**A description of the main S&T results**

Although the project CELIM has been financed in the call for an indirect support of research (FP7 Support Action Funding Scheme) with the following two supportive aims:

- to improve our capability to perform advanced feasibility studies by equipment upgrades, international networking, knowledge exchange and job stabilization of the experts,
- to enrich preliminary data in foreign established institutes and European facilities in order to create the working collaborations with top-level experts, leading to partnership in 7FP and H2020 proposals,

we have obtained several concrete important scientific results which confirm the successful accomplishment of the project objectives. The following paragraphs contain a short summary of the main research outputs delivered during the duration of the project. The specific fields in which we have performed our scientific activities and obtained results are either directly connected with the main scientific topic of the CELIM project - cell and/or bio-imaging (Monitoring of oxygen partial pressure: luminescence lifetime - based imaging method, Formation of singlet oxygen and its detection and imaging, Cellular response after photodynamic action, Construction of optically trapable SERS probes, Modeling and characterization of cancer cell heterogeneity, XFEL activities) or emerged as a consequence of the hiring of six new researchers to the CELIM team (Detection of environmental pollutants at very low concentrations by surface-enhanced Raman spectroscopy measurements - a case of organochlorine pesticides, Molecular bioenergetics – catalytic mechanism of cytochrome c oxidase, Development of nano-vehicle for targeted drug delivery, Protein engineering and stability).

1. **Monitoring of oxygen partial pressure: luminescence lifetime - based imaging method**

A monitoring of tissue oxygenation can very often signalize a malfunctioning of cellular functions. Moreover, the sufficient tissue/cellular oxygenation takes important place in the modern treatments based on photodynamic therapy (PDT), which employs oxygen, light and light-sensitive molecule. Ruthenium-based poly-pyridyl complexes are luminescent molecules which possess very intense fluorescence and phosphorescence and their triplet - state lifetimes are in the order of hundreds ns. The triplet state of these molecules can be effectively quenched by molecular oxygen, so in the absence of oxygen their luminescence lifetimes increase up to several μs. Thanks to these unique properties, Ru-complexes can be used as oxygen sensors in the time-resolved luminescence based optical techniques. There are many oxygen sensitive sensors, but only few of them are water-soluble, including dipyridyl ruthenium complexes, and can be easily used for *in vivo* bio-imaging applications.

Assessment of oxygen partial pressure ($pO_2$) by luminescence lifetime measurements of ruthenium coordination complexes has been studied intensively during the last decades. RuPhen (dichlorotris(1,10-phenanthroline) ruthenium(II) hydrate) is a water soluble molecule that has been tested previously for *in vivo* $pO_2$ detection. One minimally invasive approach to determine the $pO_2$ based on the measurement of the $[Ru(Phen)_3]^{2+}$ luminescence lifetime has been presented in our studies performed in a collaboration with prof. H. van den Bergh and Dr. G. Wagnieres from EPFL Lausanne (Switzerland) (partner in the CELIM project). We have demonstrated that the intravenous injection of this compound into the CAM model mostly leads to extracellular localization of $[Ru(Phen)_3]^{2+}$, while its topical application induces an intracellular localization at the administration site. This observation suggests that the intravascular, extravascular, intracellular and extracellular $pO_2$ can be measured. Furthermore, we have demonstrated that $[Ru(Phen)_3]^{2+}$ luminescence lifetimes in various microenvironments present linear Stern-Volmer oxygen dependences not only in the

biological liquids (CAM blood and serum), but also *in vivo* in the intra/extravascular space of CAM. Reliable and easy $pO_2$ prediction was illustrated in CAM tumors with this approach.

Further, we have demonstrated that photo-excited PpIX is able to generate PpIX photoproducts presenting a delayed fluorescence in DMSO solutions and *in vivo*. The increased concentration of these photoproducts results in a decrease of delayed fluorescence lifetimes and loss of PpIX oxygen sensitivity (**Fig. 1**). This observation has a significant impact on "point" and imaging methods based on PpIX delayed fluorescence lifetime. A significant improvement can be achieved using a detection window specific to PpIX, such as 620-640 nm, or an excitation light dose underneath a certain threshold. This leads to an increased accuracy of $pO_2$ detection. Similarly, lifetime imaging method could improve detection and real-time identification of tissue respiration, provided the set-up is tuned for the detection of PpIX luminescence and not its photoproducts.

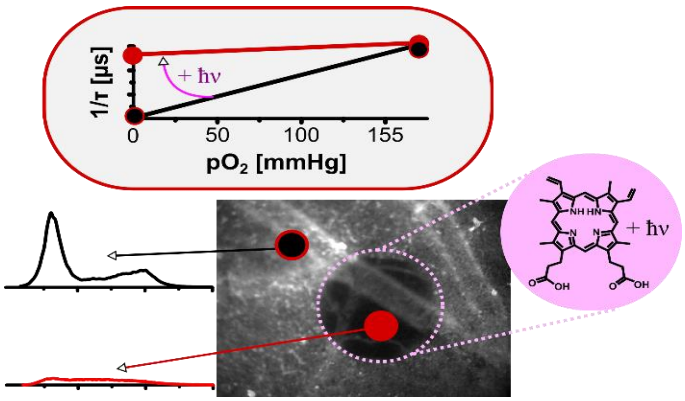


**Fig.1.** The luminescence of PpIX photoproducts in tissues affects measurements of the $pO_2$ by time-resolved delayed fluorescence of PpIX.

In conclusion, our works show that $[Ru(Phen)_3]^{2+}$ is minimally photo-toxic and pH stable, and can be used in photosensitive systems presenting important pH variations. Importantly, $[Ru(Phen)_3]^{2+}$ can be recommended for experiments where repetitive measurements of the $pO_2$ must be performed at the same location. This recommendation is also supported by the statistical parameters characterizing our datasets. However, our work also demonstrates that there is still room for improvements of the statistical analysis of the experimental findings to extract relevant information, in particular regarding the phototoxicity of the $pO_2$ sensors employed in oxygen *in vivo* detection and imaging.

**Related publications:**

1. V. Huntosova, S. Gay, P. Nowak-Sliwinska, S. Kumar Rajendran, M. Zellweger, H. van den Bergh, G. Wagnieres. In vivo measurement of tissue oxygenation by time-resolved luminescence spectroscopy: advantageous properties of dichlorotris(1, 10-phenanthroline)-ruthenium(II) hydrate. *Journal of Biomedical Optics* (2014) 19, 077004.
2. V. Huntosova, K. Stroffekova, G. Wagnieres, M. Novotova, Z. Nichtova, P. Miskovsky: Endosomes: guardians against $[Ru(Phen)_3]^{2+}$ photo-action in endothelial cells during in vivo $pO_2$ detection? *Metallomics* (2014) 6, 2279-2289.
3. V. Huntosova, E. Gerelli, D. Horvath, G. Wagnieres: Measurement of pO2 by luminescence lifetime spectroscopy: A comparative study of the phototoxicity and sensitivity of $[Ru(Phen)_3]^{2+}$ and PdTCPP in vivo. *Journal of Biophotonics* (2016), submitted.
4. V. Huntosova, E. Gerelli, M. Zellweger, G. Wagnieres: Effect of PpIX photoproducts formation on $pO_2$ measurement by time-resolved delayed fluorescence spectroscopy of PpIX in solution and in vivo. *Journal of Photochemistry and Photobiology B: Biology* (2016), submitted.

## 2. Formation of singlet oxygen and its detection and imaging

Singlet oxygen is a highly reactive molecule which causes oxidation of many biologically important compounds. From a medicinal point of view, singlet oxygen has been studied intensively because of its essential role in photodynamic therapy (PDT) of cancer. During PDT, the oxidative stress induced by reactive oxygen species (in most cases singlet oxygen in the $O_2(^1\Delta_g)$ state) initiates processes leading to cell death. Singlet oxygen is mostly produced by energy transfer between light-activated (triplet state) photosensitizer molecules and ground state (triplet, $O_2(^3\Sigma_g^-)$) oxygen molecules. In our studies, we have, together with the group of Dr. Georges Wagnieres from EPFL Lausanne (Switzerland) and dr Sergej Kruglik from P. & M. Curie University in Paris (France) (partners in the project CELIM), intended to shed more light on the kinetics of singlet oxygen formation and annihilation in various environments (organic solvents, aqueous solutions), under different physico-chemical conditions (temperature, oxygen concentration) and in the presence of several photosensitizers (hypericin, RuPhen).

Singlet oxygen ($O_2(^1\Delta_g)$) production by photo-excited hypericin (Hyp) dissolved in dimethyl-sulfoxide (DMSO) was studied by means of time-resolved phosphorescence measurements. In order to minimize photo-bleaching, the samples were excited in quasi-continuous mode using long-pulse (35 μs) laser excitation. The measured lifetime of singlet oxygen is $\tau_\Delta$ = 5.5 ± 0.3 μs. This result helps to resolve the discrepancy existing in the literature concerning singlet oxygen lifetime in DMSO. The obtained quantum yield of singlet oxygen photosensitized by Hyp in air-saturated DMSO is $\Phi_\Delta$ = 0.4 ± 0.03. The rate constant for Hyp triplet state depopulation in reaction with ground state molecular oxygen is measured to be $k_q$ = 1.6 ± 0.3 $10^9$ $M^{-1}s^{-1}$.

In another study we have investigated the production of singlet oxygen by RuPhen. The quantum yield of singlet oxygen production by RuPhen dissolved in 0.9% aqueous NaCl solution (pH=6) was measured at physiological temperatures (285-310 K) and various concentrations of molecular oxygen. In order to minimize the bleaching of RuPhen, the samples were excited with low power (<2 mW) laser pulses (20 μs long), created by pulsing a cw laser beam with an acousto-optical modulator. We show that, whereas the RuPhen phosphorescence lifetime decreases rapidly with an increase of temperature (keeping the oxygenation level constant), the quantum yield of singlet oxygen production by RuPhen is almost identical in the temperature range of 285-310 K. For air saturated conditions at 310 K the measured quantum yield is about 0.25. The depopulation rate constants of RuPhen $^3$MLCT (metal-to-ligand charge-transfer) state are determined in the absence and in the presence of oxygen. We determined that the excitation energy for the RuPhen $^3$MLCT→d-d transition is 49 kJ.mol$^{-1}$ in the 0.9% NaCl solution (pH=6).

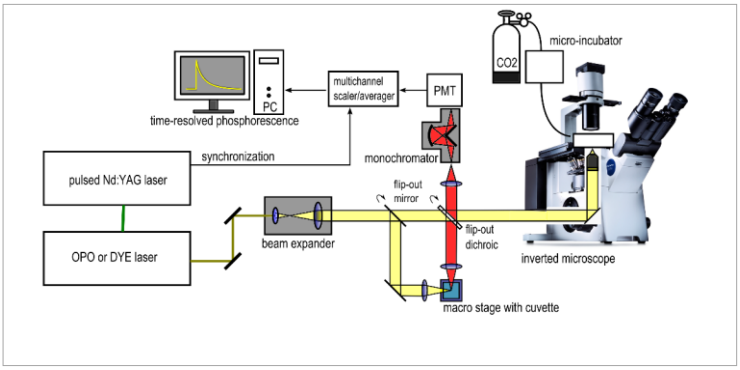


**Fig. 2.** Scheme of the apparatus for *in vivo* singlet oxygen detection and imaging.

The obtained results form a solid base for the development of singlet oxygen bio-imaging in living cells. All the major components (lasers, detectors, microscopes), needed for the construction of a new apparatus enabling in situ singlet oxygen detection, are available in the laboratories of the CELIM consortium at UPJŠ.

The schematic view of the proposed instrument, which is constructed in the collaboration with dr. Sergey Kruglik from P. & M. Curie University in Paris (France) (partner in the project CELIM), is shown on **Fig.2**.

The *in situ* production of singlet oxygen will be evaluated and compared with PDT efficacy (evaluated by flow-cytometry, which is the standard method for this type of analysis).

**Related publications:**

1. D. Petrovajova, D. Jancura, P. Miskovsky, D. Chorvat Jr, A. Chorvatova, X. Ragas, M. Garcia-Diaz, S. Nonell, Z. Nadova: Monitoring of singlet oxygen luminescence and mitochondrial autofluorescence after illumination of hypericin/mitochondria complex: a time-resolved study. *Laser Physics Letters* (2013) 10, 20131.
2. J. Varchola, V. Huntosova, D. Jancura, G. Wagnieres, P. Miskovsky, G. Bano: Temperature and oxygen-concentration dependence of singlet oxygen production by RuPhen as induced by quasi-continuous excitation. *Photochemical and Photobiological Sciences* (2014) 13, 1781-1787.
3. J. Varchola, K. Zelonkova, D. Chorvat Jr, D. Jancura, P. Miskovsky, G. Bano: Singlet oxygen produced by quasi-continuous photo-excitation of hypericin in dimethyl-sulfoxide. *Journal of Luminescence* (2016) 177, 17-21.

## 4. Cellular response after photodynamic action

Photodynamic therapy (PDT) is a promising and innovative treatment for small localized tumors, in post-surgical adjuvant protocols, and in palliative treatment of inoperable advanced tumors. PDT is based on the concept that tumor destruction occurs when a photodynamically active molecule, photosensitiser (pts), administered into a human body, accumulates within the tumor and is consequently illuminated by light at a certain time interval after pts administration. The relatively high selectivity of PDT is due to its physico-chemical mechanism (pts preferential localization in tumors and the light spatially focused on the lesion), which leads to limited side-effects in comparison to other types of cancer treatment (chemotherapy, radiotherapy). Intense research in PDT is in progress in many laboratories worldwide and is focused on solving the main challenges to widespread use of PDT. Between these challenges belong also an exploration of intracellular cell death mechanisms and molecular basis of pts-induced anticancer activity and enhancement of PDT efficacy *in vivo.* Both these topics are objects of our long-term interest.

The phototoxicity and dark toxicity of $[Ru(Phen)_3]^{2+}$ were studied *in vivo* in the CAM and *in vitro* in HCAEC. We have shown that $[Ru(Phen)_3]^{2+}$ induces minimal photo-damage after illumination with light doses larger by two orders of magnitude than those used to perform $pO_2$ measurements. Our study suggests that this low phototoxicity is due to the fact that $[Ru(Phen)_3]^{2+}$ enters endothelial cells *via* endocytosis and is then redistributed towards peroxisomes and other endosomal and secretory vesicles before it is eliminated *via* exocytosis. In addition, morphological findings observed by electron microscopy suggest that without irradiation, the presence of $[Ru(Phen)_3]^{2+}$ leads to adaptive ultrastructural changes of the endomembrane system, pointing to changes in the ER-to-Golgi transport. In contrast, the illumination induced an increased number of lysosomal vesicles with larger diameters, significant loss of ER, and disruption of $Ca^{2+}$ homeostasis accompanied by mitochondrial changes and changes in the secretory pathway. The cellular response to light and the role played by peroxisomes will be the subject of further investigations.

In another work we have shown that ROS production in cells and/or mitochondria significantly depends on light dose used for a photo-activation of a potent photosensitizer hypericin (Hyp) (**Fig. 3**). Arising oxidative stress has a strong impact on mitochondrial organization in intracellular space. Network of tubular mitochondria is under oxidative stress fragmented (**Fig. 3**, inserted images). Observed fragmentation seems to be reversible process when cells are irradiated by lover light dose (1 J/cm$^2$). Serious oxidative stress induced by Hyp photo-activation by higher light dose (4 J/cm$^2$) leads to irreversible changes in mitochondrial structure and function (dissipated mitochondrial membrane potential ($\Delta\psi_m$)).

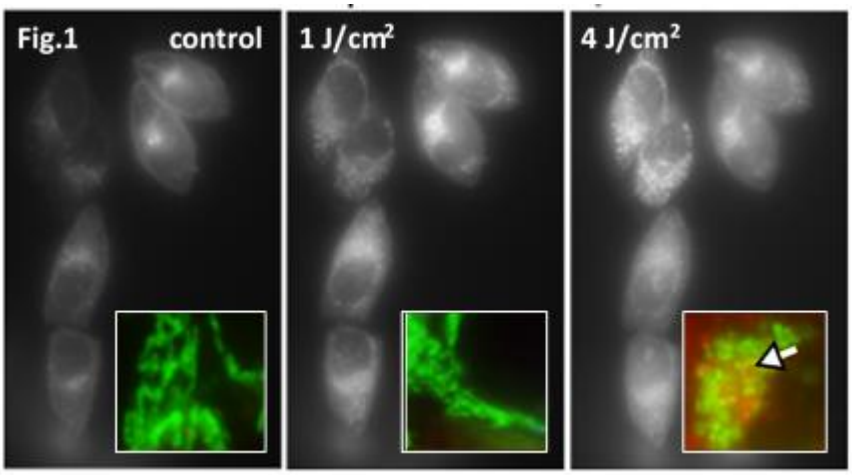


**Fig. 3:** Fluorescence images of $O_2^-$ production in mitochondria after Hyp irradiation (light dose 1J/cm$^2$ vs 4J/cm$^2$) visualized by fluorescence microscopy after MitoSOX Red stainig. Inserted images: Mitochondria after MitoTracker Red staing

Dissipation of $\Delta\psi_m$ represents the point of no return when cells start cell death program and undergo apoptosis. Observed changes in the shape, organization and functions of mitochondria under oxidative stress correlate with apoptotic program development.

To follow up our previous work and taking into account that PKCα acts at the mitochondrial level where affects anti-apoptotic functions of Bcl-2 protein, we have focused more closely on the modulation of PKCα expression and on the effects of such a modification on cell survival and cell death pathways after Hyp photo-activation. Photo-activation of Hyp strongly induced apoptosis, and moreover the level of necrosis in PKCα- cells increased significantly. We have concluded that the post-transcriptional silencing of the *pkcα* gene and the related decrease of PKCα level considerably affects the anti-apoptotic function and the anti-oxidant function of Bcl-2. This implies that PKCα, as Bcl-2 kinase, indirectly protects cells against oxidative stress and subsequent cell death.

We have also shown that Hyp has significant light-independent effects in malignant and non-malignant cells at several sub-cellular levels such as organelle ultrastructure and function, and protein synthesis and distribution. Hyp itself without light causes increased oxidative stress and ultrastructure changes in cells (slightly swollen and dilated mitochondria, fragmented the ER network, swollen cisterns of GA). Hyp significantly changed the distribution of Bcl2 and Bax proteins. Hyp interacts with Bcl2 proteins *via* hydrophobic interaction at their BH3 domain. This interaction may be one of the mechanisms underlying Hyp light independent effects in the cells. Our findings lay foundation for therapeutic use of Hyp without irradiation as BH3 domain mimetic molecule to enhance other cancer treatments similar to other small mitochondria targeting molecules (mitocans).

**Related publications**

1. L. Dzurova, D. Petrovajova, Z. Nadova, V. Huntosova, P. Miskovsky, K. Stroffekova: The role of anti-apoptotic protein kinase Cα in response to hypericin photodynamicc therapy in U-87 MG cells. *Photodiagnosis and Photodynamic Therapy* (2014) 11, 213-226.
2. J. Joniova, M. Misuth, F. Sureau, P. Miskovsky, Z. Nadova: Effect of PKCα expression on Bcl-2 phosphorylation and cell death by hypericin. *Apoptosis* (2014) 19, 1779-1792.
3. M. Misuth, J. Joniova, M. Ferencakova, P. Miskovsky, Z. Nadova: Effect of Hyp delivery system on PKCα activity: What will happen after pkcα gene silencing and Hyp photo-activation? *Proc. SPIE 9550, Biosensing and Nanomedicine VIII* (2015), art. numb. 95500J.

4. M. Misuth, J. Joniova, D. Belej, S. Hrivnak, D. Horvath, V. Huntosova: Estimation of PKCδ autophosphorylation in U87 MG glioma cells: combination of experimental, conceptual and numerical approaches. *Journal of Biophotonics* (2016), accepted.
5. M. Maslanakova, L. Balogova, P. Miskovsky, R. Tkacova, K. Stroffekova: Anti- and pro-apoptotic Bcl2 proteins distribution and metabolic profile in human coronery aorta endothelial cells before and after HypPDT. *Cell Biochemistry and Biophysics* (2016), accepted.

## 5. Detection of environmental pollutants at very low concentrations by surface- enhanced Raman spectroscopy measurements - a case of organochlorine pesticides

Surface-enhanced Raman spectroscopy (SERS) is one of the most powerful analytical techniques for the detection of trace amount of chemicals. This is due to the coupling of the Raman scattering of a molecular system to a localized plasmon resonance of silver or gold nanostructures. However, many molecules of great interest lack affinity for metal surfaces and their SERS detection is not possible. On the other hand, the affinity of an analyte towards metal surface can be increased by modifying or functionalizing the metal surface with the organic molecules. We have, together with the group of Dr. Santiago Sánchez- Cortés, IEM , CSIC, Madrid (Spain) (partner in the project CELIM), developed a nano-sensor for rapid, effective and low-cost detection of environmental pollutants, concretely organochlorine pesticides. In the following paragraphs are presented the results which have led to the construction of such nano-sensor.

The adsorption mechanism of linear aliphatic dithiols with chain lengths of 6, 8 and 10 carbon atoms on silver and gold nanoparticles has been studied by surface-enhanced Raman scattering (SERS) spectroscopy. SERS spectra provided the structural marker bands of these compounds which were employed to obtain information about the adsorption and coordination mechanism, the orientation, conformational order, and packing of the aliphatic chains on the metal nanoparticles surface. The effect of the type of metal (silver or gold) and the extent of surface coverage on all the above mentioned properties is discussed. Dithiols may act as linkers between nanoparticles and induce the formation of nanogaps with a controllable interparticle distance. The interaction through both thiol groups makes the adsorption of dithiols on metal surface substantially different from that of monothiols, in particular the orientation of dithiols is perpendicular, while monothiols adopt a tilted orientation. The nanogaps thus formed are able to produce hot spots exhibiting a large intensification of electromagnetic field in these points. This property can be potentially employed in the SERS detection of trace concentrations of chemicals which can be trapped in these gaps by an increase of the adsorption affinity induced by the presence of the polymethylene chain between two nanoparticles. The above presented results have been consequently employed for the detection of the persistent organic pollutants (POPs), concretely organochlorine pesticides aldrin, dieldrin, lindane and α-endosulfan by using SERS and for the optimization of the SERS sensing substrate. In order to overcome the inherent problem of the low affinity of the above pesticides, we have developed a above described strategy consisting of the functionalization of the metal surface with alkyl dithiols in order to achieve two different goals: i) to induce the nanoparticle linkage and create interparticle junctions where sensitive hot spots needed for SERS enhancement are present, and ii) to create a specific environment in the nanogaps between silver and gold nanoparticles making them suitable for the assembly and SERS detection of the analyzed pesticides.

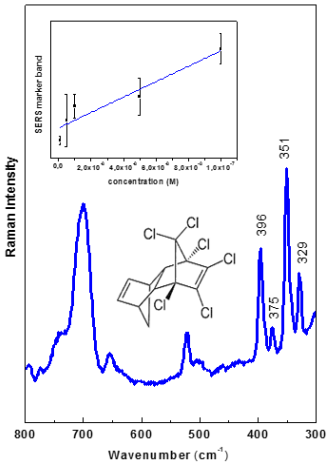


**Fig. 4**: Example of the SERS spectra and detection limit for chlorinated pollutant aldrin

Afterwards, an optimization of the sensing substrate was done by varying the experimental conditions: type of metal nanoparticles, molecular linker (aromatic vs aliphatic dithiols and the length of the intermediate chain), surface coverage, laser excitation wavelength. From the adsorption isotherms it was possible to deduce the corresponding adsorption constant and the limit of detection.

The present results confirm the high sensitivity of SERS for the detection of the organochlorine pesticides with a limit of detection about $10^{-8}$M (**Fig. 4**), thus providing a solid basis for the construction of suitable nano-sensors for the identification and quantitative analysis of this type of chemicals.

This our idea has inspired our team to create a start-up society SAFTRA photonics Ltd., with the aim to apply for a project in H2020 SME call and we have been successful in the Phase I.

**Related publications**

1. I. Izquierdo-Lorenzo, J. Kubackova, D. Manchon, A. Mosset, E. Cottancin, S. Sanchez-Cortes: Linking Ag Nanoparticles by Aliphatic α,ω-Dithiols: A Study of the Aggregation and Formation of Interparticle Hot Spots. *Journal of Physical Chemistry C* (2013) 117, 16203-16212.
2. J. Kubackova, I. Izquierdo-Lorenzo, D. Jancura, P. Miskovsky, S. Sanchez-Cortes: Adsorption of linear aliphatic α,ω-dithiols on plasmonic metal nanoparticles: a structural study based on surface-enhanced Raman spectra. *Physical Chemistry Chemical Physics* (2014) 16, 11461-11470.
3. J. Kubackova, G. Fabriciova, P. Miskovsky, D. Jancura, S. Sanchez-Cortes: Sensitive Surface-Enhanced Raman Spectroscopy (SERS) Detection of Organochlorine Pesticides by Alkyl Dithiol-Functionalized Metal Nanoparticles-Induced Plasmonic Hot Spots. *Analytical Chemistry* (2015) 87, 663-669.

**Related H2020 projects**

1. **NanoScreen: Nano-Screening for Persistent Organic Pollutants**
   SME Instrument – Phase 1: NMP-25-2014-1: Accelerating the uptake of nanotechnologies, advanced materials or advanced manufacturing and processing technologies by SMEs – **project financed**
2. **NanoScreen: Nano-Screening for Persistent Organic Pollutants**
   SME Instrument – Phase 2: NMP-25-2014-1: Accelerating the uptake of nanotechnologies, advanced materials or advanced manufacturing and processing technologies by SMEs – **project submitted**

## 6. Construction of optically trapable SERS probes

In certain cases, the presence of molecules (drugs) in biological systems is not detectable by fluorescence methods. As an example we can mention molecular aggregates with quenched fluorescence. Raman and Surface Enhanced Raman Spectroscopy (SERS) represent alternative detection methods. In the framework of the CELIM project, optically trappable SERS microprobes have been developed to facilitate the detection of non-fluorescent molecules.

In the first step, the optical tweezers apparatus was constructed (**Fig. 5**). The new setup was tested in connection with a simple micro-fluidic system for fast buffer exchange in optical tweezers experiments. Shortly, micro-shelters (i.e. thin dead-end side-arms of fluid channels) were used to aid buffer exchange in optical tweezers experiments. Particles “hidden” in micro-shelters became insensitive to extreme flow conditions in the main fluid channel, which minimized the requirements for the applied flow system.

The basic idea of the SERS micro-probes is depicted in **Fig. 6**. The microstructures made by two-photon polymerization of SU-8 photoresist were manipulated in a dual beam optical trap. The active area of the structures was covered by a SERS-active silver layer using chemically assisted photoreduction from silver nitrate solutions. Silver layers of different grain size distributions were created by changing the photoreduction parameters and characterized by scanning electron microscopy. The structures were tested by measuring the SERS spectra of emodin and hypericin.

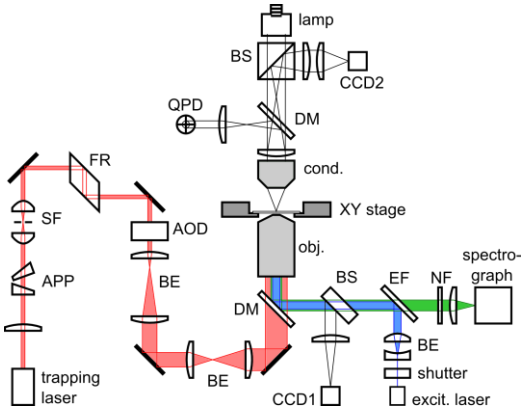


**Fig. 5.** The optical tweezers setup.

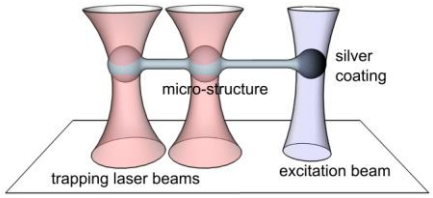


**Fig. 6.** Optically trapped SERS micro-probes.

### Related publications

1. M. A. Omar, P. Miskovsky, G. Bano: Proof-of-principle for simple microshelter-assisted buffer exchange in laser tweezers: interaction of hypericin with single cells. *Lab on a Chip* (2014) 14, 1579-1584.
2. G. Vizsnyiczai, T. Lestyan, J. Joniova, B.L. Aekbote, A. Strejckova, P. Ormos, P. Miskovsky, L. Kelemen, G. Bano: Optically Trapped Surface-Enhanced Raman Probes Prepared by Silver Photoreduction to 3D Microstructures. *Langmuir* (2015) 31, 10087-10093.

## 7. Molecular bioenergetics – catalytic mechanism of cytochrome c oxidase

In recent years, remarkable progress has been made in our understanding of the structure and function of mitochondrial as well bacterial proton transporters at the molecular level. Perhaps the most challenging and complex of these molecular energy-transducers are the terminal respiratory heme-copper oxidases. These molecular machines are designed not only to catalyze the reduction of oxygen to water, but to prevent the release of potentially toxic oxygen intermediates and, at the same time, to utilize the free energy from $O_2$ reduction to pump protons across the membrane. The resulting electrochemical proton gradient is used to drive ATP synthesis or to power a wide variety of other energy requiring reactions from metabolite transfer to mechanical movements. However, the exact mechanism of proton pumping has not been determined in any proton pump that is driven by reduction-oxidation reactions and this remains one of the major unresolved problems of molecular bioenergetics. Our effort in this area is directed to general understanding of the proton translocation mechanism by identifying the electron-proton coupling sites and establishing the mechanistic principles of pumping and electron gating in cytochrome c oxidases (CcO), a member of heme-copper oxidases.

In the absence of external electron donors oxidized bovine CcO exhibits the ability to decompose excess $H_2O_2$. Depending on the concentration of peroxide, two mechanisms of degradation were identified. At submillimolar peroxide concentrations decomposition proceeds with virtually no production of superoxide and oxygen. In contrast, in the millimolar concentration range of $H_2O_2$, CcO generates superoxide from peroxide. At submillimolar concentrations the decomposition of $H_2O_2$ occurs at least at two sites. One is the catalytic heme $a_3$-$Cu_B$ center where $H_2O_2$ is reduced to water. During the interaction of the enzyme with $H_2O_2$ this center cycles back to oxidized CcO via the intermediate presence of two oxoferryl states. We show that at pH 8.0 two molecules of $H_2O_2$ react with the catalytic center accomplishing one cycle. In addition, the reactions at the heme $a_3$-$Cu_B$ center generate the surface exposed lipid-based radical(s) that participates in the decomposition of peroxide. It is also found that the irreversible decline of the catalytic activity of the enzyme

treated by submillimolar $H_2O_2$ concentrations results specifically from the decrease in the rate of electron transfer from heme *a* to heme $a_3$ - $Cu_B$ during the reductive phase of the catalytic cycle. The rates of electron transfer from ferrocytochrome *c* to heme *a* and the kinetics of the oxidation of the fully reduced CcO with $O_2$ were not affected in the peroxide modified CcO.

This result contributes to the elucidation of the molecular mechanism of the proton pumping by heme-copper oxidases and could be helpful in the formulation of the plausible mechanism of the pumping by membrane bound electron transport complexes which is one of the key tasks of the today molecular bioenergetics.

**Related publications:**

1. D. Jancura, J. Stanicova, G. Palmer, M. Fabian: How hydrogen peroxide is metabolized by oxidized cytochrome c oxidase. *Biochemistry* (2014) 53, 564-3575.

## 8. Development of nano-vehicle for targeted drug delivery

The main hurdles associated with cancer chemotherapy include limited and non-specific accumulation of drugs in cancer tissues, which therefore requires a higher administered drug dose leading to intolerable cytotoxicity and nonspecific targeting. Thus, to mitigate the difficulty associated with chemotherapy of cancer, there is a strong need for developing a drug delivery system that will optimize the therapeutic action of drugs while reducing their toxic side effects.

In recent years, much attention has been focused on the use of endogenous lipoproteins as natural drug delivery vehicles Endogenous lipoproteins have important advantages in comparison to other drug nano-delivery systems: (i) as natural molecules, lipoproteins escape recognition by the mononuclear phagocytic system, which favors their long circulation time in the plasma, (ii) they are not immunogenic, (iii) their hydrophobic core and phospholipid shell favor binding of hydrophobic and amphiphilic drugs, respectively. The importance of lipoproteins as drug delivery vehicles is confirmed by the fact that the US Food and Drug Administration (FDA) encouraged the inclusion of lipoprotein-drug interaction studies as a part of any investigational new drug application that contains a hydrophobic compound. LDL are amongst the most important lipoproteins in terms of drug delivery, nevertheless, the importance of HDL has also been recognized and in a number of cases seems to be even higher than LDL's. The capability of both types of lipoproteins, (LDL and HDL), to bind some drugs and their functionality as drug carriers has been examined in several studies.

By means of fluorescence spectroscopy we have studied the kinetics of interaction of a photosensitizer hypericin (Hyp) with high-density HDL. Hyp is incorporated into HDL molecules as monomer till ratio Hyp/HDL~8:1 and above this ratio forms non-fluorescent aggregates. This number is different from that found in the case of Hyp incorporation into low-density lipoprotein (LDL) molecules (8:1 *vs* 30:1). The difference is mainly attributed to the smaller size of HDL in comparison with LDL molecule. Biphasic kinetics of Hyp association with HDL was observed. The rapid phase of incorporation is completed within seconds, while the slow one lasts several minutes. The kinetics of the association of Hyp molecules with free HDL, Hyp/HDL=10:1 complex and the redistribution of Hyp from Hyp/HDL=70:1 complex to free HDL molecules reveal a qualitative similar characteristics of these processes with those observed for the interaction of Hyp with LDL. However, the incorporation of Hyp into HDL in the "slow" phase is more rapid than to LDL and extend of Hyp penetration into lipoproteins in the fast phase is also much higher in the case of HDL. The lower concentration of cholesterol molecules in outer shell of HDL particles is probably the determining factor for the more rapid kinetics of Hyp incorporation to and redistribution from these molecules when comparing with LDL particles.

On the other hand, a difficult isolation of the lipoproteins in large quantity from a biological organism as well as a variability of the composition and size of these molecules makes practical application of LDL and HDL as drug delivery systems quite complicated. Synthetic LDL and HDL and large unilamellar vesicles (LUV) are potentially suitable candidates to substitute the native lipoproteins for targeted and effective drug delivery. In this work, we have studied process of an association of potent photosensitizer hypericin (Hyp) with synthetic lipid-based nano-particles (sLNP) for drug delivery and large unilamellar vesicles (LUV) containing various amount of cholesterol. Cholesterol is one of the main components of both LDL and HDL particles and its presence in biological membranes is known to be a determining factor for membrane properties. It was found that the behavior of Hyp incorporation into sLNP particles with diameter ca ~ 90 nm is qualitatively very similar to that of Hyp incorporation into LDL (diameter ca. 22 nm) and these particles are able to enter U-87 MG cells by endocytosis. The presence of cholesterol in LUV influences the capacity of these vesicles to incorporate Hyp into their structure.

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**Related publications:**

1. J. Joniova, L. Buriankova, D. Buzova, P. Miskovsky, D. Jancura: Kinetics of incorporation/redistribution of photosensitizer hypericin to/from high-density lipoproteins. *International Journal of Pharmaceutics* (2014) 475, 578-584.
2. J. Joniova, L. Blascakova, D. Jancura, Z. Nadova, F. Sureau, P. Miskovsky: Incorporation of photosenzitizer hypericin into synthetic lipid-based nano-particles for drug delivery and large unilamellar vesicles with different content of cholesterol. *Proc. SPIE9166, Biosensing and Nanomedicine VII* (2014) art. numb. 916604.

**Related H2020 project:**

1. Towards highly selective and personalized cancer treatment: DARPin – endogenous lipoprotein complexes as a new generation of targeted drug delivery vehicles, **FETOPEN-H2020**

**Remark:** This part of our results is not described in much more detail, because is confidential. Detailed description of the results and visions is provided in the above mentioned H2020 project. This is also a reason why we have not published more articles in this field. In the near future, depending on negotiations with our partners, we would like to apply for international patents in this field.

**9. Protein engineering and stability**

Thermally induced transitions of the 13-subunit integral membrane protein bovine cytochrome c oxidase (CcO) have been studied by differential scanning calorimetry (DSC) and circular dichroism (CD). Thermal denaturation of dodecyl maltoside solubilized CcO proceeds in two consecutive, irreversible, kinetically driven steps. The thermal denaturation data were analyzed according to the Lyubarev and Kurganov model of two consecutive irreversible steps. This enabled us to show for the first time that both the amphiphilic environment and the self-association state of CcO affect its kinetic stability. The important role of the amphiphilic

environment for chemical stability has been also demonstrated on mammalian and bacterial CcOs. Detailed analysis of thermal denaturation of cytochrome c in a broad range of pH values enabled us to show surprisingly complex behavior of cytochrome c at physiological pH with an implication for its physiological function.

The effect of electrostatic interaction on amyloid fibrilization of lysozyme has been studied in the presence of Hofmeister anions. Our study shows Hofmeister effect of monovalent anions on: (1) lysozyme stability; (2) ability to accelerate nucleation phase of lysozyme fibrillization; (3) amount, and (4) conformational properties of the formed fibrils (**Fig. 7**).

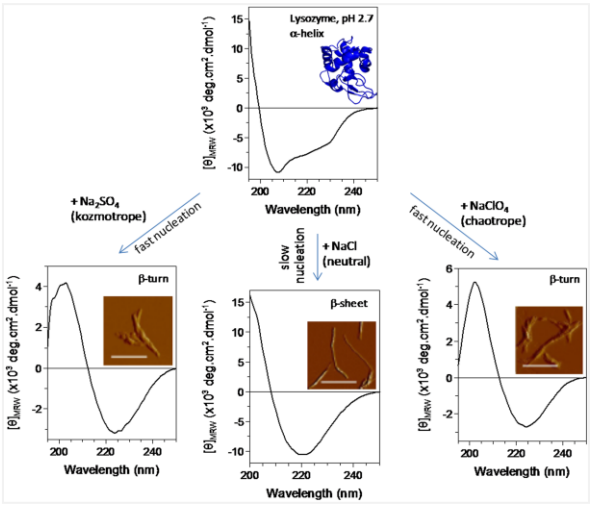


**Fig. 7.** Schematic overview of an effect of Hofmeister anions on CD spectra and shape of lysozyme fibrils.

In the collaboration with the laboratory of Prof. Plückthun's, we were able to introduce a robust method employing directed evolution of GPCRs in yeast that allows fast and efficient generation of receptor variants, which show strongly increased functional production levels in eukaryotic expression hosts. Shown by evolving three different receptors in this study, the method is widely applicable, even for GPCRs which are very difficult to express. The evolved variants showed up to a 26-fold increase of functional production in insect cells compared to the wild-type receptors. Next to the increased production, the obtained variants exhibited improved biophysical properties, while functional properties remained largely unaffected. Thus, the presented method broadens the portfolio of GPCRs accessible for detailed investigations.

Exploiting the favorable properties of the designed ankyrin repeat protein (DARPin) scaffold, we created a novel class of fluorogen activators, termed FADA, suitable for imaging of proteins on the cell surface, as well as in the cytosol. Moreover, based on the requirement of dimerization for strong fluorogen activation, a prototype FADA biosensor for in situ detection of a target protein and protein–protein interactions was developed (**Fig. 8**). Therefore, FADAs are versatile fluorescent probes that are easily produced and suitable for diverse applications and thus extend the fluorogen-activating proteins technology.

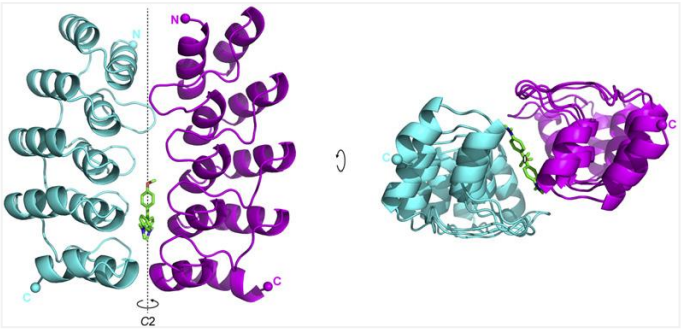


**Fig. 8.** Crystal structure of dimeric FADA-3210 binding one MG-2p molecule.

**Related publications:**

1. Sedlák et al. (2014) The Kinetic Stability of Cytochrome c Oxidase: Effect of Bound Phospholipid and Dimerization. Biophys. J. 107(12), 2932-40;
2. Sedlák et al. (2015) Advanced Analyses of Kinetic Stabilities of IgGs modified by Mutations and Glycosylation. Protein Sci. 24(7), 1100-13;

3. Poniková et al. (2015) Lysozyme stability and amyloid fibrillization dependence on Hofmeister anions in acidic pH. J. Biol. Inorg. Chem. 20, 921-33;
4. Varhač et al. (2015) Non-two-state thermal denaturation of cytochrome c at neutral and slightly acidic pH. Biophys. Chem. 203-204, 41-50;
5. Sedlák & Robinson (2015) Destabilization of the Quaternary Structure of Bovine Heart Cytochrome c Oxidase upon Removal of Tightly Bound Cardiolipin. Biochemistry 54(36), 5569-77;
6. Musatov et al. (2016) Delipidation of Cytochrome c Oxidase from Rhodobacter sphaeroides Destabilizes its Quaternary Structure. Biochimie 125, 23-31.
7. Schütz et al. (2016) Directed evolution of G protein-coupled receptors in yeast for higher functional production in eukaryotic expression hosts. Sci. Reports. 6:21508;
8. Schütz et al. (2016) Generation of fluorogen-activating designed ankyrin repeat proteins (FADAs) as versatile sensor tools. J. Mol. Biol. 428, 1272–89.

**10. Modeling and characterization of cancer cell heterogeneity**.

**TOPIC I: Development of resistance limits efficiency of present anticancer therapies**. It is accepted at an intuitive level that the resistance emerges as a consequence of the heterogeneity of cancer cells at the molecular, genetic and cellular levels. Preventing diversification of cancer cells population represents therefore a big challenge in cancer research. To understand time development of the intratumor heterogeneity, the three objectives were followed:

**i)** characterization of the intratumor heterogeneity as a complex time dependent statistical property which may be quantified by different measures, most of them coming from statistical mechanics,

**ii)** integration of intratumor heterogeneity as quantifiable and computable property of the population of cells into a conceptual evolutionary cancer model as a rigorous starting point for developing mathematical cancer models, and,

**iii)** to apply our evolutionary model to study evolution of selected diversification strategies in different environment. Different switching strategies and different dynamics of environment were investigated.

As the main results we presented:

**1)** To understand general relationship between the spatiotemporal statistics of tumor microenvironment and intratumor heterogeneity, the generalized distance based concept was applied to express distances between probabilistically described cell states and environmental conditions. As a measure of dissimilarity between the pairs of normal distributions characterizing the population and environment respectively, Hellinger distance was applied. We concluded, that as the therapy corresponds, from an evolutionary viewpoint, to a purposeful modification of the cells fitness landscape, understanding general relationship between the spatiotemporal statistics of a tumor microenvironment and intratumor heterogeneity will allow to conceive the therapy as an inverse problem and to solve it by optimization techniques.

**2)** Intratumor heterogeneity positively correlates with the development of the resistance to therapy. To address this issue, we studied evolution of a specific strategy of population diversification, the phenotype switching, at a conceptual level. For our purposes, we have devised the model of a large population of asexual organisms evolving in a time-varying environment represented by a stochastic Markov process. We demonstrated that under rapidly varying exogenous conditions organisms operate in the vicinity of the bet-hedging strategy, while the deterministic patterns become relevant as the environmental variations are less frequent.

**3)** We devised the two-state discrete-time Markovian model to study the impact of the two alternative switching strategies on the fitness of the population evolving in time varying environment. The first strategy, referred as the â responsive switching, enables the cell to make transition into the state conferring to it higher fitness in

the instant environment. If the alternative strategy, termed random switching is applied, the cell undergoes transition into the new state not regarding the instant environment. Each strategy comes with the respective cost for its physical realization. Within the framework of evolutionary model, mutations occur as random events which change parameters of the probabilistic models corresponding to the respective switching strategies. Most of the general trends of population averages can be easily understood at the intuitive level, with a few exceptions related to the cases when too low mutation noise hampers population to follow rapid environmental changes. The more detailed study of the parameter distributions reveals much more complex structure than expected.

**Topic II: Extension of the manifold-learning approach to the X-ray imaging problems**. The Manifold learning approach usually serves as a preprocessing step in familiarizing with data and for the formulation of hypotheses leading to further data analysis. It has found many important applications in biology, robotics or visual data mining. In recent years, the Manifold learning techniques have also found applications in the techniques for extracting structural information from X-ray diffraction snapshots. The part of the manifold learning process may be also viewed as a kind of stochastic optimization which is inspired by the natural systems. The main result achieved in relation to the specific topic of Manifold Learning: The research indicated that hybrid combination of the existing stochastic optimization approaches (grid search, extremal optimization, and hysteretic optimization) to the Manifold learning provided robust results superior to those when the separate single-optimization module is used.

**Related publications:**

1. B. Brutovsky, D. Horvath, Towards inverse modeling of intratumor heterogeneity, Open Phys. 13(2015), 232-241.
2. D. Horvath, B. Brutovsky, Study of selected phenotype switching strategiesin time varying environment, Physics Letters A 380(2016), 1267â 1278.
3. D. Horvath, J. Ulicny, B. Brutovsky, Self-organised manifold learning and heuristic charting via adaptive metrics, Connection Science, 2016, Vol.28/Issue 1, pages 1-26.
4. D. Horvath, B. Brutovsky, Etiology of phenotype switching strategy intime varying stochastic environment, Physica A, in press 2016

## 11. XFEL group activities

The main determinant of the scientific and technical solution is the start of XFEL Europe (E-XFEL) operation - the first lasing will not happen before the end of 2016, actual user operation for experienced users will start in June 2017, restricted access to “friendly users” is supposed to last till the end of 2018.

Before that time, the access to hard X-ray free electron lasers is very limited, based on success of ultra-competitive beamtime proposals on two existing hard X-ray FELs at USA (SLAC) and Japan (Spring-8) (we submitted two proposals, were not successful yet). All the other development work has to be done on more accessible (still scarce), but much inferior synchrotron sources ($10^{8-9}$ less intensity and no short femtosecond-scale pulse duration).

Due to our systematic work, financed from CELIM project, the group led by assoc. prof. Jozef Uličný has been qualified as “experienced users” though only with the help of other teams. In this sense, we are collaborating on instrumental and methodological development.

**User consortia**. We are participating actively in two user consortia at E-XFEL to become experienced “friendly users” with facilitated access to early-stage experiments, namely SFX consortium and XBI consortium. At the moment, we are just before signing both User consortia agreements, in which Slovak Republic express commitments to invest 1M€ to the SFX and 0.8M€ into the XBI user consortia. The investment is on

governmental level, oversaw by Ministry of Education of the Slovak Republic, and we are involved in SPB/SFX workstation construction and setup of XBI biological labs also as negotiators of Slovak Republic.

**Biological samples - suitability and preparation.** The main determinant for diffract-before-destroy suitability for SPB experiments is homogeneity of samples in case of reproducible objects (nanocrystals, viruses, etc.) or proper contrast enhancing strategy and incorporation of *apriori* known information for unique object too large for the information recorded by detectors in single-shot experiments. Our primary scientific interest (DNA architecture of living cells at mesoscopic resolution) requires development of problem specific sample preparation, contrasting and data processing strategies. We are developing with partners all relevant steps of the imaging workflow of these samples at E-XFEL.

**Contrasting**. At synchrotron (but possibly also at XFEL) sources, the resolution limit is about 10 nm due to Rose criterion, so we have to devise efficient contrasting strategy for reconstruction of 3D mesoscopic shapes of interest. For the time being, our contrasting strategy consists of use of functionalised metallic nanoparticles (from 1 nm up to cca 30 nm), possessing (for smaller particles) multimodal imaging properties (namely contrast for single nanoparticle imaging using hard X-ray imaging as well as optical domain fluorescence for traditional and superresolution imaging techniques in optical domain) (**Fig.9**). Functionalisation is done on level of small organic monolayers, preventing otherwise toxic metals from poisoning cells, and/or aptamer-based functionalisation, allowing for precision molecular targeting. The synthetic work is done at Department of Biochemistry, Faculty of Science, UPJŠ, as a part of their research program, contributing thus to overcome the fragmentation of research at our faculty.

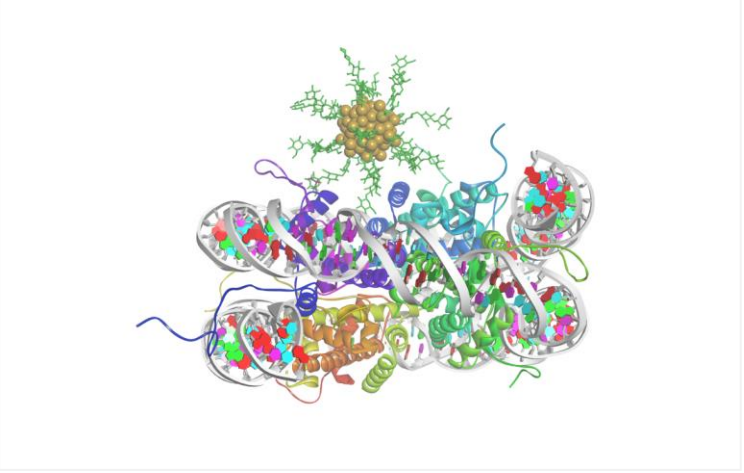

**Fig. 9** Structural motif - nucleosomal core particle densely packing inactive DNA and functionalised gold nanoparticle.

**Data processing and modeling**. DNA imaging at mesoscopic level requires predictive molecular modeling at spatial and temporal scale larger than achievable using atomistic simulations. We have developed Coarse-grain models of G-quadruplexes of DNA - structural motifs present in the native cell. (**Fig. 10**). The coarse-grain representation of the DNA constructs is sufficient for scattering simulation event, construction of support function and iterative phase retrieval part of experimental data processing.

Our coarse-grain models are enablers of technological applications for nanoconstructs based on DNA technology, including immobilised sensors as well as molecular probes for bio-imaging using optical technologies.

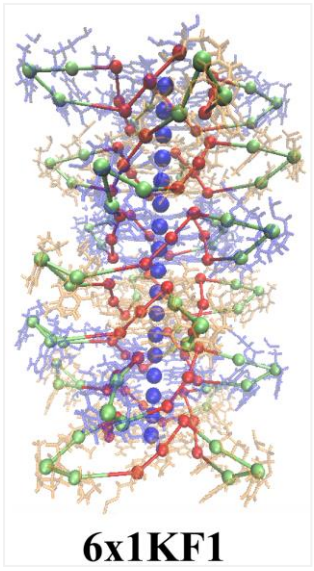


**Fig. 10** Coarse-grain model of multiple quadruplex motifs together with its all-atom representation

### Related publications

M. Rebic, F. Mocci, A. Laaksonen, J. Ulicny: Multiscale Simulations of Human Telomeric G-Quadruplex DNA. *Journal of Physical Chemistry B* (2015) 119, 105-113.

**Interpretation of experiments.** We have developed, in a collaboration with scientists from E-XFEL, propagators for imaging weakly absorbing biological objects, where most desirable modality is phase shift imaging. Using Bragg magnifier and our code, we are able to do whole organism imaging (extremophil Echiniscus) using tomographic projections of hard X-ray holograms in near-to-intermediate scattering region (**Fig. 11**).

The GPGPU accelerated code for processing small wave-front shifts, developed in our group, is actively tested and will be incorporated into Workpackage 7 PUCCA of The European Cluster of Advanced Laser Light Sources (EUCALL) H2020 project.

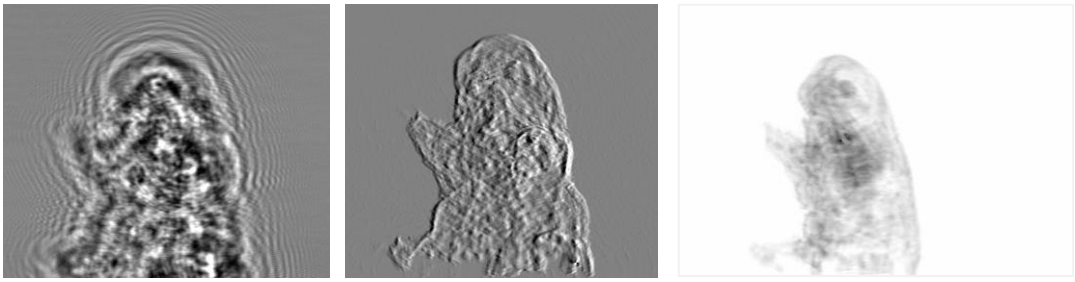

**Fig. 11** Raw hologram, iterative phase retrieval reconstruction and differential phase contrast 2D projection of Echiniscus. By combining 2D projections, full 3D microtomography (CT) is constructed. Experiments done at Diamond UK national synchrotron source.

### Practical outputs.

**Contrasting agent for macroscopic imaging of GI tract for pre-cancerous polyps**. We have submitted *via* UPJS a patent application and established start-up company (SAFTRA Imagine) aimed at commercionalization of the patent application and creating research base for contrasting agents and strategies over the whole range of X-ray and PET/SPECT imaging modalities.

We are in collaboration with research branch of Siemens Healthcare (two PhD students supervised by J. Uličný working on areas of research of common interests with Molecular Imaging group) (simulation of radiational dosage in real-time, physical processes during CT, PET and SPECT imaging, development of targeted contrasting agents for theranostics applications) as well as construction of biomolecular imaging devices using next-generation X-ray sources (XFELs and diffraction limited sources), both of which have real chance for practical applications in forms of IP and/or startup/spinoff companies.