Fluorescence Depolarization

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Small molecules rotate quickly

Large molecules rotate slowly

There is a time delay from the moment a molecule is excited to the time it fluoresces

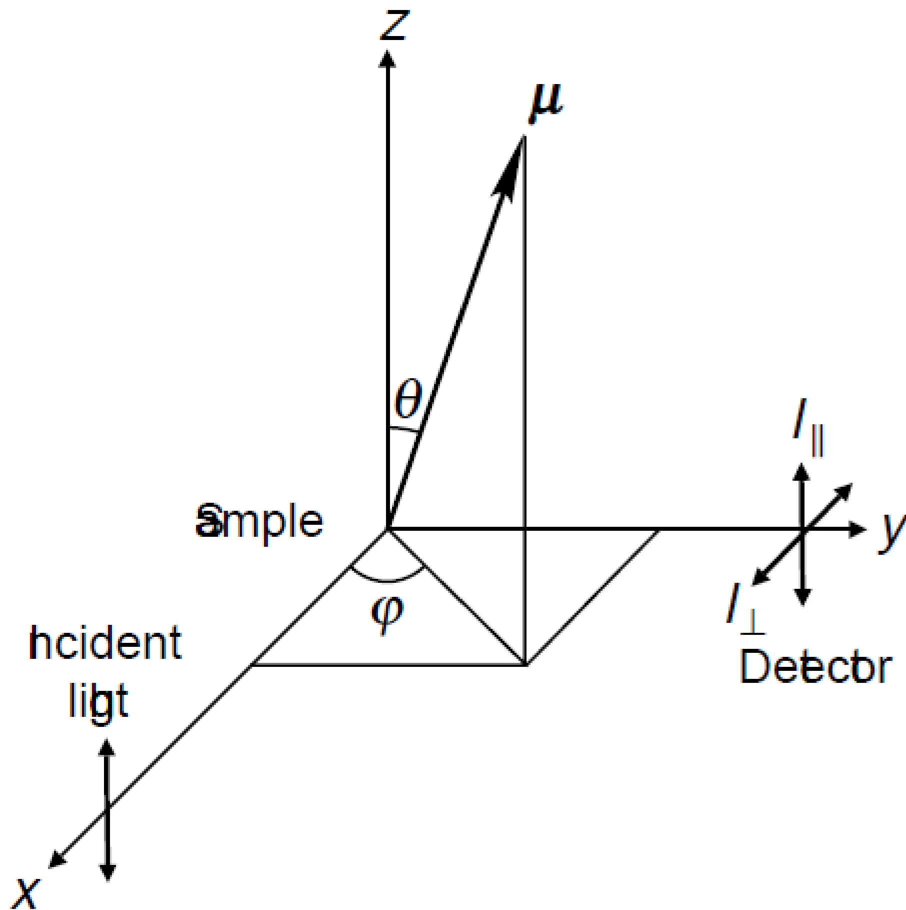
Small molecules → non polarised fluorescence

Large molecules → polarized fluorescence

Make it Glow: Fluorescence Probes

1. Intrinsic probes
   a. naturally occur - aromatic amino acids (tryptophan and tyrosine), NADH, FAD, chlorophylls...
2. Analogues of intrinsic probes
   a. 5-hydroxyltryptophan and 7-azatryptophan
3. Extrinsic probes
   a. 3rd-party structure that attaches to molecule (either covalent or non-covalent)
   b. Thousands available – dimethylaminonaphthalene sulphonyl chloride (dansyl chloride), 8-anilino-1-naphthalene sulphonats (ANS), 1,6-diphenyl-1,3,5-hexatriene (DPH), etidium bromide, sulphonyl chloride…………....soooo manyyyy

Steady-state fluorescence depolarisation



- Light comes in from the x-direction polarized in the z-direction
- molecules absorbs light along it's dipole
- fluorescence is detected along y-axis with parallel and perpendicular components

A = anisotropy (number that represents how directionally dependent the light is)

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

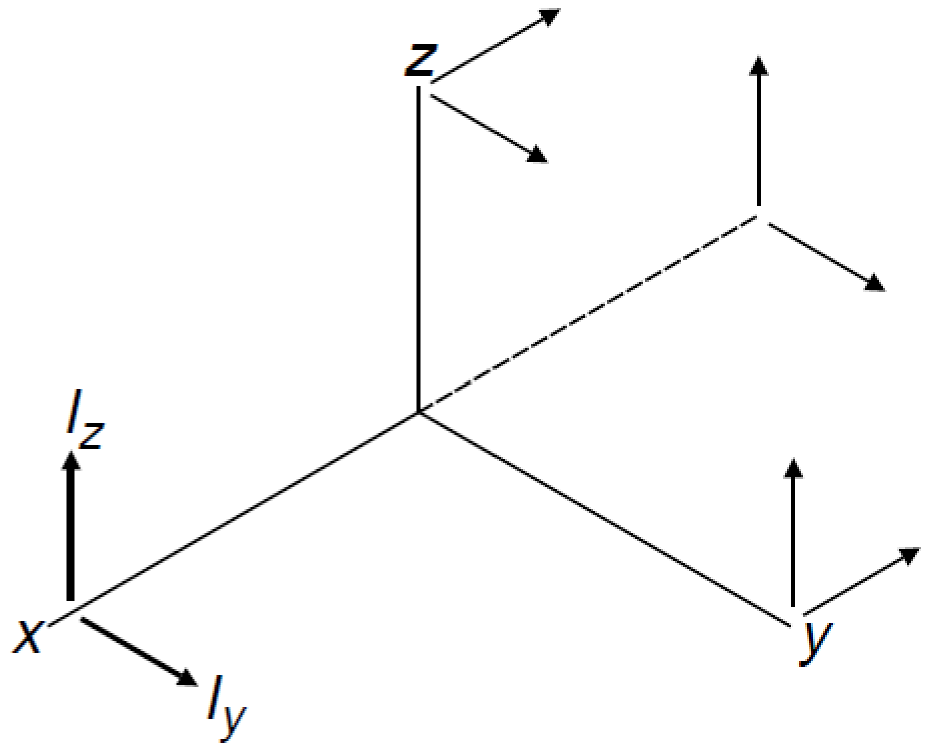
P = polarization

I = intensity

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

When parallel and perpendicular components are the same → A decreases to 0 → molecule is small and rotating randomly

Definition of anisotropy A: non-polarised light incident along x can be resolved into y- and z-polarised components. The total emission can be found by adding the emission along the three Cartesian axes.



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

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

denominator is total light if no polarization present (4 perpendicular and 2 parallel)

More generally, for the model of a rigid spherical molecule...

$$P_0 = \frac{3\cos^2\zeta - 1}{\cos^2\zeta + 3}$$

Eq (D8.3)

$$A_0 = \frac{3\cos^2\zeta - 1}{5}$$

Eq (D8.4)

ζ is the angle between the absorption and emission transition dipoles (dipole shifts upon absorption)

No rotation → $\zeta = 0$ → A goes to a max

Time-resolved fluorescence depolarization

Decay of Emission:

- Measure how long the molecule stays excited for
- From a few picoseconds to tens of nanoseconds
- Dependent on the molecular properties of the fluorophore

Anisotropy Decay:

"Monitors reorientation of the emission dipole during the excited-state lifetime and gives information on local fluorophore motion, segmental motion and the overall rotational diffusion of macromolecules"

Anisotropy Decay Continued

Observed polarization is a function of lifetime and the relaxation rate of the protein, represented in the Perrin equation:

$$A_0 = \frac{A_F}{1 + \tau_F/\tau_c}$$

A0 = steady-state

AF= A of fluorophore in the absence of rotation

TF = lifetime

TC = rotational correlation time

Anisotropy Decay Continued

$$A_0 = \frac{A_F}{1 + \tau_F/\tau_c}$$

TF >> TC → A0 approaches 0

TF << TC → A0 approaches AF

Note: fluorescence anisotropy is a ratio independent of concentration because each of the two components is proportional to the concentration

Measuring Time-resolved fluorescence

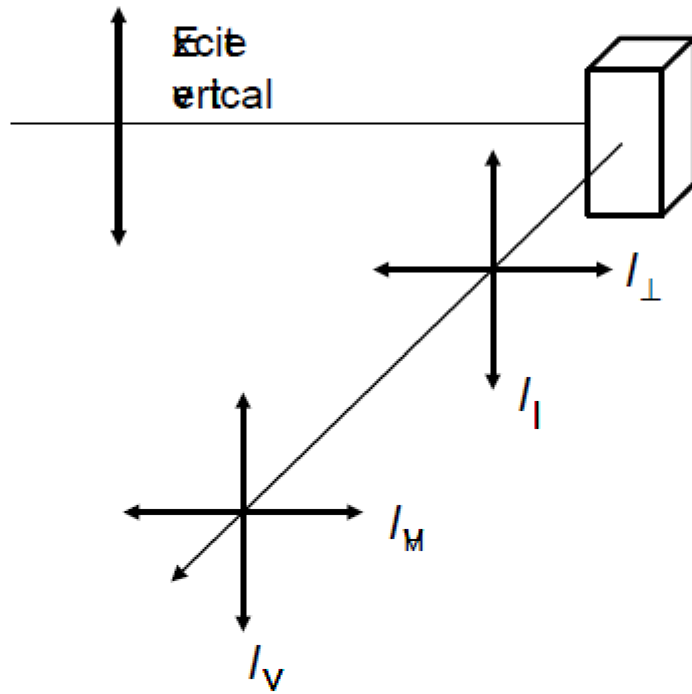
Harmonic-response method:

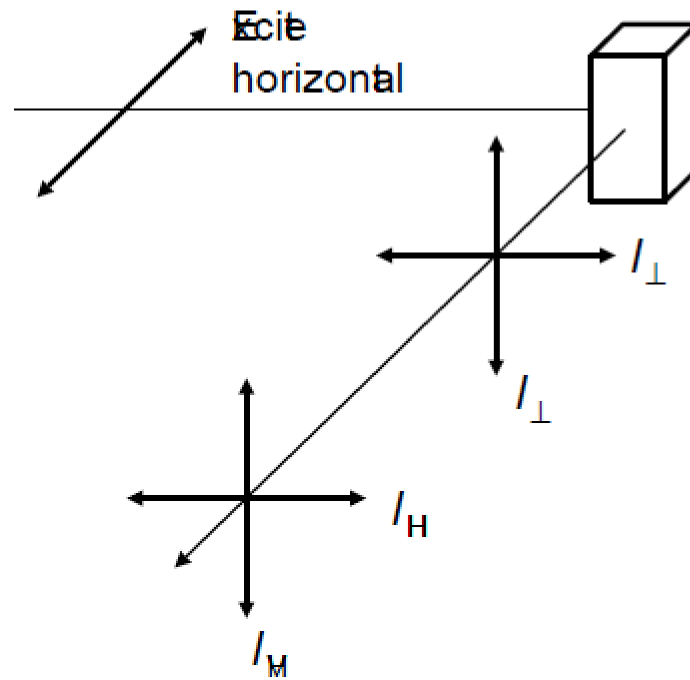
- use a modulating, sinusoidal excitation

Impulse-response method (more popular):

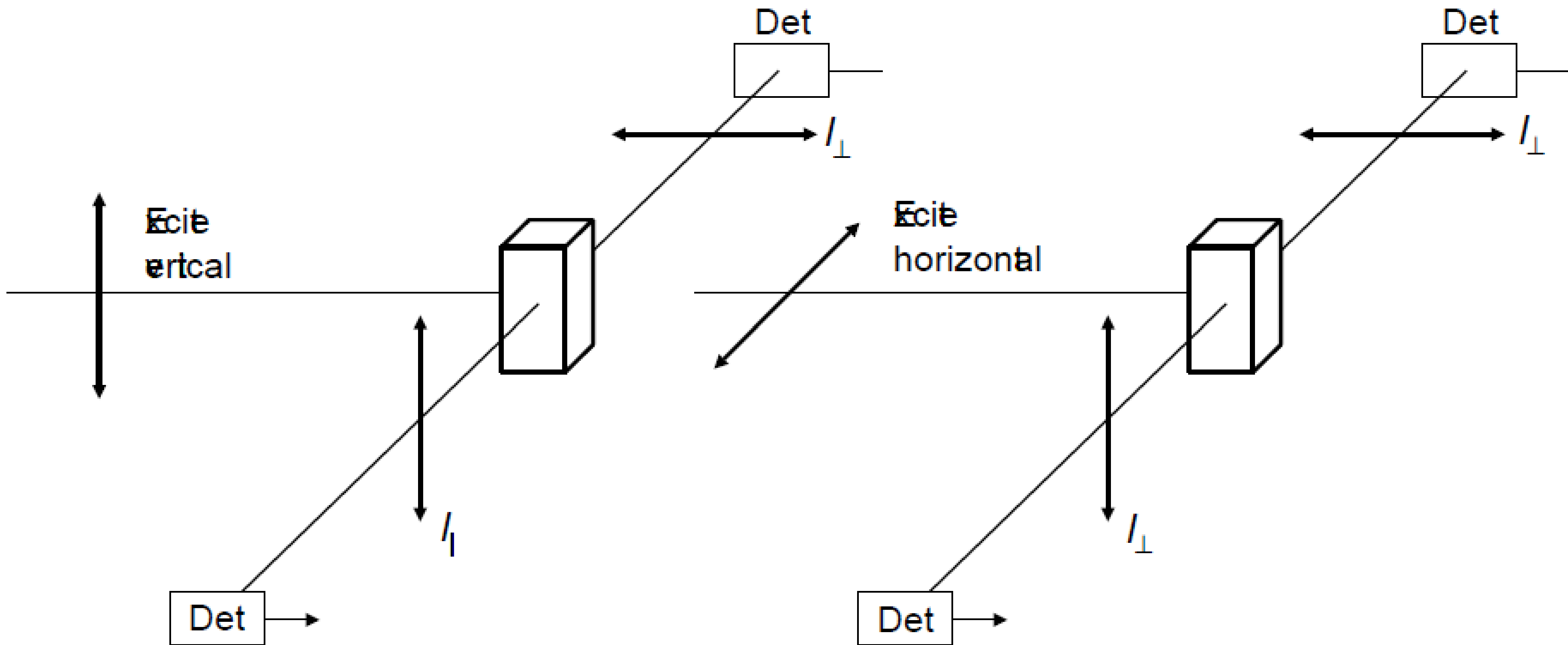
- quick pulse
- Picosecond or subpicosecond pulse from mode-locked dye lasers or titanium-sapphire lasers (visible and UV spectrums)

L-format...





…vs T-format



Devices

“Emission decay profiles are recorded by the time-correlated, single-photon-counting technique using fast photomultiplier tubes”

Photoelastic Modulator (PEM)

- uses the photoelastic effect to measure/produce polarization in light
- stress a transparent solid → polarized light passing through changes speed

Steady-State/Static Polarization

Use constant illumination to measure average polarization and anisotropy

Perrin equations

$$\frac{1}{\bar{P}} - \frac{1}{3} = \left(\frac{1}{\bar{P}_0} - \frac{1}{3}\right)\left(1 + \frac{\tau_{\mathrm{F}}}{\tau_{\mathrm{cor}}}\right) = \left(\frac{1}{\bar{P}_0} - \frac{1}{3}\right)\left(1 + \frac{\tau_{\mathrm{F}} k T}{V_{\mathrm{h}} \eta}\right)$$

$$\frac{1}{\bar{A}} = \frac{1}{\bar{A}_0}\left(1 + \frac{\tau_{\mathrm{F}}}{\tau_{\mathrm{cor}}}\right) = \frac{1}{\bar{A}_0}\left(1 + \frac{\tau_{\mathrm{F}} k T}{V_{\mathrm{h}} \eta}\right)$$

Steady-State/Static Polarization

$$\frac{1}{\bar{A}} = \frac{1}{\bar{A}_0}\left(1 + \frac{\tau_F}{\tau_{cor}}\right) = \frac{1}{\bar{A}_0}\left(1 + \frac{\tau_F k T}{V_h \eta}\right)$$

Notice the T/n relationship

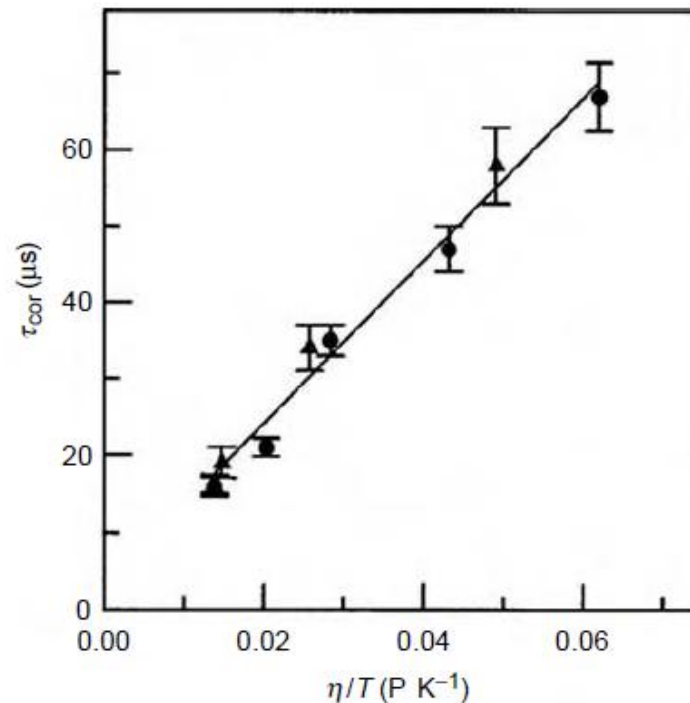
If T is small and n is large → little rotation → TF/Tc approaches 0

Then A0 becomes equation D8.4

Perrin Plot

plotting 1/A0 against T/η should result in a straight line plot

The slope yields the hydrodynamic volume (Vh), provided that the fluorescence decay time (τF) is known

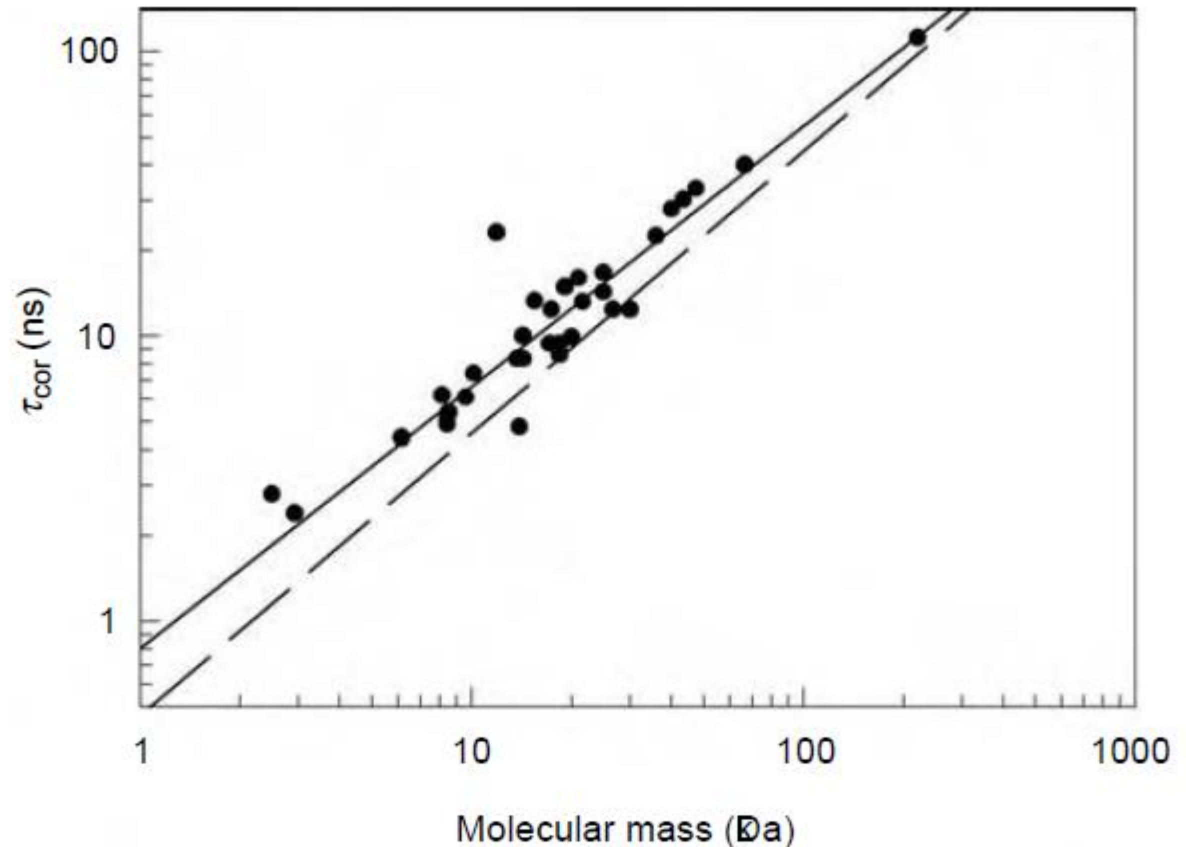


Other things to note

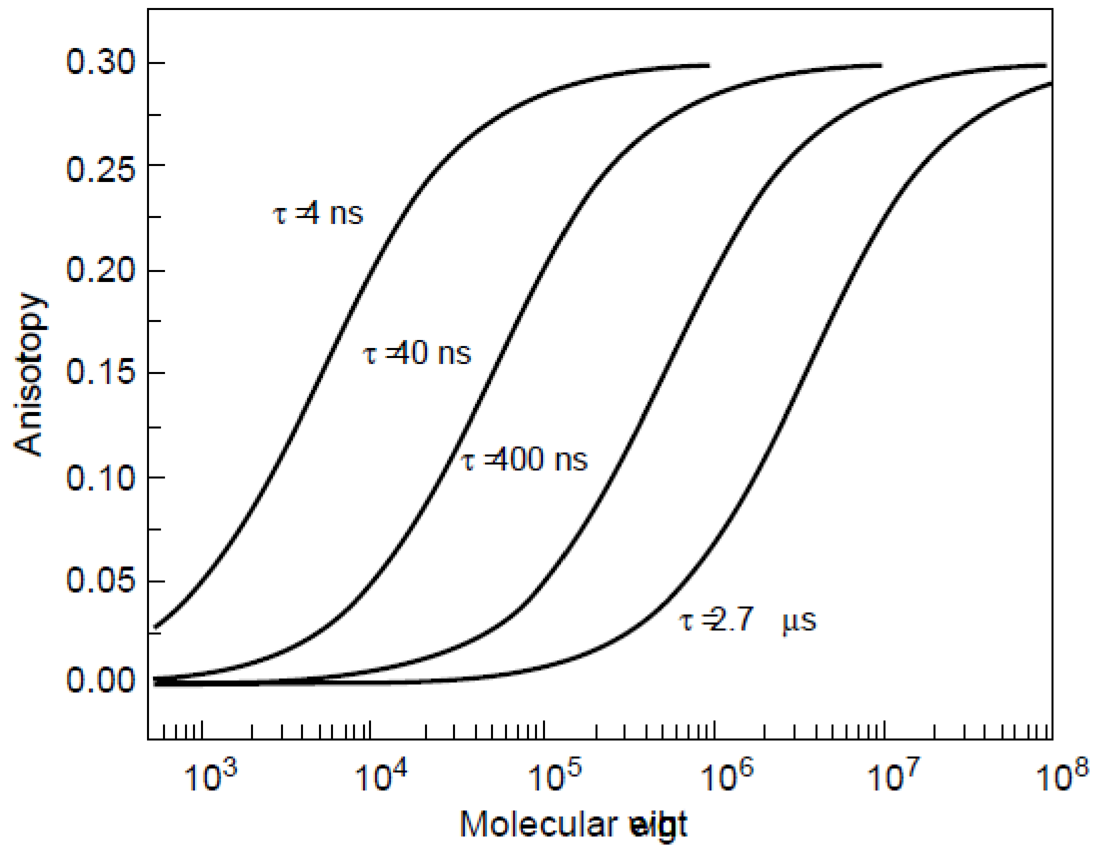
$$\tau_{\mathrm{cor}} = \frac{1}{6\Theta} = \frac{V_{\mathrm{h}}\eta_0}{kT}$$

Where Theta is rotational diffusion coefficient when modeling the molecule as a sphere

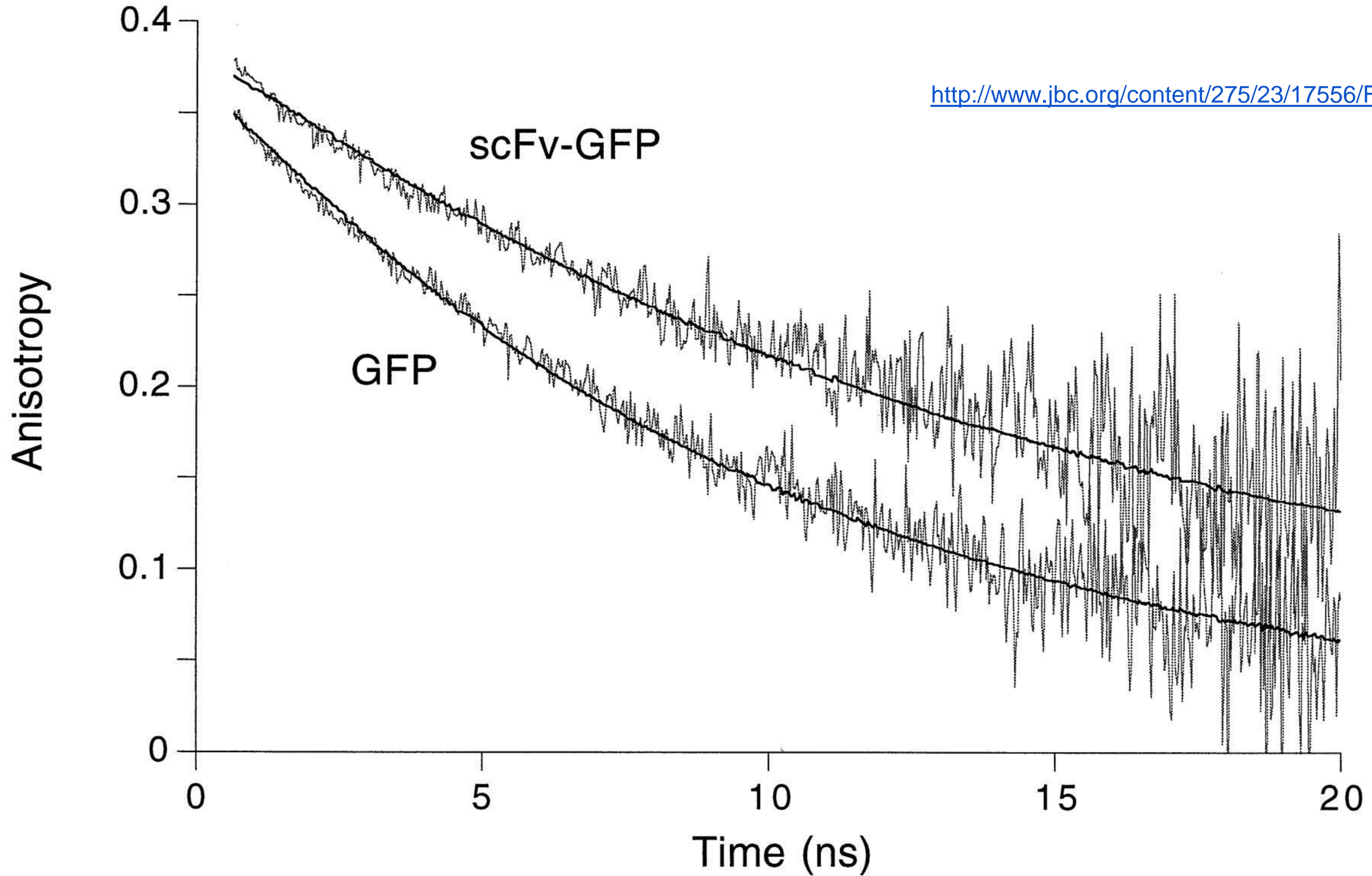
Fig. D8.12 Dependence of the rotational correlation times of 32 different proteins on molecular mass. Data are taken from Table D8.1. The dashed straight line corresponds to data predicted by Eq. (D8.24).



On page 459, note how the larger masses have higher rotational correlation times



more weight → less rotation → higher anisotropy



http://www.jbc.org/content/275/23/17556/F2.large.jpg

More time → more random motion → anisotropy decreases