LABORATORY OF BIOPHYSICS

FOR ADVANCED

Experimental exercises for III year of the First cycle studies

Field: "Applications of physics in biology and medicine"

Specialization: "Molecular Biophysics"

Absorption and emission studies on Enhanced Green Fluorescent Protein chromophore formation (Ex. 35)

HUMAN CAPITAL
NATIONAL COHESION STRATEGY

EUROPEAN UNION
EUROPEAN
SOCIAL FUND

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Experiment purpose

The purpose of experiment is to compare absorption and emission kinetics of EGFP (enhanced Green Fluorescent Protein) chromophore formation. The process should be characterised by a number of phases and relevant time constants and compared between used methods.

Introduction

Absorption and emission properties of amino acids and proteins

Proteins and peptides are composed of 20 essential amino acids, which in addition to the structural role may also be cellular oxidative substrates, neurotransmitters or hormones. Only three amino acids exhibit absorption and emission properties in near ultraviolet range: phenylalanine, tyrosine and tryptophan (Fig. 1). The conjugated π-electron system in aromatic rings is responsible for spectral properties of these amino acids.

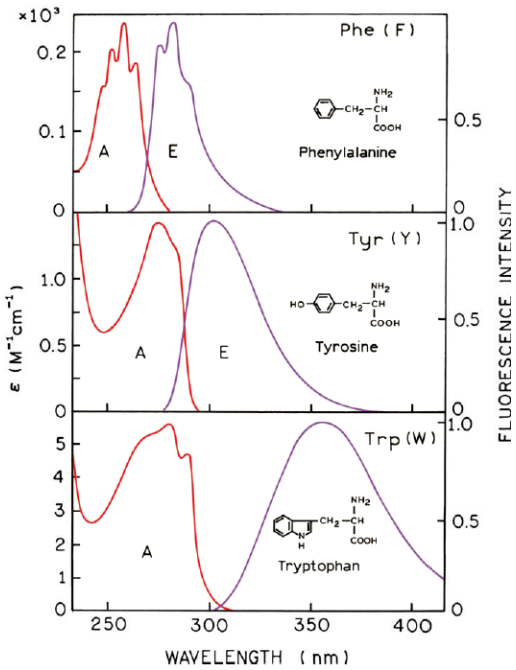

Fig. 1 Absorption (A) and emission (E) spectra of three aromatic amino acids in aqueous solution, pH 7, and their chemical structures [7]

Table 1. Spectral properties of aromatic amino acids in pH 7 (G. D. Fasman 1976 and J. R. Lakowicz 2006)

	max. λ_A [nm]	max. λ_E [nm]	ε [$M^{-1}cm^{-1}$]	Φ
Phe	257,5	282	15	0,03
Tyr	274	303	1 405	0,14
Trp	278	350	5 579	0,20

The most useful in protein studies is tryptophan, because of its unusual behaviour. It exhibits the most efficient quantum yield (Table 1) and fluorescence intensity and emission wavelength depends on hydrophobicity of its surroundings. It is a sensitive fluorescent probe of changes in protein conformation, denaturation process, in monitoring interactions of protein with ligand and subunit binding. Therefore, tryptophan is a useful tool in studies of protein function, folding and dynamics. One of its advantages is longer wavelength absorption spectrum in comparison with other aromatic amino acids. Shoulder at 295 nm makes possible to excite tryptophan independently from phenylalanine and tyrosine. Tryptophan is also very sensitive to fluorescence quenching, especially in contact with solution. Absorption and emission spectra of aromatic amino acids overlap each other, so resonance energy transfer is possible between phenylalanine and tyrosine or between tyrosine and tryptophan. Due to its properties tryptophan has a decisive influence on the shape of the absorption and emission spectra of proteins, and therefore maximum of protein absorption is observed at 280 nm.

Protein folding

It is little known about protein folding and so there is still a lot to explore to understand better that very complex process. So far our knowledge about protein folding is limited to globular proteins or globular protein domains behaviour. Probably native state is preceded by defined folding pathways, which might be divided in different mechanisms. The two most extreme models of protein folding are: framework model and hydrophobic collapse model. In framework model the secondary structure forms rapidly and is used as a scaffold for formation of the tertiary structure. Two mechanisms were proposed to describe behaviour of proteins following the framework model:

- diffusion-collision mechanism with formation of the secondary structure, which then diffuses, collides and coalesces in the tertiary structure
- nucleation mechanism in which formation of protein core is very slow, but once is formed the acquisition of the native state is fast

At early stage of folding by hydrophobic collapse model a hydrophobic nucleus forms rapidly. At this stage water molecules and hydrophilic residues are ejected from protein core, then the secondary structure is being formed. Before protein achieves native state it adopts a molten globule state consisting of hydrophobic core, high percent of overall secondary structure and possible residual tertiary structure, which is very dynamic. To become native protein must aquire correct interactions and adopt rigid structure. Realistic pathways of protein folding are between these two mentioned models.

Protein folding might be two- or multi-state (Fig. 2). The simplest example of protein folding is two-state folding (D → N), in which protein forms native structure (N) directly from denatured state (D). In multi-state folding protein forms intermediate and transition states, what affects folding rate (e.g. D→ I→ N). It is possible that intermediates exist during all types of protein folding, but in two-state folding they are not observable, because they are unstable, high-energetic (exist in high-energy minima, slightly lower than denatured state) and do not have any influence on protein folding rate.

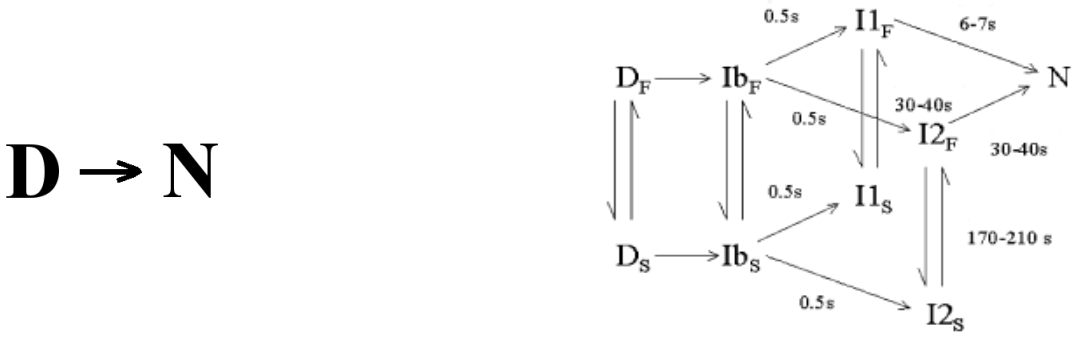

Fig. 2 Schemes of two-state folding (left panel) and multi-state folding on example of GFP mutant – Cycle3 (right panel, Enoki et al. 2004); D – denatured state, N – native state, IX_Y – different intermediates

Green Fluorescent Protein

Green Fluorescent Protein (GFP) was discovered in the 60's of XX century by a Japanese scientist Osamu Shimomura during his research on fluorescent protein – aequorin. In 2008 Osamu Shimomura, Martin Chalfie and Roger Y. Tsien received Nobel Prize in chemistry "for the discovery and development of the green fluorescent protein, GFP". GFP occurs in *Aequorea victoria* jellyfish and with aequorin forms a system that makes upset jellyfish glows. Aequorin exists in a complex with coelenterazine (pigment occurring in some coelenterates) and when protein binds calcium ions it changes structure and as a result coelenterazine is oxidised to excited form of coelenteramide. Returning to the ground state coelenteramide emits blue light or through FRET (Förster resonance energy transfer) transfers its energy to GFP chromophore obtaining green fluorescence.

Green Fluorescent Protein is a small globular protein (about 27 kDa, 238 amino acids) with characteristic β-barrel structure (Fig. 3, left panel). β-barrel consists of 11 anti-parallel β-sheets and is closed with loops and distorted helical fragments. The only α-helice is coaxial and bears chromophore (p-hydroxybenzylideneimidazolidinone). Chromophore is shielded by β-barrel structure, which prevents its visible fluorescence from quenching by solvent molecules. The proper turn in α-helice and a very complicated network of hydrogen bonds keep chromophore's ring in appropriate arrangement. The distance between rings of chromophore and only tryptophan is about 11-15 Å, which makes possible resonance energy transfer (Fig. 3, right panel).GFP chain contains 11 tyrosine, 12 phenylalanine and 2 cysteine residues, which do not form a disulphide bond.

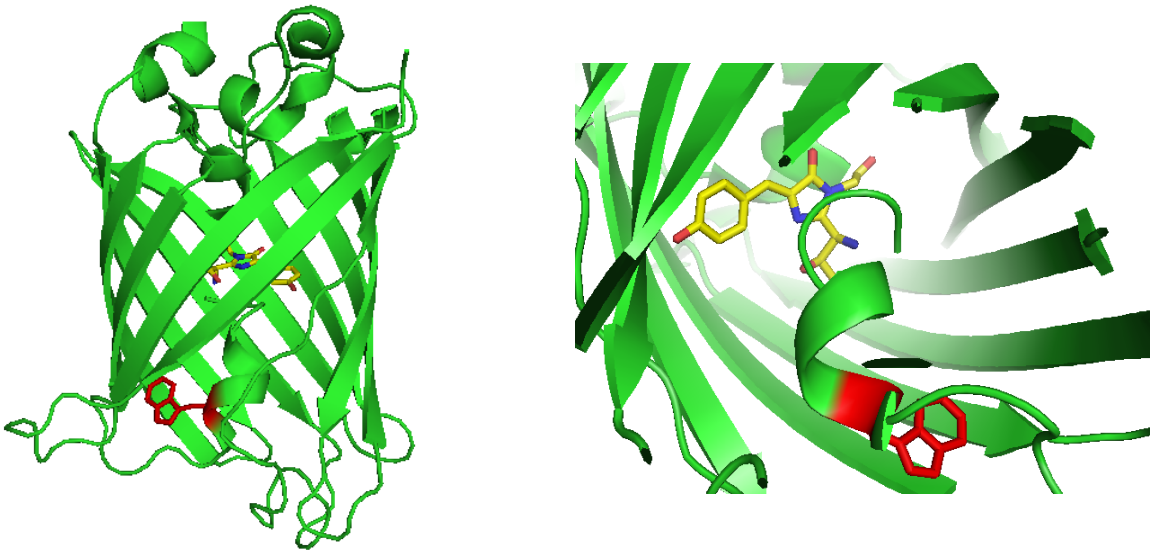

Fig. 3 Enhanced Green Fluorescent structure (left panel, PDB 2Y0G) and β-barrel interior with chromophore (yellow) and tryptophan (red).

In Enhanced Green Fluorescent Protein (EGFP) two point mutations were inserted: leucine instead of phenylalanine 64 and threonine instead of serine 65. These mutations entail more efficient protein folding, enhanced fluorescence intensity and reduction of absorption spectrum (Fig. 4). The latter is due to the change in the ionic form of chromophore, which in the wild-type protein

(wtGFP) is present both in neutral (absorption at approx. 395 nm) and anionic form (absorption at approx. 475 nm), while almost the entire population of EGFP possess chromophore in anionic form. EGFP absorption spectrum reveals two peaks with maxima at 278 nm (originates from aromatic amino acids) and at 489 nm (originates from chromophore). Extinction coefficients for aromatic amino acids and chromophore were derived experimentally by Seifert et al. (2002) and in pH 7.3 are ε_{277} = 21 000 $M^{-1}cm^{-1}$ and ε_{488} = 38 000 $M^{-1}cm^{-1}$, respectively. When excited at 280 nm there are two peaks observed: at 336 nm (aromatic amino acids) and at 509 nm (chromophore), which the latter gives protein greenish-yellow glow.

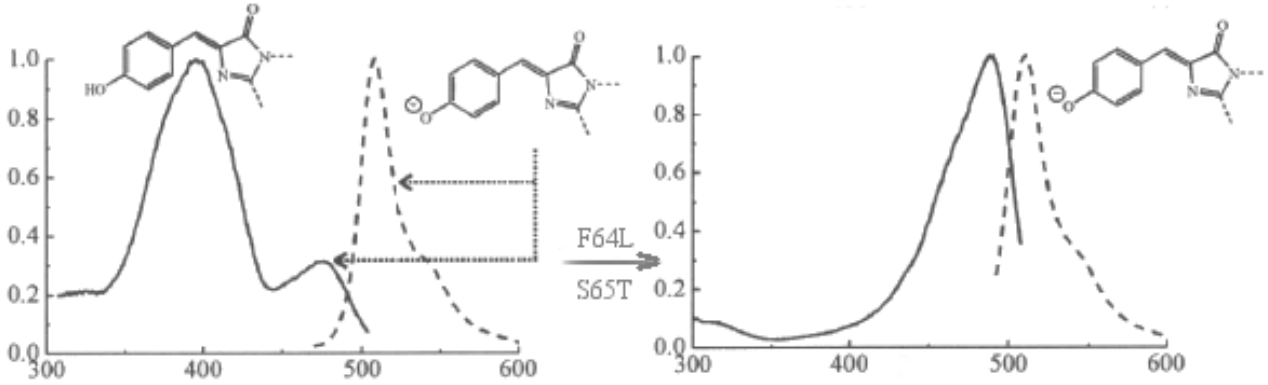

Fig. 4 Excitation (solid line) and emission (dashed line) spectrum of wtGFP (left panel) and the mutant F64L/S65T-GFP (right panel). R. Y. Tsien 1998, p. 520.

In wtGFP chromophore forms auto-catalytically from 3 amino acids: serine 65, tyrosine 66 and glycine 67. Inserting mutations in chromophore's region might change colour of the emitted light, but it is necessary to retain glycine 67 to keep protein fluorescent. After proper protein folding tripeptide is in conformation which allows internal chemical reaction resulting in cyclised ring (τ_c ~3 min.). Maturation of this chromophore pre-form includes dehydration (τ_H ~15 min.) and oxidation (τ_{ox} ~37 min.). Selection of reaction sequence depends on environmental conditions. (Fig. 5) Oxidation is a time-limiting process and usually depends on molecular oxygen concentration. However, it depends also on mutant's sequence and oxidation rate constant may vary from ~53 min for wtGFP to ~5,6 min. for GFPm (S65G/S72A/F99S/M153T/V163A-GFP). Very important role in chromophore formation play arginine 96 and glutamic acid 222 forming auxiliary hydrogen bonds and acting as a charge transporters.

Example curve representing chromophore formation can be seen on Fig. 6. Initial lag phase in increase of fluorescence intensity is due to the protein folding. Overall process is extended and maturation might be considered as completed after 24 hours.

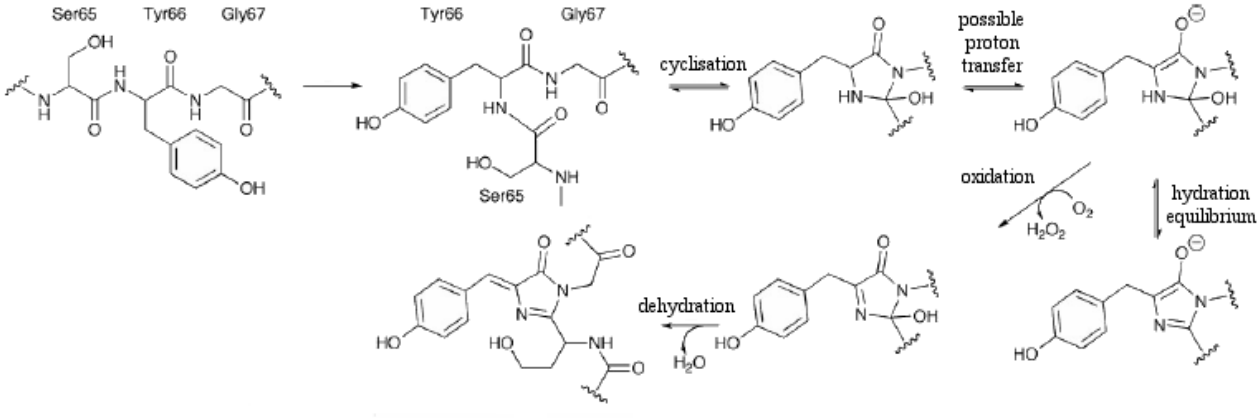

Fig. 5 Stages of chromophore formation in Green Fluorescent Protein (T. D. Craggs 2009, p. 2870.)

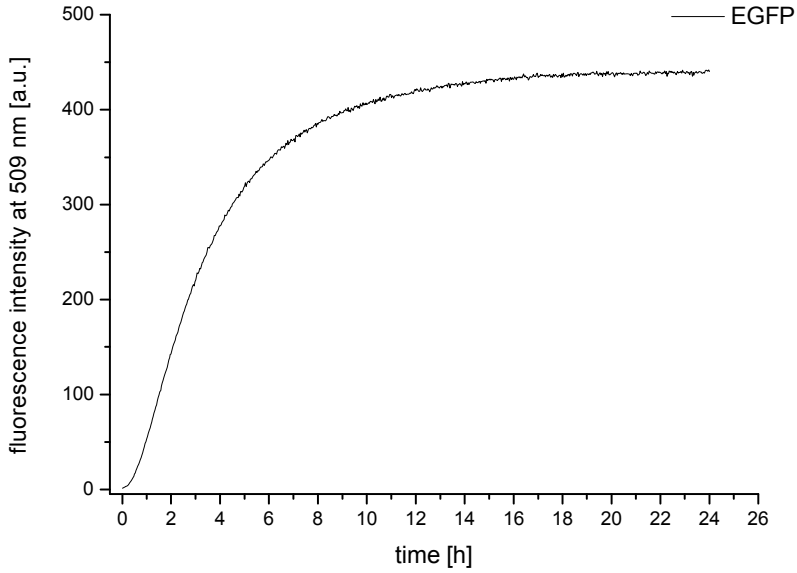

Fig. 6 Example fluorescence curve of chromophore formation in EGFP mutant, room temperature, pH 8, excitation wavelength λ_{ex} = 447 nm, observation wavelength λ_{em} = 509 nm. (J. Krasowska et al., 2014)

Data analysis

Green Fluorescent Protein folding and chromophore formation should be a multiexponential process (Reid and Flynn 1997, Enoki et al. 2004 and 2006, Zhang et al. 2008, Izuka et al. 2011) described by equation:

$$I(t) = \sum_i A_i \cdot \exp\left(\frac{-t}{\tau_i}\right) + A_{inf} \tag{1}$$

where

$I(t)$ – fluorescence intensity at time t
i – number of process phases

A_i – amplitude of phase number *i*
τ_i – time constant of phase number *i*
A_{inf} – value of fluorescence intensity in infinity

In the case when the process is not fully registered or photobleaching occurs, sometimes the phase fragment might be approximated by a linear function (experimental result). Then equation (1) is modified to:

$$I(t)=\sum_i A_i \cdot \exp\left(\frac{-t}{\tau_i}\right)+a\cdot t+b \tag{2}$$

where *a* is a slope and *b* an intercept.

Data should be fitted with equations (1) or (2). The best model with chosen number of phases should be selected on the basis of: a) analysis of fitting parameters with standard errors and b) analysis of residuals. It might happen, that mathematical analysis will be impossible, then interpretation of recorded phenomenon should be described.

Conduction of experiment

Students will receive concentrated EGFP solution (protein without chromophore) in 50 mM phosphate buffer pH 8 with 300 mM NaCl and 6 M guanidinium chloride (GdmHCl – denaturating compound). Protein folding will be initiated by dilution of EGFP in 50 mM phosphate buffer with 300 mM NaCl, pH 8, but without GdmHCl. It is necessary to add β-mercaptoethanol to folding buffer to prevent formation of disulphide bonds. The final concentration should be approx. 14 mM. Folding should be observed by absorption and emission.

For absorption measurements use quartz cuvette with optical length of 10 mm, and for fluorescence measurements use quartz cuvette with optical lengths of 10 and 4 mm remembering to set shorter optical length opposite light source. Built-in magnetic stirrer will mix added components directly in the cuvette.

Experiment will be conducted on Cary 100 or Shimadzu 2401PC dual-beam spectrophotometer and Perkin Elmer LS 55 or Shimadzu RF501PC spectrofluorometer.

Plan your experiment individually determining:

- EGFP concentration in given solution
- EGFP concentration appropriate to chromophore formation measurements
- absorption observation wavelength
- excitation and emission wavelength, slits width, photomultiplier's sensitivity

Record several chromophore formation curves using both methods and then analyse them using given equations and Origin software (preferred). Student should indicate number of phases and relevant time constants and compare them between each curve and between used methods.

Student's report

Report should consist of:

- *Introduction* describing theory of experimental subject,
- *Materials and methods* with description of solutions and apparatus used in exercise and/or description of conducting experiment,
- *Results and discussion* presenting data, its analysis and interpretation. It can be divided in two distinct paragraphs if needed. Values of parameters should be rounded and presented with standard error. If calculation of experimental error was used, appropriate equations should be presented,
- *Bibliography*,
- *Appendix* if needed.

Preliminary test

Student is allowed to perform the exercise only after passing the preliminary test. The form of preliminary test is in decision of the assistant. Range of spectroscopy subjects were presented in this instruction, during lectures “Molecular spectroscopy” and “Molecular biology” and bibliography at the end of this paper. Below there is a list of books that could be helpful in preparing to preliminary test:

- Gordon M. Barrow, *Introduction to molecular spectroscopy*
- Hermann Haken, Hans Christoph Wolf, *Molecular Physics and Elements of Quantum Chemistry: Introduction to Experiments and Theory*
- Joseph R. Lakowicz, *Principles of fluorescence spectroscopy*
- E. F. H. Brittain, W. O. George, C. H. J. Wells, *Introduction to molecular spectroscopy: theory and experiment*
- Raymond Chang, *Basic principles of spectroscopy*
- ed. D. W. Jones, *Introduction to the spectroscopy of biological polymers*
- Jeffrey I. Steinfeld, *Molecules and radiation: an introduction to modern molecular spectroscopy*
- Martin Quack and Frédéric Merkt, *Handbook of High-resolution Spectroscopy*

Before preliminary test read the following paper:
T. D. Craggs, *Green fluorescent protein: structure, folding and chromophore maturation*, Chem. Soc. Rev. 2009, 38, 2865-2875

You will receive it from the assistant.

The list of subjects that student might be asked about during preliminary test:

- Basic definitions related to electromagnetic radiation and spectroscopic measurements:
  - wavelength, frequency, wavenumber
  - radiant intensity, radiant energy density
  - molar absorptivity (molar extinction coefficient), integral absorption coefficient
  - ground state, excited state, oscillator strength
  - quantum yield
  - spectrophotometer and spectrofluorometer construction
- Emission measurements:
  - schematic principles of emission measurements
  - what is the emission spectrum?
  - what is excitation spectrum?
  - quantitative description of emission (quantum yield, how to determine it?)
  - factors determining shape and width of emission band outline
  - inner filter effect
- Electronic states of molecules:
  - molecular orbitals, what mean symbols:σ, σ^*, π, π^*, n
  - diagram of electronic states (S_0, S_1, S_2, ..., T_1, T_2,....), Jablonski diagram
  - possible absorption transitions and relative location of the bands corresponding to the frequency range of electromagnetic radiation
  - deactivation of excited states, radiative and nonradiative transitions, time constants characteristic for each process
- Electronic spectrum with vibrational structure:
  - in which region of electromagnetic spectrum electronic absorption and emission bands could be observed?
  - Jablonski diagram
  - scheme of electronic transitions allowed by selection rule
  - example of absorption and emission spectrum illustrating electronic-vibrational structure and possible transitions
- Absorption and emission properties of amino acids and proteins
  - which amino acids exhibit absorption and emission properties? why?
  - spectral characterisation of aromatic amino acids
  - particular tryptophan properties
  - how proteins can be examined spectrally?
- Green Fluorescent Protein:
  - structure
  - refolding
  - chromophore formation
  - mutation influence on GFP
- Protein folding – models and mechanisms

Bibliography

(1) O. Shimomura, *Structure of the chromophore of Aequorea Green Fluorescent Protein*, FEBS Letters 1979, 104 (2), 220-222

(2) M. Chalfie, *Green Fluorescent Protein*, Photochemistry and Photobiology 1995, 62 (4), 651-656

(3) I. Żak, *Chemia medyczna*, chapter 13 „Aminokwasy i pochodne", Śląska Akademia Medyczna, Katowice 2001

(4) V. Dagget, A. Fersht, *The present view of the mechanism of protein folding*, Nature Reviews, Molecular Cell Biology 2003, 4, 497-502

(5) J. R. Lakowicz, *Principles of fluorescence spectroscopy*, Springer, 2006

(6) T. D. Craggs, *Green fluorescent protein: structure, folding and chromophore maturation*, Chem. Soc. Rev. 2009, 38, 2865-2875

(7) R. Izuka, M. Yamagishi-Shirasaki, T. Funatsu, *Kinetic study of de novo chromophore maturation of fluorescent proteins*, Analytical Biochemistry 2011, 414, 173-178

(8) A. Bzowska, Instruction for exercise: *„Spektroskopia absorpcyjna i emisyjna aminokwasów i białek, wykorzystanie w badaniach struktury i funkcji białek"*, Laboratory of biophysics for advanced, Warsaw 2012