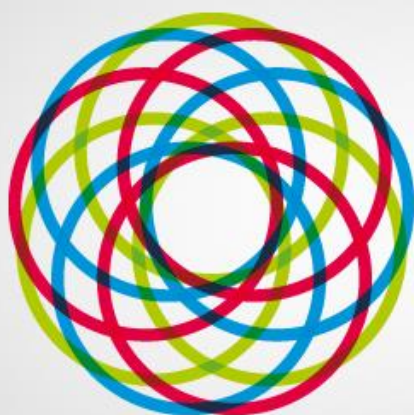


PROGRAMME



SCHOOL OF BIOPHOTONICS 2017

KOŠICE, SLOVAKIA, 22-31 MAY 2017

THEORETICAL COURSES

- I. Introduction (repetitorium in optics and spectroscopy) – *prof. P. Miškovský* (P. J. Šafárik University in Košice, *pavol.miskovsky@upjs.sk*)**
- Fundamentals of Biophotonics
 - Basic characteristics of optical radiation, polarization, reflection, refraction, interference, diffraction and diffusion of light
 - Basics principles of light interaction with matter
 - Basic principles of optical spectroscopy (absorption and emission spectroscopy, light scattering)
- II. Principles of optical experiments – *dr. G. Bánó* (P. J. Šafárik University in Košice, *gregor.bano@upjs.sk*)**
- Light sources in optical spectroscopy and microscopy
 - Principles of laser physics
 - Physical principles of optical signal detection (Photomultipliers, photodiodes and CCD cameras)
 - Optical design (optical elements, optomechanical components)
 - Laser safety
 - Magnification, spatial resolution, numerical aperture
 - Principles of image formation in light microscopy
- III. Radiometry and photometry – *MER dr. Georges Wagnières* (EPFL, Lausanne, Switzerland; *georges.wagnieres@epfl.ch*)**
- Definition of fundamental quantities
 - Overview of unit systems
 - Measurement of radiometric quantities
 - In-class exercises
- IV. Raman spectroscopy – *dr. S. Sánchez-Cortés* (IEM CSIC, Madrid, Spain; *s.sanchez.cortes@csic.es*)**
- Theoretical background of Raman effect
 - Methods of Raman spectroscopy (classical, resonance,...)

- Bio-applications

V. Surface-enhanced optical spectroscopies – *dr. S. Sánchez-Cortés*

- Introduction to Plasmonics
- Surface-enhanced optical spectroscopies (SERS – Surface-enhanced Raman spectroscopy; SEF – Surface-enhanced fluorescence; SEIRA – Surface-enhanced infrared absorption)
- Enhancement mechanisms on plasmonic nanoparticles
- Nanofabrication and functionalization of nanoparticles

VI. Fluorescence microscopy techniques – *assoc. prof. A. Marček Chorvátová* (ILC, Bratislava, alzbeta.chorvatova@ilc.sk)

- Introduction to biophotonics experiment: light interaction with molecules and tissues, emission spectroscopy and fluorescence
- Principles of microscopes: light sources, light path and filters, objectives and detectors
- Fluorescence microscopy and imaging: types of microscopes (brightfield, darkfield, reflected, phase-contrast, polarized, DIC, laser scanning confocal, etc.)
- Fluorescence probes and proteins, cell structure and function imaging
- Data analysis (image digitalization, scanning and image processing)

VII. Time-resolved fluorescence techniques – *assoc. prof. A. Marček Chorvátová*

- Time-resolved fluorescence
- Fluorescence lifetime imaging (FLIM): experimental setups, data recording and advanced data analysis
- Fluorescence resonant energy transfer (FRET) by FLIM, Bioluminescence resonant energy transfer (BRET)
- Advanced microscopy techniques: FLIM in 3D, whole animal FLIM
- Autofluorescence, endogenous pigments
- Applications in biomedical research

VIII. Advanced laser techniques – *dr. D. Chorvát* (ILC, Bratislava; Dusan.Chorvat@ilc.sk)

- Principles of nonlinear optics
- SHG and 2-photon microscopy
- Super-resolution microscopy techniques
- Light scattering and tomography

IX. Photomedicine – prof. H. van den Bergh (EPFL, Lausanne, Switzerland; hubert.vandenbergh@epfl.ch)

- Detection of early cancer by fluorescence imaging
 - i. The detection of early stage bronchial cancers by autofluorescence bronchoscopy.
 - ii. The detection of early stage bladder cancer by fluorescence cytscopy, adn the removal of bladder cancer by Trans Urethral Bladder Resection under fluorescence.
- Photodynamic therapy of cancer and eye diseases
 - i. Photodynamic therapy of early cancer in the upper aerodigestive tract (mouth, oesophagus, bronchi).
 - ii. Photodynamic therapy of cancer of wet age-related macular degeneration and PCV.
- Photodynamic drug delivery for treating cancer

X. New trends in Super-resolution microscopy and Light Sheet microscopy – Ing. Martin Kopecký, Ph.D. (Pragolab s.r.o., Prague, Czech Republic; kopecky@pragolab.cz)

- Confocal and widefield super-resolution microscopy
 - SR for live cells;
 - confocal microscopes and possible upgrades for SR;
 - STED principle and setup for live cells;
 - GSD concenpt as SR techniques;
 - application examples.
- Light Sheet systems
 - principles;
 - application examples.

XI. Introduction to MST and nanoDSF measurements – *dr. J. Nowak, PhD*
(NanoTemper, Cracow, Poland; *Jakub.Nowak@nanotemper.de*)

- Biomolecular Interactions with MicroScale Thermophoresis
 - introduction to concepts of MST;
 - experimental Set-up;
 - application examples.
- nanoDSF to measure conformational and colloidal stability of proteins
 - introduction to concepts of MST;
 - experimental Set-up;
 - application examples.

XII. Laboratory work and safety – *dr. Z. Nad'ová* (P. J. Šafárik University in Košice, *zuzana.nadova@upjs.sk*), *dr. G. Fabriciová* (P. J. Šafárik University in Košice, *gabriela.fabriciova@upjs.sk*), *dr. G. Bánó*

- Cell cultures preparation
- Lab safety
- Laser safety

PRACTICAL TRAININGS

- 1. CELL CULTURE PREPARATION and FLOW CYTOMETRY** (*dr. Z. Nad'ová*)
- 2. CONFOCAL FLUORESCENCE MICROSCOPY** (*dr. Z. Nad'ová*)
- 3. RAMAN SPECTROSCOPY** (*dr. Z. Jurašková*)
- 4. OPTICAL TWEEZERS and SIGLET OXYGEN DETECTION** (*dr. G. Bánó*)

1. CELL CULTURE PREPARATION and FLOW CYTOMETRY

dr. Z. Nad'ová

Goal: The course provides overview and practical laboratory experience of the cell cultivation and covers critical steps for cell research. Encompassing theoretical presentations and practical lab sessions, participants will learn ways of cell preparation, labeling and basic cell analysis with flow cytometry.

Material: U-87 MG (<http://www.lgcstandards-atcc.org/.../HTB-14.aspx>, human glioma cell line), fetal bovine serum, antibiotics, D-MEM, hypericin. Apoptosis assay kit (Invitrogen, USA), DiOC6, MACSQuant Analyzer (Miltenyi Biotec, Germany)

Description: Routine methods in cell cultivation, Theoretical basics of flow cytometry, Instrument setup and fluorophores, Acquisition and analysis of parameters

2. CONFOCAL MICROSCOPY

dr. Z. Nad'ová

Goal: The course provides overview and practical laboratory experience of the cell cultivation and covers critical steps for cell research. Encompassing theoretical presentations and practical lab sessions, participants will learn ways of cell preparation, labeling and basic cell analysis with fluorescence microscopy.

Material: U-87 MG (<http://www.lgcstandards-atcc.org/.../HTB-14.aspx>, human glioma cell line), fetal bovine serum, antibiotics, D-MEM, hypericin. Organelle specific markers (MitoTracker, LysoTracker, NBDC6-ceramide), primary- and secondary-antibodies, Confocal microscope system (LSM 700, Zeiss, Germany)

Description: Imaging and documenting live cell processes, Detection of cellular structures using fluorescent dyes, Immunofluorescence and fixation techniques, signal optimization, specimen preparation

3. RAMAN SPECTROSCOPY

dr. Z. Jurašková

Goal: Introduction to Raman spectroscopy (to understand principles of dispersive Raman instrument) and optimization of Raman experiment for the best possible results. Demonstration of SERS spectroscopy as an important analytical technique.

Description: Principles of Raman and SERS spectroscopy will be explained through the particular measurements:

- a) Registration of Raman spectra of different samples (solid/liquid; inorganic/organic) – optimization of instrument and software setting for the best possible results (macro/micro mode, laser power, exposure time, accumulations).
- b) Data processing and simple analysis. Illustrative interpretation of the registered Raman spectra – basic vibrational assignments. Post-registration manipulation of the data (cosmic rays, peak pick, baseline subtraction, etc.).
- c) Effect of different experimental conditions: wavelength of the excitation line, type of colloid, concentration, pH, etc.
- d) SERS experiments (fluorescence quenching, signal enhancement, detection limit, affinity of the molecule towards different SERS substrates, different experimental conditions, impurities).

Preparation for SERS experiments:

- I. Preparation of active SERS substrates using metal colloids.
- II. Characterization of metal colloids by UV-Vis and Raman Spectroscopy (absorption maximum – plasmon resonance, fwhm – particle size distribution, aggregation – activation of SERS substrates, anomalous bands, effect of pH,...).

Materials: In this demonstration, the experimental setup present in the Laboratory of Raman spectroscopy at KBF (<http://www.biophysics.sk/en/laboratory/8/laboratory-of-raman-spectroscopy-i>) will be used. There is no need of any special sample preparation. Silver and gold colloids will be prepared by chemical reduction. All the glass materials have to be previously cleaned using chromic solution and rinsed several times with tri-distilled water.

Special issues: Raman spectroscopy also possesses desirable properties for imaging applications, such as chemical specificity, high spatial resolution, low background signal, any or little sample preparation, etc. This technique will be of interest to better understand the distribution of components within the samples.

4. OPTICAL TWEEZERS and SINGLET OXYGEN DETECTION

dr. G. Bánó

a) Trap stiffness and Raman measurement in a Raman tweezers setup

Goal: to demonstrate the basic principles of a Raman tweezers setup

Materials: polystyrene and silica micro-beads, yeast cells

Description: participants will gain insight into the design of basic laser tweezers equipment. The trap stiffness will be evaluated by measuring the power spectrum of trapped particles' oscillations. Raman spectra of trapped objects will be detected.

Special issues: laser safety considerations

b) Time-resolved in-vitro detection of singlet oxygen produced in photodynamic processes

Goal: to perform measurements of singlet oxygen phosphorescence

Materials: photosensitizers (hypericin, rose bengal)

Description: phosphorescence of singlet oxygen at 1270 nm will be measured following pulsed laser excitation of photosensitizers. The signal from a PMT will be collected in photon counting mode using a multichannel scaler/averager. The lifetime of singlet oxygen will be evaluated.

Special issues: laser safety considerations

INDIVIDUAL PROJECTS

- a) **Delivery systems: Study of interaction of hydrophobic drugs with transport lipoproteins** (*dr. G. Fabriciová*)
- b) **Investigation of the molecular mechanism of the cell death pathways mediated by photodynamic therapy (PDT) in cancer cells** (*dr. Z. Nad'ová*)
- c) **Quantitative analysis of light-induced photosensitizer reactions with molecular oxygen** (*dr. G. Bánó*)
- d) **Raman spectroscopy as an analytical tool in the identification and characterization of natural dyes and pigments employed in Cultural Heritage** (*dr. Z. Jurašková*)

A. Delivery systems: Study of interaction of hydrophobic drugs with transport lipoproteins

Goal: By application of UV-VIS absorption and fluorescence spectroscopy to analyze interaction of hydrophobic photosensitizer hypericin with low-density lipoproteins (LDL).

Materials and Methods: UV-VIS absorption spectrometer (Shimadzu UV-2401), fluorimeter (Shimadzu RF-5301), hypericin, dimethyl sulfoxide (DMSO), low-density lipoproteins, phosphate buffer.

Description: Hypericin (Hyp) is a natural photosensitizing pigment from plants of the genus *Hypericum*. Hyp under light illumination causes anti-proliferative and cytotoxic effects (necrosis as well as apoptosis) in many tumor cell lines. These properties together with minimal dark toxicity, tumor selectivity and high clearance rate from the host body, make Hyp a promising agent for photodynamic therapy of cancer as well as for tumor photo-diagnosis [1,2]. Hyp is a hydrophobic molecule and is readily soluble in polar organic solvents like dimethyl sulfoxide (DMSO), acetone, ethyl acetate and ethanol. This compound is sparingly soluble in non-polar organic solvents and oil, and in aqueous environment forms insoluble non-fluorescent aggregates [3]. The formation of aggregates significantly suppresses Hyp photodynamic activity [3–5]. Moreover, this aqueous insolubility makes intravenous injection of Hyp problematic and restricts its medical applications. The study of Hyp distribution in human plasma has shown that Hyp molecules are predominantly associated with low-density lipoproteins (LDL) and only relatively small amounts are bound to high-density lipoproteins (HDL) and proteins (mainly serum albumin) [6]. In the presence of the plasma (lipo)proteins Hyp aggregates can redissociate resulting in fluorescent monomeric and biological active form of this molecule [3, 4, 7–9].

The capacity of LDL to bind some hydrophobic drugs and their functionality as drug carriers have been examined in several studies [4, 7–11]. LDLs are recognized by and internalized into the cells through specific membrane receptors that interact with the apolipoprotein B of the LDL particle [12]. The tumor cells and tumor vascular endothelial cells express the LDL receptor in higher number due to either their increased proliferation or increased membrane turnover [12]. This makes LDL particles extremely attractive vehicles for drug delivery and targeting.

Using the fact that only monomeric form of Hyp is fluorescent, the transition of Hyp from aggregated to monomeric state in the presence of LDL will be investigated by fluorescence spectroscopy [4]. Moreover, it is known that UV-VIS absorption spectra of aggregated and monomeric Hyp are different and this fact is reflected by the ratio of Hyp absorbances at 554 nm and 597 nm. The absorption spectra of Hyp at various Hyp/LDL ratios will be measured and consequently the quantification of the population of Hyp monomers at different Hyp/LDL ratios will be evaluated [4].

Fluorescence spectroscopy will be further utilized to study kinetics of Hyp incorporation into LDL particles as well as the kinetics of Hyp redistribution from Hyp/LDL complex (Hyp/LDL = 200:1) to free LDL molecules [7].

Literature:

Miskovsky P. (2002): Hypericin – a new antiviral and antitumor photosensitizer: Mechanism of action and interaction with biological macromolecules. *Curr. Drug Targets*, 3, 55–84

Kramer B., Verwinger T. (2012): Molecular response to hypericin-induced photodamage. *Current Med. Chem* 19, 793–798

Falk H., Meyer J. (1994): On the homo- and heteroassociation of hypericin. *Monatsh. Chem.* 125, 753–762

Kascakova S., Refregiers M., Jancura D., Sureau F., Maurizot J.C., Miskovsky P. (2005): Fluorescence spectroscopic study of hypericin-photosensitized oxidation of low-density lipoproteins. *Photochem. Photobiol.* 81, 1395–1403

B. Investigation of the molecular mechanism of the cell death pathways mediated by photodynamic therapy (PDT) in cancer cells

Goal: Identification of events related to cell death induced by photoactivated Hypericin (Hyp) in human glioma cells

Materials and Methods: U-87MG cells, Hypericin (Hyp), low density lipoproteins (LDL), annexin V-FITC, propidium iodide (PI), DiOC6, anti-cytochrome C antibody, anti-Bcl2 antibody, flow cytometer (Miltenyi Biotec, Germany), confocal microscope system (LSM700, Zeiss, Germany), DNA electrophoresis

Description: Low-density lipoproteins (LDL), a natural in vivo carrier of cholesterol in the vascular system will be used for targeted transport of Hyp to U87 glioma cells. For low Hyp/LDL ratio (10:1), the cellular uptake of Hyp is characterized by endocytosis of [Hyp-LDL] complex, while Hyp alone passes into the cells by passive diffusion.

We will investigate the effect of Hyp on subcellular distribution dynamics in glioma cells with/without Hyp bonding with LDL. Corresponding time evolutions of the sub-cellular distributions will be obtained in co-localization experiments by confocal fluorescence microscopy. Apoptosis induction after photoactivation will be monitored by flow-cytometry (Annexin-V-FITC/PI). Effect of photoactivated Hyp on mitochondria ($\Delta\psi_m$, cytochrome c release, Bcl-2 family proteins translocation) will be assessed by using flow cytometry and/or fluorescence microscopy.

Possible aggregation of Hyp in cells (organelles) will be assessed by steady-state and time-resolved fluorescence spectroscopy (imaging).

Literature:

1. Longobardi Givan A: Flow cytometry, First principles, Wiley-Liss, 2001
2. Davis J: Animal Cell Culture: Essential Methods, 2011
3. Lakowicz JR: Principles of Fluorescence Spectroscopy, Springer, 3rd edition, (2006)
4. Huntosova V, Nadova Z, Dzurova L, Jakusova V, Sureau F, Miskovsky P.: Cell death response of U87 glioma cells on hypericin photoactivation is mediated by dynamics of hypericin subcellular distribution and its aggregation in cellular organelles. Photochem Photobiol Sci. 2012 15; 11(9):1428–36.
5. Huntosova V, Alvarez L, Bryndzova L, Nadova Z, Jancura D, Buriankova L, Bonneau S, Brault D, Miskovsky P, Sureau F: Interaction Dynamics of hypericin with low-density lipoproteins and U87-MG cells, Int. J. Pharm. 2010, 389, 32–40.

C. Quantitative analysis of light-induced photosensitizer reactions with molecular oxygen

Goal: to get practice in time-resolved optical measurements for the characterization of singlet oxygen production during the photodynamic action

Materials: a set of different lasers, a NIR PMT, multichannel scaler/average, fast photodiodes, different photosensitizers

Description: singlet oxygen is an electronically excited state of molecular oxygen that can be produced by energy transfer between photo-excited metastable triplet states of different photosensitizer molecules and ground state molecular oxygen. The basic idea behind the photodynamic therapy is to use the highly reactive singlet oxygen for destruction of cancerous cells. In general, efficient photosensitizers have high quantum yield of singlet oxygen production. The amount of singlet oxygen produced can be monitored by detection of its phosphorescence at 1270 nm [1]. Participants will use (and/or modify) the available experimental apparatus (see practical training no. 5) in order to get extensive information about the photodynamic process from the point of view of singlet oxygen production. The most efficient singlet oxygen producer is to be chosen from a set of photosensitizer candidates and the quantum yield of singlet oxygen production is to be determined at different experimental conditions. The variables to be changed are: temperature, oxygen percentage, solvent.

Literature:

[1] P. R. Ogilby, Singlet oxygen: there is indeed something new under the sun, Chem. Soc. Rev., 2010, 39, 3181–3209

D. Raman spectroscopy as an analytical tool in the identification and characterization of natural dyes and pigments employed in Cultural Heritage

Goal:

The main goal of the project consists in an application of Raman spectroscopy and Surface-enhanced Raman spectroscopy (SERS) technique on the analysis of selected artifacts.

Description: The analysis of the colouring materials used in art objects may be a valuable tool to understand how an object originally looked like, where it comes from and how old it is. This knowledge also allows conservators to choose appropriate procedures for restoration. Raman spectroscopy has evolved as an efficient non-destructive, and even in situ, technique for identification of inorganic pigments and other materials in artworks.[1, 2] In addition, since every molecule scatters light uniquely, Raman spectroscopy is an excellent analytical tool to “fingerprint” unknown compounds. Nevertheless, the application of this technique in the characterization of organic pigments and dyes has been limited by two main drawbacks: (1) the intense fluorescence emission from organic pigments, which normally covers the corresponding Raman spectra; and (2) the minute quantities of coloured material, which, in general, are below the detection limit of the low-sensitivity Raman technique. Surface-enhanced Raman scattering (SERS) can overcome the above disadvantages because of the fluorescence quenching of the analyte, as well as the high sensitivity provided by the giant intensification of the radiation intensity in the presence of the metal nanoparticles.[3]

The successful analysis of selected artifacts requires first approximation of their composition where employed pigments/dyes will be identified and defined as target molecules. The characterization of the target molecules in their isolated state as well as in complexes with different binding molecules (for examples, proteins and mordant molecules) is crucial for their identification in real art objects. Several experimental approaches will be applied to characterize them – UV-Vis, fluorescence and Raman spectroscopy and they will be studied under different experimental conditions (pH, concentration, λ_{exc}). In the case of organic pigments/dyes (where no Raman spectra are obtained because of the strong fluorescent background), their SERS spectra will be registered. The database of the reference spectra (or at least of the specific spectral markers; i.e. individual Raman bands) for the target molecules created during the project duration will be used for final analysis of the pigments/dyes found in the studied artifacts. The obtained results will also be discussed with corresponding bibliographic sources.

Materials: The experimental setup present in the Laboratory of Raman spectroscopy at KBF be used. Silver and gold colloids employed mainly for dye characterization will be prepared by chemical reduction.[4] Silver particles for in situ detection will be prepared by photoreduction.[5–7] Particular artifacts (painting, coloured photograph, etc.) will be analysed. In consequence, different pigments/dyes, binding media, etc. will be characterized. All the glass materials have to be previously cleaned using chromic solution and rinsed several times with tri-distilled water.

Literature:

- [1] Vandenabeele, P.; Edwards, H. G. M.; Moens, L. *Chemical Reviews*, 107, 2007, 675.
- [2] Bellot-Gurlet, L.; Pages-Camagna, S.; Coupry, C. *J. Raman Spectrosc.*, 37, 2006, 962.
- [3] Aroca, R. *Surface-enhanced Vibrational Spectroscopy*: Chichester, 2006.
- [4] Cañamares, M. V.; Garcia-Ramos, J. V.; Sanchez-Cortes, S.; Castillejo, M.; Oujja, M. J. *Colloid Interface Sci.*, 326, 2008, 103.
- [5] Cañamares, M. V.; Garcia-Ramos, J. V.; Gomez-Varga, J. D.; Domingo, C.; Sanchez-Cortes, S. *Langmuir*, 23, 2007, 5210.
- [6] Jurasekova, Z.; Domingo, C.; Garcia-Ramos, J. V.; Sanchez-Cortes, S. *J. Raman Spectrosc.*, 39, 2008, 1309.
- [7] Jurasekova, Z.; del Puerto, E.; Bruno, G.; García-Ramos, J. V.; Sanchez-Cortes, S.; Domingo, C. *J. Raman Spectrosc.*, 41, 2010, 1455.