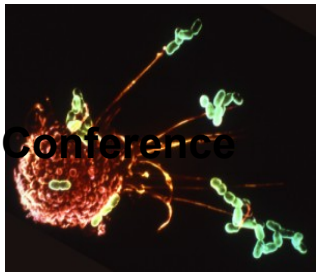

Downscaling Analysis: 3rd Münster Conference on

Single Cell and Molecule Technologies

December 4-5, 2006

Organizers: Interdisciplinary Center for Clinical Research, Core Facility Integrated Functional Genomics, Münster, Germany
Kurt Sieberns, Rita Naskar, Simone König

Chairs: Franz Hillenkamp, Stephan Ludwig, Michael Schäfers, Frank-Ulrich Müller, Simone König

Speaker Abstracts

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„Techniques for probing the brain's chemistry one cell at a time"

Understanding the functioning of networks of neurons is hampered by a lack of knowledge of the full complement of neuro-transmitters and neuromodulatory compounds used in most neuronal systems. As neurotransmitters range from gaseous molecules such as NO to large peptides that are only bioactive with particular posttranslational modifications, a variety of capillary separations and sample limited mass spectrometric approaches have been developed. Several capillary electrophoretic (CE) and capillary liquid chromatographic (cLC) methods allow neurotransmitters to be measured at individual neurons and neuronal release

sites. Mass spectrometric approaches ranging from single cell mass spectrometry (MS), single bead solid phase extraction and MS-based imaging are described.

Besides new technology, several applications of single cell separations are highlighted: novel indolamine neurochemistry, determining the role of d-Asp in the brain, and single cell peptide measurements. Specifically, new serotonin-related compounds and literally hundreds of new neuropeptides have been characterized in well-defined neuronal networks, and in several cases, the functional roles of these molecules described.

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"Fire and ice – First results from the space mission Stardust"

Comets are probably the most primitive objects in our solar system. Since the formation of the solar system some 4.6 billion years ago, these objects spent most of their lifetime in the outer, cold regions of the solar system, in the Kuiper belt or in the Oort cloud. Due to gravita-tional perturbations, they eventually get into the inner regions of the solar system, where they form remarkable tails through the interaction with solar wind and solar photons.
Although comets are believed to be ideally suited to study the early stages of our solar system and its origin, only little was known about these celestial bodies prior to NASA's space mission Stardust, the first mission to return solid samples from a specific astronomical body other than the moon.
Stardust returned its precious freight to Earth on January 15, 2006, after almost seven years of travel in interplanetary space. During the passage through the coma of comet Wild 2 in January 2004, Stardust collected cometary dust at a velocity of 6.12 km/s. During the cruise phase before the cometary encounter, a sample of contemporary interstellar dust was also col-lected. The primary collection medium for both cometary and interstellar dust was low-density silica aerogel, arranged in 132 individual cells with a total area of 1039 cm2 for each collection. In addition, ~153 cm2 of aluminum foil was exposed to each dust source. Aerogel, a solid with

extremely low densities of 5–50 mg/cm3 was used to gently decelerate the im-pinging dust grains, while particles cannot survive unaltered an impact at ~6 km/s on Al foil, but generate small craters. However, in most cases, residual material can be found associated with the craters that allow an identification of the respective impactors.

After Stardust's return to Earth and recovery of the capsule in the Utah desert, different types of samples, entire particle tracks in aerogel, individual particles extracted from aerogel, or impact craters on Al foil, were distributed to selected scientists all over the world. During a so-called preliminary examination phase, lasting for six months, these scientists addressed some of the key questions relating to the elemental, isotopic, organic, and mineralogical com-position, spectroscopic characteristics, and size distribution of Wild 2 dust.

First results showed that during formation of Wild 2 in the cold regions of the solar sys-tem, high-temperature minerals, which probably formed close to the early sun, were incorpo-rated. Such large-scale mixing in the early solar system was not expected before.

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"Cytomics and the resolution of organismal biocomplexity"

The high biocomplexity of organisms is caused to a significant degree by the functional specialization of cells as well as by the organisation of various cell types in form of tissues and organs.

Molecular disregulations in cells or cellular systems may cause disease in humans, prompting for the characterisation of molecular pathways for example during cell proliferation, differentiation, signaling or apoptosis in a *bottom-up* direction that is from the genome over the proteome up to the cell system and organism levels to find the molecular causes of a disease. Molecular pathways can be mathematically modeled in a *hypothesis-driven* way. The number of components in the model can be gradually increased or model cell systems can be perturbed, followed by molecular network analysis as in *systems biology*. The bottom-up approach is complex and it is uncertain whether clinically useful biomarker patterns can be

obtained to predict therapy outcome in individual patients (*personalised*, *individualised medicine*) in a foreseeable time.

Alternatively, available molecular patient data can be used *top-down* in a comparatively *hypothesis-free, data-driven* way (*inductive discovery* science). Data may be obtained for example by the *differential* analysis of molecular cell phenotypes as obtained by the analysis of multitudes of single cells by multiparameter flow or image cytometry followed by bioinformatic knowledge extraction from the multiparametric data (*cytomics*). Since diseases are caused by molecular aberration in cells as the elementary function units of organisms, *molecular cell phenotypes* of diseased or disease associated cells in affected body compartments correlate with the molecular disease processes as consequence of the sum of *genotypic* disposition and *exposure of the organism t*o disease inducing conditions.

Differential cell phenotype analysis may concern therapy response versus non-response, disease progression versus stationary state or survivor versus non-survivor patients. Additional patient specific clinical chemistry or clinical parameters can be included into the differential cell analysis data to initially enrich the discriminatory parameters from the highest possible number of molecular parameters. Discriminatory parameters are enriched as standardised and hospital independent *biomarker profiles* for diagnosis or *therapy dependent predictions* about disease progression of individual patients in stratified patient groups (*predictive medicine by cytomics*) while non-discriminatory parameters are eliminated by *algorithmic* "data sieving". Such biomarker profiles may serve as starting points for retrograde analysis that is *molecular reverse engineering* of underlying disease pathways by systems biology (-> *drug discovery*).

No detailed *a-priori* knowledge on specific disease processes is initially required for the analysis of the entire observable cellular heterogeneity of affected tissues downwards to the organelle and molecular network levels. Due to its mainly *inductive* nature, the cytometric approach may open so far *hidden* knowledge spaces, *inaccessible* to traditional *deductive hypothesis*. The systematic analysis of bioparameter profiles may furthermore permit to discriminate between preferentially genotypic or exposure induced disease at the individual patient level, thus facilitating early *preventive* measures in case of mainly exposure induced disease. The validity of this approach will be shown for two clinical multicenter leukemia studies.

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"Combined cell sorting and few cell gene expression profiling for the characterisation of GABAergic interneurons and neural precursors"

Gamma-aminobutyric acid (GABA)ergic neurons play a crucial role in information processing as they regulate the activity of other neurons in the central nervous system. Accordingly, the massive loss of GABAergic neurons is central to neurodegenerative diseases like Huntington disease. To extend the knowledge about GABAergic neurons and neural precursors we have isolated and characterized several populations of GABAergic neurons differing in their spatiotemporal distribution as well as neuronal precursor defined by the expression of certain surface markers. A two-step, mild enzymatic digestion protocol (Neural Tissue Dissociation Kit) was developed to produce a single cell suspensions of brain tissue, focusing on high yield of living cells and conservation of antibody epitopes for subsequent FACS and MACS applications. GABAergic neurons were isolated from different brain regions of glutamic acid decarboxylase–green fluorescence protein (GAD67-GFP) knock-in mice by microdissection or fluorescence activated cell sorting (FACS). Using surface markers like A2B5 (glial precursors), CD24 (neuronal precursors, ependymal cells) and PSA-NCAM (neuronal and oligodendroglial precursors), different populations of neural precursors were isolated by magnetic cell sorting (MACS).

As the number of isolated cells was partly very low, we have used a new single cell amplification procedure (SuperAmp) combining bead based mRNA extraction and reverse transcription, global PCR amplification and Klenow fragment labelling to generate gene expression profiles.

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„Single object tracking as tool to analyze virus entry and cell biological transport phenomenons"

Viruses are excellent objects to study not only viral pathogenesis but also cell biological processes. Viruses use a 'Trojan horse' strategy to enter their host cells. Entry, i.e. the delivery of the viral genome and accessory proteins to the cell interior, is a highly dynamic process and involves binding to cell surface, diffusion and/or transport on the cell surface, internalisation - often by endocytosis -, and intracellular transport. Here, we used high resolution time-lapse microscopy to follow single viruses during entry. As tool to analyze this data quantitatively we developed single object tracking software that we applied during various steps of virus entry. To highlight the use and limitations of this approach examples on diffusion and active transport of viruses will be presented.

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„Aquaporin 2 activity in renal epithelial cells monitored with digital holographic microscopy"

Water permeability across biological membranes is supported by aquaporins in the plasma membrane. In the kidney the final step in urine concentration occurs in the collecting duct. In this segment aquaporin-2 (AQP2) in the luminal membrane of principal cells is the final target of vasopressin (AVP). AVP binds to the vasopressin 2 receptor activating the adenylate cyclase via cAMP, which leads to activation of

PKA. Phosphorylation of AQP2 by PKA leads to translocation of AQP2 from intracellular vesicles to the luminal membrane. To further study AQP2 regulation we present a method to dynamically monitor regulation of water permeability in living cells.

In this work we examined the influence of AVP on water permeability of primary cultured principal cells of rat inner medullary collecting ducts (IMCD). IMCD-cells endogenously expressing AQP2 were grown to confluency in cell culture medium. For quantitative phase contrast imaging of living IMCD cells digital holographic microscopy was applied. As this marker-free technique is non-scanning it permits the quantification of the swelling process with a high spatial and temporal resolution (up to video repetition rate). In time-lapse measurements dynamic changes in the cell thickness and shape were investigated. A hypoosmolar change of the growth medium from 600 to 200 mosm/kg was used to stimulate water influx across the cell membranes. Cell thickness was plotted with time followed by regression analysis. Control cells were compared to cells stimulated with AVP (0.5 µM, 30 min). With digital holographic microscopy the changes in cell thickness as a result of changes of water permeability of the membrane could be recognized in IMCD cells. Kinetics of changes of the cell thickness was described by sigmoidal curve fitting allowing determination of the time constant τ and maximal changes in optical length (OPL).

τ after this hypoosmolar shock in control cells was 101 ± 4 s and ΔOPL was approximately 55%. τ and ΔOPL decreased in these first measurements after AVP by approximately 5% and 29%, respectively (n=4 each).

In conclusion, the digital holographic microscopy is a suitable method to measure changes in cell thickness in living renal cells to indicate changes in water permeability with combined high temporal and spatial resolution.

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"Following primary hyperparathyroidism from surgery to proteomic analysis"

Primary hyperparathyroidism is a serious disorder of bone metabolism that often requires surgical treatment. The main causes of primary hyperparathyroidism are a single adenoma (85%) and hyperplasia of all four glands (10-12%) These two forms of the disorder are frequently indistinguishable prior to surgery. The diagnosis is made at the time of surgery, and identifying the particular form prior to surgery remains a challenge. A proteomic study was therefore undertaken to determine if any fundamental differences between the two forms of primary hyperparathyroidism exist. Parathyroid glands removed from patients were frozen, fixed in optimal cutting temperature (OCT) matrix and cut into 8 μm slices and stained. Three groups of cells—adenomatous, hyperplastic, and normal—were selectively harvested from these slices with laser capture microdissection (LCM). These cells were then lysed with detergent, and the proteins were denatured with urea and assayed with a Ciphergen time of flight (TOF) matrix assisted laser desorption ionization mass spectrometer (MALDI-MS). The resulting proteomic profiles of the three groups were analyzed and compared via statistical tests and classification. As a proof of principle the raw data were used; an alternative method is the use of scaled spectra or calculated peak lists.

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„Combined transcriptome, proteome, and metabolome analysis at a cell type-specific level"

Knowledge of the local distribution of transcripts, proteins, and metabolites between different cells and tissues is a presupposition for understanding multi-cellular organisms like higher plants. However, most experimental approaches in plant science as yet average several different cell types.

The use of microcapillaries is one well-established method to obtain the contents of individual cells from living plants. Caused by the small volumes that can be extracted, however, highly specialised strategies for downstream analyses have to be employed. Laser microdissection coupled to laser pressure catapulting (LMPC) has only recently entered plant science and allows the enrichment of specific cell types from histological sections in a completely touch-free manner. This technique makes tissue-specific samples accessible to standard analytical strategies. However, truly comprehensive molecular profiling is still a challenge. One major difficulty is the preparation of tissue sections that retain enough histological detail to reliably identify the cell type of interest while keeping all analytes intact, extractable and localised at their in vivo position. We have implemented methods that allow a comprehensive analysis of transcripts, proteins, and metabolites in parallel samples at a cell type-specific level.

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„Multiplex miRNA profiling from single embryonic stem cells and early embryos"

MicroRNAs are short (17-25 nucleotides), non-coding RNAs that play critical roles in gene regulation and cellular differentiation during development. Recently developed miRNA microarray techniques have contributed greatly to miRNA research but require too large an RNA sample to be used in many crucial studies, such as developmental studies involving primordial tissue samples from embryos or laser-captured samples from developing tissues. Here we report a real-time PCR 220-plex miRNA expression profiling method that is sensitive and accurate enough to profile miRNA from samples as small as a single cell. MicroRNA expression profiles have been measured for single mouse embryonic stem cells and for early mouse embryos at the zygote, two-cell, and four-cell stages. This method should also be useful in cancer studies where only limited amounts of material are available from tissue biopsies or archived material.

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„Intraneuronal crystallin beta b-2 (crybb2) triggers elongation of axons"

Mature neurons of the central nervous system (CNS) fail to regenerate their axons within their natural environment after injury and are induced to degenerate. Adult retinal ganglion cells (RGCs) can regenerate their axons both in vivo and in vitro and serve as a model to examine mechanisms of brain repair. Using regenerating retina tissue in culture and proteomics, we have discovered that the supernatants of cultured retinas contain isoforms of crystallins, with crystallin beta-b2 (crybb2) being clearly upregulated in the regenerating retina when compared with controls. Immunohistochemistry revealed the expression of crybb within the retina, including in filopodial protrusions and axons of RGCs. Cloning and overexpression of crybb2 in RGCs and hippocampal neurons increased axonogenesis, which in turn could be

blocked with antibodies against beta crystallin. Conditioned medium from crybb2-transfected cell cultures also supported the growth of axons. Finally, real-time imaging of the uptake of GFP-tagged-crybb2 fusion protein in single cells showed that this protein becomes internalized and redistributed within the cell including the axons. These data are the first to show that axonal regeneration is related to crybb2 movement. The results suggest that neuronal crystallins constitute a novel class of neurite-promoting factors that likely operate through an autocrine mechanism, and that they could be used in neurodegenerative diseases.

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"Proteins in action: Monitored by tr (time-resolved) FTIR spectroscopy"

In the Postgenom era proteins are coming into the focus in the life sciences. Proteins are the nanomachines that perform the work in living organisms or are the receptors and mediators for external signals. By NMR and x-ray the three dimensional structural architecture of proteins are determined. In order to elucidate the function, time-resolved methods have to be applied.
FTIR difference spectroscopy can be used to monitor the reactions within proteins at the atomic level with ns time-resolution up to days (1). In combination with site directed mutagenesis or isotopically labelling the IR bands can be clear cut assigned to specific amino acids or ligands. This provides in combination with structural models also spatial resolution.
Based on fast scan studies on bacteriorhodopsin the key catalytic residues, asp 85 and asp 96 an their protonation kinetics are identified and summarized in a first detailed proton pump model (2). Their structural arrangement as resolved in succeeding x-ray experiments by several groups supports this proposal. The X-ray structural model at 1.55 A resolves in addition the oxygens of internal water molecules. Based on succeeding step scan FTIR measurements the interplay between these water molecules, a strong hydrogen bonded water, a dangling water and a protonated water complex is elucidated in detail. It results in a controlled Grothuis proton transfer from the central proton binding site to the protein surface. (3,4). A similar mechanism might apply in the photosynthetic reaction center (5)

and the cytochrome oxydase (6). The step scan approach is also successfully applied to the photoactive yellow protein (7).

The difference technique requires fast triggering of the protein reaction, which is easy to accomplish for the chromoproteins as described before. Progress for the investigation of non chromophoric proteins is aquired by developing a micro mixing cell for FTIR studies, allowing mixing times in the sub ms time range. This cell is used to investigate protein folding reactions (8).

Alternatively, photolabile caged compounds can be applied. Using caged GTP the GTPase mechanism of the protooncogen Ras is investigated (9, 10). Also its protein protein interaction with the GAP protein could be studied time-resolved (11,12). This provides a detailed insight into the catalytic mechanism by which GAP activates the GTPase by five orders of magnitude. The activation by GAP proteins is a central process in the signal transduction. In oncogenic ras this activation process is inhibited and involved in uncontrolled cell growth. The study proves that the approach can be extended to protein-protein interactions.

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„Single cell detection by time of flight secondary ion mass spectrometry"

Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) has proved to be a powerful tool in organic surface analysis. Meanwhile also the analysis of single cells has become within reach. Driven by instrumental advancement now two-dimensional and three-dimensional images on the distribution of organic chemical components within a cell preparation are possible. This contribution reviews the state of the art in single cell detection by TOF-SIMS.

As for all mass spectrometric techniques, for TOF-SIMS an ionization process has to be take place prior to separation and detection of the analyte. In TOF-SIMS this process occurs upon impact of primary ions (e.g. Bi1+) of a highly focussed ion beam. This impact leads, among others, to the desorption of secondary ions from the surface of the investigated sample. These secondary ions can be mass separated and detected by a Time-of-Flight analyzer. For single cell detection two TOF-SIMS application modes are relevant: The imaging mode and the so called 3D microarea analysis mode.

TOF-SIMS imaging is based on the two-dimensional mass spectrometric detection of chemical components. As the focussed primary ion beam can be rastered over the surface of the sample, the intensity of all detected analytes in each rastered pixel can be displayed along a colorscale. This procedure leads to so called mass resolved images mapping each detected signal. TOF-SIMS imaging is to date applied to tissue slides as well as cell culture samples allowing a lateral resolution ca. 0.3 µm. Sample destruction upon ion impact can nowadays be limited using cluster ions as primary ions (e.g. Bi3+, C60+).

A prolonged primary ion bombardment of the sample leads to sputter erosion of the surface. Mass analysis of the sputtered material allows to identify the vertical and horizontal composition of the sample. This so called 3D microarea analysis mode of TOF-SIMS allows a three-dimensional mapping of the detected analytes. The

vertical resolution for cell material was shown to be better than 100 nm. Nevertheless, a vertical resolution of 30 nm might be achievable, as this was already shown for organic polymer films.

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"Multiplex RT-PCR of DNA modification genes in single blastomeres of mouse preimplantation embryos"

Over the last years, RT-PCR has become the preferred method for validating results obtained from microarray analyses and other techniques that evaluate gene expression changes on a global scale. RT-PCR is the most sensitive technique for mRNA detection and quantification currently available. On the other hand, there is an increasing requirement of techniques for expression analysis at the single cell level. We are specifically interested to study the expression of known and candidate genes for nuclear reprogramming in individual cells of preimplantation embryos. In a pilot study, we are using the AmpliGrid technology (Advalytix, Brunnthal) for comparing expression profiles in mouse blastomeres.
The AmpliGrid is a glass chip with a surface structure that allows for the specific positioning of one single microliter on each of the 48 discrete reaction sites (enabeling to run 48 1 µl reactions in parallel). Single cells are placed under optical control onto these reaction sites followed by direct amplification (RT-PCR).
In a first set of experiments we have identified appropriate control genes, which are expressed at more or less constant levels in early embryonic cells, and compared expression of a dozen study genes that are involved in DNA modification and epigenetic reprogramming of the two germ line genomes after fertilization in individual cells of mouse morula-stage embryos. Preliminary results suggest that the transcriptome encoding the reprogramming machinery and, by extrapolation, nuclear reprogramming differs between blastomeres.

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"Non-contact microdissection for pure DNA, RNA, proteins and live cells"
Bridging the gap between microscopy and molecular analysis

Modern molecular biomedical research relies on the capability of pure sample preparation. Amongst various options to achieve homogeneous samples, only laser microdissection and micromanipulation offers high-resolution control of sample composition by selecting or rejecting individual cells.

Tissue preparation and extraction protocols allow the utilization of microsamples for qualitative and quantitative molecular and proteomic analyses like, e.g., PCR and RT-PCR amplification, microarray analysis, and MALDI/SELDI spectrometry. Laser Microdissection and Pressure Catapulting (LMPC) results in an eminent increase in the specificity of downstream analyses.

The PALM MicroBeam combines laser technology with high quality robotic tools for precise microdissection of specimens, whilst the patented pressure catapulting feature allows for non-contact collection with no impairment to the recovery of DNA, RNA or proteins. The integration of image analysis platforms to the LMPC technology fully automates screening, identification and finally subsequent high-throughput sample handling.

We will show our results of single cell LMPC and subsequent measurement of expression profiles of individual cells. Using LMPC and real-time PCR in combination with a new mRNA extraction kit we analyzed the expression of murine porphobilinogen deaminase (PBGD) gene from single microdissected frozen tissue cells, and of human PBGD gene from single live cells from cell culture, respectively. Furthermore, in forensics the isolation of single cells is also an important issue. Collection of spermatozoa and epithelial cells from smear preparations is feasible in a fast and convenient way.

The technology of LMPC enables a big step forward to the contamination-free analysis of small samples. It opens a wide field of interesting applications in cell and developmental biology or pharmacology where homogeneous cell clusters or defined clones are needed.

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"Molecular profiling of herbal and animal tissue by direct infrared laser desorption ionization mass spectrometry"

We have developed infrared laser desorption ionization mass spectrometry (IR-LDI-MS) to generate molecular profiles directly from native tissue or from natural products with high mass accuracy. The method requires little sample preparation besides for an eventual dissection of the areas of interest and drying of particular water-rich samples. The spatial resolution of the analysis is to first order defined by the focal laser spot size and the ablation depth of material removal of a few micrometer per pulse. Moreover, the amount of ablated material can be controlled by the laser pulse energy and focal spot size, and the laser thus employed as a precision surgical tool. Various types of small molecules are visualized in herbal tissue. Among these are carbohydrates, phospholipids, triglycerides, waxes, and flavonoids. The potential of the method for a "3-dimensional" molecular analysis is furthermore demonstrated. Substantially different molecular profiles are for example detected from the membrane surrounding and the inside of seeds.

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„From mass imaging to class imaging – New Horizons in MALDI molecular imaging"

MALDI Imaging is a powerful new tool for the proteomic characterization of tissue sections. The conventional approach towards MALDI imaging is to acquire spectra with spatial resolution over the tissue and assemble images based on the intensities of selected mass signals. This approach is usually sufficient to locate histological features (such as the tumor area) on the tissue. This approach lacks the possibility to classify unknown tissue, because single peptide/protein markers are not sufficient for classifications and the possibilities to perform statistical analyses are

limited. Here we present a fundamentally different approach to MALDI imaging: The mass spectra are used to generate statistical models to allow a classification of the spectra based on similarity. The parameter used for generating the molecular image is then the similarity of the mass spectra with the reference model. This allows the unambiguous classification of tissue by mass spectrometry, and also allows the correlation of whole mass spectra with clinical data. We estimate this approach as very relevant for the diagnostic and prognostic use of MALDI imaging of tissue samples.

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„Advances in tissue imaging using a MALDI Quadrupole oa-TOF mass spectrometer"

Imaging the spatial distribution of molecules in tissue using MALDI mass spectrometers is a rapidly developing technique. The acquisition of accurate mass data in this type of experiment can be hampered in axial MALDI Tof systems. It has long been recognised that even small changes in sample position and laser energy in the source region of this type of mass spectrometer affect mass measurement accuracy and mass spectral resolution. Here, we show how the use of an orthogonal Tof MALDI mass spectrometer circumvents these problems by decoupling the MALDI source from the mass analyser

Imaging data are acquired on a MALDI Q-Tof Premier (Waters, Manchester) mass spectrometer. The tissue sections are mounted on a target plate and moved in a raster pattern relative to the laser. To reduce interference from the biological matrix and enhance specificity the instrument is operated in MS/MS mode, a quadrupole is used for precise precursor ion selection. The sensitivity of specific ions can be further enhanced by synchronising the high voltage push of the Tof mass analyser with the arrival of ions of appropriate m/z in the acceleration region.

MALDI imaging information was obtained from thin sections of rat brain from animals doped with the well studied D_2/D_3 dopamine receptor antagonist

Raclopride. Data obtained on the spatial distribution of Raclopride and endogenous adenosine monophosphate are presented. Challenges in sample preparation are discussed.

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„Imaging of proteins and small molecules using the 4800 MALDI TOF/TOF analyzer"

Introduction

The emerging technique of MALDI mass spectrometry imaging (MALDI MSI) offers the potential of profiling biomolecules and small molecules and their metabolites directly on tissue samples. The technology involves mounting thinly sliced tissue sections onto a MALDI sample plate followed by the application of a MALDI matrix solution. The matrix facilitates the desorption and ionization of labile analytes. A two dimensional array of mass spectra is obtained, combined to an ion intensity map allowing to locate analytes of interest on the tissue.

The 4800 MALDI TOF/TOF analyzer is an ideal instrument for tissue imaging due to its high precision of sample stage positioning of about 1 um and the approx. laser beam diameter on sample of about 50 um and its high speed of acquisition.

Experimental

Target tissue were harvested from rats, snap frozen and sliced using a cryostat. Tissue surfaces were coated using a-cyano-4-hydroxycinnamic acid solution. All MALDI MSI measurements were carried out using the 4800 MALDI TOF/TOF Analyzer in MS or MSMS mode. Data were acquired using the MSI acquisition tool available at http://www.maldi-msi.org. Data evaluation was performed using BioMAP Software available from the same webpage. Some sections were kindly provided by Novartis, Basel, Switzerland.

Results

The 4800 MALDI TOF/TOF Analyzer combined with BioMAP software represents a powerful platform for the imaging of peptides, proteins and small molecules.

Results obtained for imaging from rat brain sections are presented. Imaging results in MS reflector and linear mode data is presented. The MSMS capability of the 4800 instrument allowed identification of protein features of interest from rat brain sections.

In further MSMS experiments on small molecules the highly specific transition of a selected precursor mass to a fragment ion mass is used to obtain specific ion intensity maps of the analyte of interest. Data on rat sections of a complete rat dosed with a pharmaceutical compound is presented.

POSTER

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"Application of nanotechnologies for analyzing virulence factors of pathogenic bacteria: shiga toxin 1-caused damage of human brain microvascular endothelial cells"

Shiga toxin-producing *Escherichia coli* (STEC) strains, especially of serotype O157:H7, are responsible for life-threatening zoonotic food- or water-borne illness consisting of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). Serious systemic complications of STEC infection such as HUS, and the ability of these organisms to cause large outbreaks make these bacteria one of the most important emerging pathogens. HUS is histopathologically characterized by microvascular lesions in the renal glomeruli, the gastrointestinal tract, and other

organs, e.g., pancreas, lungs, and brain, which result from the injury of microvascular endothelial cells by Shiga toxins (Stx), the major virulence factors of STEC. It is now evident that, when death occurs during HUS, it is most commonly associated with the damage to the brain. Specific therapeutic or preventive strategies are presently not available.

The integrity of the blood-brain barrier is critical for normal brain function. Human brain microvascular endothelial cells (HBMECs) form a unique, tightly interconnected, cellular monolayer. This highly selective impermeable barrier strictly controls the exchange of material between the blood circulation and the brain. Several studies suggest that Stx1 is involved in manifestation of neurological dysfunction, which is associated with endothelial cell injury in microcapillaries of the central nervous system. The processes by which Stx1 causes these pathological changes are not well understood.

Stx1 is a member of the so-called AB_5-class of bacterial toxins, consisting of a single A subunit (~33 kDa) and a pentamer of identical B subunits (~7.7 kDa each). The catalytic A subunit has RNA N-glycosidase activity and inhibits eukaryotic protein synthesis. The pentameric B subunit is responsible for binding to the functional cell-surface receptor glycosphingolipid (GSL) globotriaosylceramide (Gb3Cer/CD77). Gb3Cer (Galα1-4Galβ1-4Glcβ1-1Cer) consists of a lipophilic ceramide and a hydrophilic trisaccharide moiety. This oligosaccharide with the unusual terminal Galα1-4Gal-sequence represents the binding structure for Stx1.

Due to the clinical importance of this life-threatening toxin, a cooperation was established connecting biological and biophysical nanotechnologies for the *in vitro* and *in vivo* receptor-based functional analysis of Stx1-mediated interaction with HBMECs.

FT-ICR mass spectrometry

In comparison to other mass analyzers FT-ICR MS provides unique specifications with respect to resolving power, mass accuracy, high sensitivity, and dynamic range in molecular analysis. According to these properties FT-ICR MS, combined with immunochemical detection of GSLs, represents a perfect tool for analysis of GSLs with structural heterogeneities in the oligosaccharide and ceramide moieties. Here, we report about the structural characterization of several Gb3Cer species obtained from HBMECs. For this purpose, HBMECs were propagated on microcarriers in bench-scale bioreactors. The isolated GSLs were separated on high-performance thin-layer chromatography plates and the Stx1-receptors were

detected by an ELISA analogous procedure directly on the HPTLC plate. The Stx1-positive GSLs were extracted from the silica gel and submitted to FT-ICR MS analysis. Several chemically distinct Gb3Cer species with variations in their fatty acid chain
lengths from C16 to C24 fatty acids were fully structurally characterized and identified as the functional Stx1-receptors.

4Pi fluorescence microscopy

The subcellular localization of the Stx1 receptor Gb3Cer has been analyzed using confocal and 4Pi microscopy in HBMECs. 4Pi microscopy enables three-dimensional imaging with an axial resolution down to ~110 nm by coherently adding the spherical wavefronts of two opposing lenses and utilization of two-photon excitation. Therefore a resolution along the optical axis is obtained which is 3-7 times better than in classical confocal microscopy. Cells were grown on quartz cover slips and processed for immunofluorescence by fixation, permeabilization, and successive incubation with first and secondary antibodies. Subsequent confocal imaging revealed the subcellular distribution pattern of the Stx1 receptor. Gb3Cer was found to be highly clustered in the plasma membrane, but a significant fraction was also located inside the cell probably associated with the endomembrane system. Furthermore, these intracellular receptor association sites have been structurally characterized in more detail using 4Pi microscopy.

High-resolution scanning electron microscopy

Scanning electron microscopy (SEM) of HBMECs exhibited the typical „cobble-stone pattern" of endothelial cells with strict contact inhibition. Upon incubation of confluent grown microcarriers with Stx1, the monolayers of HBMECs were found to be partly damaged. The most obvious toxin-mediated changes of the cellular shape were indicated by the loss of the monolayer integrity due to increase of spaces between the cells and a considerable loss of cell surface microvilli. This clearly reflects a moderate toxic effect of Stx1 towards HBMECs, which have been suggested for a long time being unsusceptible to the exposure of Stx1. Thus, Stx1-mediated mild deformation of HBMECs upon treatment with Gb3Cer-binding Stx1 could be clearly demonstrated.

Modular digital holographic microscopy

An inverse modular digital holographic microscope with included incubator for stabilized temperature was applied for quantitative phase contrast imaging of Stx1-

treated living single HBMECs. Long term *in vivo* investigations of dynamic changes of the cells' shape and thickness were carried out by time lapse measurements. 16 h after the addition of the toxin, first reactions of the cells such as cell rounding were detected that effect a local increase of the cell thickness up to 50 percent. Simultaneously, a significant decrease of intracellularly induced fluctuations in the holographic phase contrast images was observed. The begin of the final cell collapse was detected after 41 h by decrease of the quantitative phase contrast due to corrosion that finished after 50 h. These findings are in accordance with the SEM results, showing that digital holographic microscopy allows minimal invasive analysis of dynamic cellular changes caused by Stx1 towards HBMECs.

Conclusions

The integrated nanotechnology-based approach reveals an appropriate strategy for analyzing morphology changes of Stx1-targeted cells, initiated and based on the biological principle of carbohydrate-protein viz. Gb3Cer-Stx1-interaction. The multidisciplinarity will likely provide new insights into the virulence factors of pathogenic bacteria. Furthermore, clinical proofs of Stx receptor analogues for preventing systemic toxemic complications of STEC infections are currently in progress. Experimental animal models of HUS that would reproduce the whole spectrum of the pathological changes seen in humans and would be thus useful for testing potential therapeutic approaches have not been developed. We believe that our attempt to analyze the interaction of Stx1 with the host cell using nanotechnology techniques will contribute to the optimizing of therapeutic strategies to defeat the life-threatening infections caused not only by STEC but also by other toxin-producing pathogenic bacteria.

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„Native UV laser induced fluorescence detection for single cell analysis in microfluidic devices"

For proteome research, effective, sensitive and quantitative separation and detection techniques are essential and thus a subject of intense investigations. Microfluidic devices have the potential to fulfil these requirements, which is impressively demonstrated by the transfer of proteom relevant separation techniques to the microfluidic format such as protein electrophoresis, isoelectric focusing and two-dimensional protein separation techniques [1, 2]. However, averaging effects from cell-cycle dependent states, the different and inhomogeneous cellular response to external stimuli, or the introduction of genomic and proteomic variability during cell proliferation, are difficult to address upon studying cell ensembles. Novel and efficient single cell analysis tools are therefore mandatory and have the potential to provide considerable insights for proteom research as well as systems nanobiology. However, low abundant proteins in a single cell require a high sensitivity with a detection limit in the range less than 100 nM (assuming a copy number of 105 proteins in a cell with a diameter of 10 µm). We chose UV-laser induced fluorescence (LIF) as detection method on chip because in capillary format, such as capillary electrophoresis, label-free LIF detectors for proteins based on the UV fluorescence of the aromatic amino acids have been reported with ultimate sensitivity in detection.
Here, we extend our previous studies on single cell analysis in poly(dimethylsiloxane) (PDMS) microfluidic devices with native label-free UV-LIF detection [3] in order to improve separation efficiency and detection sensitivity. With the use of poly(oxyethylene) based coatings we were able to improve the separation efficiency, resulting in an increase in plate height to 46 µm compared to previous studies [3]. Furthermore, we investigated the reduction of the background fluorescence by the adaptation of the confocal volume to the microfluidic channel dimensions as well as with bulk modification of PDMS by the use of carbon black particles. With the use of optimised parameters we enhanced the detection limit to 25 nM, thereby reaching the relevant concentration ranges necessary for the label-

free detection of low abundant proteins in single cells [4]. Taking these results into account in combination with a more recently proposed method of our group for single cell analysis combining navigation and steering of single cells with optical tweezers, on-chip cell lysis and electrophoretic separation of proteins with subsequent LIF detection [3, 5], we were able to perform single cell electropherograms on chip combined with UV-LIF detection. We will demonstrate the first electropherogram from an individual spodoptera frugiperda (Sf9) cell with native label-free UV-LIF detection in a microfluidic chip in comparison to an electropherogram of four Sf9 cells [4]. Detailed chip design and operations are described in references [3, 5]. Our future work is dedicated toward label-free protein fingerprinting from single cells on chip.

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„Single-cell multi-analysis (FISH, IHC, single-cell CGH) of early flat urothelial lesions"

Aims: Our aim was to investigate first genetic events in proliferating cells of early flat urothelial lesions (hyperplasia and dysplasia). Methodically multi-colour fluorescence in situ hybridisation (FISH) and Ki-67 immunohistochemistry were the basis for consequent analysis of single cells by comparative genomic hybridisation (CGH).

Methods: Double staining of fluorescence in situ hybridisation (Urovysion, Vysis/Abbott) and Ki-67 immunohistochemistry was carried out on frozen tissue sections from 15 patients with precancerous lesions (hyperplasia and dysplasia) of the bladder. In addition single-cell suspensions made of frozen tissue sections were used. Ki-67 positive and negative cells were Laser-microdissected (PALM) and analysed in addition with single-cell comparative genomic hybridisation (CGH).

Results: Combination of these three methods (FISH, IHC and CGH) at single-cell level was successful. Using this multi-method analysis tool, our results are: In early flat urothelial lesions (hyperplasia and dysplasia) genetic aberrations for the Urovysion probe set are also present in proliferating cells. For example, in dysplasia proliferating cells have more aneusomies (mono- and trisomies) for centromere probe 3 than Ki-67 negative cells. Proliferating basal cells show a normal diploid FISH signal and no loss of the chromosomal 9p21 (p16) locus. CGH data confirmed the loss of the chromosomal region 9p21 in proliferating cells of precancerous lesions as the most common event, however, other significant aberrations, e.g. amplification of the Y-chromosome, occurred.

Conclusion: The linkage of these three methods (FISH, IHC and CGH) opens up new vistas in single-cell analysis. The facility to combine these three methods (FISH, IHC, CGH) at single-cell level is apt to show that genetic aberrations detected in early bladder lesions or normal urothelium are biologically relevant since found in proliferating cells. This work has been supported by the German Science Foundation (DFG, grant no: Kn263/9-2)

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„Immune modulators expressed in laser microdissected keratinocytes during the early phase of experimental leishmaniasis and the potential role of I TAC for Th2-cell-differentiation"

Studies on Leishmania major (L. major) have been largely responsible for the characterisation of the Th1/Th2 paradigm associated with susceptibility or resistance of different inbred mice strains to intracellular infection. Little is known about early events in the skin preceding final T-cell priming in lymphnodes. In order to further clarify the role keratinocytes might play in the process of T-cell-differentiation, approximately 1000 keratinocytes were isolated from infected and non-infected skin of susceptible Balb/c and resistant C57BL/6 mice by laser microbeam microdissection and laser pressure catapulting. cDNA was preamplified by means of random based PCR amplification, prior to gene expression analysis by real time PCR. In addition, we investigated the gene expression kinetics in whole skin samples from these mice for selected genes during the first three days after L. major infection. For the first time it was shown, that keratinocytes are involved in the production of immune-modulators rapidly after infection with L. major. The expression level of the majority of genes was clearly higher in keratinocytes of resistant C57BL/6 mice than in susceptible Balb/c mice. An exception to this was the expression of interferon inducible T-cell-alpha chemoattractant (I-TAC), which was significantly stronger induced in Balb/c compared to C57BL/6 mice. This is the first report of the expression of I-TAC in experimental leishmaniasis. I-TAC expression in the epidermis was confirmed by immunofluorescence and in situ hybridization. The biological relevance of this differential I-TAC expression in Balb/c and C57BL/6 mice was underlined by a negative influence of I-TAC administration on the course of infection in C57BL/6 mice.
Differential expression of immune-modulators in keratinocytes of susceptible and resistant mice after infection with L. major indicates that early induction of

epidermal gene expression has an impact on the resulting specific immune response and hence on resistance and susceptibility in experimental leishmaniasis.

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"Neprilysin and angiotensin-converting enzyme: peripheral control of proopiomelanocortin peptide bioactivity by endothelial cell-derived peptidases"

Dermal microvascular endothelial cells (EC) are an established source and target of the immunomodulatory proopiomelanocortin (POMC) peptides adrenocorticotropin (ACTH) and α-melanocyte-stimulating hormone (α-MSH). Despite a well-characterized POMC peptide generation in intracellular secretory vesicles, much less is known on their extracellular cleavage by the neuropeptide specific zinc metalloproteases neprilysin (neutral endopeptidase; NEP) and angiotensin-converting enzyme (ACE), which may regulate the local POMC peptide bioavailability and modulate activation of ACTH/α-MSH-specific melanocortin 1 receptors (MC1). EC membranes prepared from ACEhigh/NEPlow expressing primary dermal EC (HDMEC) and the ACElow/NEPhigh expressing EC cell line HMEC-1 degraded ACTH1-39 over time resulting in temporary increased α-MSH immunoreactivity. MALDI-TOF MS peptide mapping and ESI-MS sequencing identified several stable fragments generated from ACTH1-39, ACTH1-24 and α-MSH by EC membranes or recombinant NEP and ACE. Whereas some fragments could be clearly assigned to a cell-specific NEP or ACE activity, other unexpected degradation products require additional enzyme activity. Selected peptides, such as α-MSH2-12 generated from ACTH or α-MSH by both EC membranes and recombinant NEP were equipotent in comparison to the parental peptides in inducing intracellular cAMP in MC1 receptor transfected HMEC-1 or HEK293 cells. Likewise, pharmacologic NEP inhibition promoted cAMP induction by ACTH and α-MSH. In a murine model of allergic contact dermatitis, α-MSH2-12 retained a

similar anti-inflammatory activity in comparison to α-MSH or ACTH. Thus, NEP and ACE significantly contribute to the EC processing of stress hormones (ACTH) and anti-inflammatory peptides (α-MSH), which modulates MC1 activation, but does not completely inactivate the peptide ligand. Since NEP and ACE are regulated by inflammatory mediators and UV light, this may be important for ACTH/MSH-modulated skin inflammation.

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"Hepatic targeting using transplantation of single cells "

Minimally invasive procedures for targeting liver disease using cell based approaches are currently introduced into the clinic. Most protocols involve the transplantation of non-modified cells, e. g. hepatocytes, which are, however, rarely available for clinical applications. Adult stem cells can be highly expanded *in vitro* and when differentiated into hepatocyte-like cells may have enormous potential for cell based therapy.

We hypothesize that both isolated hepatocytes and adult stem cells bioengineered *in vitro* with appropriate bioactive factors by e. g. gene therapy methods are superior targets for efficient cell based therapy of liver. We will monitor the differentiation of stem cells after expression of bioactive molecules *in vitro* as well as *in vivo* using established animal models of liver disease (LEC rat, CCL_4 rat). Animal models mimic the natural course of human liver fibrosis, a major issue in the clinical setting.

As a proof of principle we will translate the known antifibrotic effects of the factor adiponectin (244 amino acids) to cell based therapy of liver fibrosis in various animal models. Monitoring the status of cell differentiation and gene expression will be followed in long-term experiments by established state-of-the-art methodology. Characterization of the differentiation status (proteomics) and gene expression (gene array technology, qPCR) of stem cells will allow a better understanding of the

underlying mechanisms of cell therapy. *In vivo* characterization of stem cell differentiation and repopulation of liver will be carried out by intravital microscopy, immunohistology, FISH and RT-qPCR. Therapeutic effects will be determined by clinical and biochemical parameters.

The current status of this project will be presented. Our model is highly relevant to other alternative strategies in organ transplantation.