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# ABSTRACTS

# Oral Presentations

## The Sperm Chromatin Structure Assay® and its Relationship to Morphology, Fertility and DNA Integrity for Bulls and Stallions

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In a large study using a few hundred sperm samples of 50 bulls and 50 stallions as well we have compared the well known flow cytometric Sperm Chromatin Structure Assay (SCSA®) with data on sperm head morphology, DNA integrity and fertility of the animals. Multiple sperm samples have been taken at different time points at different seasons during the year. The flow cytometric data of the SCSA® assay have been analyzed using an automated algorithm to prevent influences of human interaction as much as possible. The extent of chromatin denaturation was calculated as the mean value of DFI (defragmentation index) of sperm cells [DFI=red/(red + green)]. Head morphology of sperm has been studied by visual microscopy. In some cases the gel electrophoretic COMET assay has been used to determine breakage in sperm DNA.

In a few cases testicular degeneration caused by locally X-radiated testes of healthy bulls has been related to alterations in sperm chromatin structure and its time course for 30 days has been monitored.

Fertility estimates for each bull or stallion as determined by the non-return rate are compared with the DFI of the SCSA® assay.

Our study shows, that indeed a significant relationship between sperm chromatin structure and fertility of bulls and stallions could be observed. However, differences in sperm chromatin structure between ejaculates, the intra-animal variation, are almost as high as the inter-animal variation.

## Antibody Secreting Cell Lines as Model Systems for Affinity Matrix Secretion Assay Based Cell Sorting

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The cell surface affinity matrix secretion assay, which is a well established technique for the sorting of cells according to amounts of secreted proteins, was used in this work for an efficient generation of antibody producing cell lines of different types and species.

We used recombinant CHO cells, mouse-human heterohybridomas and mouse hybridomas as model systems. From a recombinant CHO cell line different clones with properties like enhanced long time antibody production stability for more than hundred generations without selective and amplification pressure, or altered kinetics of product secretion during growth in batch culture were isolated. In a modified secretion assay application of a human-mouse heterohybridoma cell line exhibiting a positive correlation of antibody surface expression and secretion rate for cell sorting including both secretion coupled fluorescence and surface fluorescence was used for the improvement of clones.

Sorting antigen specific clones out of pools after mouse hybridoma fusion nevertheless was limited by several practical considerations. Thus the time point of sorting after fusion was shown to be important due to the outgrowth of non-producers. In addition, unknown binding affinities and specificities of matrix components, which were necessary for the identification of clones to sort, influenced the obtainable signal. In order to recover high producing cell lines derived from monoclonal wells, a strategy of two sorting and cloning rounds had to be followed.

## Apoptosis in B- and T-Cells in Patients with Lupus Erythematosis

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Lupus erythematosus (LE) is the prototype of autoimmune diseases. Although a variety of immunologic deviations have been found in patients with this disease, the underlying pathomechanisms are far from being clear. During the past it has been shown that dysregulation of apoptosis may play an important part in the development of autoimmune diseases in general and in the development of LE in particular. The aim of this study was to investigate apoptosis-related molecules in B- and T-cells.

Peripheral blood T- and B-cells either in whole blood specimens or after Ficoll density gradient isolation were stained with monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD95, CD19, CD5, CD14, active caspase 3 and Bcl-2 proteins and analyzed by

flow cytometry. For the detection of phosphatidylserin externalisation, a characteristic feature for cells undergoing apoptosis, the annexin V-binding assay was used.

Peripheral T-cells of patients with LE, showed a marked increase in annexin V-positive cells, and a drastic reduction in the number of Bcl-2-positive cells. Active caspase 3 was present in freshly isolated T-cells without additional stimulation, indicating the presence of apoptotic stimuli in vivo. In contrast, B-cells neither did bind annexin V nor anti-CD95 mAb, but were strongly positive for Bcl-2. MTX treatment induced B-cell apoptosis as shown by annexin V-binding, increased levels of activated caspase 3 protein within the cytoplasm, and DNA-laddering. Induction of apoptosis decreased B-cell counts in the peripheral blood, while other cell subsets remained stable or increased under low-dose MTX. Increasing counts could be documented for CD3+ CD4+, CD3+ CD8+ cells, monocytes, and platelets. This was accompanied by decreasing pro-apoptotic molecules and phenomenons. The exact stimulus triggering apoptosis in patients with LE still remains unknown but the present study provi!

des strong evidence that the reduced apoptosis in B-cells is associated with the production of autoantibodies. These autoantibodies due to cross-reactivity and/or epitop-specificity may bind to T-cells, which promote apoptotic cell death. Treatment with low-dose MTX induced B-cell apoptosis, decreased autoantibody production, and led to increasing T-cell counts by reducing the apoptosis rate of this subset. Thus, the results support the notion of a role for autoantibodies in T-cell apoptosis.

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## Telomeres and Telomerase in Cancer and Aging: An Overview

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The ends of the chromosomes are capped by specialized structures, the telomeres. These are comprised of tracts of hexanucleotid sequences and, in combination with specific proteins, protect the chromosome against degradation, fusion events and as being recognized as „damaged" DNA; thus they guarantee chromosomal integrity. Due to deficiencies during DNA replication, the telomeres continuously loose part of their sequences and it has been proposed that this loss is the limiting factor for the replicative capacity of a cell, i.e. telomeric loss is the counting mechanism, the internal clock of aging. In order to proliferate indefinitely, the cells must prevent telomere erosion and this is mostly achieved by up-regulation or *de novo* expression of the ribonucleoprotein complex telomerase. This enzyme which has a reverse-transcriptase activity is able to add telomeric sequences to the outer most ends of the telomeres and thereby can stabilize or even elongate the telomeres. Since telomerase is expressed in about 90% of all tumors while expression is absent in many somatic tissues, it is not surprising that the causal role of telomere erosion is presently the most favored hypothesis of cellular aging and that telomerase up-regulation is believed to be essential for tumor development.

# Fast Fluorochroming of Cellular Substances to Monitor Bioprocesses

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Flow Cytometry has been developed to a essential method for multi-parameter analysing single cells over the past 20 years. However this powerful method was mainly used for medical applications.

To monitor processes in biotechnology Flow Cytometry suffers from being too expensive. The high investments and the small size of microbes prevented the wide application of this method in the field of biotechnology.

In this study the newly developed ultra compact CyFlow® Flow Cytometer (Cytecs GmbH, Görlitz) is used for monitoring bioprocesses. Therefore an online coupling of the bioreactor to the Flow Cytometer via FIA (Flow Injection Analyses) will be set up. For online monitoring of microorganisms fast and efficient staining protocols are necessary.

New fluorescent dyes for dead-vital staining of different yeast species were tested and established. The most applicable dye system was LDS 751 (vital dye, Molecular Probes) and Propidium Iodide (dead dye, Sigma Aldrich). Employing this dye combination dead and vital cells of *Saccharomyces cerevisiae*, *Candida hämalonii, Hansenula anomala,* and *Yarrowia lipolytica* could be identified and counted within 10 min.

To realize these staining procedures it is necessary to develop a module for washing, fixing, staining, and diluting of cells. This module will be integrated within the FIA transferring the sample to the Flow Cytometer.

# The New BD LSR II Flow Cytometer: 4 Lasers, 10 Colors, Fiber Optics, Digital Electronics

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The BD LSR II flow cytometer offers more flexibility for research applications. The BD LSR II can be configured with up to four lasers (Blue 488nm, Red 638nm, Violet 405nm, UV 325nm). The new VioFlame diode laser (405nm) from COHERENT is optimal for the detection of Cascade Blue, CascadeYellow, Alexa 430, the living colour protein ECFP which is used in ECFP/EYFP Fluorescence Resonance Energy Transfer (FRET) applications and the B-Lactamase Gene Reporter Assay. The newly developed collection and detection optics guide the fluorescence light from the laser intercepts through 4 different fiber optics, coupled to the revolutionary octagonal detection arrays. Each laser has its own dedicated detection array with easily exchangeable dichroic mirrors and bandpass filters. These BD Octagons provide highly efficient light collection (less than 15 % light loss through a whole Octagon). The fluorescence is

detected simultaneously by up to 10 detectors and two additional scatter detectors. The BD LSR II uses similar digital electronics as the DiVa option on the FACSVantage SE cell sorter. The new, database based, software, dedicated to analyze the digital data, runs on a high performance PC. The electronics digitize signals at a rate of 10 million times per second into 16,384 discrete levels. As a result, logarithmic amplifiers and the traditional analog peak-and-hold circuits are no longer required. This has two main advantages: inaccuracies introduced by the logarithmic amplifiers are eliminated and there is no electronic dead time. Digital processing improves linearity, enables intra- and inter-beam compensation and multiple thresholding/triggering, and provides fully integrated pulse processing (height, area and width measurements for all parameters).

The new BD-LSR II opens exciting new possibilities to the high-end researcher while setting a new standard in the technology of flow cytometry.

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# On-line Observation of Cell-Growth with In-Situ Microscopy

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To increase quality and yield of biotechnology processes, it is useful to obtain information about the process in real-time. Furthermore, on-line information about a cultivation is a requirement for a complete automation of the cultivation process. Up to now samples are taken off the bioreactor and cell number and viability are determined off-line. It is not possible to control the process without interfering with it. In-situ microscopy is a new method to control a bioprocess on-line and in real-time. A big advantage of this method is that cultivation parameters as cell number and biomass are available on-line.

Information about the status of cell growth is an interesting parameter in modern research. One indicator of the status of cell growth is the ratio of single and double cells during cultivation. The requirement to enable the on-line determination of this parameter leads to in-situ microscopy. For the observation of a yeast batch cultivation a specially designed sampling zone was developed and integrated in the in-situ microscopy system. The sampling zone is located between a green LED behind a condensor as a light source and the objective. Two sapphire windows work as slide and cover slip of a standard light microscope. By means of several cultivations it was shown that the optimal height of the sampling zone for a yeast cultivation is between 8 and 10 µm. To clean the sampling zone it is sufficient to open the sampling zone slightly. Observation of an N-limited yeast batch cultivation with the developed sampling zone shows that the status of cell growth could be determined with the in-situ microscope. With N available, mainly double cells are monitored. With decreasing N the amount of single cells increases and at the end of the cultivation single cells predominante.

Different cultivations show that by adaptation of the sampling zone and the optical system on-line observation can be realised. This allows for complete automation of process control.

# CD203c is a Novel Marker for Basophils and Glandular Epithelial Cells in Endometrium

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Basophils and mast cells are important effector cells of inflammatory reactions. Both cell types derive from $CD34^+$ hematopoietic progenitors. We have generated a series of monoclonal antibodies, that specifically detect human basophils, mast cells (lung, skin), and their $CD34^+$ progenitors. To identify the recognized antigen, partial amino acid sequence analysis of affinity chromatography-purified and SDS-PAGE-separated KU-812 cell lysates was performed. Comparison with the data base revealed identity of 130kDa and 150 kDa proteins with ecto-pyrophosphatase/ phosphodiesterase 3, now clustered as CD203c. Sorting of $CD203c^+$ peripheral blood (PB) and bone marrow (BM) cells resulted in a pure basophil population, whereas other mature hematopoietic cells were negative for this marker. Approximately 1% of $CD34^+$ BM and PB cells were found to coexpress CD203c. Culture of sorted $CD34^+CD203c^+$ BM cells in the presence of IL-3 or SCF resulted in the generation of basophils or mast cells, respectively. Cross-linking of high affinity IgE receptors with anti-IgE antiserum for 15 minutes resulted in a 2 - 11-fold upregulation of CD203c expression. CD203c on basophils was also upregulated in patients allergic to hymenoptera venoms when activated by appropriate recombinant or purified allergens. Finally, a prominent CD203c expression in endometrium and decidua sections was observed on the surface glandular epithelia and in lumen-orientated areas surrounded by epithelial cells. These data demonstrate that CD203c is a differentiation marker for basophils and mast cells, a powerful activation marker for basophils as well as a selective marker for glandular epithelia in endometrium.

# Pure Sample Preparation with Laser Pressure Catapulting: A Prerequisite for High Quality Molecular Analyses: Preparation of Single Cells, Cell Areas or Chromosomes in a Non-Contact Way

RENATE BURGEMEISTER

PALM Microlaser Technologies AG

State-of-the-art molecular analyses need the handling with extremely pure samples to yield a good result. This is important e.g. for single cell genetic analyses (like in fetal cells in maternal blood, in preimplantation diganosis or in disseminated tumor cells), for microarray technologies and for single chromosome preparation. Thus pure sample preparations are indispensible for various fields in medicine and biology.

To obtain pure samples is one of the most thrilling tasks in modern molecular science. A modern tool in this topic is Laser Microdissection combined with Laser Pressure Catapulting. This laser technology simply utilizes the force of focused laser light to eject a selected specimen from the object plane and to directly lift it into the cap of a routine microfuge tube. This completely non-contact Laser Pressure Catapulting technology avoids any danger of contamination with unwanted specimen. In every case where the comparison of different cell types (genetic, expressional or proteomic) is important for research or diagnosis, a precise differentiation between selected cells is mandatory.

Any kind of tissue from various sources (also archival histological samples or living cells) and even subcellular structures can be captured using this laser method. Selection of a living cell of a cell culture, collection in medium and subsequent reculture is an easy way for cell cloning. Wherever precise micromanipulation is required or where the procurement of homogenous samples is obligatory for the subsequent analysis of specific genetic or proteomic alterations, the PALM MicroLaser system is a key technology.

# CDCP1 is a Novel Marker for Hematopoietic Stem Cells

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CUB domain containing protein 1 (CDCP1) is a transmembrane protein that contains three CUB domains within the extracellular region and a hexalysine stretch within the cytoplasmic region [1]. CDCP1 mRNA is highly expressed in lung and colon tumors and in the erythroleukemic cell line K562 [1]. To analyze CDCP1 protein expression, monoclonal antibodies against the extracellular domain of CDCP1 were raised. For this purpose, the complete coding sequence of CDCP1 was cloned into the pRK vector and used to transfect NIH-3T3 cells. Balb/c mice were then immunized with the resultant

cell line NIH-3T3/huCDCP1. After fusion of SP2/0 cells with immune spleen cells, hybridoma clones were selected that secreted antibodies reacting with NIH-3T3/huCDCP1 cells but not with parental cells. Four antibodies (CUB1 – 4) were obtained that fulfilled these criteria. Screening of peripheral blood cells revealed that the antibodies did not recognize mature lymphocytes, monocytes, granulocytes, erythrocytes, or platelets. In contrast, <1% of bone marrow cells were detected by our antibodies. Multi-color analyses revealed that CDCP1 protein is almost exclusively expressed on a subset of $CD34^+$ stem/progenitor cells. The antigenic profile of the $CDCP1^+$ bone marrow population was almost identical to that of the CD133 subset [2] and differed from CD34. More than 99% of isolated $CDCP1^+$ cells coexpressed high levels of CD34 and CD133. By morphology, these cells represented immature blasts. Together, our data suggest that CDCP1 is a novel stem cell marker.

References:

1] Oncogene 2001;20:4402.

2) CD133 cluster report. In: Leucocyte Typing VII. (2002), p. 622.

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# Future Perspectives of Clinical DNA Content Measurements by Flow Cytometry

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Flow cytometric DNA content measurements of nuclei isolated from solid human neoplasms were pioneered by Göhde and Dittrich. The number of publications on DNA content measurements increased due to the major methodological improvements by Vindeløv and Hedley. Numerous investigators studied the prognostic relevance of DNA-ploidy for survival of patients. For some types of cancer (e.g. ovarian cancer) it was shown that DNA content measurements may contribute to clinically relevant information. However, the clinical utility of DNA content measurements by flow cytometry appears to have been overestimated (DNA Cytometry Consensus Conference). The limited impact of DNA content measurements on clinical outcome can partly be explained by technical factors impairing DNA histogram resolution, e.g. the use of paraffin embedded material vs. fresh or frozen specimen and underestimation of intratumour DNA-index heterogeneity by one-sample, one-parameter DNA flow cytometry. Another limitation of one-parameter DNA content measurements is that DNA diploid tumour cells cannot be discriminated from DNA diploid normal cell types. This also affects the accuracy of S-phase fraction (SPF) estimation. Furthermore, the presence of a diploid tumour cell population in addition to an aneuploid population in the same sample can not reliably be demonstrated and leads to underestimation of intratumour heterogeneity.

In contrast to the limited impact of DNA ploidy measurements on clinical outcome, a number of studies demonstrated the usefulness of S-phase fraction (SPF) estimation in human tumour samples. Recently, significant progress has been made in the prognostic impact of DNA ploidy as well as S-phase estimation after reclassification adjustments. Furthermore, recent developed multi-colour staining technique fulfils the suggestions made by the *DNA Consensus* and might contribute to refined DNA-ploidy assessment as well as S-phase estimation. Simultaneously, our understanding about chromosomal

instability in cancer is increasing. The clinical value of these new developments on DNA content measurements and S-phase estimates must be awaited.

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# Irradiated Lymphoid Tumour Cells Undergo DNA Repair and are Protected from Apoptosis in the Endoreduplication-Reduction Cycle

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We have previously reported that p53 mutated lymphoid tumours respond to high dose irradiation by producing endopolyploid cells. These giant cells undergo meiotic-like processes and segregate into mitosis-competent survivors. In this study, the relevance of endoreduplication to mitotic arrest, meiotic events, DNA repair, and apoptosis was studied in three lymphoid cell lines. Three-four days after irradiation with 10 Gy, p53 mutant cells undergo profound metaphase arrest. Chromosomes remain condensed and reconstruct into a non-radial endomitotic arrangement where they initiate unscheduled DNA synthesis, reaching modal values of 8, 16, and 32C. Simultaneously, the nuclei begin reconstruction into bouquets with characteristic chromatin loops, unipolar spindles, and telomere clusters. This is accompanied by accumulation of mos and non-degradable cyclin B1 proteins, characteristic of meiosis. Subsequently, the bouquets segregate into subnuclei and subcells with radially arranged metaphases and anaphases. In the giant cell nuclei and bouquets, Rad51 was observed in juxtaposed foci, indicating that DNA repair by recombination between homologs was occuring. During the post-irradiation response, Rad 51-protein positive cells were seen mirroring the kinetics of polyploidisation. Using flow cytometry with annexin V, the reverse relationship between cell ploidy and apoptosis was found, indicating that the giant cells are not undergoing apoptosis but are instead undergoing DNA repair.

# High Resolution Far Field Light Microscopy

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A serious problem for the extension of present far field light microscopical studies of nuclear genome structure to nuclear genome *nano*structure is the limited optical resolution. In recent years, various light optical approaches to overcome this impasse have been described. A recently introduced light microscopic approach, Spectral Precision Distance Microscopy [SPDM] based on labelling of neighbouring objects with different spectral signatures, spectrally selective registration, high precision position monitoring, and careful calibration of chromatic aberrations, cross talk etc., allowed the measurement of DNA-sequence positions and mutual distances ("topology") in nuclear chromatin down to the 30 - 50-nanometer range. Theoretical considerations supported by "Virtual Microscopy" computer simulations indicated that using "Point Spread Function Engineering" approaches with a suitably narrowed Point Spread Function, even at the fluorescence photon count number typical for single molecule fluorescence emission, a topological resolution limit down to the few-nanometer range with a precision in the order of 0.1 nm might become feasible.

A similar topological resolution limit and precision range is also expected using newly developed methods of Structured Illumination Microscopy, such as Spatially Modulated Illumination [SMI] far field light microscopy; presently, experimental distance measurements in the direction of the optical axis down to the few nanometer scale, with a precision in the one-nanometer range have been realized. Furthermore, SMI-approaches have been used to measure the diameter of individual fluorescent targets down to a few tens of nanometer, corresponding to about 1/16 of the wavelength used. This "SMI-Nanosizing" technique is based on the analysis of the small pertubations of the SMI-diffraction image correlated with the object size; presently, it is being applied to measure the size of nuclear macromolecular complexes; of individual gene regions; or of the thickness of metaphase chromosomes; its

# Use of Phase-Sensitive Flow Cytometry to Measure DNA and RNA Based on Fluorescence Lifetime Differences of a Single Probe When Bound to Nucleic Acids

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The phase-sensitive flow cytometer (PS-FCM) developed in our laboratory is a conventional flow cytometer with additional capabilities analyzing the fluorescence lifetime of dyes bound to single cells in real time. The instrument allows for direct correlation of lifetime with all the parameters routinely obtained by conventional flow cytometry. Using the PS-FCM, we previously showed that the fluorescence lifetimes of ethidium bromide (EB) and propidium iodide (PI) were differently altered when intercalated into cellular double-stranded DNA or RNA (Sailer et al., European J. Histochem 42:19, 1998). Recent studies using four ethidium derivatives also demonstrated lifetime differences for each of the dyes when bound to DNA or RNA. The magnitude of the difference of the lifetime values of the individual dyes bound to DNA or to RNA was dependent on the dye structure, the staining concentration, and conditions used for analysis of stained cells. The fluorescence lifetime of both DNA- and RNA-bound fluorochromes was reduced with increasing dye concentration; however, a more significant decrease was noted for the dye bound to RNA. In addition, analysis of stained cellular DNA in dye-free solution, which favors only high affinity binding, led to elevated fluorescence lifetime values compared to values obtained from analysis of cells in the dye solution under equilibrium binding conditions. Based on the differences in the lifetime values of ethidium homodimer I (EthD 1) when bound to DNA and RNA, we applied phase-resolved, lifetime analysis techniques to discriminate the nucleic acid contents in HL-60 cells. These studies demonstrate the potential of a novel technique for distinguishing and quantifying various cellular moieties based on differential fluorescence lifetime signatures of a single fluorescent probe.

Supported by the US Department of Energy and the Los Alamos National Flow Cytometry Resource (NIH Grant P41-RR01315) and NIH Grants R01 CA92632 and R01 RR07855.

## Combined Flow Cytometric and Phylogenetic Analysis of Marine Bacterio-Plankton Communities: From Dilution Culture to the Open Ocean

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Flow cytometry is a well-established method in aquatic microbial ecology to measure the abundance and to determine the biomass/size distribution and chlorophyll content of the picoplankton community. Within seconds several parameters of thousands of single cells can be analysed simultaneously allowing the discrimination of different subpopulations of picoplankton. However the ecological function of the flow cytometrically defined bacterioplankton group remains largely unknown.

We developed a combination of flow cytometric sorting of cells based on cellular DNA - and protein content and subsequent molecular analysis with FISH, DGGE and cloning to investigate the taxonomic composition of flow cytometric defined subgroups of the marine bacterioplankton. We tested this approach on a dilution culture experiment and were able to follow the development of different bacterioplankton groups. During a field experiment in the North Sea a coccolithophore bloom was closely monitored in a $SF_6$ - labeled water patch. The bacterial uptake of the algal storage compound dimethylsulfoniopropionate (DMSP) was measured and the key bacterial group cleaning up the DMSP could be determined.

## Effect of hTERT Infection on Telomere Length and Genetics in Early and Late-Passage Human Fibroblasts: A Q-FISH Study

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Normal human cells in culture show a proliferation-dependent telomere shortening and it is believed that this is causal for their limited division potential. Furthermore, it has been shown that introduction of hTERT, the catalytic subunit and limiting factor for telomerase, leads to telomerase activity in otherwise negative cells (e.g. fibroblasts) and makes them capable of indefinite cell divisions (immortality). The aim of our study was to elucidate whether this live-extending effect of hTERT would be restricted to young fibroblasts, i.e. cells in a still actively proliferating state or would also be effective for old fibroblasts close to senescence. We, therefore, infected normal human foreskin fibroblasts in early (p10, ~35 population doubling (pd) and late (p 20, ~70 pd) passage with hTERT and studied telomerase induction (TRAP-assay), telomere length regulation (TRFL, and Q-FISH) as well as genetic changes (M-FISH)

up to 26 passages (~90 pd) after infection. Our experiments showed that the replicative potential of both cultures after hTERT infection reached far beyond the Hayflic limit (control cells senesce around passage 25 with a remaining telomere length of ~6kb). Although induction of telomerase and telomere length regulation differed significantly in early versus young fibroblasts, telomere length finally stabilized and suggested that both cell types had reached a state of homeostasis required for indefinite growth. Furthermore, and contradictory to results from others, the M-FISH results revealed genomic changes in both hTERT infected fibroblast cultures. Surprisingly, all metaphases within one population showed the same genetic aberrations strongly arguing for the selection of rare genetically altered clonal populations. Moreover, since the number of aberrations per metaphase was higher in the "old" fibroblasts, we have to conclude that the shorter telomeres at the time of telomerase up-regulation allowed a higher degree of chromosomal instability. Thus, our data show that hTERT is able to induce longevity in both young and old cells. However, other genetic changes seem to be required in addition for immortalization of the human skin fibroblasts.

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## Cell Sorting as a Straight Forward Strategy for Lifting the Veil of Obscure Tissue Array Data

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Expression profiling with DNA microarrays has been widely applied in the analysis of human diseases. The expectations associated to this cutting-edge technology in biomedical sciences are exorbitant, hoping to identify new disease-related genes and to unravel the riddles of cancer and other chronic diseases. During the last years a huge amount of array data has been produced, but only a minor fraction of these contributed to a better understanding of disease-associated mechanisms. A major reason for this ineffective application of microarray experiments is the cellular heterogeneity of tissue samples investigated. After homogenisation and/or lysis of tissue samples, a heterogeneous, quantitatively and qualitatively undefined cell population is obtained and the corresponding microarray data are either extremely hard to interpret or not at all. The explanation for differential expression of a particular gene in a heterogeneous tissue can be twofold: either the composition of the cell population has been changed, or the difference is the result of a regulatory event at the single cell level. Moreover, an unambiguous relation of a differentially regulated gene to the corresponding cell type responsible for this effect is not possible in crude tissue extracts. Consequently, the separation of well defined cell populations from tissue, exudate and blood samples should be a valuable strategy to circumscribe this methodical limitation. In this respect we describe state of the art technologies, such as magnetic cell sorting (MACS), highspeed fluorescence-activated cell sorting (FACS) and the combination of both, as examplified by the separation of multiple cell populations from blood of rheumatic and normal donors. Array data derived from

custom-made cDNA and from genome-wide Affymetrix chips were demonstrated and discussed for cells, purified by these methods.

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## Precise Distance Measurements by Means of an Automated Micro-Axialtomograph in a Standard Light Microscope

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Micro (µ-)axial tomography is a challenging technique in light microscopy improving imaging quality especially in cytogenetic applications by means of defined sample rotation under the microscope objective. The advantage of µ-axial tomography is an effective improvement of the precision of distance measurements between point-like objects. Under certain circumstances, the effective (3D-) resolution can be improved, because the spatial anisotropy typical for nearly all far-field microscopes can be overcome, if at least 3 perspectives are acquired. We present a miniaturised micro-mechanical device in the dimensions of a microscope slide. It allows computer controlled, micro-motor driven rotation of precise glass fibres perpendicular to the optical axis of a microscope lens. A fully automated programme acquires a series of images during rotation of the objects in the field of view. The images taken under different perspectives can automatically be aligned with a precision much better than the full width at half maximum of the point spread function using a newly developed fast algorithm. The theoretically feasible precision of the method was calculated using computer generated data and confirmed by tests on real image series obtained from data sets of 200 nm fluorescent nano-particles. Measurements with test-particles revealed a precision of some ten nanometer for distances in the micrometer range. For the application in cytogenetics high-resolution image information can be obtained using a special software package ("FISH 2.0") which allows automatic segmentation of cell nuclei and labelling sites, determination of intensity bary centres and distance measurements between FISH labelled sites and other nuclear coordinates.

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## Tumor-Derived Fibroblasts Differentially Affect Cell Cycle Distribution of Various Breast Cancer Cells in 3-D Culture

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**Background and Aim:** Fibroblasts are the quantitatively most abundant stromal cell type in desmoplastic ductal invasive breast tumors. These tumor-associated fibroblasts are known to exhibit a spatio-temporal abnormal phenotype and to affect tumor cell growth and differentiation. Very recently, a novel type of co-culture model was established to investigate reciprocal interactions between tumor cells and fibroblasts in a 3-D environment under well-defined conditions. We systematically investigated the impact of tumor-derived fibroblasts on tumor cell proliferation in this in-vitro system in order to identify tumor-cell type specific alterations.

**Methods:** Fibroblasts outgrown from tumor biopsy material (PF37) and three different breast tumor cell lines (BT474, T47D, SK-BR-3) were applied in spheroid mono- and coculture. Spheroids were dissociated after defined times of cocultivation, cell suspensions were fixed in MeOH, and cell cycle distribution was determined by flow cytometry using propidium iodide. A monoclonal mouse anti human cytokeratin

18 combined with a secondary Cy-5 conjugated anti-mouse IgG was applied to discriminate tumor cells and fibroblasts in mixed cell suspensions.

**Results:** Over a period of 7 days the S-phase fraction (SPF) in tumor spheroid monocultures was not altered ranging between 16% and 23% for BT474 and SK-BR-3, and between 10% and 14% for T47D cells. A systematic alteration in SPF throughout cocultivation with PF37 fibroblasts was only recorded in BT474 but not in SK-BR-3 or T47D cells. The SPF of BT474 tumor cells decreased as a function of time in coculture (22.7% +/- 1.7% at day 2 versus 14.7% +/- 4.0% at day 7 in coculture).

**Conclusions:** The effect of tumor-derived fibroblasts on tumor cell proliferation in a 3-D environment critically depends on the tumor cell type. The heterologous interaction mediated a reduced proliferative activity of BT474 cells derived from a solid breast tumor. A similar effect was not shown for T47D and SK-BR-3 cells, both originated from pleural effusions of breast tumor patients. This correlates with the observation that T47D and SK-BR-3 but not BT474 tumor cells seem to have the capacity to induce myofibroblast differentiation in the 3-D coculture model.

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# Gene Expression Profiling of Normal and Malignant Cell Populations: From Cell Isolation to Data Analysis

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DNA microarray technology has a profound impact on biological research as it allows the simultaneous monitoring of the transcription levels of tens of thousands of genes. Soon it will be possible to profile the whole transcriptome of higher organisms with only a few DNA gene chips. Gene expression profiling of the organism's various cell types will allow us to obtain a global view of their different phenotypes. These data will be extremely helpful in unraveling biological pathways and networks and, by comparison with diseased/malignant cells, also in the identification of disease-associated genes. Toward this goal, it is necessary to develop strategies to purify the individual cell types from their respective tissues, and to apply computational algorithms that can extract useful information from the corresponding microarray data sets. The following major issues that need to be addressed when planning a profiling experiment will be discussed: i.) availability of markers that allow cell isolation; ii.) purity of the cell population; iii.) minimal amount of cells required per gene chip hybridization; iv.) number of samples per cell type required to obtain statistically significant gene expression differences; v.) application of suitable biostatistical analysis methods that optimally and correctly interpret the gene expression data. These points will be discussed on the example of a study performed in the laboratory of R. Dalla-Favera at the Institute for Cancer Genetics, Columbia University. The study was aimed at comparatively analyzing gene expression profiles generated from purified normal human B-lymphocyte subpopulations and B-cell lymphomas i.) to investigate the cellular derivation of B-cell tumors and their relationship to normal B-cells, and ii.) to identify genes that are deregulated in B-cell tumors. The analysis was

performed using oligonucleotide-based DNA microarrays (Affymetrix U95A gene chip) representative of ~12,000 genes.

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# Approaching the Three-Dimensional Organization and Dynamics of the Human Genome by Virtual Microscopy

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To approach by virtual microscopy the three-dimensional organization of the human cell nucleus, the structural-, scaling- and dynamic properties of interphase chromosomes and cell nuclei were simulated with Monte Carlo and Brownian Dynamics methods. The 30 nm chromatin fiber was folded according to the Multi-Loop-Subcompartment (MLS) model, in which ~100 kbp loops form rosettes, connected by a linker, and the Random-Walk/Giant-Loop (RW/GL) topology, in which 1-5 Mbp loops are attached to a flexible backbone. Both the MLS and the RW/GL model form chromosome territories but only the MLS rosettes result in distinct subcompartments visible with light microscopy and low overlap of chromosomes, -arms and subcompartments. This morphology and the size of subcompartments agree with the morphology found by expression of histone autofluorescent protein fusions and fluorescernce in situ hybridization (FISH) experiments. Even small changes of the model parameters induced significant rearrangements of the chromatin morphology. Thus, pathological diagnoses based on this morphology, are closely related to structural changes on the chromatin level. The position of interphase chromosomes depends on their metaphase location, and suggests a possible origin of current experimental findings. The chromatin density distribution agrees with the MLS model and recent experiments. The scaling behaviour of the chromatin fiber topology and morphology of CLSM stacks revealed fine-structured multi-scaling behaviour in agreement with the model prediction. Review and comparison of experimental to simulated spatial distance measurements between genomic markers as function of their genomic separation also favour an MLS model with loop and linker sizes of 63 to 126 kbp. Visual inspection of the morphology reveals also big spaces allowing high accessibility to nearly every spatial location, due to the chromatin occupancy <30% and a mean mesh spacing of 29 to 82 nm for nuclei of 6 to 12 µm diameter. The simulation of diffusion agreed with this structural prediction, since the mean displacement for 10 nm sized particles of ~1 to 2 µm takes place within 10 ms. Therefore, the diffusion of biological relevant tracers is only moderately obstructed, with the degree of obstruction ranging from 2.0 to 4.0 again in experimental agreement.

## Slide-Based Immunophenotyping by Six Colour Laser Scanning Cytometry

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**Aim:** To further increase the capacities of slide-based immunophenotyping by detection of the near-infrared tandem-dyes PE-Cy7 and APC-Cy7.

**Material and Methods:** The filter settings for the 4 photomultipliers of the standard LSC were changed: PMT1 and PMT2 were kept for detection of FITC and PE, respectively, but PMT3 was changed for PE-Cy5 and APC, and PMT4 was optimised for PE-Cy7 and APC-Cy7. Human PBLs were incubated with biotinylated anti-CD3 and with streptavidin conjugated to one of the six dyes; this step was performed in order to calculate the resolution and the spill-over and to establish a cross-compensation matrix. Human PBLs were then incubated with a panel of six different CD-markers for lymphocyte subtyping.

**Results:** For PE-Cy7 the resolution is in the range of FITC, but for APC-Cy7 it is ~30% lower. The spill-over is prominent into the respective donor-channel. Since the LSC operates with two lasers emitting at 488nm and 633nm, we were able to simultaneously detect and differentiate FITC: CD4, PE: CD19, PE-Cy5: CD16&56, APC: CD45, PE-Cy7: CD3 (biot.), and APC-Cy7: CD8.

**Discussion:** With this adaptation it is possible to measure the tandem conjugates PE-Cy7 and APC-Cy7. This new set-up opens the way for six-colour immunophenotyping by slide-based cytometry. This potential will further be exploited by applying re-staining assays that re-analyse the same cells after incubation with a second set of CD-markers.

## Quantification of Eosinophilia in Nasal Polyps by FITC Binding

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**Aim:** Eosinophils are made responsible for the development and recurrence of nasal and paranasal polyposis in a subset of patients. Although an aggressive therapy for polyposis with dense eosinophilic infiltration is proposed there is no objective and quantitative way to determine the relative percentage of eosinophils per epithelial cells in polyps up to now. In general analysis is performed semi-quantitatively by subjective judgement of tissue sections. We were looking for a standardised method that yields exact data.

**Material and Methods:** Eosinophils are known to bind FITC with low Kd. Usually this is an unwanted feature interfering with the specific detection of antigens by FITC-conjugated antibodies. We made use of this special feature of eosinophils and established an assay that detects eosinophils by FITC. Polyps were obtained during routine paranasal surgery. The tissue was dissociated mechanically and the cell suspension was placed on conventional microscope slides. After fixation with ethanol cells are incubated with anti-cytokeratin-APC, PI, and FITC. Slides are analysed by laser scanning cytometry. For evaluation, manual counting of 3,000 cells per slide was performed, too. Quantitative data were compared with semi-quantitative analysis by routine histology.

**Results:** Manual counts of epithelial cells and eosinophils and data by LSC showed good correlation (r = 0.78). There was no correlation with routine histology. Also, analysis of the clinical data concerning history of allergy or recurrence did not show a correlation with the percentage of eosinophils.

**Discussion:** The discrepancy between LSC and routine histology might be caused by the influence of artificial oedema of the polyps; this changes the density of cells in a section and will lead to misinterpretation by routine histology. Most of the speculation on the role of eosinophils, however, is based on the subjective judgement on section analysis. By taking objective quantitative data, we did not see any correlation with the clinical course.

**Conclusion:** In future, not the percentage but the function of selected cell subtypes should be analysed. This could give new insights into the pathophysiology of polyposis.

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# Near-Ffield Scanning Optical Microscopy (NSOM): Optical Super Resolution and Single-Molecule Detection Sensitivity on the Cell Membrane

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Fluorescence microscopy has gained a firm position among the most important research tools in modern cell biology and continues to yield insight in the complex mechanisms of cell signaling. It is becoming increasingly clear, however, that understanding of the early events in cell signaling requires insight into the assembly and spatial organization of the plasma membrane on the molecular (submicroscopic) level. The maximal attainable resolution using light-microscopy – ~250 nm – is dictated by the laws of diffraction. The challenge to break this diffraction limit has led to the development of several novel imaging techniques. One of them, near-field scanning optical microscopy (NSOM) combines the high resolution of scanning probe microscopy with the contrast of optical microscopy. NSOM, therefore, allows fluorescence imaging at a resolution of only a few tens of nanometers. Importantly,

because of the extremely small excitation volume, near-field excitation dramatically reduces background fluorescence from the cytoplasm. In this lecture examples will be presented of NSOM applications in studies on distribution-function relationships of cell adhesion receptors labeled either directly, using Green Fluorescent Protein (GFP), or indirectly, by immuno-fluorescence labeling procedures. It will be shown that NSOM allows for 1) quantitative fluorescence imaging with single-molecule detection sensitivity even on the surface of cells that show severe auto-fluorescence, and 2) independent observation of molecules at physiologically relevant packing densities. So far, this level of sensitivity can only be reached on fixed cells, but similar results should be achievable under physiological conditions in the near future.

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# How Does the Fluorescence of DNA Specific Dyes Depend on Base Composition and Base Sequence? Comparison of the Sequenced Species *Oryza Sativa* and *Arabidopsis Thaliana*.

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It is well known that some of the DNA-binding dyes are base specific. DAPI and the Hoechst dyes bind preferentially to adenine and thymine (AT), mithramycin to guanine and cytosine (GC). The relation between AT or GC frequency of the nuclear DNA and fluorescence intensity of these dyes is not quite clear, but it seems that 3 – 5 consecutive base pairs of the same type are necessary for binding. This results in a non-linear relation between AT/GC ratio and fluorescence intensity.

However, because the bases are non-randomly distributed within the DNA sequence, the fluorescence intensity is expected to be not only a function of base pair ratio, but also of the base sequence.

The first sequenced species of higher plants, *Oryza sativa* and *Arabidopsis thaliana* give the opportunity to verify this relation.

The result is rather surprising: For all 4 tested dyes (DAPI, Hoechst 33258, Hoechst 33342 and mithramycin) the number of bases of the same type necessary to bind one dye molecule is probably 1, but in no way greater than 2. This is in contradiction to results obtained by other methods and may indicate that the simple theory of several consecutive bases of the same type binding one dye molecule is not correct.

## Living Cells in Depth: Analysing Cellular Processes by Ultrafast 4D Recording of two Fluorescent Tagged Proteins and High Throughput Deconvolution

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Ultrafast tri-dimensional time lapse (4D) imaging consists in recording Z-series very rapidly (typically > 15 images/sec). Deconvolved Z-series are then used to generate 3D animations and to analyze 3D movements. It can record structures labeled by fluorescence-tagged proteins moving in all directions at speeds reaching 2-3 µm/sec. It has proven potential for describing transient events of cellular processes and for studying protein function. The aproach is still in its childhood. Many studies require the recording in this very fast way of at least two fluorescent proteins within the same cell. This provides a powerful tool for studying the spatial and temporal relationships between visualized structures, the dynamic association of proteins with labelled structures, or the efficient analysis of invalidation of function phenotypes. We have built a 2 colour 4D (2C 4D) imaging system which rapidly records 2C Z-series allowing for the timelapse analysis of cellular processes visualized by one or two fluorescent chimeric proteins (e.g. GFP and DsRed). Data certifying the mechanical and optical stabilities during 2C 4D imaging will be presented. 4D imaging also requires heavy computing to perform computationally deblurring, and this is problematic for the very large data sets (1-3 Go) processed by 2C 4D imaging. Routine 4-D imaging depends on the availability of fast and automated deconvolution methods and streamlined procedures for the analysis of 3D movements and other parameters. We have adapted a constrained iterative deconvolution algorithm, not widely used in light microscopy, which yields greatly improved image contrast at much increased speed on a standard PC. Incorporated into software, it enables batch-wise deconvolution of hundreds of 2 colour stacks on a normal PC in a few hours. As example of the use of this system, the elucidation of the role of dynein/dynactin containing positional cues in spindle positioning in MDCK cells co-expressing GFP-tubulin and DsRed LIS1 will be presented. It is anticipated that our approach, and future evolutions of it will contribute to the widespread use of nC 4-D imaging for exploring cellular processes and for studying gene product functioning.

## Novel Applications of Immunohistochemistry in Diagnostic Histopathology

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Light microscopy is the main tool of diagnostic histopathology. For the clinical pathologist, a relatively simple microscope without subtilized technique remains to be sufficient not only for conventionally stained slides but also for immunohistochemistry.

There are, however, novel technical developments which relate to (1) improved methods of antigen detection in form-alin-fixed, paraffin-embedded tissue specimens and (2) the continuing emergence of new monoclonal antibodies (MAbs) against cell and tissue antigens which are not only useful for sophisticated diagnosis of tumors but, in some instances, also highly relevant for specific tumor therapies.

Formalin fixation and paraffin embedding remains the standard method for pathological analysis of clinical tissue material; it is simple, widely established and provides superior morphology. However, due to masking of antigenic epitopes by formaldehyde, immunohisto-chemistry usually requires antigen retrieval methods. Rather than the older protease diges-tion methods, heat-induced epitope retrieval (HIER; microwave-oven heating, pressure cook-er heating, combined instruments) has become the method of choice for many diagnostic antibodies. While for some antibodies [e.g. MAbs against cytokeratins (CKs) 5, 8, 20, vimentin], improved staining is obtained by HIER, other antibodies (e.g. MAbs against CKs 4, 14, 17, E-cadherin, uroplakins) require HIER to be reactive at all with paraffin sections.

There are various fields of novel applications of immunohistochemistry in pathology. One is the analysis of individual CKs in metastatic carcinomas using selective MAbs meanwhile available (Moll R, Subcellular Biochem 31:205-262, 1998). Specific CK patterns may be valuable adjuncts for the identification of an unknown primary tumor. Examples are the identification of squamous cell carcinomas ($CK5^+$), the subtyping of adenocarcinomas on the basis of their origin (e.g. colorectal carcinomas: $CK20^+$ / $CK7^-$), the identification of malignant mesotheliomas ($CK5^+$) and the distinction of Merkel cell carcinomas ($CK18^+$ / $CK20^+$) from small cell carcinomas of the lung ($CK18^+$ / $CK20^-$).

The calcium-dependent cell-cell adhesion molecule E-cadherin is widely distributed among epithelial tissues and tumors. For histodiagnosis, MAbs against E-cadherin (e.g. HECD-1, 5H9) are useful for the typing of breast cancer since ductal carcinomas are positive while the diffusely infiltrating lobular carcinomas are negative for E-cadherin. Uroplakin III, a specific differentiation marker of urothelial umbrella cells, may be helpful in the diagnosis of urothelial carcinoma in metastatic specimens (Moll R et al., Am J Pathol 147:1383-1397, 1995; Kaufmann O et al., Am J Clin Pathol 113:683-687, 2000). Although the sensitivity of uroplakin III antibodies (MAb AU1) is only moderate (50% – 60%), the specificity seems to reach 100%.

Several antigens which have become routinely detectable by immunohistochemistry have therapeutic relevance in terms of molecular targeting. The first tumor antigen to be targeted with a therapeutic MAb (Rituximab) is CD20, expressed in B-cell lymphomas. HER2, a tyrosine kinase receptor, is overexpressed (due to gene amplification) in ~25% of breast cancers which then may be treated by a humanized MAb, Trastuzumab. c-kit (CD117), a receptor tyrosine kinase, is mutationally activated in gastrointestinal stromal tumors (GISTs) which can be treated by a specific tyrosine kinase inhibitor, Imatinib. Immunohistochemical demonstration of these antigens – to prove their expression in the individual tumor – is required prior to application of these therapies.

In conclusion, applications of light microscopical immunohistochemistry in clinical pathology are still expanding. Next to valuable diagnostic information provided by cell and tissue specific MAbs, certain immunohistochemical analyses may be the basis of modern individualized tumor therapy.

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## Automating Interphase FISH Analysis in Tissue Sections

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As fluorescent cell assays have become the methods of choice for a number of applications, there is an increasing demand for fast and efficient screening systems. Over the last couple of years we developed our Metafer multipurpose hardware platform for automated screening of microscope slides and microtiter plates.

Besides the detection of rare cells at occurrence rates of as little as 1 in several millions, we are mainly interested in interphase FISH scoring of chromosome aneuploidies, gene amplifications/deletions, and chromosome translocations.

Extending these analyses from single cell preparations to tissue sections bears a number of specific problems: (a) due to the high cell density it is quite often impossible to find a sufficient number of single nuclei for the analysis, (b) signal detection suffers from a great variability in hybridization efficiency; (c) cells of interest are interspersed within the tissue; (d) large clusters of FISH spots (homogenously stained regions: HSR) cannot be resolved by "spot counting".

In order to overcome these obstacles, we devised a novel "tile sampling" strategy to obtain statistical meaningful results from tissue areas: FISH signals are measured in non-overlapping equi-sized squares ("tiles"), placed automatically according to the counterstain intensity.

In a case study on the semi-automatic scoring of *her2-neu* breast cancer gene amplification, we could demonstrate the validity and robustness of our approach. On the basis of 95 cases the excellent correlation between manual and automatic evaluation was proven. The frequent occurrence of HSR made it impossible to use conventional FISH spot counting. Samples were automatically classified as HSR and non-HSR, and either "tile sampling" or "spot counting" algorithms were applied accordingly.

Currently, we explore the possibilities of applying the "tile sampling" strategy also onto tissue arrays and living cells in microtiter plates. First results will be presented.

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# Flow Cytometric Detection of Malaria Pigment-Containing Neutrophils by Wavelength Dependent Depolarized Light Scatter

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Flow cytometric measurement of depolarized light scatter at a single wavelength has been exploited for malaria detection of blood samples of malaria patients. This procedure results in modified scatter diagrams of white blood cells when observing intensity and depolarized side scatter simultaneously. In particular, signals corresponding to monocytes with inclusions of malaria pigment, i.e. hemozoin, appear in regions usually not associated with white blood cells. We found pigment-carrying monocytes (PCM) to appear at a relative frequency of about $2 \cdot 10^{-5}$ to $10^{-2}$ with respect

to the total leukocyte count. Significantly higher relative frequencies were determined for semi-immune patients compared to non-immune patients.

During accumulation of the birefringent malaria pigment hemozoin, the intensity of depolarized light scatter of PCM and of pigment-containing neutrophilic granulocytes ($PCG_n$) increases, thus allowing to discriminate PCM from other leukocytes. On the other hand, pigment-containing neutrophilic granulocytes interfere with normal eosinophils and cannot be detected by depolarized light scatter at a single wavelength.

We observed that the dependence on wavelength of the relative degree of depolarization determined for PCM and $PCG_n$ is different from that of normal white blood cells, presumably due to the strong absorption bands of hemozoin close to 410 nm and 525 nm. Simultaneous measurement of depolarized side scatter at two suitably chosen wavelengths (413 nm, 633 nm) allows for the identification of $PCG_n$ in the corresponding correlated scatter diagram. This result was confirmed by cell sorting and subsequent microscopic evaluation of the sorted cells. Present studies aim at improving discrimination of $PCG_n$ from other leukocytes including PCM by selecting the wavelengths and at determining relative concentrations of PCM and $PCG_n$ simultaneously.

„Alternatives – A Personal View on 30 Years of Flow Cytometry"

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# "Alternative": Personal Experience of 30 Years in Flow Cytometry

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Wie können Zellen synchronisiert werden, wie kann man die synchrone Vermehrung messen? Welches Durchflusszytometer kann man dazu gebrauchen, Lampe- oder Laser-Gerät? Wie lässt sich die Zellzykluskinetik synchroner und asynchroner Zellen nach Bestrahlung messen bzw. wie kann man DNA-Verteilungen analysieren? Wie lässt sich die Messung der Zellzykluskinetik synchroner und asynchroner Zellen ausweiten (DNA-RNA mit AO)? Wie kann man G2- von M-Zellen unterscheiden? Wie lässt sich die Dauer der Zellzyklusphasen messen (mit BrdUrd)? Kann man Mikrokerne im Durchflusszytometer messen? Kann man in Mikrokerne hineinschauen? Wie kann man Chromosomenaberrationen im Durchflusszyto-meter messen? Wie ist es mit der Slit-Scanning Zytometrie zur Messung von dizentrischen Chromosomen? Haben wir alle nicht schon immer Apoptose gemessen? Jetzt machen wir es aber richtig! Wollten wir alle nicht schon immer dieselben Zellen noch einmal sehen und vermessen (Laser-Scanning-Cytometer, LSC)?

Zur Lösung dieser Fragestellungen gab es eine Reihe von Alternativen:

1. Durch die Mitte oder am Rand entlang?
2. Zellzyklus: Volumen oder DNA? –
3. Lampe oder Laser?
4. Wiegen oder Zählen?
5. Synchron oder asynchron?

6. Ein oder mehrere Parameter?

7. G2 oder M?

8. Mit oder ohne BrdUrd?

9. Debris oder Mikrokerne?

10. FISH oder FACS?

11. Karyotyp oder Flow Karyotyp?

12. Pulsfläche oder Pulsform?

13. Schrott oder Apoptose?

14. FCM oder LSC?

GSF-Flow Cytometry Group, Neuherberg

30 years of scientific research using various flow cytometric techniques and equipment will be described by means of some examples from our laboratory. The following questions will be addressed that were and still are important for the solutions of several scientific problems:

How can cells be synchronized, how can a synchronous proliferation of cells be measured? Which flow cytometer should be used, laser of Hg-arc lamp based systems? How can we analyse DNA distributions to study cell cycle kinetics after irradiation? How can the measurement of cell proliferation be enhanced using AO-techniques? How can we discriminate between G2- and M-phase cells? How can we measure duration of cell cycle phases using incorporation of BrdUrd? Can we measure micronuclei using a flow cytometer? Ca we look into a micronucleus? How can we measure chromosomal aberrations using a flow cytometer? Is it possible to detect dicentric chromosomes using a home-made slit scanning flow cytometer? How can we measure apoptotic cells, haven't we done this already 20 years ago? Let's now do it in the right way! Haven't we always tried to see and measure exactly the same cells again? What about laser scanning cytometry?

To solve these problems we had several alternatives during these 30 years:

1. Through the middle or along the periphery of an orifice?

2. Cell cycle: volume- or DNA-dependent?

3. Lamp or laser?

4. Weighing or counting?

5. Synchronous or asynchronous?

6. One or more parameters?

7. G2- or M-phase?

8. With or without BrdUrd?

9. Debris or micronuclei?

10. FISH or FACS?

11. Karyotype or flow karyotype?

12. Pulse area or pulse shape?

13. Debris or apoptosis?

14. FCM or LSC?

# Digital Image Analysis and Immunophenotyping of Lymphocytes in Normal and Pathological Conditions.

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The methods of quantitative estimation of morphocytochemical peculiarities of cells, tissues, organs are very important now. Digital image analysis belongs to such methods, and it was performed in the laboratory of immunomorphology of Institute of Clinical and Experimental Lymphology SB RAMS thanks to the special apparatus and programmes. The densito-geometric parameters of DNA, enzymes and cell components have been evaluated in the lymphocytes of healthy objects, during the induction of autoimmune experimental processes and diseases, during the pathology of the lymphatic system, during the investigation of the drugs, food supplement, sorbent action. Also immunophenotyping of leukocyte subpopulations has been made with the help of flow cytometry with monoclonal antibody.

The changes in DNA, chromatin structure, enzyme activity and immune state have been found in lymphocytes of healthy persons of different gender, which was connected with the differences in subpopulational content and immune state of lymphocytes.

It has been shown that during experimental autoummune reaction and autoimmune disease (rheumatoid arthritis) and their treatment with immunomodulators marked changes in immunomorphological parameters of lymphocytes have been observed connected with the processes of activation, proliferation, differentiation and apoptosis of immune cells

The peculiarities of immunomorphological parameters of lymphocytes did not stay constant under the pathology of lymphatic system - lymphatic edema of upper and lower extremities.

We also managed to show the influence of sorbents and food supplements on the functional state of immune system and densito-geometric peculiarities of lymphocyte.

One can say about practical use of digital image analysis and immuno-phenotyping of lymphocytes in prognosis of the efficacy of treatment of the disease and character of its duration.

The meaning and information of digital image analysis and immunophenotyping of lymphocytes proved to be very high in the medical and biological investigations.

# Analysis of the Influence of Recombinant Mistletoe Lectin 1 on Peripheral Mononuclear Blood Cells from Different Donors Using c DNA Arrays

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Various immunemodulators have been used in the past as adjuvances to enhance immune reactions after immune-supressive or -destructing therapies. In general, enhanced proliferation and activation of immune cell subpopulations and influence on the expression patterns of various cytokines was reported after application of immunestimulators. For the majority of the applied substances neither the general mode of action nor the influence on defined cell subpopulations has been described so far. The effect of proposed immunemodulators can be monitored on an transcriptional level by expression profiling. Influences on defined cell subpopulation is of special experimental intrest. Here, the influence of a immunemodulator was investigated on transcriptional level by PIQORTM cDNA arrays in an in vitro model. In short, peripheral mononuclear blood cells (PBMCs) have been isolated from different donors. PBMCs from each donor were splitted and treated with the immunemodulator or PBS as a control. Stimulation was terminated and total RNA from a part of treated or untreated PBMCs was prepared, processed and hybridized on arrays which represented 648 genes involved in immunology, cancer, apoptosis, cell-cell and cell-matrix interaction. Because monocytes are suspected to be the primary aim the investigated immunemodulator, CD14+ cells were separated from the other part of the PBMCs after treatment by magnetic cell sorting. Total RNA prepared from CD14+ cells was amplified and also hybridized on PIQOR cDNATM arrays. Expression profiles resulting from the described experiments show high comparability with previouse results on the transcriptional or translational level. We also demonstrated, that array based expression profiling of enriched cell populations is feasible. Comparison between expression profiles resulting from PBMC pools and CD14+ cells of the same donor show a major enhancement in detection sensitivity of expressed genes. These results clearly show the benefit of combining expression profiling with cell sorting.

# Telomeres and Nuclear Reorganization During Meiosis

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Somatic interphase nuclei undergo architectural changes during development and differentiation. The most striking alterations of nuclear topology occur during the extended prophase of the first of the two meiotic divisions, when homologous interphase chromosomes reorganize and extend below an intact nuclear envelope to pair and recombine with each other. Recombination provides physical links between homologues that allow them to separate from each other during the reduction division (Meiosis I). The second meiotic division follows without an intervening S-phase and ultimately creates haploid gametes or spores from diploid progenitor cells, thereby

compensating the genome doubling that occurs at fertilization. Telomeres, which occupy relatively fixed positions in the mitotic interphase nucleus, reposition to the nuclear envelope at the onset of meiotic prophase. Once attached to the inner nuclear membrane telomeres obtain a mobility that culminates in temporal telomere clustering opposite to the cytoplasmic microtubule organizing center, which is know as bouquet formation. By molecular cytology of meiosis of yeast and mouse mutants we have gained insight in the requirements for meiotic telomere dynamics. It was found that bouquet formation catalyses homologue pairing and, in synaptic meiosis, depends on the presence of the telomere protein *sc*Ndj1p. Emerging evidence will be discussed which indicates that epigenetic modifications are important for entry into prophase I, while signaling from meiotic DSB repair influences homologue pairing and the release of meiotic telomere clustering.

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# Increased Genetic Damage with Age Detected with SCSA and Comet

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**Introduction:** With advances in health care more men are fathering children at advanced ages. The male germ-line mutation rate is known to increase with age, but the mechanism and consequences are little understood. The purpose of this study was to determine the frequency of DNA strand breaks and chromatin disturbances in mouse spermatozoa increases with age.

**Materials & Methods:** Sperm samples were obtained 12 hours after treatment from 5 old (15 months of age) and 5 young (2 months of age) untreated mice and from 5 Doxorubicin treated (12 mg/kg) old and young mice. Defective chromatin packaging was quantified by the Sperm Chromatin Structure Assay (SCSA) with a flow-cytometer. DNA strand breakage were measured by the Comet Assay.

**Results:** SCSA analysis showed a significant increase in disturbed chromatin in sperm of the old untreated mice compared with the young untreated mice (4.6% vs. 3.4%, $p=0.027$) and in sperm of the old Doxorubicin treated mice compared with the young Doxorubicin treated mice (4.4% vs. 3.5%, $p=0.015$). There was no difference between treated and untreated animals in the same age group.

Using the Comet assay, a significant increase ($p<0.01$) in tail moment was found within the group of old untreated mice compared with young untreated mice (5.2 vs. 2.16) and in the group of old Doxorubicin treated mice compared with young Doxorubicin treated mice (4.93 vs. 2.81), demonstrating significantly high levels of DNA-strand breaks. There was also a significant difference between treated and untreated young mice (2.81 vs. 2.16, $p<0.01$), but not between treated and untreated old mice.

**Discussion and Conclusions:** Our data indicate that aged mice have elevated levels of sperm chromatin disturbances and sperm DNA-strand breaks, which has not previously been reported. The lack of effects of doxorubicin in the SCSA suggests that sperm nuclei are not susceptible to this agent. However, the effect seen with the

Comet Assay in young mice indicates that SCSA and Comet are detecting different types of damage and that ageing alters the susceptibility of sperm to genotoxins. These data are the first reporting the use of SCSA and Comet assays to evaluate genetic damage in mouse sperm related to advanced age.

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# Analytical Representation and Approximation of Distance Distributions of Indepently Distributed Points in Cell-like Objects

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In a manifold of applications, the distances of two points each in a cell are measured and contribute to a distribution depending on experimental parameters. An example are the different distributions of specifically labelled fluorescent sites on chromosomes with respect to different situations within the cell cycle or aberration status of a cell. In this case, the points of interest can be the intensity bary centres of the labelled sites acquired by microscopic imaging.

As a first step of evaluation the comparison of the measured distribution (e.g. intensity bary centres) to the distribution of distances of independently distributed points in the object under consideration

(e.g. cell nucleus) can give hints on the effects of the experimental treatment. As the probabilistic simulation of such a distribution can be very time consuming, it is desirable to have analytical formulae at hand.

We give a description of the general method to compute distance distributions and mention several examples of exact results for interesting geometric objects like 2-D and 3-D balls and sphere shells. The calculation in some cases involves elliptic integrals, hence analytical expressions for the final distribution cannot be obtained. Here, spline functions yield analytical approximations which are easy to evaluate. Once established, analytical formulae can eliminate costly probabilistic simulations.

## Nanosizing of Fluorescent Objects in Cytometry

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Here, a new application is described to determine the size of individual fluorescent objects far below the optical resolution limit, using Spatially Modulated Illumination Microscopy (SMI) in combination with SMI-Virtual microscopy (SMI-VIM). SMI-VIM simulations, performed using excitation wavelengths between 360nm and 647nm indicate that reliable size measurements down to about 20nm (1/30 of the conventional optical resolution, e.g. 600nm) are feasible even under relatively low photon count conditions (e.g. total number of detected photons N_tot=10000).

This method is based on the fact that the modulation of the axial Point Spread Function (PSF) of the SMI microscope is "disturbed" by the size of the object. Using appropriately calculated VIM calibration functions, this "disturbance" of the modulation can be used to determine the original size of the object.

Furthermore, experimental SMI measurements of fluorescent objects with known diameter were performed using 488nm. The results of such measurements on fluorescent spherical particles (beads) with a nominal diameter (according to the manufacturer) ranging between 25nm and 140nm indicate that experimental size measurements are, actually, feasible with an accuracy in the range of a few nanometers. This corresponds to the size of a small nucleosome cluster.

Using SMI microscopy with a cytometric setup, this "nanosizing" method allows a variety of applications, e.g. analysis of protein clusters, deformation of fluorescent objects; analysis of gene expression, transcription factories, or gene copy in 3D-intact nuclei. An important practical feature of the SMI-nanosizing method described is that such studies may be conducted using conventional object slides.

## Identification of Deficient Mitochondrial Apoptosis Signaling in Leukemia Cells by a Novel Flowcytometric Method for Detection of Mitochondrial Cytochrome C Release.

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Deficient activation of apoptosis signaling pathways may be responsible for drug resistance and treatment failure of malignant diseases. Several apoptosis pathways converge on mitochondria. Mitochondrial apoptosis signaling is initiated by changes in mitochondrial membrane integrity, delivering mitochondrial signaling molecules like cytochrome c into the cytosol. We analyzed mitochondrial apoptosis resistance in leukemia cells by a novel method for the detection of cytochrome *c* release in intact cells by flowcytometry. In CD95 receptor and cytotoxic drug induced apoptosis in

Jurkat T cell leukemia cells, mitochondrial cytochrome *c* release could be detected by flowcytometry as reduction of the intracellular cytochrome c signal after appropriate fixation and permeabilisation. The experimental system of type I and type II cells with differential requirement for mitochondrial signaling in CD95 induced apoptosis was used for analysis of mitochondrial apoptosis resistance. Interesitngly, we found deficient cytochrome c release in the presence of caspase-3 activation as a sign of mitochondrial apoptosis resistance in SKW (type I) and Jurkat (type II) cells overexpressing Bcl-2. In addition, mitochondrial apoptosis resistance was analyzed in primary leukemia samples treated with anticancer drugs in vitro. In 8 samples of patients with acute leukemia, cytochrome c release and caspase-3 activation was detected. In leukemia cells of a patient with resistant disease, cytochrome c was not released after in vitro drug treatment despite activation of caspase-3. Thus, deficient cytochrome c release analyzed by flowcytometry identifies mitochondrial apoptosis resistance. Identificiation of mitochondrial apoptosis resistance in primary leukemia cells by flowcytometry may be predictive for drug resistance and treatment failure in acute leukemia.

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## Degradation of a Xenobiotic Using a Binary Culture and Multi-Parametric Flow Cytometry

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To study the degradation of a xenobiotic that requires a mixed culture it is essential to monitor the proportions and to control the population dynamics of the component strains. For these purposes fluorochromising techniques and multi-parametric flow cytometry were used to follow *Rhodococcus erythropolis* K2-3 and *Ochrobactrum anthropi* K2-14, both of which are needed to degrade the herbizide 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB). Although the two strains can grow in constant proportions in mixed cultures on other substrates, 2,4-DB could not be degraded as a sole substrate in a continuous process and *R. erythropolis* K2-3 was clearly impaired in the binary mixture. Addition of a second, easily assimilable substrate (xylitol) in appropriate concentrations (empirically determined) helped this strain survive, and thus facilitated complete degradation of the xenobiotic. This combination of substrates was found to stabilise the growth of *R. erythropolis* K2-3 and, consequently promoted the action of *O. anthropi* K2-14. Thus, the two organisms became established in constant proportions in a continuous process until reaching steady state. Consequently, multiplication and cell division activities of the two components of the binary culture were high and reached similar values to those attained when they are grown in pure culture.

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## Renaissance of Fluorescence Resonance Energy Transfer in Mapping the Cell Surface

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The interaction of the cell surface proteins plays a key role in the process of transmembrane signaling. Fluorescence resonance energy transfer (FRET) is an excellent tool for determining distance relationships of cell surface molecules, since it can be applied on live cells without major interference with the physiologic condition of the cells. FRET is a physical process by which energy is transferred non-radiatively from and excited fluorophore (donor) to another chromophore (acceptor) via long-range dipole-dipole interaction. The FRET efficiency (E) decreases with the $6^{th}$ power of the donor-acceptor separation over the range of 1-10 nm, a distance usually separating most biologically relevant macromolecules interacting with each other.

Flow cytometric energy transfer (FCET) measurements can reveal cell-to-cell heterogeneity within the cell population with high statistical accuracy in a reasonably short timeframe. In the latest version of FCET cyanine dyes with long emission wavelength and a cell-by-cell correction of autofluorescence are applied. The increased accuracy of the new method makes cells with low receptor expression amenable to FCET investigation.

Applied in the fluorescence microscope, FRET is a very selective and sensitive tool for resolving spatial heterogeneity of molecular interactions within single cells with a spatial resolution imposed by the inherent diffraction limit of optical microscope. In a confocal microscope capable of multiple (at least three) excitations both donor and acceptor photobleaching FRET can be performed sequentially on the same sample. This dual FRET approach opens the scope of the questions, which can be addressed with image FRET technique.

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# Six and More Color Immunophenotyping on the Slide by Slide Based Cytometry

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There is always a need for new colors in immunology to detect subsets of leukocytes. Detection of minor cellular subsets such as dendritic cells, antigen specific T-cells or circulating stem-cells need a minimum of three to four colors. But for further characterization additional colors are necessary, this is however not achievable with most of the state of the art technologies. Fluorescence imaging techniques allow the simultaneous analysis of three or at maximum four colors. By Laser Scanning Cytoemtry (LSC), a relatively new slide based cytometry technology, up to five colors are detectable. The guiding principle of the instrument is immobilization of the specimen on the microscope slide. This has several advantages: cells are not lost in a fluid stream but are kept on the slide and therefor even minimal specimens as low as 1.000 cells can be analyzed. In addition, cells are available for further analyses; such as for the documentation of the morphology, FISH or single cell PCR. We have developed assays for immunophenotyping of peripheral blood leukocytes (PBLs) the LSC whereby we can analyze simultaneously the binding of up to six different

antibodies taking 10µl blood. The specimen is prepared according to routine FCM protocols. With our adaptation combinations of the following dyes can be used in a single run FITC, PE, PE/Cy5, PE/Cy7, APC and APC/Cy7. Using different antibody combinations minor subsets can be quantified and morphologically documented. This assays further improves the microanalytical immunophenotyping enhancing both, the quantity and resolution of the data. The six color immunophenotyping on a slide may pen new approaches in immunology and immune diagnostics and can be further extended to seven or eight colors.

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## CellTracks : A Multifunctional Cell Analysis Instrument

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The CellTracks cell analysis system, is based on the immuno-magnetic selection, followed by the magnetic separation, and aligning of the selected cells along ferromagnetic lines nickel (Ni). These Ni lines are present on the inside uppersurface of a capillary which is placed in a strong magnetic field.

After the aligning, a 635 nm laser is focused on the aligned cells with a regular Compact Disk (CD) objective. By moving the capillary in the direction of the Ni lines, the cells pass the laser focus one after the other. For each cell the fluorescence signal, which identifies the cell, together with its position are recorded.

After this first quick scan the aligned cells can be revisited since their location is known and a fluorescence image of the selected cell can be acquired.

The magnetic force is strong enough to hold the cells in position while replacing the buffer in which they reside. Fluorescent labels, medicines or other reagents can be added to the cells and the biological response as a function of time of a single cell, selected from the large number of aligned cells, can be followed by measuring the fluorescence intensity or acquiring fluorescence images of that specific cell.

This CellTracks cell analysis system is significantly less complex than current cell analysis equipment and provides additional functionality through its ability to subject cells to repeated and varied analysis with the possibility to acquire a fluorescence image of each specific event.

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## Clinical Application of Spectral Karyotyping in Cancer Cytogenetics

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The wealth of cytogenetic information has enabled the identification of chromosomal abnormalities and has greatly assisted the comprehension of chromosomal causation of human diseases. Fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH) techniques have added a great amount of information to that provided by conventional cytogenetics. However, none of these can resolve the problem of unidentifiable chromosomes, so called marker chromosomes, especially in cancer cytogenetics, due to poor mitosis morphology. Recently, a novel molecular cytogenetics technique name spectral karyotyping (SKY) was introduced to facilitate the more precise identification of complex aberrations that result in marker chromosomes. The simultaneous hybridization of 24 fluorescently labeled chromosome painting probes allows the visualization and analysis of each chromosome in a single image exposure. This technique is based on the measurement of complete emission spectra, r!

anging between 400 and 800 nm, using a combination of a Sagnac interferometer and a CCD camera (Applied Spectral Imaging, Migdal HaEmek, Israel). The characteristic spectral information available for each image point permits the identification of the dye combination used to label the individual chromosome and thus automatic chromosome classification.

We employed SKY for the study of secondary leukemias and childhood neuronal tumors, often characterized by poor chromosome morphology and complex karyotypes. SKY identified partially characterized rearrangements, defined markers of unknown origin, reclassified G-banding, and permitted analyses of polyploid and poor-morphology metaphase cells. This pool of additional information not only allowed us to define different clones within each malignancy but further comprehend the pathways of clonal evolution.

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# Characterization of a Binary Culture of Lactobacteriaceae Using Flow Cytometry

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*Lactobacteriaceae* are widely used for manufacturing fermented milk products. Quantitatively well-defined mixed cultures are added to get dairy products of stable quality.  On the whole the perfor-mances of the pure strains are already documented, however only minor knowledge exist about their action during growth in mixed culture.

A model system, consisting of the two strains *Lactococcus lactis* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, was established. The binary culture was grown in a chemostat under different cultivation conditions. Differentiation was performed by using *in situ* hybridisation, multiplication activity was analysed as a physiological parameter. The information acquired by flow cytometry was compared with structured population data. A complex product spectrum was monitored and com-pared with the physiological state of the respective subpopulations.

# Good Early Treatment Response in Childhood ALL is Associated with Bax Nuclear Accumulation and PARP Cleavage as Measured by Laser Scanning Cytometry.

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Bax activation plays pivotal role in triggering mitochondrial pathway of apoptosis. Its intracellular redistribution during apoptosis as well as prognostic significance in childhood ALL remain a matter of controversy. Cleavage of poly(ADP-ribose) polymerase (PARP) by activated caspases is considered to be a hallmark of programmed cell death.

The study aimed to assess changes in Bax activation and its intracellular distribution as well as number of PARP p89 fragment positive cells in response to prednisone treatment in 43 children with ALL, treated according to BFM 90 Protocol. Blood mononuclear cells, collected prior to and after 6 and 12 hours of prednisone administration were stained for Bax and for PARP fragment p89 with FITC-labelled secondary antibodies. DNA was counterstained with PI/RNase A. Green and red fluorescence intensity was measured by laser scanning cytometer.

Significant rise of Bax-FITC green integral fluorescence in cytoplasm was seen in good responders as early as 6 hours from prednisone administration followed by significant rise of Bax-FITC green integral fluorescence in nucleus after 12 hours. At the same time points of measurement Bax expression in cytoplasm and nucleus in the group of poor responders remained unchanged. Mean pretreatment rate of p89 PARP positive cells was 3.5%. After 12 hours of prednisone administration it raised significantly in the group of good treatment responders, but remained unchanged in poor treatment responders. We conclude that increased cytoplasmic expression of Bax, its nuclear accumulation and PARP cleavage during first 12 hours of prednisone therapy may predict good outcome in children with ALL.

# Individualized Pretherapeutic Risk Assessment for Acute Myeloid Leukemia (AML) Patients

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**Background:** Individualized risk assessment in disease is directly patient related and may be obtained by the analysis of suitable patterns of molecular or clinical data (Valet G JBRHA 16:164-167(2002) predictive medicine by cytomics). It is distinguished from statistical prognosis evaluation where individual molecular or clinical parameters are typically related to disease progression or outcome of large patient groups. Disease prognosis factors are not directly related to individual patients.

**Goal, Patients and Methods:** The goal of this study concerned the identification of high risk patients at AML diagnosis by data pattern analysis (http://www.biochem.mpg.de/valet/classif1.html). Clinical (n=15), malignant immunophenotype (n=36) and cytogenetic (n=25) parameters of 724 patients of the AML'96 multicenter study of the Süddeutsche Hämoblastosegruppe were analyzed.

**Results:** Patients of the learning set classified with predictive values of 100.0% and 88.6% for 5 and 2 year non-survival at predictive values of 15.1% and 38.9% for survival. This was quite similar to the classification of unknown test patients with values of 100.0% and 79.6% for 5 and 2 year non-survival and 26.9% for 2 year survival. No 5 year survivors were available amongst the test set patients. The learning sets consisted of 275 and 203  5 and 2 year non-survivors at 25 and 97 survivors with test sets of 167 and 201 non-survivors together with none available and 62 survivors. 257 and 161 patients with less than 5 or 2 year overall survival remained a-priori excluded from classification. 1.9 and 5.6% of the patients were unclassifiable due to transitional classifications between survival and non-survival. A data pattern of increased patient age, leukemic cell counts, percentage of CD2, CD4, CD13, CD36 and CD45 positive cells was indicative of 5 year non-survival while 2 year non-survival was characterized by increased patient age, percentage of CD4, CD7, CD11b, CD24, CD45 and HLA-DR positive cells at decreased percentage of CD1, CD65, and CD95 positive cells. Data columns with less than 10% of available values remained excluded from analysis such as LDH, FLT3, LEUK, CD1, CD3, cytoplasmatic CD3, CD41, CD42, CD56, CD58, CD64, CD95 and CD117 in the 5 year classification and LDH, LEUK, CD56 in the 2 year classification

**Conclusion:** High risk AML patients for 2 or 5 year non-survival can be reliably identified prior to therapy (predictive values of 88.6% and 100.0%) from clinical and malignant immunophenotype parameters. Cytogenetic parameters were not selected for the individualized risk asessment.

# Individualized Risk Assessment for Diffuse Large-B-Cell Lymphoma Patients prior to Chemotherapy

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Goal: Individualized risk assessment for diffuse large-B-cell lymphoma patients by data pattern analysis (Valet G JBRHA (2002) 16:164-167) using data communicated by Rosenwald A et al. NEJM (2002) 346:1937-1847.

Methods: Files: DLBL_patient_data_NEW.txt and NEJM_Web_Fig1data.html from http://llmpp.nih.gov/DLBCL contain patient information and risk indices as well as 7399 gene expression profiles for 160 learning set patients, 80 validation set patients, 36 further lymphoma patients and 19 gene expression profiles of CLL patients, B-cells and transformed cell lines. The gene array data were subjected to CLASSIF1 data pattern analysis (http://www.biochem.mpg.de/valet/classif1.html).

Results: Predictive values of 96.1% for non survival and 66.3% for survival were obtained for patients of the learning set together with 78.3% and 45.4% for patients of the validation set. The discriminatory data pattern contains 12 genes. Indicators for high risk non-survivor patients are increased expression of ATP synthase (Hs.25), adenine phosphoribosyltransferase (Hs.28914), nuclear receptor co-repressor 2 (Hs.287994) and of genes Hs.15106, Hs.334808, Hs.140945 together with decreased expression of CD9 antigen (Hs.1244), nuclear receptor subfamily 3, group C, member 1 (Hs.75772) and genes Hs.79741*AA830742, Hs.79741*N24822, Hs.159556, Hs.79123.

Conclusion: Classification of the multigene expression profiles against disease outcome permits the identification of high risk non-survivor diffuse large B-cell lymphoma patients in the learning and validation sets of patients prior to the envisaged chemotherapy. The prediction for survival, in contrast, is unreliable with the available data. Extension of the 12 gene discriminatory data pattern into adjacent molecular pathways may increase the predictivity levels and provide insight into molecular mechanisms of particularly aggressive malignancies.

# ProteinChip® Technology for Protein Expression Profiling and Biomarker Discovery

PETRA WESTERTEICHER

Ciphergen

An overview of the ProteinChip technology and its application will be presented. The System enables rapid protein expression profiling and biomarker discovery from crude biological samples on a single integrated platform.

The system consists of three components: ProteinChip Arrays that allow direct application of diverse biological samples like serum, urin or lysates from tissues or cell cultures. The second component, the ProteinChip Reader, is a SELDI (surface enhanced laser desorption/ionisation) time-of-flight mass spectrometer. The third

component is a specially developed software, for the comparative analysis and further evaluation of the generated data.

Arrays are available with different types of chromatographic surfaces (e.g. reverse phase, cationic/anionic exchange). On each type of surface a specific subpopulation of proteins from the sample are retained, according to their biophysical properties. Consequently, low concentrated potential biomarkers are often enriched and can be easily detected for the very first time.

Highly hydrophobic proteins and proteins with extreme isoelectric points that are often excluded from 2D gels can be analysed with this technology. Furthermore, the range below 20 kDa, normally neglected by traditional methods, is easily accessible.

Software components are incorporated that rapidly and effectively speed up the discovery and validation process for potential diagnostic markers or new therapeutic targets without the need to develop antibody arrays.

In addition to these chromatographic Arrays, pre-activated Arrays can be used to covalently link proteins of interest (e.g. antibodies or receptors) to the surface and to study a wide range of protein-protein interactions.

# Poster Presentations

## Automatic Analysis of the Sperm Chromatin Structure Assay® and its Application for the Analysis of Bull and Stallion Sperm

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The Sperm Chromatin Structure Assay (SCSA®) is an important flow cytometric tool to evaluate damage in the chromatin structure of sperm. Using this technique, the extent of in-situ denaturation of nuclear DNA can be determined. It has been shown, that for humans and bulls an increase in heterogeneity of chromatin structure is linked to fertility problems.

In general, using the metachromatic Acridine-Orange staining the amount of double-stranded DNA can be evaluated by means of green fluorescence, whereas red fluorescence is a marker for single-stranded nucleic acids.

A highly standardized protocol for staining is used in order to minimize any artificial sample-to-sample variation. To analyze the flow cytometric data, the DFI "defragmentation index" [ DFI = (red / (red+green) ] has been determined for each cell, and the resulting histogram has been analyzed for symmetry. A measure of asymetry of the DFI histogram has been evaluated for each sample and is compared to manual data analysis with classical gating. Using this automated procedure we avoid variations by the the personal working in the flow cytometric laboratory. This automated procedure allows us, together with the mentioned standard protocol to easily compare data from different flow studies in different institutions. It also prevents day-to-day variations in the same lab. This is a major step forward to use the SCSA® test for fertility prognosis and control.

We present the detailed protocol and software setup as well as the SCSA® data for few hundred sperm samples of bulls and stallions in comparison with their fertility values.

# Selective Fluorescent Dyes Used in Histological Stainings and their Applications in Confocal Laser Scanning Microscopy

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Several histological dyes, used in brightfield microscopy, are fluorescent and may be applied to label specific targets, as: a) nucleic acids; b) organelles; c) cellular components (proteins, lipids); d) functional cellular states (pH, cAMP, $Ca^{++}$); e) cellular populations; f) extracellular matrix; g) ossification; and h) macroparasites. Then, they could be examined by Laser Scanning Confocal Microscopy (LSCM), after conventional stainings, mainly with formalin-fixed and paraffin-embedded material. The items a-d are usually stained in non-fixed isolated cells or in cell culture, while the others, in paraffin-embedded specimens. This work focuses on the latter group. Picrosirius (Direct red 80), after treatment with phosphomolybdic acid, turns interstitial collagens (types I and III) in fluorescence state (543nm). Elastic system (elastic, elaunin and oxytalan fibers) becomes completely stained by Direct blue 2B (543nm), while Eosin Y (514nm) and Evans blue (520-630nm) develop only elastic fibers. Neutral glycoproteins, after placing Schiff's reagent to microwave, show fuchsin fluorescence (546nm). Areas of ossification are visualized after Alizarin (567nm) and Chromotrope 2R (510-530nm) stainings. Eosinophils are detected after Sirius red pH 10.2 (543nm) and Chromotrope 2R. Connective Tissue Mast Cells may be marked by Berberin sulfate and Safranin (530nm) and helminths by Chloride Carmine (531nm), followed by diaphaneity with creosote. DNA in paraffin embedded material is easily evidenced after Feulgen stain, even to show nuclear and kinetoplast DNA from protozoa. Lipids in fresh material are revealed by Oil Red O (518nm) and Nile Red (553nm). In conclusion, selective fluorescent dyes, although less specific than monoclonal antibodies, do not require reaction controls, are not species-specific, being technically easy and quick to reproduce and usually cheaper than immunofluorescent labeling. They also offer great fluorescence stability and low noise in image acquisition.

# Flow Cytometric Studies of DNA-Binding Characteristics of an Inorganic Layered Double Hydroxide (LDH)

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Layered double hydroxides (LDHs) consist of cationic metalhydroxide layers and exchangeable interlayer anions. Due to this negatively charged structure, biomolecules like DNA can be incorporated and a so called DNA-LDH hybrid is formed. The hydroxide layers can be removed in acidic media and the DNA will be released. CERATOFIX®*)NA belongs to the familiy of LDHs and is produced by Süd-Chemie AG. The chemical structure can be summarized as [Mg2Al(OH)6](CO3)0,5.

DNA removal from cell culture supernatants is often one of the major concerns in downstream processing. Due to the anion exchange capabilities of LDHs it seemed a very interesting approach to utilize these materials for binding of DNA for elimination purposes.

The binding capacity of different types of CERATOFIX®NA for a model DNA was determined by using flow cytometry. The intercalating process was observed after coupling of a fluorescence dye to the DNA. A linear relationship was found for the detection of the fluorescence signals and the amount of DNA applied.

Reference: Choy et al.: Angew. Chem. 2000, 112, Nr. 22; 4207-4211

*) CERATOFIX is a registered trademark of Süd-Chemie AG

# Correlation of DNA Ploidy Assessment Method with Immunocytochemival Analysis in Primary Breast Carcinoma

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Background

The goal of our study was to compare post-operative material (samples of cell suspension) for DNA ploidy assessment in neoplastic tumors using flow cytometry with Ki-67, estrogen and progesterone receptors status of investigated cells.

Methods

Samples for DNA ploidy studies were prepared in a standard manner and measured by COULTER EPICS XL cytometer. Ki-67 receptor, estrogen receptor and progesterone receptor statuses were determined using primary mouse monoclonal antibodies. Post operative material was stained with H&E

Results

Material studied comprised 117 post operative cases analyzed in 1999–2001. We found in this group 95 carcinoma cases with aneuploid type of cell cycle with CV value between 2.0 and 7.9 while the number of cells in the S phase of aneuploid cell cycle was between 1.6% and 81.9%. On the other hand, in 22 cases of primary breast carcinoma with diploid type of cell cycle the CV value ranged between 3.0 and 8.1 and the number of cells in the S phase of diploid cell cycle was between 1.6% and 20.6%.

Conclusions

DNA aneuploidy correlated with weak or lack of reaction for the presence of estrogen and progesterone receptors as well as with strong reaction for the presence of Ki-67 receptors. This approach could be useful in prognosis and treatment of patients who are candidates for subsequent chemotherapy and / or hormone therapy.

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# Quantitative phase Optical Characterization of Heat Stressed Cells Uusing Light Microscopical Data

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Quantitative interference laser phase microscopy (LPM) is an ex-perimental technique based on the conversion of laser induced inter-ferometric patterns into electric signals restored as spatial phase shift distributions. Optical phase shift of incident light wave fronts are generated when light passes through cells. The LPM technique is hampered by high technical requirements, limited wavelength vari-ability and the costly reconstruction of phase shift patterns. On the other hand observations of optical cellular changes, especially the variations in refractive index distributions caused by structural rear-rangements as well as metabolic activities do not require target spe-cific labeling (e.g. immunofluorescence). Therefore this technique provides the opportunity to monitor cellular activities under native conditions.

Here we present a fast and simple method of quantitative phase shift reconstruction using the linearized transport equation of classi-cal, partially coherent radiation. Since phase optical data are in-cluded in every light transmission micrograph the cellular phase shift distributions are tomographically restorable from sequential images in selected focus levels (Z-stacks) in every cell. Phase shift patterns can be structure correlated with the light micrographs.

Light microscopical data were acquired using an Axioplan 2 (Carl Zeiss) equipped with a high resolution digital camera AxioCam HR.

In order to study the sensitivity of the quantitative reconstruction of various phase shift distributions we used heat as stress factor. On the cellular level heat stress response is associated with genetic, morphological, and metabolic rearrangements. Generally, the kind and degree of stress induced subcellular alterations are directly cor-related with the intensity and duration of the heat stress. The phase shift data were compared with fluorescence microscopical images of heat induced cytoskelatal alterations.

The authors wish to thank Dr. G.Graschew (Surgical Research Unit OP2000, Max Delbrück Centrum für Molekulare Medizin) for providing the LPM equip-ment. This study was financed by BMBF (FKZ 01EZ0036).

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# Diagnostic Imaging of Histologic Sections of Melanocytic Skin Tumors Using Tissue Counter Analysis

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In tissue counter analysis, digital images of tissue scenes are dissected into elements of equal size and shape, and the contents of each element are evaluated by a set of grey level, color and texture features. The aim of this study was to test the applicability of tissue counter analysis and CART (Classification and Regression Tree) to the diagnostic discrimination of a large series of histologic sections of benign common nevi and malignant melanoma. Two hundred cases each of benign nevi and malignant melanoma were consecutively sampled. After creation of datasets based on 10 cases each, CART analyses of background versus tissue elements and cellular versus other tissue elements were performed. In a second step, a learning set of 120000 cellular elements obtained from 100 cases each was created. Based on the learning set, CART analysis was performed in order to differentiate between benign and malignant cellular elements. For diagnostic assessment, only the percentage of cellular elements suggestive for malignancy in each case was used. In the learning sets, CART analysis led to a correct classification of 99% of background versus tissue elements, 96% of cellular versus other tissue elements and 79.1% of benign versus malignant cellular elements. When the percentage of cellular elements suggestive for malignancy in each case was evaluated it turned out that a threshold level of 52.51% provides a correct classification of 192 nevi and 186 melanoma out of 200 each (specificity 96%, sensitivity 93%, positive predictive value 95.9%). In conclusion, an overall performance of 94.5% correctly classified cases in a total of 400 histologic sections of melanocytic skin tumors clearly suggests that tissue counter analysis combined with CART (Classification and Regression Tree) is a powerful tool for diagnostic purposes in histopathology.

# Rapid Evaluation of Cell Nuclear Morphology in Tumors by Image Cytometry

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In recent years many morphometric, densitometric and texture features have been developed for quantitative evaluation of pathological alterations in tumor cells. Unfortunately many of them are tightly correlated and therefore may be abundant.

One of possibilities to resolve this problem is to represent a digital image of cell nucleus as pseudosurface and describe "roughness of surface" as a function of perimeter of all chromatin structures versus ascending gray levels. Square under this curve will be equal to whole fractal area of nucleus, so we have named the curve as "Fractal Area Profile" (FAP). FAP contains information about size, shape and inner structure of nucleus including topological properties of chromatin grains. Further development of parameters is provided by segmentation or nonlinear approximation of FAP. For example segmentation of FAP on its global maximum gives fractal areas (FA) of such important nucleus components as eu- and heterochromatin.

We used this approach to investigate fine needle biopsies of thyroid tumors and cell cultures of thyroid and lymphoid tumors in the presence of antitumor drugs. It was revealed that FAP and FA are sensitive enough to detect changes of chromatin in almost all cell populations.

Thus we propose a simple method which makes it possible to describe morphology of cell nuclei in tumors "on the fly".

# Role of Extracellular Matrix in Human Osteosarcoma Proliferation

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Osteosarcoma is the most common primary malignancy of bone in childhood and adolescence. Its therapy might be improved from identification of novel therapeutic targets, especially seeding from understanding of the significance of extracellular matrix (ECM) in proliferation of these tumor cells. A stabile cell line was established from the biopsy fragments of an osteosarcoma patient. After stabilisation cells were cultured conventionally or on extracellular matrix gel (ECM-gel), as well as on the matrix produced by the same osteosarcoma cells (OSCORT-ECM). Cell proliferation (with cell counting and MTT assay), cell cycle distribution (with flow cytometry), expression of proliferation-related proteins (with immunoblots) were compared under the two culture conditions. ECM-gel and osteosarcoma matrix increased cell proliferation compared to cells cultured on plastic. The number of cells decreased in G1 and increased in S-phase when they were cultured on ECM-gel or OS!

CORT ECM, moreover fibronectin and HSPG had similar effect on cell cycle apportioning. These phenomena were confirmed by increase of cyclin D1 and PCNA. On the contrary, type IV collagen concentration dependently decreased proliferation, and caused G2 accumulation. This effect was further proved with increase of cyclin B1 and Ki-67 production, and increase of topoisomerase II activity. We conclude that ECM has considerable role in maintenance of proliferation of human osteosarcoma, which is presumably due to heparan sulfate proteoglycan and fibronectin.

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# Confocal Scanning Microscopy for High Sensitivity Fluorescence Detection in Cells and Tissue Sections

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Confocal microscopy is being applied to observe fluorescently labeled monoclonal antibodies (mAb) on the surface of single cells and in tissue sections with high sensitivity. In particular, we identify T-cells, B-cells and possibly activated B-cells in a frozen section of lymph node tissue by non-amplified immuno-fluorescence. The highly sensitive detection is mandatory to quantify mAb expression and to apply multiple staining techniques which is hardly possible with biochemical amplification procedures commonly used to obtain sufficiently high fluorescence signals.

To increase specificity for the detection of fluorescently labeled probe molecules, autofluorescence background was suppressed by subtraction of two images, one of which was obtained with excitation wavelength and emission filter adapted to the absorption and fluorescence of the chromophore. The second image was recorded with the excitation wavelength tuned outside the absorption band of the fluorophore, while keeping the same observation filter. Alternatively, the second image was measured by retaining the excitation wavelength and changing the emission filter to a wavelength range with negligible fluorophore fluorescence. In each case, the first image represents a superposition of specific fluorescence and autofluorescence whereas the second one is predominantly caused by autofluorescence. For both schemes, appropriate scaling and subtraction allowed to improve signal to background ratio by a factor of about six for the tissue sections investigated.

At present, further improvements of autofluorescence suppression techniques are investigated aiming at the detection and localization of single bio-molecules in tissue sections of lymph nodes.

# Flow Cytometry an Bakterien – Eine Herausforderung: Messungen mit MoFlo und MicroFlow 6.1.

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Auf Grund der meist geringen Größe und auch der geringen Menge fluoreszenzmarkierbarer Zellinhaltsstoffe stellen die flowcytometrischen Untersuchungen von mikrobiologischen Meßobjekten, speziell Bakterien, hohe Anforderungen an ein Flow Cytometer. Ein entscheidender Parameter, auch um die Bakterien auf Grund ihrer Eigenschaften sortieren zu können, ist die Empfindlichkeit des Meßgerätes als Analyzer und ein entsprechend großer Signal-Rausch-Abstand.

In der Leipziger Gruppe Flow Cytometry am UFZ/SIAB sind sowohl ein Sorter MoFlo von Cytomation als auch ein selbst entwickelter Laboraufbau eines Analyzers MicroFlow 6.1 vorhanden.

Vor- und Nachteile der beiden Systeme werden diskutiert und abgeschätzt. Dabei werden die Ergebnisse von Messungen verschiedener Einzelparameter (Vorwärtsstreulicht, DNS und weitere funktionelle und strukturelle bakterielle Parameter) und auch von Mehrfachfluoereszenzanregungen gezeigt.

# How Does the Fluorescence of DNA Specific Dyes Depend on Base Composition and Base Sequence? Comparison of the Sequenced Species Oryza Sativa and Arabidopsis Thaliana.

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It is well known that some of the DNA-binding dyes are base specific. DAPI and the Hoechst dyes bind preferentially to adenine and thymine (AT), mithramycin to guanine and cytosine (GC). The relation between AT or GC frequency of the nuclear DNA and fluorescence intensity of these dyes is not quite clear, but it seems that 3 – 5 consecutive base pairs of the same type are necessary for binding. This results in a non-linear relation between AT/GC ratio and fluorescence intensity.

However, because the bases are non-randomly distributed within the DNA sequence, the fluorescence intensity is expected to be not only a function of base pair ratio, but also of the base sequence.

The first sequenced species of higher plants, Oryza sativa and Arabidopsis thaliana give the opportunity to verify this relation.

The result is rather surprising: For all 4 tested dyes (DAPI, Hoechst 33258, Hoechst 33342 and mithramycin) the number of bases of the same type necessary to bind one dye molecule is probably 1, but in no way greater than 2. This is in contradiction to results obtained by other methods and may indicate that the simple theory of several consecutive bases of the same type binding one dye molecule is not correct.

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## Mechanical Deformation of Cells with Optical Tweezers

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Mechanical properties of living cells give important information on dynamic cellular processes such as attachment, migration or division. For the study of mechanical characteristics it is advantageous to apply controlled forces on cells in such a way that the cellular response can be visualized with high resolution. Here we describe the development of an easy to handle method for mechanical strain application based on an optical tweezers, a single beam optical gradient trap. Optical tweezers uses laser light for non-invasively manipulation of microscopic particles in a pico newton range. Because the generated forces are insufficient for measuring mechanical properties, specially prepared human erythrocytes attached unspecifically on cell surfaces were used as very effective tools for the optical force transmission. The magnitude of transmitted forces is in such a range that deformations of mouse fibroblasts (cell line L-929) and rat kangaroo epithelial cells (cell line PtK 1) were clearly detectable by phase contrast microscopy. Differences in the elastic response and local elastic properties explained by distinct structures of cytoskeleton were found between the two adherent cell lines. In addition, after vital fluorescence staining of mitochondria investigations of mechanical properties were even possible on cells grown on light impermeable surfaces such as biomaterials used in medicine and dentistry. The method offers the possibility not only to examine but also to modulate the spatial and temporal mechanical properties of cells.

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## Digital Image Analysis and Immunophenotyping of Lymphocytes in Normal and Pathological Conditions

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The methods of quantitative estimation of morphocytochemical peculiarities of cells, tissues, organs are very important now. Digital image analysis belongs to such methods, and it was performed in the laboratory of immunomorphology of Institute of Clinical and Experimental Lymphology SB RAMS thanks to the special apparatus and programmes. The densito-geometric parameters of DNA, enzymes and cell components have been evaluated in the lymphocytes of healthy objects, during the induction of autoimmune experimental processes and diseases, during the pathology of the lymphatic system, during the investigation of the drugs, food supplement,

sorbent action. Also immunophenotyping of leukocyte subpopulations has been made with the help of flow cytometry with monoclonal antibody.

The changes in DNA, chromatin structure, enzyme activity and immune state have been found in lymphocytes of healthy persons of different gender, which was connected with the differences in subpopulational content and immune state of lymphocytes.

It has been shown that during experimental autoummune reaction and autoimmune disease (rheumatoid arthritis) and their treatment with immunomodulators marked changes in immunomorphological parameters of lymphocytes have been observed connected with the processes of activation, proliferation, differentiation and apoptosis of immune cells

The peculiarities of immunomorphological parameters of lymphocytes did not stay constant under the pathology of lymphatic system - lymphatic edema of upper and lower extremities.

We also managed to show the influence of sorbents and food supplements on the functional state of immune system and densito-geometric peculiarities of lymphocyte.

One can say about practical use of digital image analysis and immunophenotyping of lymphocytes in prognosis of the efficacy of treatment of the disease and character of its duration.

The meaning and information of digital image analysis and immunophenotyping of lymphocytes proved to be very high in the medical and biological investigations.

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# Increased Genetic Damage with Age Detected with SCSA and Comet in Mouse Sperm

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**Introduction:** The increase in the male germ-line mutation rate with age is little understood. We investigated age-dependent increases in the frequency of DNA strand breaks and chromatin disturbances in mouse spermatozoa.

**Materials & Methods:** Sperm were obtained 12 hours after treatment from 5 15-month and 5 2-month old, untreated mice and 5 Doxorubicin-treated (12 mg/kg) old and young mice and analysed by the Sperm Chromatin Structure Assay (SCSA) and the Comet Assay.

**Results:** There was a significant increase in disturbed chromatin in sperm of the old untreated mice compared with the young untreated mice (4.6% vs. 3.4%, $p=0.027$) and in sperm of the old Doxorubicin-treated mice compared with the young Doxorubicin treated mice (4.4% vs. 3.5%, $p=0.015$). There was no difference between treated and untreated animals within the same age group. A significant increase ($p<0.01$) in tail moment was found among the old untreated mice compared with young untreated mice (5.2 vs. 2.16) and in the group of old Doxorubicin-treated

mice compared with young Doxorubicin-treated mice (4.93 vs. 2.81). There was also a significant difference between treated and untreated young mice (2.81 vs. 2.16, $p<0.01$), but not in old mice.

**Discussion and Conclusions:** Thus, ageing is associated with disturbances in sperm chromatin and sperm DNA-strand breaks. The lack of effect of doxorubicin in the SCSA suggests that sperm nuclei are not susceptible to this agent. The effect seen with the Comet Assay in young mice indicates that SCSA and Comet detected different types of damage.

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# Reaction-Diffusion-Dynamics in Cardiac Calcium Management: Parameter Estimation from Experimental Studies

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The Calcium dynamics in heart tissue of chicken is studied in a combined UV-microbeam fluorescence microscopic setup which photolytically cleaves cytosolic Ca$^{2+}$np-EGTA and simultaneously records the Calcium Green1 fluorescence in a group of embryonic cardiac myocytes. As a result, the concentration c(x,t) of free Ca$^{2+}$-ions in the cells is monitored as a light intensity function of space and time.

In a simple mathematical model, c(x,t) is described by a diffusion equation incorporating nonlinear refractory influx and efflux effects. Efflux and influx are defined for any location x thus allowing also inhomogenous distribution of physiological Calcium sinks and sources (like Ryanodine receptors and Calcium pumps). Refractoricity can be modelled concentration or time dependently.

For parameter estimation, we restrict to the one dimensional case. We find a lot of special situations in experiment, which can be described by this reduced approach. Parameters for the model equation are extracted from concentration curves and phase plots using smoothing B-spline approximations.

# Analysis of Cell Cycle and Differentiation in the Haploid Moss Physcomitrella patens

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Haploid moss plants like *Physcomitrella patens* are being used as a model system in the study of plant development as they display a rather simple developmental pattern. Moss spores germinate by forming tip-growing chloronema cells and depending upon age, nutrients, light and hormones tip cells differentiate into caulonema cells. *Physcomitrella patens* can be cultured photoautotrophically either on solid medium or in flasks or bioreactors with liquid medium.

We used flask cultures to compare the effect of three different media on differentiation and cell cycle: modified Knop medium (1.84 mM $KH_2PO_4$, 3.36 mM $MgSO_4 \times 7H_2O$, 1.02 mM KCl, 4.24 mM $Ca(NO_3)_2 \times 4H_2O$ and 0.045 mM $FeSO_4$, pH 5,8), Knop medium with 5 mM ammonium tartrate, and Knop medium supplemented with auxin (5 mM NAA). We could confirm previous findings that addition of ammonium results in inhibition of caulonema formation, while auxin stimulate caulonema induction. Our flowcytometric analysis showed, that chloronema cells were predominantly in 1G C2, while caulonema cells stayed in 1G C1. In a second experiment we evaluated the effect of different inoculation densities on moss differentiation, cell cycle, and endopolyploidisation during a 22-day-lasting culture period. Start of the culture with low inoculation density in Knop medium supplemented with auxin resulted in a high number of endopolyploid cells compared to the control.

Our study show, that flowcytometry is a useful tool to study the linkage between cell cycle and differentiation in *Physcomitrella patens* and specify the effect of nutrients, plant hormones and environmental factors in both processes.

This work has been performed in a joint project with BASF Plant Science GmbH.

# Tissue Microarray: Rapid Linking of Molecular Changes to Clinical Endpoints of Head and Neck Squamous Cell Carcinoma

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Advances in genomics and proteomics are dramatically increasing the need to evaluate large numbers of molecular targets for their diagnostic, predictive or prognostic value in clinical oncology. Analysis of prognostic and predictive markers in cancer has traditionally been accomplished by testing one marker at a time, starting from a relatively small sample size. Before routine clinical application, large-scale

studies of hundreds of well-characterized tissue specimens with clinical follow-up information will need to be carried out to demonstrate the independent significance of the biomarker.

The Tissue Microarray (TMA) technology has the potential to significantly accelerate studies seeking for associations between molecular changes and clinical endpoints. Gains and amplifications of target genes in 343 primary head and neck squamous cell carcinoma determined by Flourescence in situ hybridization (FISH) on TMA were correlated with patients survival using the Kaplan-Meier-Analysis. FISH was applied on tissue microarray sections with labelled BAC-probes for different genes in the region 3q26 to 3q28. Amplification of this region appears to occur frequently among HNSCC.

Cyclin L encodes a putative key regulator of the G0 to G1 transition. Cylin L overexpression was found in head and neck squamous cell carcinoma.

P63 encodes for different isoforms with distinct transcriptional activities. A frequent increased p63 gene copy number was reported in squamous cell carcinoma of head and neck and of lung.

PIK3CA is involved in multiple cancer-related function: cell survival, proliferation, cell migration, vesicle trafficking and vesicle budding.

Amplification of SNO was accompanied by significant increases in expression level in ESC cell line.

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## Tsa Has a Complex Effect on Cell Cycle Progression

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Trichostatin A (TSA) is a commonly used inhibitor of histone deacetylases. Previous results in the literature have shown that TSA induces an arrest in the early G1 and in the G2/M stage of the cell cycle. By flow cytometry of HeLa and ID13 cells it is demonstrated here that the effect of TSA on cell cycle progression is more complex. It depends strongly on the TSA concentration and incubation time since we also observed a block in S phase. Depending on the TSA concentration arrests in G1 or G2/M phase were apparent after an incubation time of 12h. Further incubation for a total of 24h caused an arrest in the S phase. The same results were obtained when the cells were synchronized by serum depletion. No significant difference was observed between the different cell types studied. Experiments were also performed with cell lines stably expressing histone H2A fused to yellow fluorescent protein (H2A-YFP). H2A-YFP is incorpoarated into chromatin resulting in an in vivo fluorescence label. The presence of TSA induced an up to twofold increase in the H2A-YFP fluorescence that was correlated with the percentage of apoptotic cells. From the relation between apoptosis and TSA concentration the existence of at least two binding sites for TSA with different affinity is inferred.

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## Analysis of P-glycoprotein (P-gp) activity in Childhood Acute Leukemia (ALL) by Laser Scanning Cytometry (LSC):

# Correlation of Rhodamine 123 Efflux Rate with Ploidy and Morphology

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P-gp reveals the ability to actively remove from the cell particles of many anticancer compounds and variety of fluorochromes of which rhodamine 123 (Rh123) is most commonly used to assess P-gp activity. Prognostic significance of P-gp expression in childhood ALL has not been unequivocally determined yet.

This study aimed to test the feasibility of LSC to measure the Rh123 efflux rate in mononuclear cells of healthy individuals and children with ALL in correlation with ploidy and cell morphology.

Slide attached mononuclear cells were incubated with 0.1 M Rh123, rinsed in PBS and immediately measured repeatedly, than rinsed in PBS, stained with PI/RNase A and measured again, finally air dried, stained with Giemsa and relocated by LM to correlate cell morphology with fluorescence parameters. Values of Rh123 and PI fluorescence were recorded as.fcs files (subsequently merged). In all studied cases interpolation of Rh123 fluorescence over the time resulted in exponential (y=a*eb*x) curves indicating that Rh123 efflux followed first order kinetics that was described by kinetic parameters (AUC, t1/2, C0) as for one compartment linear model. Kinetic analysis of curves created for individual cells in respect to its morphology and cell cycle phase revealed large heterogeneity within studied cells populations.

LSC offers the possibility to measure rapid changes in Rh123 fluorescence over time in individual cells in correlation with cells morphology, DNA content and cell cycle phases. Kinetic analysis (calculation of Rh123 t1/2, C0, AUC) of LSC data is a sensitive tool to assess P-gp activity in children with ALL.