Fluorescence anisotropy

Fluorescence methods in life sciences

Ctirad Hofr

The phenomenon of anisotropy

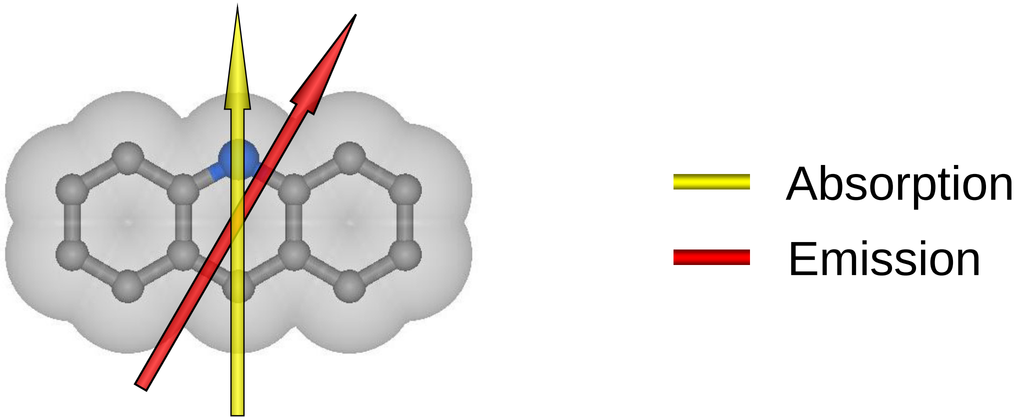
- When excitation is caused by light vibrating in one plane, it is linearly polarized, fluorescence emission becomes polarized.
- The level of emission polarization is described by anisotropy (heterogeneity). Substances that show certain degree of heterogeneity emit polarized fluorescence.
- Why is emitted light polarized?

Anisotropy around us: monitor

- Anisotropy occurs in LCD monitor
- What does cause it?

http://www.youtube.com/watch?v=imQnWrLW2i0

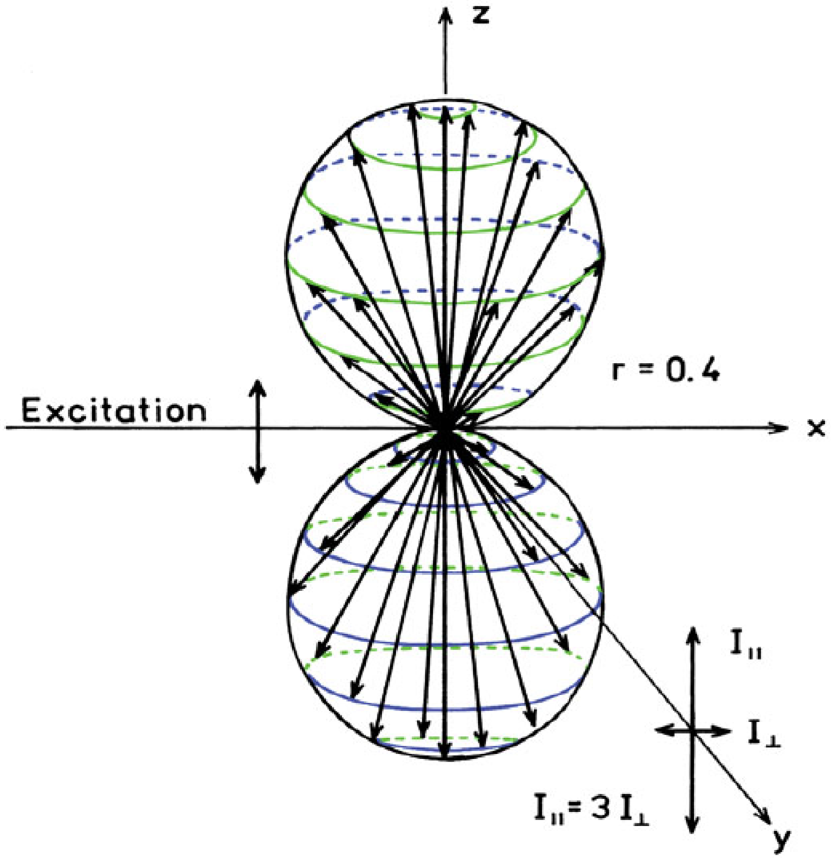
Transition dipole moment



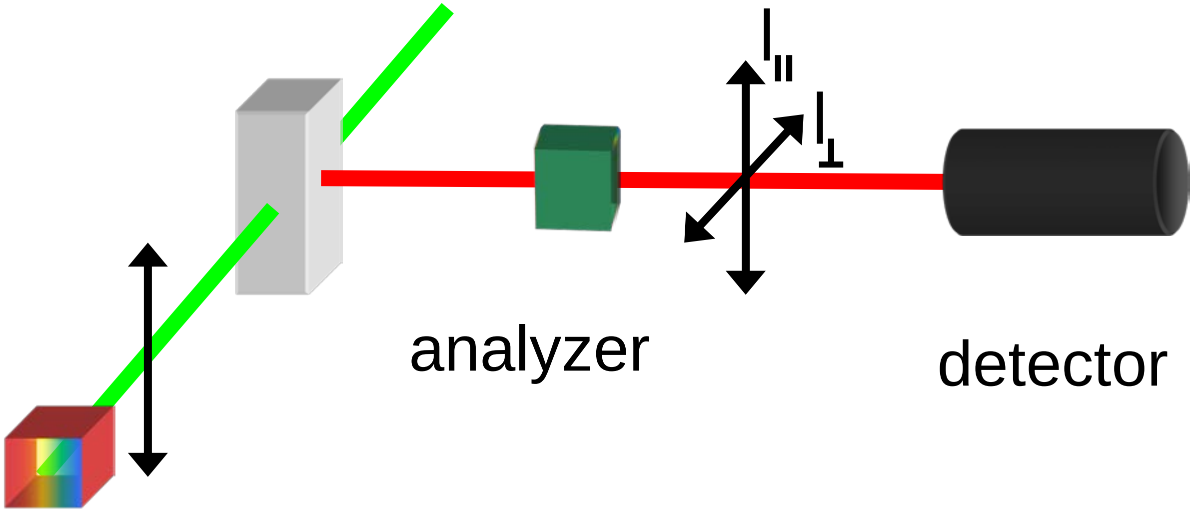
- **Transition dipole moment** is a quantum mechanical issue. It is not a real dipole moment. It is given by immediate state of electron shell of the molecule. The size of the dipole moment of transfer indicates the ability of the given molecule state to absorb or emit the light. The direction of the dipole moment of transfer indicates the direction in which the light is preferably absorbed or emitted by the molecule.
- The molecules preferentially absorb the light whose electric component oscillates in the same plane as **absorption transition dipole** of electron into a higher energetic level.
- Molecules preferably emit light in the same plane as the **emission transition dipole** of electron transition into a lower energetic level.

Photoselection

- If solution of fluorophores is excited by linearly polarized light then molecules with nonzero projection of their absorption transition moment into the direction of polarization of excitation radiation will be excited



Fluorescence anisotropy



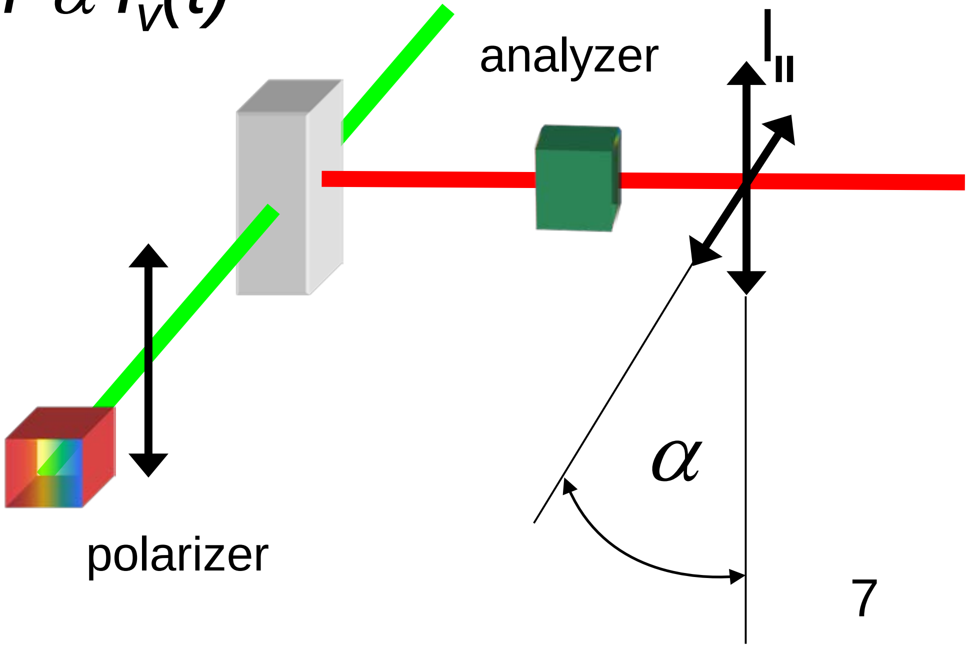
polarizer

$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}}$$

Intensity of fluorescence

The theory of depolarization of fluorescence shows that the fluorescence intensity observed using the analyzer, which is rotated by an angle and from the direction of parallel polarization is

$$I_{\alpha}(t) = \cos^2\alpha\, I_{II}(t) + \sin^2\alpha\, I_{v}(t)$$



At what angle of polarizer rotation α can we measure the total fluorescence intensity of rotation?

Total intensity of fluorescence

$$I_{total} = I_{II} + 2I_{\perp}$$

$$I_{\alpha} = \cos^2\alpha \cdot I_{II} + \sin^2\alpha \cdot I_{\perp}$$

$$\frac{\cos^2\alpha}{\sin^2\alpha} = \frac{1}{2} \qquad \sin^2\alpha + \cos^2\alpha = 1$$

$$\cos\alpha = \sqrt{\frac{1}{3}}$$

α = 54,7 °

The total intensity is measured at the magic angle

Total intensity $I(t) = I_{II}\ (t) + 2\ I_{v}(t)$ depends on the rotational motion of the fluorophore and is measured under "magic" angle of 54.7 °

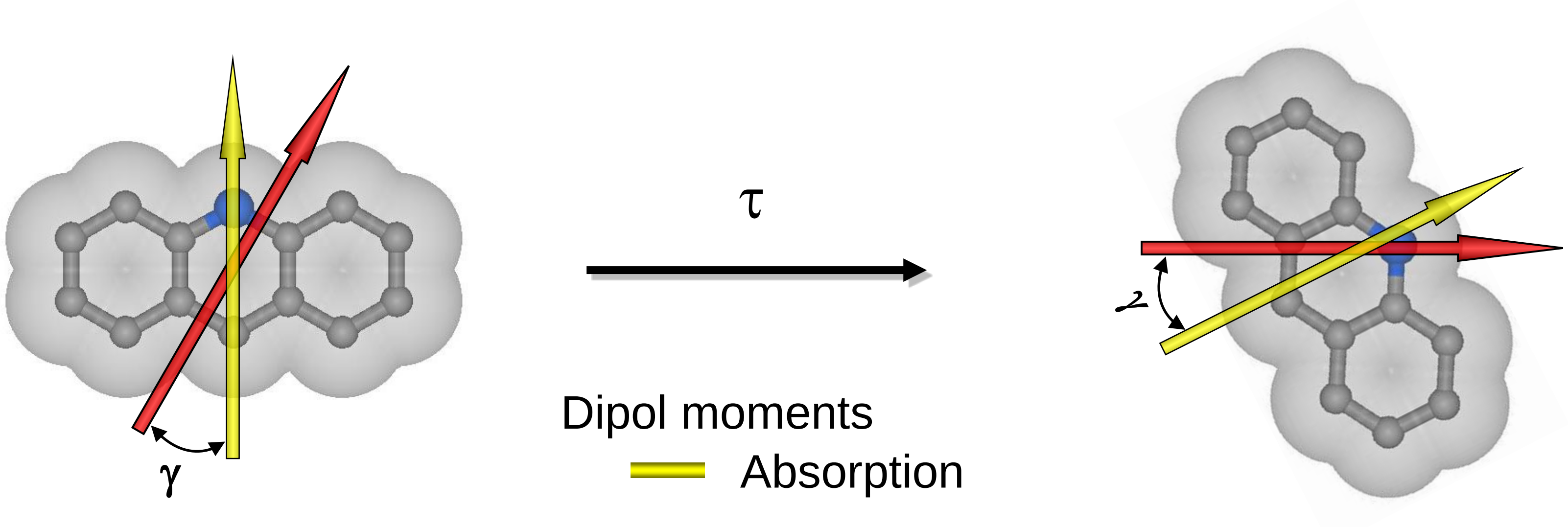
The time dependence of polarized fluorescence

and for time dependence of anisotropy we apply

$$r(t) = (3\cos^2 \gamma(t) - 1)/5$$

γ is – a dipole reorientation angle at time from 0 to t

– an angle between the dipole moment of absorption and emission



Dipol moments

🟨 Absorption

🟥 Emission

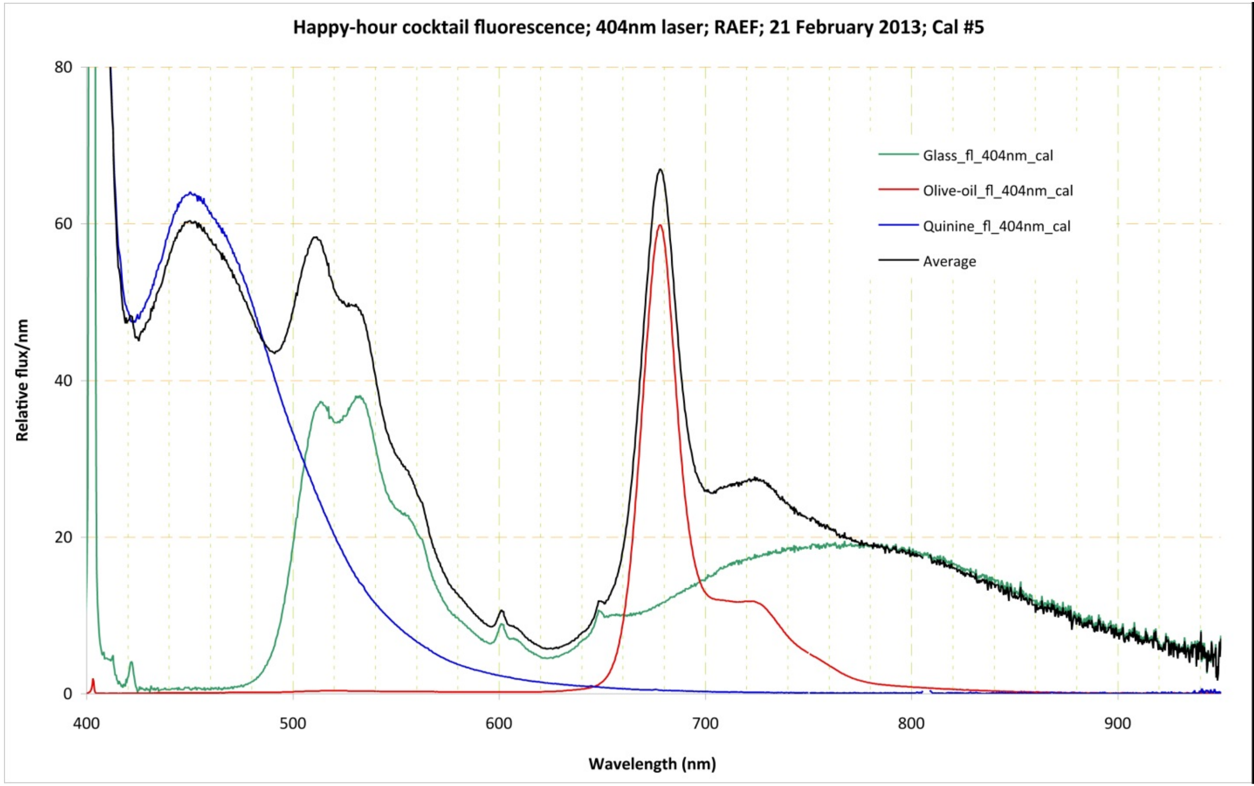
The maximal value of anisotropy

$$r_0 = \frac{(3\cos^2\gamma - 1)}{5}$$

From the equation, it follows that in the absence of depolarization (e.g. in dilute frozen solutions in the absence of depolarizing mechanisms and assuming that the transition moments of absorption and emission are parallel) the limit value of anisotropy of fluorescence excited by polarized radiation is given only by photoselection

$$r_0^{max} = 2/5 = 0.4$$

Anisotropy of frozen sample



http://thelab.photophysics.com/circular-dichroism/fluorescence-polarization-darkness-visible

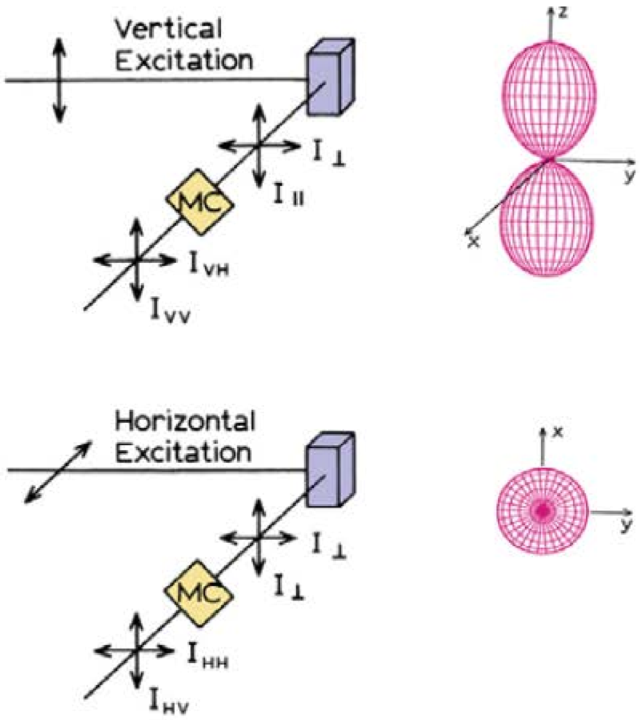
Anisotropy measurements at steady-state fluorescence

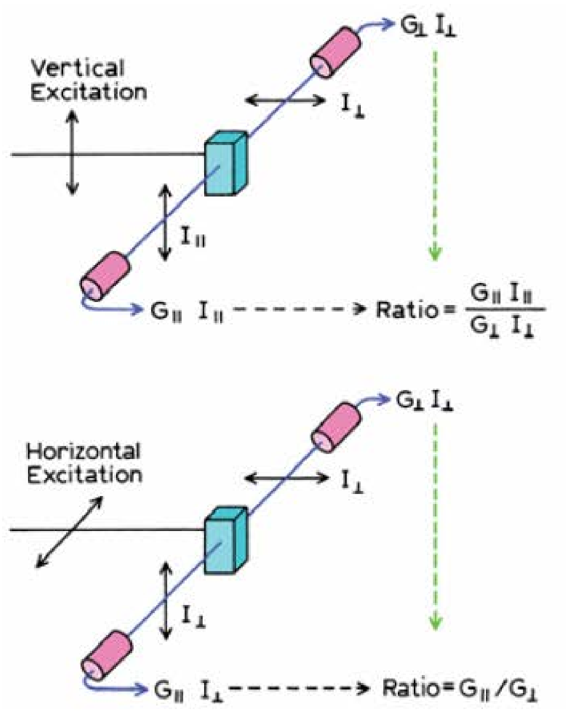
- Anisotropy – measures polarized emission
- The polarizer is on the track of the excitation light and makes it plane polarized
- Polarizer - analyzer is in the path of emitted light – fluorescence
- We measure the fluorescence intensity at rotation of both the polarizer and the analyzer vertically (VV), then at rotation of the polarizer vertically and the analyzer horizontally (VH)
- The value of anisotropy <r> is calculated from measured intensities

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2 \cdot I_{VH}}$$

http://www.youtube.com/watch?v=imQnWrLW2i0

L and T measurement arrangements



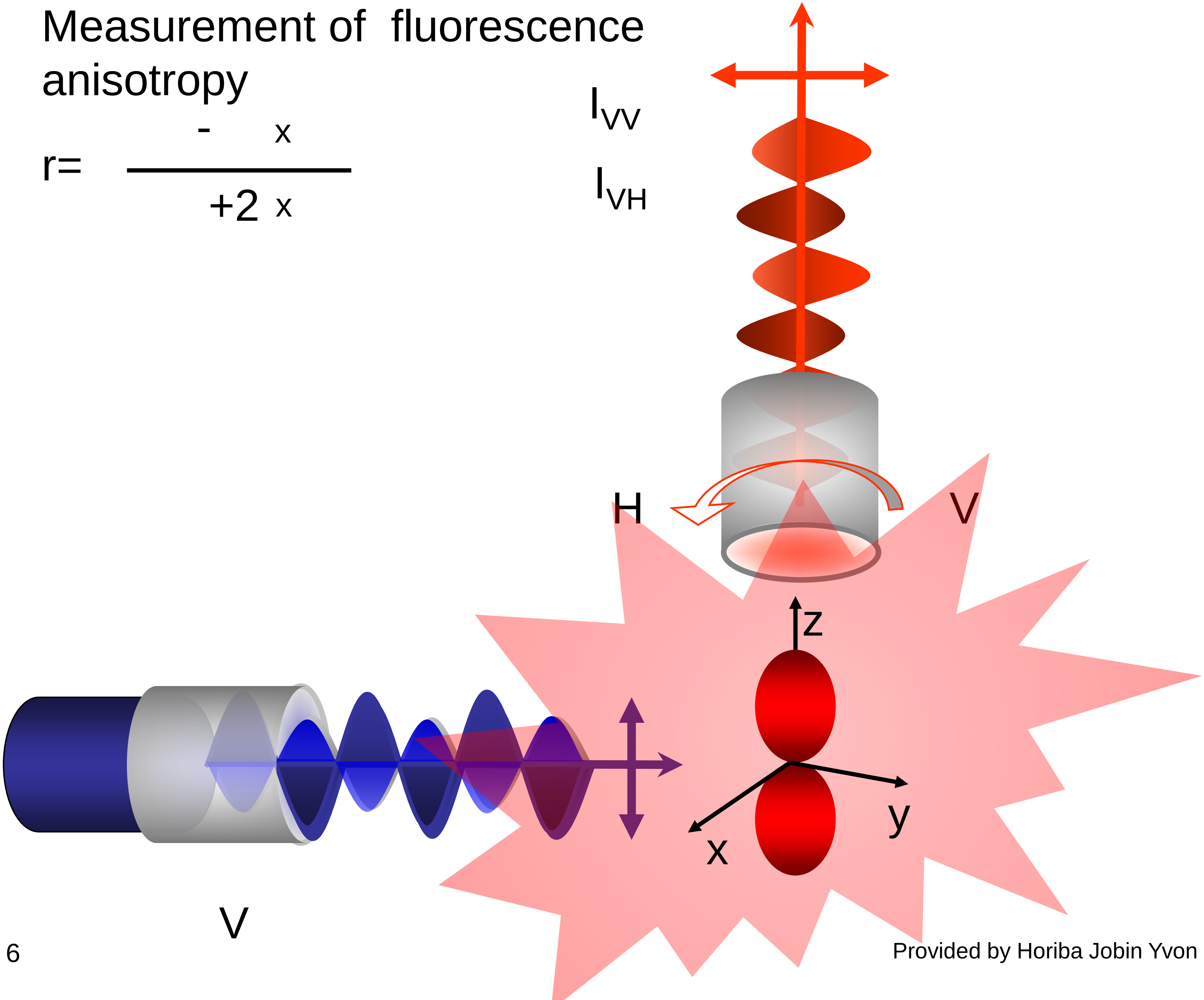


Measurement of fluorescence anisotropy

$$r = \frac{- \quad x}{+2 \quad x}$$

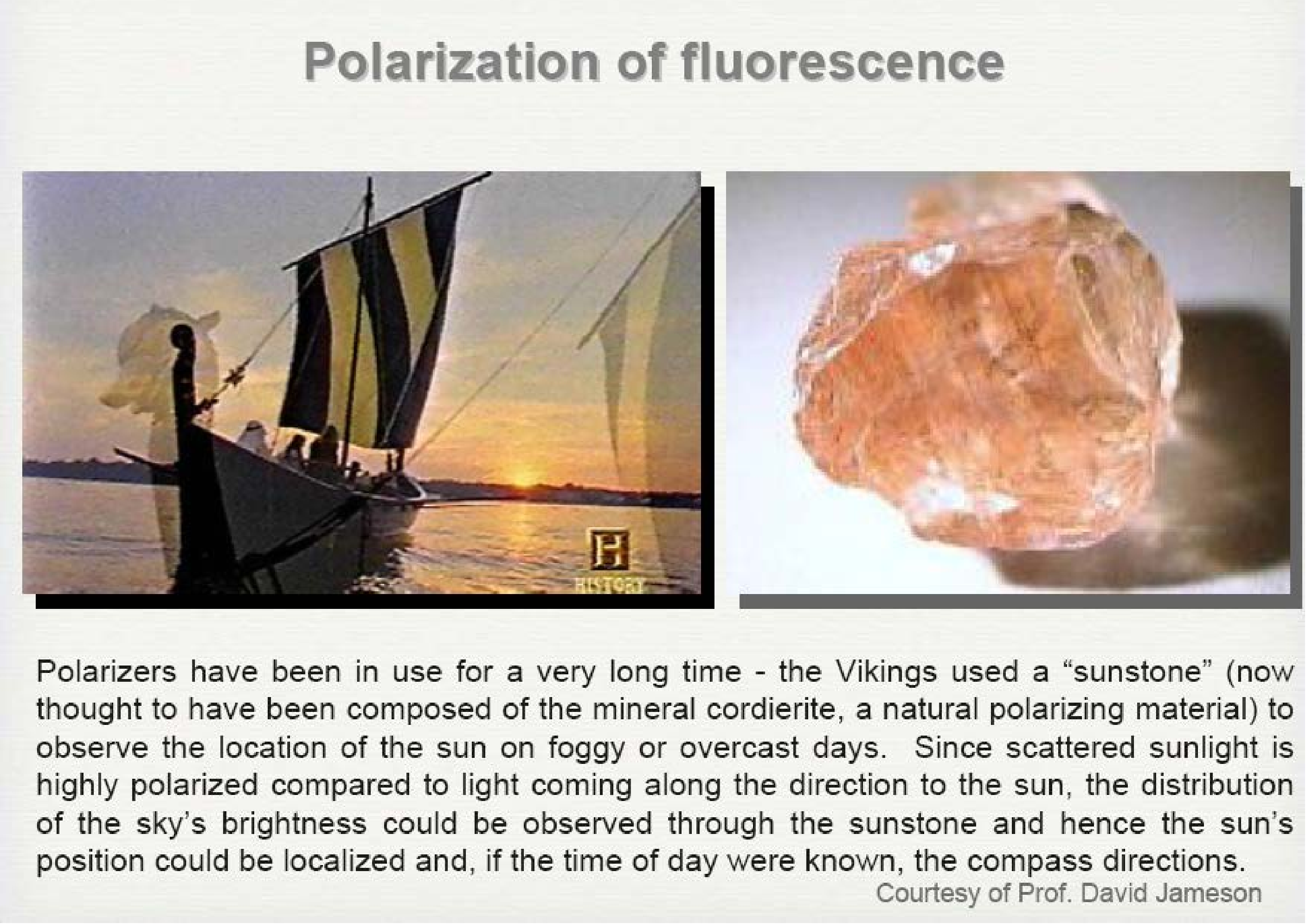
I_{VV}

I_{VH}



Provided by Horiba Jobin Yvon

Who did use the anisotropy of light for the first time?



Polarization of fluorescence

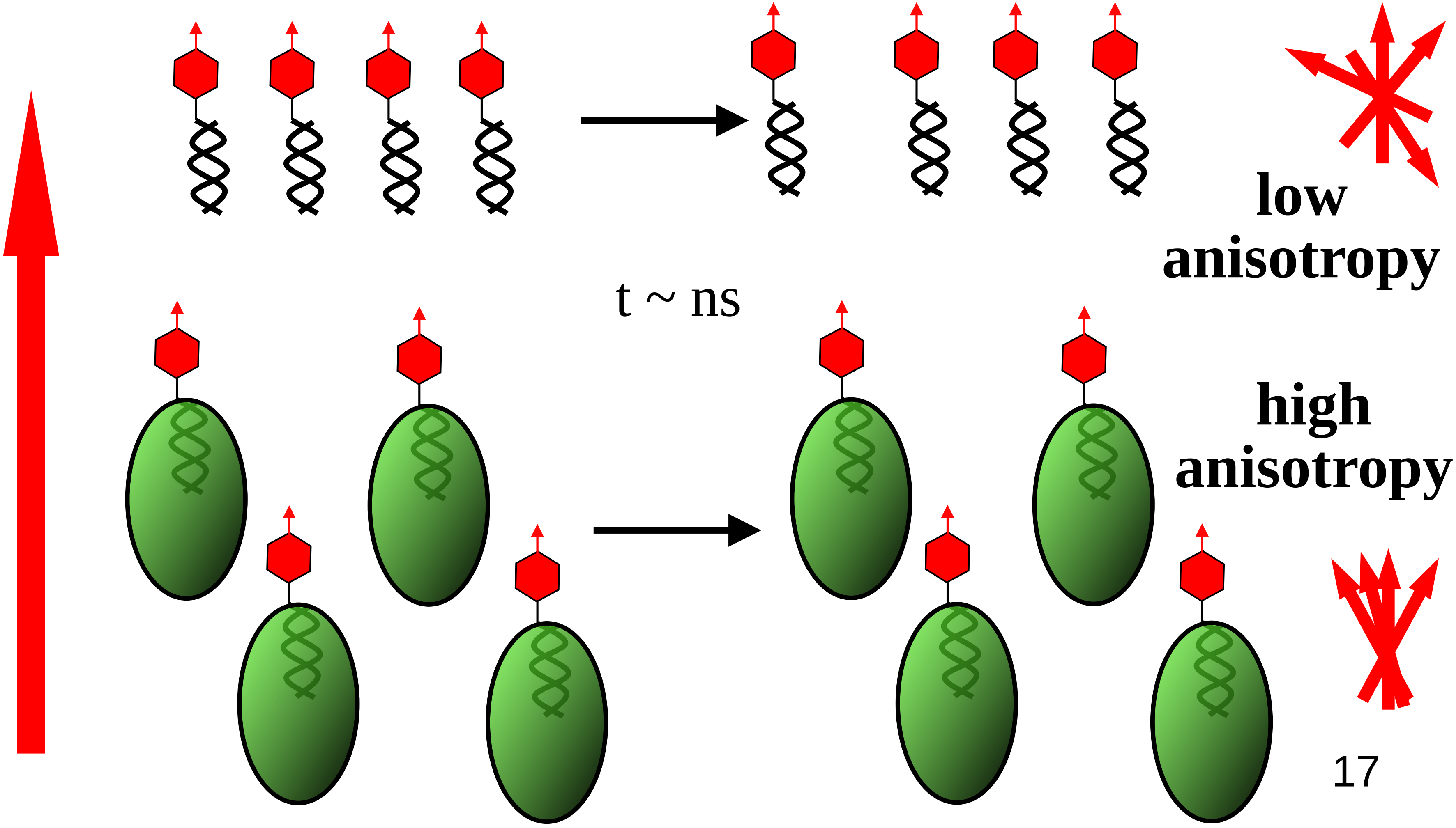
Polarizers have been in use for a very long time - the Vikings used a “sunstone” (now thought to have been composed of the mineral cordierite, a natural polarizing material) to observe the location of the sun on foggy or overcast days. Since scattered sunlight is highly polarized compared to light coming along the direction to the sun, the distribution of the sky’s brightness could be observed through the sunstone and hence the sun’s position could be localized and, if the time of day were known, the compass directions.

Courtesy of Prof. David Jameson

http://www.youtube.com/watch?v=NTpWDp_3W_4 3rd minute

http://www.nordskip.com/vfog.html

The principle of monitoring of macromolecule binding



Excitation

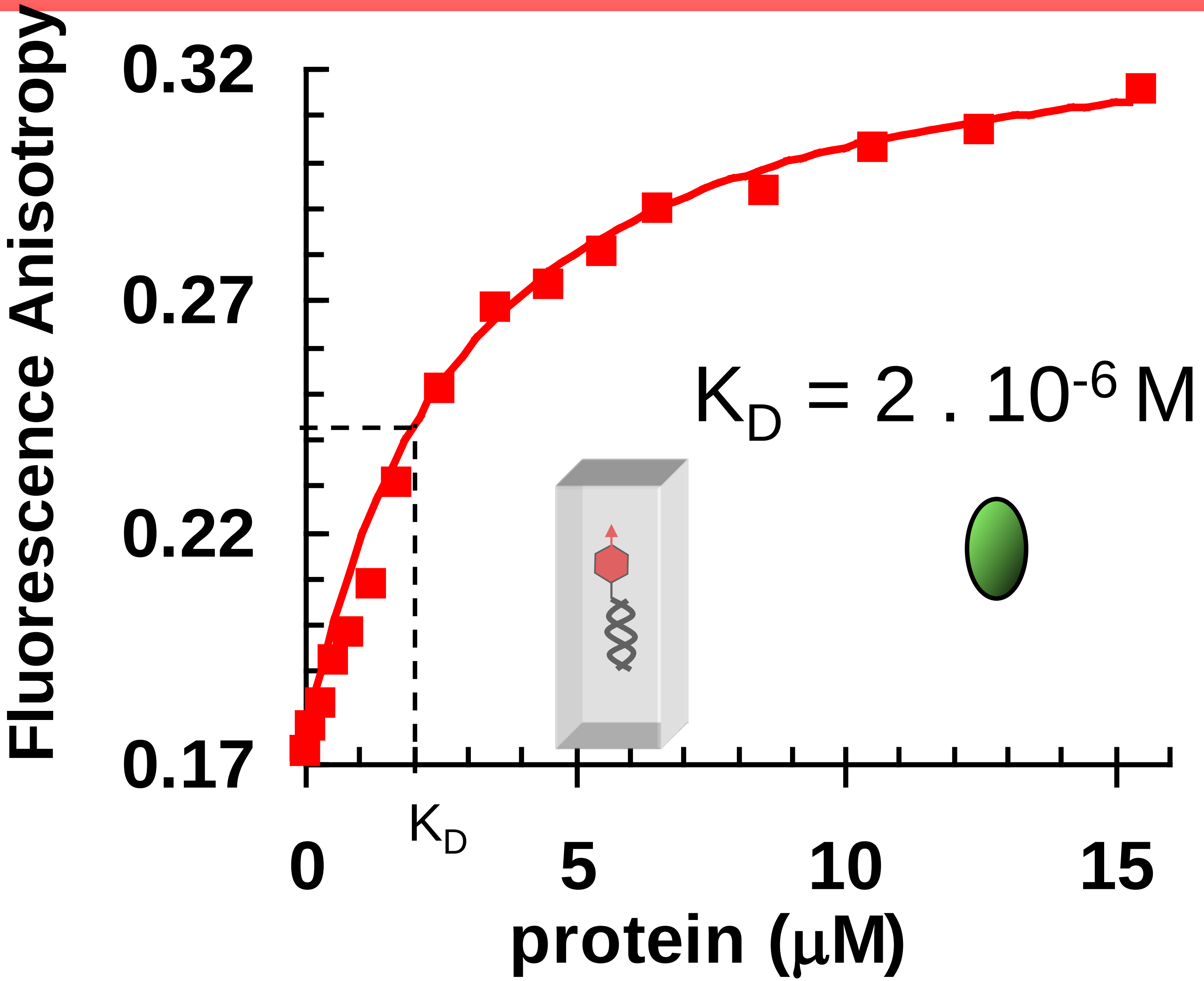
Emission

t ~ ns

low anisotropy

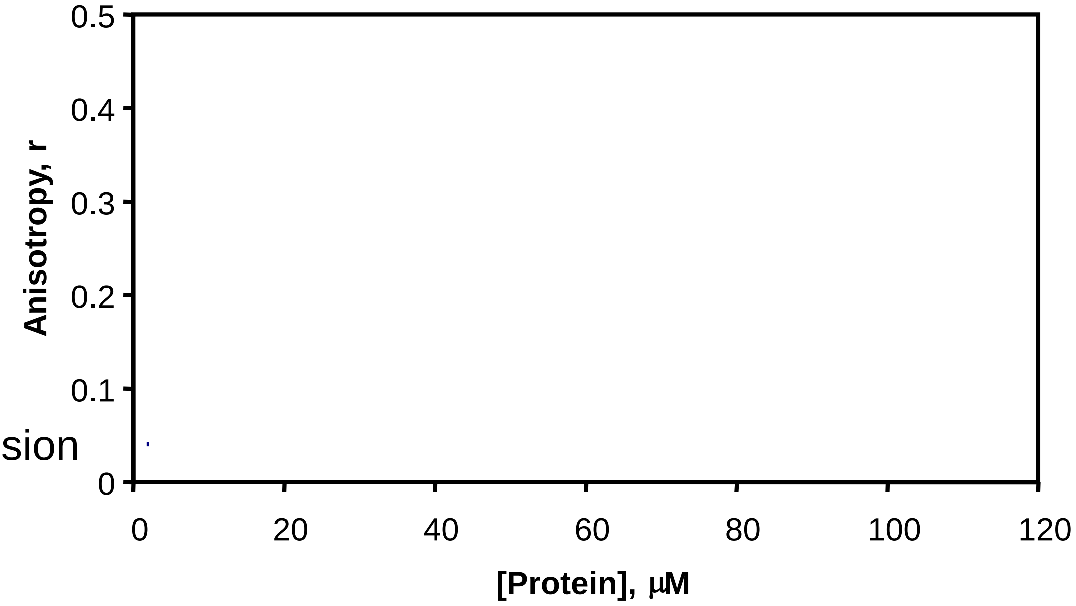
high anisotropy

AF detection of protein-DNA interaction



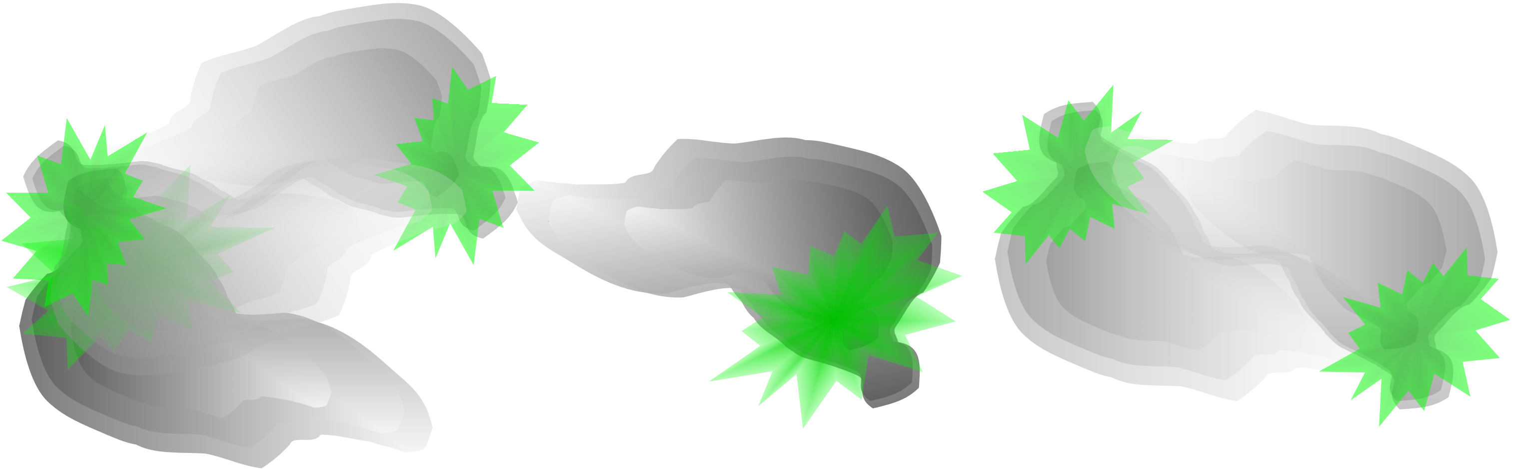
Monomers:
Small
Rapid rotation
Unhindered

Low anisotropy
Depolarized emission



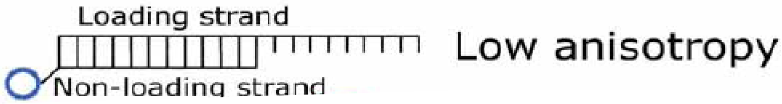
Dimers:
Larger
Slow rotation
Hindered by Viscosity

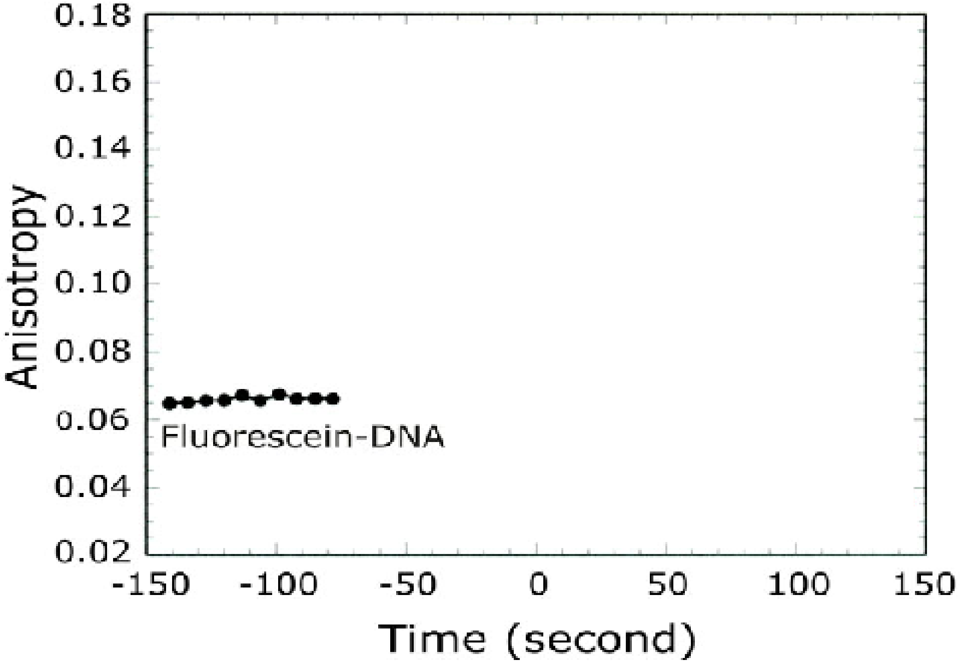
High anisotropy
Polarized emission



Poskytnuto Horiba Jobin Yvon

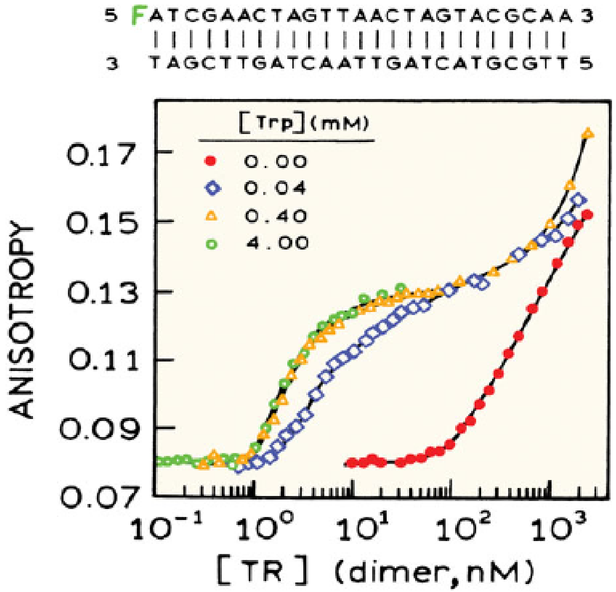
Monitoring of DNA unwinding

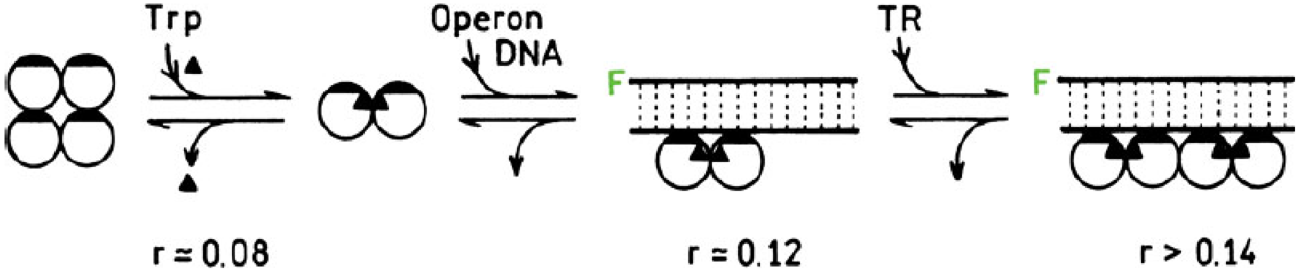




Repressor binding to DNA

- TRP repressor binds to DNA after activation by tryptophan and controls the synthesis of tryptophan.
- Anisotropy increases upon binding of TR to fluorescently labeled DNA
- At increasing concentrations of tryptophan, TRP repressor binding to DNA increases and prevents further synthesis of tryptophan





Time-resolved fluorescence anisotropy

- Measurement of time-resolved fluorescence anisotropy upon the pulse excitation gives much more information about rotational motion of the fluorophore than steady-state fluorescence.
- The steady-state fluorescence provides averaged values of the measured parameters and for their interpretation it is necessary to perform a number of measurements at different temperatures. In contrast, the method of time-resolved fluorescence anisotropy provides necessary information from measurement at a constant temperature
- When using the method of time-resolved fluorescence anisotropy, time-dependent intensity components $I_{||}$ (t) and I_v(t) are measured. Total intensity I(t) = $I_{||}$ (t) + 2 I_v(t) does not depend on the rotational motion of the fluorophore and is measured under a "magic" angle 54.7 °

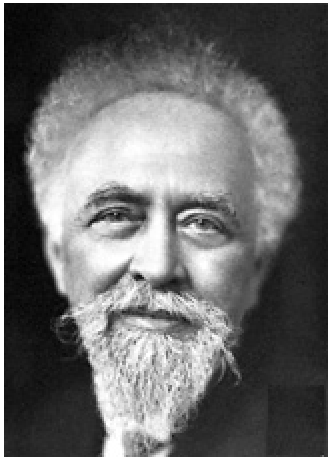
What kind of information can time-resolved fluorescence anisotropy provide?

- If the time decay of fluorescence τ is comparable with molecular reorientation rate, then the fluorescence polarization is modulated by molecular motion and analysis of time dependence of emission anisotropy will provide information about the anisotropy of the system where the fluorophore is located.
- Measurement of fluorescence polarization provides information about the molecular orientation and mobility and processes that are modulated by them, e.g.:

1. protein-DNA interaction
2. ligand-receptor interaction
3. biopolymers flexibility
4. fluidity of membranes
5. proteolysis
6. muscle contractions
7. activity of protein kinases

Perrin equation for the relationship of anisotropy and rotation of spherical molecules

$$\frac{\langle r \rangle}{r_0} = \frac{1}{1 + \left(\tau / \phi \right)}$$



r_0 anisotropy at time 0

τ fluorescence decay time

ϕ rotational correlation time – describes the rotation of molecules

6 the time in which the molecule rotates by 1 radian ~ 57.3°

When can time-resolved anisotropy give us information about the movement of molecules?

$$\frac{<r>}{r_0} = \frac{1}{1 + \left(\tau / \phi \right)}$$

τ = 10 ns

ϕ_1 = 0.1 ns
ϕ_2 = 10 ns
ϕ_3 = 1000 ns

$$\frac{<r>}{r_0} = \;?$$

0.0009
0.5
0.99

When the motion of molecules is happening at a time comparable with the time decay of fluorescence

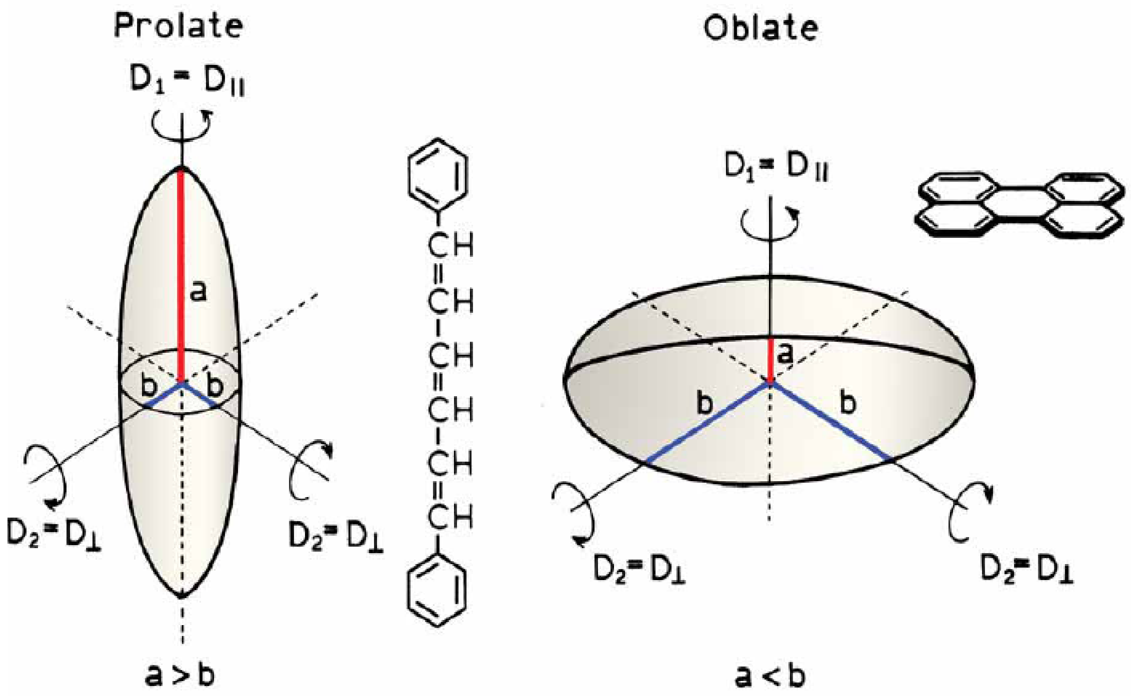
$$\phi \sim \tau$$

Use of Perrin equation for determination of molecule properties

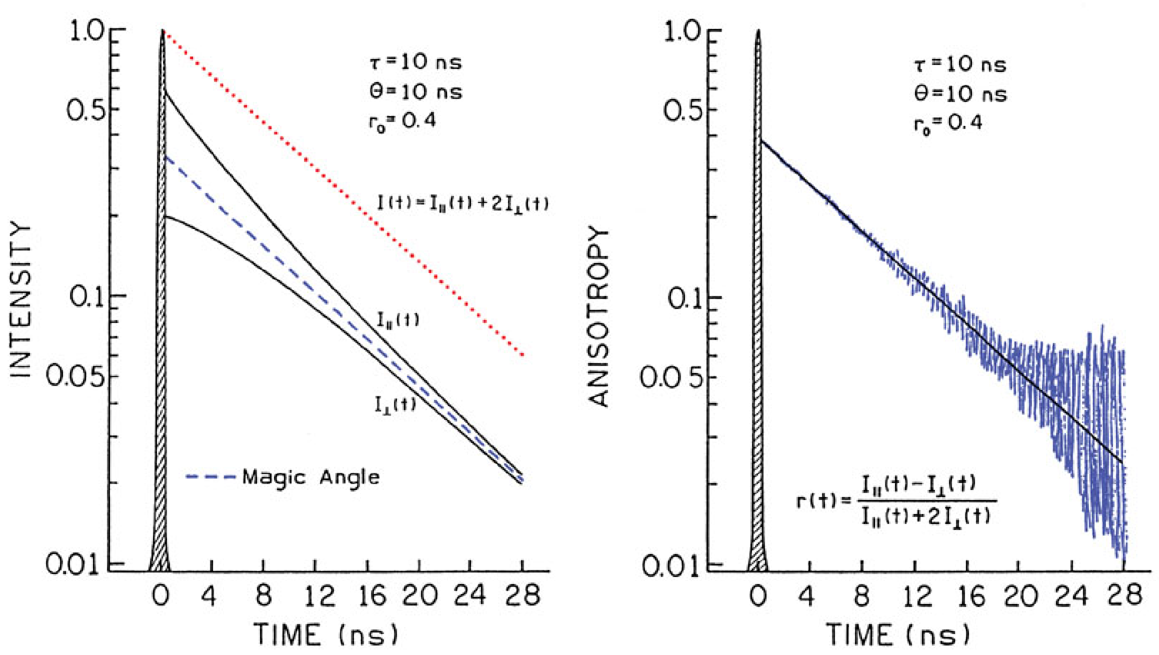
$$\frac{<r>}{r_0} = \frac{1}{1 + \left(\tau / \phi \right)} = 1 + 6D_r\tau = 1 + \frac{kT\tau}{V\eta}$$

r_0 anisotropy at time 0
τ fluorescence decay time
ϕ rotation correlation time
D_r rotation diffusion constant
V volume of a molecule
η viscosity of environment
k Boltzman constant (=1.38 .10^{-23}JK^{-1})
T absolute temperature in Kelvin

The time dependence is more complex for molecules with complex shapes

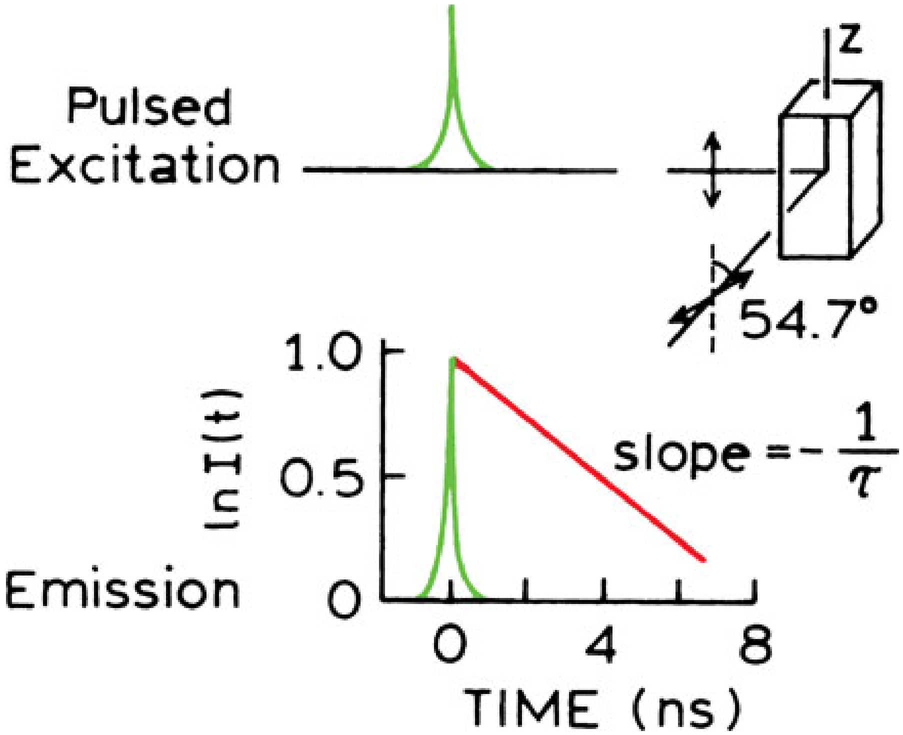


Time-resolved anisotropy



- Dependence of anisotropy is calculated from the time dependence of the vertically and horizontally polarized emission
- In the case that the absorption and emission transition moments are parallel, initial anisotropy is r_0=0.4
- Vertically polarized emission decreases faster because the number of molecules emitting in the direction decreases by rotation and decay.
- Horizontally polarized emission decreases slower, because the population of molecules is increased by molecules that change their position from vertical to horizontal arrangement by rotation.

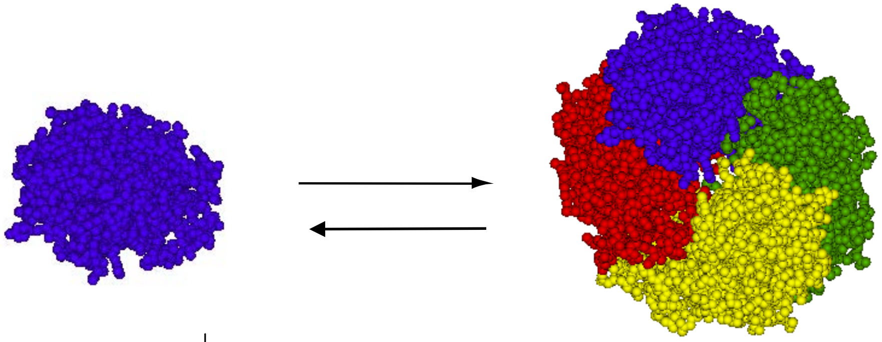
Measurement of time-resolved fluorescence anisotropy

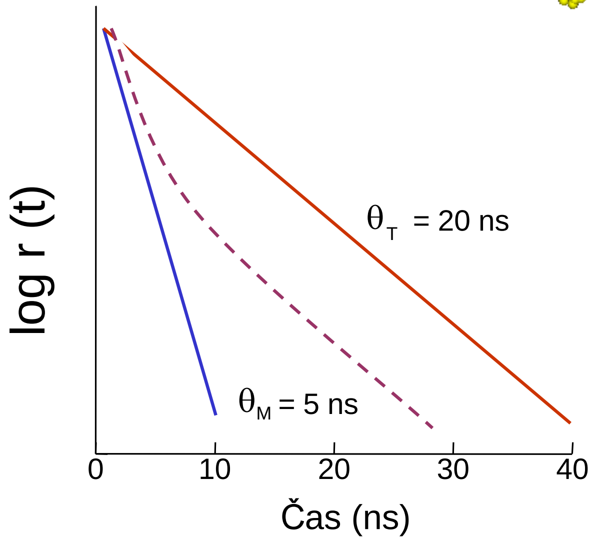


- The sample is excited by short pulses with durations much shorter than the decay time τ

Change of rotational correlation time during multimer formation

- The time dependent fluorescence anisotropy can be used to monitor changes in the dynamics of molecules
- Monitoring of formation of mutlimers by phosphofructokinase

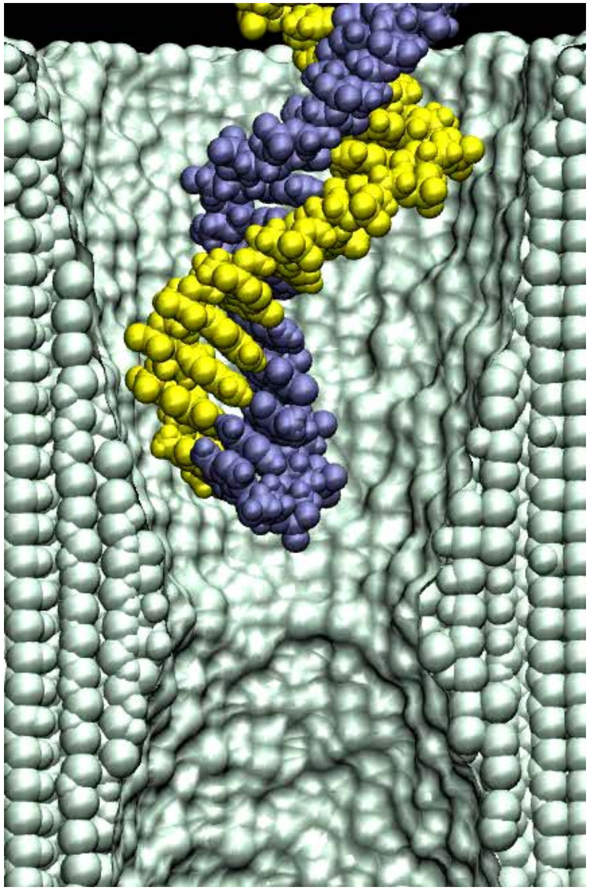




DNA flexibility

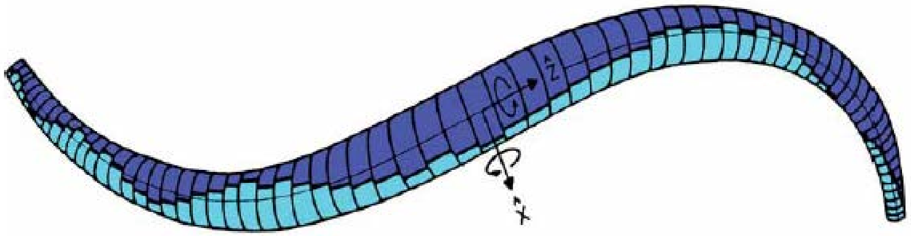
- How does the flexibility of DNA depend on environmental conditions?

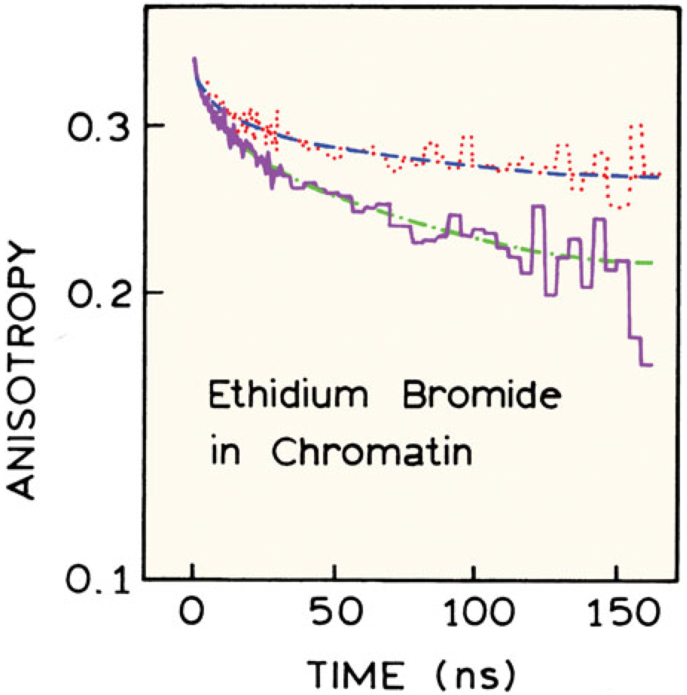
- What is the smallest pore diameter which can the DNA pass?



http://www.ks.uiuc.edu/Research/nanopore/PICTURES/ds2.0-V3.2.mpg

Monitoring of the movement of DNA after intercalation of EtBr

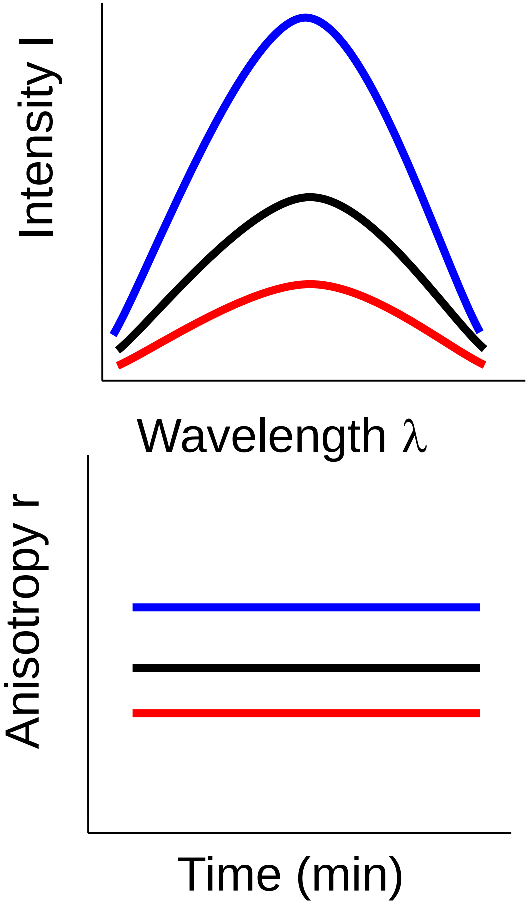




- After increasing the ionic strength (dashed) the structure of DNA is more rigid.
- The first part of the curve of the fluorescence decay describes torsional movement of DNA
- The latter part of the curve describes the bent of DNA structures

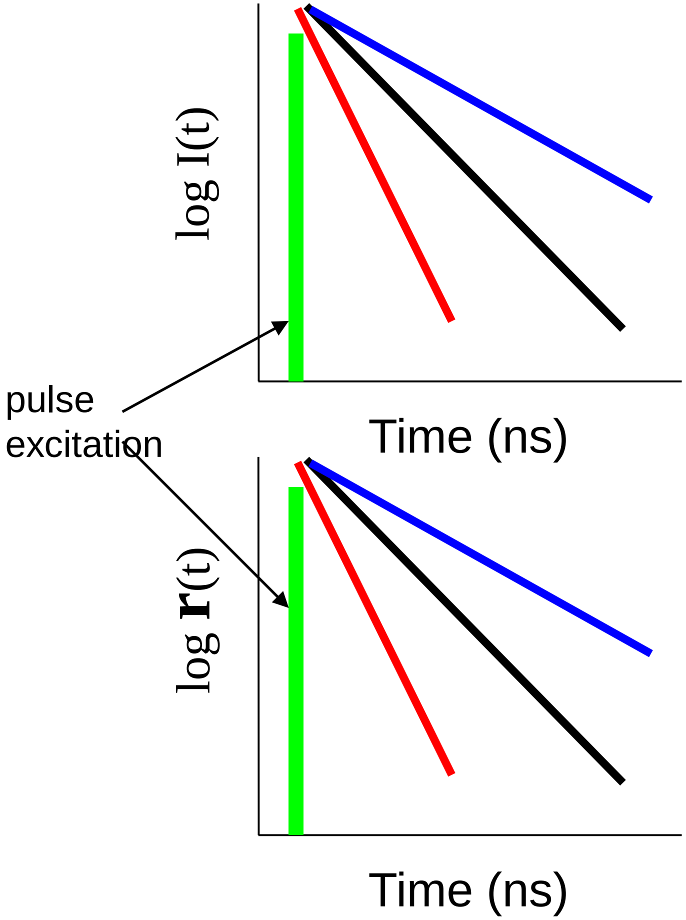
Comparison of steady-state and time-resolved intensity and fluorescence anisotropy

Steady-state



Time-resolved

More information!



Literature

- Lakowicz J.R.: Principles of Fluorescence Spectroscopy. Third Edition, Springer + Business Media, New York, 2006.

- Fišar Z.: FLUORESCENCNÍ SPEKTROSKOPIE V NEUROVĚDÁCH http://www1.lf1.cuni.cz/~zfisar/fluorescence/Default.htm

Acknowledgment

Graphics from the book of Principles of Fluorescence for the purpose of this lecture was kindly provided by Professor JR Lakowitz.