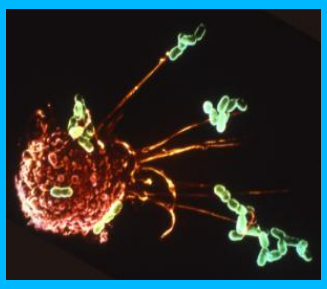

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Clemens Sorg, Peter Josten, Kurt Sieberns, Kristina Riehemann, Simone König

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"Computational systems biology: Towards 'in silico' cell models?"

After sequencing of complete genomes it has become apparent that single genes or their products do not cause most of the biological phenomena observed. These findings have drawn researchers to the conclusion that the most interesting phenomena in biology result from the interrelated actions of many components within the system as a whole. For elucidating and quantifying these molecular interrelations efficient high-throuput techniques have been developed to measure in parallel concentration profiles of many different mRNA's, proteins and metabolites (=molecular variables). The central problem is currently the causal interpretation of such 'molecular snapshots'. Computational systems biology is a rapidly growthing new field in biosciences that aims at formulating both molecular interactions and molecular states into mathematical models of various complexity. The final goal of this approach to set up computer models of whole cells that allow to the predict the temporal and spatial behavior of a large set of molecular variables at various external conditions (e.g. changes in temperature, pH or supply of substrates) or alterations on the molecular level (e.g. inherited enzyme defects, inhibition of processes with drugs). Currently there exist basically two alternative approaches to reach this ambitious goal.

The bottom-up approach continues the traditional way of settling models on a detailed kinetic description of the underlying elementary processes. The top-down approach tries to identify and quantify the relevant interrelations among molecular variables directly from large amounts of high-troughput data. In my talk I will outline the specific strengths and weaknesses of these approaches and the role that single cell analysis may have in contributing to both of them.

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“The necessity of cell- and cell type-specific expression analyses in cardiopulmonary research”

The morphological study and the identification of cell differentiation within tissues are the basis for assessment of physiological and pathological processes. Applying molecular techniques, the analysis of commonly used tissue homogenates bears the risk of masking genetic deviations or expression changes of an individual cell type by the bulk of surrounding cells. To overcome the problem of tissue heterogeneity, cells have to be harvested selectively for further analysis. Therefore, microdissection techniques were developed making retrieval of target cells simple, rapid and precise. Accordingly, the use of these techniques has increased exponentially within the last few years.
Presenting three examples from cardiopulmonary research, it is demonstrated that single cell isolation is often preconditional for the investigation of splicing isoform expression (1). Combining microdissection of few cells and real-time RT-PCR allows to determine relative mRNA expression in a cell-type specific manner to reveal gene regulation (2). Combination with RNA preamplification techniques and micro arrays results in cell type- or compartment-specific expression profiles that especially differ from those of tissue homogenates when minor represented cell types are investigated (3).
In consequence, the isolation of cells or cell types allows a more accurate investigation of complex tissues and gives deeper insight to regulation processes and crosstalk of the respective cells.

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“Immunogenetics at Single Cell Level”

The immune response is governed by a complex array of cellular populations and sub-population. Gene expression profiling is proving an important means of understanding and classifying physiologic and pathophysiologic processes by identifying genes, gene pathways and pathway networks not previously known to be associated with inflammation and autoimmunity. However, disease-associated gene expression can be obscured by surrounding 'normal' cells / tissue. The ability to study and compare gene usage at the cellular level will therefore provide valuable new insights into the biology of a disturbed immune response. A summary of developments in the area of expression profiling in single cells of the immune system will be described, and the rationale for these types of studies will be presented based on our own experience in studying the maintenance and break down of tolerance in both mice and humans.

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It is now possible to generate one- and two-dimensionalelectrophoresis data from single mammalian cells. These separations are capable of resolving hundreds of components from the cell, and the data can be correlated with cell cycle and other properties of the cells. Examples have been generated from breast, prostate, colon, andesophageal cancer cell lines, from astrocytes and neurons, and fromosteoprecursors and myoblasts. Instrumentation is under development that can characterize tens of thousands of cells per day in one-dimensional electrophoresis and thousands of cells per day in two-dimensional electrophoresis. Related technology is under development to monitor post-translation modifications of specific proteins in single cells at the single copy level.

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„Higher mass accuracy, higher lateral resolution: A closer view of single cells“

With the combination of soft ion formation techniques and ultraprecise mass spectrometers, the accurate mass of biomolecules is becoming a physical quantity of high interest in bioanalytical methodology. FT-ICR mass spectrometry with ESI or MALDI sources now allows to easily determine the amino acid composition and sequence of medium size unknown peptides. This new method, called "Composition-based Sequencing (CBS)" [1] appears to open new perspectives in proteomics. On the single cell level, another feature of FT-ICR mass spectrometry will be of importance in the future, which is the high mass resolving power for being able to analyse highly complex, non-fractionated samples.

Investigating single cells directly by MALDI requires not only the high mass resolving power and high mass accuracy, but also a sufficient lateral focusing of the laser beam. MALDI imaging [2] is a new technique of visualizing biomolecule distributions in tissue by mass spectrometry. Most instruments developed for this approach, however, lack the necessary lateral resolution for reaching the single cell level. All of them so far lack the necessary mass resolving power and accuracy to identify and characterize proteins from single cells by this approach.

A goal for the future is to combine micrometer-resolution MALDI imaging with high accuracy FT-ICR mass spectrometry. The prospects and limitations of this approach will be discussed and first results will be presented.

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„MALDI Mass Spectrometric Imaging Applied in the Race to Find New Cures for Diseases“

Molecular imaging techniques play an increasingly important role in biomedical research. With the ability to localize changes in organisms on the molecular level, we have a tremendous amount of information in hands to support the effort to find new cures for diseases. Widely applied molecular imaging techniques in research such as MRI and PET utilize molecular probes to report the presence of the analytes of interest in living organisms. With matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI MSI) a technique is presented which allows de-novo spatial analysis of biological samples. Sections of the tissues are introduced in mass spectrometer, where a laser is used to raster over the tissue while acquiring mass spectra of the ablated ions at every image point. From one array of spectra, hundreds of analyte-specific images can be generated, based on the selected masses. This allows a detailed tissue analysis with a resolution in the micrometer range for protein, compounds and metabolites. The techniques has been applied to target finding, biomarker discovery and drug delivery studies. Applications shown in this presentation cover different stages in biomedical research, from localization of high-mass proteins in tissue sections to drug imaging in dosed animals.

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„Laser micromanipulation and capture of single cells – New approaches in cell and molecular biology“

The understanding of the molecular mechanisms of cellular interactions depends on identification, isolation and finally characterization of a single cell or a population of cells and thereafter of their specific subsets of biomolecules. Thus, sample generation is a very crucial step for high-resolution downstream applications.
Laser Microdissection and Pressure Catapulting (LMPC) is a well-known method to isolate and collect selected cells from complex animal or plant tissues, resulting in an eminent increase in the specificity of downstream molecular analyses like PCR and RT-PCR amplification, DNA, RNA and protein microarray analysis, and MALDI/SELDI spectrometry.
With LMPC, only the force of focused laser light is utilized to excise selected single cells or large tissue areas of a specimen down to a resolution of subcellular components like organelles or chromosomes, respectively. After microdissection, the separated sample is directly lifted up into an appropriate collection device by using the same laser (Laser Pressure Catapulting, LPC). As both processes are working entirely without any mechanical contact, LMPC enables pure and homogeneous sample retrieval from e.g., paraffin and cryosections, cell smears, cytospins and chromosome preparations.
An important innovation is the laser driven isolation of cultured cells. The work with selected living cells is extremely facilitated with this new approach and opens a wide field of new

applications and research possibilities. Individual or small groups of cultured cells can be separated by morphology or fluorescent label for direct molecular analysis, clonal expansion or re-cultivation (e.g., stem cells), creation of mixed cell populations (e.g., tissue engineering), and maintaining specific cell type ratios in mixed cultures. The efficacy of this method was recently shown by LMPC-mediated isolation of transgenic murine embryonic stem cells, which were successfully used to generate chimeric mice.
Furthermore, the same laser system can be used to open the cell and nuclear membrane of living cells, or drill holes into the solid wall of plant cells. This enables new methods for microinjection of drugs or genetic material into living cells without any involvement of mechanical, chemical or viral components. In addition, the controlled fusion of different cell types becomes possible with Laser MicroBeams, as could be demonstrated for B-cell hybridoma.

The applied laser wavelength of 337 nm does not affect the viability of the specimens: There is no impairment of the biological information, even after several times of catapulting and recultivation, as is shown by, e.g., Comparative Genomic Hybridization (CGH) analysis.

With the specificity of LMPC, the sensitivity of downstream analyzing methods becomes a critical issue. Especially in gene expression arrays but also for protein array analysis enormous efforts have been carried out to reduce the required molecule amounts. On the one hand, biased amplification should be minimized but also biomolecule detection should become feasible below femtomolar amounts. For a prove-of-principle we extracted RNA of heat shock treated versus untreated cells, both isolated by LMPC. After one single round of T7 RNA amplification, the amplified message was hybridized on a microarray chip from Zeptosens AG (Switzerland). We could demonstrate a significant and reliable gene expression result from as few as 500 cells using the extremely sensitive planar wave-guide SensiChip™ technology.

Keywords: Laser Microdissection and Pressure Catapulting, high-resolution sample preparation and cell analysis, SensiChip™ microarray technology, stem cells

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“In situ proteomics imaging: analysing "real world" protein networks within intact cells and tissues”

The hierarchy of cell function comprises at least four distinct functional levels: genome, transcriptome, proteome, and toponome. The toponome (rule of protein arrangement) is the entirety of all proteins, protein-complexes, and protein networks traced out directly on the single cell level in the natural environment of the cells in situ (e.g. tissues). Whole cell protein-fingerprinting (WCPF/MELK) enables the quantitative mathematical description and exploration of the toponome by using large tag libraries localizing proteins (hundreds simultaneously) sub- and supracellularly.
Thereby the technology addresses the fact that each protein must be at the right time at the right place at the right concentration in a cell to interact with other proteins assembled in a spatially organized network. To encode the myriads of cellular functionalities, cells appear to having at their disposal a large, albeit finite and highly non-random repertoire of toponome units, i.e. a system of rules to construct topological hierarchies in a cell's proteome. The entirety of these toponome units represents the total functional code of a cell. The complete toponome is therefore as fundamental a data set as the genome or the proteome.

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„Nanobiology: From Single Molecule Detection and Manipulation Towards Single Cell Analysis in Microfluidic Networks“

Our concepts to detect and analyze minute amount of substance (single molecules) via mechanical and optical schemes in order to 1) fundamentally investigate the nature and related physical mechanisms of specific biomolecular interaction and 2) to ambitiously analyze the proteome of single cells in nanofluidic microdevices (lab-on-a-chip) will be presented.
a) Specific binding of single receptor and ligand molecules can be investigated in dynamic force spectroscopy experiments by atomic force microscopy (AFM) or optical laser tweezers (OT) and give access to intermolecular forces, interaction mechanism, kinetics of the reaction and the binding energy landscape. The ability to measure inter- and intramolecular forces and elasticities under physiological conditions in biological transcriptional regulators (DNA-protein) [1-3], in bioorganic synthetized peptide-DNA systems [4] and in supramolecular guest-host systems (cation-calixarene) [5] with piconewton-, nanometer- and millisecond resolution allows investigation of single ligand-receptor interaction in a broad affinity range from $K_d=10^{-5}$-10^{-15} M at a discrimination level of single point mutations.
b) The understanding and control of cellular adhesion plays an enormous role in many applications like implantation, transplantation, tissue engineering or inflammation. Our most recent results where molecular interaction and cellular adhesion was investigated on individual cells downto the single molecule level with OT [6,7] will be presented.
c) We have recently demonstrated that periodical geometric structuring of microchannels allow separation of long DNA-fragments (with sizes > 40kbp) in less than a minute [8], a fragment size range which is rarely accessible by standard separation mechanisms in microchannels. We extended our investigations on single-molecule DNA-fragment migration by means of computer simulations [9] and exploited how such structures can be used to generate DNA separation devices based on ratchet effects. In microfluidic devices governed by conditions far from thermodynamic equilibrium unexpected migration of particles and other mechanisms such as negative mobility [10,11] have been predicted theoretically. We experimentally prove this paradox migration mechanism for the first time in a structured microfluidic device with colloidal particles in solution.
d) Our concept of single-cell analytics and the first results of our microchip-integrated separation and ultra-sensitive label-free detection of minute amounts of analyte molecules is presented and discussed [12].

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"Fluorescence spectroscopic techniques to study protein/protein interactions and conformational changes on the single molecule level"

Fluorescence spectroscopy is an experimental technique with a great potential of elucidating inter- and intramolecular interactions of biomolecules. Especially the application of fluorescence techniques to the study of single (individual) molecules has several striking advantages, if complex systems (like biological systems) with static and dynamic heterogeneity have to be investigated. This technique makes it possible to directly probe distributions of static properties as well as dynamical transitions between different states (e.g. structural conformations), without the necessity to synchronize the transitions in an ensemble. This ability becomes crucial when studying biological systems where synchronizing molecules in an ensemble is problematic due to the complex or even branched reaction pathways. Although single-molecule investigations of biological systems are still in their infancy, several promising studies in this direction have already been reported.
The fluorescent dyes used in those studies change their emission properties (e.g., emission wavelength, emission intensity, life-time, anisotropy) in accordance with changes of the direct environment of the dyes (local pH, hydrophobicity, energy transfer, etc.) during the different states of the working cycle. A more sophisticated and powerful approach for studying conformational changes on a single-molecule level as well as the interaction between different molecules provides Förster resonance energy transfer (FRET), electron transfer, and fluorescence quenching mechanisms. These techniques can be employed to study conformational changes in a time-resolved manner. An overview of these techniques and some applications will be presented.

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"Towards scanning (near field) infrared laser microscopy of cells"

We have developed a chemical nanoscope in the chemically important fingerprint region to monitor label free functional groups. Using our unique cw Infrared Opto Parametrc Oscillator (with output powers of 2 W) we have been able to obtain 2-dim images of cells in the spectral fingerprint bands. As a first example we present pictures of hepatocytes scanned in the range of the lipid band at 2920 cm^{-1}. The method allows a label free detection method and provides a very fast scanning method (each pixel is scanned in less than a msec). IR spectroscopy offers moreover the advantage of yielding absolute concentrations. This was demonstrated by a near infrared microscope which offers a cheap solution for the quantitative measurement

of water in living cells. It is shown that IR imaging is able to monitor the influence of minimum dosages of pharmaceuticals on the intracellular water concentration on a single cell level.
We were able to increase the resolution considerably when measuring in the near field limit. Using the tip of an AFM as nano-antenna we sampled the infrared spectra of the surface by the evanescent field. In a first test we achieved resolutions of 30 nm (corresponding to λ/100) in our scanning near field infrared microscope (SNIM) setup.

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„Volume, surface and stiffness of individual cells measured with atomic force microscopy“

Atomic force microscopy (AFM) is a tactile instrument that can be used as a nano-scope as well as a manipulative nano-tool. In principle AFM consists of a fine tip that physically scans surfaces. At low interaction forces (low pN range) the AFM tip images surfaces at sometimes molecular resolution. At high interaction forces (nN range) the AFM tip more or less strongly interacts with the surface and thus distorts it. Since forces can be controlled and measured some unique parameters as e.g. the stiffness of living cells can be quantified. We applied AFM to human endothelial cells and measured single cell volume, single cell surface and single cell stiffness in response to steroid hormones. Aldosterone, a steroid hormone known to regulate salt and water transport in kidney and to control blood pressure in humans was found to exert dramatic effects on cell volume, surface and stiffness. AFM allows the quantification of these fundamental cell characteristics. The instrument is easy to handle, commercially available and well equipped with software that supports many different applications, from molecular imaging to force-volume measurements. In conclusion, AFM is a nanotechnique with an exciting perspective in biomedical research.

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