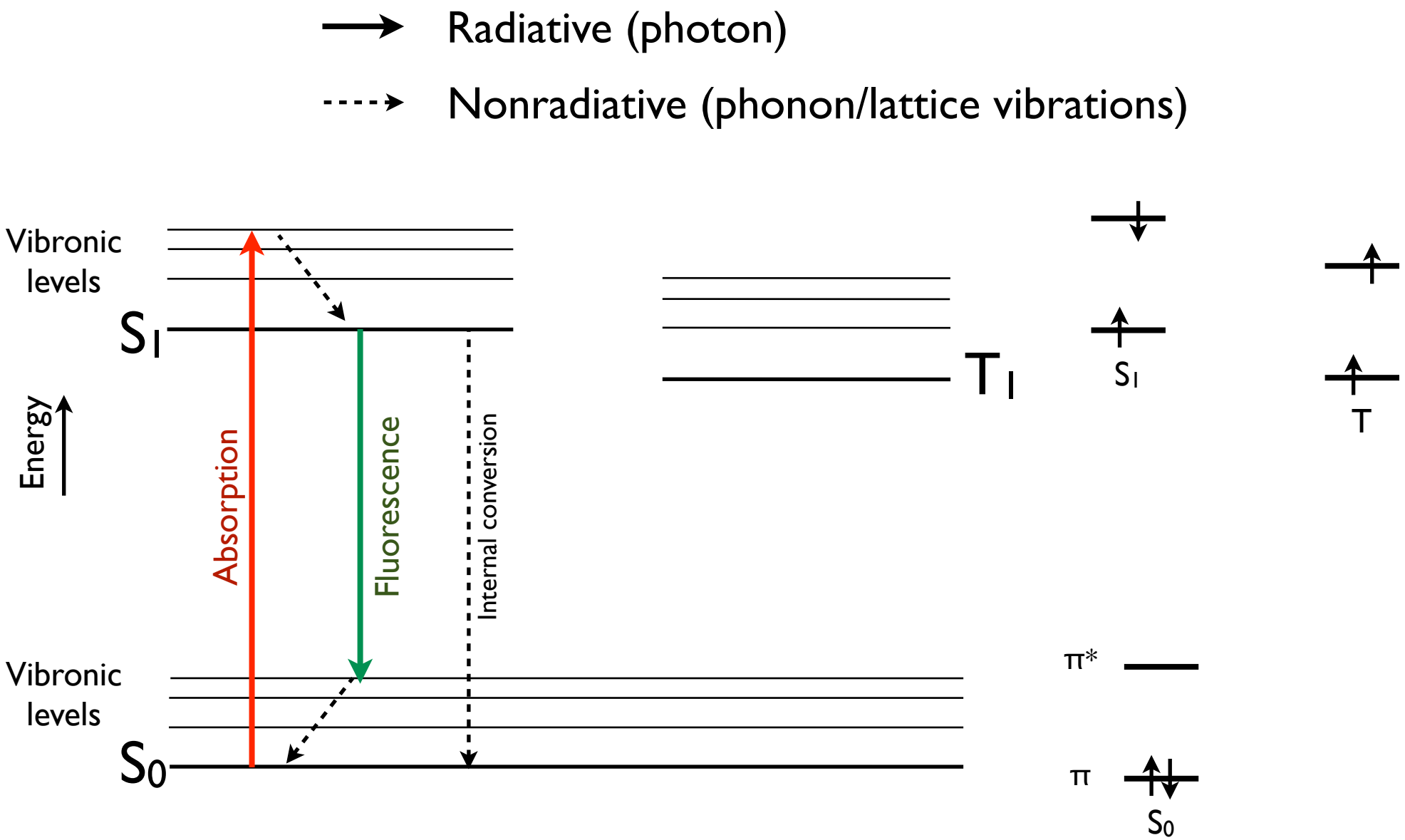
$$k_{abs}[S_0] = \{k_F + k_{ic}\}[S_1]$$

 # molecs excited *# excited states decayed*

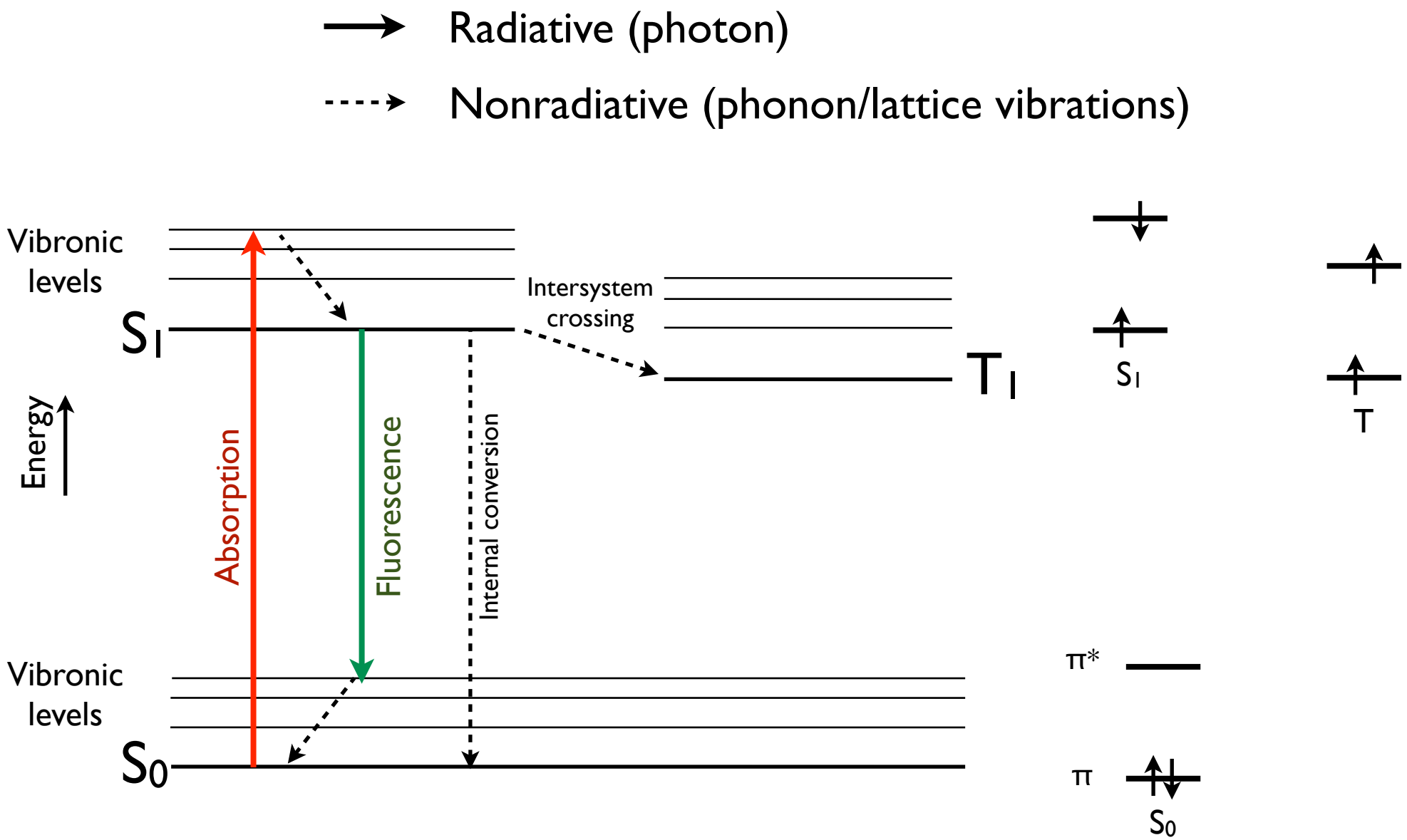
Fluorescence quantum yield:

$$(\#\text{ photons emitted})/(\#\text{ photons absorbed}) = \frac{k_F[S_1]}{\{k_F + k_{ic}\}[S_1]} = \frac{k_F}{\{k_F + k_{ic}\}}$$

Electronic Energy Levels



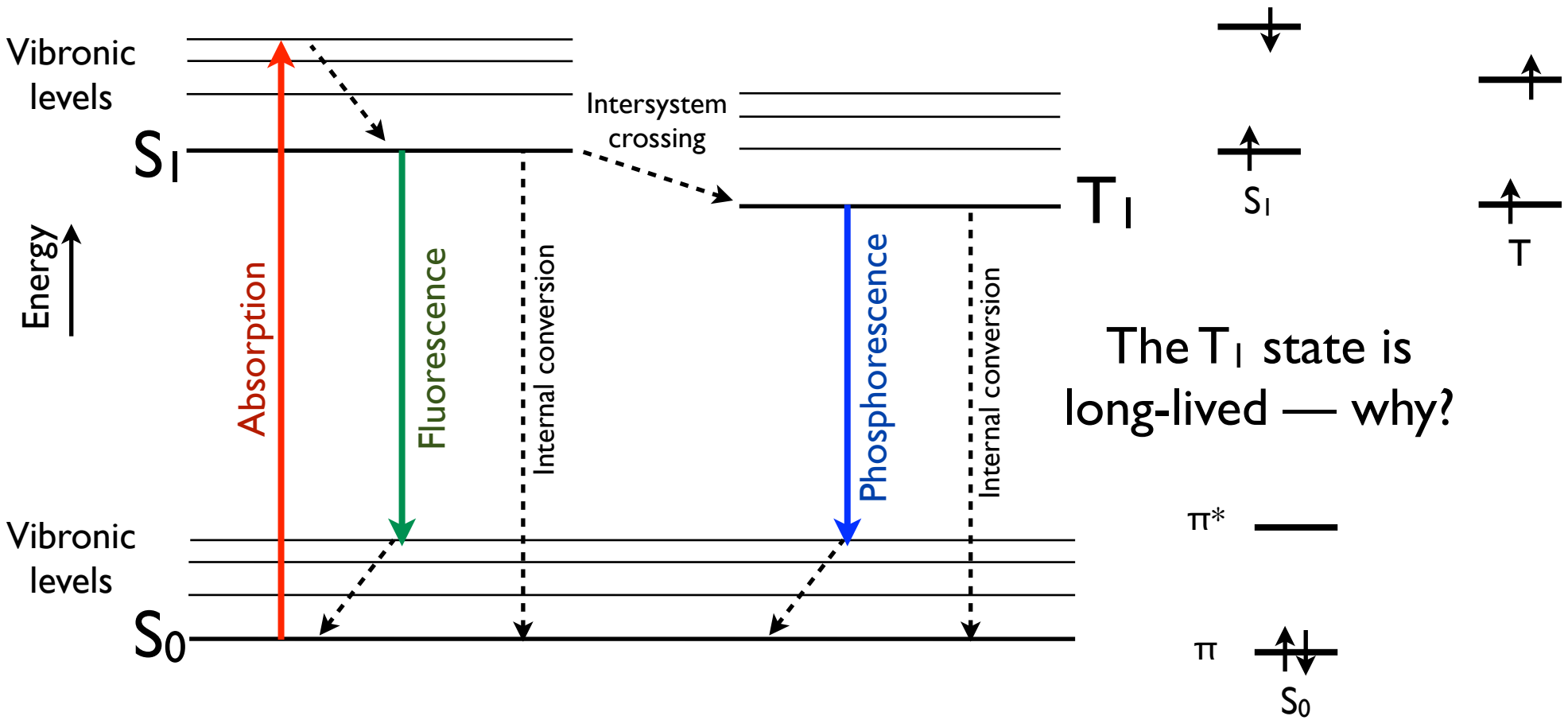
Electronic Energy Levels



Electronic Energy Levels

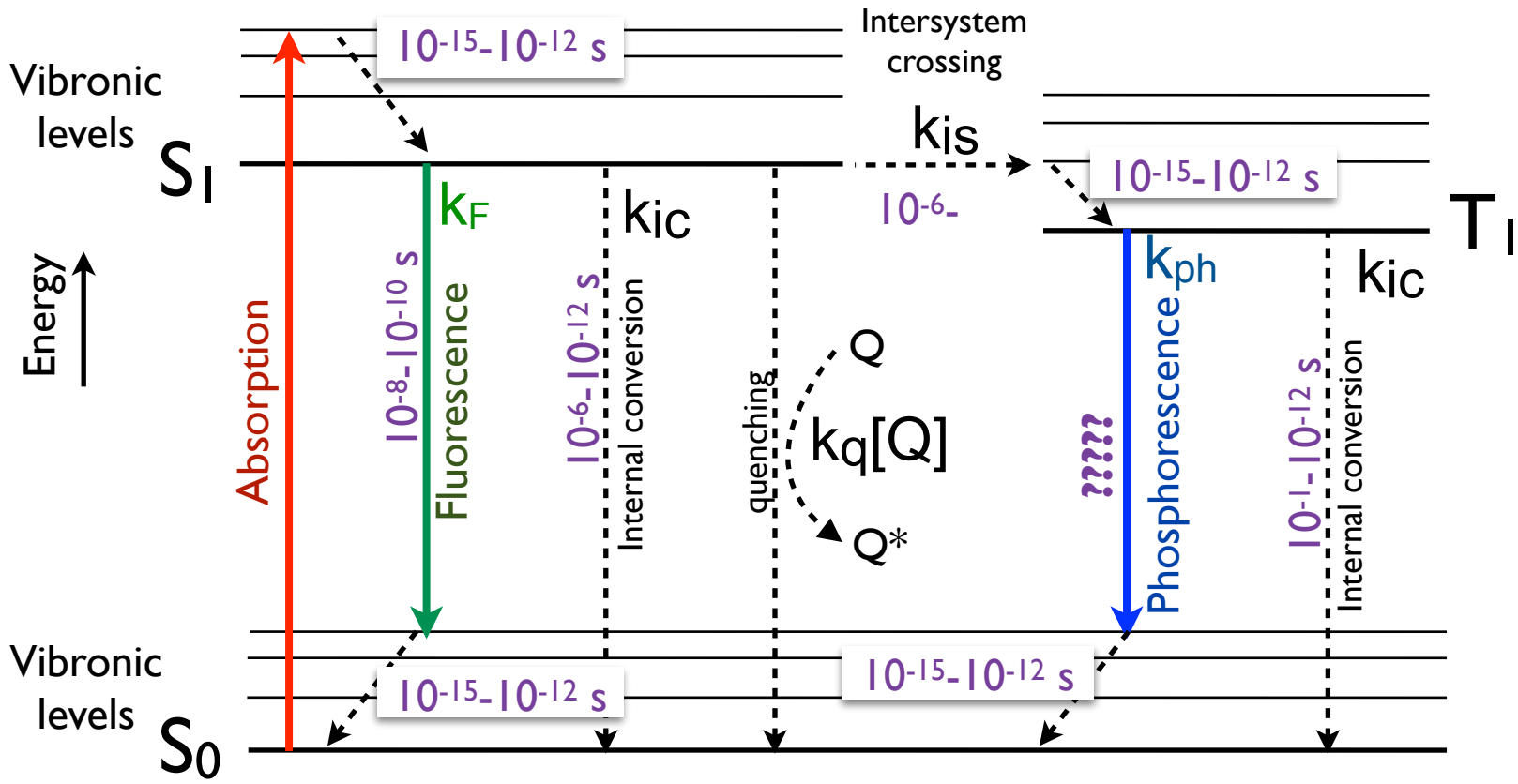
Radiative (photon)

Nonradiative (phonon/lattice vibrations)



The T_1 state is long-lived — why?

Electronic Energy Levels



What goes up, must come down

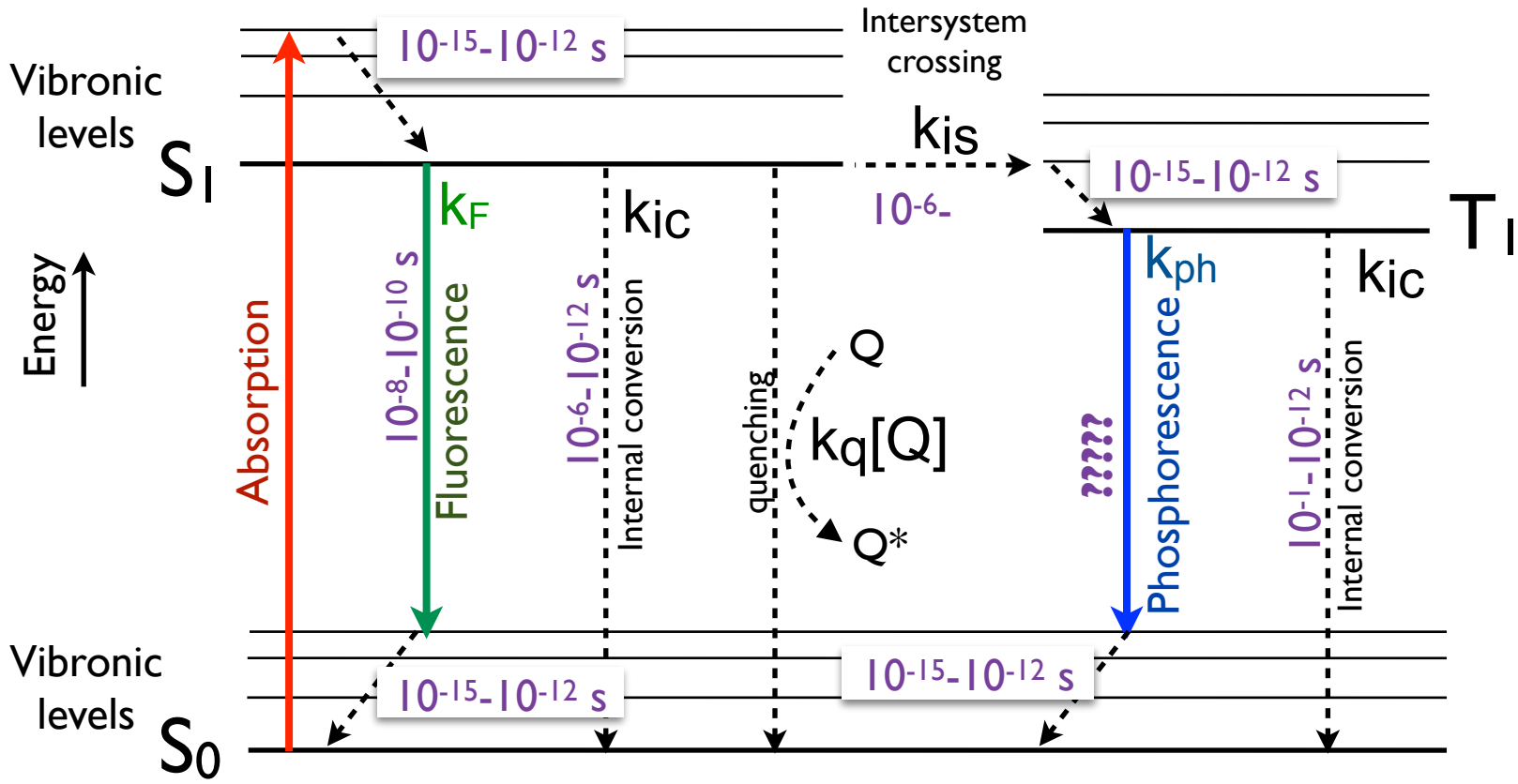
$$k_{abs}[S_0] = \{k_F + k_{ic} + k_q[Q] + k_{is}\}[S_1]$$

 # molecs excited *# excited states decayed*

Fluorescence quantum yield:

$$(\#\text{ photons emitted})/(\#\text{ photons absorbed}) = \frac{k_F}{\{k_F + k_{ic} + k_q[Q] + k_{is}\}}$$

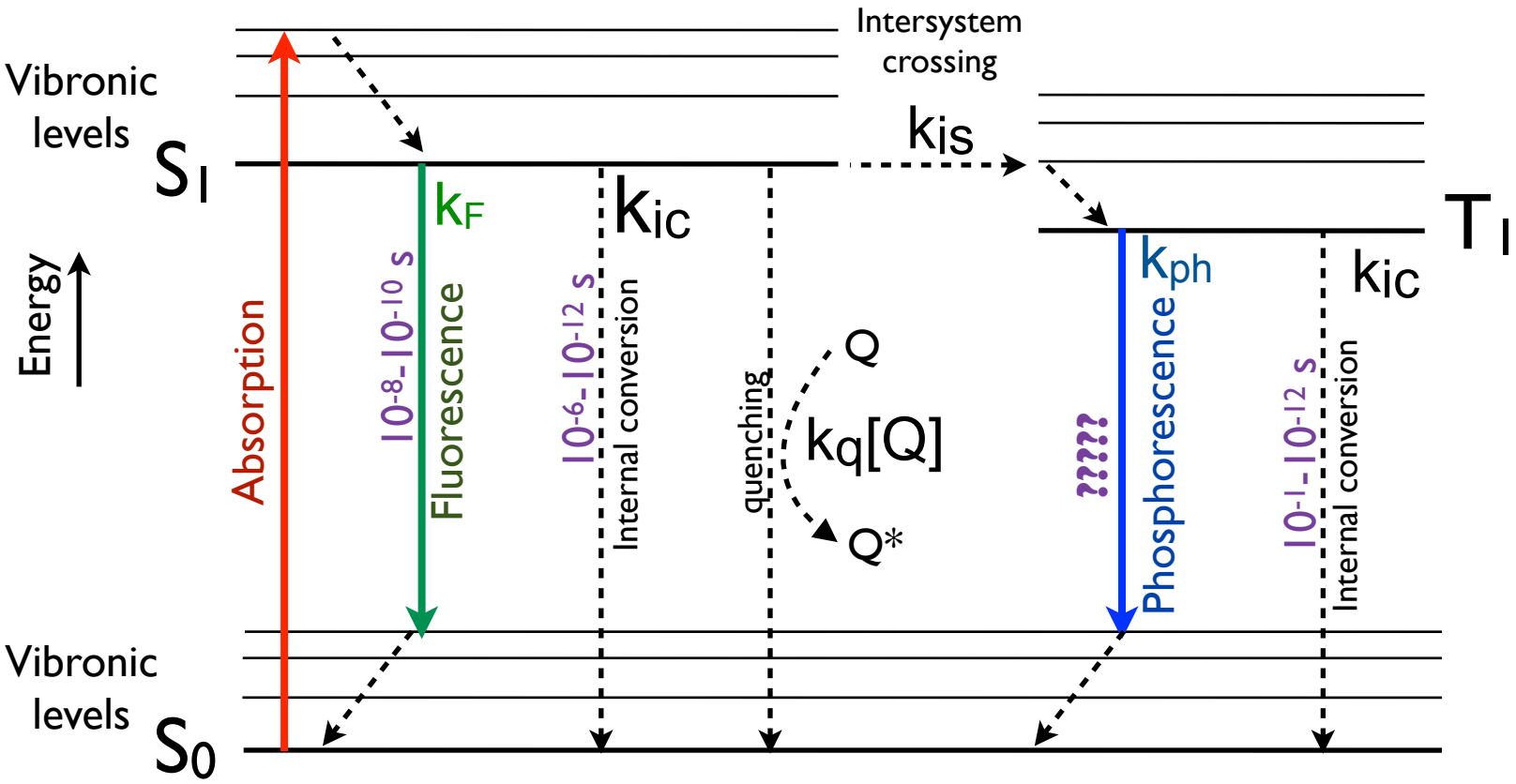
Electronic Energy Levels



$$\Phi_F = \frac{\#\text{ photons emitted}}{\#\text{ photons absorbed}} = \frac{k_F \cancel{[S_1]}}{\left[k_F + k_{ic} + k_q[Q] + k_{is}\right]\cancel{[S_1]}}$$

$$\Phi_F = \frac{\#\text{ photons emitted}}{\#\text{ photons absorbed}} = \frac{k_F}{\left[k_F + k_{ic} + k_q[Q] + k_{is}\right]}$$

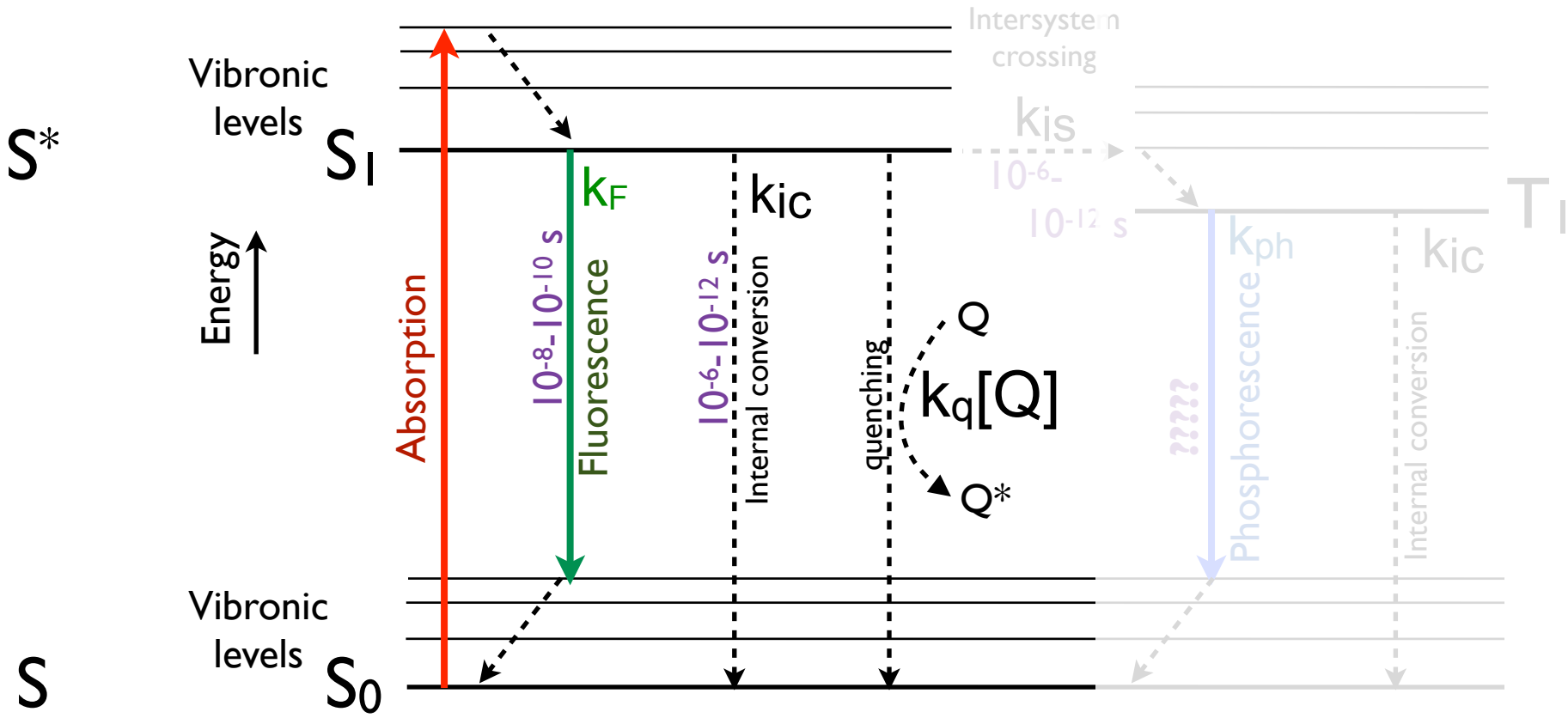
Fluorescence Quenching



k_{ic}

- Interactions with solvent/neighbor (environment, more broadly)
- Dissipation through internal vibrational modes
- increases with increasing temperature (usually)
  - practical alert: fluorescence can depend on temperature!!

Fluorescence Quenching



- Collisional deactivation $S^* + Q \longrightarrow S + Q^{vib/kin}$

k_q

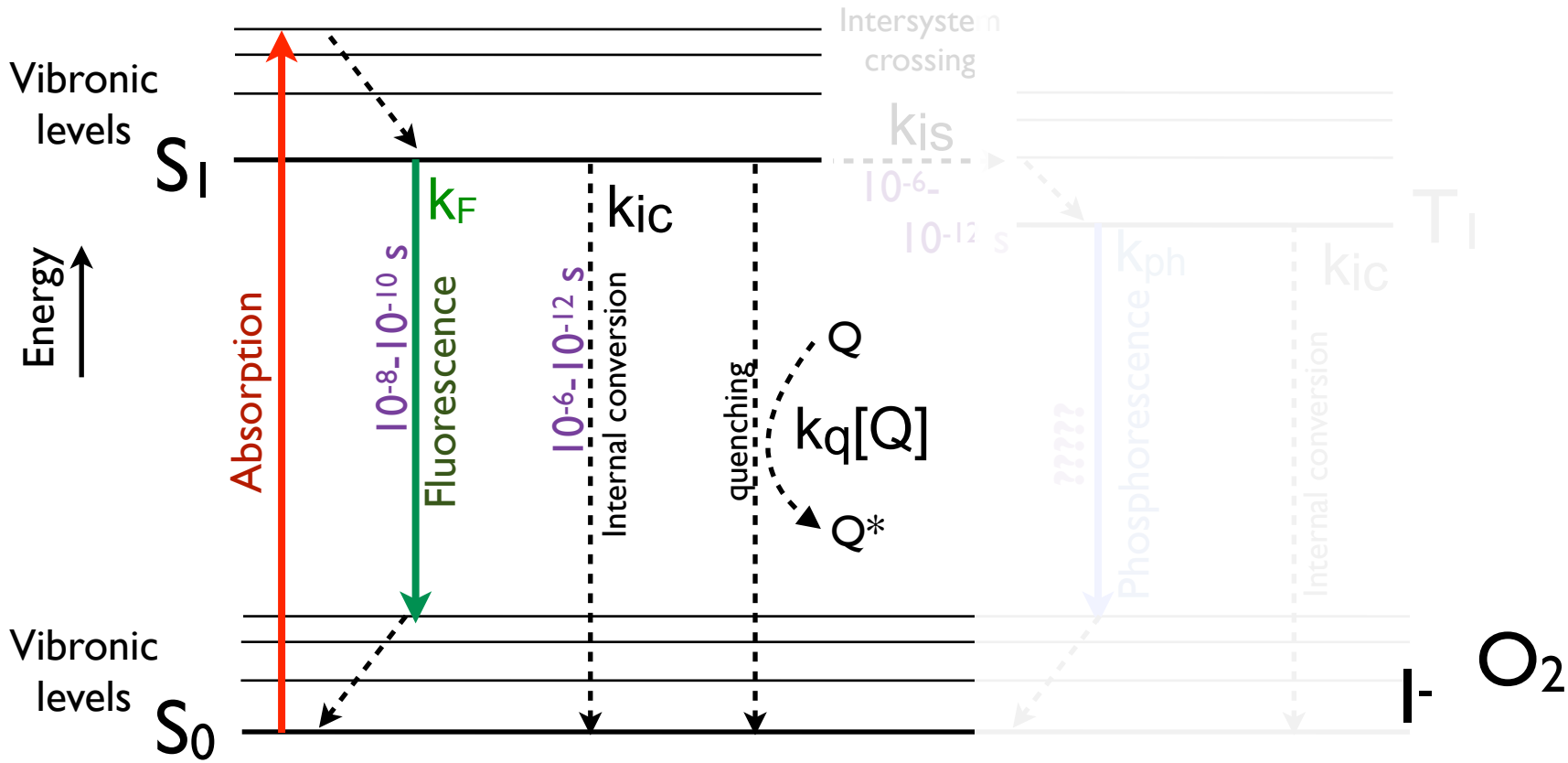
- Electron transfer $S^* + Q \longrightarrow S^+ + Q^-$

  $S^* + Q \longrightarrow S^- + Q^+$

- Resonance energy transfer $S^* + Q \longrightarrow S + Q^*$

I^- O_2

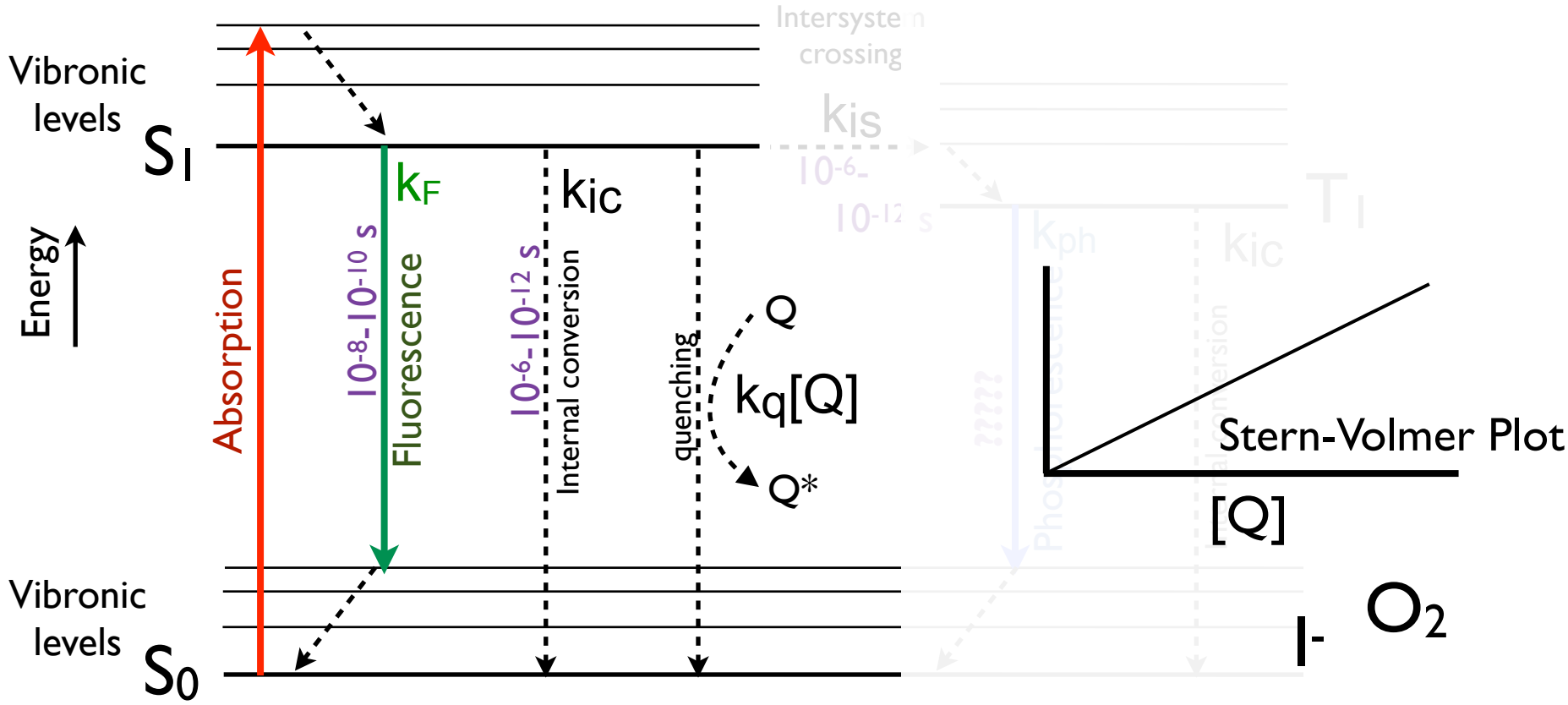
Fluorescence Quenching



- Collisional deactivation $S^* + Q \longrightarrow S + Q^{vib/kin}$

$$\frac{\Phi_F^{[Q]=0}}{\Phi_F^{Q}} = \frac{\dfrac{k_F}{k_F + k_{ic}}}{\dfrac{k_F}{k_F + k_{ic} + k_q[Q]}} = \frac{k_F + k_{ic} + k_q[Q]}{k_F + k_{ic}} = 1 + \frac{k_q}{k_F + k_{ic}}[Q]$$

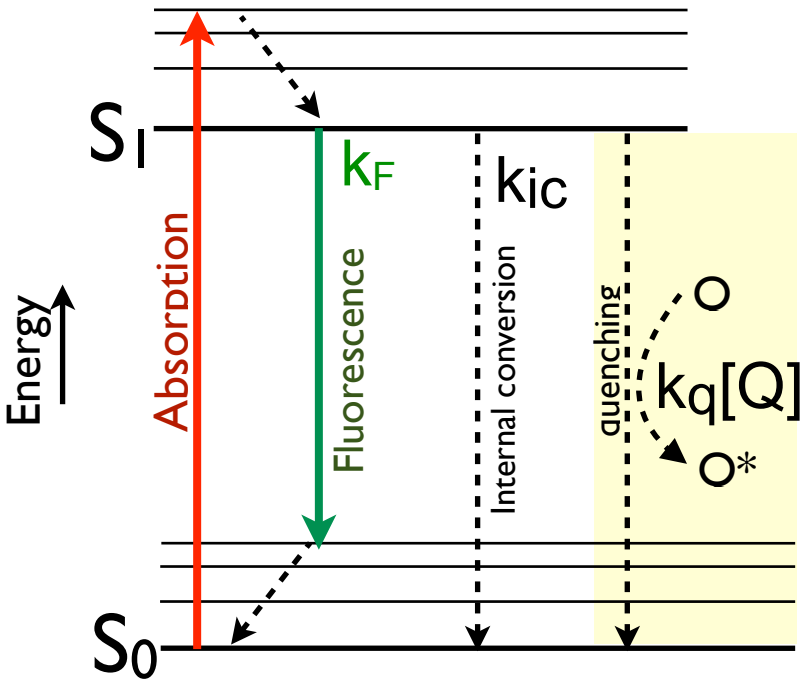
Fluorescence Quenching



- Collisional deactivation $S^* + Q \longrightarrow S + Q^{vib/kin}$

$$\frac{\Phi_F^{[Q]=0}}{\Phi_F^{Q}} = \frac{\frac{k_F}{k_F + k_{ic}}}{\frac{k_F}{k_F + k_{ic} + k_q[Q]}} = \frac{k_F + k_{ic} + k_q[Q]}{k_F + k_{ic}} = 1 + \frac{k_q}{k_F + k_{ic}}[Q]$$

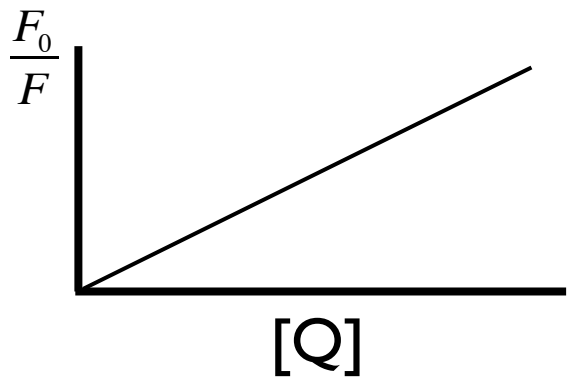
Electronic Energy Levels



<u>Collisional (dynamic)</u> assumes that interaction between fluorophore and quencher is purely "collisional" - two non-interacting billiard balls

$$S^* + Q \longrightarrow S + Q^{vib/kin}$$

Increasing collision freq



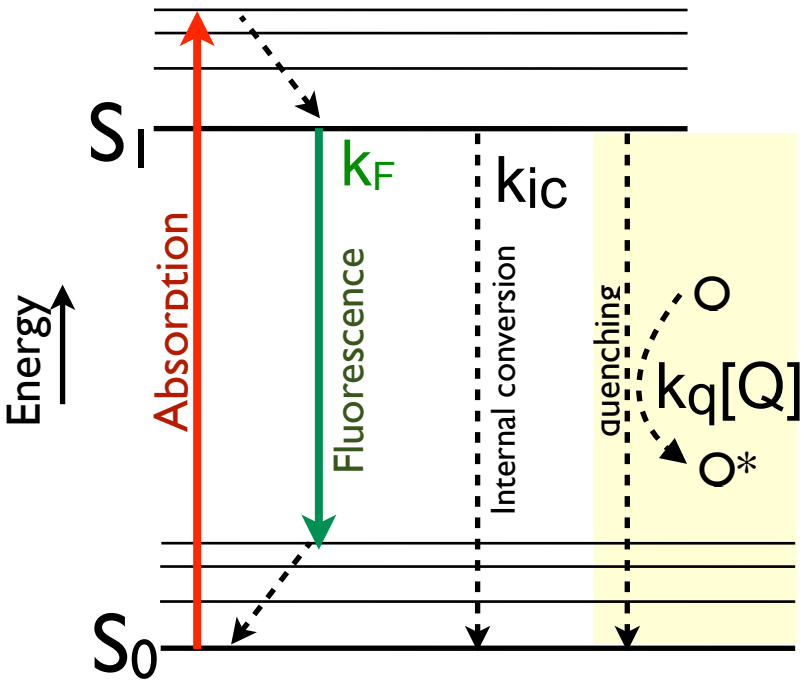
$$\frac{F_0}{F} = \frac{\Phi_F^{[Q]=0}}{\Phi_F^Q} = 1 + \frac{k_q}{k_F + k_{ic}}[Q] = 1 + K_{SV}[Q]$$

dynamic

$$A \xrightarrow{h\nu} A^* \xrightarrow{k[Q]} A + Q + heat$$

collisional - "billiard balls"

Electronic Energy Levels



Collisional (dynamic) assumes that interaction between fluorophore and quencher is purely "collisional" - two non-interacting billiard balls

$$S^* + Q \longrightarrow S + Q^{vib/kin}$$

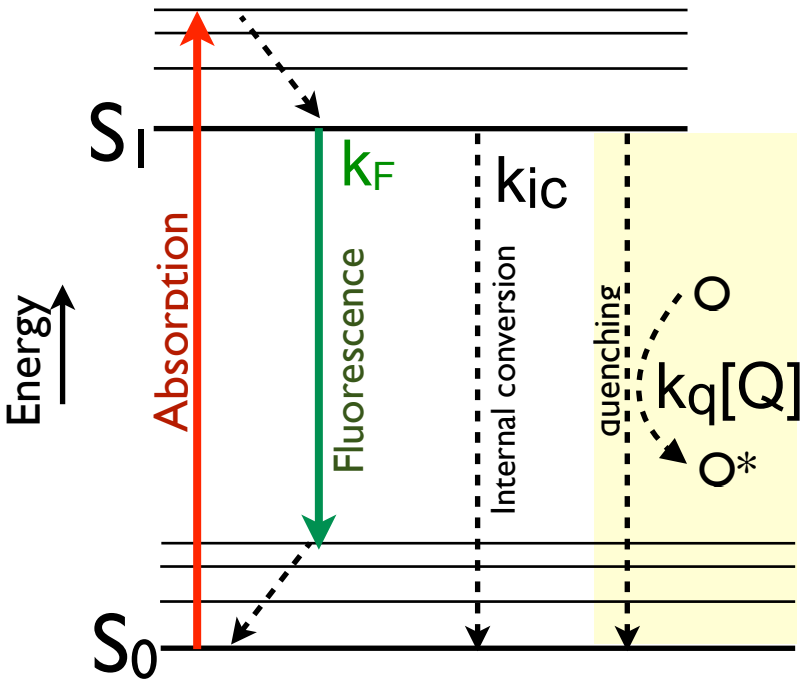
$$A + Q \underset{}{\overset{K}{\rightleftharpoons}} AQ \xrightarrow{h\nu} A^*Q \longrightarrow AQ + heat$$

static

$$A \xrightarrow{h\nu} A^* \xrightarrow{k[Q]} A + Q + heat$$

dynamic

Electronic Energy Levels



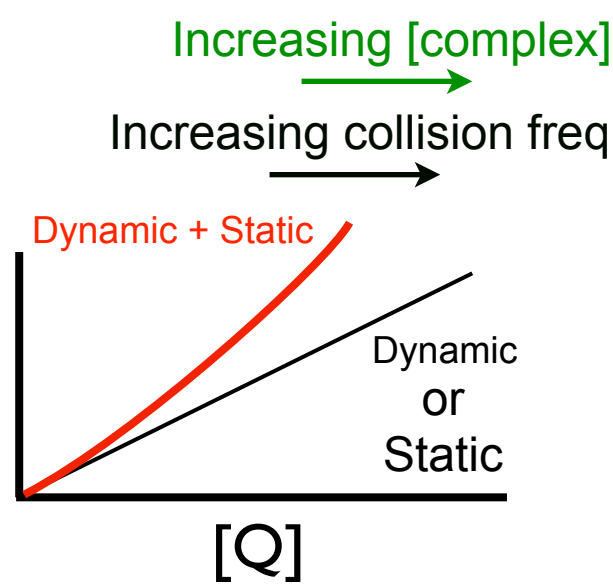
Collisional (dynamic) assumes that interaction between fluorophore and quencher is purely "collisional" - two non-interacting billiard balls

$$S^* + Q \longrightarrow S + Q^{vib/kin}$$

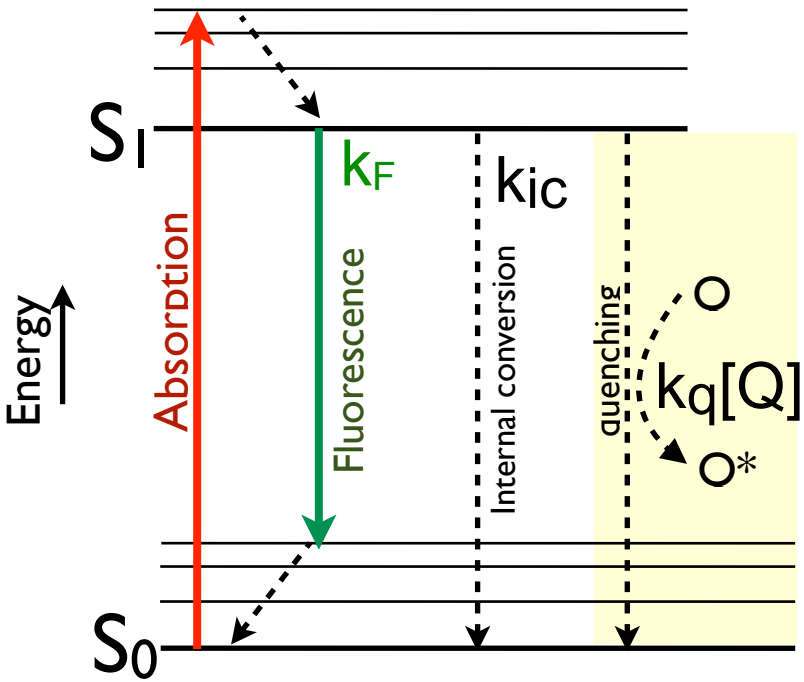
Binding (static) assumes that the quencher binds the fluorophore *in its ground state* and that the bound state has different relaxation properties.

$$K_D = \frac{[S][Q]}{[SQ]}$$

$$\frac{F_0}{F} = \underbrace{\left(1 + K_{SV}[Q]\right)}_{\text{dynamic}}\underbrace{\left(1 + K_D[Q]\right)}_{\text{static}}$$

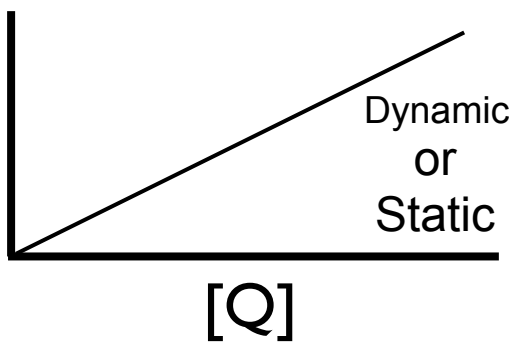


Electronic Energy Levels



Increasing [complex]

Increasing collision freq



Collisional (dynamic) assumes that interaction between fluorophore and quencher is purely "collisional" - two non-interacting billiard balls

$$S^* + Q \longrightarrow S + Q^{vib/kin}$$

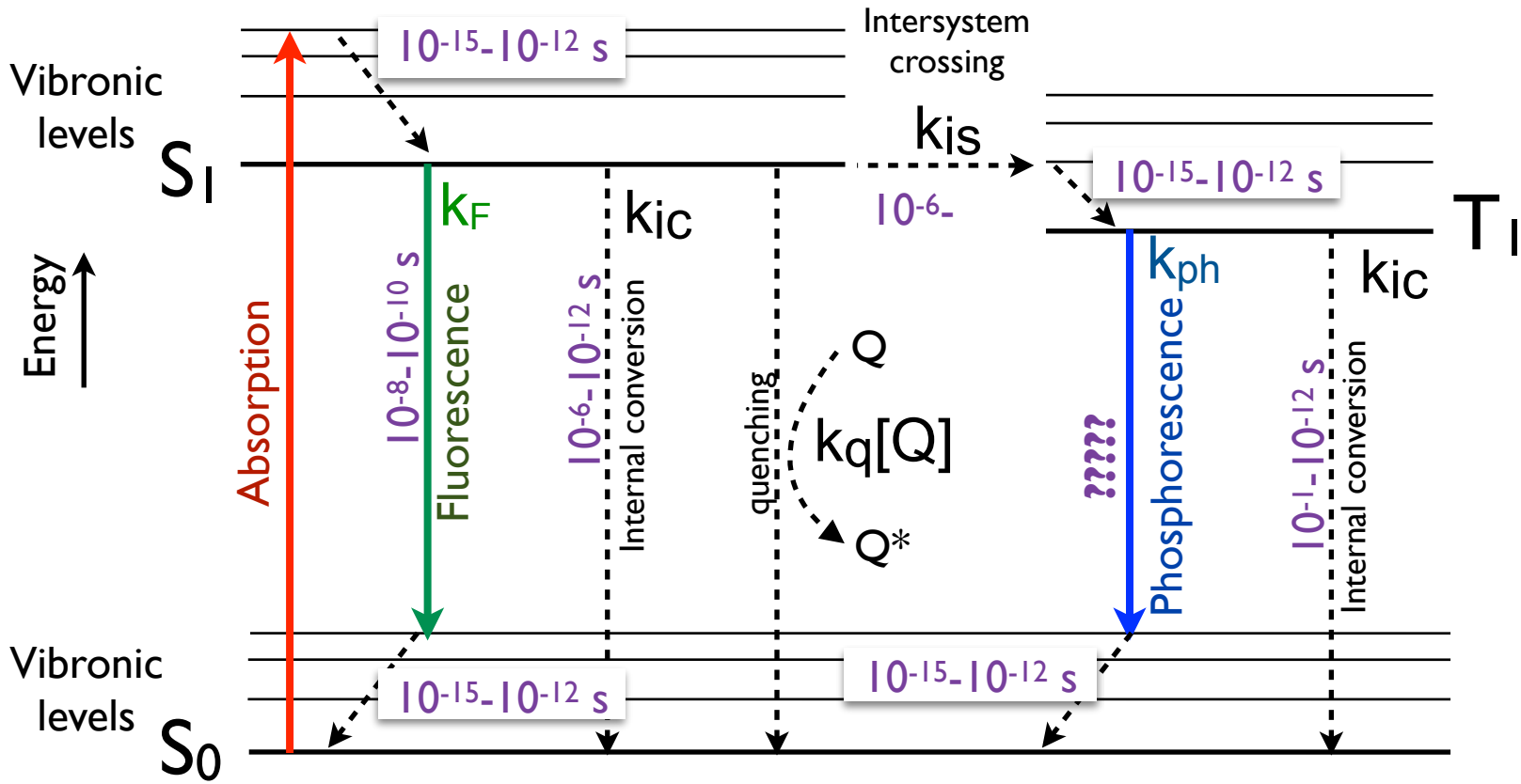
K_{SV} *usually* increases with temperature

Binding (static) assumes that the quencher binds the fluorophore *in its ground state* and that the bound state has different relaxation properties.

$$K_D = \frac{[S][Q]}{[SQ]}$$

K_D *usually* decreases with temperature

Electronic Energy Levels



Fluorescence Quantum Yield

$$\Phi_F = \frac{\#\text{ photons emitted}}{\#\text{ photons absorbed}} = \frac{k_F}{k_F + k_{ic} + k_q[Q] + k_{is}} = \tau_0 k_F$$

$$\tau_F = \frac{1}{k_F}$$

Fluorescence Lifetime (S_1 lifetime) (fluorescence decay)

$$\tau_0 = \frac{1}{k_F + k_{ic} + k_q[Q] + k_{is}}$$

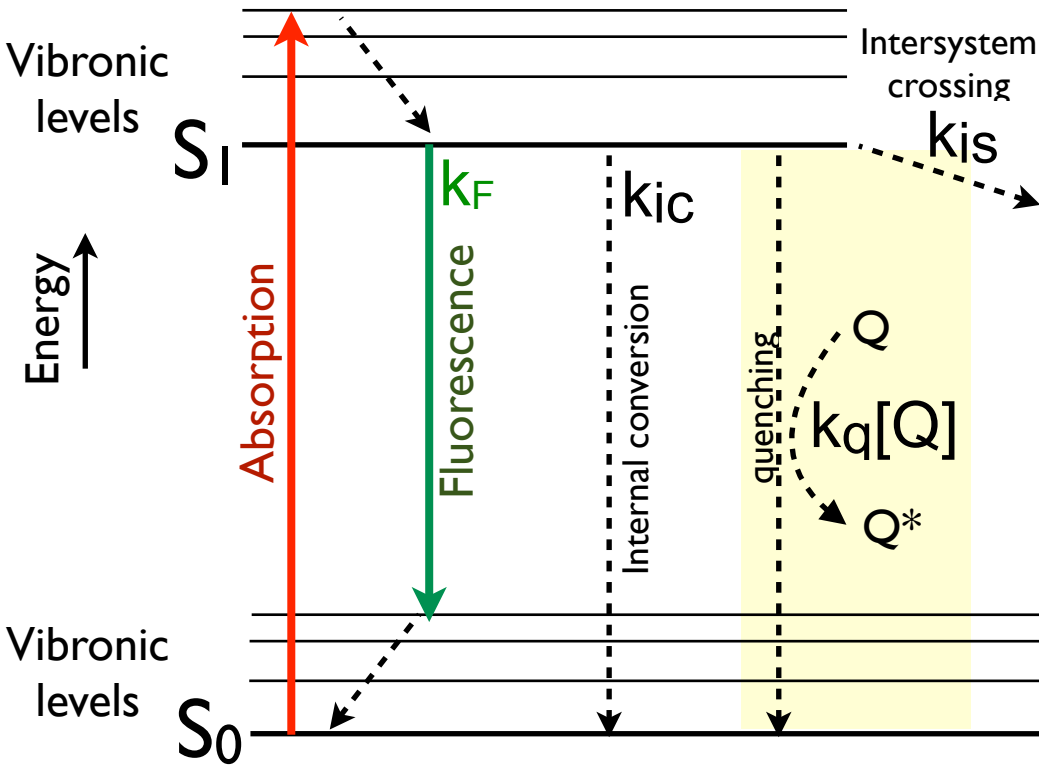
Fluorescence Lifetimes

Fluorophore	Lifetime (ns)	Excitation Max [nm]	Emission Max [nm]	Solvent
ATTO 655	3.6	655	690	Water
Acridine Orange	2.0	500	530	PB pH 7.8
Alexa Fluor 488	4.1	494	519	PB pH 7.4
Alexa Fluor 647	1.0	651	672	Water
BODIPY FL	5.7	502	510	Methanol
Coumarin 6	2.5	460	505	Ethanol
CY3B	2.8	558	572	PBS
CY3	0.3	548	562	PBS
CY5	1.0	646	664	PBS
Fluorescein	4.0	495	517	PB pH 7.5
Oregon Green 488	4.1	493	520	PB pH 9
$Ru(bpy)_2(dcpby)[PF_6]_2$	375	458	650	Water
Pyrene	> 100	341	376	Water
Indocyanine Green	0.52	780	820	Water
Rhodamine B	1.68	562	583	PB 7.8

Fluorescence Lifetime

$$\tau_0 = \frac{k_F}{k_F + k_{ic} + k_q[Q] + k_{is}}$$

Electronic Energy Levels



Excite a population, then turn off hν

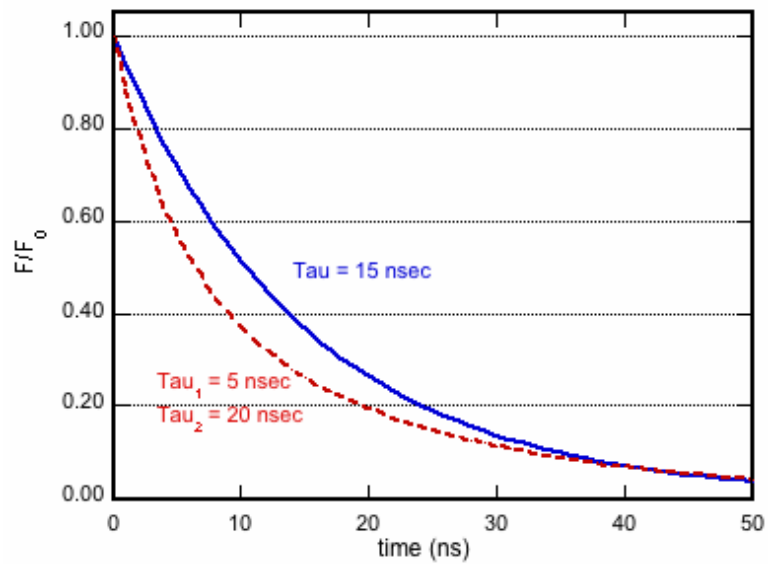
$$[S_1]_t = [S_1]_{t=0} e^{-k_{tot} t}$$

$$F = k_F [S_1]_t = k_F [S_1]_{t=0} e^{-k_{tot} t} = F_0 e^{-k_{tot} t}$$

$$\frac{F}{F_0} = e^{-k_{tot} t}$$

Exponential decay

If k_{tot} is a mix of population, decay will be multiexponential



What if multiple fluorophores with different lifetime parameters?

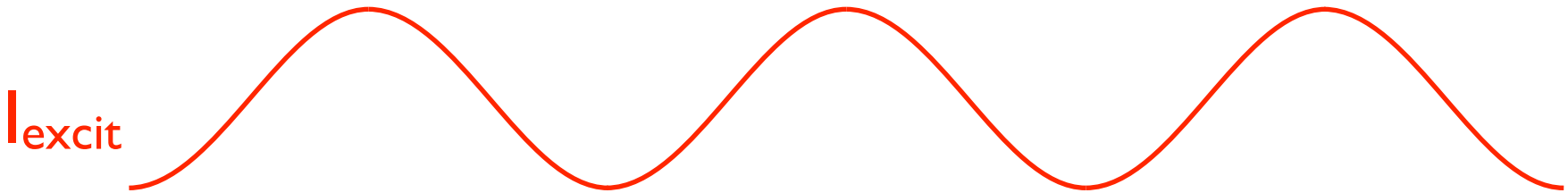
Better

Time resolved fluorescence

$$\frac{\tau_0}{\tau} = 1 + \tau_0 k_0 [Q] = 1 + K_{SV} [Q]$$

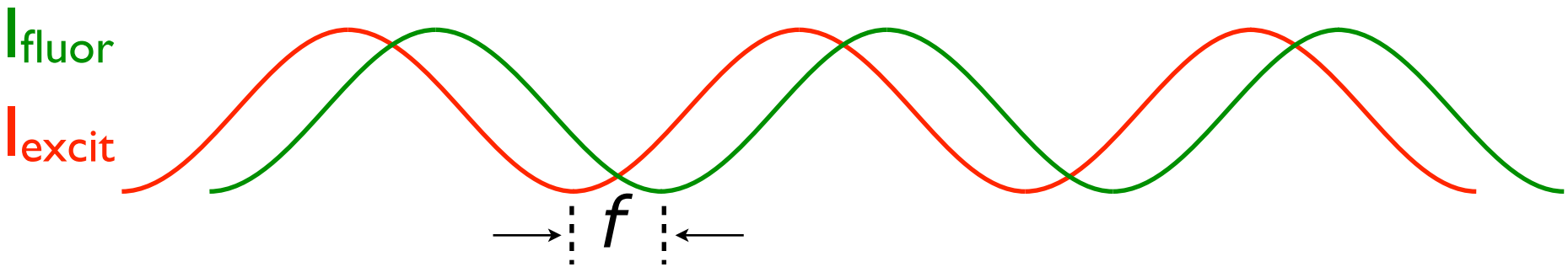
Phase Modulated Time Resolved Fluorescence

Modulate intensity of excitation light



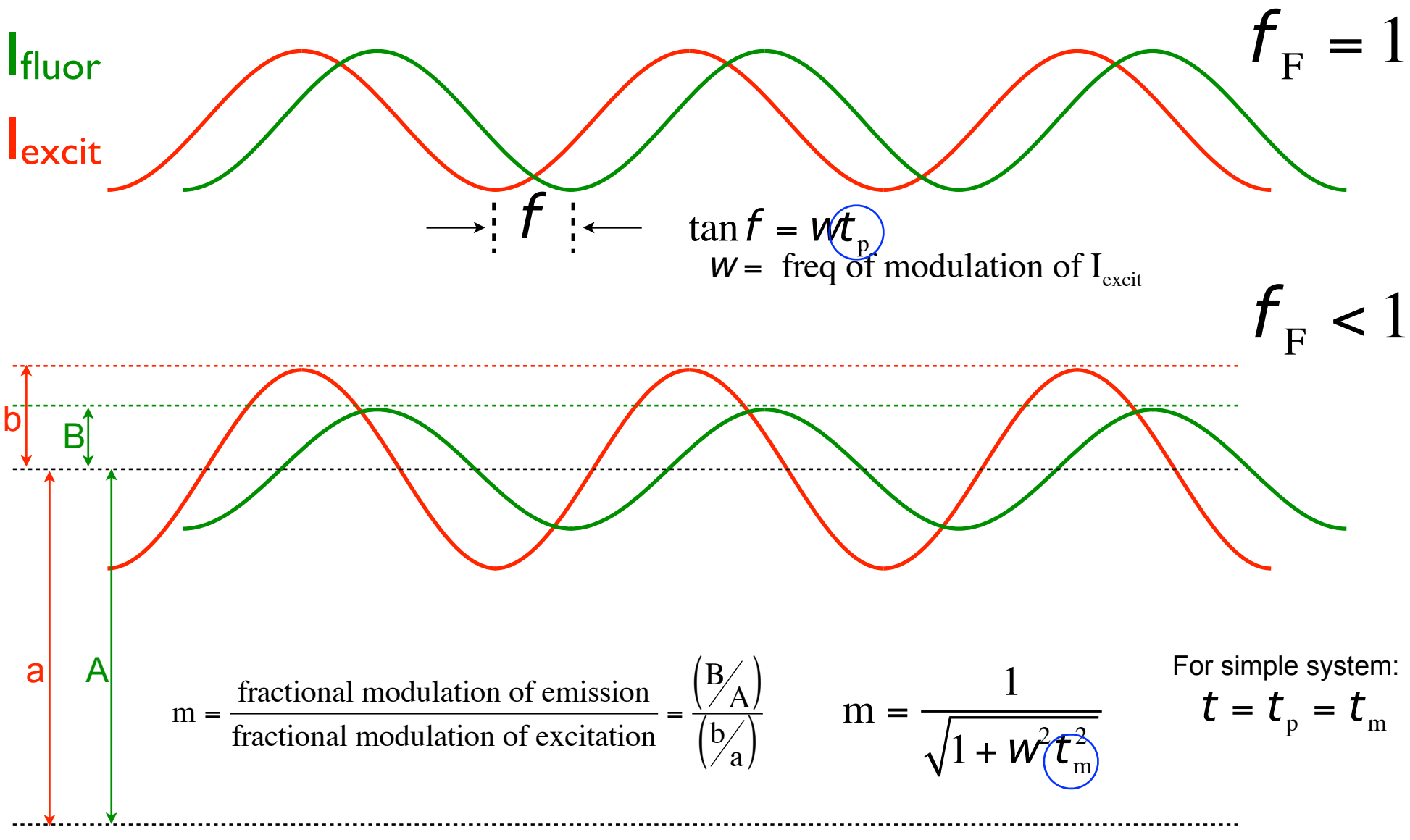
Phase Modulated Time Resolved Fluorescence

Modulate intensity of excitation light

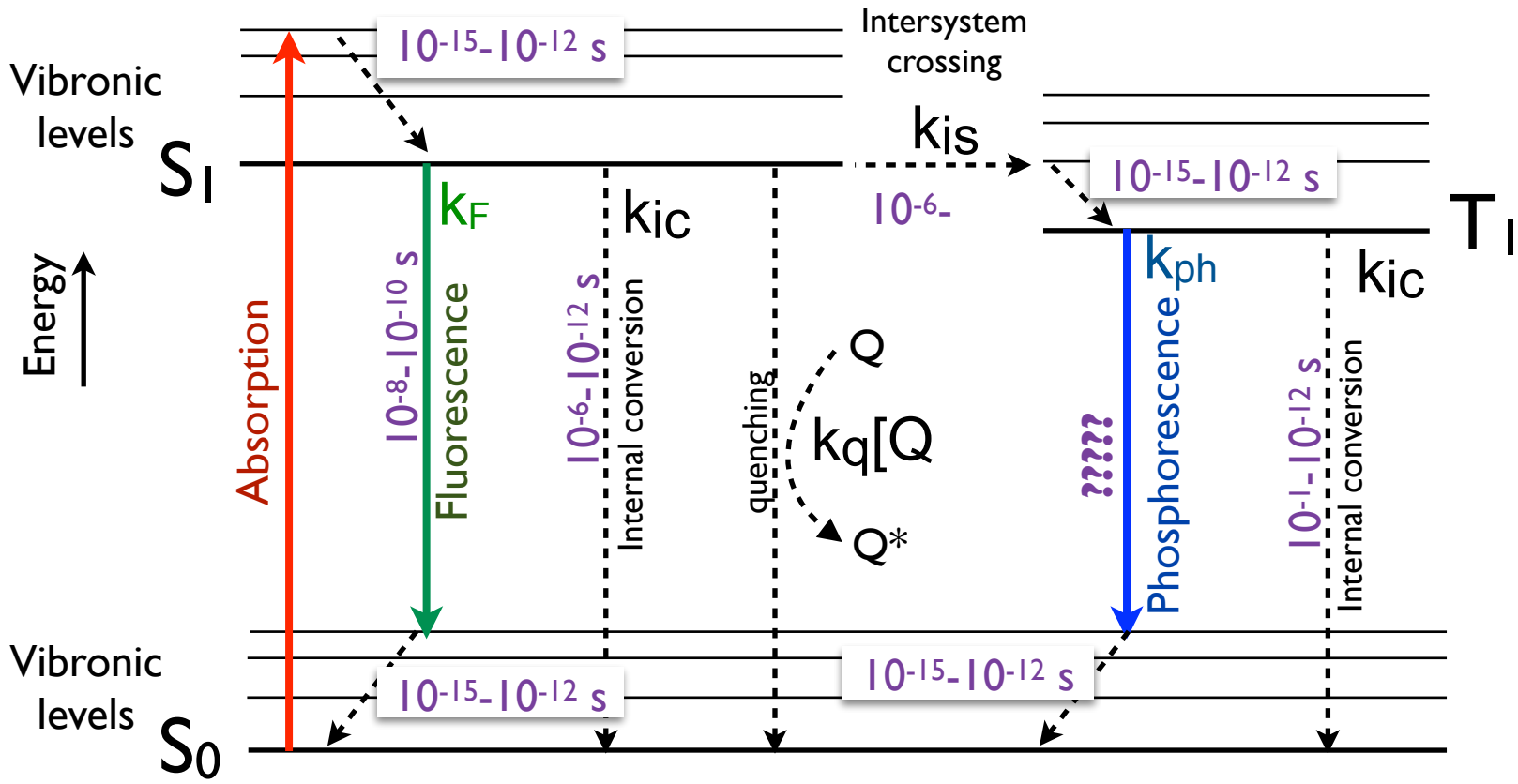


Phase Modulated Time Resolved Fluorescence

Modulate intensity of excitation light



Electronic Energy Levels

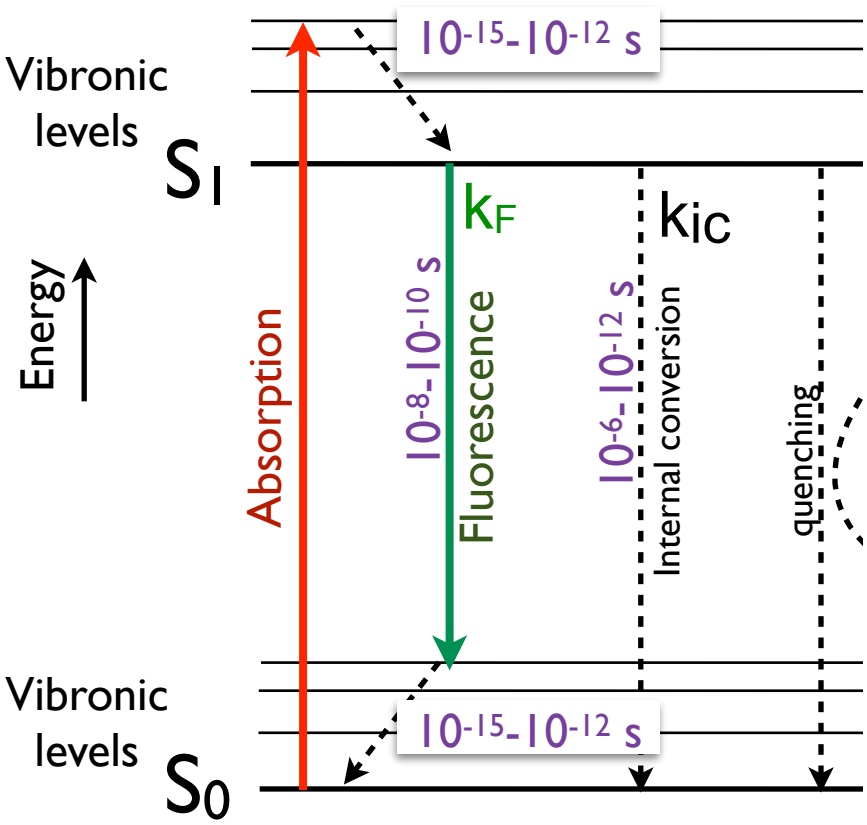


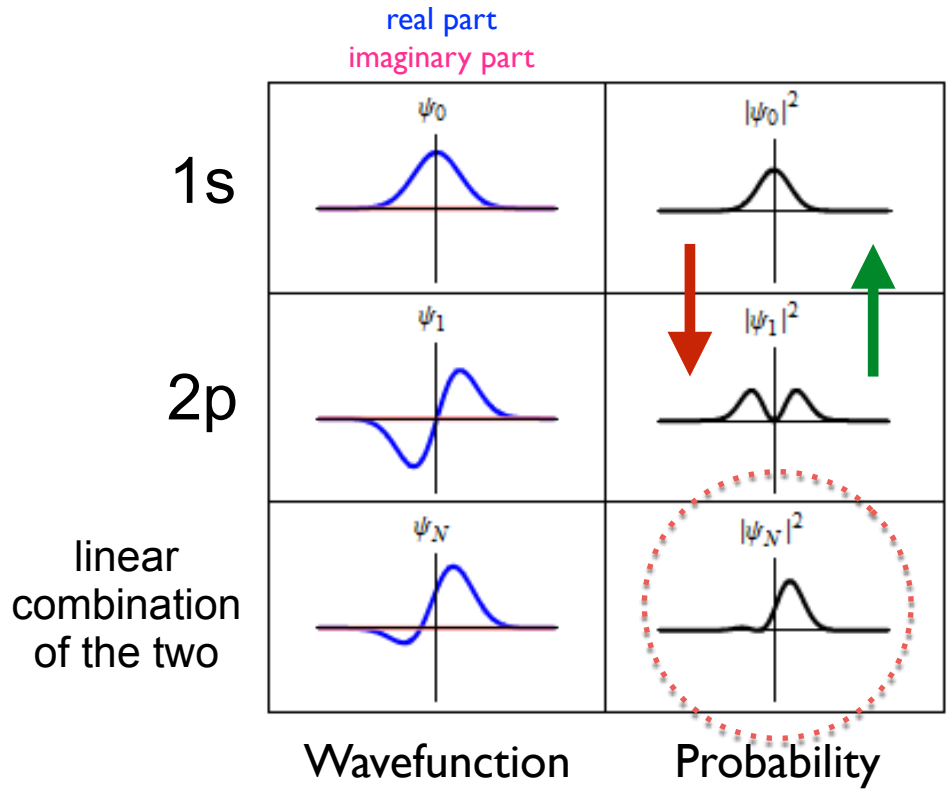
$$B_{S_0S_1} = B_{S_1S_0} = \langle \psi_{S_1} | \mu | \psi_{S_0} \rangle$$

Light emitted in fluorescence is subject to (almost) the same rules as that absorbed initially

Same polarization rules
(but any propagation direction)

Electronic Energy Levels





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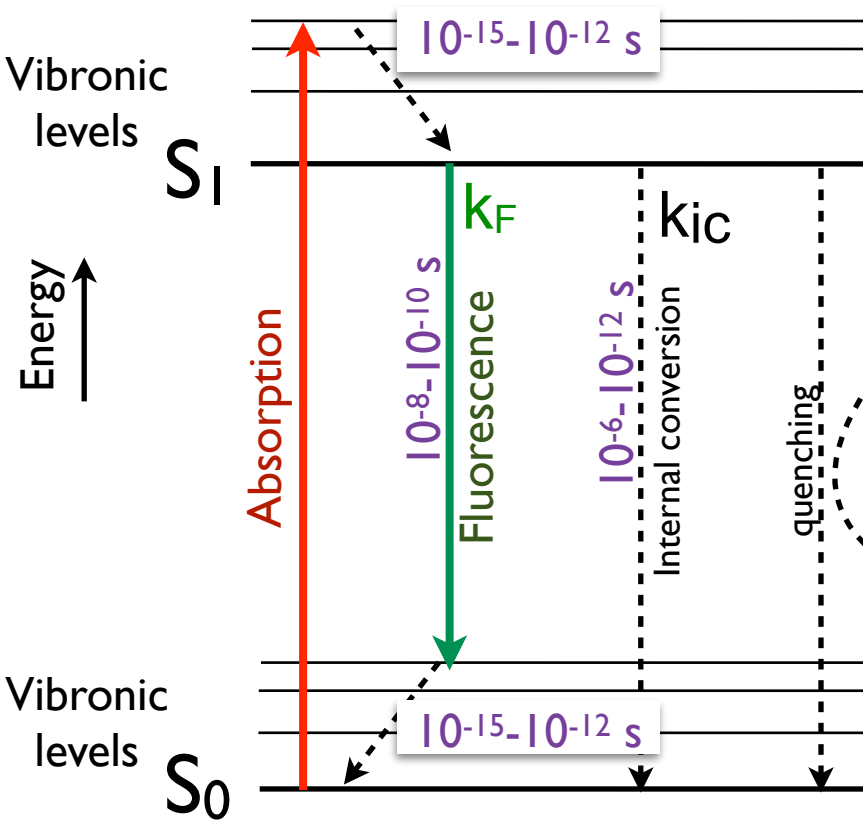
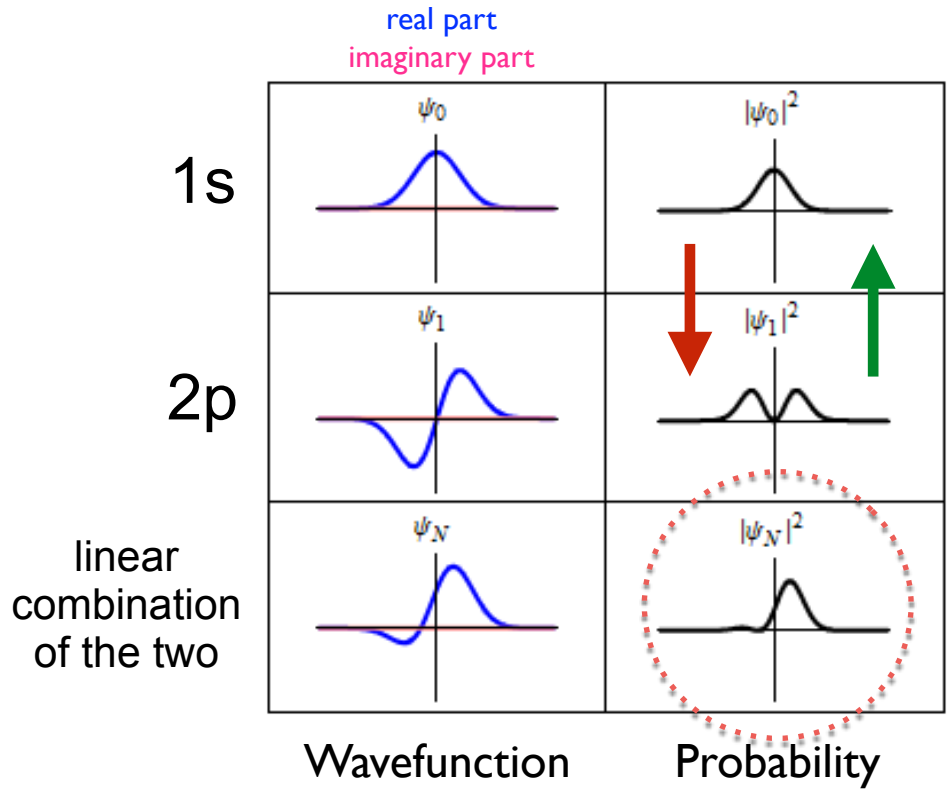
Electronic Energy Levels





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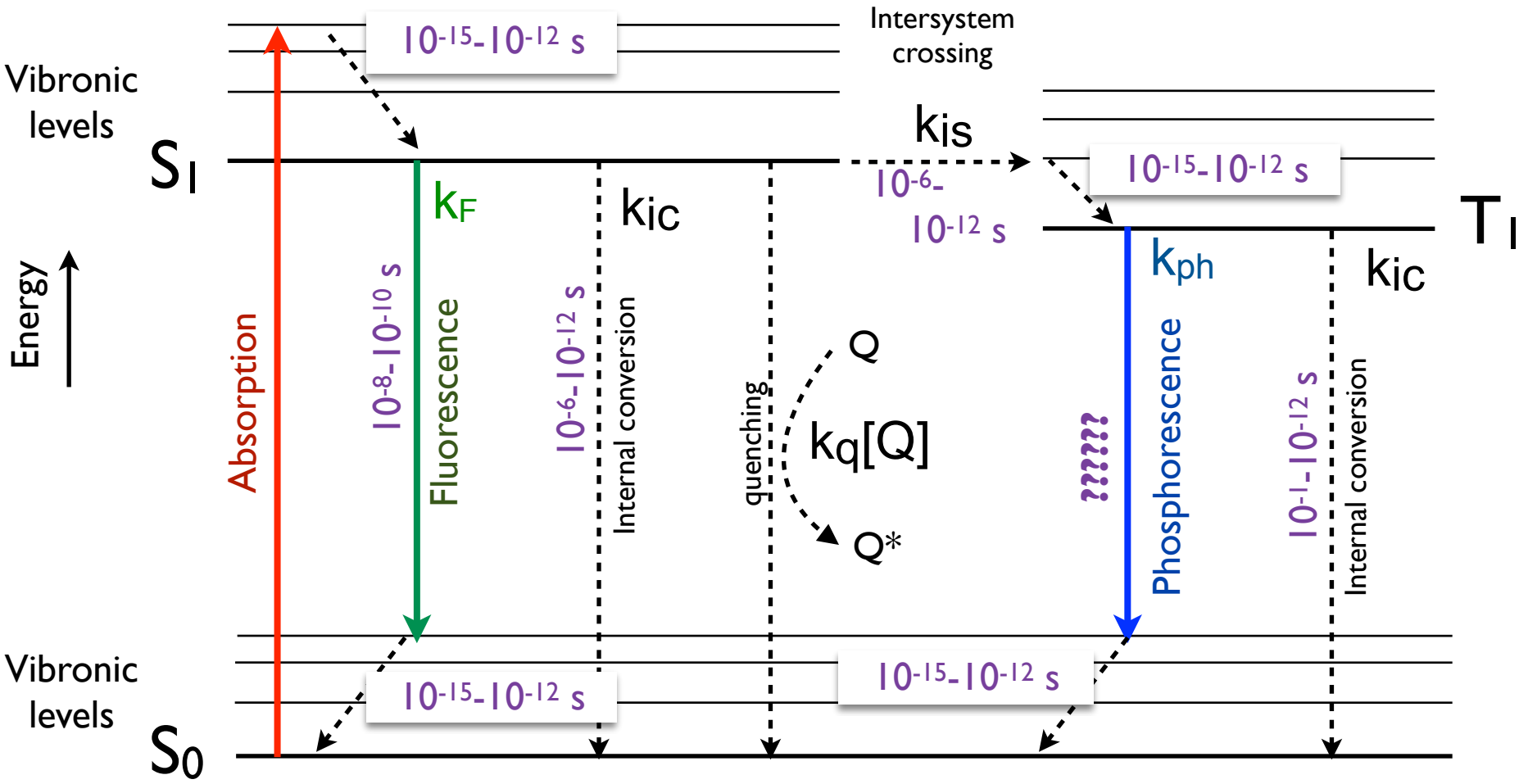
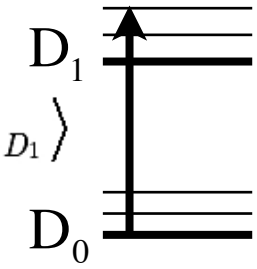
Electronic Energy Levels



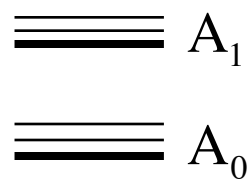
Fluorescence Resonance Energy Transfer

A particular type of quenching

$$\mu_D = \langle \psi_{D0} | \mu | \psi_{D1} \rangle$$



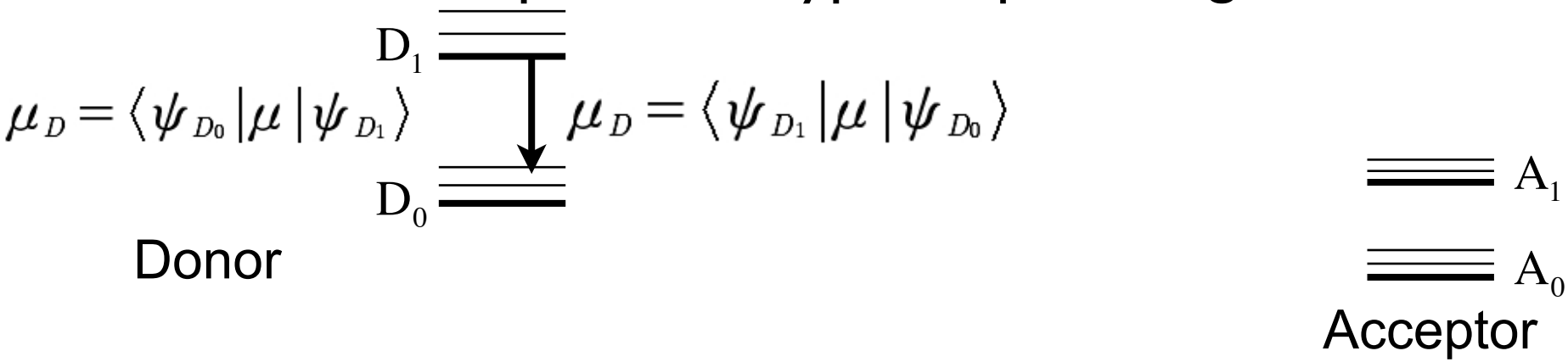
Donor



Acceptor

Fluorescence Resonance Energy Transfer

A particular type of quenching



Donor

Acceptor

Fluorescence Resonance Energy Transfer

A particular type of quenching

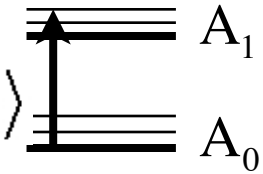
D_1

$\mu_D = \langle \psi_{D0} | \mu | \psi_{D1} \rangle$ $\quad$ $\mu_D = \langle \psi_{D1} | \mu | \psi_{D0} \rangle$

D_0

Donor

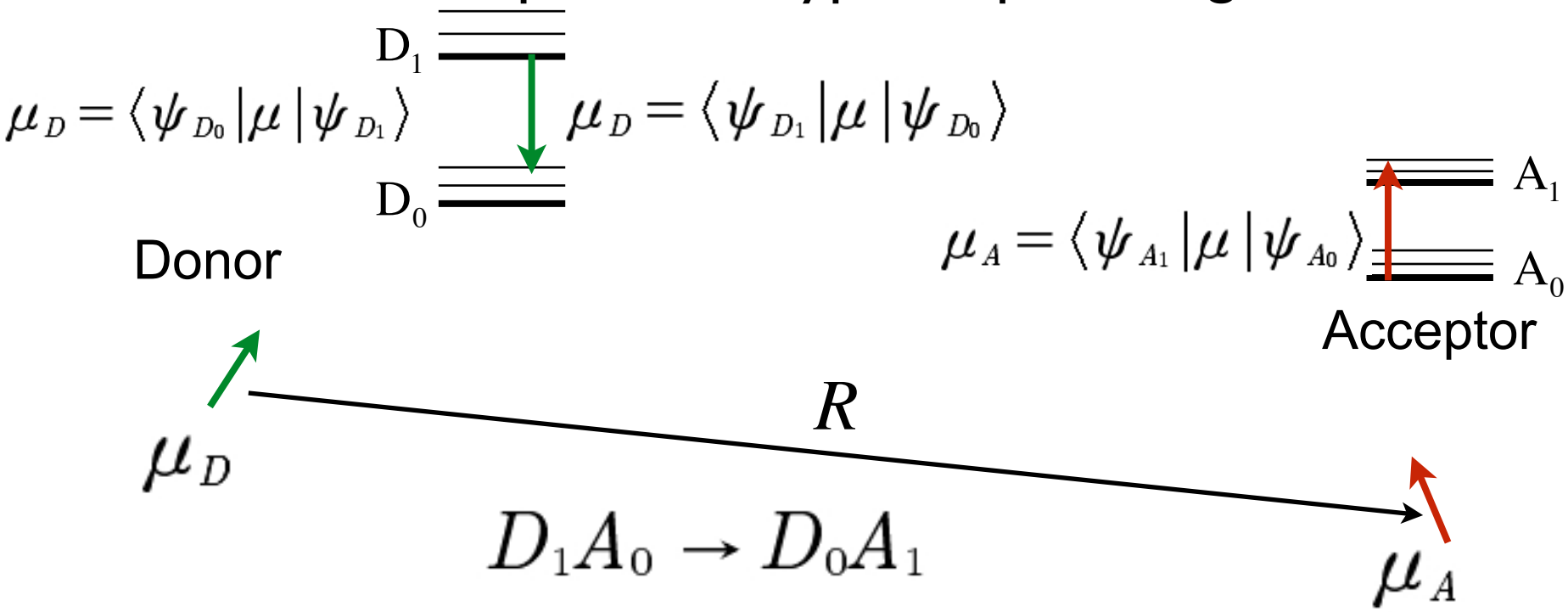
$\mu_A = \langle \psi_{A1} | \mu | \psi_{A0} \rangle$



Acceptor

Fluorescence Resonance Energy Transfer

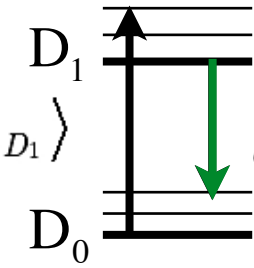
A particular type of quenching



Fluorescence Resonance Energy Transfer

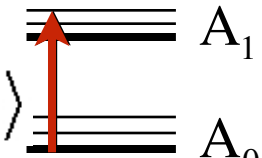
A particular type of quenching

$\mu_D = \langle \psi_{D_0} | \mu | \psi_{D_1} \rangle$ $\mu_D = \langle \psi_{D_1} | \mu | \psi_{D_0} \rangle$



Donor

$\mu_A = \langle \psi_{A_1} | \mu | \psi_{A_0} \rangle$



Acceptor

oscillating transition dipole

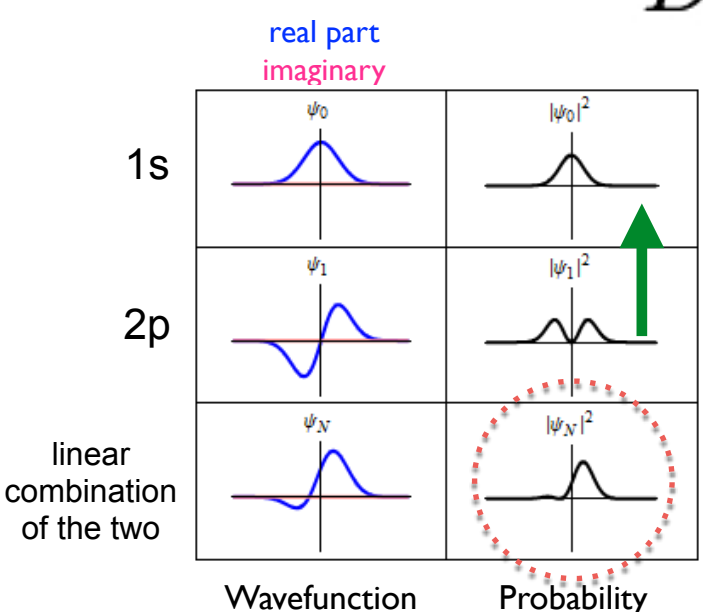
μ_D

R

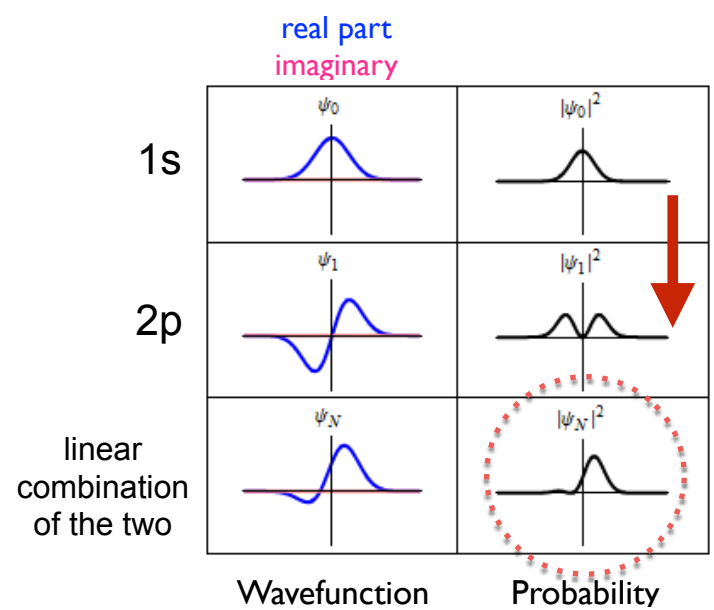
oscillating transition dipole

μ_A

$$D_1A_0 \rightarrow D_0A_1$$

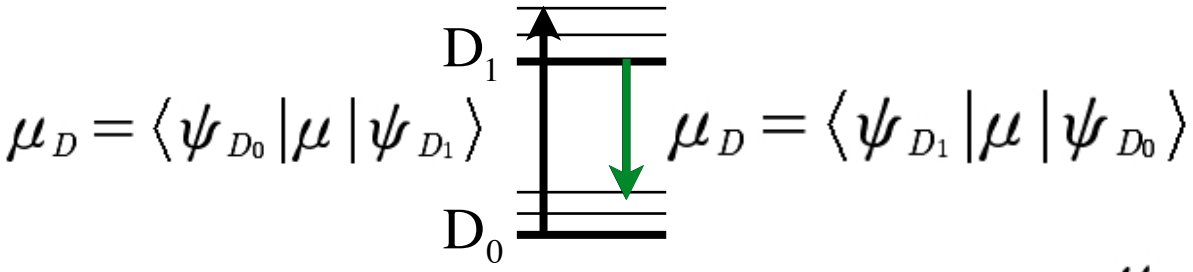


Interacting transition dipoles

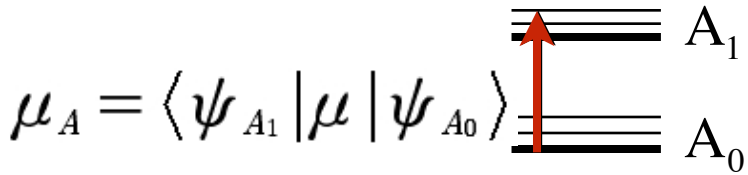


Fluorescence Resonance Energy Transfer

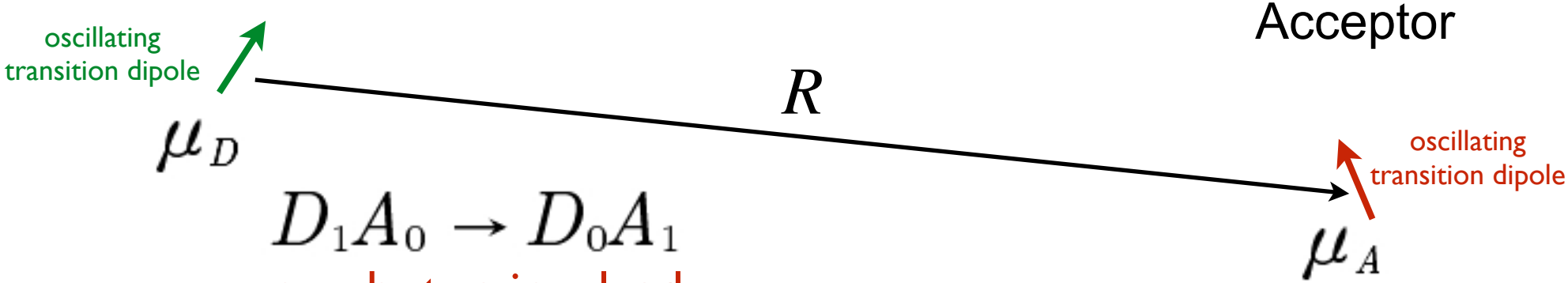
A particular type of quenching



Donor



Acceptor



$$D_1A_0 \rightarrow D_0A_1$$

no photon involved

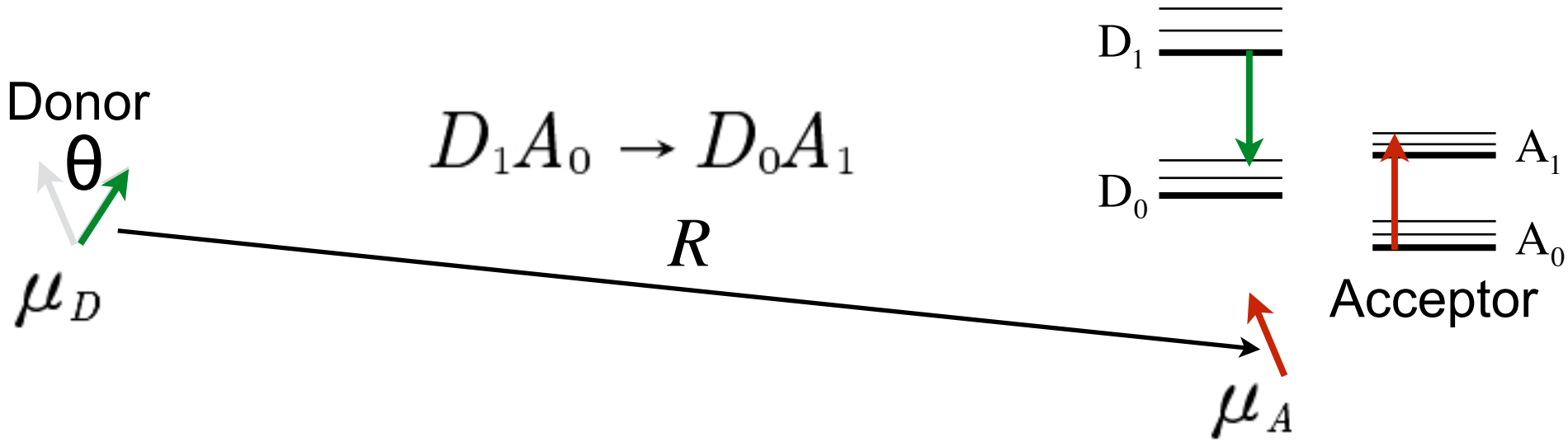
induced dipole-dipole interaction

$$Rate \propto \left| \langle \psi_{D0} \psi_{A1} | \mu_{dipole-dipole} | \psi_{D1} \psi_{A0} \rangle \right|^2$$

$$k_{ET} \propto \left| \left\langle \psi_{D0} \psi_{A1} \left| \frac{\vec{\mu}_D \bullet \vec{\mu}_A}{R^3} - \frac{(\vec{\mu}_D \bullet \vec{\mathbf{R}})(\vec{\mathbf{R}} \bullet \vec{\mu}_A)}{R^5} \right| \psi_{D1} \psi_{A0} \right\rangle \right|^2$$

Fluorescence Resonance Energy Transfer

A particular type of quenching



$$k_{ET} \propto \left| \left\langle \psi_{D0}\psi_{A1} \left| \frac{\vec{\mu}_D \bullet \vec{\mu}_A}{R^3} - \frac{(\vec{\mu}_D \bullet \vec{\mathbf{R}})(\vec{\mathbf{R}} \bullet \vec{\mu}_A)}{R^5} \right| \psi_{D1}\psi_{A0} \right\rangle \right|^2$$

$$k_{ET} \propto \left| \left\langle \psi_{D0}\psi_{A1} \left| \frac{\vec{\mu}_D \bullet \vec{\mu}_A}{R^3} \right| \psi_{D1}\psi_{A0} \right\rangle \right|^2 \dashrightarrow k_{ET} \propto \frac{\Phi_{Fl}^D}{\tau_{A1}^D} \frac{\kappa^2}{R^6}$$

$$\vec{\mu}_D \bullet \vec{\mu}_A = |\mu_D||\mu_A| \cos(\theta_{\vec{\mu}_D, \vec{\mu}_A})$$

κ^2 includes angular dependencies

If fast rotation, angular dependence averages out

κ^2 = 2/3 for very rapid relative tumbling

Fluorescence Resonance Energy Transfer

A particular type of quenching

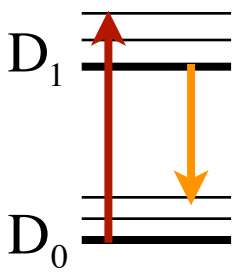
Donor

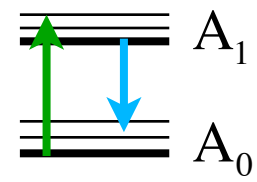
Acceptor

R

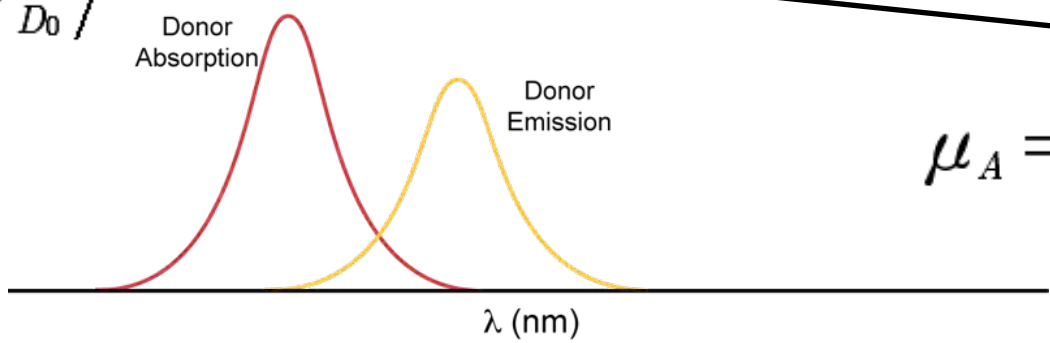
$\mu_D = \langle \psi_{D1} | \mu | \psi_{D0} \rangle$

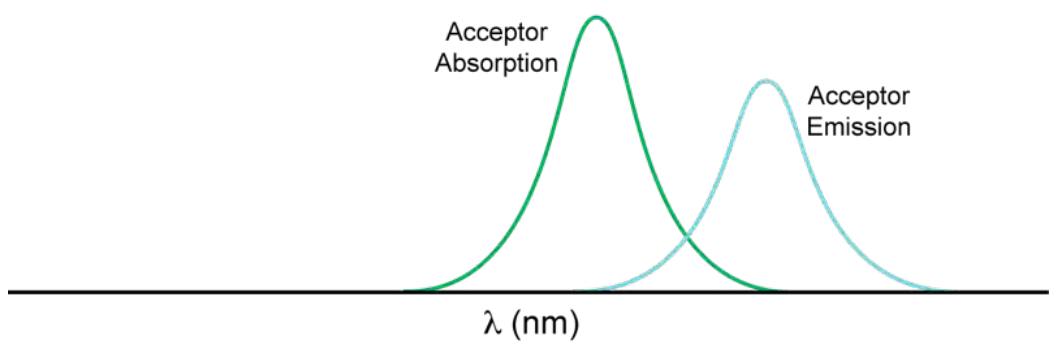
$\mu_A = \langle \psi_{A1} | \mu | \psi_{A0} \rangle$

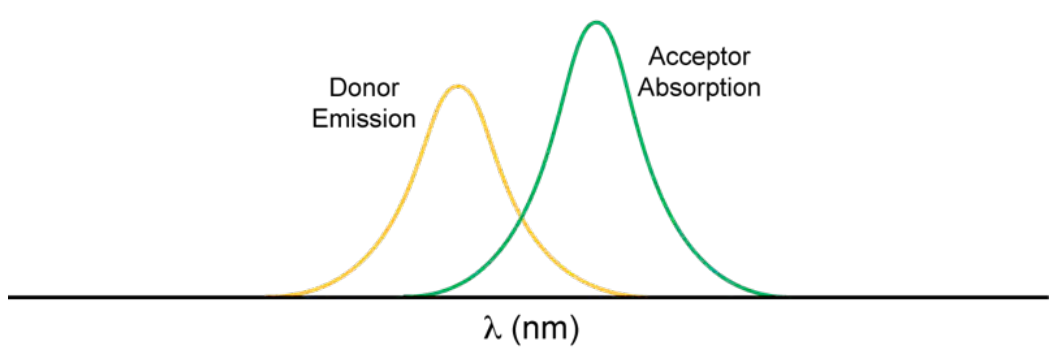




(new coloring…)





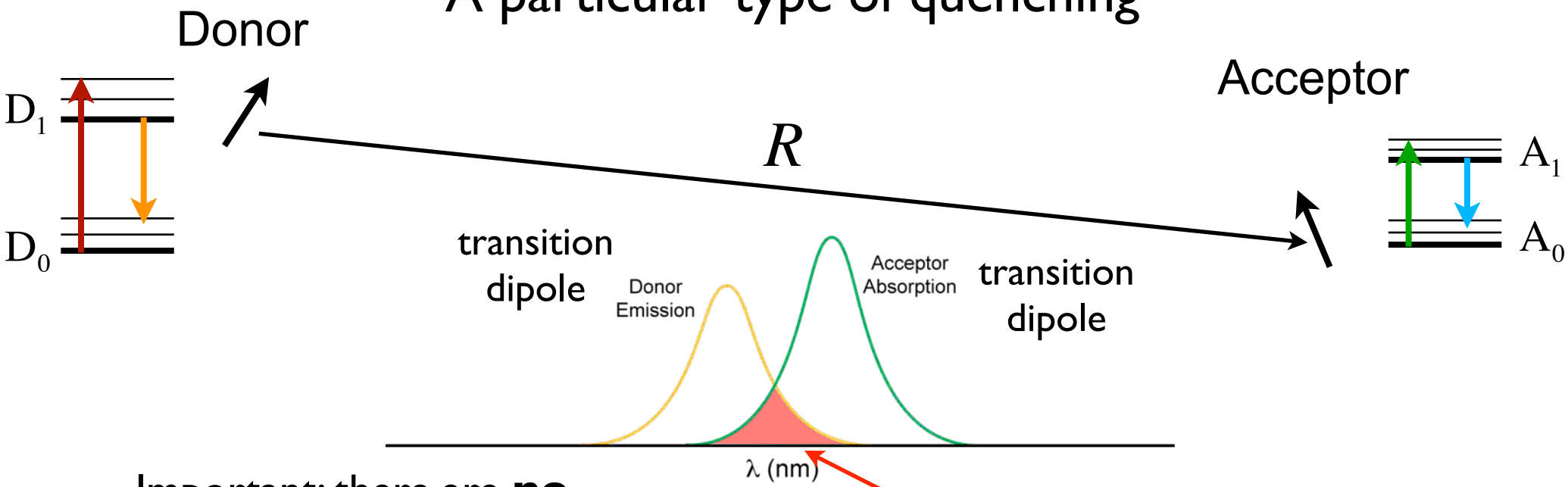


$$k_{ET} \propto \frac{\Phi_{Fl}^{D}}{\tau_{A_1}^{D}} \frac{\kappa^2}{R^6}$$

$$Rate \propto \frac{\kappa^2}{R^6}$$

Fluorescence Resonance Energy Transfer

A particular type of quenching



Important: there are **no photons** involved here!!!

Overlap integral

$$k_{ET} \propto \left| \left\langle \psi_{D0}\psi_{A1} \left| \frac{\vec{\mu}_D \bullet \vec{\mu}_A}{R^3} \right| \psi_{D1}\psi_{A0} \right\rangle \right|^2$$

$$k_{ET} \propto \frac{\Phi_{Fl}^{D}}{\tau_{A1}^{D}} \frac{\kappa^2}{R^6} \qquad Rate \propto \frac{\kappa^2}{R^6}$$

Dependencies:

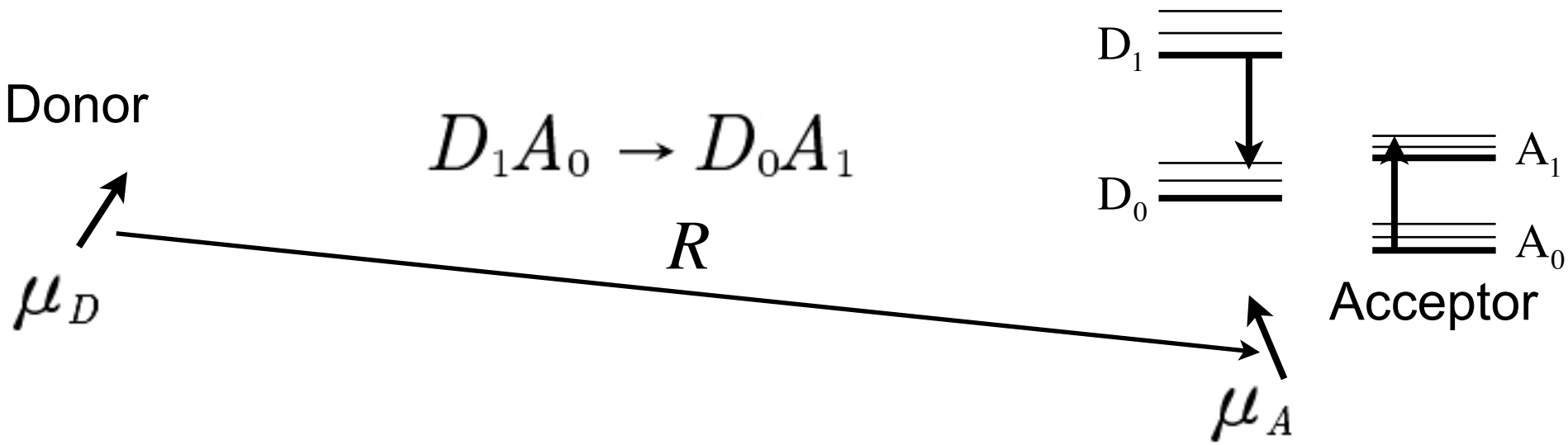
energy match

favorable angle

"short" distance

Fluorescence Resonance Energy Transfer

A particular type of quenching



FRET efficiency

$$\Phi_{ET} = \frac{k_{ET}}{k_F^D + k_{IC}^D + k_{ISC}^D + k_{ET}} = \frac{k_{ET}}{k_{All\ else}^D + k_{ET}} = \frac{k_{ET}}{\frac{1}{\tau_{S_1}^D} + k_{ET}}$$

$\tau_{S_1}^D$: lifetime of the donor in the absence of fluor energy transfer

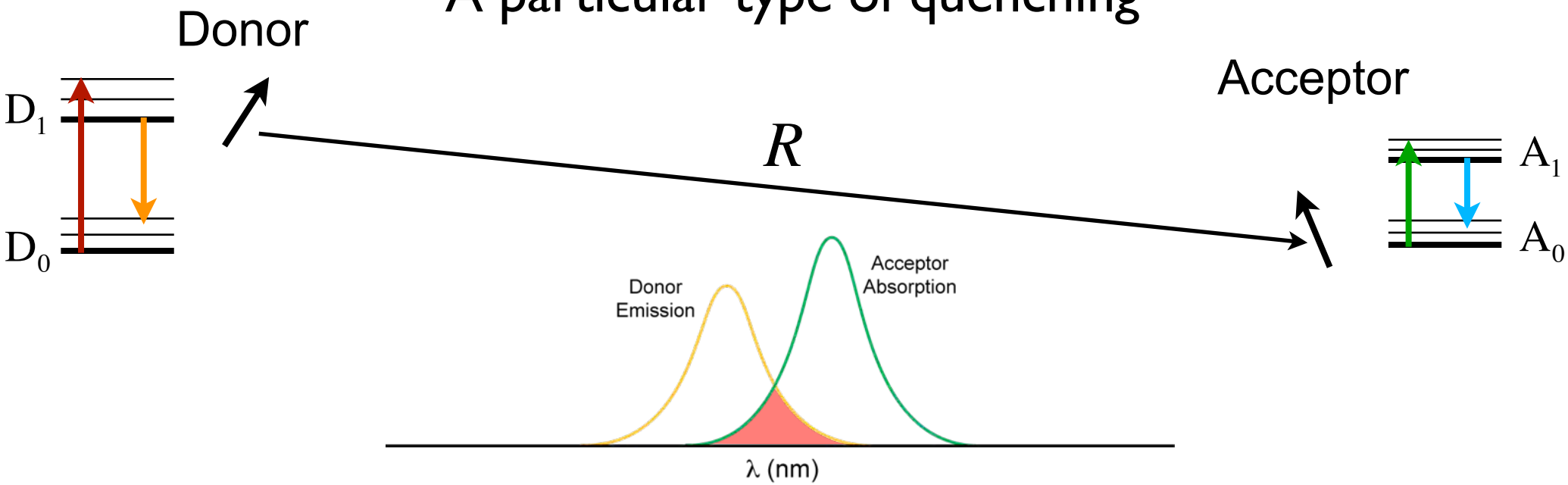
$$k_{ET} \propto \frac{\Phi_{Fl}^D}{\tau_{A_1}^D} \frac{\kappa^2}{R^6}$$

κ^2 includes angular dependencies

κ^2 = 2/3 for very rapid relative tumbling

Fluorescence Resonance Energy Transfer

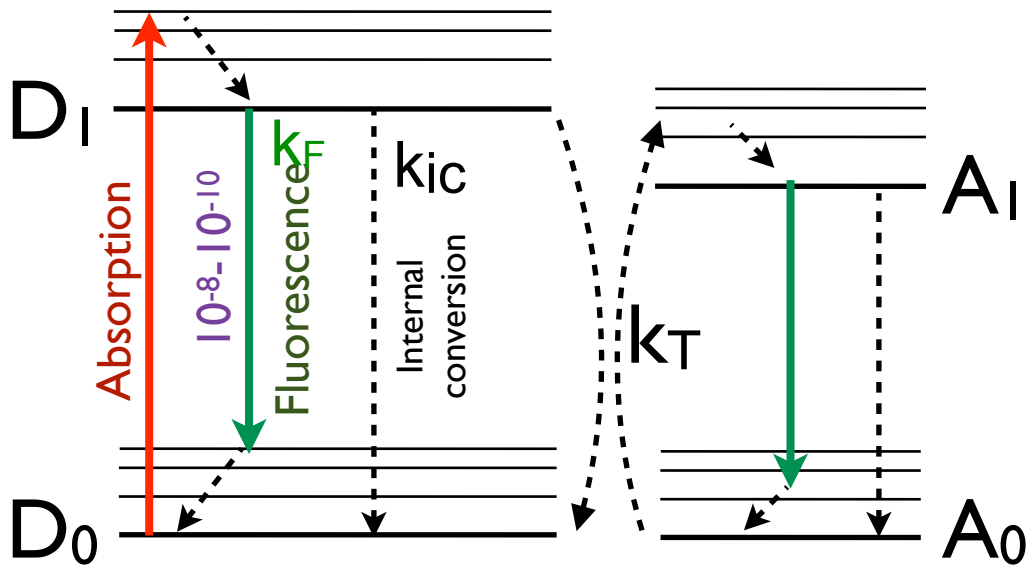
A particular type of quenching



$$k_{ET} \propto \frac{\Phi_{Fl}^{D}}{\tau_{A_1}^{D}} \frac{\kappa^2}{R^6} J$$

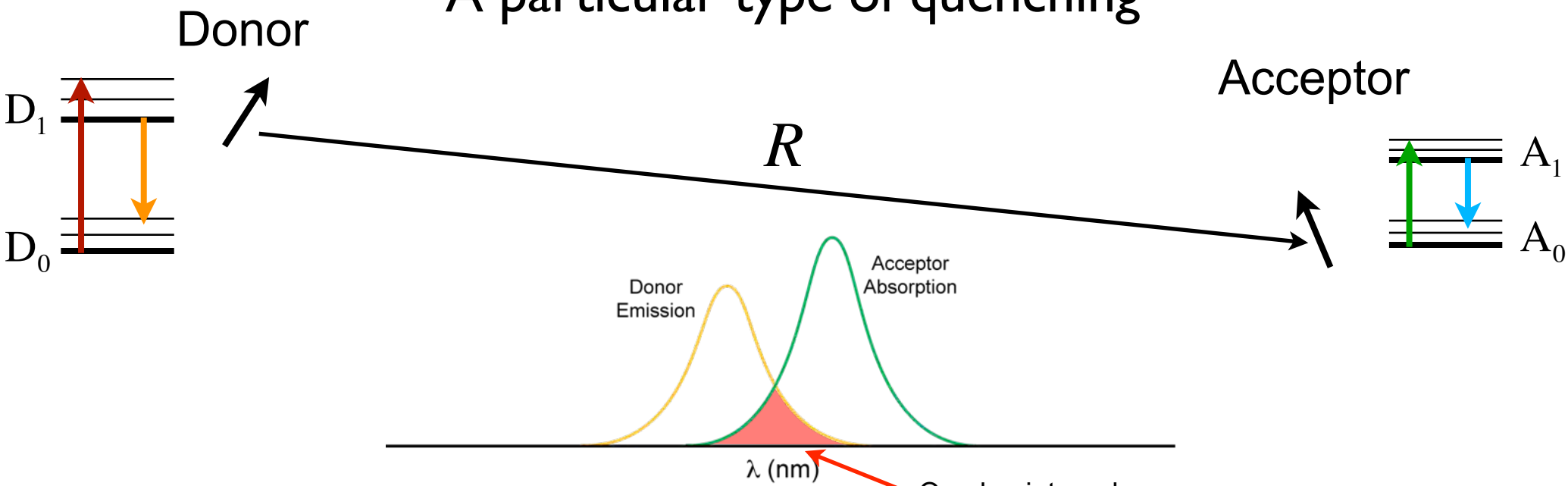
$$\Phi_{ET} = \frac{k_{ET}}{k_{All\,else}^{D} + k_{ET}} = \frac{k_{ET}}{\frac{1}{\tau_{S_1}^{D}} + k_{ET}}$$

$$\Phi_{ET} = \frac{R_0^6}{R_0^6 + R^6} = \frac{1}{1 + \frac{R^6}{R_0^6}}$$



Fluorescence Resonance Energy Transfer

A particular type of quenching



$$k_{ET} \propto \frac{\Phi_{Fl}^{D}}{\tau_{A1}^{D}} \frac{\kappa^2}{R^6} J$$

$$R_0 = 8.79x10^{-5} \left(J\kappa^2 n^{-4} \Phi_D \right)^{\frac{1}{6}} \quad \text{Å}$$

Overlap integral

Refractive index of intervening medium (polarizability)

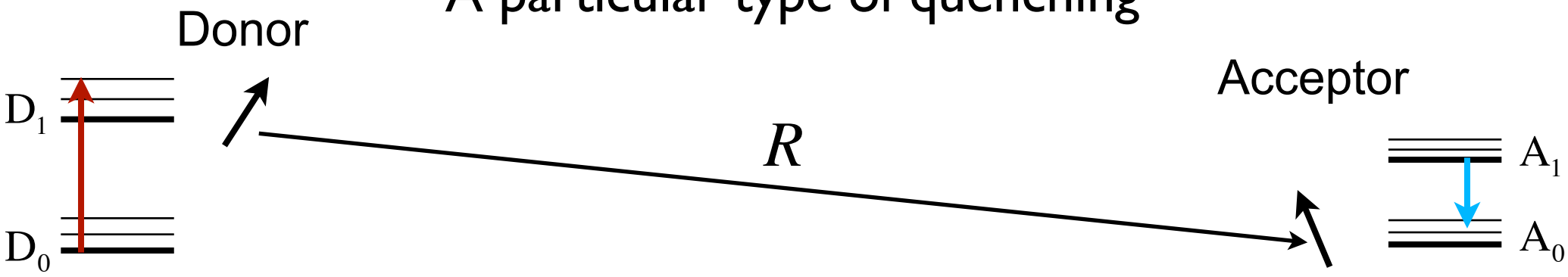
Angular Dependence (0-4; 2/3 for full averaging)

Quantum yield of donor (related to D_1 lifetime)

$$\Phi_{ET} = \frac{R_0^6}{R_0^6 + R^6} = \frac{1}{1 + \frac{R^6}{R_0^6}}$$

Fluorescence Resonance Energy Transfer

A particular type of quenching



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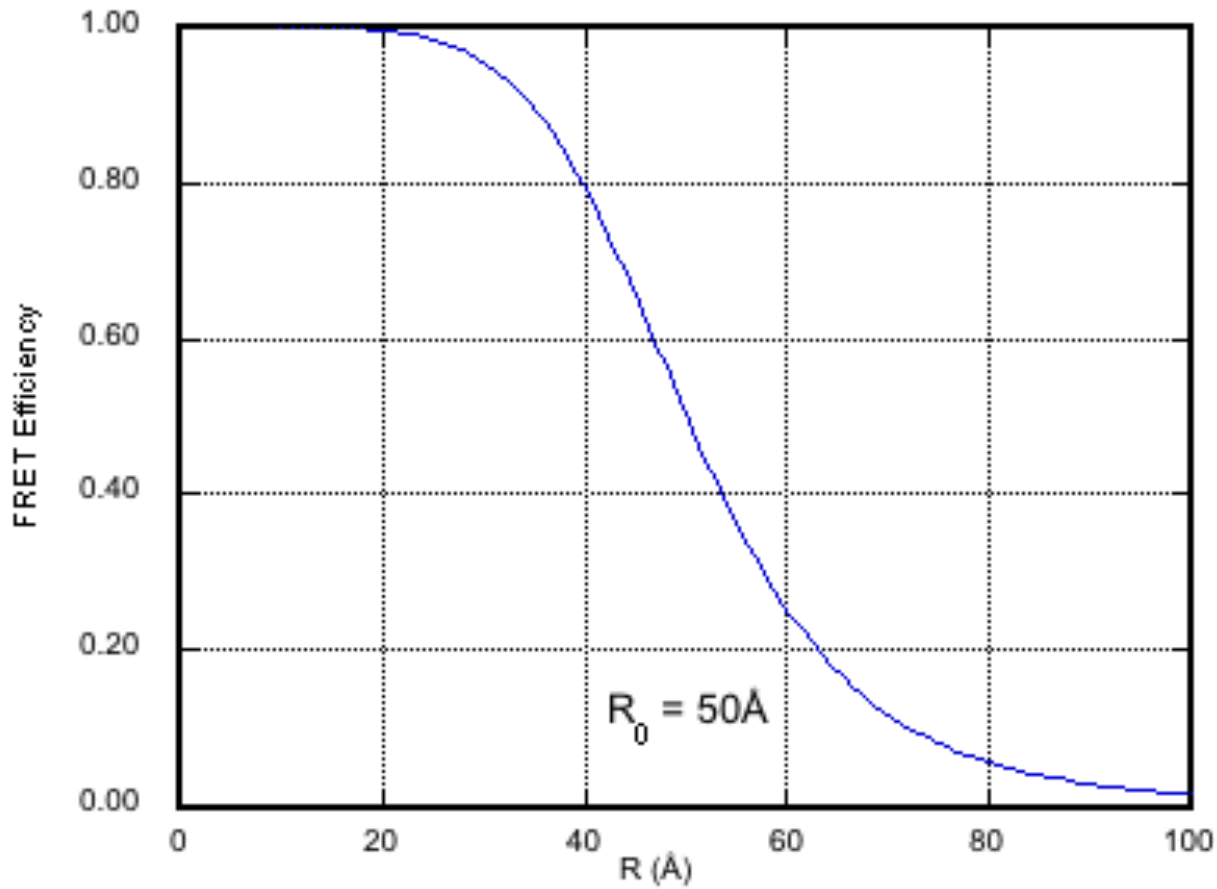
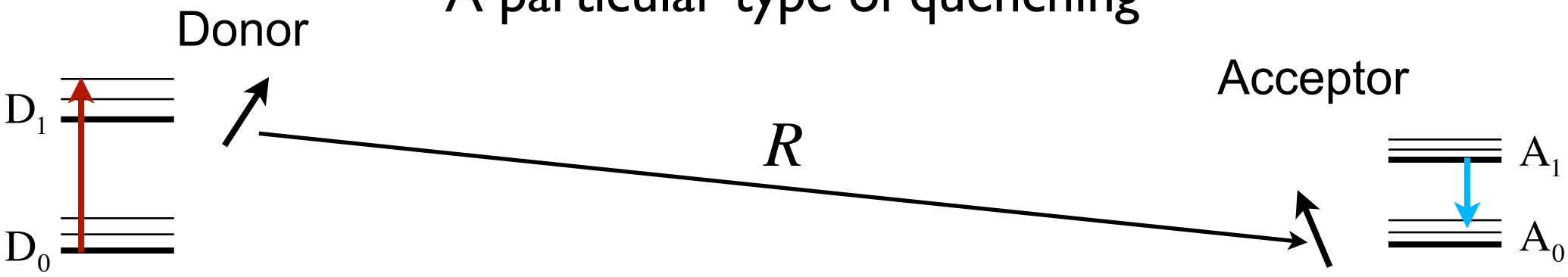


Fluorescence Resonance Energy Transfer

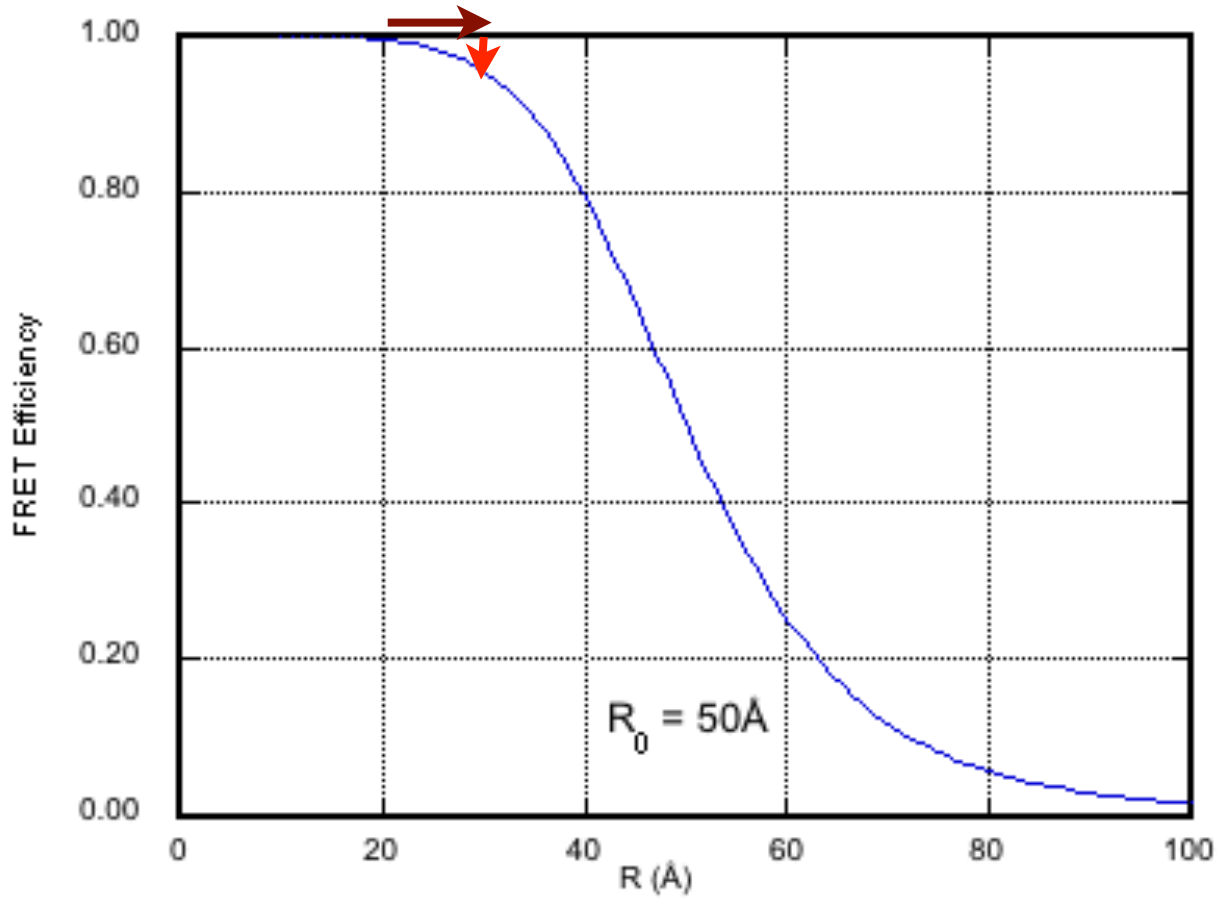
A particular type of quenching



20-30Å

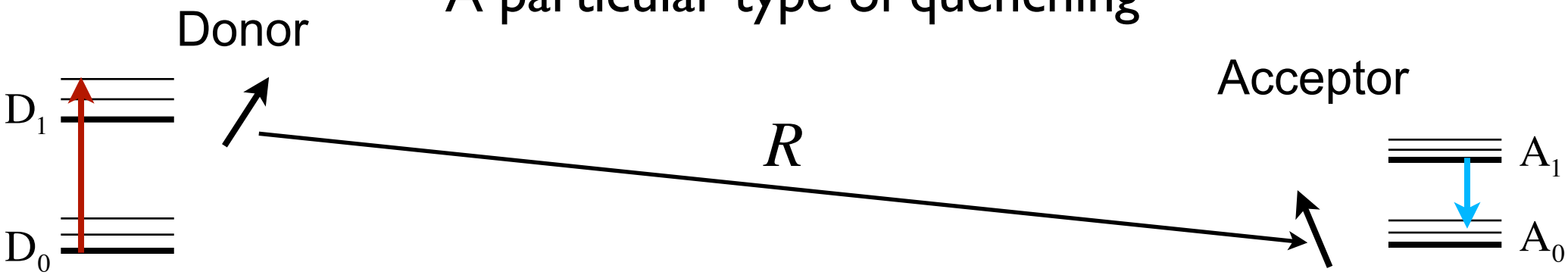
ΔE=0.996-0.955=0.040

$$\Phi_{ET} = \frac{R_0^6}{R_0^6 + R^6} = \frac{1}{1 + \frac{R^6}{R_0^6}}$$



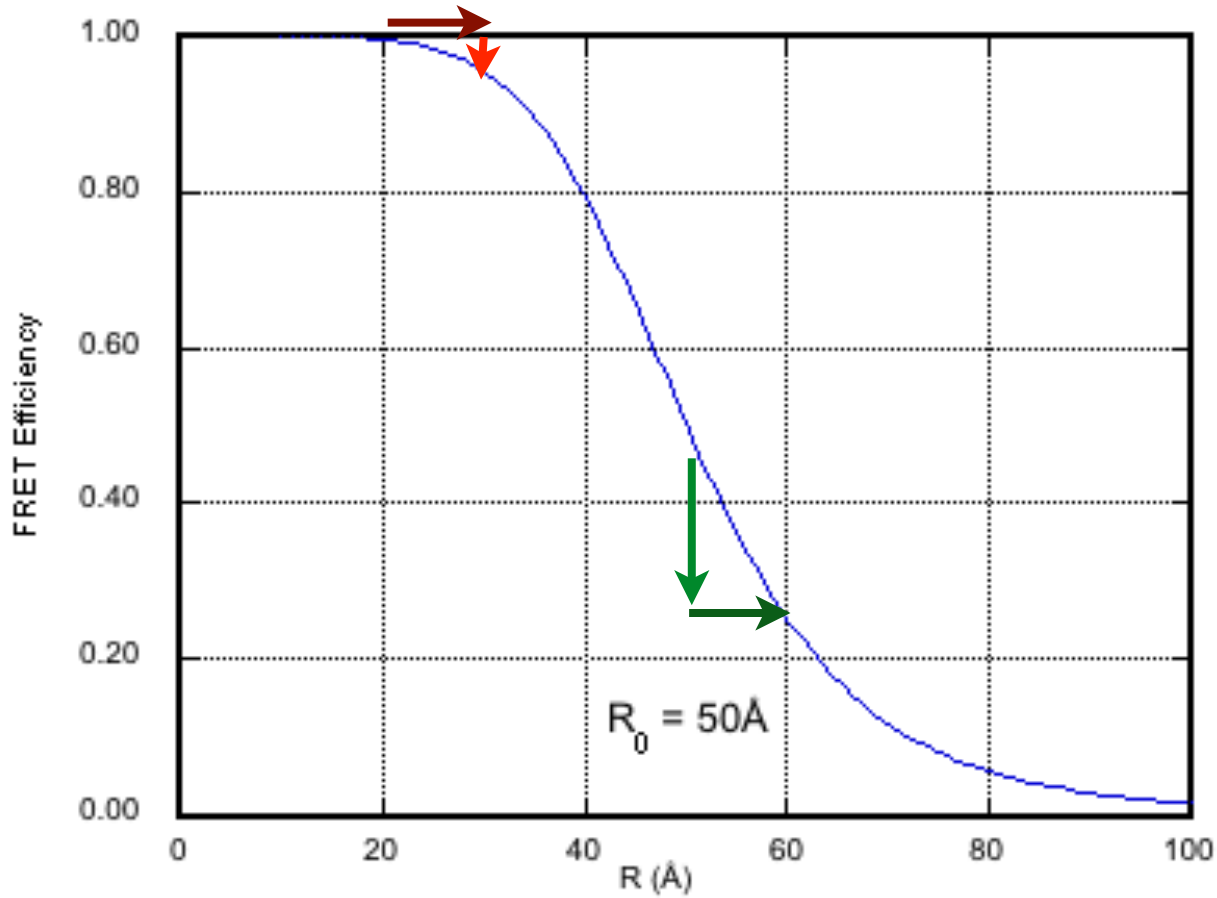
Fluorescence Resonance Energy Transfer

A particular type of quenching



20-30Å
ΔE=0.996-0.955=0.040

50-60Å
ΔE=0.500-0.251=0.249



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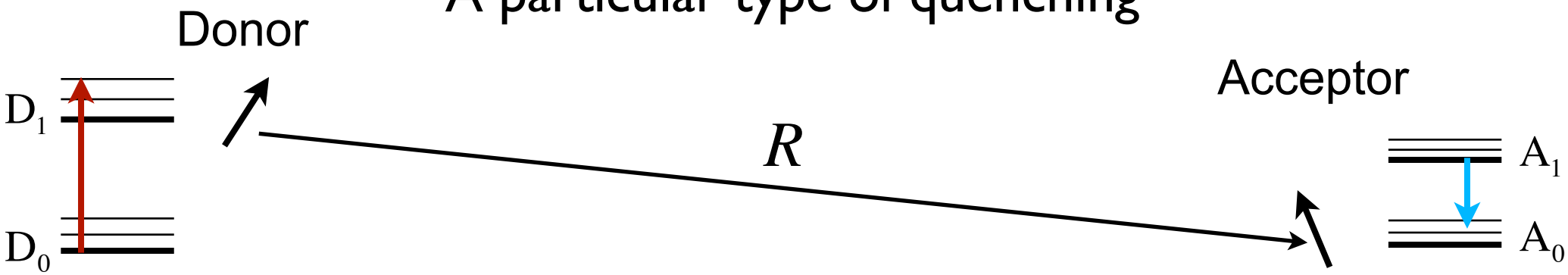
Fluorescence Resonance Energy Transfer

A particular type of quenching



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70-80Å
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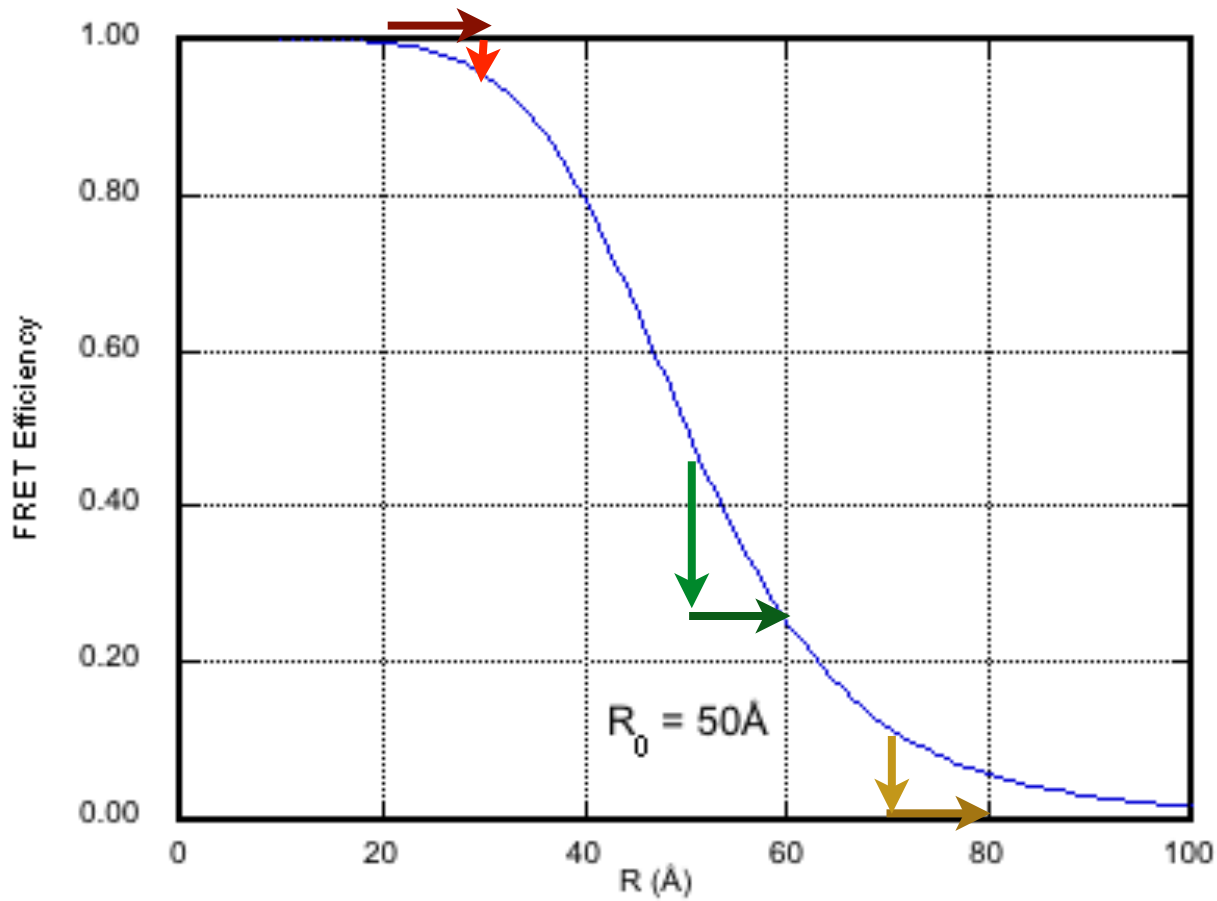

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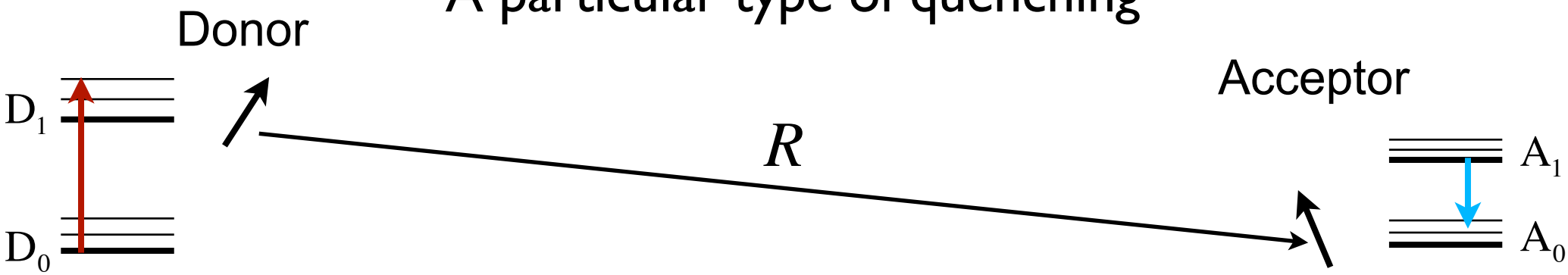
Fluorescence Resonance Energy Transfer

A particular type of quenching



20-30Å
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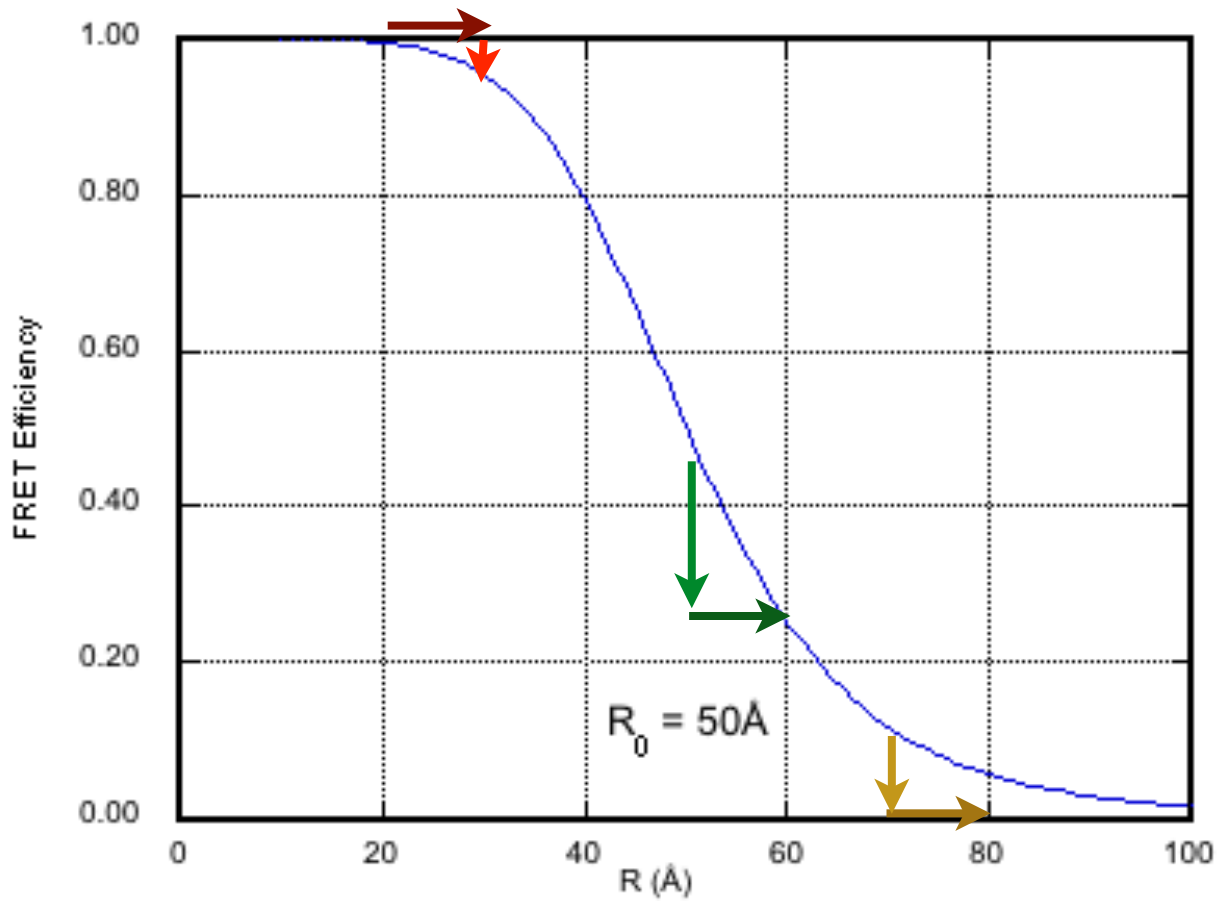

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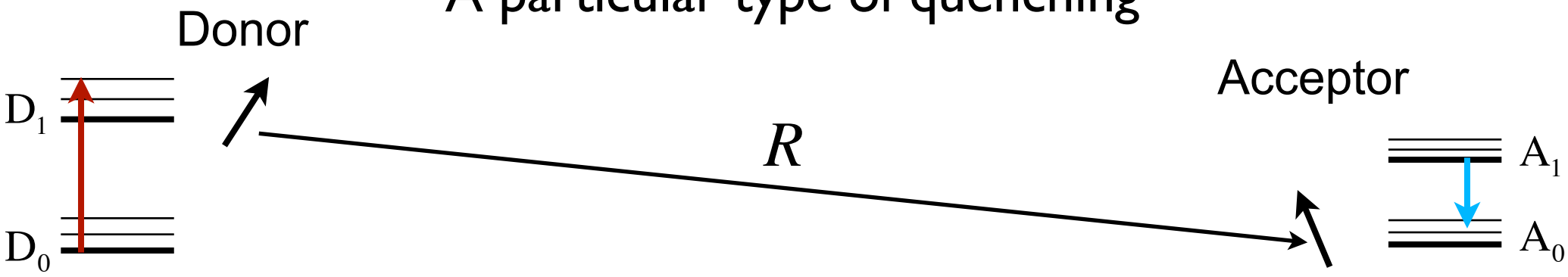
Fluorescence Resonance Energy Transfer

A particular type of quenching



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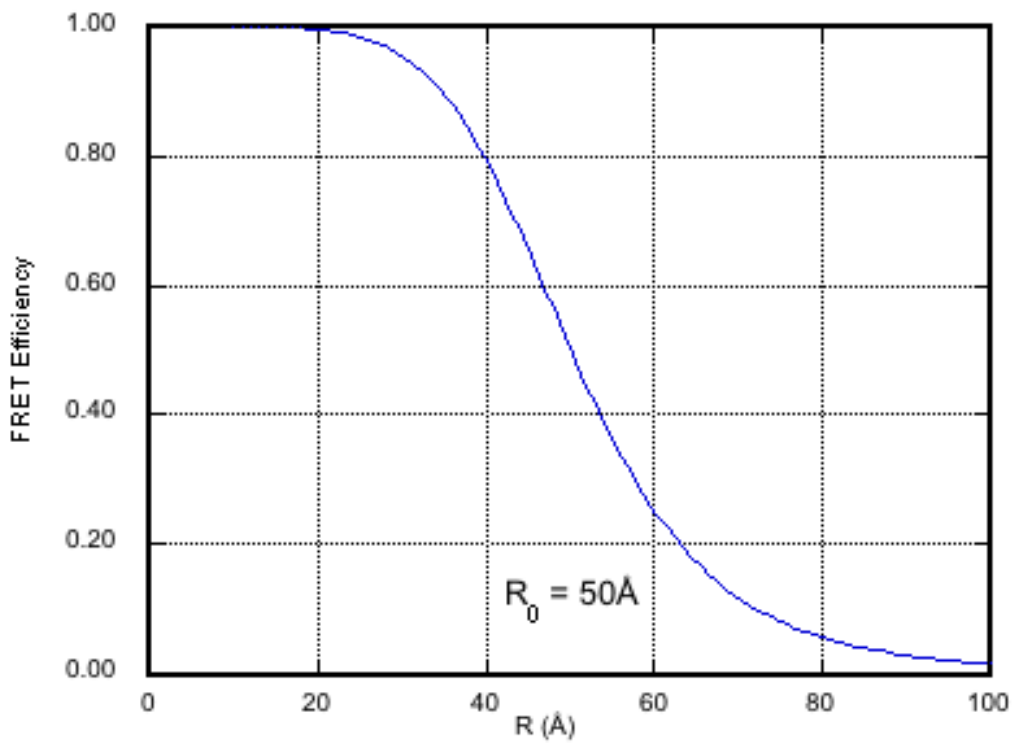

Fluorescence Resonance Energy Transfer

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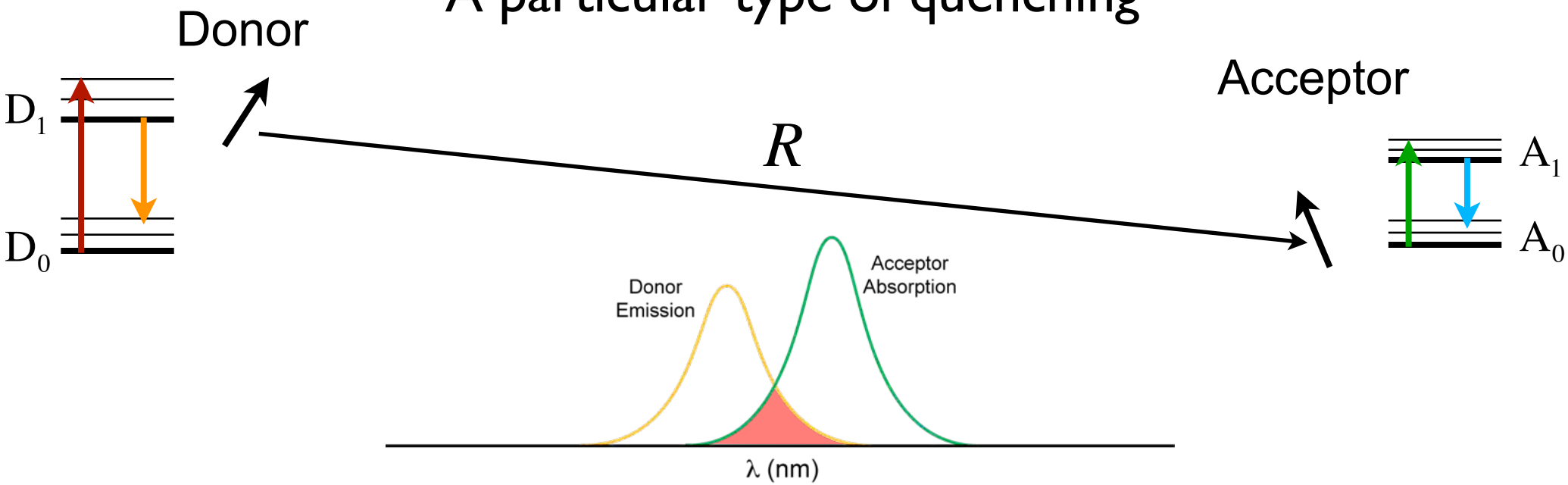


$$\Phi_{ET} = \frac{R_0^6}{R_0^6 + R^6} = \frac{1}{1 + \frac{R^6}{R_0^6}}$$

$$R_0 = 8.79x10^{-5}\left(J\kappa^2 n^{-4}\Phi_D\right)^{\frac{1}{6}} \text{ Å}$$

Overlap integral

Quantum yield of donor (related to lifetime)

Angular Dependence (0-4; 2/3 for full averaging)

Refractive index of intervening medium (polarizability)

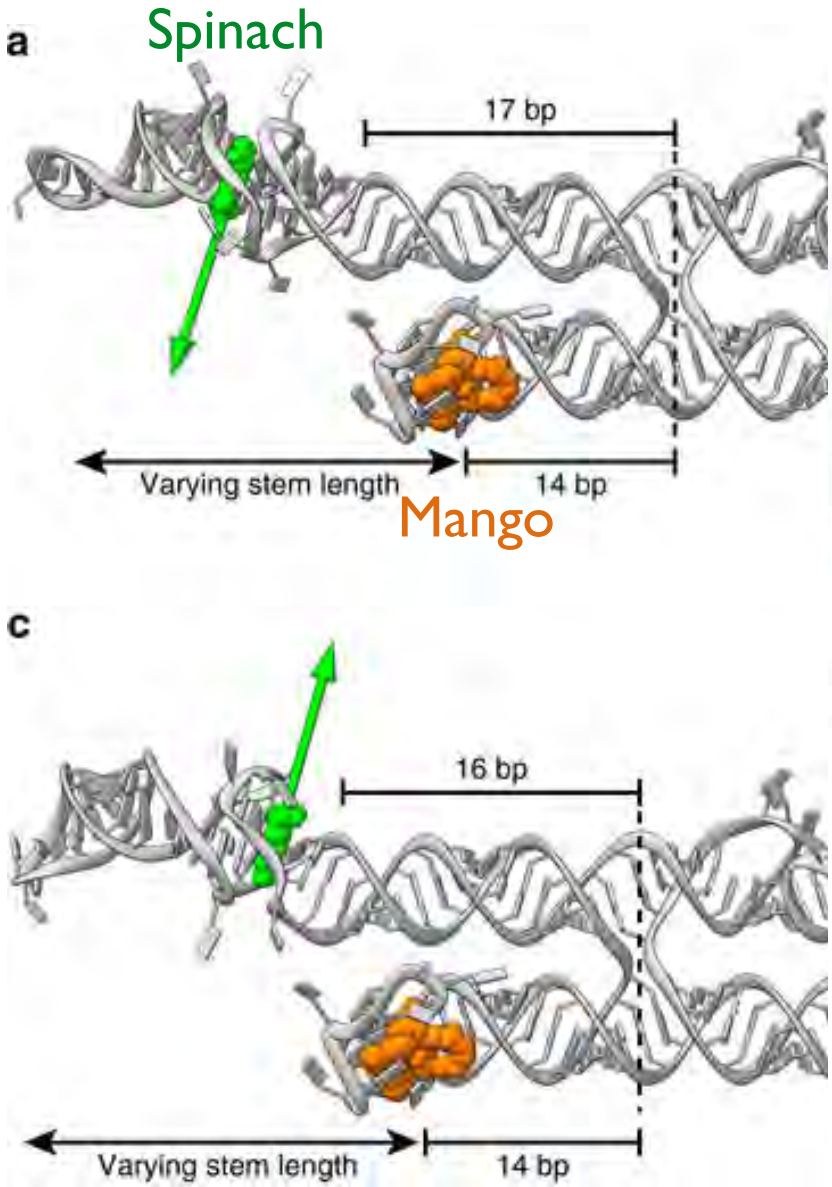
Measuring absolute distances?

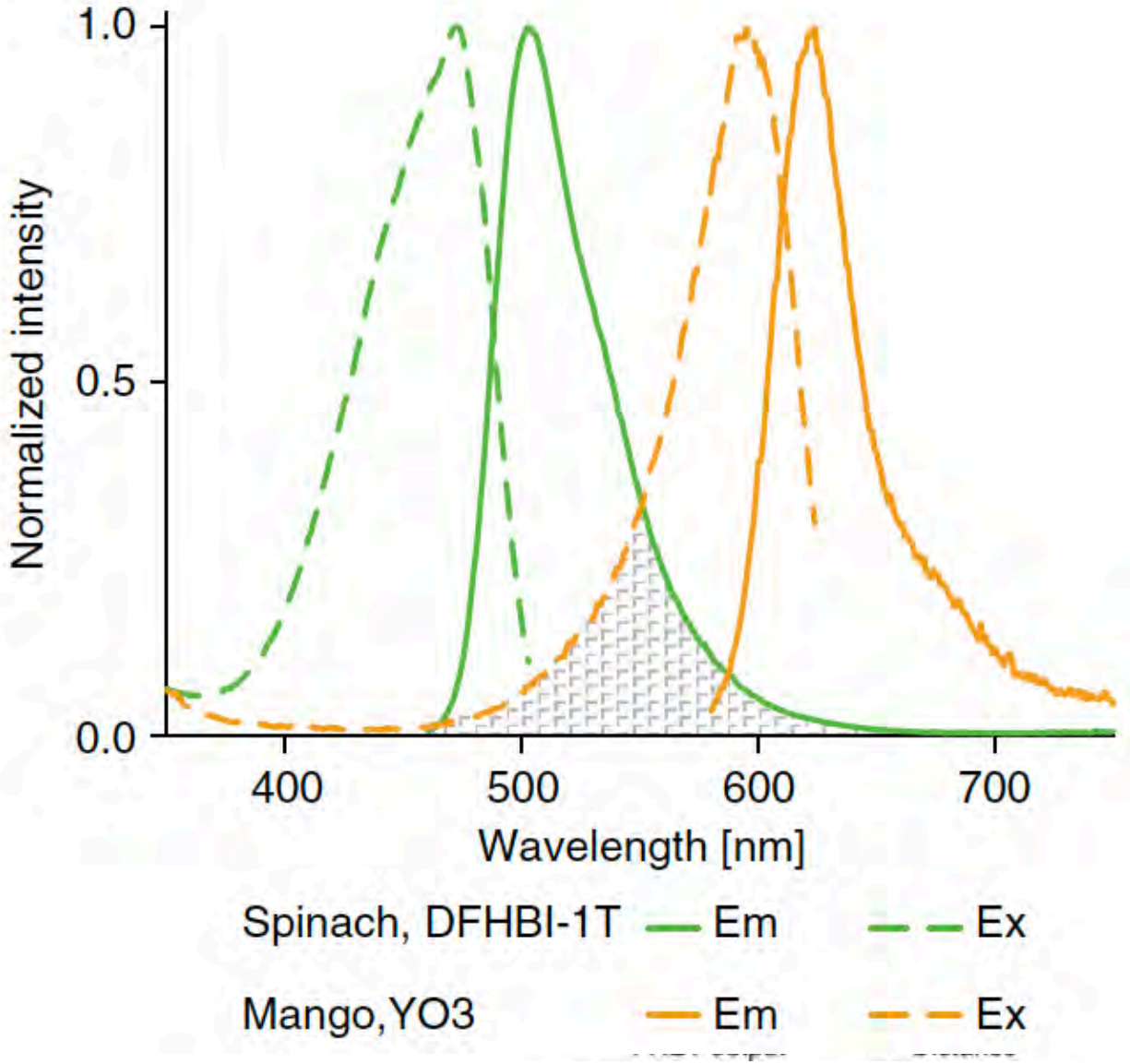
- difficult
- burden: knowing R_0

Measuring changes in distances?

- excellent, particularly if accuracy not important

FRET angle probe

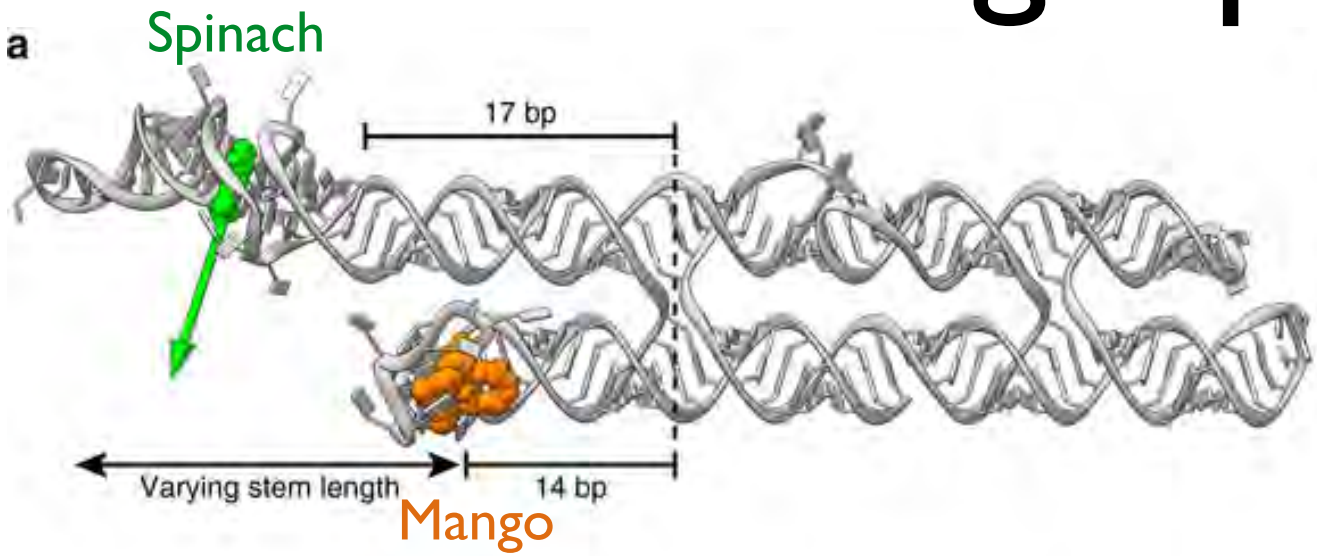


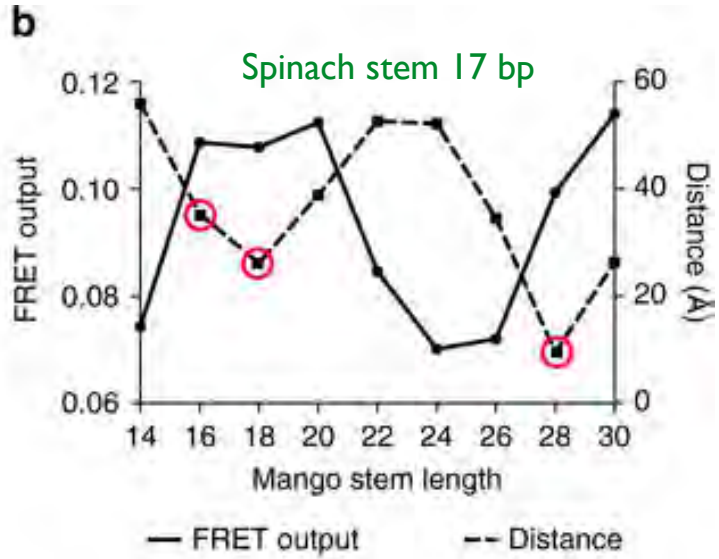


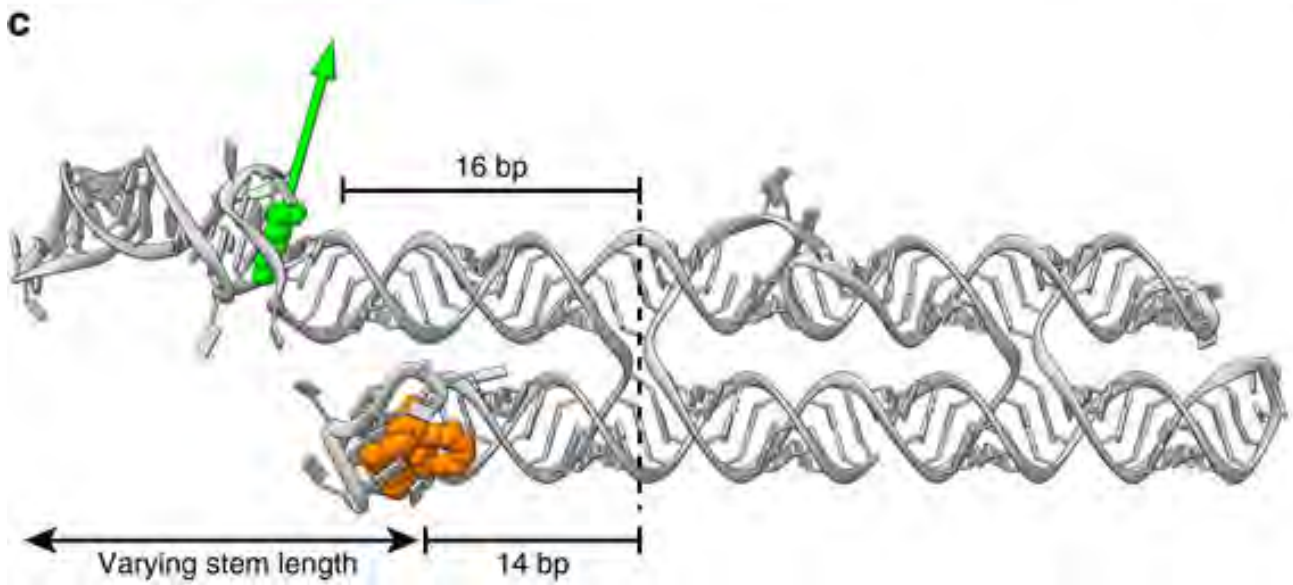
Development of a genetically encodable FRET system using fluorescent RNA aptamers

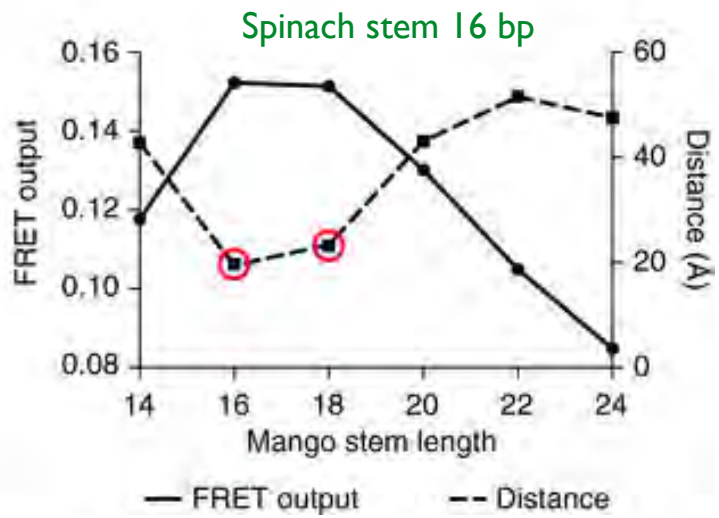
Mette D. E. Jepsen, … & Ebbe S. Andersen, *Nature Communications* **9**, 18 (2018) doi:10.1038/s41467-017-02435-x

FRET angle probe









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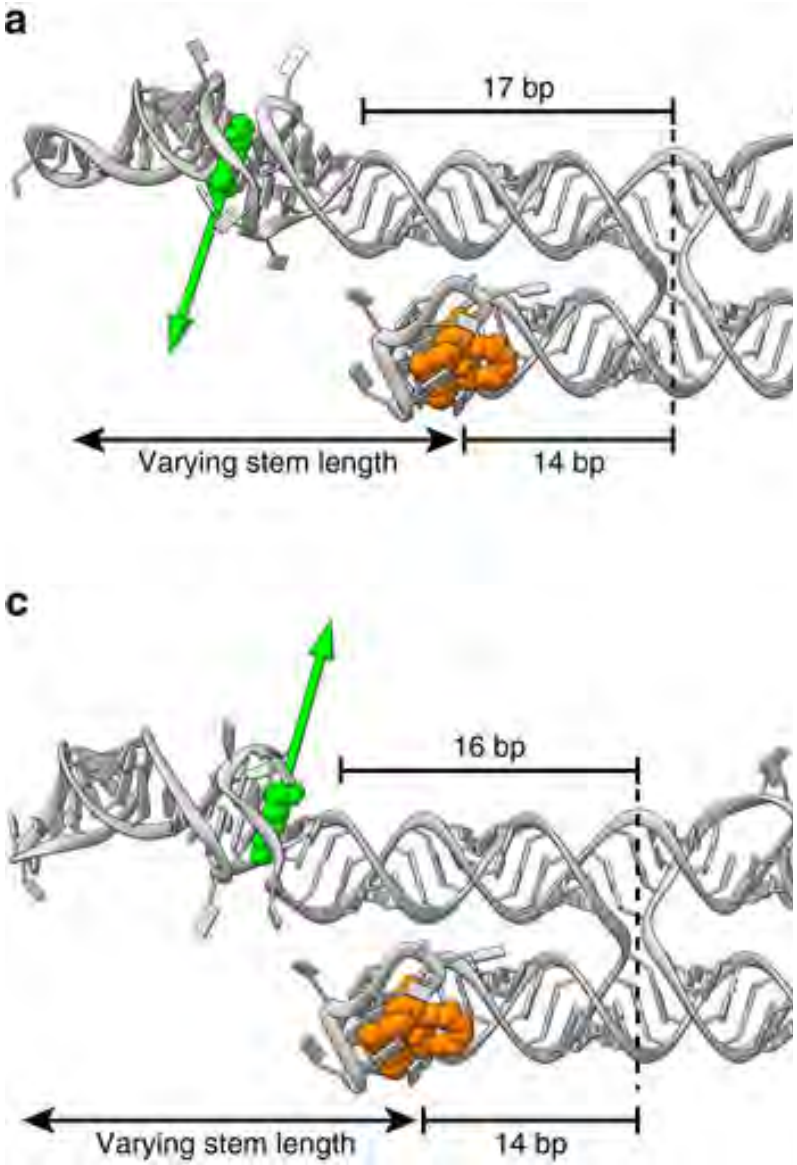
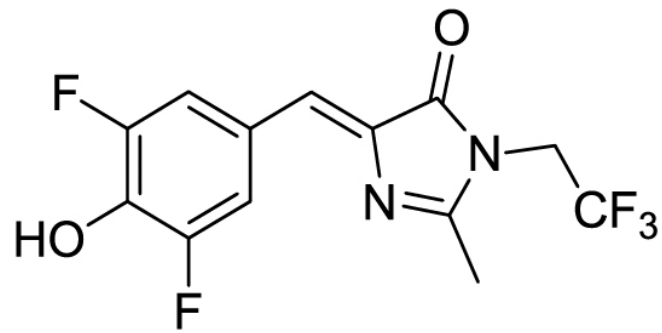
A little knowledge…



Dipole moment calculation

The dipole moment of DFHBI-1T was calculated using the Marvin software suite (version 15.10.19) and the Calculator Plugin developed by ChemAxon (http://www.chemaxon.com/). The chemical structure of DFHBI-1T was drawn in MarvinSketch, and the protonation state of the molecule was determined at pH 7.8 by using the pKa Calculator Plugin. The dipole moment of the most abundant DFHBI-1T microspecies (70.4% at pH 7.8) was calculated by using the Dipole Moment Calculator Plugin. The total dipole moment of DFHBI-1T was visualized as a vector expressed in the principal axis frame.



What's wrong with this?

Checking in with Wikipedia

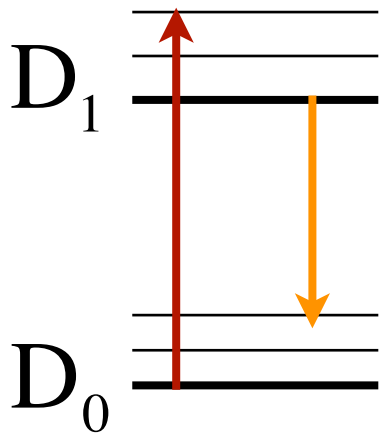
"FRET is analogous to near-field communication, in that the radius of interaction is much smaller than the wavelength of light emitted. In the near-field region, the excited chromophore emits a virtual photon that is instantly absorbed by a receiving chromophore…."

"…These virtual photons are undetectable, since their existence violates the conservation of energy and momentum, and hence FRET is known as a *radiationless* mechanism"

In physics, a **virtual particle** is a transient quantum fluctuation that exhibits some of the characteristics of an ordinary particle, while having its existence limited by the uncertainty principle

Virtual photon ≠ photon

Determining Fluorescence Quantum Yield



$$\frac{\text{photons emitted}}{\text{photons absorbed}}$$

Φ proportional to I/A

$$\Phi_{ET} = \frac{k_{ET}}{k_F^D + k_{IC}^D + k_{ISC}^D + k_{ET}} = \frac{k_{ET}}{k_{All\ else}^D + k_{ET}} = \frac{k_{ET}}{\frac{1}{\tau_{S_1}^D} + k_{ET}}$$

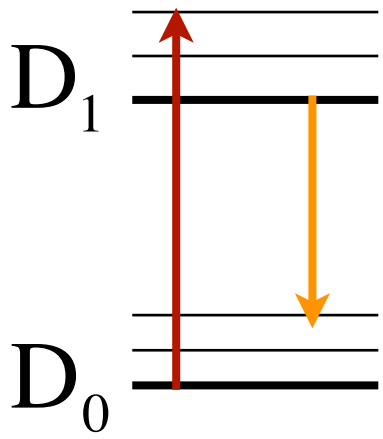
Determine relative to a known reference (R) standard

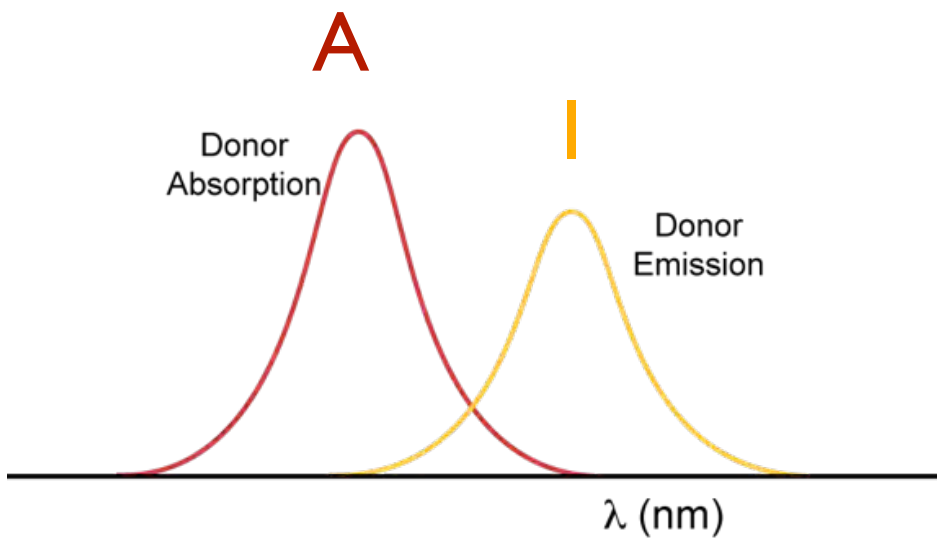
FRET efficiency

$$\Phi = \frac{I/A}{I_R/A_R}\frac{n^2}{n_R^2}\Phi_R$$

$$\Phi_D^{absence\ of\ A} = \frac{I}{I_R}\frac{A_R}{A}\frac{n^2}{n_R^2}\Phi_R$$

Determining Fluorescence Quantum Yield





Determine relative to a known reference (R) standard

For a slightly more complex, but more accurate approach, see this link

I = Integrated Fluor Emmission Intensity

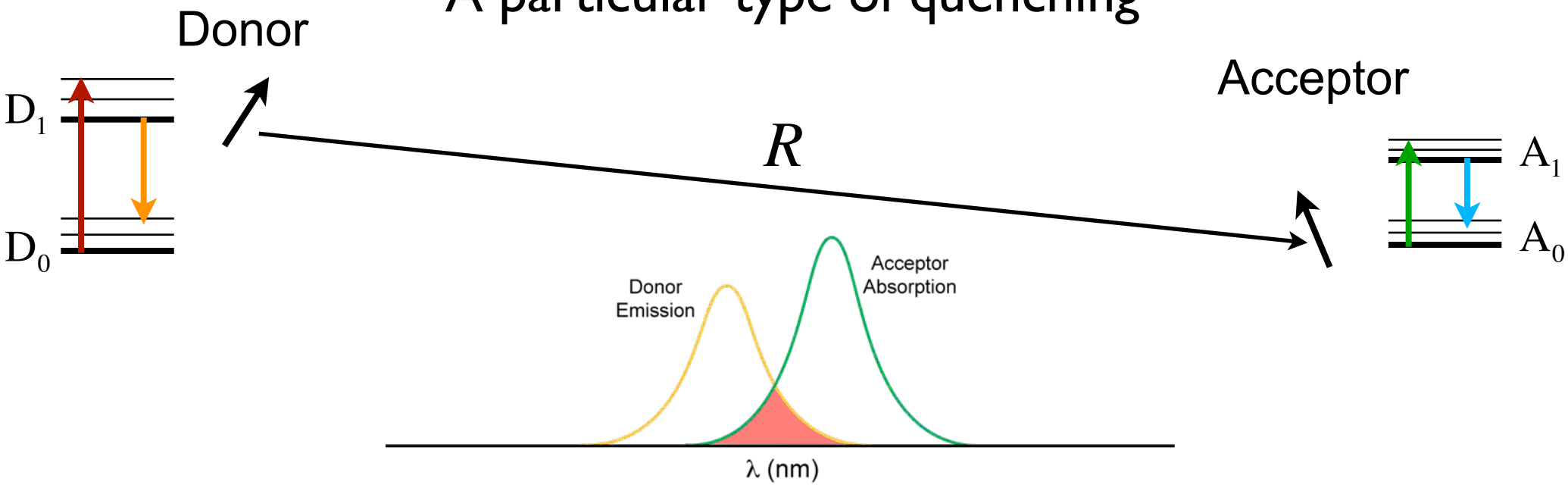
A = Absorbance at Abs Max

n = refractive index of medium

$$\Phi_D^{absence\ of\ A} = \frac{I}{I_R}\frac{A_R}{A}\frac{n^2}{n_R^2}\Phi_R$$

Fluorescence Resonance Energy Transfer

A particular type of quenching



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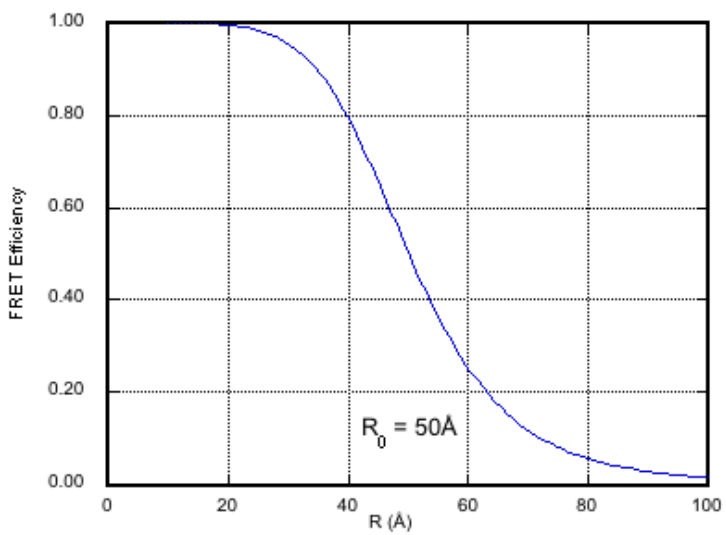


Measuring absolute distances?

- difficult
- burden: knowing R_0

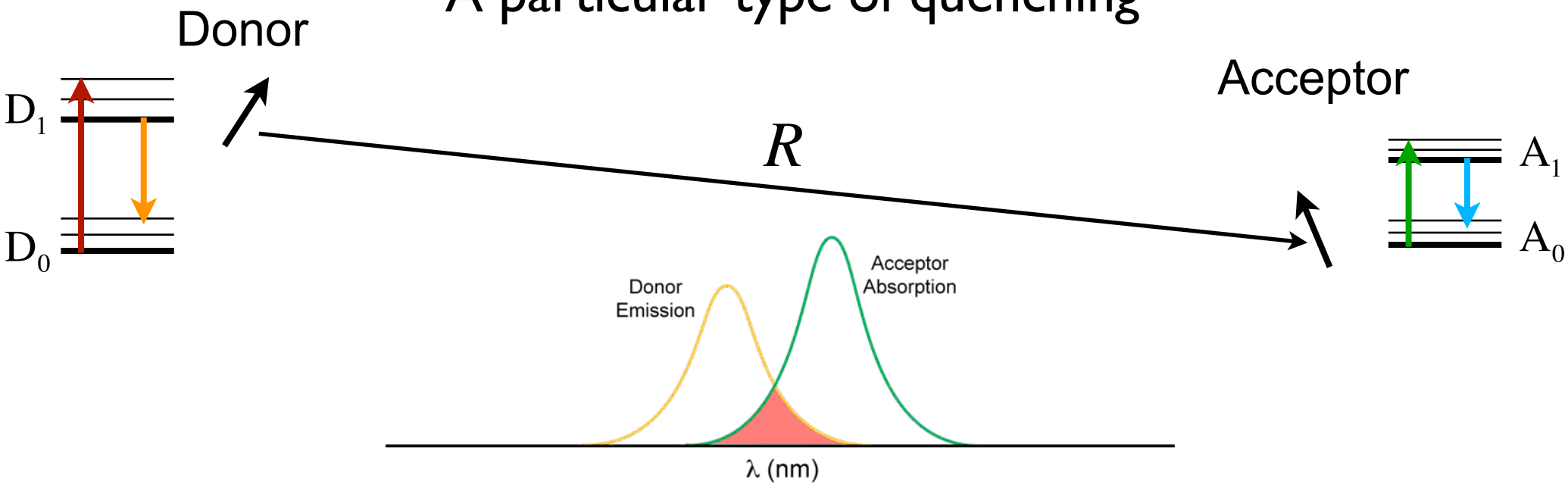
In the measured system

Measuring changes in distances?

- better, particularly if accuracy not important

Fluorescence Resonance Energy Transfer

A particular type of quenching



$$\Phi_{ET} = \frac{R_0^6}{R_0^6 + R^6} = \frac{1}{1 + \frac{R^6}{R_0^6}}$$

$$R_0 = 8.79x10^{-5}\left(J\kappa^2 n^{-4}\Phi_D\right)^{\frac{1}{6}} \text{ Å}$$

Overlap integral

Quantum yield of donor (related to lifetime)

Angular Dependence (0-4; 2/3 for full averaging)

Refractive index of intervening medium (polarizability)

Measuring absolute distances?

- difficult
- burden: knowing R_0

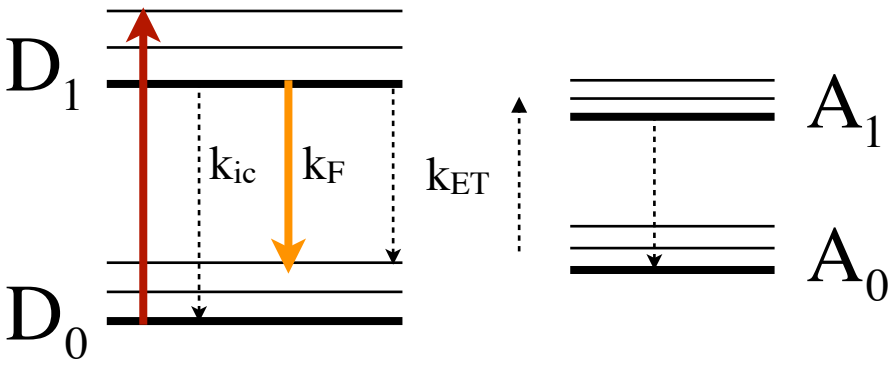
Measuring changes in distances?

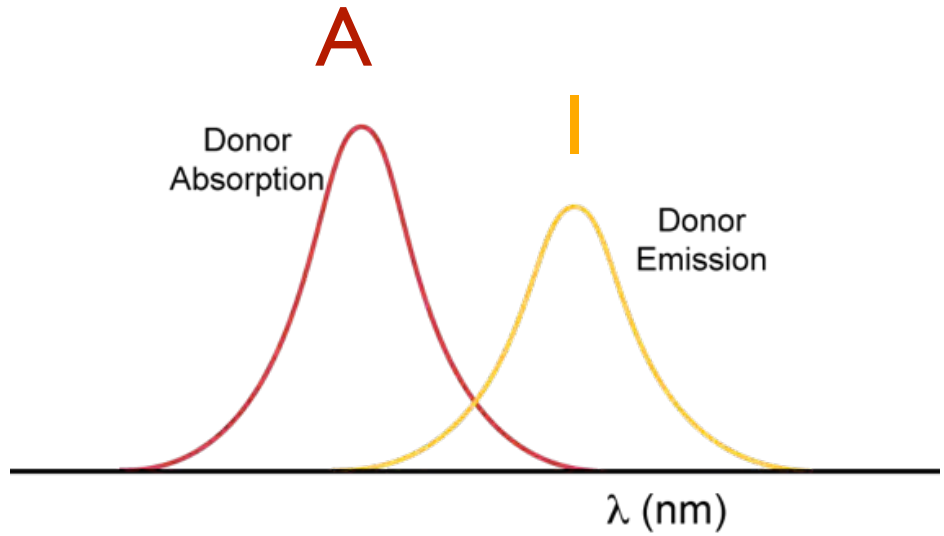
- better, particularly if accuracy not important

BUT…

Determining FRET Efficiency

Measure quantum yield of donor





$$\Phi_D^{absence\ of\ A} = \frac{k_F}{k_F + k_{ic}}$$

$$\Phi_D^{presence\ of\ A} = \frac{k_F}{k_F + k_{ic} + k_{ET}}$$

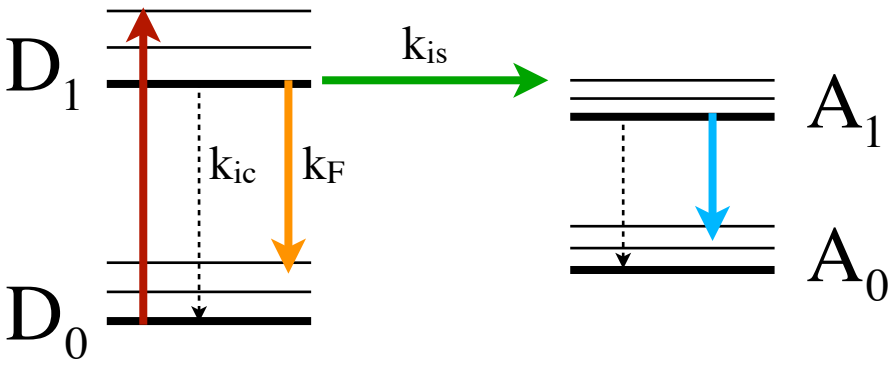
Use a separate control OR
Photobleach acceptor

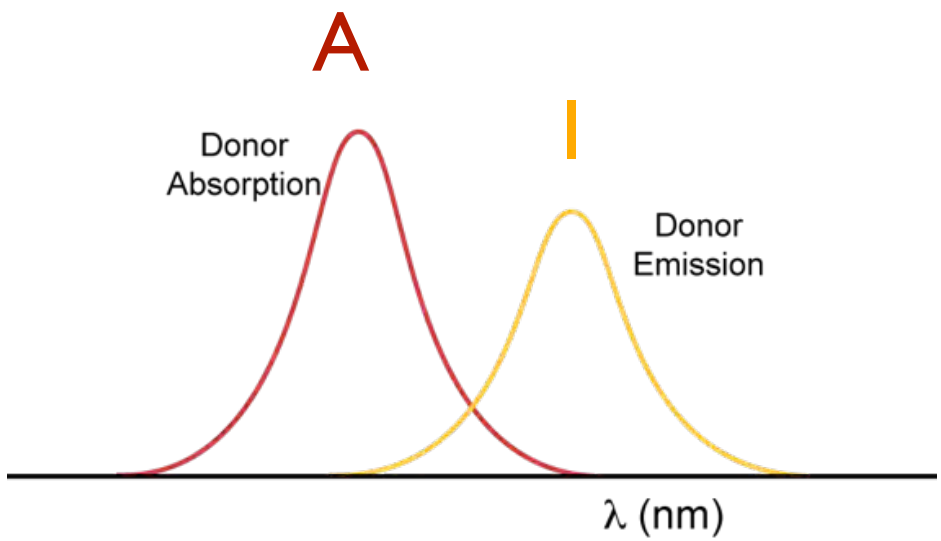
$$\Phi_{FRET}^{quenching} = \frac{\Phi_D^{absence\ of\ A} - \Phi_D^{presence\ of\ A}}{\Phi_D^{absence\ of\ A}}$$

$$\Phi_{FRET}^{quenching} = \frac{\frac{k_F}{k_F + k_{ic}} - \frac{k_F}{k_F + k_{ic} + k_{ET}}}{\frac{k_F}{k_F + k_{ic}}} = \frac{\frac{1}{k_F + k_{ic}} - \frac{1}{k_F + k_{ic} + k_{ET}}}{\frac{1}{k_F + k_{ic}}} = \frac{\tau_D^{absence\ of\ A} - \tau_D^{presence\ of\ A}}{\tau_D^{absence\ of\ A}}$$

Determining FRET Efficiency

Measure quantum yield of donor





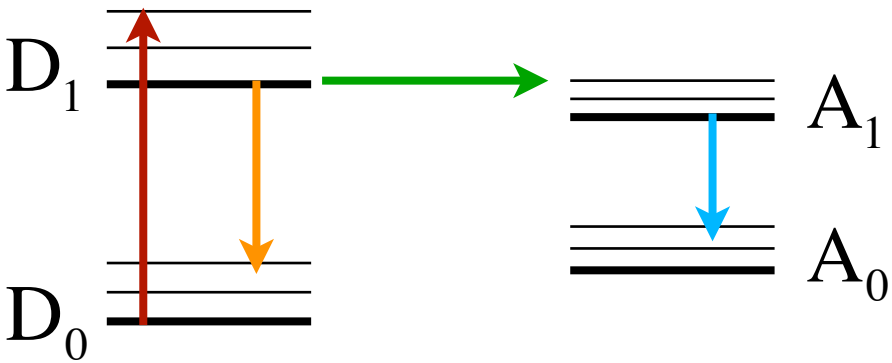
$$\Phi_{ET} = \frac{R_0^6}{R_0^6 + R^6} = \frac{1}{1 + \frac{R^6}{R_0^6}}$$

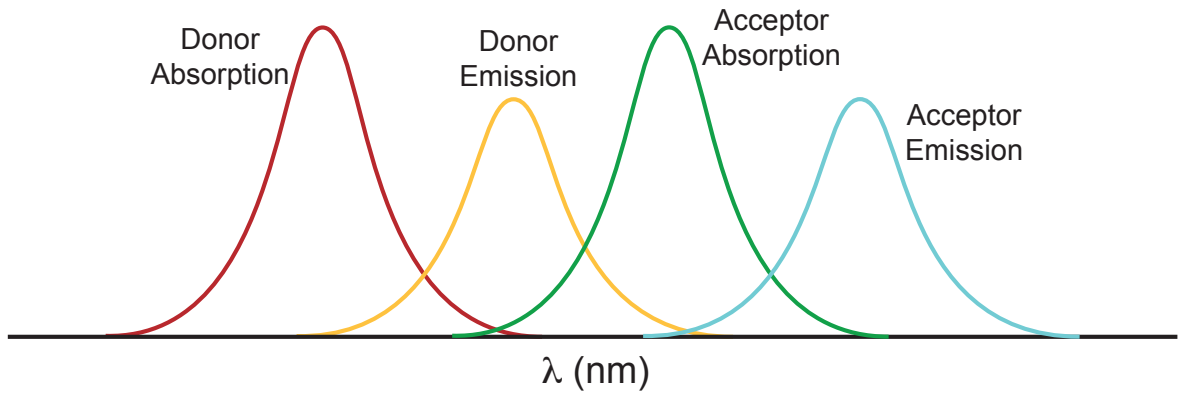
$$\Phi_{FRET}^{quenching} = \frac{\Phi_D^{absence\ of\ A} - \Phi_D^{presence\ of\ A}}{\Phi_D^{absence\ of\ A}}$$

$$\Phi_{FRET}^{quenching} = 1 - \frac{\Phi_D^{presence\ of\ A}}{\Phi_D^{absence\ of\ A}}$$

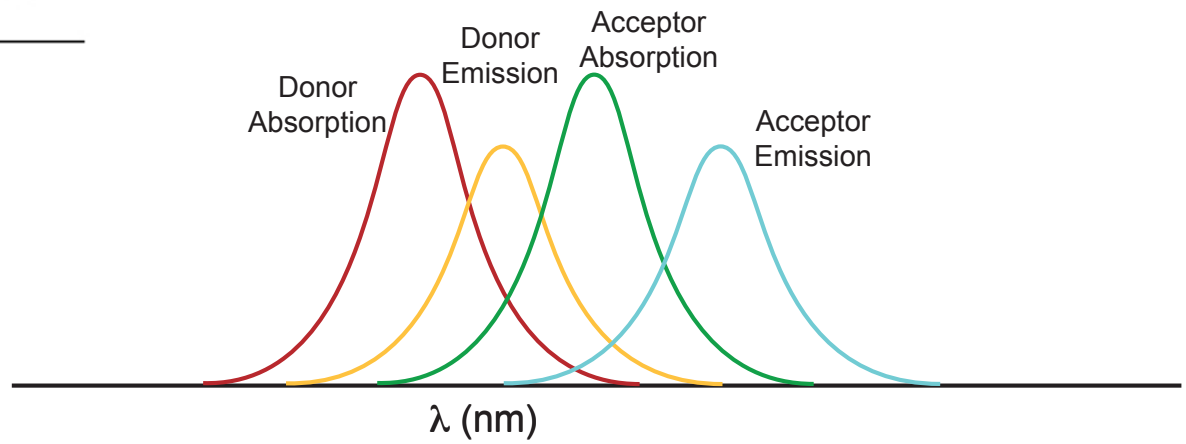
Determining FRET Efficiency

Measure quantum yield of donor





$$\Phi_{FRET}^{quenching} = \frac{\tau_D^{absence\ of\ A} - \tau_D^{presence\ of\ A}}{\tau_D^{absence\ of\ A}}$$

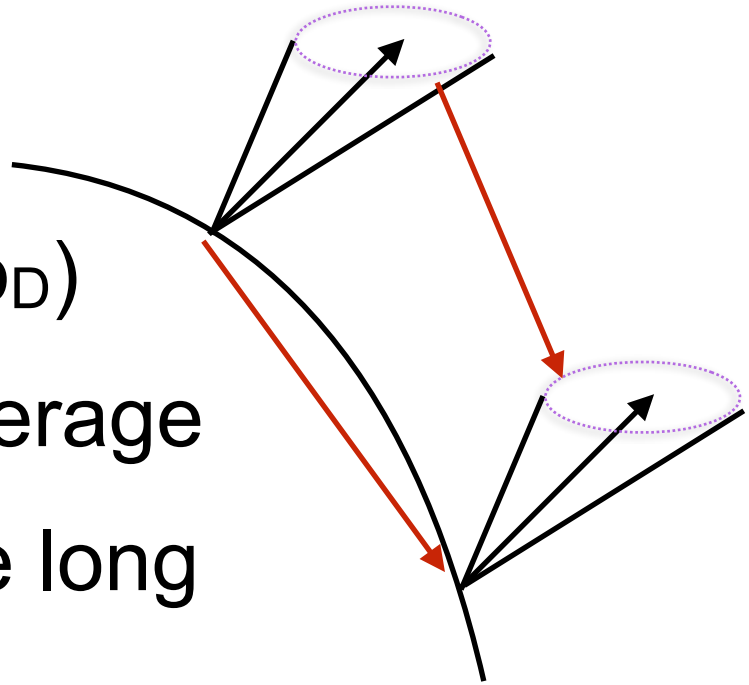


$$\Phi_{ET} = \frac{R_0^6}{R_0^6 + R^6} = \frac{1}{1 + \frac{R^6}{R_0^6}}$$

$$\Phi_{FRET}^{quenching} = 1 - \frac{\Phi_D^{presence\ of\ A}}{\Phi_D^{absence\ of\ A}}$$

Determining Distances / Distance Changes

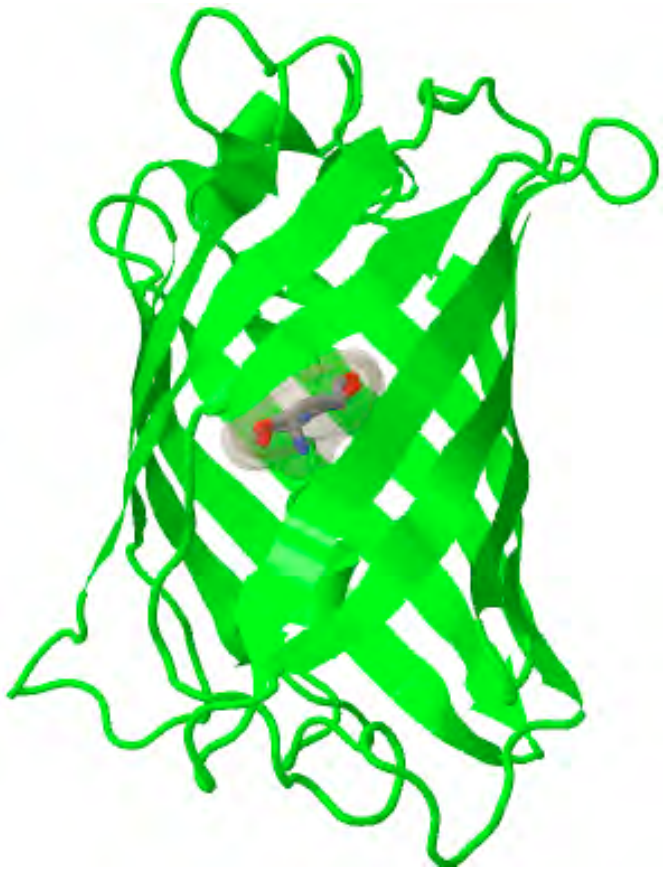
Caveats



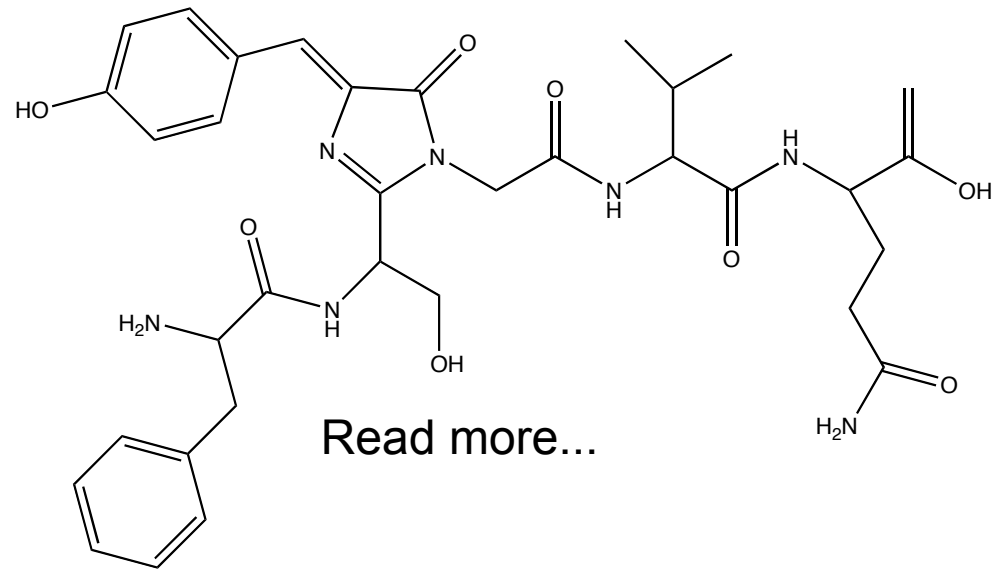
- Angular dependence (κ^2)
- Environment dependence (J, ϕ_D)
- Distance is a (complicated) average
- Probe is large, linkages can be long
- Construct complications
  - Free fluorophores
  - Donors without partner acceptors
  - Acceptors without partner donors

Determining Distances / Distance Changes
Common probes and Caveats

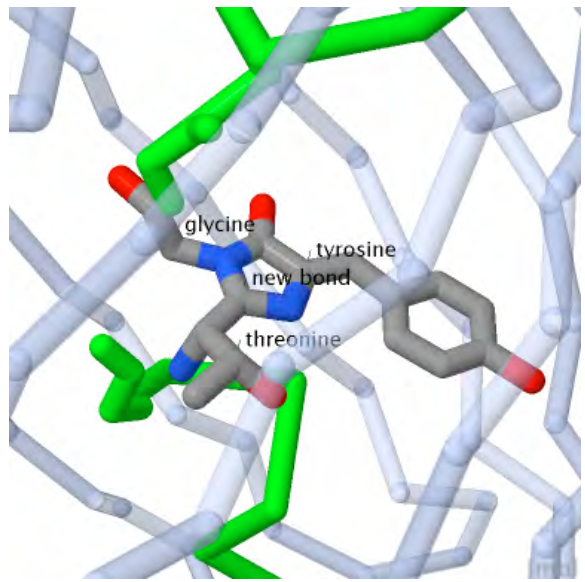
- Common probes
  - GFP/ YFP, etc



View from Proteopedia



Read more...



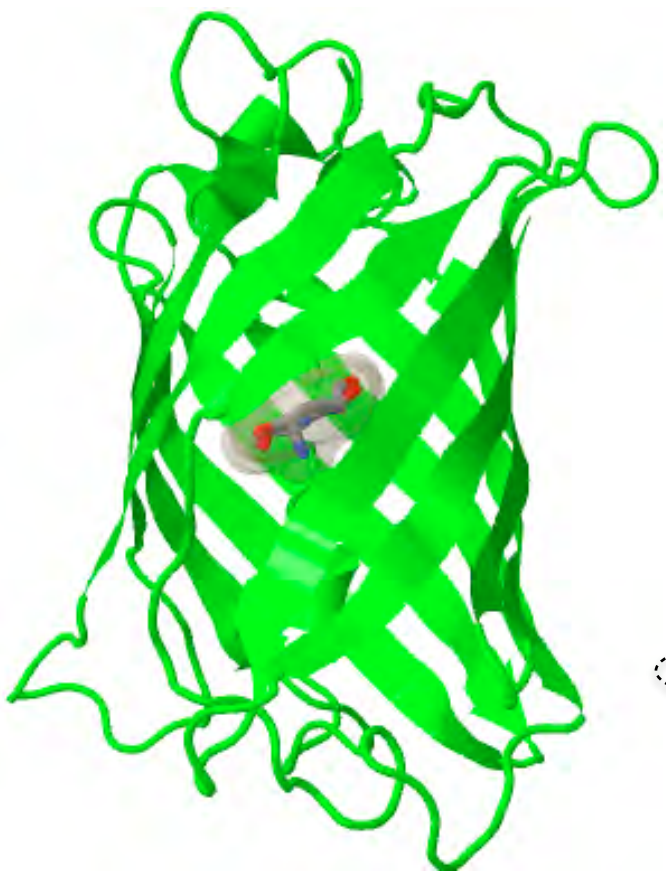
Genetically fuse onto other proteins. Provides an *in vivo* fluorescent tag

Fluorophore chemically "matures" from amino acid precursors

Determining Distances / Distance Changes
Common probes and Caveats

Improving FRET dynamic range with bright green and red fluorescent proteins

Amy J Lam[1,2], François St-Pierre[1,2], Yiyang Gong[3–5], Jesse D Marshall[3–5], Paula J Cranfill[6,7], Michelle A Baird[6,7], Michael R McKeown[8–10], Jörg Wiedenmann[11], Michael W Davidson[6,7], Mark J Schnitzer[3–5], Roger Y Tsien[8–10] & Michael Z Lin[1,2]



View from Proteopedia

Table 2 | Examples of r_0 values of fluorescent protein FRET pairs

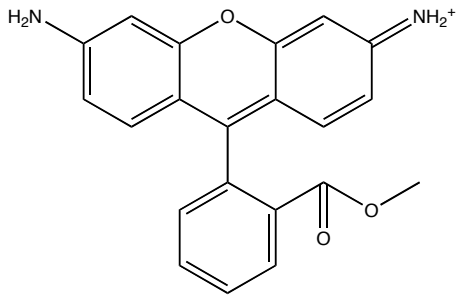
FRET pair	ϕ_D[a]	ε_A[b] (mM^{-1} cm^{-1})	r_0 (nm)
ECFP-Citrine	0.36[c]	77[d]	4.8
ECFP-Venus	0.36[c]	92[d]	5.0
Cerulean-Citrine	0.49[c]	77[d]	5.4
Cerulean-Venus	0.49[c]	92[d]	5.2
SECFP-SEYFP	0.58[e]	101[e]	5.4
EGFP-mCherry	0.60[d]	72[d]	5.4
TagGFP-TagRFP	0.59[f]	100[f]	5.7
mTFP1-Citrine	0.85[d]	77[d]	5.7
mTFP1-mOrange	0.85[d]	71[d]	5.7
Citrine-mKate2	0.76[d]	63[d]	5.8
Clover-mCherry	0.76[g]	72[d]	5.8
mTurquoise1-SEYFP	0.84[c]	101[e]	5.8
mTurquoise2-SEYFP	0.93[c]	101[e]	5.9
Clover-mRuby2	0.76[g]	113[g]	6.3

Random interfluorophore orientation is assumed. Pairs are ordered by r_0.
[a]Quantum yield of donor. [b]Extinction coefficient of acceptor. [c]Values from ref. 29. [d]Values from ref. 47. [e]Values from ref. 50. [f]Values from ref. 15. [g]Data from this study.

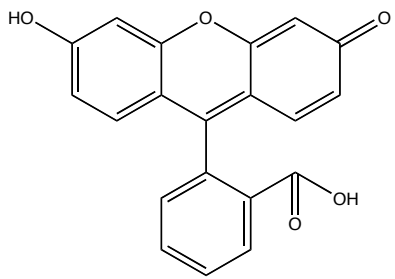
Determining Distances / Distance Changes

Common probes

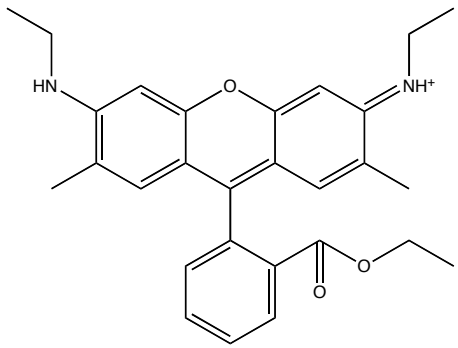
- Common probes
  - GFP/ YFP, etc
  - Rhodamine family
  - Fluorescein family



Rhodamine

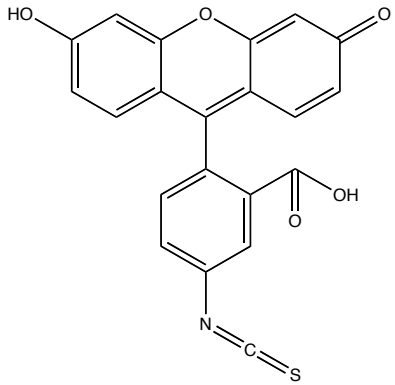


Fluorescein



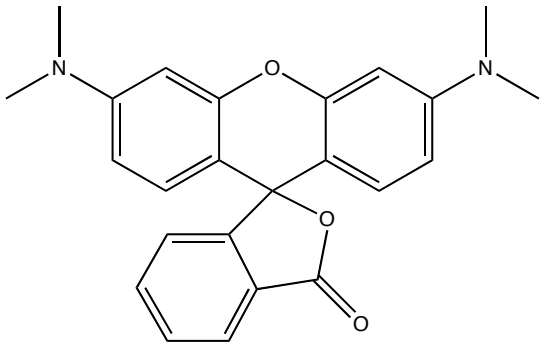
Rhodamine 6G

Other derivatives:
Texas Red, TRITC



Fluorescein isothiocyanate (FITC)

Other derivatives:
Oregon Green, Tokyo Green

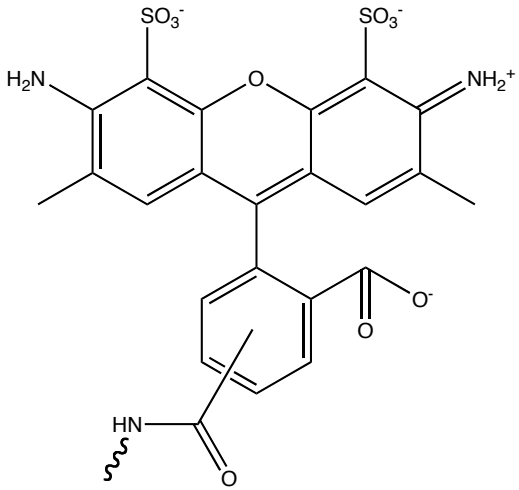


Tetramethyl Rhodamine (TAMRA)

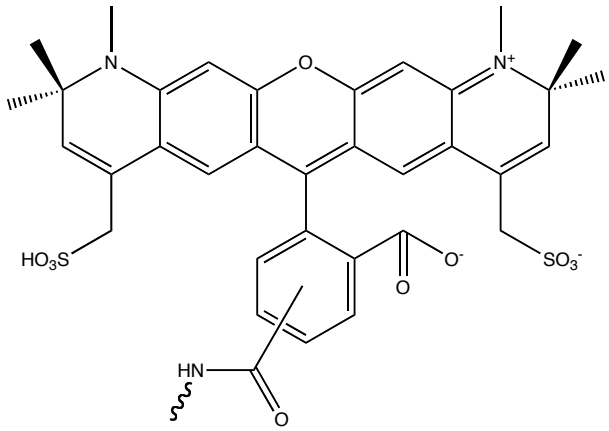
Determining Distances / Distance Changes

Common probes

- Common probes
  - GFP/ YFP, etc
  - Rhodamine family
  - Fluorescein family
  - Alexa family



Alexa Fluor 488



Alexa Fluor 594

Molecular Probes, Inc.
(Invitrogen)

less pH-sensitive & more photostable
than fluorescein, rhodamine, etc

Determining Distances / Distance Changes

Common probes

- Common probes
  - GFP/ YFP, etc
  - Rhodamine family
  - Fluorescein family
  - Alexa family
  - Cy3, Cy5 family

	Color†	Absorb (nm)	Emit (nm)	MM (g/mol)	ε ($cm^{-1}M^{-1}$)
Alexa Fluor 350	blue	346	442	410	19,000
– 405	violet	401	421	1028	34,000
– 430	green	434	541	702	16,000
– 488	cyan-green	495	519	643	71,000
– 500	green	502	525	700	71,000
– 514	green	517	542	714	80,000
– 532	green	532	554	721	81,000
– 546	yellow	556	573	1079	104,000
– 555	yellow-green	555	565	~1250	150,000
– 568	orange	578	603	792	91,300
– 594	orange-red	590	617	820	90,000
– 610	red	612	628	1172	138,000
– 633	red	632	647	~1200	100,000
– 647	red	650	665	~1300	239,000
– 660	red	663	690	~1100	132,000
– 680	red	679	702	~1150	184,000
– 700	red	702	723	~1400	192,000
– 750	red	749	775	~1300	240,000

† = approximate color of the emission spectrum

ε = extinction coefficient

Determining Distances / Distance Changes

Common probes

- Common probes
  - GFP/ YFP, etc
  - Rhodamine family
  - Fluorescein family
  - Alexa family
  - Cy3, Cy5 family

Molecular Probes, Inc.
(Invitrogen)

less pH-sensitive & more photostable
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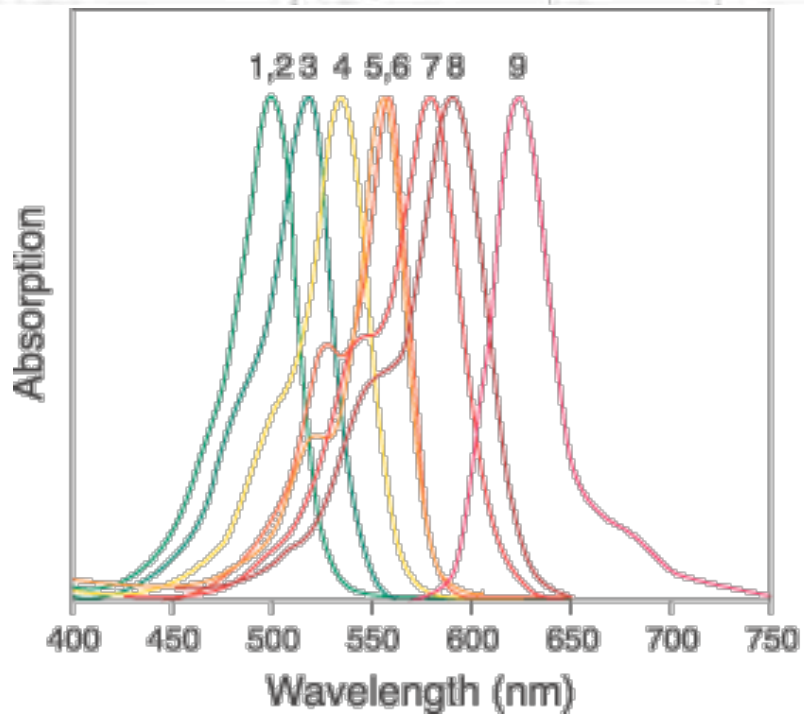
	Color†	Absorb (nm)	Emit (nm)	MM (g/mol)	ε ($cm^{-1}M^{-1}$)
Alexa Fluor 350	blue	346	442	410	19,000
— 405	violet	401	421	1028	34,000
— 430	green	434	541	702	16,000
— 488	cyan-green	495	519	643	71,000
— 750	red	749	775	~1300	240,000

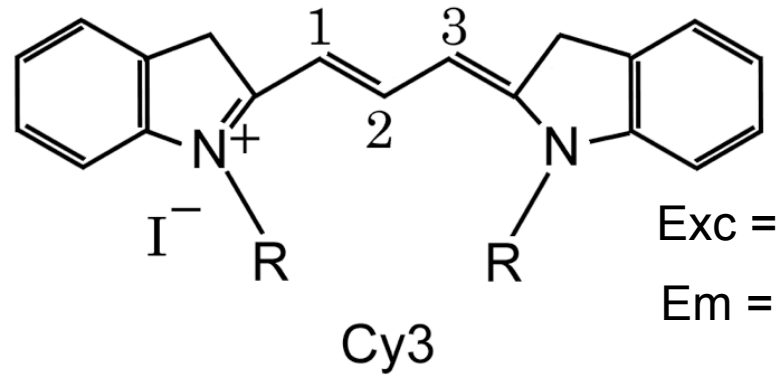
† = approximate color of the emission spectrum
ε = extinction coefficient



Determining Distances / Distance Changes

Common probes

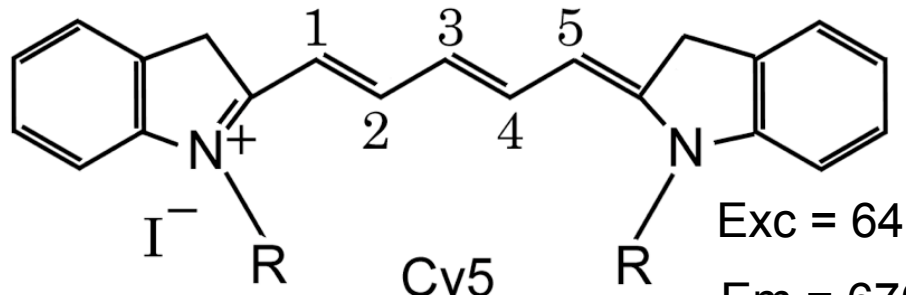
- Common probes
  - GFP/ YFP, etc
  - Spinach, Broccoli
  - Rhodamine family
  - Fluorescein family
  - Alexa family
  - Cy3, Cy5 family



Cy3

Exc = 550 nm

Em = 570 nm



Cy5

Exc = 649 nm

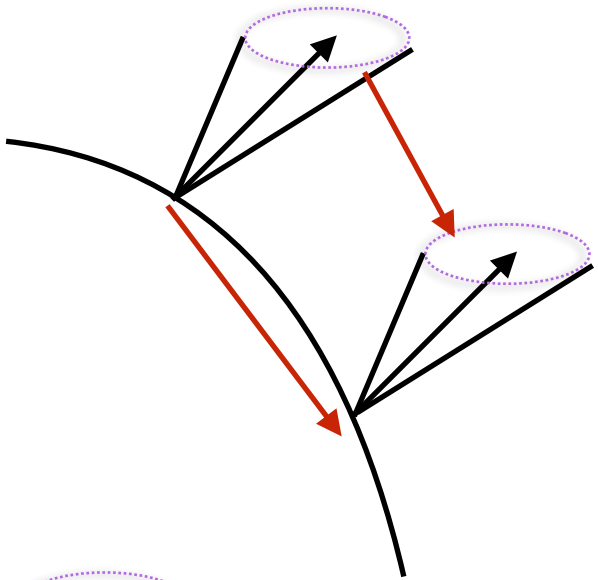
Em = 670 nm

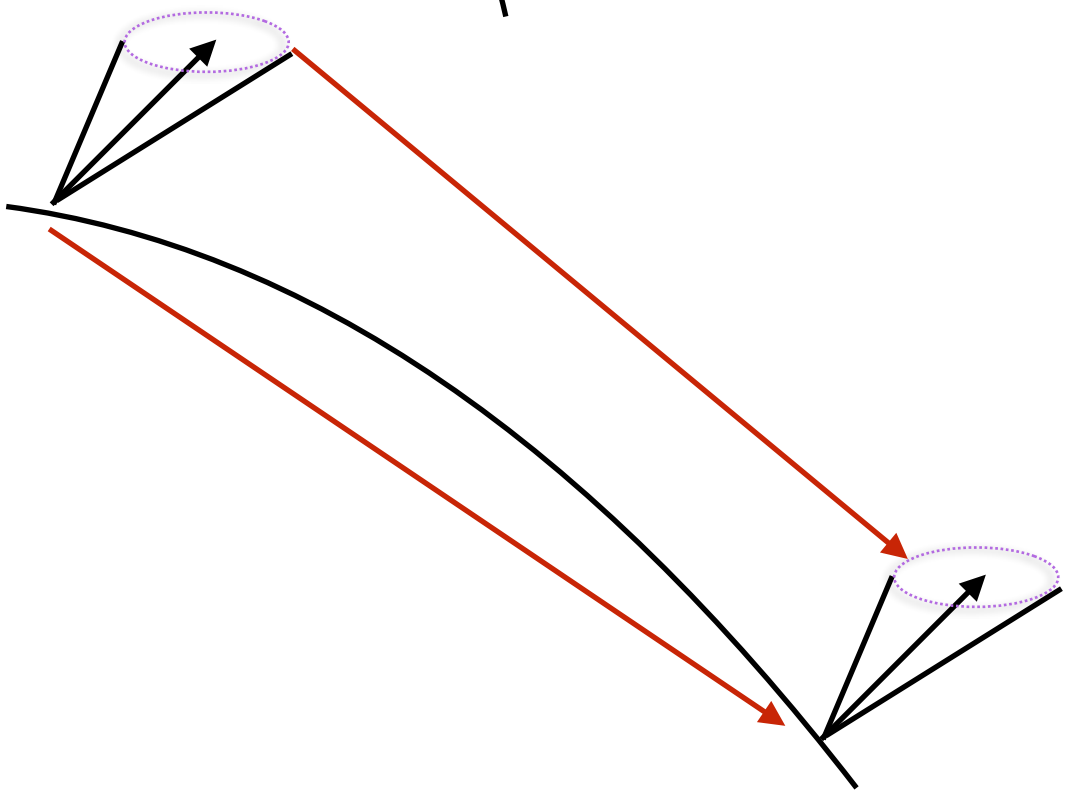
Which is donor?

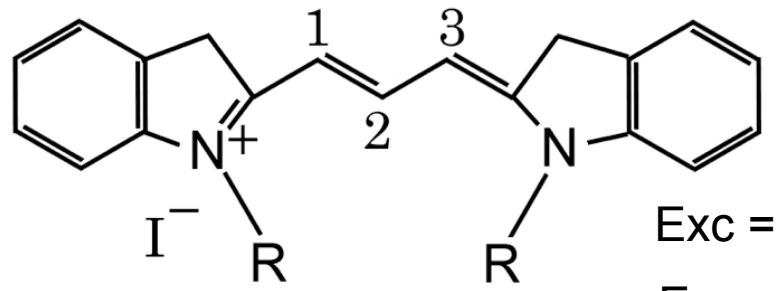
More in family: Cy3.5, Cy5.5, et al.

Determining Distances / Distance Changes

Common probes



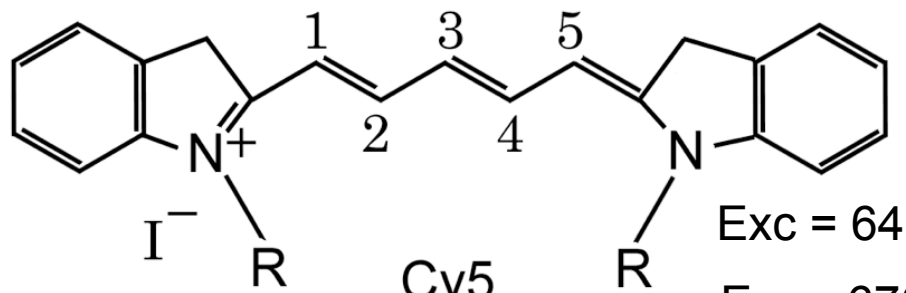




Cy3

Exc = 550 nm

Em = 570 nm



Cy5

Exc = 649 nm

Em = 670 nm

Which is donor?

Determining Distances / Distance Changes

Table 1.6 R_0 values for some Alexa Fluor dyes.

Donor	Acceptor					
	Alexa Fluor 488	**Alexa Fluor 546**	**Alexa Fluor 555**	**Alexa Fluor 568**	**Alexa Fluor 594**	**Alexa Fluor 647**
Alexa Fluor 350	50					
Alexa Fluor 488	NA	64	70	62	60	56
Alexa Fluor 546		NA		70	71	74
Alexa Fluor 555			NA		47	51
Alexa Fluor 568				NA		82
Alexa Fluor 594					NA	85
Alexa Fluor 647						NA

R_0 values in angstroms (Å) represent the distance at which fluorescence resonance energy transfer from the donor dye to the acceptor dye is 50% efficient (Förster radius). Values were calculated from spectroscopic data as outlined (Fluorescence Resonance Energy Transfer (FRET) —Note 1.2). NA = Not applicable.

Determining Distances / Distance Changes

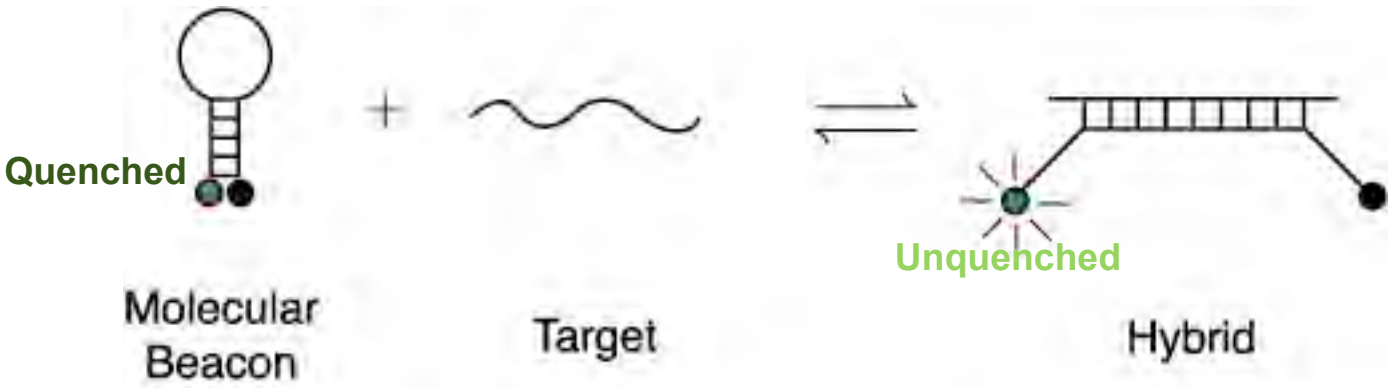
Table 2: Common donor/acceptor pairs for FRET and TR-FRET/HTRF[1]

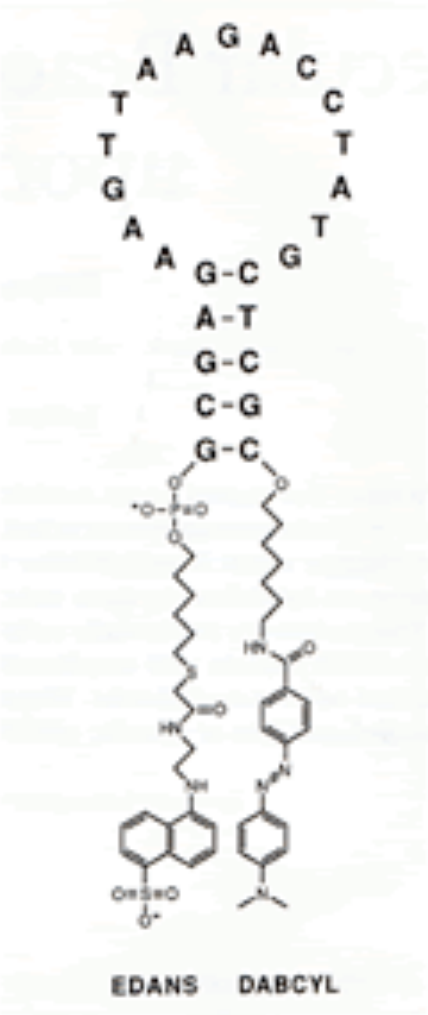
Donor	Acceptor	R_0 (Å)[2]
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46
EDANS	Dabcyl	33
BODIPY FL	BODIPY FL	57
Fluorescein	QSY 7 and QSY 9 dyes	61
Alexa Fluor 488	Alexa Fluor 555	70
Alexa Fluor 594	Alexa Fluor 647	85
Europium (III) cryptate	XL665 (allophycocyanine) or d2	90
Terbium (III)	Fluorescein	70

[1] Adapted from Invtrogen.com (FRET; Alexa dyes) and Cysbio (TR-FRET); [2]Ro is the distance at which FRET efficiency is 50%.

Molecular Beacons

a simple application of FRET quenching





Donor

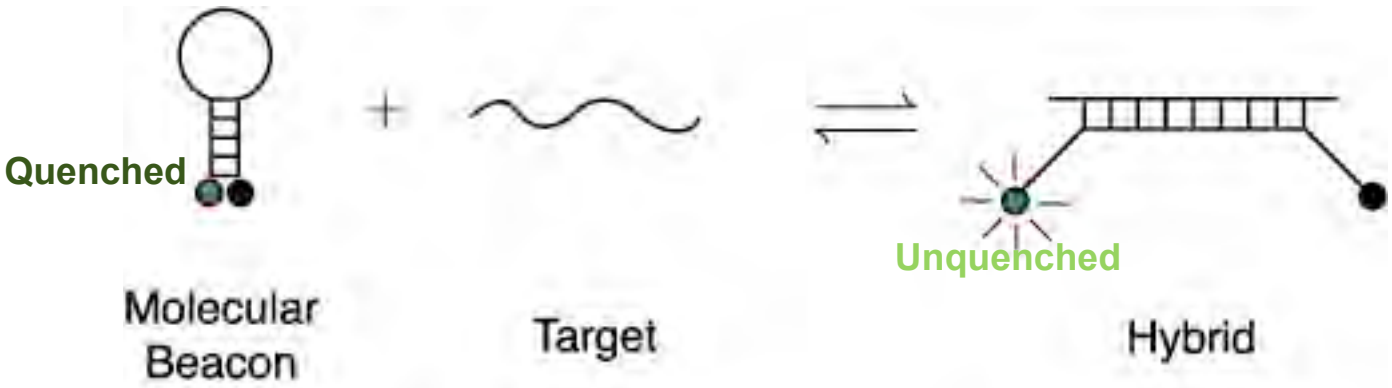
Acceptor

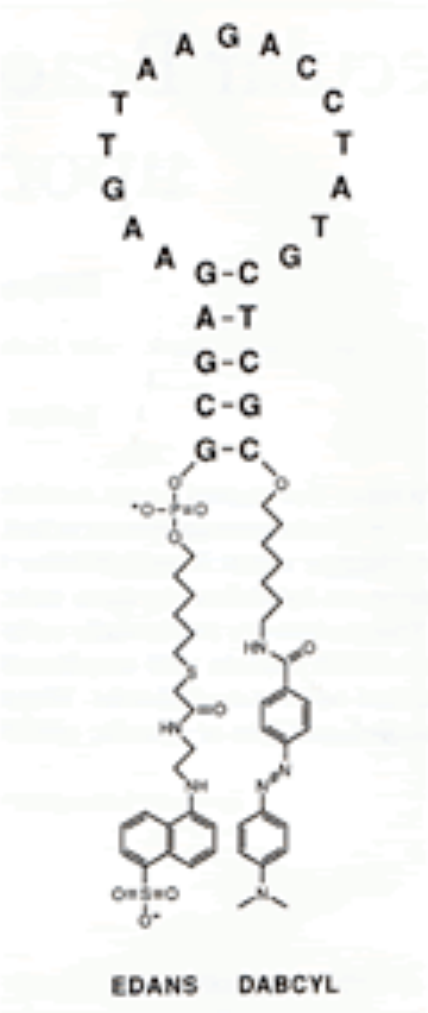
(non-fluorescent)

(aka "Quencher")

Molecular Beacons

a simple application of FRET quenching





Huge number of applications:

- Gene probing
- Real Time PCR
- Molecular switch sensors

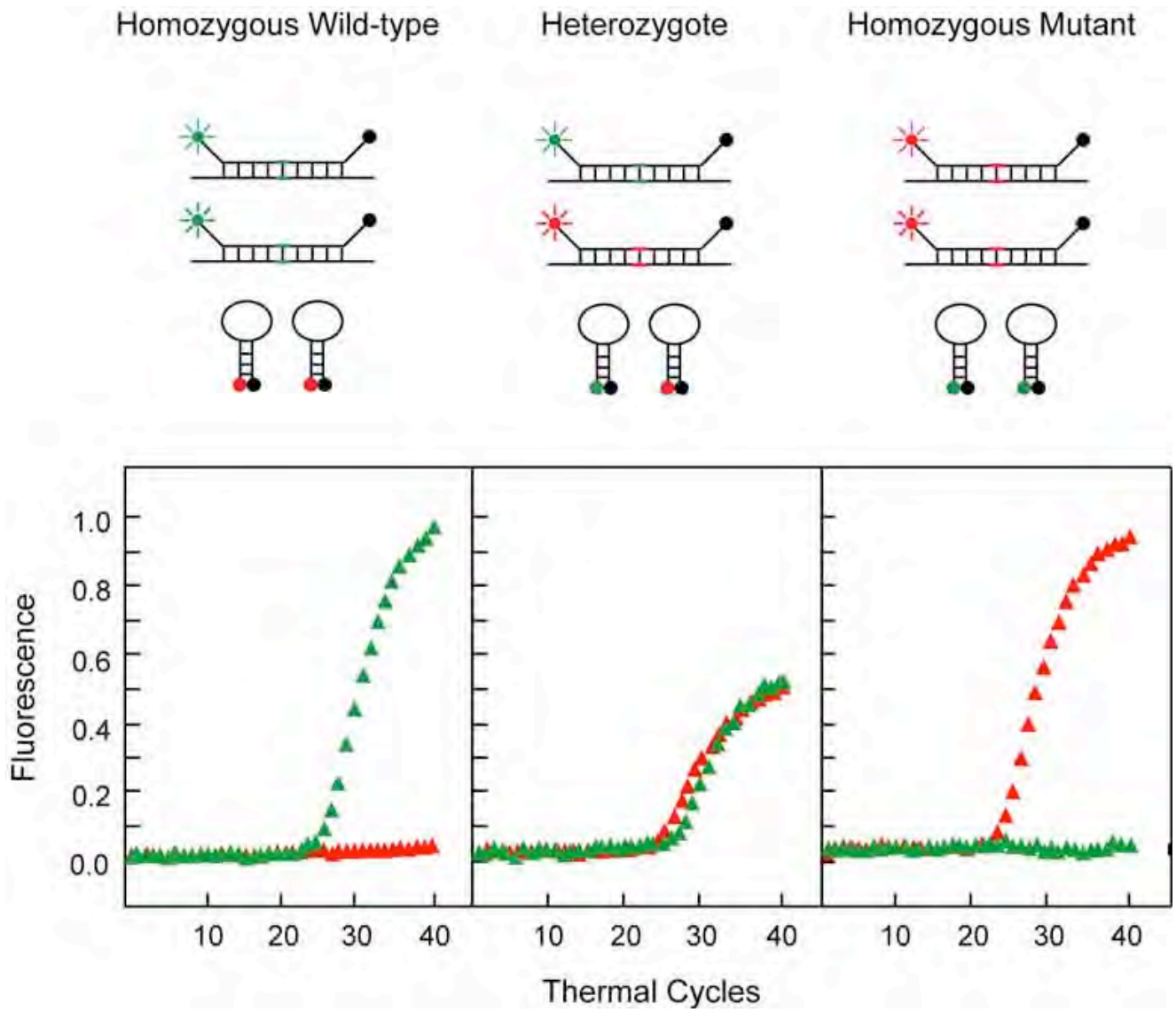
Advantages

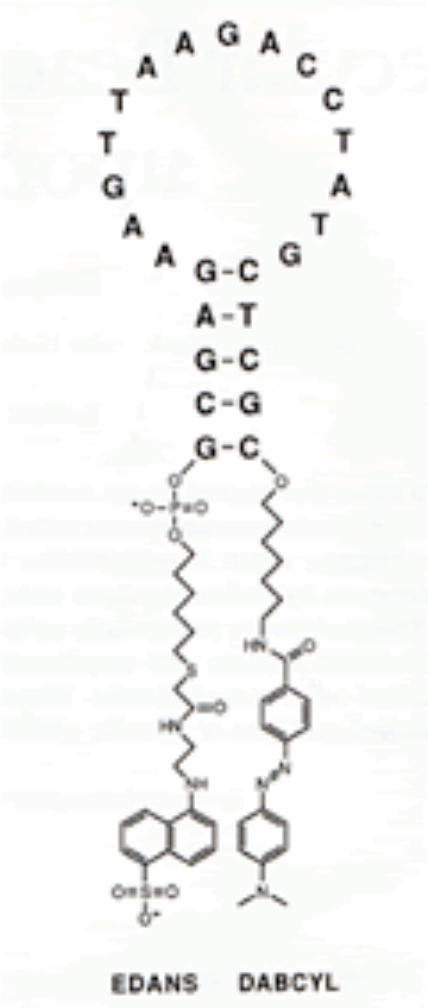
- Fluorescence - very sensitive!
  - low background
  - always better to detect signal ON
- Inexpensive

Molecular Beacons

a simple application of FRET quenching

- Gene probe diagnostics (**multiplexing**)

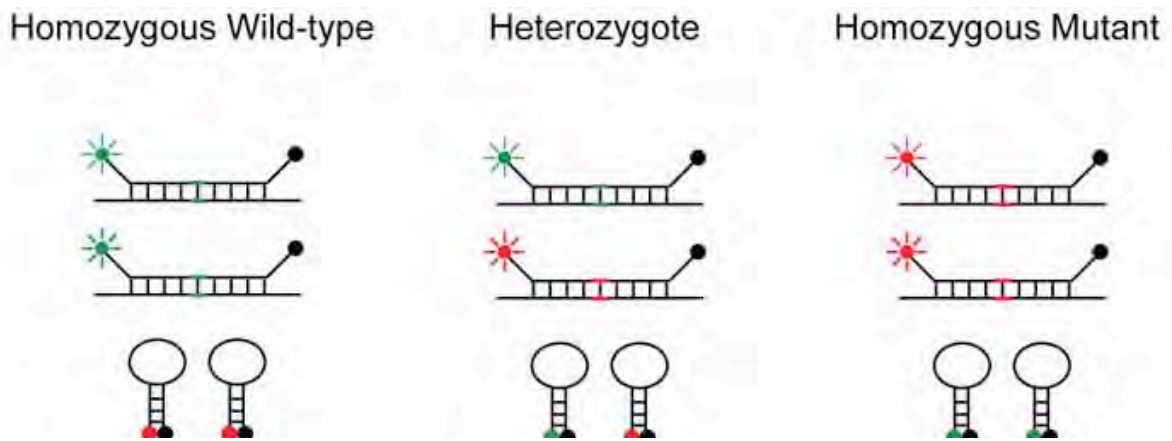


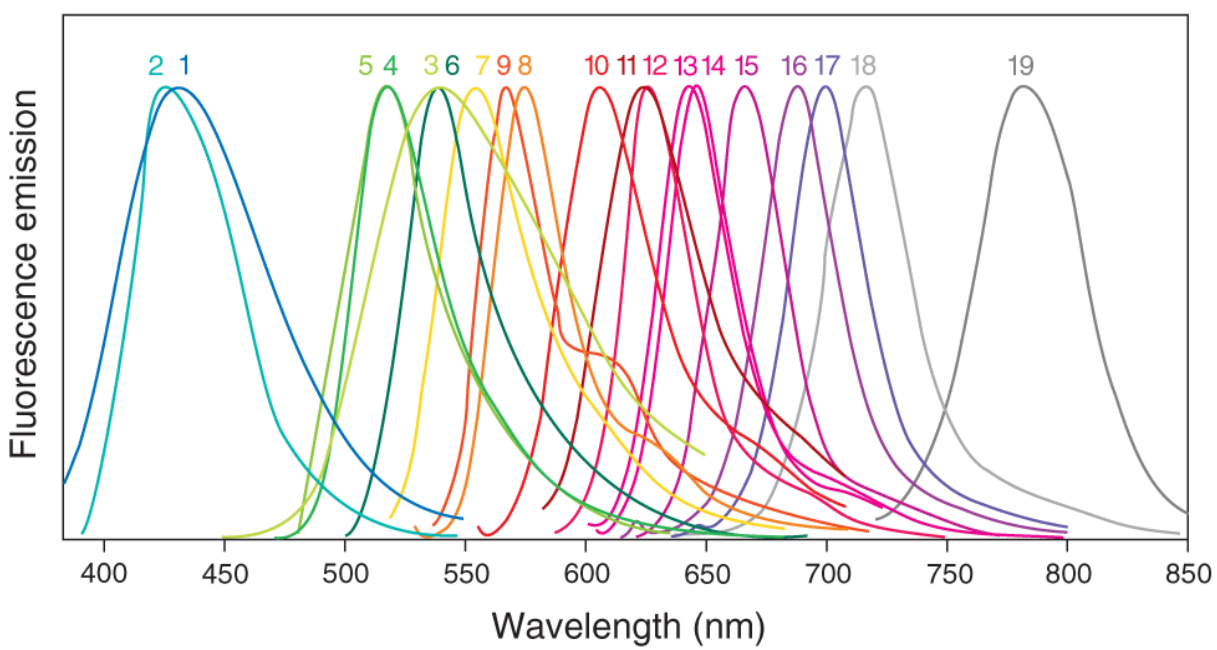


Molecular Beacons

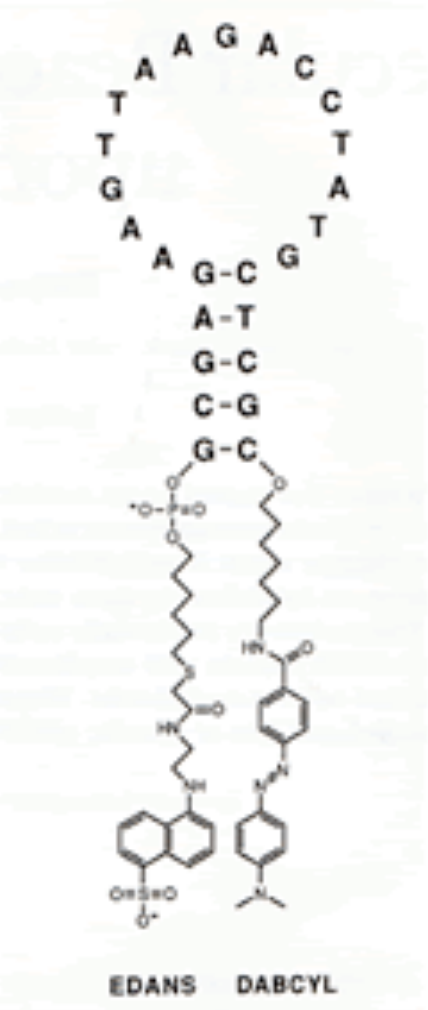
a simple application of FRET quenching

- Gene probe diagnostics (**multiplexing**)

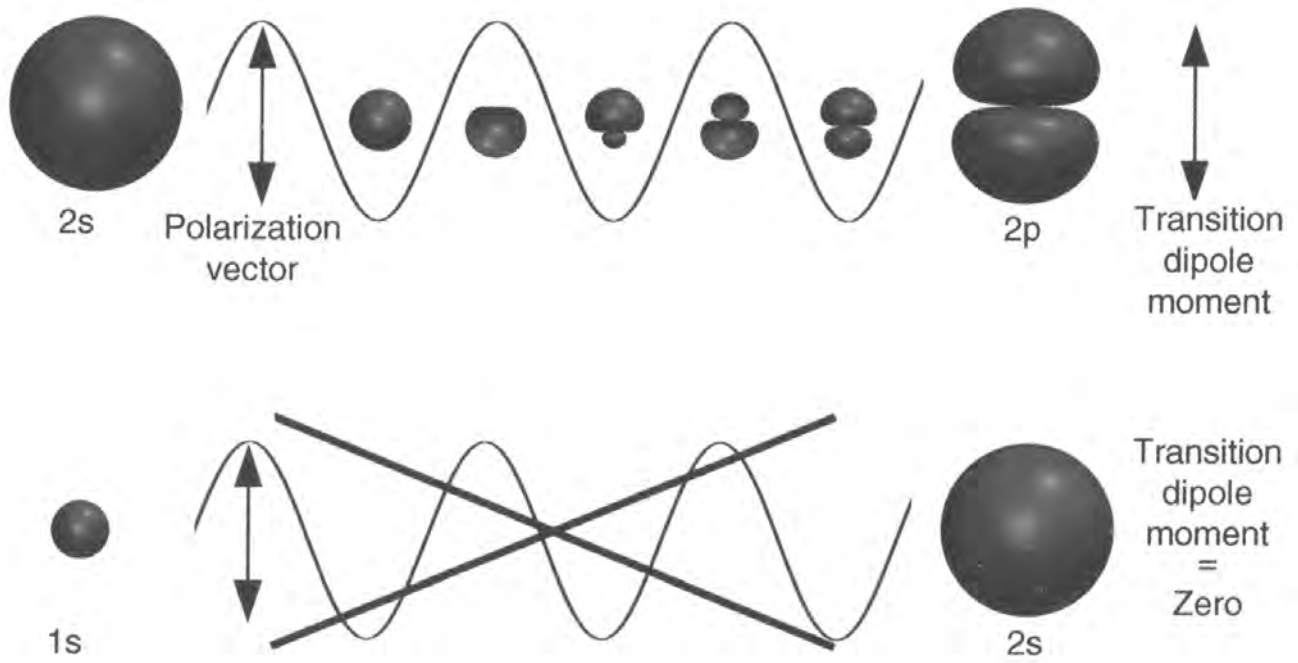




1. Alexa Fluor 350
2. Alexa Fluor 405
3. Alexa Fluor 430
4. Alexa Fluor 488
5. Alexa Fluor 500
6. Alexa Fluor 514
7. Alexa Fluor 532
8. Alexa Fluor 546
9. Alexa Fluor 555
10. Alexa Fluor 568
11. Alexa Fluor 594
12. Alexa Fluor 610
13. Alexa Fluor 633
14. Alexa Fluor 635
15. Alexa Fluor 647
16. Alexa Fluor 660
17. Alexa Fluor 680
18. Alexa Fluor 700
19. Alexa Fluor 750



Recap - Transition Dipole Moment



$$\left(\int_{-\infty}^{+\infty}\psi_2\bar{\mu}_x\psi_1\,\partial x\right)\left(\int_{-\infty}^{+\infty}\psi_2\bar{\mu}_y\psi_1\,\partial x\right)\left(\int_{-\infty}^{+\infty}\psi_2\bar{\mu}_z\psi_1\,\partial x\right)$$

$$\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\int_{-\infty}^{+\infty}\psi_2\bar{\mu}\psi_1\,\partial x\,\partial y\,\partial z$$

Transition Dipole Moment

Integrals the easy way

$$\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_x \psi_1 \, \partial x$$

even

↓

Non-zero

$$\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_x \psi_1 \, \partial x$$

odd

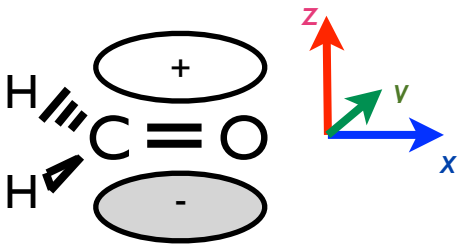
↓

Zero

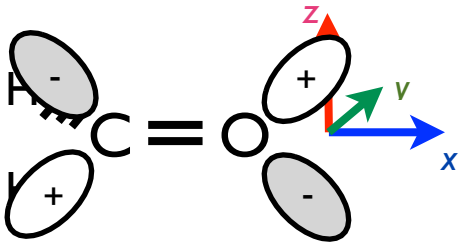
$$\left(\underset{\neq 0}{\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_x \psi_1 \, \partial x} \right) \left(\underset{0}{\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_y \psi_1 \, \partial x} \right) \left(\underset{0}{\int_{-\infty}^{+\infty} \psi_2 \bar{\mu}_z \psi_1 \, \partial x} \right)$$

even odd odd

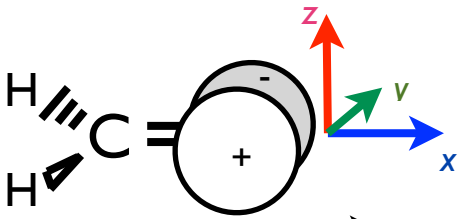
Formaldehyde: π to π*



	x	y	z
	even	even	odd



	x	y	z
	odd	even	odd



	x	y	z
	even	odd	even
μ_x	odd	even	even
μ_y	even	odd	even
μ_z	even	even	odd

$\int \pi \mu_x \pi^* dx$ e o o — *even*

$\int \pi \mu_x \pi^* dy$ e e e — *even*

$\int \pi \mu_x \pi^* dz$ o e o — *even*

$\int \pi \mu_y \pi^* dx$ e e o — *even*

$\int \pi \mu_y \pi^* dy$ e o e — *odd*

$\int \pi \mu_y \pi^* dz$ o e o — *even*

$\int \pi \mu_z \pi^* dx$ e e o — *even*

$\int \pi \mu_z \pi^* dy$ e e e — *even*

$\int \pi \mu_z \pi^* dz$ o o o — *odd*

$\langle \pi|\mu|\pi^* \rangle = \langle \pi|\mu_x|\pi^* \rangle + \langle \pi|\mu_y|\pi^* \rangle + \langle \pi|\mu_z|\pi^* \rangle$

allowed (eoo)(eee)(oeo) + (eeo)(eoe)(oeo) + (eeo)(eee)(ooo)

(eoe) zero; (ooo) zero

dx dy dz | dx dy dz | dx dy dz

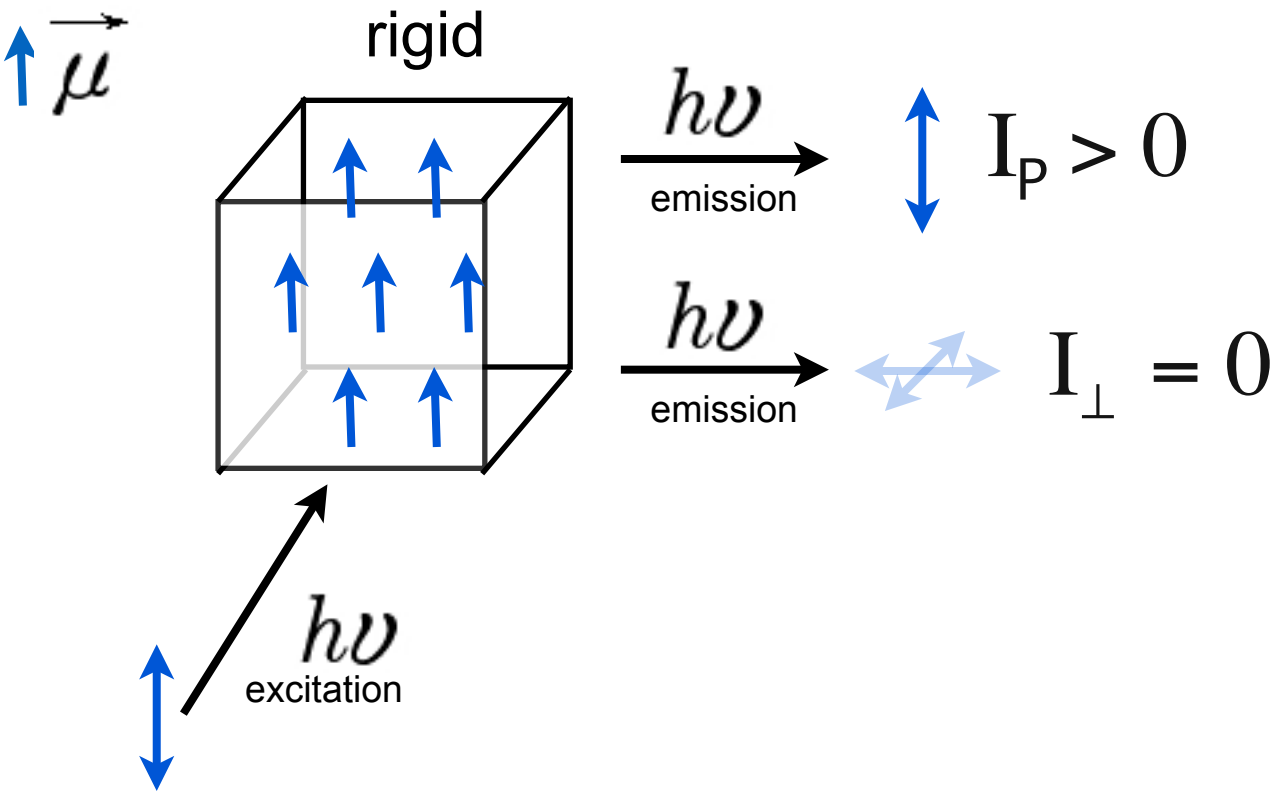
non-zero + **zero** + **zero**

Fluorescence Anisotropy

(fluorescence polarization)

$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$

$$\left(P = \frac{I_P - I_\perp}{I_P + I_\perp} \right)$$

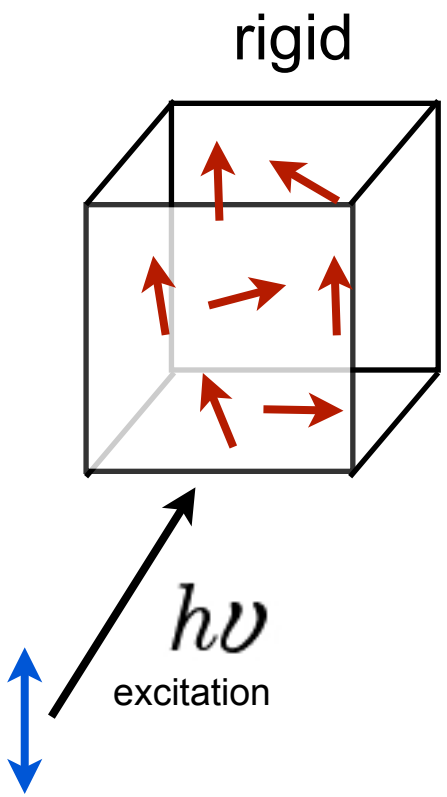


$$A > 0$$

Fluorescence Anisotropy

(fluorescence polarization)

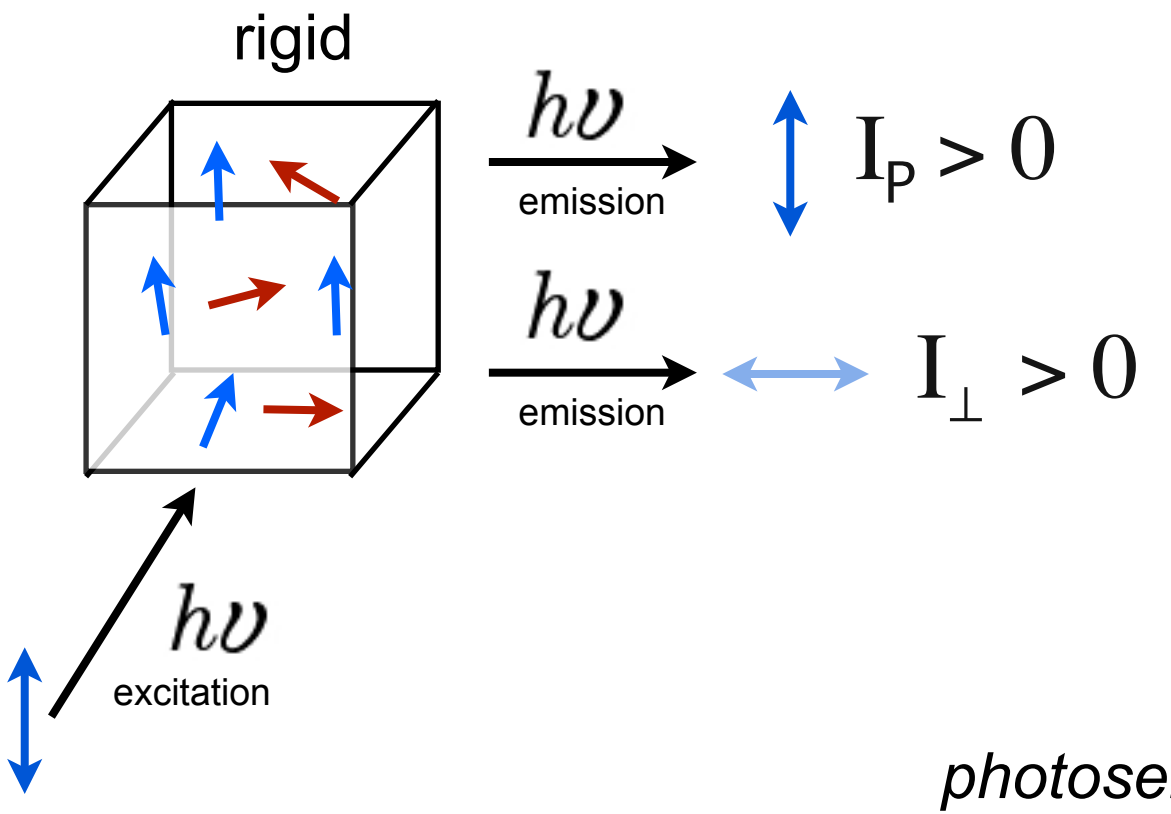
$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



Fluorescence Anisotropy

(fluorescence polarization)

$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



$$\frac{2}{5} > A > 0$$

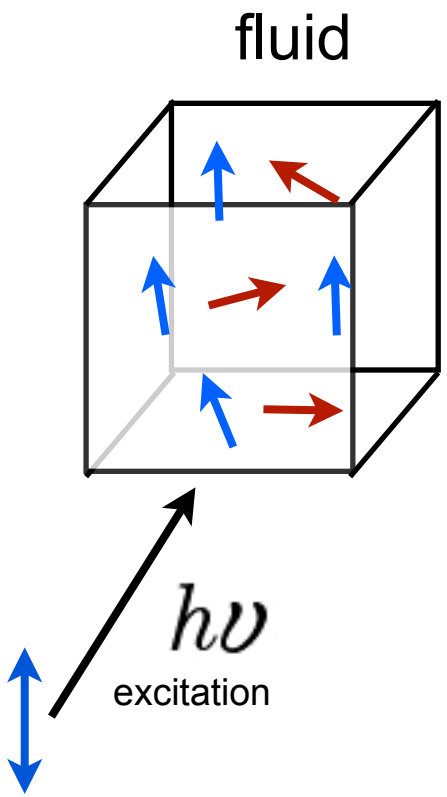
For both absorption and emission have to integrate $\cos^2$ and $\sin^2$ functions over all angles

photoselection

Fluorescence Anisotropy

(fluorescence polarization)

$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$

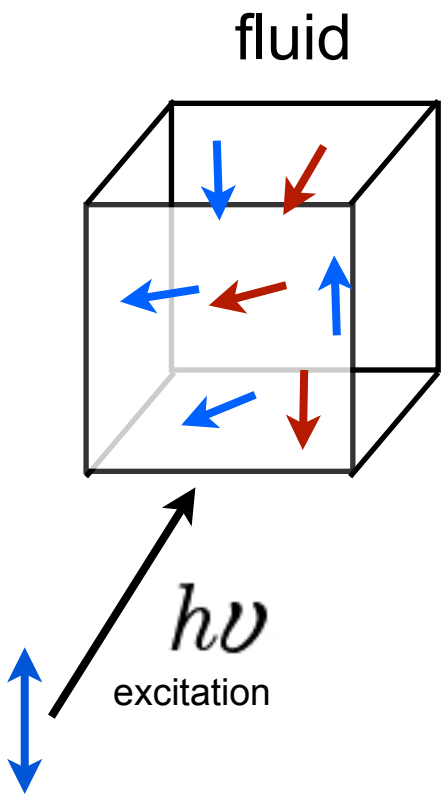


photoselection

Fluorescence Anisotropy

(fluorescence polarization)

$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



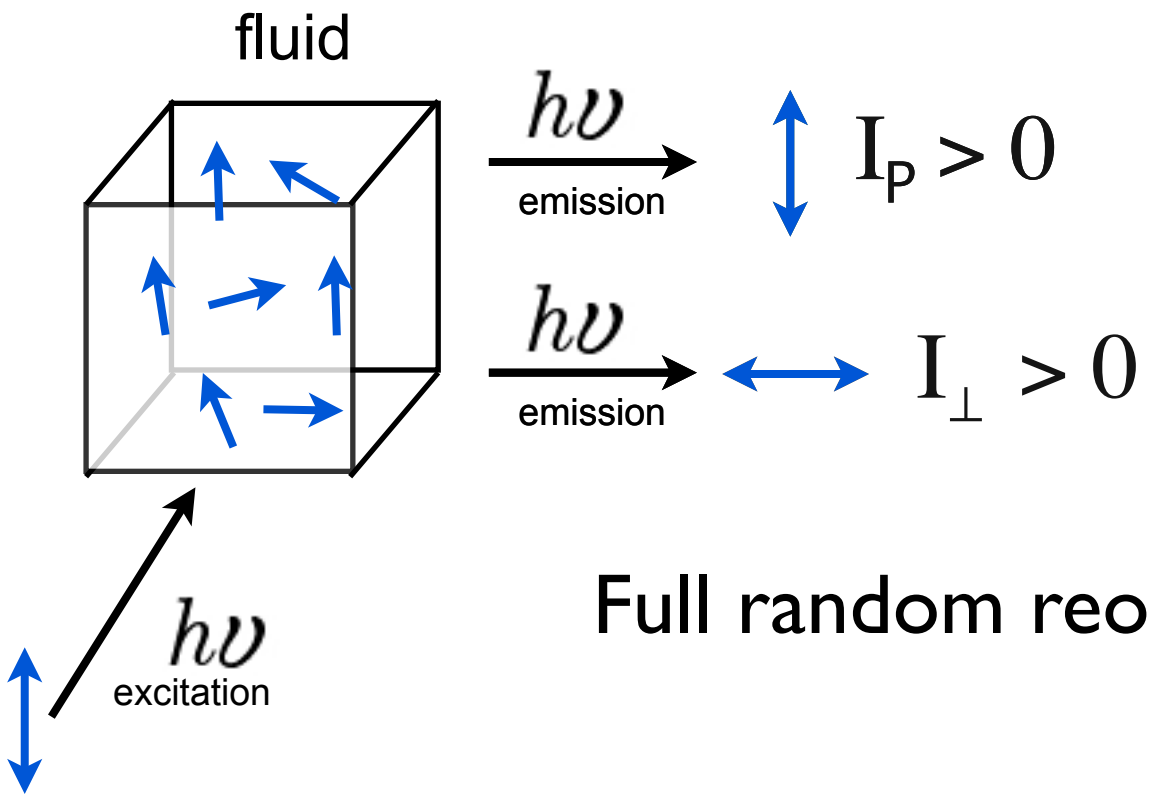
photoselection

random rotation

Fluorescence Anisotropy

(fluorescence polarization)

$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



$A \approx 0$

Full random reorientation, A=0

Fluorescence Anisotropy
(fluorescence polarization)

But wait…

This all assumes that the absorption and emission transition dipole moments are parallel (in an ideal world, they are, but we don't live in an ideal world)

The details can be more complicated, but the basic story remains the same

$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$

$$\frac{2}{5} > A > 0$$

For both absorption and emission have to integrate $\cos^2$ and $\sin^2$ functions over all angles

Fluorescence Anisotropy, Single Molecule, TIRF

February 23, 2021

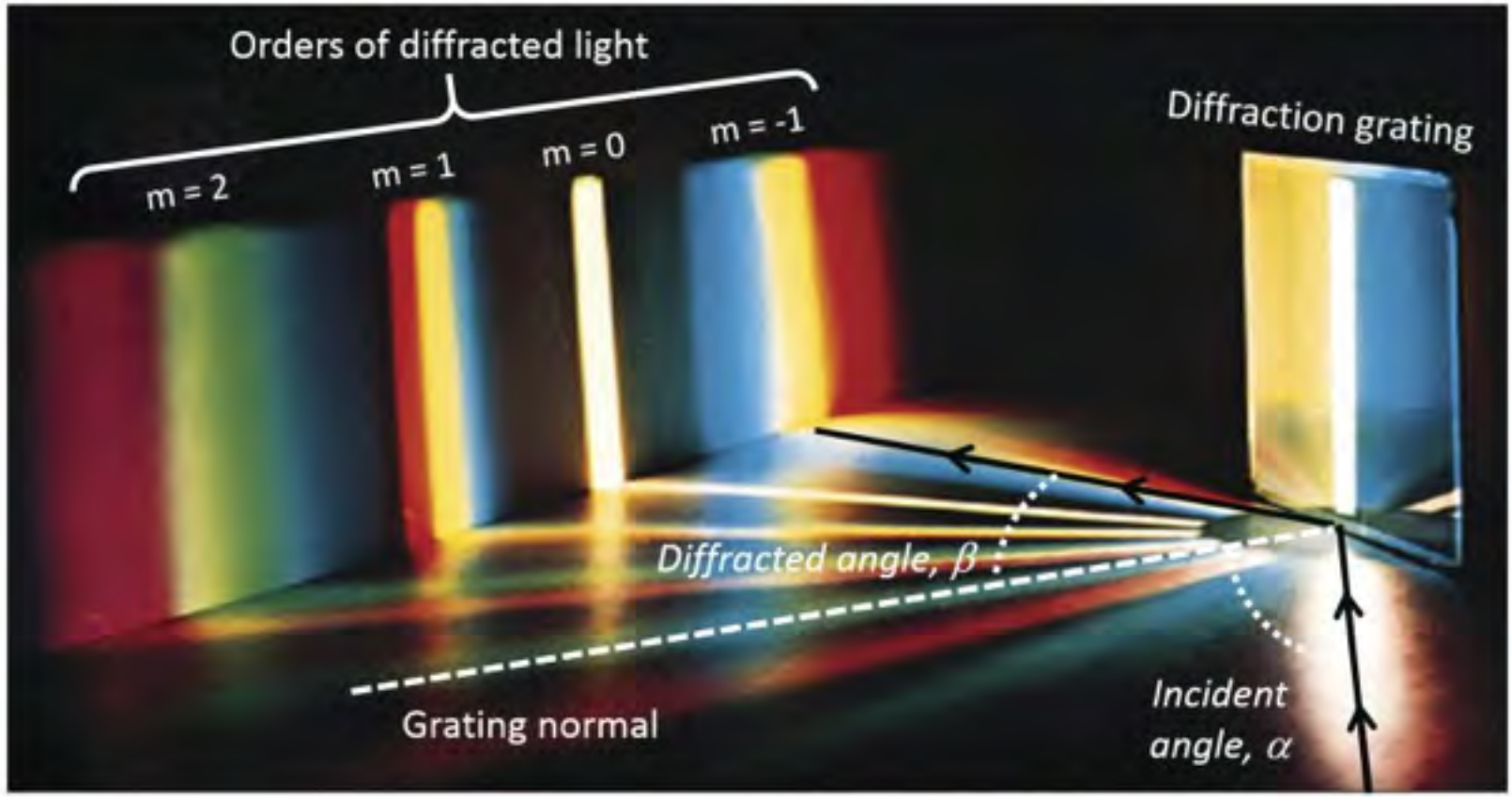
Exam/Quiz Schedule

Th	2/25	Short Quiz 1, in class; Take home exam #1 released
Tu	3/2	Take home exam #1 due before class starts
Tu	4/27	Short Quiz 2, in class; Take home exam #2 released on Moodle
Fri	4/30	Take home exam #2 due in Moodle by 11:00p

Grading

- 30% Take Home Exam 1
- 30% Take Home Exam 2
- 10% In-class Quizzes
- 20% Homework / Projects
- 10% Class participation, including in Moodle Forums (required)

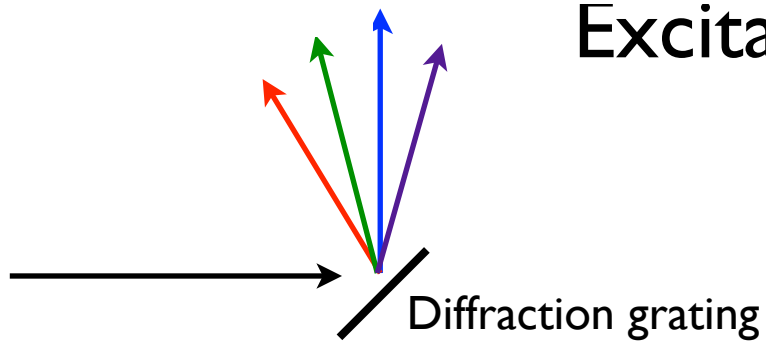
Fluorescence



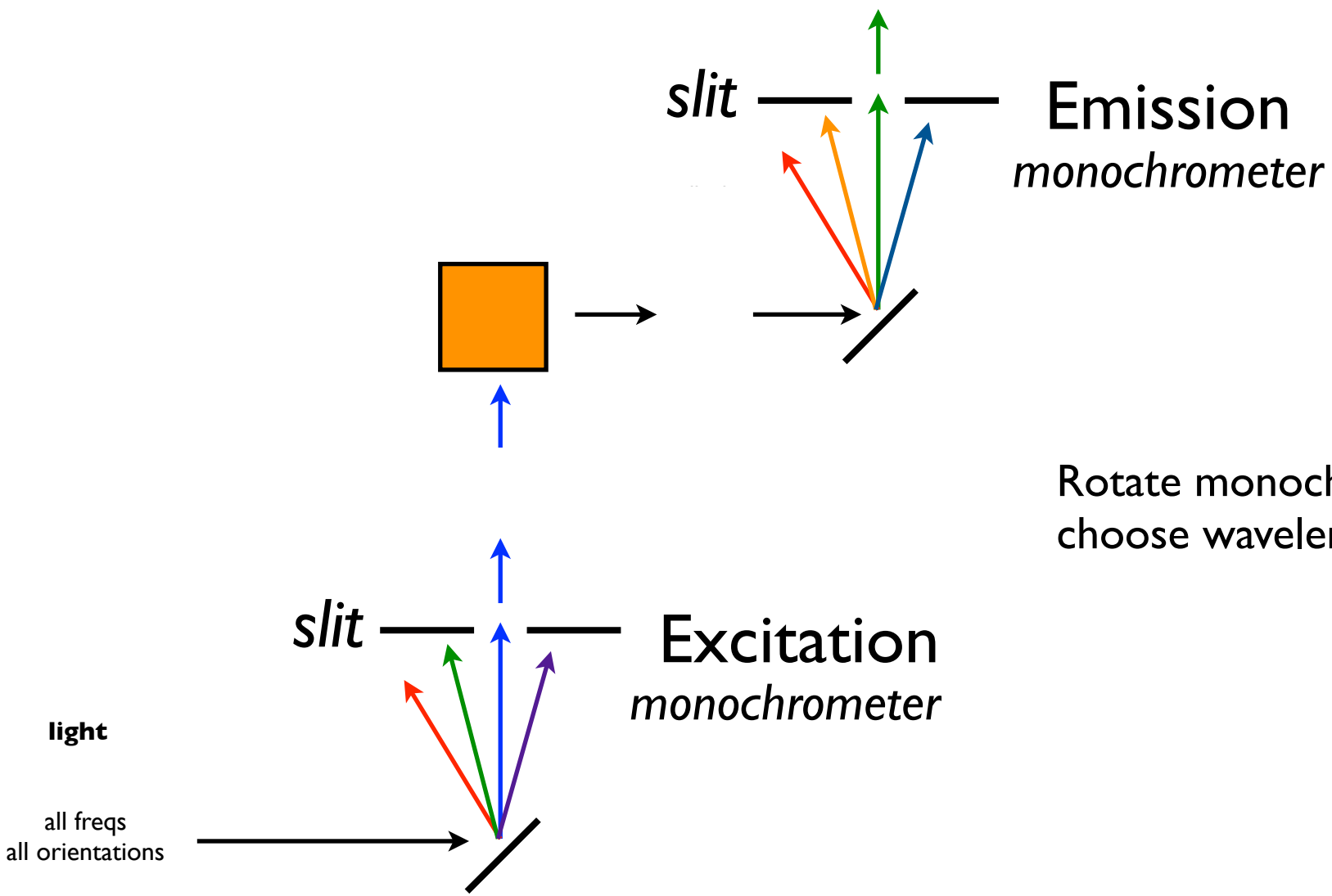
Excitation

light

all freqs
all orientations



Fluorescence

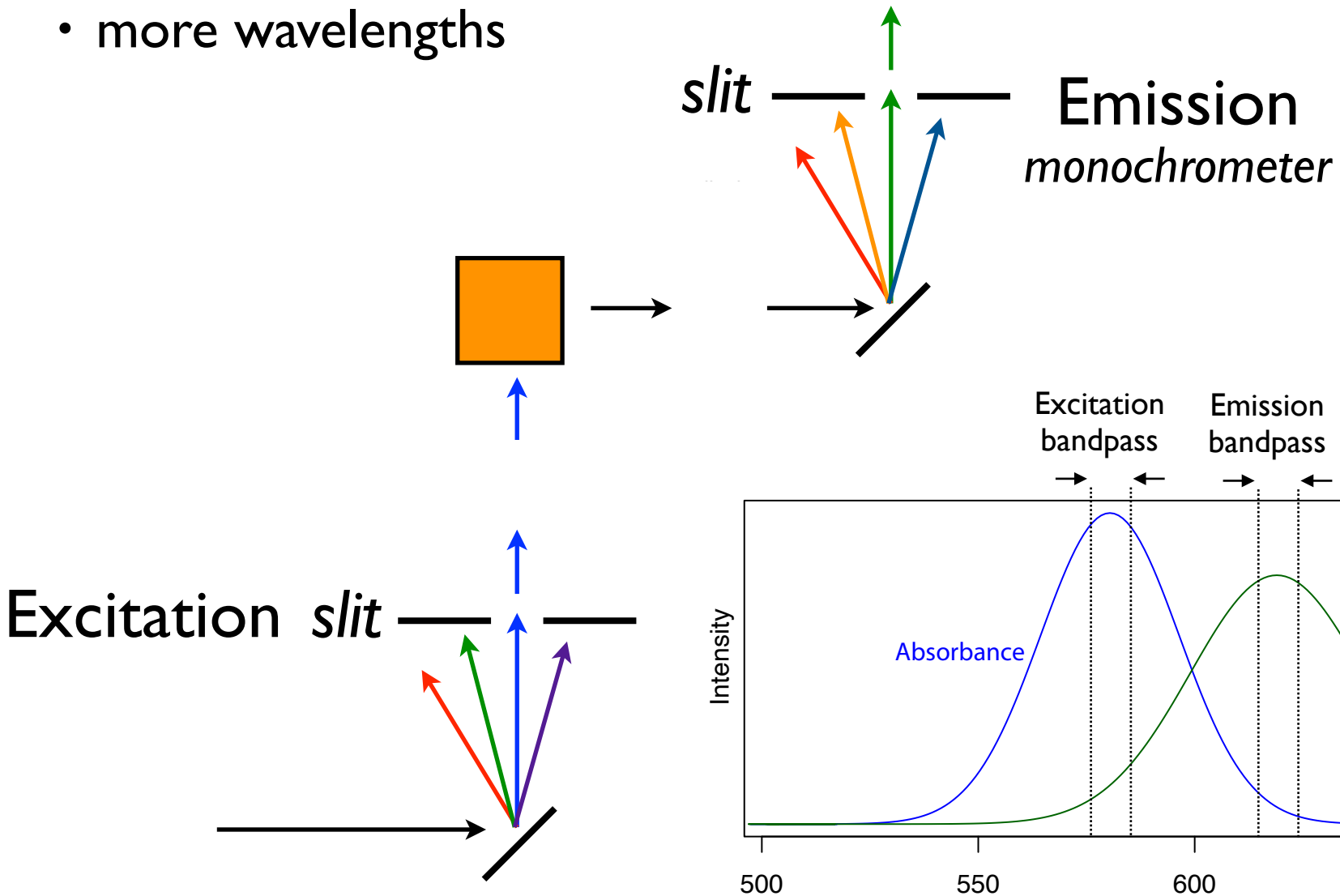


Rotate monochrometers to choose wavelength (center)

Fluorescence

Larger *slit*

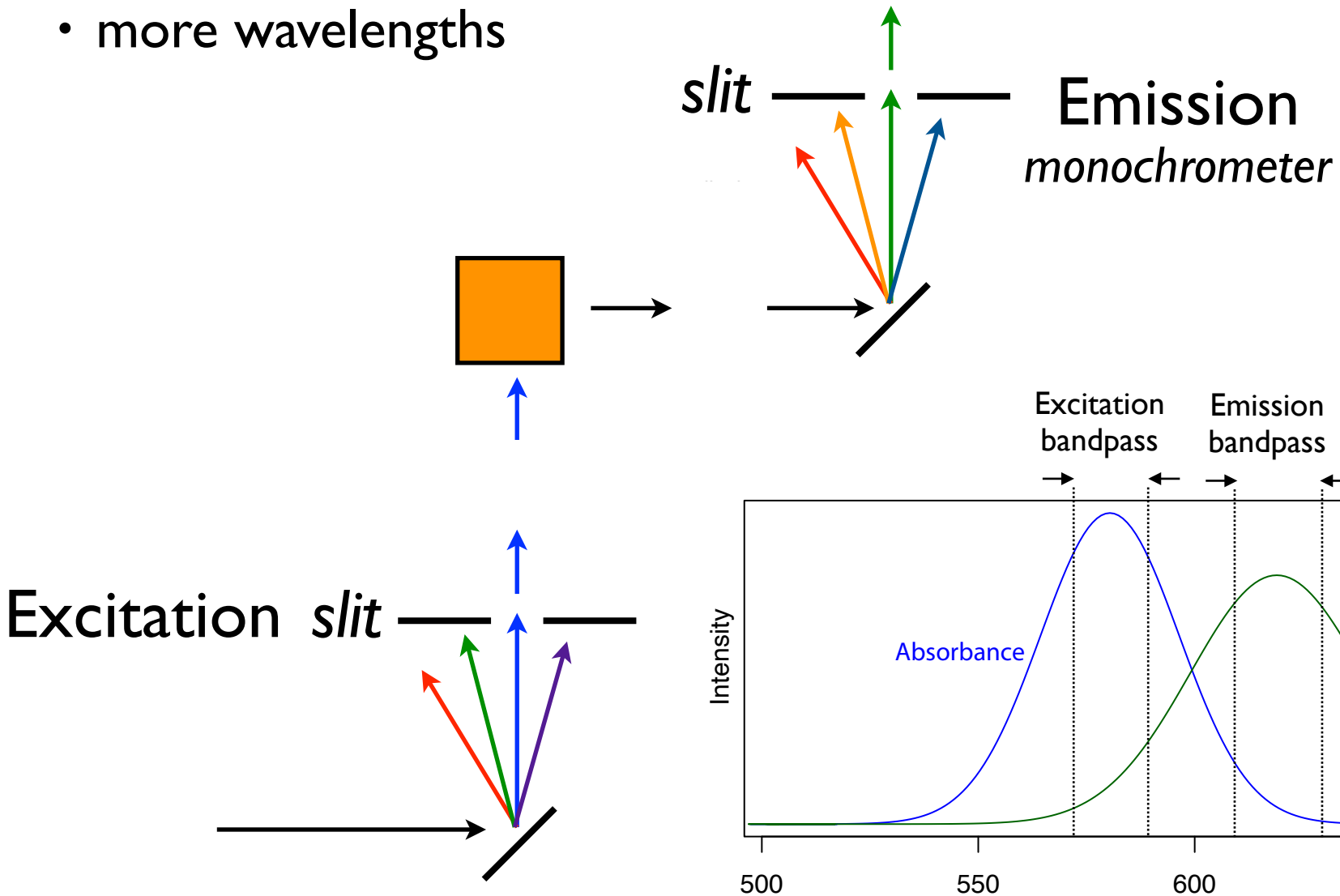
- more light!
- more wavelengths



Fluorescence

Larger *slit*

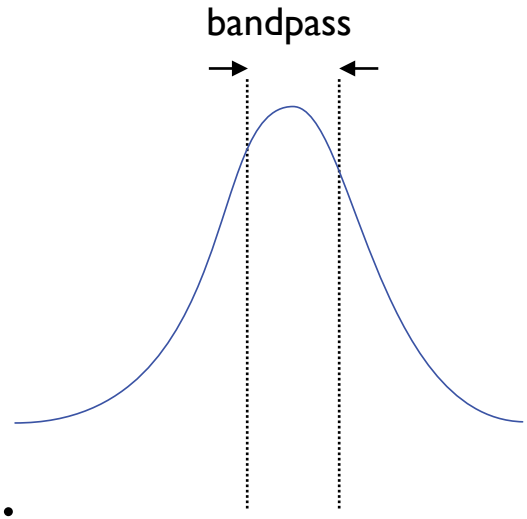
- more light!
- more wavelengths

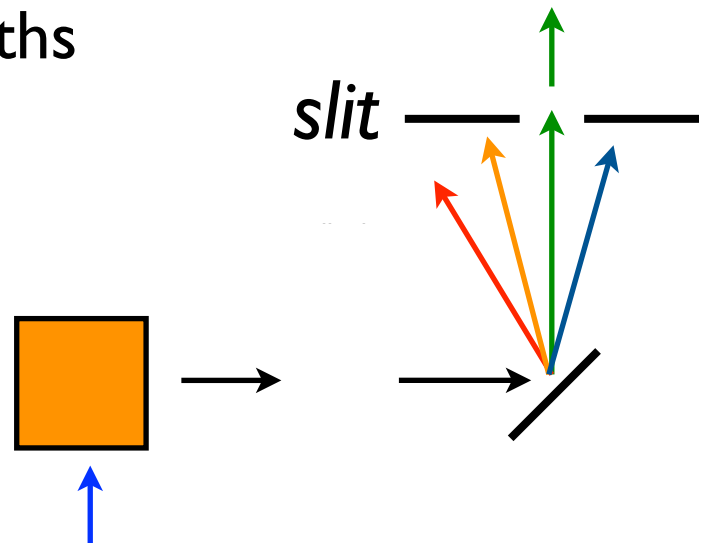


Fluorescence

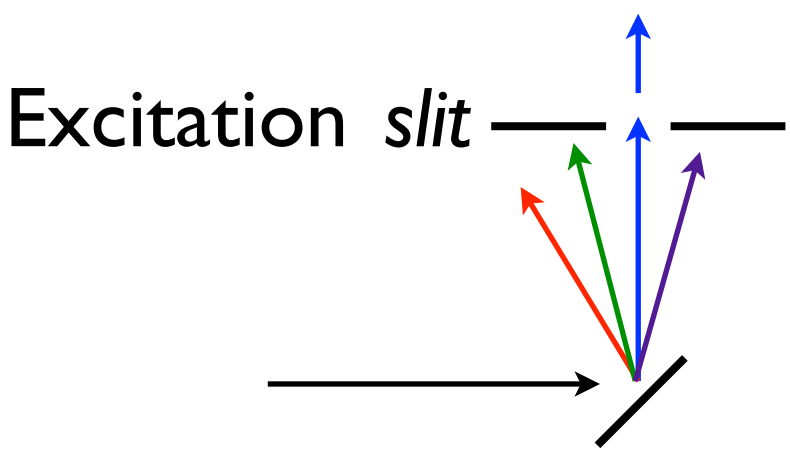
Larger *slit*

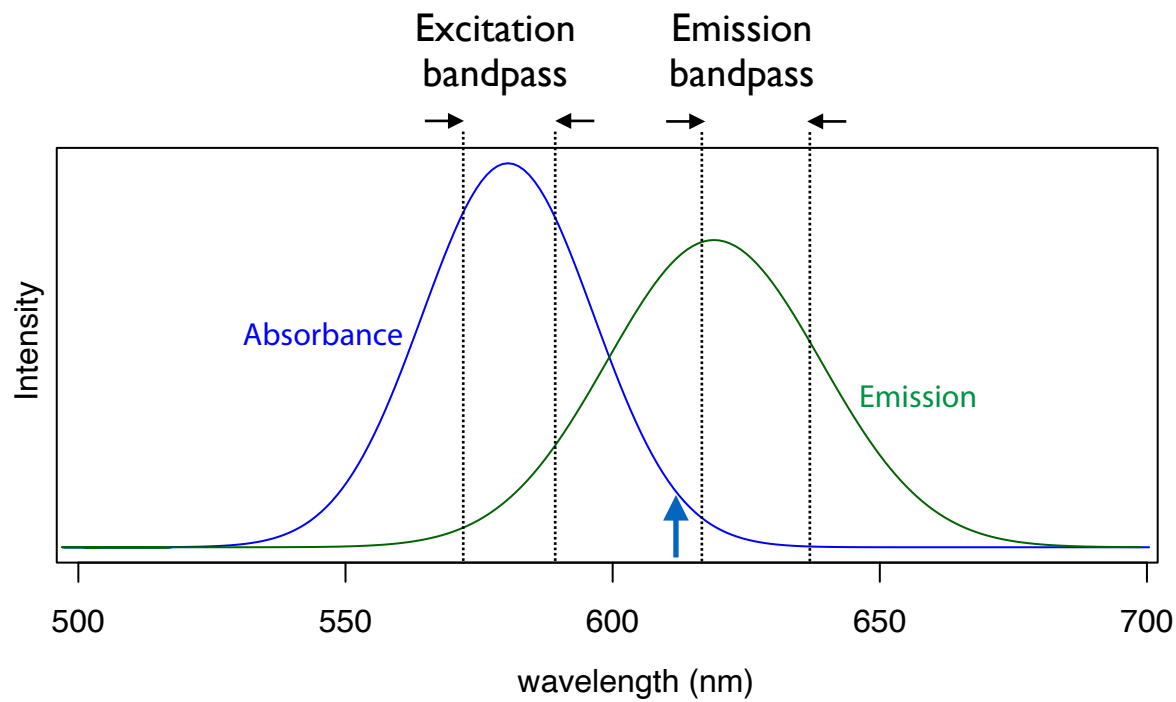
- more light!
- more wavelengths



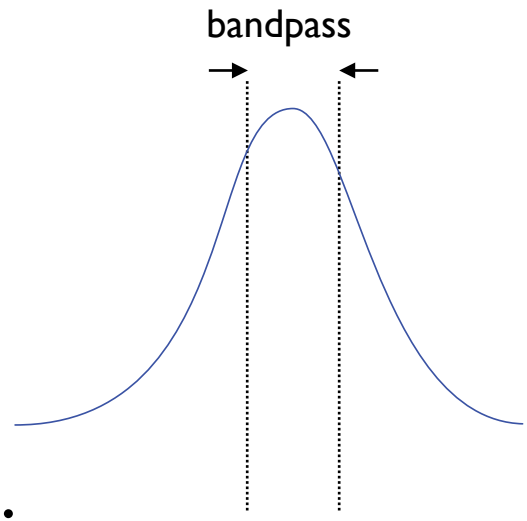


But excitation is MUCH brighter than emission!



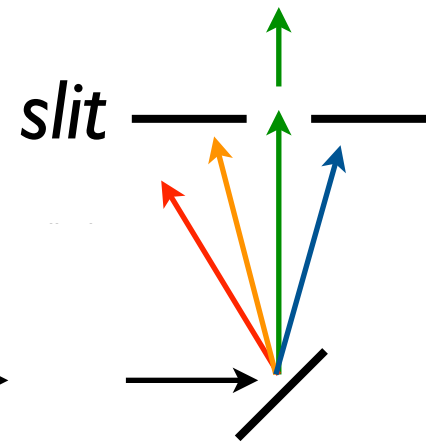


Fluorescence



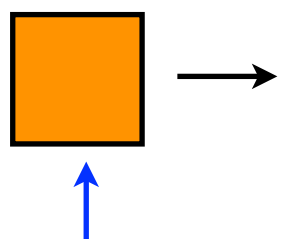
Larger *slit*

- more light!
- more wavelengths

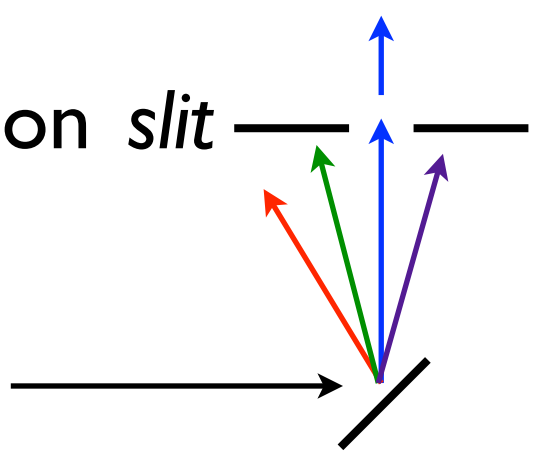


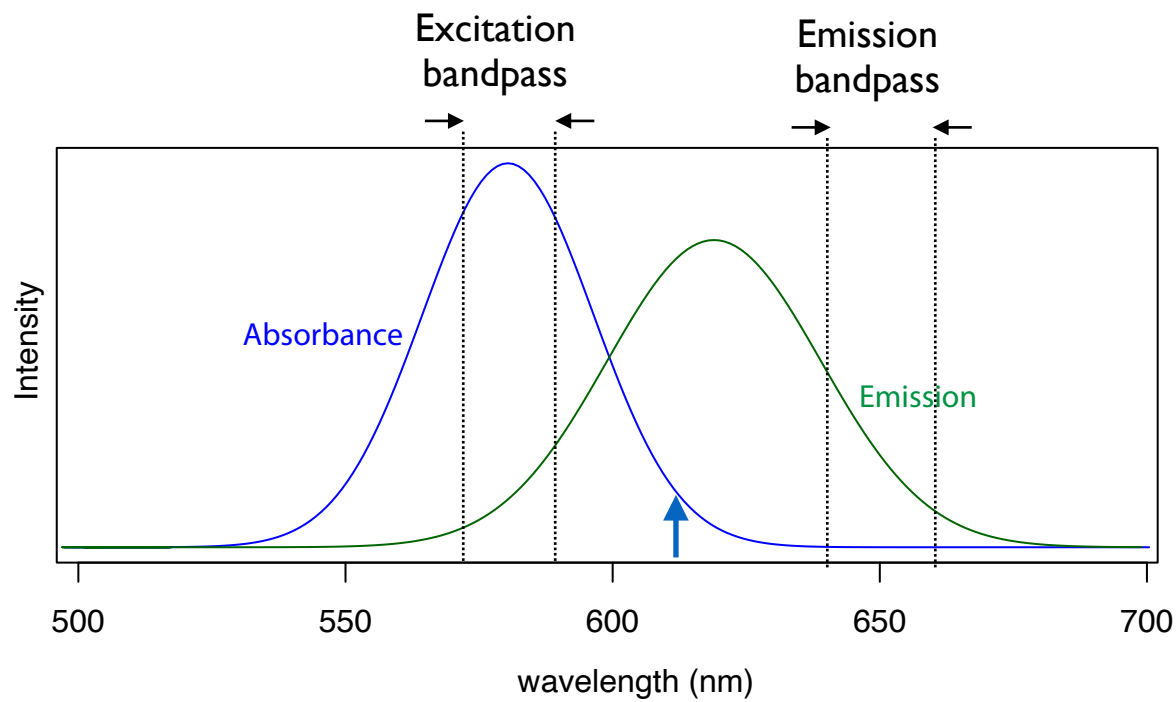
Emission
monochrometer

But excitation is MUCH brighter than emission!



Excitation *slit*



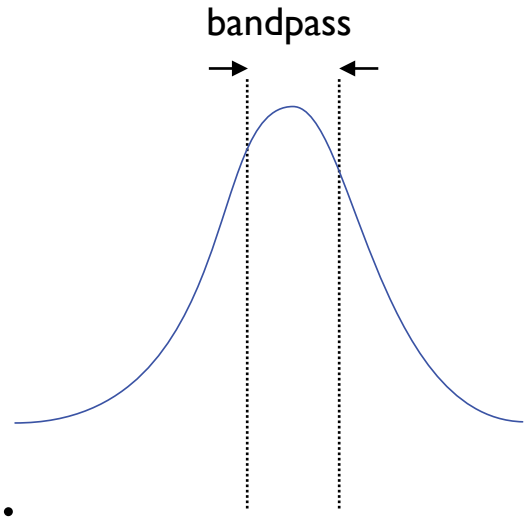


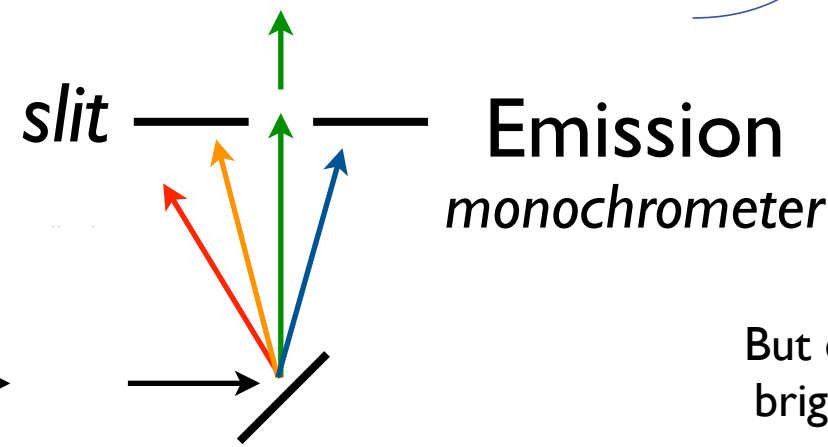
Fluorescence

Questions?

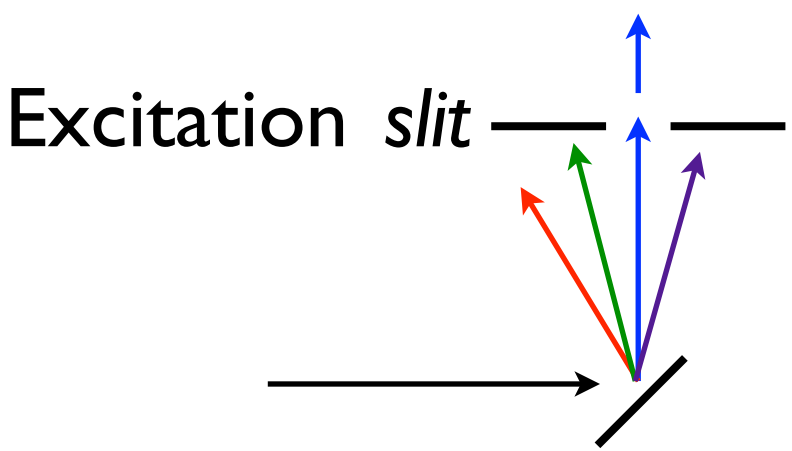
Larger *slit*

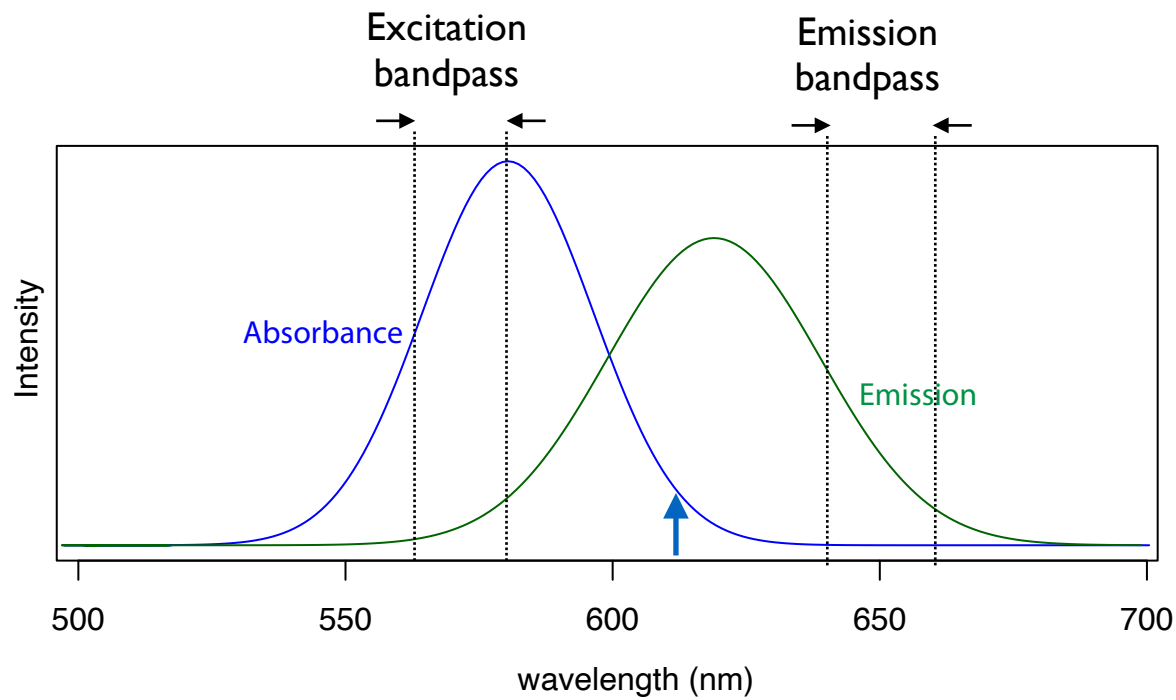
- more light!
- more wavelengths





But excitation is MUCH brighter than emission!

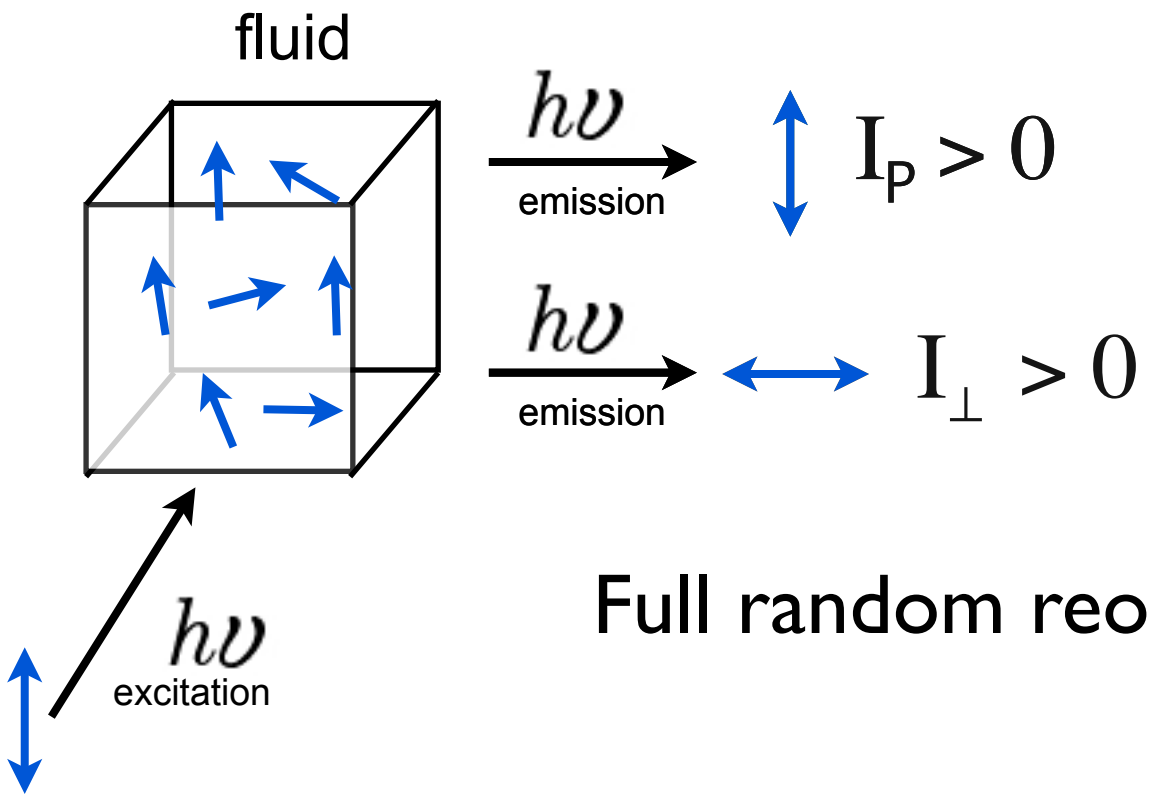




Fluorescence Anisotropy

(fluorescence polarization)

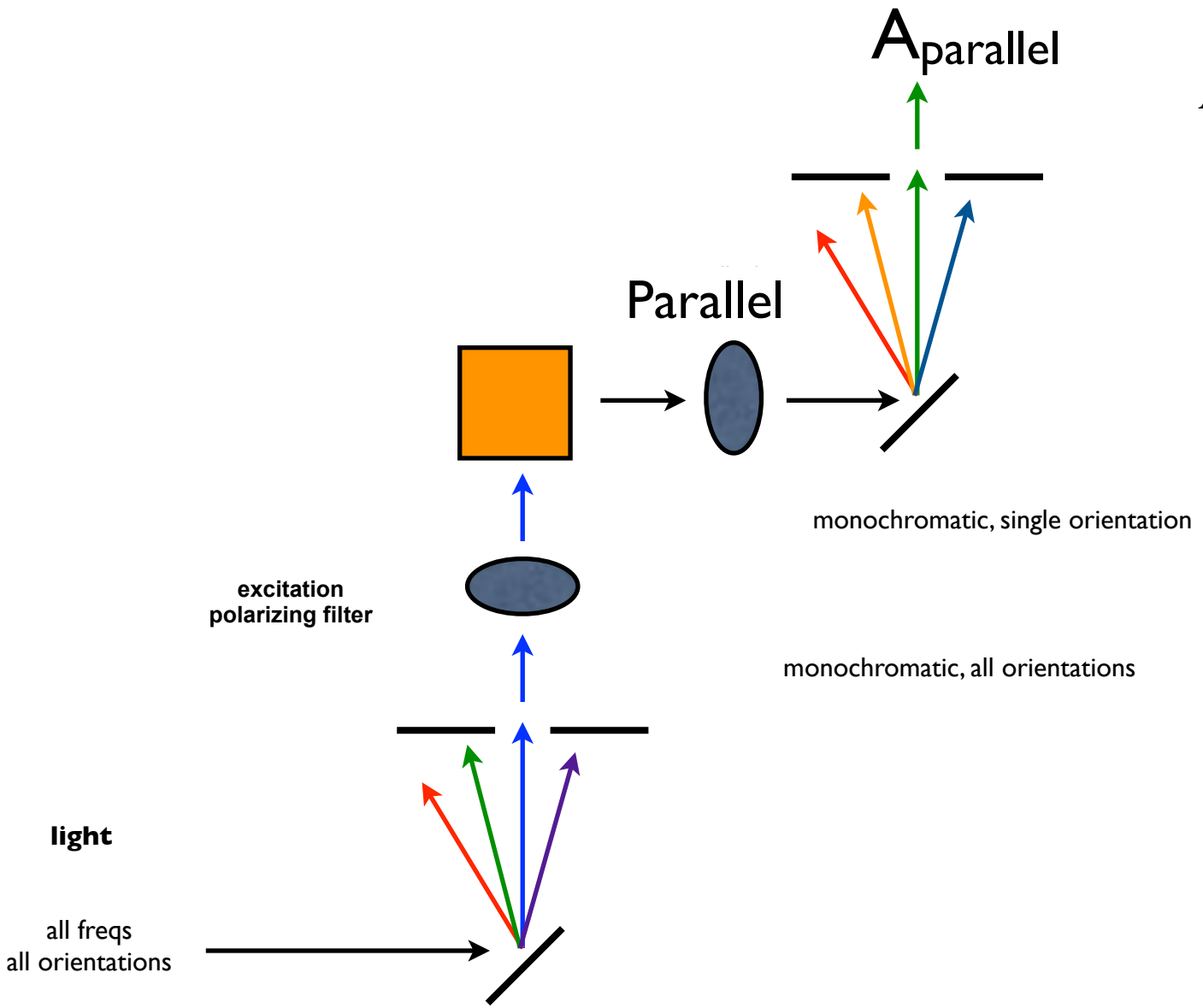
$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



Full random reorientation, A=0

Fluorescence Anisotropy

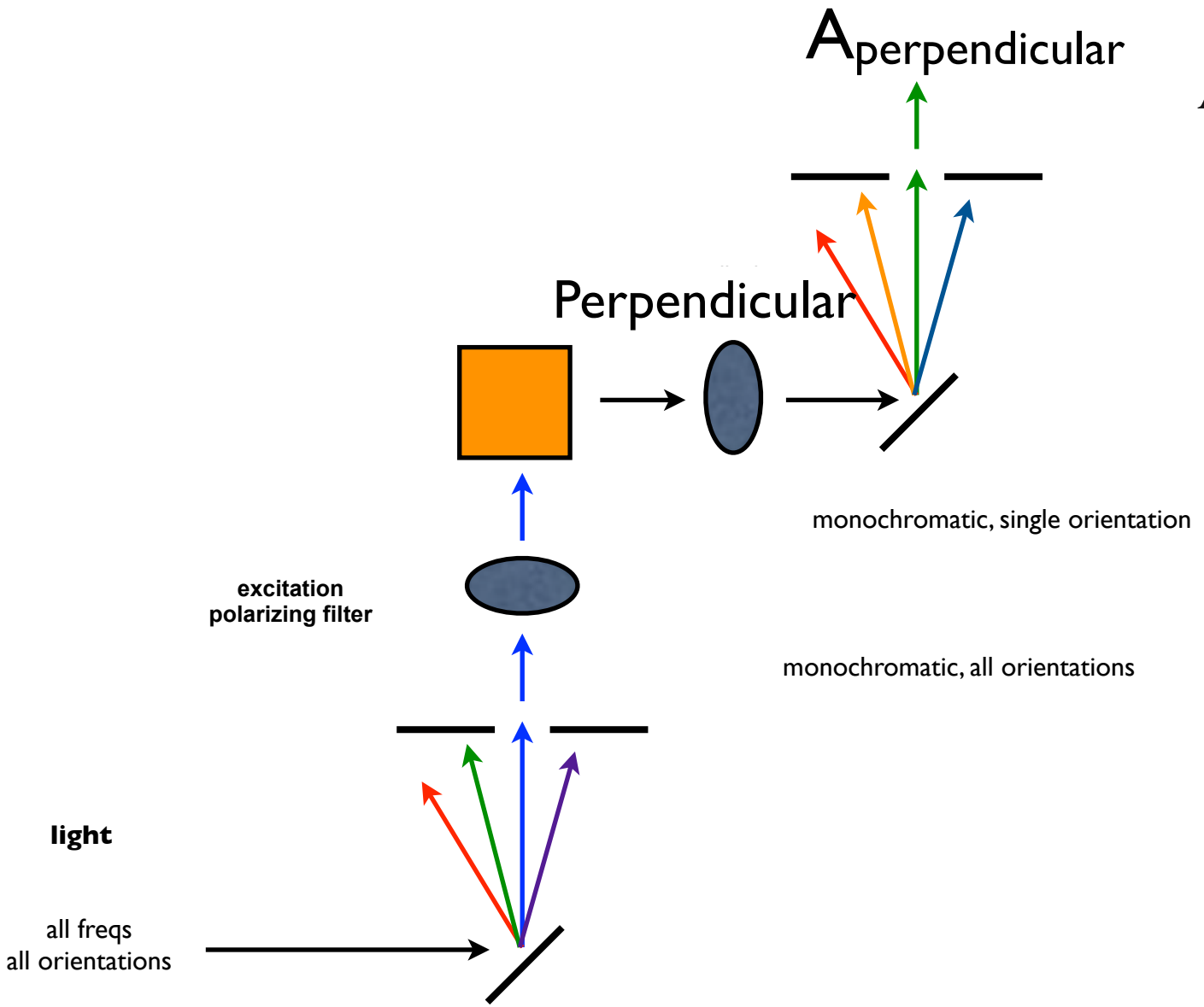
(fluorescence polarization)



$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$

Fluorescence Anisotropy

(fluorescence polarization)



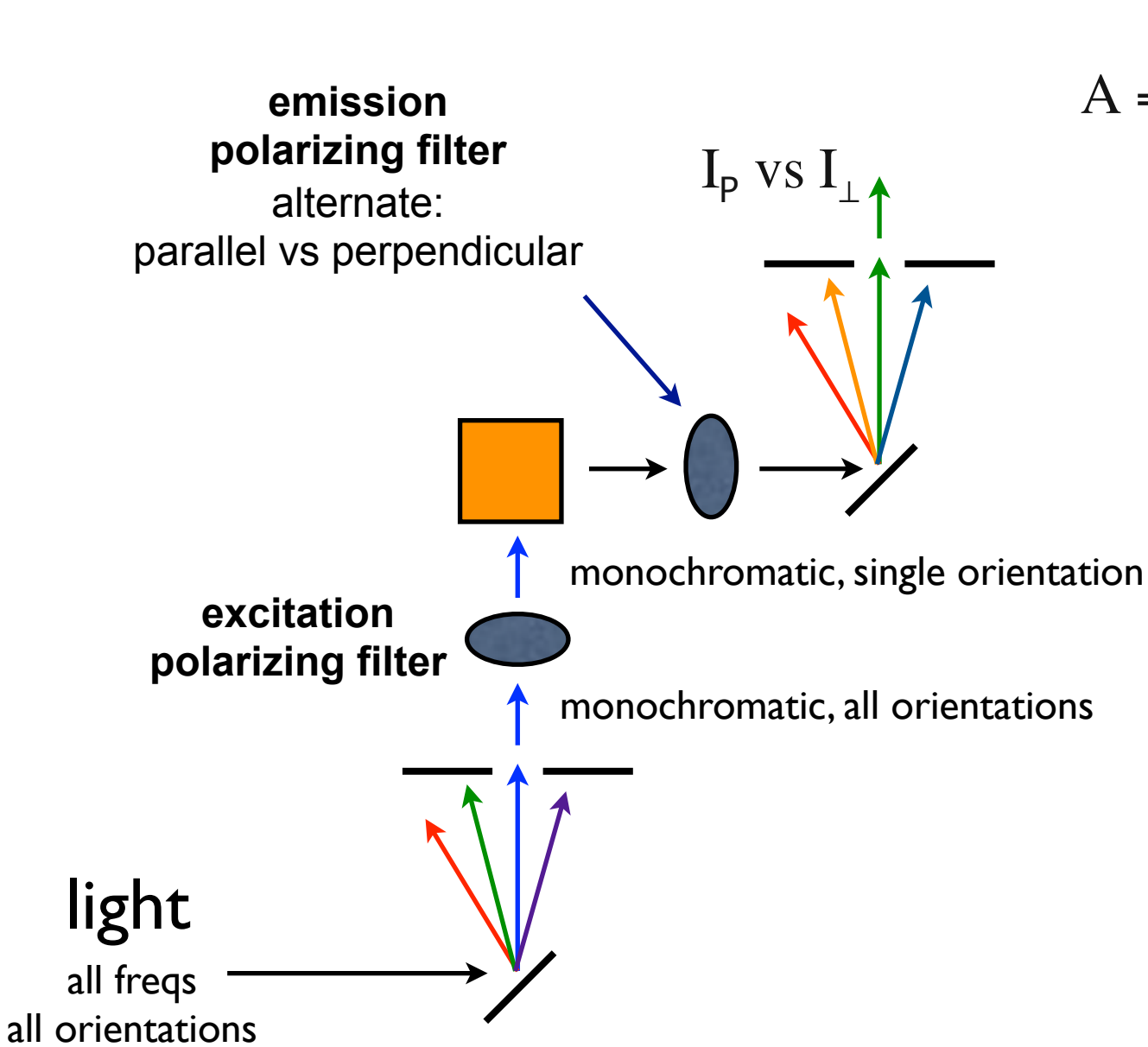
$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$

Fluorescence Anisotropy

(experimental setup)

L-format

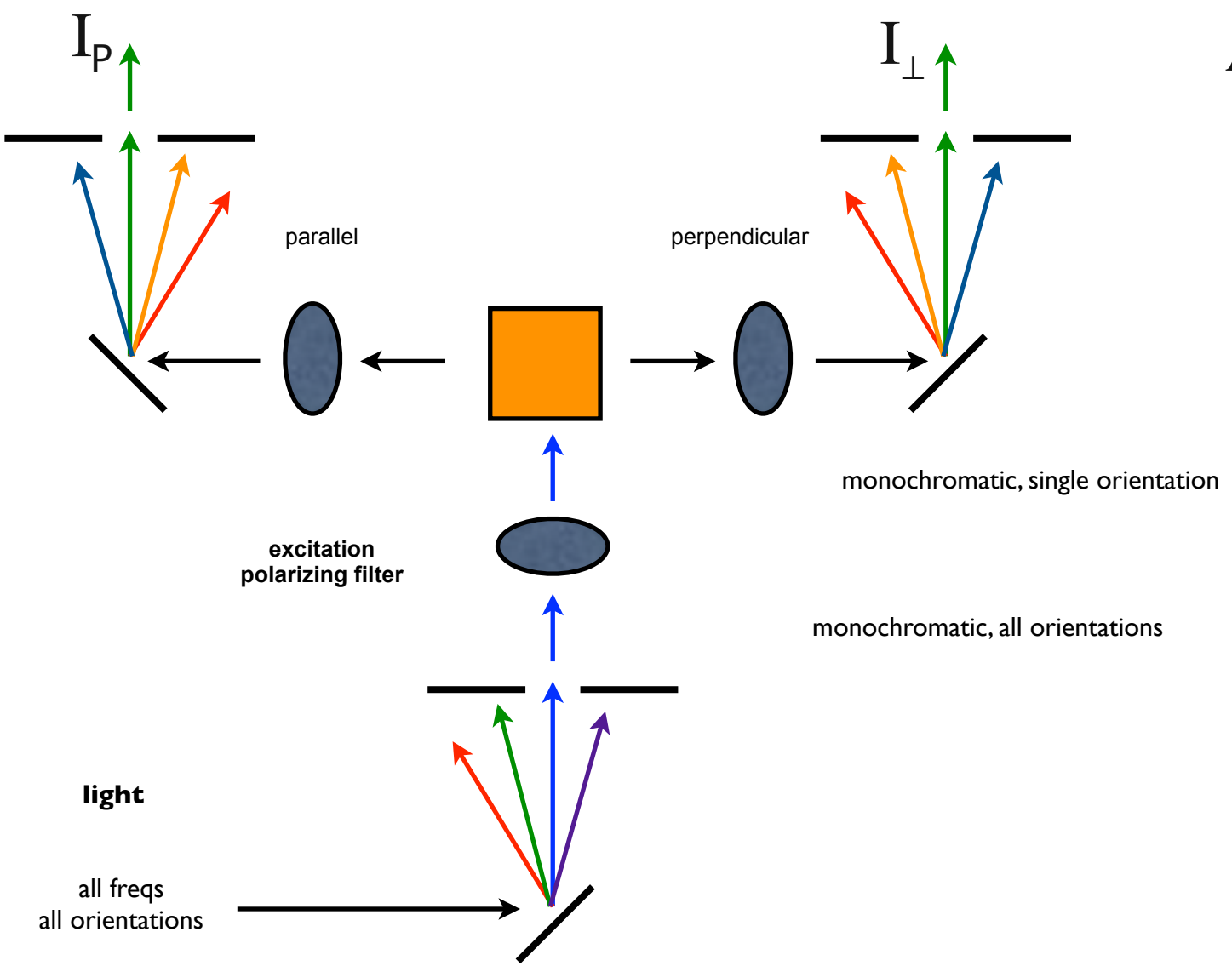
$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



Fluorescence Anisotropy

(fluorescence polarization)

T-format



$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$

Fluorescence Anisotropy

(fluorescence polarization)

$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$

Randomization/rotation - how fast is fast enough?

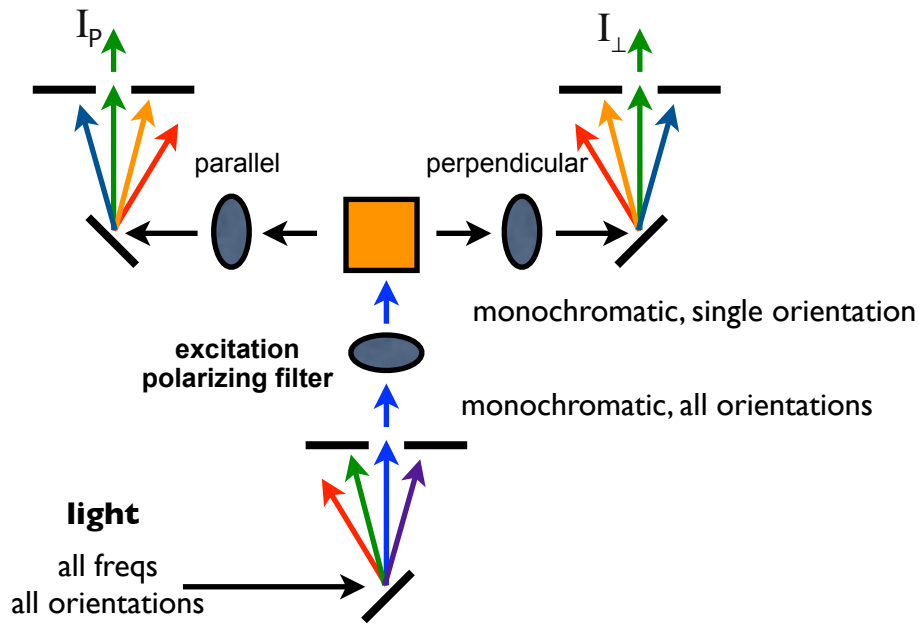
Time scale is relative to how long the molecules stays excited before emitting - their fluorescence lifetimes.

Typically 1-100 nsec

Small protein - reasonable rotation

Large protein - little rotation

Fluorophore connected via -CH_2CH_2- linkage - reasonable rotation



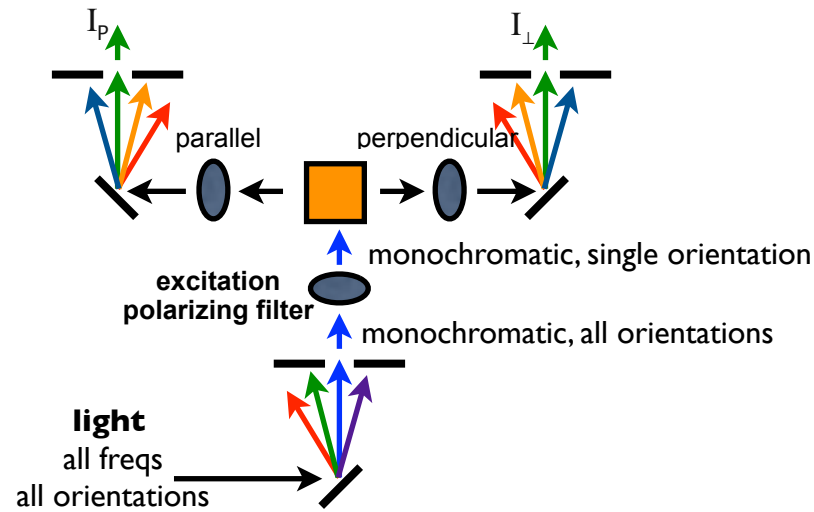
Fluorescence Anisotropy

caveats and uses

Factors effecting anisotropy

- local vs global motions
- FRET - scrambles polarization
  - why?
- light scattering
- misalignment of polarizers
  - problem for absolute measurements
- temperature dependence
- wavelength dependence
- T-format better, but more $$
  - better sensitivity (2X)
  - time dependent measurements

$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



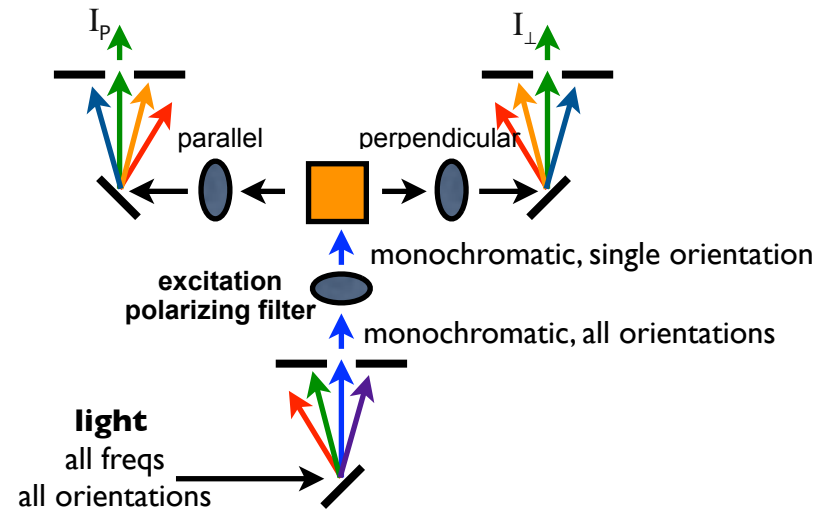
Fluorescence Anisotropy

caveats and uses

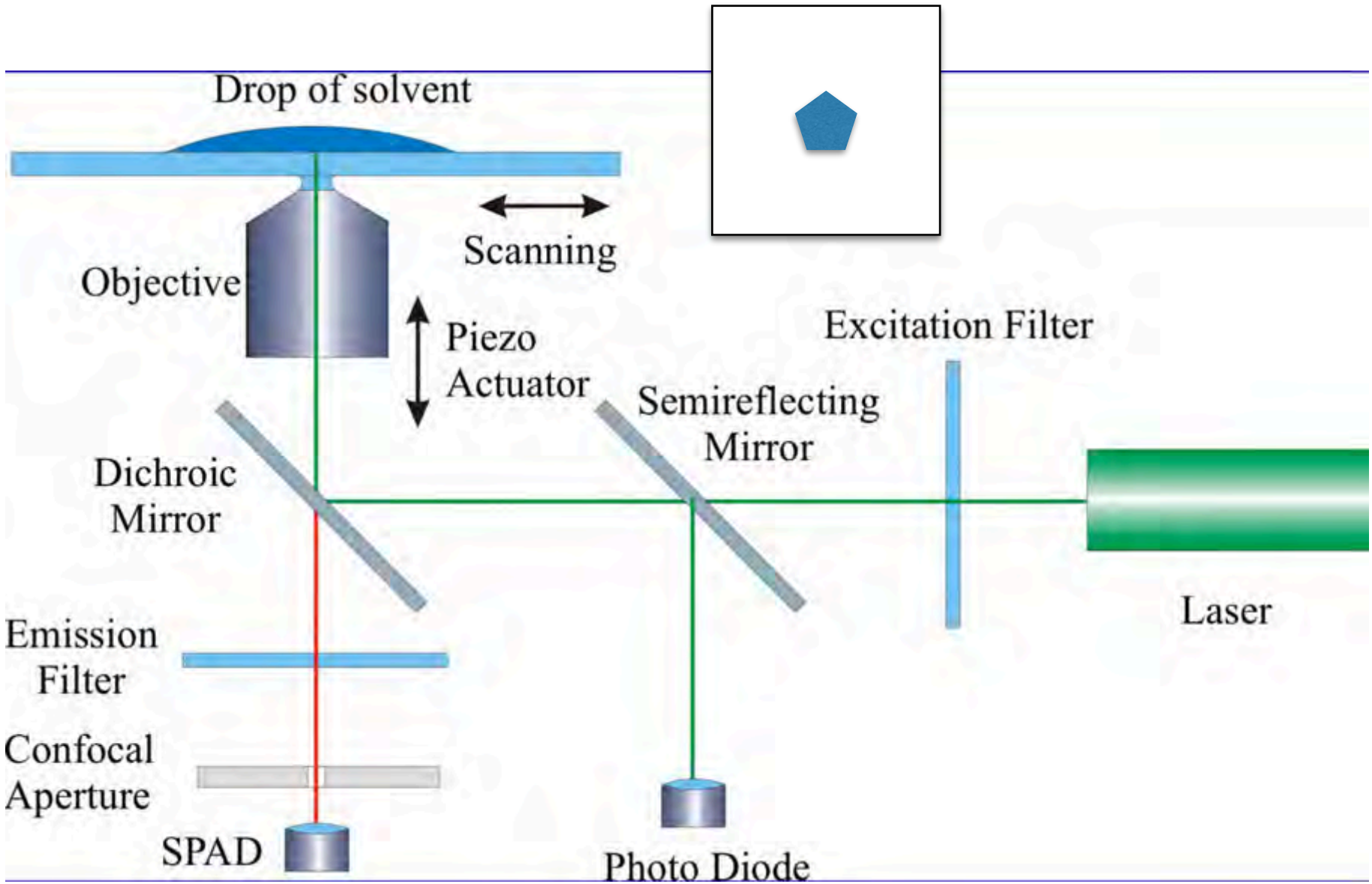
Insights gained

- Measure binding!
  - sometimes
- Measure conformational change
  - sometimes
- A spherical proteins – $r_0/r = 1 + \tau/\theta = 1 + 6D\tau$, where D is the diffusion coefficient
- Rotational correlation time $\theta = \eta V/RT$
- For a single exponential intensity decay
  - $r=r_0/(1+ \tau/\theta)$
- Can calculate anisotropy of labeled proteins in solution
  - with caveats
- $\theta = \eta V/RT = \theta = [\eta M/RT](v+h)$

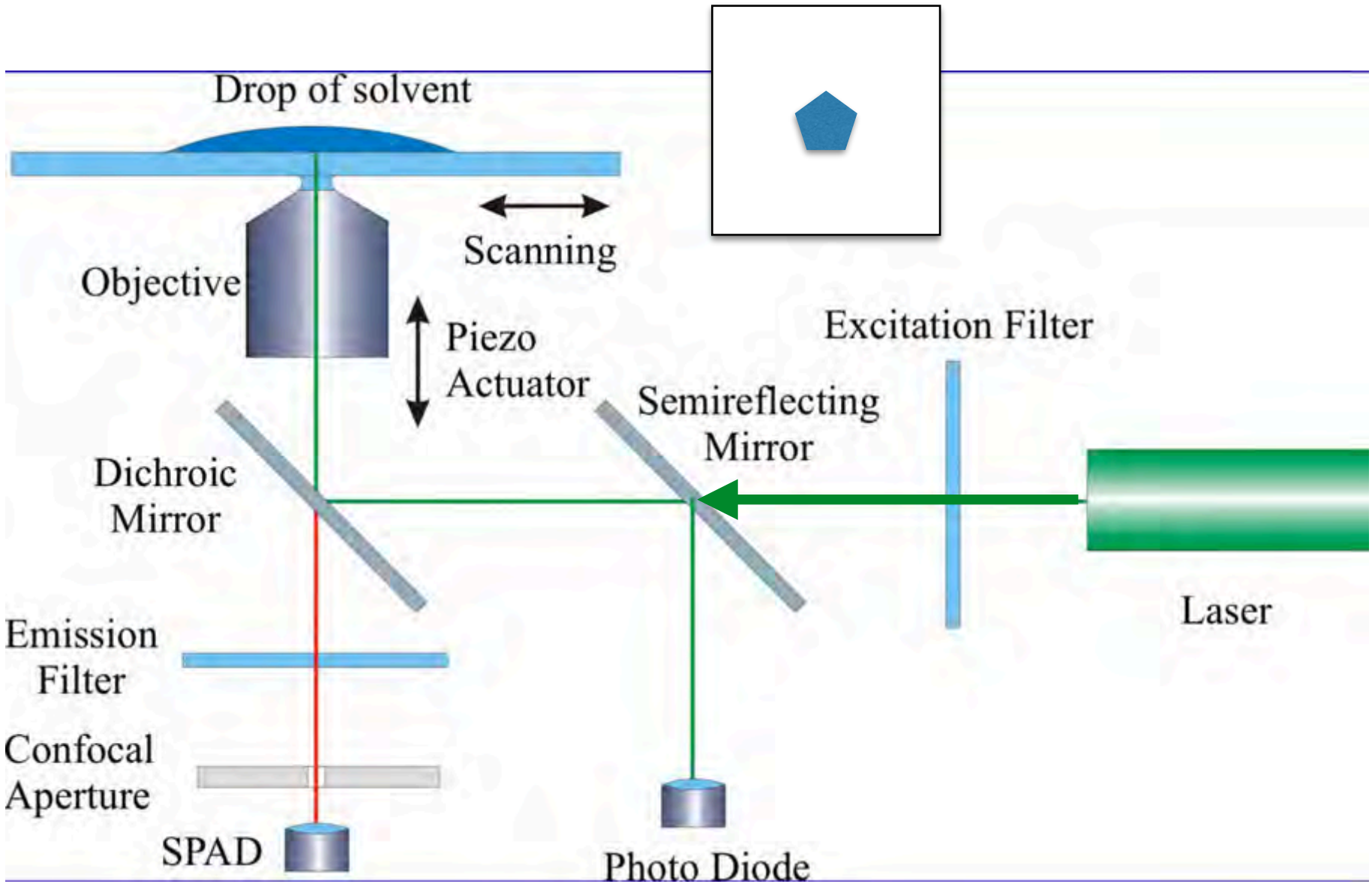
$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



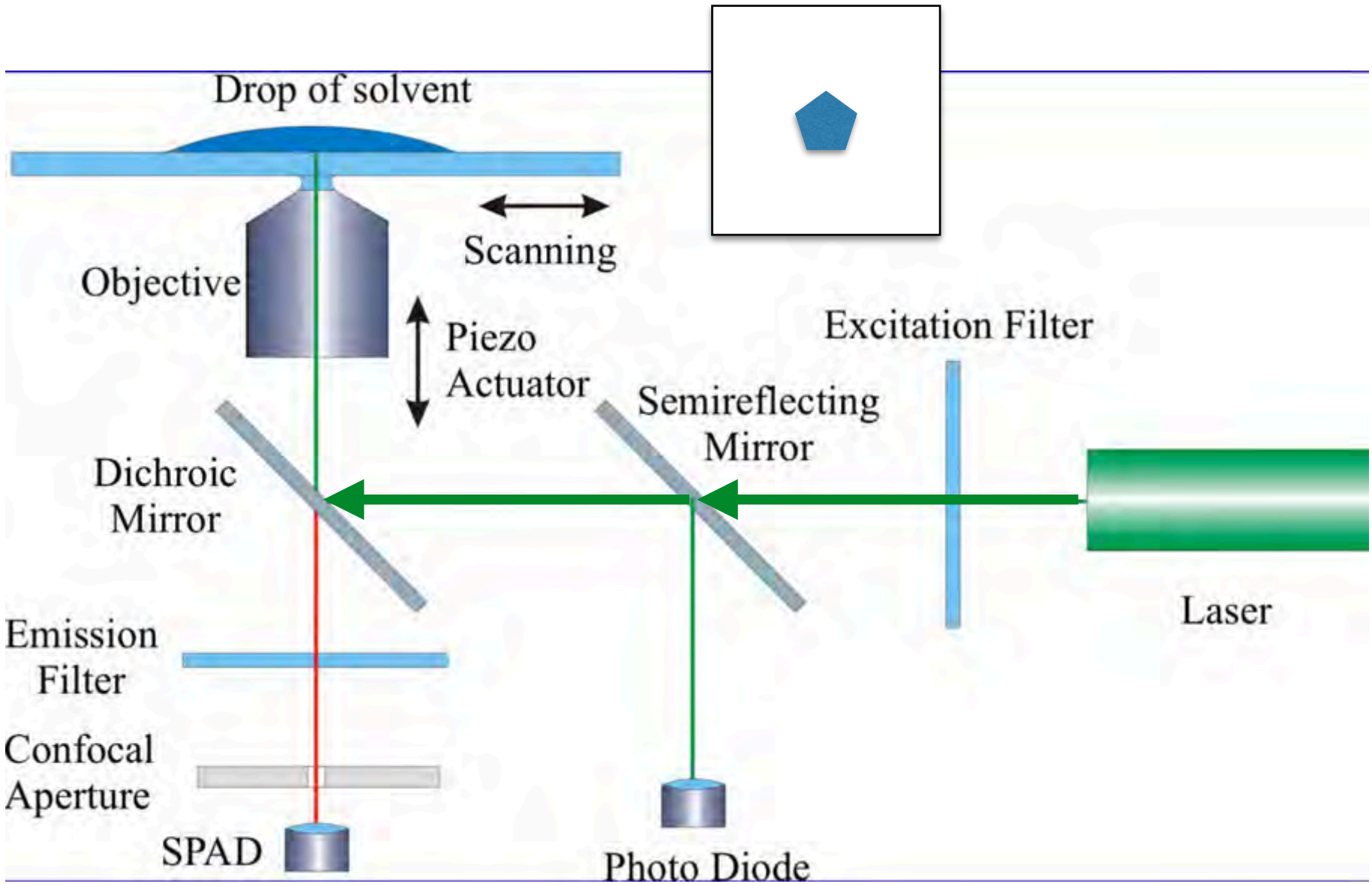
Single Molecule Fluorescence



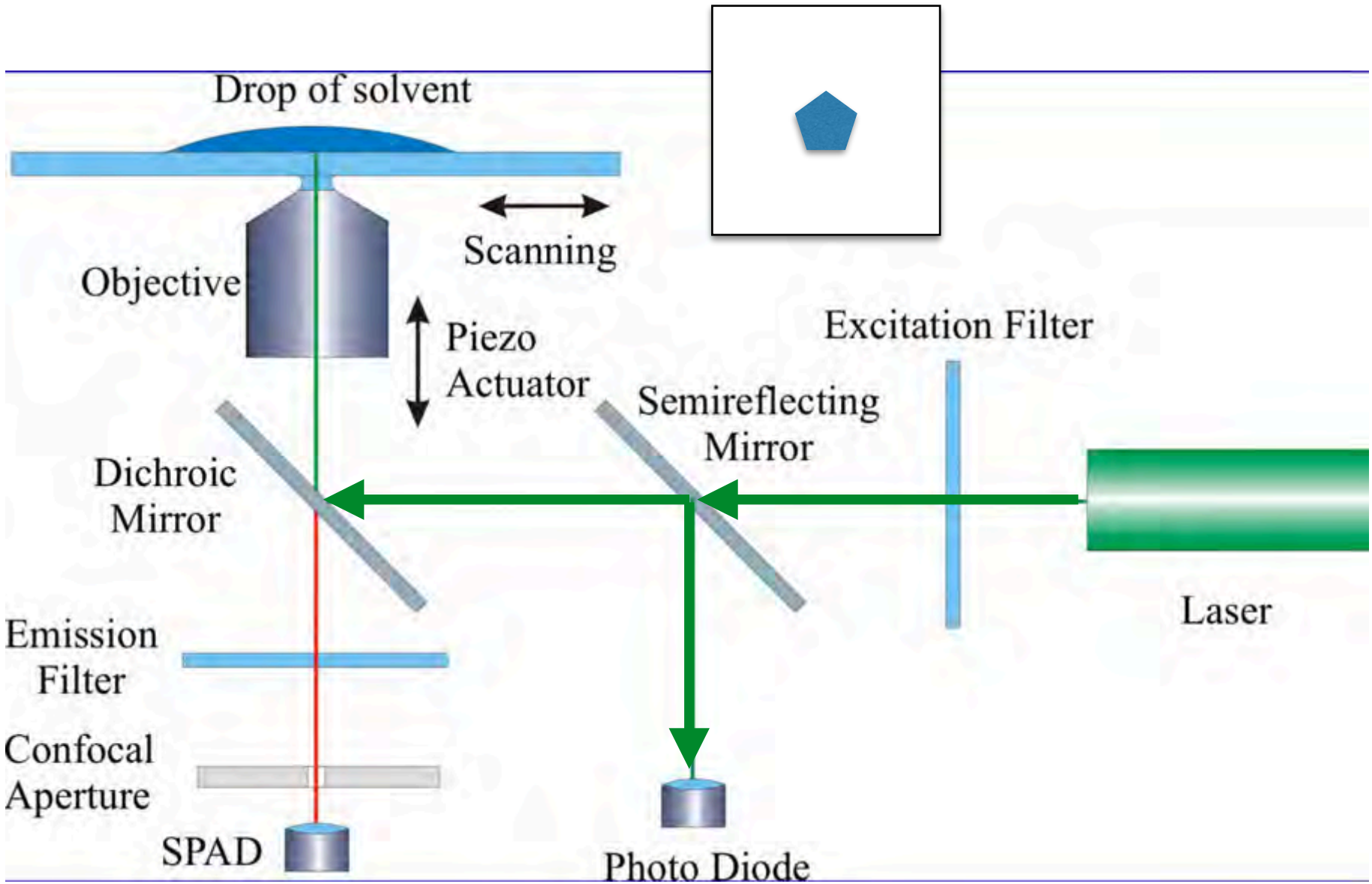
Single Molecule Fluorescence



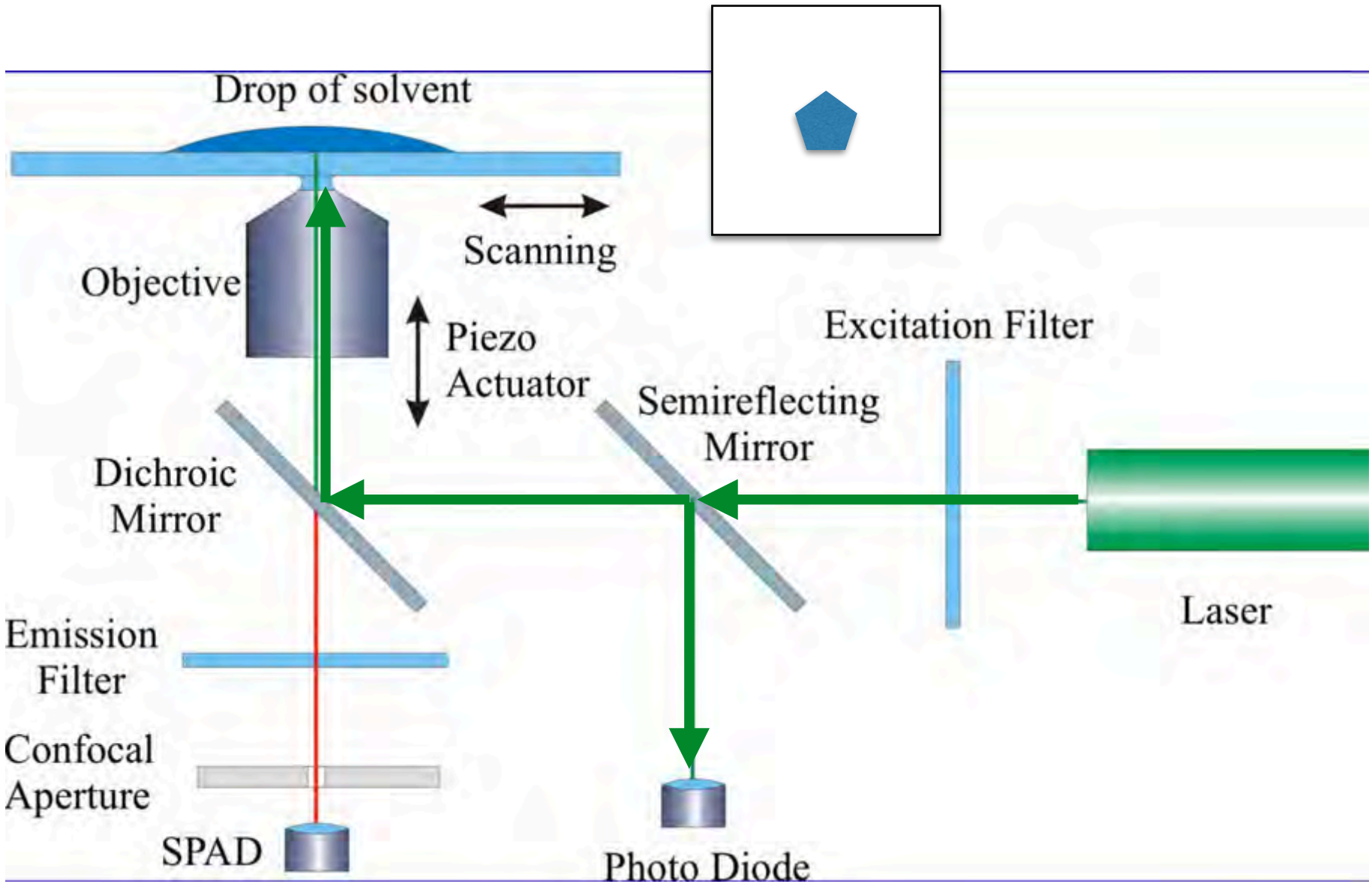
Single Molecule Fluorescence



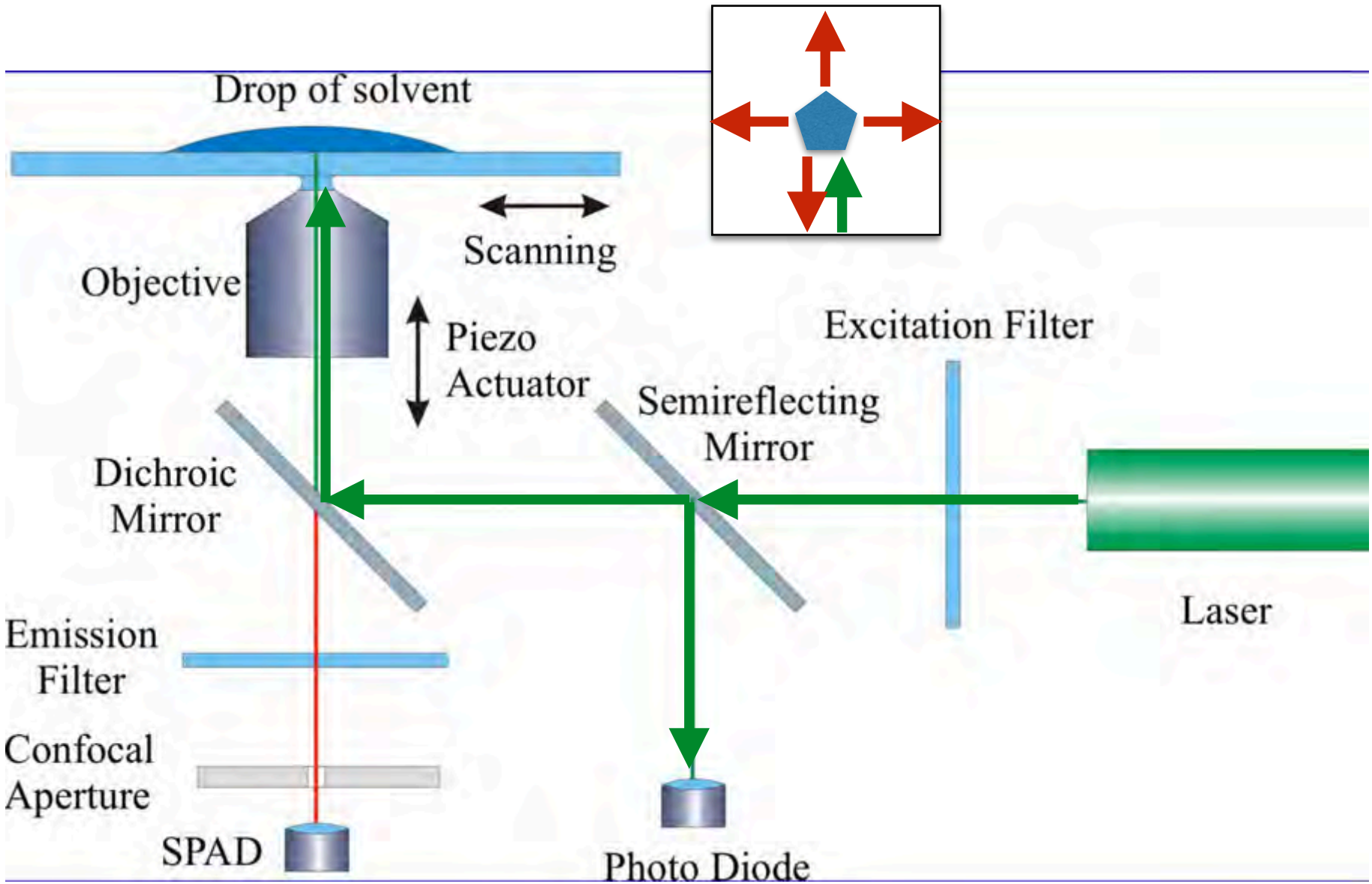
Single Molecule Fluorescence



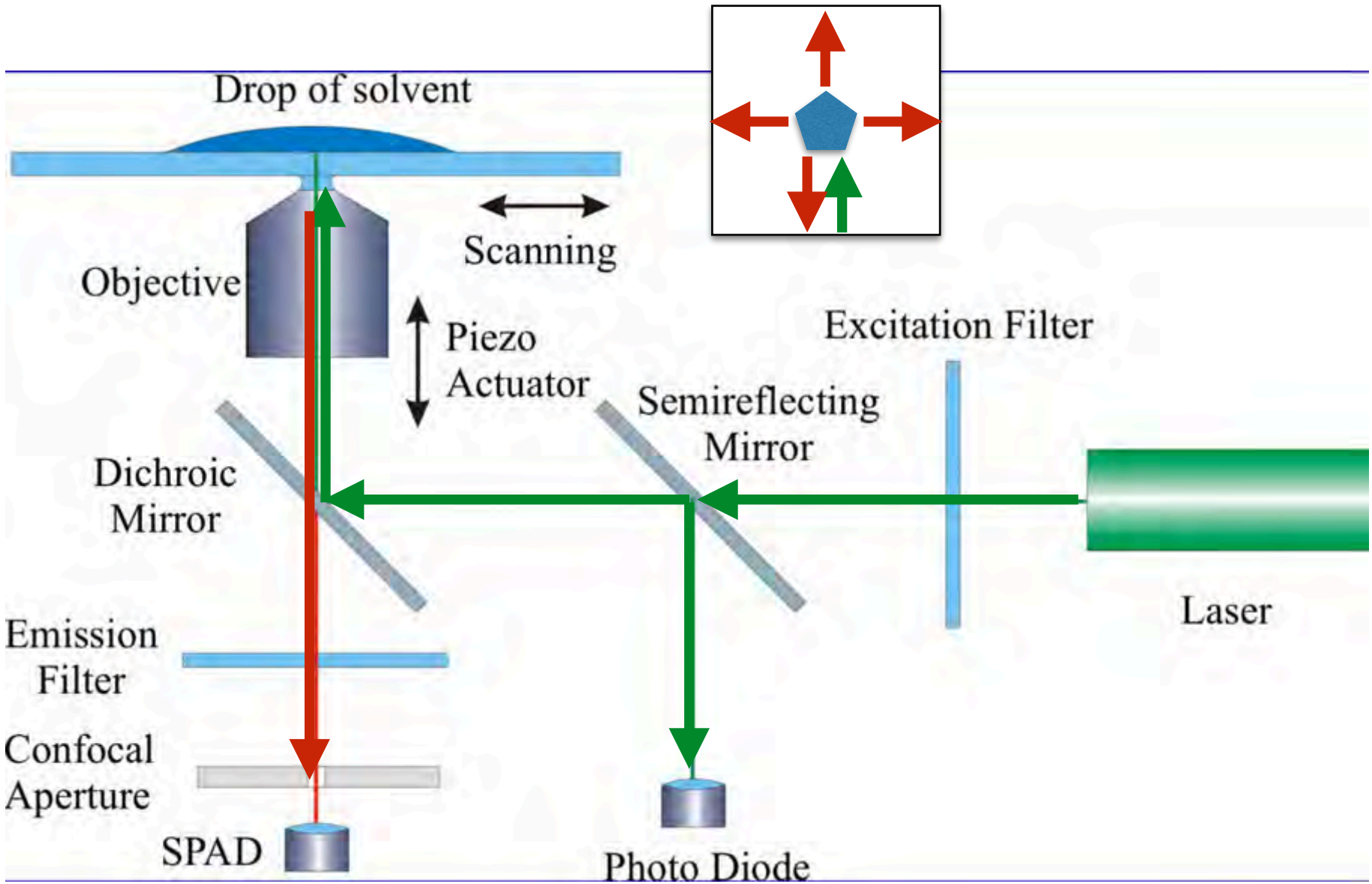
Single Molecule Fluorescence



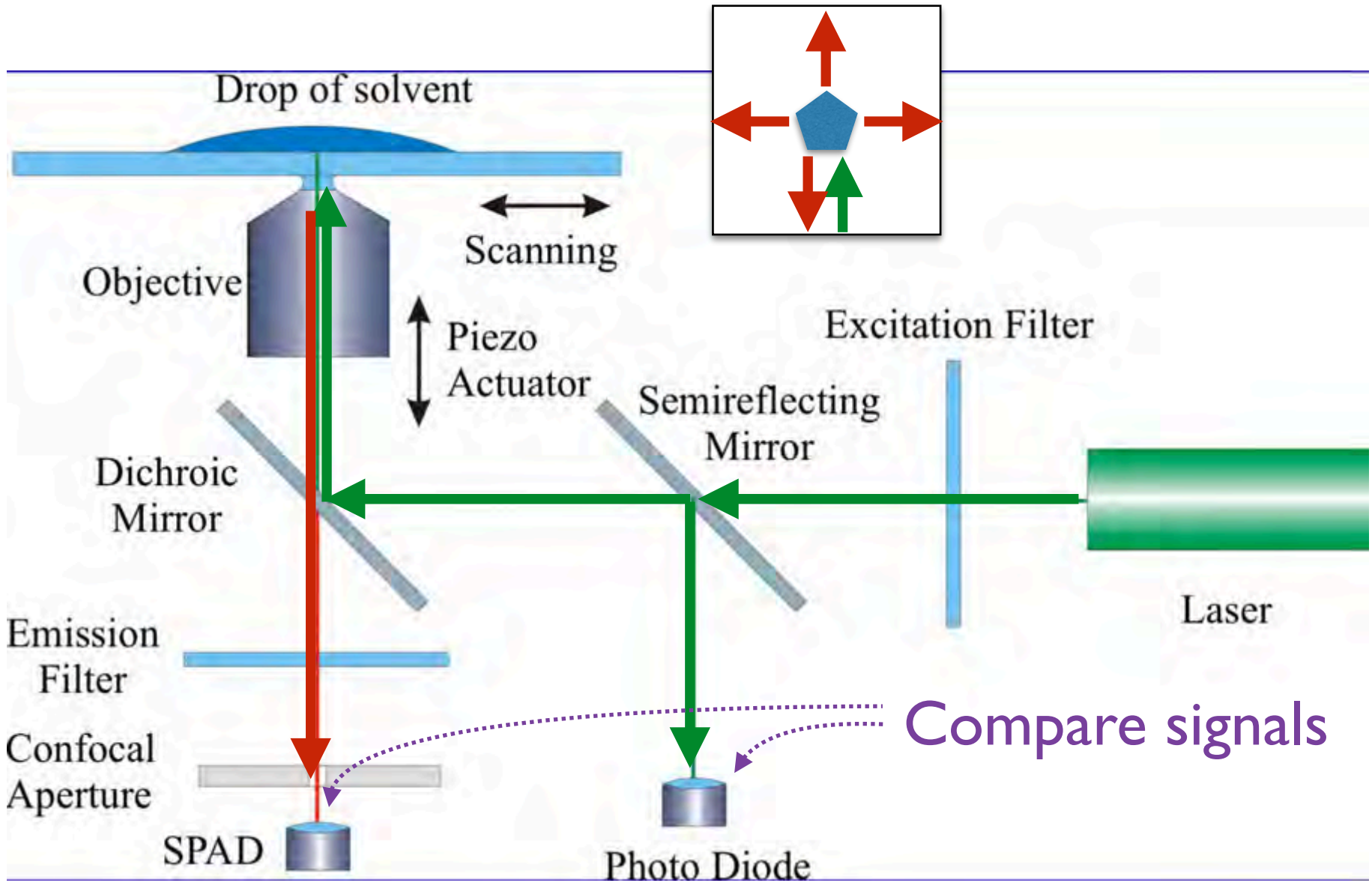
Single Molecule Fluorescence



Single Molecule Fluorescence



Single Molecule Fluorescence

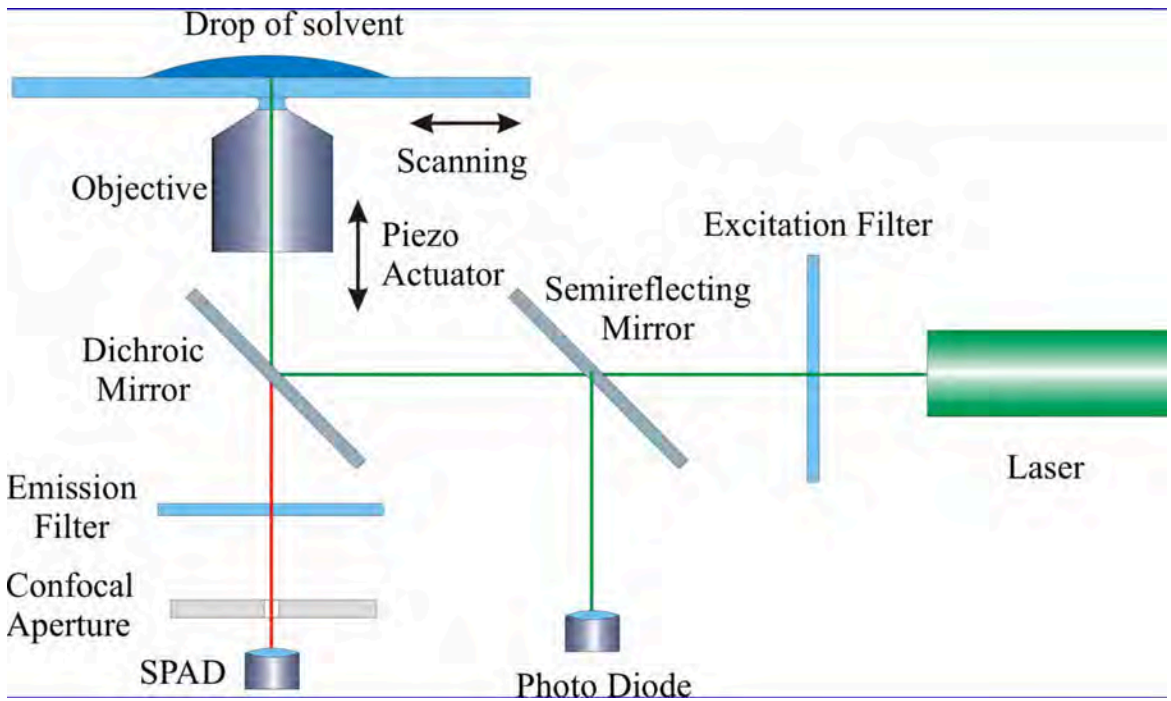


single photon avalanche detector

Single Molecule Fluorescence

Why a microscope?

- narrow x-y area
- TIRF allows thin layer (z)
- small observation volume ≤ fL (10^{-15} L)



Single Molecule Fluorescence

Wide field microscope - Dual color

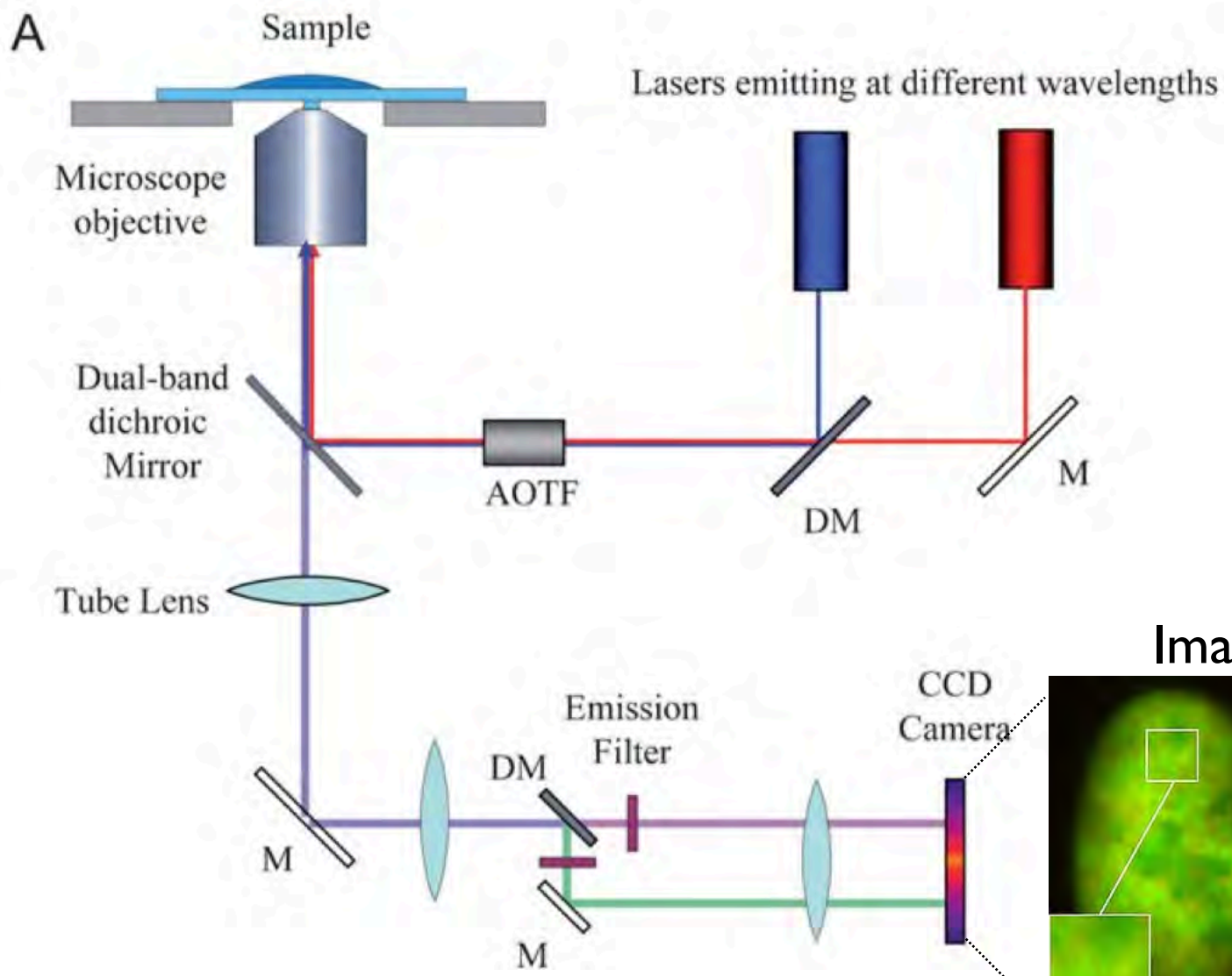
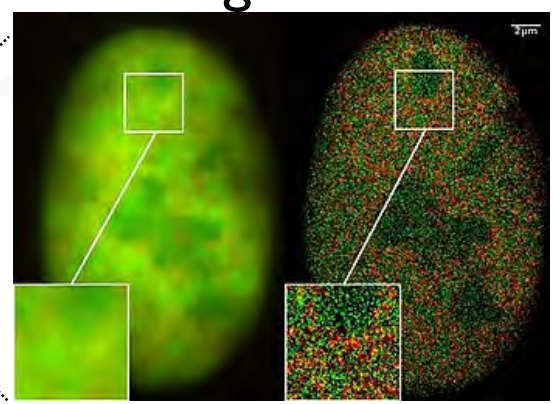
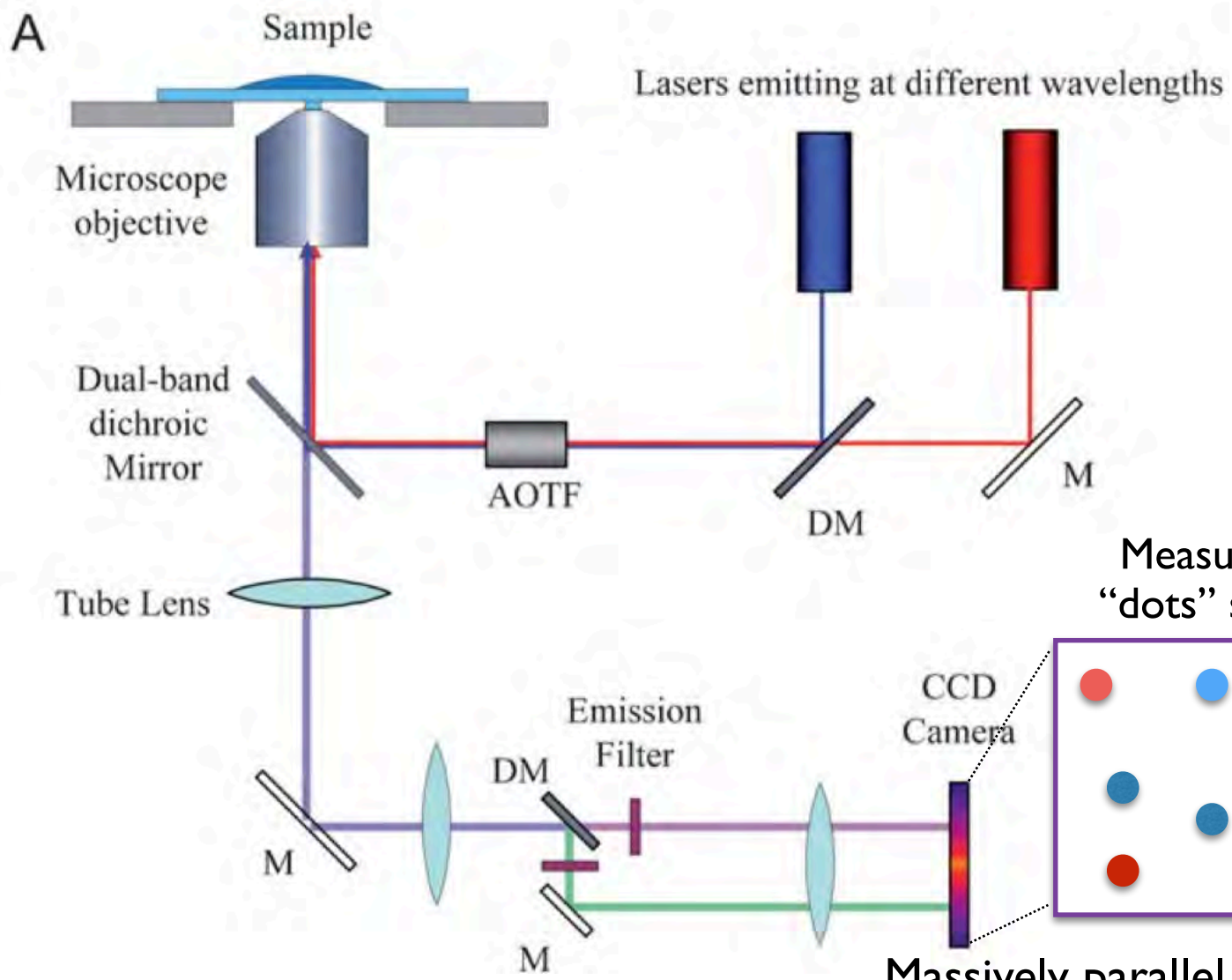


Image a cell



Single Molecule Fluorescence

Wide field microscope - Dual color



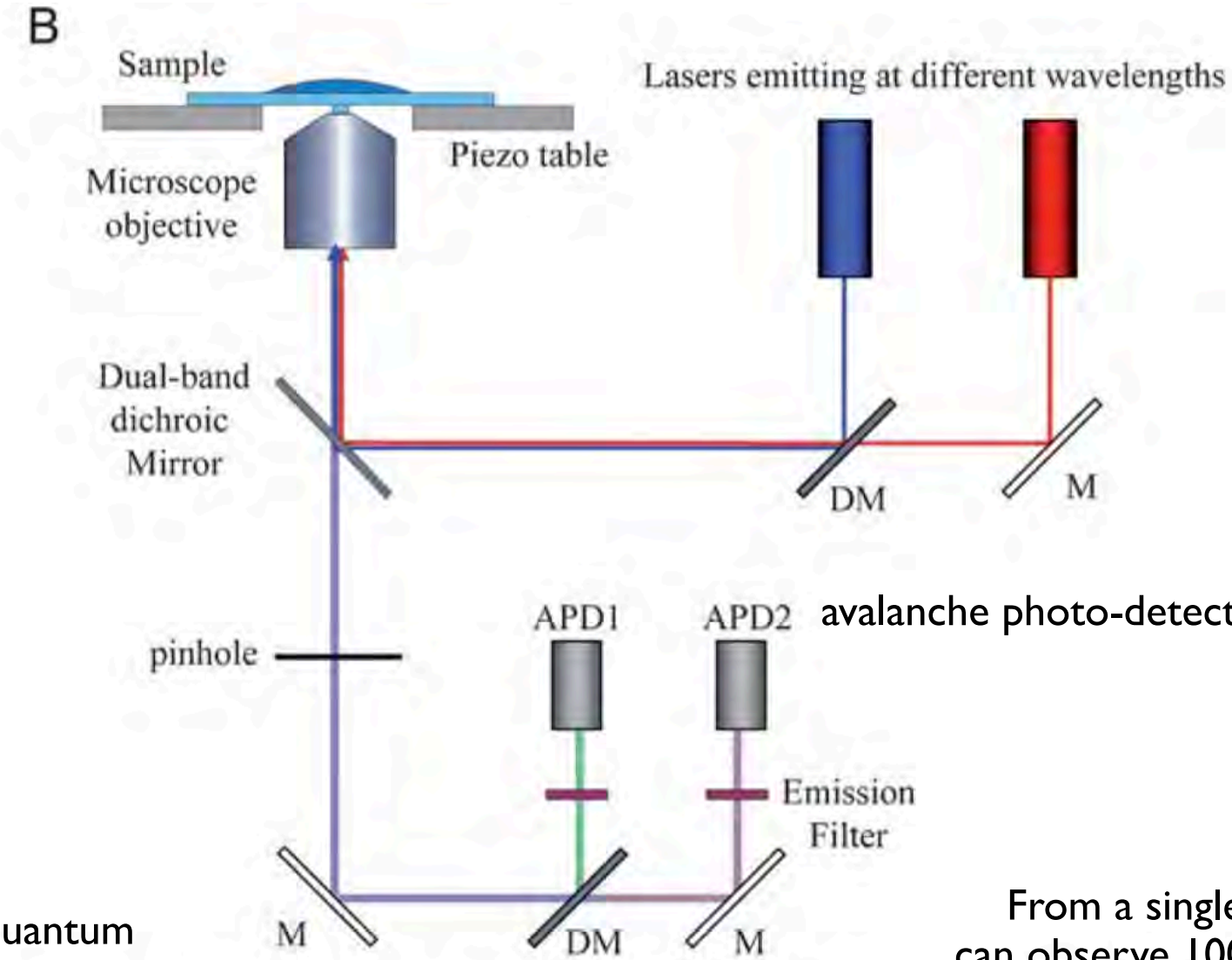
Measure millions of "dots" simultaneously

Massively parallel data collection (lifetimes, anisotropy, FRETm etc)

AOTF = acousto-optical tunable filter

Single Molecule Fluorescence

Confocal Microscope - decreases background signal



Desirable: quantum yields of 0.8-0.9

From a single molecule, can observe 100,000-200,000 photons per second

Single Molecule Fluorescence

Challenges in solution

Diffusion:

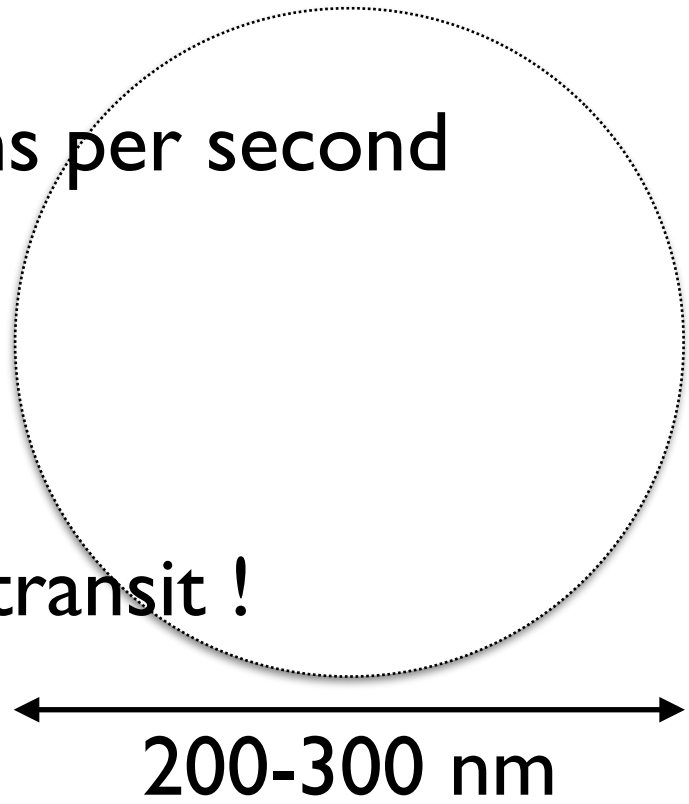
Transit time ≈50 μs

100,000-200,000 photons per second

5-10 photons / transit !

Transit time ≈5 ms

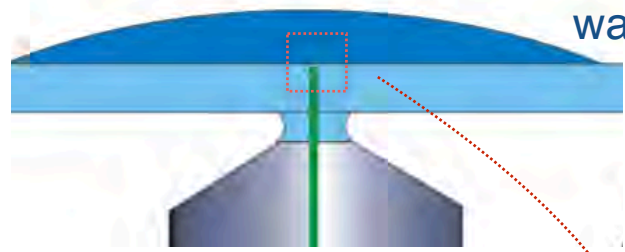
5,000-10,000 photons / transit !



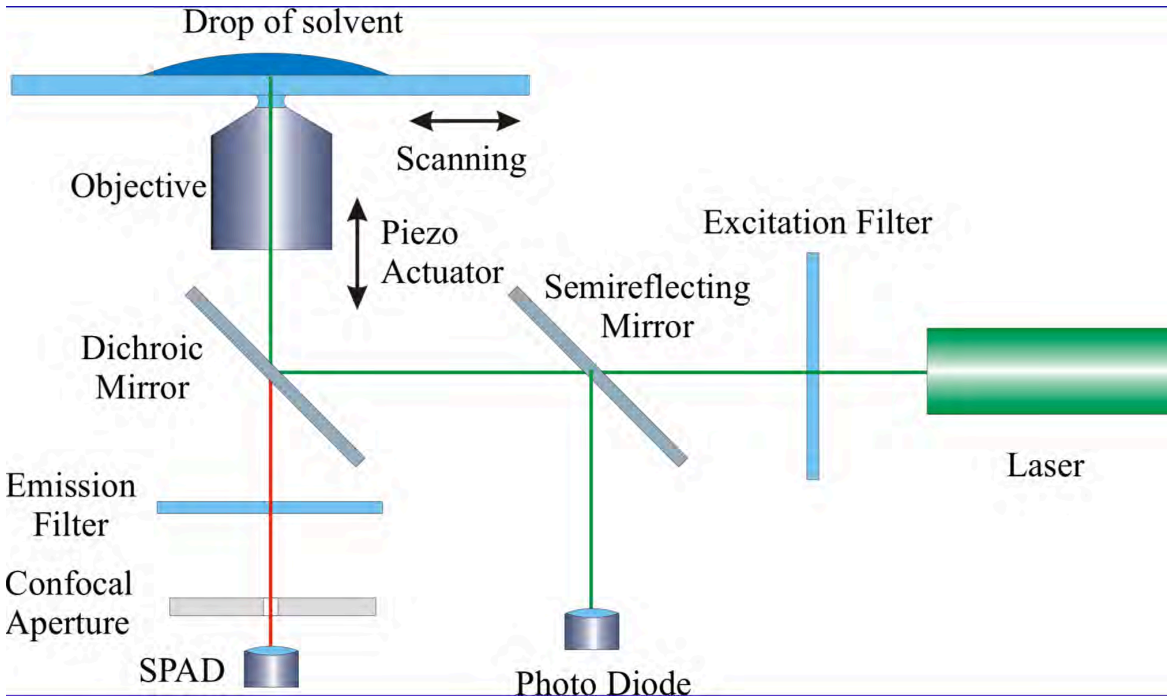
Towards Single Molecule Fluorescence

Why a microscope?

- narrow x-y area
- TIRF allows thin layer (z)
- small observation volume ≤ fL (10^{-15} L)

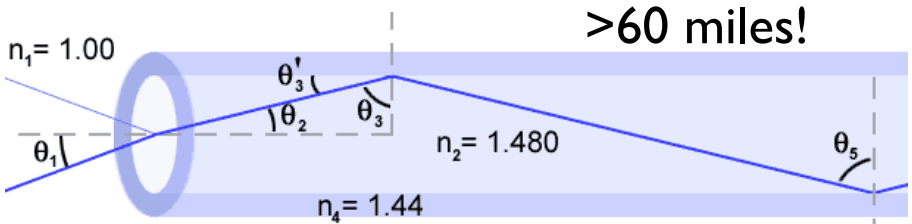


How can we limit fluorescence only from things very close to the surface?

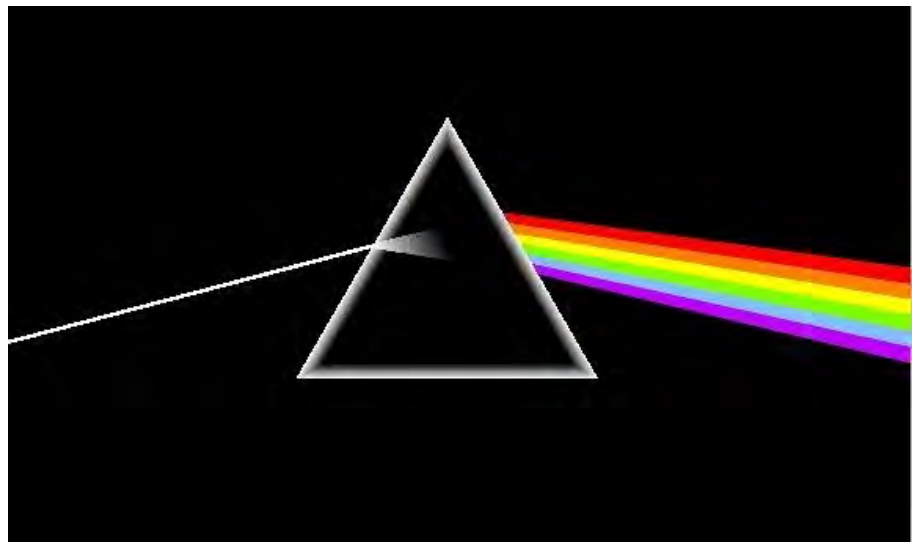


Refraction of Light

Media with different refractive indices



What common technology these days uses this?

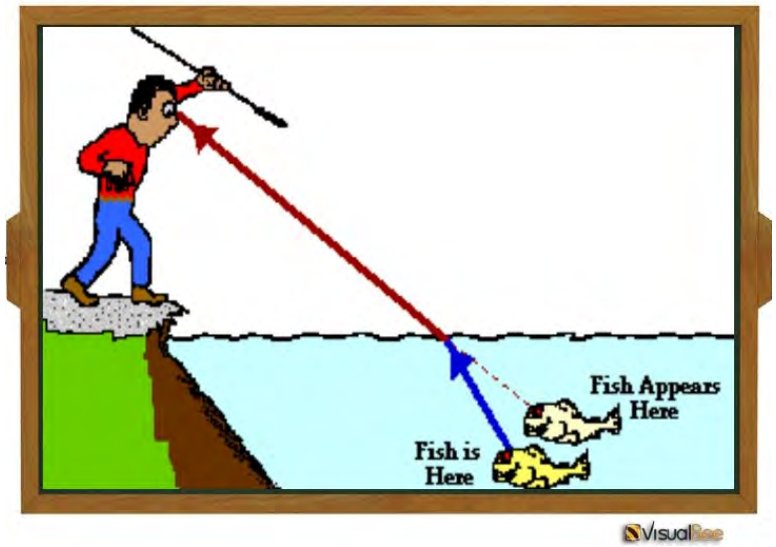


faster medium

glass w different refractive index

slower medium

Quartz



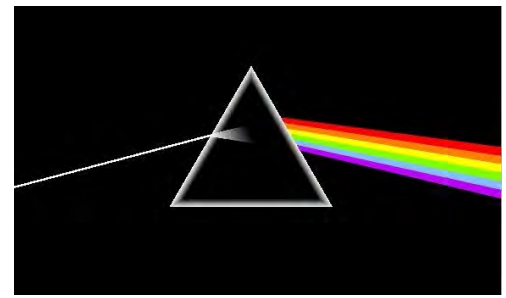
Refraction of Light

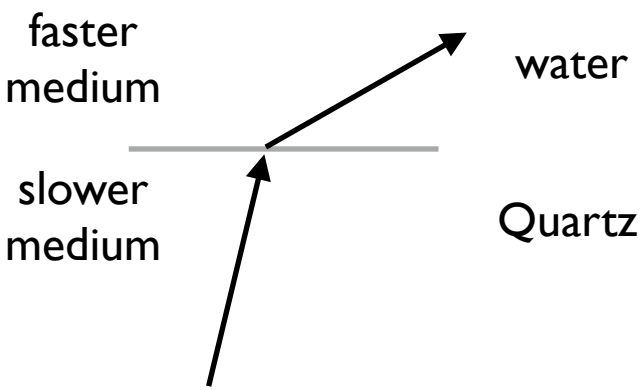
Media with different refractive indices

$$n_{vacuum} = 1.0\overline{0}$$

$$n_{air} = 1.00029$$

$$n_{water}^{20°C} = 1.333$$





index of refraction $n = \frac{c}{v}$

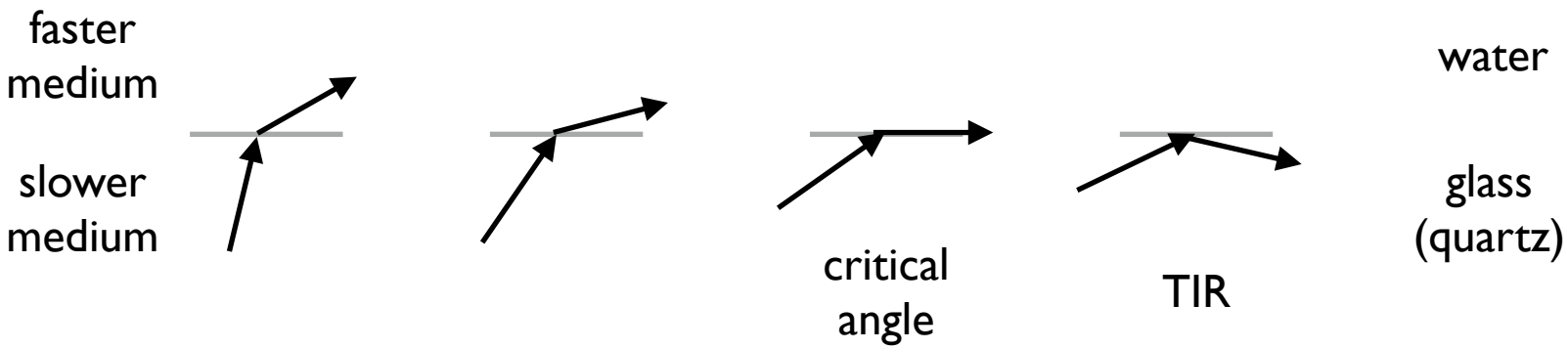
speed of light in a vacuum

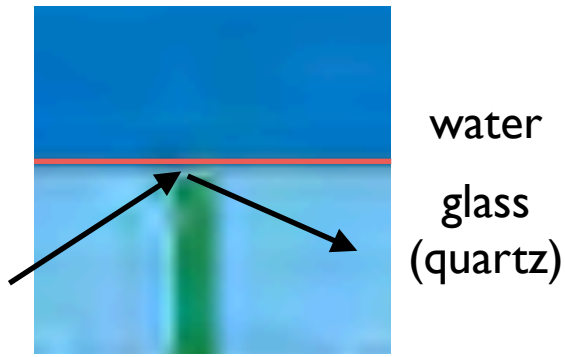
phase velocity of light in the medium

For a detailed explanation of phase velocity and refractive index, see Wikipedia

Total Internal Reflectance

Media with different refractive indices



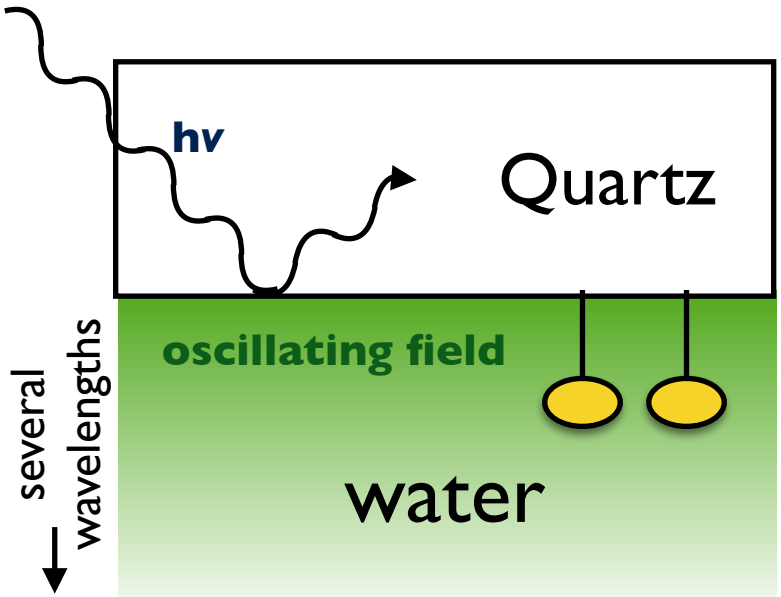


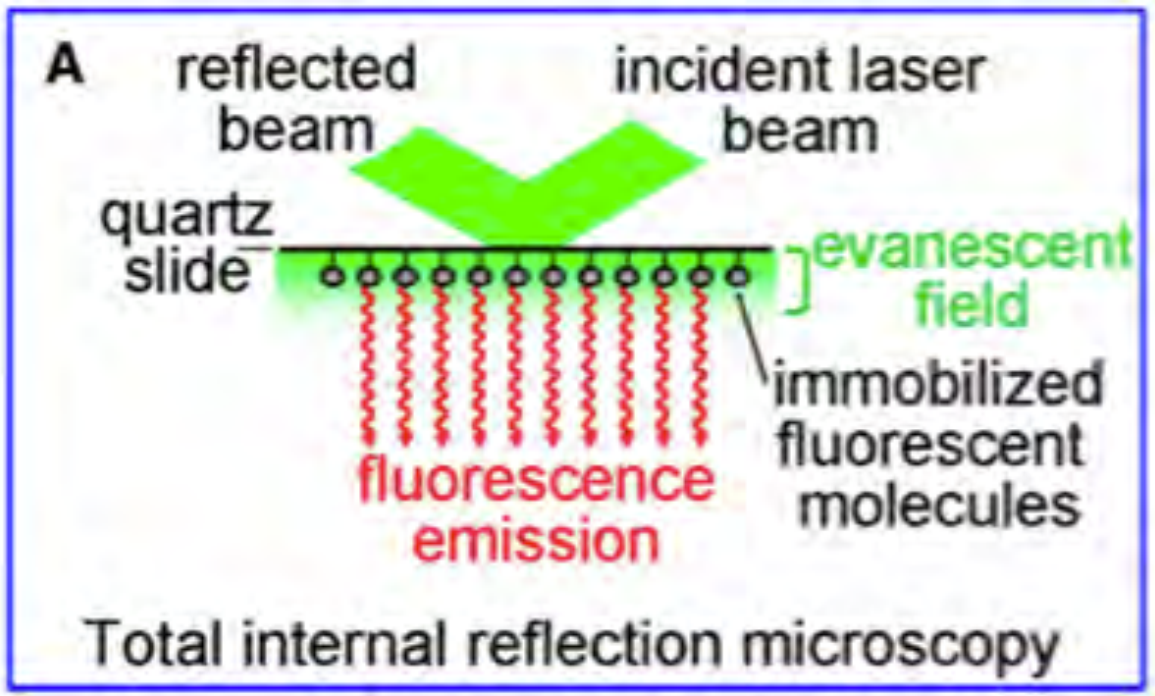
Total Internal Reflectance

Molecular Cell 24, 317–329, November 3, 2006

Single-Molecule Biology:
What Is It and How Does It Work?

Jordanka Zlatanova1, and
Kensal van Holde

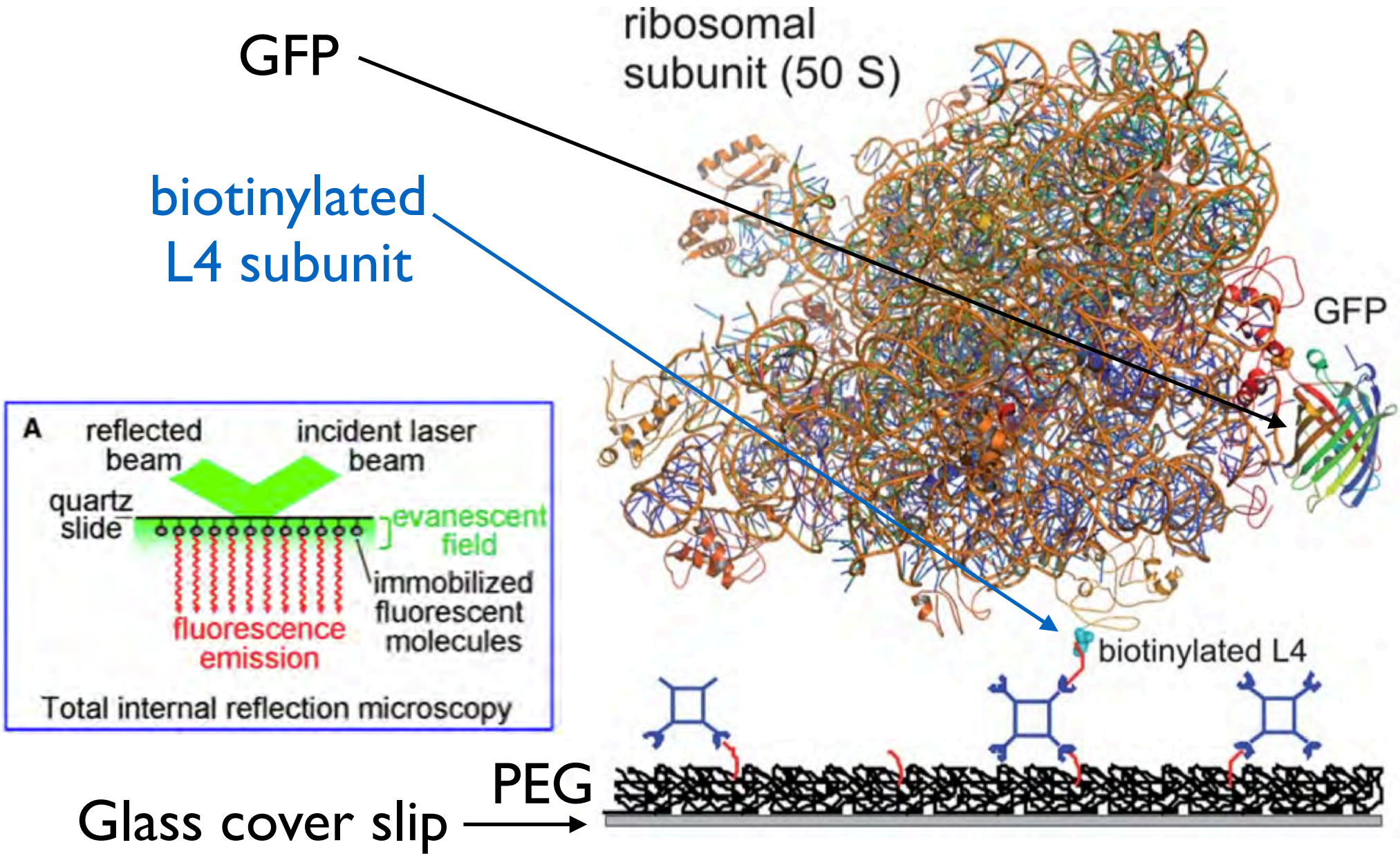




In TIR, the excitation light is directed toward an interface between two media of different refractive indices (i.e., from an optically denser medium, such as a glass slide, into a less-dense medium, such as water) (Axelrod, 2001). The incident angle of the beam is set larger than a certain critical angle, defined by the properties of the two media; all the light is reflected off the glass and does not penetrate into the solution (Figure 1A). How-ever, an electromagnetic field that oscillates with the same frequency as the incident light does form in the less-dense medium (the water on the other side). Because this electromagnetic field (also called evanescent field or wave) decays exponentially from the glass surface, it is capable of exciting fluorophores only in a very small volume close to the surface, thus effectively preventing out-of-focus fluorescence background. The excitation light itself is cleanly removed from the observation chamber, reducing the background even further.

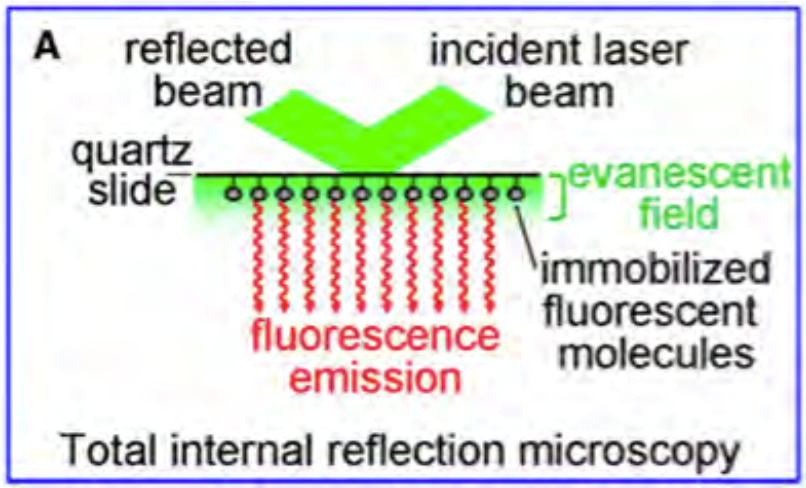
Single Molecule Fluorescence

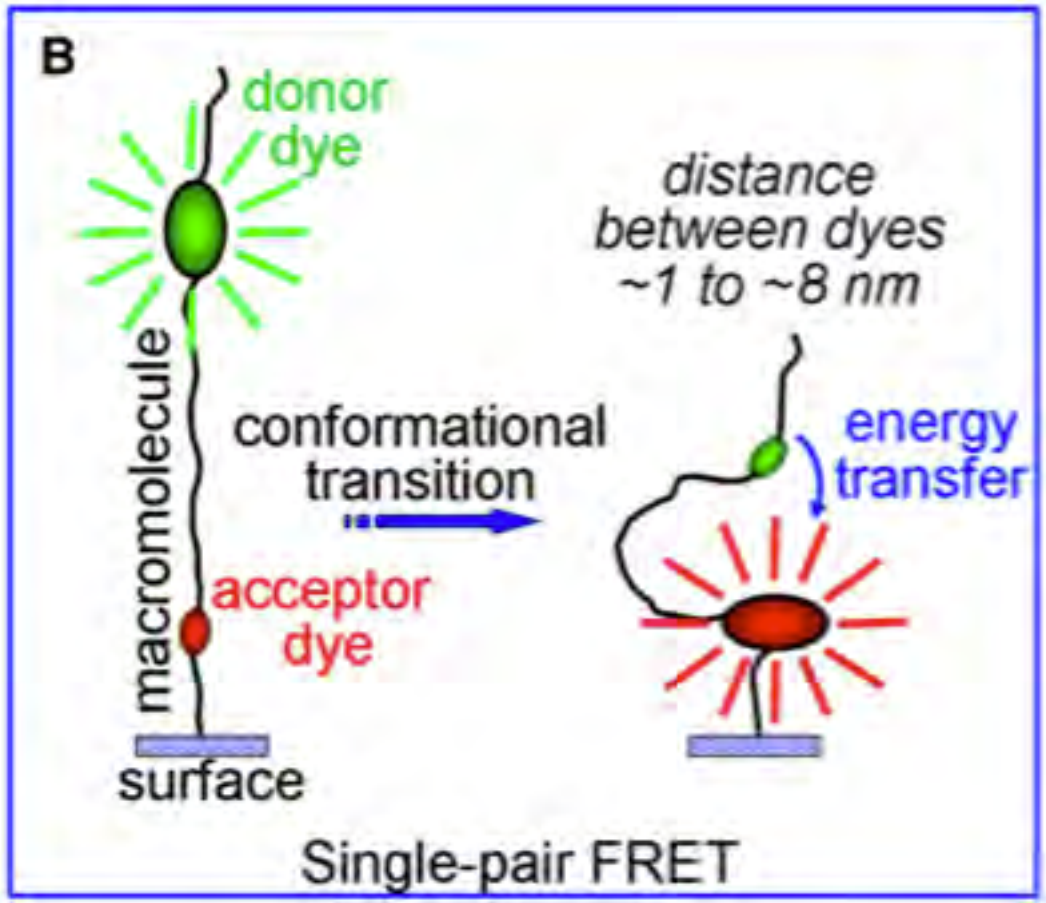
Molecular Cell 24, 317–329, November 3, 2006



Single Molecule Fluorescence

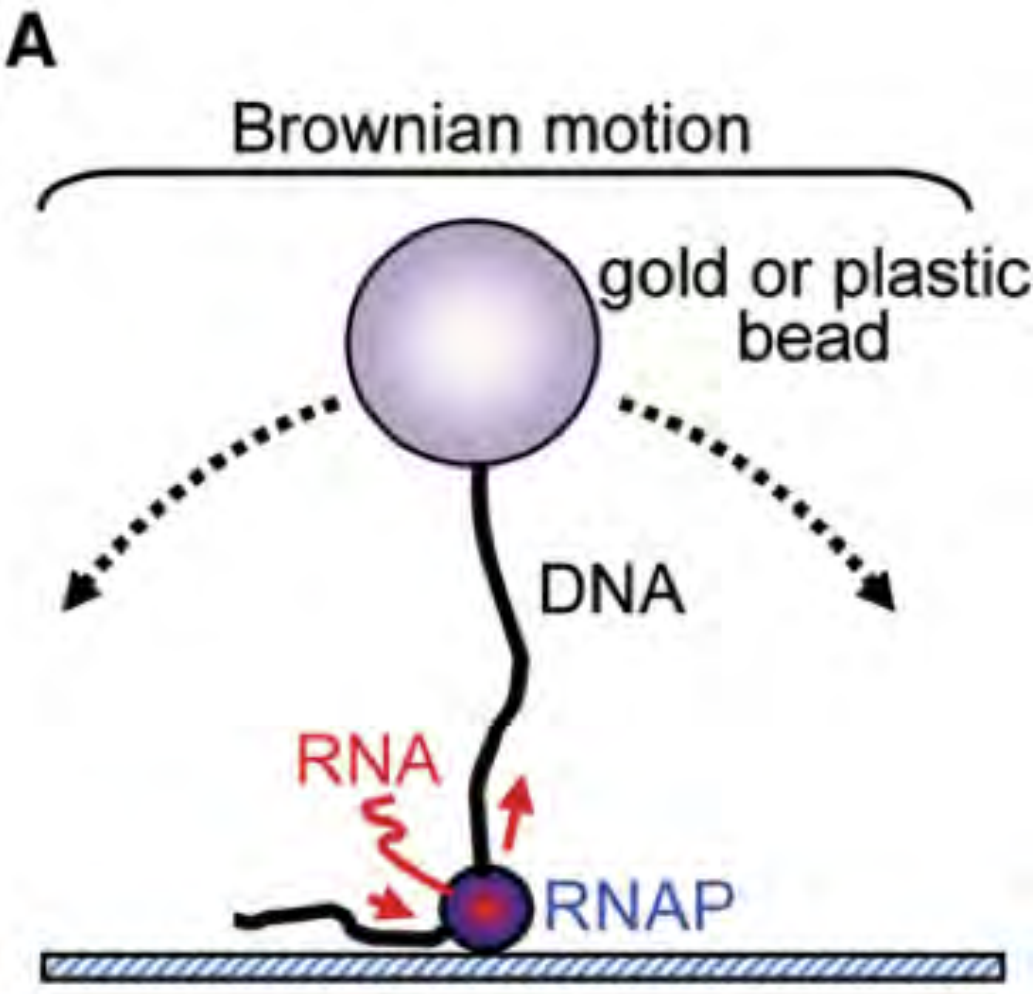
Molecular Cell 24, 317–329, November 3, 2006





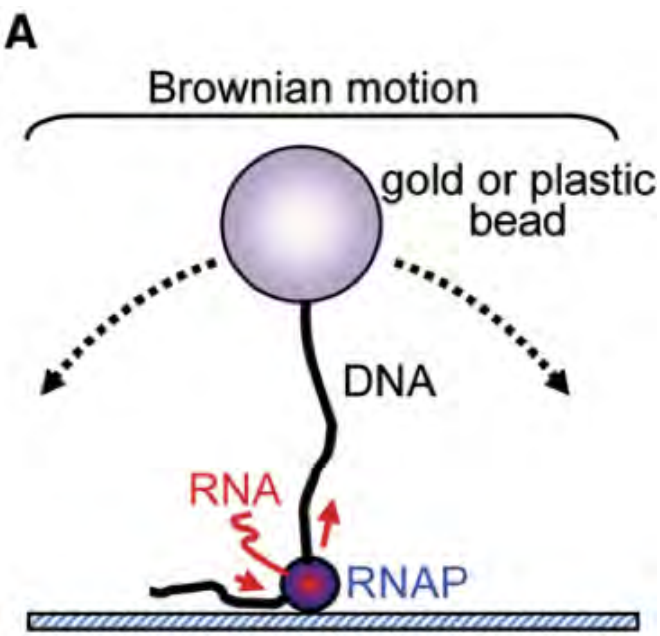
Single Molecule Fluorescence

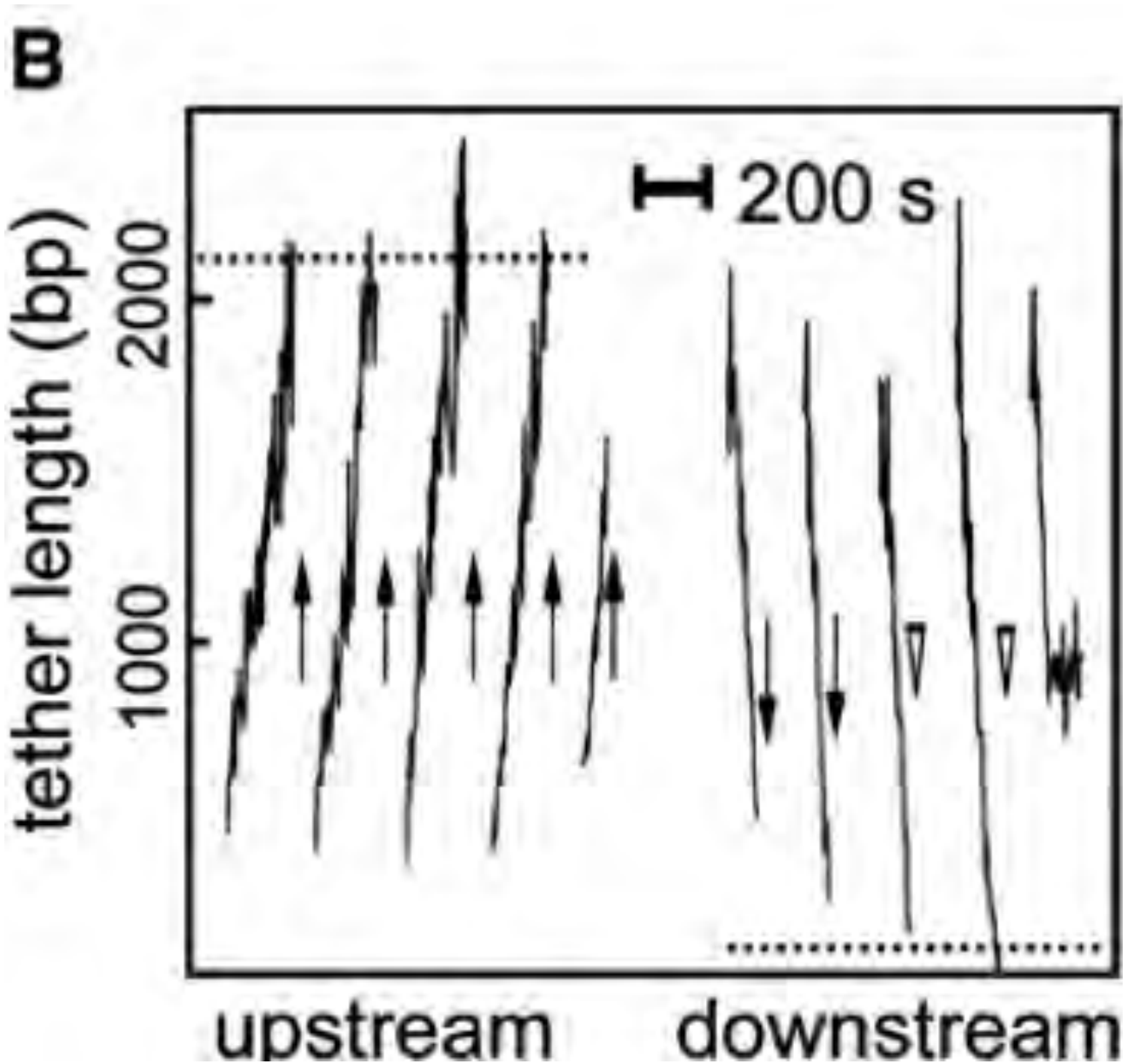
Molecular Cell 24, 317–329, November 3, 2006



Jeff Gelles, et al.

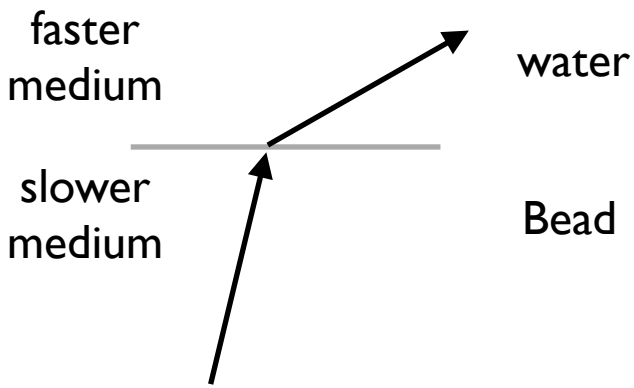
Single Molecule Fluorescence





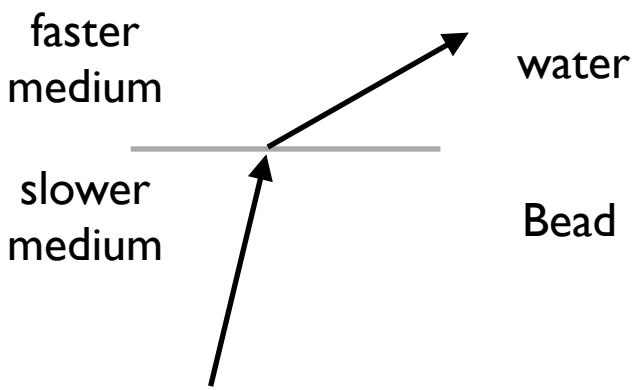
Refraction of Light

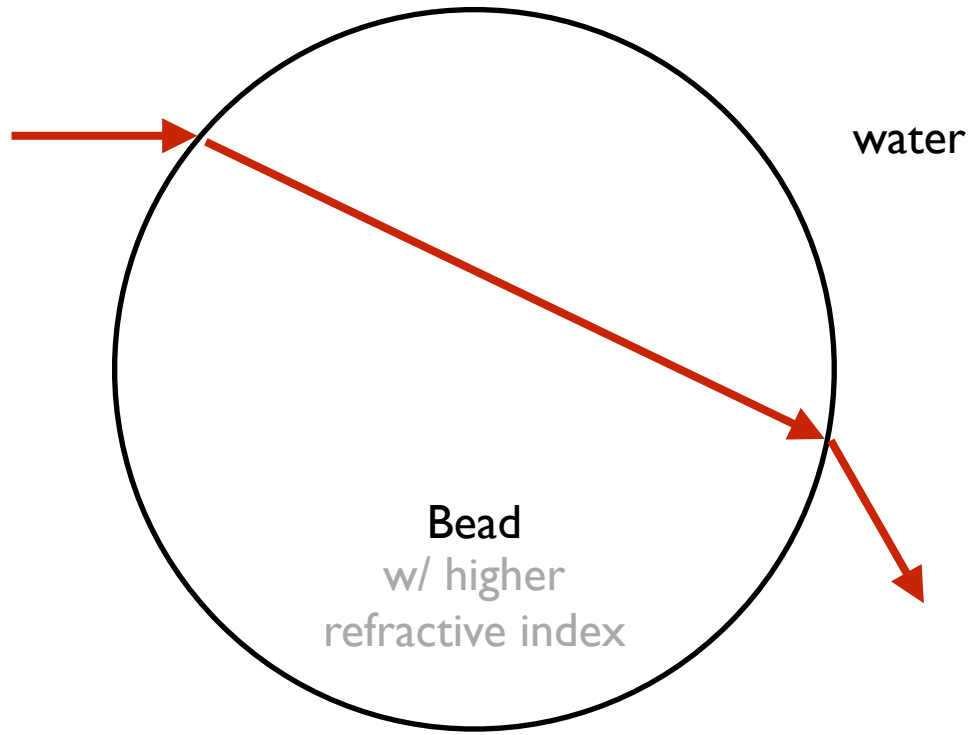
Media with different refractive indices



Refraction of Light

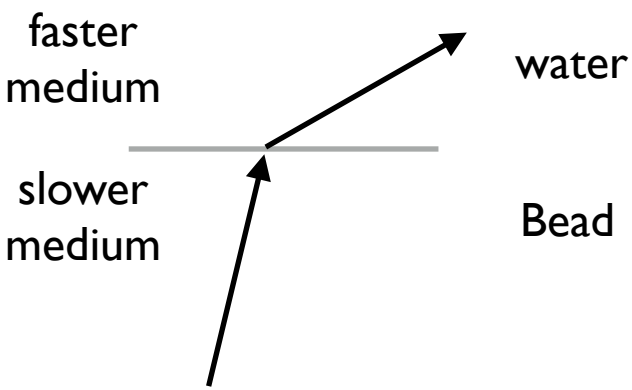
Media with different refractive indices

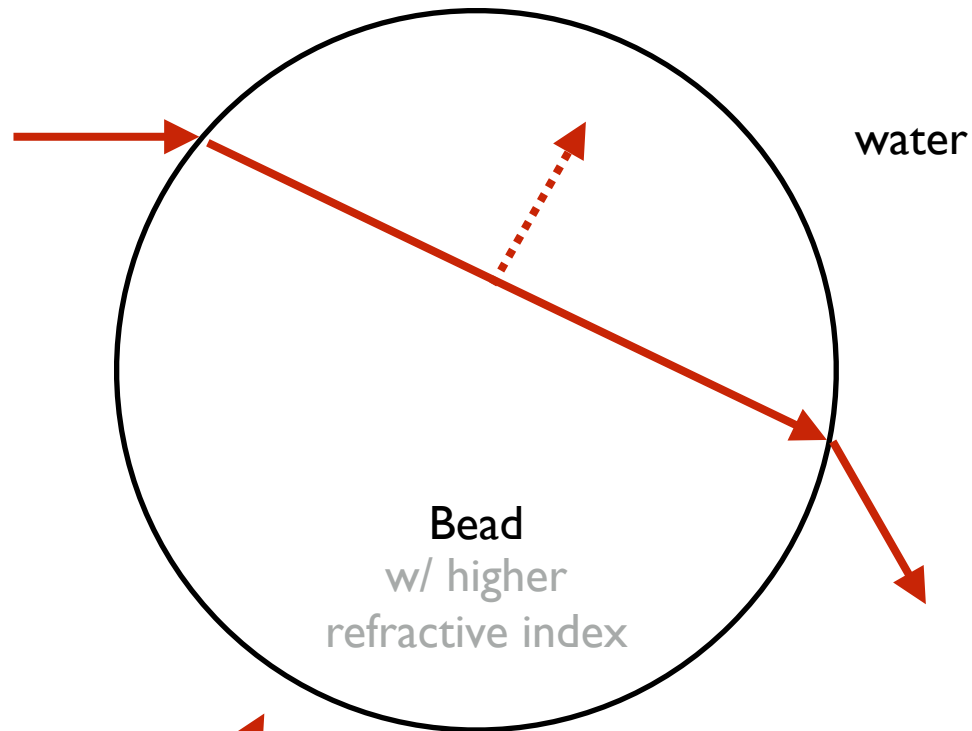




Refraction of Light

Media with different refractive indices

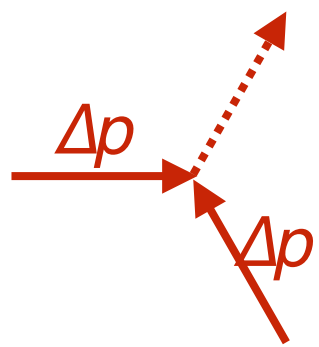




$$p = \frac{h}{\lambda}$$

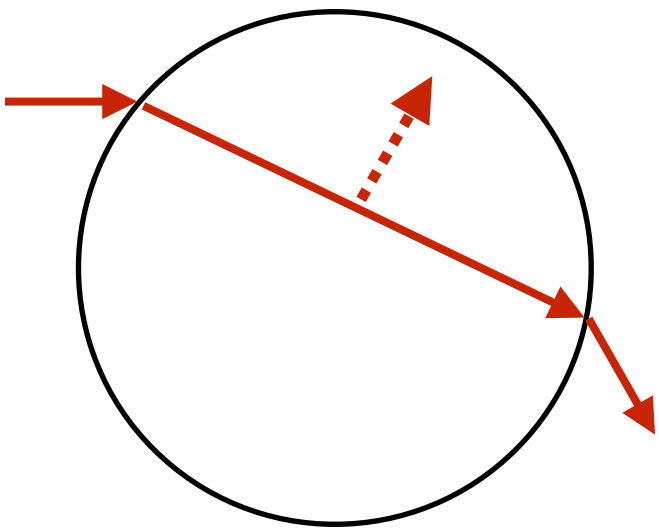
Light has momentum

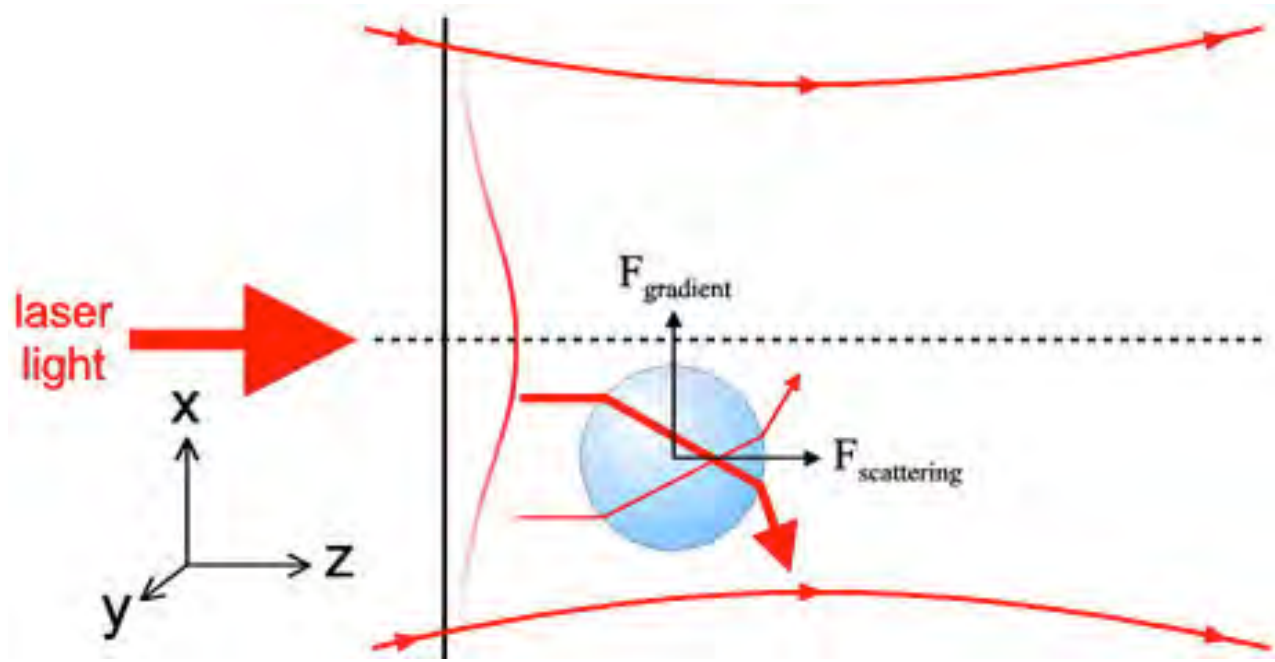
conservation of momentum



Laser (Bead) Trap

Media with different refractive indices

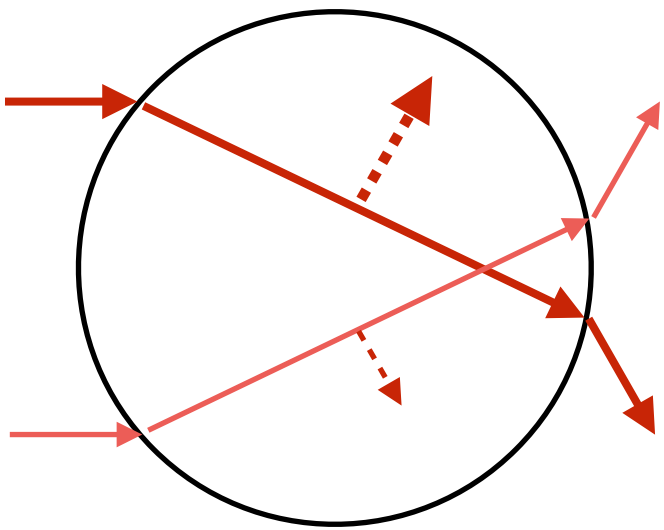


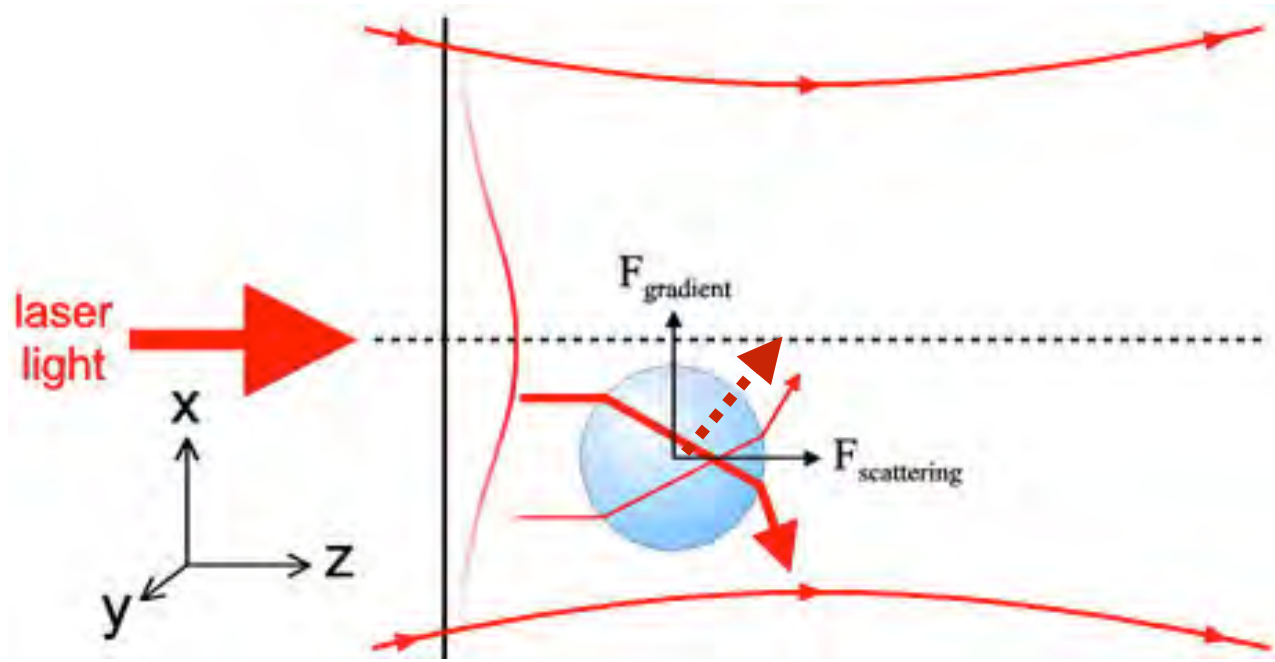


Adapted from https://blocklab.stanford.edu/optical_tweezers.html

Laser (Bead) Trap

Media with different refractive indices



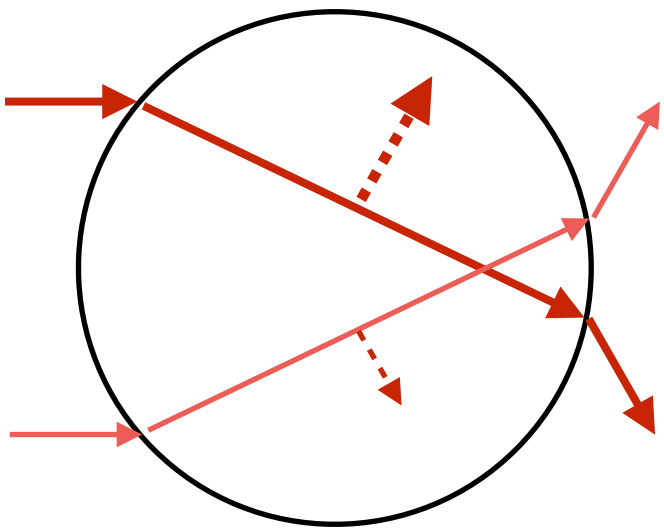


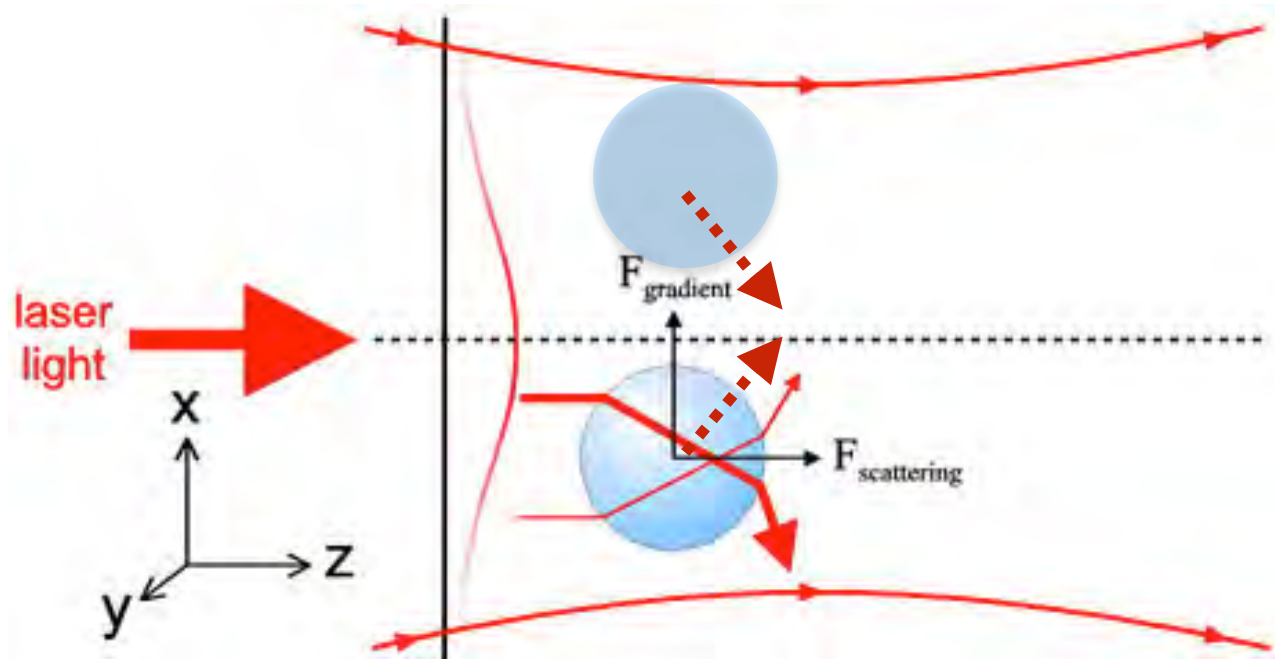
Adapted from https://blocklab.stanford.edu/optical_tweezers.html

Laser (Bead) Trap

Media with different refractive indices

force pushes bead to center of trap





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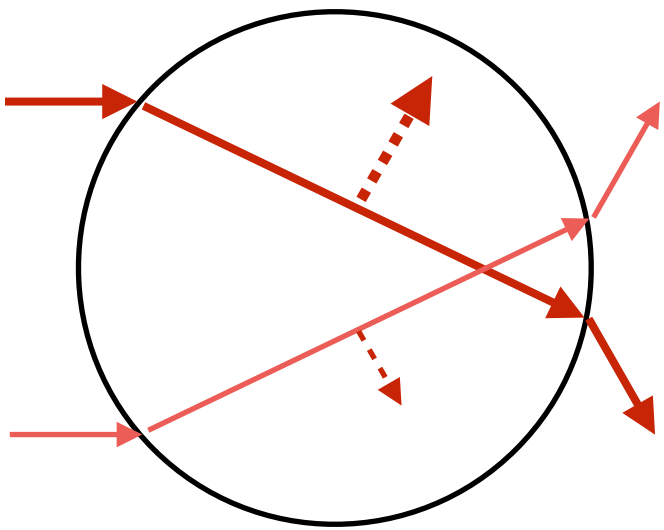
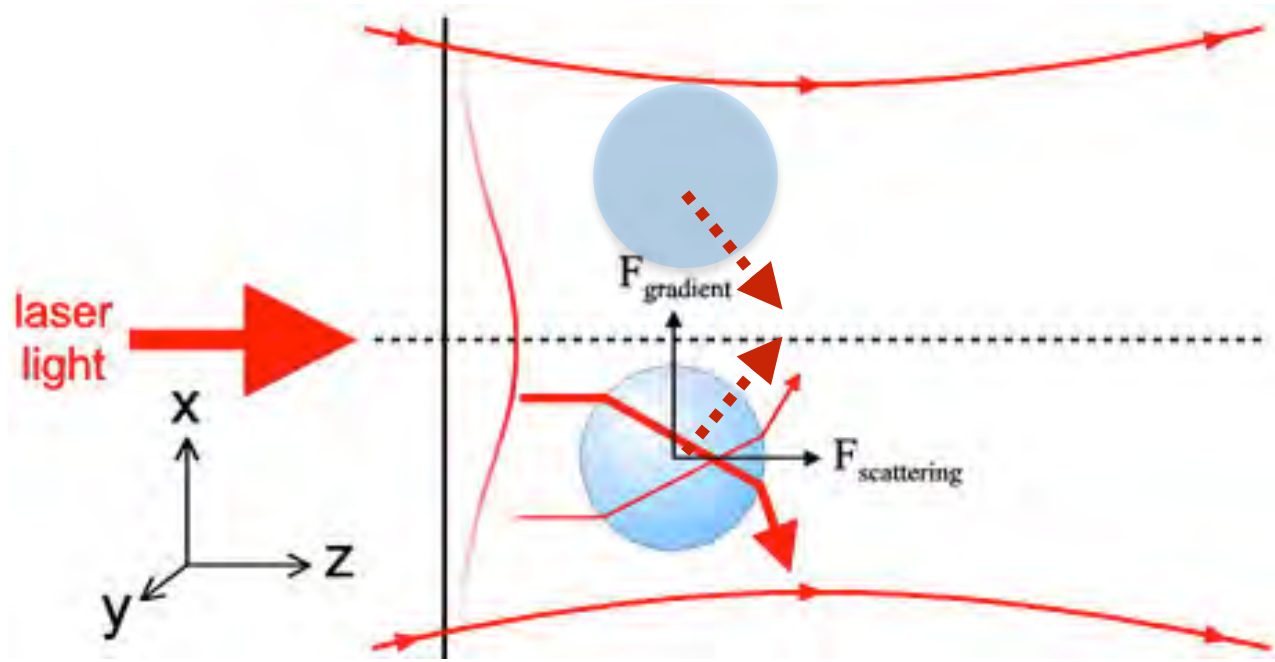
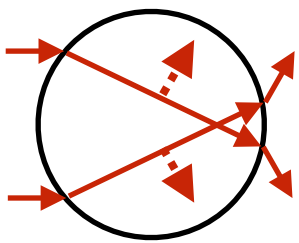






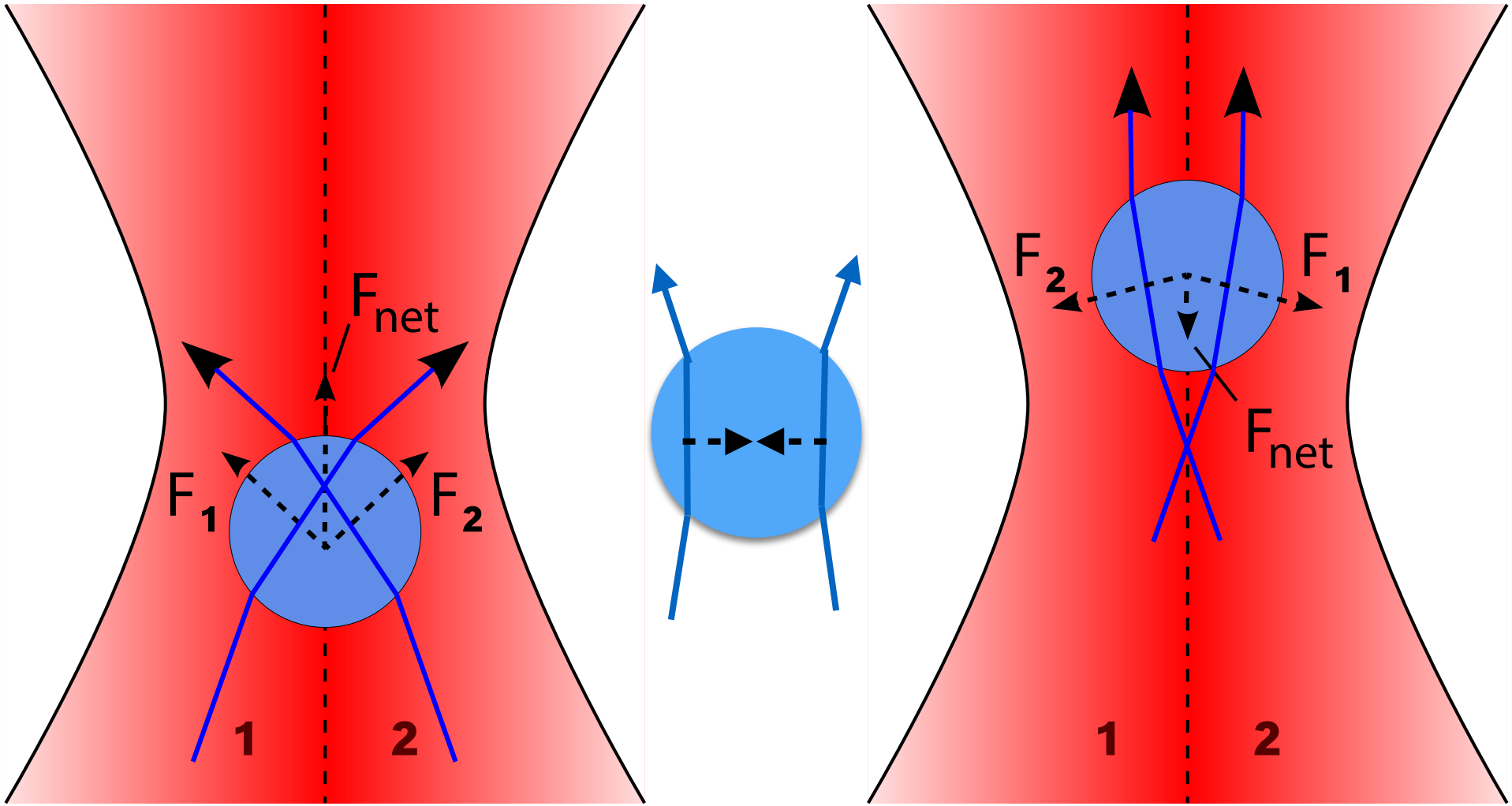
no net lateral force at center

but there is a net axial force

Adapted from https://blocklab.stanford.edu/optical_tweezers.html

Laser (Bead) Trap

Traps both laterally and axially



laser light in

laser light in

Adapted from Roland Koebler, CC BY 3.0,
https://commons.wikimedia.org/w/index.php?curid=15083883

https://en.wikipedia.org/wiki/Optical_tweezers

Single Molecule Fluorescence

Molecular Cell 24, 317–329, November 3, 2006

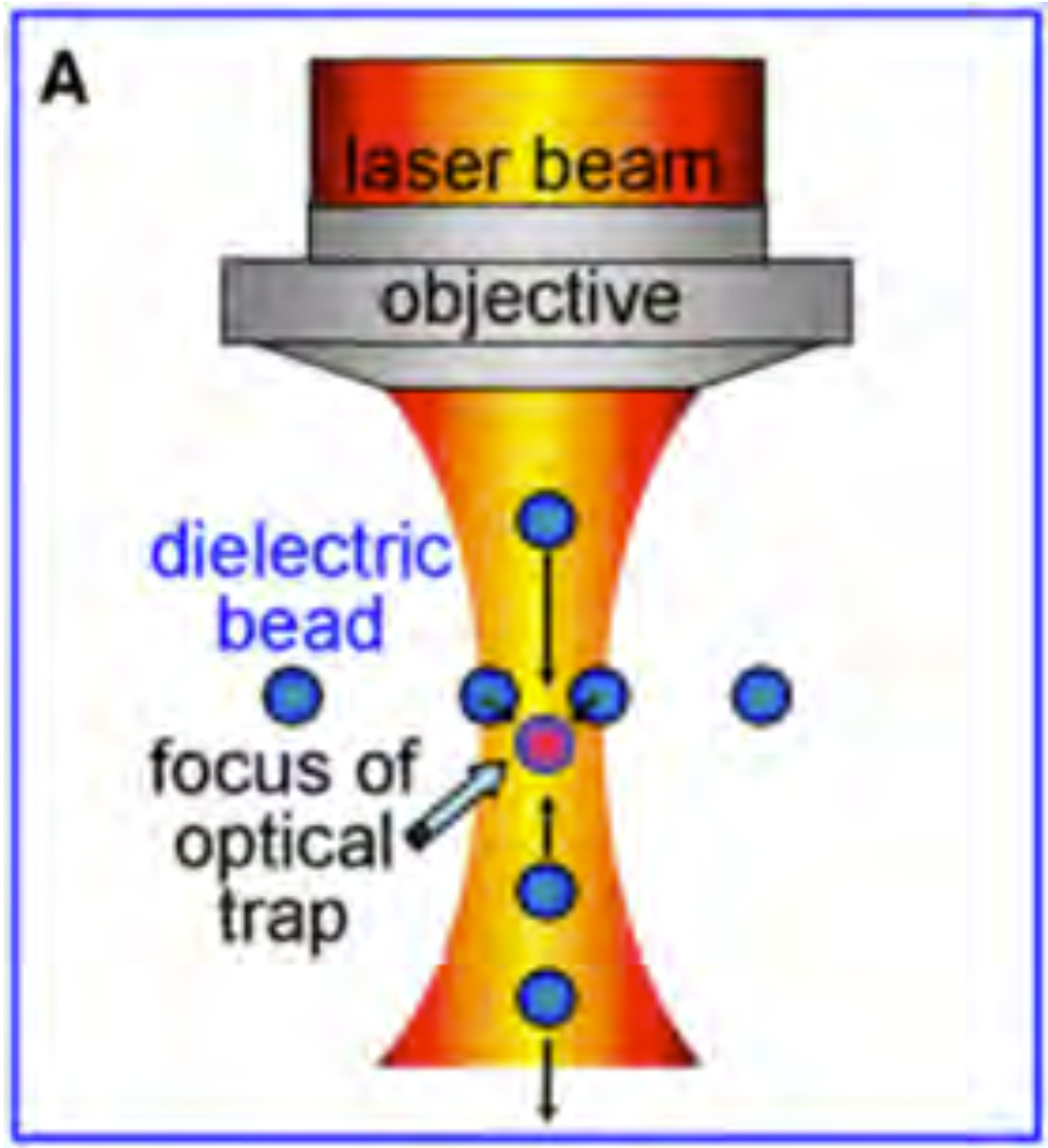
The location (focus) of the trap can be moved by adjusting the focusing of the lenses

Ashkin, A., Dziedzic, J., Bjorkholm, J. & Chu, S. (1986) Observation of a single-beam gradient force optical trap for dielectric particles. Opt. Lett. 11, 288–290

Nobel Prize in Physics 1997

Steven Chu
Prof of Physics and of Mol & Cell Bio
Stanford

U.S. Secretary of Energy
2009 - 2013



Single Molecule Fluorescence

Molecular Cell 24, 317–329, November 3, 2006

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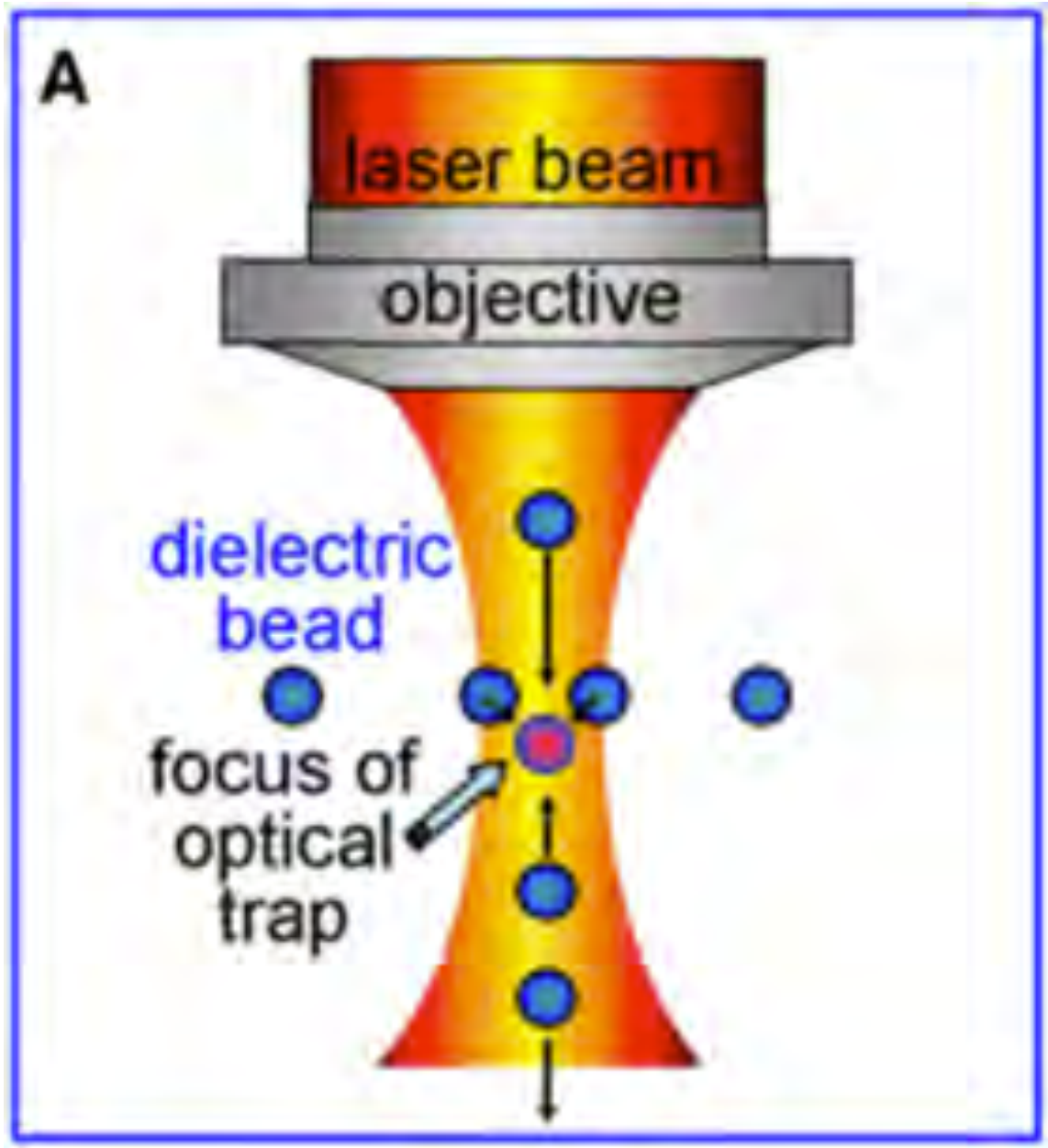
The strength of the trap can also be adjusted.



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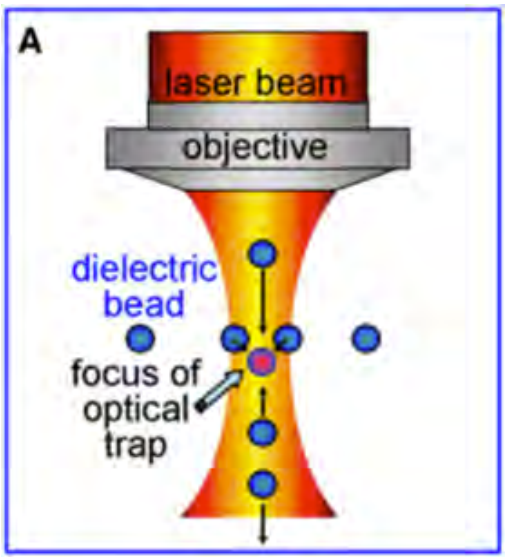
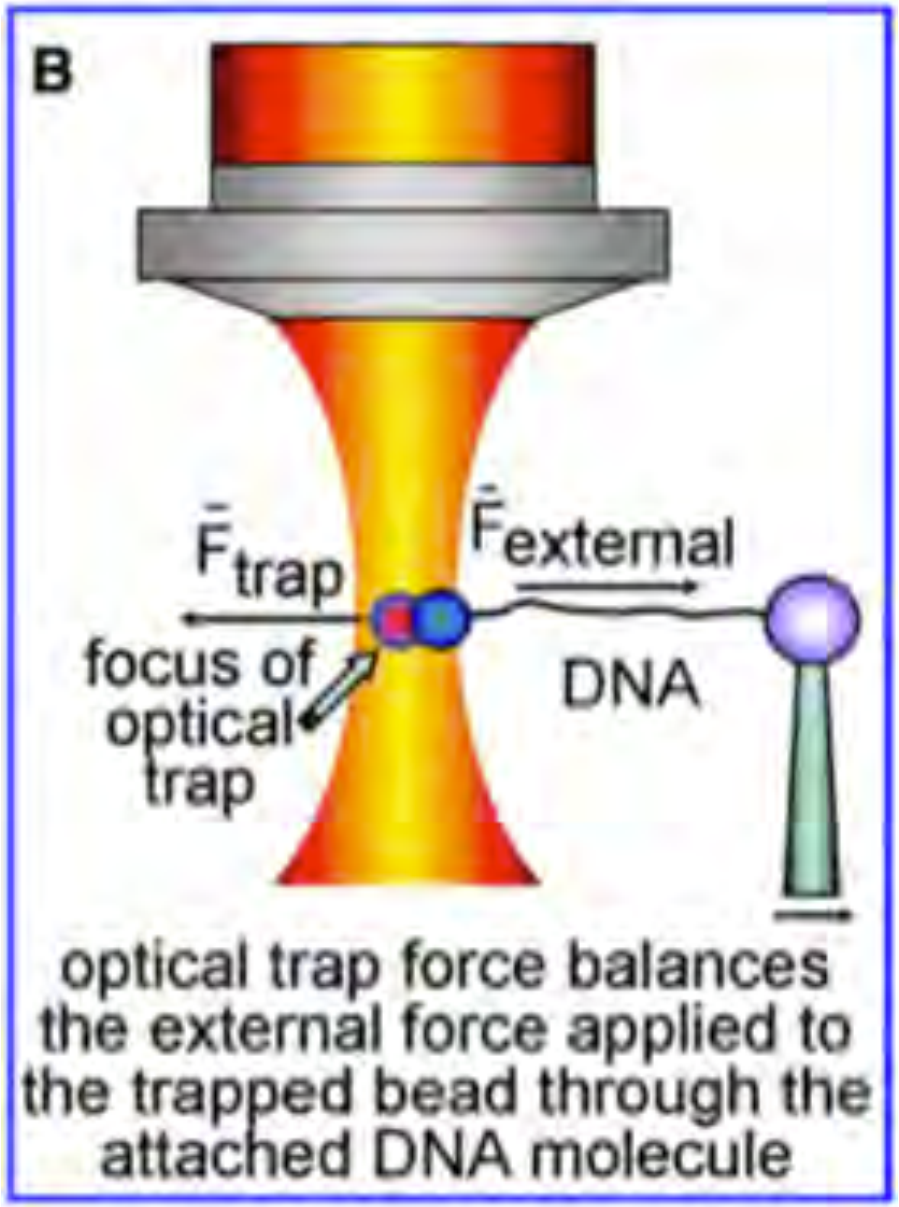
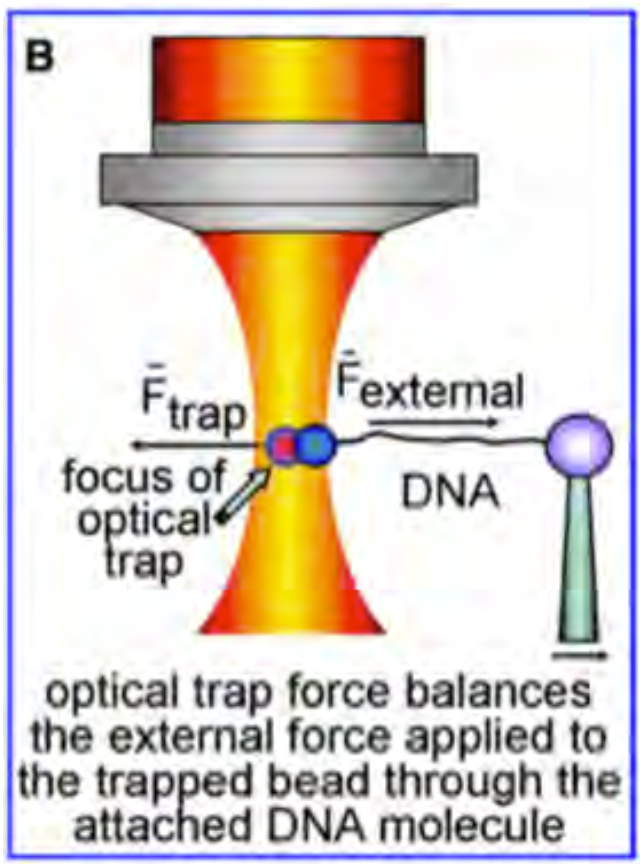


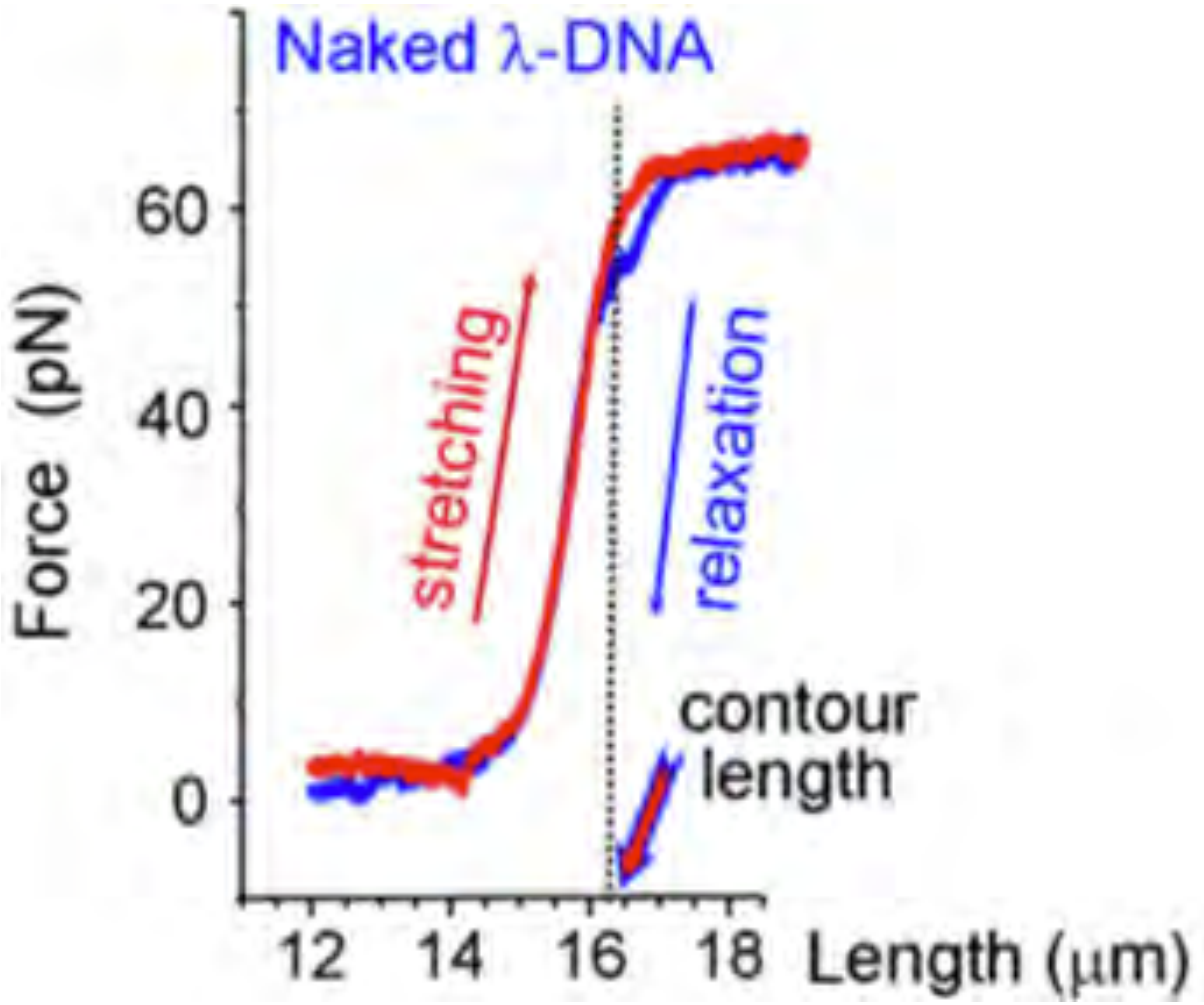


Single Molecule Fluorescence

Molecular Cell 24, 317–329, November 3, 2006

The force of the trap is varied, automatically under computer control, to maintain the bead in the trap.





Single Molecule Fluorescence

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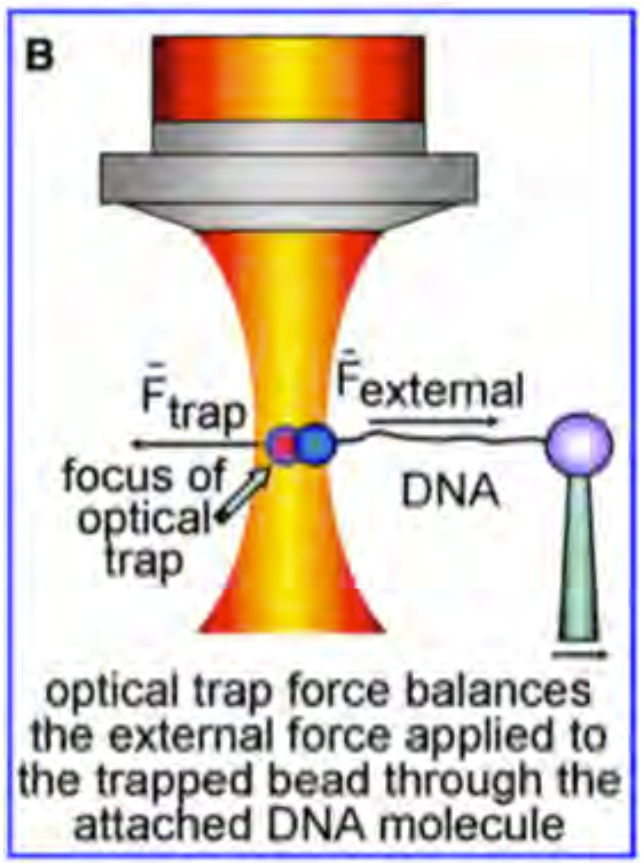
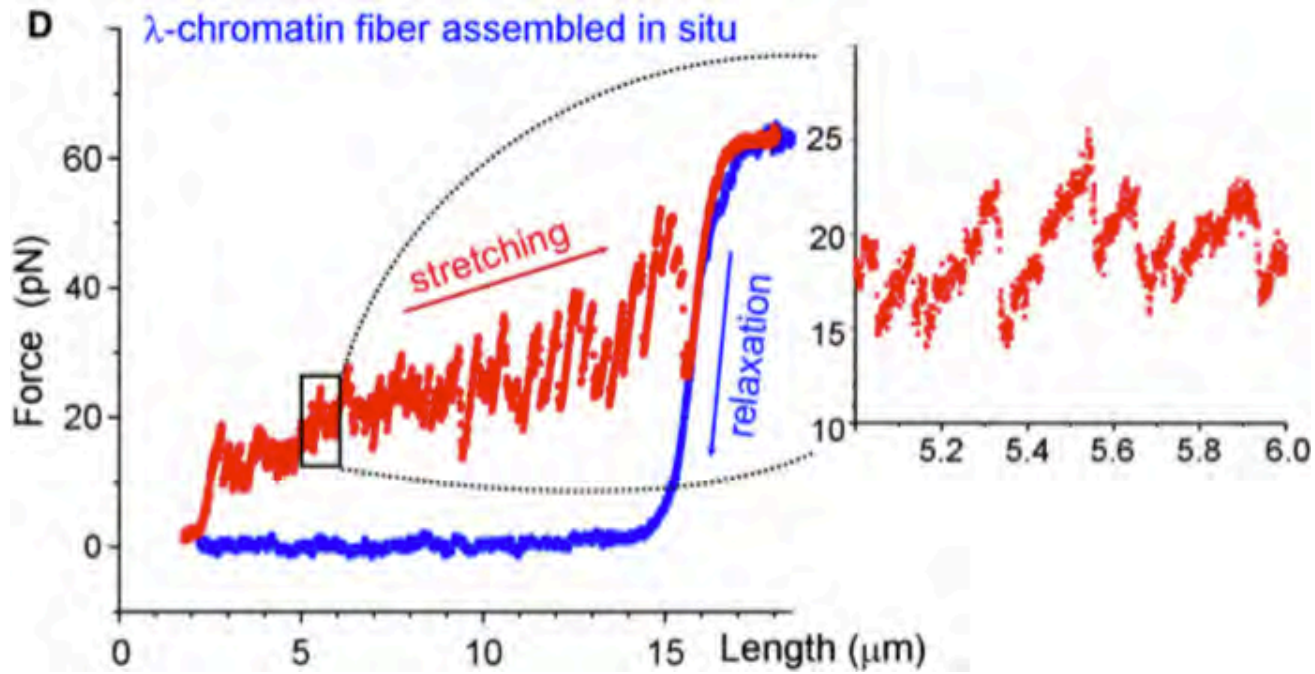
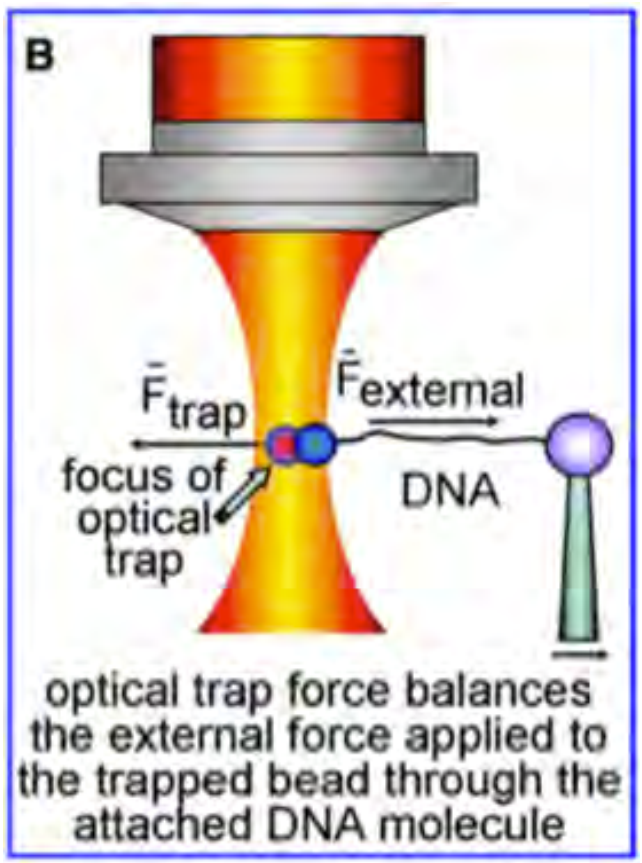


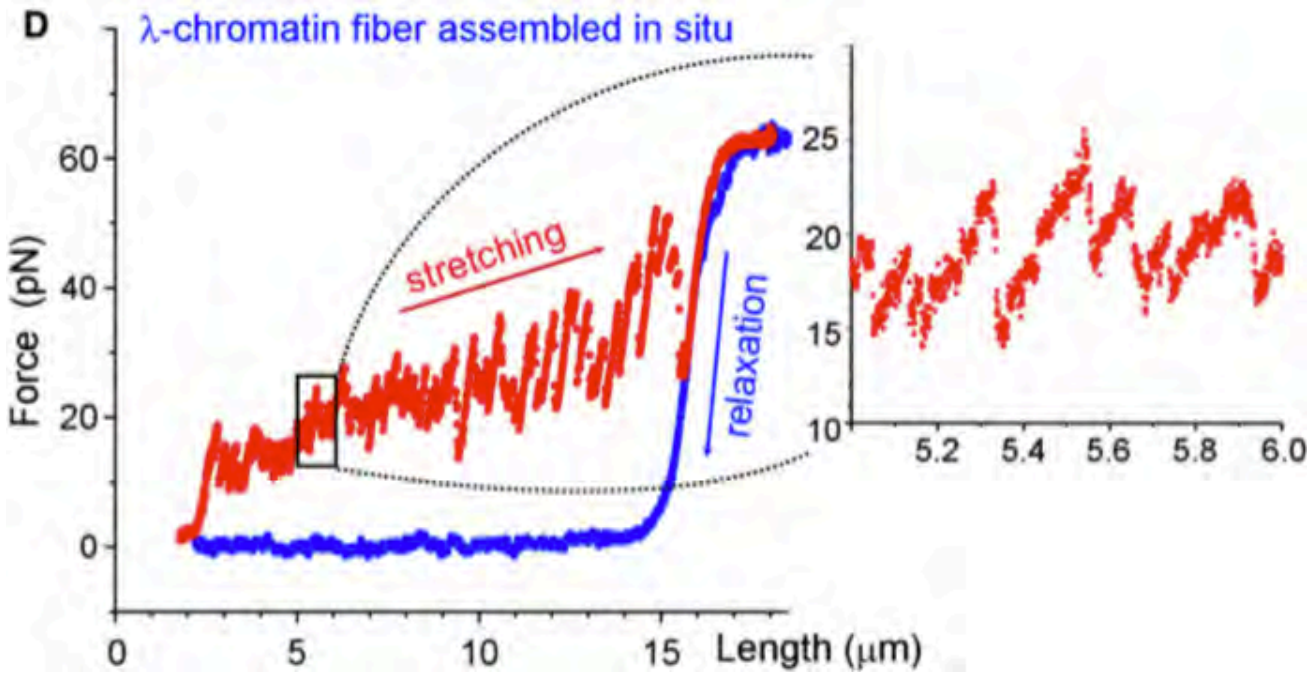


(D) Stretching of a chromatin fiber assembled on naked l-DNA molecule **by the addition of X. laevis egg extract directly into the flow cell of the instrumen**t. The extract contains core histones and protein factors needed for assembly (assembly is manifested by shortening of the distance between the two beads with time). Note the sharp discontinuities in the force-extension curve reflecting the unraveling of the DNA from around the histone octamer that forms the core of the nucleosomal particles. Nucleosomes can unwrap either individually or in groups of two, three, or four. At high extension, when all histones have been forced off, the curve approaches that of naked DNA. Note that the two force-extension curves for DNA (C) and chromatin (D) are aligned with respect to the length of the structure during stretching, so that a direct comparison of the behavior of DNA and of chromatin is possible.

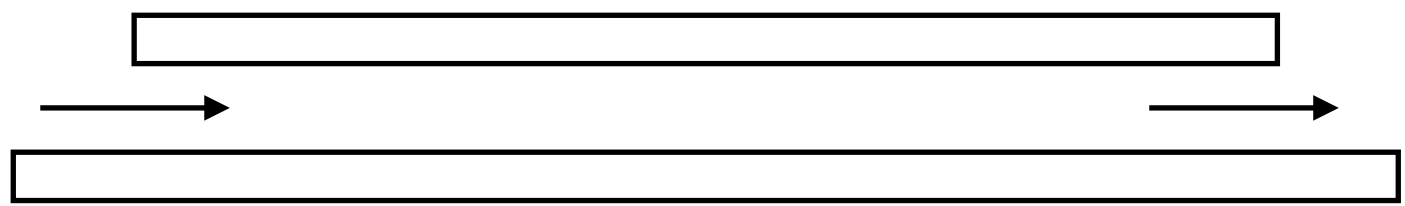
Single Molecule Fluorescence

Molecular Cell 24, 317–329, November 3, 2006





Can flow things in and out…



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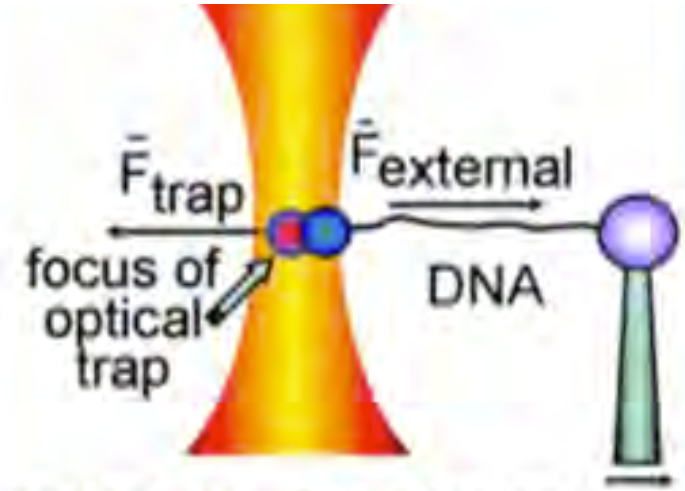
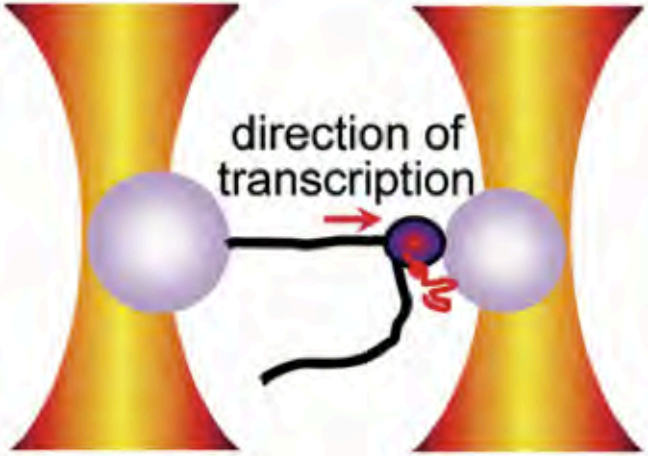
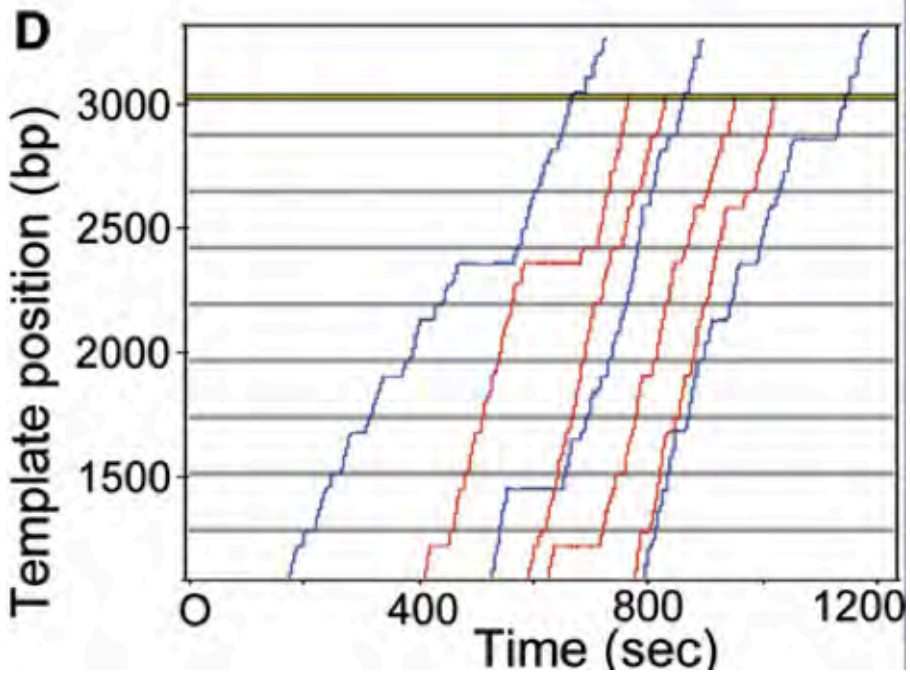




Steve Block, et al.



Atomic Force Spectroscopy

Molecular Cell 24, 317–329, November 3, 2006

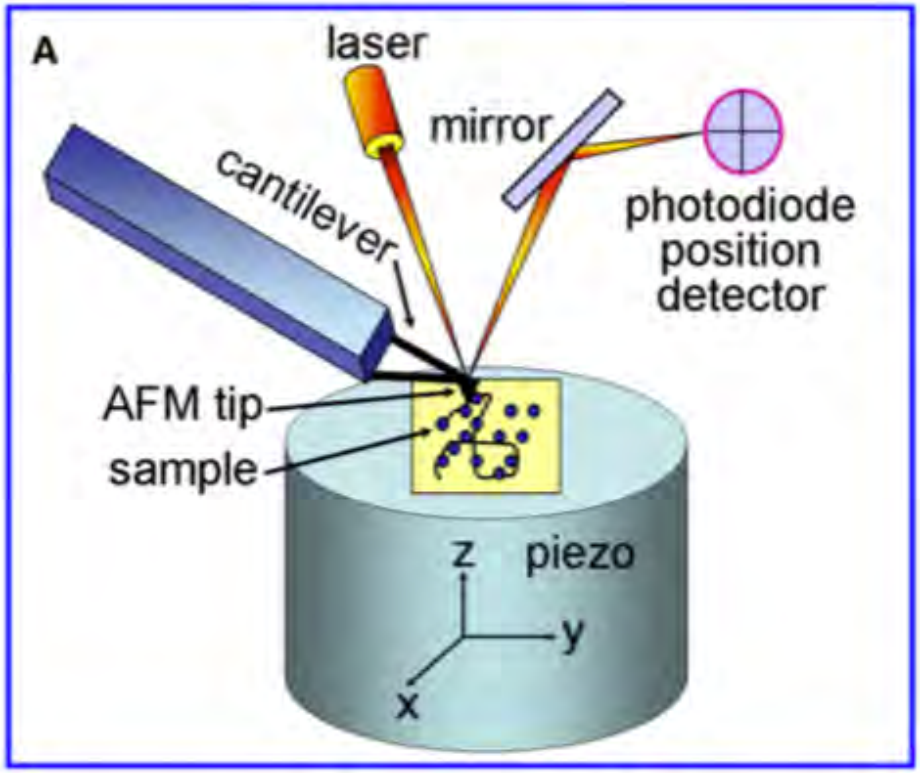
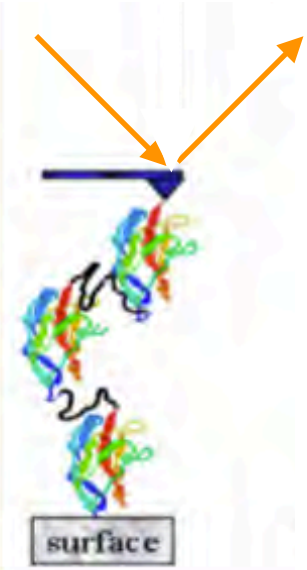


Figure 2. Use of the Atomic Force Microscope for Imaging and Force Spectroscopy

(A) Schematic of the principle of action of the AFM (see text).

(B) A schematic representation showing the structural transitions in a multidomain protein upon mechanical stretching using the AFM. The sawtooth pattern of the force-extension curves results from strings of successive enthalpic and entropic portions, reflecting the unfolding of individual domains, followed by entropic stretching of the unfolded domain. The unfolding of each domain adds significant length to the chain and relaxes the stress on the cantilever, which returns to its nondeflected state. The unfolded portion of the polypeptide chain can now undergo entropic stretching, which is accompanied by gradual deflection of the cantilever (adapted from Zlatanova et al. [2000]).



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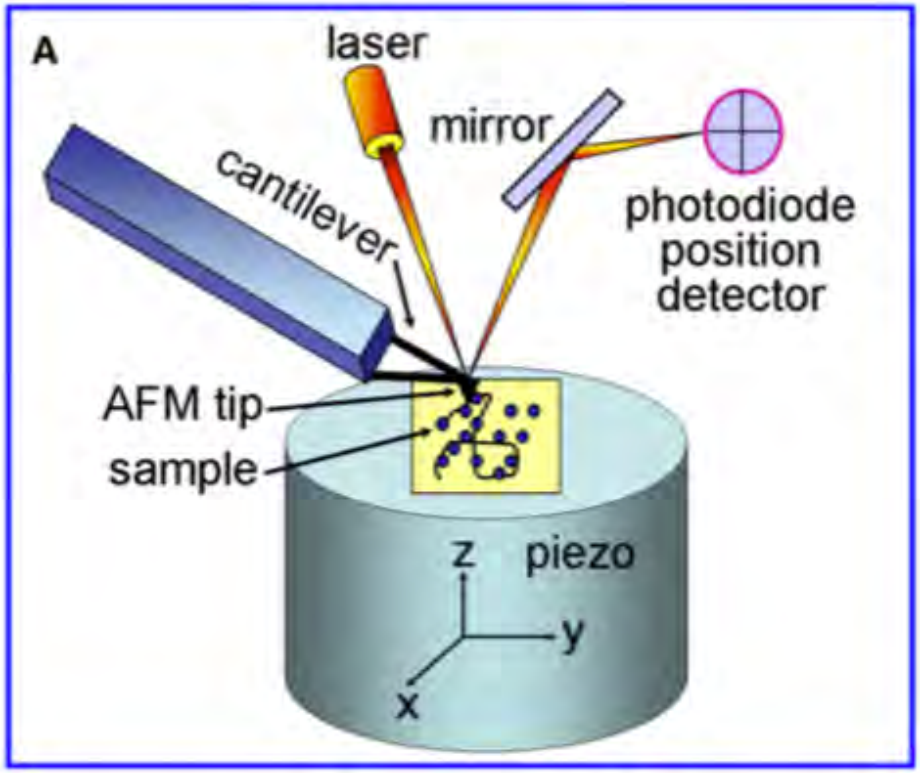

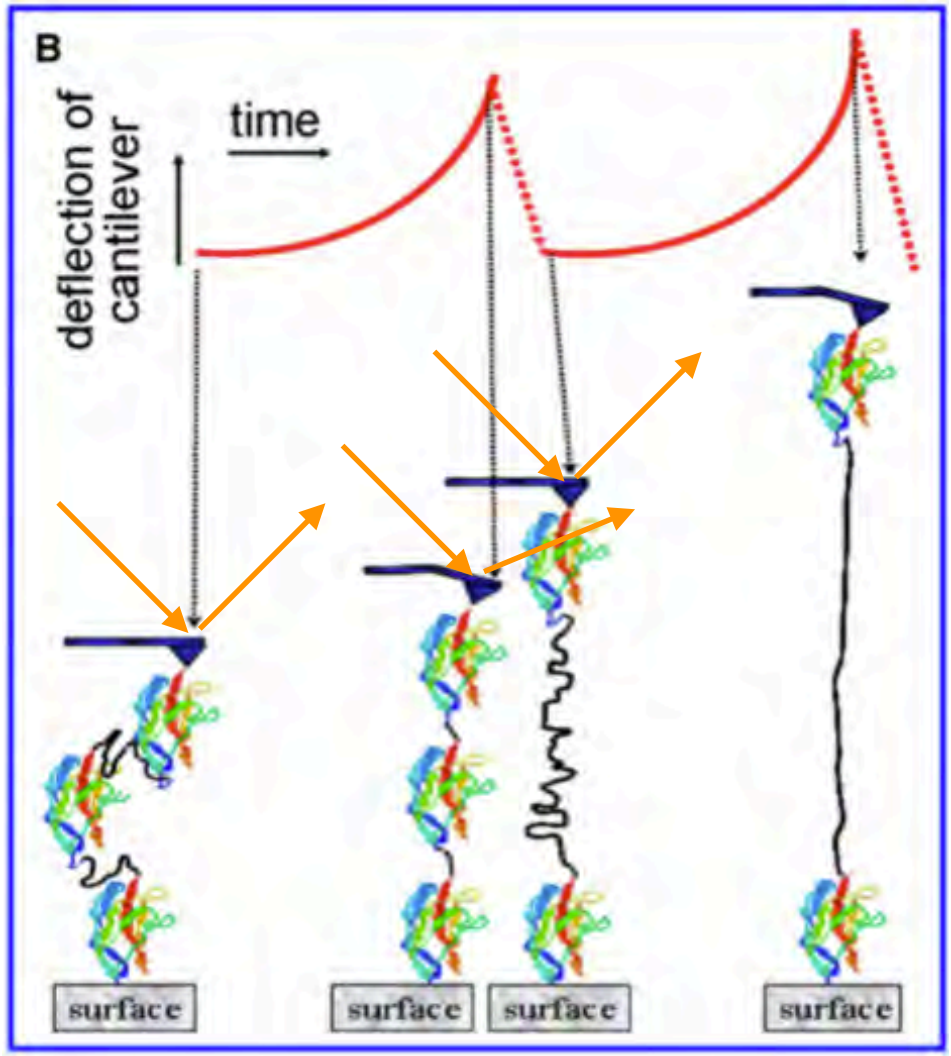

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Refraction of Light

Media with different refractive indices

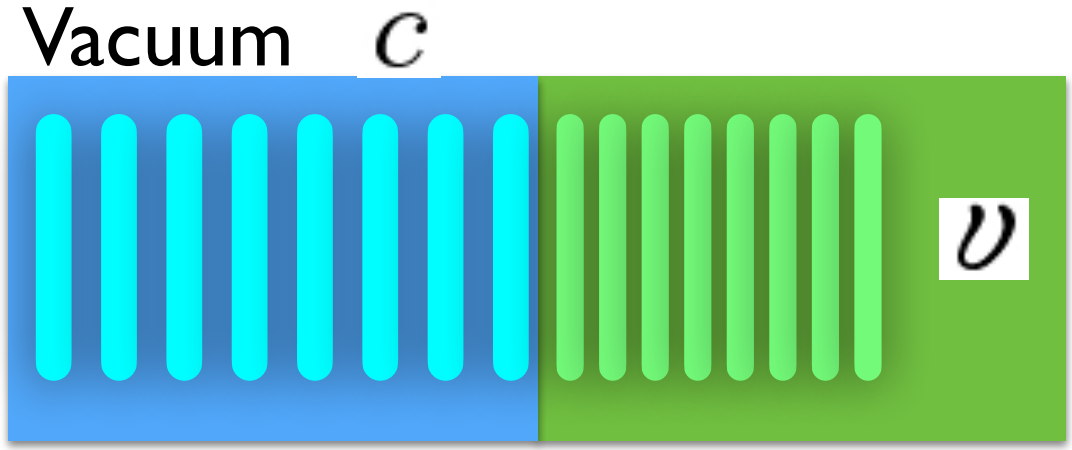
Light slows in a medium other than vacuum.

This is <u>not</u> scattering or absorption.

The oscillating electric vector of light causes electrons in atoms to oscillate.

They, in turn, generate their own electromagnetic wave (think of a radio antenna) at the same frequency, leading to constructive interference.

The resulting "combined" wave has wave packets that pass an observer at a slower rate. The light has effectively been slowed.



Electric field is oscillates at the same frequency on each side

Propagation rate has changed

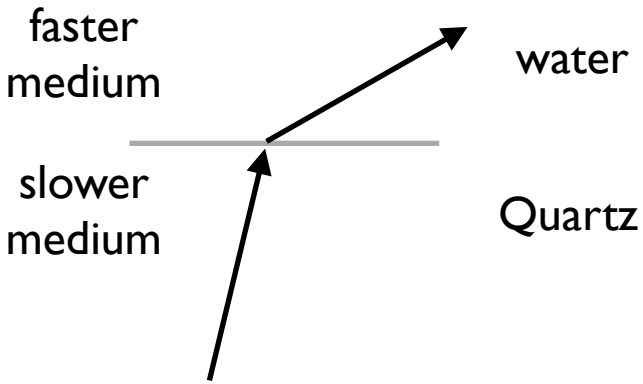
index of refraction $n = \frac{c}{v}$

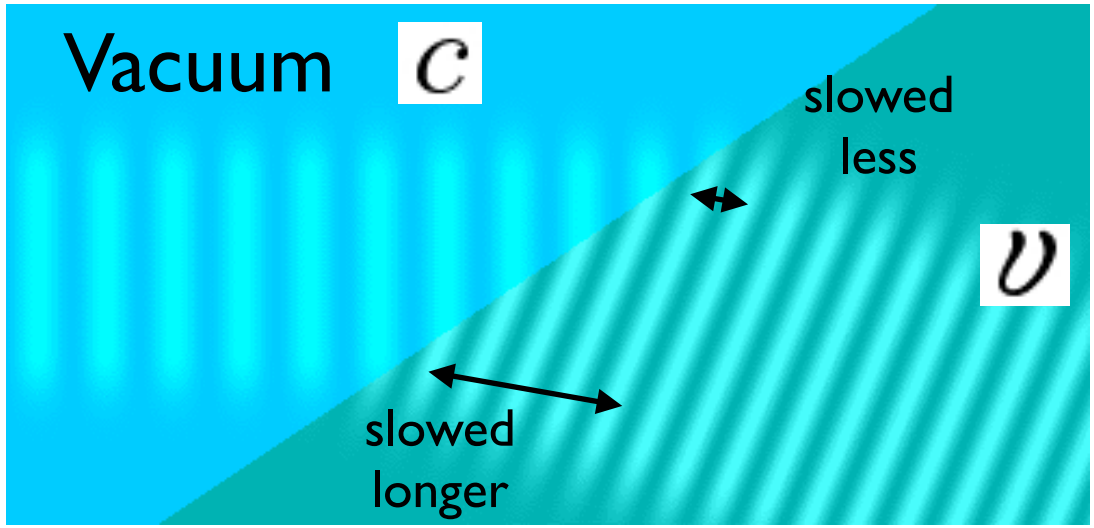
c: speed of light in a vacuum

v: phase velocity of light in the medium

Refraction of Light

Media with different refractive indices





By Ulflund - https://commons.wikimedia.org/w/index.php?curid=73784342

The amount of bending depends on:

- Change in speed (n_v/n)
- Angle of the incident ray

index of refraction $n = \frac{c}{v}$

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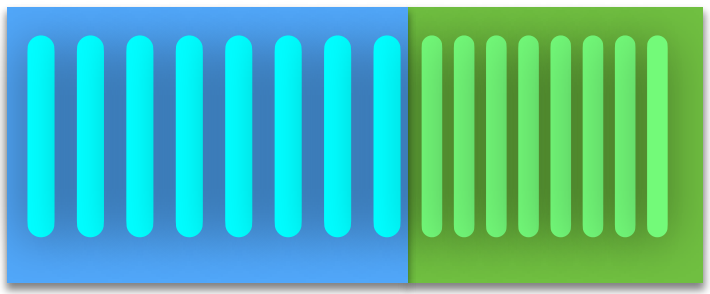
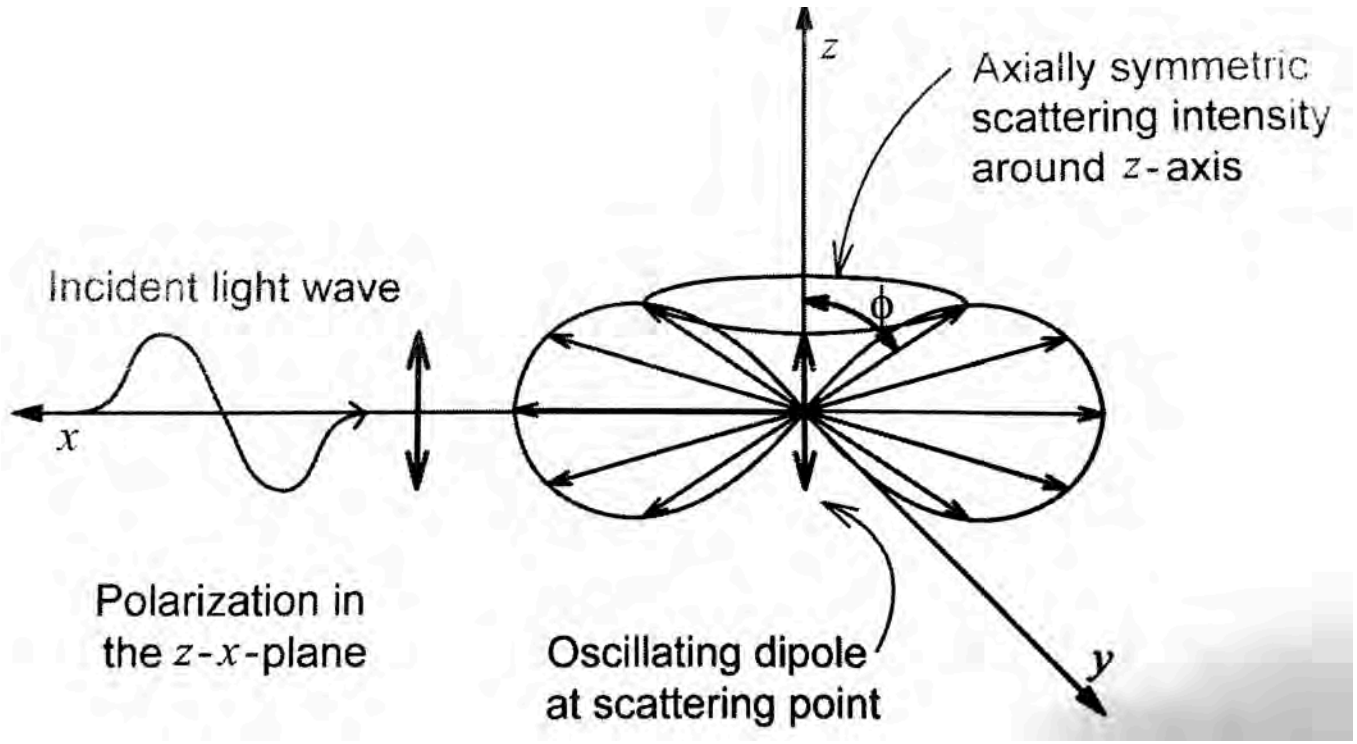
v: phase velocity of light in the medium



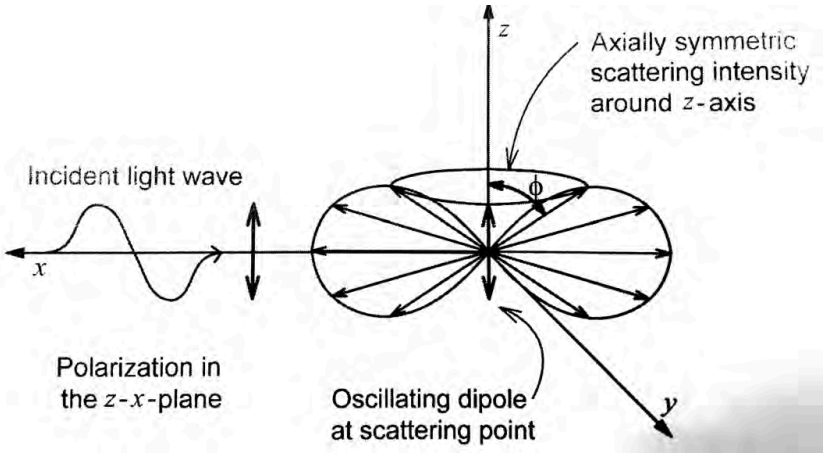
Uniform behavior

Scattering

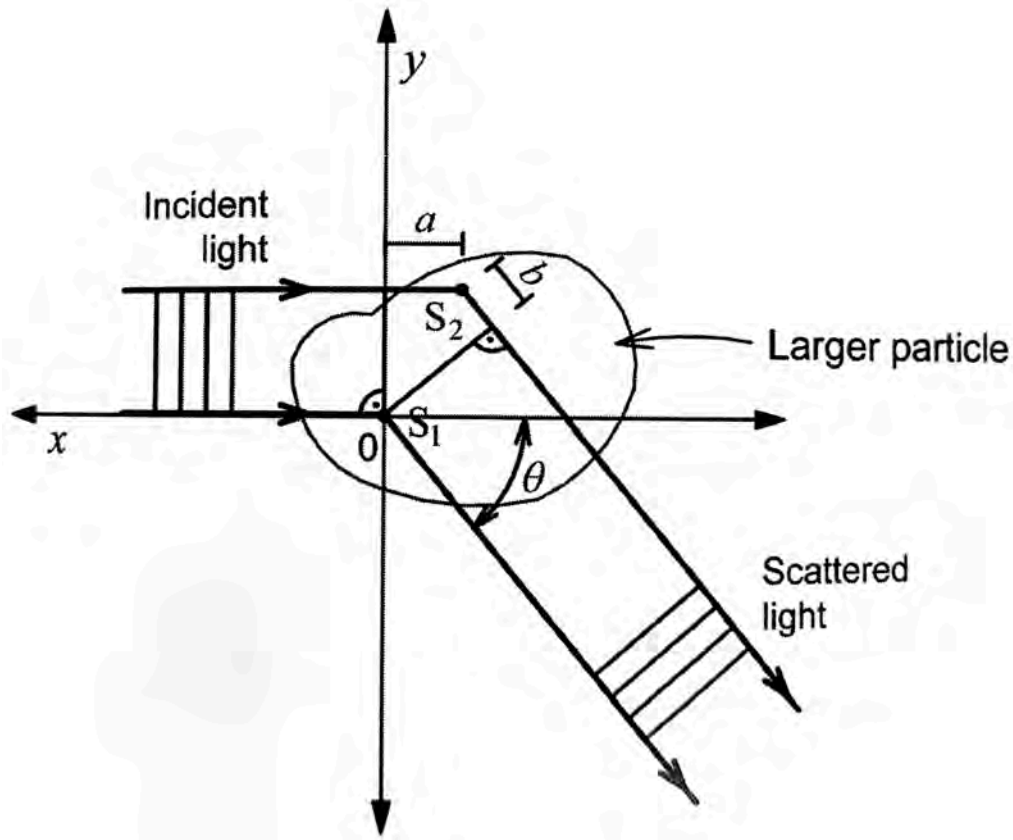
Rayleigh scattering: the oscillating electric field of a light wave acts on the charges (electrons) within a particle (atom/molecule), causing the charges to move at the same frequency (the particle is ***polarizable***). The particle, therefore, becomes a small radiating dipole whose radiation we see as scattered light.



Scattering



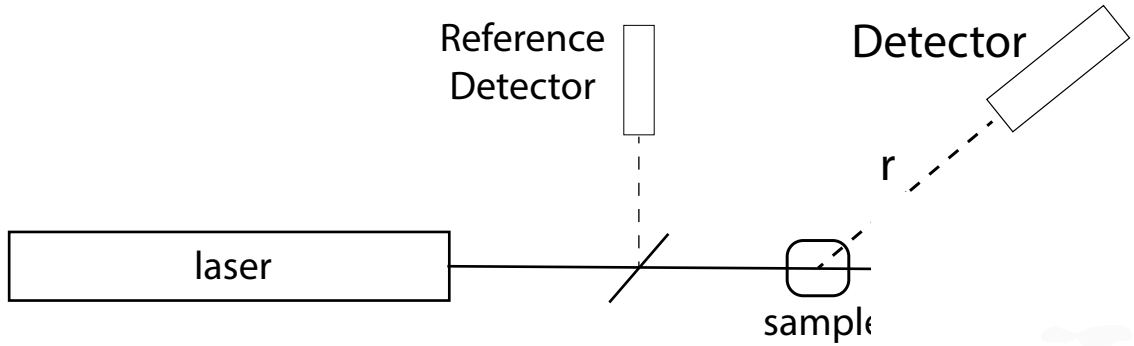
In Raleigh scattering, the particles are much smaller than the wavelength of the light.

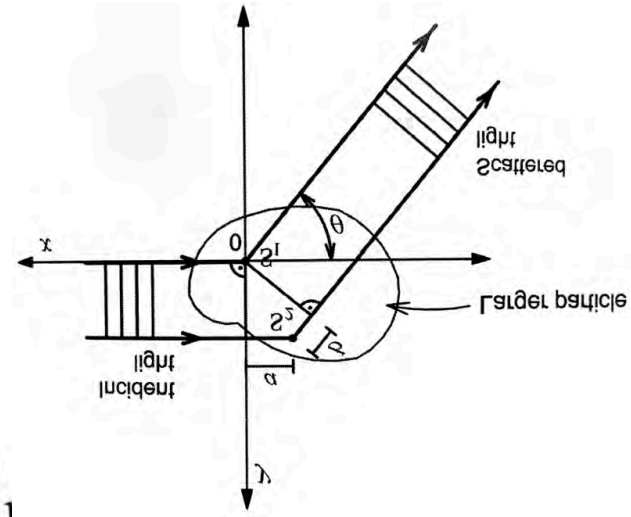


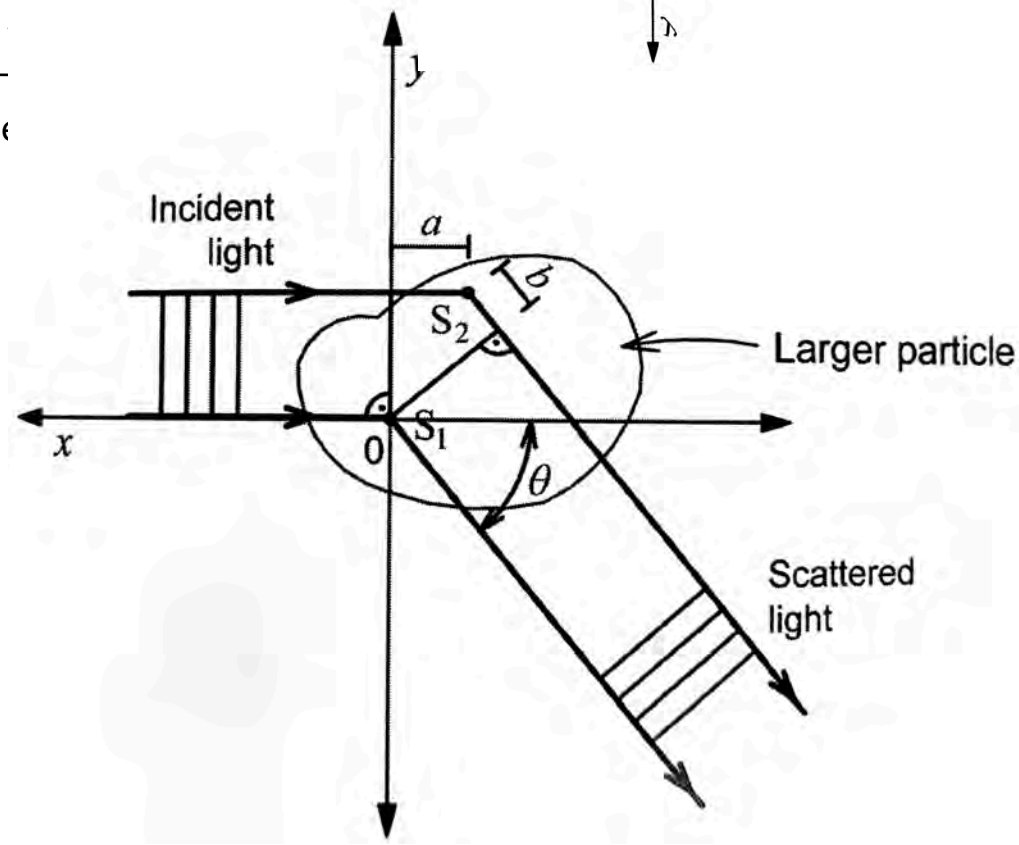
EM of the incident light induces an oscillation in the electron cloud, with the same frequency as the incident light

The oscillating electron cloud (dipole) then generates EM (of the same frequency)

Scattering







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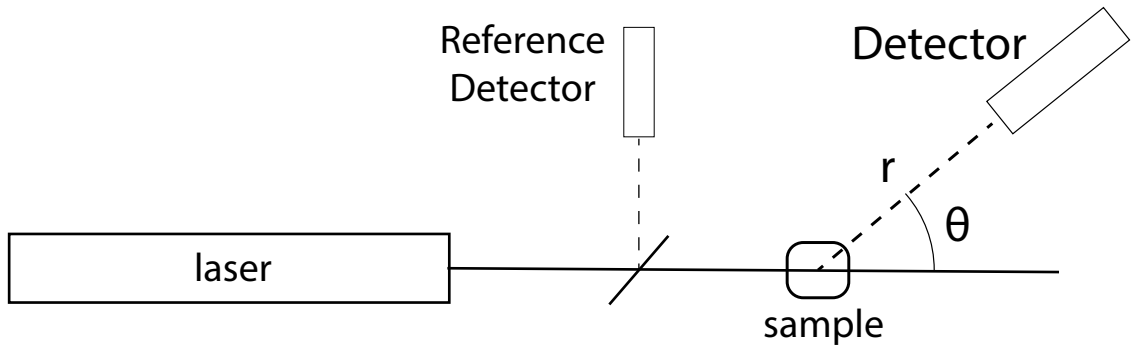


$$I_{scattered}(\theta) = K \; I_{laser} \frac{V_{scat}(\theta)}{r^2} C \; M \qquad K = \frac{(2\pi n_o)^2 \left(\frac{dn}{dC}\right)^2}{N_A \lambda^4}$$

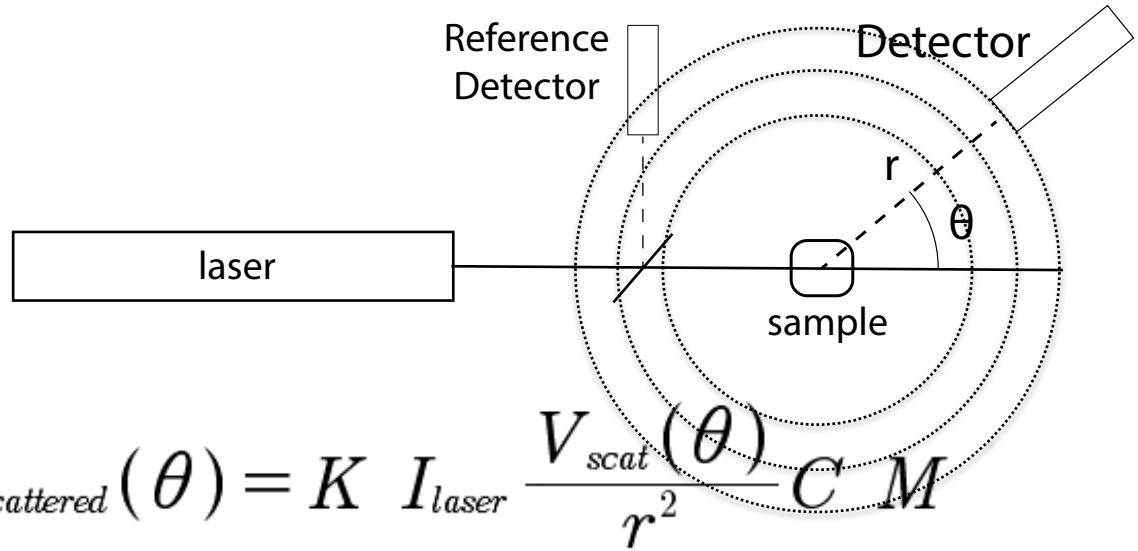
M molar mass (molecular weight) of solute (g/mol)

n_o refractive index of the solvent in the absence of solute

$\frac{dn}{dC}$ change in refractive index of the solvent vs weight concentration of solute
= 0.185 mL/g for proteins (in water)

C = weight concentration of solute (g/mL) λ = wavelength of laser light $N_A = 6.022 \cdot 10^{23}\ mol^{-1}$

Scattering



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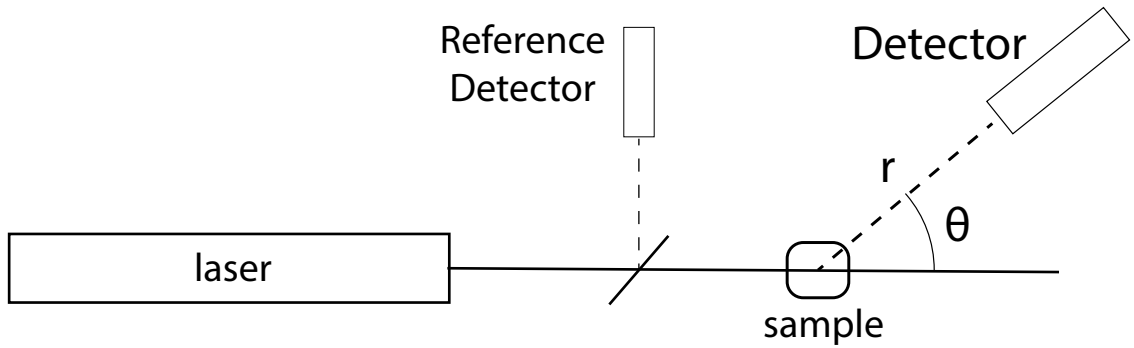
= 0.185 mL/g for proteins (in water)

$V_{scat}(\theta)$ scattering "volume" seen by the detector at angle θ and distance r

C = weight concentration of solute (g/mL) λ = wavelength of laser light $N_A = 6.022 \cdot 10^{23}\ mol^{-1}$

Scattering



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n_o refractive index of the solvent in the absence of solute

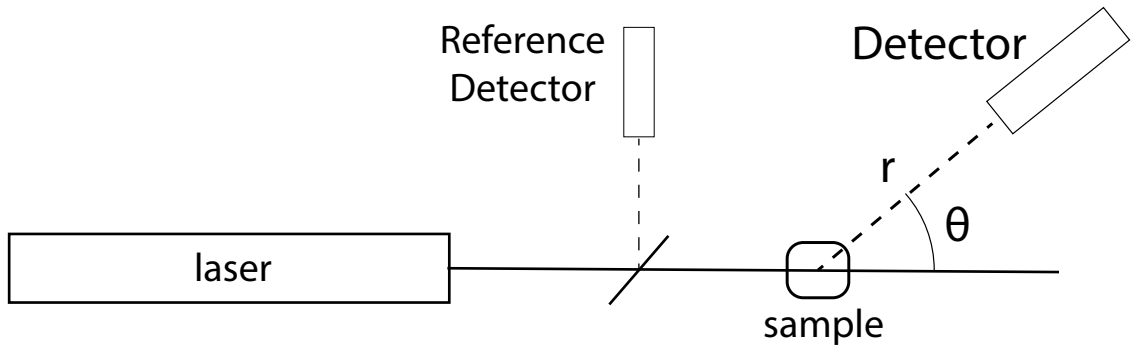
$\frac{dn}{dC}$ change in refractive index of the solvent vs weight concentration of solute
= 0.185 mL/g for proteins (in water)

$V_{scat}(\theta)$ scattering "volume" seen by the detector at angle θ and distance r

$P(\theta)$ correction factor that considers the SHAPE of the solute

C = weight concentration of solute (g/mL) λ = wavelength of laser light $N_A = 6.022 \cdot 10^{23}\ mol^{-1}$

Scattering



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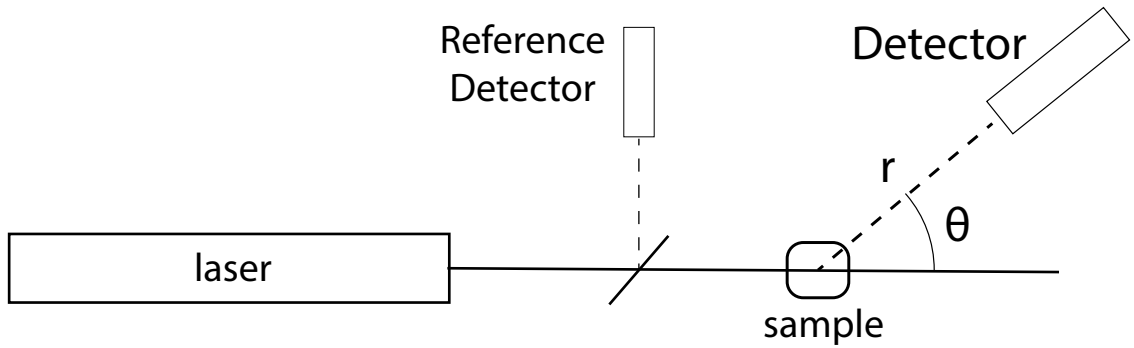

$$\frac{I_{scattered}(\theta)}{I_{laser}}\frac{r^2}{V_{scat}(\theta)} = R(\theta) = K\,C\,M\,P(\theta) \qquad K = \frac{(2\pi n_o)^2\left(\frac{dn}{dC}\right)^2}{N_A\,\lambda^4}$$

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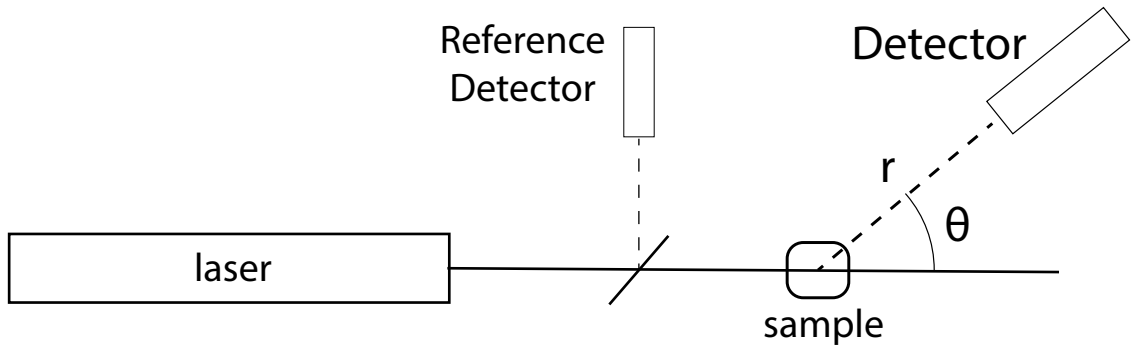

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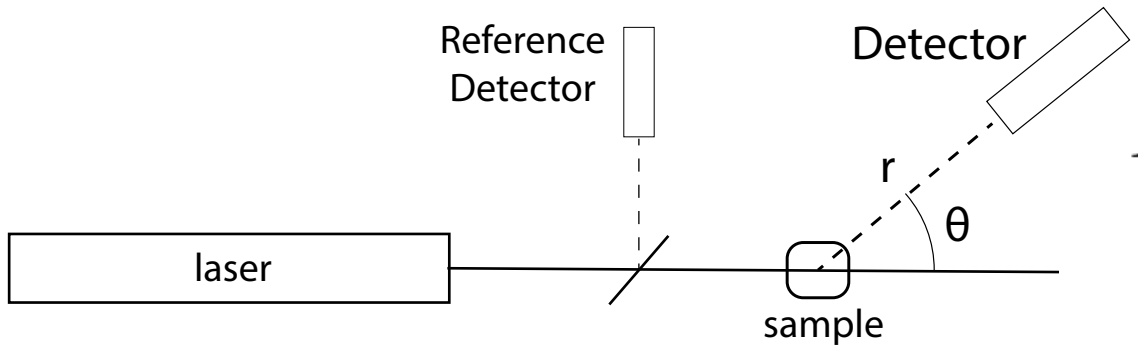

$$R(\theta) = \frac{I_{scattered}(\theta)}{I_{laser}} \frac{r^2}{V_{scat}(\theta)}$$

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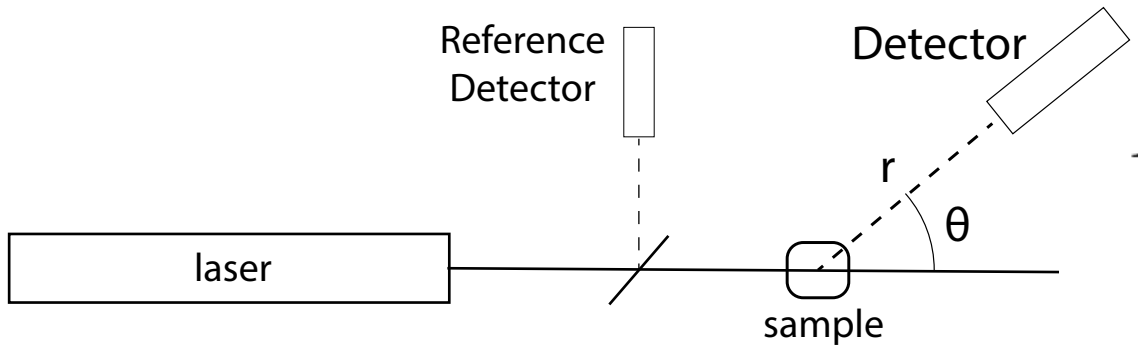

$$R(\theta) = \frac{I_{scattered}(\theta)}{I_{laser}} \frac{r^2}{V_{scat}(\theta)}$$

$$\frac{K\,C}{R(\theta)} = \frac{1}{M\,P(\theta)}$$

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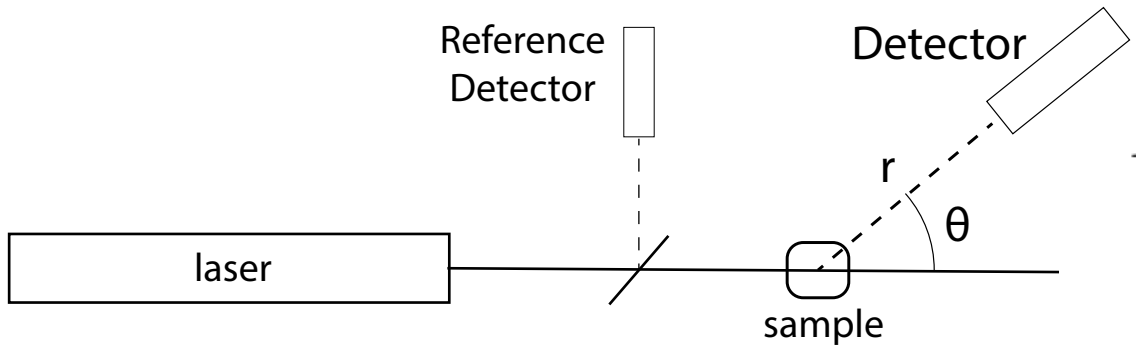

$$R(\theta) = \frac{I_{scattered}(\theta)}{I_{laser}} \frac{r^2}{V_{scat}(\theta)}$$

$$\frac{K\,C}{R(\theta)} = \frac{1}{M\,P(\theta)} + 2A_2C$$

$$K = \frac{(2\pi n_o)^2 \left(\frac{dn}{dC}\right)^2}{N_A \lambda^4}$$

M molar mass (molecular weight) of solute (g/mol)

$P(\theta)$ correction factor that considers the SHAPE of the solute

A_2 A_2 (second virial coefficient) is a measure of non-ideality - a measure of the interaction forces between dissolved particles: If A_2 is positive, the interparticle forces are repulsive. If it is negative the forces are attractive.

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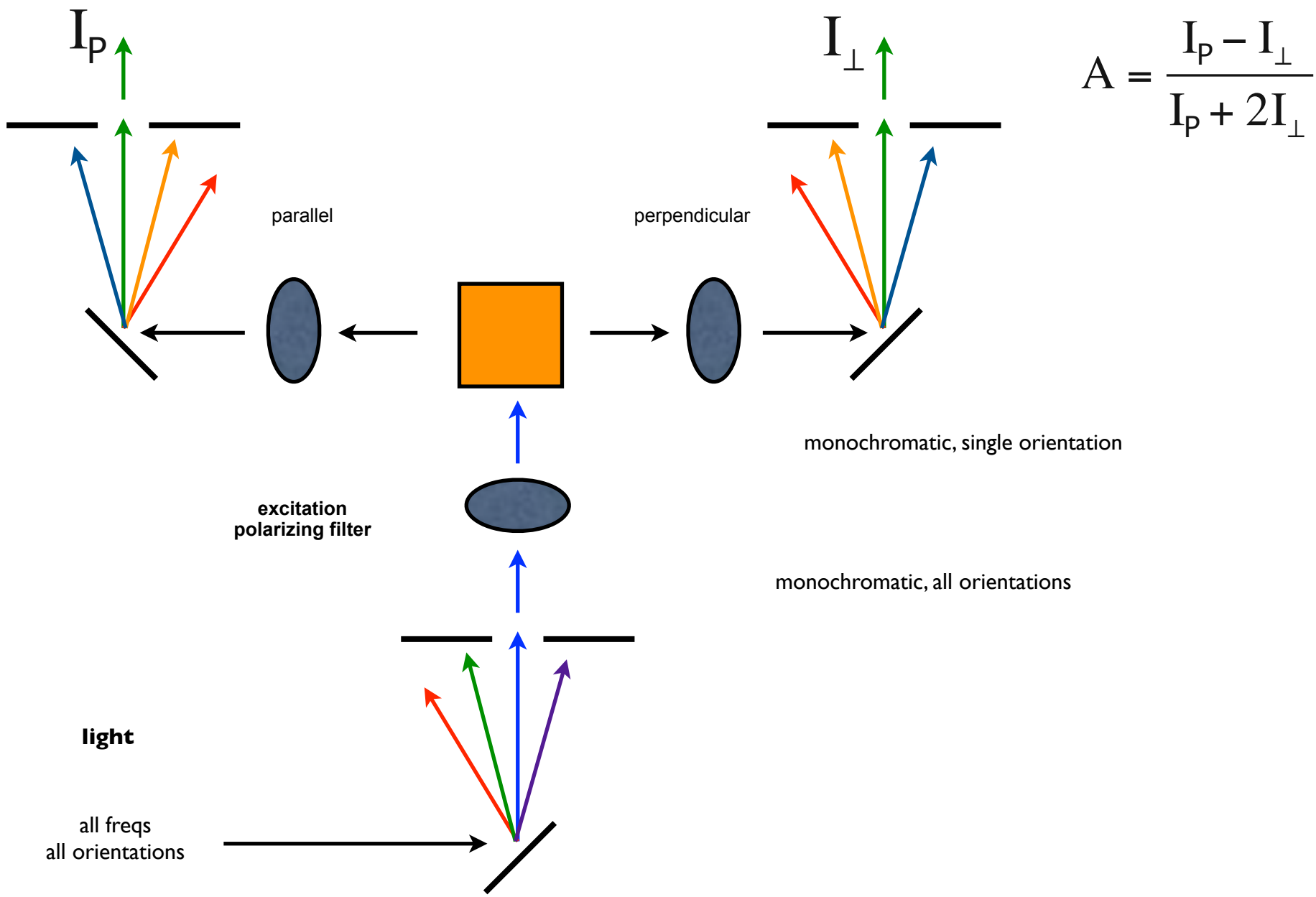
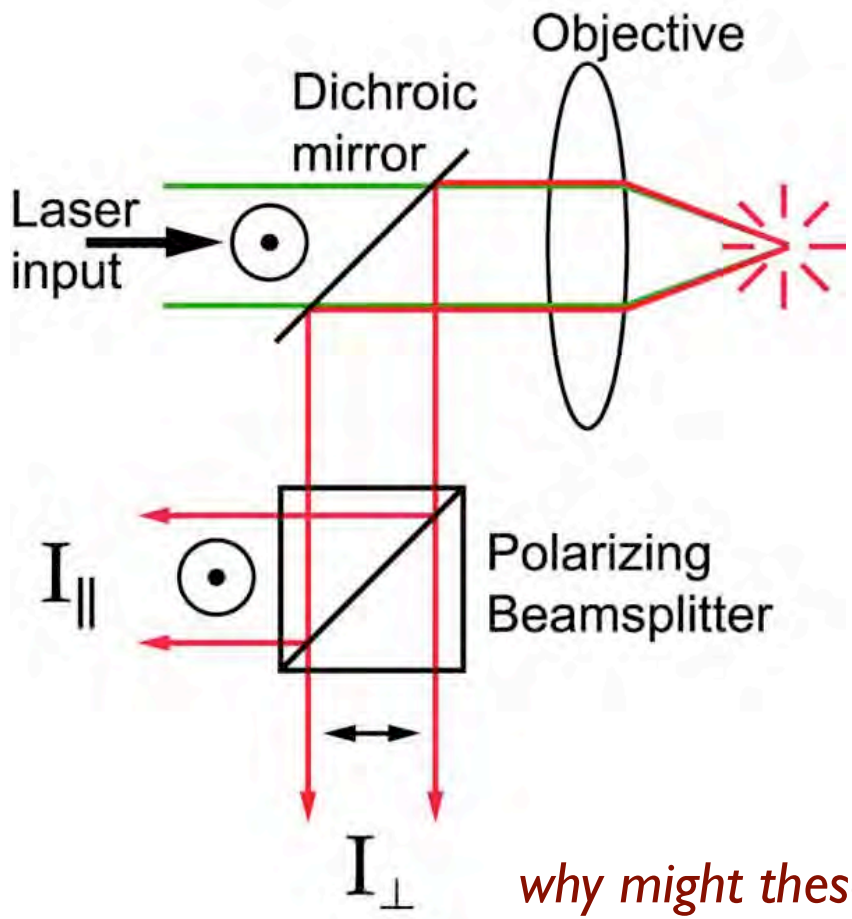
Revisit T-format Anisotropy Measurement

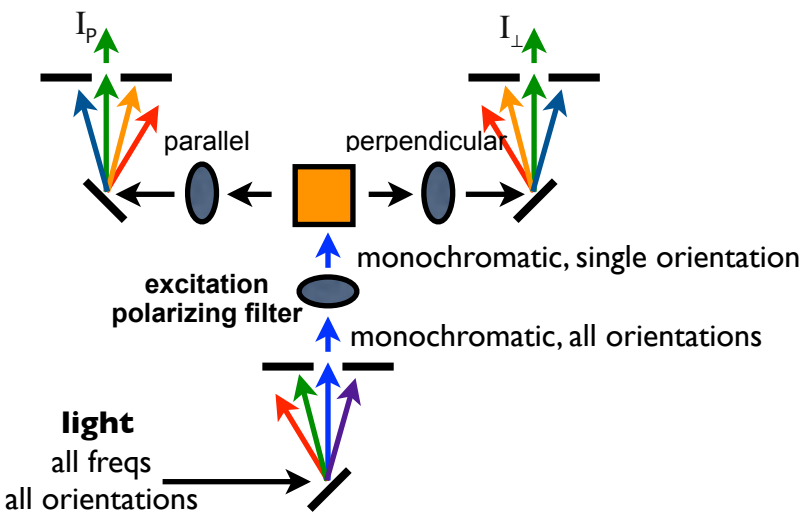


Confocal Fluorescence Microscopy Anisotropy

Polarized Monochromatic



$$A = \frac{I_P - I_\perp}{I_P + 2I_\perp}$$



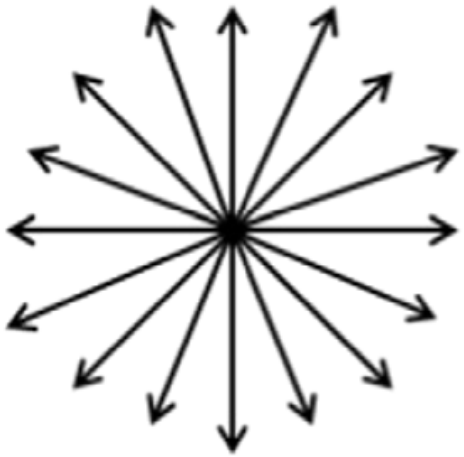
why might these have advantages over simple fluorescence or direct FRET?

Similarly, fluorescence lifetime imaging (FLIM) FRET

Scattering

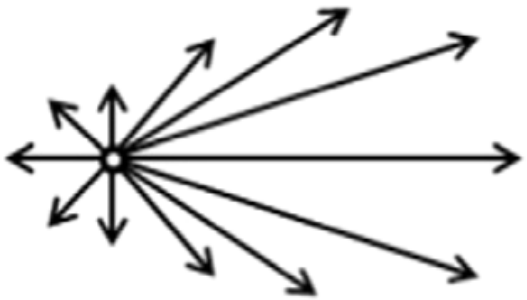
Rayleigh scattering

- Particle size < λ/10
- Not angle-dependent
- Elastic scattering



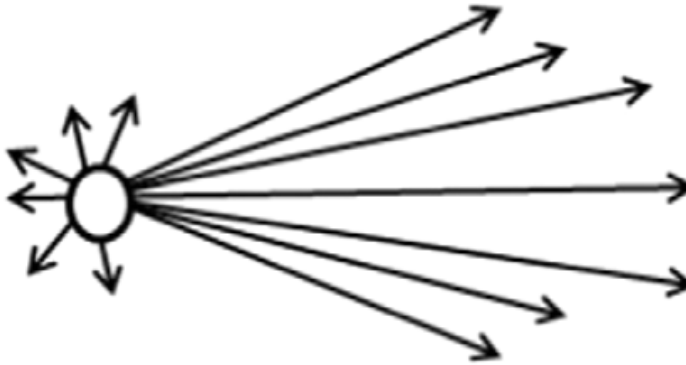
Mie scattering

- Particle size > λ/10
- Angle-dependent
- Inelastic scattering



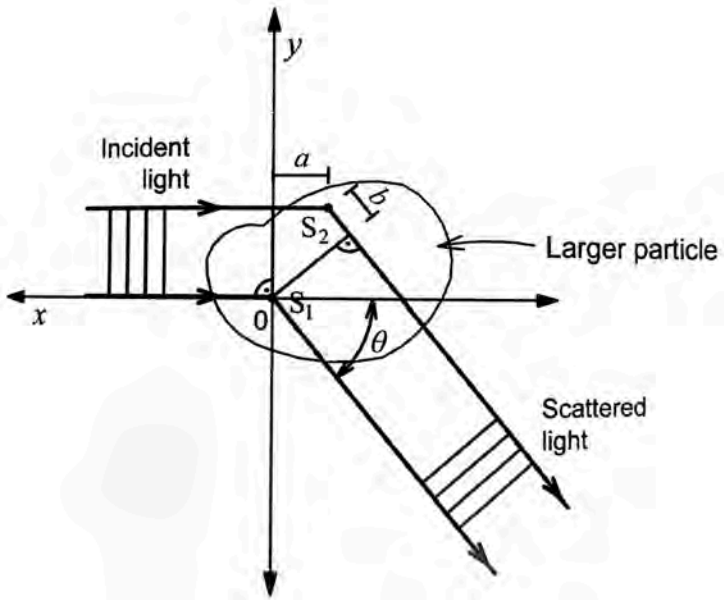
Mie scattering

angle-dependence increases with bigger particles



Incident laser

wavelength λ



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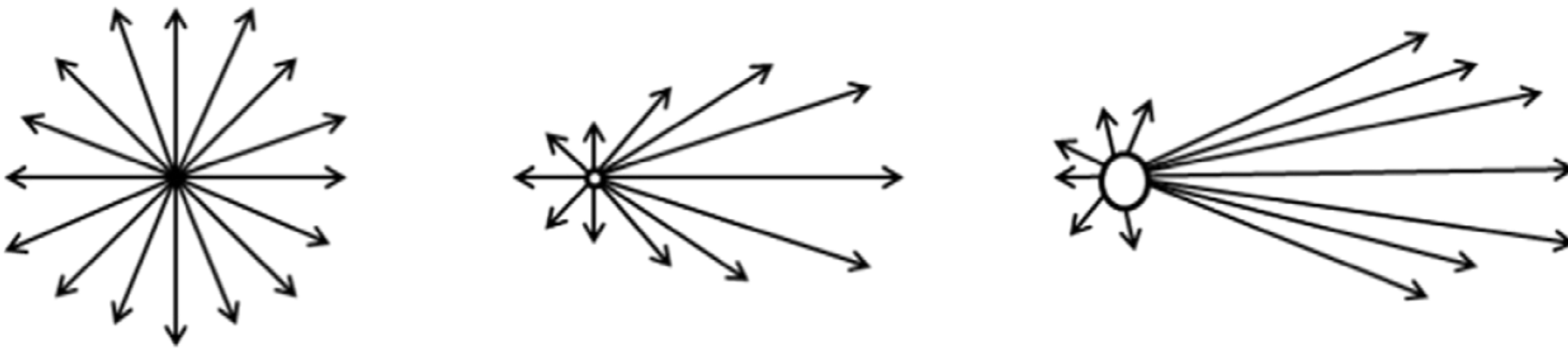

Incident laser

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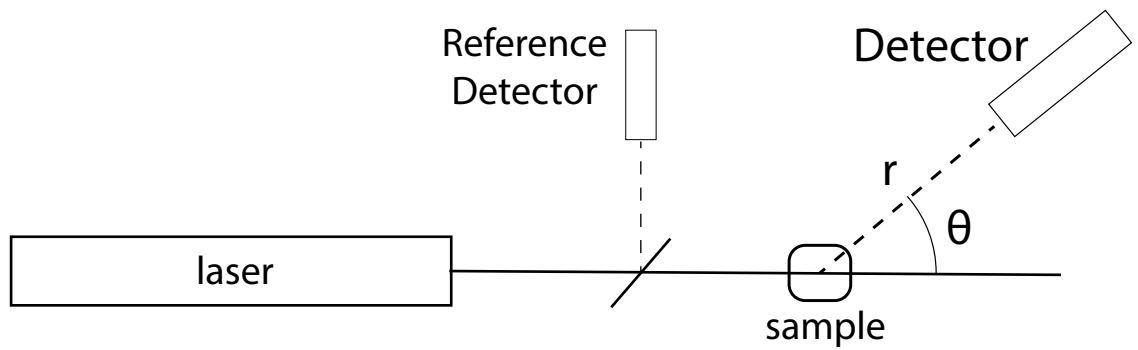
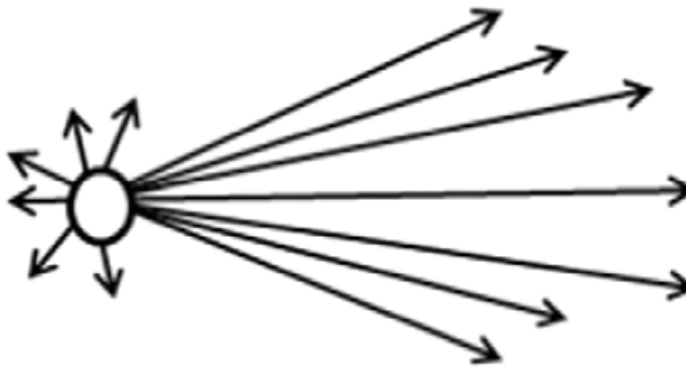


Diffusion (Brownian Motion)

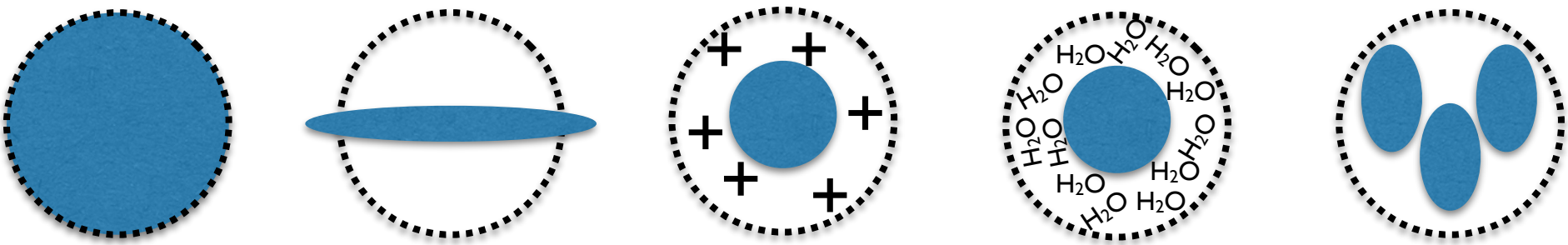
$$D = \frac{k_B T}{6\pi\eta r}$$

η = solution viscosity

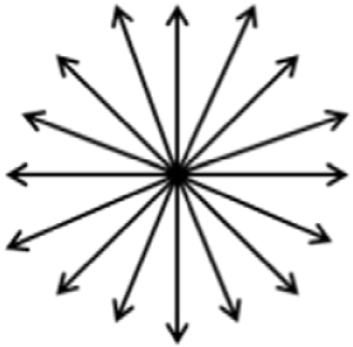


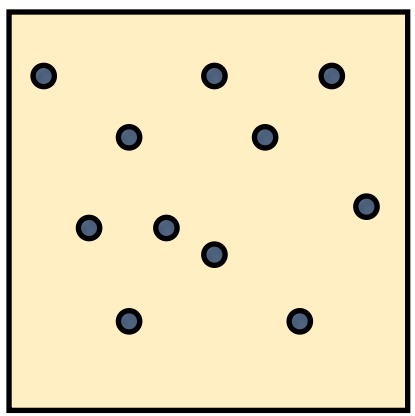
r = hydrodynamic radius

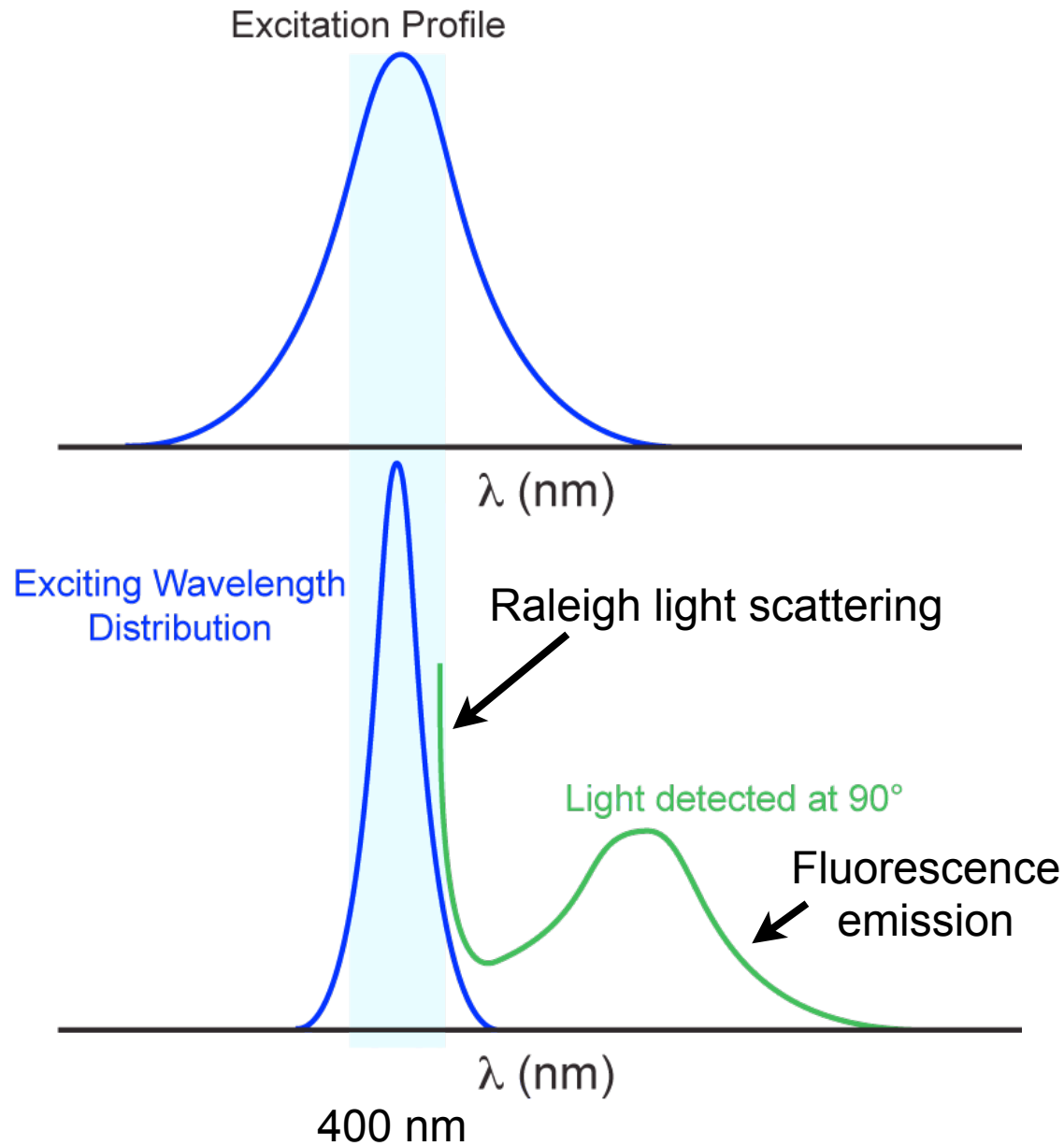
"*apparent* size"

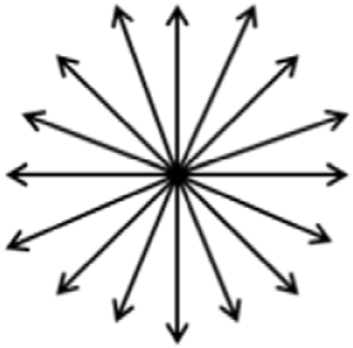


Raleigh Scattering

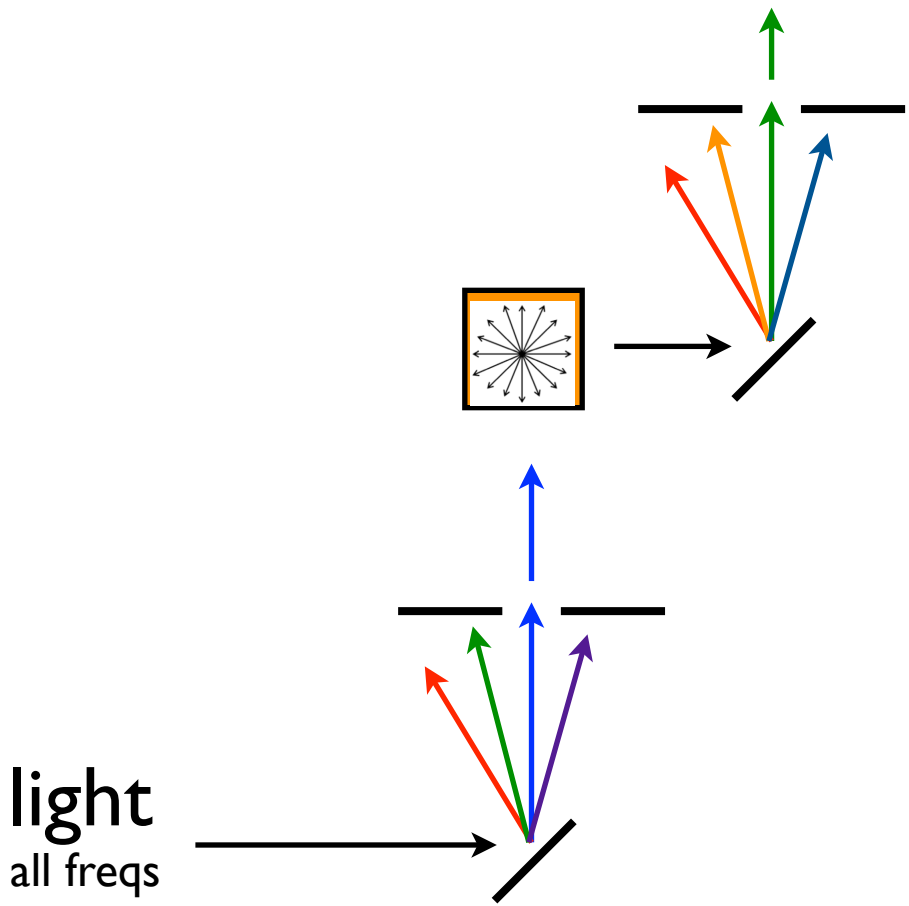


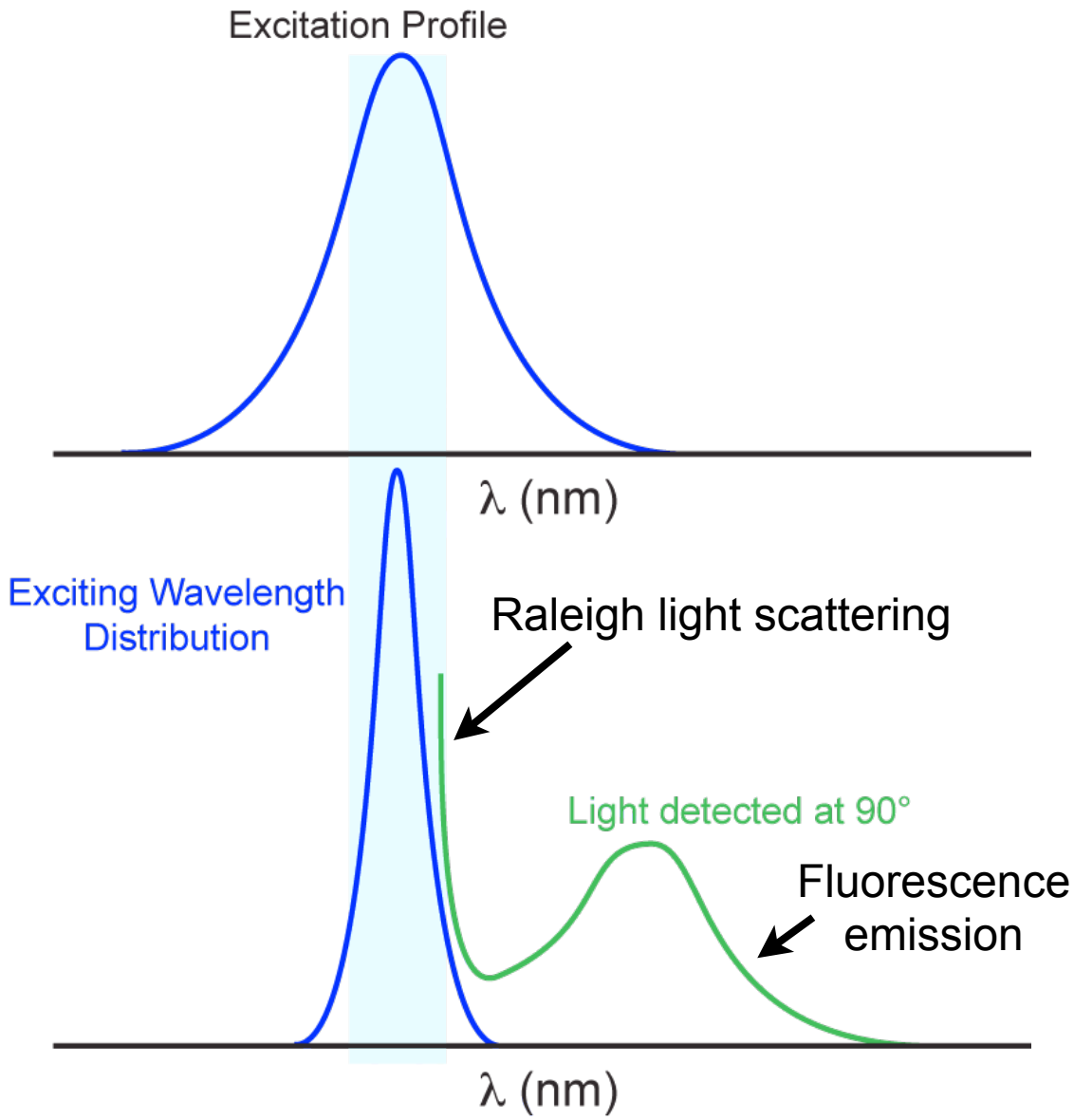




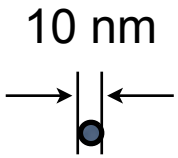


Fluorescence Spectroscopy

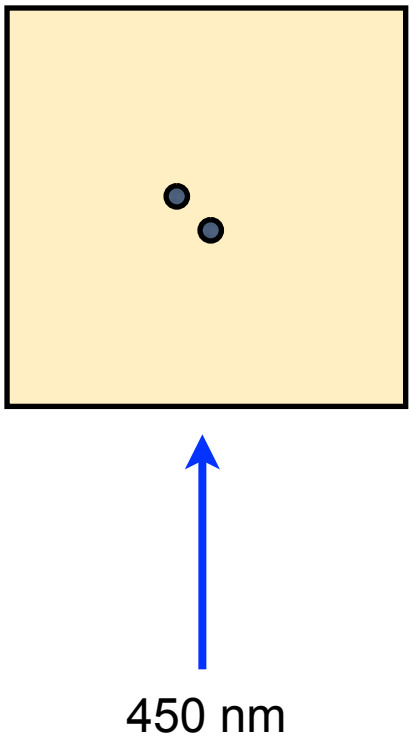




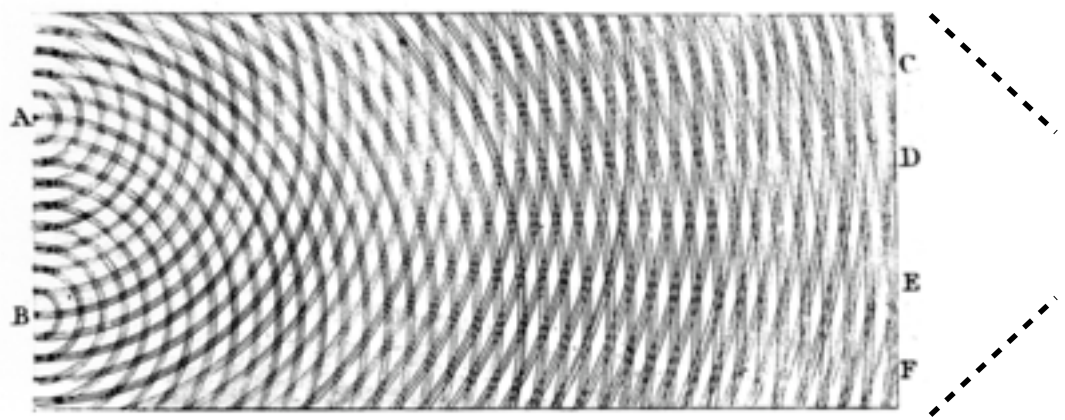
Scattering - TWO particle



Particle Pair



Diffraction Pattern



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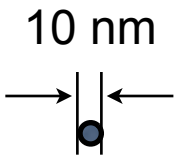
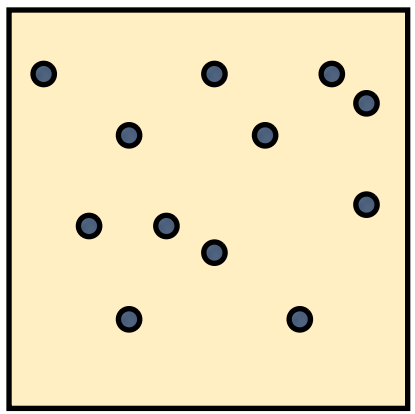
Constructive & Destructive Interference

you see a pattern of dark and light spots

Scattering - MANY particles



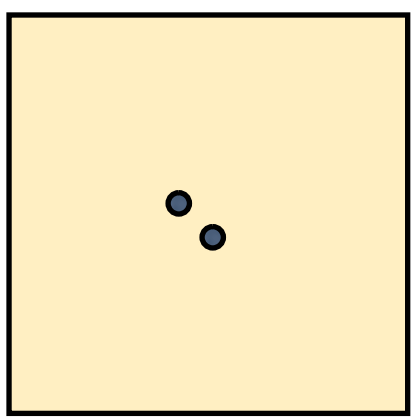


Many pairwise groups of constructive & destructive interferences

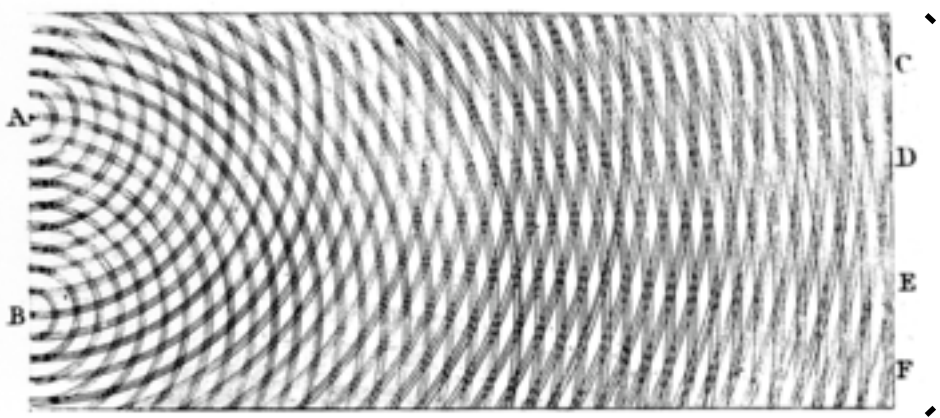
But no coherent additivity

you see dark and light spots, but no pattern

Scattering but no pattern



450 nm

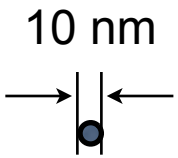


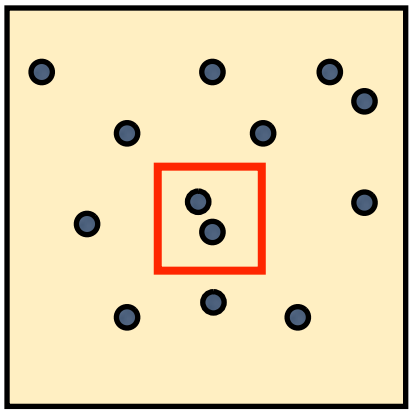
Thomas Young - Royal Society, 1803

Constructive & Destructive Interference

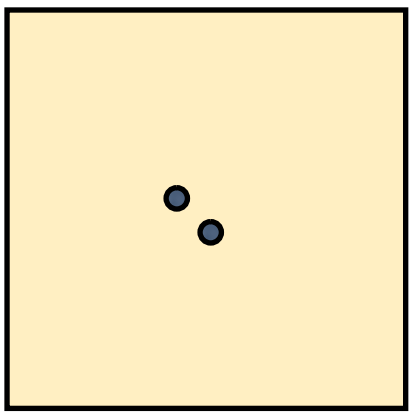
you see a pattern of dark and light spots

Dynamic Light Scattering

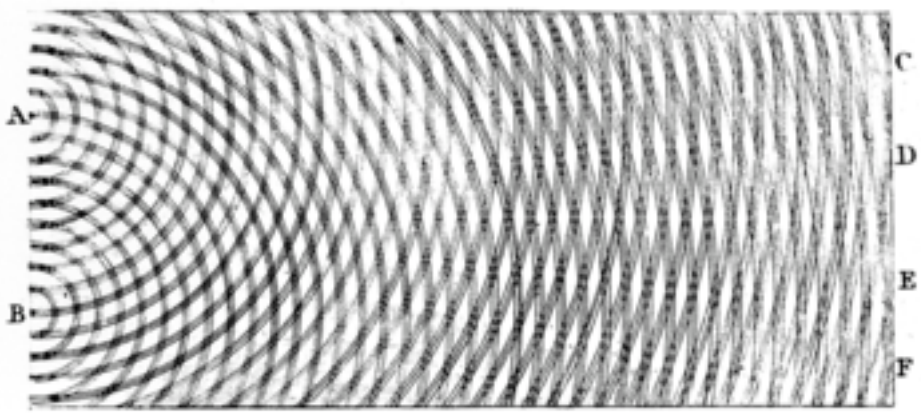




Pair of molecules yields
scattering at a specific angle and magnitude



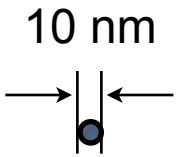
450 nm

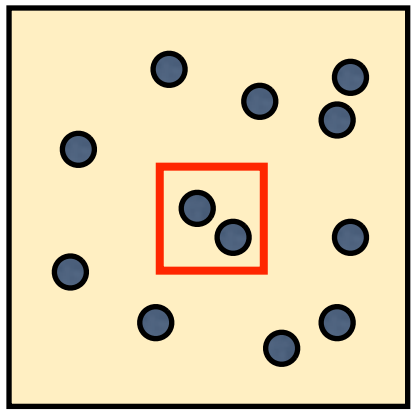


Constructive
& Destructive
Interference

Thomas Young - Royal Society, 1803

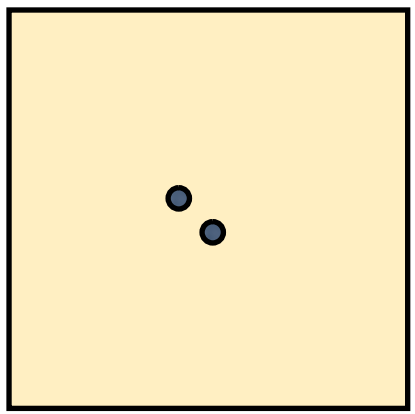
Dynamic Light Scattering



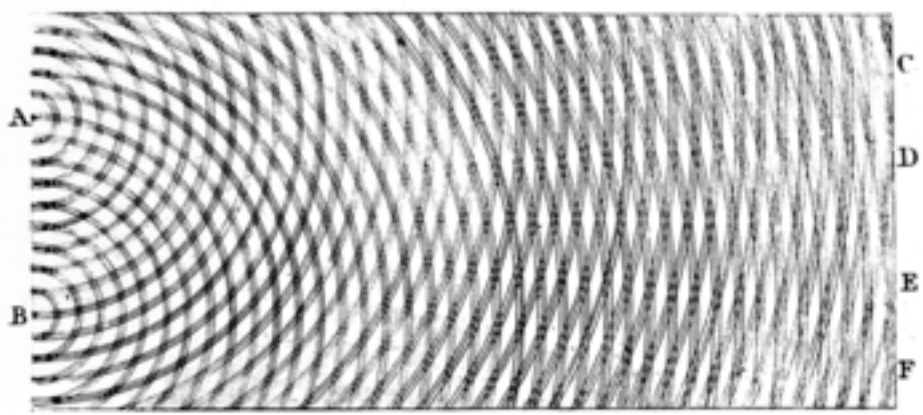


Pair of molecules yields
scattering at a specific angle and magnitude

Different sizes - a new angle & magnitude



450 nm



Constructive
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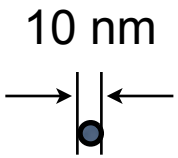
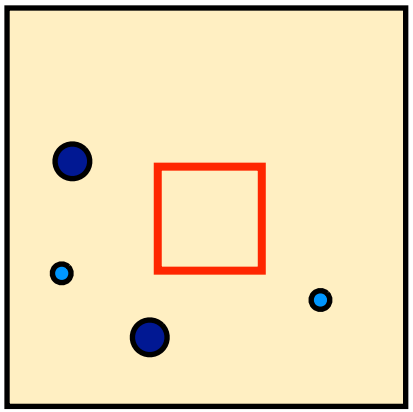
Thomas Young - Royal Society, 1803

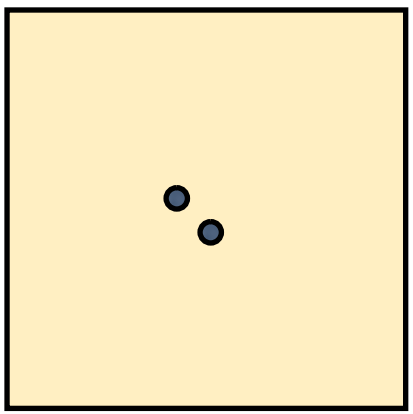
Dynamic Light Scattering

Photon Correlation Spectroscopy

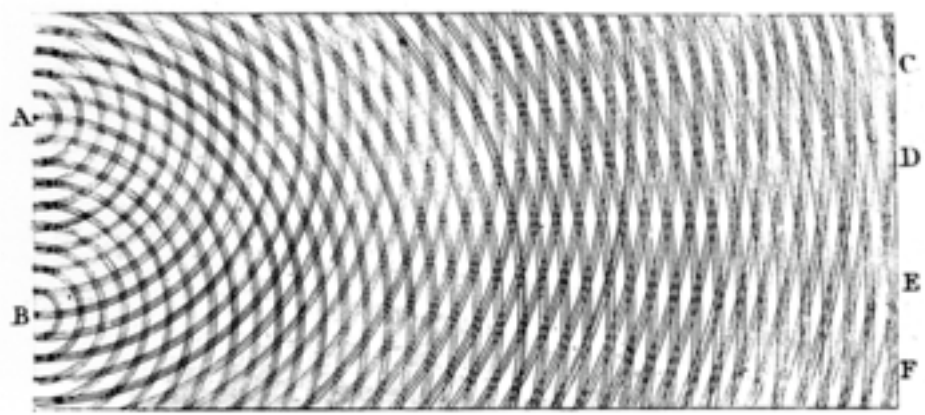




Volume empty - no scattering



450 nm

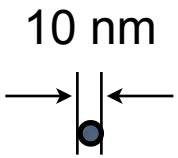


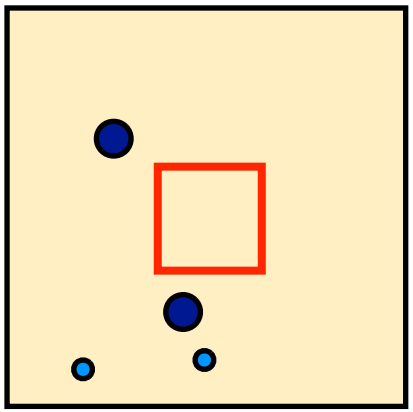
Constructive & Destructive Interference

Thomas Young - Royal Society, 1803

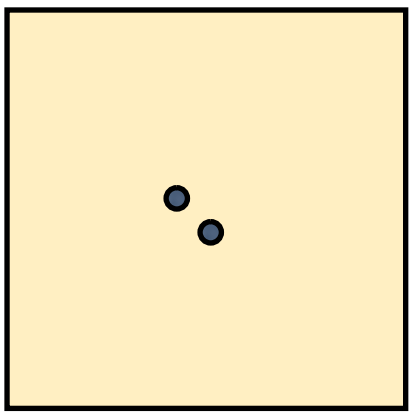
Dynamic Light Scattering

Photon Correlation Spectroscopy

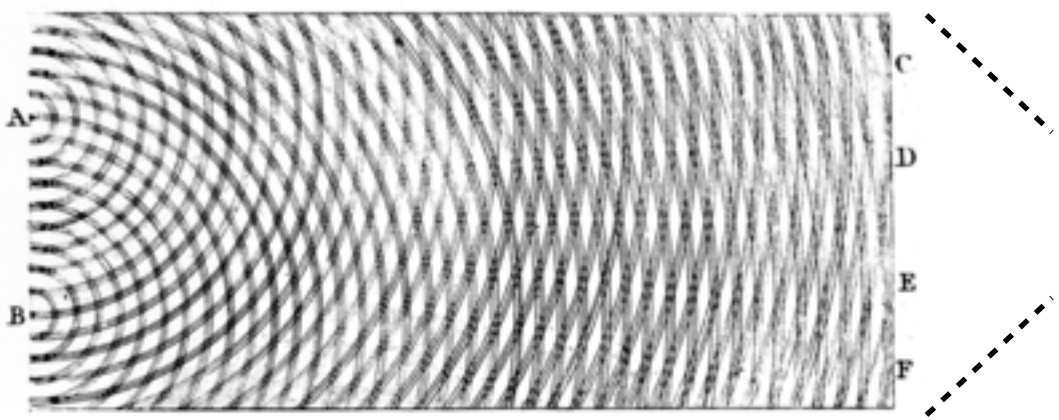




Volume empty - no scattering



450 nm

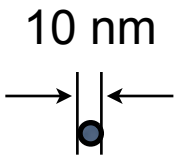


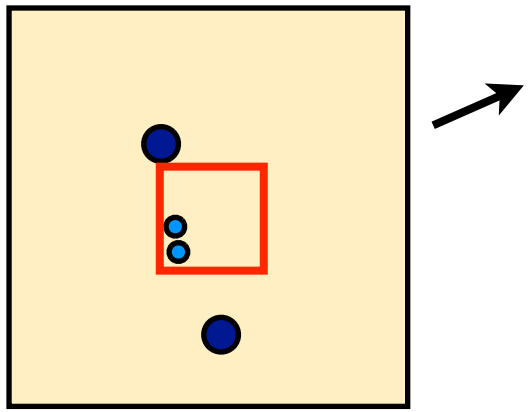
Constructive & Destructive Interference

Thomas Young - Royal Society, 1803

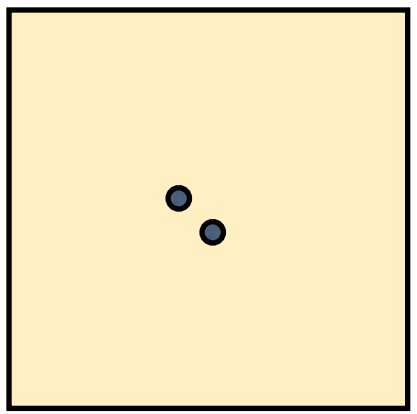
Dynamic Light Scattering

Photon Correlation Spectroscopy

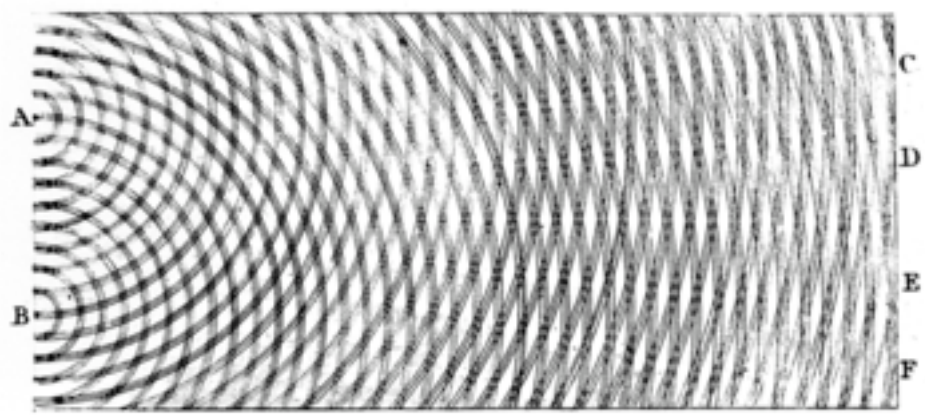




Smaller molecule scattering - angle 1



450 nm

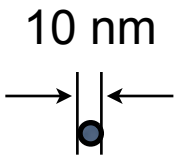


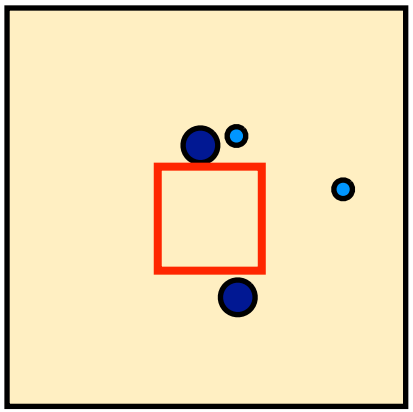
Constructive & Destructive Interference

Thomas Young - Royal Society, 1803

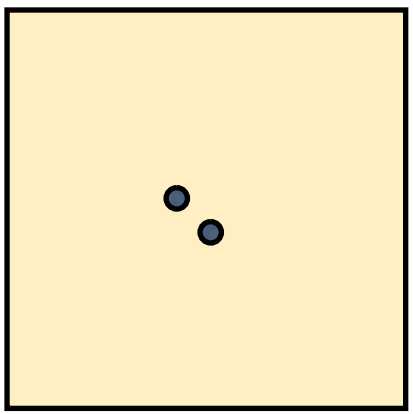
Dynamic Light Scattering

Photon Correlation Spectroscopy

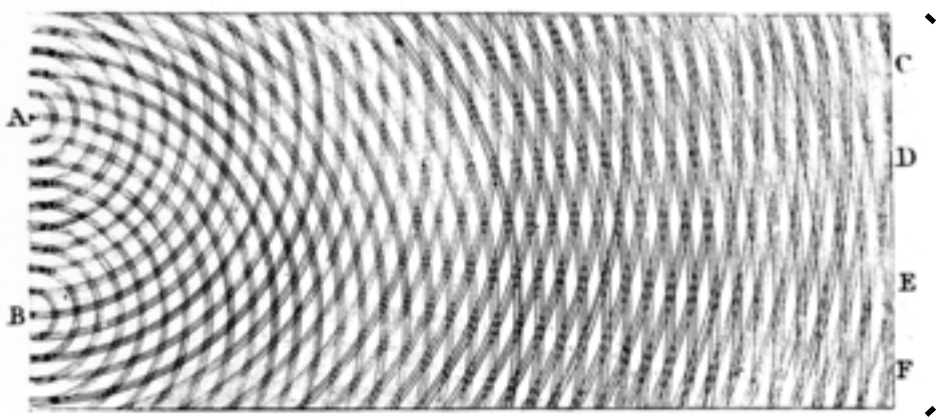




Volume empty - no scattering



450 nm

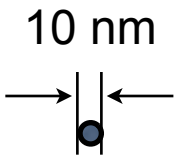


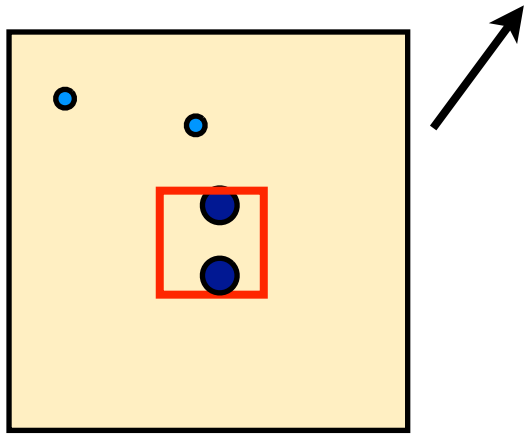
Constructive & Destructive Interference

Thomas Young - Royal Society, 1803

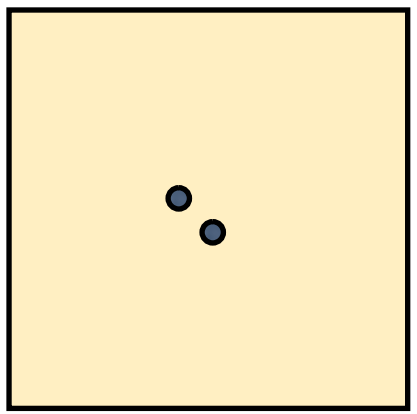
Dynamic Light Scattering

Photon Correlation Spectroscopy

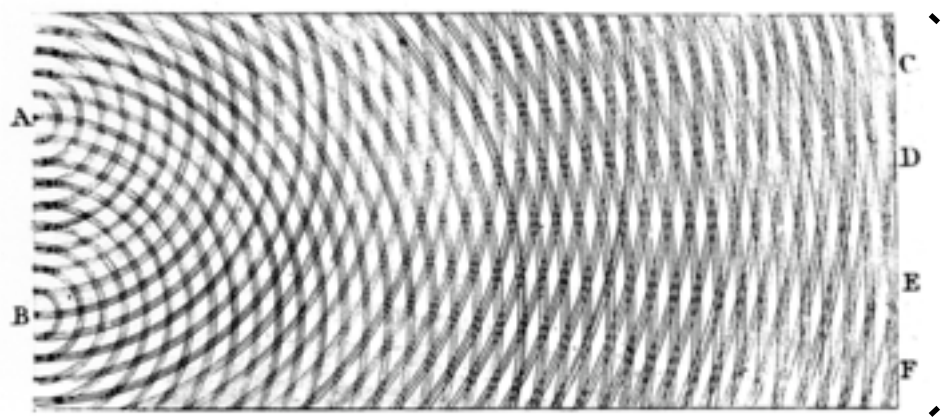




Larger molecule scattering - angle 2



450 nm



Constructive & Destructive Interference

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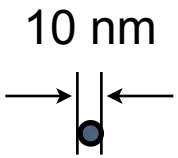
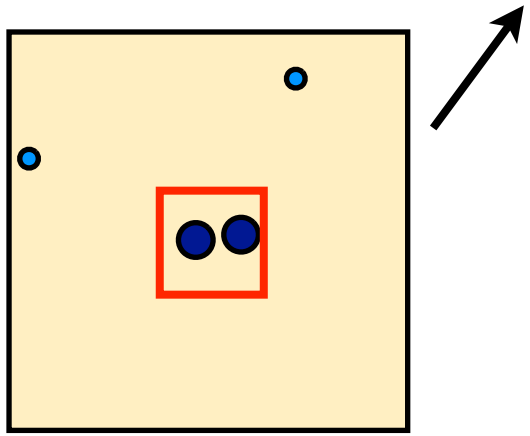
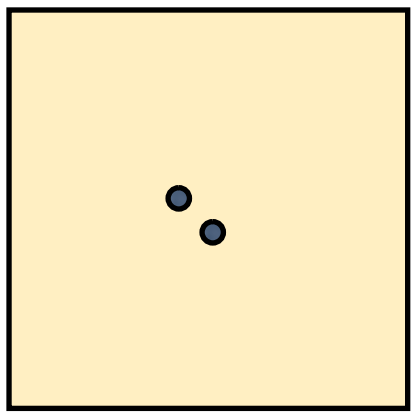
Dynamic Light Scattering

Photon Correlation Spectroscopy

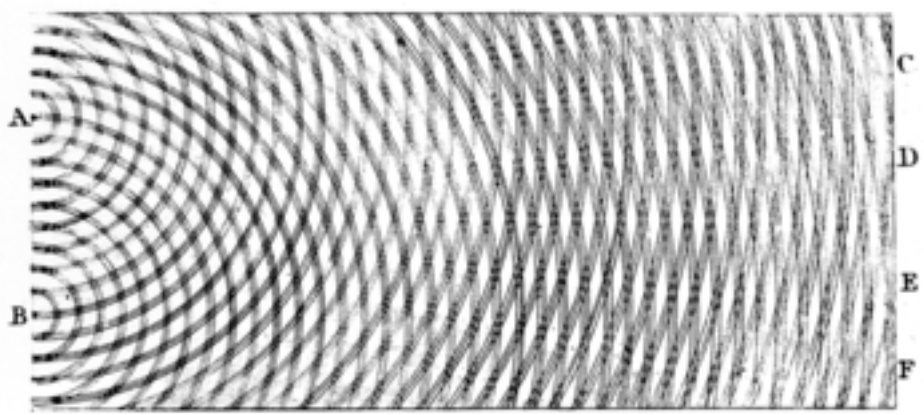




Larger molecule scattering - angle 2



450 nm



Constructive & Destructive Interference

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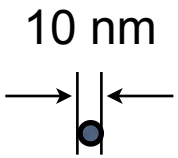
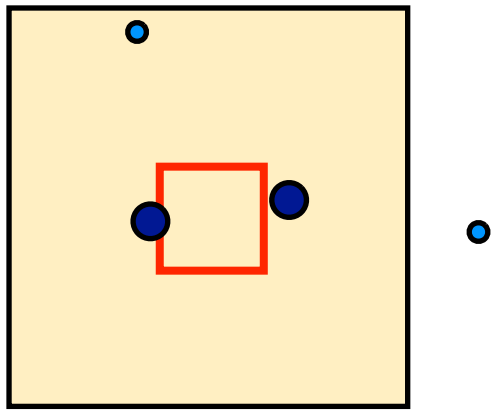
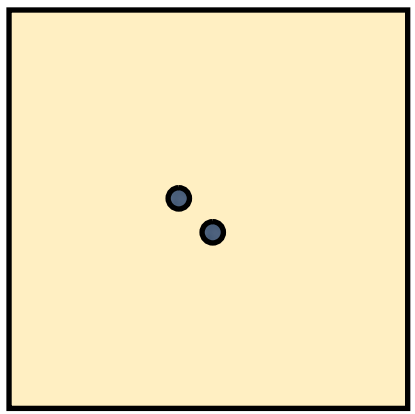
Dynamic Light Scattering

Photon Correlation Spectroscopy

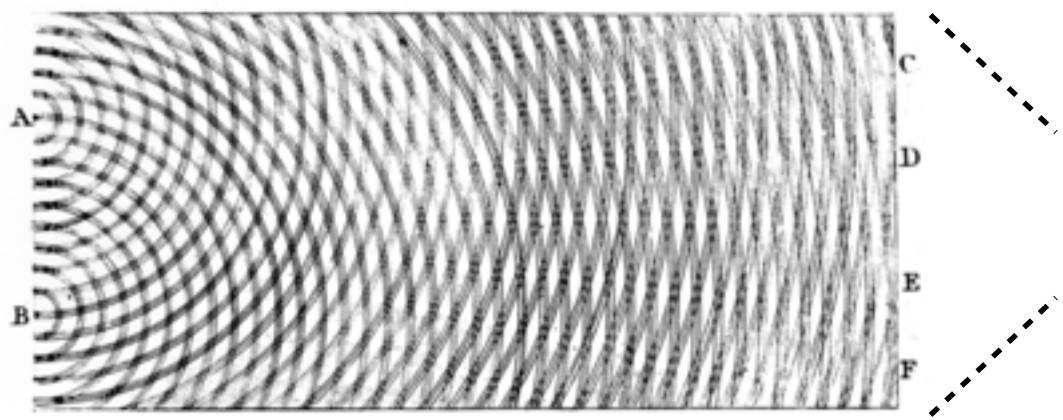




Volume empty - no scattering



450 nm



Constructive & Destructive Interference

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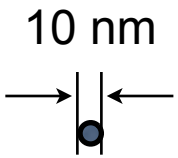
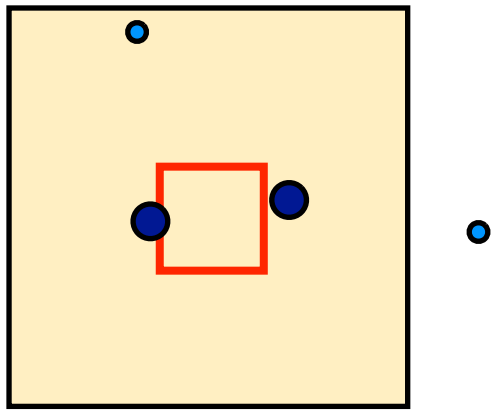
Dynamic Light Scattering

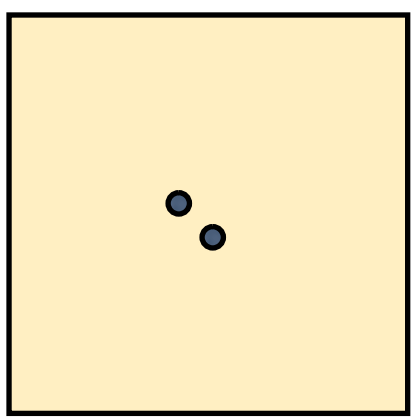
Photon Correlation Spectroscopy



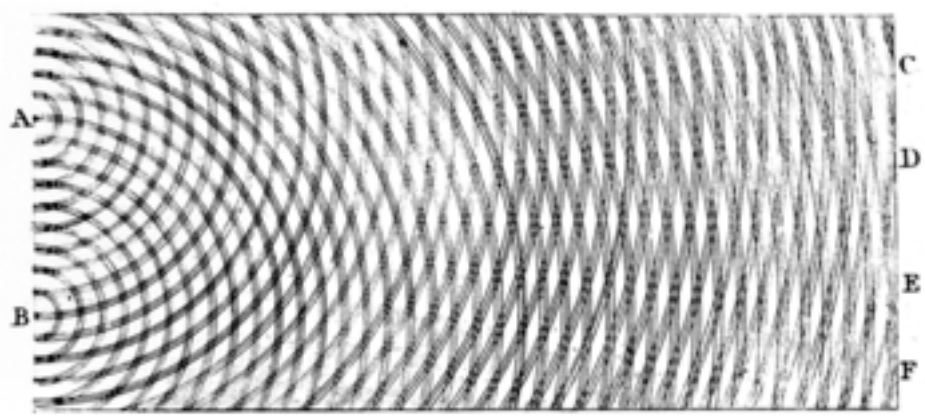
you see dark and light spots, no pattern. "**blinking**"



Volume empty - no scattering



450 nm

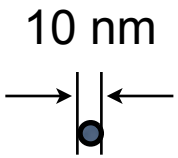


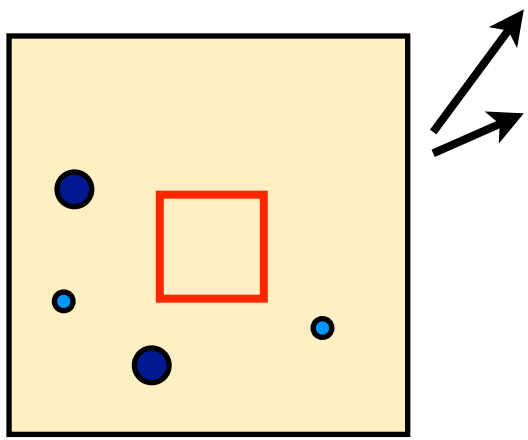
Constructive & Destructive Interference

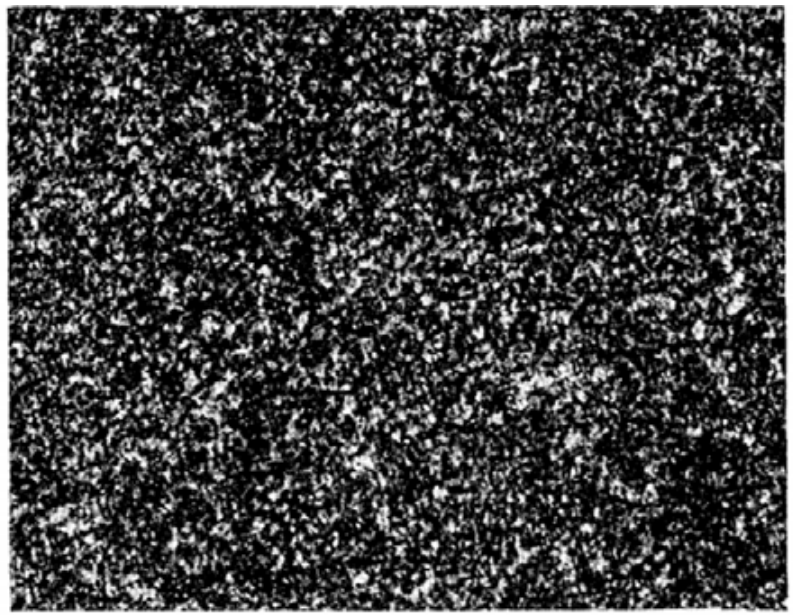
Thomas Young - Royal Society, 1803

Dynamic Light Scattering

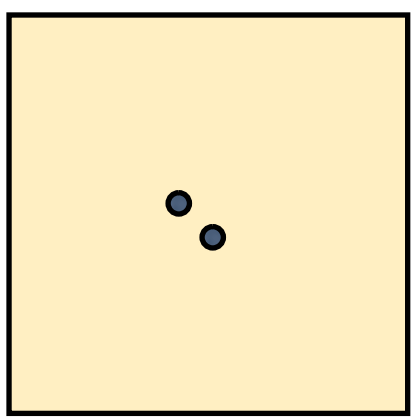
Photon Correlation Spectroscopy

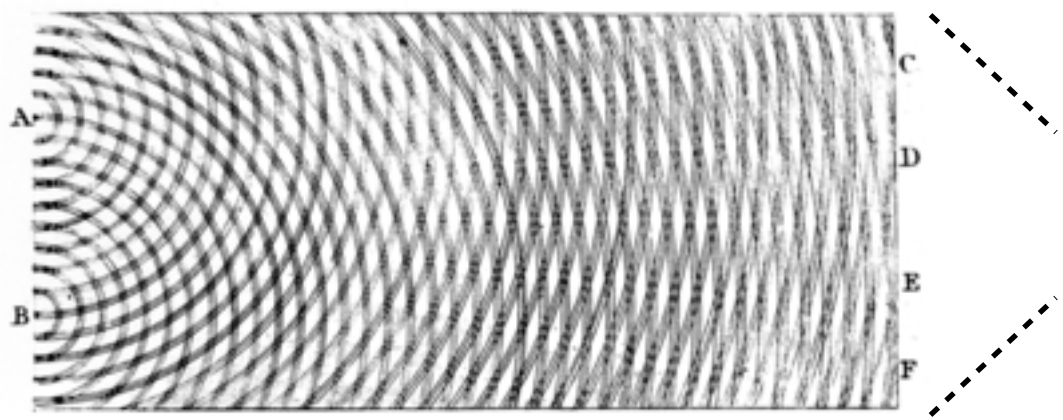






you see dark and light spots, no pattern. "**blinking**"





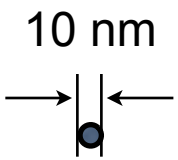
Constructive & Destructive Interference

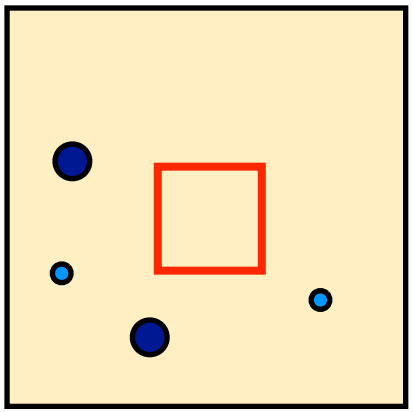
450 nm

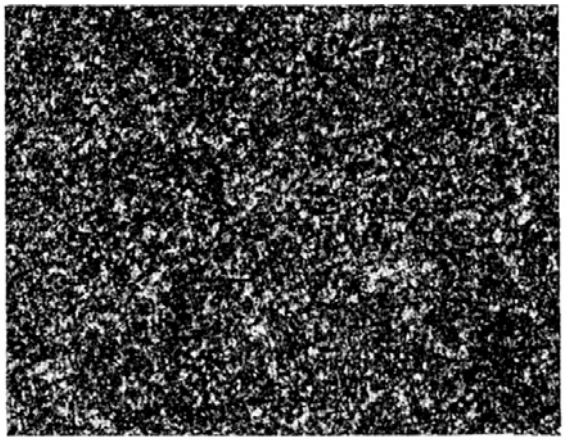
Thomas Young - Royal Society, 1803

Dynamic Light Scattering

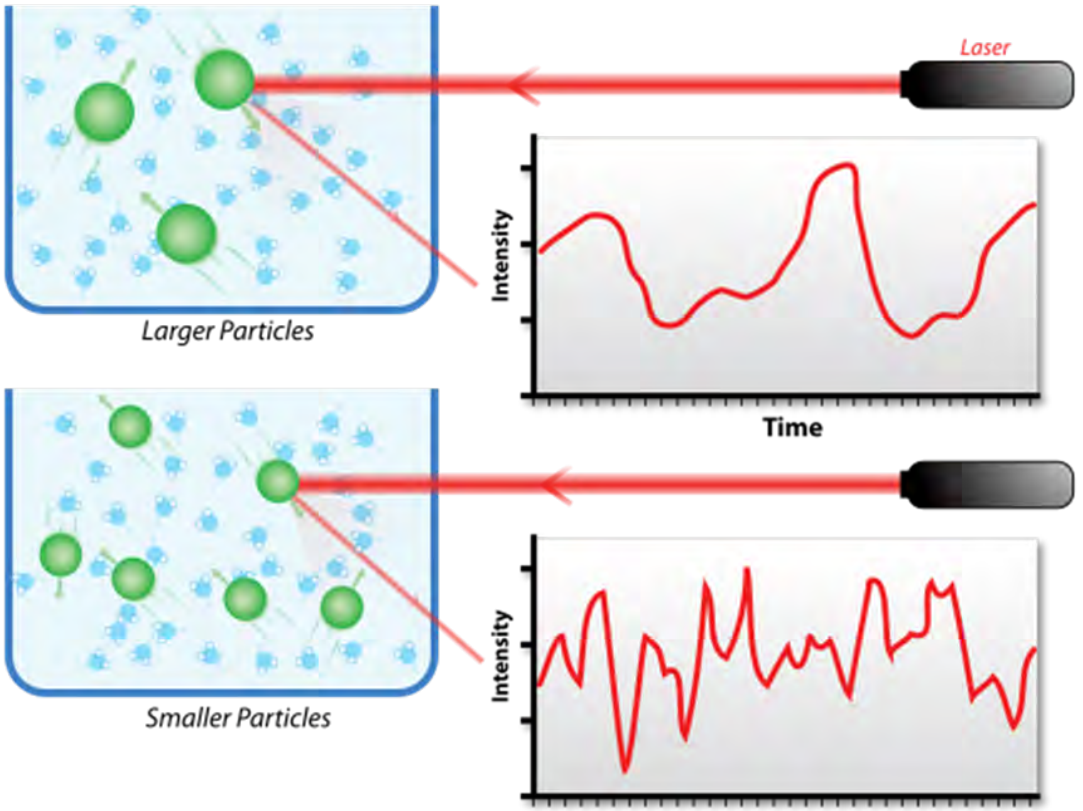
Photon Correlation Spectroscopy





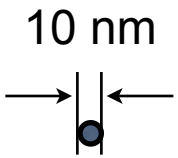


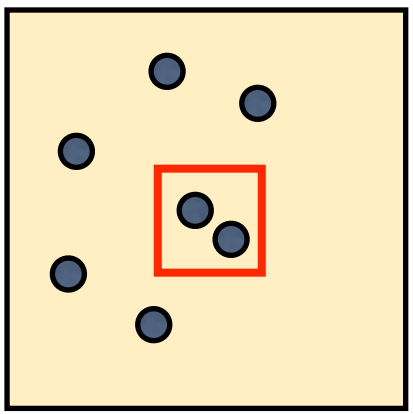
you see dark and light spots, no pattern. "**blinking**"

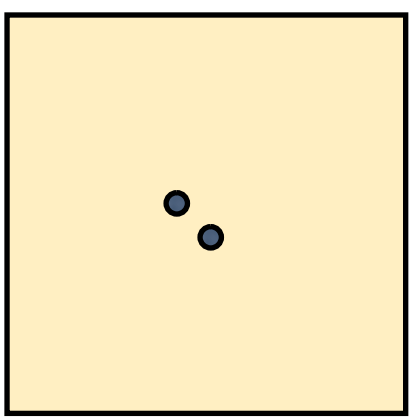


Dynamic Light Scattering

Photon Correlation Spectroscopy







Autocorrelation function

$$g^2(q,t) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

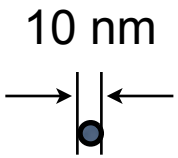
Specific wave vector

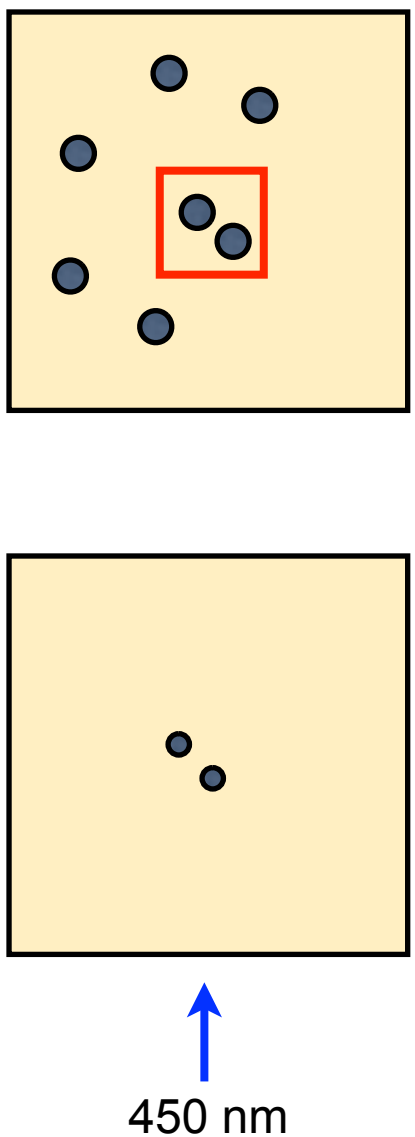
time

$\langle f(t) \rangle$ = time average of $f(t)$

Dynamic Light Scattering

Photon Correlation Spectroscopy





Autocorrelation function

small time interval

$$g^2(q,t) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

Specific wave vector

time

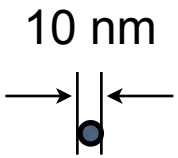
Intensity of scattered light

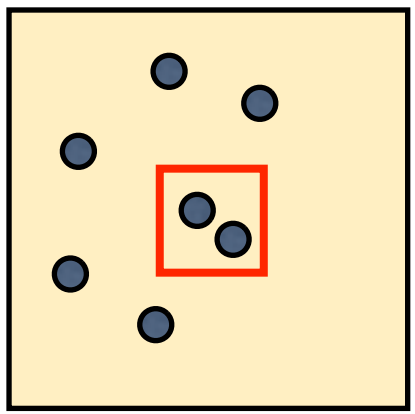
non-zero only when particles "stick around" in time τ

$\langle f(t) \rangle$ = time average of $f(t)$

Dynamic Light Scattering

Photon Correlation Spectroscopy





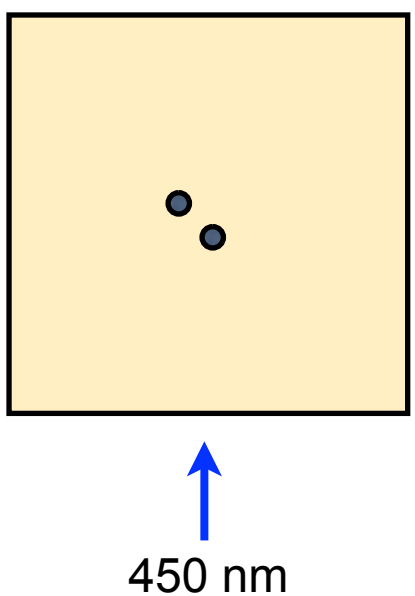
$$g^2(q,t) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

non-zero only when particles "stick around" in time τ

Relates to probability distribution function

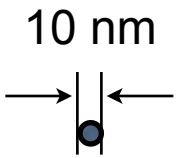
$$P(r,t \mid 0,0) = (4\pi Dt)^{-3/2} \; e^{\frac{-r^2}{4Dt}}$$

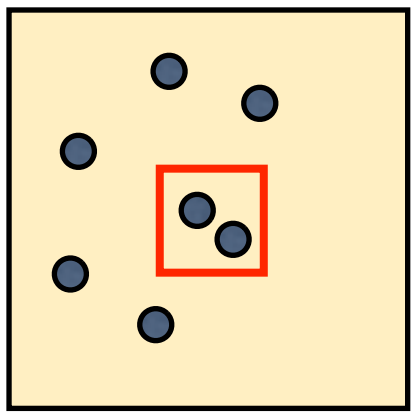
Assumes random (Brownian) motion



Dynamic Light Scattering

Photon Correlation Spectroscopy

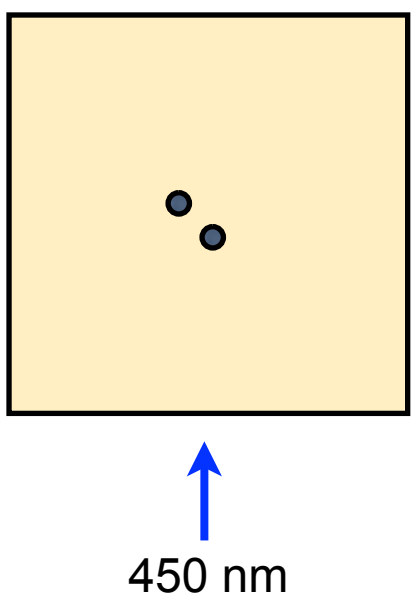




$$g^2(q,t) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

non-zero only when particles "stick around" in time τ

Diffusion relates to (hydrodynamic) size



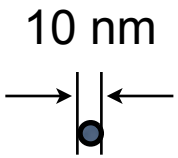
$$D = \frac{k_B T}{6\pi\eta r}$$

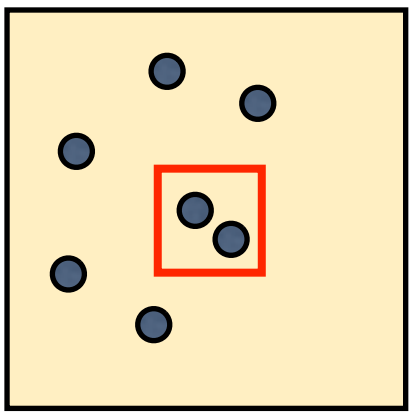
Assumes spherical particles of radius R

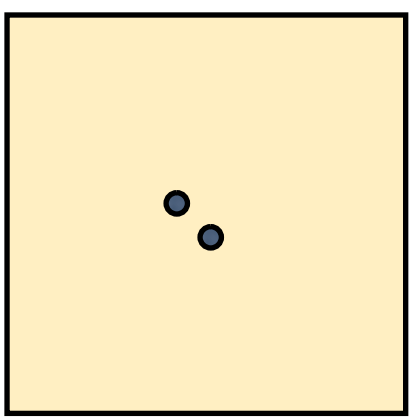
Calibrate with particles of known size

Dynamic Light Scattering

Photon Correlation Spectroscopy

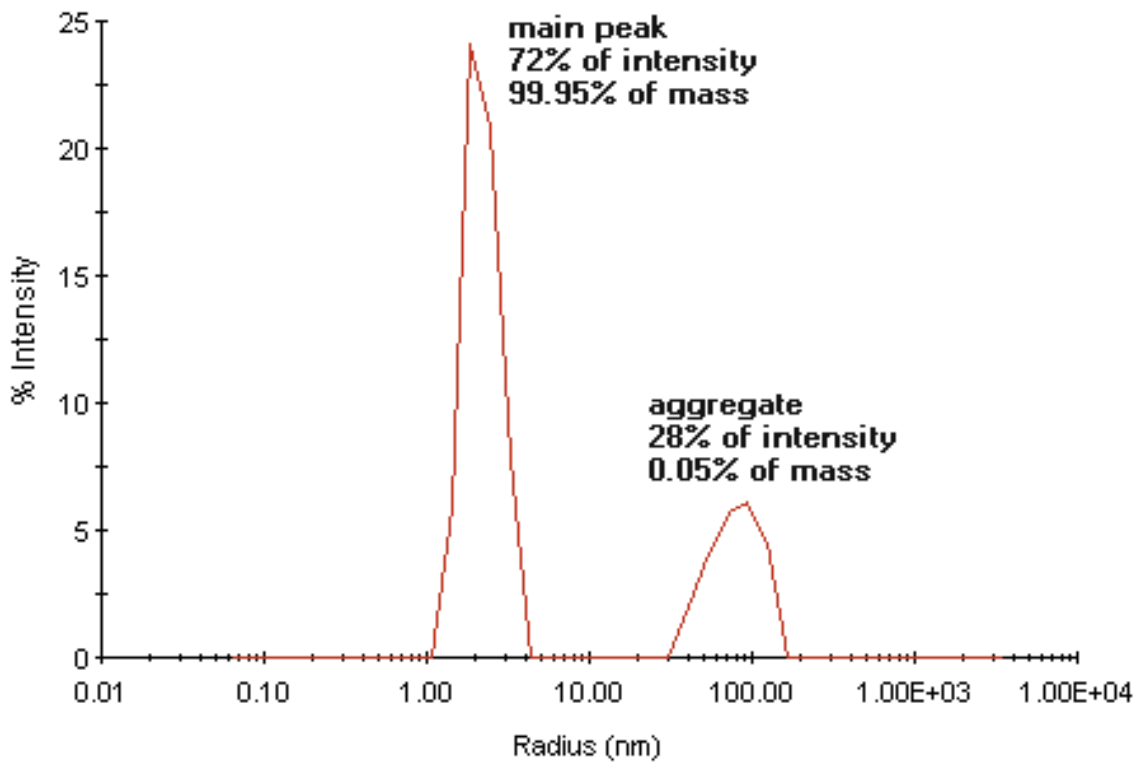






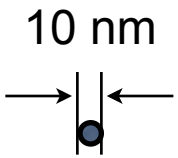
450 nm

$$g^2(q,t) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

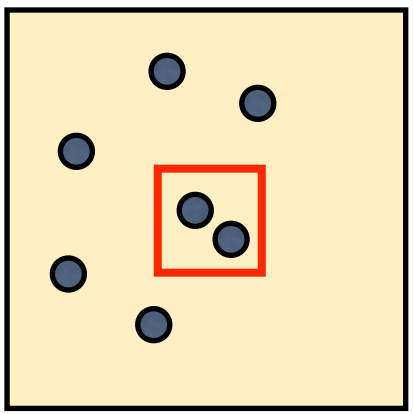


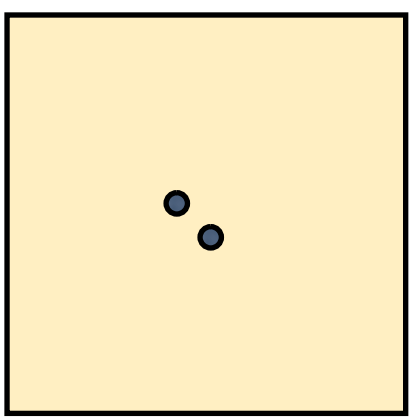
Dynamic Light Scattering

Assumptions and Caveats



$$D = \frac{k_B T}{6\pi\eta r}$$





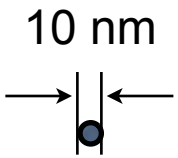
450 nm

Assumes

- Brownian motion
  - ★ non-interacting billiard balls
- Spherical scatterers
- Proper calibration for viscosity, etc
- Properly dilute solution
- No interference from other scatterers

Dynamic Light Scattering

Assumptions and Caveats



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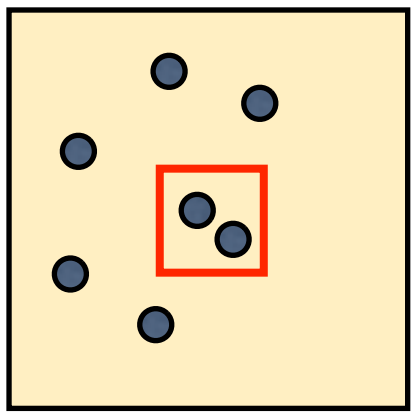

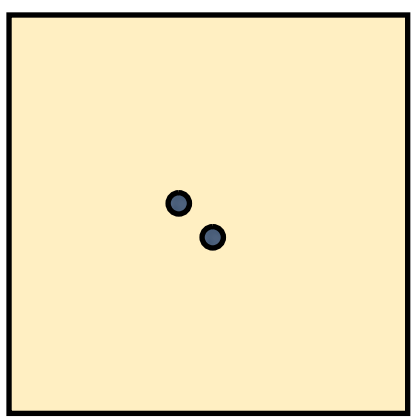

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Assumes

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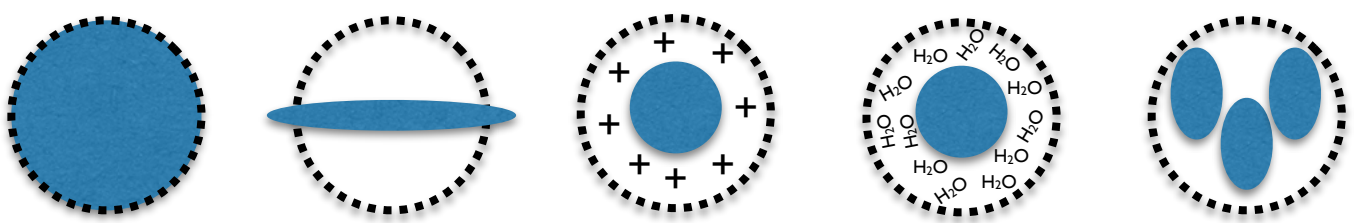
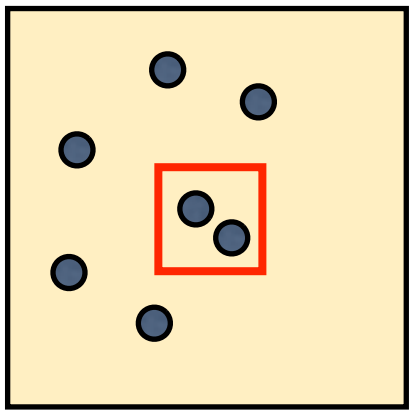


Dynamic Light Scattering



$$D = \frac{kT}{6\pi\eta R}$$

	DLS	AFM/TEM
Sample Preparation	Solution	Substrate
Measurement	Easy	Difficult
Sampling	Bulk	Small area
Shape of Particles	Sphere	No Requirement
Polydispersity	Low	No Requirement
Size Range	nm to um	nm to um
Size Info.	Hydrodynamic radius	Physical size

Table 2.4.1 Comparison between DLS, AFM, and TEM.

Read data from a file

```
>edat <- read.csv("DataGrp1.csv", header = TRUE)
```

To confirm a successful read:

```
>edat
```

```
    X  t         dat
1   1  0 10.13859677
2   2  1  8.35533476
3   3  2  6.76788472
4   4  3  5.36280912
```

```
>plot(edat$t, edat$dat)
```

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Read the data in

Plot the data

Read data from a file

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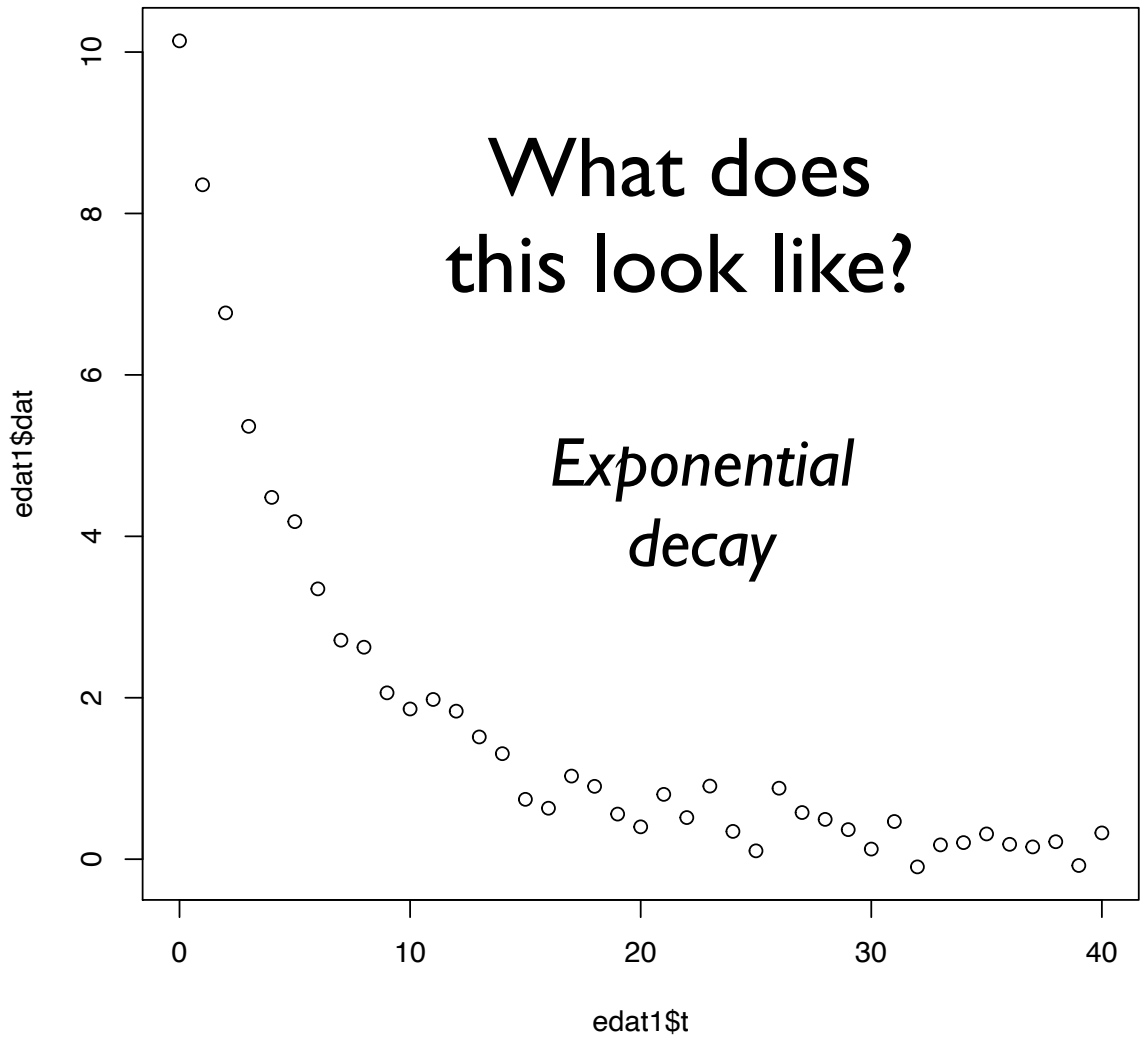
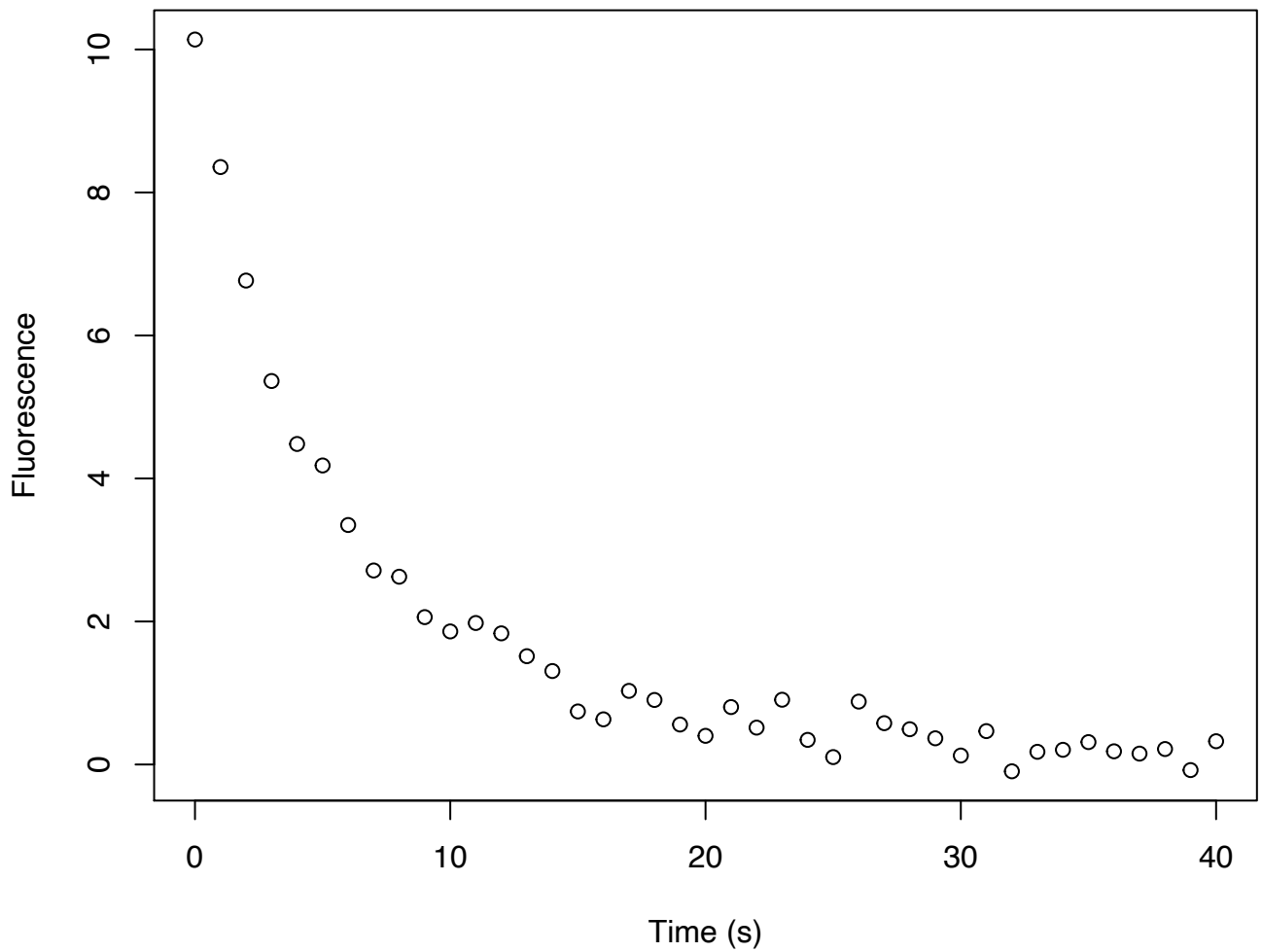
4   4  3  5.36280912
```

```
>plot(edat$t, edat$dat)
```



Let's make the plot prettier

```
>plot(edat$t, edat$dat, xlab="Time (s)", ylab="Fluorescence")
```



Return to Breakout Group

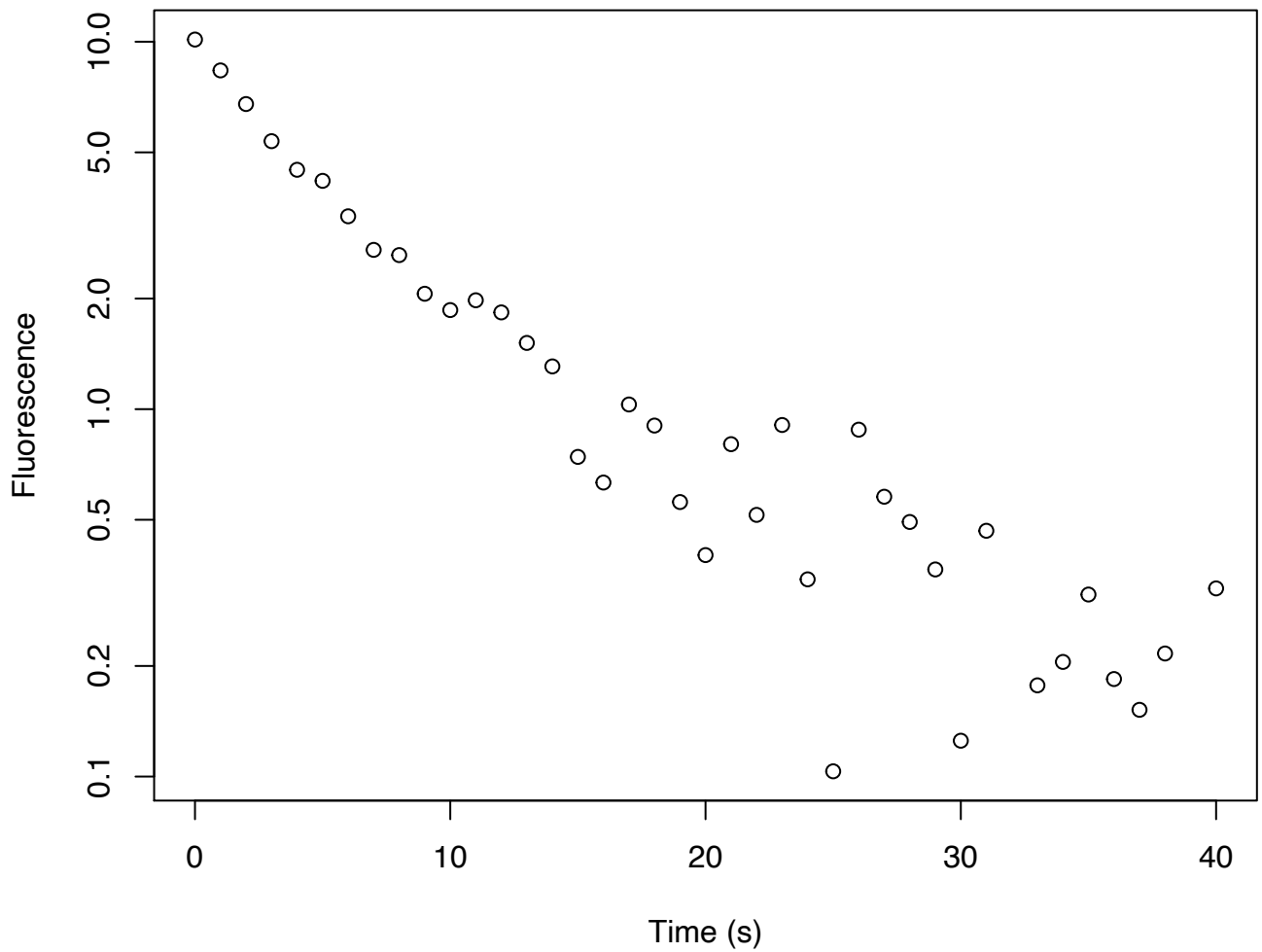
Re-plot the data as above

Remember "up-arrow"

Watch out for "curly quotes"

Log scale

```
>plot(edat$t, edat$dat, xlab="Time (s)", ylab="Fluorescence", log="y")
```

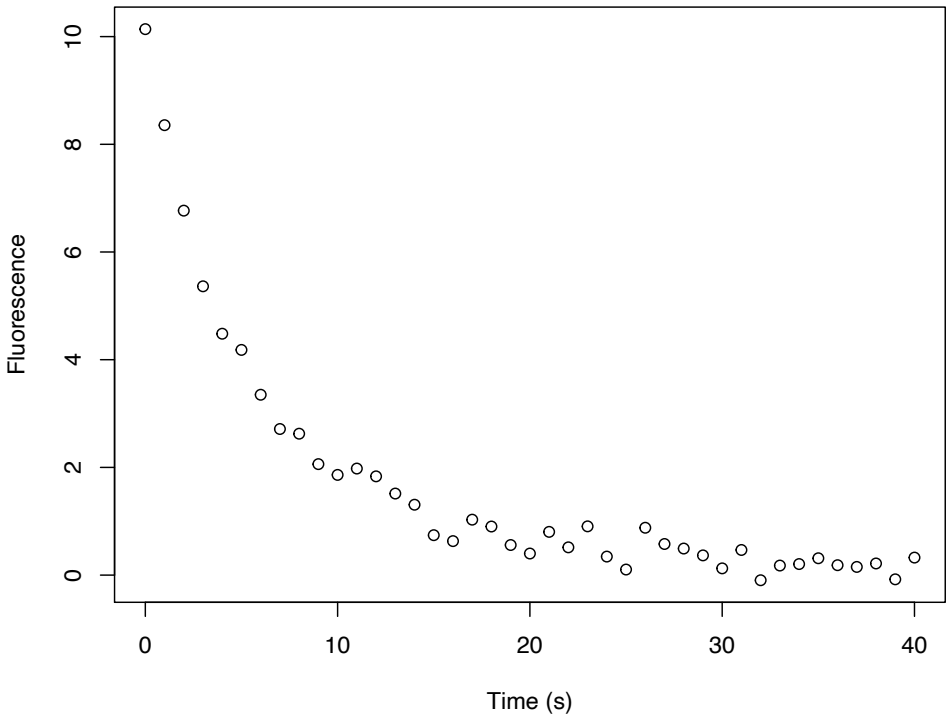


In the old days,
they'd get out a ruler

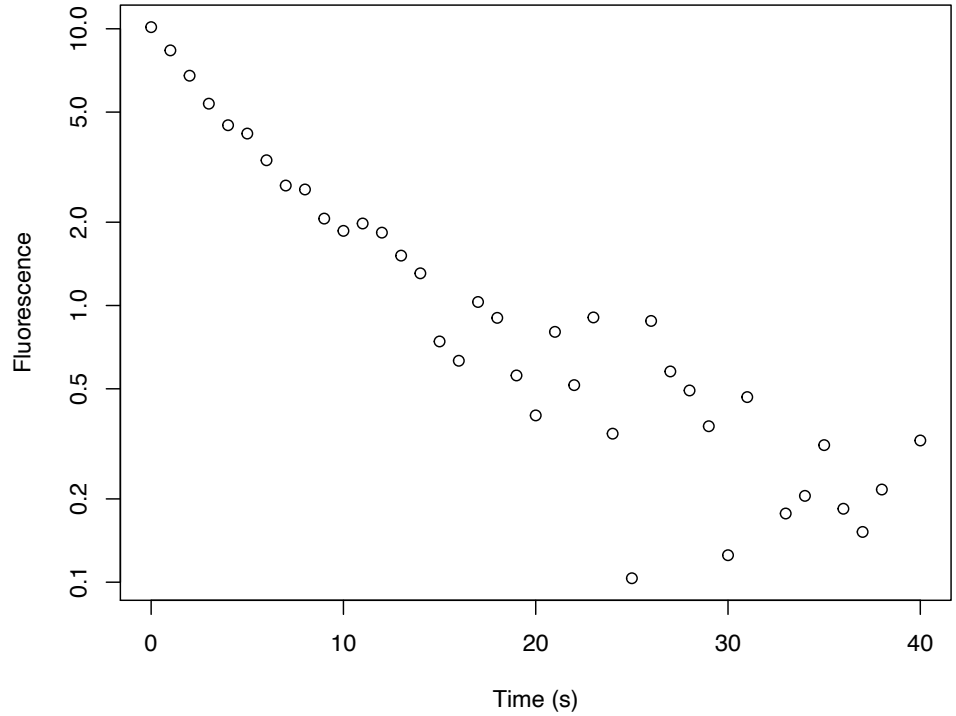
Why doesn't
Craig like this?

Come up with a function

Experiment (observations)

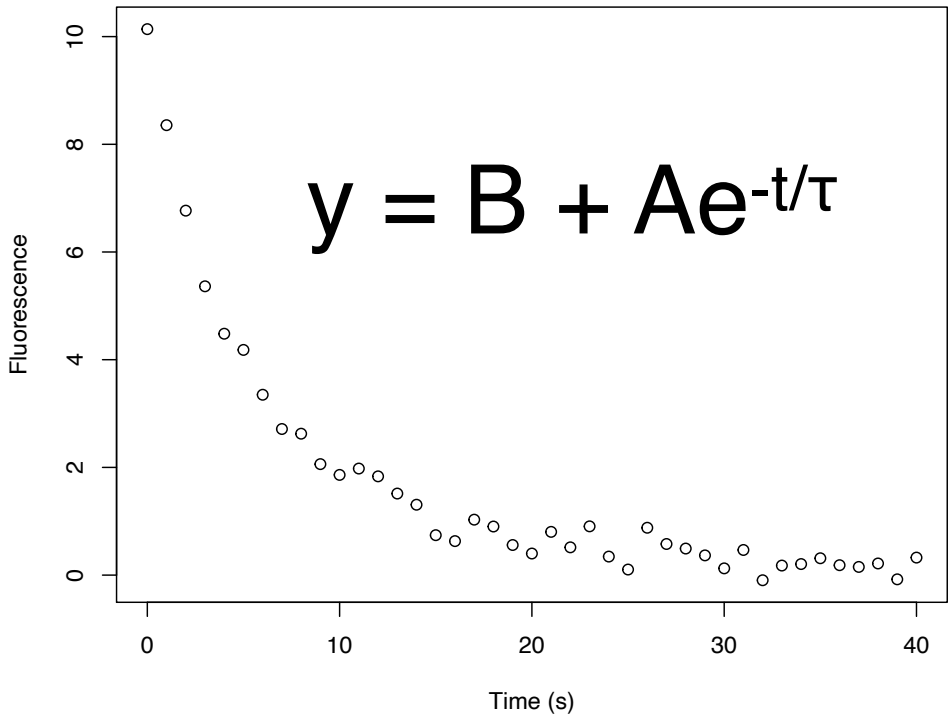


Distortion of data

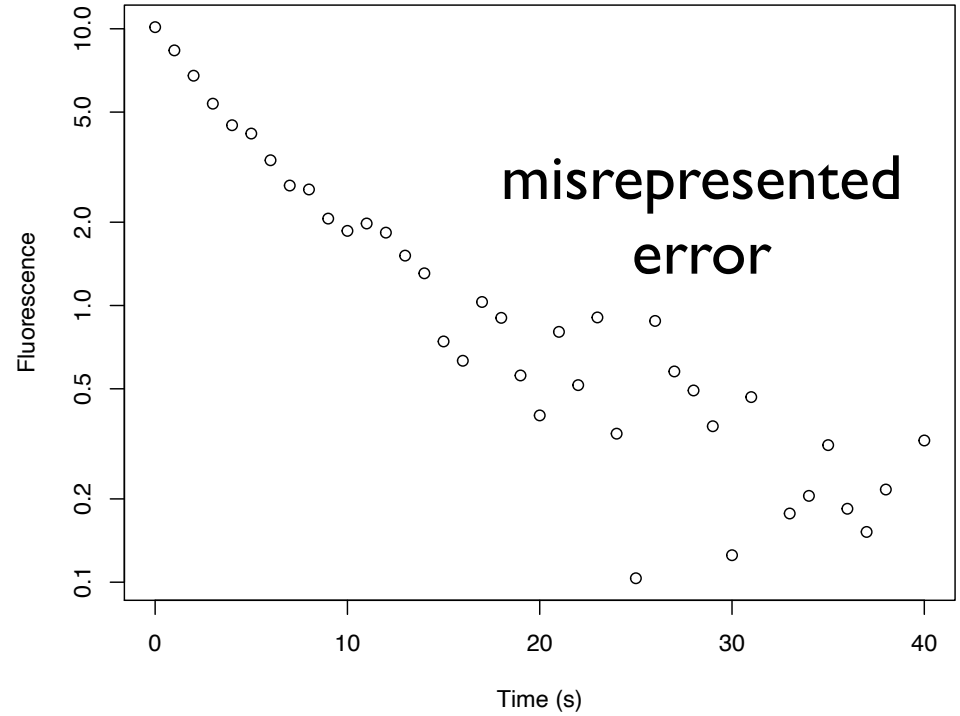


Come up with a function

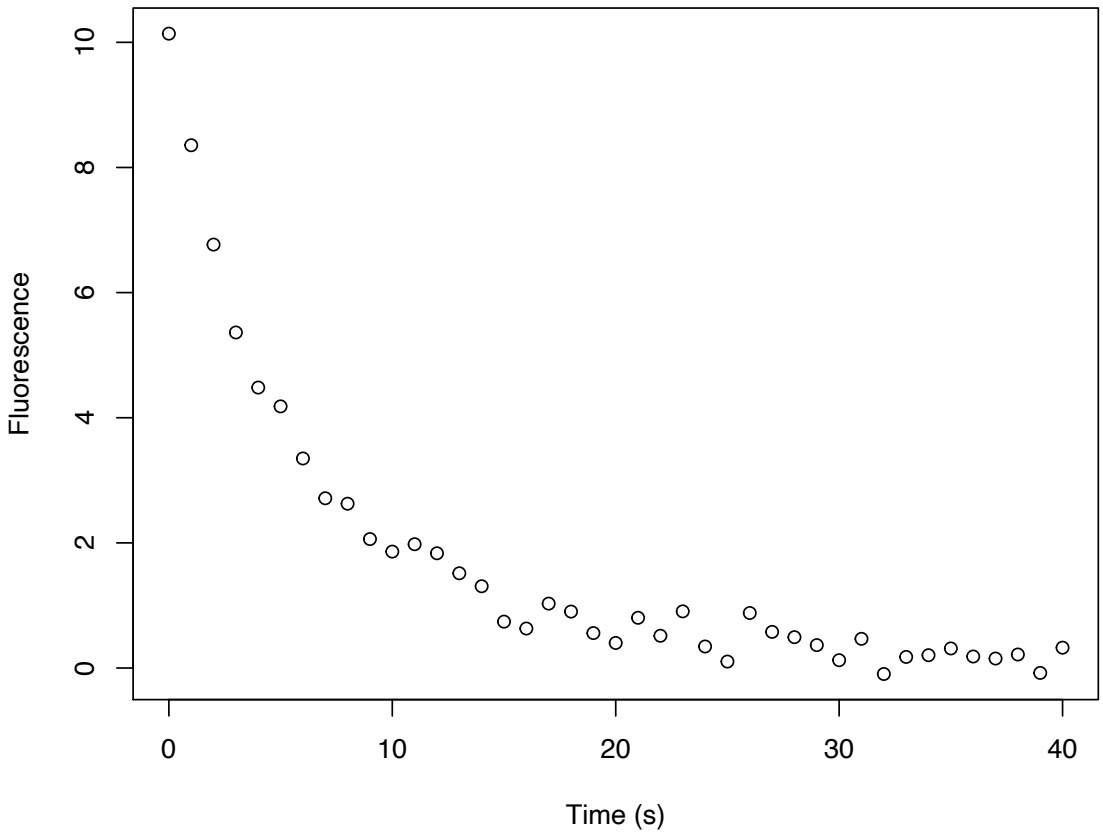
Experiment (observations)



Distortion of data



Come up with and define a function



$$y = Ae^{-t/\tau}$$

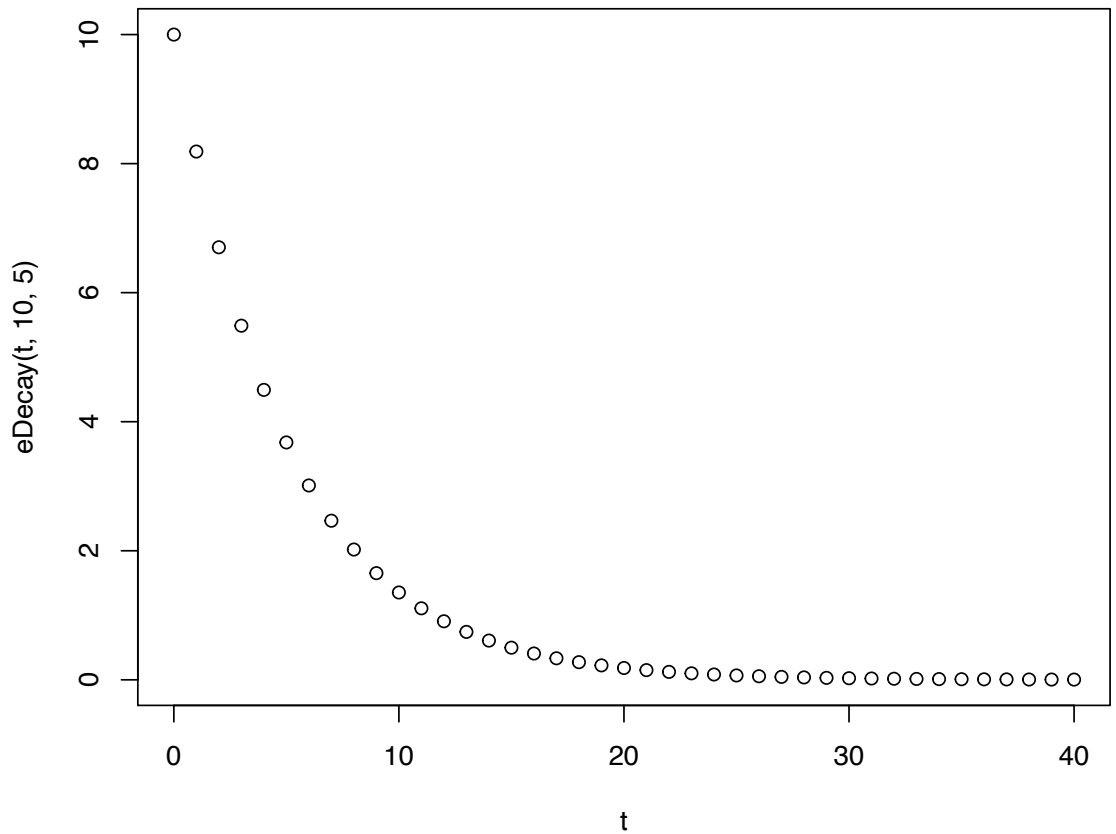
$$y = B + Ae^{-t/\tau}$$

Come up with and define a function

```
>eDecay <- function(t, ampl, tau) (ampl*exp(-t/tau))
```

func name *define parameters* *func definition*

```
>plot(t, eDecay(edat$t,10,5))
```



$$y = Ae^{-t/\tau}$$

R - nonlinear regression (nls)

```
>eDecay <- function(t, ampl, tau) (ampl*exp(-t/tau))
```

func name — *define parameters* — *func definition*

```
>model1 <- nls(edat$dat ~ eDecay(edat$t,ampl,tau), start=list(ampl=10,  tau=5), trace=TRUE)
```

fluor data — *function call* — *initial guesses*

Results stored here

Compare experimental data to theory

Return to Breakout Group

```
>summary(model1)
```

$$y = Ae^{-t/\tau}$$

R - nonlinear regression (nls)

```
>model1 <- nls(edat$dat ~ eDecay(edat$t,ampl,tau), start=list(ampl=10,  tau=5), trace=TRUE)
```

fluor data *function call* *initial guesses*

```
Formula: edat1$dat ~ eDecay(edat1$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl   9.5239     0.2294   41.52   <2e-16 ***
tau    6.2702     0.2308   27.16   <2e-16 ***


Formula: edat2$dat ~ eDecay(edat2$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl   9.3353     0.1727   54.05   <2e-16 ***
tau    6.3280     0.1788   35.39   <2e-16 ***


Formula: edat3$dat ~ eDecay(edat3$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl   9.3329     0.1938   48.16   <2e-16 ***
tau    6.4709     0.2049   31.58   <2e-16 ***


Formula: edat4$dat ~ eDecay(edat4$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl   9.1827     0.1936   47.44   <2e-16 ***
tau    6.5311     0.2098   31.12   <2e-16 ***
```

Rigor and Reproducibility?

$$y = Ae^{-t/\tau}$$

9.5 ± 0.2 6.3 ± 0.2 s

6.3 ± 0.2 s

6.5 ± 0.2 s

6.5 ± 0.2 s

R - Plot data and the fit

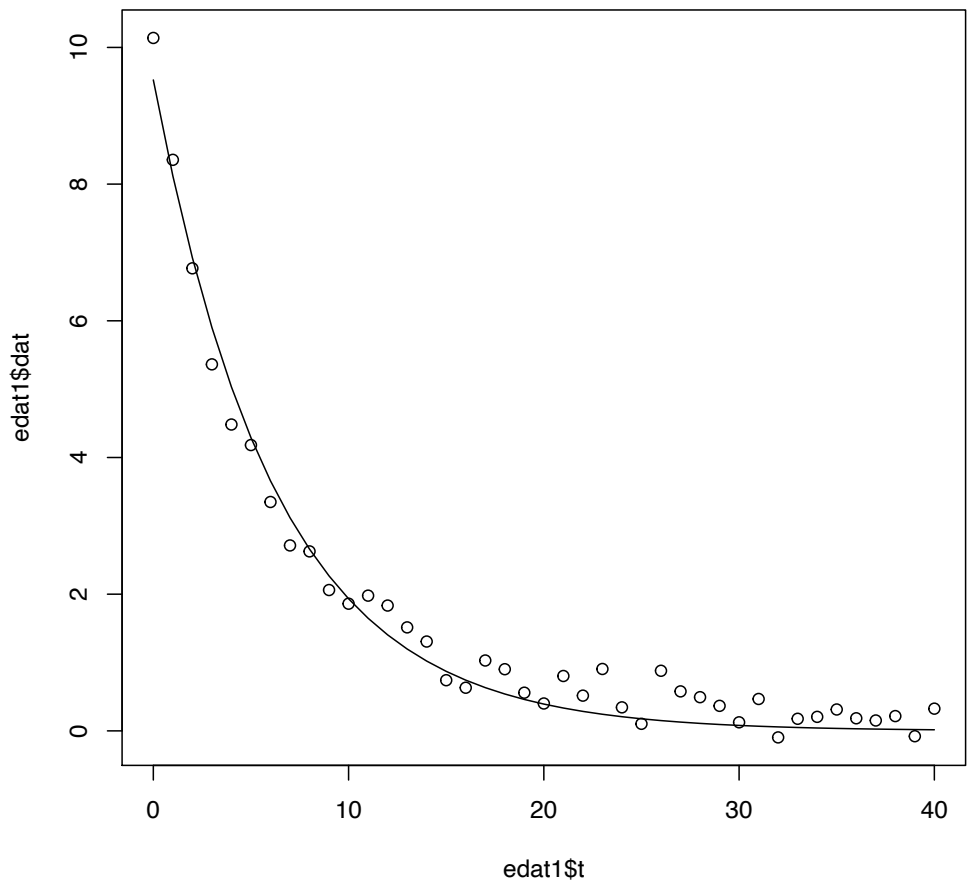
```
>model1 <- nls(edat$dat ~ eDecay(edat$t,ampl,tau), start=list(ampl=10,  tau=5), trace=TRUE)
```

fluor data *function call* *initial guesses*

```
>plot(edat$t, edat$dat)

>lines(edat$t,predict(model1))
```

R func



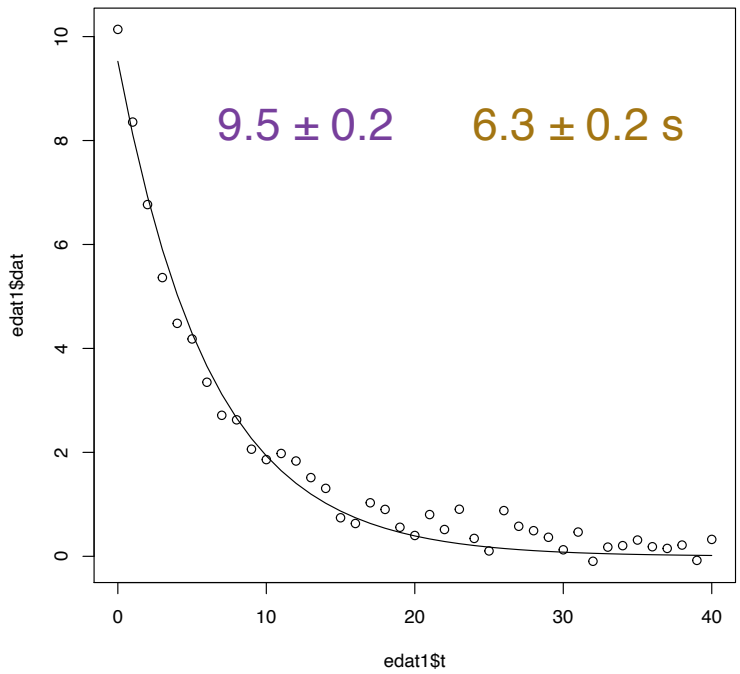
Return to Breakout Group

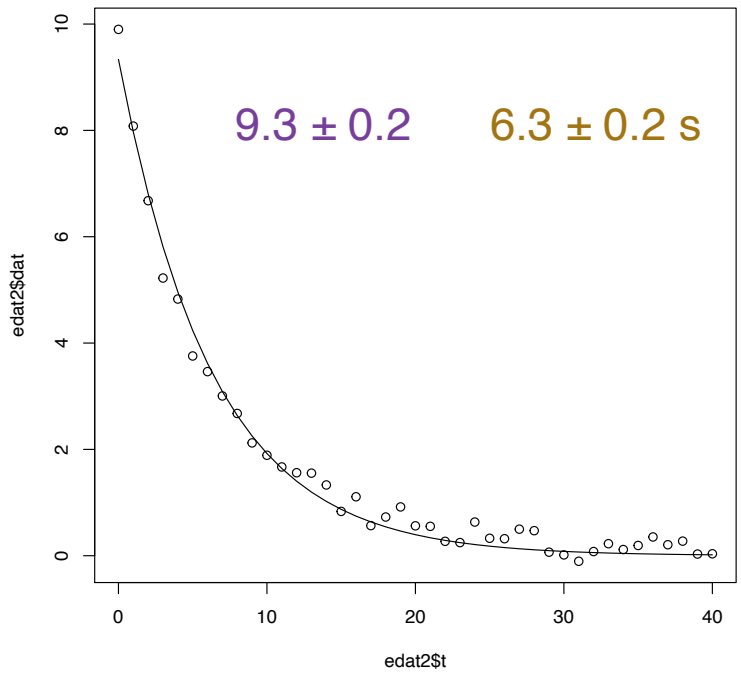
$$y = Ae^{-t/\tau}$$

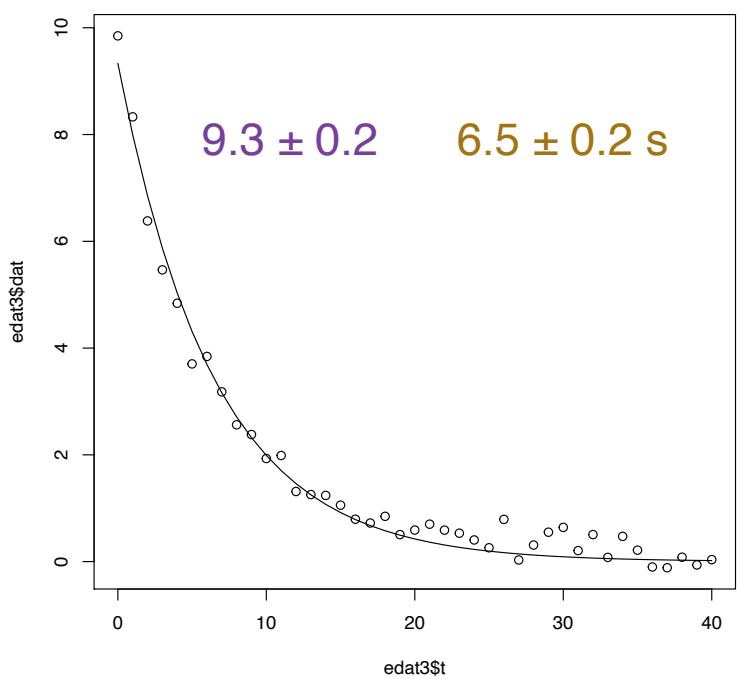
9.5 ± 0.2	6.3 ± 0.2 s
9.3 ± 0.2	6.3 ± 0.2 s
9.3 ± 0.2	6.5 ± 0.2 s
9.2 ± 0.2	6.5 ± 0.2 s

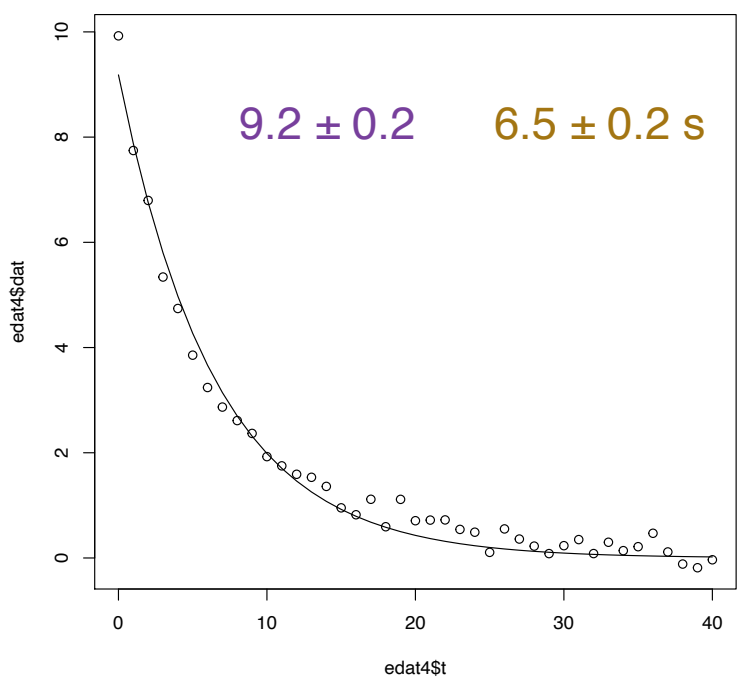
$y = Ae^{-t/\tau}$

R - nonlinear regression (nls)









R - Plot data and the fit

```
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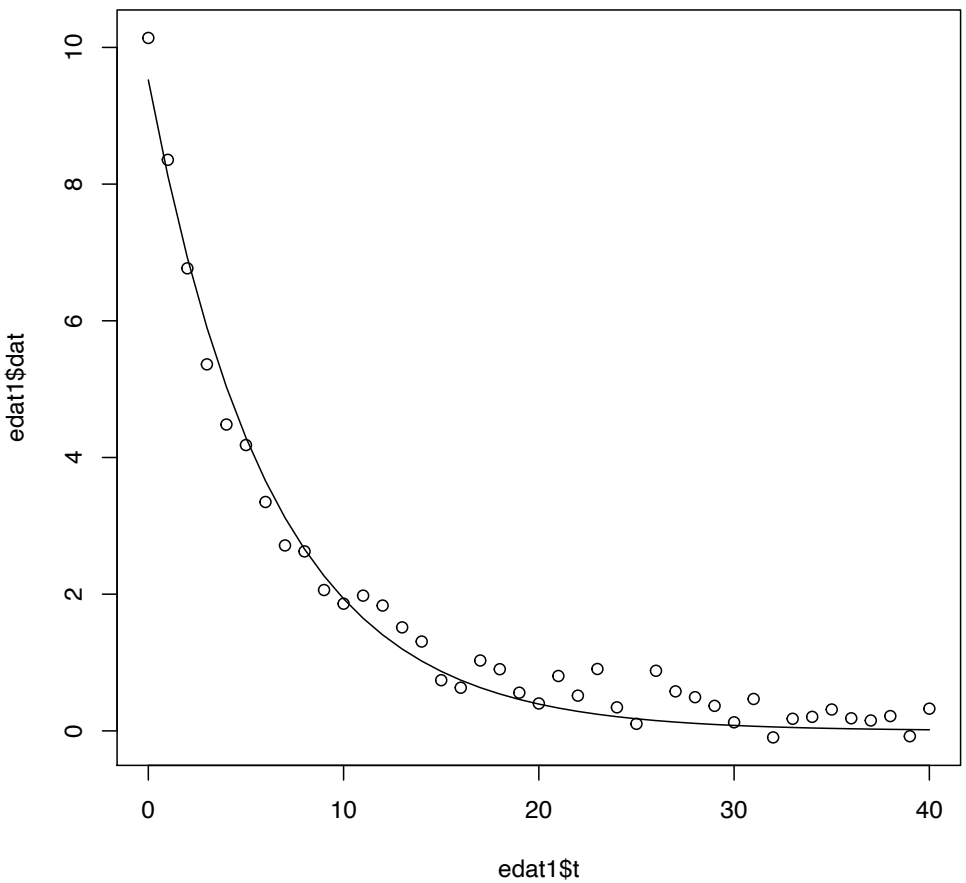
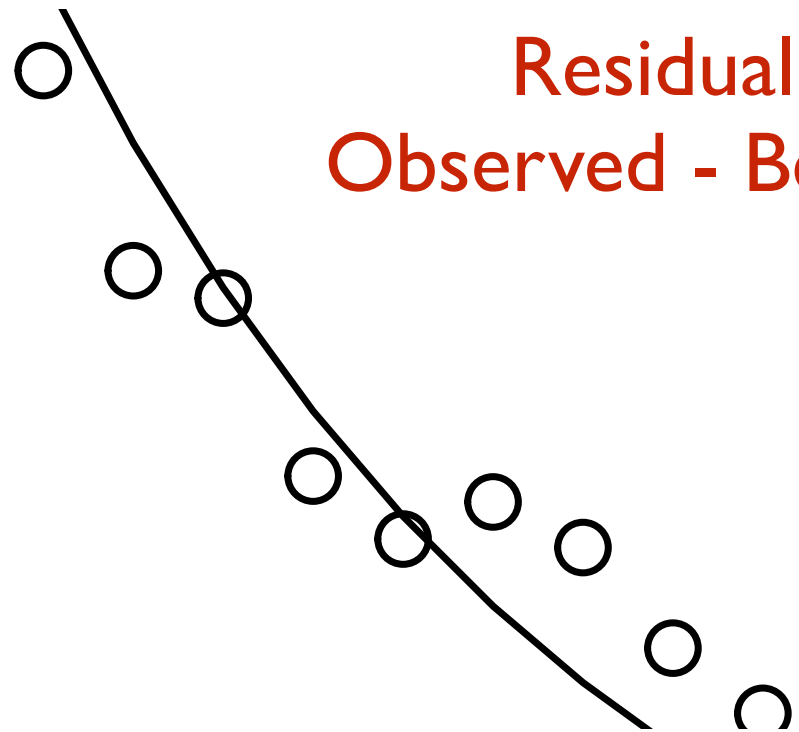
```

fluor data *function call* *initial guesses*





Residual
Observed - BestFit

Read data from a file

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To confirm a successful read:

```
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2 2  1  8.35533476
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4 4  3  5.36280912
```

```
>plot(edat$t, edat$dat)
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Plot the data

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```

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3   3  2  6.76788472
4   4  3  5.36280912
```
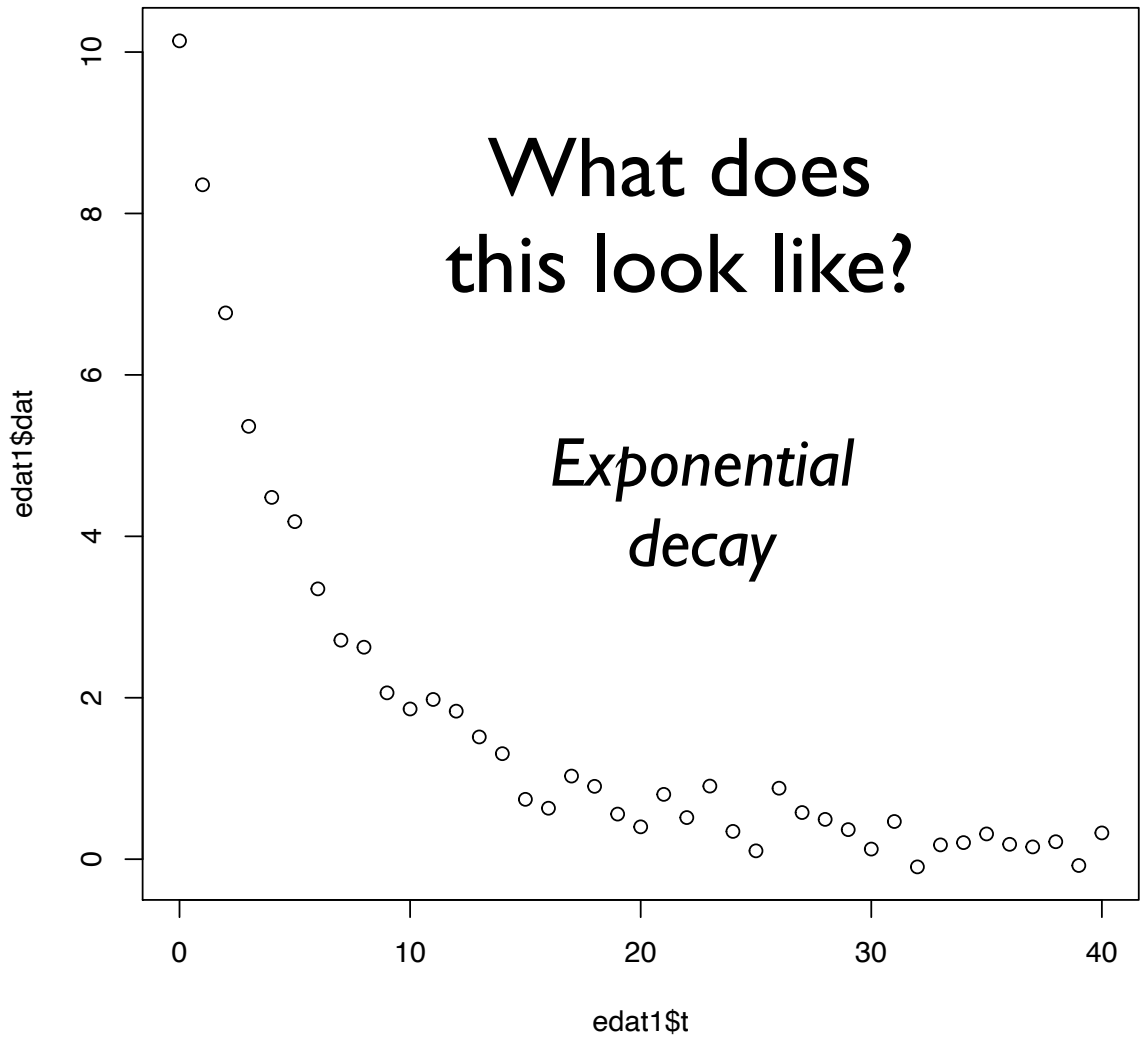
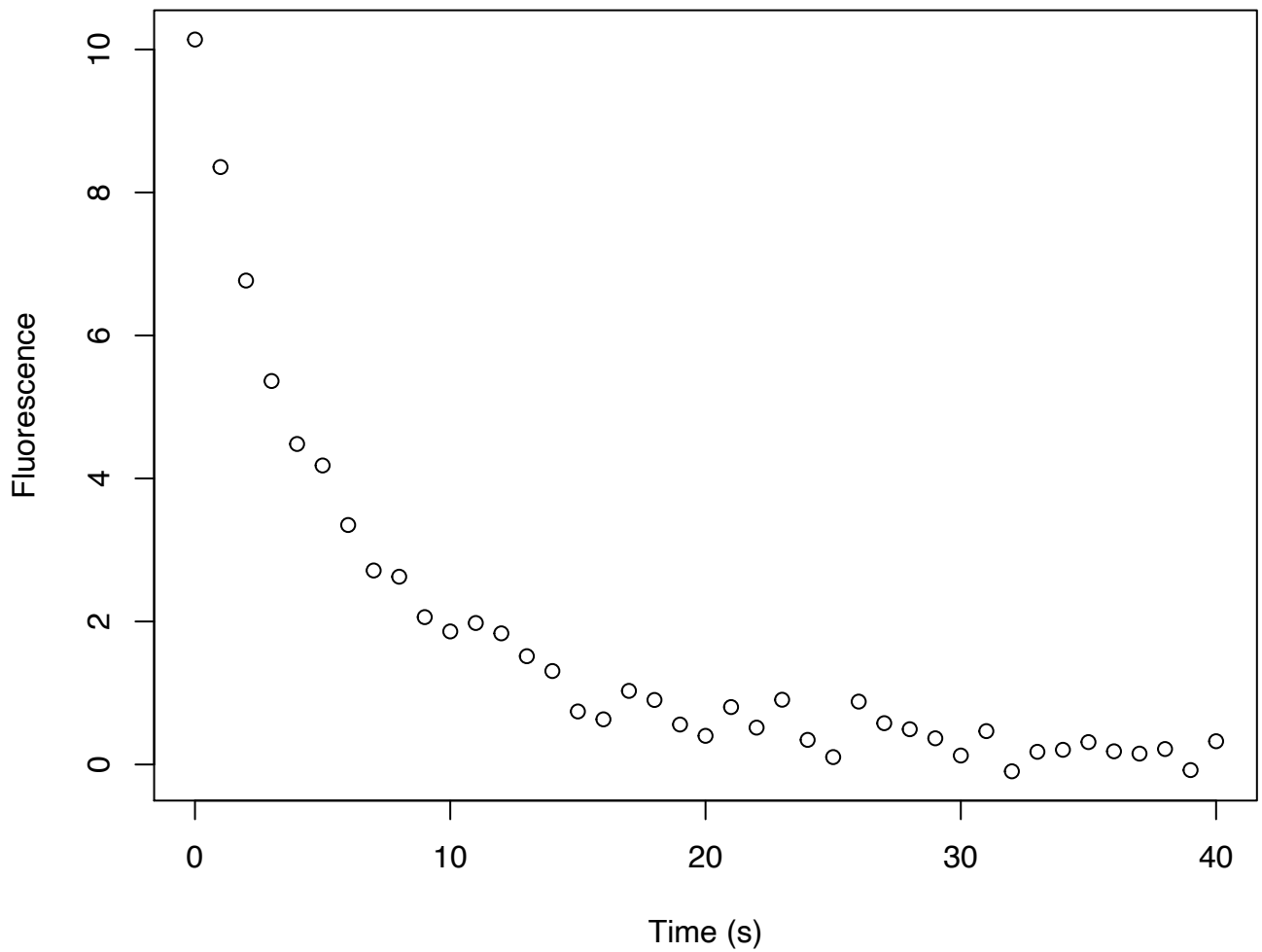

```
>plot(edat$t, edat$dat)
```



Let's make the plot prettier

```
>plot(edat$t, edat$dat, xlab="Time (s)", ylab="Fluorescence")
```



Return to Breakout Group

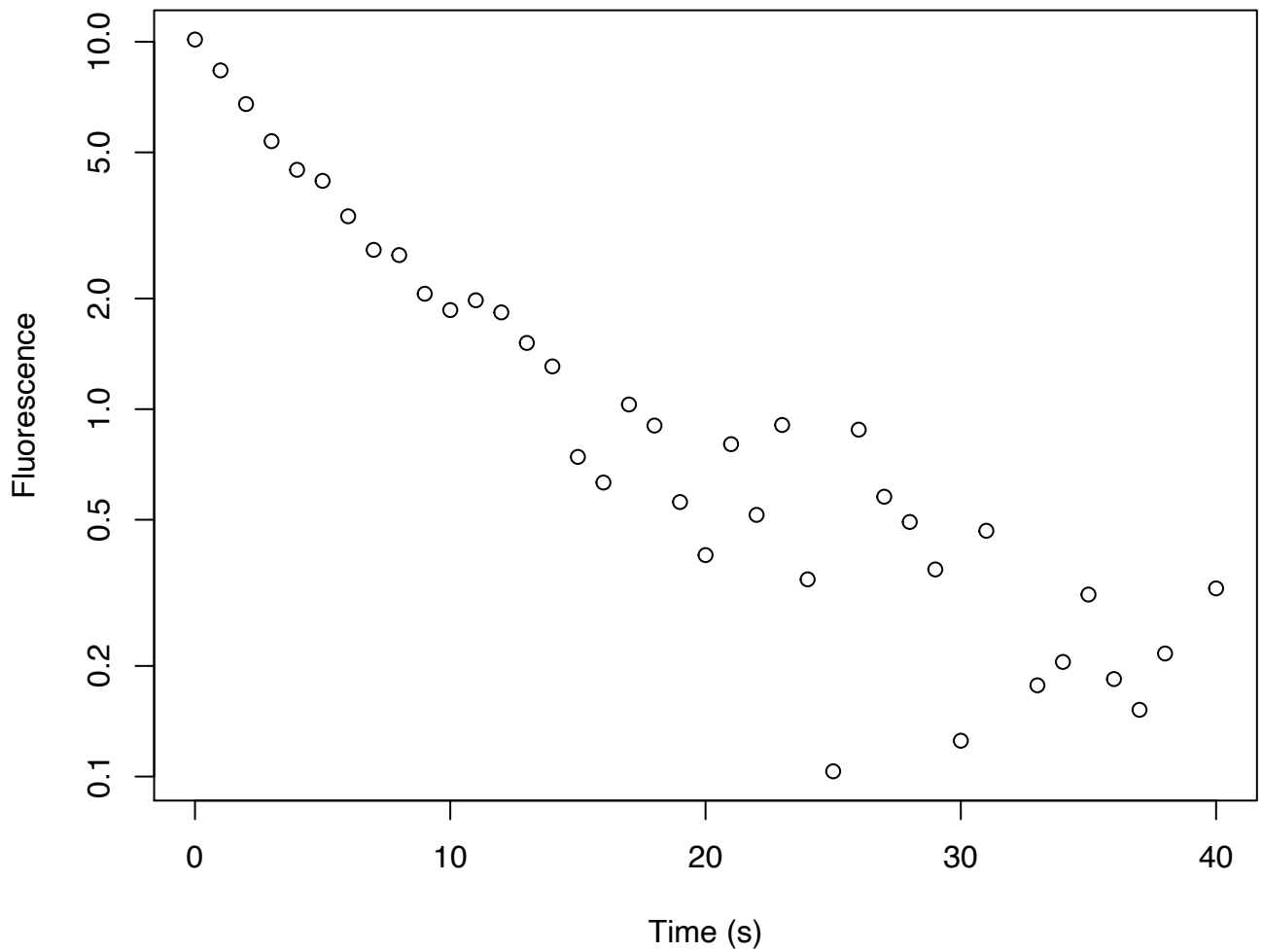
Re-plot the data as above

Remember "up-arrow"

Watch out for "curly quotes"

Log scale

```
>plot(edat$t, edat$dat, xlab="Time (s)", ylab="Fluorescence", log="y")
```

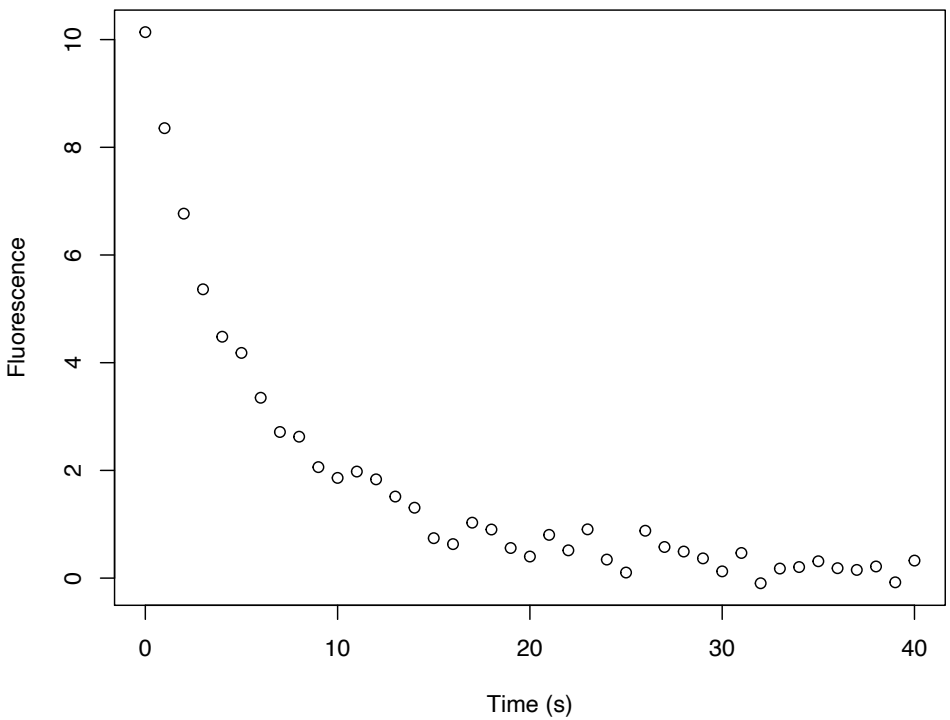


In the old days,
they'd get out a ruler

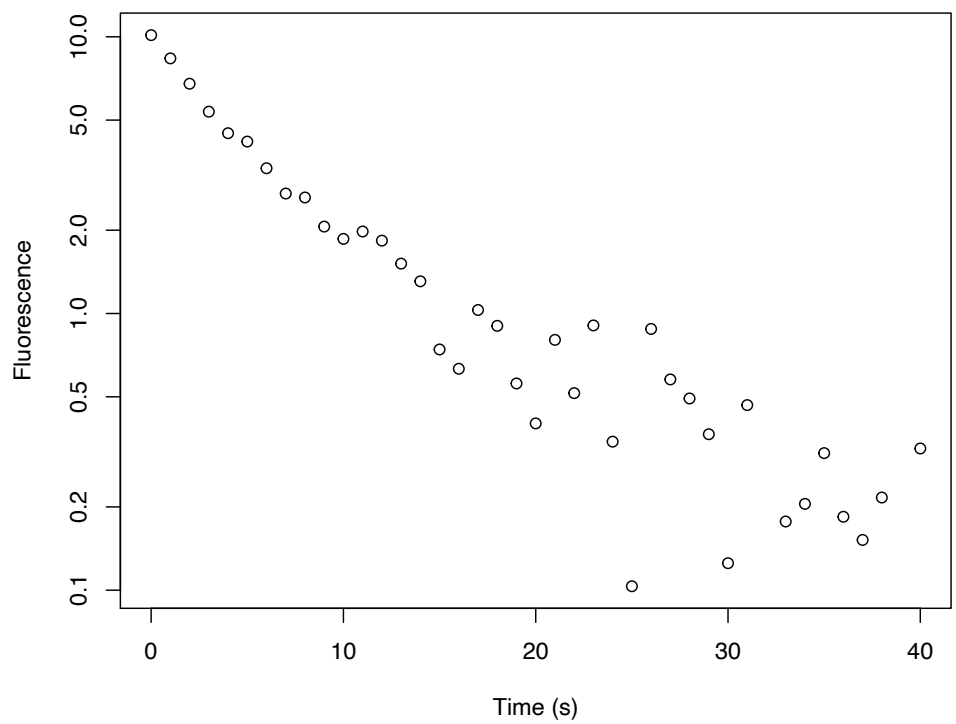
Why doesn't
Craig like this?

Come up with a function

Experiment (observations)

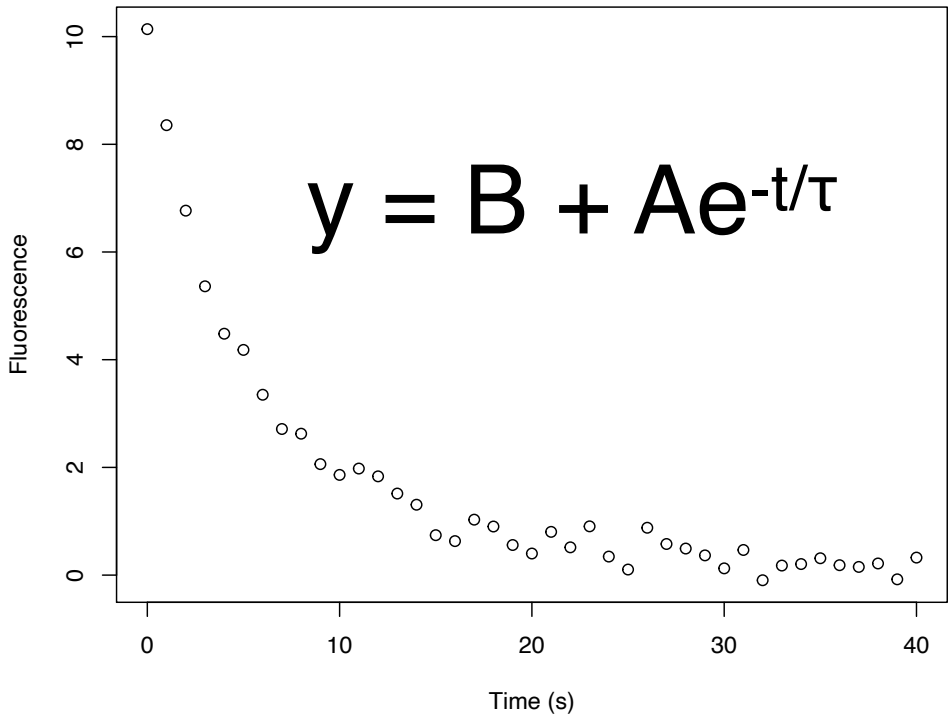


Distortion of data

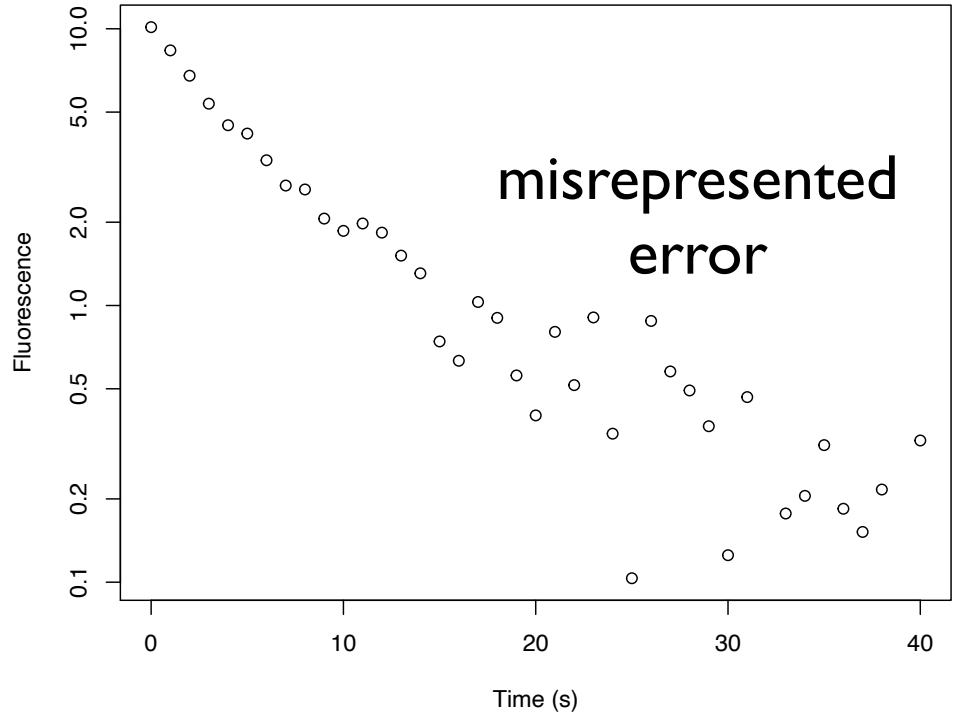


Come up with a function

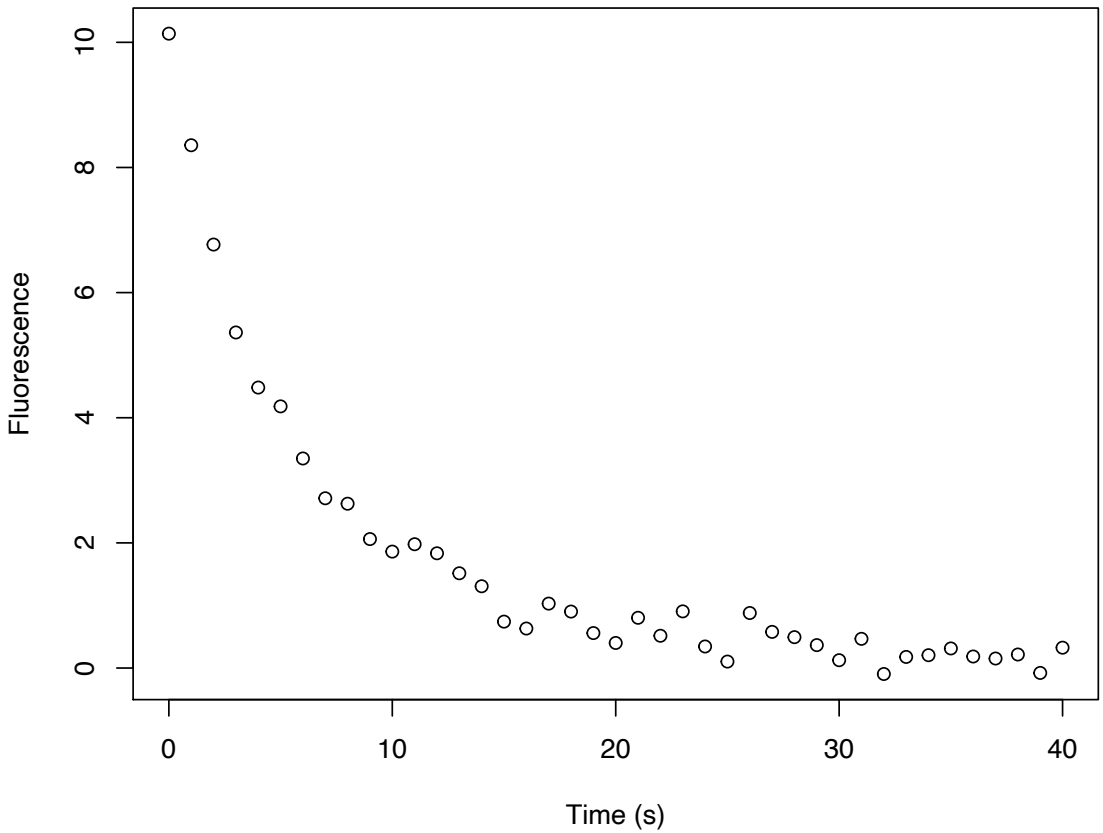
Experiment (observations)



Distortion of data



Come up with and define a function



$$y = Ae^{-t/\tau}$$

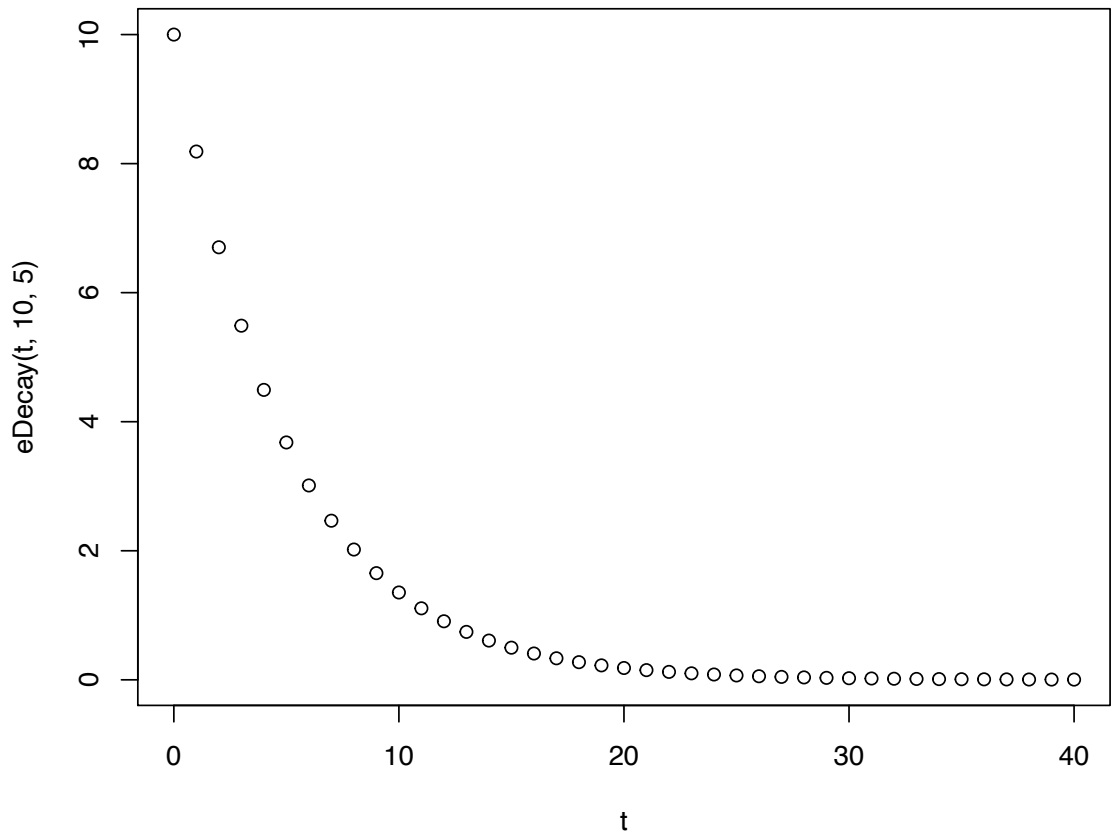
$$y = B + Ae^{-t/\tau}$$

Come up with and define a function

```
>eDecay <- function(t, ampl, tau) (ampl*exp(-t/tau))
```

func name *define parameters* *func definition*

```
>plot(t, eDecay(edat$t,10,5))
```



$$y = Ae^{-t/\tau}$$

R - nonlinear regression (nls)

```
>eDecay <- function(t, ampl, tau) (ampl*exp(-t/tau))
```

func name — *define parameters* — *func definition*

```
>model1 <- nls(edat$dat ~ eDecay(edat$t,ampl,tau), start=list(ampl=10,  tau=5), trace=TRUE)
```

fluor data — *function call* — *initial guesses*

Results stored here

Compare experimental data to theory

Return to Breakout Group

```
>summary(model1)
```

$$y = Ae^{-t/\tau}$$

R - nonlinear regression (nls)

```
>model1 <- nls(edat$dat ~ eDecay(edat$t,ampl,tau), start=list(ampl=10,  tau=5), trace=TRUE)
```

fluor data *function call* *initial guesses*

```
Formula: edat1$dat ~ eDecay(edat1$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl   9.5239     0.2294   41.52   <2e-16 ***
tau    6.2702     0.2308   27.16   <2e-16 ***


Formula: edat2$dat ~ eDecay(edat2$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl   9.3353     0.1727   54.05   <2e-16 ***
tau    6.3280     0.1788   35.39   <2e-16 ***


Formula: edat3$dat ~ eDecay(edat3$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl   9.3329     0.1938   48.16   <2e-16 ***
tau    6.4709     0.2049   31.58   <2e-16 ***


Formula: edat4$dat ~ eDecay(edat4$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl   9.1827     0.1936   47.44   <2e-16 ***
tau    6.5311     0.2098   31.12   <2e-16 ***
```

Rigor and Reproducibility?

$$y = Ae^{-t/\tau}$$

9.5 ± 0.2 6.3 ± 0.2 s

6.3 ± 0.2 s

6.5 ± 0.2 s

6.5 ± 0.2 s

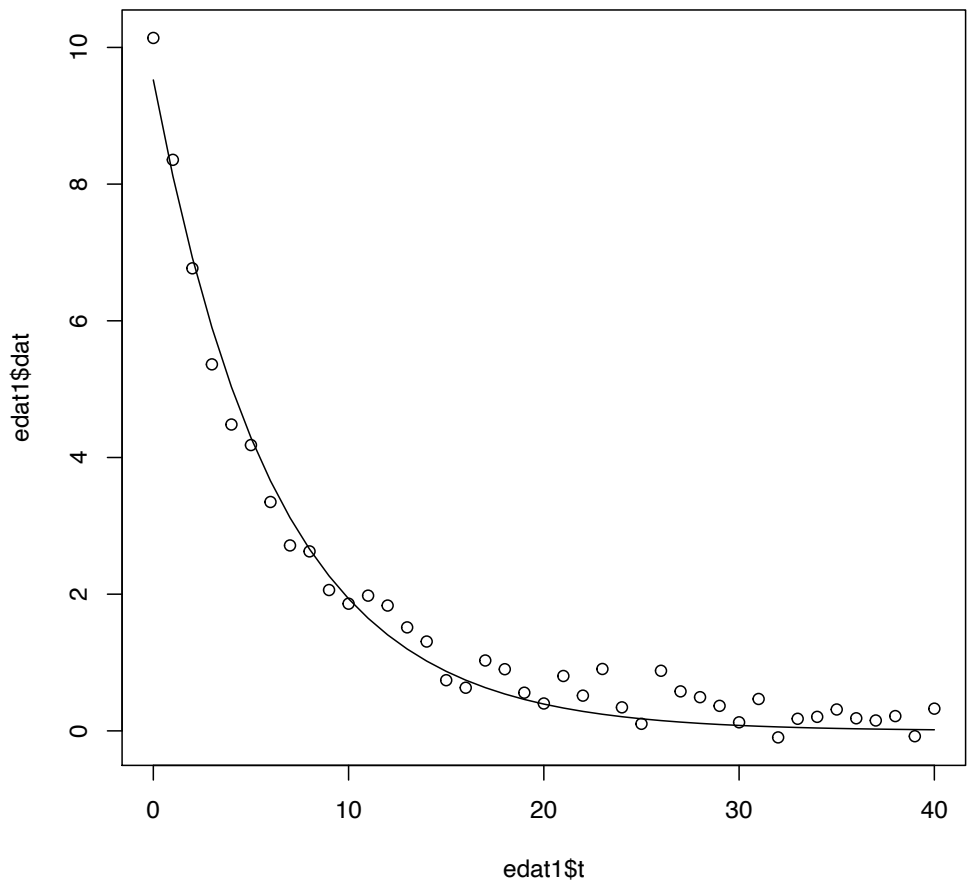
R - Plot data and the fit

```
>model1 <- nls(edat$dat ~ eDecay(edat$t,ampl,tau), start=list(ampl=10,  tau=5), trace=TRUE)
```

fluor data *function call* *initial guesses*

```
>plot(edat$t, edat$dat)

>lines(edat$t,predict(model1))
```

R func



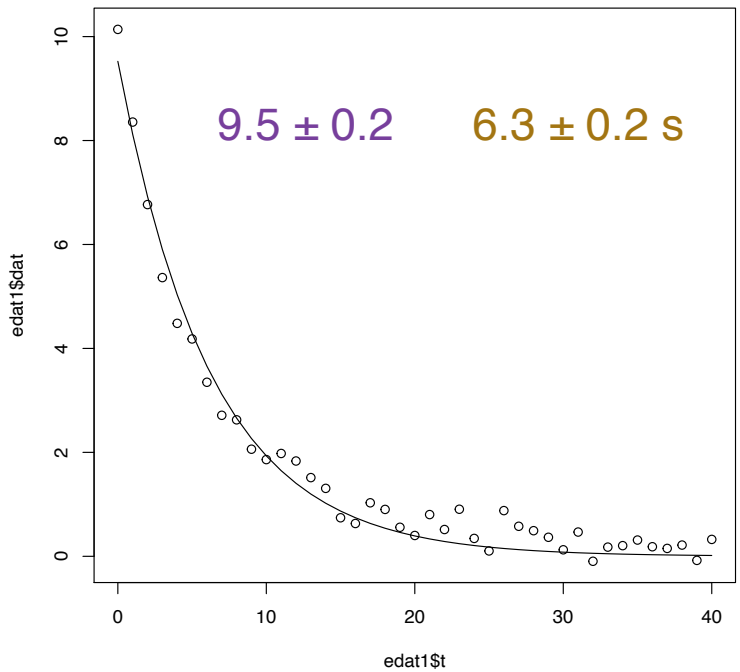
Return to Breakout Group

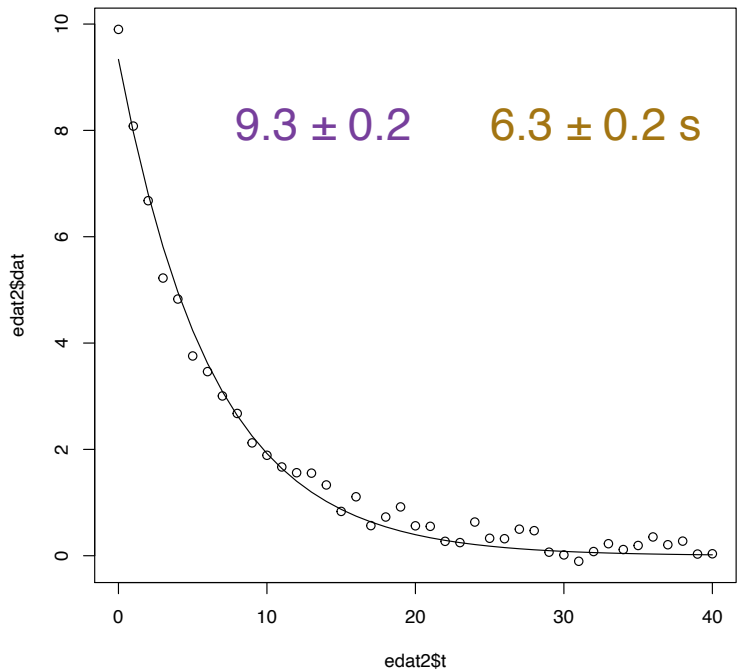
$$y = Ae^{-t/\tau}$$

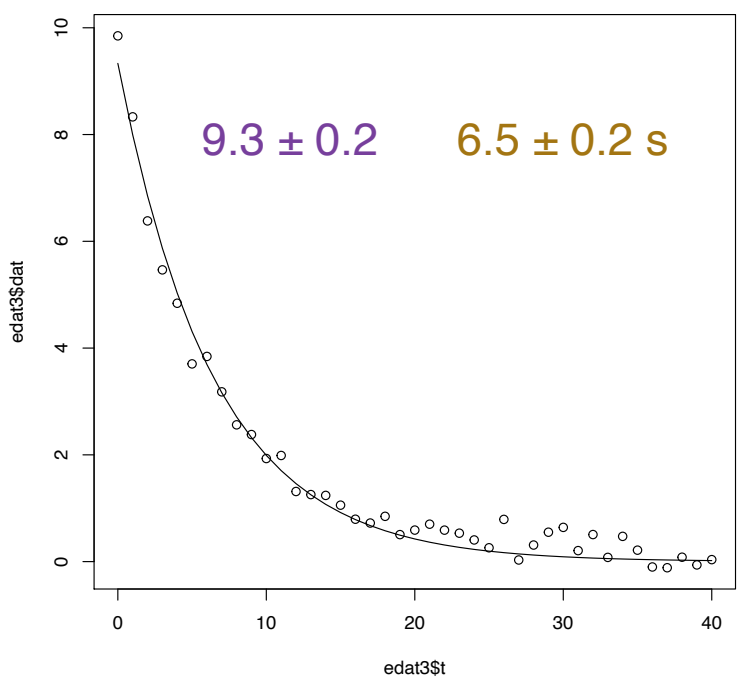
9.5 ± 0.2	6.3 ± 0.2 s
9.3 ± 0.2	6.3 ± 0.2 s
9.3 ± 0.2	6.5 ± 0.2 s
9.2 ± 0.2	6.5 ± 0.2 s

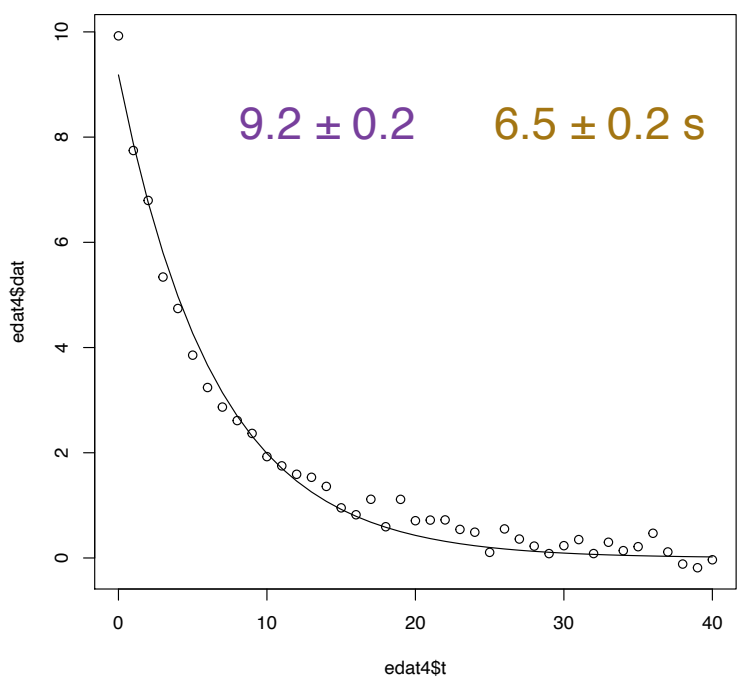
$y = Ae^{-t/\tau}$

R - nonlinear regression (nls)





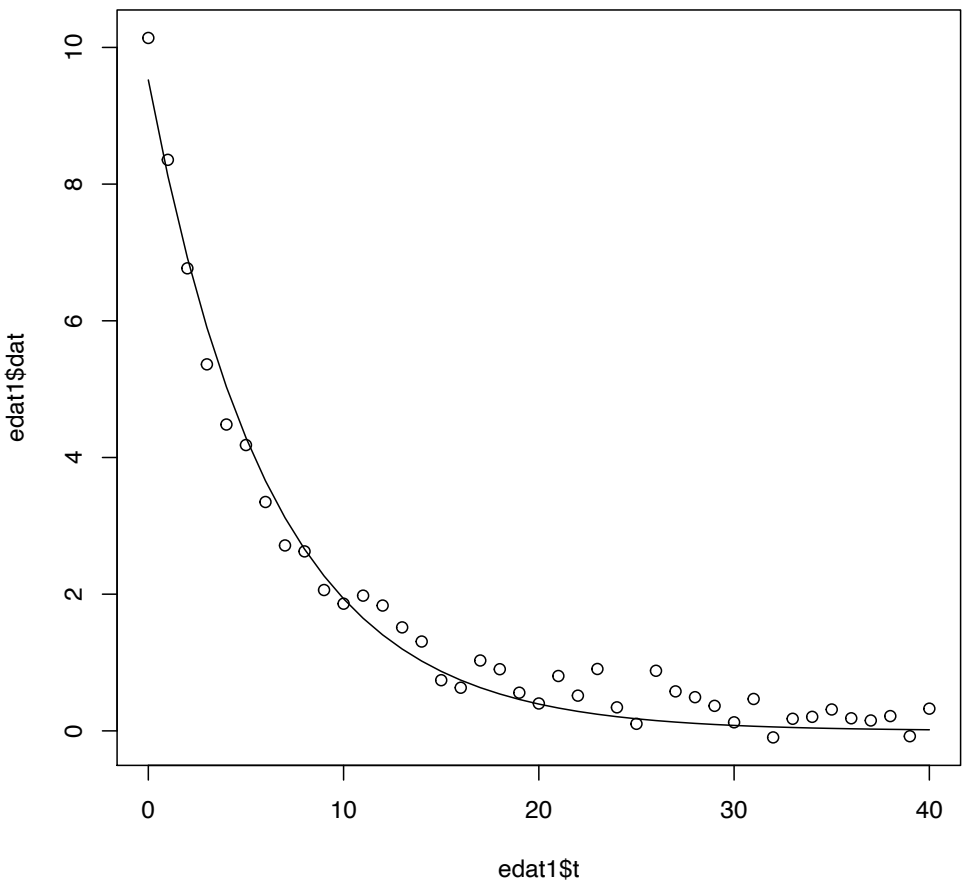


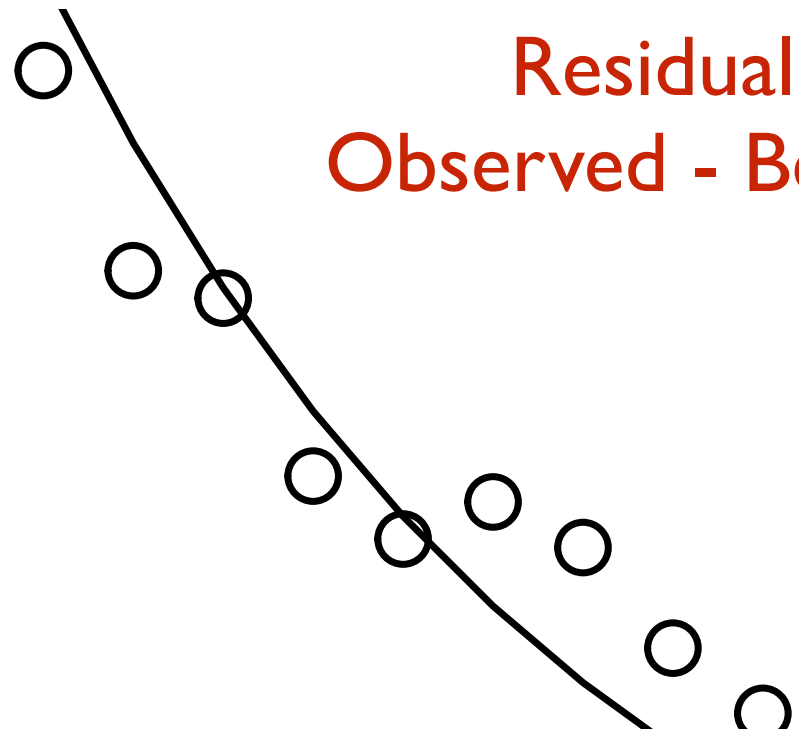


R - Plot data and the fit

```
>model1 <- nls(edat$dat ~ eDecay(edat$t,ampl,tau), start=list(ampl=10,  tau=5), trace=TRUE)
```

fluor data *function call* *initial guesses*

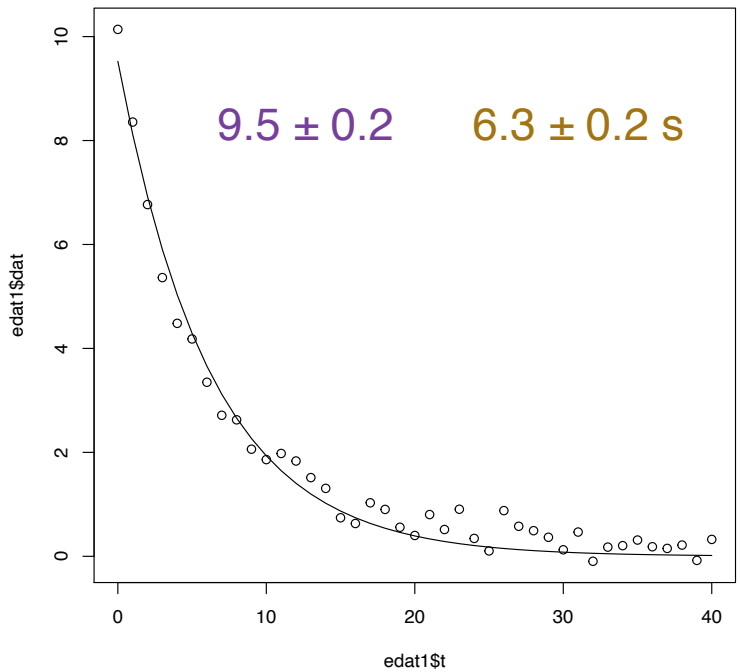


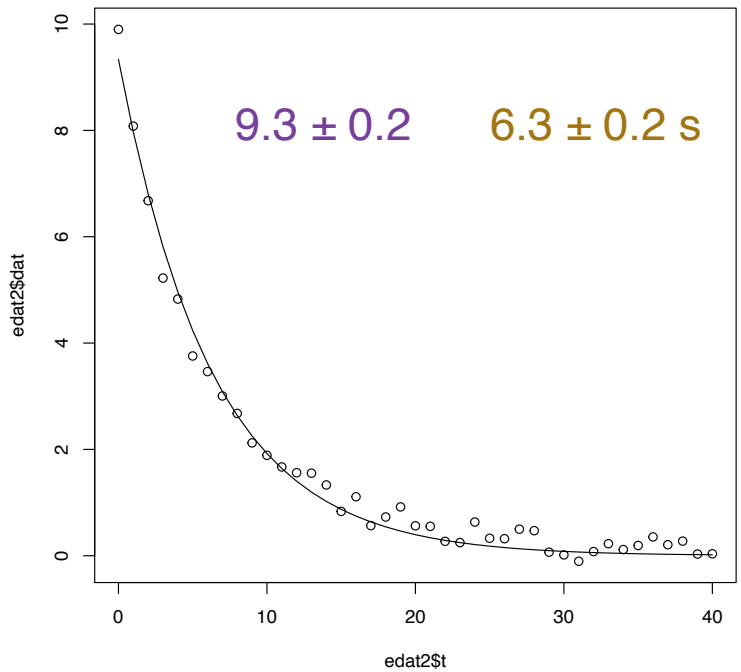


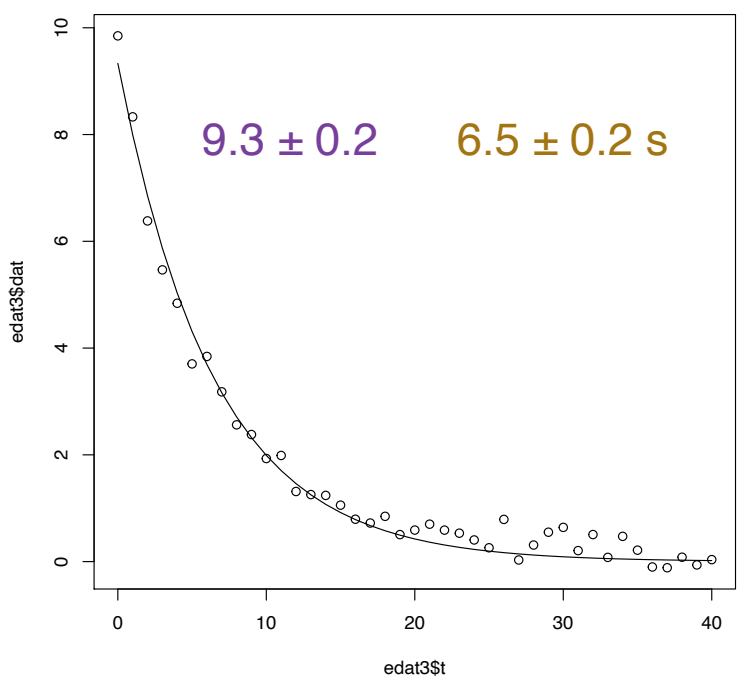
Residual
Observed - BestFit

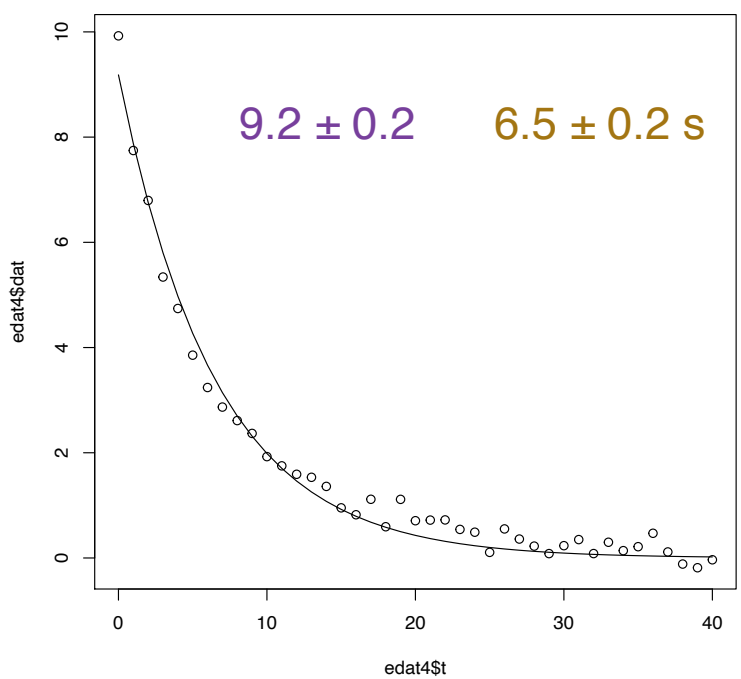
$y = Ae^{-t/\tau}$

R - nonlinear regression (nls)



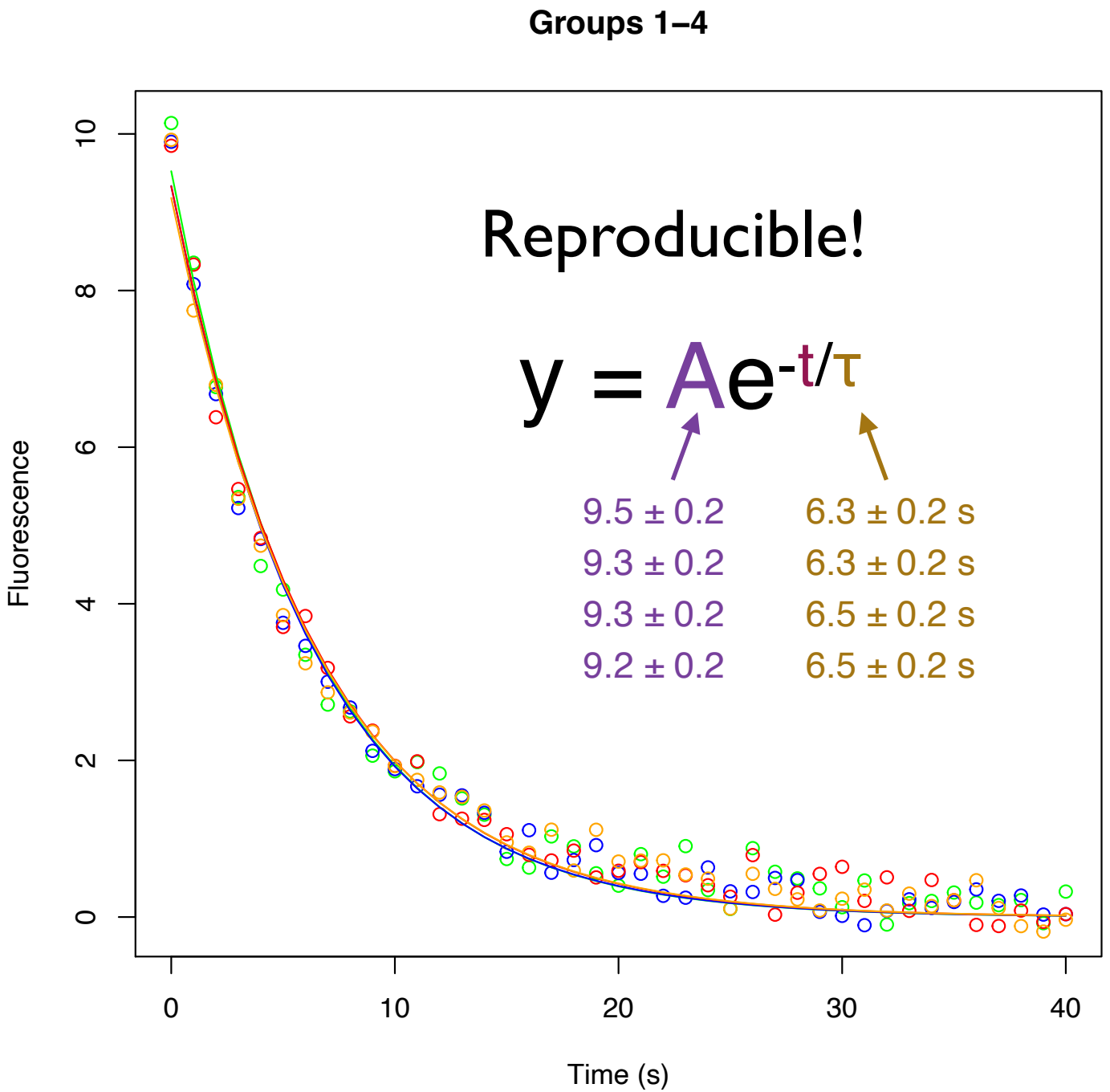






$y = Ae^{-t/\tau}$

R - nonlinear regression (nls)



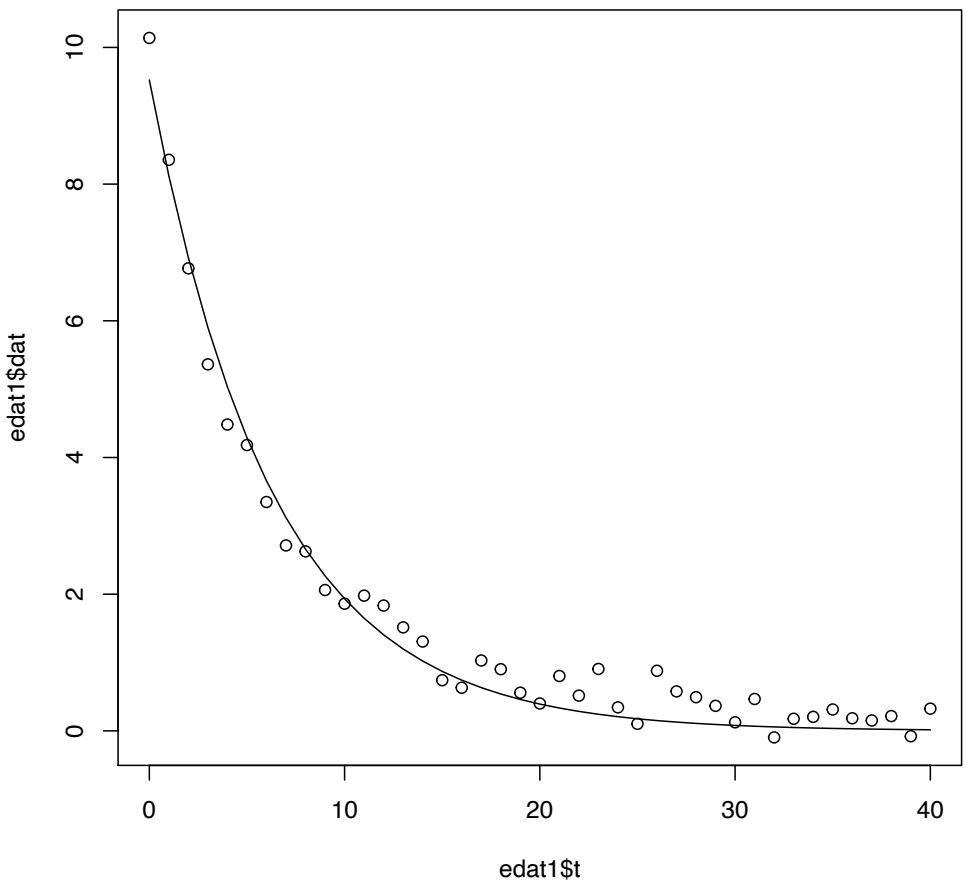
Observations?

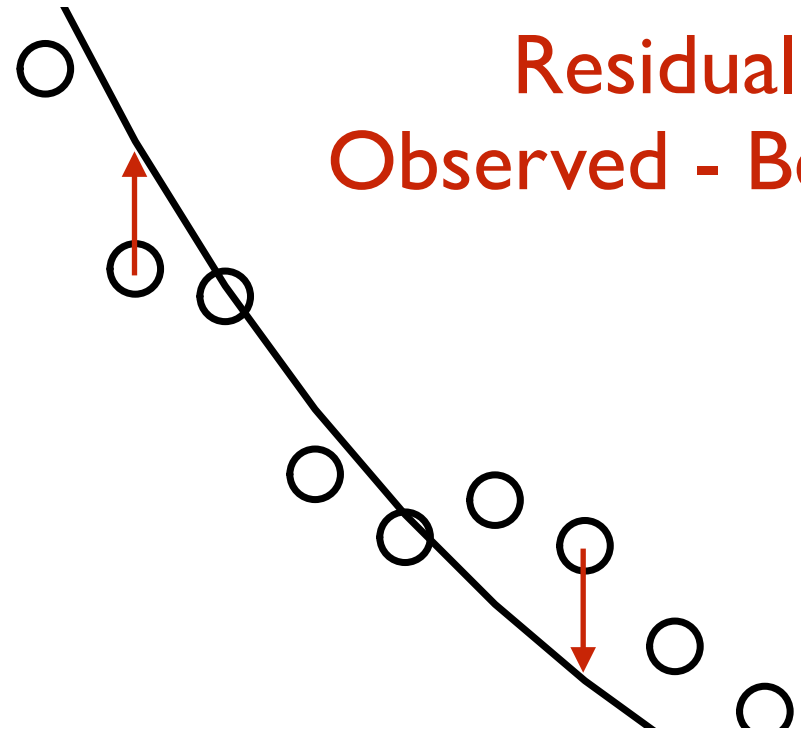
Rigorous?

R - Plot data and the fit

```
>model1 <- nls(edat$dat ~ eDecay(edat$t,ampl,tau), start=list(ampl=10,  tau=5), trace=TRUE)
```

fluor data *function call* *initial guesses*





Residual
Observed - BestFit

Mathematical goal of curve fitting

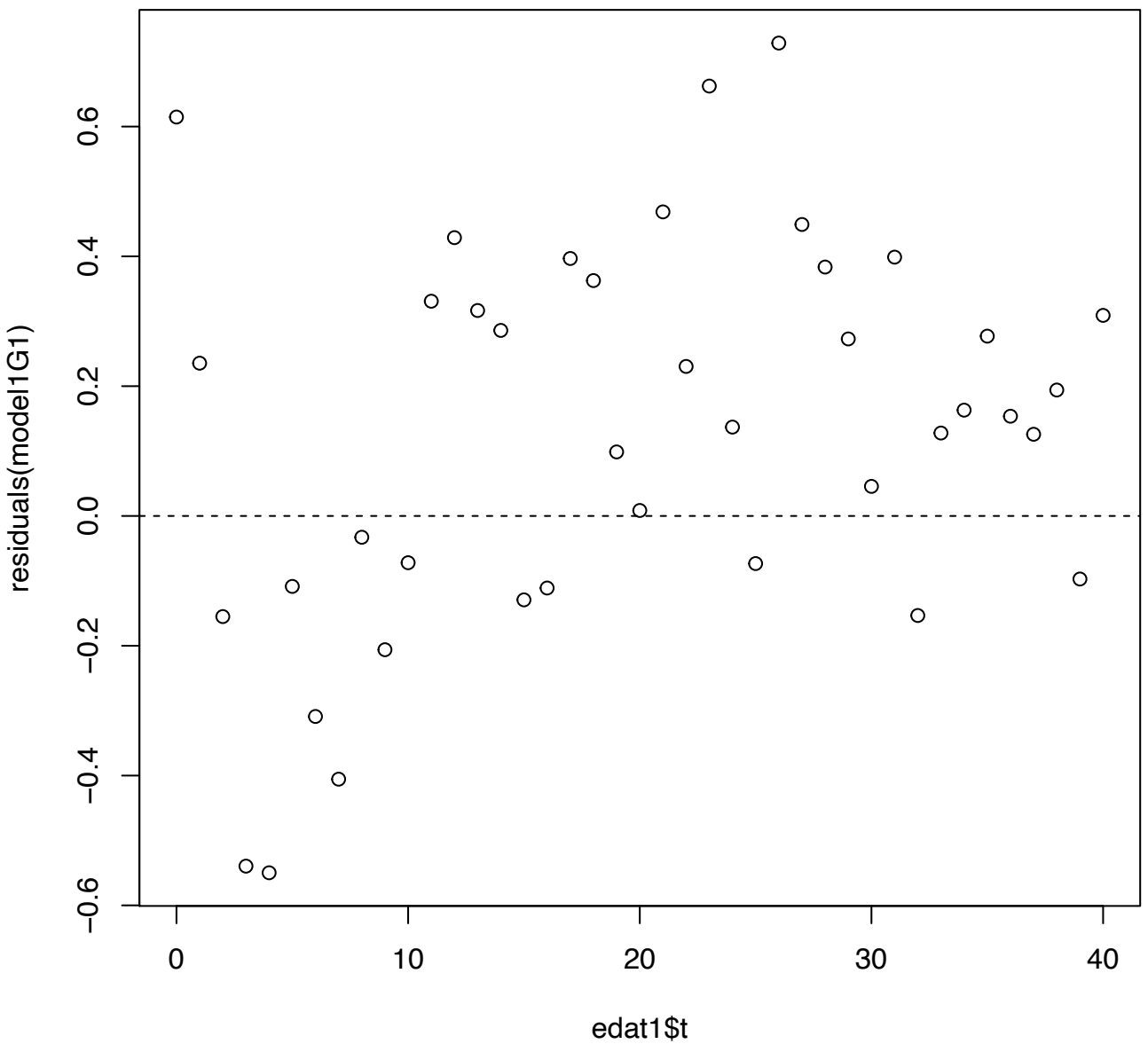
$$RSS = \sum_{i=1}^{n} (y_i - f(x_i))^2$$

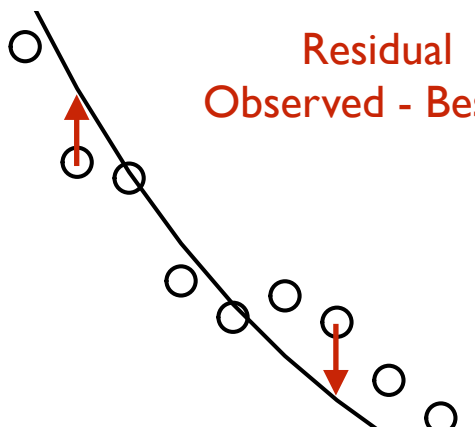
$y = Ae^{-t/\tau}$

R - Residuals

Return to Breakout Group

```
>plot(edat$t,residuals(model1))
>abline(h=c(0.0), lty=2)
```





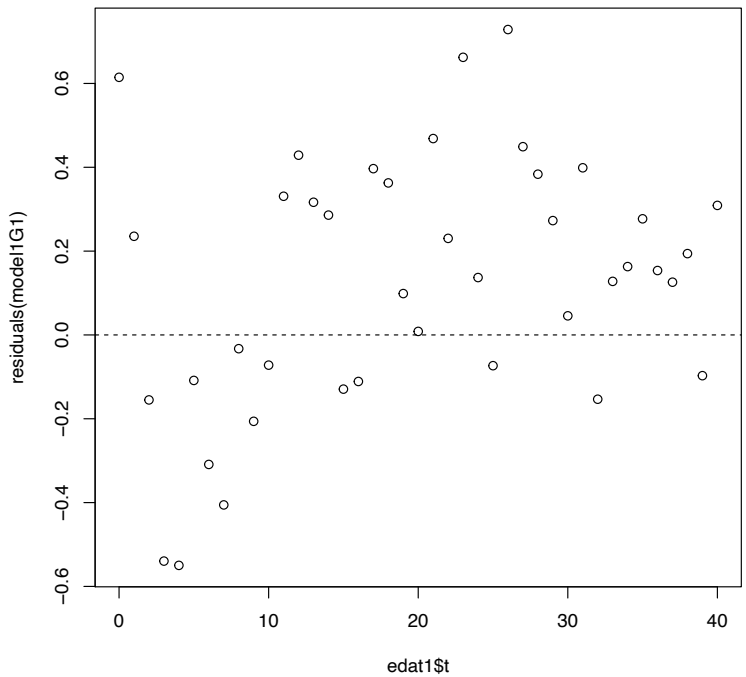
Residual
Observed - BestFit

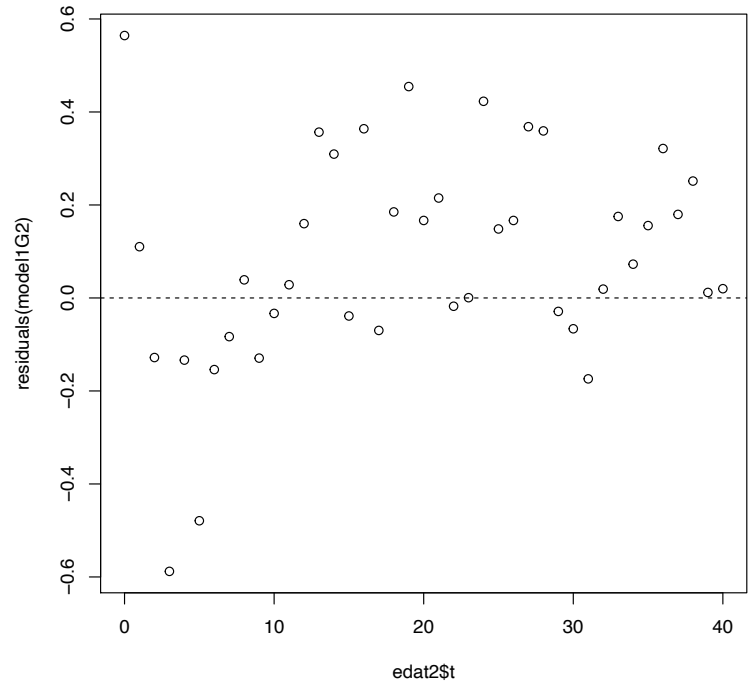
Mathematical goal of curve fitting

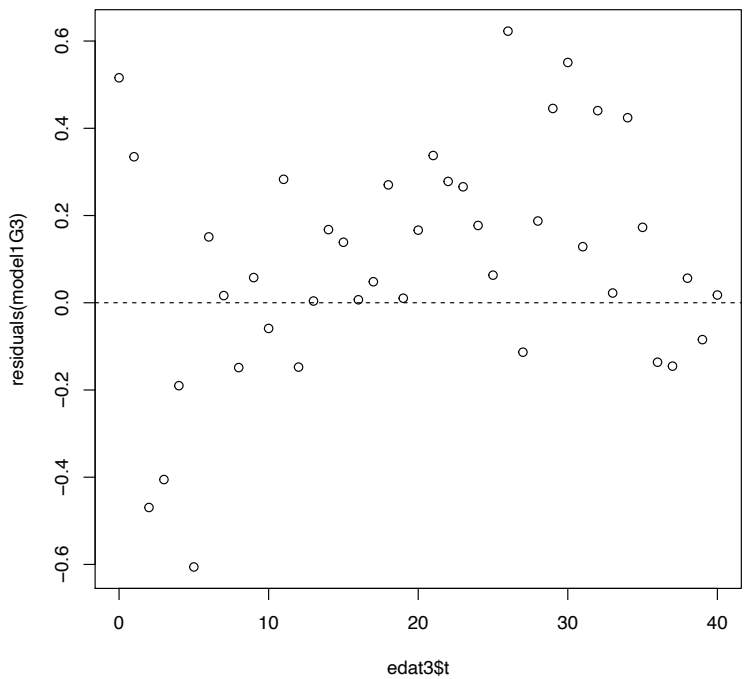
$$RSS = \sum_{i=1}^{n} (y_i - f(x_i))^2$$

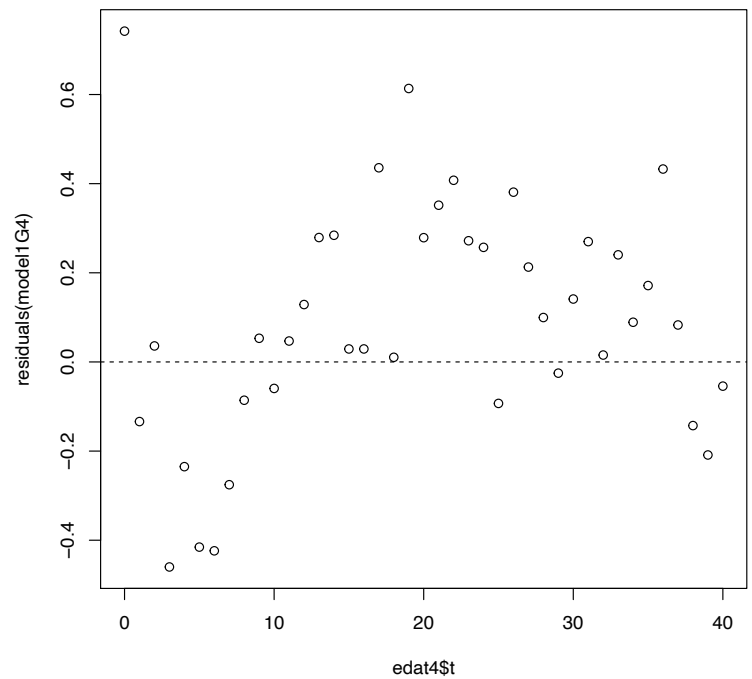
$y = Ae^{-t/\tau}$

R - Residuals





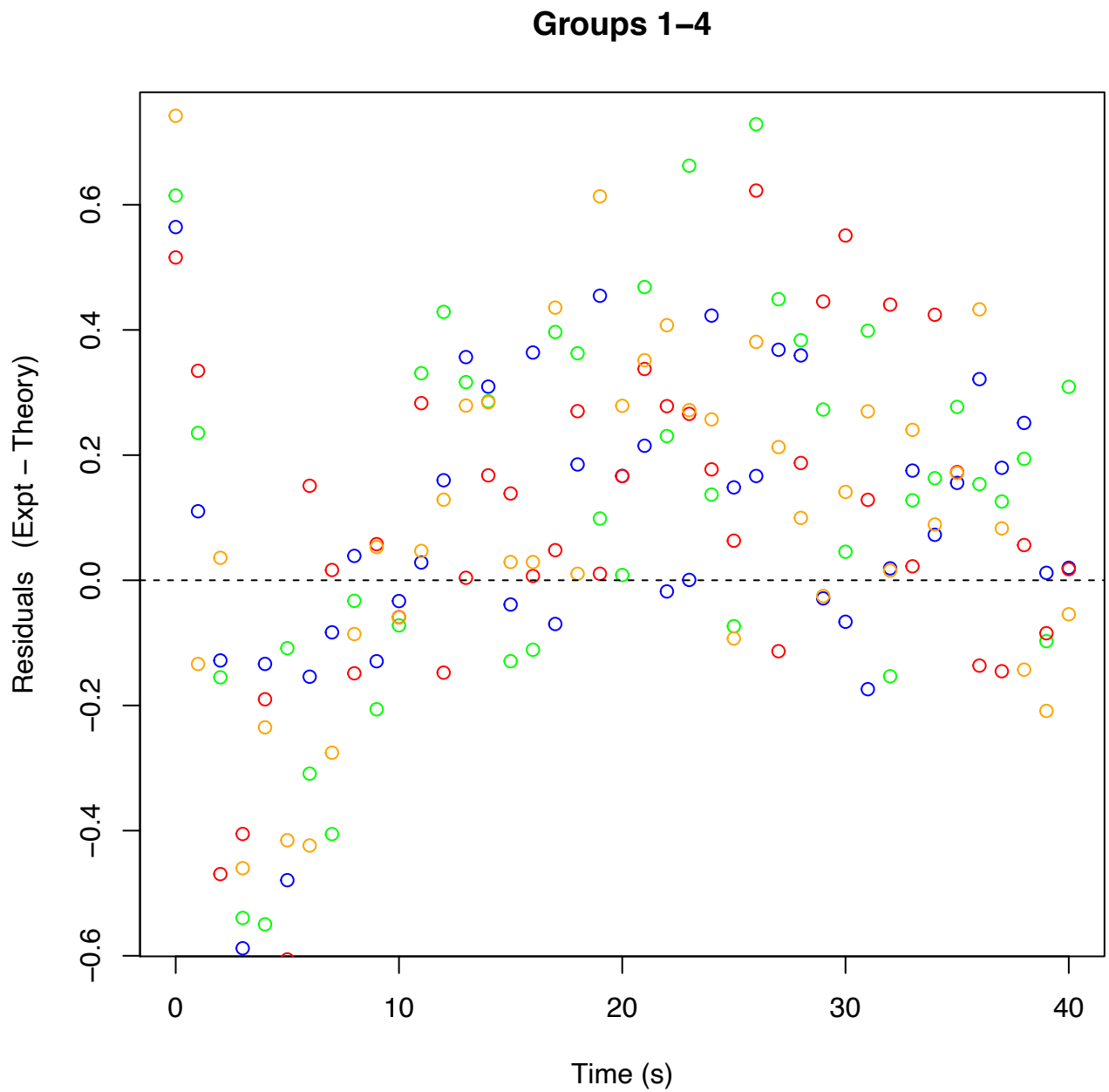




$y = Ae^{-t/\tau}$

R - Residuals

Residuals ≈ "Noise"
(or at least we supposed)



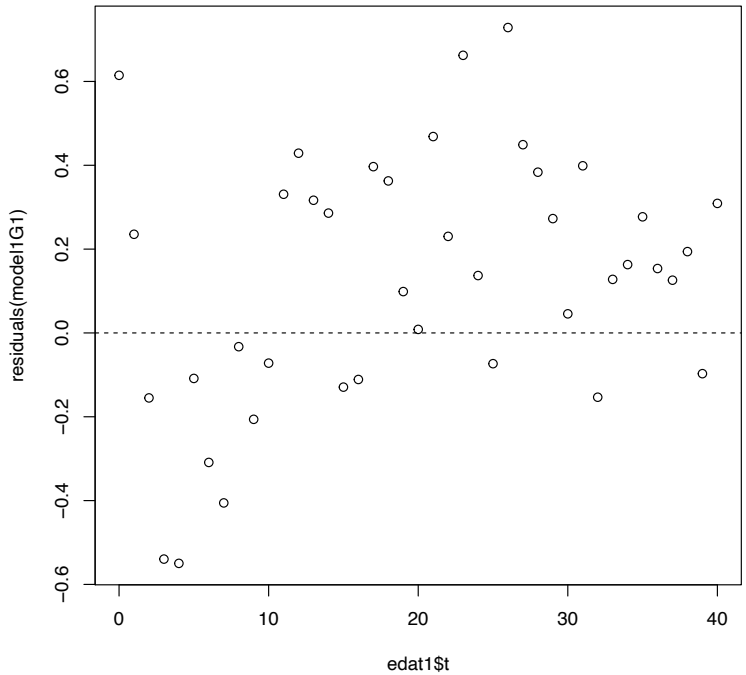
Observations?

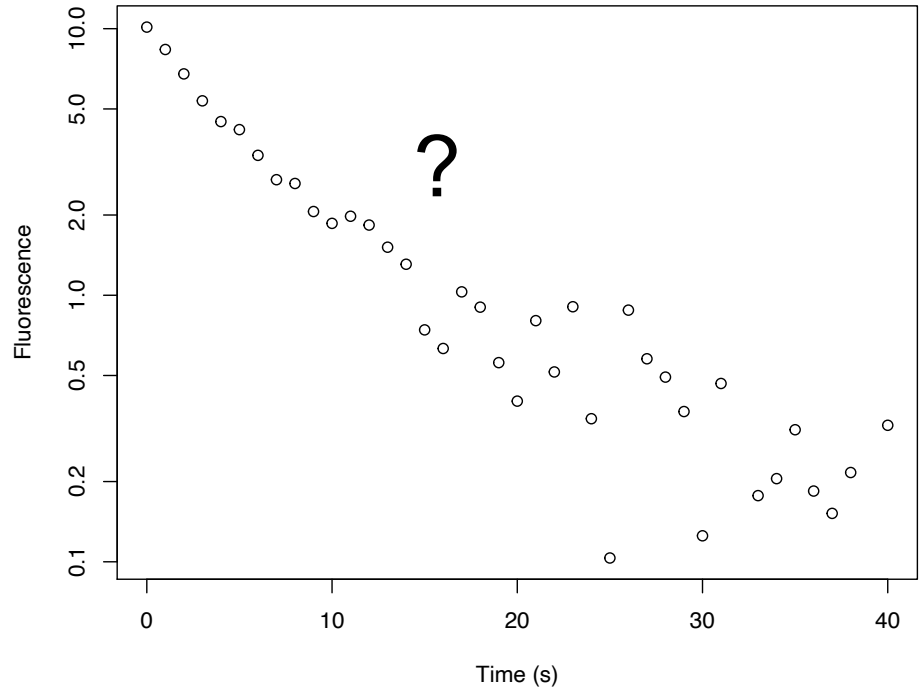
Noise should be
<u>randomly</u> distributed

In fact, the fitting *assumes* a <u>normal distribution</u> in the noise

$y = Ae^{-t/\tau}$

Wrong Model?

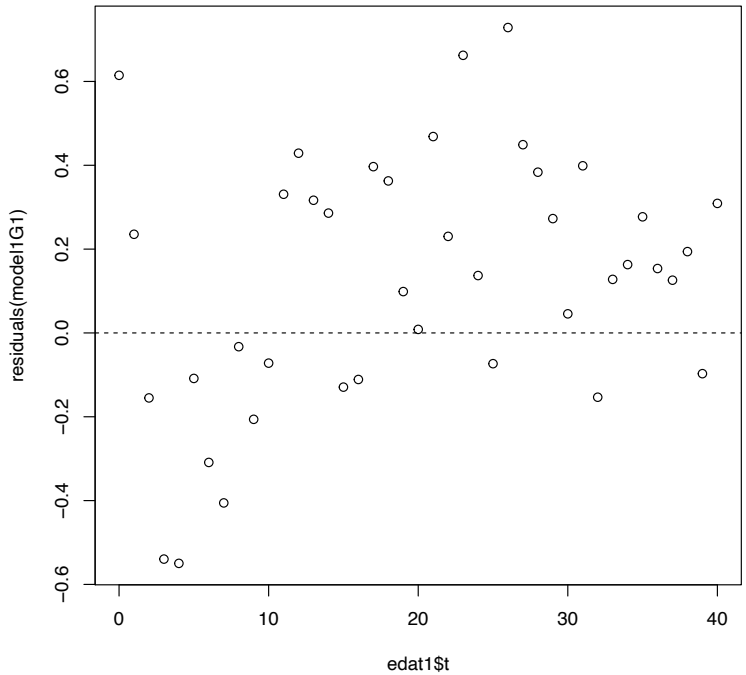


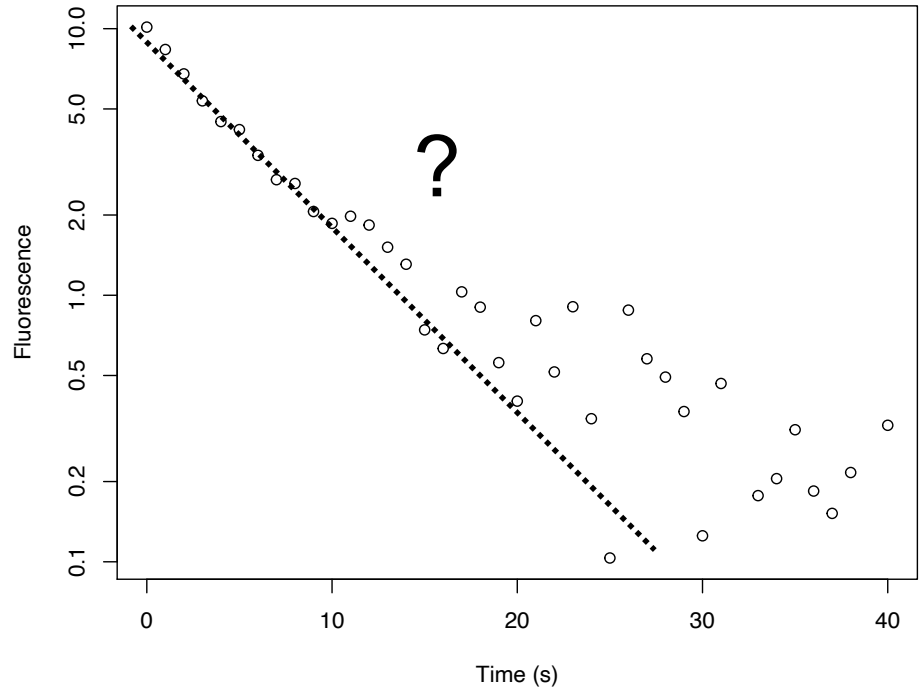



```
>model2 <- nls(edat$dat ~ eDecay2(edat$t,ampl,f,tau1,tau2), start=list(ampl=10,  f=0.5, tau1=3, tau2=8), trace=TRUE)
```

$y = Ae^{-t/\tau}$

Wrong Model?



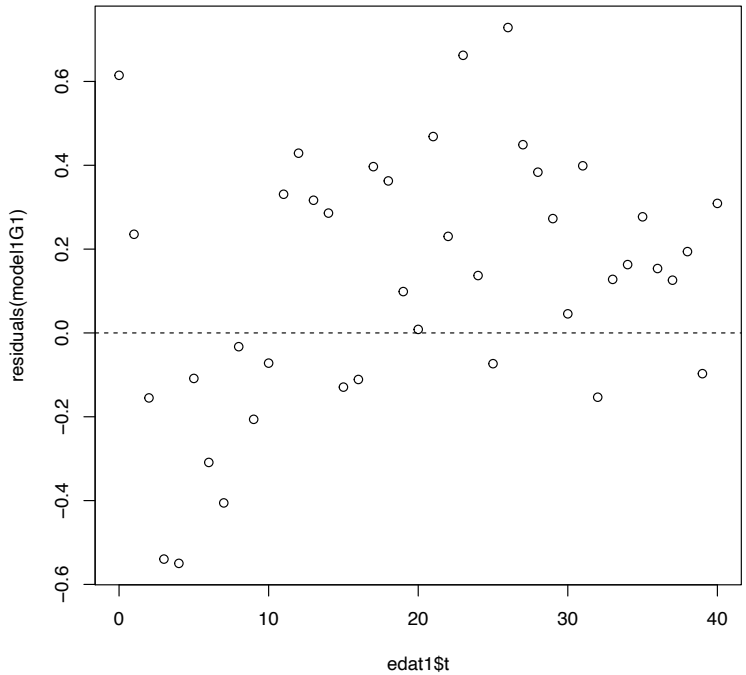


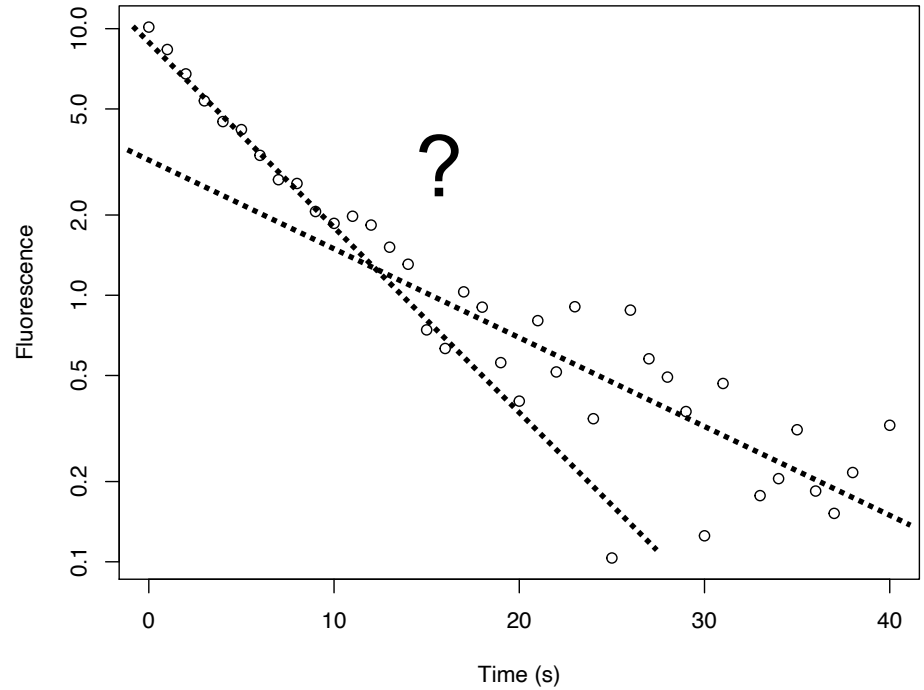

```
>model2 <- nls(edat$dat ~ eDecay2(edat$t,ampl,f,tau1,tau2), start=list(ampl=10,  f=0.5, tau1=3, tau2=8), trace=TRUE)
```

$y = Ae^{-t/\tau}$

Wrong Model?

Return to Breakout Group





$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

biexponential decay

```
>eDecay2 <- function(t, ampl, f, tau1, tau2) (ampl*((f*exp(-t/tau1))+((1-f)*exp(-t/tau2))))
```

func name — *define parameters* — *func definition*

```
>model2 <- nls(edat$dat ~ eDecay2(edat$t,ampl,f,tau1,tau2), start=list(ampl=10,  f=0.5, tau1=3, tau2=8), trace=TRUE)
```

$y = Ae^{-t/\tau}$ $y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$

R - nonlinear regression (nls)

```
>eDecay2 <- function(t, ampl, f, tau1, tau2) (ampl*((f*exp(-t/tau1))+((1-f)*exp(-t/tau2))))
>model2 <- nls(edat$dat ~ eDecay2(edat$t, ampl, f, tau1, tau2), start=list(ampl=10,  f=0.5, tau1=2.5, tau1=10), trace=TRUE)
>summary(model2)
```

Return to Breakout Group

```
Formula: edat1$dat ~ eDecay(edat1$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)

ampl   9.5239     0.2294   41.52   <2e-16 ***
tau    6.2702     0.2308   27.16   <2e-16 ***


Formula: edat2$dat ~ eDecay(edat2$t, ampl, tau)

ampl   9.3353     0.1727   54.05   <2e-16 ***
tau    6.3280     0.1788   35.39   <2e-16 ***


Formula: edat3$dat ~ eDecay(edat3$t, ampl, tau)

ampl   9.3329     0.1938   48.16   <2e-16 ***
tau    6.4709     0.2049   31.58   <2e-16 ***


Formula: edat4$dat ~ eDecay(edat4$t, ampl, tau)

ampl   9.1827     0.1936   47.44   <2e-16 ***
tau    6.5311     0.2098   31.12   <2e-16 ***
```

```
Formula: edat1$dat ~ eDecay2(edat1$t, ampl, f1, tau1, tau2)

                    Parameters:
           Estimate Std. Error t value Pr(>|t|)

ampl  10.1816     0.1912  53.241  < 2e-16 ***
f      0.6374     0.1074   5.933 7.75e-07 ***
tau1   3.4062     0.5409   6.297 2.49e-07 ***
tau2  11.8507     2.0367   5.818 1.11e-06 ***

Formula: edat2$dat ~ eDecay2(edat2$t, ampl, f1, tau1, tau2)

ampl   9.9212     0.1512  65.614  < 2e-16 ***
f      0.4342     0.1035   4.195 0.000163 ***
tau1   2.7866     0.5781   4.820 2.46e-05 ***
tau2   8.9873     0.8865  10.138 3.16e-12 ***

Formula: edat3$dat ~ eDecay2(edat3$t, ampl, f1, tau1, tau2)

ampl   9.8412     0.1867  52.709  < 2e-16 ***
f      0.5901     0.1615   3.654 0.000795 ***
tau1   3.6538     0.7715   4.736 3.18e-05 ***
tau2  10.8119     2.1369   5.060 1.17e-05 ***

Formula: edat4$dat ~ eDecay2(edat4$t, ampl, f1, tau1, tau2)

ampl  9.86679    0.15321  64.401  < 2e-16 ***
f     0.49242    0.08878   5.547 2.58e-06 ***
tau1  2.90183    0.49557   5.856 9.85e-07 ***
tau2 10.03356    1.00571   9.977 4.89e-12 ***
```

$$y = A[f e^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

R - nonlinear regression (nls)

```
>eDecay2 <- function(t, ampl, f, tau1, tau2) (ampl*((f*exp(-t/tau1))+((1-f)*exp(-t/tau2))))
>model2 <- nls(edat$dat ~ eDecay2(edat$t, ampl, f, tau1, tau2), start=list(ampl=10,  f=0.5, tau1=2.5, tau1=10), trace=TRUE)
```

0.64 ± 0.11

0.43 ± 0.10

0.59 ± 0.16 s

0.49 ± 0.09 s

$$y = A[f e^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

10.2 ± 0.2	3.4 ± 0.5 s	12 ± 2 s
9.9 ± 0.2	2.8 ± 0.6 s	9 ± 1 s
9.8 ± 0.2	3.7 ± 0.8 s	11 ± 2 s
9.9 ± 0.2	2.9 ± 0.5 s	10 ± 1 s

```
Formula: edat1$dat ~ eDecay2(edat1$t, ampl, f1, tau1, tau2)

                    Parameters:
           Estimate Std. Error t value Pr(>|t|)

      ampl  10.1816     0.1912  53.241  < 2e-16 ***
      f      0.6374     0.1074   5.933 7.75e-07 ***
      tau1   3.4062     0.5409   6.297 2.49e-07 ***
      tau2  11.8507     2.0367   5.818 1.11e-06 ***

Formula: edat2$dat ~ eDecay2(edat2$t, ampl, f1, tau1, tau2)

      ampl   9.9212     0.1512  65.614  < 2e-16 ***
      f      0.4342     0.1035   4.195 0.000163 ***
      tau1   2.7866     0.5781   4.820 2.46e-05 ***
      tau2   8.9873     0.8865  10.138 3.16e-12 ***

Formula: edat3$dat ~ eDecay2(edat3$t, ampl, f1, tau1, tau2)

      ampl   9.8412     0.1867  52.709  < 2e-16 ***
      f      0.5901     0.1615   3.654 0.000795 ***
      tau1   3.6538     0.7715   4.736 3.18e-05 ***
      tau2  10.8119     2.1369   5.060 1.17e-05 ***

Formula: edat4$dat ~ eDecay2(edat4$t, ampl, f1, tau1, tau2)

      ampl  9.86679    0.15321  64.401  < 2e-16 ***
      f     0.49242    0.08878   5.547 2.58e-06 ***
      tau1  2.90183    0.49557   5.856 9.85e-07 ***
      tau2 10.03356    1.00571   9.977 4.89e-12 ***
```

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

R - nonlinear regression (nls)

```
>eDecay2 <- function(t, ampl, f, tau1, tau2) (ampl*((f*exp(-t/tau1))+((1-f)*exp(-t/tau2))))
>model2 <- nls(edat$dat ~ eDecay2(edat$t, ampl, f, tau1, tau2), start=list(ampl=10,  f=0.5, tau1=2.5, tau1=10), trace=TRUE)
```

0.64 ± 0.11
0.43 ± 0.10
0.59 ± 0.16 s
0.49 ± 0.09 s

Less reproducible

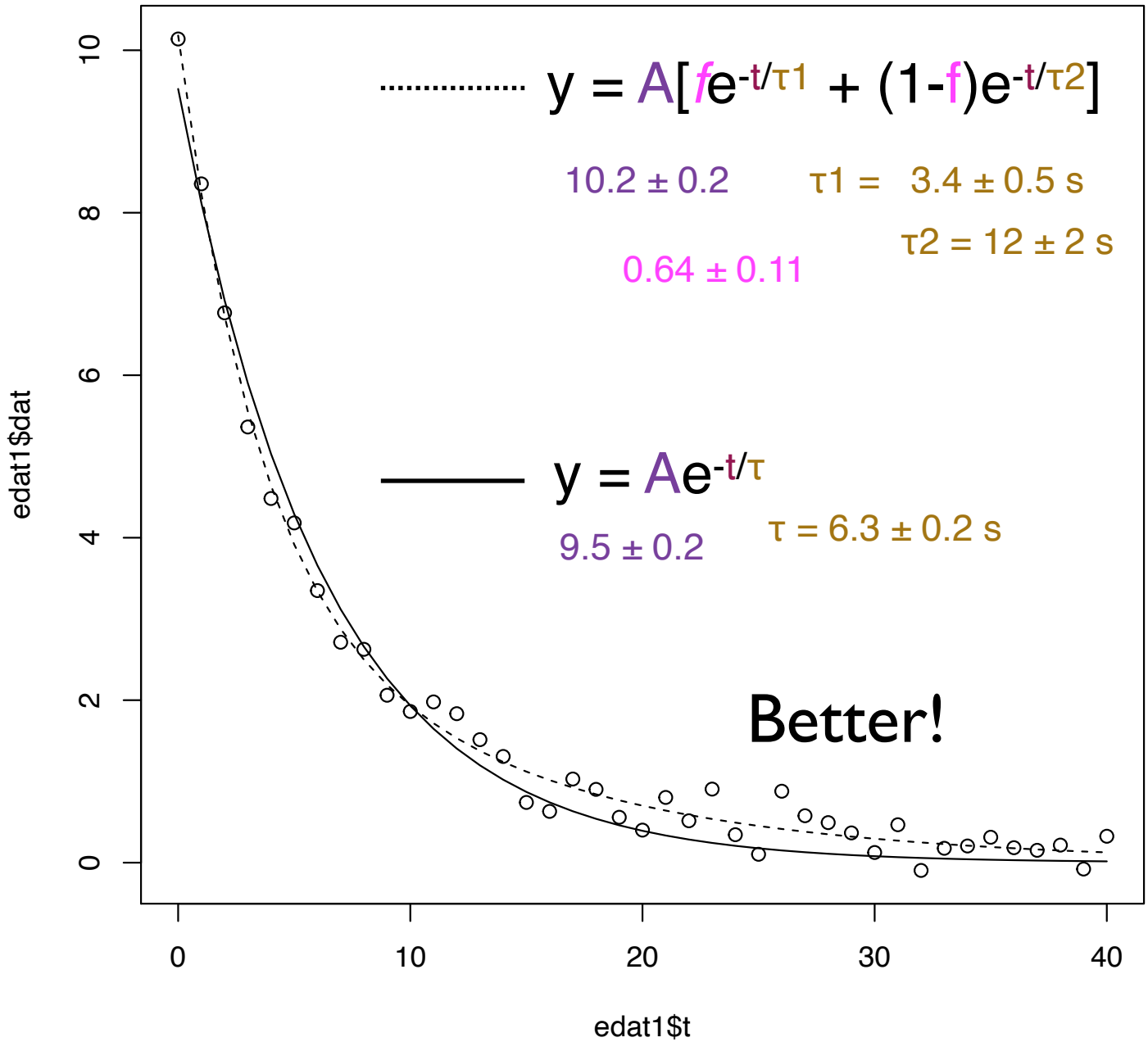
$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

10.2 ± 0.2	3.4 ± 0.5 s	12 ± 2 s
9.9 ± 0.2	2.8 ± 0.6 s	9 ± 1 s
9.8 ± 0.2	3.7 ± 0.8 s	11 ± 2 s
9.9 ± 0.2	2.9 ± 0.5 s	10 ± 1 s

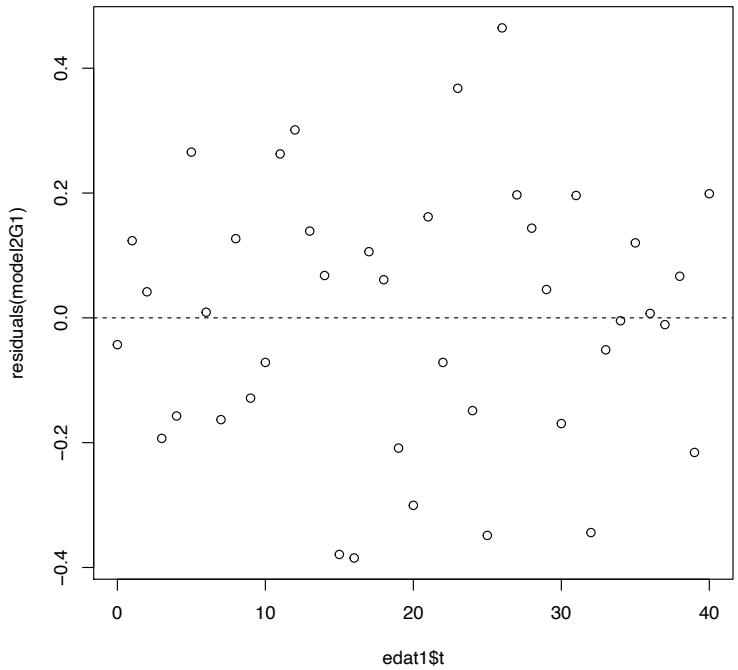
$$y = Ae^{-t/\tau}$$

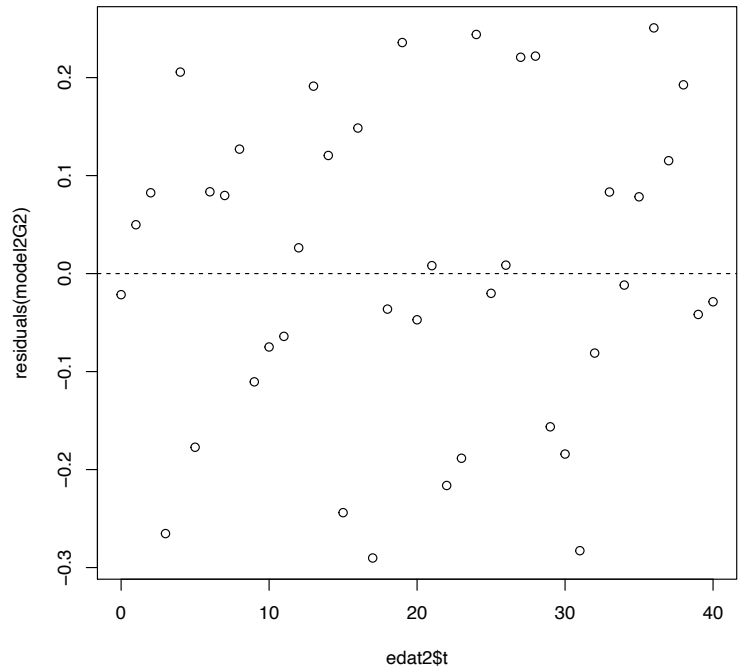
9.5 ± 0.2	6.3 ± 0.2 s
9.3 ± 0.2	6.3 ± 0.2 s
9.3 ± 0.2	6.5 ± 0.2 s
9.2 ± 0.2	6.5 ± 0.2 s

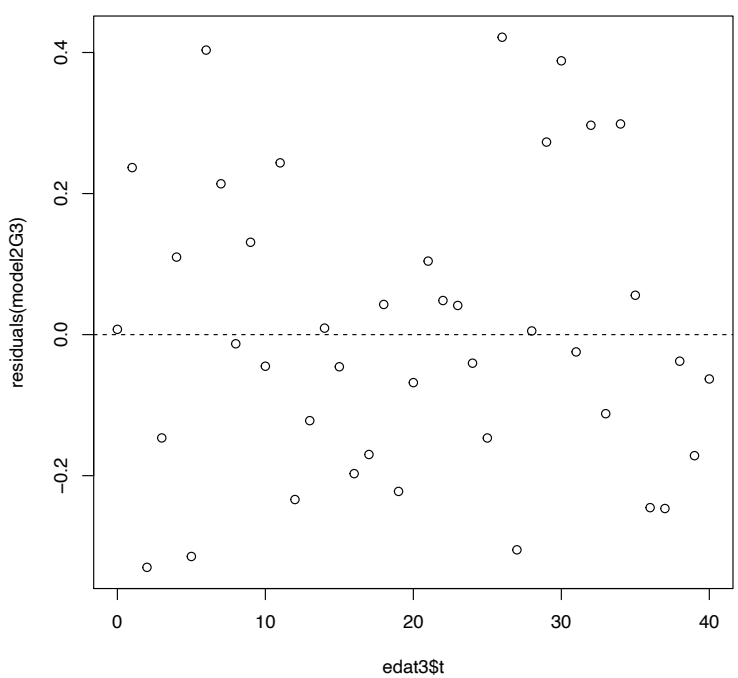
R - nonlinear regression (nls)

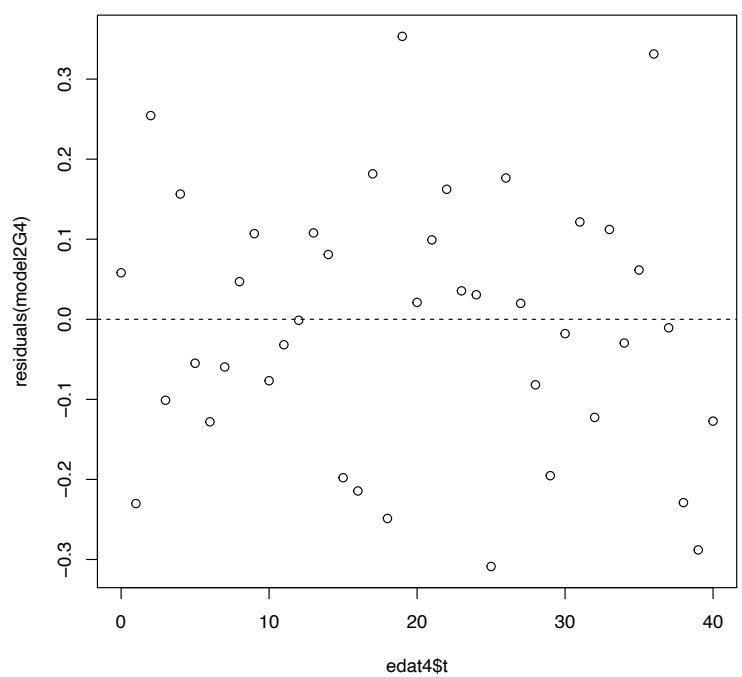


R - residuals

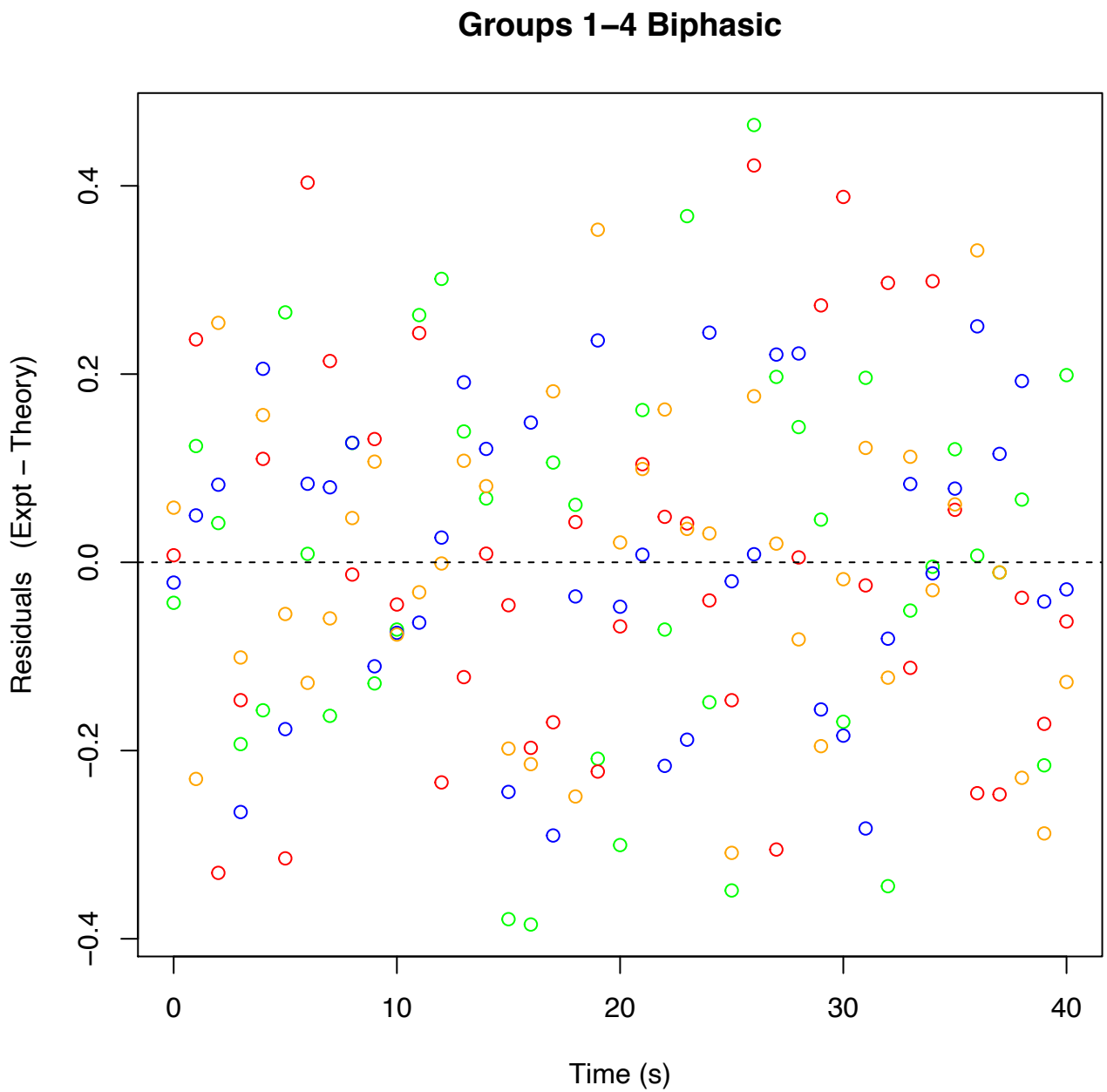

residuals(model2G1)
edat1$t


residuals(model2G2)
edat2$t


residuals(model2G3)
edat3$t


residuals(model2G4)
edat4$t

R - residuals


Groups 1–4 Biphasic
Residuals (Expt – Theory)
0.4
0.2
0.0
−0.2
−0.4
0
10
20
30
40
Time (s)

R - residuals

```
      Estimate Std. Error t value Pr(>|t|)
ampl  10.1816     0.1912  53.241  < 2e-16 ***
f      0.6374     0.1074   5.933 7.75e-07 ***
tau1   3.4062     0.5409   6.297 2.49e-07 ***
tau2  11.8507     2.0367   5.818 1.11e-06 ***
```

10.2 ± 0.2

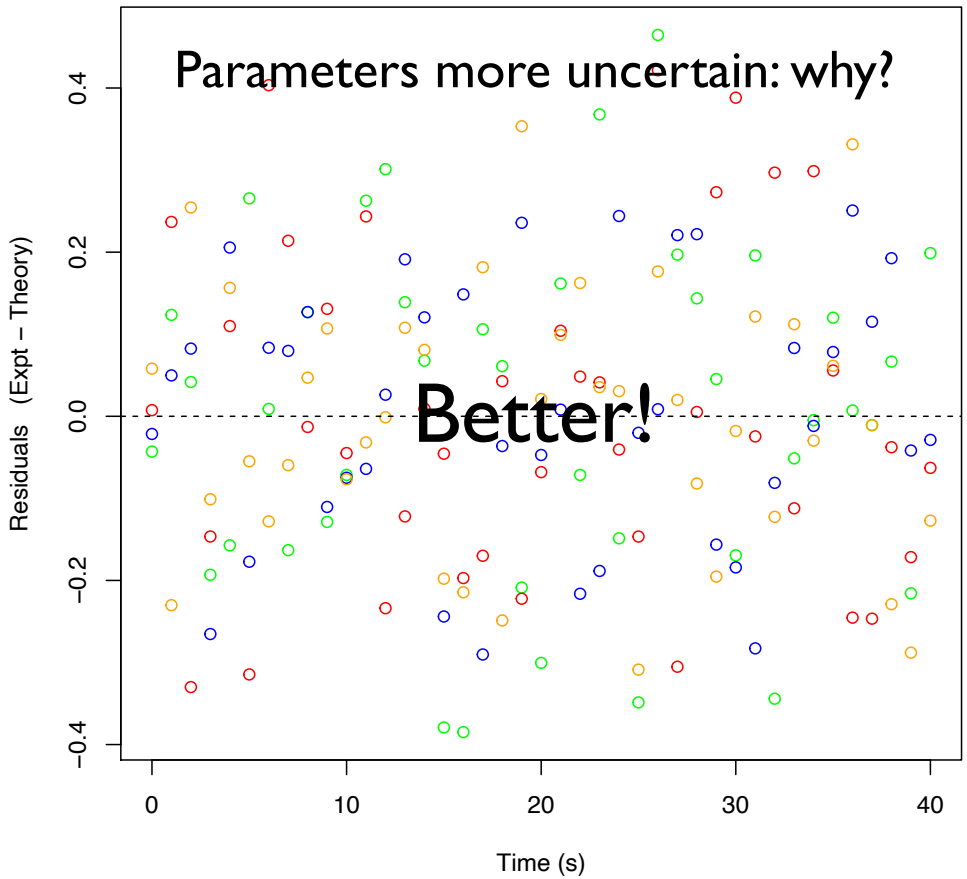
τ1 = 3.4 ± 0.5 s

0.64 ± 0.11

τ2 = 12 ± 2 s

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

Groups 1–4 Biphasic



Reproducible

More rigorous

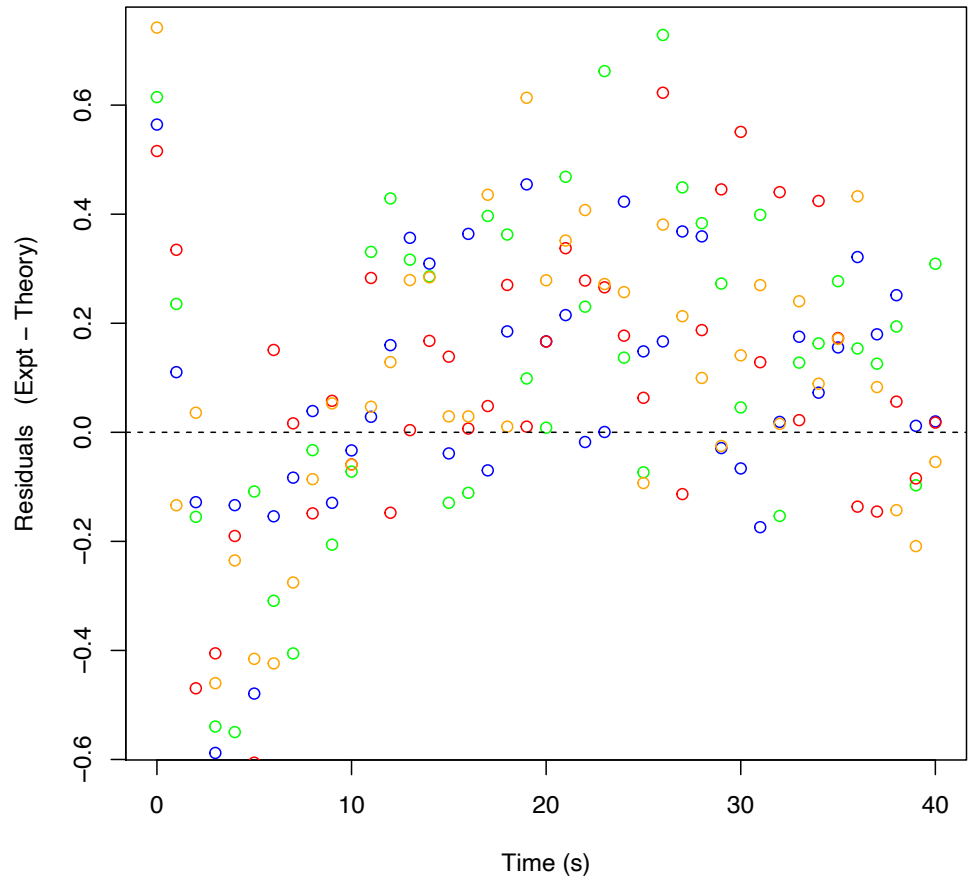
```
      Estimate Std. Error t value Pr(>|t|)
ampl   9.5239     0.2294   41.52   <2e-16 ***
tau    6.2702     0.2308   27.16   <2e-16 ***
```

9.5 ± 0.2

τ = 6.3 ± 0.2 s

$$y = Ae^{-t/\tau}$$

Groups 1–4



Reproducible

Rigorous?

R - residuals

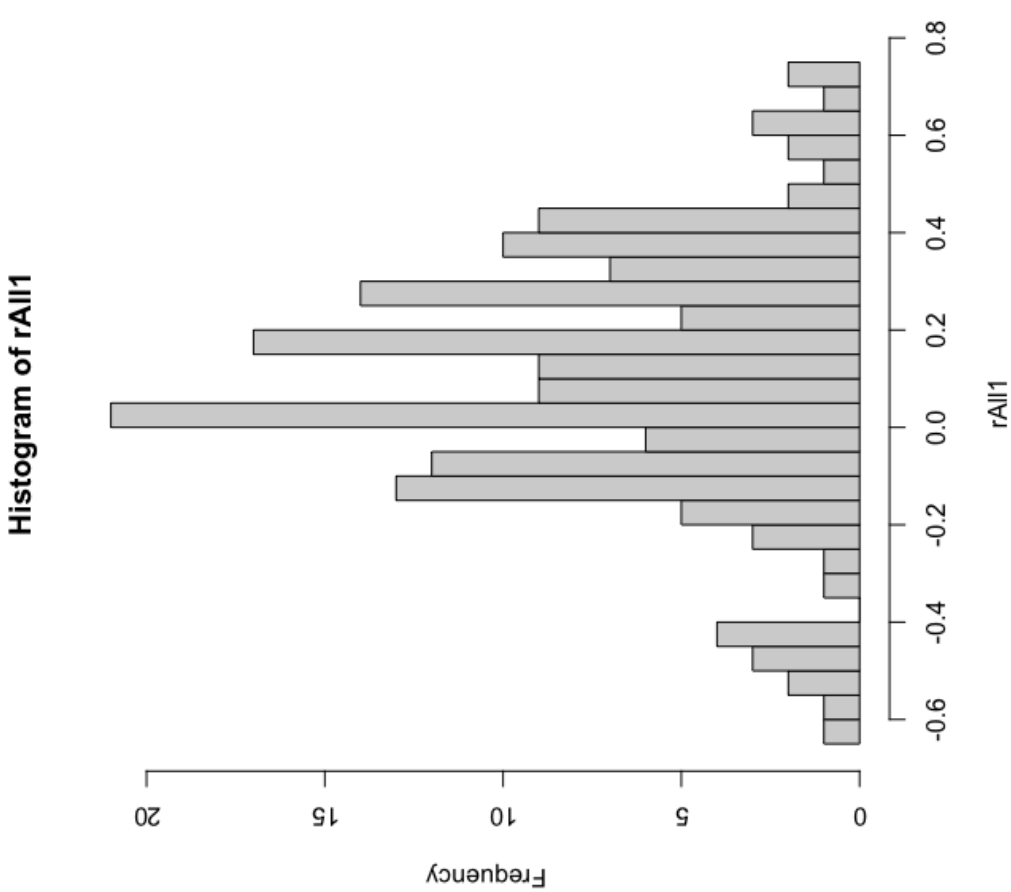
Noise should be *randomly* distributed

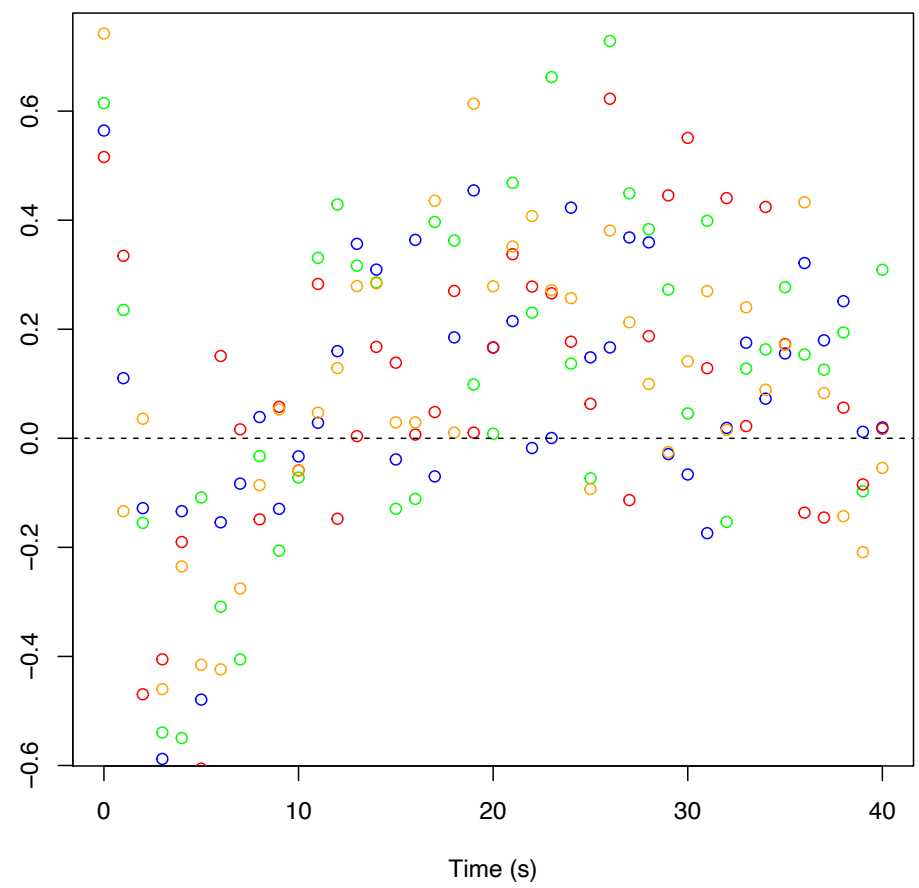
In fact, the fitting *assumes* a normal distribution in the noise

```
        Estimate Std. Error t value Pr(>|t|)
ampl    9.5239     0.2294   41.52   <2e-16 ***
tau     6.2702     0.2308   27.16   <2e-16 ***
```

9.5 ± 0.2 τ = 6.3 ± 0.2 s

$$y = Ae^{-t/\tau}$$





R - residuals

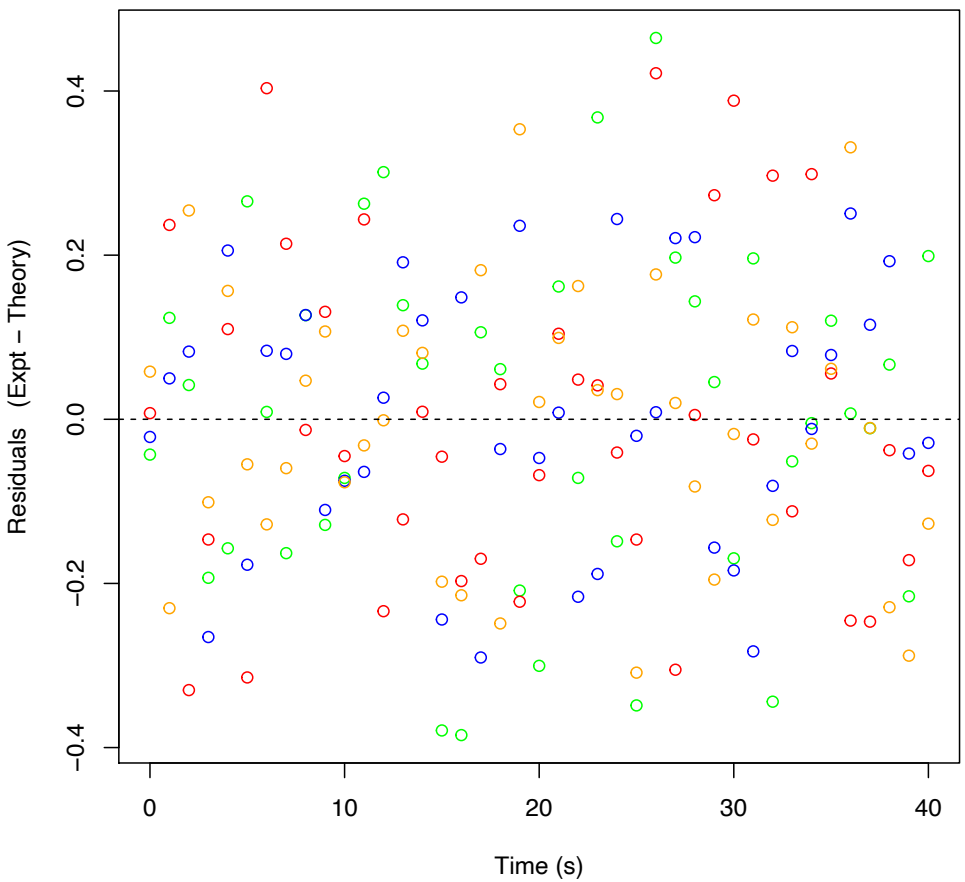
Noise should be *randomly* distributed

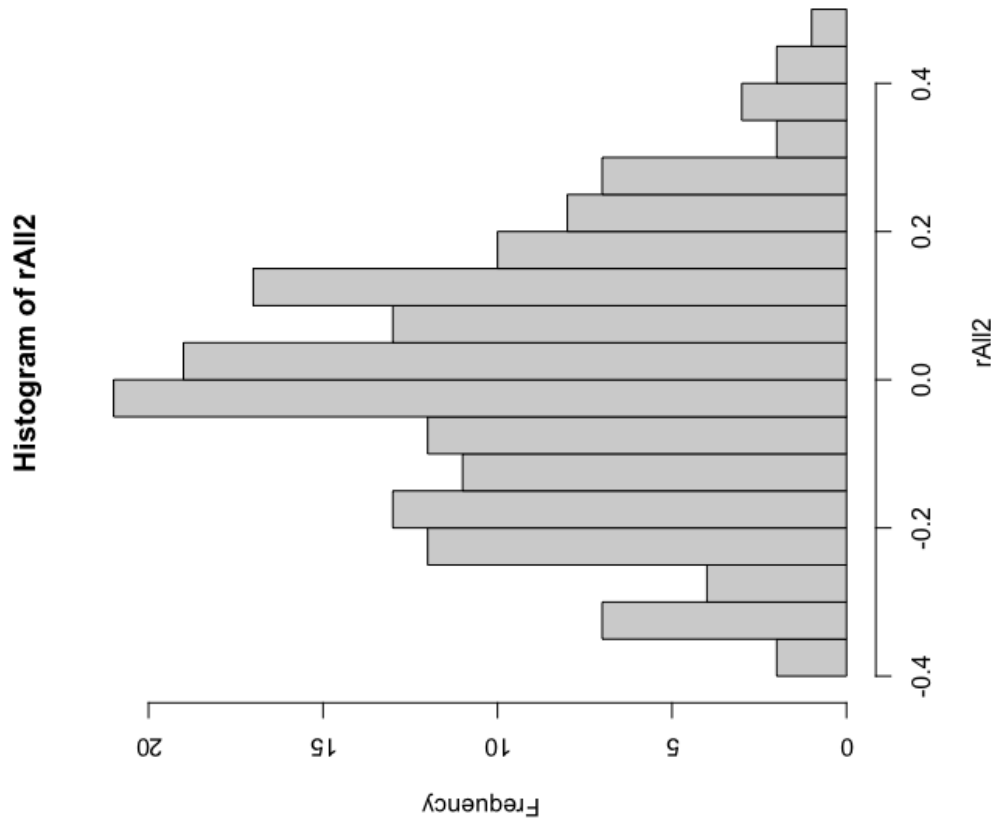
10.2 ± 0.2 τ1 = 3.4 ± 0.5 s

0.64 ± 0.11 τ2 = 12 ± 2 s

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

In fact, the fitting *assumes* a normal distribution in the noise

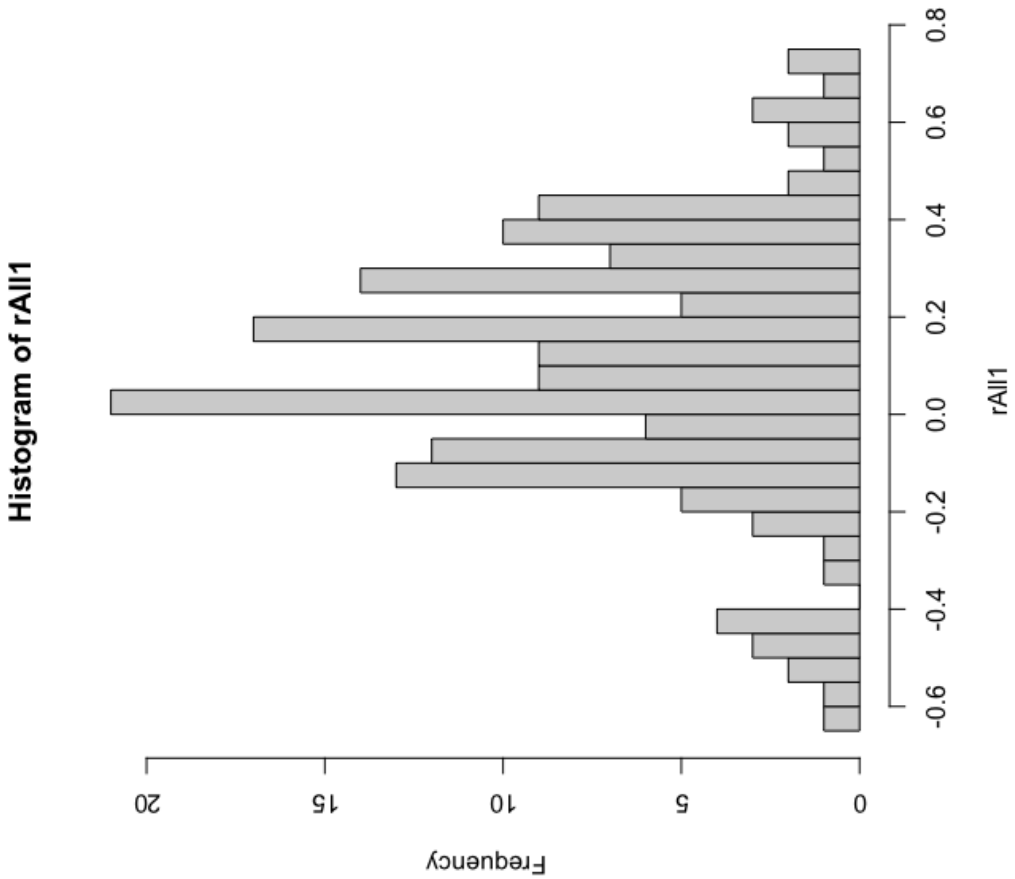


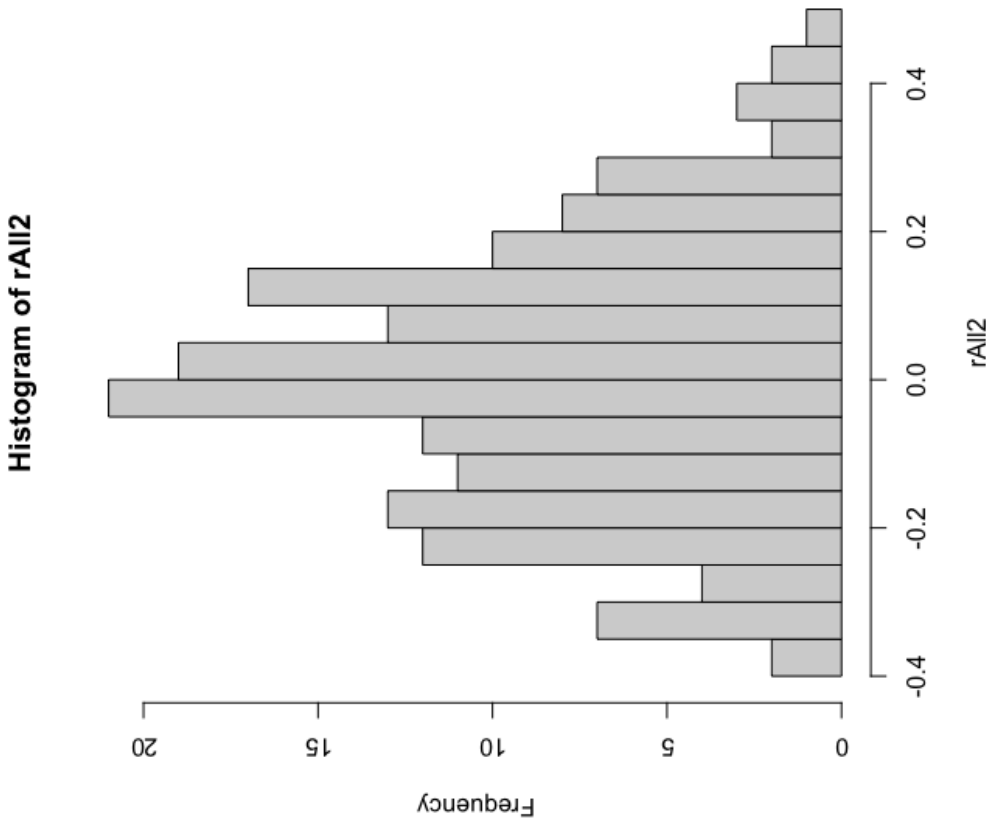


R - residuals

$y = Ae^{-t/\tau}$

$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$

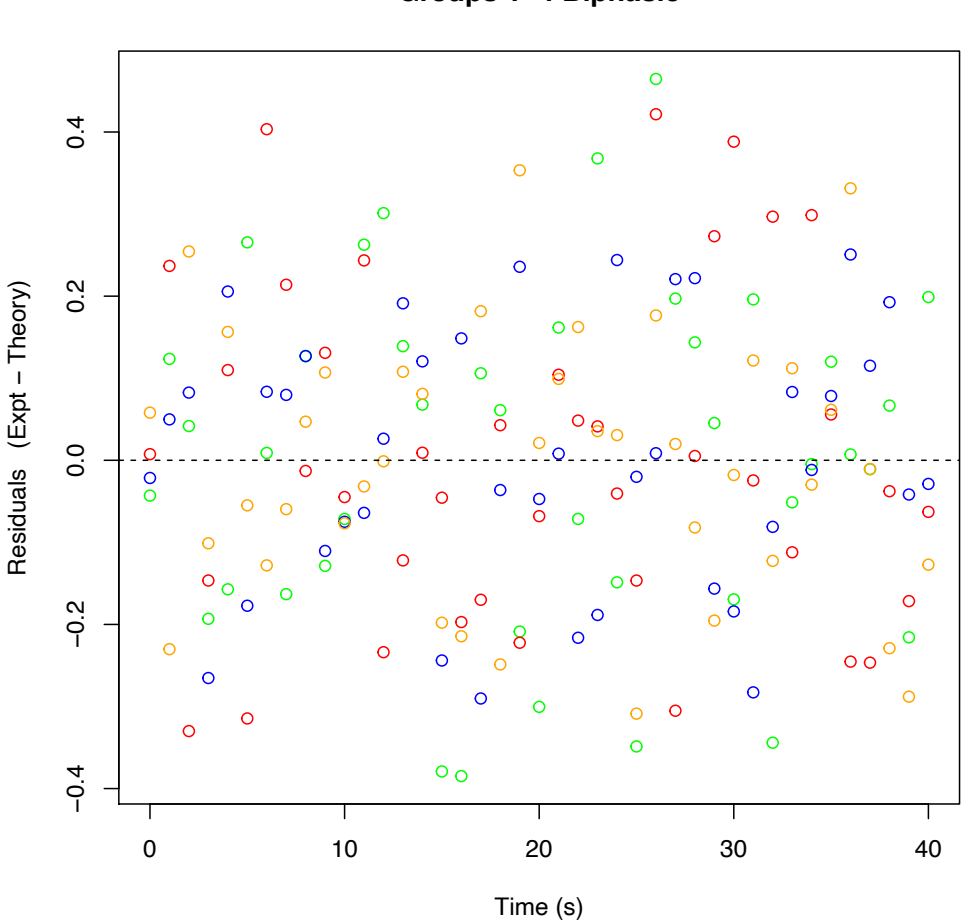




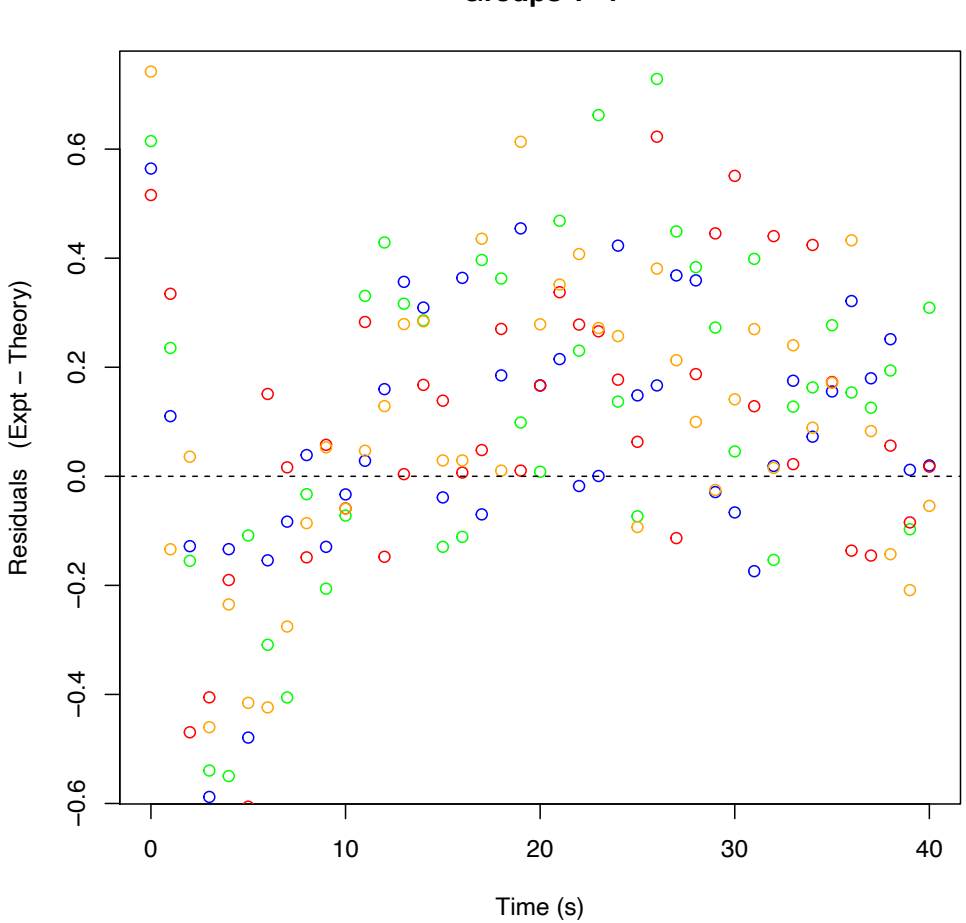
We will rarely have this much data!

R - residuals

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

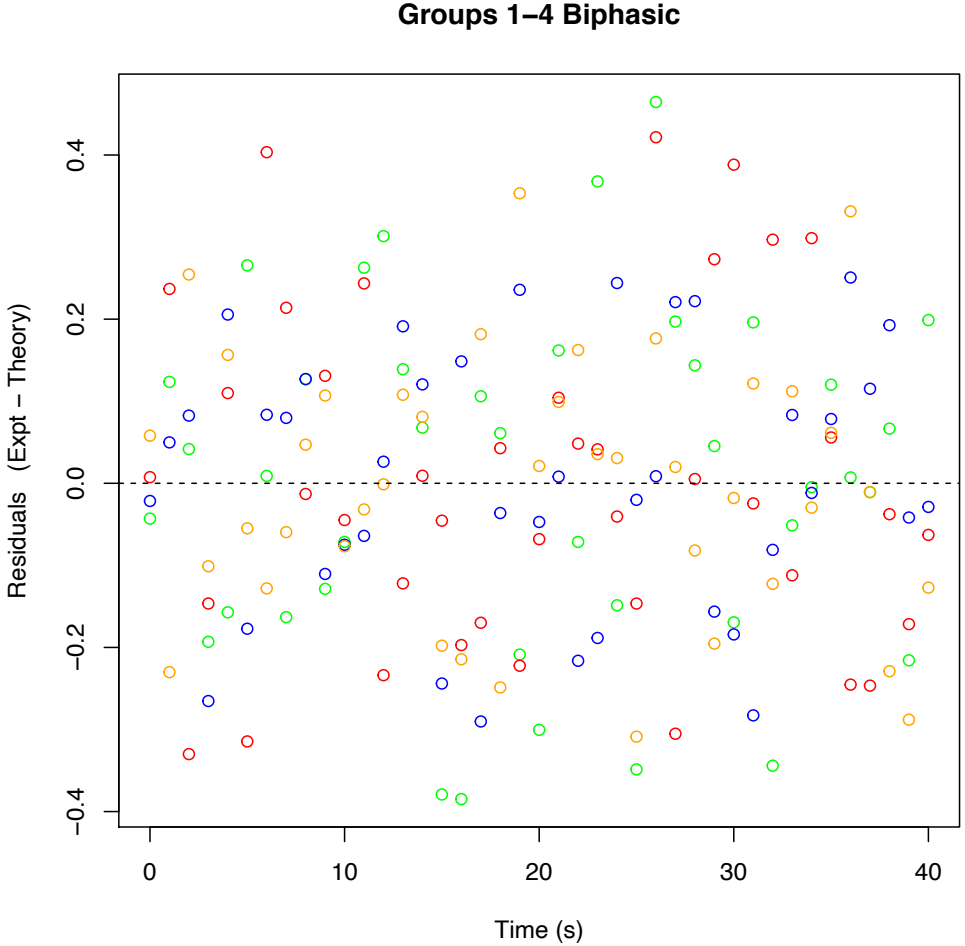


$$y = Ae^{-t/\tau}$$

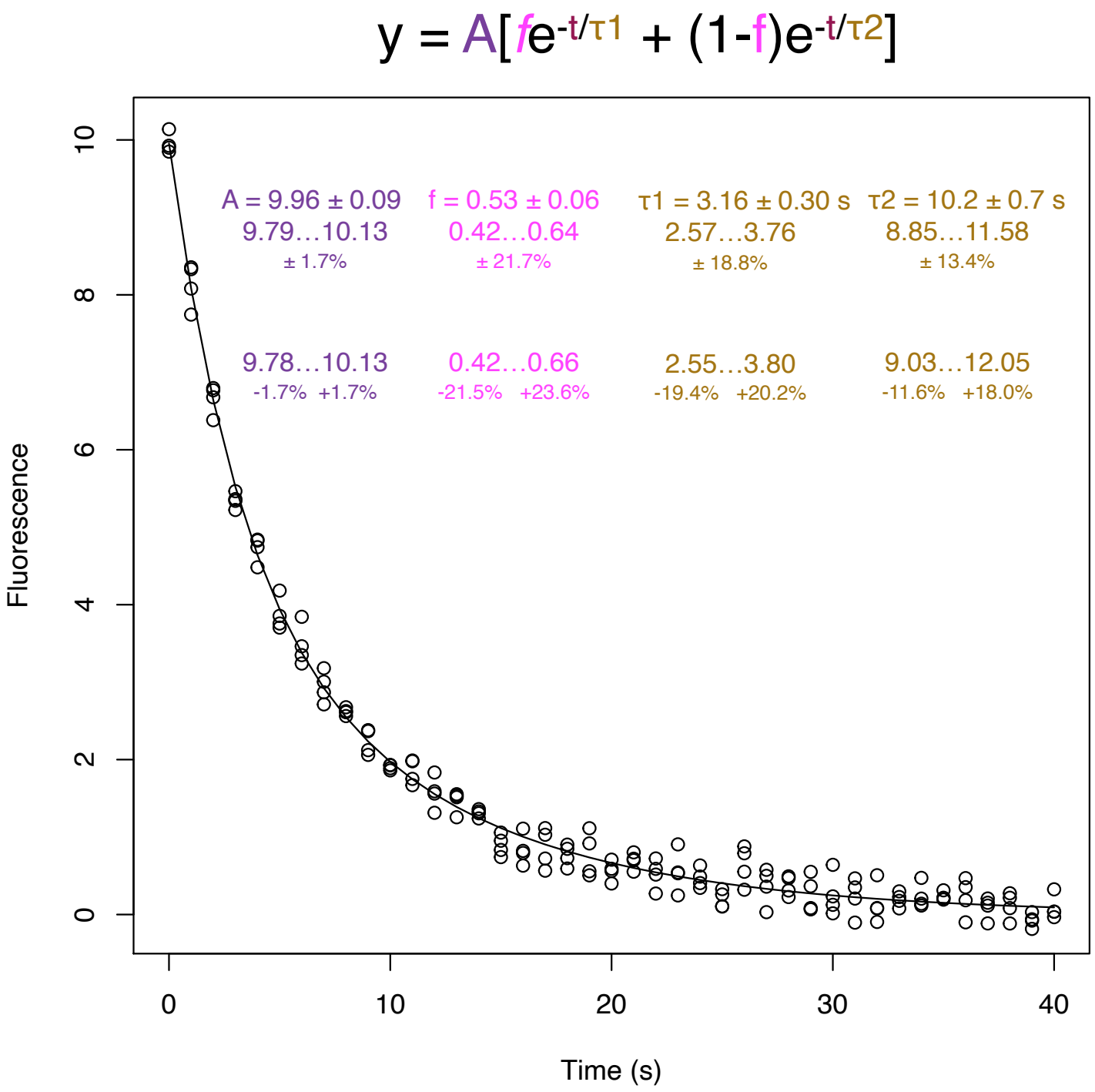


R - residuals

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$



Perhaps we should fit with a Tri-Exponential?

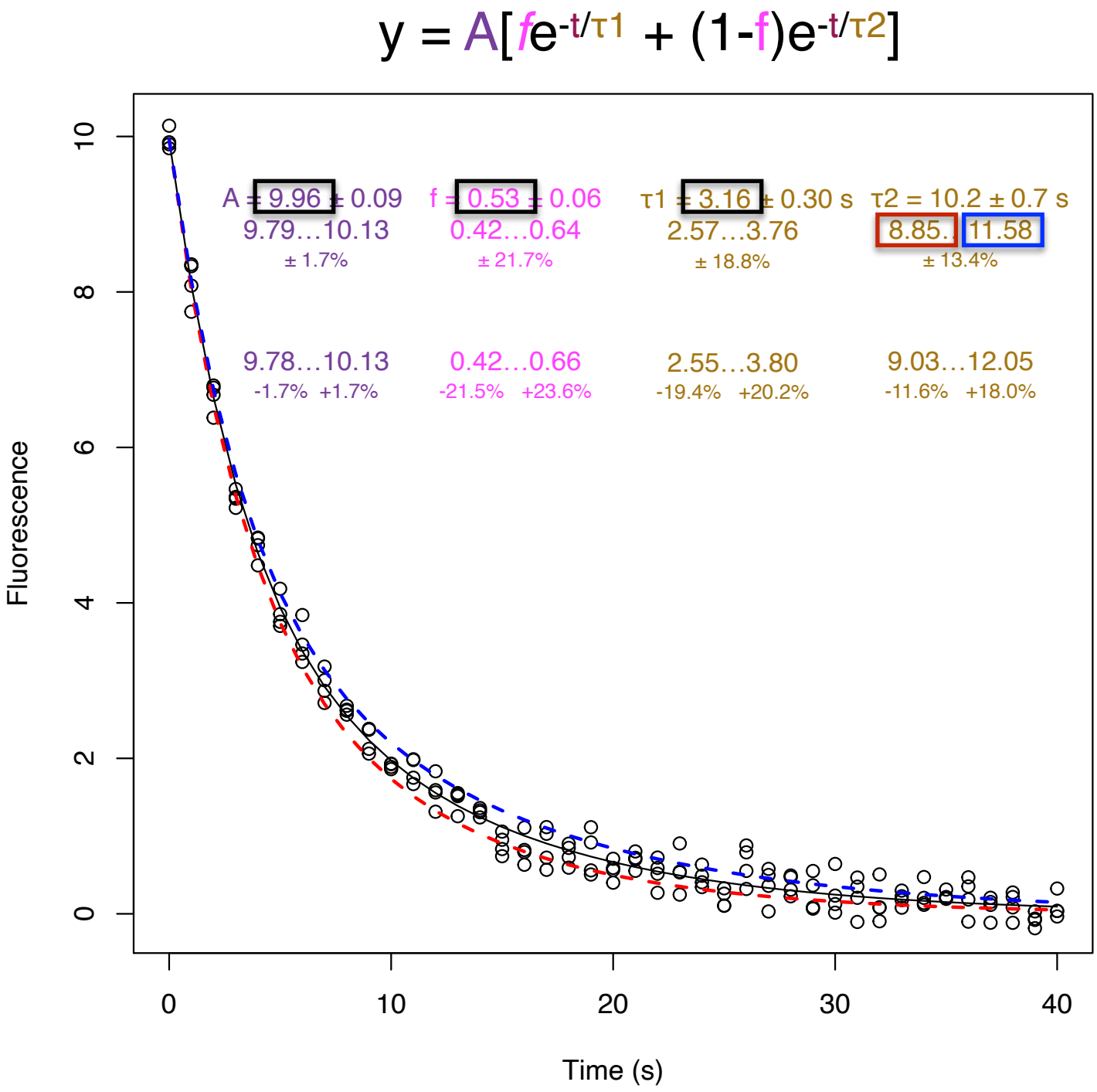


summary(model)

param ± std error
(param ± 2(st err))
percent

97.5% confidence interval
percent

confint(model)

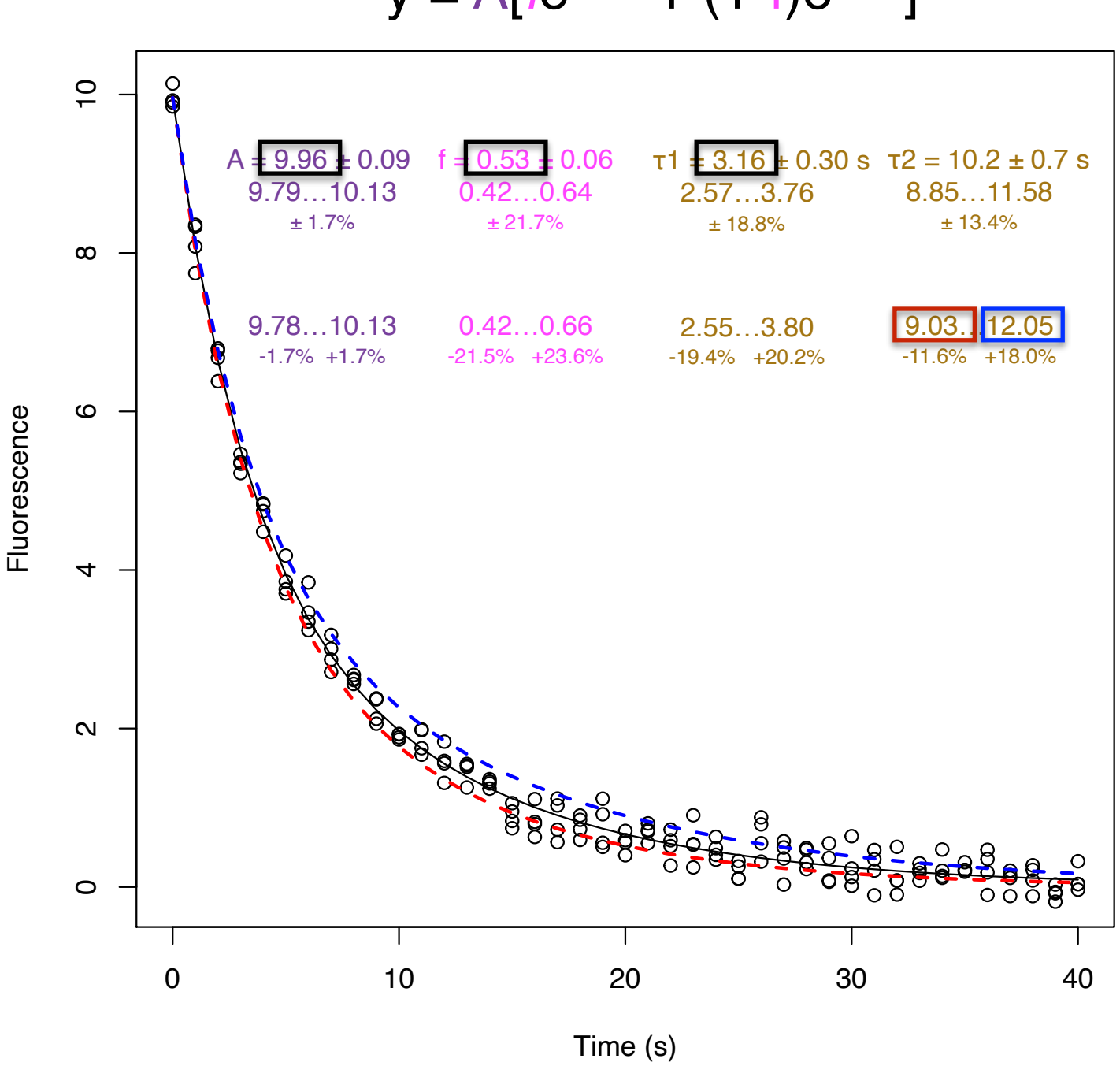


summary(model)

param ± std error
(param ± 2(st err))
percent

97.5% confidence interval
percent

confint(model)

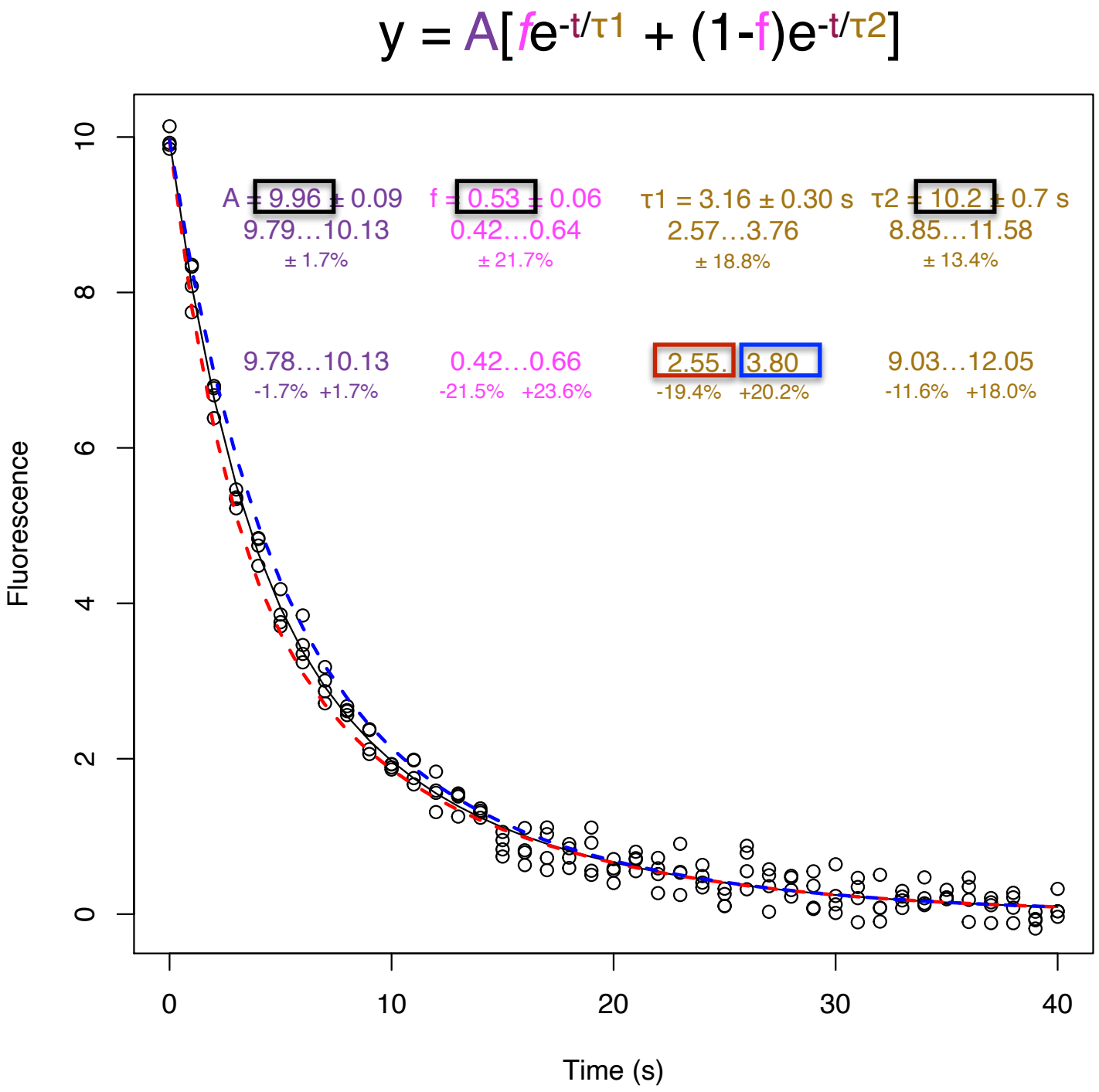


summary(model)

param ± std error
(param ± 2(st err))
percent

97.5% confidence interval
percent

confint(model)



summary(model)

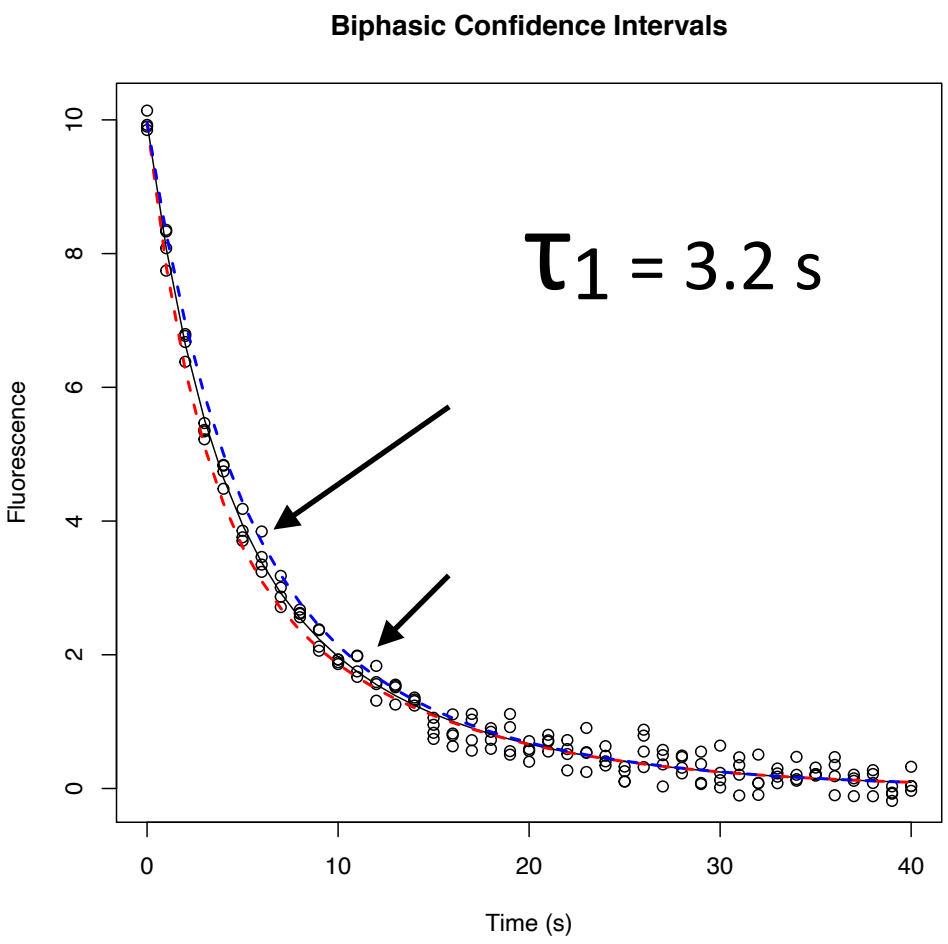
param ± std error
(param ± 2(st err))
percent

97.5% confidence interval
percent

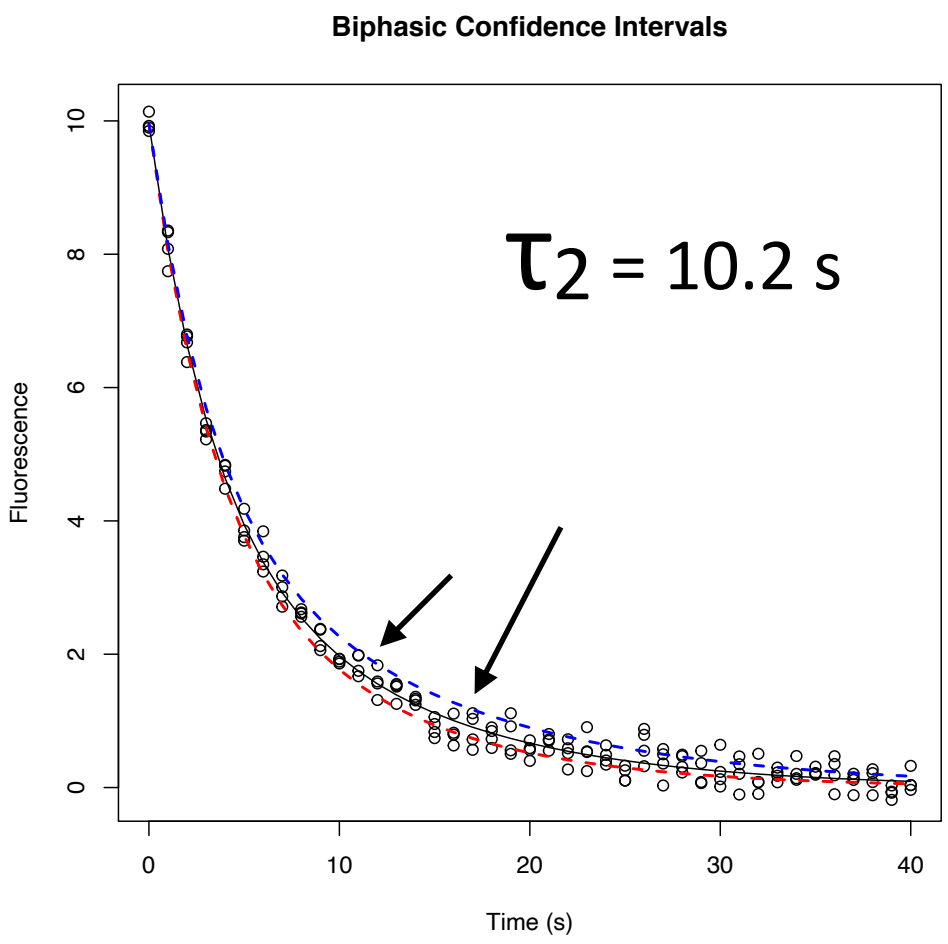
confint(model)

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

A = 9.96 ± 0.09	f = 0.53 ± 0.06	τ1 = 3.16 ± 0.30 s	τ2 = 10.2 ± 0.7 s
9.79…10.13	0.42…0.64	2.57…3.76	8.85…11.58
9.78…10.13	0.42…0.66	2.55…3.80	9.03…12.05



A = 9.96 ± 0.09	f = 0.53 ± 0.06	τ1 = 3.16 ± 0.30 s	τ2 = 10.2 ± 0.7 s
9.79…10.13	0.42…0.64	2.57…3.76	8.85…11.58
9.78…10.13	0.42…0.66	2.55…3.80	9.03…12.05



$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

A = 9.96 ± 0.09	f = 0.53 ± 0.06	τ1 = 3.16 ± 0.30 s	τ2 = 10.2 ± 0.7 s
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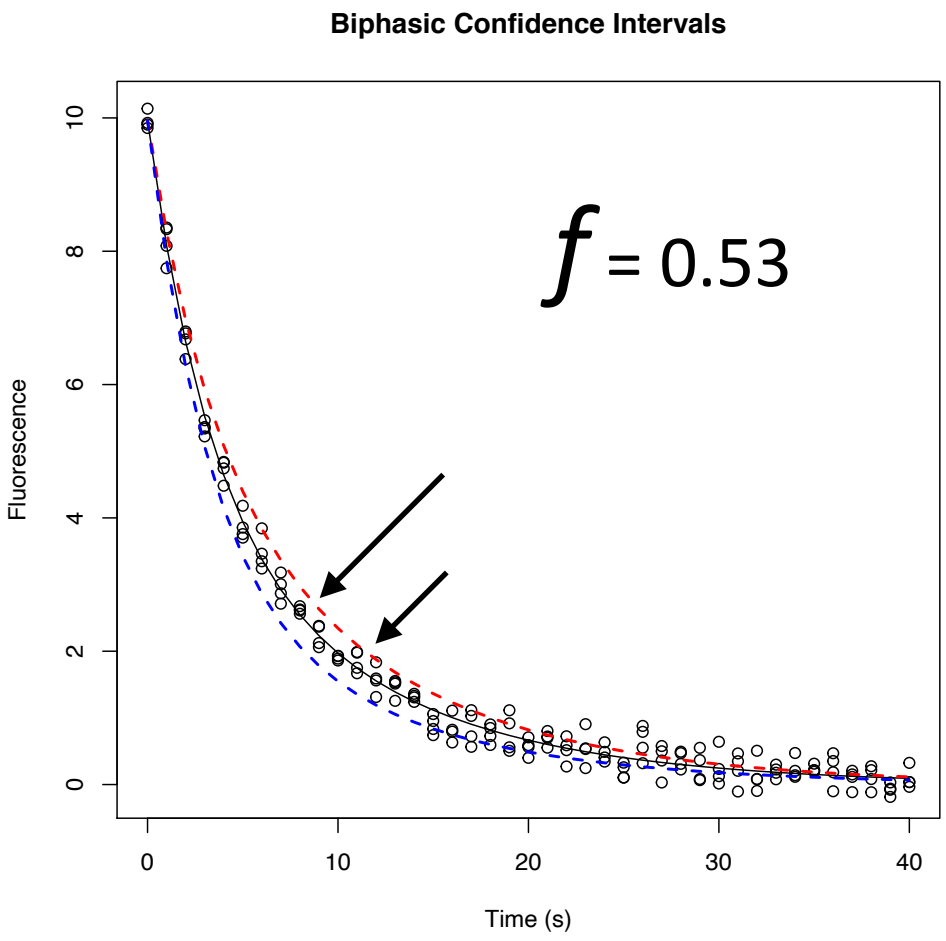

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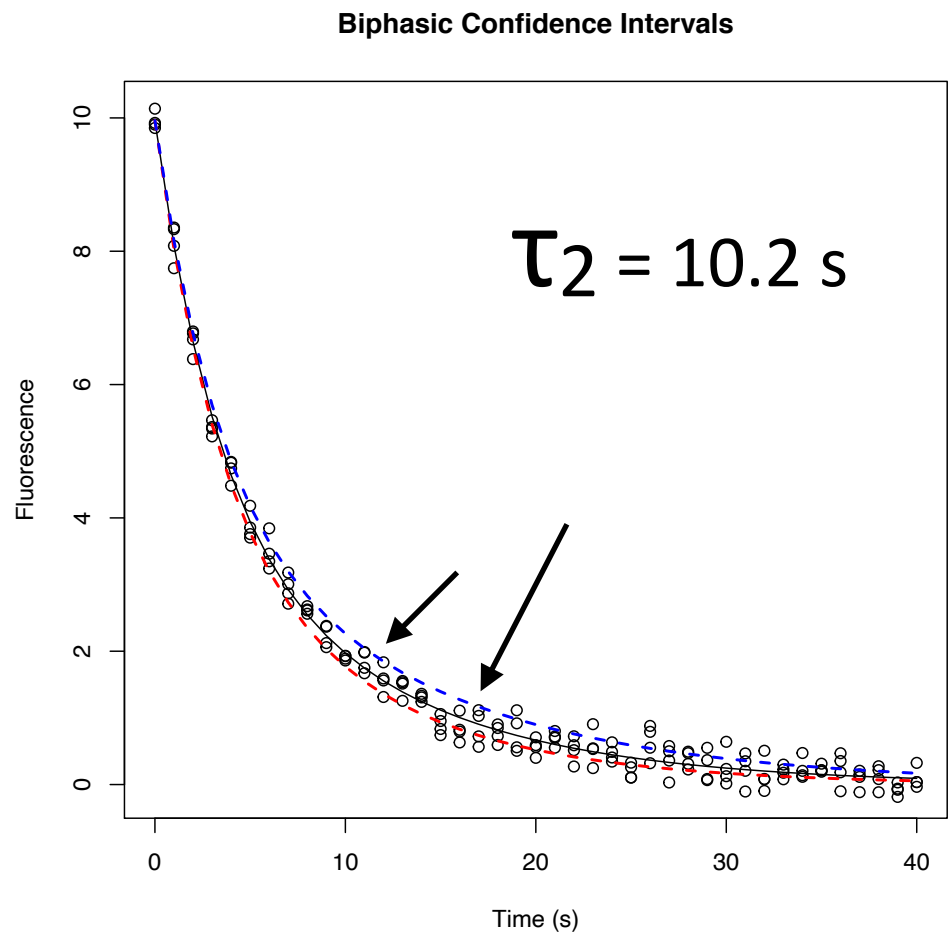
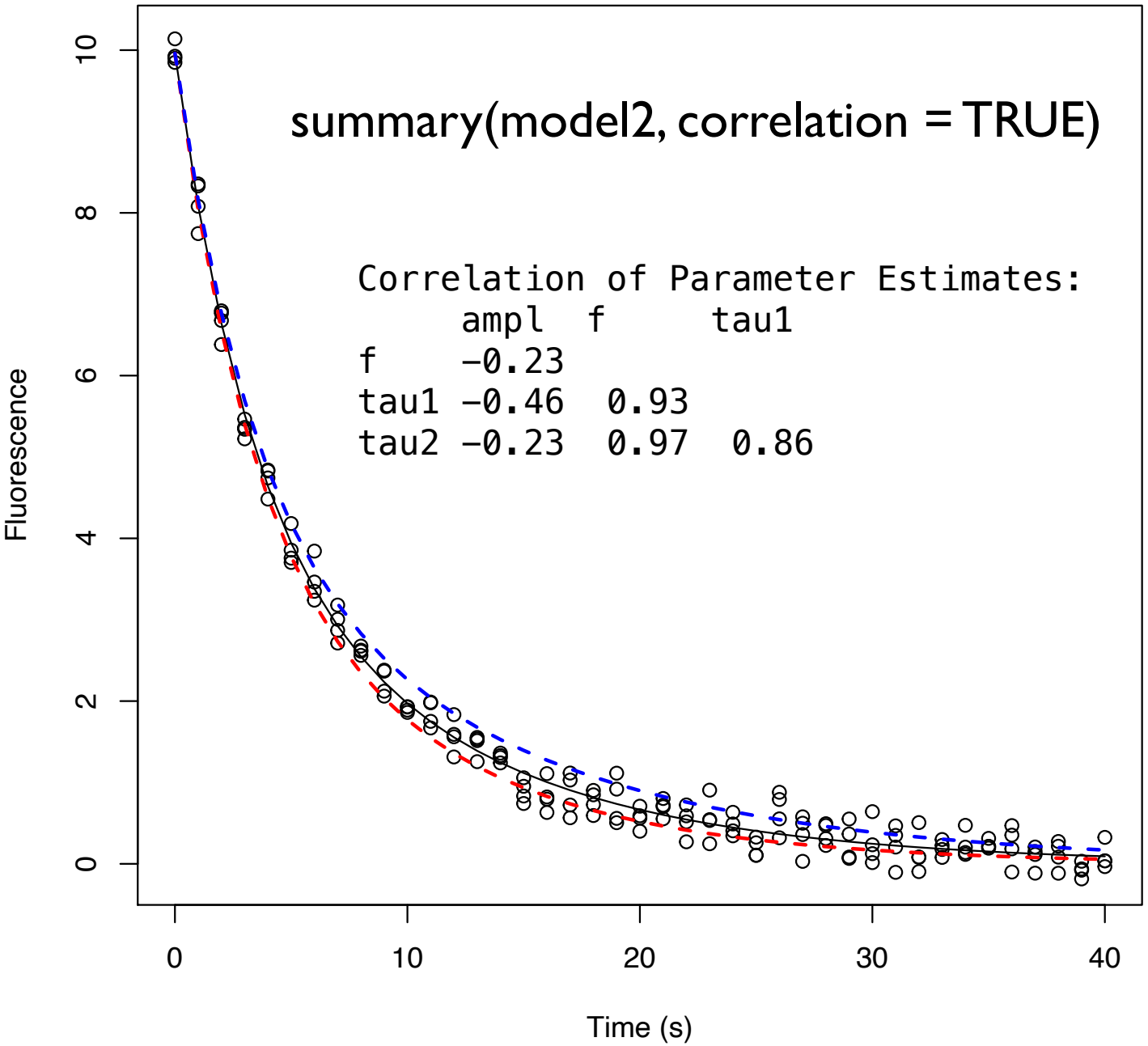


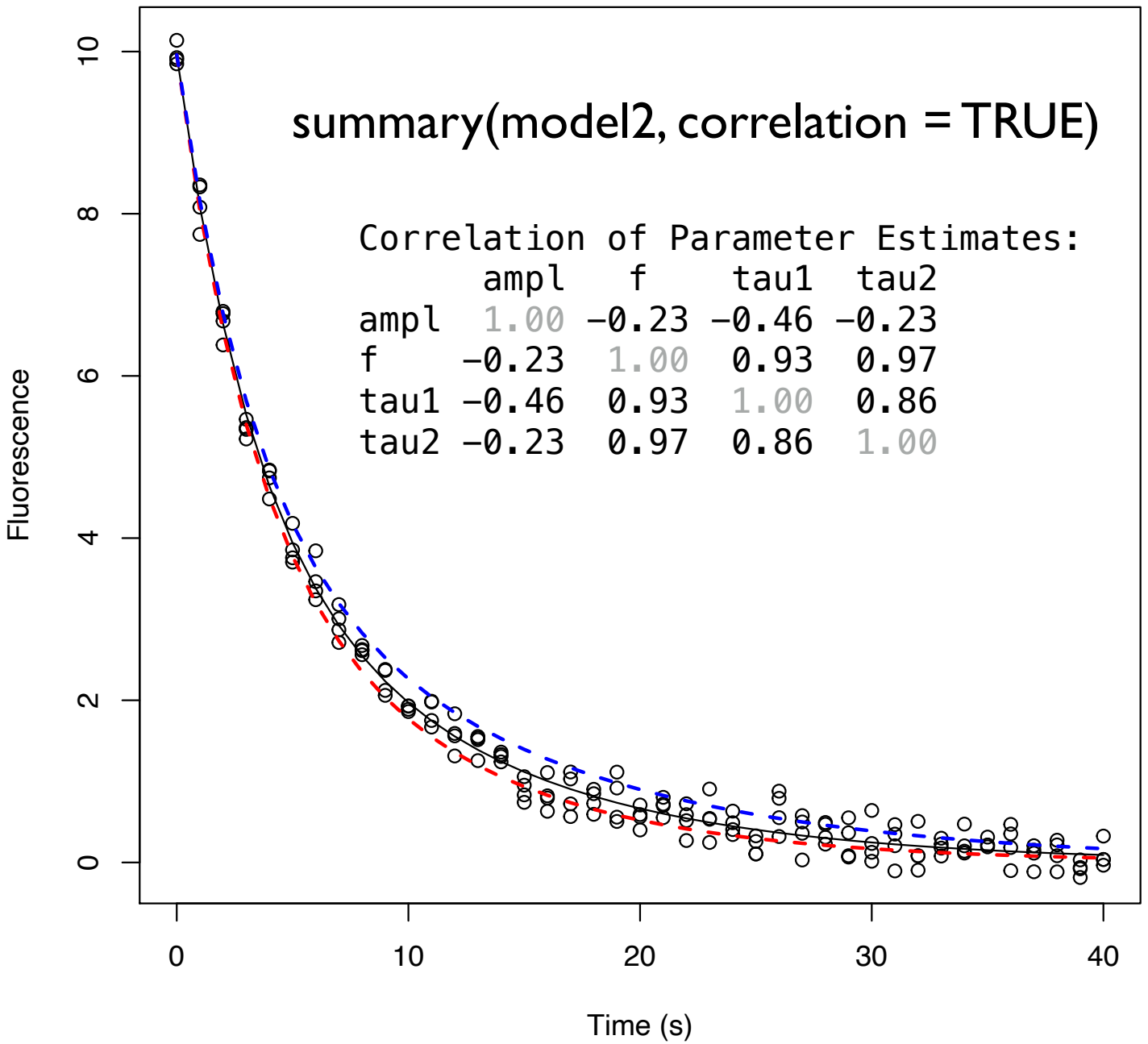
$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$



R command

R output

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$



R command

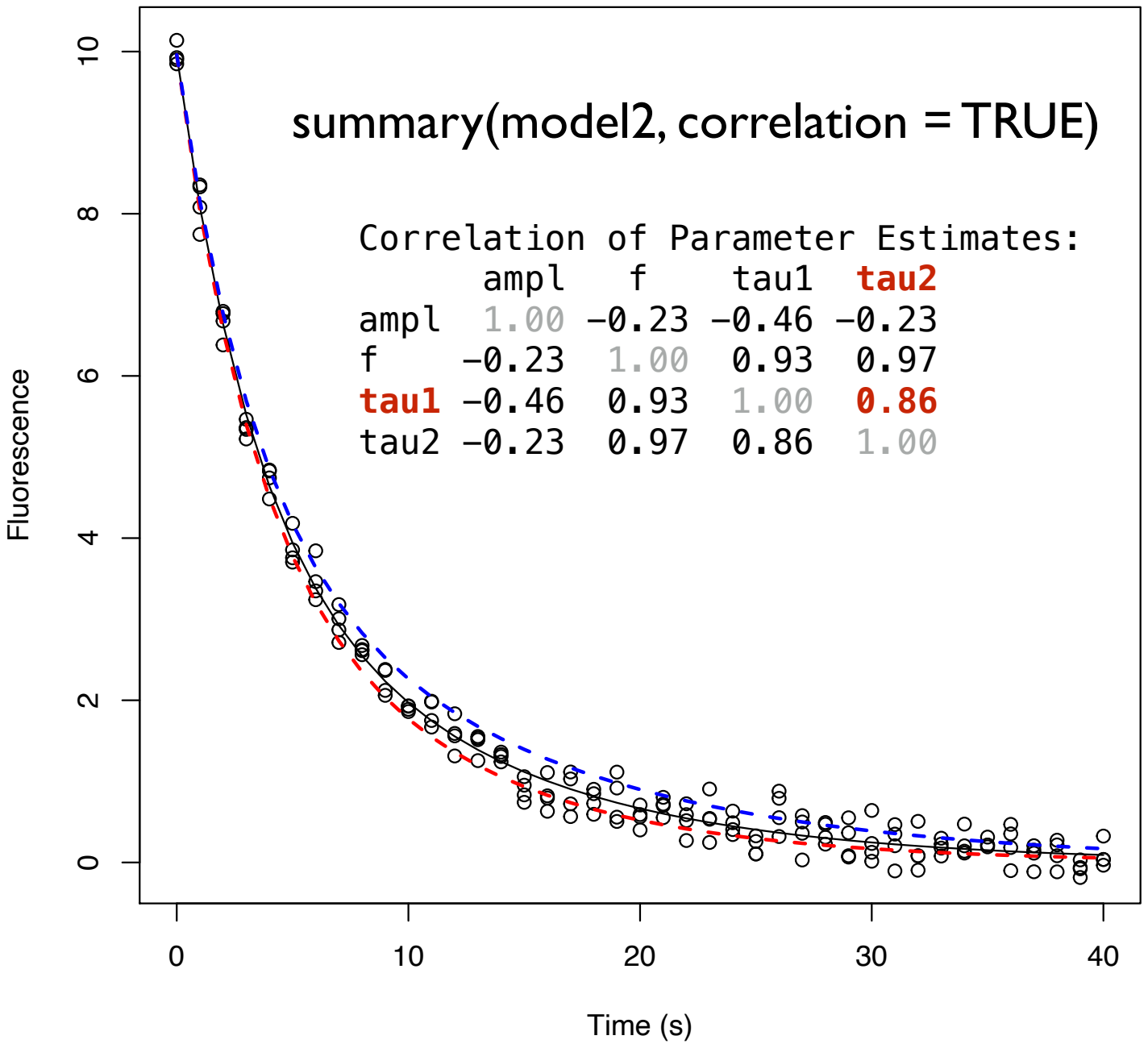
R output

-1 = full anti-correlation

0 = no correlation

1 = full correlation

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$



R command

R output

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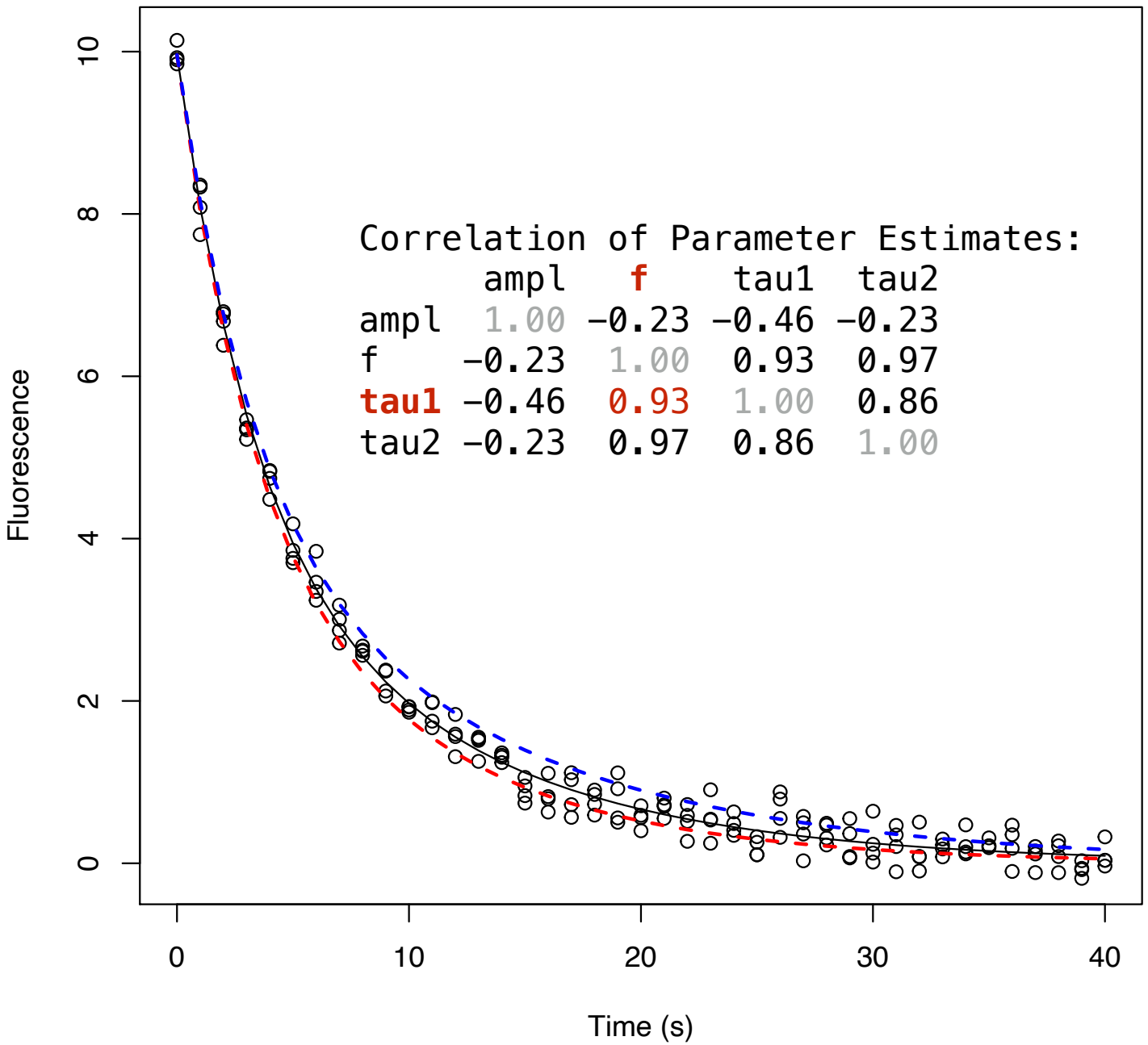
$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$



R command

R output

-1 = full anti-correlation

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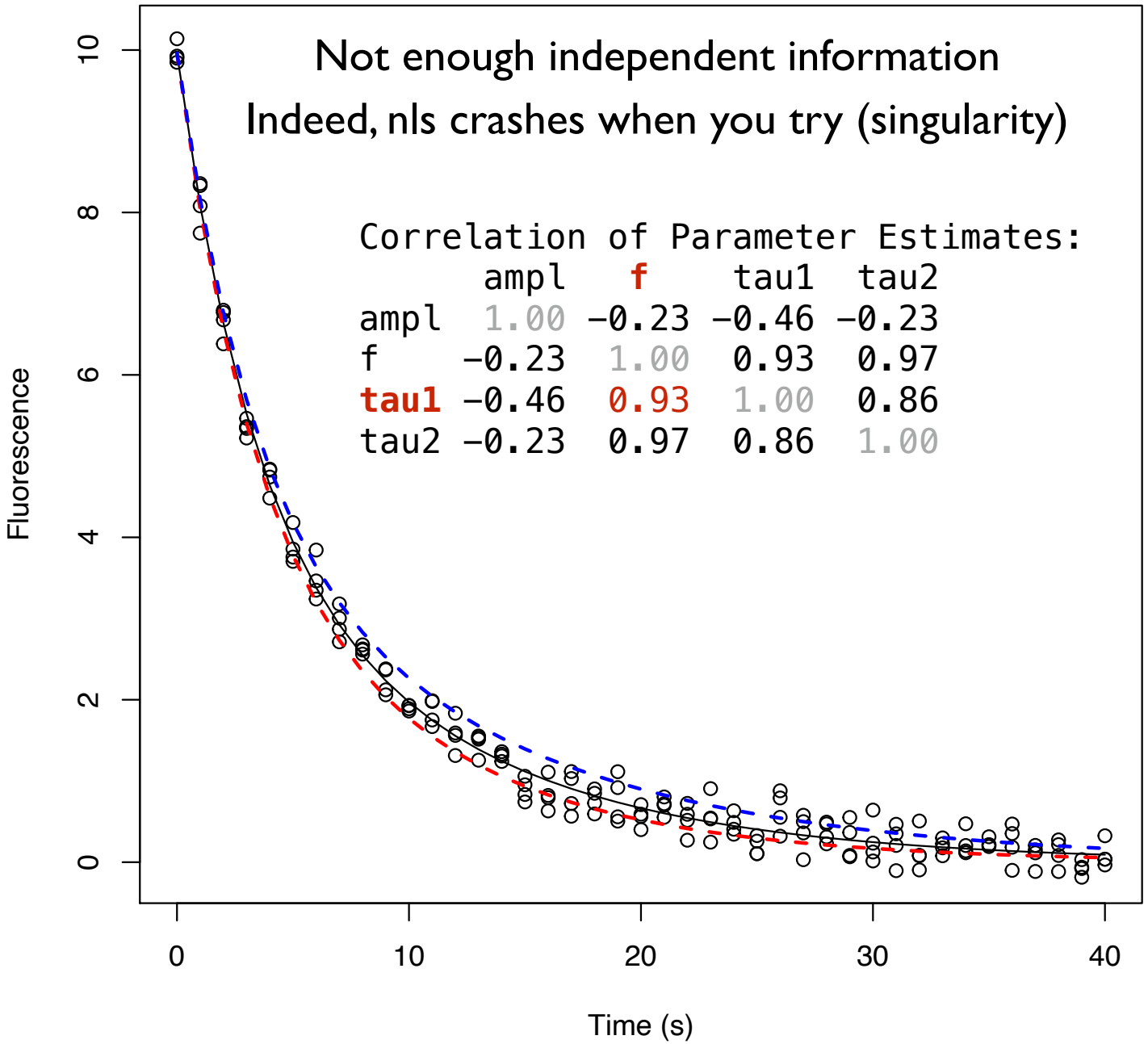
Perhaps we should fit with a Tri-Exponential?

$$y = A[f e^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$



R command

R output

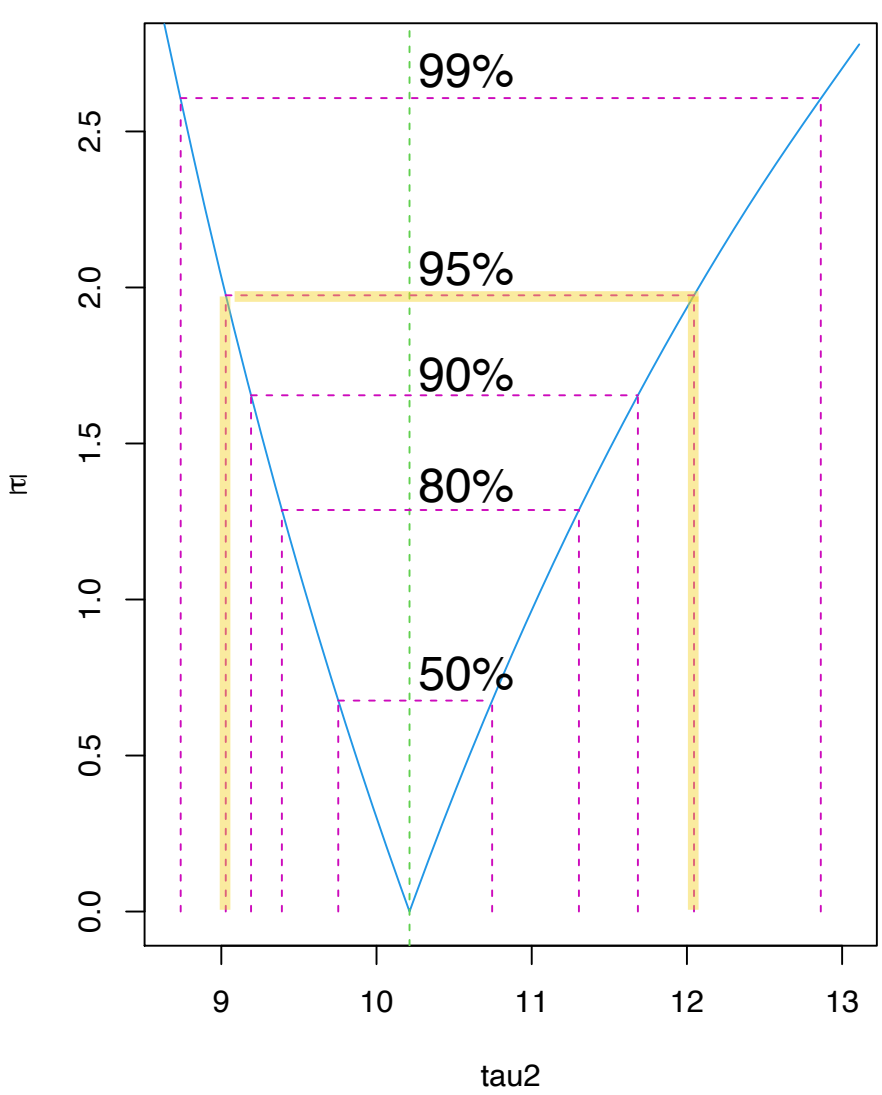
-1 = full anti-correlation

0 = no correlation

1 = full correlation

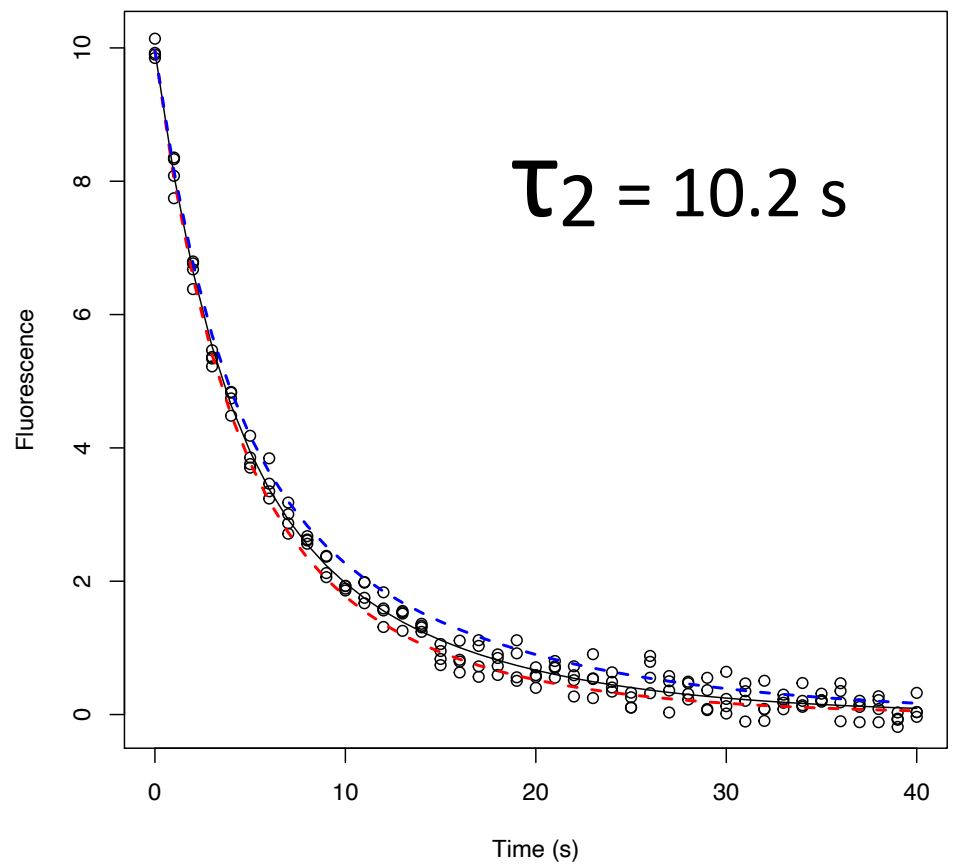
$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

```
pf=profile(model2)
plot(pf, conf = c( 99, 95, 90, 80, 50)/100,  absVal = TRUE, ylab = NULL, lty = 2)
```

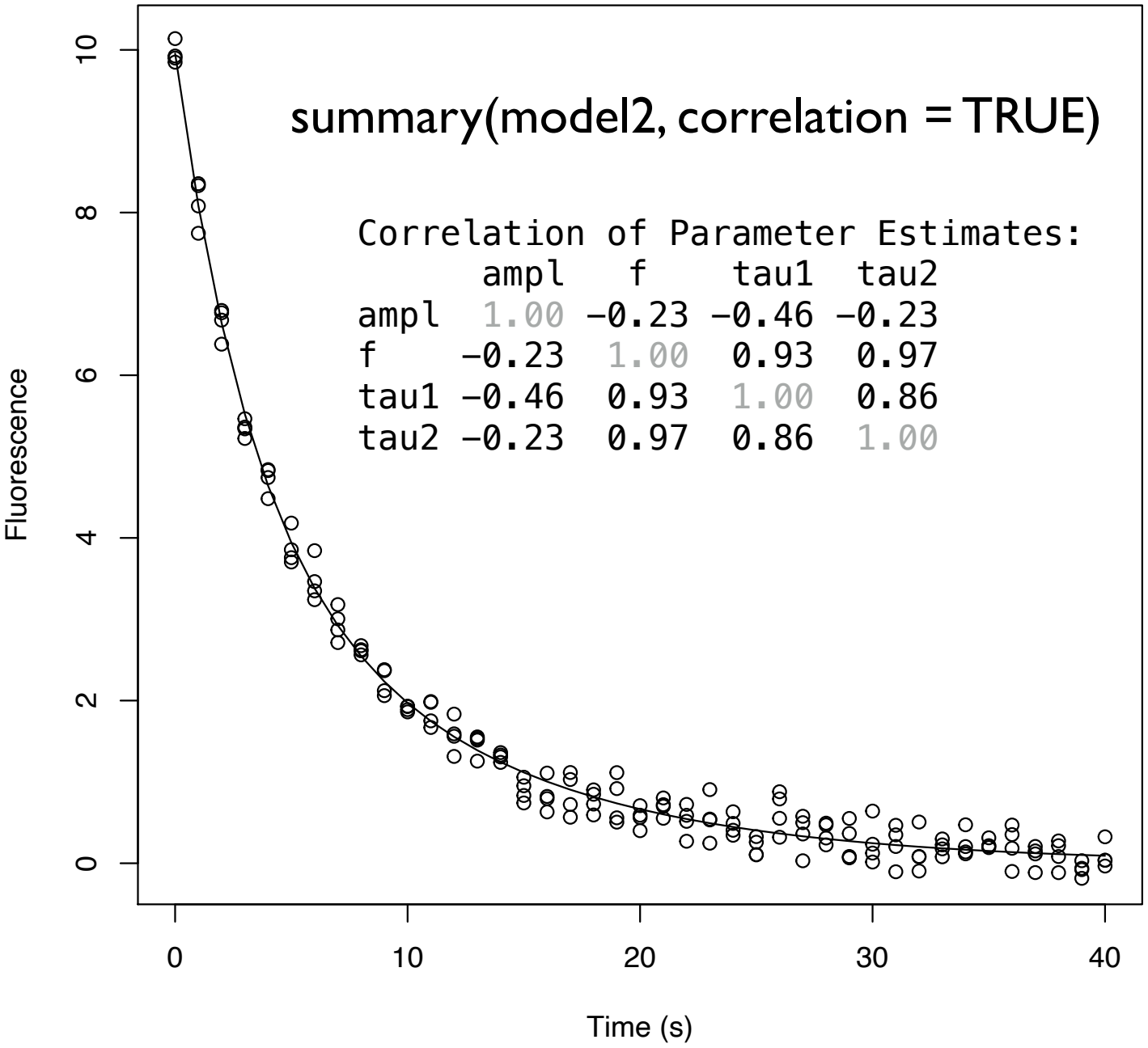


A = 9.96 ± 0.09 | f = 0.53 ± 0.06 | τ1 = 3.16 ± 0.30 s | τ2 = 10.2 ± 0.7 s

9.79…10.13	0.42…0.64	2.57…3.76	8.85…11.58
9.78…10.13	0.42…0.66	2.55…3.80	9.03…12.05



$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$



R command

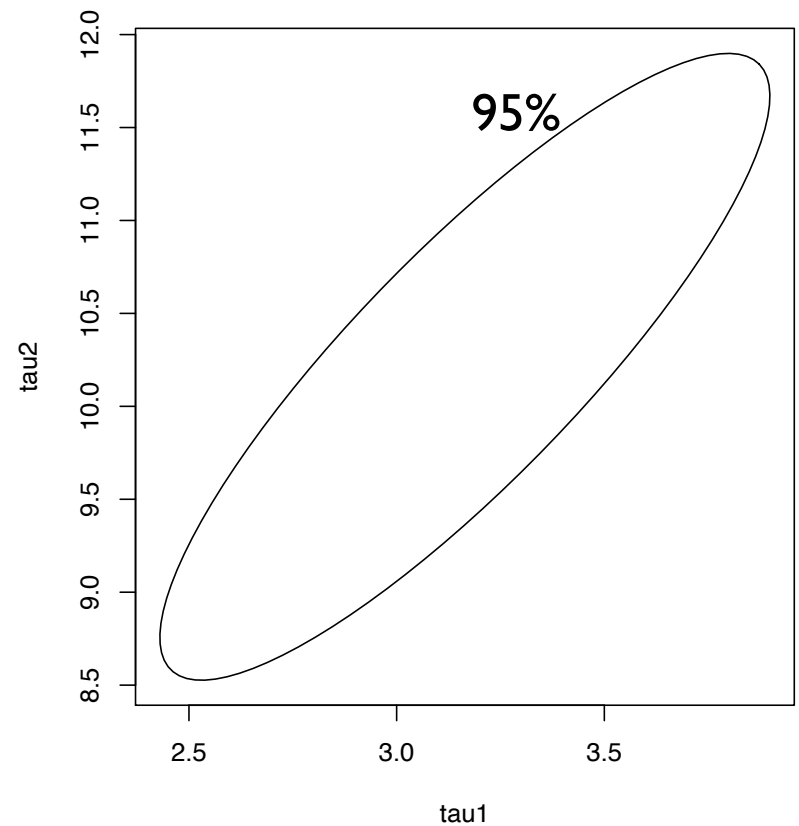
R output

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

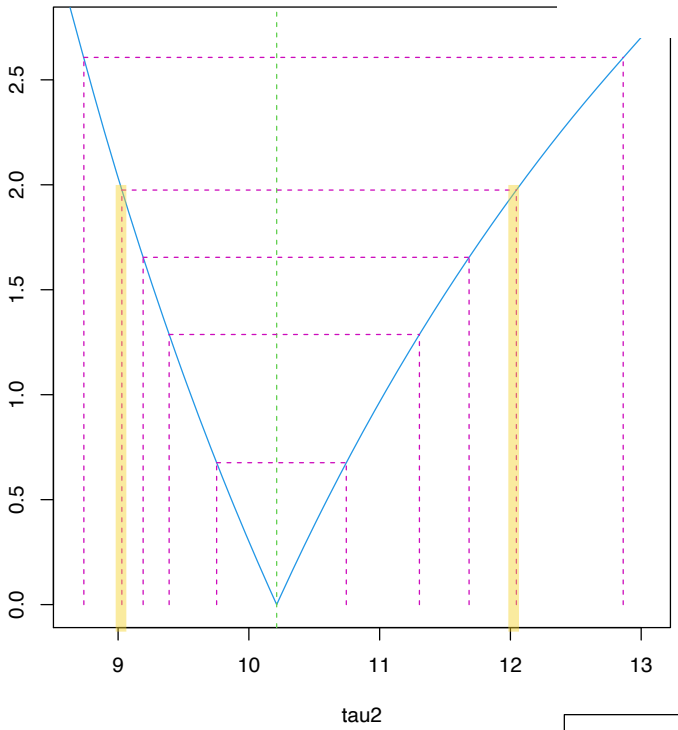
```
plot(ellipse(model2,level=c(0.95),which=c('myTau1','myTau2')), type = 'l')
```

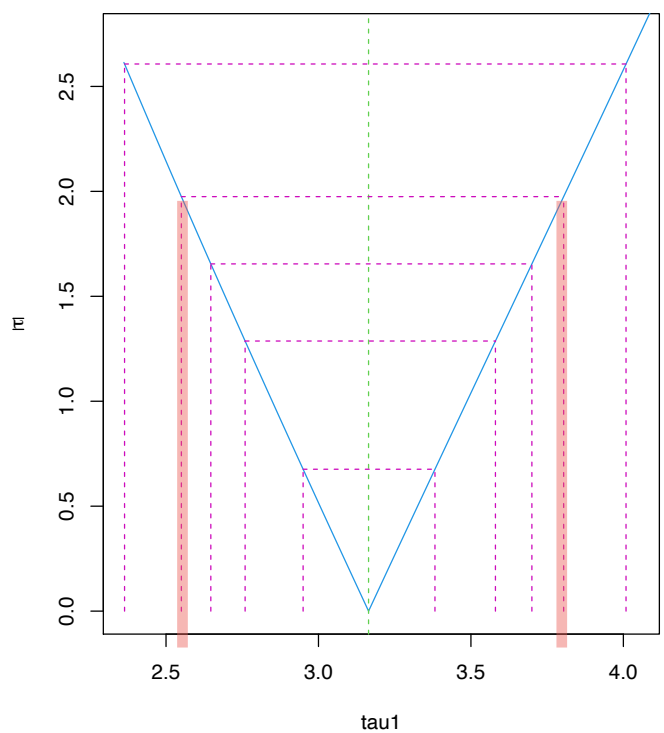
```
Correlation of Parameter Estimates:
      ampl    f     tau1  tau2
ampl  1.00 -0.23 -0.46 -0.23
f    -0.23  1.00  0.93  0.97
tau1 -0.46  0.93  1.00  0.86
tau2 -0.23  0.97  0.86  1.00
```

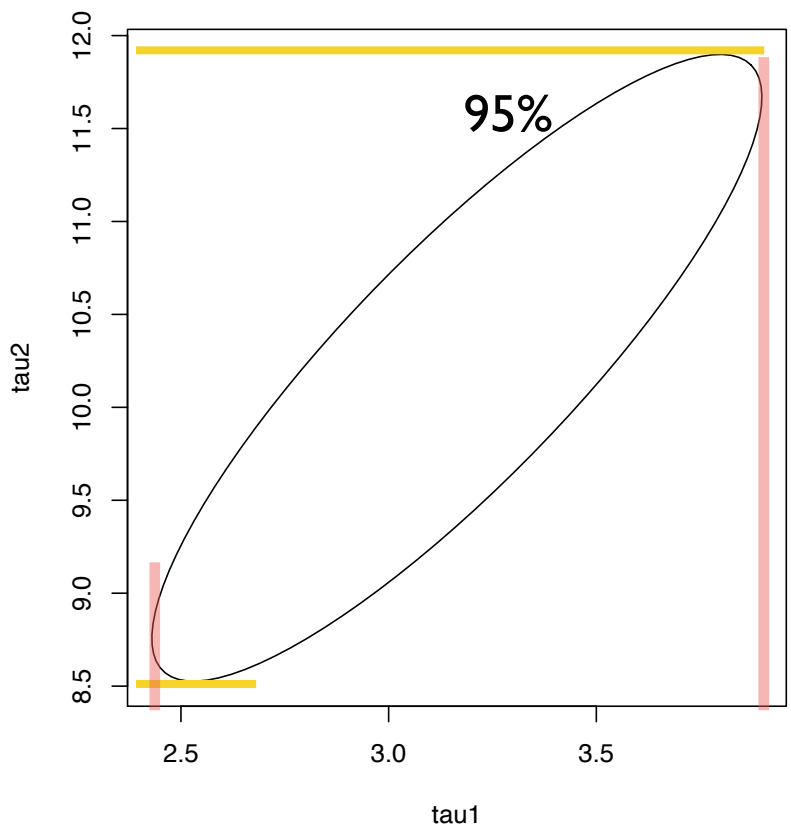
```
Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl  9.95572    0.08520 116.856   <2e-16 ***
f1    0.53314    0.05767   9.244   <2e-16 ***
tau1  3.16427    0.29720  10.647   <2e-16 ***
tau2 10.21256    0.68244  14.965   <2e-16 ***

tau1 2.57 …  3.75
tau2 8.85 … 11.58

ampl 9.78 … 10.139
f1   0.42 …  0.66
tau1 2.55 …  3.80
tau2 9.03 … 12.05
```



$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$







$$y = Ae^{-t/\tau}$$

$$y = A[fe^{-t/\tau 1} + (1-f)e^{-t/\tau 2}]$$

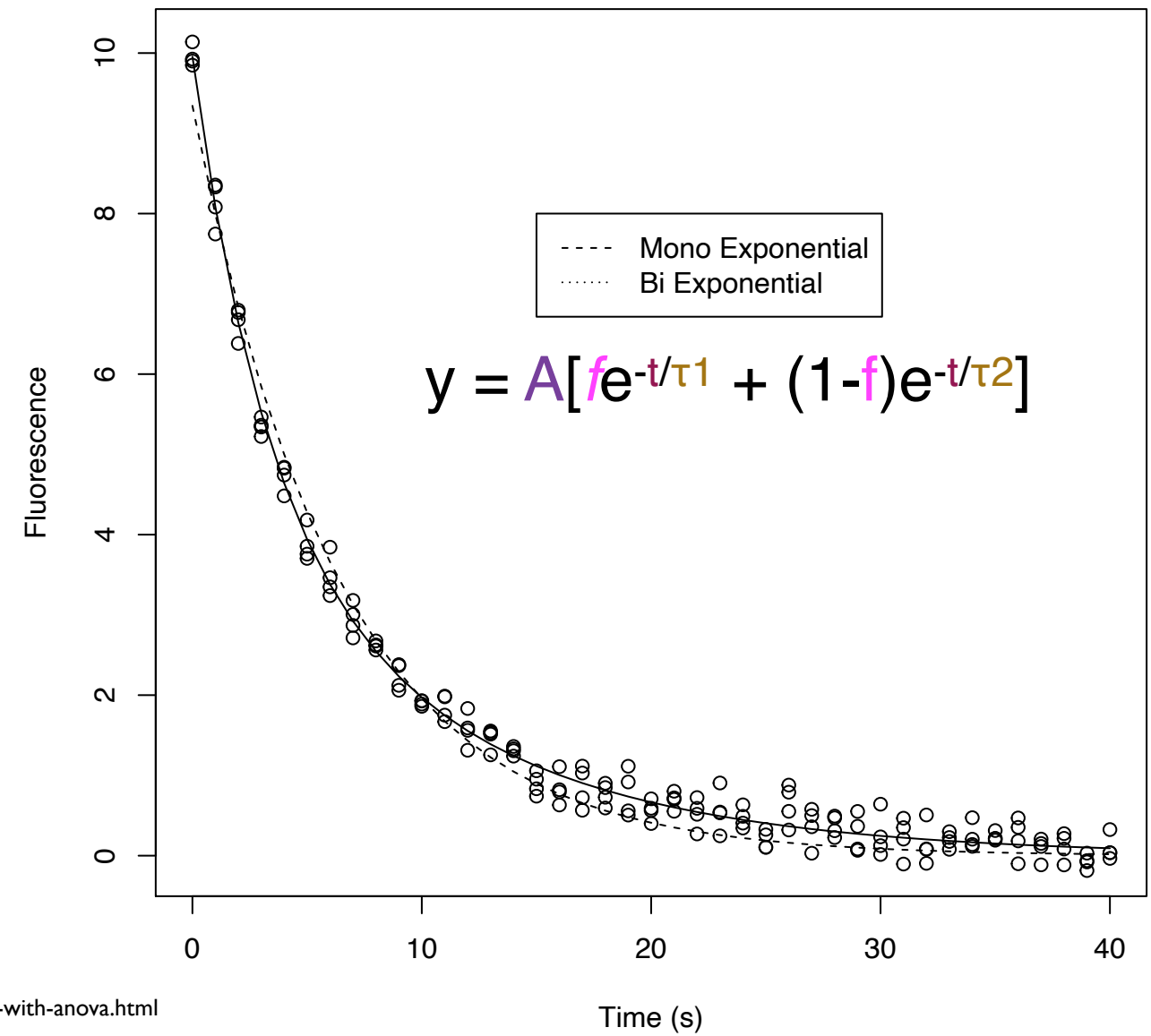
anova(model1, model2)

A good model not only needs to fit data well, it also needs to be *parsimonious*. That is, a good model should be only be as complex as necessary to describe a dataset.

If you are choosing between a simple model with 2 parameters, and a more complex model with, say, 4 parameters, the complex model needs to provide a much better fit to the data in order to justify its increased complexity. If it can't, then the simpler model should be preferred.

To compare the fits of two models, you can use the anova() function with the regression objects as two separate arguments. The anova() function will take the model objects as arguments, and return an ANOVA testing whether the more complex model is significantly better at capturing the data than the simpler model. If the resulting p-value is sufficiently low (usually less than 0.05), we conclude that the more complex model is significantly better than the simpler model, and thus favor the more complex model. If the p-value is not sufficiently low (usually greater than 0.05), we should favor the simpler model.

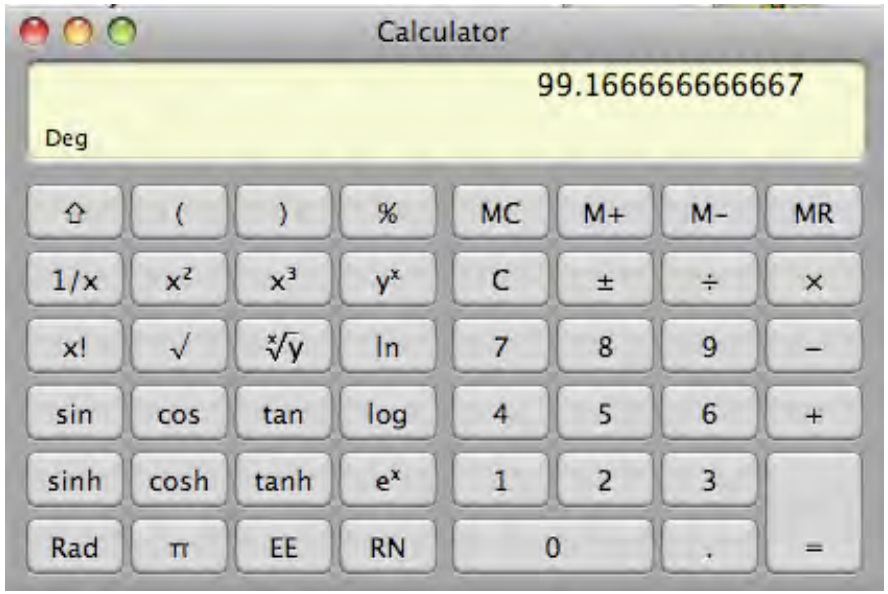
```
Analysis of Variance Table

Model 1: edt$dat ~ eDecay(edt$t, ampl, tau)
Model 2: edt$dat ~ eDecay2(edt$t, ampl, f1, tau1, tau2)

  Res.Df Res.Sum Sq Df Sum Sq F value    Pr(>F)
1    162    13.3479
2    160    5.7151  2 7.6328  106.84 < 2.2e-16 ***

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```



Adapted from:
https://bookdown.org/ndphillips/YaRrr/comparing-regression-models-with-anova.html

Question: I drove to New Haven recently.
It took me 1 hr 25 min, driving at 70 mi/hr.
How far is it from Amherst to New Haven?

$$\left(\frac{70\,mi}{hr}\right)(85\,\text{min})\left(\frac{hr}{60\,\text{min}}\right) =$$



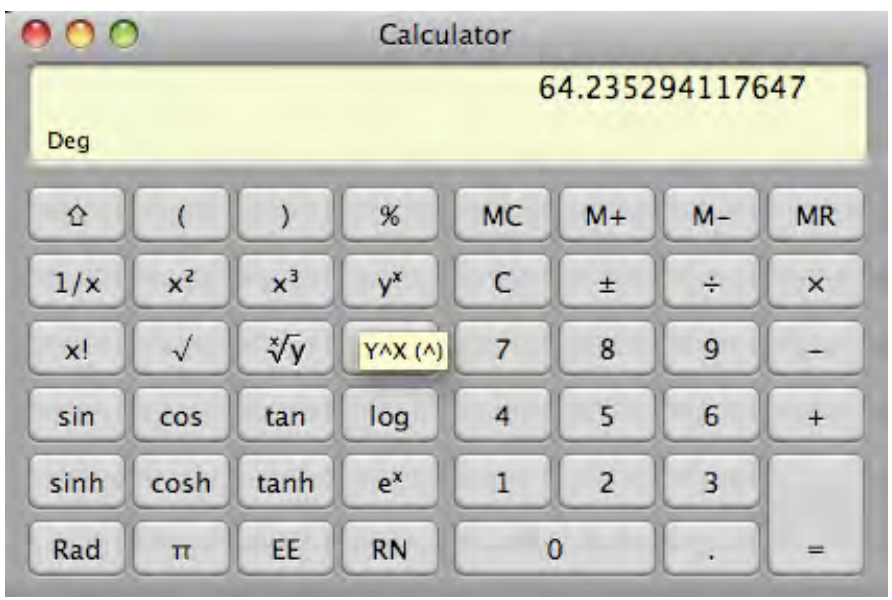
Write your answer on a piece of paper

99.167 miles

0.001 miles = 5 ft

Question: I drove to New Haven recently.
Google Maps says that it's 91 miles.
It took me 1 hr 25 min
How fast was I driving, on average?

$$\frac{91mi}{85\,\text{min}}\left(\frac{60\,\text{min}}{hr}\right) =$$



64.2353 miles/hour

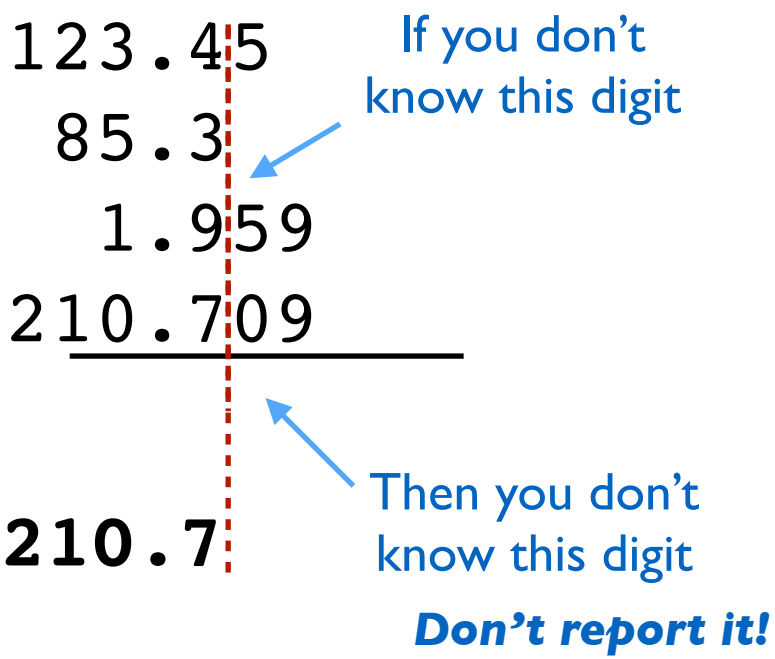
Alexa: will it rain tomorrow?

"You can expect 1.34 inches of rain tomorrow"

```
Parameters:
     Estimate Std. Error t value Pr(>|t|)
ampl  9.95572    0.08520 116.856   <2e-16 ***
f1    0.53314    0.05767   9.244   <2e-16 ***
tau1  3.16427    0.29720  10.647   <2e-16 ***
tau2 10.21256    0.68244  14.965   <2e-16 ***
```

Significant Figures - Rules

#2) Addition / Subtraction



Significant Figures - Rules

#2) Addition / Subtraction

```
123.45
 85.3
  1.959
210.709
```

If you don't know this digit

Then you don't know this digit

210.7

Don't report it!

#3) Multiplication / Division

123.45 x 1.95 = 240.7275

5 sig figs 3 sig figs

241

3 sig figs

Significant Figures - Rules

#1) Think - be reasonable! Always!

How much do you believe that last digit?

#2) Addition / Subtraction

```
123.45
 85.3
  1.959
210.709
```

If you don't know this digit

Then you don't know this digit

210.7

Don't report it!

#3) Multiplication / Division

```
123.45 x 1.95 = 240.7275
```

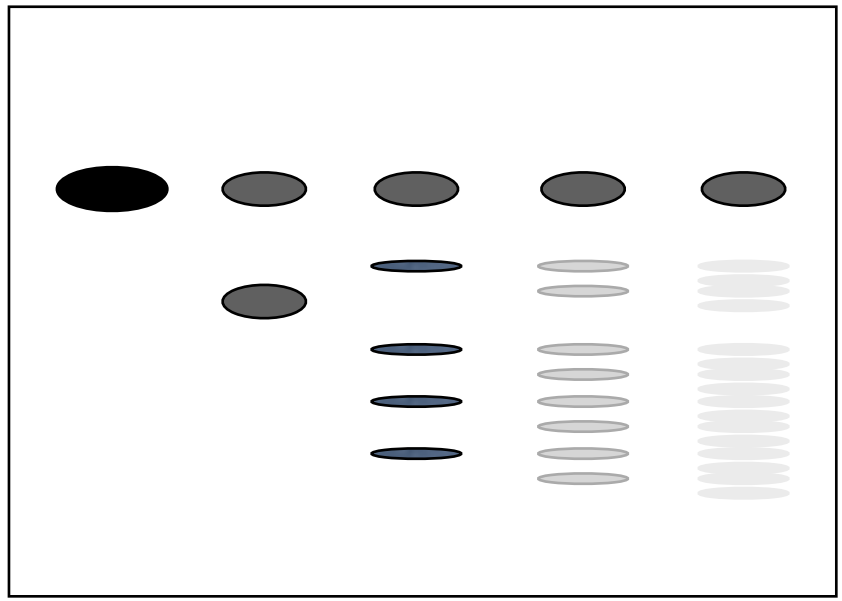
5 sig figs 3 sig figs

241

3 sig figs

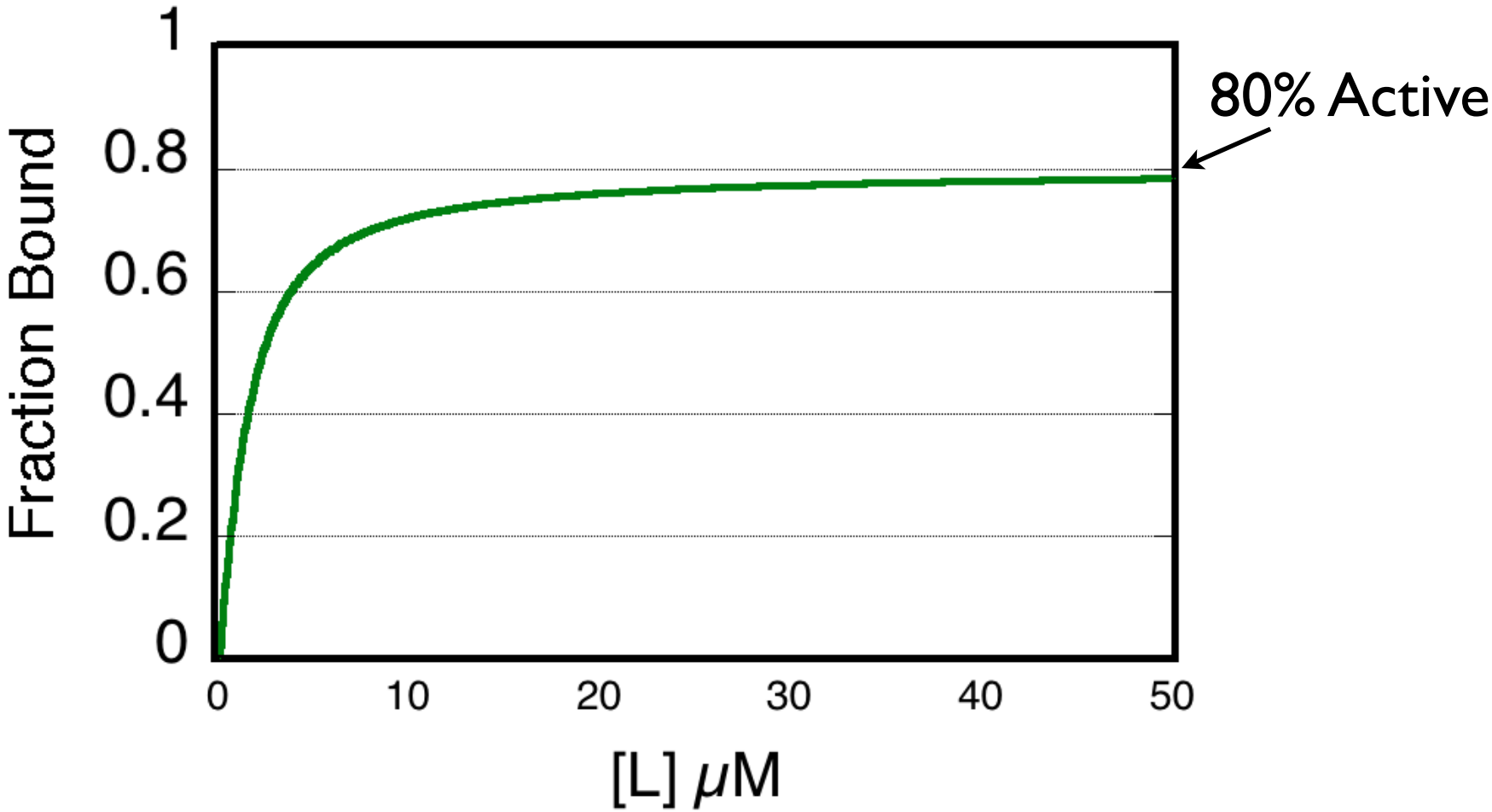
Sources of Error

- Sample concentration
  - A_{280}, Bradford, etc
- Sample purity
  - Other proteins, absorbers
- Sample activity



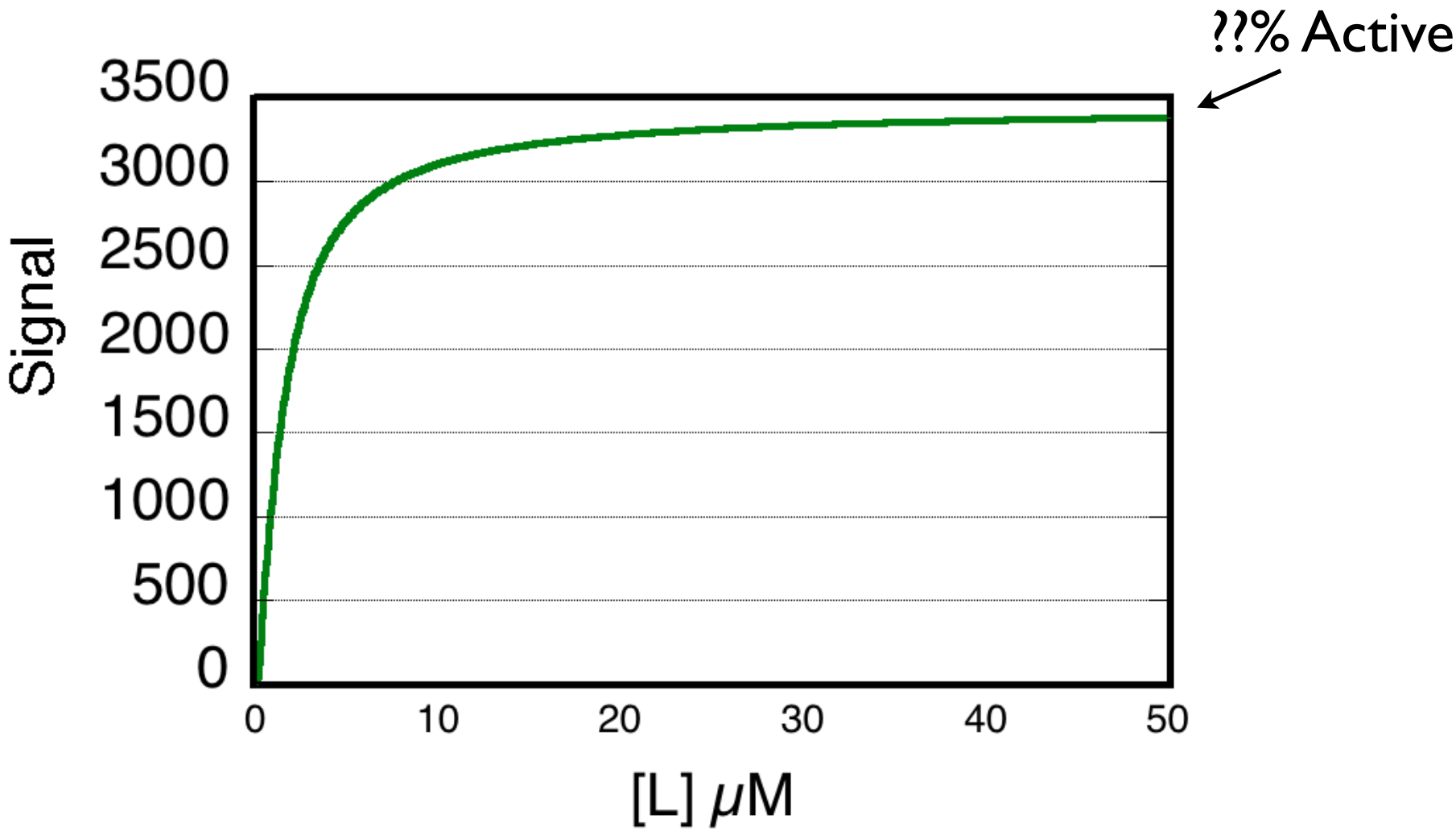
Sources of Error

- Sample activity



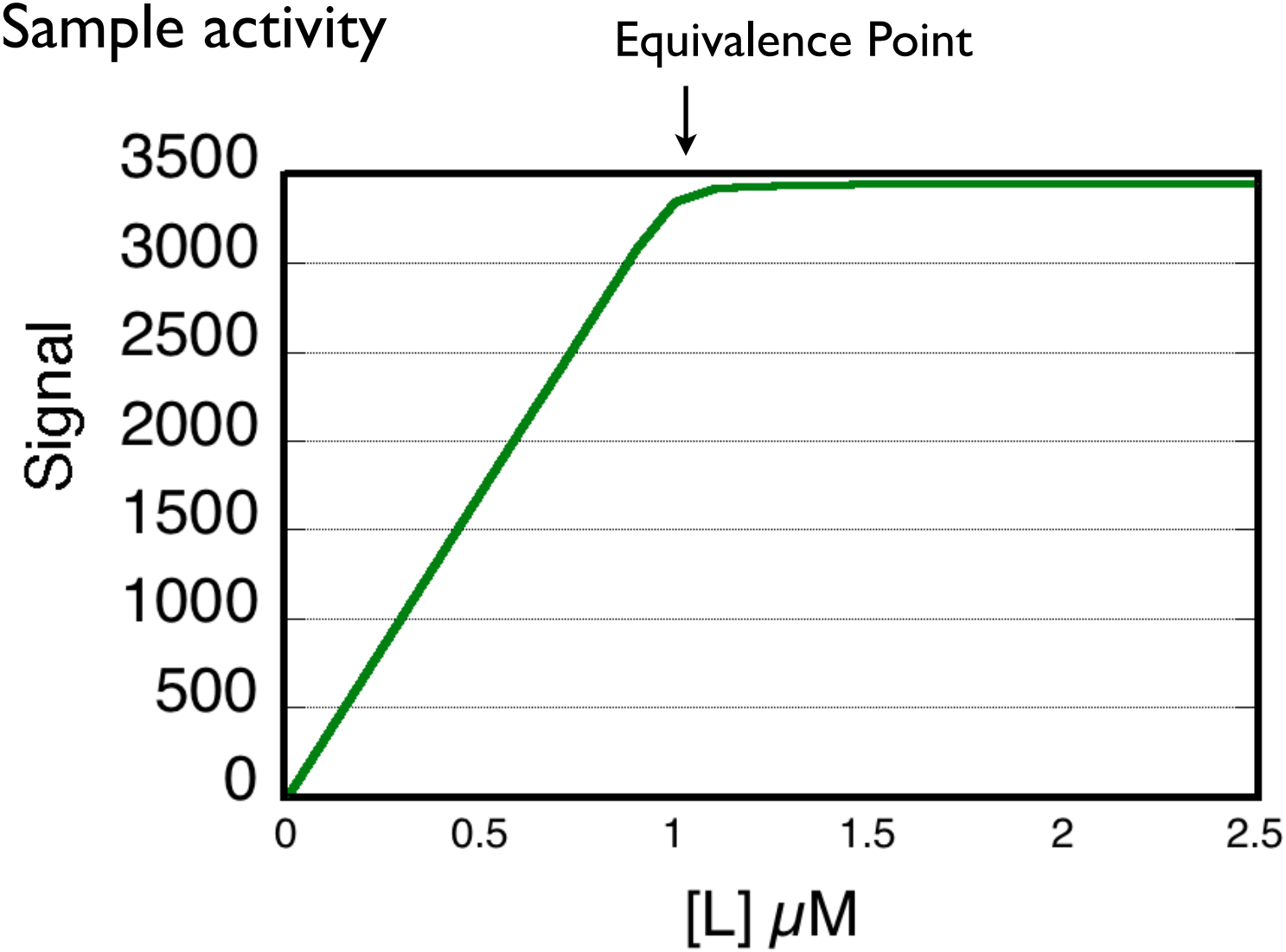
Sources of Error

- Sample activity



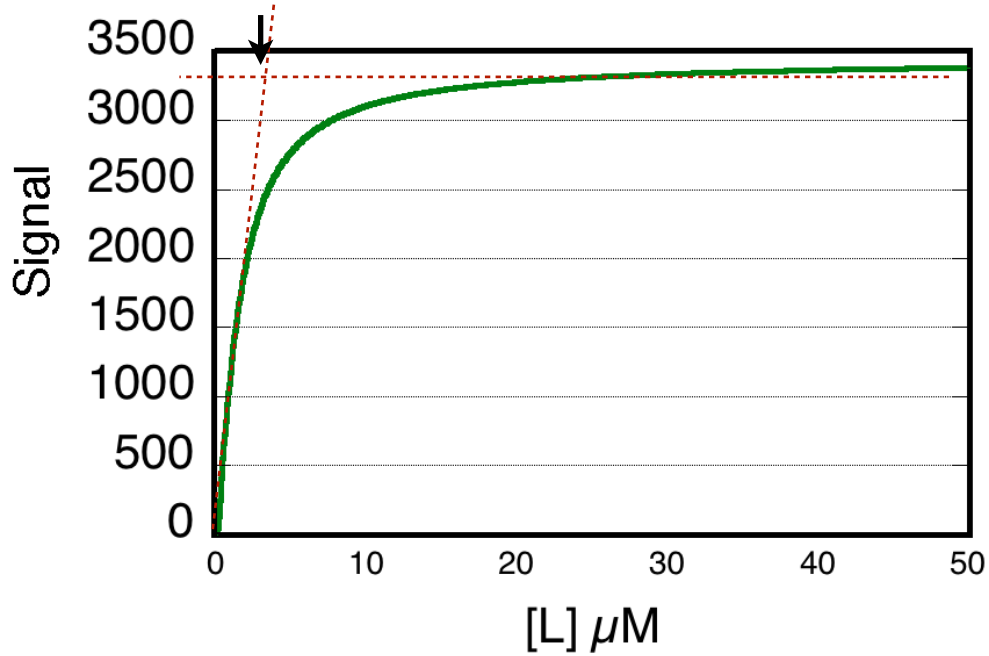
Sources of Error

- Sample activity

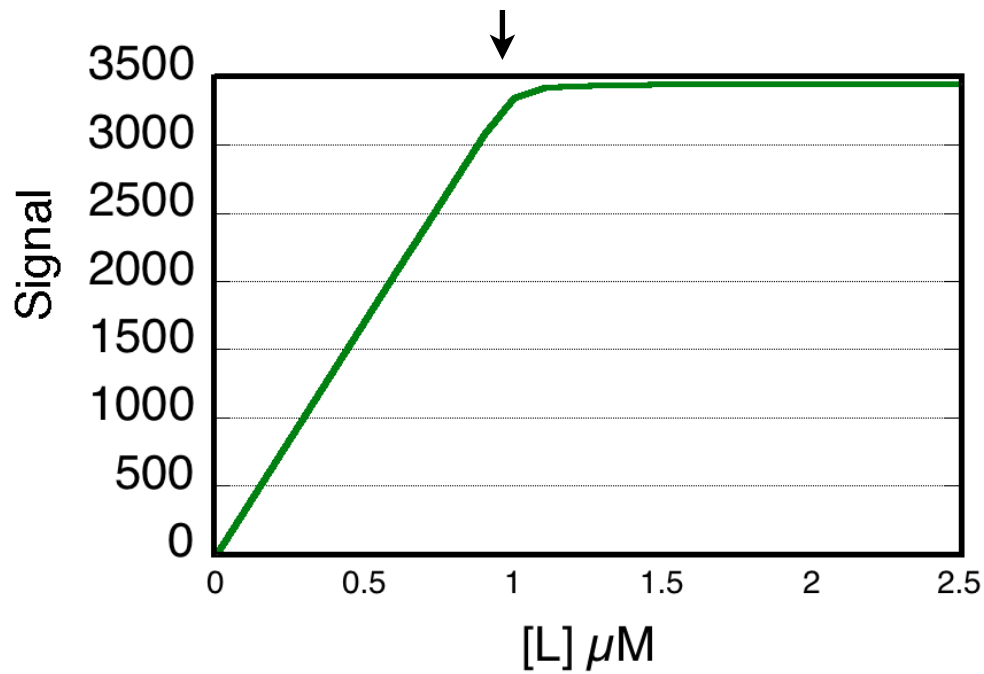


Sample Activity

Fitting all data is better than the approach shown at right

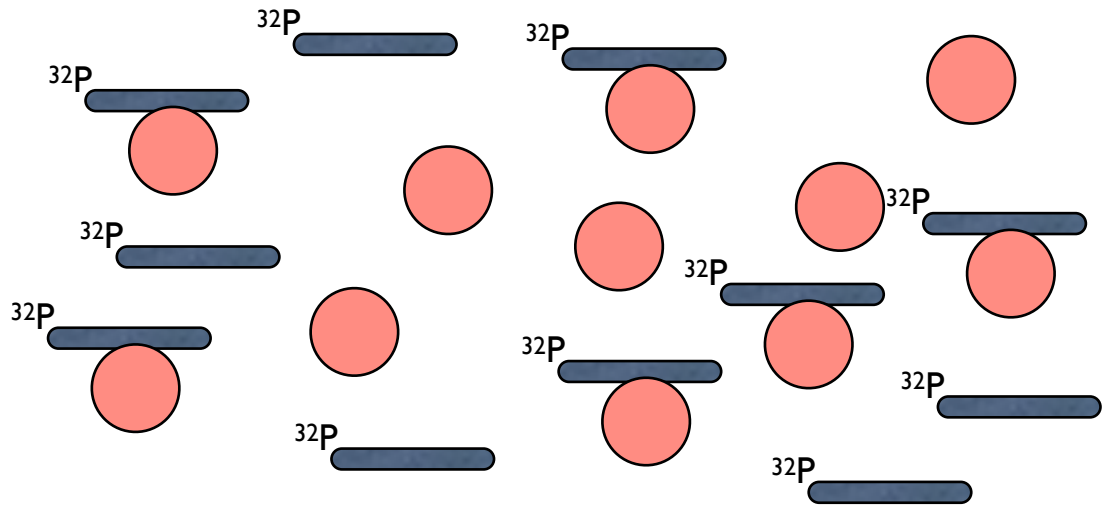


These approaches only work for tight-binding interactions



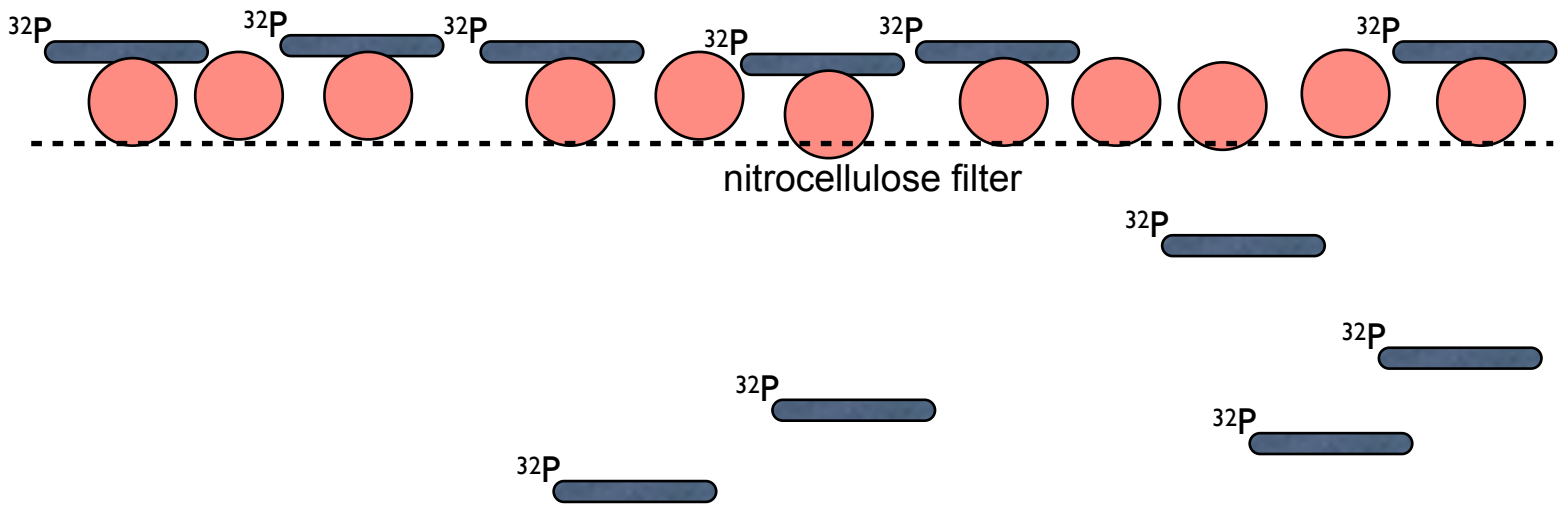
Methods to Measure Binding
Filter Binding

Equilibrate



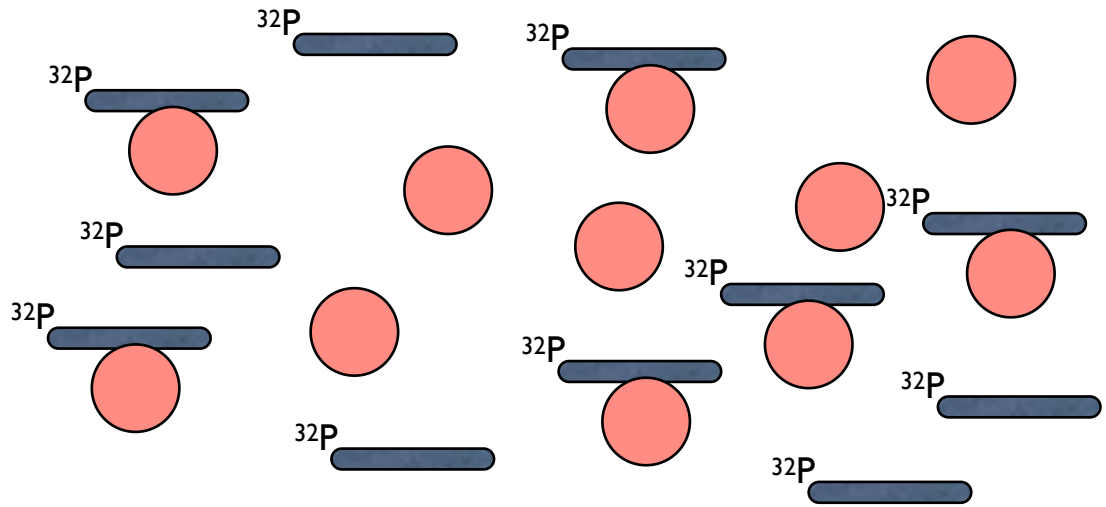
This shouldn't work: why?

Flow, wash



Methods to Measure Binding
Filter Binding

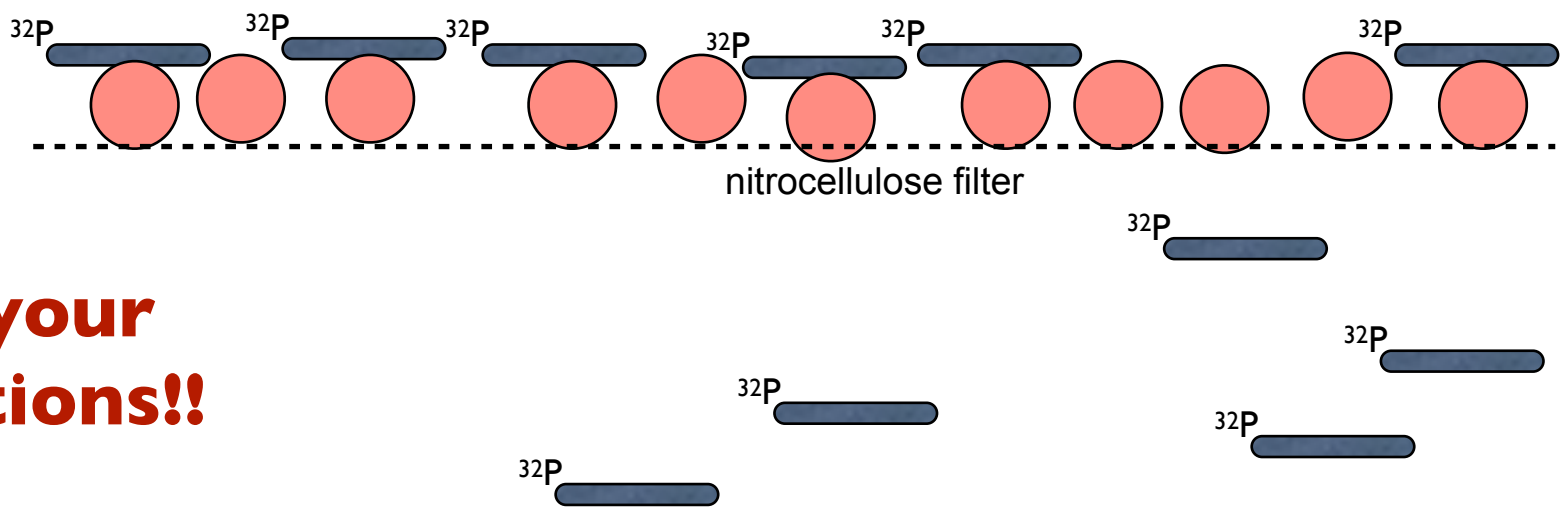
Equilibrate



This shouldn't work: why?

Assumes no re-equilibration

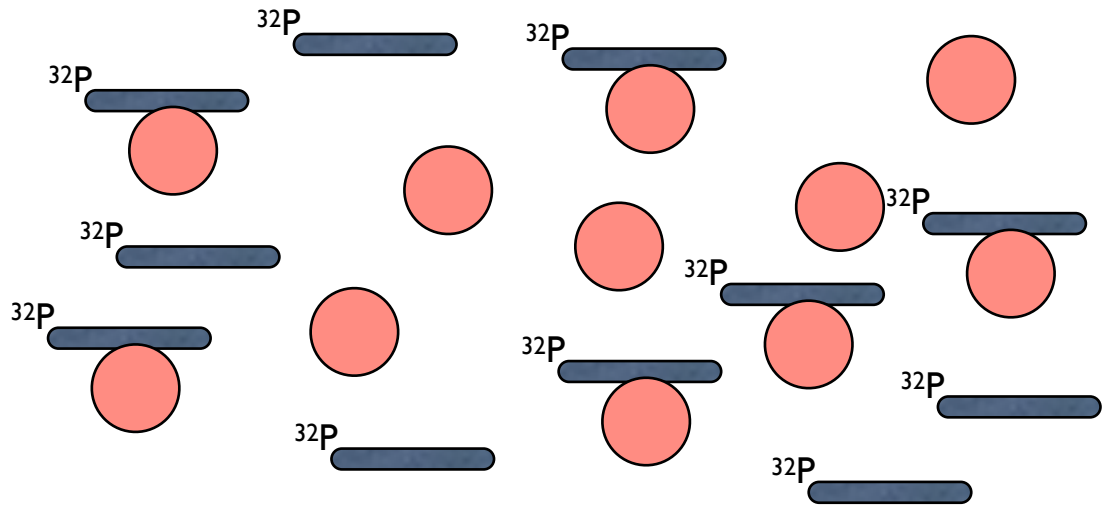
Flow, wash



Know your assumptions!!

Methods to Measure Binding
Filter Binding

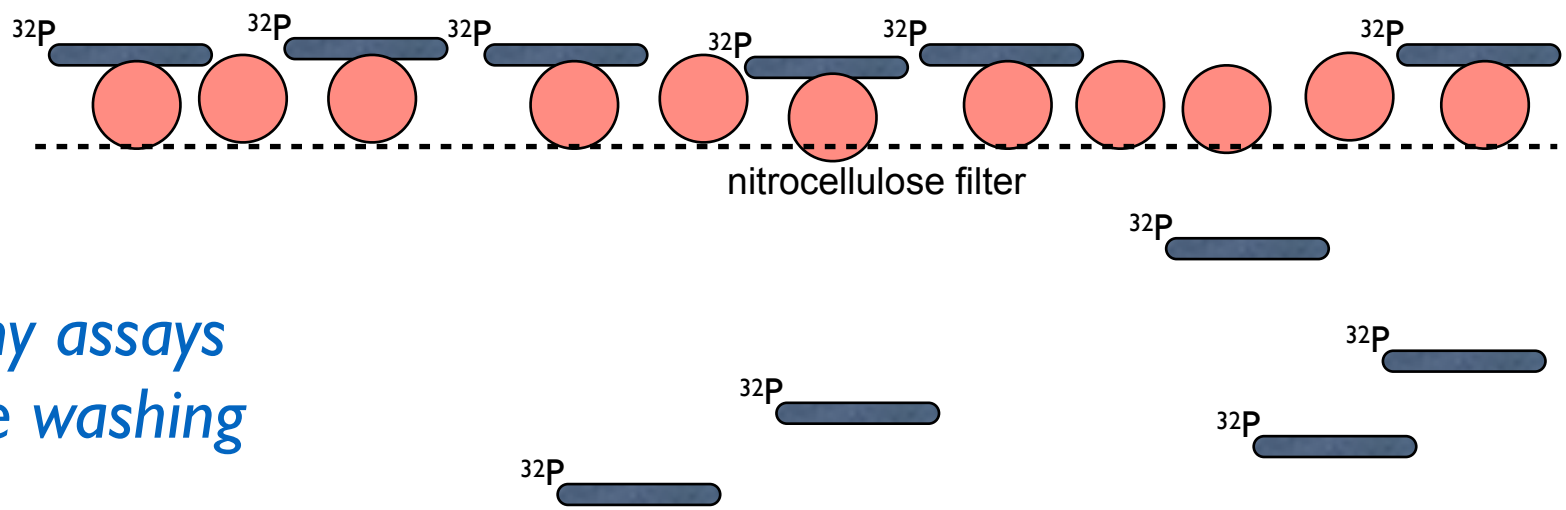
Equilibrate



Similar protocols?

- pull down assay
  (antibody based)

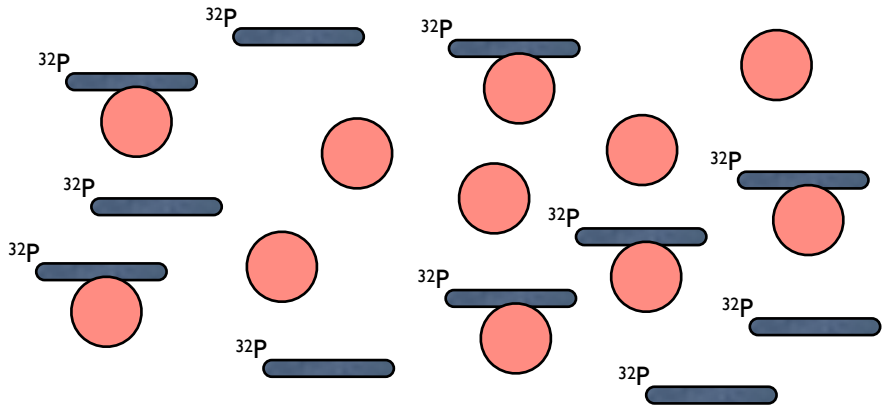
Flow, wash



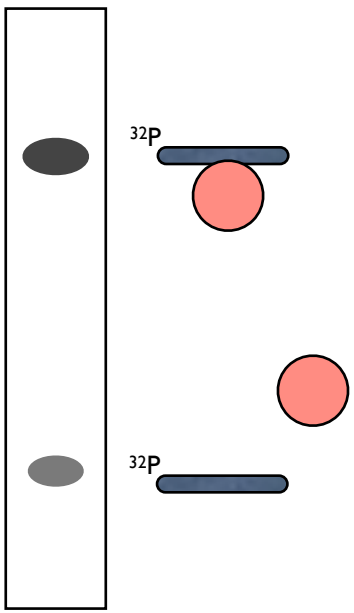
distrust any assays that involve washing

Methods to Measure Binding
Gel shift assay

Equilibrate

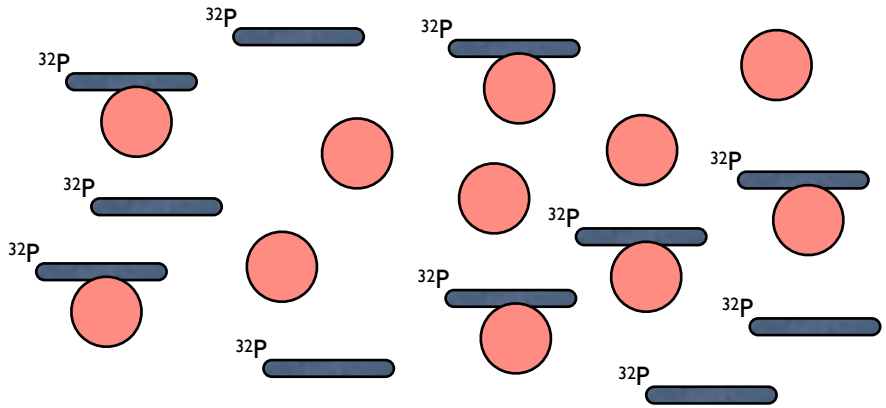


Nondenaturing gel



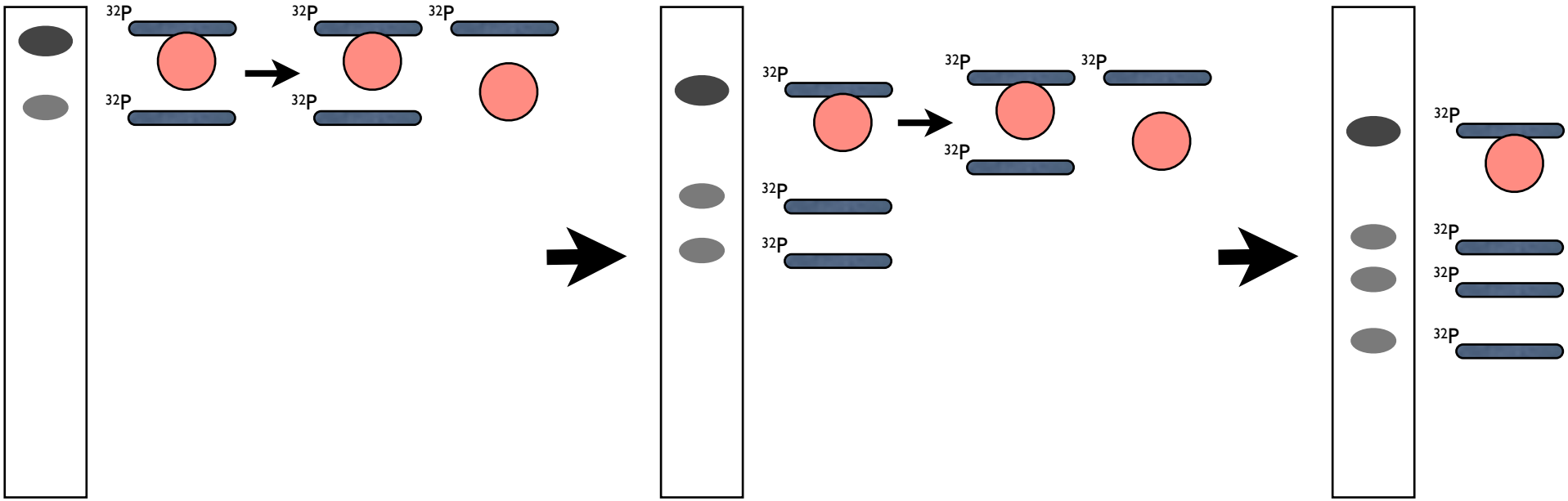
Methods to Measure Binding
Gel shift assay

Equilibrate

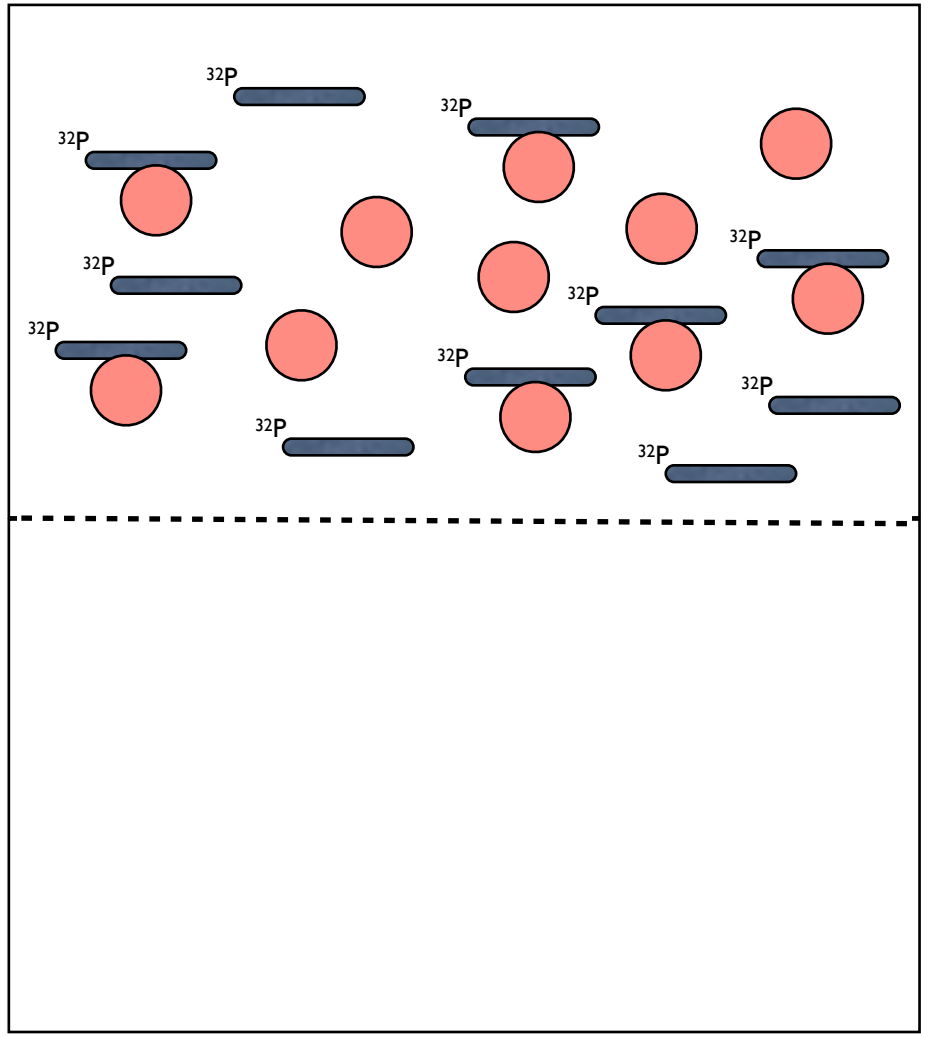


This shouldn't work: why?

Nondenaturing gel

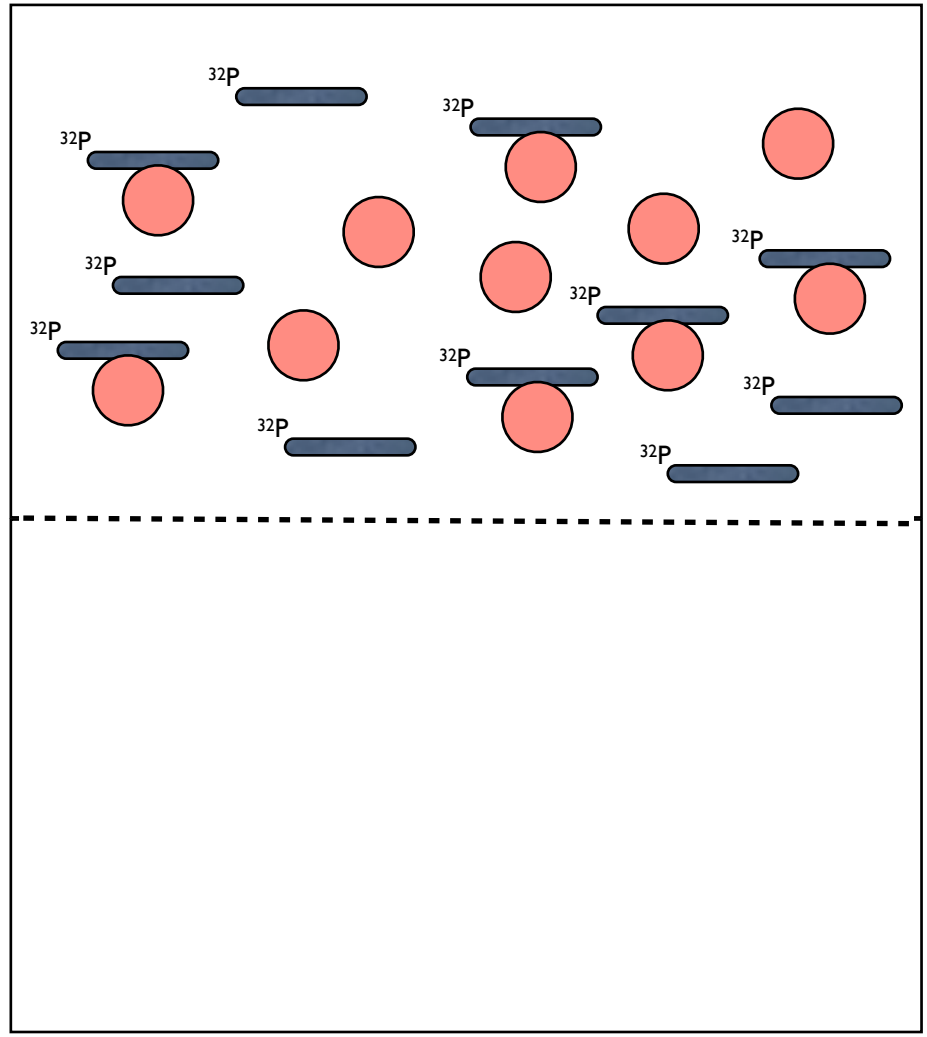


Methods to Measure Binding
Equilibrium Dialysis



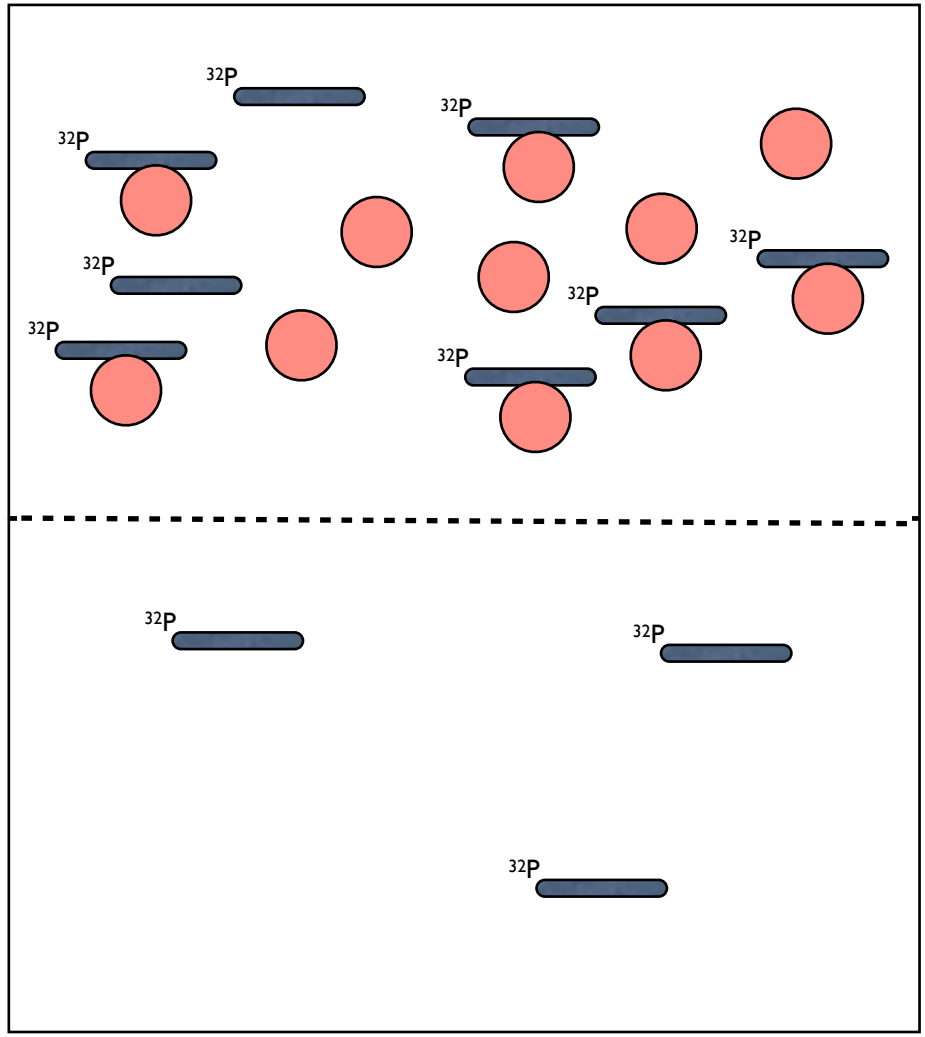
Methods to Measure Binding
Equilibrium Dialysis

Equilibrate



Methods to Measure Binding
Equilibrium Dialysis

Equilibrate

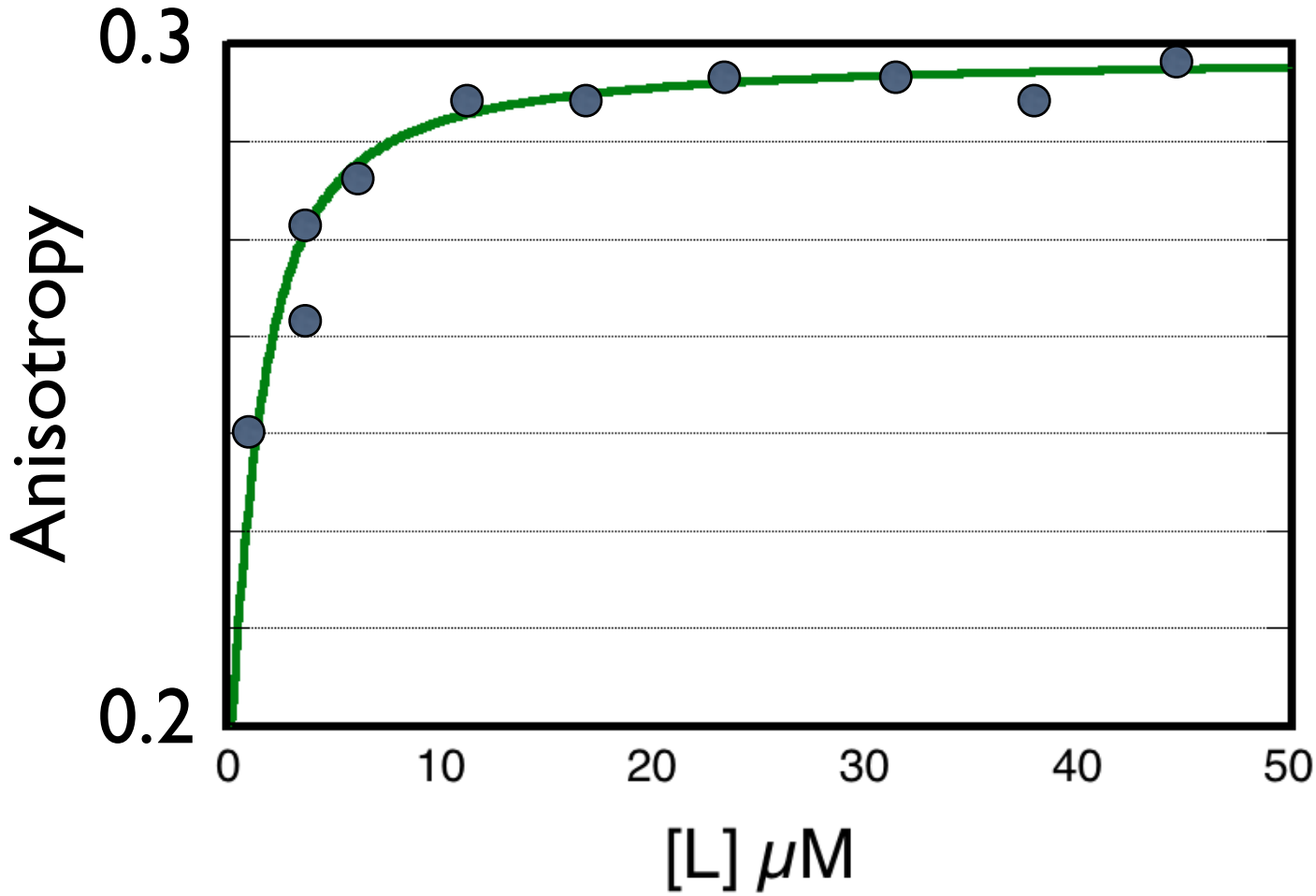


Binding Assays

- Non-equilibrium assays that more or less separate complexes
  - Filter binding
  - Pull-down
  - Gel shift
- Equilibrium assays
  - Fluorescence
    - Changes in quantum yield
    - Changes in wavelength maxima
    - Changes in anisotropy
  - Protection assays (quantitative footprinting, etc)

Methods to Measure Binding
Fluorescence Anisotropy

Equilibrium!!



Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$
$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$
$$K_d x = (A_T - x)(B_T - x)$$

Assume $B_T >> x$

$$K_d x \approx (A_T - x) B_T$$
$$(B_T + K_d) x \approx A_T B_T$$

$$x = [AB] \approx \frac{A_T B_T}{B_T + K_d}$$

Fraction Bound

$$\frac{[AB]}{A_T} \approx \frac{B_T}{B_T + K_d}$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$A_T = [A] + [AB]$$

$$B_T = [B] + [AB]$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB] \longleftarrow A_T = [A] + [AB]$$

$$[B] = B_T - [AB] \longleftarrow B_T = [B] + [AB]$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$
$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$

$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$

$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$
$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$

$$K_d x = (A_T - x)(B_T - x)$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$
$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$
$$K_d x = (A_T - x)(B_T - x)$$

Assume $B_T >> x$

$$K_d x \approx (A_T - x) B_T$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$
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$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$
$$K_d x = (A_T - x)(B_T - x)$$

Assume $B_T >> x$

$$K_d x \approx (A_T - x) B_T$$
$$(B_T + K_d) x \approx A_T B_T$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$
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$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$
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Assume $B_T >> x$

$$K_d x \approx (A_T - x) B_T$$
$$(B_T + K_d) x \approx A_T B_T$$

$$x = [AB] \approx \frac{A_T B_T}{B_T + K_d}$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
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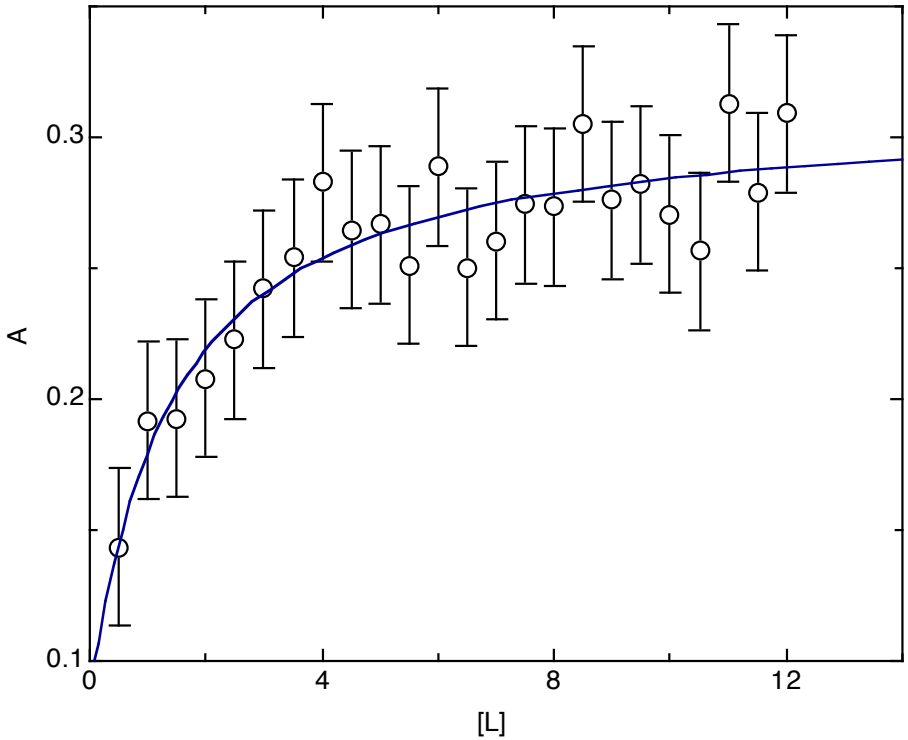
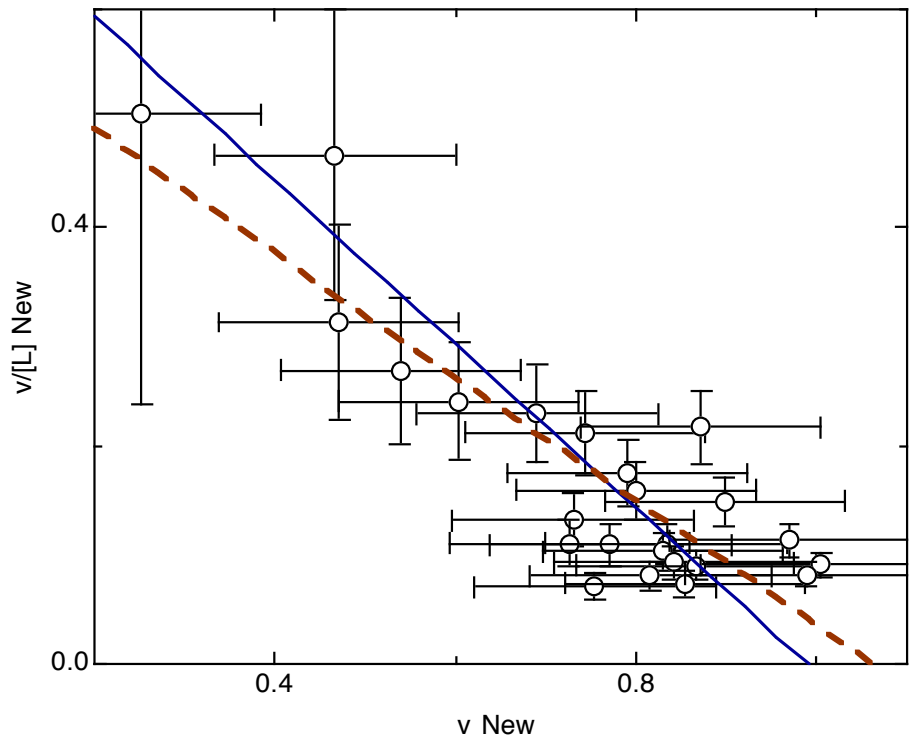
$$x = [AB] \approx \frac{A_T B_T}{B_T + K_d}$$

Fraction Bound

$$\frac{[AB]}{A_T} \approx \frac{B_T}{B_T + K_d}$$

$$\nu \approx \frac{B_T}{B_T + K_d}$$





Direct fit

K = 1.35 ± 0.66 μM

A_{bound} = 0.312 ± 0.012

$A_{unbound}$ = 0.087 ± 0.040

Weighted fit

K = 0.75 ± 0.20 μM

n = 0.99

Unweighted fit

K = 0.57 ± 0.07 μM

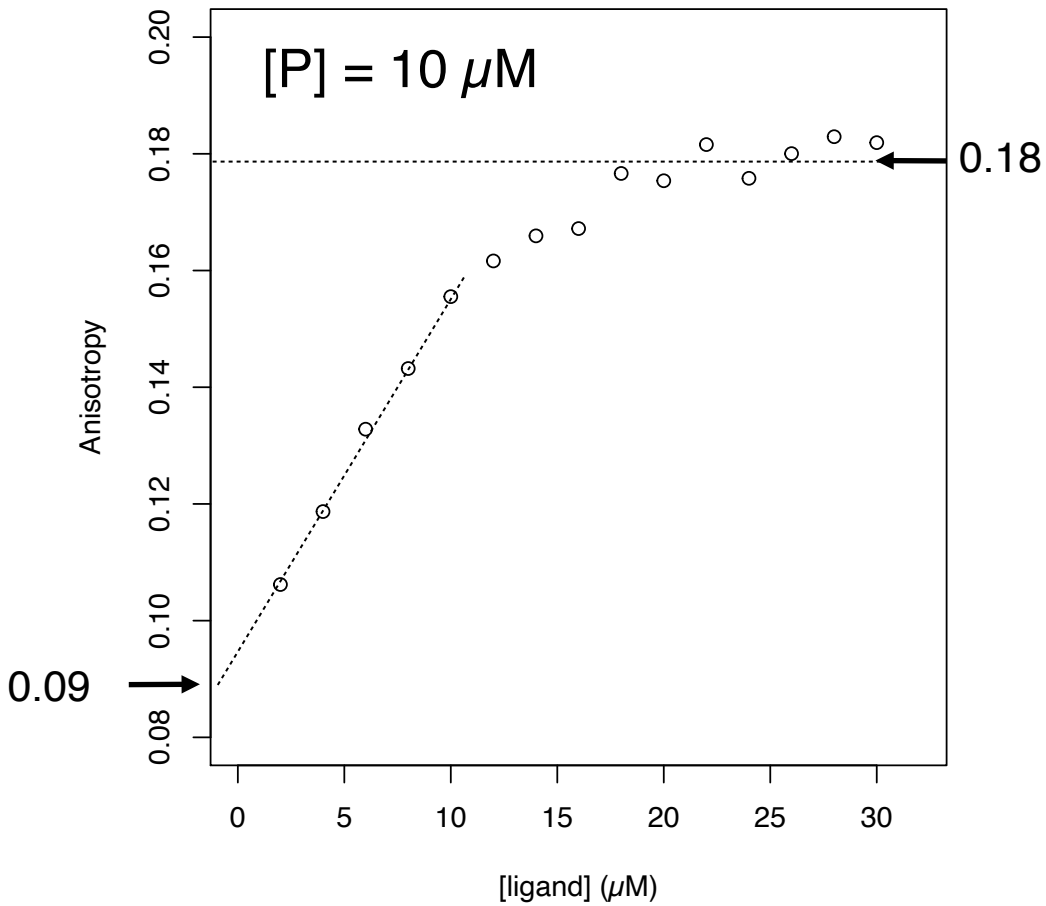
n = 1.06

Fixed

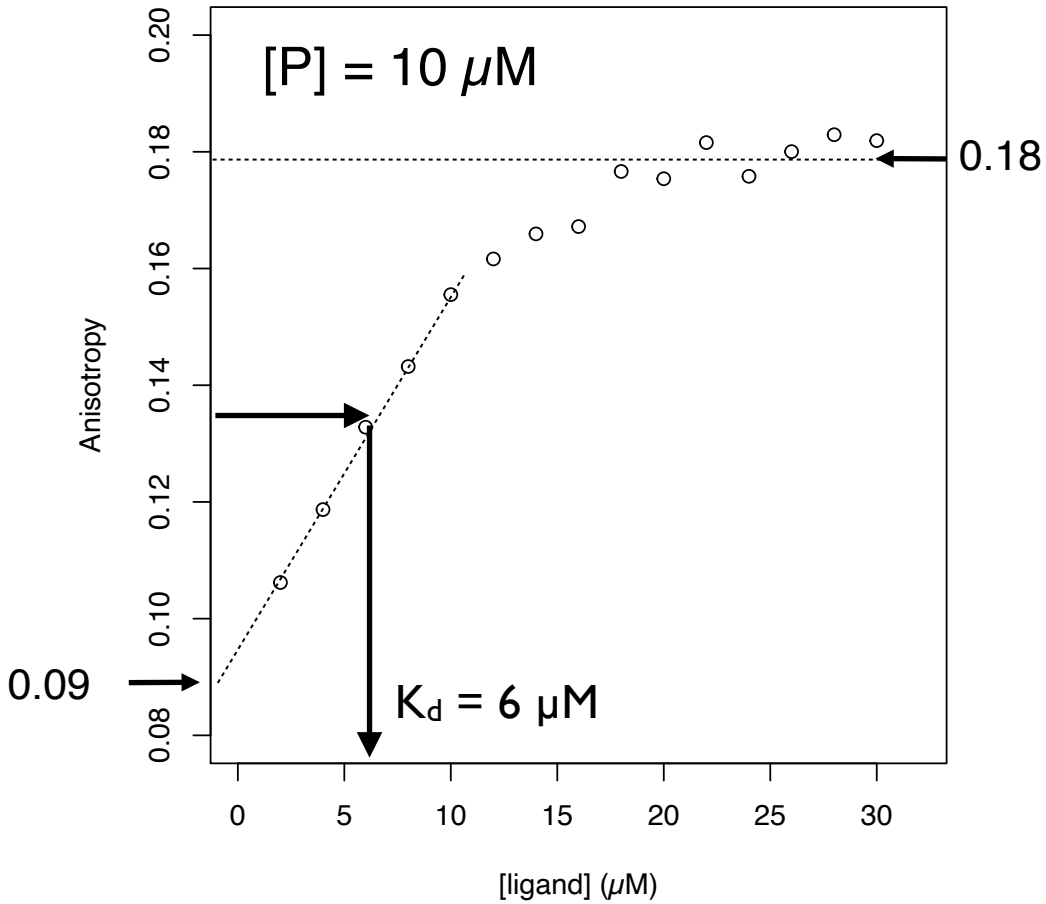
A_{bound} = 0.312

$A_{unbound}$ = 0.087

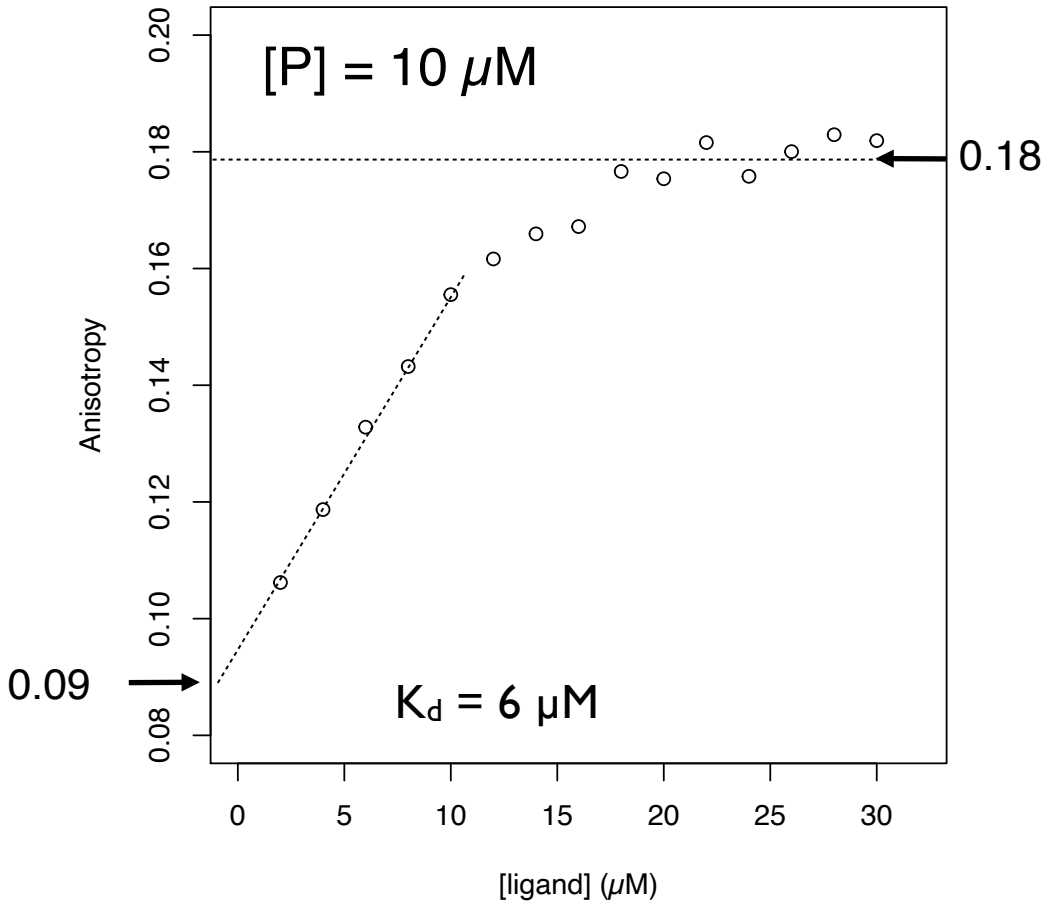
Fluorescence Anisotropy Titration



Fluorescence Anisotropy Titration



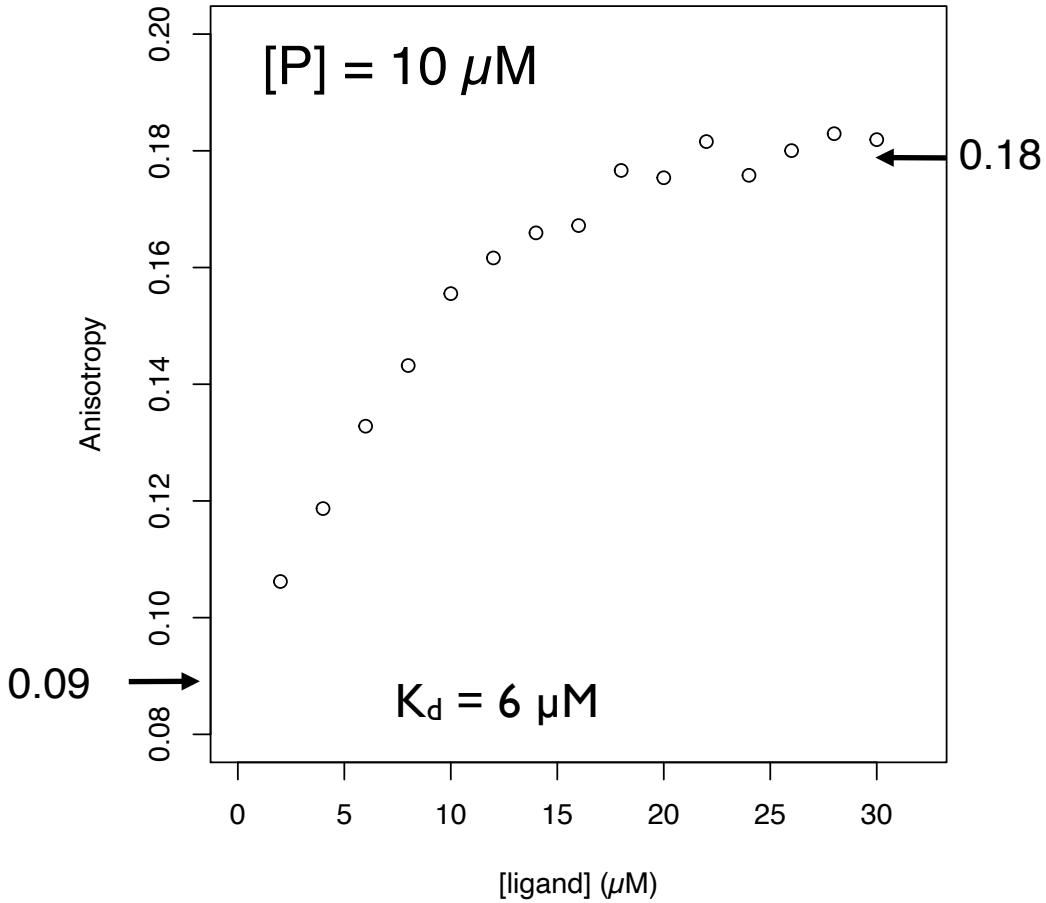
Fluorescence Anisotropy Titration

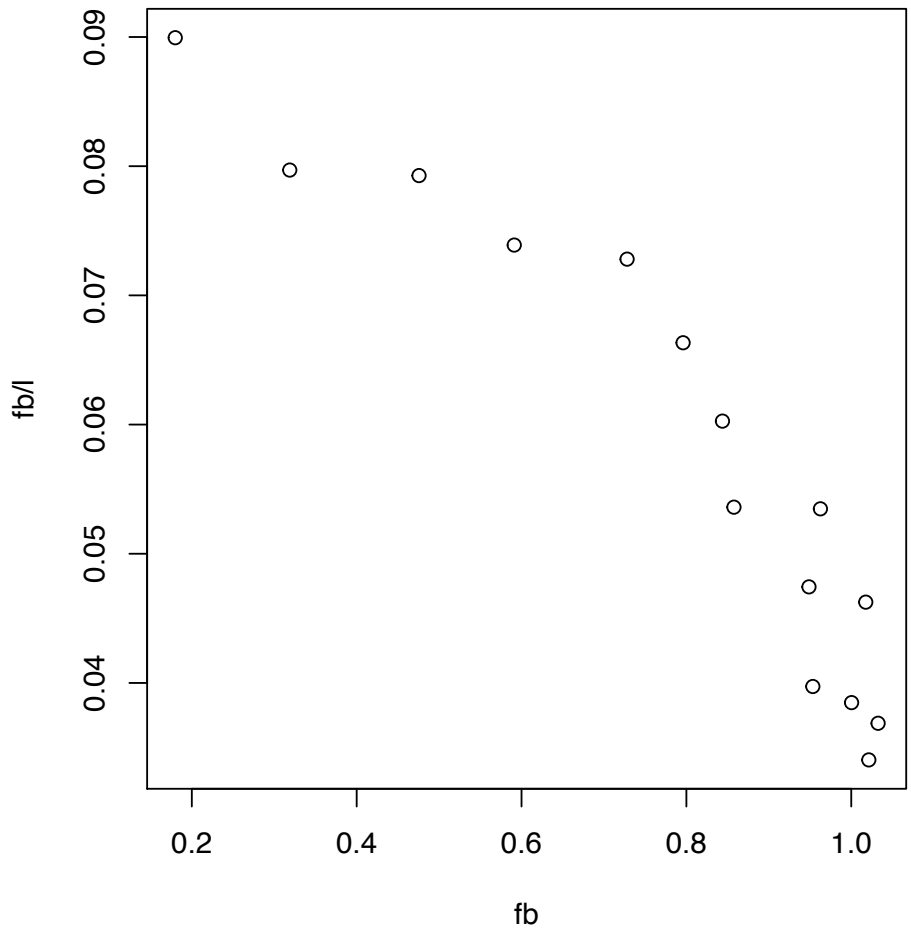


- Scatchard Analysis
  - Pick beginning and end values

- Scatchard Analysis
  - Calculate v & v/[L]
  - Plot v/[L] vs v

Fluorescence Anisotropy Titration





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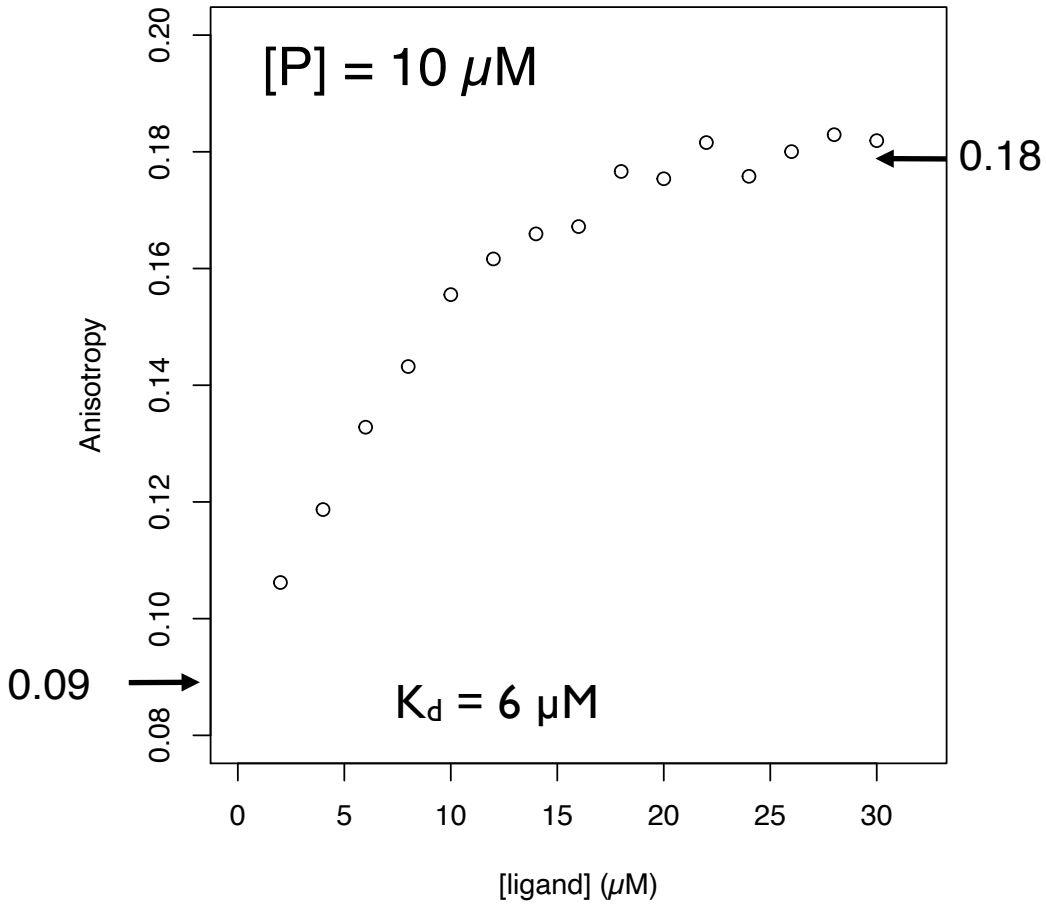
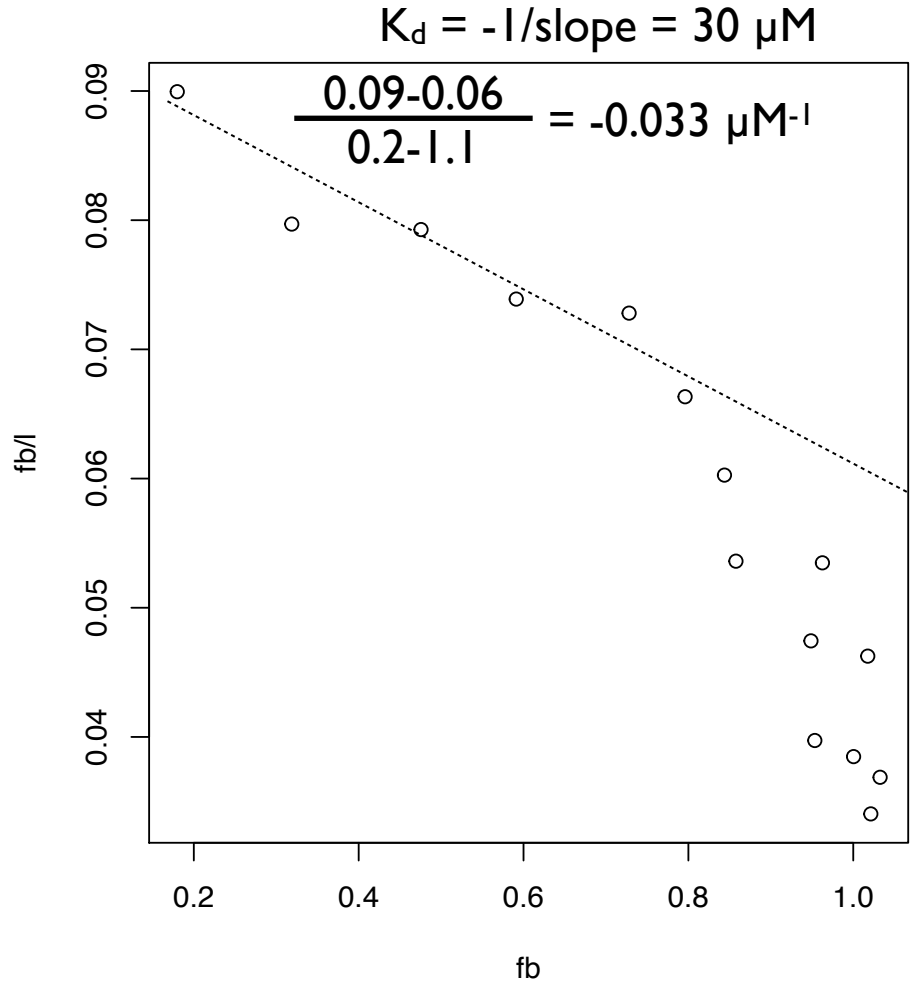
Fluorescence Anisotropy Titration





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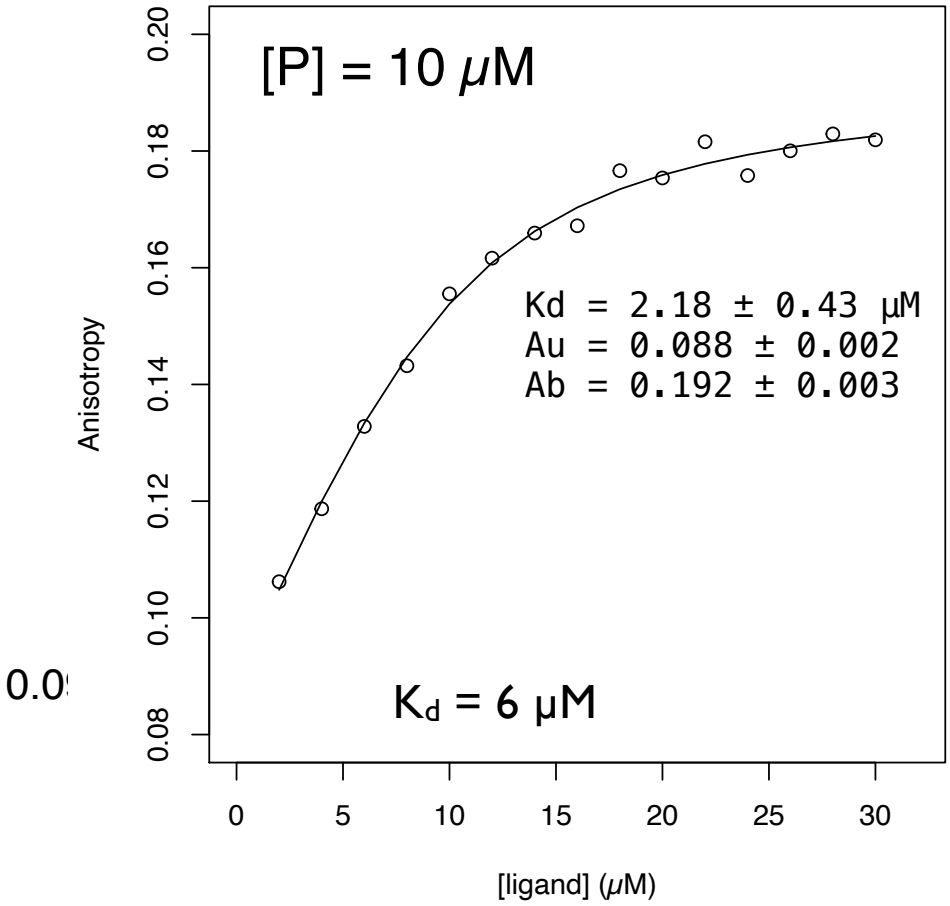
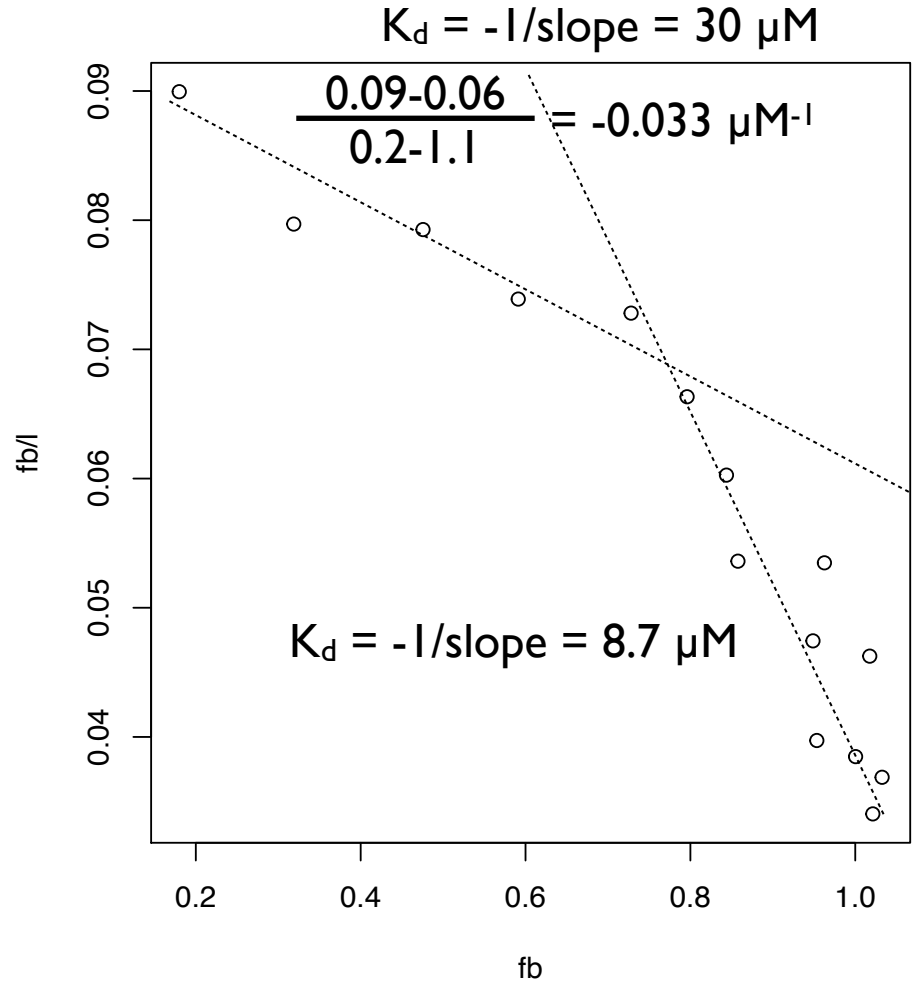
Fluorescence Anisotropy Titration





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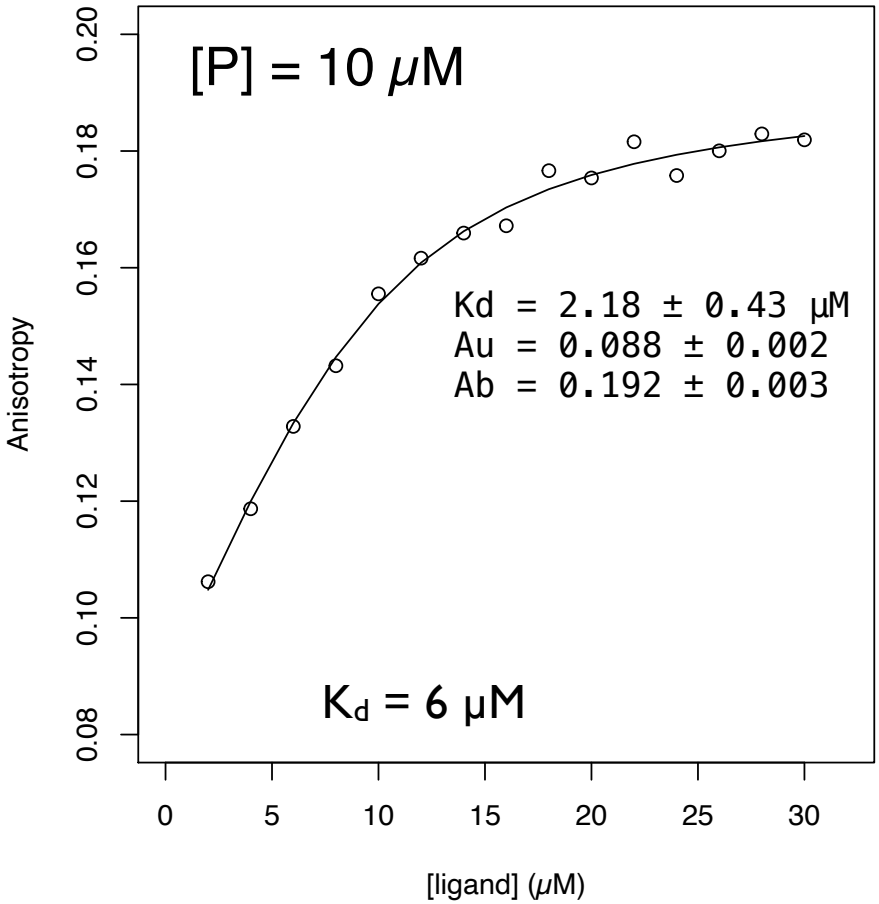
Fluorescence Anisotropy Titration



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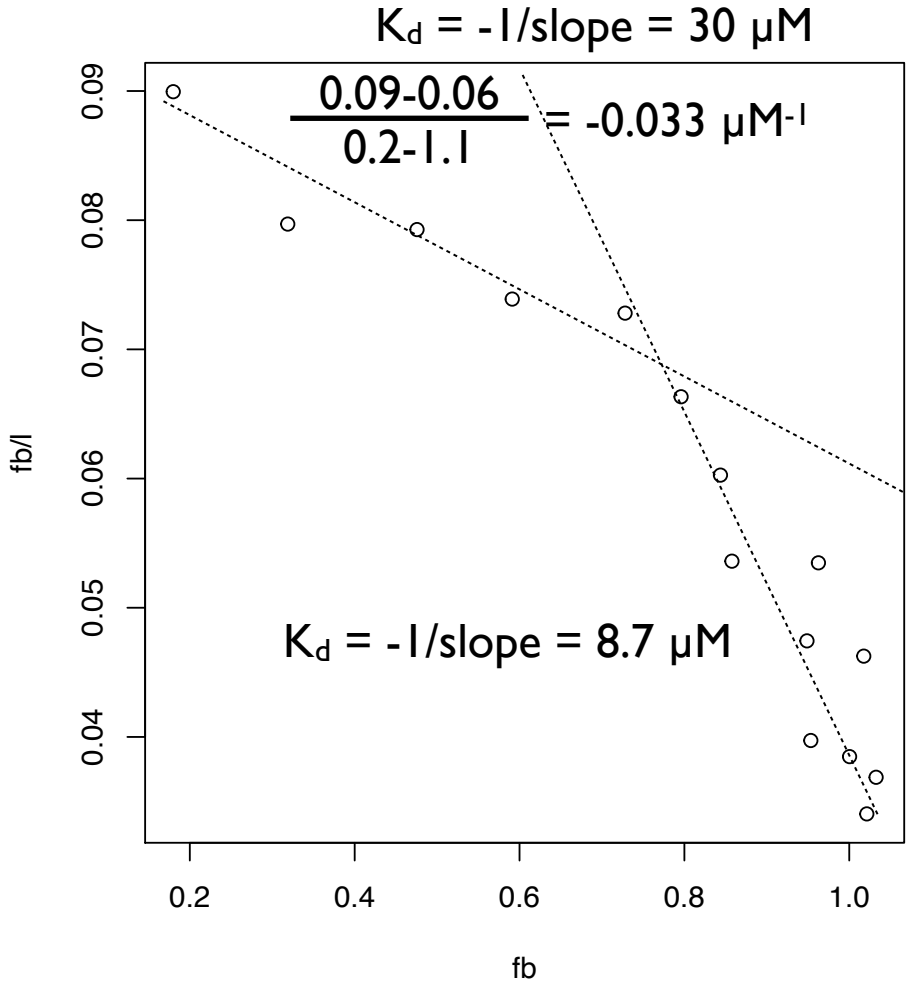

Why did the linearized approaches fail miserably?

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

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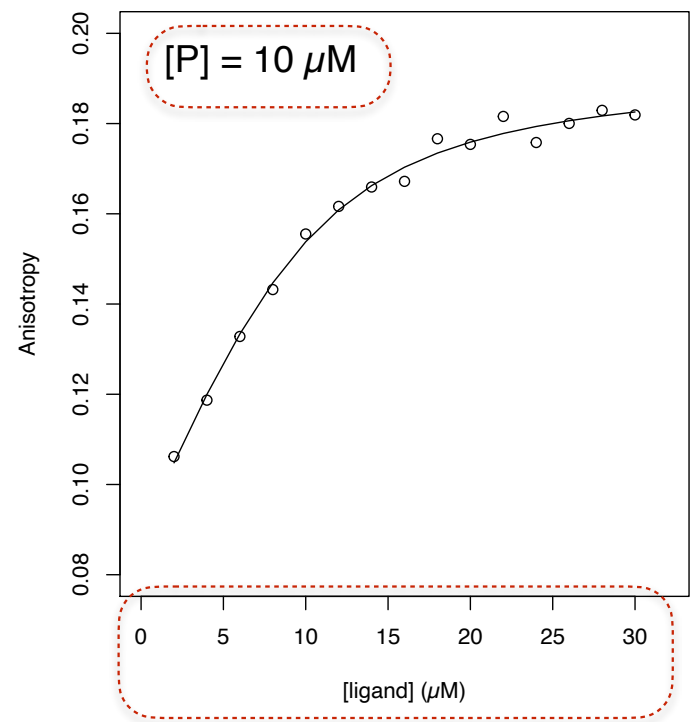
~~Assume $B_T >> x$~~



Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

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$$K_d = \frac{[A][B]}{[AB]}$$

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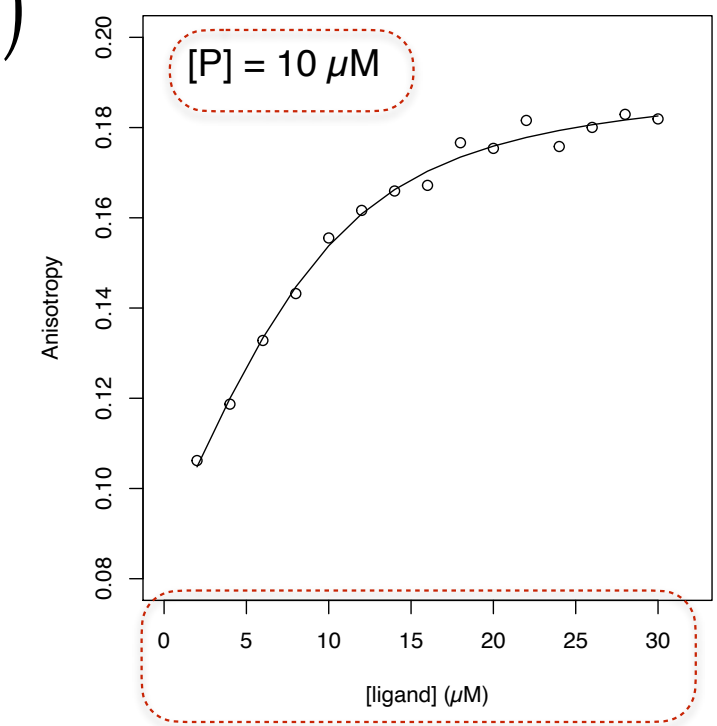
$$K_d x = (A_T - x)(B_T - x)$$

~~Assume $B_T >> x$~~

$$x^2 - (A_T + B_T + K_d)x + A_T B_T = 0$$

$$ax^2 + bx + c = 0 \qquad x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$



Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

$$[A] = A_T - [AB]$$

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Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

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$$x = \frac{(A_T + B_T + K_d) - \sqrt{(A_T + B_T + K_d)^2 - 4A_T B_T}}{2} = [AB]$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

$$[A] = A_T - [AB]$$

$$[B] = B_T - [AB]$$

Knowns

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$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$

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$$x = \frac{(A_T + B_T + K_d) - \sqrt{(A_T + B_T + K_d)^2 - 4A_T B_T}}{2} = [AB]$$

Fraction Bound

$$\frac{[AB]}{A_T}$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

$$x = \frac{\left(A_T + B_T + K_d\right) - \sqrt{\left(A_T + B_T + K_d\right)^2 - 4A_T B_T}}{2} = \left[AB\right]$$

Fraction Bound

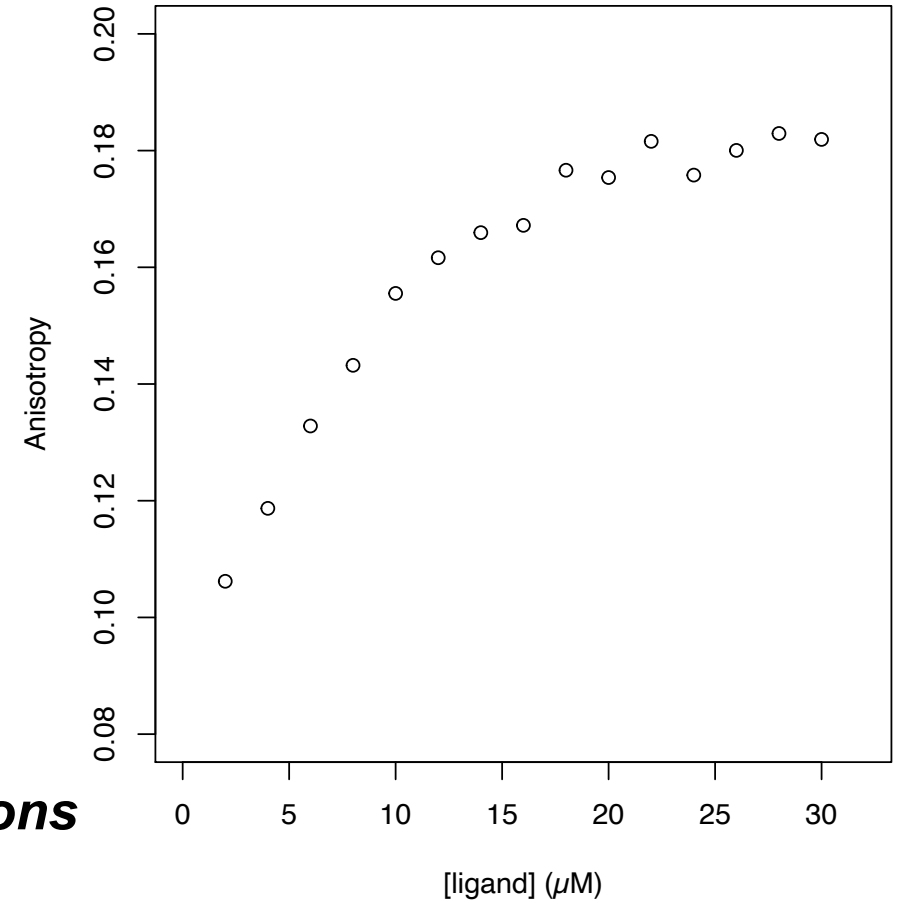
$$f = \frac{\left[AB\right]}{A_T} = \frac{\left(A_T + B_T + K_d\right) - \sqrt{\left(A_T + B_T + K_d\right)^2 - 4A_T B_T}}{2A_T}$$

At half-saturation, f=0.5

$$0.5 = \frac{\left[AB\right]}{A_T} = \frac{\left(A_T + B_T + K_d\right) - \sqrt{\left(A_T + B_T + K_d\right)^2 - 4A_T B_T}}{2A_T}$$

Equilibrium Math

$$P + L \underset{}{\overset{K_d}{\rightleftharpoons}} PL$$



Assume L >> P

Fraction Bound

$$f \approx \frac{1}{1 + \frac{K_d}{L_T}}$$

At half-saturation, f=0.5

$$L_T = K_d$$

No assumptions

Fraction Bound

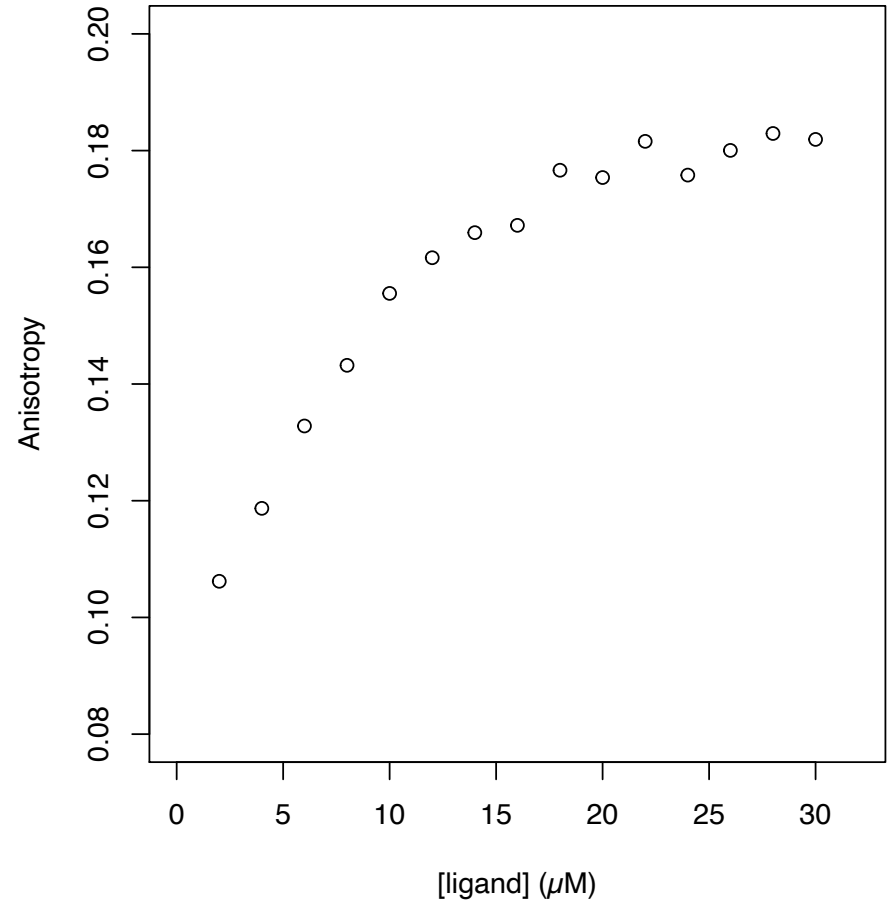
$$f = \frac{[PL]}{P_T} = \frac{\left(P_T + L_T + K_d\right) - \sqrt{\left(P_T + L_T + K_d\right)^2 - 4P_T L_T}}{2P_T}$$

At half-saturation, f=0.5

$$L_T = \frac{1}{2}P_T + K_d$$

Equilibrium Math

$$P + L \underset{}{\overset{K_d}{\rightleftharpoons}} PL$$



$$f = \frac{[PL]}{P_T} = \frac{(P_T + L_T + K_d) - \sqrt{(P_T + L_T + K_d)^2 - 4P_T L_T}}{2P_T}$$

(Au + (Ab-Au)*((Kd+Pt+Lt) - sqrt[(Kd+Pt+Lt)^2 - 4*Pt*Lt] / (2*Pt)))

$$A = A_u + f(A_b - A_u)$$

```
LigBndGen <- function(Lt, Kd, Pt, Au, Ab) (Au + (Ab-Au)*(((Kd+Pt+Lt)-sqrt((Kd+Pt+Lt)^2-4*Pt*Lt))/2)/Pt )

model <- nls(Anis ~ LigBndGen(l,myKd,10,myAu,myAb), start=list(myKd=4,myAu=0.15,myAb=0.35))
```

Equilibrium Math

[P] = 10 μM

$$P + L \underset{}{\overset{K_d}{\rightleftharpoons}} PL$$

Assume L >> P

Fraction Bound

$$f \approx \frac{1}{1 + \frac{K_d}{L_T}}$$

At half-saturation, f=0.5

$$L_T = K_d$$

No assumptions

Fraction Bound

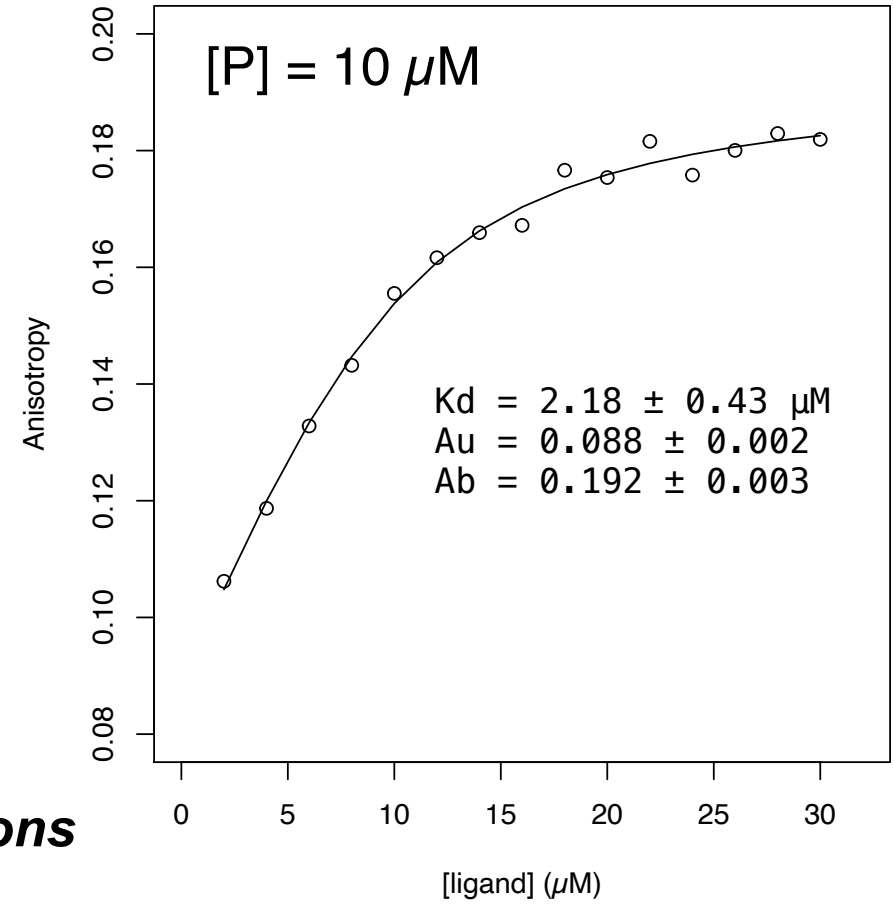
$$f = \frac{[PL]}{P_T} = \frac{\left(P_T + L_T + K_d\right) - \sqrt{\left(P_T + L_T + K_d\right)^2 - 4P_T L_T}}{2P_T}$$

At half-saturation, f=0.5

$$L_T = \frac{1}{2}P_T + K_d$$

Equilibrium Math

$$P + L \underset{}{\overset{K_d}{\rightleftharpoons}} PL$$



Assume L >> P

Fraction Bound

$$f \approx \frac{1}{1 + \frac{K_d}{L_T}}$$

At half-saturation, f=0.5

$$L_T = K_d$$

No assumptions

Fraction Bound

$$f = \frac{[PL]}{P_T} = \frac{\left(P_T + L_T + K_d\right) - \sqrt{\left(P_T + L_T + K_d\right)^2 - 4P_T L_T}}{2P_T}$$

At half-saturation, f=0.5

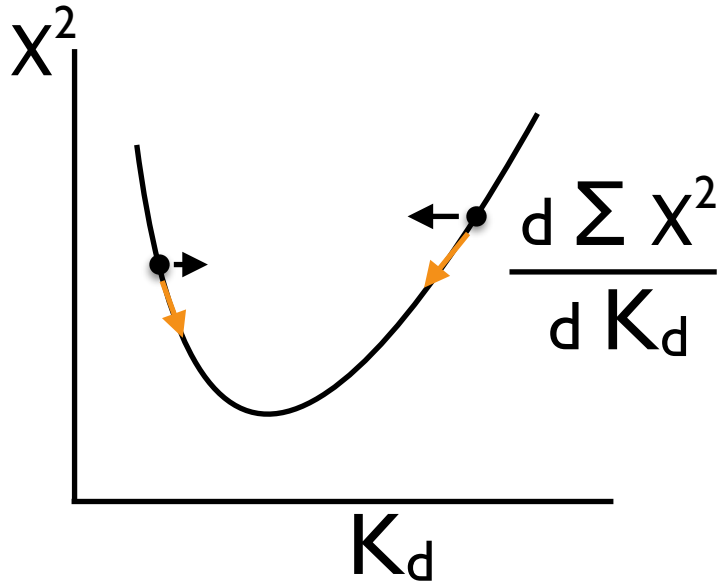
$$L_T = \frac{1}{2}P_T + K_d$$

Levenberg - Marqardt

The Levenberg-Marquardt algorithm

The Levenberg-Marquardt algorithm is derived directly from the mean square deviation expressions (8) or (10) and cannot be used with deviation functions *R* other then the square deviation *R*(*d*) = *d*2.

The Levenberg-Marquardt algorithm is a very fast fitting algorithm. Its performance, however, depends strongly on the behavior of the function to be fitted as well as on the selected starting parameters.



The classical version of the Levenberg-Marquardt algorithm does not allow for x-errors and minimizes the mean square deviation (10). The algorithm can be described in words as follows:

Starting from a given set of parameters, the mean square deviation X^2 is calculated. Then **the parameters are varied slightly to observe their influence on X^2**. From this, **the direction in which X^2 decreases most rapidly** can be evaluated and a new set of parameters is chosen. This procedure is reiterated with this new set of parameters . When the minimum is near, the algorithm goes over to a more deterministic “guessing” at the position of the minimum and solves some equations to find it. The fitting stops when the value of X^2 does not decrease anymore between successive steps.

The algorithm is described in W.H. Press, B.P. Flannery, S.A. Teukolsky, W.T. Vetterling, *Numerical Recipes - the Art of Scientific Computing*, Second Edition, University Press, Cambridge, 1992. When x-errors are specified, the algorithm is modified in such a way that it minimizes (8). It finds at the same time the set of x-coordinates $x\hat{}\, i$ and the function parameters that minimize the mean square deviations between the data points $(xi\,, yi\,)$ and the function values $(x\hat{}\, i\,, f(x\hat{}\, i\,))$.

Levenberg - Marqardt

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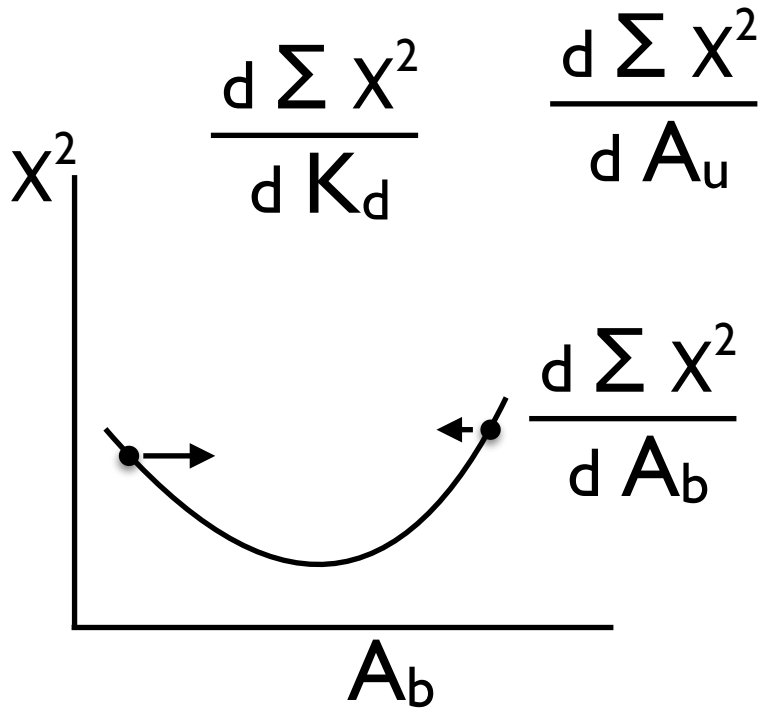

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Levenberg - Marqardt

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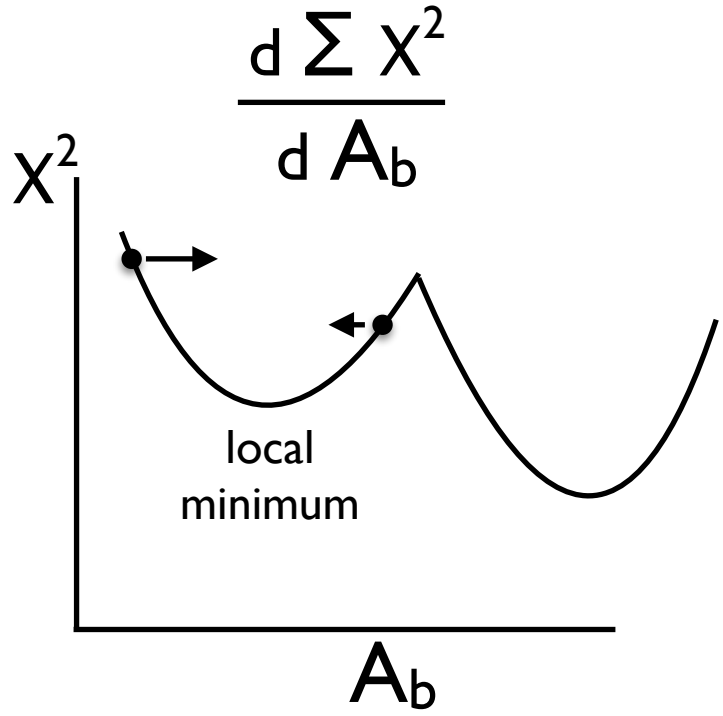

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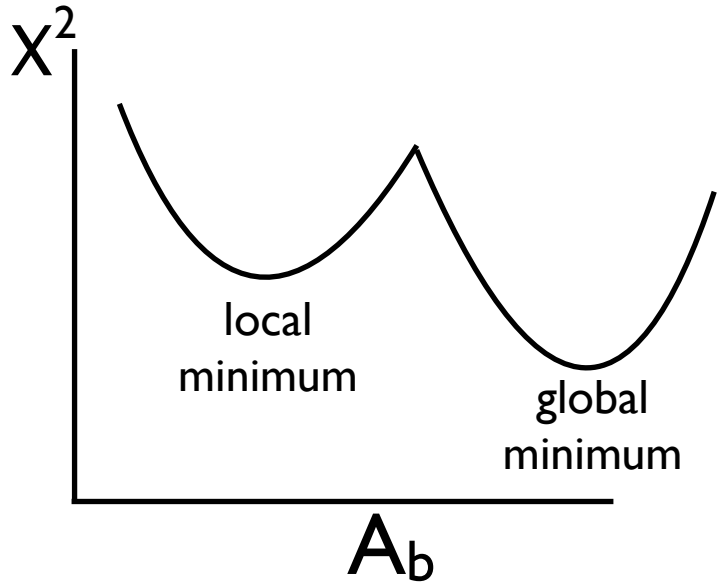
Monte Carlo

The Monte Carlo algorithm
This method randomly varies the parameters of a function within given intervals. When x-errors are defined, the algorithm also varies randomly the set of x-coordinates $x\hat{}\,i$ while observing the given errors and error distributions.



For each random guess, $\mathbf{X}$R is calculated according to Eq. (2) and the parameter sets corresponding to the smallest values of $\mathbf{X}$R are remembered.

The strength of this method is also its biggest disadvantage. It looks for the best parameter set by shooting blindly inside the given region of parameter space. Although there is an option of letting this parameter space region follow the position of the currently best parameter set, this algorithm can only converge very slowly towards the best parameter set.

Its main use is to “scan” parameter space in order to find good parameter starting values for one of the deterministic fit algorithms, or to try to “jump out” of a local minimum where a deterministic fitting algorithm is stuck.

Since the algorithm is normally used for a first estimate of fitted parameters, it is not recommended to run it with non-zero x-errors – this merely slows down the algorithm without substantially increasing the accuracy of the estimates

Multiple Binding Sites
Overview

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

$^{+}H_3NCH_2COOH$

$^{+}H_3NCH_2COOH \rightleftharpoons {}^{+}H_3NCH_2COO^{-} + H^{+}$ k_1

$^{+}H_3NCH_2COOH \rightleftharpoons H_2NCH_2COOH + H^{+}$ k_2

Multiple Binding Sites
Overview

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

$^{+}H_3NCH_2COOH$

$^{+}H_3NCH_2COO\mathbf{H}$	⇌	$^{+}H_3NCH_2COO^{-}$ + $\mathbf{H^{+}}$	$\mathbf{k_1}$	H-Gly^{+}-H	⇌	H-Gly	+	$\mathbf{H^{+}}$
$^{+}\mathbf{H}_3NCH_2COOH$	⇌	H_2NCH_2COOH + $\mathbf{H^{+}}$	$\mathbf{k_2}$	H-Gly^{+}-H	⇌	Gly-H	+	$\mathbf{H^{+}}$
			$\mathbf{K_1}$	$^{+}$Gly-H_2	⇌	Gly-H	+	$\mathbf{H^{+}}$

$^{+}\mathbf{H}_3NCH_2COO^{-}$	⇌	$H_2NCH_2COO^{-}$ + $\mathbf{H^{+}}$	$\mathbf{k_3}$	H-Gly	⇌	Gly^{-}	+	$\mathbf{H^{+}}$
$H_2NCH_2COO\mathbf{H}$	⇌	$H_2NCH_2COO^{-}$ + $\mathbf{H^{+}}$	$\mathbf{k_4}$	Gly-H	⇌	Gly^{-}	+	$\mathbf{H^{+}}$

Multiple Binding Sites
Overview

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

$^{+}H_3NCH_2COOH$

$^{+}H_3NCH_2COO\mathbf{H}$	$\rightleftharpoons$	$^{+}H_3NCH_2COO^{-}$ + $\mathbf{H^{+}}$	$\mathbf{k_1}$	H-Gly^{+}-H	$\rightleftharpoons$	H-Gly +	$\mathbf{H^{+}}$
$^{+}\mathbf{H}_3NCH_2COOH$	$\rightleftharpoons$	H_2NCH_2COOH + $\mathbf{H^{+}}$	$\mathbf{k_2}$	H-Gly^{+}-H	$\rightleftharpoons$	Gly-H +	$\mathbf{H^{+}}$
			$\mathbf{K_1}$	$^{+}$Gly-H$_2$	$\rightleftharpoons$	Gly-H +	$\mathbf{H^{+}}$

$^{+}\mathbf{H}_3NCH_2COO^{-}$	$\rightleftharpoons$	$H_2NCH_2COO^{-}$ + $\mathbf{H^{+}}$	$\mathbf{k_3}$	H-Gly	$\rightleftharpoons$	Gly^{-} +	$\mathbf{H^{+}}$
$H_2NCH_2COO\mathbf{H}$	$\rightleftharpoons$	$H_2NCH_2COO^{-}$ + $\mathbf{H^{+}}$	$\mathbf{k_4}$	Gly-H	$\rightleftharpoons$	Gly^{-} +	$\mathbf{H^{+}}$
				Gly-H	$\rightleftharpoons$	Gly^{-} +	$\mathbf{H^{+}}$

Multiple Binding Sites
Overview

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

$^{+}H_3NCH_2COOH$

$^{+}H_3NCH_2COOH \rightleftharpoons {}^{+}H_3NCH_2COO^{-} + H^{+}$ $\quad k_1$ $\quad$ H-Gly^{+}-H $\rightleftharpoons$ H-Gly + H^{+}

$^{+}H_3NCH_2COOH \rightleftharpoons H_2NCH_2COOH + H^{+}$ $\quad k_2$ $\quad$ H-Gly^{+}-H $\rightleftharpoons$ Gly-H + H^{+}

K_1 $\quad$ $^{+}$Gly-H$_2$ $\rightleftharpoons$ Gly-H + H^{+}

$$k_1 + k_2 = K_1$$

$^{+}H_3NCH_2COO^{-} \rightleftharpoons H_2NCH_2COO^{-} + H^{+}$ $\quad k_3$ $\quad$ H-Gly $\rightleftharpoons$ Gly^{-} + H^{+}

$H_2NCH_2COOH \rightleftharpoons H_2NCH_2COO^{-} + H^{+}$ $\quad k_4$ $\quad$ Gly-H $\rightleftharpoons$ Gly^{-} + H^{+}

Gly-H $\rightleftharpoons$ Gly^{-} + H^{+}

Multiple Binding Sites
Overview

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

$^{+}H_3NCH_2COOH$

$^{+}H_3NCH_2COO\mathbf{H} \rightleftharpoons {}^{+}H_3NCH_2COO^{-} + \mathbf{H^{+}}$ $\quad \mathbf{k_1}$ $\quad$ H-Gly^{+}-H $\rightleftharpoons$ H-Gly + $\mathbf{H^{+}}$

$^{+}\mathbf{H}_3NCH_2COOH \rightleftharpoons H_2NCH_2COOH + \mathbf{H^{+}}$ $\quad \mathbf{k_2}$ $\quad$ H-Gly^{+}-H $\rightleftharpoons$ Gly-H + $\mathbf{H^{+}}$

$\mathbf{K_1}$ $\quad$ $^{+}$Gly-H$_2$ $\rightleftharpoons$ Gly-H + $\mathbf{H^{+}}$

$$\mathbf{k_1 + k_2 = K_1}$$

$^{+}\mathbf{H}_3NCH_2COO^{-} \rightleftharpoons H_2NCH_2COO^{-} + \mathbf{H^{+}}$ $\quad \mathbf{k_3}$ $\quad$ H-Gly $\rightleftharpoons$ Gly^{-} + $\mathbf{H^{+}}$

$H_2NCH_2COO\mathbf{H} \rightleftharpoons H_2NCH_2COO^{-} + \mathbf{H^{+}}$ $\quad \mathbf{k_4}$ $\quad$ Gly-H $\rightleftharpoons$ Gly^{-} + $\mathbf{H^{+}}$

$\mathbf{K_2}$ $\quad$ Gly-H $\rightleftharpoons$ Gly^{-} + $\mathbf{H^{+}}$

$$\mathbf{1 \,/\, (1/k_3 + 1/k_4) = K_2}$$

Multiple Binding Sites Overview

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

$$k_1 = \frac{[\text{H-Gly}]\ [\text{H}^+]}{[\text{H-Gly}^+\text{-H}]} \qquad k_2 = \frac{[\text{Gly-H}]\ [\text{H}^+]}{[\text{H-Gly}^+\text{-H}]}$$

$^+H_3NCH_2COOH$

$^+H_3NCH_2COOH \rightleftharpoons {}^+H_3NCH_2COO^- + H^+$ $\quad k_1$ $\quad$ H-Gly$^+$-H $\rightleftharpoons$ H-Gly + H^+

$^+H_3NCH_2COOH \rightleftharpoons H_2NCH_2COOH + H^+$ $\quad k_2$ $\quad$ H-Gly$^+$-H $\rightleftharpoons$ Gly-H + H^+

$\quad K_1$ $\quad {}^+$Gly-$H_2 \rightleftharpoons$ Gly-H + H^+

$$k_1 + k_2 = K_1$$

$^+H_3NCH_2COO^- \rightleftharpoons H_2NCH_2COO^- + H^+$ $\quad k_3$ $\quad$ H-Gly $\rightleftharpoons$ Gly^- + H^+

$H_2NCH_2COOH \rightleftharpoons H_2NCH_2COO^- + H^+$ $\quad k_4$ $\quad$ Gly-H $\rightleftharpoons$ Gly^- + H^+

$\quad K_2$ $\quad$ Gly-H $\rightleftharpoons$ Gly^- + H^+

$$1 \ / \ (1/k_3 + 1/k_4) = K_2$$

Multiple Binding Sites Overview

$$k_1 = \frac{[\text{H-Gly}]\ [\text{H}^+]}{[\text{H-Gly}^+\text{-H}]}$$

$$k_2 = \frac{[\text{Gly-H}]\ [\text{H}^+]}{[\text{H-Gly}^+\text{-H}]}$$

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

$^+H_3NCH_2COOH$

$^+H_3NCH_2COOH \rightleftharpoons {}^+H_3NCH_2COO^- + H^+$

$^+H_3NCH_2COOH \rightleftharpoons H_2NCH_2COOH + H^+$

microscopic

k_1	H-Gly$^+$-H	⇌	H-Gly + H^+
k_2	H-Gly$^+$-H	⇌	Gly-H + H^+
K_1	$^+$Gly-H_2	⇌	Gly-H + H^+

$$k_1 + k_2 = K_1$$

$^+H_3NCH_2COO^- \rightleftharpoons H_2NCH_2COO^- + H^+$

$H_2NCH_2COOH \rightleftharpoons H_2NCH_2COO^- + H^+$

microscopic

k_3	H-Gly	⇌	Gly$^-$ + H^+
k_4	Gly-H	⇌	Gly$^-$ + H^+
K_2	Gly-H	⇌	Gly$^-$ + H^+

$$1 \,/\, (1/k_3 + 1/k_4) = K_2$$

Multiple Binding Sites Overview

$$k_1 = \frac{[\text{H-Gly}]\ [\text{H}^+]}{[\text{H-Gly}^+\text{-H}]}$$

$$k_2 = \frac{[\text{Gly-H}]\ [\text{H}^+]}{[\text{H-Gly}^+\text{-H}]}$$

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

$^+H_3NCH_2COOH$

$^+H_3NCH_2COOH \rightleftharpoons {}^+H_3NCH_2COO^- + H^+$

$^+H_3NCH_2COOH \rightleftharpoons H_2NCH_2COOH + H^+$

microscopic

k_1	H-Gly$^+$-H	$\rightleftharpoons$	H-Gly + H^+
k_2	H-Gly$^+$-H	$\rightleftharpoons$	Gly-H + H^+
K_1	$^+$Gly-H$_2$	$\rightleftharpoons$	Gly-H + H^+

MACROSCOPIC

$k_1 + k_2 = K_1$

Two ways to deprotonate

$^+H_3NCH_2COO^- \rightleftharpoons H_2NCH_2COO^- + H^+$

$H_2NCH_2COOH \rightleftharpoons H_2NCH_2COO^- + H^+$

microscopic

k_3	H-Gly	$\rightleftharpoons$	Gly^- + H^+
k_4	Gly-H	$\rightleftharpoons$	Gly^- + H^+
K_2	Gly-H	$\rightleftharpoons$	Gly^- + H^+

MACROSCOPIC

$1 / (1/k_3 + 1/k_4) = K_2$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$
$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$
$$K_d x = (A_T - x)(B_T - x)$$

Assume $B_T >> x$

$$K_d x \approx (A_T - x) B_T$$
$$(B_T + K_d) x \approx A_T B_T$$

$$x = [AB] \approx \frac{A_T B_T}{B_T + K_d}$$

Fraction Bound

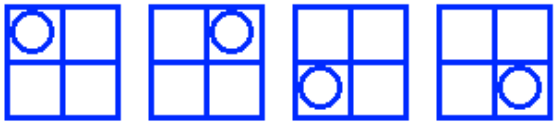
$$\frac{[AB]}{A_T} \approx \frac{B_T}{B_T + K_d}$$

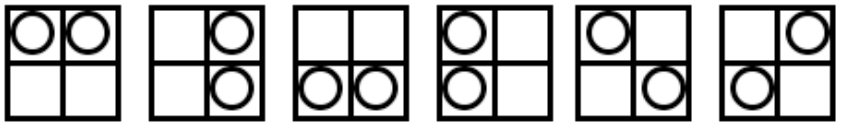
Multiple Binding Sites
Identical, Independent Sites

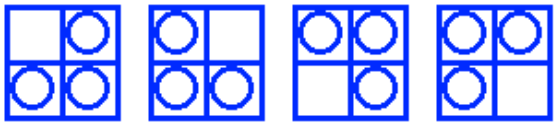
$$\bar{v} = \frac{nk[L]}{1 + k[L]}$$

$$\Omega_{n,i} = \frac{n!}{(n-i)!i!}$$

$M_o =$ 

$M_o + L \xrightarrow{k} M_1$ $\quad M_1 =$ 

$M_1 + L \xrightarrow{k} M_2$ $\quad M_2 =$ 

$M_2 + L \xrightarrow{k} M_3$ $\quad M_3 =$ 

$M_3 + L \xrightarrow{k} M_4$ $\quad M_4 =$ 

$$\Omega_{n,i} = \frac{4!}{4!0!} = 1$$

$$\Omega_{n,i} = \frac{4!}{3!1!} = 4$$

$$\Omega_{n,i} = \frac{4!}{2!2!} = 6$$

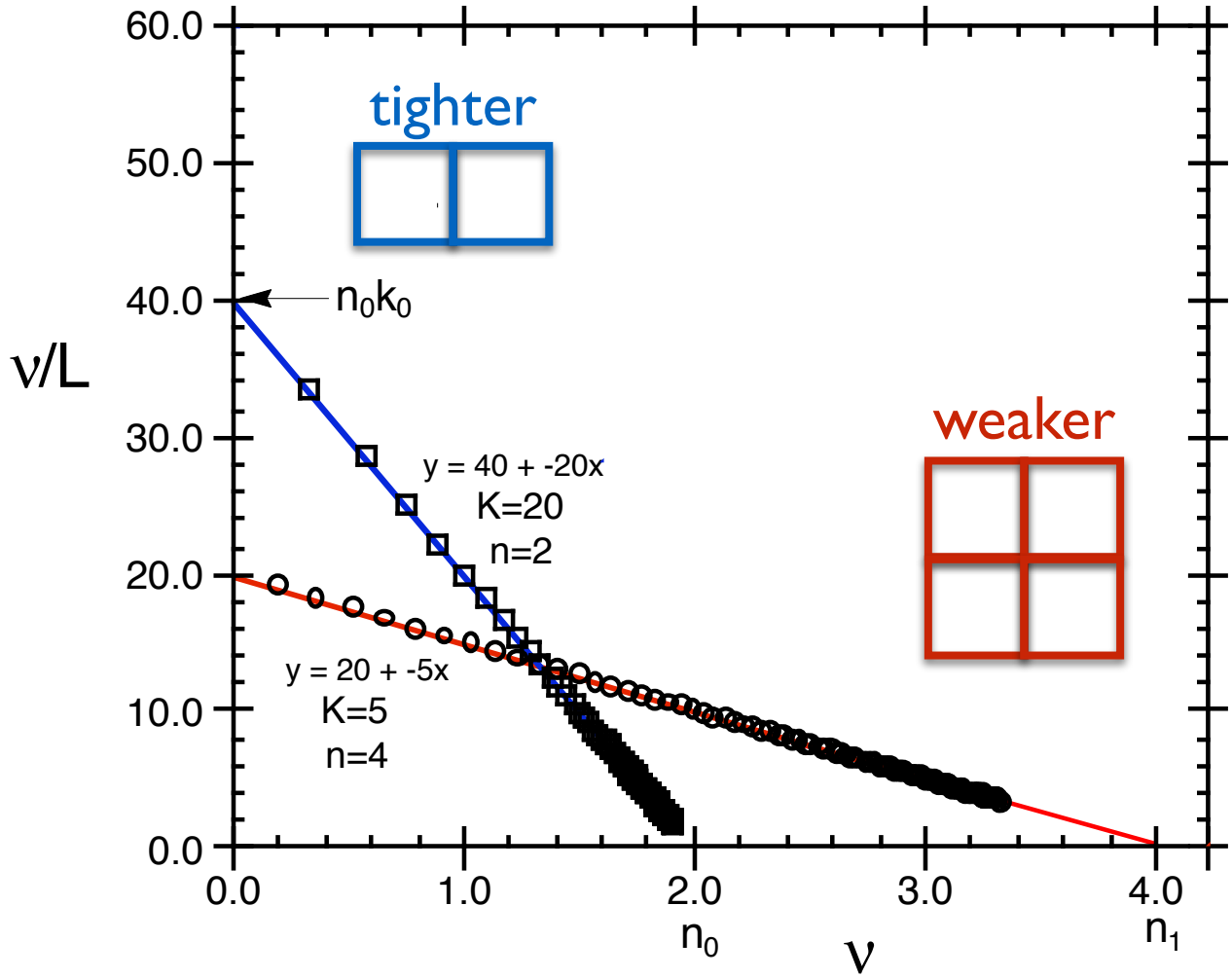
$$\Omega_{n,i} = \frac{4!}{1!3!} = 4$$

$$\Omega_{n,i} = \frac{4!}{0!4!} = 1$$

Multiple, Identical, Independent Binding Sites

Assuming L in excess

$$\frac{\bar{\nu}}{[L]} = nK_a - K_a\bar{\nu}$$

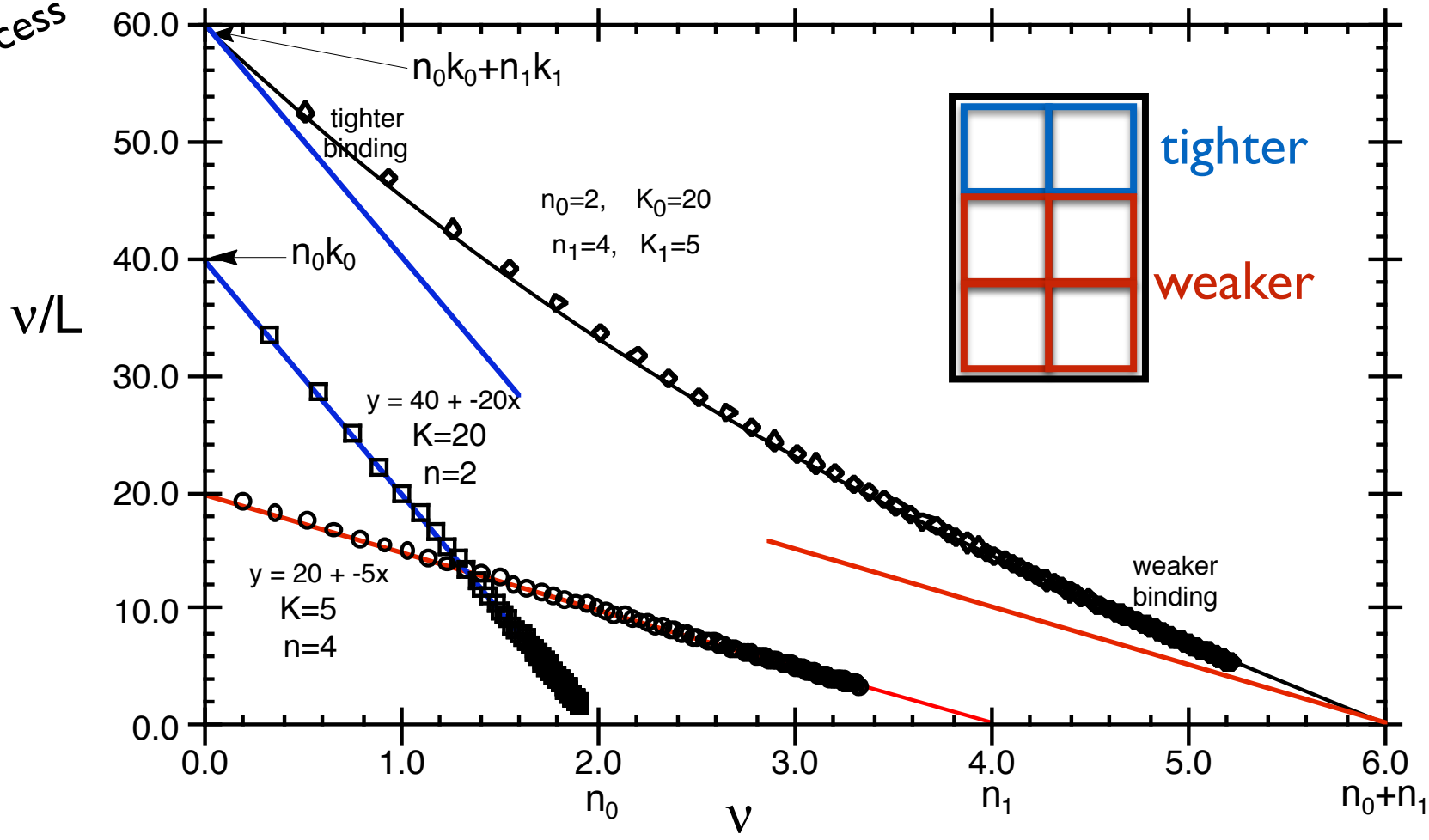


Multiple Classes of Identical, Independent Binding Sites

$$\frac{\bar{v}}{[L]} = \sum_{i=1}^{m} \frac{n_i k_i}{1 + k_i [L]}$$

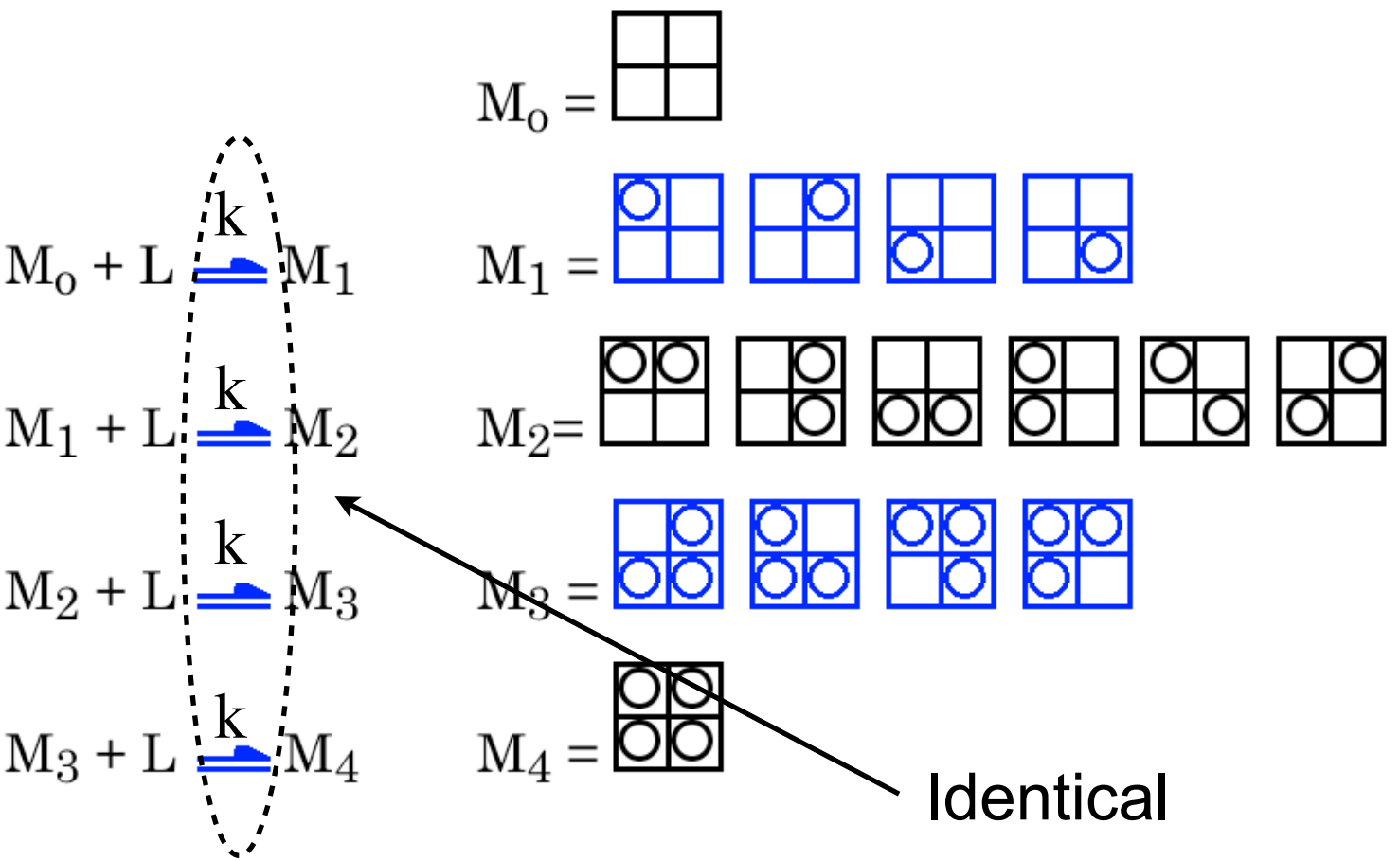
$$\frac{\bar{v}}{[L]} = n_1 k_1 + n_2 k_2 - K_a(\bar{v}) \cdot \bar{v}$$

Assuming L in excess



Multiple, Identical, Independent Binding Sites

$$\bar{v} = \frac{nk[L]}{1 + k[L]}$$



$$\Omega_{n,i} = \frac{4!}{4!0!} = 1$$

$$\Omega_{n,i} = \frac{4!}{3!1!} = 4$$

$$\Omega_{n,i} = \frac{4!}{2!2!} = 6$$

$$\Omega_{n,i} = \frac{4!}{1!3!} = 4$$

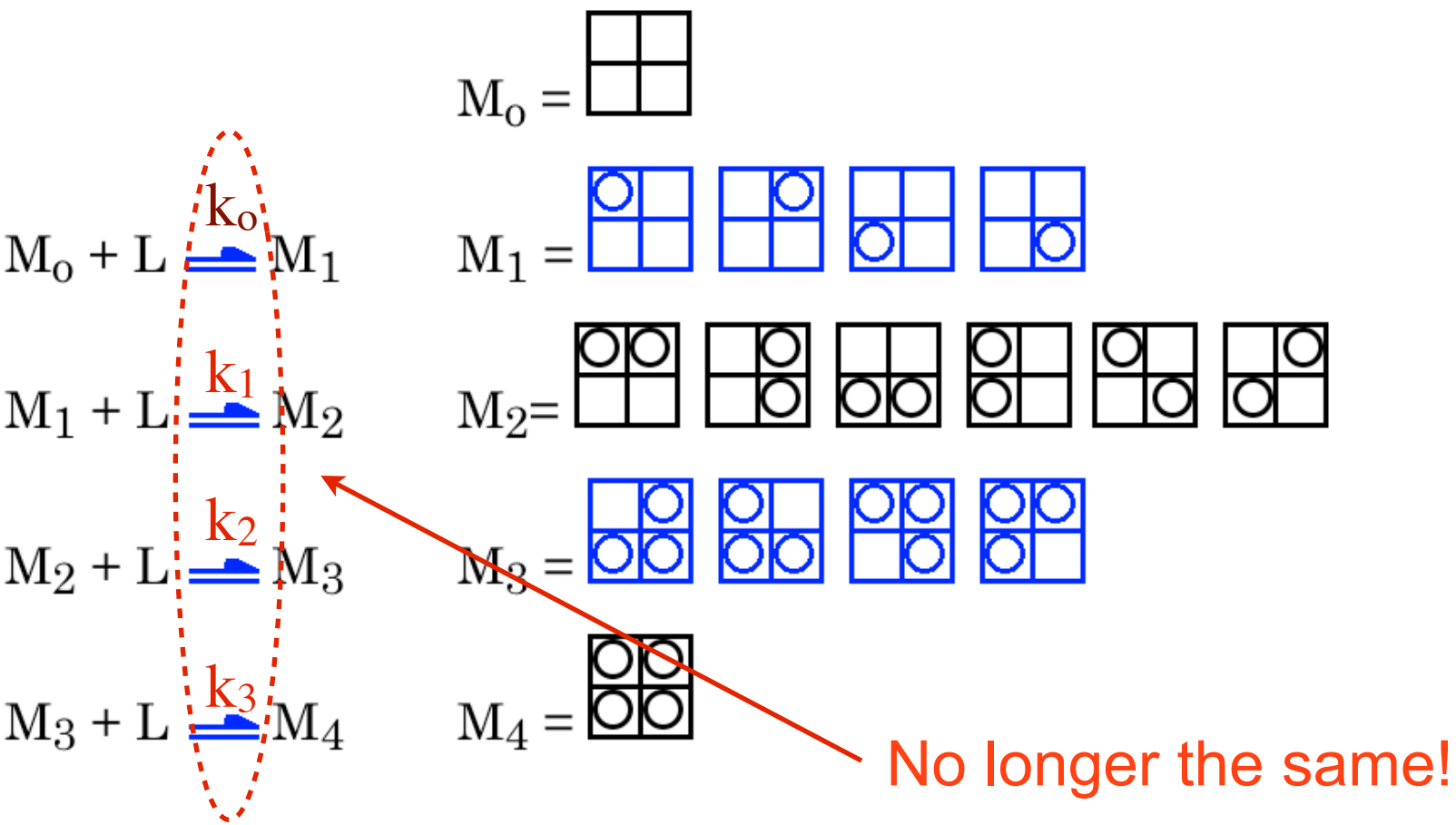
$$\Omega_{n,i} = \frac{4!}{0!4!} = 1$$

Before:

$$\bar{v} = \frac{nk[L]}{1 + k[L]}$$

Multiple Binding Sites

Identical, **Dependent** Sites



Before:

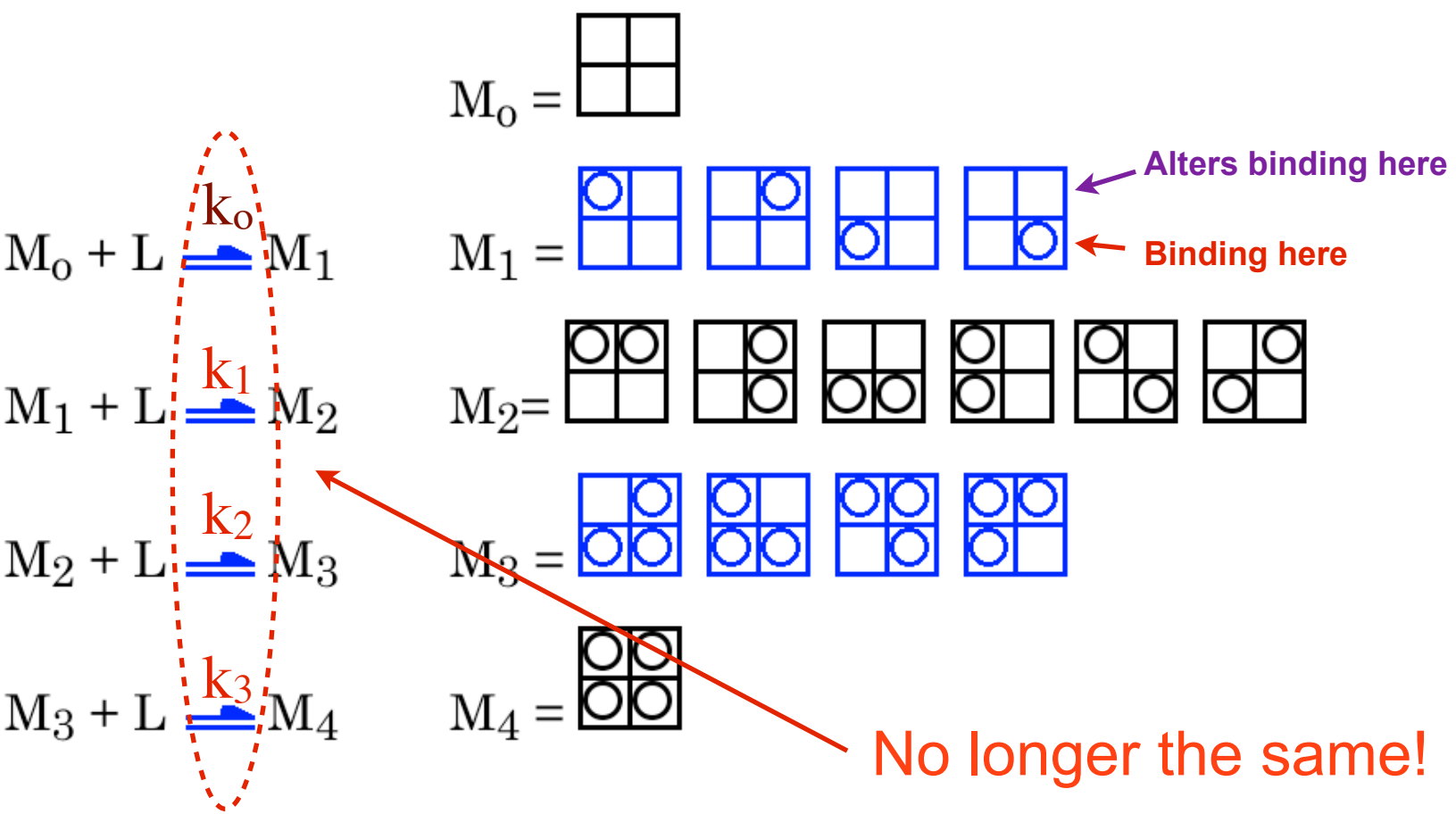
$$\bar{v} = \frac{nk[L]}{1 + k[L]}$$

Multiple Binding Sites

Identical, Dependent Sites

$$\Delta G^o = \Delta G^o_o + RTf_n \quad \text{where} \quad \Delta G^o_o = -\text{RTlnk}_o$$

$$k(n) = e^{-\Delta G^o / RT} = e^{-\left(\Delta G^o_o + RTf_n\right)/RT} = e^{-\Delta G^o_o / RT} e^{-f_n} = k_o e^{-f_n}$$



Multiple Binding Sites
Identical, **Dependent** Sites

Before:

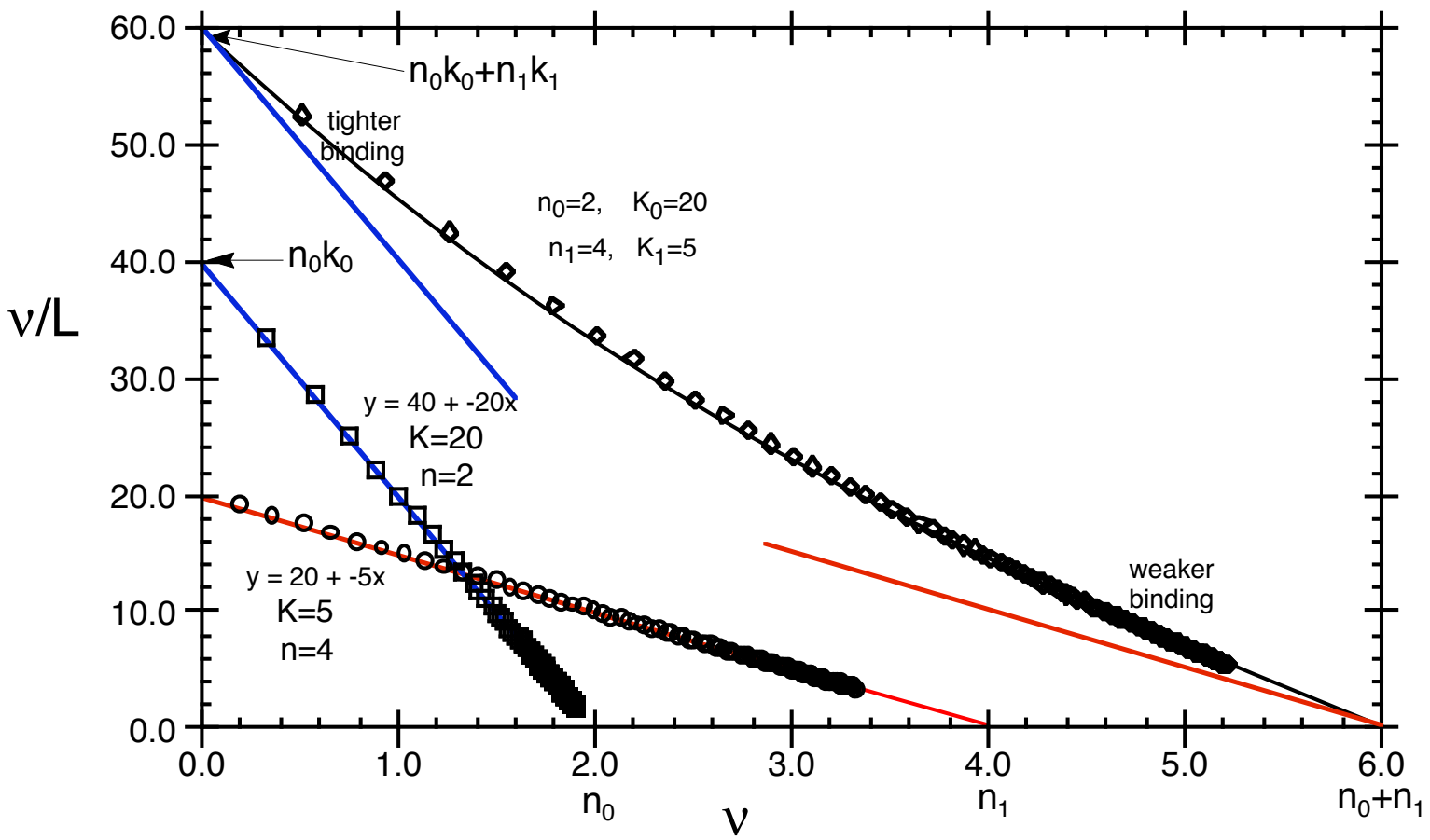
$$\frac{\bar{v}}{[L]} = \sum nK_a - K_a(\bar{v})\cdot\bar{v}$$

$$\Delta G^o = \Delta G_o^o + RTf_n \quad \text{where} \quad \Delta G_o^o = -\text{RTlnk}_o$$

$$k(n) = e^{-\Delta G^o/RT} = e^{-\left(\Delta G_o^o + RTf_n\right)/RT} = e^{-\Delta G_o^o/RT} e^{-f_n} = k_o e^{-f_n}$$

$M_o + L \underset{}{\overset{k_0}{\rightleftharpoons}} M_1$

$M_1 + L \underset{}{\overset{k_1}{\rightleftharpoons}} M_2$



Multiple Binding Sites
Identical, **Dependent** Sites

Before:

$$\frac{\bar{v}}{[L]} = \sum nK_a - K_a(\bar{v})\cdot\bar{v}$$

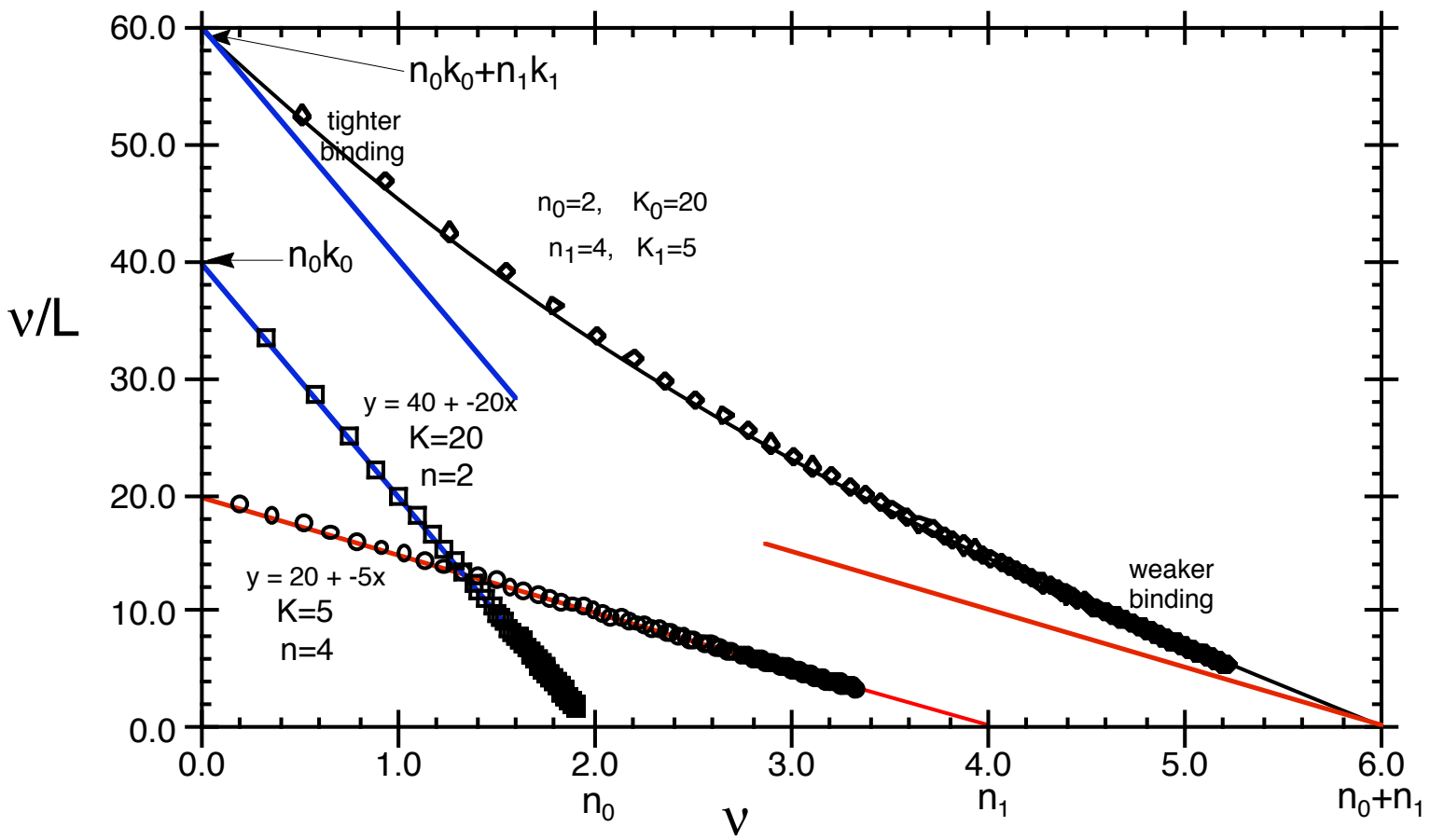
$$\frac{\bar{v}}{[L]} = n_1k_1 + n_2k_2 - K_a(\bar{v})\cdot\bar{v}$$

$$\Delta G^o = \Delta G_o^o + RTf_n \quad \text{where} \quad \Delta G_o^o = -RT\ln k_o$$

$$k(n) = e^{-\Delta G^o/RT} = e^{-\left(\Delta G_o^o + RTf_n\right)/RT} = e^{-\Delta G_o^o/RT}e^{-f_n} = k_o e^{-f_n}$$

$M_o + L \underset{}{\overset{k_0}{\rightleftharpoons}} M_1$

$M_1 + L \underset{}{\overset{k_1}{\rightleftharpoons}} M_2$



Mu[illegible]Binding Sites
Identi[illegible]ependent Sites

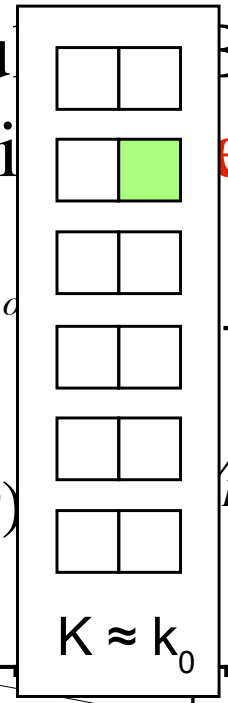
Before:

$$\frac{\bar{v}}{[L]} = \sum nK_a - K_a(\bar{v})\cdot\bar{v}$$

$$\frac{\bar{v}}{[L]} = n_1k_1 + n_2k_2 - K_a(\bar{v})\cdot\bar{v}$$

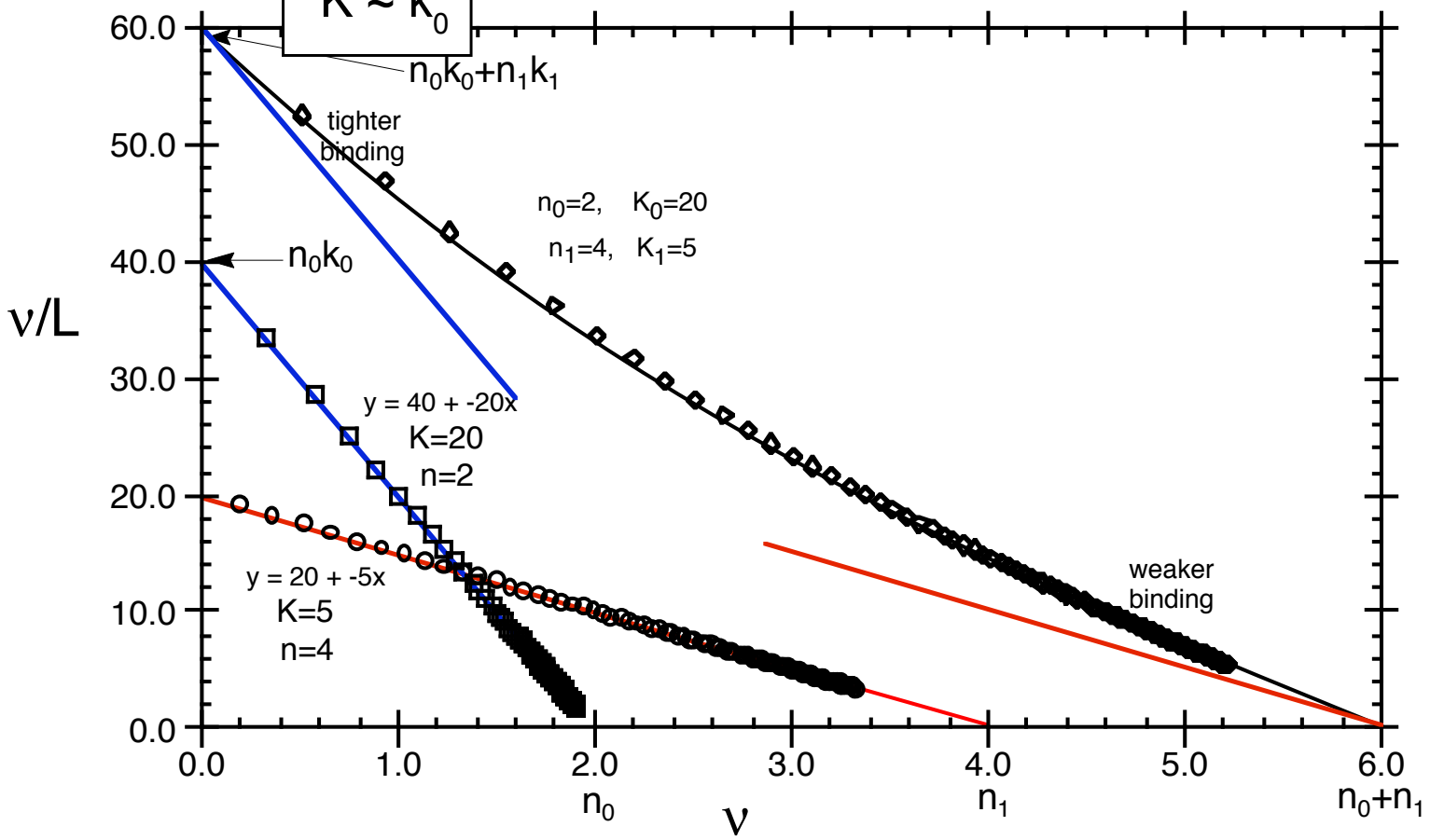
ΔG^{o} [illegible] $+ RTf_n$ where $\Delta G^{o}_{o} = -RT\ln k_o$

$k(n) = e^{-\left(\Delta G^{o}_{o} + RTf_n\right)/RT} = e^{-\Delta G^{o}_{o}/RT} e^{-f_n} = k_o e^{-f_n}$



$M_o + L \underset{}{\overset{k_0}{\rightleftharpoons}} M_1$

$M_1 + L \underset{}{\overset{k_1}{\rightleftharpoons}} M_2$



Mu[illegible]Binding Sites
Identi[illegible]ependent Sites

Before:

$$\frac{\bar{v}}{[L]} = \sum nK_a - K_a(\bar{v})\cdot\bar{v}$$

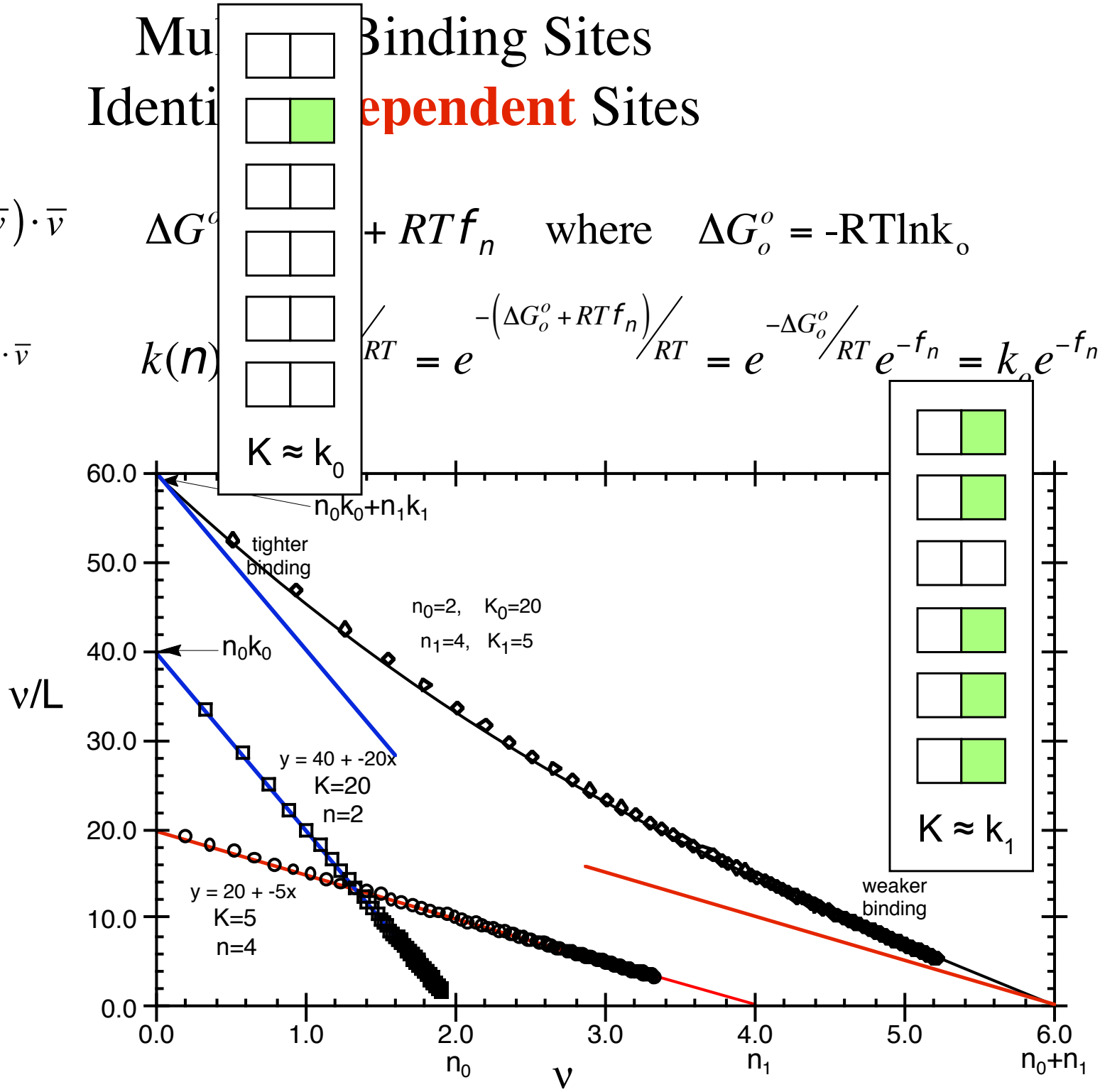
$$\frac{\bar{v}}{[L]} = n_1k_1 + n_2k_2 - K_a(\bar{v})\cdot\bar{v}$$

ΔG^{o} [illegible] $+ RTf_n$ where $\Delta G^{o}_{o} = -RT\ln k_o$

$k(n) = e^{-\left(\Delta G^{o}_{o} + RTf_n\right)/RT} = e^{-\Delta G^{o}_{o}/RT} e^{-f_n} = k_o e^{-f_n}$

$M_o + L \underset{}{\overset{k_0}{\rightleftharpoons}} M_1$

$M_1 + L \underset{}{\overset{k_1}{\rightleftharpoons}} M_2$

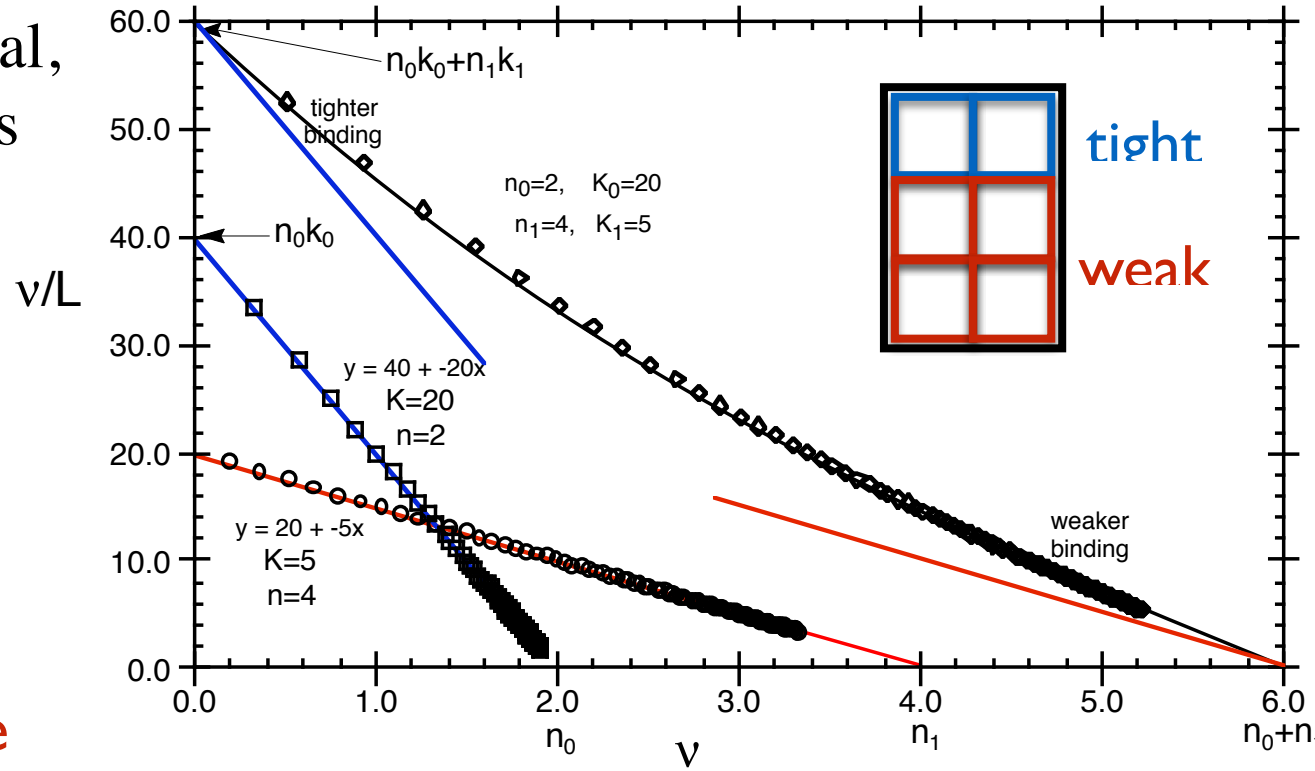


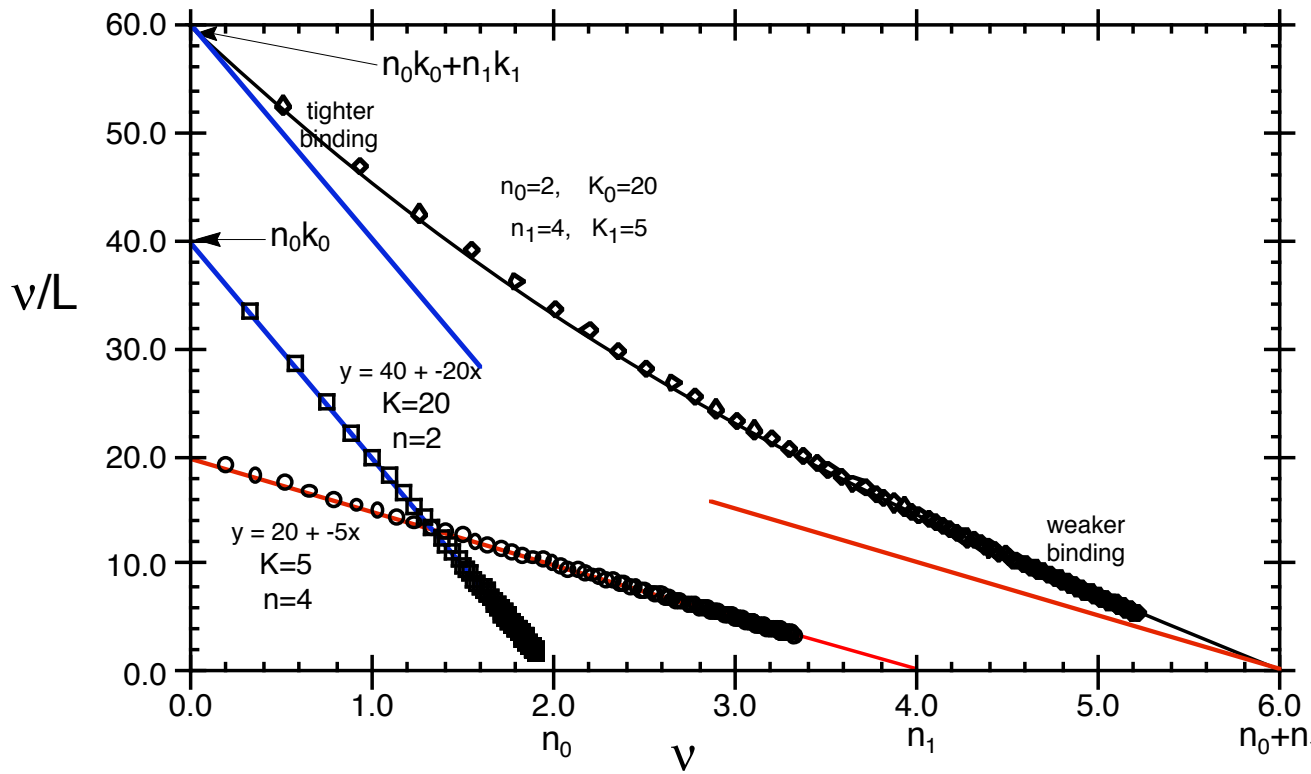
Multiple **Classes** of Identical, Independent Binding Sites

Be Careful!

You can't tell the difference

Multiple Binding Sites Identical, **Dependent** Sites





Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$A_T = [A] + [AB]$$

$$B_T = [B] + [AB]$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB] \longleftarrow A_T = [A] + [AB]$$

$$[B] = B_T - [AB] \longleftarrow B_T = [B] + [AB]$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

$$A_T = [A] + [AB]$$
$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

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$$K_d x = (A_T - x)(B_T - x)$$

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$$K_d x = (A_T - x)(B_T - x)$$

Assume $B_T >> x$

$$K_d x \approx (A_T - x) B_T$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

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$$(B_T + K_d) x \approx A_T B_T$$

Equilibrium Math

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Assume $B_T >> x$

$$K_d x \approx (A_T - x) B_T$$
$$(B_T + K_d) x \approx A_T B_T$$

$$x = [AB] \approx \frac{A_T B_T}{B_T + K_d}$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
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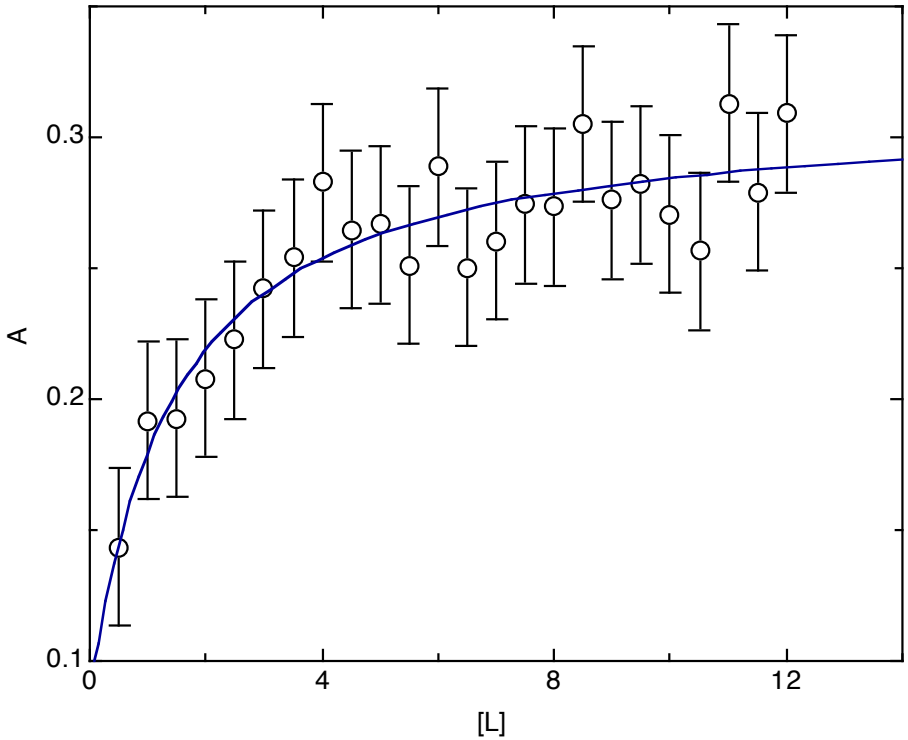
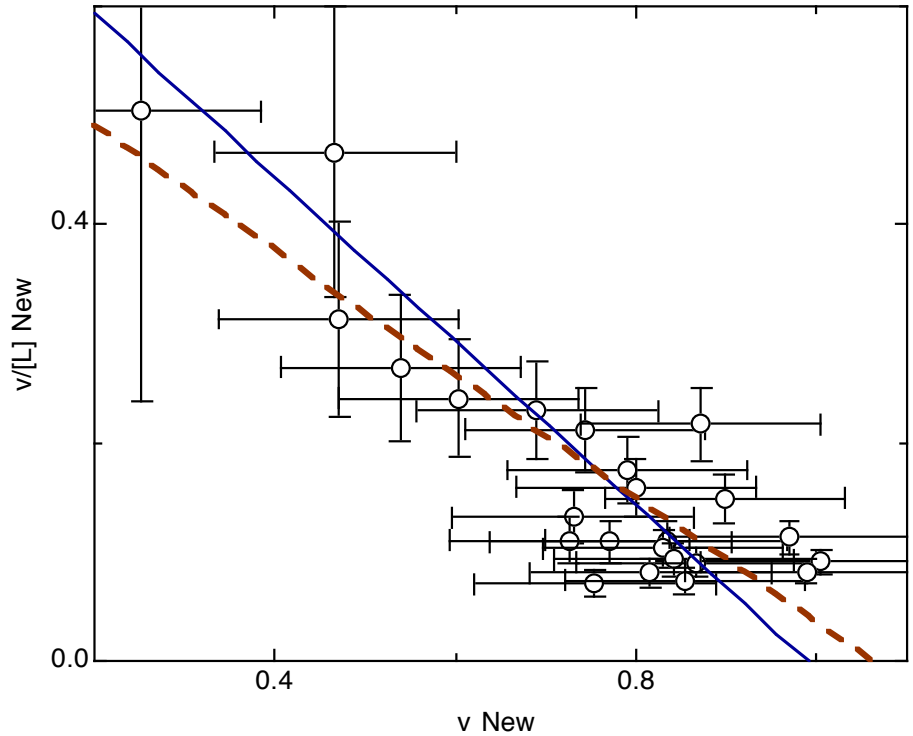
Fraction Bound

$$\frac{[AB]}{A_T} \approx \frac{B_T}{B_T + K_d}$$

$$\nu \approx \frac{B_T}{B_T + K_d}$$





Direct fit

K = 1.35 ± 0.66 μM

A_{bound} = 0.312 ± 0.012

$A_{unbound}$ = 0.087 ± 0.040

Weighted fit

K = 0.75 ± 0.20 μM

n = 0.99

Unweighted fit

K = 0.57 ± 0.07 μM

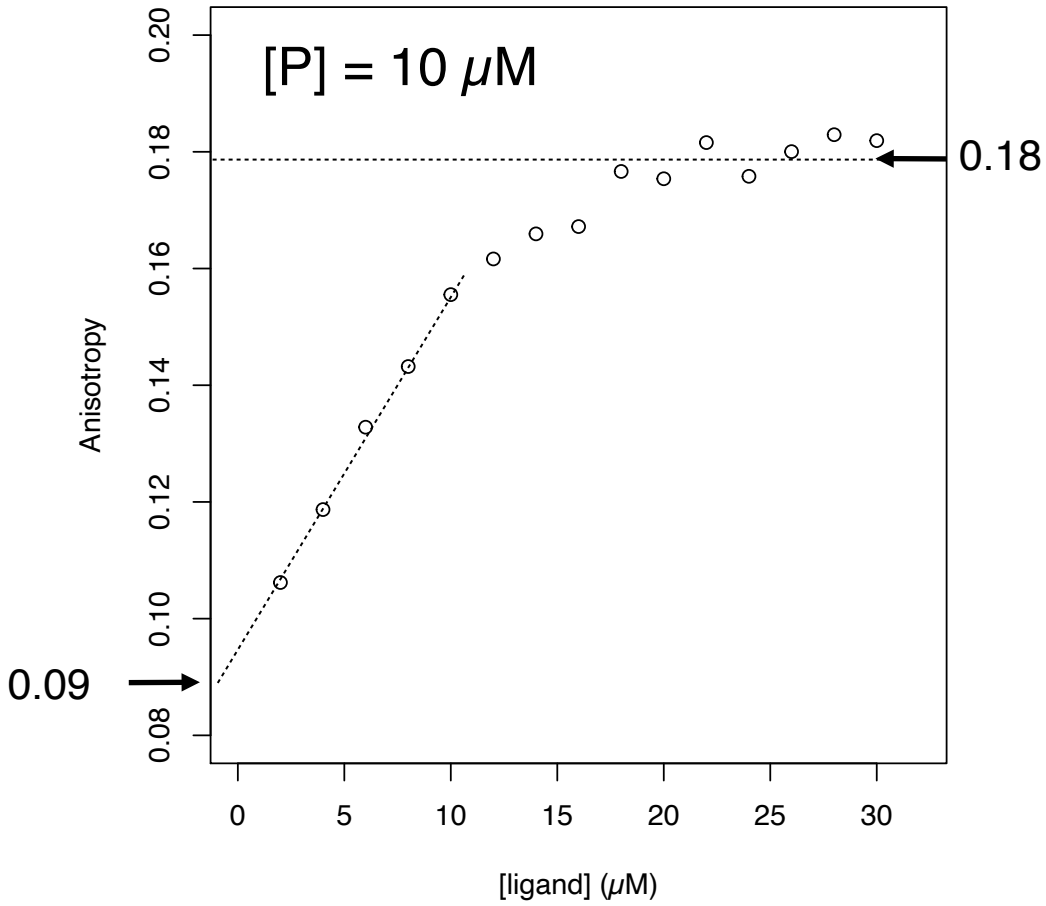
n = 1.06

Fixed

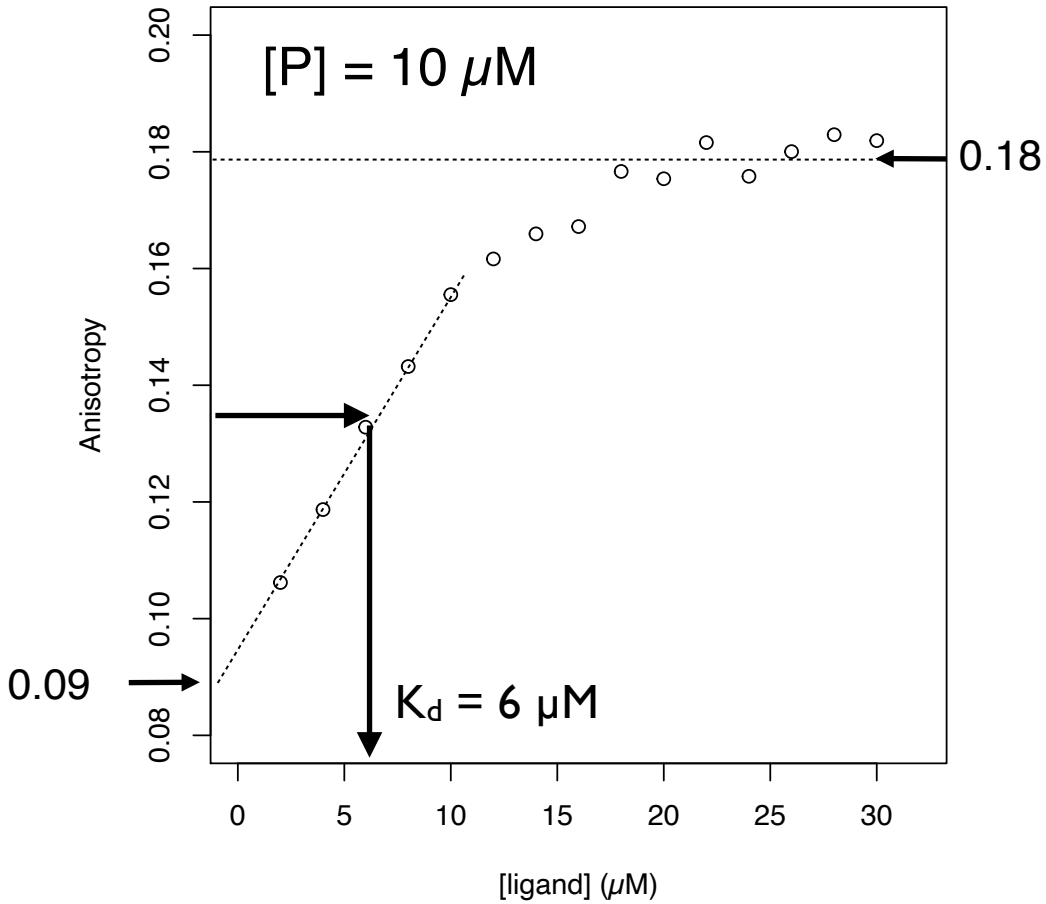
A_{bound} = 0.312

$A_{unbound}$ = 0.087

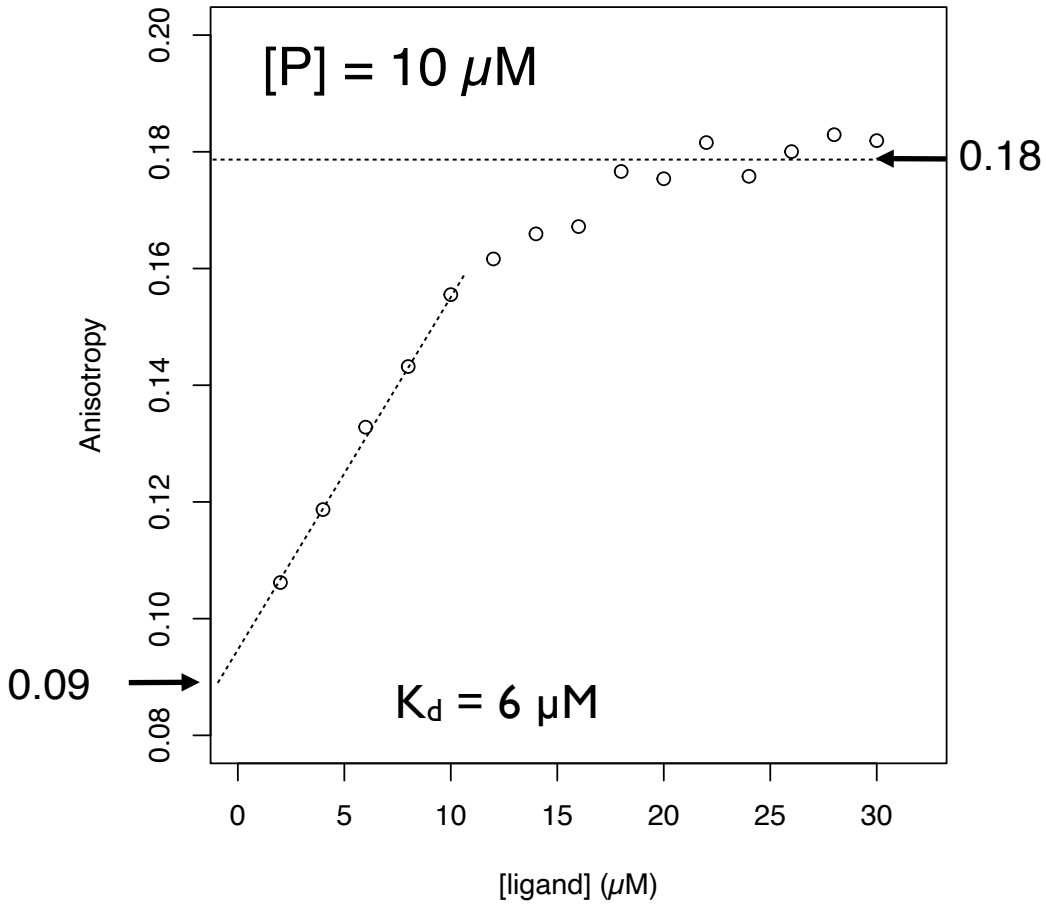
Fluorescence Anisotropy Titration



Fluorescence Anisotropy Titration



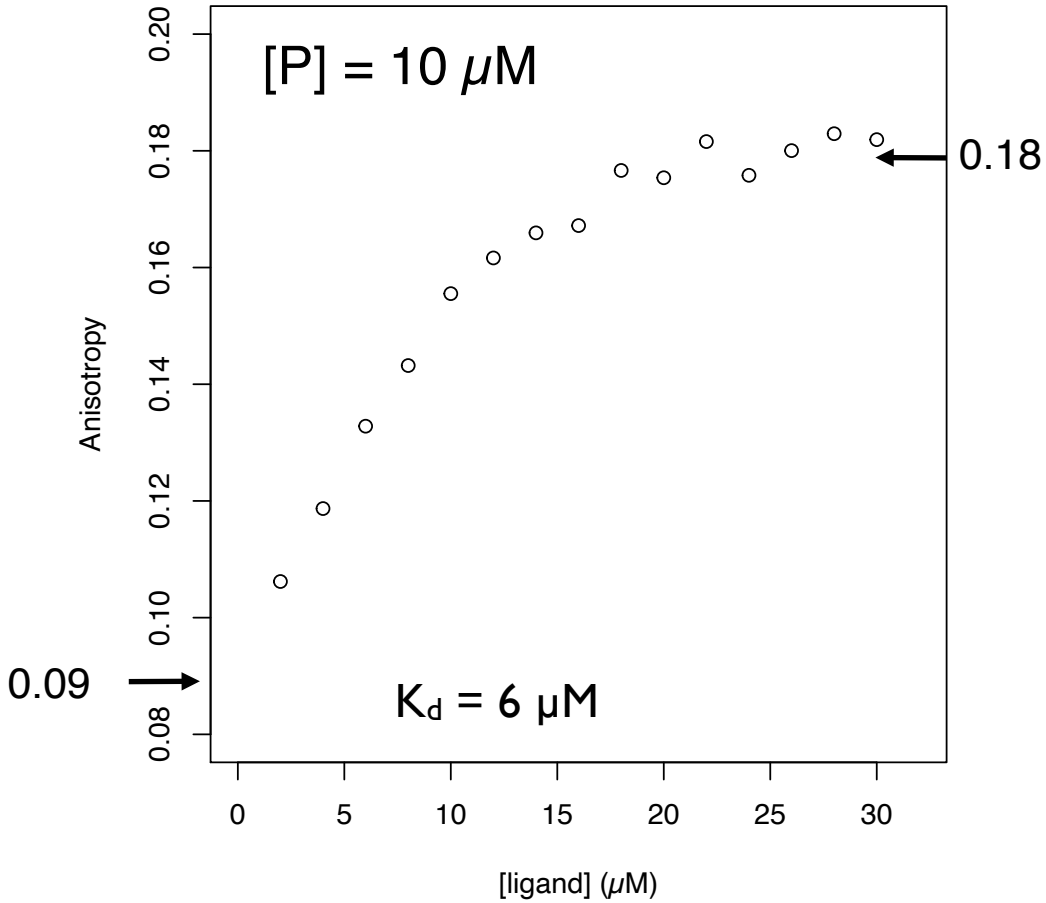
Fluorescence Anisotropy Titration

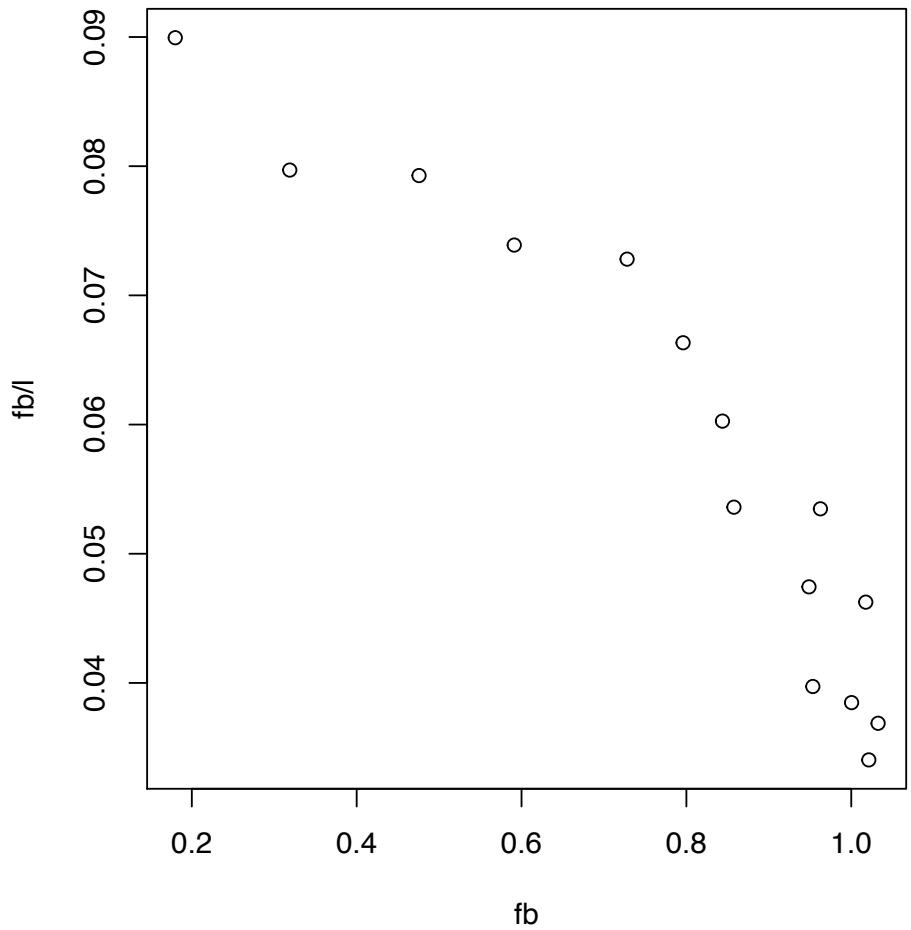


- Scatchard Analysis
  - Pick beginning and end values

- Scatchard Analysis
  - Calculate v & v/[L]
  - Plot v/[L] vs v

Fluorescence Anisotropy Titration





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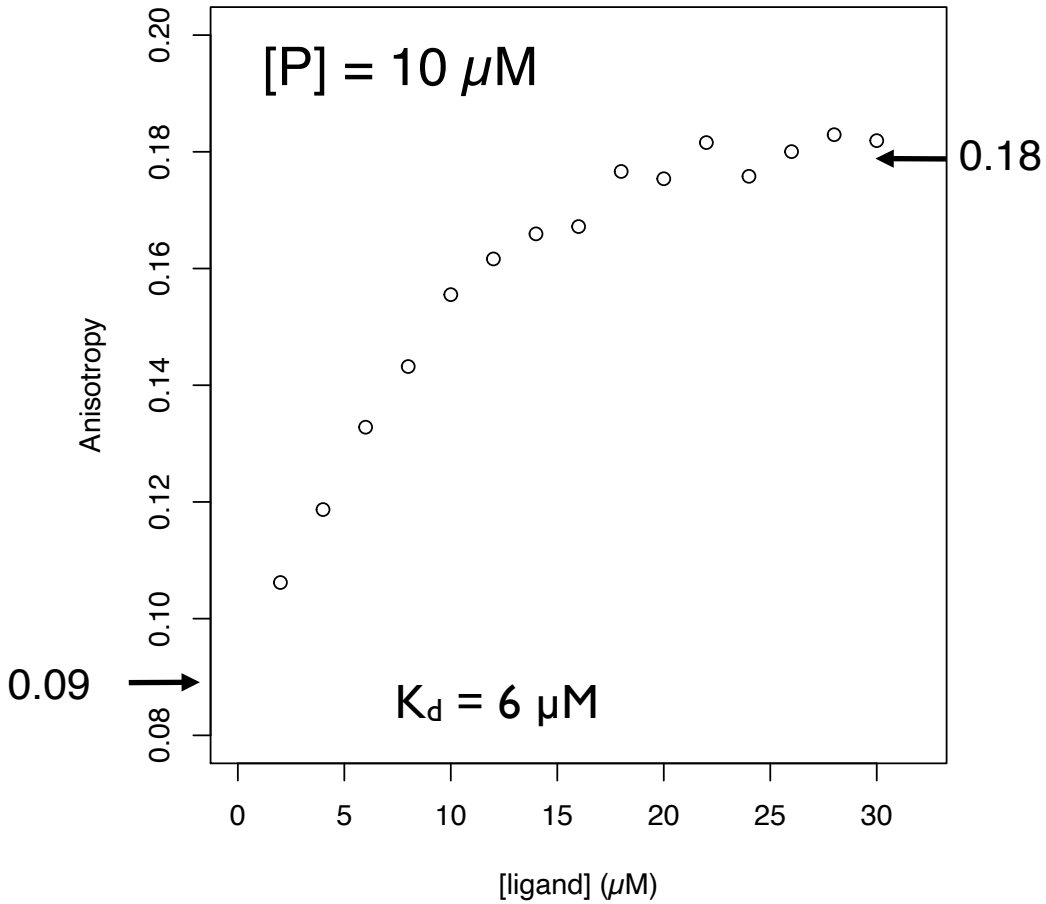
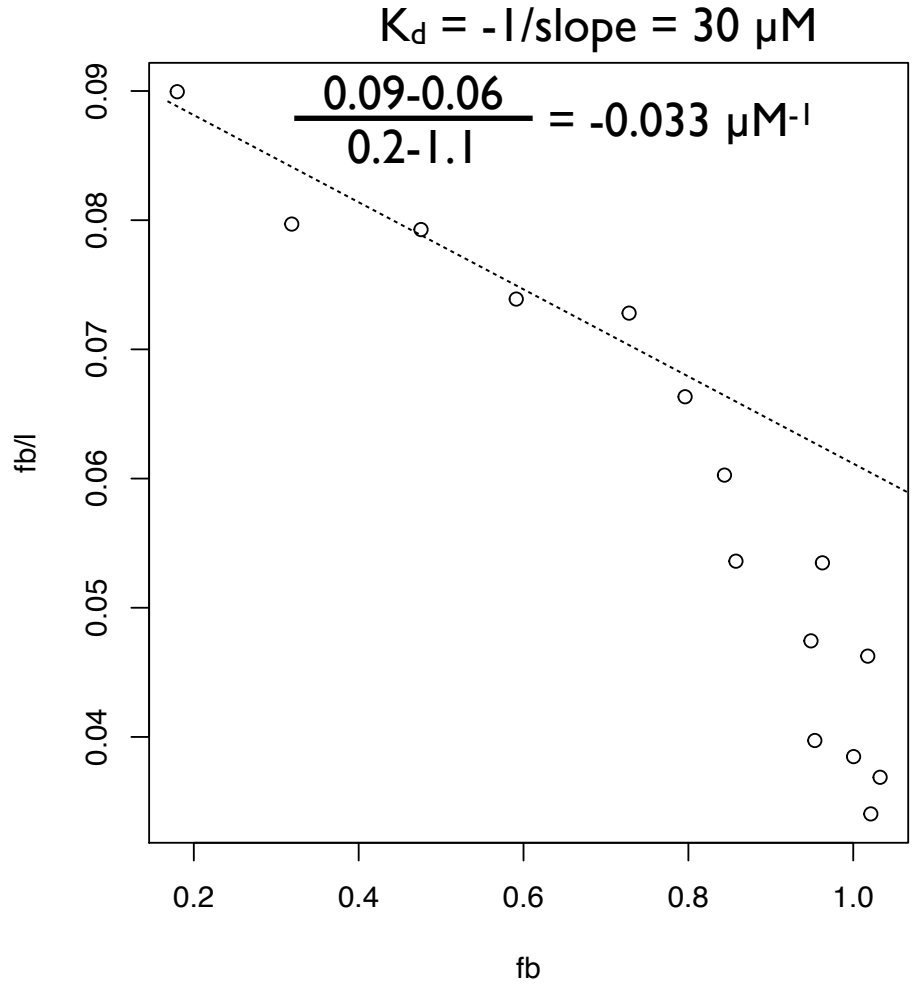
Fluorescence Anisotropy Titration





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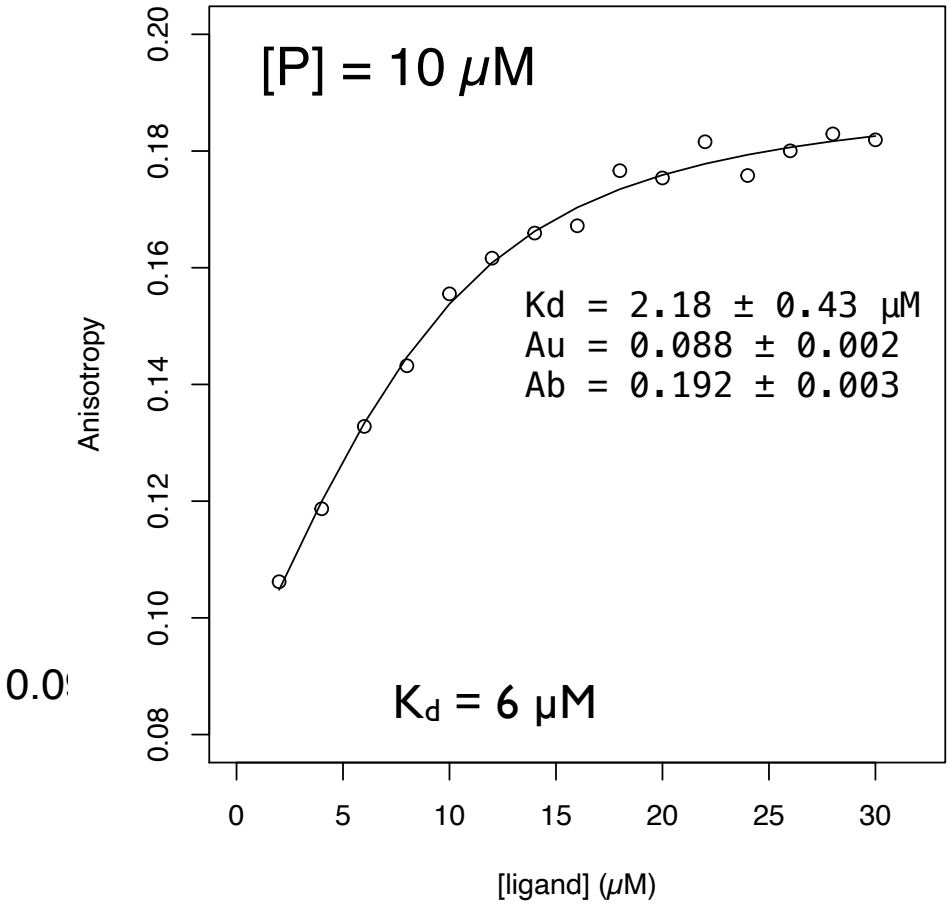
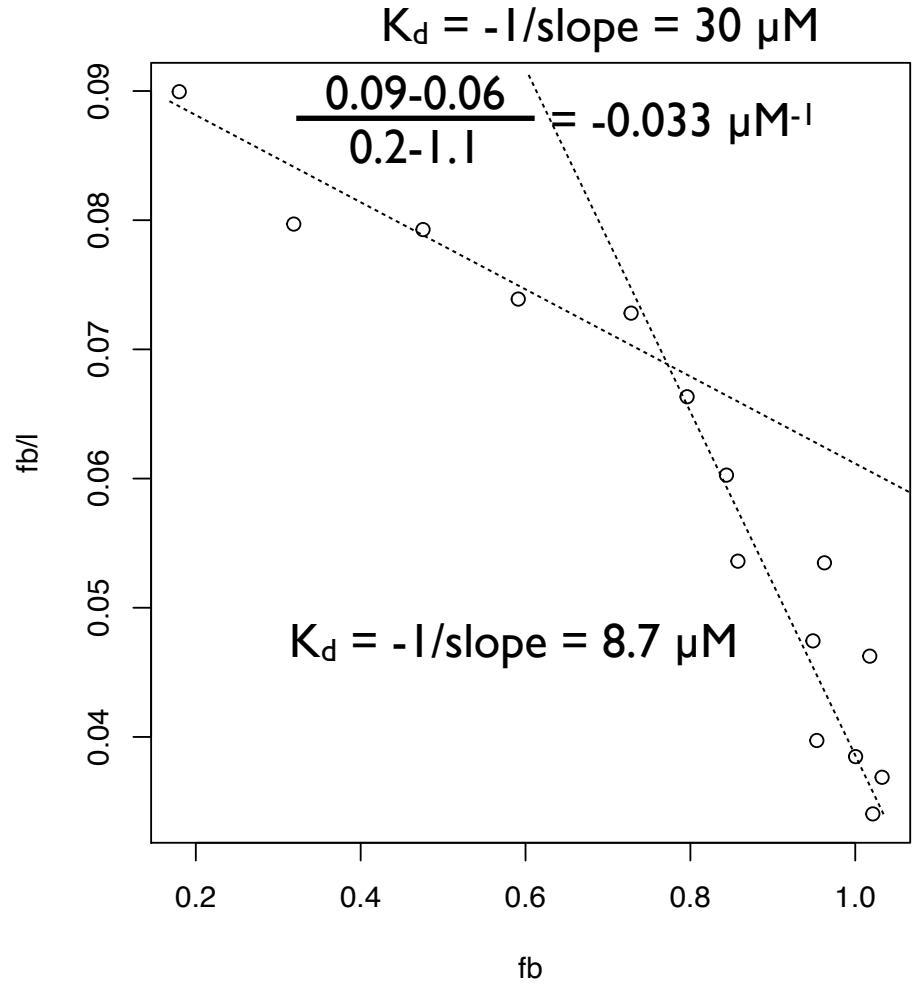
Fluorescence Anisotropy Titration





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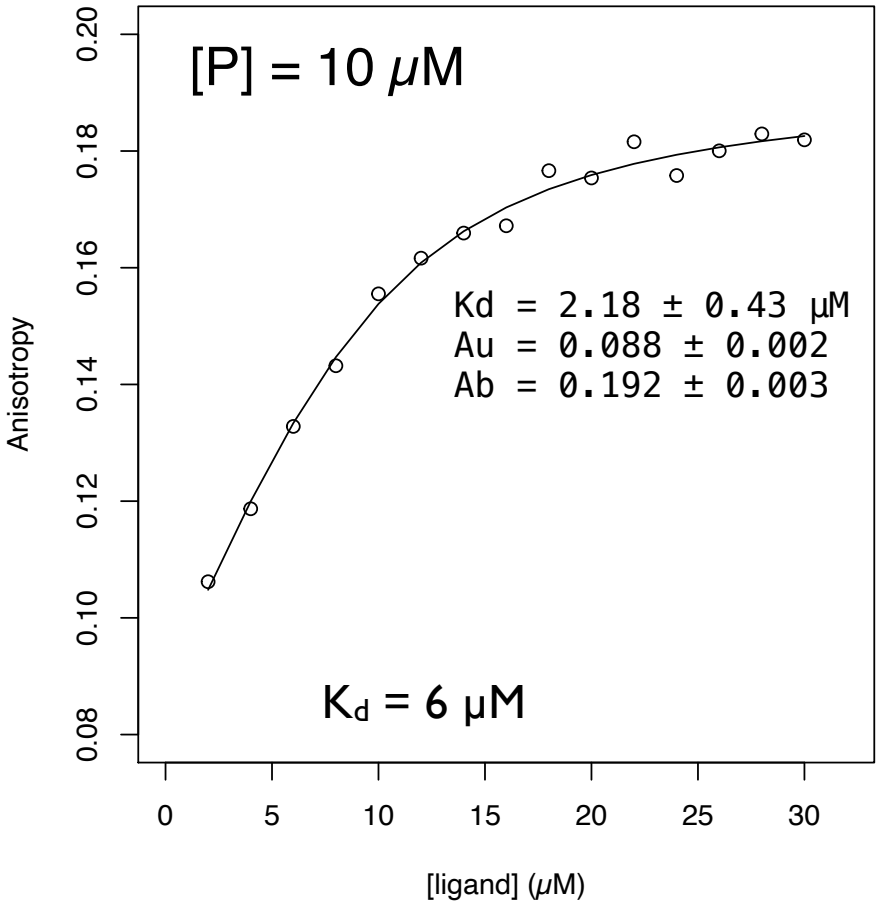
Fluorescence Anisotropy Titration



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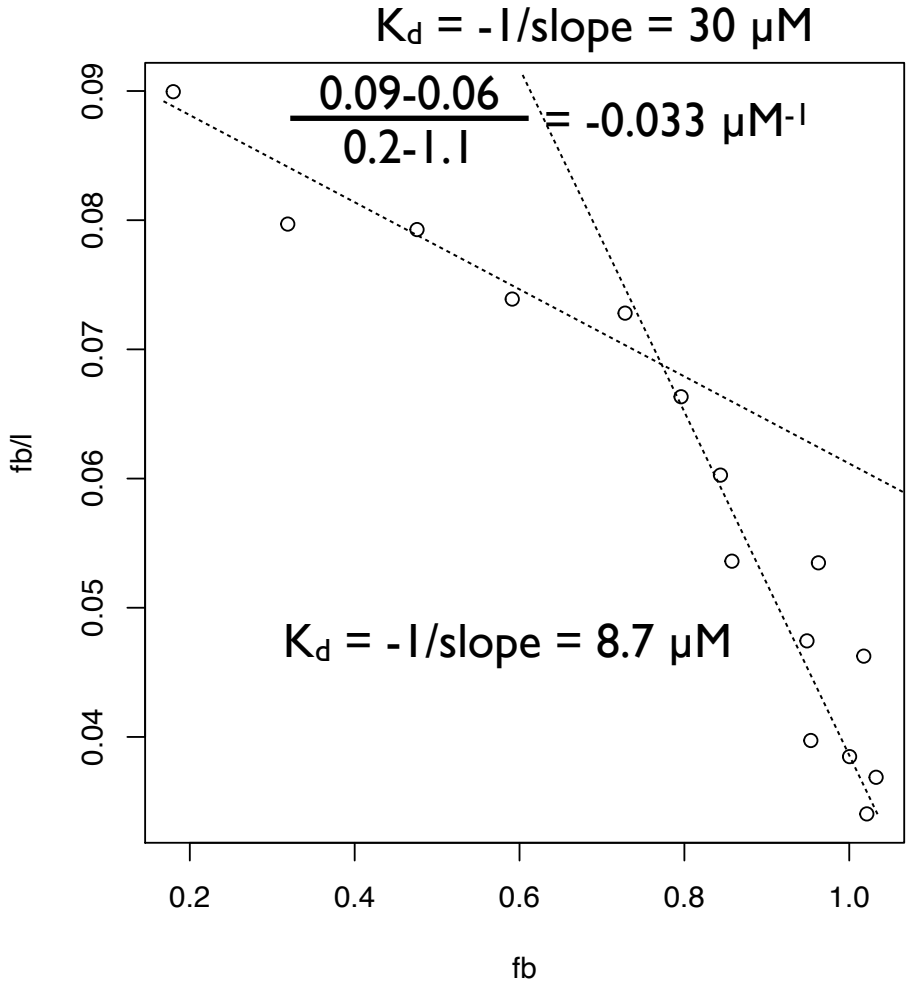

Why did the linearized approaches fail miserably?

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

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$$[A] = A_T - [AB]$$
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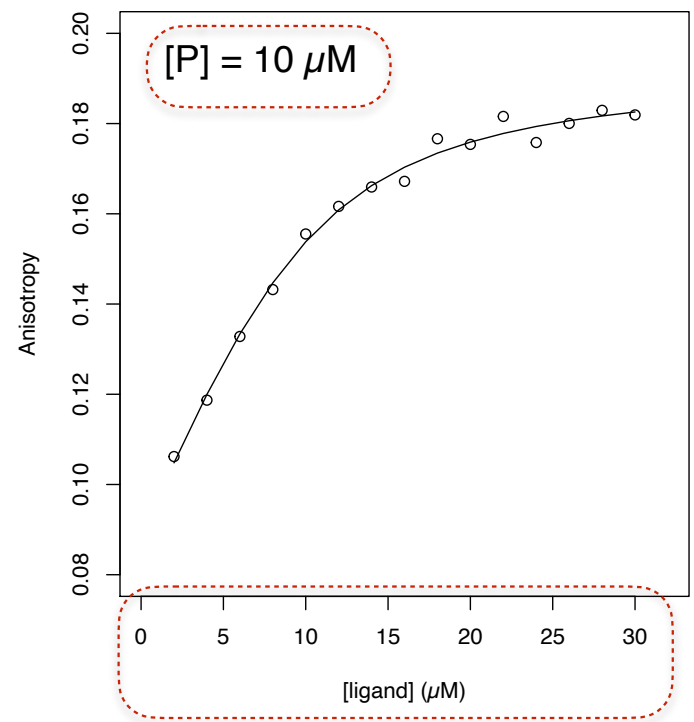
~~Assume $B_T >> x$~~



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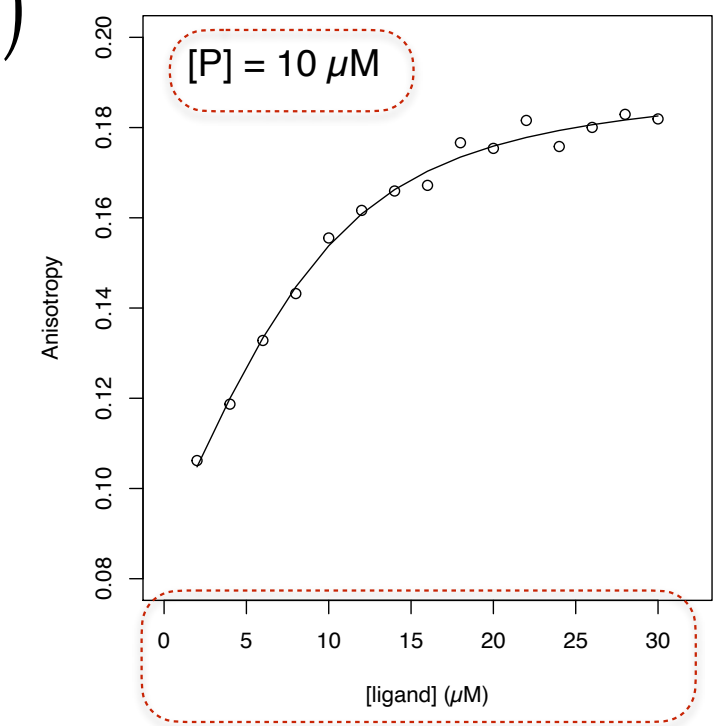
$$K_d x = (A_T - x)(B_T - x)$$

~~Assume $B_T >> x$~~

$$x^2 - (A_T + B_T + K_d)x + A_T B_T = 0$$

$$ax^2 + bx + c = 0 \qquad x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$



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$$x = \frac{(A_T + B_T + K_d) - \sqrt{(A_T + B_T + K_d)^2 - 4A_T B_T}}{2} = [AB]$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

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$$x = \frac{(A_T + B_T + K_d) - \sqrt{(A_T + B_T + K_d)^2 - 4A_T B_T}}{2} = [AB]$$

Fraction Bound

$$\frac{[AB]}{A_T}$$

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

$$x = \frac{\left(A_T + B_T + K_d\right) - \sqrt{\left(A_T + B_T + K_d\right)^2 - 4A_T B_T}}{2} = \left[AB\right]$$

Fraction Bound

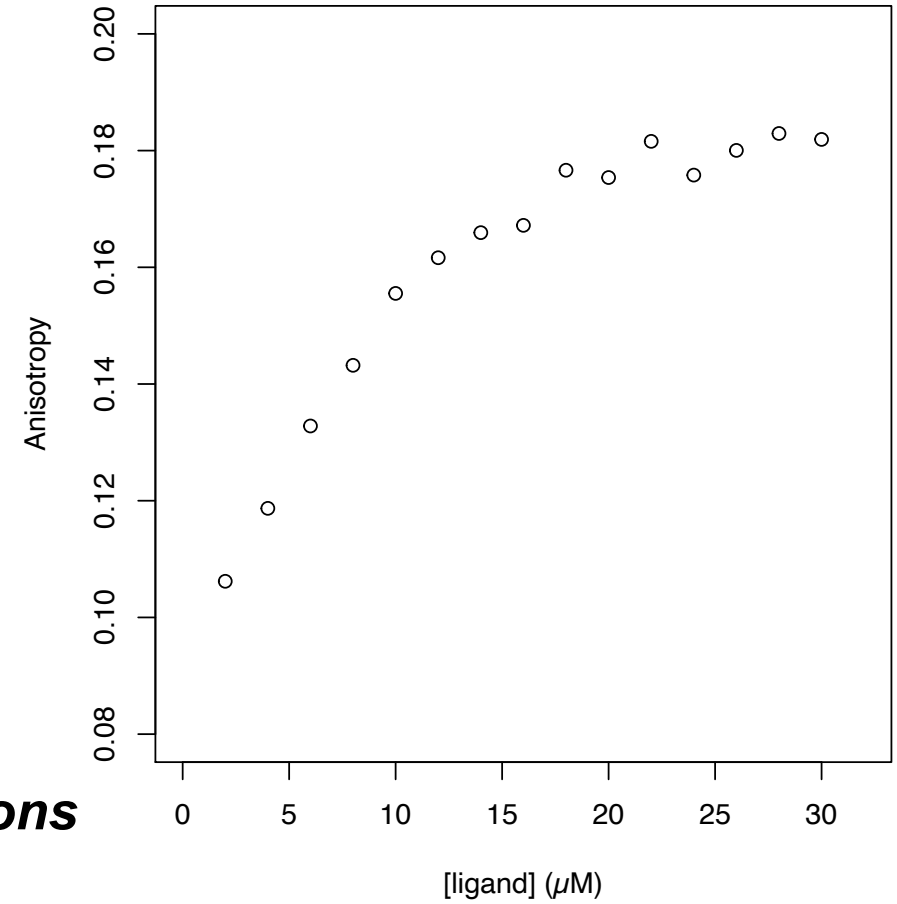
$$f = \frac{\left[AB\right]}{A_T} = \frac{\left(A_T + B_T + K_d\right) - \sqrt{\left(A_T + B_T + K_d\right)^2 - 4A_T B_T}}{2A_T}$$

At half-saturation, f=0.5

$$0.5 = \frac{\left[AB\right]}{A_T} = \frac{\left(A_T + B_T + K_d\right) - \sqrt{\left(A_T + B_T + K_d\right)^2 - 4A_T B_T}}{2A_T}$$

Equilibrium Math

$$P + L \underset{}{\overset{K_d}{\rightleftharpoons}} PL$$



Assume L >> P

Fraction Bound

$$f \approx \frac{1}{1 + \frac{K_d}{L_T}}$$

At half-saturation, f=0.5

$$L_T = K_d$$

No assumptions

Fraction Bound

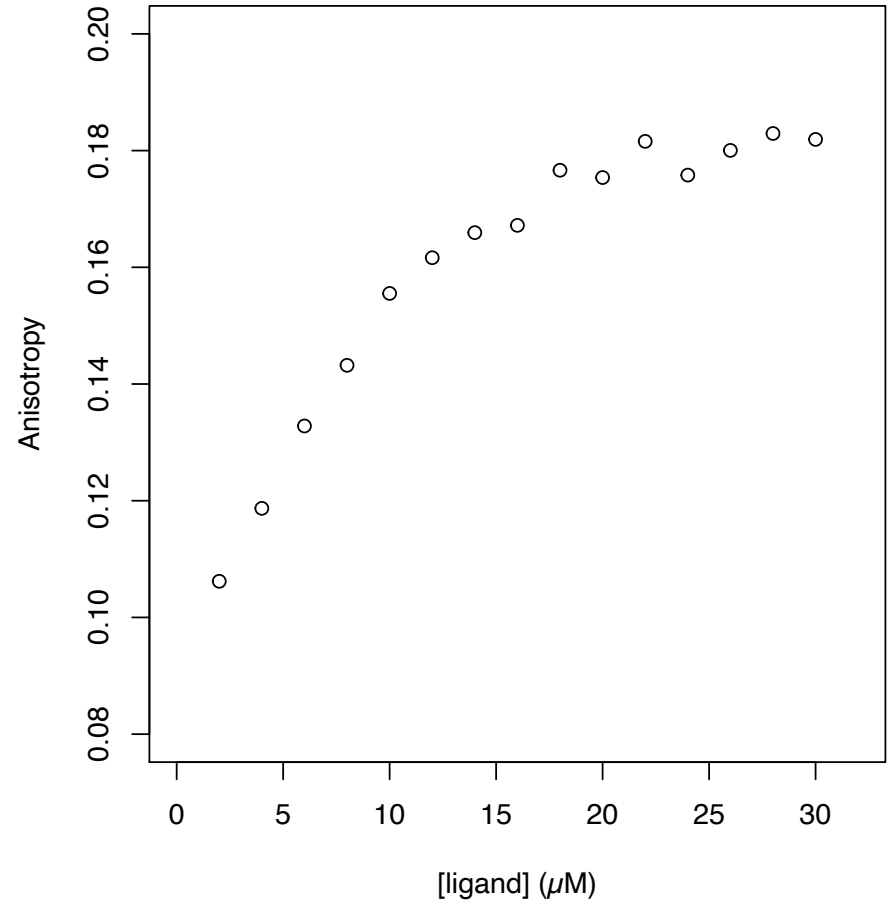
$$f = \frac{[PL]}{P_T} = \frac{\left(P_T + L_T + K_d\right) - \sqrt{\left(P_T + L_T + K_d\right)^2 - 4P_T L_T}}{2P_T}$$

At half-saturation, f=0.5

$$L_T = \frac{1}{2}P_T + K_d$$

Equilibrium Math

$$P + L \underset{}{\overset{K_d}{\rightleftharpoons}} PL$$



$$f = \frac{[PL]}{P_T} = \frac{(P_T + L_T + K_d) - \sqrt{(P_T + L_T + K_d)^2 - 4P_T L_T}}{2P_T}$$

(Au + (Ab-Au)*((Kd+Pt+Lt) - sqrt[(Kd+Pt+Lt)^2 - 4*Pt*Lt] / (2*Pt)))

$$A = A_u + f(A_b - A_u)$$

```
LigBndGen <- function(Lt, Kd, Pt, Au, Ab) (Au + (Ab-Au)*(((Kd+Pt+Lt)-sqrt((Kd+Pt+Lt)^2-4*Pt*Lt))/2)/Pt )

model <- nls(Anis ~ LigBndGen(l,myKd,10,myAu,myAb), start=list(myKd=4,myAu=0.15,myAb=0.35))
```

Equilibrium Math

[P] = 10 μM

$$P + L \underset{}{\overset{K_d}{\rightleftharpoons}} PL$$

Assume L >> P

Fraction Bound

$$f \approx \frac{1}{1 + \frac{K_d}{L_T}}$$

At half-saturation, f=0.5

$$L_T = K_d$$

No assumptions

Fraction Bound

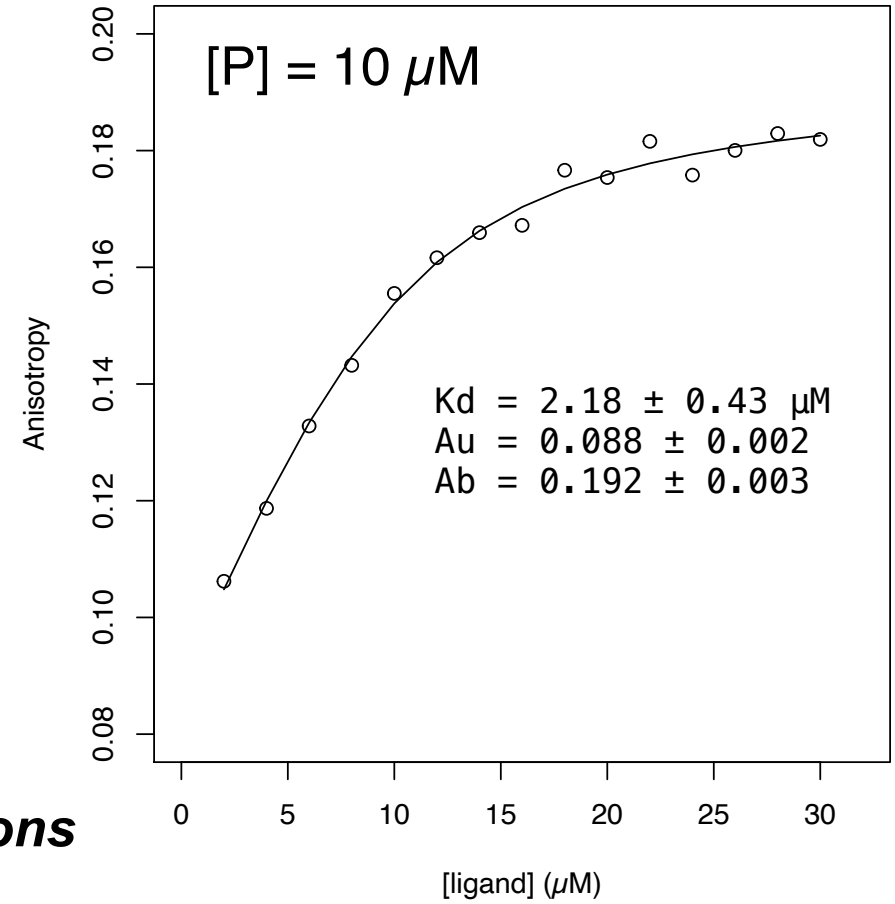
$$f = \frac{[PL]}{P_T} = \frac{\left(P_T + L_T + K_d\right) - \sqrt{\left(P_T + L_T + K_d\right)^2 - 4P_T L_T}}{2P_T}$$

At half-saturation, f=0.5

$$L_T = \frac{1}{2}P_T + K_d$$

Equilibrium Math

$$P + L \underset{}{\overset{K_d}{\rightleftharpoons}} PL$$



Assume L >> P

Fraction Bound

$$f \approx \frac{1}{1 + \frac{K_d}{L_T}}$$

At half-saturation, f=0.5

$$L_T = K_d$$

No assumptions

Fraction Bound

$$f = \frac{[PL]}{P_T} = \frac{\left(P_T + L_T + K_d\right) - \sqrt{\left(P_T + L_T + K_d\right)^2 - 4P_T L_T}}{2P_T}$$

At half-saturation, f=0.5

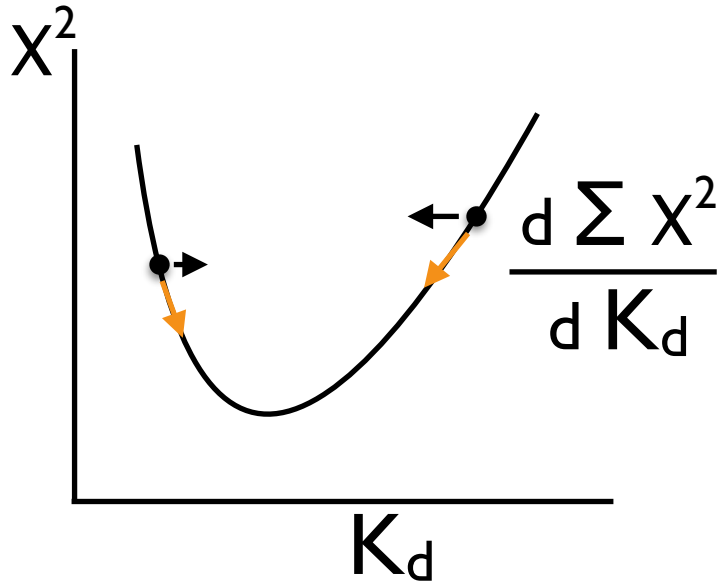
$$L_T = \frac{1}{2}P_T + K_d$$

Levenberg - Marqardt

The Levenberg-Marquardt algorithm

The Levenberg-Marquardt algorithm is derived directly from the mean square deviation expressions (8) or (10) and cannot be used with deviation functions *R* other then the square deviation *R*(*d*) = *d*2.

The Levenberg-Marquardt algorithm is a very fast fitting algorithm. Its performance, however, depends strongly on the behavior of the function to be fitted as well as on the selected starting parameters.



The classical version of the Levenberg-Marquardt algorithm does not allow for x-errors and minimizes the mean square deviation (10). The algorithm can be described in words as follows:

Starting from a given set of parameters, the mean square deviation X^2 is calculated. Then **the parameters are varied slightly to observe their influence on X^2**. From this, **the direction in which X^2 decreases most rapidly** can be evaluated and a new set of parameters is chosen. This procedure is reiterated with this new set of parameters . When the minimum is near, the algorithm goes over to a more deterministic “guessing” at the position of the minimum and solves some equations to find it. The fitting stops when the value of X^2 does not decrease anymore between successive steps.

The algorithm is described in W.H. Press, B.P. Flannery, S.A. Teukolsky, W.T. Vetterling, *Numerical Recipes - the Art of Scientific Computing*, Second Edition, University Press, Cambridge, 1992. When x-errors are specified, the algorithm is modified in such a way that it minimizes (8). It finds at the same time the set of x-coordinates $x\hat{}\, i$ and the function parameters that minimize the mean square deviations between the data points $(xi\,, yi\,)$ and the function values $(x\hat{}\, i\,, f(x\hat{}\, i\,))$.

Levenberg - Marqardt

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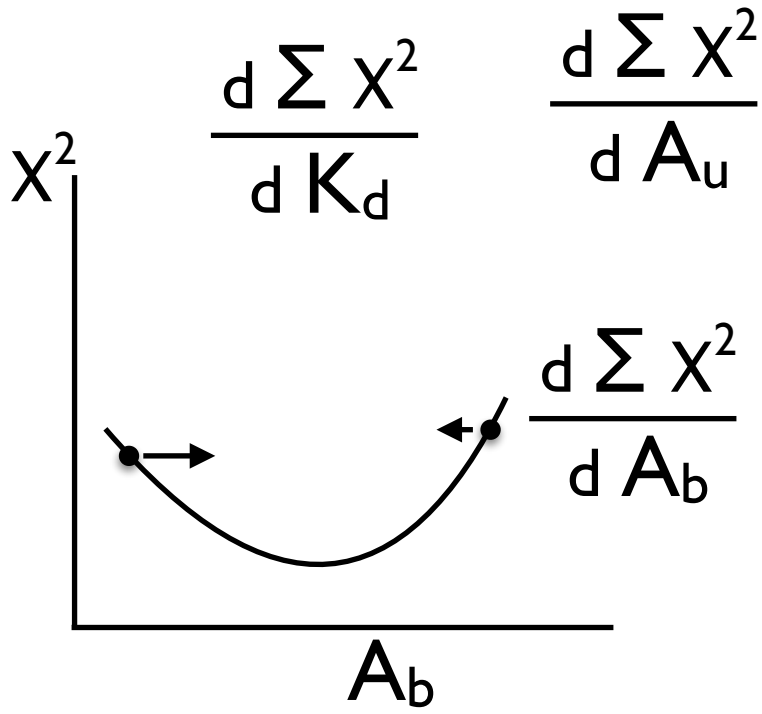


The classical version of the Levenberg-Marquardt algorithm does not allow for x-errors and minimizes the mean square deviation (10). The algorithm can be described in words as follows:

Starting from a given set of parameters, the mean square deviation X^2 is calculated. Then **the parameters are varied slightly to observe their influence on X^2**. From this, **the direction in which X^2 decreases most rapidly** can be evaluated and a new set of parameters is chosen. This procedure is reiterated with this new set of parameters . When the minimum is near, the algorithm goes over to a more deterministic “guessing” at the position of the minimum and solves some equations to find it. The fitting stops when the value of X^2 does not decrease anymore between successive steps.

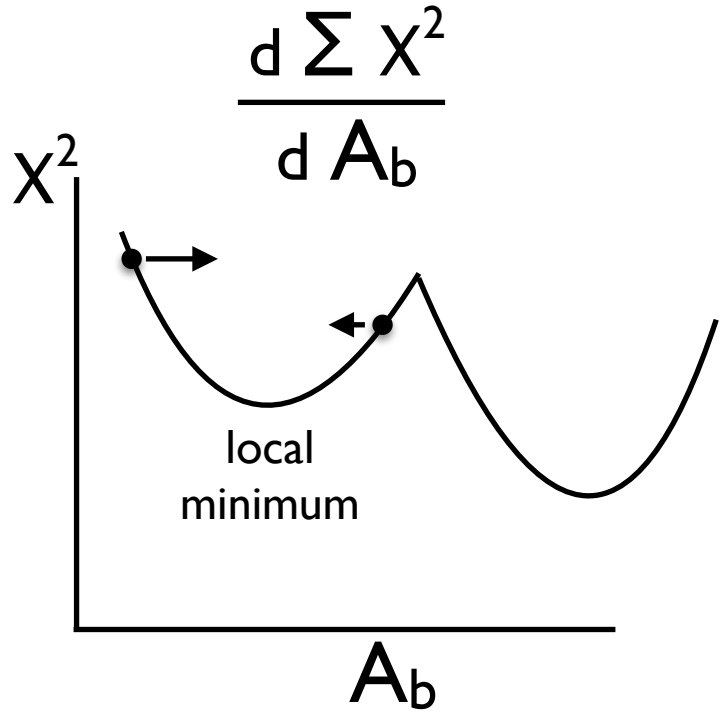
The algorithm is described in W.H. Press, B.P. Flannery, S.A. Teukolsky, W.T. Vetterling, *Numerical Recipes - the Art of Scientific Computing*, Second Edition, University Press, Cambridge, 1992. When x-errors are specified, the algorithm is modified in such a way that it minimizes (8). It finds at the same time the set of x-coordinates $x\hat{}\, i$ and the function parameters that minimize the mean square deviations between the data points $(xi\,, yi\,)$ and the function values $(x\hat{}\, i\,, f(x\hat{}\, i\,))$.

Levenberg - Marqardt

The Levenberg-Marquardt algorithm

The Levenberg-Marquardt algorithm is derived directly from the mean square deviation expressions (8) or (10) and cannot be used with deviation functions *R* other then the square deviation *R*(*d*) = *d*2.

The Levenberg-Marquardt algorithm is a very fast fitting algorithm. Its performance, however, depends strongly on the behavior of the function to be fitted as well as on the selected starting parameters.



The classical version of the Levenberg-Marquardt algorithm does not allow for x-errors and minimizes the mean square deviation (10). The algorithm can be described in words as follows:

Starting from a given set of parameters, the mean square deviation X^2 is calculated. Then **the parameters are varied slightly to observe their influence on X^2**. From this, t**he direction in which X^2 decreases most rapidly** can be evaluated and a new set of parameters is chosen. This procedure is reiterated with this new set of parameters . When the minimum is near, the algorithm goes over to a more deterministic “guessing” at the position of the minimum and solves some equations to find it. The fitting stops when the value of X^2 does not decrease anymore between successive steps.

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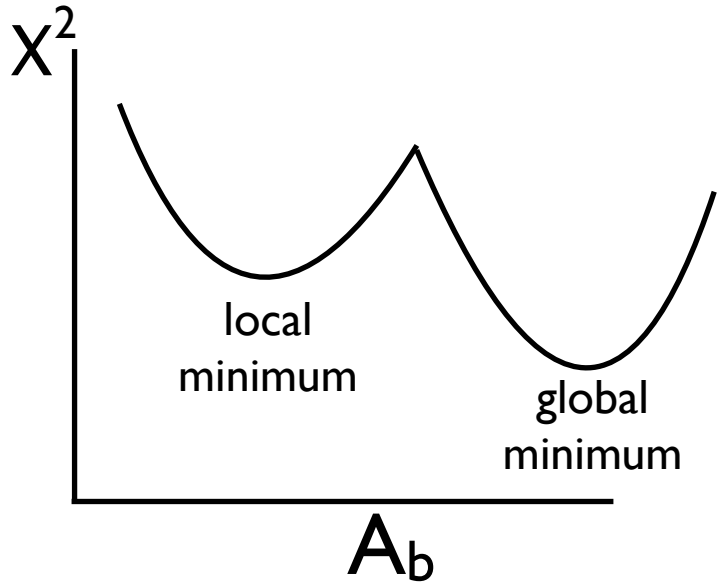
Monte Carlo

The Monte Carlo algorithm
This method randomly varies the parameters of a function within given intervals. When x-errors are defined, the algorithm also varies randomly the set of x-coordinates $x\hat{}\,i$ while observing the given errors and error distributions.



For each random guess, χR is calculated according to Eq. (2) and the parameter sets corresponding to the smallest values of χR are remembered.

The strength of this method is also its biggest disadvantage. It looks for the best parameter set by shooting blindly inside the given region of parameter space. Although there is an option of letting this parameter space region follow the position of the currently best parameter set, this algorithm can only converge very slowly towards the best parameter set.

Its main use is to “scan” parameter space in order to find good parameter starting values for one of the deterministic fit algorithms, or to try to “jump out” of a local minimum where a deterministic fitting algorithm is stuck.

Since the algorithm is normally used for a first estimate of fitted parameters, it is not recommended to run it with non-zero x-errors – this merely slows down the algorithm without substantially increasing the accuracy of the estimates

Complex, accurate, and *easier** kinetics

you can have it all

March 23, 2021

Well, *easier** is relative…

Michaelis-Menten Kinetics

What assumptions do we make

$$E + S \rightleftharpoons ES \rightarrow E + P$$

$$\frac{d\left[ES\right]}{dt} = 0$$

substrate in excess

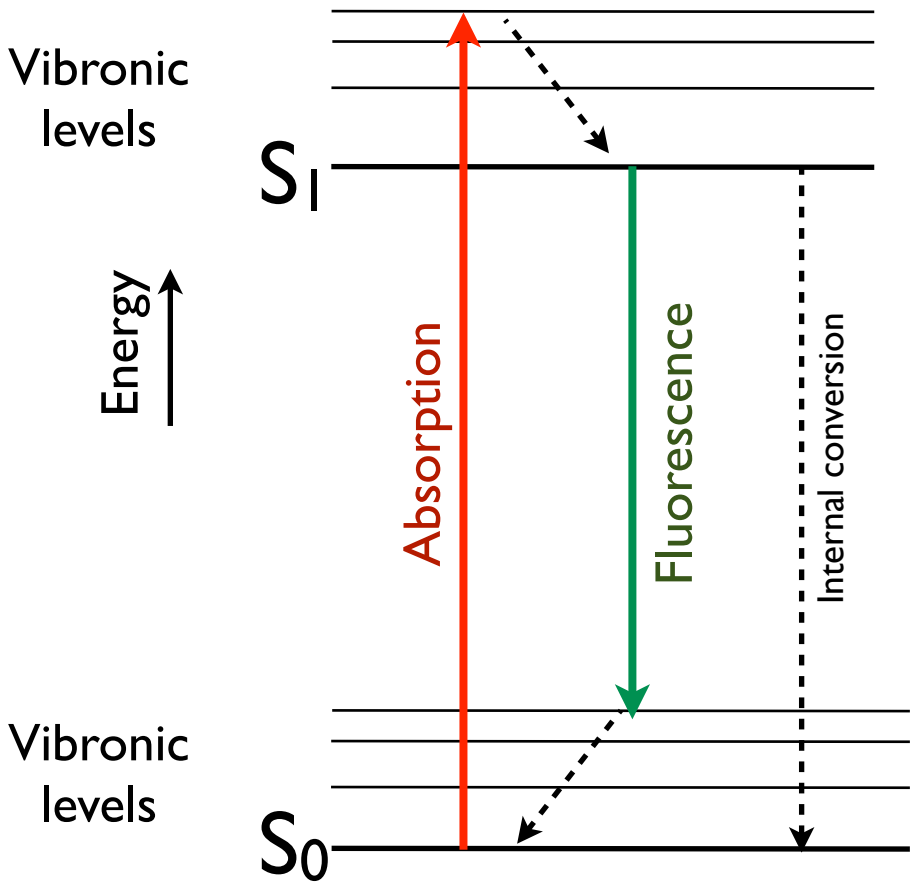
Rate Equations

First Order Decay

$$-\frac{\partial[A]}{\partial t} = k[A]$$

$$\int \frac{\partial[A]}{[A]} = -k\int \partial t$$

$$A = A_0 e^{-kt} = A_0 e^{-t/\tau}$$



Rate Equations

First Order Decay

$$-\frac{\partial[A]}{\partial t} = k[A]$$

$$\int \frac{\partial[A]}{[A]} = -k\int \partial t$$

$$A \xrightarrow{k} B$$

$$A = A_0 e^{-kt} = A_0 e^{-t/\tau}$$

$$B = B_0\left(1 - e^{-t/\tau}\right)$$

Rate Equations

Bimolecular Association

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

$$-\frac{\partial[AB]}{\partial t} = -k_1[A][B] + k_{-1}[AB]$$

Rate Equations

Bimolecular Association

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

$$-\frac{\partial[AB]}{\partial t} = -k_1[A][B] + k_{-1}[AB]$$

Approximations

≈easy

Early time [AB] << [A], [B]

$$-\frac{\partial[AB]}{\partial t} \approx -k_1[A][B]$$

$$[AB] = [AB]_{\max}\left(1 - e^{-k_{obs}t}\right)$$

k_{obs} depends on [B]

And excess [B]

$$-\frac{\partial[AB]}{\partial t} \approx -\left(k_1[B]\right)[A] \approx -\left(k_1'\right)[A]$$

Rate Equations

Bimolecular Association

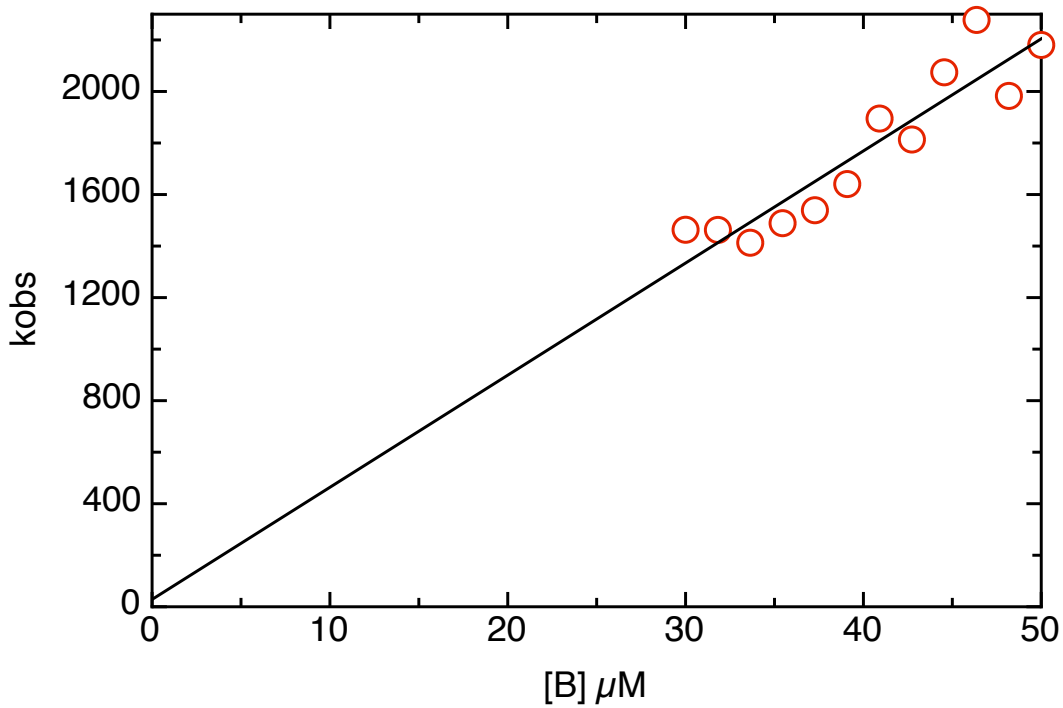
$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

$$-\frac{\partial[AB]}{\partial t} = -k_1[A][B] + k_{-1}[AB]$$

OK, what assumptions?

B in large excess

$$k_{obs} = k_1[B] + k_{-1}$$



Rate Equations

Bimolecular Association

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

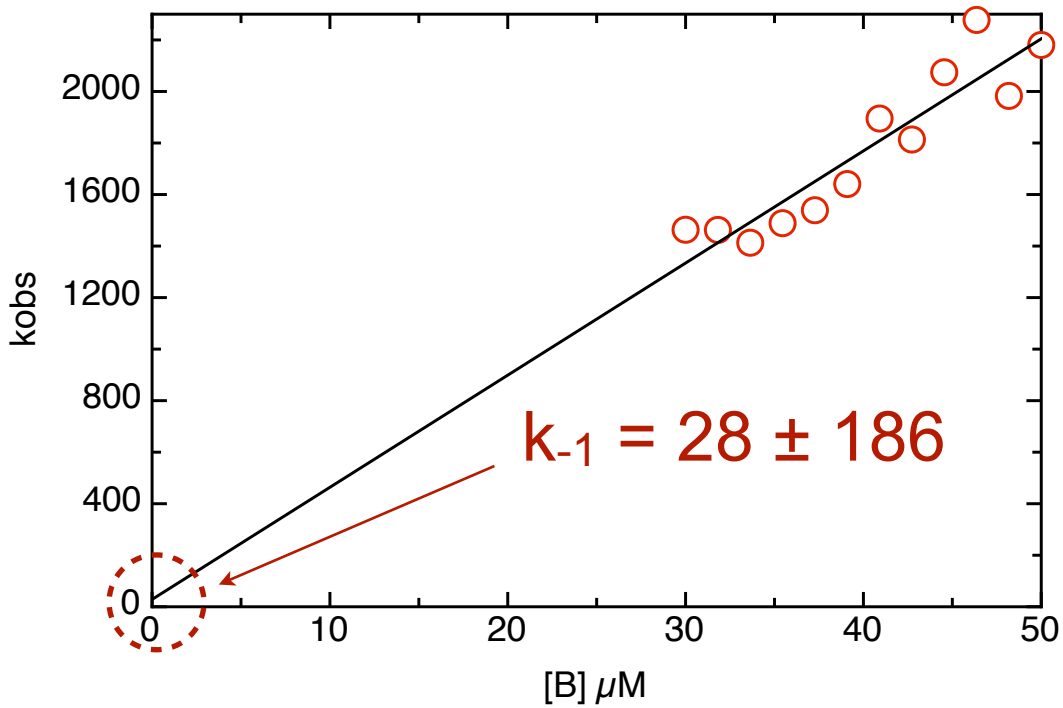
$$-\frac{\partial[AB]}{\partial t} = -k_1[A][B] + k_{-1}[AB]$$

OK, what assumptions?

B in large excess

$$k_{obs} = k_1[B] + k_{-1}$$

What is the problem here?



Iterations: 6

Chi squared = 15.1578
Goodness of fit = 0.1264

Parameters:		Standard deviations:	
deg =	1.0000		
const =	28.4998	Δconst =	186.2243
a1 =	43.5194	Δa1 =	4.5993

Confidence intervals (95.000%):

const	-336.7976	...	427.8580
a1	33.3910	...	52.5907

(based on 500 converging iterations)

Complex Kinetics

Exact* and Easy*

In the 20th century, we looked for one equation to fit

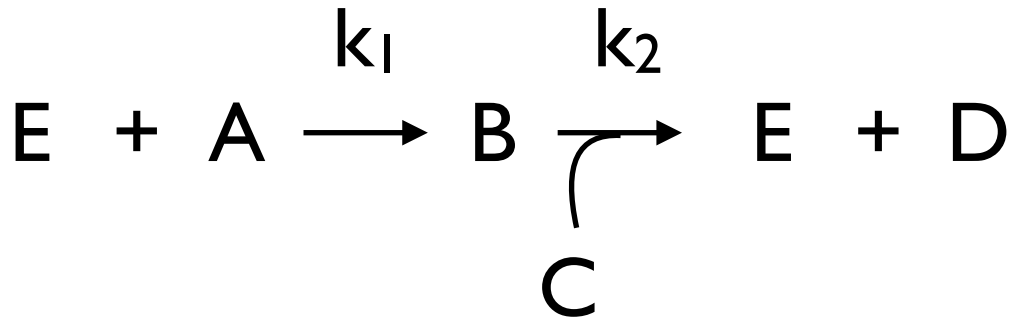
A single equation predicts [Y] at time t

But maybe we have a *program* predict [Y] at t

How?

Complex Kinetics

Exact* and Easy*



$$\frac{\partial[A]}{\partial t} = -k_1[E][A]$$

$$\frac{\partial[D]}{\partial t} = k_2[B][C]$$

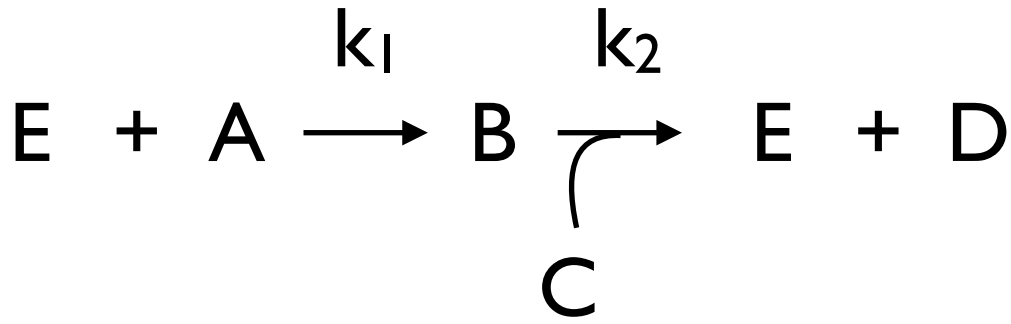
$$\frac{\partial[B]}{\partial t} = k_1[E][A] - k_2[B][C]$$

$$\frac{\partial[C]}{\partial t} = -k_2[B][C]$$

$$\frac{\partial[E]}{\partial t} = k_2[B][C] - k_1[E][A]$$

Complex Kinetics

Exact* and Easy*



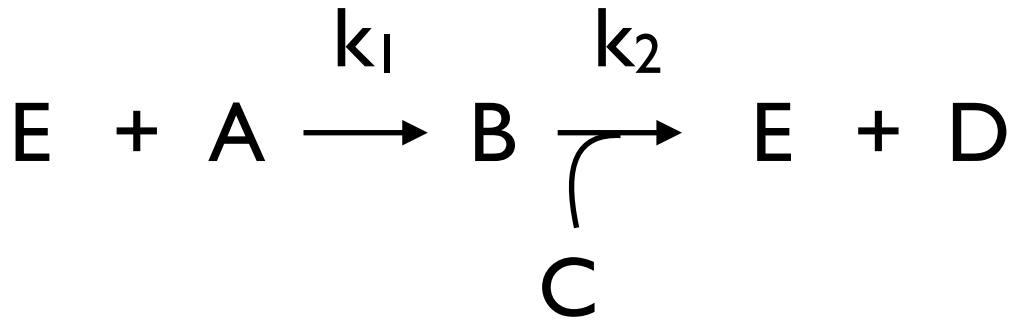
$$\frac{\partial[A]}{\partial t} = -k_1[E][A]$$

$$[A]_{t_{final}} = \int_{t=0}^{t_{final}} \partial[A] = \int_{t=0}^{t_{final}} -k_1[E]_t[A]_t\,\partial t$$

$$[A]_{t+\Delta t} = [A]_t + \frac{\partial[A]}{\partial t}\Delta t = [A]_t - k_1[E]_t[A]_t\,\Delta t$$

Complex Kinetics

Exact* and Easy*

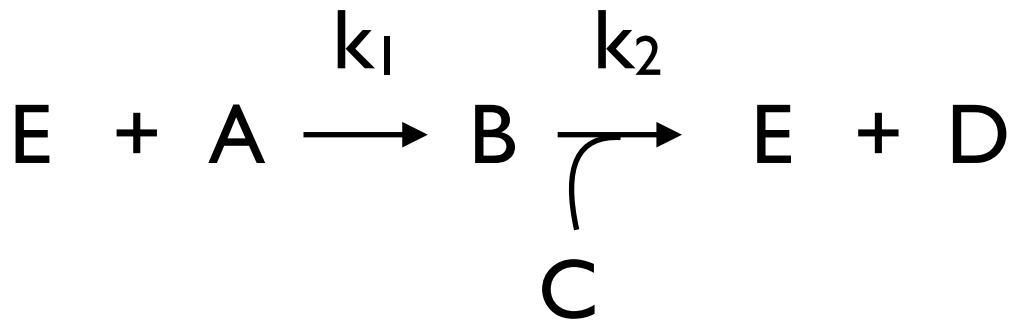


$$\frac{\partial[B]}{\partial t} = k_1[E][A] - k_2[B][C]$$

$$[B]_{t+\Delta t} = [B]_t + \frac{\partial[B]}{\partial t}\Delta t = [B]_t + \left(k_1[E]_t[A]_t - k_2[B]_t[C]_t\right)\Delta t$$

Complex Kinetics

Exact* and Easy*

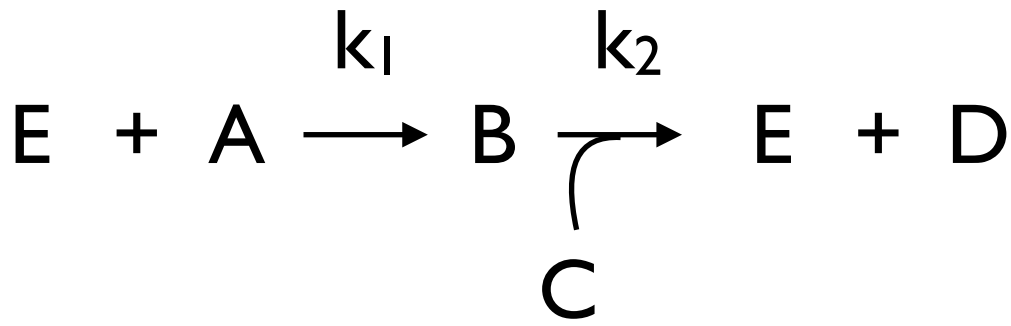


$$\frac{\partial[D]}{\partial t} = k_2[B][C]$$

$$[D]_{t+\Delta t} = [D]_t + \frac{\partial[D]}{\partial t}\Delta t = [D]_t + k_2[B]_t[C]_t\,\Delta t$$

Complex Kinetics

Exact* and Easy*

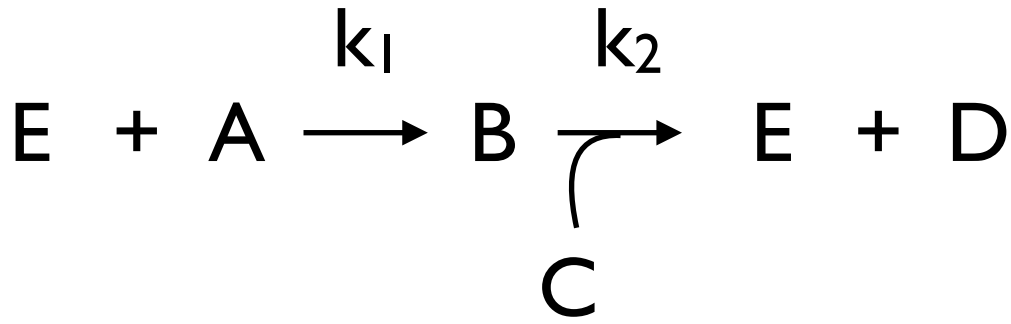


$$\frac{\partial[C]}{\partial t} = -k_2[B][C]$$

$$[C]_{t+\Delta t} = [C]_t + \frac{\partial[C]}{\partial t}\Delta t = [C]_t - k_2[B]_t[C]_t\,\Delta t$$

Complex Kinetics

Exact* and Easy*

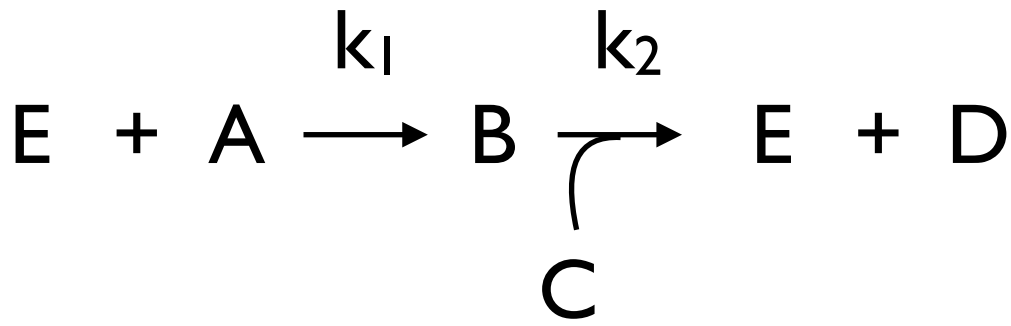


$$\frac{\partial[E]}{\partial t} = k_2[B][C] - k_1[E][A]$$

$$[E]_{t+\Delta t} = [E]_t + \frac{\partial[E]}{\partial t}\Delta t = [E]_t + \left(k_2[B]_t[C]_t - k_1[E]_t[A]_t\right)\Delta t$$

Complex Kinetics

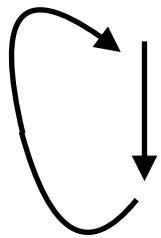
Exact* and Easy*



$$[A]_{t+\Delta t} = [A]_t - k_1[E]_t[A]_t\,\Delta t$$
$$[B]_{t+\Delta t} = [B]_t + \left(k_1[E]_t[A]_t - k_2[B]_t[C]_t\right)\Delta t$$

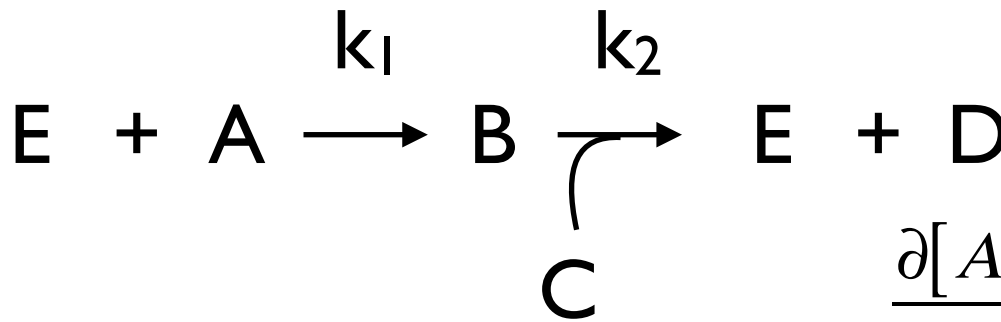
$$[D]_{t+\Delta t} = [D]_t + k_2[B]_t[C]_t\,\Delta t$$
$$[C]_{t+\Delta t} = [C]_t - k_2[B]_t[C]_t\,\Delta t$$
$$[E]_{t+\Delta t} = [E]_t + \left(k_2[B]_t[C]_t - k_1[E]_t[A]_t\right)\Delta t$$

Starting concentrations E_0, A_0, B_0, C_0, D_0

repeat t_{total} / Δt times  increment by $\Delta E, \Delta A, \Delta B, \Delta C, \Delta D$ in Δt

Complex Kinetics

Exact* and Easy*




```
time <- seq(0, 50, by = 0.01)

# parameters
parameters <- c(k1=0.08, k2=0.02)

# initial conditions
state <- c(A=5,B=0,C=50,D=0,E=5)

# R function to calculate the value of the derivatives at each time value
# Use the names of the variables as defined in the vectors above

multiKin <- function(t, state, parameters){
  with(as.list(c(state, parameters)), {
    dA = -k1*E*A
    dB = k1*E*A - k2*B*C
    dC = -k2*B*C
    dD = k2*B*C
    dE = k2*B*C - k1*E*A
    return(list(c(dA, dB, dC, dD, dE)))
  })
}
## Integration with 'ode' - ordinary differential equations
out <- ode(y = state, times = time, func = multiKin, parms = parameters)
```

$$\frac{\partial[A]}{\partial t} = -k_1[E][A]$$

$$\frac{\partial[B]}{\partial t} = k_1[E][A] - k_2[B][C]$$

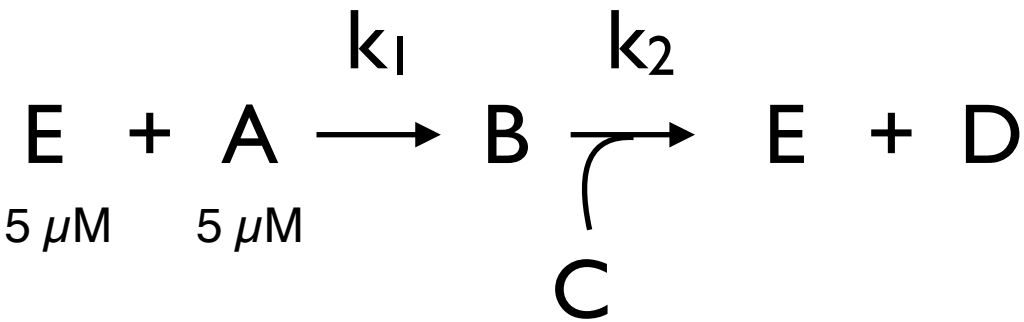
$$\frac{\partial[C]}{\partial t} = -k_2[B][C]$$

$$\frac{\partial[D]}{\partial t} = k_2[B][C]$$

$$\frac{\partial[E]}{\partial t} = k_2[B][C] - k_1[E][A]$$

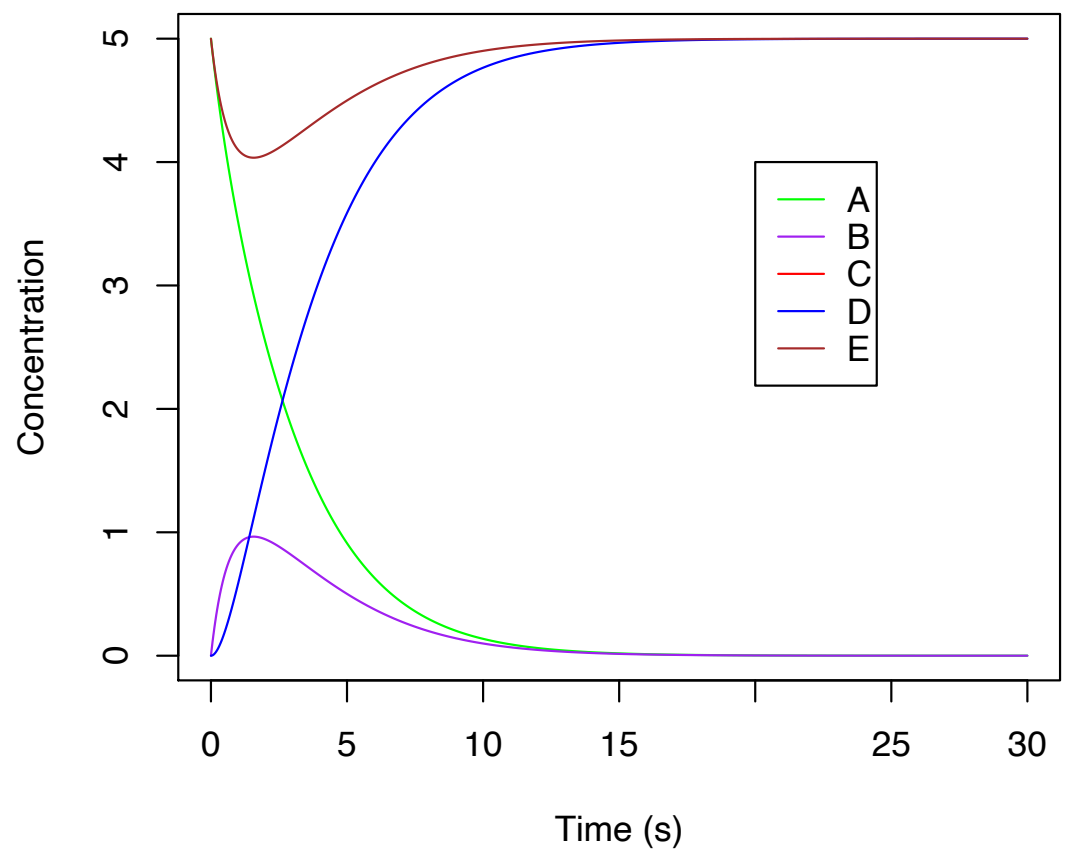
Complex Kinetics

Exact* and Easy*



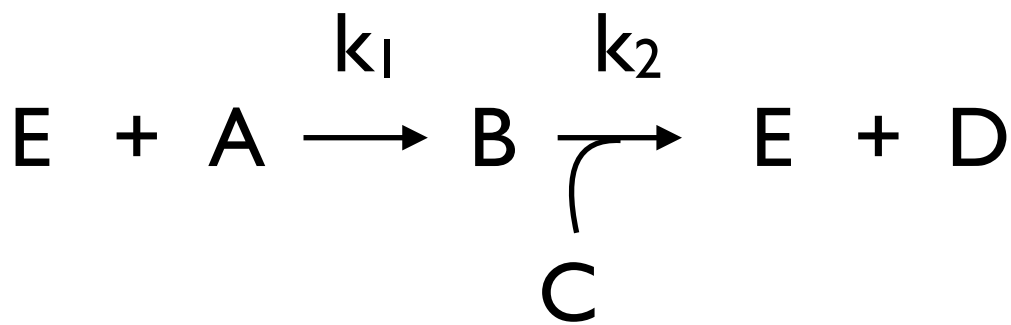
$$\frac{d[B]}{dt} = 0$$

```
time <- seq(0, 50, by = 0.01)

# parameters: a named vector
parameters <- c(k1=0.08, k2=0.02)

# initial condition: a named vector
state <- c(A=5,B=0,C=50,D=0,E=5)

# R function to calculate the value of the derivatives at each time value
# Use the names of the variables as defined in the vectors above
multiKin <- function(t, state, parameters){
  with(as.list(c(state, parameters)), {
    dA = -k1*E*A
    dB = k1*E*A - k2*B*C
    dC = -k2*B*C
    dD = k2*B*C
    dE = k2*B*C - k1*E*A
    return(list(c(dA, dB, dC, dD, dE)))
  })
}
## Integration with 'ode' - ordinary differential equations
out <- ode(y = state, times = time, func = multiKin, parms = parameters)

## Ploting
out.df = as.data.frame(out)

plot(out.df$time, out.df$A, type="l", col="green")
lines(out.df$time, out.df$B, col="purple")
lines(out.df$time, out.df$C, col="red")
lines(out.df$time, out.df$D, col="blue")
lines(out.df$time, out.df$E, col="brown")
```



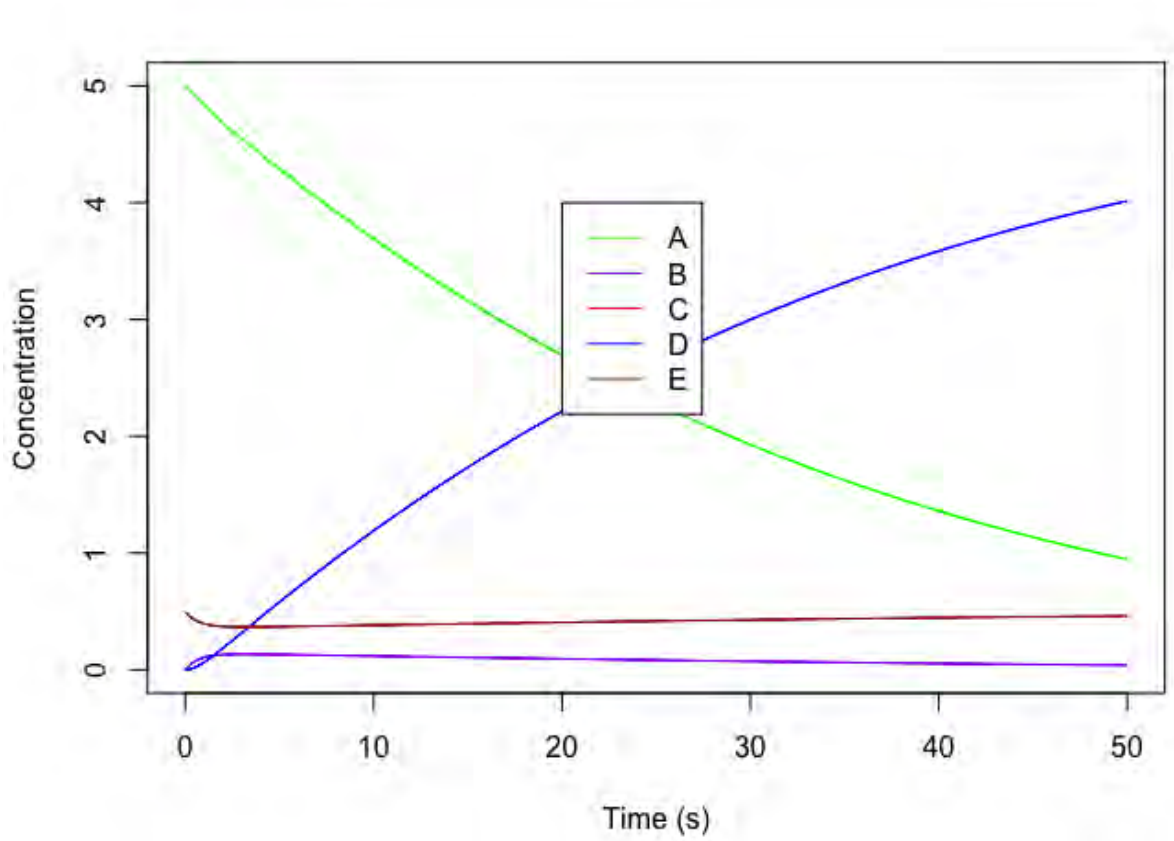
Complex Kinetics

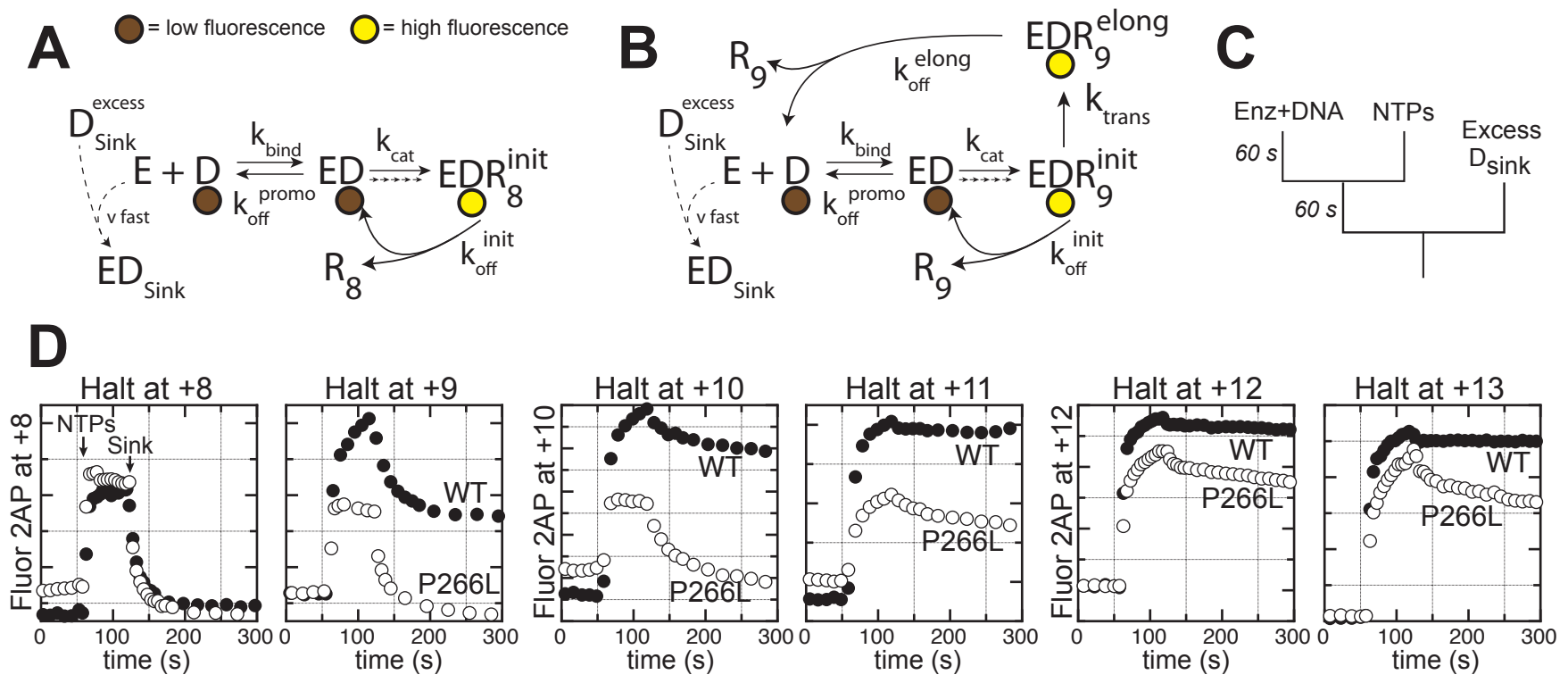
Exact* and Easy*

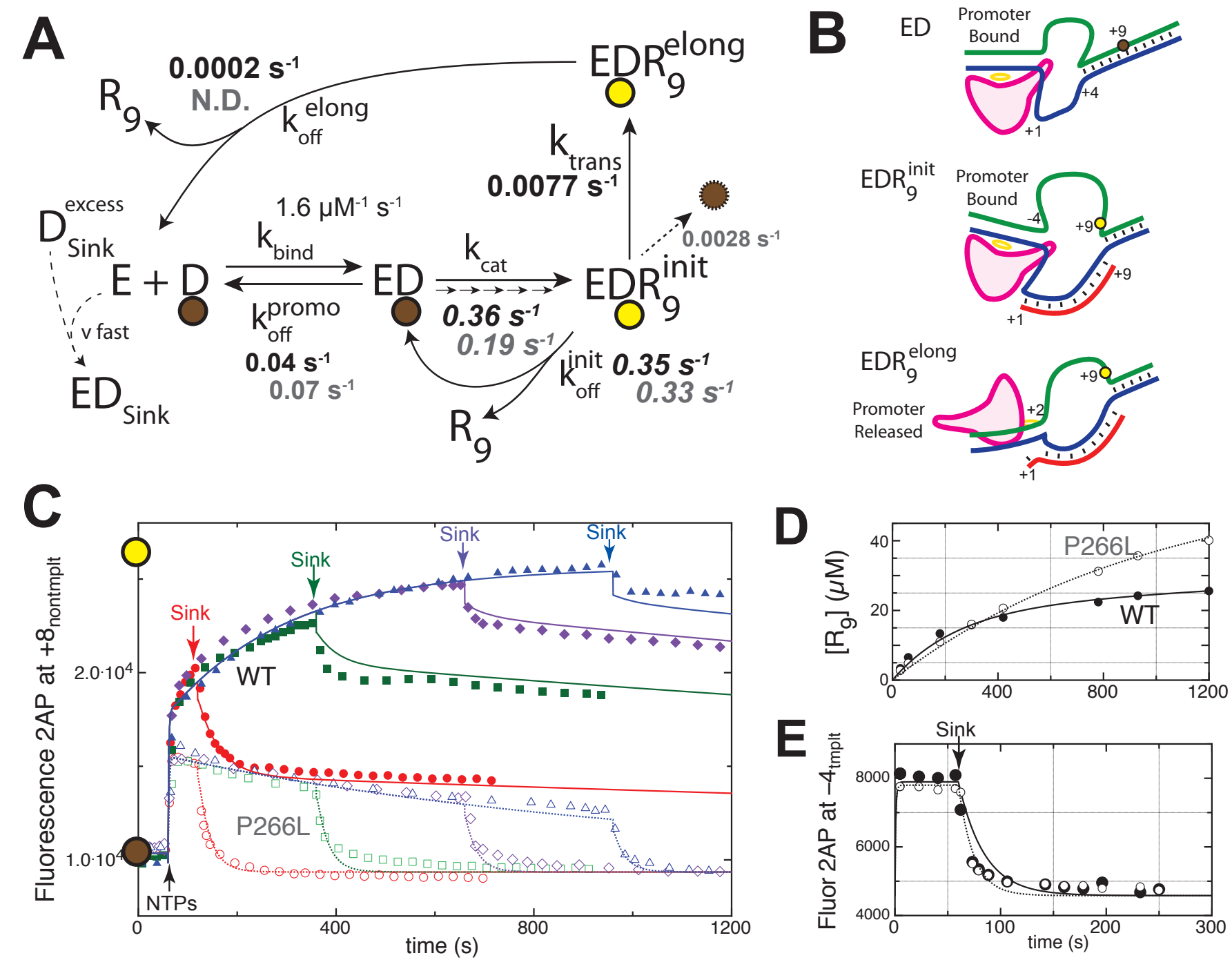


$$\frac{d[B]}{dt} = 0$$

```
time <- seq(0, 50, by = 0.01)

# parameters: a named vector
parameters <- c(k1=0.08, k2=0.02)

# initial condition: a named vector
state <- c(A=5,B=0,C=50,D=0,E=0.5)

# R function to calculate the value of the derivatives at each time value
# Use the names of the variables as defined in the vectors above
multiKin <- function(t, state, parameters){
  with(as.list(c(state, parameters)), {
    dA = -k1*E*A
    dB = k1*E*A - k2*B*C
    dC = -k2*B*C
    dD = k2*B*C
    dE = k2*B*C - k1*E*A
    return(list(c(dA, dB, dC, dD, dE)))
  })
}
## Integration with 'ode' - ordinary differential equations
out <- ode(y = state, times = time, func = multiKin, parms = parameters)

## Ploting
out.df = as.data.frame(out)

plot(out.df$time, out.df$A, type="l", col="green")
lines(out.df$time, out.df$B, col="purple")
lines(out.df$time, out.df$C, col="red")
lines(out.df$time, out.df$D, col="blue")
lines(out.df$time, out.df$E, col="brown")
```




A
= low fluorescence
= high fluorescence
D excess Sink
E + D
k bind
k off promo
ED
k cat
EDR8 init
v fast
ED Sink
R8
k off init
B
R9
k off elong
EDR9 elong
k trans
D excess Sink
E + D
k bind
k off promo
ED
k cat
EDR9 init
v fast
ED Sink
R9
k off init
C
Enz+DNA
NTPs
Excess Dsink
60 s
60 s
D
Halt at +8
Halt at +9
Halt at +10
Halt at +11
Halt at +12
Halt at +13
NTPs
Sink
Fluor 2AP at +8
Fluor 2AP at +10
Fluor 2AP at +12
WT
P266L
0
100
200
300
time (s)


A
0.0002 s-1
N.D.
R9
k elong off
EDR9 elong
k trans
0.0077 s-1
D excess Sink
1.6 μM-1 s-1
k bind
E + D
k promo off
0.04 s-1
0.07 s-1
ED
k cat
0.36 s-1
0.19 s-1
EDR9 init
0.0028 s-1
v fast
ED Sink
k init off
0.35 s-1
0.33 s-1
R9
B
ED
Promoter Bound
+9
+4
+1
EDR9 init
Promoter Bound
-4
+9
+9
+1
EDR9 elong
Promoter Released
+9
+2
+1
C
Fluorescence 2AP at +8nontmplt
2.0·10^4
1.0·10^4
Sink
Sink
Sink
Sink
WT
P266L
NTPs
0
400
800
1200
time (s)
D
[Rg] (μM)
40
20
0
P266L
WT
0
400
800
1200
E
Fluor 2AP at –4tmplt
Sink
8000
6000
4000
0
100
200
300
time (s)

Happy Biophysics Week!!!

Hands on with R - simulating kinetics

Mar 25, 2021

Enzyme Kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_c} E + P$$

$$E_T = [ES] + [E] \qquad [E] = E_T - [ES]$$

$$S_T = [S] + [ES] + [P] \qquad [S] \approx S_T - [ES]$$

$$\frac{d[ES]}{dt} = k_1 [E][S] - k_{-1}[ES] - k_c [ES] + k_r [E][P]$$

Assume [P] negligible

$$k_1 [E][S] - k_{-1}[ES] - k_c [ES] \approx 0$$

Assume steady state

$$k_1 [E][S] \approx (k_{-1} + k_c)[ES]$$

$$\frac{[E][S]}{[ES]} \approx \frac{k_{-1} + k_c}{k_1} = K_M \qquad \frac{[P][L]}{[PL]} = K_d$$

Enzyme Kinetics

Assume [P] negligible

Assume steady state

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_c} E + P$$

$$\frac{[E]\,[S]}{[ES]} \approx \frac{k_{-1} + k_c}{k_1} = K_M$$

$$[E] = E_T - [ES]$$

$$[S] \approx S_T - [ES]$$

$$[E]\,[S] \approx K_M\,[ES]$$

$$(E_T - [ES])(S_T - [ES]) \approx K_M\,[ES]$$

Assume substrate in excess

$$(P_T - [PL])(L_T - [PL]) = K_d\,[PL]$$

Assume ligand in excess

$$(E_T - [ES])S_T \approx K_M\,[ES]$$

Enzyme Kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_r}{\overset{k_c}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

```
dE <-  -k1*E*S + km1*ES + kc*ES - kr*E*P
dS <-  -k1*E*S + km1*ES
dES <- k1*E*S - km1*ES - kc*ES + kr*E*P
dP <-  kc*ES - kr*E*P
```

```
EnzKin <- function(t0=0, tf, tincr=(tf-t0)/50, k1p, km1p, kcp, krp=0, E0, S0, ES0=0, P0=0) {

        # create a time range
        time <- seq(t0, tf, by = tincr)

        # parameters:
        parameters <- c(k1=k1p, km1=km1p, kc=kcp, kr=krp)

        # initial condition:
        state <- c(E=E0, S=S0, ES=ES0, P=P0)

        # R function to calculate the value of the derivatives at each time value
        # Use the names of the variables as defined in the vectors above
        # define in same order as specified above in state
        multiKin <- function(t, state, parameters){
          with(as.list(c(state, parameters)), {
                dE <-  -k1*E*S + km1*ES + kc*ES - kr*E*P
                dS <-  -k1*E*S + km1*ES
                dES <- k1*E*S - km1*ES - kc*ES + kr*E*P
                dP <-  kc*ES - kr*E*P

                # return derivatives in same relative order as specified above in state
                return(list(c(dE, dS, dES, dP)))
          })
        }  ## end of function multiKin

        ## Integration with 'ode' - ordinary differential equations
        out1 <- ode(y = state, times = time, func = multiKin, parms = parameters)

        # get results as a dataframe
        df <- as.data.frame(out1)

        # return a list with things we might want to access directly in plotting, etc
        outl <- list("output"=out1, "t"=time, "E"=df$E, "S"=df$S, "ES"=df$ES, "P"=df$P )

        return(outl)
 }
# call the above function (you can omit optional parameters)
 out <- EnzKin(tf=15,  tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2, E0=0.2, S0=20.0)
```

optional

~~Assume [P] negligible~~

~~Assume steady state~~

0.1 μM^{-1} s^{-1} 0.2 s^{-1}

$$E + S \rightleftharpoons ES \rightleftharpoons E + P$$

0.1 s^{-1} 0.001 μM^{-1} s^{-1}

```
dE <-  -k1*E*S + km1*ES + kc*ES - kr*E*P
dS <-  -k1*E*S + km1*ES
dES <- k1*E*S - km1*ES - kc*ES + kr*E*P
dP <-  kc*ES - kr*E*P
```

```
# plot the results (this will auto-scale to the product rang
plot( out$t, out$P, col="green", type="l", xlab="Time (s)"
lines(out$t, out$E, col="purple")
lines(out$t, out$ES, col="red")
lines(out$t, out$S, col="blue")

legend(200, 3, c("P", "E", "ES", "S"), col = c("green", "pu
```

Enzyme Kinetics

~~Assume [P] negligible~~

~~Assume steady state~~

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=15, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2,
E0=0.2, S0=20.0

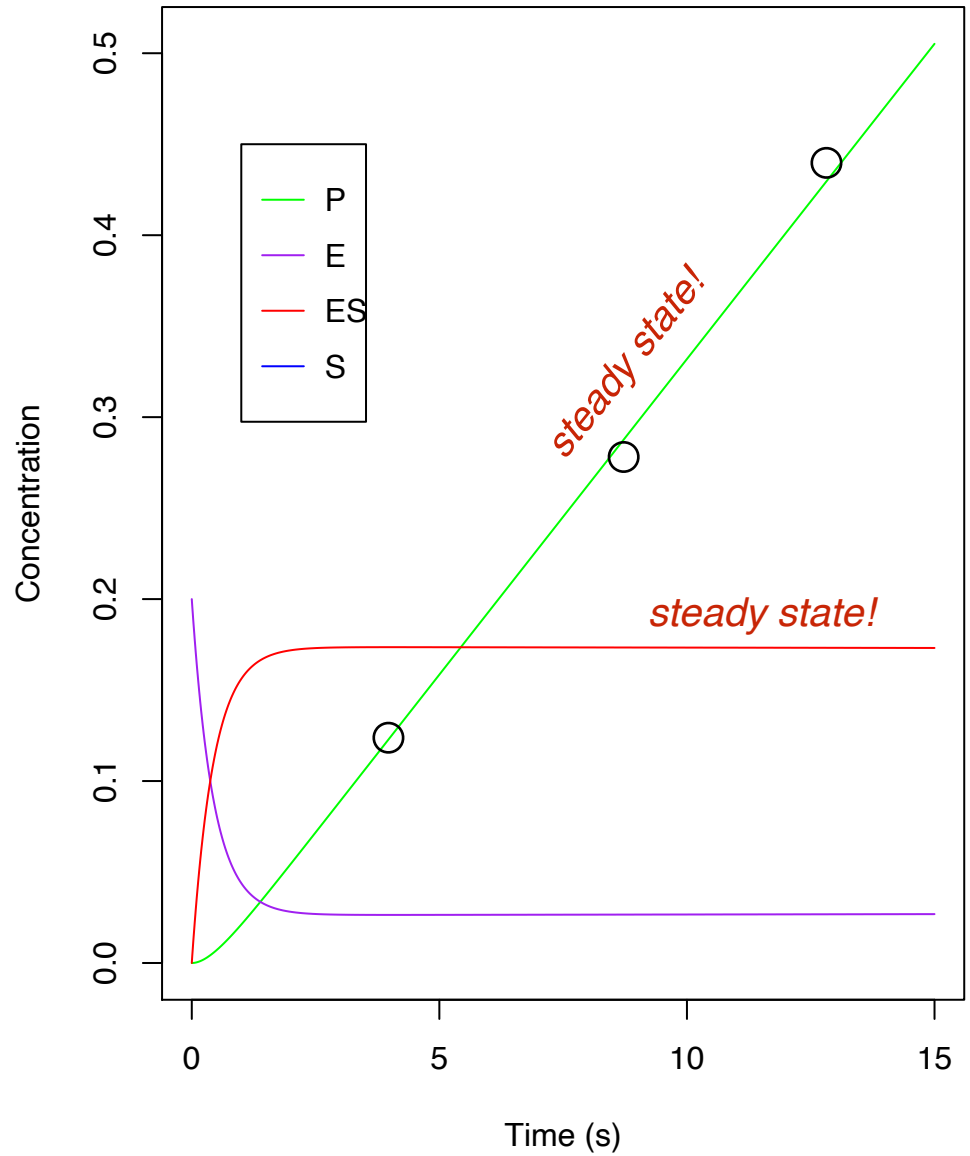
k_1 = 0.1 $\mu M^{-1}\ s^{-1}$

k_{-1} = 0.1 $\mu M^{-1}\ s^{-1}$

k_{cat} = 0.2 s^{-1}

k_r = 0.001 s^{-1}

(K_m = 1.0 μM)



Enzyme Kinetics

~~Assume [P] negligible~~

~~Assume steady state~~

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=15, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2,
E0=0.2, S0=20.0

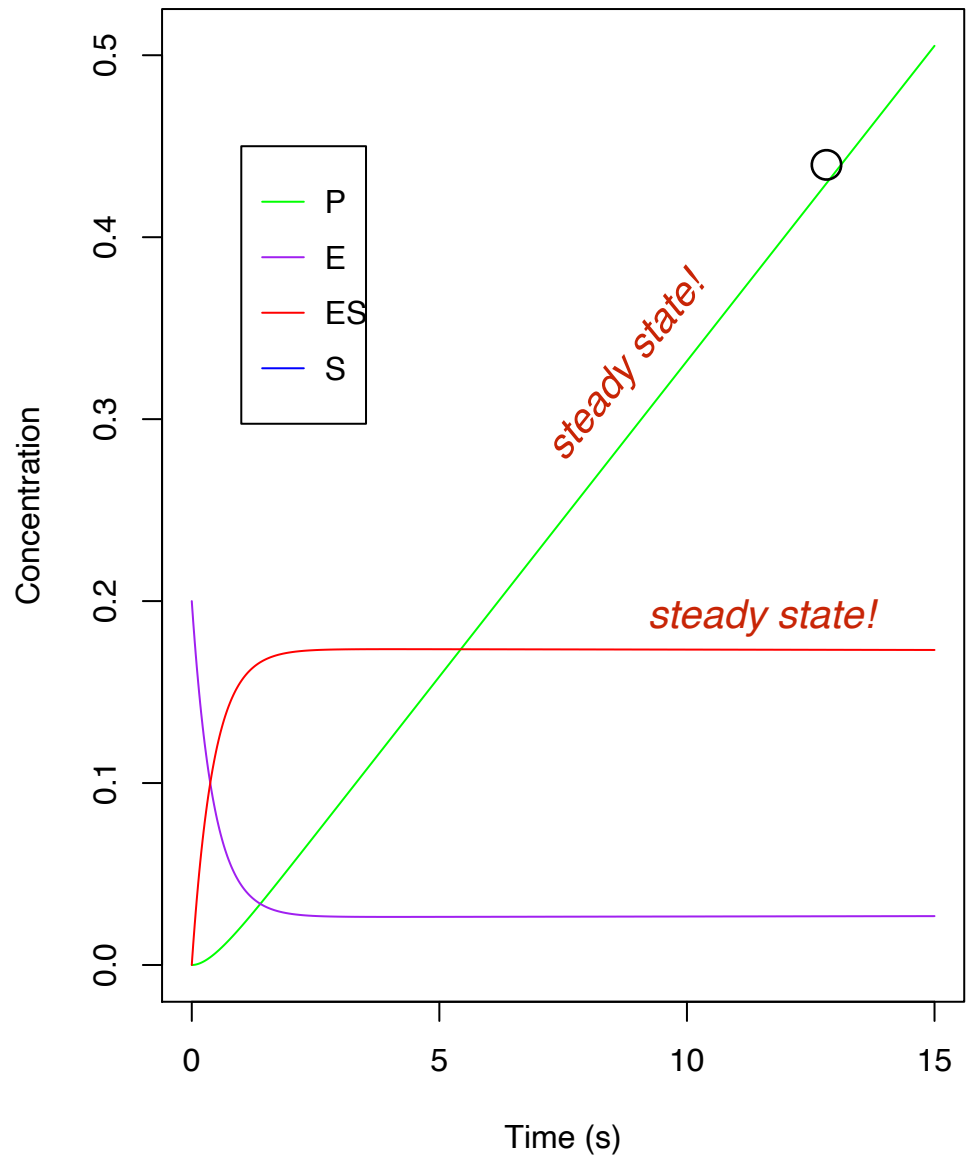
k_1 = 0.1 μM^{-1} s^{-1}

k_{-1} = 0.1 μM^{-1} s^{-1}

k_{cat} = 0.2 s^{-1}

k_r = 0.001 s^{-1}

(K_m = 1.0 μM)



Break out exercise

- Go to Moodle and download today's R script
- Run it in R to generate the graph we just saw
- You will have to install the "deSolve" package
  - For help go to the bottom of the "R – fitting data to a mathematical model" web site created for this course.
- Then just run the script
  - source("EnzymeKinetics.txt")
  - (remember not to use "curly" quotes)

Enzyme Kinetics

~~Assume [P] negligible~~

~~Assume steady state~~

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=15, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2, E0=0.2, S0=20.0

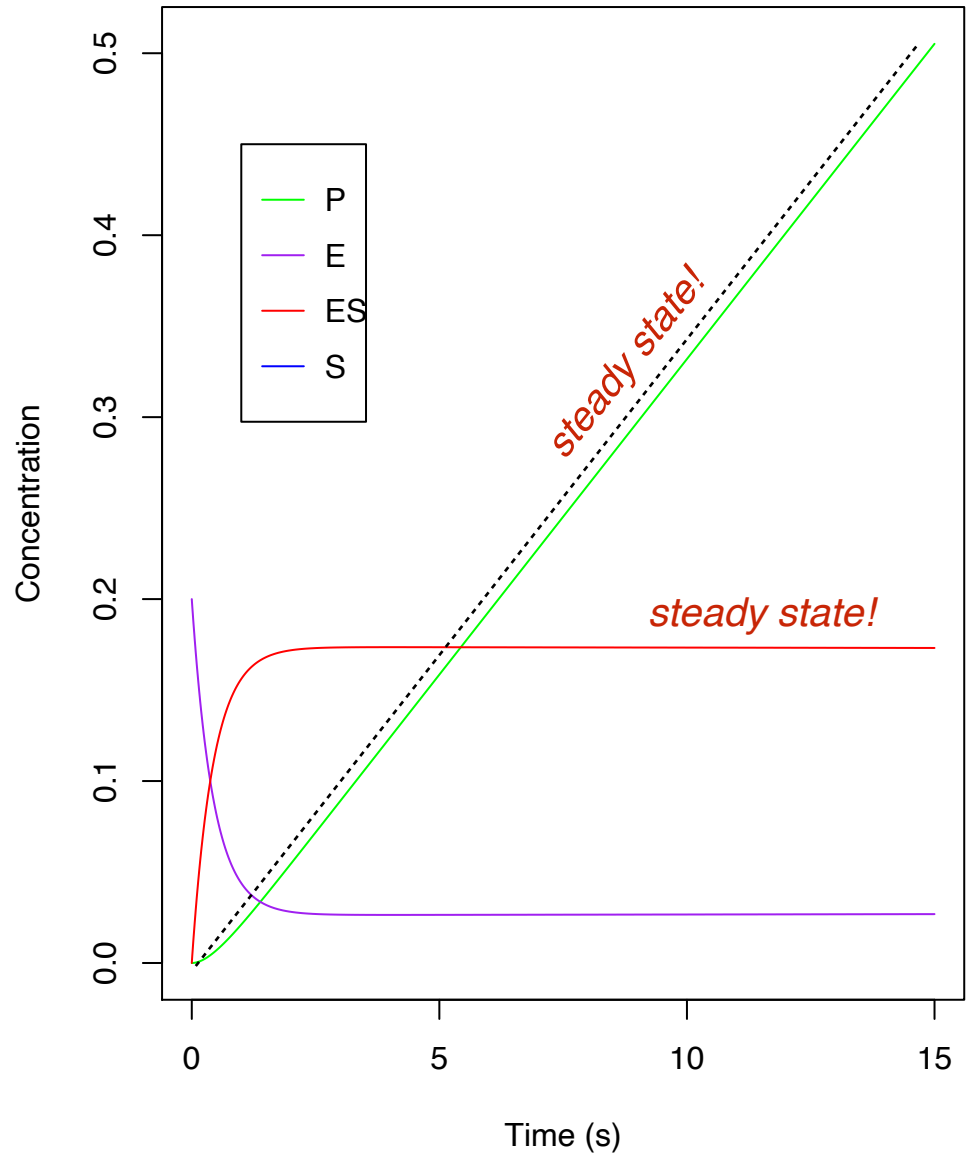
k_1 = 0.1 μM^{-1} s^{-1}

k_{-1} = 0.1 μM^{-1} s^{-1}

k_{cat} = 0.2 s^{-1}

k_r = 0.001 s^{-1}

(K_m = 1.0 μM)



Break out exercise

- Drop the substrate concentration from 20 μM to 2 μM
- Replot

Enzyme Kinetics

~~Assume [P] negligible~~

~~Assume steady state~~

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=15, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2,
E0=0.2, S0=2.0

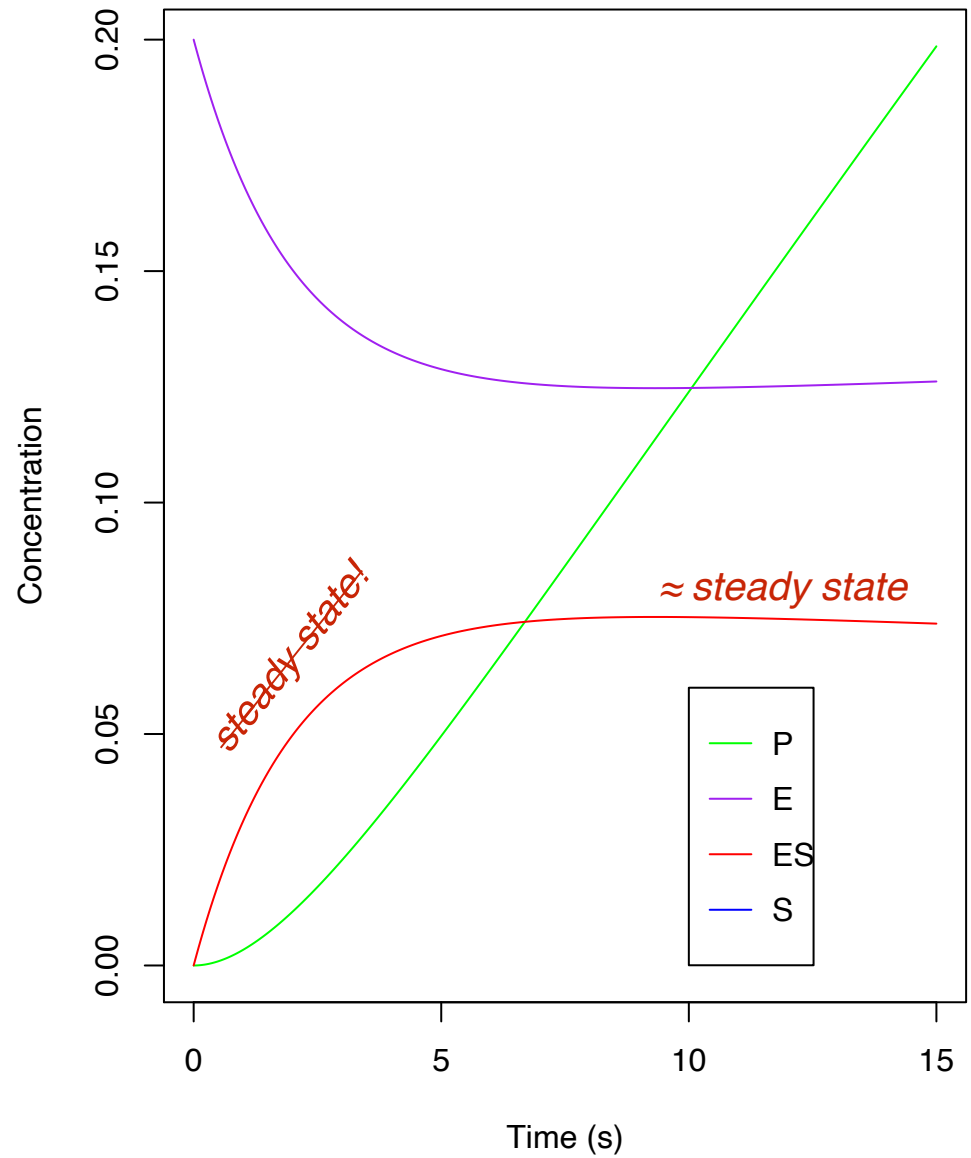
k_1 = 0.1 μM^{-1} s^{-1}

k_{-1} = 0.1 μM^{-1} s^{-1}

k_{cat} = 0.2 s^{-1}

k_r = 0.001 s^{-1}

(K_m = 1.0 μM)



Break out exercise

- Keep substrate at 2 μM
- Run the reaction for 150 s
- Replot

Enzyme Kinetics

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=150, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2,
E0=0.2, S0=2.0

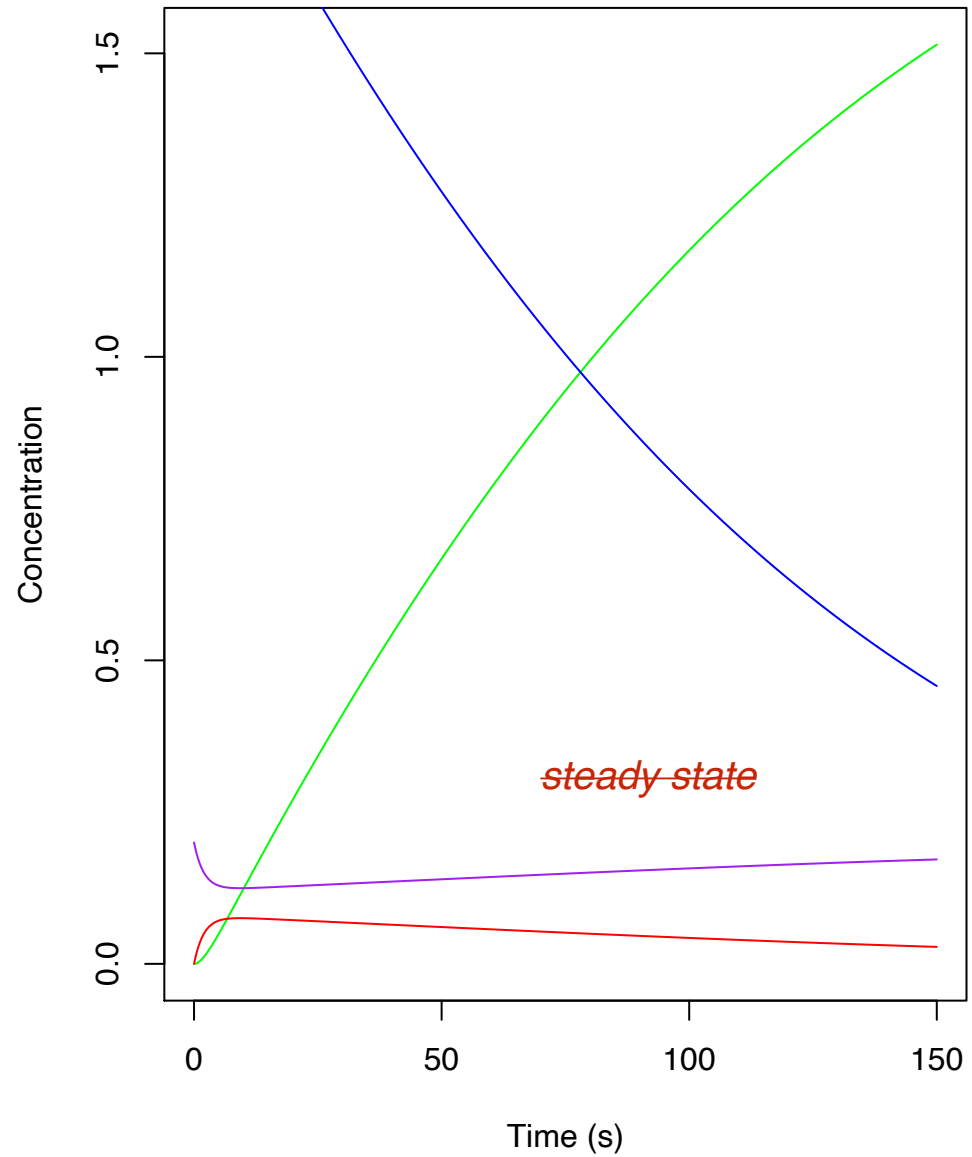
k_1 = 0.1 μM^{-1} s^{-1}

k_{-1} = 0.1 μM^{-1} s^{-1}

k_{cat} = 0.2 s^{-1}

k_r = 0.001 s^{-1}

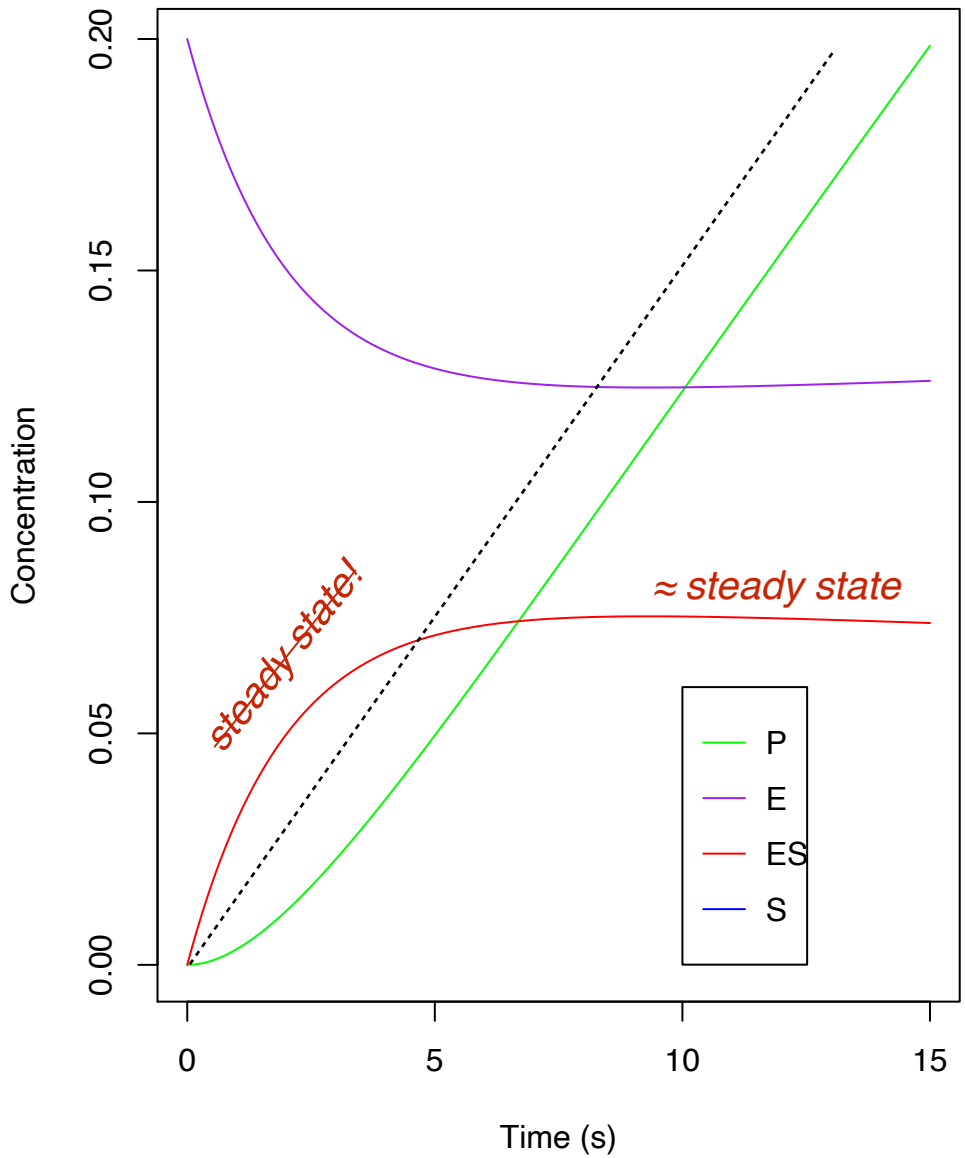
(K_m = 1.0 μM)

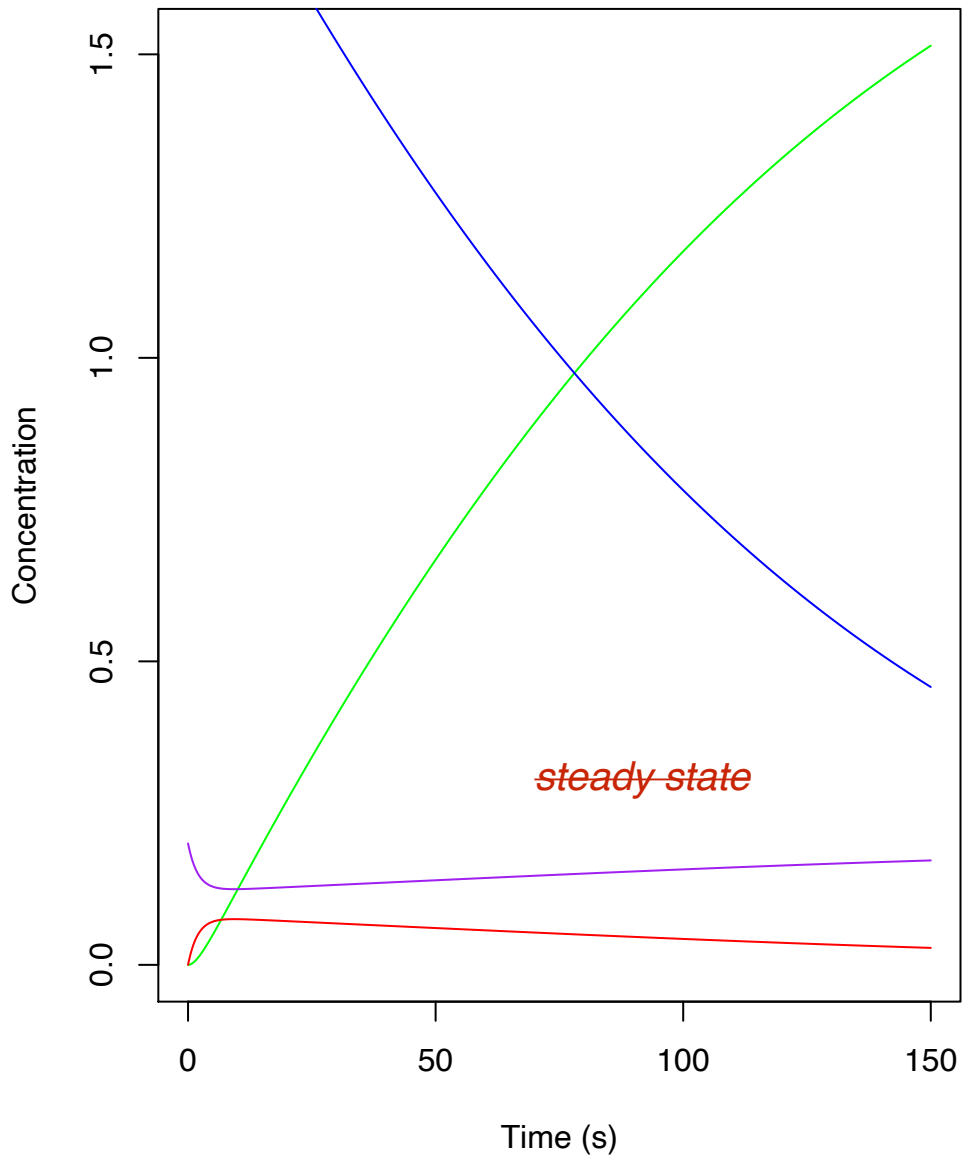


Enzyme Kinetics

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$





Break out exercise

- Let's an inhibitor
- Replot

$$E + I \rightleftharpoons EI$$

Happy Biophysics Week!!!

Hands on with R - simulating kinetics

Mar 25, 2021

Enzyme Kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_c} E + P$$

$$E_T = [ES] + [E] \qquad [E] = E_T - [ES]$$

$$S_T = [S] + [ES] + [P] \qquad [S] \approx S_T - [ES]$$

$$\frac{d[ES]}{dt} = k_1 [E][S] - k_{-1}[ES] - k_c [ES] + k_r [E][P]$$

Assume [P] negligible

$$k_1 [E][S] - k_{-1}[ES] - k_c [ES] \approx 0$$

Assume steady state

$$k_1 [E][S] \approx (k_{-1} + k_c)[ES]$$

$$\frac{[E][S]}{[ES]} \approx \frac{k_{-1} + k_c}{k_1} = K_M \qquad \frac{[P][L]}{[PL]} = K_d$$

Enzyme Kinetics

Assume [P] negligible

Assume steady state

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_c} E + P$$

$$\frac{[E]\,[S]}{[ES]} \approx \frac{k_{-1} + k_c}{k_1} = K_M$$

$$[E] = E_T - [ES]$$

$$[S] \approx S_T - [ES]$$

$$[E]\,[S] \approx K_M\,[ES]$$

$$(E_T - [ES])(S_T - [ES]) \approx K_M\,[ES]$$

Assume substrate in excess

$$(P_T - [PL])(L_T - [PL]) = K_d\,[PL]$$

Assume ligand in excess

$$(E_T - [ES])S_T \approx K_M\,[ES]$$

Enzyme Kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_r}{\overset{k_c}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

```
dE <-  -k1*E*S + km1*ES + kc*ES - kr*E*P
dS <-  -k1*E*S + km1*ES
dES <- k1*E*S - km1*ES - kc*ES + kr*E*P
dP <-  kc*ES - kr*E*P
```

```
EnzKin <- function(t0=0, tf, tincr=(tf-t0)/50, k1p, km1p, kcp, krp=0, E0, S0, ES0=0, P0=0) {

        # create a time range
        time <- seq(t0, tf, by = tincr)

        # parameters:
        parameters <- c(k1=k1p, km1=km1p, kc=kcp, kr=krp)

        # initial condition:
        state <- c(E=E0, S=S0, ES=ES0, P=P0)

        # R function to calculate the value of the derivatives at each time value
        # Use the names of the variables as defined in the vectors above
        # define in same order as specified above in state
        multiKin <- function(t, state, parameters){
          with(as.list(c(state, parameters)), {
                dE <-  -k1*E*S + km1*ES + kc*ES - kr*E*P
                dS <-  -k1*E*S + km1*ES
                dES <- k1*E*S - km1*ES - kc*ES + kr*E*P
                dP <-  kc*ES - kr*E*P

                # return derivatives in same relative order as specified above in state
                return(list(c(dE, dS, dES, dP)))
          })
        }  ## end of function multiKin

        ## Integration with 'ode' - ordinary differential equations
        out1 <- ode(y = state, times = time, func = multiKin, parms = parameters)

        # get results as a dataframe
        df <- as.data.frame(out1)

        # return a list with things we might want to access directly in plotting, etc
        outl <- list("output"=out1, "t"=time, "E"=df$E, "S"=df$S, "ES"=df$ES, "P"=df$P )

        return(outl)
 }
# call the above function (you can omit optional parameters)
 out <- EnzKin(tf=15,  tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2, E0=0.2, S0=20.0)
```

optional

~~Assume [P] negligible~~

~~Assume steady state~~

0.1 μM^{-1} s^{-1} 0.2 s^{-1}

$$E + S \rightleftharpoons ES \rightleftharpoons E + P$$

0.1 s^{-1} 0.001 μM^{-1} s^{-1}

```
dE <-  -k1*E*S + km1*ES + kc*ES - kr*E*P
dS <-  -k1*E*S + km1*ES
dES <- k1*E*S - km1*ES - kc*ES + kr*E*P
dP <-  kc*ES - kr*E*P
```

```
# plot the results (this will auto-scale to the product rang
plot( out$t, out$P, col="green", type="l", xlab="Time (s)"
lines(out$t, out$E, col="purple")
lines(out$t, out$ES, col="red")
lines(out$t, out$S, col="blue")

legend(200, 3, c("P", "E", "ES", "S"), col = c("green", "pu
```

Enzyme Kinetics

~~Assume [P] negligible~~

~~Assume steady state~~

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=15, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2,
E0=0.2, S0=20.0

k_1 = 0.1 $\mu M^{-1}\ s^{-1}$

k_{-1} = 0.1 $\mu M^{-1}\ s^{-1}$

k_{cat} = 0.2 s^{-1}

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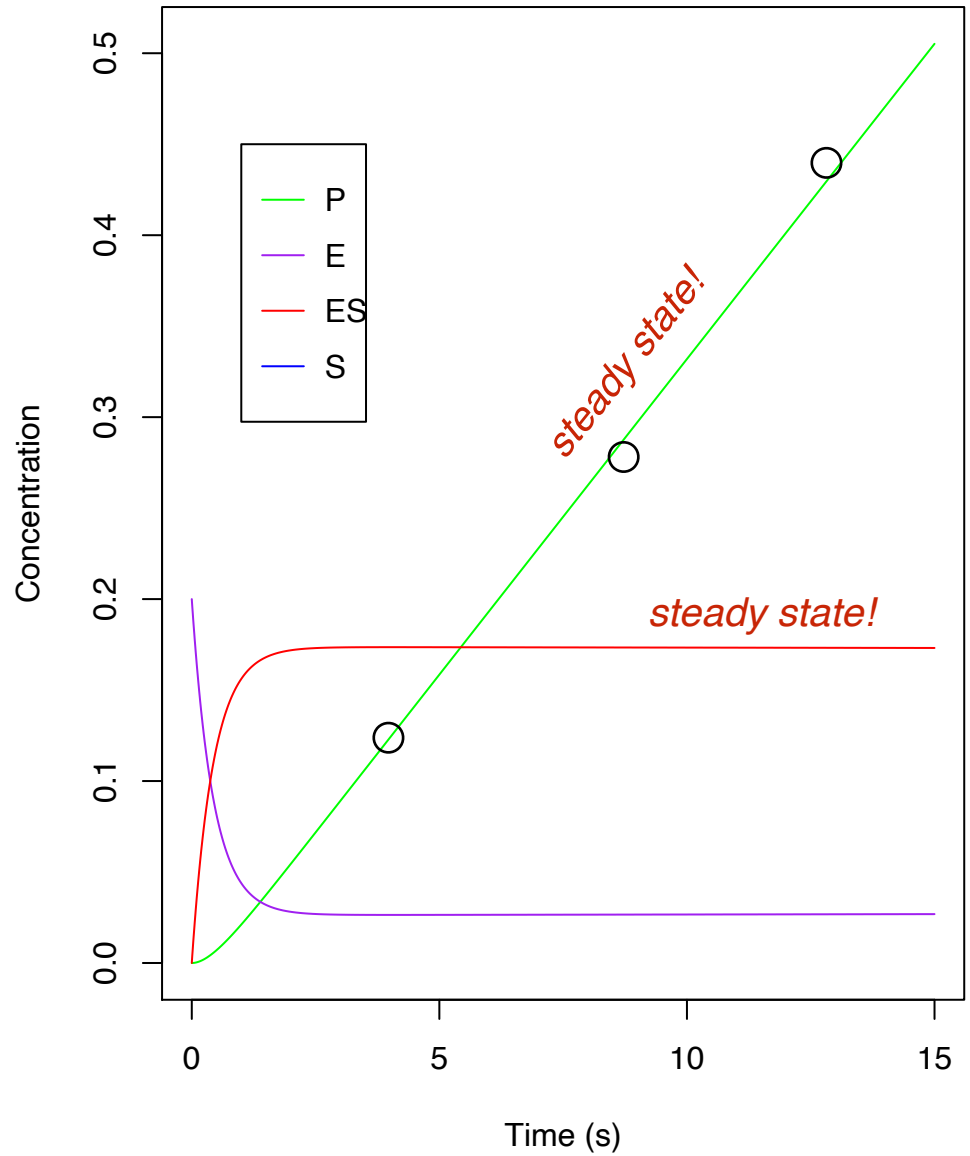
(K_m = 1.0 μM)



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$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=15, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2, E0=0.2, S0=20.0

k_1 = 0.1 μM^{-1} s^{-1}

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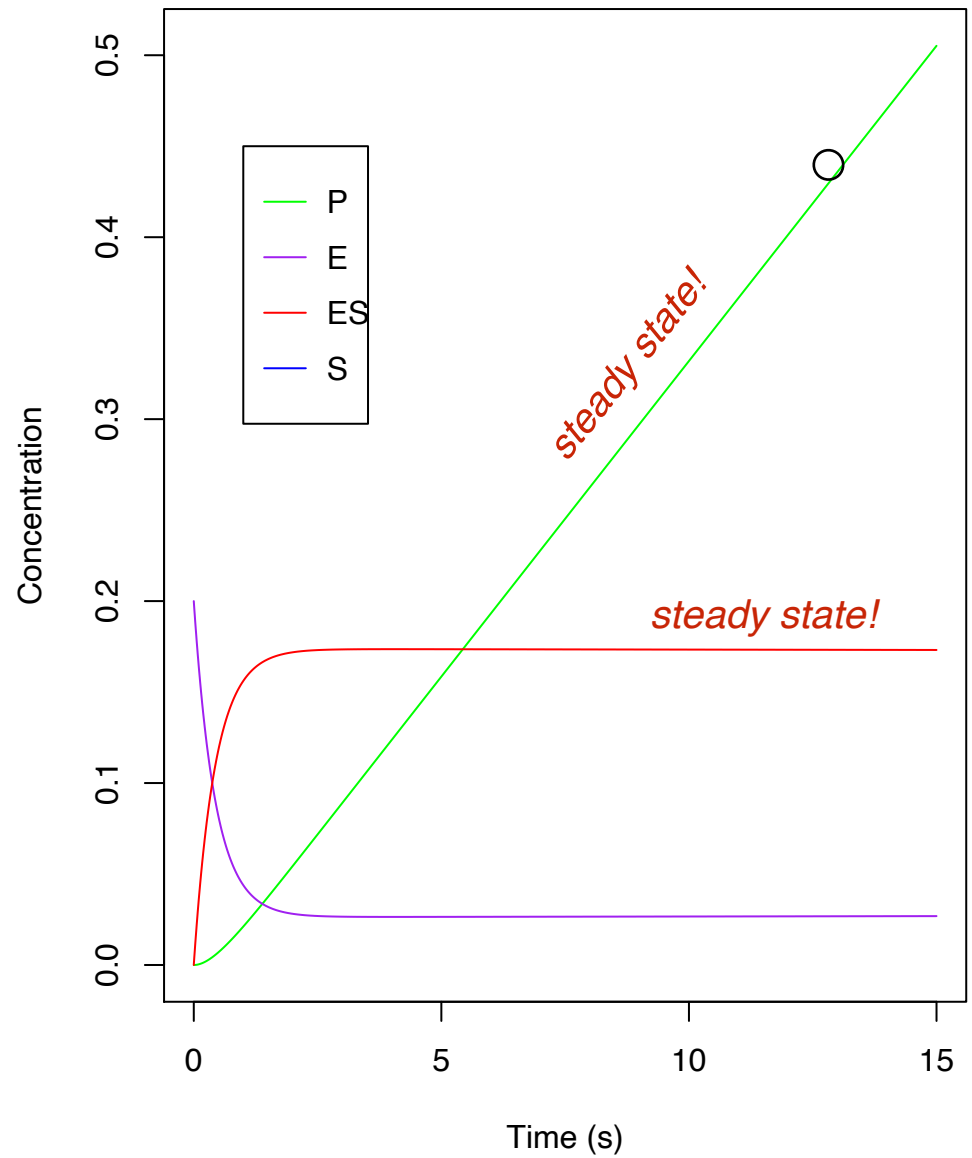

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- Then just run the script
  - source("EnzymeKinetics.txt")
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Enzyme Kinetics

~~Assume [P] negligible~~

~~Assume steady state~~

[Enz] = 0.2 μM

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$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=15, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2, E0=0.2, S0=20.0

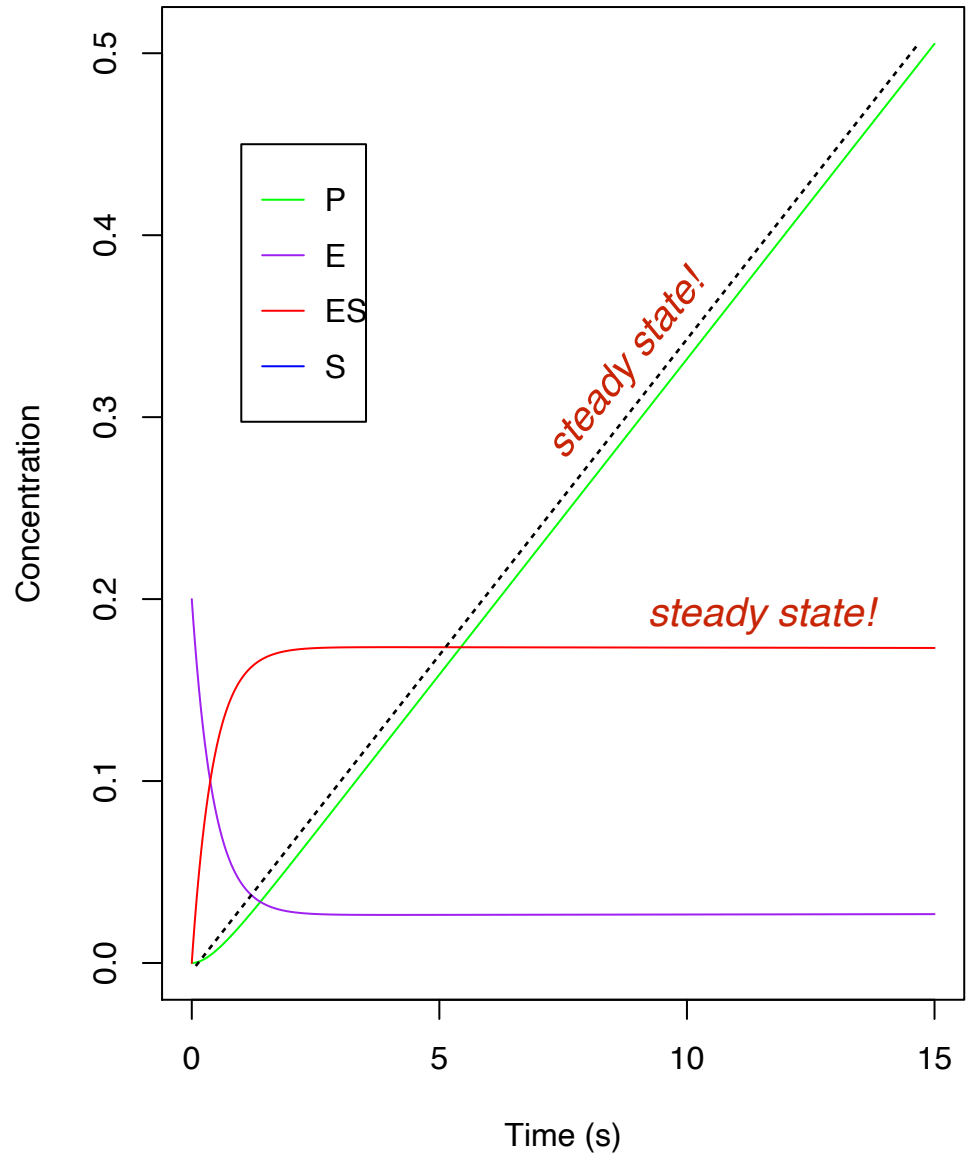
k_1 = 0.1 μM^{-1} s^{-1}

k_{-1} = 0.1 μM^{-1} s^{-1}

k_{cat} = 0.2 s^{-1}

k_r = 0.001 s^{-1}

(K_m = 1.0 μM)



Break out exercise

- Drop the substrate concentration from 20 μM to 2 μM
- Replot

Enzyme Kinetics

~~Assume [P] negligible~~

~~Assume steady state~~

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=15, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2,
E0=0.2, S0=2.0

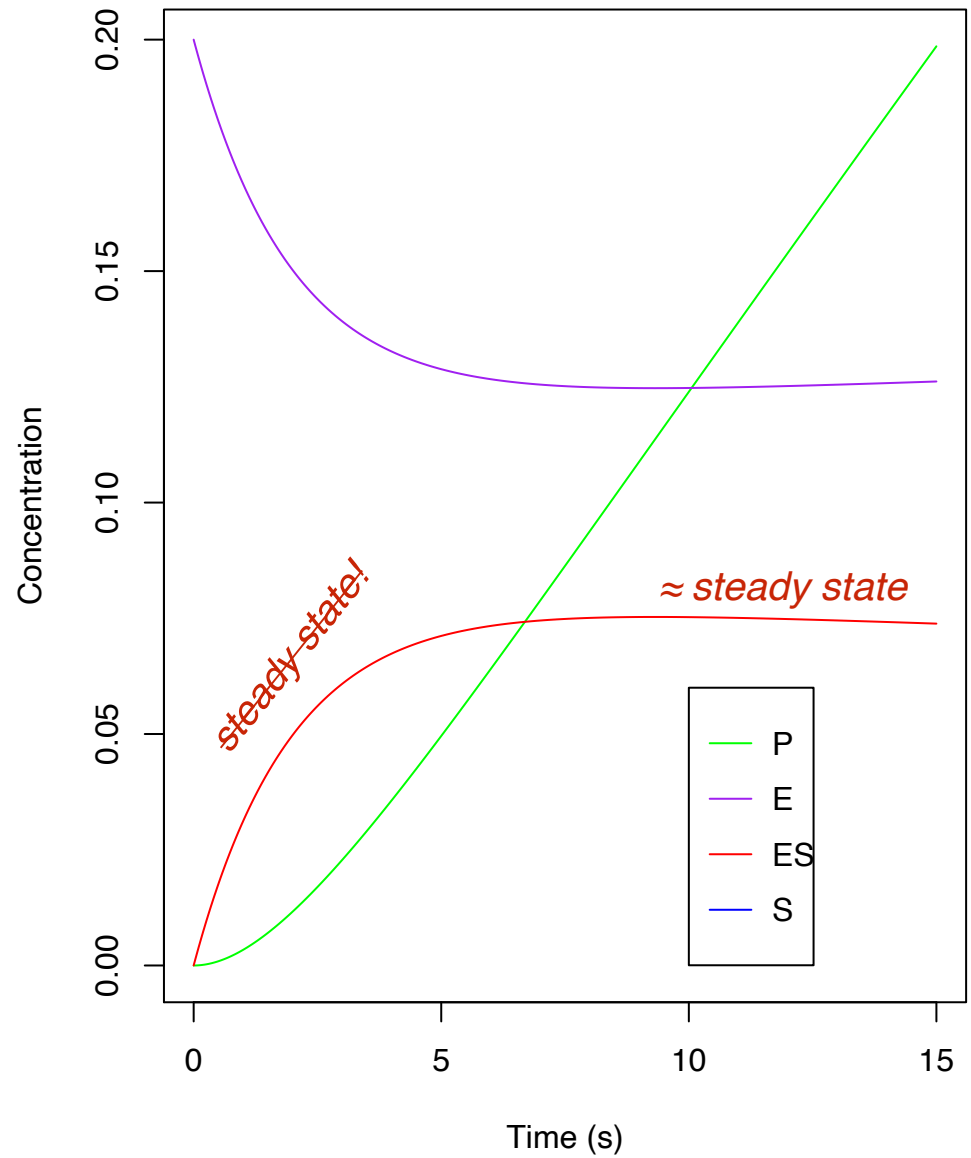
k_1 = 0.1 μM^{-1} s^{-1}

k_{-1} = 0.1 μM^{-1} s^{-1}

k_{cat} = 0.2 s^{-1}

k_r = 0.001 s^{-1}

(K_m = 1.0 μM)



Break out exercise

- Keep substrate at 2 μM
- Run the reaction for 150 s
- Replot

Enzyme Kinetics

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_c[ES] + k_r[E][P]$$

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_c[ES] - k_r[E][P]$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$

$$\frac{d[P]}{dt} = k_c[ES] - k_r[E][P]$$

tf=150, tincr=0.01, k1p=0.1, km1p=0.1, kcp=0.2,
E0=0.2, S0=2.0

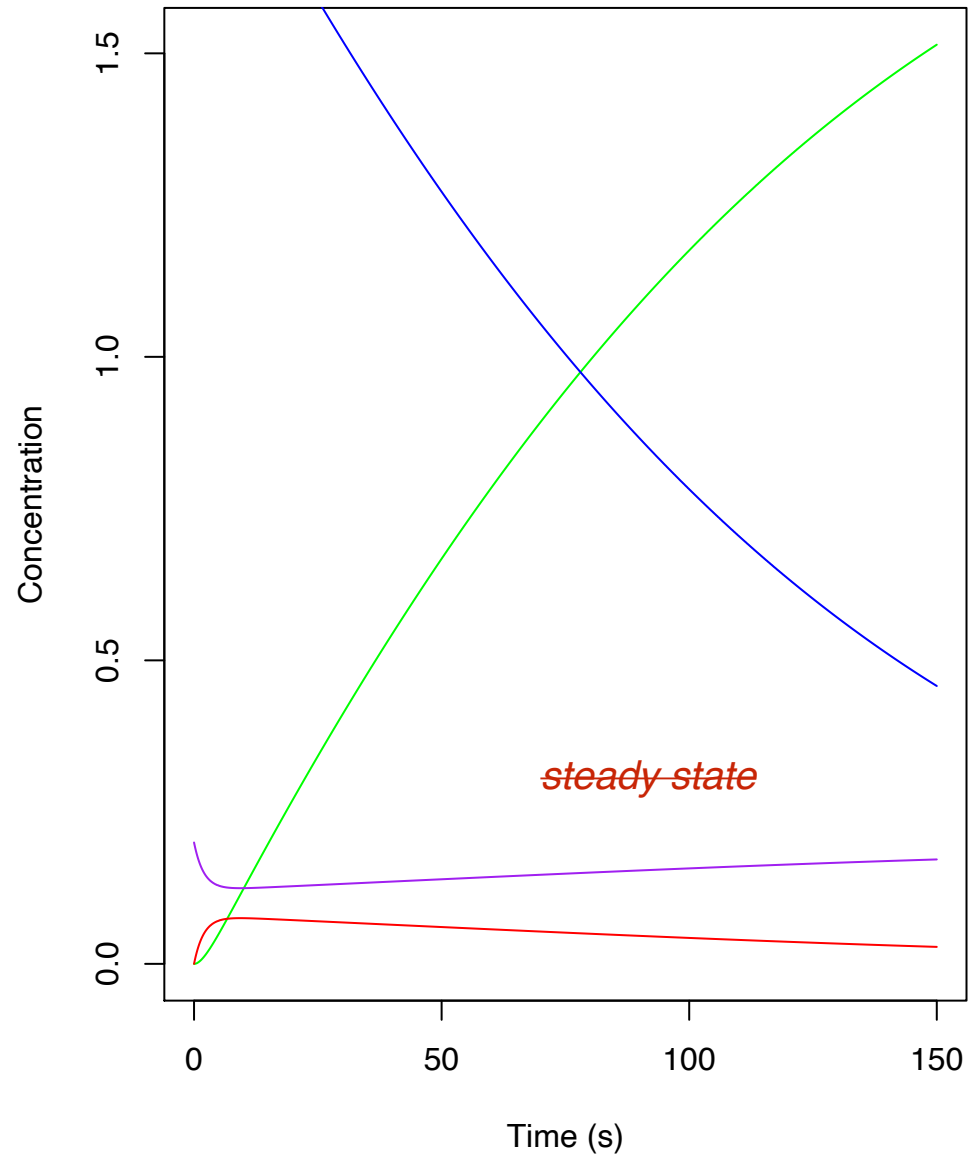
k_1 = 0.1 μM^{-1} s^{-1}

k_{-1} = 0.1 μM^{-1} s^{-1}

k_{cat} = 0.2 s^{-1}

k_r = 0.001 s^{-1}

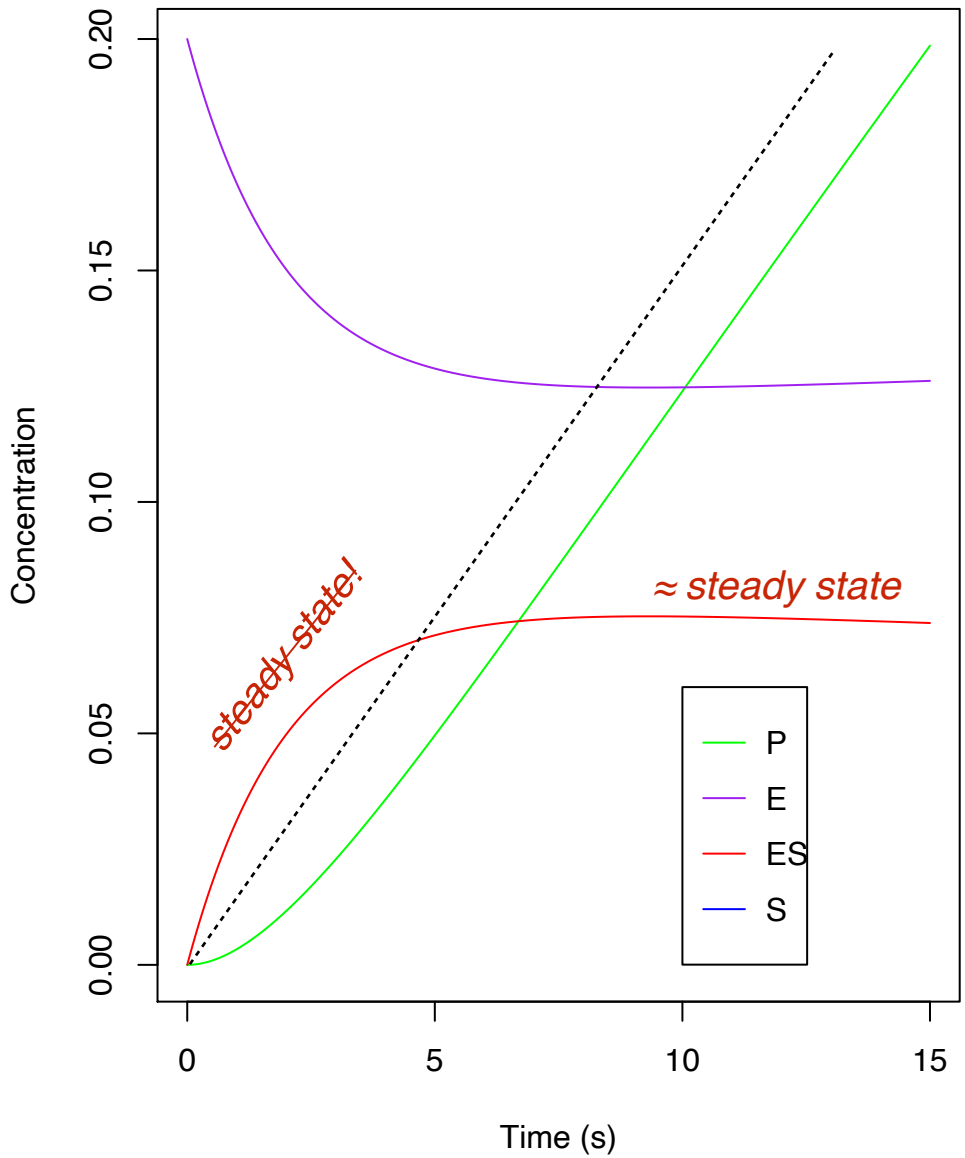
(K_m = 1.0 μM)

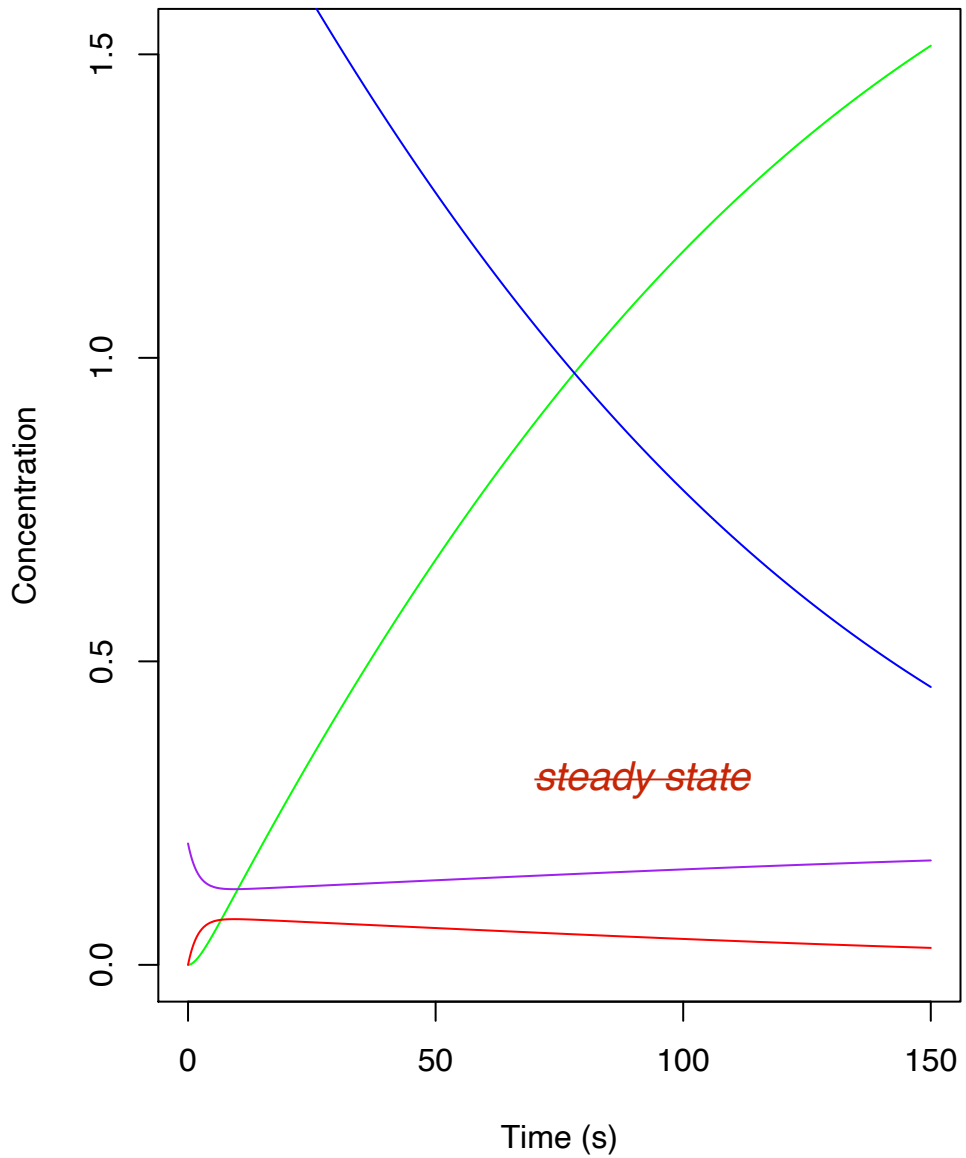


Enzyme Kinetics

[Enz] = 0.2 μM

$$E + S \underset{0.1\ s^{-1}}{\overset{0.1\ \mu M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{0.001\ \mu M^{-1}\ s^{-1}}{\overset{0.2\ s^{-1}}{\rightleftharpoons}} E + P$$





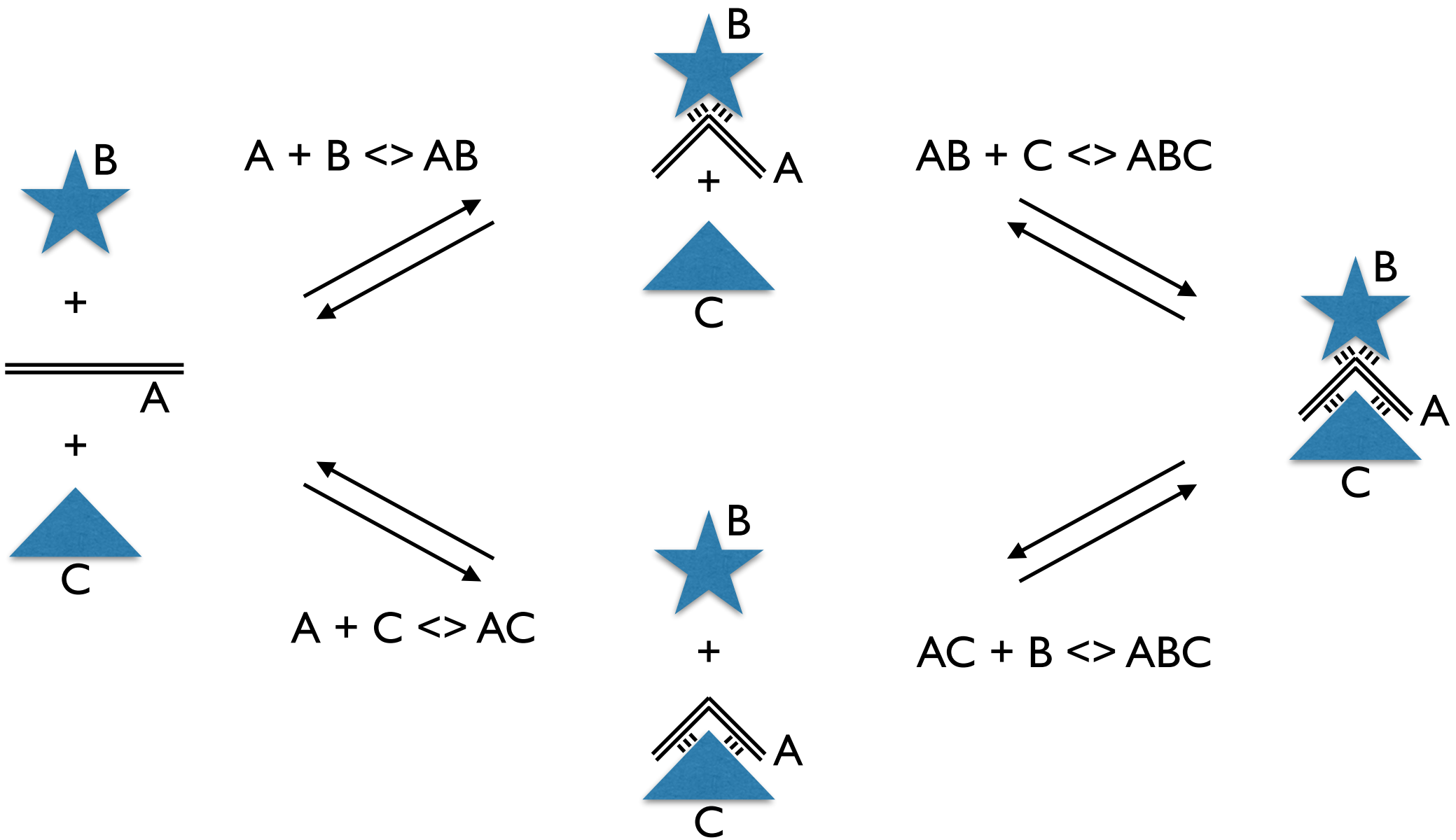
Break out exercise

- Let's an inhibitor
- Replot

$$E + I \rightleftharpoons EI$$

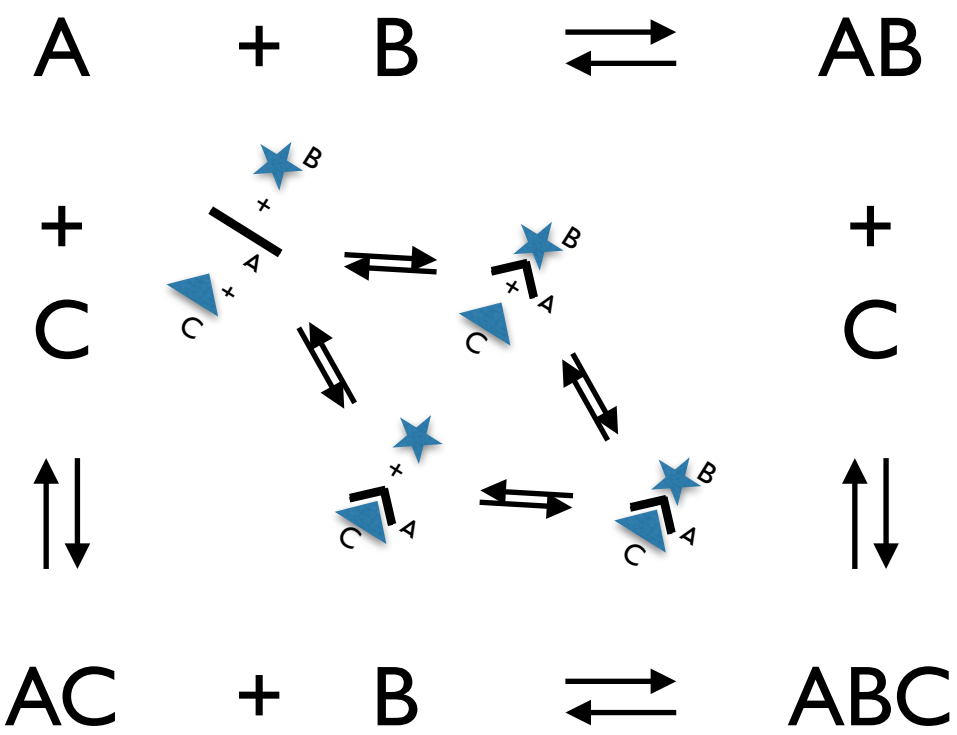
Cooperativity

Structural/chemical adaptation

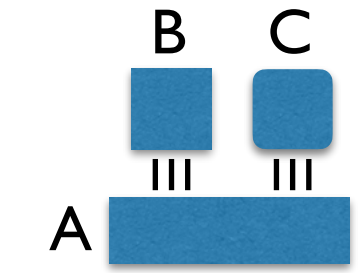


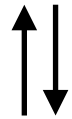
Cooperativity

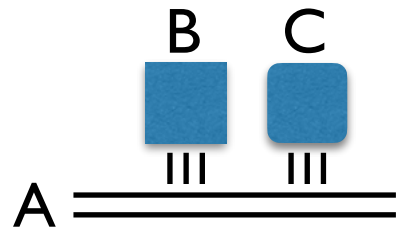
Structural/chemical adaptation

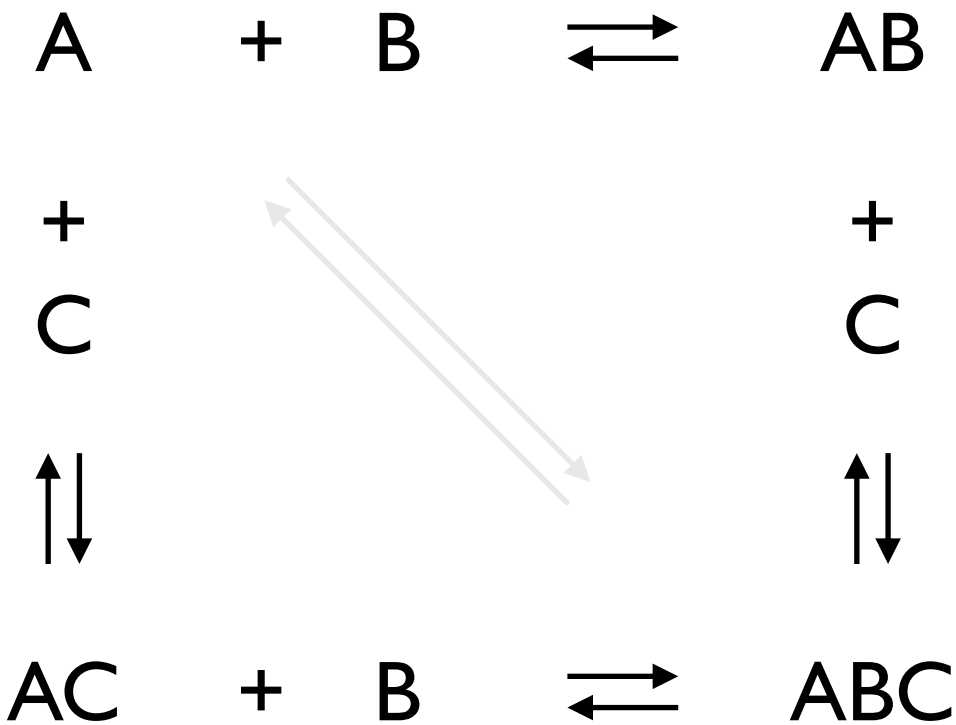


Independent Binding



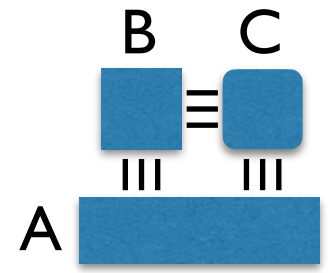


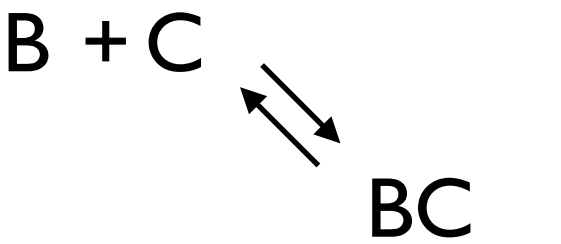


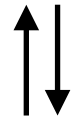


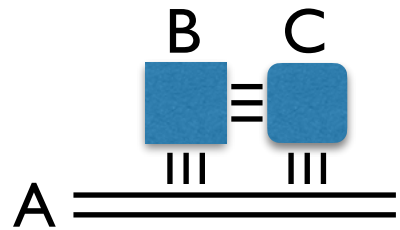
Cooperativity

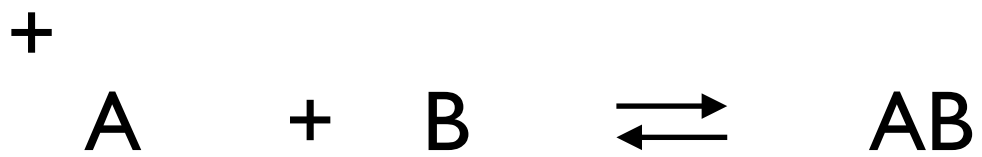
Local tethering





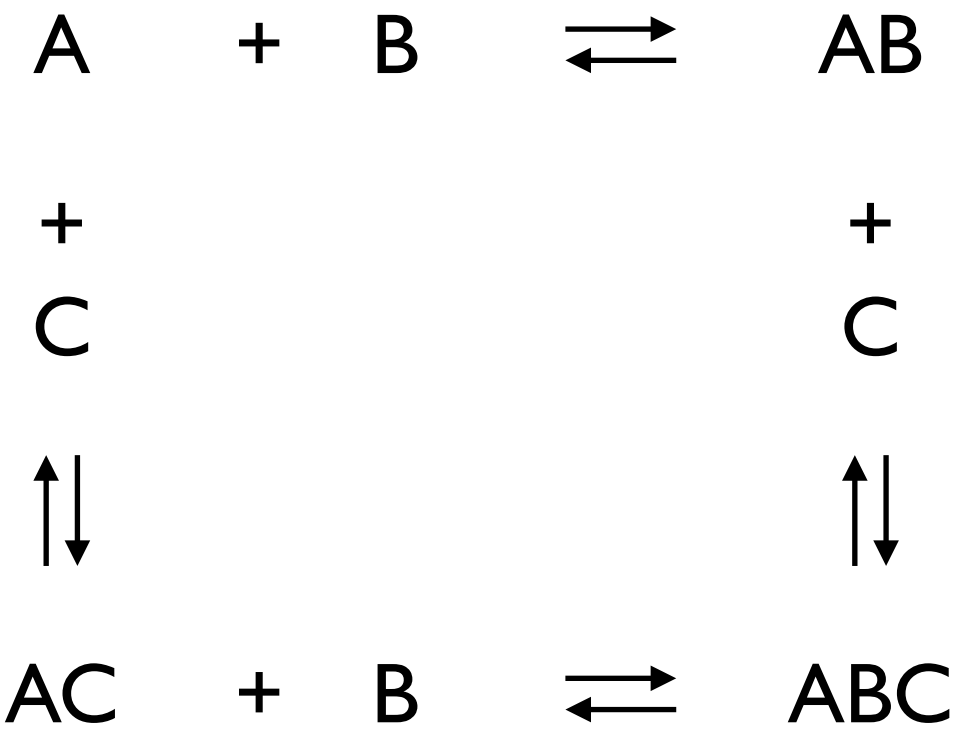


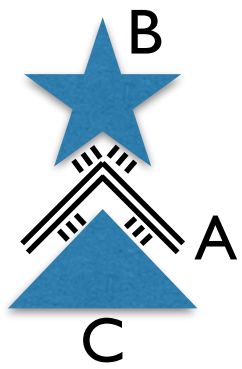




Cooperativity

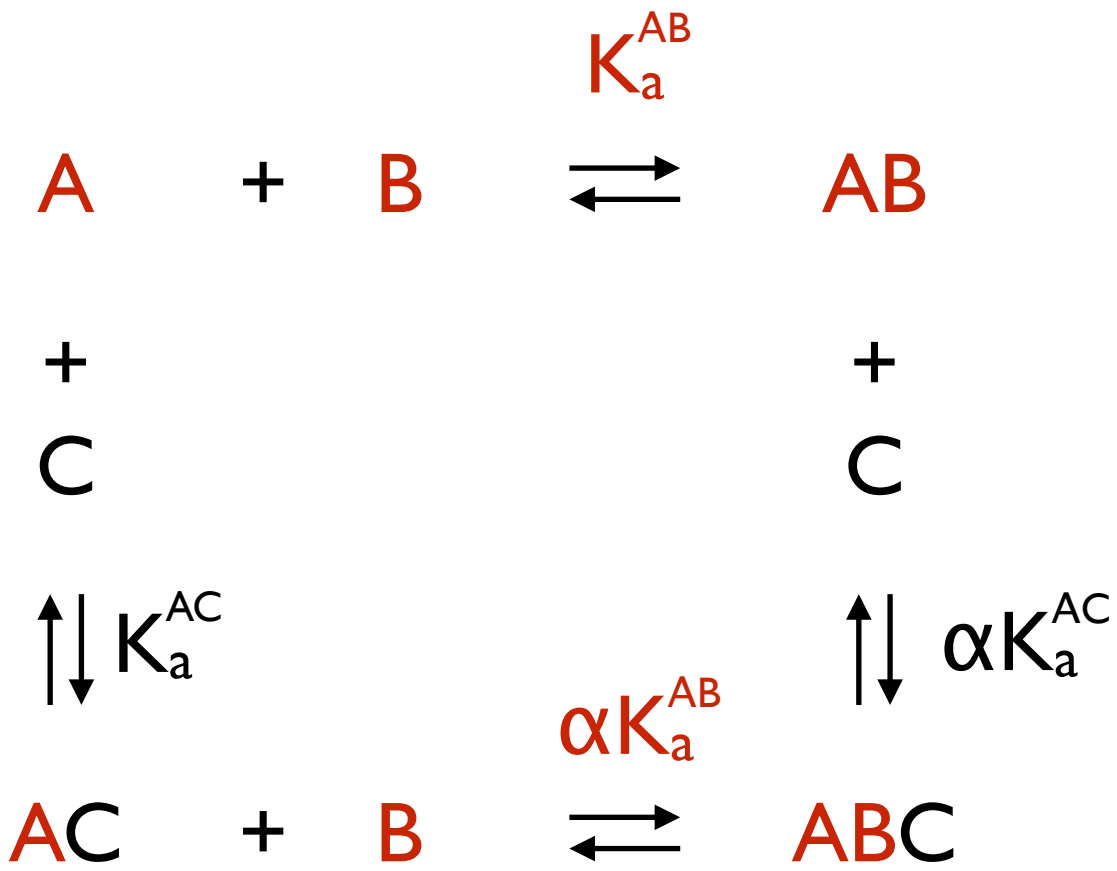
Simple 3 component





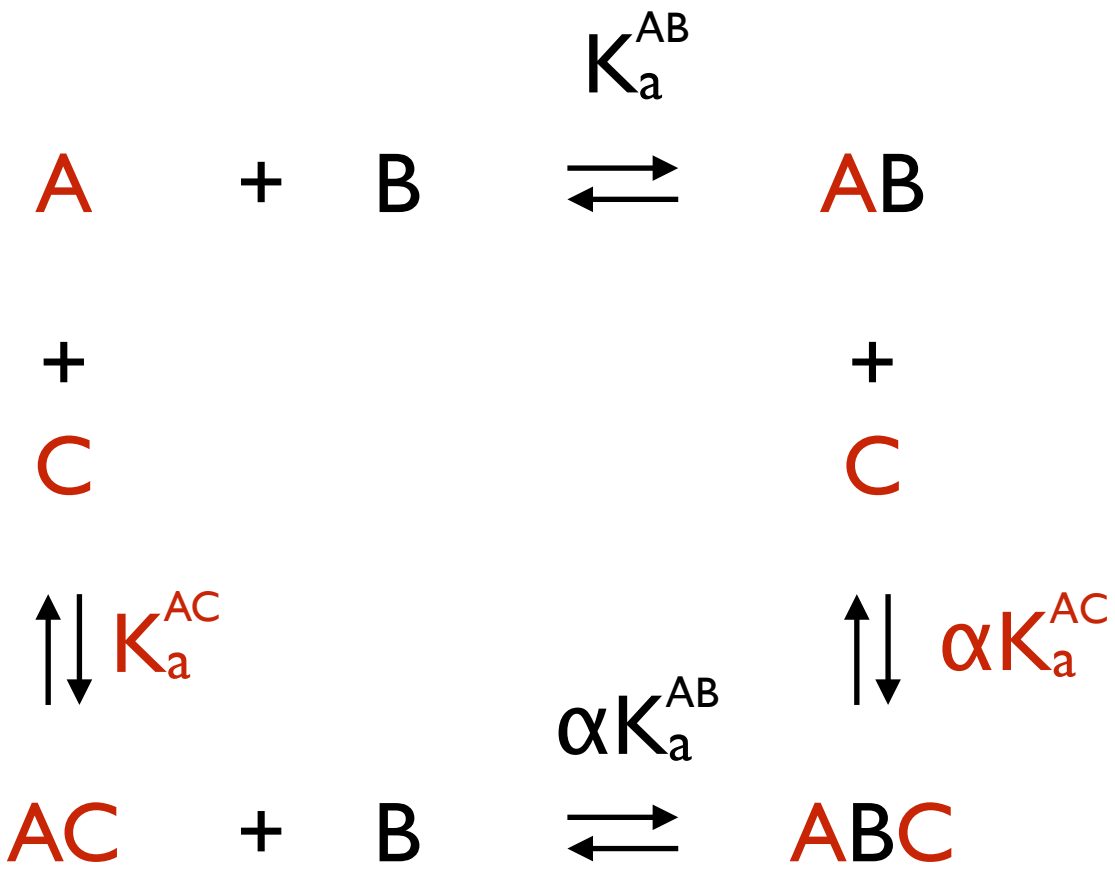
Cooperativity

Simple 3 component



Cooperativity

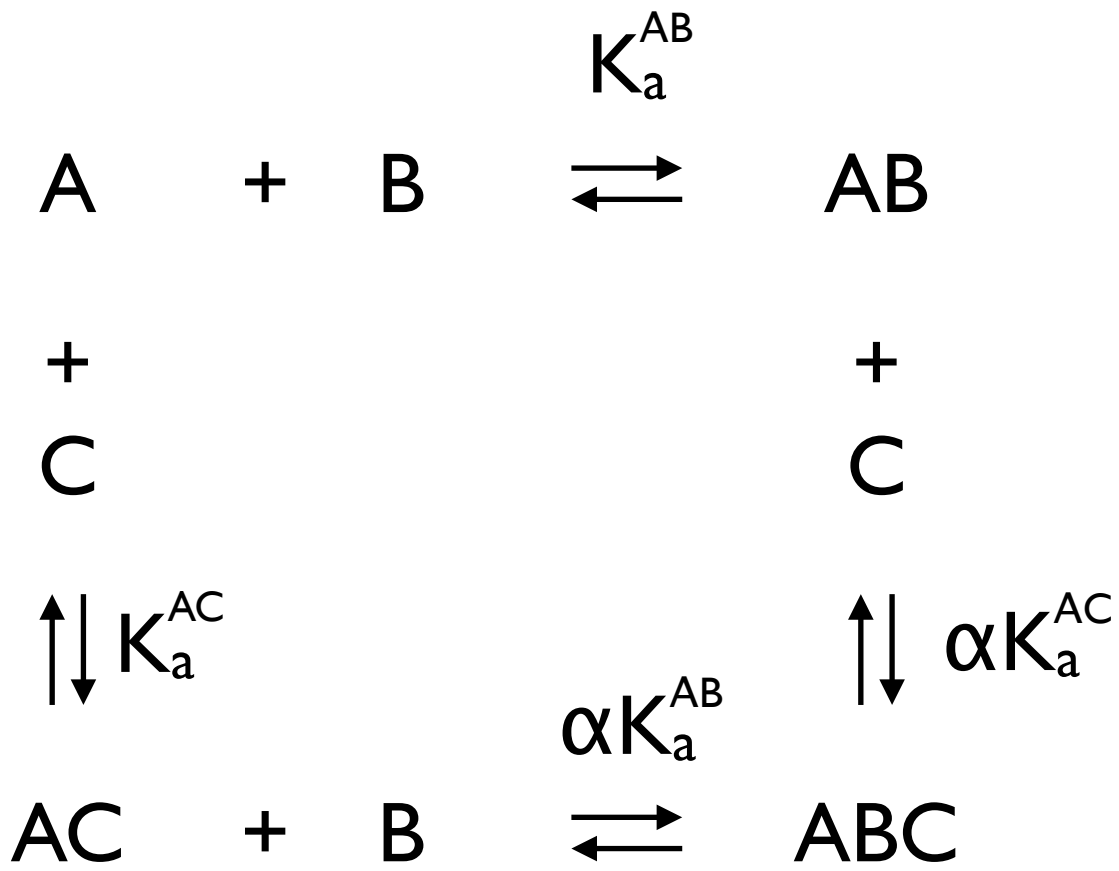
Simple 3 component



Cooperativity

Simple 3 component

How do I know that α applies to BOTH?



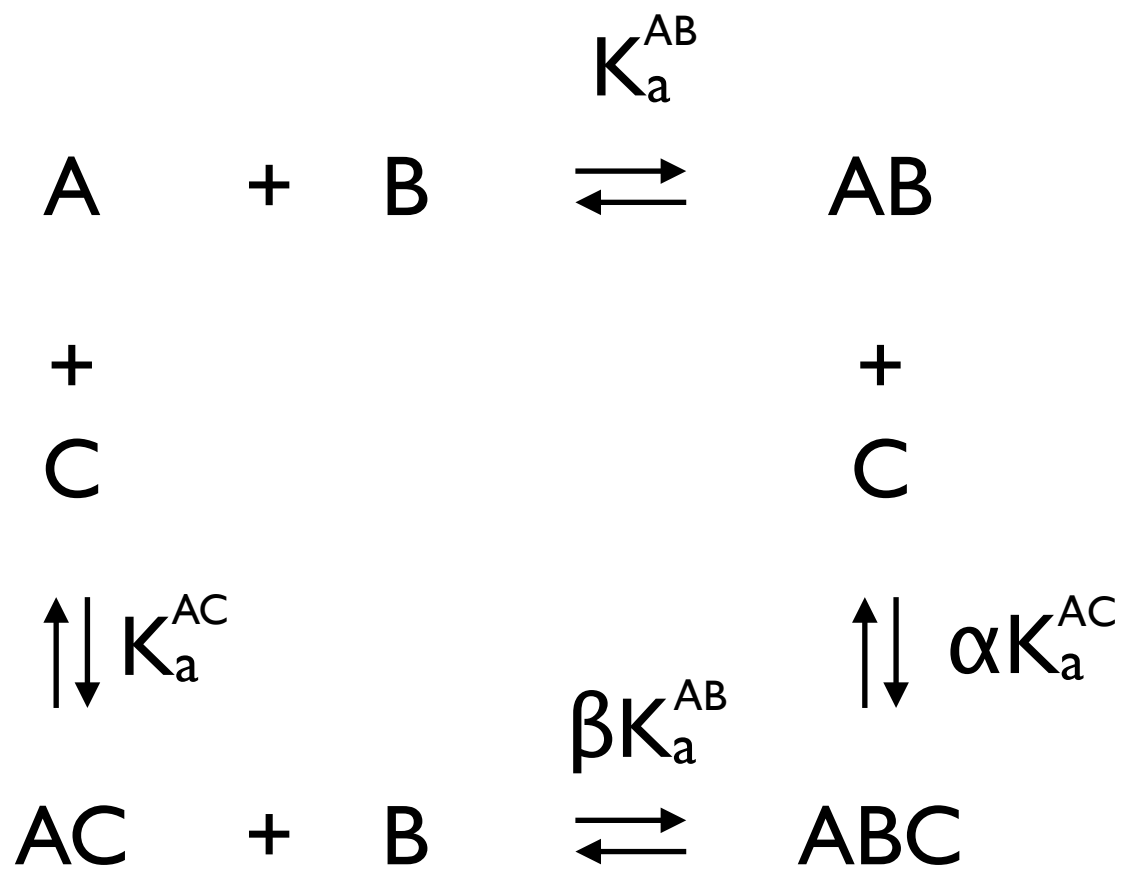
$$K_a^{AB} = \frac{[AB]}{[A][B]}$$

$$\beta K_a^{AB} = \frac{[ABC]}{[AC][B]}$$

$$K_a^{AC} = \frac{[AC]}{[A][C]}$$

$$\alpha K_a^{AC} = \frac{[ABC]}{[AB][C]}$$

$$[ABC] = \alpha K_a^{AC} [AB][C]$$



$$K_a^{AB} = \frac{[AB]}{[A][B]}$$

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$$K_a^{AC} = \frac{[AC]}{[A][C]}$$

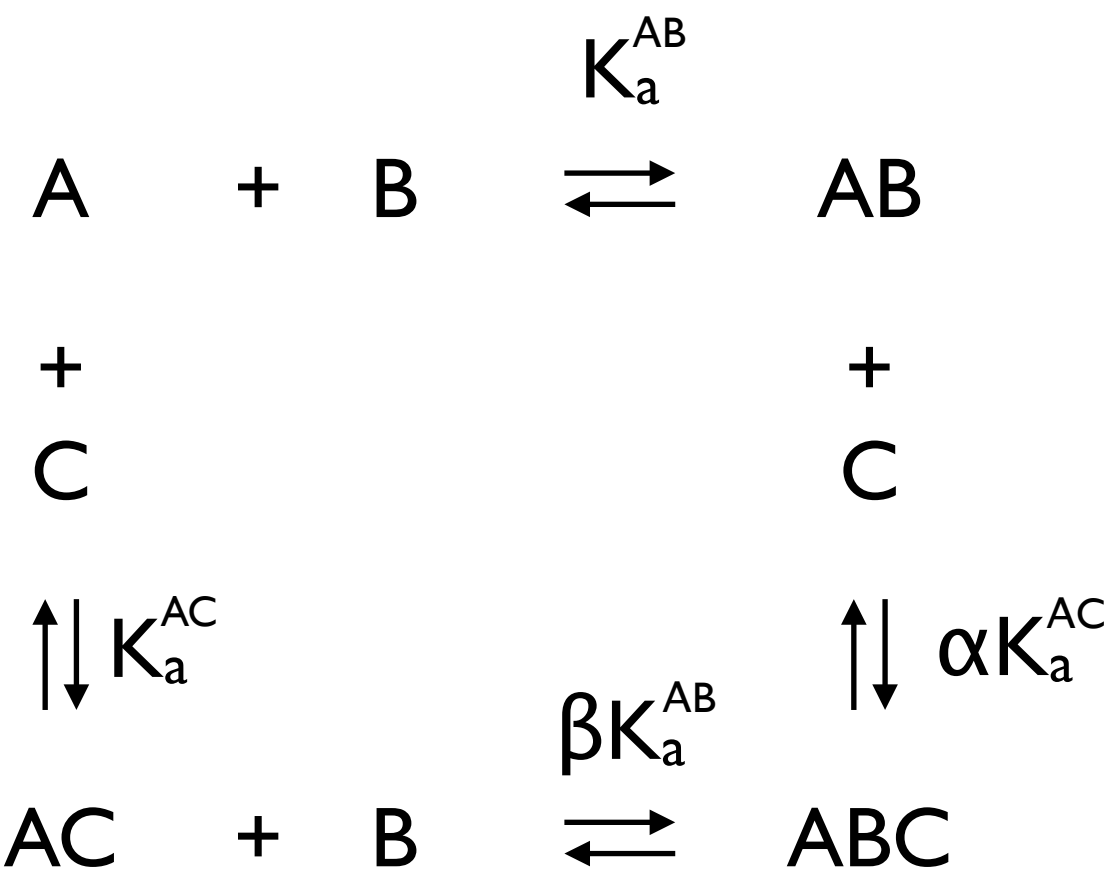
$$\alpha K_a^{AC} = \frac{[ABC]}{[AB][C]}$$

$$K_a^{AB} = \frac{[AB]}{[A][B]}$$

$$\beta K_a^{AB} = \frac{\alpha K_a^{AC}[AB][C]}{[AC][B]}$$

$$K_a^{AC} = \frac{[AC]}{[A][C]}$$

$$[ABC] = \alpha K_a^{AC}[AB][C]$$



$$K_a^{AB} = \frac{[AB]}{[A][B]}$$

$$\beta K_a^{AB} = \frac{[ABC]}{[AC][B]}$$

$$K_a^{AC} = \frac{[AC]}{[A][C]}$$

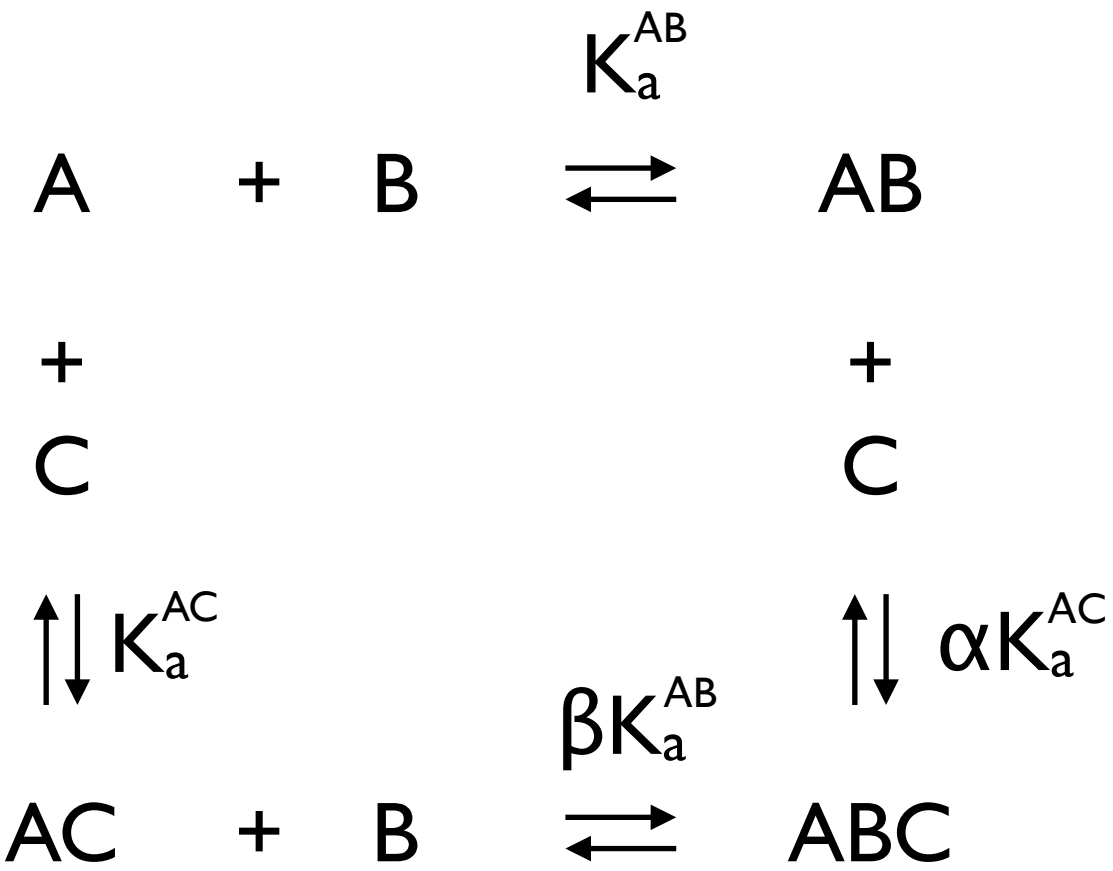
$$\alpha K_a^{AC} = \frac{[ABC]}{[AB][C]}$$

$$K_a^{AB} = \frac{[AB]}{[A][B]}$$

$$\beta K_a^{AB} = \frac{\alpha K_a^{AC}[AB][C]}{[AC][B]}$$

$$K_a^{AC} = \frac{[AC]}{[A][C]}$$

$$\beta K_a^{AB} = \frac{\alpha K_a^{AC} K_a^{AB}[A][B][C]}{K_a^{AC}[A][C][B]}$$



$$K_a^{AB} = \frac{[AB]}{[A][B]}$$

$$\beta K_a^{AB} = \frac{[ABC]}{[AC][B]}$$

$$K_a^{AC} = \frac{[AC]}{[A][C]}$$

$$\alpha K_a^{AC} = \frac{[ABC]}{[AB][C]}$$

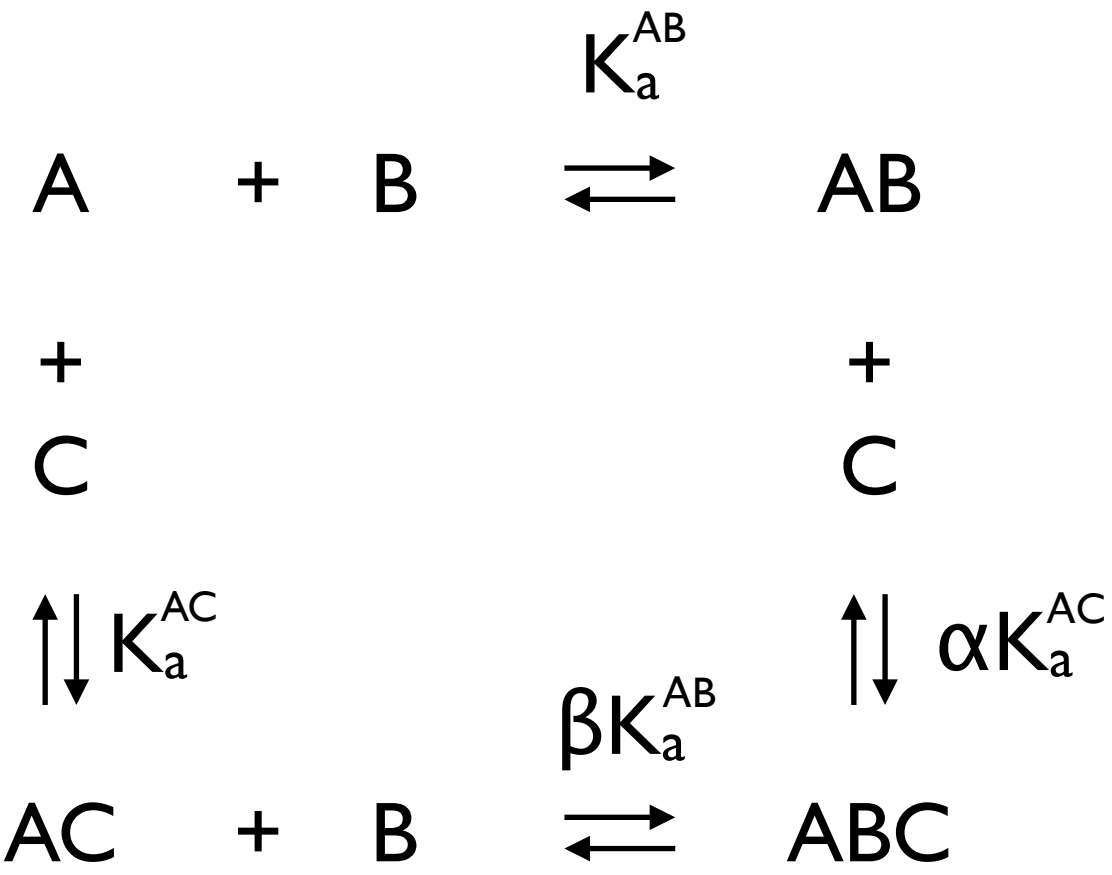
$$K_a^{AB} = \frac{[AB]}{[A][B]}$$

$$\beta K_a^{AB} = \frac{\alpha K_a^{AC}[AB][C]}{[AC][B]}$$

$$K_a^{AC} = \frac{[AC]}{[A][C]}$$

$$\beta K_a^{AB} = \frac{\alpha K_a^{AC} K_a^{AB}[A][B][C]}{K_a^{AC}[A][C][B]}$$

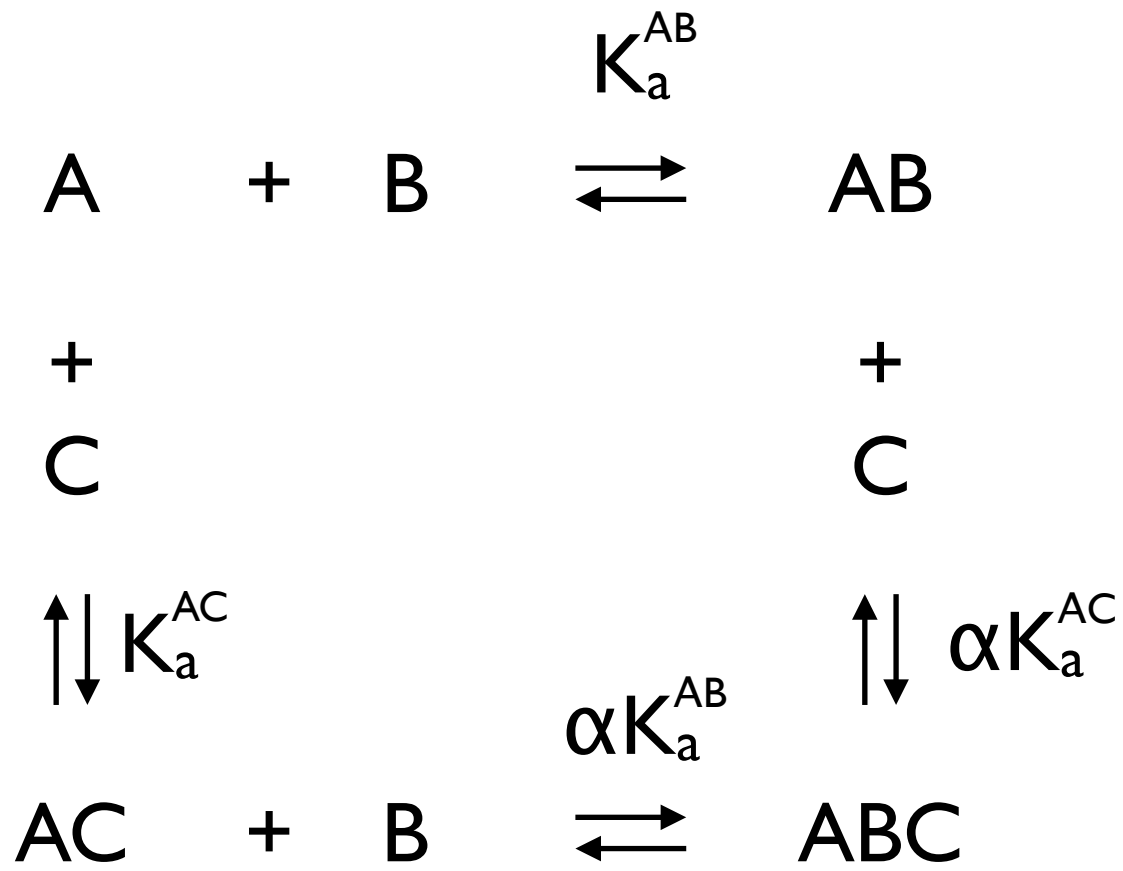
$$\beta = \alpha$$



Cooperativity

applies to K_{assoc}

	Cooperativity	Effect on Affinity
α>1	positive	increases
α=1	none	none
α<1	negative	decreases



Cooperativity

	Cooperativity	Effect on Affinity
α>1	positive	increases
α=1	none	none
α<1	negative	decreases

at [C]=0

Ligand in excess

$$[B] \approx [B]_{Total}$$

$$frac = \frac{[AB]}{[A]_{Total}} \approx \frac{[B]}{[B] + 1/K_a^{AB}}$$

$$A + B \rightleftharpoons AB \quad (K_a^{AB})$$

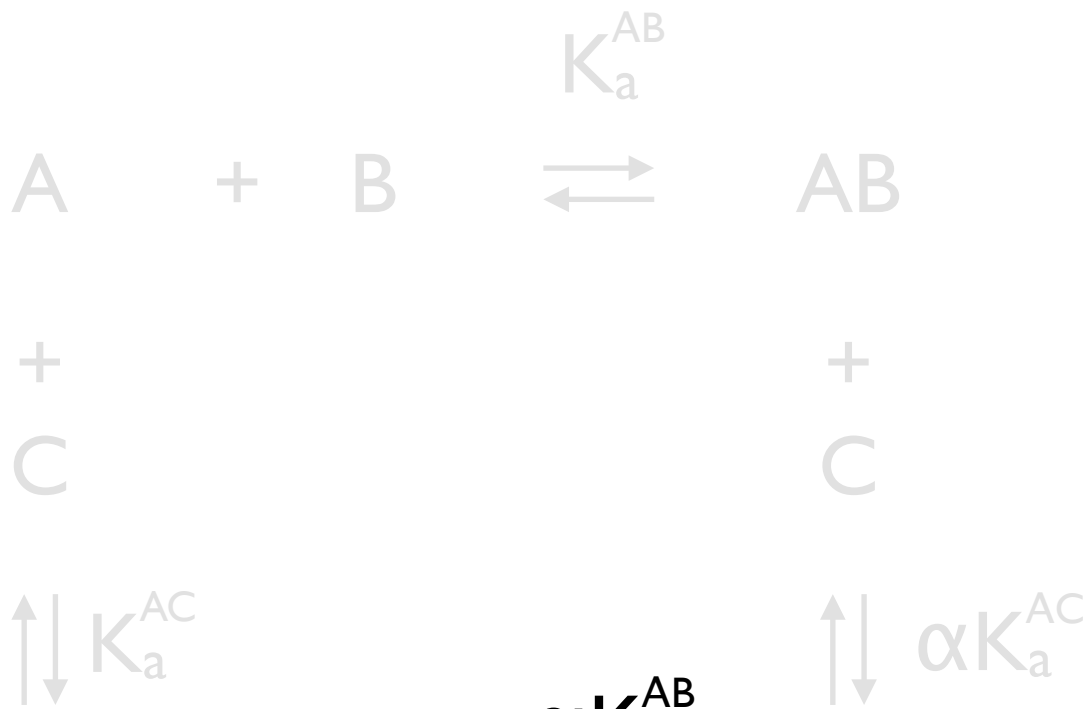
$$+ C \quad \updownarrow K_a^{AC}$$

$$AC + B \rightleftharpoons ABC \quad (\alpha K_a^{AB})$$

$$+ C \quad \updownarrow \alpha K_a^{AC}$$

Cooperativity

	Cooperativity	Effect on Affinity
α>1	positive	increases
α=1	none	none
α<1	negative	decreases



at saturating [C]

Ligand in excess

$$[B] \approx [B]_{Total}$$

$$frac = \frac{[AB]}{[A]_{Total}} \approx \frac{[B]}{[B] + {}^{1}\!/\!_{\alpha K_a^{AB}}}$$

$$AC + B \rightleftharpoons^{\alpha K_a^{AB}} ABC$$

Cooperativity

	Cooperativity	Effect on Affinity
α>1	positive	increases
α=1	none	none
α<1	negative	decreases

at [C]=0

$$[B] \approx [B]_{Total}$$

$$frac = \frac{[AB]}{[A]_{Total}} \approx \frac{[B]}{[B] + 1/K_a^{AB}}$$

$$A + B \rightleftharpoons AB \quad (K_a^{AB})$$

$$+ C \qquad + C$$

$$\updownarrow K_a^{AC} \qquad \updownarrow \alpha K_a^{AC}$$

at saturating [C]

$$[B] \approx [B]_{Total}$$

$$frac = \frac{[AB]}{[A]_{Total}} \approx \frac{[B]}{[B] + 1/\alpha K_a^{AB}}$$

$$AC + B \rightleftharpoons ABC \quad (\alpha K_a^{AB})$$

Cooperativity - Multiple identical sites

	Cooperativity	Effect on Affinity
α>1	positive	increases
α=1	none	none
α<1	negative	decreases

at very low [B]

$K_{obs} \approx K_a$

$$AB + B \rightleftharpoons AB \qquad K_{obs} \approx \frac{[AB]}{[A][B]}$$

$$AB_i + B \rightleftharpoons AB_{i+1}$$

at very high [B]

$K_{obs} \approx \alpha K_a$

$$AB_{n-1} + B \rightleftharpoons AB_n \qquad K_{obs} \approx \frac{[AB_n]}{[AB_{n-1}][B]}$$

Multiple independent, identical sites

$$K_d = \frac{[P]\,[L]}{[PL]}$$

$$K_d = \frac{[P]\,[L]^n}{[PL_n]}$$

$$P + L \rightleftharpoons PL \qquad P + L \rightleftharpoons PL + PL_2 + \ldots + PL_n$$

$$\nu = \frac{[bound]}{[protein]} = \frac{[bound]}{[unbound] + [bound]} \qquad \theta = \frac{[boundsites]}{[totalsites]} = \frac{[boundsites]}{[unboundssites] + [boundssites]}$$

from Scatchard et al., single binding site (n=1)

$[B] \approx [B]_{Total}$

Ligand in excess

$$\nu = \frac{[L]}{K_d + [L]}$$

$$\frac{\nu}{1-\nu} = \frac{[L]}{K_d}$$

$$log(\frac{\nu}{1-\nu}) = log([L]) - log(K_d)$$

Multiple independent, identical sites

$$K_d = \frac{[P]\,[L]}{[PL]}$$

$$K_d = \frac{[P]\,[L]^n}{[PL_n]}$$

$$P + L \rightleftharpoons PL \qquad P + L \rightleftharpoons PL + PL_2 + \ldots + PL_n$$

$$\nu = \frac{[bound]}{[protein]} = \frac{[bound]}{[unbound] + [bound]} \qquad \theta = \frac{[boundsites]}{[totalsites]} = \frac{[boundsites]}{[unboundssites] + [boundssites]}$$

from Scatchard et al., single binding site (n=1)

$[B] \approx [B]_{Total}$
Ligand in excess

$$\nu = \frac{[L]}{K_d + [L]}$$

purely empirical!

$$\theta = \frac{[L]^n}{K_d + [L]^n}$$

$$\frac{\nu}{1-\nu} = \frac{[L]}{K_d}$$

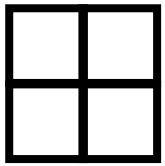
$$\frac{\theta}{1-\theta} = \frac{[L]^n}{K_d}$$

$$log(\frac{\nu}{1-\nu}) = log([L]) - log(K_d)$$

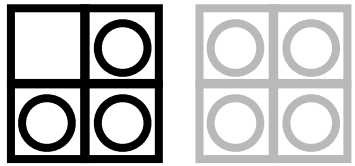
$$log(\frac{\theta}{1-\theta}) = n\ log([L]) - log(K_d)$$

$$K_d = \frac{[P]\,[L]^n}{[PL_n]}$$

$$P + L \rightleftharpoons PL + PL_2 + \ldots + PL_n$$



Sites all empty



Other sites all full

Max coop

No coop Early

No coop Late

independent sites

$$log(\frac{\theta}{1-\theta}) = n\ log([L]) - log(K_d)$$

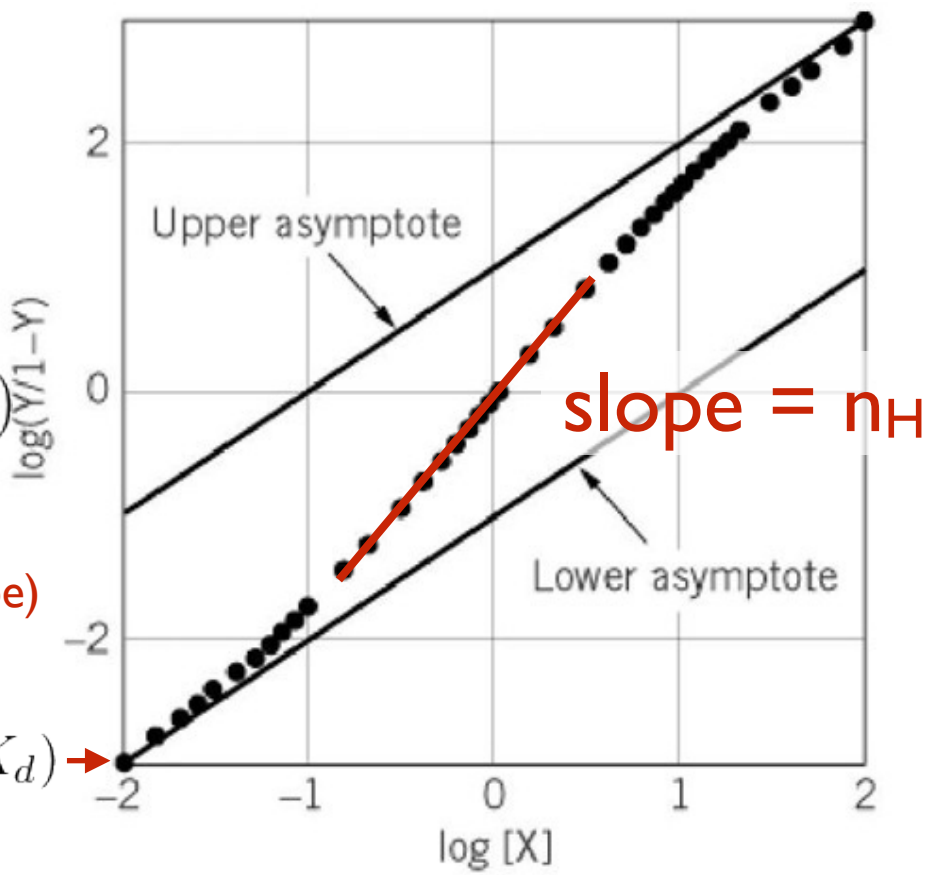
constant

dependent (cooperative) sites

$$log(\frac{\theta}{1-\theta}) = n_H\ log([L]) - log(K_d)$$

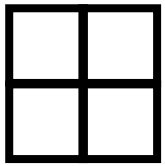
varies with [L]

The reported Hill coefficient is the maximal n_H (slope)

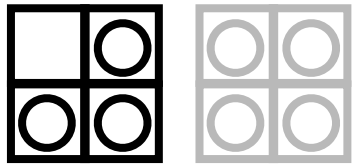


$$K_d = \frac{[P]\,[L]^n}{[PL_n]}$$

$$P + L \rightleftharpoons PL + PL_2 + \ldots + PL_n$$



Sites all empty



Other sites all full

Max coop

No coop Early

No coop Late

dependent (cooperative) sites

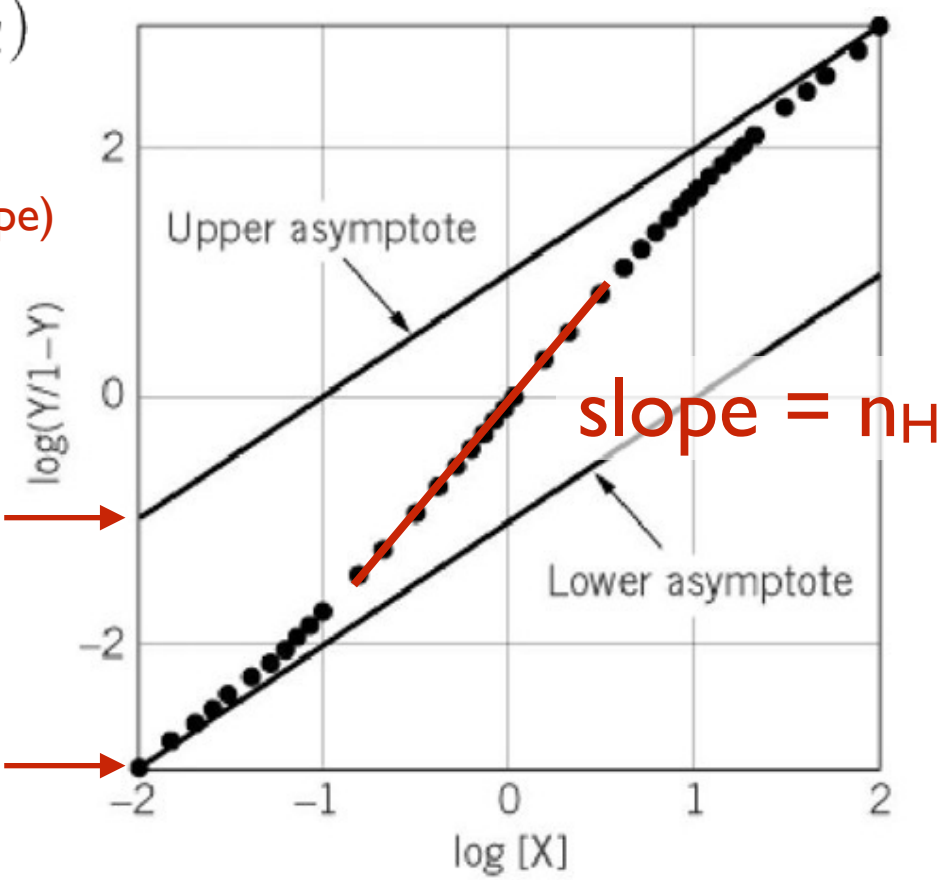
$$log(\frac{\theta}{1-\theta}) = n_H \; log([L]) - log(K_d)$$

varies with [L]

The reported Hill coefficient is the maximal n_H (slope)

$-log(K_d^{coop})$

$-log(K_d^{empty})$



$$K_d = \frac{[P]\,[L]^n}{[PL_n]}$$

$$P + L \rightleftharpoons PL + PL_2 + \ldots + PL_n$$

dependent (cooperative) sites

$$log(\frac{\theta}{1-\theta}) = n_H \; log([L]) - log(K_d)$$

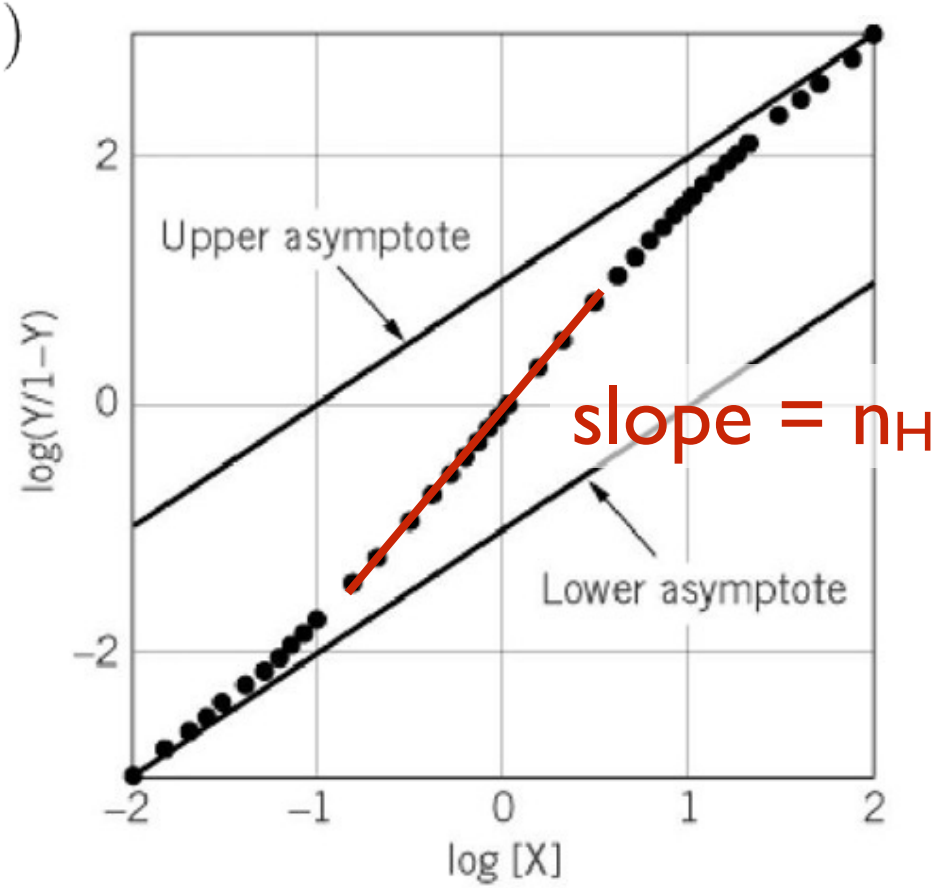
varies with [L]

From Wikipedia article on the Hill Equation:

"All of these formulations assume that the protein has *n* sites to which ligands can bind. In practice, however, the Hill Coefficient n_H rarely provides an accurate approximation of the number of ligand binding sites on a protein.[5] [7] Consequently, it has been observed that the Hill coefficient should instead be interpreted as an "interaction coefficient" describing the cooperativity among ligand binding sites.[5]"

From Wikipedia article on the Hill Equation:

"Transformations of equations into linear forms such as this were very useful before the widespread use of computers, as they allowed researchers to determine parameters by fitting lines to data. However, these transformations affect error propagation, and this may result in undue weight to error in data points near 0 or 1. [nb 2] This impacts the parameters of linear regression lines fitted to the data. Furthermore, the use of computers enables more robust analysis involving nonlinear regression."

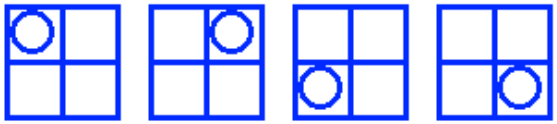


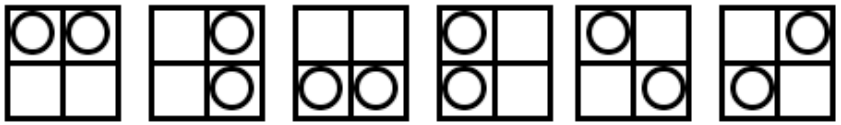
Multiple Binding Sites
Identical, Independent Sites

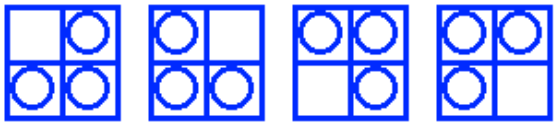
$$\bar{v} = \frac{nk[L]}{1 + k[L]}$$

$$\Omega_{n,i} = \frac{n!}{(n-i)!i!}$$

$M_o =$ 

$M_o + L \xrightarrow{k} M_1$ $\quad M_1 =$ 

$M_1 + L \xrightarrow{k} M_2$ $\quad M_2 =$ 

$M_2 + L \xrightarrow{k} M_3$ $\quad M_3 =$ 

$M_3 + L \xrightarrow{k} M_4$ $\quad M_4 =$ 

$$\Omega_{n,i} = \frac{4!}{4!0!} = 1$$

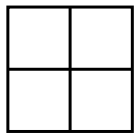
$$\Omega_{n,i} = \frac{4!}{3!1!} = 4$$

$$\Omega_{n,i} = \frac{4!}{2!2!} = 6$$

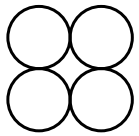
$$\Omega_{n,i} = \frac{4!}{1!3!} = 4$$

$$\Omega_{n,i} = \frac{4!}{0!4!} = 1$$

Models for Cooperativity
MWC - Monod, Wyman, Changeux



R - stronger ligand binding (k_r)

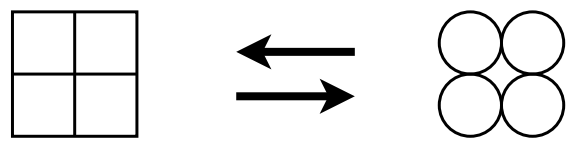


T - weaker ligand binding (k_t)

Mixed R & T - not allowed - two state only

Models for Cooperativity
MWC - Monod, Wyman, Changeux

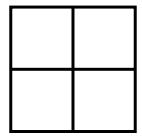
$$L_{RT} = \frac{T}{R}$$



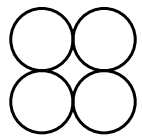
R - stronger (k_r)

T - weaker (k_t)

Models for Cooperativity
KNF - Koshland, Nemethy, Filmer

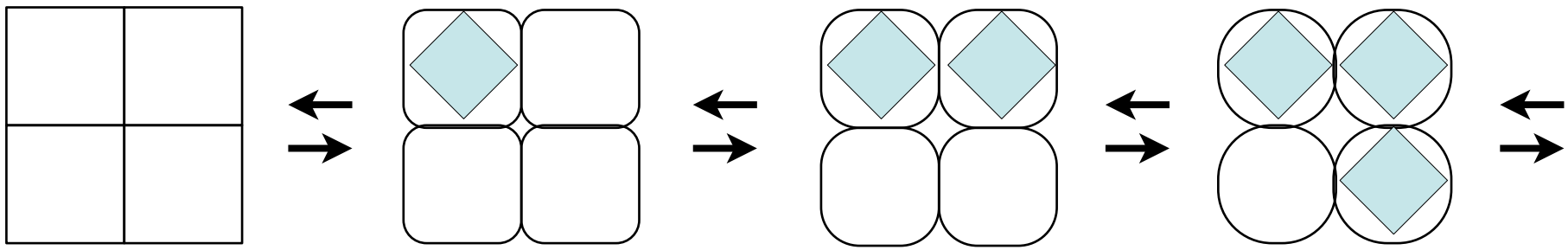


R - stronger ligand binding (k_r)



T - weaker ligand binding (k_t)

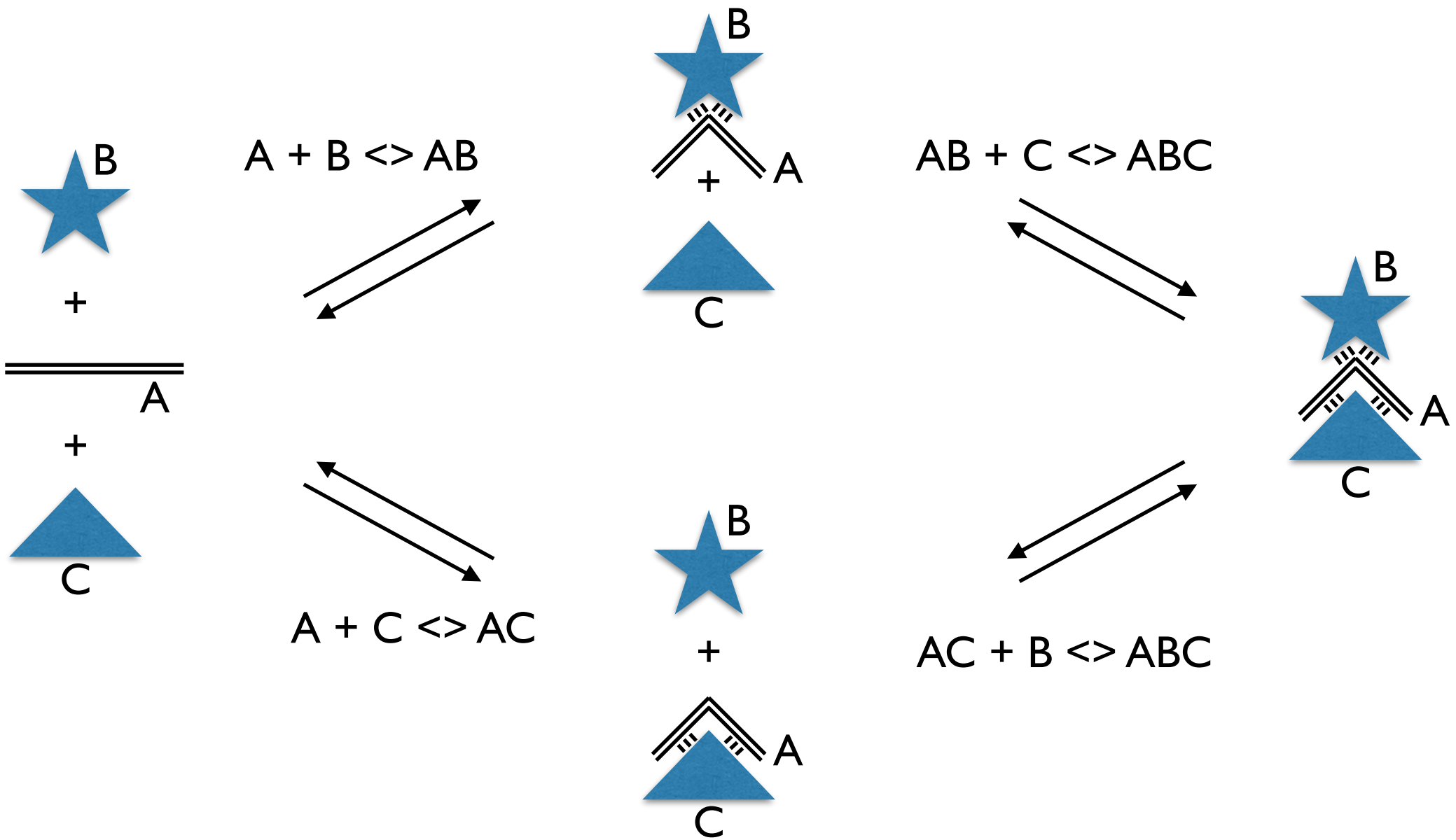
Intermediate states allowed



AKA - the Sequential Model

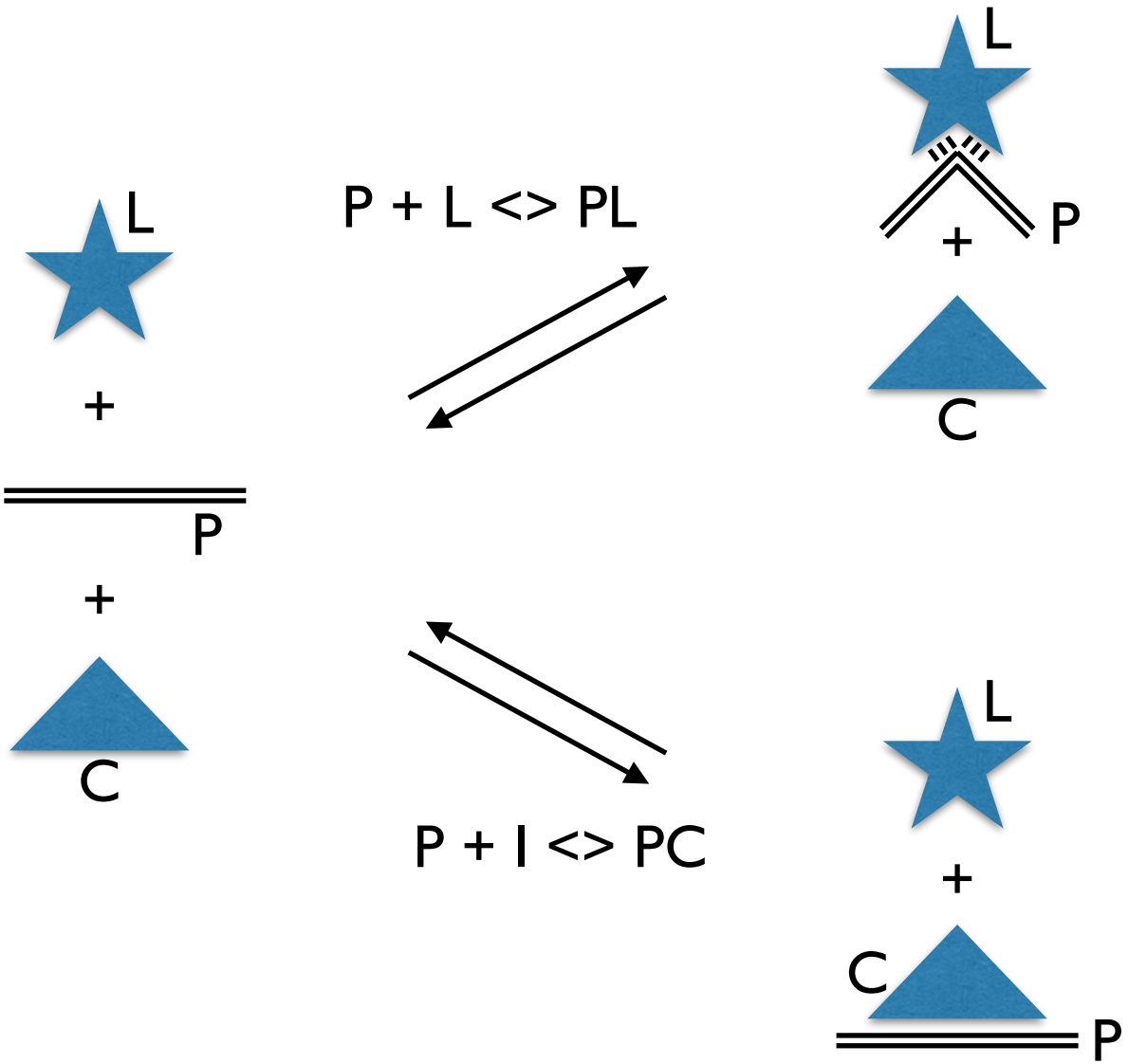
Cooperativity

Structural/chemical adaptation



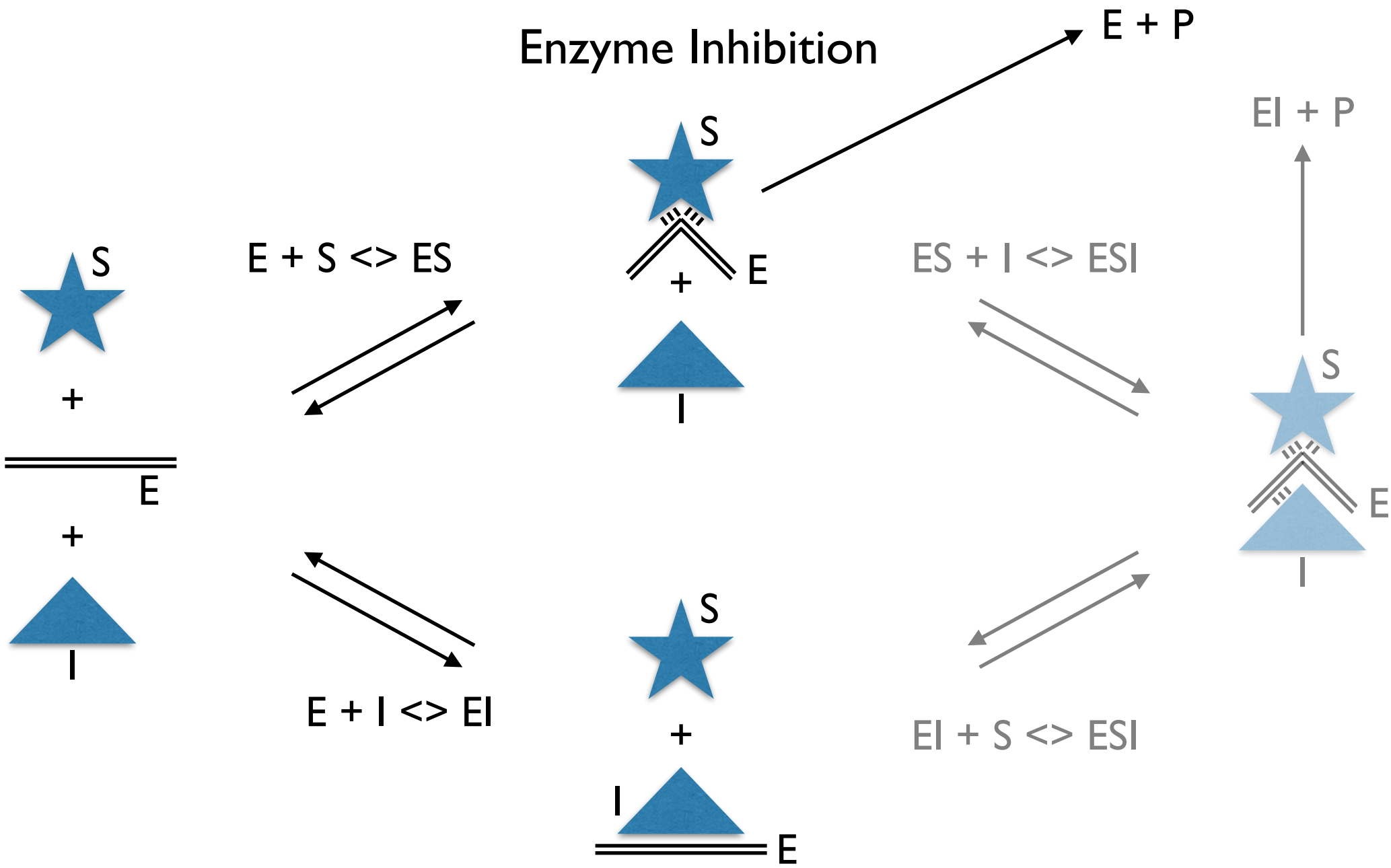
Cooperativity

Inhibition of Ligand Binding



Cooperativity

Enzyme Inhibition



Thermodynamics Overview

$$-RT\ln K = \Delta G^0 = \Delta H^0 - T\Delta S^0$$

$$K = e^{-\frac{\Delta G^0}{RT}}$$

$$\ln K = -\frac{\Delta G^0}{RT}$$

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R}$$

$$\frac{\partial \ln K}{\partial T} = \frac{\Delta H^0}{RT^2}$$

Assuming?

van't Hoff Equation

ΔS°

ΔH°

$$A + B \rightleftharpoons C$$

$$\ln K_{T_2} - \ln K_{T_1} = -\frac{\Delta G^0_{T_2}}{RT} + \frac{\Delta G^0_{T_1}}{RT}$$

$$= -\frac{\Delta H^0_{T_2}}{RT_2} + \frac{\Delta H^0_{T_1}}{RT_1} + \frac{\Delta S^0_{T_2} - \Delta S^0_{T_1}}{R}$$

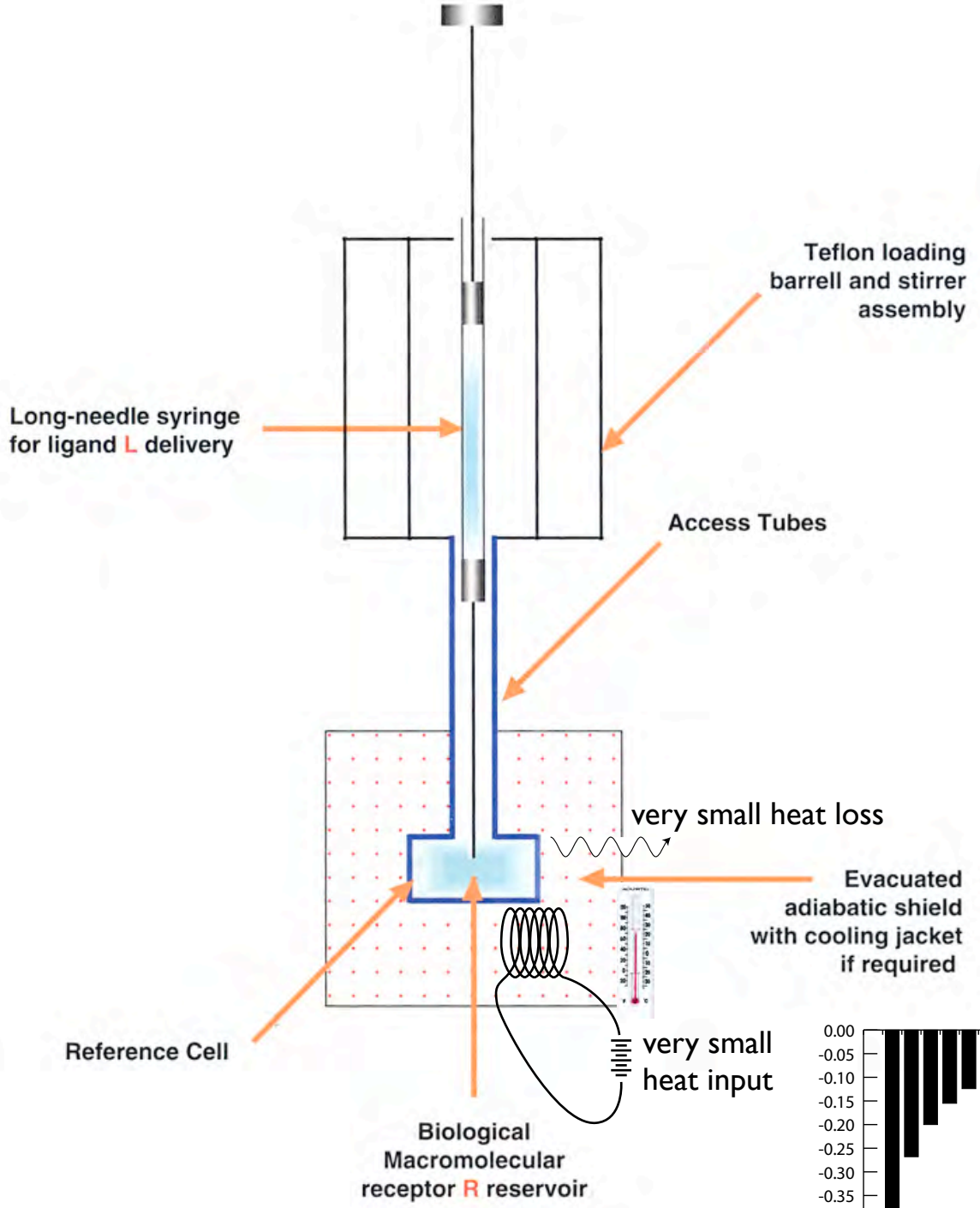
$$= \frac{\Delta H^0_T}{R}\left(-\frac{1}{T_2} + \frac{1}{T_1}\right) = \frac{\Delta H^0_T}{R}\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

Assuming?

Heat capacity

$$C_P = \left(\frac{\partial q_{rev}}{\partial T}\right)_P$$

ITC - Isothermal Titration Calorimetry



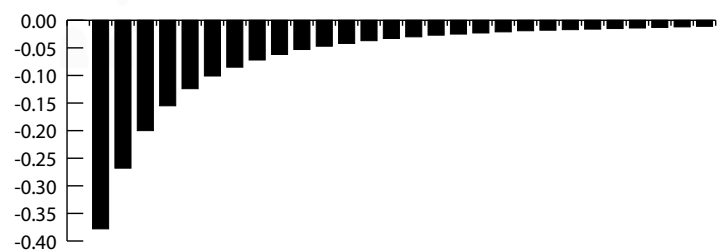
$$P + L \underset{}{\overset{K_a = 1/K_d}{\rightleftharpoons}} PL$$

$$\Delta G = RT \ln Q - RT \ln K_a$$

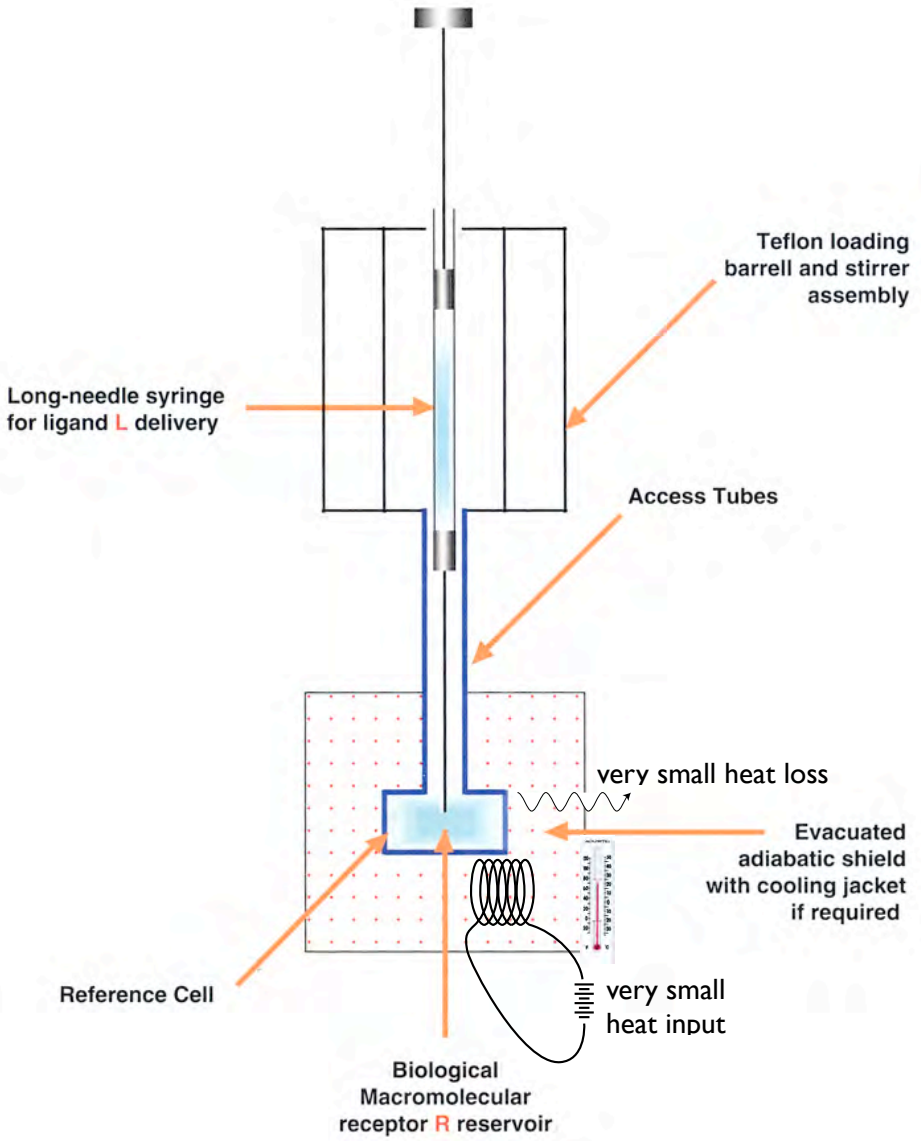
$$\Delta G = 0 = RT \ln \frac{[PL]}{[P][L]} - RT \ln K_a$$

$$\Delta[PL] = -\Delta[P] = -\Delta[L]$$

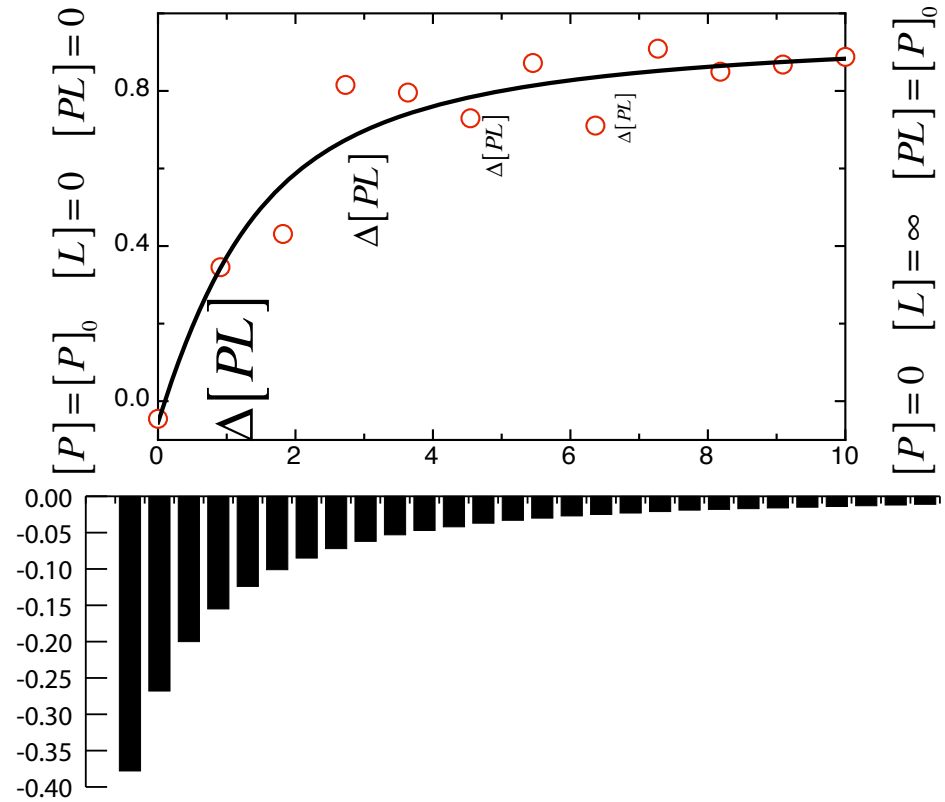
$$q = \Delta H \Delta[PL]$$



ITC - Isothermal Titration Calorimetry



$$P + L \underset{}{\overset{K_a = 1/K_d}{\rightleftharpoons}} PL$$

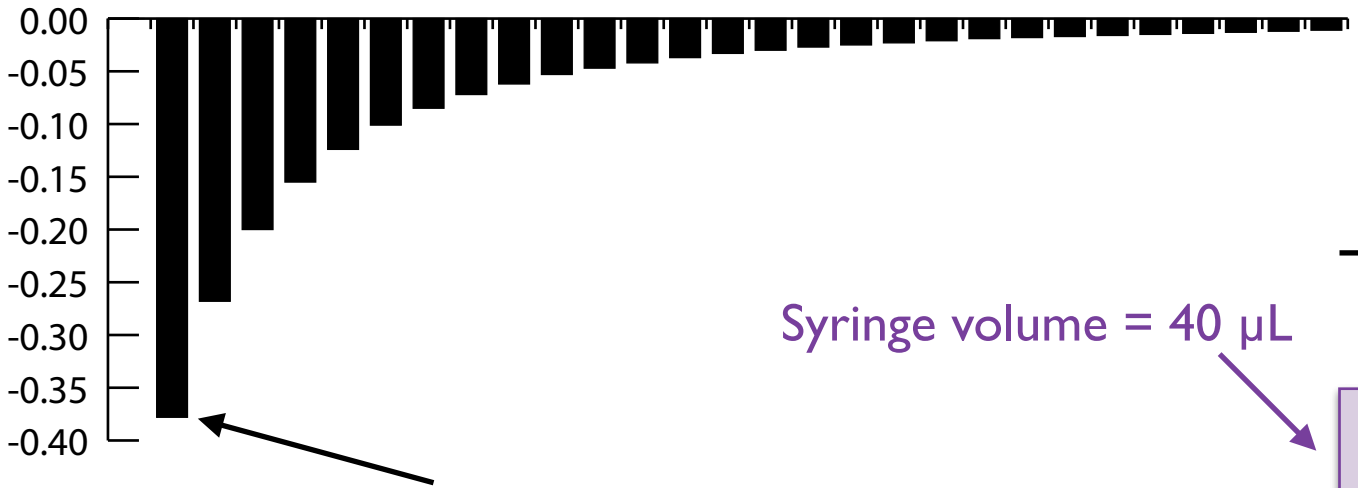


$$q = \Delta H \Delta[PL]$$

ITC

K_d = 100 μM

ΔH = -12 kcal/mol



Integrate each peak

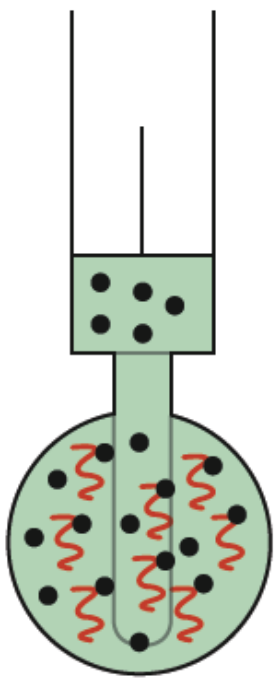
Syringe volume = 40 μL

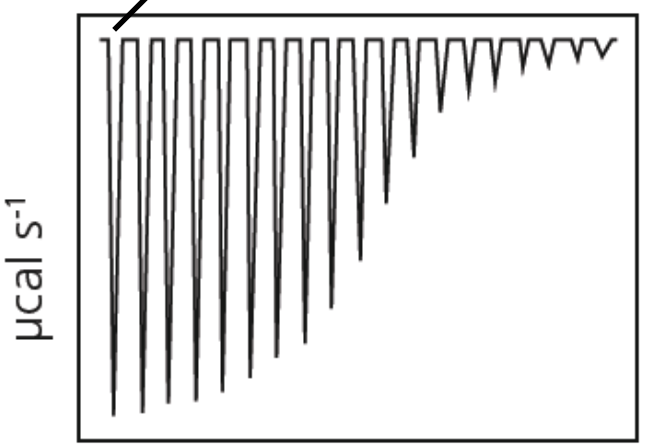
$[L]_{stock}$ = 3804 μM

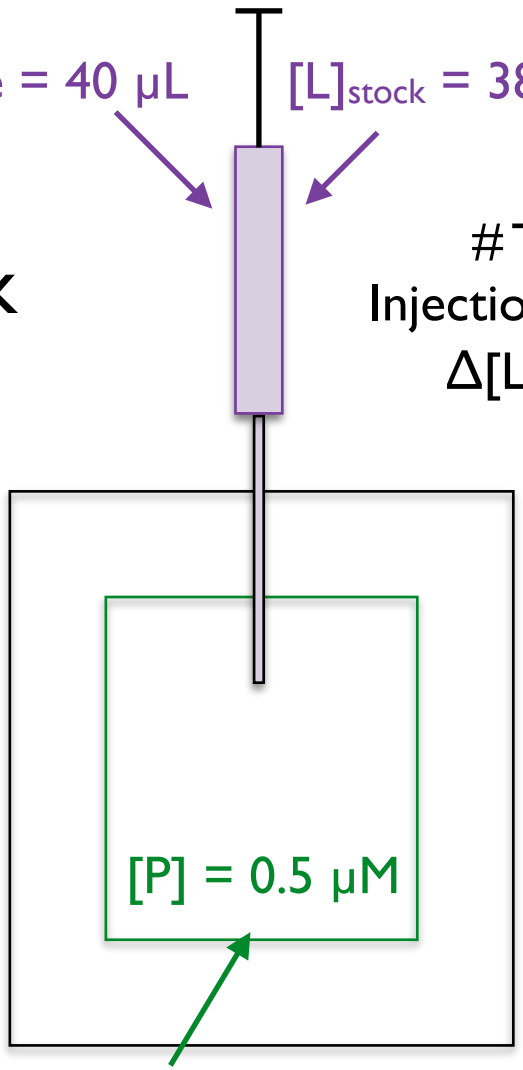
Titrations = 15

Injection volume = 2 μL

$\Delta[L]_{Tot}$ = 38 μM







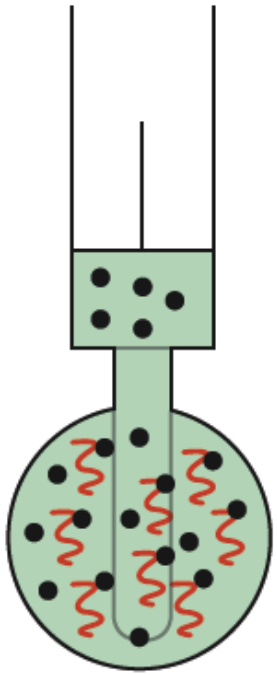
Cell volume = 200 μL

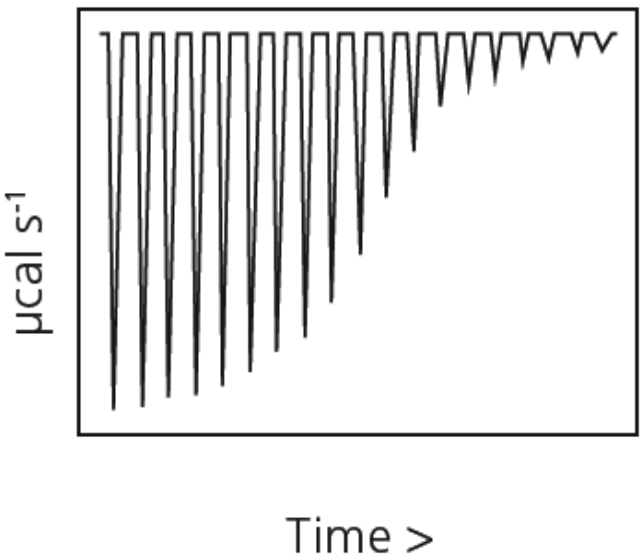
Isothermal Titration Calorimetry (ITC)

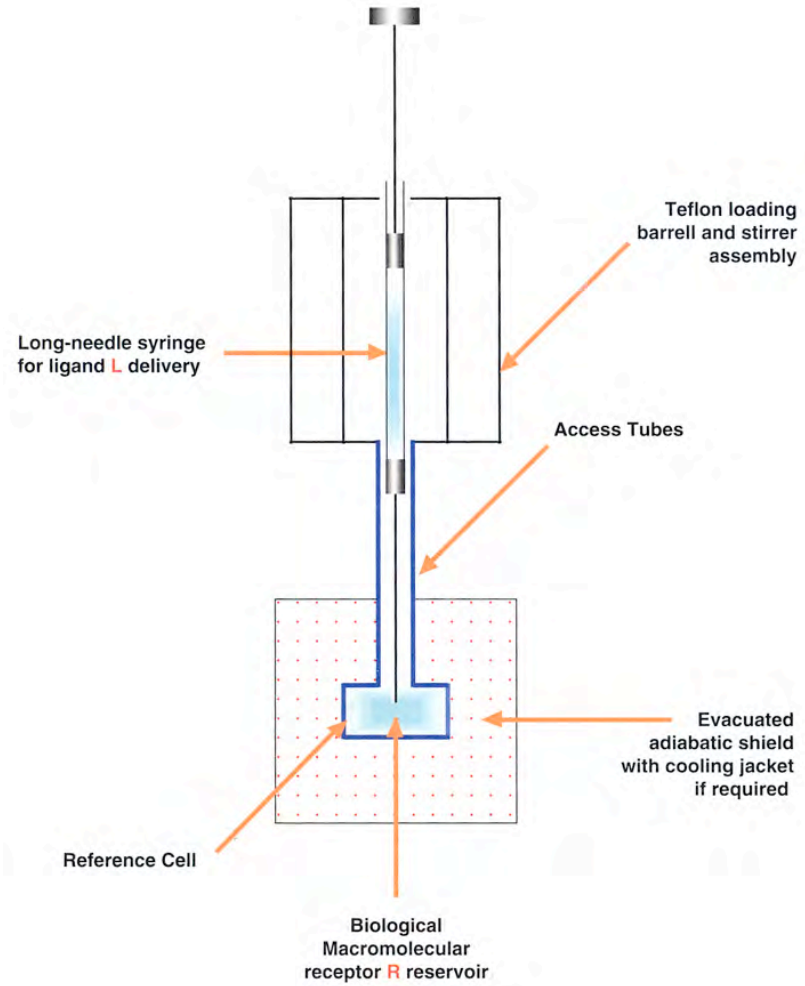
q = heat energy added to system

Negative peak: exothermic

Integrate peak to get q (µcal/injection)





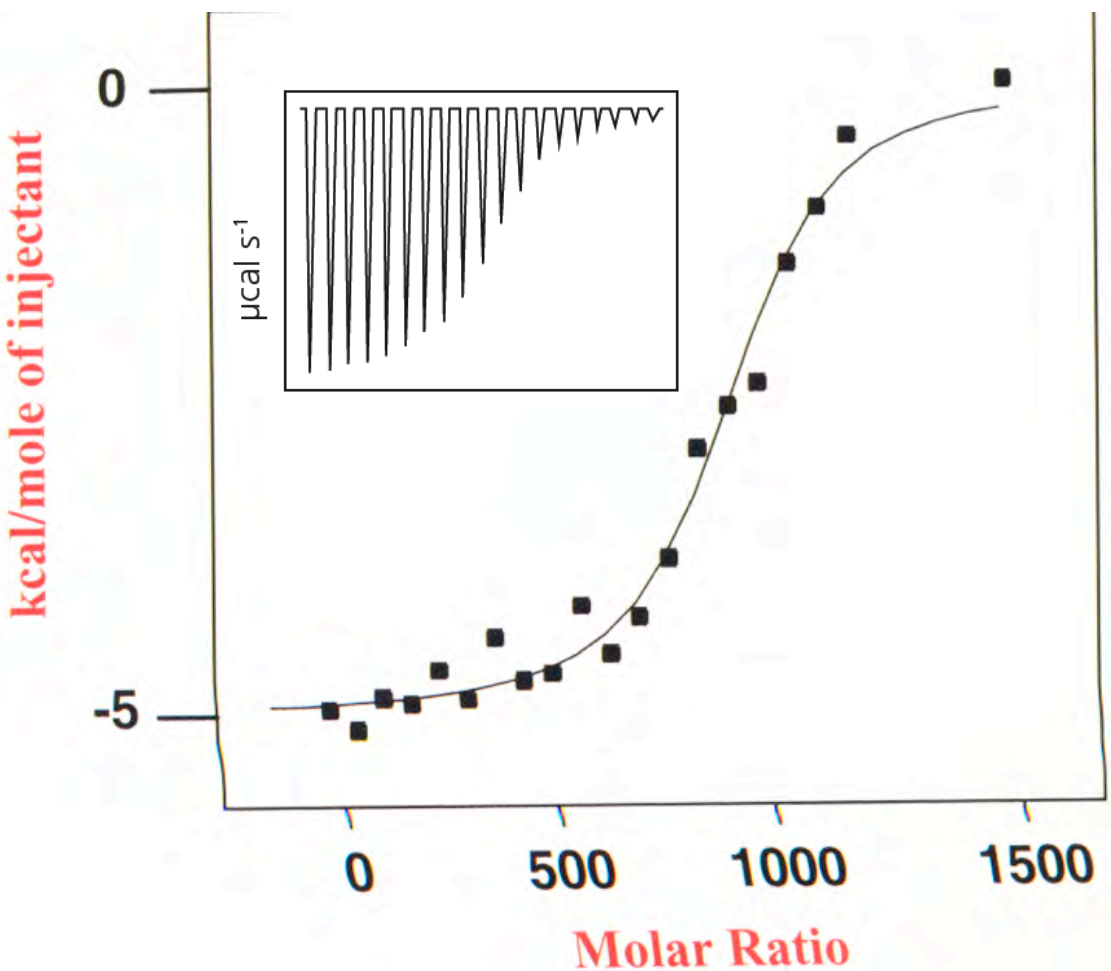


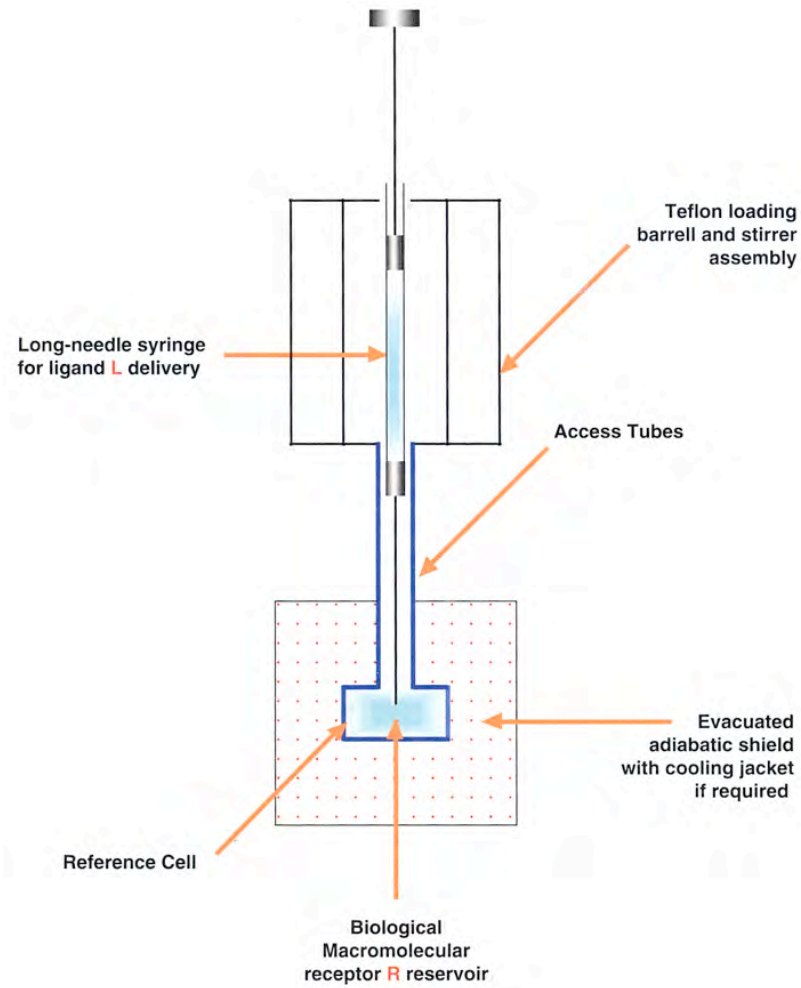
Isothermal Titration Calorimetry (ITC)

q = heat energy added to system

$$\Delta G^0_{bind}(T) = -RT \ln K_a = \Delta H^0_{bind} - T\Delta S^0_{bind}$$

$$\ln K_a = \frac{-\Delta H^0_{bind}}{RT} + \frac{\Delta S^0_{bind}}{R}$$

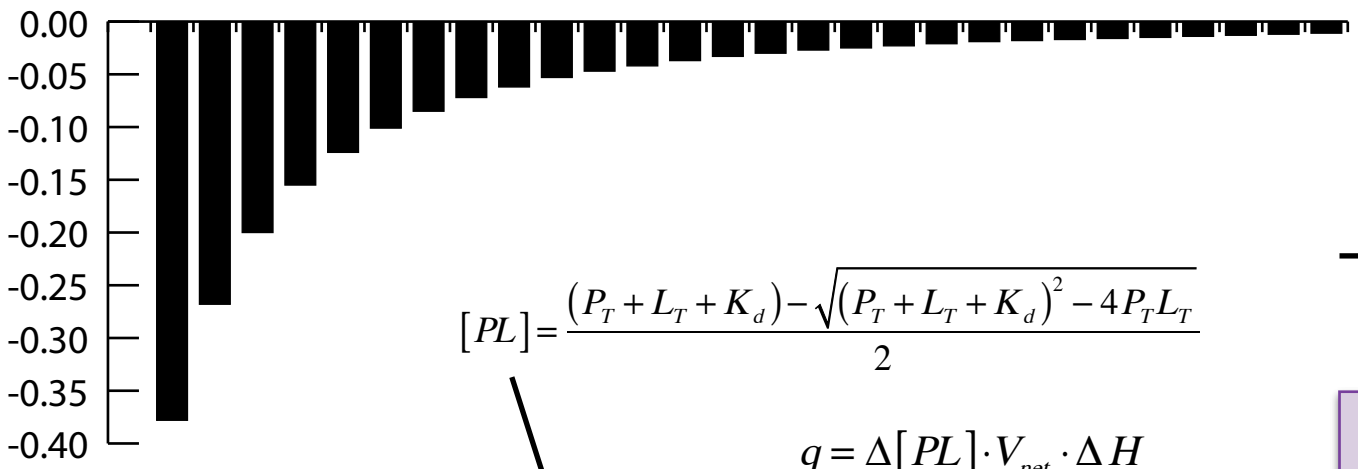




ITC

K_d = 100 μM

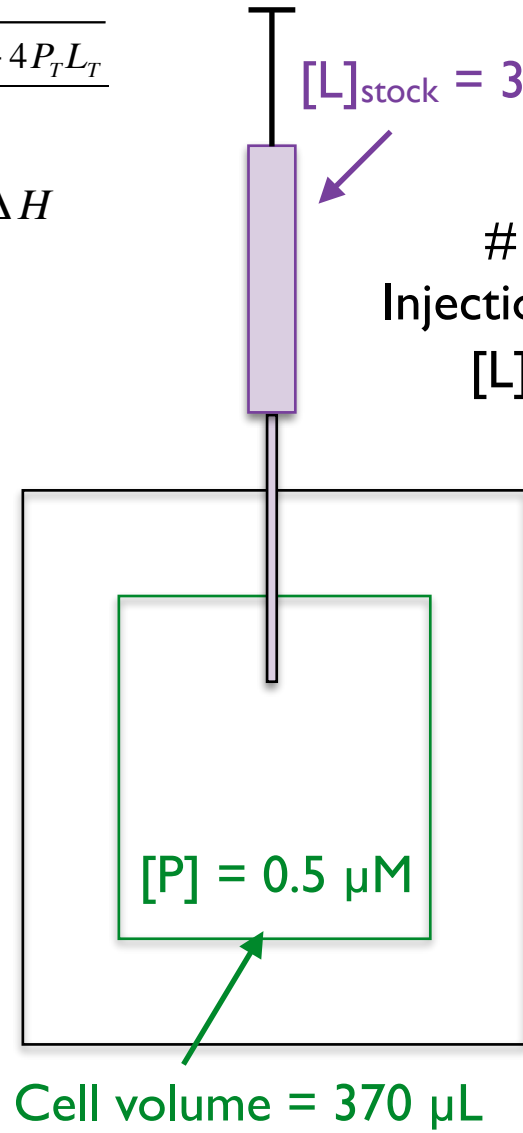
ΔH = -12 kcal/mol



$$[PL] = \frac{(P_T + L_T + K_d) - \sqrt{(P_T + L_T + K_d)^2 - 4P_T L_T}}{2}$$

$$q = \Delta[PL] \cdot V_{net} \cdot \Delta H$$

Titration	Vadded	Vnet	[L]tot	[PL]	Δ[PL]	q (μcal)
0	0	370	0	0		
1	2	372	20	0.085	0.085	-0.378
2	4	374	41	0.144	0.060	-0.268
3	6	376	61	0.188	0.044	-0.200
4	8	378	80	0.223	0.034	-0.155
15	30	400	285	0.370	0.006	-0.030
28	56	426	500	0.417	0.002	-0.011



Titrations = 28

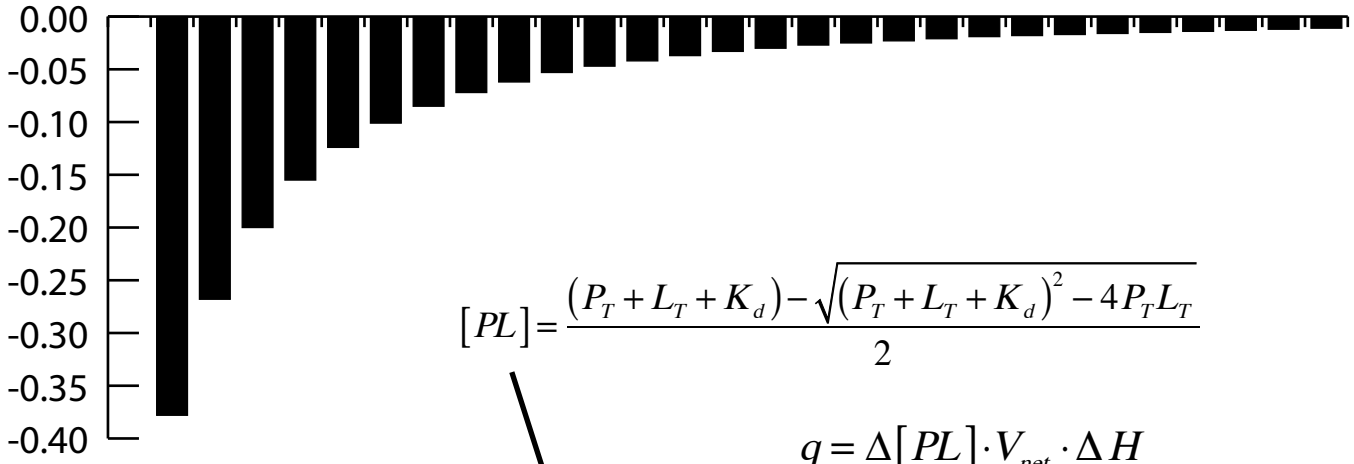
Injection volume = 2 μL

$[L]_{Tot}^{final}$ = 500 μM

ITC

K_d = 100 μM

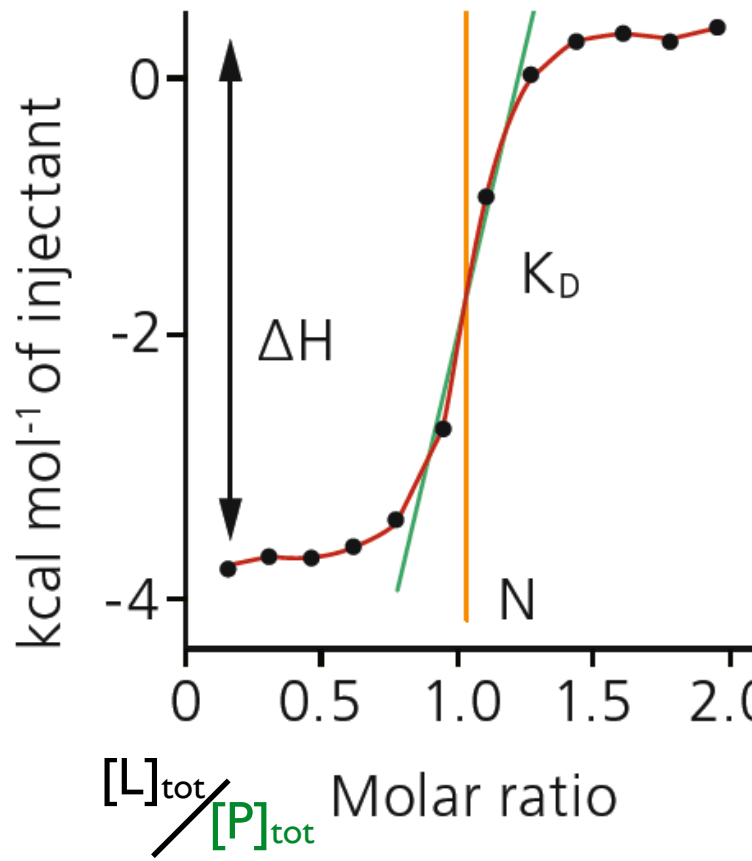
ΔH = -12 kcal/mol



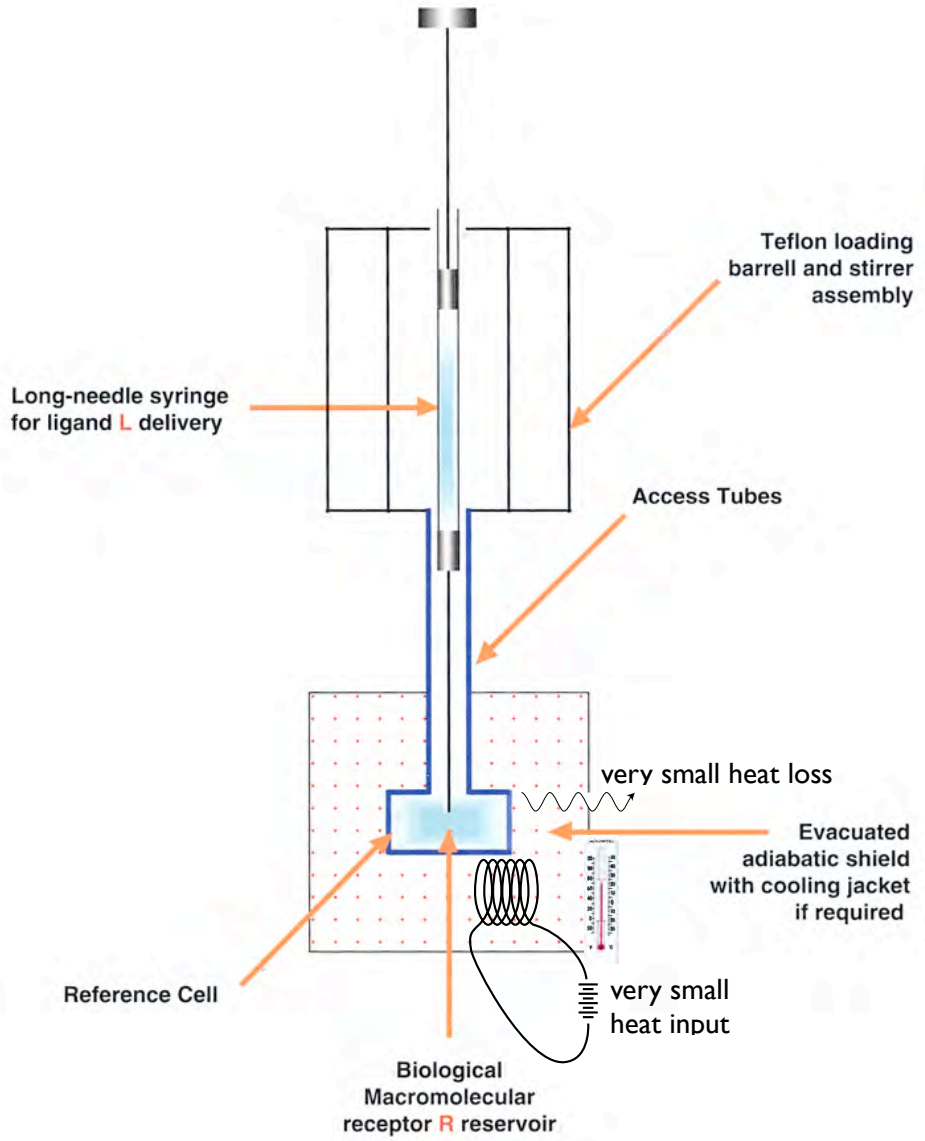
$$[PL] = \frac{(P_T + L_T + K_d) - \sqrt{(P_T + L_T + K_d)^2 - 4P_T L_T}}{2}$$

$$q = \Delta[PL] \cdot V_{net} \cdot \Delta H$$

Titration	Vadded	Vnet	[L]tot	[PL]	Δ[PL]	q (μcal)
0	0	370	0	0		
1	2	372	20	0.085	0.085	-0.378
2	4	374	41	0.144	0.060	-0.268
3	6	376	61	0.188	0.044	-0.200
4	8	378	80	0.223	0.034	-0.155
15	30	400	285	0.370	0.006	-0.030
28	56	426	500	0.417	0.002	-0.011



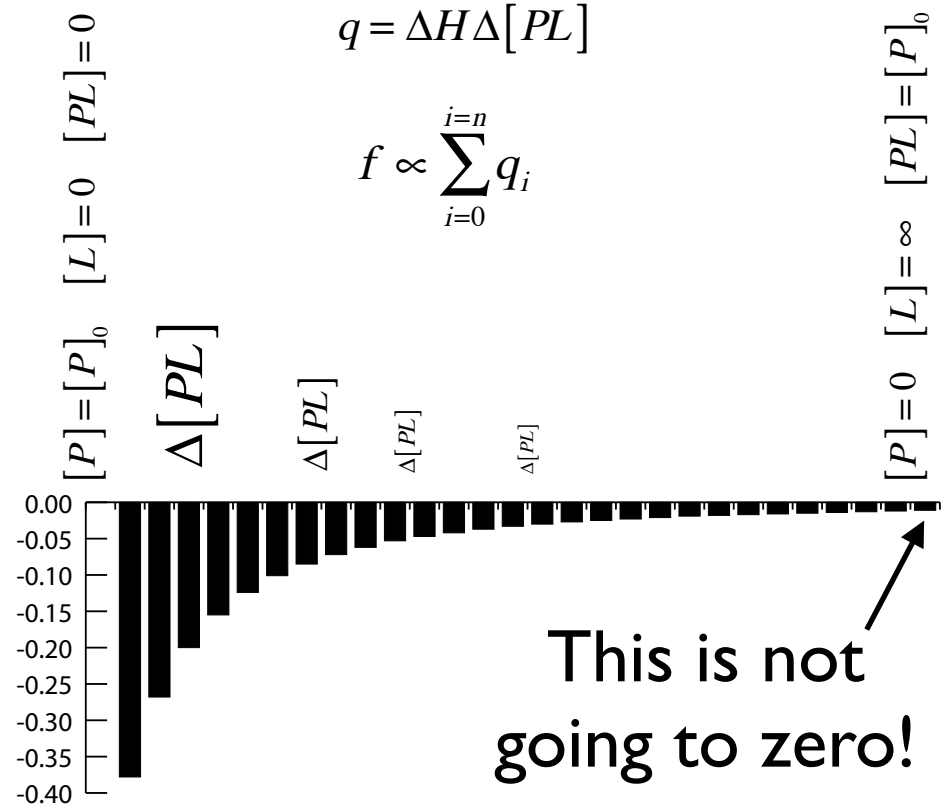
ITC - Isothermal Titration Calorimetry



$$P + L \underset{}{\overset{K_a = 1/K_d}{\rightleftharpoons}} PL$$

$$q = \Delta H \Delta[PL]$$

$$f \propto \sum_{i=0}^{i=n} q_i$$



This is not going to zero!

…why not?

Caveats

ΔH = -12 kcal/mol

Protein is 0.5 μM

ΔH measured is for the entire sample

Titration	V_{added}	V_{net}	$[L]_{tot}$	[PL]	Δ[PL]	q (μcal)
4	8	378	80	0.223	0.034	-0.155

Change in [PL] is 0.034 μM

NaCl is10 mM

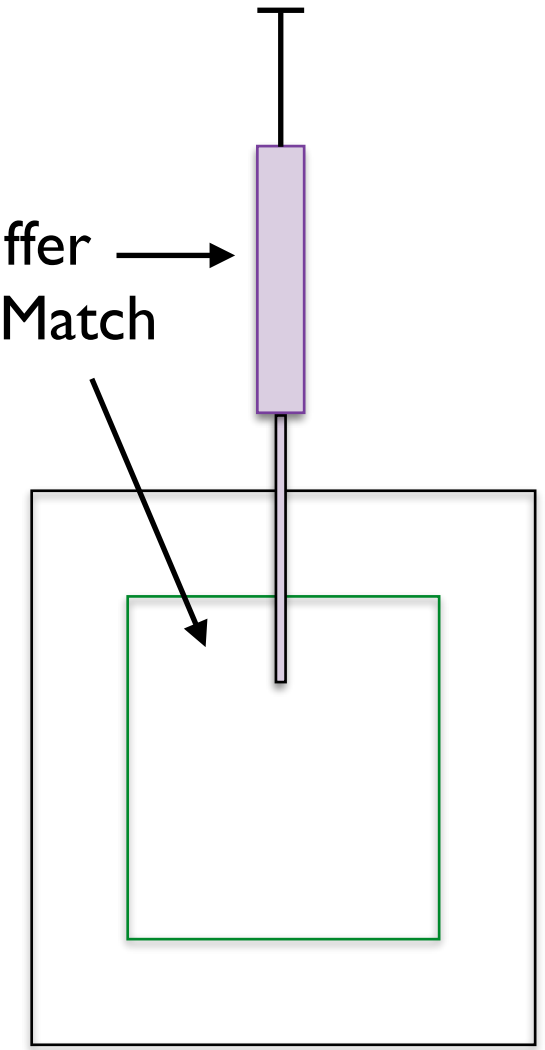
ΔH for dissolution of NaCl is +0.9 kcal/mol

Dilution per injection is 8/378= 0.022

NaCl is more than 20,000X that of protein

Assume buffer mismatch = 0.1%

dilution = $0.001 \times 0.022 \times 10\,mM = 0.22\,\mu M$



$$q = \left(\frac{0.22\times10^{-6}\,mol}{L}\right)\left(\frac{0.9\,kcal}{mol}\right)\left(370\times10^{-6}\,L\right)\left(\frac{10^{9}\,\mu cal}{kcal}\right) = 0.073\,\mu cal$$

ITC

Another caveat

Back to Gen Chem & PChem

Definition of "system"

ΔH measured is for the *entire* sample

Ligand binding might lead to indirect heats

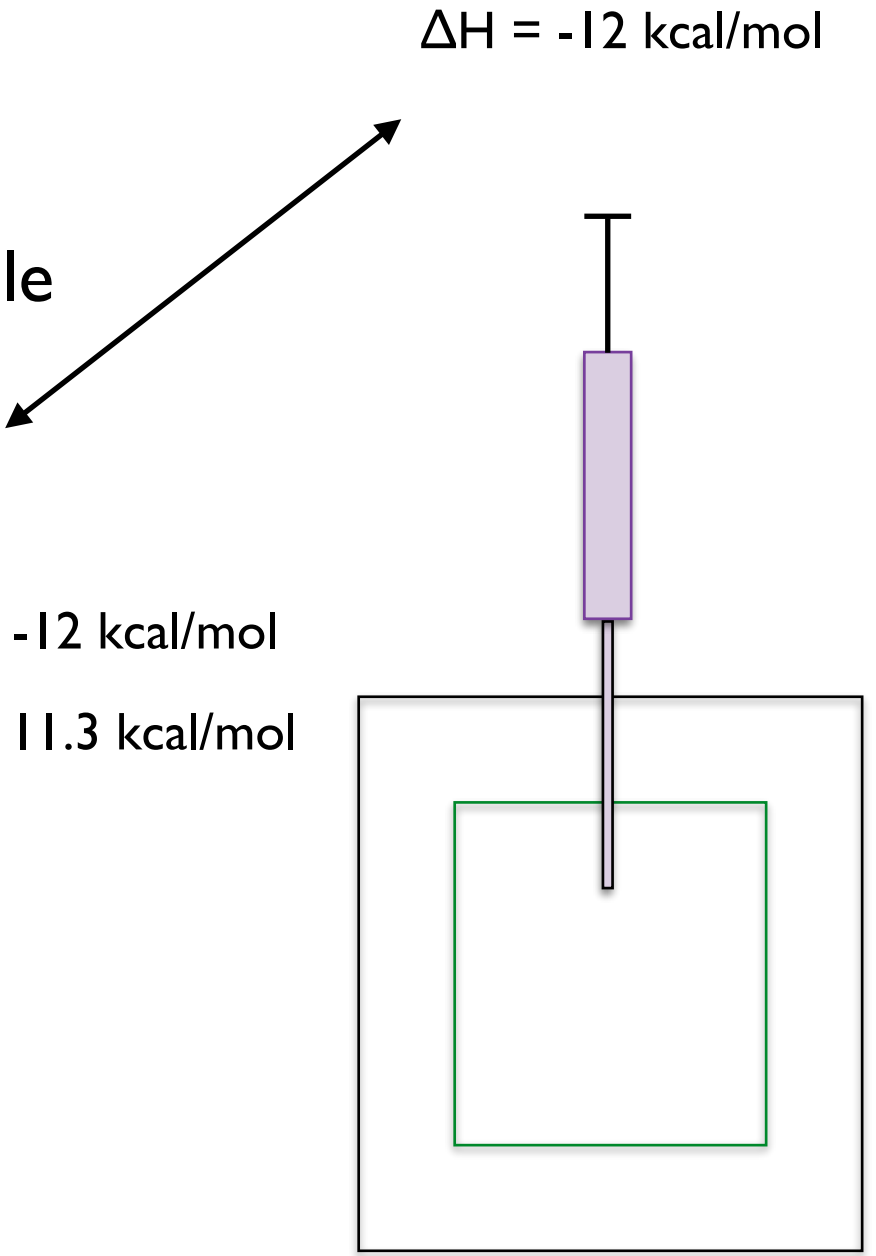
Protonation of Tris buffer ΔH = 11.3 kcal/mol

$P + L \Longleftrightarrow PL$

$PH^+ + L \Longleftrightarrow PL + H^+$ ΔH = -12 kcal/mol

$H^+ + Tris^- \Longleftrightarrow TrisH$ ΔH = 11.3 kcal/mol

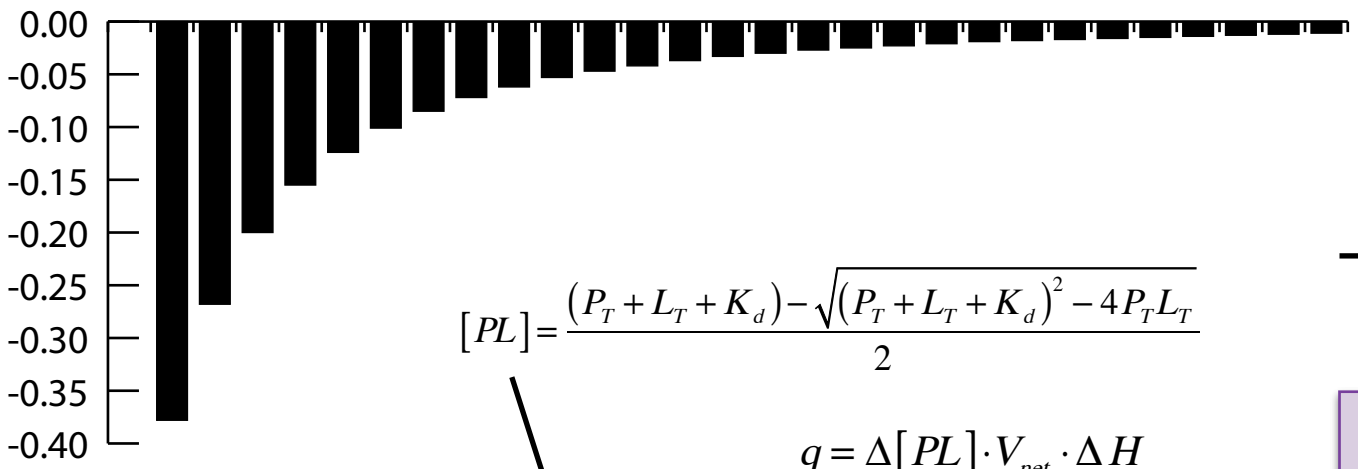
30 mM Tris



ITC

K_d = 100 μM

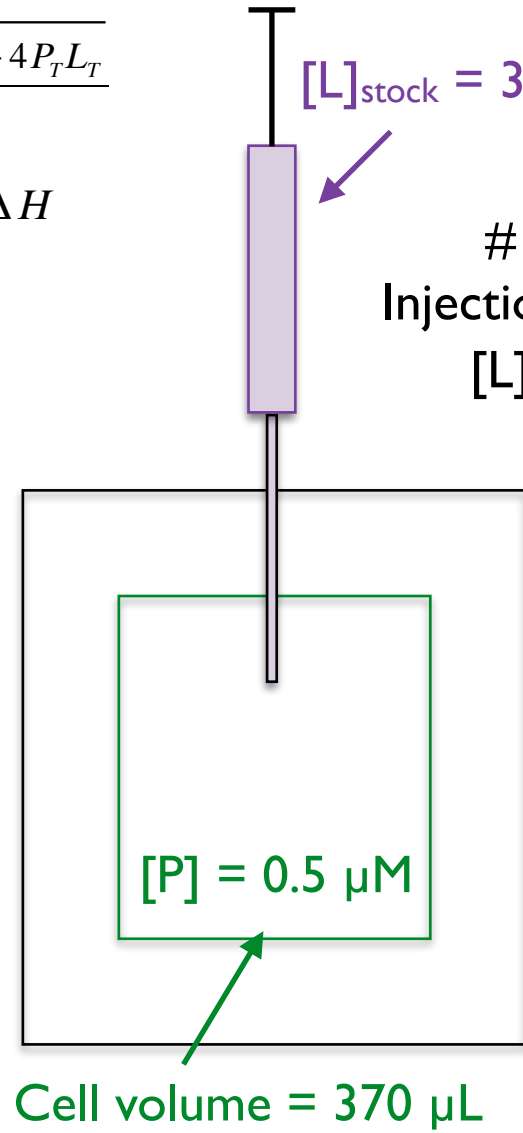
ΔH = -12 kcal/mol



$$[PL] = \frac{(P_T + L_T + K_d) - \sqrt{(P_T + L_T + K_d)^2 - 4P_T L_T}}{2}$$

$$q = \Delta[PL] \cdot V_{net} \cdot \Delta H$$

Titration	Vadded	Vnet	[L]tot	[PL]	Δ[PL]	q (μcal)
0	0	370	0	0		
1	2	372	20	0.085	0.085	-0.378
2	4	374	41	0.144	0.060	-0.268
3	6	376	61	0.188	0.044	-0.200
4	8	378	80	0.223	0.034	-0.155
15	30	400	285	0.370	0.006	-0.030
28	56	426	500	0.417	0.002	-0.011



Titrations = 28

Injection volume = 2 μL

$[L]_{Tot}^{final}$ = 500 μM

ITC

q per injection (µcal) (the measurement!)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

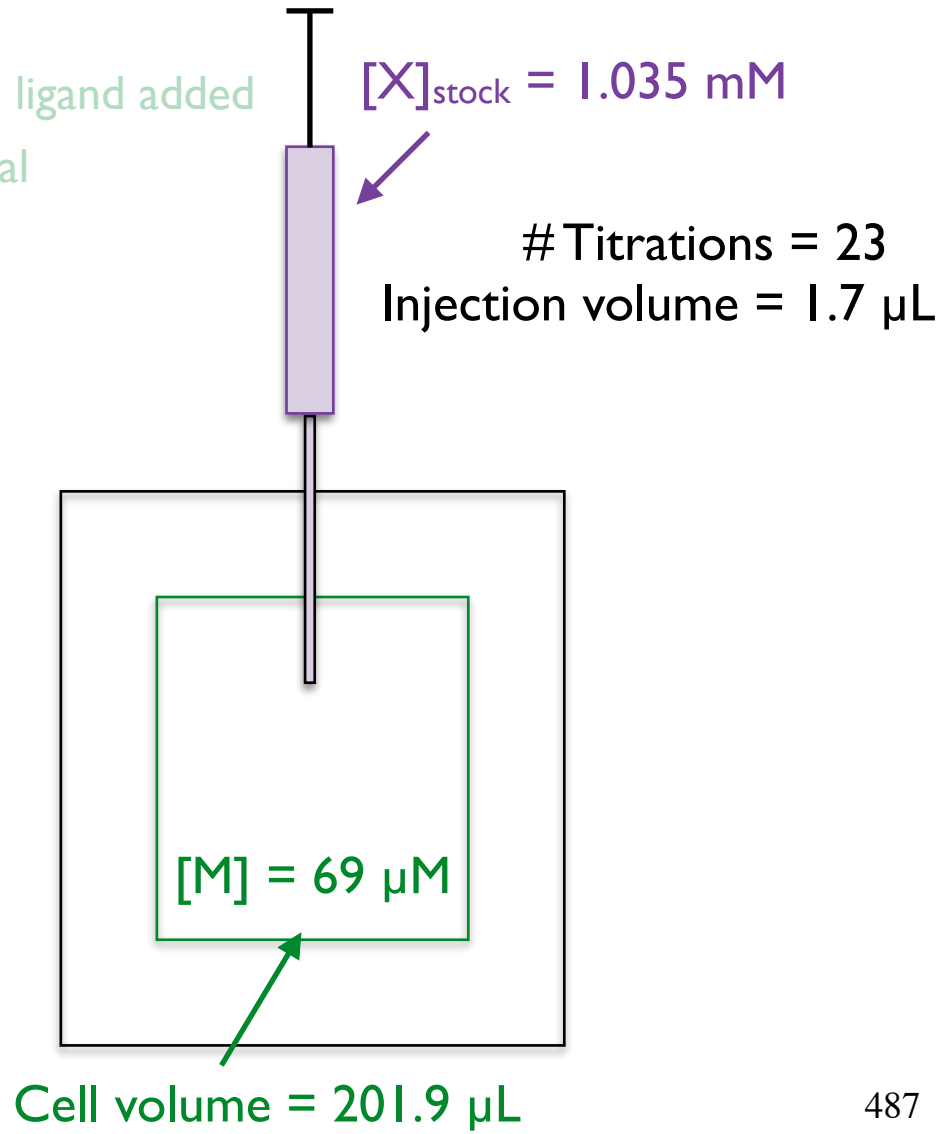
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.03619	--	2299.99	--
4.01479	1.7	0.00249	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	1.7	0.01092	0.06827	0.28458	2316.65	2276.50	40.14832
3.9874	1.7	0.01927	0.06772	0.41028	2266.21	2247.79	18.41812
3.86433	1.7	0.02756	0.06716	0.53699	2196.27	2191.38	4.88868
3.54838	1.7	0.03577	0.06661	0.66471	2016.70	2063.29	-46.5927
3.021	1.7	0.04392	0.06607	0.79344	1716.96	1739.43	-22.46873
1.96747	1.7	0.052	0.06553	0.92317	1118.20	1096.36	21.83654
0.91076	1.7	0.06	0.065	1.05391	517.62	501.20	16.42658
0.402	1.7	0.06794	0.06447	1.18565	228.47	227.82	0.65576
0.14295	1.7	0.07581	0.06394	1.31841	81.24	120.26	-39.02183
0.02726	1.7	0.08361	0.06342	1.45217	15.50	72.24	-56.74176
0.0026	1.7	0.09134	0.0629	1.58693	1.48	47.53	-46.04968
-0.04201	1.7	0.099	0.06238	1.7227	-23.88	33.35	-57.22539
-0.00329	1.7	0.10659	0.06187	1.85948	-1.87	24.51	-26.37896
-0.04848	1.7	0.11411	0.06137	1.99727	-27.55	18.65	-46.20145
-0.02632	1.7	0.12156	0.06086	2.13606	-14.96	14.57	-29.5273
-0.03007	1.7	0.12894	0.06037	2.27586	-17.09	11.61	-28.69484
-0.01286	1.7	0.13626	0.05987	2.41667	-7.31	9.39	-16.69765
0.01504	1.7	0.1435	0.05938	2.55849	8.55	7.68	0.86992
0.02926	1.7	0.15067	0.05889	2.70131	16.63	6.33	10.29665
-0.02331	1.7	0.15778	0.05841	2.84513	-13.25	5.24	-18.49181
0.00538	1.7	0.16481	0.05793	2.98997	3.06	4.35	-1.29615
	--	0.17178	0.05745	--			



ITC

q per injection (µcal) (the measurement!)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

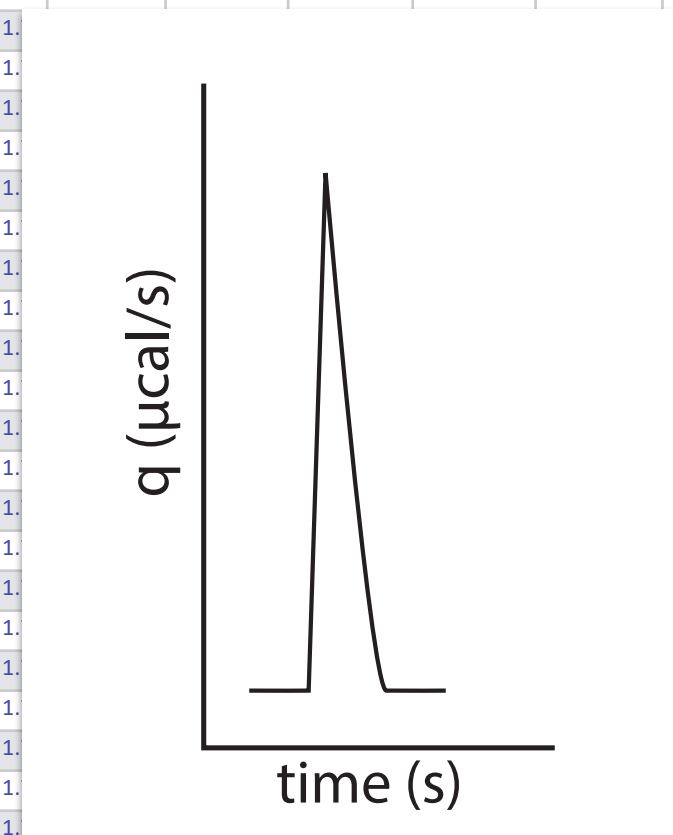
Ratio $[X]_{tot}/[M]_{tot}$

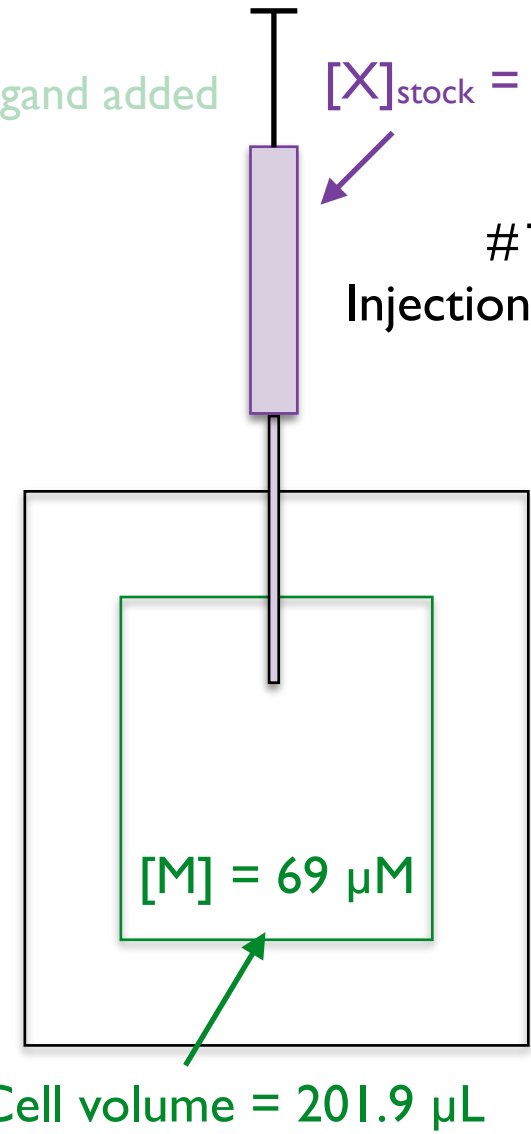
q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.03619	--	2299.99	--
4.01479	1.7	0.00249	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	1.						40.14832
3.9874	1.						18.41812
3.86433	1.						4.88868
3.54838	1.						-46.5927
3.021	1.						-22.46873
1.96747	1.						21.83654
0.91076	1.						16.42658
0.402	1.						0.65576
0.14295	1.						-39.02183
0.02726	1.						-56.74176
0.0026	1.						-46.04968
-0.04201	1.						-57.22539
-0.00329	1.						-26.37896
-0.04848	1.						-46.20145
-0.02632	1.						-29.5273
-0.03007	1.						-28.69484
-0.01286	1.						-16.69765
0.01504	1.						0.86992
0.02926	1.						10.29665
-0.02331	1.						-18.49181
0.00538	1.						-1.29615
	--	0.17178	0.05745	--			





Titrations = 23

Injection volume = 1.7 µL

Cell volume = 201.9 µL

ITC

q per injection (µcal)

injection volume (µL) (note: increases Volume)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

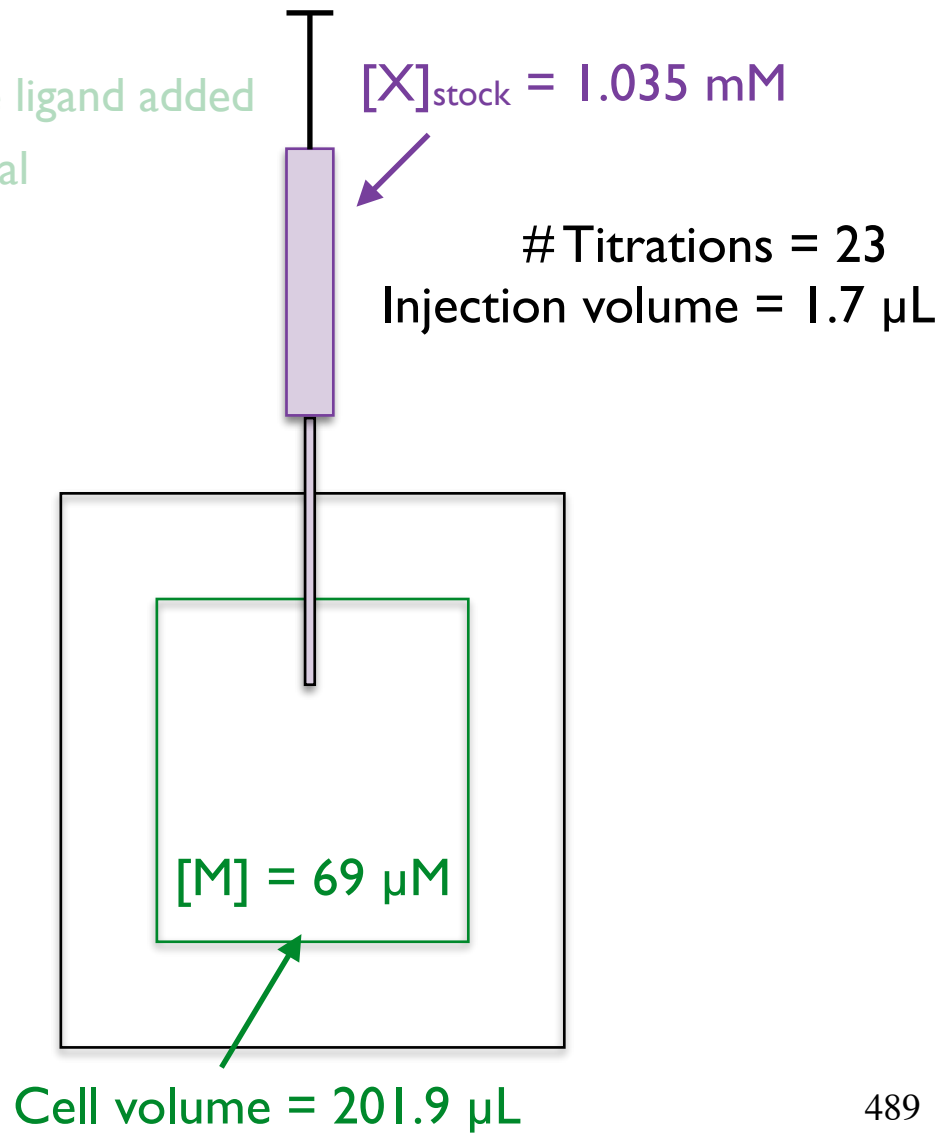
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	**0.5**	0	0.069	0.03619	--	2299.99	--
4.01479	**1.7**	0.00249	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	**1.7**	0.01092	0.06827	0.28458	2316.65	2276.50	40.14832
3.9874	**1.7**	0.01927	0.06772	0.41028	2266.21	2247.79	18.41812
3.86433	**1.7**	0.02756	0.06716	0.53699	2196.27	2191.38	4.88868
3.54838	**1.7**	0.03577	0.06661	0.66471	2016.70	2063.29	-46.5927
3.021	**1.7**	0.04392	0.06607	0.79344	1716.96	1739.43	-22.46873
1.96747	**1.7**	0.052	0.06553	0.92317	1118.20	1096.36	21.83654
0.91076	**1.7**	0.06	0.065	1.05391	517.62	501.20	16.42658
0.402	**1.7**	0.06794	0.06447	1.18565	228.47	227.82	0.65576
0.14295	**1.7**	0.07581	0.06394	1.31841	81.24	120.26	-39.02183
0.02726	**1.7**	0.08361	0.06342	1.45217	15.50	72.24	-56.74176
0.0026	**1.7**	0.09134	0.0629	1.58693	1.48	47.53	-46.04968
-0.04201	**1.7**	0.099	0.06238	1.7227	-23.88	33.35	-57.22539
-0.00329	**1.7**	0.10659	0.06187	1.85948	-1.87	24.51	-26.37896
-0.04848	**1.7**	0.11411	0.06137	1.99727	-27.55	18.65	-46.20145
-0.02632	**1.7**	0.12156	0.06086	2.13606	-14.96	14.57	-29.5273
-0.03007	**1.7**	0.12894	0.06037	2.27586	-17.09	11.61	-28.69484
-0.01286	**1.7**	0.13626	0.05987	2.41667	-7.31	9.39	-16.69765
0.01504	**1.7**	0.1435	0.05938	2.55849	8.55	7.68	0.86992
0.02926	**1.7**	0.15067	0.05889	2.70131	16.63	6.33	10.29665
-0.02331	**1.7**	0.15778	0.05841	2.84513	-13.25	5.24	-18.49181
0.00538	**1.7**	0.16481	0.05793	2.98997	3.06	4.35	-1.29615
	--	0.17178	0.05745	--			



ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc) (corrected for dilution)

$[M]_{tot}$ prior to injection

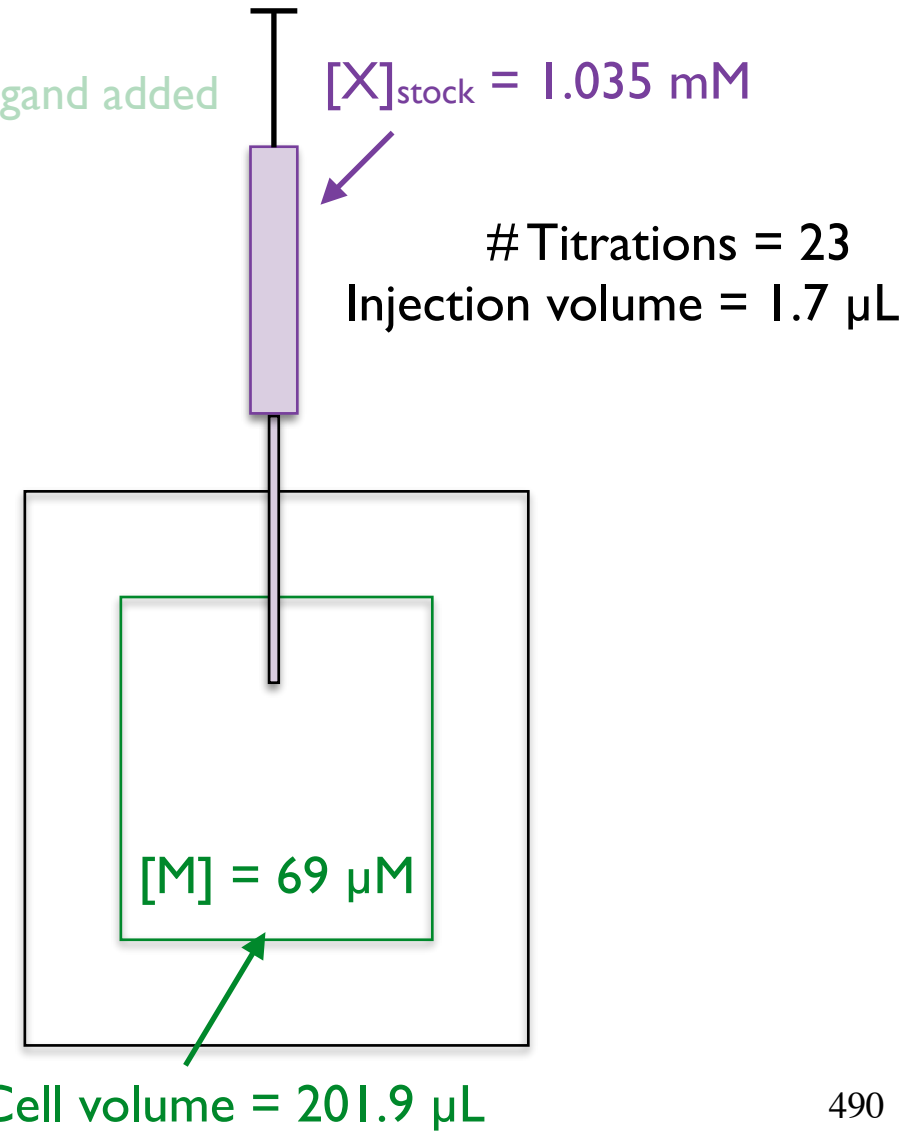
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	**0**	0.069	0.03619	--	2299.99	--
4.01479	1.7	**0.00249**	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	1.7	**0.01092**	0.06827	0.28458	2316.65	2276.50	40.14832
3.9874	1.7	**0.01927**	0.06772	0.41028	2266.21	2247.79	18.41812
3.86433	1.7	**0.02756**	0.06716	0.53699	2196.27	2191.38	4.88868
3.54838	1.7	**0.03577**	0.06661	0.66471	2016.70	2063.29	-46.5927
3.021	1.7	**0.04392**	0.06607	0.79344	1716.96	1739.43	-22.46873
1.96747	1.7	**0.052**	0.06553	0.92317	1118.20	1096.36	21.83654
0.91076	1.7	**0.06**	0.065	1.05391	517.62	501.20	16.42658
0.402	1.7	**0.06794**	0.06447	1.18565	228.47	227.82	0.65576
0.14295	1.7	**0.07581**	0.06394	1.31841	81.24	120.26	-39.02183
0.02726	1.7	**0.08361**	0.06342	1.45217	15.50	72.24	-56.74176
0.0026	1.7	**0.09134**	0.0629	1.58693	1.48	47.53	-46.04968
-0.04201	1.7	**0.099**	0.06238	1.7227	-23.88	33.35	-57.22539
-0.00329	1.7	**0.10659**	0.06187	1.85948	-1.87	24.51	-26.37896
-0.04848	1.7	**0.11411**	0.06137	1.99727	-27.55	18.65	-46.20145
-0.02632	1.7	**0.12156**	0.06086	2.13606	-14.96	14.57	-29.5273
-0.03007	1.7	**0.12894**	0.06037	2.27586	-17.09	11.61	-28.69484
-0.01286	1.7	**0.13626**	0.05987	2.41667	-7.31	9.39	-16.69765
0.01504	1.7	**0.1435**	0.05938	2.55849	8.55	7.68	0.86992
0.02926	1.7	**0.15067**	0.05889	2.70131	16.63	6.33	10.29665
-0.02331	1.7	**0.15778**	0.05841	2.84513	-13.25	5.24	-18.49181
0.00538	1.7	**0.16481**	0.05793	2.98997	3.06	4.35	-1.29615
	--	**0.17178**	0.05745	--			



ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc) (corrected for dilution)

$[M]_{tot}$ prior to injection

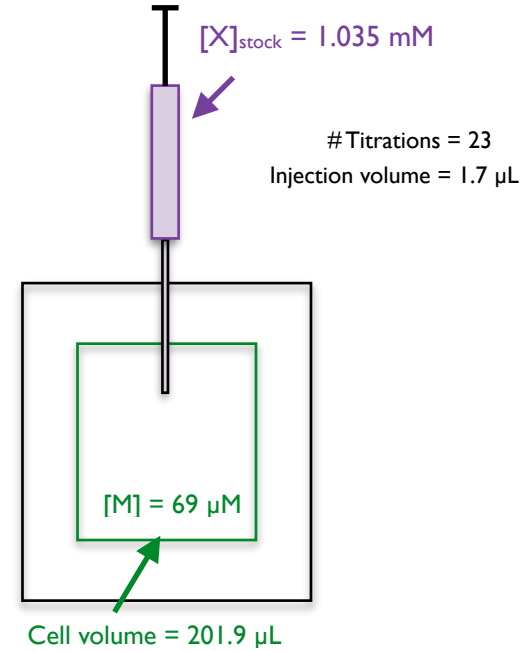
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

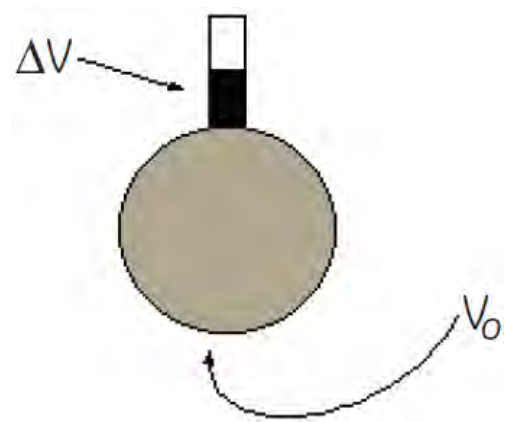
Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	**0**	0.069	0.03619	--	2299.99	--
4.01479	1.7	**0.00249**	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	1.7	**0.01092**	0.06827	0.28458	2316.65	2276.50	40.14832
3.9874	1.7	**0.01927**	0.06772	0.41028	2266.21	2247.79	18.41812
3.86433	1.7	**0.02756**	0.06716	0.53699	2196.27	2191.38	4.88868
3.54838	1.7	**0.03577**	0.06661	0.66471	2016.70	2063.29	-46.5927
3.021	1.7	**0.04392**	0.06607	0.79344	1716.96	1739.43	-22.46873
1.96747	1.7	**0.052**	0.06553	0.92317	1118.20	1096.36	21.83654
0.91076	1.7	**0.06**	0.065	1.05391	517.62	501.20	16.42658
0.402	1.7	**0.06794**	0.06447	1.18565	228.47	227.82	0.65576
0.14295	1.7	**0.07581**	0.06394	1.31841	81.24	120.26	-39.02183
0.02726	1.7	**0.08361**	0.06342	1.45217	15.50	72.24	-56.74176
0.0026	1.7	**0.09134**	0.0629	1.58693	1.48	47.53	-46.04968
-0.04201	1.7	**0.099**	0.06238	1.7227	-23.88	33.35	-57.22539
-0.00329	1.7	**0.10659**	0.06187	1.85948	-1.87	24.51	-26.37896
-0.04848	1.7	**0.11411**	0.06137	1.99727	-27.55	18.65	-46.20145
-0.02632	1.7	**0.12156**	0.06086	2.13606	-14.96	14.57	-29.5273
-0.03007	1.7	**0.12894**	0.06037	2.27586	-17.09	11.61	-28.69484
-0.01286	1.7	**0.13626**	0.05987	2.41667	-7.31	9.39	-16.69765
0.01504	1.7	**0.1435**	0.05938	2.55849	8.55	7.68	0.86992
0.02926	1.7	**0.15067**	0.05889	2.70131	16.63	6.33	10.29665
-0.02331	1.7	**0.15778**	0.05841	2.84513	-13.25	5.24	-18.49181
0.00538	1.7	**0.16481**	0.05793	2.98997	3.06	4.35	-1.29615
	--	**0.17178**	0.05745	--			



V_{tot} = 201.9 µL



Both the cell and the cell stem are filled with macromolecule solution, but only the active volume is monitored calorimetrically. Each injection drives liquid from the active volume into the cell stem (darkened portion representing ΔV). Consequently, in a typical experiment, Mt decreases slightly (~1%) with each injection. We assume no mixing occurs between the active volume and the cell stem, so the average bulk concentration of macromolecule in ΔV is the computed to be the average of Mt(t=0) and Mt

V_{tot} = 238.1 µL (+ 20%)

ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection (corrected for dilution)

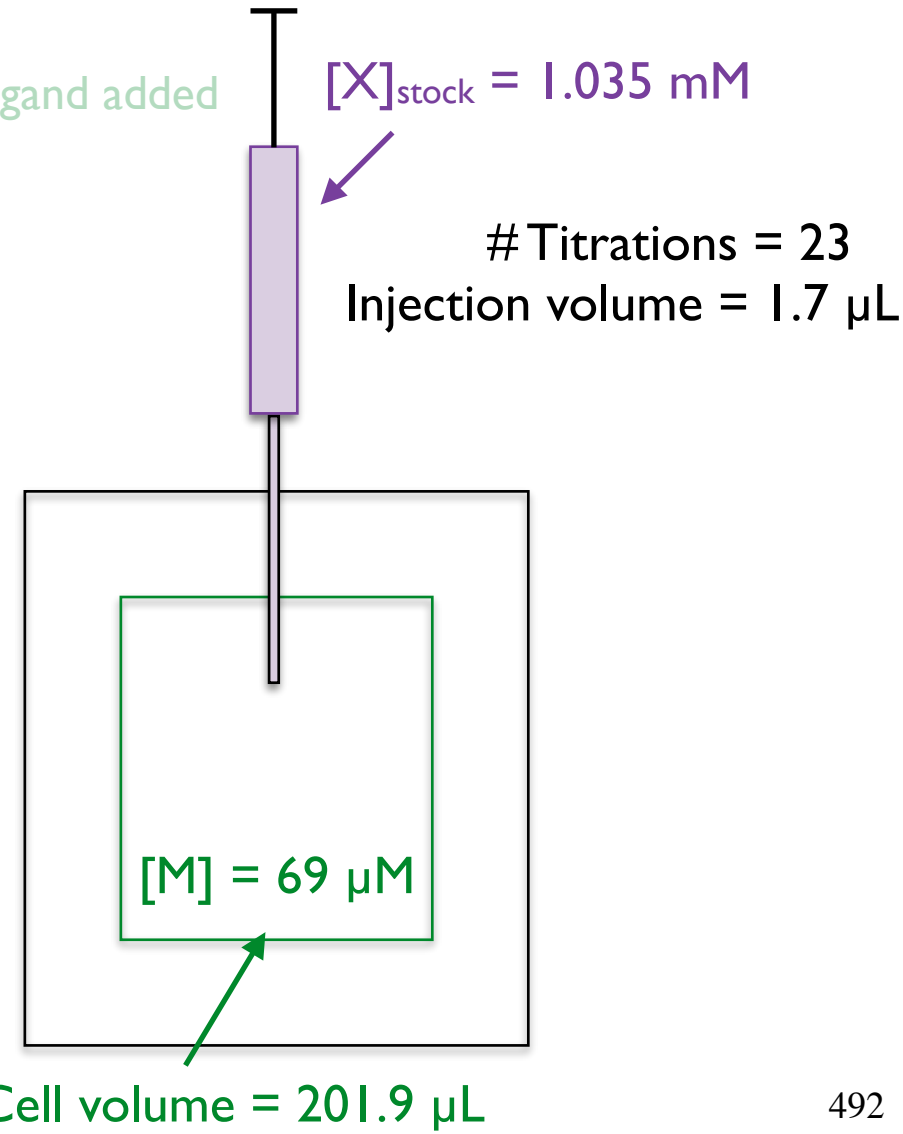
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	**Mt**	XMt	NDH	Fit	DY
0.98819	0.5	0	**0.069**	0.03619	--	2299.99	--
4.01479	1.7	0.00249	**0.06883**	0.15988	2281.78	2292.82	-11.04176
4.07614	1.7	0.01092	**0.06827**	0.28458	2316.65	2276.50	40.14832
3.9874	1.7	0.01927	**0.06772**	0.41028	2266.21	2247.79	18.41812
3.86433	1.7	0.02756	**0.06716**	0.53699	2196.27	2191.38	4.88868
3.54838	1.7	0.03577	**0.06661**	0.66471	2016.70	2063.29	-46.5927
3.021	1.7	0.04392	**0.06607**	0.79344	1716.96	1739.43	-22.46873
1.96747	1.7	0.052	**0.06553**	0.92317	1118.20	1096.36	21.83654
0.91076	1.7	0.06	**0.065**	1.05391	517.62	501.20	16.42658
0.402	1.7	0.06794	**0.06447**	1.18565	228.47	227.82	0.65576
0.14295	1.7	0.07581	**0.06394**	1.31841	81.24	120.26	-39.02183
0.02726	1.7	0.08361	**0.06342**	1.45217	15.50	72.24	-56.74176
0.0026	1.7	0.09134	**0.0629**	1.58693	1.48	47.53	-46.04968
-0.04201	1.7	0.099	**0.06238**	1.7227	-23.88	33.35	-57.22539
-0.00329	1.7	0.10659	**0.06187**	1.85948	-1.87	24.51	-26.37896
-0.04848	1.7	0.11411	**0.06137**	1.99727	-27.55	18.65	-46.20145
-0.02632	1.7	0.12156	**0.06086**	2.13606	-14.96	14.57	-29.5273
-0.03007	1.7	0.12894	**0.06037**	2.27586	-17.09	11.61	-28.69484
-0.01286	1.7	0.13626	**0.05987**	2.41667	-7.31	9.39	-16.69765
0.01504	1.7	0.1435	**0.05938**	2.55849	8.55	7.68	0.86992
0.02926	1.7	0.15067	**0.05889**	2.70131	16.63	6.33	10.29665
-0.02331	1.7	0.15778	**0.05841**	2.84513	-13.25	5.24	-18.49181
0.00538	1.7	0.16481	**0.05793**	2.98997	3.06	4.35	-1.29615
	--	0.17178	**0.05745**	--			



ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

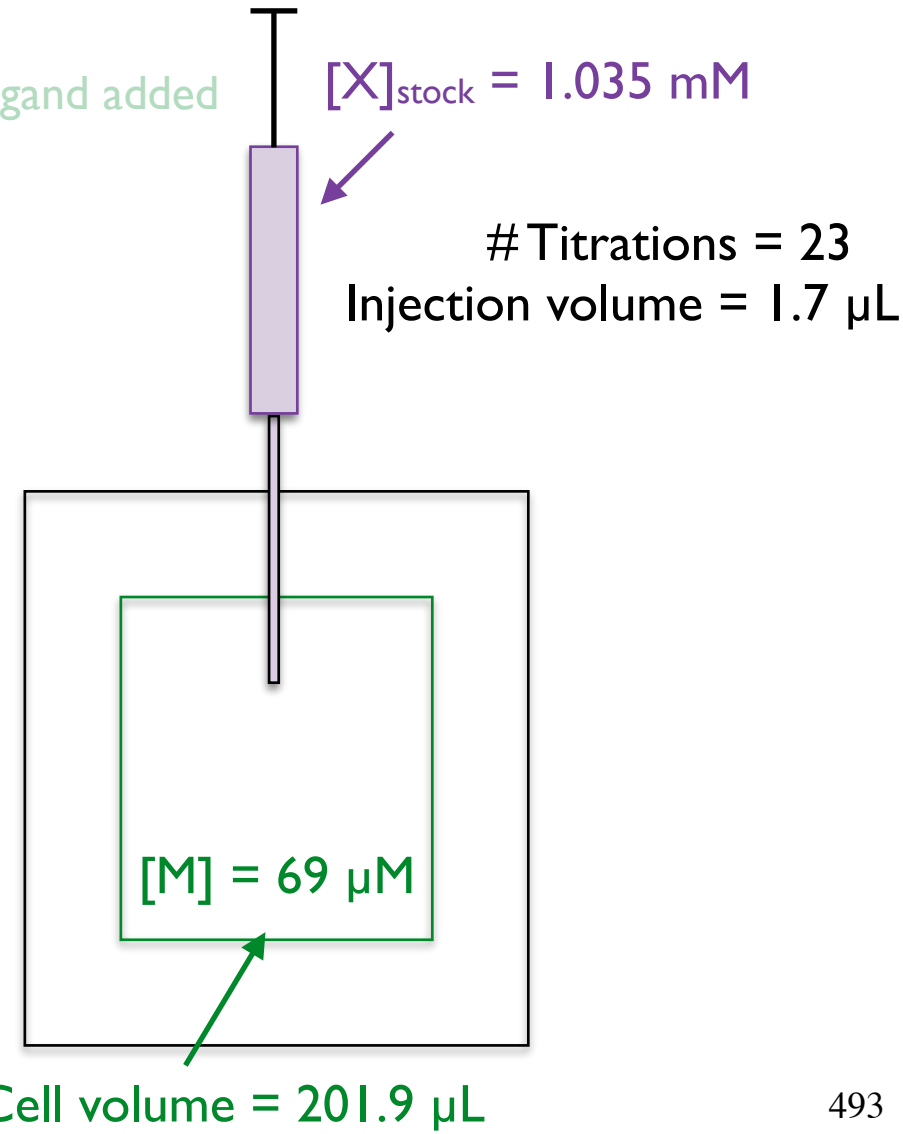
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	**0.03619**	--	2299.99	--
4.01479	1.7	0.00249	0.06883	**0.15988**	2281.78	2292.82	-11.04176
4.07614	1.7	0.01092	0.06827	**0.28458**	2316.65	2276.50	40.14832
3.9874	1.7	0.01927	0.06772	**0.41028**	2266.21	2247.79	18.41812
3.86433	1.7	0.02756	0.06716	**0.53699**	2196.27	2191.38	4.88868
3.54838	1.7	0.03577	0.06661	**0.66471**	2016.70	2063.29	-46.5927
3.021	1.7	0.04392	0.06607	**0.79344**	1716.96	1739.43	-22.46873
1.96747	1.7	0.052	0.06553	**0.92317**	1118.20	1096.36	21.83654
0.91076	1.7	0.06	0.065	**1.05391**	517.62	501.20	16.42658
0.402	1.7	0.06794	0.06447	**1.18565**	228.47	227.82	0.65576
0.14295	1.7	0.07581	0.06394	**1.31841**	81.24	120.26	-39.02183
0.02726	1.7	0.08361	0.06342	**1.45217**	15.50	72.24	-56.74176
0.0026	1.7	0.09134	0.0629	**1.58693**	1.48	47.53	-46.04968
-0.04201	1.7	0.099	0.06238	**1.7227**	-23.88	33.35	-57.22539
-0.00329	1.7	0.10659	0.06187	**1.85948**	-1.87	24.51	-26.37896
-0.04848	1.7	0.11411	0.06137	**1.99727**	-27.55	18.65	-46.20145
-0.02632	1.7	0.12156	0.06086	**2.13606**	-14.96	14.57	-29.5273
-0.03007	1.7	0.12894	0.06037	**2.27586**	-17.09	11.61	-28.69484
-0.01286	1.7	0.13626	0.05987	**2.41667**	-7.31	9.39	-16.69765
0.01504	1.7	0.1435	0.05938	**2.55849**	8.55	7.68	0.86992
0.02926	1.7	0.15067	0.05889	**2.70131**	16.63	6.33	10.29665
-0.02331	1.7	0.15778	0.05841	**2.84513**	-13.25	5.24	-18.49181
0.00538	1.7	0.16481	0.05793	**2.98997**	3.06	4.35	-1.29615
	--	0.17178	0.05745	--			



ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

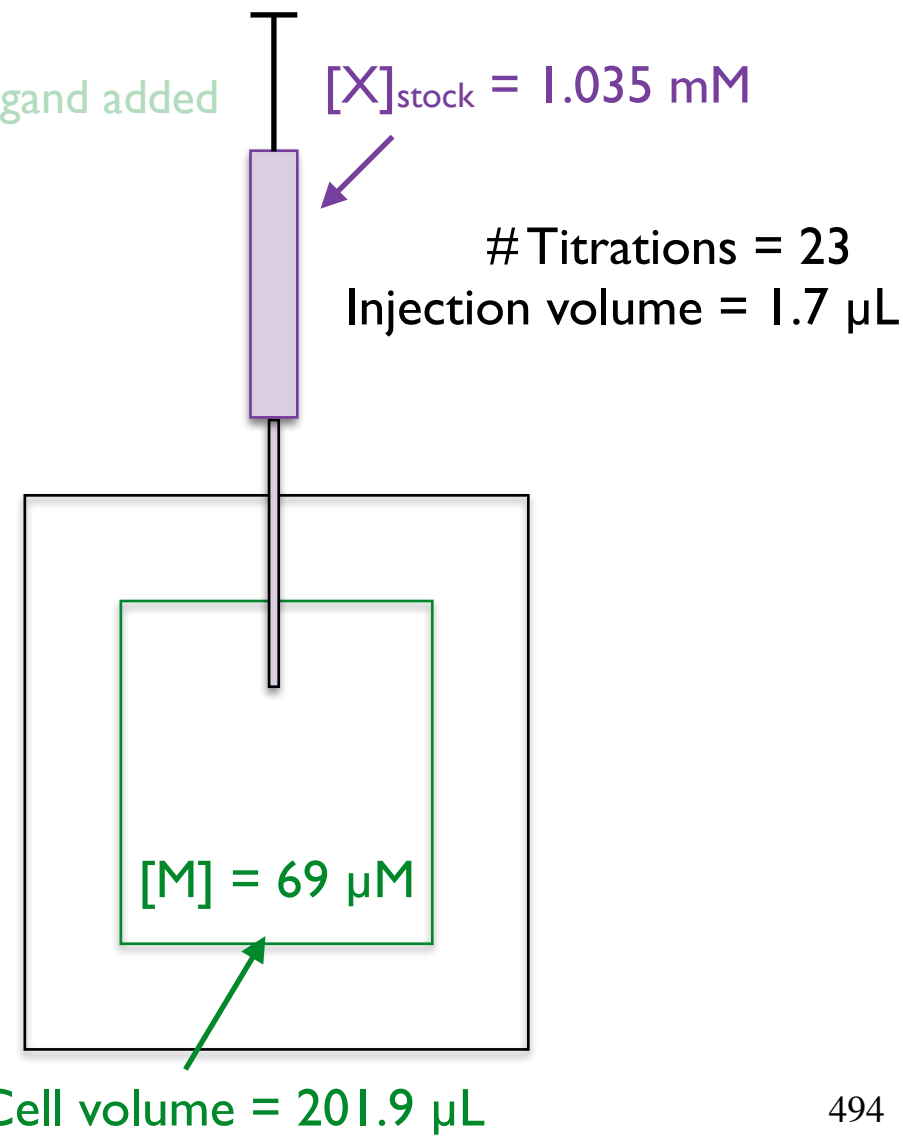
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

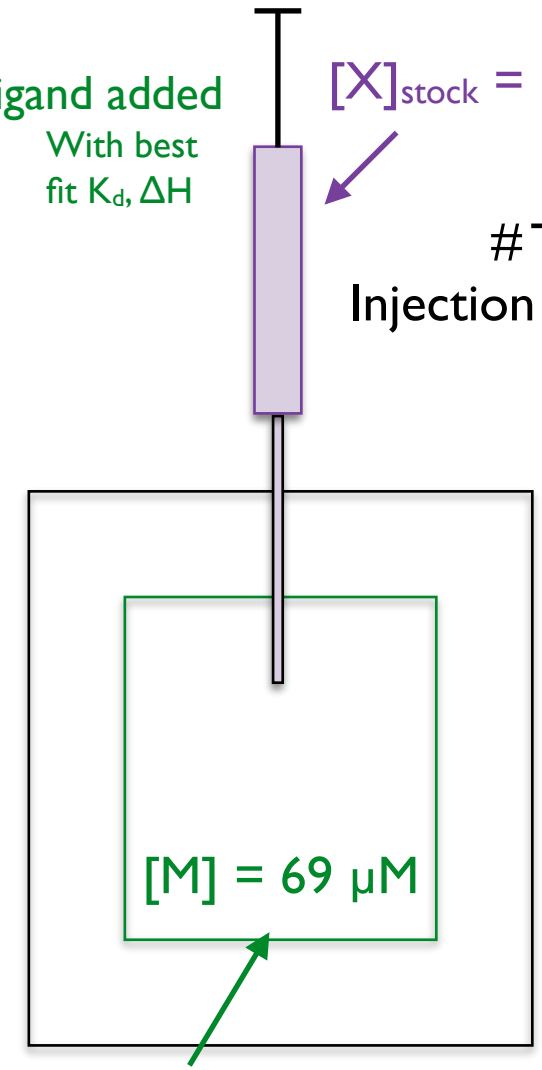
DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.03619	--	2299.99	--
4.01479	1.7	0.00249	0.06883	0.15988	**2281.78**	2292.82	-11.04176
4.07614	1.7	0.01092	0.06827	0.28458	**2316.65**	2276.50	40.14832
3.9874	1.7	0.01927	0.06772	0.41028	**2266.21**	2247.79	18.41812
3.86433	1.7	0.02756	0.06716	0.53699	**2196.27**	2191.38	4.88868
3.54838	1.7	0.03577	0.06661	0.66471	**2016.70**	2063.29	-46.5927
3.021	1.7	0.04392	0.06607	0.79344	**1716.96**	1739.43	-22.46873
1.96747	1.7	0.052	0.06553	0.92317	**1118.20**	1096.36	21.83654
0.91076	1.7	0.06	0.065	1.05391	**517.62**	501.20	16.42658
0.402	1.7	0.06794	0.06447	1.18565	**228.47**	227.82	0.65576
0.14295	1.7	0.07581	0.06394	1.31841	**81.24**	120.26	-39.02183
0.02726	1.7	0.08361	0.06342	1.45217	**15.50**	72.24	-56.74176
0.0026	1.7	0.09134	0.0629	1.58693	**1.48**	47.53	-46.04968
-0.04201	1.7	0.099	0.06238	1.7227	**-23.88**	33.35	-57.22539
-0.00329	1.7	0.10659	0.06187	1.85948	**-1.87**	24.51	-26.37896
-0.04848	1.7	0.11411	0.06137	1.99727	**-27.55**	18.65	-46.20145
-0.02632	1.7	0.12156	0.06086	2.13606	**-14.96**	14.57	-29.5273
-0.03007	1.7	0.12894	0.06037	2.27586	**-17.09**	11.61	-28.69484
-0.01286	1.7	0.13626	0.05987	2.41667	**-7.31**	9.39	-16.69765
0.01504	1.7	0.1435	0.05938	2.55849	**8.55**	7.68	0.86992
0.02926	1.7	0.15067	0.05889	2.70131	**16.63**	6.33	10.29665
-0.02331	1.7	0.15778	0.05841	2.84513	**-13.25**	5.24	-18.49181
0.00538	1.7	0.16481	0.05793	2.98997	**3.06**	4.35	-1.29615
	--	0.17178	0.05745	--			



ITC

q per injection (µcal)
injection volume (µL)
$[X]_{tot}$ prior to injection (ligand conc)
$[M]_{tot}$ prior to injection
Ratio $[X]_{tot}/[M]_{tot}$
q per mole ligand injected
Best fit q per mole ligand added
Residual
With best fit K_d, ΔH

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.03619	--	2299.99	--
4.01479	1.7	0.00249	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	1.7	0.01092	0.06827	0.28458	2316.65	2276.50	40.14832
3.9874	1.7	0.01927	0.06772	0.41028	2266.21	2247.79	18.41812
3.86433	1.7	0.02756	0.06716	0.53699	2196.27	2191.38	4.88868
3.54838	1.7	0.03577	0.06661	0.66471	2016.70	2063.29	-46.5927
3.021	1.7	0.04392	0.06607	0.79344	1716.96	1739.43	-22.46873
1.96747	1.7	0.052	0.06553	0.92317	1118.20	1096.36	21.83654
0.91076	1.7	0.06	0.065	1.05391	517.62	501.20	16.42658
0.402	1.7	0.06794	0.06447	1.18565	228.47	227.82	0.65576
0.14295	1.7	0.07581	0.06394	1.31841	81.24	120.26	-39.02183
0.02726	1.7	0.08361	0.06342	1.45217	15.50	72.24	-56.74176
0.0026	1.7	0.09134	0.0629	1.58693	1.48	47.53	-46.04968
-0.04201	1.7	0.099	0.06238	1.7227	-23.88	33.35	-57.22539
-0.00329	1.7	0.10659	0.06187	1.85948	-1.87	24.51	-26.37896
-0.04848	1.7	0.11411	0.06137	1.99727	-27.55	18.65	-46.20145
-0.02632	1.7	0.12156	0.06086	2.13606	-14.96	14.57	-29.5273
-0.03007	1.7	0.12894	0.06037	2.27586	-17.09	11.61	-28.69484
-0.01286	1.7	0.13626	0.05987	2.41667	-7.31	9.39	-16.69765
0.01504	1.7	0.1435	0.05938	2.55849	8.55	7.68	0.86992
0.02926	1.7	0.15067	0.05889	2.70131	16.63	6.33	10.29665
-0.02331	1.7	0.15778	0.05841	2.84513	-13.25	5.24	-18.49181
0.00538	1.7	0.16481	0.05793	2.98997	3.06	4.35	-1.29615
	--	0.17178	0.05745	--			



ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

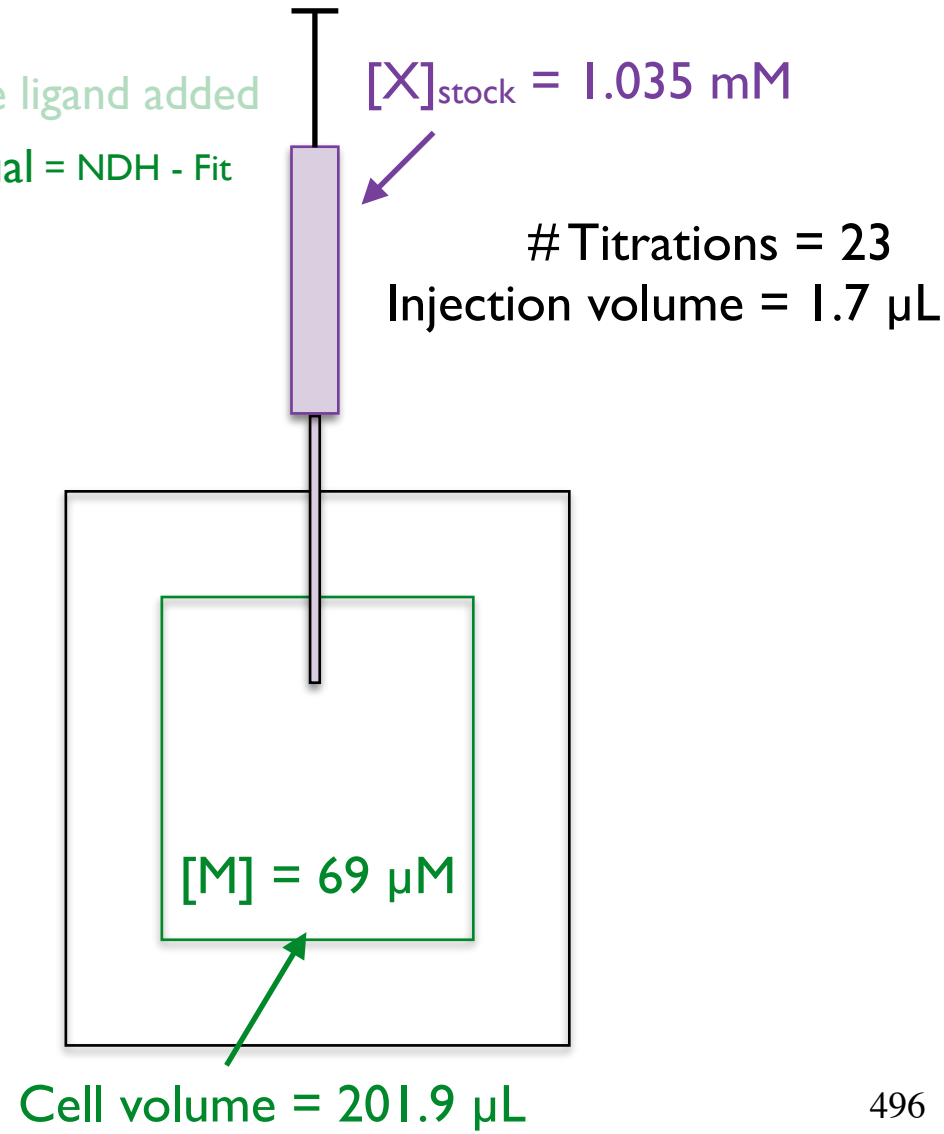
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual = NDH - Fit

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.03619	--	2299.99	--
4.01479	1.7	0.00249	0.06883	0.15988	2281.78	2292.82	**-11.04176**
4.07614	1.7	0.01092	0.06827	0.28458	2316.65	2276.50	**40.14832**
3.9874	1.7	0.01927	0.06772	0.41028	2266.21	2247.79	**18.41812**
3.86433	1.7	0.02756	0.06716	0.53699	2196.27	2191.38	**4.88868**
3.54838	1.7	0.03577	0.06661	0.66471	2016.70	2063.29	**-46.5927**
3.021	1.7	0.04392	0.06607	0.79344	1716.96	1739.43	**-22.46873**
1.96747	1.7	0.052	0.06553	0.92317	1118.20	1096.36	**21.83654**
0.91076	1.7	0.06	0.065	1.05391	517.62	501.20	**16.42658**
0.402	1.7	0.06794	0.06447	1.18565	228.47	227.82	**0.65576**
0.14295	1.7	0.07581	0.06394	1.31841	81.24	120.26	**-39.02183**
0.02726	1.7	0.08361	0.06342	1.45217	15.50	72.24	**-56.74176**
0.0026	1.7	0.09134	0.0629	1.58693	1.48	47.53	**-46.04968**
-0.04201	1.7	0.099	0.06238	1.7227	-23.88	33.35	**-57.22539**
-0.00329	1.7	0.10659	0.06187	1.85948	-1.87	24.51	**-26.37896**
-0.04848	1.7	0.11411	0.06137	1.99727	-27.55	18.65	**-46.20145**
-0.02632	1.7	0.12156	0.06086	2.13606	-14.96	14.57	**-29.5273**
-0.03007	1.7	0.12894	0.06037	2.27586	-17.09	11.61	**-28.69484**
-0.01286	1.7	0.13626	0.05987	2.41667	-7.31	9.39	**-16.69765**
0.01504	1.7	0.1435	0.05938	2.55849	8.55	7.68	**0.86992**
0.02926	1.7	0.15067	0.05889	2.70131	16.63	6.33	**10.29665**
-0.02331	1.7	0.15778	0.05841	2.84513	-13.25	5.24	**-18.49181**
0.00538	1.7	0.16481	0.05793	2.98997	3.06	4.35	**-1.29615**
	--	0.17178	0.05745	--			



ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

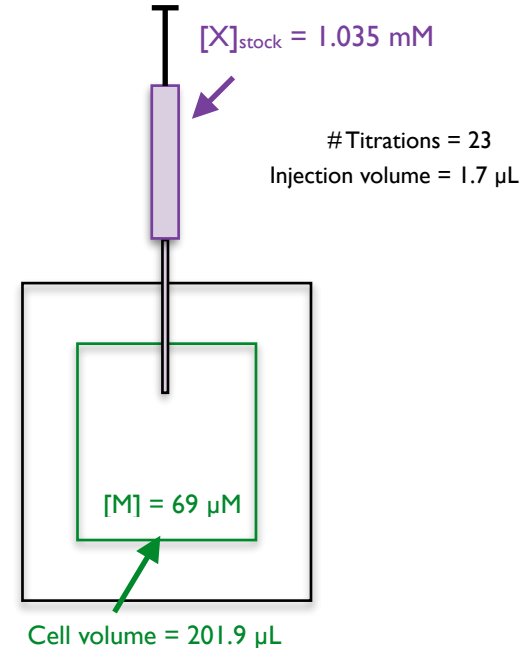
Ratio $[X]_{tot}/[M]_{tot}$

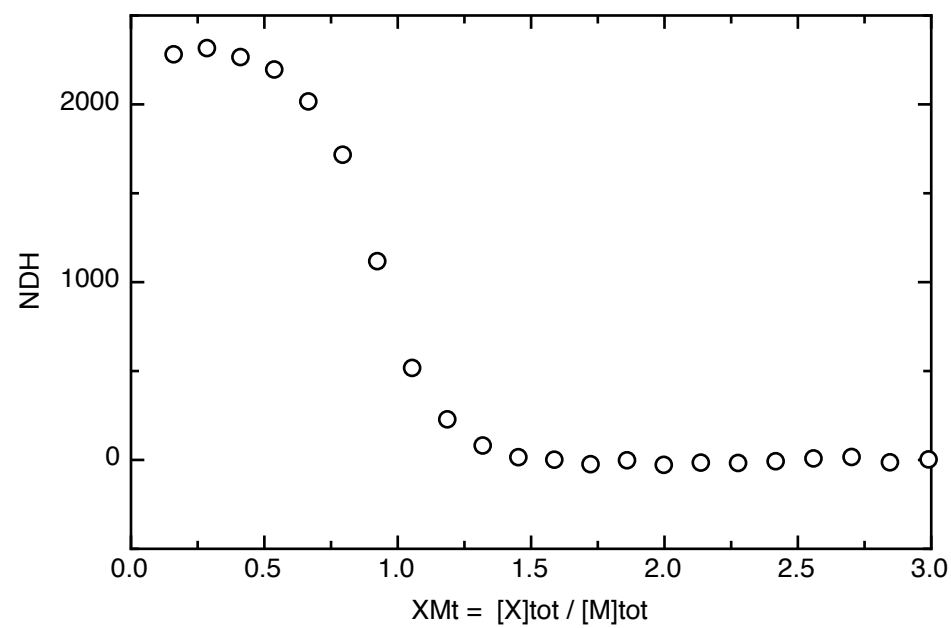
q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.0[illegible]9	--	2299.99	--
4.01479	1.7	0.00249	0.06883	**0.15988**	**2281.78**	2292.82	-11.04176
4.07614	1.7	0.01092	0.06827	**0.28458**	**2316.65**	2276.50	40.14832
3.9874	1.7	0.01927	0.06772	**0.41028**	**2266.21**	2247.79	18.41812
3.86433	1.7	0.02756	0.06716	**0.53699**	**2196.27**	2191.38	4.88868
3.54838	1.7	0.03577	0.06661	**0.66471**	**2016.70**	2063.29	-46.5927
3.021	1.7	0.04392	0.06607	**0.79344**	**1716.96**	1739.43	-22.46873
1.96747	1.7	0.052	0.06553	**0.92317**	**1118.20**	1096.36	21.83654
0.91076	1.7	0.06	0.065	**1.05391**	**517.62**	501.20	16.42658
0.402	1.7	0.06794	0.06447	**1.18565**	**228.47**	227.82	0.65576
0.14295	1.7	0.07581	0.06394	**1.31841**	**81.24**	120.26	-39.02183
0.02726	1.7	0.08361	0.06342	**1.45217**	**15.50**	72.24	-56.74176
0.0026	1.7	0.09134	0.0629	**1.58693**	**1.48**	47.53	-46.04968
-0.04201	1.7	0.099	0.06238	**1.7227**	**-23.88**	33.35	-57.22539
-0.00329	1.7	0.10659	0.06187	**1.85948**	**-1.87**	24.51	-26.37896
-0.04848	1.7	0.11411	0.06137	**1.99727**	**-27.55**	18.65	-46.20145
-0.02632	1.7	0.12156	0.06086	**2.13606**	**-14.96**	14.57	-29.5273
-0.03007	1.7	0.12894	0.06037	**2.27586**	**-17.09**	11.61	-28.69484
-0.01286	1.7	0.13626	0.05987	**2.41667**	**-7.31**	9.39	-16.69765
0.01504	1.7	0.1435	0.05938	**2.55849**	**8.55**	7.68	0.86992
0.02926	1.7	0.15067	0.05889	**2.70131**	**16.63**	6.33	10.29665
-0.02331	1.7	0.15778	0.05841	**2.84513**	**-13.25**	5.24	-18.49181
0.00538	1.7	0.16481	0.05793	**2.98997**	**3.06**	4.35	-1.29615
	--	0.17178	0.05745	--			





ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.03[illegible]9	--	2299.99	--
4.01479	1.7	0.00249	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	1.7	0.01092	0.06827	0.28458	2316.65	2276.50	40.14832
3.9874	1.7	0.01927	0.06772	0.41028	2266.21	2247.79	18.41812
3.86433	1.7	0.02756	0.06716	0.53699	2196.27	2191.38	4.88868
3.54838	1.7	0.03577	0.06661	0.66471	2016.70	2063.29	-46.5927
3.021	1.7	0.04392	0.06607	0.79344	1716.96	1739.43	-22.46873
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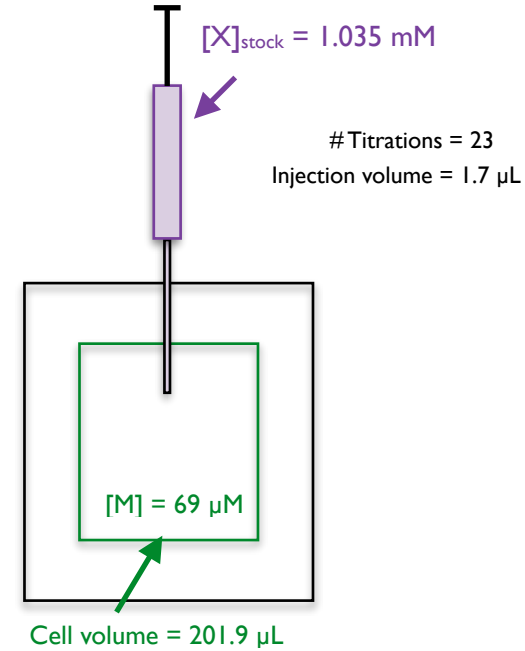
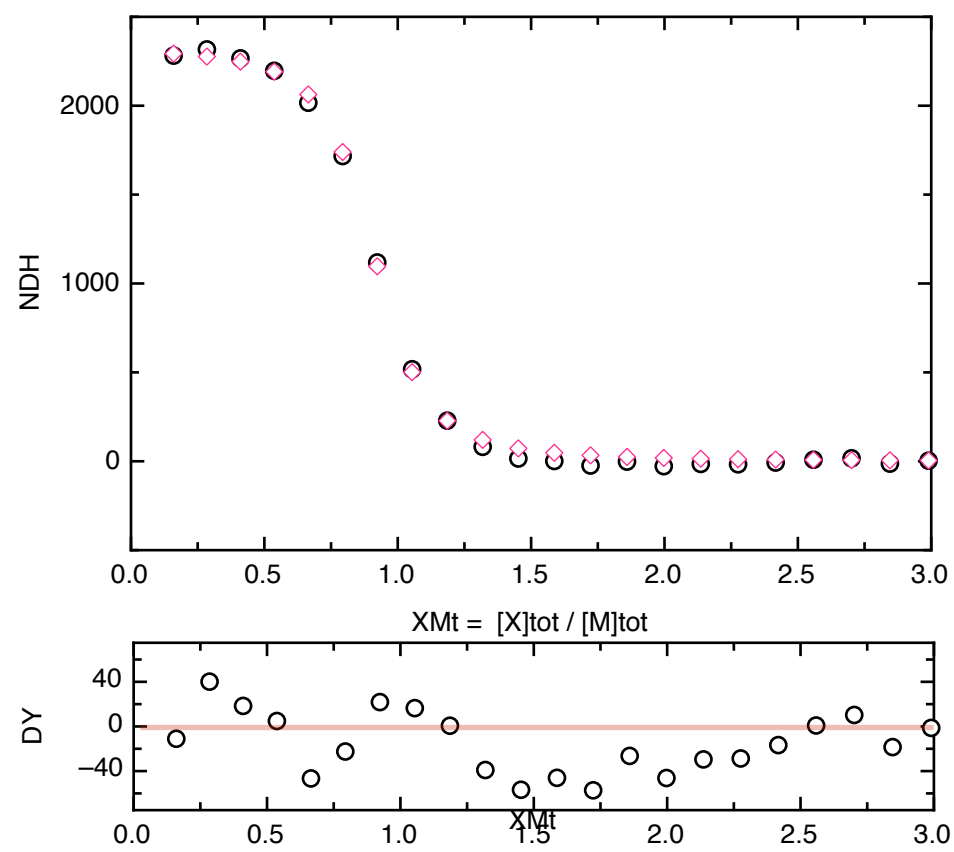
0.402	1.7	0.06794	0.06447	1.18565	228.47	227.82	0.65576
0.14295	1.7	0.07581	0.06394	1.31841	81.24	120.26	-39.02183
0.02726	1.7	0.08361	0.06342	1.45217	15.50	72.24	-56.74176
0.0026	1.7	0.09134	0.0629	1.58693	1.48	47.53	-46.04968
-0.04201	1.7	0.099	0.06238	1.7227	-23.88	33.35	-57.22539
-0.00329	1.7	0.10659	0.06187	1.85948	-1.87	24.51	-26.37896
-0.04848	1.7	0.11411	0.06137	1.99727	-27.55	18.65	-46.20145
-0.02632	1.7	0.12156	0.06086	2.13606	-14.96	14.57	-29.5273
-0.03007	1.7	0.12894	0.06037	2.27586	-17.09	11.61	-28.69484
-0.01286	1.7	0.13626	0.05987	2.41667	-7.31	9.39	-16.69765
0.01504	1.7	0.1435	0.05938	2.55849	8.55	7.68	0.86992
0.02926	1.7	0.15067	0.05889	2.70131	16.63	6.33	10.29665
-0.02331	1.7	0.15778	0.05841	2.84513	-13.25	5.24	-18.49181
0.00538	1.7	0.16481	0.05793	2.98997	3.06	4.35	-1.29615
	--	0.17178	0.05745	--			





ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

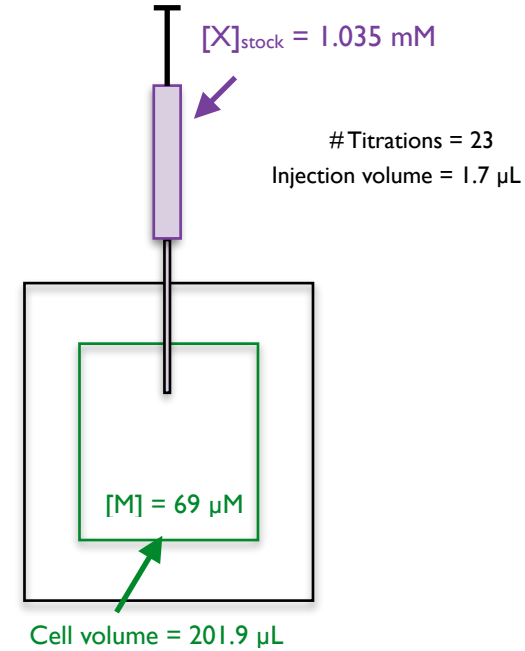
Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.0359	--	2299.99	--
4.01479	1.7	0.00249	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	1.7	0.01092	0.06827	0.28458	2316.65	2276.50	40.14832
3.9874	1.7	0.01927	0.06772	0.41028	2266.21	2247.79	18.41812
3.86433	1.7	0.02756	0.06716	0.53699	2196.27	2191.38	4.88868
3.54838	1.7	0.03577	0.06661	0.66471	2016.70	2063.29	-46.5927
3.021	1.7	0.04392	0.06607	0.79344	1716.96	1739.43	-22.46873
1.96747	1.7	0.052	0.06553	0.92317	1118.20	1096.36	21.83654
0.91076	1.7	0.06	0.065	1.05391	517.62	501.20	16.42658
0.402	1.7	0.06794	0.06447	1.18565	228.47	227.82	0.65576
0.14295	1.7	0.07581	0.06394	1.31841	81.24	120.26	-39.02183
0.02726	1.7	0.08361	0.06342	1.45217	15.50	72.24	-56.74176
0.0026	1.7	0.09134	0.0629	1.58693	1.48	47.53	-46.04968
-0.04201	1.7	0.099	0.06238	1.7227	-23.88	33.35	-57.22539
-0.00329	1.7	0.10659	0.06187	1.85948	-1.87	24.51	-26.37896
-0.04848	1.7	0.11411	0.06137	1.99727	-27.55	18.65	-46.20145
-0.02632	1.7	0.12156	0.06086	2.13606	-14.96	14.57	-29.5273
-0.03007	1.7	0.12894	0.06037	2.27586	-17.09	11.61	-28.69484
-0.01286	1.7	0.13626	0.05987	2.41667	-7.31	9.39	-16.69765
0.01504	1.7	0.1435	0.05938	2.55849	8.55	7.68	0.86992
0.02926	1.7	0.15067	0.05889	2.70131	16.63	6.33	10.29665
-0.02331	1.7	0.15778	0.05841	2.84513	-13.25	5.24	-18.49181
0.00538	1.7	0.16481	0.05793	2.98997	3.06	4.35	-1.29615
	--	0.17178	0.05745	--			



Sources of error?

Buffer mismatch, etc?

Is this a problem here?

No, but maybe….?

ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

Residual

DH	INJV	Xt	Mt	XMt	NDH	Fit	DY
0.98819	0.5	0	0.069	0.0[illegible]9	--	2299.99	--
4.01479	1.7	0.00249	0.06883	0.15988	2281.78	2292.82	-11.04176
4.07614	1.7	0.01092	0.06827	0.28458	2316.65	2276.50	40.14832
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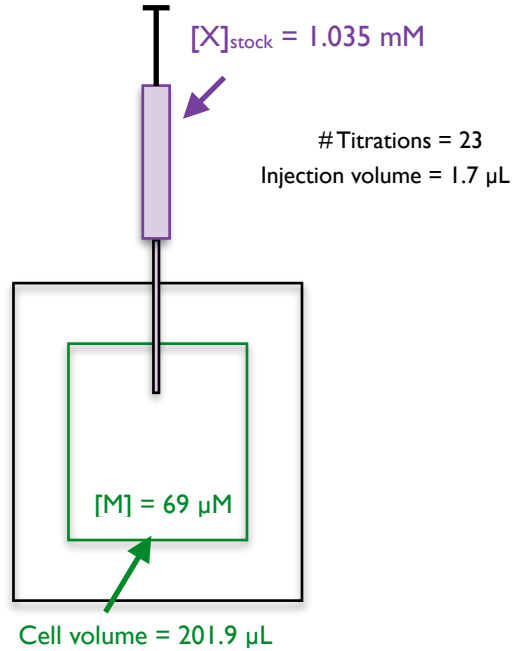
	--	0.17178	0.05745	--			



Sources of error?

- Errors in $[X]_{stock}$
- Errors in [M]

ITC

q per injection (µcal)

injection volume (µL)

$[X]_{tot}$ prior to injection (ligand conc)

$[M]_{tot}$ prior to injection

Ratio $[X]_{tot}/[M]_{tot}$

q per mole ligand injected

Best fit q per mole ligand added

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-0.04201	1.7	0.099	0.06238	1.7227	-23.88	33.35	-57.22539
-0.00329	1.7	0.10659	0.06187	1.85948	-1.87	24.51	-26.37896
-0.04848	1.7	0.11411	0.06137	1.99727	-27.55	18.65	-46.20145
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-0.03007	1.7	0.12894	0.06037	2.27586	-17.09	11.61	-28.69484
-0.01286	1.7	0.13626	0.05987	2.41667	-7.31	9.39	-16.69765
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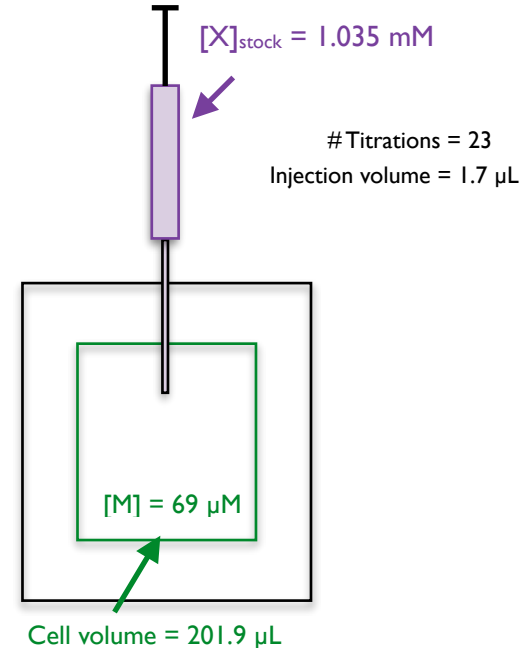
-0.02331	1.7	0.15778	0.05841	2.84513	-13.25	5.24	-18.49181
0.00538	1.7	0.16481	0.05793	2.98997	3.06	4.35	-1.29615
	--	0.17178	0.05745	--			

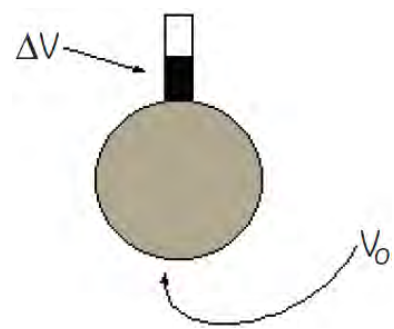


Sources of error?

Injection volume

Random

Systematic



How would that present?

Volume — cumulative

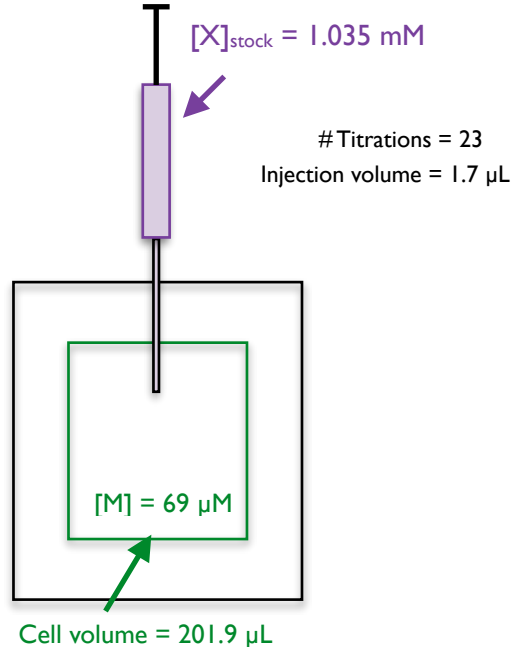
Impacts *both* x and y

in nonlinear ways

ITC

$$K = \frac{[MX]}{[M][X]} = \frac{\theta}{(1-\theta)[X]}$$

$$[X]_{tot} = [X] + [MX] = [X] + \theta[M]_{tot}$$

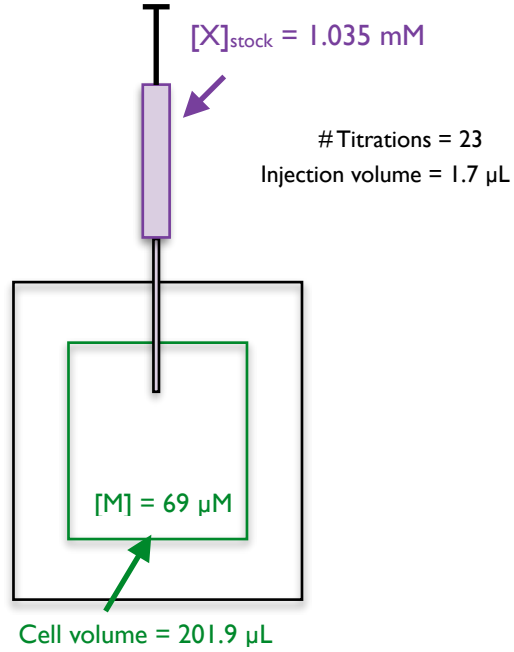


ITC

$$K = \frac{[MX]}{[M][X]} = \frac{\theta}{(1-\theta)[X]}$$

$$[X]_{tot} = [X] + [MX] = [X] + \theta[M]_{tot}$$

$$K(1-\theta)[X] = \theta$$



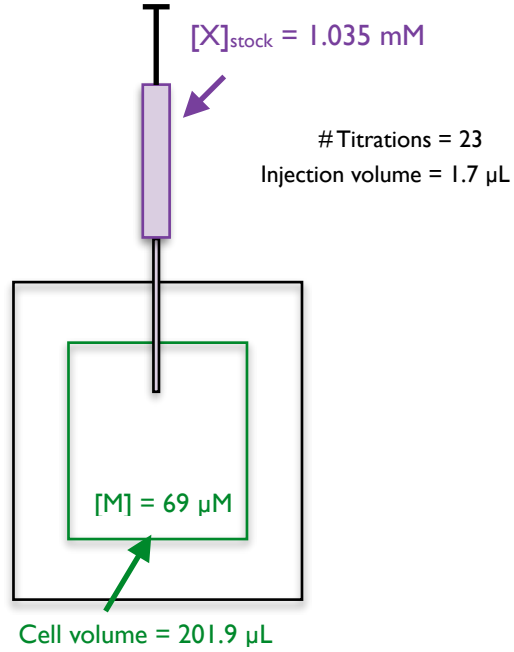
ITC

$$K = \frac{[MX]}{[M][X]} = \frac{\theta}{(1-\theta)[X]}$$

$$[X]_{tot} = [X] + [MX] = [X] + \theta[M]_{tot}$$

$$K(1-\theta)[X] = \theta$$

$$K(1-\theta)\left([X]_{tot} - \theta[M]_{tot}\right) = \theta$$



ITC

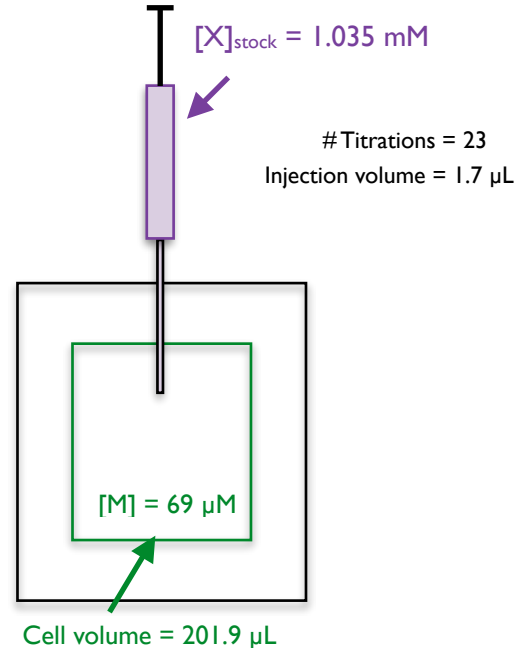
$$K = \frac{[MX]}{[M][X]} = \frac{\theta}{(1-\theta)[X]}$$

$$[X]_{tot} = [X] + [MX] = [X] + \theta[M]_{tot}$$

$$K(1-\theta)[X] = \theta$$

$$K(1-\theta)\left([X]_{tot} - \theta[M]_{tot}\right) = \theta$$

$$\theta^2 - \theta\left(1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}} + \frac{X_{tot}}{M_{tot}}\right) = 0$$



ITC

$$K = \frac{[MX]}{[M][X]} = \frac{\theta}{(1-\theta)[X]}$$

$$[X]_{tot} = [X] + [MX] = [X] + \theta[M]_{tot}$$

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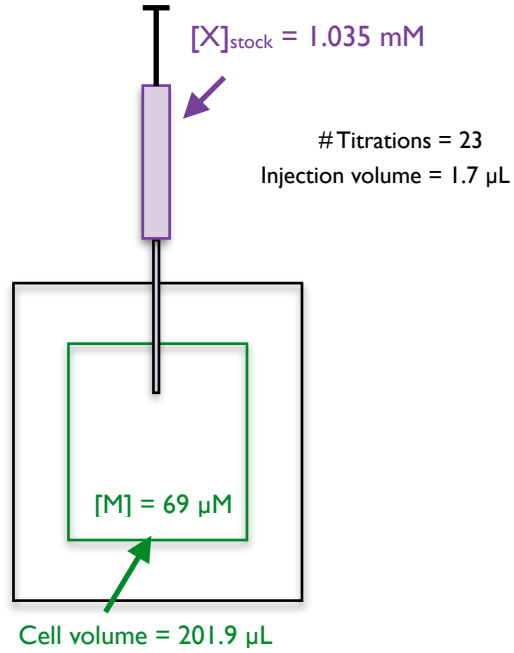
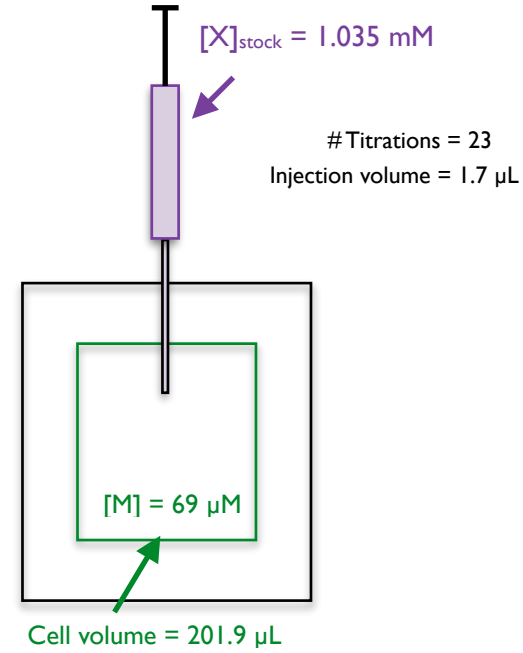
$$\theta^2 - \theta\left(1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}} + \frac{X_{tot}}{M_{tot}}\right) = 0$$



$$Q = \theta \times M_{tot} \times V_0 \times \Delta H$$

ITC



$$K = \frac{[MX]}{[M][X]} = \frac{\theta}{(1-\theta)[X]}$$

$$[X]_{tot} = [X] + [MX] = [X] + \theta[M]_{tot}$$

$$K(1-\theta)[X] = \theta$$

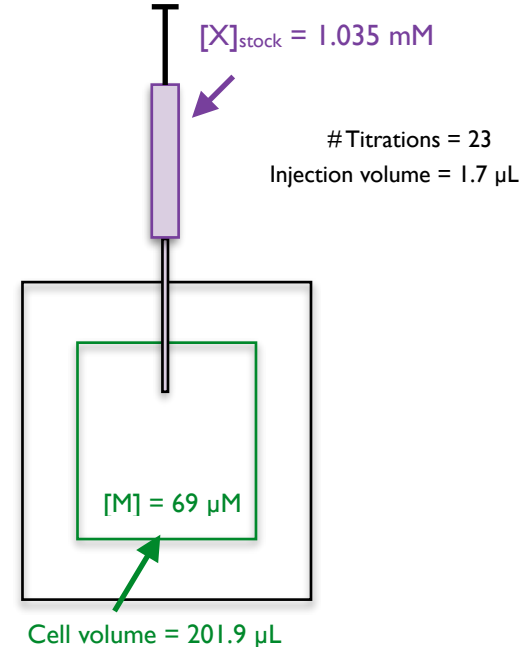
$$K(1-\theta)\left([X]_{tot} - \theta[M]_{tot}\right) = \theta$$

$$Q = \theta \times M_{tot} \times V_0 \times \Delta H$$

$$\theta^2 - \theta\left(1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}} + \frac{X_{tot}}{M_{tot}}\right) = 0$$

$$Q = \frac{M_{tot} \times V_0 \times \Delta H}{2}\left[1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}} - \sqrt{\left(1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}}\right)^2 - 4\frac{X_{tot}}{M_{tot}}}\right]$$

ITC



$$K = \frac{[MX]}{[M][X]} = \frac{\theta}{(1-\theta)[X]}$$

$$[X]_{tot} = [X] + [MX] = [X] + \theta[M]_{tot}$$

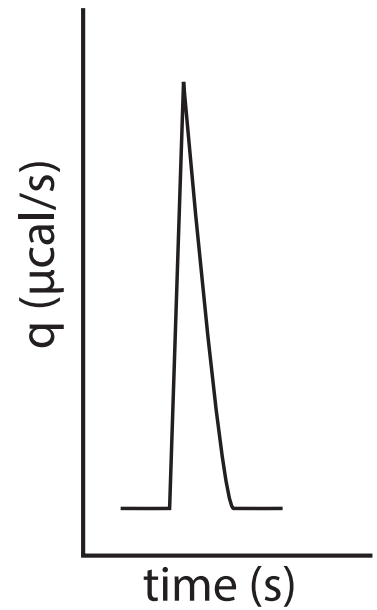
$$K(1-\theta)[X] = \theta$$

$$K(1-\theta)\left([X]_{tot} - \theta[M]_{tot}\right) = \theta$$

$$Q = \theta \times M_{tot} \times V_0 \times \Delta H$$

$$\theta^2 - \theta\left(1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}} + \frac{X_{tot}}{M_{tot}}\right) = 0$$

$$Q = \frac{M_{tot} \times V_0 \times \Delta H}{2}\left[1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}} - \sqrt{\left(1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}}\right)^2 - 4\frac{X_{tot}}{M_{tot}}}\right]$$

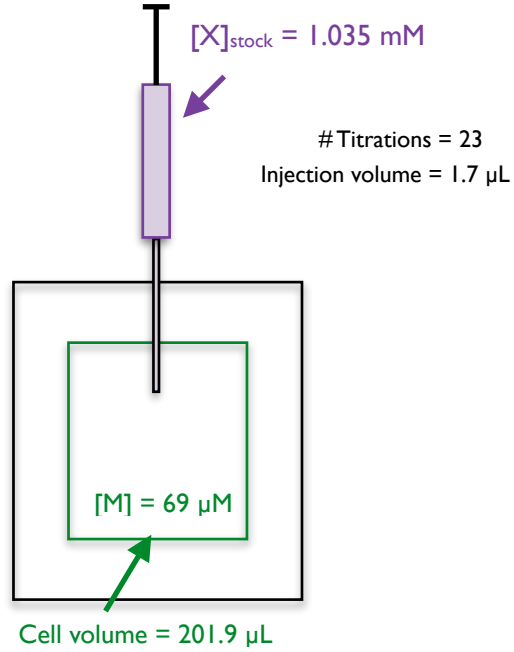


$$\Delta Q(i) = Q(i) - Q(i-1)$$

ITC

$$Q = \frac{M_{tot} \times V_0 \times \Delta H}{2}\left[1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}} - \sqrt{\left(1 + \frac{X_{tot}}{M_{tot}} + \frac{1}{KM_{tot}}\right)^2 - 4\frac{X_{tot}}{M_{tot}}}\right]$$

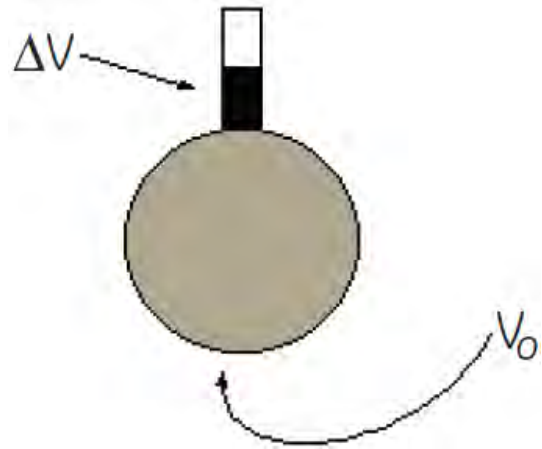
$$\Delta Q(i) = Q(i) - Q(i-1)$$



$$Q = \theta \times M_{tot} \times V_0 \times \Delta H$$

But remember that there is this exclude volume stuff

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1)$$



ITC

12.3.3 Two sets of independent sites

Using the same definition of symbols as above for set 1 and set 2, we have

$$K_1 = \frac{\Theta_1}{(1 - \Theta_1)[X]}$$

$$K_2 = \frac{\Theta_2}{(1 - \Theta_2)[X]}$$

Equation (11)

$$X_t = [X] + M_t(n_1\Theta_1 + n_2\Theta_2)$$

Equation (12)

Solving Equation (11) for Θ_1 and Θ_2 and then substituting into Equation (12) gives

$$X_t = [X] + \frac{n_1 M_t[X]K_1}{1 + [X]K_1} + \frac{n_2 M_t[X]K_2}{1 + [X]K_2}$$

Equation (13)

Clearing Equation (13) of fractions and collecting like terms leads to a cubic equation of the form

$$[X^3] + p[X^2] + q[X] + r = 0$$

Equation (14)

where,

$$p = \frac{1}{K_1} + \frac{1}{K_2} + (n_1 + n_2)M_t - X_t$$

$$q = (\frac{n_1}{K_2} + \frac{n_2}{K_1})M_t - (\frac{1}{K_1} + \frac{1}{K_2})X_t + \frac{1}{K_1K_2}$$

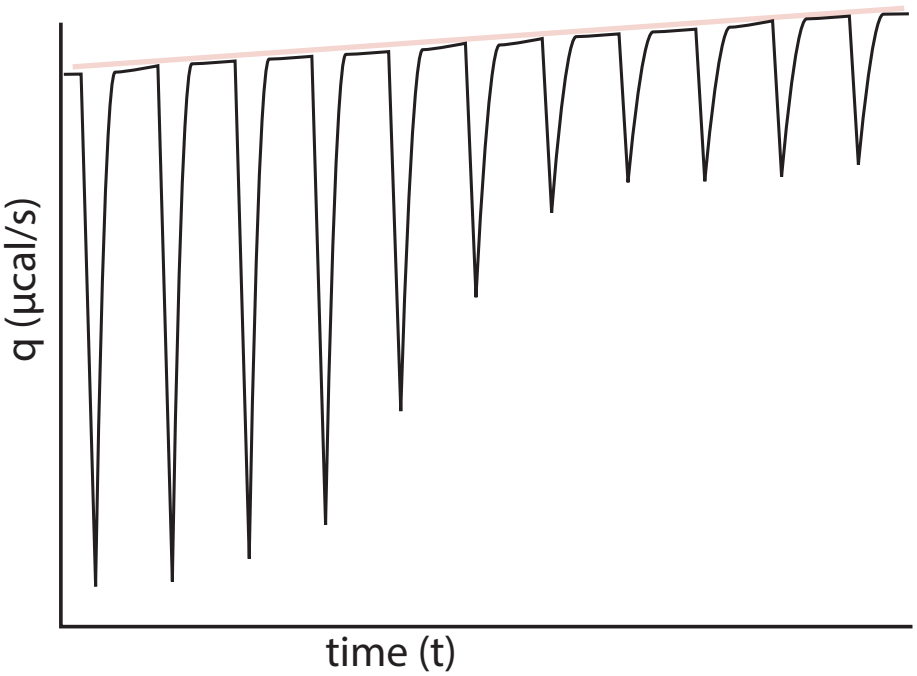
$$r = \frac{-X_t}{K_1K_2}$$

Equation (15)

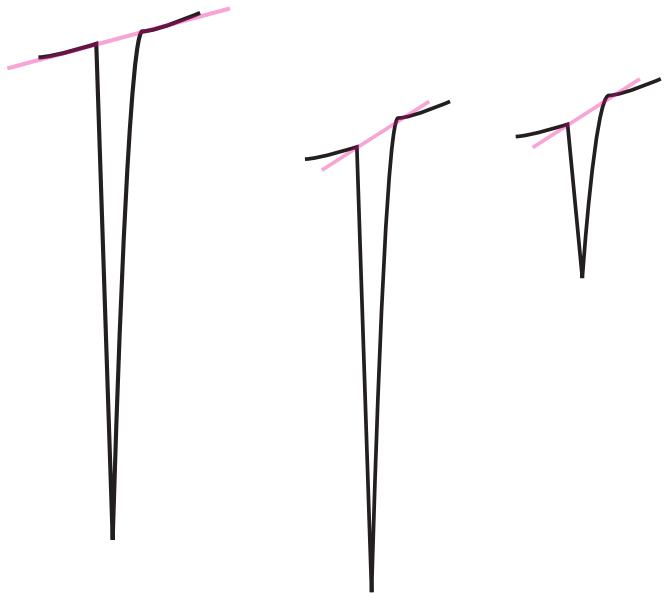
From the Manual

"Equation 14 and Equation 15 can be solved for [X] either in closed form or (as done in MicroCal ITC Software) numerically by using Newton's Method if parameters n1, n2, K1, and K2 are assigned. Both θ1 and θ2 may then be obtained from Equation 11 above."

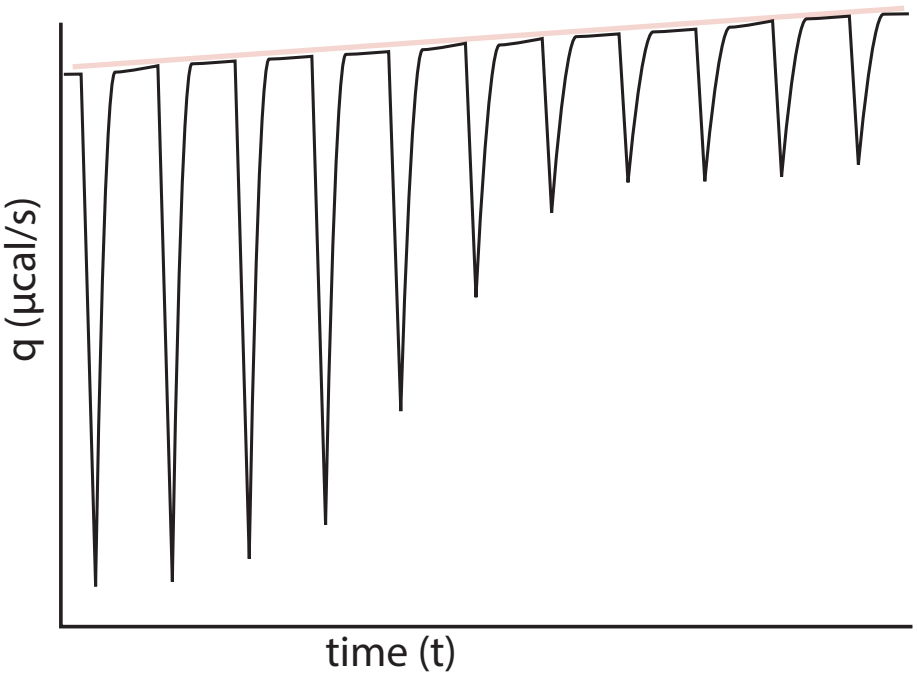
ITC
Practical



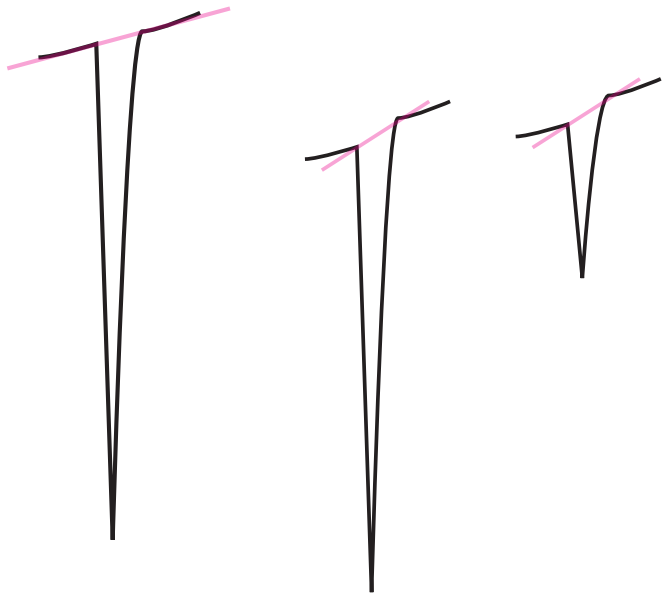
Baseline subtraction



ITC
Practical

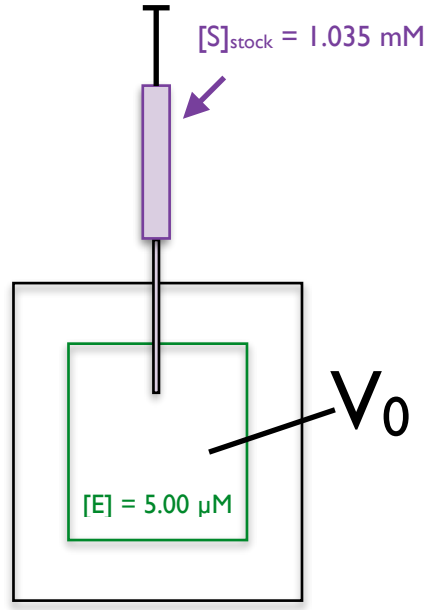


Baseline subtraction



Over-smoothing

ITC
Enzyme Kinetics



$$Velocity = R_t = \frac{P}{\Delta H \times V_0} \quad = \frac{\mu cal/s}{\left(\mu cal/mol\ S\right) \times (Liters)}$$

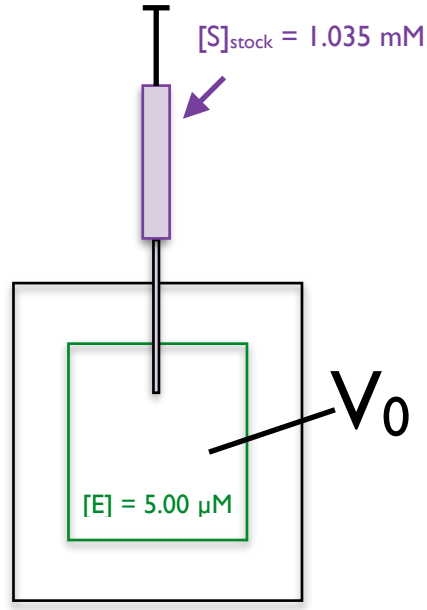
$$R_t = \frac{k_{cat}[E]_T[S]_T}{[S]_T + K_m}$$

Assuming $[S]_T >> [E]_T$

Assuming steady state kinetics

$$E + S \underset{k_r}{\overset{k_f}{\rightleftarrows}} ES \xrightarrow{k_{cat}} E + P$$

ITC
Enzyme Kinetics



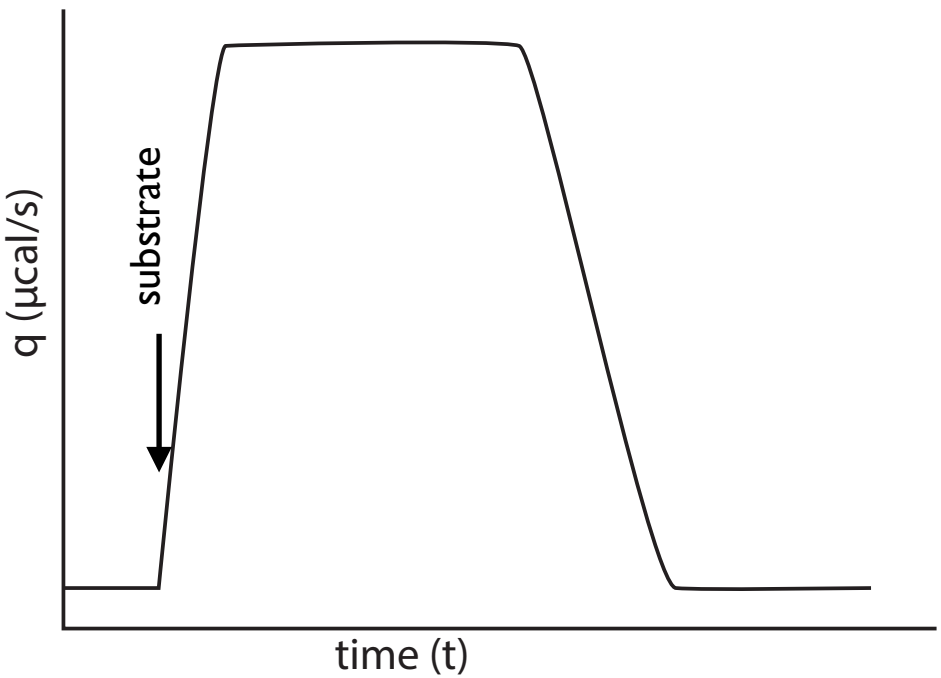
$$Velocity = R_t = \frac{P}{\Delta H \times V_0} = \frac{\mu cal/s}{\left(\mu cal/mol\ S\right) \times (Liters)}$$

$$R_t = \frac{k_{cat}[E]_T[S]_T}{[S]_T + K_m}$$

Assuming $[S]_T >> [E]_T$

Assuming steady state kinetics

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$



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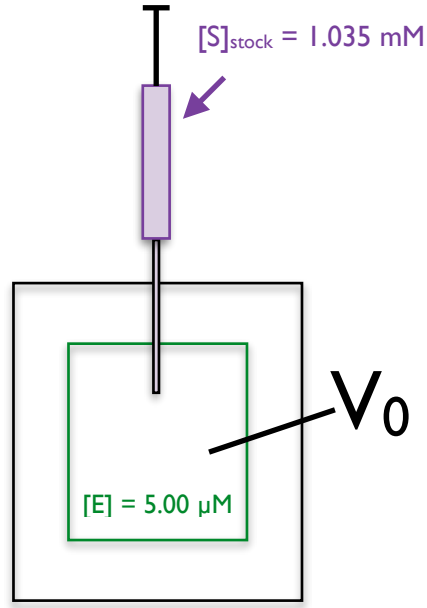

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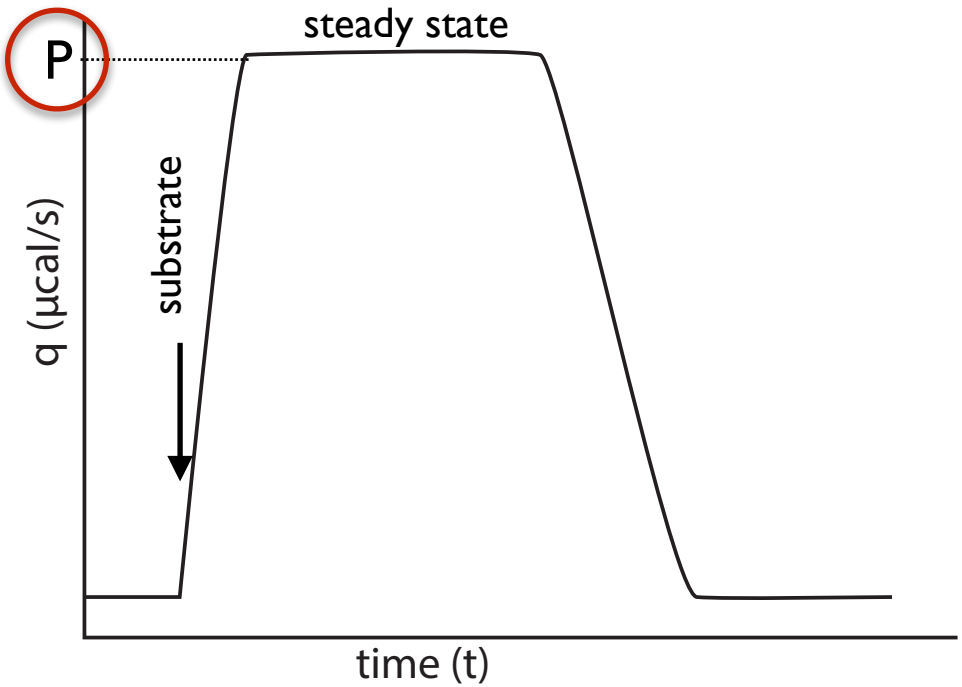
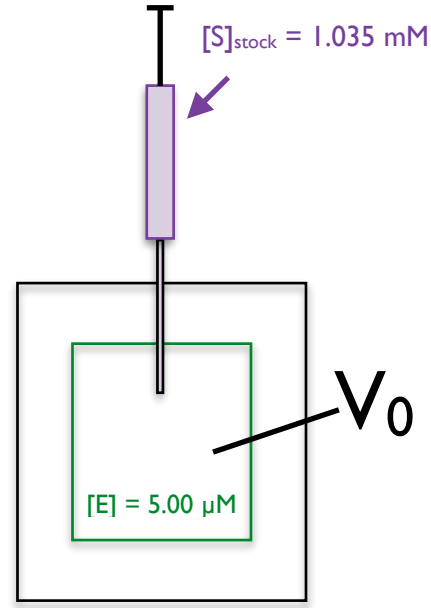


ITC
Enzyme Kinetics



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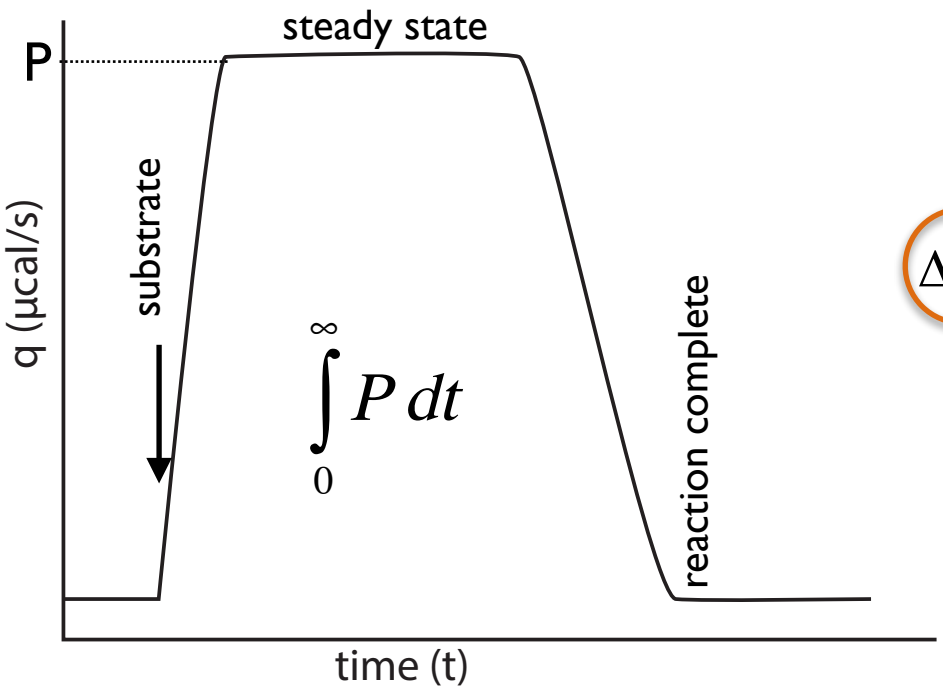
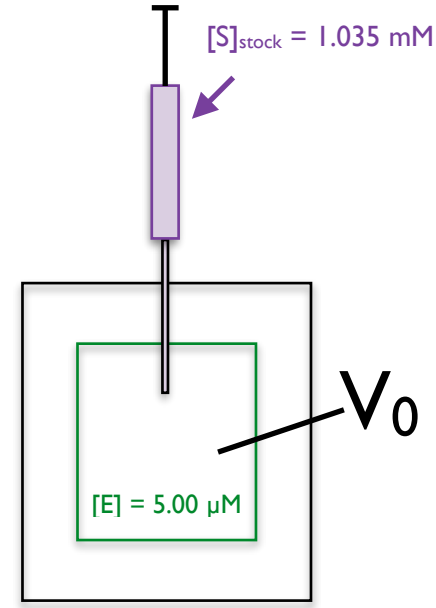


$$\Delta H = \frac{\int_0^\infty P\,dt}{[S]_0 \times V_0}$$

Heat generate in consuming all of S

ITC
Enzyme Kinetics



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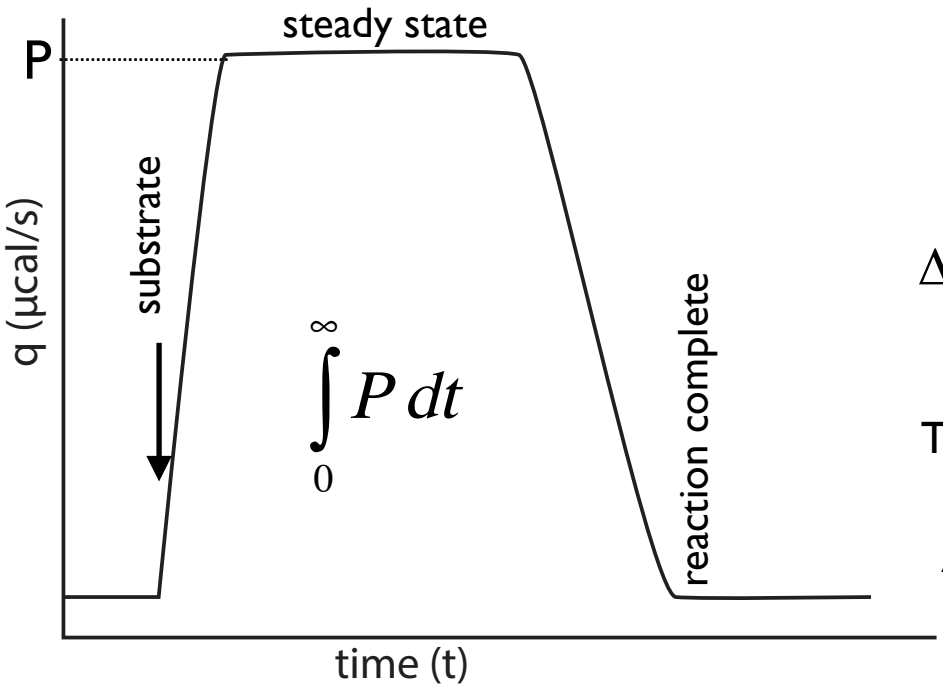
Assuming $[S]_T >> [E]_T$

Assuming steady state kinetics



$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

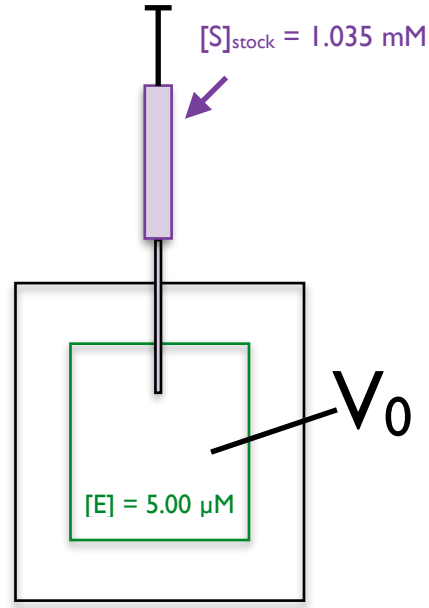
$$\Delta H = \frac{\int_0^\infty P\,dt}{[S]_0 \times V_0}$$

Heat generate in consuming all of S

Then fit Velocity (R_t) vs $[S]_T$ as with traditional kinetics

Assumes no back or background reaction (e.g. hydrolysis)

ITC
Enzyme Kinetics



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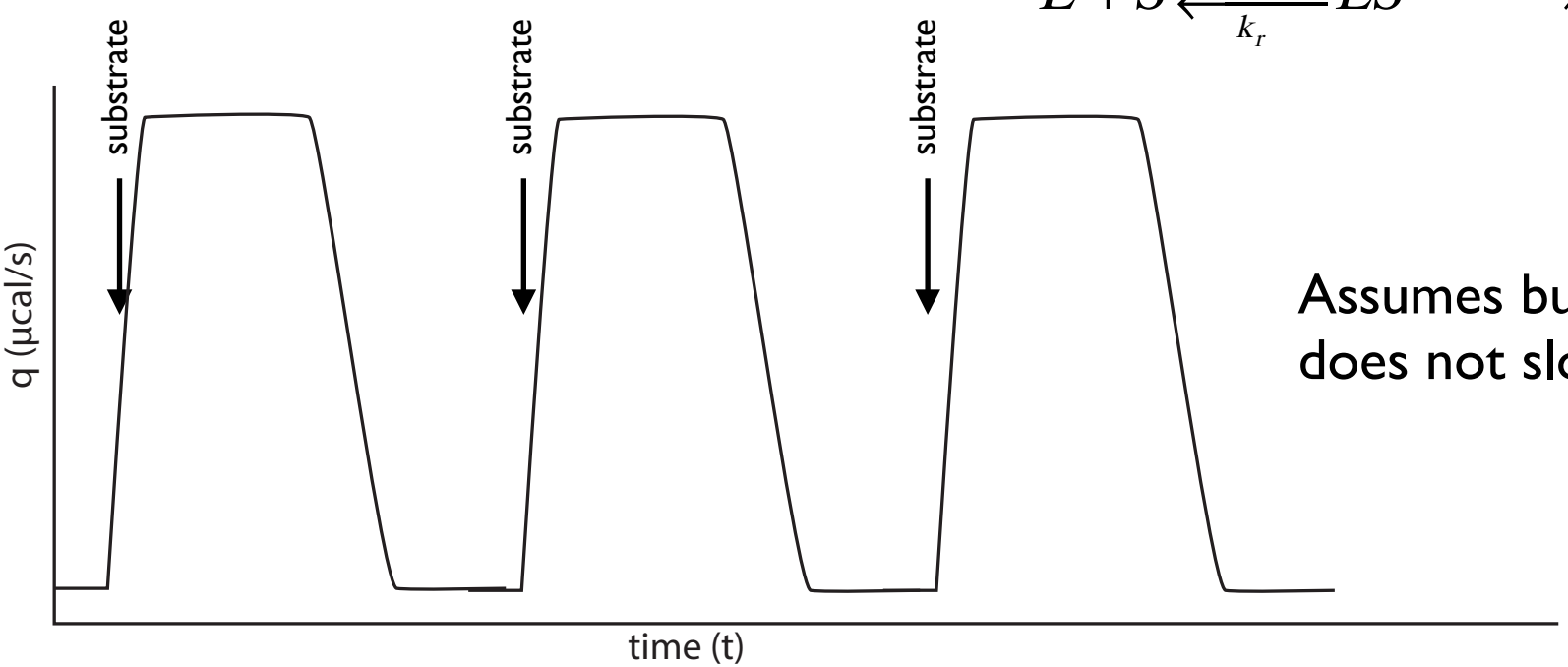


Assumes build up of product does not slow reaction

Abs = nnn

Conc = xxx

NanoDrop

(routine UV-Vis more generally)

Let's simulate a spectrum in R

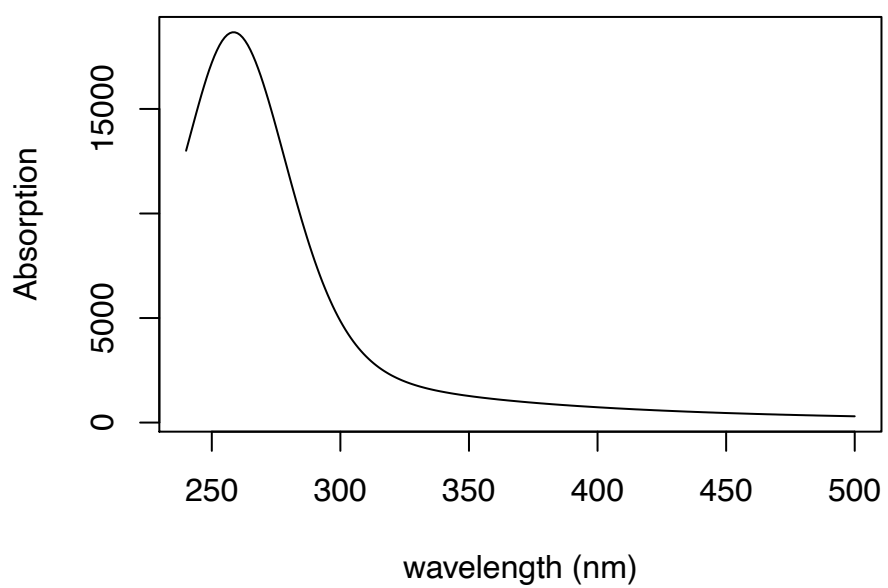
Normal (Gaussian) distribution $f(x) = \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{(x - x_{max})^2}{2\sigma^2}}$

x = seq(240,500, by=1)

y = dnorm(x, mean, sd) y = dnorm(x, 260, 10)

plot(x, y, type="l")

Incorrect - why?



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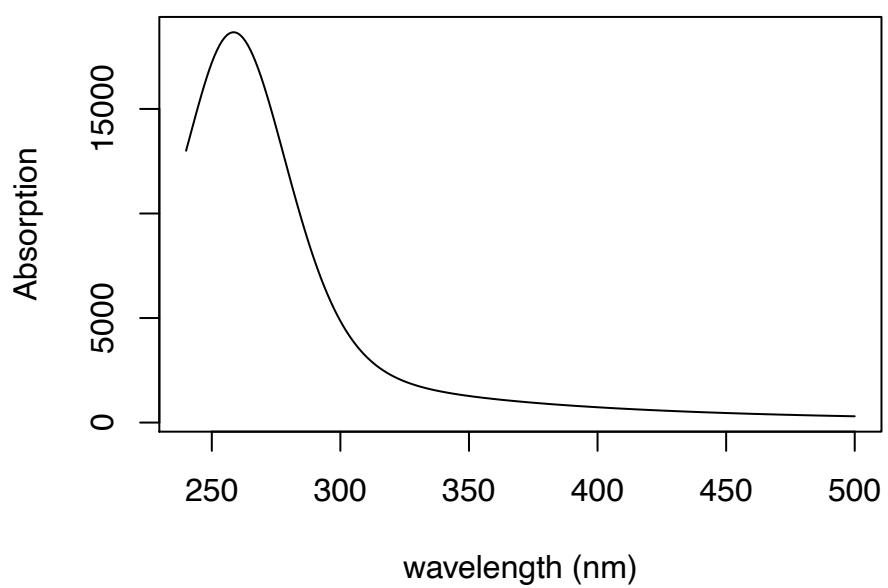

hypothesis: scattering!

NanoDrop

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(routine UV-Vis more generally)

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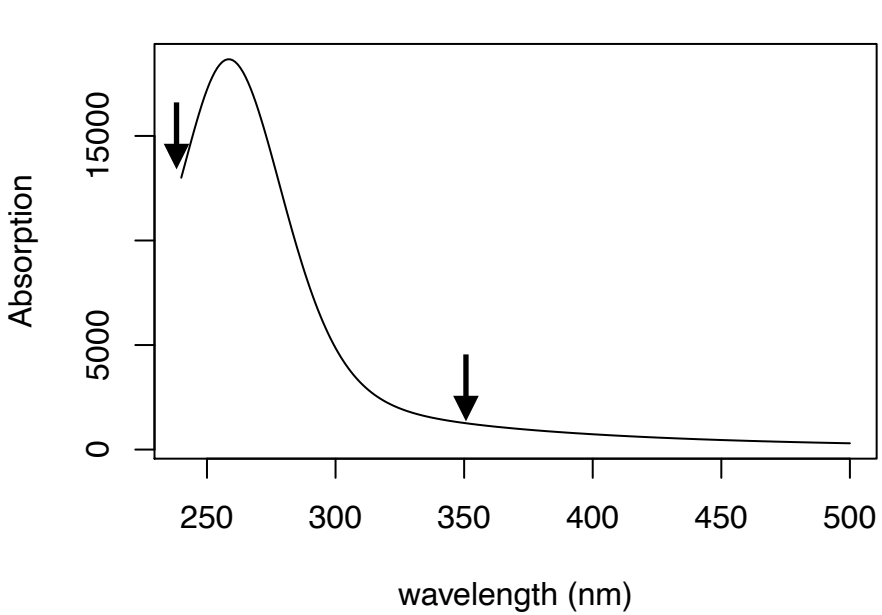
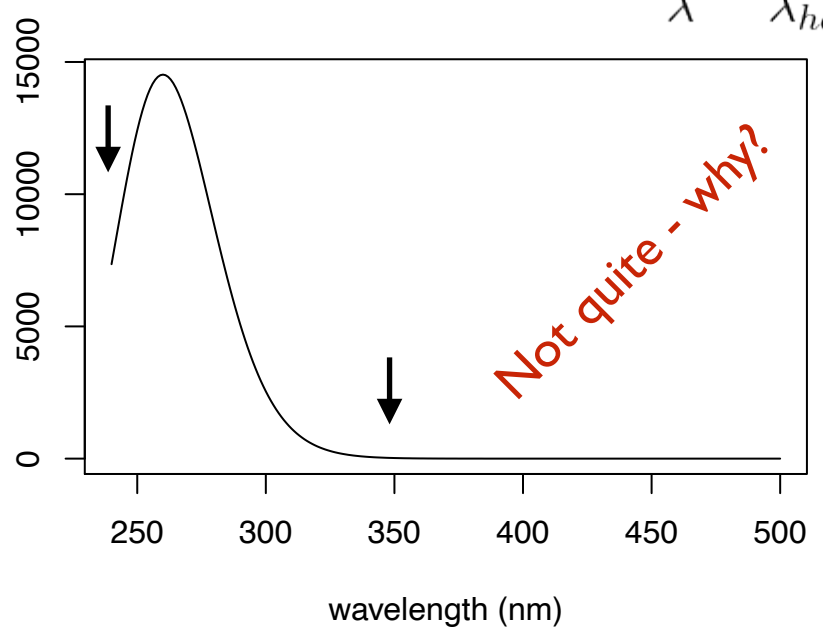
x = seq(240,500, by=1)

```
spectrm <- function(x, lmax, lwid, inten) ( inten * dnorm(1/x, 1/lmax, sd=(1/lmax - 1/(lmax+lwid))) )
```

plot(x, spectrm(x, 260, 20, 10), type="l")

$$\sigma = \frac{1}{\lambda} - \frac{1}{\lambda_{halfwidth}}$$





NanoDrop

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(routine UV-Vis more generally)

Let's simulate a spectrum in R

Normal (Gaussian) distribution

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x = seq(240,500, by=1)

$$f_{scatter}(\lambda) = A_{scatter}\frac{\bar{\lambda}^4}{\lambda^4}$$

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scattr <- function(x,intenSc) (intenSc * mean(x)^4 / x^4)

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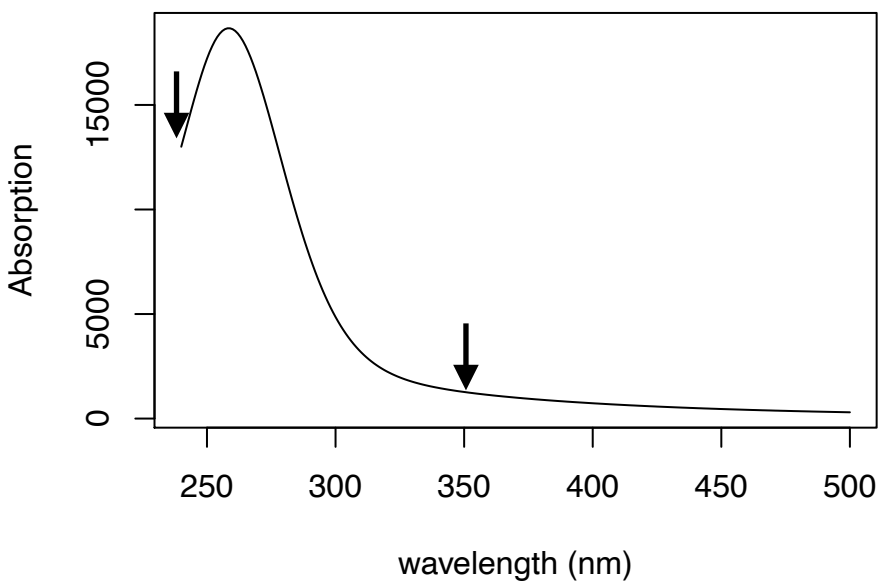
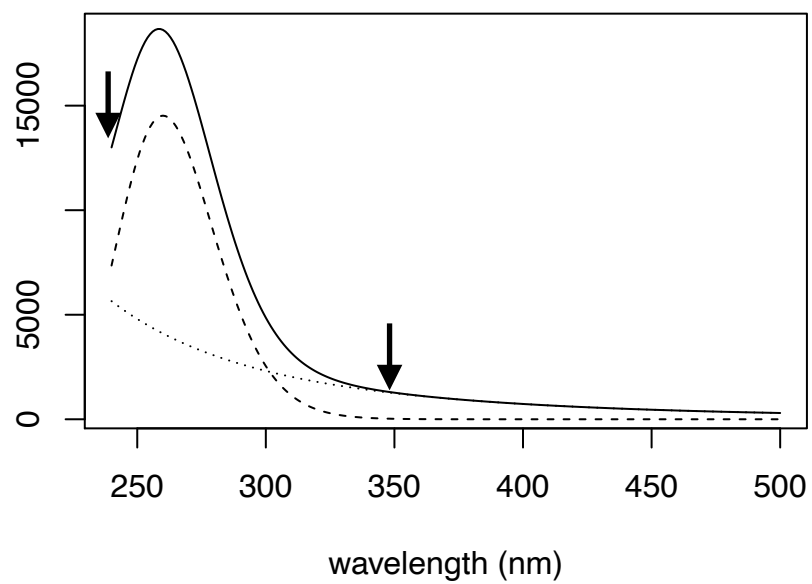




NanoDrop

$$Energy \propto \frac{1}{\lambda}$$

T037 – TECHNICAL BULLETIN
NanoDrop Spectrophotometers

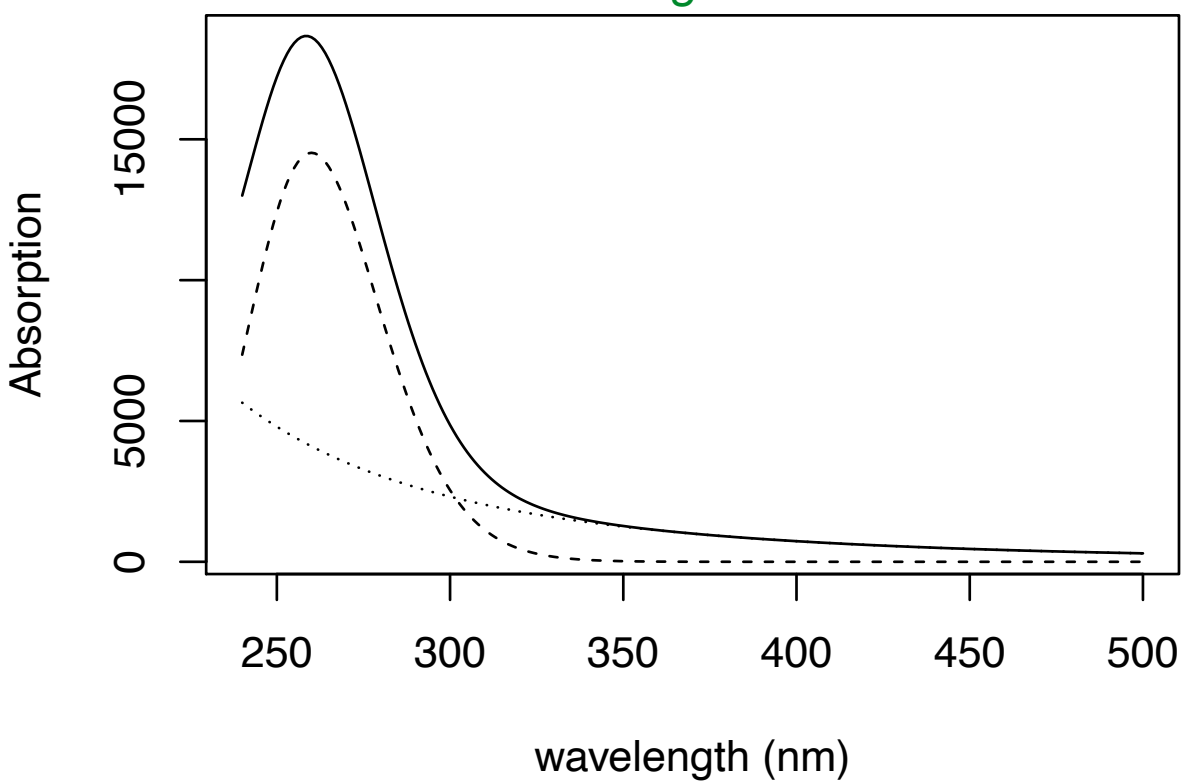
(routine UV-Vis more generally)

Thermo Scientific NanoDrop Spectrophotometers use a bichromatic absorbance correction for nucleic acid and protein A280 measurements. This type of correction is performed to offset the effect of instrument noise and light scattering particulates on low concentration nucleic acid and protein sample measurements. Due to the lack of absorbance by nucleic acids or proteins at higher UV wavelengths, it has been our general observation that any UV wavelength at or above 320 nm can be utilized for this bichromatic correction.

$$f(\lambda) = A\frac{1}{\sqrt{2\pi}\sigma}e^{-\frac{(\frac{1}{\lambda}-\frac{1}{\lambda_{max}})^2}{2\sigma^2}}$$

$$f_{scatter}(\lambda) = A_{scatter}\frac{\bar{\lambda}^4}{\lambda^4}$$

This is good!



$$\sigma = \frac{1}{\lambda} - \frac{1}{\lambda_{halfwidth}}$$

The software for the NanoDrop 1000 and NanoDrop 8000 Spectrophotometers automatically subtracts the 340 nm absorbance from the entire spectrum. The NanoDrop 2000/2000c software allows for the selection of any wavelength for this bichromatic correction (the default setting is 340 nm) or for de-selection of this function. The NanoDrop Lite subtracts the absorbance at 365 nm from the 260 nm and 280 nm wavelength absorbance, respectively.

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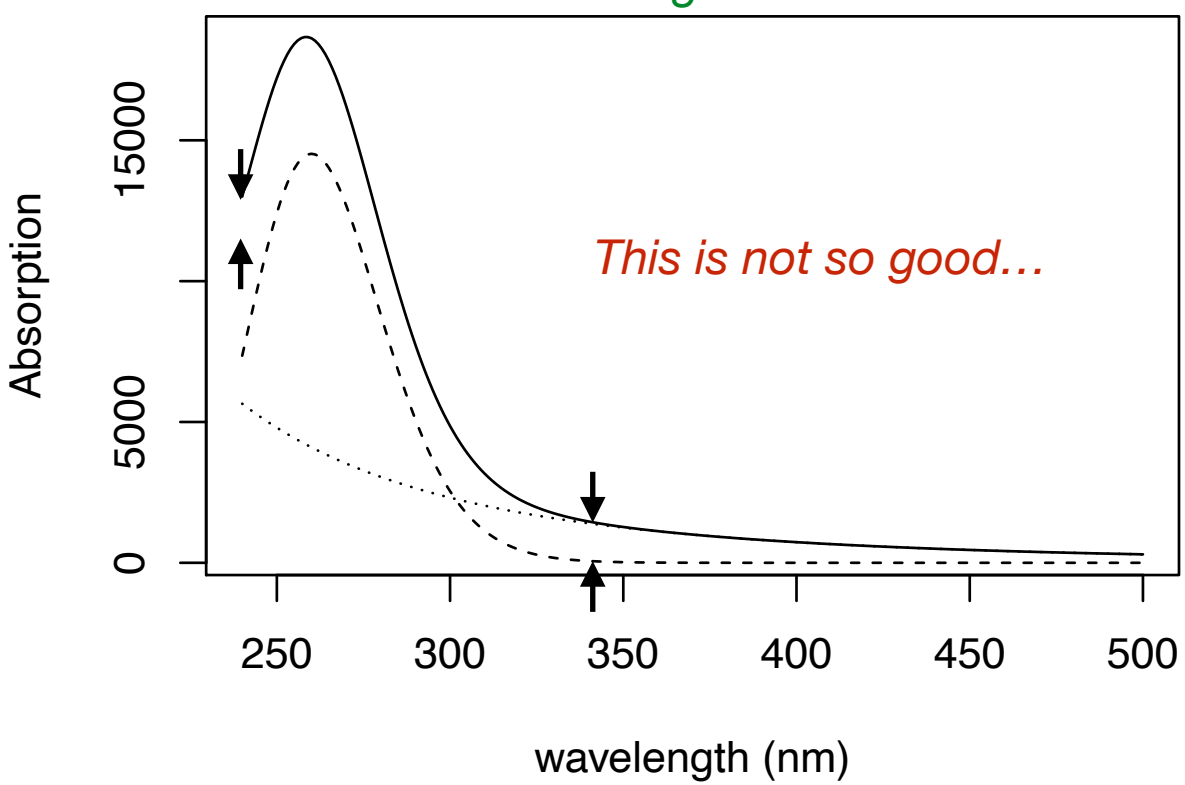

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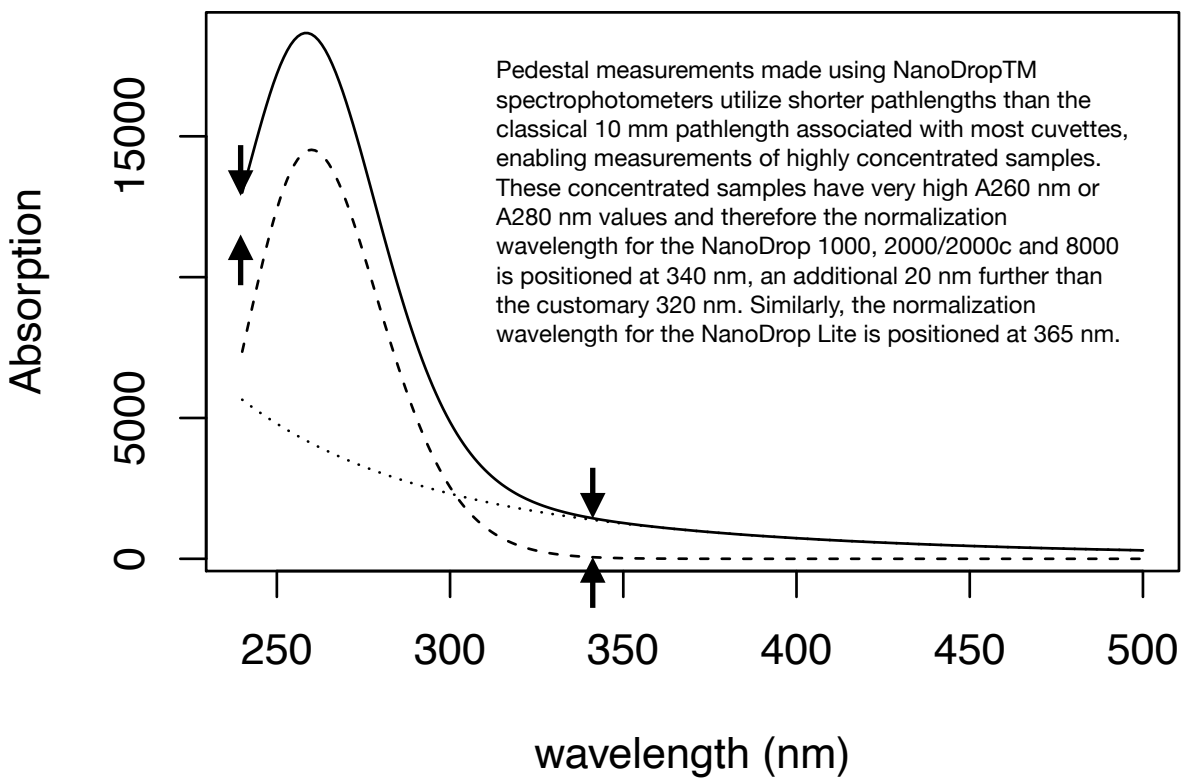

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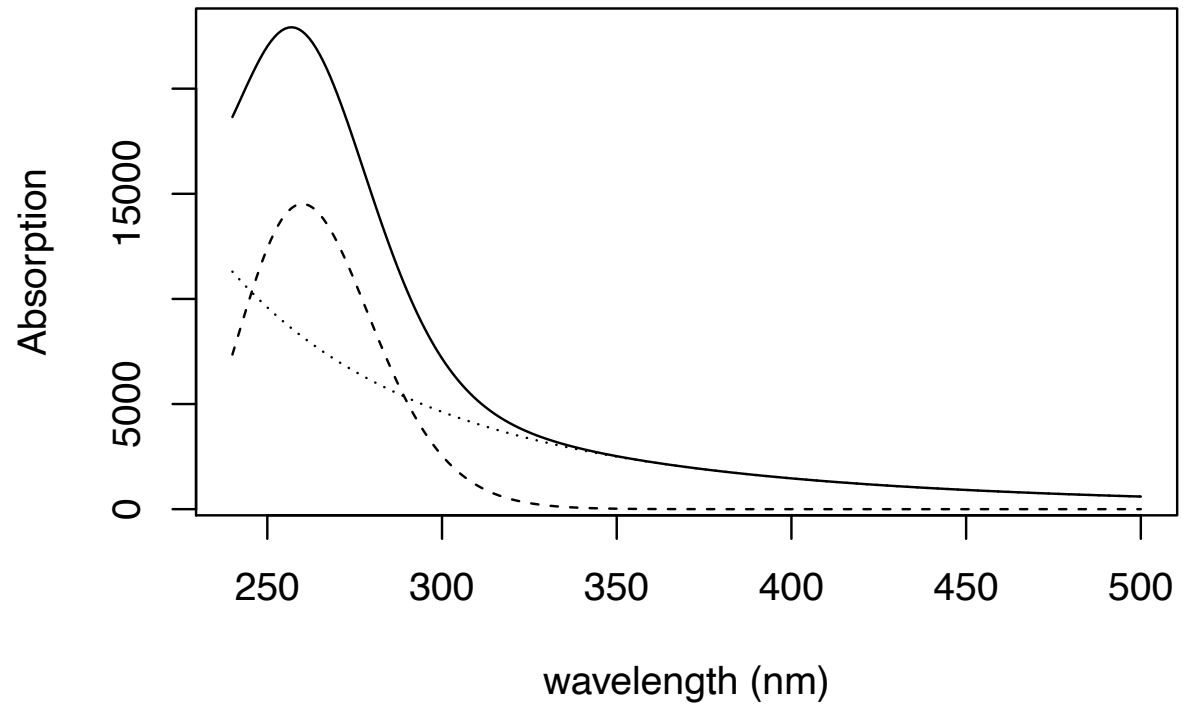
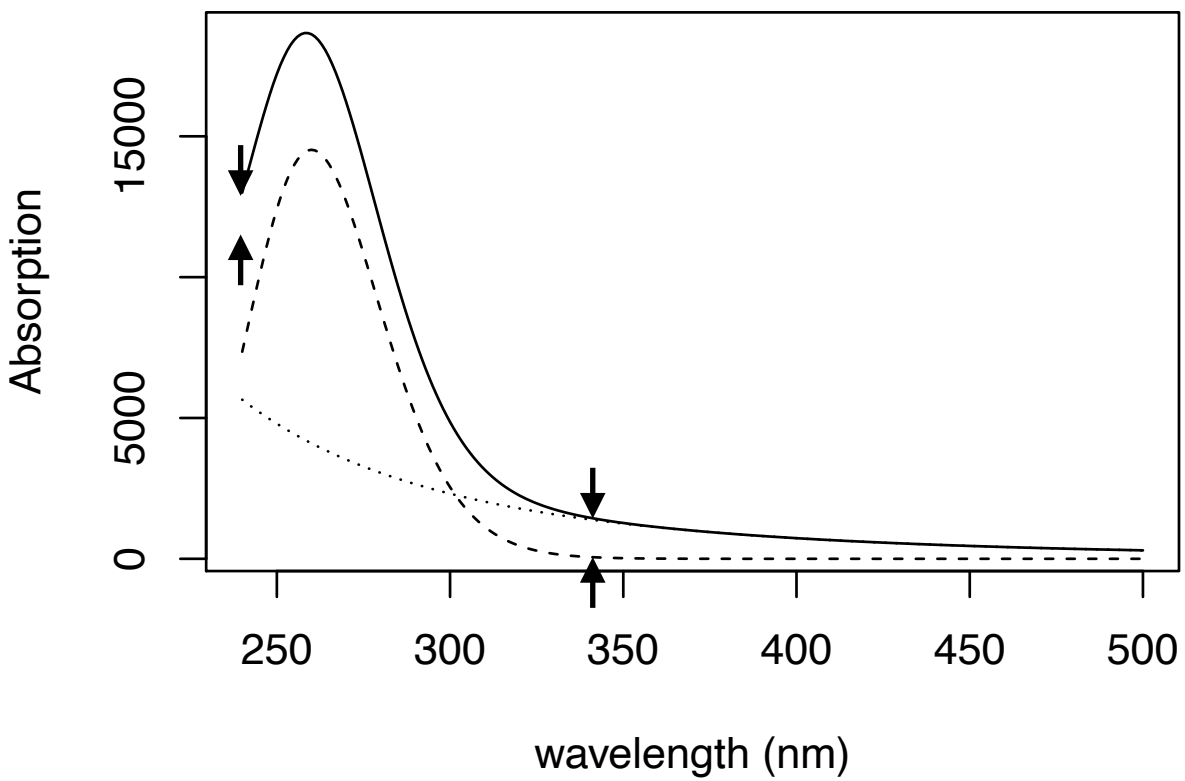
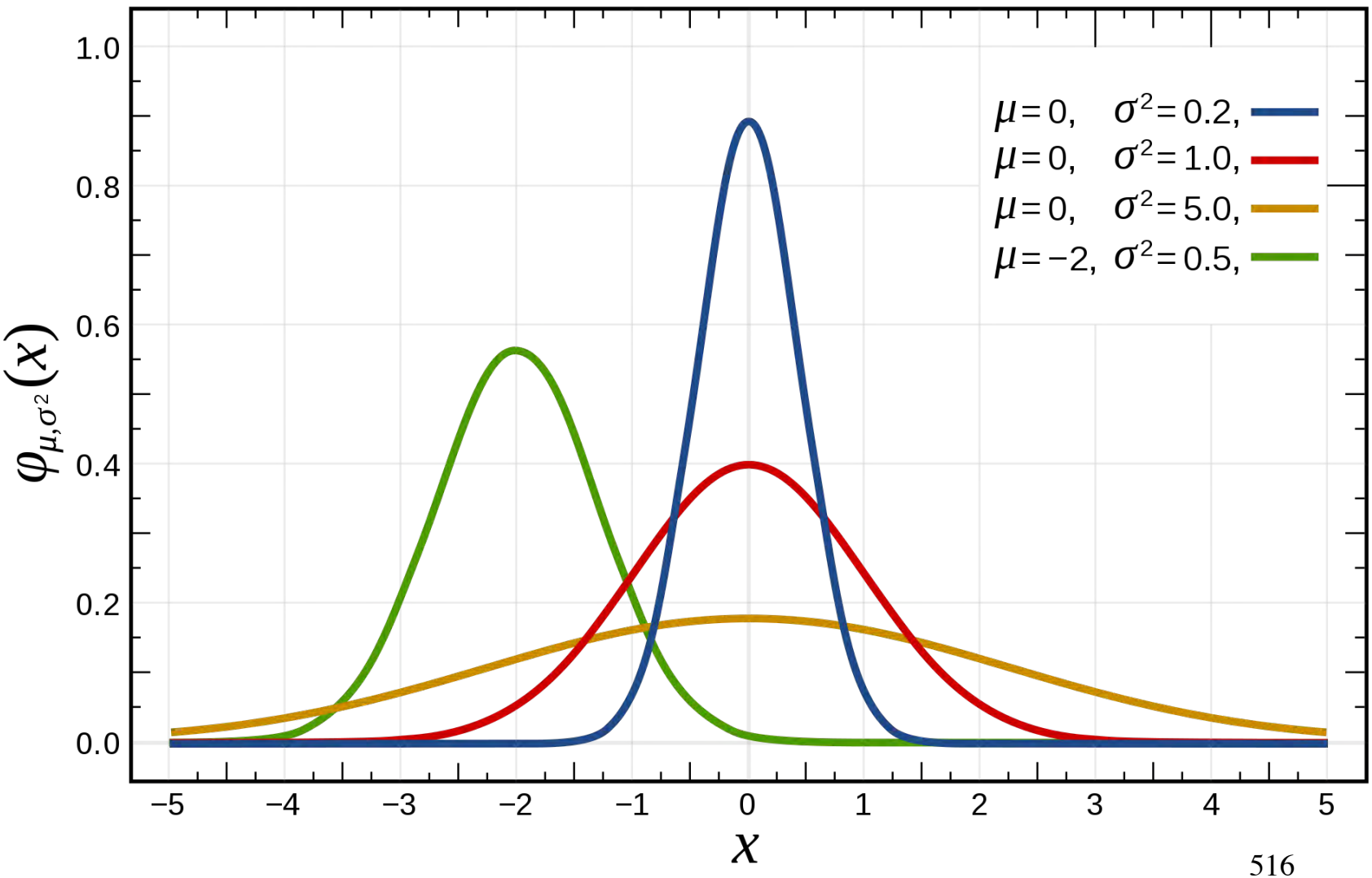

Absorption
0
5000
15000
250
300
350
400
450
500
wavelength (nm)


Absorption
0
5000
15000
250
300
350
400
450
500
wavelength (nm)

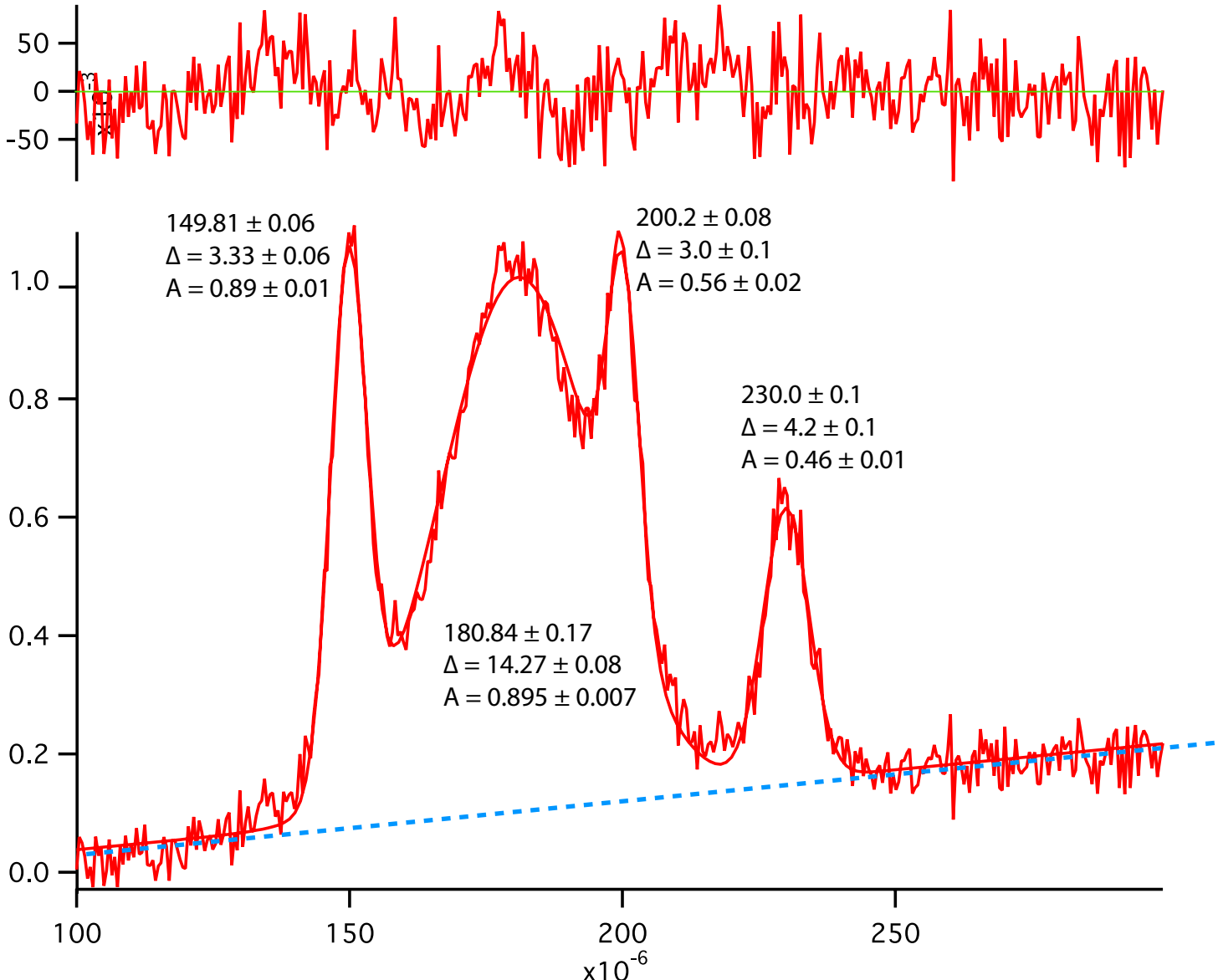
Fitting Spectra

$$Y = Ae^{-\frac{(x-\mu)^2}{2\sigma^2}}$$



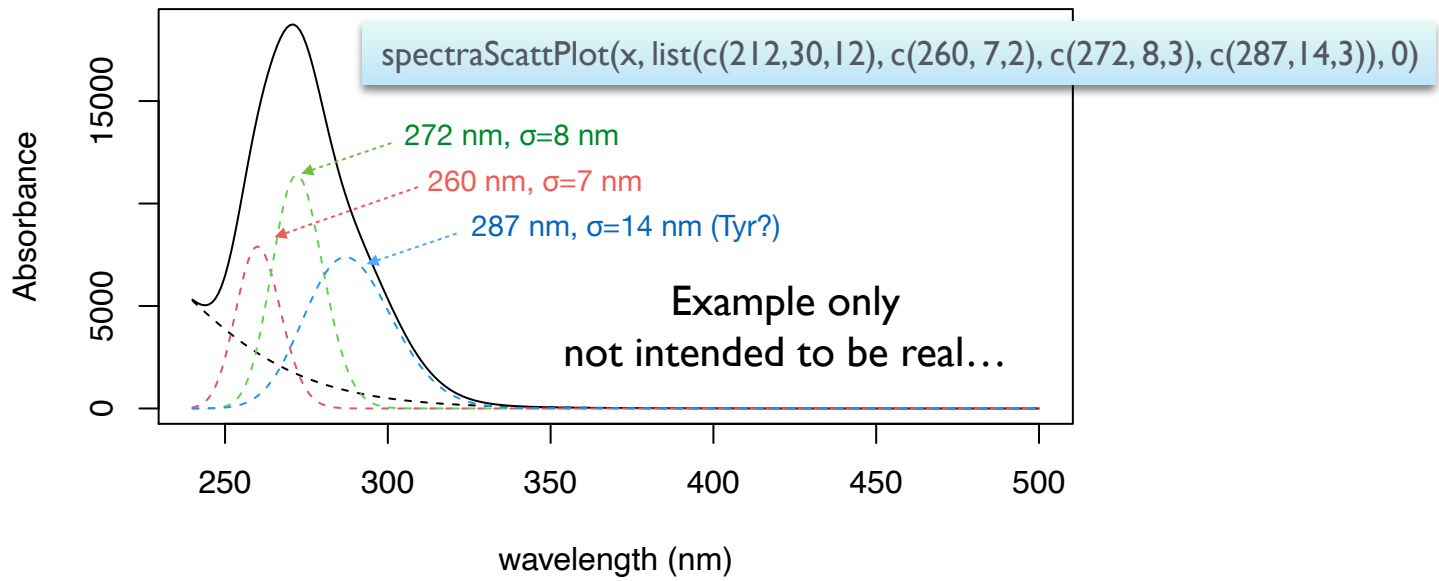
Spectral Deconvolution

$$f(x) = A_1 e^{-\frac{(x-x_1)^2}{2\Delta_1^2}} + A_2 e^{-\frac{(x-x_2)^2}{2\Delta_2^2}} + A_3 e^{-\frac{(x-x_3)^2}{2\Delta_3^2}} + A_4 e^{-\frac{(x-x_4)^2}{2\Delta_4^2}} + mx + b$$



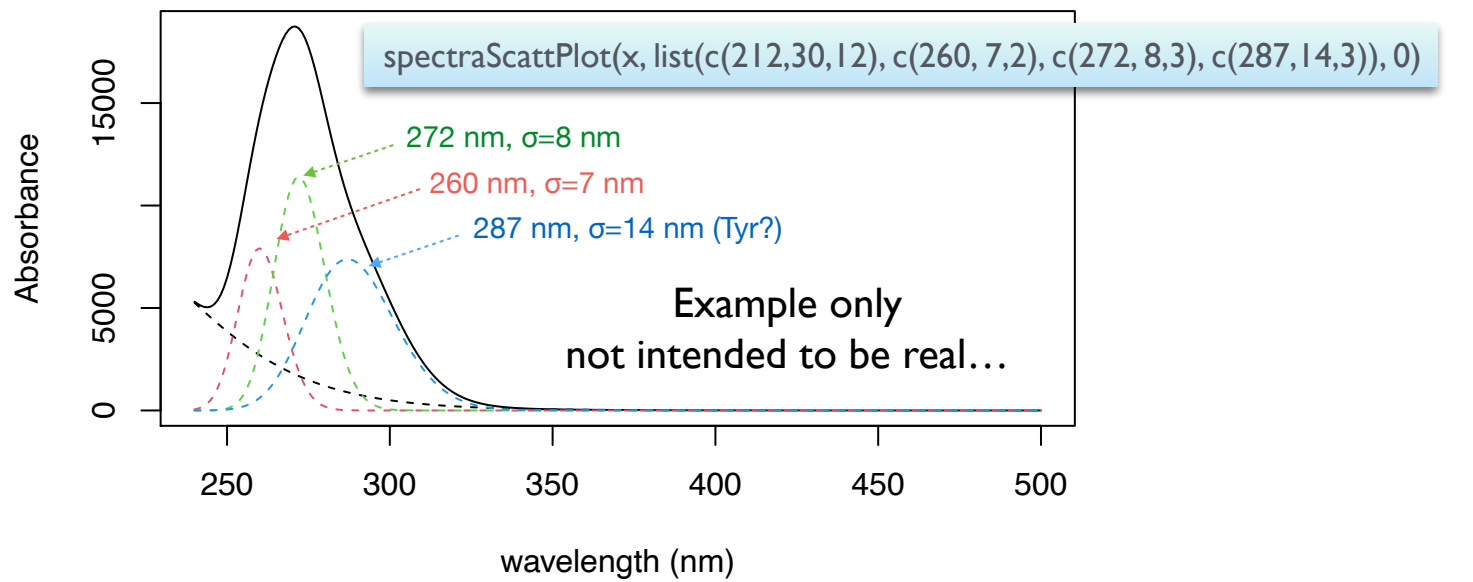
UV-Vis Proteins

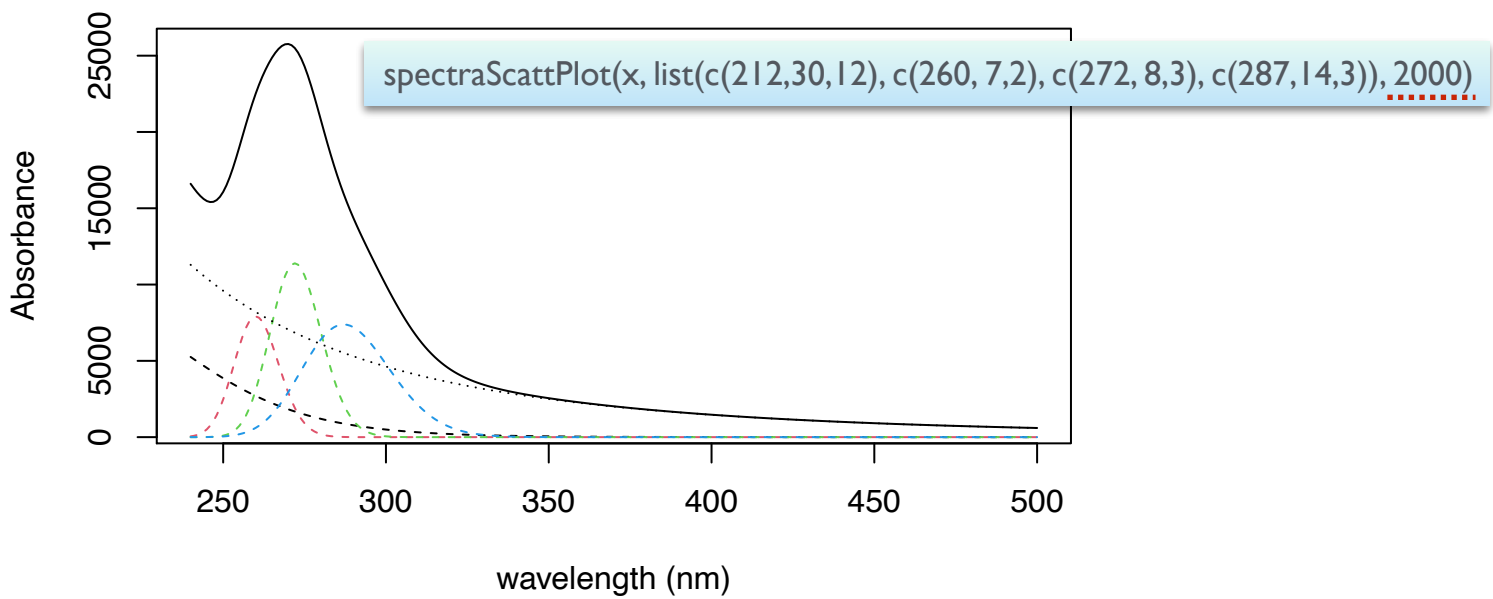
(routine UV-Vis more generally)



UV-Vis Proteins

(routine UV-Vis more generally)





Oligonucleotide Extinction Coefficients

5’–AGGCACGGTCACGTGGCAC–3’

ε260 - not the absorption maximum for each!

$$\epsilon_{260}^{A} = 15,400 \qquad \epsilon_{260}^{T} = 8,700$$

$$\epsilon_{260}^{G} = 11,500 \qquad \epsilon_{260}^{C} = 7,400$$

By how much does sequence composition affect extinction coefficient?

Would you believe that the extinction coefficient for a sequence as short as 6 bases can vary just by arranging the bases in a different order? Additionally, base composition can lead to significant differences in the extinction coefficient. See Table 1 for examples.

Greatest accuracy is therefore achieved when the exact value of ε260 is calculated for each oligo. Further, it is necessary to take into account the presence of oligo modifications, such as fluorescent dyes, which may have significant absorbance at 260 nm.

For these reasons, IDT calculates the extinction coefficient for every oligo synthesized using a nearest neighbor method. This value is then used to measure the yield for each oligonucleotide produced.

Nearest neighbor values **for ε260** of dNTPs are:

5′→3′	dA	dC	dG	dT
dA	27,400	21,200	25,000	22,800
dC	21,200	14,600	18,000	15,200
dG	25,200	17,600	21,600	20,000
dT	23,400	16,200	19,000	16,800

$$\varepsilon_{260} = \sum_{1}^{N-1} \varepsilon_{Nearest\ Neighbor} - \sum_{2}^{N-1} \varepsilon_{Individual\ Bases} + \sum_{1}^{N} \varepsilon_{Modifications}$$

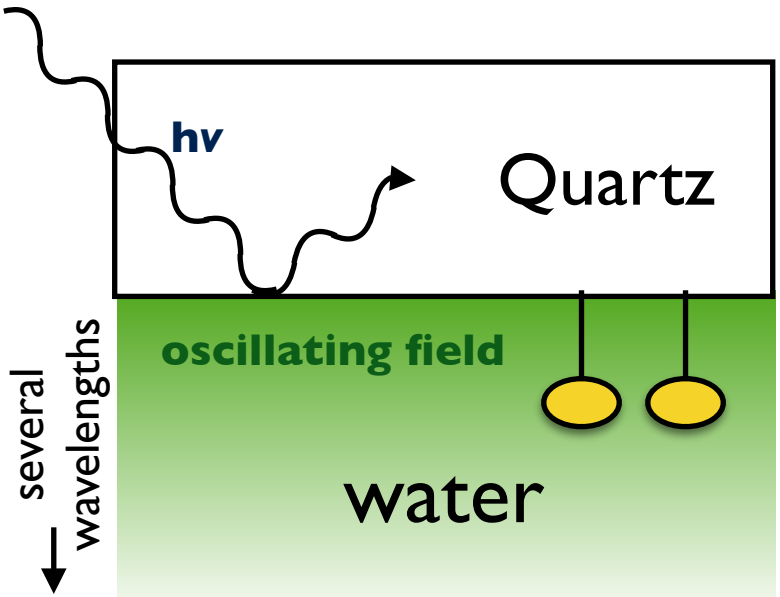
https://www.idtdna.com/pages/tools

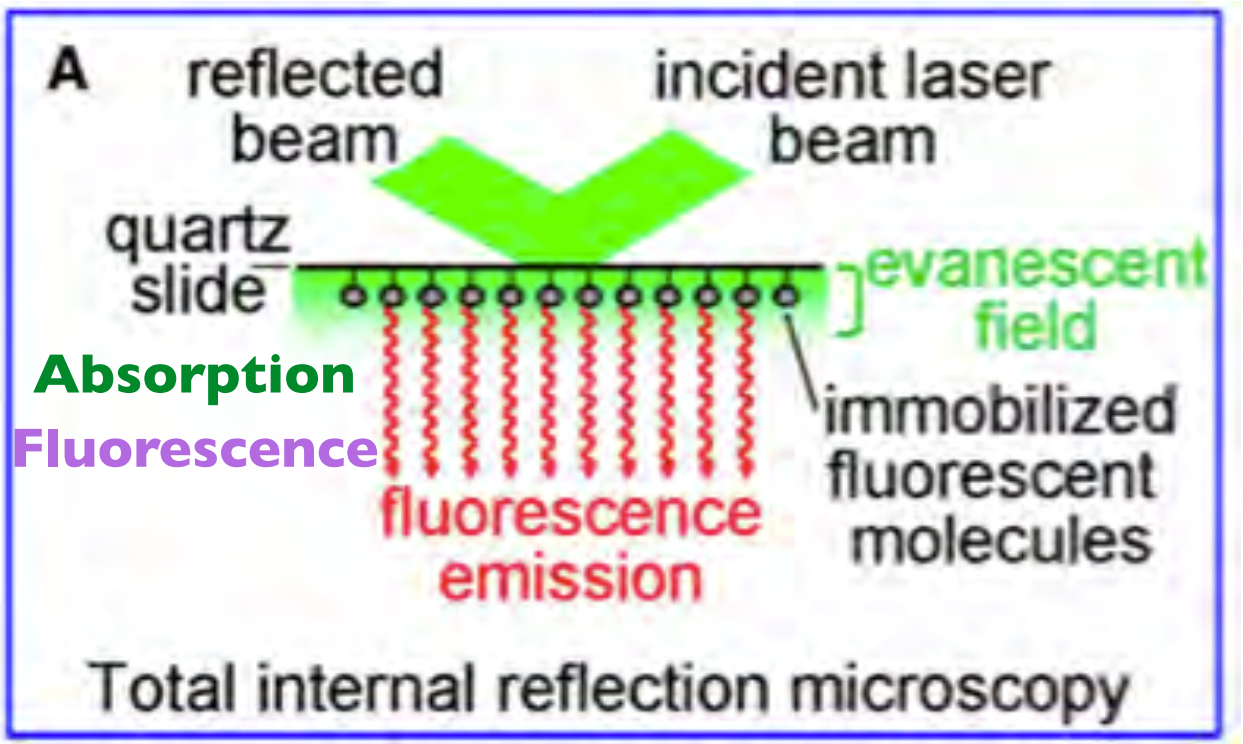
Total Internal Reflectance

Molecular Cell 24, 317–329, November 3, 2006

Single-Molecule Biology:
What Is It and How Does It Work?

Jordanka Zlatanova1, and
Kensal van Holde





In TIR, the excitation light is directed toward an interface between two media of different refractive indices (i.e., from an optically denser medium, such as a glass slide, into a less-dense medium, such as water) (Axelrod, 2001). The incident angle of the beam is set larger than a certain critical angle, defined by the properties of the two media; all the light is reflected off the glass and does not penetrate into the solution (Figure 1A). How-ever, an electromagnetic field that oscillates with the same frequency as the incident light does form in the less-dense medium (the water on the other side). Because this electromagnetic field (also called evanescent field or wave) decays exponentially from the glass surface, it is capable of exciting fluorophores only in a very small volume close to the surface, thus effectively preventing out-of-focus fluorescence background. The excitation light itself is cleanly removed from the observation chamber, reducing the background even further.

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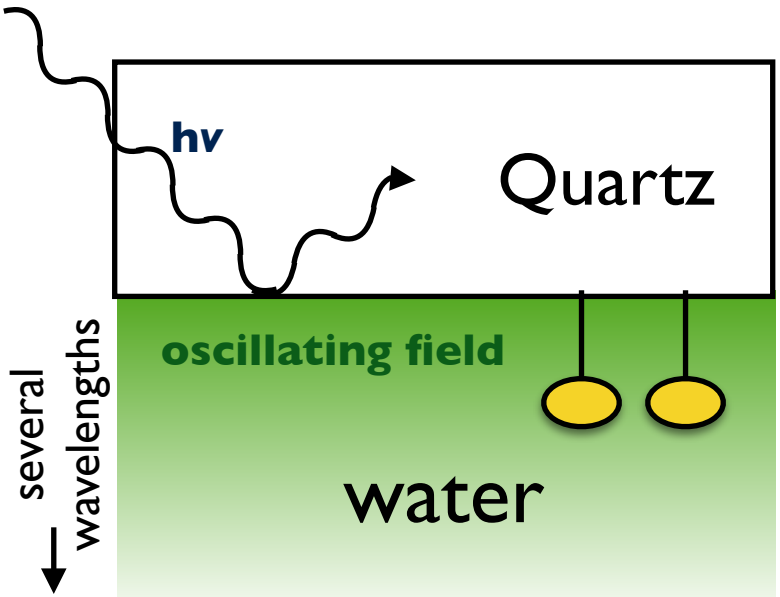
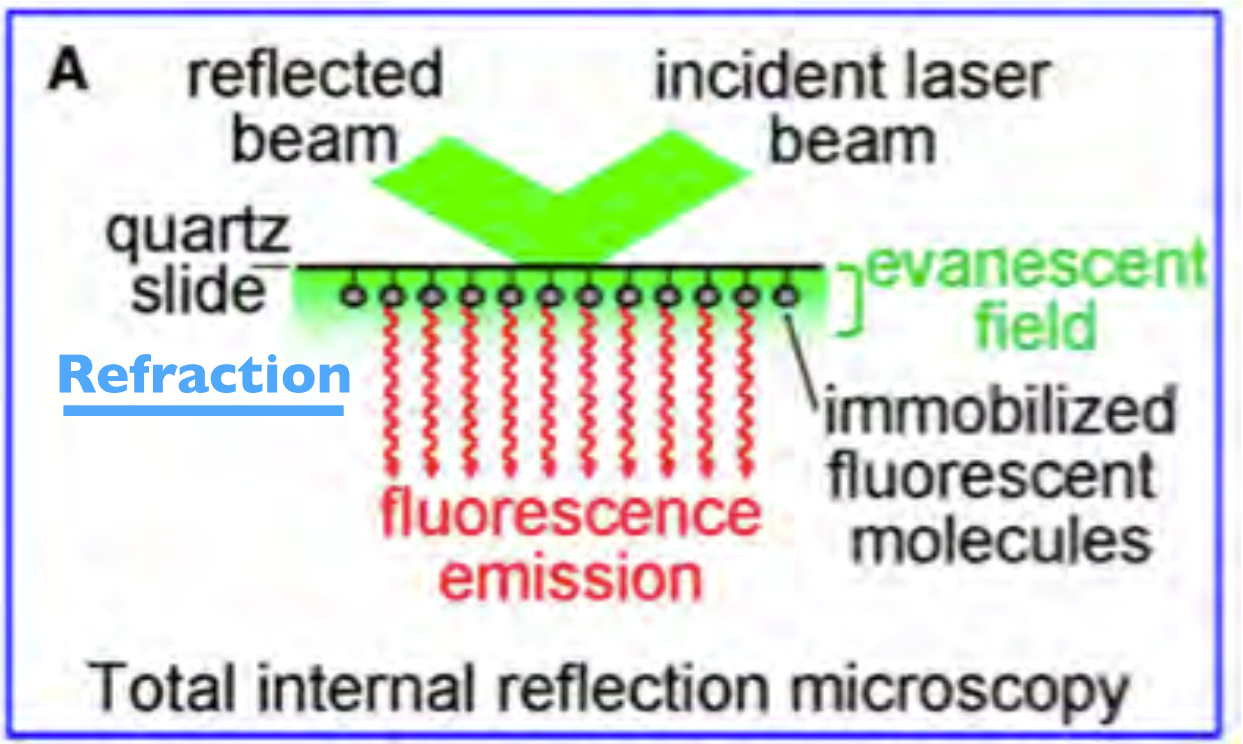
Kensal van Holde

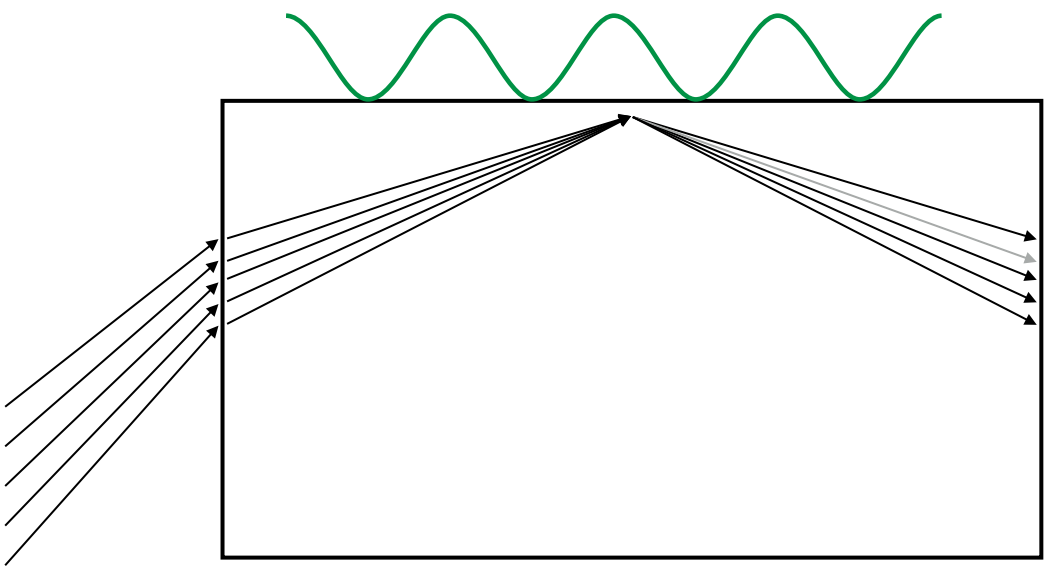




In TIR, the excitation light is directed toward an interface between two media of different refractive indices (i.e., from an optically denser medium, such as a glass slide, into a less-dense medium, such as water) (Axelrod, 2001). The incident angle of the beam is set larger than a certain critical angle, defined by the properties of the two media; all the light is reflected off the glass and does not penetrate into the solution (Figure 1A). How-ever, an electromagnetic field that oscillates with the same frequency as the incident light does form in the less-dense medium (the water on the other side). Because this electromagnetic field (also called evanescent field or wave) decays exponentially from the glass surface, it is capable of exciting fluorophores only in a very small volume close to the surface, thus effectively preventing out-of-focus fluorescence background. The excitation light itself is cleanly removed from the observation chamber, reducing the background even further.

Surface Plasmon Resonance

As before, the E field penetrates a short (tens of nm) distance into a medium of a lower refractive index creating an exponentially attenuated evanescent wave.



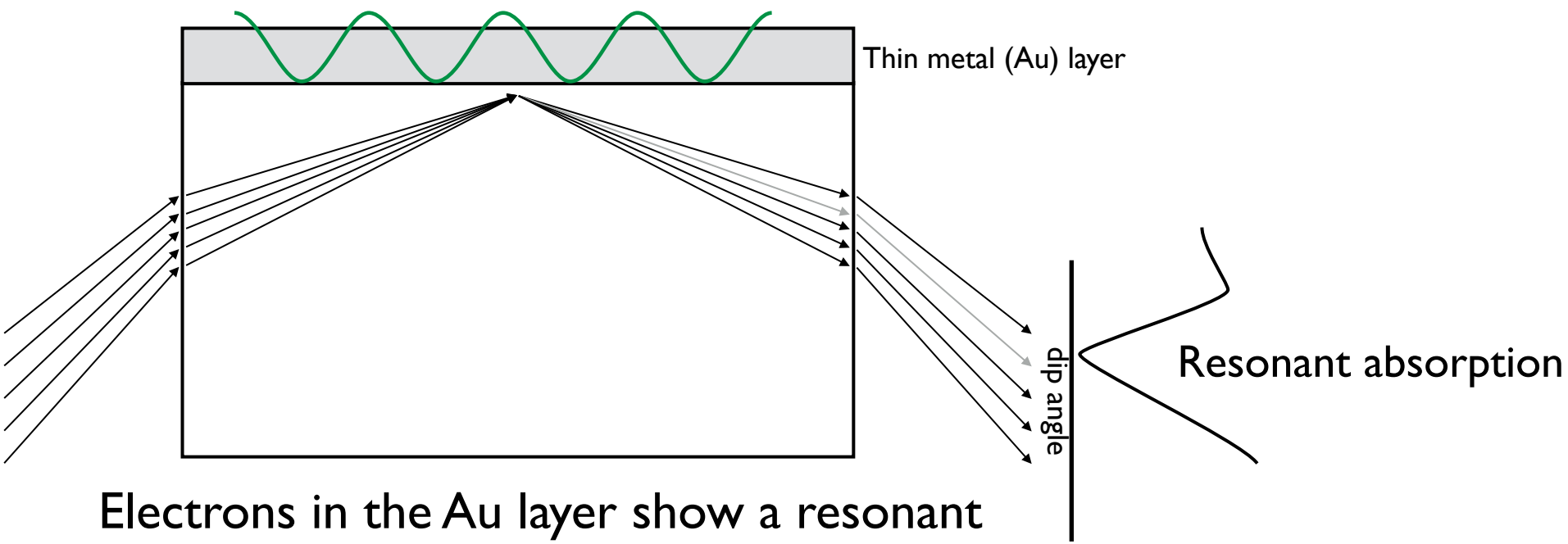
Surface Plasmon Resonance

Surface Plasmons - collective, quantized oscillations of conduction band electrons in a metal

As before, the E field penetrates a short (tens of nm) distance into a medium of a lower refractive index creating an exponentially attenuated evanescent wave.

If the quartz (glass) is coated with a thin layer of metal (gold), and light is monochromatic and p-polarized, the intensity of the reflected light is reduced at a specific incident angle producing a sharp shadow (called surface plasmon resonance) due to the resonance energy transfer between evanescent wave and surface plasmons.

The resonance conditions are influenced by the material adsorbed onto the thin metal film. A linear relationship exists between the resonance energy and the mass concentration of biochemically relevant molecules such as proteins, sugars and DNA.



Electrons in the Au layer show a resonant absorption at a specific frequency

Surface Plasmon Resonance

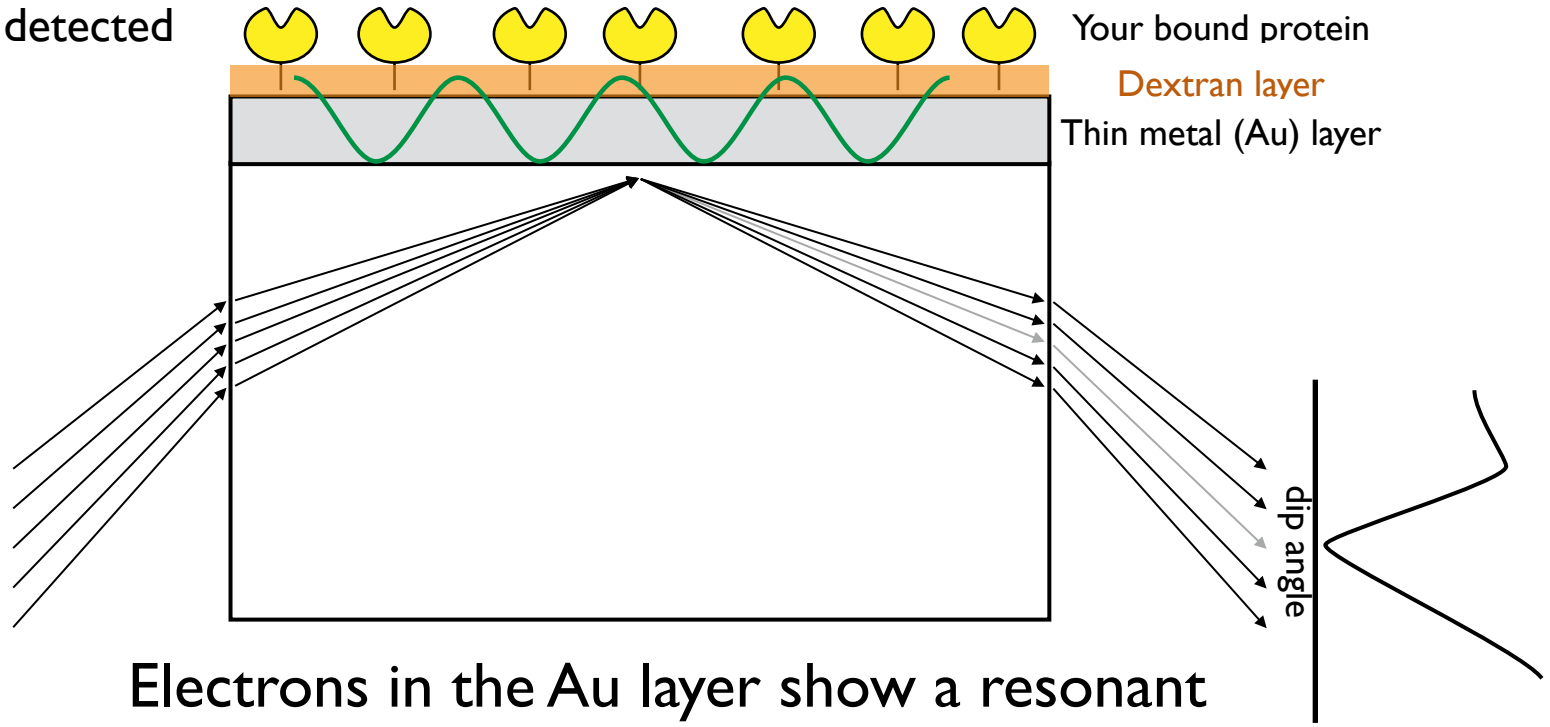
Surface Plasmons - collective, quantized oscillations of conduction band electrons in a metal

Have wave/particle duality like photons, electrons

The SPR signal which is expressed in resonance units is therefore a measure of mass concentration at the sensor chip surface.

not detected

detected



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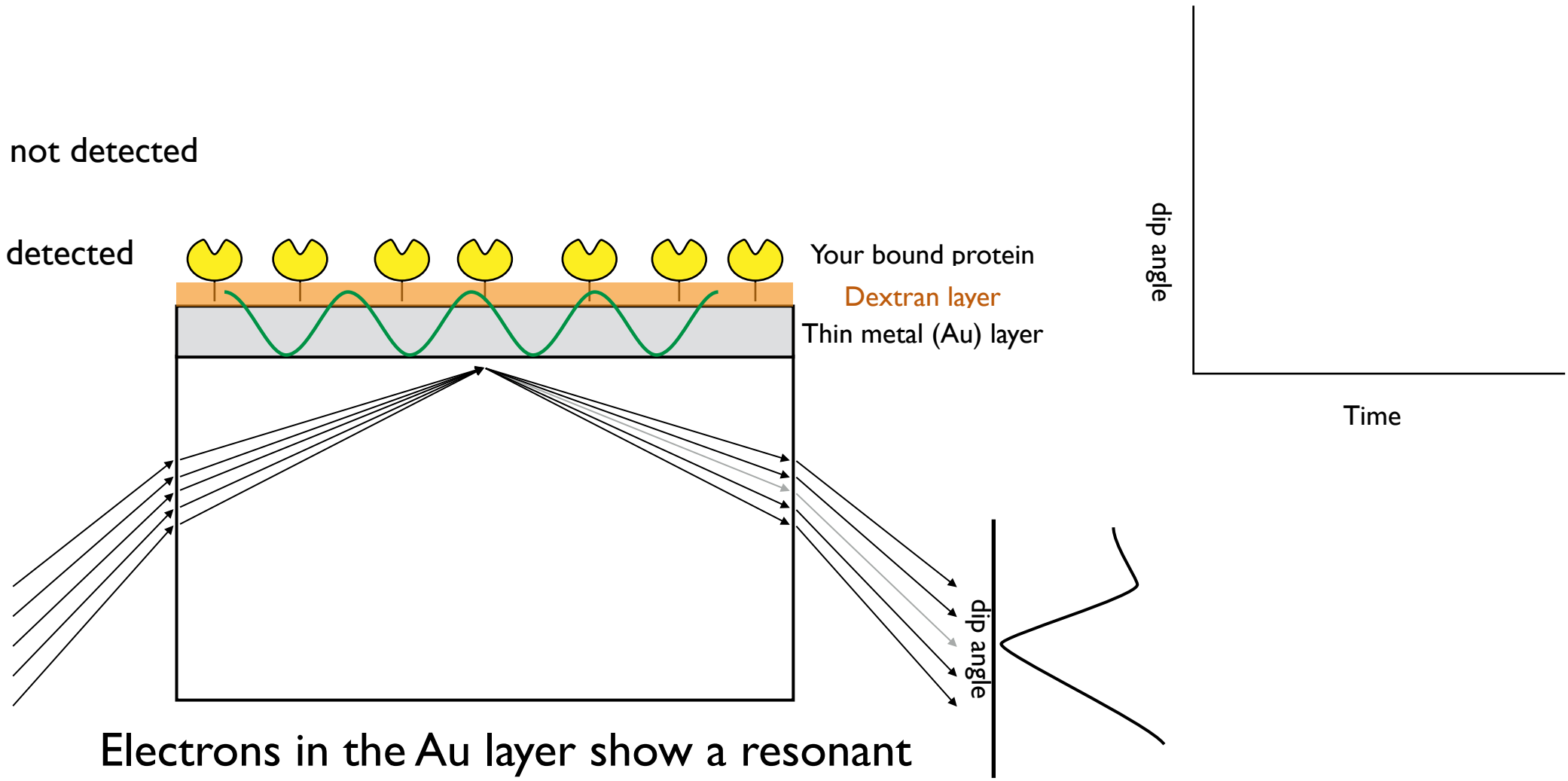
Have wave/particle duality like photons, electrons



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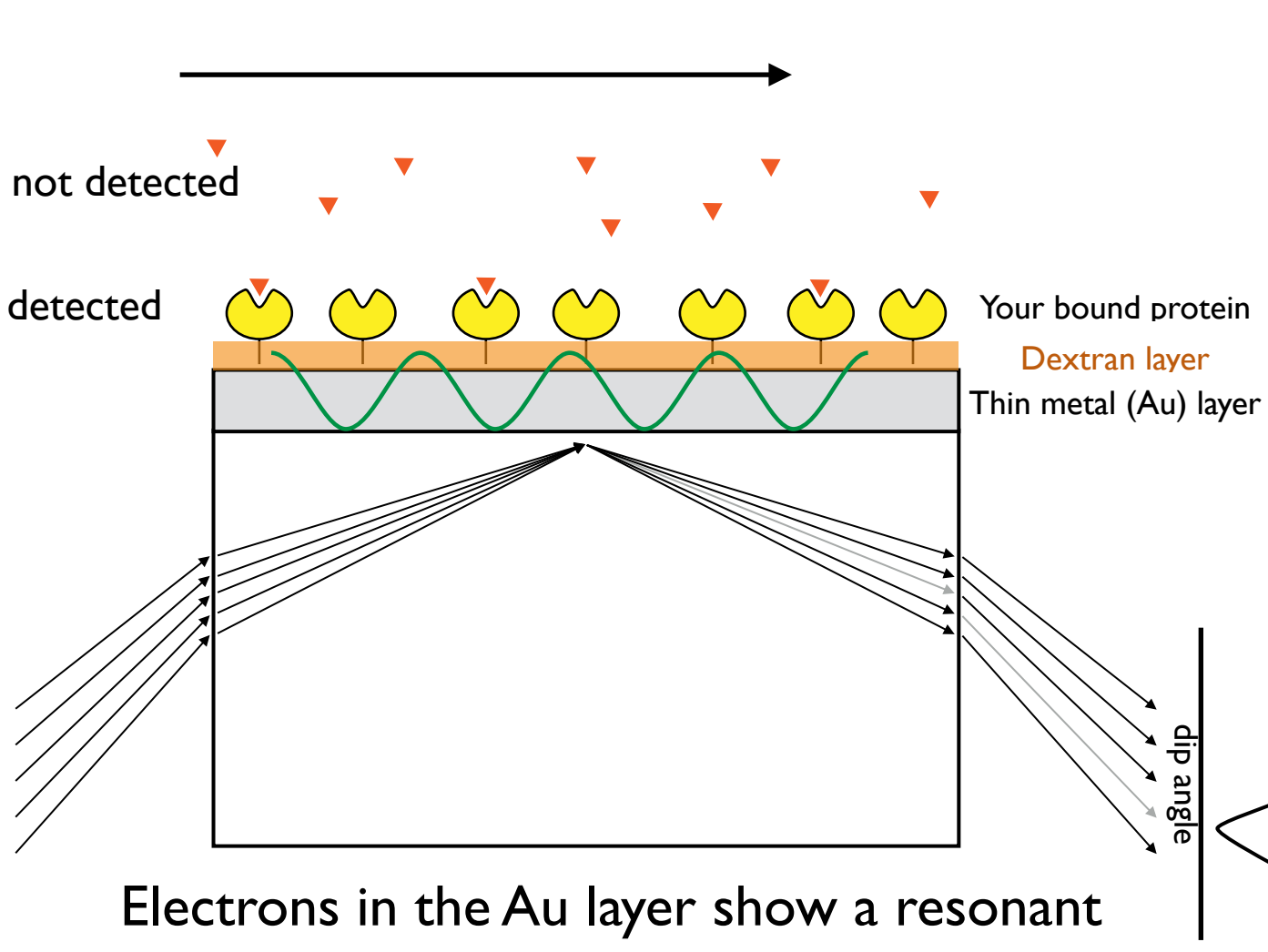

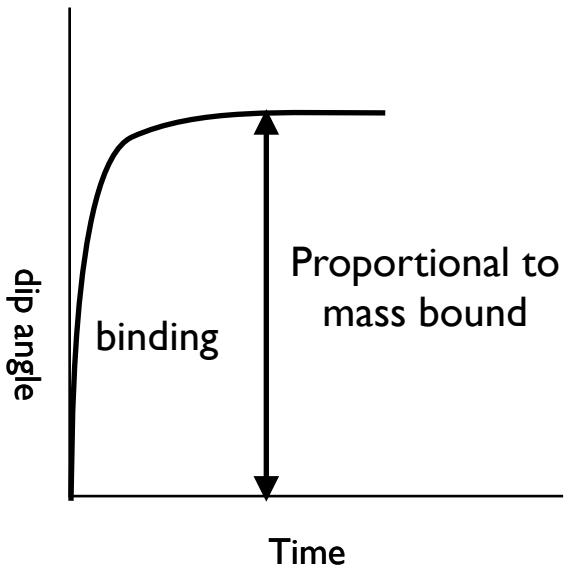

$$Y_t = \left(Y_\infty - Y_0\right)\left(1 - e^{-k_{on}' t}\right) + Y_0$$

$$k_{on}' = k_{on}\left[L\right]$$

Electrons in the Au layer show a resonant absorption at a specific frequency

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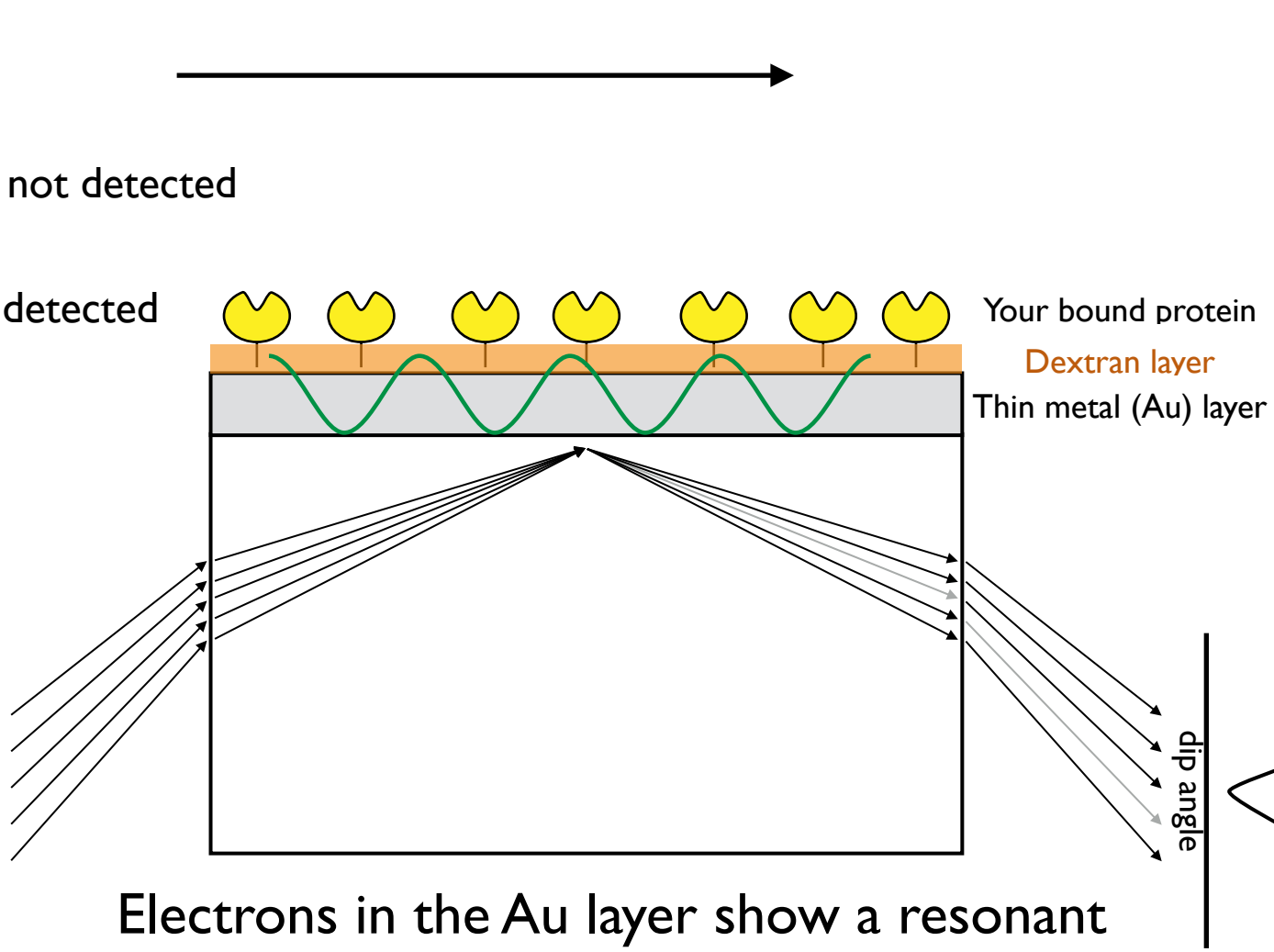

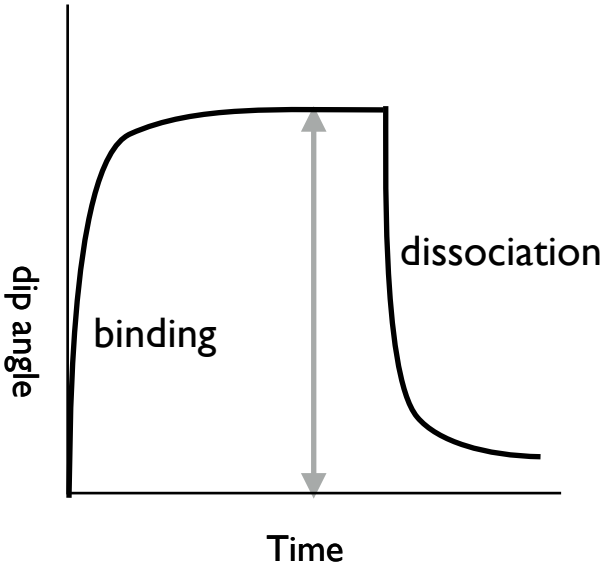

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Electrons in the Au layer show a resonant absorption at a specific frequency

Surface Plasmon Resonance

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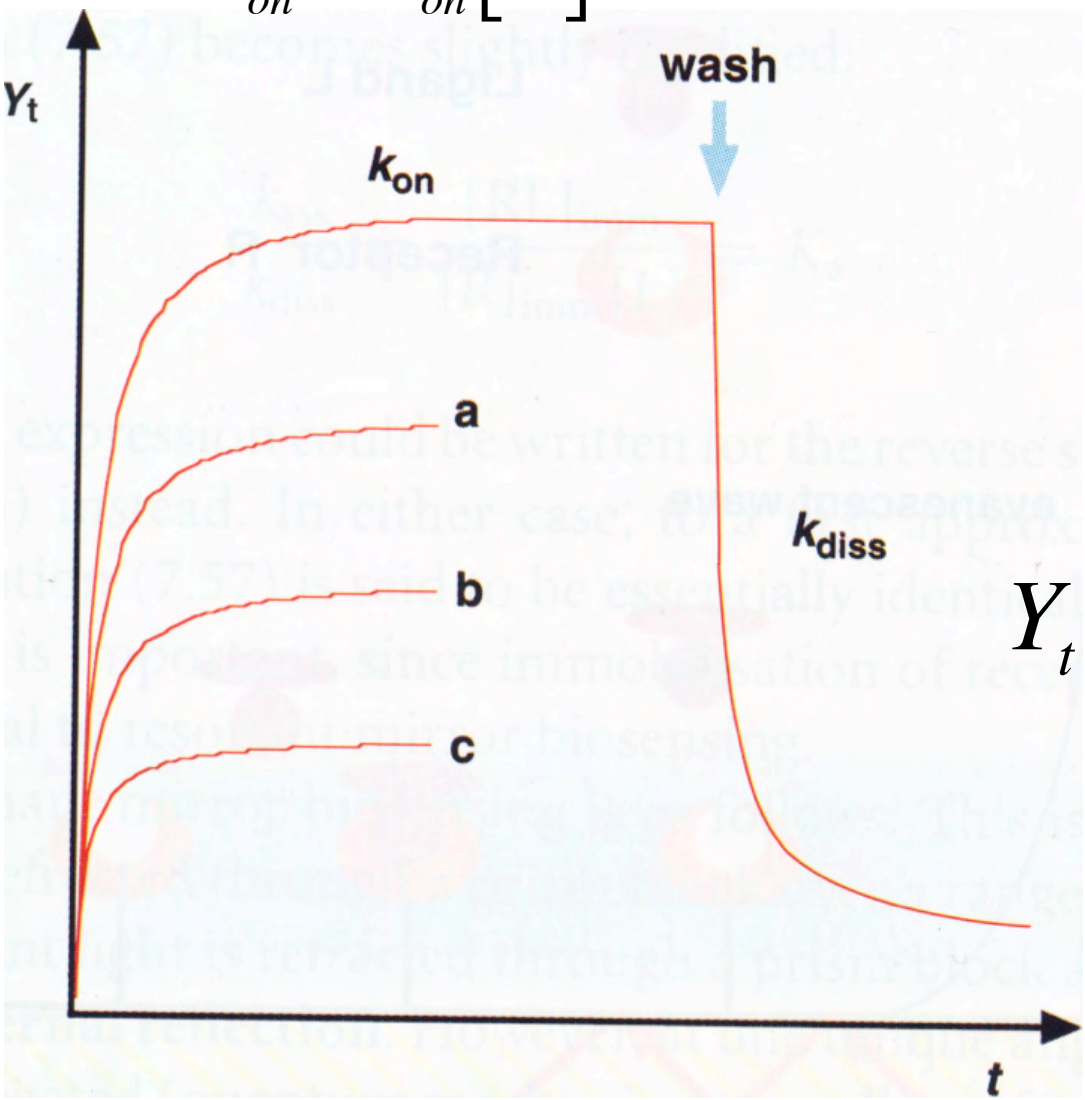
$$K_{equilib} = \frac{k_{on}}{k_{diss}}$$



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Surface Plasmon Resonance

Surface Plasmons - collective, quantized oscillations of conduction band electrons in a metal

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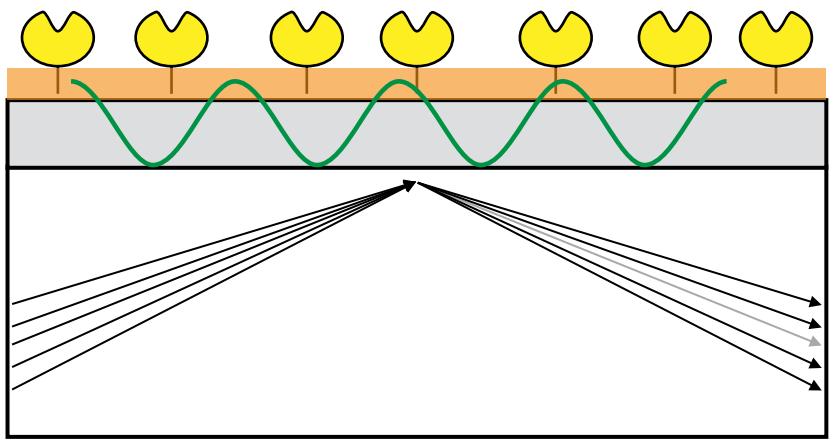
detected



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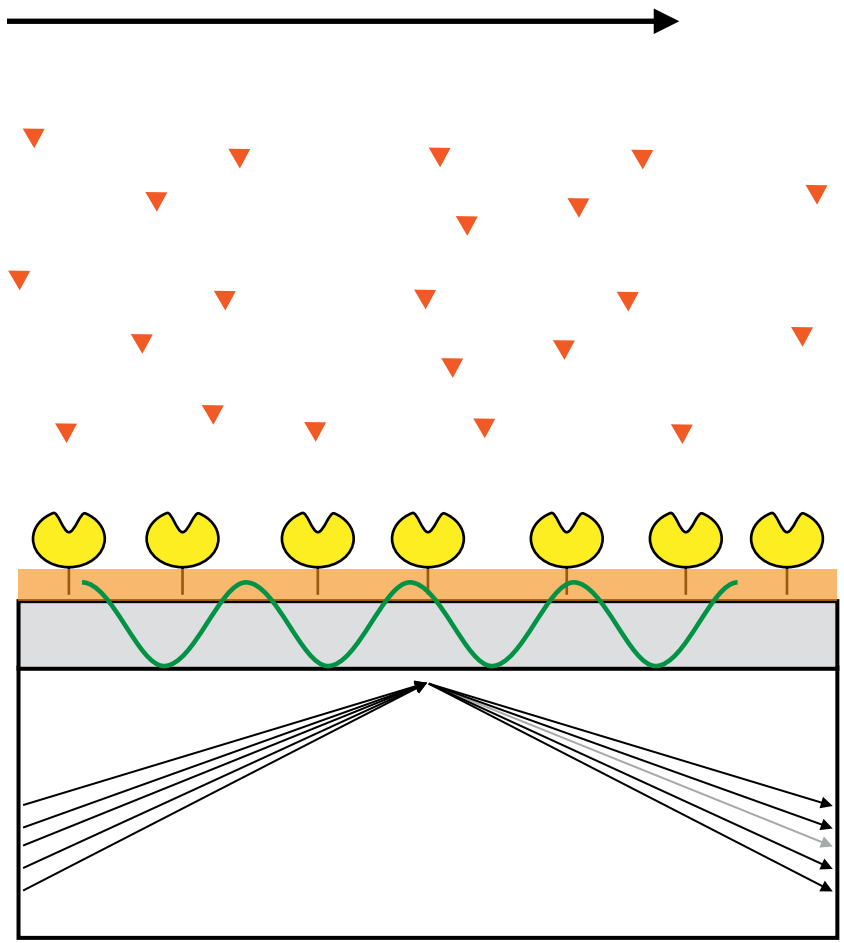

detected

Time

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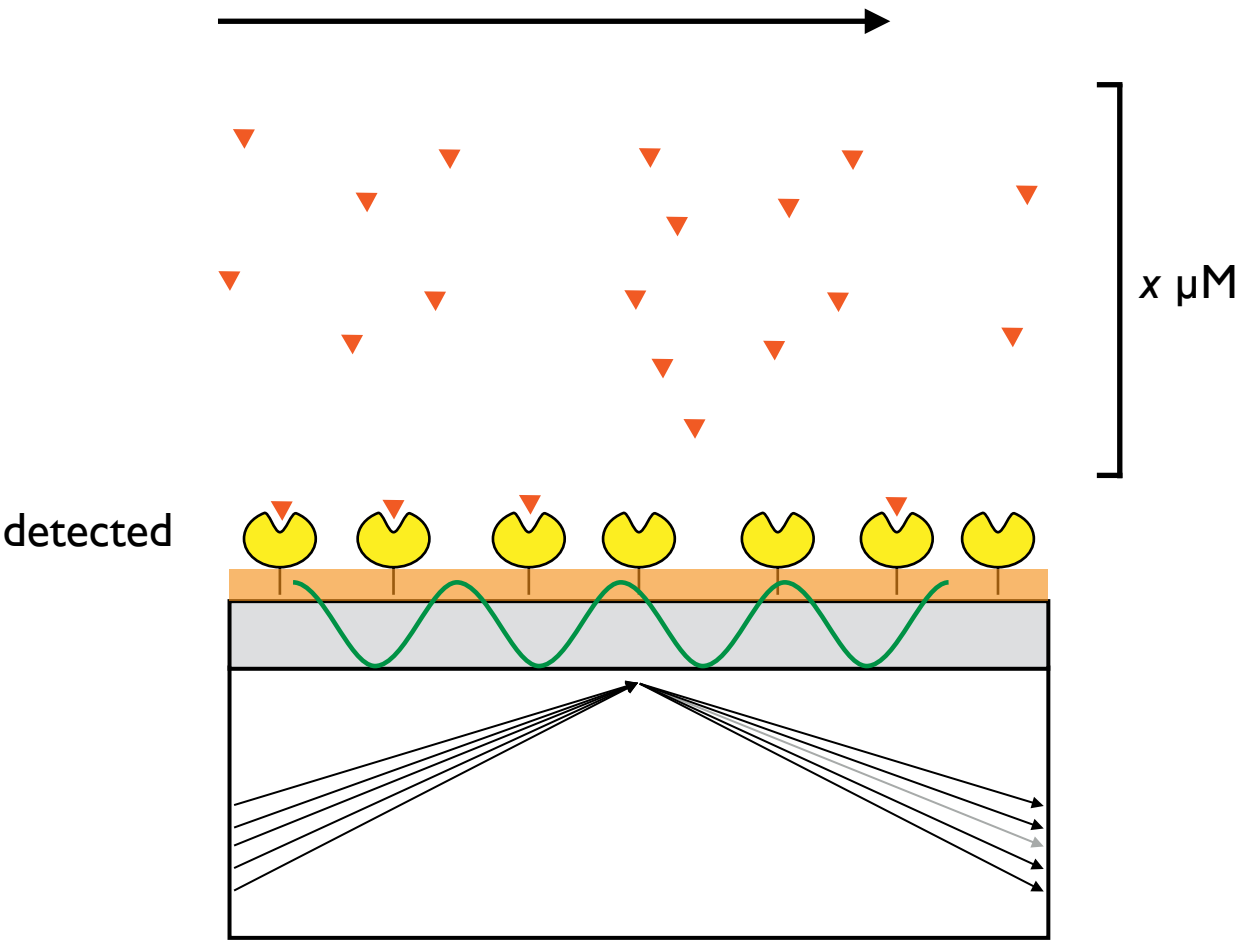

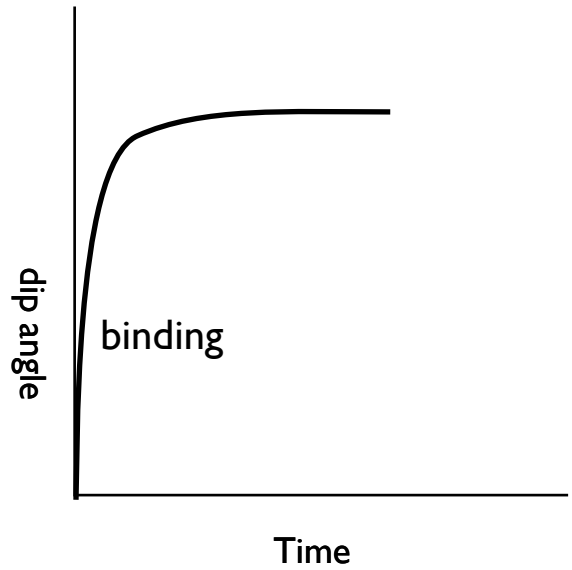

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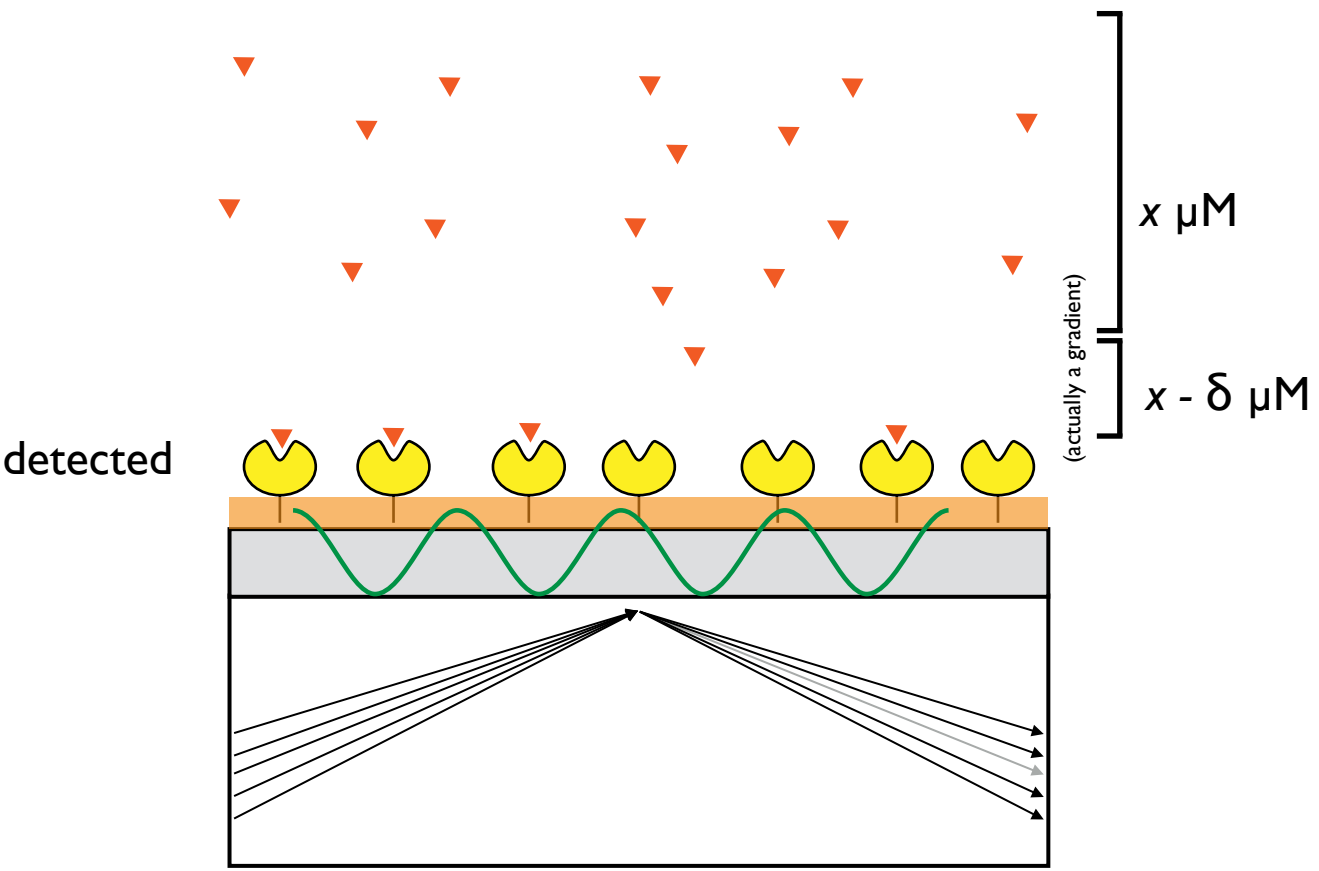
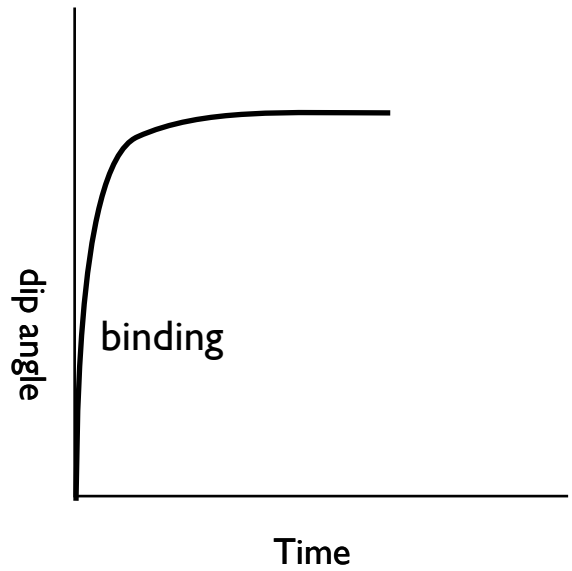
If diffusion of ligand is much faster than binding, then this is not a problem, but….





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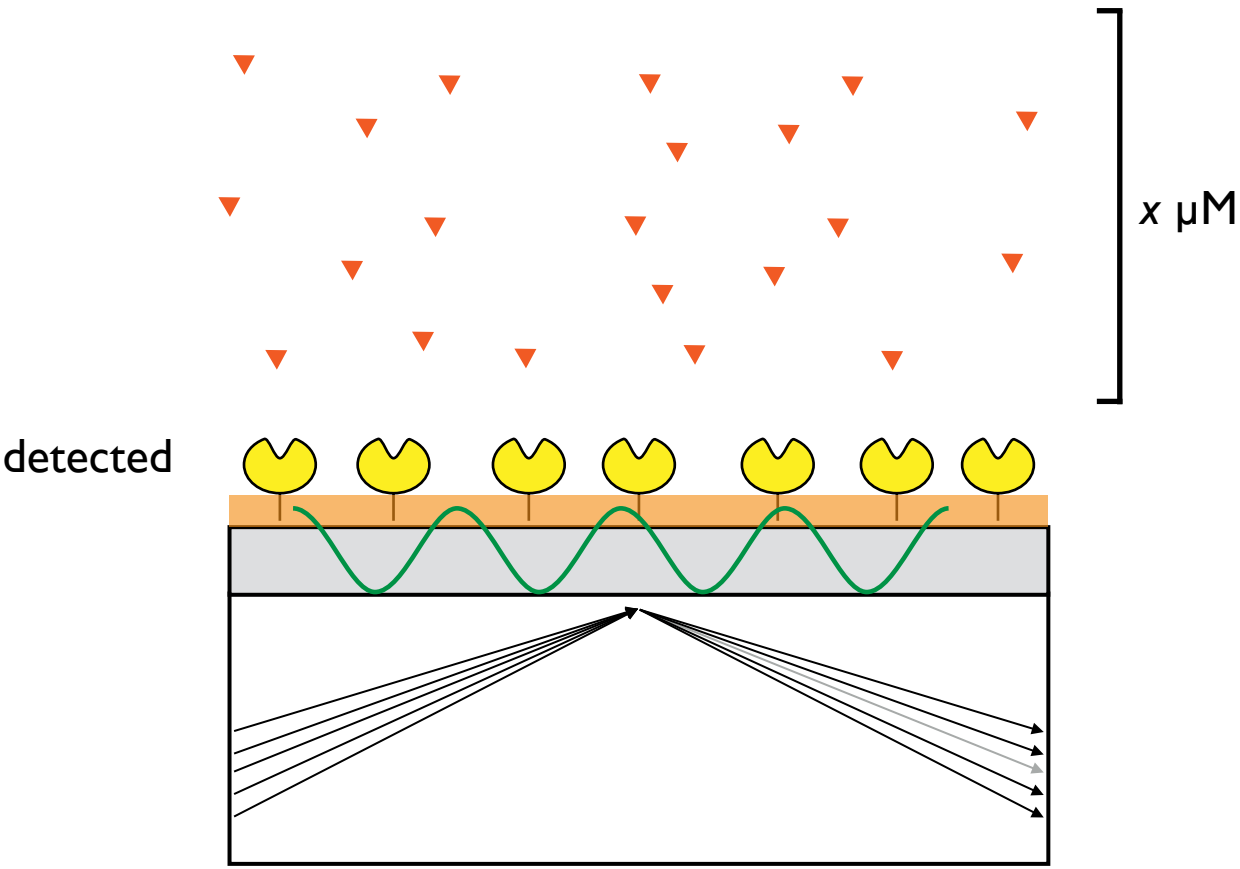
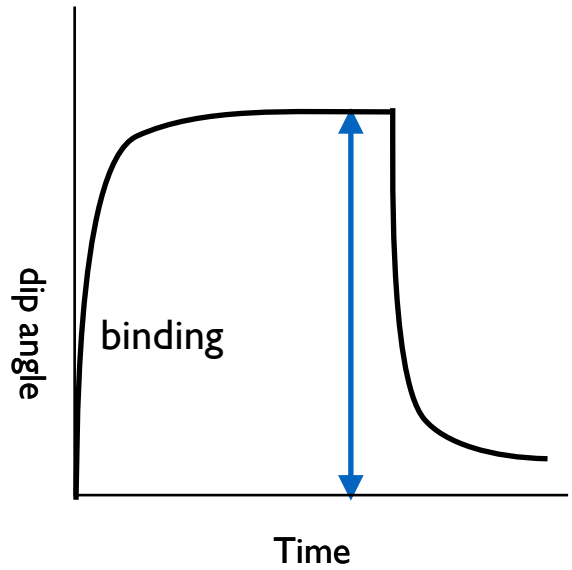
Surface Plasmon Resonance

Advantages:

measures K_d, k_{on} and k_{off} (in principle)





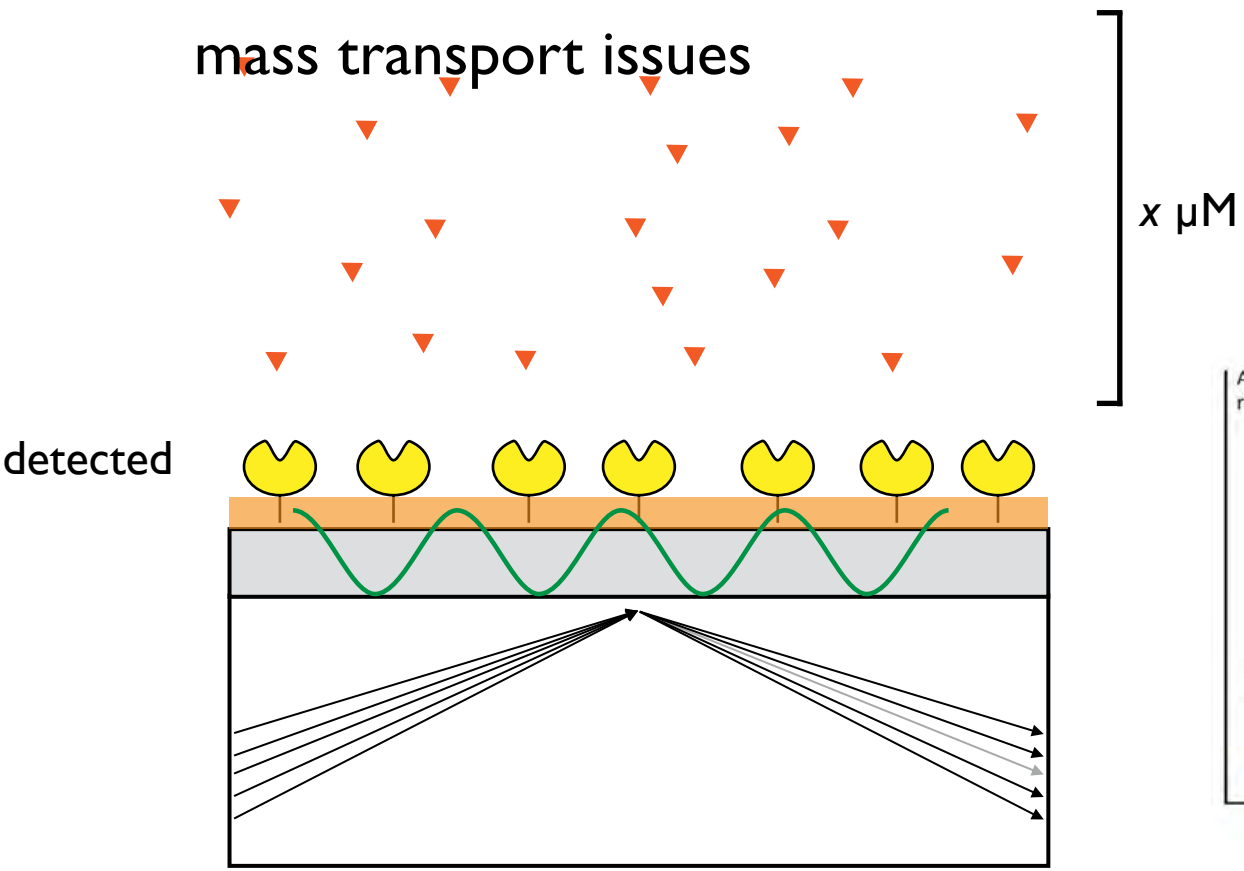
Surface Plasmon Resonance

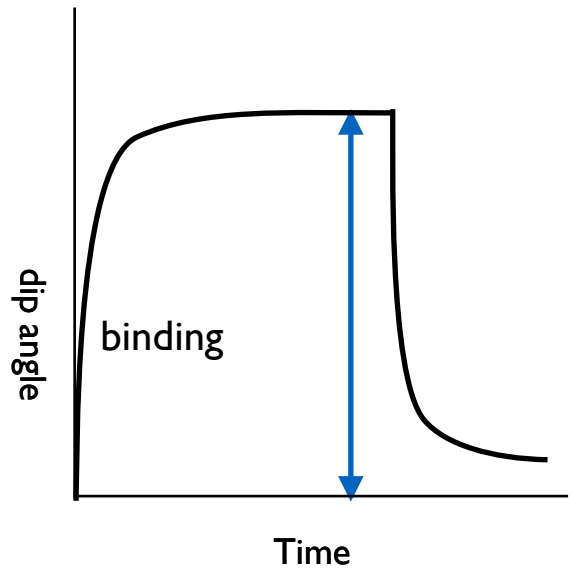
Advantages:

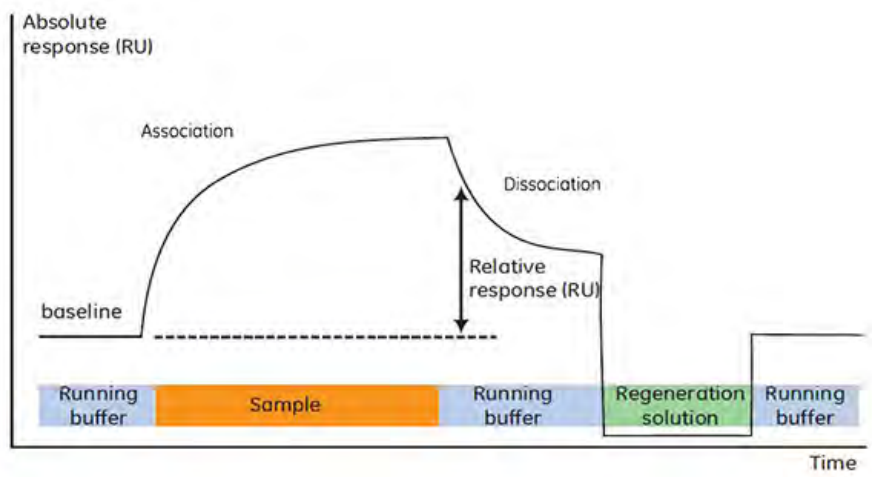
- measures K_d, k_{on} and k_{off} (in principle)
- fluidics allows high throughput (in principle)
- label free / works in complex environments

Disadvantages:

- surface immobilization
- mass transport issues

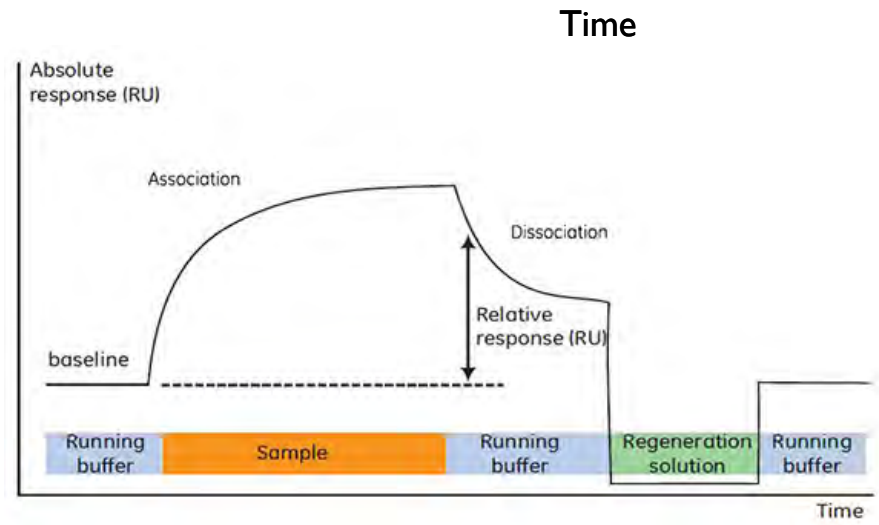






Surface Plasmon Resonance

https://www.cytivalifesciences.com/en/us/solutions/protein-research/knowledge-center/surface-plasmon-resonance/surface-plasmon-resonance



Competing Technologies

Surface Plasmon Resonance

Cytiva

Carterra

"Proprietary HT-SPR™ technology" targeting monoclonal antibody screening…

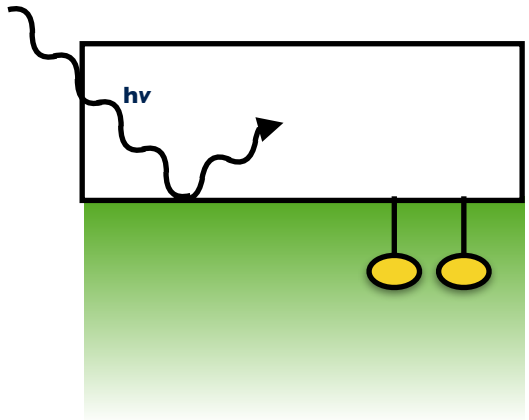
A high-resolution CCD camera images the entire chip surface simultaneously, enabling up to 384 real-time interactions to be monitored in parallel with local referencing via neighboring interspots.

Complete kinetic data processing and analysis can be automated, with referencing, zeroing, cropping, blank subtraction, and kinetic model fitting all executed as a single operation for up to 1152 samples per unattended run.

Bio-Layer Interferometry

Sartorius / BioForte

Measures change in refractive index at the surface



Thermophoresis

but first…

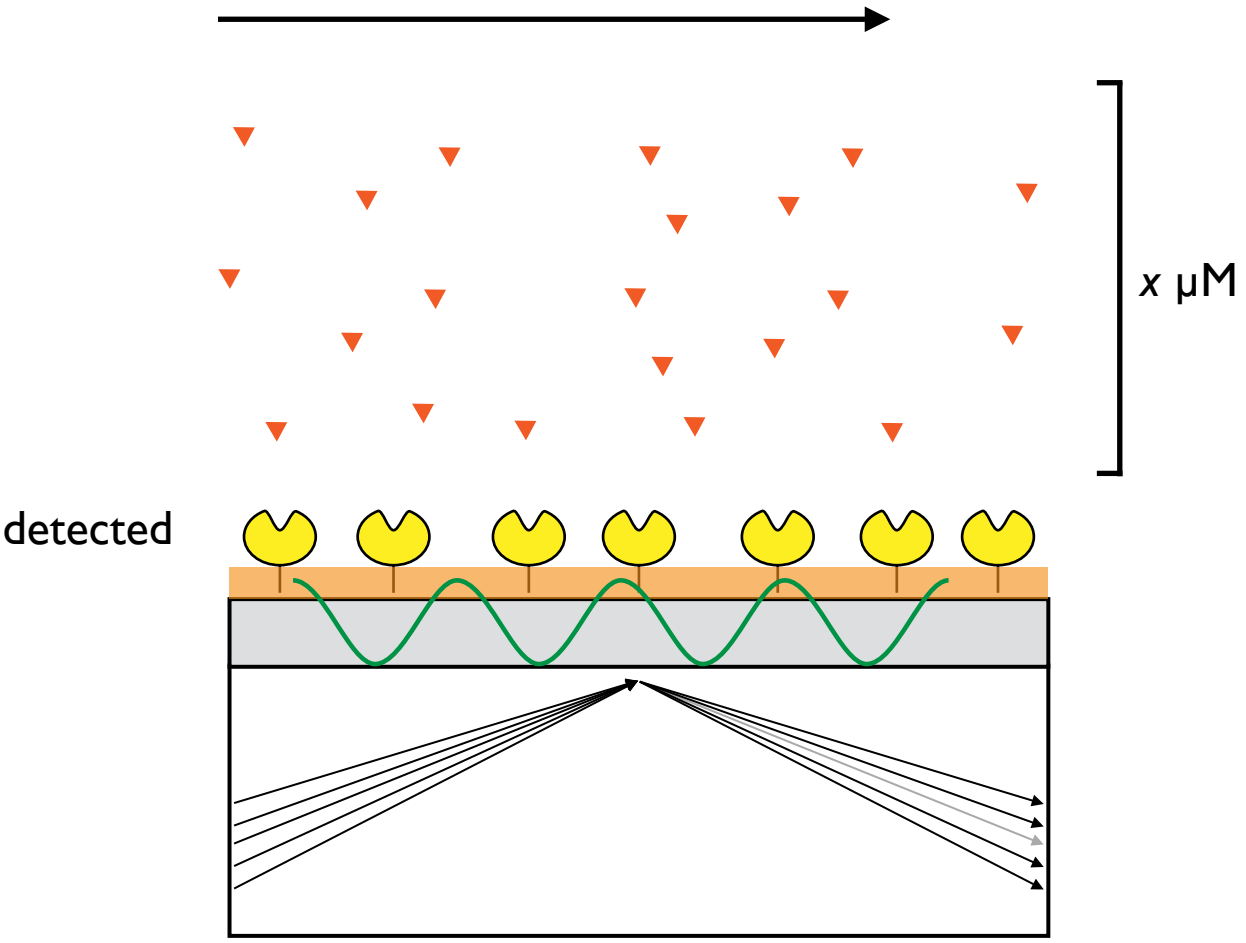
Recap / Revisit

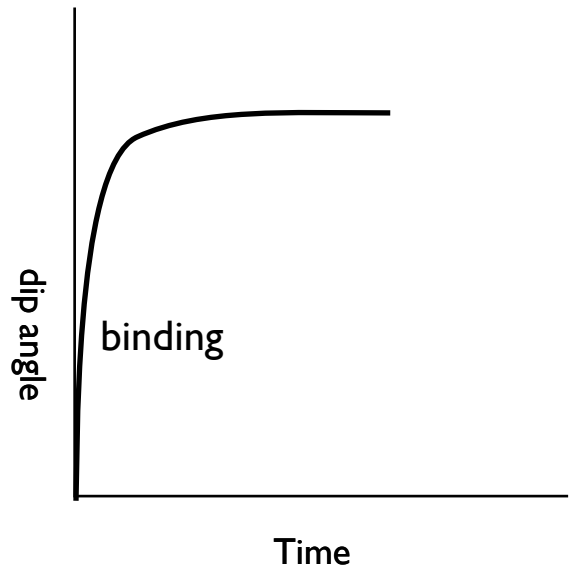
April 13, 2021

Surface Plasmon Resonance - recap

Surface Plasmons - collective, quantized oscillations of conduction band electrons in a metal

Have wave/particle duality like photons, electrons





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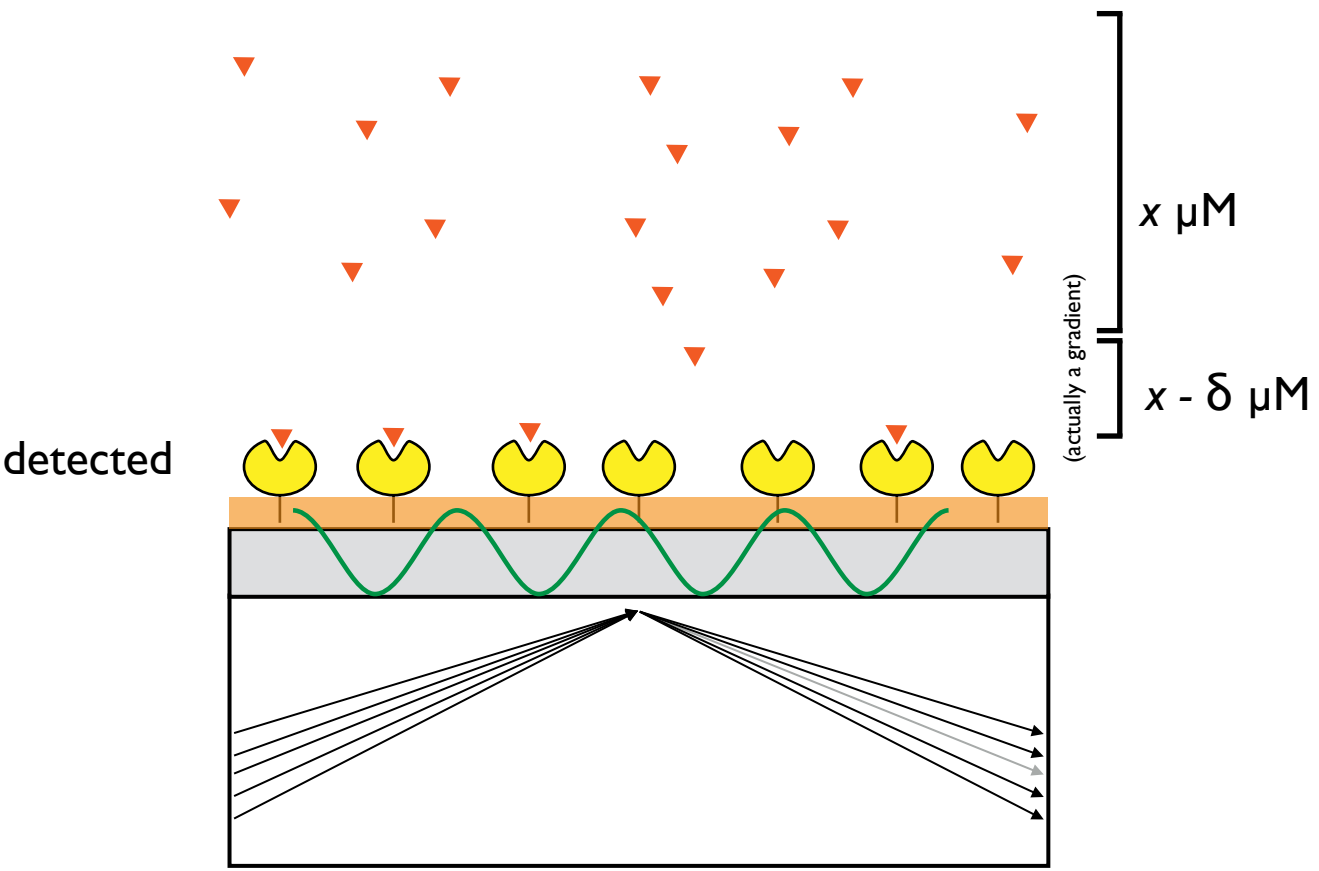

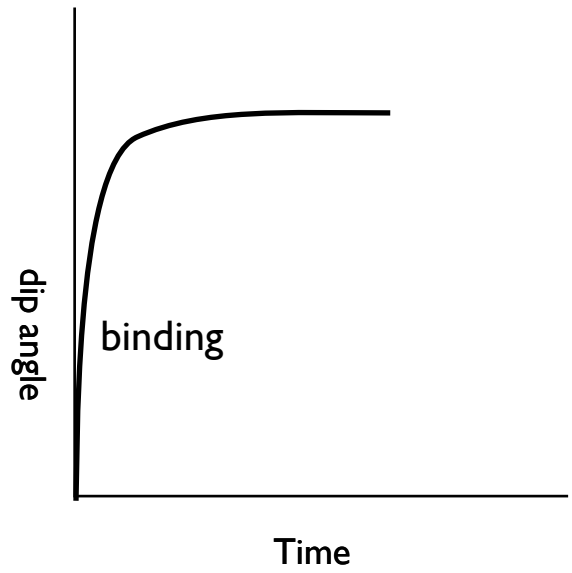

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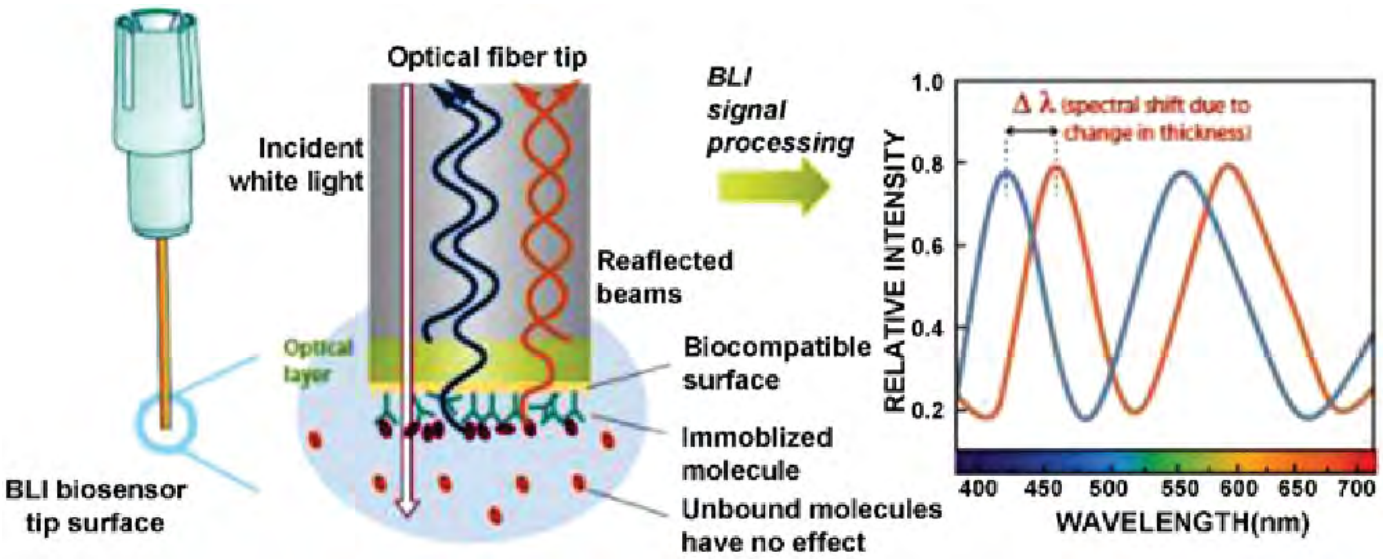
Competing Technologies

Bio-Layer Interferometry

Sartorius / BioForte

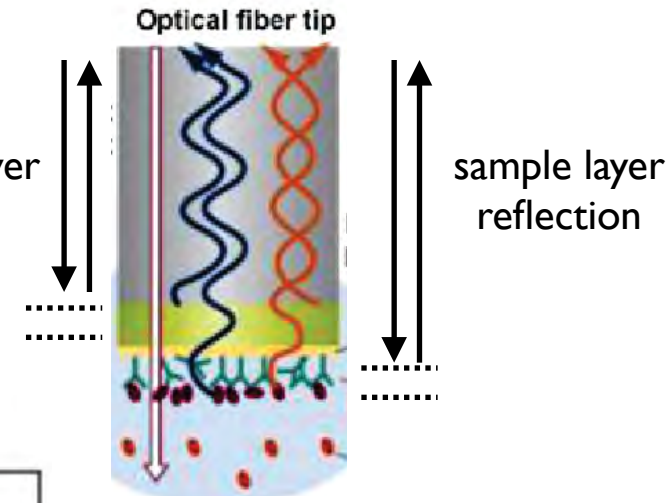


Not a flow system, so not subject to mass transfer issues

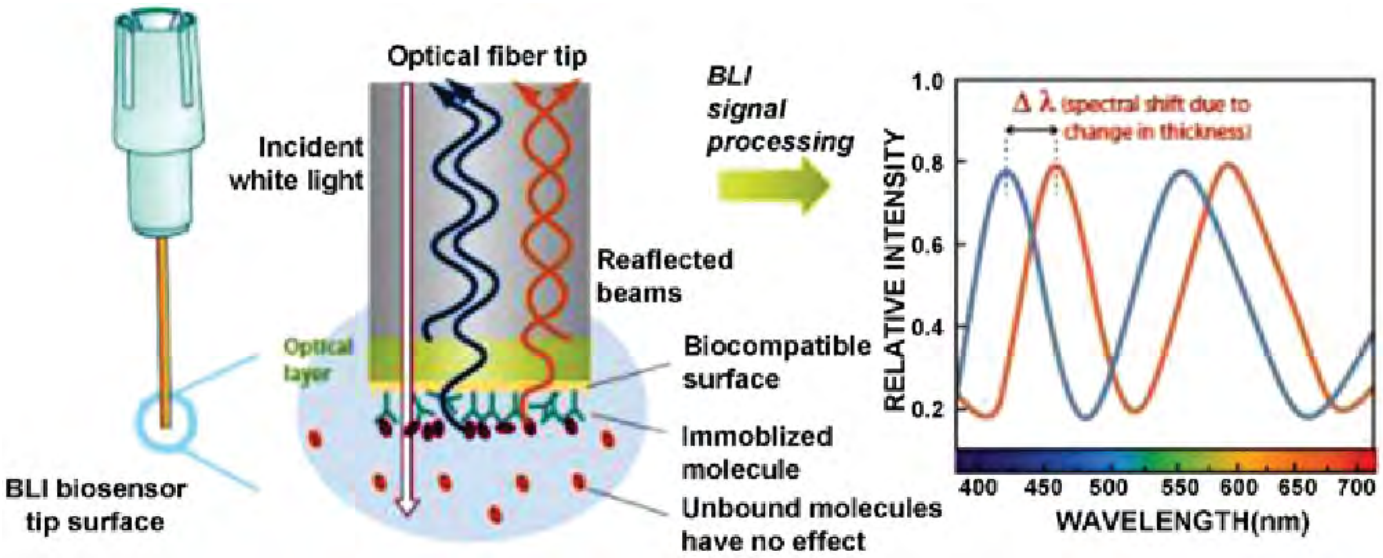
Competing Technologies

Bio-Layer Interferometry

Sartorius / BioForte



White light incident/reflected, but some wavelengths shifted on reflection. Shift is proportional to thickness of perturbing layer



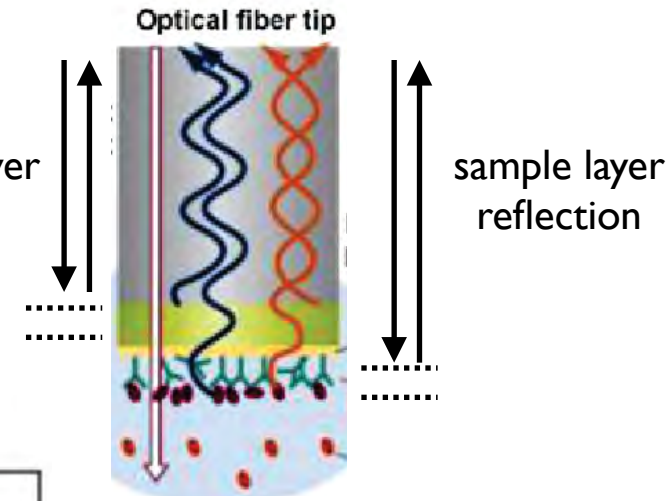
The well plate was on a rapid shaker, why?

Not a flow system, so not subject to mass transfer issues

Competing Technologies

Bio-Layer Interferometry

Sartorius / BioForte



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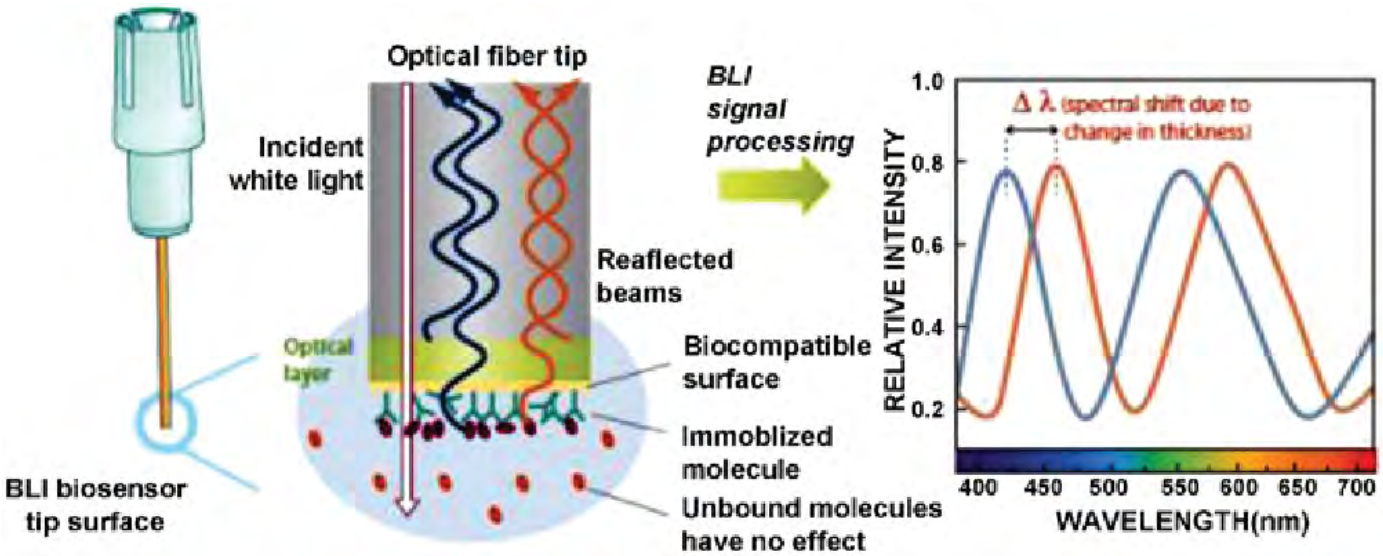
https://www.youtube.com/watch?v=Jv0CVIgaZ9s



And of course, this is immobilization on a surface, which should always raise concerns

The well plate was on a rapid shaker, why?

Not a flow system, so ~~not~~ subject to mass transfer issues

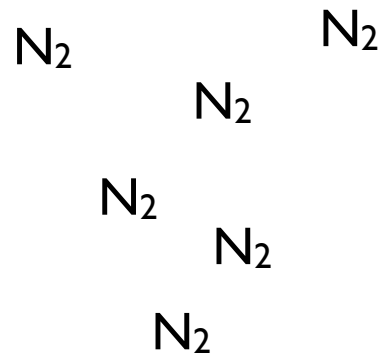
How would the fluidics SPR experimenter do the same?

Thermophoresis

April 13, 2021

Aerosol Thermophoresis

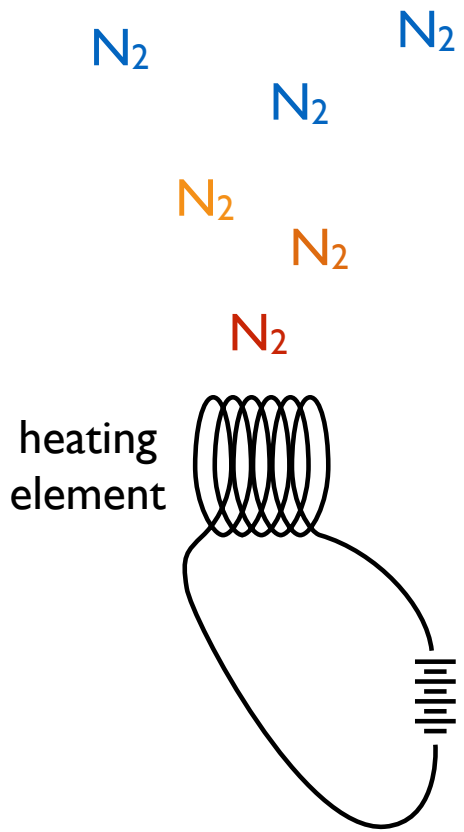
… not for biology …



all are in motion, described by kinetic theory of gases

Aerosol Thermophoresis

… not for biology …



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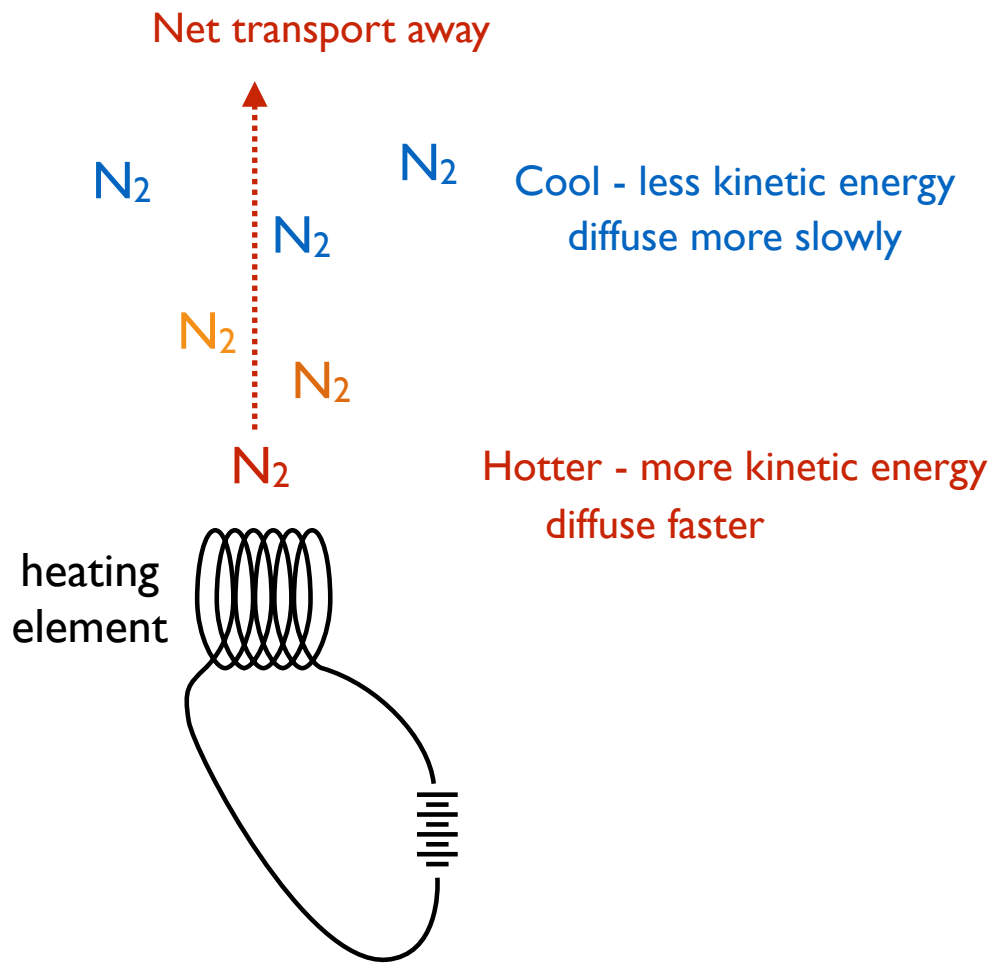
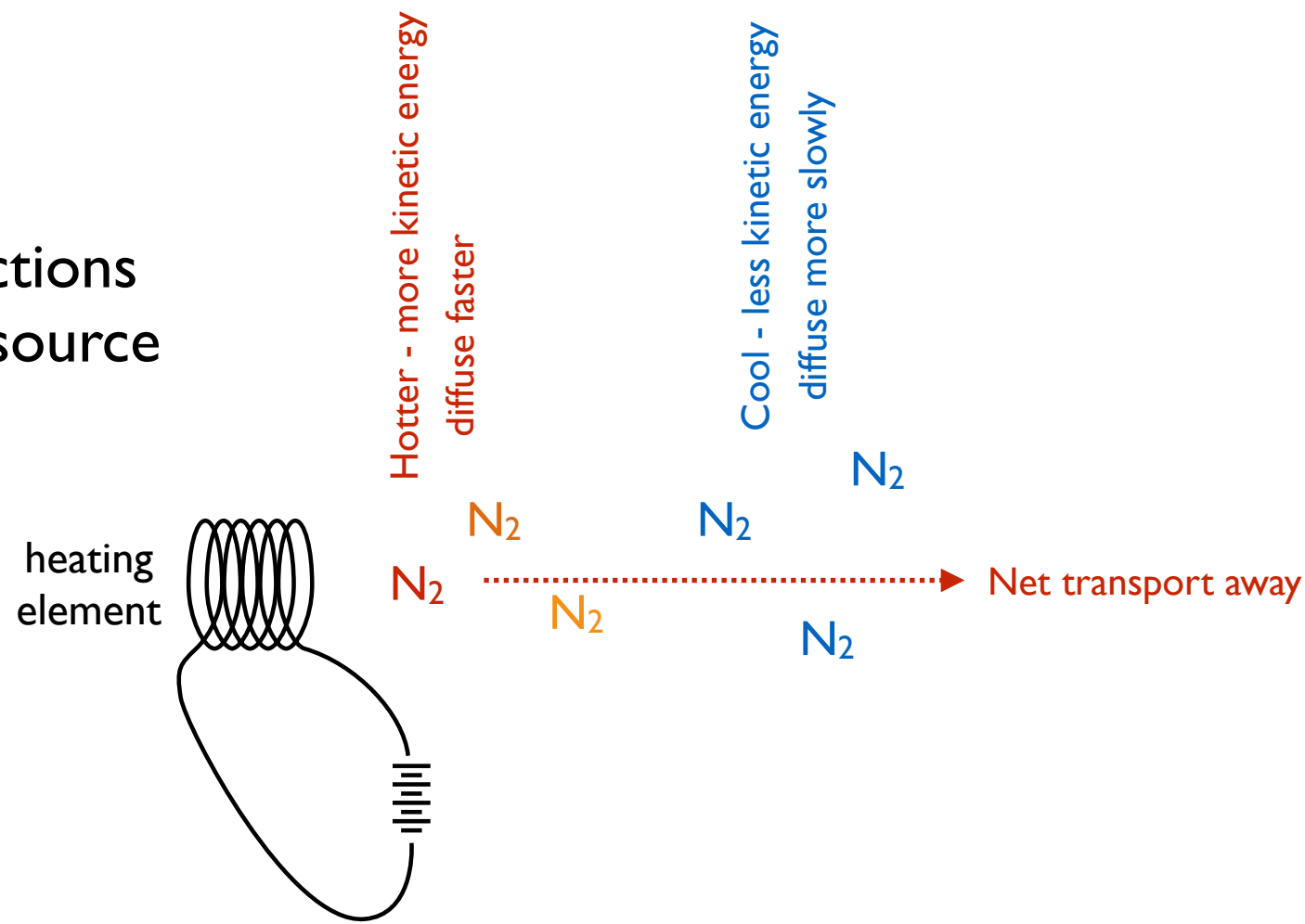


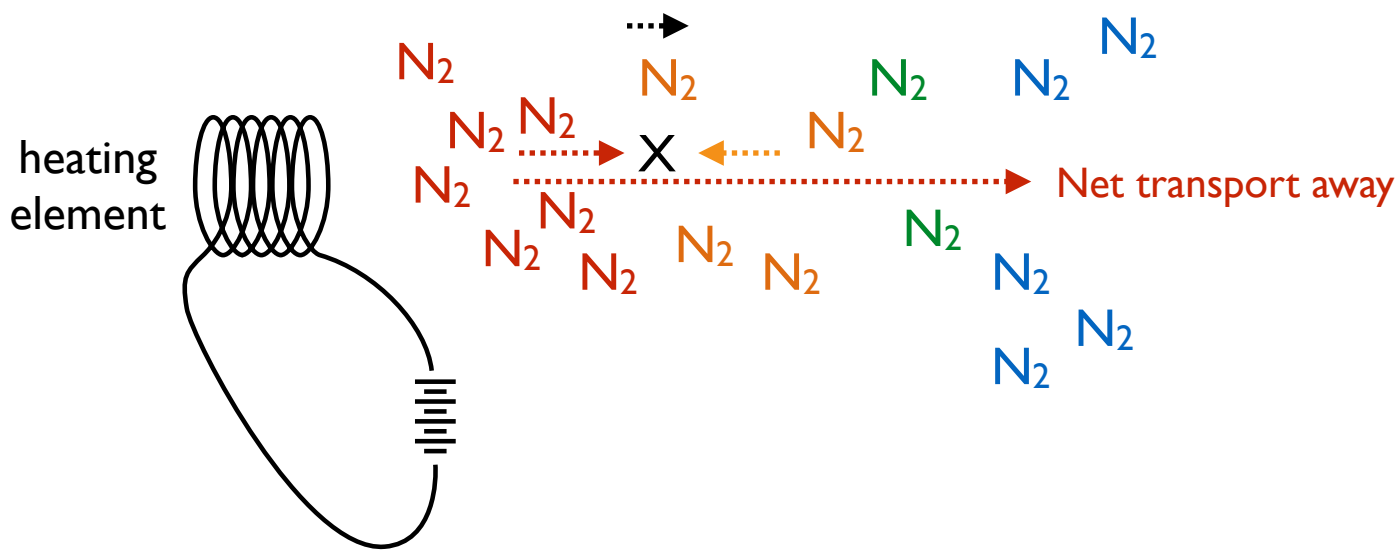
Aerosol Thermophoresis

Diffusion in <u>all</u> directions away from the heat source

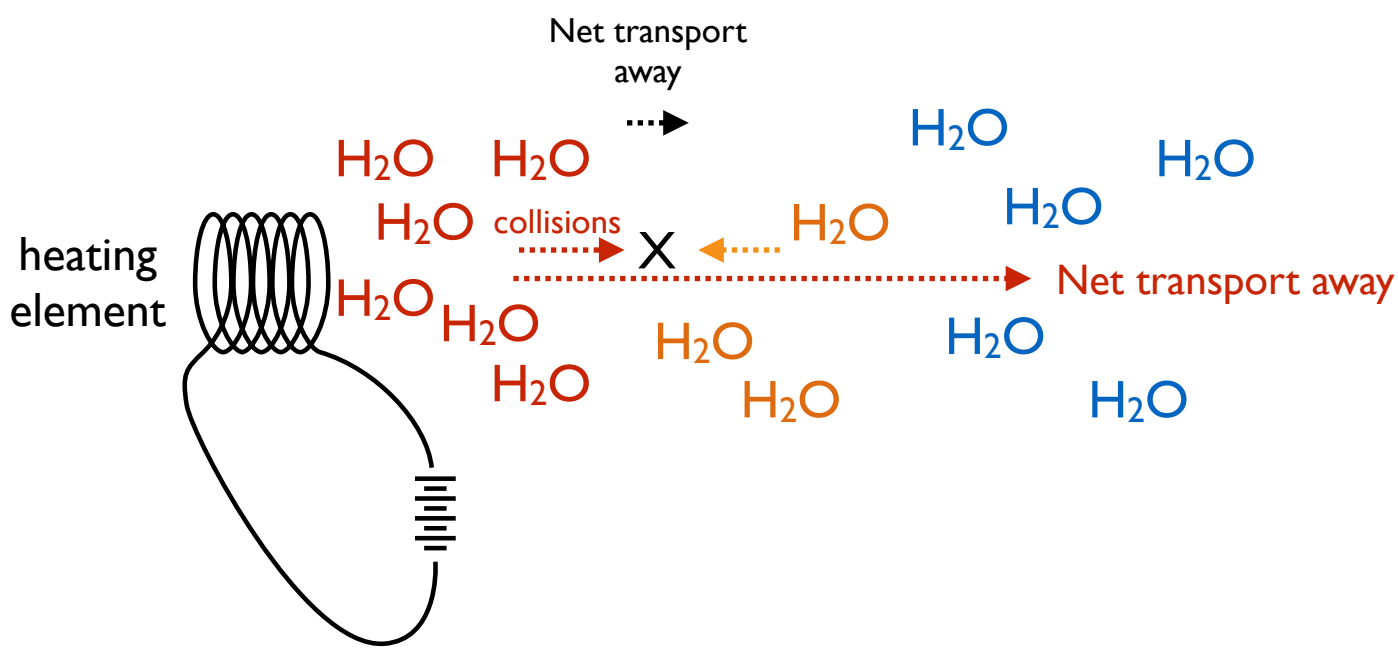


Aerosol Thermophoresis

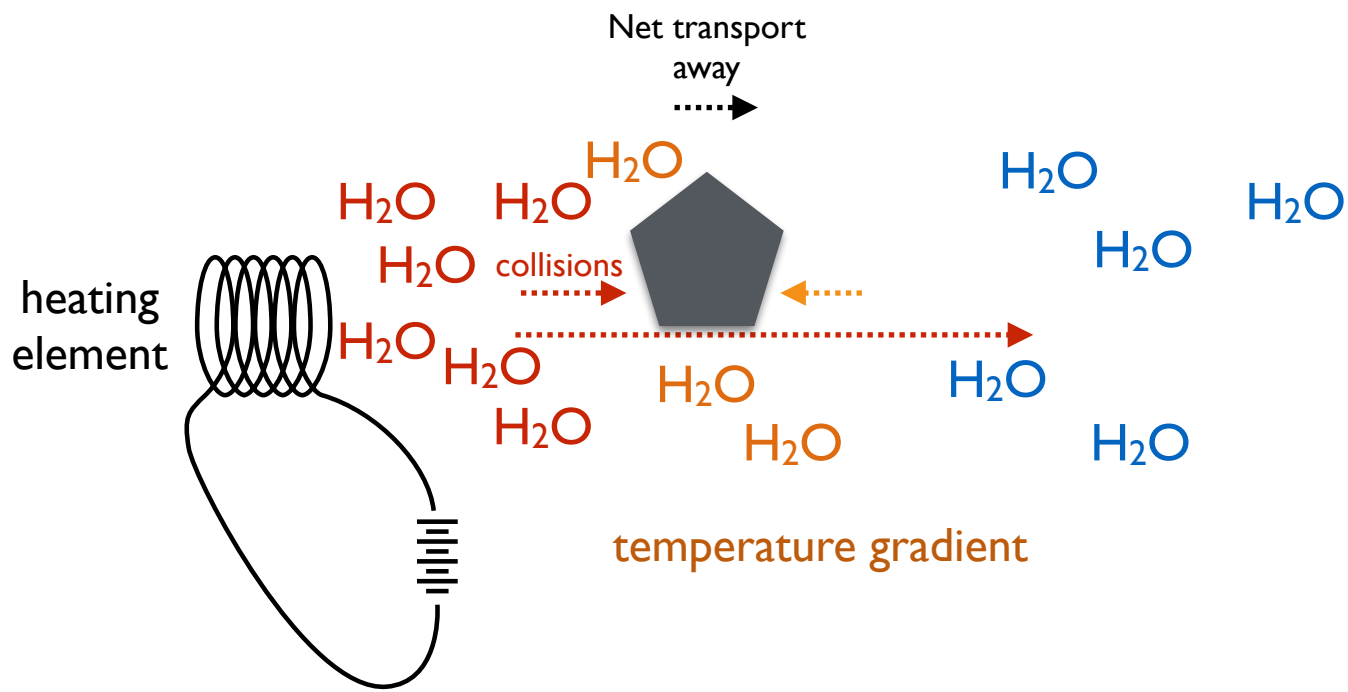
The phenomenon is observed at the scale of one millimeter or less. An example that may be observed by the naked eye with good lighting is when the hot rod of an electric heater is surrounded by tobacco smoke: the smoke goes away from the immediate vicinity of the hot rod. As the small particles of air nearest the hot rod are heated, they create a fast flow away from the rod, down the temperature gradient. They have acquired higher kinetic energy with their higher temperature. When they collide with the large, slower-moving particles of the tobacco smoke they push the latter away from the rod. The force that has pushed the smoke particles away from the rod is an example of a thermophoretic force.



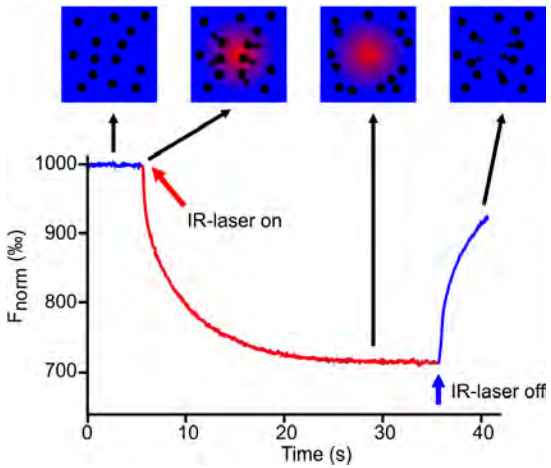
Microscale Thermophoresis



Microscale Thermophoresis



Microscale Thermophoresis

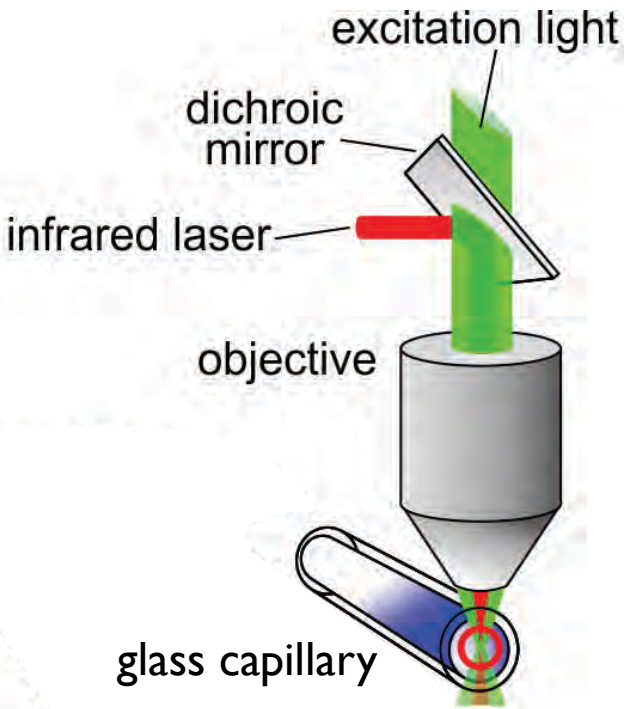


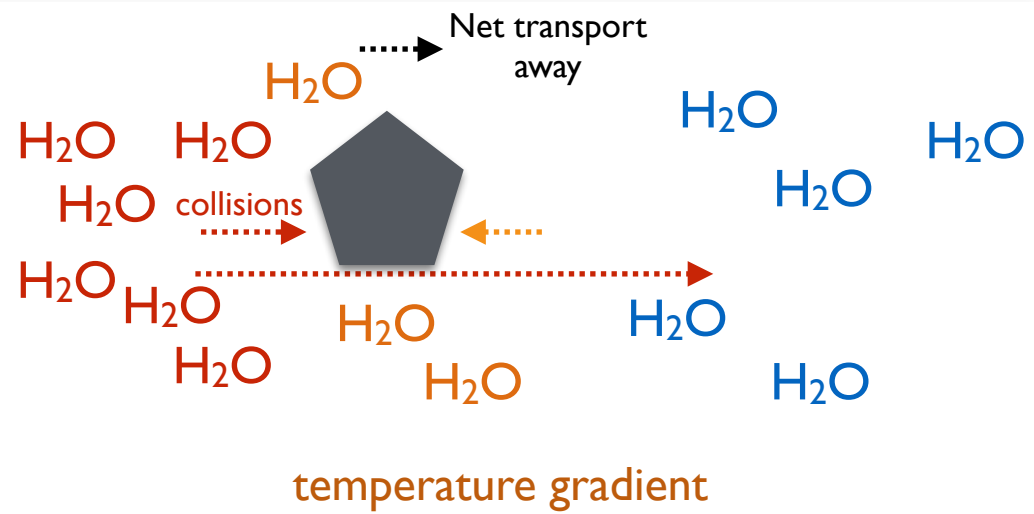
Fluorescence Emission
e.g., 503 nm

Fluorescence Excitation
e.g., 420 nm

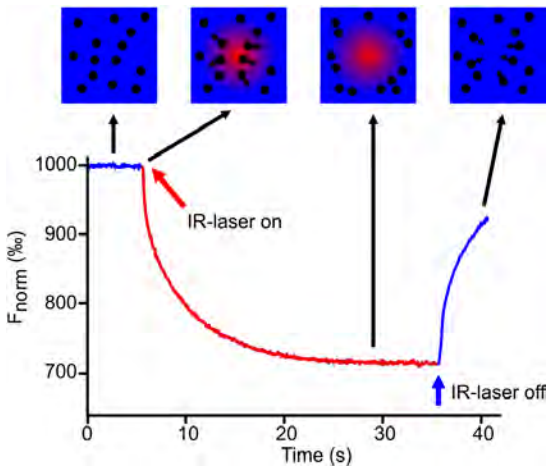
Laser heating
1480 nm

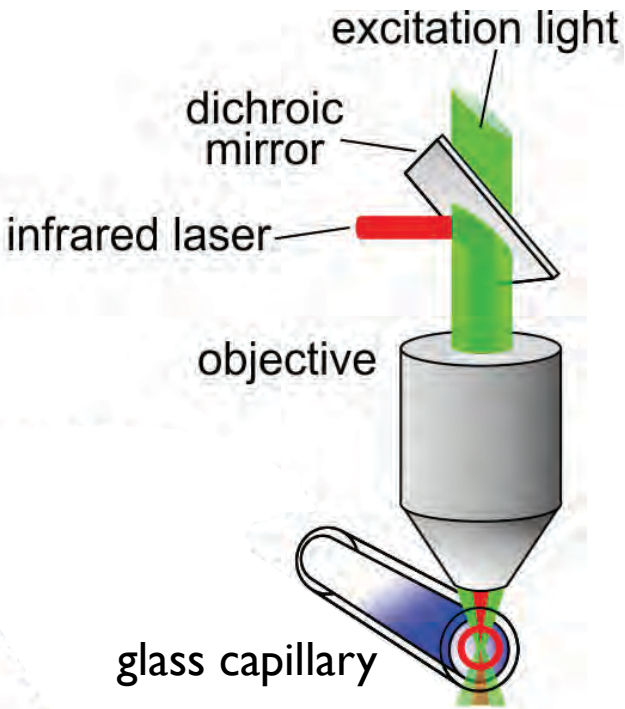
$\Delta T \approx$ 2-6 °C
(local)

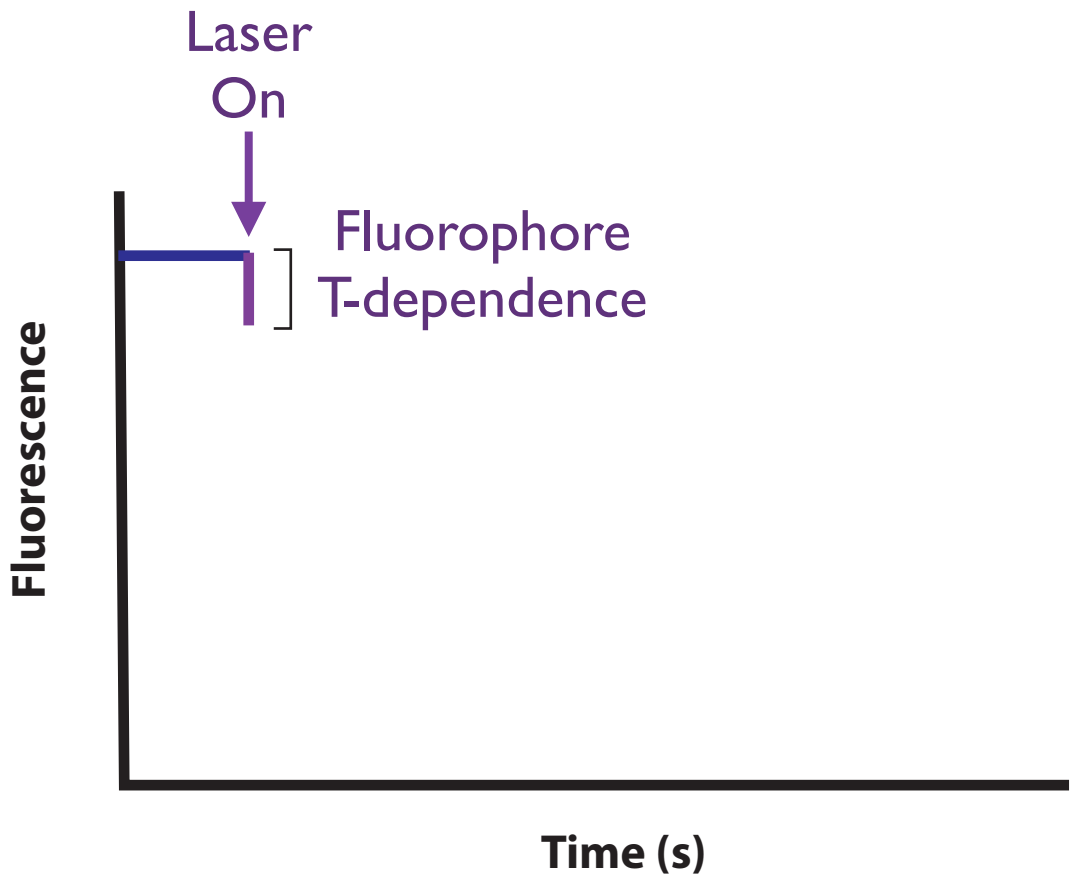




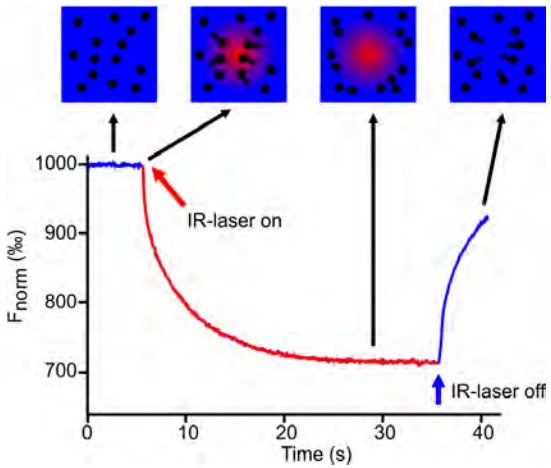
Microscale Thermophoresis

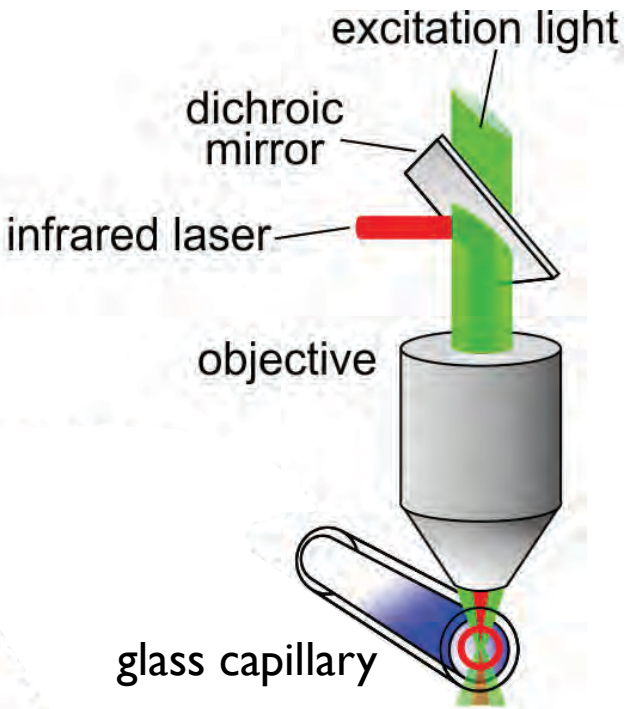


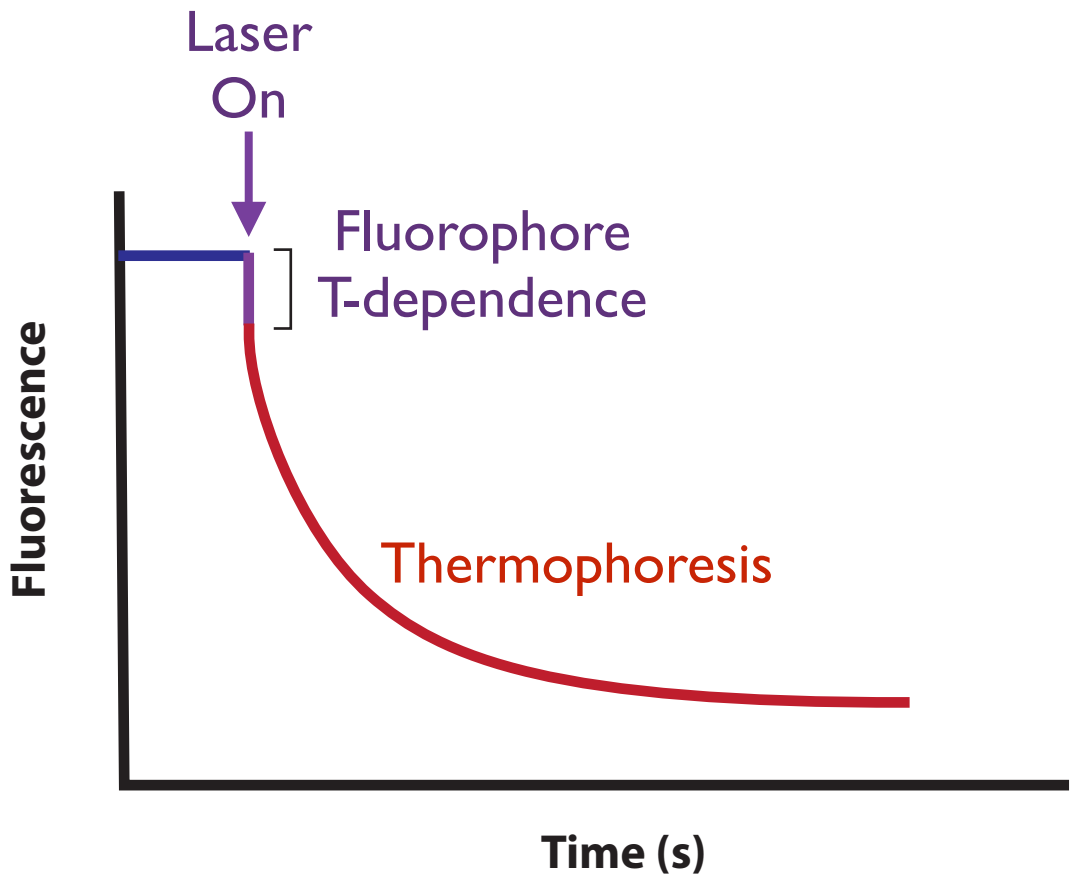




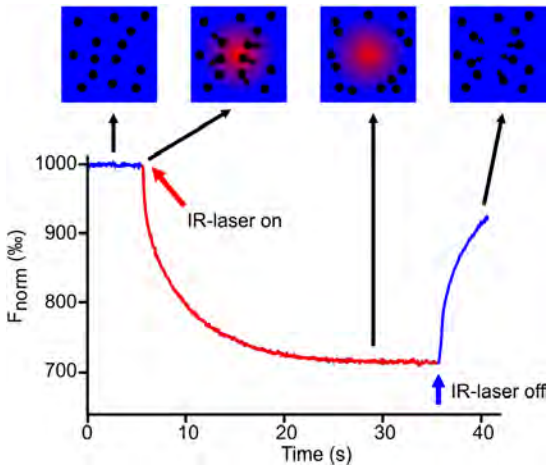
Microscale Thermophoresis

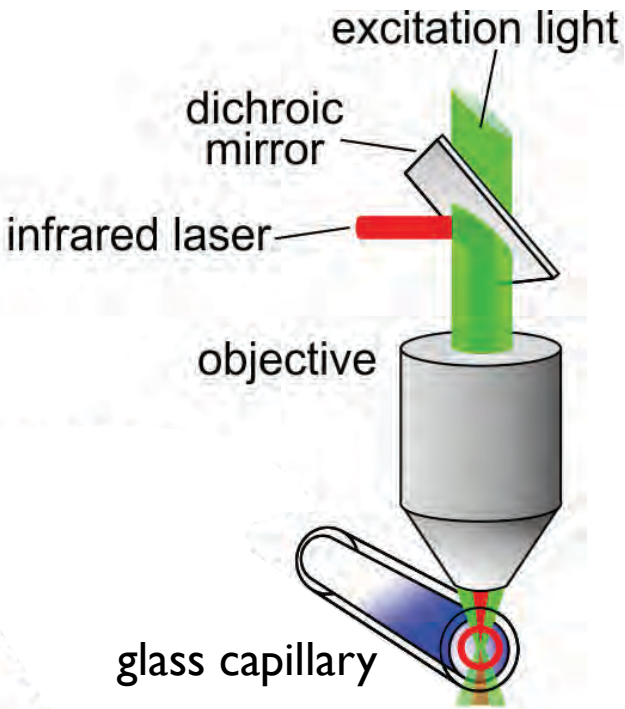


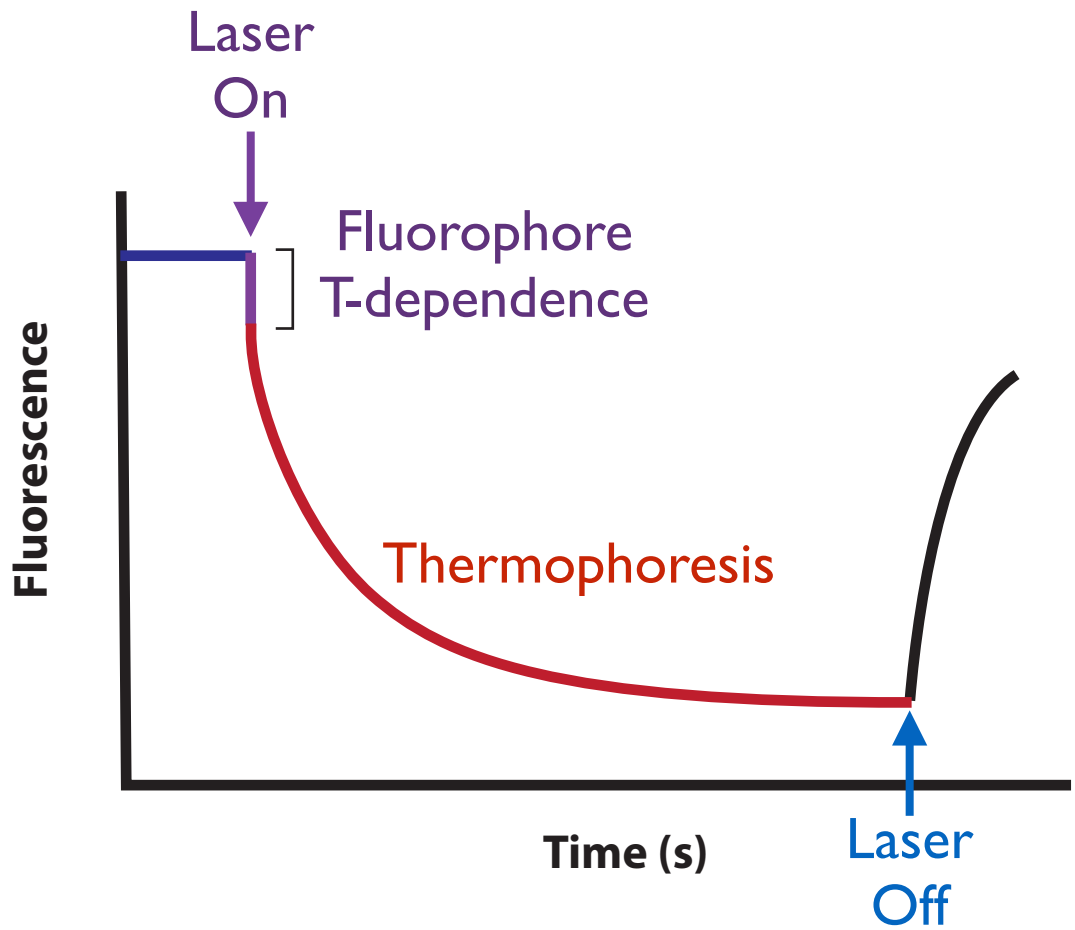




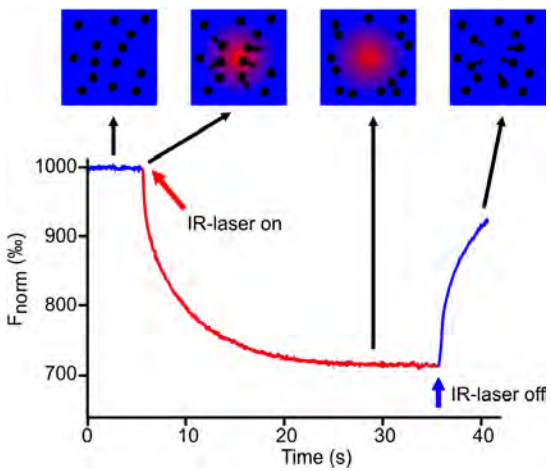
Microscale Thermophoresis

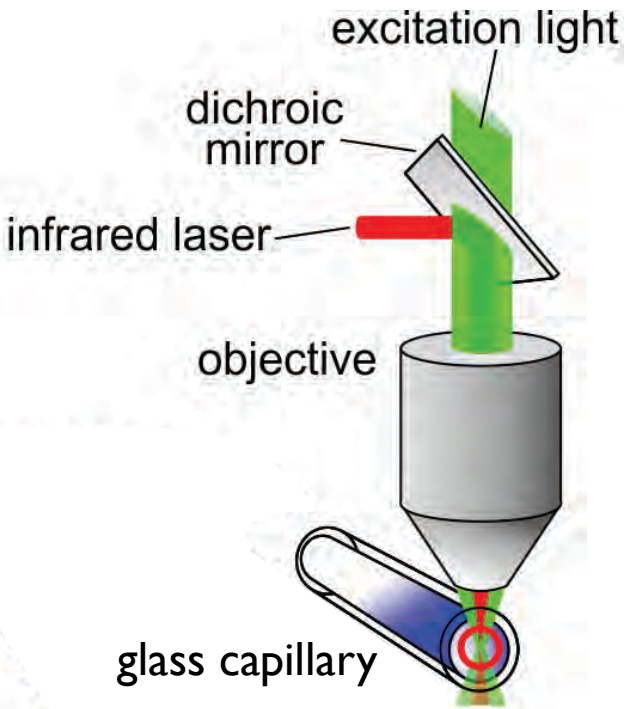






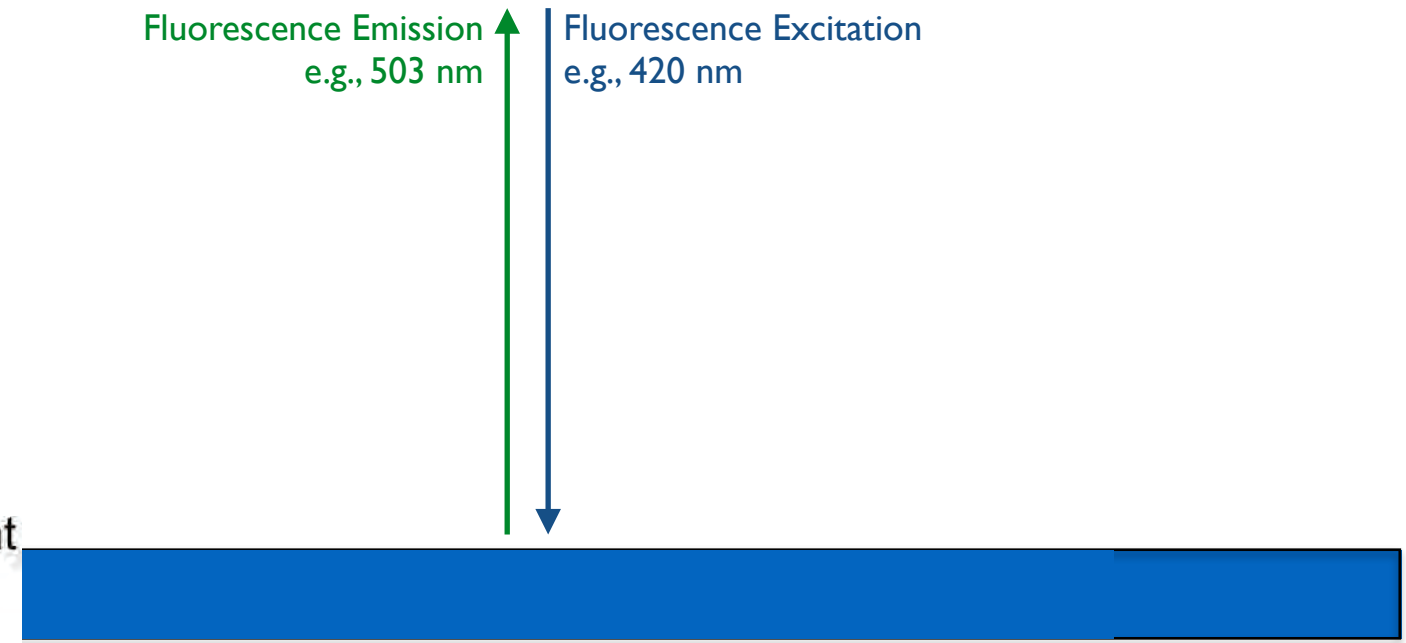
Microscale Thermophoresis





Fluorescence Emission
e.g., 503 nm

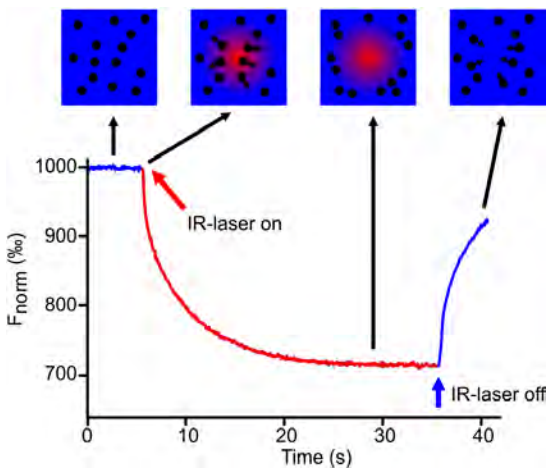
Fluorescence Excitation
e.g., 420 nm

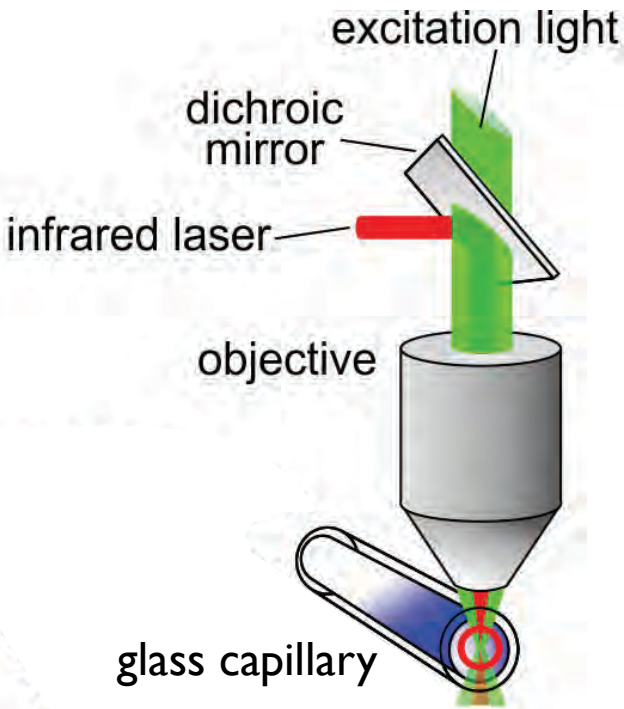


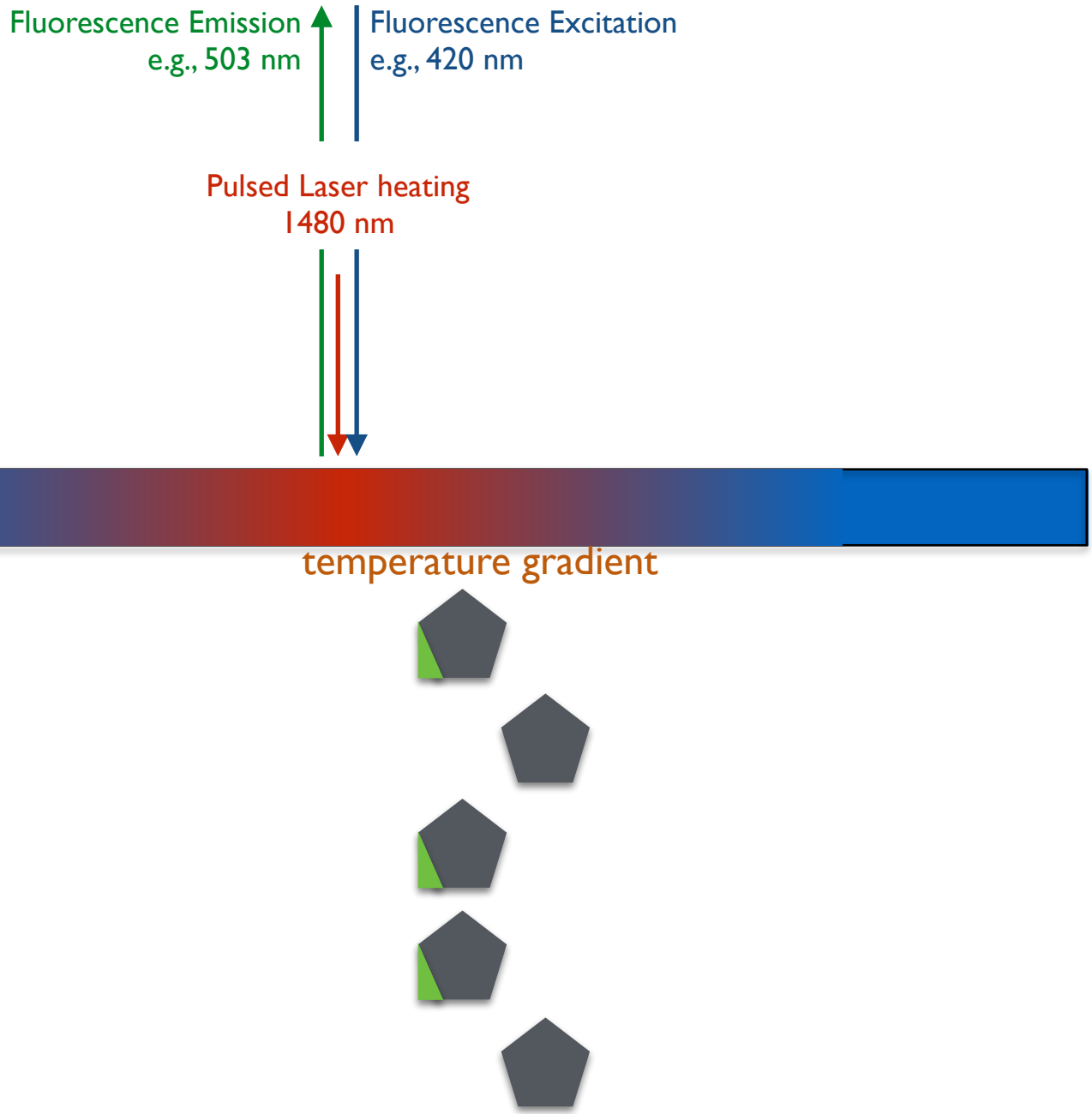
Ligand binding at equilibrium

K_d, P_{tot}, L_{tot}

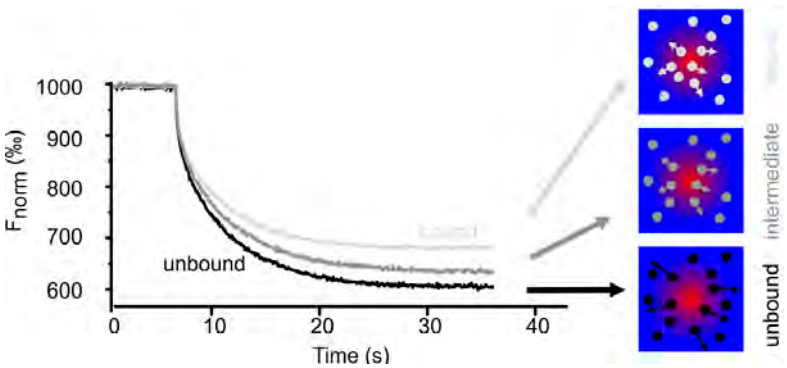
Microscale Thermophoresis





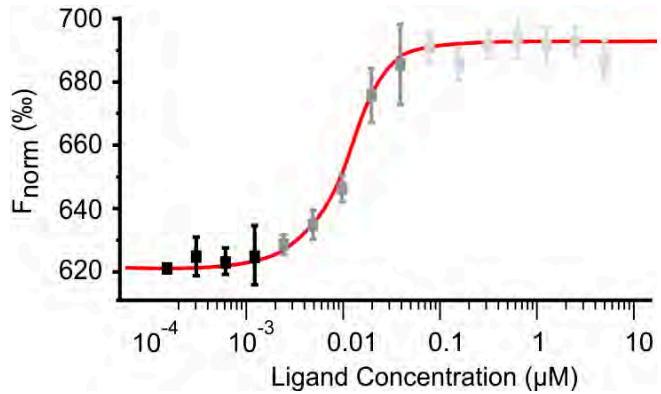


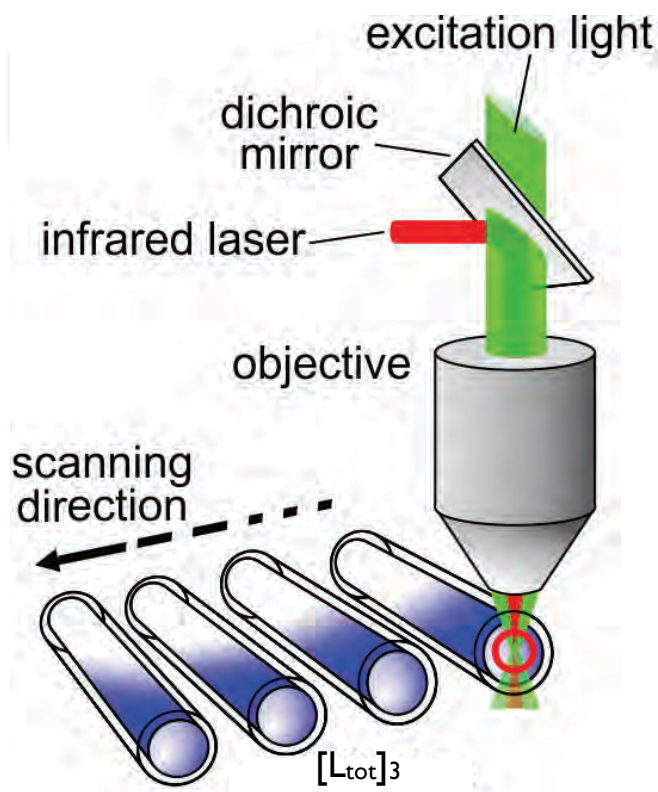
Microscale Thermophoresis



Fluorescence Emission
e.g., 503 nm

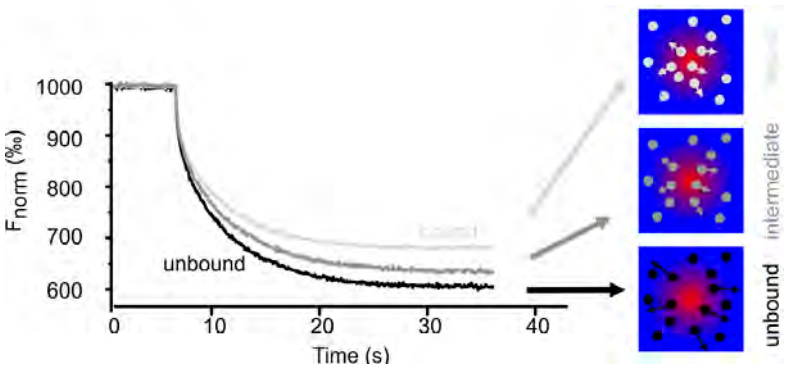
Fluorescence Excitation
e.g., 420 nm





temperature gradient

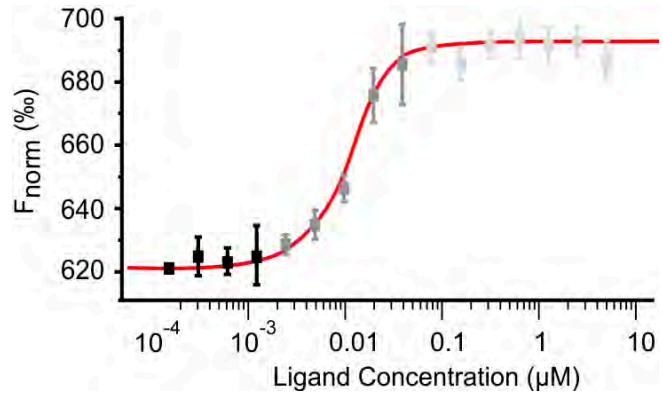
Microscale Thermophoresis

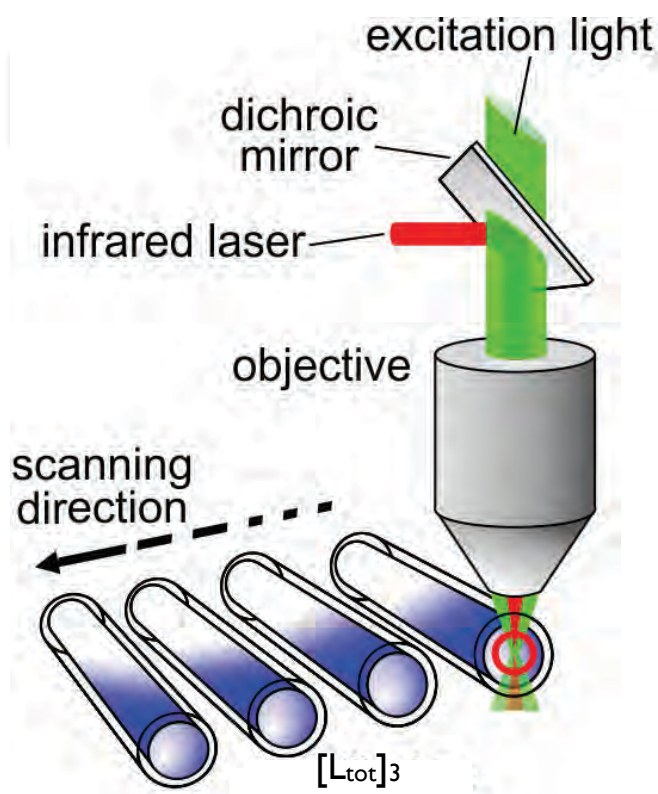


Fluorescence Emission
e.g., 503 nm

Fluorescence Excitation
e.g., 420 nm

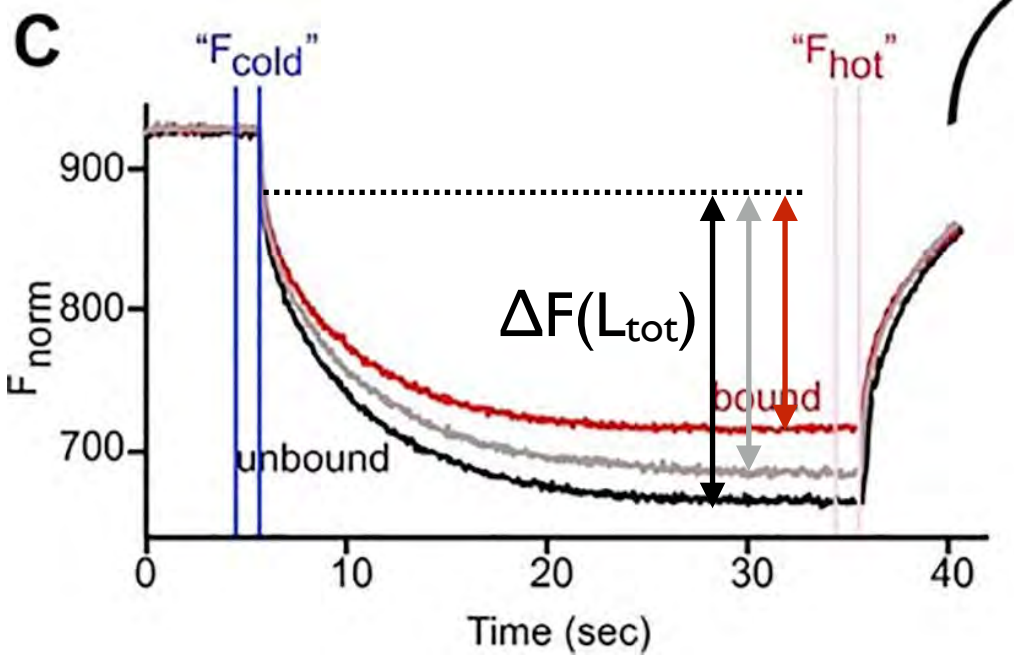
Pulsed Laser heating
1480 nm

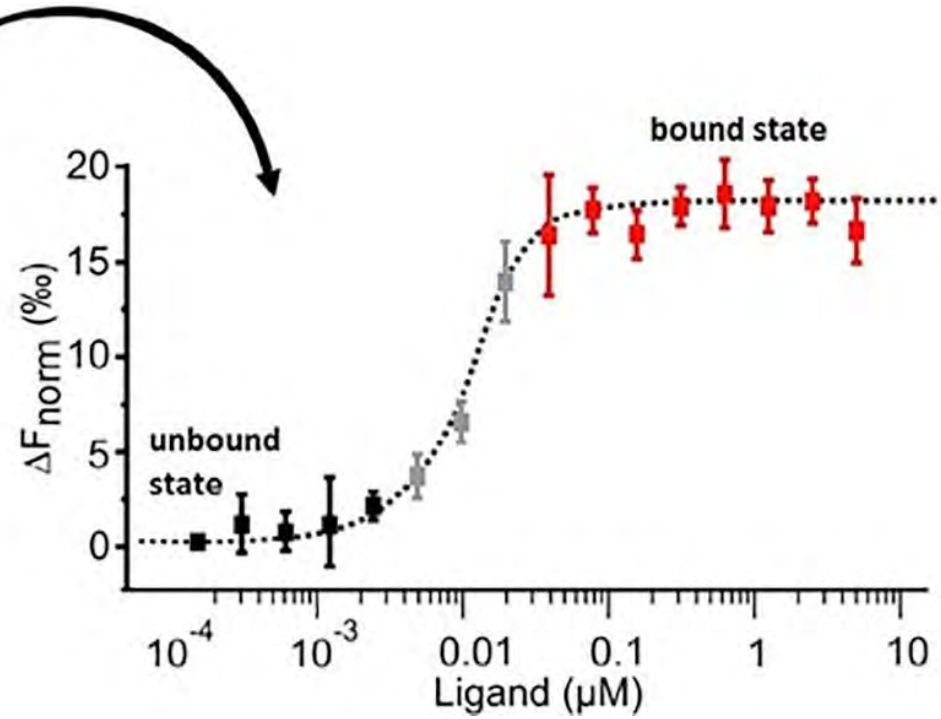


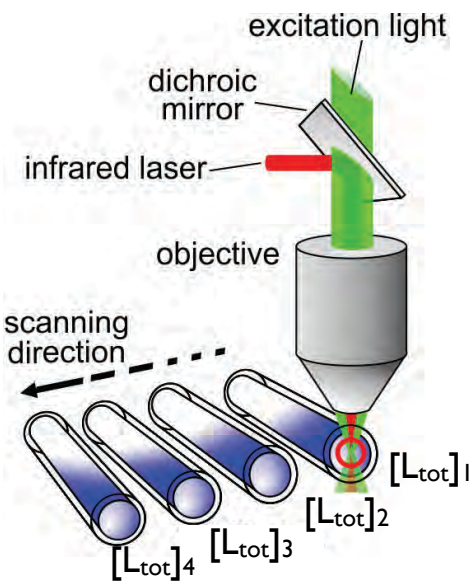


temperature gradient

Microscale Thermophoresis



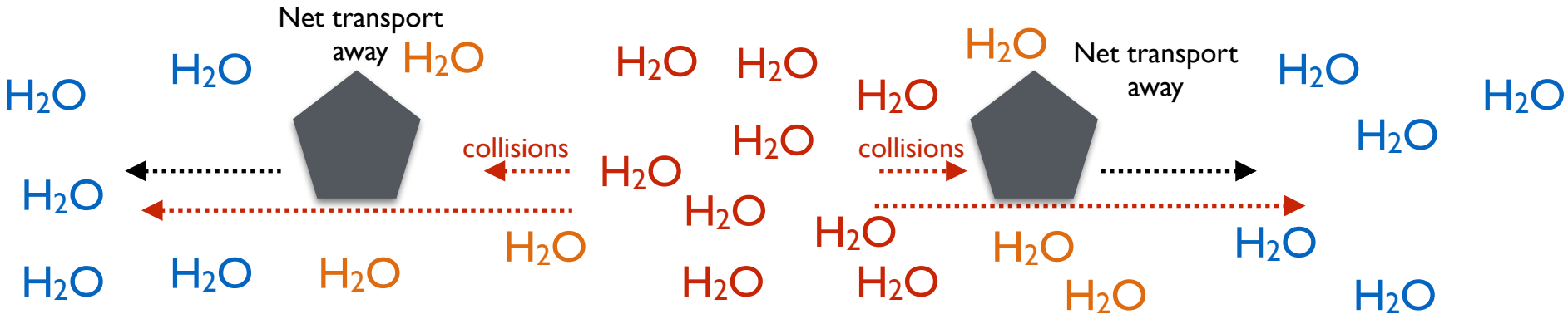


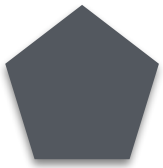


$$f = \frac{[PL]}{[P]_{tot}}$$ ← K_d, P_{tot}, L_{tot}

$$F_{norm} = \frac{F_{hot}}{F_{cold}} = (1-f)F_{norm}^{unbound} + (f)F_{norm}^{bound}$$

Microscale Thermophoresis





Steady state position (and ΔF) depends on "size"

complicated

Under constant buffer conditions, thermophoresis probes the size, charge, and solvation entropy of the molecules.

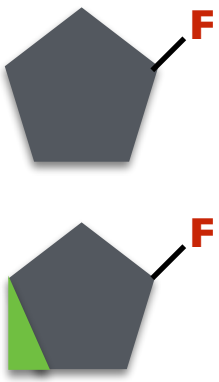
Ligand does not necessarily have to be large relative to the macromolecule

Binding may close an open cleft

Binding may change solvation shell

Microscale Thermophoresis

Fluorescence



<u>Strengths</u>

(measure Kd's at the low pM level)

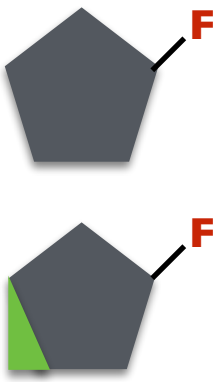
fluorescence can be highly sensitive (laser excitation!)

can be in a complex mixture

even cell lysate!

Microscale Thermophoresis

Fluorescence



Strengths

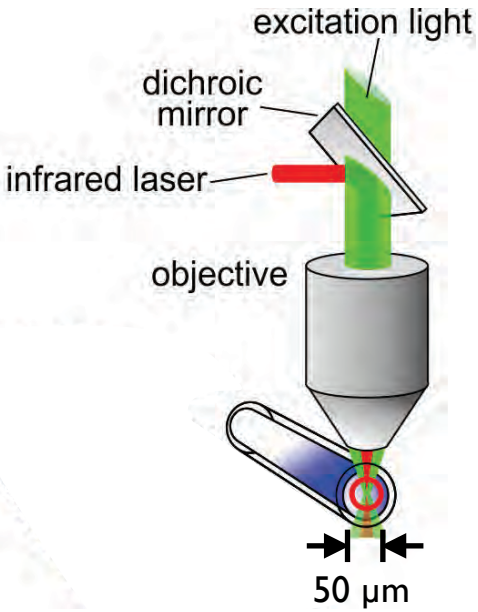
(measure Kd's at the low pM level)

fluorescence can be highly sensitive (laser excitation!)

can be in a complex mixture

even cell lysate!

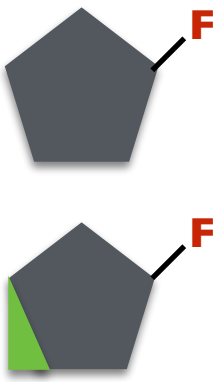
uses very small volumes



<1 nL (!) (but realistically more, since we can't mix at that level!)

Microscale Thermophoresis

Label-free Fluorescence



Use *intrinsic* protein fluorescence

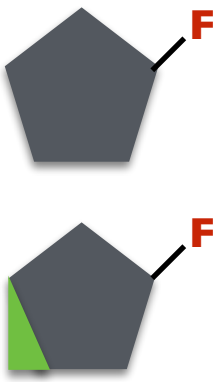
2-3 Trp residues - detect down to 100 nM

fluorophore details don't matter

Trp, Tyr, Phe…

Microscale Thermophoresis

Fluorescence



Strengths

ligand need not be large

may induce change in the protein

shape/hydration

that change can be in either direction

"negative" thermophoresis

example?

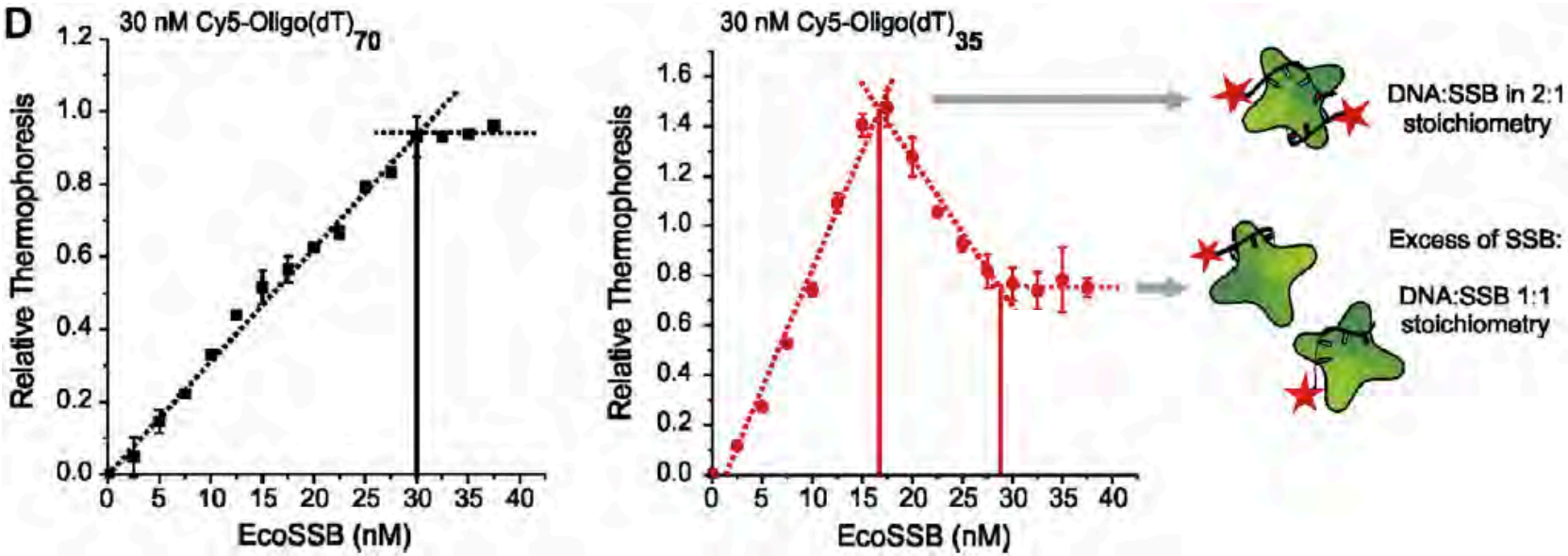
Caveats

The effects of two (or more) binding sites is *not additive*

extreme example: one positive, one negative thermophoresis

Microscale Thermophoresis

Titration at *high* concentrations



SSB: a homotetramer

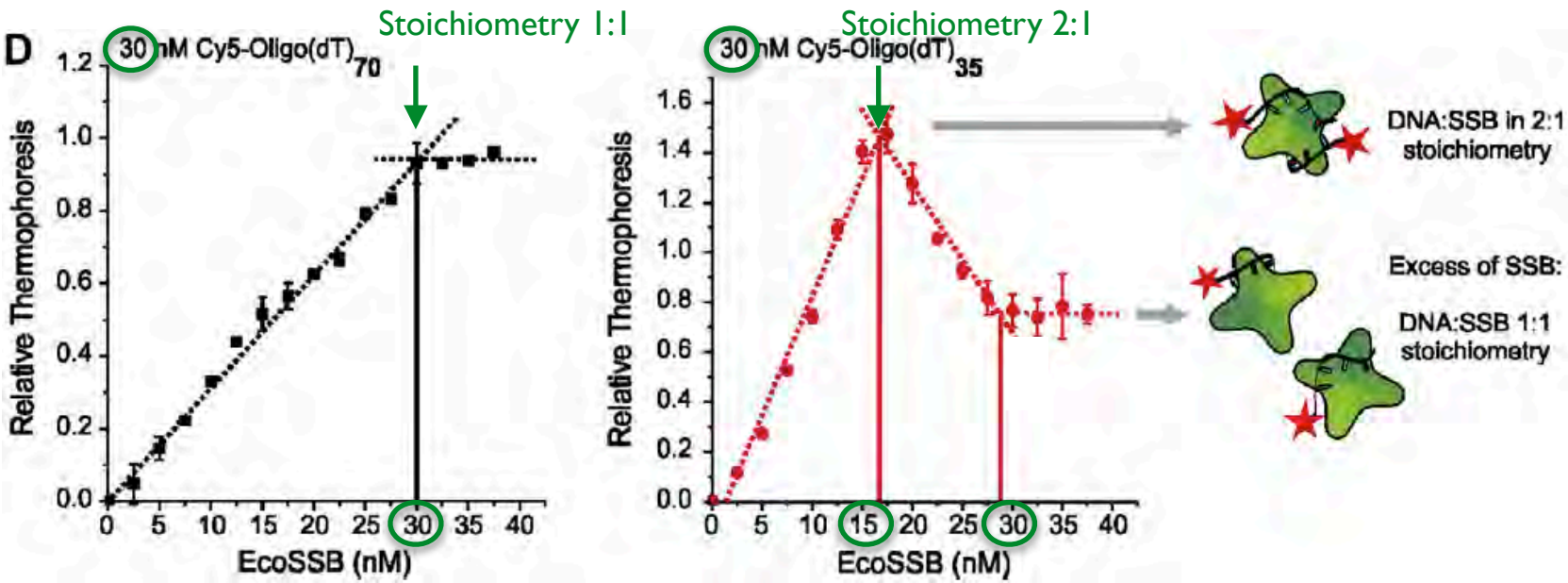
- *cooperatively* binds dT_{70} oligo

The effects of two (or more) binding sites is *not additive*

extreme example: one positive, one negative thermophoresis

Microscale Thermophoresis

Titration at *high* concentrations



SSB: a homotetramer

- *cooperatively* binds dT_{70} oligo

at 17.5 nM SSB, each SSB has two 35mers bound

adding more SSB, some 35mers bind instead to new/extra SSB

at 30 nM SSB, 35mers are distributed 1:1

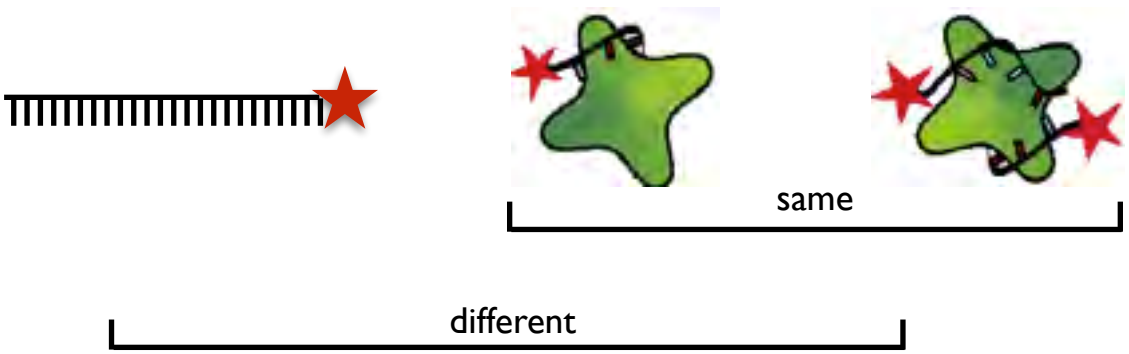
The effects of two (or more) binding sites is *not additive*

extreme example: one positive, one negative thermophoresis

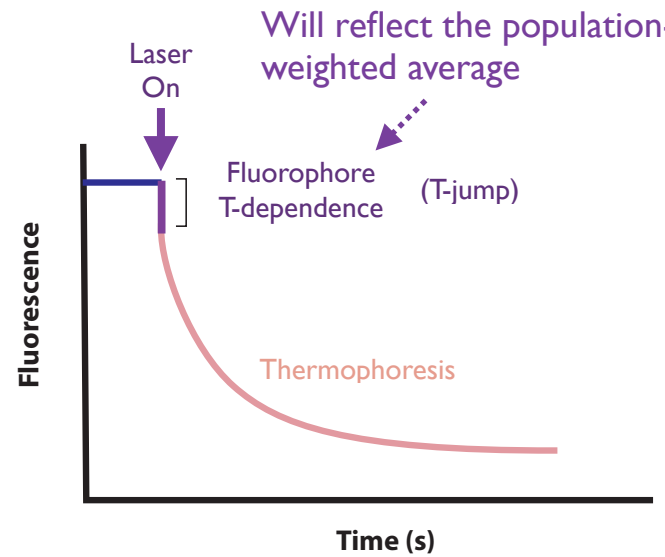
Microscale Thermophoresis

But wait…

What if the fluorophore shows environment-dependent temperature dependence?



Then we can use this as a separate measure of binding!

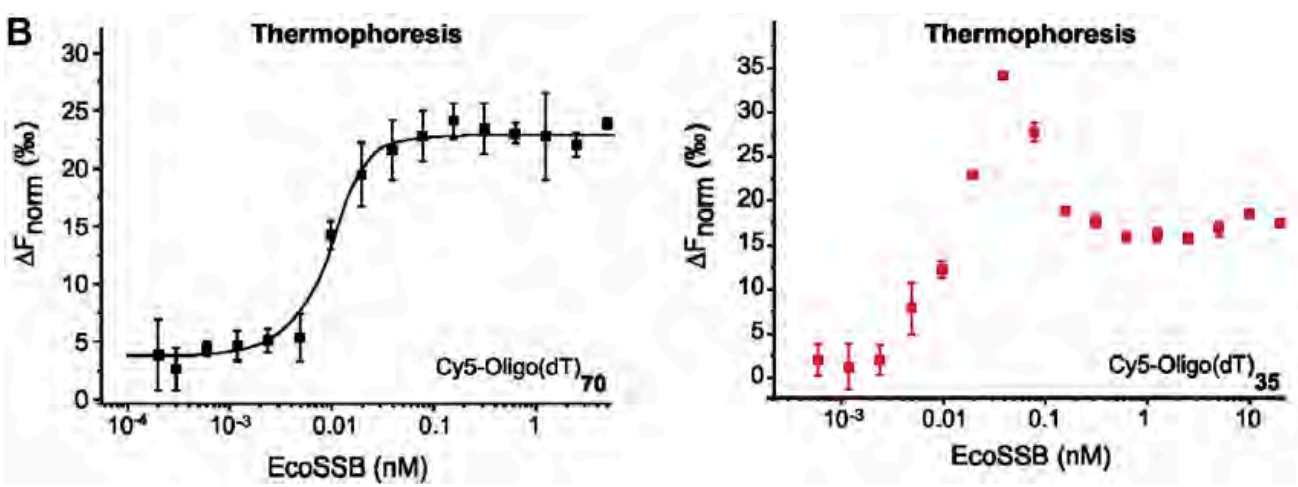


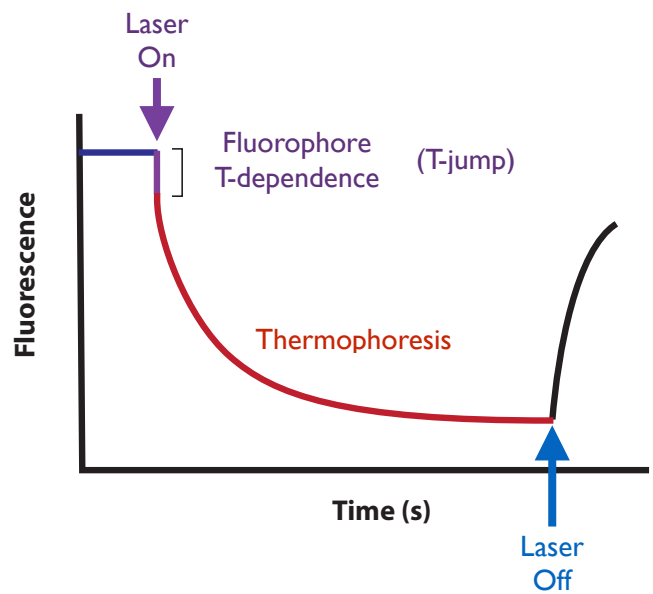
Microscale Thermophoresis

Two separate measures of binding!

SSB: a homotetramer

- *cooperatively* binds dT_{70} oligo



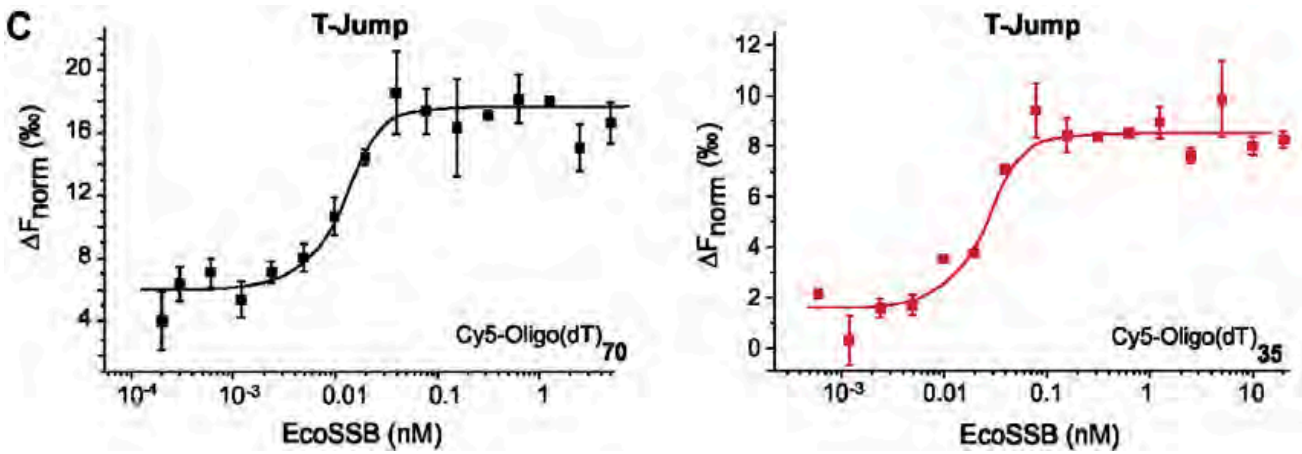


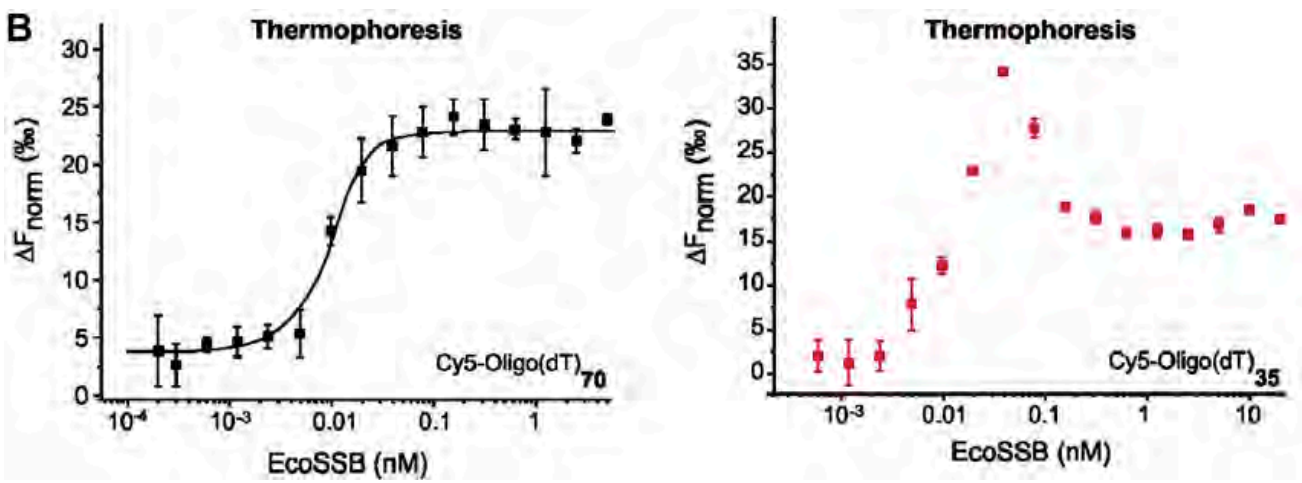
Microscale Thermophoresis

Two separate measures of binding!

SSB: a homotetramer

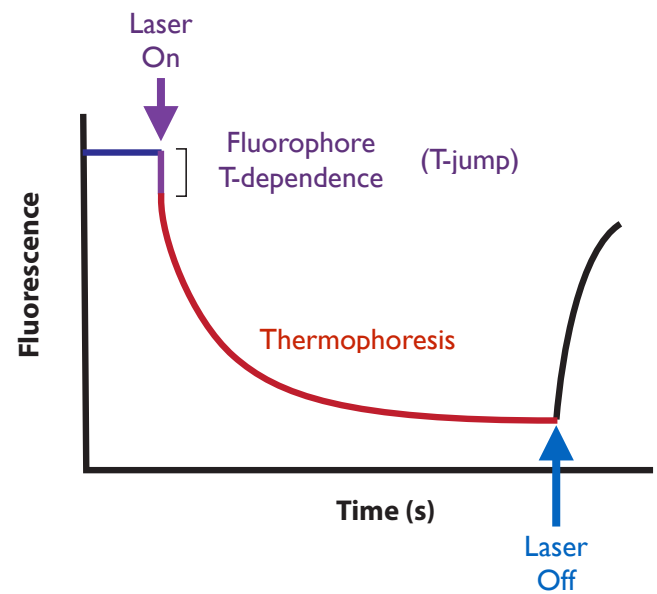
- *cooperatively* binds dT_{70} oligo





Binding of fluorphore-DNA to the protein changes the environment of the fluorophore, imparting a different temperature dependence

Can use this to *separately* assess binding



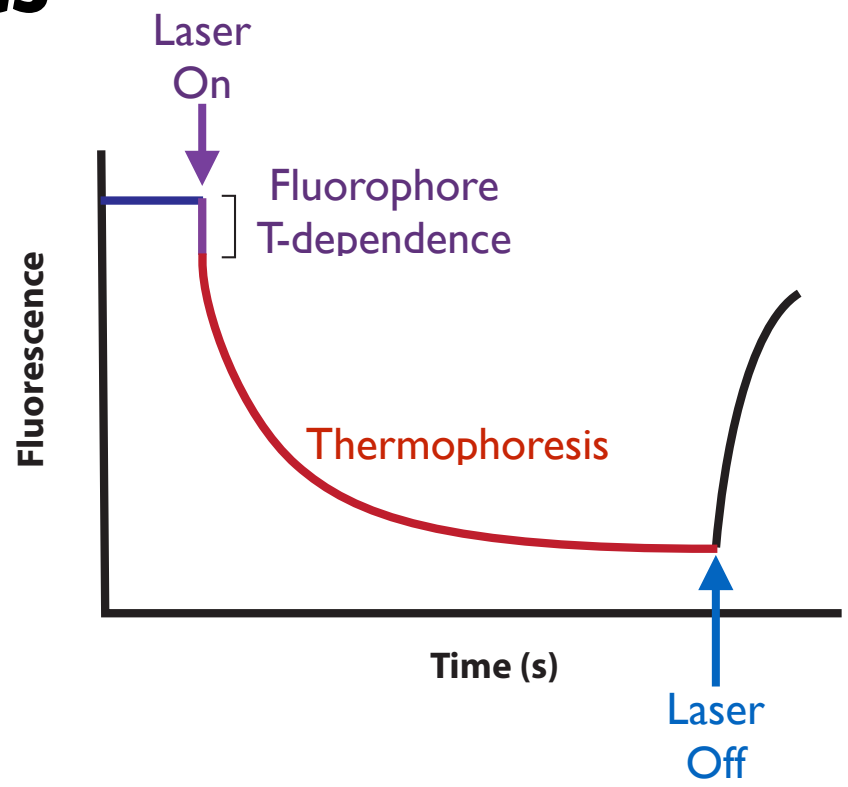
Microscale Thermophoresis

Kinetics

Yes, but…

complications?

What else contributes to kinetics?



Multi-Angle Light Scattering - recap

When laser light impinges on a macromolecule, the oscillating electric field of the light induces an oscillating dipole within it. This oscillating dipole will re-radiate light, much like the antenna for a radio station sends out radio waves. The intensity of the radiated light depends on the magnitude of the dipole induced in the macromolecule. The more polarizable the macromolecule, the larger the induced dipole, and hence, the greater the intensity of the scattered light.

"Molecule" = your favorite molecule

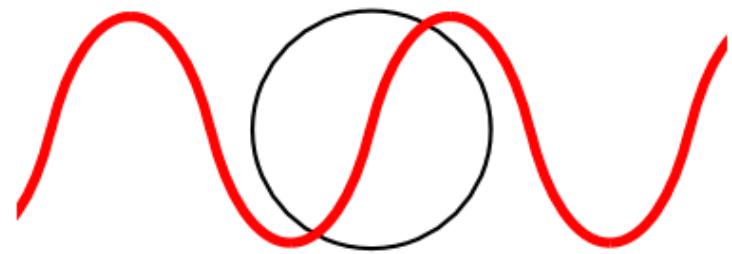
"Molecule" = plus all of the solvent molecules (including water)

Therefore, must know your molecule's polarizability relative to the surrounding medium.

$$\frac{\partial n}{\partial [C]}$$

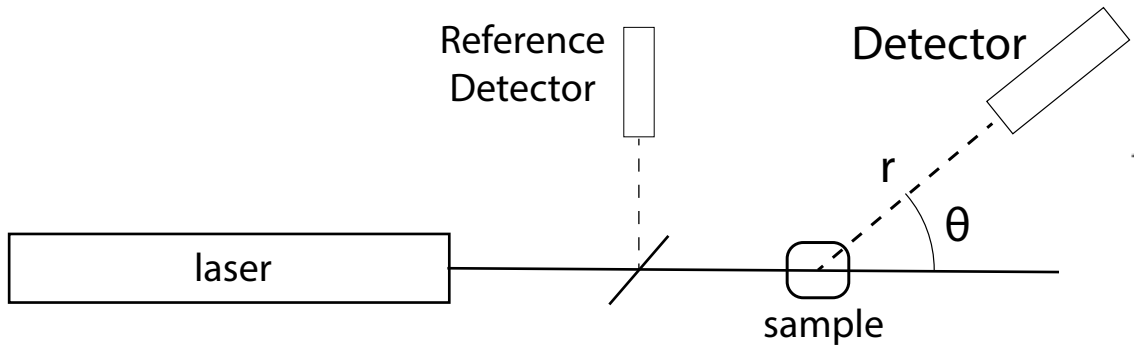
n = refractive index of entire solution

[C] = concentration of your protein



https://www.wyatt.com/library/theory/understanding-multi-angle-static-light-scattering.html

Multi-Angle Light Scattering - recap



$$R(\theta) = \frac{I_{scattered}(\theta)}{I_{laser}} \frac{r^2}{V_{scat}(\theta)}$$

$$\frac{K\,C}{R(\theta)} = \frac{1}{M\,P(\theta)} + 2A_2C$$

$$K = \frac{(2\pi n_o)^2 \left(\frac{dn}{dC}\right)^2}{N_A \lambda^4}$$

M molar mass (molecular weight) of solute (g/mol)

$P(\theta)$ correction factor that considers the SHAPE of the solute

A_2 A_2 (second virial coefficient) is a measure of non-ideality - a measure of the interaction forces between dissolved particles: If A_2 is positive, the interparticle forces are repulsive. If it is negative the forces are attractive.

n_o refractive index of the solvent in the absence of solute

$\frac{dn}{dC}$ change in refractive index of the solvent vs weight concentration of solute
= 0.185 mL/g for proteins (in water)

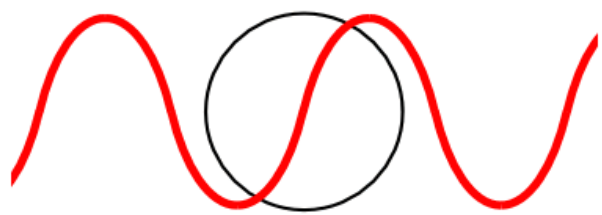
C = weight concentration of solute (g/mL) λ = wavelength of laser light $N_A = 6.022 \cdot 10^{23}\ mol^{-1}$

Multi-Angle Light Scattering - recap

"MALS"

When there are many macromolecules in solution, each macromolecule scatters light via the aforementioned induced dipole mechanism. Hence, the intensity of the scattered light is proportional to the concentration of the macromolecules in solution; twice as many molecules scatter twice as much light.

The intensity of light scattered by a molecule, measured by means of a DAWN HELEOS II multi-angle light scattering (MALS) detector, is directly proportional to the molar mass.

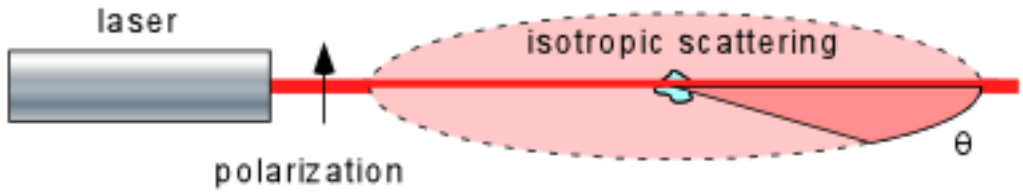


https://www.wyatt.com/library/theory/understanding-multi-angle-static-light-scattering.html

Multi-Angle Light Scattering - recap

MALS - Multi-Angle Light Scattering

Macromolecules much smaller than the wavelength of the incident light can be treated as though they were essentially point scatterers. For such very small molecules, the light scattered into the perpendicular plane is independent of scattering angle. It is the same at every scattering angle – the macromolecule scatters light isotropically.



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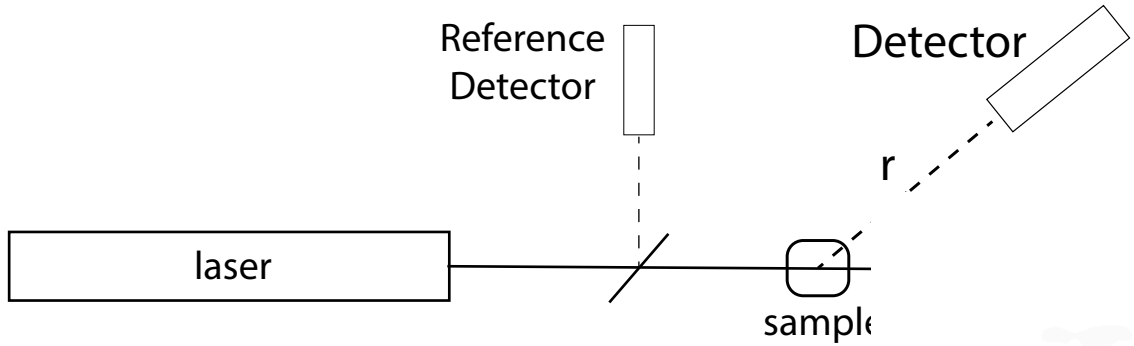
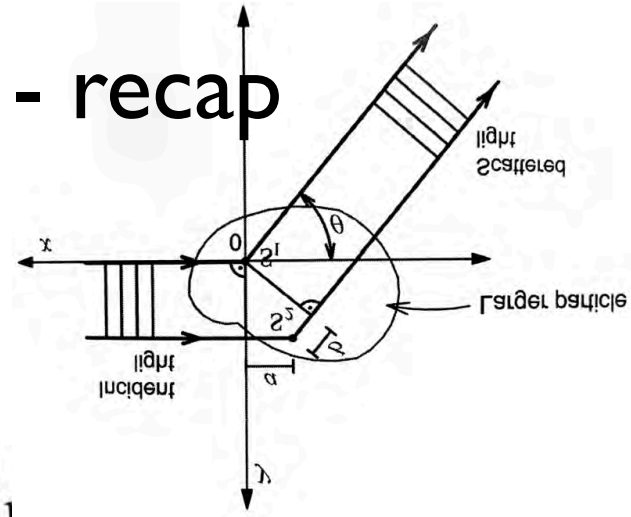
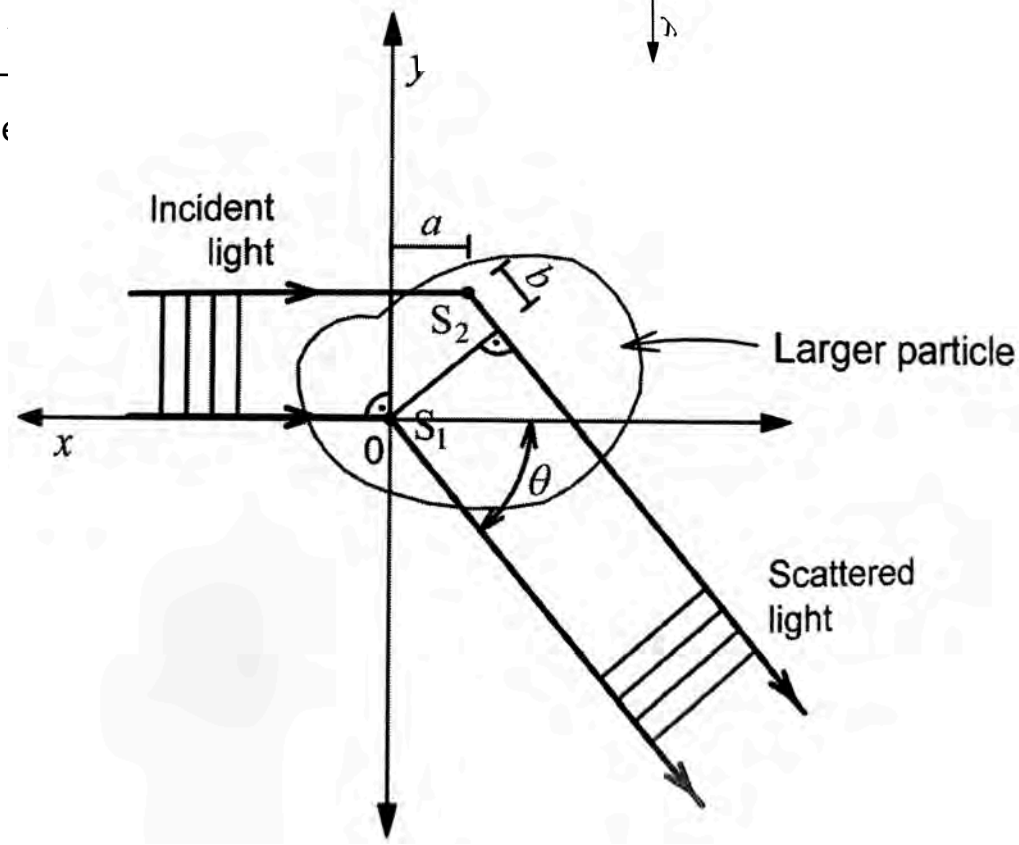
Multi-Angle Light Scattering - recap







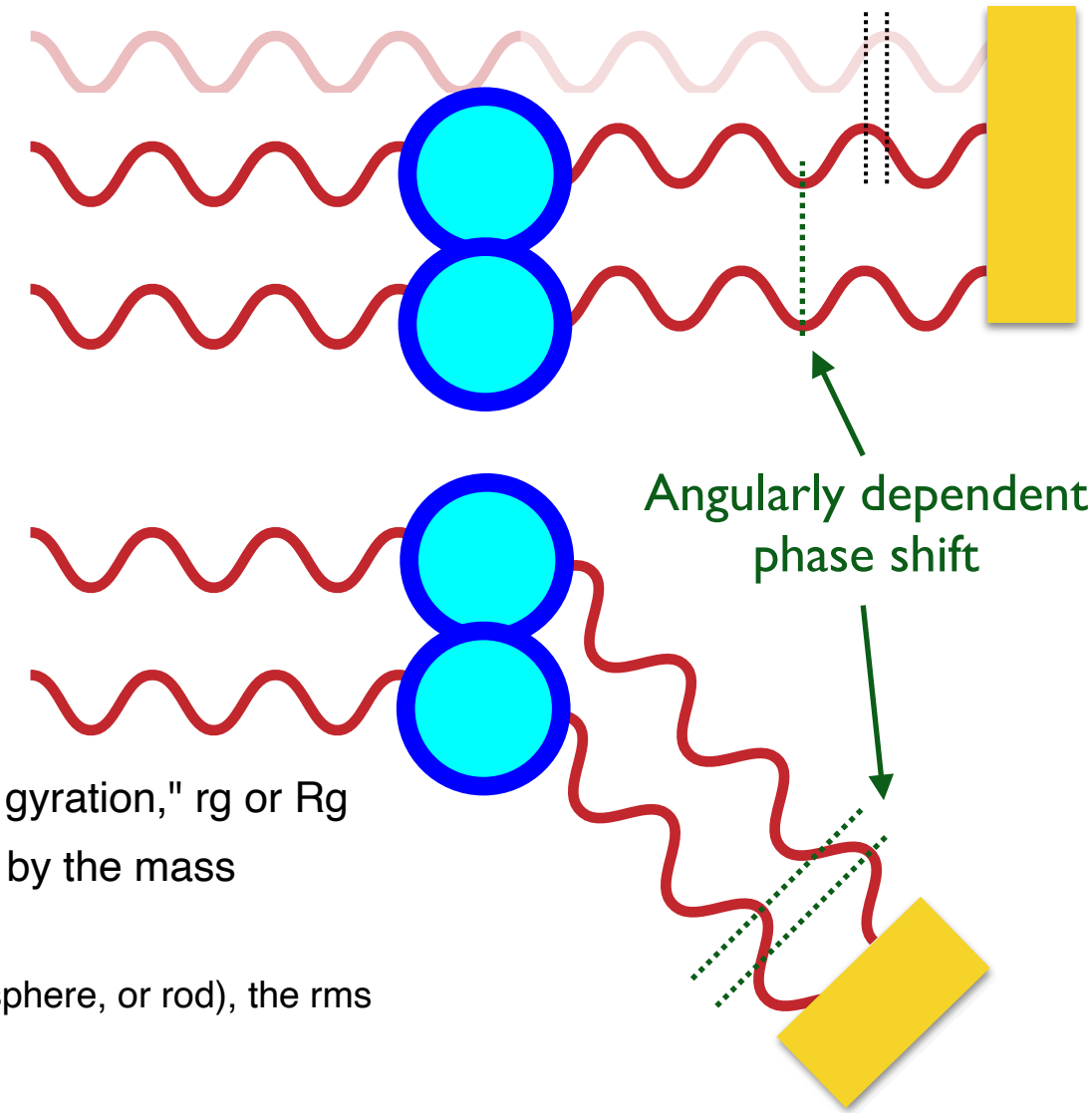
EM of the incident light induces an oscillation in the electron cloud, with the same frequency as the incident light

The oscillating electron cloud (dipole) then generates EM (of the same frequency)

SEC-MALS

MALS - Multi-Angle Light Scattering

Refractive index



Light scattered from different parts of the macromolecule reach the detector with different phases, leading to the destructive or constructive interference.

The net light intensity is reduced relative to light scattered in the forward direction, varying with angle.

The angular dependence of the scattered light reflects the (geometrical) size of the molecule

- root mean square (rms) radius, aka "radius of gyration," rg or Rg
  - a measure of the molecule's size weighted by the mass distribution about its center of mass.

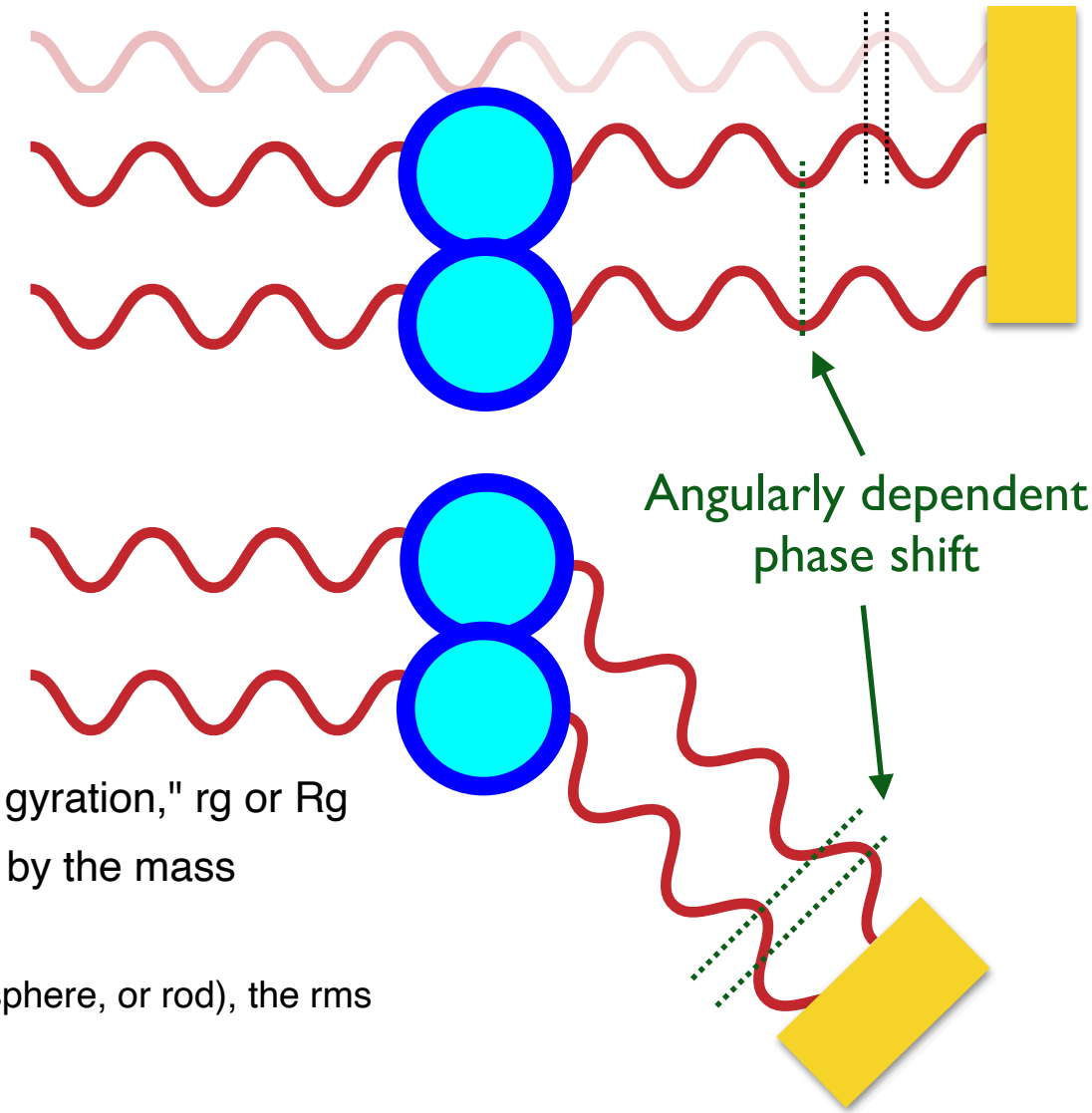
Assuming a specific conformation (e.g., random coil, sphere, or rod), the rms radius can be related to its geometrical dimensions.

https://www.wyatt.com/library/theory/understanding-multi-angle-static-light-scattering.html

SEC-MALS

MALS - Multi-Angle Light Scattering

Refractive index



Light scattered from different parts of the macromolecule reach the detector with different phases, leading to the destructive or con

The
light ying
with

The light
refl cule

But assumes a uniform solution (one protein type)

- root mean square (rms) radius, aka "radius of gyration," rg or Rg
  - a measure of the molecule's size weighted by the mass distribution about its center of mass.

Assuming a specific conformation (e.g., random coil, sphere, or rod), the rms radius can be related to its geometrical dimensions.

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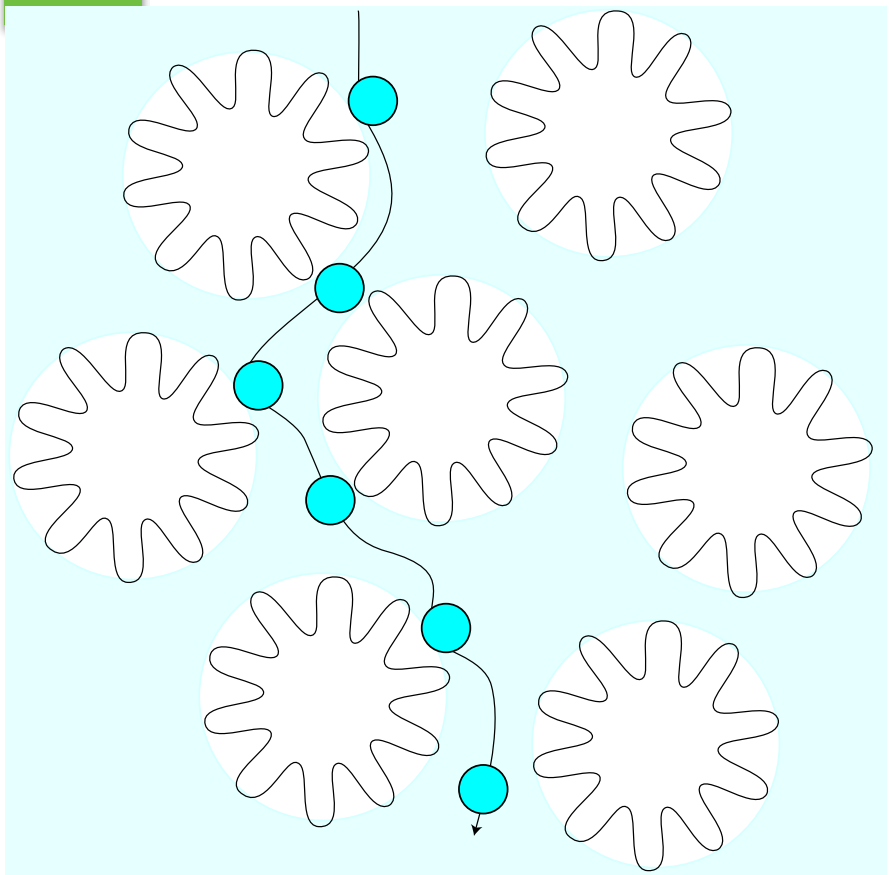
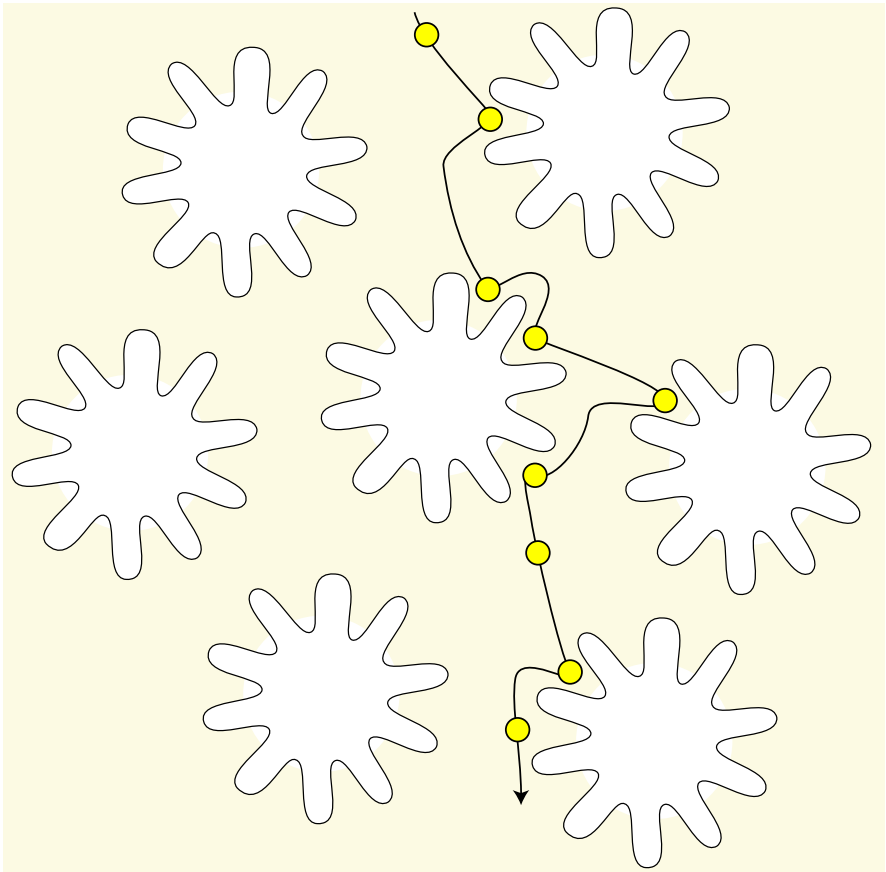
SEC-MALS

SEC - Size Exclusion Chromatography

HPLC



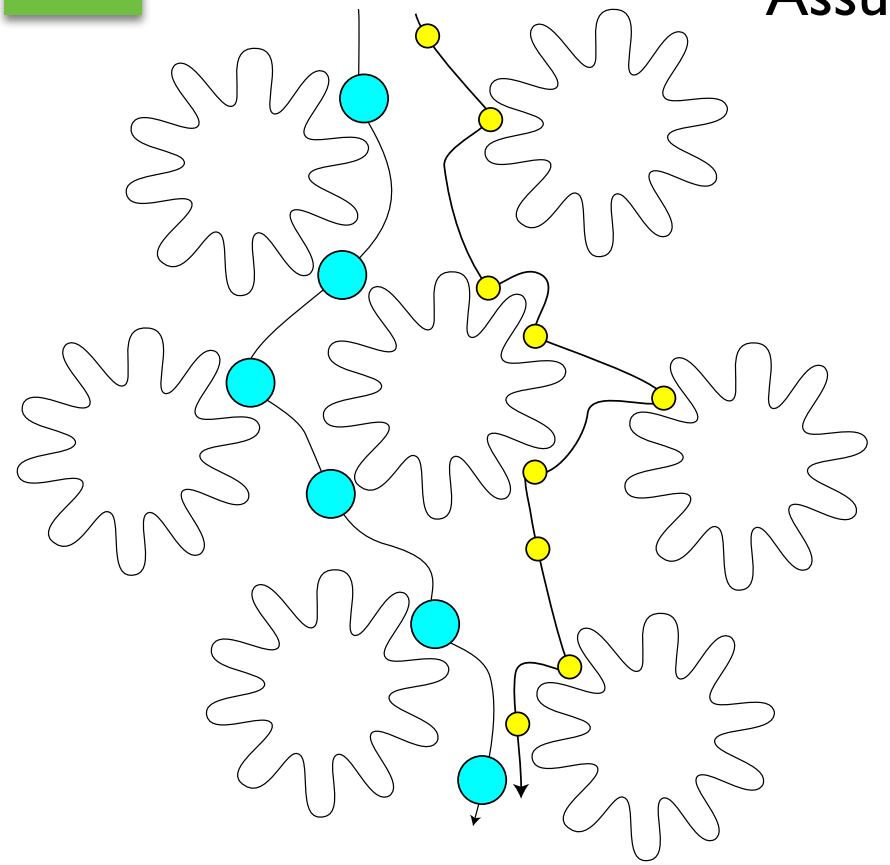


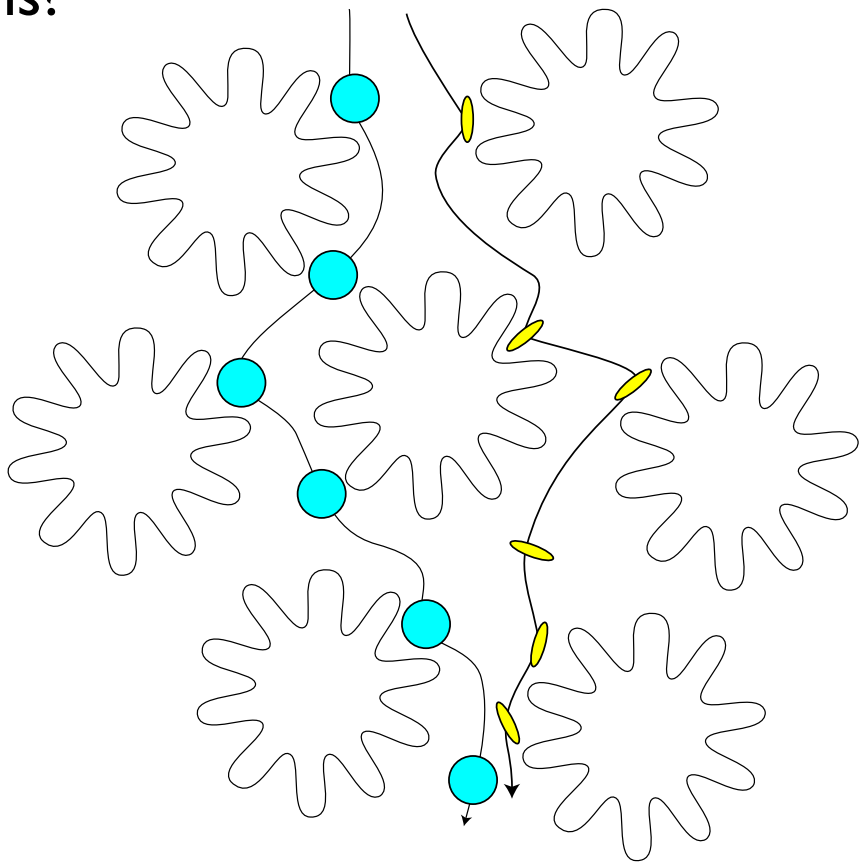
SEC-MALS

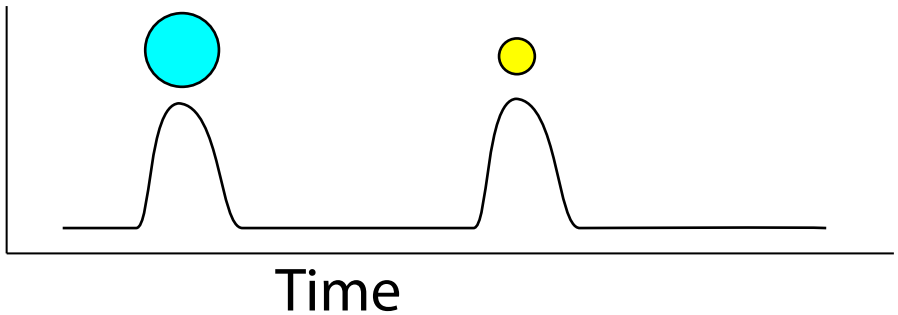
SEC - Size Exclusion Chromatography

HPLC

Assumptions?





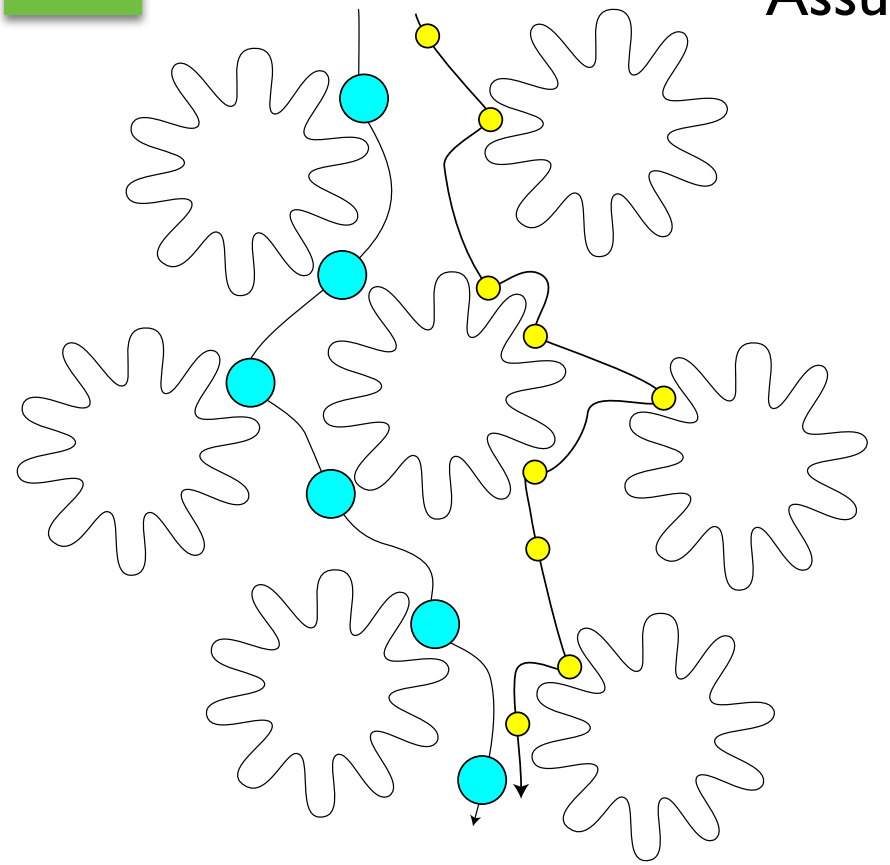


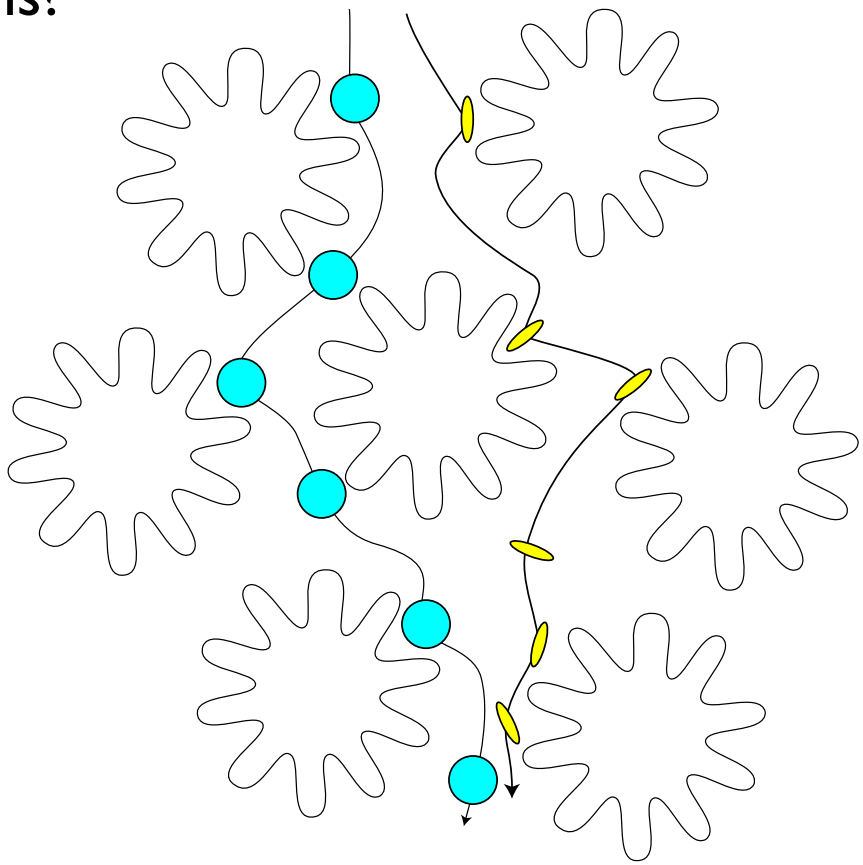
SEC-MALS

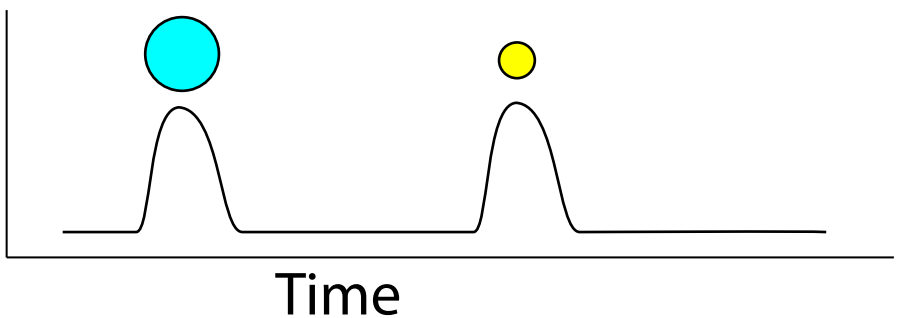
SEC - Size Exclusion Chromatography

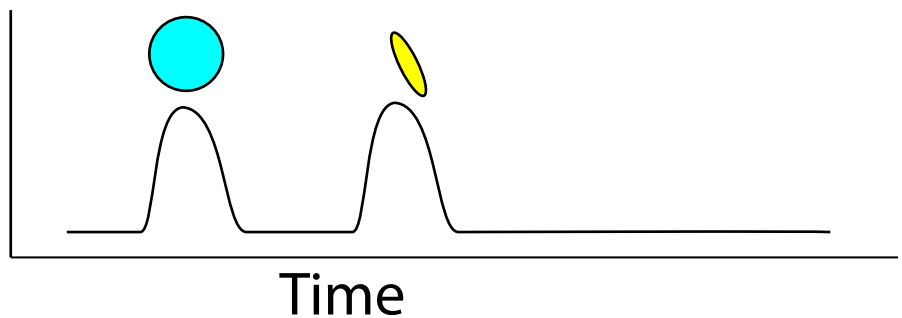
HPLC

Assumptions?





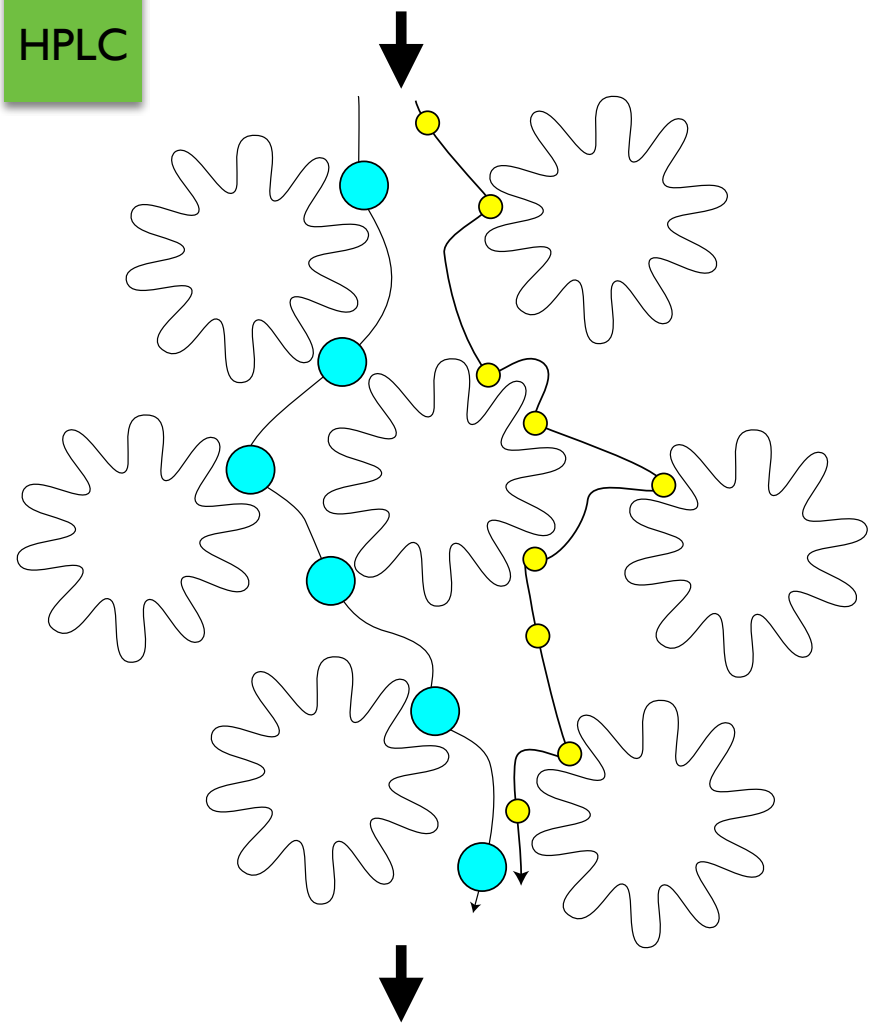


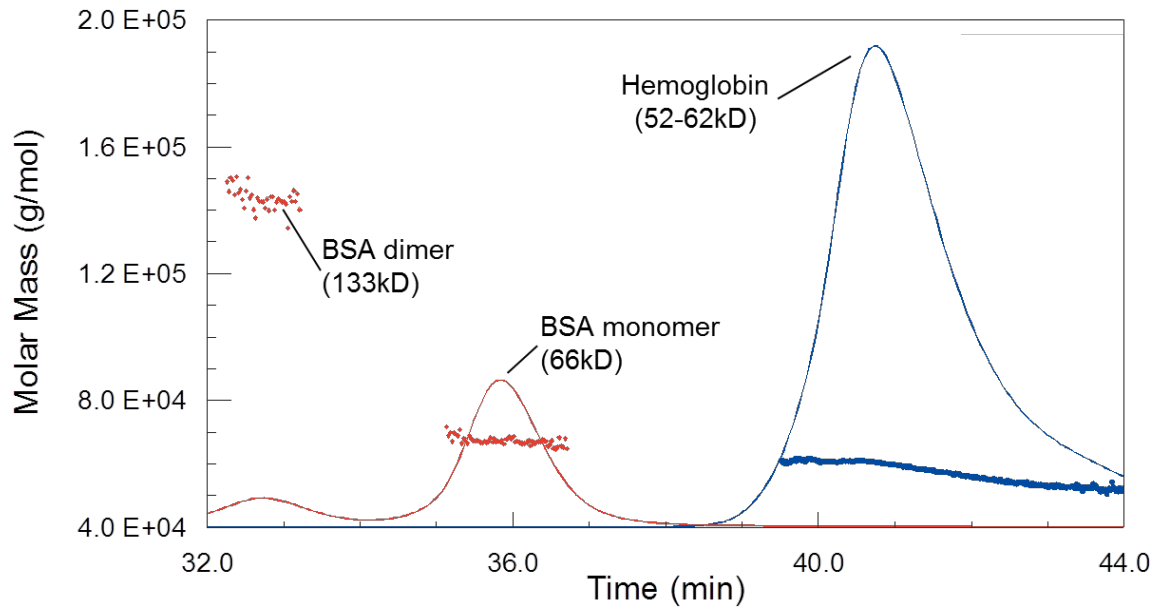


SEC-MALS

SEC - Size Exclusion Chromatography

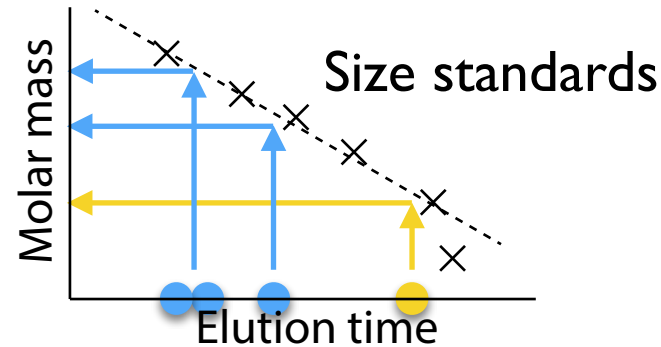
HPLC





Larger Proteins

Smaller Proteins



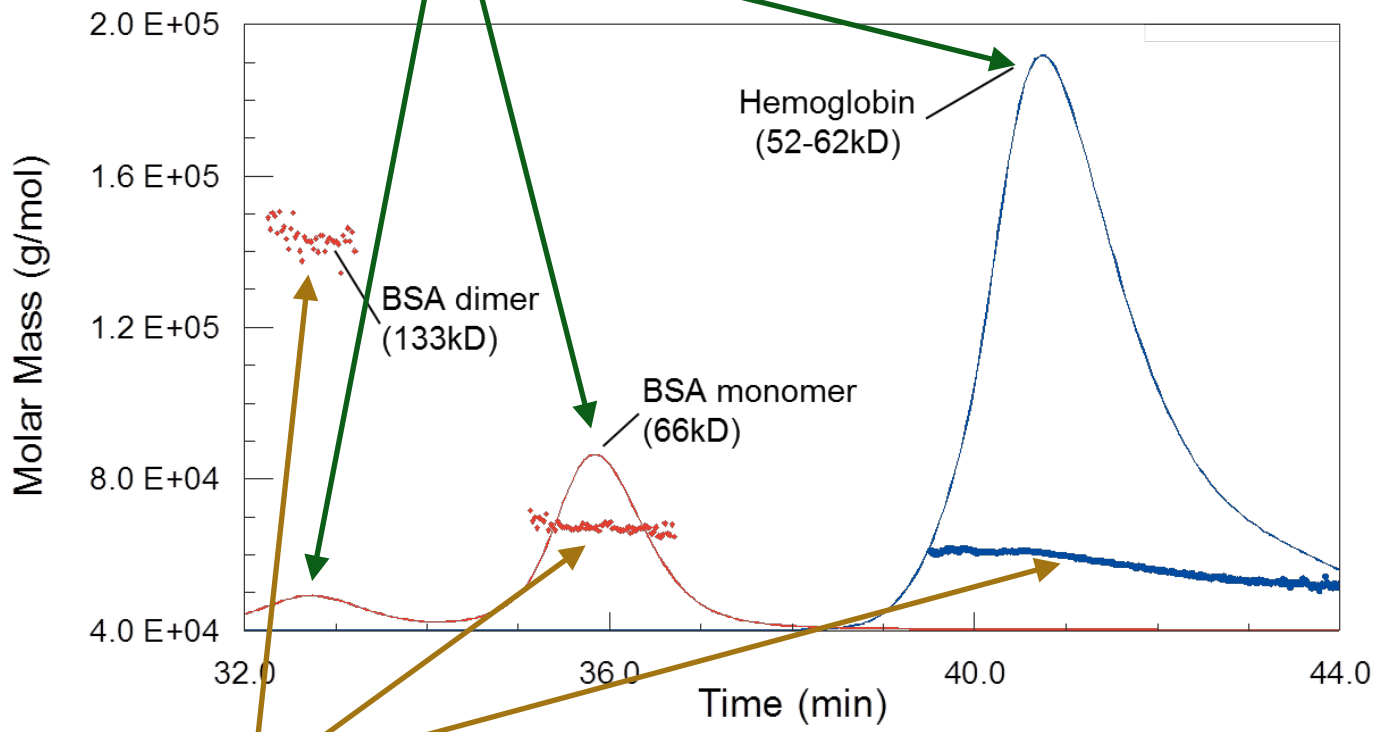
Concerns

- choose proper column (G25, G50…)
- ensure protein does not "stick"
- assumes globular (or…) protein

SEC-MALS

HPLC

SEC - Size Exclusion Chromatography



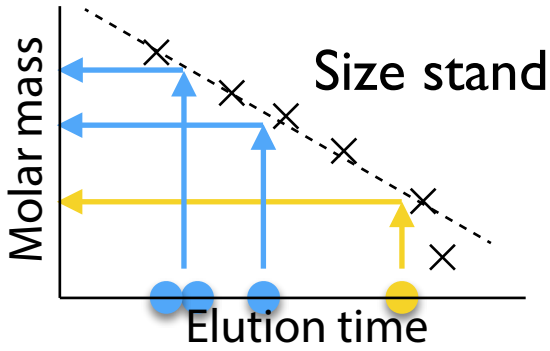
MALS - Multi-Angle Light Scattering

A sample containing a broad distribution of molecular masses may be separated by SEC or GPC, and light scattering data acquired at each elution volume to determine molar mass Mw and rg. The measured rms radius may be plotted against the correspondingly measured molar mass to determine the sample's conformation.

SEC-MALS *at UMass*

HPLC

SEC - Size Exclusion Chromatography



…bring your own column

MALS - Multi-Angle Light Scattering

Wyatt Dawn Heleos II

Determine molar masses from 200 Da to 1GDa and radii from 10-500 nm
Temperature control -15°C to 150°C

dRI - Differential Refractive Index Detector

Wyatt Optilab T-rEX

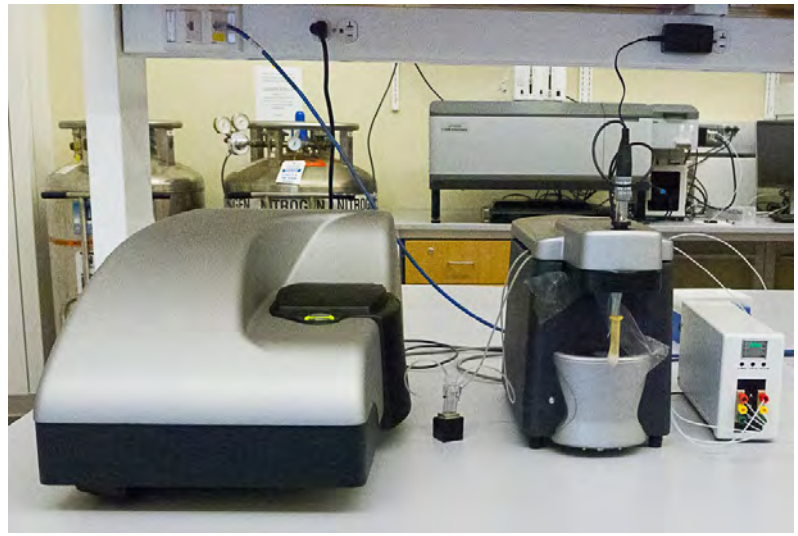
Measures protein concentration by refractive index
Measures solvent absolute refractive index (required for MALS analysis)
Temperature control 4°C to 65°C

Malvern Zetasizer ZSP - dynamic light scattering (DLS)

- Dynamic Light Scattering is used to measure particle and molecule size. DLS measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship.

- Measurement of size as a function of concentration enables the calculation of kD, the DLS interaction parameter.

- Laser Doppler Micro-electrophoresis is used to measure zeta potential. An electric field is applied to a solution of molecules or a dispersion of particles, which then move with a velocity related to their zeta potential. This velocity is measured using a patented laser interferometric technique called M3-PALS (Phase analysis Light Scattering). This enables the calculation of electrophoretic mobility and from this the zeta potential and zeta potential distribution.This technique is very demanding on the sensitivity and stability of the whole system, and requires that every element of the design is optimized to ensure accuracy and repeatability.



- Static Light Scattering is used to determine the molecular weight of proteins and polymers. The scattering intensity of a number of concentrations of the sample is measured, and used to construct a Debye plot. From this the weight average molecular weight and second virial coefficient can be calculated, which provides a measure of protein solubility.

~~Static~~ Dynamic Light Scattering

Malvern Zetasizer ZSP - dynamic light scattering (DLS) system

Dynamic Light Scattering is used to measure particle and molecule size. DLS measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship.

Measurement of size as a function of concentration enables the calculation of kD, the DLS interaction parameter.

The Microrheology option uses the DLS measurement of tracer particles to probe the structure of dilute polymer and protein solutions.

Laser Doppler Micro-electrophoresis is used to measure zeta potential. An electric field is applied to a solution of molecules or a dispersion of particles, which then move with a velocity related to their zeta potential. This velocity is measured using a patented laser interferometric technique called M3-PALS (Phase analysis Light Scattering). This enables the calculation of electrophoretic mobility and from this the zeta potential and zeta potential distribution.

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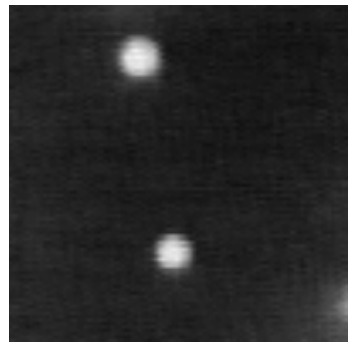
Dynamic Light Scattering measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship.



As light scatters from the moving macromolecules, this motion imparts a randomness to the phase of the scattered light, such that when the scattered light from two or more particles is added together, there will be a changing destructive or constructive interference. This leads to time-dependent fluctuations in the intensity of the scattered light.

Time-dependent fluctuations in the scattered light are measured by a *fast photon counter*. The fluctuations are directly related to the rate of diffusion of the molecule through the solvent, which is related in turn to the particles' hydrodynamic radii

Stokes-Einstein

$$r_h = \frac{kT}{6\pi\eta D}$$

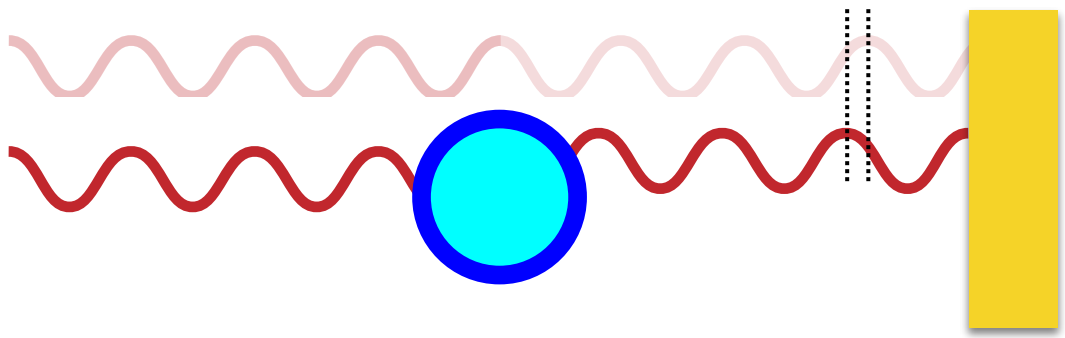
radius hydrodynamic — r_h

k — *Boltzmann constant*

T — *temperature*

η — *solvent viscosity*

D — *diffusion constant*



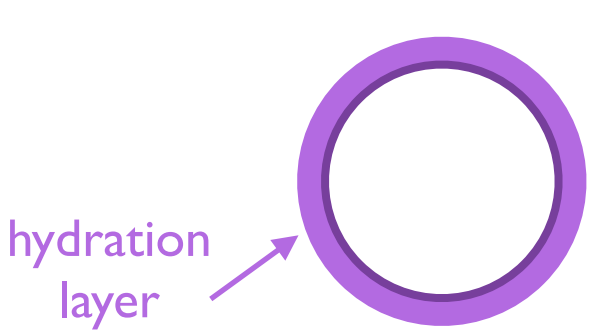
~~Static~~ Dynamic Light Scattering

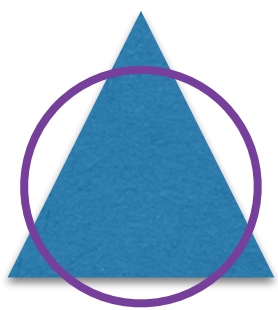
Malvern Zetasizer ZSP - dynamic light scattering (DLS) system

Hydrodynamic radius

bigger than the actual molecule

assumes everything is a sphere





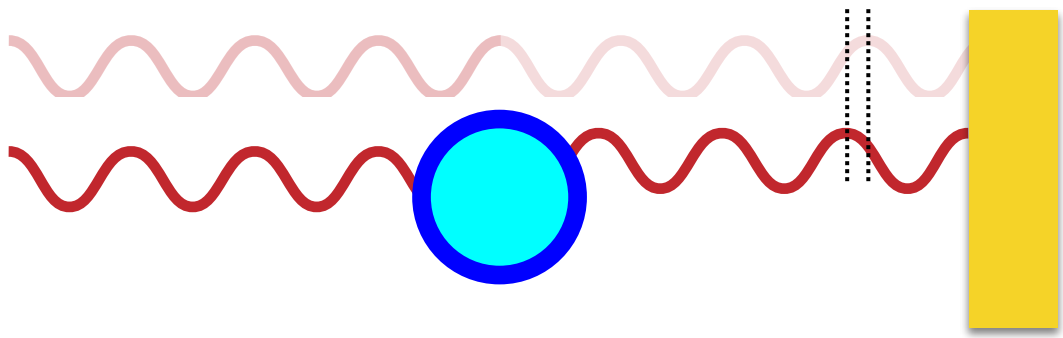
Stokes-Einstein

$$r_h = \frac{kT}{6\pi\eta D}$$

r_h: radius hydrodynamic

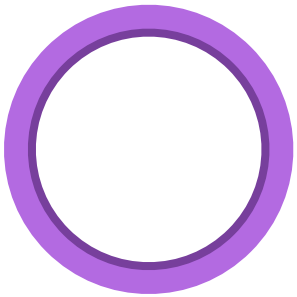
η: solvent viscosity

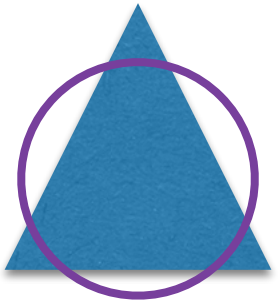
D: diffusion constant



~~Static~~ Dynamic Light Scattering

Malvern Zetasizer ZSP - dynamic light scattering (DLS) system





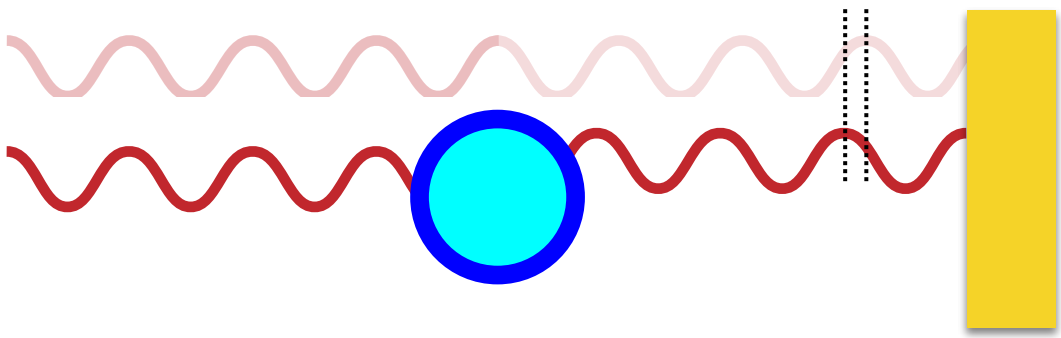
Assumes

- Brownian motion
  - ★ non-interacting billiard balls
- Spherical scatterers
- Proper calibration for viscosity, etc
- Properly dilute solution
- No interference from other scatterers

Stokes-Einstein

$$r_h = \frac{kT}{6\pi\eta D}$$

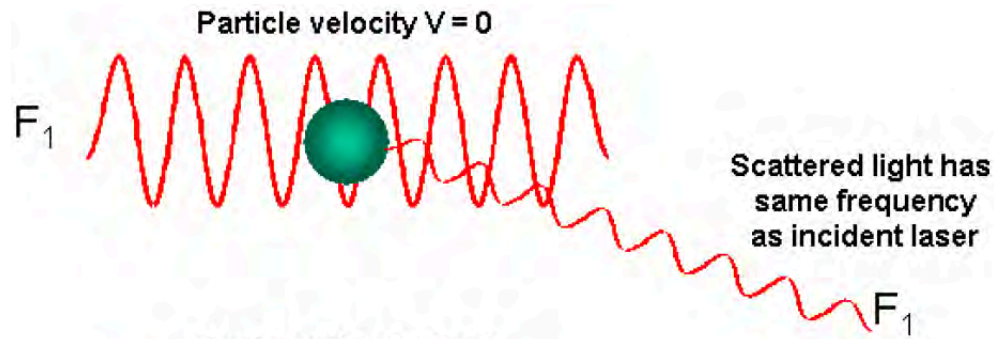
radius hydrodynamic — *solvent viscosity* — *diffusion constant*



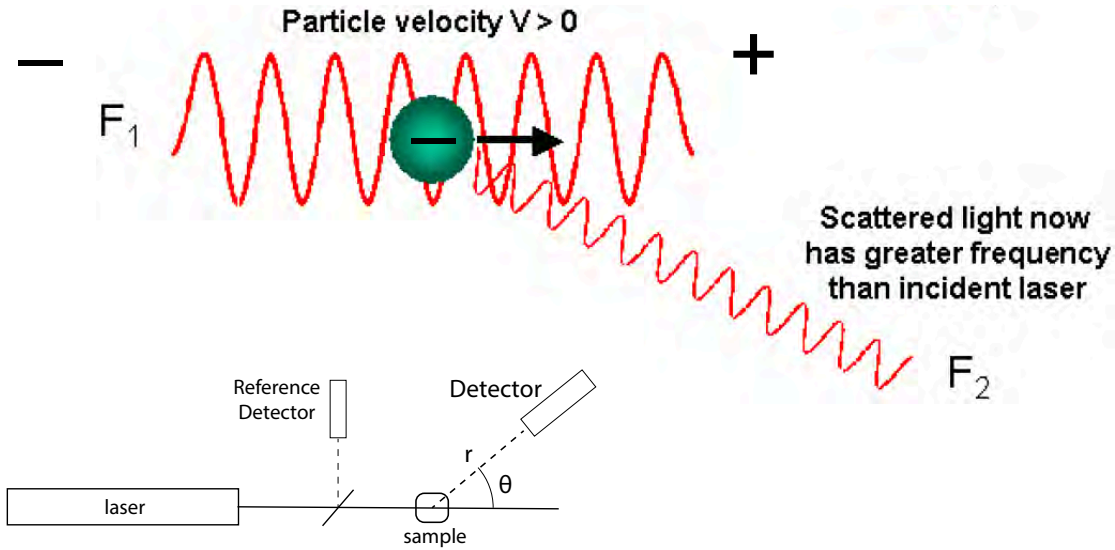
Electrophoretic Light Scattering

Malvern Zetasizer ZSP - dynamic light scattering (DLS) system

aka Laser-Doppler Electrophoresis



actually, this approach uses an alternating pulsed field



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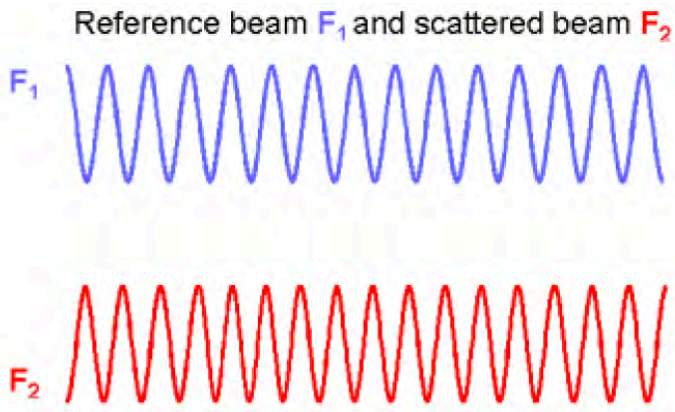
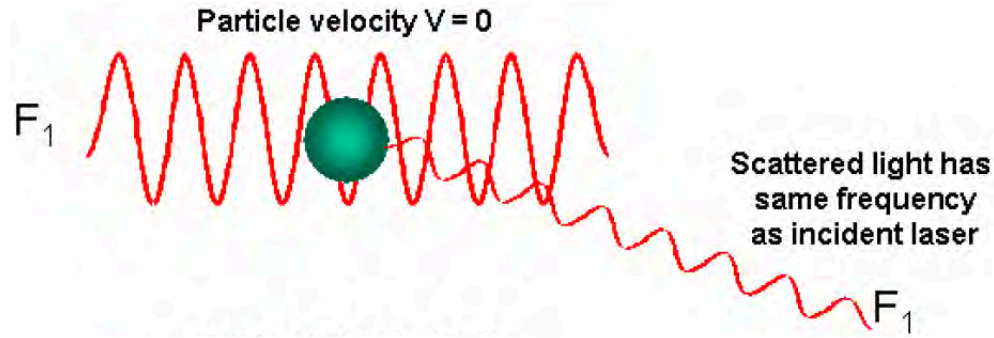
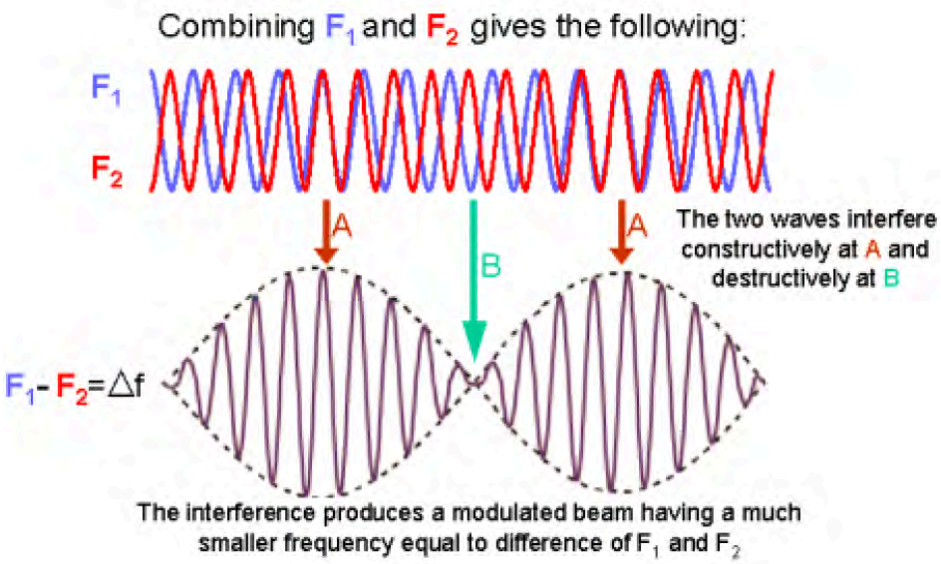
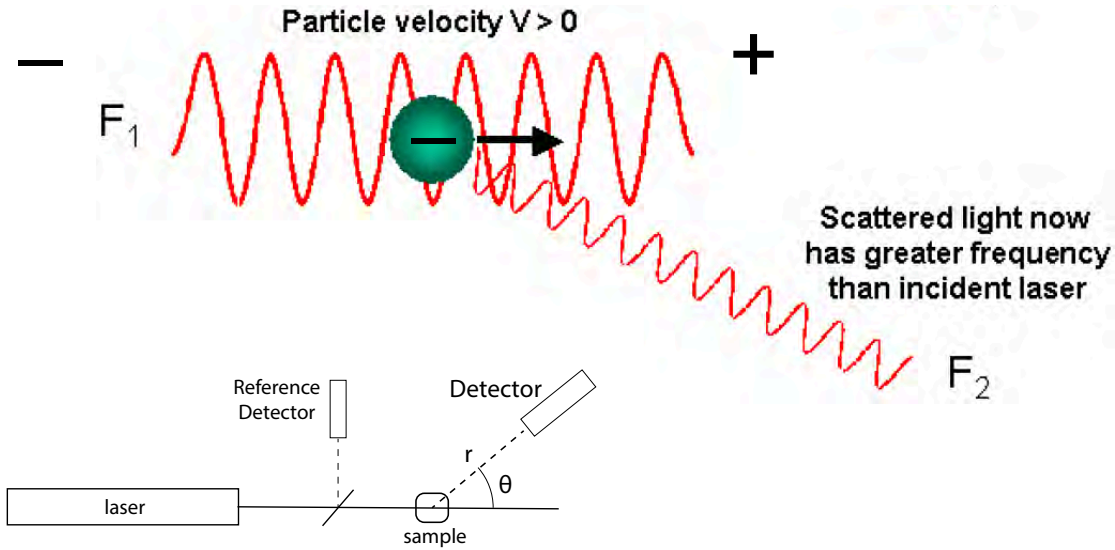




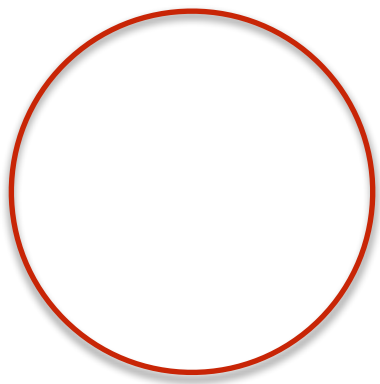
actually, this approach uses an alternating pulsed field





Zeta potential

6 Lys, 5 Arg, 2 His, 6 Glu, 7 Asp



But 1 Glu is internal and protonated

2 Asp are internally salt bridged with 2 Lys

1 His is protonated, on the surface

There's a bound sulfate, forming a salt bridge with a Arg

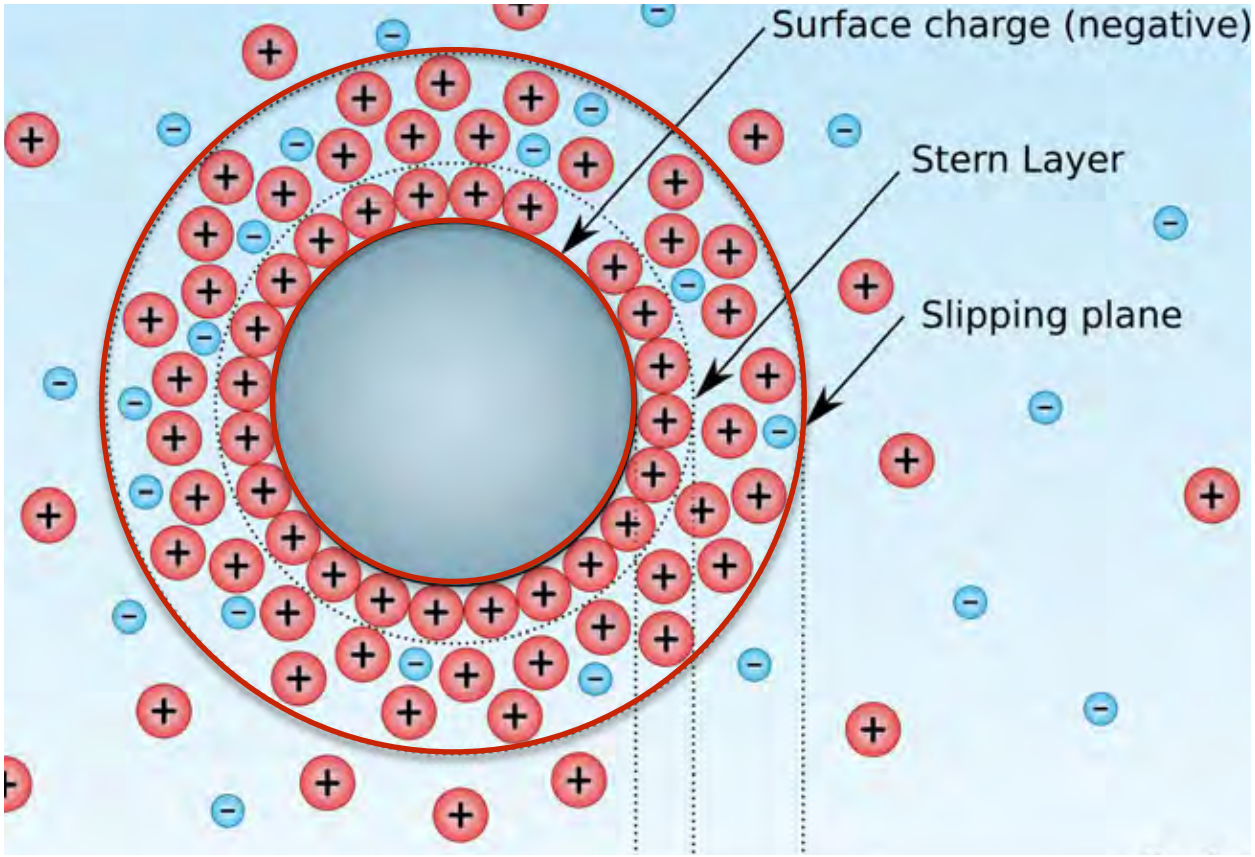
Everything else is on the surface

OK, now what's the net charge of this protein?

Zeta potential

Highly dependent on

- pH
- ions in solution (concentration and identities)
- bound ligands
- conformational changes
- just about everything…



6 Lys, 5 Arg, 2 His, 6 Glu, 7 Asp

But 1 Glu is internal and protonated

2 Asp are internally salt bridged with 2 Lys

1 His is protonated, on the surface

There's a bound sulfate, forming a salt bridge with a Arg

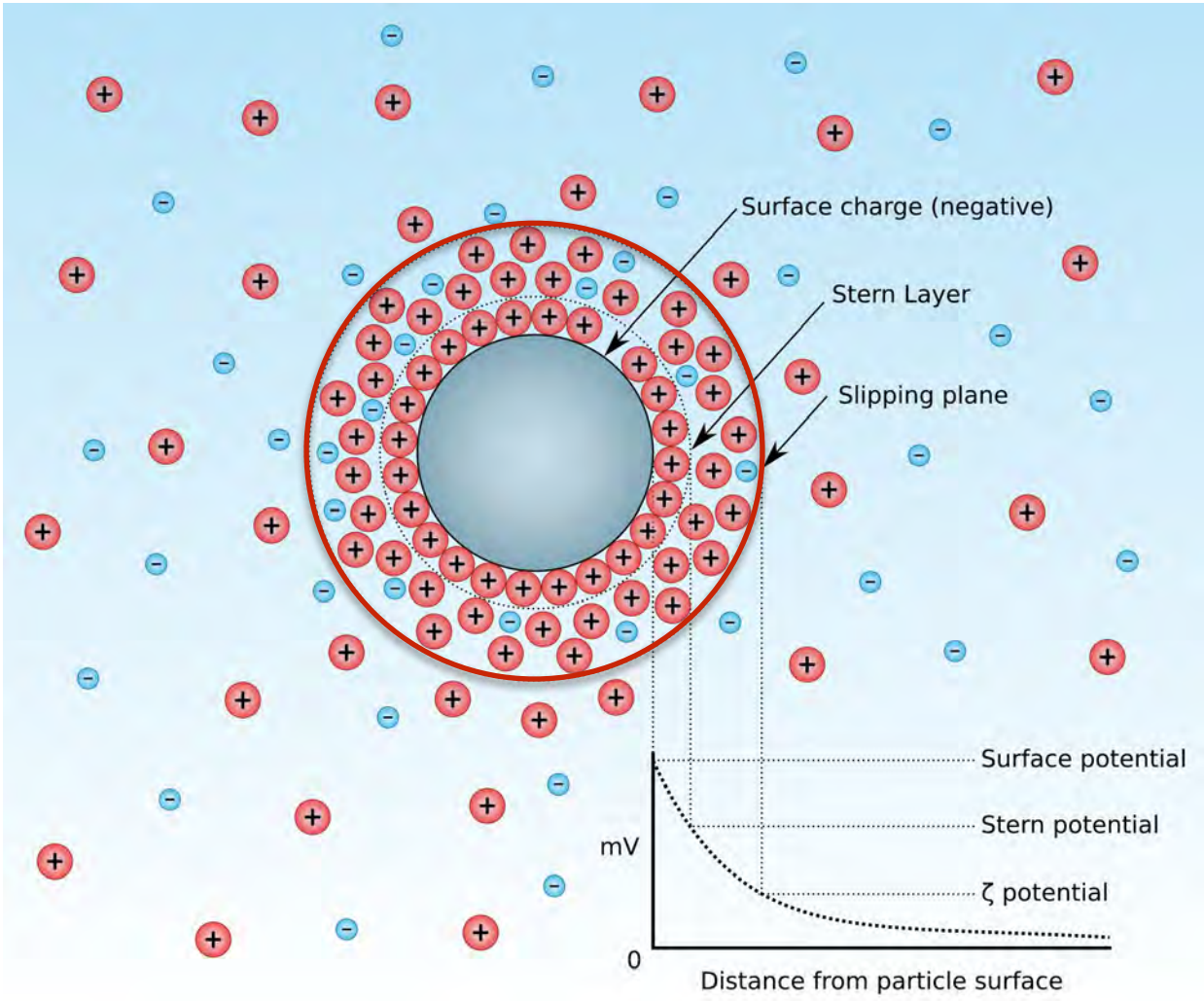
Everything else is on the surface

But ions in solution are tightly bound to some of the surface anions/ cations.

Some more than others…

OK, now what's the net charge of the hydrated protein?

Zeta potential



Surface charge density at the slipping (or shear) plane.

The magnitude of the zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in a dispersion.

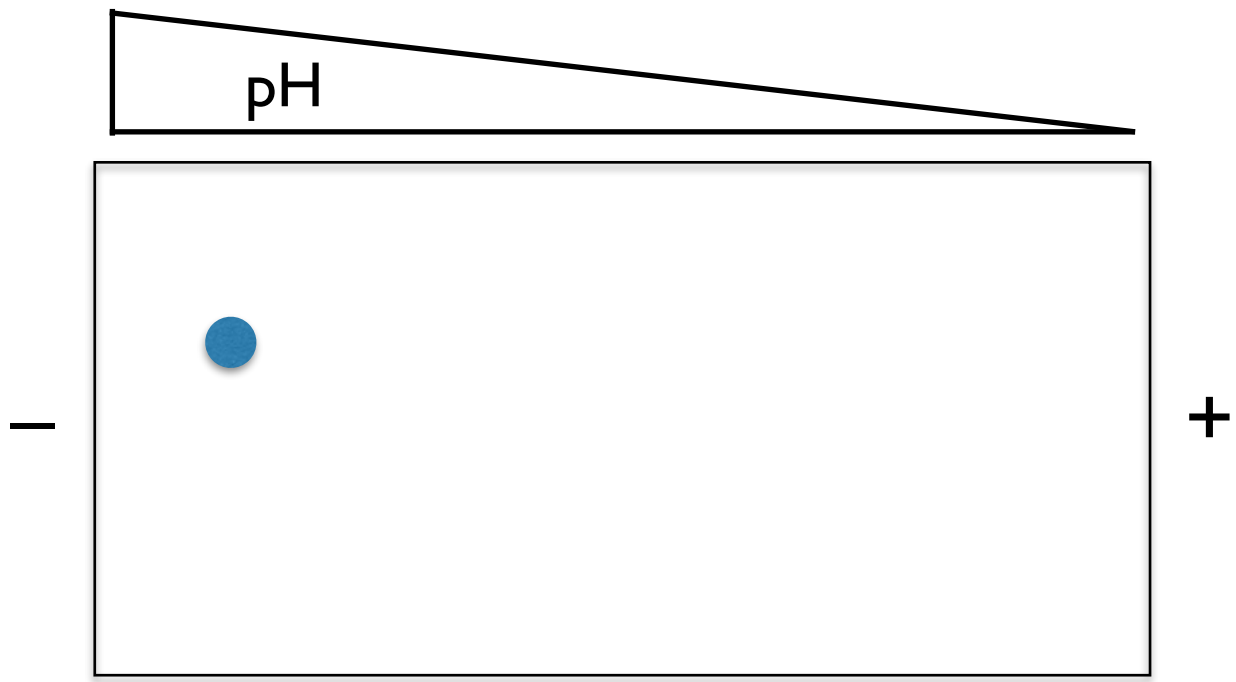
For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation.

When the potential is small, attractive forces may exceed this repulsion and the dispersion may break and flocculate.

Zeta potential

Isoelectric Point

(Isoelectric focusing)



Zeta potential

Isoelectric Point

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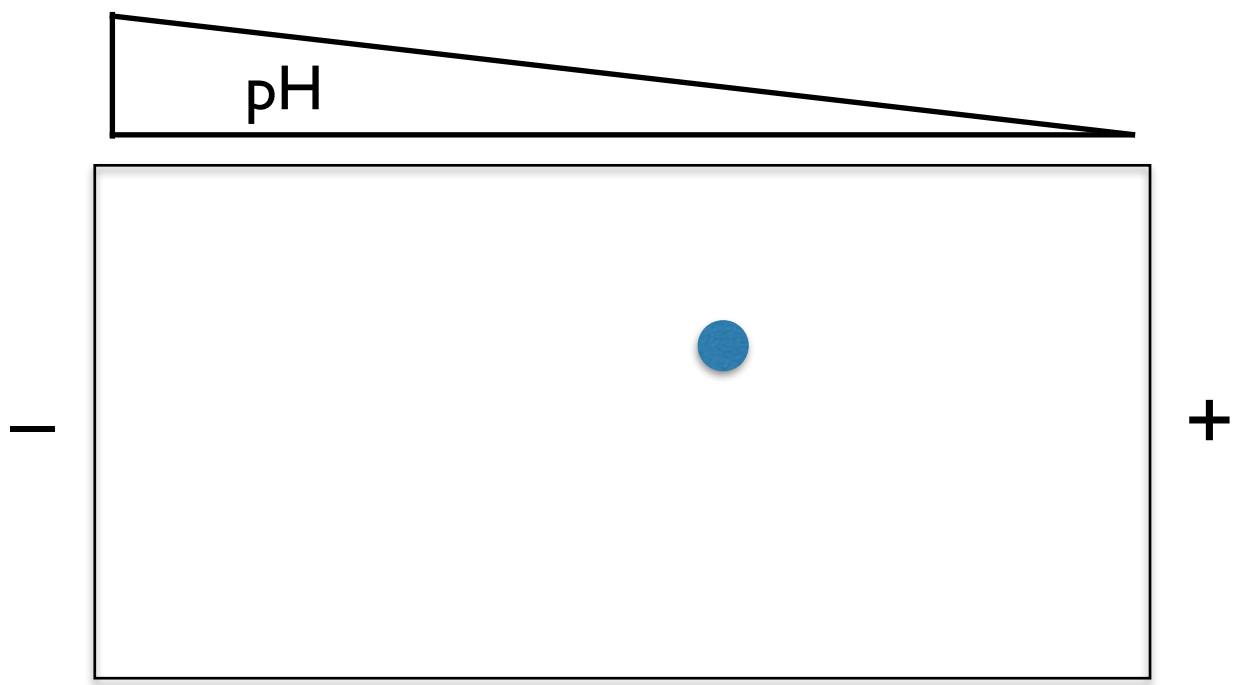

Zeta potential

Isoelectric Point

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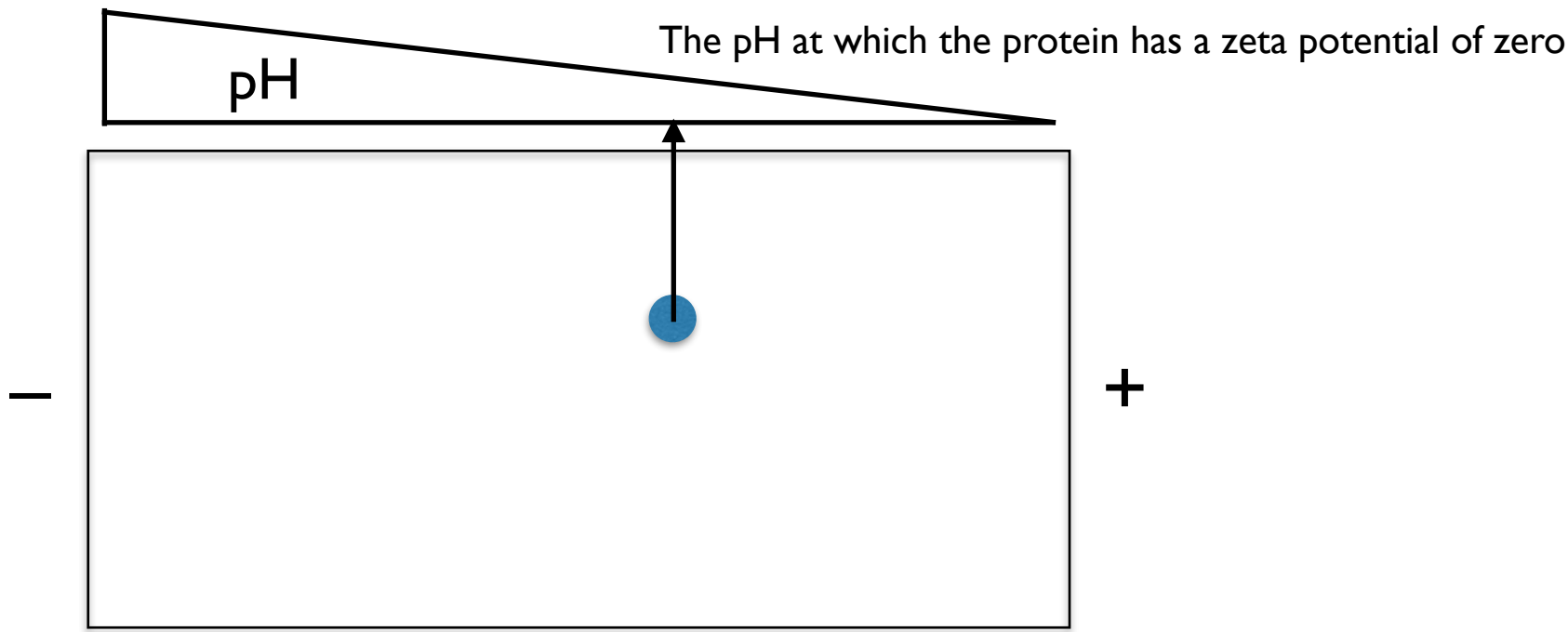


Odyssey LI-COR

Next generation imaging

- stable near-infrared fluorescent signals and over 6 logs of linear dynamic range
- capture images with exactly the same settings every time with the AutoScan feature, for the most consistent and reproducible results.
- analyze up to nine miniblots or six microplates in a single scan with a large imaging area.

near-IR fluorescence

- near-IR stains, labels, etc (700-800 nm)

BioTek Plate Reader

fluorescence plate reader

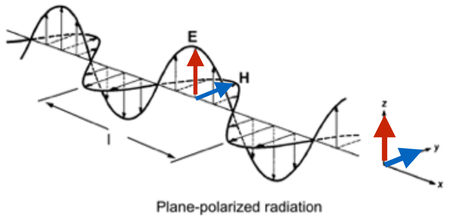
- fluorescence detection w deep blocking filters and dichroic mirrors for the best level of performance
- dedicated absorbance detection system is monochromator-based
- dedicated optical elements for each individual detection technique
- detection modes include
  - Fluorescence Intensity
  - Fluorescence Polarization
  - Time-Resolved Fluorescence
  - AlphaScreen
  - Luminescence
  - UV-visible Absorbance
- three broad-spectrum light sources have been chosen for optimal illumination and excitation in all applications.

MicroCal Auto-iTC200

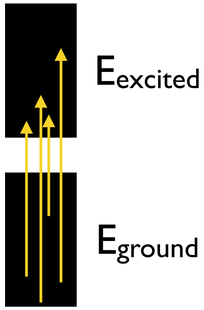
automated titration microcalorimeter

- fully automated, low volume, highly sensitive isothermal titration calorimeter
- delivers direct, label-free in solution measurement of all binding parameters in a single experiment
- applications include characterizing molecular interactions of small molecules, proteins, antibodies, nucleic acids, lipids and other biomolecules
- can also be used to measure enzyme kinetics.

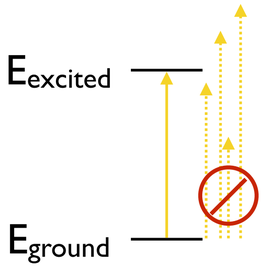
Chem 728 Recap



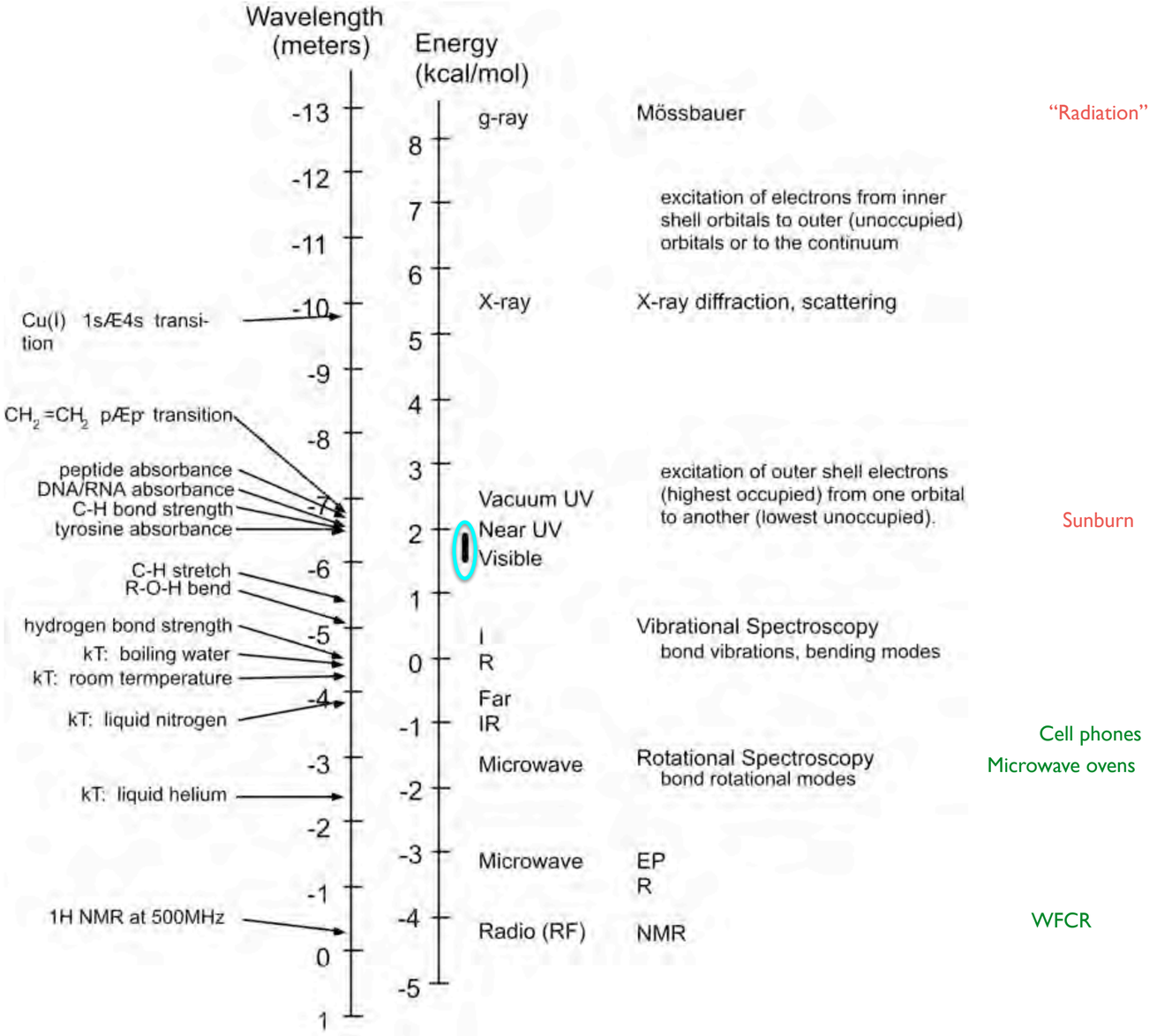
Plane-polarized radiation



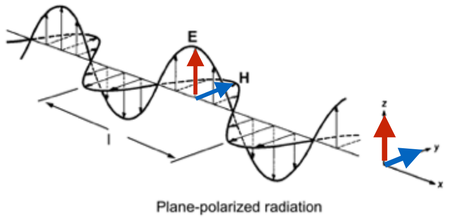
Classical: electrons can have any energy



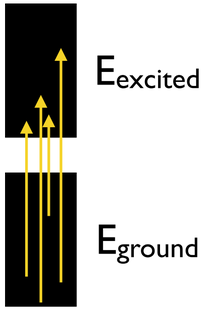
QM: electrons can have only discrete (quantized) energies



Chem 728 Recap



Plane-polarized radiation



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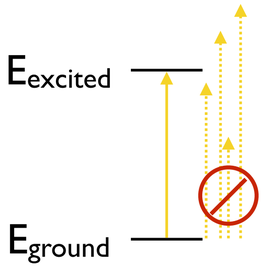
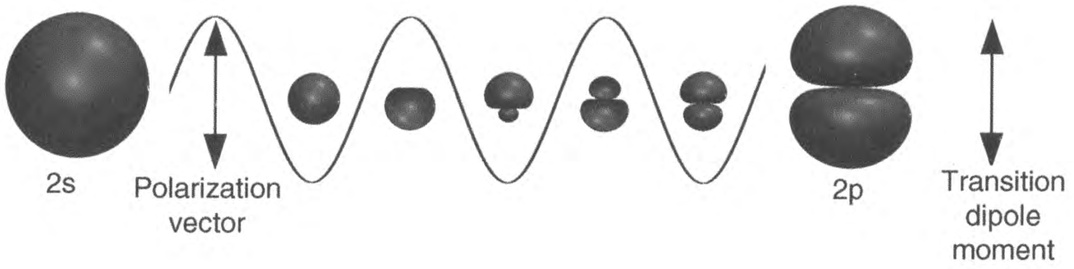


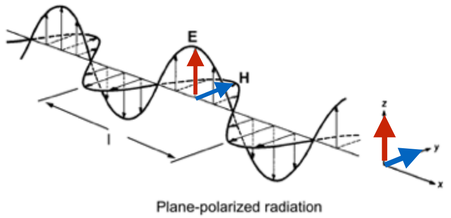
QM: electrons can have only discrete (quantized) energies

Energy must approximately match



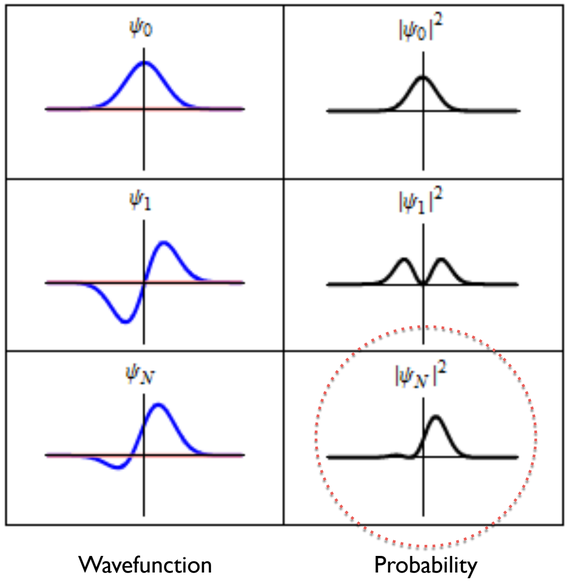
There must be a mechanism

Chem 728 Recap



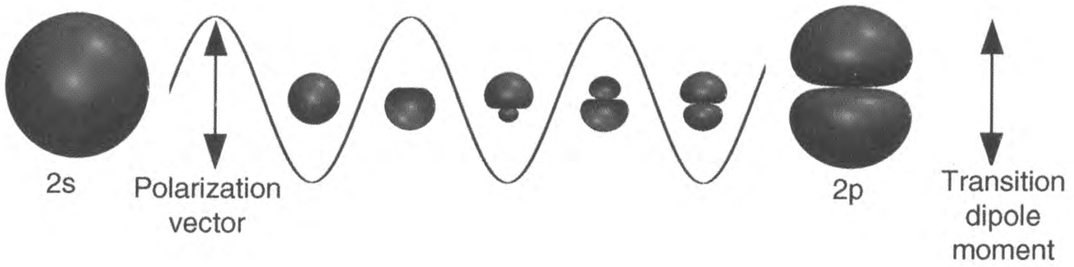
Plane-polarized radiation

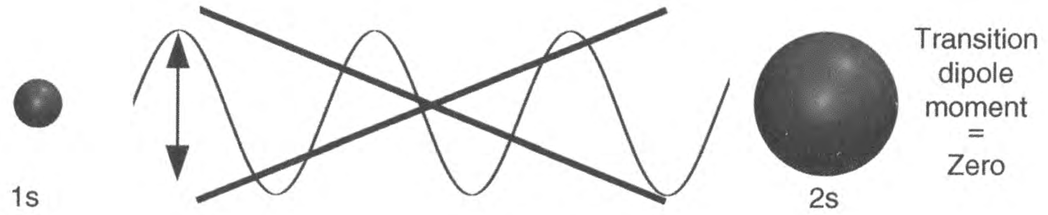
real part
imaginary part



linear combination of the two

Transition dipoles can be complicated





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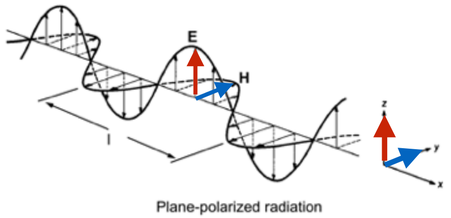
Chem 728 Recap



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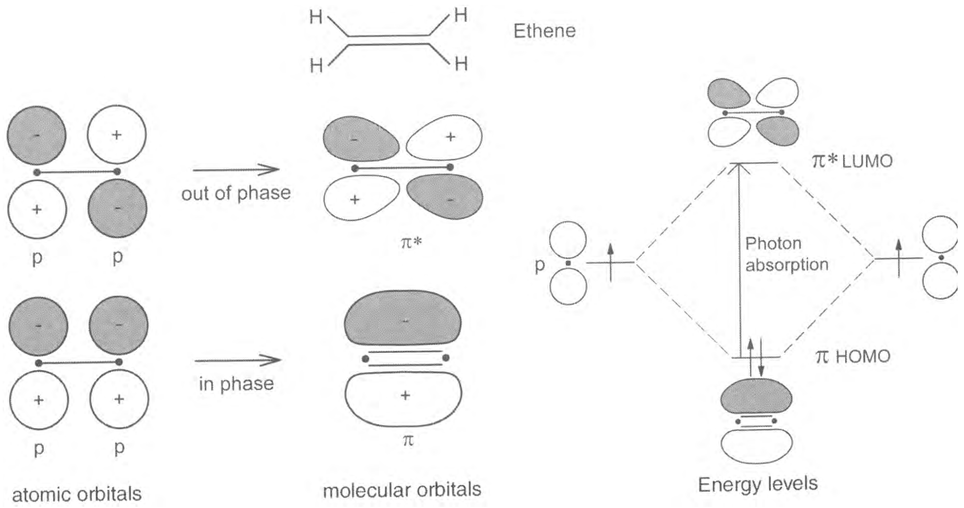

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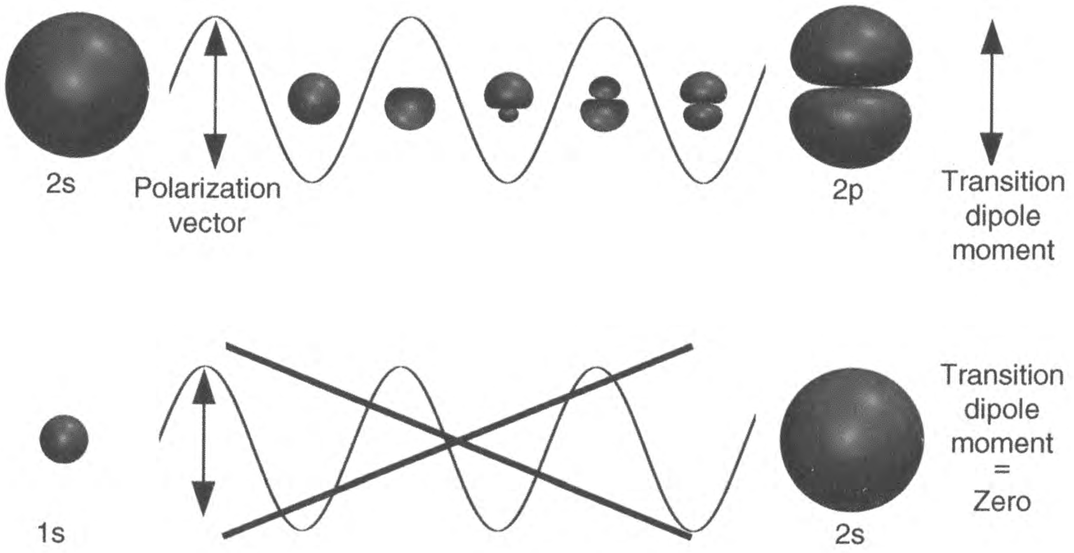

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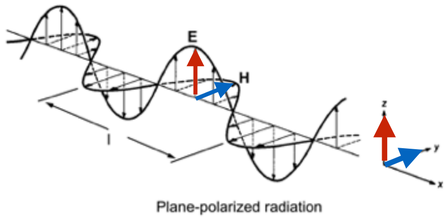
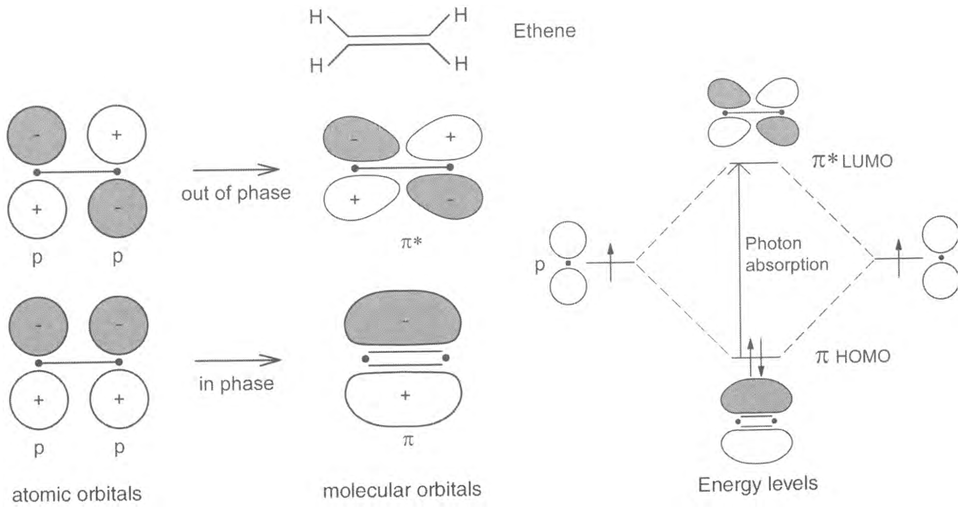


Plane-polarized radiation



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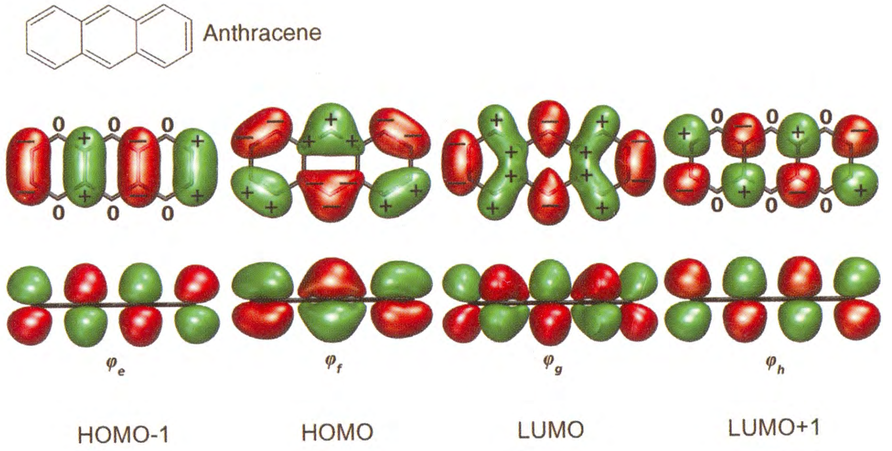

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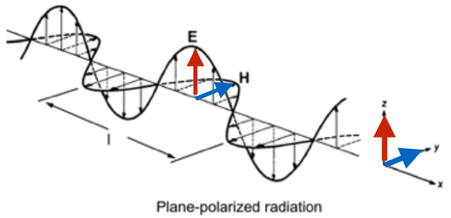
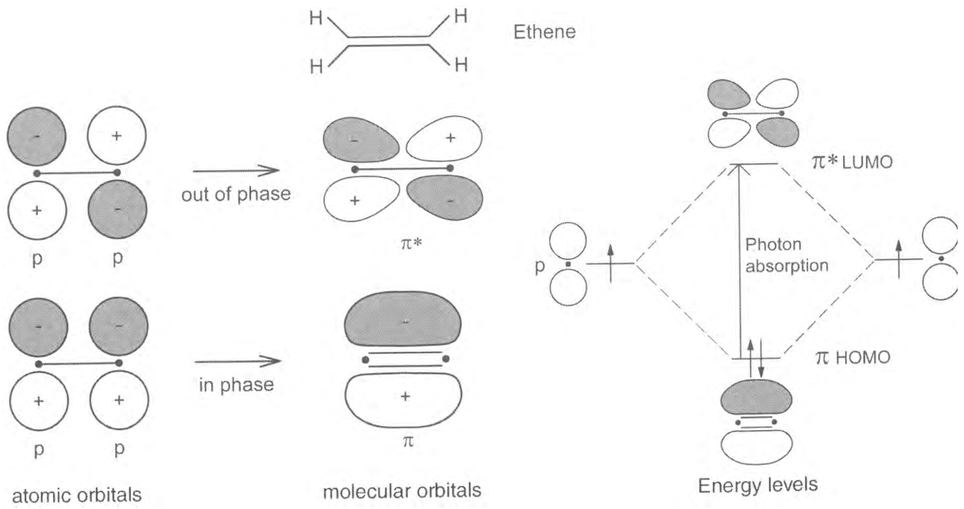
Chem 728 Recap

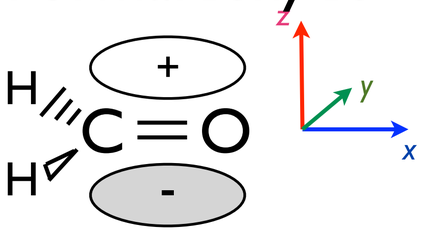


Plane-polarized radiation

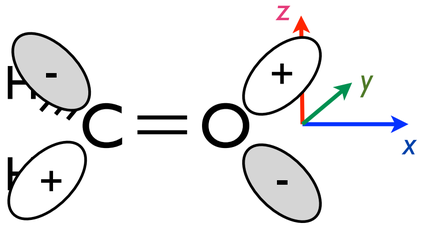


Transition dipoles can be complicated

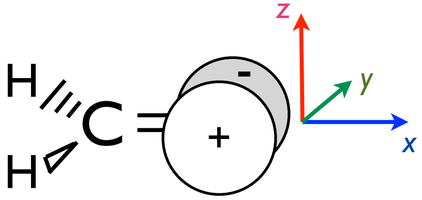
Formaldehyde



	x	y	z
	even	even	odd



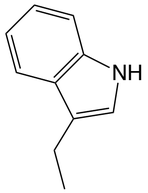
	x	y	z
	odd	even	odd



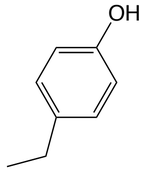
	x	y	z
	even	odd	odd

But symmetry CAN lead to zero or low probabilities

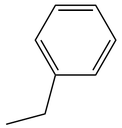
Chem 728 Recap



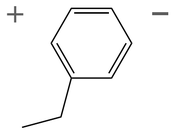
Trp 280 nm 5050 M^{-1} cm^{-1}



Tyr 274 nm 1440 M^{-1} cm^{-1}



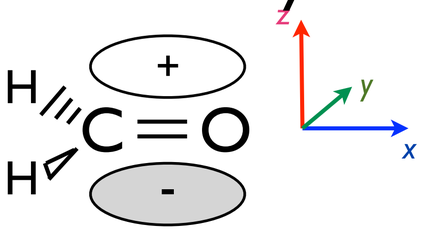
Phe 257 nm 220 M^{-1} cm^{-1}



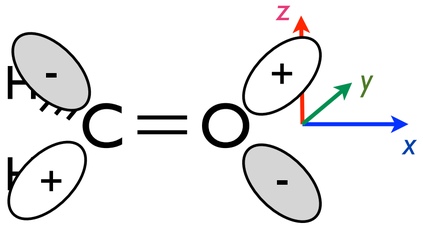
>220 M^{-1} cm^{-1}

Transition dipoles can be complicated

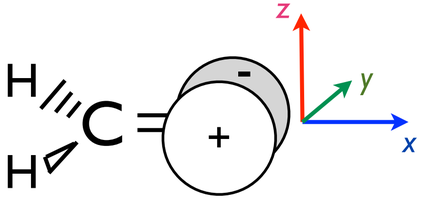
Formaldehyde



x	y	z
even	even	odd



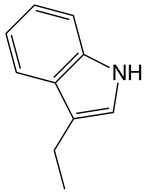
x	y	z
odd	even	odd



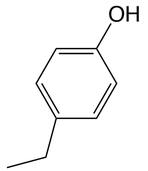
x	y	z
even	odd	odd

But symmetry CAN lead to zero or low probabilities

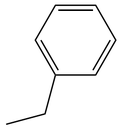
Chem 728 Recap



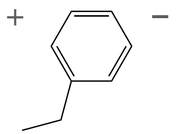
Trp 280 nm 5050 M^{-1} cm^{-1}



Tyr 274 nm 1440 M^{-1} cm^{-1}



Phe 257 nm 220 M^{-1} cm^{-1}

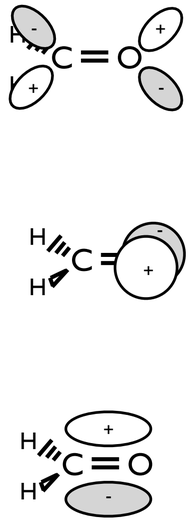


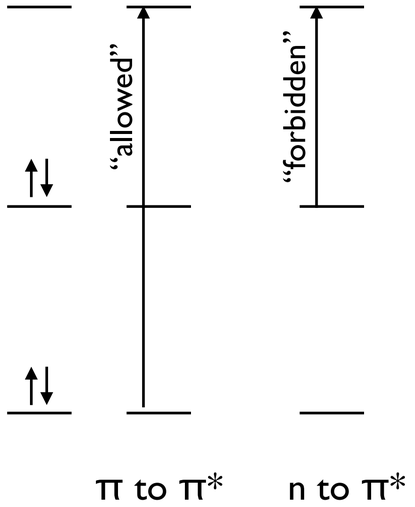
>220 M^{-1} cm^{-1}

Transition dipoles can be complicated

Which orbitals are more "extended?"

Which orbitals are more "compact?"

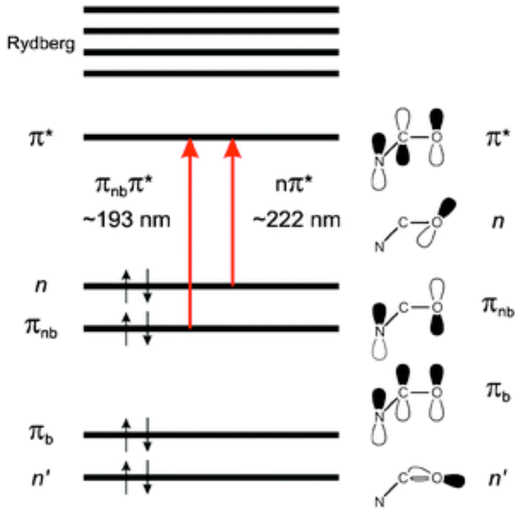


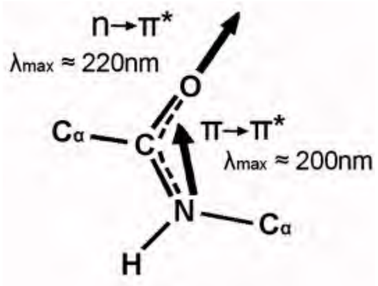


But symmetry CAN lead to zero or low probabilities

Chem 728 Recap

Peptide bond

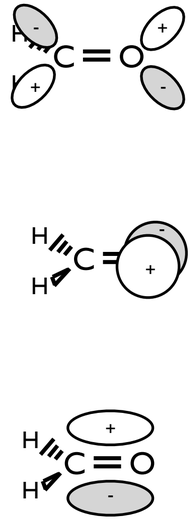


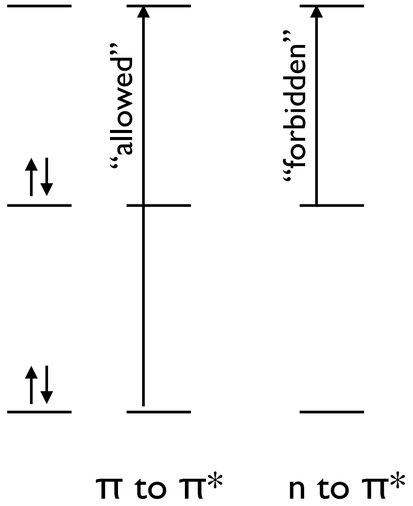


Whitmore & Wallace (2007)
Biopolymers 89, 392-400

Which orbitals are more "extended?"

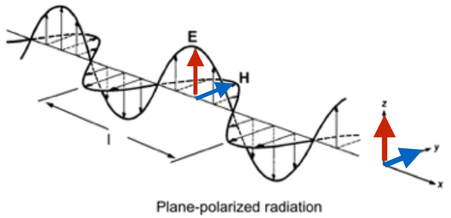
Which orbitals are more "compact?"





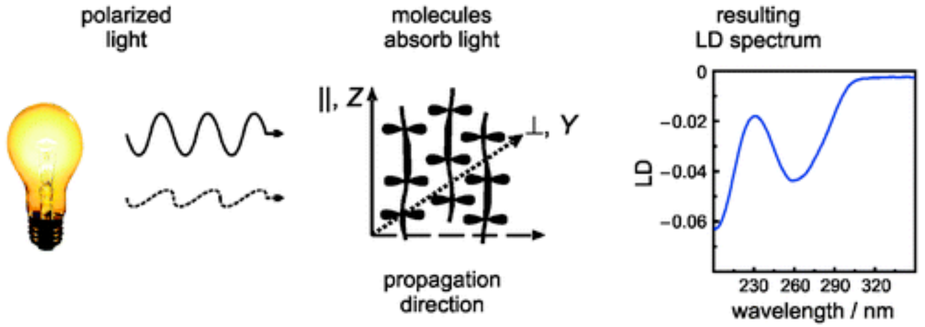
But symmetry CAN lead to zero or low probabilities

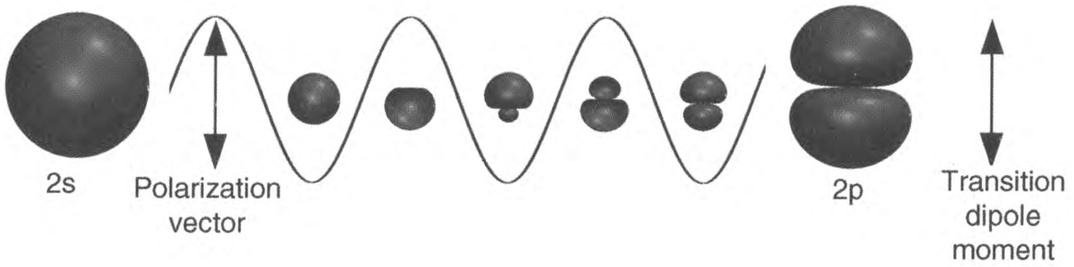
Chem 728 Recap

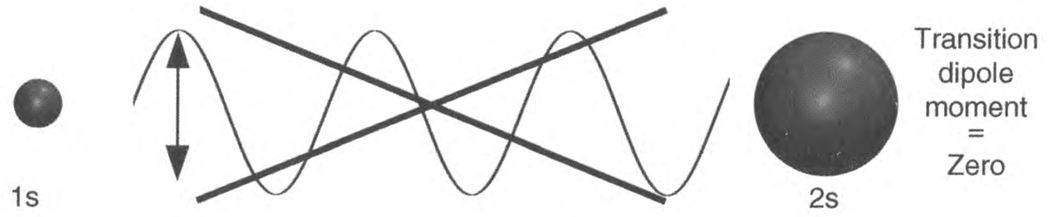


Plane-polarized radiation

Linear dichroism

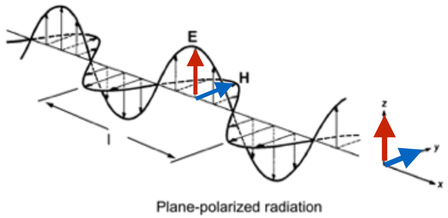


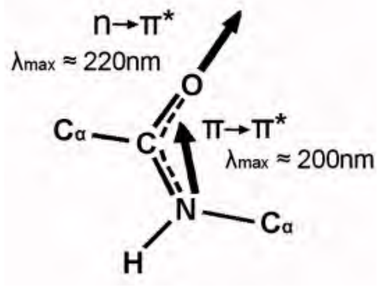


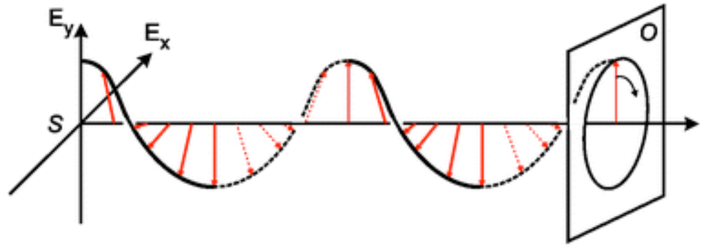


There must be a mechanism

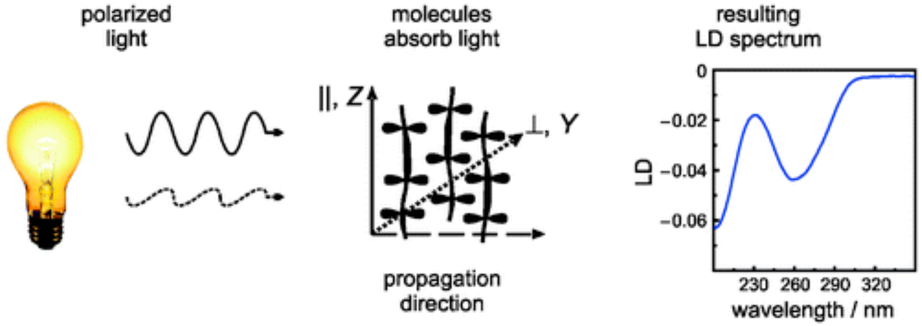
Chem 728 Recap

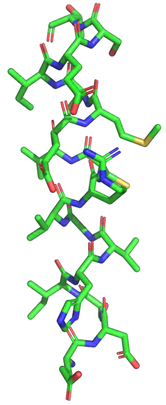




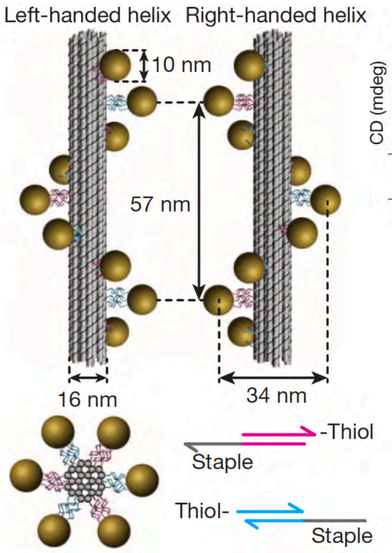


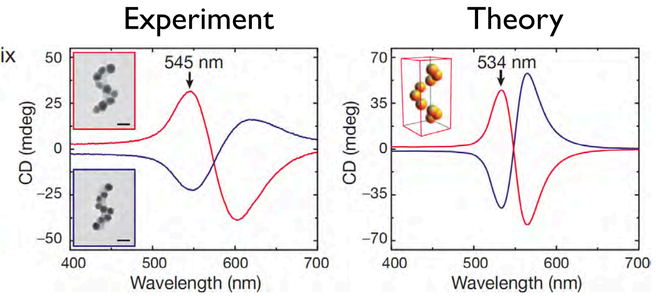
Linear dichroism



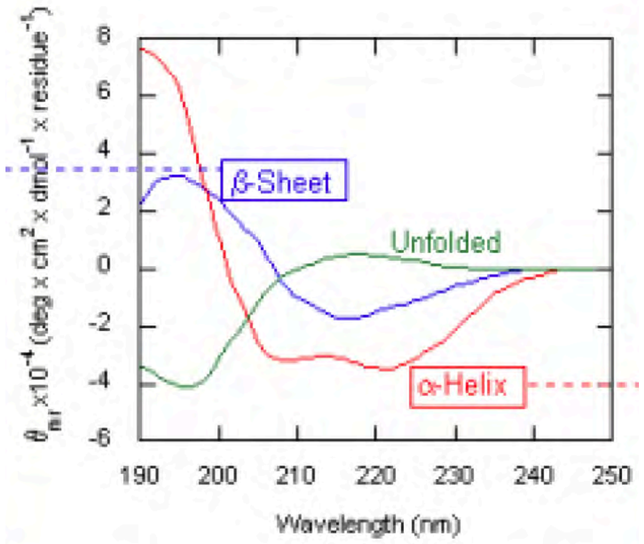


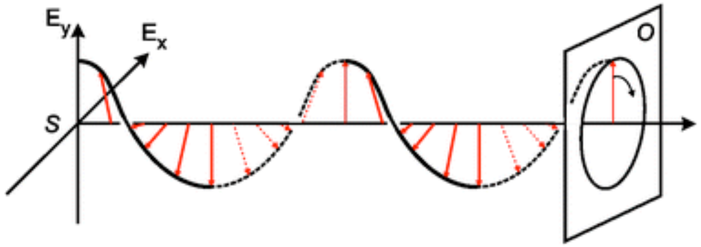
Circular dichroism

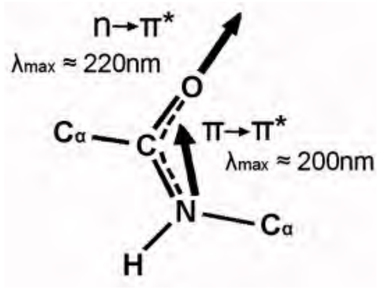




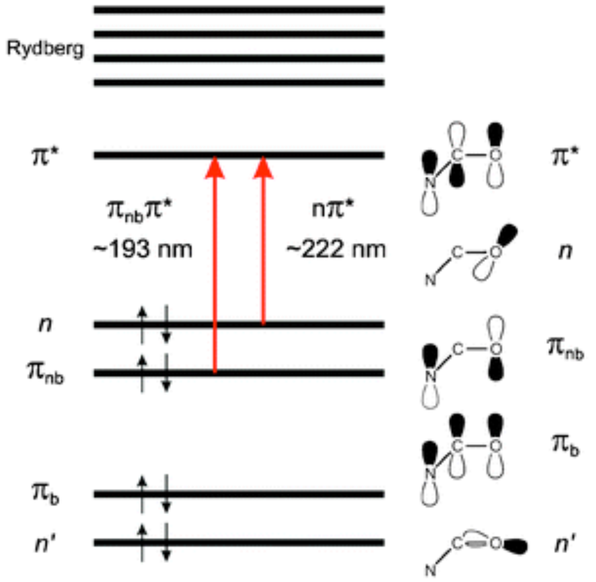
Chem 728 Recap

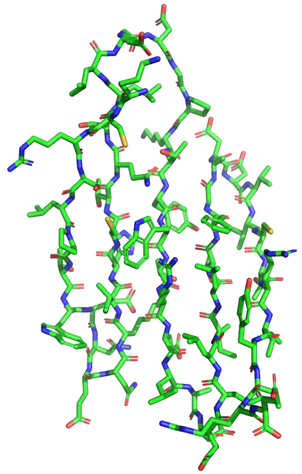


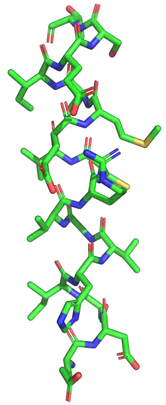


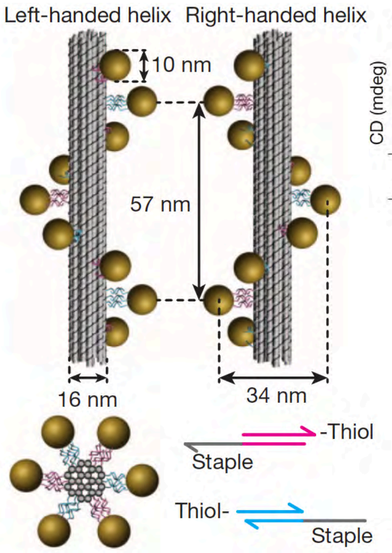


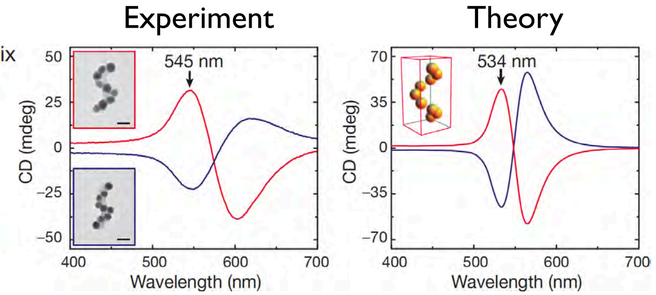
Circular dichroism



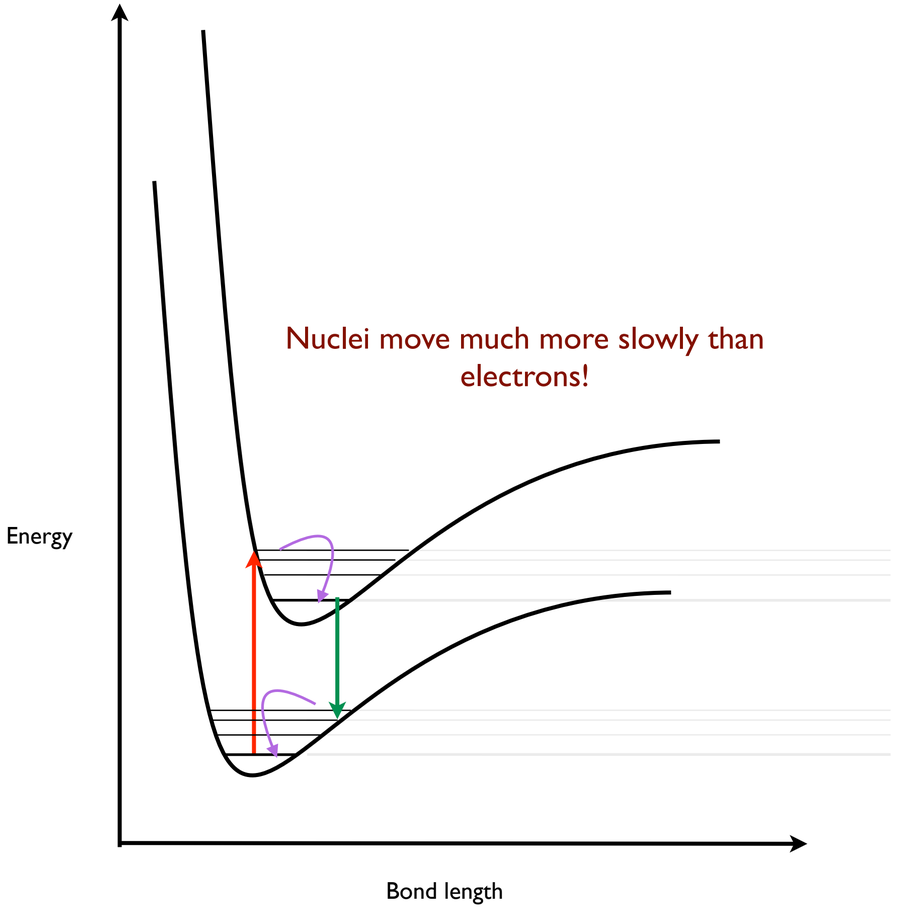


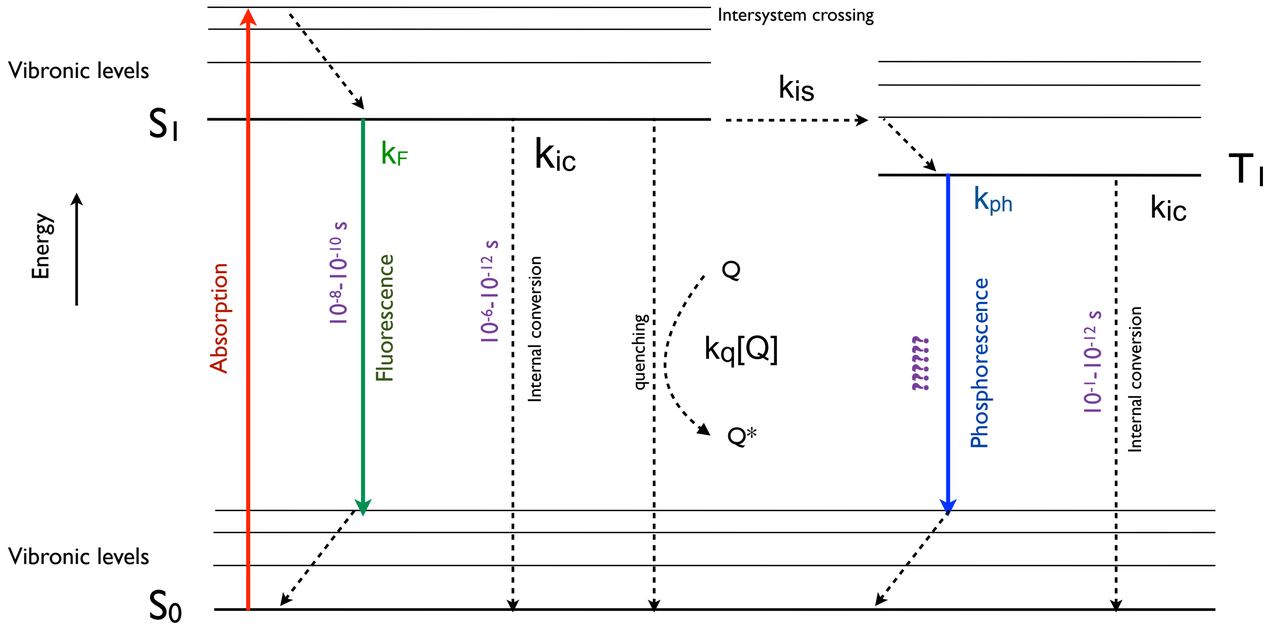






Chem 728 Recap

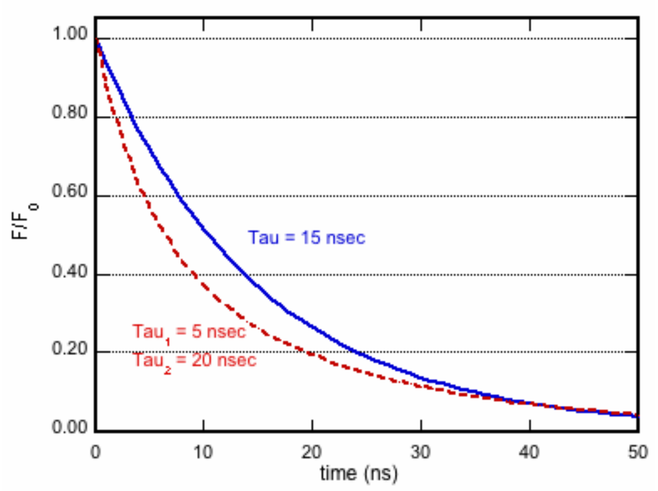


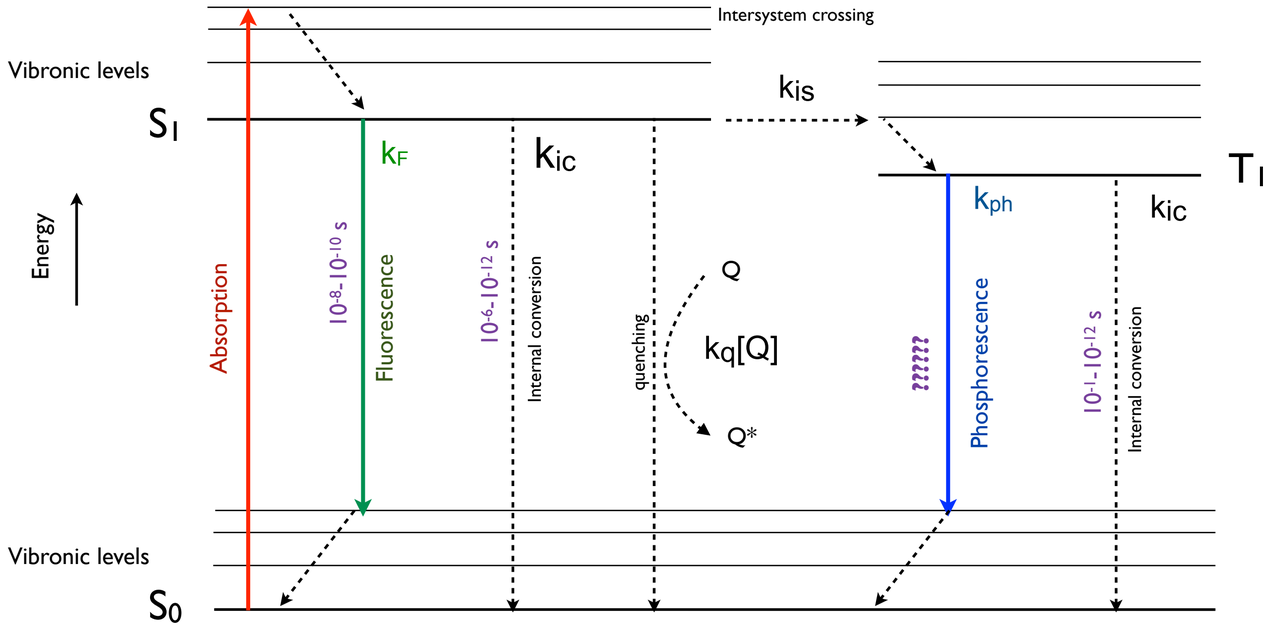


Chem 728 Recap

Exponential decay

If k_{tot} is a mix of population, decay will be multiexponential

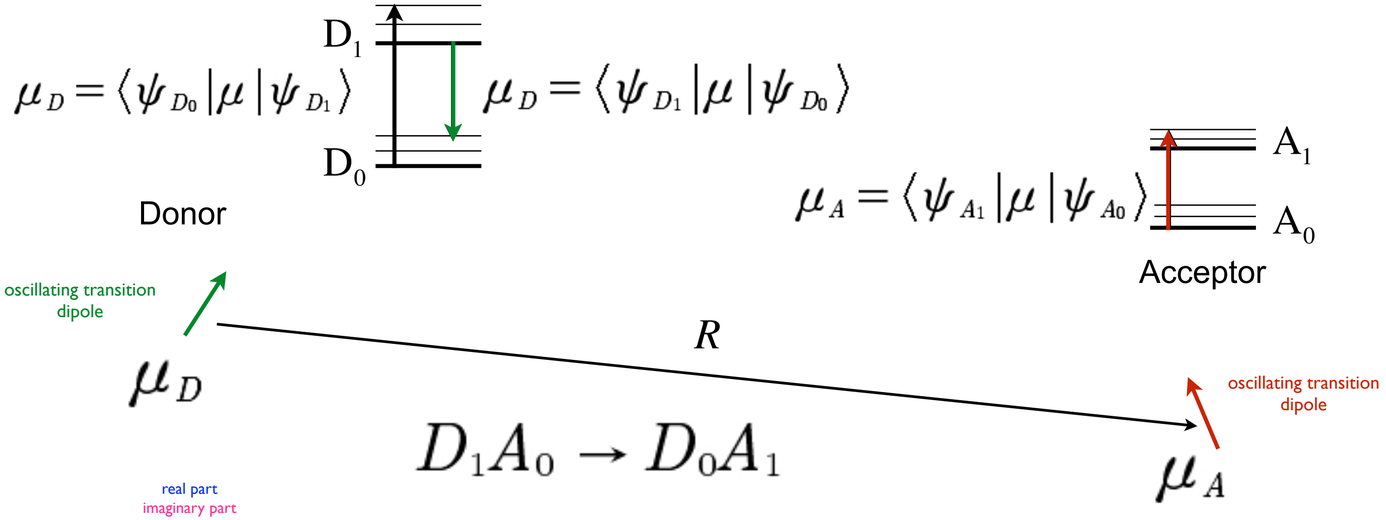


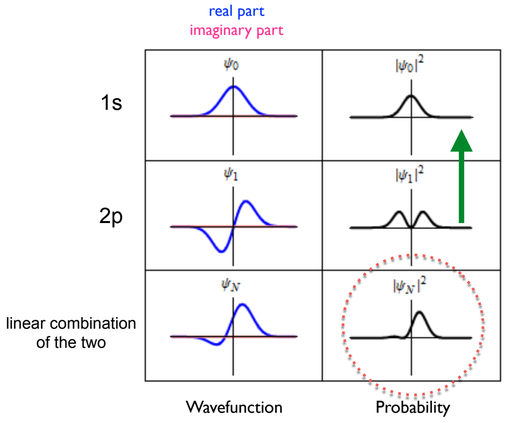


Chem 728 Recap

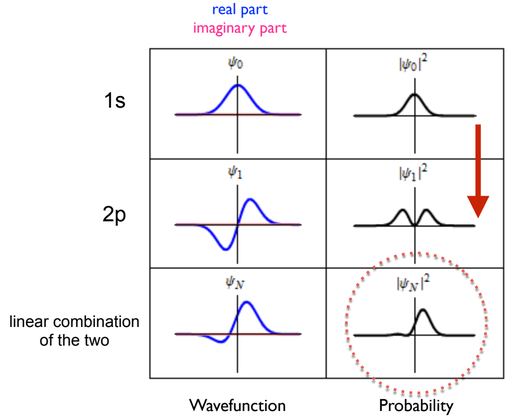
Fluorescence Resonance Energy Transfer

A particular type of quenching





Interacting transition dipoles



Distance dependent

Angular dependence

Energy match

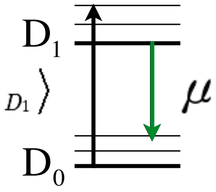
Competes w/ other mechs

Chem 728 Recap

Fluorescence Resonance Energy Transfer

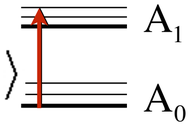
A particular type of quenching

$\mu_D = \langle \psi_{D_0} | \mu | \psi_{D_1} \rangle$ $\mu_D = \langle \psi_{D_1} | \mu | \psi_{D_0} \rangle$



Donor

$\mu_A = \langle \psi_{A_1} | \mu | \psi_{A_0} \rangle$



Acceptor

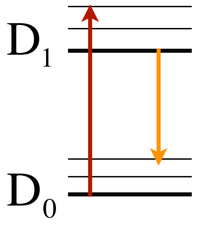
Distance dependent

Angular dependence

Energy match

Competes w/ other mechs

Fluorescence Quantum Yield



$$\frac{\text{photons emitted}}{\text{photons absorbed}}$$

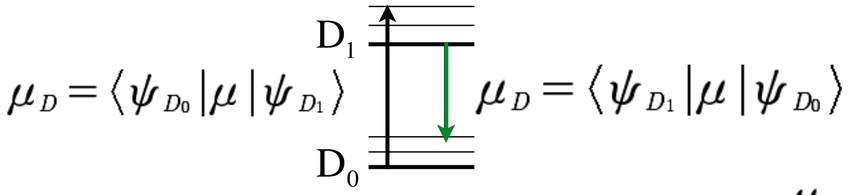
Φ proportional to I/A

$$\Phi_{ET} = \frac{k_{ET}}{k_F^D + k_{IC}^D + k_{ISC}^D + k_{ET}} = \frac{k_{ET}}{k_{All\ else}^D + k_{ET}} = \frac{k_{ET}}{\frac{1}{\tau_{S_1}^D} + k_{ET}}$$

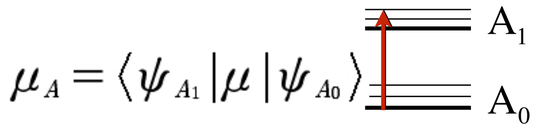
Chem 728 Recap

Fluorescence Resonance Energy Transfer

A particular type of quenching



Donor



Acceptor

Quenching of Donor Fluorescence

Fluorescence of acceptor

Can Measure

Distance dependent

Angular dependence

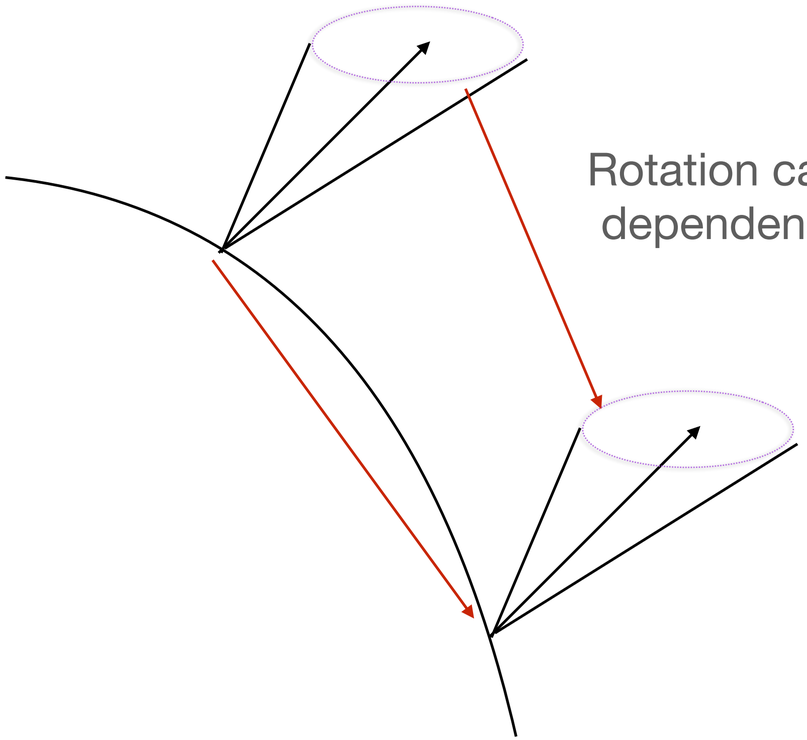
Energy match

Competes w/ other mechs

Chem 728 Recap

Fluorescence Resonance Energy Transfer

A particular type of quenching



Rotation can average out angular dependence, but BE CAREFUL!

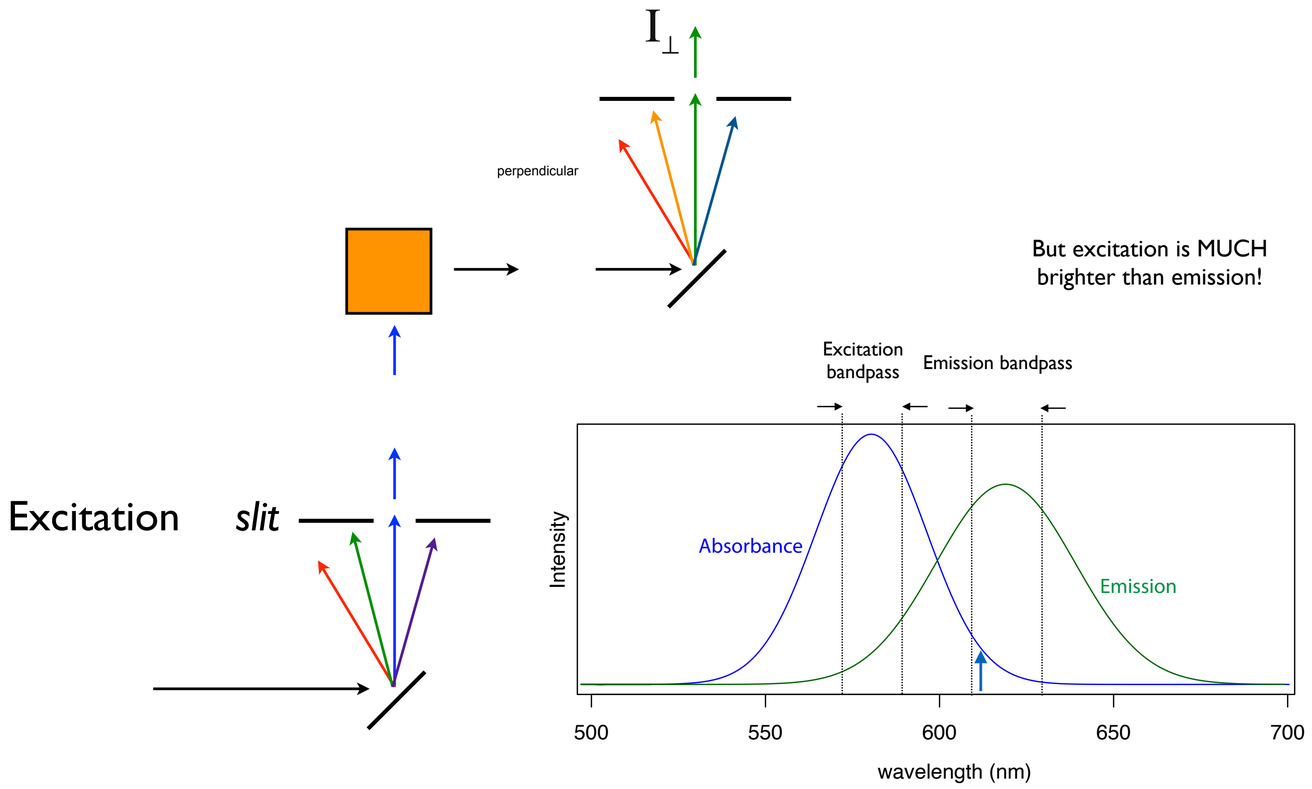
Distance dependent

Angular dependence

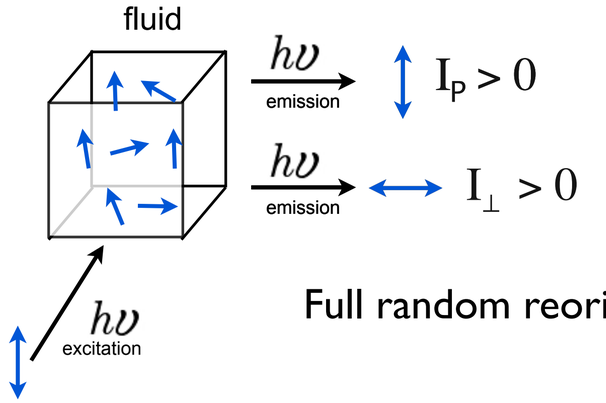
Energy match

Competes w/ other mechs

Chem 728 Recap



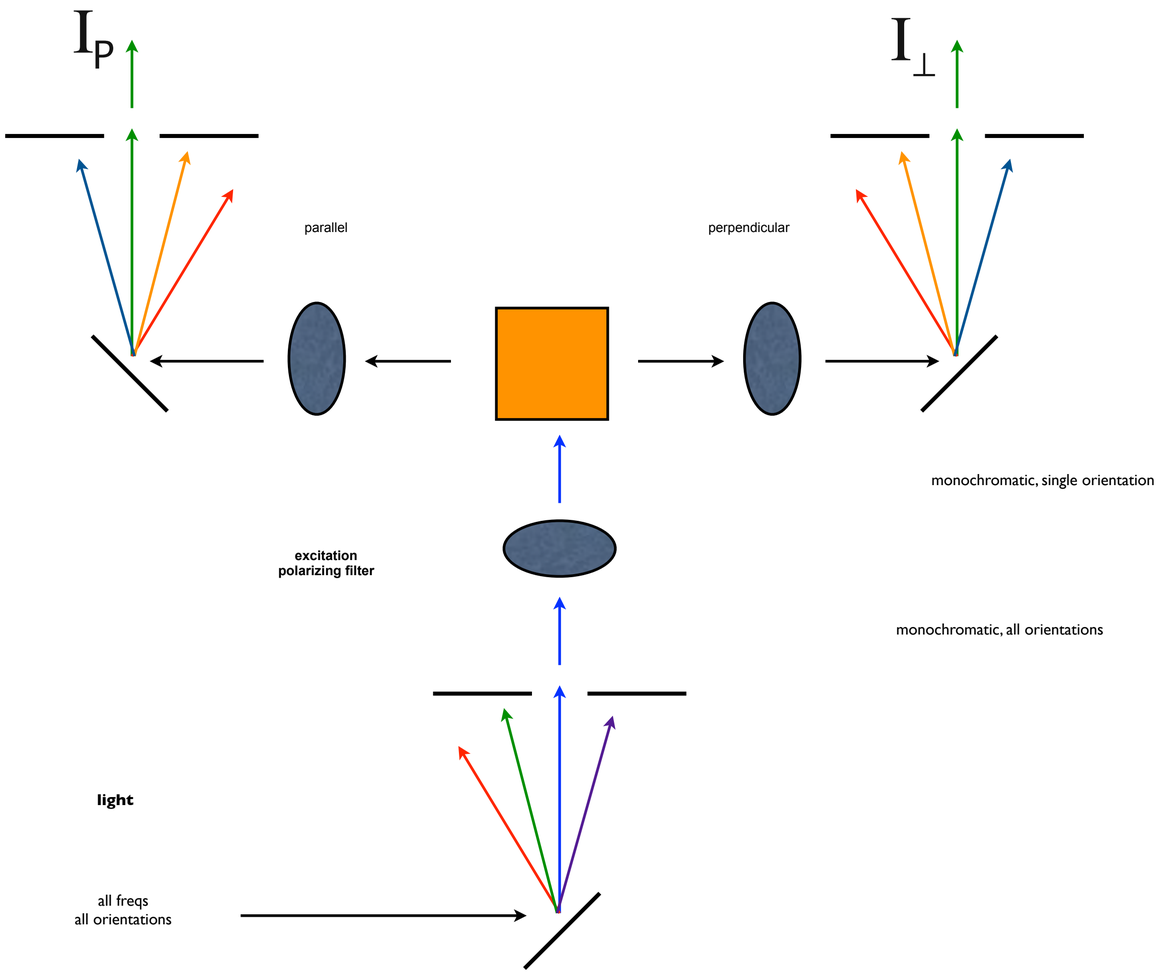
Anisotropy / Polarization



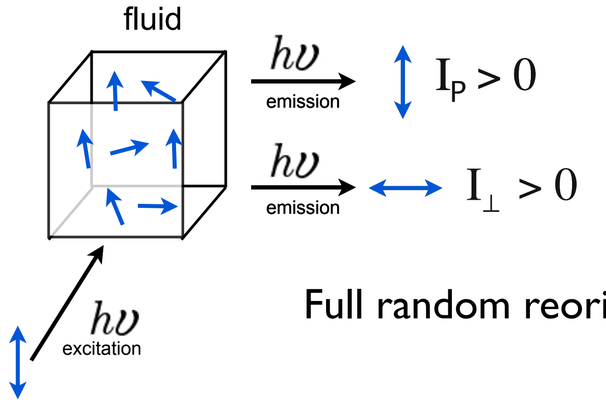
$A \approx 0$

Full random reorientation, A=0

Chem 728 Recap



Anisotropy / Polarization

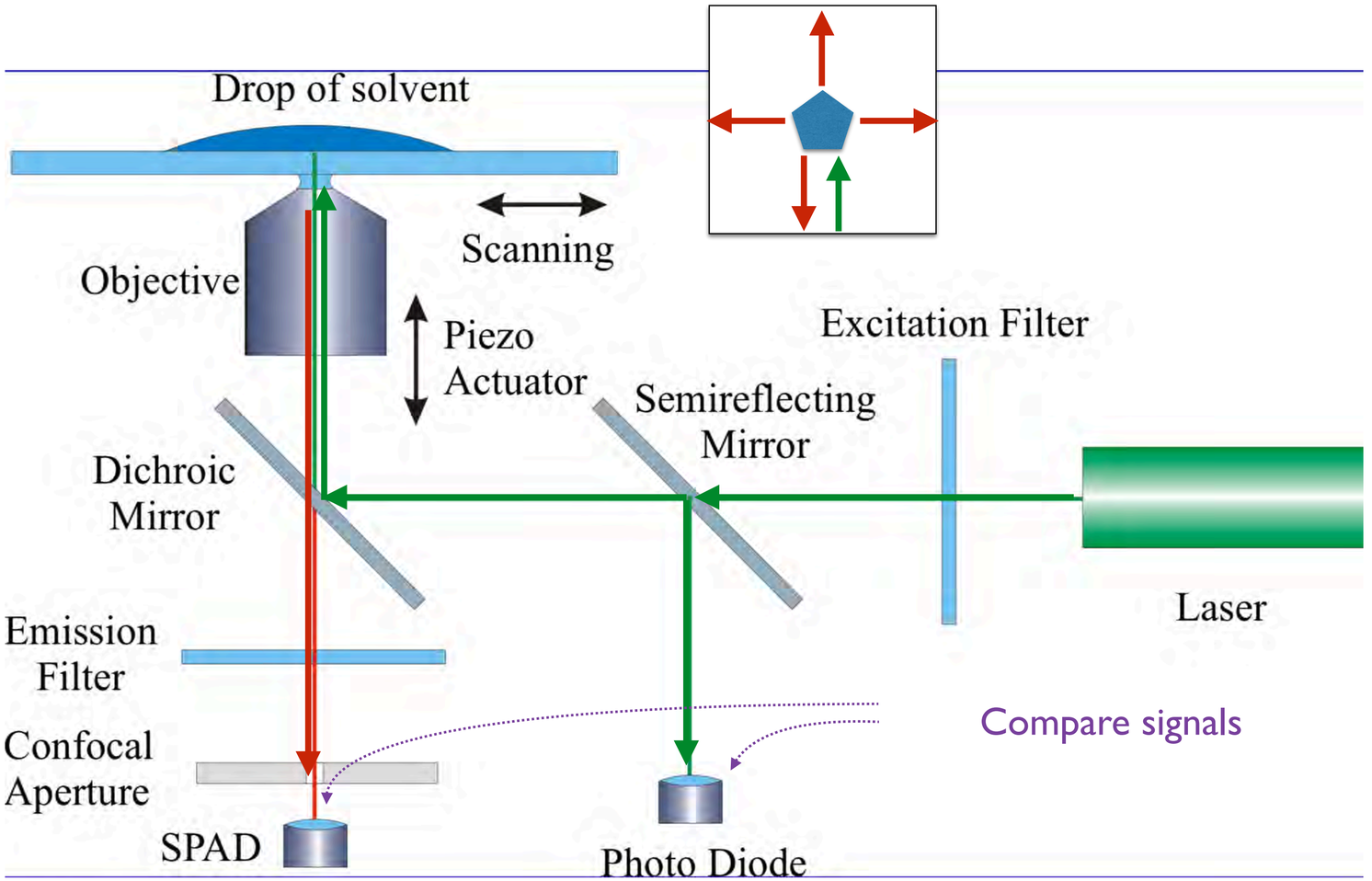


$A \approx 0$

Full random reorientation, A=0

Chem 728 Recap

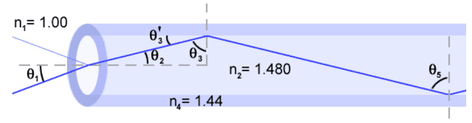
Lasers and Imaging - A Whole New World!

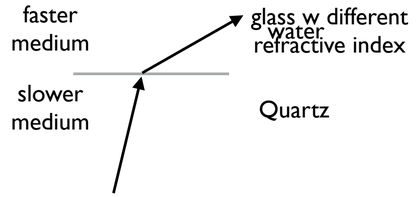


single photon avalanche detector

Chem 728 Recap

Lasers and Imaging - A Whole New World!





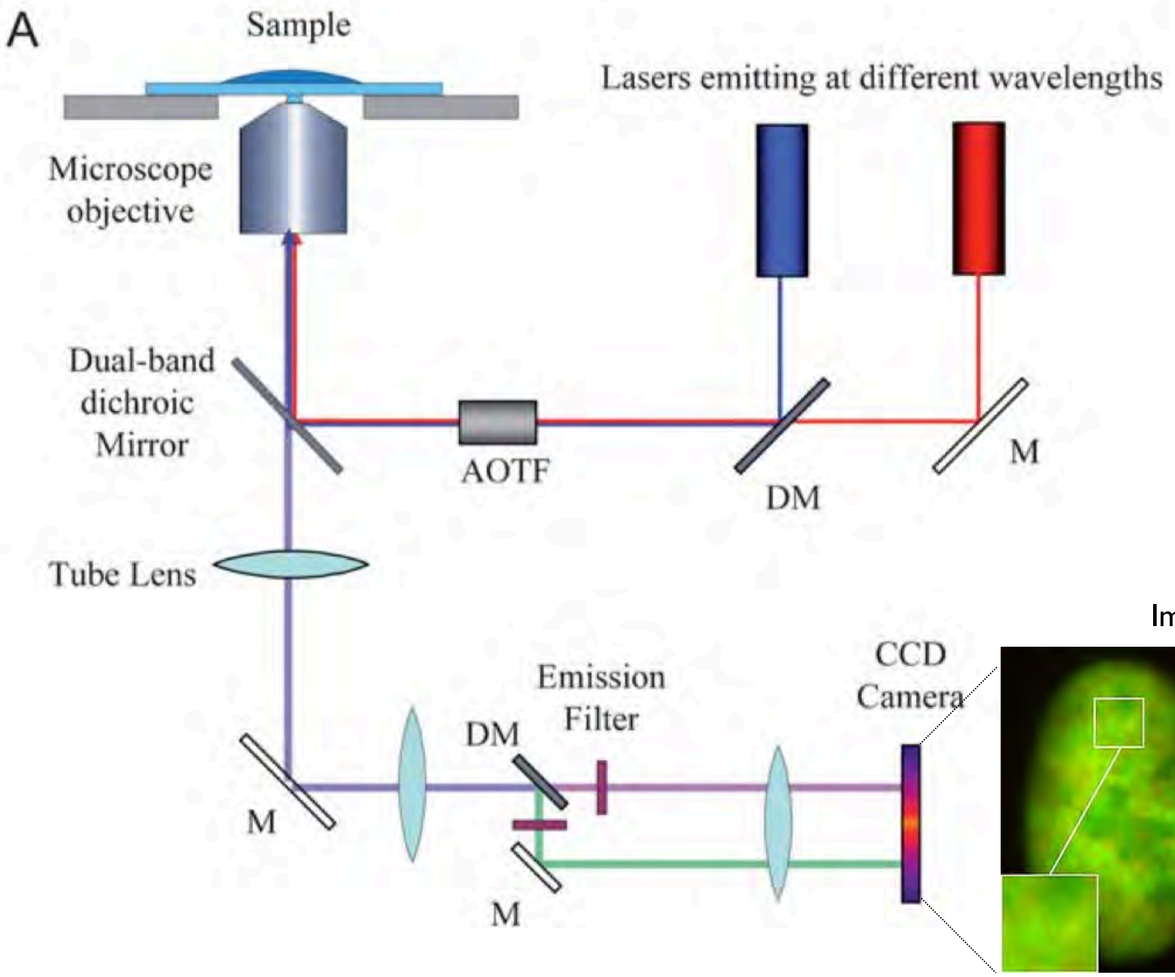
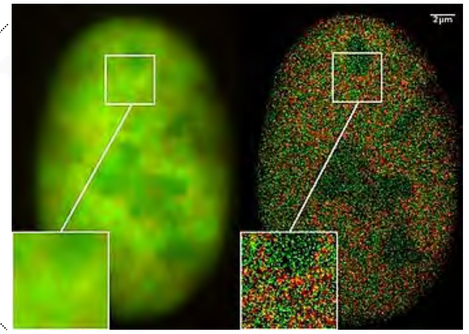
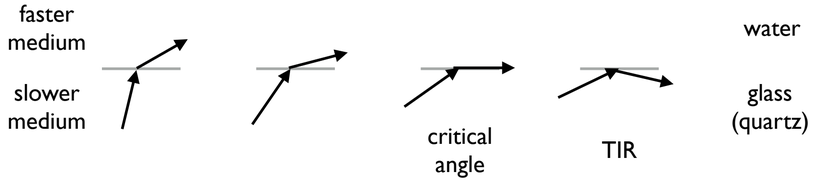
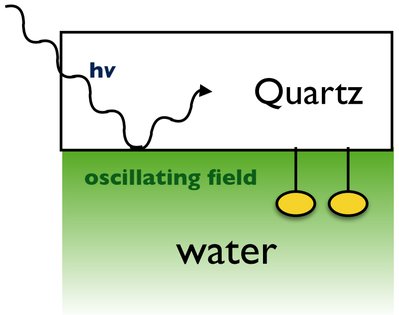


Image a cell



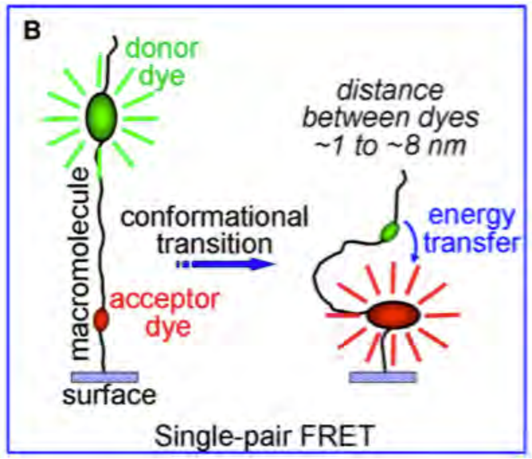
Total Internal Reflectance



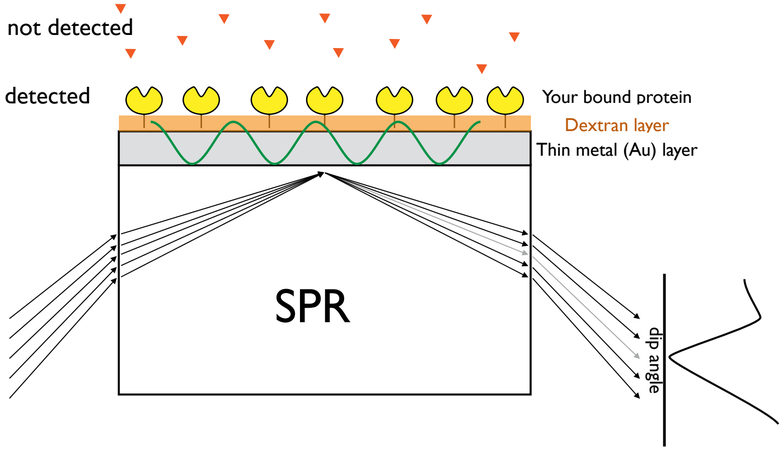


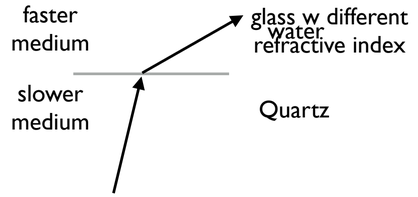
Chem 728 Recap

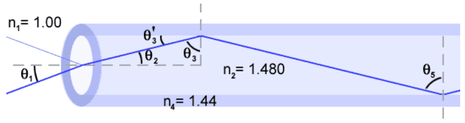
Surface Detection / Immobilization / Localization



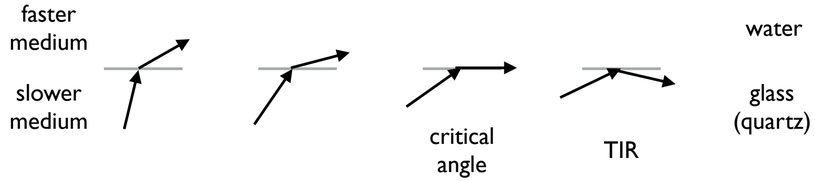
Fluorescence at Surfaces

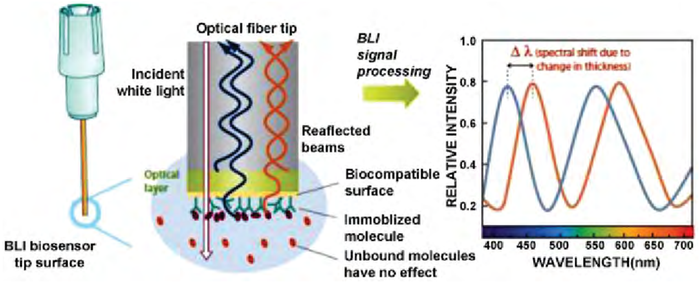




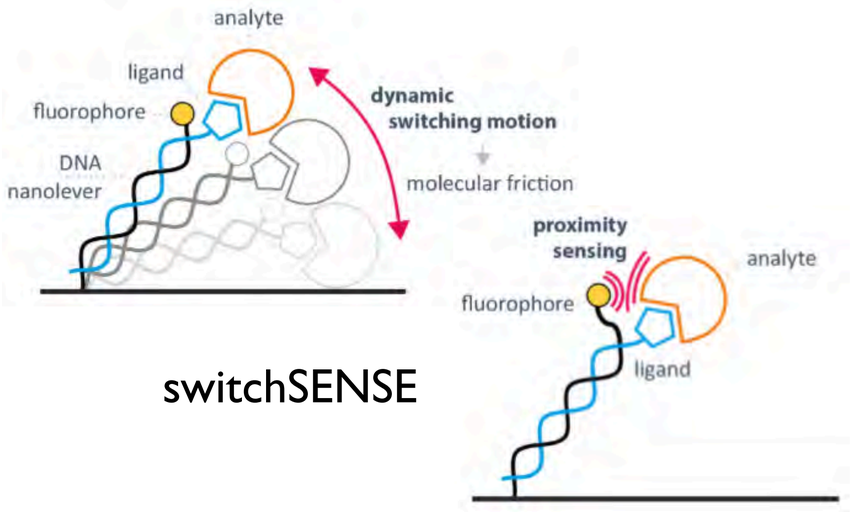


Total Internal Reflectance

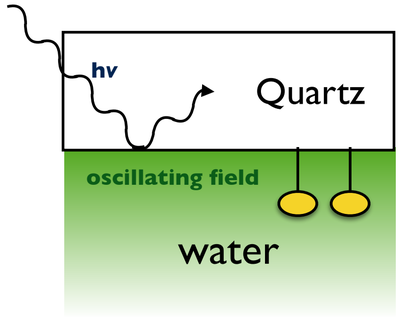




Bio-Layer Interferometry

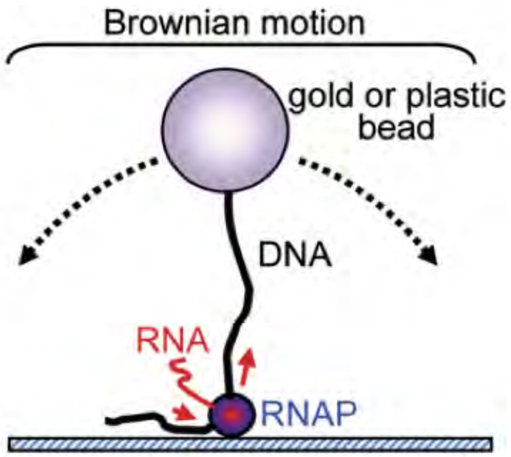


switchSENSE

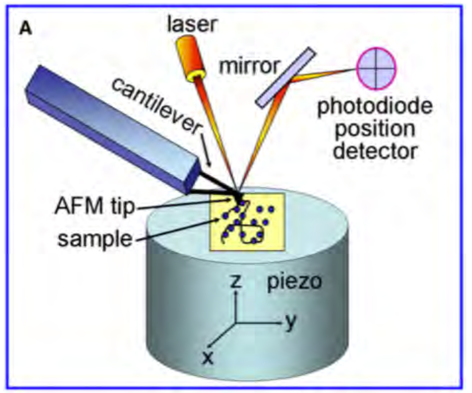


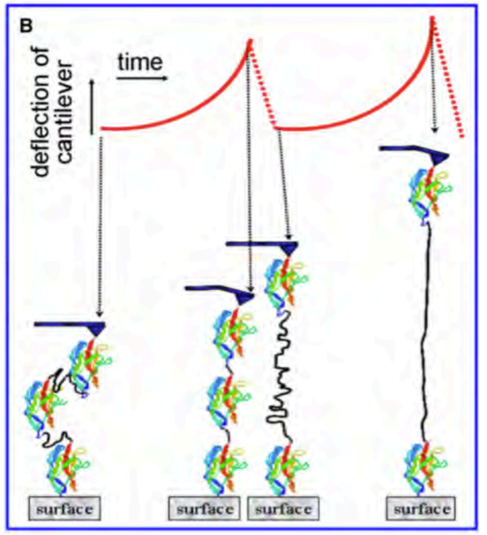
Chem 728 Recap

Single Molecule Approaches



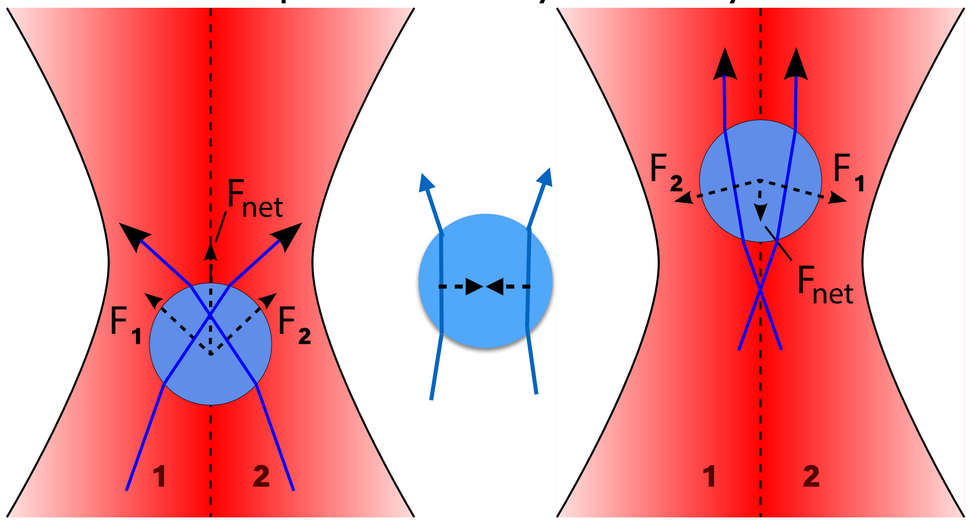
NOT Single photon!!





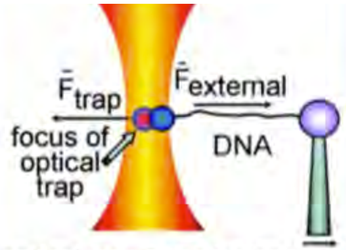
Laser (Bead) Trap

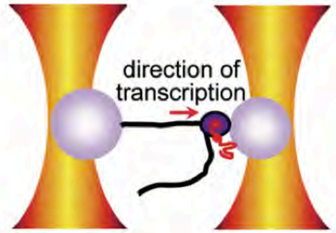
Traps both laterally and axially



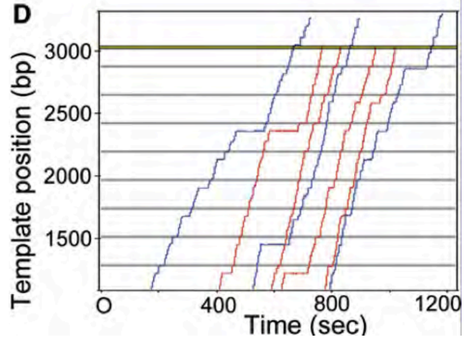
laser light in

laser light in



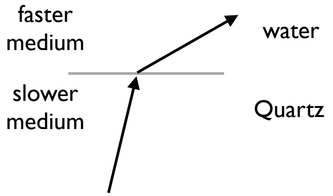


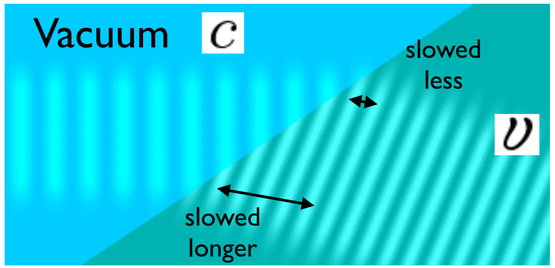
Steve Block, et al.



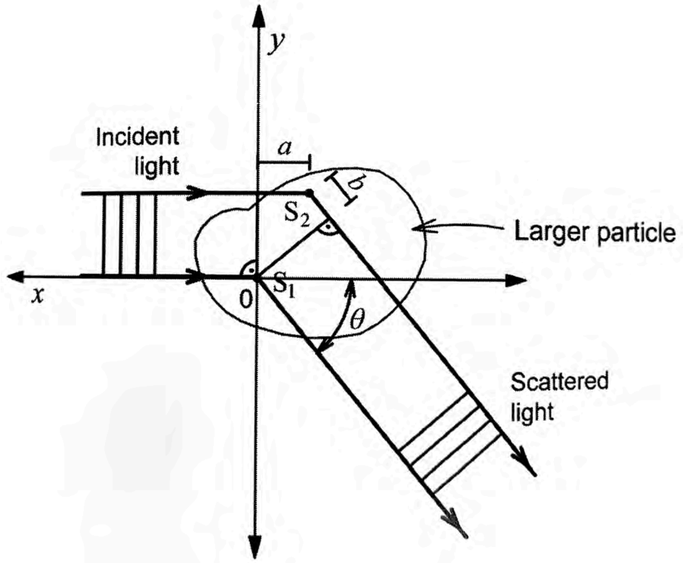
Chem 728 Recap

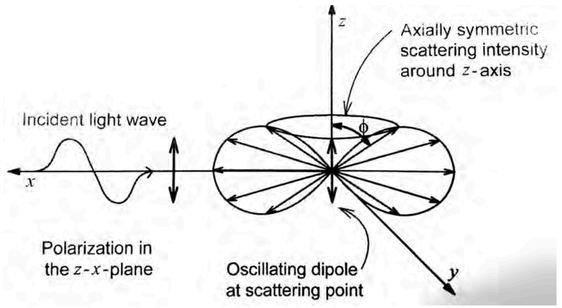
More with refraction





By Ulflund - https://commons.wikimedia.org/w/index.php?curid=73784342

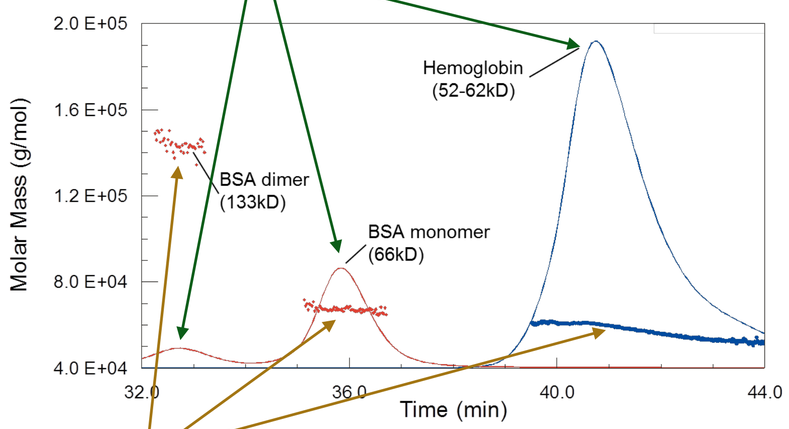




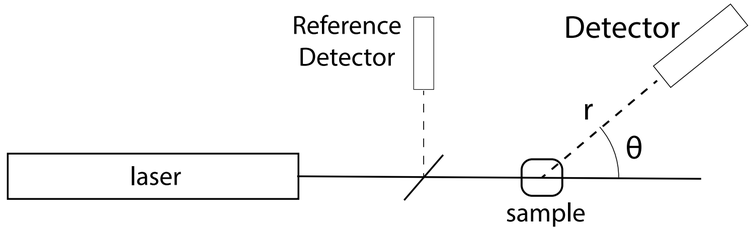
SEC-MALS

HPLC

SEC - Size Exclusion Chromatography



MALS - Multi-Angle Light Scattering



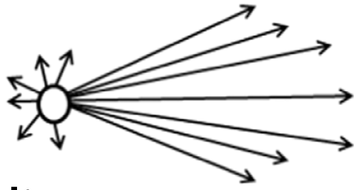
Chem 728 Recap

Diffusion of Molecules

Diffusion (Brownian Motion)

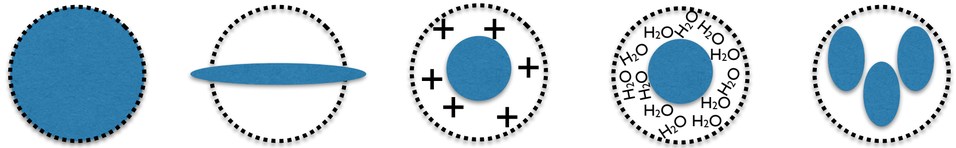
$$D = \frac{k_B T}{6\pi\eta r}$$

η = solution viscosity



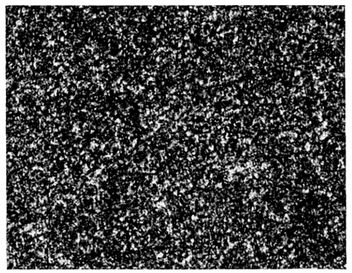
r = hydrodynamic radius

"*apparent* size"

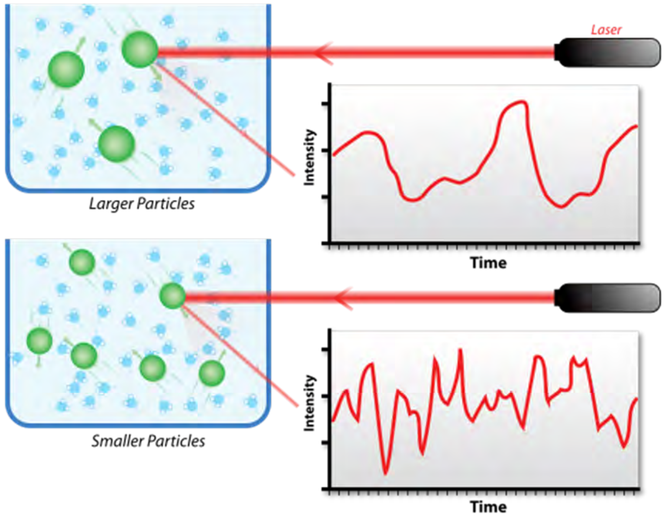


Dynamic Light Scattering

Photon Correlation Spectroscopy



you see dark and light spots, no pattern.
"**blinking**"



Chem 728 Recap

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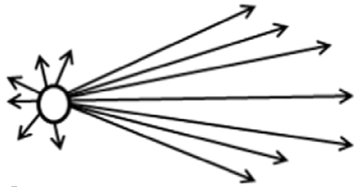
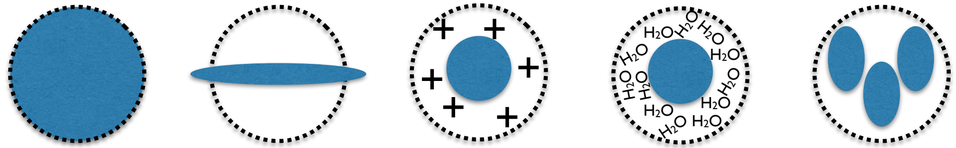


r = hydrodynamic radius

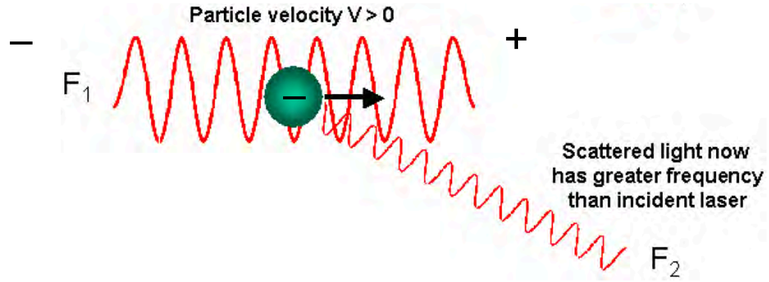
"*apparent* size"

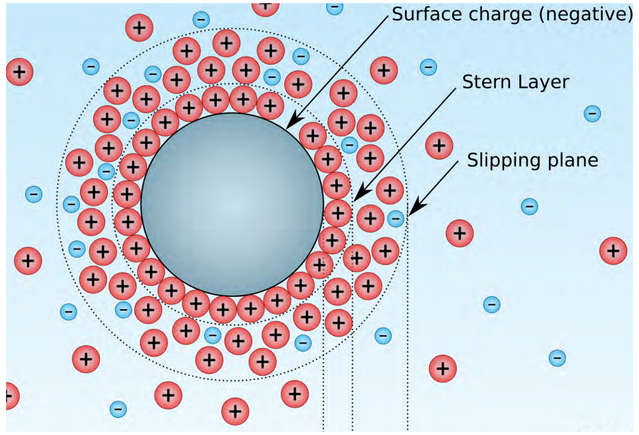


Electrophoretic Light Scattering

Malvern Zetasizer ZSP - dynamic light scattering (DLS) system

aka Laser-Doppler Electrophoresis

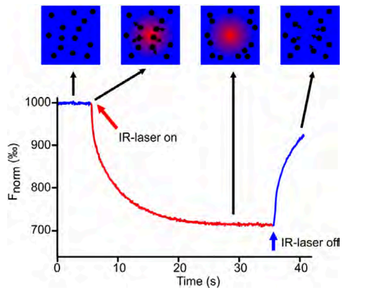


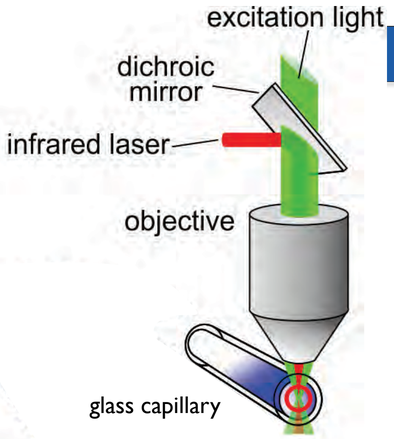


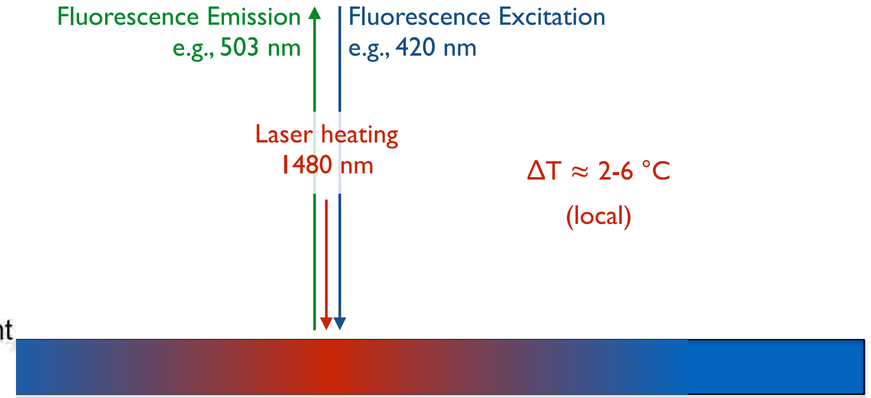
Chem 728 Recap

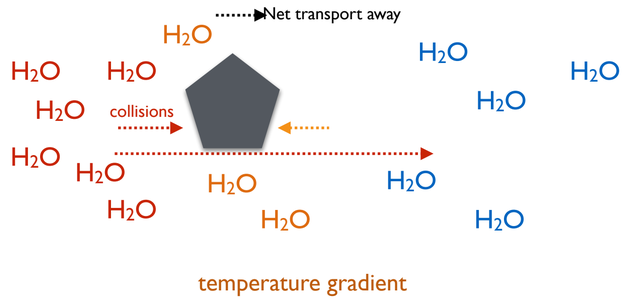
More fun with fluorescence

Microscale Thermophoresis





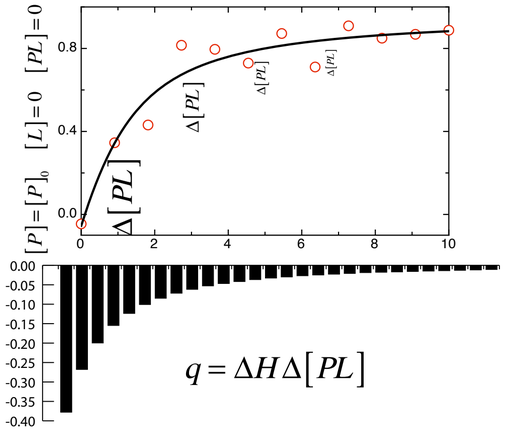




Chem 728 Recap

Measuring Heats

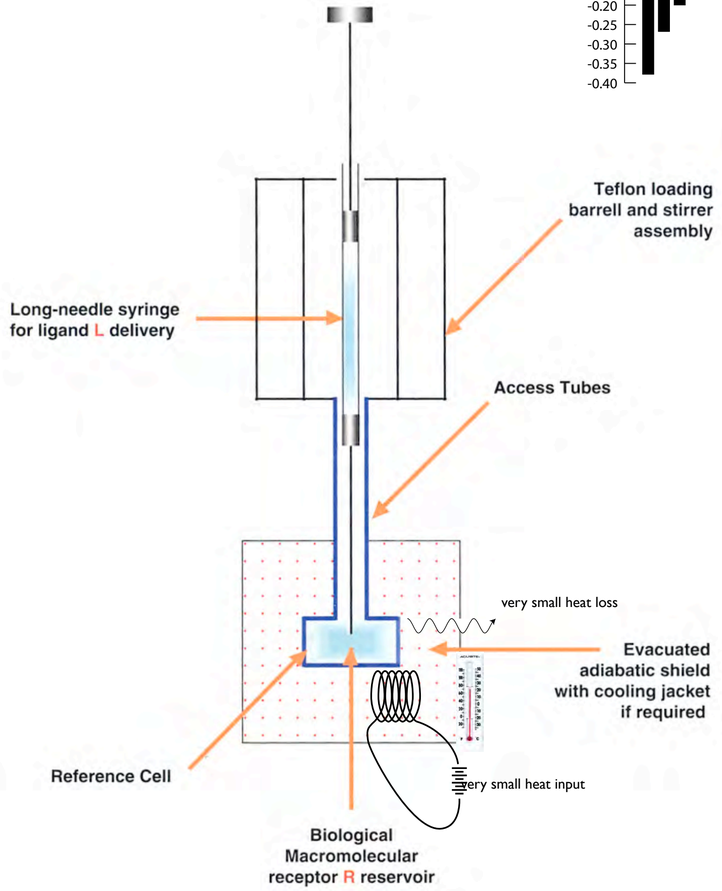
$$P + L \underset{}{\overset{K_a = 1/K_d}{\rightleftharpoons}} PL$$



$$q = \Delta H \Delta[PL]$$

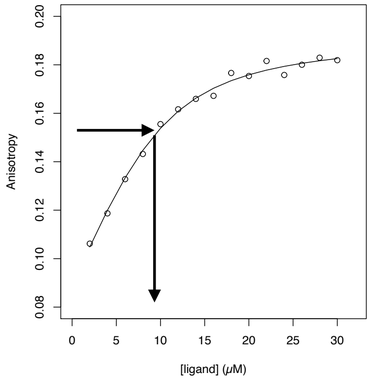
But remember that you can always assess van't Hoff enthalpies from the T-dependence of K

In either case, ΔH is for the entire system, not just your intended system, so be careful not to over-interpret

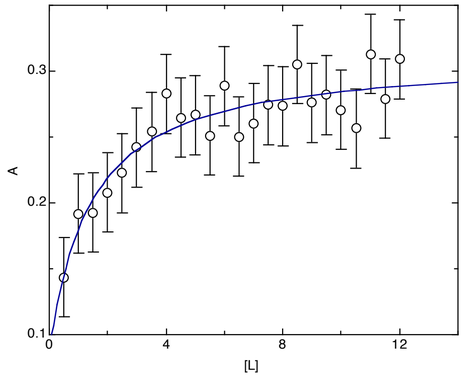


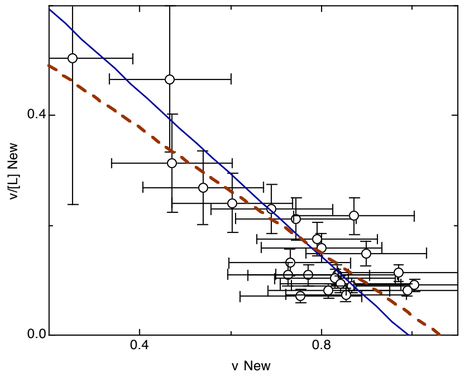
Chem 728 Recap

Ignore the old textbooks



Never Do Unnecessary Manipulations!





Never Make Unnecessary Assumptions!

Equilibrium Math

$$A + B \underset{}{\overset{K_d}{\rightleftharpoons}} AB$$

Knowns

$$K_d = \frac{[A][B]}{[AB]}$$

$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

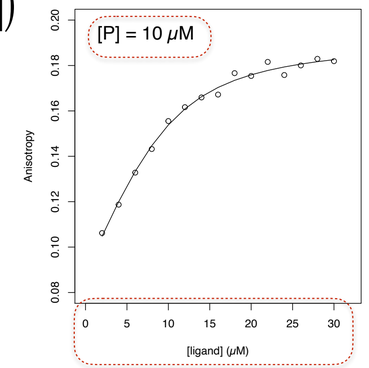
$$A_T = [A] + [AB]$$
$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$
$$K_d x = (A_T - x)(B_T - x)$$

~~Assume B_T >> x~~

$$x^2 - (A_T + B_T + K_d)x + A_T B_T = 0$$

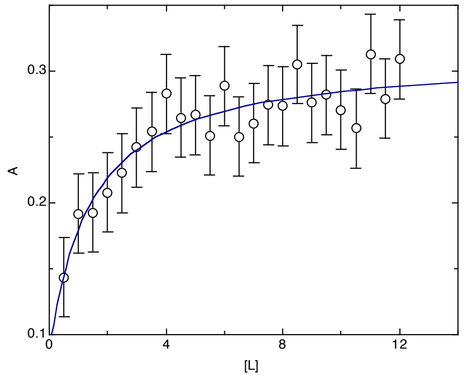
$$ax^2 + bx + c = 0 \qquad x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$



Chem 728 Recap

Ignore the old textbooks

Learn to Love R!



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$$[A] = A_T - [AB]$$
$$[B] = B_T - [AB]$$

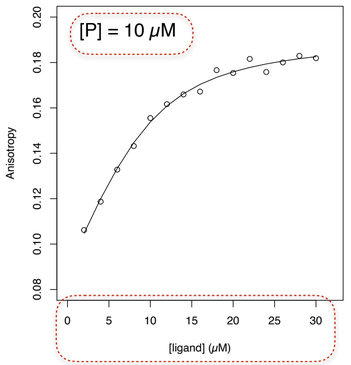
$$A_T = [A] + [AB]$$
$$B_T = [B] + [AB]$$

$$K_d[AB] = [A][B] = (A_T - [AB])(B_T - [AB])$$
$$K_d x = (A_T - x)(B_T - x)$$

~~Assume $B_T >> x$~~

$$x^2 - (A_T + B_T + K_d)x + A_T B_T = 0$$

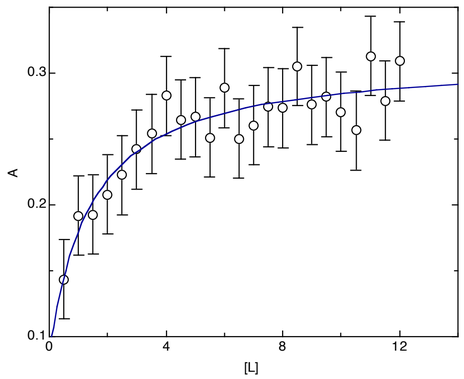
$$ax^2 + bx + c = 0 \qquad x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$



Chem 728 Recap

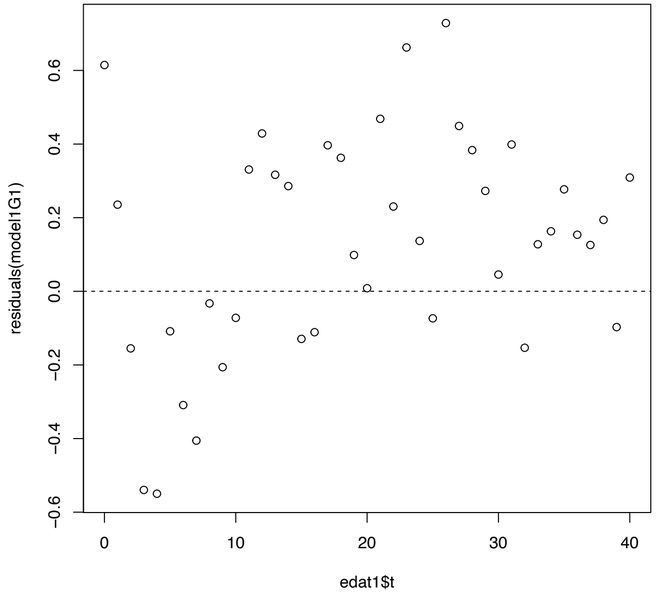
Ignore the old textbooks

Learn to Love R!



$$ax^2 + bx + c = 0 \qquad x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

Always Plot and Analyze Your Residuals!



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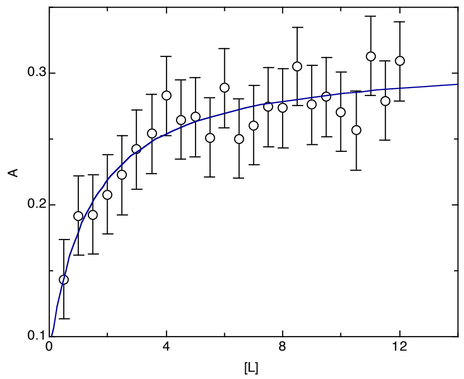

$$ax^2 + bx + c = 0 \qquad x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

R can simulate kinetics!

Always remember that reported errors rely on assumptions

```
Formula: edat1$dat ~ eDecay(edat1$t, ampl, tau)

Parameters:
     Estimate Std. Error t value Pr(>|t|)

ampl   9.5239     0.2294   41.52   <2e-16 ***
tau    6.2702     0.2308   27.16   <2e-16 ***
```

Is the model the correct one?

Are errors “normally distributed”?

Always do a “gut check” on your results