Discussion Session prior to the Second Examination:

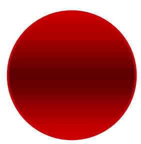
Sunday evening April 13
6 to 8 pm

161 Noyes Laboratory

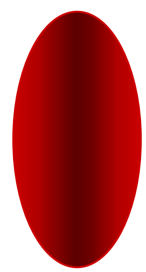
Determination of the Stokes Radius by measuring the Rotational Diffusion Coefficient: D_{rot}

$$D_{rot} = \frac{kT}{f_{rot}} = \frac{kT}{8\pi\eta R_s^3}$$

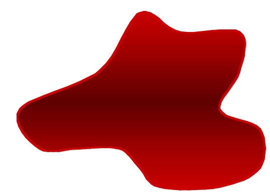
Isotropic rotation



$D_x = D_y = D_z$



$D_x = D_y \neq D_z$



$D_x \neq D_y \neq D_z$

Measure D_{rot}

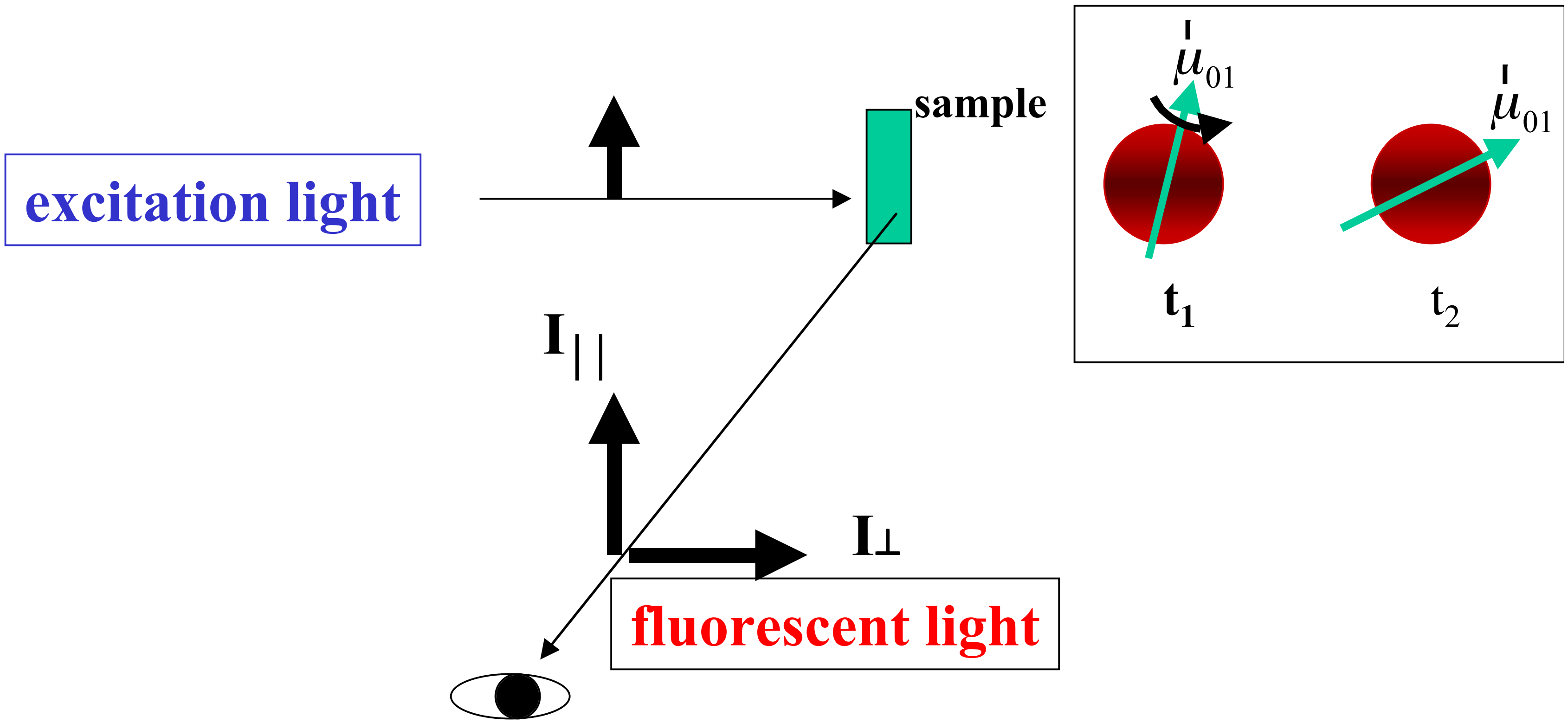
$$D_{rot} = \frac{kT}{8\pi\eta R_s^3}$$

If you know $R_s \Rightarrow \eta$ microviscosity

If you know $\eta \Rightarrow R_s$ molecular size, shape

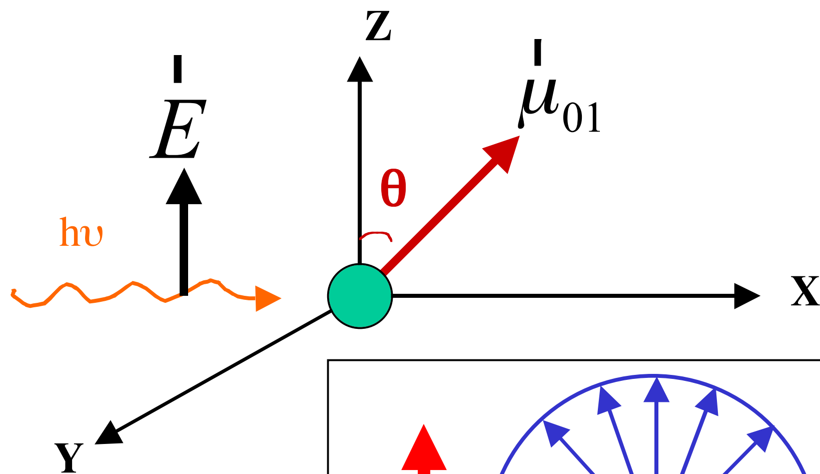
Rotational Diffusion Rate can be determined by Fluorescence Spectroscopy

Fluorescence polarization (anisotropy)
can measure how rapidly a molecule is tumbling in solution

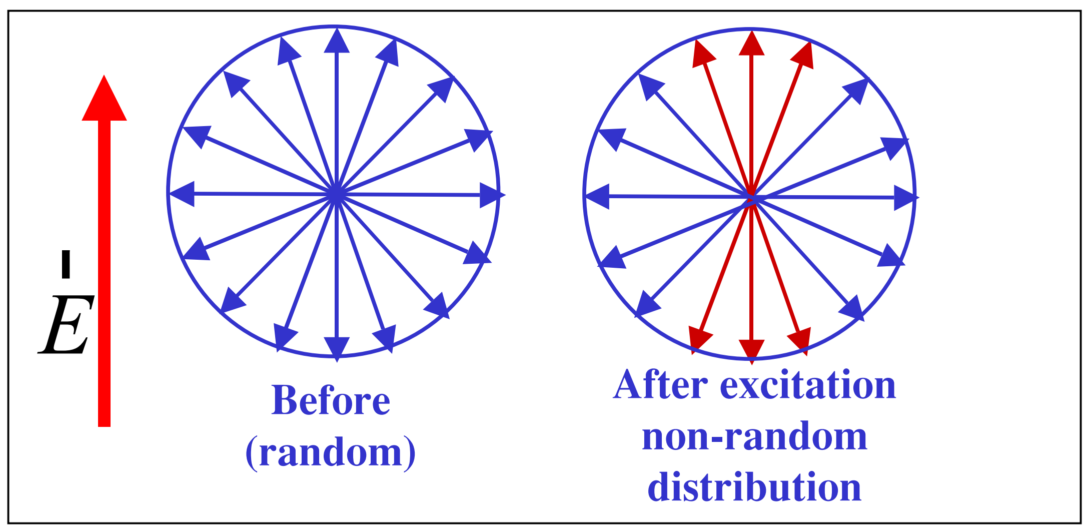


Fluorescence anisotropy

For polarized excitation light, the probability of absorption of a photon depends on $\cos^2\theta$

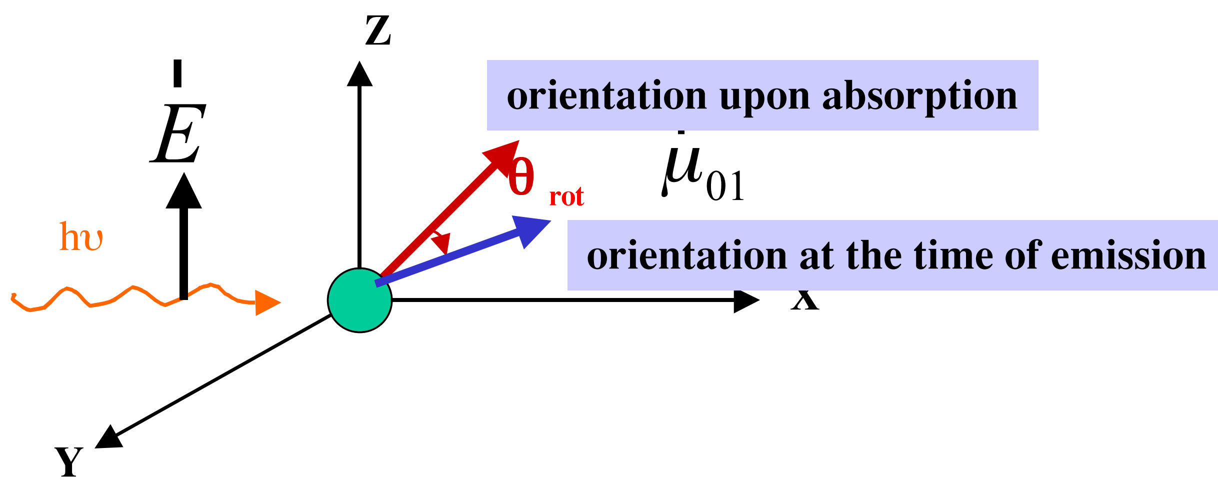


preferential excitation of molecules whose $\vec{\mu}_{01}$ is parallel to the electric field vector of the excitation light

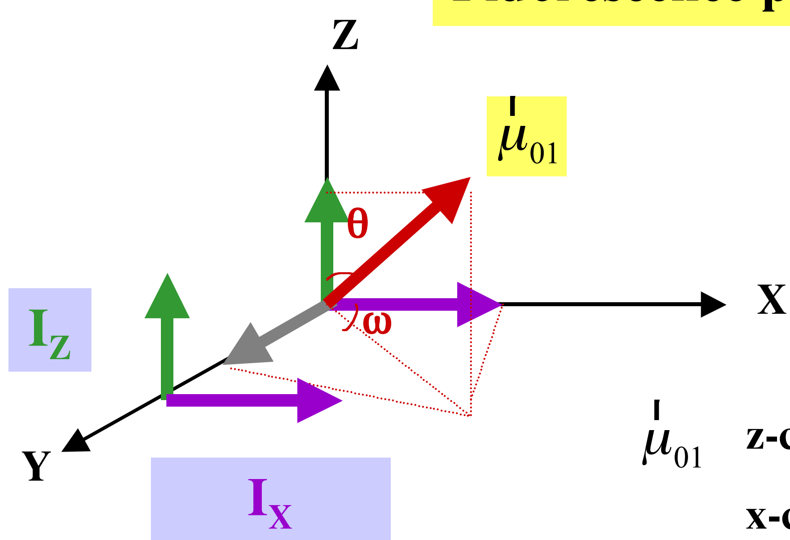


Fluorescence anisotropy

After formation of the excited state, the molecule can rotate during the time prior to photon emission



Fluorescence polarization



The probability of detecting the emitted photon depends on the orientation of the transition dipole moment of the excited state molecule $\vec{\mu}_{01}$ AT THE TIME OF EMISSION and the orientation of the polarizer in the detection device

$\vec{\mu}_{01}$

z-component = $\vec{\mu}_{01}\cos\theta$

x-component = $\vec{\mu}_{01}\sin\theta\cos\omega$

y-component = $\vec{\mu}_{01}\sin\theta\sin\omega$

probability of detecting the photon with polarization along the

z-axis = $|\mu|^2\cos^2\theta$

x-axis = $(\sin\theta\cos\omega)^2$

y-axis = $(\sin\theta\sin\omega)^2$

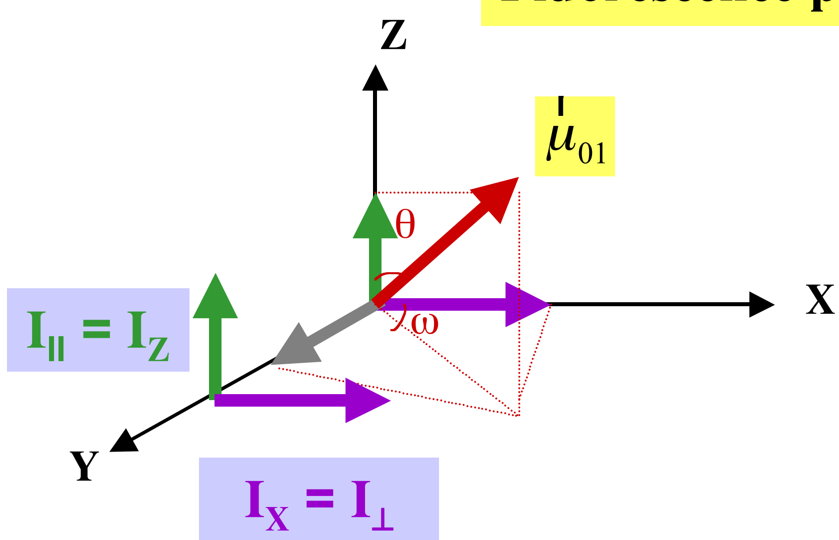
Average over population

I_z proportional to $<\cos^2\theta>$

I_x proportional to $<\sin^2\theta><\cos^2\omega>$

I_Y proportional to $<\sin^2\theta><\sin^2\omega>$

Fluorescence polarization



Define $I_{\parallel} = I_Z$

If we start with excitation light polarized along the z-axis, then

$$I_x = I_y = I_{\perp}$$

The total light intensity at the detector is $I_{tot} = I_{\parallel} + 2I_{\perp}$

Define: polarization, $P = \dfrac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$

anisotropy, $A = \dfrac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$

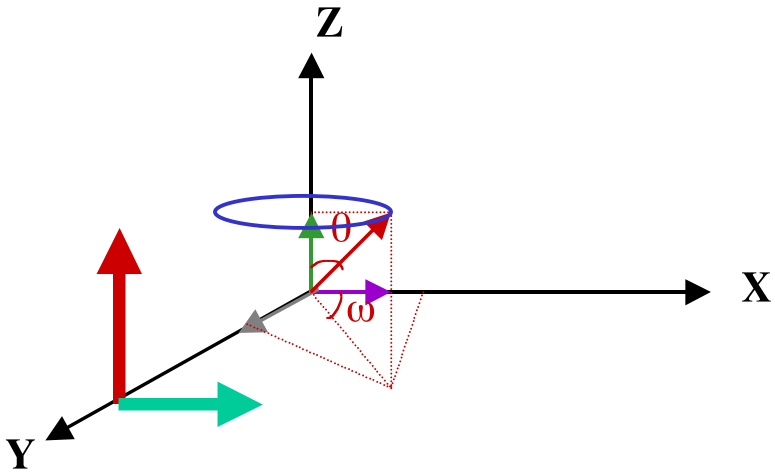
$$\boxed{A = \frac{2}{3}\left[\frac{1}{P} - \frac{1}{3}\right]^{-1}}$$

Polarization

Relate Anisotropy to $< \cos^2\theta >$

$$A = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} = \frac{< \cos^2\theta > - < \cos^2\omega > < \sin^2\theta >}{< \cos^2\theta > + 2 < \cos^2\omega > < \sin^2\theta >}$$

^



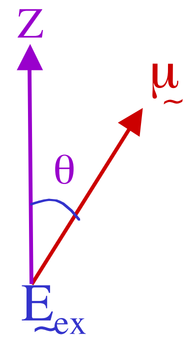
Since we excite with light polarized along Z, we must have symmetry about this axis - so ω will always be random => $< \cos^2\omega > = 1/2$

$$\boxed{A = \frac{3 < \cos^2\theta > - 1}{2}}$$

By measuring I_X and I_Z we can compute $< \cos^2\theta >$ for the emitting molecules

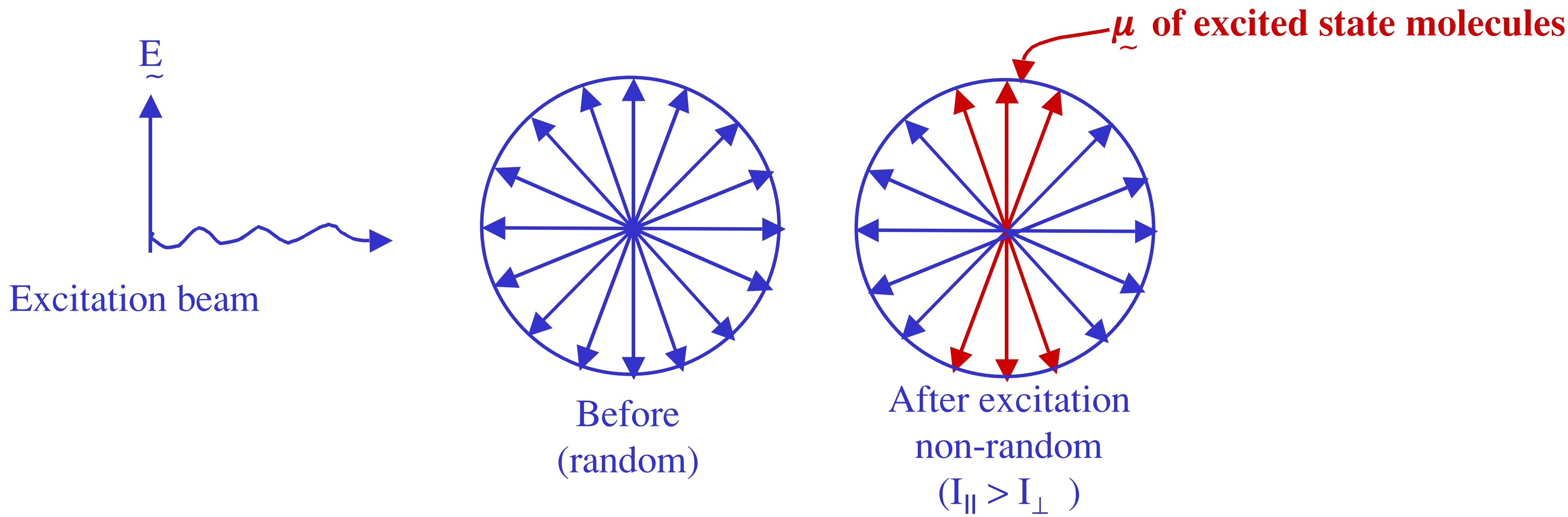
What goes into $< \cos^2\theta >$?

I **Photoselection**



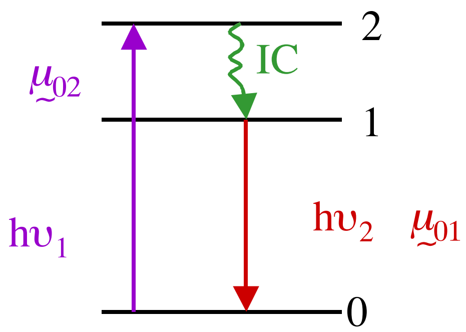
Probability of absorption $\propto \cos^2\theta$

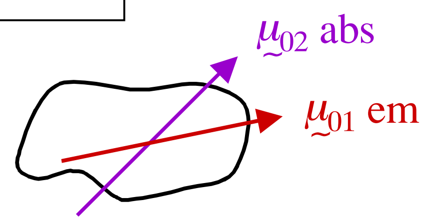
The excited state population is not randomly oriented immediately after excitation



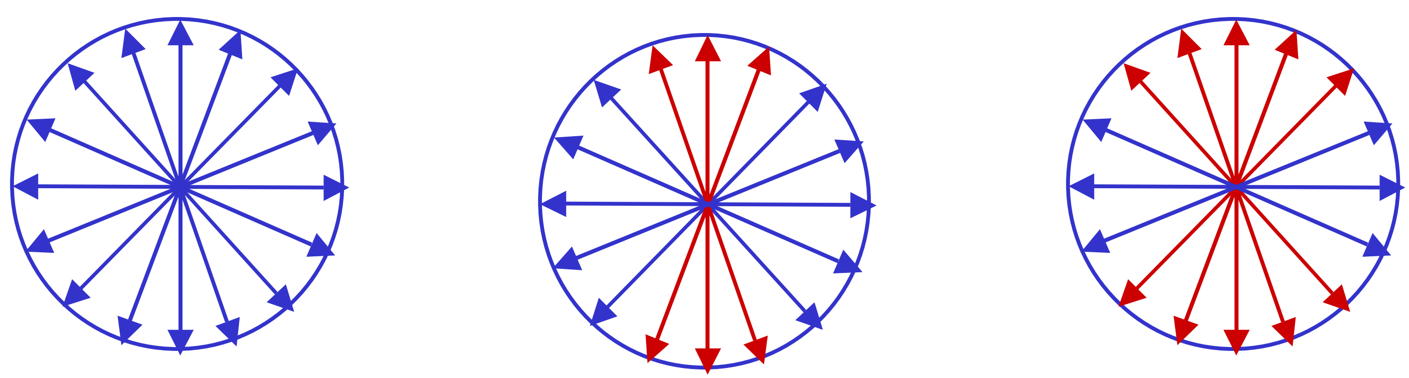
What goes into $< \cos^2\theta >$?

II Change in $\underset{\sim}{\mu}$ for absorption + emission





Fixed angle λ

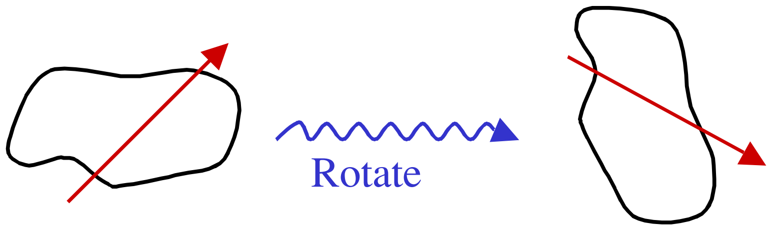


photoselection

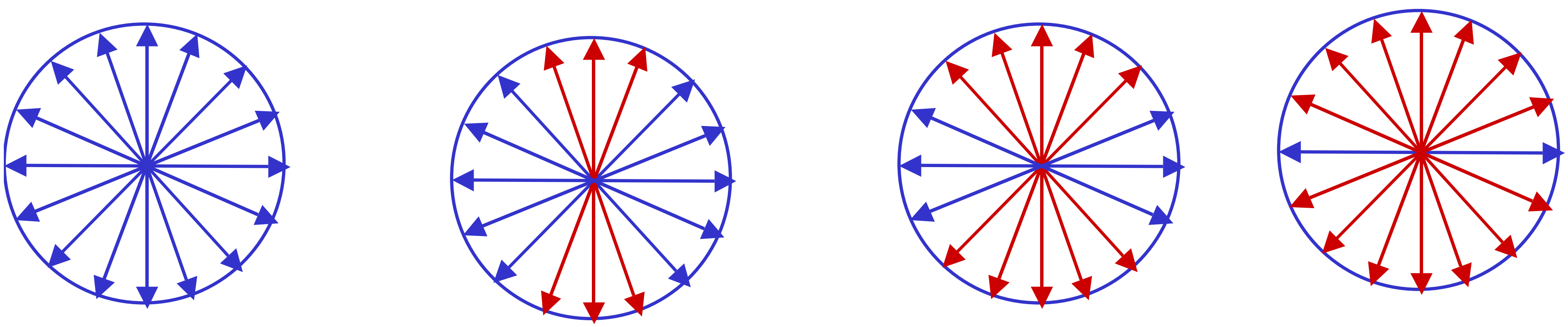
Change μ

What goes into $< \cos^2\theta >$?

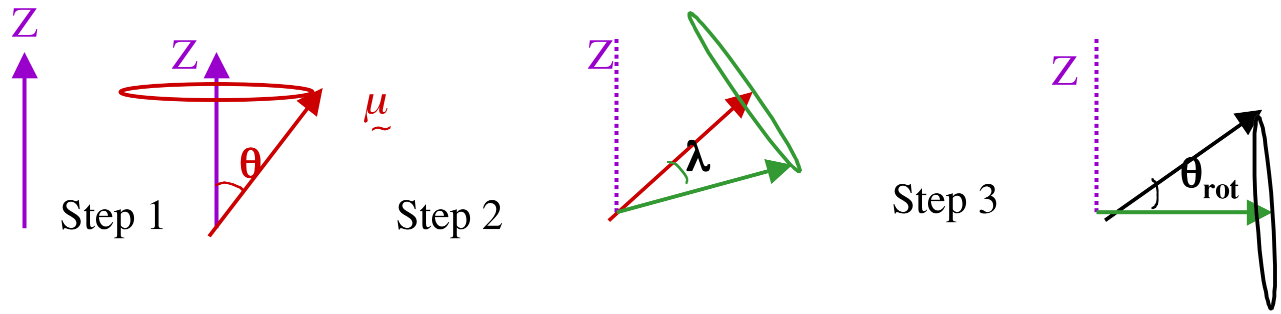
III Rotational diffusion:



Rotational diffusion coefficient D_{rot}



View each of these as a series of isotropic displacements_of the average direction of the transition dipole moment



photoselection

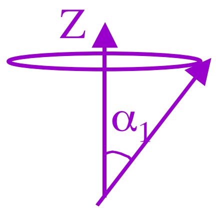
Change μ

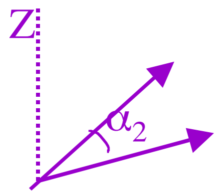
rotation

<u>Goal:</u> Find average displacement (θ) in terms of known parameters

Use Soleillet's equation - from geometry, describing a series of isotropic displacements of a vector

$$\frac{3 < \cos^2\theta > - 1}{2} = \frac{3 < \cos^2\alpha_1 > - 1}{2} \cdot \frac{3 < \cos^2\alpha_2 > - 1}{2} \cdots\cdots \frac{3 < \cos^2\alpha_N > - 1}{2}$$





etc.

Perrin's equation:

$$A = \frac{3 < \cos^2\theta > - 1}{2} = \underset{1}{\frac{3 < \cos^2\theta_0 > - 1}{2}} \cdot \underset{2}{\frac{3 < \cos^2\lambda > - 1}{2}} \cdot \underset{3}{\frac{3 < \cos^2\theta_{rot} > - 1}{2}}$$

Measure this

Find this

$\langle|\theta_{rot}|\rangle \longrightarrow \mathbf{D_{rot}} \longrightarrow$ **Stokes radius**

Photoselection

Photoselection:

$$A = \frac{3 < \cos^2\theta > - 1}{2} = \underbrace{\frac{3 < \cos^2\theta_0 > - 1}{2}}_{1} \cdot \underbrace{\frac{3 < \cos^2\lambda > - 1}{2}}_{2} \cdot \underbrace{\frac{3 < \cos^2\theta_{rot} > - 1}{2}}_{3}$$

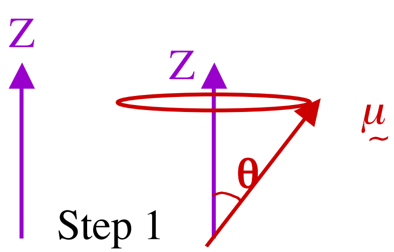
Measure this

Find this

Probability of absorption $\propto \cos^2\theta_0$

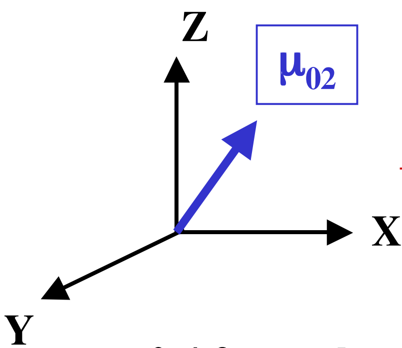
$< \cos^2\theta_0 > = 3/5$ This is derived in the 346 Class Notes

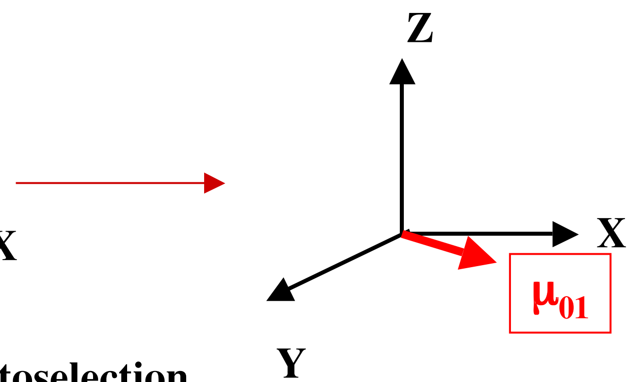
1 **So $A = 0.4$** / **$P = 0.5$** } **Maximum values of Anisotropy and Polarization**

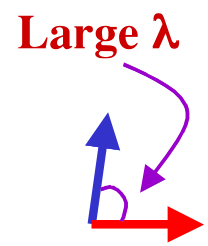


Emit from a different transition after internal conversion

2 Change in $\underset{\sim}{\mu}$:







0.4 from photoselection

$$A_0 = 0.4\left[\frac{3 < \cos^2\lambda > - 1}{2}\right]$$

A_0 is the anisotropy obtained in the absence of molecular rotation - e.g., using frozen solutions or solutions with high viscosity

Depends on excitation + emission wavelengths

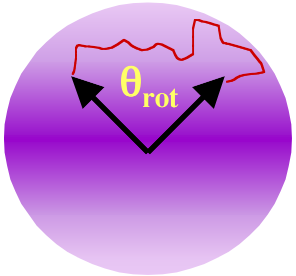
Maximum value for $\lambda = 90°$ so limits on A_0 and P_0 are

$-0.2 \leq A_0 \leq 0.4$

$-0.33 \leq P_0 \leq 0.5$

Rotational Diffusion: will always tend to bring A → 0 or P → 0

$$A = A_0 \left[\frac{3 < \cos^2\theta_{rot} > - 1}{2} \right]$$



Anisotropy of frozen sample

$< \cos^2\theta_{rot} >$ = 1 if there is **no rotation**: $A = A_o$

$< \cos^2\theta_{rot} >$ =1/3 for random orientation: $A = 0$

Rotational Diffusion

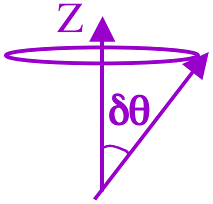
3 **Rotational Diffusion: will always tend to bring A → 0 or P → 0**

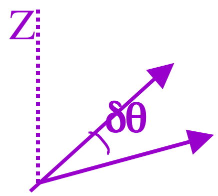
A_o value from photoselection plus change in μ

$$A = A_0 \left[\frac{3 < \cos^2\theta_{rot} > - 1}{2} \right]$$

Break down rotation into a series of N isotropic steps of size $\delta\theta$

$$\frac{3 < \cos^2\theta > - 1}{2} = \frac{3 < \cos^2\delta\theta > - 1}{2} \cdot \frac{3 < \cos^2\delta\theta > - 1}{2} \ldots\ldots \frac{3 < \cos^2\delta\theta > - 1}{2}$$





etc.

$$\cos^2\delta\theta = 1 - \sin^2\delta\theta \approx 1 - \delta\theta^2$$

For a small "step" Rotational Diffusion: $< \delta\theta^2 > = 4\, D_{rot}\, \delta t$

$$= 1 - 4\, D_{rot}\, \delta t$$

$$\boxed{\frac{3 < \cos^2\theta_{rot} > - 1}{2} = (1 - 6\, D_{rot}\, \delta t)^{\overbrace{t/\delta t}^{N}}}$$

Rotational Diffusion

$$\frac{3 < \cos^2\theta_{rot} > - 1}{2} = (1 - 6\, D_{rot}\, \delta t)^{\overbrace{t/\delta t}^{N}}$$

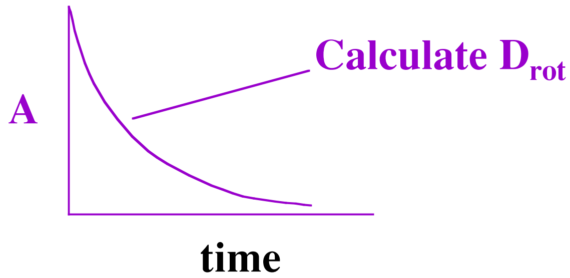
But $(1\text{-}x) \approx e^{-x}$ for $x << 1$

$$\boxed{\frac{3 < \cos^2\theta_{rot} > - 1}{2} = e^{-6\, D_{rot}\, t}}$$

$$\boxed{A = A_0 e^{-6\, D_{rot}\, t}}$$

Time-resolved Anisotropy yields D_{rot}

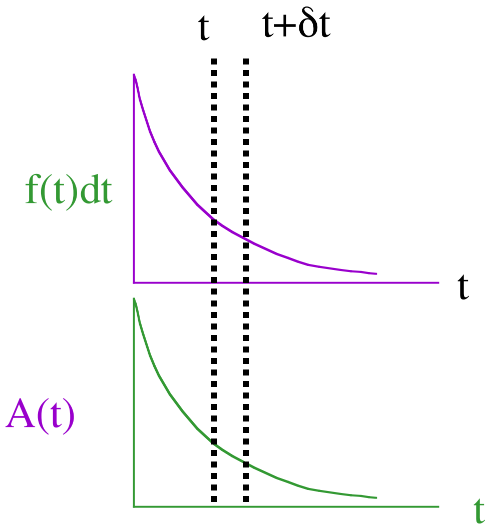
Monitor anisotropy following a pulse of excitation light:



Steady State Measurement of Fluorescence Anisotropy

Fraction of photons emitted in (t, t+dt) = f(t)dt

$$f(t) = \frac{e^{-t/\tau}}{\tau} dt$$



Average anisotropy $$A = \int_0^\infty A(t) f(t) dt$$

$$A = \int_0^\infty A_0 e^{-6D_{rot}} e^{-t/\tau} (1/\tau) dt$$

$$\boxed{A = A_0 (1 + 6\, D_{rot}\, \tau)^{-1}}$$

Perrin's equation

Various forms of Perrin's equation

(1) $$\frac{A_0}{A} = 1 + 6\,D_{rot}\tau$$

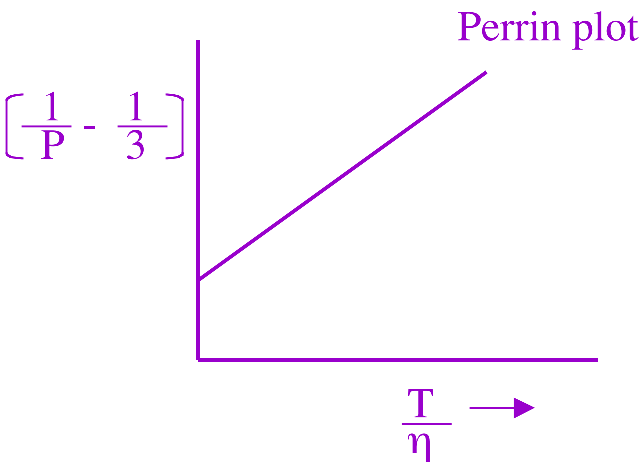
(2) $$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)(1 + 6D_{rot}\tau)$$

substitute : $$D_{rot} = \frac{kT}{8\pi\eta R_s^3} = \frac{kT}{6\eta V}$$

V ≡ molecular volume

(3) $$\frac{A_0}{A} = 1 + \frac{k}{V}\left[\frac{T\tau}{\eta}\right] = 1 + C\frac{T\tau}{\eta}$$

(4) $$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{\tau k}{V}\left[\frac{T}{\eta}\right]\right)$$



Define: rotational diffusion time = $$\rho = \frac{1}{2D_{rot}}$$

(5) $$\frac{A_0}{A} = 1 + \frac{3\tau}{\rho}$$ $$\rho = \frac{4\pi\eta R_s^3}{kT} \leftarrow \text{seconds}$$

Result for steady state fluorescence from a rotating molecule

$$\boxed{\frac{A_0}{A} = 1 + 6\,D_{rot}\,\tau}$$

Measure this

$$\boxed{\frac{kT}{8\pi\eta R_s^{3}}}$$

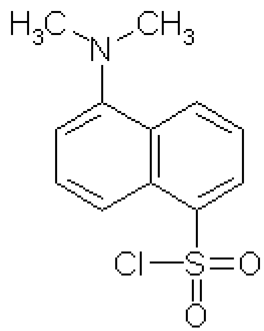
Fluorescence lifetime

Define: rotational diffusion time = $$\rho = \frac{1}{2D_{rot}}$$

$$\frac{A_0}{A} = 1 + \frac{3\tau}{\rho} \qquad \rho = \frac{4\pi\eta R_s^3}{kT} \leftarrow \text{seconds}$$

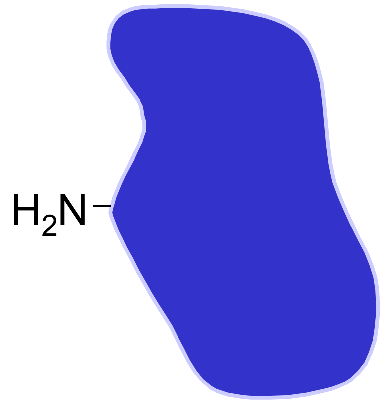
Protein Rotational Diffusion

I. Extrinsic Probes

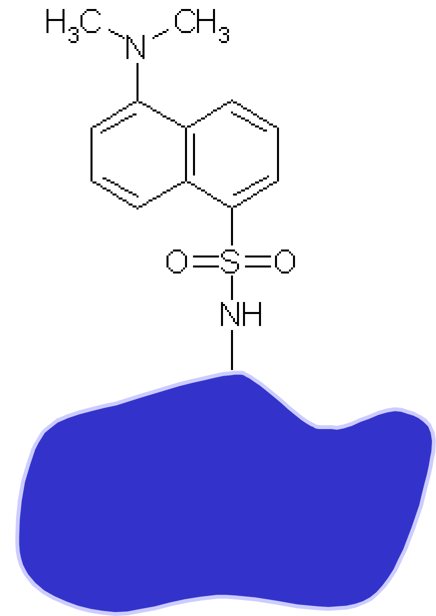


Dansyl chloride

+



τ = 14 nsec

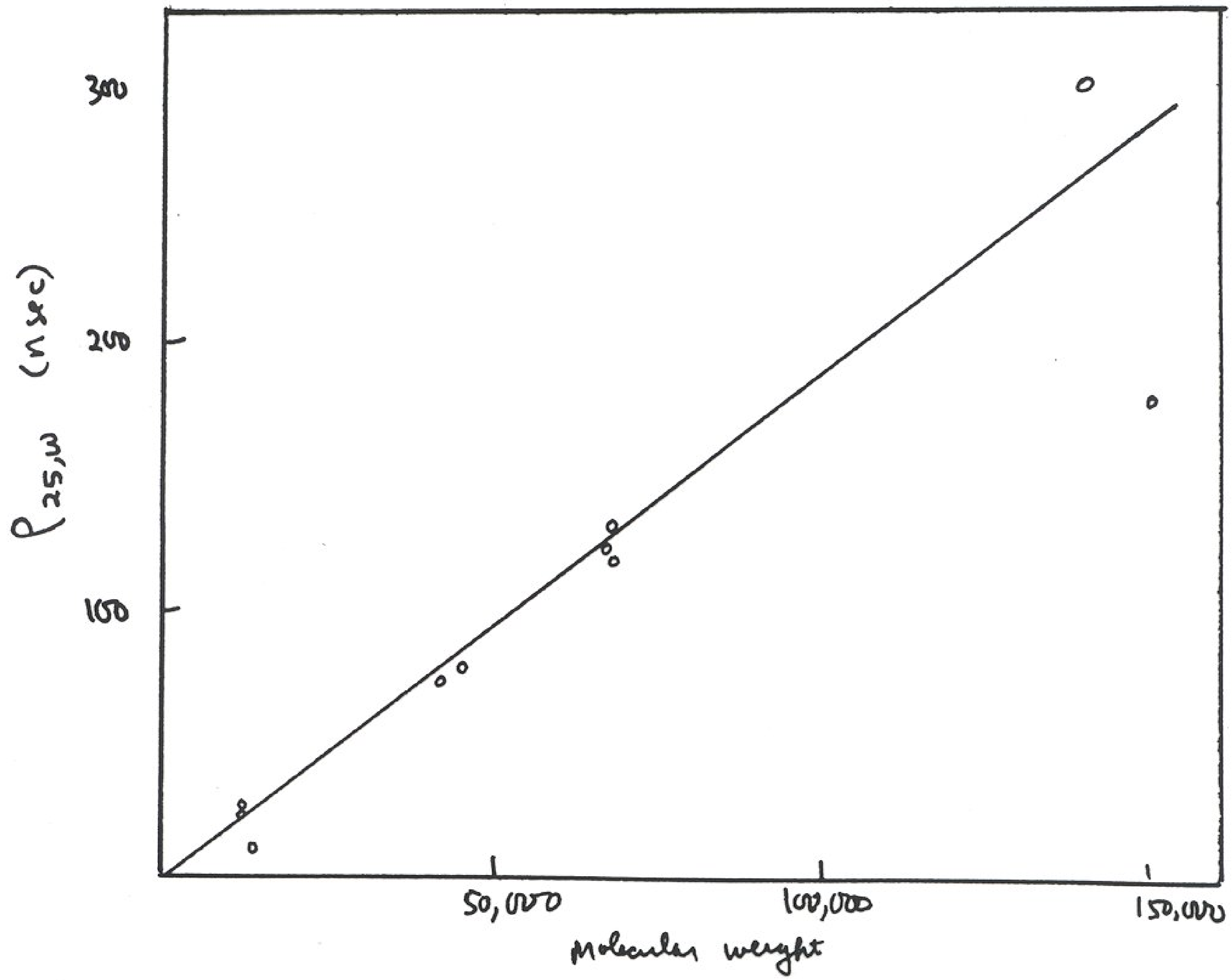


II. Results

Protein	*M*	*ρ*	*ρ/ρ₀*
Avidin	71,000	100	1.78
BSA	67,000	127	2.39
Enolase	82,000	90	1.35
rIgG	160,000	127	1.0
LDH	138,000	188	1.65

$$\boldsymbol{\rho_0 = (3\eta V) / (kT) = (3hV_2M) / (NkT) \approx [(\mathrm{mol.wt}) / 1.15] \times 10^{-3}\ \mathrm{nsec}}$$

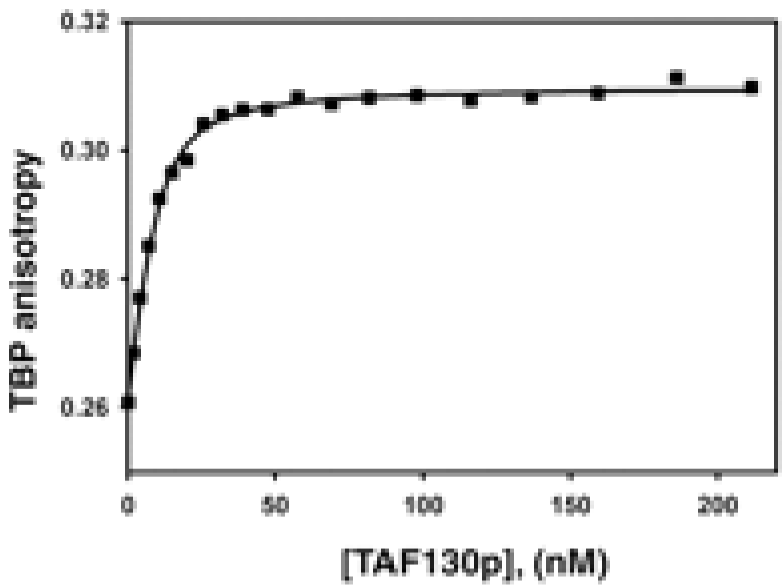
$(A_0 / A) = 1 + (3\tau / \rho)$ Measure A_0, A, τ $\longrightarrow$ find ρ



Rotational diffusion times of globular proteins measured using dansyl conjugates.

Example: Fluorescence Anisotropy Measurement of Protein-Protein Interactions

TATA-box Binding Protein (TBP) binds with high affinity to TBP-associated Factor subunit TAF130p



1. Label TBP with tetramethyl rhodamine at a reactive cysteine residue
2.To a solution of 100 nM TBP (labeled) titrate increasing amounts of TAF130p (0-255 nM)
3. Exitation at 540 nm; emission monitored at 575 nm

Conclude: High affinity binding (K_d = 0.5 nM)

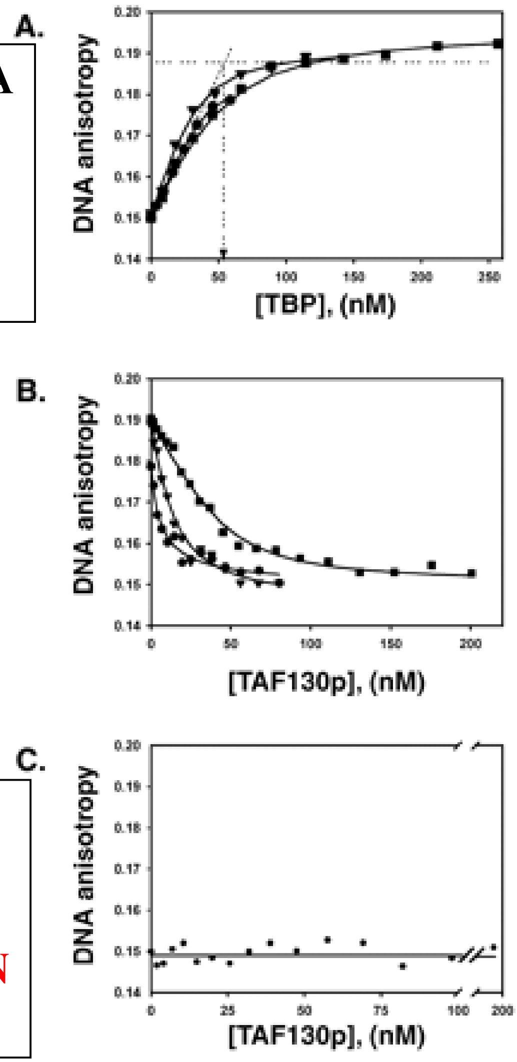
Biological consequence: binding of TAF130p to TBP competes with DNA binding to TBP

J. Biol. Chem., Vol. 276, Issue 52, 49100-49109, December 28, 2001

Fluorescence Anisotropy used to monitor Protein-DNA Interactions

1. 50 nM Rhodamine labeled DNA
2. Titrate TBP
3. Excitation at 580 nm/Emission at 630 nm

SHOWS 1:1 COMPLEX



1. 1:1 DNA:TBP complex, with labeled DNA (50, 100, 250 nm TBP)
2. Titrate TAF130p

SHOWS ELIMINATION OF TBP-DNA COMPLEX BY COMPETING TAF130p

1. 50 nm labeled DNA
2. Titrate with TAF130p

SHOWS NO COMPLEX BETWEEN DNA AND TAF130p

J. Biol. Chem., Vol. 276, Issue 52, 49100-49109, December 28, 2001

Fluorescence anisotropy used to measure microviscosity

The rotational diffusion coefficient can be related to
Molecular volume: V

$$D_{rot} = \frac{kT}{8\pi\eta R_s^3} = \frac{kT}{6\eta V}$$

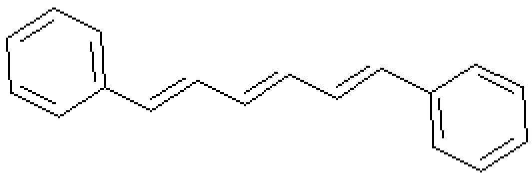
V ≡ molecular volume

$$\frac{A_0}{A} = 1 + \frac{k}{V}\left[\frac{T\tau}{\eta}\right]$$

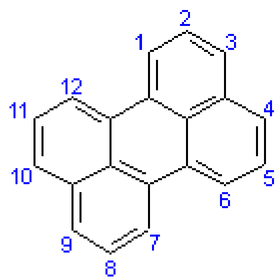
Measure this

If you know V
You can determine η

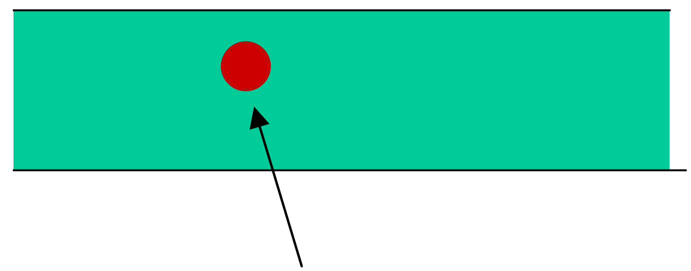
Fluorescent Probes dissolved are used to measure Membrane microviscosity



Diphenyl hexatriene (DPH)



Perylene



Probe dissolved in membrane bilayer

<u>Hydrophobic probes:</u>

— partition into membrane bilayer
— do not bind to protein
— rotation reports <u>local</u> viscosity

$$\frac{A_0}{A} = 1 + \frac{k}{V}\left[\frac{T\tau}{\eta}\right]$$

Measure for specific probe

Molecular constant: determine for probe in known viscosity (η)

Then: A => η

Typically: for biological membranes

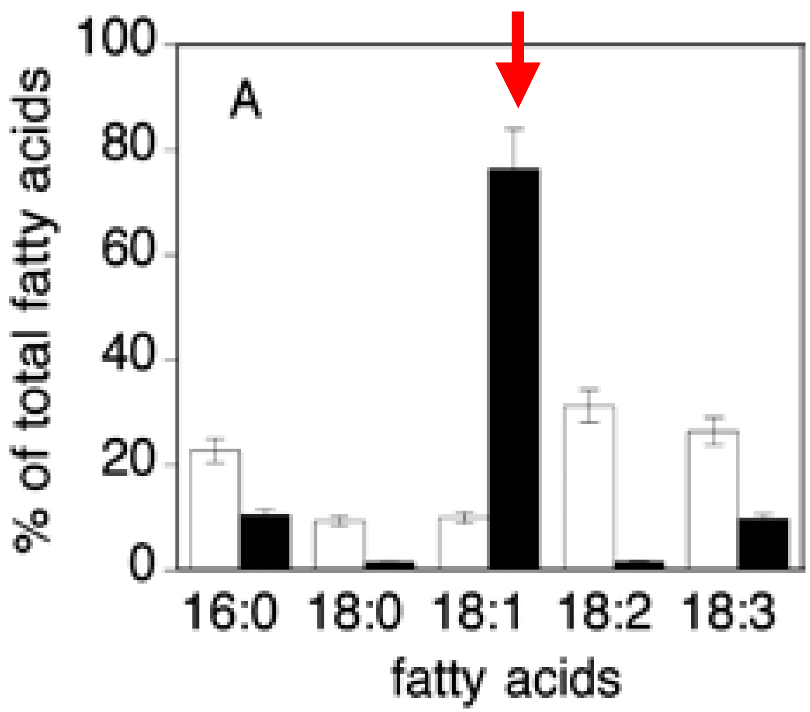
$\eta \approx 1$ Poise

100-times the viscosity of water

Note: $(1 / \eta) =$ fluidity of membrane

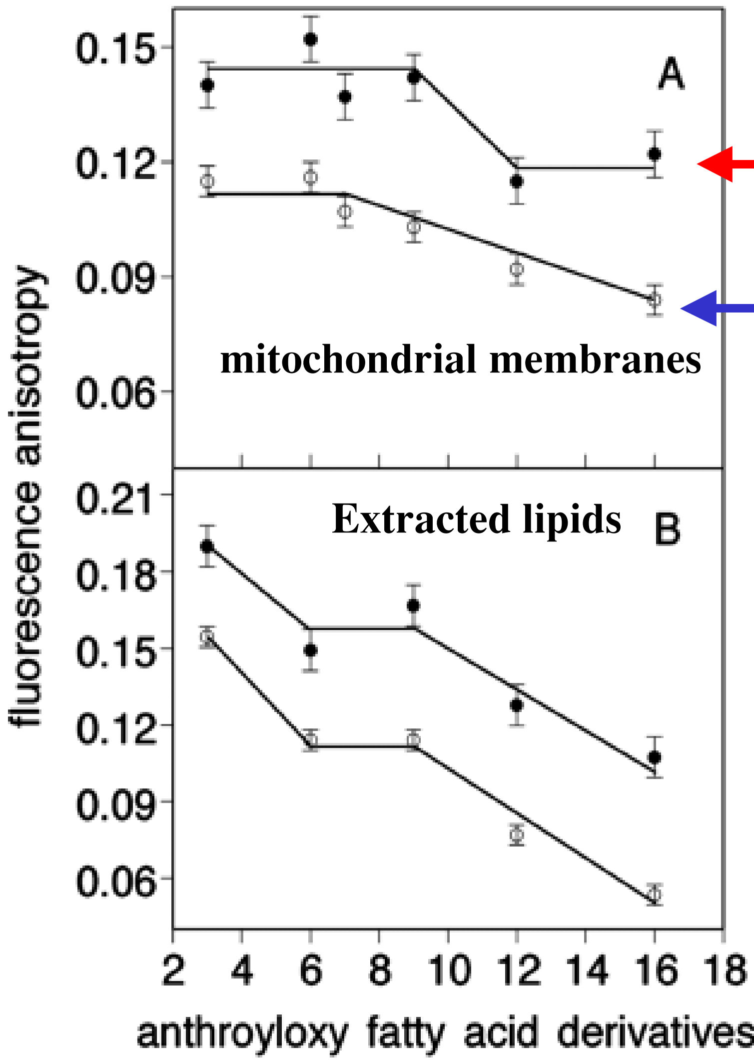
Example: The effect of deletion of the gene encoding ω-6-oleate desaturase in *Arabidopsis thaliana*

Changes the fatty acid composition of the mitochondrial membrane: mostly oleic acid is present in the mutant (*fad*2)



J. Biol. Chem., Vol. 276, Issue 8, 5788-5794, February 23, 2001

Membrane Fluidity monitored by the fluorescence Anisotropy of anthroyloxy fatty acid derivatives: the fluorophore is located at different depths in the membrane bilayer



depth in the membrane bilayer/position in fatty acid chain)

mutant

wild-type

CONCLUDE: Decrease in unsaturation of the fatty acid components in the membrane results in increased anisoptropy, or increase in membrane viscosity

Biological Consequence: decreased respiration and altered bioenergetics of the mitochondria