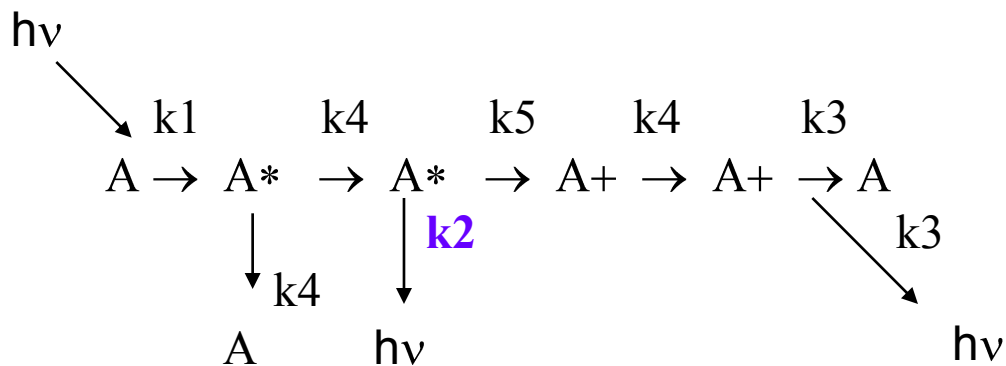
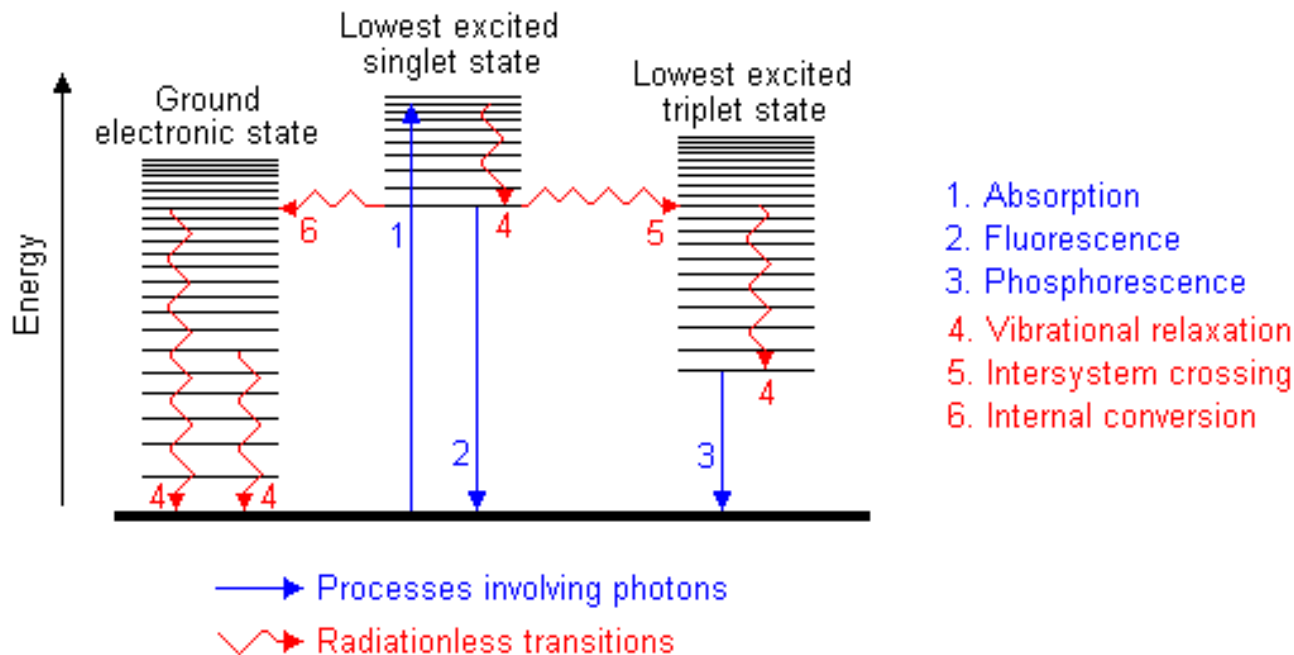
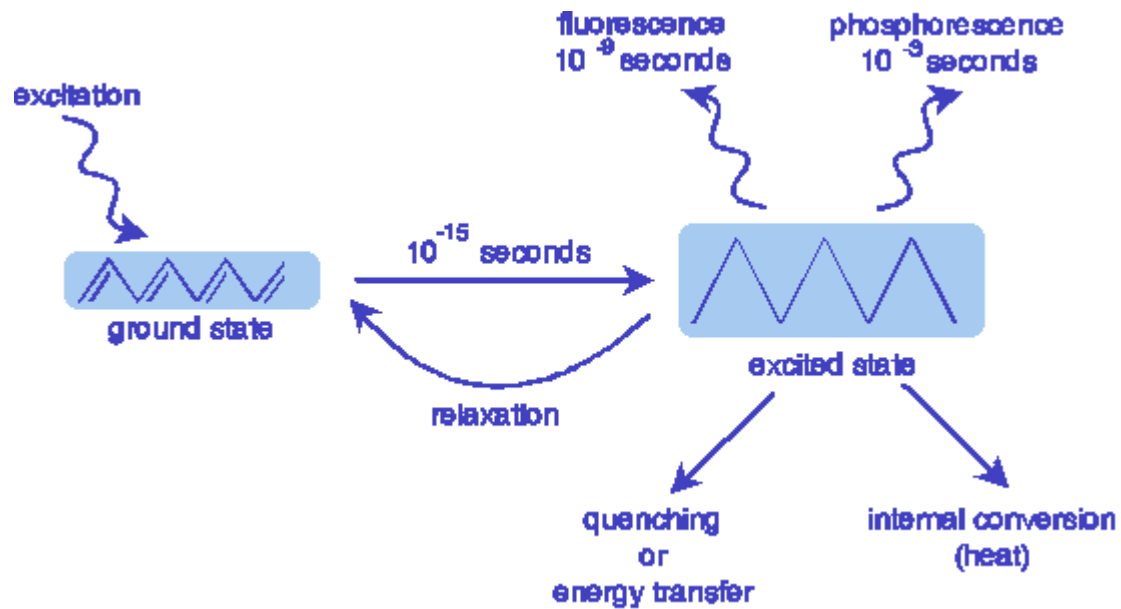


# Luminiscenční spektroskopie



# Luminiscenční spektroskopie



# Luminiscenční spektroskopie

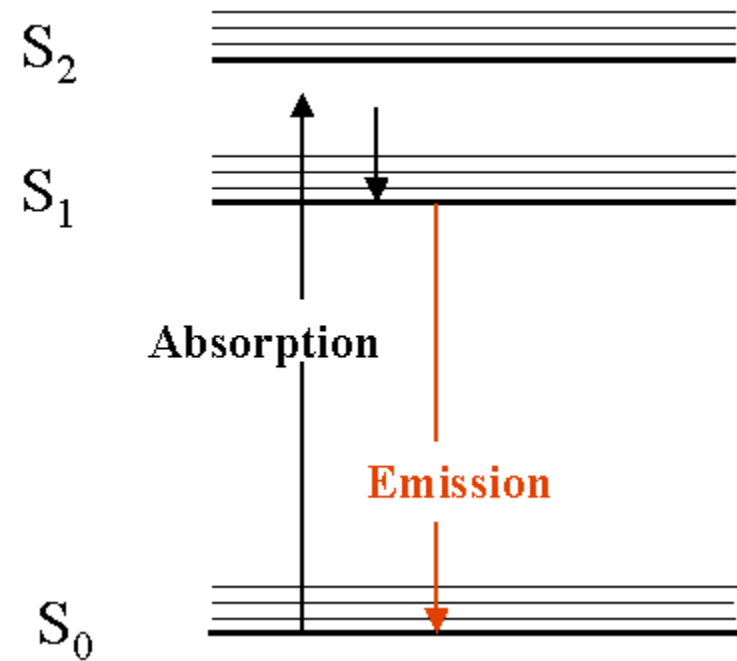
Fluorescenční spektroskopie

Fosforescenční spektroskopie

Chemiluminiscenční spektroskopie

# Základní pojmy

Excitace a emise

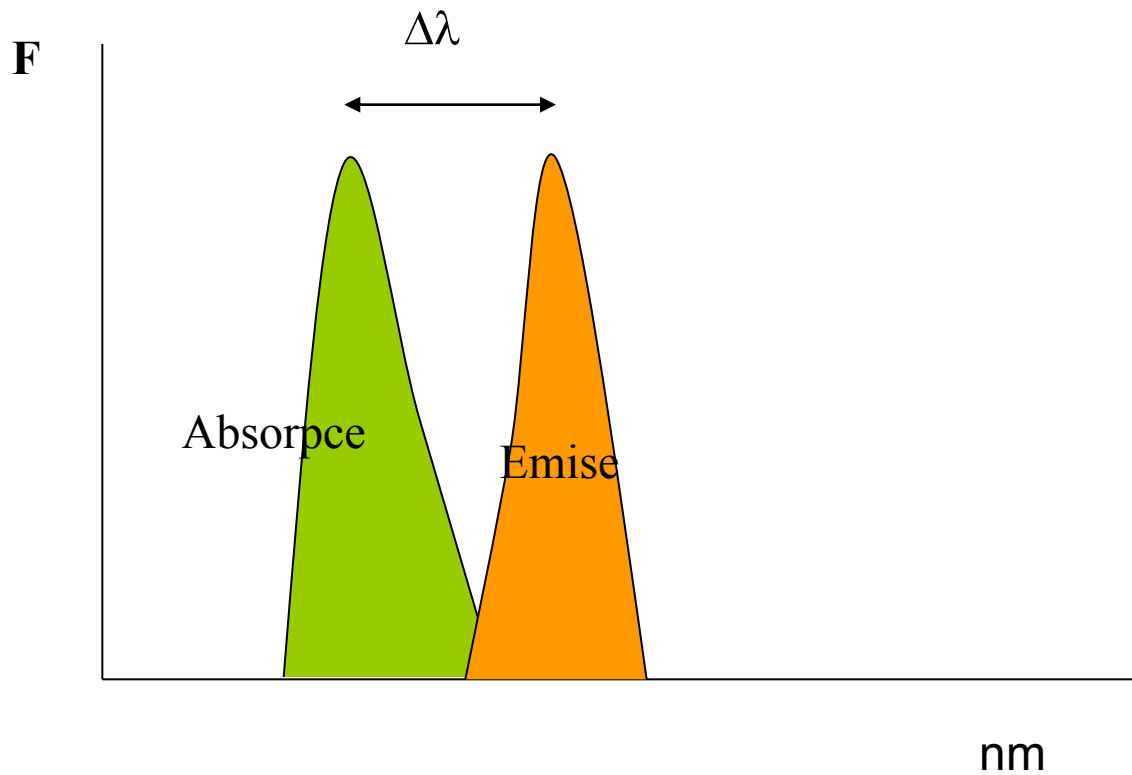


Interakce s rozpouštědlem  
Singletový excitovaný stav

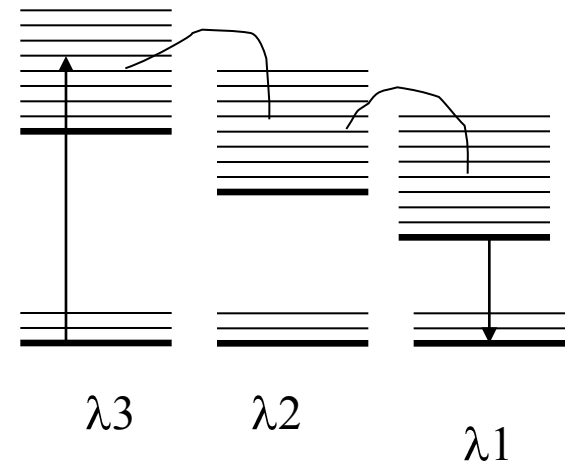
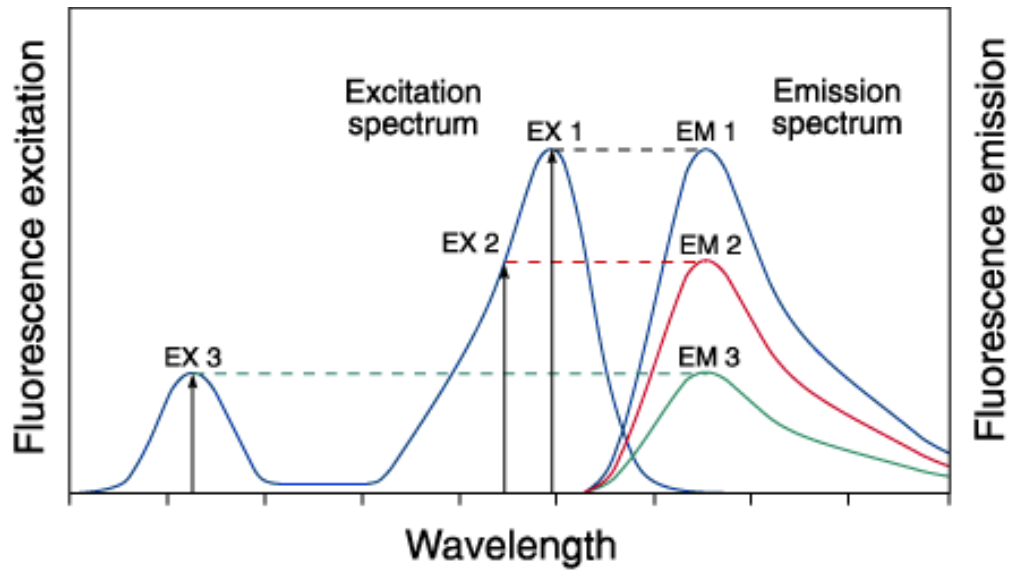
Singletový základní stav

# Základní pojmy

Stokesův posun – ztráty energie po dobu excitovaného stavu

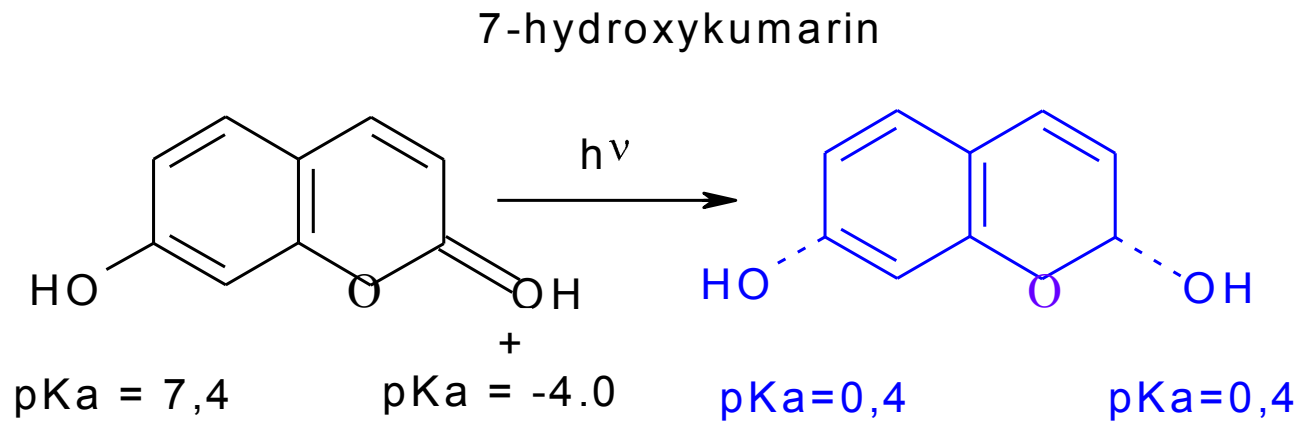


# Základní pojmy



# Základní pojmy

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



# Základní pojmy

Kvantový výtěžek fluorescence

$\Phi$  = 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  $1/e I_0$

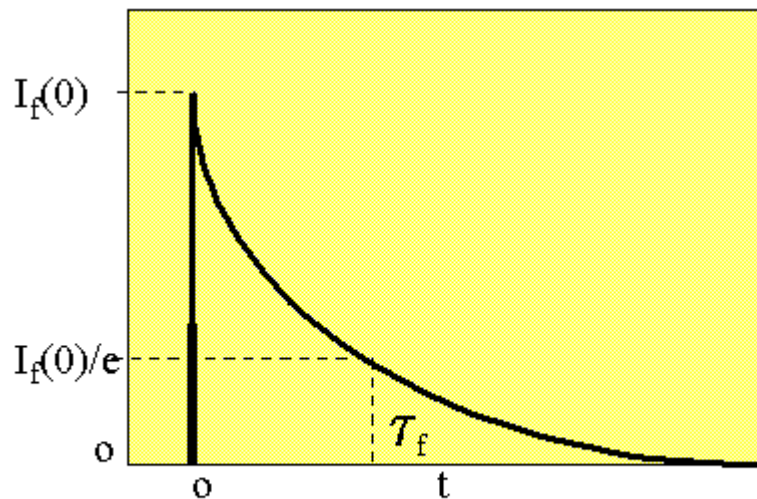
Střední doba života  $\tau$   
 $\tau = 1/k_f$

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

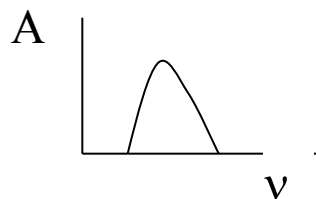
Přirozená doba života  $\tau_0$

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  
 $\epsilon$  - molární abs. Koeficient  
 $\nu$  - vlnčet abs. maxima



# Základní pojmy

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

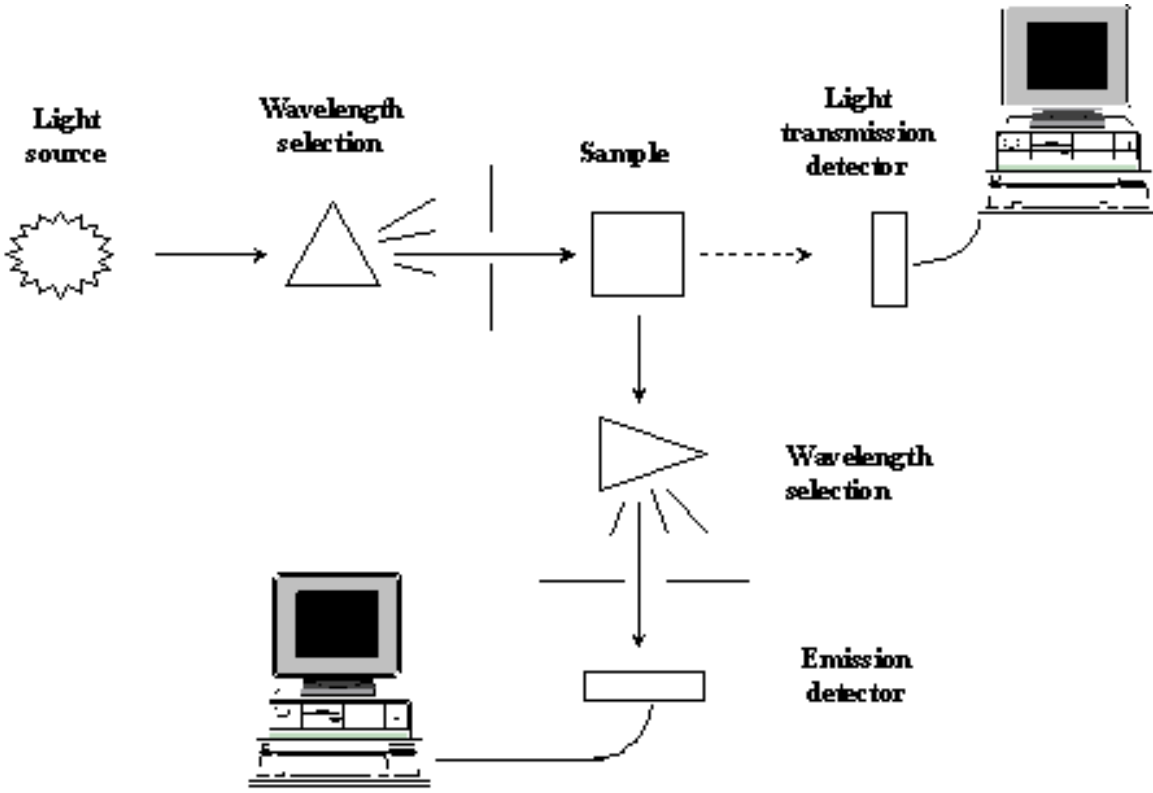
Střední doba života fluorescence

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

# Biochemicky významné fluorofory

|               | $\lambda_{exc}$ | $\lambda_{em}$ | 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 <sub>2</sub> SO <sub>4</sub> ) |
| Pyridoxamine  | 324             | 392            | 0.11 (pH=8.2)                             |
| Vitamine A    | 325             | 470            | - (ethanol)                               |
| Aminobenzoate | 294             | 345            | -   |

# Instrumentace



# Instrumentace

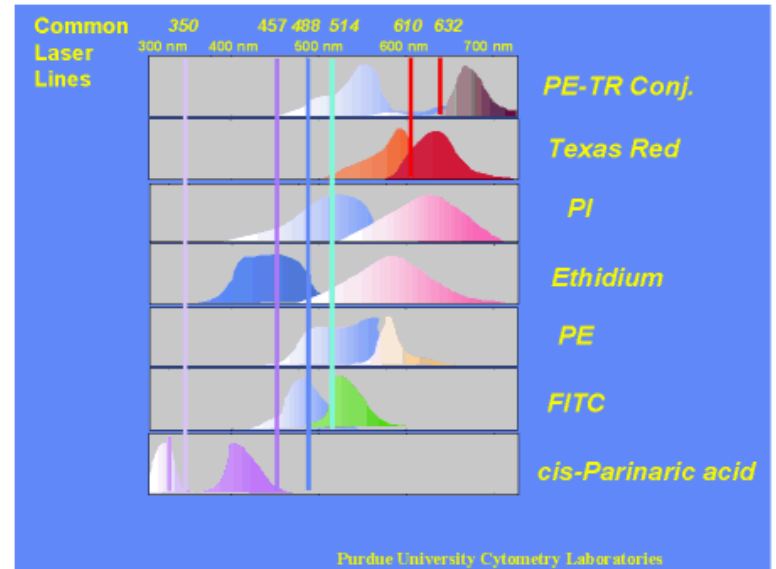
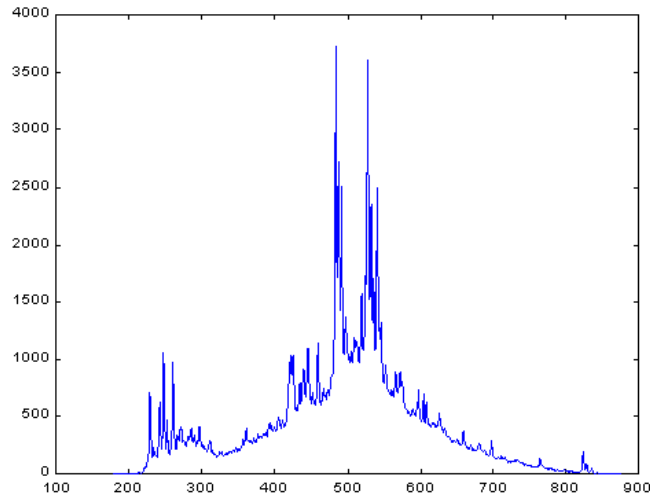
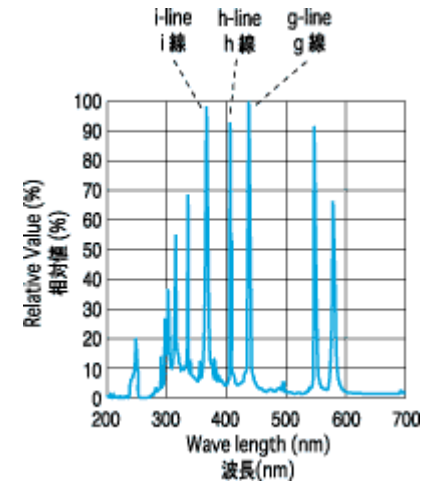
Zdroj:

Xenonová lampa

Rtuťová výbojka

Laser

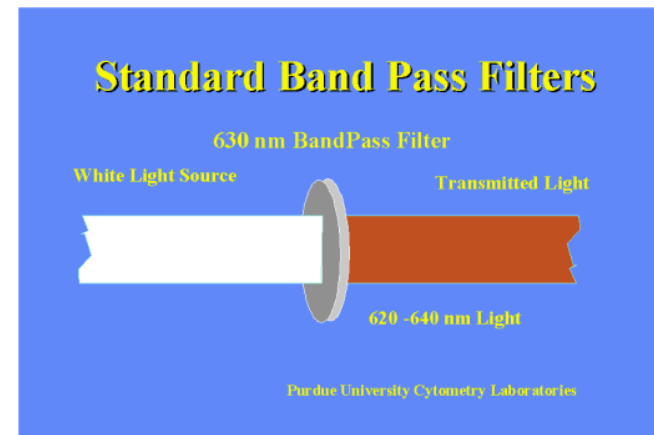
Světelné diody - LED (430, 450, 505, 592, 612 and 637 nm)



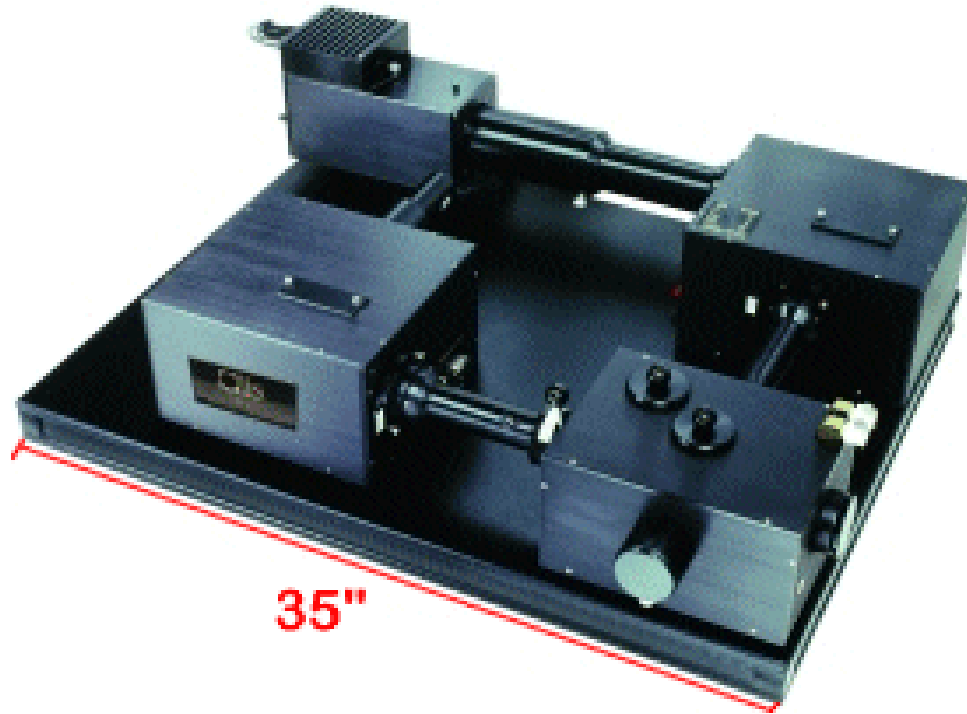
# Instrumentace

Monochromátor

- mřížka
- filtry

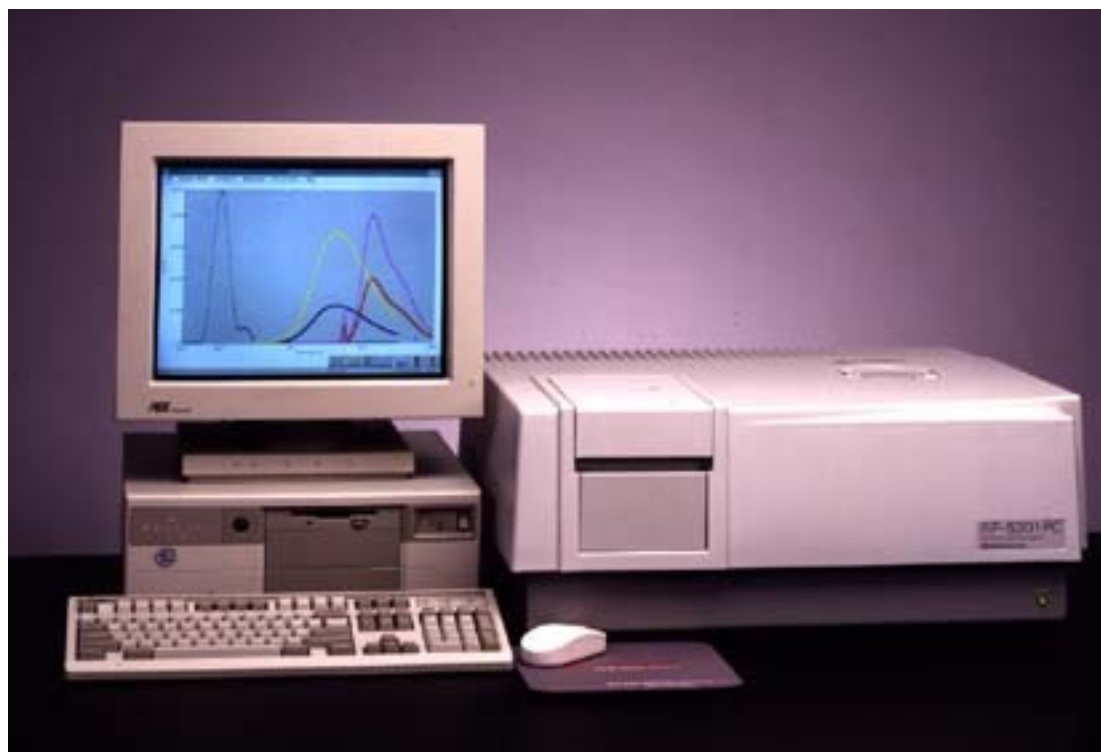


# Instrumentace



# Instrumentace

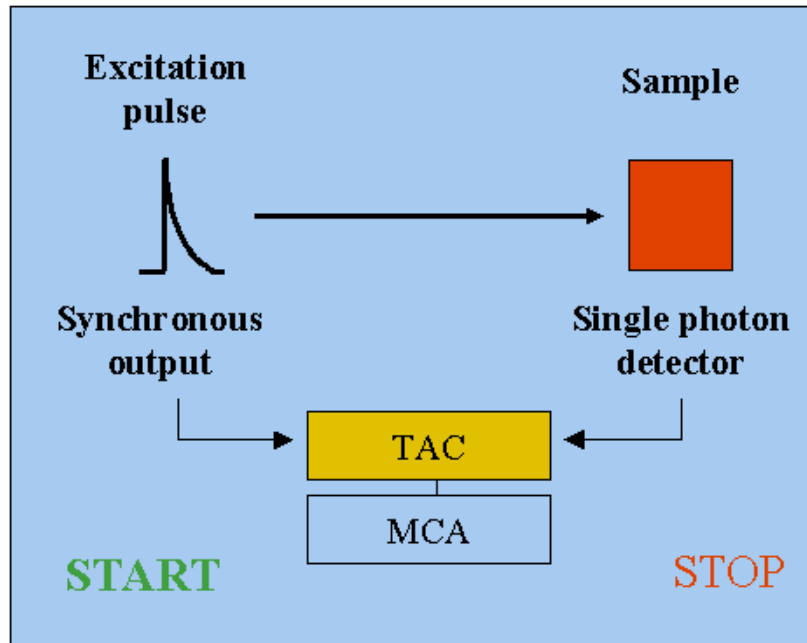
## RF-5301PC – spektrofluorimetr Shimadzu





# Instrumentace

Měření střední doby života fluorescence



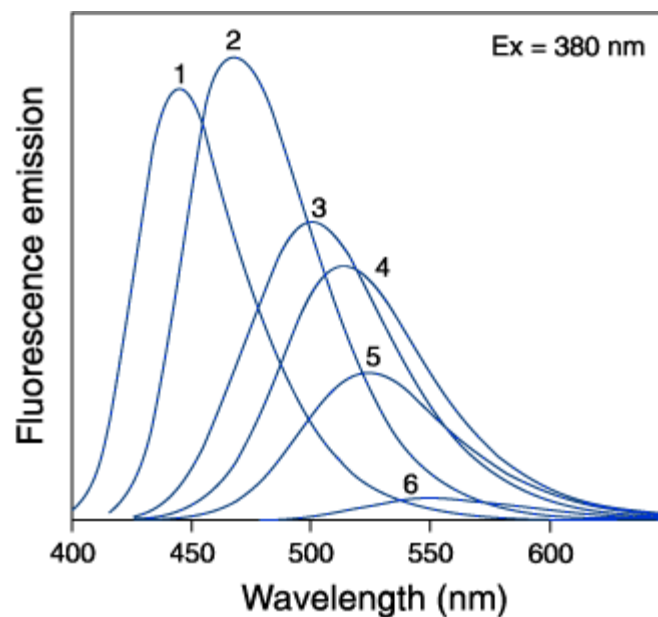
# Podmínky fluorescence

Závislost na polaritě a viskozitě

Nitrobenzoxadiazol

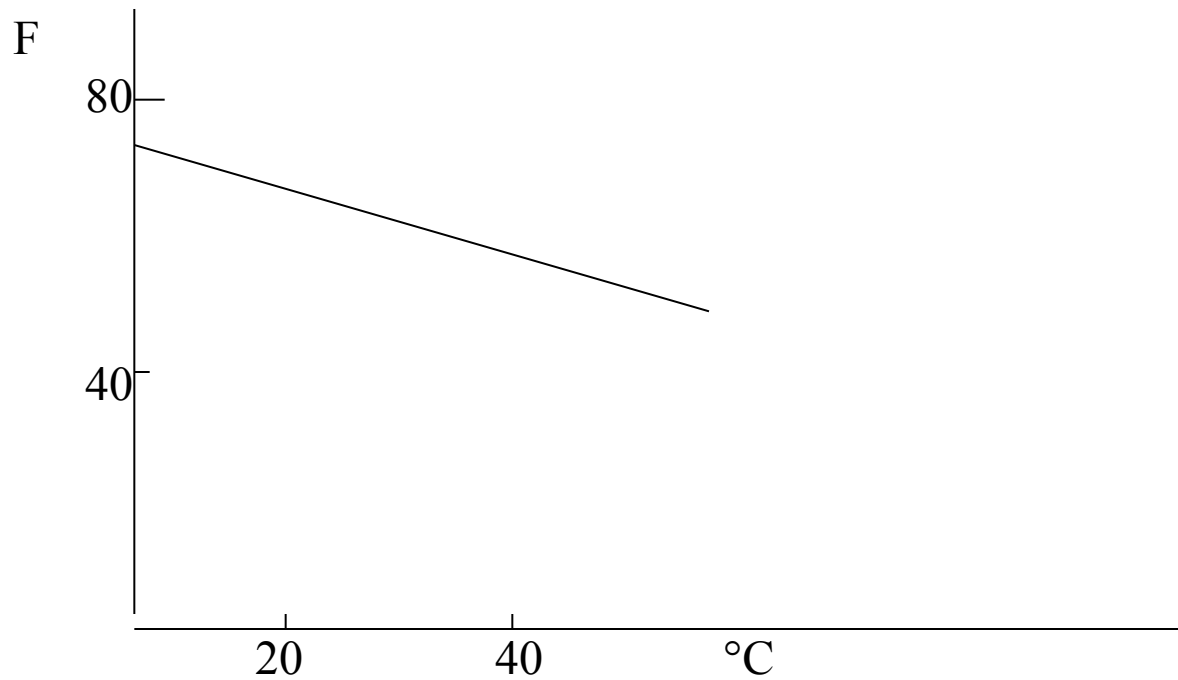
| Solvent          | Freq. domain (ns) | TCSPC (ns) | Literature (ns) |
|------------------|-------------------|------------|-----------------|
| H <sub>2</sub> O | 0.92              | 0.97       | 0.93            |
| Methanol         | 5.35              | 5.31       | 5.64            |
| DMSO             | 7.15              | 7.54       | 7.48            |
| Ethyl acetate    | 10.93             | nd         | 10.5            |

Pokles polarity 6 – 1.



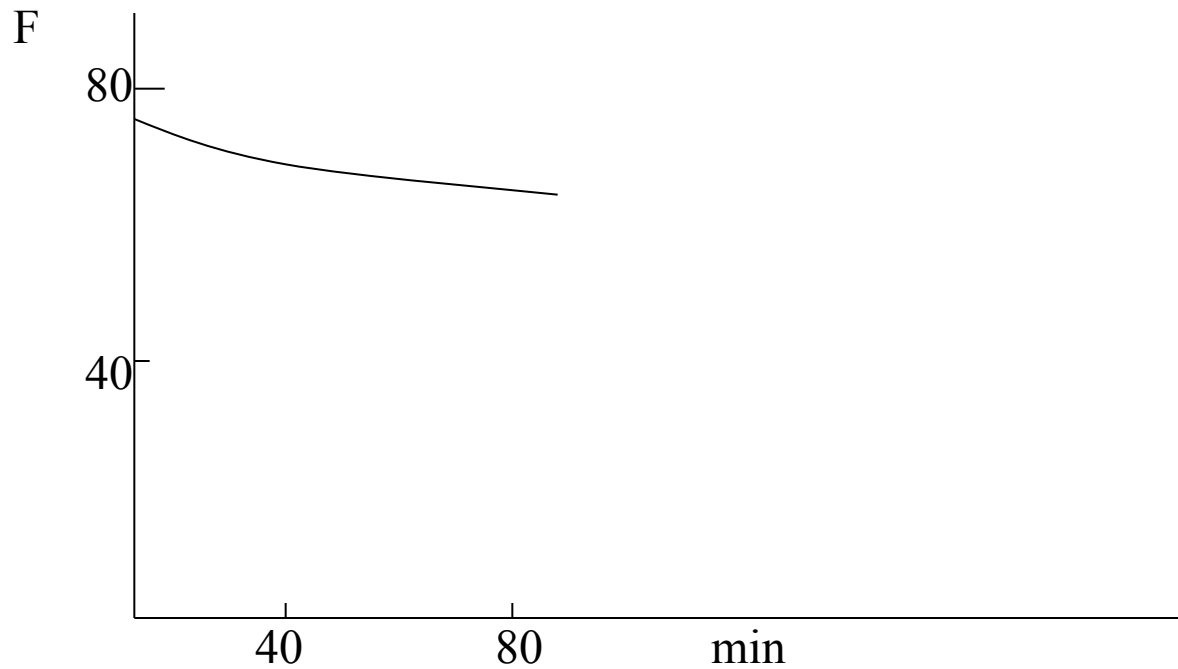
# Podmínky fluorescence

Závislost fluorescence na teplotě

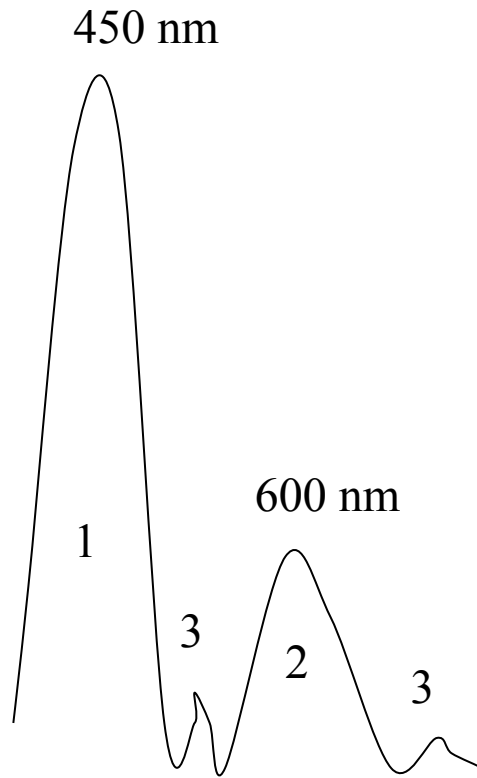


# Podmínky fluorescence

Stabilita fluorescenčního signálu  
chininsulfátu



# Podmínky fluorescence



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

Excitace 450 nm

Emisní spektrum

# Kvantitativní fluorimetrie

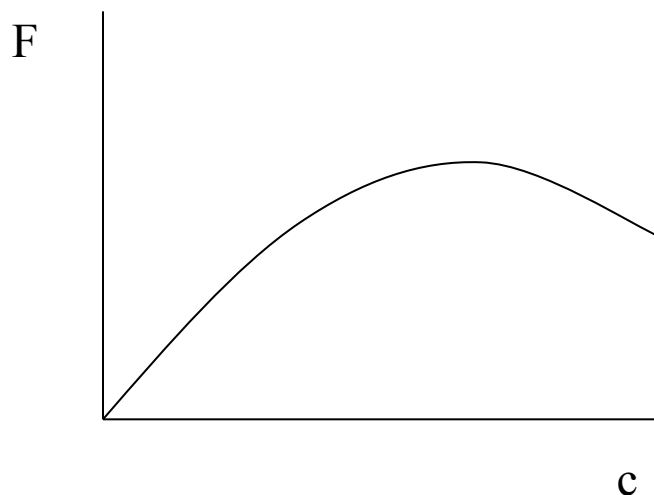
Závislost intenzity fluorescence na koncentraci látky

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

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

jestliže  $c \rightarrow 0$

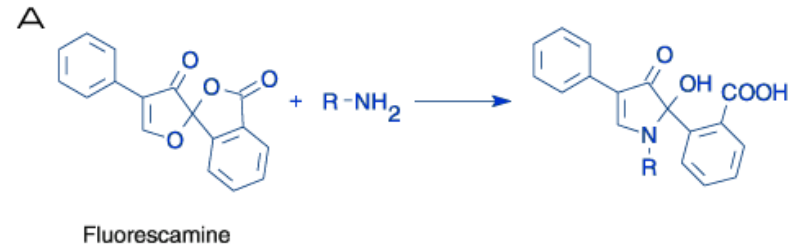
$$F = I_0 \Phi \cdot 2,3 \cdot \epsilon d \cdot c$$



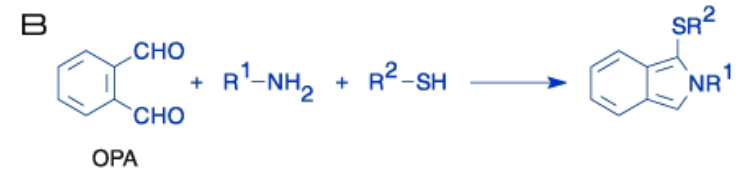
# Kvantitativní fluorimetrie

Stanovení koncentrace aminokyselin

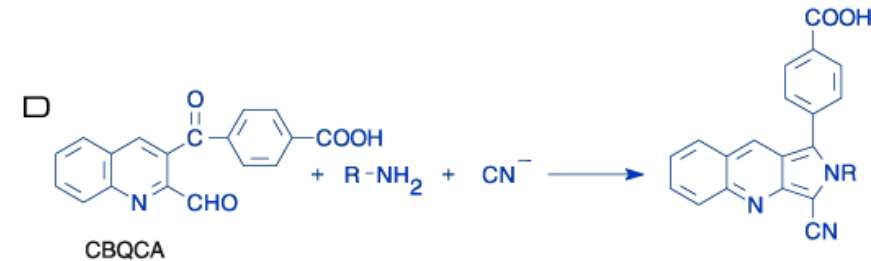
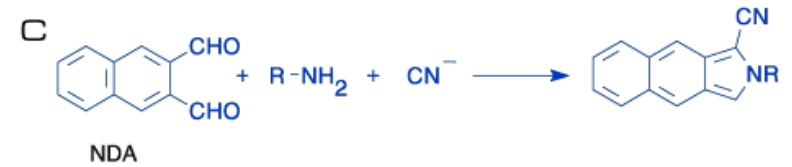
390/464 nm



340/455 nm



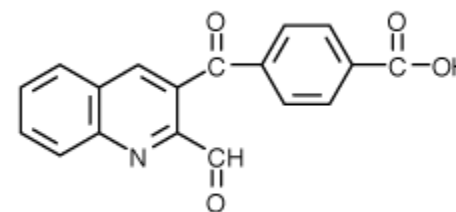
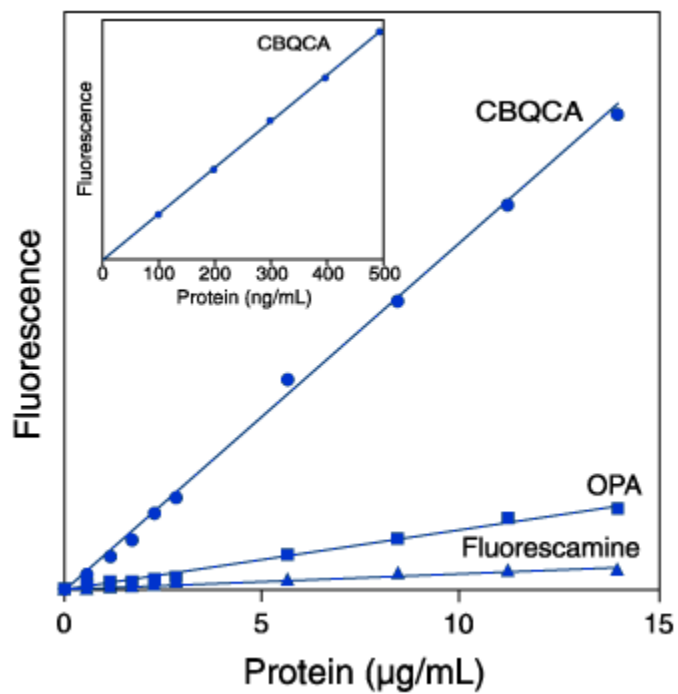
450/550 nm



# Kvantitativní fluorimetrie

Stanovení bílkovin

CBCQA





# Kvantitativní fluorimetrie

Detekce bílkovin v gelu

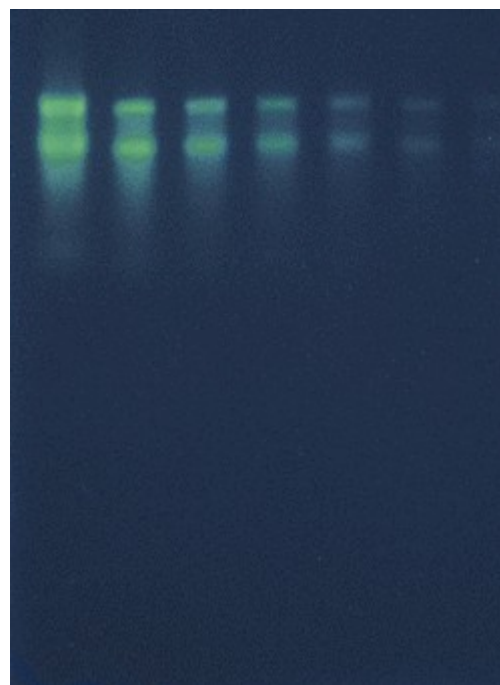
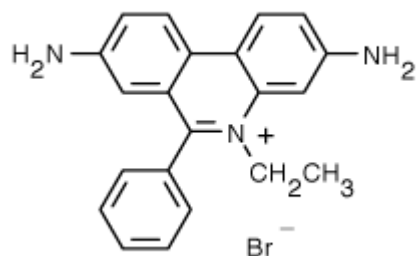
(barevně: Coomasie blue, stříbrné barvení)

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

# Kvantitativní fluorimetrie

Detekce nukleových kyselin

Ethidium bromid

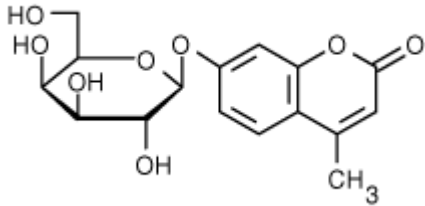


rRNA 16 a 23s barvená SYBR Green II  
Molecular Probes

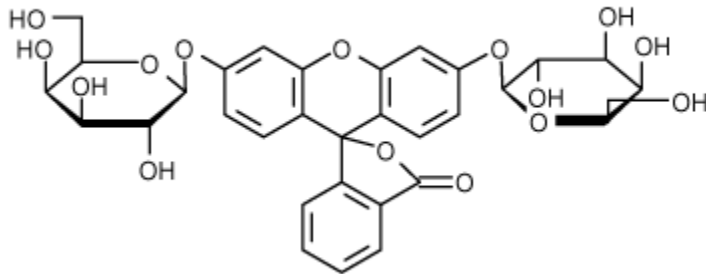
# Fluorogenní substráty

## Galaktosidasy

4-methylumbelliferyl- $\alpha$ -galaktosid

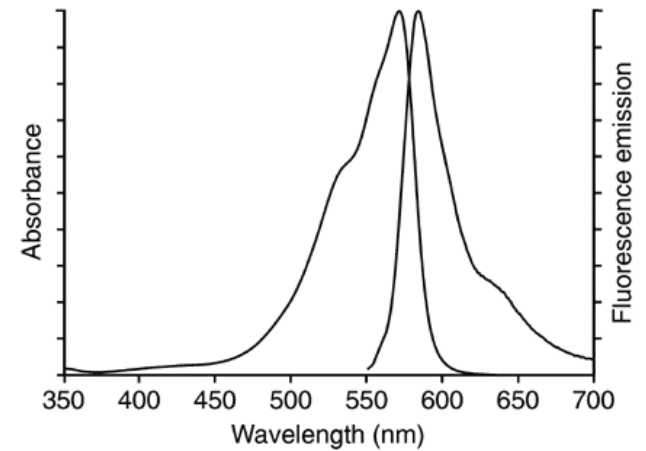
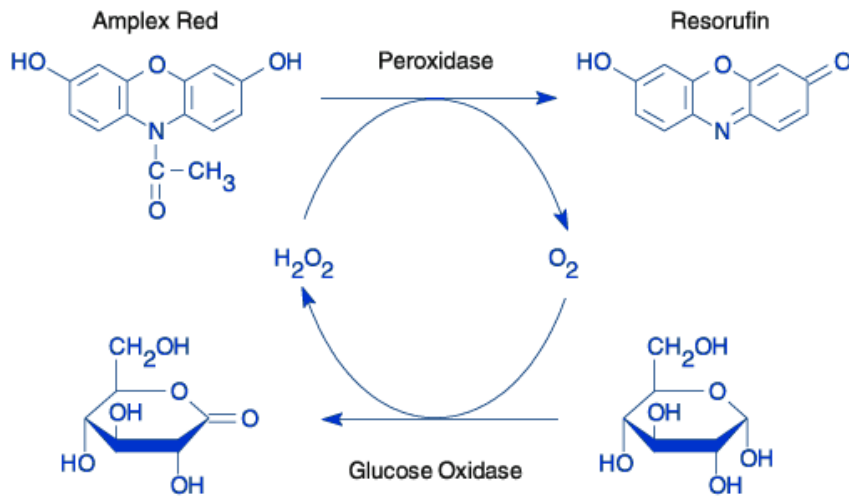


Fluorescein-digalaktosid



# Fluorogenní substráty

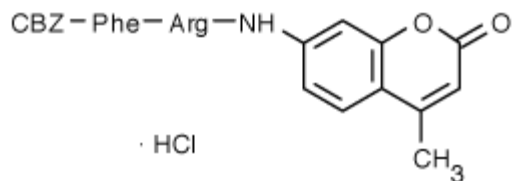
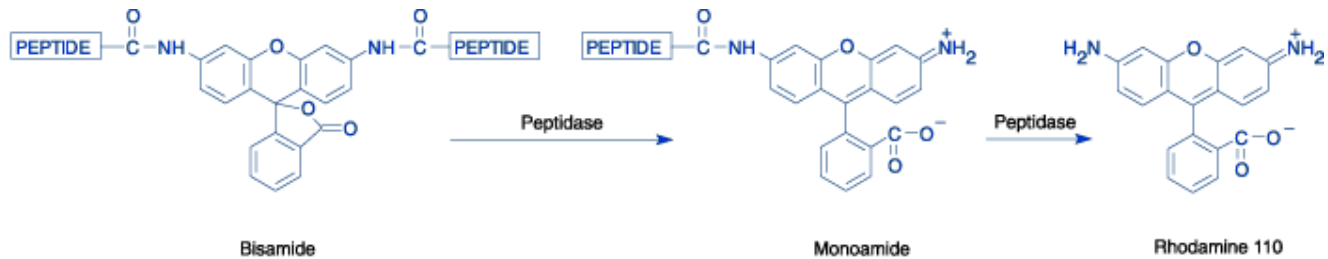
Peroxidasy – amplex red, vznik resorufinu



# Fluorogenní substráty

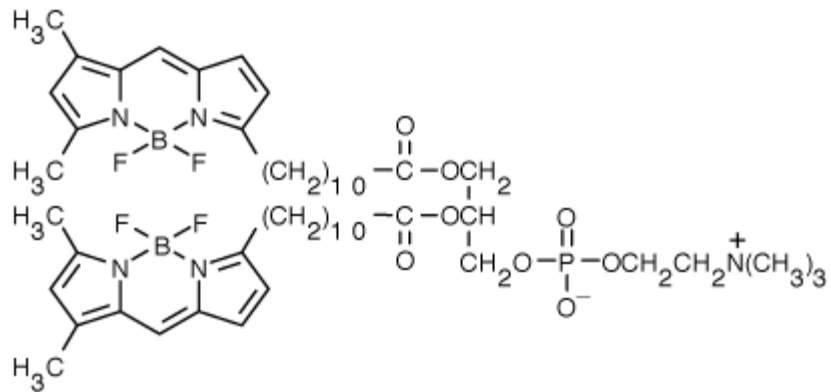
## Proteinasy, peptidasy

### 1) Fluorescenční konjugáty proteinů a peptidů



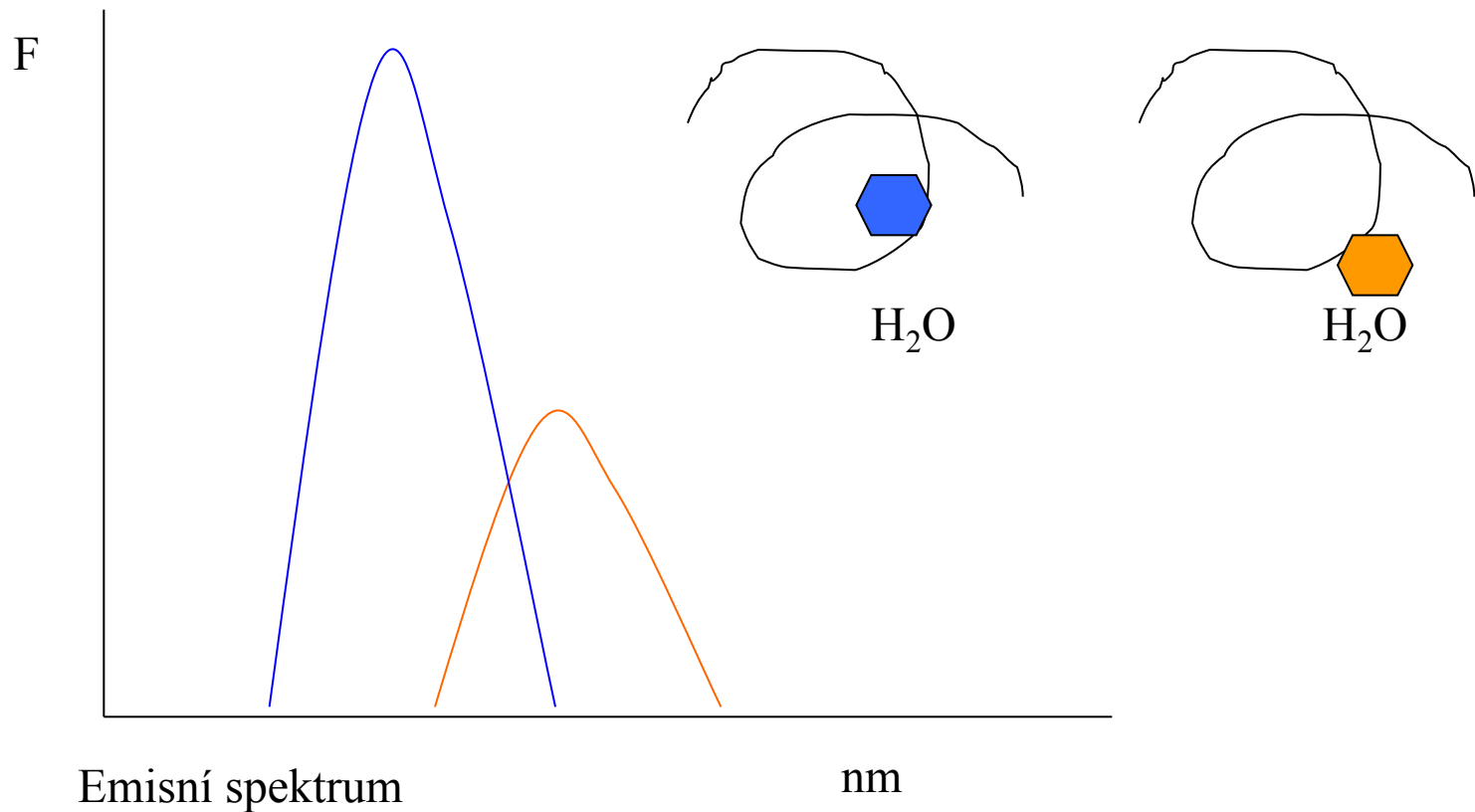
# Fluorogenní substráty

## Fosfolipasa A

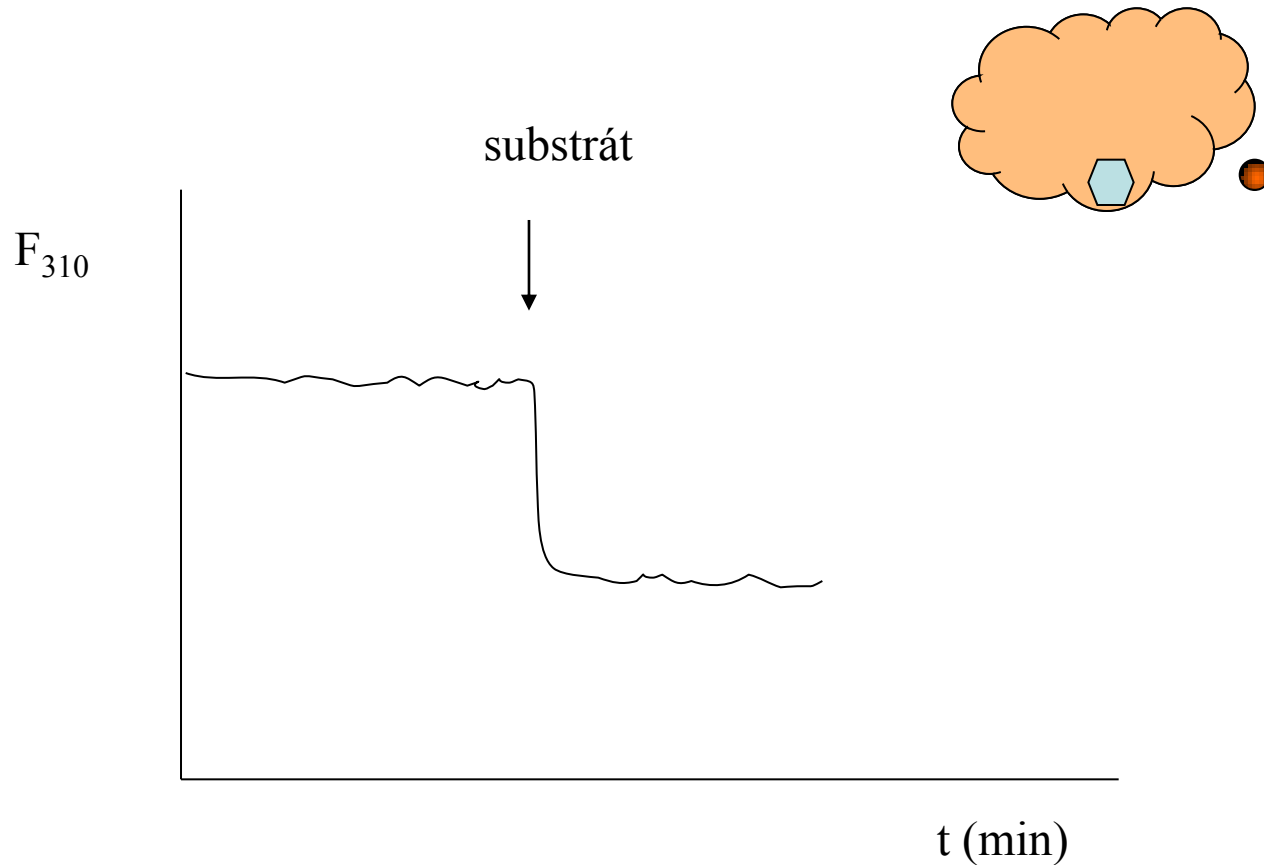


# Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

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



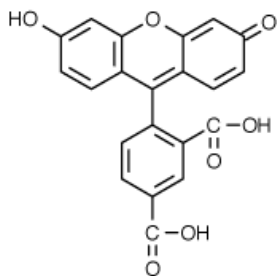
# Použití fluorimetrie ke sledování struktury biopolymerů a interakcí



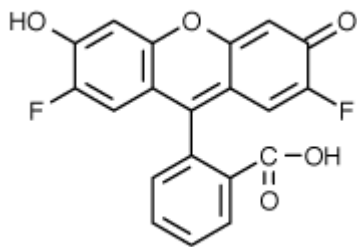


# Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

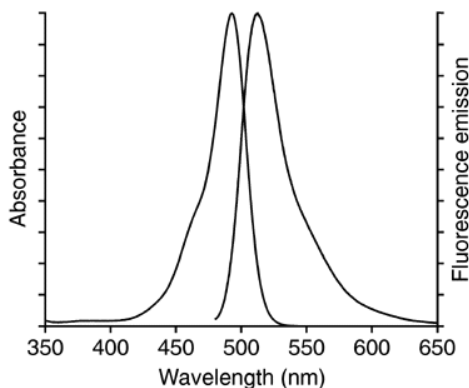
Fluorescenční konjugáty



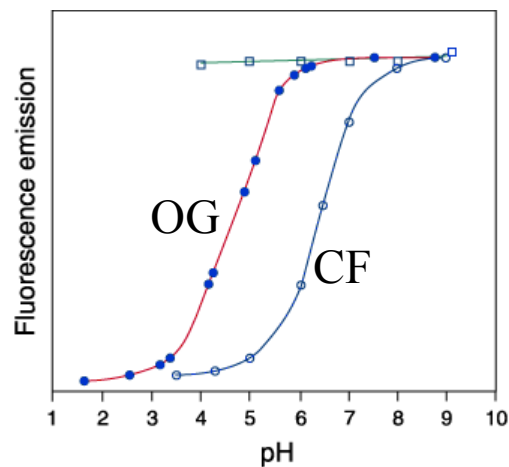
Karboxyfluorescein –  
(494/520 nm)



Oregon Green - (496/524 nm)



Absorpce/emise  
fluoresceinu  
při pH 9

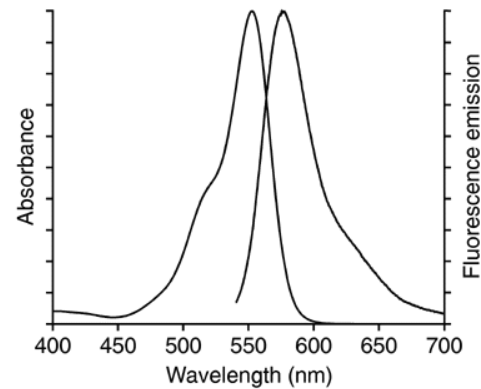
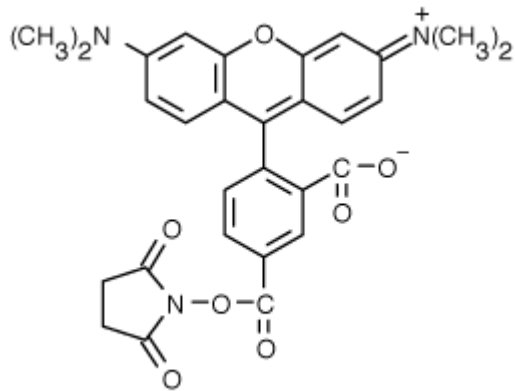


CF-karboxyfluorescein  
OG oregon green

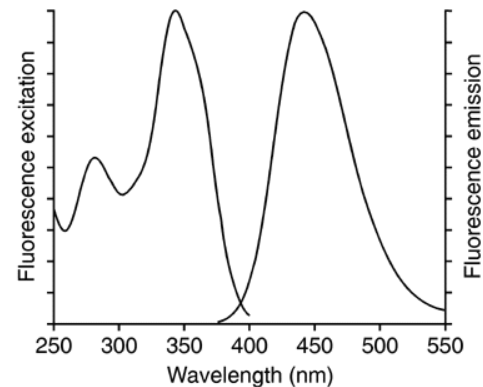
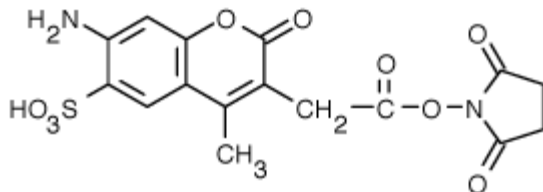
# Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

## Fluorescenční konjugáty

Teramethylrhodamin – 545/580 nm)

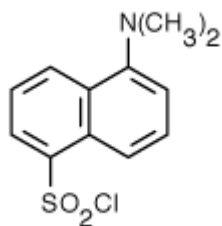


Kumariny – 350/450 nm)

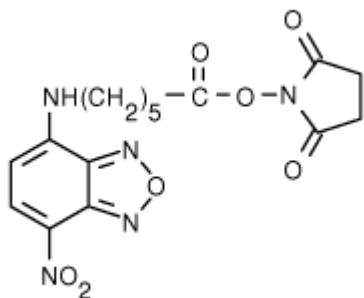
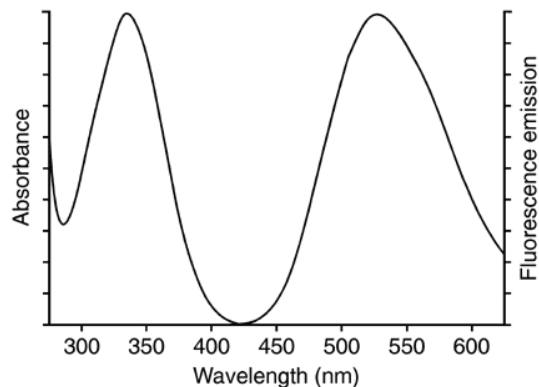


# Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

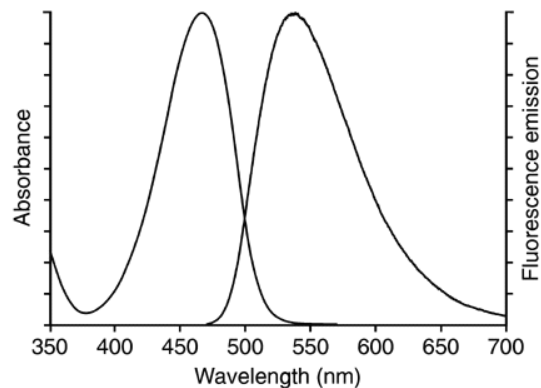
## Fluorescenční konjugáty



Dansyl



Benzoxadiazol –N-sukcinimid



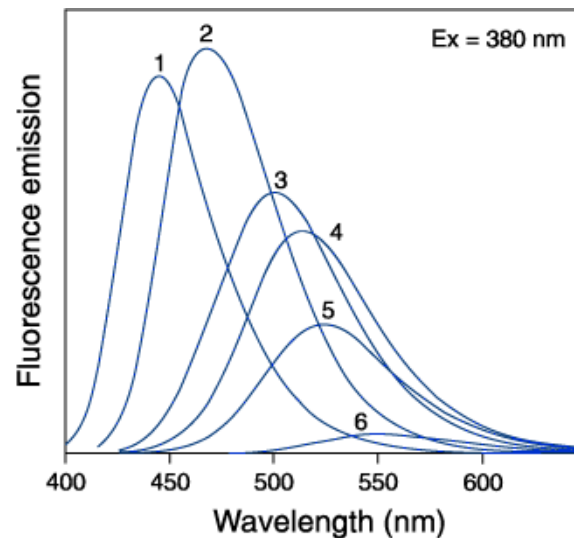
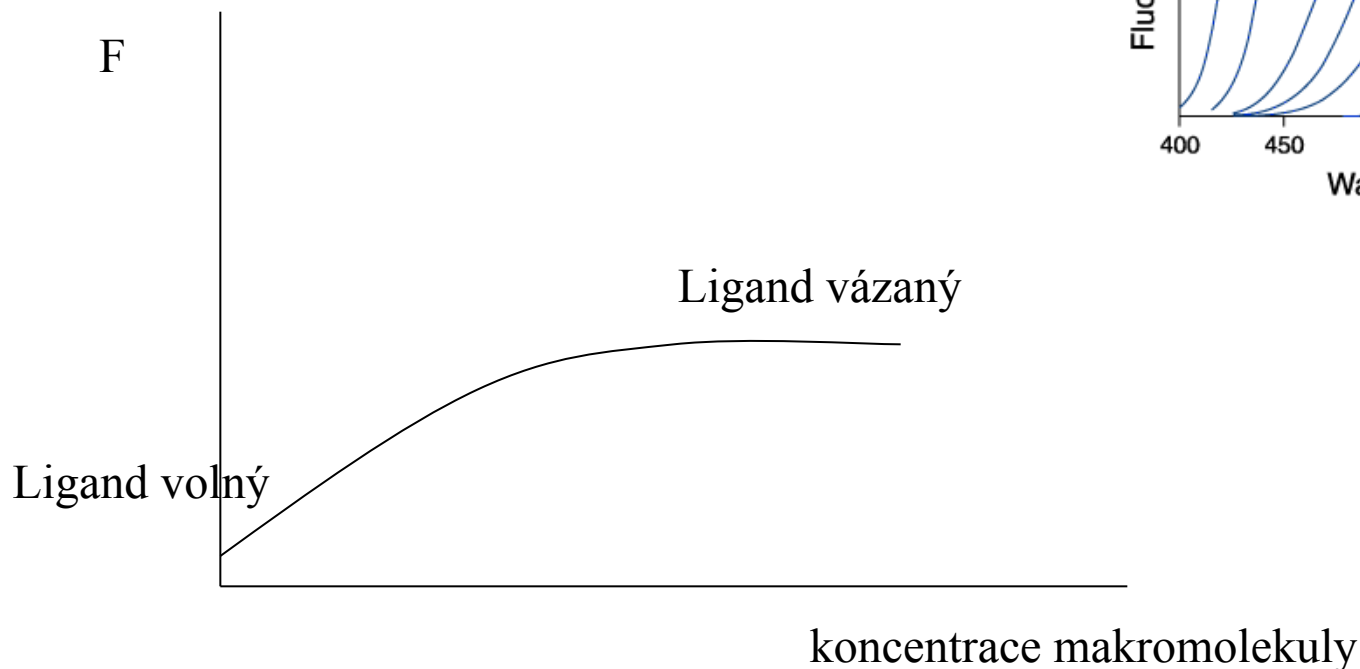


# Použití fluorimetrie ke sledování struktury biopolymerů a interakcí



$$K_d = L_f \cdot M_f / LM$$

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

$\Phi_b, \Phi_f$  – kvant. Výtěžek fluorescence vázaného, volného ligandu

$C_b, C_f$  – koncentrace vázaného, volného Ligandu

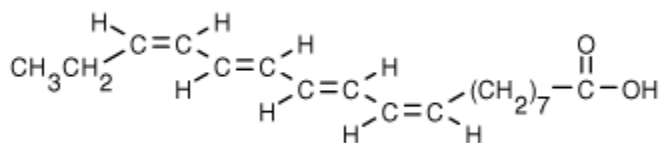
$C$  – celková koncentrace ligandu

$F$  – celková fluorescence

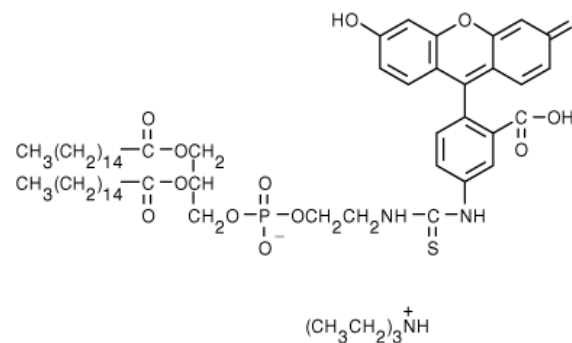
# Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Interakce makromolekul s ligandy

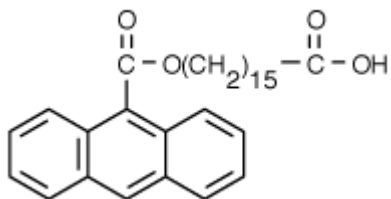
Použití fluorescenčních analogů



Kys. cis-parinarová



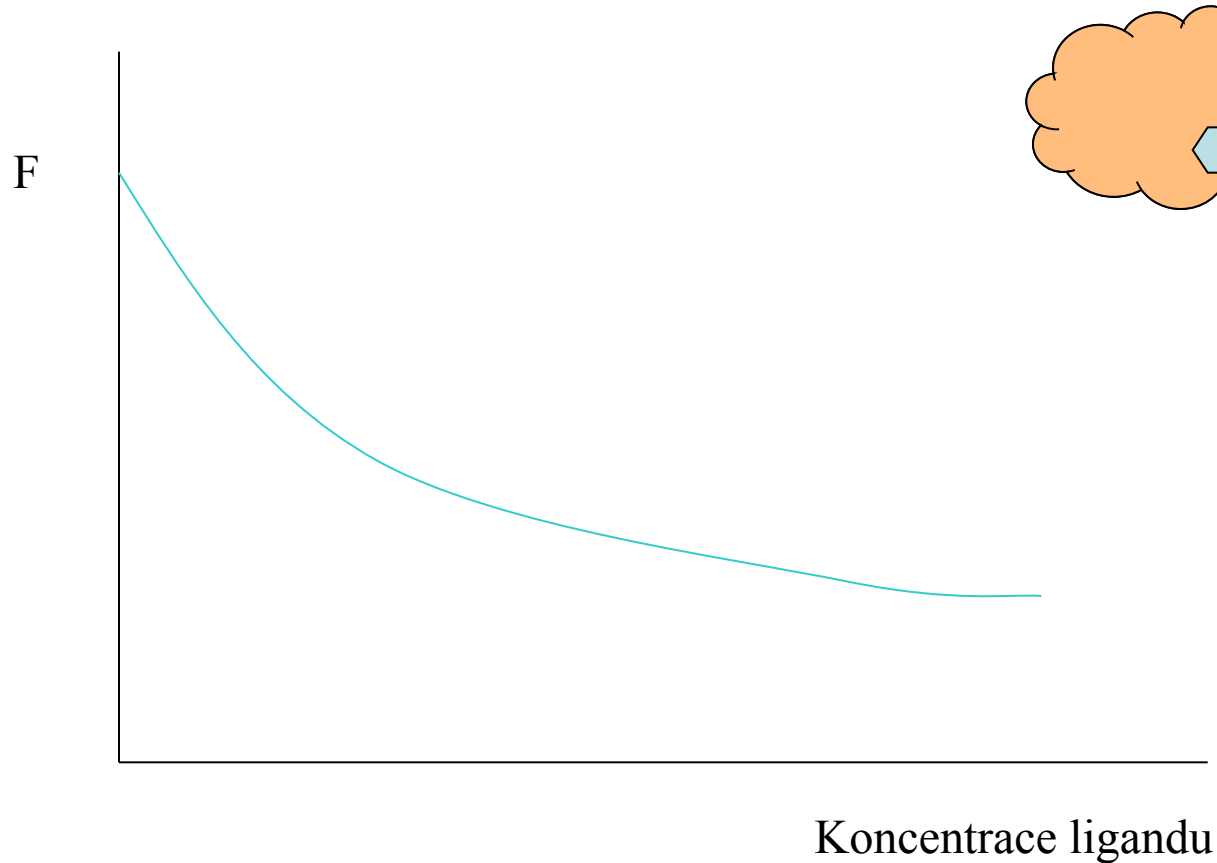
Fluorescein-PE



Anthroyloxypalmitát

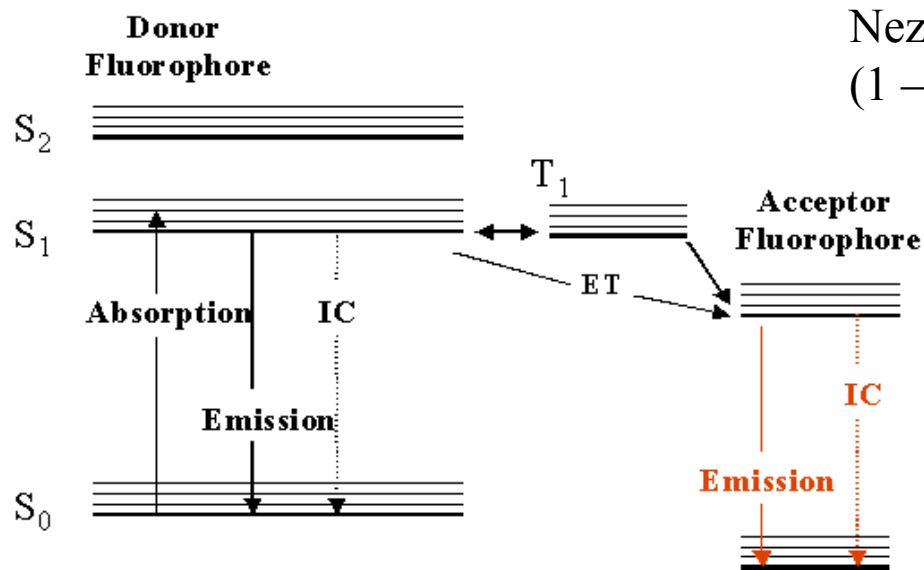
# Použití fluorimetrie ke sledování struktury biopolymerů a interakcí

Použití značené makromolekuly  
Fluorescence značené bílkoviny



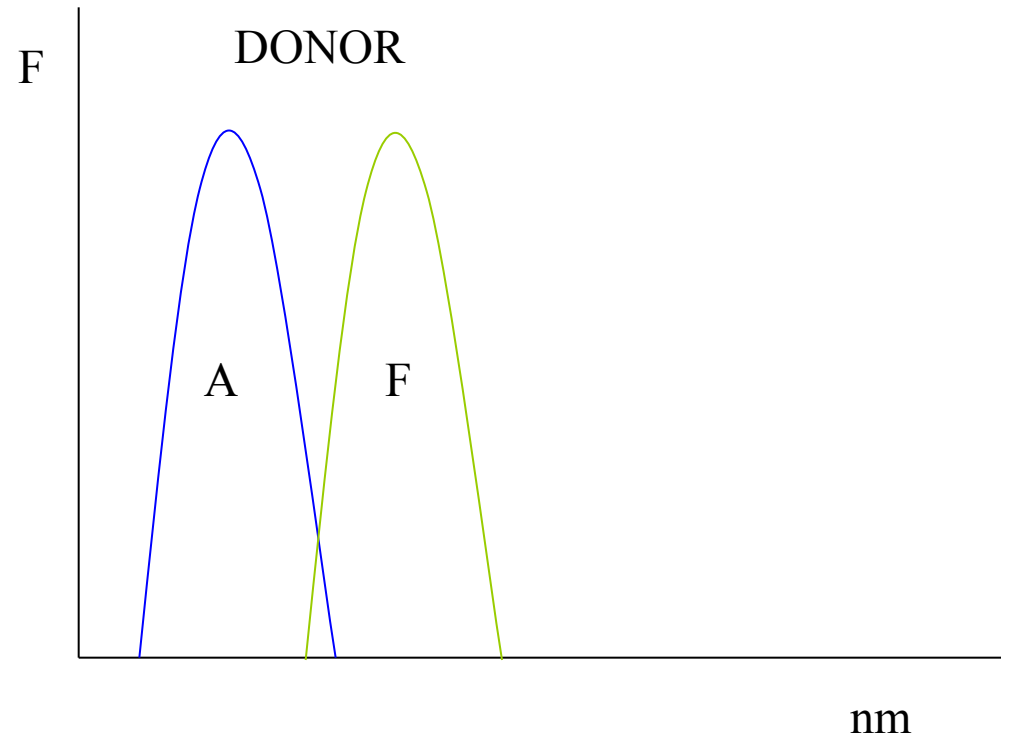
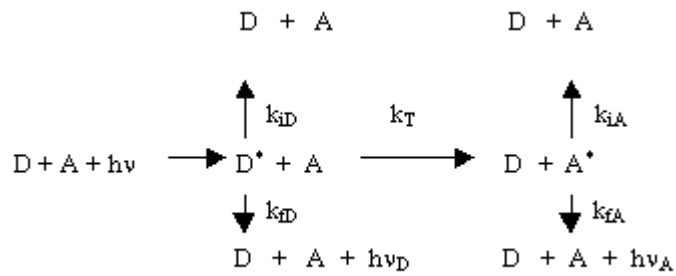


# Fluorescenční rezonanční transfer energie (Försterův přenos)

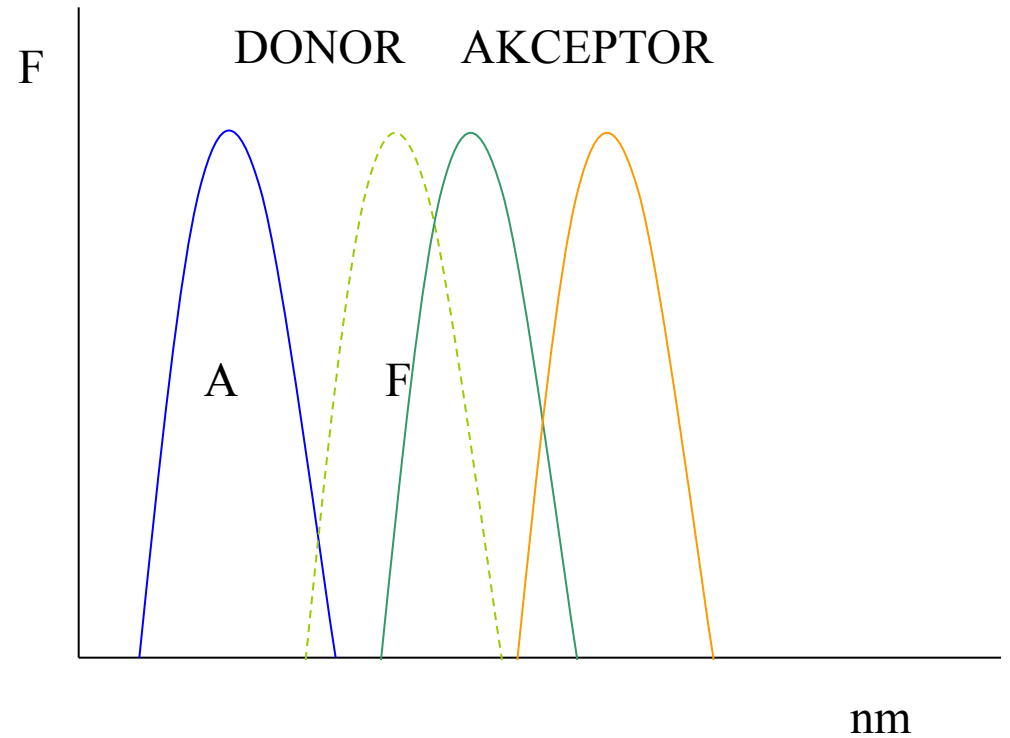
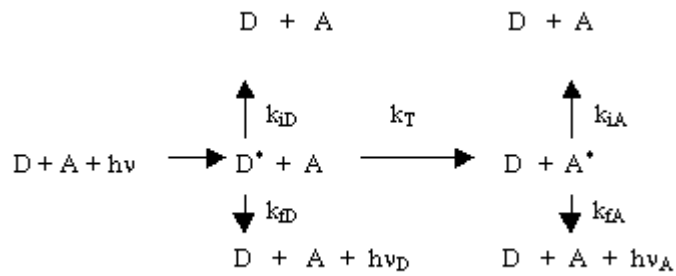


Nezářivý přenos energie z donoru na akceptor  
(1 – 10 nm)

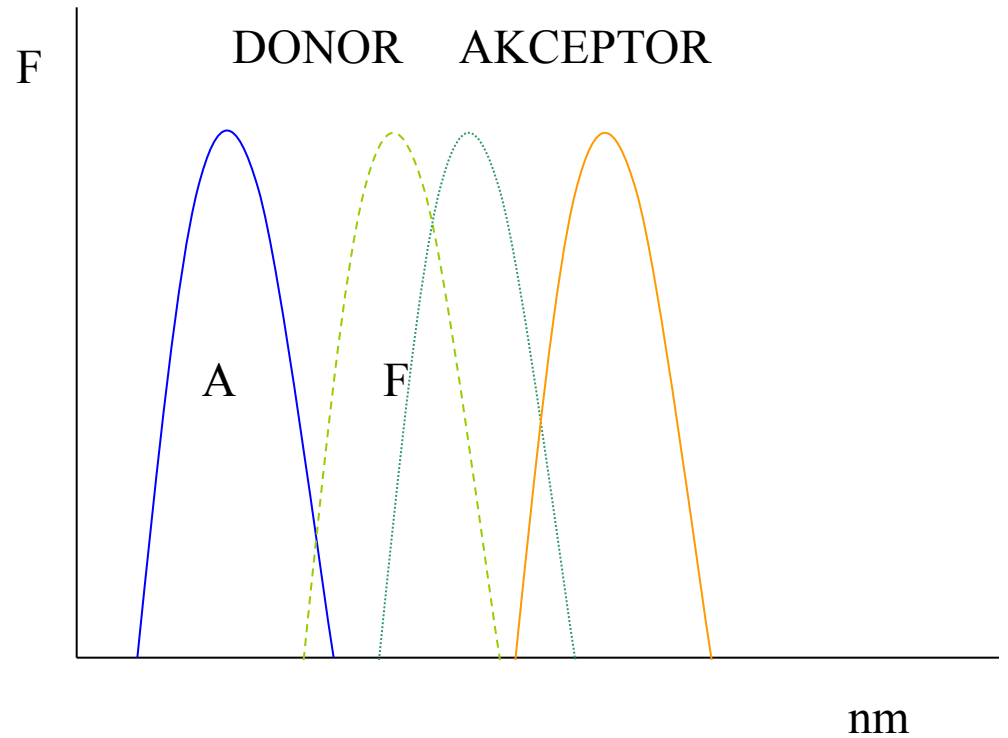
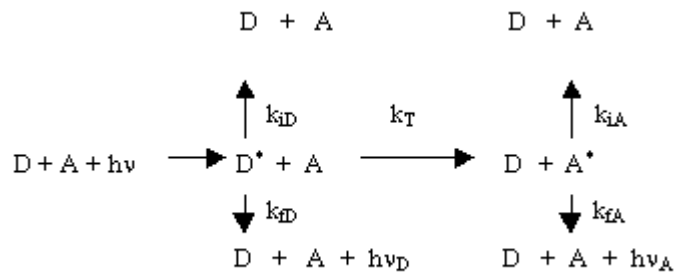
# Fluorescenční rezonanční transfer energie



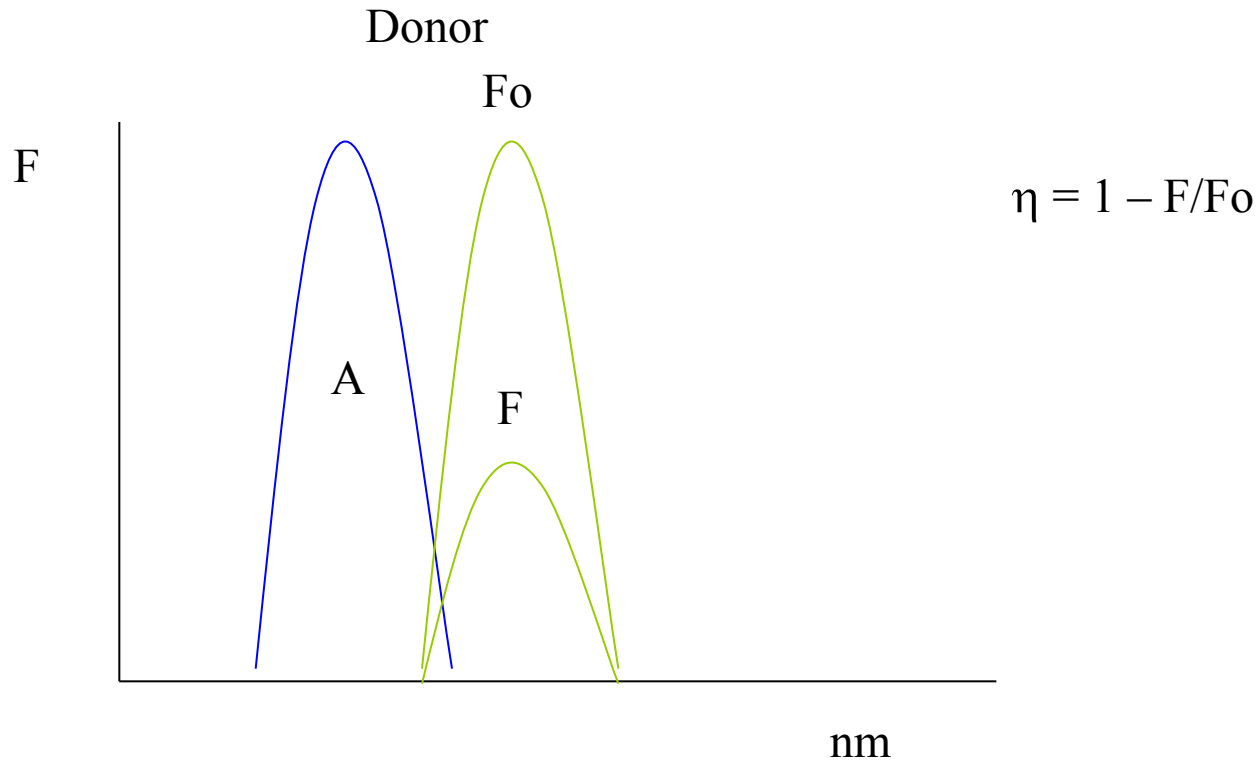
# Fluorescenční rezonanční transfer energie



# Fluorescenční rezonanční transfer energie



# Fluorescenční rezonanční transfer energie



# Fluorescenční rezonanční transfer energie

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

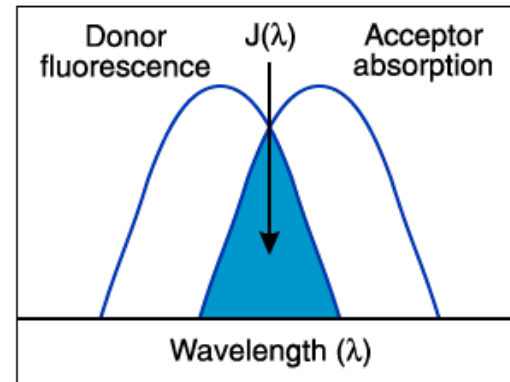
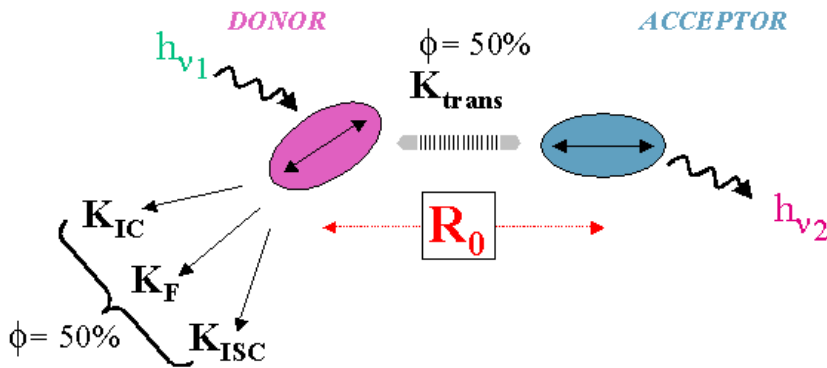
$$R_0^6 = \sqrt{1,66 \cdot 10^{-33} \cdot \tau \cdot J / n^2 \nu_0^2}$$

$\tau$  – doba života exc. stavu

$J$  – překryvový integrál

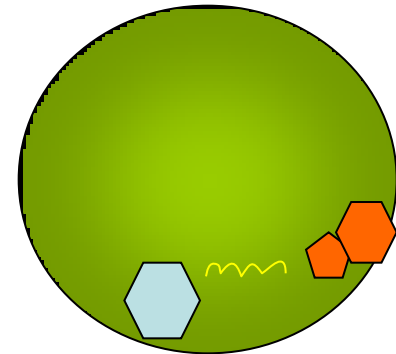
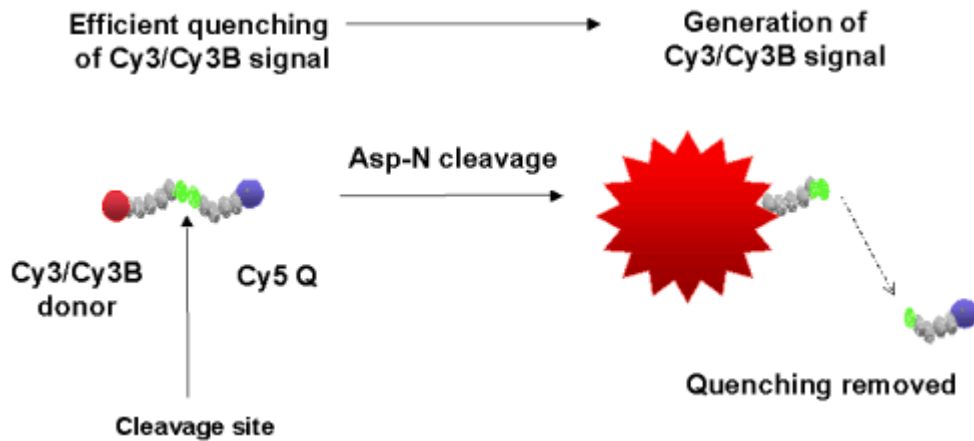
$n$  – refraktivní index rozpuštědla

$\nu$  – vlnočet emise donoru

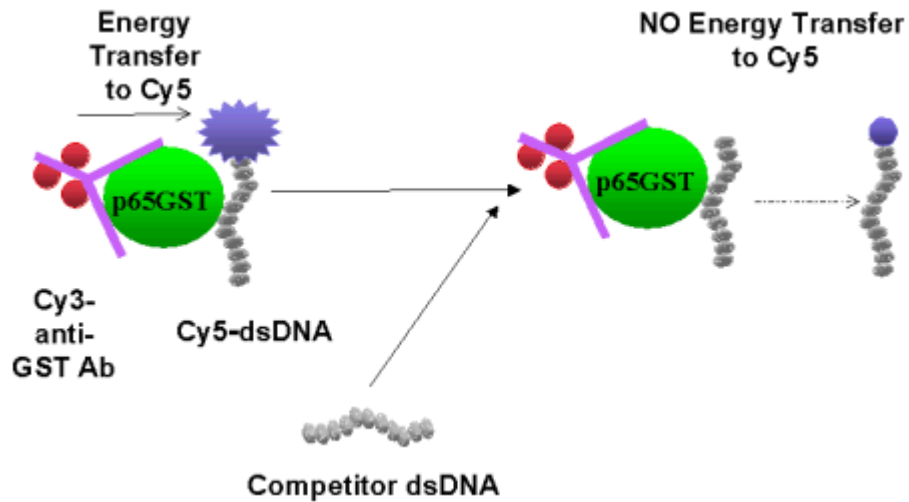


# Fluorescenční rezonanční transfer energie

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

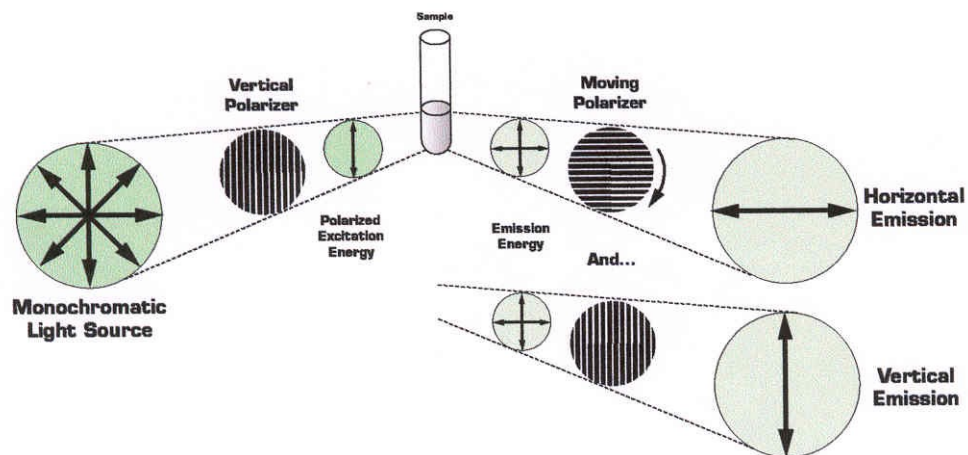


# Fluorescenční rezonanční transfer energie



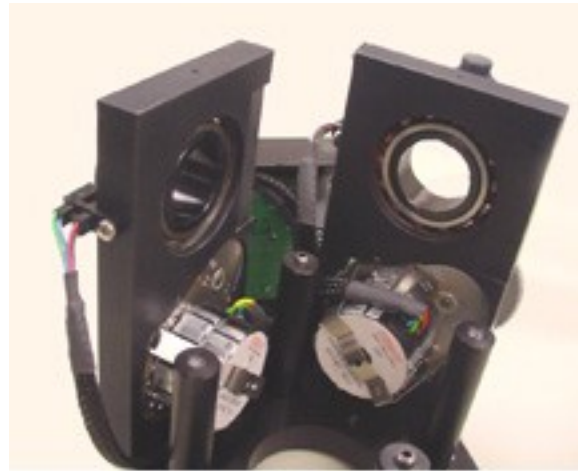


# Fluorescenční anizotropie



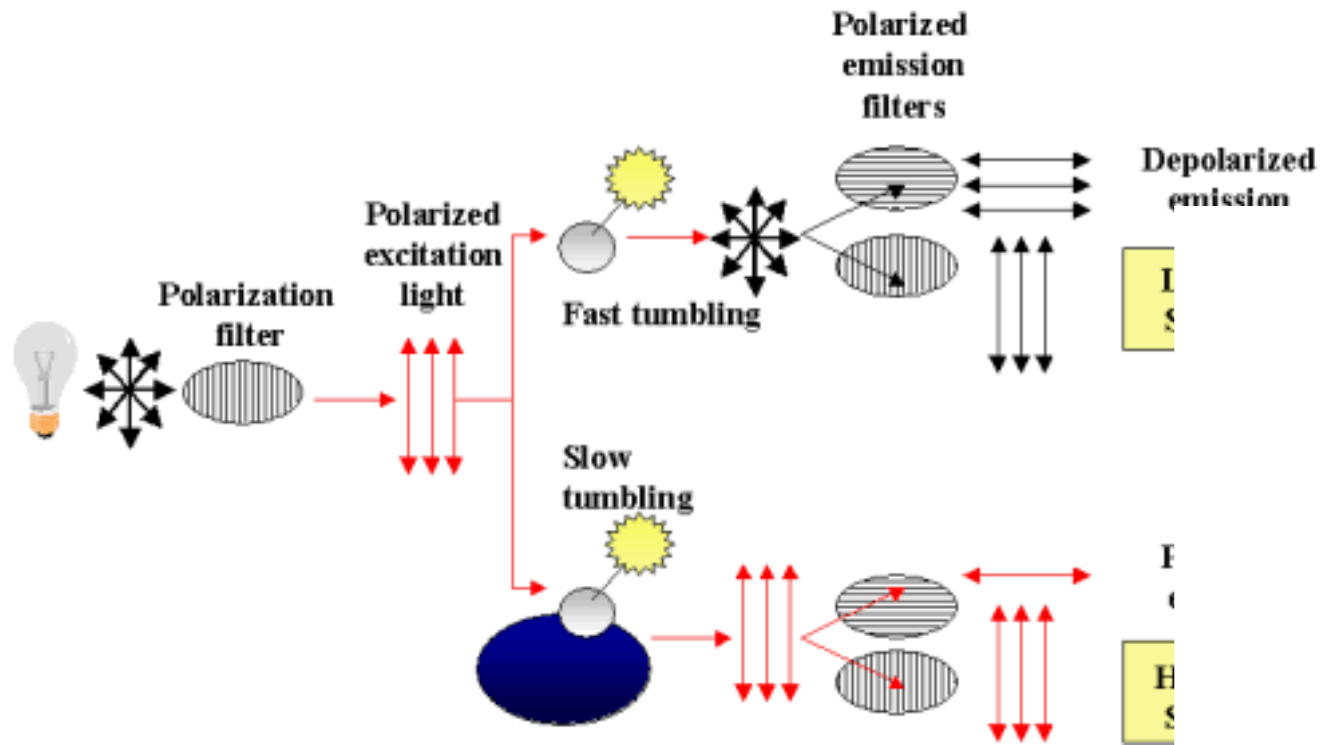
# Fluorescenční anizotropie

Polarizační filtry

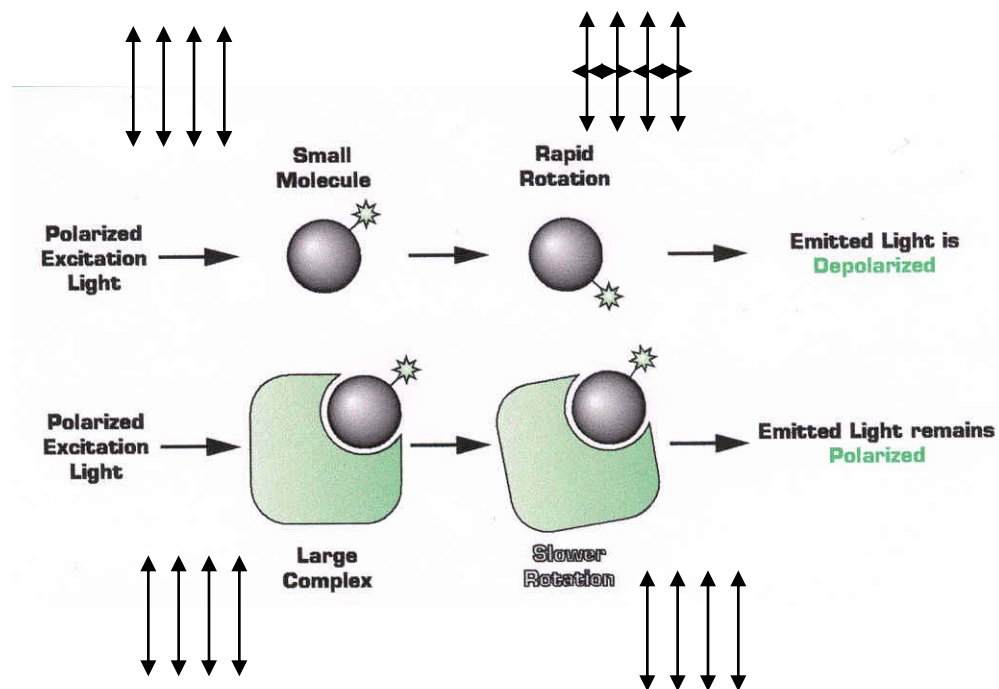


**Auto-Polorizer Accessory**

# Fluorescenční anizotropie



# Fluorescenční anizotropie

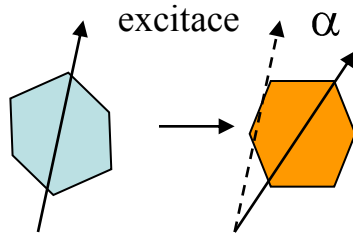


# Fluorescenční anizotropie

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

$$I = I_v + 2I_h$$

Rotační relaxační čas



$$r_0 = (3 \cos^2 \alpha - 1) / 5$$

$$r_0/r = 1 + 3\tau/\rho$$

$\tau$ , střední doba života fluorescence  
 $\rho$ , rotační relaxační čas molekuly  
 $r_0$  – anizotropie nepohyblivé molekuly

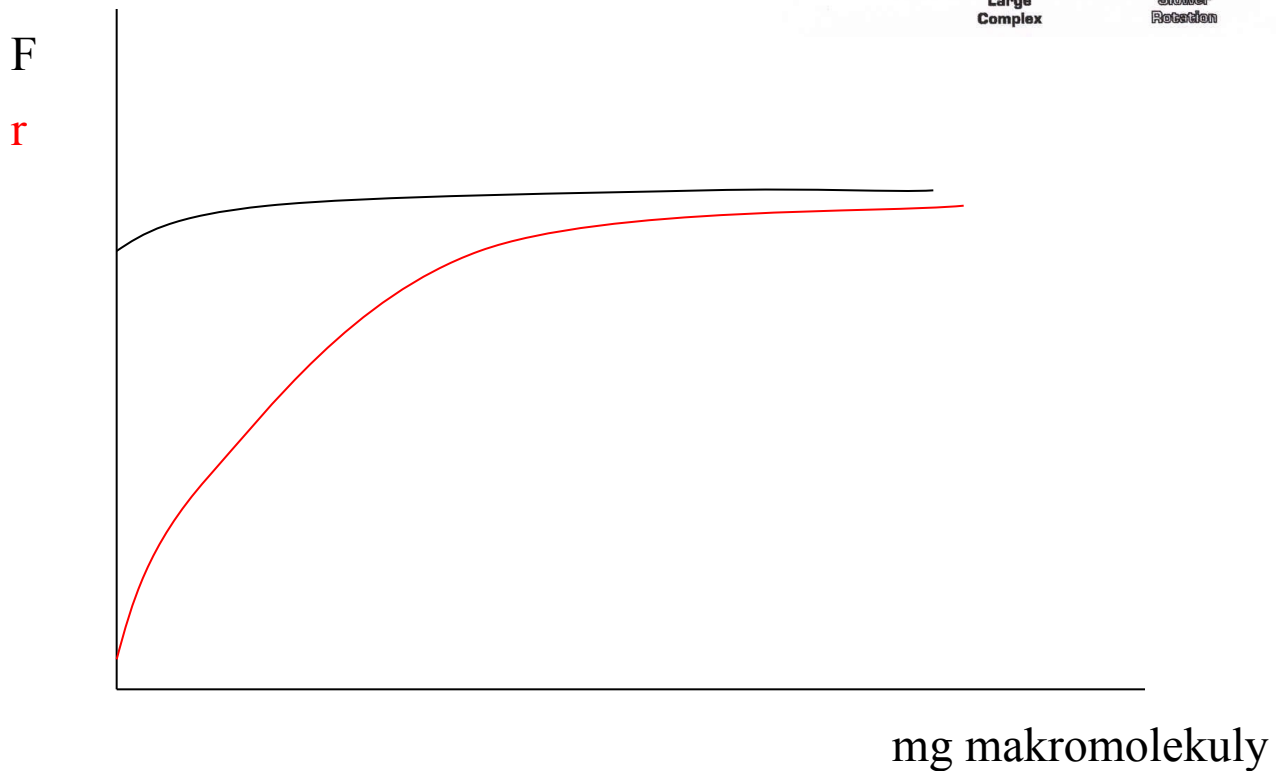
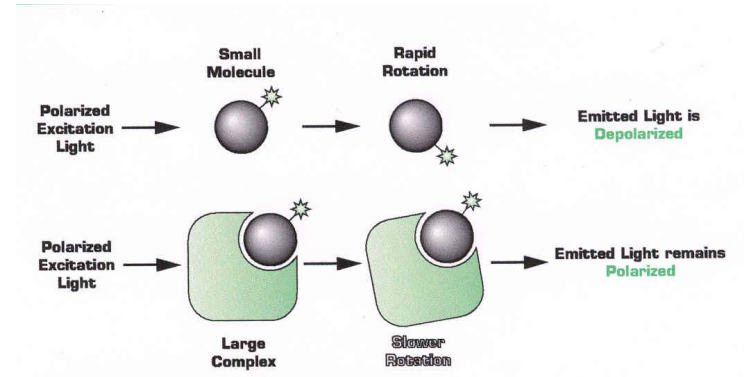
$$\rho = V\eta/RT$$

$V$  objem  
 $\eta$  viskozita

$$r_0/r = 1 + 3\tau RT/V\eta$$

# Fluorescenční anizotropie

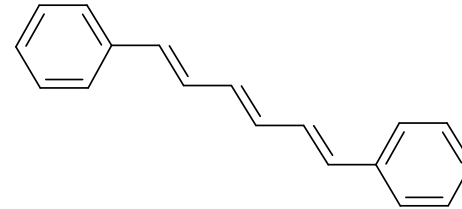
Využití:  
Interakce makromolekuly s ligandem



# Fluorescenční anizotropie

Využití:

Měření viskozity prostředí

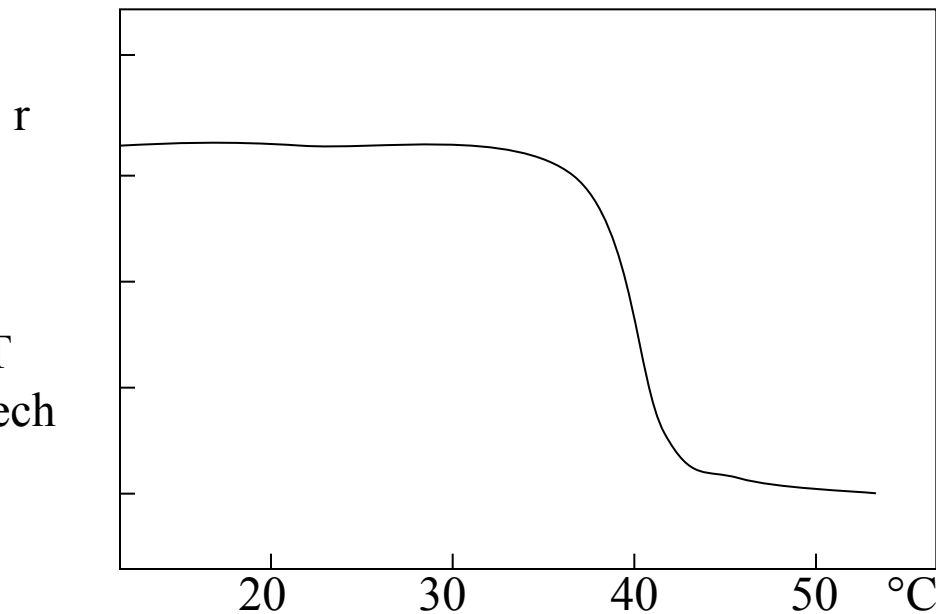


$$r_0/r = 1 + 3\tau RT/V\eta$$

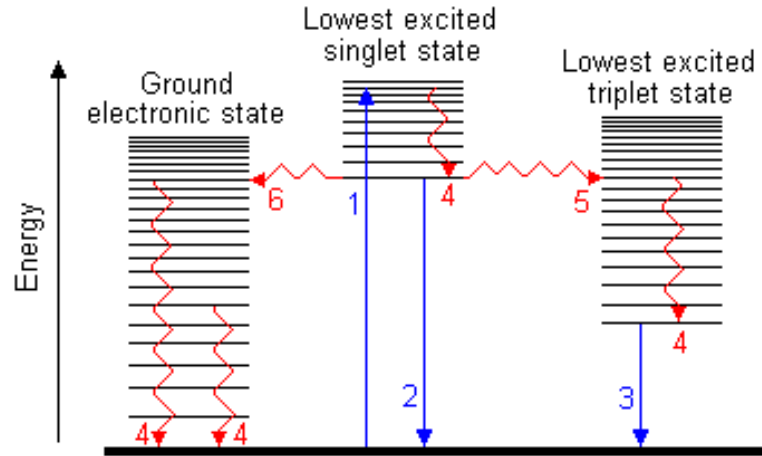
$$r_0/r = 1 + K/\eta$$

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

Fl. anizotropie DPHT  
Vázaného v liposomech  
DPPC



# Fosforescence

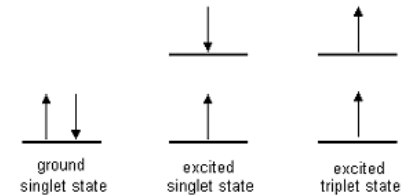
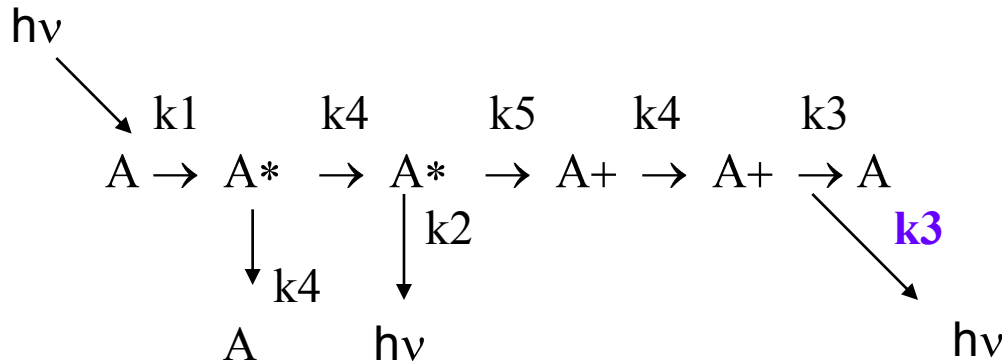


1. Absorption
2. Fluorescence
3. Phosphorescence
4. Vibrational relaxation
5. Intersystem crossing
6. Internal conversion

→ Processes involving photons  
~→ Radiationless transitions

Multiplicita

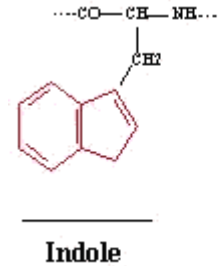
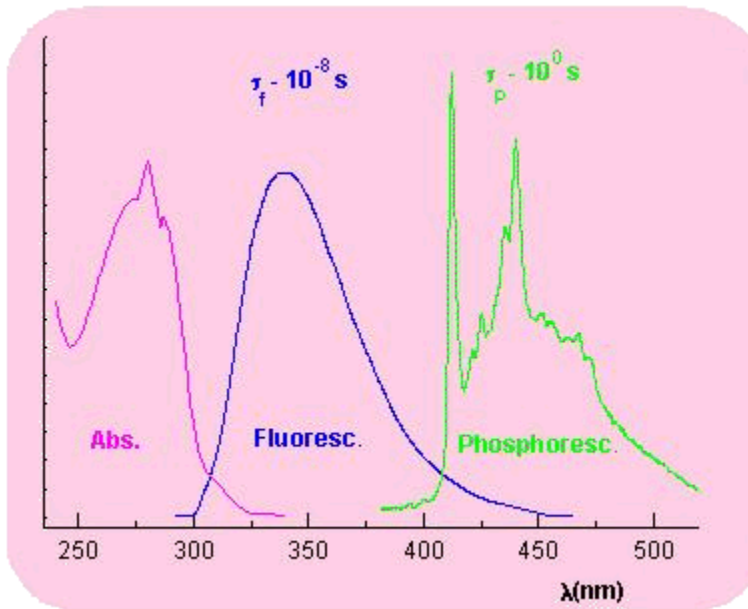
$$M = 2S + 1$$





# Fosforescence

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



Tryptophan

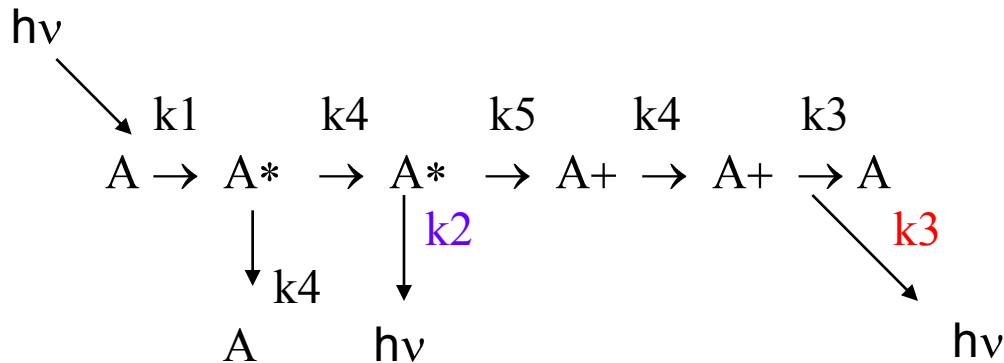
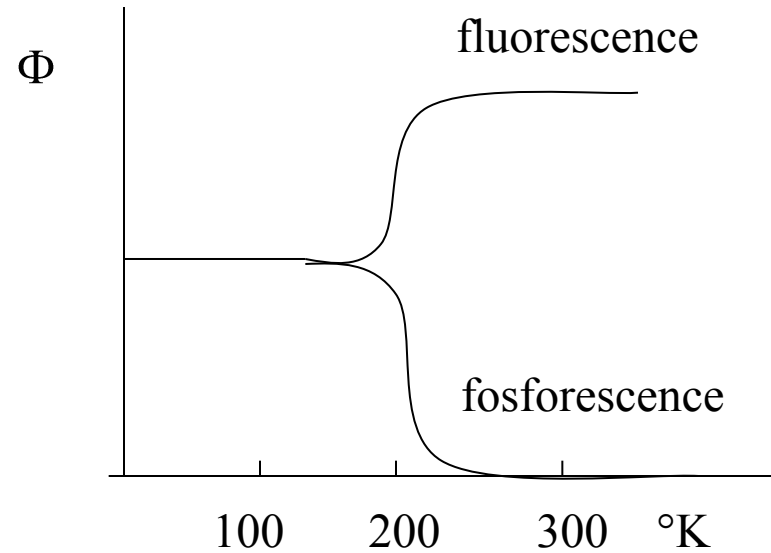
# Fosforescence

Kvantový výtěžek fosforescence

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

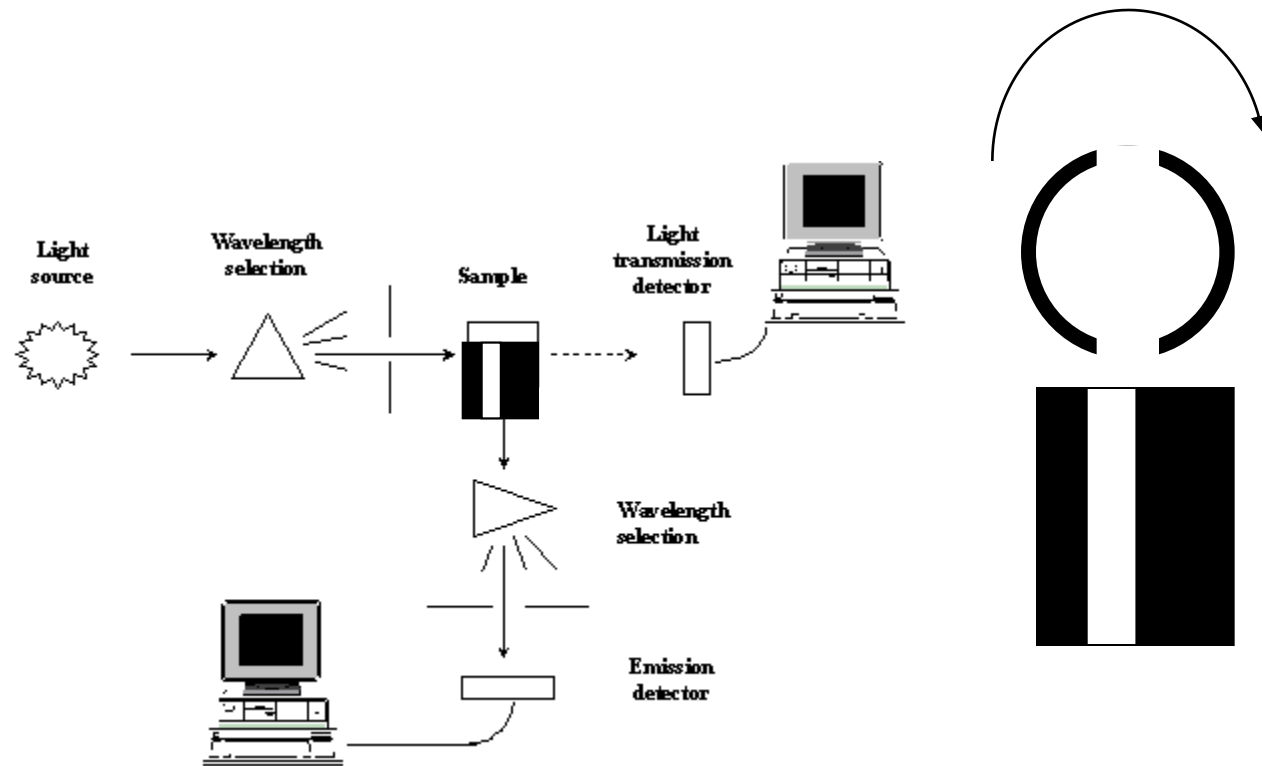
$$\Phi_f = k_2 / (k_3 + k_2 + k_4)$$

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

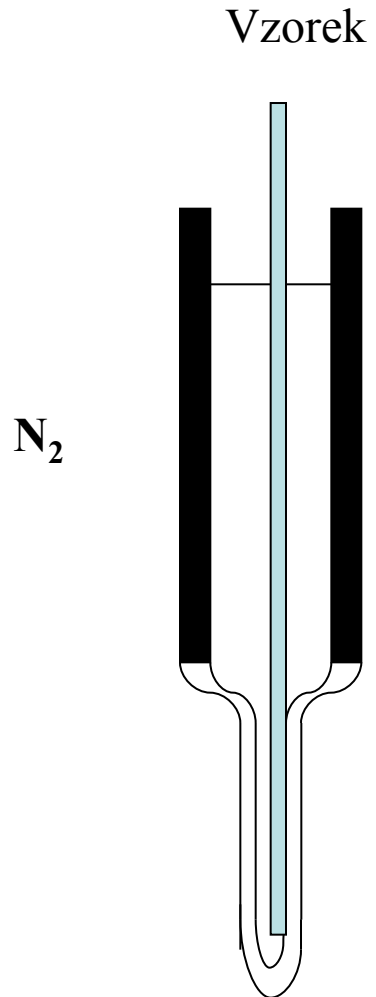


# Fosforescence

Experimentální uspořádání



# Fosforescence



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

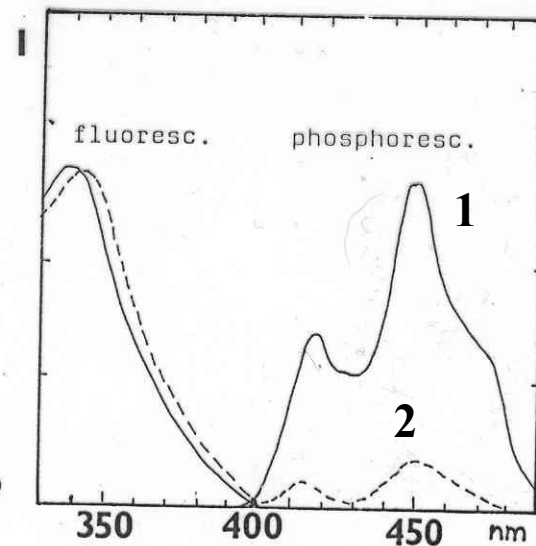
1 – nativní enzym

2 – enzym po odstranění Zn

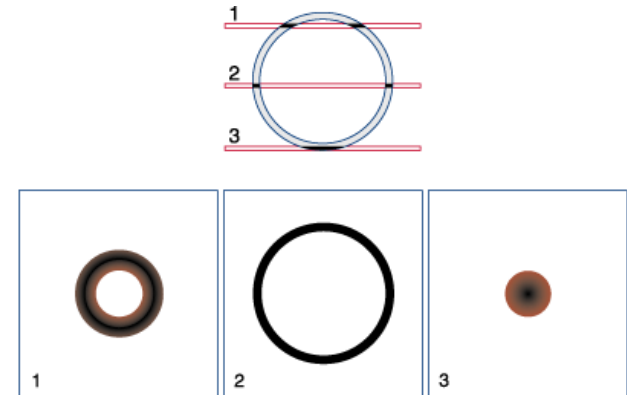
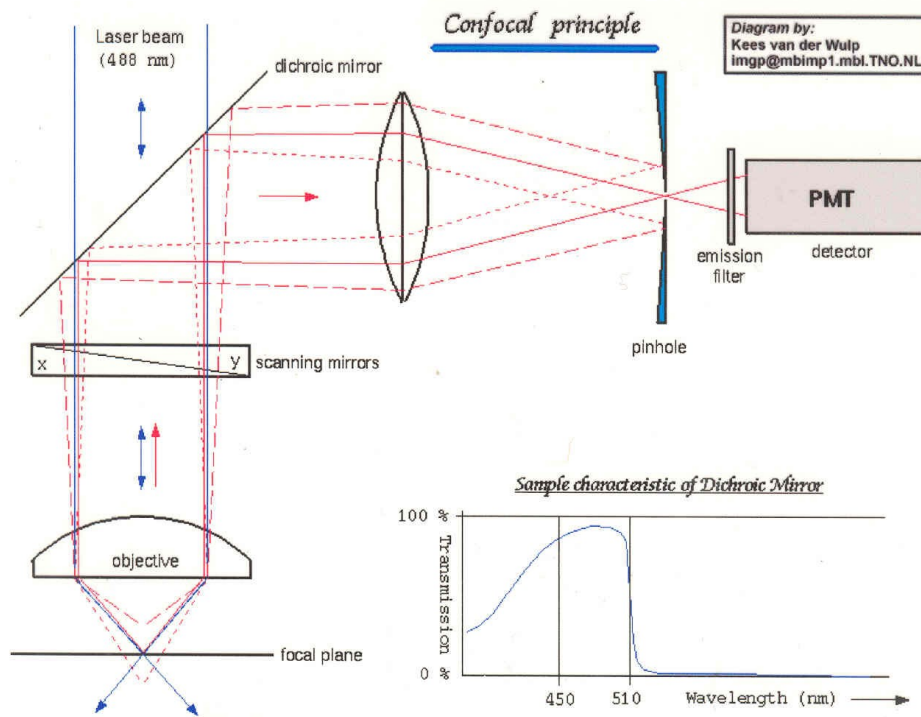
Phosphatase  
alcaline

Spectra de fluor.  
et de phosphoresc.  
de tryptophane

Cioni et al. (1989)  
Eur. J. Biochem.

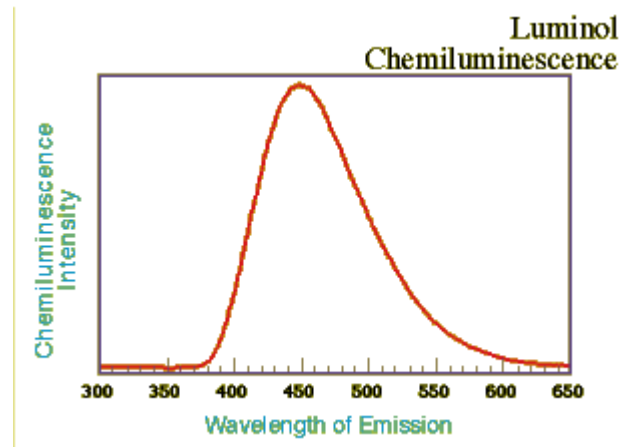
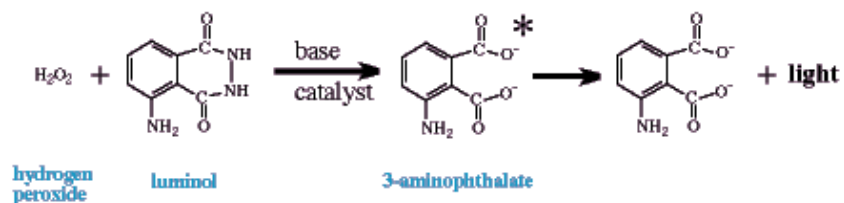


# Princip fluorescenční konfokální mikroskopie

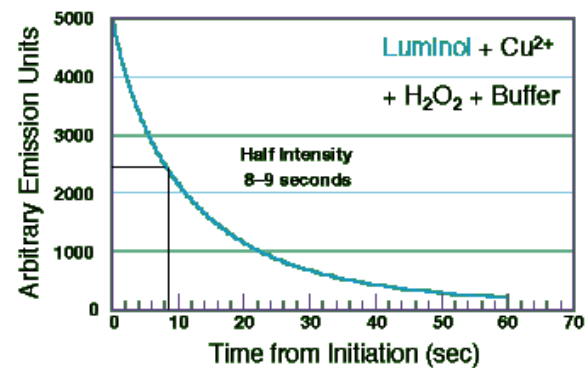


# Chemiluminescence

## Luminol



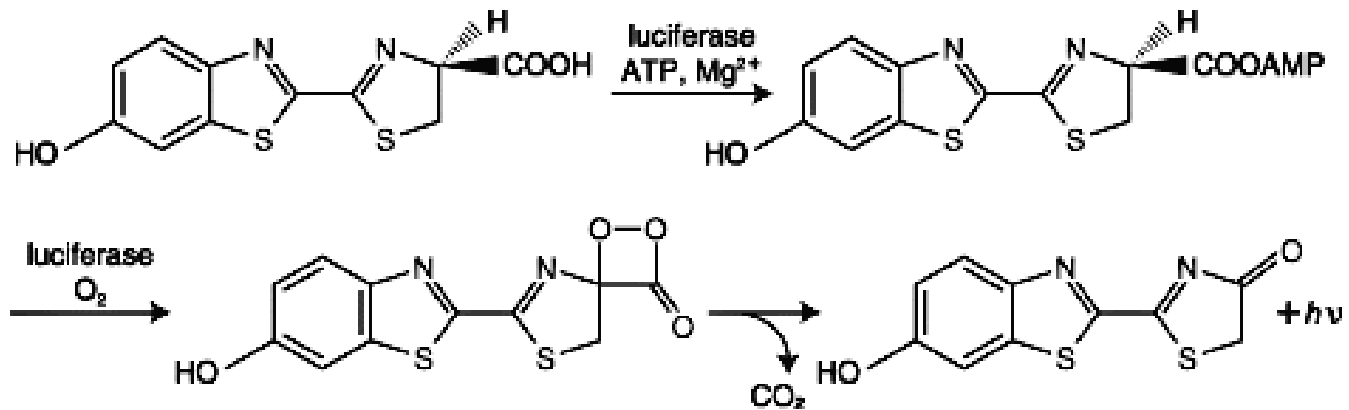
## Luminol Emission Time Profile





# Chemiluminescence

## Luciferin



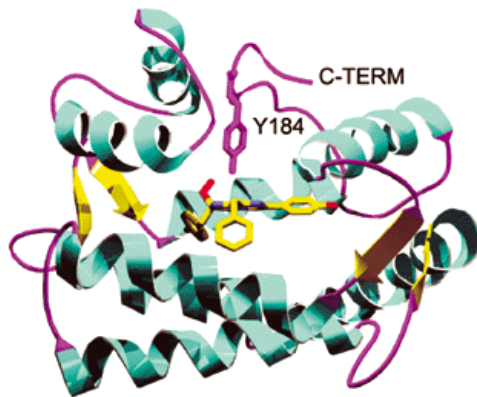
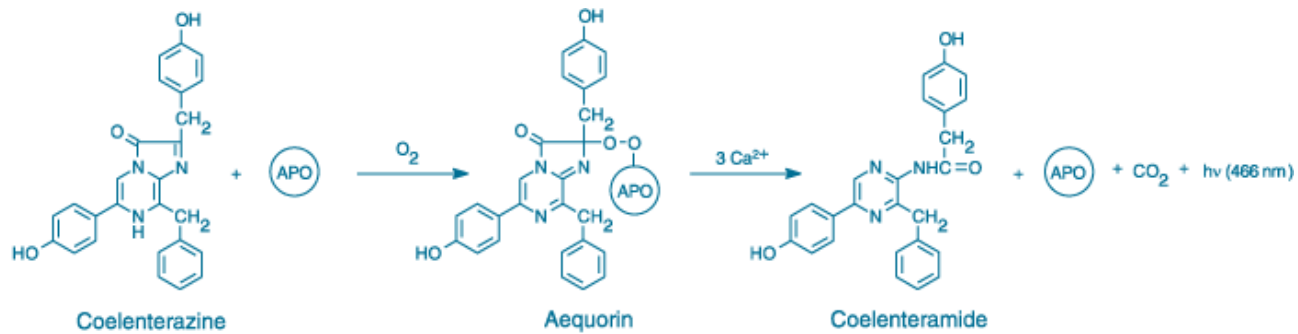
# Chemiluminescence

**Aequorin – *Aequoria victoria***



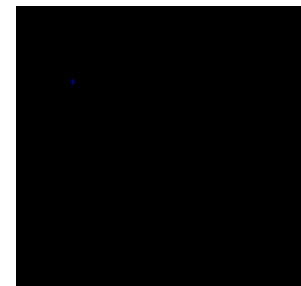
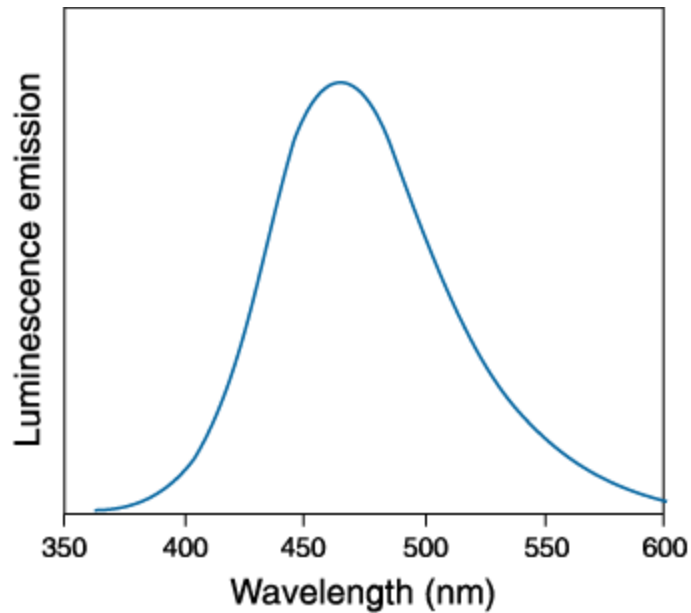
# Chemiluminescence

## Aequorin – *Aequoria victoria*



# Chemiluminescence

## Aequorin – *Aequoria victoria*



Průnik vápníku do mitochondrií  
Aktivuje oxidaci