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Abstracts

Oral presentations (alphabetical order)

Correlative microscopy of virus infected cells: The dilemma of recognition and resolution

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To relate the structural analysis of cells with functional parameters, live cell imaging should be correlated with high resolution microscopy. This approach, however, requires the serial employment of a great variety of methods and types of microscopes and to concert their focus on a singular structural element. Ideally, biological samples should primarely be monitored in the living state by time-lapse imaging. The cells then are fixed and processed by methods including immunohistochemical and/or other staining procedures. Light microscopy at this stage allows an exhaustive 2-D, 3-D as well as a spectral analysis of fluorescence signals. In a last step, cells are processed for electron microscopy (EM) to examine regions of interest (ROI) at high resolution.

We have employed correlative microscopy to study virus infected cells. Sequential observations of ROI`s by wide field microscopy, confocal microscopy and transmission electron microscopy were made to characterize cytopathical effects, transport of viral elements and the morphogenesis of virus particles. This will be illustrated by various examples including: 1) time-lapse monitoring by video-enhaced contrast microscopy (VECM) of cells infected with herpes simplex virus (HSV) followed by immunofluorescence and identification of HSV-particles by TEM. 2) imaging of a GFP-fusion protein in transfected cells followed by identification of GFP-tagged products by TEM. 3) VECM of the morphogenesis of respiratory syncytial virus and monitoring of immunogold reactions in real time.

The observation of viruses and cells cover a formidable range of dimensions which can be bridged only by extending the limited resolution of light-microscopy by electron microscopy. Moreover, modern light microscopy in conjunction with image processing techniques are often revealing structures below the classical resolution of light optics; their identification is of greatest importance an is achieved most convincingly by subsequent imaging with the EM.

Scanning fluorescent microscopy analysis is applicable for absolute and relative cell frequency determinations

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Background: Flow cytometry(FCM) and laser scanning cytometry (LSC) are the routine techniques for fluorescent cell analysis. Recently, we developed a scanning fluorescent microscopy (SFM) technique. This study compares SFM to LSC and FCM in experimental and clinical setting.
Materials and methods: For the relative cell frequency determinations HT29 colorectal cancer cells and Ficoll eparated blood mononuclear (FSBM) cells were serially diluted (from 1:1 to 1:1000). For the absolute cell number determinations FSBM cells were cytocentrifuged then HT29 cells were placed on the slide with a micromanipulator (1 to 50 cells). As clinical samples, circulating tumor cells were isolated from colorectal cancer patients blood. All samples were double stained by CD45 and CAM antibodies. TOTO3 and Hoechst 33258 DNA dyes were applied as nuclarcounter staining.

Results: In the relative cell frequency determinations, the correlation between t he calculated value and the SFM, LSC and FCM values was r2 = 0.79, 0.62, 0.84, respectively. In the absolute cell frequency determinations, SFM and LSC correlated by r2 = 0.97. Conclusions: SFM proved to be a reliable alternative method as compared to LSC and FCM, however further methodological improvements are necesseryto increase the scanning speed.

Disturbed apoptosis induction in peripheral mononuclear cells by anti-dsDNA antibodies

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F-actin cleavage was studied in PBMC after treatment with anti-dsDNA antibodies. Significant changes in F-actin disruption detected by decrease of FITC-phalloidin staining occurred after apoptosis induction with anti-dsDNA antibodies ($p < 0.006$). Despite of similar F-actin disruption the switch of phosphatidylserine (PS) to the outer leaflet of the cell membrane as detected by annexin V binding was lower after anti-dsDNA antibody than without antibody treatment (58.4% ± 11.0% vs. 81.9% ± 7.7%). F-actin disruption was accompanied by activation of caspase 3 within the cytoplasm ($r = -0.92599$; $p < 8.87446 \times 10^{-10}$) under both conditions with and without autoantibodies. These findings indicate that anti-dsDNA antibody-induced apoptosis is more marked within the cell than upon the cell surface. The diminished externalization of PS might result in a decreased phagocytosis. Thereby, the reduced clearance of apoptotic cells could induce autoantibody production possibly against epitopes which arise due to the apoptotic disruption of cells.

Continuous Bromodeoxyuridine (BrdU) exposition differentially affects cell cycle progression of human breast and bladder cancer cell lines

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Background:
Incorporation of Bromodeoxyuridine (BrdU) during DNA replication is frequently used for cell cycle analysis. The flow cytometric BrdU/Hoechst quenching technique allows high resolution assessment of cell cycle kinetics but requires continuous BrdU treatment which may have cytostatic or cytotoxic effects.
Methods:
We examined the impact of BrdU on the proliferation of four different tumor cells lines (bladder carcinoma: RT4, J82; breast carcinoma: BT474, SK-BR-3). Both one- and two-parametric DNA measurements were performed to identify BrdU-induced alterations in the S-phase fraction and in cell cycle progression. An annexinV/propidium iodide (PI) assay was used to identify potential induction of apoptosis by BrdU.
Results:
Proliferative activity in BT474, SK-BR-3, and RT4 cultures is reduced in different cell cycle phases due to continuous treatment with 60, 5.0, and 3.5 µM BrdU. This effect which was not found in J82 cultures is dependent on exposure time (96 h vs. 48 h) and is also dose-dependent for RT4 and SK-BR-3. BrdU application does not induce apoptosis or necrosis as revealed with the annexinV/PI assay.
Conclusion:

Continuous BrdU treatment does not affect cell viability but essentially alters cell cycle progression in three out of four cell lines tested. Cell-type specific validation of the feasibility of the powerful BrdU/Hoechst quenching technique is required and recommended.

Functional assessment of probe-molecular target complxes by fluorescence lifetime analysis and multiparameter flow cytometry

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Recent development of a novel Phase-Sensitive Flow Cytometer (PS-FCM) that measures the absolute value of fluorescence lifetime in real-time adds a new dimension to multiparameter analysis. Fluorochrome labels have characteristic lifetime values determined by their molecular structure; however, the probe lifetime can be altered by a number of environmental factors, including the interaction of the probe with a target molecule. Monitoring the changes in the absolute lifetime value of the probe yields information relating to the changes in molecular conformation and the functional activity of the molecular target. Lifetime values also provide unique signatures for resolving the emissions of multiple fluorochrome labels with overlapping spectra, thereby increasing the number of fluorochrome combinations using a single excitation source. This approach was used to separate overlapping fluorescence of PI and PE/Tx red (tandem)-labeled antibody by PS-FCM. Lifetime analysis of cells stained with different nucleic acid-binding fluorochromes revealed several other unique observations and demonstrated the accuracy of the PS-FCM methodology. Our lifetime studies provided the discrimination of DNA and dsRNA based on differences in the lifetime value of either PI or EB bound to the respective nucleic acids. Differences in lifetime values relate to the differences in the structure of the nucleic acid complexes, as well as the dissimilarities in the dye-intercalation into DNA or dsRNA. Similar lifetime data were obtained with fluorescent chemotherapeutic agents, including ellipticine and adriamycin, thereby allowing, potentially, for discriminating and quantitating binding of these drugs to either DNA or RNA. Bivariate profiles of lifetime versus DNA content, obtained from analysis of EB stained, HL-60 cell populations induced into apoptosis by 3h treatment with camptothecin, showed a 3.0 ns reduction in the lifetime of EB bound to apoptotic cells compared to the non-apoptotic subpopulation. DNA content and lifetime analysis revealed a unique subpopulation of human skin fibroblasts cells in very early S phase with a significantly reduced EB-lifetime. Multiparameter DNA content, EB lifetime and immunofluorescent antibody analysis of cyclin D and cyclin E levels for asynchronous HSF cells demonstrated that the subpopulation of cells contained elevated levels of both cyclin D and cyclin E, characteristic of cells in very early S phase. Following release of synchronized cells from G1/S phase, the subpopulation entered mid-S phase with EB lifetime values elevated above G1 phase cells and a progressive increase in EB lifetime was noted as cells proceed to the G2/M phase.

Collectively these studies demonstrate various applications of lifetime measurements for the analysis of the binding of different fluorochromes to DNA or RNA in single cells. Data also show the utility of lifetime measurements for monitoring changes in chromatin structure associated with cell cycle progression, cellular differentiation, or DNA damage, as in the early stages of apoptosis. Potential modifications of the PS-FCM will provide for simultaneous measurement of multiple lifetimes, thereby enhancing detection and quantitation of fluorescent compounds, including chemotherapeutic agents, bound to multiple subcellular complexes in viable cells.

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Competition for IL-2 is essential for CD4+CD25+ regulatory T cell function

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Regulatory T cells are involved in regulation of different immune responses in vivo and mediate protection from autoimmunity. Although CD4+CD25+ T cells (Treg) are the best characterized population of regulatory T cells, the functional role of CD25 and the mechanism of suppression has not been clarified yet. Here, we analysed the role of IL-2 and the IL-2 receptor (IL-2R) for the suppressive activity of CD4+CD25+ T cells in vitro. We show that expression of CD25 and consumption of IL-2 is essential for the activation and the suppressive activity of regulatory T cells. Co-culture of regulatory and responder T cells results in an up-regulation of CD25 on regulatory T cells, but simultaneously in a down-regulation of CD25 on the target T cells. We can show that this effect is mediated by selective IL-2 uptake by Tregs during the co-culture. This uptake of IL-2 by Tregs results in a strong increase of their suppressive activity as shown by pre-activation with anti CD3 and IL-2. To directly assess the role of IL-2 and the IL-2 receptor for regulatory T cells we established a chimeric culture system using murine regulatory T cells and human target cells. This system allows to selectively block or saturate the IL-2 receptor on the Treg cells without affecting the human responder T cells. The blocking of the IL-2R with antibodies completely reverses the suppressive effect. In addition the saturation of the IL-2 receptor using murine IL-2, which does not react with the human IL-2R, reverses suppression in a range between 30% and 60% indicating that a major mechanism of suppression by Tregs is mediated by competition for IL-2. In summary, we show here that IL-2 is an essential activator of Treg function and the suppressive activity is mediated to a large extent by competition for IL-2.

Correlative light and electron microscopy in analysing vascular permeability

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Failure of the structural and metabolic barriers normally regulating the passage of materials across the blood vessel wall is of considerable clinical interest. In early diagnosis and in monitoring of therapy, attention focusses on how substances gain entry to the blood from the surrounding tissue (e.g., the PSA molecule in prostate cancer). In the reverse direction, the extravasation of water and proteins into the perivascular tissues underlies a range of pathophysiological states of urgent clinical importance (e.g., brain oedema) and is considered to underlie the dose-limiting side-effects of radiotherapy. Our work has aimed to elucidate these vascular responses to radiotherapy, focussing on radiation-induced vascular hyperperme- ability. This question has a century-long history. Early elegant experiments demonstrated the occurrence of radiation-induced vascular leakage by use of vital dyes, the results being visible to the naked eye. Further progress has required detailed examination of the endothelial cell wall: since light microscopical imaging has insufficient resolution to resolve the endothelial wall, ultrastructural analysis is essential. Vascular hyperpermeability could in theory be caused by paracellular leakage through opened interendothelial junctions, or by transendothelial passage for example via caveolae, or by release from disintegrated microvessels following endothelial cell death. After high-dose radiation, endothelial death does indeed account for considerable leakage of blood proteins into the tissue. However, modern fractionated radiotherapy applies a sequence of small doses (3 Gy) that leaves endothelial cells in apparently vital condition after accumulated 60 Gy, yet the vessels are leaky. Ultrastructural analysis shows that this hyperpermeability results at least in part from transendothelial extravasation. Further progress in understanding requires quantitating the development of vascular leakiness over time after defined small-dose irradiation, best by quantifying protein tracers by fluorescence microscopy and simultaneously demonstrating

the structural basis of the extravasation by electron microscopy. Fluorescence quantitative analysis presents numerous pitfalls, and our approach to these problems and their solutions will be discussed briefly. Our progress in this direction will be illustrated by reference to work with placental structures and with different tumour models.

Inhibitory and stimulatory effects of growth factors and Herceptin on cell proliferation mediated by specific erbB-receptor interaction and activation

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Background:
The erbB-receptor tyrosine kinases (EGFR, c-erbB2/Her-2, c-erbB3, and c-erbB4) fundamentally regulate cell proliferation. Upon ligand binding to the extracellular domain, the erbB-receptors assemble homo- and heterodimers and subsequently stimulate the intrinsic tyrosine kinase activity. EGFR and c-erbB2 are described to predominately control cell proliferation and define malignancy of human breast cancer. We investigated the effect of growth factors and Herceptin on a cellular basis and examined interaction and activation of EGFR and c-erbB2 in c-erbB2 overexpressing breast cancer cells.
Methods:
BT474 and SK-BR-3 breast cancer cell lines were treated with EGF, Heregulin and Herceptin in different combinations. Cell proliferation was evaluated with the flow cytometric anti-BrdU technique. Fluorescence-Resonance-Energy-Transfer and ELISA experiments were performed to investigate erbB-receptor interaction and phosphorylation.
Results:
EGF induced EGFR/c-erbB2 interaction and EGFR-phosphorylation correlates with stimulation of cell proliferation in BT474. In contrast, cell proliferation of SK-BR-3 cells upon EGF treatment was inhibited. Herceptin derogates receptor interaction with c-erbB2 involvement and effectively inhibits cell proliferation of BT474 both in the presence and absence of growth factors. However, Herceptin has no inhibitory effect on SK-BR-3 in the presence of Heregulin. Heregulin application stimulates cell proliferation extensively both in BT474 and in SK-BR-3 without increased c-erbB2-phosphorylation. Activation of EGFR is increased upon application of both EGF and EGF/Herceptin. Herceptin enhances the phosphorylation of c-erbB2 in both cell lines independently of growth factor treatment. Herceptin induced phosphorylation of c-erbB2 correlates with inhibition of cell proliferation.
Conclusion:
The growth inhibitory effect of Herceptin is defined by erbB-receptor coexpression and interaction rather than exclusively by c-erbB2 receptor overexpression. However, Herceptin does not inhibit cell proliferation of c-erbB2 overexpressing tumor cells per se. The optimization and specification of diagnostic and therapeutic approaches based on erbB-receptor targeting should manly account for erbB-receptor coexpression.

Characterization of the microbial community structure in activated sludge systems with biological phosphorus elimination by molecular techniques

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Today activated sludge systems with enhanced biological phosphorus removal (EBPR) are the most widely used technology for phosphate removal from wastewater. But it is still not exactly known which microorganisms are responsible for the EBPR.
The aim of these investigations was to compare the bacterial composition of activated sludge from two bench scale plants (BSP) and from three full scale wastewater treatment plants (WTP1, WTP2, WTP3). To identify possible polyphosphate accumulating organisms (PAO), two specific oligonucleotide probes for the *Rhodocyclus*-group and for two clones associated to the genus *Tetrasphaera* were applied.
Despite the quite different modes of operation, no significant differences in the bacterial composition of the activated sludge of the BSP1 (with EBPR, without nitrification [Ni]), BSP2 (with EBPR and Ni) and WTP 1 (without EBPR and Ni) on the level of major taxonomical groups were detected by FISH. All of the communities were dominated by bacteria belonging to the β-Proteobacteria (about 20%). The α-Proteobacteria, the Cytophaga-Flavobacteria cluster and the *Gram*-positive bacteria with a high DNA G+C content had similar parts (about 15%). On the other hand, WTP 2 and WTP 3 with a higher sludge age (about 70d and 80d; with EBPR and Ni) than WTP1 were dominated by bacteria belonging to the α-Proteobacteria (about 40%). The other groups had proportions of about 10%. In all plants each of the two groups of possible PAOs had an abundance of 4-8%.

To examine the bacterial composition of the BSP1 and BSP2, two 16S rDNA clone libraries were constructed. In contrast to the investigations by FISH, great differences could be detected between the clone libraries. Within 147 sequenced clones, 84 different sequences were found. BSP 1, with 58 different sequences from 77 clones, seemed be more heterogeneous than BSP 2. In this plant only 37 different sequences were found in 70 clones. The operational taxonomic unit (OTU) richness, estimated by the Rarefact.For program, was around 210 OTUs in case of BSP1 and 110 OTUs for BSP2. Only 11 of the 84 sequences could be found in both plants.

Automated histological evaluation of gastric biopsy specimen using digital slide

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AbstractText:
Background: Digital slides and virtual microscopy was shown to be alternative techniques for routine gastric biopsy specimen analysis. Image analysis techniques should support the automated, quantitative, reproducible classification of digital slides into major histological diagnostic groups.
Materials and methods
: From routine gastric biopsy, histological material 3 healthy, 17 gastritis and 7 adenocarcinoma, H/E stained routine sections were selected. Using the Hi-Scope slide digitiser system, digitalization was performed. In C++ automatic histological evaluation modules were developed. Altogether 36 parameters described the area, cell density, cellular characteristics of the basic tissue components: the surface epithelium, the glands, the muscle and connective tissue and the inflammatory cell compartment in the biopsy. In each compartment the cell morphometric features were calculated, as well. Area ratios of the different tissue compartments (biopsy area to epithelia, gland, connective tissue area, epithelium to connective tissue, epithelium to muscle tissue, epithelium to glands, epithelium and glands to non epithelial tissue) were also calculated.
Results:
Significant differences were found between the ratios of the biopsy/gland area (4.68+1.65 in normal, 4.04+1.05 in gastritis, and 67.5+48.5 in adenocarcinoma ($p < 0.05$)), and in the

biopsy/connective tissue area (4.4+3.9, 1.73+0.37, 2.75+1.32, p 0.05). Significant difference were also found in the ratio of cell numbers in the different cell compartments (5.65+2.65 in healthy,16.7+7.4 gastritis, 180.1,+114.2 adenocarcinoma p<0.01).
Conclusions:
This preliminary study proved that the development and evaluation of quantitative tissuemetric features can be used in the automated classification of histological gastric biopsy specimen.

COMBO-FISH: Specific labeling of chromosome regions by computer selected oligo-probe combinations

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The principle of Fluorescence *In Situ* Hybridization (FISH) with COMBinatorial Oligo (COMBO) probes is presented as a new approach that permits specific labeling of any given genomic sites for all species with an established genome data base. COMBO-FISH takes advantage of homopurine/homopyrimidine oligo-nucle- otides that form triple helices with intact duplex genomic DNA without the need for prior thermal or chemical denaturation of the target sequence, usually applied for probe binding in standard FISH protocols. An analysis of human and mouse genome data bases has shown that homopurine/homopyrimidine sequences longer than 14 DNA bases are nearly homogeneously distributed over the genome and that they represent about 1-2 % of the entire genome. Considering that the minimum observation volume in a confocal laser scanning microscope equipped with a high numerical aperture lens corresponds on average to a ~250 kb chromatin domain in a normal mammalian cell nucleus (e.g. lymphocyte), this volume should typically contain 150 - 200 homopurine/homopyrimidine stretches. Using DNA data base information, a set of distinct, uniformly labeled oligo-nucleotide hybridization probes can be configurated from these stretches. This set is expected to exclusively co-localize within a 250 kb chromatin domain, although some of the oligonucleotides have additional binding sites somewhere else in the genome. Due to the diffraction limited resolution of a microscope, the fluorescence signals of the joined oligo probe set merges into a typical, nearly homogeneous FISH “spot”. Typical sets are introduced for tumor correlated genome loci. Experiments in the ABL region of human chromosome 9 were performed as a very first “proof of principle” of COMBO-FISH. The technique was applied to human peripheral blood lymphocytes, paraffin embedded tissue sections, and routine bone marrow smears. Besides DNA-oligo probes also PNA-oligo probes as well as “smart probes” were used according to the COMBO-FISH protocol. The protocol offers the advantage of gentle specimen treatment in contrast to standard protocols. Although the experimental protocol applied so far contains several steps that are not compatible with live cell conditions, the theoretical approach may be a first methodological advance towards specific FISH in high-resolution fluorescence microscopy of *vital* cells.

Bringing flow cytometry to the limit: abundance and activity of small aquatic bacteria

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In marine and freshwater systems, ca. 10^6 bacteria per millilitre are found in a heterogenous microbial community together with viruses, protists, and algae. As aquatic bacteria are decomposers and the food base for other organisms, they play a major role in the carbon-cycle. Flow cytometry (FC) has entered aquatic microbial ecology as a tool that firstly increases the number of samples and the number of cells per sample that can be analyzed, and secondly facilitates multi-parameter analysis. In this study we investigated the small-scale vertical structure of the bacterial community of a high mountain lake. For this, 5 depth profiles, 4 in summer and 1 in winter, were made at the 9m-deep Lake Gossenkölle at 10 cm-intervals. We used a MoFlo cell sorter (DakoCytomation, Denmark) for measurement of cell number, cell size (side scatter), DNA-, RNA-, and protein content (DAPI, SYTO 13, SYPRO red, respectively) and activity (FDA +). In addition, the protocol to obtain absolute bacterial cell counts by FC was improved and the counting error optimized. The results showed significant changes in abundance of total bacteria and bacterial subgroups along the water column. In contrast, vertical distribution of bacteria was strongly similar among the 4 depth profiles in summer, but different from that in winter. Data on nucleic acid and protein content, and activity of bacteria will be discussed.

Predominant T cell activation in mesenteric lymph nodes versus Peyers Patches in response to orally applied proteins

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High doses of orally applied dietary antigens are known to induce systemic hyporesponsiveness by induction of anergy and deletion of specific T cells while administration of low doses leads to the generation of regulatory T cells. However, still little is known about the antigen-presenting cell (APC) involved in these processes and also the site of antigen presentation/ T cell activation remains unclear. To address these questions we used flow-cytometry to analyse the antigen-specific T cell response and the presentation of a specific peptide in various lymphoid organs after oral antigen application. Antigen presentation was measured by high sensitivity flow-cytometry to detect peptide presentation even at very low level (<100 peptides cell).
We show that after feeding of the Ova-peptide presentation is maximal on DC of the PP followed by DC in the mesenteric lymph nodes. At low doses presentation is restricted to DC of the PP and at higher doses peptide presentation can also be detected in the peripheral lymph nodes and spleen. Similarly Ova-specific T cells proliferate mainly in PP and to a lesser extent in mLN depending on the peptide dose fed. In contrast, after feeding of ovalbumin protein T cell activation preferentially occurs within the mLN but only at higher doses also in PP. This lack of T cell proliferation in the PP towards ovalbumin was also not reversed by infection with Salmonella which induces a strong inflammatory environment in the PP. Taken together these data highlight the importance of mLN for low dose oral tolerance against proteins and may suggest a defect in protein uptake or processing by APC of the PP.

„GFP-Walking“: Artifical Construct Conversions Caused by Simultaneous Co-Transfection Analysed by Spatially and Intensity Resolved Planeometric Microscopy (SIRPM)

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Several GFP variants have been developed for multicolor labeling *in vivo*. Here we report that simultaneous co-transfection of fluorescent protein chimeras can give falsepositive results caused by the conversion of spectral properties. The cDNA of the cysteine protease cathepsin B (CB) tagged with the enhanced yellow fluores- cent protein (eYFP) and the cDNA of the histone H2A was tagged with the enhanced cyan fluorescent protein (eCFP) and were cotransfected into lung carcinoma cells. Stable clones with converted fluorescence properties were esta- blished by G418 selection and prooven on the DNA sequence level by genomic PCR. Thus, conversion is based on homologous recombination/repair/replication processes that occur between the nucleotide sequences of the fluorescent proteins. To quantify the abundance of conversion high-throughput spatially and intensity resolved planeometric microscopy (SIRPM) was applied: The fluorescent nuclei imaged with an epifluorescence microscope were segmented according to their spatial and intensity properties in both the eCFP and eYFP channels and the conversion rate was calculated from the respective number of fluorescent nuclei. Under standard transfection conditions, approximately 8% of cells produce false-positive results, but, depending on the conditions, up to 26% of the cells permanently express altered fusion proteins. The conversion is independent of transfection methods or cell types. Generally, this compromises the interpretation of results obtained by dual-colour cotransfection using auto-fluorescent proteins. Consecutive transfection or low sequence similarities, however, avoided recombination. The appearance of conversion facilitates exchanges of spectral properties in fusion proteins, the creation of libraries, or the assembly of DNA fusion constructs *in vivo*. The detailed quantification of the conversion rate allows the investigation of recombination/repair/ replication processes in general.

High-resolution cytometry of the large-scale genome organization during cell differentiation and transformation

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High-resolution cytometry showed that some features of the global structure of cell nuclei does not change dramatically during the cell differentiation or transformation. Radial arrangements of chromosome territories (CTs) or their parts in terminally differentiated as well as in cancer cells are CT-specific with central localization of the highly expressed CTs. In addition, radial positions of extra copies of genetic elements in trisomic cells show similar distribution as compared with original distribution of diploid cells. Chimeric chromosomes that are typical of some leukaemia were found approximately midway between the nuclear positions of both original CTs, which seems to correspond to the rule that the radial positions of chromosome regions inside the cell nucleus are determined by the level of their transcription. Nuclear topography of genetic loci in colon cancer cells showed pronounced disorganization of CTs. Several types of topographic changes have been distinguished: doublets, chains, clusters. Some of amplified loci were detected on their CTs, the other were translocated on other CTs. To find changes of the chromatin structure, the nuclear distances between two BAC clones with short genomic separation (1-2 Mb) were measured. Larger nuclear distances reflecting decondensation of chromatin were found in tumour as compared with epithelial cells for the same genomic separation. The degree of chromatin decondensation decreased with the amplification of genetic loci. The influences of heterochromatin on gene silencing during the cell differentiation and transformation have been also investigated. During myeloid differentiation cell silencing correlates to gene distances to nearest centromeric heterochromatin. The influence of heterochromatin on RB1 gene silencing was investigated in retinoblastoma tumour cells with an (X;13) translocation. The potential role of the large-scale genome structure in diagnostics is discussed.

Protein-losing enteropathy after Fontan surgery: is assessment of risk patients with immunological data possible?

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Background:
Protein-losing enteropathy (PLE) is a late complication of the Fontan type surgery for univentricular heart characterised by massive enteric protein loss. The pathogenesis of PLE is not fully understood, and it is unclear why the onset of PLE varies widely and occurs months or even years after surgery. Besides characteristic laboratory findings, a typical cellular feature concerns the almost selective loss of CD4+ lymphocytes and an only slightly changed CD8+ lymphocyte count. The present pilot study aimed to test whether immunological or laboratory parameters differ in patients at risk for PLE.
Methods:
From children (n=15) with Fontan type circulation, extensive cellular, humoral and clinical laboratory data were analysed. Patients without enteric protein loss (group I, n=8), with transient phases of enteric protein loss in the absence of gastric infections (group II, n=6), and one PLE patient were distinguished. The 90 data columns obtained in phases with normal serum protein levels were compared. Results:
Clear differences were apparent between patients prior to PLE onset (group III), patients that in at least one occasion exhibited PLE signs (group II), and patients without detectable signs for PLE (group I). The most discriminatory parameters between the three patient groups were NK, $CD8TCRab^{+}$, $CD8TCRgd^{+}$ cell counts, as well as sL-selectin, IgE and Ca^{2+} (average recognition index = 91.5% negative/positive prediction/sensitivity/specificity > 83%).
Conclusions:
The results of this study seem to provide access to the pre-symptomatic assessment of PLE-risk patients.

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Improvement of the routine and automated evaluation technique in electron microscopic immunolocalization applications involved in correlative microscopy

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When one uses immunomarkers, three factors should be noticed: affinity, resolution and visibility. Affinity is one very important factor and means that the antibody you are using should have good or excellent affinity to the specific antigen you are looking for. Several factors can affect the affinity of an antibody complex to an antigen. Sometimes a complex that works well in fluorescence experiments not necessarily will work perfectly in electron microscopy assays. One problem related to the EM immunolocalization experiments is the association of particles to the antibodies which are “visible” for the electron beam. Gold markers are still very good particles to be used in this context. The other important aspect is resolution. Gold particles are in the range of several nanometers. The maximal resolution in ordinary electron microscopes is around 0.2nm, but for several reasons we can assume that, in practice, it is around 1nm. So, if a particle presents a diameter greater than this value, it will be detected by the electron microscope. However, this does not necessarily mean that very small particles will be viewed by the operator at the electron microscope. It is important to note that the maximal resolution of any part of the equipment cannot be assumed to be the final resolution of the system. In other words, if we are observing a field previously marked with gold labels of 3nm diameter, our microscope certainly will resolve the particles, but we

will need to magnify the image several times before they become visible to our eyes. This is the moment when the third factor starts to play a role: visibility. The final goal of an immunolocalization is to give the operator or scientist the opportunity to observe the distribution of the desired antigens, by means of labels to localize them. When one uses particles of a small diameter one needs to magnify by several times the image restricting the field of view, thus loosing the possibility to have a panoramic view of the sample and, of course, the overview of the particle distribution in a large area of the sample. If one decides to use big particles of 30 – 50nm to improve visibility at low magnifications, the affinity of the antibody complex will decay. This problem becomes even more severe, if one wants to perform double or multiple labeling. For our sake, the modern digital cameras are able to obtain images with very good resolution and high dynamic range (gray scales). To overcome all the problems listed above, one can use such digital cameras and image processing techniques to prepare image montages, with frames acquired at higher magnifications and then stitch them together to create large fields of view, allowing the researcher to easily observe and evaluate the distribution of small label particles over a large microscopic field. At this point, the electron microscopic observation touches the field of fluorescence light microscopy because the size of the largest wide-field views that you can get with this stitching and evaluation method for electron microscopy is at the upper limit of what light microscopy can manage, at least as far as beneficial magnification is concerned.

Complementary reading: Monteiro-Leal L.H., Tröster H., Campanati L., Spring H., Trendelenburg M.F. (2003): *Gold Finder*: A computer method for fast automatic double gold labelling detection, counting and colour overlay in electron microscopic images. Journal of Structural Biology 141 (3), 228-239.

System-Oriented Modelling of Saccharomyces cerevisiae: Coupling Cell Cycle Progression and Energy Metabolism

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Baker's yeast currently represents the best characterized eucaryotic organism and thus serves as an important model system in systems biology. Here we present an integrated model describing the coordination of yeast cell growth and proliferation. The second messenger cyclic AMP (cAMP) plays a central role in this context, since cAMP influences both energy metabolism and cell cycle progression via a protein kinase A-dependent signaling cascade.

Experiments have been performed in synchronous and continuous yeast cultures to quantitatively assess cAMP cell cycle dynamics and its regulatory effects on energy metabolism. These results are incorporated into a mathematical model comprising mutually interlinked submodules for metabolism (glycolysis and storage carbohydrates), cell growth, cell cycle progression, and cAMP signal transduction. Model parameterization and validation is performed on the basis of own and published experimental data. This guarantees the intimate connection of experiments and model development typical of the systems biology approach.

The integrated single cell model yields a dynamic description of the cAMP-dependent regulation of metabolism and cell cycle progression during the different cell cycle phases. The chosen modular approach is potentially transferable to systems of medical importance, e.g. when modeling tumor cell behavior. Moreover, the model can also serve as a basis for a segregated description of heterogeneous cell populations, e.g. in bioreactors.

Ultrastructural and flow cytometric monitoring of differentiation of cord blood derived dendritic cells under the influence of hormones regulating the female reproductive system

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The generation of dendritic cells (DCs) from human cord blood is of pivotal interest in respect to several clinical applications such as anti-tumor vaccination or immune tolerance induction. We investigated the morphological changes using light and electron microscopy during differentiation and maturation in relation to the immunophenotype of the respective cells. In addition, the influence of hormones influencing the reproductive system on the maturation of DCs was investigated.

Cord blood stem cells were obtained by MACS separation of $CD34^+$ cells from isolated MNCs. Cells were cultivated in IMDM medium supplemented with 20% fetal calf serum, antibiotics and the cytokines GM-CSF, TNF-α, SCF (20ng/ml each) and TGF-β1 (0.5ng/ml). In addition, cells were inoculated in shell vials containing coverslips for TEM and SEM. The cultures were maintained for 2 weeks and medium change was performed twice a week. Progesterone or beta-estrogen was added to the culture medium in a concentration of 10^{-5}M. Flow cytometry of cells was performed on day 7 and 14 using fluorochrome-labeled monoclonal antibodies: against CD1a, CD14, CD45, HLA-DR, HLA-DQ and. CD83.

After 7 days of culture clusters with outspreading cells occurred showing knob-like protrusions and branching cell projections. The majority of cells were immature DCs ($CD1a^+/CD83^{dim}$) with intracellular Birbeck granules. About 17% were mature DCs ($CD83^{bright}$) which increased in number after 14 days of culture to 30% and were characterized by numerous cell processes. Progesterone in the culture medium inhibited while beta-estrogen stimulated the maturation of DCs. Possible implica- tions of these results for the immune status of pregnant women are discussed.

Standardization and Quantification of Circulating Tumor Cells

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Background:
In order for detection and quantitation of circulating tumor cells from solid tumors to become a valuable parameter for therapy monitoring, the procedure needs to be standardised.
Methods:
In the present work we have analysed the influence of preanalytical handling of the specimen with respect to storage, white blood cell isolation, access to the population of epithelial cells with and without enrichment procedures, and the sensitivity of the analytical process.
Findings:
Storage of whole blood samples over 2-3 days had little influence on the number of recovered epithelial cells whereas sucrose densitiy separation of mononuclear cells did not enrich epithelial cells but even depleted and deselected these cells leading to a gross underestimation of the number of such cells in hämatological specimens. The effectiveness of magnetic bead enrichment procedures was dependent on the initial numnber of the cells sought-after and the volume of the original material used for enrichment.
Interpretation:
We therefore propose a simple and easy method of cell preparation leading to highly

reproducible results which will allow repeated analysis of the number of circulating tumor cells during treatment. We show that tight monitoring of patients during therapy enables real-time surveillance of treatment response and thus will contribute to more individually tailor therapy.

Investigations of cell contacts to solid surfaces using TIRF microscopy

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Adherent cells develop complex surface contacts commonly known as focal adhesions. Besides their function in cell anchoring focal adhesions transfer information from the substrate surface into the cell by integrin dependent signal transduction.
Through the manipulation of topographical and chemical properties of a surface it is possible to influence the behavior of adherent eucaryotic cells. Here we look on the focal adhesions and the basal cell contact pattern on materials structured under controlled conditions.
In Total Internal Reflection Fluorescence (TIRF) microscopy a laser beam is totally reflected at the interface between a glass supporter and an aqueous cell culture medium. The resulting evanescent field has a decay distance of 80-150nm towards the fluid medium. As a consequence the surface associated compartments of an entirely labelled cell is exclusively excited. This way it is possible to examine the focal adhesions of adherent cells and to check the cell reaction on particular surfaces and coatings.
The pattern of focal adhesions changes with the cell type, the overall cell condition and the local surface structure. This suggests that distinct surface patterns of abiotic materials and 2-dimensionally arranged coatings of signal peptides or components of the extracellular matrix can induce a particular behavior in adherent cells. We will make use of this strategy in order to stimulate the cell motion along defined pathways, to restrict the cell spreading, and to align the cell body.

Correlative microscopy of consecutive ultra-thin specimens using reflection contrast light microscopy (RCM), confocal laser scanning light microscopy (CLSM) and electron microscopy (TEM)

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Reflection contrast microscopy (RCM) can be described as the bridge between light microscopy (LM) and electron microscopy (TEM). An ultra-thin section cut from a tissue block can be transferred - using a wire loop - to a microscope glass slide for LM/RCM, or reflection mode CLSM.Then the next, consecutive, ultra-thin section cut from the same tissue block is put on a grid for TEM. Ultra-thin cryo-sections can be brought directly on the glass slide and stretch very well. The RCM-image looks like an inverted low magnification TEM image. When the same section is examined with a reflection mode CLSM a similar image can be obtained. Compared with the CLSM fluorescence mode, the CLSM reflection mode permits a more than 5-10x smaller pinhole (better resolution and no fading). It is ,however, not easy to examine the small ultra-thin sections with CLSM. It would be easier if the CLSM would be also equipped with the optical parts for RCM. A valid comparison between RCM / CLSM and TEM images is possible since the same embedding and staining methods can be used. In immune-studies the use of sequential sections permits the use of multiple antigens using only one type antibody for each ultra-thin section. This avoids possible antibody competition. Most immune-labels like gold, silver-enhanced gold, peroxidase, phosphatase give strong

reflectance signals. The same section can also be stained with conventional histochemical stains for morphological orientation. A large image contrast between the bright immune marker and the morphology staining can be achieved, making digital image analysis easy. RCM permits the examination of very small (< 1 mm) biopsy, enabling a micro-biopsy from e.g. the kidney, lung and tissue culture. RCM fulfils all requirements for high definition microscopy. The image contrast is extremely high and with ultra-thin sections no pre- and post- focal images disturb the RCM image formation, resulting in a microscope resolution near the very maximum achievable with LM.

Detection of rare nuclei showing DNA-heterogeneity in populations of 50.000 cervical cells, using a high speed hyperthreaded scanning and cell-analysis program (HSCA)

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The purpose of this study is to describe an instrumental strategy to detect rare (frequency below 0.01%) suspect cells which may be missed by visual evaluation in e.g. ASCUS specimens in cervical cytology. Modern high speed PC processors using hyperthreading techniques can now facilitate applications that have to handle simultaneous piezo-electric focusing, image acquisition by CCD, cell segmentation and classification.

Nuclear DNA analysis with this method is not used primarily for diagnostic or prognostic purposes, but only as a screening method to be followed by colposcopy and histopathology for further diagnostic evaluation.

Increased DNA content is one of the most robust cellular parameters in cytometry for cytology screening. In large previous studies (1,2) it was found that cervical specimens from healthy woman very rarely showed high nuclear DNA values. These investigations were performed with earlier instruments such as the MIAC system from LEICA and the DISCOVERY system from BECTON and DICKINSON .The screening of nuclei with increased DNA-content took about 45 minutes per specimen. For practical application in a routine cytology laboratory this was not a cost effective procedure. Fairfield Imaging Ltd (Nottingham, England) has now developed an instrument that can analyse a sufficient number of cells in approximately 10 minutes using HSCA of liquid cytology slides with well flattened nuclei (these specimens require only few focussing steps). The clinical importance of the detection of rare nuclei with abnormal DNA content in cervical ASCUS cases has been shown by Bertino (3). In a significant number of specimens a few nuclei with suspect DNA values were found among 50.000 nuclei present on a Feulgen stained ASCUS slide.

(1) van Driel-Kulker et al., Cytometry 1985;6:268
(2)Strohmeier et al., Cytopathology 1993; 4: 139
(3) Bertino et al. 2001:http://rex.iutcaen.unicaen.fr/7esacp/abstracts/A012.html

Correlative light and electron microscopy for the structural investigation of nuclear architecture

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Gene activity is regulated by the primary sequence of DNA of e.g. promoters and enhancers, and the modification of chromatin by e.g. histon acetylation and methylation. As a third level

of control it is discussed that the structural organization of chromatin influences gene activity by regulation of the accessibility of transcription factors to their gene-target.

To investigate diffusion-limited spaces in the nucleus, we analyzed by correlative fluorescence and electron microscopy the distribution of organic compounds ectopically loaded into nuclei of human cell-culture systems (SW13, HeLa, MCF7): the intermediate filament protein vimentin from *Xenopus* (nucleus-targeted by an NLS and tagged with GFP), the mural interferon-induced protein Mx1 (tagged with YFP), and dextran-FITC of high molecular weight (2.5 MD). Obviating the need to preserve antigenicity, correlative microscopy allows the use of strong fixation for good ultrastructural preservation. The fluorescently labeled tracer-compounds are located on electron micrographs by comparison with the fluorescence signal observed in the identical nucleus beforehand by fluorescence light-microscopy.

The nucleus targeted *Xenopus* vimentin polymerizes into filaments at 28°C which arranged into bundles looping throughout the nucleus. Bridger et al. (1998, J. Cell Sci. 111) showed that vimentin bundles follow the contours of chromosome territories. Nuclear bodies frequently localized adjacent to these vimentin bundles. This was also observed for YFP-tagged Mx1, which formed small elongated crystals. Since these bodies were never found embedded within vimentin bundles, which would suggest kind of affinity-interaction, limitation of space forcing the structural entities to locate in close proximity may rather be the reason for the observed adjacent localization. Thus, the mechanism of space-restriction may be based on the inaccessibility of large chromatin sectors due to higher order chromatin organization. Accordingly, 2.5 MDa FITC-dextran microinjected into cell nuclei did not distribute homogenously: The FITC-signal rather concentrated in foci and lacuna-like spaces. Electron microscopy showed the dextran excluded from perinuclear and perinucleolar heterochromatin.

Single cell and tissue based microarrays for pharmacotoxicological and therapeutical biomonitoring

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Automated screening of the selectivity, toxicity, metabolism and absorption of drugs could support the development of novel innovative diagnosis and therapeutics. Today scientists are able to assay compounds on living cells or tissues on bioelectronic chips. Therefore, we developed biohybrid microstructures for optoelectronic single cell monitoring and microfluidic capillary modules contacted by three-dimensional in vitro tissues for life time and longterm pharmaceutical, gene therapeutical and pharmacotoxicological screening. Planary microstructures with suction holes and ring electrodes (average diameter, 6µm) have been designed, developed and fabricated for selection, characterisation of single cells, monitoring of expression patterns and protein kinetics in real-time. Furthermore, a novel 3D tissue based sceening system designed as a microcapillary array was realized by plastic forming technology with the following components: microcapillaries with diameters of 100 µm, 200µm, 300 µm, and 400 µm and implemented platinum electrodes for impedance spectroscopy and/or potential recording. Biohybrid Microstructures were established in combination with two models of an embryonic 3D in vitro retina – model 1 represents a simulation of an in vivo retina, model 2 represents a pathogen retina. Signals of neurons, alterations of the tissue or molecular parameters could be measured via integrated microelectrodes. Further microarrays with embryonic cardiomyocytes and breast cancer tumour cell aggregates have been generated and applied for detection of risk factors and drug screening. The progress of all of these biosensors is focused on the ultra-fast synchronized detection and monitoring of cellular alterations in realtime and online.

Design of specific labeling probes for COMBO-FISH

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In Fluorescence In Situ Hybridization with Combinatorial Oligo probes (COMBO-FISH, cf. M. Hausmann et. al., this abstract booklet), any given genomic site can be labeled by a specific set of oligonucleotides that is co-localizing within a 250 kb region at the desired location, but nowhere else. The specific set consists of homopurine- or homopyrimidine-stretches hybridizing to intact duplex genomic DNA. In accordance with a theoretic model based on an independent distribution of nucleotides with their natural genomic requencies, about 1-2 % of a chromosome consist of such sequences of a length of more than 14 bases. We discuss the model distributions and the bioinformatical findings for human, mouse and rat genomes concerning homoP- and homoY-sequences also including a small number of mis- matches. To design a clustering set for a specific gene locus, we extract the posi- tion of the gene from an NCBI annotation file by exact search for key words and select the candidate set from the respective contig. This set, usually in the order of 50 stretches, is searched for in the total genome sequence taken from Golden Path chromosome assembly using a finite automaton. This also relocates the contig within the respective chromosome. A cluster analysis follows, and clusters of 6 or more sequences within 250 kb are removed by rejecting selected sequences. For the target locations tested so far, a set of 30 to 40 homooligos remains. In addition, clusters with 5 sequences per 250 kb are in the order of 10 or less. Improved ver- sions of the search algorithms allow the whole procedure to be performed within 15 minutes interacting time. Open problems concern treatment of SNP's, annoying tan- dem replications, and optimization of algorithms within the trade off of running time and storage space.

Predictive medicine in cardiology

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Predictive Medicine aims at the detection of changes in patient's disease state prior to the manifestation of deterioration or improvement of the current status. Patient-specific, disease-course predictions with >95% or >99% accuracy during therapy would be highly valuable for everyday medicine. If these predictors were available, disease aggravation or progression, frequently accompanied by irreversible tissue damage or therapeutic side effects, could then potentially be avoided by early preventive therapy. The molecular analysis of heterogeneous cellular systems (Cytomics) by cytometry in conjunction with pattern-oriented bioinformatic analysis of the multiparametric cytometric and other data provides a promising approach to individualized or personalized medical treatment or disease management. Predictive medicine is best implemented by cell oriented measurements e.g. by flow or image cytometry.
Nowadays there are several examples of clinical applications of predictive medicine by Cytomics in cardiology and cardiovascular surgery. Important aspects of predictive medicine in pediatric cardiology concern the preoperative identification of patients with a tendency for postoperative complications based on leukocyte activation marker expression and serological data, pre-symptomatic recognition of children at risk for protein losing enteropathy, a life threatening complication after heart surgery of children with univentricular heart or adult coronary artery disease patients with an increased tendency for restenosis. As a consequence, better patient care and new forms of inductive scientific hypothesis development based on the interpretation of predictive data patterns are at reach.

Detection of carcinoma of the upper aerodigestive tract by laser-scanning-cytometry of mucosal swabs

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Up to now minimal sample volumes such as mucosal swabs of the larynx are difficult to analyse by multiparametric cytometry. This is first of all due to the minute cell number obtained by such approaches. Nevertheless, objective data such as the DNA ploidy of the tumour could prove to be a useful tool in the preoperative management of the patient.

We adopted laser-scanning-cytometry (LSC) for the multiparametric analysis of swabs from the larynx and other regions. Cells were immobilised on a conventional slide, and their DNA-content (DNA-ploidy) and cytokeratin expression were determined. By re-localisation of cells of interest their morphology was documented; to this aim slides were counterstained with H&E after LSC-analysis. All specimens were obtained during routine microlaryngoscopies and were additionally analysed by conventional cytology; excisional biopsies of the same lesions were analysed by histology as gold standard.

Out of 112 swabs there were 15 classified as insufficient. From the remaining 97 there were 63 classified as DNA aneuploid; in routine histology 61 turned out to be carcinoma and 2 were leukoplakias. The positive predictive value for LSC-analysis is 96.8%, it’s specificity is 92%, and it’s sensitivity is 84.7%.

Analysing cytological specimens by LSC obtains objective and quantitative data in addition to routine cytology. This is of special relevance where only minimal sample volumes are available. It’s clinical use might be as part of the follow-up of cancer patient in order to early detect recurrent disease.

An improved method for determining and correcting glare in a microscope

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Glare is an important source of error in microdensitometry. Traditionally, images of cell nuclei in cytometry are believed to gain some extra light due to glare. The “amount of glare” usually is determined by measuring apparent transmission of opaque objects, e.g. carbon particles. Methods currently adopted for glare correc- tion are based on a subtraction of the predetermined “glare amount” from the mea- sured transmission of cell nuclei. However, it is wrong to consider glare merely as some additional light. Glare as a physical phenomenon represents a redistribution of the light within the field of view (FoV). As a consequence, the mean brightness (transmittance) of the FoV remains unchanged, but light areas within the FoV loose their brightness, whereas dark areas gain in brightness due to glare. The amount of light gained or lost by a particular object due to glare depends on the relation between the true transmittance of the object and the transmittance of the entire FoV. For example, empty spaces between opaque particles loose their brightness due to glare, the loss being linearly dependent on the amount of the particles in the FoV, i.e., the mean brightness of the FoV. Methods for measuring and correcting glare should thus account for the brightness of the FoV. All statements above are proven experimentally. An improved formula for glare correction, which is universally appropriate for both light and fluorescence microscopy, is provided. Glare correc- tion is performed on each pixel of an image resulting in “glare-free” images. This allows measuring correctly not only integrated optical density, but also any other densitometric or textural features.

Towards the individualization of systemic Lupus Erythematodes (SLE) therapy

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Background:
The majority of SLE patients benefit from the various available therapeutic options. Some suffer, however, from side effects or therapy resistance. It is presently not possible to identify those patients pretherapeutically for case adapted individualized therapy. The goal of this study was to determine whether therapy specific patterns of clinical, laboratory or cellular parameters exist which are useful for the pretherapeutic identification of therapeutic susceptibility or resistance.
Material and Methods:
15 clinical and clinical chemistry, 11 serological and 11 cellular parameters from 44 SLE patients were classified by a mathematically assumption free data sieving algorithm (http://www.biochem.mpg.de/valet/classif1.html). The algorithm selects the most discriminatory parameters patterns in an iterative way to pretherapeutically discriminate between various therapy schemes at the level of the individual patient.
Results:
Patients requiring high therapeutic intensity were pretherapeutically identified with a predictive value of 89.5% and patients with active disease who profited from therapy could be distinguished with predictive values of 87.2%. Sensitivity and resistance to cyclophosphamide therapy was pretherapeutically predictable with 87.5% and 100.0% accuracy in a subgroup of 12 patients. Patients during Quensyl, Imurek and Sandimun therapy were discriminated by specific parameter patterns with predictive values of 83.3%, 90.0% and 100.0% while low Prednison and CellCept therapy showed relatively similar parameter patterns with lower predictive values of 55.5% and 75.0%.
Conclusion:
The present results suggest that specific patterns of clinical, serological an cellular data contain pretherapeutic information on therapy outcome and optimal configuration of therapy for the individual patient. The findings are encouraging in the effort to provide an individually optimized therapy for SLE patients.

Scanning fluorescent microscopy is an alternative for quantitative fluorescent cell analysis

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Background:
Fluorescent measurements on cells are performed today with flow and laser scanning cytometry. The scientific community dealing with quantitative cell analysis would benefit from the development of a new digital multichannel and virtual microscopy based scanning fluorescent microscopy technology and from its evaluation on routine standardization fluorescent beads and clinical specimen.
Methods:
We applied a commercial motorized fluorescent microscope system. The scanning was done at 20x magnification, on 3 channels (Rhodamine, FITC, Hoechst). The SFM (Scanning Fluorescent Microscope) software included the following features: scanning area, exposure time, and channel definition, autofocusedscanning, densitometric and morphometric cellular feature determination, gating on scatter plots and frequency histograms, preparation of

galleries of the gated cells. For the calibration and standardization Immuno-Brite beads were used.
Results:
With the application of the shading compensation the CV of the fluorescence of the beads decreased from 24.3% to 3.9%. Using JPEG image compression method until 1:150 compressionno significant change was observed in the CV of the beads fluorescence. The change of focus influenced the CV significantly only after ± 5 µm error.
Conclusions:
Scanning fluorescent microscopy is a valuable method for the evaluation of fluorescent labeled cells.

Monitoring of biotechnology processes via online flow cytometry

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Flow cytometry which has for many years been indispensable for research in haematology, immunology, and oncology, is now attracting the attention of more biotechnology scientists.

The ability of flow cytometry to provide direct, quantitative information about cellular physiologic processes makes it a powerful tool to monitor bioprocesses.

However, process monitoring by Flow Cytometry in biotechnological applications suffered from being too expensive. The high investments and the small size of the yeast prevented the wide application of this method in the field of biotech sciences. Furthermore the manual multi- step preparation of the samples consisting of e.g. cell- washing, diluting, fixation of the cells, and fluorescence dying, makes this method somewhat time consuming and hard to automate.

Therefore a newly developed FIA (Flow Injection Analysis) was used to couple an ultra compact CyFlow® SL Flow Cytometer (Cytecs GmbH, Görlitz) online to a bioreactor.

The FIA executes sampling, all necessary preparation steps, and delivers the sample to the CyFlow® SL automatically. The modular design of the FIA, consisting of three subsystems, (i) sample delivery (ii) sample handling, (iii) sample injection and analysis, makes it a versatile instrument to perform highly automated process monitoring by flow cytometry.

To reduce the sampling intervals and enhance the situation of process data, fast and robust dying procedures are essential. For this reason two dying procedures were optimized for the online FIA Flow Cytometry. A procedure for dead-vital staining and another for cell cycle analysis could be optimized or newly developed, respectively. Both procedures can be performed using *Saccharomyces cerevisiae* within 10 minutes or less.

Relationship among chemical structure of polyphenols, affinity to formaldehyde and their apoptosis inducing effect on tumor cell lines

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Chemical Technology Introduction Earlier studies of reactions in cell free solutions at 20C and pH=7,4 showed that among H2O2, HCHO, and biological amines (lysine, guanosine) intensive radical excitation (N-formylation) reactions occur. On the basis of literature data we presumed that these reactions could be modified by polyphenols and take place also in cell culture and affect cell proliferation and cell death. Methods Tumor cell cultures (HT29 and Oscort) in 24 well plates were treated for 24 hours by 60 mM a H2O2, HCHO and their combination with polyphenols (gallic-acid, myricetin, floroglucin, (±) catechin, (-)epigallocatechin-gallate (EGCG) and trans-resveratrol). These polyphenols are commonly occur in seeds fruits or plant products (green tee wine). With unfixed cells necrosis %, with ethanol fixed cells cell cycle distribution and apoptosis % were determined by flow cytometric analysis. Results The combinational treatment by H2O2 and HCHO, induced increased necrosis and apoptosis % compared to H2O2 or HCHO alone. Among polyphenols the EGCG and myricetin and also resveratrol boosted the effect of the combination of H2O2 and HCHO. However the (±) katechin and gallic-acid and floroglucin did not have observable additive effects. Conclusions by our hypotheses the polyphenols in the Mannich condensation reaction (the characte- ristic reaction of phenols, formaldehyde and amino-groups) according to their molecule size and affinity react with DNA. The reactive multi-cyclic, bigger mole- cules as EGCG and myricet in cross-link the DNA double helix (establish a bridge between the two DNA chains) and cause increased necrosis and apoptosis %.

Intracellular localisation of Hepatitis B virus core and surface proteins encoded by recombinant Semliki Forest virus replicons

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Semliki Forest virus (SFV) vectors have been developed recently to provide a convenient system to express different proteins in virtually any animal cells. We adapted this SFV expression system for hepatitis B virus (HBV) investigation. Genes of HBV structural proteins were inserted into the SFV replicon and recombinant SFV/HBV was produced for the infection of BHK cell line. The result of the infection was successful expression of all HBV structural proteins that was proved by the electrophoresis of specific immunoprecipitates in PAAG and by immunocytochemical method.
The aim of the work was to show the intracellular localisation of HBV structural proteins: three surface antigens (HBs: large, L; middle, M and small, S) encoded by gene S and the capsid protein (HBc antigen) encoded by gene C by immunocytochemistry method. BHK-21 cells grown on tissue culture chamber slides (Nagle Nunc International) were infected with recombinant SFV, and incubated at
37 ^{0}C (5% CO_2) for 24 h. The expressed HBV structural proteins were tested using appropriate mouse monoclonal antibodies and alkaline phosphatase conjugated anti-mouse

IgG secondary antibodies. Sigma FAST reagent (Fast Red TR/Naphtol AS-MX) developed alkaline phosphatase activity.
HBc antigen was found in almost all cells, it covered all the cytoplasm. HBc was found to locate also in the nucleus of small proportion of cells (4-8%). On the contrary, the surface antigens always were concentrated in the Goldgi region, in several cells also as a narrow ring in the perinuclear cytoplasm or expanding from the paranuclear Goldgi region to the periphery covering, however, only one segment of the cytoplasm. Immunoreactive material filled up also quite all the cytoplasm of several cells in the case of HBs M and 25-30% of cells in the case of HBs L.
Studies on intracellular kinetics of different kinds of subviral particles could promote the understanding of HBV pathogenesis at the molecular level.

Online analysis of microcarrier cultivations

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Microcarriers are being used in high-density cell cultures in several bioprocesses, which can be divided in three categories, a) high-yield production of cells or cell products, b) studies of cells in vitro, c) routine cell culture techniques. The advantage of this technique is that anchorage-dependend cells are growing on the surface of small spheres, so the disadvantages of a standard monolayer culture can be avoided by increasing the surface area with the use of microcarriers. Our aim was to develop an analytical system to monitor a microcarrier cultivation process. Therefore a model cell line (NIH-3T3) was grown on different microcarrier spheres. The most important criterion for the microcarriers was the size and size distribution, as flow cytometry is limited to smaller objects not exceeding 150 µm.

Several different microcarriers were used for the usability testing (Cytodex 1 and 3, 130-215 µm, Amersham Pharacia; Glass Microcarriers, 90-150 µm and Plastic Microcarriers 150-210 µm, Cellon SA; PMMA Beads 83 µm, Bangs Labs; Polystyrene Beads 45.6 µm, Polyscience). The inoculation density of the microcarriers was 2 g per liter culture medium with a cell density of 2,5*10E4 cells per ml. Cultivations were carried out in 500 ml spinner flasks, RPMI 1640, 5 % NCS. The large scale cultivation process was performed in a self-build 5 l steel tank reactor with an adjusted 25 mm port for the in situ microscope. Flow cytometric analysis was done using MacroSort of the FACS Vantage SE (BD Biosciences) with a 200 and 400 µm nozzle.

In-situ microscopy has proven to be a suitable tool for monitoring the plating efficiency and the cell density for every chosen microcarrier. The pictures obtained during fermentation had a similar quality with the phase contrast pictures. All chosen standard microcarriers caused tube or nozzle blocking. The 45.6 µm polystyrene spheres have shown a good plating efficiency and due to the small diameter were easily detectable by flow cytometry. Flow cytometric analysis has shown a good correlation in judging the plating efficiency with the in-situ microscopy and microscopic analysis. By plotting sidewards scattered light against fluorescence intensity four separate populations were distinguishable corresponding to single cells, microcarriers with no cells and an increasing amount of cells attached to the surface.

Significance of cytotoxic T cells bearing CD3+CD8+CD28- phenotype in patients with respiratory tract cancers

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In patients diagnosed with bronchial and head and neck cancer, qualified for radical surgery, long term survival and prognosis are still very poor. Therefore searching for the biological background of this phenomenon is of great clinical importance. The aim of the study was to determine differences in phenotype of T lymphocyte isolated from peripheral blood and regional lymph nodes in patients with and without metastases. The study group consisted of 12 laryngeal and 8 bronchial cancer patients. All patients were diagnosed as squamous cell carcinoma. Apoptosis, expression of CD28 and TCR zeta chain were determined by means of flow cytometry on the backgated T lymphocytes. Apoptosis was detected using Annexin V binding, and Caspase-3 activation. In a few patients the expression of Granzyme B was detected. All antibodies used were commercially available: CD3, CD4, CD8, CD28 and anti-Caspase3 (Becton Dickinson), TCR zeta (Beckman- Coulter) and Granzyme B. In peripheral blood lymphocytes from cancer patients the decrease or even absence of TCR zeta expression was observed mainly in CD8 subpopulation as compared to control. In PBL higher content of CD3+CD8+CD28- lymphocytes was also detected. The CD3+CD8+CD28- subset in lymph nodes was very small, but there was a tendency for its increase in lymph nodes bearing metastases. This CD3+CD8+CD28- subpopulation both, isolated from T lympho-. cytes and lymph nodes presented also the highest rate of apoptosis. The CD3+CD8+CD28- T cells are supposed to be the "true" cytotoxic T cells and may undergo apoptosis in the AICD (activation induced cell death), mechanism of their elimination. They may also constitute population eliminated as a consequence of lack of costimulation. Thus the appearance of metastases in regional lymph nodes may be evidence not only of tumor progression, but also impaired immune function. Higher content of CD3+CD8+CD28 T cell subset, down regulation of TCR zeta and increased apoptosis of T cells may play important role in this chain of events.

Preparation of cells on glass fibres for micro axial tomography

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Micro Axial Tomography allows cells to be rotated in a far-field light microscope and thus to be viewed under different perspectives (different rotation angles). In practice, this results in an improvement of the 3D-resolution so that true distances between fluorescent labelled objects can be measured much more precisely (Lit.1, 2).
In contrast to object slide based microscopy an obvious shortcoming of micro axial tomography appeared to be the attachment and fixation of cells or cell nuclei on the glass fibre necessary for the object rotation. The use of standard capillaries for cell preparation and specific labelling of subcellular structures (e.g. fluorescence in situ hybridisation of genome regions in intact cell nuclei) requires careful handling of the fibres and relatively high amounts of material.
The recently developed automatic micro axial tomography setup (Lit. 3, 4) usually works with glass fibres with a diameter of 125 micron which have been made “sticky” for cells by coating them with a polymer with a high positive charge density. To handle those coated fibres carefully and to attach cells or cell nuclei, a fixture has been constructed where the fibre is firmly positioned in the centre of a glass capillary of 2 mm inner diameter and 20 mm length. The capillary can be filled from one side with cell suspension using a standard 100 microlitre pipette, taking advantage of the capillary action. Cells touching the fibre adhere due to the stickiness of the polymer coating of the fibre.
For fluorescence in-situ hybridisation (FISH) experiments, especially in biological or diagnostic routine, commercial DNA probe sets are applied which are designed as probe combinations specific for the detection of certain disease correlated chromosome aberrations. Such probe sets are expensive. Therefore, a further fixture, offering a much smaller volume of 10 microlitres for FISH of cells sitting on the fibre has been designed.

Here, the fibre with the adhered cells is placed into a narrow channel formed by two hemocytometer cover slips glued side by side onto a standard object slide. On both ends of the channel, provisions have been made to firmly hold the fibre in place, avoiding a touch of any surface of the channel. During the hybridisation process, the channel can be covered with a longitudinal slice of a standard cover slip.
These two setups are presently under laboratory test. With appropriate care, the two sample preparation devices can be handled successfully avoiding undue loss of cells on the fibre.
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Complete assessment of human antigen-specific CD4-Th-cell responses *ex vivo* according to antigen-reactive CD154 expression

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One of the major aims for both basic science and therapeutical applications is still to gain access onto the entire repertoire of human antigen specific $CD4^+$ Th-cells *ex vivo*. However, currently available methods suffer from various limitations as lacking knowledge about immune dominant peptides in the case of MHC-II-peptide multimers or dependence on antigen-reactive cytokine expression in the case of the cytrometric cytokine assay (CCS). We have here established methods to overcome these drawbacks. For the analysis of antigen-specific Th-cells we employed the detection of CD154 intracellular in fixed $CD4^+$ Th-cells and the activation of Th-cells in the presence of a secretion inhibitor Bref-A as has been described recently. For the isolation of live antigen-specific Th-cells the assay utilizes the antigen-driven *in vitro* activation of specific Th-cells in the presence of anti-CD40 monoclonal antibodies, which are able to block interaction of CD154 with its counterpart CD40 expressed by activated B-cells or antigen presenting cells. Based on this strategy we show that the highly efficient enrichment of antigen-specific Th-cells from complex cell mixtures is feasible. Our results offer novel perspectives to analyse and isolate antigen specific human $CD4^+$ Th-cells in an easy and fast way e.g. to generate highly specific Th-cell lines.

Diagnostic tissue elements in melanocytic skin tumors in automated image analysis

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In tissue counter analysis (TCA), digital images are divided into subregions (elements), and the digital information in each element is used for statistical analysis. In this study we assessed the morphologic details of tissue elements, which have turned out to be of diagnostic significance in the discrimination of benign common nevi and malignant melanoma. Analysis was performed using an Axioskop 2 bright field microscope with a scanning table (Zeiss, Oberkochen, Germany) mounted with a CCD three-chip colour video

camera (Sony, Tokyo, Japan) connected to a KS 400 3.0 image analysis unit (Zeiss Vision, Hallbergmoos, Germany). After creation of a data set based on a total of 12.000 cellular elements obtained from 100 benign common nevi and 100 malignant melanomas, CART (Classification and Regression Tree) analysis was performed in order to differentiate between cellular elements of nevi and melanoma. In a second step, the slides were re-evaluated by the decision!
tree and cellular elements suggestive either for benign common nevi or for malignant melanoma were highlighted on zoomed images of the whole sections and the individual elements were displayed in galleries. 8 groups of elements (so-called terminal nodes) seemed to indicate benign common nevi, whereas 7 terminal nodes were suggestive for malignant melanoma. The elements of nodes suggestive for benign nevi largely contained nevus cells with amphiphilic cytoplasm, intermingled with fibrillary material, while the elements of the nodes suggestive for malignant lesions often showed hyperchromatism, perinuclear halos, heavy pigmentation, or a lymphohistiocytic infiltrate. In conclusion, tissue counter analysis automatically detects tissue elements for diagnostic discrimination, which are in accordance with morphologic criteria used in conventional histopathology.

Synthetic deoxyglyco-conjugate (JJ34) induces apoptosis in carcinoma cells

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Four libraries of substances have been screened by the so called MTT-cell culture test for their cell toxicity properties: A juice of the American Venus flytrap (Dionaea Musicpula), extracts of the European thistle (Carthamus lanatus), extracts of the marine bacteria Halomonas Marina and different derivates of synthetic deoxyglyco-conjugates.

For the testing of the dose-effect relationship cell lines of human cervix carcinoma (HeLa) and human lung carcinoma (A549) have been used. The deoxyglyco-conjugate JJ34 has been identified to be the most effective substance. JJ34 causes a verifiable decrease of the living cells at 200 nmolar concentration.

Further investigations have been carried out by flow cytometry to investigate how JJ34 affects the metabolism of the cell. The vitality and the cell number have been investigated and it shows that the vitality decreases during incubation with JJ34. Additionally the effect of JJ34 on the cell cycle has been investigated and an abnormal increase of the cell count in the G2 phase has been observed. Annexin V – FITC combined with PI staining has been used to prove evidence of apoptosis in JJ34 treated cells.

At present further research aims at BrdU techniques to further examine the effect of JJ34 on the cell cycle.

What happens with highly purified monocytes on their way from blood to the gene-chip?
Gene expression profiling of monocytes isolated by different sorting strategies

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It has been shown for various chronic diseases that disease-specific gene expression patterns are reflected at the level of blood cells. But if analysing whole blood samples by

genome-wide microarrays the following problems have to be taken into account: (1) the contribution of rare cells to an expression profile will be lost; (2) the undefined cellular composition of a crude tissue extract allows no destinction between change in gene activity or change in cell numbers; (3) the allocation of differentially expressed genes and the corresponding celltype seems to be an insurmountable barrier. Therefore, it seems advisable to analyse purified subpopulations instead of whole blood samples. For this purpose several cell separation techniques are available, such as fluorescence activated cell sorting (FACS), magnetic cell sorting (MACS), density gradient centrifugation and the combination of them. Unfortunately, leukocytes behave very sensible against experimental manipulations and partial activation of cells is almost not avoidable. In many cases the degree of activation is difficult to quantify and only a very limited number of activation markers has been monitored.

In the present report a systematic analysis of preparation-induced alterations in monocytes that have been isolated by different procedures was performed by gene expression profiling experiments with custom-made cDNA arrays. Principally, monocytes can be isolated by a positiv labeling of cells, mostly via the CD14 antigen or by an untouched approach were all cells other then monocytes were labeled and depleted. The experimental design presented in this study allowed the determination of the influence of (1) CD14-labeling, (2) Ficoll density centrifugation, (3) hypoosmotic treatment, (4) magnetic and (5) fluorescence activated cell sorting by monitoring approximately 900 genes in parallel at the transcriptional level. The results of this study should help to assess the suitability of different monocyte separation strategies on the way to minimize preparation-induced artefacts and may be valuable for designing experimental setups in the fields of functional genomics and proteomics.

Role of extracellular matrix biopolymers in proliferation of osteosarcoma cells

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Introduction:
The proliferation and metastatic potential of human osteosarcoma may also be determined by the signal transduction pathways related to extracellular matrix components. Since the extracellular matrix construction is principally different in tumours from normal tissue, this point of view may earn significance in understanding of growth properties of this malignancy.
Aims: In this study involvement of extracellular matrix and its components in osteosarcoma cell proliferation and tumour progression was investigated.
Materials and methods:
A stabile osteosarcoma cell line (OSCORT) was established from the biopsy fragments of a 17-year-old boy. Osteosarcoma cells were cultured conventionally, or on extracellular matrix gel (ECM-gel). Cell proliferation, cell cycle analysis, expression of proliferation-related proteins and collagenase IV activity was compared within these culture conditions.
Results:
ECM-gel increased cell proliferation (cell numbers and thymidine incorporation), which was confirmed by increased cyclin D1 and PCNA protein expression. Among ECM components heparan sulfate proteoglycan (HSPG) and fibronectin was the most responsible for these effects. On the other hand, laminin did not change, and collagen IV definitively decreased cell proliferation. Representation of S-phase of cell cycle has double increased among cells kept on ECM-gel or treated with HSPG or fibronectin. Collagen IV instead, significantly decreased G1 and S-phase and increased G2-phase counts. Expression of cyclin B1 decreased, while, PCNA and Ki-67 increased in cells cultured on ECM-gel. The decrease of cyclin B1 and cdc2 was also observed in presence of all studied ECM components. Collagenase IV activity was induced by ECM-gel, which was most enhanced for HSPG.
Conclusion:
We conclude that ECM has significant role in maintenance of malignant phenotype of human

osteosarcoma, and the heparan sulfate proteoglycan may partly be responsible for this effect.

Investigating the intracellular fate of liposomes by flow cytometry and spectral bio-imaging

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Liposomes are drug delivery systems that are used to transfer macromolecules or other substances to target cells. To improve the efficiency of the drug targeting it is necessary to understand the mechanism of liposome uptake by the cell and to follow the intracellular fate of internalized liposomes and their contents.

After adsorption to the cell surface liposomes are taken up into cytoplastic vesicles (endosomes, phagosomes, caveolae) by different modes of internalization, mainly by endocytosis. Endocytosis is described as a diverse set of processes that can be further categorized. Clathrin-dependent endocytosis is probably the mechanism best understood. Other types of endocytosis include those mediated by caveolae, and so called clathrin-independent pathways that involve neither clathrin-coated pits nor caveolae. Finally, some cell types are capable of internalizing large amounts of fluid via macropinocytosis or large particulates by phagocytosis.

To investigate the initial mode of internalization of liposomes (fluorescently labeled) we apply a combination of several inhibitors that block selectively different pathways. In addition a set of fluorescent model substances is used to highlight the different endocytotic pathways.

To characterize liposomal uptake two different methods are applied to gain both quantitative data from flow cytometry and qualitative data from spectal bio-imaging.

Spectral bio-imaging is a powerful method for measuring the spectrum of light at every picture element (pixel) and is particularly useful in investigations with fuorescent probes. The SepectraCube™ system combines spectroscopy, CCD imaging, light microscopy and analysis software and can be used to identify and map several fluorophores simultaneously in one measurement. The inherently high spectral resolution of spectral bio-imaging is ideally suited to distinguish between fluorophores with overlapping spectra.

The combination of flow-cytometry and spectral bio-imaging offers the possibility to examine the initial mode of internalization and to follow the intracellular fate of liposomes.

Geometric properties of cellular adhesion patterns

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The skeletal muscle is a dynamic tissue that regulates the number of fibers in response to the mechanical forces. For this purpose the muscle contains stem cells that keep their proliferative state, but can differentiate to fully developed muscle fibres, too. Differentiated cells merge and form long myotubes that are only fixed at the ends.

Using the mouse skeletal muscle cell line C2C12 we found some regularities concerning the geometric composition of focal adhesions. Analysing the focal adhesions of the cell ends, we investigate the changes in adhesion patterns in response to a known force that acts towards the direction of contraction.

The Focal Adhesion Kinase (FAK) is a marker to visualize adhesion patterns. FAK binds to activated integrins, autophosphorylates itself and organizes the assembly of the focal adhesion. Its cleavage by calpain enables the cell to dissolve the focal contact from the substrate. In order to distinguish the activated FAK from the nonactivated cytosolic protein that is also stained we used TIRF microscopy. The images obtained represent only the active focal adhesions and their geometric patterns. After detailed analysis of this data we find regularities concerning size and distribution. Thus we expect that surface structures that reflect geometric adhesion parameters will influence the cell adhesion or spreading. Currently electron beam lithography is used to produce surface patterns with such geometric properties.

Eight-color immunophenotyping by Laser Scanning Cytometry (LSC)

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Objective:
For a more profound immunophenotyping or due to a lack of sample volume a measurement with a maximum of detectable colours is basically aimed. Currently it is possible to differentiate six fluorescence colours in the laser scanning cytometer (LSC) [Cytometry 48:115,2002]. Aim of the study was to increase the number of measurable colours to eight. The possibility to distinguish these eight fluorescences was enabled due to the use of the new fluorescence dyes PECy5.5 and APCCy5.5 combined with a filter change and a subsequent remeasuring. Materials and methods:
Staining of human peripheral blood was performed as described previously [J Immunol Methods, 246:175,2000]. Following fluorochromes were used: FITC, PE, PECy5, PECy5.5, PECy7, APC, APCCy5.5 and APCCy7. The first measurement was performed with a 670/20nm-filter in PMT3 to detect Cy5. Afterwards the filter is replaced by a 710/20nm-filter which allows to detect Cy5.5 but not Cy5. Between the two measurements the slide is not removed from the microscope. Due to the registration of the exact position of each event the two data files can be combined. This so obtained merged data file contains the information of the first and the second measurement. An eight-colour panel was set up using anti CD3, CD4, CD8, CD14, CD16, CD19, CD45, and CD56 antibodies. With this panel >10 sub sets could be quantified.
Results and Conclusion**:**
By filter changing two fluorescences are measurable in one channel (Cy5 and Cy5.5). With this eight-color panel it is now possible to distinguish between several leukocyte subsets per specimen. This assay could be useful in hypocellular specimen analysis and the detection of novel leukocyte subsets.
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Critical steps during the ontogeny of bone marrow in mice

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Although bone marrow (BM) is the major hematopoietic organ in adult mice, the stroma assembly, as well as the implantation of the first hematopoietic cells during its ontogeny is scarcely studied.

To approach this, fetuses with 15; 15,5; 16 and 17 days post-coitum (dpc) were fixed in Carson's Millonig formalin and embedded in paraffin. Serial sections (7 μm) were stained with HE, Lennert's Giemsa and PAS-AB pH=1.0 or 2.5, and analyzed by brightfield microscopy. Immunofluorescence to fibronectin, laminin, tropomyosin, α-smooth muscle actin (α-SMA) and thrombomodulin was analyzed by confocal laser microscopy.

The results were: a) 15 dpc: the ossification did not begin and hematopoiesis was restricted to the liver; b) 16 dpc: some cartilaginous templates showed hypertrophic degeneration together with perichondrial calcified zones; c) 17 dpc: a progressive substitution of some cartilaginous templates by collagen, PAS(+) glycoproteins and low-sulfated proteoglycans was detected; d) Fibronectin occurred in calcified regions, perichondrial mesenchyme and inside degenerating chondrocytes, while laminin was detected only in the endosteum and around small vessels; e) Tropomyosin and α-SMA were seen only in arterial muscle; f) Thrombomodulin has endosteal and interstitial distribution and was also detected in adventicial cells of penetrating vessels.

In conclusion, a) BM ontogeny in mice presents ossification, angiogenesis and stroma establishment steps; b) proteoglycans and fibronectin seem to be major extracellular matrix components of the developing BM; c) some stromal cells showed the phenotype: thrombomodulin(+), tropomyosin(-), α-SMA(-), suggesting a mesenchymal-endothelial origin.

Changes in intracellular calcium of different cell types as a response of micromechanical force loading

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All living cells are subjected to mechanical forces of internal and / or external origin. Cells respond to such forces via a cascade of biochemical signals witch may result in cytoskeleton reorganisation or modifications of shape and adhesive properties. Thus a response to the mechanical stimuli is important for many physiological processes such as differentiation, migration or attachment.

A multitude of techniques producing mechanical loads of different magnitudes and frequencies either to an individual cell or to a cell population have been introduced. For a controlled force loading to single cells of different cell lines we used a newly developed method based on optical tweezers. This method allows a bright field microscopic observation of the cells elastic behaviour and additionally a simultaneous investigation of other parameter e.g. intracellular calcium in the fluorescent mode. We tested several adherent cell tissue types (connective tissue fibroblasts, kidney epithelial cells, cervix carcinoma epithelial cells, pancreatic acinar carcinoma cells). After mechanical manipulation an elevation in intracellular calcium (an universal signal transduction element) of the stimulated cell followed by propagation of the calcium signal to neighbouring cells were found for all investigated cell types .The presented data support the quasi ubiquitous importance of calcium signalling in mechanotransduction. Also it is shown that the existence of active gap junctions is not an essential condition for intercellular calcium signal propagation.

Nanosizing of fluorescent objects by 458 nm SMI microscopy using a simplified size calibration algorithm

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The determination of individual object sizes is of special importance in biological research and thus one of the main applications of microscopy. Of special interest in cell biology is the topological analysis of three-dimensional nanostructures of biomolecular complexes (biomolecular machines, BMM). Such biomolecular machines usually have a size in the range of some 100 nm or less. Furthermore, the analysis of individual specific chromatin regions is very important for a better understanding of the functional topology of the genome [1-3]. In recent years substantial progress in the analysis of biomolecular machines was achieved by using electron, atomic-force and scanning-nearfield microscopy. However, these methods require special preparations of the specimens not allowing the use of conventional object slides and cover slips. Furthermore, with these methods only surface structures can be studied. The analysis of structures in the interior of three- dimensionally (3D) conserved cells would be possible by using far-field light microscopy only. To study sizes of and distances between small subwavelength-sized objects far-field microscopic methods like standing wave and spatially modulated illumination (SMI) microscopy [4-7], and several other point spread function (PSF) engineering methods, e.g. 4Pi [8-11] and stimulated emission depletion microscopy [12] have been developed recently. Nanosizing of individual fluorescent, optically isolated small objects using SMI microscopy requires only one spectral signature; conventional object preparation can be used. The number of photons contributing to the information is as high as in epifluorescence microscopy, so it is also possible to do nanosizing of weakly fluorescent objects. Previously, nanosizing was based on computer simulations (virtual microscopy) of the emission/detection process [13]. Here, a new method to derive the calibration curves is described. These methods assume a gaussian dye distribution within the object. Size measurements of fluorescent objects using SMI microscopy with an excitation wavelength of λ_{ex}= 458 nm are presented. This wavelength is adequate to excite cyan fluorescent protein (CFP) and other fluorophores commonly used in the biological research.

Fluorescent beads with actual diameters between 200 nm and 57 nm (according to the manufacturer) were measured by 458 nm SMI microscopy. The results show that using this method, size ranges considerably below the conventional optical resolution can be estimated.

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On-line nanosizing by spatially modulated illumination microscopy

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Current progresses in far field light microscopical methods encourage the use of new non-invasive instruments for the analysis of biological nanostructures and their dynamics, in particular including the interior of three-dimensionally conserved cells, based on fluorescence microscopy. We show an extension of the recently described method of "nanosizing" to measure within the acquisition time frame the sizes of small, subwavelength sized, fluorescent objects using Spatially Modulated Illumination (SMI) microscopy [Failla et al., ComPlexUs (2003); Failla et al., Appl. Optics (2002); Albrecht et al., Appl. Optics (2002)]. With the present set-up, on-line determination of the diameters of such objects in one (axial) direction has been achieved. Comparison with conventional far field light microscopical methods revealed a substantial improvement in the determination of sizes of single subwave-length sized objects. A necessary condition for this is the optical isolation of the objects. This condition, however, may be realised in a large variety of biological applications. As a fluorescence microscopy method, SMI approaches allow not only size and topology measurements of optically isolated objects down to the nm-range, but also their specific identification e.g. by molecular labelling.

A new acoustic microscopy to measure elastic properties of cells on a sub cellular level

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Mechanical properties of living cells give important information on dynamic cellular processes such as attachment, migration or division. Using 1GHz ultrasound it is possible to image these properties in a micrometer regime. Sound is focused into the sample with a sapphire lens and the reflected sound is detected with the same lens in a confocal setup. The image is recorded while mechanically moving the lens over the sample area. Using short pulses it is possible to measure the local sound velocity.
Acoustic microscopy was mainly used for opace samples. When studying transparent biological samples a combination of acoustical and optical microscope offers many benefits. We build a new scan head that can be mounted on a inverted optical microscope. The acoustic lens is moved with a piezo stage and excited with short pulses. Approach of the lens is done under visual control in a semi automatic fashion. At every lens position the received

echo transient is amplified, feed into a fast transient recorder and stored onto hard disk for later processing.
Synchronous fluorescence microscopy helps identify structures in the cell and study dynamic processes. The optical microscope can be used to measure the cell thickness. Quantitative measurements of the bulk sound velocity are derived from the time of flight of the sound pulse with and without the sample to the substrate and the local cell thickness.
Pictures showing the longitudinal arrangement of fibrous structures in C2C12 myotubes in fluorescence and acoustic microscopy will be given.

New long wavelength fluorochromes for cytometric analysis

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Background:
Fluorescent bioanalytics comprises powerful technologies for cytometric analysis based on the cellular and molecular level. Cytometric techniques as e. g. flow cytometry, microscopy, array technologies and others require fluorochromes with unique (bio-)physical properties. The development or derivatization of fluorochromes with specific chemical and (bio-)physical properties is of peculiar interest for the optimization of research and diagnostic applications. Here we introduce three new long wavelength fluorochromes characterized by individual qualities: Chromeon 642-lipophilic, Chromeon 537-Acid, and Chromeon 670-Alcohol.
Methods:
Both fresh and MeOH fixed J82 tumor cells were stained with 10 and 50 µM of each fluorochrome. Emission and excitation wavelengths as well as emission intensities were analyzed on a luminescence spectrometer (Aminco Bowman, Series 2, SLM) with a standard 150 W xenon lamp as excitation source. The applicability in flow cytometry was tested on a FACS-Calibur instrument (BD Biosciences) equipped with standard optics. J82 stained cells were excited with 488 and 635 nm laser line, respectively. Cellular staining localization was microscopically determined with a Zeiss Axiovert S100 inverted microscope. Pictures were generated with a CCD camera (Princeton Instruments), collected and stored using the MetaMorph software (Universal Imaging Corp.). Potential toxicity i. e. number of dead cells induced by a 12 hrs period of dye incubation was tested using a flow cytometric BCECF and PI exclusion assay.
Results:
Fluorescence emission maxima were detected at 580, 670 and 710 nm in the case of Chromeon 537-Acid, Chromeon 642-lipophilic and Chromeon 670-Alcohol, respectively. Fluorescence intensity of Chromeon 670-Alcohol is three times higher compared to Chromeon 537-Acid. All dyes had no toxic effect on J82 tumor cells during 12 hrs incubation in vitro, neither at 10 nor at 50 µM. Chromeon 537-Acid is exclusively located nuclear whereas Chromeon 670-Alcohol could be found in the cytoplasm without nuclear staining. Chromeon 642-lipophilic has a diffuse staining pattern in both cell compartments. Flow cytometric analysis revealed a specific excitation of Chromeon 642-lipophilic by the 635 diode laser resulting in strongfluorescence emission that can be detected on FL4. In contrast Chromeon 537-Acid was excited by both laser lines producing signals that appear in FL2, FL3 and FL4. Chromeon 670-Alcohol can be measured on the third channel and alternatively on FL4.
Conclusion:
The three new long wavelength fluorochromes presented here are characterized by unique biophysical properties. They show specific excitation and emission maxima and are suitable for flow cytometric and/or microscopical cell analysis. Cellular staining can be found in

different cell compartments. Since the dyes are non-toxic they can be applied for cellbiological assays.