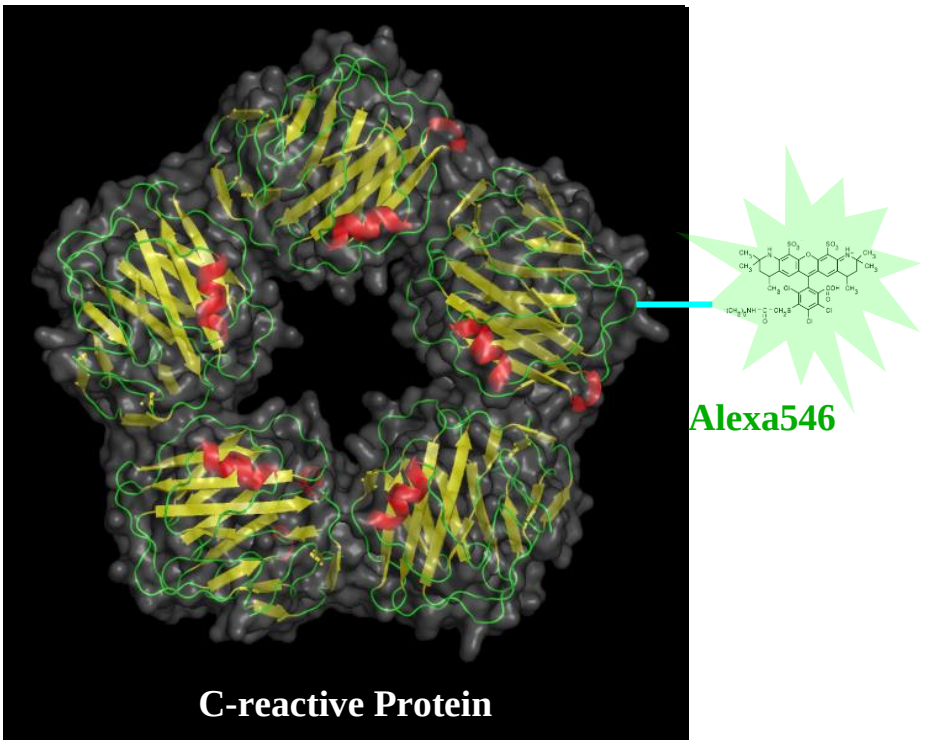
Biophysics Lab Course

ulm university universität ulm

Fluorescence labelling of proteins

Specific fluorescence labelling and characterization
with absorption and fluorescence spectroscopy

Subjects: Fluorescent labelling, amino- and thiol- modification, separation by gel filtration, absorption spectroscopy, protein quantification, fluorescence spectroscopy



Experiment:

The plasma protein CRP (C-reactive protein, an "acute-phase protein", the concentration is strongly increased during inflammation) is labelled with an amino-reactive fluorescent dye.

After separation of the excess of free dye the degree of labelling is characterized by absorption spectroscopy. The fluorescence excitation and emission spectra of the labelled protein can be determined.

Please bring your lab coat with you for this experiment

Fluorescent labelling

The fluorescence-based research methods, due to their high sensitivity, are gaining increasing importance in biochemical analysis, cell biology and other fields of life sciences. In the analysis this methods are allowing the usage of small amounts of sample together with very short measurement times, which is especially important for “high-throughput screening” in the pharmaceutical industry. In basic research, the high sensitivity allows using confocal laser microscopy also for the study of the properties and interactions of biomolecules on a single molecule level.

For example, a protein molecule can be labelled with two different fluorophores, which are suitable as a donor or acceptor for fluorescence resonance energy transfer (FRET). Because of the distance dependence of FRET, the conformational changes within a single molecule can be examined [1].

For this purpose, the protein molecules must be first labelled with fluorophores such as organic chemical fluorescent dyes. Nowadays a large variety of amino- or thiol-reactive fluorophores for coupling to the lysine or cysteine side chains is available. A number of fluorophores, which are sold under the commercial name "Alexa Fluor" (Molecular Probes, Inc.), are listed in the "fluorescent dyes" table. Since all fluorophores can perform only a limited number of excitation cycles before, due to a photochemical reaction, they will lose fluorescence ("photobleaching"), they should be protected from light (container wraped in aluminium foil).

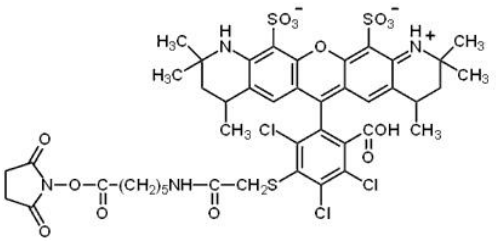
Fluorescent dyes

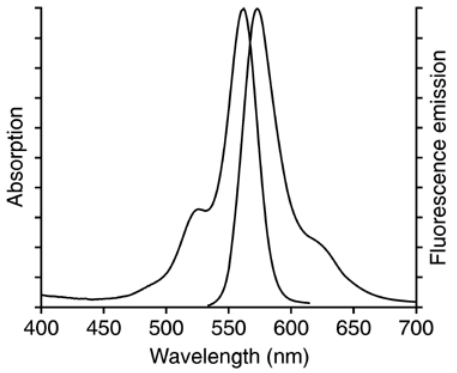
Table 3. Physical characteristics of the Alexa Fluor dyes.

Dye	Molecular Weight	Abs *	Em *	ε †	CF_{280} ‡	CF_{260} §
Alexa Fluor 350	410	346	442	19,000	0.19	0.25
Alexa Fluor 405	1028	401	421	34,000	0.70	0.23
Alexa Fluor 430	702	434	541	16,000	0.28	ND
Alexa Fluor 488	643	495	519	71,000	0.11	0.30
Alexa Fluor 500	700	502	525	71,000	0.18	0.32
Alexa Fluor 514	714	517	542	80,000	0.18	0.31
Alexa Fluor 532	721	532	554	81,000	0.09	0.24
Alexa Fluor 546	1079	556	573	104,000	0.12	0.21
Alexa Fluor 555	~1250	555	565	150,000	0.08	0.08
Alexa Fluor 568	792	578	603	91,300	0.46	0.45
Alexa Fluor 594	820	590	617	73,000	0.56	0.43
Alexa Fluor 610	1172	612	628	138,000	0.46	0.31
Alexa Fluor 633 **	~1200	632	647	100,000	0.55	ND
Alexa Fluor 647 **	~1300	650	665	239,000	0.03	0.00
Alexa Fluor 660 **	~1100	663	690	132,000	0.10	0.00
Alexa Fluor 680 **	~1150	679	702	184,000	0.05	0.00
Alexa Fluor 700 **	~1400	702	723	192,000	0.07	0.00
Alexa Fluor 750 **	~1300	749	775	240,000	0.04	0.00

* Absorbance and fluorescence emission maxima, in nm, conjugated to an IgG antibody. † Extinction coefficient at λ_{max} in $cm^{-1}M^{-1}$. ‡ Correction factor for absorbance readings (Abs_{280}) at 280 nm; e.g., $Abs_{280,actual} = Abs_{280,observed} - (CF_{280} \times Abs_{max})$. § Correction factor for absorbance readings (Abs_{260}) at 260 nm; e.g., $Abs_{260,actual} = Abs_{260,observed} - (CF_{260} \times Abs_{max})$. ** Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red–fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope.

In the experiment AlexaFluor546 is used:





AlexaFluor546 succinimidyl ester

Labelling reactions

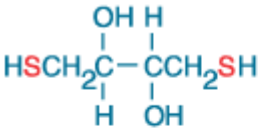
1. Thiol- modification:

For the specific labelling of protein molecules the thiol- modification is suitable, because smaller proteins usually contain very few cysteines. By point mutations cysteines can be introduced precisely where the label should be attached.

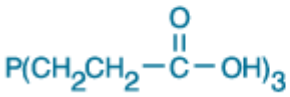
Maleimide Thioether

During the thiol- modification maleimide coupled to the dye (R1) reacts with a free thiol group, such as with the cysteine side chain in a protein (R2), creating a thioether, as shown above. The reaction proceeds optimally at neutral pH around 7.0-7.5.

Before this reaction will be performed on the protein, one must ensure that the thiol groups are presented in the reduced state. This can be achived, for example, by pre-incubation with reducing agents such as dithiothreitol (DTT) or Tris (2-carboxyethyl) phosphine (TCEP).



(A) dithiothreitol (DTT)

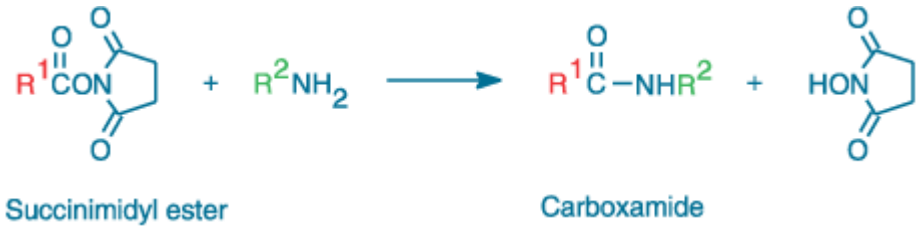


(B) tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP)

TCEP compared to DTT has the advantage that it does not have to be removed from the reaction tube before adding maleimid conjugate, as it does not contain a thiol group.

2. Amino- modification:

The modification of the amino groups of a protein is often used in conventional fluorescence microscopy. The conjugates are very stable, because of the frequency of the lysine in proteins; this method is, however, unsuitable for side specific labelling.
As labelling reagents for amino modification usually succinimidyl ester (NHS ester) derivates of fluorophores are used:



R1 in this example could again be a fluorophore, the R2 is the protein to be labelled. In proteins, there is an α-amino group at the beginning of the polypeptide and, if present, ε-amino groups in the side chains of the lysines. Amino reactive samples as the succinimidyl ester need deprotonated amino groups for reaction. The ε-amino group in lysine has a pKa of about 10.5, so that one should work in the slightly basic range in order to have some amino groups in the deprotonated state. If a more neutral buffer would be used, the α–amino group preferentially reacts with succinimidyl ester, as it has a lower pKa value than the ε-amino group.

Separation of conjugates

The gel filtration chromatography separates molecules by size. The solid phase of the column consists of bloated, small porous beads. Small molecules (in this case buffer molecules or fluorescent dye) can penetrate into the pores of the beads, where they are retained longer than larger molecules (in this case the protein). The large molecules flow in between the beads is much faster and it causes the big molecule to elute early. In case of the "spin columns" used in our experiment, the flow through the chromatography column is increased by centrifugal force.

Characterisation of protein conjugates

RNase H

RNase H is an endonuclease (155AA, M_w = 17.5 kDa), which can cut an RNA strand when it is hybridized to a complementary DNA strand. In this experiment, the mutant "C135" is used, which contains only one cysteine at position 135 and thus can be specifically labelled by thiol- modification. The amino acid sequence of the protein used is:

```
MLKQVEIFTDGSALGNPGPGGYGAILRYRGREKTFSAGYTRTTNNRMELMAAIVALEALK
EHAEVILSTDSQYVRQGITQWIHNWKKRGWKTADKKPVKNVDLWQRLDAALGQHQIKWEW
VKGHAGHPENERADCLARAAAMNPTLEDTGYQVEV
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C-reactive Protein (CRP)

CRP is one of the “acute phase proteins” whose concentration in the blood increases greatly during inflammation. The serum concentration of CRP is thus used as a nonspecific inflammatory parameter. CRP is a homopentameric protein belonging to the family of pentraxins, which are characterized by five identical subunits arranged in a disk shape. Each of the subunits of CRP consists from 206 amino acids (mainly two antiparallel β- sheets with a short α-helix) with a molecular mass of 23 kDa, and the following amino acid sequence (UniProt: P02741):

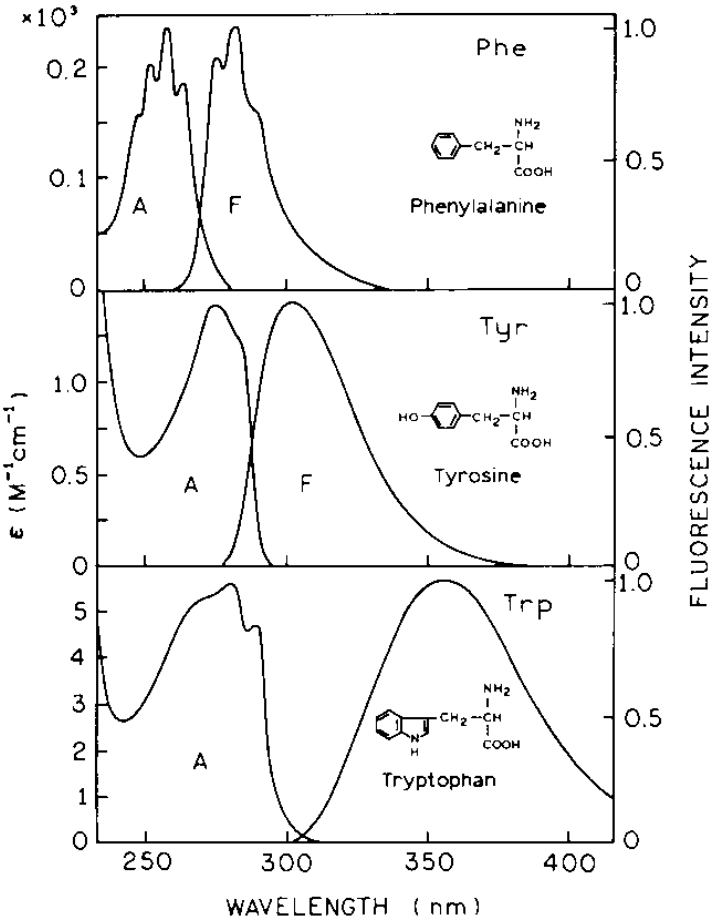
```
QTDMSRKAFVFPKESDTSYVSLKAPLTKPLKAFTVCLHFYTELSSTRGYSIFSYATKRQD
NEILIFWSKDIGYSFTVGGSEILFEVPEVTVAPVHICTSWESASGIVEFWVDGKPRVRKS
LKKGYTVGAEASIILGQEQDSFGGNFEGSQSLVGDIGNVNMWDFVLSPDEINTIYLGGPF
SPNVLNWRALKYEVQGEVFTKPQLWP
```

UV-Absorption of proteins

Proteins contain three amino acids, which mainly contribute to their UV absorption: tyrosine, tryptophan and phenylalanine.

Absorption and emission spectra for these amino acids in aqueous solution at pH 7.0 are shown in the graph on the right.

The absorbance at 280 nm is used for the characterization of the protein concentration, so only the tyrosine and tryptophan absorption essentially contribute.



As the environment of an amino acid in a folded polypeptide influences its extinction coefficient, average extinction coefficients, which were determined by absorbance measurements of native proteins [2], are used here:

Tyrosine (Y): ε_{280} (Y) = 1480 $M^{-1}cm^{-1}$

Tryptophan (W): ε_{280} (W) = 5540 $M^{-1}cm^{-1}$

The amount of tyrosine (n) and tryptophan (m) residues in a protein results in the extinction coefficient of the protein:

$$\varepsilon_{280}\,(\text{Protein}) = n \cdot \varepsilon_{280}\,(Y) + m \cdot \varepsilon_{280}\,(W)$$

According to the Lambert-Beer law, the concentration (c) of the protein can be determined using the absorption (A) of protein solution:

$$A = \log(I_0/I) = \varepsilon \cdot c \cdot d,$$

where the thickness (d) of the cuvette is typically 1 cm.

Characterization of labelling

From the absorption spectrum, the concentrations of the protein and dye can be calculated. During determination of the protein concentration from the UV absorption one must also remember that the dye itself shows UV absorption.
For this we define a correction factor:

$$CF_{280} = \varepsilon_{280}(\text{dye}) / \varepsilon_{556}(\text{dye})$$

Thus, the pure protein absorption at 280 nm is given by:

$$\begin{aligned} A_{280}(\text{prot.}) &= A_{280} - A_{280}(\text{dye}) \\ &= A_{280} - A_{556} \cdot CF_{280} \end{aligned}$$

The correction factor for CF_{280} AlexaFluor 546 is indicated in the table above.
The protein concentration is calculated from the following equation:

$$c(\text{prot.}) = (A_{280} - A_{556} \cdot CF_{280}) / (\varepsilon_{280}(\text{prot.}) \cdot d)$$

Using the dye absorption in the chromophore peak (here 556nm), the dye concentration, and thus the ratio of dye molecules to protein molecule (labelling efficiency), can be determined.

How likely it is to find non-labelled protein molecules? How likely is it for protein molecules to have one or two dye labels? (Poisson distribution!)

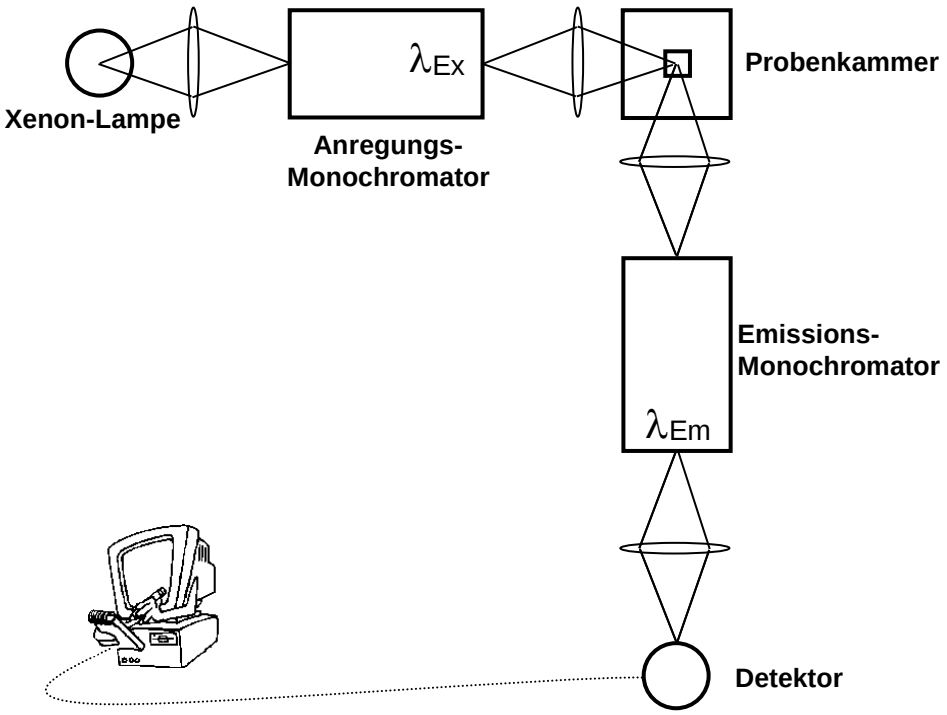
FCS to verify the conjugation

While the characterization based on absorption only describes the ratio of the components, fluorescence correlation spectroscopy (see experiment “FCS”) can be used to show that the fluorophores are in fact associated with the large protein, as the mobility of the dye molecules is drastically reduced. This experiment will be performed in the summer semester. Therefore, store your protein conjugate at 4 °C until the FCS experiment, and then characterize the hydrodynamic radius and the molecular mass of the protein conjugate.

Fluorescence Spectroscopy

After excitation of a fluorescent sample with light of wavelength $\lambda_{Ex,}$ part of the absorbed photons is emitted again in the form of photons, but with less energy and thus longer wavelength λ_{Em} [3]. The emitted radiation is detected in a fluorescence spectrometer (spectrofluorometer) in general at an angle of 90° to the excitation direction. For recording spectra either the excitation wavelength λ_{Ex} is fixed and the emission wavelength can be varied (emission spectrum) or the emission wavelength λ_{Em} is fixed and the excitation wavelength varies (excitation spectrum). If the fractional part of the emitted photons to absorbed photons (fluorescence quantum yield) does not depend on the excitation wavelength, then the absorption spectrum and the excitation spectrum should be identical.

The schematic structure of a spectrofluorometer is outlined below. The spectral resolution of the device can be changed by varying the slit widths of the excitation and emission monochromators.



Assignment

1 + 2) Label one of the two presented proteins (we choose CRP) with the fluorescent dye AlexaFluor 546 – NHS ester and purify the reaction product of the excess of free dye.

3) Determine the absorption spectrum of your product and the labelling efficiency. How likely is it to have non-labeled, single-labeled and double-labeled protein molecules? First calculate the extinction coefficient ε_{280} of your protein from its amino acid sequence. Determine the protein yield by comparison with the amount of used protein.

4) Measure a fluorescence excitation spectrum and an emission spectrum of your labelled protein, and another emission spectrum with excitation in the UV band of the protein. Compare the excitation spectrum and the absorption spectrum and discuss the relationship between excitation and emission spectrum.

5) Save your protein sample for future mobility test using FCS. To be prepared for the FCS measurement, solve the following task and add the calculation to your protocol: With the help of known data for Rhodamine 6G (M= 470 Dalton, R_H = 0.59 nm) and the relation $\frac{R_H^{Produkt}}{R_H^{Rh6G}} = \sqrt[3]{\frac{M^{Produkt}}{M^{Rh6G}}}$ answer the question: which hydrodynamic radius of the tagged proteins (RNaseH, CRP monomer CRP pentamer) is to be expected, what is expected for the free dye?

In the experiment "FCS" you will use the measured diffusion correlation times for your product and for Rh6G and the equation: to determine the hydrodynamic radius of your product in order to verify the fluorescence labelling of the protein.

$$\frac{R_H^{Produkt}}{R_H^{Rh6G}} = \frac{\tau_D^{Produkt}}{\tau_D^{Rh6G}}$$

Literature:

[1] EV Amirgoulova, J. Groll, CD Heyes, T. Ameringer, C. Röcker, M. Möller, GU Nienhaus, ChemPhysChem **2004**, 5, 552

[2] H. Mach, CR Middaugh, RV Lewis, Anal. Biochem. **1992**, 200, 74

[3] CR Cantor, PR Schimmel, Biophysical Chemistry: Part II, WH Freeman, **1980**.

Experimental procedure

1) Labelling of CRP using amino-modification

- transfer of the protein into reaction buffer (100 mM phosphate buffer, pH 8.5):
  - equilibrate spin column 2x (small Eppi centrifuge 4200 rpm, 2 min) with 150 µl reaction buffer, then 1x with 20 µl reaction buffer
  - transfer 20 µl protein solution (8.7 µM, does it already have the right concentration?) to spin column, centrifuge into new Eppi tube
- labelling of the protein:
  - add 0.2 µl of a 2 mM dye solution in DMSO to the protein solution
  - incubate for 1 h at RT in the dark

2) Separation

- equilibrate 2 spin columns 2x each with 150 µl PBS buffer (pH 7.5), then 1x with 20 µl
- transfer reaction solution to spin column, centrifuge in a new Eppendorf tube
- repeat the procedure with the second spin column

For 1) and 2) see also spin column scheme on next page

3) Absorption spectrum and degree of labelling

- record baseline 260-650 nm with 180 µl of PBS buffer in microcuvette
- add 20 µl of reaction solution, measure absorption spectrum
- determine absorption of the protein (280 nm) and of the dye (556 nm).
- dye concentration: c (dye) = 10 * A_{556} / ε_{556}
- protein concentration: c (prot.) = 10 * (A_{280} - A_{556} * CF_{280}) / ε_{280}(prot.)
- labelling efficiency r = c (dye) / c (prot.)

4) Fluorescence spectra of the conjugate

- fluorescence excitation spectrum (260 - 600 nm) at 610 nm emission wavelength
- emission spectrum (540 nm-700 nm) at 520 nm excitation
- emission spectrum (290 nm-450 nm) at 280 nm excitation
- transfer the protein solution into low binding reaction tubes and store the protein at -20 °C for later mobility test

5) Mobility (performed while "FCS")

- *Perform FCS at 20-fold diluted solution:*
  Determination of the hydrodynamic radius using the mobility of the product (Measurement of the diffusion correlation time) and comparison with the expected values for the conjugate and the free dye.

Spin column scheme

(sketched by former physics students Florian Bergmann und Michael Griener)