Steady State Fluorescence

Fluorescence methods in life sciences

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Steady state fluorescence

1. Definition
2. Fluorescence sensitivity to environment
3. Effects on fluorescence spectrum
4. Fluorescence quenching

The steady state and time-resolved fluorescence

- **Steady state fluorescence** is measured during by continuous irradiation by a source of excitation light. The fluorescence intensity is time-averaged.

- **Time resolved fluorescence** is measured using pulse excitation (pulse length is usually shorter than the fluorescence decay time of the sample) or phase-modulated excitation radiation. TR fluorescence enables us to analyze the time dependence of the fluorescence intensity or anisotropy.

4

The influence of environment on the absorption and emission spectrum

In solutions, solvation of fluorescent molecules occurs between fluorophore molecules and solvent due to electrostatic interactions dipole-dipole or dipole-induced dipole. Because, in general, the molecules differ in their **dipole moments** and **polarizability** in the ground and excited state, changes in the optical spectra of different solvation molecules occur during fluorescence measurements in solutions by different **solvation** of molecules. The time required for **molecular relaxation** (10^{-10}s) is much longer than the transition rate of the electron - **absorption** (10^{-15}s) but usually shorter than the lifetime of the excited state (10^{-8}s). The emission therefore occurs from the state where equilibrium configuration has been reached. As part of the absorbed energy is spent for relaxation of solvent molecules around the molecules of the fluorophore in the excited and ground state, energy of emitted fluorescent radiation is lower than would correspond only to a electron transition

From. Fišar: http://www1.lf1.cuni.cz/~zfisar/fluorescence/Default.htm

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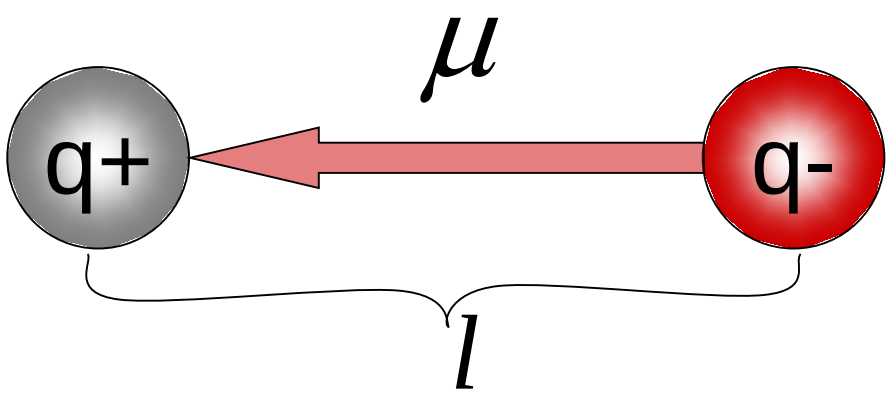
Electric dipole



- It consists of a pair of opposite polarity charges in the distance l
- Dipole moment $\boldsymbol{\mu = q \,.\, l}$

  vector pointing from the negative charge to positive charge

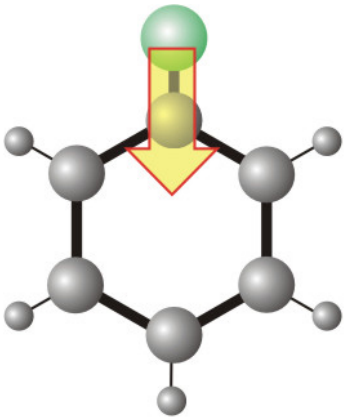
  Unit: Debye; 1D = 3.3 x 10^{-30} C.m

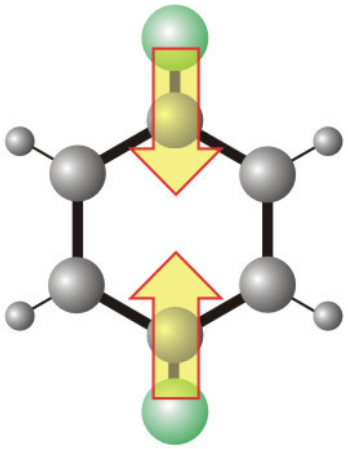
Molecular dipoles

- A molecule is a dipole when the distribution of positive and negative charges overlap. In the case where the molecule is not mirror-symmetrical, charge distribution is irregular and the molecule is a dipole.
- A molecule having a dipole moment is **polarized.**
- Molecules (generally mirror symmetrical - CO_2) that are not dipoles may be transformed to dipoles when the molecule occurs in an electric field - induced dipole is formed.

The dipole moment of polyatomic molecules

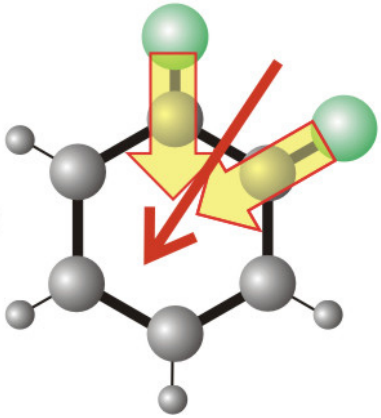
(a) $\mu_{obs} = 1.57$ D

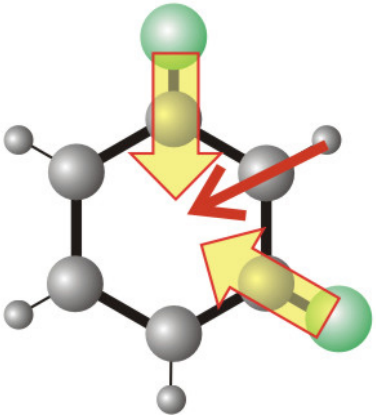




(b) $\mu_{calc} = 0$
$\mu_{obs} = 0$

(c) $\mu_{calc} = 2.7$ D
$\mu_{obs} = 2.25$ D

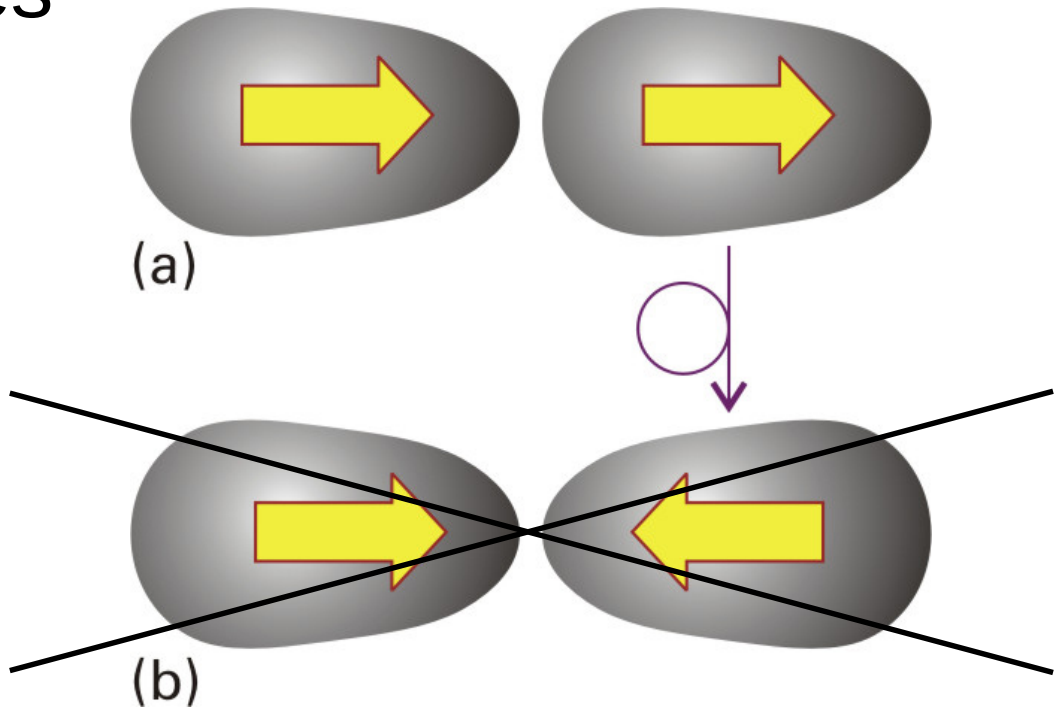




(b) $\mu_{calc} = 1.6$ D
$\mu_{obs} = 1.48$ D

Interaction of dipoles

- The polarized molecules prefer the arrangement with the minimum energy of dipoles



Polarizability of molecules

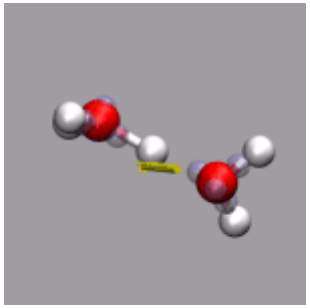
- The ability of molecules to create an induced dipole due to an external electric field
- The induced dipole is proportional to the intensity of the electric field ***E***
- **Induced dipole** μ^*

$$\mu^* = \alpha \boldsymbol{E}$$

α is **polarizability** of molecules

The greater the polarizability of the molecule, the greater the influence of electric field on the molecule.

Change of the dipole during interaction of molecules

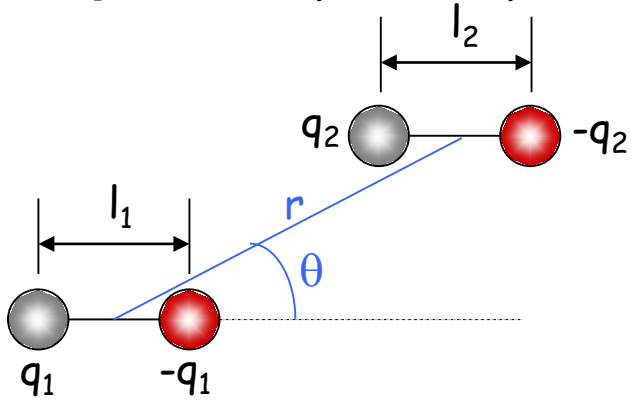


http://www.theochem.ruhr-uni-bochum.de/~axel.kohlmeyer/cpmd-vmd/part3.html

Interaction energy of two dipoles

- Interaction between two dipoles $\mu 1$ a $\mu 2$

$$V = -\frac{\mu_1 \mu_2 \,(1-3\cos^2\theta)}{4\pi\varepsilon_0 r^3}$$



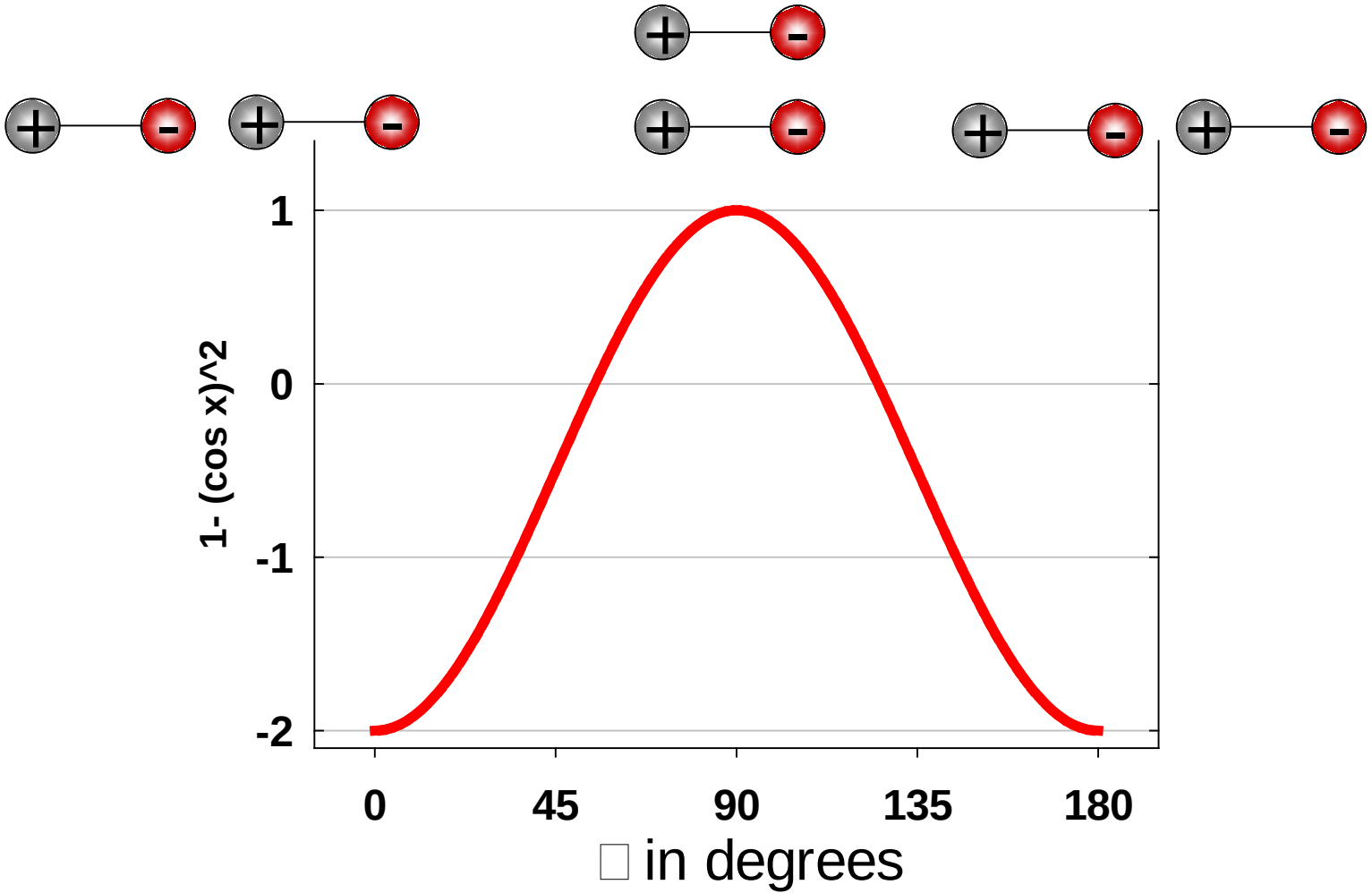
For the dipole-dipole interaction, potential energy V depends on the relative orientation.

Minimal energy is at $\theta = 0°$
attractive interaction (opposite charges are together) $\theta < 54.7°$

Maximal energy is at $\theta = 90°$
repulsive interaction (same charges are together) $\theta > 54.7°$

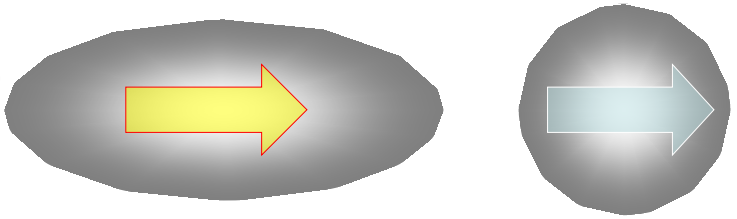
4 Zero potential energy is at the "magic" angle $\theta = 54.7°$

Dependence of potential energy on position of dipoles

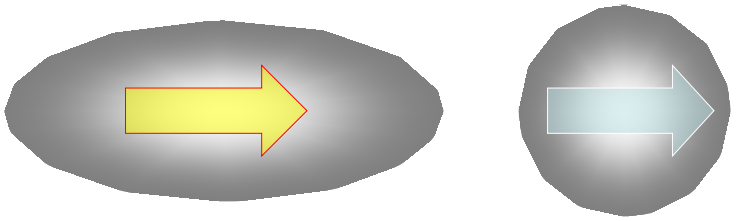


Dipole-induced dipole interaction

- Polar molecule with a dipole moment μ1 can induce a dipole moment in a polarizable molecule



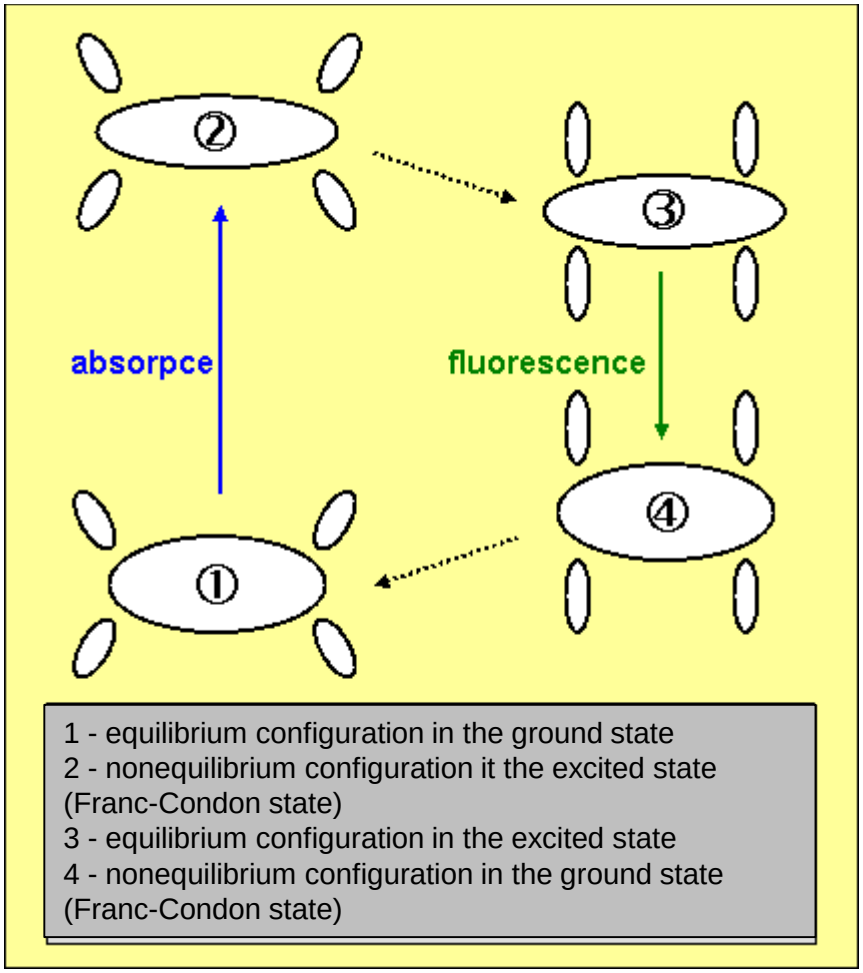
- An induced dipole interacts with a permanent dipole of the first molecule and they are mutually attracting



- An induced dipole (blue arrows) follows changes in a permanent dipole orientation (yellow arrow)

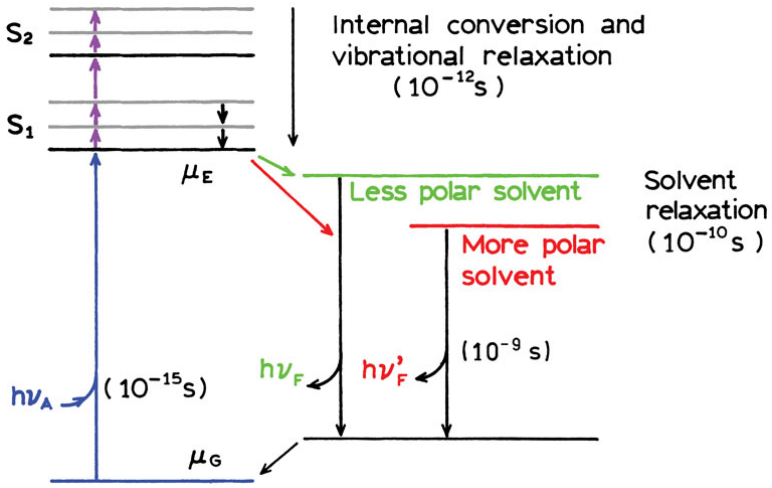
$$V = -\frac{\mu_1^2 \alpha_2}{\pi \varepsilon_0 r^6}$$

Solvation of the fluorophore during absorption and emission in solutions



In solutions, solvation of fluorescent molecules occurs between fluorophore molecules and solvent due to electrostatic interactions dipole-dipole or dipole-induced dipole. Because, in general, the molecules differ in their **dipole moments** and **polarizability** in the ground and excited state, changes in the optical spectra of different solvation molecules occurs during fluorescence measurements in solutions by different **solvation** of molecules. The time required for **molecular relaxation** (10^{-10}s) is much longer than the transition rate of the electron - **absorption** (10^{-15}s) but usually shorter than the lifetime of the excited state (10^{-8}s). The emission therefore occurs from the state where equilibrium configuration has been reached. As part of the absorbed energy is spent for relaxation of solvent molecules around the molecules of the fluorophore in the excited and ground state, energy of emitted fluorescent radiation is lower than would correspond only to a electron transition

Influence of solvent polarity



The dipole moment of the molecule in the excited state μ_E is greater than in the ground state μ_G

After excitation, solvent molecules are oriented (relaxed) around μ_E, which reduces the energy of the excited state

The greater the polarity of the solvent, the greater the influence of orientation of dipoles and the more energy is consumed for their orientation and then less energy left for the emitted light, i.e. the greater the wavelength of the emitted light

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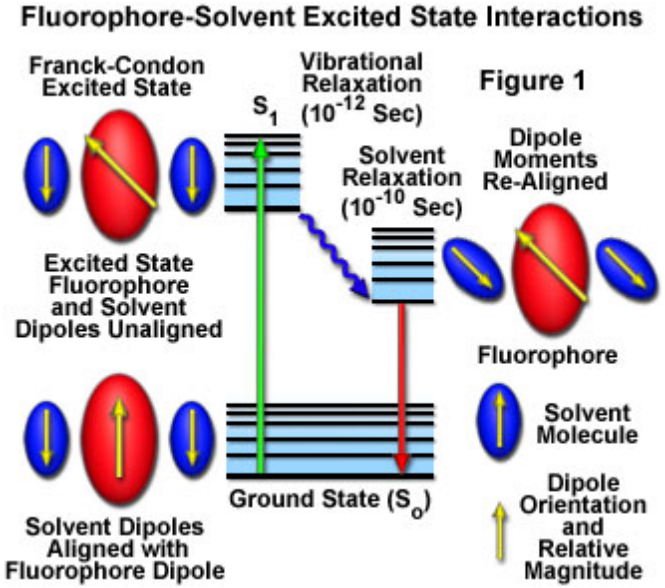
Interaction of the excited fluorophore and solvent

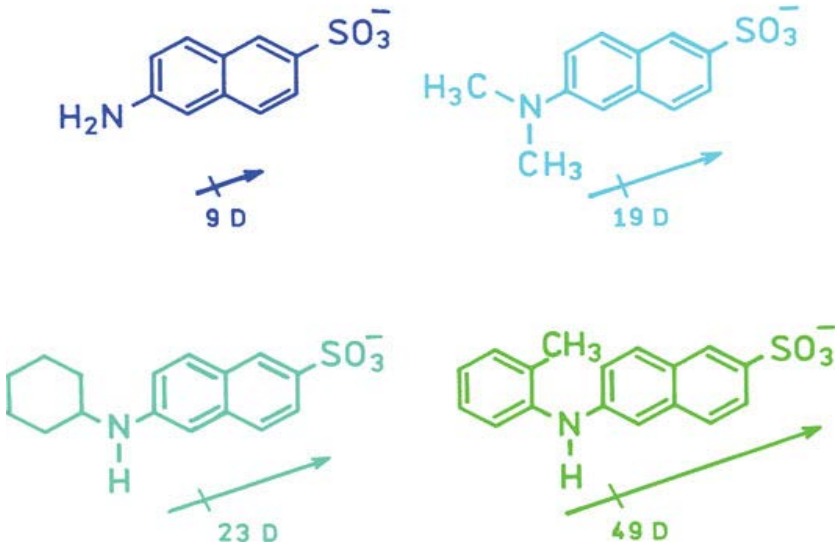


The greater the polarity of the solvent, the greater the influence of orientation of dipoles, the smaller the energy of the emitted radiation and the larger the shift of emitted light λ .

Polar fluorophores are the most sensitive to solvent polarity.

[4] Nonpolar fluorophores are less sensitive.

The dependence of the dipole moment on the shape of the molecule



Change of dipole moment is higher for longer fluorophores

Aminonaphtalene derivatives with a phenyl group exhibit greater sensitivity to the solvent and greater dipole moments in an excited state probably due to greater charge separation along the long aromatic system

Change of dipole moment upon excitation is higher for longer molecules.

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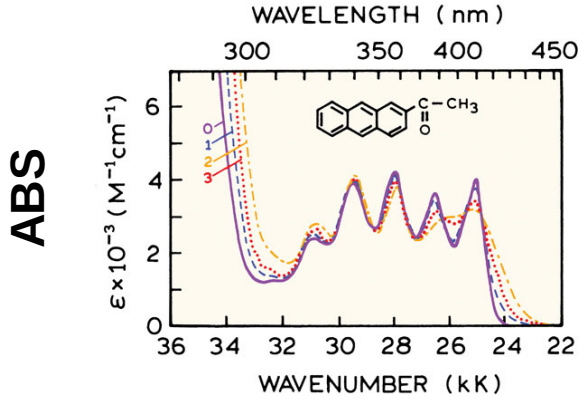
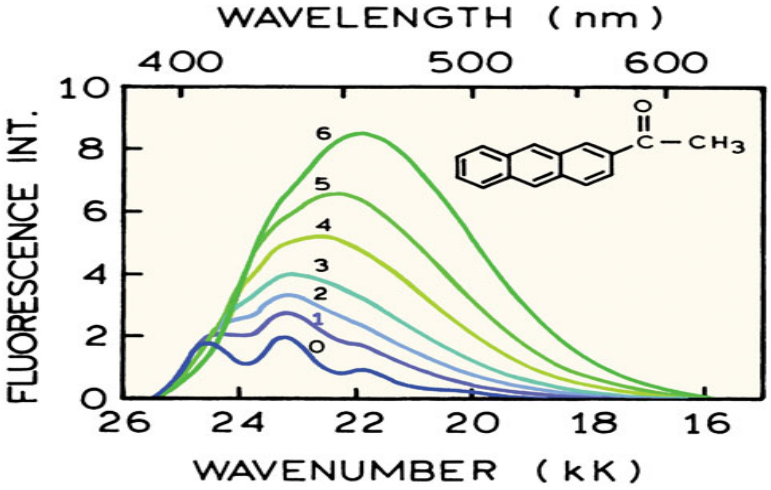
Difference of the solvent polarity effect on absorption and emission spectrum

2-acetylantracene



Absorption spectrum of 2-acetylantracene in pure hexane (0), 200mM solution of methanol in hexane (1) and pure methanol (2).



Raising of molar concentration of methanol in hexane in the range of 0-340 mM (0 -> 6)

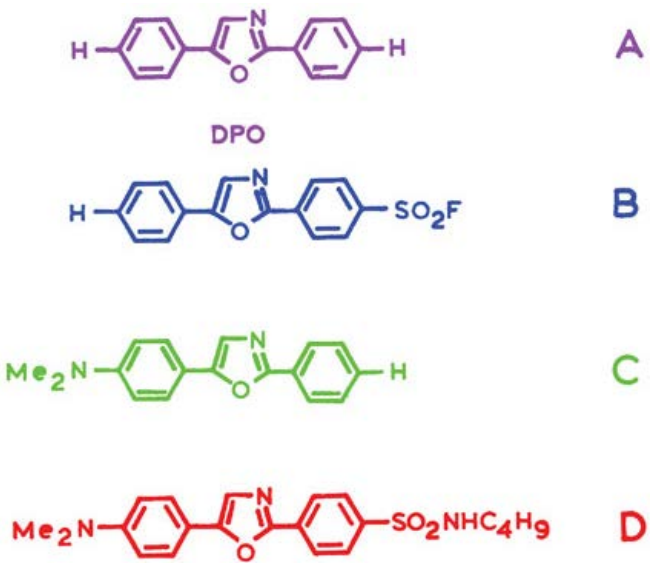
Fluorescence spectrum varies more with increasing polarity of solvents than the absorption spectrum.

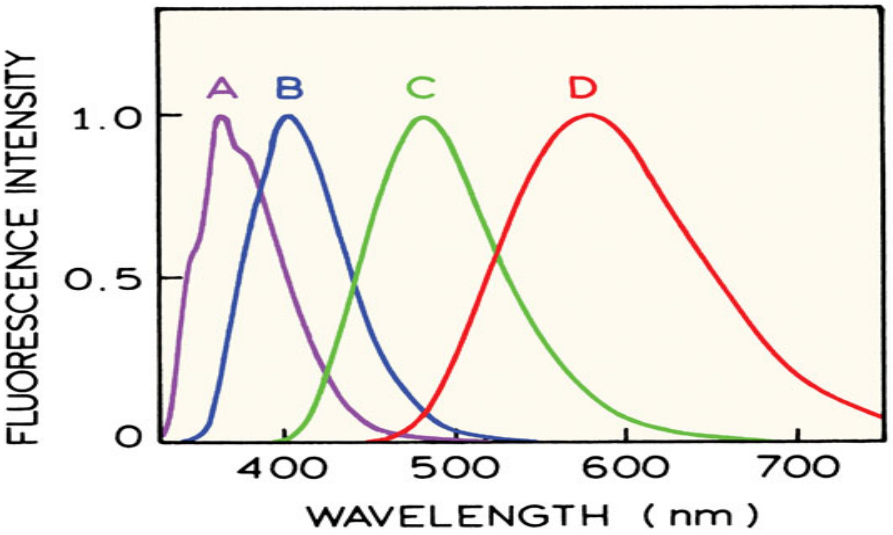
4

Probe for monitoring of environment polarity

- Addition of polar groups to fluorophore increases its sensitivity to solvent polarity
- Addition of more polar groups also increases the Stokes shift

Derivates of DOP (2,5-difenyloxazol) and their emission spectrums





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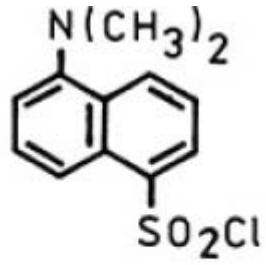
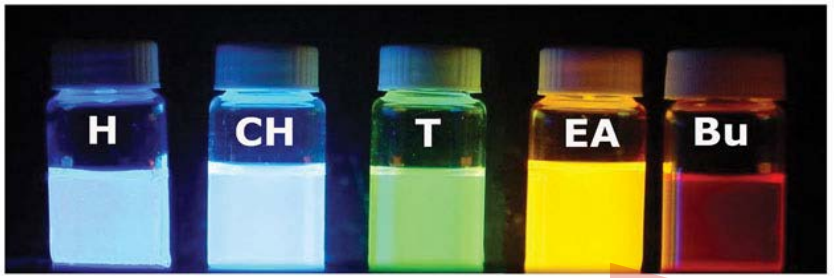
Why is emission spectrum more sensitive to environment polarity than the absorption spectrum?

- Because absorption is faster than emission which is slower than the relaxation of molecules
- chronological order : **Absorption (10^{-15}s)** -> **environment relaxation (10^{-10}s)** ->**emission (10^{-8}s)**
- absorption can not detect changes in the local environment of the molecule because it is quicker than they occur
- Molecule environment is the same before and after the absorption
- In contrast, the fluorophore molecule is already surrounded by relaxed (changed) environment during emission

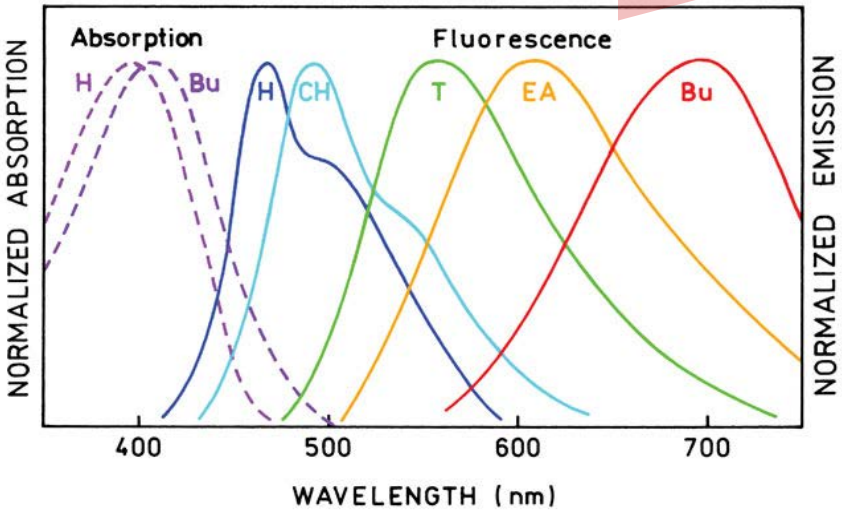
Dependence of emission spectrum on solvent polarity



DNS-Cl



polarity

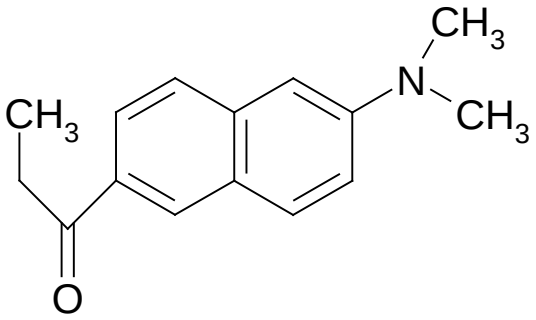


By increasing polarity :

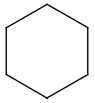
H – hexane
CH- cyklohexane
T- toluene
EA – ethylacetate
Bu – n-buthanol

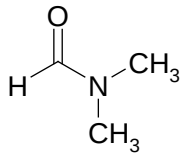
Practical demonstration

- Prodan (*N,N*-Dimethyl-6-propionyl-2-naphthylamine)



polarity

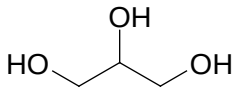
C - Cyklohexane 

D - dimethylformamide 

E - Ethanol CH_3CH_2OH

V - water H_2O

Bu - n- Buthanol $CH_3CH_2CH_2CH_2OH$

G – Glycerol 

Temperature effect on the emission spectrum

- Reducing the temperature generally causes an increase in viscosity of the solvent thereby increas the time required for orientation of molecules of the solvent

- The lower the temperature, the less the molecules return to the ground state with relaxed surrounding solvent molecules - the less energy is consumed and thus the shift is smaller

Change of emission spectrum upon molecule binding

Sensitivities of fluorescent probes to environment are used for monitoring of binding and quantifying of the amount of biological molecules.

Quantum yield is often increased when the fluorophore binds to the protein or DNA. This is used in monitoring of binding.

ANS to HSA

Prodan to protein

DAPI to DNA

EtBr to DNA

ANS fluorescence intensity increase upon binding to human serum albumin

ANS (1-anilinonaftalén-8-sulfonic acid):

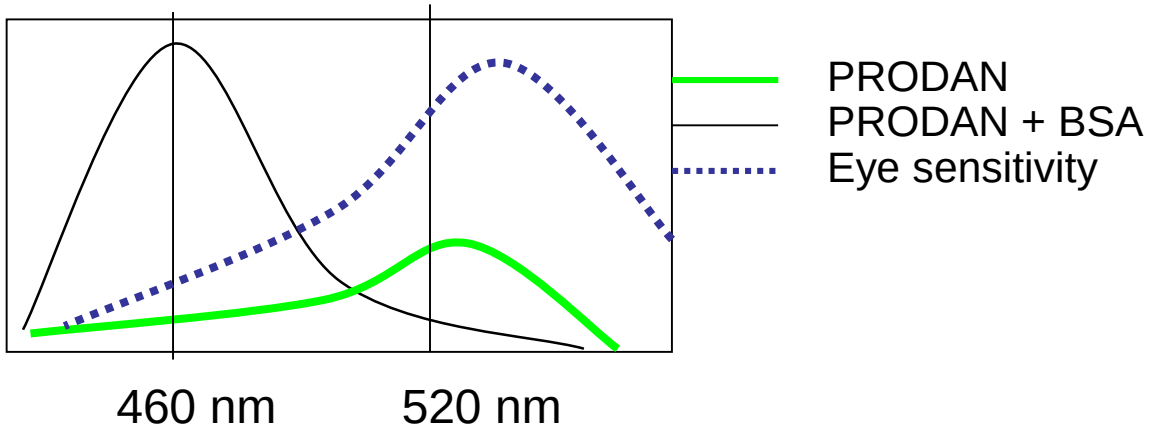
- MW = 321,33
- solvent for the stock solution: dimethylformamide(DMF)
- solvent for spectroscopic measurement: methanol (MeOH)
- longwave absorption maximum in methanol : λ_{exmax} = 372 nm (molar extinction coefficient: 7800 cm-1M-1)
- fluorescent emission maximum in methanol : λ_{emmax} = 480 nm
- quantum yield of fluorescence depends on the environment and is particularly sensitive to the presence of water; emission depends on the solvent
- detailed description of the characteristics of the ANS can be found in [Slavík J.: Anilinonaphthalene sulfonate as a probe of membrane composition and function. Biochim. Biophys. Acta 694, 1-25 (1982)]

The environment polarity change is shown by the emission intensity change in the visible region of the spectrum

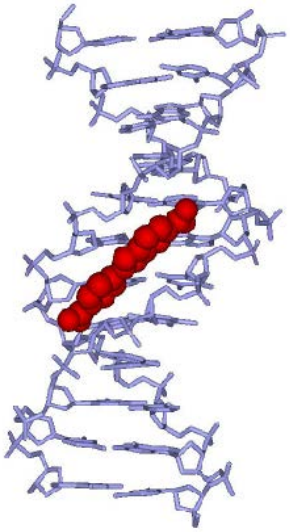
What happens when PRODAN binds to BSA ?

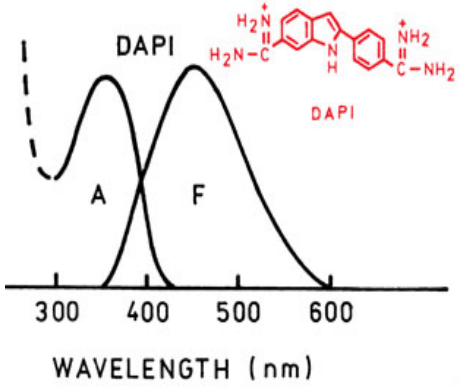
Maximum wavelength of 520 nm moves to 460. Although, the emission intensity increases, we are not able to observe it because the eye has a lower sensitivity to light with λ=460 nm.

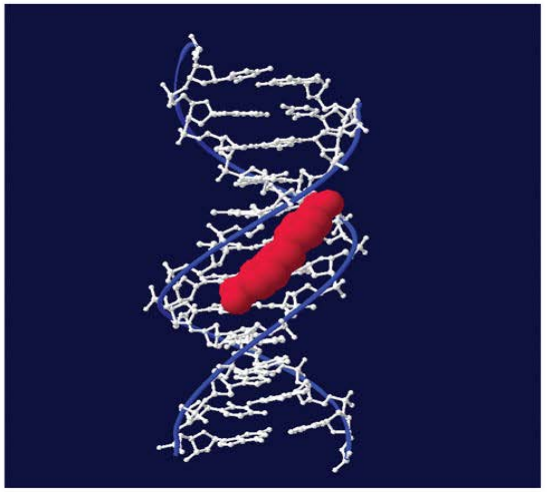
Gradual bond of PRODAN to BSA can be better monitored as emission decrease of a free fluorophore

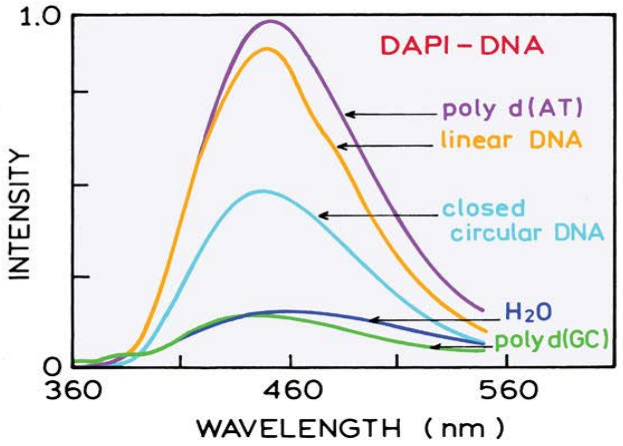


DAPI binding to DNA



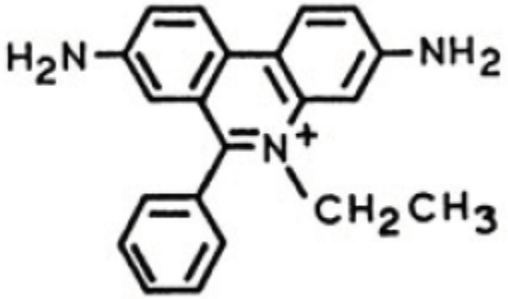






- DAPI (4,6-diamidino-2-phenylindole)
- Ex. 355 nm / Em. 461 nm
- Binding to the minor groove
- The biggest increase in intensity during binding near the AT-rich regions
- Use for DNA labeling for fluorescence microscopy specimens (sensitivity of the order of ng of DNA)

EtBr binding to DNA

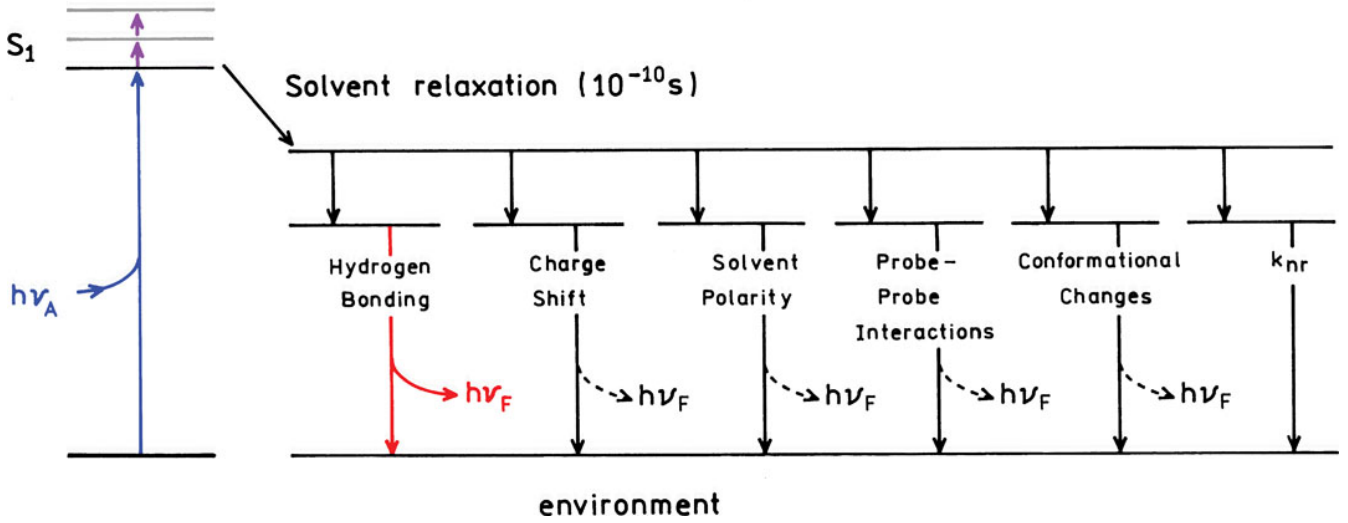


Probe characteristics

MW = 394,31

- soluble in water
- it emits fluoresces a little in water. Fluorescence increases about 30 times upon binding to DNA
- time decay of fluorescence is about 1.7 ns in water and increases to 20 ns after binding to double-stranded DNA
- binding to DNA is performed by intercalation of the aromatic ring between base pairs of double-stranded DNA
- absorption maximum in DNA: λ_{exmax} = 523 nm
- fluorescent emission maximum in DNA: λ_{emmax} = 604 nm
- Sensitivity: in the order of ng of DNA can be detected on the gel

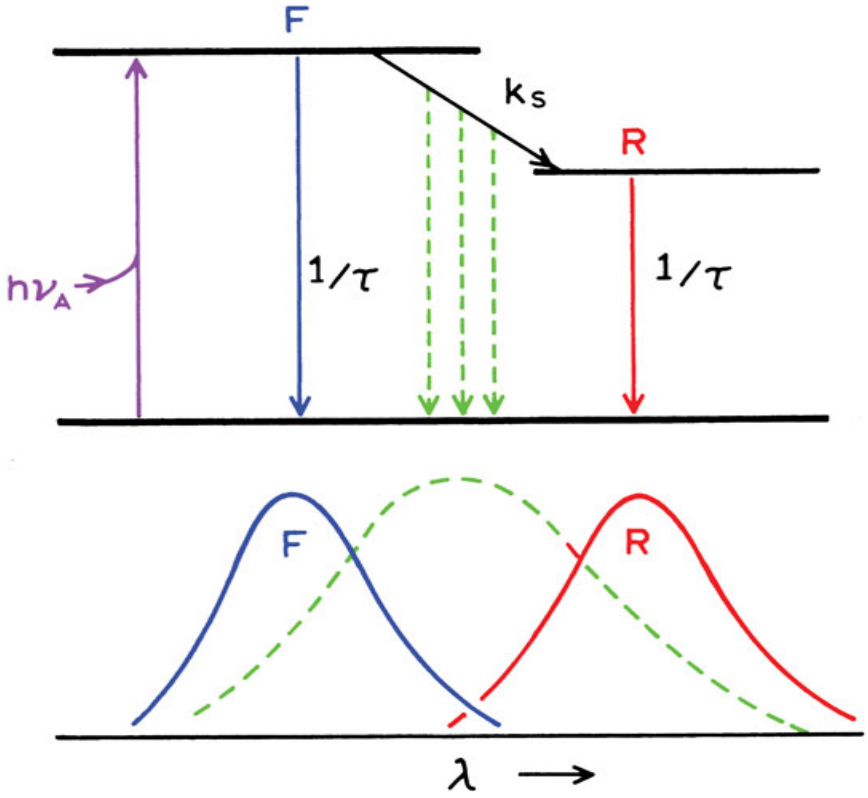
Other mechanisms of spectral shift

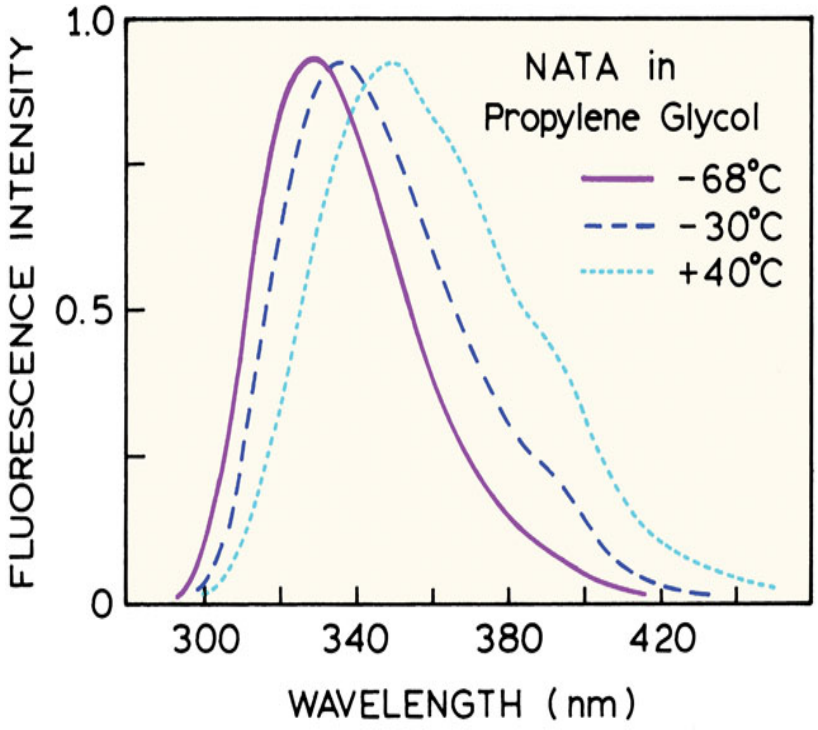


- Hydrogen bonds in a solvent
- Inner transfer of charge (within a molecule)
- Solvent molecules relaxation rate
- Interaction of probe – probe
- Conformational changes of the fluorescent probe
- Changes in rates of radiative and non-radiative processes

Dashed arrows represent that the transitions can be iradiative or non-radiative

The dependence of the emission spectrum on temperature





Temperature reduction increases the time needed to relax solvents.

4 Temperature reduction has similar effects as reduction of solvent polarity

Interaction of probe-probe excimer fluorescence

Fluorophore molecules can create together excited complex excimer.

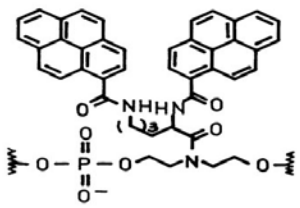
Excimer is **exc**ited di**mer** shortly.

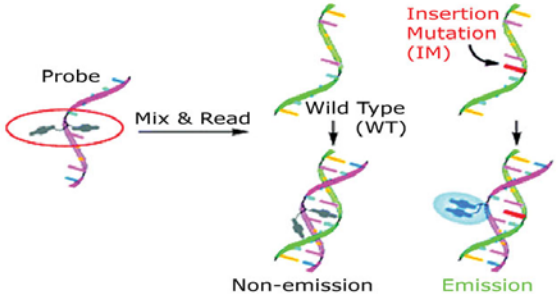
It is exiples in case of two different molecules.

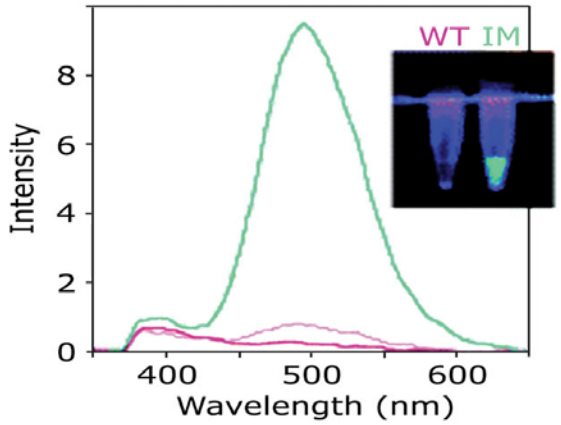
Molecules must be in contact to create an excimer .

Excimer fluorescence emission band is shifted to longer wavelengths compared to the fluorescence of isolated molecules.

Use of excimer upon detection of DNA insertion mutations







- Oligonucleotide with attached pyrene residues instead of single base
- When it binds to WT unmutated DNA: one pyrene residue intercalates, the latter is outside the helix
- When it binds to mutated DNA which contains one extra base: excimer will create.
- Excimer emission shows that it is an insertion mutant

Fluorescence quenching

- **Fluorescence quenching** can be defined as bimolecular process which reduces the quantum yield of fluorescence (ie. the intensity of fluorescence) without changing the fluorescence emission spectrum. It can be the result of different processes.
- **collisional (dynamic) quenching** occurs when the fluorophore is deactivated in the excited state (i.e. it returns to the ground state non-radiatively) during collision with quenching molecule. The molecules in this process are not chemically changed in contrast to
- **static quenching**, when non-fluorescent complex is created after contact of the fluorophore and the quencher.
- **selfquenching** is the quenching of the fluorophore by itself; occurs at high concentrations or high degree of labeling.

[4]

Dynamic quenching

Reducing of the fluorescence intensity by dynamic quenching is described in Stern-Volmer equation **:**

$$F_0/F = \tau_0/\tau = 1 + k_q\ \tau_0\ C_q$$

F_0 – fluorescence quantum yield in the absence of a quencher, F – the same in the presence of a quencher at C_q, τ_0 – fluorescence time decay without a quencher, τ – time decay in the presence of a quencher, k_q – bimolecular quenching constant (= bimolecular rate constant determined by diffusion multiplied by the efficiency of quenching). kq value gives concentration of a quencher at which the fluorescence intensity is reduced by half.

Molecular oxygen (O_2) is the most common quencher of fluorescence and phosphorescence. Furthermore, fluorescence is quenched (due to intersystem conversion) by halogen atoms such as **bromine** and **iodine**. Acrylamide is also a frequently used quencher.

Static quenching

- A fluorophore and a quencher creates a complex, which does not emit fluorescence
- The Stern-Volmer equation is also applied :

$$F_0/F = 1 + K_a \tau_0 C_q$$

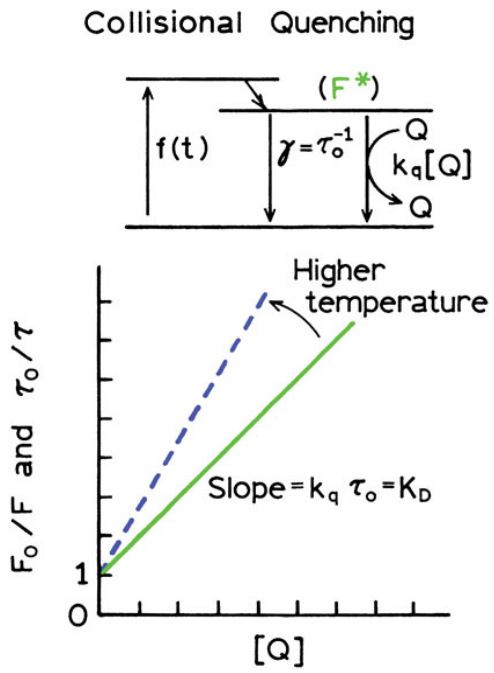
K_a is association constant of the fluorophore and the quencher

Typical static quenchers:

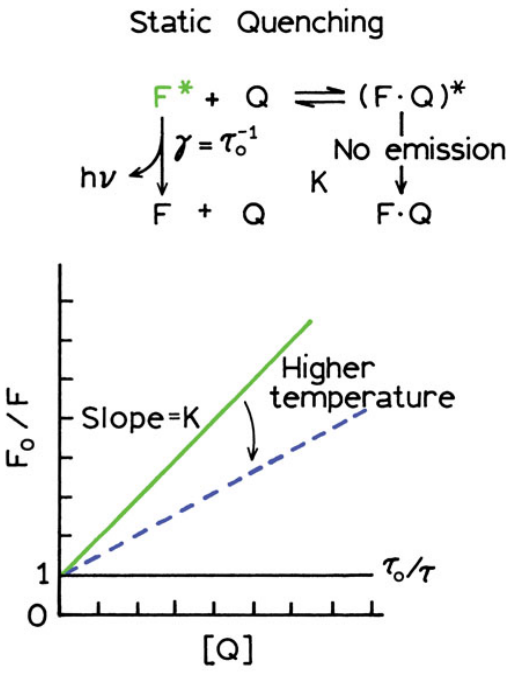
Nucleic acid bases
Nicotinamide
Heavy metals
Guanine

Different dependence of dynamic and static quenching

Dynamic quenching



Static quenching

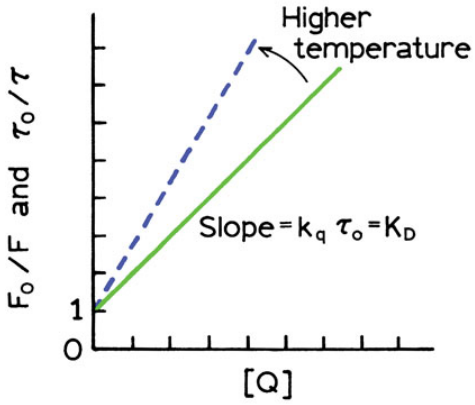


- Both types of quenching show the same dependence on the concentration of the quencher.
- A part of fluorophores forming complexes is only "invisible„ during static quenching. Fluorescence time decay τ does not alter.
- During dynamic quenching, time decay alters $\tau_0 = \tau$

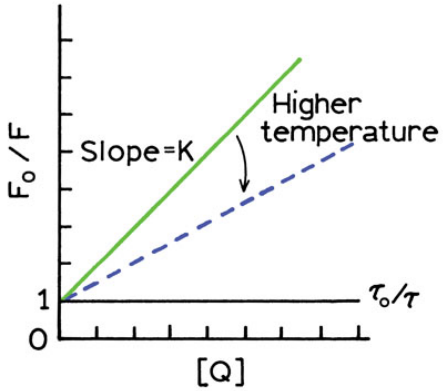
4

Dependence of both types of quenching on temperature

Dynamic quenching

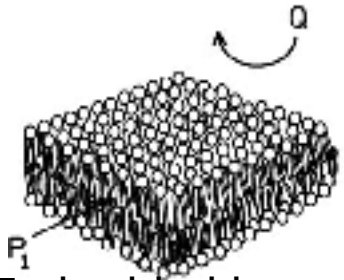


Static quenching

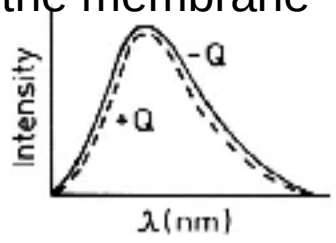


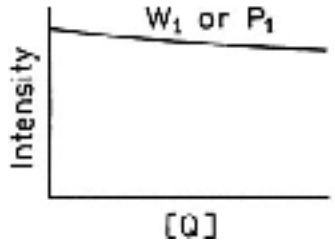
- Dynamic quenching increases with increasing temperature. The mobility of quencher molecules increases, thereby they "quench" more fluorophore molecules in the same time .
- Static quenching decreases with increasing temperature because it is easier to dissociate weakly bound complexes of a flourophore and a quencher.

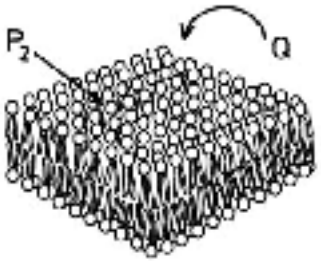
Use of quenching during fluorophore localization



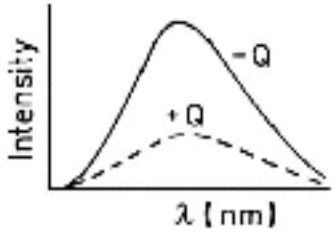
Embedded in the membrane

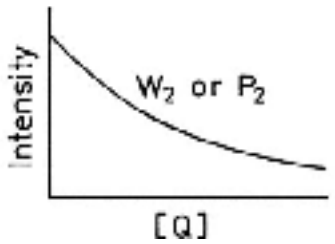






Exposed on the surface





In the membrane

- If the fluorophore P1 is embedded in the membrane, it is unavailable for the quencher Q and it almost does not lead to quenching.
- Fluorescence intensity is almost unchanged with increasing concentration of the quencher.

On the surface

- If the fluorophore P2 is on the surface, it leads to efficient quenching.
- Fluorescence intensity declines significantly with increasing concentration of the quencher .

4

Literature

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Next

- What does time resolved fluorescence tell us more than stable fluorescence?