



REPUBLIC OF SLOVENIA
MINISTRY OF EDUCATION,
SCIENCE AND SPORT



Fourth Annual Conference

»Superresolution Microscopy«

Institute of Pathological Physiology,
University of Ljubljana, Medical Faculty, Ljubljana

10-12 June 2013

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Ljubljana, 10-12 June 2013

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Preface

The fourth annual conference of Centre of Excellence for integrated approaches in chemistry and biology of proteins (CIPKeBiP) focuses on **super resolution microscopy**. The super resolution microscope installed in Ljubljana is the cutting edge technology. The key instrument is STED technology based instrument. It represents the most advanced technology in the field of optical microscopy, comprising of most advanced optics, mechanics and electronics. It enables observation of living objects at resolution between 35 and 40 nm with the potential to visualize objects beyond the declared limits.

Due to the introduction of technological novelty, this year conference includes a workshop with practical demonstration of the super-resolution microscopy. At the workshop the applications of microscopy on cells, tissues, and organisms will be shown.

The lectures of the first day are focused on presentation of new super resolution technics and their applications in research in different systems: exocytosis in astrocytes, surface dynamics of GLT-1 in astrocytes, astrocytes role in brain function, and traumatic brain injury and study of gliotransmitter vesicles.

The workshop with practical demonstrations will use the following instrumentation:

ELYRA – Enter to the super-resolution microscopy

LSM 780 – The highest level of detection in laser confocal microscopy

AxioZoom – The motorized fluorescence stereo zoom microscope

Apo.Tome – Optical slices at all magnifications

LSM 700 – The compact laser scanning confocal microscopy

STED – STED on the inverted stand Axio Observer Z1

The second day of the conference is devoted to presentations of research activities of CIPKEBIP partners: Center of Excellence for Research on Aging from Italy, NCP Cluster of Medical and Ecological Instrument Engineering and Biotechnologies from Saint-Petersburg and Saint-Petersburg Pavlov State Medical University.

Welcome to the conference and enjoy in the science and practical demonstrations of super-resolution microscopy.

Robert Zorec, Marko Kreft, Livija Tušar, Dušan Turk
June, 2013

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Program

10 June 2013

13:00 -18.00 Arrival, Registrations and Installation of demo equipment

11 June 2013

9:00-9:10: Conference openings

Dušan Turk, Scientific Director of CIPKEBIP

Urban Krajcar, Acting Director General, Ministry of education, science and sport

Robert Zorec, CIPKEBIP, Organizer of the Conference

Section I: Overview of super-resolution microscopy (Part A)

9:10-9:15 **Robert Zorec**, Chair, Introduction

9:15-9:30 **Peter Amend**, Significance of high-resolution microscopy in live-cell imaging research

9:30-10:10 **Vladimir Parpura**, Exocytosis in astrocytes: Lessons learned from evanescent wave and atomic force microscopy approaches

10:10-10:40 **Stéphane Oliet**, Surface dynamics of GLT-1 in astrocytes

10:40-11:10 **Alexander Egner**, Superresolution Microscopy (Nanoscopy)

11:10-11:30 Coffee break

11:30-12:00 **Caludia Geisler**, Two color STED microscopy

12:00-12:30 **Jaques Paysant**, SIM superresolution microscopy

12:30-12:45 **Marko Kreft**, Advanced microscopy and cell physiopathology

12:45-13:00 **Jernej Jorgačevski**, Gliotransmitter vesicles studied by STED microscopy

13:00-14:00 Lunch

Section II Hands-on Experience

Instruments and titles of demonstrations:

ELYRA – Enter to the super-resolution microscopy

LSM 780 – The highest level of detection in laser confocal microscopy

AxioZoom – The motorized fluorescence stereo zoom microscope

Apo.Tome – Optical slices at all magnifications

LSM 700 – The compact laser scanning confocal microscopy

STED – STED on the inverted stand Axio Observer Z1

The participants will join the workshops regarding their log in at the conference.

14:00-15.30 Practical demonstration of the superresolution

15:30-15:45 Coffee break

12 June 2013

08:45 -09:00 Registrations

Section I: Overview of super-resolution microscopy (Part B)

09:00-11:00 **Robert Zorec**, Chair, Astroglia: an introduction
Alexei Verkhratsky, History of Neurophysiology. Emphasis on Glia
Patrizia D'Adamo, Genetics of mental retardation and animal models
Sergei Kirov, Window into the injured brain: Neurons, astrocytes and microglia in early stroke and traumatic brain injury

11:00-11:15 Coffee break

Section III: Presentations of CIPKEBIP project collaborators and CIPKEBIP researchers

11:15-12:15 **Dušan Turk**, Chair, Scientific Director of CIPKEBIP
Saverio Alberti, Center of Excellence for Research on Aging, Unit of Cancer Pathology
Anastasia Egorkina, NCP Cluster of Medical and Ecological Instrument Engineering and Biotechnologies
Prof. Nataliia Artiushenko, Saint-Petersburg Pavlov State Medical University

12:15-13:00 Mechanisms and pathways of immune response, Chair: **Maja Rupnik**
Maja Rupnik, Workpackage Mechanisms and pathways of immune response – the overview
Jure Škraban, Studies of gut microbiota associated with *C. difficile* in humans and animals
Franč Strle, University Medical Centre Ljubljana

13:00-14:30 Intra in inter-cellular communication response, Chair: **Enej Kuščer**
Gregor Kosec, Promiscuity of an unusual acyltransferase domain enables incorporation of extender units with chemically amenable side chains into polyketide chains
Marjan Slak Rupnik, Calcium and membrane depolarization waves in islets of Langerhans in pancreas tissue slices.
Jernej Iskra, Synthesis and bioactivity of synthetic peroxides
Anja Pucer, Group X Secreted Phospholipase A₂ Induces Lipid Droplet Formation and Promotes the Survival of Breast Cancer Cells
Eva Žerovnik, Oligomerization and amyloid fibril formation of stefin B in vitro and in cells.
Matej Vizovišek, N- and C-terminomic approaches for discovery of substrates of cysteine cathepsins

14:30-15:15 Lunch

15:15 -16:45 Adaptation mechanisms of extremophiles to environment response,
Chair: **Nina Gunde Cimerman**
Nina Gunde Cimerman, Adaptation mechanisms of extremophiles to environment

Monika Novak Babič, Dishwashers as an example of the indoor extreme environment for survival and propagation of *Exophiala dermatitidis* and *E. phaeomuriformis*

Janja Zajc, Cene Gostinčar, Metka Lenassi, Genomes of fungi from hypersaline environments

Ajda Ota, Archeosomes as vehicles for recombinant protein delivery into skin cells

16:45-18:15 Protein Bank: Proteins, their production, current use and perspectives, Chair: Dušan Turk
Dušan Turk, WorkPackage 4 Protein Bank: Proteins, their production, current use and perspectives

Šnajder Marko, Calcium modulated mechanism of structural stability of pernisine

Miha Renko, Structural Insight into the Lysosomal Degradation of Glycans

Marko Mihelič, "The Same" Enzymes with Different Activities - Comparison of *S. aureus* Bifunctional Autolysin and SAV2307/Autolysin E Activity

Organizing Committee: Closure of the conference

Organizing Committee: Robert Zorec, Marko Kreft, Dušan Turk, Livija Tušar



ABSTRACTS

Section I: Overview of super-resolution microscopy (Part A)

Exocytosis in astrocytes: Lessons learned from evanescent wave and atomic force microscopy approaches

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The mechanism underlying Ca^{2+} -dependent release of various transmitters from astrocytes is exocytosis. Astrocytes express the protein components of the SNARE complex, including synaptobrevin 2, syntaxin and SNAP-23, but not SNAP-25. Using astrocytes expressing synapto-pHluorin, exocytotic sites can be fluorescently imaged. Fusions of synapto-pHluorin labeled vesicles with the plasma membrane can be observed using evanescent wave microscopy (also referred to as total internal reflection fluorescence microscopy); the time course of fusion events (burst vs. sustained), their type ("kiss-and-run" vs. full fusion) and spatial relationship between different fusion sites is discussed. Single molecule investigations of the SNARE complex using atomic force microscopy in force spectroscopy mode show that ternary complexes containing SNAP-23 have a shorter spontaneous lifetime than those containing SNAP-25B. Thus, the spatio-temporal characteristics of astrocytic exocytosis might be in part due to intrinsic properties of the ternary SNARE complex in astrocytes.

Surface dynamics of GLT-1 in astrocytes

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Glutamate is the major excitatory transmitter in the central nervous system. Once released in the synaptic cleft, most of its clearance is ensured by GLT-1, a highly specific glutamate transporter expressed on astrocytes. Due to the slow transport cycle of glutamate transporters (approx. 70ms), it has been hypothesised that these transporters effectively remove glutamate from the synaptic cleft by binding and buffering glutamate thereby maintaining point-to-point transmission and preventing excitotoxicity. To date, it is unknown whether lateral diffusion of glutamate transporters can contribute to this functional buffering and removal of glutamate from the synapse. Here we show that GLT-1 transporters are highly mobile on the surface of astrocytes. Such membrane diffusion is dependent upon both neuronal and transporter activity. Most importantly, the surface diffusion of GLT-1 transporters is greatly decreased in the vicinity of glutamatergic synapses. Finally, strong impairment of lateral diffusion through cross linking of these transporters has a direct impact on the kinetics of excitatory postsynaptic currents, revealing the important role played by surface diffusion of GLT-1 in neuronal synaptic transmission.



Super-Resolution Microscopy

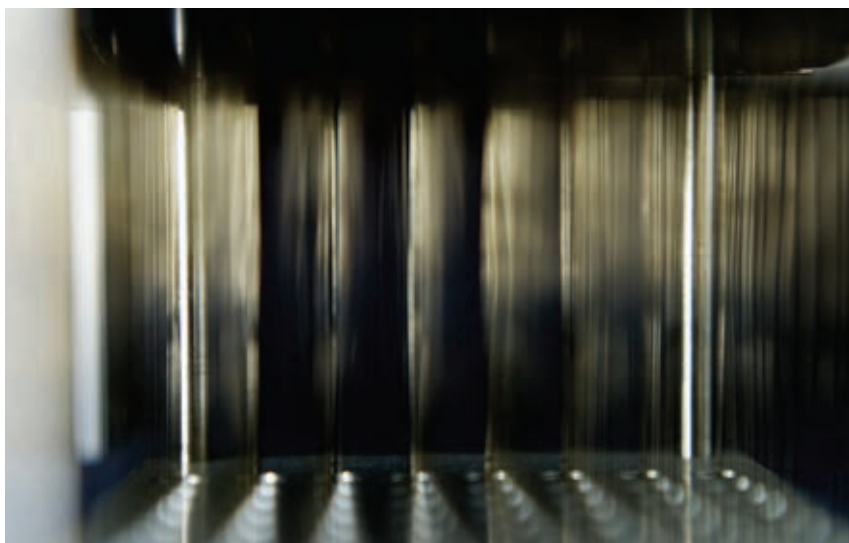
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Far field optical microscopy is a well established method for the non-invasive 3D-investigation of cellular structures. However, the resolution of conventional light microscopy is limited by diffraction to $\sim 200\text{nm}$ in the focal plane and $\sim 600\text{nm}$ along the optic axis. In order to discern identical labels which are much closer than this, one has to overcome the diffraction barrier. The utilization of optical switching events allows one to circumvent Abbe's diffraction limit: The switching of only markers within an area which is much smaller than the size of a diffraction limited spot to a visible "bright" state while all other markers are switched to a non-visible "dark" state defines a sub-diffraction area. By sequentially recording all areas within the diffraction spot, it is possible to assemble a sub-diffraction image.

The first radical concept for improving the resolution of a far field microscope was Stimulated Emission Depletion (STED) microscopy. In this concept the saturated depletion of the excited state of the fluorescent molecule is used to generate a fluorescent spot that is narrower than the diffraction limit. Another method utilizing molecular switching events for achieving nanoscale resolution in microscopy uses a more pointillist approach. Single molecules which are initially in a dark state are sequentially activated, located and deactivated. The localization accuracy of each molecule depends, of course, on the number of detected photons per molecule and can be as high as 2 nm. Over the whole field of view, these methods provide an average resolution in the order of several tens of nanometers.



Two-color STED microscopy

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Images in shades of gray (single color images) show the spatial distribution of marker molecules, however they usually do not differentiate between different molecular species. When a microscope is additionally equipped with the ability to recognize two or more colors and distinguish between them, it will provide decisive new information, for example knowledge about the relative spatial distribution of two different types of molecules. The resulting color coded image of protein locations allows for example to draw conclusions about the proteins' interaction or their function.

The specific technical implementation of a two-color STED (Stimulated Emission Depletion) microscope is presented and examples for its scope of application are given. The system combines two-color detection with a high spatial resolution beyond the diffraction barrier which is achieved by using stimulated emission to sequentially read out markers inside a diffraction-limited volume.

[1]

Two pairs of excitation and STED beams which are provided by a pulsed white light source are aligned in space. They are additionally delayed in time such that the excited fluorescence can not only be spectrally but also temporally separated at the detector. In this way, the cross-talk between the two color channels can be assessed and compensated later on. The system runs at 20 MHz and provides two-color high resolution images of emitters with emission peaks around 620 and 670 nm.

[1] S. W. Hell and J. Wichmann, Opt. Lett. 19(11), 780–782 (1994).

Advanced microscopy and cell physiopathology

Marko Kreft^{1,2,3}, Maja Potokar^{1,2}, Matjaž Stenovec^{1,2}, Marko Muhič¹, Tina Pangršič¹, Mateja Prebil¹, Jernej Jorgačevski^{1,2}, Nina Vardjan^{1,2}, Helena Chowdhury^{1,2}, Robert Zorec^{1,2}

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Astrocytes are increasingly viewed as crucial cells in supporting and integrating the brain function. An important process of communication between astrocytes and neurons is exocytotic release of gliotransmitters from astrocytic membrane-bound vesicles into the extracellular space. High resolution patch-clamp membrane capacitance measurements were used to monitor changes in membrane area of a single astrocyte while the photolysis of caged calcium compounds by a UV flash was used to elicit steps in $[Ca^{2+}]_i$ to determine the exocytotic properties of astrocytes. Experiments show that astrocytes exhibit Ca^{2+} -dependent increases in membrane capacitance. Prior to fusing with the plasma membrane, membrane-bound vesicles are transported through the cytoplasm. Electrophysiology experiments were complemented by high resolution fluorescence measurements of vesicle trafficking and the consequent release of vesicular content may be changed in altered physiological conditions, therefore affecting the physiological status of neurons. We developed a method to study the mobility of fluorescently labeled peptidergic, glutamatergic, purinergic and other vesicles in the cytoplasm of single rat and mouse astrocytes in culture and in brain slices. The results show that the delivery of vesicles to the plasma membrane for membrane merger involves an interaction with the cytoskeleton, in particular microtubules, actin and intermediary filaments. Astrocytes also play a significant role in the brain energy metabolism. Their anatomical position between blood vessels and neurons makes them an interface for effective glucose uptake from blood. The dynamics of their energy metabolic response to neurotransmitter application is not known. We used a FRET glucose nanosensor to dynamically measure the cytosolic glucose concentration in single astrocytes. We show that following the adrenaline or noradrenaline stimulation the availability of cytosolic glucose is increased promptly after stimulation. This indicates, that astrocytic cytosolic glucose metabolism responds to neuronal activity in the time-domain of tens of seconds.

Gliotransmitter vesicles studied by STED microscopy

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The perisynaptic astrocytes tightly enwrap synapses and most likely influence synaptic signaling. Non-vesicular and vesicular-based mechanisms appear to co-exist in astrocytes. The latter is based on vesicle gliotransmitter release, which is mediated by regulated exocytosis. The diameters of these vesicles were reported to be in the range from 30 to 700 nm, as measured by electron microscopy.

The resolution of far-field fluorescence microscopy techniques, including confocal microscopy (CM), is limited by the diffraction limit to ~200 nm. To overcome the diffraction limit one can use structured illumination microscopy (SIM) or/and stimulated emission depletion (STED) microscopy, which both surpass the diffraction limit at list by a factor of two. We performed a complete screening of diameters of vesicles containing vesicular glutamate transporter (VGLUT1), atrial natriuretic peptide (ANP), D-serine and brain derived neurotropic factor (BDNF), by utilizing CM, SIM and STED microscopy.

The results show that the average diameters of vesicles, containing the afore mentioned gliotransmitters, are similar.



Section I: Overview of super-resolution microscopy (Part B)

Window into the injured brain: Neurons and astrocytes in early stroke and traumatic brain injury

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Disturbance of brain water homeostasis during stroke and traumatic brain injury (TBI) leads to a life-threatening state of brain edema. Spreading depolarizations (SDs) are waves of sustained near-complete neuronal and glial depolarization that actively propagate a collapse of ion gradients through the brain with associated dramatic neuronal and glial swelling that entails *cytotoxic edema*. While short-lasting SDs are withstood in healthy tissue, longer-lasting SDs are harmful in metabolically challenged tissue of the injured brain. In the ischemic penumbra, recurring SDs combine with compromised blood supply to increase the metabolic load, thereby expanding the initial infarct. Similarly to the peri-infarct tissue SDs occurring in the peri-contusional cortex after TBI can be detrimental and contribute to maturation of cortical lesions. The full spectrum from short- to very long-lasting SDs has been recorded in the evolution of stroke and TBI not only in animals but also in the human brain. Moreover, patients with multiple or prolonged SDs have very poor prognoses for recovery pointing to SD as the important *mechanism* in acute human brain injury. Yet, this area of research is still largely neglected. One critical step is to identify how SDs induce damage to neurons, glia and fine synaptic circuitry. Using *in vivo* two-photon laser scanning microscopy and transgenic mouse strains with intrinsic fluorescent neurons and glia we directly distinguish and quantify neuronal and glial components of cytotoxic brain edema during ischemic and traumatic injury in experimental settings relevant to clinical conditions. I will present and discuss data pointing to SD as a specific mechanism that significantly accelerates neuronal and astroglial injury in the metabolically compromised peri-lesional cortex, worsening secondary damage following stroke and TBI.

Supported by NIH NS057113 and NS062154.

History of Neurophysiology. Emphasis on Glia

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The neuronal doctrine, which shaped the development of neuroscience was born from a long-lasting struggle between reticularists (led by Camillo Golgi), who assumed internal continuity of neural networks and neuronists (championed by Santiago Ramon y Cahal), who defined the brain as a network of physically separated cellular entities, defined as neurones. Today, however, we know that integration and information processing in the brain occurs through close interactions of two cellular circuits represented by neuronal networks embedded into internally connected astroglial syncytium. Our understanding of glial function changed dramatically over last two decades. This change concerns the whole concept of how the brain is organized, and how the development, life and death of neural circuits are controlled. There is compelling evidence demonstrating that these are the astrocytes that are creating the compartmentalisation in the CNS, and these are the astrocytes that are able to integrate neurones, synapses, and brain capillaries into individual and relatively independent units. Astroglial syncytium allows intercellular communication route, which permits translocation of ions, metabolic factors and second messengers. The resulting potential for parallel processing and integration is significant and might easily be larger, but also fuzzier, than the binary coded electrical communication within the neuronal networks. The neuronal-glia circuitry endowed with distinct signalling cascades, form a "diffuse nervous net" suggested by Golgi, where millions of synapses belonging to very different neurones are integrated first into neuronal-glia-vascular units and then into more complex structures connected through glial syncytium. These many levels of integration, both morphological and functional, presented by neuronal-glia circuitry ensure the spatial and temporal multiplication of brain cognitive power.

Neuroglial cells are intimately involved in all forms of neurological diseases and this are neuroglia, which, to a very large extent, determine the progression and outcome of neuropathological process. Astrocytes are specifically involved in various neurodegenerative diseases including Alzheimer's disease, Amyotrophic lateral sclerosis, Parkinson's disease and various forms of dementia. Recent evidence suggest that early stages of neurodegenerative processes are associated with atrophy of astroglia, which causes disruptions in synaptic connectivity, disbalance in neurotransmitter homeostasis and neuronal death through increased excitotoxicity. At the later stages astrocytes became activated and contribute to neuro-inflammatory component of neurodegeneration.

Genetics of mental retardation and animal models

Patricia d'Adamo

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Intellectual Disability (ID), formerly known as Human Mental Retardation (MR), is a common disorder characterized by an IQ lower than 85. A poor cognitive ability can be the only visible symptom in non-specific ID, whereas syndromes (e.g. Down syndrome) with ID accompanied by behavioral deficits and other clinical signs. In some cases, ID may be associated with metabolic, mitochondrial or developmental disorder. Symptoms appear early in life and affect between 2-3% of the population, with devastating effects on the quality of life for patients and huge burden on healthcare systems. Family studies have demonstrated a relatively large number of X-linked forms of ID (XLID) with an incidence of about 0.9-1.4 over 1,000 males. The work of many laboratories has identified more than 100 different genes encoding proteins with a large variety of functions; i.e. chromatin remodelling, synaptic function, intracellular trafficking, etc. Although, compelling cell paradigms of dysfunction are still missing, XLID likely represents the final phenotypic outcome of a constellation of abnormal cellular processes leading to pre- and/or post-synaptic neuronal dysfunction.

One of the first genes, to be mutated in XLID patients, was GDI1. GDI1 encodes for α GDI, a protein physiologically involved in retrieving inactive, GDP bound RABs from the membrane. The identification of GDI1 as one of the genes causing human ID, suggested that vesicular traffic in neuronal cells is an important pathway for development of cognitive functions. Although the importance of α GDI in neuronal function has been demonstrated, it is unclear whether this protein has a direct impact on the role of glial cells and whether mutations in GDI1 lead to functional deficits vesicle trafficking and contribute to the aetiology of ID diseases. Abnormalities in GDI1 and other genes associated with ID may impair neuron-glial crosstalk that plays a key role in brain function, including cognition.

We propose that human ID represents not only the final phenotypic outcome of many different types of abnormal neuronal processes, but also derives from alterations in glial cell function which affects neuron-glial crosstalk and neuronal cell viability.

Section II Hands-on Experience

Instruments and titles of demonstrations:

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The participants will join the workshops regarding their log in at the conference.

Section III:

Presentations of CIPKEBIP project collaborators and CIPKEBIP researchers

Higher-order aggregates for cell signaling for cell growth

Romina Tripaldi, Marco Trerotola, Pasquale Simeone, Anna Laura Aloisi, Emanuela Guerra and Saverio Alberti
Unit of Cancer Pathology, CeSI, Foundation University 'G. d'Annunzio', and Department of Neuroscience and Imaging, BAMS, Chieti, Italy.
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High temporal and spatial resolution techniques have provided fundamental insight into cell-membrane signaling-platform organization. However, growth induction typically requires highly-multiplexed signaling events, acting over long time scales. Hence, to explore growth-signaling macromechanics, we performed multi-pronged, long-term, quantitative analyses of fluorescent proteins-signal-transducers chimeras in living cells. This led to the discovery of higher-order aggregates of cell-membrane signaling molecules. The latter were shown to be required for signaling for growth, were induced by signaling activators and disappeared with growth-factor deprivation. The discovery of higher-order membrane signaling protein aggregates in living, unperturbed cells, as transduction sites for cell growth shifts current paradigms on mechanisms of cell signaling for growth. These results may also help unraveling the mechanics of space-time transitions from nanoscopic to macroscopic signaling platforms.

1. Mechanisms and pathways of immune response, Chair: Maja Rupnik

Workpackage Mechanisms and pathways of immune response – the overview

Maja Rupnik^{1,2,3}

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This workpackage (WP) has three subareas: Generic mechanisms of immune response, Modulation of immune response by small molecules and Pathogens and their interactions with host. This talk will focus on the research within area pathogens-host interactions, while some other aspects of the WP such as bacterial PKS enzymes, immunosuppressive effect of the produced chemical compounds, crystal structures and studies of endosomal enzymes will be covered in presentations of other WPs.

Differentiation of pathogen strains by clinical manifestation – Clostridium difficile is one of the model pathogens selected for this work. *C. difficile* strains can be further differentiated into ribotypes and it is known that some ribotypes are associated with outbreaks and more severe disease. During the large outbreaks with type 027 in Slovenia we have studied some of their properties, mainly the interactions with gut microbiota. Other studied bacterial pathogens will be covered in separate talk (see abstract by Strle et al.).

Identify potential targets for diagnostics of severe infections – Unique protein targets specific for severe infection were not identified. However, we have improved detection of specific ribotypes by description of new ribotyping method which gives results in 24 h (as compared to min 72 h with previously known methods).

Reveal interaction mechanisms of specific combinations of host and pathogen factors that are prerequisite for development of severe forms of diseases – Two combination of factors were studied: gut microbiota (see abstract by Skraban et al) and Slp proteins. *C. difficile* surface layer proteins (Slp) are important in adhesion to the host cell and are also common target of host immune response during *C. difficile* infection (CDI). We have studied *C. difficile* strains with several Slp variants and their competition properties in the gnotobiotic mouse model.

Newly identified bacterial proteins may prove their potential as candidates for vaccines – Atypical cytotoxic activity of some *C. difficile* strains negative for all three known toxins (A, B, CDT) was studied and found to be associated with cell cycle arrest. However, initial characterization did not show this activity to be linked with protein fraction(s).

STUDIES OF GUT MICROBIOTA ASSOCIATED WITH *C. difficile* IN HUMANS AND ANIMALS

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Clostridium difficile is a major pathogen responsible for antibiotic-associated diarrhoea and pseudomembranous colitis in humans. Recently, its presence is often reported in animals as well. *C. difficile* infection/colonization is typically associated with disturbed gut microbiota and changes in individual bacterial taxa have been described for humans. Until now, no data were available for farm animals. We have used a simple molecular method (DHPLC - denaturing high pressure liquid chromatography) combined with machine learning tools, to identify changes in bacterial, fungal and archaeal gut microbiota associated with *C. difficile* colonisation of humans and poultry.

We have analysed 208 human faecal samples, of which 171 were routine samples (105 were *C. difficile* positive and 66 negative) and 37 were from healthy volunteers. In poultry, 143 faecal samples from a single poultry farm were collected in seven consecutive samplings. Eighty-six samples were *C. difficile* positive and 57 were *C. difficile* negative. The total DNA was isolated from the faecal samples by a standard procedure and bacterial, archaeal and fungal genes (16S rRNA or ITS2) were amplified. Faecal microbiota was analysed using DHPLC (which separates DNA amplicons based on fragment size and sequence) and different numbers of bacterial, fungal and archaeal groups were identified. To the results of this analysis, we applied statistics and the machine learning tool WEKA J48, where the resulting decision tree relates the presence or absence of different microbial groups and specific combinations thereof to the *C. difficile* status of a sample.

The microbes associated with *C. difficile* colonization were different in humans and poultry. In humans, the key predictor associated with *C. difficile* negative samples was *Bifidobacterium longum*. The presence of *Streptococcus sp.* - *Enterococcus sp.* was linked to the faecal samples colonised with the PCR ribotype 027. In poultry, the absence of *Acidaminococcus intestini* was recognised as the main predictor for the good *C. difficile* growth. In addition to these key predictors, we have identified specific microbial combinations predictive of *C. difficile* colonization in humans and poultry. Some of these patterns were specifically associated with certain *C. difficile* subgroups (ribotypes) or with the amount of *C. difficile* present.

2. Intra in inter-cellular communication response, Chair: Enej Kuščer

Promiscuity of an unusual acyltransferase domain enables incorporation of extender units with chemically amenable side chains into polyketide chains

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Polyketides, biosynthesized by the enzymes termed polyketide synthases (PKS), present a large and diverse class of bioactive natural products that includes numerous medically important compounds. PKS-derived compounds are synthesized by ordered condensation of acylthioesters, biosynthetic process similar to the synthesis of fatty acids. PKS (type I) are organized in enzymatic modules in a manner that each module catalyzes one step of polyketide chain extension. Each module consists of at least three protein domains, namely β -ketoacyl synthase, acyltransferase (AT) and acyl carrier protein, where the AT domain determines the choice of the extender unit for the relevant chain elongation step.

Specificity of the PKS modules can be exchanged entirely by AT domain replacement or their substrate specificity modified partially by the modification of key specificity-determining motifs of AT domains using site-directed mutagenesis. However, incorporated acylthioester extender units are typically limited to a small group of malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA and only very rarely an AT domain of a PKS module selects other extender units with more diverse structural characteristics, thus limiting the structural diversity of polyketide backbones. Therefore, a technology, which would enable introduction of more synthetically amenable side chains would represent an extremely powerful tool in drug development.

We have previously elucidated the promiscuous nature of the AT domain of module 4 of the FK506 PKS (AT4) which naturally incorporates allylmalonyl-CoA into the polyketide chain. We have now demonstrated that this promiscuity extends beyond the naturally present extender units to externally added synthetic extenders, such as propargylmalonyl-SNAC, enabling the incorporation of chemically amenable triple bonds into polyketide chains. In addition, the AT4 domain excludes abundantly available smaller extender units such as malonyl-CoA and methylmalonyl-CoA, giving rise to a very attractive system for generating potentially useful polyketide analogues. Taking advantage of these features of the AT4 domain we have produced a novel FK506 derivative - 21-desallyl-21-propargyl FK506. In addition, we have cloned the AT4 domain into different modules of erythromycin PKS enzymes - DEBS1 and DEBS2 - resulting in enzymatically active hybrid PKS systems.

Calcium and membrane depolarization waves in islets of Langerhans in pancreas tissue slices.

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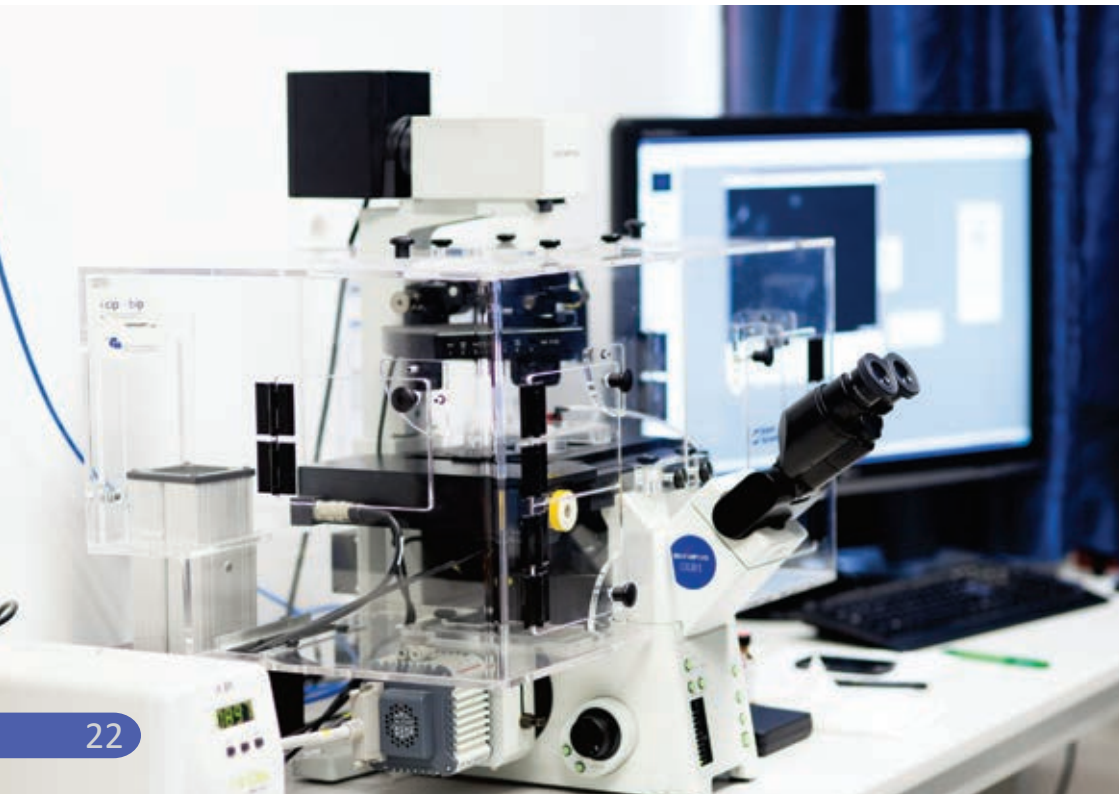
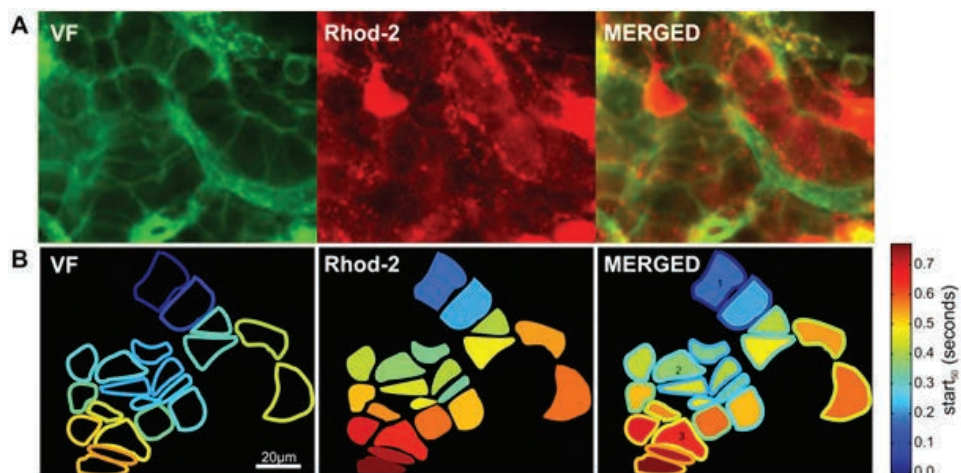
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Plasma glucose concentration drives insulin release from pancreatic beta cells. The glucose stimulus triggers an increase in cytosolic calcium, followed by regular oscillations that spread across the islets of Langerhans in the form of waves [1]. The functional connectivity within the electrically coupled functional syncytium of beta cells of the islet has network properties [2]. Glucose-dependent beta cell activation also induces changes in the electrical activity indicating tight coupling between cytosolic calcium and membrane potential changes, however the interdependence between these two parameters is not yet understood. We used confocal microscopy and specific fluorescent probes to simultaneously measure both cytosolic calcium and membrane potential changes in a large number of beta cells within intact islets in fresh pancreas tissue slices. High temporal and spatial resolution measurement of both cytosolic calcium and with the use of a novel fluorescent probe [3] also membrane potential changes, in a majority of beta cells within an optical section of an islet. We demonstrated for the first time that in addition to calcium waves the existence of membrane depolarization waves. These novel findings about the beta cell networks within the intact islets will enable us further insights into functioning of endocrine pancreas and develop novel strategies for diabetes mellitus therapy.

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Group X Secreted Phospholipase A₂ Induces Lipid Droplet Formation and Promotes the Survival of Breast Cancer Cells

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Tumour cells display progressive changes in metabolism that support their increasing demands for growth and proliferation. Changes in lipid metabolism have been recognized as an important part of the neoplastic transformation. The human group X secreted phospholipase A₂ (hGX sPLA₂) is an enzyme that releases fatty acids (FAs) from cell membranes and lipoproteins and has been recently implicated in the regulation of lipid metabolism, obesity and cancer. Here we demonstrate that hGX sPLA₂ induces lipid droplet (LD) formation (Figure) in invasive breast cancer cells, stimulates cell proliferation and prevents cell death on serum deprivation. These effects are differentially expressed in breast cancer cell lines with different tumorigenic potential and are dependent on the products of hGX sPLA₂ enzymatic activity. The central metabolic sensor AMP-dependent protein kinase (AMPK) was activated during LD formation in proliferating cells, which is consistent with the observed increase in the expression of FA oxidation enzymes and the LD-associated protein perilipin 2, and with the reciprocal decrease in the levels of major lipogenic enzymes. Inhibition of FA oxidation by etomoxir reduced both hGX-induced LD formation and cell survival, suggesting a critical role for LD lipolysis and FA oxidation for the survival of hGX-treated cells. Our results reveal a novel role for hGX sPLA₂ as a modulator of lipid metabolism that stimulates LD formation and prolongs the survival of breast cancer cells.

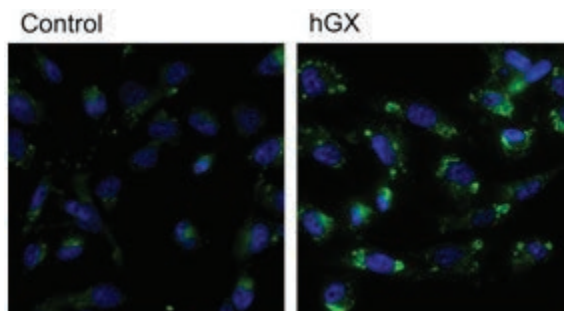


Figure: hGX induces lipid droplet formation in MDA-MB-231 breast cancer cells. After incubation of the cells with hGX sPLA₂ for 48 h, Nile red was used to visualize lipid droplets (green) and nuclei were stained with DAPI (blue).

Oligomerization and amyloid fibril formation of stefin B *in vitro* and in cells.

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Mutations in the gene of human stefin B are responsible for the primary defect underlying Unverricht-Lundborg disease, a rare progressive myoclonic epilepsy (EPM1). Stefin B functions as a cysteine proteases inhibitor, however, other physiological functions are likely.

Human stefin B is the most labile and amyloidogenic protein among the members of the family of cystatins. We have used it thus far for studies of the mechanism of amyloid fibril formation [1-5], oligomers interaction with biomimetic membranes [6-8] and interaction with other amyloidogenic peptides, such as A-beta [9]. By studying protein concentration effects on the oligomerization and fibrillation reactions as assayed by SEC and ESI MS, we have come to a new, improved model, which I will briefly present.

In the absence of stefin B (KO mice), there is increased cell apoptosis and sensitivity to oxidative stress. The protein is over-expressed in status epilepticus and after seizures, further implying its protective role; however over-expression could in turn cause its aggregation and gain in toxic function. It proved as a copper binding protein [10]. As many other amyloidogenic proteins it binds and perforates biomimetic membranes [6-7], even the wild-type were shown to bind and perforate lipid bilayers [8]. Of interest, it interacts with APP in cells and it binds A-beta in oligomer specific manner, where the tetramers bind strongest and inhibit amyloid fibril formation of A-beta [9].

Cell studies [11] have shown that abundant aggregates form in cells expressing the four EPM1 missense mutants. The aggregates cause increase in oxidative stress and are cytotoxic, dependant on the aggregate type; where scattered smaller aggregates are more toxic than the aggresome like, fibrillar perinuclear aggregates. New studies have shown that the number of intracellular aggregates increases after induction of autophagy, which suggests impairment of this cellular process and accumulation of protein aggregates in autophagosomes, especially in KO cells (astrocytes). We hypothesize that lower stability and higher propensity to aggregate of the missense EPM1 mutants of stefin B can lead to a toxic gain-in-function and to autophagy impairment. This also implies that the wild type stefin B could directly or indirectly regulate autophagy.

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N- AND C-TERMINOMIC APPROACHES FOR DISCOVERY OF SUBSTRATES OF CYSTEINE CATHEPSINS

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Cysteine cathepsins are papain-like proteases found to be upregulated in several human disease conditions including cancer (1), where they play a crucial role by exerting irreversible modifications on proteins thereby changing their structure and function. Elevated cathepsin activity is often connected with bad prognosis for cancer outcome since they are thought to promote tumour growth and metastasis (2). Stable cleavage products formed in the complex network of disease-related proteases could be considered as disease markers, yet following the proteolytic events in high complexity samples is technically challenging. Since there is an urge in the proteomic field to develop reliable yet convenient proteomic approaches for in-depth identification of substrates, we are

developing new strategies for protease cleavage site discovery employing chemical labeling of neo N- or C-termini, that could be used as a platform for tracking proteolytic events. Our strategies take advantage of combining FASP and Stage Tip (3) with in-solution labeling method for sample preparation prior to MS-analysis. Our N-terminal methods for protease cleavage site discovery are based on (D₃)-acetylation of free amino groups (4), C-terminal methods are based on carbodiimide coupling of ethanolamine (5) or aniline to free carboxyl groups. We tested our methods on myoblobin tryptic peptides to estimate labeling efficiency and select the optimal labeling strategy for our applications. Furthermore, we tested our method on lysates prepared from human breast cancer cells (MDA-MB-231) which were treated with cathepsins K, L, S or V to search for substrates and cleavage sites. The strategy enabled the identification of several substrates of cysteine cathepsins and construction of sequence logos based on the detected cleavages, that are in good agreement with the current status of knowledge on cysteine cathepsins.

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Synthesis and bioactivity of synthetic peroxides

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Organic peroxides are receiving increasing interest as bioactive compounds. Since 2001 WHO has recommended artemisinin-based combination therapies (ACT, combination of artemisinin or its derivative with another antimalarial) as first-line treatment for uncomplicated *P. falciparum* malaria. Artemisinin is a sesquiterpene trioxane lactone, containing an endoperoxide bridge essential for its bioactivity. Due to low bioavailability, semi-synthetic derivatives were developed (artemeter and artesunate are currently used in therapy). Simplification of the basic structure resulted in development of synthetic peroxides, of which the most bioactive are trioxolanes and tetraoxanes. Last year the first ACT containing synthetic peroxide (arterolane in combination with piperazine) was registered. Beside antimalarial activity, artemisinins are also antitumor, antiviral, antibacterial and antifungal agents. Due to similar mode of action of artemisinins and synthetic peroxides, the latter also may exhibit wide range of bioactivity.

1,2,4,5-Tetraoxanes are promising derivatives that have two endoperoxide groups in a structure and the unsymmetrical derivatives could be specifically designed. Symmetrical tetraoxanes could be prepared directly from carbonyl compounds, while unsymmetrical ones can only be prepared in two-step procedure with *gem*-dihydroperoxides as intermediate products. Our work focuses on chemical synthesis of structurally diverse tetraoxanes under mild and simple reaction conditions with emphasis on using hydrogen peroxide for peroxidation.¹

The antimalarial activity for several symmetrical 3,3,6,6-tetraalkyl-1,2,4,5-tetraoxanes was measured on FCB1 *Plasmodium* strains (resistant to chloroquine). Even though it is known that dispiro tetraoxanes have better bioactivity, selective introduction of alkyl chain onto the tetraoxane scaffold enables to study the effect of polarity and steric encumbrance on antimalarial activity. We observed that the antiparasitic activity of structurally related tetraoxanes was enhanced by decreasing logP value. Tetraoxanes with substituents that sterically hindered peroxide bond were several times less active.

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3. Adaptation mechanisms of extremophiles to environment response

Adaptation mechanisms of extremophiles to environment

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The extremophiles are adapted to a life at extremely harsh conditions. Its survival depends on macromolecules (proteins, nucleic acids, lipids), that unlike their mesophilic homologues retain the native structure and activity in such extreme environments. In the recent years, extremophilic microorganisms became an interesting source of hydrolytic enzymes, among which many are of an industrial importance.

In comparison with other hyperthermophilic microorganisms archaea *Aeropyrum pernix* has at least two advantages. Biotechnological use of archaea *A. pernix* might be more convenient due to its aerobic metabolism. Moreover, the living conditions with which the microorganism has to cope with probably led to evolution of macromolecules with not only unusual tolerance to extremely high temperature but also with resistance to elevated ionic strength. In addition, this archaea has a unique membrane composition. Its membrane is composed of only C25,25-archaeols, while the membranes of other species of archaea contain standard C20,20-archaeols and C40,40-caldarchaeols. Liposomes prepared from archaeal ether lipids (archaeosomes) turned out to be chemically (resistance to oxidation and hydrolysis) and physically (no fusion or aggregation of vesicles) considerably more stable than conventional liposomes.

Hypersaline waters in salterns are not populated only by halophilic archaea and bacteria. The polymorphic halotolerant black yeasts: *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimastix salinum*, *Aureobasidium pullulans* and *Cladosporium* spp. were detected with the highest frequency just before the peak of halite (NaCl) concentration. Obligate halophilic fungus *Wallersteigia ichthyophaga* was also isolated from extremely salty environments and added to the list of model organism to study molecular adaptation to high NaCl concentrations. Genomes of *H. werneckii*, *W. ichthyophaga* and four varieties of *Aureobasidium* were recently sequenced and will be presented separately. The state of the art of the molecular adaptation to low water activity will be discussed, focusing on kinetic studies on Hal2 proteins from *H. werneckii* and production of MAP kinase Hog1 from *H. werneckii*, using different expression systems. Recently, a potentially pathogenic fungus has found a home living in extreme conditions in some of the most common household

appliances. The discovery of this widespread presence of extremophilic fungi (polyextremotolerant black yeasts *Exophiala dermatitidis* and *E. Phaeomuriformis*) in some of our common household appliances suggests that these organisms have embarked on an extraordinary evolutionary process that could pose a significant risk to human health in the future.

Dishwashers as an example of the indoor extreme environment for survival and propagation of *Exophiala dermatitidis* and *E. phaeomuriformis*

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Extreme environments are not found only in nature, but are also created by human. For example, in our homes, many extreme ecological niches are generated inside of several household appliances. Such niches can be occupied by extremotolerant microorganisms, which may tolerate stress factors as high temperature, high or low pH and high concentration of salt inside the appliances.

Dishwashers are globally spread devices due to better economical standard. For example in Slovenia, 52 % of households had dishwashers in 2010. In our global study the presence of fungi in dishwashers is presented. On samples taken from dishwashers at various continents, we frequently found several human opportunistic pathogenic fungi. Among others, also black yeasts belonging to the genus *Exophiala* were isolated and identified. Different *Exophiala* species are known to cause skin infections, in East Asia also a few fatal brain infections and systemic infections have been recorded. These black yeasts are rarely detected in nature and it has been speculated that *E. dermatitidis* originates from tropical rain forest. Our findings imply that species of *Exophiala* and specifically *E. dermatitidis* are present in dishwashers and may be transferred into the dishwashers with water.

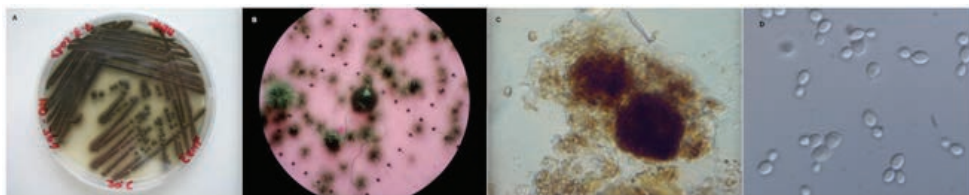


Figure: *Exophiala dermatitidis* growing on MEA medium (A), on DRBC with chloramphenicol (B), *Exophiala* spp. meristematic growth in biofilm from dishwasher (C) and yeast-like form in liquid ME medium (D)

Genomes of fungi from hypersaline environments

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Halophilic *Wallemia ichthyophaga*, extremely halotolerant *Hortaea werneckii* and halotolerant *Aureobasidium pullulans* are three fungal species that are able to thrive in environments with high concentrations of salt. They are important as models for studying the mechanisms of eukaryotic salt tolerance.

We performed *de novo* genome sequencing and analysis on all three species. The genomes vary substantially in their size (10 Mbp – 50 Mbp) and predicted number of genes (4884 – 23333). In *W. ichthyophaga*, the P-type ATPase cation transporter and the hydrophobin groups are expanded. Transcription of all but three cation transporters is salt in-dependent, despite of presumed importance of these proteins in hypersaline environments. Hydrophobins are cell-wall proteins with multiple cellular functions and in *W. ichthyophaga* they contain an unusually large number of acidic amino acids. This haloadaptation is of particular interest due to the numerous applications of these molecules (industry, pharmaceuticals, medicine).

The genome of *H. werneckii* appears to have experienced a recent whole genome duplication, and contains two highly identical gene copies for almost every protein. This is consistent with some previous studies that reported increases in genomic DNA content triggered by exposure to salt stress. Additionally, most types of cation transporters experienced several gene duplications at various points during their evolution. Consequently they are present in much higher numbers than expected. The resulting diversity of transporters presents interesting biotechnological opportunities, since some of these genes could be used to improve the halotolerance of salt-sensitive species.

For *A. pullulans* four genomes have been sequenced (one for each of the four varieties that are recognised in the species). The most recent results of the ongoing comparative transcriptomic analyses will be presented. The differences between the genomes will be discussed in light of the individual environments and conditions preferred by each variety.

The genomes of halotolerant/halophilic fungi reflect their peculiar lifestyles and reveal some shared, but also several unique traits. Availability of the genomic sequence is expected to facilitate further research into these species, and this should help to tackle some of the major problems caused by high salinity and osmotic stress in agriculture and the biofuel industry.



Figure: *Wallemia ichthyophaga* grown at 25% NaCl (left), *Hortaea werneckii* grown without NaCl (middle) and *Aureobasidium pullulans* grown without NaCl (right); all on YNB medium.

Archeosomes as vehicles for recombinant protein delivery into skin cells

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Archeosomes are a type of lipid bilayer vesicles (liposomes) that are made from extracted archaeal lipids. These have unique structural characteristics that increase the lipid bilayer's stability even under high temperatures, low or high pH, presence of phospholipases and bile salts. Consequently this has led to the development of new potential drug, gene and vaccine delivery systems.

Here we present the data obtained on large unilamellar archeosomes (400 nm size) extracted from *Aeropyrum pernix* K1, as a potential new method for drug/therapy delivery to skin cells. The core lipid of *A. pernix* consists solely of C_{25,25}-archaeol(2,3-di-sesterpanyl-*sn*-glycerol). C_{25,25}-archaeol(2,3-di-sesterpanyl-*sn*-glycerol) (AGI), with its glucosylinositol polar head-group accounts for 91 mol%, while C_{25,25}-archaeol(2,3-di-sesterpanyl-*sn*-glycerol) (AI) with its myoinositol polar head-group, accounts for 9 mol%.

The potential cytotoxic effect of archeosomes on skin cells was measured using an *in vitro* cell metabolic activity assay (Figure 1). HaCaT keratinocytes grown in culture proved unaffected by archeosomes, even when higher concentrations were used (500 µg/ml). Subsequently, large (400 nm in size) unilamellar archeosomes were prepared, which were packed with calcein as reporter dye. Calcein is a fluorescent dye that can be readily detected by spectrophotometer or under an epifluorescent microscope. Archeosomes containing calcein were simply supplemented to keratinocyte growth medium and cells were incubated for 24hrs. After 24hrs cells were fixed and examined under the microscope. Our results show that in a confluent keratinocyte monolayer a large

number of cells (30%) displayed diffuse fluorescence due to archeosome intake and calcein release (Figure 2). Using the same approach we have also packed archeosomes with recombinant human keratin 14 (K14) and succeeded in delivering the protein into SW13 (human adrenal carcinoma) cells grown in culture. SW13 cells do not normally express keratins, so a positive signal after immunofluorescent staining with monoclonal antibodies specific to K14 presents proof-of-principle for the potential use of this method in a wide range of applications (Figure 2). We are now testing different types of archeosomes as possible new tools for drug, protein and DNA delivery in a variety of cell culture *in vitro* model systems.

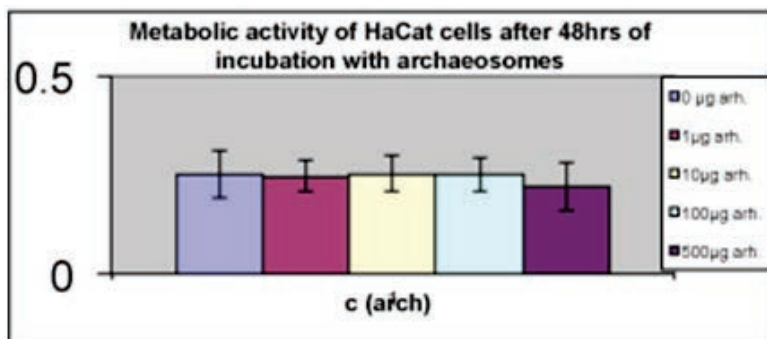


Figure 1: Archaeosomes have no cytotoxic effect on HaCat keratinocytes grown in culture.

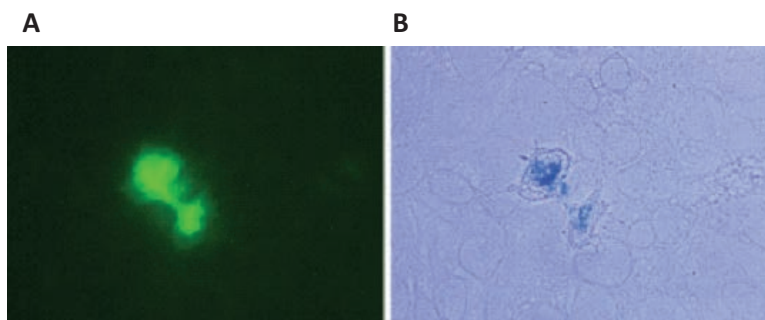


Figure 2: (A) Archaeosomes successfully delivered fluorescent calcein into HaCat keratinocytes grown in culture. (B) Archaeosomes successfully delivered recombinant human keratin 14 (K14) into SW13 cells grown in culture. K14 detection was performed by immunofluorescence, using monoclonal antibodies against K14.

4. Protein Bank: Proteins, their production, current use and perspectives, Chair: Dušan Turk

WorkPackage 4: Protein bank: Protein production, use and perspectives

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High throughput protocols for *E. coli* and insect and mammalian cell lines are established. The protein production platform setup is almost complete: static light scattering analyzer (in process of being purchased), mammalian cell line facility (purchase in preparation). Crystallization platform for automatic of crystal growth (installation in July/August).

Through the high throughput protein production and the medium throughput structure determination we have reached the low throughput stage of functional analysis of targeted proteins and their chemistry.

The following groups of proteins are investigated:

- ✧ lysosomal proteins (hydrolases and MHC class II molecules) (Marko Mihelic, Andreja Dobersek, Miha Renko, Masa Cernic, Piotr Sosnowski) The crystal structures of two human hydrolases Alpha fucosidase I and di-N-acetylhitobiase were determined. Characterization of the active site residues by mutagenesis and cocrystallization with substrates is in progress. (report by M. Renko)
- ✧ surface proteins from human pathogens
 - ✧ *S. aureus* (Marko Mihelic, Andreja Dobersek, Miha Renko) From final 27 targeted proteins on the surface of the bacteria understanding of involvement of autolysins in biofilm formation is most advanced. Collaborations with groups abroad are established. (report by M. Mihelic).
 - ✧ *C. difficile* (Sasa Usenik, Miha Renko, Gregor Pretnar, Maja Rupnik) For the 12 targeted surface proteins we are in the process of domain identification and characterization.
- ✧ proteins from extremophiles
 - ✧ *H. Wernecki* (Marko Mihelic, Metka Lenasi, Miha Renko, Ana Plemenitas) Crystal structures of Hal2a and Hal2b were determined, yet did not provide insight into the role of the specific peptide, which is assumed to be crucial for the adaption to the environment of high salt concentration. Work in progress.

- ✦ *Aeropyrum pernix* (Marko Snajder, Marko Mihelic, Narasa Poklar Urlih, Hrvoje Petkovic) (A patent application was submitted). Further developments are planned to improve the high temperature stability of pernizin expressed in *E. coli*. (Report by M. Snajder.)

CALCIUM MODULATED MECHANISM OF STRUCTURAL STABILITY OF PERNISINE

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Hyperthermophilic archaeon *Aeropyrum pernix* K1 grows optimally in a sea water at temperatures higher than 90°C. *A. pernix* secretes thermostable serine protease pernisine in an extracellular media. Thermostable proteases are potentially biotechnologically interested for chemical industry and pernisine could be also used for degradation of infective prion proteins.

Aminoacid sequence comparison of pernisine with subtilisin from *Thermococcus kodakaraensis* showed that they share most of the aminoacids residues that are involved in Ca²⁺ binding sites. There is high probability that pernisine has also seven binding sites for Ca²⁺. Using program for determining 3D model structure from homologous protein with known 3D structure, we presented 3D model with all Ca²⁺ binding sites for pernisine.

PrP^{Sc} degradation and afterwards immunodetection of remaining PrP^{Sc} showed that pernisine is capable to degradate infections prion protein aggregates from different organisms as well protein aggregates connected with Alzheimer disease at high temperatures in less than 10 minutes.

Biochemical characterization using azocasein or small chromogenic peptide substrates specificity showed Ca²⁺ dependence of pernisine at higher temperature. Activity was preserved for more than two hours at temperatures 70°C or 90°C with addition of CaCl₂, while without CaCl₂ it lost activity in 40 or 20 min, respectively. Pernisine can be exploited in different extreme reaction conditions that contain different reducents, denaturants or detergents.

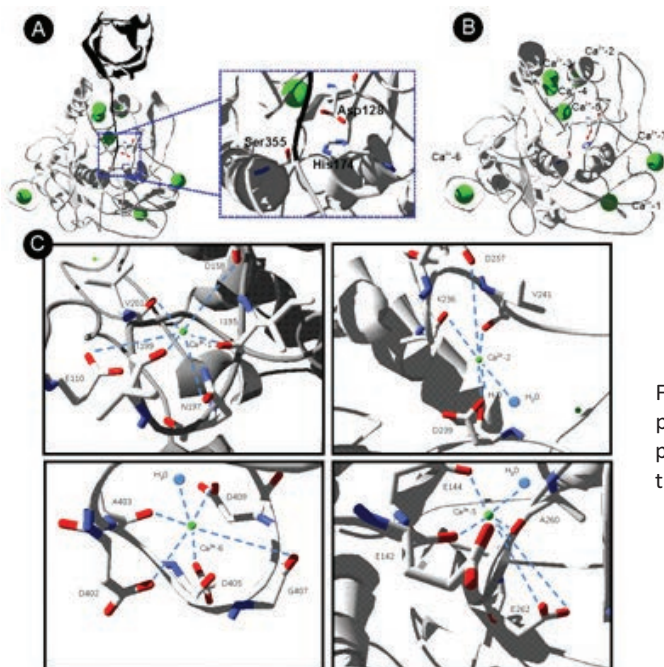


Figure 1: 3D model structure of pernisine using Geno3D program with shown catalytic triad and Ca^{2+} binding sites.

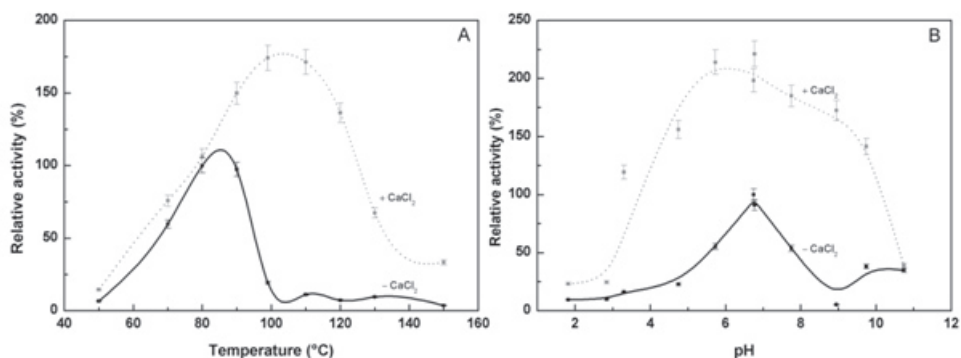
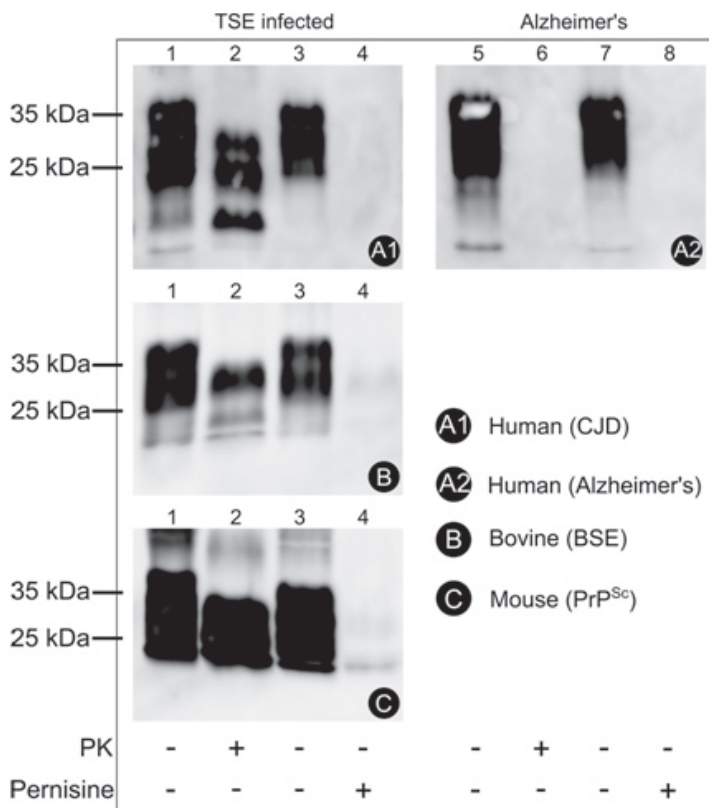


Figure 2: The results presents pernisine enzymatic activity evaluated with azocasein assay. (A) Temperature dependence of pernisine in presence (grey) and absence (black) of CaCl_2 under 50 mM Tris-HCl pH 8.0. (B) pH dependence (50 mM buffers: Glycine/HCl (pH 2 to 5), HEPES (pH 6 to 8) and Glycine/NaOH (pH 9 to 11)) of pernisine in presence (grey) and absence (black) of CaCl_2

Figure 3:
Immunodetection of
PrP fragments after
treatment with the
R30 fraction and
purified pernisine



Structural Insight into the Lysosomal Degradation of Glycans

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Degradation of the proteins in lysosomes is a random process, performed by often redundant and nonspecific proteases. In contrast, degradation of glycoconjugates appears a highly ordered and specific process which includes multiple non-redundant glycosidases. Absence of a lysosomal glycosidases due to genetic disorders cause lysosomal storage diseases (LSD). The signs of these diseases are accumulation of undegraded material in tissues and secretion of fragments into the urine. The LSD are group of approximately 50 rare inherited disorders, which usually manifest in severe mental and motor retardation and premature death.

We studied tissue α -L-fucosidase 1 (FUCA1) and di-N-acetylchitobiase (CTBS), responsible for the removal of the L-fucosyl residues from the nonreducing end of the glycoconjugates and splitting the reducing-end GlcNAc from chito oligosaccharides. Mutations in FUCA1 gene leading to inactive protein cause LSD termed fucosidosis, whereas an LSD caused by the defects in CTBS was still not identified, even though the mice lacking CTBS gene do accumulate undegraded glycoconjugates.

We have succeeded to determine the 3-dimensional structures of both enzymes and are in the process to determine the structures of these two enzymes in the complex with their substrates. This work provides the basis for understanding the mechanism of their activity and the potential roles of the enzymes in the specific degradation of fucose-containing glycans.

“The Same” Enzymes with Different Activities - Comparison of *S. aureus* Bifunctional Autolysin and SAV2307/Autolysin E Activity

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Autolysins are group of the bacterial cell-wall modifying enzymes. They play an essential role in a cell growth and division and are considered as primary candidates for development of novel strategies in combating the spread of bacterial infection. They can be divided in three broad groups: amidases, glucosaminidases and peptidases.

Several different Autolysins from *S. aureus* have already been characterized including Bifunctional Autolysin (BFAtI). It is believed that BFAtI is the predominant staphylococcal Autolysin. It consists of two enzymatic domains, one with the amidase and the other one with the glucosaminidase activity. The silencing of the BFAtI gene causes severe defects in the cell growth and division. Sequence analysis of various *S. aureus* strains, however, showed presence of at least 4 additional genes with the high degree of sequence homology (50 %) to glucosaminidase domain of BFAtI. The activity and function of these sequences, which are present as single domain proteins or as part of multidomain proteins, is still unclear.

To examine the activity of *S. aureus* glucosaminidases, we have developed a system for production of recombinant glucosaminidase domains and adopted protocols for assessment of their activity. Comparison of the activity of BFAtI glucosaminidase domain and SAV2307/Autolysin E showed striking differences - not only at the level of protein stability and activity, but also on the ability of the living *S. aureus* cells to form biofilms. Our data suggest that these high-sequence gene homologues do not play redundant roles, but are more likely involved in specialized cellular processes.

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Guest Editors

FEMS Microbiology Ecology: Special Thematic Issue on Polar and Alpine Microbiology
Editors: **Nina Gunde-Cimerman**, Dirk Wagner and Max M. Häggblom

Patent

1. Authors: Robert Zorec, Matjaž Stenovec, Saša Trkov, Nina Vardjan, Maja Potokar, Marko Kreft, Mateja Gabrijel, Jernej Jorgačevski; Patent application no. PCT/EP 2012/001759, Title: Screening methods based on vesicle mobility; Involved institutions: University of Ljubljana, Kongresni trg 12, Ljubljana, Celica d.o.o., Technology park 24, Ljubljana; CIPKEBIP Centre of excellence for integrated approaches in chemistry and biology of proteins, Jamova cesta 39, Ljubljana; Short explanation: The present invention relates to methods and systems for screening for pharmaceutically active substances. The invention also relates to methods, systems and a reference compound for studying subcellular organelle traffic and related disease states.
2. Authors: Marko Šnajder, Marko Mihelič, Dušan Turk, Nataša Poklar Ulrih; Patent application no. ; Title: The expression of recombinant pernisine (serine protease) using *E. coli*; Involved institutions: University of Ljubljana (Biotechnical faculty), Kongresni trg 12, Ljubljana, CIPKEBIP Centre of excellence for integrated approaches in chemistry and biology of proteins, Jamova cesta 39, Ljubljana, Jožef Stefan Institute, Jamova cesta 39, Ljubljana; Short explanation: The

gene Ape263.1 for pernisine in *E. coli* was successfully expressed. The histidine tag for easier detection and purification of pernisine was added. Codon optimization of Ape263.1 on a host translation system *E. coli* was crucial for the expression of pernisine. Activity determination was confirmed with zymography. Pernisine mutation of a proposed catalytic site S355A completely loss activity.



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