Extrinsic fluorescence

Fluorescence methods in life sciences

Ctirad Hofr

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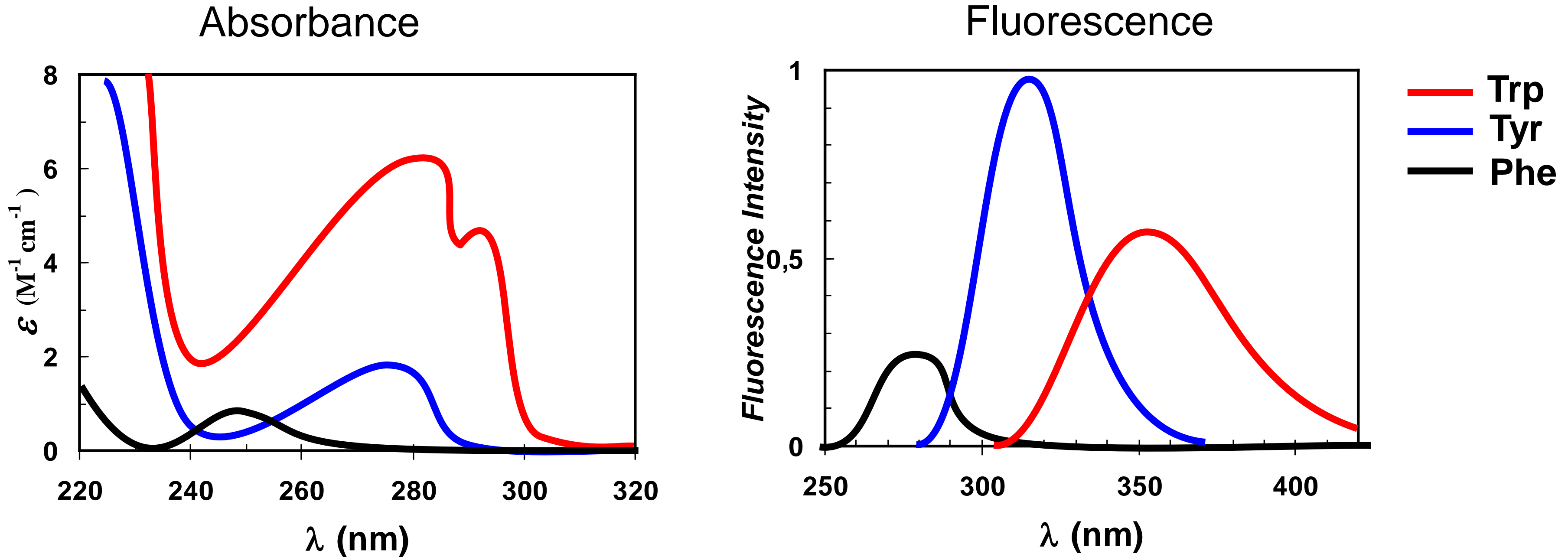
Is intrinsic protein fluorescence usefull for concentration determination?



- Only to a very limited extent and for certain proteins (dependence of tryptophan emission on the position in a protein structure, mutual fluorescence quenching of amino acids by energy transfer)
- Concentration determination is more accurate with external labeling

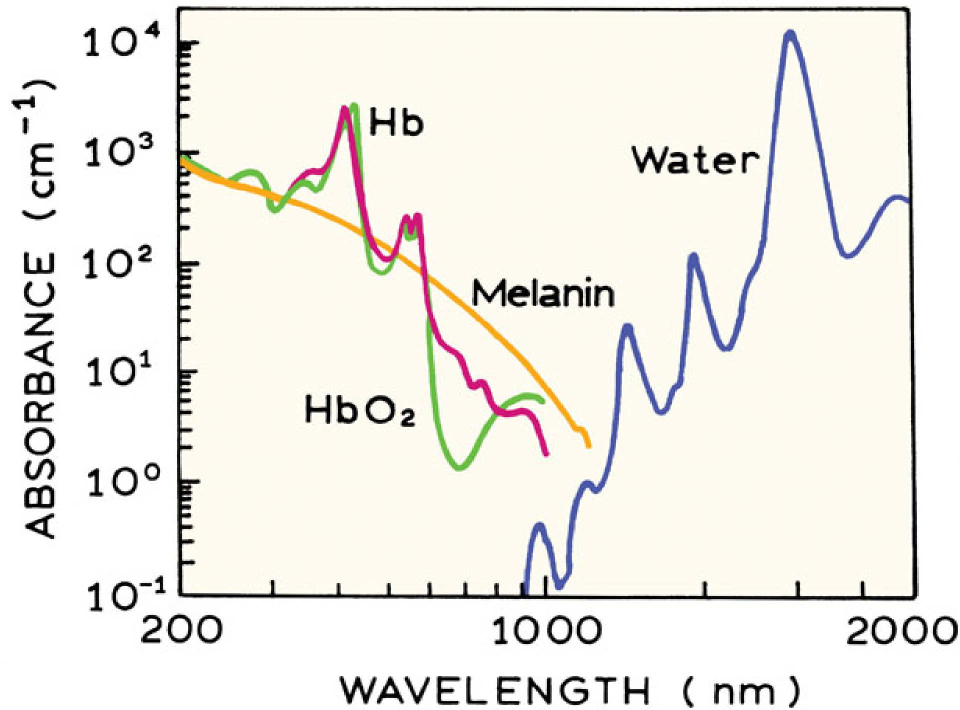
Extrinsic fluorophores

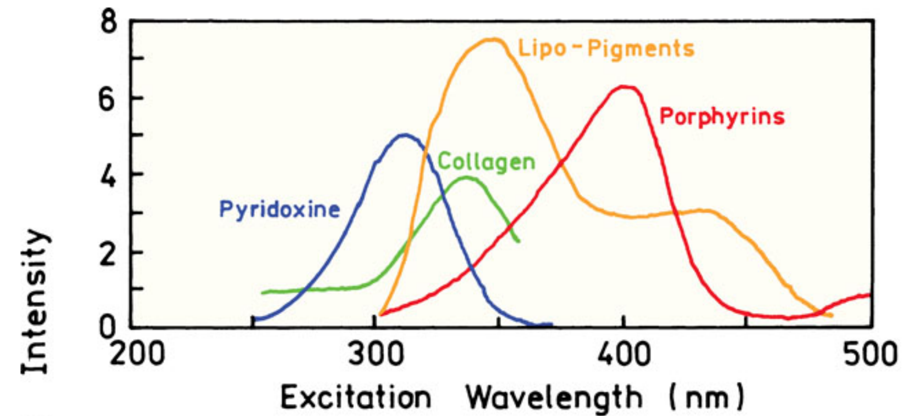
External, alias extrinsic fluorophores are more used than intrinsic fluorophores.

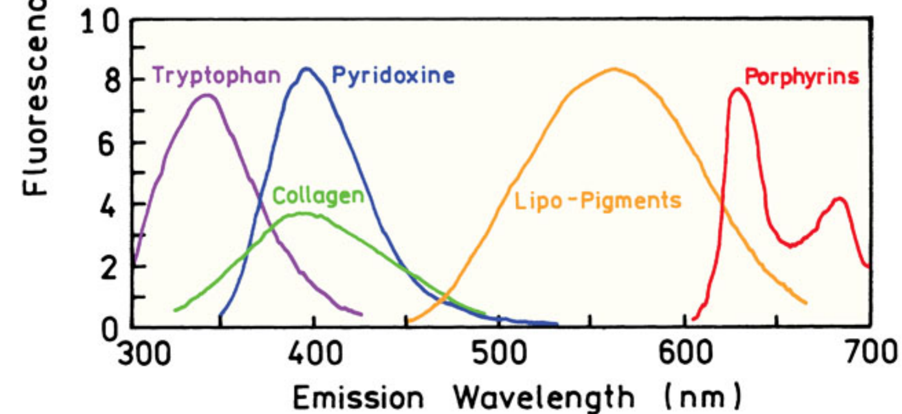
Fluorescent labels – are added and covalently bound to a sample. They bind to proteins and nucleic acids via amine, sulphydryl or histidine side chains and thiol groups.

Fluorescent probes – are bound noncovalently to a sample and then change their fluorescent properties (e.g. intensity, position of em. maximum)

Absorbance of biological material







- Biological material absorbs relatively less in the range 500 - 600 nm
- The lowest natural fluorescence background is in the range 400 - 500 nm
- That is also the reason why probes and labels with exc. and em. maximum in this range are used
- To enable study of labeled biomolecules even in the presence of unlabeled proteins, excitation and emission wavelength of labels and probes must be higher than that of intrinsic fluorophores (aromatic AA): 400-600 nm

Fluorescent labels

A proper label should have following parameters to be covalently bound to a biomolecule:

- high fluorescence intensity
- stability during continuous light exposure
- minimal effect on biological properties of a molecule

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Brightness

- Is given by a product of quantum yield and molar extinction coefficient ε

$$Bs = Q\ \varepsilon$$

- Describes efficiency of a label to transform excitation light to fluorescence
- A covalent bond of a label to a biomolecule leads often to a significant change of brightness
- Proper brightness of a label is:

$$Bc>5000$$

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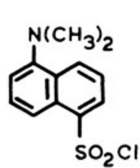
Examples of extrinsic fluorophore brightness

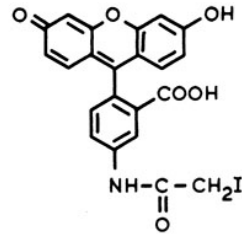
Fluorophore	ε (cm^{-1} M^{-1})	Quantum yield (Q)	Brightness (Bs)
Oregon Green® 488	87 000	0.9	78 300
BODIPY FL	91 000	0.9	81 900
Fluorescein (FAM)	79 000	0.9	71 100
JOE	71 000	0.6	42 600
TAMRA	103 000	0.2	20 600
Rhodamine Red-X (ROX)	82 000	0.7	57 400
Texas Red	139 000	0.9	125 100

http://www.promega.com/geneticidproc/ussymp8proc/21.html

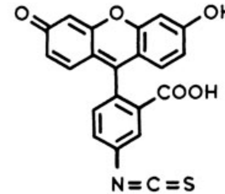
Examples of fluorescent labels



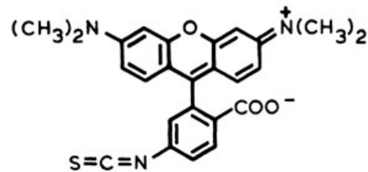
DNS-Cl



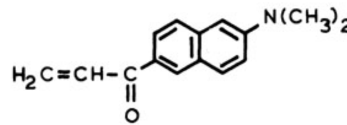
5-IAF



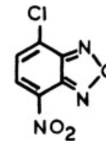
FITC



TRITC



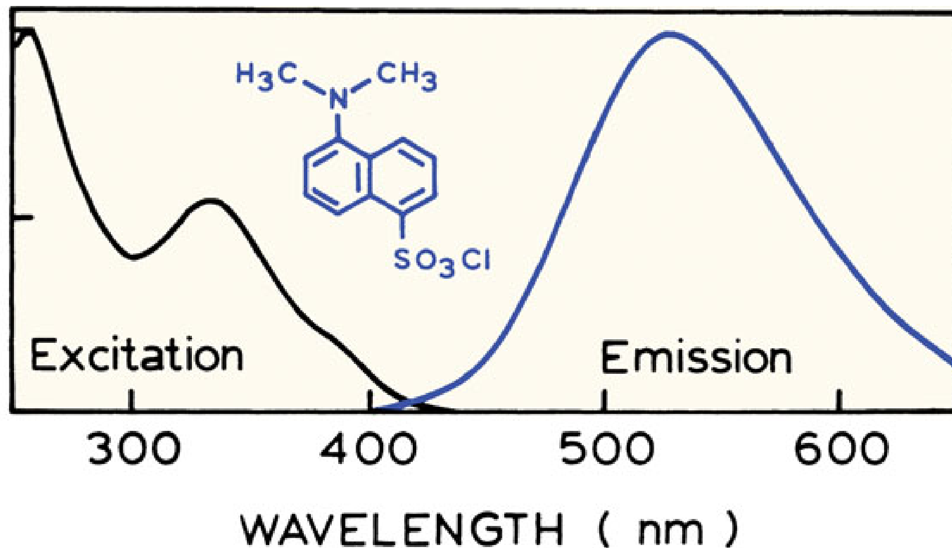
Acrylodan

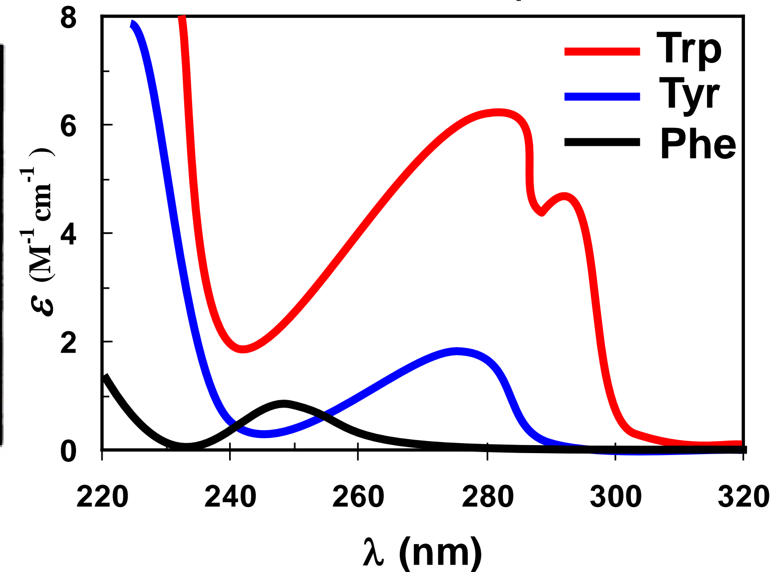


NBD-Cl

- dansyl chloride (DNS-Cl; 5-dimethylaminonapthalene-1-sulfonyl chloride)
- fluorescein-5-isothiocyanate (FITC)
- 5-iodoacetamidofluorescein (5-IAF)
- tetramethylrhodamine-5(a 6)-isothiocyanate (TRITC)
- 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl; 4-chloro-7-nitrobenzofurazan)
- 6-acryloyl-2-dimethylaminonapthalene (Acrylodan)

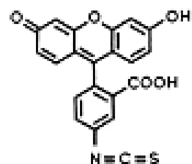
Dansyl chloride



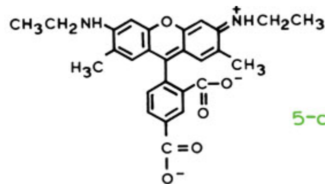


- One of the first and for this reason also the most representative fluorescent labels in literature
- Is used often for protein labeling, it is useful especially for measuring of anisotropy
- Very suitable fluorescence decay time $\tau \sim 10$ ns
- Is excited at 350 nm, where proteins almost do not absorb
- The emission spectrum is sensitive to solution polarity and has mostly a maximum around 520 nm
- Reacts with free amino groups of proteins

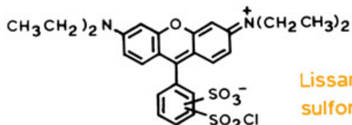
Fluorescein and rhodamines



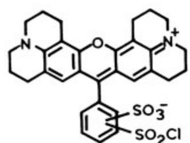
FITC



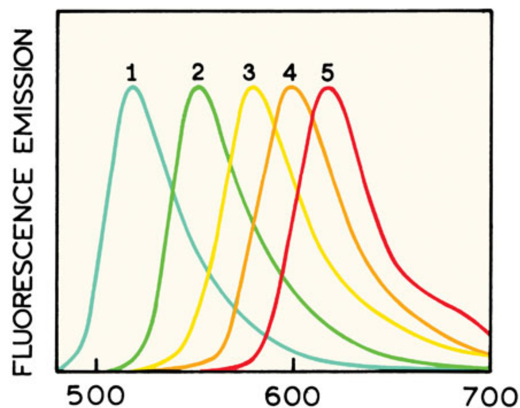
5-carboxyrhodamine 6G hydrochloride



Lissamine rhodamine B sulfonyl chloride

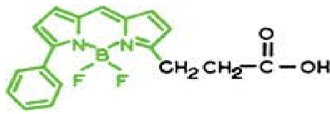


Texas Red sulfonyl chloride



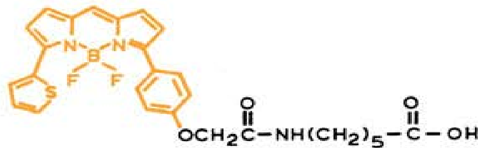
- Belong to the most widely used fluorescent labels Abs.max. Em. max.
  fluorescein (490nm) (520)
  rhodamines (500-600 nm) (530-620)
- Sensitive to solvent polarity and pH
- High value ε ~ 80 000 $M^{-1}cm^{-1}$
- High quantum yield Q ~ 0.3-0.9
- Fluorescence decay time ~ 4 ns
- A large number of derivatives is synthesized, derivates that are used for labeling of proteins and DNA through NH_2 nebo SH groups
- The fluorescence intensity depends on pH
- They tend to photobleaching

BODIPY

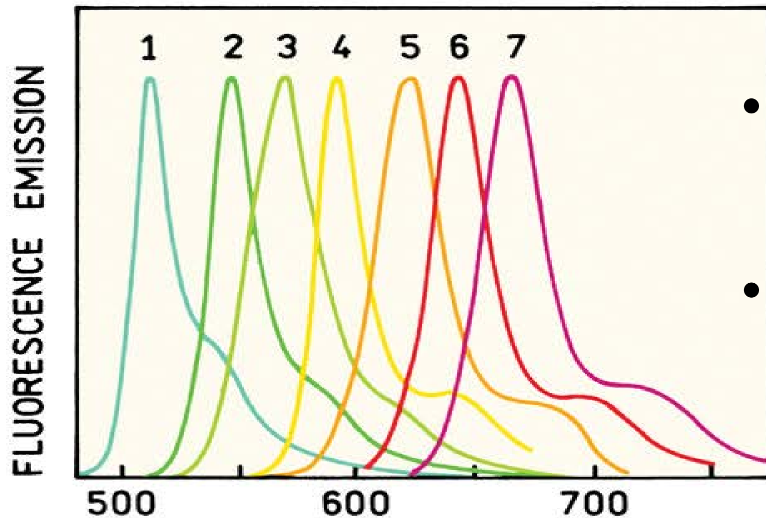


BODIPY-R6G

BODIPY- 581/591



BODIPY- Texas Red



- Successor to fluorescein and rhodamine labels
- Derived from the fluorophore which contains Boron
- Emission maxima 510-675 nm
- Extremely high quantum yield Q~ 1!
- They are not sensitive to solvent polarity and pH
- Emission spectrum is narrow and emission is thus concentrated on a narrow range of wavelengths and more different labels in the mixture can be differentiated
- Disadvantage: small Stokes shift resulting in the relatively small value of Förster distance at resonance energy transfer (R_0=57 A)
- Thanks to significant overlap of emission and absorption spectrum, selfquenching occurs at high concentrations of labeling (when the molecules of fluorophores are closer than R_0)
- Not suitable for FRET applications

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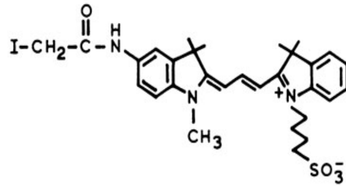
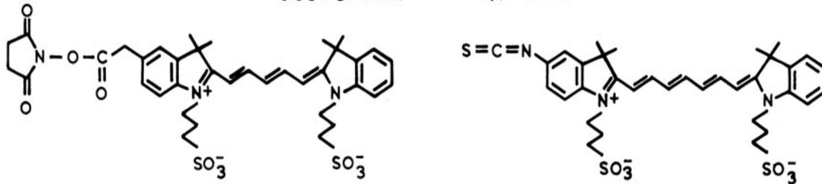
Cy labels



Cy-3 Iodo Acetamide

565/590 nm QY≈0.07

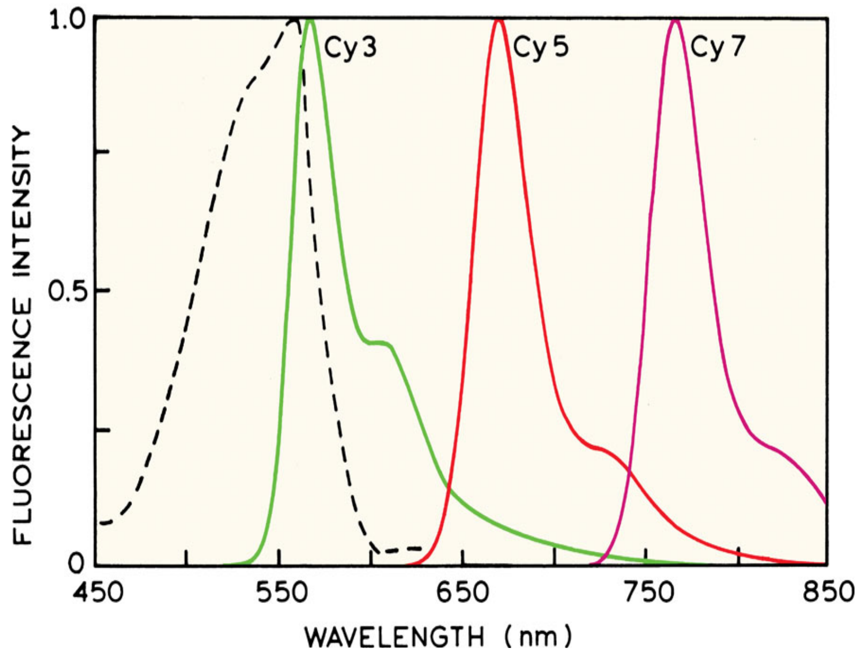


Cy-5-N-Hydroxysuccinimide

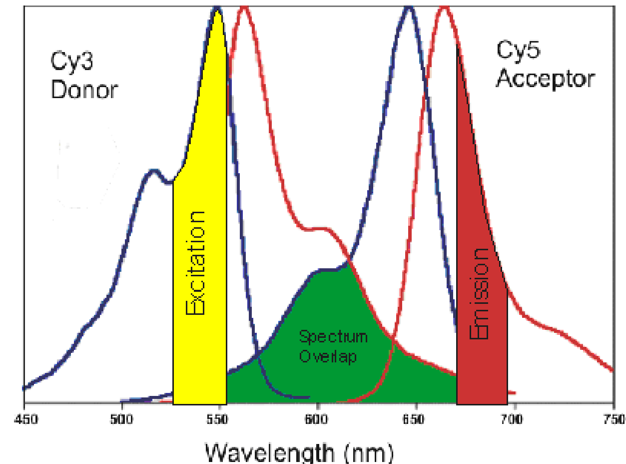
648/669 nm QY≈0.10

Cy-7-Isothiocyanate

750/777 nm QY≈0.10

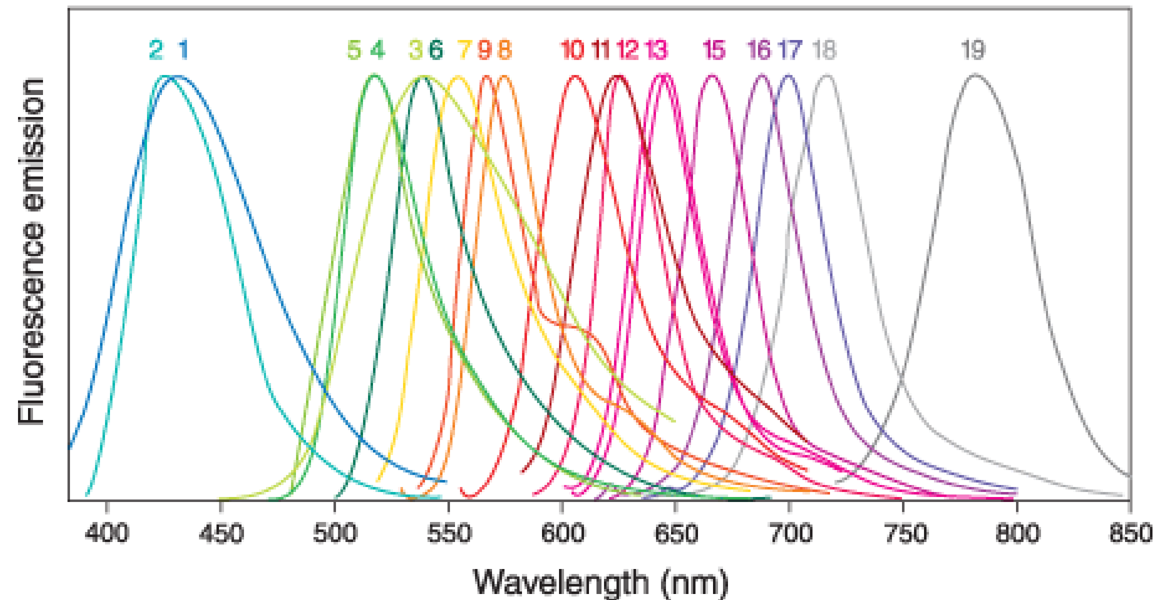


- Very popular
- The number means the length of the chain of conjugated bonds between two aromatic rings
- Suitable for areas from 550 nm further
- Relatively small Stokes shift
- They are used for FRET studies



http://www.cytographica.com/animations/Cy3Cy5FRET.html

Alexa Fluor



- High quantum yield-> high brightness
- Improved water solubility
- Small dependence of fluorescence on pH
- **Photostable !**

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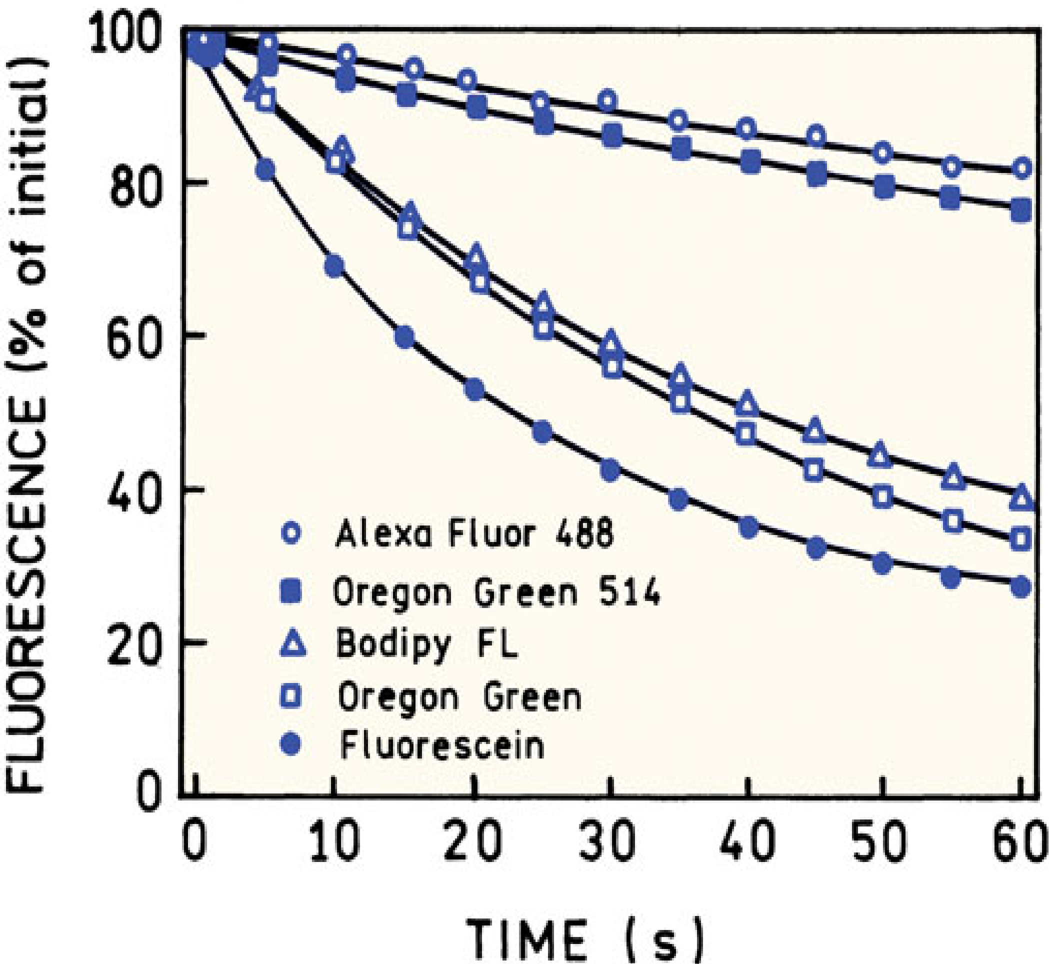
Photostability of fluorophores



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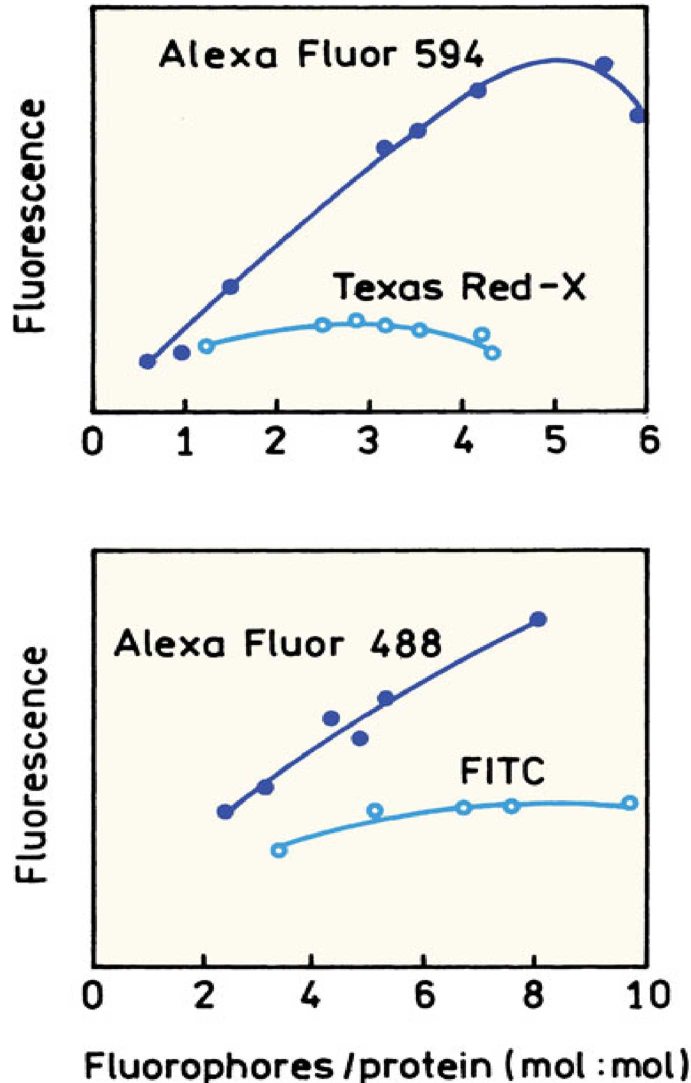
- Photobleaching comes to each fluorophore after a certain time
- Photostability is the most important in microscopy, where high intensities of excitation light are used
- Alexa probes show the highest photostability
- There has not been observed any connection between the structure of fluorophores and their photostability yet

The effect of degree of labeling on the intensity of fluorescence



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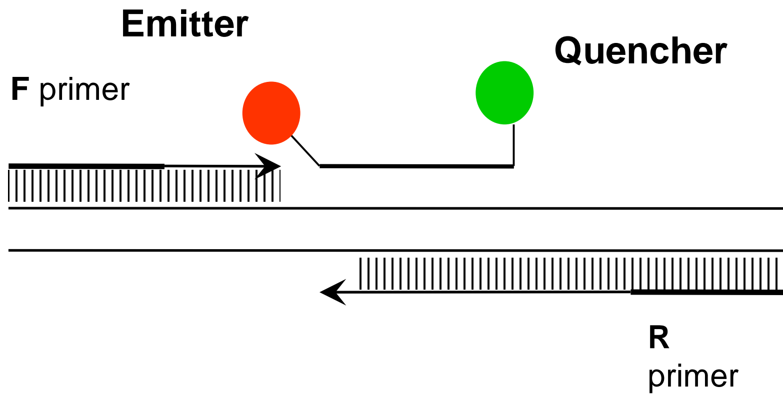
- Photobleaching occurs often in a classic fluorescein and rhodamines at the high degree of labeling (fluorophore molecules are located at distance about R_0)
- In the case of Alexa fluorophores, photobleaching does not occur in such extent and hence the emission intensity is higher in the case of higher degree of labeling

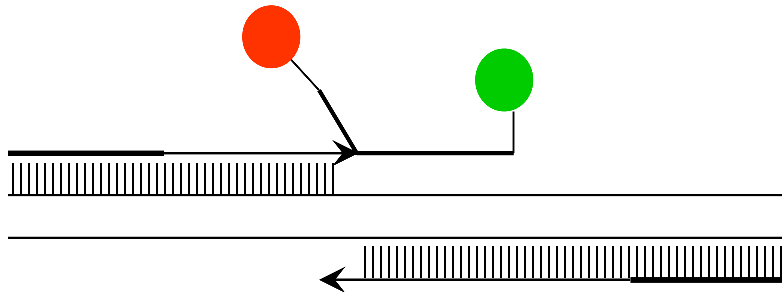
Real-time PCR

detection of DNA amplification

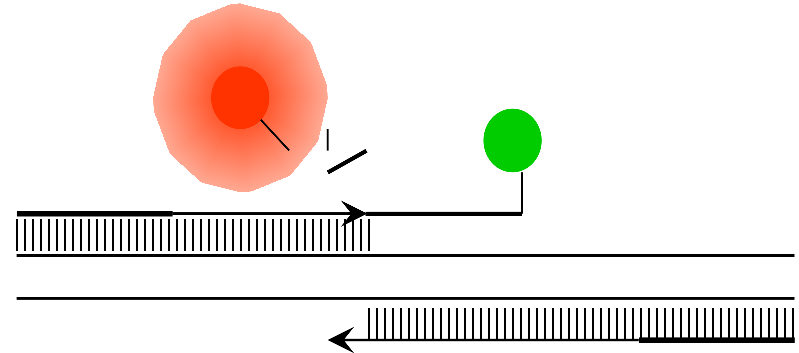
Bi9310



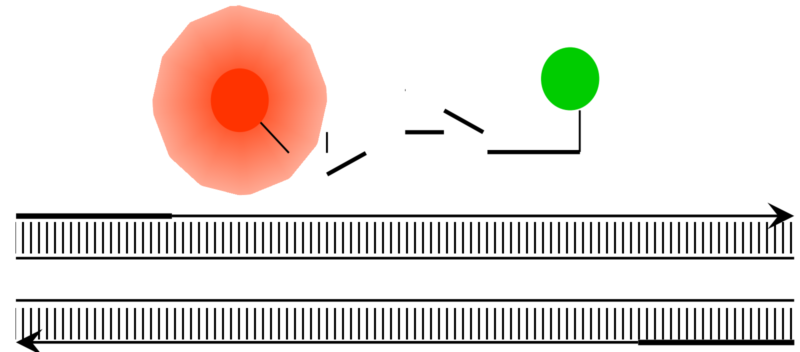
1. The labeled probe hybridizes to a complementary sequence. Radiation of emitter is quenched and is not observed.



2. The probe is replaced by a new chain during polymerization.



3. At each amplification cycle, the polymerase cleaves the emitter. The emission intensity increase is detected.



4. Polymerization is completed. The intensity of the emitted radiation is directly proportional to the quantity of amplified DNA.

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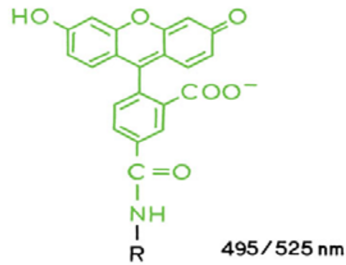
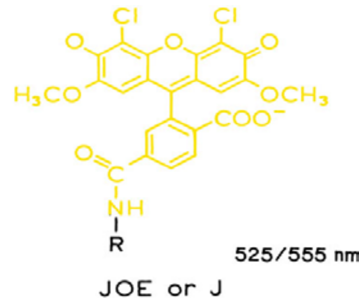
Fluorescent labels for RT-PCR



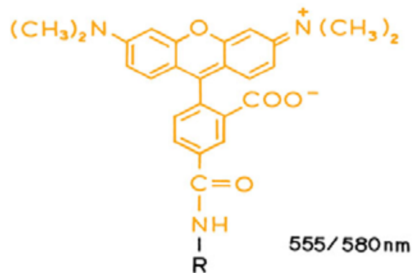
FAM or F

5-carboxyfluorescein



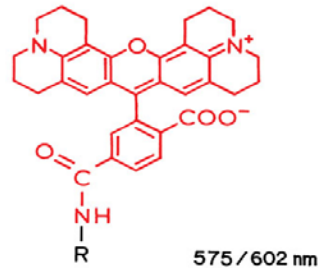
JOE or J

2',7'-dimethoxy-4',5'-dichloro-6-carboxy-fluorescein



TAMRA or T

N,N,N',N'-tetramethyl-6-carboxyrhodamine



ROX or R

5-carboxy-X-rhodamine

- Which of these labels are the best to use?

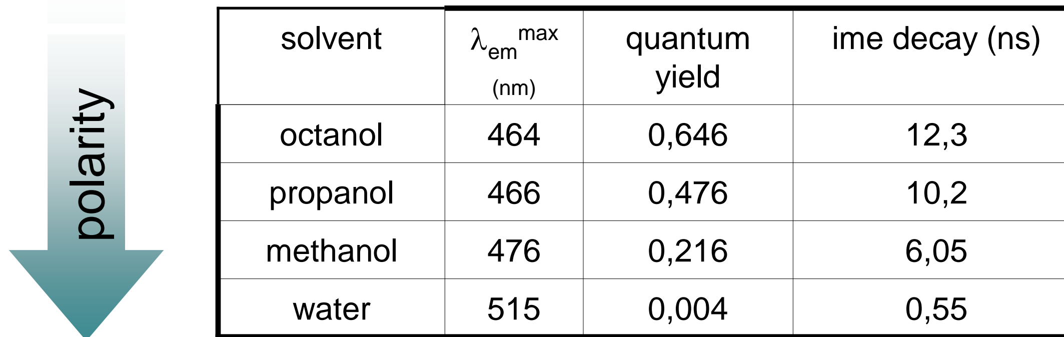
Fluorescent probes

- **Fluorescent probes** are extrinsic fluorophores, that are bound non-covalently to a monitored structure and alter often their fluorescent properties.

Fluorescent probes are themselves very little fluorescent in the solution usually. However, their fluorescence significantly increases after binding to proteins or DNA.

Probes sensitive to environment polarity

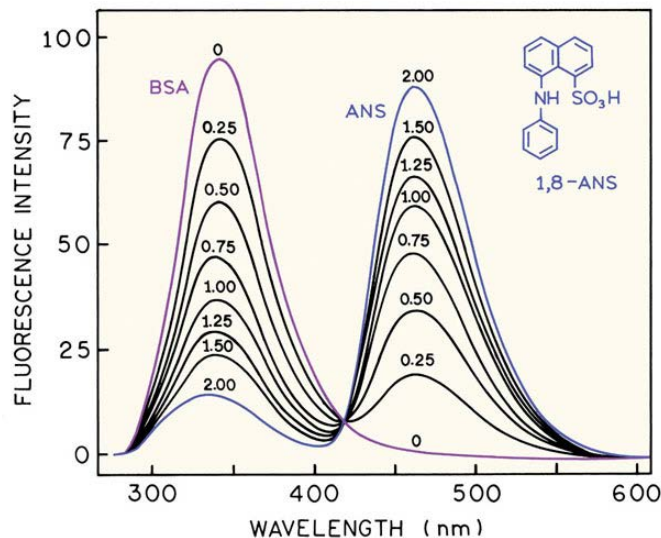
1-anilinonapthalene-8-sulfonate (ANS) a **2-*p*-toluidinonapthalene-6-sulphonate (TNS)** are typical probes for dynamic polarity. The table shows the ANS fluorescence parameters in various solvents, it follows that with increasing polarity of the solvent the ANS fluorescence emission maximum shifts to red region and simultaneously quantum yield and time decay decreases. When ANS binds to apomyoglobin, ANS is bound into a non-polar binding site for heme, emission maximum shifts to 454 nm and fluorescence quantum yield increases to 0.98. In this way, it is possible to study the structure and degree of polarity of different binding sites on proteins including possible displacing of fluorescent probes from this bond or changes induced by e.g. enzyme activation, etc. ANS was used e.g. for studying of polarity of the binding site for heme in apomyoglobin and apohemoglobin or conformational changes in muscle and in nerve endings during action potential. TNS was used eg. to study conformational changes after activation of chymotrypsinogen and changes of conformation of nerve membranes.



solvent	λ_{em}^{max} (nm)	quantum yield	ime decay (ns)
octanol	464	0,646	12,3
propanol	466	0,476	10,2
methanol	476	0,216	6,05
water	515	0,004	0,55

Parameters of fluorescent probe 1-anilinonapthalene-8-sulphonate (ANS) at different solvent polarity

Change of fluorescence of serum albumin in the presence of ANS



- By increasing the ratio of molecules ANS: SA there is a shift of the emission maximum from 350 nm to 480 nm
- This will increase the intensity of light which we see by the eye at excitation of 280 nm

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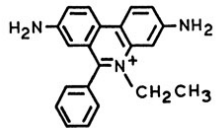
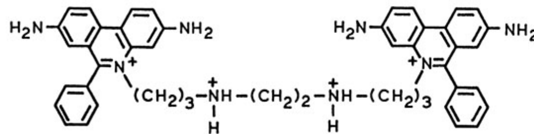
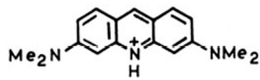
DNA probes



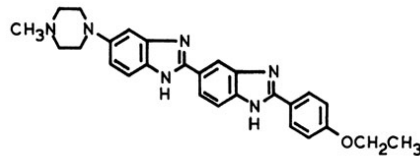
Ethidium Bromide
518/605 nm



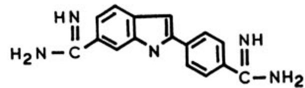
Ethidium Homodimer
528/617 nm



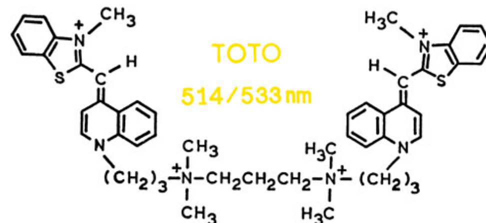
Acridine Orange
500/526 nm DNA
460/650 nm RNA



Hoechst 33342
350/460 nm

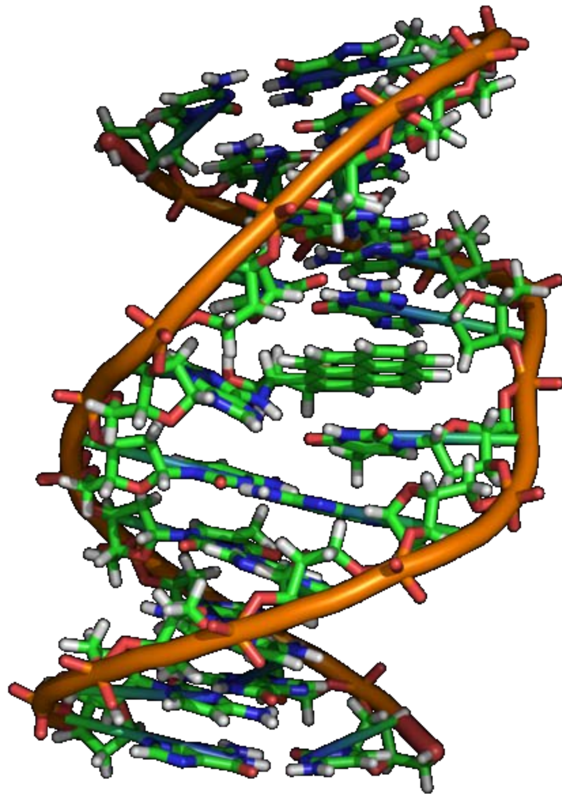


DAPI
355/461 nm

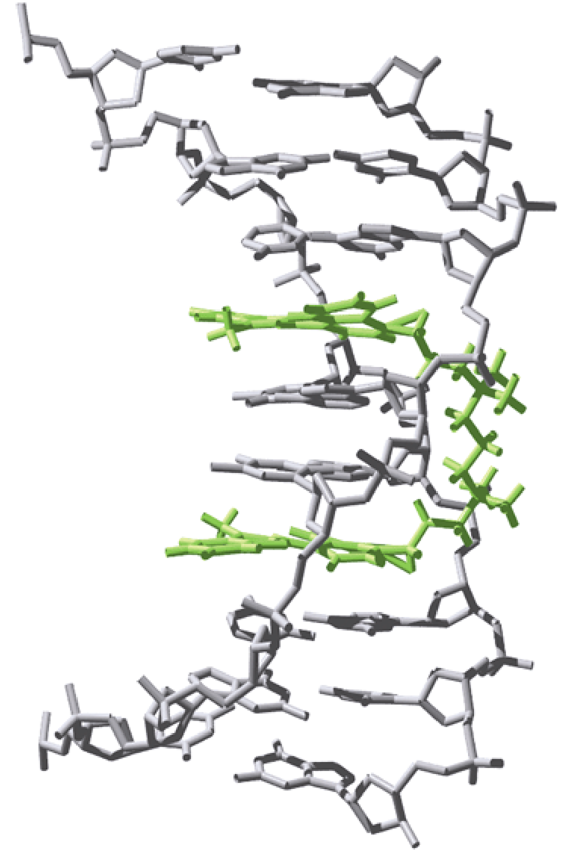


- Intercalators EB, AO, TOTO intercalate between base pairs
- Hoechst, DAPI bind to a DNA minor groove
- EB increases the intensity of fluorescence after binding 30x and τ extends from 2 to 20 ns
- DAPI increases the intensity of fluorescence most near AT pairs
- TOTO (Thiazole Homodimer) increases the intensity of fluorescence after binding 1100x
- Probes with high affinity as EB homodimer (binds 10 000x tighter than monomer EB) and positively charged TOT0 remain bound to the DNA during electrophoresis and are used for visualisation of DNA on a gel to help increase sensitivity upto 500x compared to conventional EB staining
- **How is it possible to reduce the consumption of the probe**?

Intercalation



Benzpyrene



TOTO

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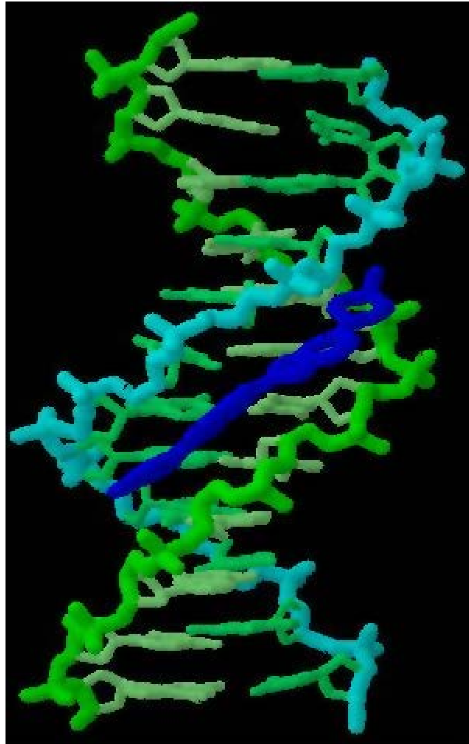
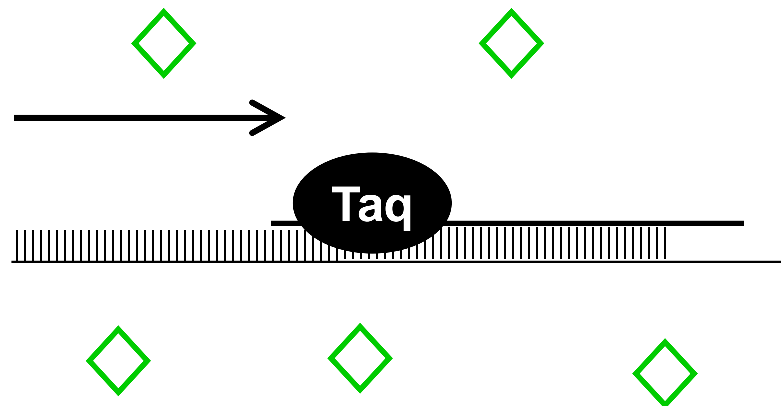
Syber Green



- Selectively binds to ds DNA into minor groove
- Detection from 1 ng/mL
- Use in RT-PCR to quantify amplified DNA



Comparison of probes for quantification of dsDNA

Probe	**Sensitivity for dsDNA**	**Extinction Coefficient (cm^{-1} M^{-1})**	**Quantum yield after binding to dsDNA**	**Increase in fluorescence intensity upon binding to dsDNA**
PicoGreen	25 **pg**/mL	70,000	0.53	~2000x
Hoechst 33258	1-10 ng/mL	40,000	0.59	~100x
Ethidium bromid	1-10 ng/mL	5,000	<0.3	~30x

The extinction coefficients were determined for the free probe in aqueous solution

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http://www.promega.com/geneticidproc/ussymp8proc/21.html

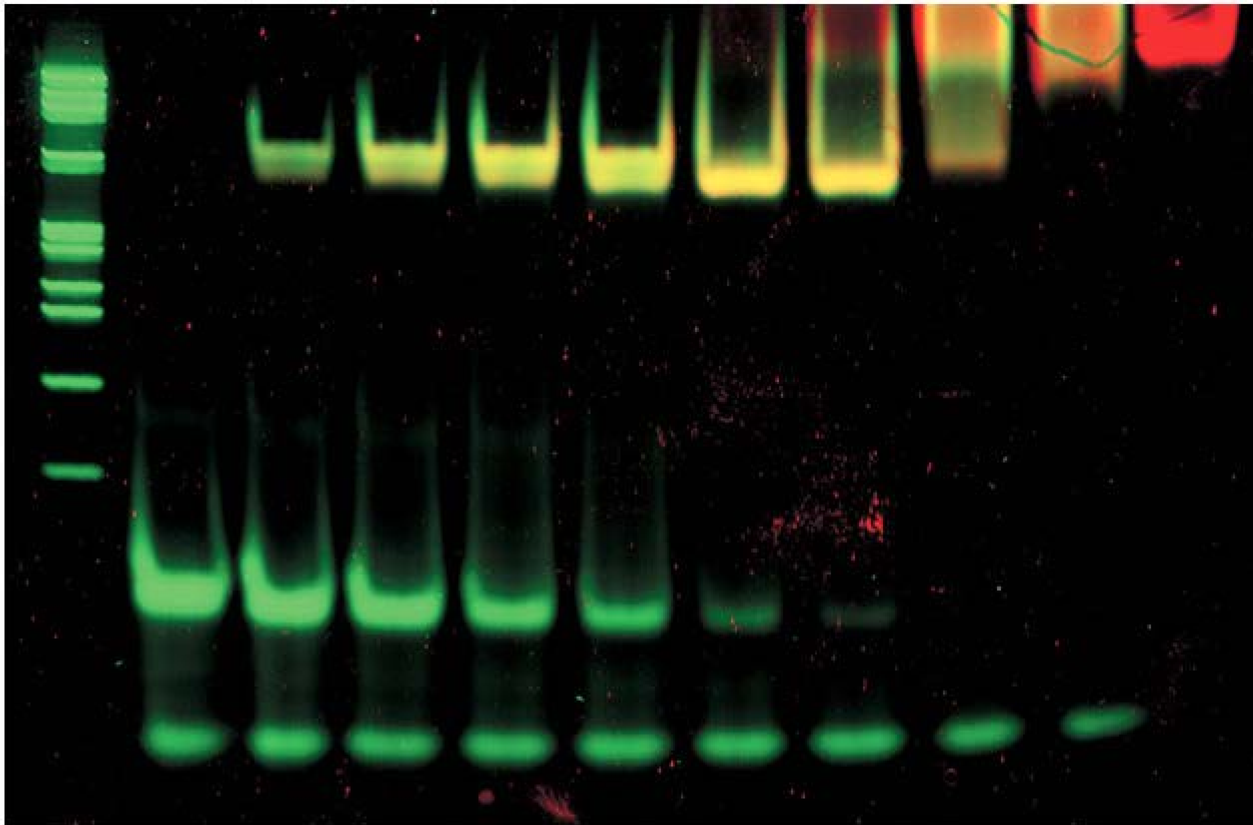
Protein probe

- Increase fluorescence intensity upon binding to the protein
- The most sensitive fluorescent probes for staining proteins in the gel are from the group of organometallic compounds SYPRO
- SYPRO Red, Orange, Tangerine, Rose
- High sensitivity ~ ng/mL
- Acidic fixation is important before use

Sypro probes

- Primarily used for staining of proteins in the gel
- Use in criminology

Simultaneous staining of DNA and proteins in the gel

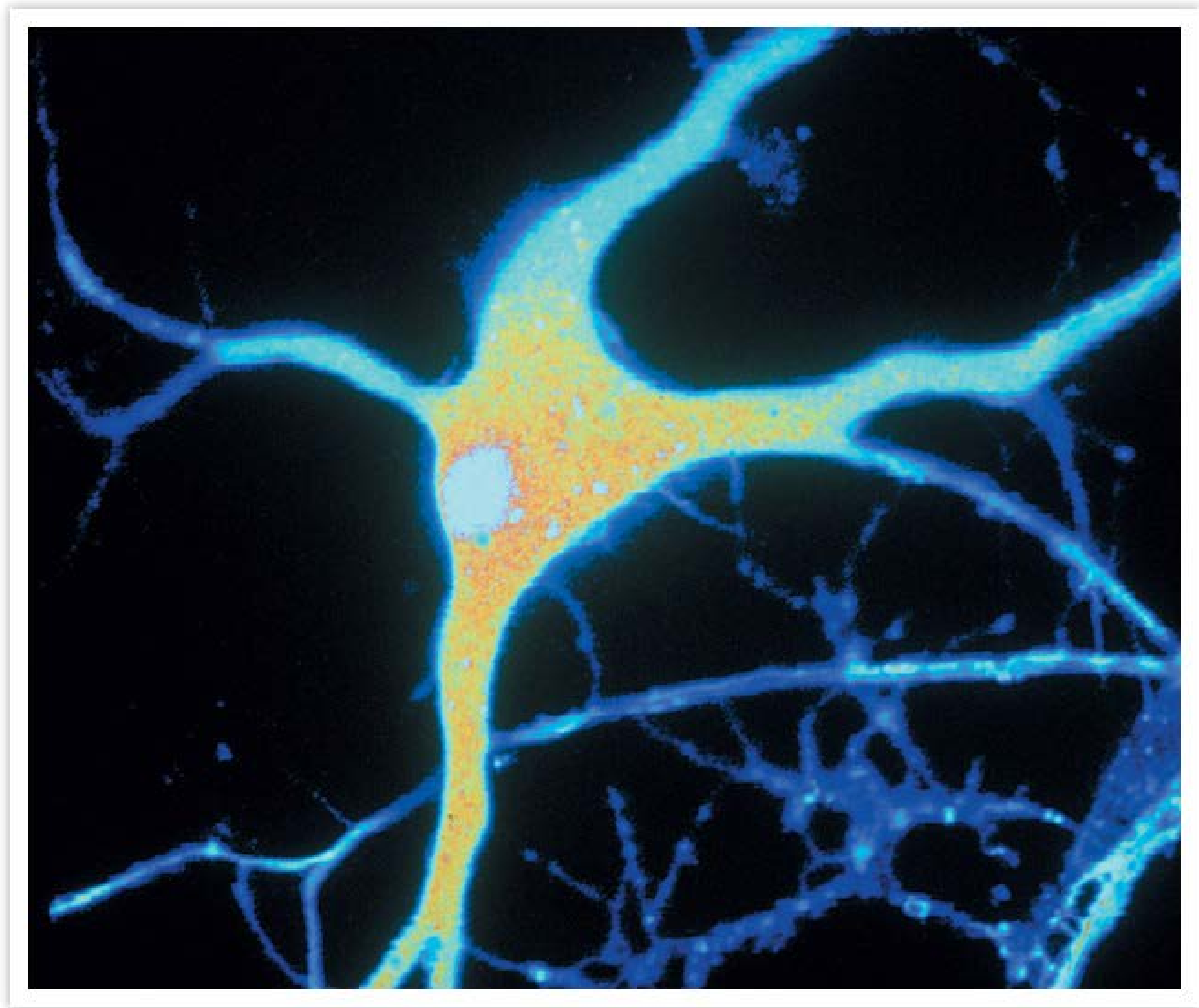


- DNA is stained by Syber Green
- Protein SYPRO Ruby

Ion indicators

- Fluorescence measurement of changes of intracellular ions is possible through probes that change their spectral properties upon binding of the ion. Ca^{2+} is measured most commonly and is related to a number of books. The indicators are usually derivatives of chelators Ca^{2+}, Mg^{2+}, Na^{+} or K^{+} as EGTA and BAPTA that have appropriate affinity for the studied ion. When selecting a suitable indicator we take into account :
- **form of the indicator** (salt, acetoxymethyl ester, dextran conjugate) that affects the way how to get into cells (microinjection, electroporation, infusion from the patch pipette, passive diffusion) and intracellular distribution
- **measurement method** - some indicators exhibit spectral absorption or emission shift upon binding of the ion (measure the ratio of intensities at different wavelengths of excitation or emission), other exhibit change in fluorescence intensity
- **dissociation constant** - must be comparable with the measured concentration of cations (concentration less than one tenth or bigger than ten times of the dissociation constant cause too small changes in the observed signal)

Indication of Ca^{2+} in nerve cells using Fluoro-3



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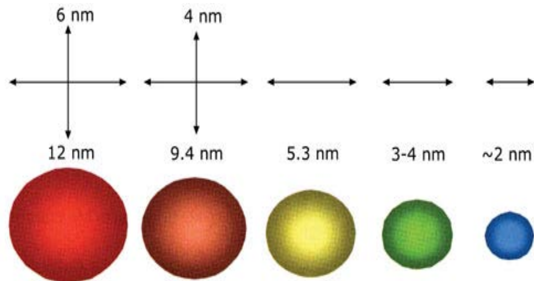
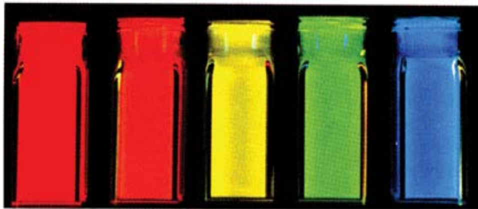
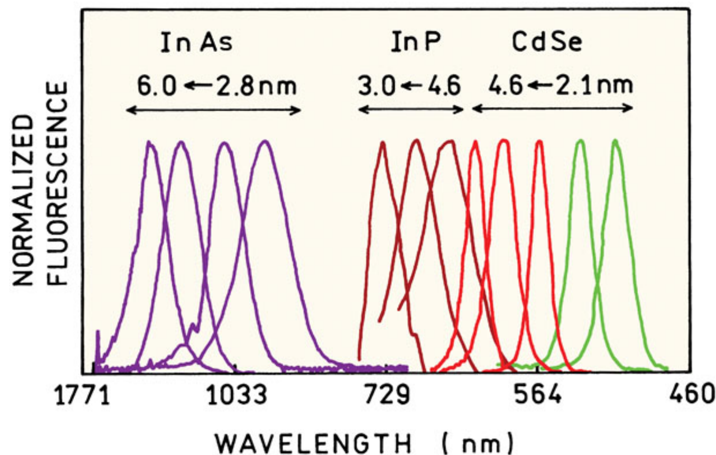
Quantum dots







- Semiconductor material is the base
- Particles in the order of nm
- They have a narrow range of symmetric
- No photobleaching!
- The emission wavelength is determined by the diameter and by the particles material
- Wide range of emission from UV to IR

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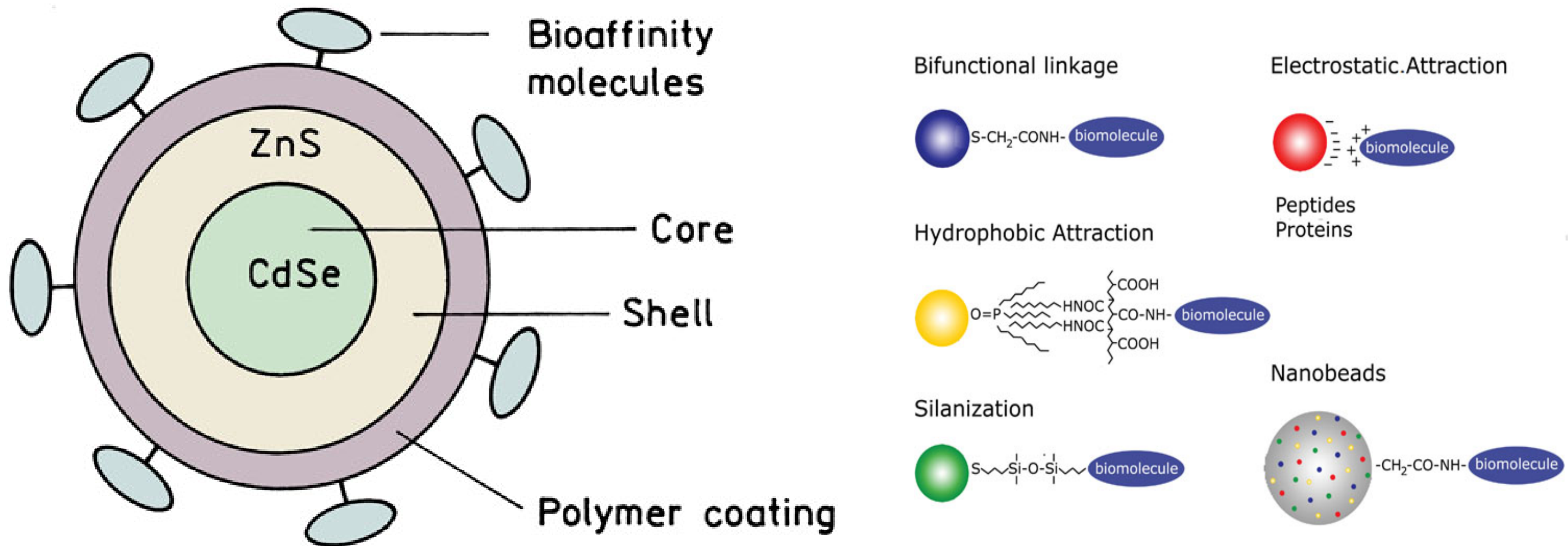
The binding of biomolecules to Qdots

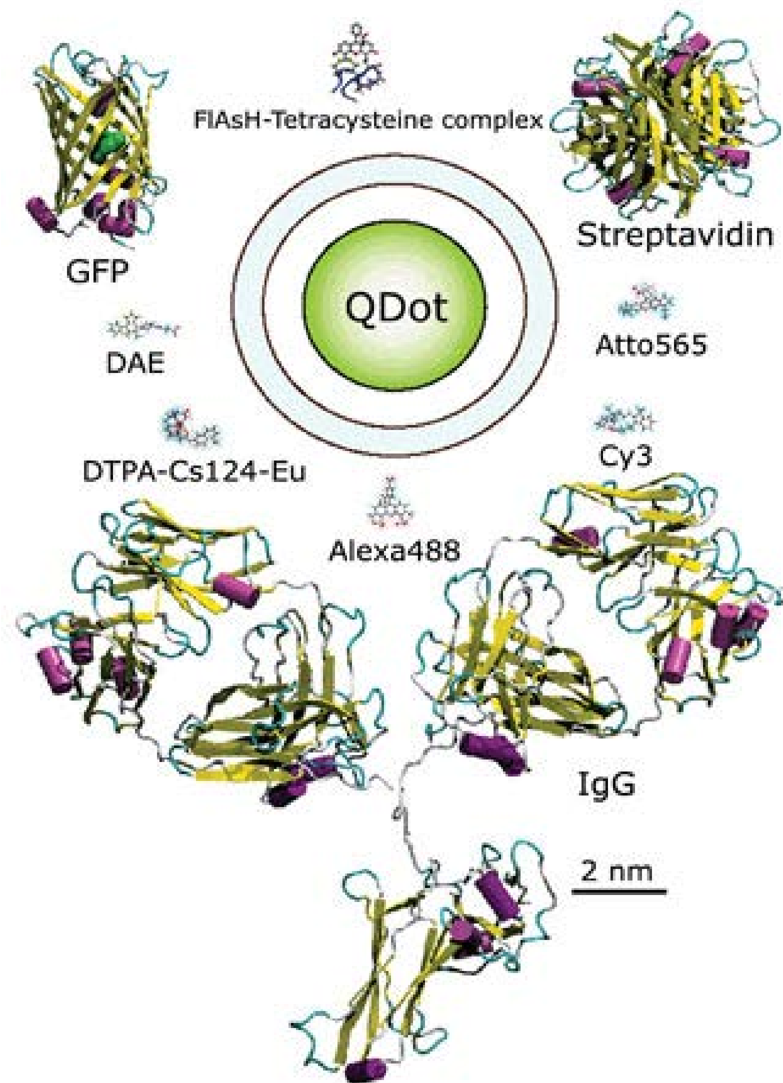


- The core is made of the semiconductor CdS for UV, CdSe for Vis and CdTe for IR
- Its size is comparable with the dimension of GFP
- The core is covered with a casing (shell), which allows connection of the core with outer hydrophilic layer (Polymer coat)
- The outer hydrophilic layer provides solubility and enable binding of biological molecules

Properties QDots

- The width of the emission spectrum is 20 to 30 nm, which is about 1/3 of the value of "classic" fluorophores
- Quantum yield 0.35-0.5
- Absorb at each wavelength (semiconductor)
- They may emit at different wavelength at the same excitation radiation!
- **Ultraphotostable** 100 times more resistant to photobleaching than the "classic" organic fluorophores
- Decay time ~ 100 ns
- High $\varepsilon \sim 10\,000\,000\ M^{-1}cm^{-1}$ [10]

Size comparison of QDot



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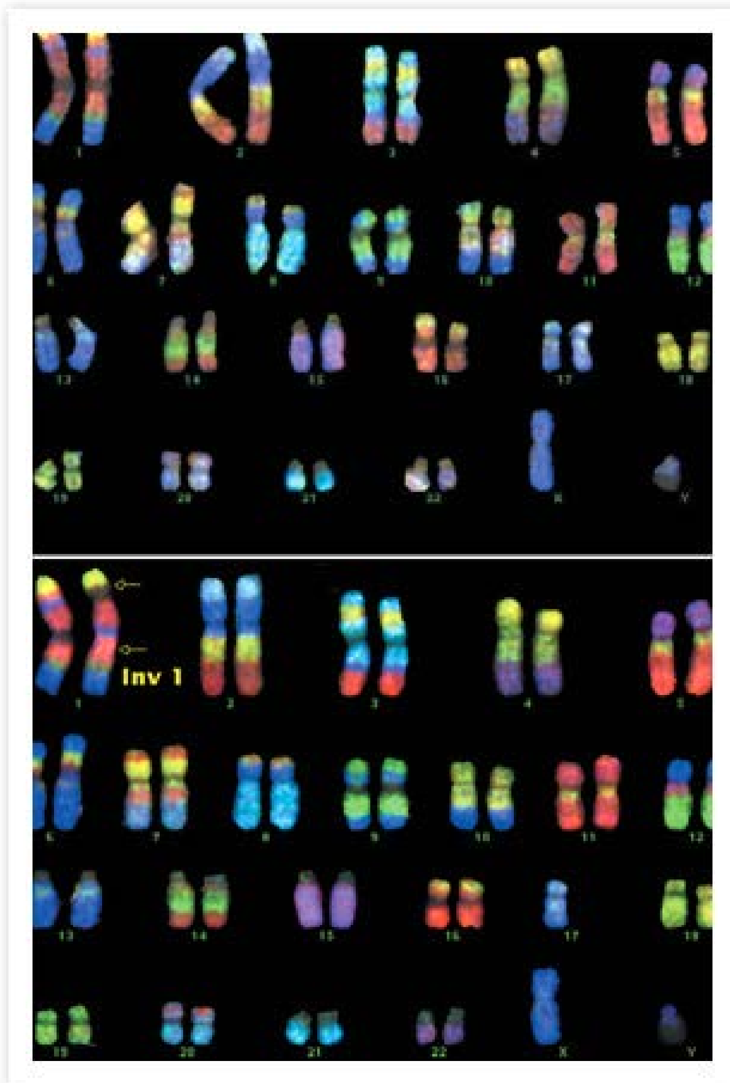
Applicability of fluorescent labeling



- The difference between the human and monkey karyotype
- Fluorescent labeling helps to answer the basic scientific questions

Literature

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- Haugland R.P.: Handbook of Fluorescent Probes and Research Products. Ninth Edition, Molecular Probes, 2002
- Fišar Z.: FLUORESCENČNÍ SPEKTROSKOPIE V NEUROVĚDÁCH http://www1.lf1.cuni.cz/~zfisar/fluorescence/Default.htm

Acknowledgement

Graphics from the book Principles of Fluorescence was for the purpose of this lecture kindly provided by Professor JR Lakowitzem.

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