Fluorescent Dyes and Proteins

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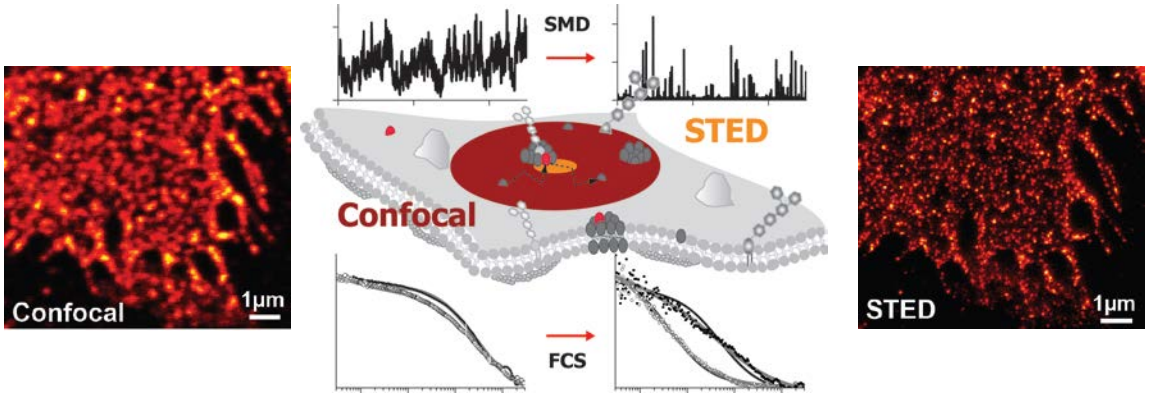


Fluorescence Microscopy

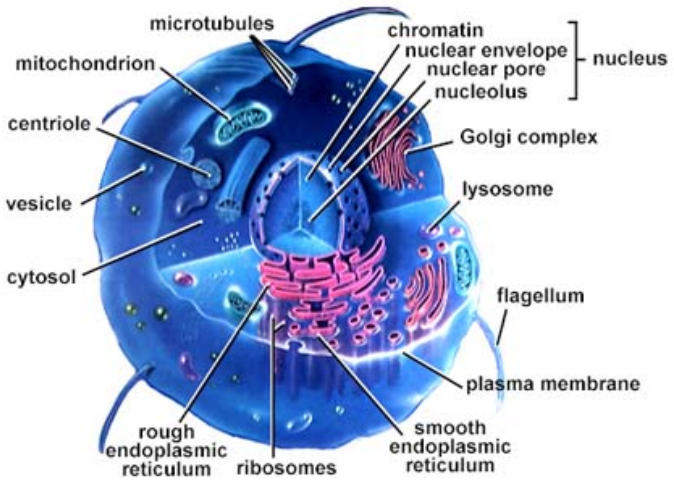
Label molecule of interest

with signaling label

Live Cell Far-Field Microscopy

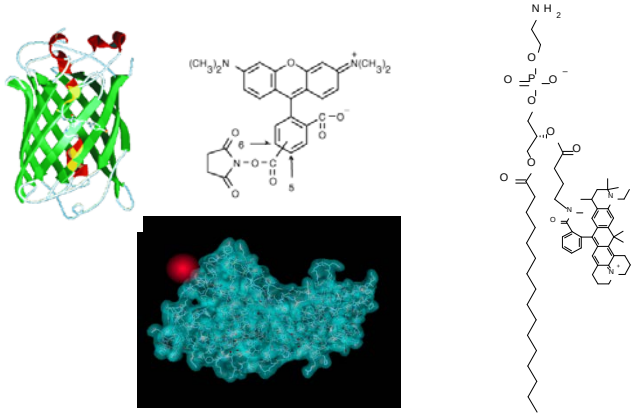
Fluorescence

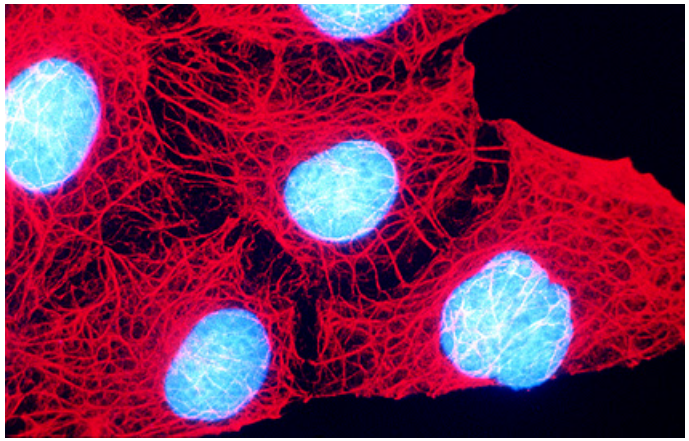
Study specific molecular processes in the living cell:



Fluorescence microscopy

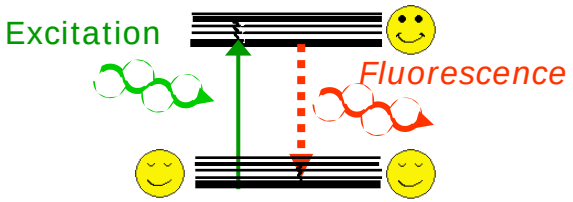
Label specific protein/molecule





Liver-Cells: Nucleus and Cell-skeleton

Excite fluorescence by laser light



Fluorescence

Classification of **Fluorescencent probes** in three types:

Intrinsic probes

- ideal but rare (e.g. tryptophan in proteins)

Extrinsic associating probes

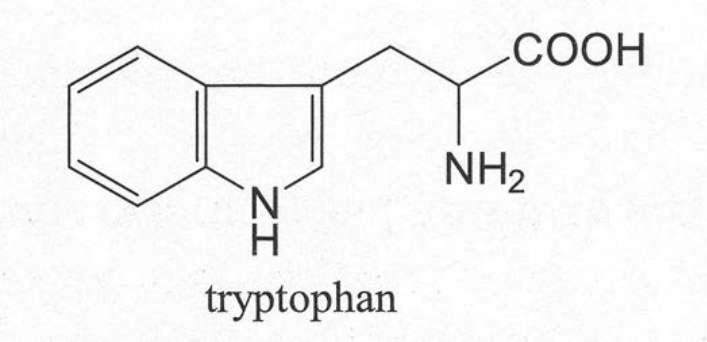
- non-covalent attachment (e.g. adsorption, …)
- hydrophylic, hydrophobic, amphiphilic character, …

Extrinsic covalently bound probes (fluorophore, dye)

- covalently attached dye-label (synthesize)
- fluorescent protein
- advantage: generally known location

Fluorescence

Examples of Fluorophores



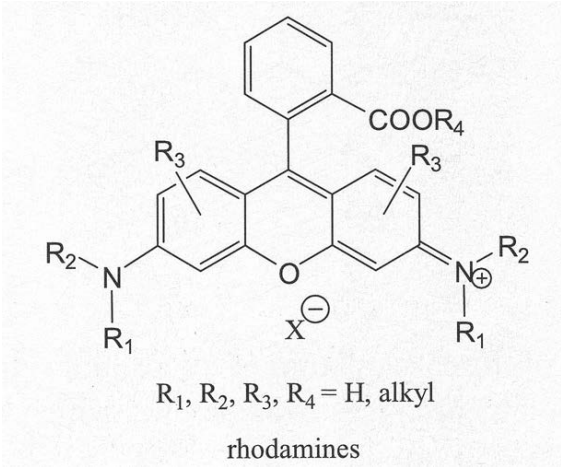
tryptophan

Important for investigations of proteins

UV absorption

Tryptophan (intrinsic)

Rhodamines
(extrinsic)
- first laser dyes
- small stokes shift
  (20 – 30 nm)
- fluorescence window
  500 – 700 nm

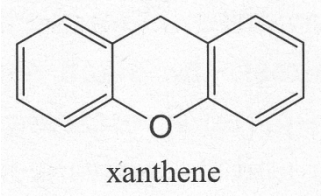


R_1, R_2, R_3, R_4 = H, alkyl

rhodamines

High Extent of delocalization in π-electron system ($\pi \rightarrow \pi^*$ transition)

... is what is needed for fluorescent molecules

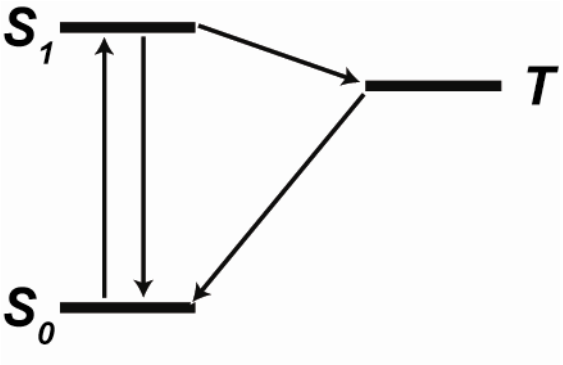


xanthene

Fluorescence

Absorption of a photon ⇒ electronic transition

Electronic transition: Promotion of an electron from an orbital of a molecule in the ground state to an (antibonding) orbital higher in energy

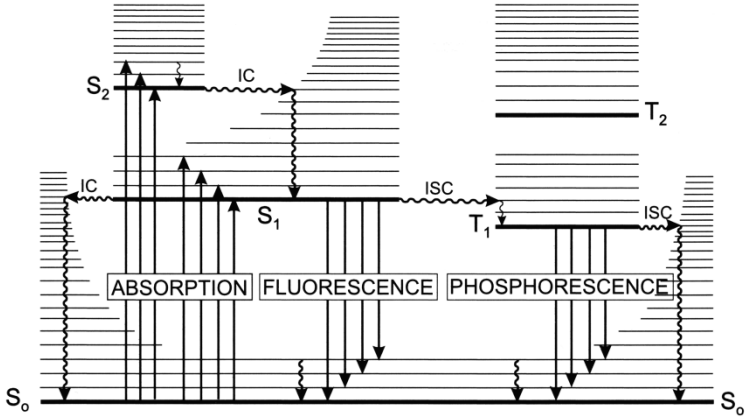


Electronic States:
- S_0, S_1, …

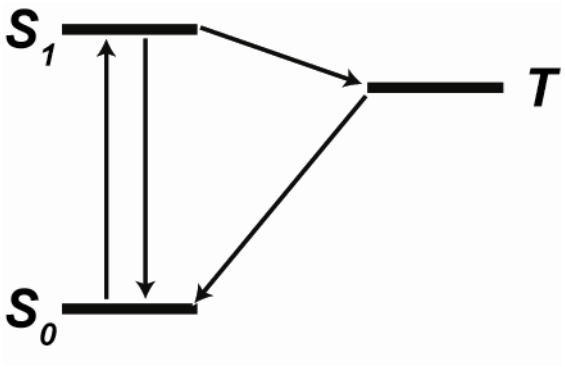
Vibronic sub-states
in each electronic state (molecular vibrations)

Visualisation:

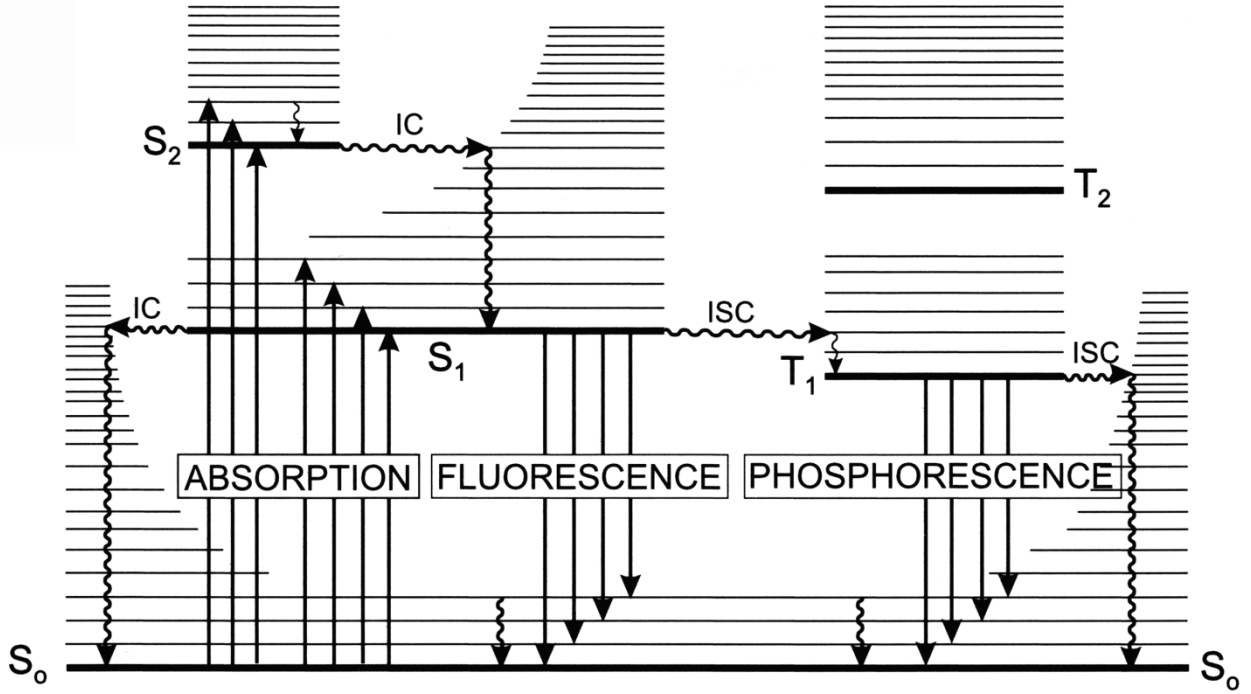
Perrin-Jablonski diagram



Fluorescence: Transitions after Absoprtion



First Step - Absorption



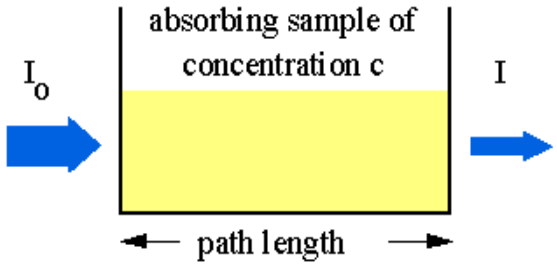
Fluorescence

The probability of transitions - Absorbance

absorbance = efficiency of absorption at wavelength λ:

$$A(\lambda) = \log \frac{I_\lambda^0}{I_\lambda} = \varepsilon(\lambda) l c$$

absorbance

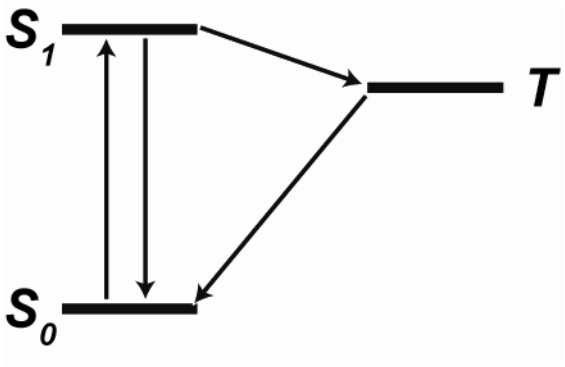


Beer-Lambert law

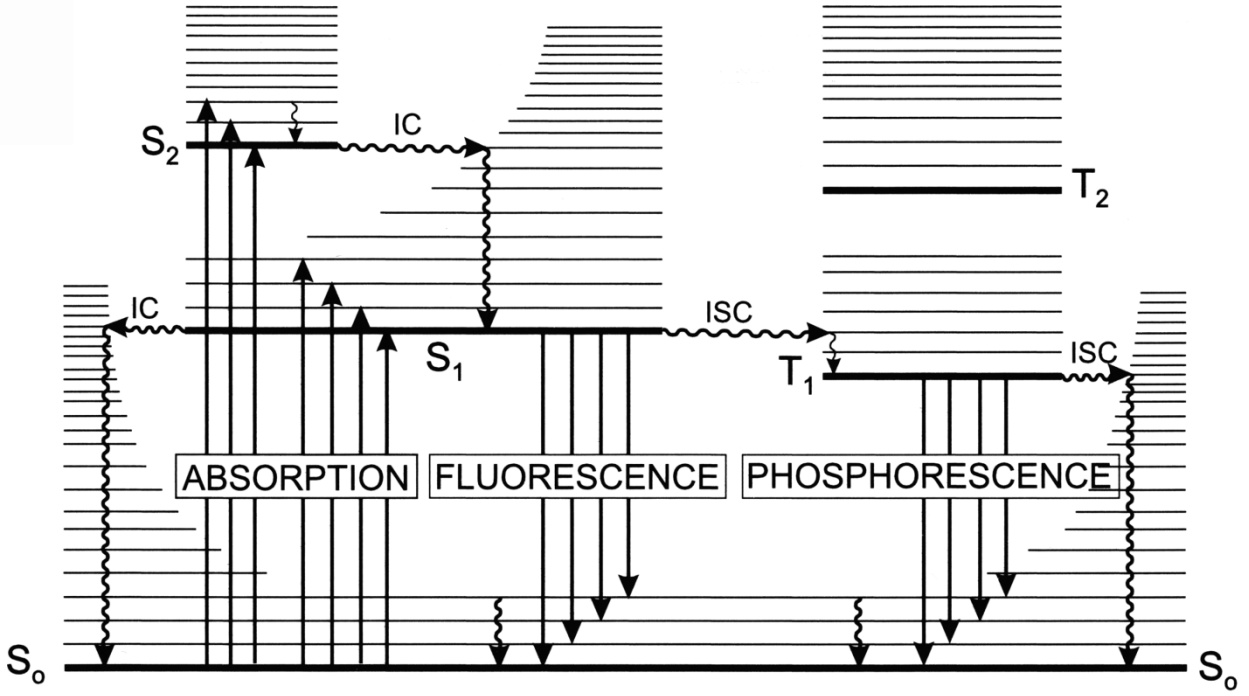
ε(λ): molar (decadic) absorption coefficent [L mol^{-1} cm^{-1}]
***c*: concentration [mol L^{-1}]**
***l*: absorption path length [cm]**

Examples: ε(max) = Benzene ~10^2 / Anthracene ~10^4 / Rhodamine B ~10^5

Fluorescence: Transitions after Absoprtion



After Absorption -
Transitions between electronic states

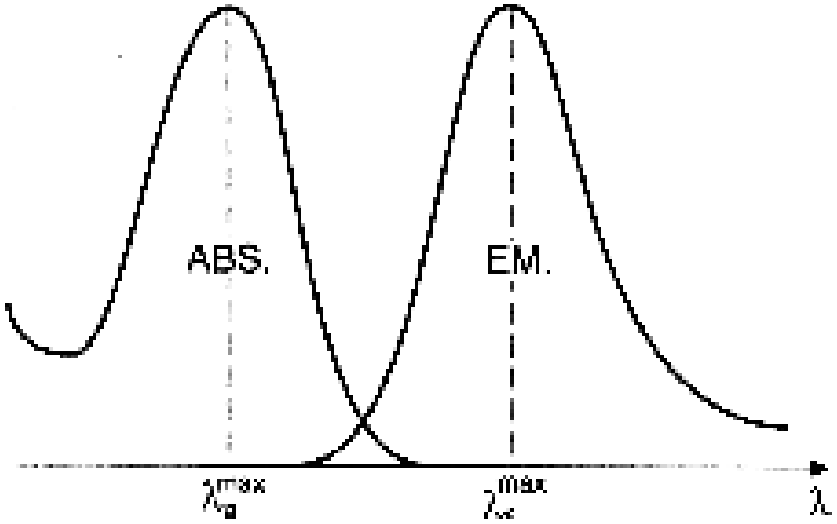


- Fluorescence

Fluorescence: Stokes Shift + Spectral Broadening

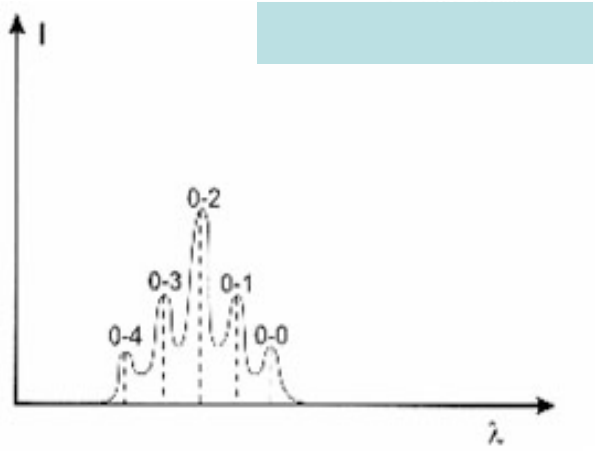
Stokes shift: gap between max. of first absorption band and max. of fluorescence

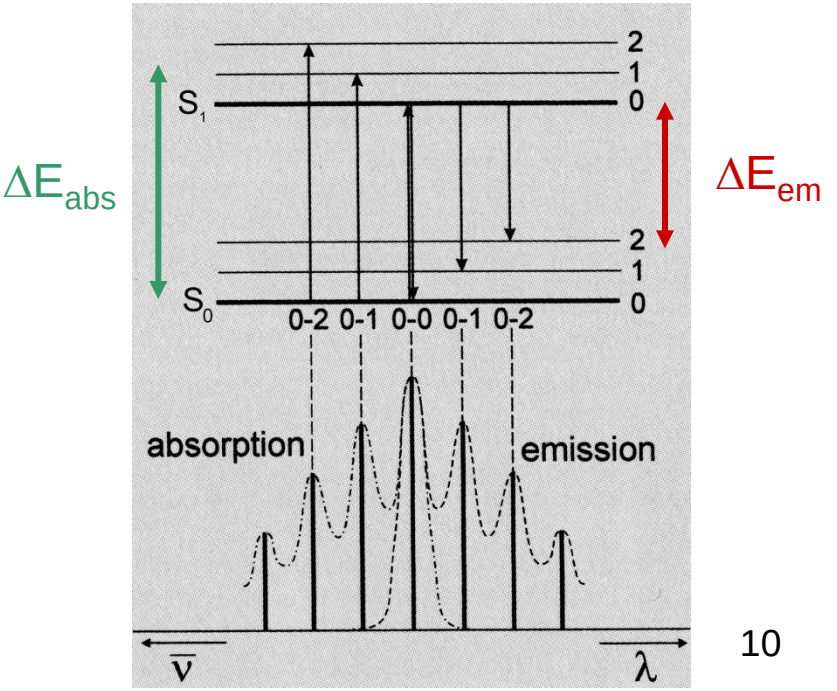
$\lambda_{em.} > \lambda_{abs.}$



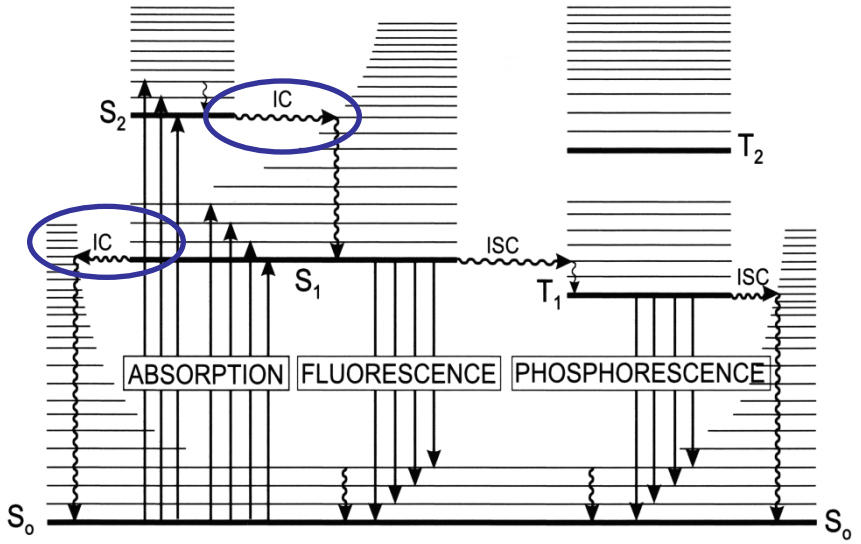
vibrational relaxation: $\Delta E_{abs} > \Delta E_{em}$

(in)homogeneous spectral broadening
vibrations/ fluctuations of the structure





Internal conversion (IC)

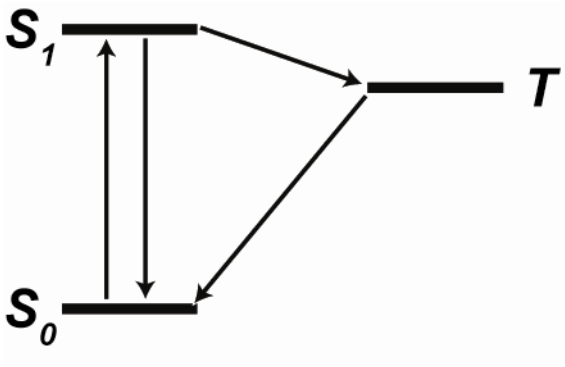


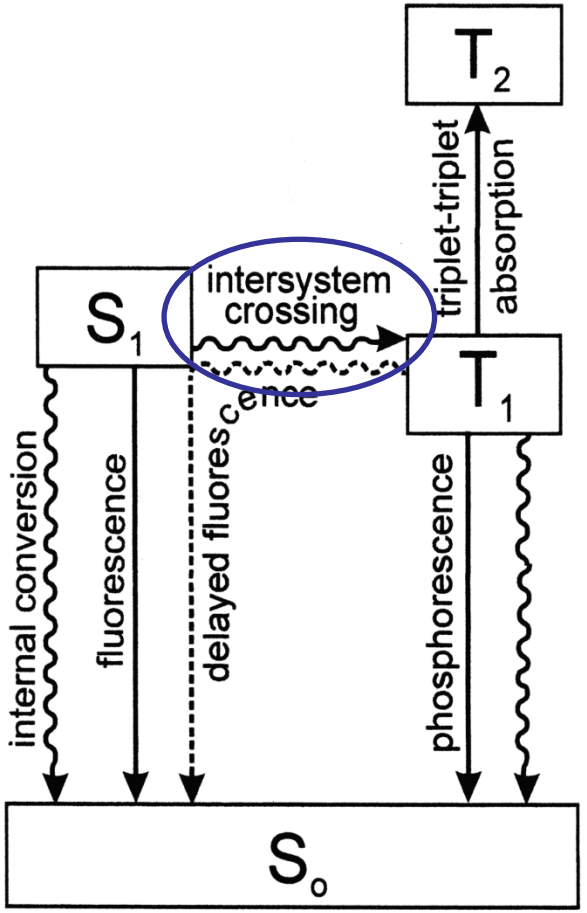
Non-radiative transition between two electronic states ($S_2 \rightarrow S_1$ or $S_1 \rightarrow S_0$)

any transition followed by fast vibrational relaxation (e.g. collision) to vibronic ground state

- $S_1 \rightarrow S_0$ is less efficient than $S_2 \rightarrow S_1$ (larger energy gap)
- $S_1 \rightarrow S_0$ IC competes with fluorescence
- picoseconds

Intersystem crossing





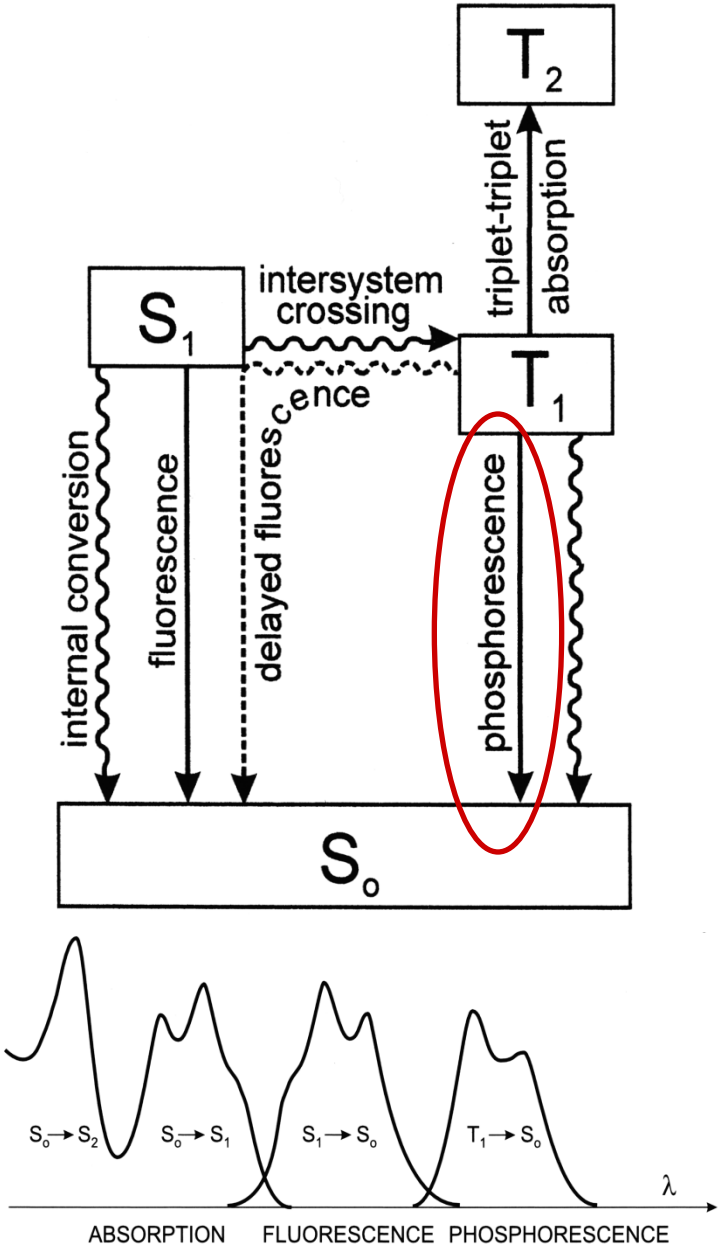
Non-radiative transition between two isoenergetic vibrational levels of different spin multiplicities ($S_1 \rightarrow T_1$) – spin conversion

- Competes with fluorescence and IC
- Forbidden - slow process: 10^{-10} – 10^{-8} s

Phosphorescence

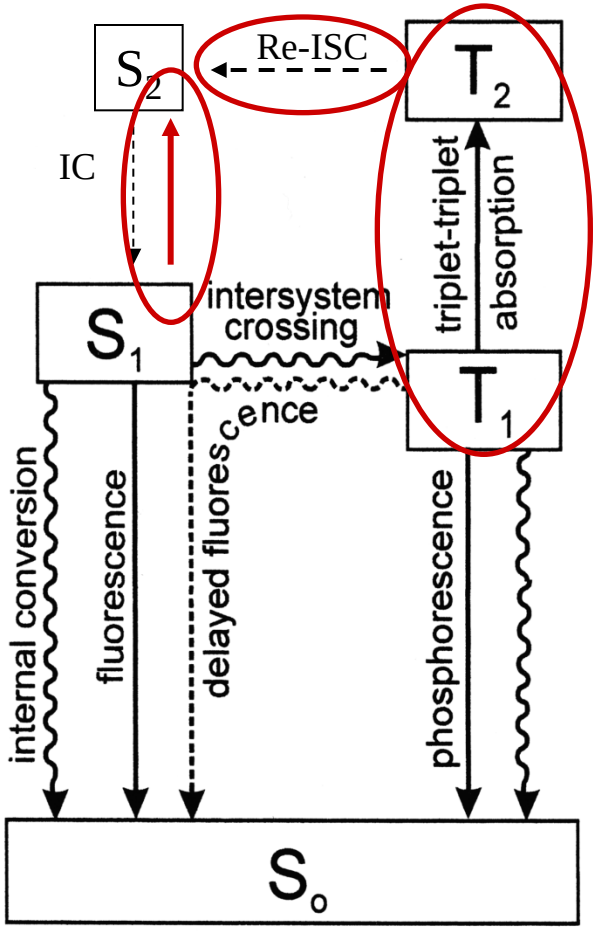
radiative de-excitation ($T_1 \rightarrow S_0$)

- Forbidden - slow process (long lifetime µs to s)
- Higher wavelengths than those of fluorescence
  (Hund's rule – energy(triplet) < energy(singlet))
- Competes with non-radiative processes
  (especially in solution, collisional de-excitation)
  (mainly visible at low T or in rigid medium)



Triplet-triplet absorption

- $T_1 \rightarrow T_{2/n}$
- Absorption of a second photon of same or different wavelength
- When T_1 is highly populated



- Possibility of re-intersystem crossing to $S_n \rightarrow S_1$
- $S_1 \rightarrow S_{2/n}$ absorption also possible

- usually ~ 1/10-1/100 $\varepsilon(0 \rightarrow 1)$

Fluorescence Parameters:

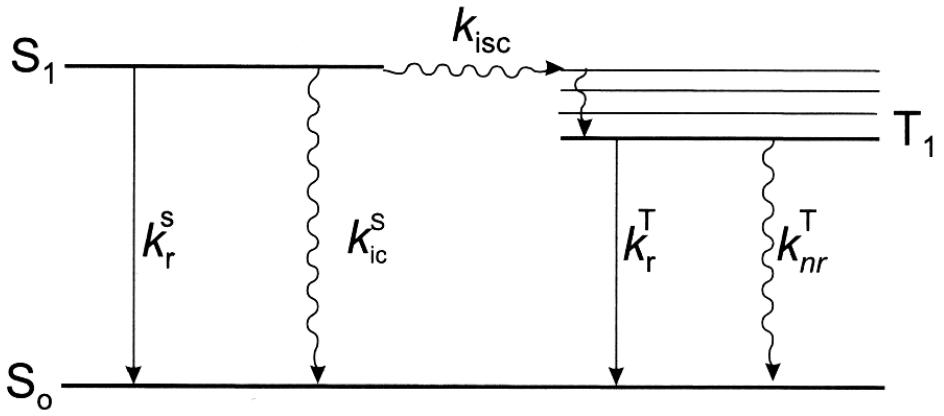
Lifetime

Quantum Yield

Excited-state lifetimes

Definition of rate constants:

k_{nr}: Non-radiative decay (ic + isc)
k_r: radiative decay (flu. + phosph.)
k_{isc}: intersystem-crossing



$$k_{nr}^{S} = k_{ic}^{S} + k_{isc}$$

Lifetime of excited state:
sum of all de-excitation rate constants

Singlet $$\tau_S = \frac{1}{(k_{ic\,ic}^S + k_{isc}^S) + k_r^S}$$

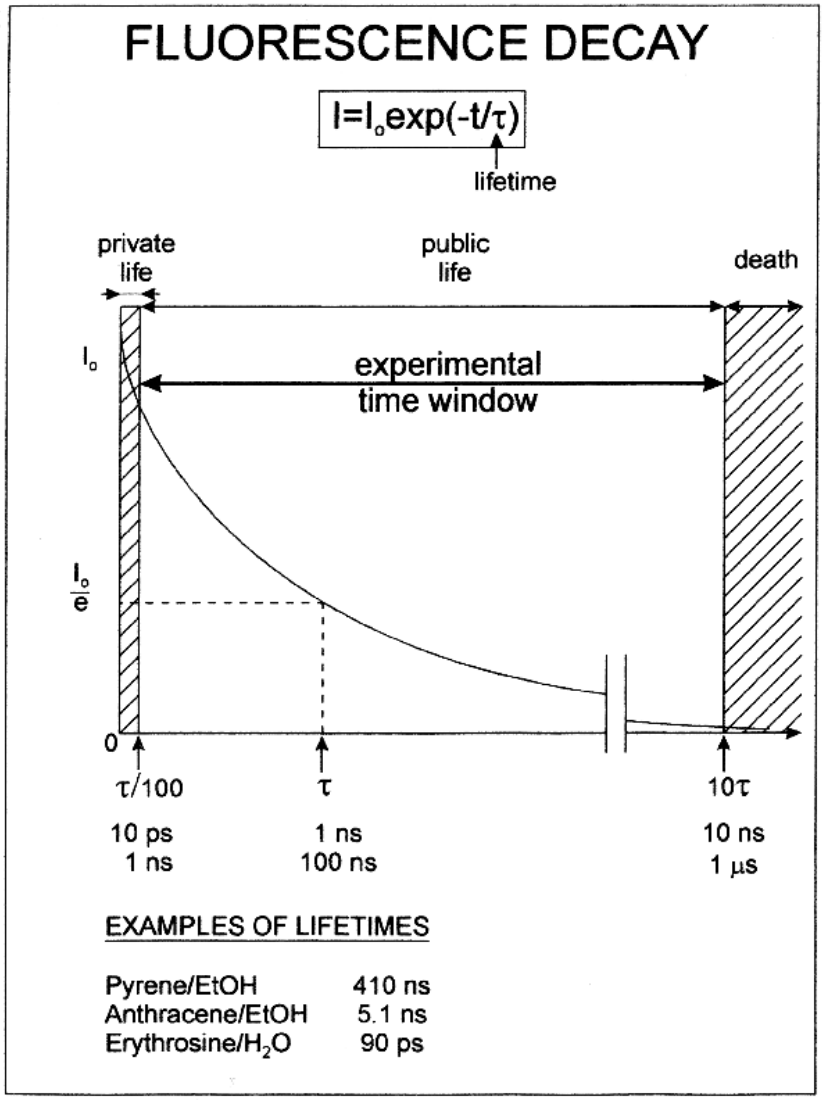
Triplet $$\tau_T = \frac{1}{k_{nr}^T + k_r^T}$$

Exponential decay:

$$\frac{dS_1}{dt} = -\left[(k_{ic\,ic}^S + k_{isc}^S) + k_r^S\right]S_1 = -(1/\tau_S)S_1$$

$$\boxed{S_1(t) = S_1(0)\exp(-t/\tau_S)}$$

Fluorescence lifetime



Fluorescence lifetime (S_1) important

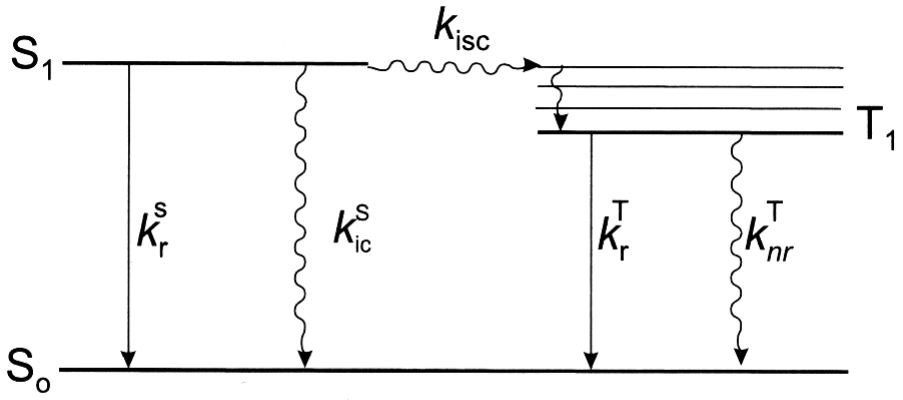
-defines the time window of observation
-sensitive on environment (solvent !!!)

Laser dyes: 1 – 4 ns

Pyrene: 410 ns

Ruthenium: 14 µs

Fluorescence: Quantum Yield



$$k_{nr}^S = k_{ic}^S + k_{isc}$$

$$\Phi_F = \frac{k_r^S}{k_r^S + k_{nr}^S} = k_r^S \tau_S$$

Fluorescence quantum yield

Fraction of photon absorptions/excitations to S_1 that return to S_0 by fluorescence (and not by internal conversion...)

Fluorescence Labels

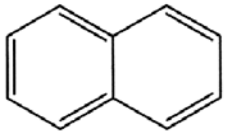
Effects of molecular structure on fluorescence

Extent of π-electron system

Nature of lowest lying transition

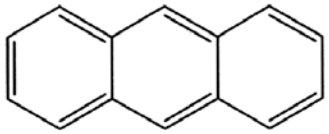
Most fluorescent compounds are aromatic

generally: increase in extent of π-electron system = shift of spectra to longer λ + higher Φ_F



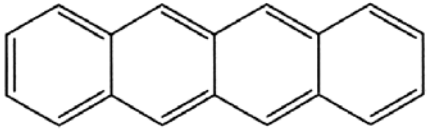
naphthalene

Fluorescence: ultraviolet



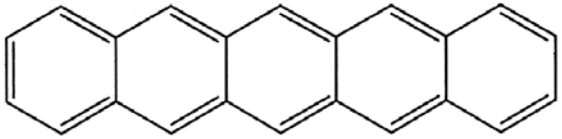
anthracene

blue



naphthacene

green



pentacene

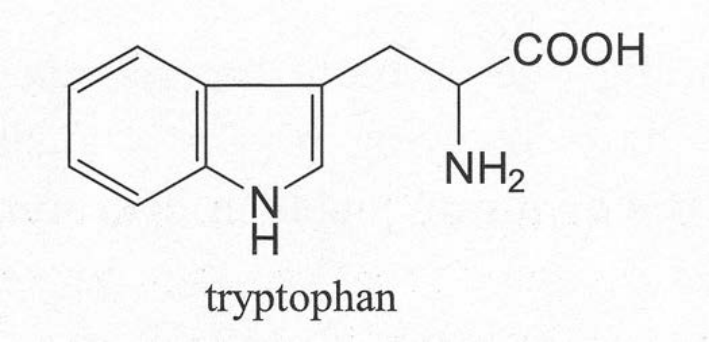
red

wavelength λ

fluorescence quantum yield Φ_F

Energy gap π → π*

Examples of Fluorophores

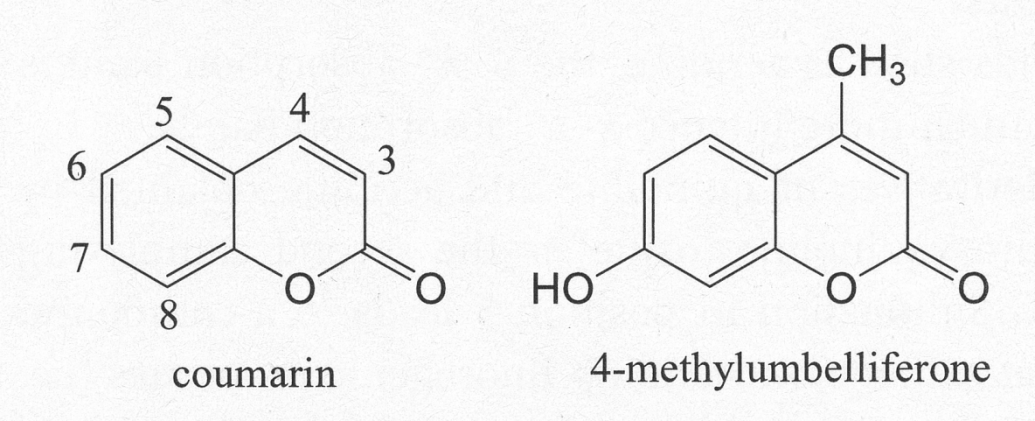


tryptophan

Important for investigations of proteins

UV absorption

Tryptophan



coumarin

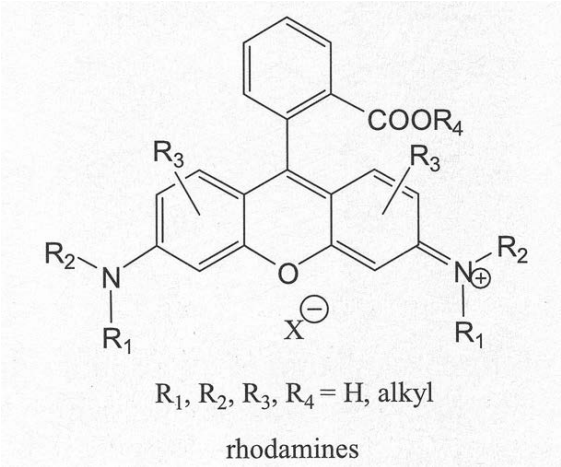
4-methylumbelliferone

Coumarins

blue-green fluorescence
400 – 550 nm
pH-sensitive

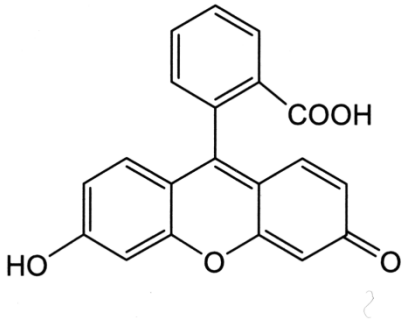
Large Stoke Shift: 50- 100 nm

Rhodamines
- first laser dyes
- small stokes shift
  (20 – 30 nm)
- fluorescence window
  500 – 700 nm



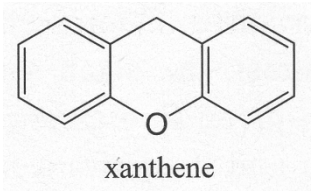
R_1, R_2, R_3, R_4 = H, alkyl

rhodamines

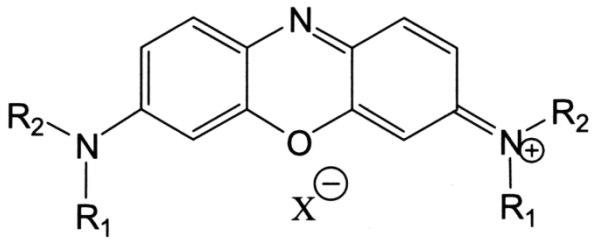


fluorescein

Fluorescein



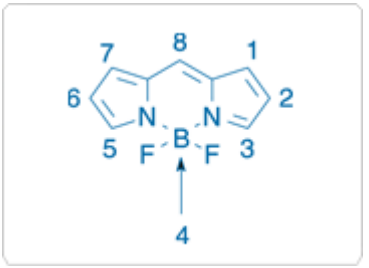
xanthene



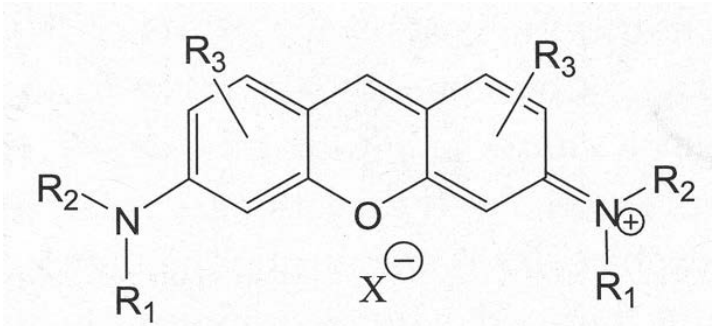
R_1, R_2 = H, alkyl

oxazines

Oxazines



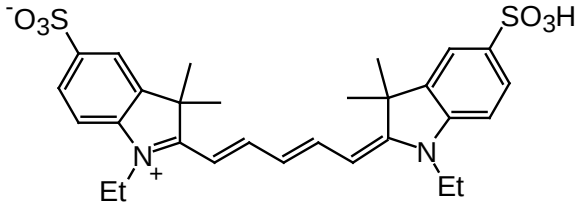
Bodipy series
500 – 700 nm



R_1, R_2, R_3, R_4 = H, alkyl

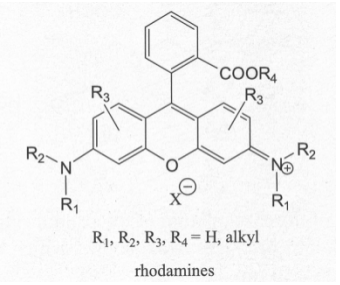
pyronines

Pyronines



Cyanin series
Cy5 (647/670 nm)
cis/trans

Substituted aromatic hydrocarbons



Nature and position of substituent can influence fluorescence characteristics

Electron-donating substituents: -OH, -OR, -NH_2, -NHR, -NR_2

- lon electron pair involved directly in π-bonding of aromatic system (increase of conjugated system)

increase in molar absorption coefficient

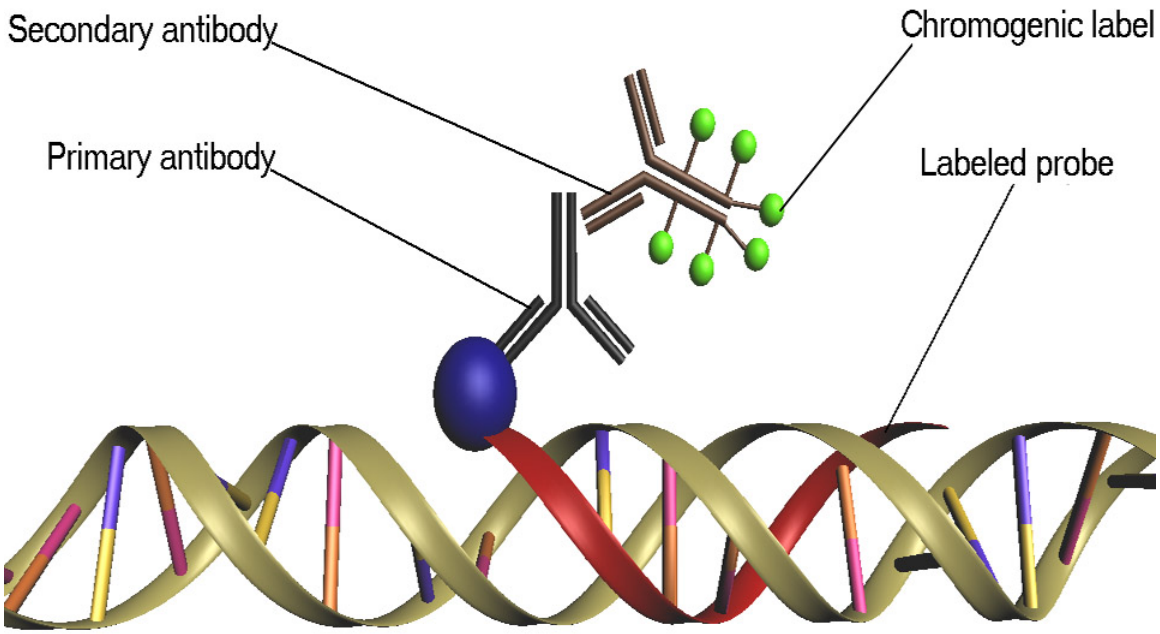
absorption and fluorescence spectra shifted + broad, structure-less

Cellular

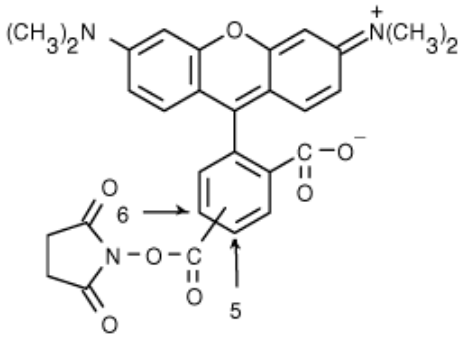
Fluorescence Microscopy

Cellular Labeling - Immunolabeling

- Labeling of cellular object (protein, organelle, DNA, …) via **labeled antibodies** (primary + secondary): recognizes object
- **Use bright + photostable organic dyes!**



Dye



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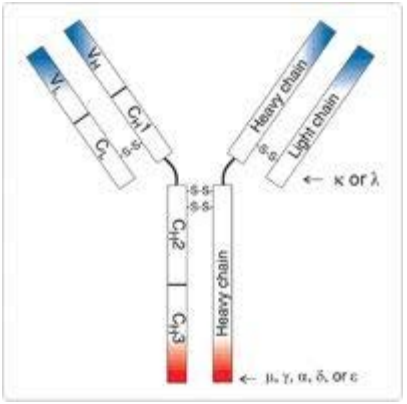
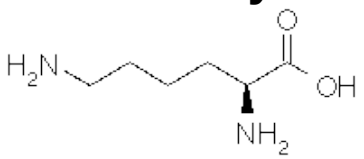


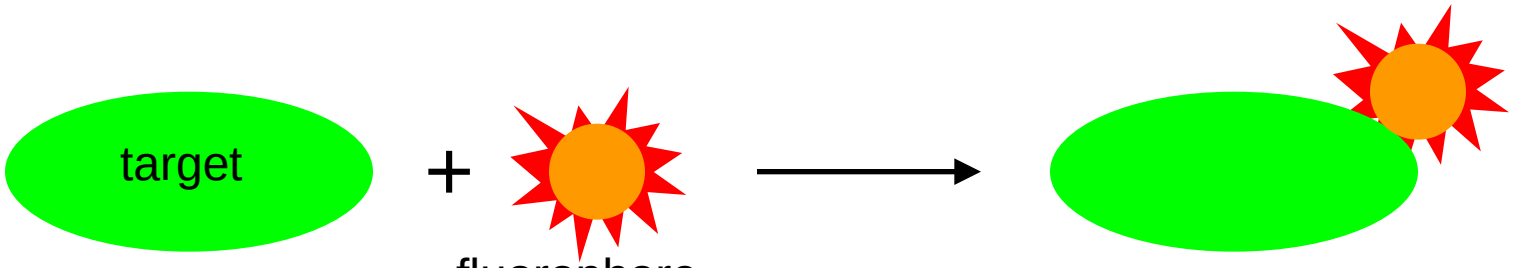
Antibody



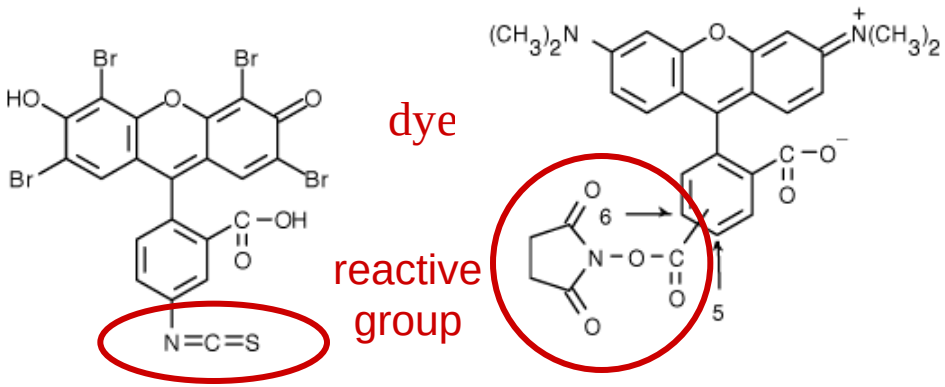
lys k Lysin

Fluorescence Labeling

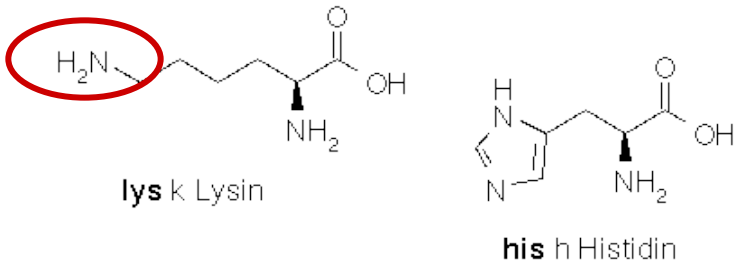
Attachment of fluorophore to target molecule (e.g. protein)

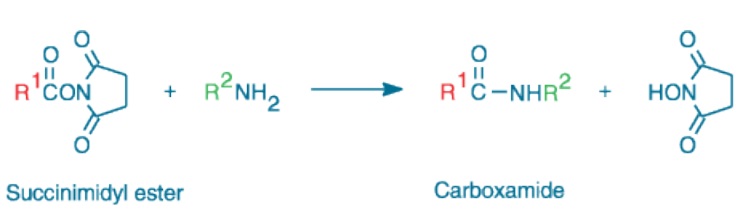


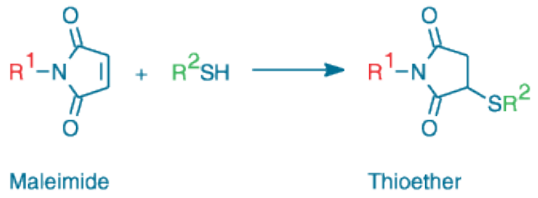
Dye with reactive group



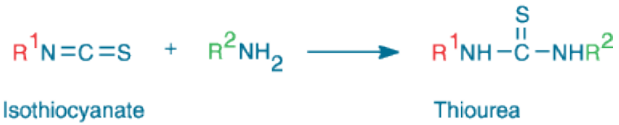
Reactive (amino) group at protein







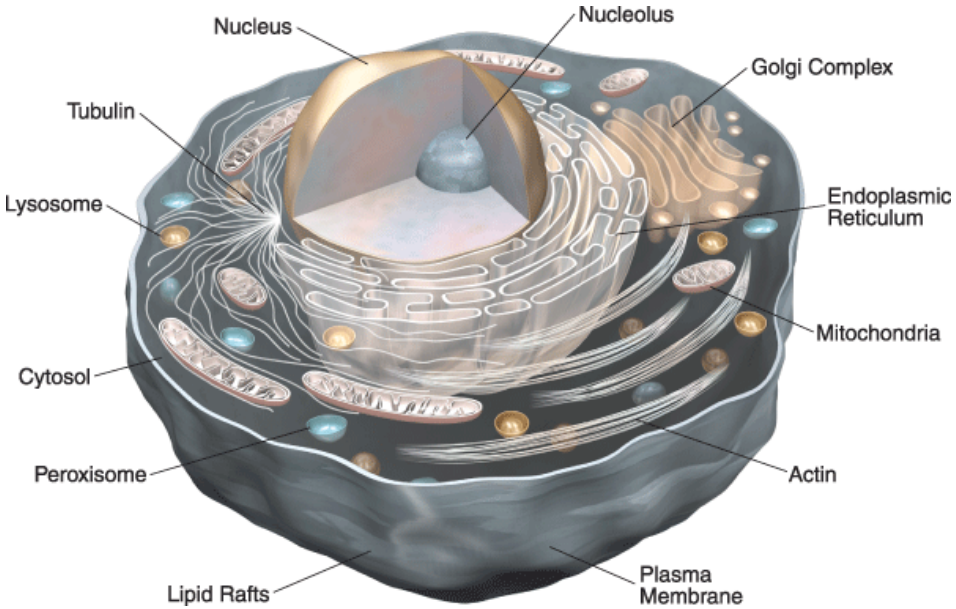
Amine-Reactive

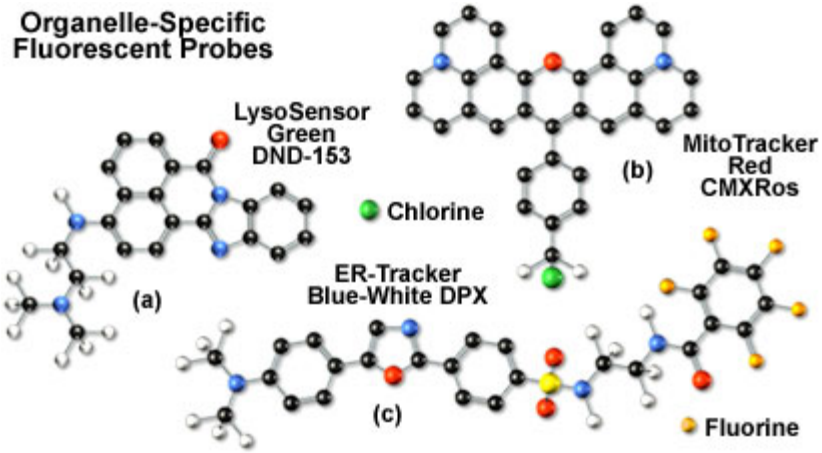


Thiol-Reactive

Cellular Labeling

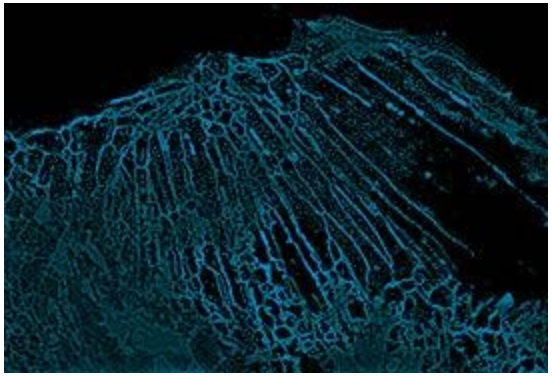
Organelle-specific fluorescent probes





Membrane permeable

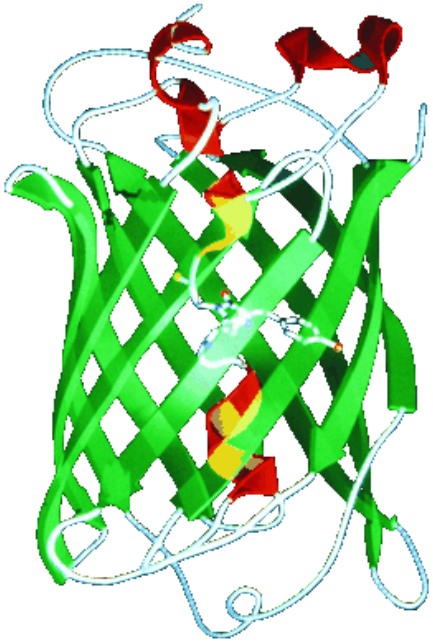
- Example: Endoplasmatic Reticulum (ER)

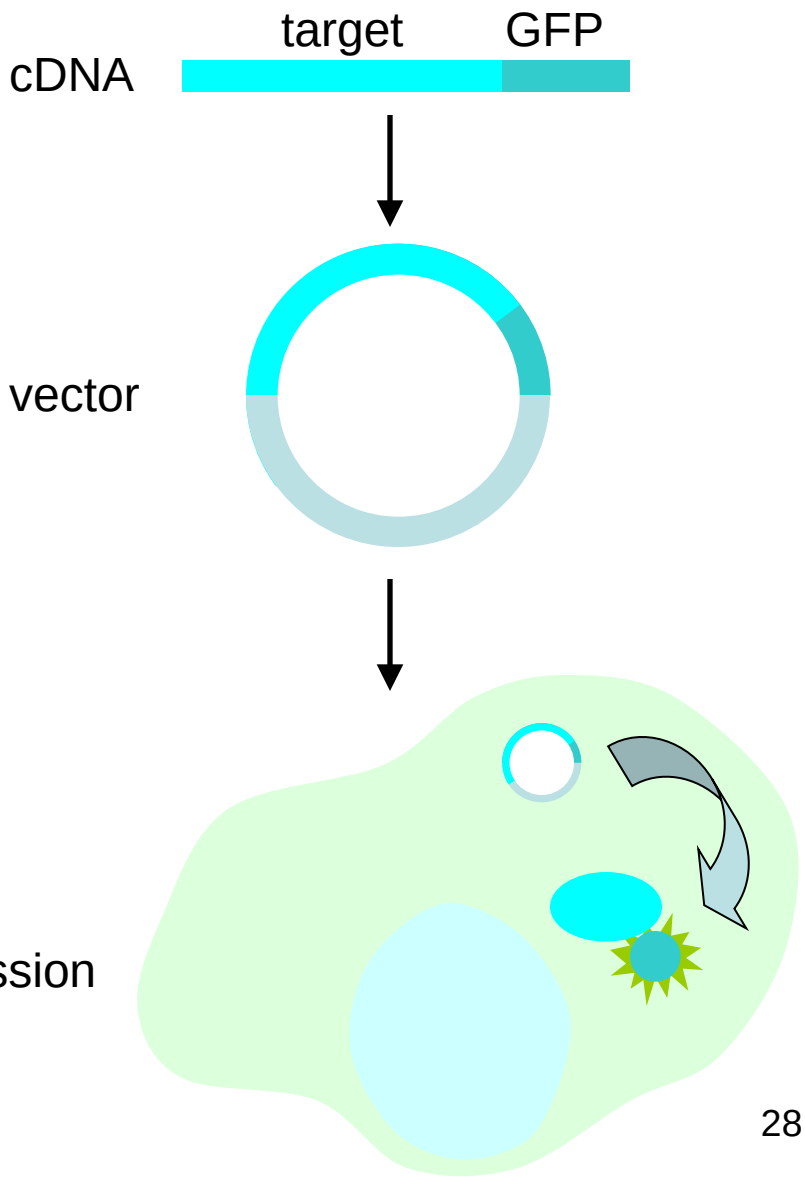


Liive Cell Labeling: Fluorescent Proteins

Genetically encoded fluorescent probes

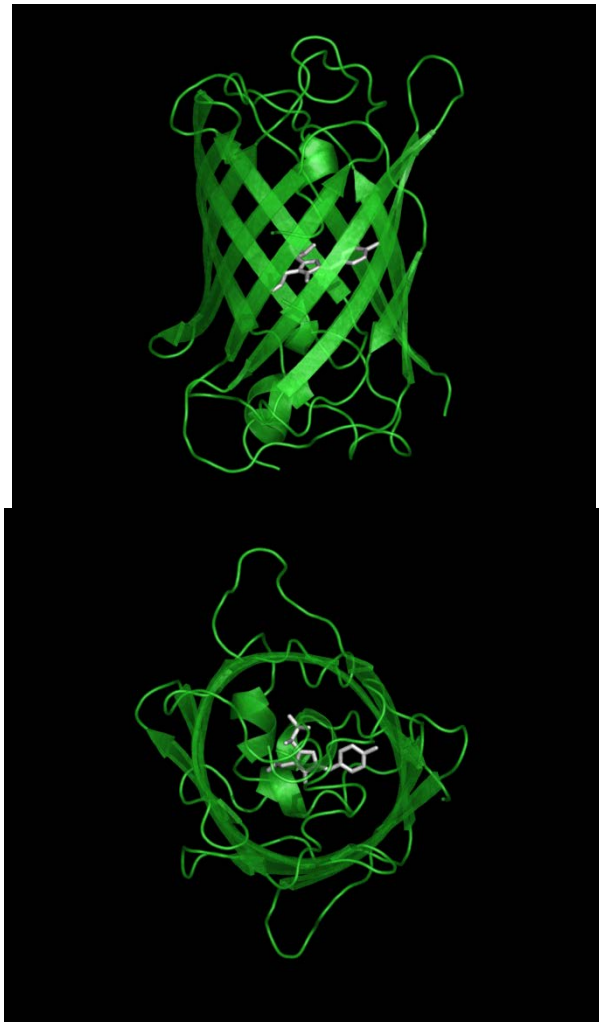
Green fluorescent protein (GFP)





Fluorescent Proteins

Green-Fluorescent Protein (GFP)



GFP 1,9Å (1EMA, Ormo, *Science*, 1996)

-GFP from water jellyfish (*Aequorea victoria*)
absorption: 395 + 475 nm
fluorescence: 508 nm

- variety of Mutants:

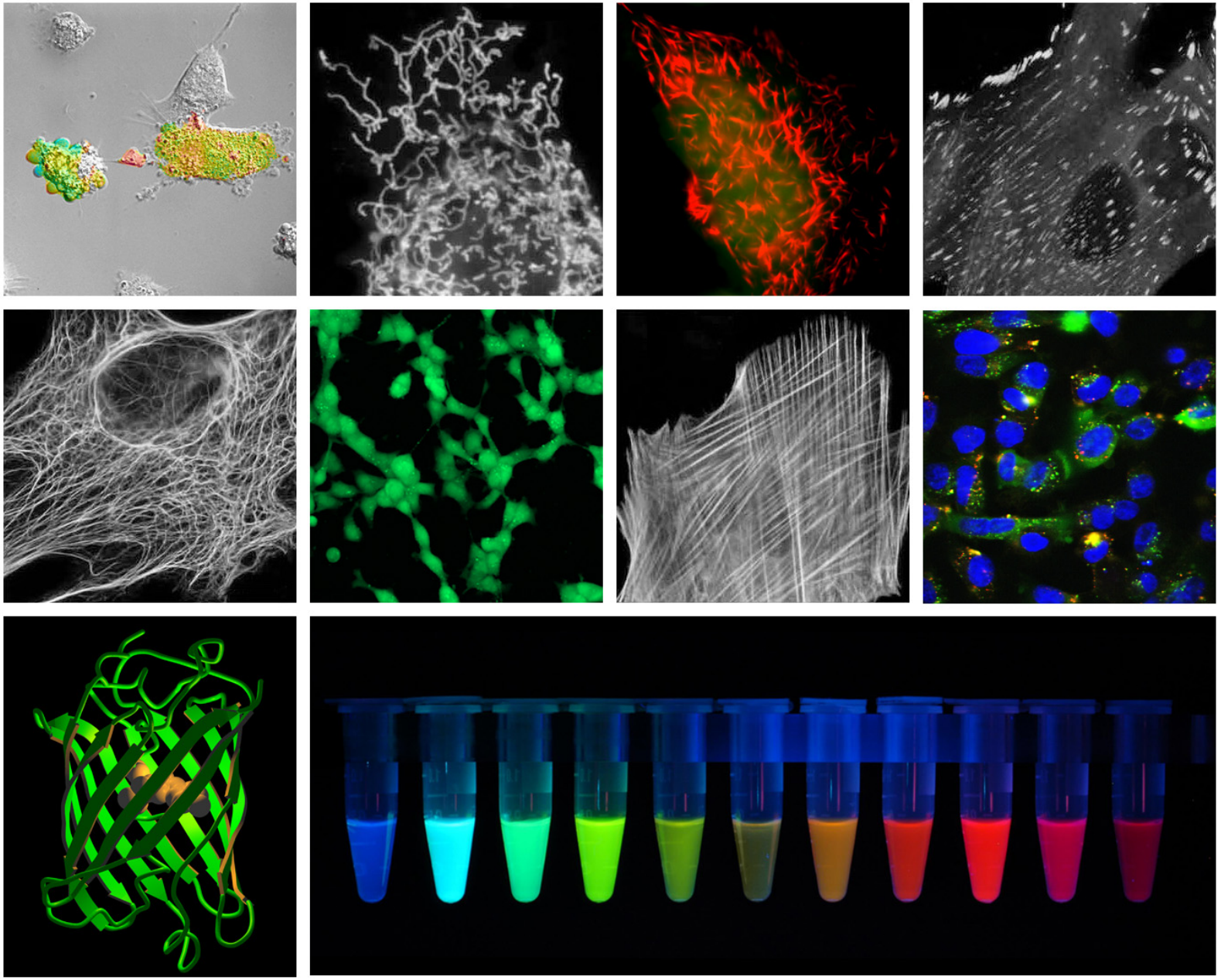
pH-senitive (pHGFP)

enhanced fluorescence (EGFP)

shifted emission (e.g. EYFP)

- simple expression, folding and cloning

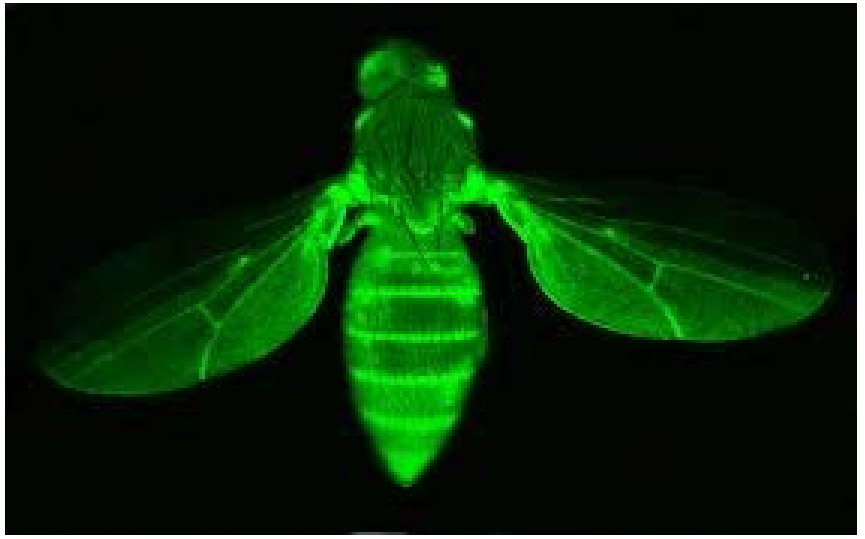
Fluorescent proteins



Tsien home page

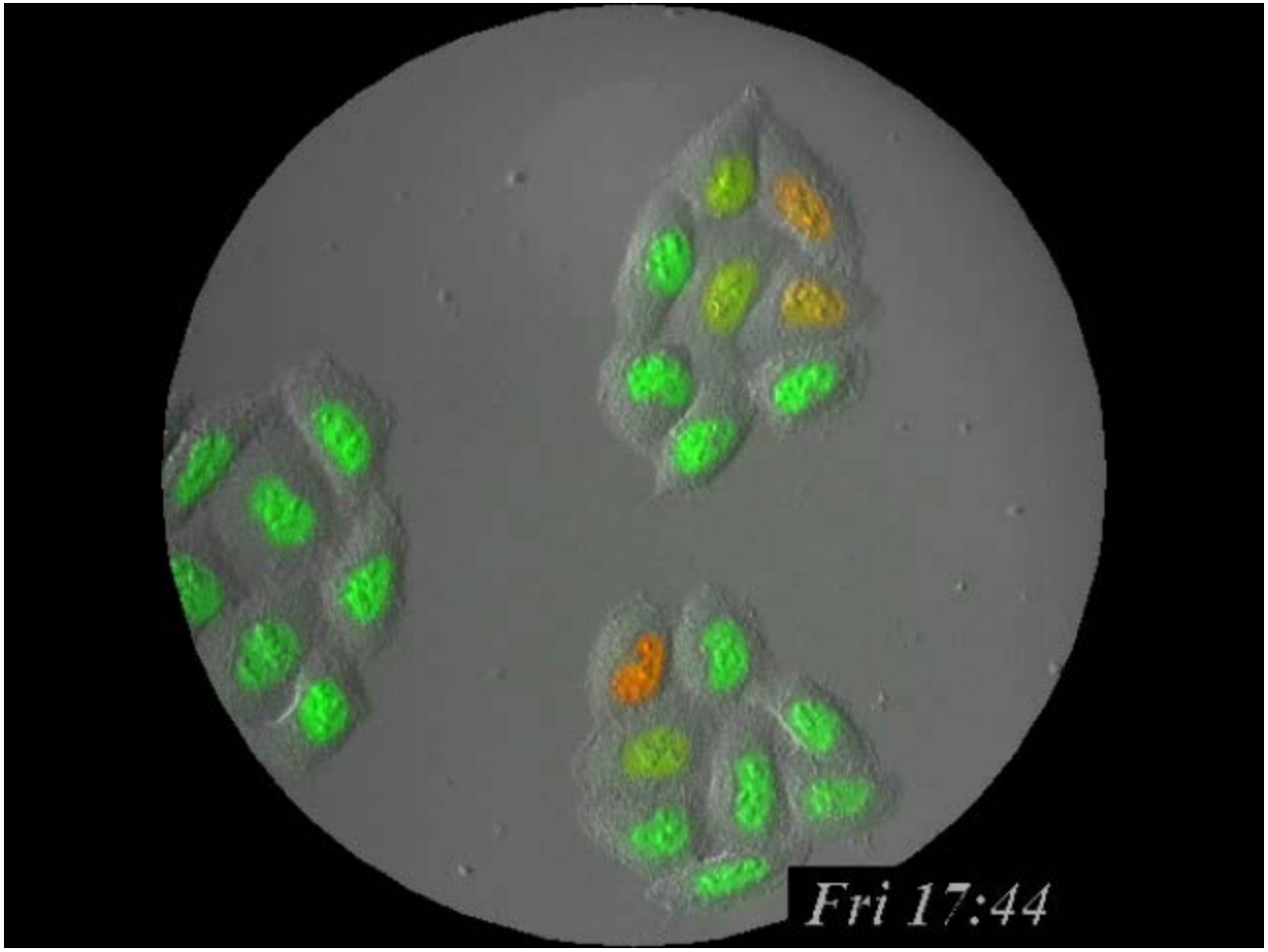
Fluorescent proteins – living species

Transgenic GFP mouse
brainwindows.wordpress.com



GFP in flies
tursiops-biology.com

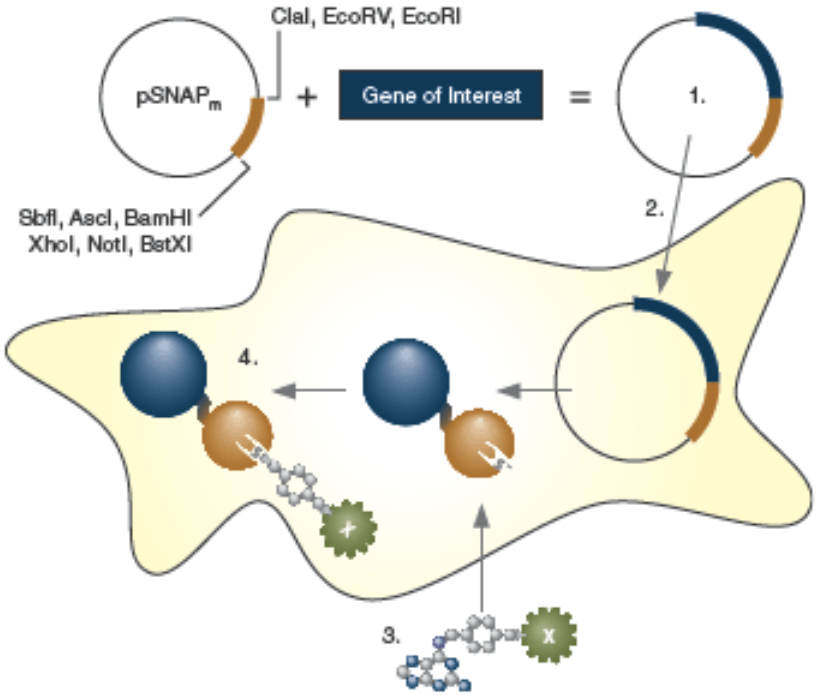
Fluorescent proteins - Dynamics



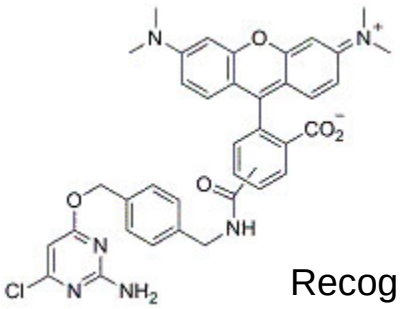
Miyawaki and coworkers, Cell 2008, 132, 487-498

Live Cell Labeling: Organic Dyes (SNAP/HALO/CLIP/…)

Genetically encoded tags – recognizing functionalized dyes

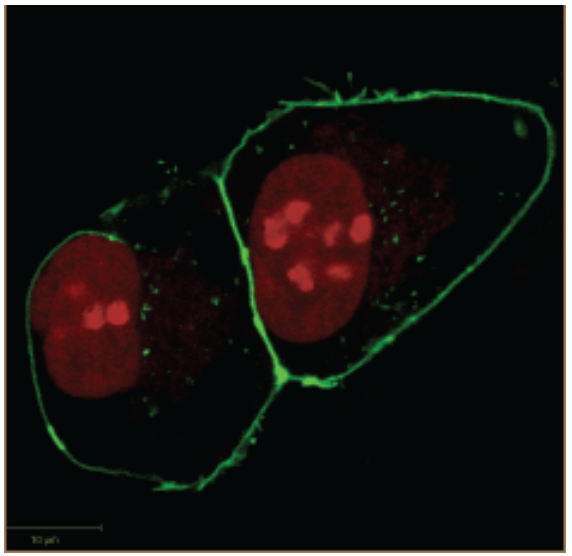


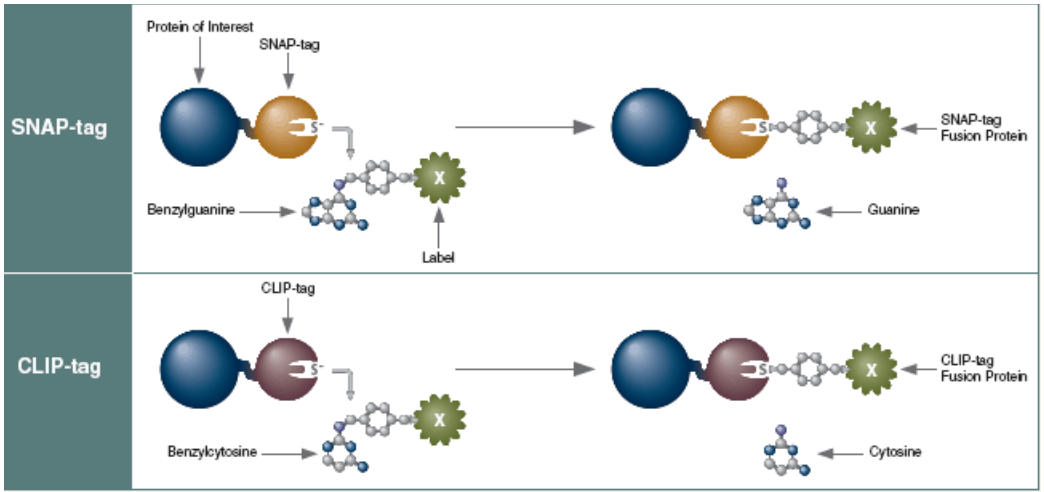
Membrane permeable



Dye: TMR

Recognizing group





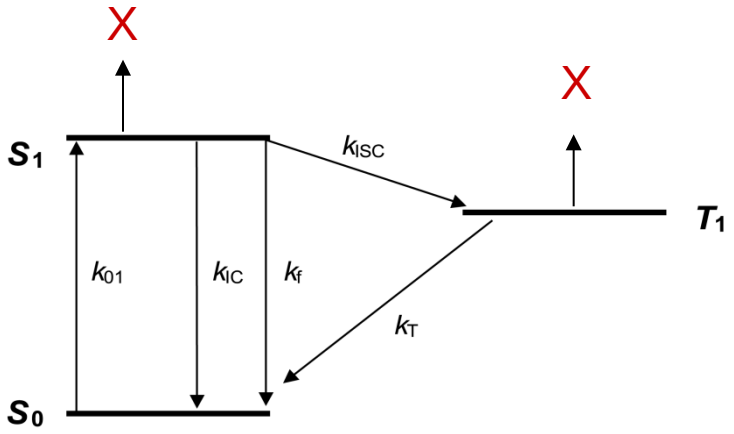
http://www.neb.com/

Optimization
of
Fluorescence Signal

Fluorescence - *Limits*

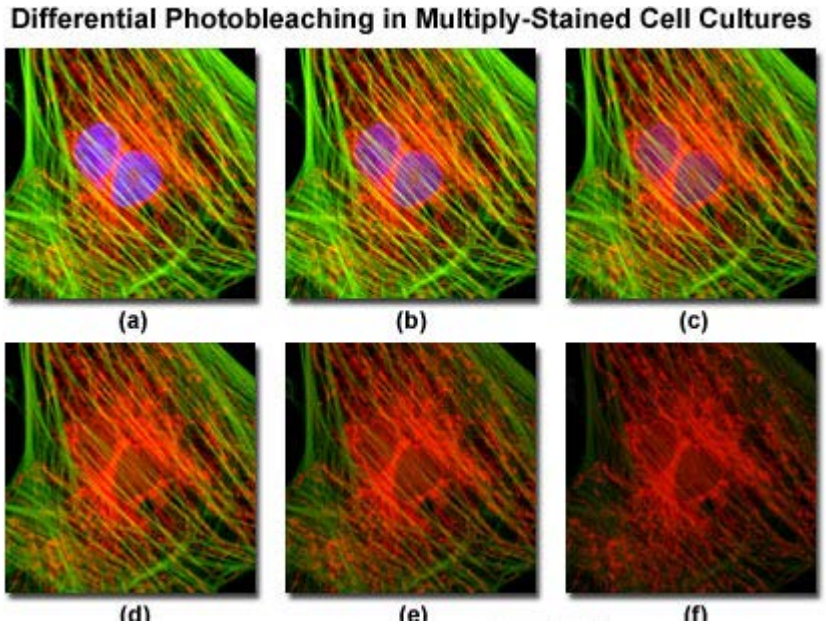
Photophysics: Dark (triplet) states + Photobleaching

After Excitation:



Transition into long-lived dark (triplet) states
DARK PERIODS (reversible loss of signal)

Enhanced reactivity (state of higher energy)
PHOTOBLEACHING
IRREVERSIBLE LOSS OF SIGNAL



Limits

fluorescence signal/yield

signal to background ratio

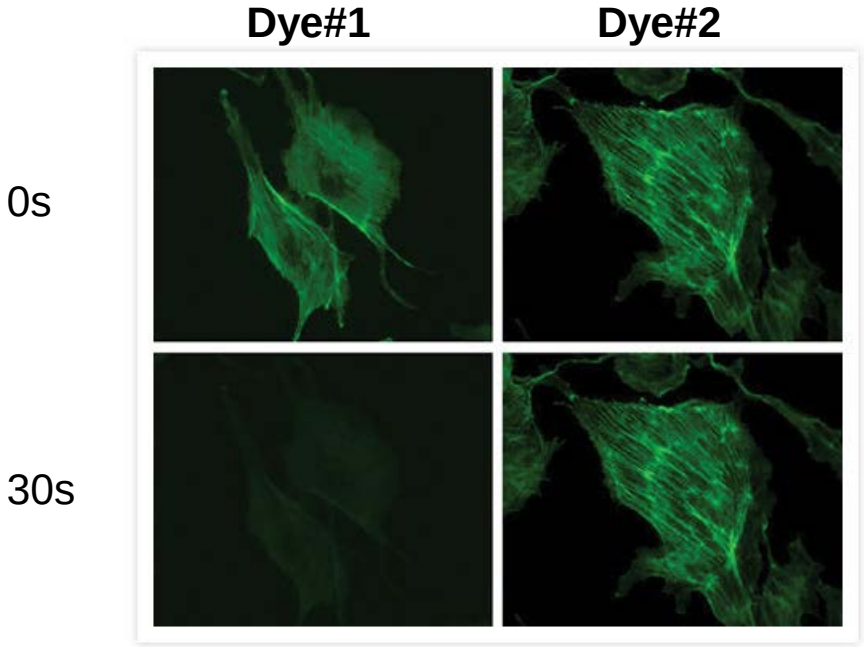
observation time

Optimization of Fluorescence Signal

Fluorophore

A good fluorophore

- Large absorption/extinction coefficient (≈ 10^5 $cm^{-1}M^{-1}$)
- High fluorescence quantum yield (> 0.8)
- Large shift of the fluorescence vs. absorption, Stokes shift (> 40 nm)
- Low quantum yield of photobleaching (< 10^{-6})



Immunolabeled actin

Invitrogen

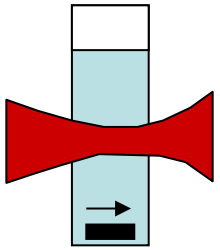
Optimization of Fluorescence Signal

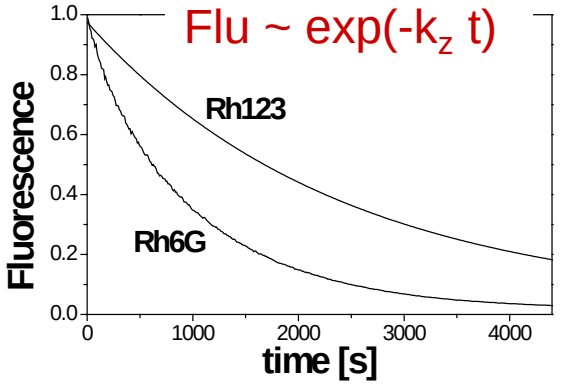
Photobleaching at Low Laser Irradiances (Widefield)

Simple Experiments – Cell Bleaching

Irradiate dye solution in cuvette

exponential decay over time due to photobleaching





Probability of photobleaching

p_b =

photobleached molecules / # excitations to S_1

$p_b = k_z / (k_{01} S_0) = k_b \tau_{Flu}$

Number of survived absorption cycles

$\mu = 1/p_b$

Dye	p_b	μ	Exc/Em [nm]
Coumarins (C-120, C-307, Carbostyril)	10^{-3}-10^{-4}	~1000 – 5000	350/450
Rhodamines (Rh6G,TMR,Rh123)	10^{-6}-10^{-7}	~1,000,000	500/570
Fluorescein	$3x10^{-5}$	~30,000	500/520
Texas Red	$5x10^{-5}$	~20,000	590/620
Cyanin 5	$5x10^{-6}$	~200,000	650/670
EGFP	~10^{-7}	~100,000	490/520

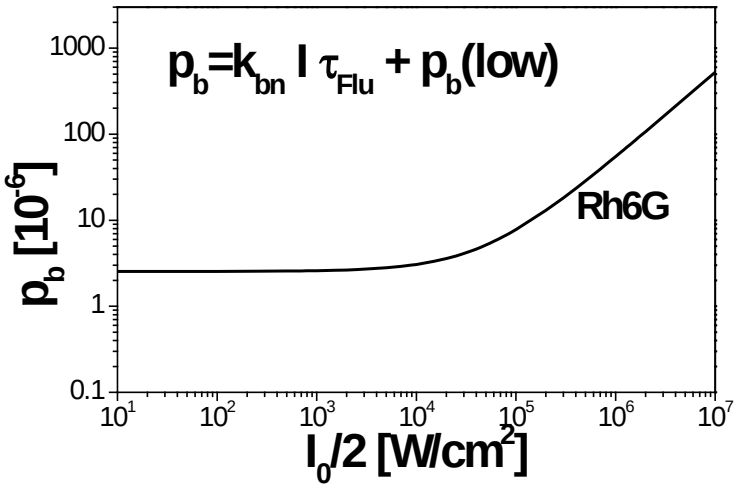
Optimization of Fluorescence Signal

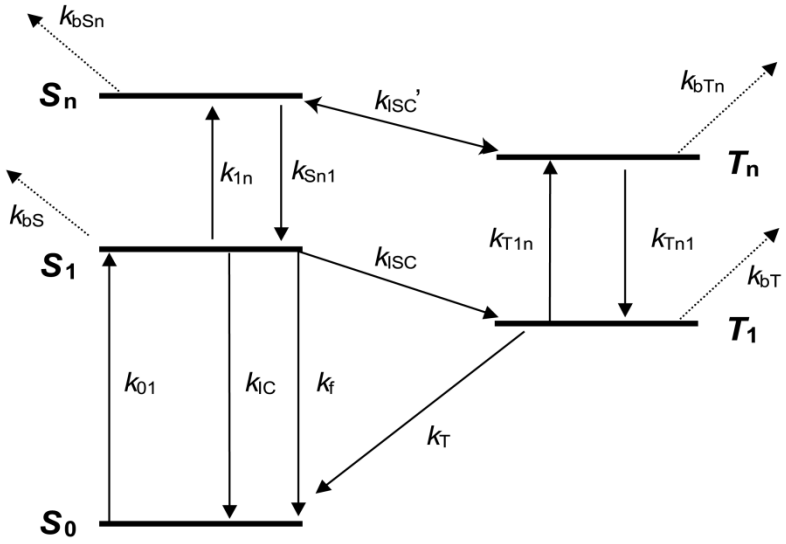
Photobleaching at High Laser Irradiances (Confocal)

Low irradiances (W/cm^2)
⇒ wide-field microscopy

Confocal microscopy
⇒ High irradiances (kW/cm^2)

⇒ Increase of photobleaching (p_b) with irradiance I
⇒ Decrease of survived absorption cycles μ





Photobleaching from higher excited states

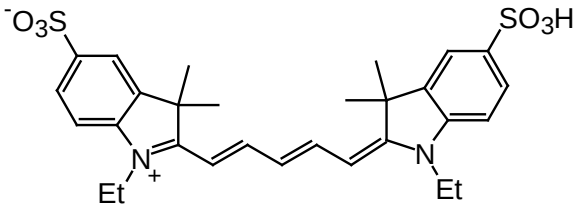
- S_n and T_n
  - -effective photobleaching – ionic states
  - -population ~ irradiance I (additional absorption)
  - -dominant at high irradiance
  - $-\sigma_{(T)1n}$ ~ (1/100 – 1/10) σ_{01}

- efficient via triplet (long-lived)

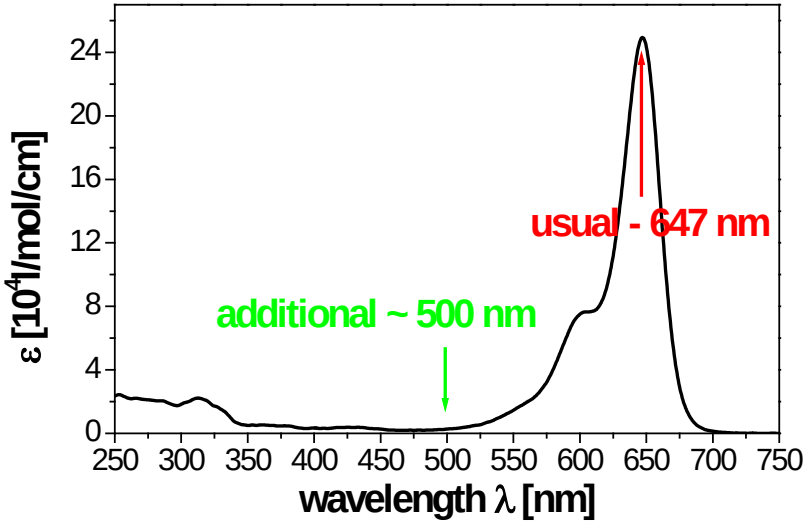
Optimization of Fluorescence Signal

Multi-Colour

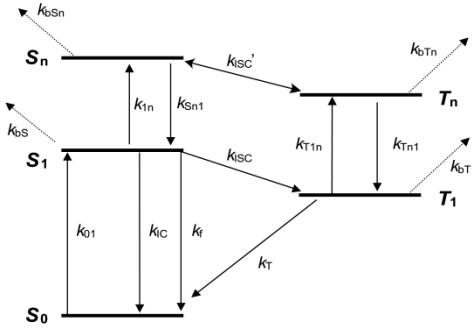
Simultaneous excitation with green/red light
or green excitation in FRET-experiments
for red dye

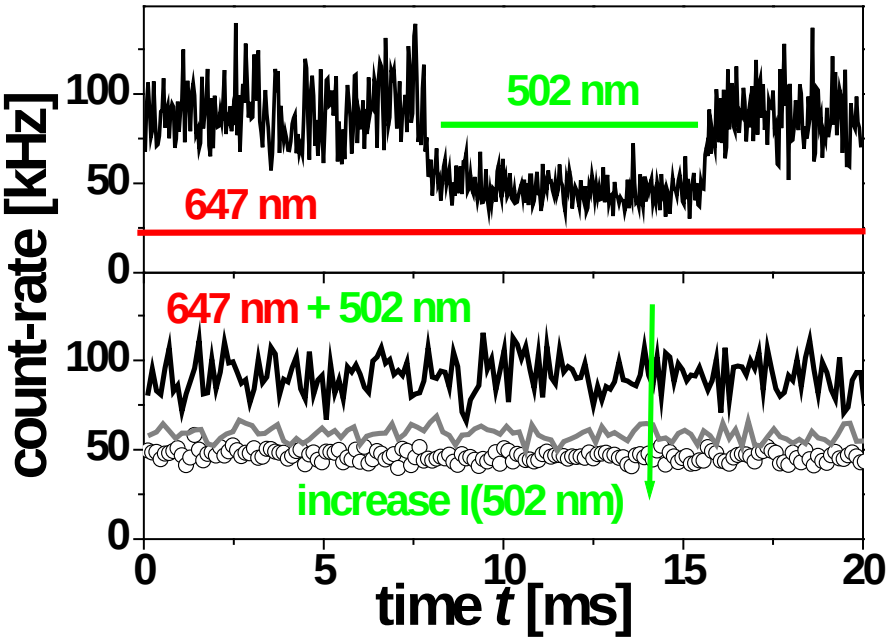


Cyanin5
water



⇒Additional photobleaching
due to excitation to S_n/T_n by
green light



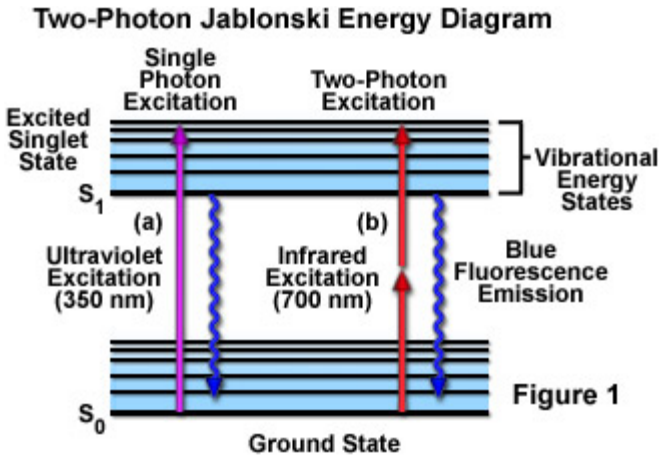


⇒ Adapt green irradiance in
Multiple laser experiments

Two-photon microscopy

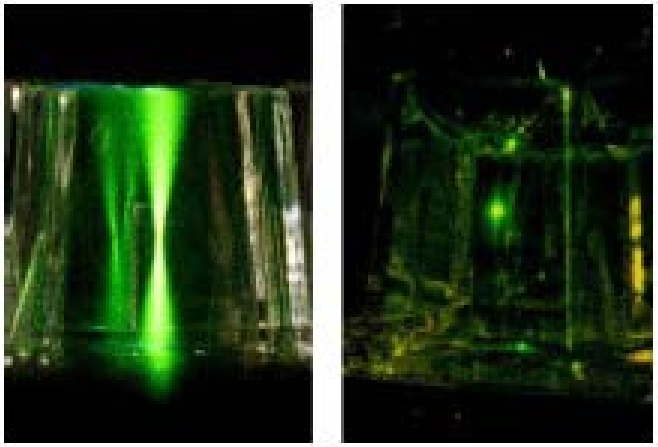
Two-Photon Microscopy - Principle

Two-photon excitation microscopy



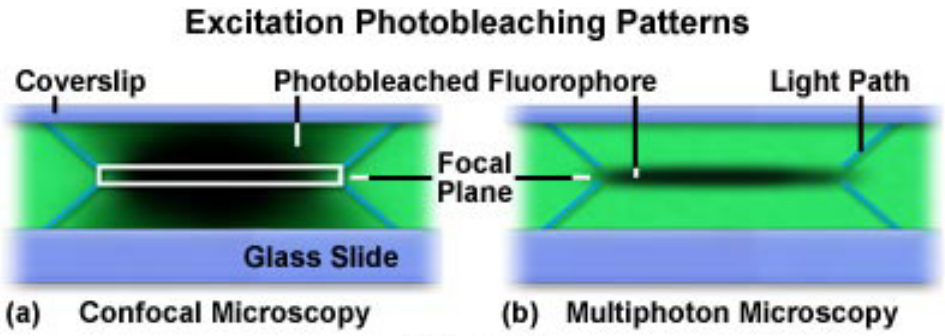
Only excitation at region of high irradiance

-localization to focus



Schwille, Appl.Phys.B 2001, 73

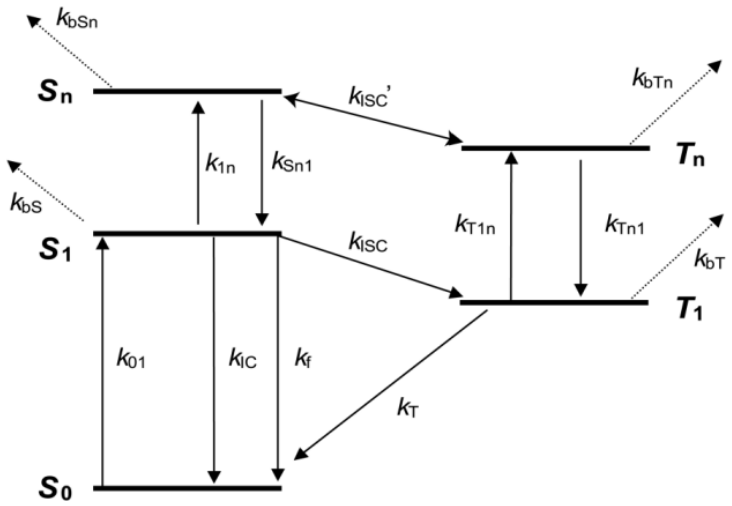
-No out-of focus photobleaching



Optimization of Fluorescence Signal
Two-Photon Excitation

Two-photon excitation microscopy – photobleaching in focal region

Enhanced / saturated bleaching from S_n / T_n
⇒ too high photon density
⇒ one- (or more step - k_{nn}) absorption steps
⇒ ionization (more in water)



⇒ extreme photobleaching inside focus (no bleaching outside focus)

⇒ 100/1000-times less count-rate per molecule than one-photon excitation

Alternatives

Fluorescent nanoparticles

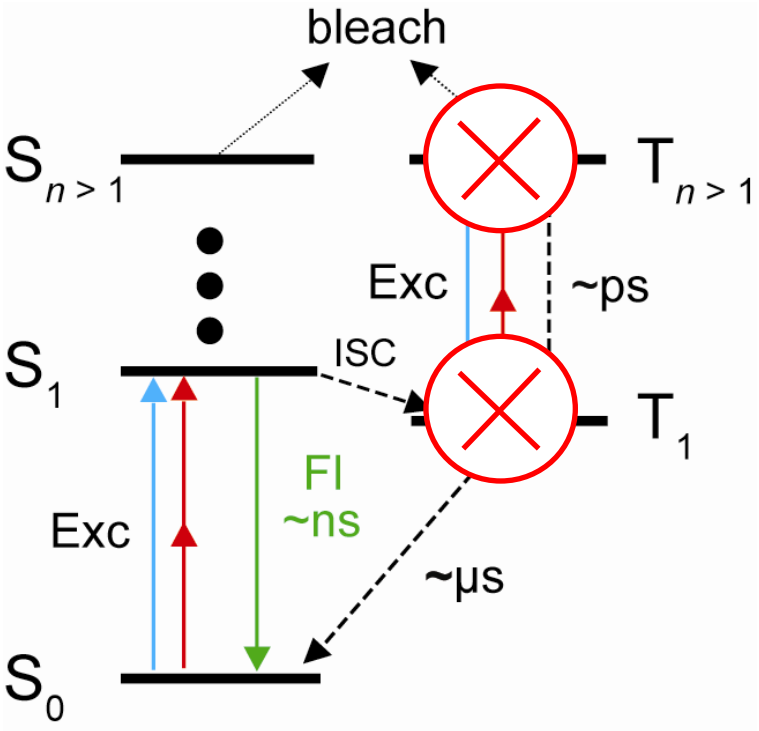
- Quantum dots

CdSe, CdTe, InAs, ….

Michalet X, FF Pinaud, LA Bentolila, JM Tsay, S Doose, JJ Li, G Sundaresan, AM Wu, SS Gambhir and S Weiss. (2005) Quantum dots for live cells, in vivo imaging, and diagnostics. Science 307: 538.

Optimization of Fluorescence Signal

Dark/Triplet-State Relaxation



Saturation:

Long-lived triplet (dark) state > µs

Photobleaching:

Higher excited (triplet) states

⇒ Wait for the triplet state to relax between successive excitation events

⇒ No triplet population
No higher-order photobleaching

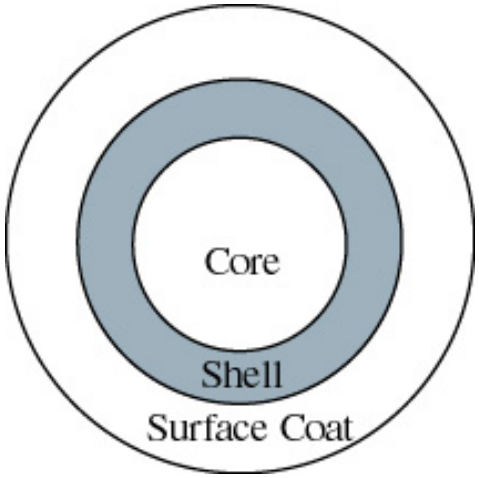
⇒ Low-Repetition Rate Excitation
(> µs - < 1 MHz)

⇒ Fast Scanning

D-Rex Microscopy
(dark-state relaxation)

Donnert, Eggeling, Hell *Nat Meth* 2006

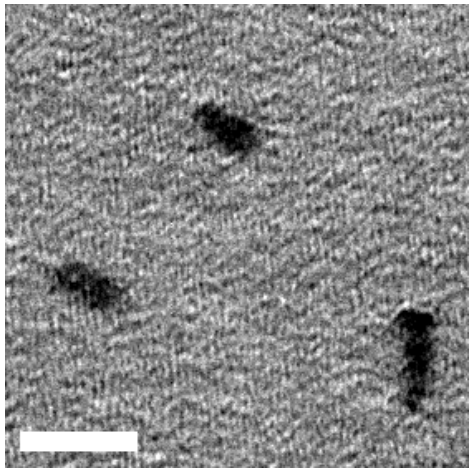
Quantum dot basics



Qdots are inorganic nanocrystals
(~10^2-10^3 atoms)

Core/Shell: Material determines wavelength range
CdSe/ZnS (visible)

Size determines exact wavelength
3 nm CdSe -> 520 nm emission
5.5 nm CdSe -> 630 nm emission



Surface Coat: Renders water solubility
Facilitates bioconjugation

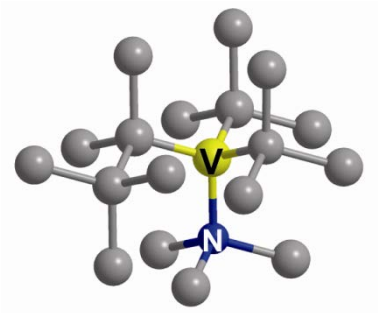
Overall size of 10 nm – 20 nm diameter

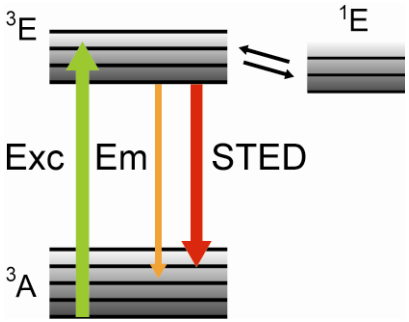
Fluorescence Nanoscopy

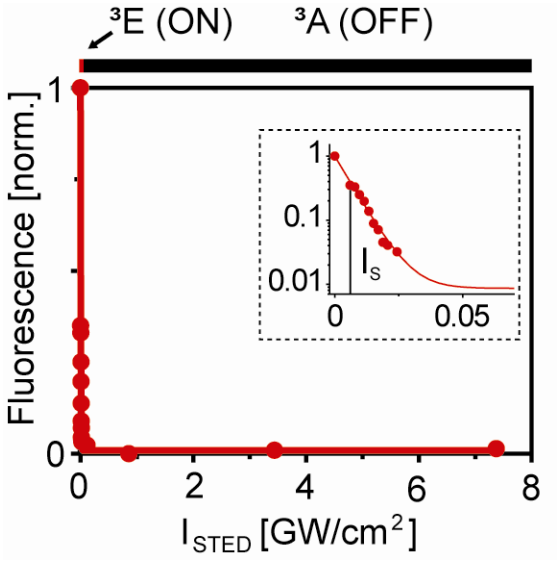
NV centers in diamond

Nitrogen vacancies (NV) centers in diamond

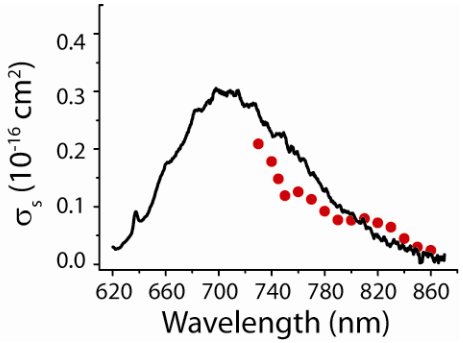
Ultrastable luminescence sources (Gruber et al. Science 1996)







Excitation 532nm – Luminescence ~700nm

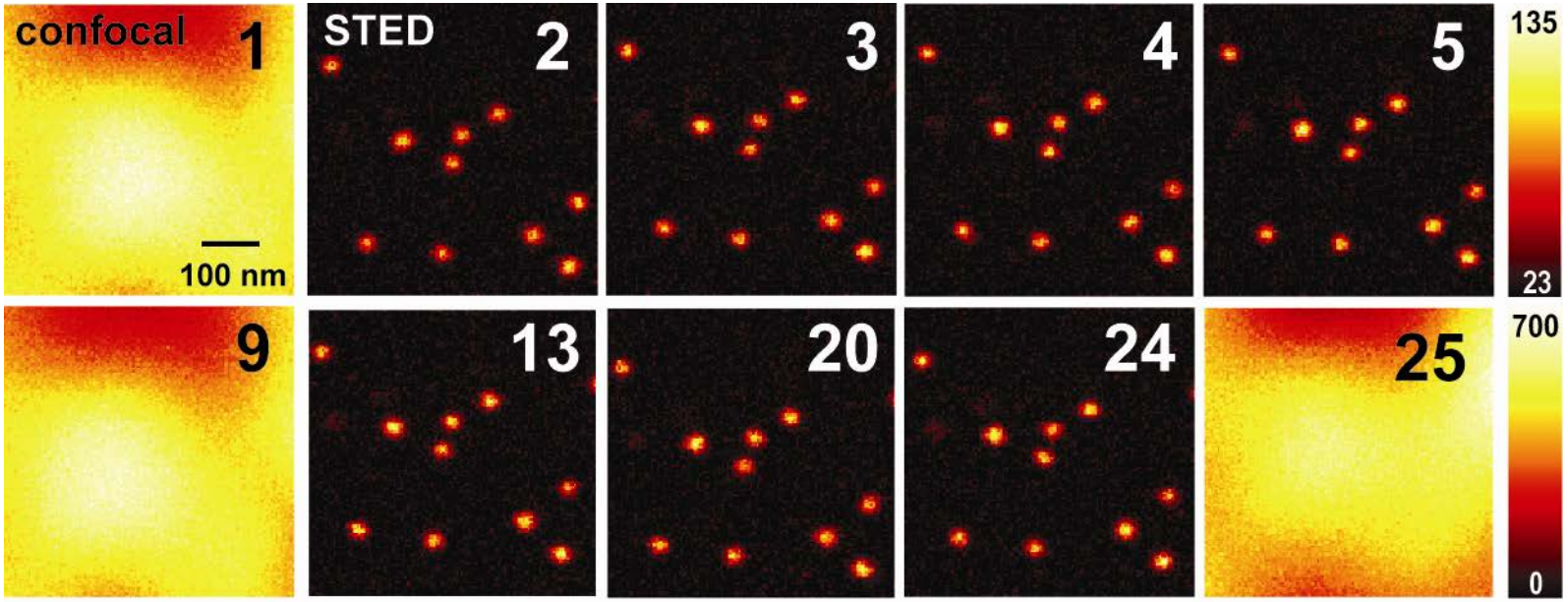


Fluorescence Nanoscopy

STED Imaging on single NV centers

STED on “isolated“ NV centers
in diamond of type IIa grown by chemical vapour deposition (Jelezko, Wrachtrup (Stuttgart))

exc: 532nm - STED: 775nm 8MHz

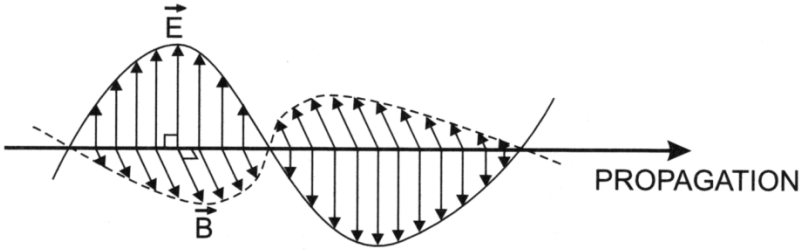


E. Rittweger, K.Y. Han et al, Nature Photonics 2009

Fluorescence

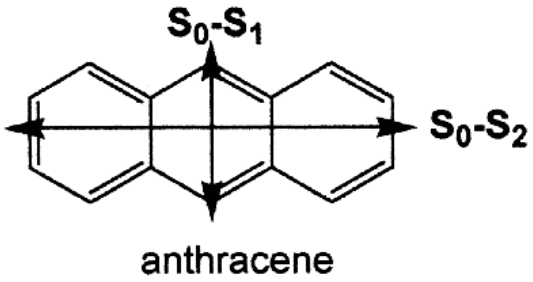
Anisotropy

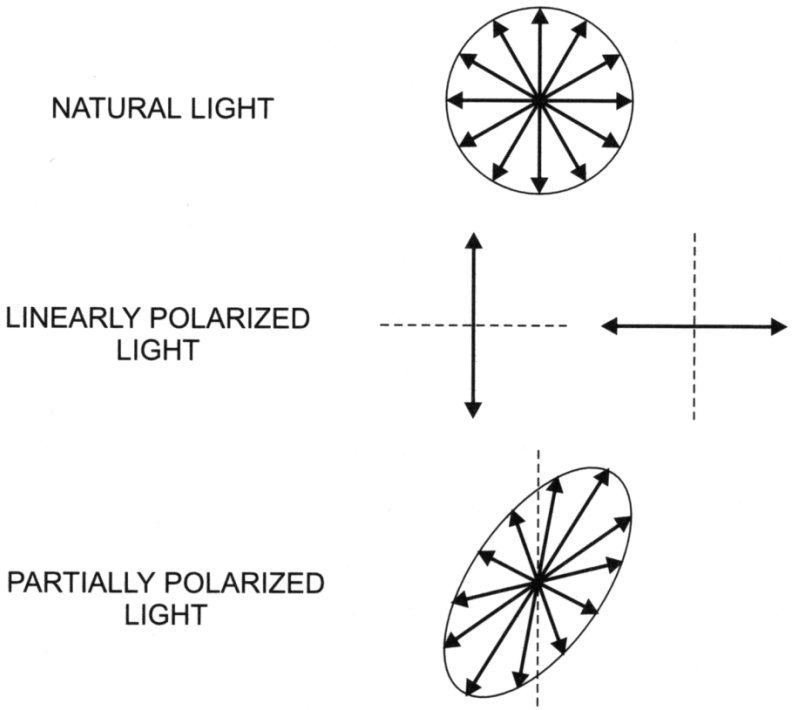
Polarized excitation - Anisotropy



Absorption Transition Moment

Most chromophores absorb light along a preferred direction, depending on electronic state

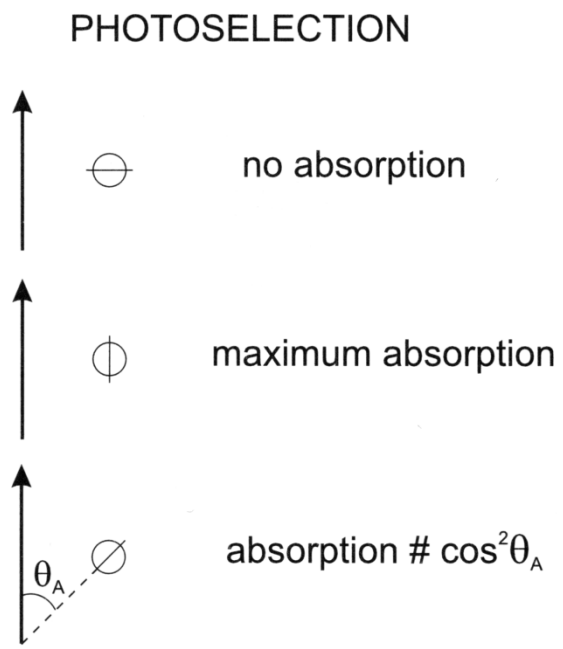




Light is an electromegnetic wave consisting of electric field E and magnetic field M

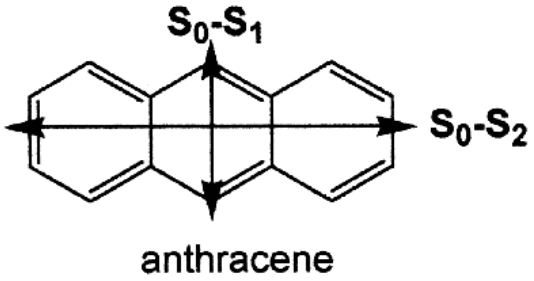
Oscillation direction!

Polarized excitation - Photoselection



θ_A: angle between electric vector E of incident light and absorption transition moment M_A

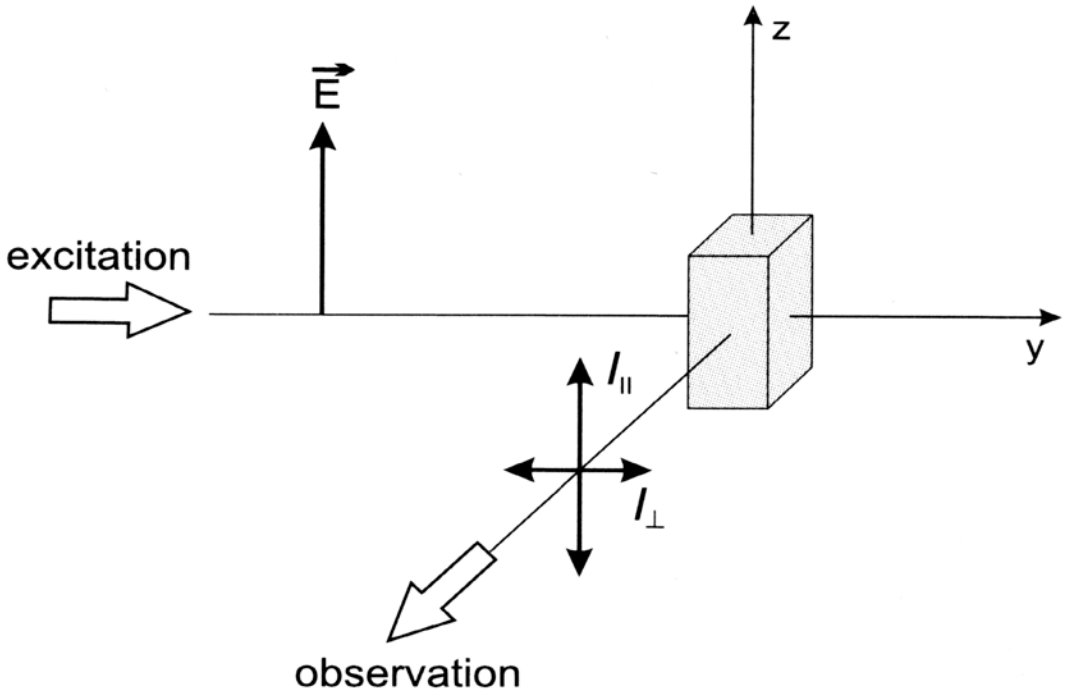
Linearly polarized incident light:
Excitation probability ~ $\cos^2\theta_A$



Fluorescence polarization measurements provide information about mol. mobility, size, shape, orientation and flexibility of molecules and fluidity of medium

Configuration for measuring fluorescence polarization

measure I_{par} and I_{perp} separately



Configuration for measuring fluorescence polarization

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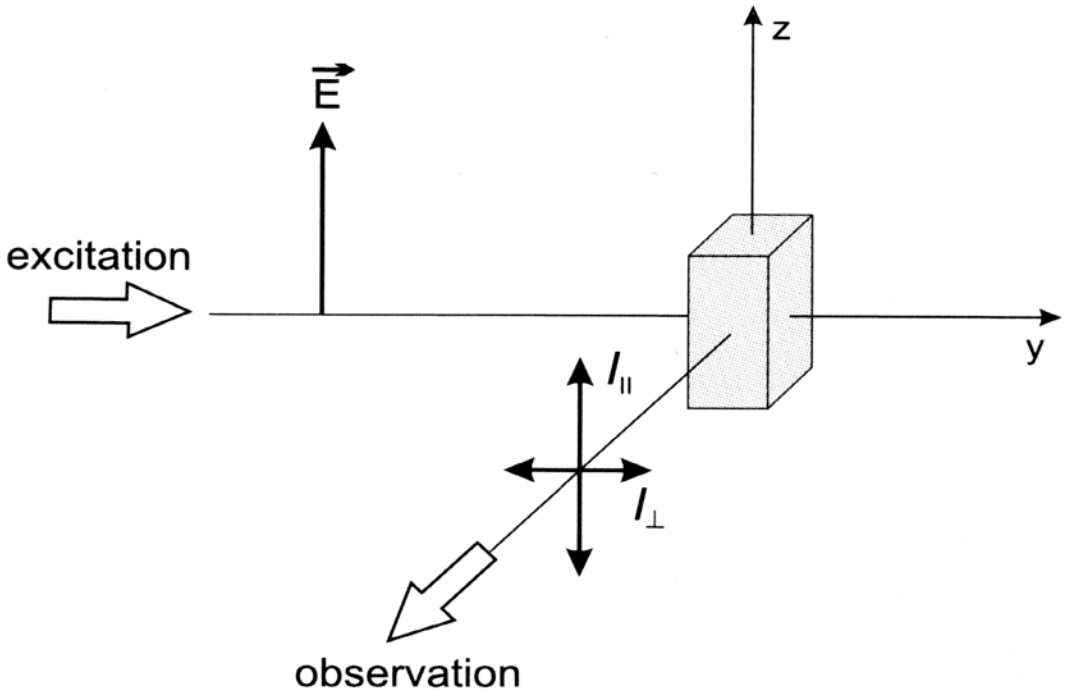


Anisotropy

polarization ratio

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

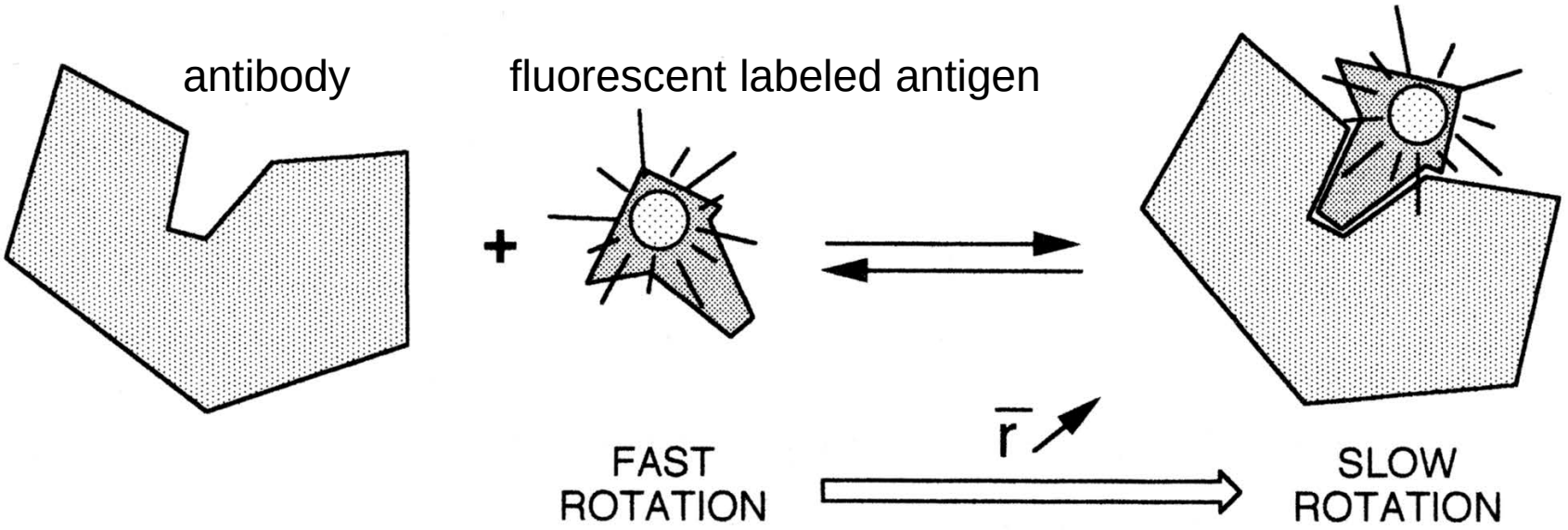
emission anisotropy

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

$I_{tot} = I_{\parallel} + 2I_{\perp}$

$$r = \frac{2p}{3 - p}$$

applications:



equilibrium studies:
free and bound species have different rotational rates / anisotropies