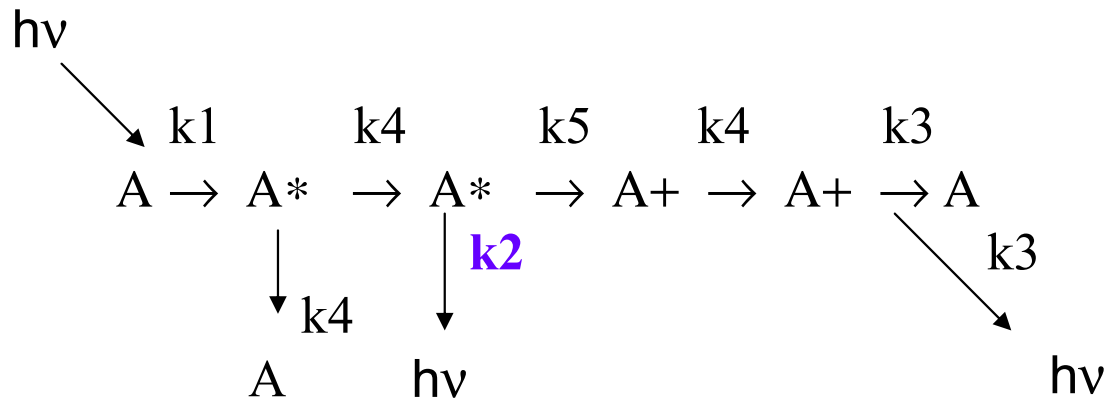
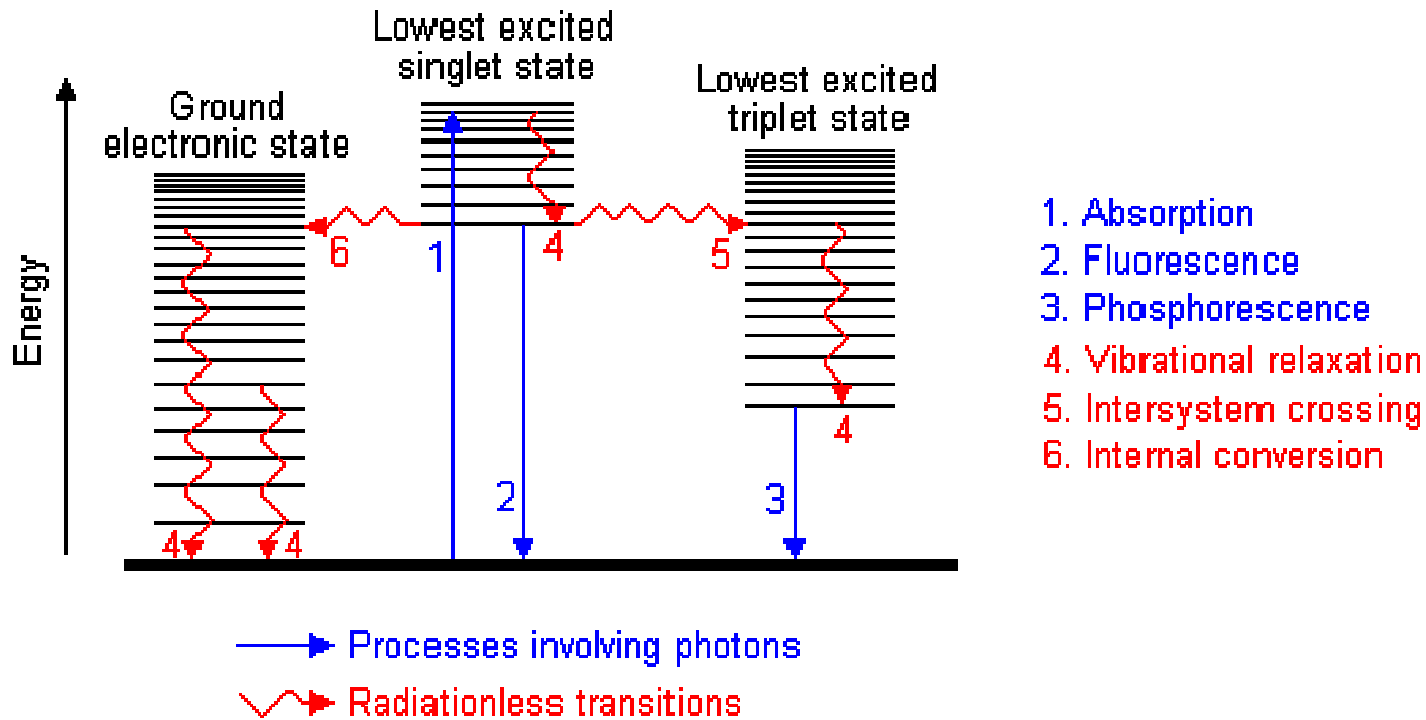
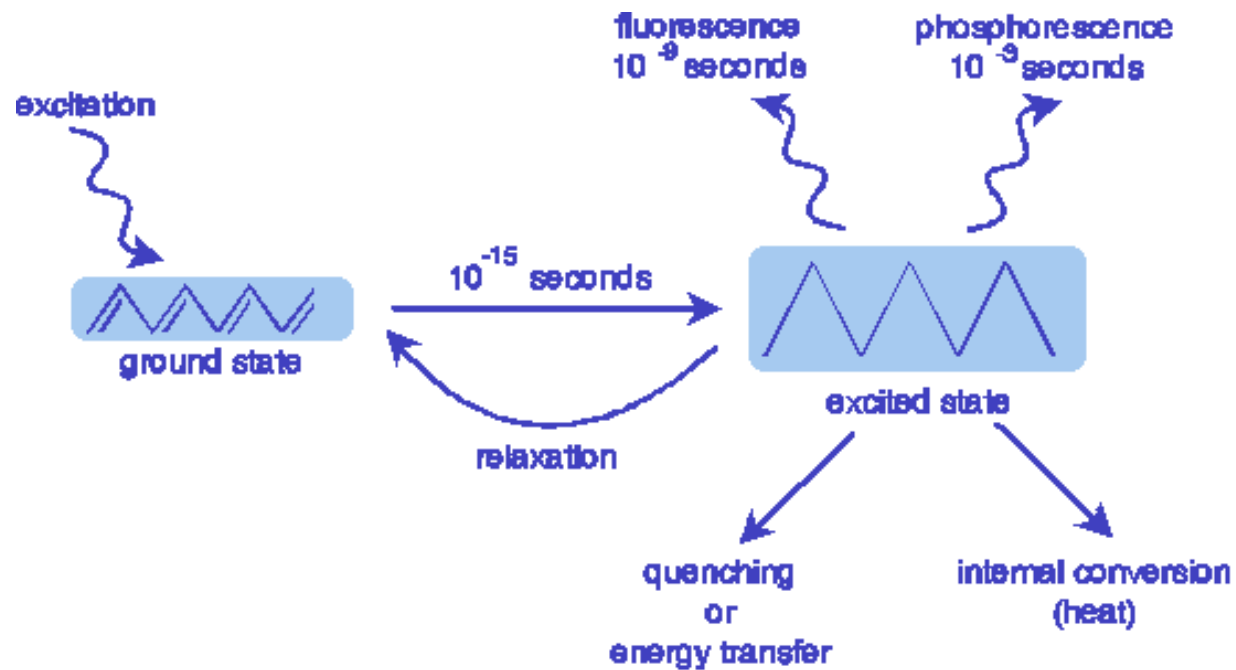


Luminiscenční spektroskopie



Luminiscenční spektroskopie

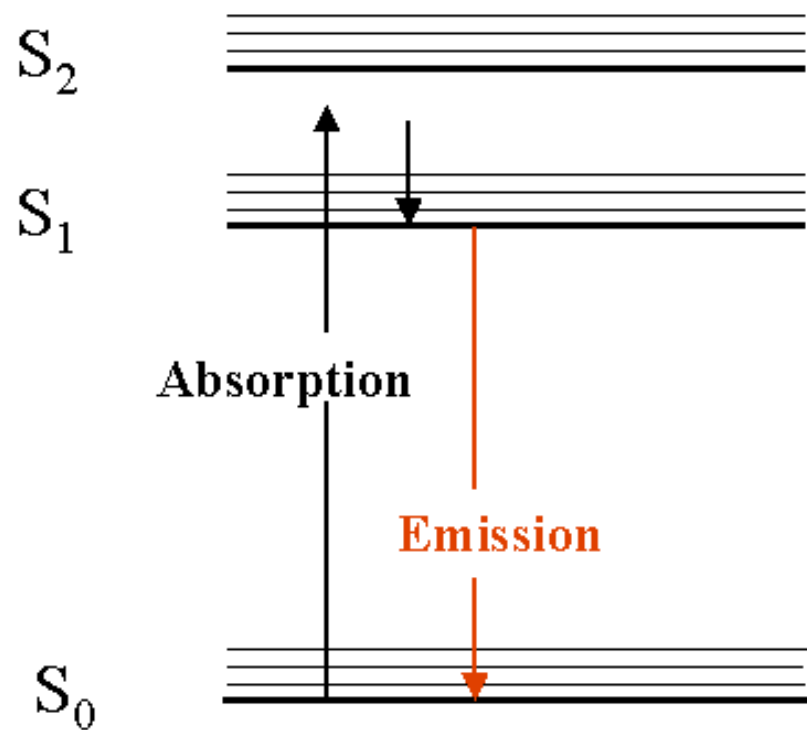


Luminiscenční spektroskopie

Fluorescenční spektroskopie
Fosforescenční spektroskopie
Chemiluminiscenční spektroskopie

Základní pojmy

Excitace a emise

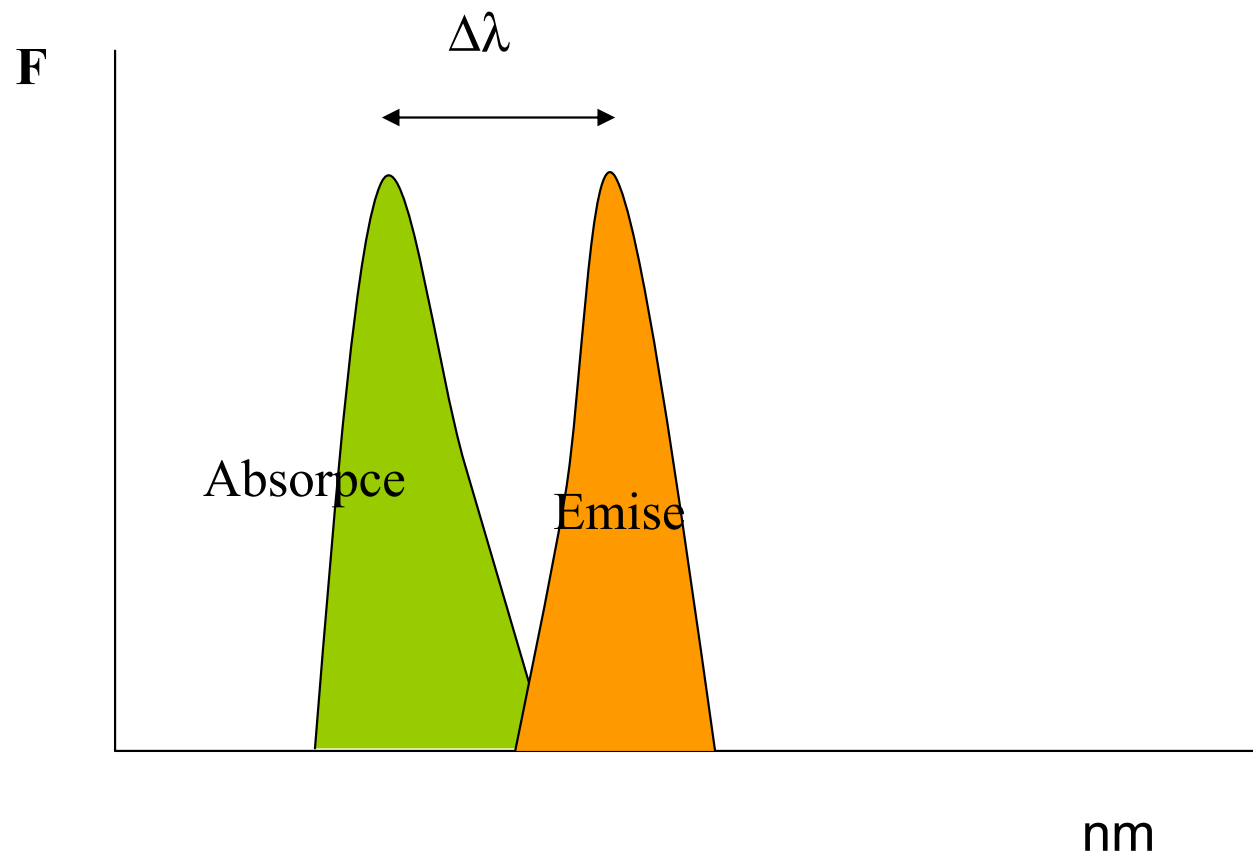


Interakce s rozpouštědlem
Singletový excitovaný stav

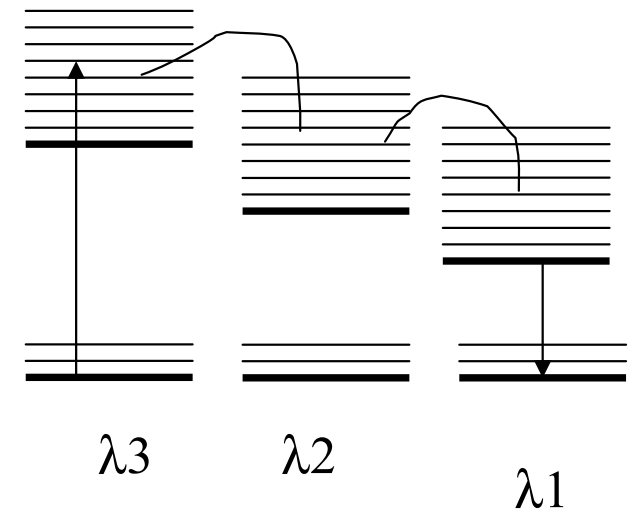
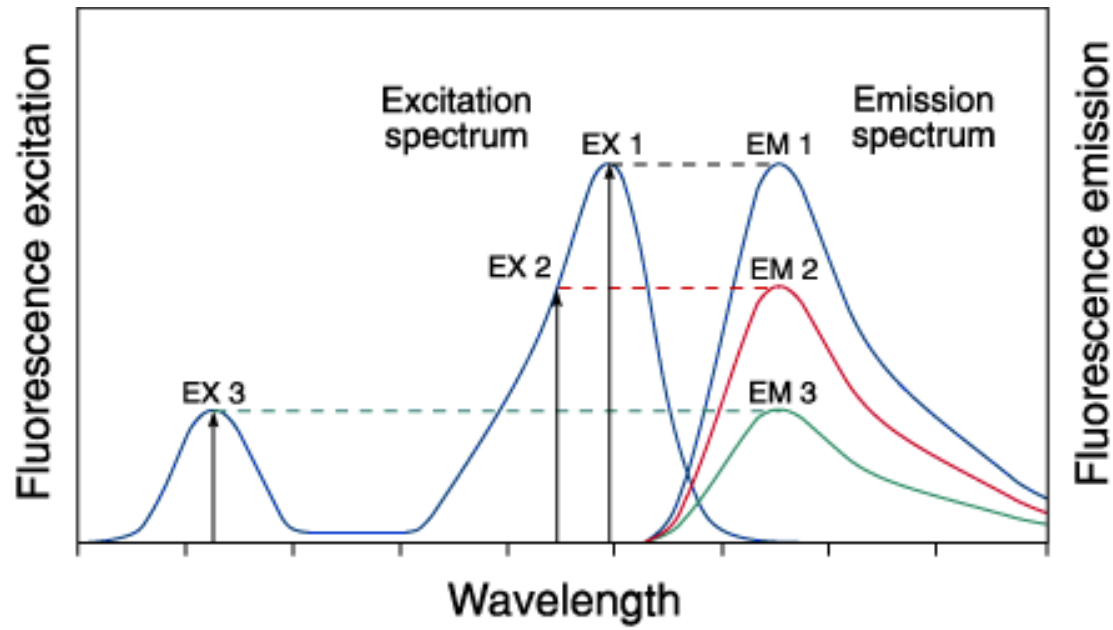
Singletový základní stav

Základní pojmy

Stokesův posun – ztráty energie po dobu excitovaného stavu



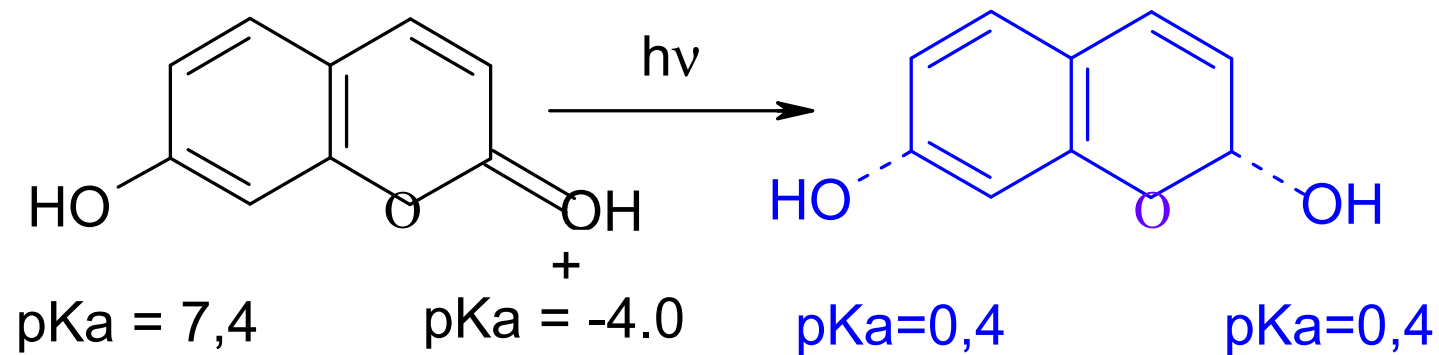
Základní pojmy



Základní pojmy

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

7-hydroxykumarin



Základní pojmy

Kvantový výtěžek fluorescence

Φ = počet kvant emitovaných/počet kvant absorbovaných

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

k_e = rychlost emise

k_k = rychlost konverzních
procesů

Intenzita fluorescence látky = $f(\epsilon, \Phi, N)$

Základní pojmy

Doba života excitovaného stavu
 Doba potřebná k poklesu
 fluorescence na hodnotu $1/e$ I_0

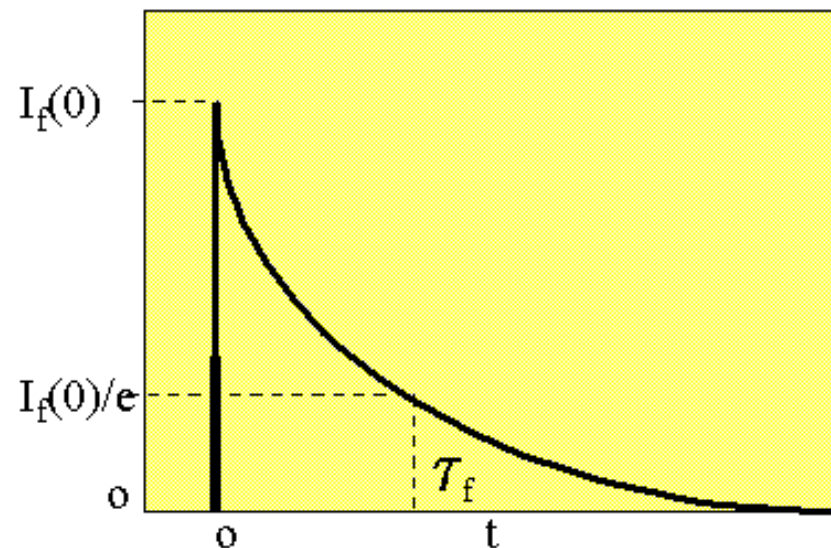
Střední doba života τ
 $\tau = 1/k_f$

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

Přirozená doba života τ_0

Definovaná pro $\Phi = 1$

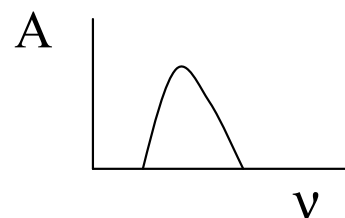
$$\tau_0 = 2,88 \cdot 10^{-9} \cdot n^2 \cdot \nu_A^2 \cdot \int_0^{\infty} \epsilon(\nu) d\nu$$



n - refrakční index rozpouštědla

ϵ - molární abs. Koeficient

ν - vlnčet abs. maxima



Základní pojmy

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

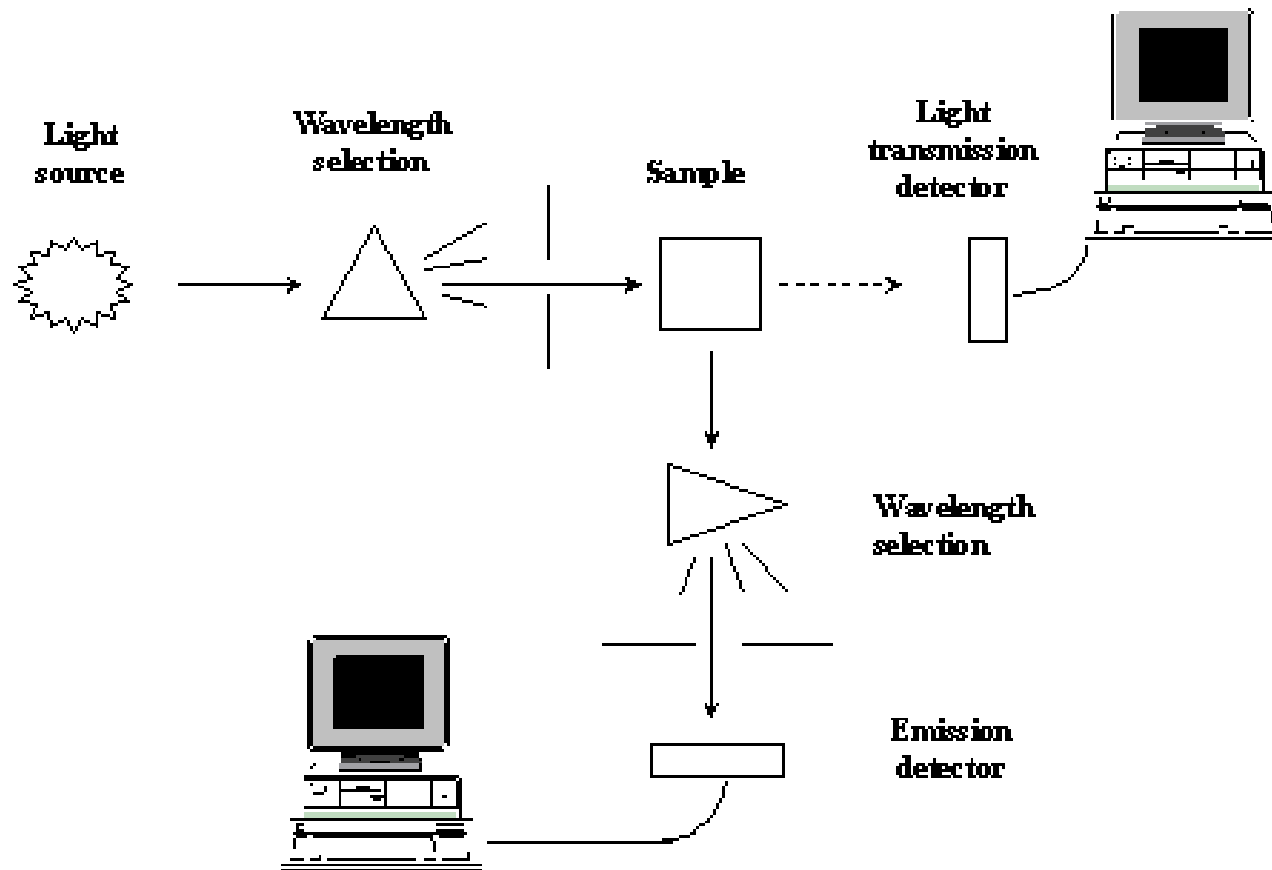
Střední doba života fluorescence

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 ₂ SO ₄)
Pyridoxamine	324	392	0.11 (pH=8.2)
Vitamine A	325	470	- (ethanol)
Aminobenzoate	294	345	-

Instrumentace



Instrumentace

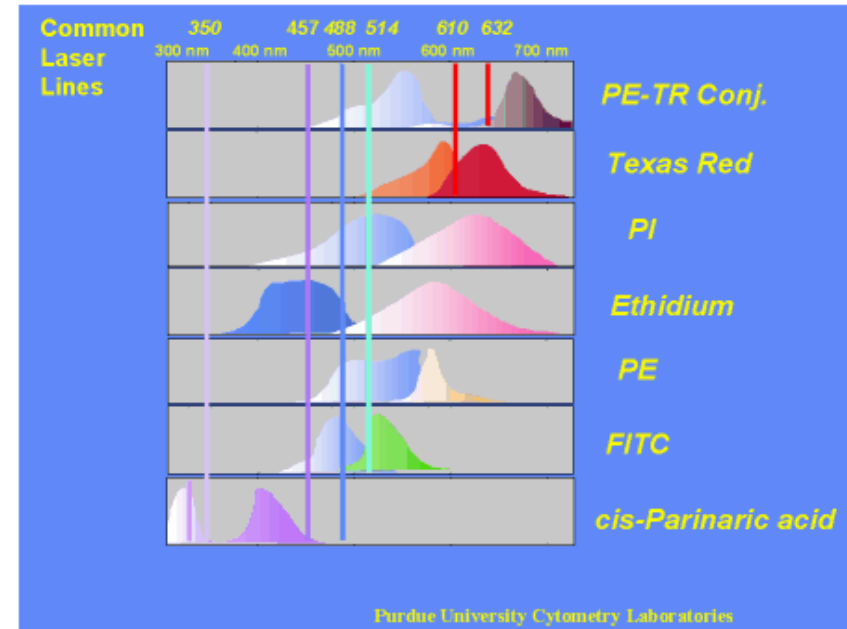
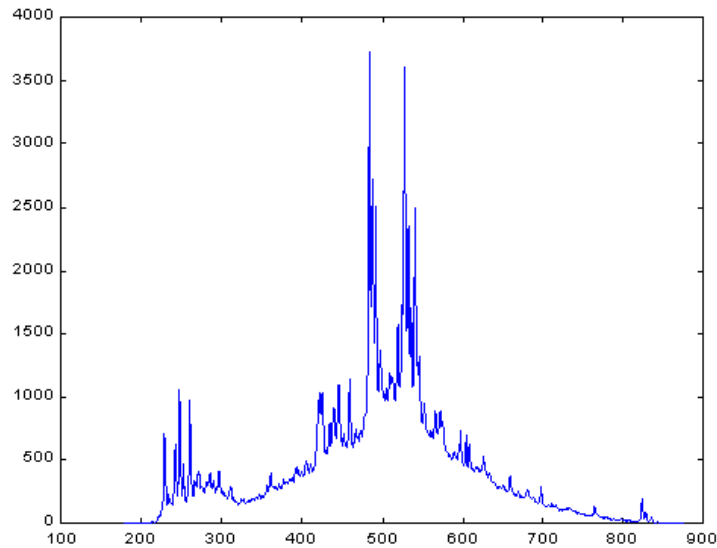
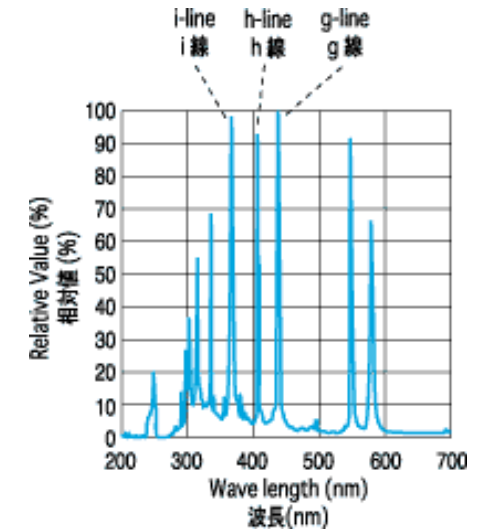
Zdroj:

Xenonová lampa

Rtuťová výbojka

Laser

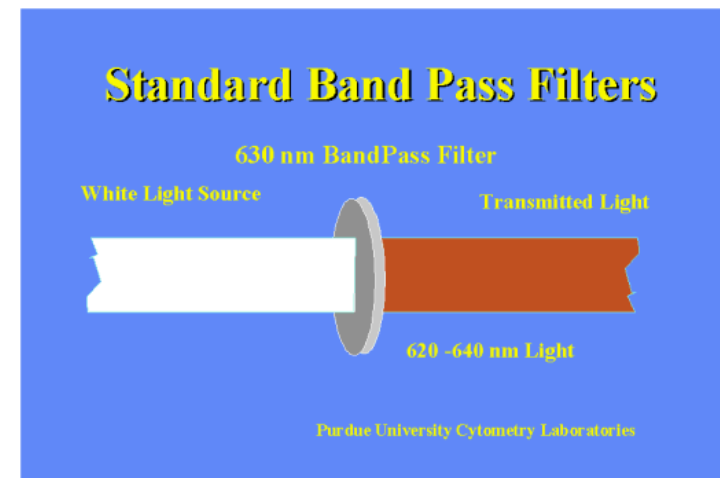
Světelné diody - LED (430, 450, 505, 592, 612 and 637 nm)



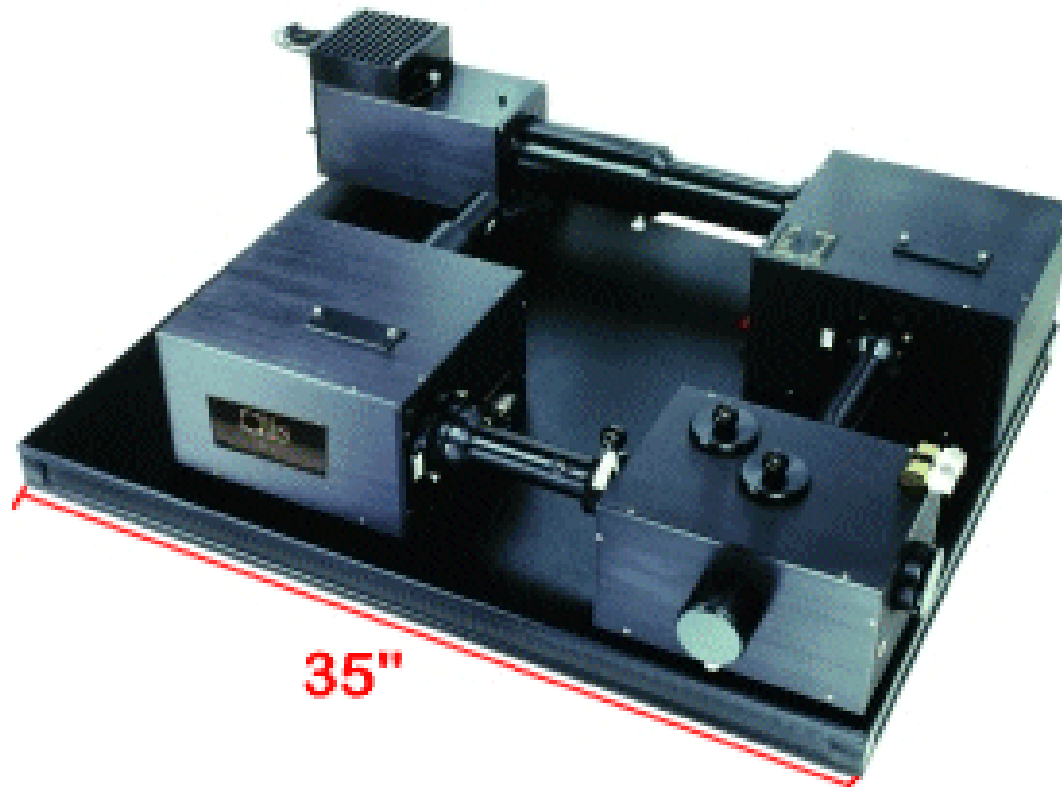
Instrumentace

Monochromátor

- mřížka
- filtry



Instrumentace



35"

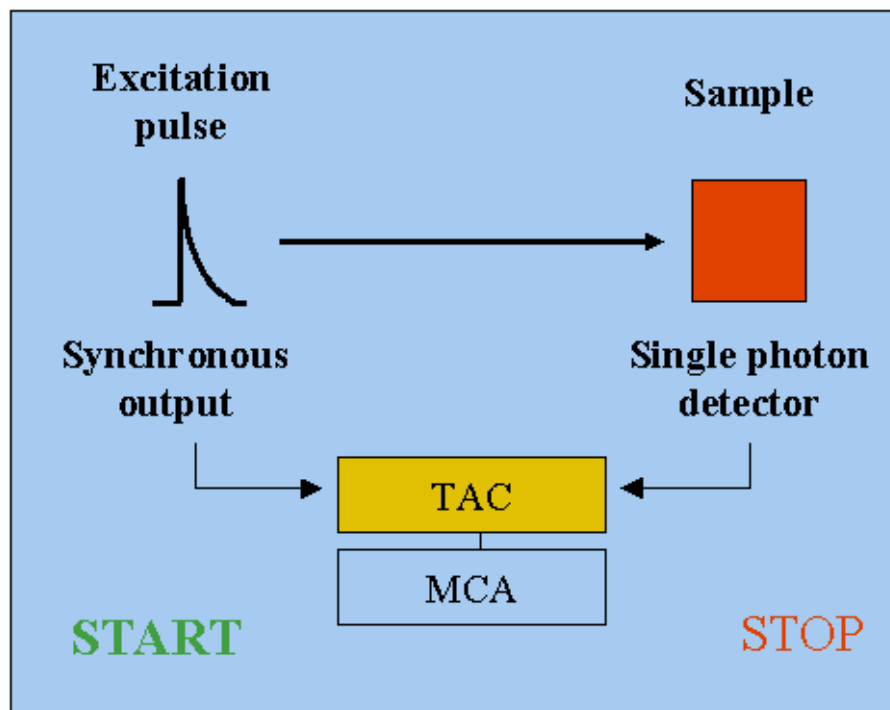
Instrumentace

RF-5301PC – spektrofluorimetr Shimadzu



Instrumentace

Měření střední doby života fluorescence



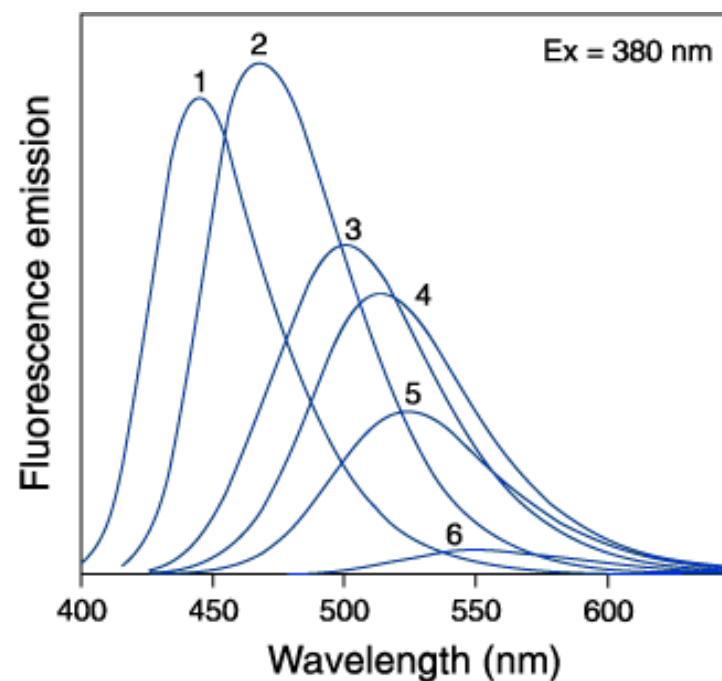
Podmínky fluorescence

Závislost na polaritě a viskozitě

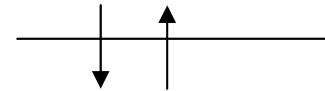
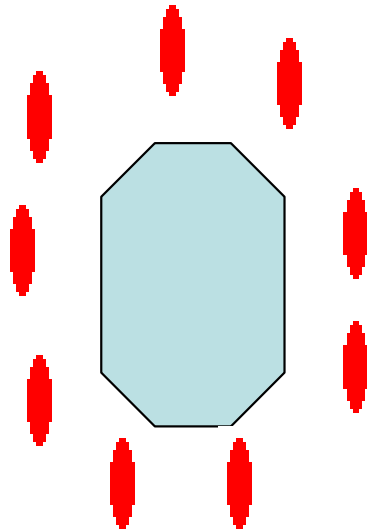
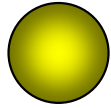
Nitrobenzoxadiazol

Solvent	Freq. domain (ns)	TCSPC (ns)	Literature (ns)
H ₂ O	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

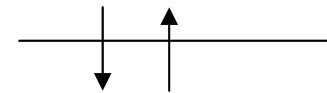
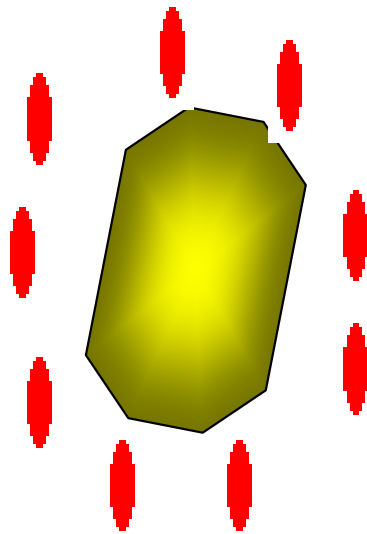
Pokles polarity 6 – 1.



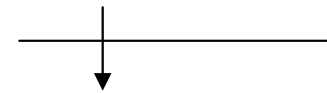
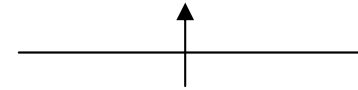
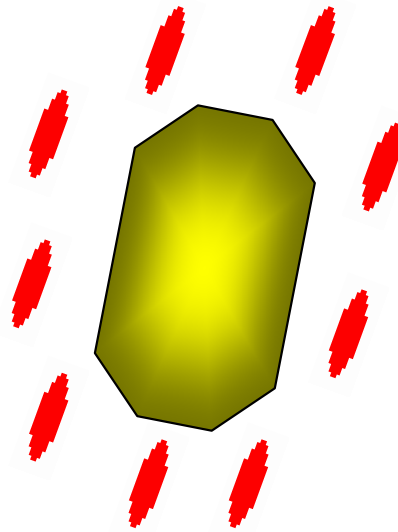
Podmínky fluorescence



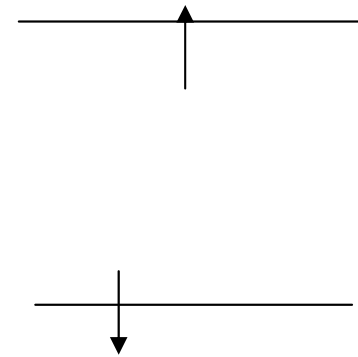
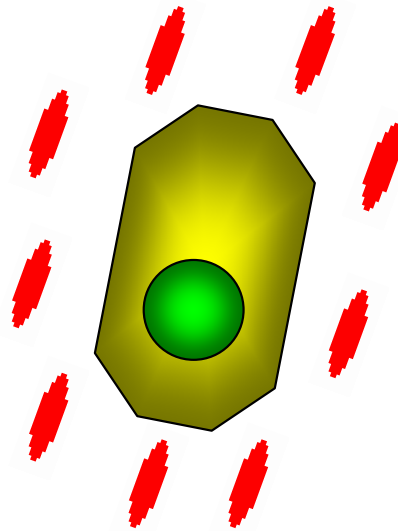
Podmínky fluorescence



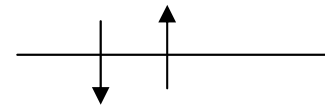
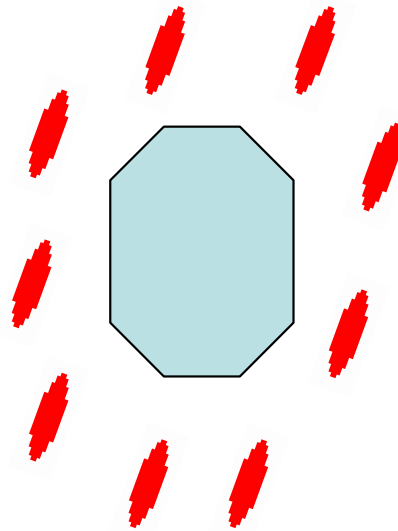
Podmínky fluorescence



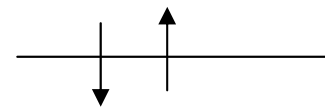
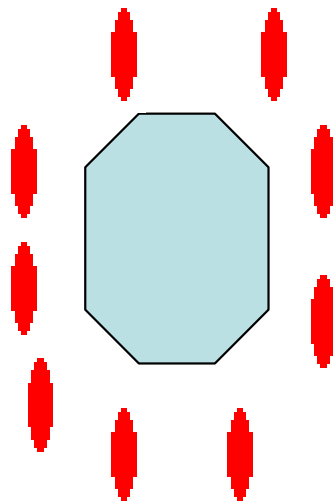
Podmínky fluorescence



Podmínky fluorescence

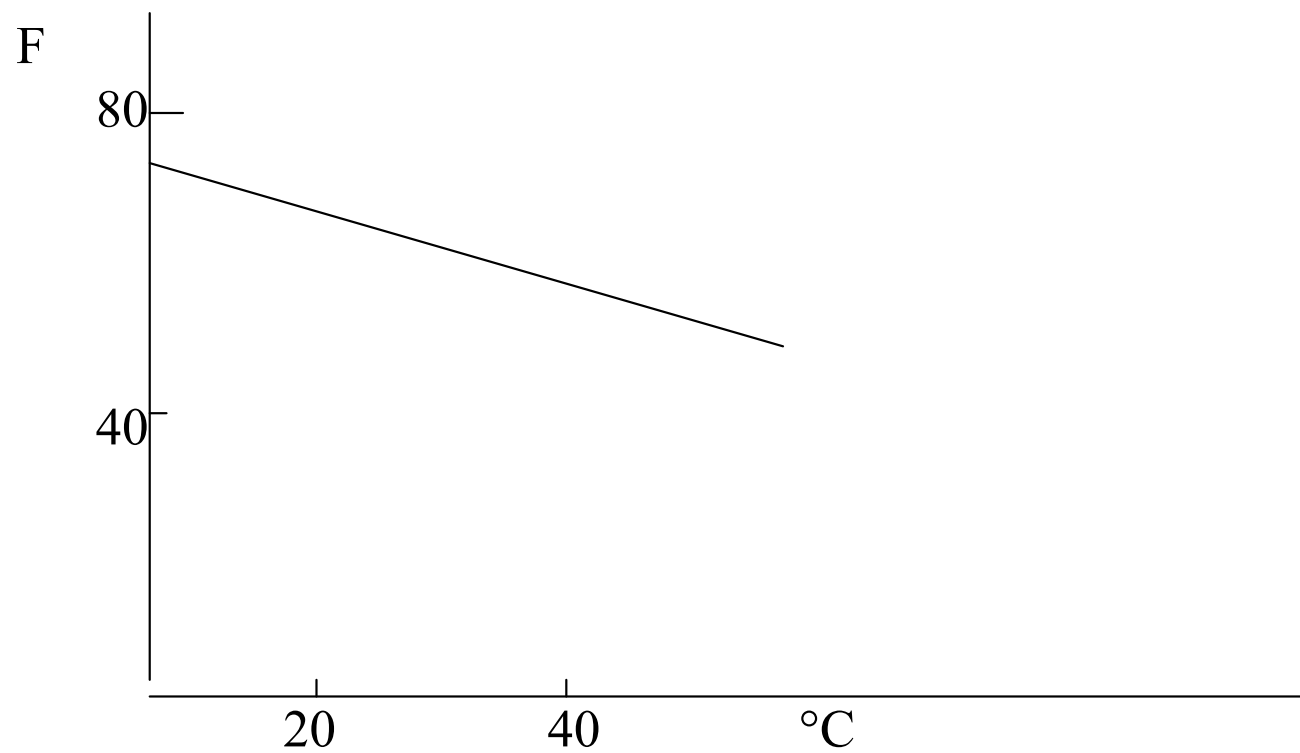


Podmínky fluorescence



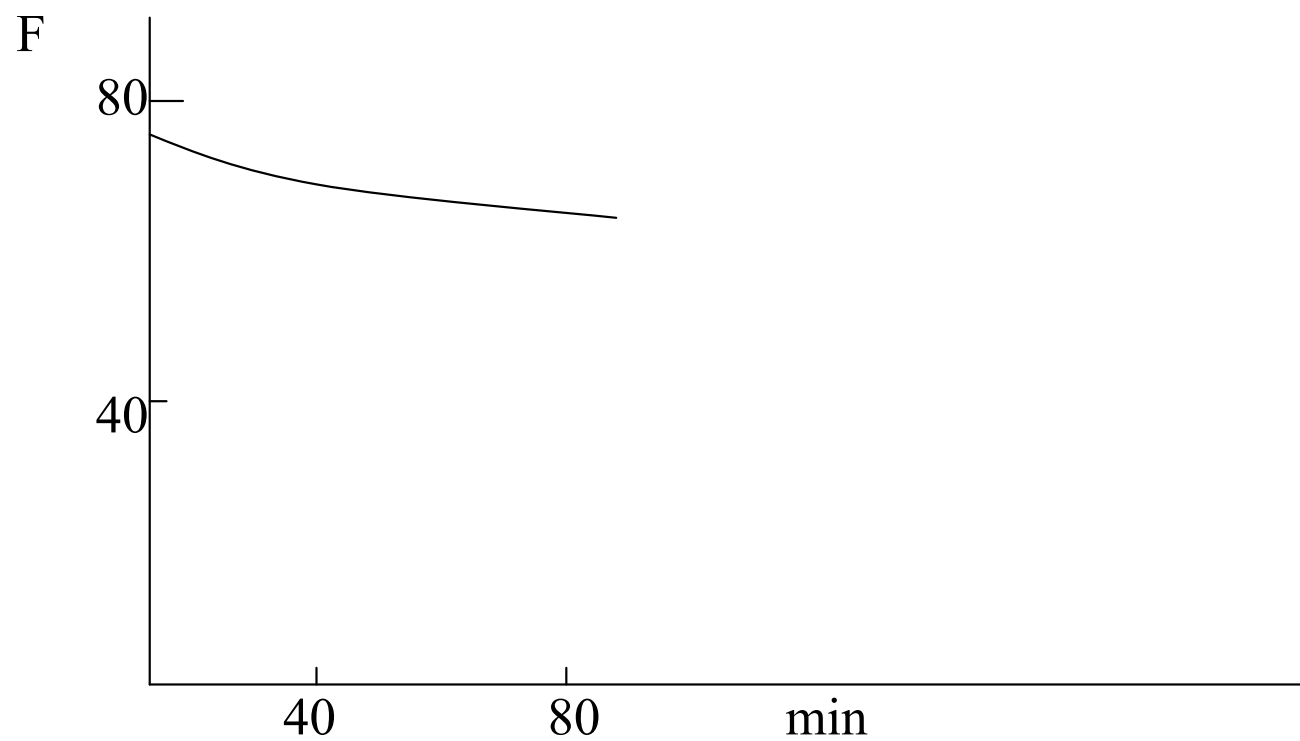
Podmínky fluorescence

Závislost fluorescence na teplotě

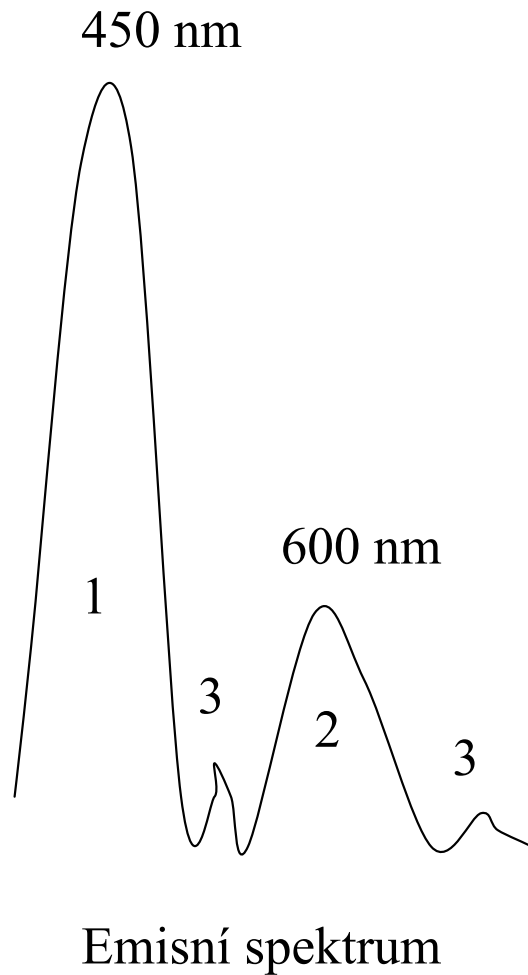


Podmínky fluorescence

Stabilita fluorescenčního signálu
chininsulfátu



Podmínky fluorescence



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

Excitace 450 nm

Kvantitativní fluorimetrie

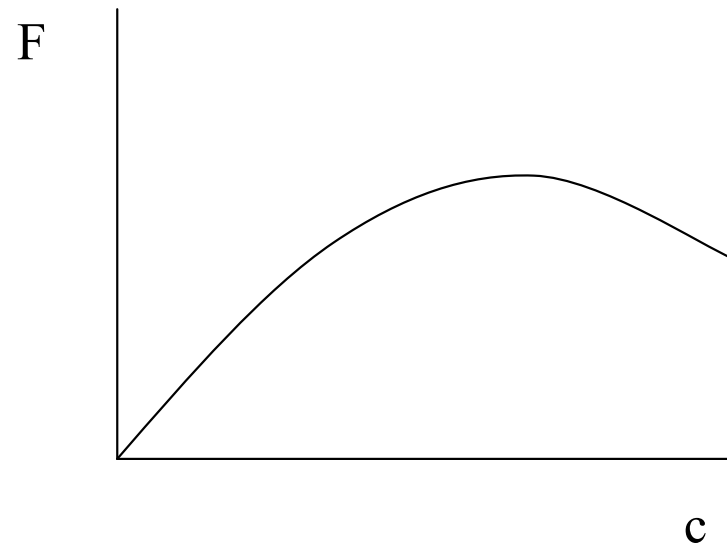
Závislost intenzity fluorescence na koncentraci látky

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

$$F = I_0 \Phi [1 - 10^{-\epsilon cd}]$$

jestliže $c \rightarrow 0$

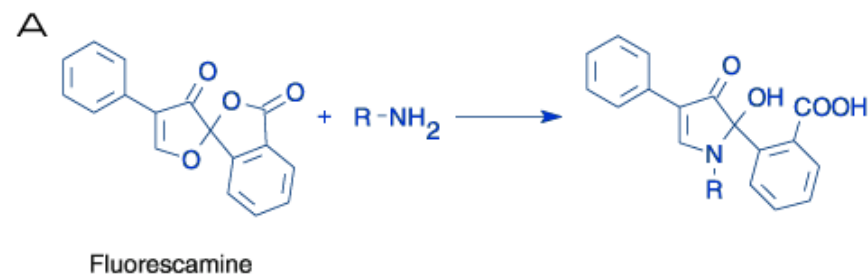
$$F = I_0 \Phi \cdot 2,3 \cdot \epsilon d \cdot c$$



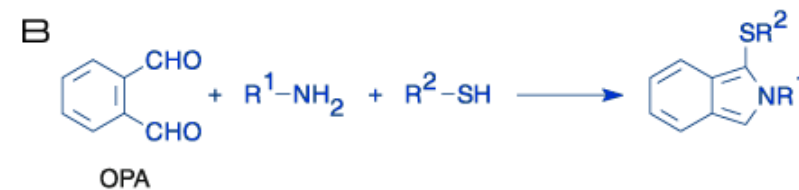
Kvantitativní fluorimetrie

Stanovení koncentrace aminokyselin

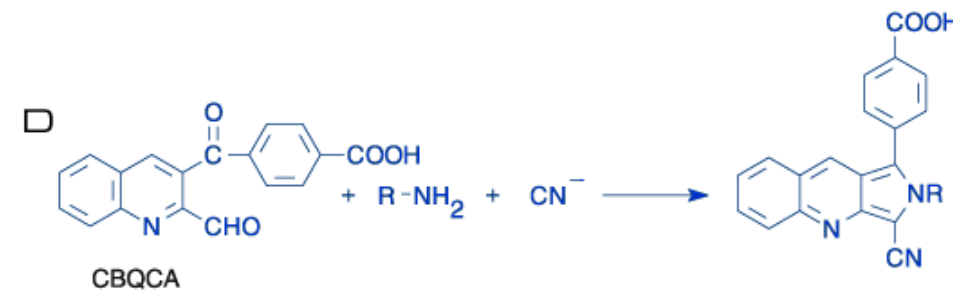
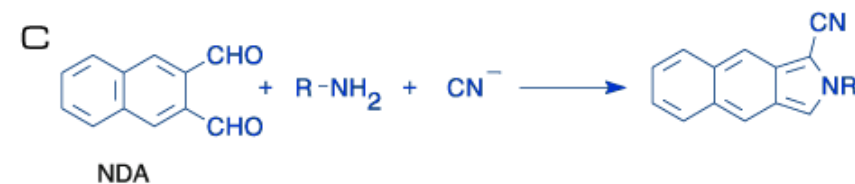
390/464 nm



340/455 nm



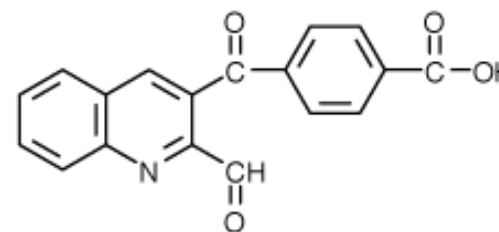
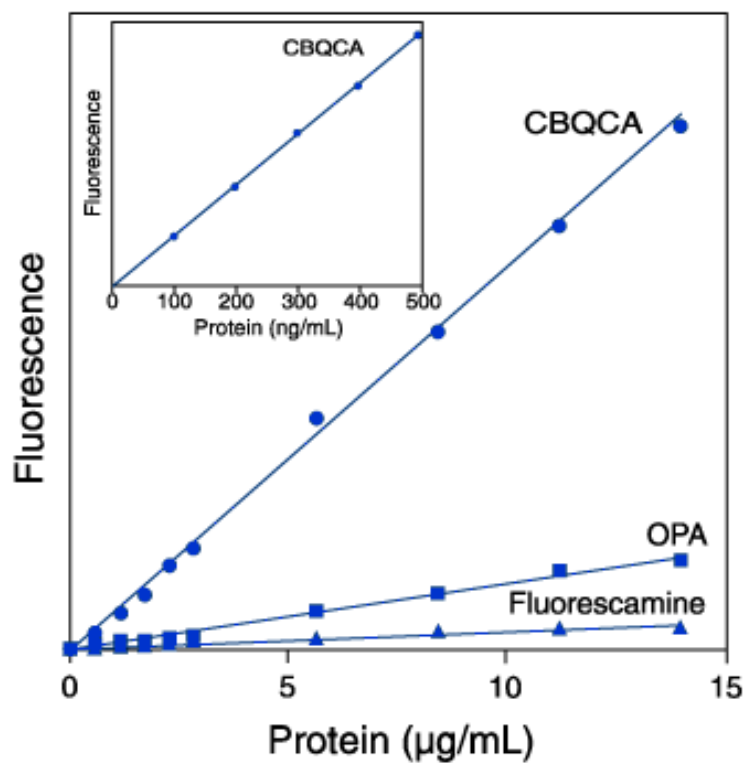
450/550 nm



Kvantitativní fluorimetrie

Stanovení bílkovin

CBCQA



Kvantitativní fluorimetrie

Detekce bílkovin v gelu

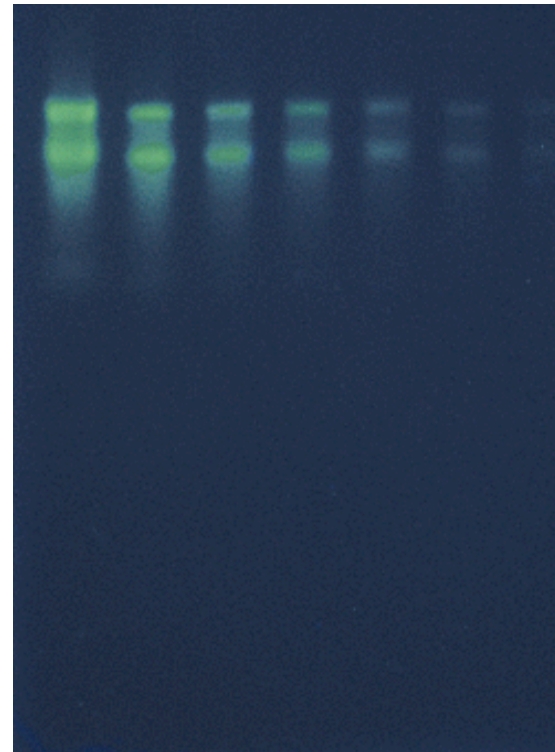
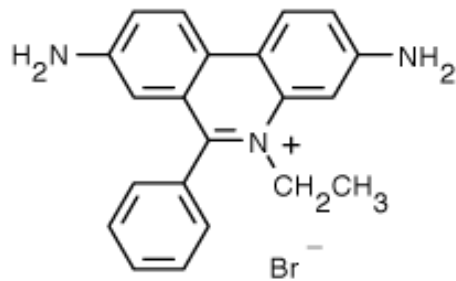
(barevně: Coomassie blue, stříbrné barvení)

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

Kvantitativní fluorimetrie

Detekce nukleových kyselin

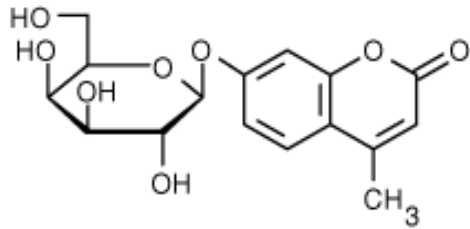
Ethidium bromid



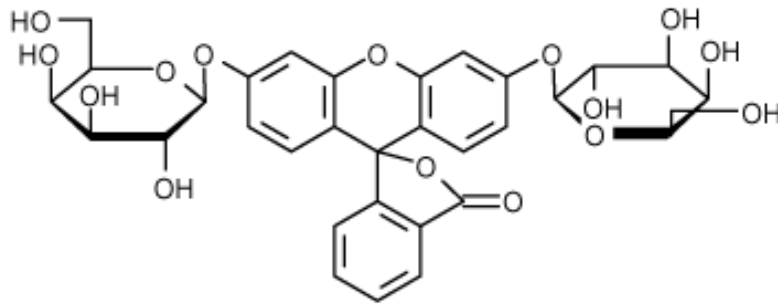
rRNA 16 a 23s barvená SYBR Green II
Molecular Probes

Fluorogenní substráty

Galaktosidasy



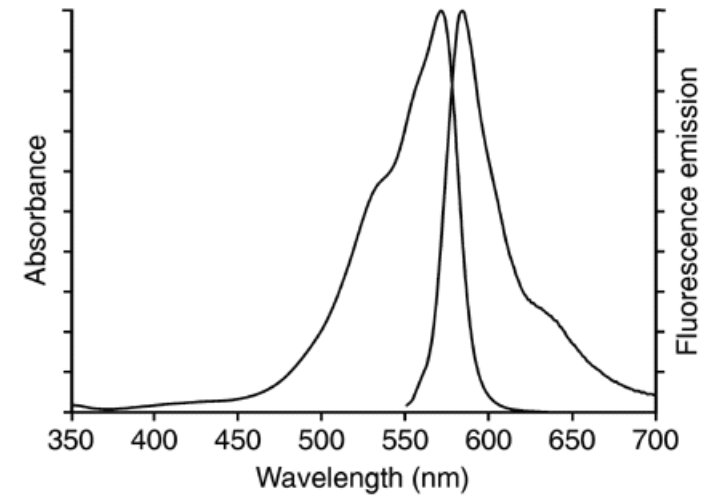
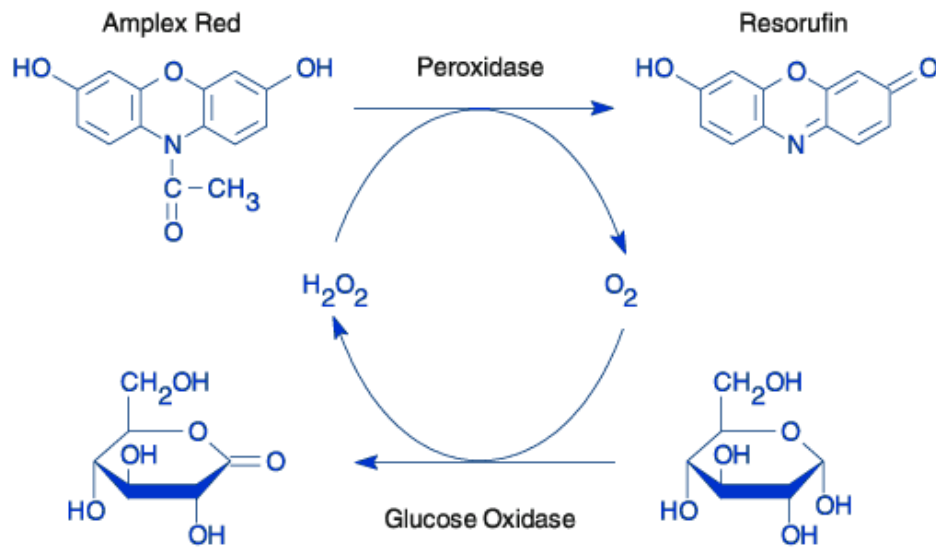
4-methylumbelliferyl- α -galaktosid



Fluorescein-digalaktosid

Fluorogenní substráty

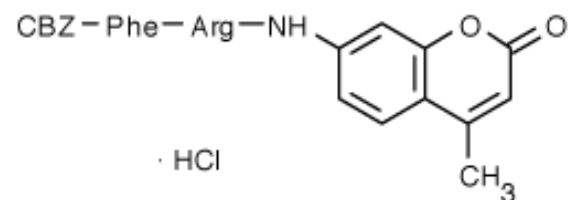
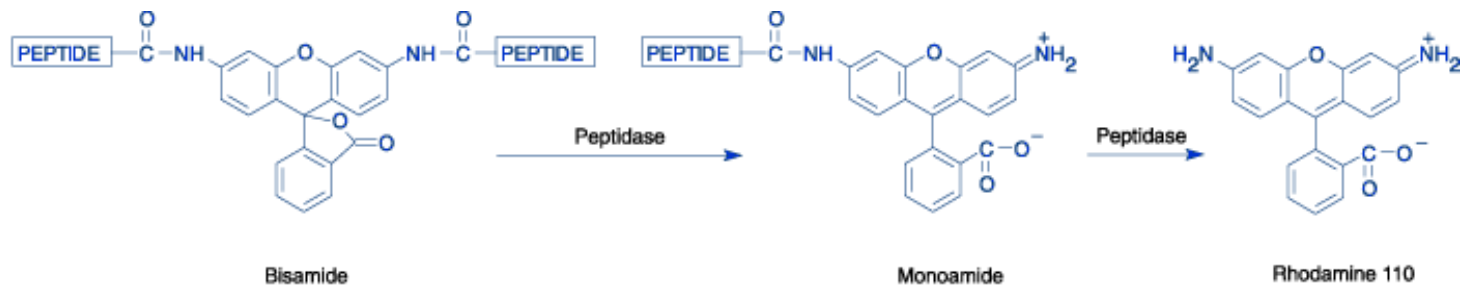
Peroxidasy – amplex red, vznik resorufinu



Fluorogenní substráty

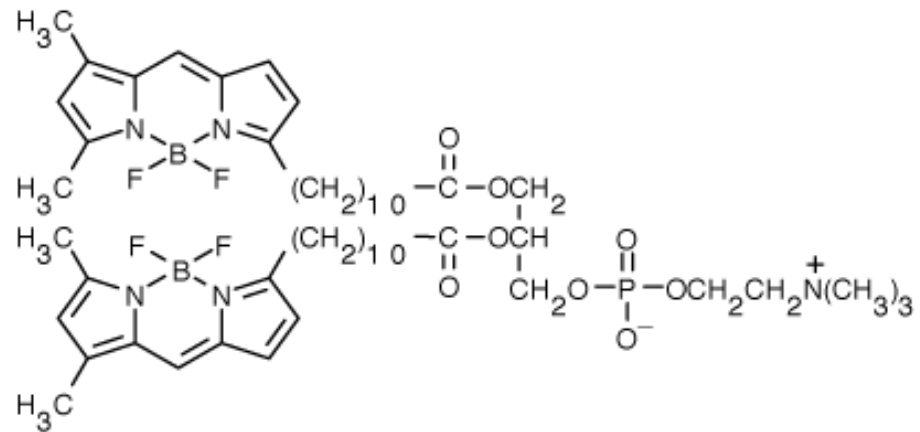
Proteinasy, peptidasy

1) Fluorescenční konjugáty proteinů a peptidů



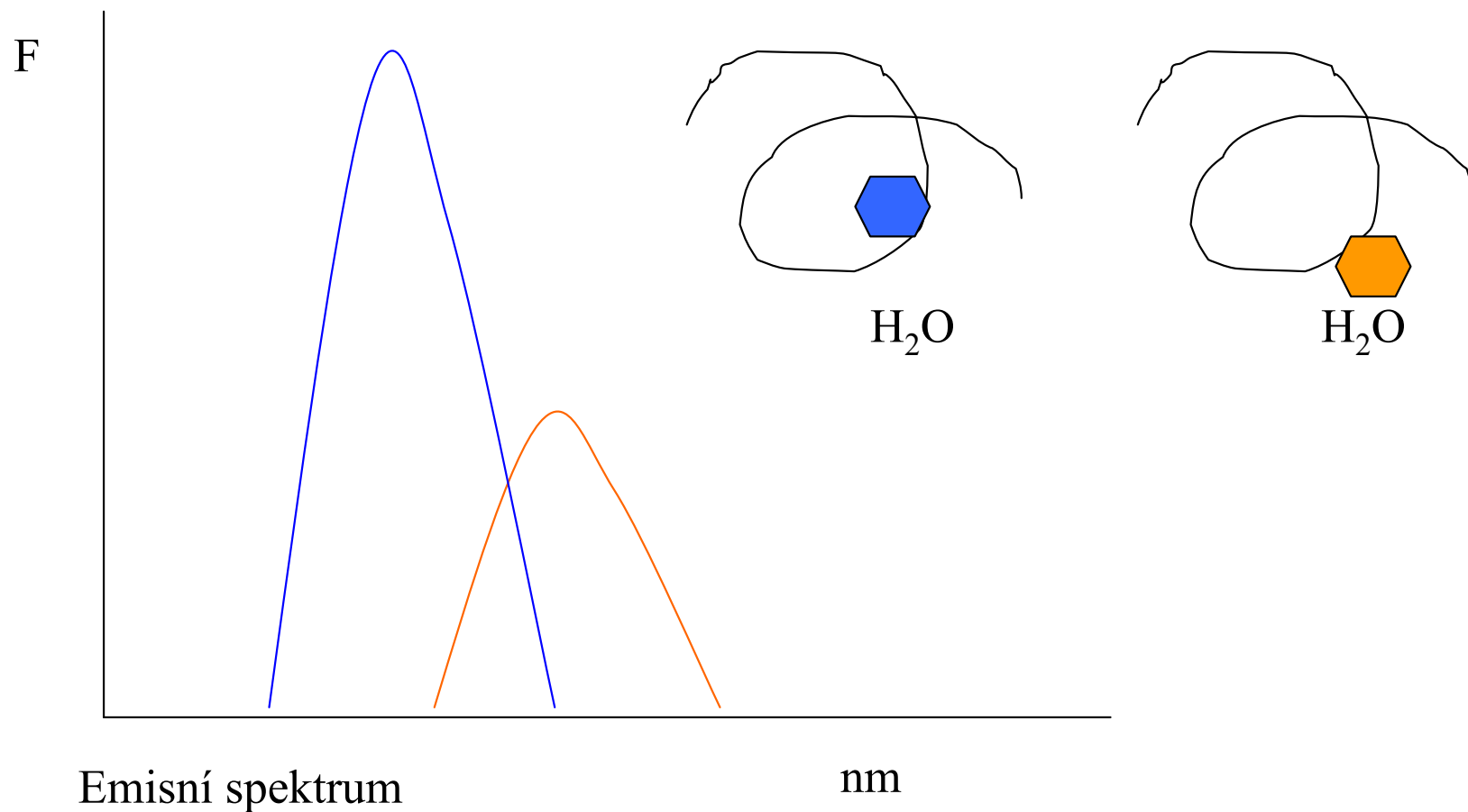
Fluorogenní substráty

Fosfolipasa A

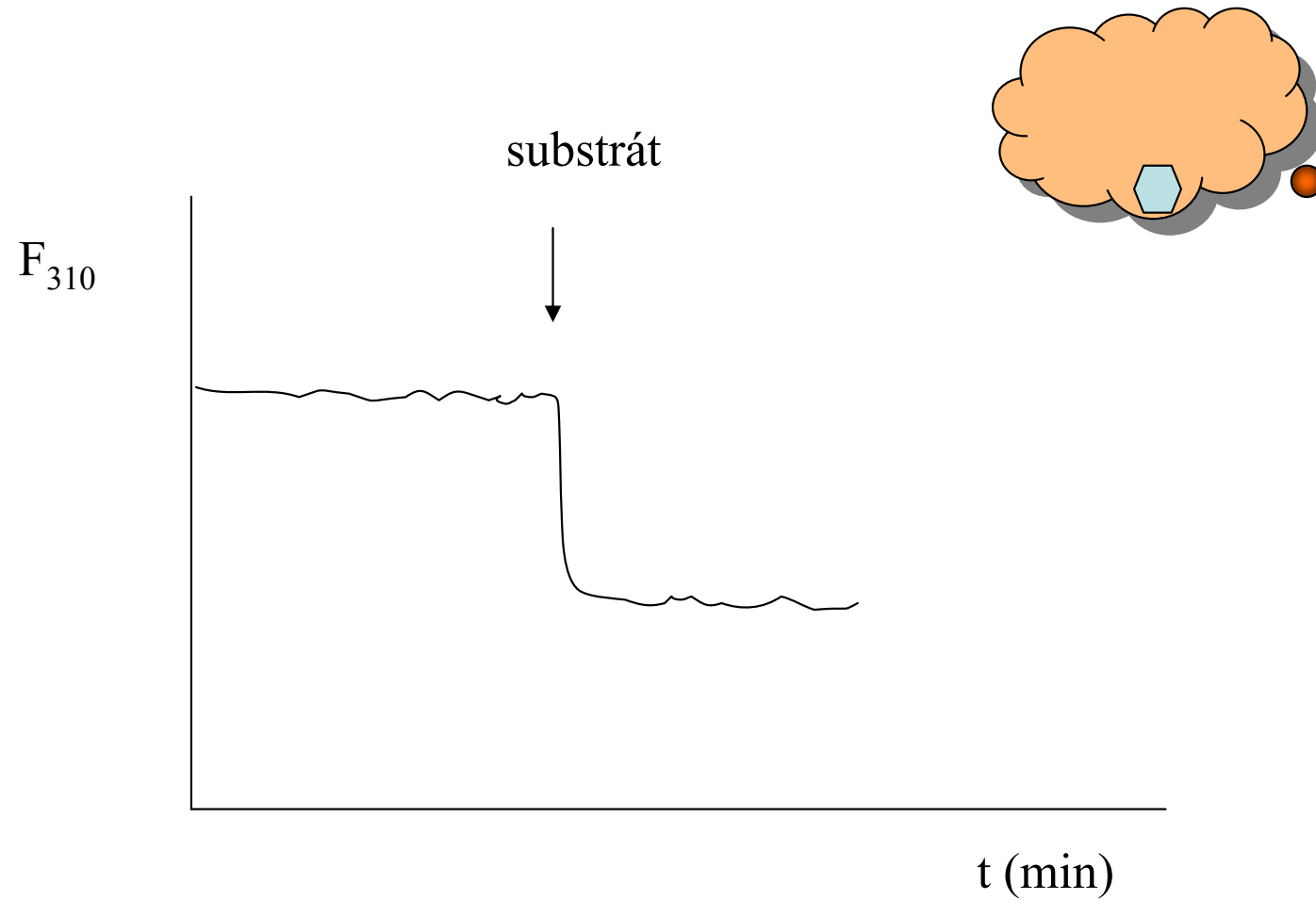


Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

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

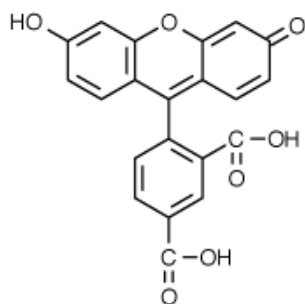


Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

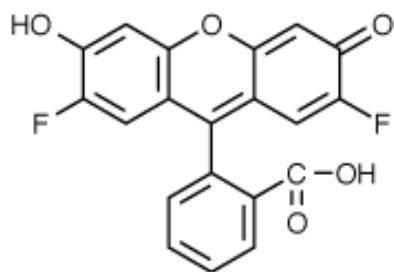


Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

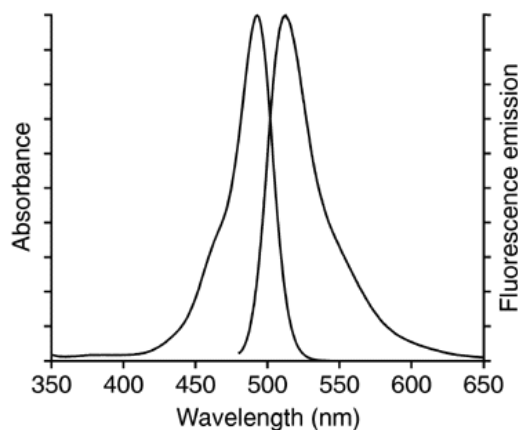
Fluorescenční konjugáty



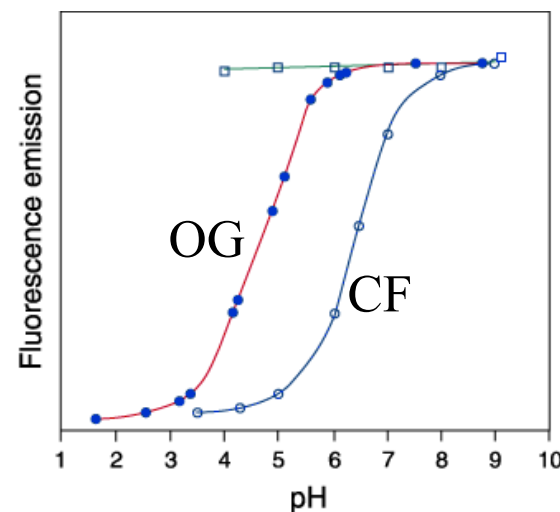
Karboxyfluorescein –
(494/520 nm)



Oregon Green - (496/524 nm)



Absorpce/emise
fluoresceinu
při pH 9

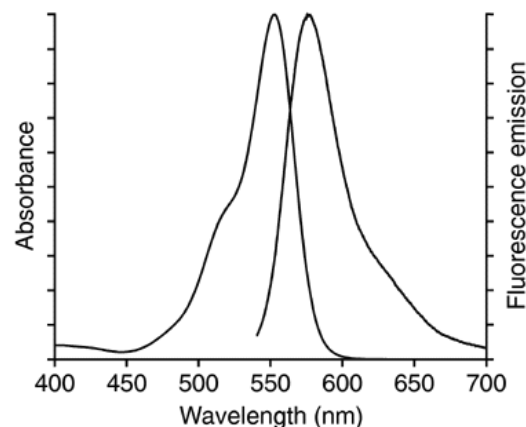
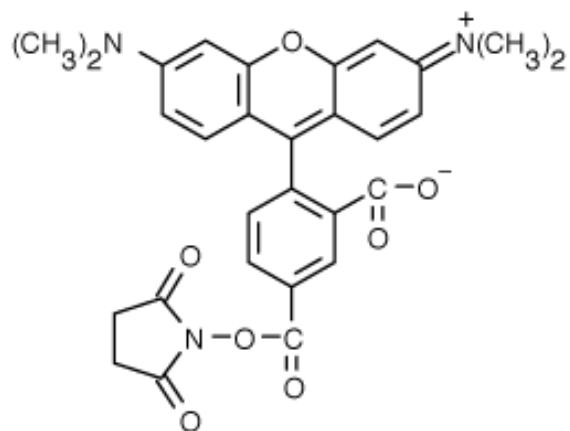


CF-karboxyfluorescein
OG oregon green

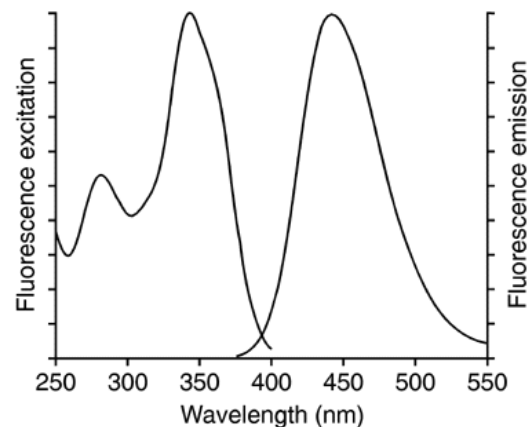
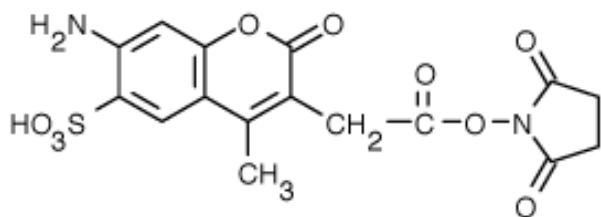
Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Fluorescenční konjugáty

Teramethylrhodamin – 545/580 nm)

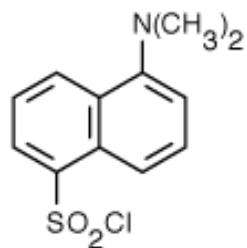


Kumariny – 350/450 nm)

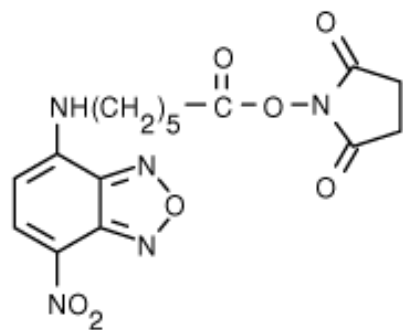
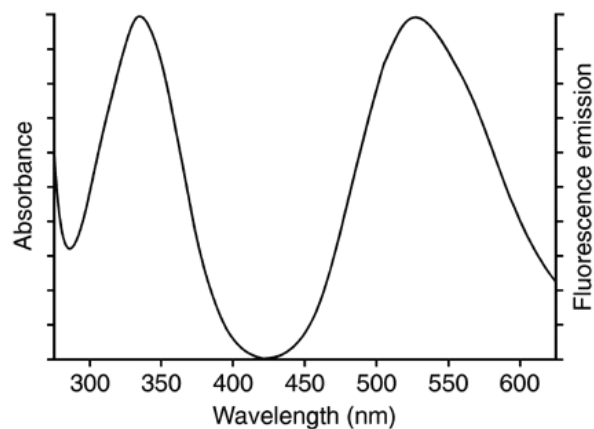


Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

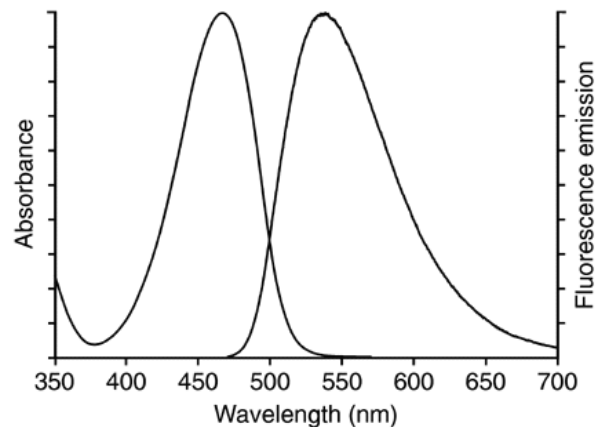
Fluorescenční konjugáty



Dansyl

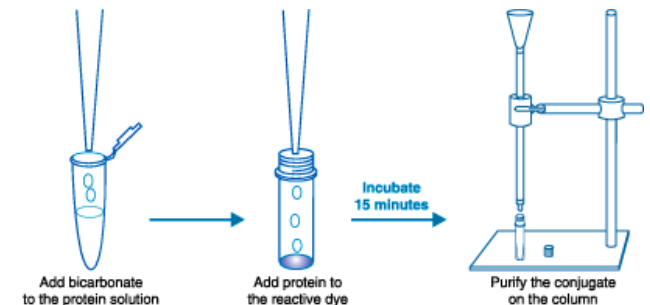
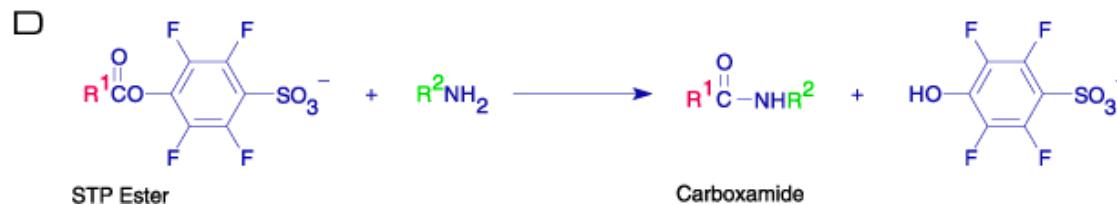
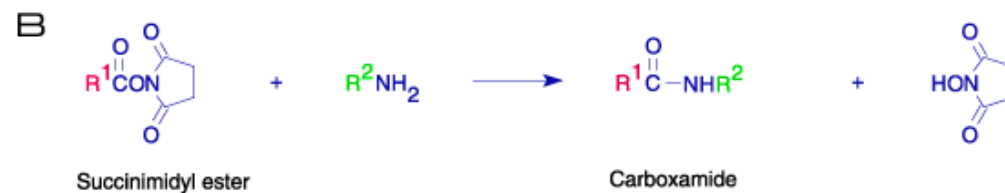
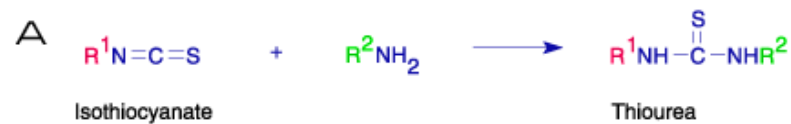


Benzoxadiazol –N-sukcinimid



Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Fluorescenční konjugáty

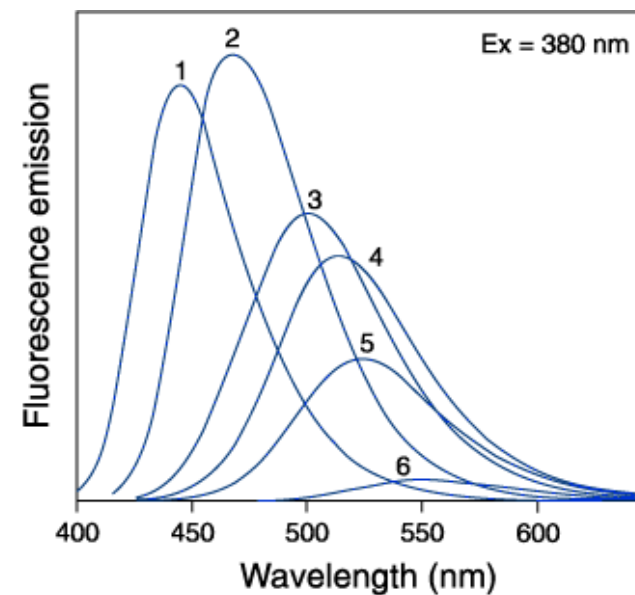
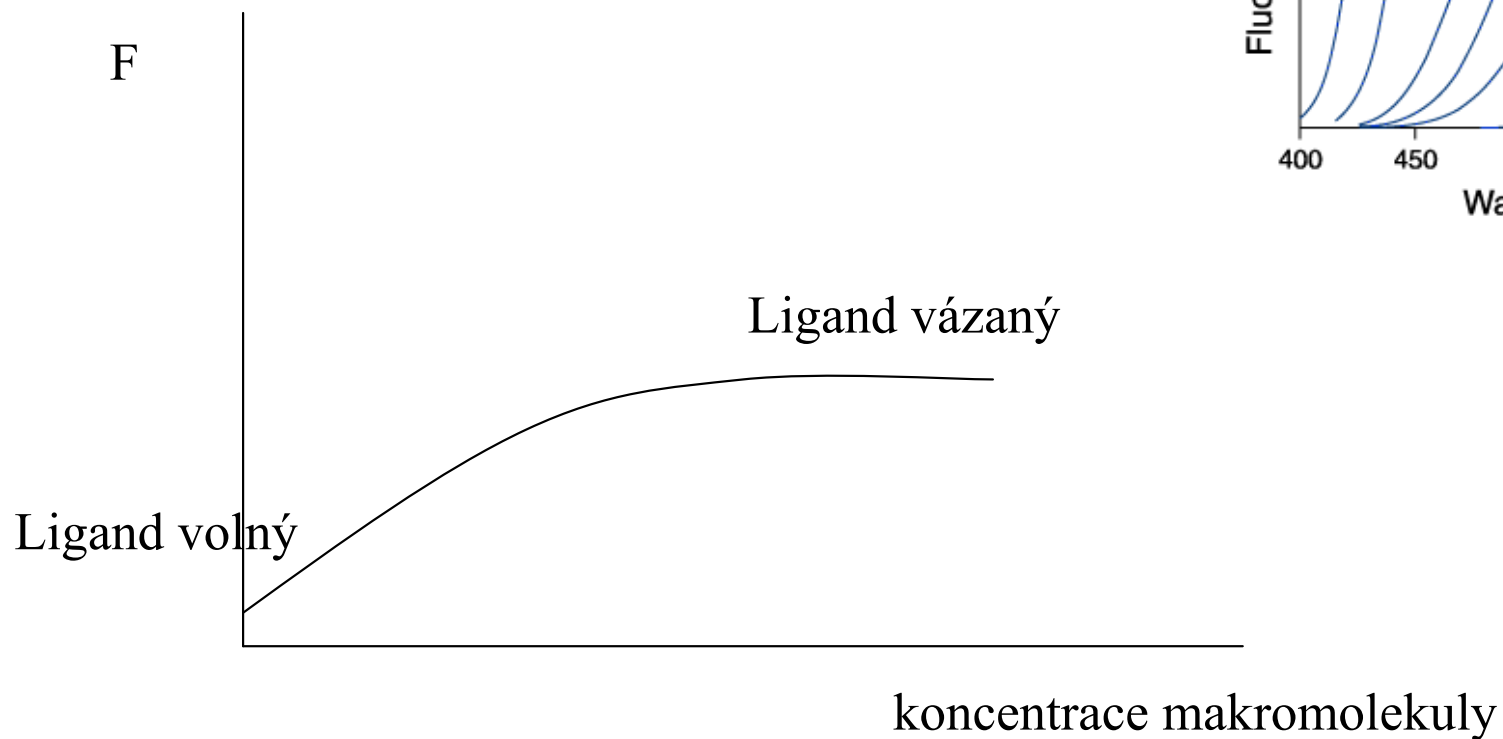


Použití fluorimetrie ke sledování struktury biopolymerů a interakcí



$$K_d = \frac{L_f \cdot M_f}{LM}$$

Interakce makromolekul s ligandy



Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

$$F = F_f + F_b$$

$$F = C_f \cdot \Phi_f + C_b \cdot \Phi_b$$

$$F = (C - C_b) \Phi_f + C_b \cdot \Phi_b$$

$$F = C\Phi_f - C_b\Phi_f + C_b \cdot \Phi_b$$

$$F = F_0 + C_b (\Phi_b - \Phi_f)$$

$$C_b = (F - F_0) / (\Phi_b - \Phi_f)$$

F_f, F_b – fluorescence volné, vázané frakce

Φ_b, Φ_f – kvant. Výtěžek fluorescence vázaného, volného ligandu

C_b, C_f – koncentrace vázaného, volného Ligandu

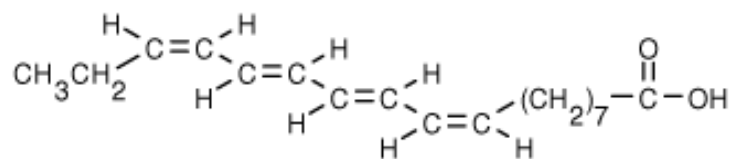
C – celková koncentrace ligandu

F – celková fluorescence

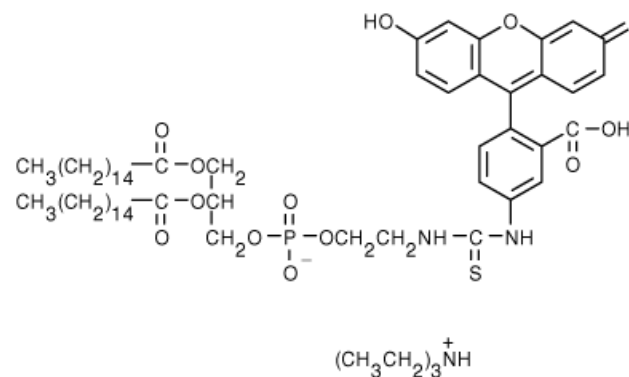
Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Interakce makromolekul s ligandy

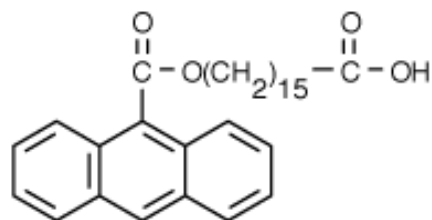
Použití fluorescenčních analogů



Kys. cis-parinarová



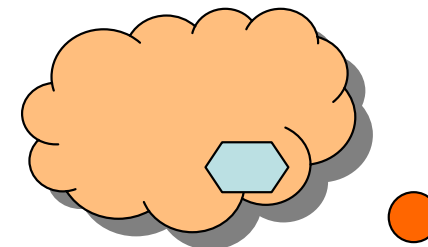
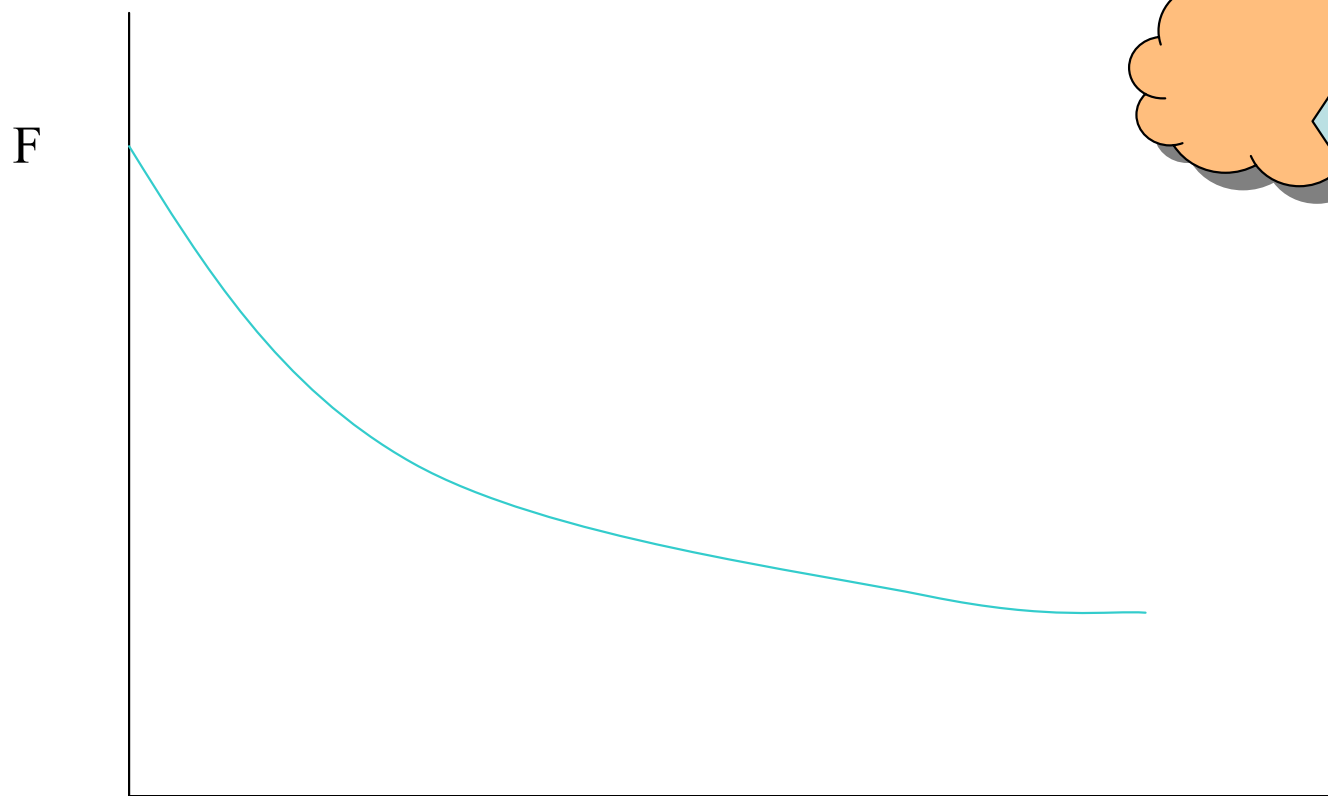
Fluorescein-PE



Anthroyloxypalmitát

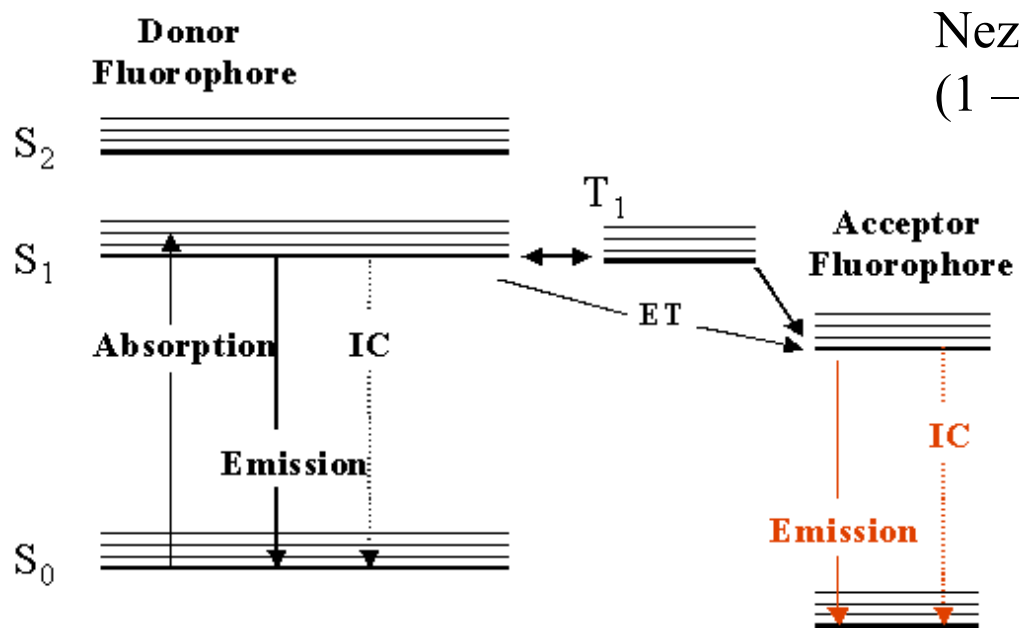
Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Použití značené makromolekuly
Fluorescence značené bílkoviny



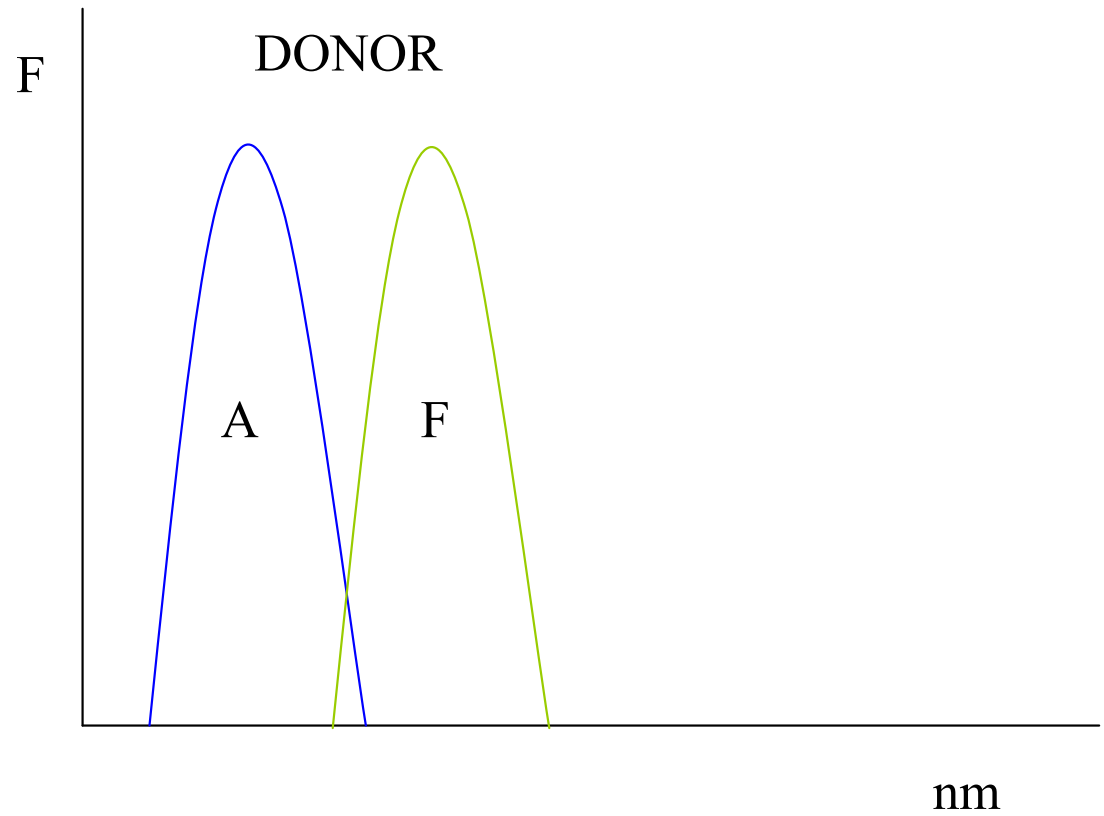
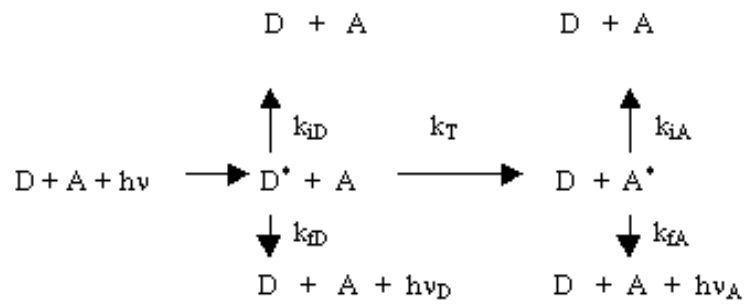
Koncentrace ligandu

Fluorescenční rezonanční transfer energie (Försterův přenos)

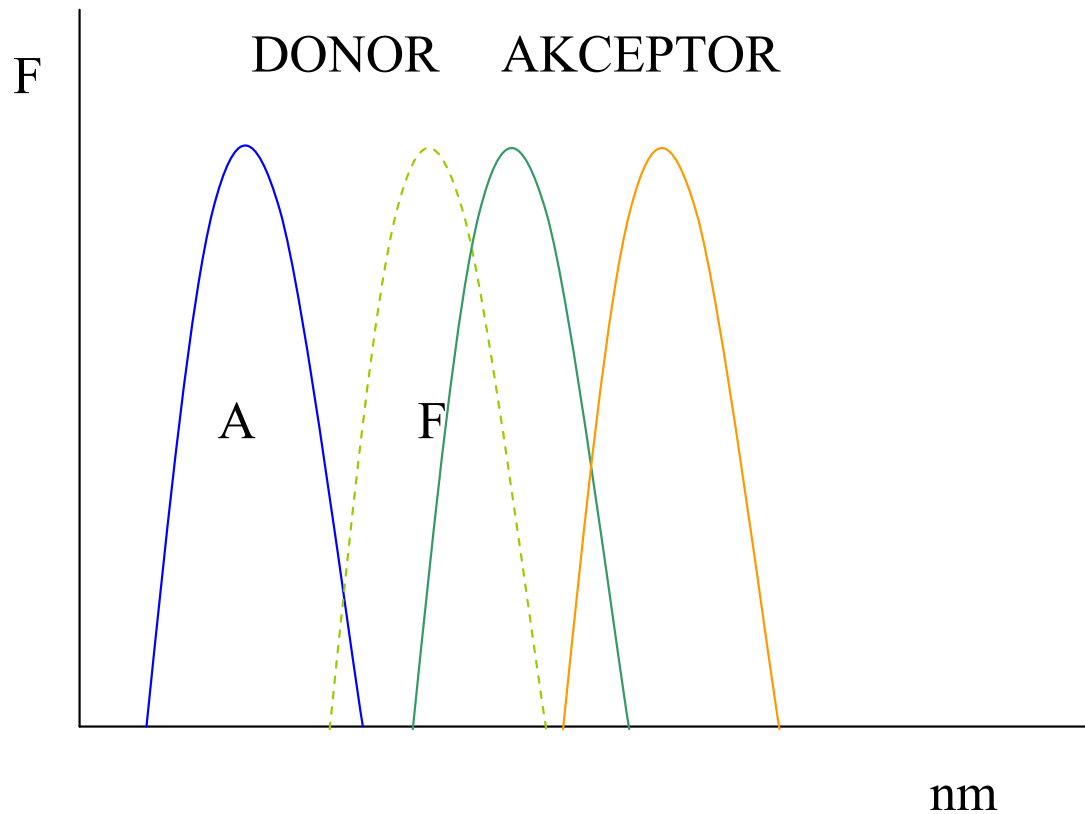
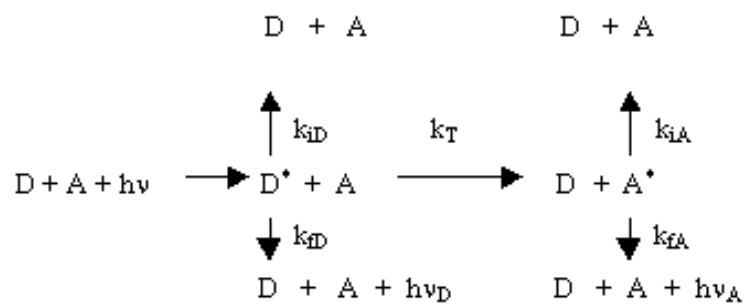


Nezářivý přenos energie z donoru na akceptor
(1 – 10 nm)

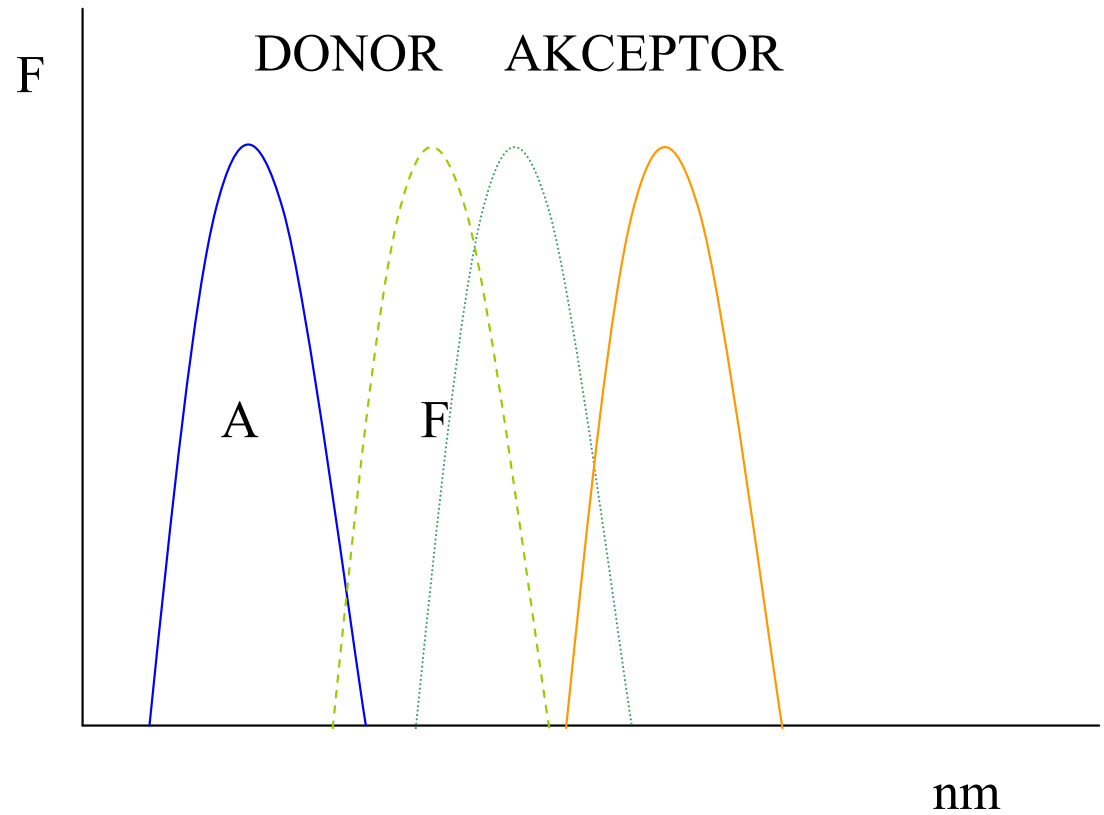
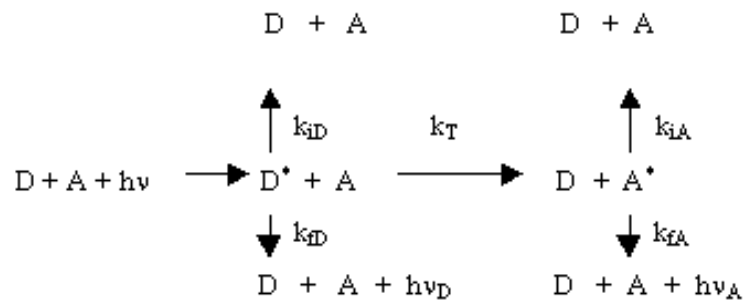
Fluorescenční rezonanční transfer energie



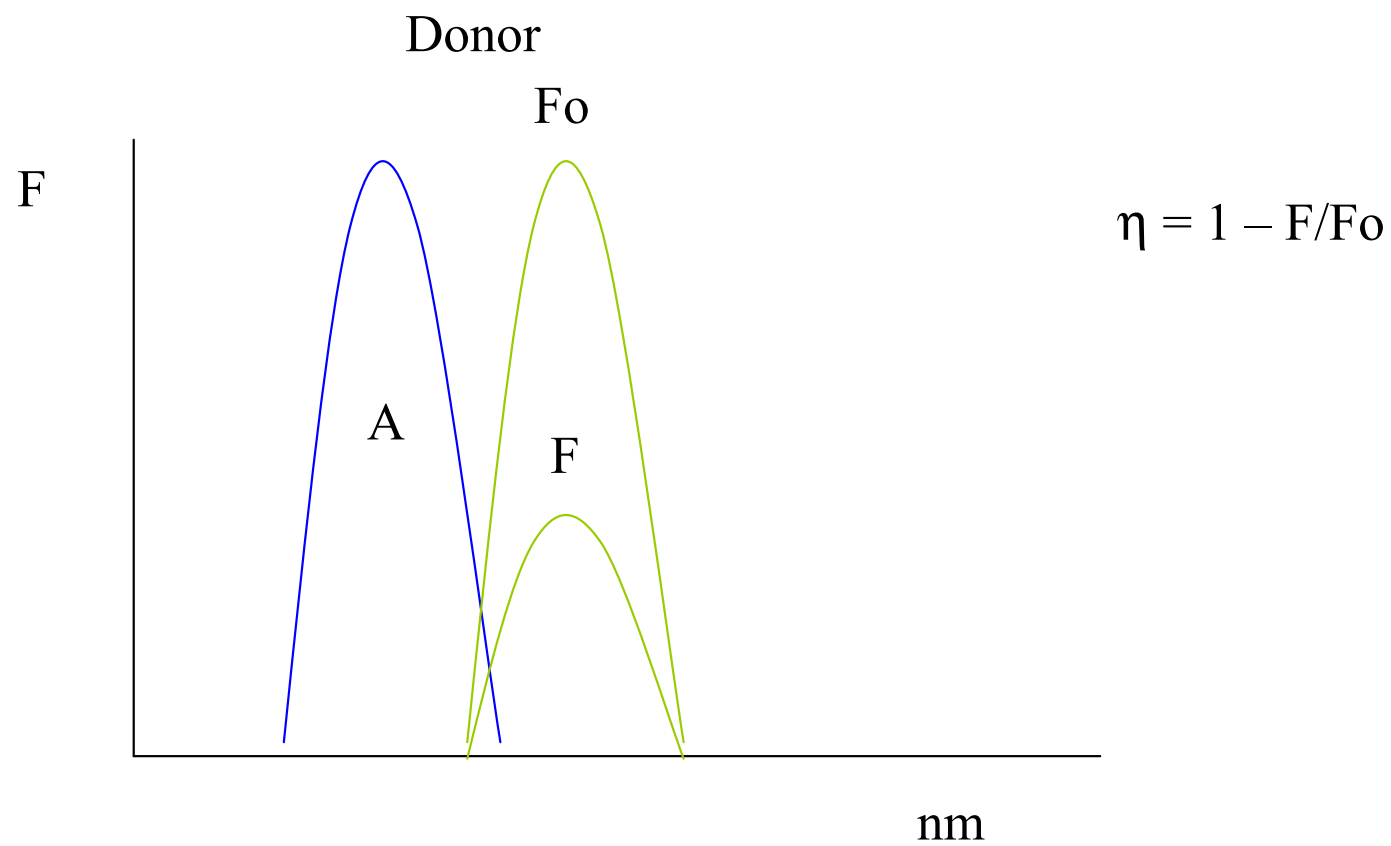
Fluorescenční rezonanční transfer energie



Fluorescenční rezonanční transfer energie



Fluorescenční rezonanční transfer energie



Fluorescenční rezonanční transfer energie

$$\eta = R_0^6 / (R_0^6 + R^6)$$

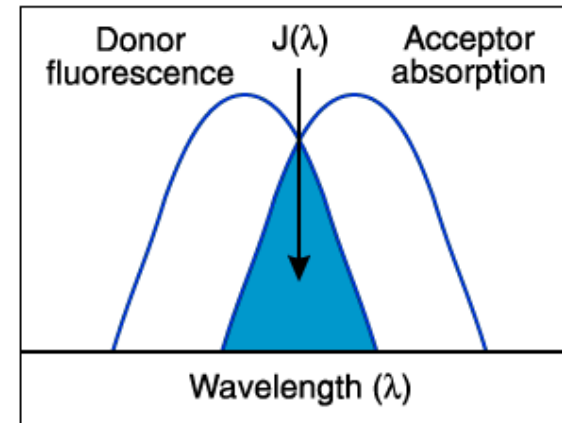
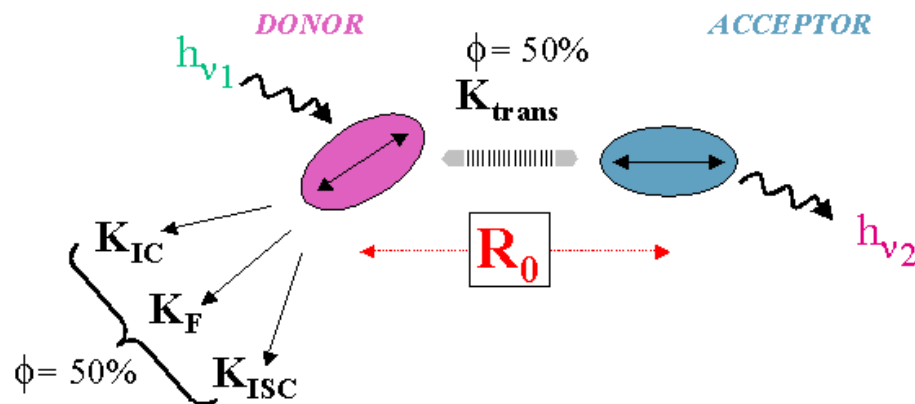
$$R_0^6 = \sqrt{1,66 \cdot 10^{-33} \cdot \tau \cdot J / n^2 \nu_0^2}$$

τ – doba života exc. stavu

J – překryvový integrál

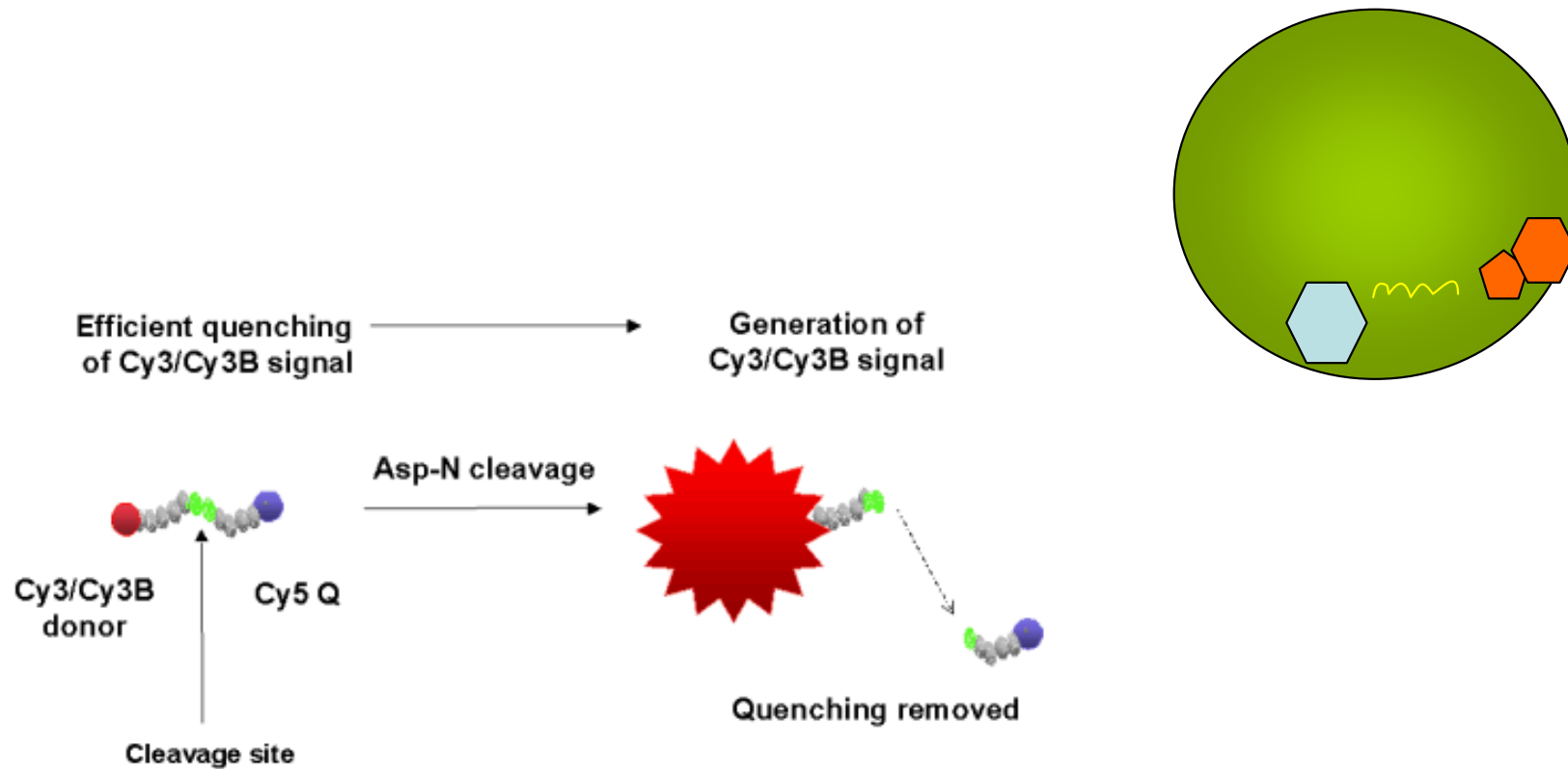
n – refraktivní index rozpuštědla

ν – vlnčet emise donoru

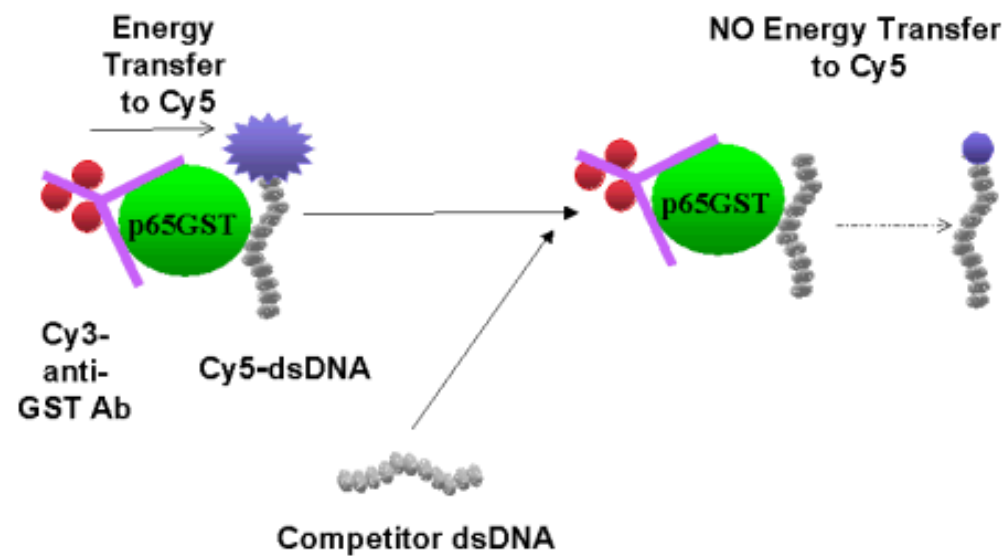


Fluorescenční rezonanční transfer energie

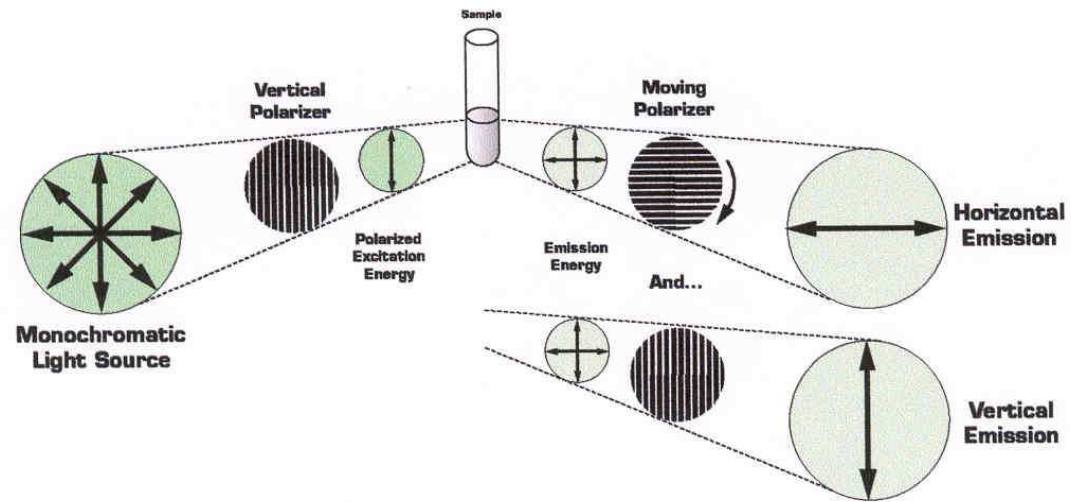
Použití – změření vzdálenosti mezi dvěma molekulami v bílkovině
Tryptofan (290/340) vs. NADH (340/450 nm)



Fluorescenční rezonanční transfer energie



Fluorescenční anizotropie



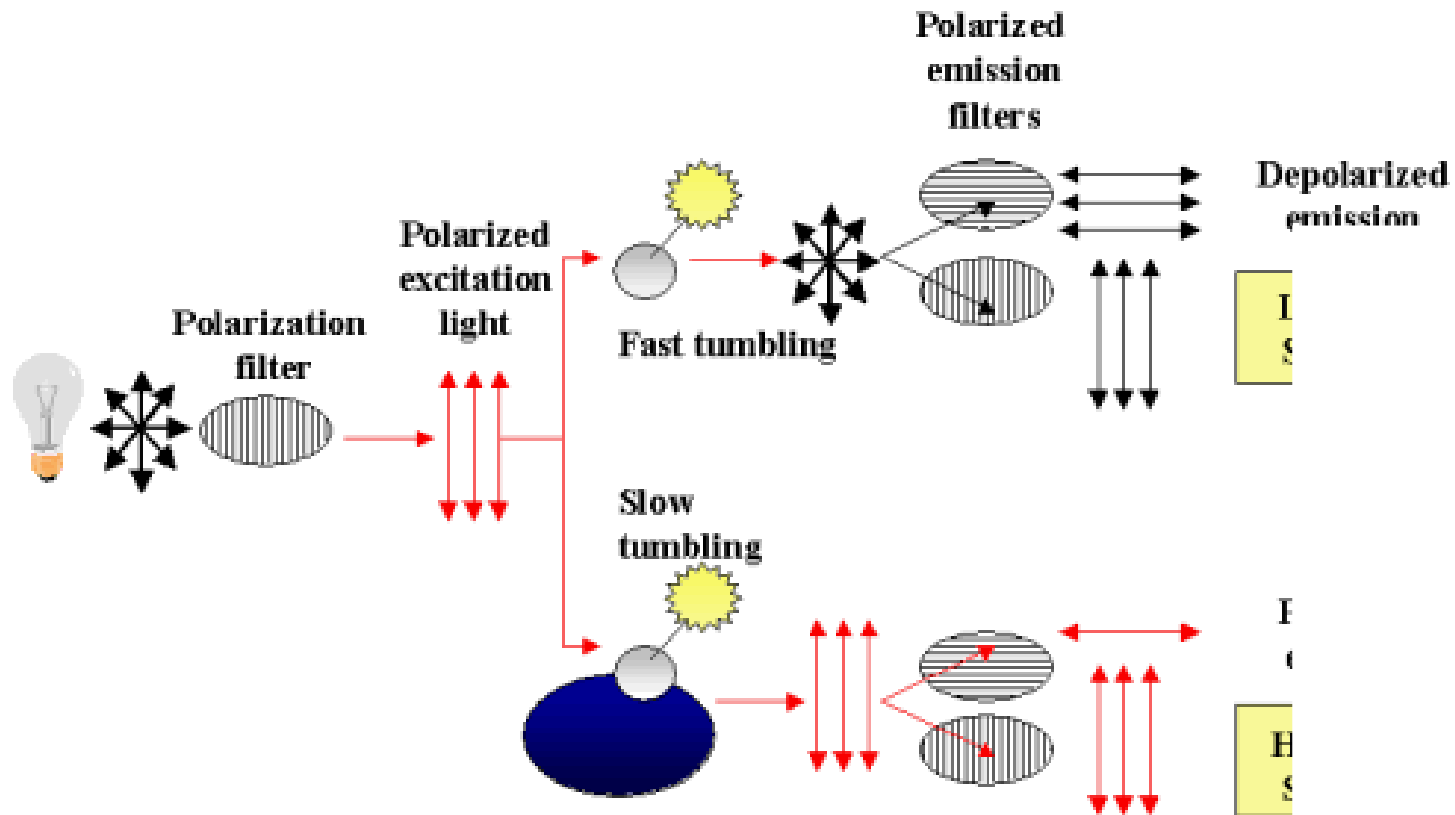
Fluorescenční anizotropie

Polarizační filtry

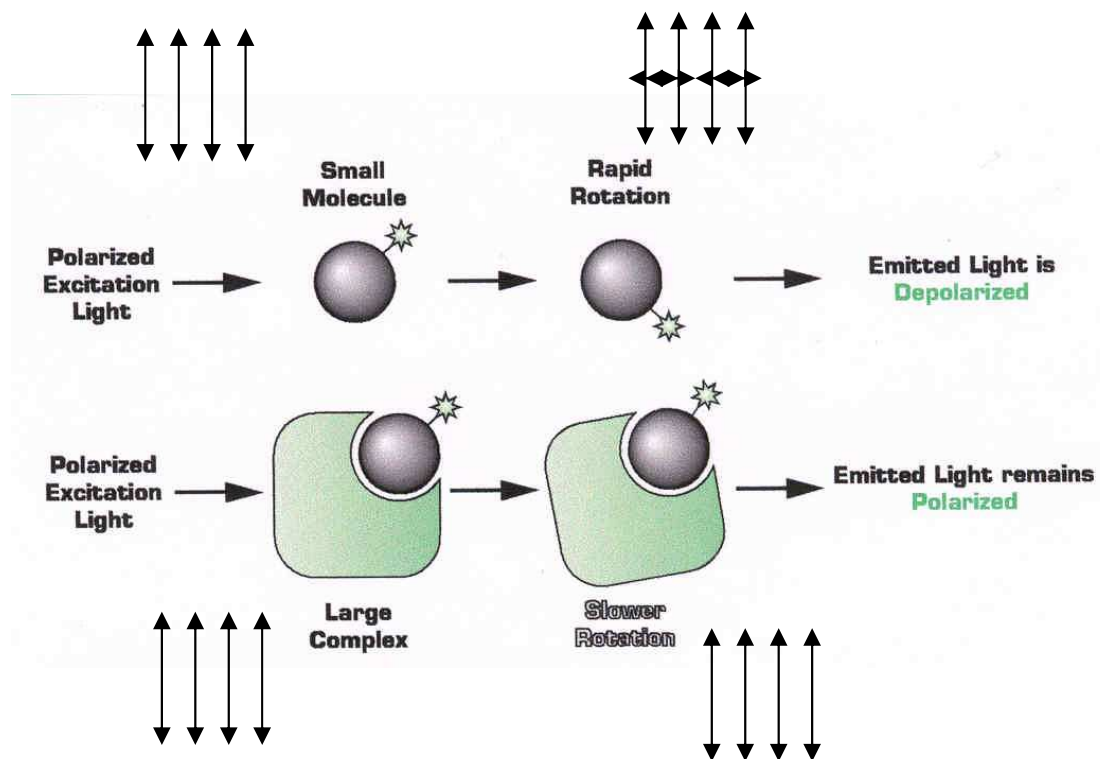


Auto-Polarizer Accessory

Fluorescenční anizotropie



Fluorescenční anizotropie

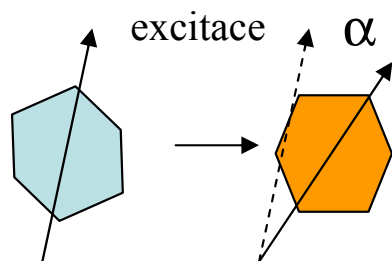


Fluorescenční anizotropie

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

$$I = I_v + 2I_h$$

Rotační relaxační čas



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

$$r_o/r = 1 + 3\tau/\rho$$

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

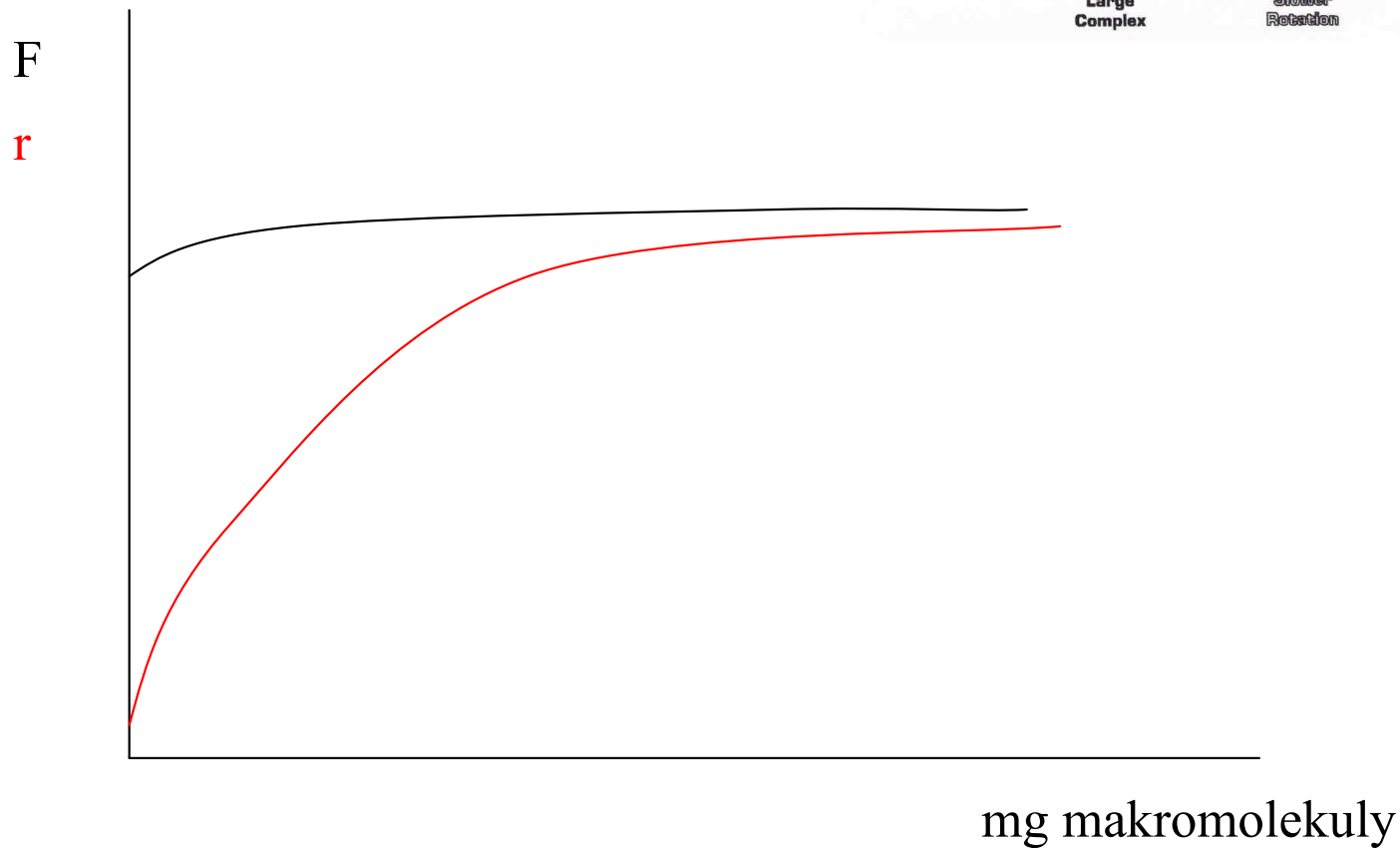
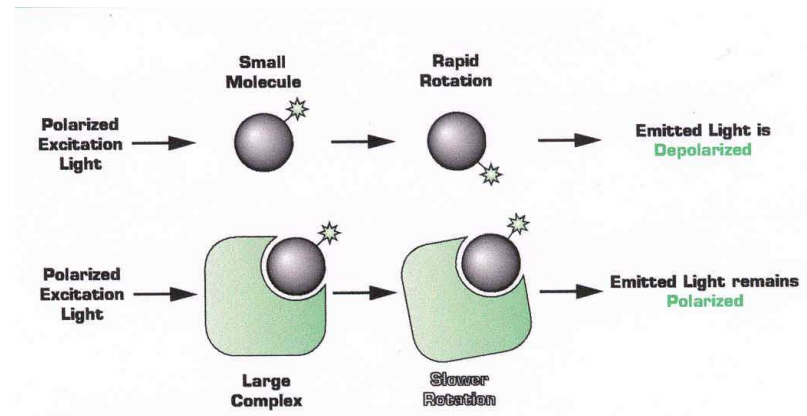
$$\rho = V\eta/RT$$

V objem
 η viskozita

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

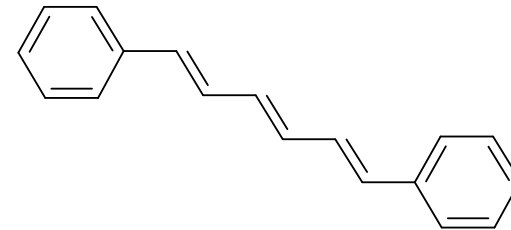
Fluorescenční anizotropie

Využití:
Interakce makromolekuly s ligandem



Fluorescenční anizotropie

Využití:
Měření viskozity prostředí

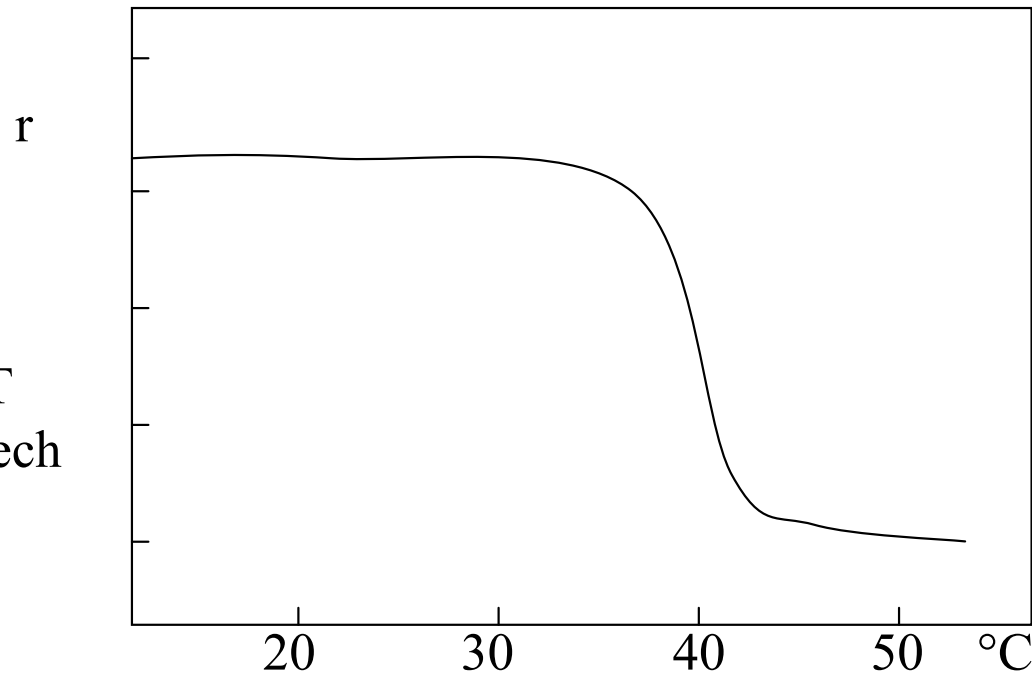


$$r_o/r = 1 + 3\tau RT/V\eta$$

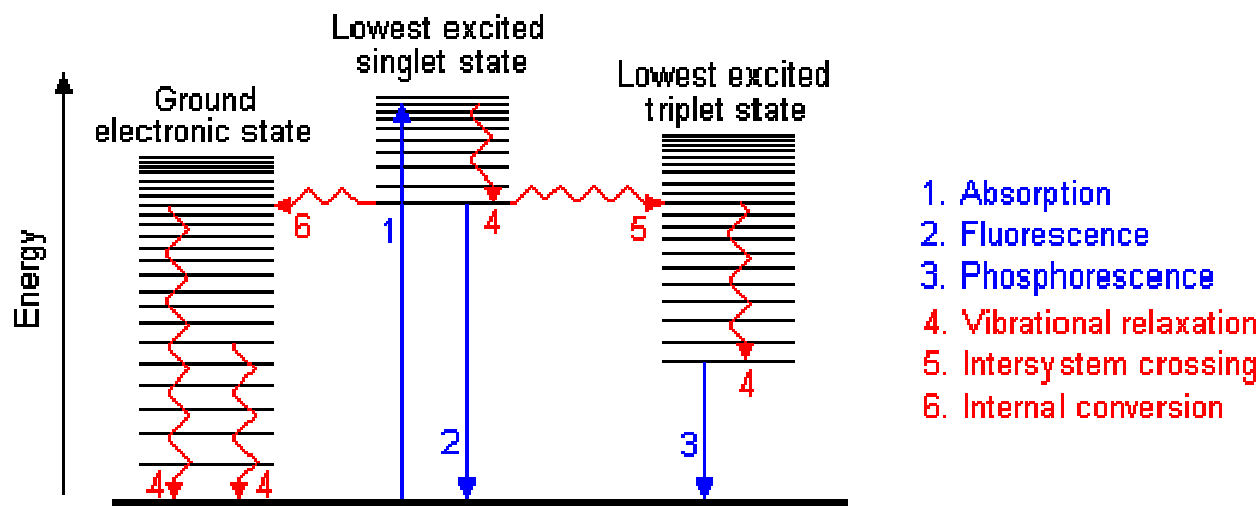
$$r_o/r = 1 + K/\eta$$

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

Fl. anizotropie DPHT
Vázaného v liposomech
DPPC



Fosforescence

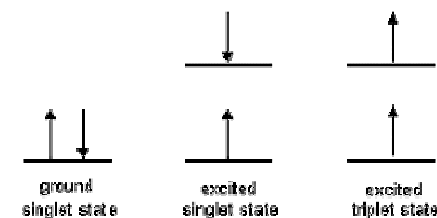
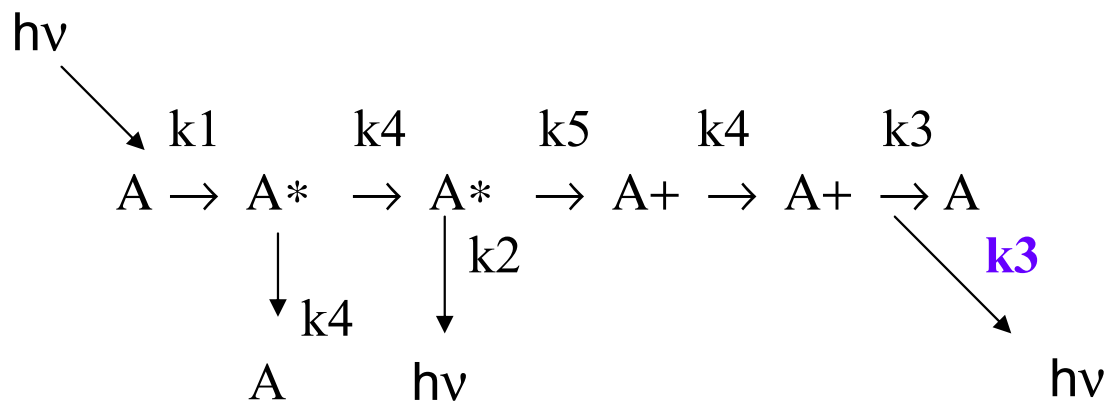


1. Absorption
2. Fluorescence
3. Phosphorescence
4. Vibrational relaxation
5. Intersystem crossing
6. Internal conversion

→ Processes involving photons
~> Radiationless transitions

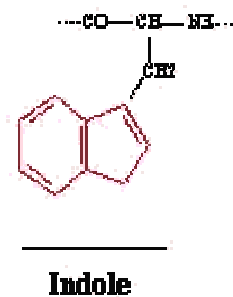
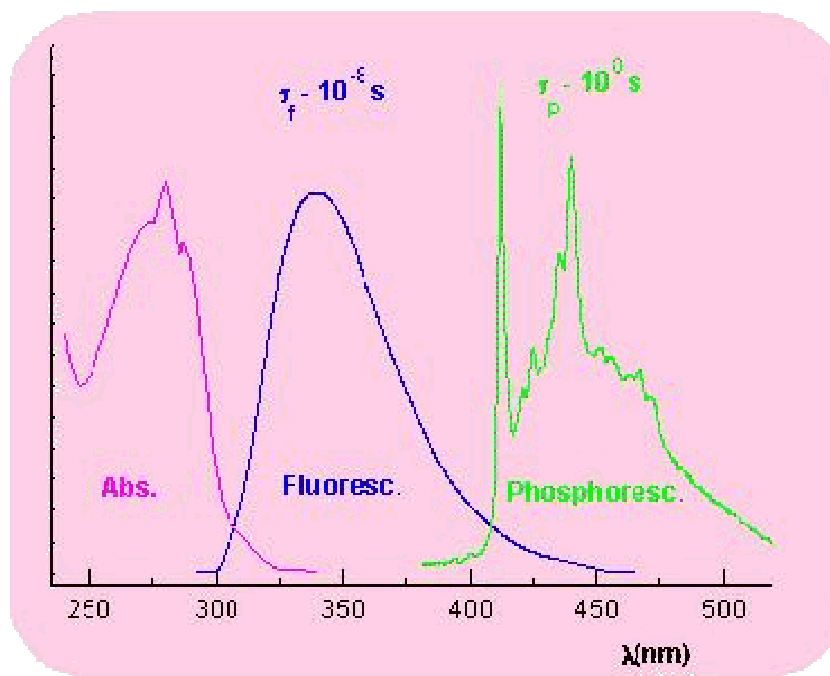
Multiplicita

$$M = 2S + 1$$



Fosforescence

Střední doba života τ $10^{-4} - 100$ s



Tryptophan

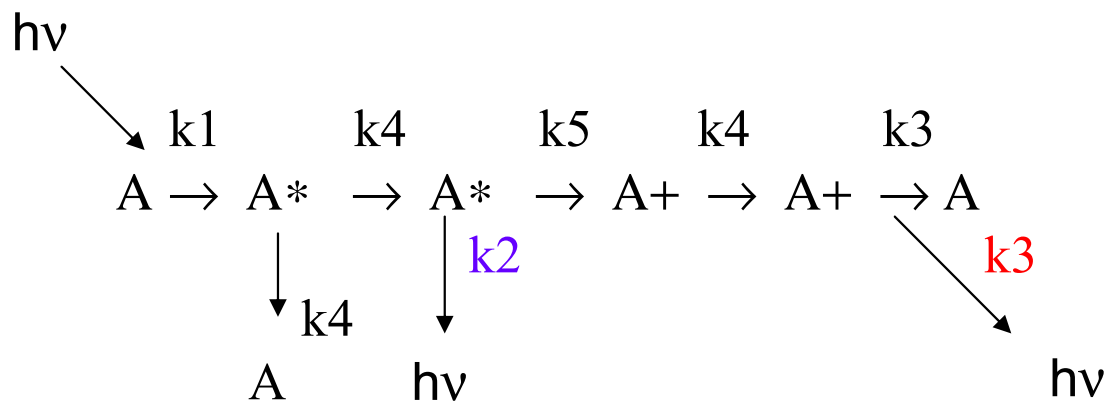
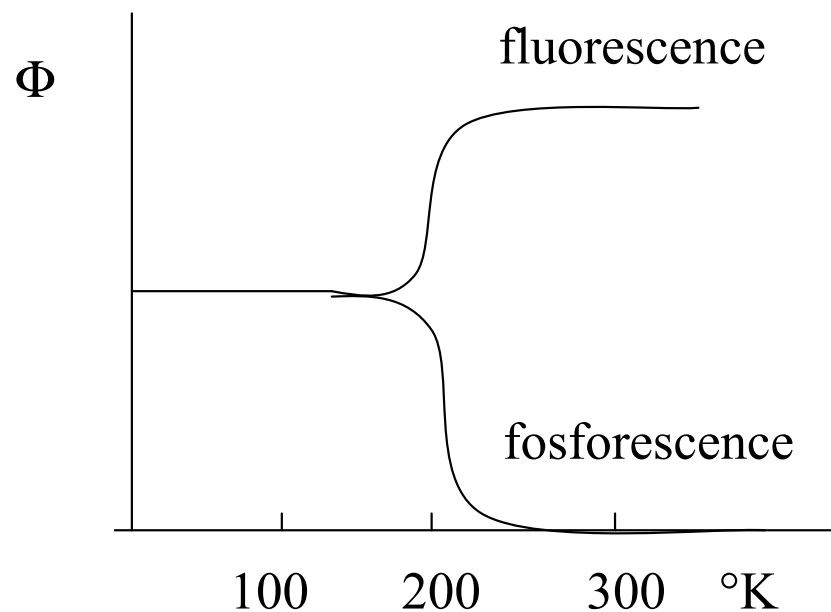
Fosforescence

Kvantový výtěžek fosforescence

$$\Phi_p = k_3 / (k_3 + k_2 + k_4)$$

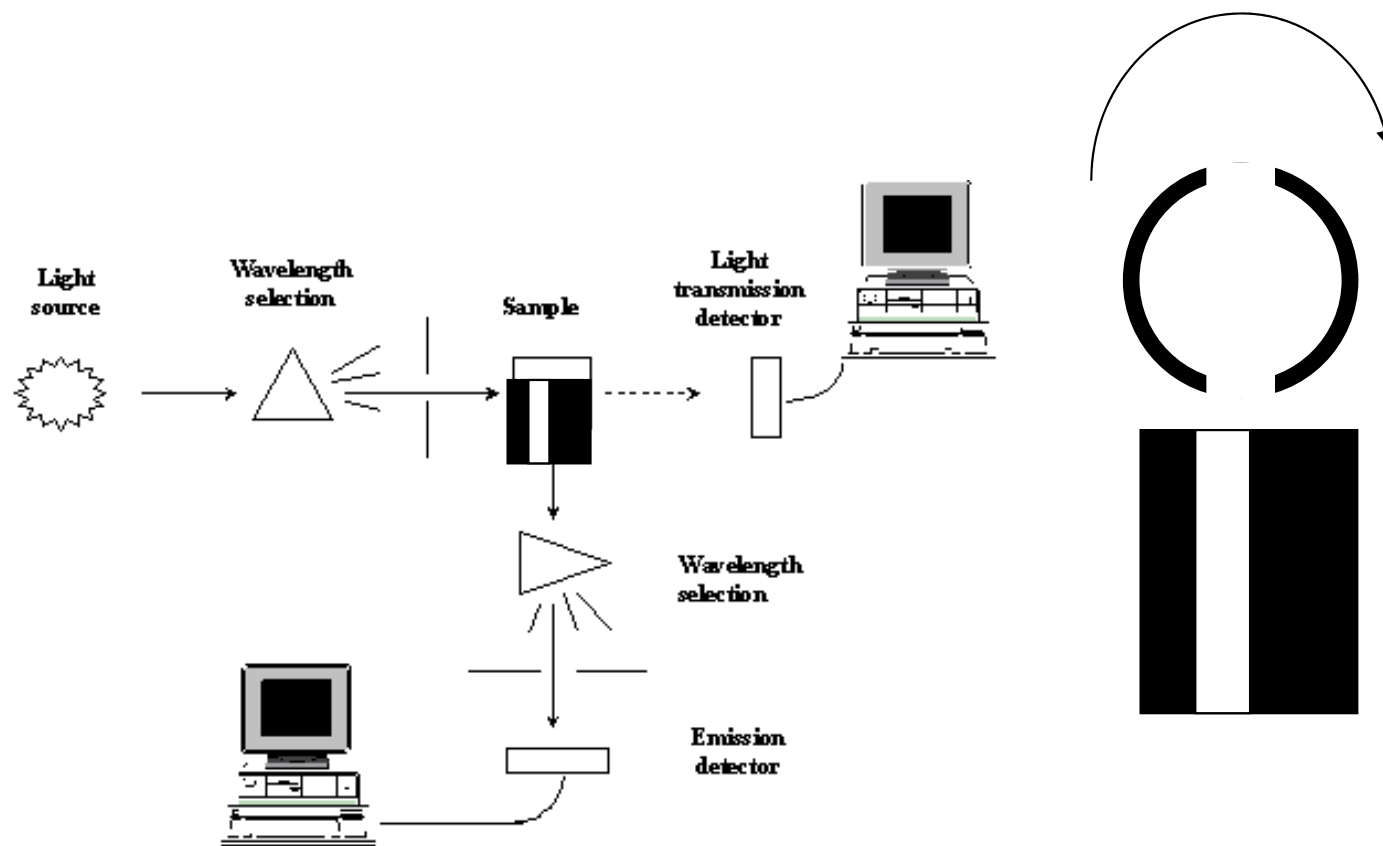
$$\Phi_f = k_2 / (k_3 + k_2 + k_4)$$

$$\Phi_f / \Phi_p = k_2 / k_3$$



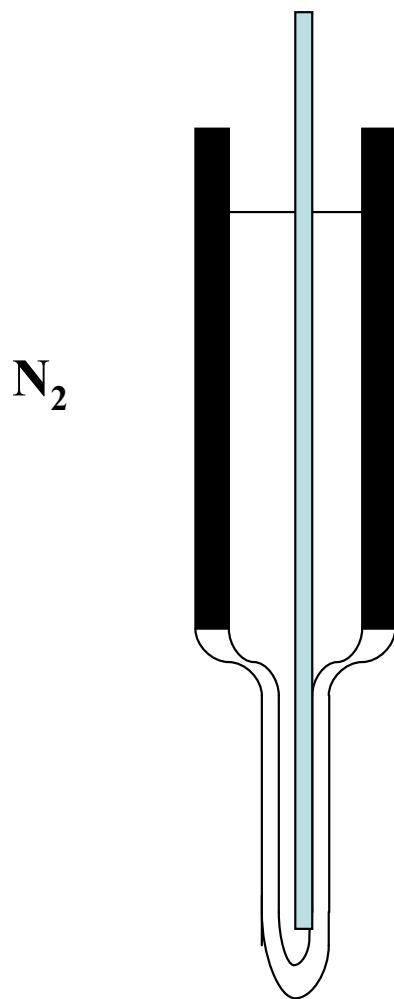
Fosforescence

Experimentální uspořádání



Fosforescence

Vzorek



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. salicylique	315	430	6.2
Quinine	340	500	1.3
Naphtalène	290	505	
Codéine	275	505	0.3
Caféine	285	440	2.0

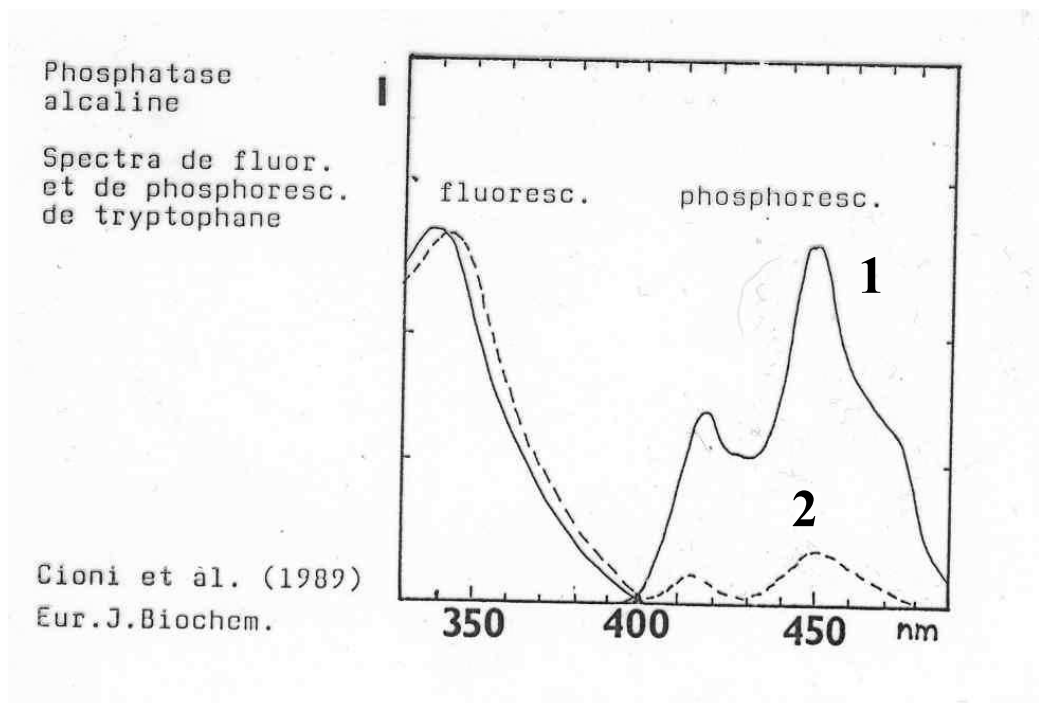
Fosforescence

Fosforescence alkalické fosfatasy

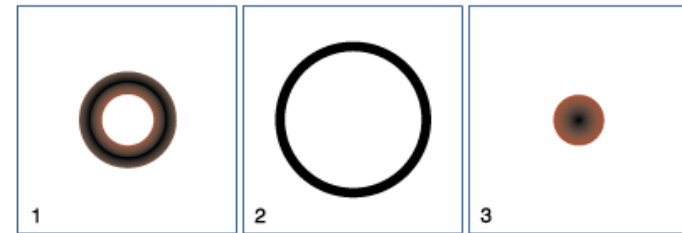
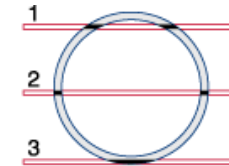
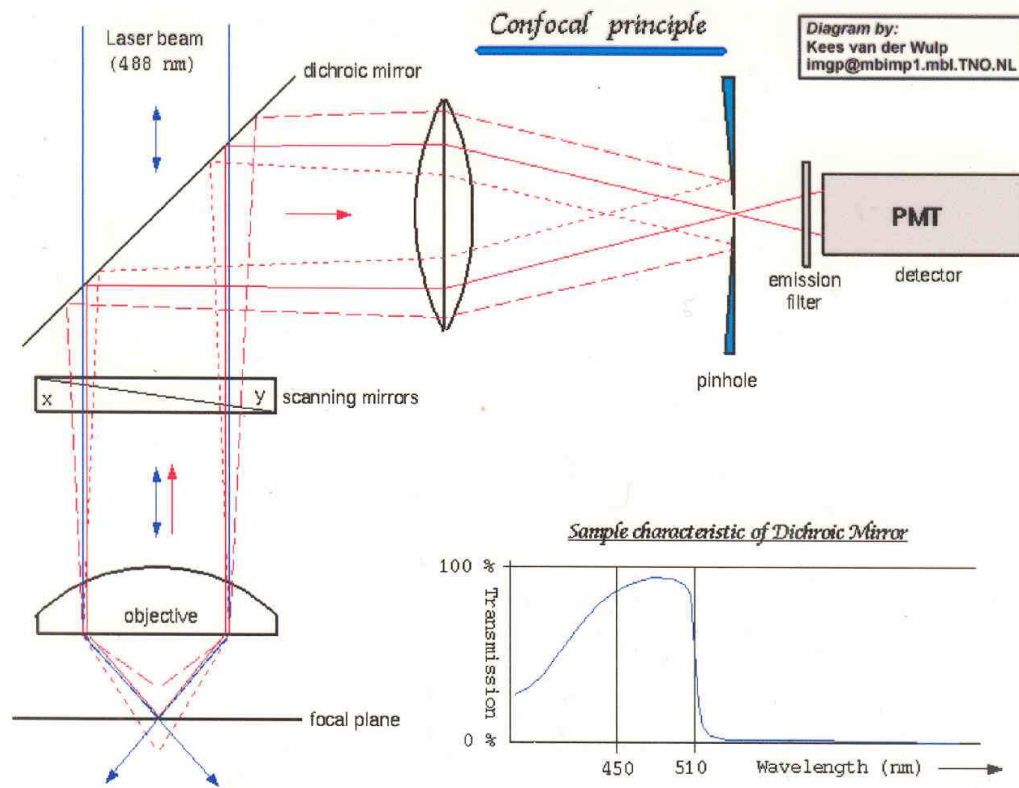
3 Try, pouze Try 109 fosforeskuje

1 – nativní enzym

2 – enzym po odstranění Zn

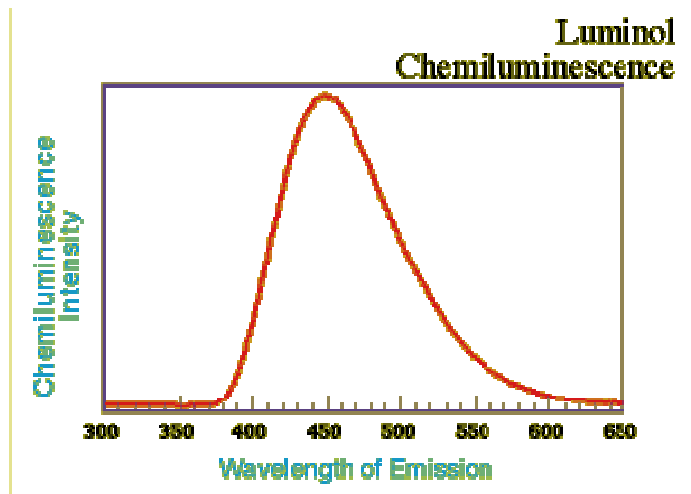
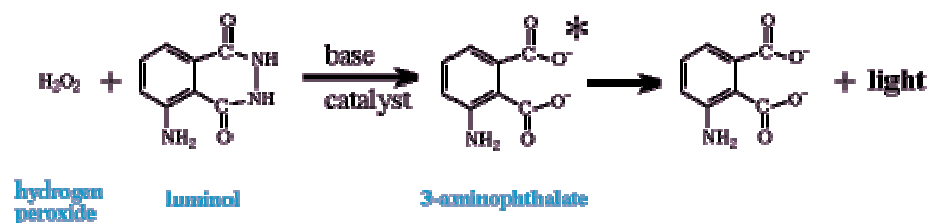


Princip fluorescenční konfokální mikroskopie

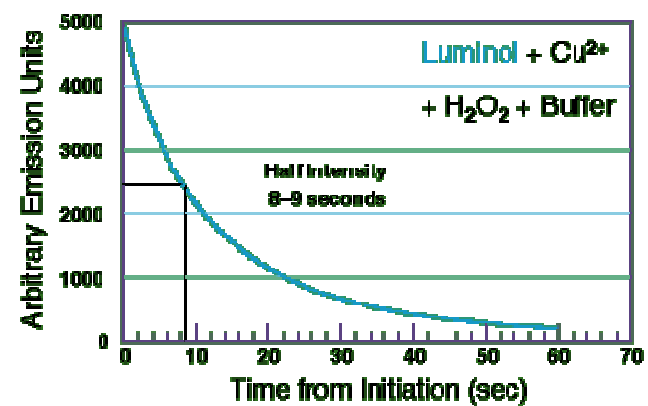


Chemiluminescence

Luminol

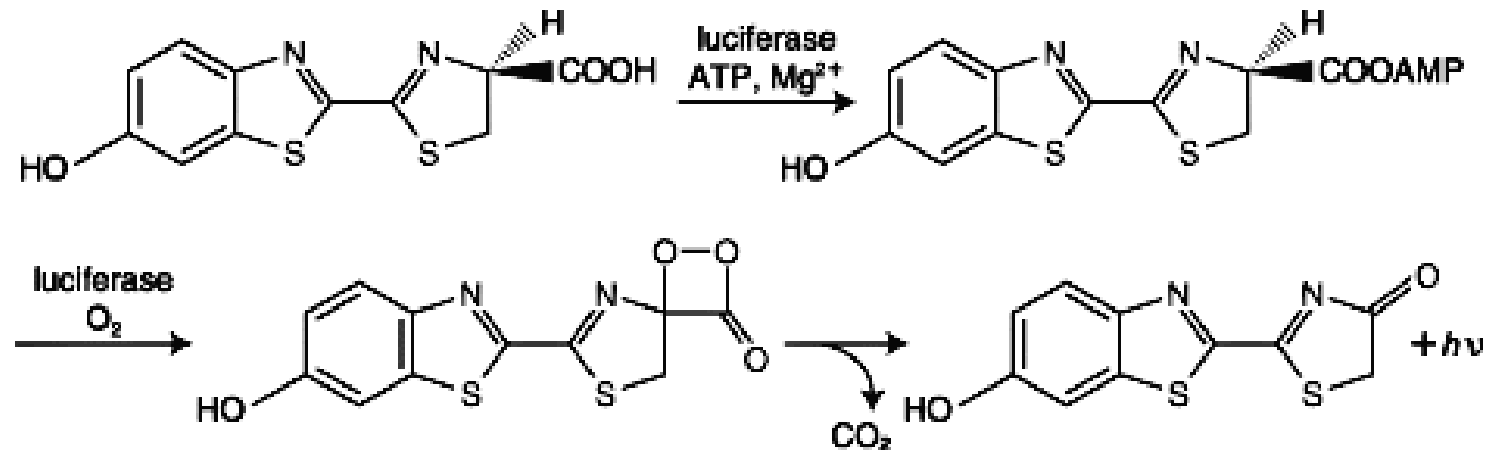


Luminol Emission Time Profile



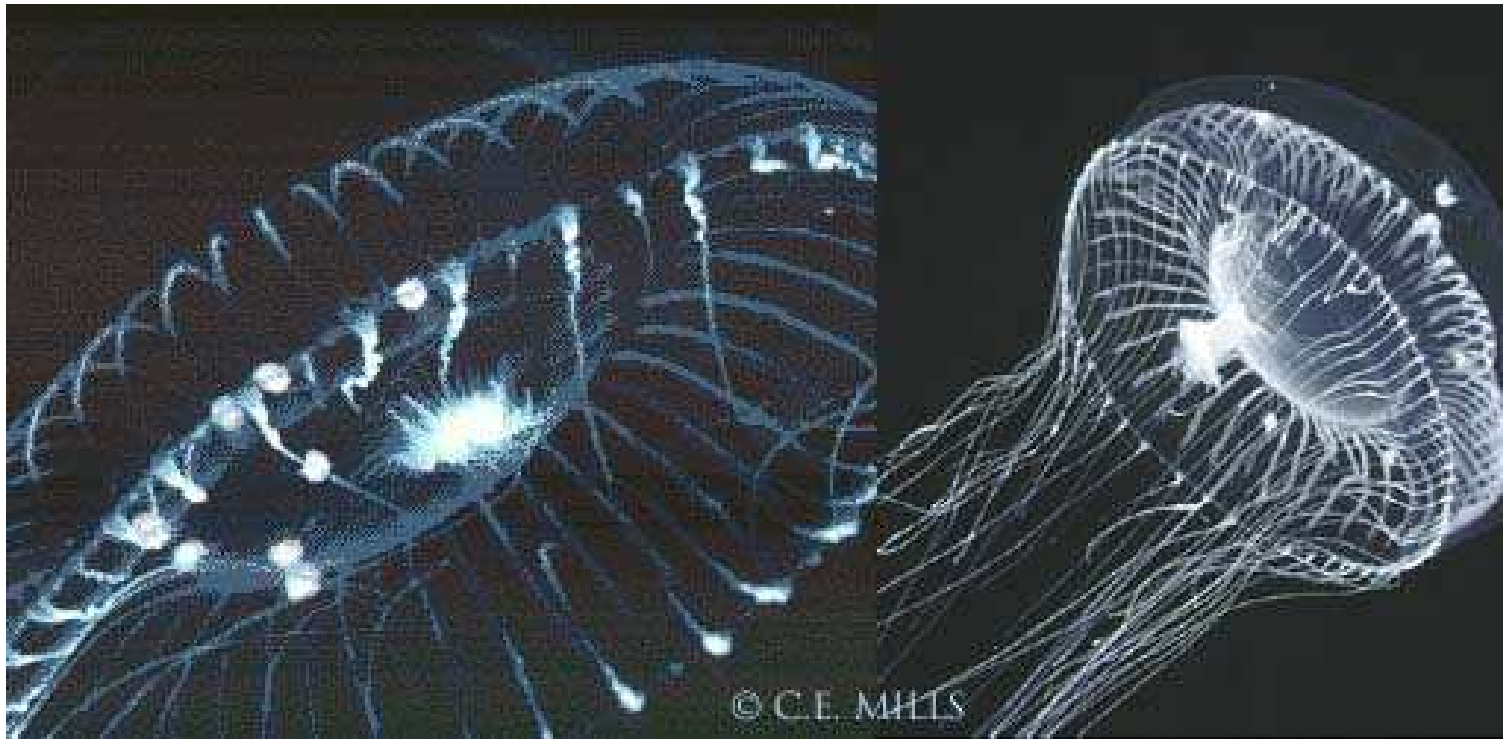
Chemiluminescence

Luciferin



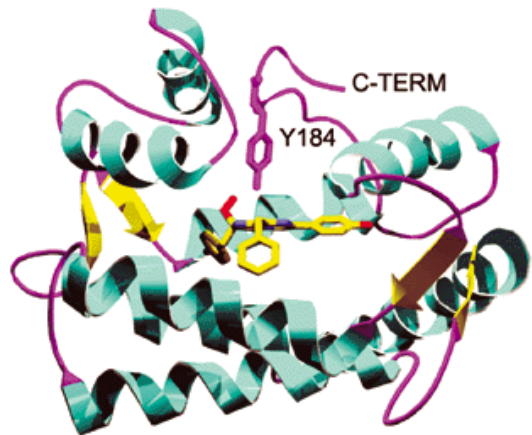
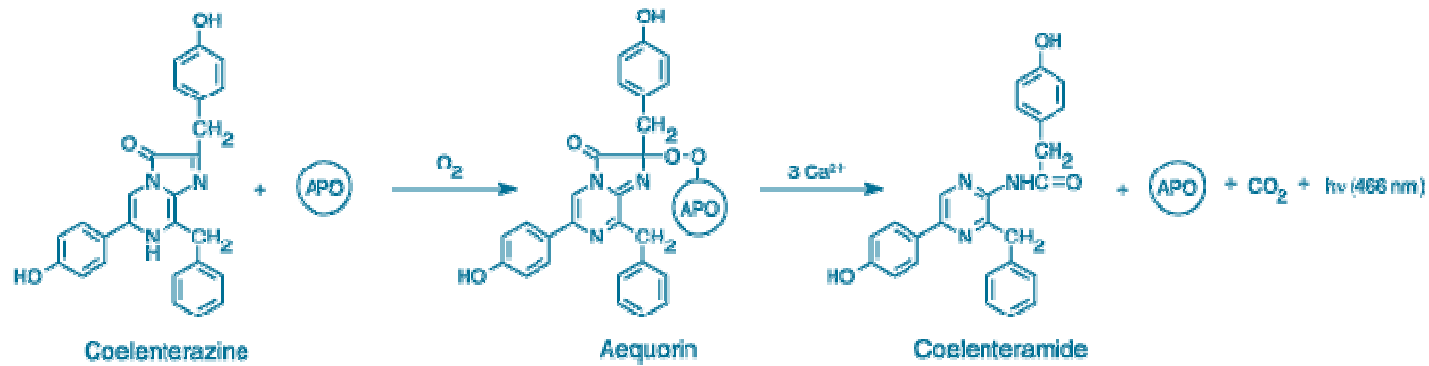
Chemiluminescence

Aequorin – *Aequoria victoria*



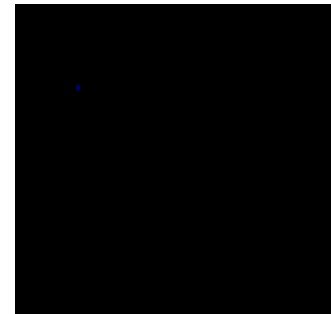
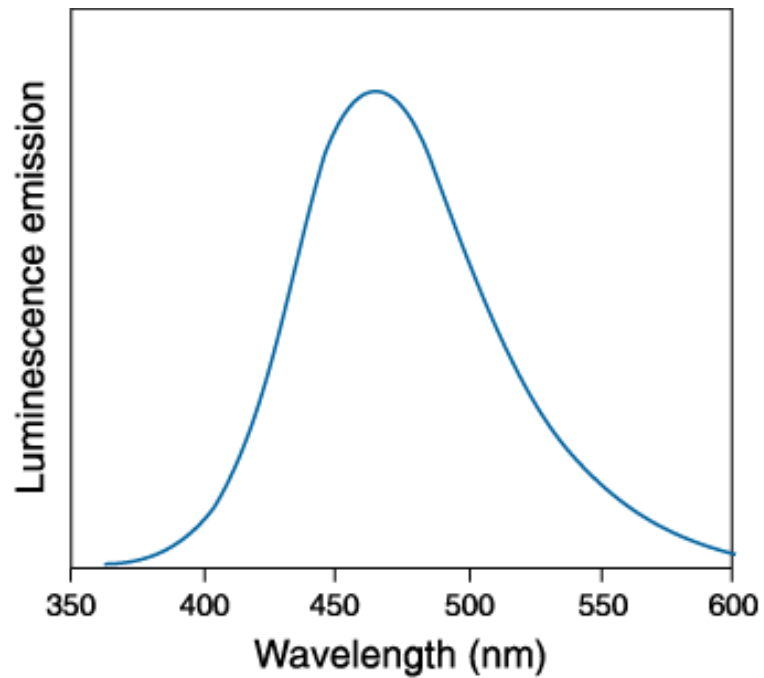
Chemiluminescence

Aequorin – *Aequoria victoria*



Chemiluminescence

Aequorin – *Aequoria victoria*



Průnik vápníku do mitochondrií
Aktivuje oxidaci