

Systems Biology and Clinical Cytomics: The 10th Leipziger Workshop and the 3rd International Workshop on Slide-Based Cytometry

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Despite very significant technical and software improvements in flow cytometry (FCM) since the 1980's, the demand for a cytometric technology combining both quantitative cell analysis and morphological documentation in Cytomics became evident. Improvements in microtechnology and computing permit nowadays similar quantitative and stoichiometric single cell-based high-throughput analyses by microscopic instruments, like Slide-Based Cytometry (SBC). SBC and related techniques offer unique tools to perform complex immunophenotyping, thereby enabling diagnostic procedures during early disease stages. Multicolor or polychromatic analysis of cells by SBC is of special importance not only as a cytomics technology platform but also because of low quantities of required reagents and biological material. The exact knowledge of the location of each cell on the slide permits repetitive restaining and reanalysis of specimens. Various separate measurements of the same specimen can be ultimately fused to one database increasing the information obtained per cell. Relocation and optical evaluation of cells as typical SBC feature, can be of integral importance for cytometric analysis, since artifacts can be excluded and morphology of measured cells can be documented.
Progress in cell analytic: In the SBC, new horizons can be opened by the new techniques of structural and functional analysis with the high resolution from intracellular and membrane (confocal microscopy, nanoscopy, total internal fluorescence microscopy (TIRFM), and tissue level (tissomics), to organ and organism level (in vivo cytometry, optical whole body imaging).
Predictive medicine aims at the detection of changes in patient's state prior to the manifestation of the disease or the complication. Such instances concern immune consequences of surgeries or noninfectious posttraumatic shock in intensive care patients or the pretherapeutic identification of high risk patients in cancer cytostatic therapy. Preventive anti-infectious or anti-shock therapy as well as curative chemotherapy in combination with stem cell transplantation may provide better survival chances for patient at concomitant cost containment. Predictive medicine-guided optimization of therapy could lead to individualized medicine that gives significant therapeutic effect and may lower or abrogate potential therapeutic side effects.
The 10th Leipziger Workshop combined with the 3rd International Workshop on SBC aimed to offer new methods in Image- and Slide-Based Cytometry for solutions in clinical research. It moved towards practical applications in the clinics and the clinical laboratory. This development will be continued in 2006 at the upcoming Leipziger Workshop and the International Workshop on Slide-Based Cytometry. © 2005 International Society for Analytical Cytology

Key terms: predictive medicine; cytomics; biocomplexity; laser scanning cytometry; scanning fluorescence microscopy

Key aspects of the 10th Leipziger Workshop from April 7–9, 2005 in Leipzig, were Systems Biology and Clinical Cytomics, thematically continuing earlier workshops (1–3). Diagnostic, intensive care, and surgical improvements in the treatment of cardiovascular diseases (4,5), whether congenital or acquired, have led to a decreased mortality of these diseases. Over the last years, flow cytometry (FCM) has become the standard tool for analyzing immunological changes in clinical diagnosis and research. The field of predictive medicine by cytomics has become another important application of FCM, facilitating individualized therapy. Thus, the improved sensitivity of predictive panels permits differentiation between patients at risk and those for whom treatment may be reduced in order to avoid adverse drug effects (6–9).

This journal was piloting in pushing forward the cytomics approach by bringing the idea of Predictive Med-

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icine by Cytomics (6) and the essential technologies needed to perform cytomics research (7) to a broader audience. This idea is now taken up by several scientists of the field of Systems Biology (10). In fact, Systems Biology and Cytomics are two sides of the same coin aiming at the understanding of intracellular signaling networks with the major goal of understanding and predicting the response of an organism (diseased/healthy etc.) at the individual level.

In spite of very significant technical and software improvements in FCM since the 1980s, the demand for a cytometric technology that combined both quantitative cell analysis and morphological documentation in Cytomics became evident. Improvements in microtechnology and computing technologies enable nowadays to perform similar quantitative and stoichiometric single cell-based high-throughput analyses by microscope-based instruments, like Slide-Based Cytometry (SBC). An upcoming focus issue on Slide Based Cytometry in Cytomics research in this journal will further expose this challenging development.

The 3rd workshop for SBC has gained attentive interest. Researchers and clinicians from many different countries discussed the usefulness of modern SBC imaging methods in clinical research. Participants of the industry introduced instrumentation novelties and improvements in image analysis and laboratory work.

The workshop combined

- practical hands-on courses,
- examples and user-defined questions in SBC presented by different cytometer providers,
- immunological aspects of cardiopulmonary bypass surgery and transplant rejection, and
- new developments of commercial exhibitors.

The 3rd International Workshop on SBC was followed by the 10th Leipziger Workshop being focussed on Systems Biology and Clinical Cytomics as well as the emerging field of Regenerative Medicine. The abstracts of the workshop are published in this issue of Cytometry (see pages 000-000, referred to as lecture abstracts or as poster abstracts in the following and demonstrate the versatility of cytometric topics from technical innovation to clinical applications. The abstracts of highest impact were selected by a peer-reviewing committee and short articles on these topics will be published in an upcoming issue of Cytometry Part A.

The workshops stood under the patronage of the International Society for Cytology (ISAC; www.isac-net.org), the German Society for Cytometry (DGfZ; www.dgfz.org), and the German Foundation for Heart Research (www.herzstiftung.de).

The Leipziger Workshop is embedded in a rich academic and biotechnological environment that mutually supports and fertilizes each other. Leipzig made in the last years substantial progress to become one of the leading biotechnology centers in Germany and Europe. Furthermore, Leipzig harbors, for example, the newly founded BIO CITY. This biotechnological–biomedical center, where industry, science, and research live and work together, has a strong academic backbone, thus providing a unique potential for industrial networking. The newly established Fraunhofer Institute for Cell Therapy and Immunology, IZI, in Leipzig, in addition ensures an important new platform for technology development and medical application of cell analysis.

SLIDE-BASED CYTOMETRY

SBC bridges the gap between high-throughput multiparametric cytometry and morphological analysis and was represented by two instruments: The LSC (Laser Scanning Cytometer) and the SFM (Scanning Fluorescence Microscope). LSC is a slide-based analytical technique that allows rapid quantitative analysis of cells and other specimens tagged with fluorescent dyes. The LSC setup is built around a conventional epifluorescence microscope. Technology and applications are described elsewhere (11–14). The SFM is a computer-controlled technique with automated object scanner and data storage (P1) (15,16), applicable for multiparametric fluorescence-activated cytometry.

The 3rd International Workshop on Slide-based cytometry (SBC) aimed to summarize both, presentation of the newest measuring instruments and recently developed methods. Its main intention was to highlight the plethora of applications for SBC systems, innovative analysis technologies and to demonstrate multicolor analysis. The workshop on SBC introduced new possibilities for physicians to improve their quality of analysis, especially concerning multicolor and tissue cytometry.

PROGRESS IN CELL ANALYTIC

New horizons can be opened by the new SBC techniques of structural and functional high-resolution analysis from intracellular and membrane components (confocal microscopy, L1, nanoscopy, L18) (17), total internal fluorescence microscopy (TIRFM, L32) (18), and tissue level (tissomics, P10, L21) (19–22), to organ and organism level (in vivo optical imaging, L22) (23).

In the field of leukocyte phenotyping and marker expressions analysis of immune competent cells, multiparametric or polychromatic cytometry is the most important technique. More and more parameters become simultaneously measurable by the new developments (iterative restaining, L28, (24); detection of 9 parameter on a 4 PMT flow cytometer, P3; sequential photobleaching, P2).

Discrete cell populations of cellular systems can be either uniform or split to particular subpopulations while changes in low-frequency cell subpopulations may be lost by dilution (25). Essential progress in single-cell molecular analysis has been achieved by the continuous development of new image and flow cytometric instrumentation. Multicolor measurements (19,24), spectral imaging (26), confocal (27), and laser scanning cytometry (12,28,29), fluorescence resonance energy transfer (FRET) (26,30), fluorescence lifetime imaging (FLIM) (31), and second harmonic imaging (32) mark the progress in optical bioimaging. Also, a family of concepts has been developed that

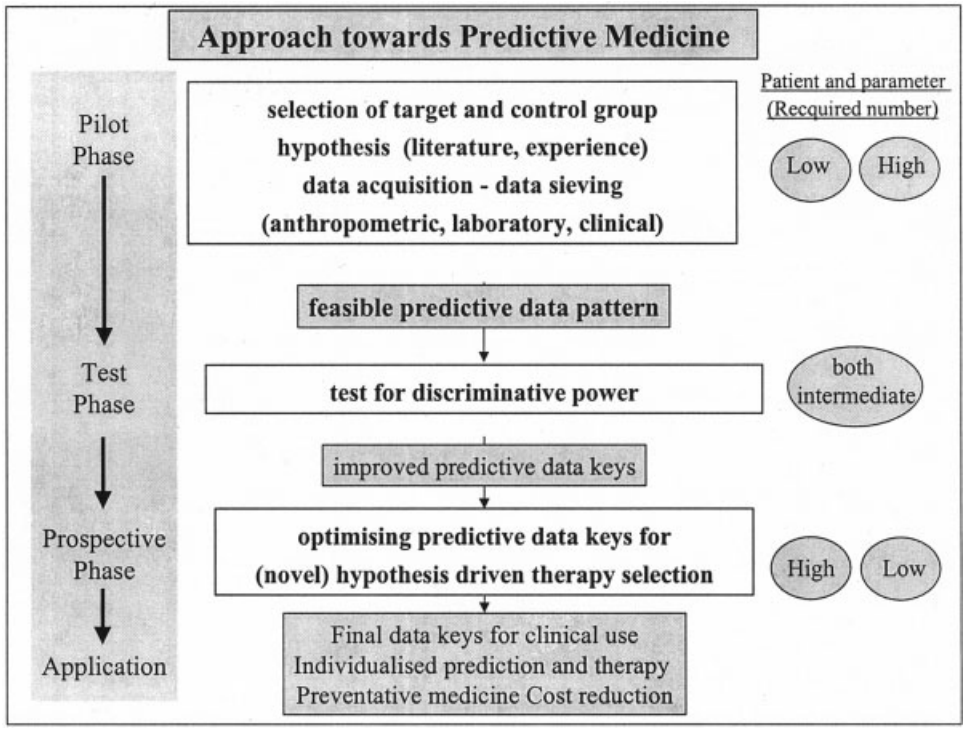

FIG. 1. Generalized schematic outline for development and evaluation of a predictive medicine platform. The concept consists of three levels (phases). Phase 1 is intended to test for a specific disease course or therapy if with a taken panel of data individualized prediction seems feasible. In this phase, a broad number of parameters on a small number of patients (control and diseased) is acquired. If individualized recognition of patients is feasible based on the data pattern analysis, the second study phase is conducted. Here a larger number of patients is prospectively enrolled and in parallel the number of patients analyzed is reduced for cost reduction. In this phase, the data key is tested and optimized, if necessary. In the third phase, the data key from phase two is tested on a large prospective group. If the prospective results fulfill the demand of >90% correct recognition, then this data pattern is appropriate for use in individualized prediction. The resulting predictive data may lead to new hypothesis of the disease and innovative therapy. On the right it is shown that with increasing predictive capacity of the data key the number of patients required for the study increases whereas the number of acquired parameters decreases.

allows image acquisition far beyond traditional resolution limit down to the nm-range, including multiphoton excitation (33), stimulated emission depletion (STED) microscopy (17), spectral distance microscopy (34), and image restoration techniques (35). Fast fluorescence imaging in flow (36), optical stretching in flow (37), miniaturized flow cytometry within laboratories on a chip (38,39) constitute essential progress in flow cytometry and flow imaging. Biomolecular analysis techniques like bead arrays (40), laser microdissection (41), layered expression imaging (42), single-cell polymerase chain reaction (PCR) (41), tyramide signal amplification (43) or biomolecule labeling by quantum dots (44), magnetic nanobeads (45), and aptamers (46) open new horizons of sensitivity, molecular specificity and multiplexed analysis at the single-cell level. Metabolomics (L30) (47) and Lipidomics (L3, L6, L8, L12, L16, L19) are emerging new subdisciplines that will increase substantially the focus of Cytomics.

PREDICTIVE MEDICINE

The goal of predictive medicine consists in the detection of changes in patient's condition for example prior to clinical deterioration to counteract early on by preventive medicine. Therefore, both the diagnosis of diseases like cancer, coronary artherosclerosis, or congenital heart failure as well as the prediction of the effect of specific therapies on patients' disease course are major fields of predictive medicine. Predictive medicine aims at the recognition of the "fate" of each individual patients in order to yield unequivocal indications for clinical decision making (i.e., how does the patient respond to therapy, react to medication etc.). This individualized prediction is based on the predictive medicine by cytomics concept (48) (Brief explanation in Fig. 1). These considerations have recently stimulated the idea of the Human Cytome Project (8,9).

Application of this concepts may concern, but obviously are not limited to, multiorgan failure in sepsis or noninfectious posttraumatic shock in intensive care (49,50), or the pretherapeutic identification of high-risk patients in cancer cytostatic therapy (51–53). Accurate predictive measures would more effectively guide early anti-infectious or anti-shock therapy as well as curative chemotherapy in combination with stem- or progenitor-cell transplantation, potentially providing better survival chances for individual patients with concomitant cost containment. In the emerging field of stem cell therapy, either by embryonic stem cells (54) or stem for regenearation of liver (55), lung (56), gastrointestinal tract (57) or the cardiovascular system (5,58). Early reduction on cessesation of

therapy could be guided by predictive medicine and there by lower or abrogate potential therapeutic side effects.

Other potentially important aspects of predictive medicine concern the recognition of preasthmatic and early rheumatic disease patients as well as the preoperative identification of patients with a tendency for postoperative complications (59) or coronary artery disease patients with an increased tendency for restenosis or other complications (60–62).

Once initiated, one realizes that everyday medicine is full of predictive issues. They are present in a variety of severe diseases like cancer, leukemias, rheumatoid diseases, diabetes, and asthma. However, they are also present in complex disease syndromes (63) or infections in newborns, pediatric patients (64,65), adults, or elderly patients, as well as bleeding risks during surgery etc. In many instances, individualized disease-course predictions for currently envisaged standard therapies would definitively allow early curative interaction by specific therapeutic measures prior to the occurrence of irreversible tissue destruction with its inherent potential to incapacitate, or compromise the patient later on.

CONCLUDING REMARKS

Over the last eight years, the Leipziger Workshops have concentrated on cutting edge developments of Cytomics and Predictive Medicine at the basic research to clinical interface (6–8,52,59,60,66–68). The 10th Leipziger Workshop and the 3rd International Workshop on Slide-Based Cytometry advocate for the intensified use of cytomics in future clinical studies to enhance feedback into the technological developments and data evaluation software for modeling molecular disease pathways by systems biology. This development will be continued at the upcoming 11th Leipziger Workshop and the 4th International Workshop on Slide-Based Cytometry in 2006.

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Abstracts from

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LECTURE ABSTRACTS

1

IN VIVO CYTOMETRY: A SPECTRUM OF POSSIBILITIES

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The importance of optical imaging in biomedical research is growing rapidly. In modern clinical medicine, relying on molecular signatures, noninvasive diagnostics, gene therapies, and minimally invasive surgery, there is a rising interest in tools capable of analyzing tissues at the (sub)cellular level, preferably in vivo. This can best be achieved by using light as the investigational tool, given its versatility, resolution, and noninvasiveness. Optical contrast can be achieved by many means, including intensity, wavelength, polarization, coherence, lifetime, and nonlinear effects, and sub-micron spatial and sub-microsecond temporal resolutions are attainable. We explored whether optical imaging can detect abnormalities in tissue, in a range of specimens (fixed, live in vitro, fresh ex-vivo, and in vivo), without the use of added contrast agents. This is a critical step towards real-time, high resolution intrasurgical diagnostics, and their tight spatio-temporal coupling into intervention. Cancer is our main experimental focus.We will review and present new results on transmittance, reflectance, fluorescence, two-photon, multispectral, and fluorescence lifetime imaging, performed with instrumentation we developed or modified, with special emphasis on spectral methods. The results demonstrate that optical imaging can detect and localize cellular signatures of cancer in real-time, in vivo, without the use of contrast agents.

2

ITERATIVE RE-STAINING AS A PIVOTAL TOOL ON THE WAY TO N-COLOR IMMUNOPHENOTYPING BY SLIDE-BASED CYTOMETRY

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Slide-based cytometry (SBC) offers the chance to "ask a cell a second time". A prerequisite for this effort is the fact that cells do not change their position on the slide between repetitive analyses. Especially in small sample volumes (e.g. peripheral blood from critically ill neonates) this can be used to analyze a cell sample again and again and to combine the data of every analysis step to one file on a single cell level based on the fixed *x*- and *y*-co-ordinates of every cell. We exploited this approach for the analysis of minute samples of peripheral blood. To intensify the data density obtained from a single sample we have performed iterative re-staining steps with respective analyses by a laser scanning cytometer (LSC, CpomyCyte) in between. We used an LSC equipped with an argon- and a helium-neon laser tuned to 488- and 633-nm emission respectively. Our protocol uses a primary staining with anti-CD3-FITC, anti-CD14-PE, anti-Cd45-APC, and 7AAD as DNA stain to obtain a stable trigger signal. After the first analysis, which defines thymocytes and monocytes, a secondary staining with anti-CD4-PE is performed; the following second analysis defines the helper T-cell subset. Then a tertiary staining with anti-CD8-PE and anti-CD19-PE is performed; the third analysis defines the cytotoxic T-cell subset and the B-cells. Finally, the cells are stained with H&E and single cells from the different subtypes are re-localized again and micrographs are taken. In addition to the immunologically defined subsets, eosinophils, basophils, and neutrophils are also distinguished. In conclusion, iterative re-staining and re-analysis generates virtual new colors that can be distinguished by the LSC; in our example the repeated use of PE allows us to detect four markers that could not be distinguished if all markers would be stained at a single step. This might prove for be a pivotal tool on the way to n-color immunophenotyping exclusive to slide-based cytometric concepts.

3

NEW QUANTITATIVE ANALYTICAL CAPABILITIES OF CONFOCAL MICROSCOPY

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Confocal microscopy has become to be known as a technique capable of delivering multicolour images of cells and subcellular structures with dramatically better contrast and resolution than those obtained in conventional wide field fluorescence microscopy. An additional advantage was the ability to create 3D digital images amenable to computer analysis and animation. Consequently, a confocal microscope has originally been perceived as a sophisticated technique capable of 3D imaging of biological objects that were difficult to visualize by conventional optical microscopy. In recent years, however, confocal microscopy has witnessed a dramatic technological expansion. Currently, confocal microscopy embraces a family of refined analytical techniques that employ the original principle supplemented with various other new technological advancements, and offers broad analytical quantitative capabilities (Dobrucki, 2004; and cited references). These analytical techniques include FRAP (Fluorescence Recovery After Photobleaching), FLIP (Fluorescence Loss in Photobleaching), FCS (Fluorescence Correlation Spectroscopy), FRET (Fluorescence Resonance Energy Transfer), multispectral imaging, and FLIM (Fluorescence Lifetime Imaging Microscopy). A modern confocal microscope can measure mobility of proteins within live cells, estimate a mobile and a bound fraction of a protein, measure nanometer distances between interacting molecules or resolve spectral signatures of several overlapping fluorescent species in the same location within a cell. A capability to image small struc-

tures has been dramatically improved. With the advent of 4Pi and STED (Stimulated Emission Depletion) techniques the achievable spatial resolution has been pushed beyond "the diffraction limit," to values several times lower than the wavelength of visible light. Distances and sizes of small structures can be measured with spectral precision microscopy and SMI (Spatially Modulated Illumination microscopy). Moreover, large objects a few millimetres in diameter can now be imaged with high spatial resolution using SPIM (Selective Plane Illumination Microscope). Optical microscopy is finding applications in high-throughput studies, aiming at collecting and analyzing large numbers of cell images. Thus, modern optical microscopy, including confocal microscopy, is now recognized as a broad range of techniques specialized in various types of subcellular measurements of biologically relevant parameters, and is reaching far beyond the applications of standard 3D imaging.

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ASSESSMENT OF CYTOKINE PRODUCTION OF T HELPER 1 AND 2 CELLS WITH THE CYTOMETRIC BEAD ARRAY TECHNIQUE IN PATIENTS BEFORE AND AFTER HEART TRANSPLANTATION

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Differential expression of cytokine production of T helper 1 (Th1) and 2 (Th2) cells is known to play a major role in allograft rejection. First, this study was designed to compare the cytokine production of Th1 and 2 cells in patients before and after heart transplantation (HTx). Second, the effects of immunosuppressive therapy on cytokine expression were compared between unstimulated and mitogen-stimulated cultures in HTx recipients at different time points. In group I, blood was drawn from 50 HTx recipients at Ctrough and from 47 patients with dilated cardiomyopathy (DCM), and 20 healthy volunteers (HV). In group II, blood from 17 HTx was drawn at Ctrough and 2 h after dosing (C2). All HTx recipients received cyclosporin and mycophenolate mofetil immunosuppressive therapy. Cytometric bead array (CBA) technique was used to asses cytokine production of Th1 (INF-g, IL-2, TNF-a) and Th2 cells (IL-4, IL-10, IL-6) in serum with FACS. In group II, cytokine production was additionally assessed after mitogen-stimulation of whole blood through the T cell pathway. In group I, cytokine production (pg/ml ± SEM) in HTx recipients was significant different for TNF-a and IL-6 compared with DCM and HV ($P < 0.05$): TNF-a: HTx: 9 ± 2, DCM: 2 ± 0.5, HV: 1 ± 0.1; IL-6: HTx: 75 ± 11, DCM: 30 ± 7, HV: 32 ± 6. In group II, there as a significant enhanced production of TNF-a and IL-6 at C2 compared with production at Ctrough in unstimulated blood ($P < 0.05$): Ctrough: TNF-a: 3 ± 1, IL-6: 192 ± 76; C2: TNF-a: 32 ± 6, IL-6: 426 ± 87. After mitogen-stimulation cytokine production was significantly decreased compared with production at Ctrough for the following cytokines ($P < 0.05$): Ctrough: TNF-a: 26 ± 6, IFN-g: 103 ± 26, IL-6: 605 ± 162, IL-2: 227 ± 11; C2: TNF-a: 12 ± 3, IFN-g: 23 ± 10, IL-6: 259 ± 74, IL-2: 44 ± 17. For the first time, the CBA technique was used to assess differences in cytokine production of Th1 and 2 cells in HV and patients before and after HTx. Furthermore, our results show that it is important for this assay to activate T cells prior to analyzing the effects of immunosuppressive drugs. Future studies in HTx recipients have to show if cytokines of Th1 and Th2 cells could be biomarkers for clinical outcomes like rejection or infection.

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A NEW METHOD TO QUANTIFY PHAGOCYTOSIS AND INTRACELLULAR DEGRADATION OF BACTERIA BY FLOW CYTOMETRY: A COMPARISON OF CORD BLOOD MACROPHAGES (MF) AND PERIPHERAL BLOOD MF OF HEALTHY ADULTS

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Background: Neonates are more susceptible for systemic bacterial infections than are adults. Although neonatal MF are deficient in tasks of adaptive immunity, e.g. their ability to activate T cells, no clarity on their capacity to maintain innate immunity functions, e.g. bacterial phagocytosis and intracellular killing exists.

Hypothesis: Basal phagocytotic activity and intracellular killing of cord blood MF (CBMF), as detected by a single cell assay, are not reduced to MF of adults (PBMF).

Methods: PBMF and CBMF of healthy term neonates without signs of early onset infection were isolated. *E. coli* DH5α, expressing a prokaryotic variant of green fluorescent protein (*E. coli*-gfp) were added in a constant ratio (bacteria:-cells = 50:1) for 5–180 min or for constant intervals (45 min) with increasing bacterial concentrations (1:5 to 500:1). After removal of free bacteria, cells were either fixed and phenotyped or seeded in new medium for 5–120 min to detect intracellular degradation by loss of gfp-fluorescence. Phagocytosis Index (PI; $CD14^+gfp^+$: $CD14^+$ cells) and Phagocytosis Capacity (PC; mean gfp-fluorescense intensity, MFI of $CD14^+$) as well as phenotypes were analyzed. Extracellular membrane-bound bacteria were identified by Cytochalasin D.

Results: Basal PI of PBMF versus CBMF after 15 min was 5.2% (SD 1.4) versus 4.8% (SD 1.2); PI rose to 40% (SD 11) versus 46% (SD 8) after 45 min and reached a maximum of 53% (SD 14) versus 56% (SD 7) after 180 min. PBMF (1.3%) versus 1.8% of CBMF were conjugated with bacteria extracellularly. Intracellular localization of *E. coli*-gfp was proven by confocal microscopy. PC of PBMF rose linearly and reached its maximum after 180 min at 477 MFI (SD 120) versus 375 MFI (SD 70) of CMF. CD14 expression of PBMF decreased in a time-dependent manner by 75 versus 74% of CBMF after 180 min. PI and PC correlated positively with the amount of bacteria. PI of PBMF rose from 1% (SD 0.74) versus 3% of CBMF (SD 0.39; bacteria: cell-ratio 1:1) to 79% (SD 10) versus 84% (SD 10; ratio 500:1). Triple staining revealed 20% (SD 6) of PBMΦ to be $CD16^+$ versus 7% (SD 3) of CBMΦ. $CD16^+$ MΦ showed no phagocytic activity. *E. coli*-gfp degradation was completed at 120 min in both, PBMF and CBMF.

Conclusion: The basal phagocytic activity and the degradation activity of CBMF and PBMF are comparable. PBMΦ- and

CBMΦ-populations differ in their amount of $CD14^+$ $CD16^+$ cells. $CD14^+CD16^+$ cells do not take part in the clearance of *E. coli*-gfp in vitro. *E. coli*-gfp is a suitable tool to measure phagocytosis and intracellular degradation and has to be evaluated for functional assays during systemic inflammatory reactions.

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PROCEDURE FOR THE OBJECTIVE QUALITY EVALUATION OF FLUORESCENCE IMAGES FOR AN OPTIMIZED AUTOMATIC IMAGE ACQUISITION

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The automated analyzes of fluorescence images by image-analytic methods become more important in medical laboratories. The acquired image data thereby often orient themselves at the human subjective quality feeling and are therefore just conditionally suitable from information-technical view for an automatic computer-assisted evaluation. Different spectral sensitivities of human eye and image sensor lead to different perception of fluorescence signals. The often subjective selected image acquisition parameters are accordingly not optimal and cause with the computer-assisted analysis to errors. Particularly the data generation is an important step in the image analysis, because following work steps depend on it and errors affect directly the following analysis steps. We determine always existing image parameters that can be computed simply allowing an objective appraisal of quality of image data as well as a separation of object and background. The calculated parameters can be used for an automated adjustment of the camera parameters in image analysis systems to the optimization of the image processing chain. An improvement of the analysis quality can be achieved.

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IDENTIFICATION OF NOVEL TARGETS IN ACTIVATED T LYMPHOCYTES

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T cell activation is controlled by a coordinated web of TCR/MHC interaction, costimulation, and cytokines. These extracellular stimuli activate a series of a signal transduction pathways in T lymphocytes. To achieve a better insight in the cellular mechanisms of T cell activation proteome-based profiling of T cells activated by PMA, cytokines and/or the B7 costimulatory signal was performed. Using this approach, we identified distinct sets of proteins in T cells whose expression is either elevated or reduced upon PMA and cytokine treatment or B7-1 and/or B7-2 costimulation. The proteins involved in T cell activation include regulators of the cell cycle and cell proliferation, signal transducers, components of the antigen processing machinery, cytoskeletal proteins, and metabolic enzymes. Interestingly, some of the proteins regulated have not yet been recognized in T cells. In addition, a number of differentially expressed proteins are further modified by distinct phosphorylation patterns. Our analysis provides novel insights into the complexity of activation pathways of T cells and their modulation and might lead to novel targets of therapeutic interventions in order to enhance or limit T cell responses.

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TOPONOMICS: EXPLORING PROTEIN NETWORKS DIRECTLY IN THE CELL

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The hierarchy of cell function comprises at least four distinct functional levels: genome, transcriptome, proteome, and toponome. The Toponome is the entirety of all protein networks traced out directly as patterns on the single cell level in the natural environment of cells in situ (e.g. tissues). We have developed a photonic microscopic robot technology (MELK) capable of tagging and imaging hundreds (and possibly thousands) of different molecular components (e.g. proteins) in morphologically intact fixed cells and tissue. MELK data sets represent multidimensional vectors of the topologically determined arrangements of proteins within the cell. These vectors are translated as geometric objects, which are visualized as functional images of the whole cell, referred to as toponome maps. Toponome maps are direct labels of the organized proteome, providing essential information on the protein network architectures in the cell. It will be shown in this paper that Toponome maps can be experimentally explored, thereby uncovering the precise cellular function of each given protein as element of the hierarchically ordered network structure. The data, assembled in a Toponome dictionary of the cell, give rise to a new concept for target and drug lead discovery.

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Ox-LDL AND E-LDL DIFFERENTIALLY REGULATE CERAMIDE AND CHOLESTEROL RAFT MICRODOMAINS IN HUMAN MACROPHAGES

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A hallmark in atherosclerosis is the generation of lipid-loaded macrophage foam cells. To study the effects of differ-

ent lipoprotein modifications, monocyte-derived macrophages from apolipoprotein E3 donors were incubated with two types of atherogenic lipoproteins, enzymatically modified LDL (E-LDL) and mildly oxidized LDL (Ox-LDL) followed by subsequent deloading with antiatherogenic HDL3 and apoA-I particles. Total cell analysis using lipidomics, genomics, and proteomics based on flow cytometry, image microscopy, and fluorescent 2D-gelelecrophoresis was applied to unravel key mechanisms underlying the regulation of cellular lipid influx, raft formation, lipid efflux, and storage. The cellular lipid content of the cells was determined with ESI-MS/MS. The surface expression of cholesterol, ceramide, and glycosphingolipids and the surface lipid distribution was analyzed by flow cytometry and confocal microscopy using different fluorescent-labeled antibodies or toxins (e.g. antibodies against ceramide backbone lipids, theta-toxin, cholera-toxin). We also investigated gene expression profiles of sphingolipid and cholesterol metabolism by Affymetrix microarrays and quantitative TaqMan RT-PCR. We have identified that E-LDL loading predominantly increased the cellular cholesterol content, while Ox-LDL loading preferentially increased the cellular ceramide content. Ox-LDL in comparison to E-LDL also led to a higher cell surface expression of ceramide and glycosphingolipids. Confocal microscopy confirmed an induction of ceramide rafts with Ox-LDL loading while E-LDL generated cholesterol-rich membrane microdomains, which was directly related to changes in gene expression of sphingolipid and sterol metabolism. Using this approach, we were also able to demonstrate distinct abnormal regulations of the cellular cholesterol and phospholipid influx/efflux rheostat in cells of three different lipid trafficking disorders: apoE4 homozygosity, ABCA1 deficiency, and Niemann-Pick C disease with implications, for e.g., to apoptosis and cholesterol homeostasis in macrophages during atherogenesis.

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PREPARATION OF MULTIFUNCTIONAL PARTICLES AND CAPSULES BY MEANS OF THE LAYER BY LAYER TECHNOLOGY

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The alternating adsorption of oppositely charged polymers (polyelectrolytes) on monodisperse colloidal spheres of sizes from 100 to 10,000 nm allows the stepwise deposition of stable layers. The thickness per layer can be controlled in the nanometer range (1–10 nm). In these layers several functions could be incorporated simultaneously. This is realized either by covalent linkage of functional groups on the polyelectrolytes or by assembling charged materials as for example nanoparticles as layers in between an oppositely charged polyelectrolyte. To avoid cross-talk between different functions a defined number of "dummy" layers can be assembled in between the functional layers. We will demonstrate as example for covalent coupling the introduction of multiplex dyes and as examples for nanoparticles the assembling of magnetite and of quantum dots. Furthermore, peptides or proteins can be attached on the surface of such particles via carboxyl or amino groups by standard coupling reactions, which we will demonstrate by hand of streptavidine. Finally, the core particle can be dissolved in order to have either a thin-walled flexible capsule, which can penetrate biological systems better than solid particles or to use the interior for the transport of active materials.

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MICROSCOPY-BASED MULTICOLOR TISSUE CYTOMETRY (MMTC)

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In the horizon of cytomics and a Human Cytome Project reliable, observer-independent, and fast quantification of cells and tissues is a prominent requirement and the necessity of a standard for automated microscopic evaluation of single cells either in immunohistology or confluent cell cultures is being increasingly emphasized among the scientific community. MMTC is the microscopic equivalent to flow cytometry and is used to quantify tissues and confluent cell layers on the single cell level. In particular, it is a means of analytical cytology and tissue cytometry. MMTC allows looking quantitatively at many cells undergoing the same phenomenon, while taking full account of heterogeneity. Further, complex interactions on the cellular as well as subcellular level can be addressed. MMTC is appropriate for determining complex interactions among and between single cells in tissue and cell monolayers with respect to how many of which cells are where and what is their (functional) status. This technique is a versatile analytical tool of research and—with respect to current development—could also be established in clinical routine diagnostics in the future.

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HUMAN CYTOME PROJECT, CYTOMICS, AND SYSTEMS BIOLOGY

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Background: Molecular alterations in cells as elementary units of cell systems (cytomes), tissues, and organs may manifest as disease at the organism level. They are detectable as altered molecular cell phenotypes by single-cell flow, image, or chemical cytometry. Molecular cell phenotypes result from genotype and exposure influences within the heterogeneity of various cell types but also within given cell types, according to cell cycle or functional status.

Concept: Molecular cell phenotypes carry information on the present (diagnosis) and future (prediction) disease course of patients in relation to therapy. Multiparametric single cell cytometric differentials of diseased patients versus reference individuals (normals, stationary disease, survivors) characterize disease-induced molecular changes. The parameter selection is hypothesis-driven using the genome sequence as biomolecular inventory list. The subsequent exhaustive differential knowledge extraction from all cells (cytomics) is hypothesis-free and can access information on molecular pathways or particular regulatory networks being hidden to traditional hypothesis formulation for lack of *a priori* knowledge. The multiparametric

results of differential cytometric and other measurements can be investigated for discriminatory bioparameter patterns according to the predictive medicine by cytomics concept. It provides (i) individualized disease course predictions for patients in relation to therapy, (ii) disease-specific access for molecular reverse engineering of disease pathways by systems biology, (iii) new potential for drug discovery by the identification of new drug targets.

Potential: The concept of a human cytome project systematizes this approach by analyzing in a first step the multitude of multiparametric cytometric data from large patient groups available at many clinical sites and establishing a structure for public databases for the standardized relational knowledge. In a second step, molecular disease pathways are investigated and mathematically modeled by biomedical cell systems biology. Leukemia/lymphomas, cancers, rheumatoird diseases, allergies, and infections are of high medical interest while stem cell differentiation, cell cycle regulation, cell proteomics, and cell organelle functionality are attractive for basic research.

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CYTOMICS AND DRUG DISCOVERY

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Pharmaceutical and biotech companies, try to develop new drugs that have a high success rate of reaching the market. Pharmaceutical companies try to bring down the attrition rates late in drug development by improving the predictive power of early stage drug discovery. The cost to develop a single drug which reaches the market has increased tremendously in recent years and only 3 out of 10 drugs, which reached the market in the nineties, generated enough profit to pay for the investment. This is mainly due to the low efficiency and high failure rate of the drug discovery and development process. Improving disease models is an important issue to improve success rates in drug discovery and development. Using cell-based disease models, which should take into account the molecular diversity of the human cytome, will improve the predictive value of drug discovery. Current disease models lack a strong correlation to clinical reality, because of the underestimation of the complexity and variability of clinical disease processes.

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TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY (TIRFM) IN CELL BIOLOGY

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Total internal reflection fluorescence microscopy (TIRFM) permits selective detection of cell surfaces, e.g. plasma membranes, with an axial resolution of a few nanometers. In addition to an overview on TIRFM principles and techniques, measurements of membrane dynamics as a function of temperature, cell age, and cholesterol content are presented. In particular, membrane stiffness revealed to decrease with temperature and to increase with the content of cholesterol. In addition, stiffness of the plasma membrane always exceeded that of intracellular membranes. For future applications of in vitro diagnostics or screening of pharmaceutical agents, a fluorescence reader based on total internal reflection of a laser beam was developed and tested with membrane associated fluorescent proteins.

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EVALUATION OF A FLUORESCENCE-BASED CELLULAR ASSAY TO ANALYZE THE PHARMACOLOGICAL MODULATION OF LIPID HOMEOSTASIS IN HUMAN MACROPHAGES

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In atherosclerosis, macrophages progressively accumulate large amounts of cholesterol leading to foam cell formation, cell death, and lesional lipid deposition. Therefore, we have intensively investigated cholesterol and phospholipid efflux pathways mediated by ABCA1, which is induced in cholesterol-loaded macrophages. ABCA1 gene transcription is regulated by the nuclear receptor heterodimer liver X receptor/retinoid X receptor (LXR/RXR). We are currently working on fluorescence-based High-Content Screens to evaluate new agents that target LXR or RXR as potential regulators of ABCA1 expression and inducers of cholesterol efflux. To establish a fluorescence-based assay, differentiated monocytes were loaded with enzymatically modified LDL (E-LDL) and subsequently incubated for 16 h in the absence or presence of HDL3. Cells were stained either with the saturated, fluorescent lipid probe DMPE-TMR or other raft-marker fluorophores. A recent study indicated a high partition coefficient of DMPE-TMR into membrane domains that are in the liquid-ordered phase and most probably represent the in vivo correlate of Triton-resistant membrane domains. In unloaded macrophages areas of less than 1 μm size possessing a weak and homogeneous peripheral fluorescence pattern representing single lipid microdomains were observed. In contrast to this, after cholesterol-loading, the overall fluorescence intensity increased and larger confluent areas with a diameter of ~5–10 μm were visible, indicating that cholesterol-loading promotes the confluence of relatively small rafts into larger domains. HDL3 incubation of cholesterol-loaded cells reversed this effect and reduced the overall fluorescence intensity, demonstrating that HDL3 modulates the lipid microdomains at the plasma membrane in vivo. To study the impact of compounds that target LXR or RXR as potential cholesterol efflux promoters, several substances of interest were included into the incubation scheme. Our first results of a fluorescence-based quantitative assay for substances modulating the ABCA1-mediated cholesterol transport in macrophages based on a high-content screening platform and conventional 96-well plates will be reported.

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INFLAMMATORY CYTOKINES MODEL AS A PREDICTOR MARKER OF ACUTE PANCREATITIS

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Introduction: Numerous attempts have been made to detect early prognostic markers in acute pancreatitis (AP). Particularly cytokines, which have been considered markers of leucocyte activation and inflammation, may have clinical relevance.

Objectives: To analyze by flow cytometry (multiplex assay) cytokine pattern and to evaluate its relationship with AP's evolution.

Materials and Methods: We simultaneously quantified six cytokines using BD Cytometric Bead Array kit (CBA). Samples were extracted the post-diagnostic day. We analyzed samples of 10 controls and subjects with AP (26 with good and 23 with bad prognosis AP). Latex microspheres covered with specific antibodies for each cytokine and distinct fluorescence intensities (FL3) were incubated with 50 ml serum (or standard) and a second specific antibody (FL2) for each cytokines to form sandwich complexes. Analysis was performed by CBA Analysis software and by comparison with standard curve (1–10,000 pg/ml).

Results: in pg/ml

	Control	Good prognosis	Bad prognosis
IL-12p70	1.89	3.75	1.40
TNF-α	4.46	4.80	16.09
IL-10	16.13	32.96	4.92
IL-6	25.71	145.43	80.96
IL1-β	2.11	40.82	9.32
IL-8	3.31	38.06	6.21

Conclusions: The CBA system applied sensitivity of fluorescence detection and specificity of immunoassays to quantified soluble cytokines and is a rapid and cheaper technique than immunochemistry. In patients with AP, we can suspect torpid evolution when its IL's model at the diagnosis shows TNF-a, IL-6, and IL-8 higher and IL-10 lower than control. Clinical evolution was better when levels of IL12p70, IL-10, and IL-1b were elevated with regard to grave AP.

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MODULATION OF PLATELET AND NEUTROPHIL FUNCTION BY HIGH DENSITY LIPOPROTEIN

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Hyperactivity of platelets is accompanied by an enhanced risk of atherosclerosis and arterial thrombosis. Lipoprotein disorders affect platelet functions and hypersensitive platelets have been observed in hyperlipidemia. Moreover, platelets and also neutrophils participate in the acute phase response, which is accompanied by alterations especially in the metabolism of apolipoprotein A-I and high density lipoproteins (HDL). However, the role of HDL as a modulator of platelet and neutrophil function is not well established. We therefore analyzed the effects of HDL3 and Apo-AI on platelet and neutrophil function by flow cytometry, mRNA and protein expression analysis as well as imaging techniques. The ATP cassette transporter A1 (ABCA1), which facilitates the cellular release of cholesterol and phospholipids to HDL-precursors, might be regarded as a candiate receptor, mediating HDL-associated signaling. ABCA1 expression was analyzed in platelets and neutrophils as well as in in vitro differentiated umbilical cord blood stem cells. ABCA1 expression was upregulated during megakaryocytic differentiation of human cord blood stem cells and ABCA1 could also be detected in peripheral blood platelets, partially associated to Lubrol WX-resistant membrane domains. The plasma HDL-cholesterol in healthy blood donors negatively correlated to the microviscosity of platelet membranes, indicating that ABCA1 governs platelet membrane cholesterol. Moreover, platelets from a patient with ABCA1 deficiency (Tangier disease) were characterized with regard to platelet reactivity and HDL effects on platelet activation. Pre-incubation of platelets with HDL enhanced the effects of low-dose collagen or ADP, e.g. on dense granule secretion, in ABCA1-deficient platelets, suggesting that HDL modulates vesicular transport pathways in platelets, such as the AP-3-mediated vesicle trafficking, necessary for degranulation as well as cholesterol homeostasis. This hypothesis is supported by preliminary data, suggesting that HDL and ApoAI also modulate the degranulation of neutrophils upon activation by pro-inflammatory cytokines and fMLP. In summary, our data suggest that HDL modulates platelet and neutrophil function and that vesicular transport mechanisms involved in cholesterol efflux and activation associated degranulation might be linked to HDL-induced signal transduction.

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BRIDGING BIOLOGICAL SCALES BY STATE-SPACE ANALYSIS USING MOLECULAR, TISSUE CYTOMETRIC, AND PHYSIOLOGICAL DATA

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Combining data streams across different levels of biological observation such as molecular, cellular, and physiological responses support to a system-wide view in biomedicine. Recent progress in slide-based cytometry has led to the development of Tissomics, a high-throughput and high-content phenotyping methodology that provides data-rich profiles of cells and multicellular relationships. The introduction of robust machine vision software that can discern the histomorphology of tissues in a comprehensive fashion can be

used to support and confirm histopathological assessment of tissue, allowing a more complete quantitative evaluation of phenotypical responses and the identification of structural markers of normality, injury and disease on the tissue level (1,2. Phenotypical data provide co-variants that can be used to identify relevant candidate genes by associating molecular with phenotypical cellular and physiological states. Methods to identify and visualize such associations in state space are introduced, with a particular focus on the preservation of individual responses suited for computational e-diagnostic applications.

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PREDICTION OF SEPSIS WITH AID OF IMMUNOLOGICAL PARAMETERS
Sepsis-Prädiktion mit Hilfe immunologischer Kenngrößen

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In an attempt to summarize relevant genetic, humoral, and cellular immunological parameters for the prediction of sepsis a search in the literature was performed for the last 10 years. One major problem for the comparison of the results is the definition of sepsis in particular regarding neonates, and children. Therefore, sepsis was assumed if a positive blood culture was obtained. Based on this definition genetic risk factors for sepsis in infants could not be defined yet. The combination of increased serum levels of C-reactive protein and interleukin (IL)-6 appears to provide the most reliable indication of neonatal sepsis. Elevated procaltionin serum concentrations seem suitable to identify septic children. In adults the analysis was restricted to trauma patients with bacterial sepsis defined according to the Society of Critical Care Medicine and the American College of Chest Physicians criteria. The most useful genetic parameter in predicting sepsis seems to be the TNF-β-252A polymorphism. For the analyzed serum parameters (procalcionin; IL-2,4,6,10,12,18; TGF-β) no clear results were obtained regarding prediction of sepsis. A variety of presumed immunological cellular parameters have been shown to be depressed after major trauma. But especially HLA-DR expression on monocytes had gained growing attention for prediction of sepsis during the last ten years. Because a standardized test kit to measure monozytic HLA-DR expression is now available multi-center trials could now be performed. Although a variety of immunologival parameters are available for the prediction of sepsis no one had gained wide acceptance. There are some explanations for this fact: the sensitivity of any sepsis prediction rule must be exteremely high to be widely accepted by practicing clinicans under consideration of the high costs for analytics and only view therapeutical consequences.

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AN AUTOMATIC SYSTEM FOR THE ANALYSIS OF HEP-2 IMAGE PATTERN

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The kinds of cells that are considered are Hep-2 cells, which are used for the identification of antinuclear autoantibodies (ANA). ANA testing for the assessment of systemic and organ-specific autoimmune disease has increased progressively since immunofluorescence techniques were first used to demonstrate antinuclear antibodies in 1957. Hep-2 cells allow for recognition of over 30 different nuclear and cytoplasmic patterns, which are given by upwards of 100 different autoantibodies. The identification of the patterns has been done manually till date by a human inspecting the slides with the help of a microscope. The lacking automation of this technique has resulted in the development of alternative techniques based on chemical reactions, which have not the discrimination power of the ANA testing. We present a decision support system that can automatically inspect and classify the cell patterns (1,2). We describe the image acquisition unit, the image analysis and the feature extraction unit as well as the classification unit. The system was evaluated on a data set of 600 cell image samples. Finally, we give result on the classification accuracy and describe the final system.

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SLIDE-BASED LYMPHOID IMMUNOPHENOTYPING BY CLATCH'S METHOD: REVIEW OF RESULTS IN 500 CLINICAL CYTOLOGY SAMPLES

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In slide-based laser scanning cytometry (LSC) triggering on nuclear fluorescence ensures that all nucleated cells will be analyzed. But use of bright nuclear dyes precludes sequential labeling of unfixed cells adherent to a glass slide, desirable for analysis of paucicellular cytology samples. Clatch's method (Cytometry 1998;34:3-16) uses application of a three colour-conjugated antibody (Ab) mixture to unfixed cells on a glass slide and triggering by forward light scatter (FLS). We reviewed our data to determine the success of this method in clinical practice.

Methods: Cytologic samples (500) of lymphomas in needle aspirates and body fluids were analyzed by Clatch's method in the routine cytology laboratory. Cells (20,000 to 120,000)

were used for analysis of 10–20 antigens/sample. In 90 cases unfixed cells were re-scanned after secondary application of Ab, or fixed and permeabilized for assessment of cytoplasmic antigens. Results were reviewed for technical quality and concordance with immunohistochemistry (IHC) or flow cytometry (FC). Results: In 479 cases (96%) abnormal cell populations were identified and their immunophenotype determined. Results were concordant with FC and IHC except for random differences in brightness of individual antigens attributable to Ab clone or conjugates. There were no discrepancies affecting diagnosis. LSC characterized more antigens at lower cost, but showed a negative bias in the estimation of the proportion of neoplastic cells. The dynamic range of LSC was less than flow cytometry, but relationships of dim, moderate, and bright fluorescence were preserved. In 21 cases (4%) of T-cell rich B-cell lymphoma or diffuse large B-cell lymphoma LSC showed only the reactive T-cell population. Flow cytometry showed the abnormal population in 1/10 of these cases where it was performed. Intracellular antigens requiring permeabilization (TdT, cCD3, bcl-2, cIg) were more reliably shown by LSC. Conclusion: Clatch's method is a rapid and reliable means of qualitative characterization of surface and cytoplasmic antigens in paucicellular cytology samples. Triggering by FLS is unsuitable for quantitative applications because of underestimation of some cell types. Both LSC and FC may fail to identify small populations of neoplastic cells in large cell lymphoma.

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HEMOGLOBIN–HAPTOGLOBIN COMPLEX UPTAKE INDUCES OXIDATIVE BURST AND PROINFLAMMATORY CYTOKINE SECRETION IN HUMAN MACROPHAGES

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Iron metabolism is suggested to play a role in the formation of atherosclerotic lesions, although clinical studies could not consistently confirm a correlation between the body iron status and the progression of atherosclerotic disease. Therefore, local dysregulation of iron metabolism is suggested to be more important for lesion development and the progression of atheromas, probably triggered by bleeding from lesion associated microvessels as well as hemolysis of erythrocytes passing over the rough surface of the sclerotic lesion. Free hemoglobin is rapidly bound by haptoglobin, forming hemoglobin–haptoglobin (HbHp) complexes, which are taken up by macrophages via CD163-antigen-mediated endocytosis. The aim of this study was to evaluate the effects of HbHp-uptake on oxidative burst, inflammatory cytokine secretion and surface antigen expression of human monocyte-derived macrophages. Macrophages were incubated in vitro with HbHp complexes and the secretion of pro and anti-inflammatory cytokines (IL-6, TNF-α, IL-1β and IL-10), the oxidative burst activity as well as the surface antigen expression of CD163 and CD14 were measured by flow cytometry. HbHp-complexes induced a concentration dependent release of the pro-inflammatory cytokines TNF-α, IL6 and IL-1β in macrophages. Moreover, we could show an increase in spontaneous and PMA-induced oxygen radical generation especially at low concentrations of HbHp-complexes. At higher concentrations, HbHp-complexes also induced the secretion of the anti-inflammatory IL-10, although the secretion of the pro-inflammatory cytokines persisted. Uptake of HbHp-complexes by macrophages also resulted in the up-regulation of surface CD163 expression without modulating the expression of CD14. Our results suggest that within arteriosclerotic lesions HbHp uptake may also strongly modulate the phenotype of lesion associated macrophages with regard to oxidative burst activity and cytokine secretion, thereby contributing to the progression of the arteriosclerotic plaques.

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CLINICAL CYTOMICS

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The goal of predictive medicine is the detection of changes in patient's state prior to the clinical manifestation of the deterioration of the patients current status. Therefore, both the diagnostic of diseases like cancer, coronary artherosclerosis or congenital heart failure and the prognosis of the effect specific therapeutics on patients outcome are the main fields of predictive medicine. Predictive medicine aims at the recognition of the "fate" of each individual patients in order to yield unequivocal indications for decision making (i.e. how does the patient respond to therapy, react to medication etc.). This individualized prediction is based on the Predictive Medicine by Cytomics concept. These considerations have recently stimulated the idea of the Human Cytome Project. A major focus of the Human Cytome Project is multiplexed cytomic analysis of individual cells of the patient, extraction of predictive information and individual prediction that merges into individualized therapy. Although still at the beginning, Clinical Cytomics is a promising new field that may change therapy in the near future for the benefit of the patients.

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SERUM LEVELS OF CYTOKINES AND SOLUBLE ADHESION MOLECULES IN CHILDREN WITH CONGENITAL HEART DISEASE IN COMPARISON WITH AGE-MATCHED CONTROLS

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Little is known on the immunological processes in children with congenital heart disease, systematic studies of this subject have not been published so far. The aim of the present study was to find out, whether the alterations of the immune system described in adults occur similarly in children. The values of various cytokines (IL-6, IL-8, IL-10, and TNF a) and levels of soluble adhesion molecules (sE-Selectin, sICAM-1, sPECAM-1) were determined in the sera of children with congenital heart disease. The results were compared with values from a group of children and adolescents without cardiac disease. We found an age-depending decrease of the concentrations of PECAM-1 ($r = -0.72$; $P < 0.001$) and sE-

Selectin ($r = -0.54$; $P < 0.001$) in the control group. It shows that different morphology, i.e. different type of congenital heart disease, has no influence on the parameters examined, with one exeption in the group of patients with complex cyanotic lesions. The latter showed a significant increase of the adhesion molecule sICAM-1 (263.9 ng/ml; SD 16.3 vs. 352.3 ng/ml; SD 29.8; $P = 0.007$). As well there were higher values for sICAM-1 in patients with more severe symptoms (270.9 ng/ml; SD 13.6 vs. 329.5; SD 26.9; $P = 0.04$).

Conclusion: Patients with congenital cardiac defects have other pathophysiological mechanisms than adult patients with coronary heart disease, hypertension or cardiomyopathy.

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IMMUNE SURVEILLANCE OF CENTRAL NERVOUS SYSTEM (CNS): INDICATIONS FOR DIFFERENT MIGRATION PATHWAYS OF LYMPHOCYTES THROUGH THE BLOOD–BRAIN BARRIER (BBB) AND BLOOD–CEREBROSPINAL FLUID BARRIER (BCSFB) IN ANIMALS AND HUMANS

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Different pathways of lymphocyte migration from blood into brain were analyzed by cytometry establishing lock-and-key model A in brain endothelial cells, locked by two tight junctions in bbb, and model B in choroid plexus epithelial cells, locked by one tight junction in BCSFB. Model A in animals, mostly in humans (key on lymphocytes/lock on endothelial cells) for lymphocytes: A1: LFA-1(CD11a/CD18)/ICAM-2(CD102); A2: LFA-1 (CD11a/CD18)/ICAM-1 (CD54); for T lymphocytes A3: LFA-2 (CD2)/LFA-3 (CD58), A4: VLA-4 (CD49d)/VCAM-1 (CD106); for natural killer cells ($CD16^+ 56^+3^-$): A3, A4, A5: CD11b/CD18(Mac-1)/ICAM-1(CD54), A6: Integrin avβ3 (CD51/CD61)/PECAM-1 (CD31). B cells migrated only via A4. Tethering and rolling of the immune cells (using CD162, CD62L) occurred with P and E selectins on endothelial cells of low affinity (possible exception: A4 without rolling); lock-and-key avidity increased by cell activation with A2, A5. Model B: Passing animal BCSFB, immune cells first have to extravasate capillary endothelium (fenestrated in choroid plexus stroma) before migrating through the choroideal epithelial monolayer. Some pathways, similar to model A, were detected for lymphocytes B1 = A2; for T, B, NK cells B2 = A4, B3: VLA-4 (intgrin α4, CD49d)/MAdCAM-1 (unique for BCSFB); lock-and-key avidity increased after cell activation. No indications were reported for B1, B2, B3 pathways in corresponding endothelial cells of animals; but A2 was detected in human small vessels of choroid plexus villi, large venules of choroidal stroma, and postcapillary endothelial cells of the meninges.

In lumbar CSF of human controls blood/CSF cell ratio of $CD3^+4^+$ and $CD3^+8^+$ cells was lower than the ratio of NK cells, of NK subset $CD8^+3^-$ and B cells ($CD19^+3^-$) indicating a privileged passage of helper/inducer and suppressor/cytotoxic T cells through human CNS barriers during immune surveillance, which was reported to be carried out mainly with naïve and memory $CD4^+$ and $CD8^+$ cells, activated extracerebrally.

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HIGH-CONTENT BIOLOGY: SOLUTIONS FOR FULLY AUTOMATED IMAGING AND DATA GENERATION

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Automated imaging combined with sophisticated computerized data generation has meanwhile become a well-established method for analyzing large numbers of cell samples and acquiring functional cell data. This strategy has become particularly important in the modern drug discovery process.

To a significant degree this development has also been stimulated and influenced by developments of Evotec Technologies resulting in the Opera, a truly confocal new generation microplate imaging reader for fully automated high speed imaging of live cells and on-the-fly data generation. The Opera is a device allowing a wide range of multi-colour cell imaging applications comprising receptor ligand binding and activation studies, membrane potential, and transporter studies, recruitment of signalling molecules, organelle trafficking, calcium flux, cell viability, apoptosis as well as all kinds of siRNA applications but to mention a few. Some examples hereof shall be discussed in this presentation. Needless to say that a prerequisite for high-speed imaging in combination with on-the-fly data analysis requires intelligent software solutions. Such solutions are provided by a very flexible and user-controlled image analysis package as inherent part of the Opera, called Acapella. In this regard image analysis aspects will be discussed as well in order to complete the picture on high throughput analysis of cellular samples.

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LIPOPOLYSACCHARIDE-INDUCED CO-ASSEMBLY OF CD81 TETRASPANIN TO LIPID-RAFTS IN MONOCYTES: A RAPID FLOW CYTOMETRIC SCREENING FOR DETERGENT RESISTANT SURFACE ANTIGENS

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Lipid rafts are cholesterol- and glycosphingolipid-enriched plasma membrane microdomains, implicated in various cellular processes (vesicular trafficking, receptor clustering, signal transduction). Rafts show detergent insolubility at 4°C, and contain lipid-anchored membrane antigens and few transmembrane proteins. Such domains have frequently been investigated in artificial model membranes, and the results are difficult to extrapolate to biological membranes. Thus, one of the greatest clues is to discern detergent-resistant domains in living cells. In the present study, we have established a rapid flow cytometric assay to assess detergent-resistance of membrane proteins in peripheral blood monocytes. Surface antigens of isolated human monocytes were stained by fluorochrome-conjugated monoclonals. Detergents were added directly to the cells prior to flow cytometry. Two types of detection for detergent solubility were performed: end-point and kinetic mode acquisitions. Detergent solubility or

resistance of a given antigen was clearly distinguished by treatment with reduced concentration of Triton X-100 (0.01–0.1%) at 4°C or Brij 98 (0.1–0.5%) at 37°C, as time kinetic analysis showed that a short time (5 min) detergent treatment is efficient to solubilize antigens excluded of rafts (such as CD71), while raft-associated proteins (CD14, CD55, CD59) retained detergent resistance even after 30 min. Detergent resistance was abolished by cholesterol depletion of the plasma membrane (e.g. raft-disruption by methyl-β-cyclodextrin). Confocal imaging has confirmed intact cells after short time detergent treatment. In addition, ligand-induced receptor clustering was investigated on lipopolysaccharide (LPS) stimulated monocytes, and LPS is known to induce receptor coassembly. LPS induced the translocation of tetraspanin CD81 into lipid rafts, while CD81 was excluded in resting monocytes (without LPS). Data, regarding to detergent resistance of antigens detected by this rapid flow cytometric approach, were in accordance with previous data obtained by fluorescence-resonance energy transfer. The stimulation-dependent translocation of CD81 into rafts is also clearly demonstrated.

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QUANTITATION AND CHARACTERISATION OF ANTIGEN-SPECIFIC T CELL RESPONSES

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T lymphocytes play an essential role in the host defence against invading microbial pathogens. They mediate their function as T-helper cells or cytotoxic T cells via MHC-restricted recognition of processed peptide antigens. Using a rapid stimulation assay, antigen-specific T cells can be identified directly in human whole blood by intracellular cytokine detection. Both CD4- and CD8-positive T lymphocytes can be analyzed simultaneously in frequency and function (Sester et al., 2000; Heintel et al., 2002). The assay is not restricted to the knowledge of the human leukocyte antigens (HLA) of the blood donor and the corresponding antigenic peptide epitopes. Therefore, the presented technique can be used in clinical monitoring of a total cellular immune response to complex antigens during an infection or vaccination. Different applications of the intracellular cytokine staining from whole blood have been shown in studies which determine: (i) functional differences between HIV- and CMV-specific cytotoxic T cells (CTL) of double-infected patients by measuring the intracellular perforin amount (Heintel et al., 2002), (ii) the role of CMV-specific CD4-positive T cells in protection against CMV-reactivation in transplant recipients (Sester et al., 2001), (iii) monitoring of polio-specific T lymphocyte migration by staining for gut homing receptors after vaccination (Krieg et al., 2004), and (iv) *Candida*-specific T cell responses in immunocompromised individuals with high risk of invasive yeast infections (Breinig et al., manuscript in preparation). Various clinical aspects will be discussed.

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FLUORESCENCE NANOSCOPY

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Since its discovery by Abbe in 1873, the diffraction barrier has received a lot of attention. However, the subdiffraction microscopy concepts of the mid 20th century remained either too vague or subject to unrealistic physical conditions. Consequently, far-field fluorescence microscopes remained conceptually and practically diffraction-limited. In this lecture, we discuss the principle of breaking the diffraction barrier through reversible saturable optical (fluorescence) transitions (RESOLFT). This principle was put forward in the form of Stimulated Emission Depletion (STED) (1,2) and Ground State Depletion (GSD) microscopy (2,3). In these concepts, the diffraction barrier is broken by a saturated transition (depletion) between two states of a marker, whereby the transition is effected with an intensity distribution featuring one or more intensity minima (zero). The saturation level defines the size of the ultrasharp focal spot and/or the concomitantly enlarged bandwidth of the optical transfer function (OTF). We show that in a RESOLFT concept the resolution can be approximated by $\Delta x = \lambda(\pi n\sqrt{I/I_{sat}})$, whereby I_{sat} is the characteristic intensity required for saturating the transition, and I denotes the intensity applied (4). If the minima are produced by focusing optics with a numerical aperture $n \sin \alpha$, the minimal distance at which two identical objects can be discerned can be approximated by

$$\Delta x \approx \frac{\lambda}{2n \sin \alpha \sqrt{1 + I/I_{sat}}}$$

which can be regarded as an extension of Abbe's equation (4,5). The diffraction-unlimited nature of the RESOLFT family of concepts is reflected by the fact that the minimal resolvable distance can be continuously decreased by increasing $V = I/I_{\mathrm{sat}}$ (1–4,6,7). Hence the quest for nanoscale resolution comes down to maximizing $V = I/I_{\mathrm{sat}}$. This is possible by increasing I or by lowering I_{sat} (4,5,8). We give evidence of STED-microscopy displaying PSF of 10–20 nm FWHM, corresponding to a 15-fold enlargement of the OTF over Abbe's barrier. The success of STED stems from the fact that the saturation of the single-photon transition of stimulated emission provides strong nonlinearities at comparatively *low* intensities. The reason for that is simple but critical: Unlike in multiphoton events, satura-

tion is *not* effected by the joint action of multiple photons, but stems from the population of the fluorophore states (4,5). Therefore, transitions that are easy to saturate (i.e. low I_{sat}), allow huge V at low intensities. Examples include the saturation of the triplet state (2,3), which reduces Isat by ~103 as compared to STED. Of similar interest is the 'switching' between conformational fluorophore states (5,7–9), which gives a factor of >106. Suitable candidates for saturable switches are encountered in photochromic compounds (5,7–9) and photoswitchable GFP-like proteins (4,7,8), which should ultimately give nanoscale resolution at intensities provided by a lamp.

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INVESTIGATING PHYSIOLOGY OF LIVING MICROBIAL CELLS—A MULTICOLOUR APPROACH

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The activity, physiological state and, product formation of microbial individuals very often depend on the state of the cells in cell cycle and on their capacity to access and metabolise a carbon source. Rapid information on substrate uptake by single living cells, the simultaneous analysis of their proliferation activity and the determination of the amount of dead cells will facilitate reliable information about their impact on their microenvironment. Though the solitary parameters were already regarded in living microbial individuals, here the whole information is provided at the same time. Flow cytometry and Hoechst 33342 were involved to follow proliferation activity in Sacharomyces cerevisiae, whereas the fluorescent glucose analogue 2-NBD-glucose was employed to analyze the cells' affinity to glucose. Propidium iodid was used for accessory determination of dead cells. Calibration and verification experiments were performed using cells grown batch-wise as well as in continuous or transient state regimes to provide stable microenvironmental living conditions. As a result a three-colour flow cytometric assay was developed to replace single parameter approaches in order to get i) close physiological information of subsets of populations within a population and ii) to provide a reliable and rapid method for analysis of living cells within a few minutes. Information is obtained about proliferation activity, substrate affinity and the amount of cells which do not contribute actively to the performances of the population. In future we want to use this validated tool for rapid analysis of living microbial cell states and activities during the respiratory and/or fermentative mode of metabolism, which promisingly might enlighten phenomena like the metabolic or cell cycle dependent stable synchrony oscillations of S. cerevisiae.

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FLOW CYTOMETRIC ANALYSIS OF THE ''IN SITU'' HYBRIDIZATION OF CYCLOOXIGENASE (COX) ISOFORMS IN MESANGIAL CELLS (MC) TREATED WITH CYCLOSPORINE (CSA)

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Introduction: CsA increases synthesis of ROS, Thromboxane (Tx) and lipid peroxidation in kidney. The mechanisms implicated are unknown but COX may be involved. OBJECTIVES: We performed "in situ" hybridization in MC suspension to detect mRNA expression of COX isoforms (COX-1, constitutive and COX-2, inducible).

Materials and Methods: Male Wistar rats (n = 5 for group) were treated with CsA (50 mg/kg/day) for 4, 7, or 10 days. (1) Glomeruli and MC: Glomeruli were obtained by differential sieving and filtered (35-mm mesh) to obtain a MC suspension. MC (3×10^5) were fixed, treated with 10% DEPC in ethanol/1 h/22°C, centrifugated and diluted in 50 ml PBS/0.5% Tween20. (2) Preparation of labelled probe: COX probes were diluted (25 ng/ml in TE) and labelled with "The Gene Image Random Prime Labelling Protocol" (Amersham Life Science) with nonamers of random sequence and Fluorescein-11-dUTP catalyzed by the exonuclease-free Klenow fragment of E. coli DNA polymerase (Fluorescein-11-dUTP partially replaces dTTP so that FITC-labelled probes were generated). (3) Hybridization in suspension: Probes (250 ng) were denatured by boiling, snaped cool in ice and mixed with MC and 20 ml hybridization solution (10 ml formamide, 5× SSC, 5× Denhardt's, 0.1 ml SDS). The hybridization was carried out at 48°C/12 h in a shaking water bath. Cells were incubated 45 min/48°C with stringency wash solution and they were washed in PBS with 0.5% Tween20. (4) Analysis: We used a FACScan (Becton Dickinson). MC were identified by a monoclonal specific antibody (Thy 1.1-PE) and COX expression were measured by FITC fluorescence of probes labelled. RESULTS: Our results showed CsA increased COX-2 expression along time (Index with regard to Control fluorescence: 4 days = 1.35, 7 days = 1.57*, 10 days = 1.72*). However, COX-1 expression only increased at 10 days (1.25 in relation to Control fluorescence).

Conclusions: Flow cytometer analysis of "in situ" hybridization in suspension cells is an available method to detect mRNA. CsA increased the expression of inducible COX (but not constitutive COX) in mesangial cells, in a time-dependent way (and parallel to the increase of ROS, TX and MDA synthesis).

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MULTIPLEX ANALYSIS OF CYTOKINES IN EXHALED BREATH CONDENSATE

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Examination of exhaled breath condensate (EBC) has become an important procedure in diagnosis and monitoring of acute inflammatory diseases. Beside pH and leukotriene measurement, analysis of pro-inflammatory cytokines promises improved monitoring of inflammation and cellular interaction. Main problem in measurement of cytokines in EBC is the low protein content requiering concentration steps that is conflicting with need of much fluid in commonly used kits. Here, six different cytokines (IL-12p70, TNF-a, IL-10, IL-6, IL-1β, IL-8) were analyzed in the same EBC sample (2 ml) from lyophilized and partially reconstituted EBC by a multiplex bead array with a flow cytometer. Assay characteristics when applied to EBC were determined. Furthermore, 12 smoking and 21 non-smoking healthy volunteers and 11 ventilated patients with acute lung injury by a severe pneumonia were investigated. The multiplex bead array could be established and validated for application on EBC. In patients' samples, we found significant differences in EBC cytokines between patients with acute inflammatory reaction compared to controls for all investigated cytokines. These results suggest that multiplexed immunoassays in high sensitive approaches allow detection of multiple cytokines in EBC. Patients with acute lung injury can be investigated by this method in a non-invasive manner for the presence of cytokines inside airways and lung.

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ANALYSIS OF THE TWO- AND THREE-DIMENSIONAL DISTRIBUTION OF NUCLEI AND NEURONS IN BRAIN TISSUE USING LASER SCANNING CYTOMETRY (LSC)

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The Laser Scanning Cytometer (LSC) is a microscope-based cytofluorometer closing the gap between flow cytometry and fluorescence microscopy. This affords a quantitative fluorescence analysis of tissue sections. The tissue architecture and morphological details remain conserved and using immunohistochemistry, individual cell populations can be distinguished and analyzed. Thereby both, the two- and the threedimensional distribution of the cells and nuclei can be depicted. Using sections stained with propidiumiodide, the LSC enables us furthermore to assess the DNA content of single cells. Therewith the distribution of cells through the cell cycle phases can be determined and conclusions on the DNA replication and proliferation become possible. The feature of the LSC, to measure many cells and many cases in a relative short time, rounds up the picture of the LSC as a powerful tool not only for scientific but also for diagnostical use. In our study we adapted the LSC technique to brain slices. On one hand we used immunohistochemistry and different cell markers and we were able to display morphological details such as different cell layers in the human entorhinal cortex and to analyze the DNA content in neurons in healthy and Alzheimer's disease brain. On the other hand we developed a method depicting the three-dimensional distribution of the nuclei in the solid tissue by means of thick brain sections (120 μm).

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THE CHOLESTEROL ABSORPTION INHIBITOR EZETIMIB INFLUENCES THE RAFT ASSEMBLY THROUGH CD13 IN HUMAN ENTEROCYTES AND MACROPHAGES

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Our group was involved in the recent identification of aminopeptidase N (CD13) as a receptor for Ezetimib, a novel cholesterol absorption inhibitor, in enterocytic brush border. Regarding the extensive involvement of CD13 in receptor-mediated endocytosis and the dynamics of the deep apical tubule compartment (e.g. rafts), we have investigated the influence of Ezetimib on binding of the WM-47 (mAB against an extracellular domain of CD13) to CD13 in differentiated Caco-2 cells by confocal microscopy. The intense fluorescence staining of CD13 on Caco-2 cells shown by FITC-conjugated WM-47 was almost completely abolished when the cells were treated with Ezetimib prior to CD13 staining. By contrast, if Ezetimib was applied after binding of WM-47 to CD13 no significant change in the cellular fluorescence staining of CD13 occurred. Thus, Ezetimib likely induces a conformational change on CD13 (in enterocytic raft) that the WM-47 cannot recognize its binding site on the Ezetimib-pretreated CD13. Since CD13 is also expressed in macrophage-rafts, it is tempting to speculate that Ezetimib may influence raft assembly in these cells. Human monocyte-derived macrophages were loaded in vitro with enzymatically modified LDL (eLDL) or oxidized LDL (oxLDL), in order to induce a foam cell-like phenotype and modified macrophage rafts, in the presence/absence of Ezetimib. Detergent-resistant membrane fractions (DRMs) were isolated by Lubrol-WX and DRM-localization of proteins were followed by immunoblot. Expression, localization and cellular distribution of constitutively raft-associated antigens (such as CD13, CD36, CD14) or induced raft-associated antigen such as the ATP-binding cassette transporter A1 protein (ABCA1), were detected by confocal imaging and flow cytometry. The presence of ABCA1 protein in DRMs was enhanced upon eLDL-load, but diminished upon Ezetimib administration. Furthermore, Ezetimib down-regulated the protein expression of CD13, CD14 or CD36 as detected by flow cytometry, or altered the cellular distribution of macrophage rafts as assessed by confocal microscopy. Thus, Ezetimib modifies the raft assembly in macrophages, likely through a CD13-dependent dysclustering mechanism.

POSTER ABSTRACTS

1

AUTOMATED THREE COLOR CD4/CD8 ANALYSIS OF LEUKOCYTES BY SCANNING FLUORESCENT MICROSCOPY USING QUANTUM DOTS

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Background: Flow cytometer and Laser scanning cytometer are usually used for the three color phenotyping of leukocytes. The Scanning Fluorescent Microscope (SFM), the new technique for automated motorized microscopes could also be able to measure triple fluorescent labeled slides (Bocsi et al. Cytometry 2004;61(1):1–8). $CD4^+$-$CD8^+$ lymphocyte cell count and ratio are important features of the immune system for immune diagnistics. With the spread of HIV there is emerging demand for automatic methods to determine the T-Helper/T-suppressor cell ratio.

Aims: Development of triple fluorescent labeling method for leukocytes and application of SFM for their automated analysis and counting on peripheral blood specimen. Study the bleaching of some currently developed dyes.

Materials and Methods: EDTA anticoagulated blood samples were stained by the whole blood method by CD4 PE-Alexa-610 (Caltag) and CD8 FITC (BD-Biosciences) antibodies, parallel staining was made by CD4 FITC (BD-Biosciences) and CD8 biotin/Streptavidin Qdot-605 or Qdot-655. An aliquot was measured by flow cytometer. The remaining suspension was transferred to glass slides and antifade mounting medium was used for saving the fluorescence. The specimens were scanned and digitized in the three fluorescent channels. Automated cell detection, CD4 and CD8 detection was performed, ratio was calculated.

Results: Fluorescence signals were well separable. Bleaching of dyes was affected by mounting. Significant correlation between the SFM and FCM CD4/CD8 ratio results could be observed ($P < 0.05$).

Conclusions: Triple fluorescent labeling and automated SFM is an applicable tool for the CD4/CD8 ratio determination in peripheral blood samples.

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NINE FLUORESCENCE ANALYSIS ON A DUAL LASER FLUORESCENCE ACTIVATED FLOW ANALYSER

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An experiment which requires a steady control of parameters like the quality and quantity of blood cells during an infection period is limited by the availability of blood cells. Therefore it would be a great benefit to investigate as many parameters in as little volume as possible.

For this, we developed for the analysis of leukocyte populations, either from peripheral blood or lymphoid organs, a robust flow cytometric method which uses a single staining procedure containing a mixture of nine directly coupled fluorochrome labeled antibodies against CD4 (PE-Cy5), CD8 (PE-Cy5), CD19 (APC), IgM (PE), IgD (FITC), F4/80 (PE), Gr-1 (FITC and PE), and CD49b (FITC). With a special gating strategy, this method allows the determination of mature and immature B cells, CD4 and CD8 T cells, NK cells, granulocytes, and macrophages in a volume of 50 μl blood or with 1exp6 cells respectively.

In the course of the European mouse phenotyping programme (EUMORPHIA) we analyzed with the method described above the peripheral blood of four inbred mouse strains (CBA/JHsd, C3H/HeN, BALB/c, C57BL/6J), male as well as female. It became apparent that there is a clear difference in the percentages of the various subpopulations between these strains. In addition, we observed differences in male versus female mice. We now use this method to follow infection experiments in mice and to phenotype mouse mutants.

3

RETINOIC ACID (tRA) PROVOKES APOPTOSIS AND MITOCHONDRIAL DYSFUNCTION IN HUMAN MESANGIAL CELLS (HMC)

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Introduction: Vit A derivatives such as all-*trans* tRA are implicated in several essential processes (cellular proliferation, development and differentiation carcinogenesis). Apoptosis is a complex programmed cell death process and mitochondria seems to be actively (disruption of mitochondrial electronic transport, increase oxidative stress and passively (cyt. c release, caspase activation) implicated. The mechanism of tRA-induced apoptosis has not been defined, but mitochondrial dysfunction could be implicated.

Aims: To analyze functional and morphologic alterations that occur at mitochondrial level in HMC treated with tRA by multiparameter flow cytometry.

Material and Methods: (1) HMC was treated 24 h with 10-6 M tRA. (2) Apoptosis was determined using AnnV-FITC/PI (Apoptosis Detection Kit RD System). (3) Mitochondrial function was examined by staining with mitochondrial membrane potential (ÄØm)-sensitive fluorochrome (CMXRos); results were normalized by mitochondrial mass unit

(CMXRos/MTG). NAD(P)H level was determined by UV excitation and the determination of cellular autofluorescence. (4) Mitochondrial structure was studied by using the cardiolipin specific dye Nonyl Acridine Orange (NAO) and levels of reduced thiols by using Monobromobimane (MBB).

Results: (1) Control cell cultures showed 5% apoptotic cells while in tRA treated cultures appeared 30%. (2) After treatment, CMXRos/MitoTracker Green and NAD(P)H levels were decreased in apoptotic cells (six and twofold respectively), but not in non-apoptotic cells. (3) tRA-treated cells (normal and apoptotic) showed a six-fold decrease in NAO fluorescence versus control cells. However, only apoptotic cells showed lower MBB fluorescence (17 vs. 56 in control cells, arbitrary fluorescence units).

Conclusions: tRA exposure induced depletion in thiol reserve, which induces apoptosis through an alteration in cellular redox state and activates death signaling pathways. Cardiolipin contents decrease in tRA-treated cells which suggest that mitochondrial membrane lipid peroxidation is produced; then, cytochrome c release, caspase activation and mitochondrial ÄØm per cell and NAD(P)H decreased. We concluded tRA-treatment induces apoptotic process in HMC through alterations in mitochondrial metabolic and structural state.

4

NOVEL WHOLE BLOOD ASSAY TO ASSESS THE INFLUENCE OF IMMUNSUPPRESSIVE DRUGS ON HUMAN LYMPHOCYTE APOPTOSIS

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Objective: Most studies used purified lymphocytes to determine the effects of immunosuppressive drugs on apoptosis. We establish FACS assays of lymphocyte function using whole blood to determine the pharmacodynamics (PD) effects on immunosuppressive therapy in human heart transplanted (HTx) recipients. Therefore, this study was designed to develop a whole blood assay to assess the effects of different immunosuppressive drugs on apoptosis.

Methods: Peripheral blood for six experiments for each drug was drawn from healthy men. Whole blood was treated either with 1 μM cyclosporin A (CsA), 10 μM mycophenolate acid (MPA), 100 nM tacrolimus (TRL) or with 100 nM rapamycin (RAPA). Whole blood was stimulated with eight different concentrations of staurosporine (0–5 μM), an apoptosis inductor through inhibition of protein kinase C pathway, for 4 and 24 h. Apoptotic lymphocytes were measured by expression of Annexin V using FACS. Drug effects were calculated (% cells ± SEM) by taking the effects of staurosporine as baseline values.

Results: 24 h MPA and RAPA (in a lower extent TRL), but not CsA, significantly increased ($P < 0.05$) the numbers of apoptotic (MPA: 20.9 ± 3.7; RAPA: 17.5 ± 3.3; TRL: 16.4 ± 2.9 vs. control: 15.2 ± 2.8) and dead cells (MPA: 26.7 ± 4.3; RAPA: 20.9 ± 4.7; TRL: 18.5 ± 4.3 vs. control 11.9 ± 2.0) accompanied with significantly declining of intact lymphocytes compared to control (MPA: 37.8 ± 8.8; RAPA: 44.7 ± 9.2 TRL: 42.2 ± 8.6 vs. control: 52.3 ± 8.7). All drug effects were reached on the maximum expression of apoptotic cells after stimulation with 3 μM staurosporine.

Conclusion: We developed a whole blood assay to assess the effects of immunosuppressants on apoptosis. Our results showed that MPA has a stronger effect on apoptosis than RAPA and TRL, whereas CsA had no effect. These assays may be helpful to distinct between the mechanisms of action of different immunosuppressants in a combination therapy. Future studies in human HTx recipients are needed to show if such whole blood assay could be used to monitor the PD of an immunosuppressive therapy.

5

TRANSIENT INTRAOPERATIVE Th1/Th2 SHIFT CORRELATES WITH POSTOPERATIVE EFFUSIONS AND EDEMA AFTER CARDIOPULMONARY BYPASS IN CHILDREN

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Background: Major surgery including surgery with cardiopulmonary bypass (CPB) was reported to be predominated by a Th2 response in adults. In children CPB surgery induced substantial release of the anti-inflammatory cytokine IL-10. However yet no studies are available verifying if Th2 response and IL-10 release are specific to CPB or surgical trauma and if Th2 response is correlated with post-operative complications such as effusions or oedema (POEE).

Methods: Children (age range 3–16 years) undergoing cardiac surgery with CPB ($n = 50$) or without CPB ($n = 20$) were enrolled. EDTA anticoagulated blood was obtained 24 h preoperatively, after anaesthesia, thoracotomy, CPB contact, CPB end, immediately postoperatively, 4, 24, 48 h after surgery, at discharge and at least 2–3 months later. The Th1-Th2 reaction was monitored by the intracellular IL-4, IFN-g production of T-cells determined by flow cytometry and by soluble serum components: IL-4, IL-10, IFN-g, IgE, IgG2. Detailed statistical analysis was performed.

Results and Discussion: Surgery with CPB induced a transient immunosuppression and humoral immune response with an increased proportion of Th2 cells. This is in part due to Th2 cells as a source for IL-10. Proinflammatory activation of monocytes is not substantially involved. These changes begin during surgery and go back to pre-operative baseline around discharge. Conclusion: The shift of the immune system to the humoral Th2 immune response correlates with postoperative morbidity and resembles to immune sequel observed in sepsis (immune paralysis) or an allergic response. Our findings are in agreement with earlier results that patients at risk for POEE exhibit a Th2/allergic predisposition.

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6

ESTABLISHMENT OF HEP-2 CELL PREPARATION FOR AUTOMATED ANALYSIS OF ANA FLUORESCENCE PATTERN

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Identification of anti-nuclear antibodies (ANA) has large clinical importance for the assessment of systemic and organ-specific autoimmune disease. Cells of a human epidermoid carcinoma, HEp-2, are well established as a diagnostic tool for evaluation of ANA-positive patient sera. For this purpose, cells are grown as monolayers on microscope slides and used as antigenic substrates to detect autoantibodies in patients sera. Using immunofluorescence techniques positive sera reveal a nuclear fluorescence pattern that is specific for different ANAs. However, the methods used for cell preparation and immunohistological staining are mostly not known for commercially available products or there is a lack of validating different preparation steps and materials prior to analysing special ANAs.

For an automated imaging and analysis system we now develop appropriate methods for cell cultivation and preparation, thereby establishing the optimal conditions for fixations and immunocytochemical staining of HEp-2 cells.

Phase contrast microscopy and haematoxylin-eosin-staining revealed considerable changes in HEp-2 cell morphology and histological stainability between different chemical fixatives (alcohols, ketones, and aldehydes). Variations in concentrations and incubation times of the used reagents influence the staining outcomes also. These results range from a nearly homogenous staining of the cell nuclei up to a highly structured nucleus. Staining of cell nuclei with DAPI after different fixation procedures showed also a high variability in detecting intranuclear structures.

These results demonstrate that it is absolutely necessary to validate fixatives, fixation procedures and immunohistological techniques to reduce artificial events with regard to a precise diagnosis of autoimmune diseases.

7

ANALYSIS OF HYPOXIA-INDUCIBLE FACTOR-1α (HIF-1α) ACCUMULATION AND CELL CYCLE IN GELDANAMYCIN-TREATED HUMAN CERVICAL CARCINOMA CELLS BY LASER SCANNING CYTOMETRY

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Background: Tumor hypoxia has been linked to increased disease aggressiveness and poorer treatment outcomes, and the transcription factor hypoxia-inducible factor-1 (HIF-1) has been identified as the key molecule mediating the cellular response to hypoxic microenvironments. The α-subunit of this factor is accumulated under hypoxia and rapidly degraded during re-oxygenation rendering the reliable measurement of HIF-1α a difficult task. Heat shock protein 90 (Hsp90) is an essential protein that controls the activity, turnover, and trafficking of a broad variety of other proteins including HIF-1α and cell cycle transition regulators. Inhibitors of Hsp90 like geldanamycin therefore have the potential to target tumor cell survival by at least two mechanisms compromising the accumulation of HIF-1α and cell proliferation.

Methods: We describe here the simultaneous measurement of HIF-1α and cell cycle parameters by Laser Scanning Cytometry (LSC) after exposure of two different human cervical carcinoma cell lines, SiHa and ME180, to hypoxia and geldanamycin. LSC allows the investigation of short-lived processes in adherent cell lines because cells can be grown, fixed, and analyzed directly on slides without preparation a single-cell suspension as for flow cytometry.

Results: Our analysis demonstrates that the cell lines react to hypoxia and drug-treatment in a distinct way with SiHa being more affected by low oxygen concentrations (higher HIF-1α levels, decreased S-phase) than ME180 which was more sensitive to geldanamycin. Both cell lines respond to geldanamycin with a G2/M-phase arrest and a slight decrease in HIF-1α accumulation. Cell death due to the drug treatment occurs in close association with mitosis, presumably through mitotic catastrophe.

Conclusion: Our results indicate that Laser Scanning Cytometry can significantly contribute to the evaluation of in vitro drug effects particularly with respect to tumor hypoxia and the measurement of HIF-1α.

8

COMPLEX PROINFLAMMATORY ACTIVATION OF PHAGOCYTIC CELLS UPON CORONARY ARTERY BYPASS SURGERY

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Cardiopulmonary bypass surgery (CPB) is frequently associated with development of a systemic inflammatory response syndrome (SIRS). In order to minimize the deleterious effects of CPB, several approaches for conventional extracorporeal circulation (ECC) have been developed. The influence of different operative techniques on coronary artery bypass grafting (CABG), however, has not yet been evaluated.

We studied 30 patients underwent elective CABG, either by conventional ECC (ECC, $n = 10$), or miniaturized ECC (MECC, $n = 10$), or beating heart (OPCAB, $n = 10$), to assess the impact of different techniques on the activation of phagocytic cells. EDTA-blood samples were taken before and after the surgical intervention. Expression of surface antigens,

implicated in proinflammatory activation of phagocytes, was detected by flow cytometry.

In accordance to previous studies, expression of CD11b, CD14 and CD45 was down-regulated on monocytes following CABG, but irrespective of the art of intervention. Furthermore, the expression of CD16 after CABG was also decreased, but only on granulocytes. The down-regulation of CD16 was the most prominent in the group underwent CABG with ECC. The decreased expression density of CD16 on granulocytes indicates a relative immaturity of this cell population. In addition, the expression of CD32 on phagocytic cells was also decreased upon CABG, and the strongest down-regulation was observed in the ECC group. By contrast, the CD59 expression was significantly up-regulated by ECC and MECC, without significant change by OPCAB. In parallel to the above mentioned changes in the antigen expressions of phagocytic cells, there was also a significant increase of leukocyte counts in each group.

Taken together, these results demonstrate a complex proinflammatory activation of phagocytic cells upon CABG, that likely involves mobilization of vessel wall-associated resting phagocytes, as well as immature cells from the bone marrow. In addition, the certain techniques (ECC, MECC or OPCAB) are associated with different proinflammatory activation of phagocytic cells, and this may influence the severity of a resulting SIRS. The OPCAB seems to be associated with minor proinflammatory changes, and therefore this technique should be preferred.

9

SEQUENTIAL PHOTOBLEACHING OF FLUOROCHROMES FOR POLYCHROMATIC SLIDE BASED CYTOMETRY

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For immunophenotypic analysis more measurable parameters for the discrimination of leukocyte subsets are necessary. Due to the number of PMTs the amount of simultaneously measurable fluorescences per scan is limited. Nevertheless, the amount of measurable colors can be improved to eight by appropriate change of the filter settings and two scans per specimen. Aim of this study was to use the special features of Slide based Cytometry (SBC) beyond filter change, remeasurement and merging to distinguish fluorochromes with similar emission spectra. The photosensitivity of fluorochromes that are excited and emit in a similar wavelength range may be very different. The number of measurable parameters per PMT was increased using photosensitivity of different fluorochromes as additional criteria. Peripheral blood leukocytes were stained with antibodies conjugated to the fluorochromes APC, APC-Cy5.5 and Alexa-Fluor 633 and mounted on conventional uncoated glass slides with Fluorescence mounting medium. Specimens were excited in the LSC with the HeNe (633 nm) Laser and measured at different filter settings (670/20-nm-filter for APC/ALEXA633 and 710/20-nm-filter for APC-Cy5.5). At this point, APC-Cy5.5 and APC/ALEXA633 were already distinguishable. In order to differentiate between APC and ALEXA633 photobleaching was performed by repeated excitation with the laser at 633 nm. Control measurements proved that APC is much more sensitive against laser excitation, i.e. looses much more fluorescence intensity than ALEXA633. The separate measurements (before/after filter change and before/after bleaching) were merged into one file. The photostability of Alexa-Fluor 633 (1.02% bleach per scan) and APC (5.74% bleach per scan) are substantially different. Therefore, after bleaching and merging both fluorochromes can be distinguished and are regarded by the software as separate parameters. The fluorochromes APC/ALEXA633 and APC-Cy5.5 can be discriminated by changing the emission filters before bleach. By **sequential photobleaching**, change of filters and subsequent merging of the data the number of simultaneously measurable "colors" is substantially increased.

10

CHRONIC INFLAMMATION WITH OR WITHOUT FONTAN PALLIATION IN UNIVENTRICULAR HEART PATIENTS

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Background: Protein-losing enteropathy (PLE) is characterized by massive enteric protein loss in the absence of chronic enteric infections. PLE could be a late complication of patients with Fontan circulation. In the background hemodynamic alterations and immunological participation are presumed. The present study aimed to examine whether there are cellular and humoral signs of inflammation in univentricular heart patients without PLE symptoms.

Methods: Ten patients with univentricular heart were analyzed before and after the Fontan operation. Results were compared to a group of age-matched ASD II control persons ($n = 25$). Cellular parameters (cell count and activation parameters), major serum proteins, complement components, electrolytes as well as soluble serum (s) components: sIL-2R, IL-6, IL-8, IL-10, TNFα, sE-, sL-, sP-selectins, sPSGL, sICAM-1, sPECAM-1, sHistamine, sNeopterin were measured. Detailed statistical analysis was performed.

Results: Many parameters indicating increased inflammation were found which differ to control group both before as well as after surgery. Moreover, parameters were determined differing to healthy control persons only before surgery or only after surgery. Our data suggest a relation between some laboratory parameters and vena cava superior or inferior blood pressure.

Conclusions: The results show that these changes in the immune system do not only appear because of the new circu-

lation. Some of them appear earlier and are in part strengthened after or even by the operation.

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CYTOKINES IN SERUM AND PLEURA EFFUSION OF PATIENTS AFTER TCPC COMPLETION AND OTHER CARDIAC OPERATIONS

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Introduction: After the Fontan surgery (TCPC) pleura effusions often arise. The pathogenesis is not fully understood yet. Aim of this study was to compare cytokine levels of serum and the effusion fluid to gain information about the etiology of this phenomenon which is still a therapeutical problem.

Material and Method: Levels of Protein, Erythrocytes, IL-6, IL-8, IL-10, sE-, sL-Selectin and also complement (C3, C3d) were analyzed quantitatively in serum and pleura effusions of 21 patients after TCPC operation (aged: 2.5–5.3 years) according to a definite timetable.

The same parameters we analyzed in eight patients with other diagnosis as a compare group.

Results: Levels of IL-6 and IL-8 (proinflammatory) in the pleura effusion were significantly higher ($P < 0.05$) than in the serum, whereas the course showed affinities.

Levels of IL-10 (antiinflammatory) in the pleura effusion is significantly higher than in the serum at postoperative day 1. In the following time the levels are almost similar in serum and pleura effusion.

Conclusions: The results show a strengthened cytokine reaction in the thorax region after the Fontan type surgery. Analyzed cytokines represent pro- as well as antiinflammatory reactions. This could be the basis for further research aiming to influence the exudation therapeutically. Furthermore, due to the higher concentrations in the effusion, this kind of examination could be more reliable to screen immunological reactions after the Fontan surgery.