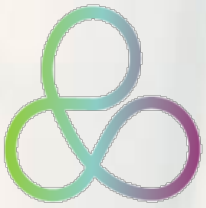

INNOMOL

Innovation Pipeline

**May 11-12, 2016**

**Ruđer Bošković Institute**

# InnoMol New Platforms for Molecular Solutions in Research and Development

InnoMol - Enhancement of the Innovation Potential in SEE through new Molecular Solutions in Research and Development


INNOMOL
Innovation Pipeline

# About InnoMol

The InnoMol project will foster a research pipeline at the Ruđer Bošković Institute (RBI) and facilitate new avenues of innovation and technology for the investigation of relevant diseases.

Over three years, a 4.8 million EUR worth InnoMol project will have an impact on around 300 scientists from seven divisions of the RBI.

## R&D TOWARDS AN INNOVATIVE DRUG

As the InnoMol goals are oriented towards the future development of possible diagnostic, preventive and/or therapeutic approaches for the major diseases of the world, it is essential to assemble a critical mass of cross-functional and project-oriented resources and expertise so as to achieve the appropriate alignment of elements along the value chain leading towards an innovative drug.

## ASSEMBLING CRITICAL MASS OF RESOURCES AND EXPERTISE

The project will bring together and reinforce 3 major elements of life-science research, namely Medicine, Biology, and Chemistry, to create a productive, state-of-the-art environment that is unique in the region and which will bridge the gap between the pre-commercial and commercial phases of R&D, thus enhancing the relevance of the RBI's position in the European Research Area (ERA).

The fully assembled research pipeline will enable inter- and, multidisciplinary research in the field of Molecular Biosciences with focus on DNA-Protein, Protein-Protein, Protein-RNA, DNA-RNA and DNA-DNA interactions.

## EXPLOITATION FOR SUSTAINABILITY

The tangible outcomes of the Action will be innovative molecular solutions for investigating major global diseases in order to transfer the knowledge to the community for the generation of wealth and public wellbeing.

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# TIMELINE

**Day 1 (May 11, 2016)**

**09:00 - 09:30 Registration (coffee/tea)**

**09:30 - 09:40** Opening by Oliver Vugrek, Project Coordinator

*Bioinformatics: chair S. Tomić*

**09:40 - 10:00** Ivica Kopriva
**10:00 - 10:20** Maria Brbić
**10:20 - 10:40** Petar Ozretić
**10:40 - 11:00** Marina Grabar Branilović

**11:00 - 11:10 Short break**

*Molecular Interactions: chair I. Jerić*

**11:10 - 11:30** Ivo Piantanida
**11:30 - 11:50** Josipa Matić

**12.00 - 13.30 Lunch break** (with the compliments of the Organizing Committee)

**13:30 - 13:50** Filip Šupljika
**13:50 - 14:10** Marija Matković
**14:10 - 14:30** Ivo Crnolatac

**14:30 - 14:50 Coffee/tea break**

*Bioimaging: chair M. Herak Bosnar*

**14:50 - 15:10** Lucija Horvat
**15:10 - 15:30** Igor Weber
**15:30 - 15:50** Marko Šoštar
**15:50 - 16:10** Adriana Lepur

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# TIMELINE

**Day 2 (May 12, 2016)**

*Genomics/Transgenics: chair T. Balog*

**09:30 - 09:50** Oliver Vugrek
**09:50 - 10:10** Rober Belužić
**10:10 - 10:30** Jelena Knežević
**10:30 - 10:50** Brankica Mravinac
**10:50 - 11:10** Andreja Ambriović Ristov

**11:10 - 11:30 Coffee/tea break**

*Proteomics: chair M. Kralj*

**11:30 - 11.50** Branka Bilić
**11.50 - 12.10** Vedrana Vidulin
**12:10 - 12:30** Martina Malnar
**12:30 - 12:50** Tina Paradžik

**13:00 Lunch (with the compliments of the Organizing Committee)**

## Bioinformatics

1. Ivica Kopriva:
   **Nonlinear Sparse Component Analysis with a Reference: Variable Selection in Genomics and Proteomics**
2. Maria Brbić
   **A comprehensive atlas of microbial phenotypes and associated genetic features**
3. Petar Ozretić
   **Bioinformatic Resources for microRNA Research**
4. Marina Grabar Branilović
   **Development of the Manganese, Mn2+, ion parameters for simulation by the AMBER Force Fields**

## Genomics/Transgenics

1. Oliver Vugrek
   **RNAseq using Illumina sequencing technology**
2. Robert Belužić
   **Whole-exome Sequencing and Comparative Genomic Hybridization: complementary approaches in advanced genomics**
3. Jelena Knežević
   **Innate Immunity of COPD and lung cancer: genetic and epigenetic approach**
4. Brankica Mravinac
   **Epigenetic aspects of centromere identity**
5. Andreja Ambriović Ristov
   **Integrin αvβ3 Expression in Tongue Squamous Carcinoma Cells Cal27 Confers Anticancer Drug Resistance Through Loss of pSrc(Y418)**

## Proteomics

1. Branka Bilić
   **Hydrogen/Deuterium Exchange Mass Spectrometry-tool for protein conformation dynamics analysis**
2. Vedrana Vidulin
   **Predicting Gene Function on a Massive Scale Reveals an Extensive Complementarity between Genome-based Methodologies**
3. Martina Malnar
   **Increased BACE1 dependent proteolysis in Niemann-Pick type C (NPC) disease – the link between Alzheimer's and NPC disease**

4. Tina Paradžik
   **Where SsbA meets SsbB?**

## Bioimaging

1. Lucija Horvat
   **Revealing functions of photosynthetic proteins with advanced transmission electron microscopy**
2. Igor Weber
   **A versatile system for confocal microscopy at RBI**
3. Marko Šoštar
   **Processing of images acquired by confocal microscopy: Huygens and Quimp**
4. Adriana Lepur
   I**maging and image analysis in life sciences**

## Molecular Interactions

1. Ivo Piantanida
   **Design of small molecule probes for proteins and DNA/RNA**
2. Josipa Matić
   **Synthesis of Peptide-Based Fluorescent Probes for Biomacromolecules**
3. Filip Šupljika:
   **Isothermal Titration Calorimetry (ITC): biomacromolecule-ligand interaction study and enzyme kinetics**
4. Marija Matković
   **Circular and linear dichroism: a (re)view on protein and DNA structures**
5. Ivo Crnolatac
   **Getting charged for the mitochondrial uptake; di-cation cyanine dyes for fluorescent labelling of mitochondria**

**Nonlinear Sparse Component Analysis with a Reference: Variable Selection in Genomics and Proteomics**

*Ivica Kopriva*

*Laboratory for information systems, Division of Electronics*

Many scenarios occurring in genomics and proteomics involve small number of labeled data and large number of variables. To create prediction models robust to overfitting variable selection is necessary. We propose variable selection method using nonlinear sparse component analysis with a reference representing either negative (healthy) or positive (cancer) class. Thereby, component comprised of cancer related variables is automatically inferred from the geometry of nonlinear mixture model with a reference. Proposed method is compared with 3 supervised and 2 unsupervised variable selection methods on two-class problems using 2 genomic and 2 proteomic datasets. Obtained results, which include analysis of biological relevance of selected genes, are comparable with those achieved by supervised methods. Thus, proposed method can possibly perform better on unseen data of the same cancer type.

**A comprehensive atlas of microbial phenotypes and associated genetic features**

*Maria Brbić*

*Laboratory for Information Systems, Division of Electronics*

Bacteria and Archaea display a wide variety of phenotypic traits and can adapt to diverse ecological niches. However, there is a shortage of databases that systematically and with broad coverage assign particular traits to individual prokaryotic taxa. We thus establish a computational pipeline that can automatically assign hundreds of different phenotypes to bacterial and archaeal genomes by text-mining the scientific literature and by drawing on genomics data.

**Bioinformatic Resources for microRNA Research**

*Petar Ozretić*

*Laboratory for Hereditary Cancer, Division of Molecular Medicine*

MicroRNAs are a family of small single-stranded noncoding RNAs which post-transcriptionally regulate gene expression. Functional studies have shown that microRNAs participate in virtually every cellular process, and changes in their expression might underlie many human pathologies such as cancer, in which we are particularly interested in. One of the main research tools used for identifying microRNAs involved in specific cellular processes is microRNA expression profiling using microarray technology, which is available at our Institute.

In this talk I will present what I have learned during my secondment at the Division of Bioinformatics at Innsbruck Medical University, Innsbruck, Austria. After short introduction about general microRNA biology and microarray technology, I will first present statistical software used for normalization, clustering, and differential expression analysis of Agilent Technologies microRNA microarray data. Next I will present various freely available bioinformatic resources (databases and web-based tools) used for various aspects of microRNA research such as microRNA sequence and expression repositories, predicted and experimentally validated microRNA:target interactions, and microRNA:function/phenotype associations. All presented resources will be demonstrated using our own experimental data obtained by microRNA microarray expression profiling of high-grade serous carcinoma samples from Croatia.

# Development of the Manganese, $Mn^{2+}$, ion parameters for simulation by the AMBER Force Fields

*Marina Grabar Branilović*

*Laboratory for Protein Biochemistry and Molecular Modelling, Division of Organic Chemistry and Biochemistry*

About 40% of all proteins are metalloproteins. Metal cations take part in enzymatic reactions and/or they stabilize protein structure. The aim of our research has been to determine reliable $Mn^{2+}$ parameters, which can be implemented in AMBER Force Fields in order to perform molecular dynamic simulations of manganese-containing metalloproteins and their complexes. We have used the methicillin-resistant *Staphylococcus aureus* aminohydrolase (PDB ID: 4EWT) active site with two manganese ions as a template for the initial systems. To determine force-field parameters, quantum mechanical calculations have been performed and a few groups of Mn-parameters were derived and tested.

One set of these parameters was used in study of *Brassica rapa* auxin-amidohydrolase (BrILL2). BrILL2 belongs to the M20D metallopeptidase subfamily, related to the amidohydrolase superfamily (M20) of enzymes which hydrolyze a number of different substrates, including amino acids, sugars, nucleic acids, and organophosphate esters. It plays a key role in homeostasis of the plant hormone auxin in a way to hydrolases amino acid conjugates of auxins. A large concentration of free auxins, of which the most common is indole-3-acetic acid (IAA), is toxic for plants, so only about 5% of the total concentration of auxin molecules in plants is in the free (active) form, while the rest is stored in inactive forms, mostly as amino acid and sugar conjugates. In order to hydrolyze the amide bond of amino acid conjugated auxins (inactive, storage forms), and release the free auxin, BrILL needs manganese. The active site structure of BrILL2, which similarly to 4EWT contains two manganese cations, was interdisciplinary studied by different experimental: biochemical, spectroscopic, calorimetric and computational approaches.[1] Further on, we performed molecular modelling studies of the ligand free enzyme, wild type BrILL2 and the Cys139Ser mutant, in the presence and absence of manganese ions. The obtained results are in agreement with the experimental data.

[1]Smolko, A., Šupljika, F., Martinčić, J., Jajčanin-Joyić, N., Grabar-Branilović, M., Tomić, S., Ludwig-Müller, J., Piantanida, I. & Salopek-Sondi, B. ***Phys. Chem. Chem. Phys.***, 2016, **18**, 8890-8900

**RNAseq using Illumina sequencing technology**

*Robert Belužić, Lucija Kovačević, Pau Marc Munoz Torres, Adriana Lepur, Oliver Vugrek*
*Laboratory for Advanced Genomics, Division of Molecular Medicine*

Transcriptome analysis is becoming more accessible due to advances in high-throughput technologies, such as microarrays or Next-Generation-Sequencing (NGS) approaches. The transcriptome is the set of all messenger RNA molecules in one cell or a population of cells and one may distinguish between a total set of transcripts in a given organism, or to the specific subset of transcripts present in a particular cell type. Because it includes all mRNA transcripts in the cell, the transcriptome reflects the genes that are being actively expressed at any given time. Thus, transcriptomics, or expression profiling, has been revolutionized by NGS, or RNA-seq that represents a highly sensitive and accurate tool for measuring expression across the transcriptome. RNA-seq provides visibility to previously undetected changes occurring in disease states, in response to therapeutics, under different environmental conditions and across a broad range of other study designs. RNA-Seq allows researchers to detect both known and novel features in a single assay, enabling the detection of transcript isoforms, gene fusions, single nucleotide variants, allele-specific gene expression and other features without the limitation of prior knowledge. Here we present a first insights into RNA-seq at the RBI, including an overview of the advanced NGS platform for established during the InnoMol project.

**Whole-exome Sequencing and Comparative Genomic Hybridization: complementary approaches in advanced genomics**

*Robert Belužić*

*Laboratory for Advanced Genomics, Division of Molecular Medicine*

Here I will present an example of a full implementation of equipment obtained through IPA (microarray platform) and Innomol (NGS) projects in Laboratory for Advanced Genomics. Array-based Comparative Genomic Hybridization (aCGH) and next-generation whole-exome sequencing (WES) present two complimentary approaches in genome research: while CGH allows for easier detection of larger genomic rearrangements, NGS can detect changes at a level of a single DNA base, covering the entire coding region of human genome. Practical application of this unified platform for discovery of novel genetic aberrations in difficult-to-diagnose (neuro)developmental disorder will be discussed.

This real-life example will demonstrate both advantages and limitations of each individual approach, and will emphasize the potential of these techniques in modern genetic testing and diagnostics.

**Innate Immunity of COPD and lung cancer: genetic and epigenetic approach**

*Jelena Knežević*

*Laboratory for Advanced Genomics, Division of Molecular Medicine*

Chronic obstructive pulmonary disease (COPD) is the third most common cause of death worldwide. Epidemiological studies have linked the presence of COPD to the development of lung cancer, the most severe comorbidity of COPD. Environmental risk factors, like smoking, have strong impact on development of both diseases, but the fact that only 20% of smokers develop COPD and 20% of COPD patients develop lung cancer suggest that genetics could play a key role in the pathogenesis of disease. Respiratory epithelium is the first line of pulmonary defence and innate immune response is essential for maintaining this process. Chronic inflammation in lungs is associated with both diseased conditions, and in general is recognized as major risk factor for the most type of cancer. Several lines of evidences are linking cancer, inflammation and infection. For example, transcription factor NF-κB, major inducer of inflammation, is activated by many cancer risk factors (cigarettes smoke) and is constitutively active in most cancer. We have shown that several mutations in innate immunity genes are associated with COPD and LC, indicating that this could be yet unknown mechanistic link of respiratory epithelial cells and control of innate immunity in COPD and LC.

On the other hand, aberrant DNA methylation of promoter region CpG islands is associated with gene silencing and serves as an alternative to mutation-induced inactivation of tumour suppressor genes in human cancers. Many epidemiological studies have shown that inflammatory/infection conditions precede most cancers. Activation and controlling mechanisms of inflammation and infection are regulated by NF-κB and almost exclusively relied on receptors of innate immunity. Thus, suppression of these proinflammatory pathways may provide opportunities for both prevention and treatment of cancer. The aim of our study is to evaluate methylation status of ASC/TMS1 and MyD88 genes, key adaptor molecules in innate immunity signalling.

We were using pyrosequencing approach, method that is still golden standard in methylation analysis, to define methylation status of promoter regions of genes of interest. We found that both MyD88 and ASC/TMS1 exhibit reduced methylation status of promoter regions in tested tumour tissues, comparing to healthy tissue. Also, we found different methylation pattern of specific tested CpG islands characteristic for lung and larynx cancer.

**Epigenetic aspects of centromere identity**

*Brankica Mravinac*

*Laboratory for Structure and Function of Heterochromatin, Division of Molecular Biology*

The centromere is a chromosomal locus responsible for the faithful segregation of genetic material during cell division. In addition to understanding its role as a biological structure, studying the centromere also is highly relevant from a biomedical point of view, because abnormalities in centromeric function are often lethal or associated with various congenital and acquired diseases, such as cancer, infertility, and birth disorders. A critical question in centromere biology is how centromere identity is established and successfully propagated from one generation to the next, maintaining the integrity of the genome. Although centromere function is fundamental in all eukaryotic organisms, no universal DNA sequence has been linked to centromere identity. Despite extreme heterogeneity of centromeric DNA, the most common centromeric DNA components in many animal and plant species are repetitive sequences such as satellite DNAs and transposable elements. The absence of primary DNA sequence determinants has strengthened the notion that epigenetic, or sequence-independent, components specify centromere assembly. CenH3, a histone H3 variant that replaces canonical H3 to create unique centromeric nucleosomes, physically marks functional centromeres. However, replacement of canonical histones by histone variants is just one aspect of epigenetic control of chromatin organization. Another pathway for specifying different chromatin domains within centromere region involves post-translational, covalent modifications (acetylation, phosphorylation, and methylation) of the amino-terminal tails of nucleosomal core histones. By studying the three different invertebrate model organisms, our group is trying to address genetic and epigenetic features that define differently organized centromeres: satellite DNA-rich, satellite DNA-poor, and centromeres dispersed on holocentric chromosomes. Within this talk, there will be presented our recent results achieved by the state-of-the-art equipment and strengthened by exchange of know-how with a high-profile partnering organization in the framework of the InnoMol project.

# Integrin αvβ3 Expression in Tongue Squamous Carcinoma Cells Cal27 Confers Anticancer Drug Resistance Through Loss of pSrc(Y418)

*Nikolina Stojanović[a], Anamaria Brozović[a], Dragomira Majhen[a], Maja Herak Bosnar[b], Gerhard Fritz[c], Maja Osmak[a], and Andreja Ambriović-Ristov[a*]*

*[a] Laboratory for Cell Biology and Signalling, Division of Molecular Biology, Ruđer Bošković Institute; [b] Laboratory for Protein Dynamics, Division of Molecular Medicine, Ruđer Bošković Institute, [c] Heinrich Heine University Düsseldorf, Medical Faculty, Institute of Toxicology, Düsseldorf, Germany*

Integrins play key roles in the regulation of tumor cell adhesion, migration, invasion and sensitivity to anticancer drugs. In the present study we investigate the mechanism of resistance of tongue squamous carcinoma cells Cal27 with *de novo* integrin αvβ3 expression to anticancer drugs. Cal27-derived cell clones, obtained by transfection of plasmid containing integrin subunit β3 cDNA, as compared to control cells demonstrate: expression of integrin αvβ3; increased expression of integrin αvβ5; increased adhesion to fibronectin and vitronectin; resistance to cisplatin, mitomycin C, doxorubicin and 5-fluorouracil; increased migration and invasion, increased amount of integrin-linked kinase (ILK) and decreased amounts of non-receptor tyrosine kinase (Src) and pSrc(Y418). Knockdown of ILK and integrin β5 in cells expressing integrin αvβ3 ruled out their involvement in drug resistance. Opposite, Src knockdown in Cal27 cells which led to a reduction in pSrc(Y418), as well as treatment with the pSrc(Y418) inhibitors dasatinib and PP2, conferred resistance to all four anticancer drugs, indicating that the loss of pSrc(Y418) is responsible for the observed effect. We identified differential integrin signaling between Cal27 and integrin αvβ3-expressing cells. In Cal27 cells integrin αv heterodimers signal through pSrc(Y418) while this is not the case in integrin αvβ3-expressing cells. Finally, we show that dasatinib counteracts the effect of cisplatin in two additional head and neck squamous cell carcinoma (HNSCC) cell lines Cal33 and Detroit562. Our results suggest that pSrc(Y418) inhibitors, potential drugs for cancer therapy, may reduce therapeutic efficacy if combined with chemotherapeutics, and might not be recommended for HNSCC treatment.

# Hydrogen/Deuterium Exchange Mass Spectrometry-tool for protein conformation dynamics analysis

*Branka Bilić*

*Laboratory for Mass Spectrometry, Division of Physical Chemistry*

Peptide bond hydrogen's are distributed along the entire protein primary structure (with exception of proline) and they can be exchanged with deuterium from solvent at slower or faster rate depending on their solvent accessibility and the strength of internal hydrogen bonding. By monitoring exchange reaction rate kinetics with mass spectrometry it is possible to infer information about protein structure dynamics, conformation, and interactions. Temperature and pH also influence H/D exchange reaction kinetics and during experiment they must be tightly controlled. Information gathered and experience gained during project secondment activities allowed IRB to update H/DX instrumentation platform with cooling box for temperature control and improve reproducibility of the collected H/DX data. In part of activities related to application of the H/DX method, two protein systems are analyzed.

Human Dipeptidyl-peptidase III protein (DPP III, EC 3.4.14.4) is chosen as a model system for initial demonstration of H/DX method capabilities. It is a monozinc metalloexopeptidase that hydrolyzes dipeptides from the N-terminal of its substrates consisting of three or more amino acids. Homologues of the human protein were identified from a variety of prokaryotic and eukaryotic species indicating importance of this molecule activity for life (Chen and Barrett 2004). So far, just one crystal structure of human DPP III complex was determined and it showed large conformational change upon ligand-inhibitor binding (Bezerra, Dobrovetsky et al. 2012). In this presentation H/DX analysis of conformation change induced by inhibitor binding to human DPPIII protein and other 5 structural homolog's will be shown.

Purine nucleoside phosphorylase (PNP) from *Escherichia coli* (EC 2.4.2.1) is the other protein system investigated by H/DX method. The biologically active form of PNP is a homohexamer unit, assembled as a trimer of dimers. Crystallography showed that upon binding of phosphate, neighboring monomers adopt different active site conformations, described as open and closed. When the virtually inactive Arg24Ala mutant is complexed with phosphate, all active sites are found to be in the open conformation. To understand how the sites in neighboring monomers communicate with each other, we carried out H/DX experiment. Although method wasn't able to distinguish conformation of each monomer unit separately it was able to indicate the dynamic mechanism of cross-talk between them. Importance of the Arg24 residue for productive phosphate binding and consequences binding has for protein backbone motion within a monomer will be explained (Kazazić, Bertoša et al. 2015).

Bezerra, G. A., et al. (2012). "Entropy-driven binding of opioid peptides induces a large domain motion in human dipeptidyl peptidase III." Proceedings of the National Academy of Sciences **109**(17): 6525-6530.

Chen, J.-M. and A. J. Barrett (2004). Handbook of Proteolytic Enzymes. Amsterdam, Elsevier Acad. Press.

Kazazić, S., et al. (2015). "New Insights into Active Site Conformation Dynamics of E. coli PNP Revealed by Combined H/D Exchange Approach and Molecular Dynamics Simulations." Journal of the American Society for Mass Spectrometry **27**(1): 73-82.

**Predicting Gene Function on a Massive Scale Reveals an Extensive Complementarity between Genome-based Methodologies**

*Vedrana Vidulin*

*Computational Biology and Bioinformatics Group, Division of Electronics*

The number of sequenced genomes rises steadily, but we still lack the knowledge about the biological roles of many genes. Automatic function prediction (AFP) is thus a necessity. We hypothesize that AFP approaches which draw on distinct genome features may be useful for predicting different types of gene functions, motivating a systematic analysis of the benefits gained by obtaining and integrating such predictions. Our AFP pipeline amalgamates 5,133,543 genes from 2,071 genomes in a single massive analysis that evaluates five established genomic AFP methodologies. Our results showed pronounced complementarity between the AFP methods, both gene-wise and function-wise: the majority of Gene Ontology functions were accessible to only one or two of the methods and different methods tend to assign a GO term to non-overlapping sets of genes. Because of this, often the best integration strategy is to rely on a single most-confident prediction per gene/function, instead of enforcing agreement across multiple AFP methods. Furthermore, we estimated the current level of knowledge in functional annotation already assigned to *Escherichia coli* genes and the level of knowledge in additional annotations assigned by the individual AFP methods and integration schemes. We also estimated the potential profit of AFP methods and integration schemes from newly sequenced genomes.

**Increased BACE1 dependent proteolysis in Niemann-Pick type C (NPC) disease – the link between Alzheimer's and NPC disease**

*Martina Malnar*

*Laboratory for Neurodegenerative Disease Research, Division of Molecular Medicine*

BACE1 is the critical protease involved in the pathogenesis of the most common neurodegenerative disorder - Alzheimer's disease (AD) as it initiates the production of the toxic amyloid-β peptides (Aβ) that accumulate in the brain of AD patients. We have previously shown that increased cholesterol, a risk factor for AD, leads to increased BACE1 cleavage of amyloid precursor protein (APP) resulting in Aβ accumulation in Niemann-Pick type C disease (NPC), a rare neurodegenerative lipid storage disorder (Malnar et al. 2010; 2012).

Collaboration with an InnoMol partner, the German Centre for Neurodegenerative Diseases in Munich, as well as the new Leica confocal microscope at Rudjer Boskovic Institute, enabled us to further investigate the involvement of BACE1 in the neuropathogenesis of NPC disease. We analysed BACE1 dependent proteolysis, expression and cellular localization of its main substrates in the central nervous system - proteins Sez6, Sez6L and APP. The obtained results will be important for a future investigation of the changes in these proteins upon neurodegeneration.

**Where SsbA meets SsbB?**

*Tina Paradžik[1], Mladen Paradžik[1], Paul Herron[2], Igor Weber[1], Dušica Vujaklija[1]*

*[1] Laboratory for Molecular Genetics, Division of Molecular Biology, Ruđer Bošković Institute, Zagreb; [2] University of Stratchclyde, Glasgow, UK*

SSB proteins are found in all living organisms, and are essenital in the processes of DNA metabolism. Antibiotic producing bacteria *Streptomyces coelicolor* possesses two paralogous *ssb* genes on their chromosomes. These genes are expressed differentialy during streptomyces complex life cycle. SsbA protein is essential for the survival, while SsbB is involved in processes of chromosome seggregation during sporulation of these bacteria. Paralogous SSB proteins bind ssDNA with different affinity. Both SSB proteins showed interesting variations in 3D structures. SSBs were tagged with fluorescent proteins; SsbA with GFP, and SsbB with mCherry. Recombinant genes were integrated into streptomyces chromosomes and expressed from its natural promotor regions. Bacteria were cultivated in rich and minimal media with different time of incubation to detect localisation of both proteins using confocal microscopy. Localisation of fluorescently labelled SSB proteins was observed in all stages of bacterial growth. These proteins are rarley found to co-localise. While SsbA had strongest flurescent foci in young vegetative hyphae, SsbB was mostly expressed in young spore chains, which is in agreement with proposed biological roles of these proteins.

**Revealing functions of photosynthetic proteins with advanced transmission electron microscopy**

*Lucija Horvat*

*Laboratory for Molecular Plant Biology and Biotechnology, Division of Molecular Biology*

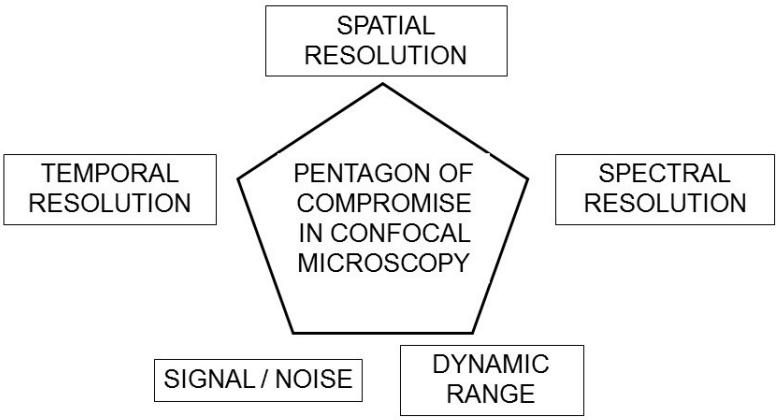
Reverse genetics is a powerful set of tools that enable us to address the functions of genes with unknown functions. During our previous work we have generated a number of knock out and knock in mutants of genes implicated in photosynthetic energy conversion in a model plant Arabidopsis thaliana. In addition to biochemical and molecular biology analyses, we employ transmission electron microscopy (TEM) to study possible changes in cellular and chloroplast ultrastructures induced by those genetic alterations. Further, small antioxidants, such as ascorbate can be visualized and quantified by using advanced immuno-gold labbeling. Application of those bioimaging techniques to address the function of recently characterized photosynthetic proteins will be presented.

**A versatile system for confocal microscopy at RBI**

*Igor Weber*

*Laboratory of Cell Biophysics, Division of Molecular Biology*

The ongoing renaissance introduces novel concepts and approaches to fluorescence light microscopy on the yearly basis. Despite of the astonishing developments, point-scanning confocal microscopes remain the working horses of contemporary bioimaging. I will describe features of the confocal system purchased through the InnoMol project, and compare its features with other imaging systems available on the market. Versatility and the overall sensitivity of the system will be particularly highlighted.



**Processing of images acquired by confocal microscopy: Huygens and Quimp**

*Marko Šoštar*

*Laboratory of Cell Biophysics, Division of Molecular Biology*

Because of the continuous decrease in time required to obtain scientific information, there is a growing need for capable data processing solutions. This is especially true in the field of bioimaging, where modern systems can produce tens of gigabytes of data daily. So, to keep pace with this information 'overload', we need equally capable tools for image analysis. Here I will present two software packages that we found very helpful in addressing this issue.

*Huygens Professional* is acquired within the InnoMol project. It is tailored for performing deconvolution of microscopic images. It has very efficient algorithms that can process large batches of images in the matter of minutes. With the plethora of other features, such as automatic estimation of the background, automatic bleaching correction, correction for spherical aberration, PSF measurement, viewer for inspecting and comparing multi-channel images in 2D, 3D and time, it's truly a very capable software for image analysis.

*QuimP software*, a set of plugins for ImageJ, has been developed to quantify spatio-temporal patterns of fluorescently labeled proteins in the cortex of moving cells. QuimP utilises an active contour algorithm to segment cells from the background of an image, so even the time series of shape changing cells can be effectively and quickly analyzed. Obtained information include centroid displacement, distance traveled, speed, elongation and circularity of the cell, cell area, cytoplasmic and cortex fluorescence etc. All data is easily exportable so it can be additionally processed if needed. We have built strong connections with the developers of the software at the Univesity of Warwick through secondments on the InnoMol project, and are actively participating in further development of this great little software package.

**Imaging and image analysis in life sciences**

*Adriana Lepur*

*Laboratory for Advanced Genomics, Division of Molecular Medicine*

Development of various imaging methods is transforming our ability to visualize biological processes. In life sciences, seeing is just a first step in believing. Optical illusions reveal how easy it is to fool a human brain. Therefore, increased emphasis is placed on image analysis before allowing images to answer any biological question.

For studying protein interactions, bi-molecular fluorescent complementation (BiFC) emerged as a powerful technique in a last decade. Two non-fluorescent parts of a fluorescent protein are genetically fused to proteins of interest, and if they interact the two parts of a fluorescent protein are brought together resulting in a gain of fluorescence. Bi-molecular fluorescent complementation is suitable for high-throughput studies, but its major advantage over similar methods is that the protein interactions can be visualized live in human cells. Large amounts of imaging data resulting form high-throughput screens would be impossible to tackle without a great computing power and image analysis software. Often, images contain information in more than 4 dimensions. Cells alone are three-dimensional objects, stained with different fluorescent markers, imaged over a certain time course. Typical image analysis pipeline uses different software in sequence before reaching an answer. For example, in a BiFC protein interaction screen we would first use ImageJ to check the dynamic range, subtract background, and adjust threshold. Next we would evaluate the % of cell transfection using Cell Profiler, powerful software for batch image analysis. Then we would classify protein interactions using Cell Cognition, a machine learning software that can process thousands of images after initial training. Finally, we can preform a co-localization analysis, again using ImageJ, to precisely place a protein interaction inside a cell.

This is just a taste of how current fluorescent microscopy and computing power can help us understand complex cellular processes.

**The design of small molecular probes for proteins and DNA / RNA**

*Ivo Piantanida*

*Laboratory for Biomolecular Interactions and Spectroscopy, Division of Organic Chemistry and Biochemistry*

Methods of synthetic organic and inorganic chemistry allow preparation of almost unlimited number of new compounds. There are many approaches to design of novel compounds, the most of them relying on a particular target (e.g. DNA, RNA, protein), mostly neglecting other possible targets. Upon synthesis, further studies are often pursued as fast as possible, loooking for the cheaper and simpler way to get an answer: "sufficiently/insufficiently active" toward foreseen target. This resulted in the development of a series of fast screening techniques, sharing common drawback of a very narrow focus. The result - a lot of compounds have never been tested against a wide range of different targets/uses. Good examples are a) fluorescent compounds that have proven insufficiently bioactive, but have never been tested as fluorescent markers; b) low-active compounds, for which the interaction with the presumed target (protein, DNA/RNA) was never characterized. This would indicate the reason for the low activity (steric hindrance binding, aggregation with a target with the emergence of colloids, mixed mode of binding).

Here will be presented somewhat more time-consuming, but simple approach to the novel compound or small series of derivatives, where a combination of generally available methods allow characterisation of new molecules in biologically relevant conditions and basic parameters of their interaction with the most common biological targets - proteins and DNA/RNA. Although this general strategy is generally not profitable for pharmaceutical industry as there is no directly visible economic impact, presented approach is of significant interest to academic institutions. Namely, in addition to a wide range of scientific results it offers a good chance for cooperation with industry on the level of highly specialised expertise.

Expertise of such research lines are multidisciplinary, demanding knowledge of chemistry, molecular biology and molecular medicine. In order to reinforce such research pipeline platform at the RBI, in 2013-2016 REGPOT project Innomol (http://www.innomol.eu/) was implemented. A part of the platform focused mainly on chemical methods will be presented in this lecture.

**Synthesis of Peptide-Based Fluorescent Probes for Biomacromolecules**

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Phenanthridine dyes are a promising group of small molecule fluorophores which specifically interact with DNA and RNA polynucleotides. Additionally, interactions with proteins are possible as well. Most familiar representative of the group is ethidium bromide, the classical double helix intercalator (Figure). It has been confirmed that addition of different substituents on the phenanthridine moiety can alter its binding mode. On the other hand, peptide derivatives are known for their particular interaction with DNA and RNA grooves which provides selectivity. Peptide backbone enables relatively simple functionalization through different synthetic methods. Aromatic moieties attached on peptide side chains offer additional interacting possibilities, while the length of the peptide linker can control the flexibility of the molecule.

In our case, phenanthridine moiety serves as an anchor to biological target but also as a fluorescent reporter group. Additional interacting groups combined with the length of the peptide linker increase selectivity and enable development of molecular probes with tunable properties.

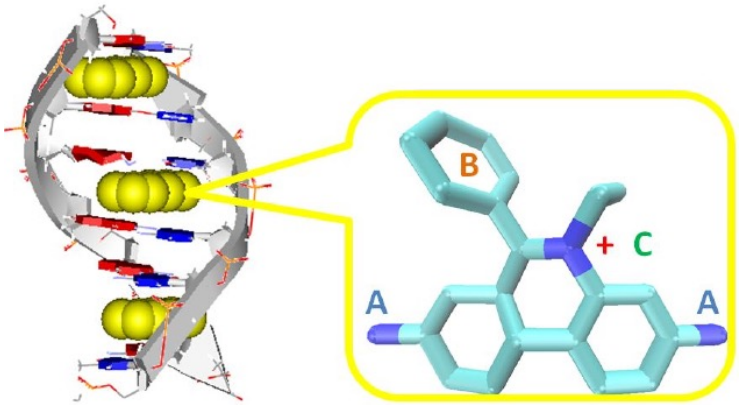


Figure. Schematic presentation of the intercalative binding mode by the neighbour exclusion principle and important structural features of ethidium bromide: A) amino substituents responsible for fluorescence increase upon DNA intercalation; B) phenyl substituent for steric control and also impact on fluorimetric properties; C) permanent positive charge for aqueous solubility and electrostatic attraction to the DNA or RNA phosphate backbone.

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# Isothermal Titration Calorimetry (ITC): biomacromolecule-ligand interaction study and enzyme kinetics

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This presentation will cover the theory, operation and maintenance of Isothermal Titration Calorimetry (ITC) and give a broad range of applications enabled by the direct nature of the technique. Isothermal Titration Calorimetry (ITC) is the ideal technique for the measurement of biological binding interactions since the data provided does not rely on the presence of chromophores or fluorophores, nor requires an enzymatic assay. It is also a good technique for studying enzyme kinetics and determining kinetic parameters.

ITC relies only on the detection of the heat effect upon binding or reaction, enabling scientists to better understand nature of binding and/or kinetics of the process.

**Circular and linear dichroism: a (re)view on protein and DNA structures**

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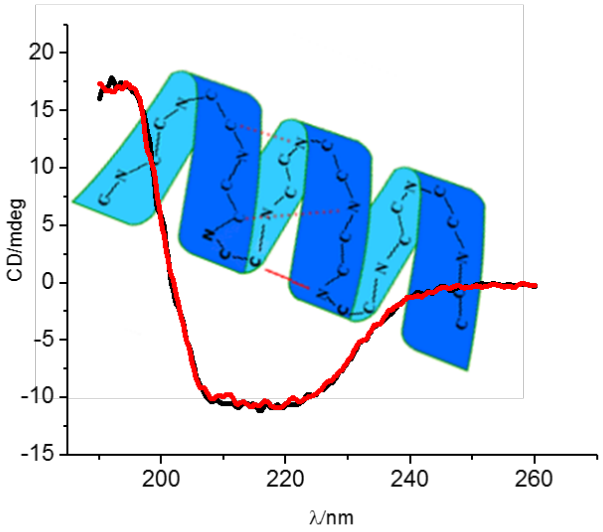
Circular (CD) and linear dichroism (LD) are very useful methods for observing the structures and ligand binding modes of biomacromolecules, such as proteins and DNA/RNA-s, in solution. Both methods are non-invasive, and need relatively low concentrations (1-100 uM) of observed species. While the CD method is based on different absorption of chiral light, the LD method depends on different absorbance of vertically and horizontally polarized light. Therefore CD can give a spectrum of a chiral molecule/complex while LD will signalize a molecule/complex that can obtain a specific orientation toward the electromagnetic field of the incoming polarized light.

Herein are presented several examples of UV-CD and UV-LD application to view changes of DNA/RNA structure upon ligand binding, or for example to view changes in the protein structure after amino acid sequence change.




a-helix picture from http://biolishl.blogspot.hr/2013/09/7.html

Figure: UV-CD spectrum of α-helix protein segment

**Getting charged for the mitochondrial uptake; di-cation cyanine dyes for fluorescent labelling of mitochondria.**

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The normal mitochondrial function is crucial for the cellular homeostasis. Hence, the mitochondrial welfare is the subject of numerous studies, which usually employ fluorescent probes to monitor the mitochondrial membrane potential. Majority of such fluorescent probes exhibit serious drawbacks in terms of their media solubility, fluorescence stability or intensity, mitochondrial or general cellular toxicity. Two series of di-cationic cyanine dyes were synthesized to specifically target mitochondria. They possess almost no intrinsic fluorescence, however upon binding to the polynucleotides the fluorescence of the resulting complex increases dramatically. By introduction of relatively long and bulky substituents on the longer arm of cyanine dye, we tried, not only to direct their non-covalent binding interactions with the polynucleotide towards groove binding, but also to limit their antiproliferative effect. Double charge was also purposely introduced to target the mitochondria, because of the relatively high membrane potential ($\Delta\psi m$) of mitochondrial membrane. Apart from colocalization studies, using confocal microscopy, results of the comparative studies of the influence of novel dyes and the commercially available ones on mitochondrial function and cell proliferation will be presented.

***Participants of The InnoMol New Platforms for Molecular Solutions in Research and Development Workshop, May 11-12, 2016 at Ruđer Bošković Institute***

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