Fluorescent labeling of molecules

Fluorescence methods in life sciences

Ctirad Hofr

Extrinsic fluorophores

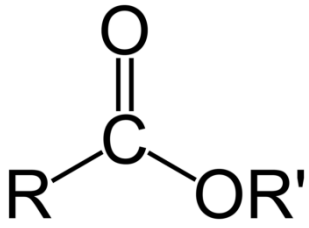
Fluorescent dyes - are added to the studied sample and bind to it covalently. They bind to proteins and nucleic acids through the amino or thiol groups and side chains.

Fluorescent probes - bind to the studied sample non-covalently and after binding they change their fluorescence properties (e.g. intensity, position of emission maximum)

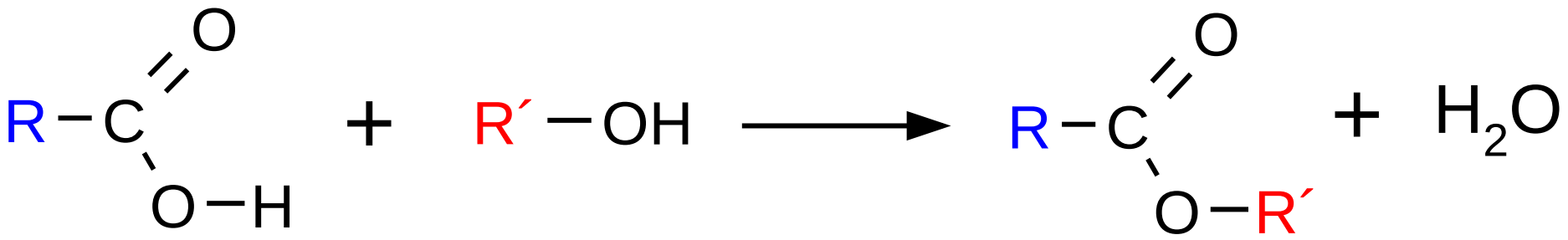
Possibility of fluorophore introducing

- **Covalent bond**
  it uses a chemical reaction of fluorophore derivative, during which a covalent bond with biomolecule is created
- **Non-covalent bond**
  fluorophore binds to a biomolecule through non-covalent (e.g. electrostatic) interactions
- **Fluorogenic reactions**
  it uses a chemical reaction, during which a non-fluorescent precursor is changed to the fluorophore

Esters



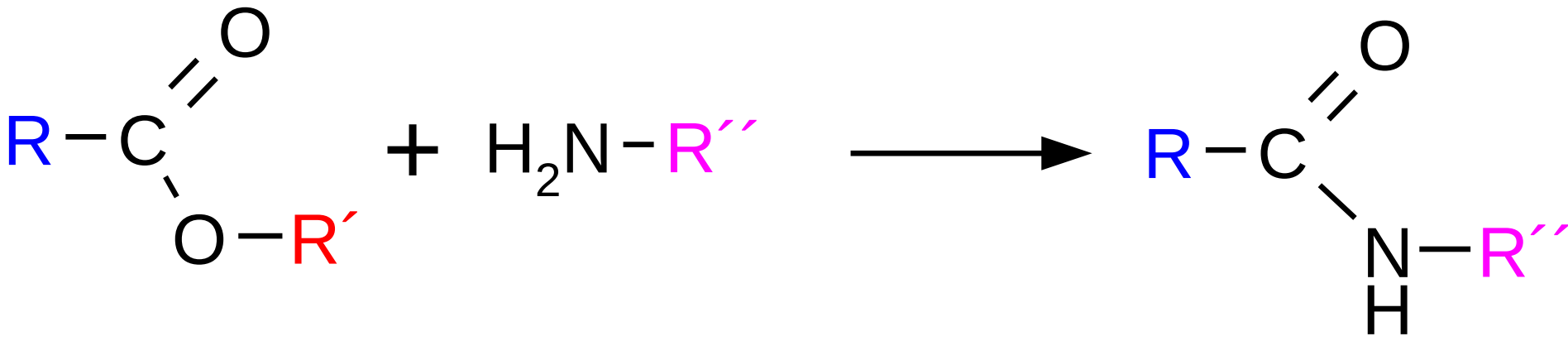
They are formed by reaction of carboxylic acid and alcohol



carboxylic acid alcohol ester

Amide formation

Amides are formed by reaction of ester and amine

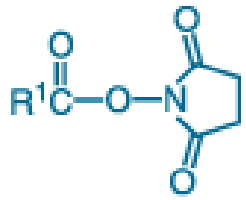


ester amine amide

Reaction of esters for covalent labeling of molecules with NH_2 group

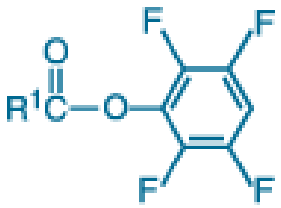
STP Ester

Carboxamide



Succinimidyl Ester

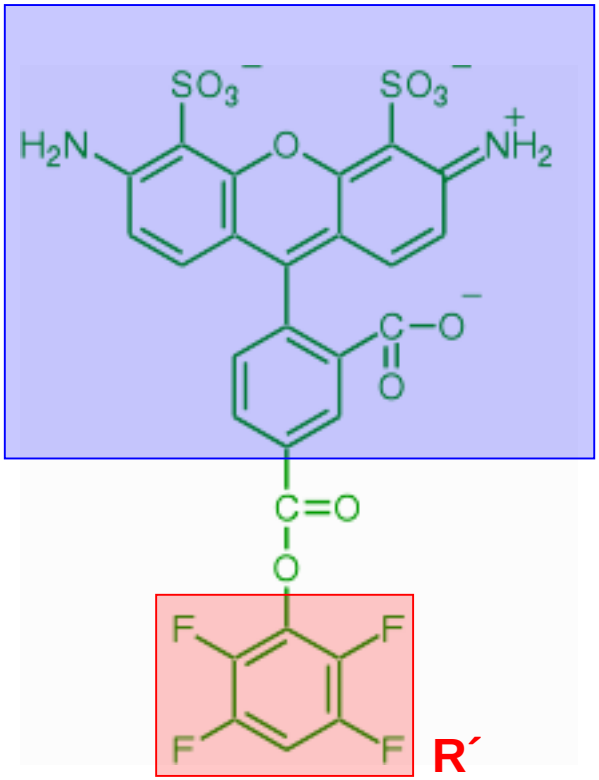
Carboxamide



TFP Ester

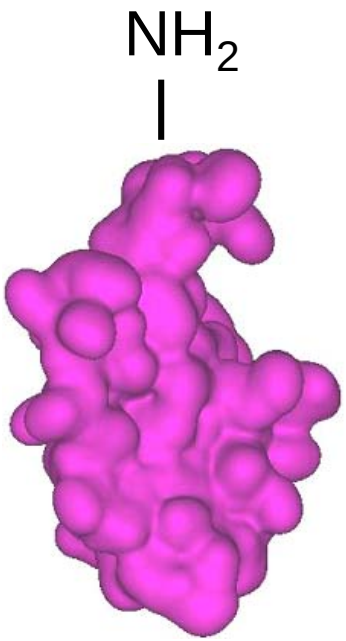
It uses a reaction of ester with amine to form amide

Reaction of carboxylic acid of dye Alexa 488 with NH_2 group of protein

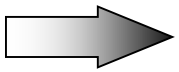


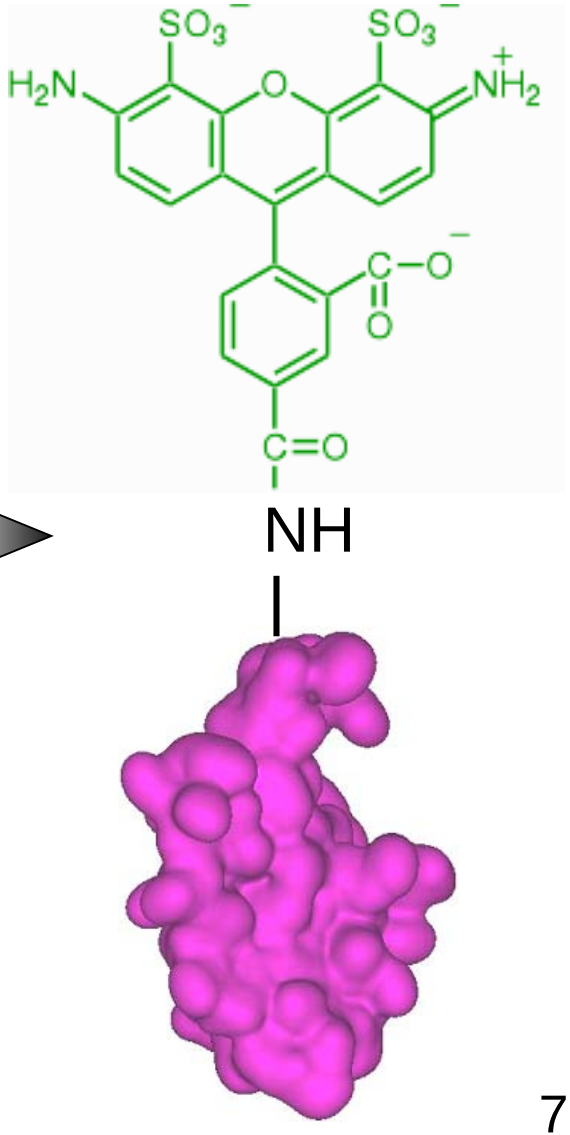
R

+



R´´





Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Alexa Fluor® 488 5-TFP)

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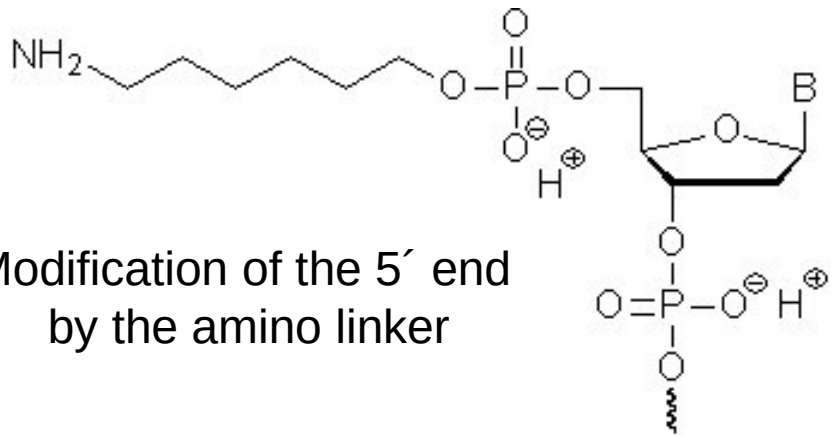
How to add NH_2 group to DNA?

Aliphatic chain terminated with NH_2 group (amino-linker) is attached directly in the synthesis of oligonucleotide



Modification of the 5´ end
by the amino linker

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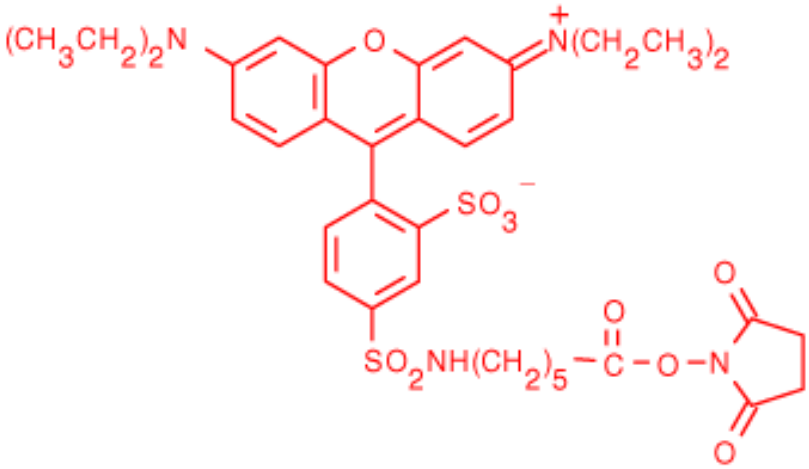
DNA labeling through NH_2 group



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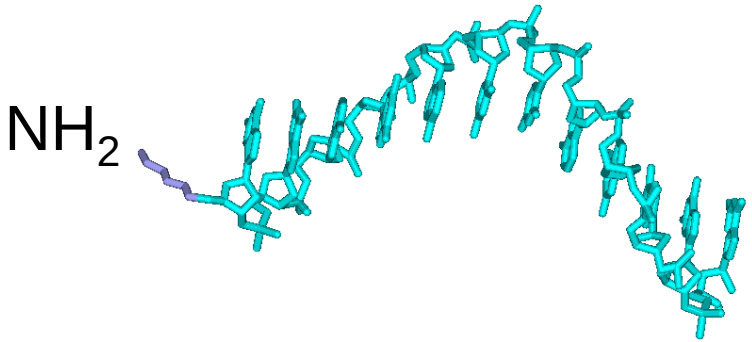

Rhodamine Red™-X, succinimidyl ester

DNA with „amino-linker“

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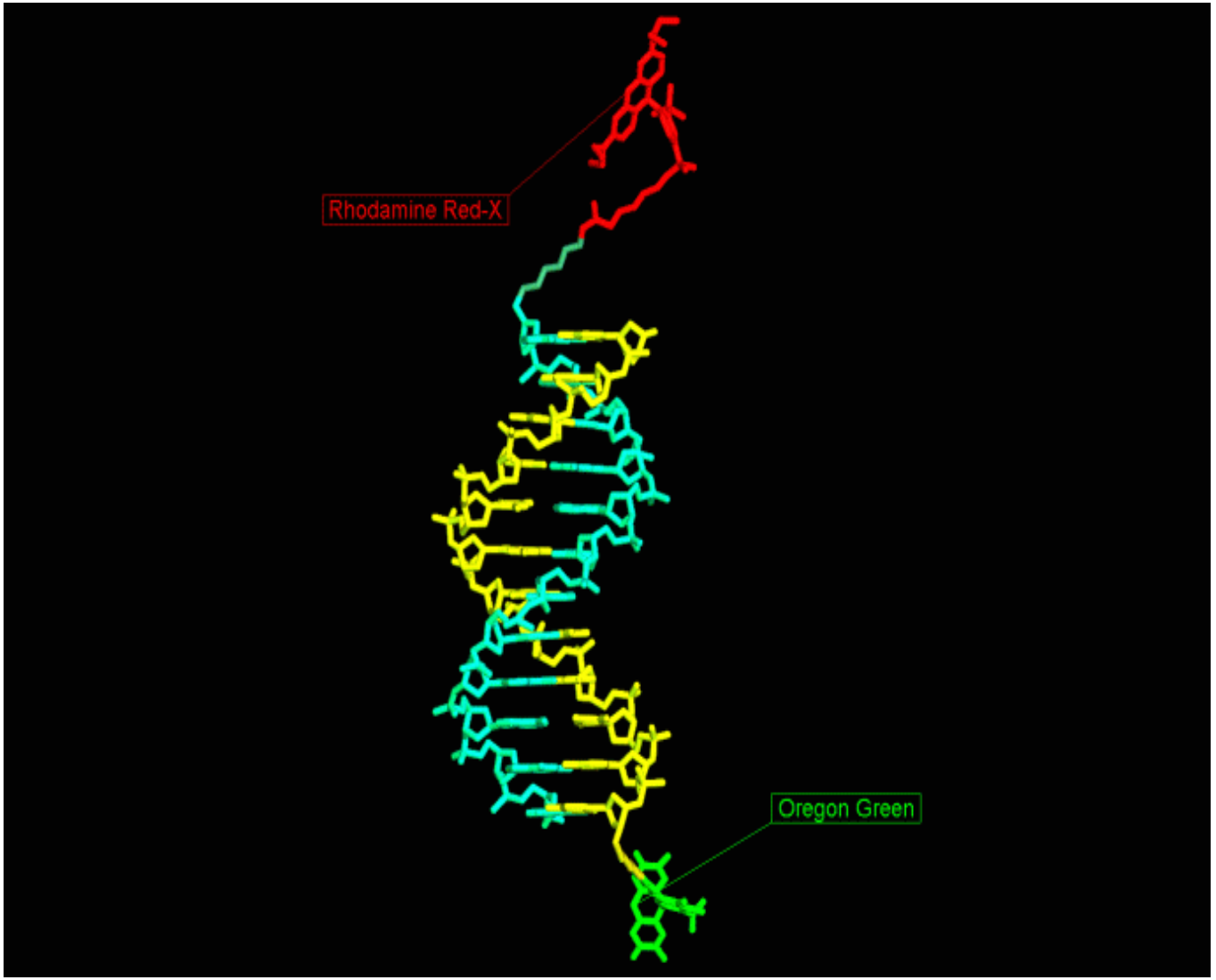
DNA labeled with Rhodaminem Red-X and OregonGreen



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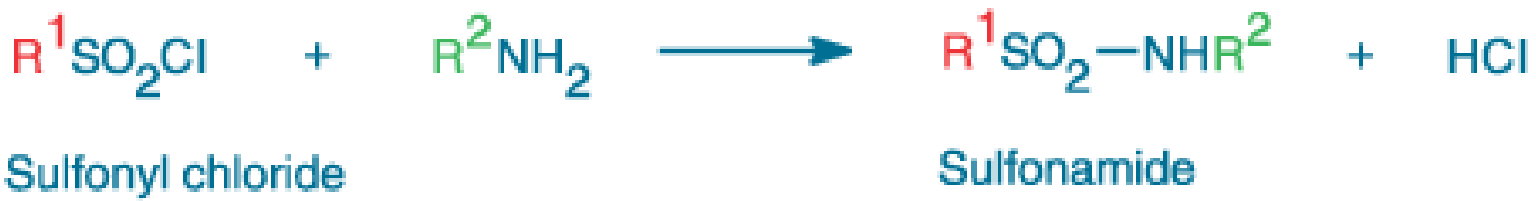
Other reactions for labeling through the amino group

Reaction of an isothiocyanate with a primary amine

Reaction of sulfonic acid chloride with an amine



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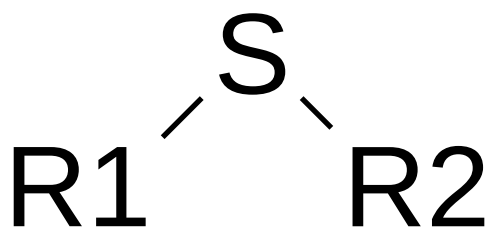
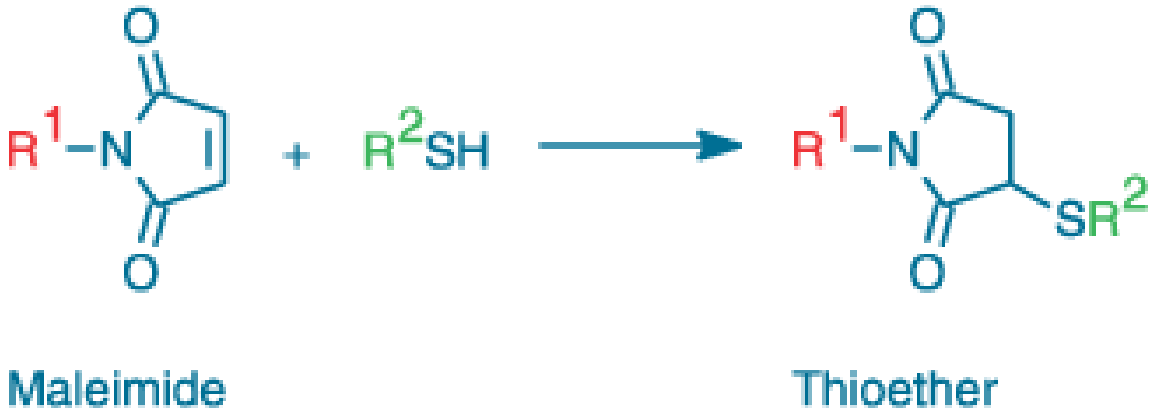
Connection through SH thiol group to form a thioether



- Thioether is similar to ester, except the O is replaced by S
- Thioether is formed by reaction of alkylating agents (e.g. halogen, maleimide) with thiols (contain SH group)

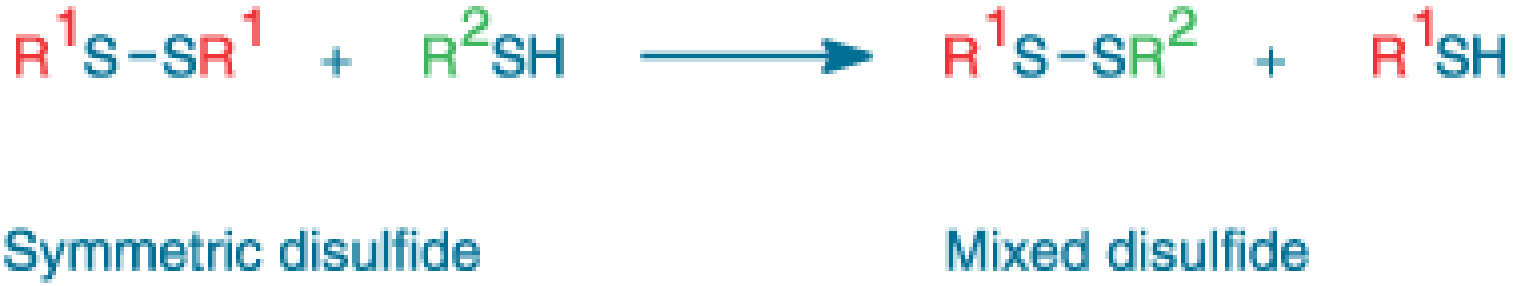
Reaction for covalent labeling of molecules with SH group

- Reaction of thiol group with maleimide to form a thioether
- The double bond of the maleimide reacts with SH thiol group to form a thioether

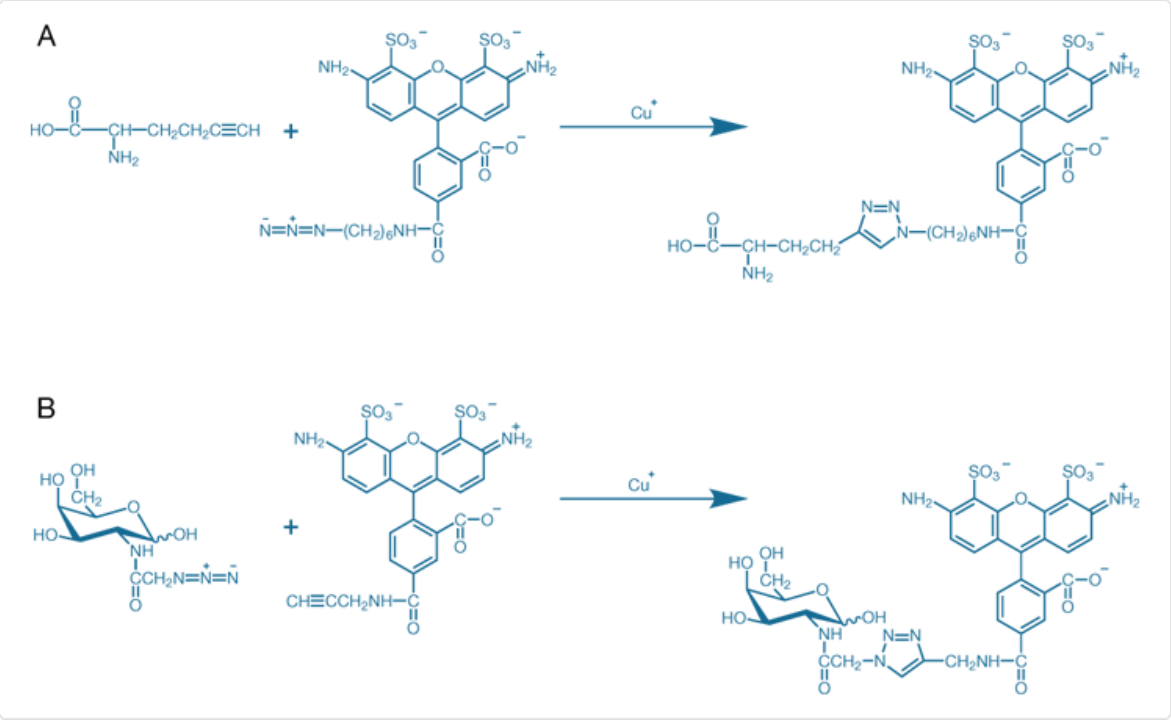


Other reactions for labeling of molecules with SH group

Disulfides

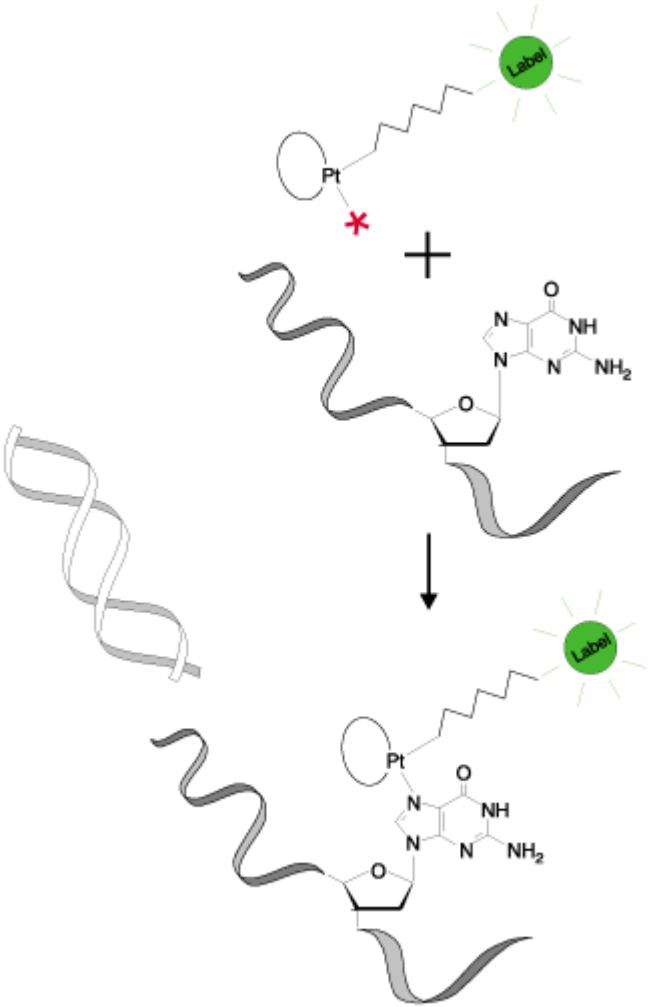


“Click” chemistry



- Reaction catalyzed by Cu^{2+}
- Azide-alkyne cyclization chemistry for the detection of A) proteins and B) sugars
- Reaction partners A) L-homopropargylglycine (HPG) and Alexa Fluor 488 azide and B) *N*-azidoacetylgalactosamine and Alexa Fluor 488 alkyne
- Partner on the left is introduced into proteins by de novo synthesis or posttranscriptional modifications

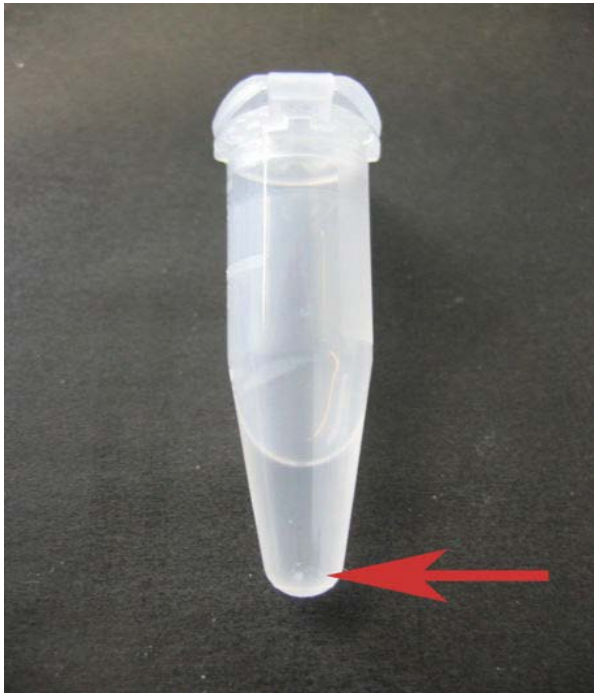
Other methods for labeling



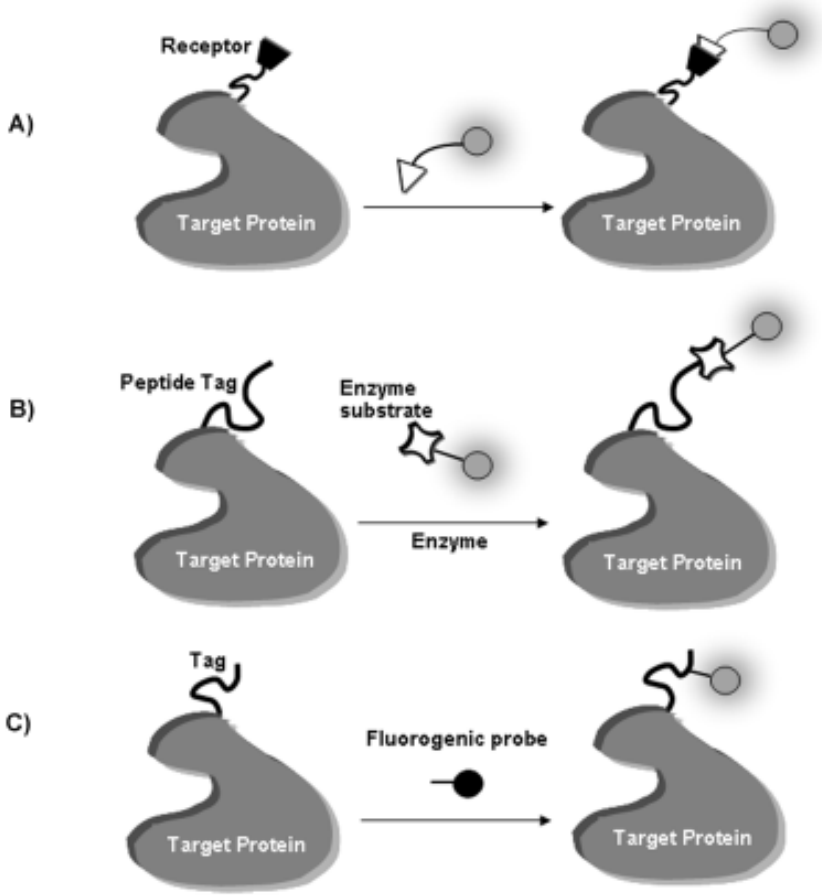
- DNA can be covalently labeled without connection of NH_2 group in the synthesis
- It uses a reactivity of N7 guanine with platinum complexes
- Platinum complex contains a fluorescent dye which is covalently attached to guanine after the reaction

Separation of unbound dye

- Chromatography (protein) gel filtration
- Ultrafiltration
- Dialysis
- Precipitation (DNA)



Chemical labeling of proteins *in vivo*



- A)Receptor-protein

– fluorescent dye or probe is attached to a molecule that has high affinity to receptor (avidin - biotin)

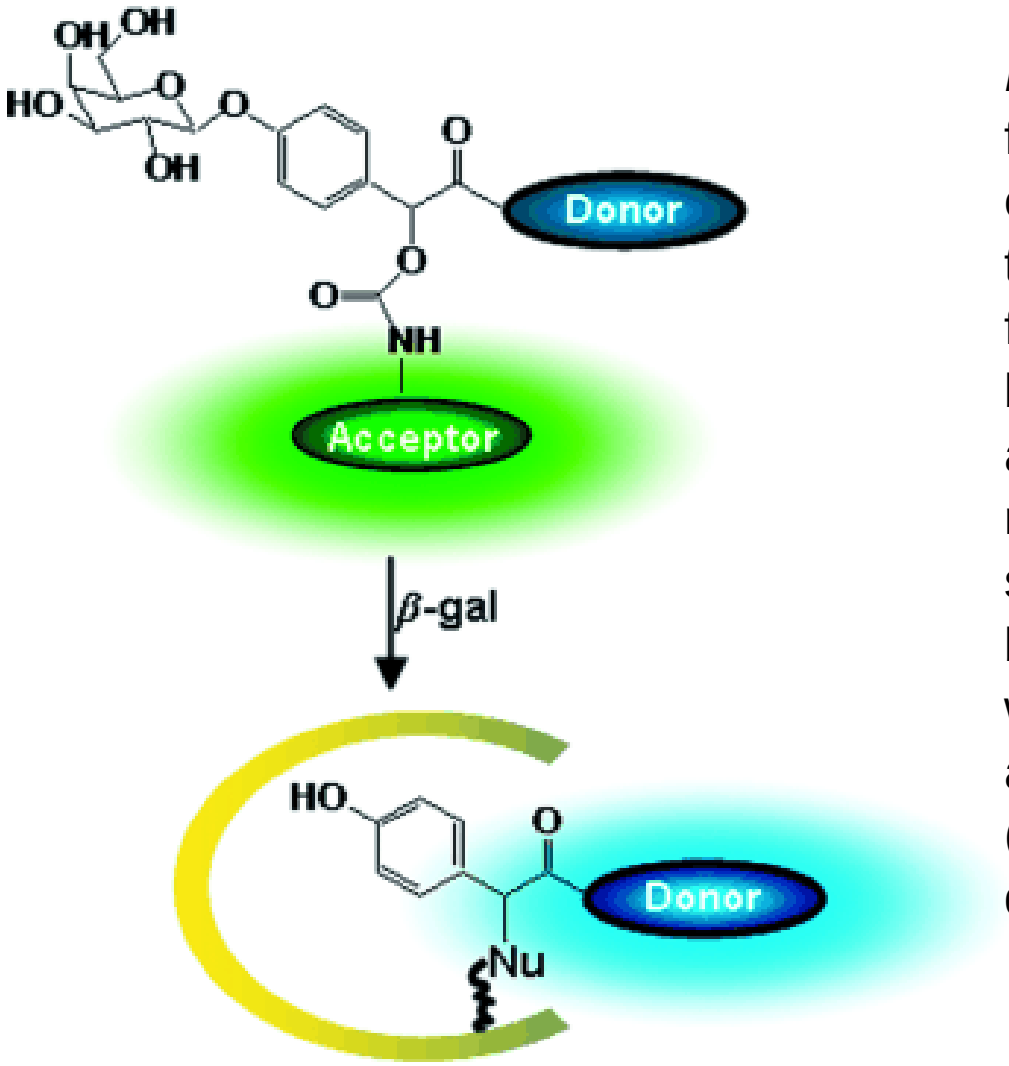
- B) Binding mediated by the enzyme

– fluorophore is attached to the substrate that can be covalently bound to a peptide "tag" of protein through another protein – enzym

- C) Probe does not emit fluorescence originally. After its binding to "tag", activation of fluorescence occurs (fluorogenic reaction)

Dragulescu-Andrasi, A., and Jianghong Rao, ChemBioChem, 2007

Fluorogenic detection



In vivo labeling of β-gal by a fluorogenic probe with spectral change. The labeling is proposed to take place in two steps. The first step involves *O*-galactoside bond cleavage, which generates an active intermediate quinone methide. This intermediate is susceptible to nucleophilic attack by a nearby amino acid residue, which leads to covalent attachment of the FRET donor (D) to the enzyme and displacement of the acceptor (A).

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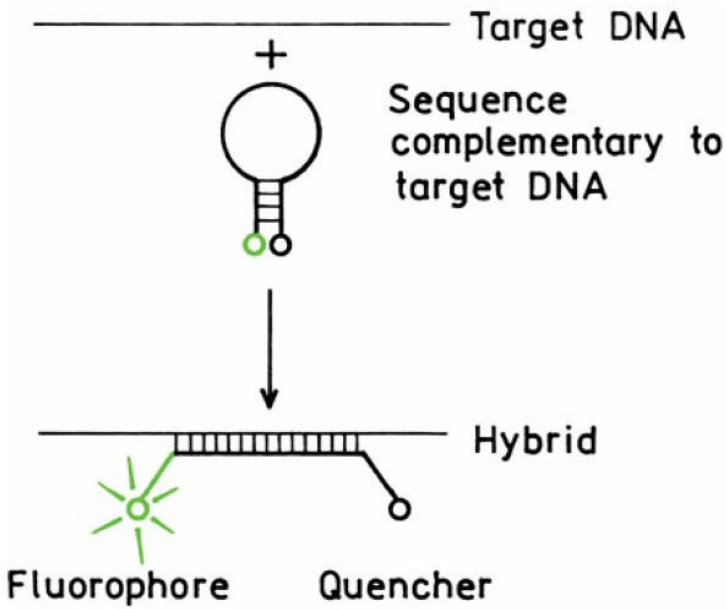
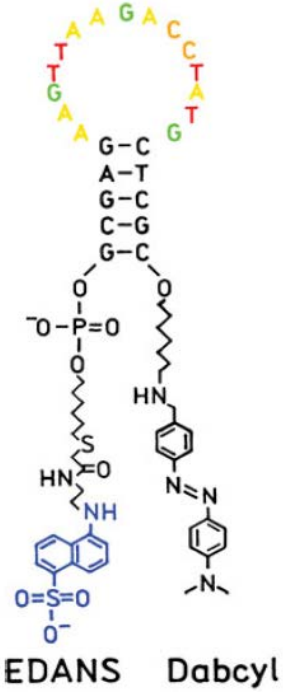
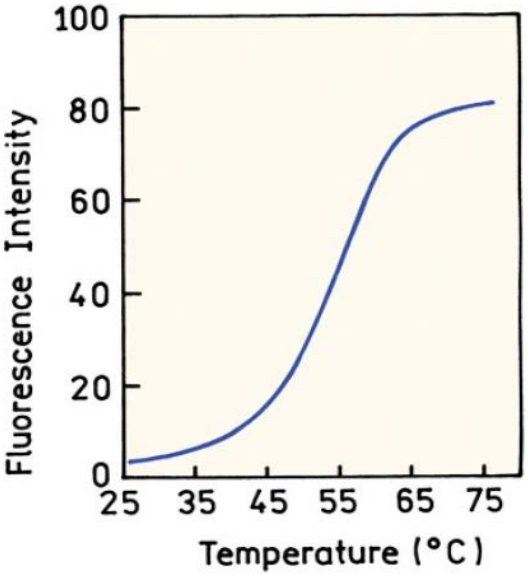
DNA beacons



- Beacon in English means signal fire (no bacon)
- DNA beacon consists of a DNA molecule that is able to form a hairpin structure
- DNA has a fluorophore attached at one end and a quencher at the other end
- In hybridization with a complementary DNA, the quencher is displaced and fluorescence is emitted subsequently

Structure and properties „DNA beacon“





Increasing temperature leads to melting of hairpin structure, which results in increase of fluorescence intensity

Note: In case of Dabcyl, it was found an unexpected ability to quench a wide range of fluorophores regardless of degree of spectral overlap. The reason for this universal quenching is probably formation of non-fluorescent complex of Dabcyl with fluorophore. Anyway, it is an advantage because one quencher can be used for a wide range of fluorophores.

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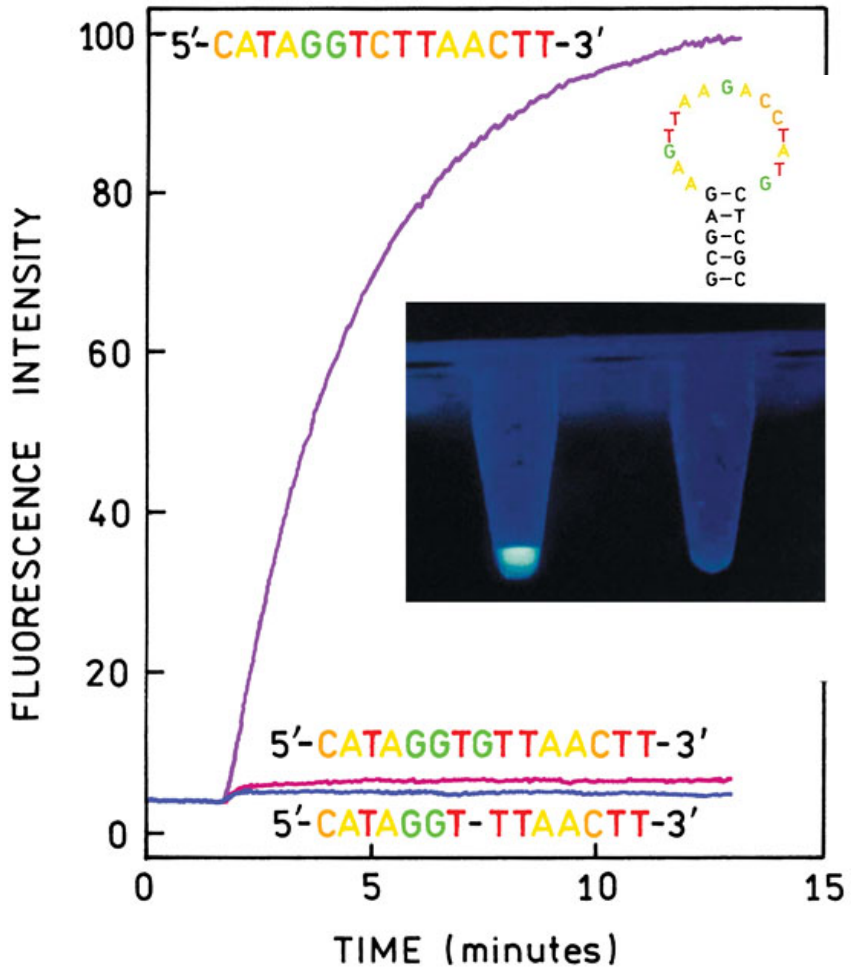
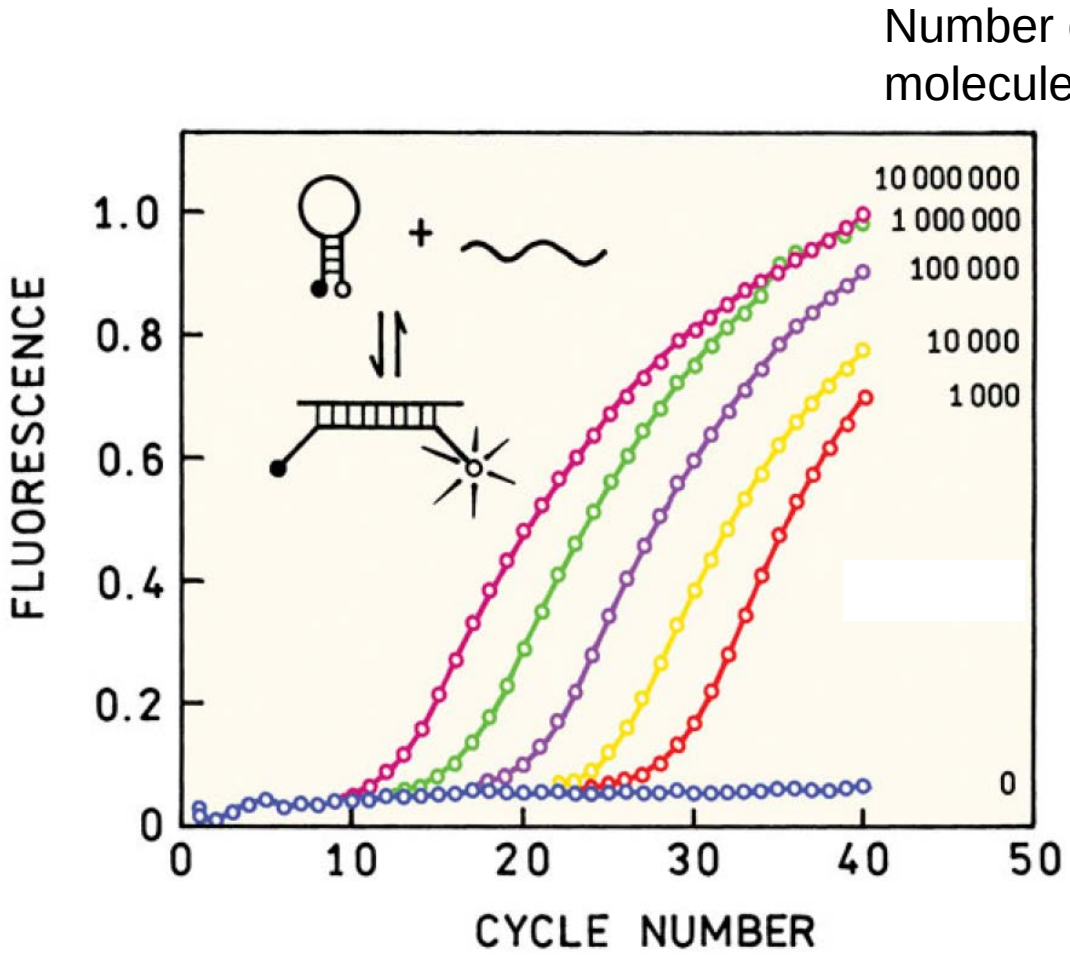
High sensitivity of DNA beacon



- In the case of hybridization with the DNA that differ only in one nucleotide, there is no increase in fluorescence signal

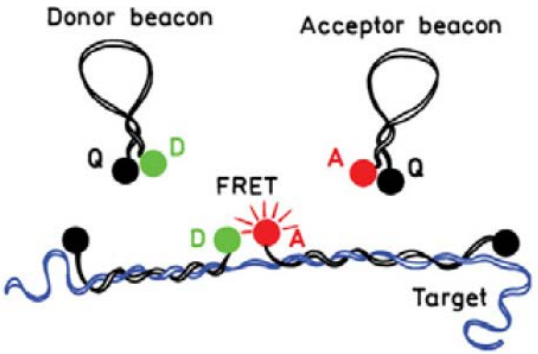
- High sensitivity and the ratio **on/off** signal **in the presence/ in the absence** of complementary DNA

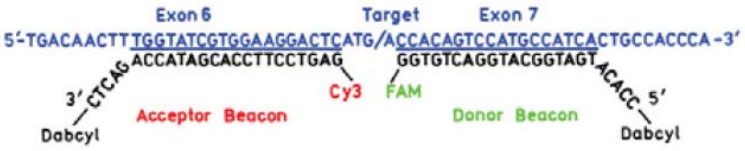
Using of Dabcyl-Fluorescein couple for „DNA beacon“ to monitor PCR

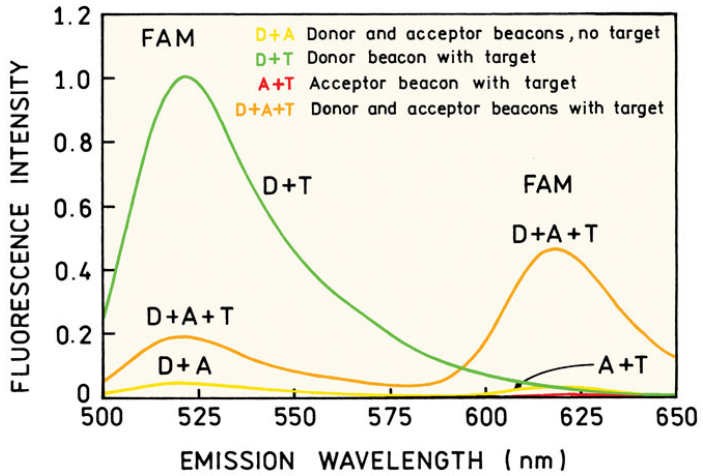


Fluorescence intensity is directly proportional to the amount of amplified DNA

Hybridization in neighboring areas

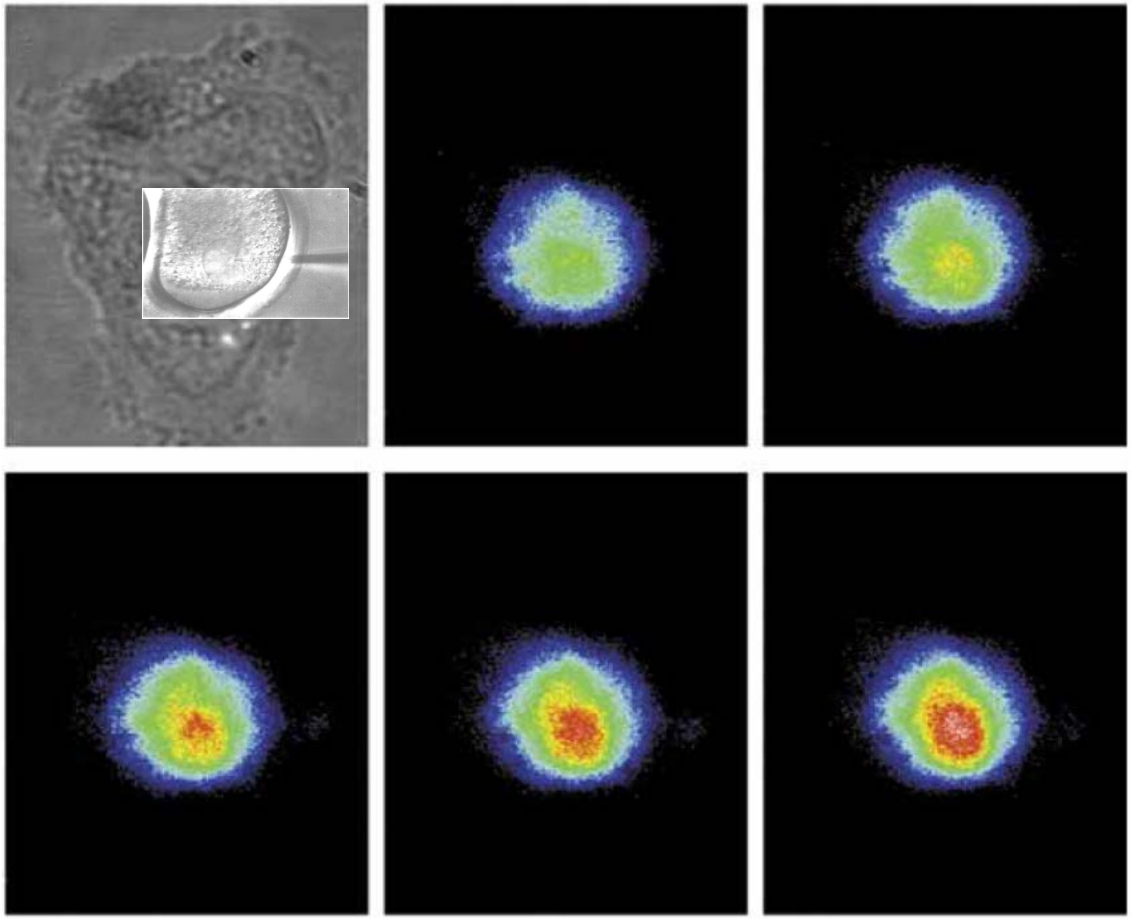






- A preferred arrangement to reduce false positive results
- It use two hairpins
- One hairpin contains donor and the second contains acceptor of FRET
- Without target DNA, both fluorophores at the hairpin are quenched
- If the correct hybridization with target DNA occurs, FRET is observed
- If only one hairpin is hybridized, although the increase in intensity occurs, FRET is not increased
- This increases the specifity of the analysis

Detection of mRNA in living cells



- Optical and fluorescent display of kidney cells
- Increase in fluorescence intensity of "DNA Beacon" against the mRNA for β-actin demonstrated an increase in the concentration of mRNA and consequently an increase in production of β-actin

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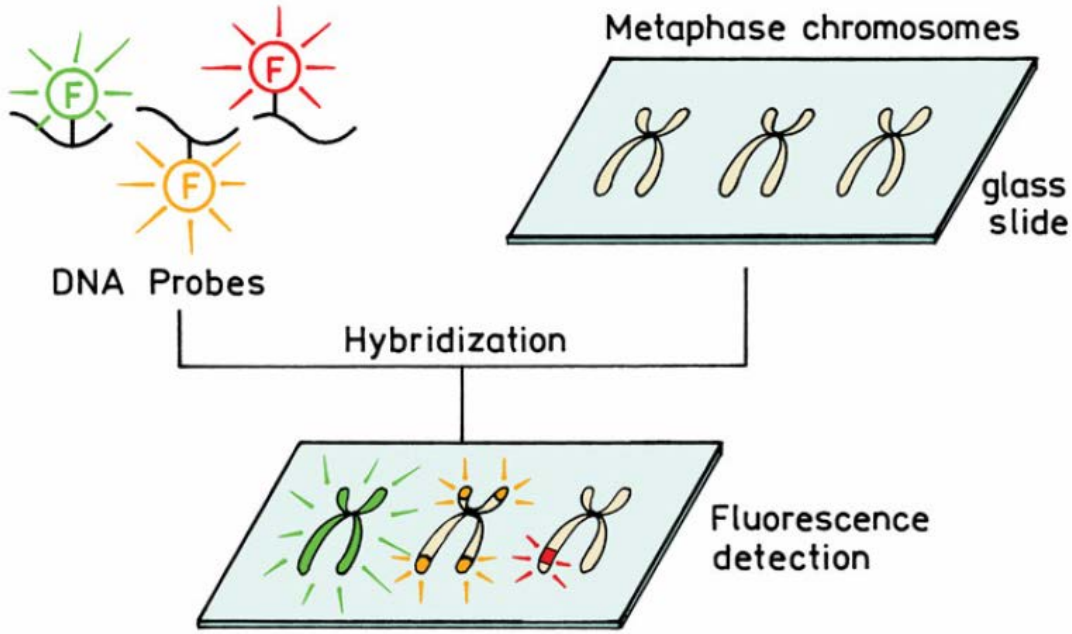
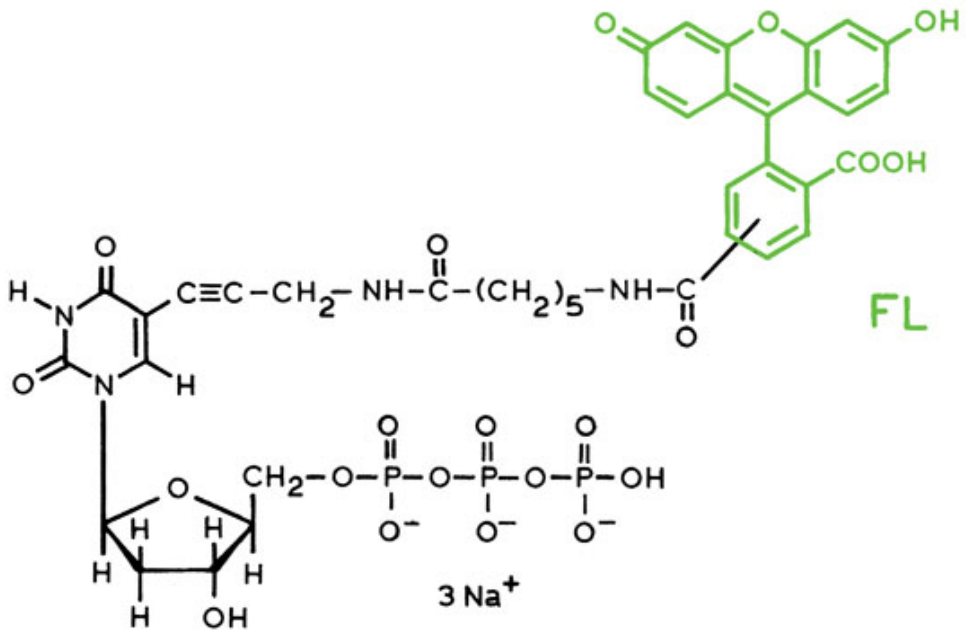
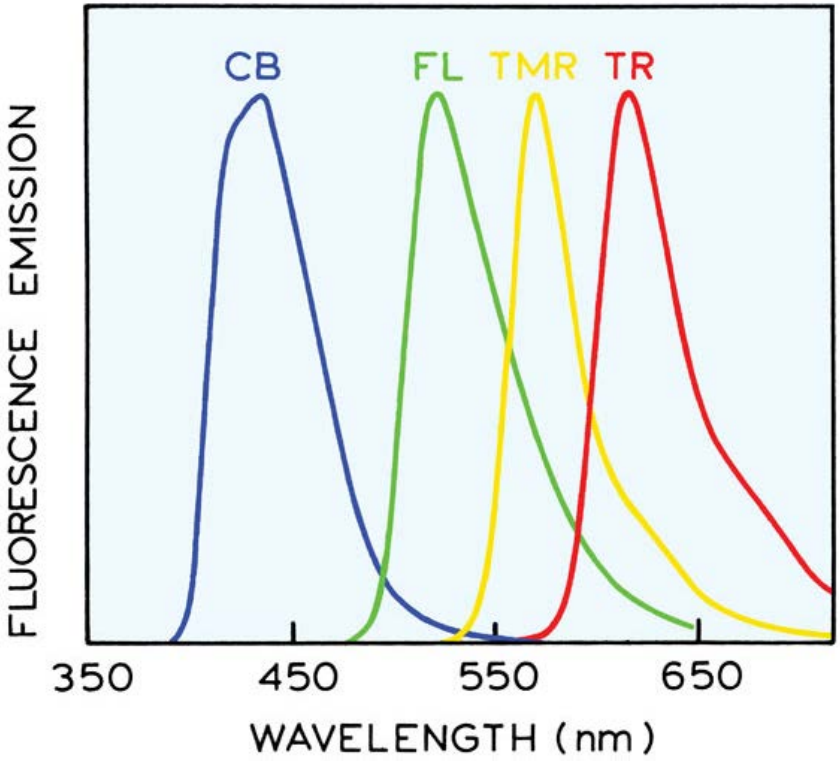
FISH (fluorescence *in situ* hybridization)



- DNA in the form of metaphase chromosomes is exposed to hybridization with fluorescently labeled DNA probe
- DNA probe has a sequence complementary to the target DNA on the chromosome
- After hybridization, a part containing the target DNA is localized based on fluorescence

Fluorescent deoxyribonucleotides for DNA synthesis of FISH probes





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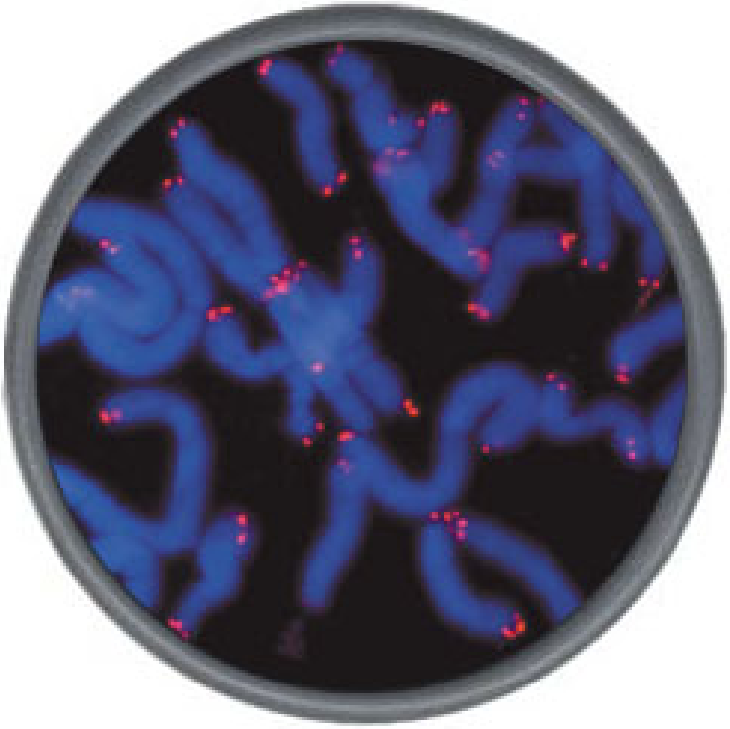
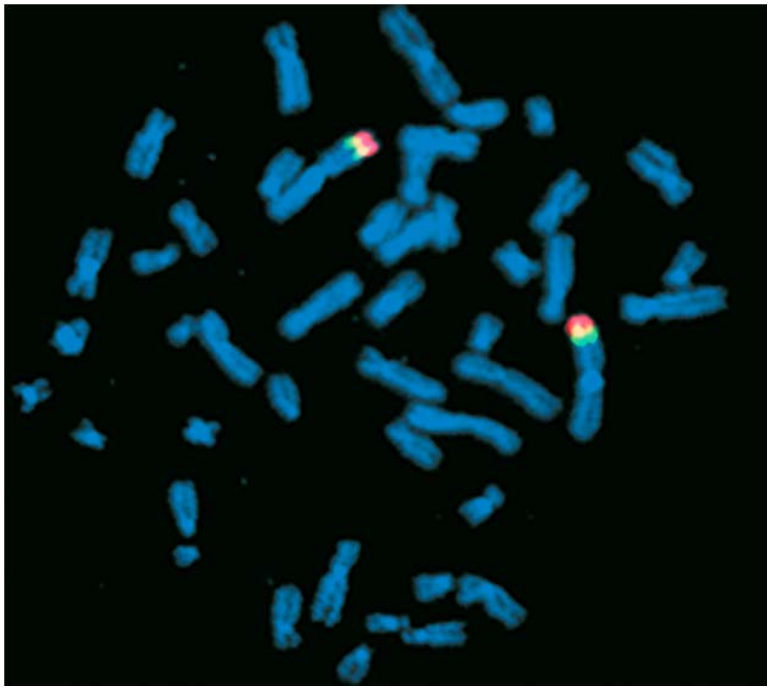
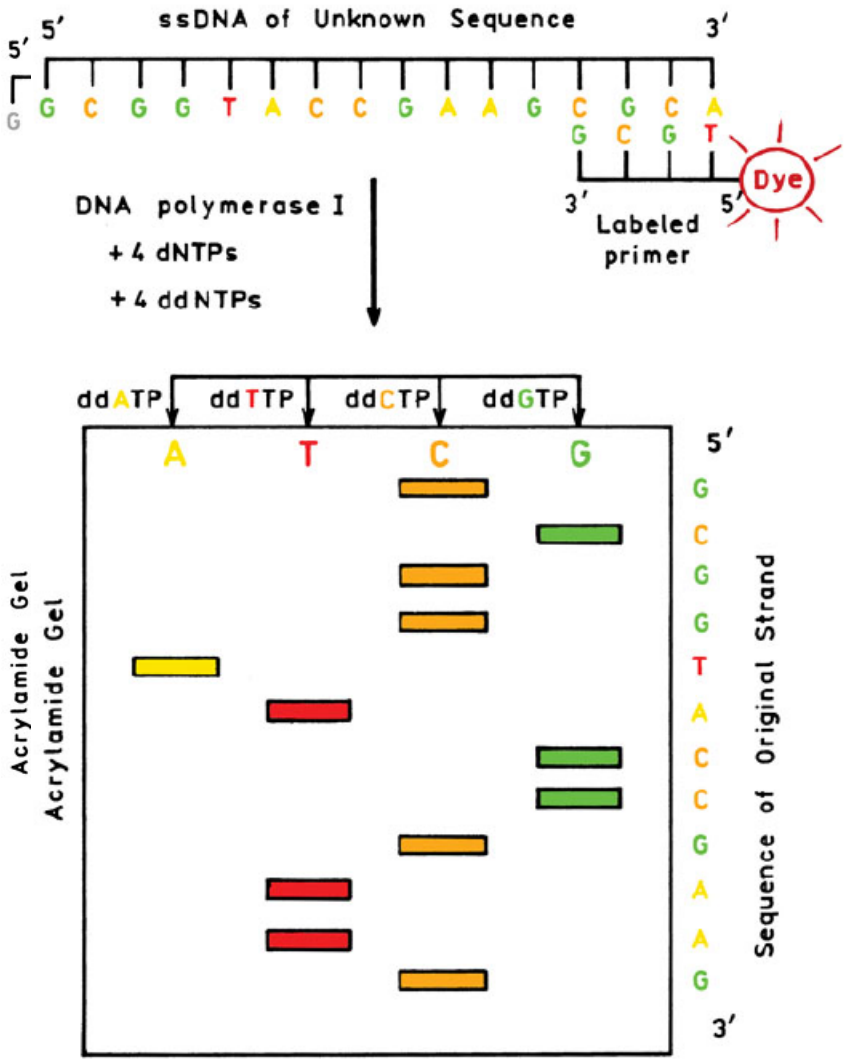
FISH examples





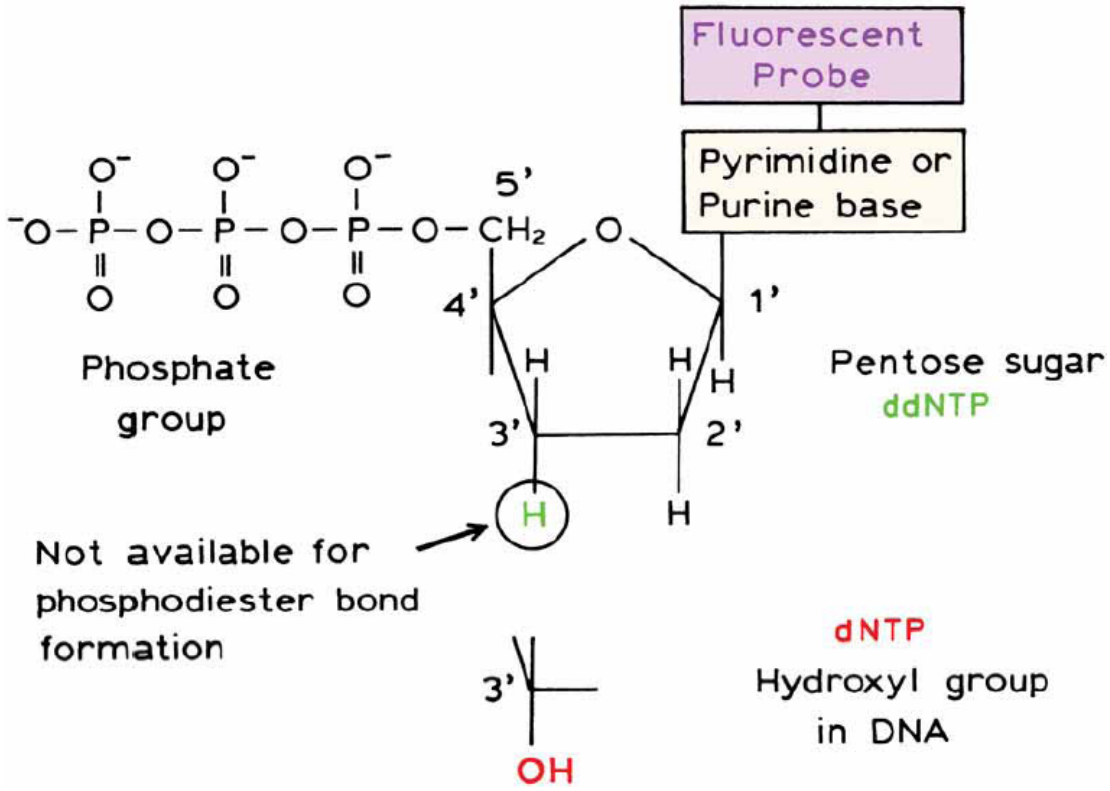
- Chromosomes hybridized with fluorescently labeled probes
- Chromosomal DNA was stained with DAPI nonspecifically

Radioactive sequencing



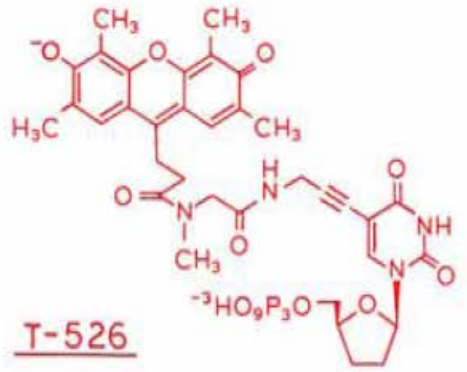
- Initial sequencing used a radioactively labeled primer
- The primer was extended by DNA polymerase according to sequenced chain
- To a mixture of normal nucleotides (dNTP) there was always added one type of dideoxynucleotide (ddNTP) that prevents further elongation of synthesized chain
- This generates a mixture of DNA chains with variable length that always ends by a given ddNTP
- Each reaction mixture for a given ddNTP was analyzed in one electrophoretic well (total in 4 wells)
- The sequence was determined from the position of band for the corresponding ddNTP on the gel

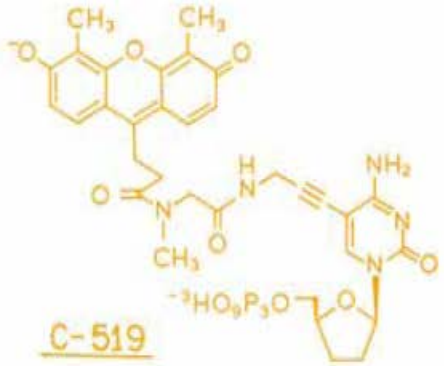
Scheme of DNA ddNTP

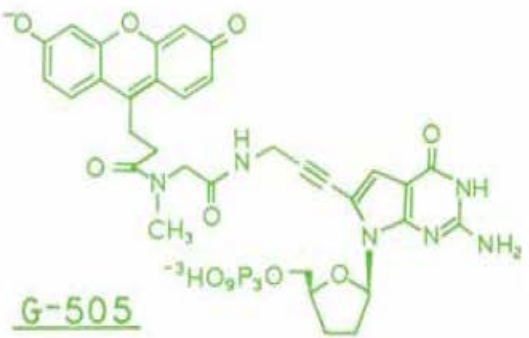


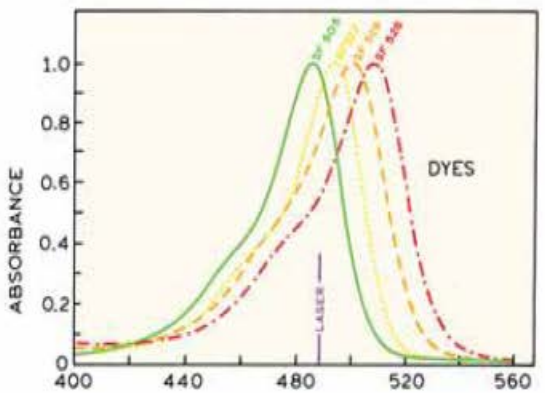
- Dideoxynucleotide triphosphate ddNTP
- At position 3´ of ribose, OH group is replaced by an H, which prevents further DNA chain elongation

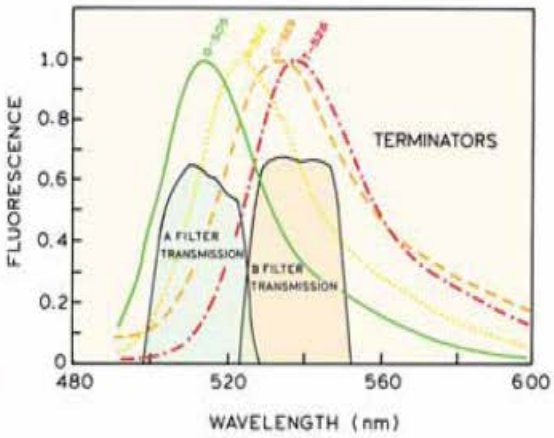
Fluorescent labels for sequencing







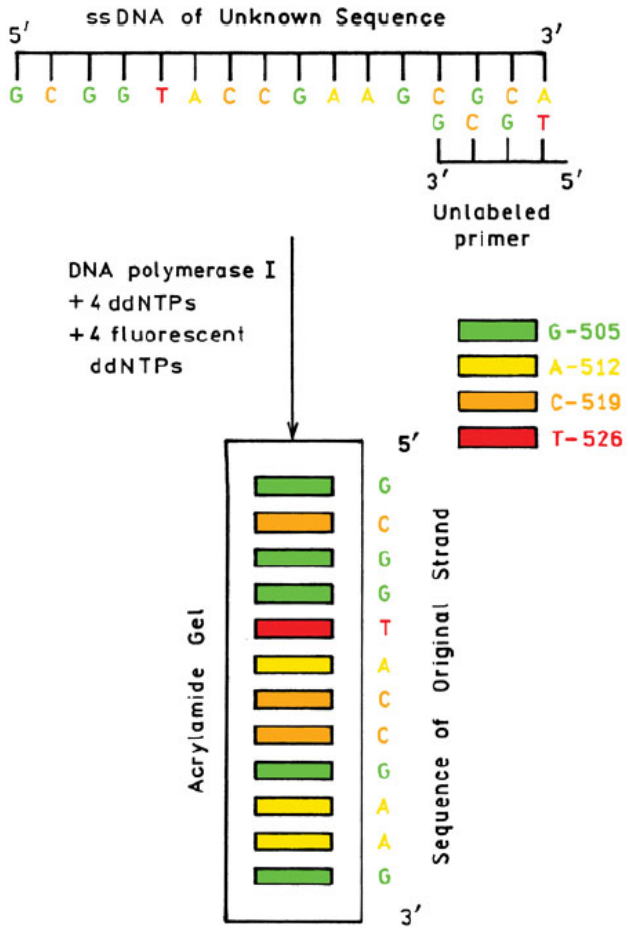




The labels are connected through an acetylene triple bond
All labels are excited by one laser (488nm)

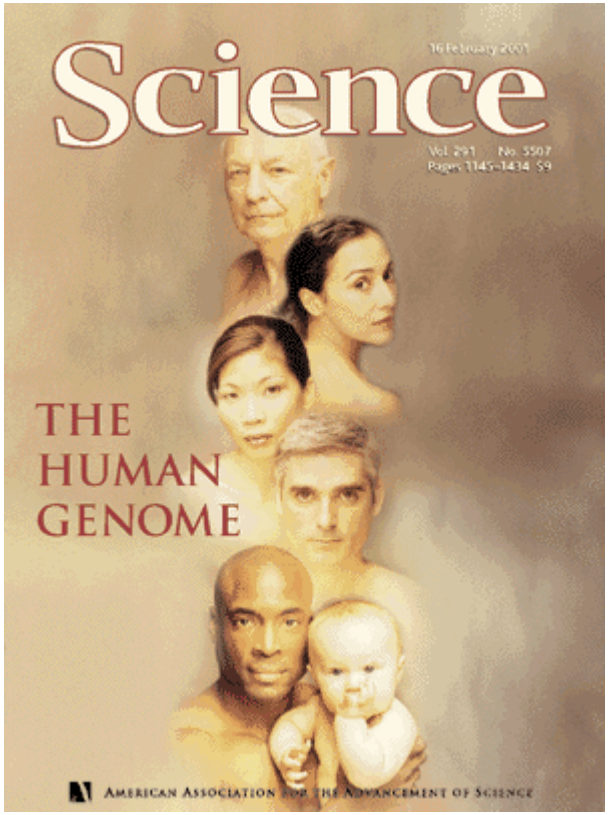
A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides
JM Prober, GL Trainor, RJ Dam, FW Hobbs, CW Robertson, RJ Zagursky, AJ Cocuzza, MA Jensen, and K Baumeister
Science 16 October 1987 238: 336-341

Fluorescent labeling accelerated the sequencing



- Using four fluorescent labels allowed to use only one lane in the gel for identification of all nucleotides => 4-fold acceleration
- Fluorescence scanning enabled to complete genome sequencing projects of whole organisms in a significantly shorter time

Human genome



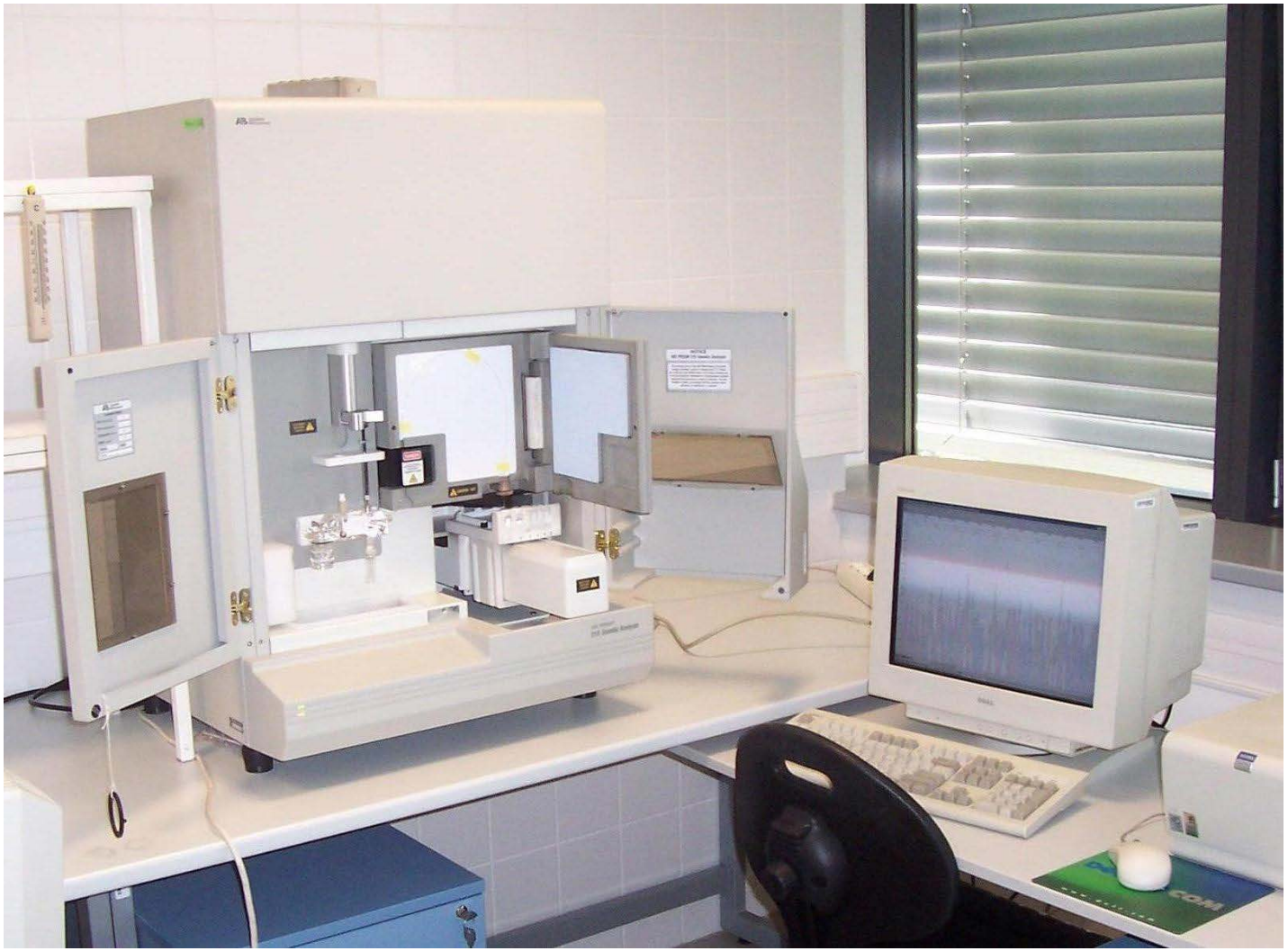
DNA sequencing

- The most common sequencing primer is M13:

5'- gTA AAA CgA Cgg CCA gTg -3´

http://www.wiley.com/college/pratt/0471393878/student/animations/dna_sequencing/index.html

Fluorescence sequencer



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Literature

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- Fišar Z.: FLUORESCENČNÍ SPEKTROSKOPIE V NEUROVĚDÁCH
  http://www1.lf1.cuni.cz/~zfisar/fluorescence/Default.htm

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