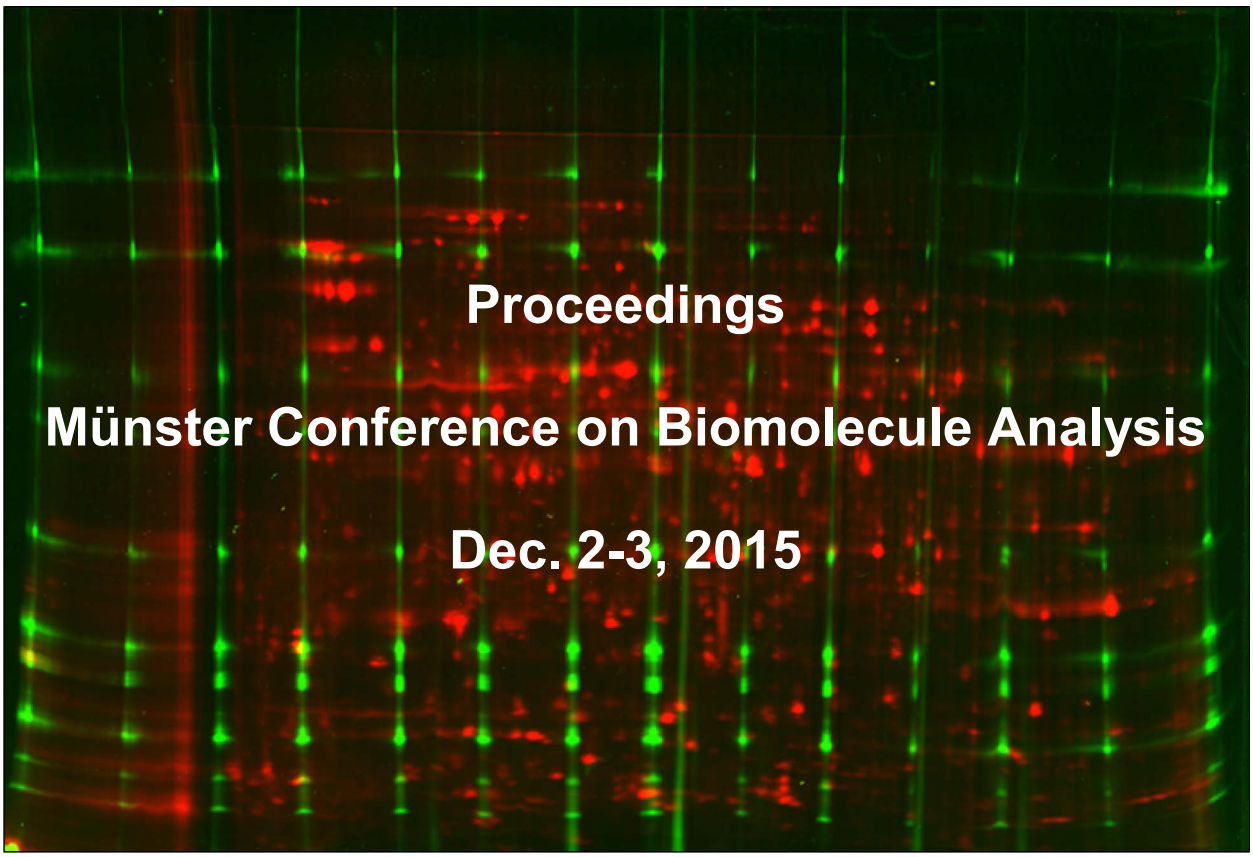

Proceedings

Münster Conference on Biomolecule Analysis

Dec. 2-3, 2015

Organized by the Core Unit Proteomics
of the Interdisciplinary Center for Clinical Research Münster
Prof. Simone König & Dr. Rita Naskar
and SERVA Electrophoresis GmbH
Günter Theßeling

Content

Preface

The Münster Conference on Biomolecule Analysis 2015 was the second with a focus on proteomics. Already in 2014, the Core Unit Proteomics (CUP) of the Interdisciplinary Center for Clinical Research Münster had organized the event as the eleventh of an annual series of bioanalytical conferences. As a result of the award of the Transfer Prize 2014 of the University of Münster to CUP and SERVA Electrophoresis GmbH, the company had joined the organizing team. Together, CUP and SERVA assembled a program with high-ranking speakers from the protein analysis field and workshops covering basic methodologies. The conference also provided a platform for companies to showcase their products. The international event successfully met the increasing interest in proteomic technologies and provided a valuable information source in particular for Ph.D. students.

Münster, Dec. 7, 2015
Simone König

Keynote Lecture

Quantitative measurement of proteins and proteomes by targeted mass spectrometry: Reproducibility matters

Ruedi Aebersold

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The reproducibility (or lack thereof) of results in experimental biology is increasingly moving into the focus of journals, funding and regulatory bodies. The basis for generating reproducible results is reproducible and accurate measurements.

In proteomics the requirements for reproducible and accurate measurements of peptides and modified peptides are fulfilled by targeted proteomics methods, which were named Method of the Year 2013 by the journal *Nature Methods*. Targeted proteomics is exemplified by selected reaction monitoring (SRM), the massively parallel SWATH-MS method and by measurements based on affinity reagents. SRM is the gold standard quantitative mass spectrometry technique in terms of accuracy, dynamic range and limit of detection, but it is limited to the quantification of ca. 100 proteins per injection. SWATH-MS approaches the performance characteristics of SRM but offers a much higher degree of multiplexing to a level of several thousand proteins per injection. In essence, SWATH-MS converts all physical specimens in a sample, in the case of proteomics the proteolytic peptides of a protein extract, into a digital file that can be perpetually interrogated for the presence and quantity of any protein that is in the detection range of the system. The thus generated complete and permanent fragment ion records are then, in a second step, queried for the presence and quantity of specific peptides, using spectral libraries as prior information.

In this presentation, I will discuss the principles and performance of these targeting mass spectrometry methods and illustrate their performance across laboratories from controlled multi-laboratory studies. I will also indicate the utility with selected examples of biological or clinical research projects.

Orals

Dissecting the cell cycle control in fission yeast by quantitative phosphoproteomics

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To quantify cell cycle-dependent fluctuations on a proteome-wide scale, we performed integrative analysis of the proteome and phosphoproteome during the four major phases of the cell cycle in *Saccharamyces pombe*. In highly synchronized cells, we identified 3,753 proteins and 3,682 phosphorylation events and relatively quantified 65 % of the data across all phases. Quantitative changes during the cell cycle were infrequent and weak in the proteome, but prominent in the phosphoproteome. Protein phosphorylation peaked in mitosis, where the median phosphorylation site occupancy was 44 %, about two-fold higher than in other phases. We measured copy numbers of 3,178 proteins, which together with phosphorylation site stoichiometry enabled us to estimate the absolute amount of protein-bound phosphate, as well as its change across the cell cycle.

In a related study, we applied site-specific quantitative phosphoproteomics in combination with chemical inhibition to identify mitotic substrates of Aurora kinase, essential for the proper execution of mitosis in eukaryotes. We detected 8,000 phosphorylation events, of which almost 6,000 were assigned to a specific residue and 220 were downregulated upon inhibitor treatment. After controlling for unspecific effects of the inhibitor, 70 sites (on 42 proteins) were classified as highly probable Aurora kinase targets. This unprecedented number of detected *in vivo* Aurora substrate candidates in a single organism allowed us to re-define the consensus sequence for phosphorylation by Aurora. Among the substrate candidates are several known targets of Aurora, validating the approach. Importantly, the most are newly detected Aurora substrates and their involvement in diverse aspects of chromatin dynamics suggests that, in addition to its established role, Aurora influences genome architecture and function during mitosis.

Finally, we observed that fission yeast cannot be labeled with a specific form of arginine, $^{13}C_6{}^{15}N_4$-arginine (Arg-10), which limits the exploitation of SILAC technology in this model organism. We hypothesized that in the fission yeast the guanidinium group of $^{13}C_6{}^{15}N_4$-arginine is catabolized by arginase and urease activity to $^{15}N_1$-labeled ammonia that is used as a precursor for general amino acid biosynthesis. We showed that disruption of Ni^{2+}-dependent urease activity, through deletion of the sole Ni^{2+} transporter Nic1, blocks this re-cycling in ammonium-supplemented EMMG medium to enable $^{13}C_6{}^{15}N_4$-arginine labeling for SILAC strategies in *S. pombe*. We are currently using the Nic1 strain to apply classical SILAC labeling and study other cell cycle relevant kinases and phosphatases in *S. pombe*.

A day in the life of a 'bioinformatics for proteomics' researcher

Martin Eisenacher

Medical Bioinformatics, Medical Proteome Center, Ruhr University Bochum, Germany

This talk elucidates aspects of the daily work in the research area Medical Bioinformatics of the Medical Proteome Center. It also presents some concepts and tools linking the applied Proteomics performed with gels / mass spectrometry and the theoretical aspects of bioinformatics / biostatistics. Besides infrastructural aspects (such as the virtual server system, and archiving and backup solutions) the talk will present some of our scientific methods, algorithms, evaluation workflows and software solutions for a statistically valid interpretation of proteomics data.

Topics are for example the decoy approach for the estimation of the false-discovery-rate (FDR) in spectrum identification, protein inference / protein ambiguity, protein list comparison, cross-platform comparison of OMICS data (mRNA <-> protein quantities), and biobanking databases (with data privacy aspects). Additionally biostatistics methods are summarized for the evaluation of the generated data using univariate (t-test) and multivariate methods (hierarchical clustering, PCA). Furthermore a machine learning method is described used for feature selection and sample classification (random forests and support vector machines). Furthermore we are very active in the development of standards for the description and storage of proteomics data and conversion of data and results into these formats.

All these activities will be available for the proteomics community in a BMBF-funded "German Network for Bioinformatics Infrastructure (de.NBI)" in a "Bioinformatics for Proteomics" infrastructure centre located in Bochum and Dortmund.

Quantitative proteomics at a global scale – methods and applications in bacteria

Dörte Becher

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Department of Microbial Proteomics

During the last decade, a rapid development of methods and techniques can be observed in the field of proteomics. Because of their low complexity, bacteria are extremely attractive model systems to test such newly developed methods. Despite the fact that 2D-gel based proteomics is still a useful approach to gain a global view of bacterial physiology, many proteins escape detection by gel-based proteomics. Thus, it is not surprising that especially mass spectrometry based proteomics had a great impact on bacterial proteomics. Through the parallel implementation of gel based and gel free approaches the maximal potential of recent technologies can be utilized. Newly developed methods are used to enlarge the coverage of accessible proteins. Analysing the single subproteomes from the cytosol, via the membrane proteins up to the surface associated and extracellular proteins we could show that almost the entire set of proteins of a model bacterium can be identified and even quantified by a combination of these techniques. The application of new MS-based analysis methods like SWATH allows for higher throughput and enables the analysis of pan proteomes as shown for different *Staphylococcus aureus* strains.

Host genetics influence on virulence of E.coli pathogens

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Microbial resistance to antibiotics is currently one of our major threats to human health, and is a direct consequence of massive use of antibiotics in livestock production. Treating *Escherichia coli* diarrhoea in weaning pigs is by far the single largest consumer of global antibiotics, and resistant *E. coli* is common in both humans and animals.

A natural gene variant in the porcine FUT1 gene protects pigs from *E. coli* F18 strains, and provides a unique opportunity to study host pathogen interactions that rule *E. coli* virulence. This talk will present our studies of how variants of the porcine FUT1 gene influence health, growth, bacterial communities and glycan structures in the pig gut epithelium. We found that FUT1 gene variants influence normal colonization of young piglets gut, and influence the glycan structures that are essential for adhesion of both pathogens and commensal bacteria in the pig gut.

Our research is directly aimed to reduce the need for antibiotics in pig production, but the knowledge we create is essential also for developing pig models that are useful for human biomedical research. Pig models that allow studies of metagenomics, nutrigenomics and host-pathogen interactions is gaining popularity because of the close physiological and genetic similarities between human an pig. This talk will present some relevant pig model models we have studied in the recent years.

MS analysis of 'difficult' protein modifications

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Tandem mass spectrometry (MS/MS) has become the method of choice for analysis of post-translational modifications (PTMs) of peptides or proteins because of the high sensitivity and high-throughput capabilities when coupled to nanoliquid chromatography. However, the most commonly used collision-based fragmentation techniques CID and HCD, which have been applied successfully in many studies, are often accompanied by a partial cleavage or even complete elimination of the side-chain modification, thus preventing the unambiguous assignment of the modification site, for example in glyco or phosphoproteomic projects. This complication is even more severe in the case of the more labile modifications such as N-phosphorylation of arginine.

For the analysis of labile protein modifications, radical-driven fragmentation techniques such as electron capture dissociation (ECD) and electron-transfer dissociation (ETD) were found to be more useful than CID due to reduced neutral losses. Based on new synthetic methods for the synthesis of site-specifically phosphorylated peptides, we present a study on the application of ETD for analysis of phospholysine (pLys) and phosphocysteine (pCys) peptides. We demonstrate that ETD tandem mass spectrometry can be used for unambiguous assignment of phosphorylated lysine and cysteine residues within proteins and that these uncommon protein modifications can be detected in cell lysates using a bottom-up proteomic approach.

Archaeoproteomics

Anna Shevchenko and Andrej Shevchenko

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Organic materials are commonly encountered during archaeological excavations. They are often attributed to foods, illuminants, pigments, etc., but this assignment mostly relies on circumstantial archeological and ethnographic evidence, rather than direct analytical determinations. Organic materials originate from diverse organisms, heavily contaminated with microorganisms (like growing mold) and degraded by intentional processing (cooking, fermentation) or natural aging. Not surprisingly, current analytical technologies mostly rely upon comparative profiling or isotopic analyses of most abundant and chemically stable fatty acids or triacylglycerols. We hypothesized that, in contrast to common lipids, proteins are information-rich molecules: their identification may directly elucidate the compositional origin and possible functional significance of ancient materials. Furthermore, protein sequences may bear hallmarks of sample processing practices, such as specific fermentation. Using SDS extraction and GeLC-MS/MS, from a few milligrams of organic masses we were able to identify hundreds of individual proteins from a large palette of organisms from bacteria and fungi to agricultural plants and cattle. The analyses determined the composition and established manufacturing recipes of Bronze Age dairy and bakery products, such as kefir cheese found on the mummies of Xiaohe [1] or sourdough bread from Subeixi [2]. We argue that the characterization of proteomes of ancient specimen is an important and accessible resource complementing already established archaeometric methodologies.

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Posters

Quantitative bioimaging of platinum *via* on-line isotopic dilution-laser ablation-inductively coupled plasma-mass spectrometry

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Platinum-based anti-cancer drugs play an important role in modern chemotherapy. Cisplatin is the most commonly applied compound due to its unmatched curing chances for certain types of cancer like testicular, ovarian, bladder and lung cancer.

To understand the mechanism of action and metabolism of these pharmaceuticals, the development of novel analytical techniques is essential and involves research on both speciation analysis and elemental bioimaging. The visualization of elemental distributions in biological tissues may provide valuable information about biological and medical correlations, thus the need for spatially resolved and quantitative information is increasing. Often, concentrations in the trace range are encountered and therefore, sensitive and reliable quantification methods are required. Regarding these demands, one of the most promising analytical methods is laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). It features high sensitivity, robustness, spatially resolved and multi-elemental analysis and enables quantification over a broad concentration range.

Quantitative analysis using imaging mass spectrometry often is impractical as there is a lack of certified reference materials (CRM) and in some cases; severe matrix effects make reliable quantification impossible. The most common quantification strategy is external calibration, which includes matrix matching of the standards. However, the preparation of matrix-matched standards is laborious and sometimes homogeneous distribution of the spiked element and similar behavior of the standard tissue and the specimen is difficult to achieve. Isotopic dilution analysis (IDA) is an absolute quantification method, which is generally traceable to SI units and less affected by drift effects and statistical errors compared to external calibration. The main principle of isotopic dilution is the change of the natural isotopic pattern of the sample by a known quantity of an isotopically enriched spike. Through subsequent measurement of a specific isotope ratio ($^{194}Pt/^{195}Pt$), the original concentration of the sample can be determined.

Within the scope of this work, the combination of *on*-line IDA and bioimaging based on LA-ICP-MS shall be presented. Therefore, the method was applied to rat kidney samples from Cisplatin perfusion experiments. The results show a high accordance with the conventional external calibration approach and demonstrate the applicability of the developed *on*-line IDA method for elemental bioimaging.

Investigating the interaction between implant metals and proteins – Speciation analysis by means of liquid chromatography coupled to elemental and molecular mass spectrometry

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The implantation of hip joint endoprosthesis has developed to one of the most important routine procedures in the field of medical surgery in the 20th century. Worldwide, approximately one million patients undergo a total hip arthroplasty annually, as such surgeries are conducted more and more frequently due to an aging society. The materials used for implants are required to ensure a good integrity and robustness of the artificial components within the environment of the biological tissue.

Beside *ceramic-on-ceramic* (CoC) and *metal-on-polyethylene* (MoP) tribological pairings, *metal-on-metal* (MoM) hip endoprosthesis based on cobalt/chromium/molybdenum (CoCrMo) are frequently used. These materials are associated with excellent mechanical properties as well as adequate corrosion resilience and friction resistance. Nevertheless, in some cases, high concentrations of the alloy constituents in blood and urine of patients with CoCrMo-hip implants occur because of abrasion effects due to friction and corrosion. Here, nanoparticles may be generated and distributed by means of blood proteins within the body resulting in high concentrations in liver, kidney, heart or lymph nodes. In addition to local effects like osteolysis, inflammation, pain or pseudotumors, systemic side effects such as cardiomyopathy or damages of the nervous system ending in deafness and blindness can appear in rare cases.

In this work, the interactions between cobalt and human serum albumin (HSA) and hemoglobin are investigated in order to gain deeper insight into toxicological mechanisms associated with metals originating from implants. Simulated samples as well as real samples of an implant patient were analyzed. Speciation analysis by means of high-performance liquid chromatography (HPLC) and inductively-coupled plasma mass spectrometry (ICP-MS) as well as electrospray ionization high resolution mass spectrometry (ESI-HRMS) has evolved as method of choice. First, the bioavailability and the uptake of respective metals has been studied by means of ICP-MS revealing high concentrations of cobalt of approximately 700 ppb in blood samples of a patient with a CoCrMo-hip implant showing high wear of friction. Present metal-protein species were separated and detected using HPLC-ICP-MS. Subsequently, the found species were further investigated using HPLC-ESI-HRMS. Based on high mass resolution and mass accuracy, different protein-metal complexes were observed and characterized.

Accurate determination of cellular protein copy numbers for a membrane-localized signal transduction network

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Systems biology interpretation requires accurate quantification of cellular components. Achieving this goal for integral membrane proteins poses additional challenges, such as selecting of an adequate standard for protein quantification. Here we describe for sperm from the sea urchin *A. punctulata,* from cell lysis to MRM-based peptide quantification with a focus on a PSAQ membrane protein standard, how recovery can be quantified and high accuracy can be achieved in each sample preparation step.

Determination of sperm cell number and lysis efficiency were determined with a Neubauer chamber and a CASY cell counter. Sample recovery upon SDS-PAGE and tryptic protein digestion was assessed by means of introducing a stable isotope-labeled guanylate cyclase (GC) and external standard proteins. GC, standard proteins (e.g. ovalbumin), and the cyclic-nucleotide-gated channel (CNGK) were quantified with AQUA peptides, using RP-LC separation and MRM analysis on a TSQ vantage, and Skyline software for quantification.

It was found that cell counting with both methods did not result in significant differences. Quantification of GC with PSAQ in comparison to AQUA peptides resulted in higher accuracy, since copy numbers were underestimated by a factor of about four with AQUA. Comparing quantification results for each peptide, usage of PSAQ delivered higher precision, reflected in a smaller standard deviation, too. It was found that the GC receptor covers about 15% of the sperm flagellar surface. An important finding for interpreting the signal transduction mechanism was the about 20fold excess of GC over CNGK, which excludes the tight association of both proteins in a signalosome complex. Reliable cell-counting methods and addition of a PSAQ standard early in the experimental workflow are mandatory for accurate quantification of cellular (membrane) protein copy numbers.

Protein biochemical characterization of human neuromelanin granules in the context of dementia with Lewy bodies

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Dementia with Lewy bodies (DLB) is an atypical parkinsonism, which is the second most common cause of dementia worldwide. The prevalence of this disease is 0.7% among the population older than 65 years. Together with Parkinson's disease (PD) and Multisystem Atrophy (MSA) DLB is classified as a synucleinopathy due to the presence of Lewy bodies. Among other characteristics a loss of dopaminergic neurons containing neuromelanin (NM) in the *substantia nigra pars compacta* (SNc) during the course of DLB is found. NM is a dark, insoluble pigment, which forms so called NM granules together with lipids and proteins. The question whether those granules are neuroprotective or neurodegenerative is widely debated since there is evidence for both sides. A protein biochemical characterization of NM granules could allow deeper insight in the function of these granules in the pathogenesis of DLB. Hence, neuromelanin granules from *post mortem* SNc tissue of DLB patients and control patients without any indications for a neurodegenerative disease were donor-specifically enriched via laser microdissection and analyzed by mass spectrometry. A pilot study with four DLB and five control cases revealed 536 proteins to be considered as a protein biochemical essential composition of the NM granules while 116 proteins were identified as significantly differential. To affirm the results and increase the statistical significance the study will be extended by 18 DLB cases.

Preparation of semisynthetic macrocycles using split inteins

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Cyclic peptides are best representatives of peptide-based drugs because of their unique properties like stability towards pH change, heat denaturation, protease degradation as compared to their linear counterparts. The cyclic backbone also has less conformational flexibility and small entropy which increase their binding affinity towards the peptide or protein interface [1]. The demand of using semisynthetic macrocycles for therapeutic applications is getting more attention as it can give access to diverse functionalities and unnatural amino acids through the synthetic part. Here in, we have developed a novel method to make semisynthetic macrocycles which is accessible to any unnatural amino acid or functionality. We make use of protein trans-splicing followed by bioorthogonal oxime ligation to join two ends of synthetic and recombinant parts of the macrocycle. The synthetic part is accessible through solid phase peptide synthesis whereas the recombinant part is expressed in *E. coli.* Therefore, the semisynthetic macrocyclic backbone can simultaneously be randomized recombinantly along with any therapeutically important non-natural synthetic moiety. We have shown the synthesis of not only a number of macrocycles of different ring sizes, but also of a number of semisynthetic lipopeptides that represent structural analogues of the nonribosomal lipopeptide antibiotic daptomycin [2]. This method promises a powerful tool for generation and selections of wide varieties of semisynthetic macrocycles of therapeutic importance.

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Laser microdissection and differential proteomic profiling of neuronal subpopulations associated with neurodegenerative diseases

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Specific subpopulations of neurons are affected by neurodegenerative diseases (NDDs), but the particular reason of this selective vulnerability remains elusive[1]. The entirety of proteins represents a prominent group of compounds with specific biochemical activities which may be somehow affected in disease both origin and progression. Ubiquitously altered proportions in protein expression patterns hold the potential of revealing biomarkers for early diagnosis, improving therapy and drug development to the level of treatment instead of symptomatic medication. Hence, sophisticated methods for the sensitive, specific and robust characterization of thousands of proteins in a complex biological matrix is necessary and needs to contemplate relative quantities as well as post translational modifications. Mass spectrometry (MS) has become the instrumental gold standard for this challenging task. However, investigating the neuroproteome remained elusive to a certain extend since traditional methods fail to provide a steady separation of different cell types from neuronal tissue. Concealing the important differences between the relevant and remaining cell types, the complex biological matrix needs to be unraveled. Recent advances in technology hold the promise of bypassing costly artificial systems (e.g. cell culture) offering a direct insight into molecular homeostasis pathways at cellular level. Using tissue sections from human or animal models leads to more meaningful results because the systemic influences of different cell types within a heterogeneous tissue are not neglected. The emerging technique of laser microdissection (LMD) proves to be a powerful tool in neuroscience combined with high resolution mass spectrometry and is thus likely to reveal novel biomarkers[2]. Using a laser, distinct single cells can be dissected from the heterogeneous neuronal tissue and isolated in a contact free manner. Isolating the neuronal subpopulations from mouse brain associated with Alzheimer´s disease (AD), Parkinson´s disease (PD), Huntington´s disease (HD) and cerebellar disorders we want to expand the common understanding of mechanisms shared and not shared by these diseases. Using liquid chromatography (LC) coupled tandem mass spectrometry (MS/MS) we pursue the relative quantification of proteomic differences between neuronal subpopulations. Focusing on the proteostasis which could make the NDDs selective to certain neurons, we seek to elucidate divergent signaling pathways contributing to the comprehension of selective neuronal vulnerability.

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Dynamics of osteoblasts and polystyrene beads on an agarose-coated culture dish

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New therapeutic approaches for bone regeneration require a comprehensive understanding of the cellular and tissue level mechanisms that underlie bone healing. With respect to the task of tissue engineering, scaffold-free techniques promise significant advantages. Experiments with osteoblast-like cells indicate that in the absence of an anchoring material, the cells form spheroid micro-masses. While it is assumed that this behavior is controlled by intercellular signaling, the influence of gravity and the topography of the substrate remain unclear.

In order to investigate these factors the dynamic behavior of vital osteoblasts (from periosteal layer of calf metacarpus) was compared with that of osteoblasts which had been devitalized with formalin and with that of polystyrene (PS) beads. Additionally, two different surface topographies of the agarose substrate were used, one which was approximately plane and one with a pronounced concave shape. The motion of the cells and beads was recorded optically and analyzed with software which has been developed by our group (Quantitative Analysis of Dynamic Behavior of Osteoblasts during in vitro Formation of Micro-Mass Cell Cultures. *Journal of Biophotonics.* Aug 2013, Vol. 6, 8, pp. 637-44). It uses an image correlation technique for tracking image structures and provides statistical information on the observed dynamics.

The evaluation of the dynamics of non-vital cells and PS beads on concave and flat substrate surfaces revealed that there is a detectable gravitational influence, depending on the shape of the substrate surface. However, it is small when compared with the observed motions of vital cells, which thus can be attributed to intercellular signals.