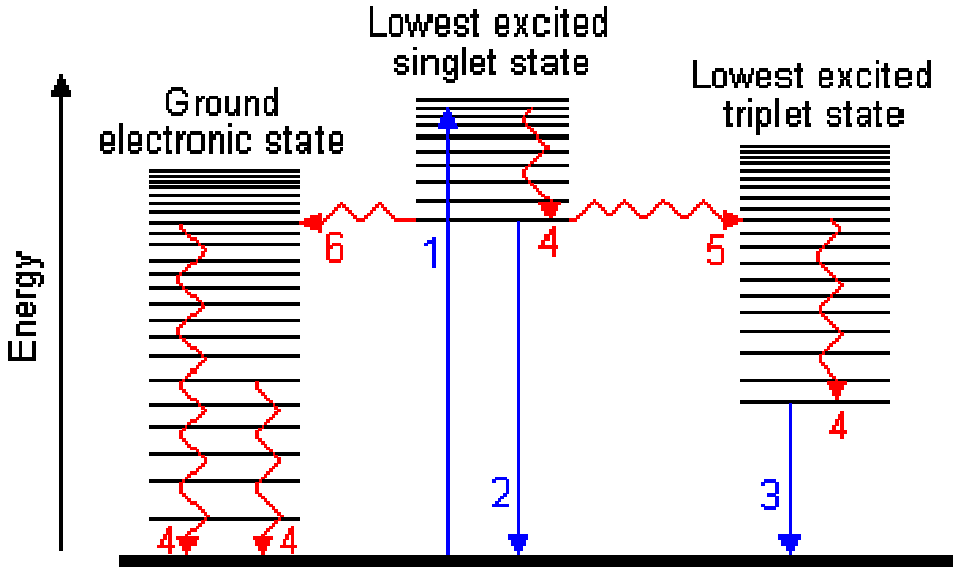
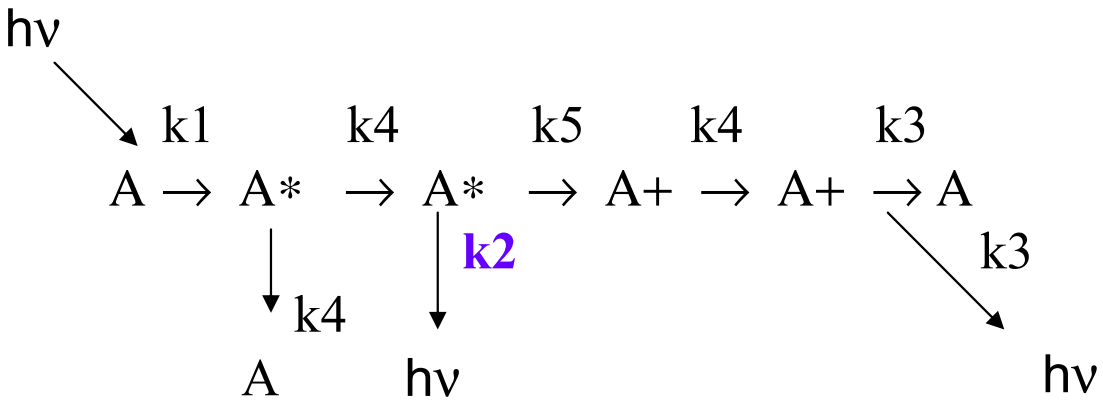
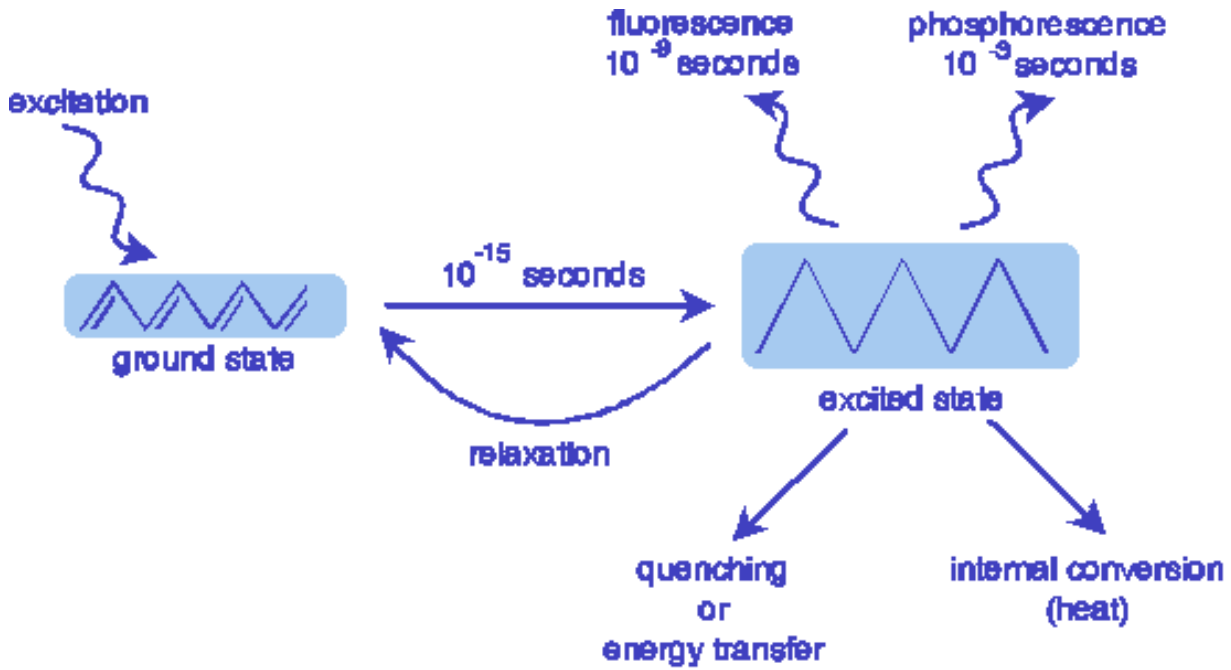
Luminiscenční spektroskopie



1. Absorption
2. Fluorescence
3. Phosphorescence
4. Vibrational relaxation
5. Intersystem crossing
6. Internal conversion



Luminiscenční spektroskopie

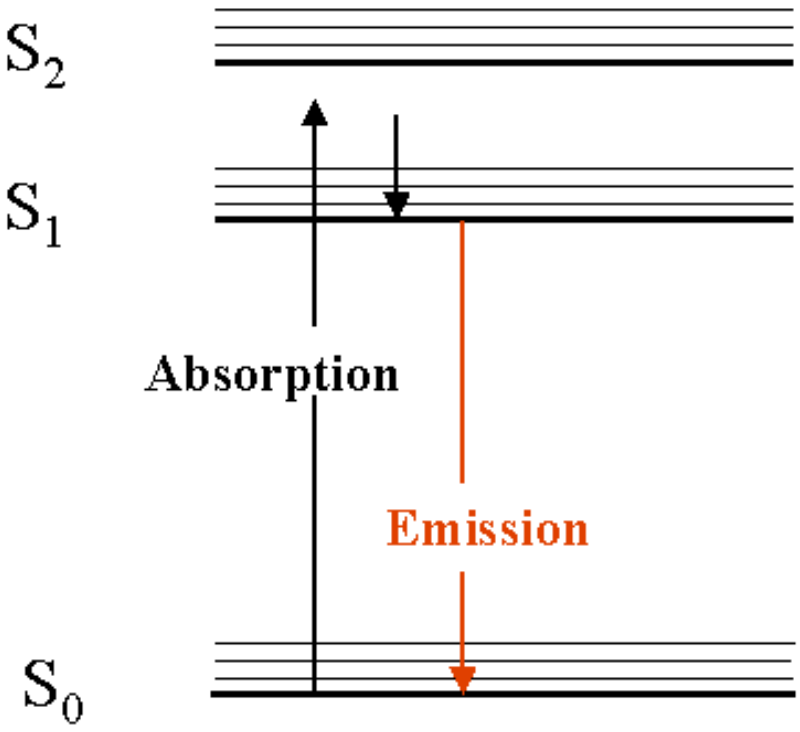


Luminiscenční spektroskopie

Fluorescenční spektroskopie
Fosforescenční spektroskopie
Chemiluminiscenční spektroskopie

Základní pojmy

Excitace a emise

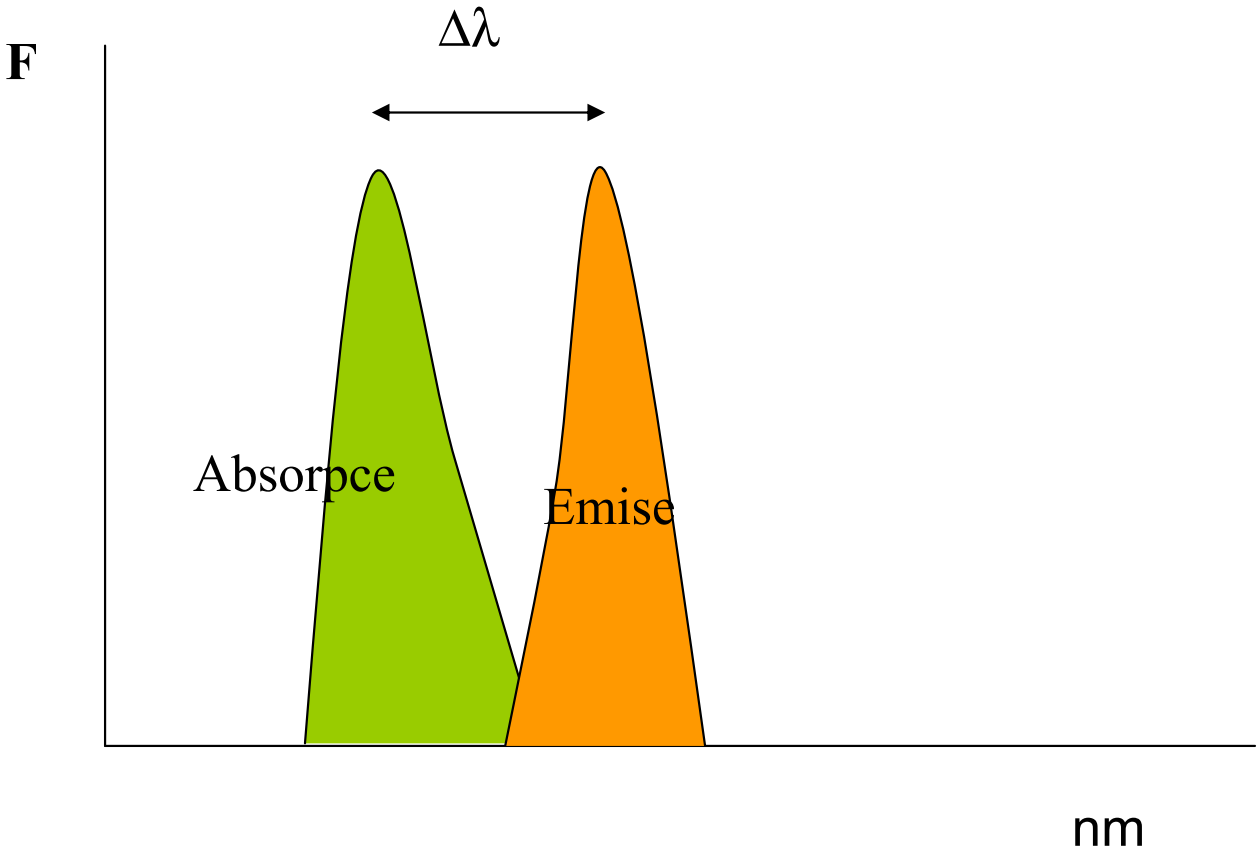


Interakce s rozpouštědlem
Singletový excitovaný stav

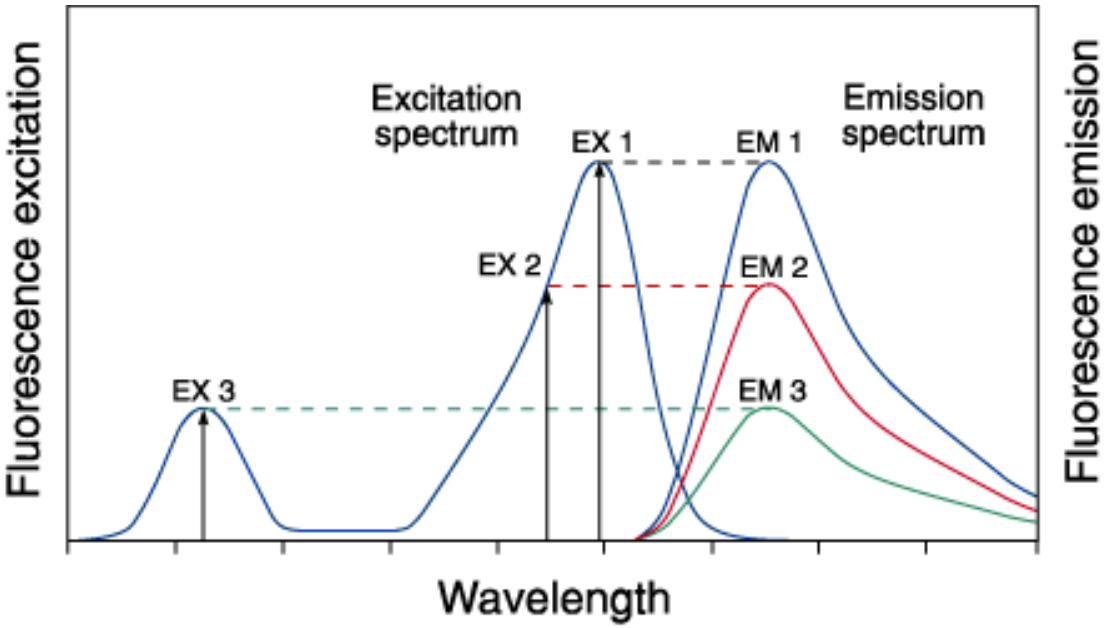
Singletový základní stav

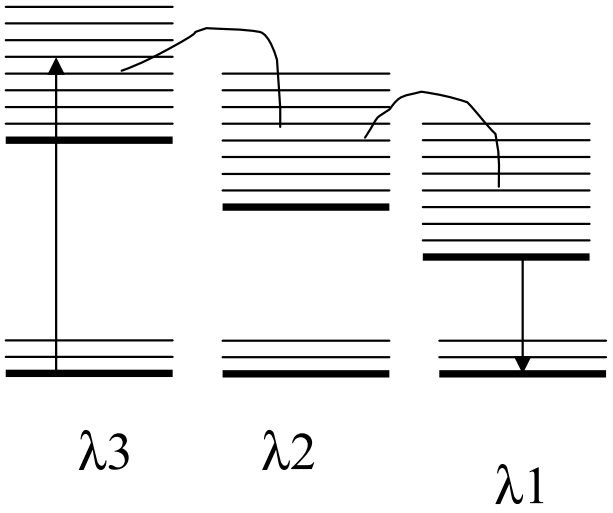
Základní pojmy

Stockesův posun – ztráty energie po dobu excitovaného stavu



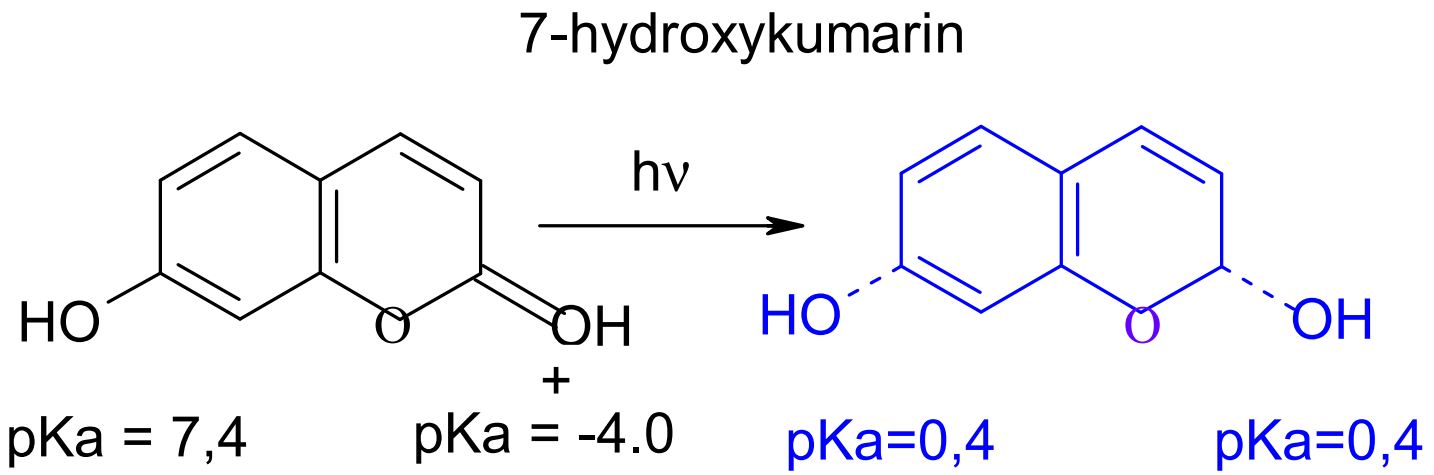
Základní pojmy





Základní pojmy

Excitovaný stav – střední doba života 10^{-7} – 10^{-9} s.



Základní pojmy

Kvantový výtěžek fluorescence

Φ = počet kvant emitovaných/počet kvant absorbovaných

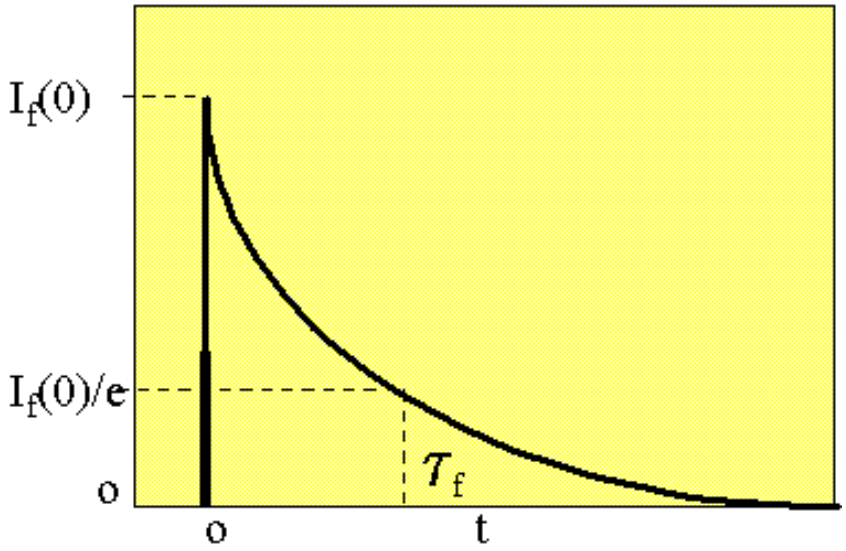
$\Phi = k_e/(k_e + \Sigma k_k)$

k_e = rychlost emise
k_k = rychlost konverzních procesů

Intenzita fluorescence látky = f (ε, Φ, N)

Základní pojmy

Doba života excitovaného stavu
Doba potřebná k poklesu fluorescence na hodnotu 1/e Io



Střední doba života τ
$\tau = 1/k_f$

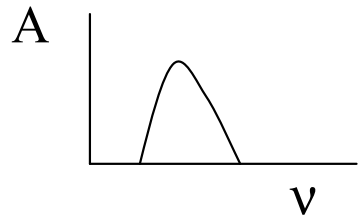
$I_f = I_0 \, e^{-t/\tau}$

Přirozená doba života τ_o

Definovaná pro $\Phi = 1$

$$\tau_0 = 2{,}88.10^{-9}.n^2.\nu_A^{\,2} \int_0^{\infty} \varepsilon(\nu)d\nu$$

n- refrakční index rozpouštědla
ε – molární abs. Koeficient
ν – vlnočet abs. maxima



Základní pojmy

$$\Phi = \tau/\tau_0$$

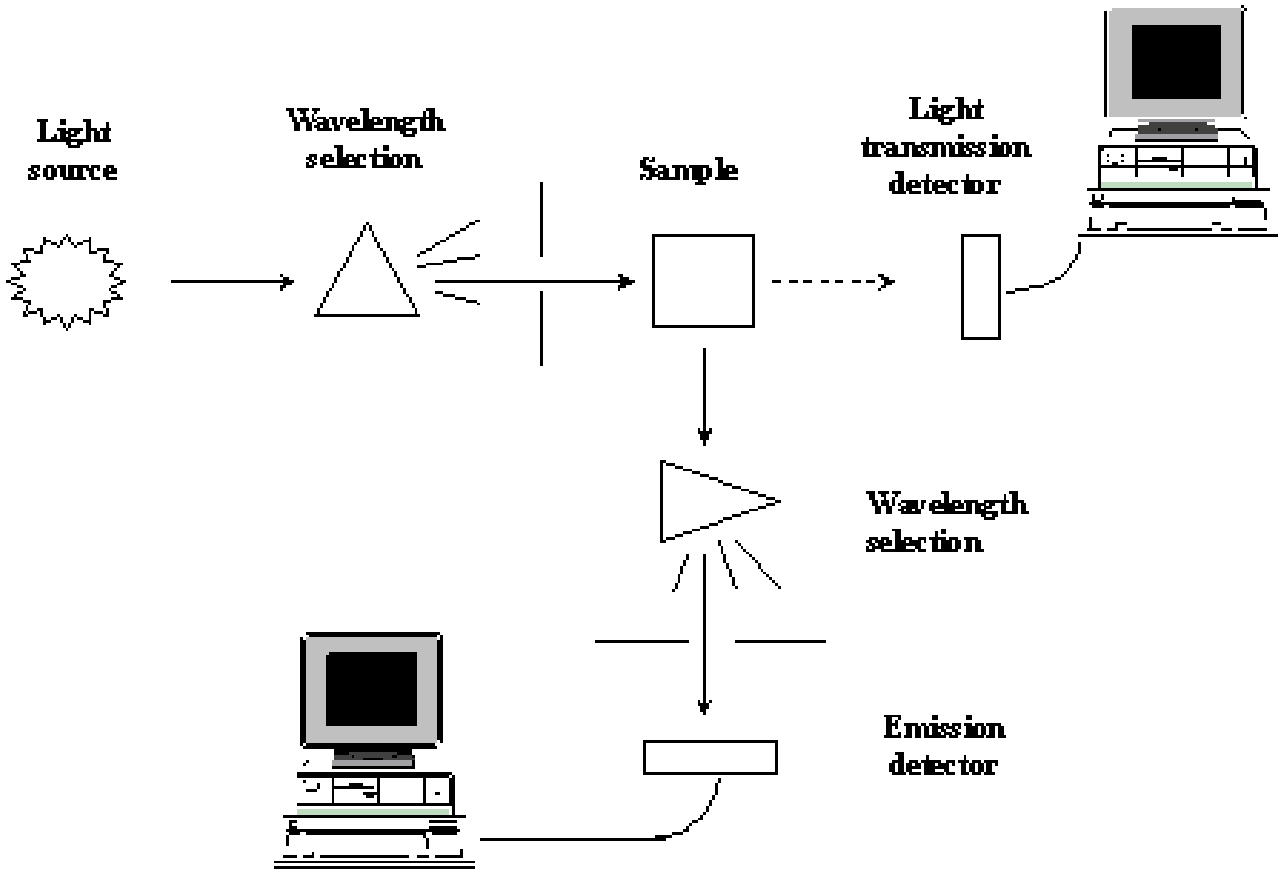
Střední doba života fluorescence

Fluorescein	4,6 ns
Chininsulfát	15 – 40 ns
NADH	0,5 ns

Biochemicky významné fluorofory

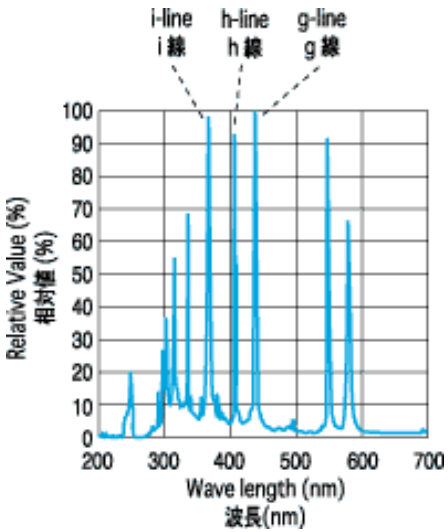
	λ_{exc}	λ_{em}	Q (25°C)
Tyrosine	275	303	0.14
Tryptophane	287	348	0.13
Indole	287	348	0.45
NADH	350	460	0.03
Riboflavine	450	535	-
Chlorophylle	436	670	0.30 (acétone)
Quinine	250	450	0.51 (1M H_2SO_4)
Pyridoxamine	324	392	0.11 (pH=8.2)
Vitamine A	325	470	- (ethanol)
Aminobenzoate	294	345	-

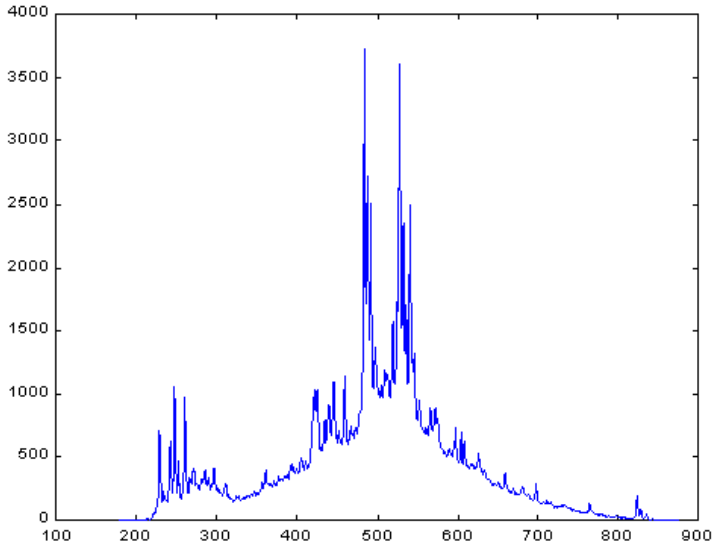
Instrumentace

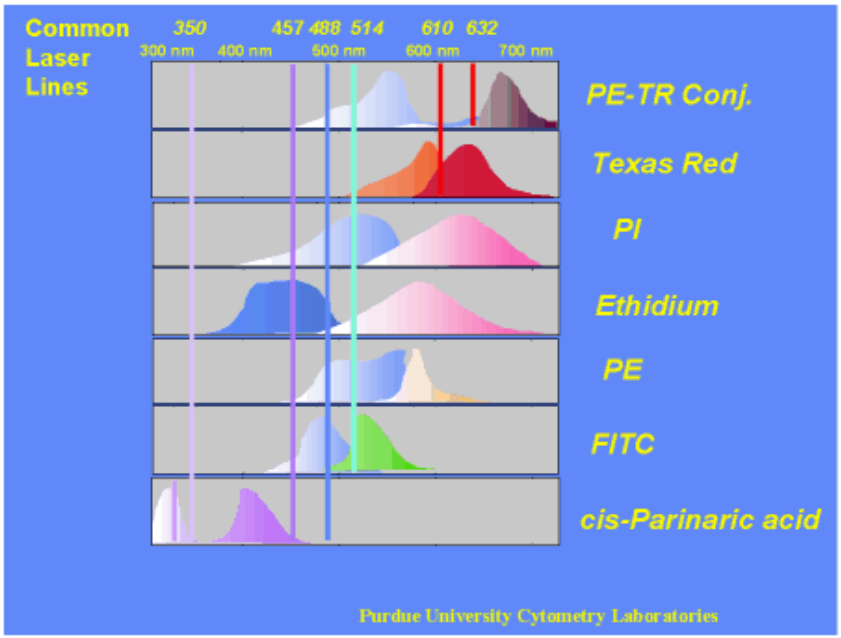


Instrumentace

Zdroj:
Xenonová lampa
Rtuťová výbojka
Laser
Světelné diody - LED (430, 450, 505, 592, 612 and 637 nm)



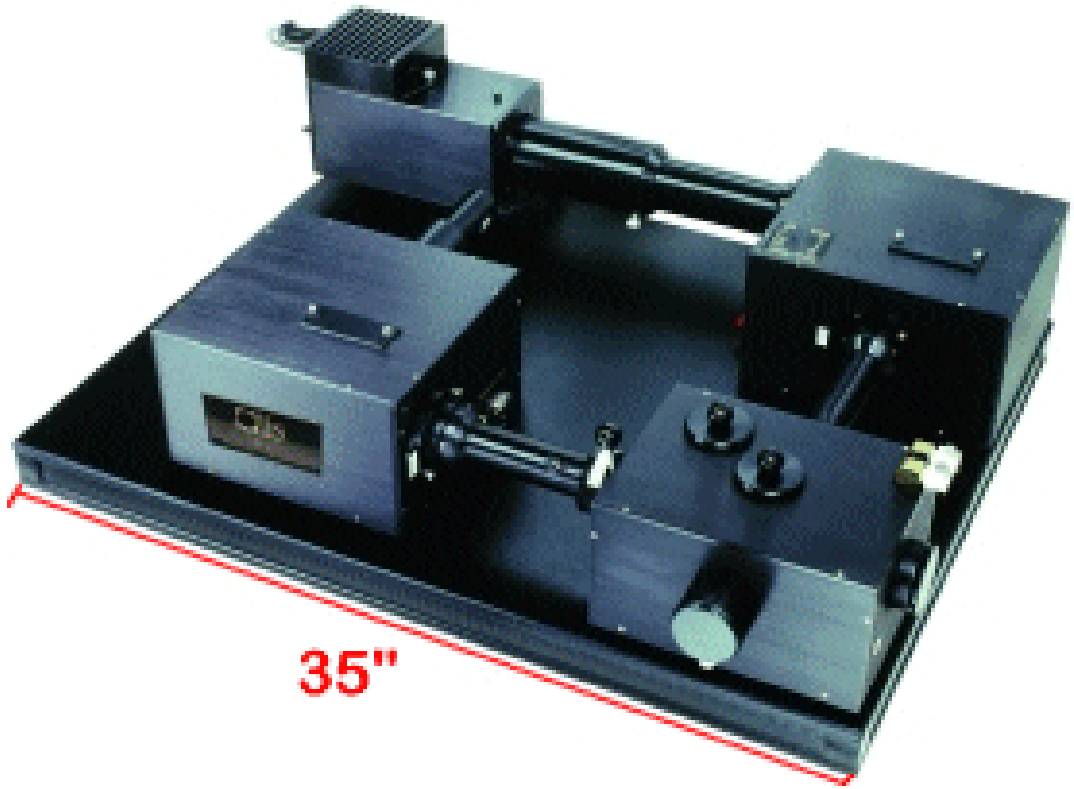




Instrumentace

Monochromátor
- mřížka
- filtry

Instrumentace

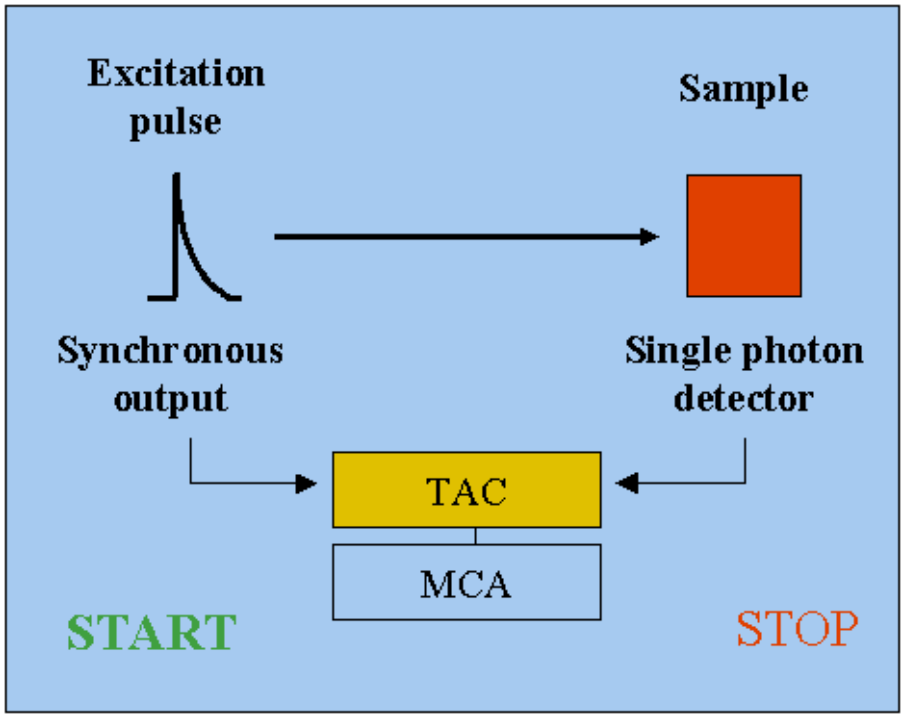


Instrumentace

RF-5301PC – spektrofluorimetr Shimadzu

Instrumentace

Měření střední doby života fluorescence



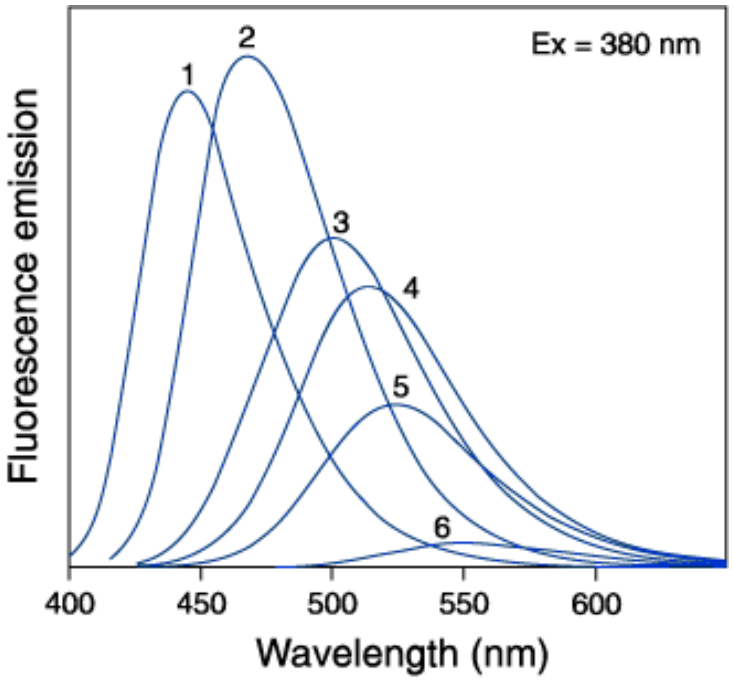
Podmínky fluorescence

Závislost na polaritě a viskozitě

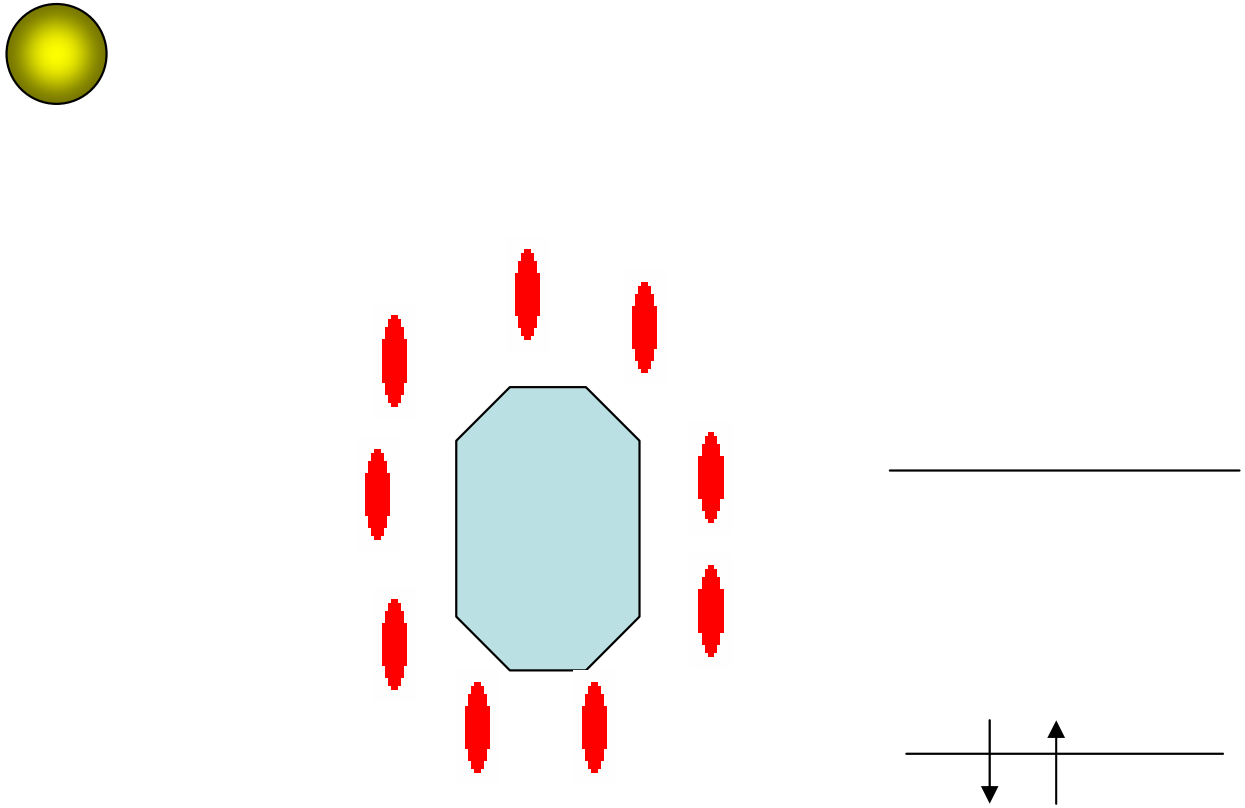
Nitrobenzoxadiazol

Solvent	Freq. domain (ns)	TCSPC (ns)	Literature (ns)
H_20	0.92	0.97	0.93
Methanol	5.35	5.31	5.64
DMSO	7.15	7.54	7.48
Ethyl acetate	10.93	nd	10.5

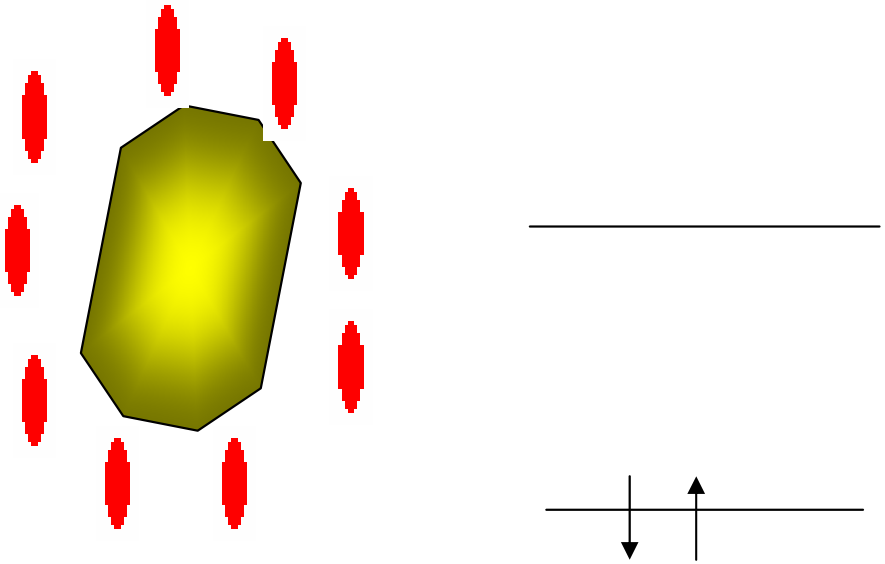
Pokles polarity 6 – 1.



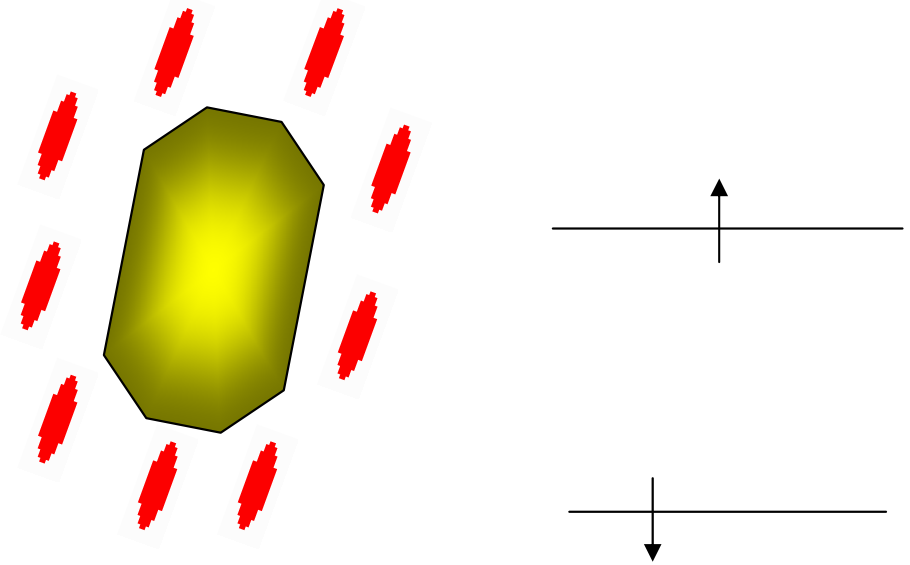
Podmínky fluorescence



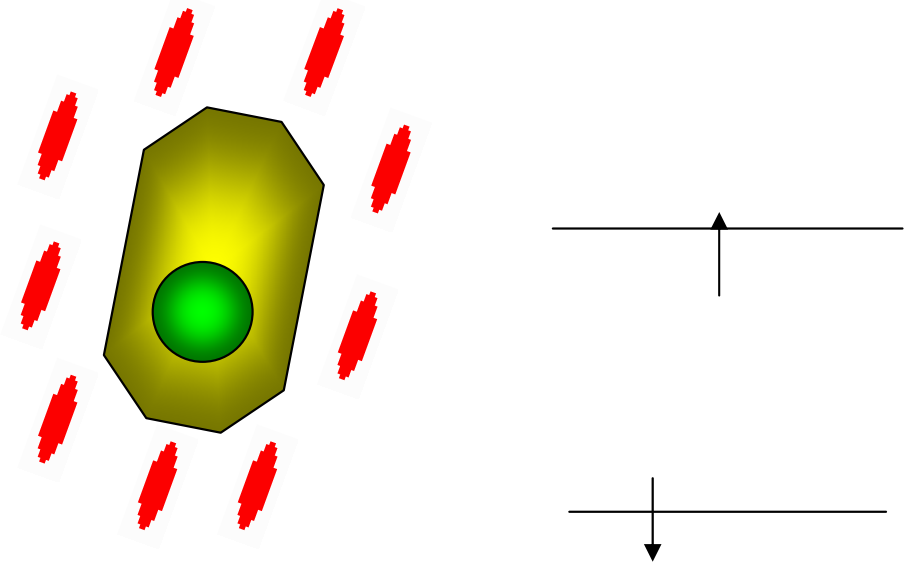
Podmínky fluorescence



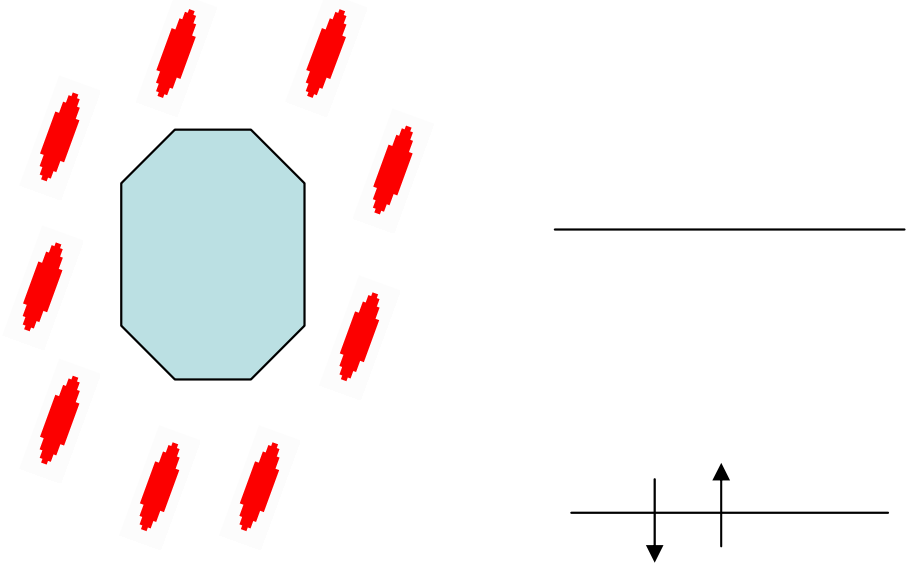
Podmínky fluorescence



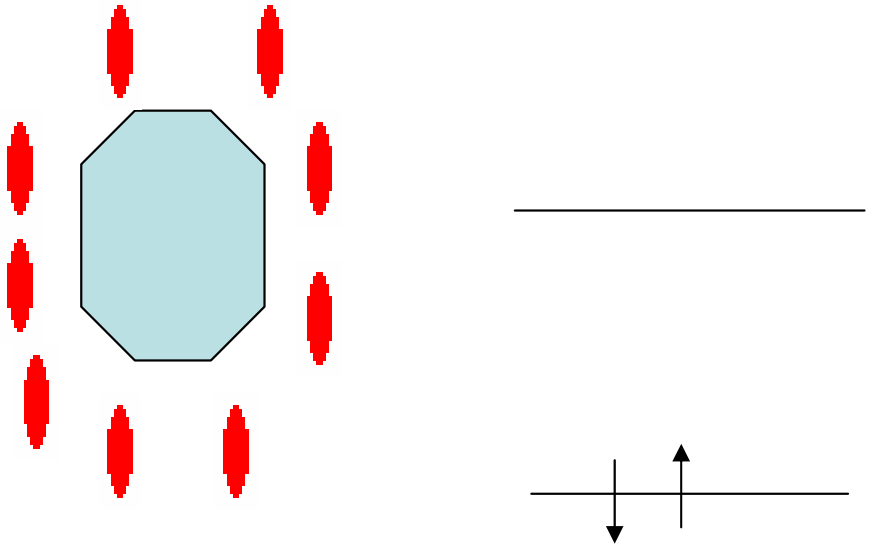
Podmínky fluorescence



Podmínky fluorescence

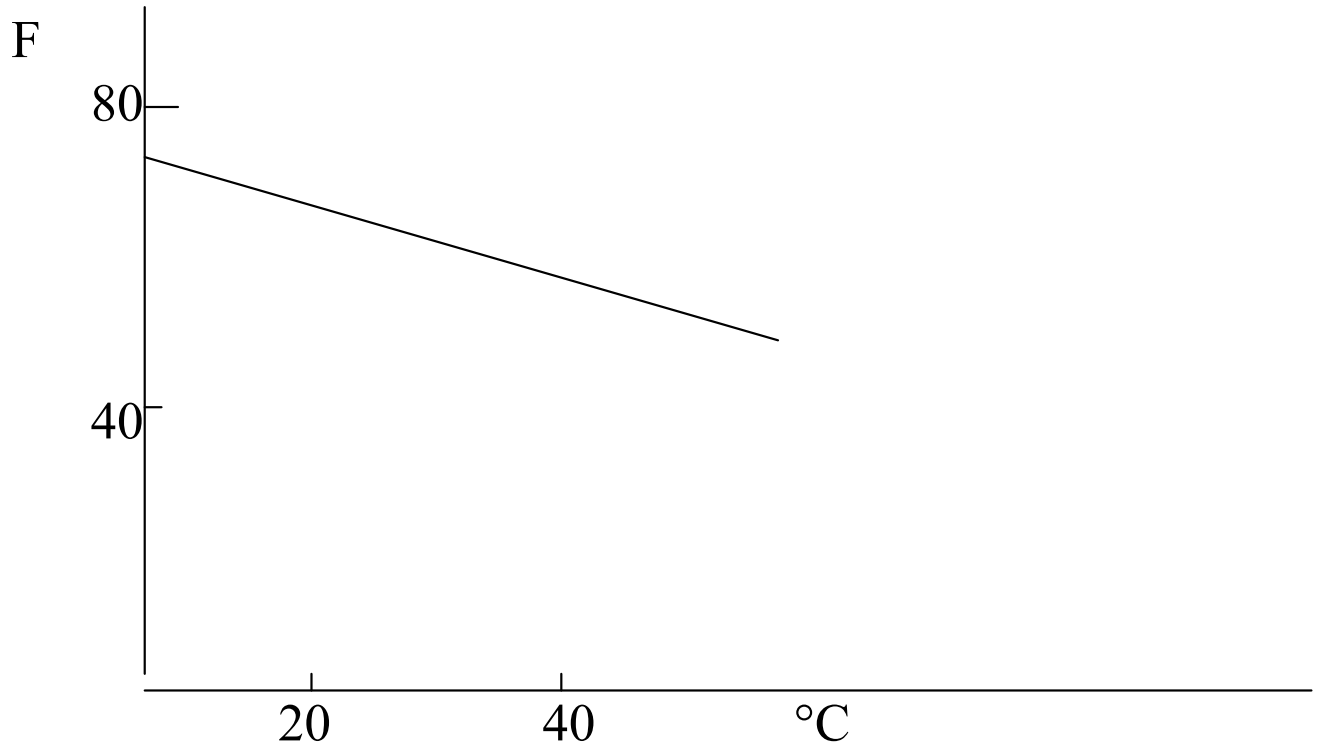


Podmínky fluorescence



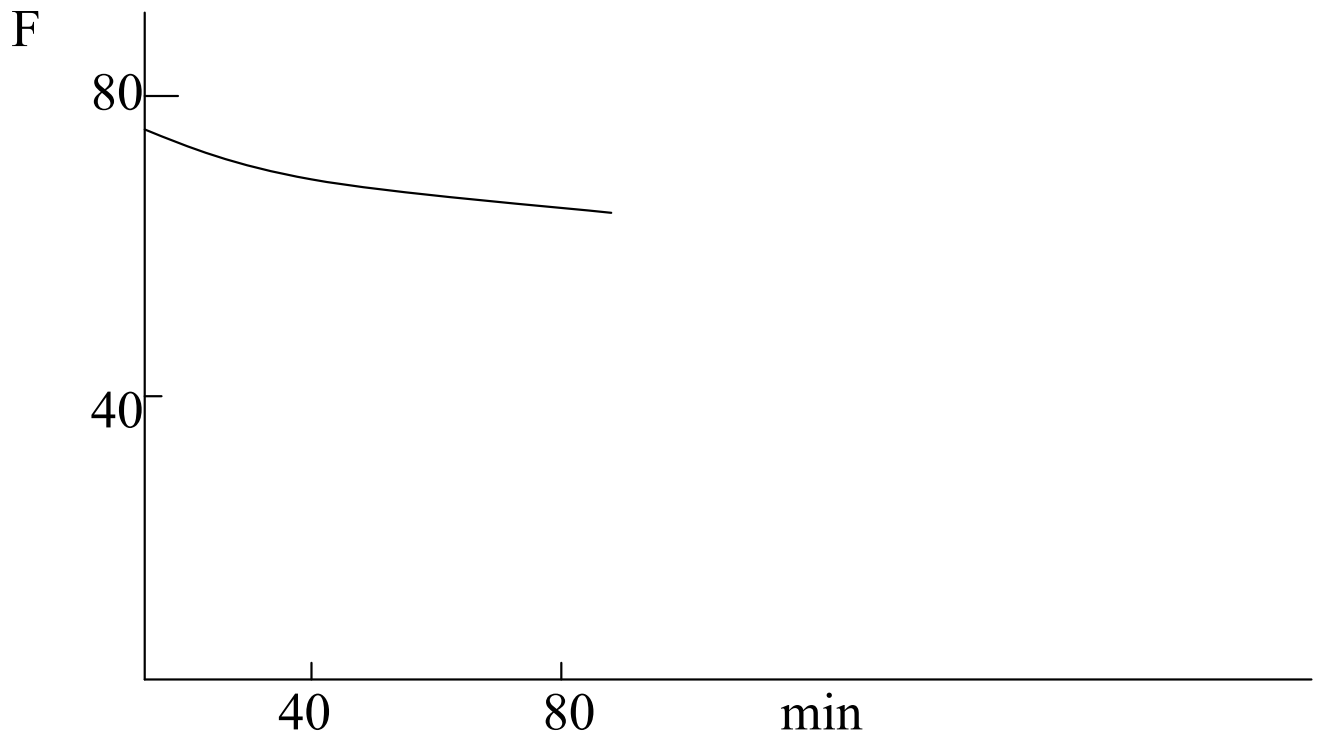
Podmínky fluorescence

Závislost fluorescence na teplotě

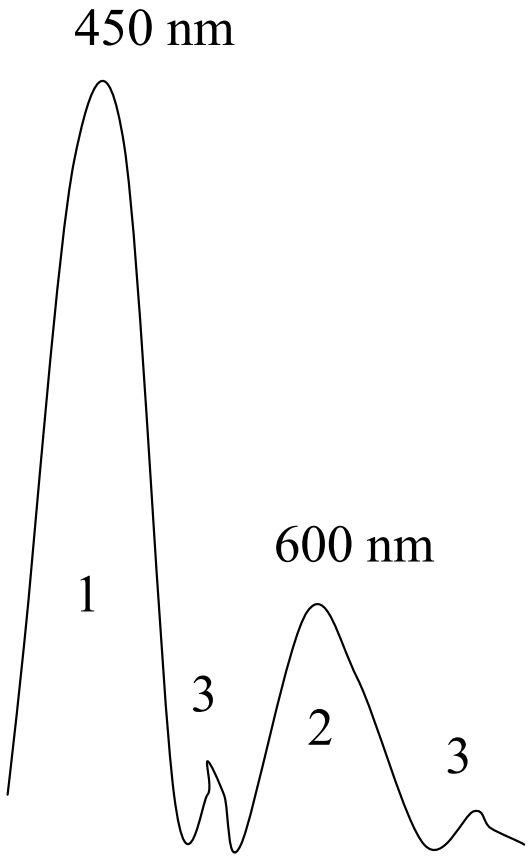


Podmínky fluorescence

Stabilita fluorescenčního signálu chininsulfátu



Podmínky fluorescence



Emisní spektrum

1 Rayleighův rozptyl (Tyndalův rozptyl)
2 Fluorescenční emise
3 Ramanův rozptyl

Excitace 450 nm

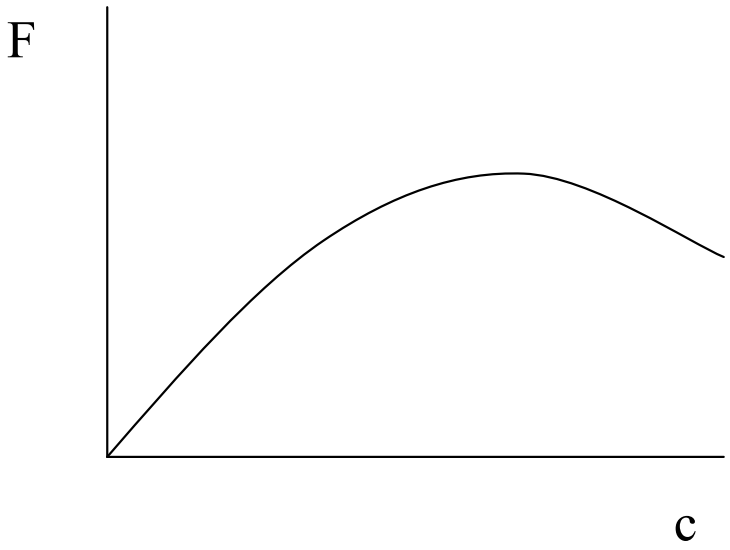
Kvantitativní fluorimetrie

Závislost intenzity fluorescence na koncentraci látky

$F = f(I, \varepsilon, c, \Phi)$

$F = Io\ \Phi\ [1 - 10^{-\varepsilon cd}]$ jestliže $c \rightarrow 0$

$F = Io\ \Phi.2,3.\varepsilon d.c$



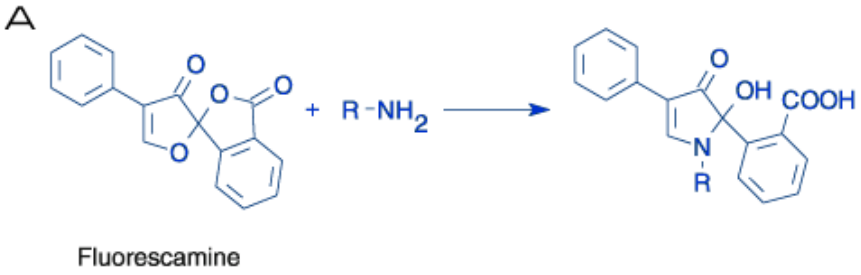
Kvantitativní fluorimetrie

Stanovení koncentrace aminokyselin

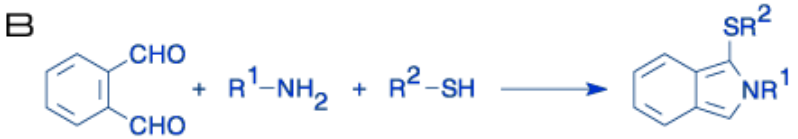
390/464 nm

340/455 nm

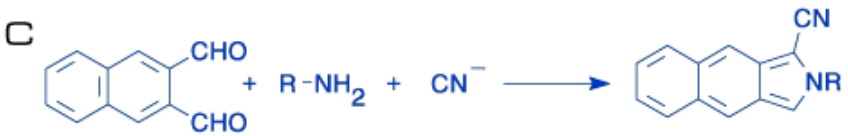
450/550 nm



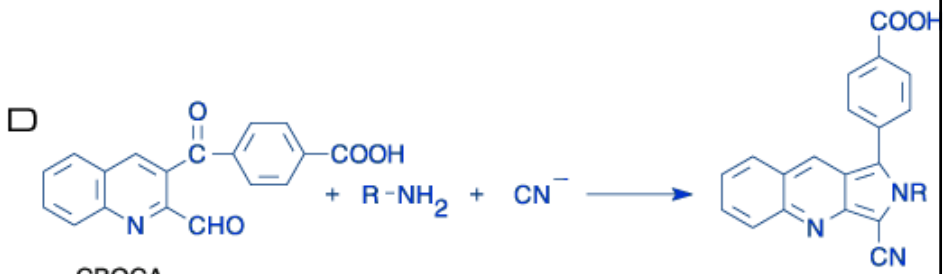
Fluorescamine



OPA



NDA

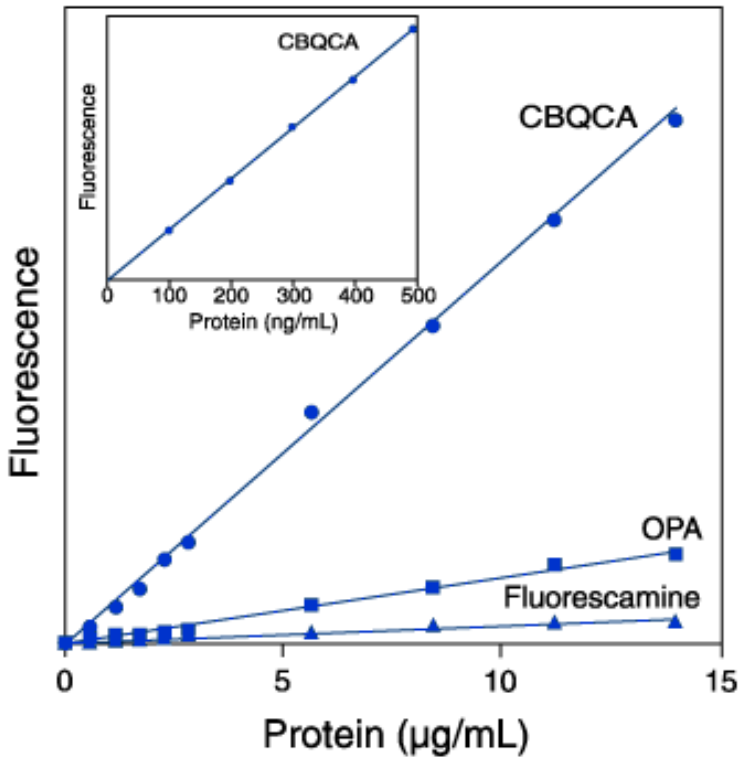


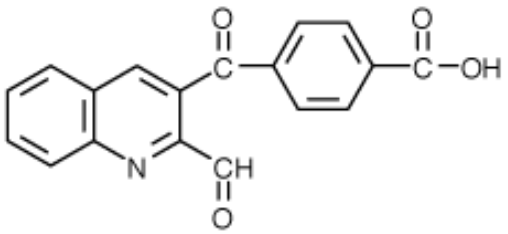
CBQCA

Kvantitativní fluorimetrie

Stanovení bílkovin

CBCQA





Kvantitativní fluorimetrie

Detekce bílkovin v gelu

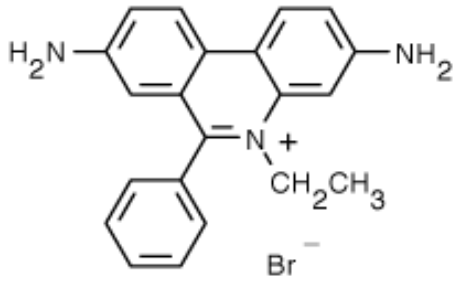
(barevně: Coomasie blue, stříbrné barvení)

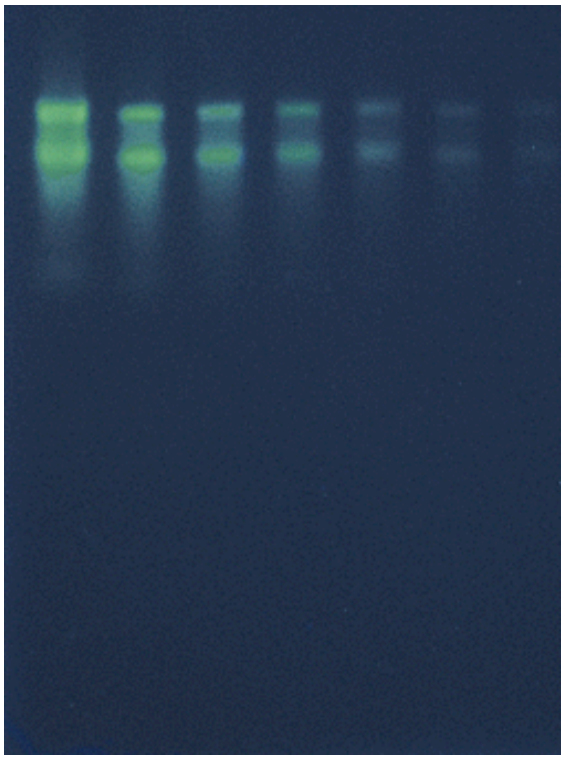
Fluorimetricky: SYPRO Orange (Molecular probes) – citlivost 1 – 2 ng

Kvantitativní fluorimetrie

Detekce nukleových kyselin

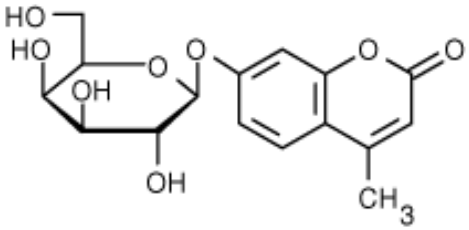
Ethidium bromid





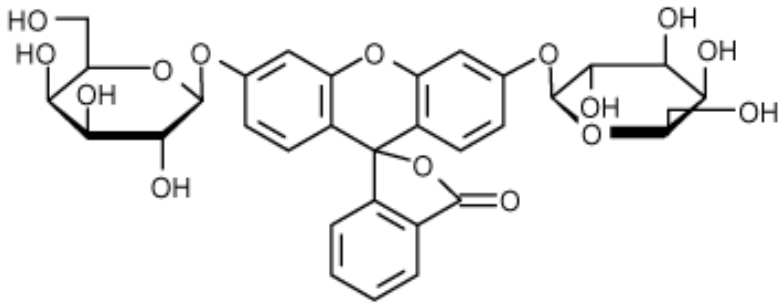
rRNA 16 a 23s barvená SYBR Green II
Molecular Probes

Fluorogenní substráty



Galaktosidasy

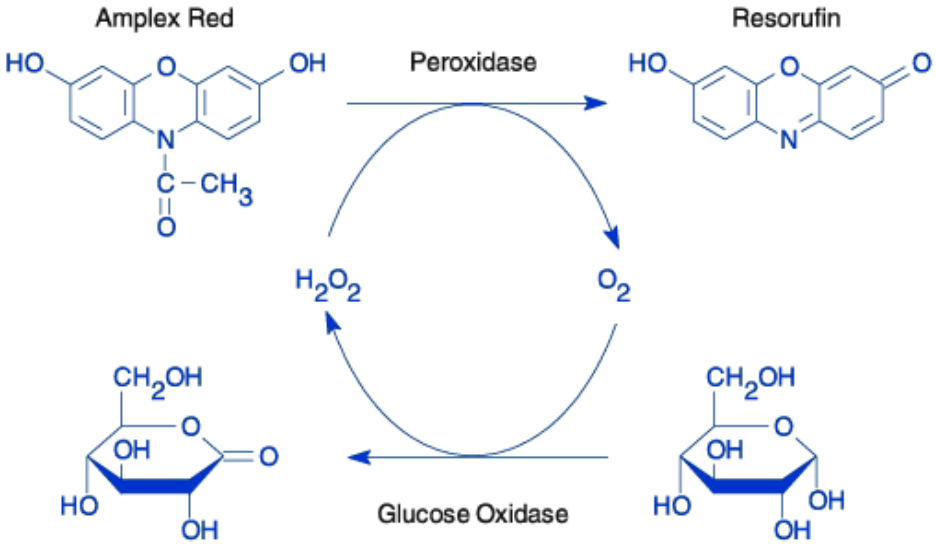
4-methylumbelliferyl-α-galaktosid

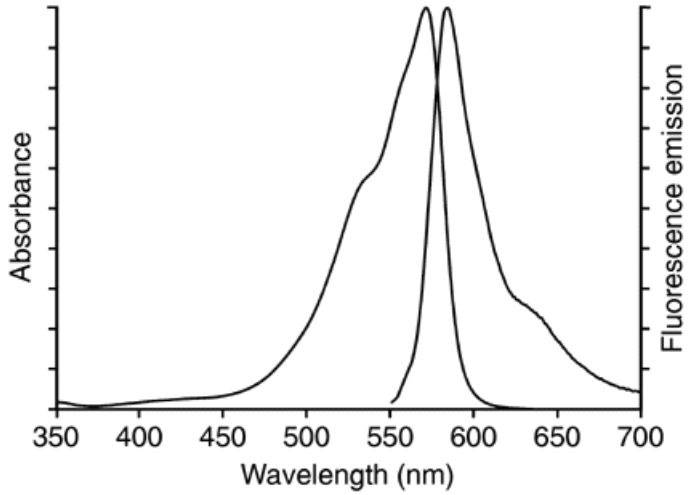


Fluorescein-digalaktosid

Fluorogenní substráty

Peroxidasy – amplex red, vznik resorufinu

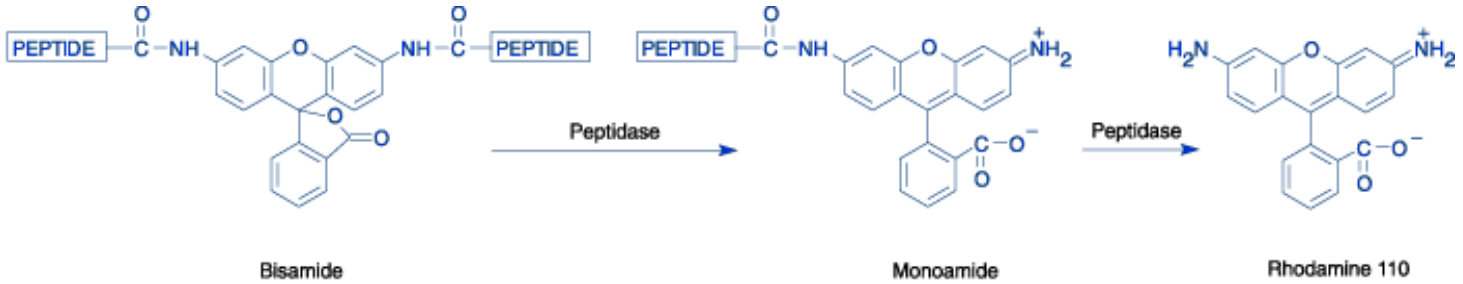


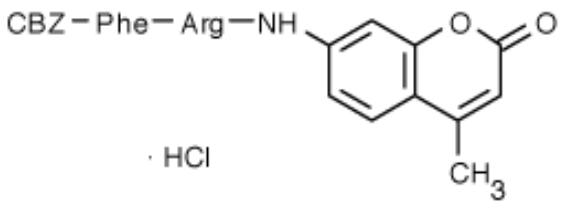


Fluorogenní substráty

Proteinasy, peptidasy

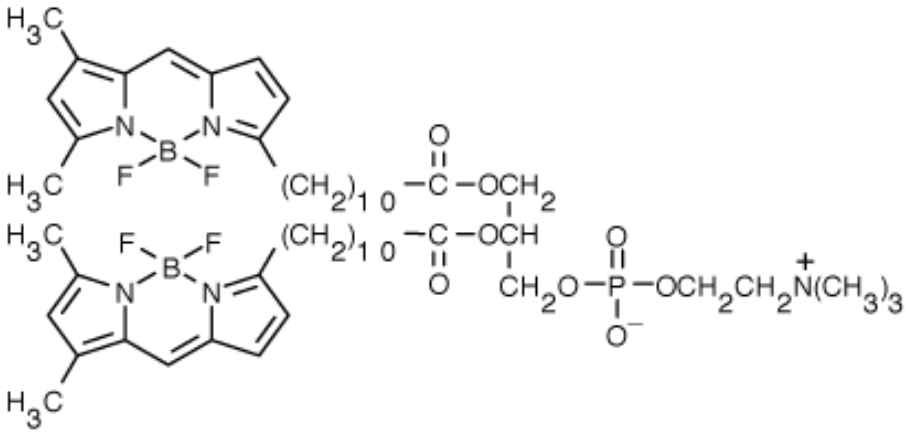
1) Fluorescenční konjugáty proteinů a peptidů





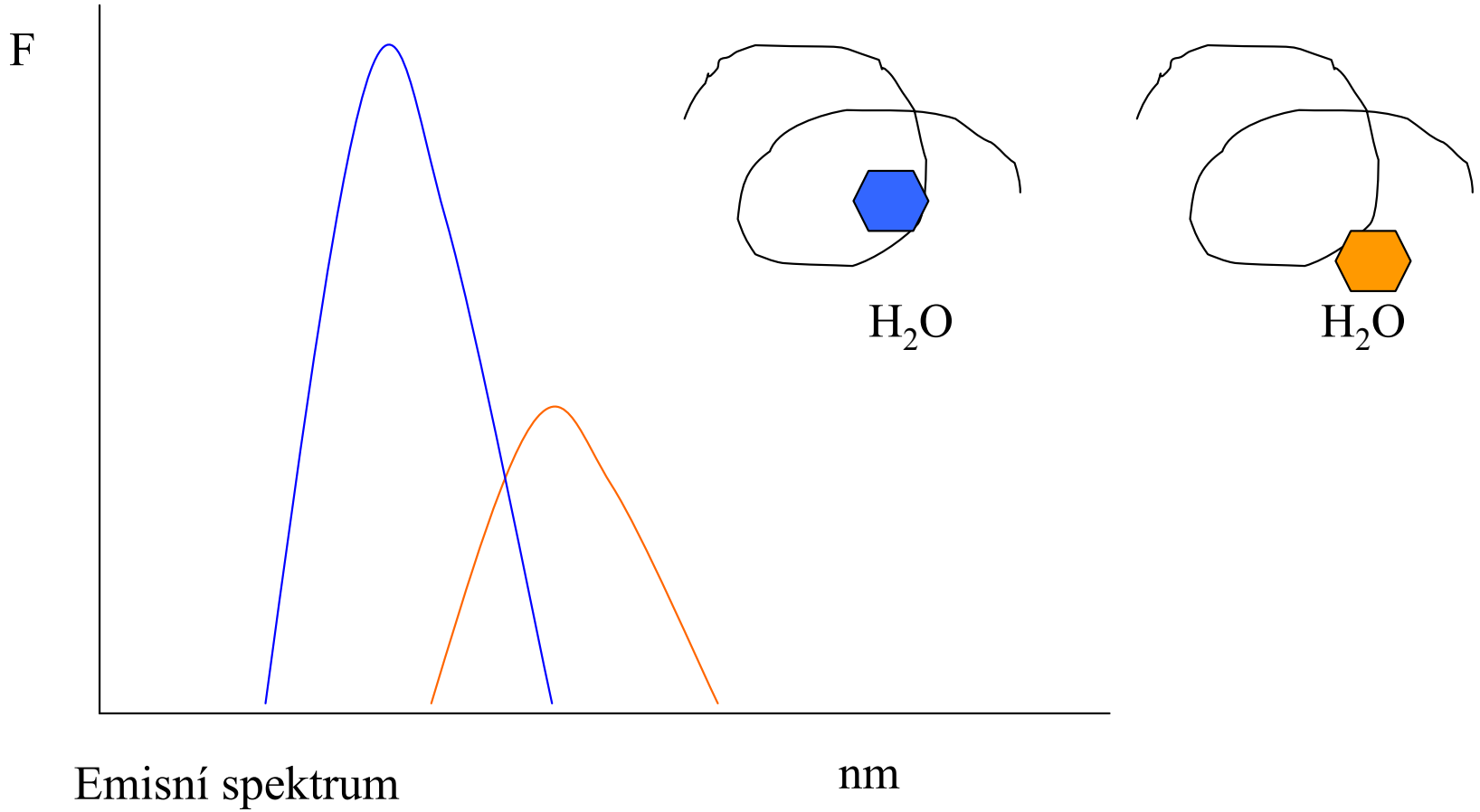
Fluorogenní substráty

Fosfolipasa A

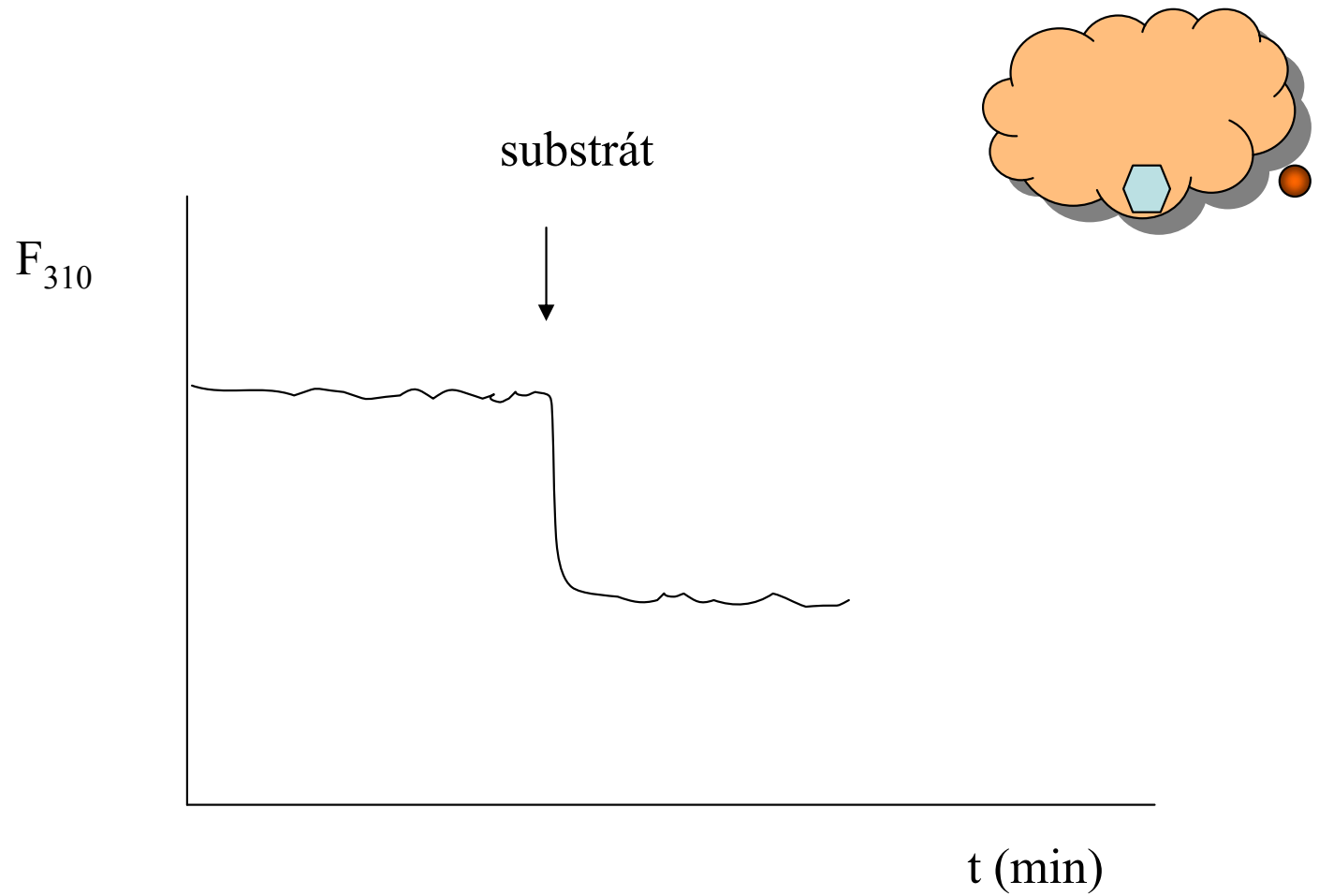


Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Přirozené fluorofory (Tyr, Try) – fluorescence závislá na polaritě prostředí obklopující fluorofor

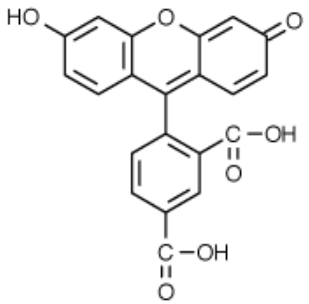


Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

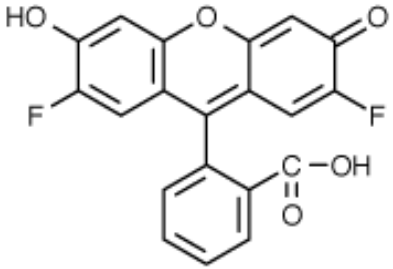


Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

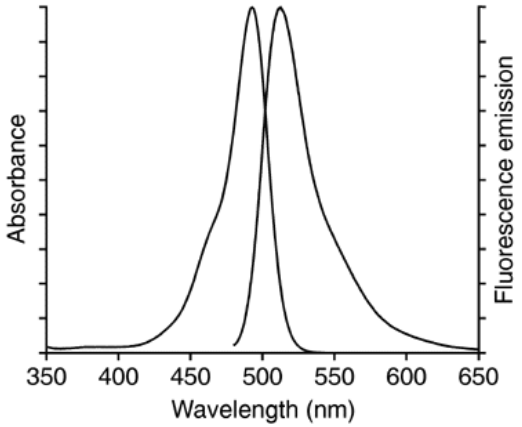
Fluorescenční konjugáty



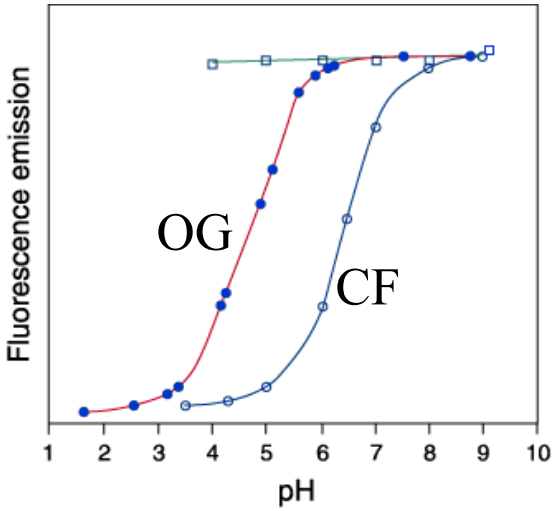
Karboxyfluorescein – (494/520 nm)



Oregon Green - (496/524 nm)



Absorpce/emise fluoresceinu při pH 9

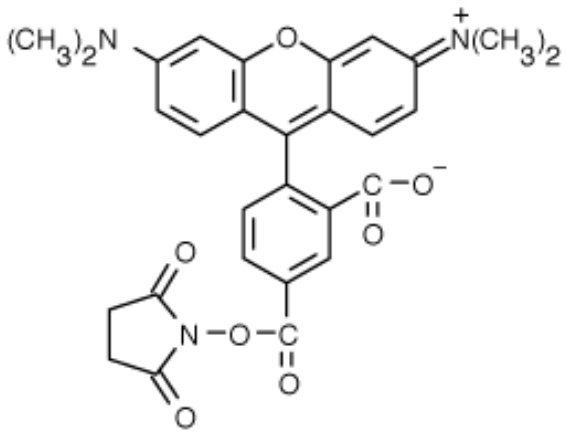


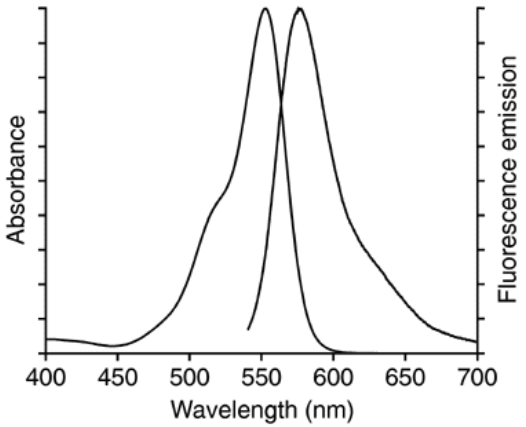
CF-karboxyfluorescein
OG oregon green

Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

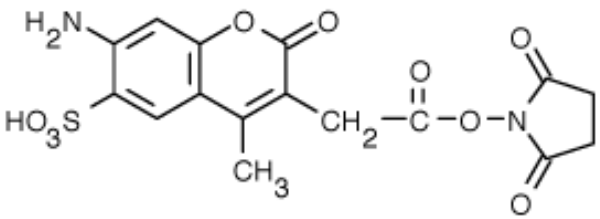
Fluorescenční konjugáty

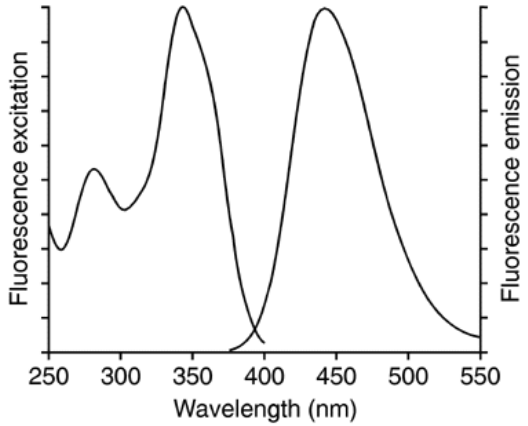
Teramethylrhodamin – 545/580 nm)





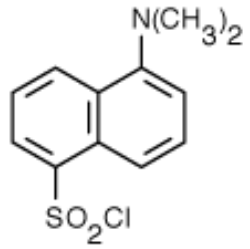
Kumariny – 350/450 nm)



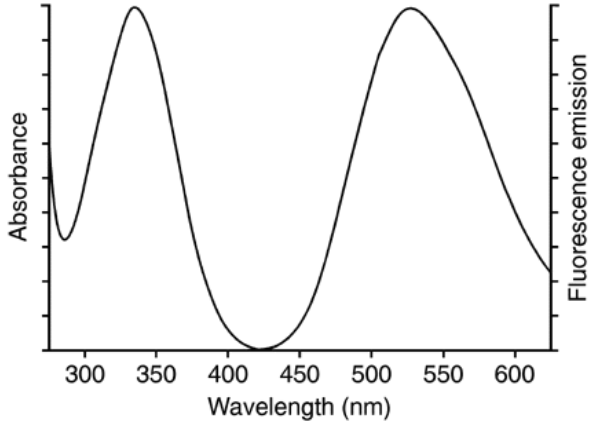


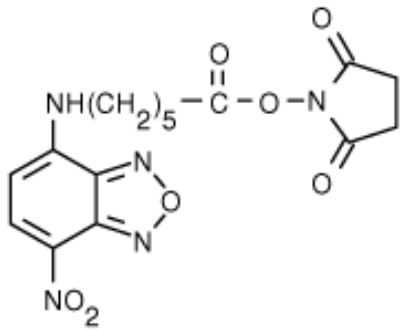
Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Fluorescenční konjugáty

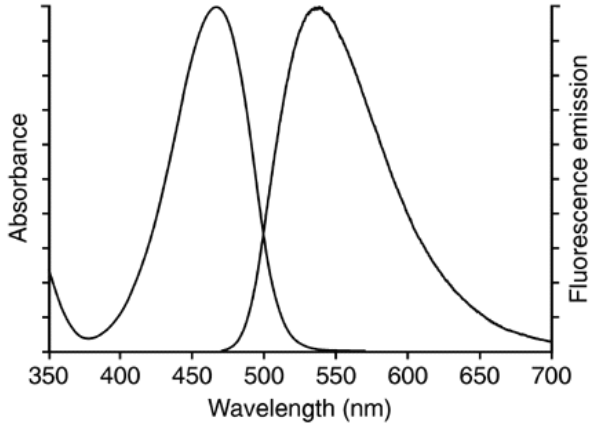


Dansyl



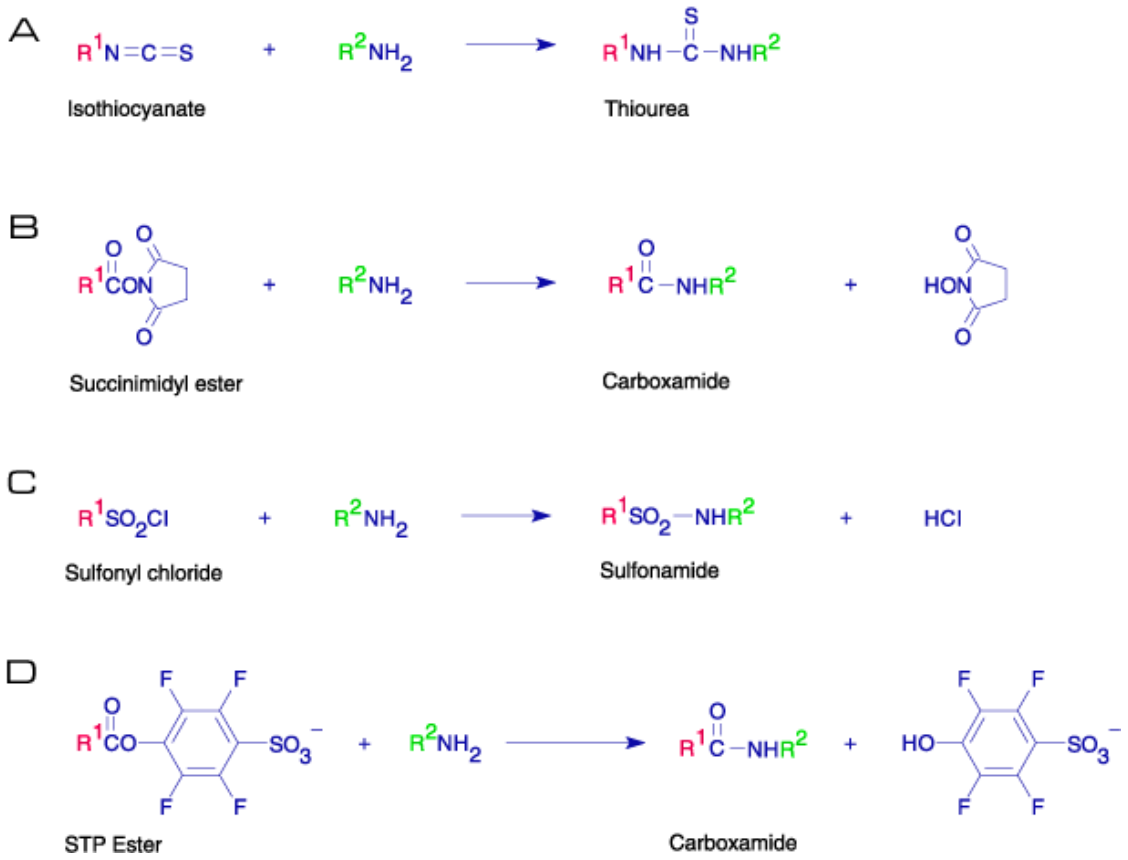


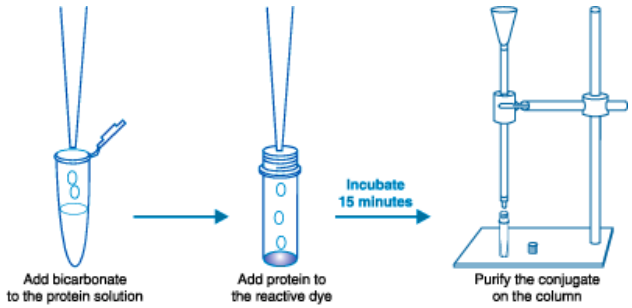
Benzoxadiazol –N-sukcinimid



Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Fluorescenční konjugáty



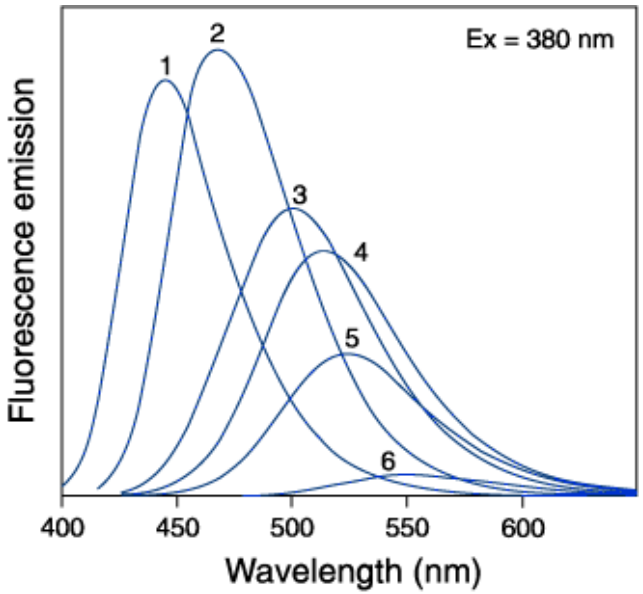


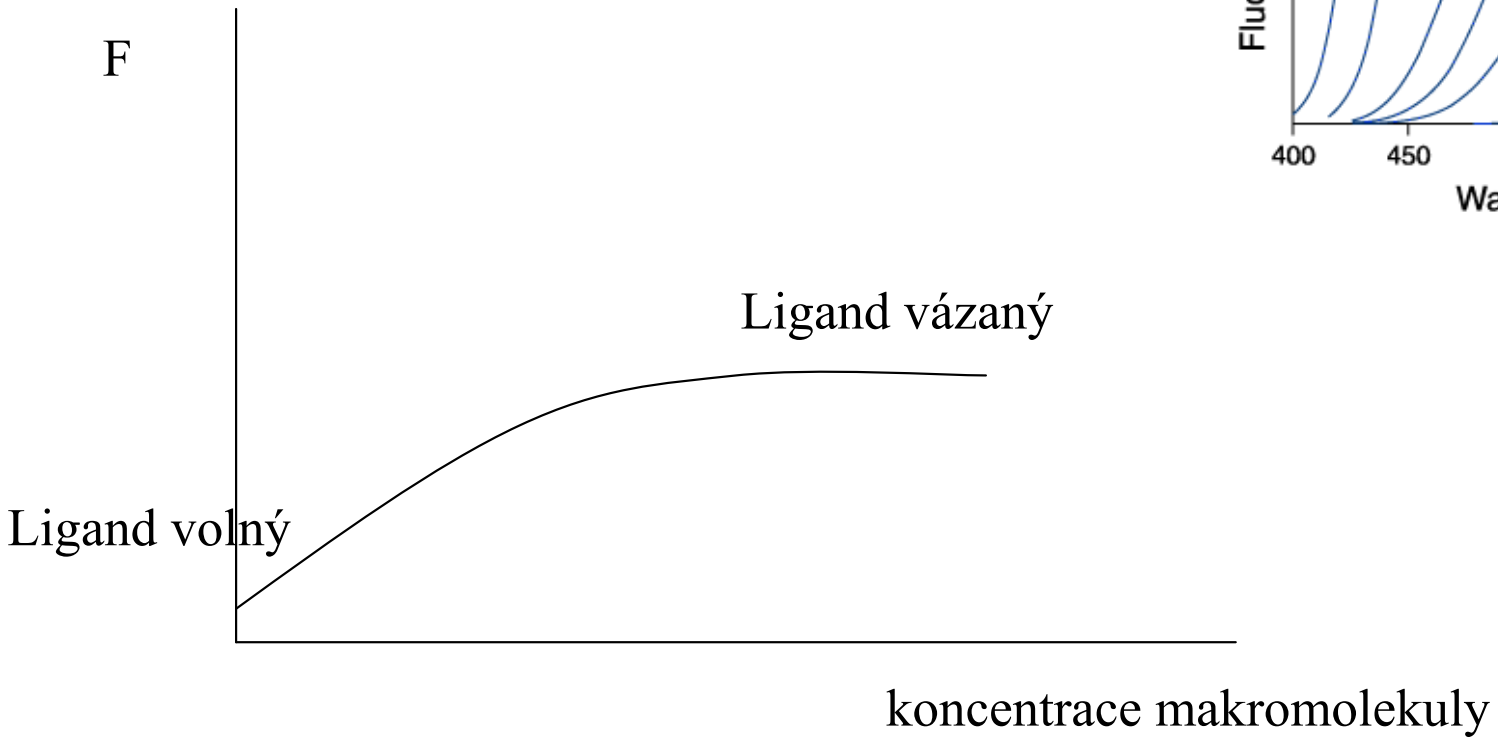
Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

$$L + M \leftrightarrow LM$$

$$Kd = L_f.M_f/LM$$

Interakce makromolekul s ligandy





Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

$F = F_f + F_b$

$F = Cf \,.\Phi_f + C_b.\ \Phi_b$

$F = (C\text{-}C_b)\ \Phi_f + C_b.\ \Phi_b$

$F = C\Phi_f - Cb\Phi_f + C_b.\ \Phi_b$

$F = Fo + C_b\ (\Phi_b - \Phi_f)$

$Cb = (F - Fo)/\ (\Phi_b - \Phi_f)$

Ff, Fb – fluorescence volné, vázané frakce

Φb, Φf –kvant. Výtěžek fluorescence vázaného, volného ligandu

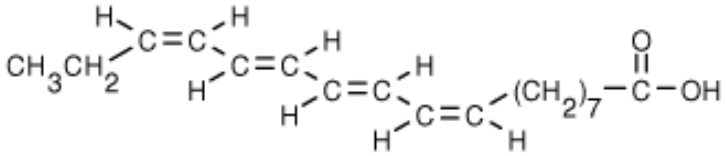
Cb,Cf – koncentrace vázaného, volného Ligandu

C – celková koncentrace ligandu

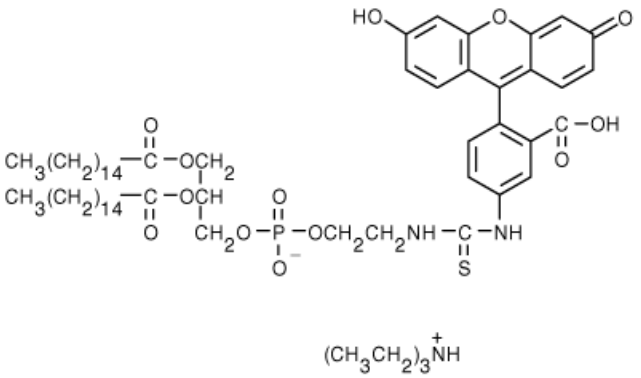
F – celková fluorescence

Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

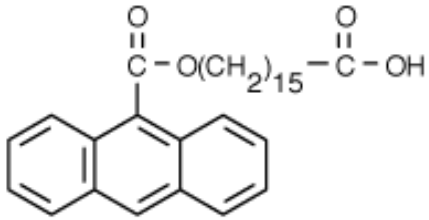
Interakce makromolekul s ligandy
Použití fluorescenčních analogů



Kys. cis-parinarová



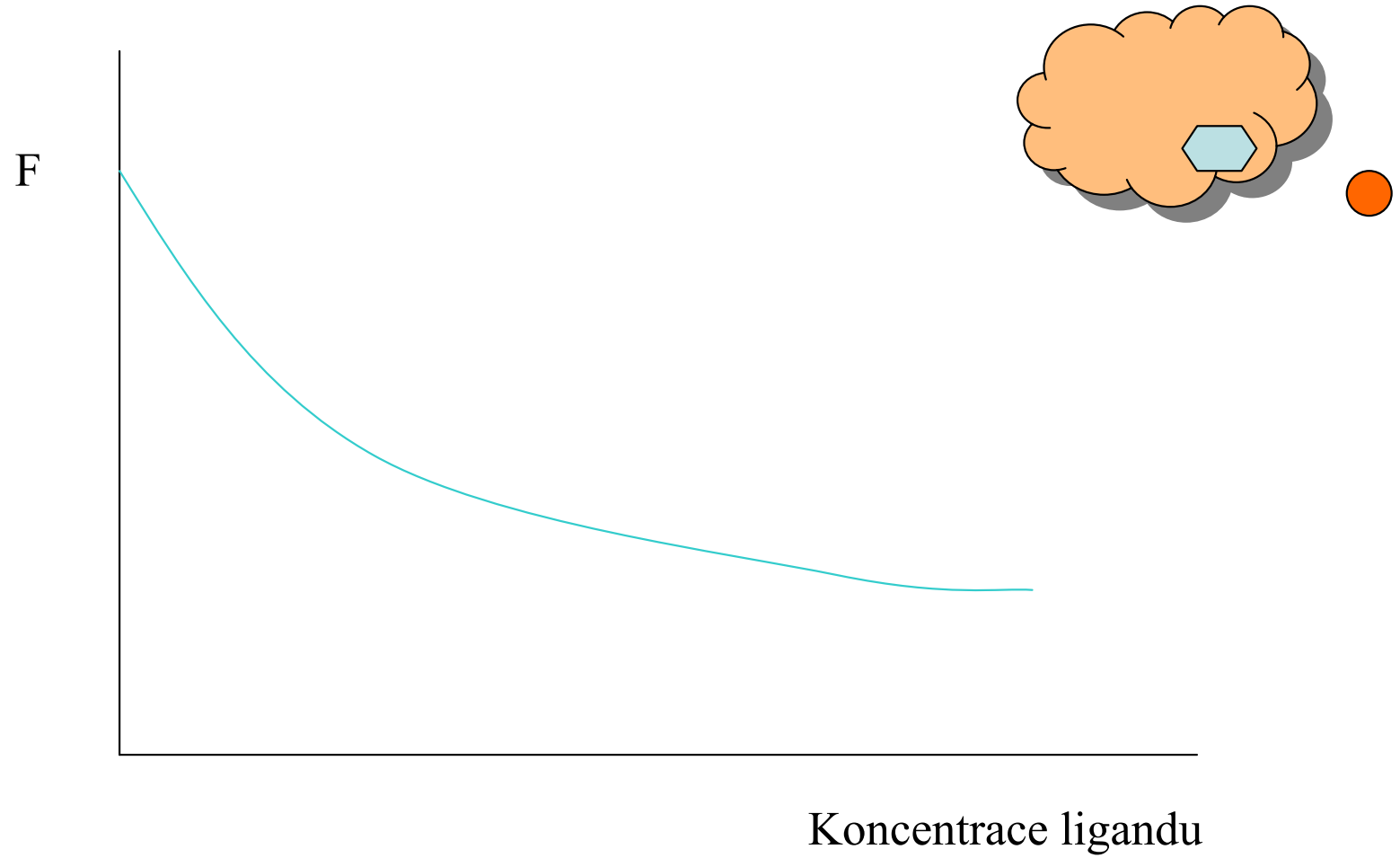
Fluorescein-PE



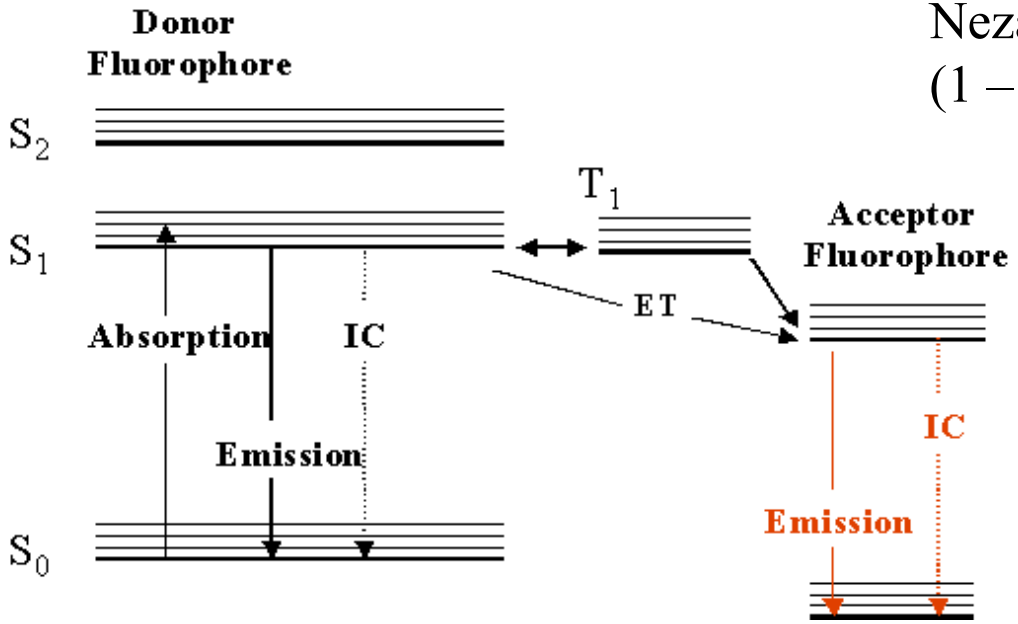
Anthroyloxypalmitát

Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Použití značené makromolekuly
Fluorescence značené bílkoviny

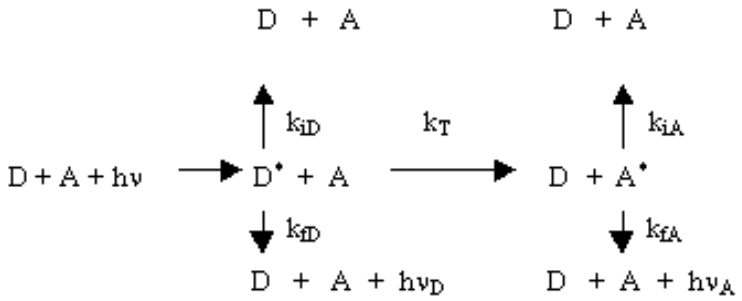


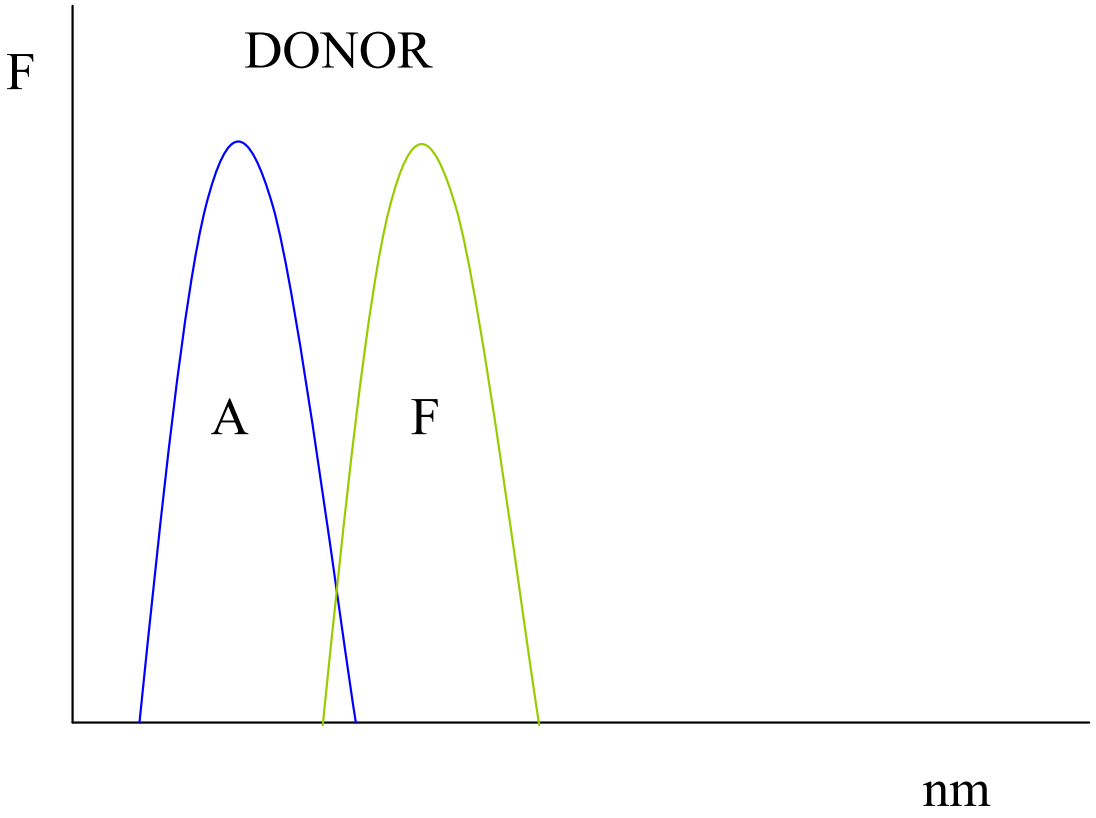
Fluorescenční rezonanční transfer energie (Försterův přenos)



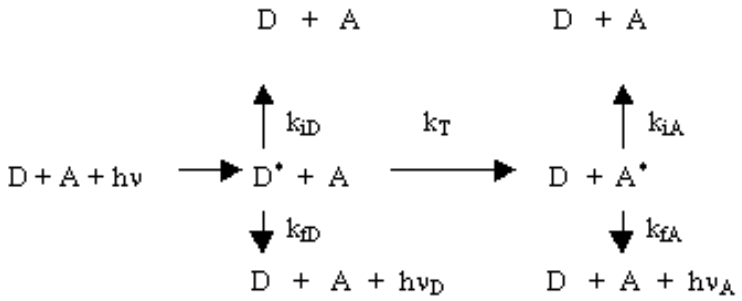
Nezářivý přenos energie z donoru na akceptor (1 – 10 nm)

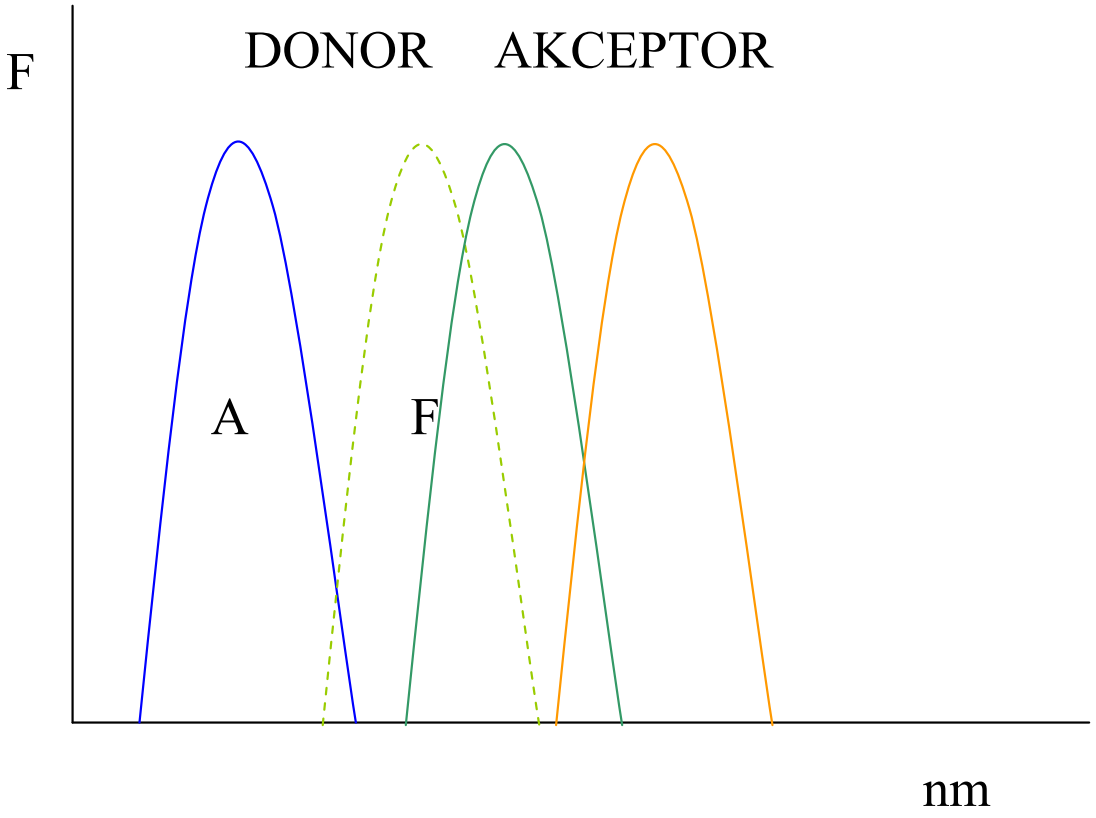
Fluorescenční rezonanční transfer energie



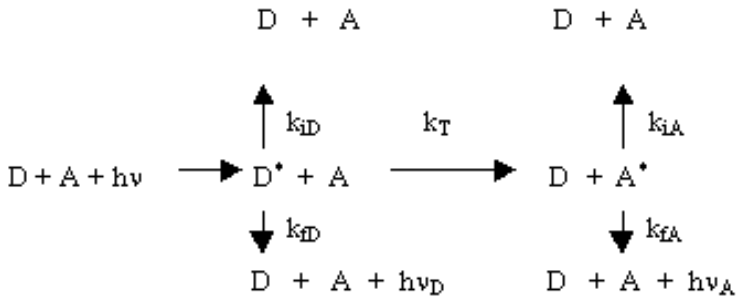


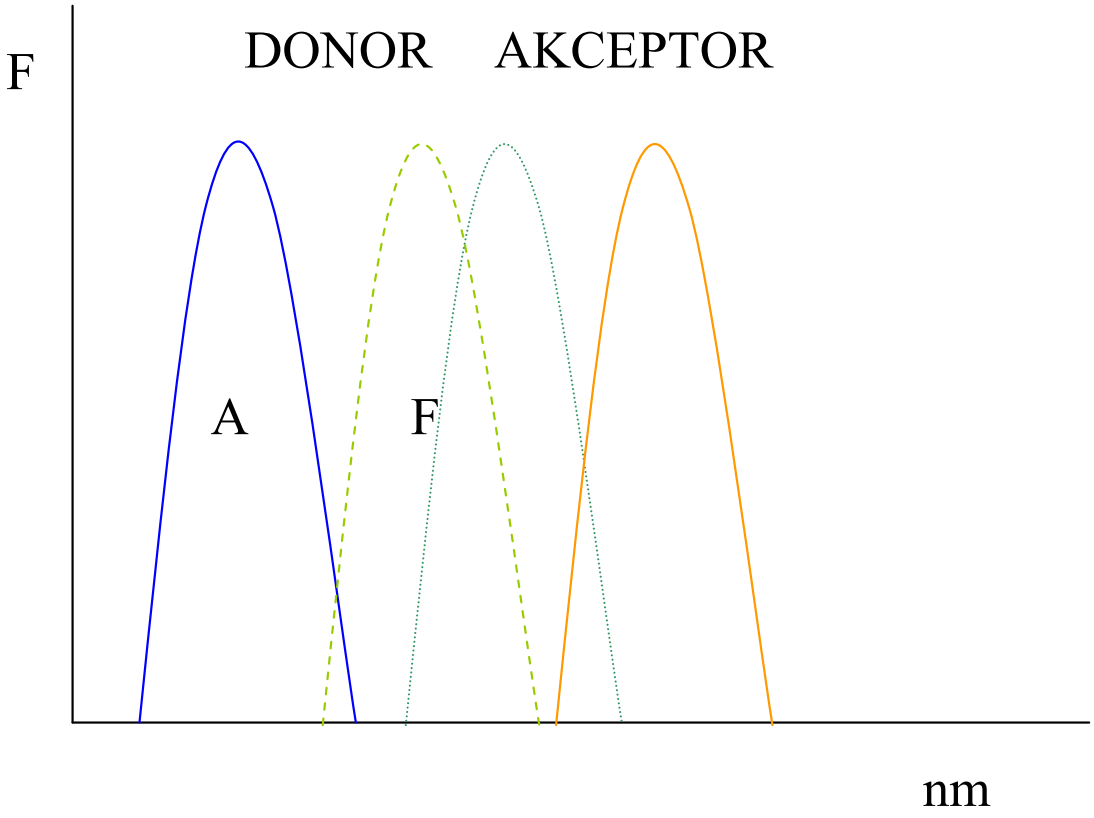
Fluorescenční rezonanční transfer energie



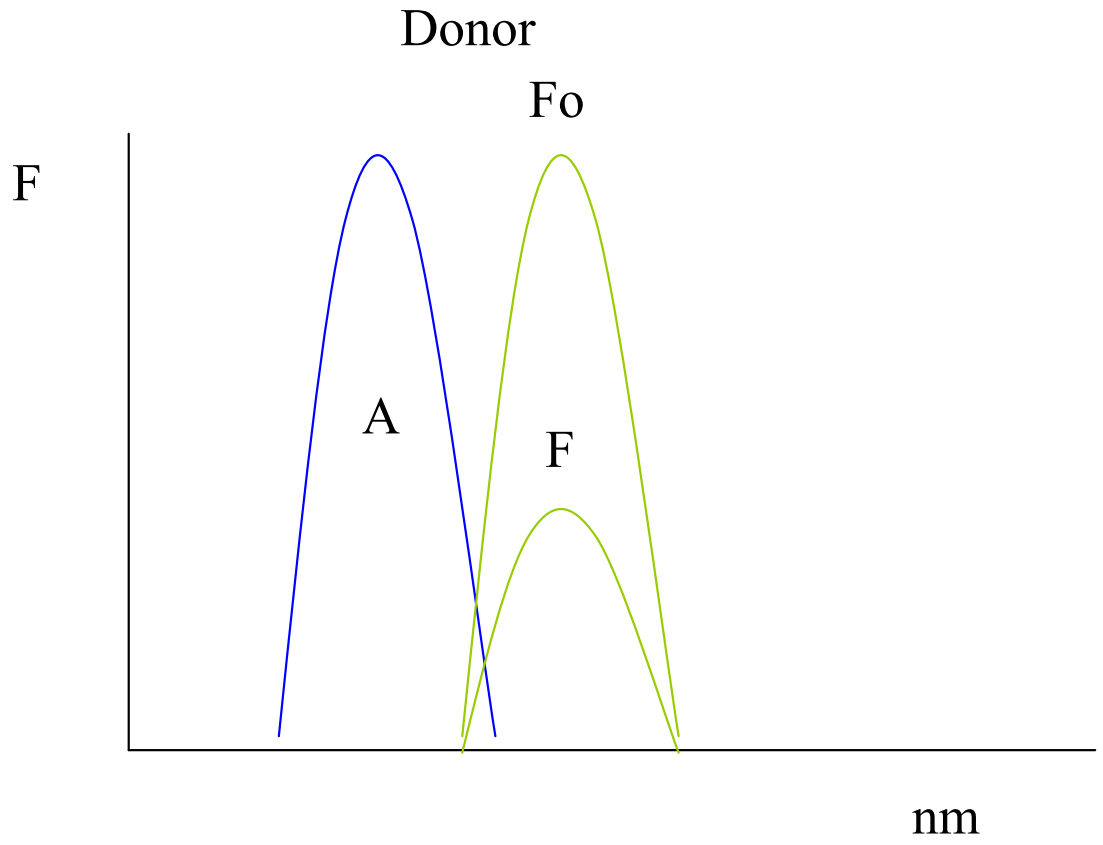


Fluorescenční rezonanční transfer energie





Fluorescenční rezonanční transfer energie



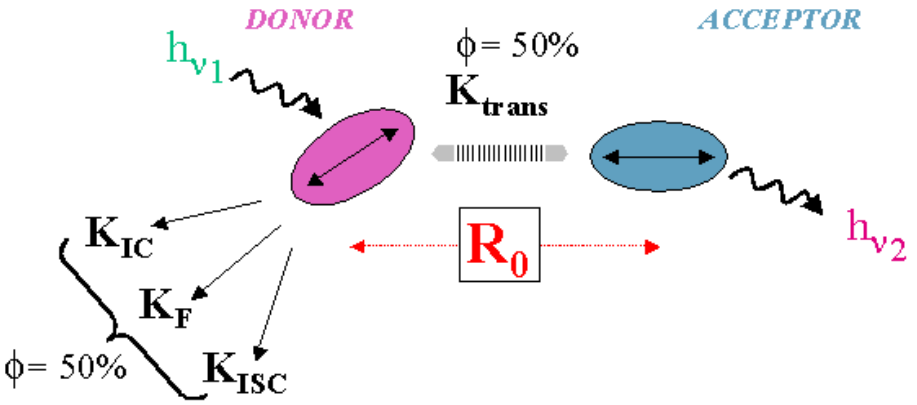
$\eta = 1 - F/Fo$

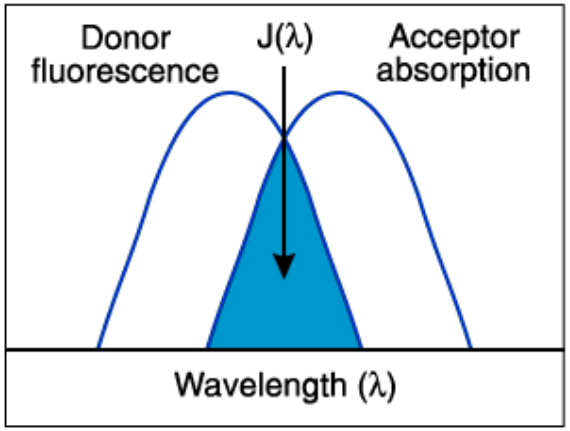
Fluorescenční rezonanční transfer energie

$$\eta = Ro^6/(Ro^6 + R^6)$$

$$Ro^6 = \sqrt{1{,}66.10^{-33}.\tau.J/n^2\nu o^2}$$

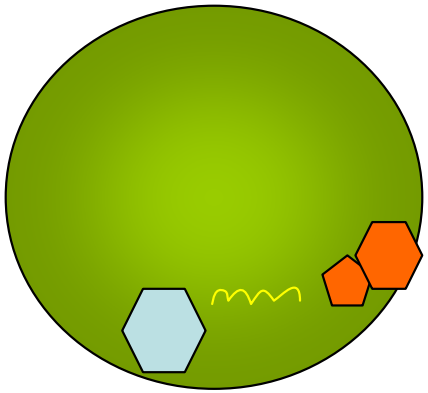
τ – doba života exc. stavu
J – překryvový integrál
n – refraktivní index rozpuštědla
ν – vlnočet emise donoru

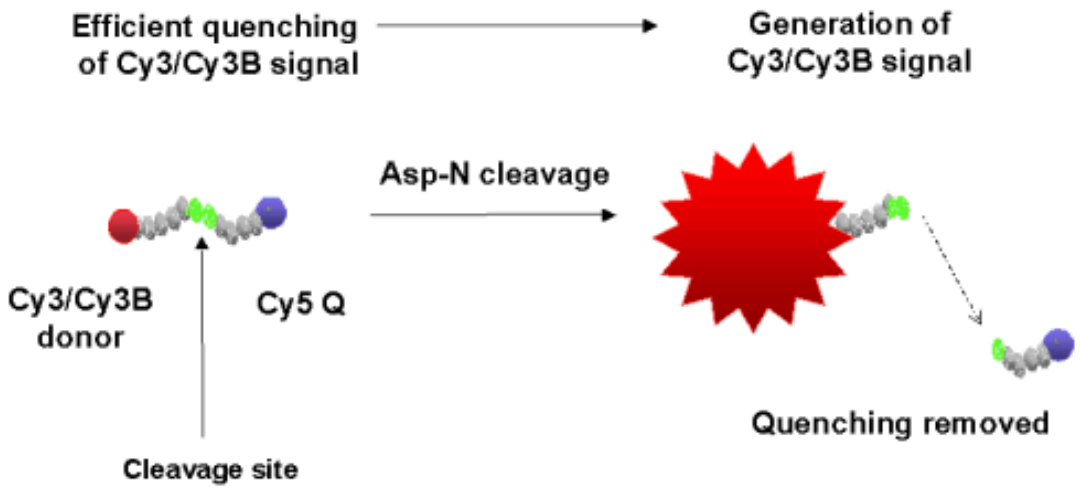




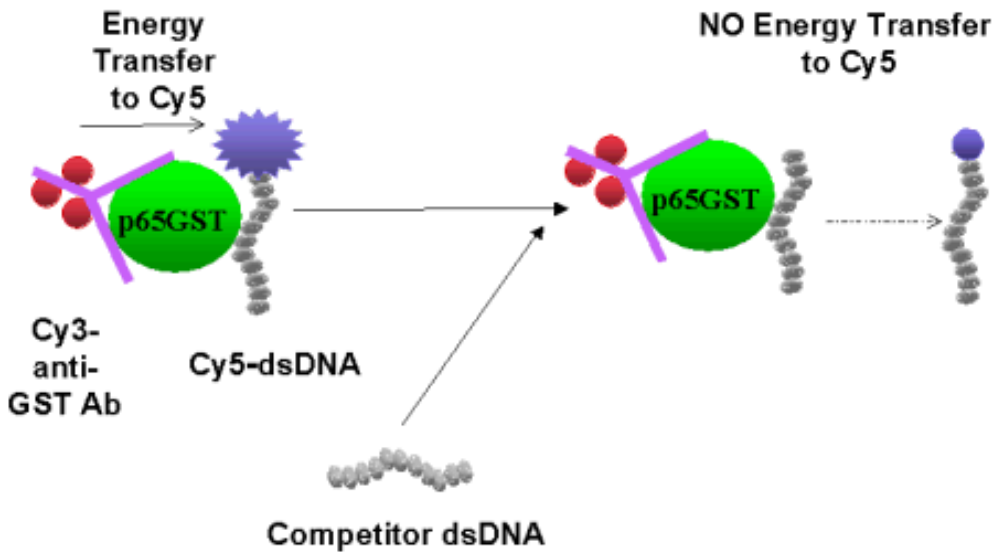
Fluorescenční rezonanční transfer energie

Použití – změření vzdálenosti mezi dvěma molekulami v bílkovině
Tryptofan (290/340) vs. NADH (340/450 nm)

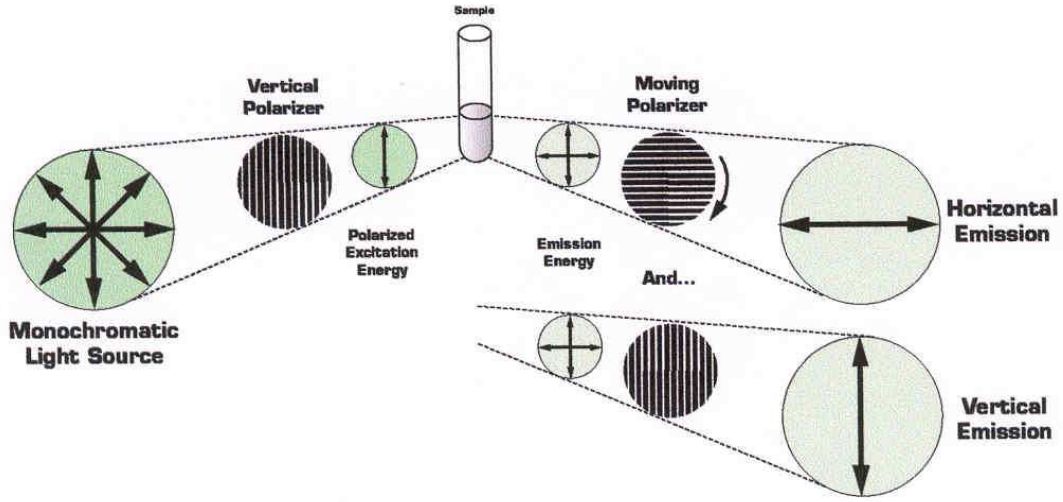




Fluorescenční rezonanční transfer energie



Fluorescenční anizotropie

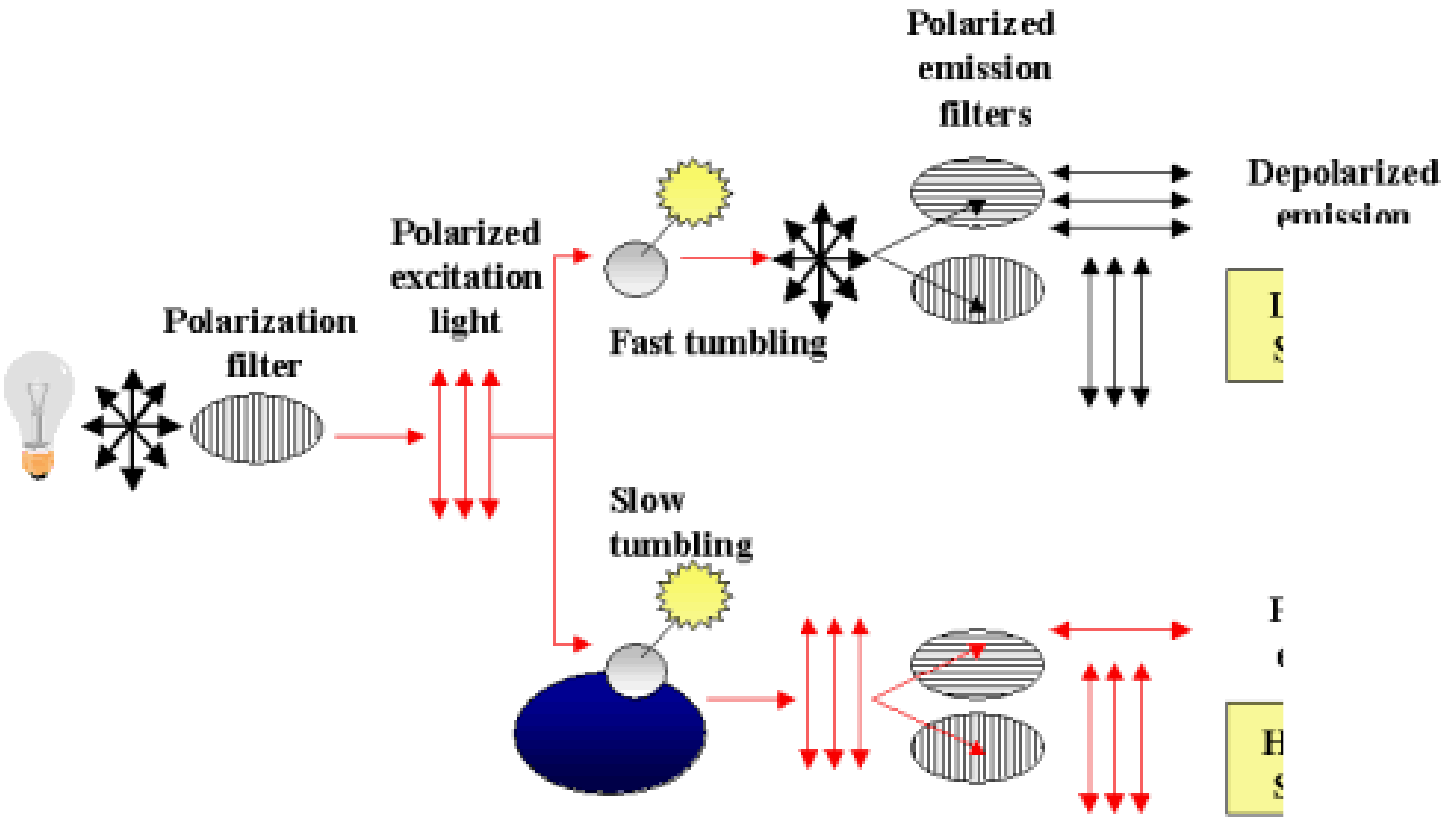


Fluorescenční anizotropie

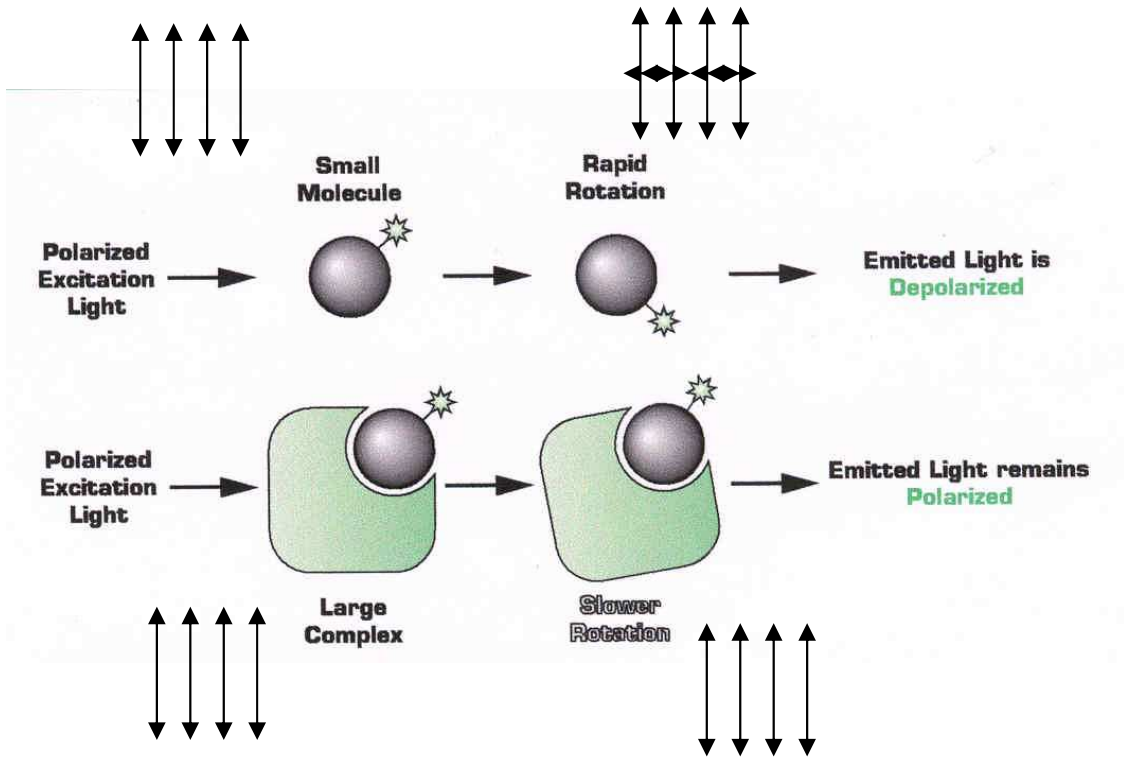
Polarizační filtry

Auto-Polorizer Accessory

Fluorescenční anizotropie



Fluorescenční anizotropie

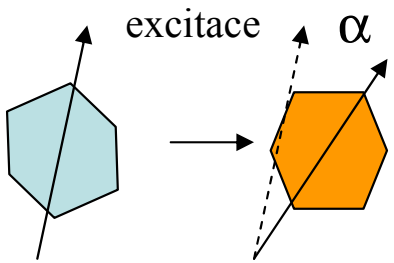


Fluorescenční anizotropie

Fluorescenční anizotropie $\mathbf{r = \frac{I_v - I_h}{I}}$

$I = I_v + 2I_h$

Rotační relaxační čas



$r_o = (3\cos^2\alpha - 1)/5$

$\mathbf{ro/r = 1 + 3\tau/\rho}$

τ, střední doba života fluorescence
ρ, rotační relaxační čas molekuly
ro – anizotropie nepohyblivé molekuly

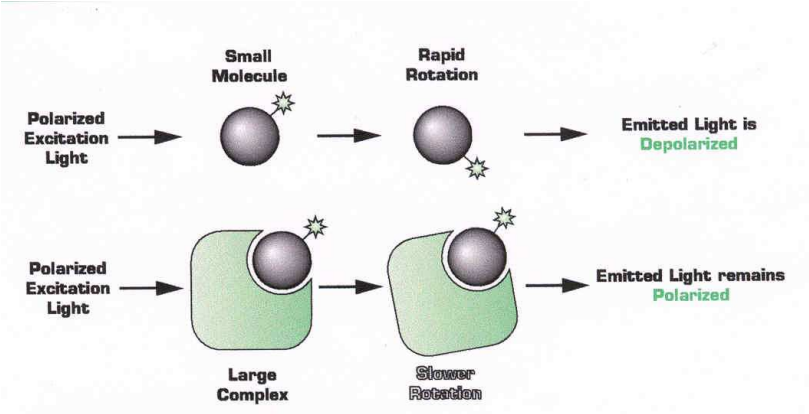
$\rho = V\eta/RT$

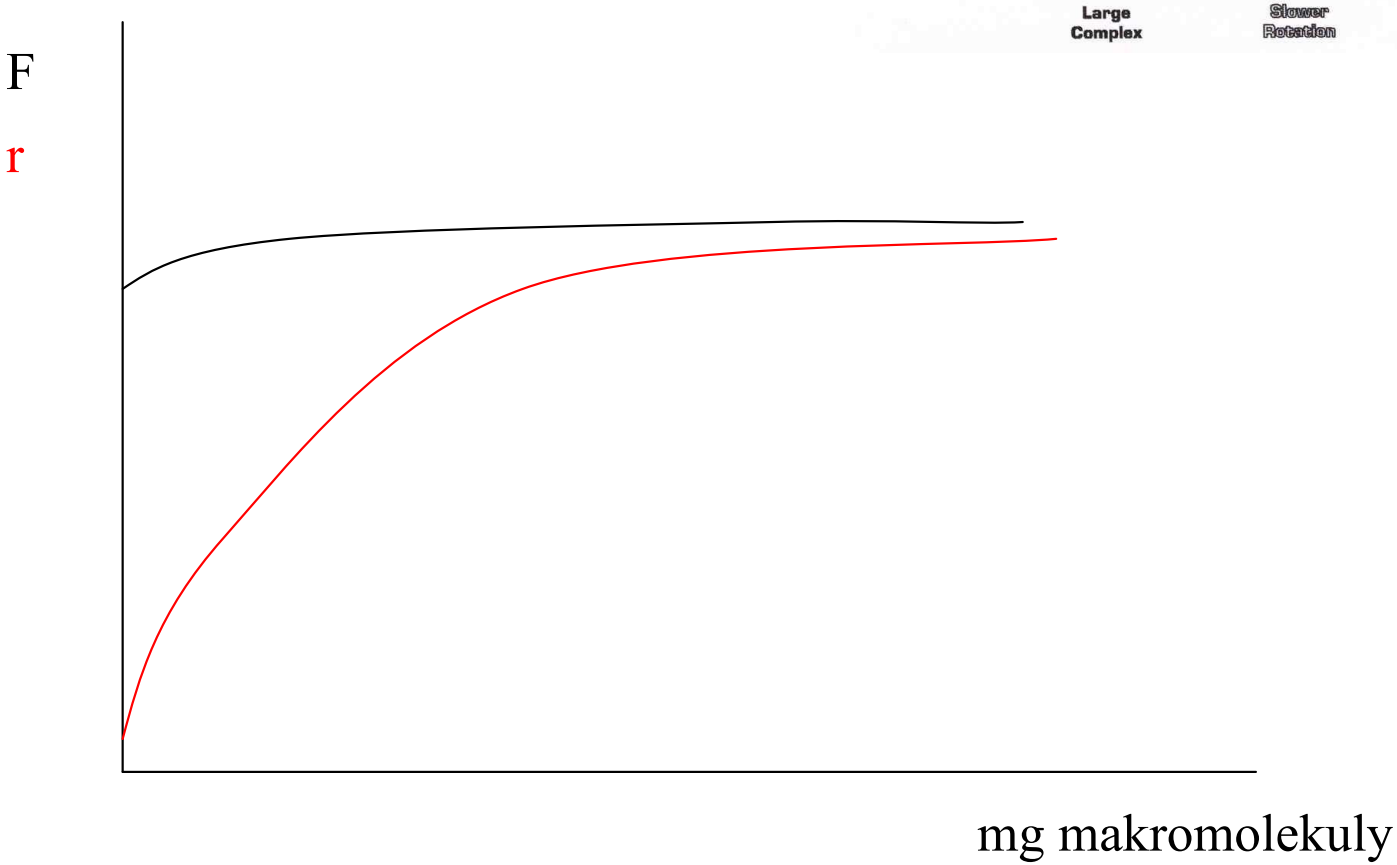
V objem
η viskozita

$\mathbf{ro/r = 1 + 3\tau RT/V\eta}$

Fluorescenční anizotropie

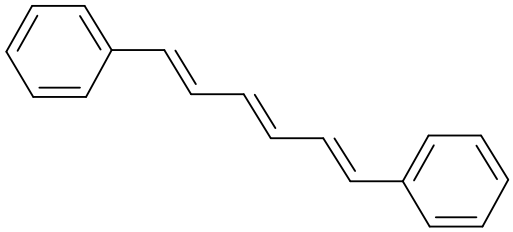
Využití:
Interakce makromolekuly s ligandem





Fluorescenční anizotropie

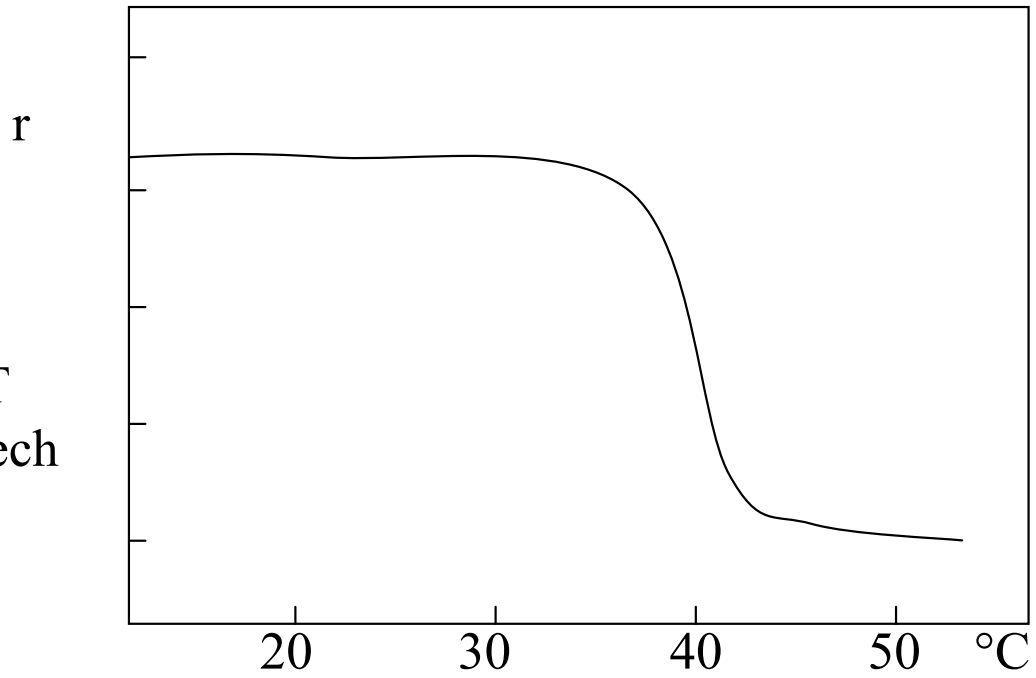
Využití:
Měření viskozity prostředí



$$r_o/r = 1 + 3\tau RT/V\eta$$

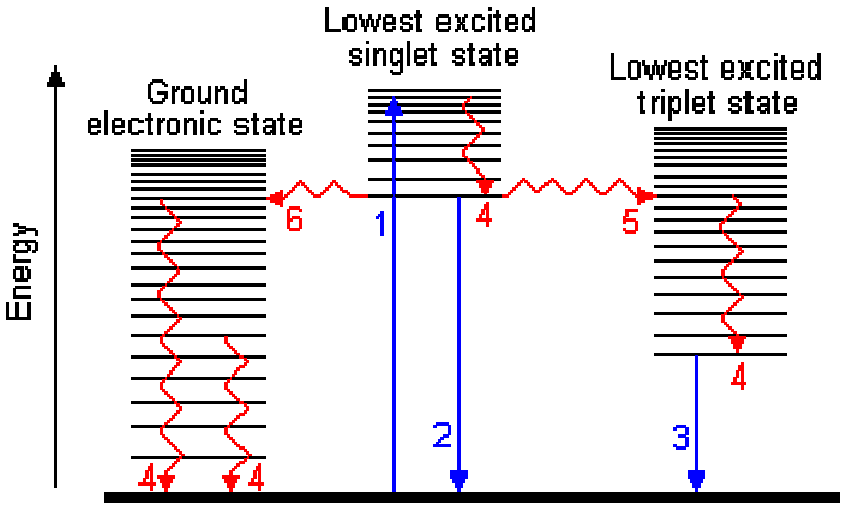
$$\eta = 2{,}4r/(0{,}362 - r)$$

$$r_o/r = 1 + K/\eta$$



Fl. anizotropie DPHT
Vázaného v liposomech
DPPC

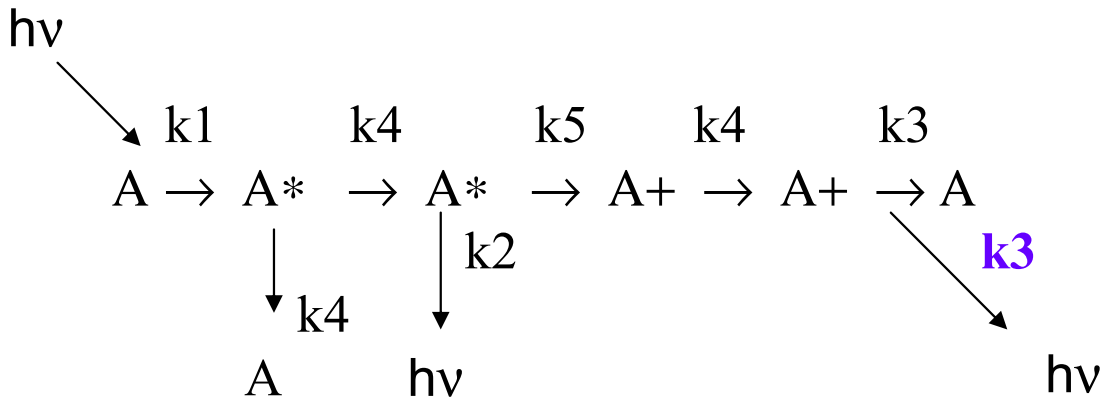
Fosforescence

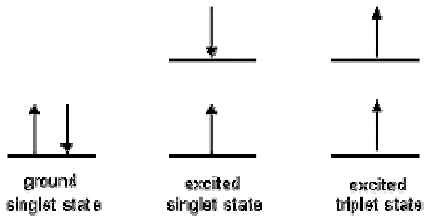


1. Absorption
2. Fluorescence
3. Phosphorescence
4. Vibrational relaxation
5. Intersystem crossing
6. Internal conversion

Multiplicita

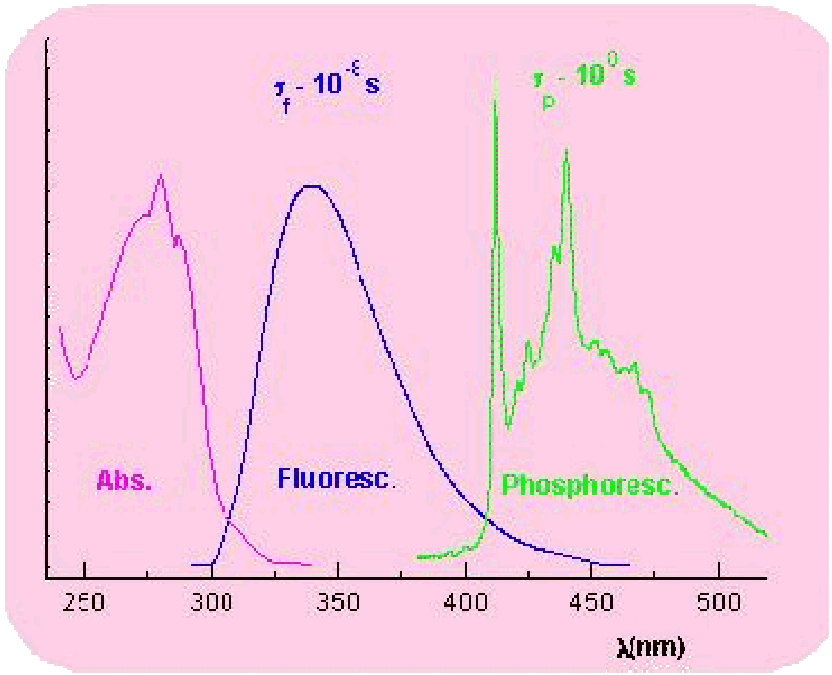
$M = 2S + 1$

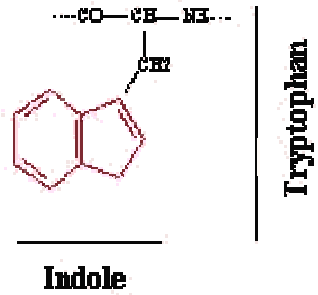




Fosforescence

Střední doba života τ 10^{-4} – 100 s





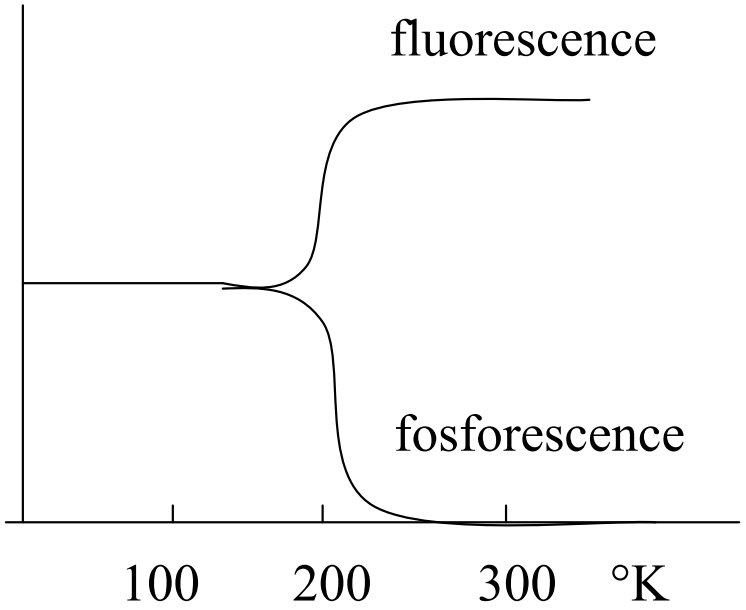
Fosforescence

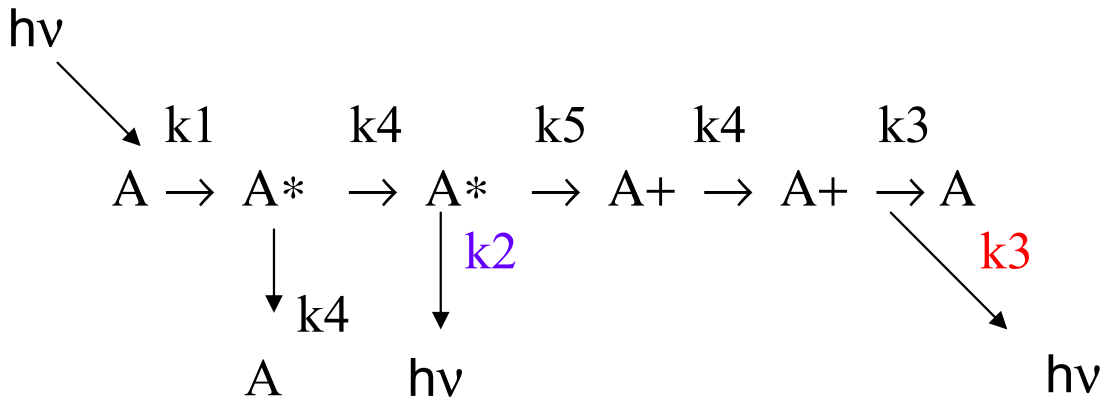
Kvantový výtěžek fosforescence

$\Phi_P = k3/(k3 + k2 + k4)$

$\Phi_f = k2/(k3 + k2 + k4)$

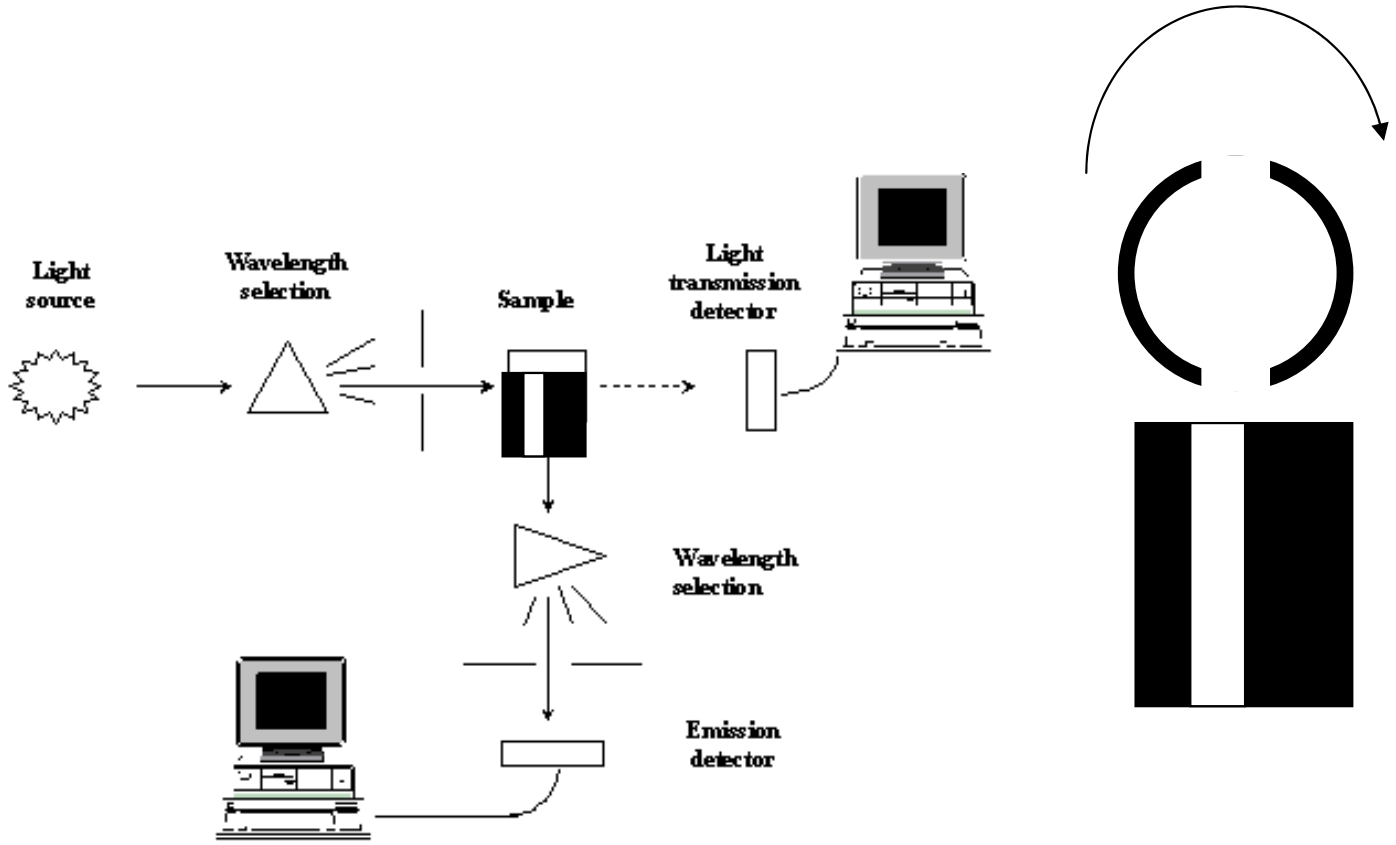
$\Phi_f / \Phi_P = k2/k3$



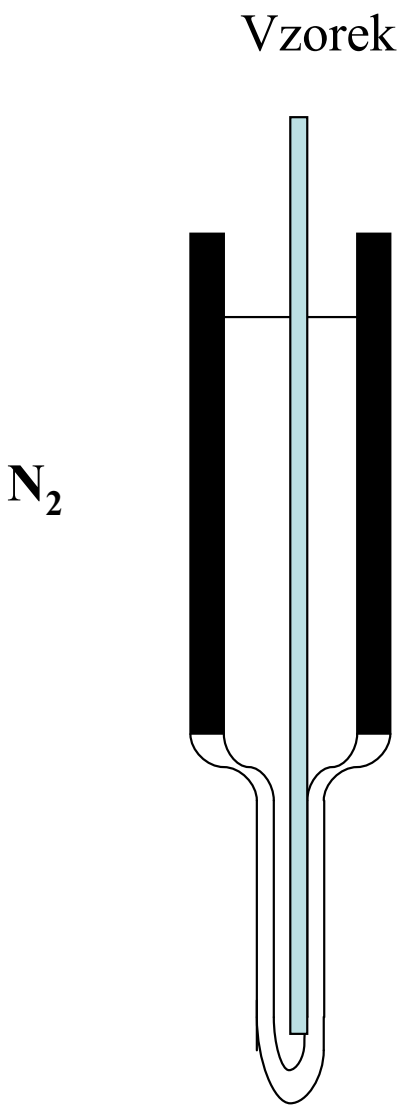


Fosforescence

Experimentální uspořádání



Fosforescence



Rozpouštědla
rigidní skla bez krystalů
(ethanol, metanol, voda:ethylenglykol., atd

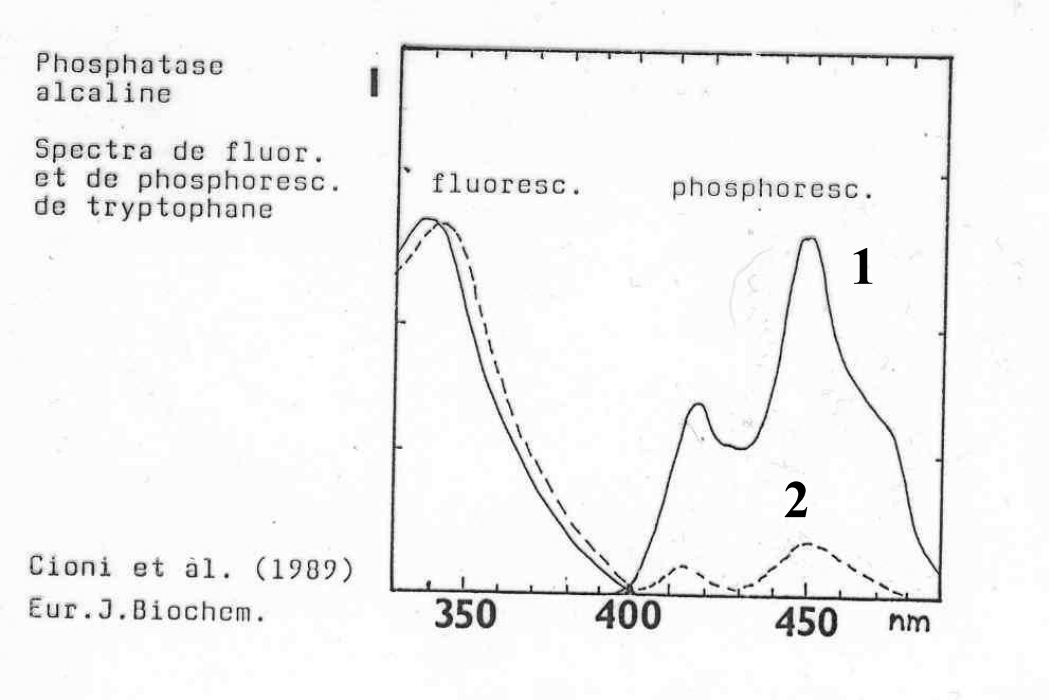
Fosforescence

Aplikace fosforescence

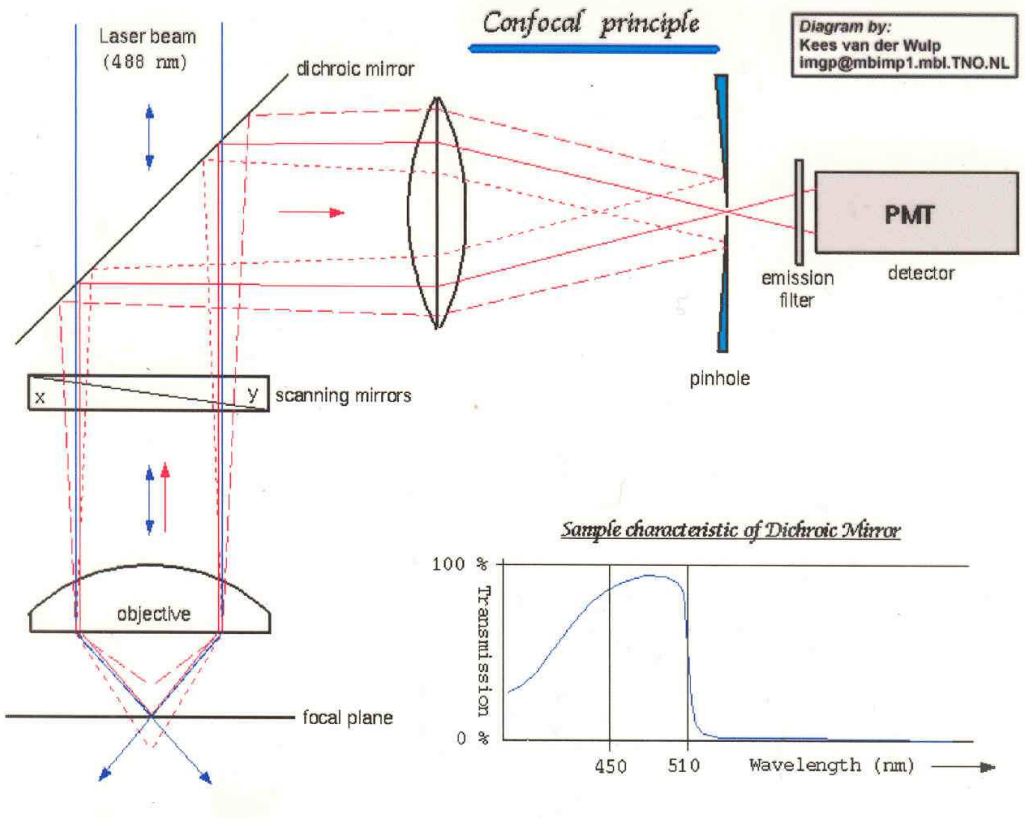
	exc	em	(sec)
Tyrosine	300	405	5.3
Tryptophane	295	440	1.5
DOPA	270	420	0.4
Phenylalanine	270	420	-
Ac. benzoïque	240	400	2.4
Ac. aminobenzoïque	310	430	3.2
Ac. indolylacétique	300	440	7.1
Ac. salycilique	315	430	6.2
Quinine	340	500	1.3
Naphtalène	290	505	
Codéine	275	505	0.3
Caféine	285	440	2.0

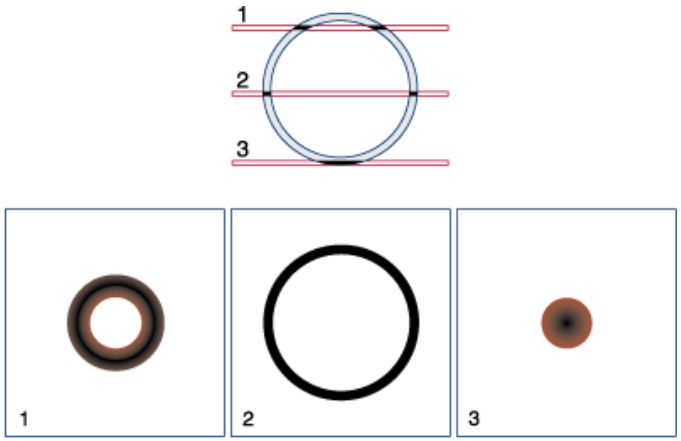
Fosforescence

Fosforescence alkalické fosfatasy
3 Try, pouze Try 109 fosforeskuje
1 – nativní enzym
2 – enzym po odstranění Zn



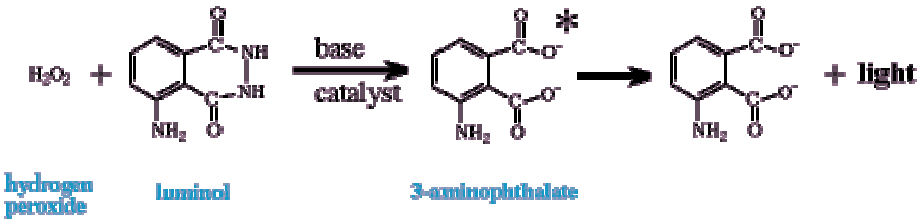
Princip fluorescenční konfokální mikroskopie

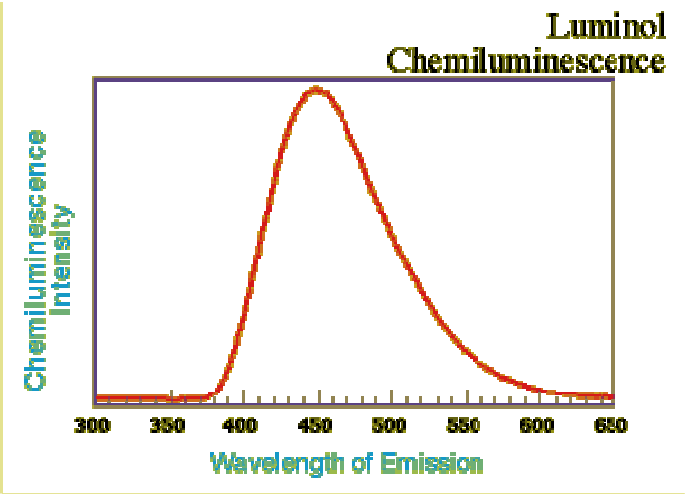


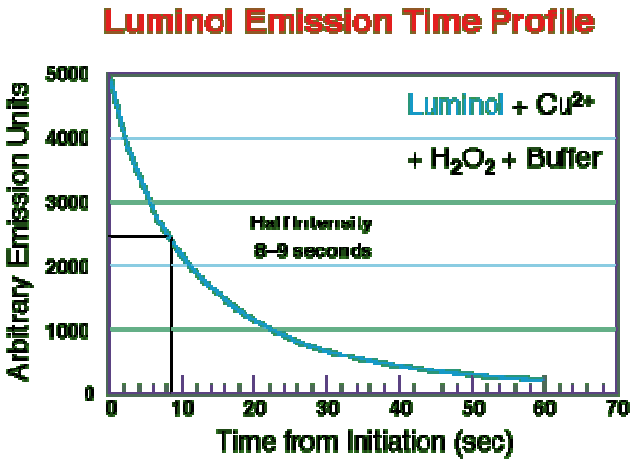


Chemiluminiscence

Luminol

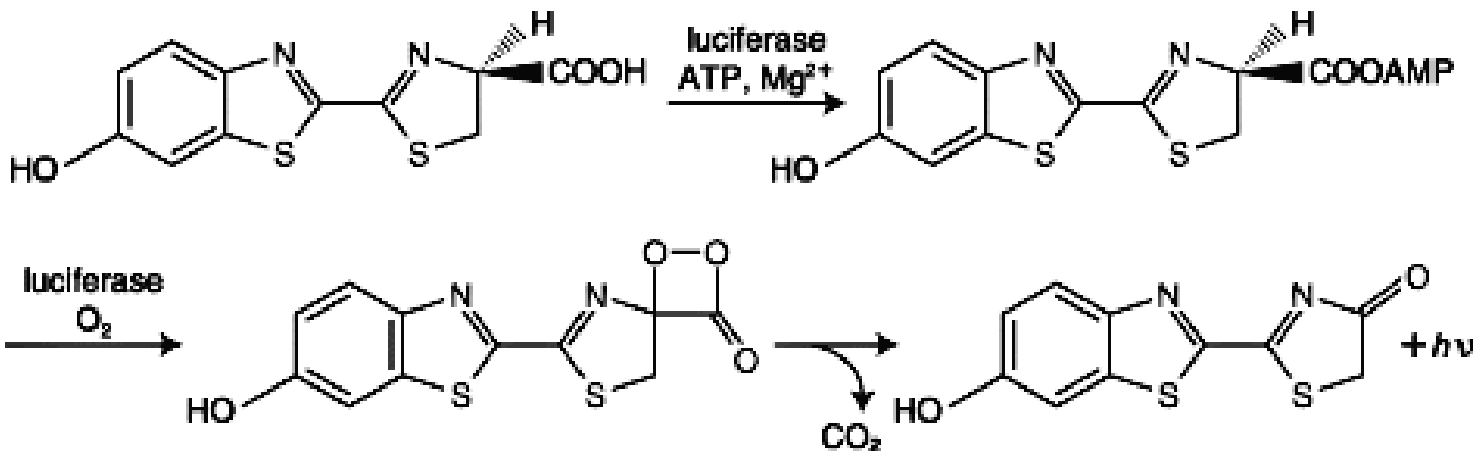






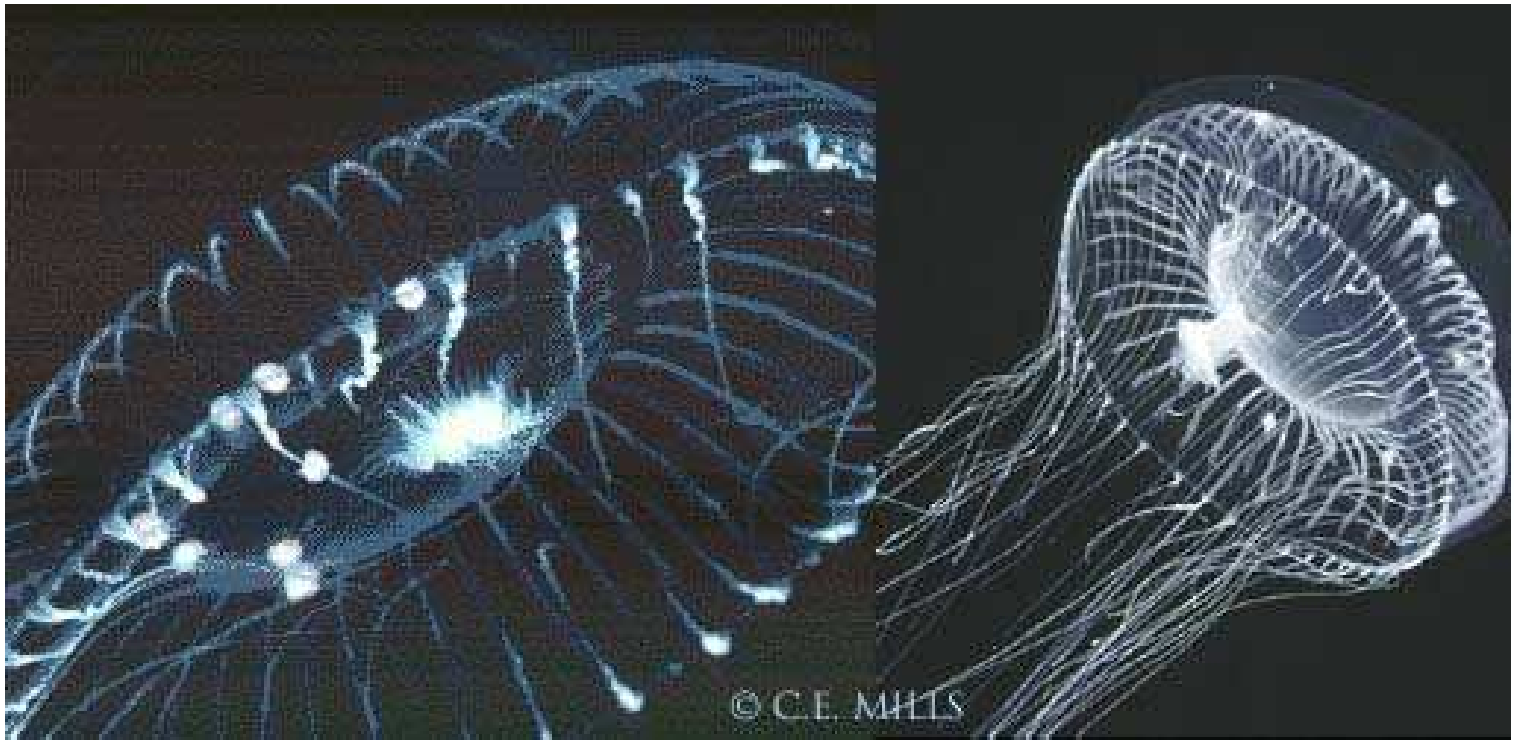
Chemiluminiscence

Luciferin



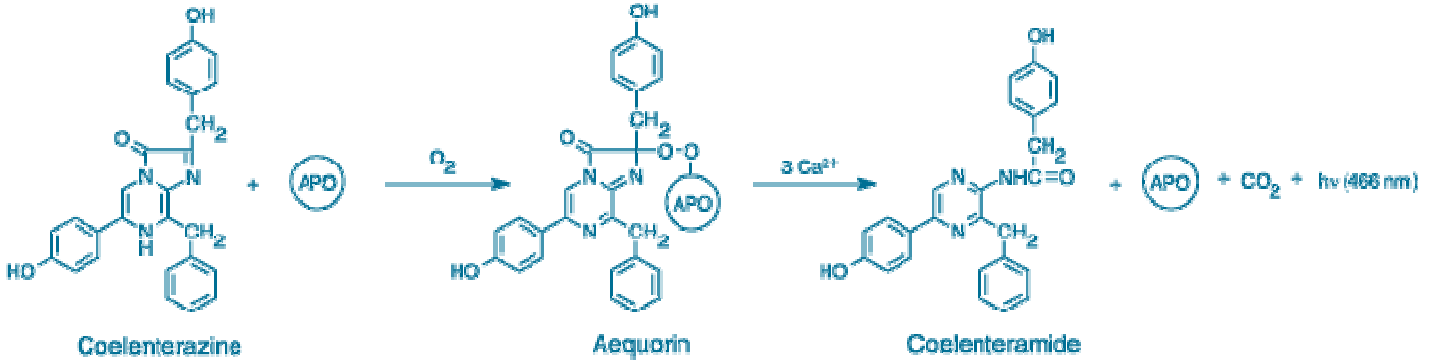
Chemiluminiscence

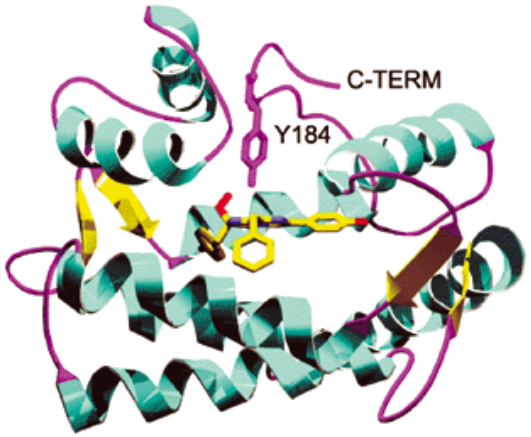
Aequorin – Aequoria victoria



Chemiluminiscence

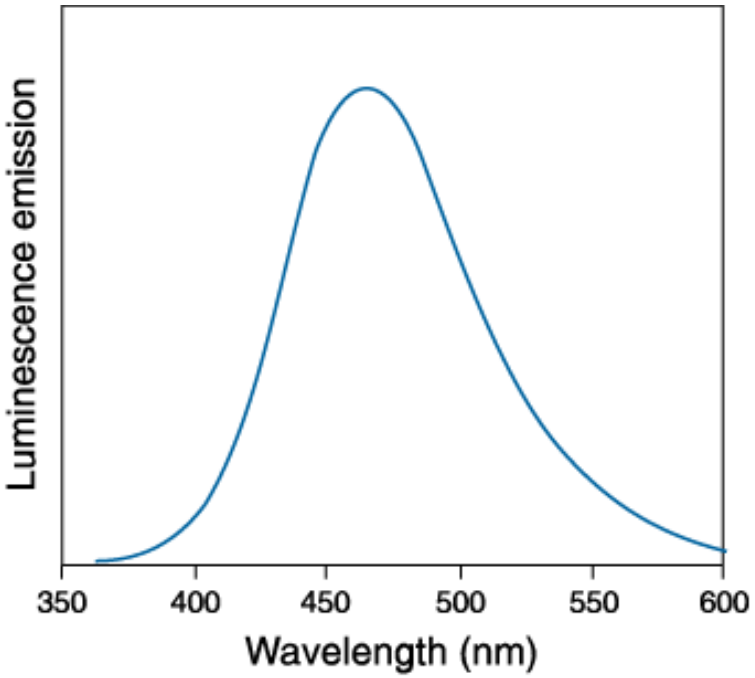
Aequorin – Aequoria victoria





Chemiluminiscence

Aequorin – Aequoria victoria



Průnik vápníku do mitochondrií
Aktivuje oxidaci