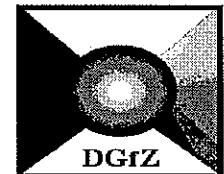




## *Abstracts*

12<sup>th</sup>



# Heidelberg Cytometry Symposium

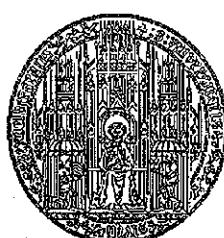
**Annual Conference of the  
German Society of Cytometry e.V.**

**Heidelberg, 21<sup>st</sup> - 23<sup>rd</sup> October 1999**

### *Location*

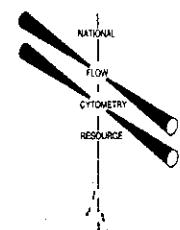
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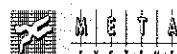
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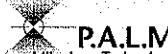
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**Thursday, 21. October 1999**

**14.00 Welcome Address**

**14.10 - 16.30 SESSION I: Antigen Specific Cytometry  
CHAIRS BAUMGARTH - RADBRUCH**

- |                      |   |
|----------------------|---|
| <b>14.10 - 14.30</b> | <b>1</b> SCHEFFOLD A, THIEL A, RADBRUCH A.<br>Why did it take so long? The cytometric identification of antigen specific T and B lymphocytes  |
| <b>14.30 - 14.50</b> | <b>2</b> HUNZELMANN N, LEYENDECKERS H, SCHMITZ J.<br>Cytometry of allergen specific memory B lymphocytes in allergic diseases   |
| <b>14.50 - 15.10</b> | <b>3</b> BUSCH DH.<br>Direct ex-vivo analysis of epitope specific T cell populations using MHC tetramers  |
| <b>15.10 - 15.30</b> | <b>4</b> THIEL A, WU P, NITSCH S, HIEPE F, SIEPER J, RADBRUCH A.<br>Identification of antigen specific T lymphocytes by cytokine-provocation and intracellular cytokine staining          |
| <b>15.30 - 15.50</b> | <b>5</b> BROSTERHUS H, LEYENDECKERS H, BRINGS S, MILTENYI S, RADBRUCH A, ASSENMACHER M, SCHMITZ J.<br>Analysis and isolation of live antigen specific T cells based on cytokine secretion |
| <b>15.50 - 16.10</b> | <b>6</b> MUZZULINI T, KUNKEL D, RADBRUCH A, SCHEFFOLD A.<br>A simple method for ex-vivo identification and sorting of viable antigen reactive T cells                                     |
| <b>16.10 - 16.30</b> | <b>7</b> FRIEDRICH SO, ROTHE G, MEIERHOFF G, SCHMITZ G.<br>Optimization of a whole blood overnight assay for the detection of antigen reactive T cells                                    |

**16.30 - 16.45 coffee break**

**16.45 - 18.30**    **SESSION II: Novel Cytometric Techniques**  
CHAIRS: HAUSMANN - ROTHE

- 16.45 - 17.15** 1 BAUMGARTH N, PARKS DR, BIGOS M, STOVEL RT, ROEDERER M, NOZAKI T, HERZENBERG LA.  
Eleven-color, thirteen parameter flow cytometry for dissecting the immune system.
- 17.15 - 17.30** 2 CRISSMAN HA, VALDEZ JG, STEINKAMP JA.  
Flow cytometric fluorescence lifetime analysis of DNA-binding probes
- 17.30 - 17.45** 3 GERSTNER A, BOOTZ F, TÄRNOK A.  
A new method for immunophenotyping of peripheral blood by laser scanning cytometry (LSC)
- 17.45 - 18.00** 4 HOPPE K, HAASE M, EYCHMULLER A, WELLER H.  
Lumineszierende Nanokristalle als nichtradioaktive Markierungssubstanzen
- 18.00 - 18.15** 5 RAPP A, DITTMAR H, MONAJEMBASHI S, HAUSMANN M, GREULICH KO.  
Development of a two colour hybridization protocol for radiation sensitivity mapping using COMET-FISH
- 18.15 - 18.30** 6 RAUCH J, HAUSMANN M, BORNFLETH H, SOLOVEI I, HORSTMCKE B, CREMER T, KNÜCHEL R, CREMER C.  
Spectral precision distance microscopy for the study of the 3D-topology of selected point-like markers

**18.30 - 19.00**    *coffee break and poster mounting*

**19.00 - 20.00**    **SESSION III: Poster Exhibition**  
CHAIR: HUTTER

**20.00 - End**    *RECEPTION AT THE GERMAN CANCER RESEARCH CENTRE*

**Friday, 22. October 1999**

**08.30 - 11.30**    **SESSION IV: Analyses of Cellular DNA**  
CHAIRS: KNUCHEL-CLARKE - OTTO

- 08.30 - 08.45** 1 MEHES G, LÖRCH T, AMBROS PF.  
Quantitative analysis of rare tumour cells by automated microscopy
- 08.45 - 09.00** 2 AMBROS PF, MEHES G, HATTINGER C, LUEGMAYR A, WITT A, PLESCH A, LÖRCH T.  
Fully automated detection and genetic characterization of rare tumor cells
- 09.00 - 09.15** 3 HEMMER J.  
High resolution DNA flow cytometry in the management of head and neck cancer.
- 09.15 - 09.30** 4 BEYER M, BLUM S, KUNZE KD, MEYER W, KAYSER K.  
Telemetrische DNA Messungen (Statische DNA Zytometrie) maligner Pleuraergüsse
- 09.30 - 09.45** 5 BAUMGARTNER A, SCHMID TE, ADLER LD, NÜSSE M.  
Evaluation of the frequency of aneuploid sperm of mice and humans by laser scanning cytometry (LSC)
- 09.45 - 10.00** 6 NOWAK R, OELSCHLÄGEL U, HERBST R, NIKLISCH M, HÄNEL A, TEICH M, EHNINGER G.  
The detection of aneuploid malignant hematopoietic cells with flow cytometry after therapy
- 10.00 - 10.15** 7 MEHES G, HATTINGER CM, LÖRCH T, ADNER H, AMBROS PF.  
Proliferative potential of tumour cells disseminated in the hematopoietic system
- 10.15- 10.30**    *coffee break*
- 10.30 - 10.45** 8 SKIERSKI JS, KORONKIEWICZ M, GRIEB P.  
The effect of FMdC on the cell cycle of three leukemia cell lines *in-vitro*
- 10.45 - 11.00** 9 BÖCKER W, PILS S, WERNER F, STREFFER C.  
Quantification of DNA telomere sequences in single chromosomes using fluorescence image cytometry

- 11.00 - 11.15** 10. HAUSMANN M, WINKLER R, DÜRM M, ESA A, CREMER C.  
Fluoreszenzmarkierung des ABL-Gens mit Computer  
selektierten Oligo-DNA-Sonden

- 11.15 - 11.30** 11. DOBRUCKI J, SLUPCZYN SKA A, KRZESZOWIEC W,  
DARZYNKIEWICZ Z.  
Imaging of *in situ* DNA sensitivity to denaturation by confocal  
microscopy

**11.30 - 12.30** *lunch*

**12.30 - 14.30** *ANNUAL GENERAL MEETING OF THE DGfZ MEMBERS*

**14.30 - 14.45** *KLAUS GOERTTLER AWARD: TALK OF THE PRIZE WINNER*

**14.45- 15.00** *coffee break*

**15.00 - 17.30** **SESSION V:** *Cytometric Investigations of Receptor  
Complexes*  
**CHAIRS:** CRISSMAN - NUESSE

- 15.00 - 15.15** 1. BECKE FM, SCHWARZ H, HOFSTÄDTER F, BROCKHOFF G.  
CD137 (ILA/4-1BB) reduces phagocytosis of human  
monocytes

- 15.15 - 15.30** 2. LEHLE K, KUNZ-SCHUGHART LA, PREUNER J, BIRNBAUM D.  
Differences in tumor cytotoxicity of human monocytes:  
cultivation and isolation of endothelial cells from large vessel  
preparations

- 15.30 - 15.45** 3. KONUR A, KREUTZ M, ANDREESEN R, BROCKHOFF G.  
Differences in tumor cytotoxicity of human monocytes  
depends on the type of tumor target: analysis in a  
threedimensional spheroid model

- 15.45 - 16.00** 4. TÁRNOK A, GERSTNER A, ADAMS V, RACZ P, RACZ K,  
SCHNEIDER P.  
Quantification of apoptosis and leukocyte subsets in tissue sections  
by laser scanning cytometry (LSC)

- 16.00 - 16.15** 5. GOTZ A, KAPINSKY M, ORSÓ E, ROTHE G, SCHMITZ G.  
The cholesterol and sphingomyelin content of the plasma  
membrane as determinants of CD14 dependent signal  
transduction

**16.15 - 16.30** *coffee break*

- 16.30 - 16.45** 6. KAPINSKY M, TORZEWSKI M, SCHINDLER G, ROTHE G,  
SCHMITZ G.  
Enzymatically modified LDL in contrast to oxidized or  
acetylated LDL induces foam cell formation independent from  
scavenger receptor expression

- 16.45 - 17.00** 7. SÄNGER N, STROHMEIER R, KAUFMANN M, KUHL H.  
Periphere Benzodiazepinrezeptoren in Relation zu  
Zellzyklusphase und Mitochondriengehalt bei Mamma-  
Carcinom-Zellkulturen

- 17.00 - 17.15** 8. LEVINA VV, VARFOLOMEEVA EY, SUKHAREVA EB,  
DROBCHENKO EA, FILATOV MV.  
Flow cytometry study of DNA clearing from noncovalently  
bound agents which is a new mechanism of drug resistance  
in mammalian cells.

- 17.15 - 17.30** 9. LÖHRKE B, VIERGUTZ T, GOLDAMMER T, KRÜGER N.  
Association between development-dependent ectopic  
expression of the transcription factor peroxisome proliferator-  
activated receptor subtypes and apoptosis in lutein cells

**17.30 - 18.30** *small dinner*

**18.30 - 21.00 Session VI: Industrial Talks**  
CHAIRS: BEISKER - GREULICH

- 18.30 - 18.45** 1. MHC-Tetramere:  
Nachweis von antigen-spezifischen CD8+ T Zellen mit Hilfe  
der Flußzytometrie  
*Beckman-Coulter*
- 18.45 - 19.00** 2. BD LSR, the 6-Color benchtop research flow cytometer  
from BD Biosciences  
*Becton Dickinson*
- 19.00 - 19.15** 3. Biomagnetic separation: Methods and applications  
*Deutsche Dynal*
- 19.15 - 19.30** 4. Kooperatives Arbeiten in der Mikroskopie über Netze  
(HISTKOM)  
*Deutsche Telekom*
- 19.30 - 19.45** 5. Continuous Gating® von medac, eine Expertensoftware  
*medac Diagnostica*
- 19.45 - 20.00** 6. Recent developments in rare cell scanning and multi  
fluorochrome imaging  
*MetaSystems*
- 20.00 - 20.15** 7. autoMACS  
For ultra high speed automated magnetic cell separation  
*Miltenyi Biotech*
- 20.15 - 20.30** 8. PAS, ein kompaktes Flowzytometer mit Färbeautomat für die  
Routine-Immunologie.  
*Partec*
- 20.30 - 20.45** 9. Das Till-Photonics Standard Imaging System  
*Till-Photonics*
- 20.45 - 21.00** 10. Multiple fluorescence microscopy and optoelectronic imaging:  
Possibilities and limits  
*Zeiss Oberkochen*

**Saturday, 23. October 1999**

**09.00 - 10.15 Session VII : Cellular Function, Differentiation and Disease**

**Part 1:**  
Chairs: ASSENMACHER - VALET

- 09.00 - 09.15** 1. BEDNER E, LI X, DARZYNKIEWICZ Z.  
Assays of cell functions by laser scanning cytometry
- 09.15 - 09.30** 2. TORZEWSKI M, KAPINSKY M, ROTHE G, SCHMITZ G.  
C-reactive protein (CRP) in atherogenesis
- 09.30 - 09.45** 3. PIPEK M, HAMBSCH J, SCHNEIDER P, TÁRNOK A.  
Increased apoptosis of circulating lymphocytes during cardiac  
surgery with cardiopulmonary bypass
- 09.45 - 10.00** 4. KRIEG R, MESSMANN H, HOFSTAEDTER F, KNUECHEL R.  
Effects of 5-aminolevulinic acid (ALA) induced intracellular  
protoporphyrin IX (PPIX) - content and - localisation on  
photodynamic therapy (PDT)
- 10.00 - 10.15** 5. BERNAS T, DOBRUCKI J.  
The role of cellular plasma membranes in reduction of tetrazolium  
salts, MTT and CTC
- 10.15 - 10.30** 6. BARLAGE ST, WIMMER A, ROTHE G, SCHMITZ G.  
Effects of the GPIIB/IIIA receptor antagonist MK-383 on  
receptor conformation and function

**10.30 - 11.00 coffee break and small breakfast**

**11.00 - 12.30 Session VII: Cellular Function, Differentiation and Disease**

**Part 2 :**

**Chairs:** TARNOK - SZÖLLÖSI

- 11.00 - 11.15** 8. SCHNEIDER EM, DOBRUCKI J, STAHL-HAEGE B, GEORGIEFF M.  
Subcellular CD95-ligand expression in microvascular endothelial cells, Ewing's tumors and long term cultured T cells.
- 11.15 - 11.30** 9. SEIDL J, KRIEG R, KNUECHEL R.  
Cellular effects of 5-aminolevulinic acid (ALA) - induced protoporphyrin XI (PPIX) - mediated photodynamic therapy (PDT) on human urothelial cell lines
- 11.30 - 11.45** 10. STARK R, ROTHE G, SCHÄFER B, SCHMITZ G.  
Flow cytometric characterisation of platelet membrane microviscosity as a determinant of abnormal platelet activation in hypercholesterolemia
- 11.45 - 12.00** 11. BÖHM I, REICHARDT A, SCHNAUTZ S, BAUER R.  
Flow cytometric detection of CD3<sup>low</sup> cells in the peripheral blood of patients with cutaneous T-cell lymphoma
- 12.00 - 12.15** 12. SZÖLLÖSI J, NAGY P.  
Mapping of cell surface distribution of ErbB proteins in breast cancer cells.
- 12.15 - 12.30** 13. VALET G, KAHLE H, TARNOK A, VAN DRIEL B, VAN NOORDEN.  
Predictive medicine by pattern analysis (CLASSIF1) of cytometric and other data
- 12.30 - 12.45** **coffee break**

**12.45 - 13.45 Sitzung VIII: Microorganisms**

**CHAIRS:** BLEY - MÜLLER

- 12.45 - 13.00** 1. BEISKER W, BRUCKMEIER B, SCHADE C, SCHÄFER H, NUESSE M.  
The characterization of phytoplankton populations using flow cytometry and laser scanning cytometry
- 13.00 - 13.15** 2. FRERICHS JG, JOERIS K, SCHAPER J, SCHEPER TH.  
*In-situ* Mikroskopie bei der Kultivierung von Mikroorganismen
- 13.15 - 13.30** 3. GROSSE-UHLMANN R, MÜLLER S, BLEY TH.  
Fluß Zytometrie als Methode zur Prozeßidentifikation am Beispiel der Synthese von Poly-β-Hydroxy-Buttersäure (PHB) durch *Methylobacterium rhodesianum* MB126
- 13.30 - 13.45** 4. ULLRICH S, LÖSCHE A, MÜLLER S.  
Durchflußzytometrische Bestimmung der Fluidität zur Charakterisierung der Strukturen bakterieller Membranen

End

**POSTER EXHIBITION**

1. ALBRECHT J, DABETIC-POPESCU C, ECKART M.  
Evaluation der Quantifizierung der CD38-Expression (Antibody Binding Capacity) auf T-Suppressorzellen unter den besonderen Bedingungen des Einsendelabors
2. BÖHM I, PEGELOW K, VON RÜCKER A, KISTLER V, BAUER R.  
Immunological differences between idiopathic CD4+ T lymphocytopenia (ICL) and HIV-infection
3. FILIPIAK K, JEDRZEJCZAK P, KOTWICKA M, PAWELECZYK L, SKIERSKI JS, WARCHOL JB.  
DNA content in human spermatozoa and *in vitro* fertilisation effects
4. GREVE B, SEVERIN E, HACKER-KLOM U, GÖHDE W.  
Mikronuklei und Zellzyklusverzögerung als Parameter zur Bestimmung der individuellen Strahlenempfindlichkeit
5. KLEINE TO.  
Indication for an altered transfer of lymphocyte subsets from blood into cerebrospinal fluid (CSF) in aging humans

6. KLEINE TO.  
Different blood/cerebrospinal fluid (CSF) ratios of human lymphocyte subsets
7. KORCZAK-KOWALSKA G, WIERZBICKI P, MICHALSKA K, DOBRZYSKA E, CHMURA A, KOSOWSKA D, PODOBISKA I, ROWISKI W, GÓRSKI A.  
The flow cytometric crossmatch in renal transplantation
8. KOTWICKA M, FILIPIAK K, WARCHOL JP.  
Study on association between DNA content and sperm morphology of males with unexplained infertility.
9. MEYER ZU HÖRSTE G, HOFFMANN A, MONAJEMBASHI SH, UHL V, PILARCZYK G, GREULICH K-O.  
Optical tools in laser scanning microscopy of cultured mammalian cells
10. MÜLLER S, ULLRICH S, LÖSCHE A, BABEL W.  
Flow cytometric techniques to characterize physiological states of *Acinetobacter Calcoaceticus*
11. NEUMÜLLER S, DUNKY A, BURTSCHER H, JILCH R, MENZEL JE.  
Interaktionen zwischen Monozyten aus dem peripheren Blut von Patienten mit Arthritis Psoriatica und kultivierten humanen dermalen mikrovaskulären Endothelzellen.
12. NUDING S, MÜLLER HAG, BODE CH.  
Flußzytometrie in der mikrobiologischen Diagnostik
13. PILARCZYK G, SCHMITT E, GREULICH KO.  
Cardiac calcium homeostasis ends up in oscillations:  
micro- cytometric measurements and numeric simulations
14. POZAROWSKI P, ROLINSKI J, SURDACKA A, KRAWCZYK P.  
Examination of inner mitochondrial transmembrane potential ((DYm)) using Chlormethyl-X-Rosamine dye
15. RIESEBERG M, SCHEPER T.  
On-Line Monitoring an Bioprozessen mittels fließinjektionscytometrischer Messungen
16. SCHMID TE, BAUMGARTNER A, NÜSSE M, ADLER ID.  
The effect of chemicals on the duration of male meiosis in mice detected with laser scanning cytometry (LSC)
17. SCHÜLER C, HOFER A, WITT M, KASPER M, WOLF C, SCHULZE E, FUNK RHW.  
Flow cytometry for assessing biocompatibility of type 1 collagen-coated titanium alloy

## Antigen Specific Cytometry

## ANALYSIS AND ISOLATION OF LIVE ANTIGEN-SPECIFIC T-CELLS BASED ON CYTOKINE SECRETION.

H. BROSTERHUS, H. LEYENDECKERS, S. BRINGS, S. MILTENYI, A. RADBRUCH, M. ASSENMACHER AND J. SCHMITZ

Miltenyi Biotec GmbH, Bergisch Gladbach, Germany Deutsches RheumaForschungszentrum, Berlin, Germany

Antigen-specific T-cells with specific functions might be powerful tools for immunotherapy and their monitoring can provide important information about immune responses. Here we have used a new, universal approach to detect and isolate live antigen-specific memory/effector T-cells following a short specific restimulation with peptides or proteins *in vitro* to induce cytokine secretion. For the cytokine secretion assay bispecific antibodies against a cell surface antigen and a cytokine are used to create an affinity matrix for a cytokine on the cell surface. Cells are allowed to secrete the cytokine which is relocated to the surface of the secreting cells and there is labeled with a second cytokine-specific antibody for enrichment and flow cytometric analysis. Using peptides or proteins of Influenza virus (FLU), Cytomegalovirus (CMV) or Tetanus toxoid (TT) for stimulation we have detected and isolated IL-4 secreting as well as IFN- $\gamma$  secreting Antigen-specific CD4+ as well as CD8+ T-cells from normal PBMC. For the occasionally encountered, non-persisting Influenza virus 1 in 1.000 to 600.000 PBMC can be isolated as IFN- $\gamma$ + FLU-specific CD8+ T-cells. Most of them are memory-type cells expressing CD27 and CD28, but not CD57. Following isolation and expansion with IL-2, recovered cells show FLU-specific cytotoxicity. The frequencies of antigen-specific T cells were lower for the infrequently encountered and only moderately immunogenic antigen TT (1 in 10.000 to 1.000.000), but much higher for the persisting virus CMV (1 in 100 to 10.000 PBMC of seropositive donors). This technique could become an invaluable tool in the analysis and isolation of T-cells specific for various pathogens, according to their functional potential.

## DIRECT EX-VIVO ANALYSIS OF EPITOPE-SPECIFIC T CELL POPULATIONS USING MHC TETRAMERS

DIRK H. BUSCH

Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, Germany

*In-vivo* investigations of T cell responses have been limited by the difficulty of identifying antigen-specific T cells among a plethora of non-specific cells. This difficulty is largely due to the low affinity of interactions between the T cell receptor (TCR) and its natural ligand, the MHC/peptide complex. Multimerization of MHC/peptide complexes can overcome these technical problems by increasing the overall affinity of the TCR-MHC interaction to an extent that such complexes can be used as reagents for epitope-specific detection of T cells.

Multimeric MHC class I/peptide complexes are usually generated by the expression of recombinant  $\alpha_2$ -microglobulin ( $\alpha_2$ m) and heavy chain (HC) molecules in bacteria. The HC is mutated to remove the transmembrane region and to add a specific biotinylation sequence at the C-terminus. Purified proteins can be refolded *in vitro* in the presence of high concentrations of peptide/epitope to form stable and soluble MHC/peptide complexes. After enzymatic biotinylation, these complexes are multimerized with streptavidin (SA), which will bind four biotin molecules. Use of fluorescence-conjugated SA allows the visualization of stained cells by flow cytometry.

The generation of soluble MHC class II-peptide complexes is not as well established, perhaps due to the more complex structure of the class II peptide binding groove. *In vivo* expression and refolding in insect cells as well as the use of covalently linked peptides/epitopes are promising approaches to overcome these technical problems.

Tetramer staining is highly epitope-specific, and even very small populations can be identified directly *ex vivo* with this technique. In addition to the precise frequency analysis, these reagents allow detailed phenotypical and functional characterization of epitope-specific T cell populations at the single cell level, e.g. the expression of surface markers, determination of cytokine profiles, and TCR repertoire analyses. The binding kinetics of tetrameric MHC/peptide complexes appear to be a useful tool to measure relative affinities of epitope-specific T cells for their ligand. In addition to the basic insights into T cell-mediated immune responses that have been made possible with tetramers, this technology may prove invaluable for adoptive immune transfer, the elimination of autoreactive T cells in autoimmune diseases, and other clinical applications.

## OPTIMIZATION OF A WHOLE BLOOD OVERNIGHT ASSAY FOR THE DETECTION OF ANTIGEN REACTIVE T CELLS

SVEN O. FRIEDRICH, GREGOR ROTHE, GUIDO MEIERHOFF, GERD SCHMITZ

Institute for Clinical Chemistry and Laboratory Medicine,  
University of Regensburg, Franz-Josef-Strauß-Allee 11,  
D-93053 Regensburg, Germany

The expansion of autoantigen reactive CD4 cells is an early process in the development of diseases such as insulin-dependent diabetes mellitus. For the development of a simple diagnostic test system for the quantification and functional characterization of antigen-specific T cells the tetanus toxoid response was selected as a model system. This weak stimulus was difficult to detect based on published whole blood cytokine procedures for CMV reactive cells (J Immunol Methods 1998; 212 (1):89-98).

Several parameters were tested with regard to discrimination of the antigen reactive T cells. Whole blood was stimulated with 5 µg/ml tetanus toxoid for 24h. The comparison of three anticoagulants showed that heparinized whole blood gave the best results based on the analysis of the activation antigens CD25 and CD69 as well as the cytokines interleukin-2 and interferon-γ. A combination of secretion blockers, furthermore, was superior when compared to brefeldin A probably based on a reduced toxicity. The 1:3 dilution of whole blood with RPMI1640 was optimal for increased CD25 expression. The simultaneous detection of activation antigens by cell surface and intracellular staining improved the detection of antigen reactive cells. Harvesting the diluted blood with 2mM EDTA and incubation for 15 minutes on ice did not improve the recovery of activated cells. These optimization steps altogether (compared to the published methods) increased the detectable reactive cells from about 2% to 4% according to the CD25 antigen, 2,5% to 5% according to the CD69 antigen, whereas the number of interleukin-2 and interferon-γ positive cells did not increase noticeably. The expression of CD25 and CD69 was specific for CD4 T cells excluding non-specific bystander activation.

The four-color flow cytometry method enables the reproducible detection of tetanus toxoid reactive cells also using 20-meric peptides as agonists and is currently being transferred to an autoantigen model.

## CYTOMETRY OF ALLERGEN-APECIFIC MEMORY B LYMPHOCYTES IN ALLERGIC DISEASES

<sup>1</sup> NICOLAS HUNZELMANN, <sup>2</sup> HEIKE LEYENDECKERS, <sup>2</sup> JÜRGEN SCHMITZ

<sup>1</sup> Dept. of Dermatology, University of Cologne;  
<sup>2</sup> Miltenyi Biotec, Bergisch Gladbach, Germany

The generation of memory B cells is believed to be critical for long term persistence of humoral immunity and allergy. The direct analysis of allergen specific memory B cells has been hampered by the rarity of these cells in the peripheral circulation and it is not clear whether the frequency of allergen-specific memory B cells in the circulation correlates with the amount of allergen-specific antibody in the serum. Insect venom allergy is a well characterized disease which is often used as a model system to study immune regulation in allergic diseases. We have used the main allergen of wasp venom allergic patients i.e. phospholipase A1B (PLA1B) in a new assay combining two step immunomagnetic enrichment with multiparameter flow cytometry to detect, enumerate and characterize allergen-specific memory B cells. In a first magnetic separation B cells are isolated from PBMC using releasable microbeads conjugated to CD19 antibody. Allergen-specific cells are then positively selected using microbeads directly conjugated with PLA1B. To proof the specificity of the enrichment, PLA1B specific B cells were cultured and the supernatant analyzed for phospholipase A1B specific IgG. Our results show that there is no statistically significant linear correlation between the frequencies of circulating allergen specific IgG bearing memory B cells and the serum titers of allergen specific IgG in wasp venom allergic patients undergoing allergen specific immunotherapy. This lack of a correlation favours a model in which memory B cells and plasma cells represent independently controlled forms of immunologic memory.

## A SIMPLE METHOD FOR EX VIVO IDENTIFICATION AND SORTING OF VIABLE ANTIGEN-REACTIVE T-CELLS

TILL MUZZULINI, DÉSIRÉE KUNKEL, ANDREAS RADBRUCH AND ALEXANDER SCHEFFOLD

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The flow-cytometric analysis of antigen-specific T cells has always been hampered by their low frequency and the low affinity of the T cell receptor to its ligand the MHC/peptide complex. We have established a simple assay to directly analyse antigen-reactive cells ex vivo by measuring the specific proliferative activity on the single cell level following antigen-restimulation in vitro. All cells are uniformly labelled with the stable fluorescent marker carboxyfluoresceine-diacetate-succinimidylester (CFDA-SE) and cultured with antigen for 72 hours. Proliferating T cells can then easily be identified by flow-cytometry according to their loss of fluorescence intensity which is halved with each single division step. In a model system of OVA-TCR transgenic T cells mixed with normal Balb/c spleen cells, we could show that it is possible to detect specific cells at frequencies as low as 0.1-0.01% and that all specific cells react in this assay. Importantly the specific cells can be separated alive and they can directly be used for further functional studies. This method can be used for the fast isolation, expansion and functional analysis of antigen-specific T cells reacting to specific peptides or proteins or even less defined antigenic mixtures.

## WHY DID TAKE SO LONG? THE CYTOMETRIC IDENTIFICATION OF ANTIGEN-SPECIFIC T AND B LYMPHOCYTES

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In the past 20 years, immunofluorescence-based cytometry and cell sorting have found their place in the repertoire of state-of-the-art technologies, mostly serving to identify subsets of lymphocytes and systemic changes of the immune system. Although certainly of value for diagnosis and analysis of immunopathology, cytometry then still had one major limitation: except for a few experimental situations it was not possible to focus analysis on those lymphocytes specifically recognizing a particular antigen. This drawback has been overcome recently both for B and T lymphocytes, using antigen to identify the cells. Today, a number of exciting new technologies offer options to analyse and isolate specifically those lymphocytes that are directly involved in the immune reaction to given antigens. Why did this take so long? The problem is two-fold. First, the diversity of the immune system implies that lymphocytes recognizing a particular antigen are rare. For a number of biological and physical reasons, immunofluorescence either with antigens or antibodies does show a considerable variation of intensity. This makes it technically difficult to identify unambiguously rare cells of interest at frequencies below  $10^{-3}$  to  $10^{-4}$ . Nevertheless, experimental work has shown that it is possible to identify and analyse cytometrically rare B memory lymphocytes and plasma cells using native antigens conjugated to haptens or fluorochromes. A major challenge for antigen-specific T cell cytometry has been that the antigen receptors of T lymphocytes recognize fragments of antigen only in the context of either MHC class I or MHC class II molecules. Recently, not only the direct labeling of T cells with MHC-peptide complexes has finally been established, but also alternative technologies which identify T cells reacting to particular antigens by DNA synthesis, proliferation or cytokine expression. In combination or alone, those technologies now offer unique options to analyse antigen-specific T lymphocytes directly ex vivo and to isolate them for molecular and functional studies. Most promising appears to be a combination of the tetramer- and cytokine-secretion technologies, allowing simultaneous identification of antigen-specific and antigen-reactive cells. To that end, both technologies will have to be advanced considerably, but even now they can help answer questions that we have wanted answers to for a long time.

## **IDENTIFICATION OF ANTIGEN-SPECIFIC T LYMPHOCYTES BY CYTOKINE-PROVOCATION AND INTRACELLULAR CYTOKINE STAINING**

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T cells play a central role in initiating and triggering autoreactive immune responses. We have analysed here directly antigen-specific T lymphocytes specific for various autoantigens associated with systemic lupus erythematosus (SLE) and for autoantigens and bacterial antigens associated with reactive arthritis (ReA). Direct cytometric analysis was performed on fixed cells after 6h in vitro stimulation of whole blood or synovial fluid cells. The short activation period and the use of CD69 as a fast activation marker efficiently reduce background and exclude bystander cells from analysis. By applying this technology, we could determine specificity and frequency of pathogenic Th cells among peripheral blood cells and synovial fluid cells in ReA during acute chlamydial infection. Up to 1 in 200 CD4<sup>+</sup> T cells among synovial fluid cells and up to 1 in 1000 peripheral blood CD4<sup>+</sup> T cells reacted with IFNg production after brief (6-8h) ex vivo stimulation with recombinant bacterial antigens. We also analysed peripheral blood Th cells reactive for the SLE-associated autoantigens Ro, La, RSmD and nucleosomes. CD4<sup>+</sup> T cells reacting with IFNg and TNFa production could be detected more frequently in untreated patients compared to patients treated with highly immunosuppressive drugs (e.g. cyclophosphamide). SLE-autoantigen reactive Th cells could also be detected among peripheral blood cells from healthy individuals, neither differing in frequency (1 in 2000 up to 1 in 500 specific Th cells) nor in the pattern of secreted cytokines from reactive Th cells from SLE-patients. Antigen specific Th cells reacting with the secretion of IFNg were also isolated viable by the recently described affinity matrix technology for further functional analysis. The direct flow cytometric analysis of antigen-specific T cells after brief ex vivo stimulation allows an easy identification of immunodominant epitopes and to determine reactive T cell frequencies. Moreover, the affinity matrix technology permits the isolation of viable reactive T cells according to secreted cytokines and makes these cells accessible to direct functional studies.

Antigen-specific T cells are important in the initiation and triggering of autoreactive immune responses. We have analysed here directly antigen-specific T lymphocytes specific for various autoantigens associated with systemic lupus erythematosus (SLE) and for autoantigens and bacterial antigens associated with reactive arthritis (ReA). Direct cytometric analysis was performed on fixed cells after 6h in vitro stimulation of whole blood or synovial fluid cells. The short activation period and the use of CD69 as a fast activation marker efficiently reduce background and exclude bystander cells from analysis. By applying this technology, we could determine specificity and frequency of pathogenic Th cells among peripheral blood cells and synovial fluid cells in ReA during acute chlamydial infection. Up to 1 in 200 CD4<sup>+</sup> T cells among synovial fluid cells and up to 1 in 1000 peripheral blood CD4<sup>+</sup> T cells reacted with IFNg production after brief (6-8h) ex vivo stimulation with recombinant bacterial antigens. We also analysed peripheral blood Th cells reactive for the SLE-associated autoantigens Ro, La, RSmD and nucleosomes. CD4<sup>+</sup> T cells reacting with IFNg and TNFa production could be detected more frequently in untreated patients compared to patients treated with highly immunosuppressive drugs (e.g. cyclophosphamide). SLE-autoantigen reactive Th cells could also be detected among peripheral blood cells from healthy individuals, neither differing in frequency (1 in 2000 up to 1 in 500 specific Th cells) nor in the pattern of secreted cytokines from reactive Th cells from SLE-patients. Antigen specific Th cells reacting with the secretion of IFNg were also isolated viable by the recently described affinity matrix technology for further functional analysis. The direct flow cytometric analysis of antigen-specific T cells after brief ex vivo stimulation allows an easy identification of immunodominant epitopes and to determine reactive T cell frequencies. Moreover, the affinity matrix technology permits the isolation of viable reactive T cells according to secreted cytokines and makes these cells accessible to direct functional studies.

## **Novel Cytometric Techniques**

## ELEVEN-COLOR, THIRTEEN PARAMETER FLOW CYTOMETRY FOR DISSECTING THE IMMUNE SYSTEM.

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Over the last decade we developed novel instrumentation, software, and fluorescent dyes to extend the capabilities of multi-parameter flow cytometry. Currently, our three-laser instrumentation set up allows us to measure simultaneously up to thirteen parameters (eleven colors and two scatter measurements). The lasers and dyes used in this system are: fluorescein, phycoerythrin (PE), Cy5PE, Cy7PE, and Cy5.5PE, excited at 488 nm by an argon laser; Texas Red (TR), allophycocyanin (APC), Cy7APC and Cy5.5APC excited at 595 nm by a pumped dye laser; and cascade blue and cascade yellow excited at 407 nm by a violet-enhanced krypton laser. Considerable spectral overlaps exist between the various fluorescent dyes, which need to be corrected by intra- and inter-laser compensation. Our system uses analog compensation to partially correct significant same laser spectral overlaps, followed by post-hoc software compensation. This strategy results in cleaner measurements than the use software compensation alone. The design of multi-color staining combinations, for example those used to identify various cell subsets, requires not only a good knowledge on the expression levels of the markers under study, but also on the spectral characteristics of the dyes used to detect them. Once a particular staining combination is established, however, the assessment of expression of up to eleven markers on one sample is relatively simple and allows for the most accurate quantitation of subset representation and sample heterogeneity, and the potential identification of novel cell subsets. With the development of "tetramer" reagents that mark antigen-specific T cells immediately *ex vivo*, "green fluorescent proteins" with differing excitation and emission spectra, and the development of intra-cytoplasmic cytokine staining, multicolor flow cytometry has become increasingly powerful, since it can now also be used for a large number of functional measurements on individual cells within heterogeneous cell populations.

## FLOW CYTOMETRIC FLUORESCENCE LIFETIME ANALYSIS OF DNA-BINDING PROBES

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Recent developments in FCM that provide for the measurement of fluorescence lifetime have added a new dimension to multiparameter flow cytometric analysis. Many unbound-fluorochromes have a characteristic lifetime value that is somewhat related to molecular structure; however, the lifetime is influenced by a number of factors that affect the probe interaction with a target molecule. Alterations in lifetime often signal variations in fluorochrome-target interactions, brought about by structural modifications in the target molecule that modify the mode of fluorochrome binding. Monitoring the changes in the lifetime of the probe yields information relating to the molecular conformation, or the functional state or activity of the biological target. In this presentation, we demonstrate various applications of lifetime measurements for the analysis of the binding of different fluorochromes to DNA in single cells. Data presented show the application of lifetime measurements for monitoring changes in chromatin structure associated with cell cycle progression, cellular differentiation, or DNA damage, such as noted during apoptosis. Additional studies show that non-fluorescent DNA probes that compete with fluorochromes for binding to DNA potentially induce structural changes in chromatin, that can be detected by changes in the lifetime of a fluorochrome probe. The flow cytometer, in its current stage of development, is a multiparameter instrument, capable of performing lifetime measurements in conjunction with all the conventional FCM measurements. Future developments in the technology will provide multiple lifetime assays and thereby allow for detection and quantitation of selected subcellular probe-complexes with different lifetime signatures. These novel assays will expand the applications for quantitative studies on the binding of various chemical agents to DNA and other molecular targets in cells, and further improve methods for rapid screening of chemotherapeutic agents or environmentally toxic compounds.

Research supported by United States Department of Energy and Los Alamos Flow Cytometry Resource (NIH Grant p41-RR01315) and NIH Grants R01 RR06758 and R01 RR07855.

## A NEW METHOD FOR IMMUNOPHENOTYPING OF PERIPHERAL BLOOD BY LASER SCANNING CYTOMETRY (LSC)

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In many clinical situations only very restricted amounts of peripheral blood can be taken, e.g. in neonates with low birth weight, during extensive operations on young children, or in patients with restricted bone marrow function. Nevertheless, sometimes repeated analyses of the leukocyte function and subset distribution have to be carried out for these patients. We developed a new assay for immunophenotyping of peripheral blood by LSC. A major advantage of LSC as compared to flow cytometry is the fact that the cells are fixed on the slide. This allows the direct morphological control of the measured events by relocating them on the slide, and reduces the amount of peripheral blood and of reagents needed.

DNA is stained by 7-AAD and the different surface antigens are detected by direct immunofluorescence. For data acquisition triggering is computed on the 7-AAD-fluorescence. Data are obtained for forward scatter (FSC), green, orange, and long red fluorescence after excitation with the Ar-laser. 100 $\mu$ l of peripheral blood are heparinized, erythrocytes are lysed, leukocytes are resuspended in PBS and are then transferred onto a glass slide to pre-marked analysis fields. The cells are air-dried and acetone-fixed and then incubated with both 7-AAD and the different antibodies. The slides are covered with a glycerol-based mounting medium and stored at 4°C in the dark. Analysis is performed with the 20x objective. Up to 1000-cells are scanned per minute. Eosinophilic and basophilic granulocytes are separated by their characteristic FSC. The relative amount of different leukocyte subsets, e.g. CD3+CD4+, CD3+CD8+, CD19+, can easily be computed on the corresponding dotplots. This assay is easy to prepare and needs only minimal volumes of peripheral blood and reagents. Therefore we expect that immunophenotyping by LSC will prove to be the ideal method in clinical situations when the immune system must be monitored repeatedly but only limited amounts of blood should be taken.

## LUMINESZIERENDE NANOKRISTALLE ALS NICHTRADIOAKTIVE MARKIERUNGSSUBSTANZEN

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Die Markierung biologischer Systeme bildet die Grundlage einer Reihe von Analysemethoden, die unter anderem in der Molekularbiologie, der DNA-Sequenzierung und der klinischen Diagnose zum Einsatz kommen. Eine der bekanntesten nichtradioaktiven Markierungsmethoden beruht auf der Anbindung lumineszierender Verbindungen an die biologischen Zielmoleküle. Unter Anregung mit Licht geeigneter Wellenlänge können markierte Systeme anhand der Lumineszenz der an sie gebundenen Markierungsmoleküle identifiziert werden. Bisher werden für diese Art der Markierung vor allem organische Fluoreszenzfarbstoffe verwendet.

Die Nachweisempfindlichkeit solcher fluorimetrischer Methoden ist unter anderem durch die Eigenfluoreszenz und Lichtstreuung biologischer Proben begrenzt. Als mögliche Alternative zu organischen Fluoreszenzfarbstoffen werden neuerdings kleinste lumineszierende Festkörperpartikel untersucht, deren Durchmesser nur wenige Nanometer beträgt und die damit nur wenig größer als große organische Farbstoffmoleküle sind. Solche anorganischen „Nanokristalle“ oder „Nanopartikel“ zeichnen sich durch hohe Absorptionskoeffizienten, gute Quantenausbeuten und hohe Photostabilität aus. Für die Verwendung als Markierungssubstanz wird die Oberfläche der Partikel chemisch modifiziert, um eine spezifische Kupplung der Nanopartikel an biologische Systeme zu ermöglichen.

Erste Markierungsversuche wurden mit Nanopartikeln aus Halbleitermaterialien beschrieben. Bei dieser besonders gut untersuchten Substanzklasse hängt die Lage der Lumineszenzbande, d.h. die Farbe des emittierten Lichtes, nicht nur vom Material selbst sondern auch von der Partikelgröße ab und kann entsprechend den Erfordernissen ausgewählt werden. Halbleitermaterialien zeigen außerdem einen breiten Absorptionsbereich, so daß für die Lichtanregung eine Wellenlänge ausgewählt werden kann, bei der die Eigenfluoreszenz der Probe vernachlässigbar klein ist. Ferner lassen sich durch geeignete Syntheseverfahren Partikel mit sehr hoher Lumineszenzausbeute herstellen. Wegen dieser Vorteile wurden Halbleiternanopartikel bei allen bisherigen Markierungsversuchen verwendet.

Als weitere interessante Substanzklasse werden lanthanidendotierte oxidische Nanopartikel vorgestellt, deren Synthese erst kürzlich gelang. Bei diesen Systemen findet nach Anregung der Partikel mit Licht eine Energieübertragung auf in die Partikel eingebettete Dotierungsatome statt. Letztere geben die Energie dann als charakteristische Lumineszenz ab. Diese Systeme zeichnen sich durch enorme chemische Stabilität, große Stokes-Verschiebungen und sehr enge Lumineszenzbanden (Linienspektren) aus. Die Lage der Lumineszenzbanden kann über die Auswahl der Dotierungsatome variiert werden. Geeignete Wirtsgitter wie Vanadate zeigen sehr hohe Absorptionskoeffizienten. Die Lumineszenz der Nanopartikel kann darüber hinaus aufgrund der sehr langen Lumineszenzlebensdauer (Millisekunden) der Lanthanidenionen von der kurzlebigen Eigenlumineszenz der biologischen Probe unterschieden werden.

Im Vortrag werden die verschiedenen Systeme vorgestellt, ihre Eigenschaften verglichen und ihre Eignung als Markierungssubstanzen diskutiert.

## DEVELOPMENT OF A TWO COLOUR HYBRIDISATION PROTOCOL FOR RADIATION SENSITIVITY MAPPING USING COMET-FISH

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The COMET-assay, a single cell based electrophoretic technique, is a sensitive tool to measure the relative amount of single and double strand breaks in genomic DNA. Recently, the combination with fluorescence *in situ* hybridisation called COMET-FISH has been developed in our laboratory. This technique has been used to measure radiation sensitivity of specific genomic regions for UV-A damage. These measurements have shown, that UV-A induced damage is not distributed randomly in the human genome, but occurs preferably at gene poor regions. For UV-A damage an inverse relationship between the breakage sensitivity and the density of expressed sequence tags (ESTs), meaning the density of active genes, have been found.

Using a second DNA-probe with a second fluorochrome further improvements for routine applications were achieved. First examinations of different sequences or genomic regions were handled parallel in one experiment, reducing the number of required experiments. In this study two whole chromosome painting probes, a combination of several centromeres and regionspecific probes on the X chromosomes were applied. In addition with an appropriately localised second differently labelled sequence the region between the two fluorescent sequences were also monitored. Thus, observation of breakpoint clusters compatible to metaphase chromosome analyses were possible by means of COMET-FISH.

## SPECTRAL PRECISION DISTANCE MICROSCOPY FOR THE STUDY OF THE 3D-TOPOLOGY OF SELECTED POINT-LIKE MARKERS

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To study the three-dimensional (3D) structure of the human genome and its functional significance, quantitative conformation measurements of multi-spectrally labelled chromatin regions are required. Fluorescence *in situ* hybridization (FISH) offers the appropriate technique for specific chromatin labelling. So far, by means of confocal laser scanning microscopes, quantitative measurements in three dimensionally conserved nuclei can be performed in the resolution regime larger than 200 nm. Here, a recently developed light microscopical approach, "Spectral Precision Distance Microscopy" (SPDM), was used. The principle of SPDM is based on the fact that the intensity center of a labelling site can be localized with higher precision than the resolution (= full width at half maximum of the point spread function). If different labelling sites with distances below the resolution are discriminated by different spectral signatures, they can be localized independently and their distances can be determined. Confocal SPDM was applied to study the distances among four different clones of the Prader-Willi-Region of chromosome 15 by means of two-colour FISH. After a careful correction of chromatic shifts by *in situ* calibration, 3D-distances could be obtained with a "resolution equivalent" less than 100 nm. A comparison to experiments using one-colour labelling of two neighbouring clones showed the superiority of the new method. From the distance measurements, conformation angles could be determined and a model of the 3D-topology of the region among the labelling sites could be reconstructed. Moreover, it was demonstrated that the SPDM method is suitable to study a possible correlation between structure and function in an imprinted genomic region.

## **Analysis of Cellular DNA**

Abstract: A new computer assisted microscopic system (MRD Detect) has been developed for the detection and quantification of rare tumor cells in bone marrow (BM), peripheral blood (PB) and apheresis products (AP). The system uses immunological staining and sequential FISH analyses to detect tumor cells. The genetic analysis is based on the detection of specific chromosomal aberrations. The system is able to relocate the immunolabelled cells and to identify them by their specific chromosomal aberrations. This allows an exact quantification of tumor cells. The system also includes a cell counter function which allows an exact quantitative analysis of all cells contained in PB, BM or AP samples. The technique was standardized by mixing a known number of tumor cells with normal PB. Virtually all cells intermixed into the PB samples were discovered, thus resulting in a detection limit which is only restricted by the number of cells available for analysis. Quantitative analysis of tumor cells was carried out in over 200 clinical samples. Automatic search, quantification and genetic analysis of low tumor cell infiltrates in routine bone marrow preparations can be carried out efficiently and reliably with this computer assisted microscopic system.

## **FULLY AUTOMATED DETECTION AND GENETIC CHARACTERIZATION OF RARE TUMOR CELLS**

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The detection and quantification of rare tumor cells present in the bone marrow (BM), peripheral blood (PB) and apheresis products (AP) is becoming increasingly significant in the treatment of cancer patients. Limitations by the current techniques (immunological techniques, and PCR) are: lack of appropriate specificity and lack of accurate quantification. To overcome these problems and to enable an exact quantification, we have fully automated the screening and relocation procedure and combined an immunological and genetic approach (MRD Detect). The system detects and exactly relocates immunolabelled cells allowing a gallery display of all positive events and a microscopic evaluation. To verify whether the immunological cells are indeed tumor/leukemic cells, sequential FISH analyses, identifying tumor specific genetic aberrations e.g. MYCN amplification or del1p in GD2 stained neuroblastomas, t(11;22) in Ewing tumors (CD99), gain1q or 8q in breast carcinomas (e.g. CK-8,-9,-19), numerical/structural chromosome aberrations in ALL (e.g. CD34 or CD10) can be applied. This simple genetic test is enabled by the automatic reposition function of the system. In addition, a cell counter function allows an exact quantitative analysis of all cells contained in PB, BM, or AP samples (cytospins, BM smears). The technique was standardized by mixing a known number of tumor cells with normal PB. Virtually all cells intermixed into the PB samples were discovered, thus resulting in a detection limit which is only restricted by the number of cells available for analysis. Quantitative analysis of tumor cells was carried out in over 200 clinical samples. Automatic search, quantification and genetic analysis of low tumor cell infiltrates in routine bone marrow preparations can be carried out efficiently and reliably with this computer assisted microscopic system.

## EVALUATION OF THE FREQUENCY OF ANEUPLOID SPERM OF MICE AND HUMANS BY LASER-SCANNING-CYTOMETRY (LSC)

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The study was designed to evaluate the frequency of spontaneous aneuploidy in sperm of humans and young adult mice. Fluorescence *in situ* hybridization (FISH) with chromosome-specific DNA-probes for chromosomes X, Y and 8 in mice and X, Y and 13 in human was applied to detect hyperhaploid sperm. Microscopic scoring of aneuploidy in 10,000 sperm per individual is laborious and time-consuming. An improvement of sperm analysis may be achieved by automated scoring using Laser-Scanning-Cytometry (LSC). The LSC technique allows to score aneuploid sperm on slides normally used for microscopic analysis. Color domains in sperm of young adult mice and humans were evaluated by manual fluorescence microscopy and LSC to compare the frequencies of spontaneous aneuploidy rates. Preliminary LSC data showed no significant differences from the expected ratio of 1:1 between normal sperm carrying an X or a Y chromosome. The frequencies of hyperhaploid sperm in mouse obtained by LSC were 0.030% for chromosome 8, 0.010% for the X chromosome and 0.020% for the Y chromosome. The corresponding frequencies obtained by manual microscopy were 0.018%, 0.016% and 0.006%, respectively. The frequencies of hyperhaploid sperm obtained by LSC in human sperm were 0.080% for chromosome 13, 0.048% for the X chromosome and 0.026% for the Y chromosome. The corresponding frequencies obtained by manual microscopy were 0.050%, 0.044% and 0.010%, respectively. It can be concluded that LSC analysis has the potential to replace microscopic analysis of aneuploidy in sperm and to facilitate the studies of aneuploidy induction in male meiosis by aneugenic chemicals.

Research funded by EU-contract ENV4-CT97-0471

## TELEMETRISCHE DNA MESSUNGEN (STATISCHE DNA ZYTOMETRIE) MALIGNER PLEURAERGÜSSE

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**Zielsetzung:** Die DNA-Zytometrie rezidivierender Pleuraergüsse mit Hilfe des EUROQUANT Servers wurde auf ihre Anwendbarkeit zur diagnostischen Unterstützung im histopathologischen/zytologischen Routinebetrieb untersucht.

**Material und Methodik:** In einer prospektiven Studie wurden insgesamt 120 nicht selektierte Pleuraergüsse unterschiedlicher Dignität mit Hilfe des EUROQUANT-Servers und dessen Meßprogramm analysiert. Die endgültige Diagnose wurde anhand zusätzlich entnommener Biopsien/Operationspräparate und der klinischen Daten einschließlich der Entwicklung des Krankheitsbildes gestellt. Unter den 120 Ergüssen befanden sich 17 Mesotheliome, 53 Malignome unterschiedlicher Ätiologie und 50 akut und chronisch entzündlich bedingte Pleuraergüsse. An Messparametern wurden die Ploidität, die S-Phase-Fraktion und der CV-Wert der Peaks im Histogramm bestimmt. Pro analysierten Erguß wurden mindestens 50 Lymphozyten als interne Referenzzellen und mindestens 300 zu analysierende Zellen ausgemessen. Um diese Zellzahl zu erreichen, waren durchschnittlich 8 Bildaufnahmen erforderlich.

**Ergebnisse:** Von den 17 Mesotheliomen waren 8 euploid und 9 aneuploid, bei den 53 Malignomen waren 4 euploid und 49 aneuploid. Unter diesen befanden sich 11 metastasierende Mammakarzinome, die in 100 % der Fälle aneuploid waren. Von 26 Bronchialkarzinomen waren 22 aneuploid. Die überwiegende Anzahl der benignen Pleuraergüsse enthielt euploide Stammlinien, jedoch wurden auch 10 Ergüsse mit aneuploiden Zellpopulationen nachgewiesen. 2 von 5 tuberkulösen sowie 3 von 5 akut entzündlichen Ergüssen waren aneuploid. Die Bildaufnahmen, der Filetransfer via Internet und der Meßvorgang ergaben pro gemessenem Fall einen mittleren Zeitaufwand von etwa einer Stunde.

**Schlußfolgerung:** Hinsichtlich der Sensitivität und Spezifität können benigne von malignen Pleuraergüssen mit Hilfe des Ploidiestatus nicht mit eindeutiger Sicherheit unterschieden werden. Der Ploidiestatus gibt keine eindeutige Auskunft über die Natur des malignen Pleuraergusses, obwohl Mesotheliome häufiger diploid als metastasierende Adenokarzinome sind. In der Routinediagnostik kann die statische D N A - Zytometrie, wenn sie unter engen standardisierten Bedingungen durchgeführt wird, die diagnostische Zuverlässigkeit der zytologischen und immunhistologischen Verfahren unterstützen, jedoch keine eindeutige Aussage ergeben.

## QUANTIFICATION OF DNA-TELOMERE SEQUENCES IN SINGLE CHROMOSOMES USING FLUORESCENCE IMAGE CYTOMETRY

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Telomeres are highly repetitive DNA sequences that have important functions for the stability and replication of chromosomes. Each chromosome carries telomeres which are especially located at the terminal regions of the respective chromatids. The number of telomeric repeats in human somatic cells ranges from a few hundreds to several thousand base pairs depending on different parameters such as cell type, number of cell divisions and age. Quantification of telomere sequence lengths could be possible by labeling the telomere DNA with specific fluorescent- Peptide Nucleic Acid(PNA) probes. Under well-defined experimental conditions (optimized fluorescence microscope, cooled CCD image acquisition system) the amount of fluorescent light collected from each labeled spot should be proportional to the corresponding telomere sequence length.

Automatic analysis is desirable in order to investigate large numbers of cell samples. In this presentation we will introduce an image analysis system developed for automatic telomere sequence quantification. The analysis is divided into three stages: 1. scoring of metaphases; 2. detection of specific chromosomes within the metaphase; 3. detection and quantification of the telomere signal spots within the specific chromosomes and the whole metaphase. At each stage different images are acquired using different interference excitation and emission filters. Image preprocessing involves corrections for optical imaging imperfections and shading illumination. The major part of image analysis is based on nonlinear filtering with mathematical morphology algorithms. Three pattern recognition procedures are necessary to classify the metaphases, the chromosomes and the telomeres. After successful classification, integrated grey level intensities and areas are stored for each telomere.

## IMAGING OF IN SITU DNA SENSITIVITY TO DENATURATION BY CONFOCAL MICROSCOPY

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It has been shown before that sensitivity of DNA *in situ* to denaturation correlates with changes in chromatin condensation and varies during the cell cycle. DNA stained with a metachromatic dye acridine orange (AO) in condensed chromatin of mitotic or apoptotic cells was shown to have higher sensitivity to denaturation in comparison with DNA in interphase cells. Analysis of DNA denaturation by flow cytometry or standard fluorescence microscopy, however, could not reveal local differences in DNA denaturability at higher resolution or detect the initial points of chromosome condensation during initiation of chromatin condensation in G<sub>2</sub>. We have investigated green (530 nm) and red (640nm) fluorescence (exc. 457nm) of the AO stained normal human fibroblasts, HL-60 lymphoma cells in different stages of the cell cycle and apoptosis and polytene chromosomes from insect larvae. DNA in these samples was partially denatured by acid treatment, stained with AO and imaged using confocal microscopy with blind deconvolution. Photobleaching of AO fluorescence and chromatic aberration was carefully controlled. Nuclei of interphase cells exhibited predominantly green fluorescence representing double-stranded DNA. However, small areas of red staining were present in all interphase nuclei, representing denatured DNA. The proportion of highly condensed DNA increased in cells approaching mitosis. Mitotic chromosomes exhibited red fluorescence indicating the highest susceptibility to denaturation. The presence of areas of highly condensed DNA in interphase chromosomes was confirmed by imaging polytene chromosomes. Several distinct areas (bands) comprising highly condensed DNA were found in all polytene chromosomes. Areas of high transcriptional activity (puffs) exhibited green fluorescence only. These studies provide new information about the structure of chromatin in interphase and mitotic cells and the stability of DNA helix *in situ*.

## **FLUORESZENZMARKIERUNG DES ABL-GENS MIT COMPUTER SELEKTIERTEN OLIGO-DNA-SONDEN**

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Der Mechanismus der (Fluoreszenz) *in situ* Hybridisierung (FISH) beruht darauf, daß ein DNA Hybriddoppelstrang aus einzelsträngiger DNA-Sonde und einzelsträngigem DNA-Target hergestellt wird. Unter der Annahme, daß die Probe bereits einzelsträngig vorliegt, setzt dies immer noch eine Denaturierung (üblicherweise thermisch unter Zusatz chaotroper Agenzen) des doppelsträngigen Targets voraus. Es wird eine neue FISH-Technik vorgestellt, die speziell auf eine Target-Denaturierung vollständig verzichten kann und darauf beruht, daß poly-Purine eines DNA Doppelstranges in der Lage sind, über Hoogsteen-Bindungen dreifache DNA-Stränge mit entsprechenden poly-Purinen oder poly-Pyrimidinen auszubilden. Eine Computeranalyse des bisher sequenzierten Teils des menschlichen Genoms zeigt, daß in allen Genomregionen (kodierend oder nicht-kodierend) 1-2% aller DNA-Basen solche poly-Purinsequenzen bilden mit einer typischen Länge von 10-30 Nukleotiden. Man kann abschätzen, daß es im Mittel ca. 2 hoch 15 Variationen dieser Sequenzen gibt. Somit sollten sich per Computeranalyse solche Sequenzen bestimmen lassen, die an einem Genomlokus kolokalisieren, während sie im Rest des Genoms eventuell in Kopien aber nicht in derselben Kombination innerhalb eines Objektvolumens vorkommen. Ein solcher Genomlokus ist typischerweise gleichzusetzen mit dem Objektvolumen einer effektiven Halbwertsbreite der Punktbildfunktion des zur Analyse verwendeten Mikroskops. Die synthetisierten Oligo-Sequenzen bilden dann bei entsprechender Markierung mit Fluorochromen nach tripelhelikaler Bindung an dem gewünschten Genomlokus ein Fluoreszenzsignal, das in seiner Intensität aufgrund der Kolokalisation aller Sonden vom Fluoreszenzhintergrund zu diskriminieren ist. Diese neue FISH-Technik wurde für die spezifische Markierung des abl-Gens in Kernen humaner Lymphozyten eingesetzt. Hierzu wurden per Computerprogramm 32 geeignete Oligo-Pyrimidinsequenzen aus der EMBL-Sequenzdatenbank ermittelt, synthetisiert und mit je einem FITC-Molekül am 3'- und 5'-Ende markiert. Die Sonden wurden ohne Denaturierung des Targets hybridisiert. Die Signale im Zellkern wurden mittels konfokaler Laser-Scanning-Mikroskopie detektiert. Die Methode eröffnet neue Perspektiven für eine spezifische FISH-Technik in vitalen Zellen.

## **HIGH-RESOLUTION DNA FLOW CYTOMETRY IN THE MANAGEMENT OF HEAD AND NECK CANCER.**

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The assessment of aneuploidy in cancer was among the first applications of FCM. The early enthusiasm has disappeared, mainly because many studies failed to delineate prognostically different subgroups on DNA ploidy profiles. However, our 14-years experience with clinical routine application provides proof that DNA-FCM significantly contributes to the clinical management of head and neck cancer patients if high-resolution techniques are employed. Coefficients of variation not exceeding 2% are currently achieved in daily routine application. Clinical data are provided demonstrating that the risk of false-diploid classification dramatically increases if the CV exceeds 3%. Using high-resolution FCM, patients with diploid carcinomas turned out to only occasionally suffer from regional metastasis and local recurrence development. An overall survival rate of approximately 90% is achieved by local surgery alone if done before the development of aneuploid cell lines. Diploidy is therefore used as a decisive factor for local intervention and save the patients the burden of adjuvant treatment. Furthermore, aneuploidy of the primary tumor is the only significant prognosticator of occult lymph node metastasis in head and neck carcinoma. Aneuploidy is therefore used as an indicator for elective neck dissection in patients without clinical evidence of neck disease. Approximately 40% of clinically node-negative patients actually presented with micrometastasis. Patients with diploid tumors evade overtreatment of the neck. High-resolution DNA-FCM is thus a consequential, cheap and rapid diagnostic tool, ideal for clinical routine application.

## QUANTITATIVE ANALYSIS OF RARE TUMOUR CELLS BY AUTOMATED MICROSCOPY

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**Introduction:** Accurate quantification of disseminated tumour cells in haematological samples is of fundamental clinical interest. In a newly developed automated slide scanning system (MRDetect, MetaSystems), the benefits of fluorescence microscopy are taken into consideration. Different cell features *i.e.* immunophenotype, genetic information and proliferation status can be demonstrated in a sequential manner as a prerequisite for a reliable detection and characterisation of single neoplastic cells. Beside this application, a cell counter approach - designed to determine the number of nucleated (DAPI stained) cells - is incorporated. The system not only provides a tool to verify the tumour typical immunological and genetic features but it also allows an accurate figure of the tumour cells and the total of cells analysed in the given sample.

**Aims:** As previous experiments disclosed a significant difference in the cell number per slide, even in situations where a given number of cells was applied per slide, we established a cell counting system. In this study, we demonstrate the reliability and necessity of microscopical cell quantification of diagnostic bone marrow samples.

**Methods and Results:** Cytospin preparations from Ficoll separated bone marrow (BM) and cells were stained with the antiganglioside antibody G<sub>D2</sub>. (Dr. R. Reisfeld, La Yolla). To demonstrate the reliability of the cell counting approach, DAPI stained images with 5-1500 nucleated cells were counted both manually and automatically according to their DAPI positivity. An excellent correlation between the manually and automatically gained results was found. To prove or disprove an inter-slide variation, routine BM cytopsin preparations from neuroblastoma patients were immunostained for G<sub>D2</sub>/FITC and counterstained with DAPI. Automatic cell counting of at least three cytopsin preparations ( $> 2 \times 10^6$  MNCs) from the same samples showed significant differences in the total cell number (up to 50% inter-slide differences). High discrepancies were also observed, comparing slides from different sampling sites of the same patients as well as materials from different patients. **Conclusions:** To enable an accurate measurement of the tumour cell content in haematopoietic samples a reliable cell counting system was developed. This approach allows an exact monitoring of the tumour cell content.

## PROLIFERATIVE POTENTIAL OF TUMOUR CELLS DISSEMINATED IN THE HEAMATOPOIETIC SYSTEM

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**Introduction:** Minimally disseminated tumour cells can be detected in the haematopoietic system of cancer patients, even if the neoplasm is less extended. Rare tumour cells at diagnosis and especially following chemotherapy are therefore of special clinical interest. Our understanding of the biological impact of circulating tumour cells is, however, incomplete.

**Aims:** In this study, automatic microscopical analyses were performed to elucidate the proliferative status (Ki-67 expression) of neuroblastoma cells disseminated to the bone marrow.

**Methods:** Cytospins were prepared from 44 bone marrow (BM) samples of 12 neuroblastoma patients. Samples before and after chemotherapy were analysed in 5 cases. G<sub>D2</sub> and Ki-67 double immunostaining was performed to demonstrate the proliferating fraction of disseminated neuroblasts present in the BM. The slides were automatically scanned for G<sub>D2</sub>-Cy3<sup>+</sup>/Ki-67-FITC<sup>-</sup> or G<sub>D2</sub>-Cy3<sup>+</sup>/Ki-67-FITC<sup>+</sup> tumour cells by the MRDetect system (MetaSystems, Altlussheim, Germany). False positive G<sub>D2</sub> immunostaining was ruled out by sequential FISH analysis of the selected cells after automatic relocation.

**Results:** The overall frequency of Ki-67 expression in tumour cells was between 0% and 78%, however, differences in the expression level correlated with differing amounts of tumour cells in the sample. The samples with tumour cells between 100 to  $10^4$  tumour cells per  $10^6$  MNC (mononuclear cells) showed the highest Ki-67 positive fractions (19.3-78%, mean 35.97%; SD 9.66). In the samples with rare G<sub>D2</sub> positive tumour cells - in the range between one to 100 tumour cells/ $10^6$  MNCs - a highly variable Ki-67 expression was seen (0-55%; mean 16.18%; SD 17.52). In patients undergoing chemotherapy (CT), whose BM still contained circulating G<sub>D2</sub><sup>+</sup> cells, a similar distribution of Ki-67 expressing cells was observed as compared to the BM samples before CT.

**Conclusions:** Minimally disseminated tumour cells can have a high proliferative potential. The differences in Ki-67 expression might be of therapeutical importance for stage 4 tumours.

## THE DETECTION OF ANEUPLOID MALIGNANT HEMATOPOIETIC CELLS WITH FLOW CYTOMETRY AFTER THERAPY

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The DNA aneuploidy occurs frequently in solid tumors and in malignant diseases of hematopoiesis. An aneuploid cell population is reported in about 30% of patients with acute lymphoblastic leukemias (ALL) and in 70% of patients with multiple myelomas (MM). The analysis of DNA-ploidy offers interesting opportunities for the detection of minimal residual disease (MRD) in the aforementioned diseases. But only 1% to 2% malignant cells are detectable with one parameter DNA quantification. Using additional immunophenotyping the sensitivity could be improved detecting one malignant cell in  $10^3$  to  $10^4$  normal bone marrow cells. In a first study of patients with ALL in complete remission 16 pts. with < 0.1% aneuploid cells have a more favorable clinical outcome than 6 pts. with > 0.1% residual aneuploid cells ( $p=0.0067$ ). Another application of the proposed method is the detection of MRD in bone marrow of pts. with multiple myeloma after autologous transplantation of peripheral blood stem cells (PBSC) and the contamination of PBSC transplants with aneuploid myeloma cells. Investigating PBSC harvests we have detected aneuploid myeloma cells in 13 of 23 samples (range: 0.02% to 0.63%). In summary, we propose this relative simple flow cytometric method for the detection of residual aneuploid cells after therapy.

## THE EFFECT OF FMdC ON THE CELL CYCLE OF THREE LEUKEMIA CELL LINES IN VITRO

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### Background:

(E)-2'-deoxy-2'-(fluoromethylene)-cytidine (FMdC), an irreversible inhibitor of ribonucleotide reductase, displays a strong toxicity towards many cell lines derived from human solid tumors, while its activity on leukemia lines is less well-known. The aim of this study was to assess the effect of FMdC on the cell cycle and cell death of human leukemia lines HL-60 and MOLT-4, and murine leukemia L-1210 in vitro. It has been assumed that a prerequisite of FMdC cytotoxicity is intracellular phosphorylation by deoxycytidine kinase (dCK).

### Methods:

Cell cultures in the exponential phase of growth were exposed to different concentrations of FMdC (10 nM to 10  $\mu$ M) for 6 and 24 hours. In a parallel set of experiments 1 mM deoxycytidine was added to prevent phosphorylation of the drug by dCK. The DNA and protein content in the cells, as well as Annexin V/PI binding were assessed by flow cytometry. The cell cycle was analyzed by the MacCycle software.

### Results:

The cytotoxic effects of FMdC, i.e., G<sub>1</sub>/S block and cell death were observed, associated with pronounced changes in the protein content. These effects were of variable intensity among the cell lines studied (HL-60 being the most susceptible), and in some cases, were not completely reversed by deoxycytidine excess.

### Conclusions:

FMdC is a potent cytotoxic/cytostatic agent against human leukemia cell lines in vitro. It also changes the cellular protein content. Unphosphorylated FMdC may slightly influence the cell cycle of some leukemic lines.

CD137 (ILA/4-1BB) belongs to the TNF/NGF-Receptor superfamily that

## **CD137 (ILA/4-1BB) REDUCES PHAGOCYTOSIS OF HUMAN MONOCYTES**

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regulate diverse physiological programs such as proliferation, differentiation and programmed cell death. CD137 is expressed as a Type I transmembrane protein or as an alternatively spliced soluble protein. It has been shown previously that CD137 is a potent activator of monocytes. CD137 promotes a proinflammatory status of monocytes by inducing the production of proinflammatory cytokines such as IL-6 and TNF and by inhibiting the production of the antiinflammatory cytokine IL-10. Furthermore CD137 induces adhesion and prolongs survival of human monocytes. Our recent results imply a function for CD137 in monocyte phagocytosis. We show that stimulation of human monocytes with immobilised CD137 reduces phagocytosis especially of the adherent cell-fraction. After coincubation of green-fluorescent microspheres with CD137 stimulated monocytes we identify adherent and non-adherent subpopulations flow-cytometrically via scatter signals and calculate a decreased number of incorporated microbeads for the adherent fraction.

## **CYTOMETRIC INVESTIGATIONS OF RECEPTOR COMPLEXES**

## THE CHOLESTEROL AND SPHINGOMYELIN CONTENT OF THE PLASMA MEMBRANE AS DETERMINANTS OF CD14 DEPENDENT SIGNAL TRANSDUCTION

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Disturbances of the lipid metabolism connected to hypercholesterolemia, play a major role in accumulation of monocytes in the vessel wall which is an early hallmark in the pathogenesis of atherosclerosis. Cholesterol and sphingolipid rich membrane domains (rafts) have been postulated to be important regulatory principles for signal transduction based on the association of specific receptor complexes. In several cell models glycosylphosphatidylinositol (GPI) anchored receptors such as the u-PA-receptor CD87 have been shown to be associated with rafts. The goal of the present study was, the investigation whether also the expression and signal transduction of the LPS receptor CD14, which is a GPI-anchor molecule on monocytes, is regulated in correlation to the lipid composition according to the raft model. Exogenous depletion of cholesterol and sphingomyelin induced a dose and time dependent decrease of the CD14 expression. Similarly to the enzymatic treatment following cholesterol loading of the membrane using methylcyclodextrin the CD14 expression was decreased.

Further to the expression of CD14, the activation dependent coassembly of CD14 with CD11b which we recently described using fluorescence resonance energy transfer (FRET) as was analysed as an indicator of CD14 dependent signal transduction. Sphingomyelinase or cholesterol oxidase treatment similarly to LPS induced a spontaneous co-assembly of CD11b and CD14. Cholesterol loading of the plasma membrane totally blocked the LPS-induced co-assembly of both receptors while cholesterol depletion reduced the coassociation of CD11b and CD14.

These data in conclusion indicate a strong correlation of an intact raft structure and formation of a multimeric receptor complex to CD14 dependent signal transduction and thus may explain the altered function of monocytes in hypercholesterolemia.

## ENZYMATICALLY MODIFIED LDL IN CONTRAST TO OXIDIZED OR ACETYLATED LDL INDUCES FOAM CELL FORMATION INDEPENDENT FROM SCAVENGER RECEPTOR EXPRESSION

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Peripheral blood monocyte heterogeneity is characterized by specific patterns in membrane antigen expression allowing discrimination of distinct subpopulations. The more mature CD14<sup>dim</sup>CD16a<sup>+</sup> monocyte subset was shown to be expanded in association with atherogenic risk factors such as plasma cholesterol and triglycerides. The aim of this study was to investigate the role of Fc<sub>y</sub> receptor CD16a and scavenger receptors in foam cell formation from human peripheral blood monocytes using either LDL treated with trypsin, cholestryll esterase and neuraminidase (E-LDL), ac-LDL or ox-LDL. Modified lipoproteins were obtained from the same donor LDL in each set of experiments. Antigen expression was determined by flow cytometric measurement. Cellular lipid content and composition was assessed by high performance thin layer chromatography (HPTLC).

Using the model of M-CSF dependent differentiation, during the first 24h of incubation ac-LDL or ox-LDL did not induce a significant lipid loading of monocyte-derived macrophages. Accordingly, scavenger receptors SR-A1 and CLA-1 did not increase in expression until 3 days of culture. In contrast, E-LDL led to a significant accumulation of cholesterol and cholestryll ester that was cytochalasin D-sensitive but remained unchanged under treatment with the microtubule disrupting agent nocodazole. At the same time a significant upregulation of CD16a was observed within the first 24-30 hours of M-CSF dependent differentiation that was strongly enhanced under treatment with E-LDL.

Competition experiments with Dil-labelled E-LDL revealed insensitivity to ac-ldl, ox-ldl or the scavenger receptor inhibitor polyinosinic acid. Interestingly, uptake of Dil-E-LDL was enhanced by its unlabelled analogue suggesting autocatalytic activity of E-LDL.

These results underline the potency of LDL modified by trypsin, cholestryll esterase and neuraminidase (E-LDL) in inducing monocyte-derived foam cell formation and suggest that this process might be closely related to expression of Fc<sub>y</sub> receptors such as CD16a rather than scavenger receptors.

**DIFFERENCES IN TUMOR-CYTOTOXICITY OF HUMAN MONOCYTES  
DEPENDS ON THE TYPE OF TUMOR TARGET: ANALYSIS IN A  
THREEDIMENSIONAL SPHEROID MODEL**

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Activated monocytes and in vitro differentiated macrophages are potent effector cells against tumor cells. In order to investigate the mechanism by which the cytotoxic effect occurs we co-cultured human monocytes with multicellular spheroids (MCS) of two clonal urothelial-carcinoma cell lines. J82 was derived from a poorly differentiated invasive carcinoma, and RT4 from a recurrent papillary G1 tumor. Co-culturing monocytes with these two types of MCS resulted in an inhibition of proliferation in RT4 MCS as determined by 3H-Thymidin incorporation. The decrease in proliferation was transferable by supernatants of IFNg/LPS stimulated monocytes. Accordingly, RT4 tumor cells showed an arrest in cell cycle demonstrated by an decrease in the percentage of RT4 cells in the S-phase. J82 tumor cells also showed an inhibition of 3H-Thymidin incorporation after monocyte co-culture but in contrast to RT4 tumor cells this inhibition was cell-contact dependent and only to a limited degree transferable by supernatants. However, J82 cells underwent apoptotic death as estimated by TUNEL-staining of J82/monocyte co-cultures. The cytokines TNF- $\alpha$  and IL-1 $\beta$  seem to play a major role in both, the cell cycle arrest of RT4 and apoptosis of J82 cells, respectively. These data demonstrate that the mechanism of monocyte tumor cytotoxicity is dependent on the tumor target.

**CULTIVATION AND ISOLATION OF ENDOTHELIAL CELLS FROM LARGE VESSEL PREPARATIONS**

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**Background and Aim:** Monolayer cultures of human endothelial cells (EC) are widely used as an in-vitro model to study pharmacological and immunological phenomena influencing the endothelium. Macrovascular EC preparations from coronary arteries, vena saphena magna or aorta mammaria interna are often contaminated by fibroblasts requiring a suitable EC isolation technique. Even if the proportion of fibroblasts is < 5% of the total cell count in the confluent primary culture, transfer into passage one results in a dramatic fibroblast growth induction leading to an overgrowth of the EC layer. **Material and Methods:** Fluorescence-activated (FACS) and magnetic cell separation (MACS; Miltenyi Biotec) have been applied using diverse antibodies (e.g. anti-CD31-FITC, AS02-FITC, anti-CD105-microbeads, anti-fibroblast-microbeads) and different labeling strategies. **Results:** With a mixture of EC:fibroblasts of 1:1 MACS separation is an easy-to-handle technique to enrich EC up to 84  $\pm$  3 % with one and 93  $\pm$  3 % with two separation cycles if positive selection using anti-CD105-microbeads is performed. The application of anti-fibroblast-microbeads for fibroblast depletion yields an EC purity of > 96 % with only one separation cycle. For both sorting schemes, more than 30 % of the original EC population is found in the fibroblast fraction. FACS separation was most efficient using anti-CD31-FITC. Dependent upon the sort modus it is not only possible to receive a purity of 99 % but we were also able to grow EC clones in 96-well plates under optimal, defined culture conditions following single cell sorting. **EC function and viability are preserved after MACS and FACS.** **Conclusion:** MACS separation is recommended for rapid EC enrichment and may be applied prior to FACS while the latter method is required if EC populations with a high purity or EC clones from large vessel preparations are used as an in-vitro test system. (This work was supported by Miltenyi Biotec Inc.)

**FLOW CYTOMETRY STUDY OF 'DNA CLEARING' FROM  
NONCOVALENTLY BOUND AGENTS WHICH IS A NEW MECHANISM OF  
DRUG RESISTANCE IN MAMMALIAN CELLS.**

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The process of DNA clearing or the cell ability to dissociate from DNA the substances that noncovalently binds with DNA has been previously reported. This process is energy-dependent and can be suppressed by topoisomerase-2 inhibitors and DNA breaks. The cytofluorimetric technique and the vital fluorescent dye Hoechst 33342, which binds to DNA in the minor groove, is used for the registration of the DNA clearing. It was shown that step by step selection with increasing concentration of the dye was resulted in the series of Hoechst -- resistant rodent and human cell lines. Some of them were characterised by an enhanced dissociation of the bisbenzimidazole dye -- DNA complex. Two cell lines from this group have been analysed in details. These lines were shown to be also cross-resistant to netropsin and Mitomycin C --clinically used minor groove alkylated agent. The DNA clearing in these lines and in Syrian hamster tumour cell lines selected for "typical" mdr-1 mediated multidrug resistance was compared. The differences in DNA clearing allow us to conclude that we obtained a group of MDR cell lines characterised by new mechanism of drug resistance based on an enhanced ability of DNA clearing.

**DEVELOPMENT-DEPENDENT ECTOPIC EXPRESSION OF THE  
TRANSCRIPTION FACTOR PEROXISOME PROLIFERATOR-ACTIVATED  
RECEPTOR SUBTYPES AND APOPTOSIS IN LUTEIN CELLS**

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Cellular self-destruction plays an import role in the formation of developmental-dependent functions of tissues, including the corpus luteum. This ovarian tissue develops from postovulatory follicular cells in the mammalian ovary and is regressed when fertilization did not take place. Apoptogenic pathways may be activated during functional regression. To elucidate the role of oxidative stress for apoptosis, bovine lutein cells of the postovulatory (day 5) and midluteal phase (day 12) were exposed to the antioxidant N-acetylcysteine (NAC). In contrast to day 5 cells, midluteal phase cells responded dose-dependently to NAC with significant rise in the portion ( $57\pm10$  vs.  $11\pm3$  %) of cells with apoptotic hallmarks determined by flow cytometry and nuclear morphology. Bcl-2 protein was highly expressed in apoptotic cells and NAC did not impair mitochondrial functions but induced a rise in oxidized glutathione (GSSG) and an enlargement of structural compartments, fluorescent by the oxidation of dihydrorhodamine. Fluorescence analysis via flow cytometry and light microscopy as well diaminobenzidine cytochemistry and electron microscopy suggest peroxisomes as the fluorescent structures. A reversal of the NAC effects on peroxisomal activity by aurintricarboxylic acid also reduced the GSSG level and strongly decreased the apoptotic sub-G<sub>0</sub>/G<sub>1</sub> area of the flowcytometric histogram, providing evidence for a link of the pathways which trigger peroxisomal activity and apoptosis. Analysis of ectopic expression of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) subtypes revealed a marked reduction of PPAR $\gamma_1$  in midluteal phase cells (9.7-fold the level of postovulatory phase cells). That the reduction was accompanied by a changed peroxisomal defence from oxidative stress suggests a downregulation of this PPAR $\gamma$  subtype as a mechanism involved in apoptogenic regression of the corpus luteum.

**PERIPHERE-BENZODIAZEPINREZEPTOREN IN RELATION ZU  
ZELLZYKLUSPHASE UND MITOCHONDRIENGEHALT BEI MAMMA-  
CARCINOM-ZELLKULTUREN**

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**Einleitung:** Periphere Benzodiazepinrezeptoren (PBR) unterscheiden sich in Aufbau, Funktion und pharmakologischen Eigenschaften von den GABA-assoziierten zentralen Benzodiazepinrezeptoren und sind überwiegend an der Mitochondrienmembran lokalisiert. Sie konnten an humanen ER- und PR-rezeptorpos. MCF-7 Mamma-Ca-Zellkulturen in geringerer Menge als an ER- und PR- rezeptorneg. BT20 Zellen nachgewiesen werden. **Fragestellung:** I. Beeinflusst die Gabe des PBR-Agonisten Ro5-4864 das Zellwachstum der Zellkulturen? II. Ist diese Beeinflussung auf eine bestimmte Zellzyklusphase begrenzt? III. Besteht ein Zusammenhang zwischen der PBR-Expression und dem Mitochondriengehalt? **Methode:** Die Zellen wurden durch Serumengzug (0,5% FKS) synchronisiert und mit dem PBR-Agonisten Ro5 inkubiert. Die Zellzyklusphasen wurden anhand des DNA-Gehaltes der Zellen ermittelt. Der Rezeptornachweis erfolgte mit Hilfe eines monoklonalen PBR-spezifischen AK und eines fluoreszenzkonjugierten 2.AK. Der Mitochondriengehalt wurde über einen spezifischen Farbstoff (NAO) bestimmt. Alle Parameter wurden durchflusszytometrisch am FACScan gemessen. **Ergebnisse:** I. Das Zellwachstum von MCF-7 und BT20-Zellen konnte unter Inkubation mit Ro5 inhibiert werden. Ein antiproliferativer Effekt zeigte sich bei 10 nM und war zeitlich auf 6-8 Std. begrenzt. II. Die Inhibition war in der S- und G2M-Phase signifikant. III: MCF-7 und BT20 zeigten sowohl in der Kontrollgruppe als auch in der Versuchsgruppe analog zu der Zunahme des Mitochondriengehaltes eine vermehrte PBR-Expression. Unter Inkubation mit Ro5 waren allerdings im Vergleich zur Kontrolle die Rezeptorexpression und der Mitochondriengehalt vermindert. **Diskussion:** Erfolgt eine Zunahme der Mitochondrien für die bevorstehende Zellteilung, so wird der PBR vermehrt exprimiert, der Rezeptorgehalt ist also an den Mitochondriengehalt gekoppelt. Bindet der Agonist Ro5 an den Rezeptor, so erfolgt sowohl eine verminderte Rezeptorenexpression als auch Mitochondrienzunahme. Der PBR besitzt somit einen antiproliferativen Effekt im Zellwachstum von MCF-7 und BT20 Zellen.

**QUANTIFICATION OF APOPTOSIS AND LEUKOCYTE SUBSETS IN TISSUE  
SECTIONS BY LASER SCANNING CYTOMETRY (LSC)**

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SCHNEIDER P 1

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Quantitative analysis of apoptosis in solid tissues is difficult to perform. Nevertheless it would yield important information in a wide variety of clinical and experimental settings. Also, the analysis of the spatial distribution of leukocytes would give relevant information, e.g. about the organism's immunological reaction at the tissue infiltrating tumor front.

We are looking for an automated analysis method suitable for archival material which would enable the analysis of these rare events (apoptotic cells, dendritic cells). The capacities offered by laser scanning cytometry (LSC) make it the ideal method for the required analyses. For analysis of apoptosis we have established an assay suitable for formalin-fixed paraffin-embedded tissue. Sections are stained with PI after RNase-treatment. Apoptotic cells are recognized by TUNEL-assay. For data acquisition triggering is done on PI-fluorescence. Data are collected for the various wavelengths after excitation with the Ar-laser. When analyzing ischaemic and non-ischaemic rat muscle the green autofluorescence allows for differentiation of cells in the fibrous tissue (weak) versus in the muscle (high). This enables us to specifically analyze apoptotic muscle cells. Finally, morphological confirmation of the analyzed cells can easily be obtained by relocation.

For analysis of the spatial distribution of leukocyte subsets in lymph nodes the material is fresh frozen. Immunofluorescent staining is performed by indirect immunofluorescence, and DNA-staining is performed by 7-AAD or PI. By this assay we were able to quantify CD4+, CD8+, or CD1a+ -cells in different compartments of lymph nodes (germinal center, mantle zone).

We expect that LSC will prove to be an efficient, cost-effective, and time-saving method to quantitate apoptotic cells and leukocyte subsets in solid tissues with additional information on the spatial distribution.

## Industrial Talks

### MHC-TETRAMERE: NACHWEIS VON ANTIGEN-SPEZIFISCHEM CD8+ T-ZELLEN MIT HILFE DER DURCHFLUSSZYTOMETRIE

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Die B-Zell vermittelte, humorale Immunantwort kann einfach analysiert werden, da Antikörper, die B-Zell-Rezeptoren, eine hohe Affinität für ihr lösliches Antigen aufweisen. Der Antikörernachweis wird seit langem in der klinischen Routine eingesetzt. Die T-Zell vermittelte, zelluläre Immunantwort ist dagegen viel schwieriger zu erfassen, zum einen, da T Zellen keine löslichen, sondern zellgebundene Antigene erkennen - in Form von Peptiden, die von MHC-Komplexen auf Antigen-präsentierenden Zellen gebunden sind -, und zum anderen, da die Affinität des T-Zell Rezeptors für das entsprechende Antigen um Größenordnungen niedriger ist.

Eine technologische Neuerung von der Universität Stanford, die exklusiv von Beckman-Coulter weiterentwickelt wird, erlaubt es jetzt, diese Probleme zu umgehen. Die Methode besteht darin, rekombinante, lösliche MHC-Moleküle herzustellen, die ein bekanntes Antigen gebunden haben, und deren Affinität durch Multimerisierung erhöht wird. Dafür werden die Komplexe spezifisch biotinyliert und mit Streptavidin tetramerisiert. Das Streptavidin ist Fluorochrom-markiert, und die so erhaltenen Reagenzien können - ganz analog zu Antikörpern - zur Zellmarkierung und zur Analyse in der Durchflusszytometrie eingesetzt werden.

In den 3 Jahren seit der ersten Veröffentlichung haben MHC-Tetramere bereits eine kleine Revolution in der Immunologie ausgelöst, da mit ihrer Hilfe gezeigt werden konnte, dass die Zahlen an Antigen-spezifischen T-Zellen erheblich höher liegen als bisher angenommen wurde. Es sind Fälle beschrieben, in denen z.B. während einer akuten EBV Infektion, zwischen 10% und 40% der CD8+ Zellen ein einziges Epitop eines viralen Proteins erkennen.

Die Liste der Anwendungsmöglichkeiten ist lang, aber ein besonders interessantes Feld ist die Evaluierung der T-Zell-Antwort nach Vakzinierung und bei immuntherapeutischen Ansätzen. So wurde bereits gezeigt, dass T-Zellen, die Tumorantigene erkennen, mit dieser Methode nachgewiesen werden können und dass das Sortieren und die genaue Analyse dieser Zellen durch MHC-Tetramere möglich geworden ist.

## **BD LSR, THE 6-COLOR BENCHTOP RESEARCH FLOW CYTOMETER FROM BD BIOSCIENCES**

**BECTON DICKINSON GMBH, HEIDELBERG**

The BD LSR is facilitating complex multicolor flow cytometric analysis by combining benchtop ease-of-use with the flexibility and performance of high-end flow cytometers in an alignment-free 488/UV laser-based system. The BD LSR supports a wide range of multicolor cell analysis applications with standard 488-nm-excited dyes and UV-excited dyes such as Hoechst 33342, DAPI, and Indo-1.

With this instrument one can free up high-performance cell sorters for high-performance cell sorting by moving routine UV applications to the BD LSR flow cytometer. The BD LSR features an air-cooled 8-mW, 325-nm He-Cd laser in addition to a 15-mW, 488-nm argon-ion laser. It provides up to six fluorescence and two scatter parameters.

Building on the ease-of-use standard set by the FACSCalibur™ flow cytometry system, the BD LSR offers software instrument control, push-button fluidics, and fine-adjust sample flow-rate control so that the user can concentrate on his science and not on the technology.

## **BIOMAGNETIC SEPARATION: METHODS AND APPLICATIONS**

**JÜRGEN LEWALD**

Deutsche Dynal GmbH, Schaartorstr. 1 20459 Hamburg, Germany

Since its startup in 1986 Dynal has developed a range of magnetic particle-based products (Dynabeads®-products). Dynal's products are used to magnetically separate cells, subcellular organelles, proteins and nucleic acids for a wide range of applications in Immunology, Cell Science, Molecular Biology and Microbiology. Recently the product range has been expanded to include two non-magnetic particle based Tissue Typing methods (SSP and SSO). Dynal's applications offer the advantages of speed, simplicity and reliability.

### **Bioscience / Molecular Biology:**

For cellular biology applications specific antibodies are bound to Dynabeads®. After incubation of mixed cell suspensions with the coated to Dynabeads a magnet is used to separate cells of interest from irrelevant cells. The to Dynabeads® are non-toxic allowing viable cells to be separated in high yield. The separation process is highly specific. In molecular biology applications Dynal uses the streptavidin-biotin system to attach DNA to the surface of the to Dynabeads® as well as oligo(dT) Dynabeads® where the oligonucleotide is directly bound to the surface. These products are used for single-strand, solid phase DANN-sequencing, mRNA isolation, RT-PCR, hybridizations specific captures and sample preparations of DNA and RNA.

### **Microbiology:**

Dynabeads® for microbiology are 2,8μm in diameter and covalently coated with affinity purified antibodies against specific surface markers on the microorganisms. The immunomagnetic selective enrichment of microorganisms increases the sensitivity and thus leads to more accurate results, giving culture confirmation in 24-48 hours. Products for the isolation of *Salmonella*, *E. coli* 0157, *Listeria*, *Cryptosporidium* and *Giardia* are currently available.

### **Tissue Typing/Diagnostics:**

Apart from simplifying and speeding up the classical method for tissue typing Dynal has succeeded in developing the market in molecular biology techniques - DNA amplification using Sequence Specific Primers (SSP) and amplified DNA detection using Sequence Specific Oligonucleotides (SSO). Dynal leads the market in SSP and is the exclusive worldwide distributor for Roche Molecular Systems, Inc. Amplicor® HLA products in SSO.

## KOOPERATIVES ARBEITEN IN DER MIKROSKOPIE ÜBER NETZE (HISTKOM)

DEUTSCHE TELEKOM AG, GB MULTIMEDIA, BONN

Präparative und mikroskopische Untersuchungen in der Patho-logie werden immer spezieller und aufwendiger, sie erfordern zunehmend mehr Kenntnisse sowie Erfahrung. Um die vollen Möglichkeiten heutiger Methoden und Geräte auszuschöpfen, sind daher Kooperationen zwischen Arbeitsgruppen, welche oft räumlich getrennt sind, angezeigt. Angestoßen durch Bedürfnisse von Radiologen und Pathologen, ihre Dienstleistungen näher an den Patienten heranzubringen, bilden sich gegenwärtig Dienstleistungsnetze für Anbieter und Nachfrager von Dienstleistungen in der Medizin. Neben der Routinepathologie könnten solche Arbeitsweisen auch Arbeitsgruppen der Forschung in Medizin und Biologie zugute kommen.

Für Vernetzungen von Arbeitsgruppen finden wir heute bereits eine ganze Reihe von Komponenten vor:

- Die Übertragungsnetze selbst: Analoges Telefon, ISDN-Telefon, Breitbandnetze (LAN's, xDSL), Internet, etc.
- Dienste in Telemedizinnetzen: Administrative Dienste, Informationsdienste, Teleradiologie, Telepathologie, Tele-DNA-Analyse, Datenbanken, Elektronische Patientenakte.
- Juristische und technische Sicherheitsvorkehrungen, wie sie die klinische Medizin erfordert ('Secure Net'). Dazu gehören: Zugangskontrolle, Authentifikation, Änderungssperren etc.). Für Anwendungen, welche im Gegenteil eine möglichst große Öffentlichkeit anstreben, können Inhalte in HTML-Formate übersetzt werden und können damit weltweit über das Internet übertragen und mit jedem gängigen Browser betrachtet werden.

Es stellt sich die Frage, ob diese bereits bestehenden Infrastrukturen auch für die Einbindung weiterer Untersuchungsmethoden geeignet erscheinen, und ob sie für die Nutzer einen Vorteil beim kooperativen Arbeiten bieten. Kandidaten für eine solche Einbindung in Netze sind natürlich zuerst alle Geräte, welche bereits über Rechner bedient oder durch Rechner gesteuert bzw. deren Ergebnisse in einem Rechner vorliegen. Zum Anschluß solchermaßen vorbereiteter Analysegeräte mitsamt ihrer Bedienoberfläche sind bereits Softwarekonzepte allgemein verfügbar, die es erlauben eine Benutzeroberfläche eines Gerätes vollständig auf einen sich irgendwo im Netz befindlichen anderen Rechner zu spiegeln (z.B. Timbuktu-Software, X-View). Um solche Anwendungen zu fördern finden gegenwärtig weltweit Anstrengungen zur Standardisierung solcher Techniken statt.

## CONTINUOUS GATING® VON MEDAC, EINE EXPERTENSOFTWARE

MEDAC DIAGNOSTICA

In der Leukamiediagnostik hat die durchflußzytometrische Analyse ihren Stammpunkt. In der Stammzellseparation muß eine Qualitätskontrolle der Aphereseprodukte erfolgen. Die Durchflußzytometrie ist auch hier fest etabliert und kann zum Auffinden von minimalen Tumorrestpopulationen genutzt werden.

Daten aus der Durchflußzytometrie kann die Expertensoftware **Continuous Gating®** von medac um das Zehnfache verbessern. Im Apheresematerial von Patienten mit Multiplem Myelom lassen sich so über den DNA-Gehalt noch 7 Tumorzellen unter 10.000 gesunden Zellen erfassen.

Erweiterbar ist dies auf alle Tumorzellen, deren DNA-Gehalt sich von dem DNA-Gehalt der gesunden Zellen unterscheidet (DNA-Index) oder deren Antigenexpression Unterschiede aufweist.

Sollte man den DNA-Index nicht kennen, bietet **Continuous Gating®** die Möglichkeit zur vollautomatischen Suche nach unbekannten DNA-Indices.

**Continuous Gating®** wurde zum Auffinden "seltener Ereignisse" entwickelt, wodurch sich weitere Anwendungen im Bereich „Stammzellseparation“ automatisch ergeben werden.

## RECENT DEVELOPMENTS IN RARE CELL SCANNING AND MULTI FLUOROCHROME IMAGING

METASYSTEMS GMBH, ALTLUSSHEIM, WWW.METASYSTEMS.DE

MetaSystems is a supplier of cytogenetic image analysis and automated microscopy solutions. We present two novel imaging solutions that were recently introduced: 1. MRDetect, an automatic detection system for rare events like micrometastases or residual tumour cells in a large population of normal cells, and 2. mBAND, a technique for high resolution color banding analysis of chromosomes.

The quantitative assessment of minimal residual disease and of micrometastases requires both high sensitivity and specificity. MRDetect is based on a motorized fluorescence microscope combined with fast image analysis. It scans microscope slides to find anti-body fluorescence-labelled tumor cells. The number of candidate cells as well as the total number of cells are scored. To achieve the required specificity candidate cells can be relocated after performing an additional assay, e.g. using FISH with tumor-specific genetic markers. Alternatively, simultaneous detection of several antibodies labelled with distinguishable fluorochromes is possible. The correlation of subsequent or simultaneous phenotypic or genotypic results is a major advantage of the fluorescence approach as compared to bright field imaging systems.

mBAND is a high resolution color banding technique that allows the identification of intra-chromosomal rearrangements like deletions, duplications, and inversions. A set of partially overlapping DNA libraries labelled with distinguishable combinations of fluorescent dyes results in characteristic intensity variations of each fluorochrome along the chromosome axis. Fluorescence ratio analysis is used to classify areas of similar ratios into bands which are then displayed in appropriate pseudo colors. The resulting multi color banding pattern is highly reproducible and independent of chromosome condensation. Ratio analysis effectively multiplies the resolution of the DNA probe set: for human chromosome 5 a probe set of 7 different libraries (and hence 7 different fluorochrome combinations) yields more than 25 distinct color bands.

## AUTOMACS

### FOR ULTRA HIGH SPEED AUTOMATED MAGNETIC CELL SEPARATION

MILTENYI BIOTECH GMBH

THE AUTO<sup>MACS</sup> IS A BENCH-TOP COMPUTER CONTROLLED MAGNETIC CELL SORTER designed for use in cell separation, in a variety of research applications, within the hematology / immunology field. The autoMACS is used with a wide range of direct and indirect MACS reagents for the isolation of virtually any cell type. Employing the MACS magnetic cell separation technology, the autoMACS is capable of sorting over 10 million cells per sec from samples up to  $4 \times 10^9$  total cells.

A touch screen is used for all computer interactions, which makes the autoMACS an extremely easy to use system. A number of different programs are available, giving you the flexibility to choose a selection strategy for enrichment or depletion, for rare cell sorting or sensitive markers. autoMACS is a robust system that requires low maintenance. A number of different rinsing and cleaning programs help you to maintain the columns and the instrument. As MACS technology is fully compatible with flow cytometry, the autoMACS is an ideal solution for pre-enrichment of cells prior to FACS® sorting.

The MACS technology is based on specific cell labeling with MACS magnetic MicroBeads, followed by a separation using high gradient magnetic separation columns placed in a strong magnetic field. This gentle sorting results in fully functional cells capable of further growth. Specific reagent kits for a whole range of applications such as T-cell, B-cell, NK cells, tumor cells, stem cells sorting are available. For separations using your own primary antibody, indirect beads are available.

# PAS, EIN KOMPAKTES FLOWZYTOMETER MIT FÄRBEAUTOMAT FÜR DIE ROUTINE-IMMUNOLOGIE.

WOLFGANG GÖHDE JUN. UND VOLKER ÖST

Partec GmbH, 48161 Münster, Germany

Eine Reihe von aktuellen technischen Entwicklungen und neue Färbemethoden eröffnen der Durchflußzytometrie immer mehr Anwendungen in der medizinischen Routine. Partec hat hierzu in der letzten Zeit folgende neue Vorschläge beigetragen:

## 1. Integrierte Software für Windows 98

Komplette Software für die ultraschnelle Datenakquisition von bis zu acht unabhängigen Parametern (bis zu 10.000 Zellen pro Sekunde). Funktionen für alle erforderlichen Anwendungsoptionen, wie z.B. Software-Kompensation bereits während der Messung oder danach (die Rohdaten stehen für nachträgliche beliebige Auswertungen immer zur Verfügung), Quadrantenstatistik mit Prozentanteilen und Absolutzahlen (Zellen pro ml), Histogrammauswertung, Zellzyklus- und Peakanalyse. Alle Analysen basieren auf einer Kanalauflösung bis zu 65000 Kanälen pro Parameter. Das PAS lässt sich problemlos über Ethernet (10/100 Mbit/s) in bestehende Netzwerke integrieren. Die Software automatisiert die Routineuntersuchung von Meßserien (Panels) und liefert automatisch Reports im MS-Word Format für das komplette Panel. Für die Datenauswertung sind keine weiteren Softwareprogramme erforderlich.

## 2. Absolutzählung zur Konzentrationsbestimmung beliebiger Zellen

Die Partec-Geräte erfassen das Suspensionsvolumen und die darin befindlichen Zellen. Referenzpartikel oder anderweitige Kalibrierungen sind nicht erforderlich. Absolutzählungen sind daher hochgenau und schnell auszuführen.

## 3. Walk-away Probenautomat

Der Partec-Färbeautomat bereitet 36 Blutproben pro Karussell mit bis zu 14 verschiedenen Antikörpern und beliebigen Lysemethoden innerhalb von wenigen Minuten vor (zuzüglich der für das Färbeprotokoll erforderlichen Inkubationszeit). Der Automat übergibt die Proben selbstständig an das Flow-Cytometer. Immunologische Messungen können damit in der klinischen Routine als echte "walk away" Protokolle ausgeführt werden. Einsatzgebiete sind Anwendungen mit großem Probenanfall und solche Messungen, bei denen die Handbedienung eine Gefährdung des Personals zur Folge haben könnte (Hepatitis, HIV etc.).

## 4. Neue Anwendungen

Neue immunologische Möglichkeiten werden durch die gleichzeitige Anwendung von Laser und HBO-Quecksilberdampf-Lampe zur Fluoreszenzanregung auch in der klinischen Routine erschlossen: z. B. Messung der Zellproliferation von immunologisch definierten Leukozytenpopulationen. Die Messung der DNA erfolgt bei UV-Anregung (DAPI, Hoechst) bei Wellenlängen unterhalb der 488 nm-Anregung der Immunmarker. Damit lässt sich im Lichtspektrum zusätzlich mindestens ein weiterer Parameter platzieren. Diese echte Zweiwellenlängenanregung ist deshalb besonders attraktiv, weil es nahezu keinen Überlapp (Crosstalk) wie bei der Verwendung von PI oder anderen mit 488 nm angeregten Fluoreszenzfarbstoffen gibt.

# DAS TILL-PHOTONICS STANDARD IMAGING SYSTEM

PETER MESSLER, JOACHIM WIETZORREK

Till-Photonics GmbH, Martinsried, Deutschland

Mittlerweile gehört die Fluoreszenzmikroskopie zu den Standardmethoden für die Messung intrazellulärer Ionenkonzentrationen (Calcium, pH, ...) und die quantitative DNA-Analyse (Comet-Assay, Fluoreszenz In Situ Hybridisierung (FISH)). Im zunehmenden Maße wird die Fluoreszenzmikroskopie auch zum Nachweis des Green Fluorescent Proteins (GFP) einschließlich seiner Derivate sowie zur Analyse der Koexpression von Proteinen mittels Fluorescence Resonance Energy Transfer eingesetzt.

Das „Till-Photonics Standard Imaging System“ bestehend aus Monochromator, gekühlter CCD-Kamera mit adaptiertem Frame Grabber, prozessorgesteuertem Systemcontroller und Software schließt alle genannten Applikationen ein. Der Polychrome II Monochromator mit einer Bandbreite von 8-15nm ermöglicht die Selektion der Anregungswellenlänge zwischen 320 und 680nm innerhalb von weniger als 3ms. Die Imago CCD-Kamera liefert Vollbilder in Videorate oder Teilbilder mit bis zu 150Hz mit 12 Bit Genauigkeit in VGA oder SVGA Auflösung. Die hohe Datenrate wird durch zeitliche Überlagerung von Integrations- und Datenübertragungsphase ohne Zuwachs an Systemrauschen erzielt. Der Imaging System Controller gewährleistet eine vom Computer unabhängige, präzise zeitliche Steuerung aller Systemkomponenten. Die TillVision Software beinhaltet einen Protokolleditor mit dem der zeitliche Ablauf komplexer Experimente definiert werden kann. Mit dem Protokoll können neben den Komponenten des Imaging Systems über analoge und digitale Ports auch externe Geräte angesteuert werden. Umgekehrt ist es möglich, das Imaging System über Eingangssports von außen zu kontrollieren.

Neben der Erläuterung des technischen Konzepts des Imaging Systems präsentieren wir aktuelle Applikationen wie z.B. Echtzeit Vesicle Tracking und Multi-Color GFP Imaging.

# MULTIPLE FLUORESCENCE MICROSCOPY AND OPTOELECTRONIC IMAGING: - POSSIBILITIES AND LIMITS

HEINZ GUNDLACH, CARL ZEISS, OBERKOCHEM

## Introduction

The last 20 years have seen an unexpected great renaissance and a partial revolution in light microscopy. This recent progress is due to new design in optics and instrumentation as well as improvement of optical contrast enhancement techniques. Recent progress in fluorescence microscopy is achieved by multiparameter fluorescence techniques, by improvement of conventional photomicrography as well as by optoelectronic imaging, confocal laser scanning microscopy, image processing and analysis. Due to the increase in number of fluorescence dyes, double and triple bandpass filter sets permit a rapid changeover between different fluorochromes simultaneously.

### Double and triple filter sets...

The new high-precision multiple bandpass filter sets now permits the simultaneous view and documentation of two or three different fluorescence dyes. The main features and benefits for the user are:

- = simultaneous view and documentation
- = high precision imaging without image shift
- = quick evaluation
- = reduced bleaching of the fluorochromes
- = more space for additional filter sets in the FI- slider or FI-turret

### Single filter sets...

Problems may occur if one type of fluorescence fades during exposure or if the two or three types of fluorescence exhibit very different intensities. In this case and also if the various fluorescence signals are in different focal planes, multiple exposure with single filter sets are recommended. To avoid pixel shift, filter sets with wedge free emission filters are recommended. Compared to double and triple filter sets, these filters have the following advantages:

- = general more intensity
- = individual exposure for each fluorescence signal
- = optimized focus
- = unlimited filter combinations

### Filter set according to Prof. Dan Pinkel

This filter set is an improvement of multiple fluorescence filter technology. Similar to the triple filter set, a polychromatic beam splitter and a triple bandpass emission filter is used. The excitation is done by single filters for e.g. UV (DAPI), Hoechst, AMVA), blue (FITC) and green (TRITC, Texas Red), which are inserted in a filter slider (manual mode) or in a computer controlled filter wheel. The benefits are:

- = multiple documentation and digital imaging without image shift.
- = differentiated and effective representation of DAPI, AMCA, Hoechst
- = individual exposure for each fluorescence signal
- = optimized focus for each fluorescence
- = avoid bleaching during the evaluation of long-wave fluorescence types.

Compared to multiple fluorescence filter sets, the fluorescence image taken by pinkel filter set has less background noise and the fluorescence signals are more sharp and crisp.

### Multicolor Fluorescence Photomicrography- Possibilities and Limits:

Photomicrography of multilabeled specimens can be done by various methods:

- = double exposure of double labeled specimens by means of single filter sets.
- = single exposure by using double band pass filter sets.

If the intensities of the two fluorochromes are more or less the same, the exposure is done like a single channel fluorescence by means of spot or integrated measurement according to the distribution of the fluorescence.

= double exposure by means of double band pass filter and single band pass filter set. With this procedure, three different fluorochromes can be exposed on one slide.

= single exposure by means of triple band pass filter set. In this case we have the same situation when using double band pass filters. When working with three different probes, very often the three fluorescence signals are not in the same focal plane. The result is that one or two fluorescence signals are not sharp. In this case, we have to use single filters or Pinkel filter sets. Trials and practical experience to date have shown, that triple exposures on high-speed color film still provide acceptable results in conventional photomicrography. Because of signal to noise ratio, the extended exposure time by small and weak fluorescence signals e.g. in cytogenetic fluorescence in situ hybridization (FISH), and the increase of fading, the application of multicolor fluorescence photomicrography is more or less limited to single filter sets, i.e. double exposure with double bandpass filters and including wedge-free emission filters.

### Fluorescence Digital Imaging Microscopy

Because of the increase of background noise, caused by the long exposure time and the fluorescence signals no longer display maximum sharpness, digital imaging techniques are more and more being used to supplement conventional fluorescence documentation. Very weak fluorescent signals can be documented more readily, image reproduction problems often occur when conventional multiexposure photomicrography is applied. The potential of image processing by means of gain and offset facilities the optimized image and analysis of a labeled specimen and quantitative data on signal intensities or measurements can be easily assessed. For high resolution real color digital imaging 3 - CCD cameras are used. For low-light-level applications, e.g. weak fluorescence or small FISH signals, integrating and/or cooled CCD cameras (black and white as well as color) are now available. The cooling system is necessary to reduce the dark current, which will increase by long exposure time. New fluorescence probes like Cyanin 5, Cyanin 5.5, and Cyanin 7 expand the field of fluorescence microscopy into the red and infrared range, where only high sensitive CCD sensors or laser scanner can detect and image this fluorescence signals. These fluorochromes will more or less not fade and no unspecific background fluorescence will appear.

### Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy is designed for obtaining and imaging optical sections through a labeled specimen. By means of optical sectioning, three-dimensional reconstructions and analysis of the specimen can be accomplished by applying appropriate computer software to a stack of digitized images.

### Conclusion and Summary

The wide selection of probes from ultraviolet to the range of red and infrared in combination with CCD sensors, laser scanning microscopy, image processing, and image analysis enable the simultaneous visualization, documentation and analysis of more than three DNA regions in 2 and 3 dimensions. These methods will supplement conventional fluorescence microscopy also by means of ratio imaging. Karyotyping of human chromosomes now by means of 24 color FISH technique.

# **Cellular Function, Differentiation, and Disease**

## EFFECTS OF THE GPIIB/IIIA RECEPTOR ANTAGONIST MK-383 ON RECEPTOR CONFORMATION AND FUNCTION

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The alpha<sub>IIb</sub>beta<sub>3</sub> integrin (Gpllb/IIIa) is expressed on platelets, where it acts mainly as a receptor for fibrinogen playing a critical role in platelet aggregation and thrombus formation, such as in myocardial infarction. Activation of the platelet and the subsequent conformational activation of the Gpllb/IIIa is a prerequisite for ligand binding, and specific antagonists have been developed, in order to block the receptor-ligand interaction. We have characterised the in-vitro effects of the Gpllb/IIIa antagonist MK-383 (tirofiban) on platelets. In a whole blood assay, at a concentration of 10<sup>-7</sup> mol/L, we could demonstrate an inhibition of fibrinogen binding to in vitro stimulated platelets. Simultaneously, although the expression density of the Gpllb/IIIa increased upon incubation with MK-383 similar to increases during platelet activation, no effect on the von Willebrand factor receptor complex and P-selectin expression or the formation of platelet/leukocyte aggregates could be observed indicating that MK-383 binding does not induce platelet activation via outside-in-signaling. We further analyzed the conformation of the Gpllb/IIIa upon in vitro activation or binding of MK-383 using an fluorescence resonance energy transfer (FRET) methodology. We could demonstrate, that the conformation of the Gpllb/IIIa induced by the binding of MK-383 differs from the resting state of the receptor as well as from the conformation induced by activation using ADP or TRAP-6 in absence or presence of fibrinogen binding. These data demonstrate, that Gpllb/IIIa receptor antagonists induce complex conformational changes that may influence the outside-in-signaling function of these receptors by mechanisms independent of receptor activation. Fluorescence resonance energy transfer techniques, therefore, may help to characterize the molecular effects of GPIIb/IIIa receptor antagonists on receptor conformation and its influence on fibrinogen binding in more detail.

## ASSAYS OF CELL FUNCTIONS BY LASER SCANNING CYTOMETRY

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Laser scanning cytometer (LSC) measures rapidly and with high accuracy fluorescence of individual cells at different wavelength and their forward light scattering property. The cells are attached to microscope slides and fluorescence cytoplasm, as well as the fluorescence intensity represented by maximal pixel, all coordinates and time of each measurement. It is possible, therefore, to correlate fluorescence or light scatter parameters with cell morphology and repeatedly measure the same cells. In this study we present the unique utility of LSC for: 1. ANALYSIS L-AMINOPEPTIDASE ACTIVITY AND LYSOSOMAL PROTON PUMP IN PERIPHERAL BLOOD CELLS. LSC measurements have been carried out on individual cells of large population to reveal intercellular variability or the presence of a cell subpopulation with different kinetic properties. The kinetic curves constructed for each measured cell were matched with the respective cells, identified by their position on the slide or classified by their morphology. 2. DETECTION OF ACTIVATION NF-KB, P53 AND BAX. Activation of NF-kB or p53 leads to their rapid translocation from cytoplasm to nucleus while activation of Bax is associated with its translocation from cytosol to mitochondria. Immunocytochemical detection of NF-kB or p53 combined with analysis of the ratio of nuclear to cytoplasmic fluorescence allowed us to measure their translocation and correlate it with cell cycle position. Mitochondrial translocation of Bax, which occurs early during apoptosis, was assayed by the increase in its local density reflected by high maximal pixel values of Bax immunofluorescence 3. ANALYSIS OF MITOCHONDRIAL PERMEABILITY TRANSITION (PT) AND OXIDATIVE STRESS DURING APOPTOSIS. Drop of mitochondrial transmembrane potential and the presence of the reactive oxygen intermediates (ROI) were measured in camptothecin (CPT) treated HL-60 cells. Subsequently, the cells were fixed and subjected to analysis of their cell cycle position and the presence of DNA strand breaks. Multivariate analysis revealed that CPT induced PT or ROI and DNA strand breaks were preferentially in S phase cells. Several attributes of LSC, in particular the possibility of cells relocation after the initial measurement for their morphological examination and/or their subsequent secondary analyses, makes this instrument exceptionally useful in studies of cell function.

## THE ROLE OF CELLULAR PLASMA MEMBRANES IN REDUCTION OF TETRAZOLIUM SALTS, MTT AND CTC

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Cell-mediated reduction of tetrazolium salts to corresponding formazans is considered to be a measure of activity of either intracellular oxidoreductases or redox enzymes located at plasma membranes of cells. Bioreduction of tetrazolium compounds is regarded as an alternative to a clonogenic assay, a thymidine incorporation assay and a plasma membrane integrity assay. However, despite a wide use of tetrazolium viability assays it has not been fully elucidated whether the reduction of tetrazolium salts can affect cell metabolism or plasma membrane integrity and thus influence the parameters that are measured. Moreover it is still uncertain whether different tetrazolium salts may penetrate through biological membranes and consequently if they are reduced by intra- or extracellular redox enzymes. We report evidence indicating that two tetrazolium salts MTT and CTC are reduced to corresponding formazans by HepG2 human hepatoma cells. In intact cells CTC-formazan is formed within or at the outer surface of plasma membranes. However, accumulation of CTC-formazan may inflict damage on plasma membranes. Consequently, loss of plasma membrane integrity is followed by intracellular reduction of CTC. We hypothesise that CTC cannot enter intact cells under optimal growth conditions and is reduced by redox enzyme systems associated with plasma membranes. In contrast to CTC-formazan, crystals of MTT-formazan appear to be formed in the interior of cells with intact plasma membranes. Preliminary data indicate that reduction of MTT is not followed by disruption of plasma membrane integrity. Therefore we hypothesise that MTT may penetrate through intact plasma membranes and is reduced by intracellular oxidoreductases.

## FLOW CYTOMETRIC DETECTION OF CD3<sup>LOW</sup> CELLS IN THE PERIPHERAL BLOOD OF PATIENTS WITH CUTANEOUS T-CELL LYMPHOMA

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While CD4+ CD7- T-cells are well known, other aberrant expressions of T-cell surface molecules have not been described in cutaneous T-cell lymphoma (CTCL). Our purpose was to find out, whether patients with CTCL might have similar defects as in those with other non-Hodgkin lymphomas. Therefore, freshly obtained circulating blood lymphocytes of 11 patients with biopsy proven CTCL were isolated and analyzed with a panel of several mAbs including SK7 directed against CD3ε, a 20kDa transmembrane signaling protein, by using FACS-analysis before starting therapy with retinoid plus photochemotherapy (RePUVA), during and afterwards. 5/11 patients had two distinct T-cell populations as detected by mean fluorescence intensity: CD3ε<sup>high</sup> (normal) and CD3ε<sup>low</sup> cells. These cells could be characterized as follows TCRαβ+ CD3ε<sup>low</sup> CD2+ CD4+ CD5+ CD7- CD8- CD19- CD25- CD45RO+ CD45RA-. Moreover, FACS-analysis of blood samples of patients with non-infectious inflammatory skin diseases ( $n = 256$ ) such as psoriasis, atopic dermatitis, lichen planus, drug induced exanthema, pityriasis lichenoides etc. did not reveal CD3ε<sup>low</sup> cells. During therapy with RePUVA we noticed a reduction and finally disappearance of CD3ε<sup>low</sup> cells, whereas CD3ε<sup>high</sup> cell counts remained stable or increased. Parallel to the cellular restoration clinical improvement with complete response could be observed. The expression of the CD3/TCR complex is under control of PKC, which mediates positive regulation of this multichain receptor complex. Previously it has been shown that retinoids can modulate T cell-derived PKC activity in vitro. Our data suggest an induction of CD3ε expression by RePUVA in vivo, as well.

## EFFECTS OF 5-AMINOLEVULINIC ACID (ALA) INDUCED INTRACELLULAR PROTOPORHYRIN IX (PPIX) -CONTENT AND LOCALISATION ON PHOTODYNAMIC THERAPY (PDT)

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In order to establish guide lines for photodynamic therapy (PDT) *in vivo*, *in vitro* testing of gastrointestinal cell lines was applied. Methods: Three human colon carcinoma cell lines (SW480, HT29, CaCo2) were incubated with ALA. PPIX accumulation was quantified after extraction and localization was visualized by fluorescence microscopy. PDT was performed with an incoherent light source. Cells, irradiated with LD50, were examined by fluorescence microscopy for morphological alterations using a double staining technique, and analysis of DNA strand breaks was performed by the laddering method. For further PDT experiments, each cell line was incubated with defined ALA concentrations, resulting in identical intracellular PPIX. Results: Cell line CaCo2 obtained highest PPIX content, followed by HT29 and SW480. In HT29 PPIX is localized in association to the cell membrane, in SW480 and CaCo2 PPIX colocalizes with mitochondria. Due to rapid photobleaching of PPIX, it is difficult to obtain high performance fluorescence images; by addition of specific iron chelator desferrioxamine (DEF) PPIX content increases, and digital confocal images can be achieved. For all cells, PDT leads to a complete kill at a dose of 15 J/cm<sup>2</sup>. For lower doses, CaCo2 as well as SW480 show a stronger response to PDT than HT29. Phototoxic effects lead to specific cellular morphological alterations. Preliminary data indicate a higher rate of apoptosis in SW480 and CaCo2 in comparison to HT29. At PDT with similar PPIX content, SW480 as well as CaCo2 still showed higher PDT response than HT29. Conclusions: It is shown for the first time that besides absolute PPIX concentration also intracellular localization of the photosensitizer plays an important role in resulting PDT effects. This work was supported by the Wilhelm-Sander-Stiftung (96.081.1)

## INCREASED APOPTOSIS OF CIRCULATING LYMPHOCYTES DURING CARDIAC SURGERY WITH CARDIOPULMONARY BYPASS

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Surgical trauma has been reported to be associated with elevated apoptotic rate of circulating leukocytes. Increased apoptosis leads to temporary anergy of monocytes and macrophages and specific removal of immunocytes and could therefore in part be responsible for reduced immune defense. Effect of cardiac surgery on leukocyte apoptosis has not been shown yet.

In a retrospective study of flow-cytometric data we analyzed blood samples from 90 children (age 3-16 yr.) who underwent cardiac surgery with (65) or without (25) cardiopulmonary bypass based on light scatter and surface antigen (CD45/CD3) expression (*Blood* 83:1268). Additionally, in an *in vitro* assay isolated leukocytes from healthy volunteers were incubated with blood obtained from the same patients before, during and after surgery for 1h at 37°C. Apoptotic rate was measured by AnnexinV using flow cytometry. DNA condensation by laser scanning cytometry LSC.

Patients who underwent surgery with CPB had elevated lymphocyte apoptosis. In particular, T-cell apoptosis increased from 0.45% (baseline) to 1.34% (4h postoperative, ANOVA p=0.0034). No effect was found during and after surgery without CPB. These results were in accordance with *in vitro* findings demonstrating elevated apoptotic activity for lymphocytes and neutrophils in serum from patients with CPB at reperfusion up to 3d after surgery (p<0.01) as measured by flow and laser scanning cytometry. No activity was found without CPB. At present the source for this apoptotic activity is not clear.

Increased apoptosis of circulating lymphocytes and neutrophils further contributes to the immune suppressive response to surgery with CPB by e.g. inactivating phagocytes via CD36 uptake of apoptotic cells. Elevated apoptotic activity in the blood of patients during CPB might also contribute to the destruction of cardiomyocytes during and after pediatric cardiac surgery. (Support: Deutsche Herzstiftung)

## SUBCELLULAR CD95-LIGAND EXPRESSION IN MICROVASCULAR ENDOTHELIAL CELLS, EWING'S TUMORS AND LONG TERM CULTURED T CELLS.

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The Ligand of the CD95 receptor is constitutively expressed in a number of tissues and is involved in the delivery of a death signal via the CD95 receptor. The membrane-bound form of the receptor is unstable since metalloproteinases rapidly cleave off the extracellular part. The soluble CD95-Ligand is less active in stimulating apoptosis. CD95-Ligand is readily detectable by western blotting, ELISA as well as by RT/PCR but screening for surface and cytoplasmic expression by flow cytometry results in a weak and often doubtful staining. Supernatants of cultured endothelial cells lack measurable CD95-Ligand (<10 pg/ml CD95-L), Ewings Tumor cells secrete about 40 pg/ml and T cells secrete 120-1000 pg/ml of soluble CD95-L (measured by MBL ELISA). Using the CD95-L antibody, M33 (Transduction Laboratories), we compared the CD95-Ligand in fixed cells using flow cytometry (FACScalibur, BD), standard immunohistochemistry and confocal laser scanning microscopy (TCS-NT Leica). In microvascular endothelial cells, the immuno-histochemical evaluation gave a strong staining of CD95-Ligand in a characteristic net-like pattern that resembled the localisation of mitochondria or secretory vesicles following structures of the cytoskeleton. This pattern was less prominent in Ewing's tumor cells as well as long-term cultured T cells especially when considering the perinuclear web. Following treatment with actinomycin D, the structural organization of CD95-Ligand staining disappeared in endothelial cells and perinuclear vesicles stained for CD95-L, instead. According to these morphological criteria, CD95-Ligand may constitute a secretory molecule rather than a membrane associated receptor. Possibly, soluble CD95-Ligand is active in protecting cells against CD95 triggered apoptosis.

## CELLULAR EFFECTS OF 5-AMINOLEVULINIC ACID (ALA) - INDUCED PROTOPORPHYRIN XI (PPIX) - MEDIATED PHOTODYNAMIC THERAPY (PDT) ON HUMAN UROTHELIAL CELL LINES

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**Aim:** PPIX-mediated photodynamic treatment of dysplastic lesions of the urothelium is an intriguing alternative to current intravesical chemotherapy. The aim of our study was to investigate cellular PDT effects as well as pathways to cell death in order to define optimal treatment modalities. **Methods:** Highly differentiated RT4 cells (as a model for a papillary tumor) and UROtsa cells (representing normal urothelium) were incubated with ALA (100-200  $\mu$ g/ml, 1-3 h) to determine cellular PPIX content, PPIX localisation as well as the response to photodynamic treatment under various illumination conditions. Parameters such as plasma membrane integrity (via propidium iodide exclusion) and potential (via the potential sensitive dye DiBAC<sub>4</sub>(3)) as well as mitochondrial activity (using the J-aggregate forming dye JC-1) were investigated. Alterations of these parameters are correlated to the mode of cell death (apoptosis or necrosis) utilizing annexin V-, tunel-assay and/or acridinorange staining for nuclear fragmentation. **Results:** PPIX accumulation was higher in RT4 cells than in UROtsa, while sensitizer distribution was very similar (3 h), and therefore the response to PDT was stronger for RT4 cells. No significant improvement of PDT could be achieved by applying fractionated light doses, neither for short nor for longer dark times. Preliminary data indicate that alterations of the plasma membrane and mitochondria are both light dose and incubation time dependent with the plasma membrane being more involved at longer incubation periods. **Conclusion:** Following PDT, alterations in plasma membrane or mitochondria are dose dependent and lead to distinct modes of cell death. Based on these facts optimal treatment modalities can be defined including both complete destruction of tumor cells as well as minimized urothelial damages.

## MAPPING OF CELL SURFACE DISTRIBUTION OF ERBB PROTEINS IN BREAST CANCER CELLS.

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The erbB proteins belong to the type I transmembrane tyrosine kinase family receptors. Out of the four members (erbB1 or epidermal growth factor receptor, erbB2, erbB3 and erbB4) erbB2 molecules play central roles in signal transduction processes involving these receptors.

Overexpression of erbB2 is linked to higher transforming activity, increased metastatic potential, and angiogenesis and drug resistance of breast tumors in laboratory experiments. As corollary of these properties, erbB2 amplification is generally thought to be associated with a poor prognosis of breast cancer patients.

The non-stochastic cell surface distribution of erbB2 was analyzed with various biophysical techniques such as flow cytometric and image cytometric fluorescence resonance energy transfer (FRET) methods and scanning near-field optical microscopy (SNOM). While FRET provides information about molecular associations in the 2-10 nanometer range, SNOM is a useful tool for investigating a long-range lateral distribution of membrane proteins in the 100-1000 nanometer range.

Combination of these techniques revealed that erbB2 showed high degree of homo- and heteroassociations in the nanometer scale. In addition, erbB2 was found to be concentrated in irregular membrane patches with a mean diameter of approximately 500 nanometer in non-activated human breast tumor cells. Activation of cells with epidermal growth factor, heregulin as well as a partially agonistic anti-erbB2 monoclonal antibody led to an increase in the mean cluster diameter to 600-900 nanometer. It is assumed that an increase in cluster size may constitute a general phenomenon in the activation of erbB molecules.

## C-REACTIVE PROTEIN (CRP) IN ATHEROGENESIS

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CRP is emerging as a key mediator (complement activation, lipoprotein opsonization, monocyte chemotaxis) of inflammatory processes already in early atherogenesis. CRP binding studies *in vitro* have demonstrated the presence of a specific receptor on leukocytes (CRP-R) in addition to CRP binding to the Fc $\square$ RI (CD64). In the present study we have investigated peripheral blood monocyte subpopulations of healthy donors and patients with acute phase reactions by flow cytometry using a mAb against the leukocyte CRP-R as well as a polyclonal anti-CRP antibody. A distinct subpopulation of CD14<sup>bright</sup> monocytes showed enhanced CRP binding and expression of the CRP-R, in particular under acute phase reactions. Furthermore, we assessed CRP-binding during loading of freshly isolated monocytes with enzymatically (trypsin, cholesterol esterase, neuraminidase) modified LDL (E-LDL). Binding of CRP but not expression of CD64 was markedly enhanced on E-LDL-loaded monocyte-derived macrophages compared to non-loaded cells. These results demonstrate that CRP binds to the CD14<sup>bright</sup> peripheral blood monocyte subpopulation carrying the CRP-R as well as E-LDL loaded macrophages. Thus, modulation of CRP binding to monocytes/ macrophages might be an important therapeutic target for inhibition of inflammatory mechanisms induced by CRP deposition in the arterial wall.

## PREDICTIVE MEDICINE BY PATTERN ANALYSIS (CLASSIF1) OF CYTOMETRIC AND OTHER DATA

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**Predictions** of disease development for **individuals** patient is frequently very important for therapy choice, early detection of therapy success or failure and estimation on final disease outcome. **Prognosis** in medicine is unfortunately mostly based on **statistical** analysis of many patients with a probability and confidence limit for the individual patients future disease development, it is clear that individual predictions are preferable to statistical prognosis.

Biochemical alterations in cellular systems or organs are the basis for disease development i.e. the cytometric determination of cell biochemical changes should provide **predictive** as well as **diagnostic** information which is more directly linked to the disease process as e.g. the change of humoral parameters in the peripheral blood or in the urine.

The flow cytometric determination of cell function parameters in granulo- and monocytes (DHR123 burst, proteinases) in combination with the exhaustive list mode analysis and data pattern analysis provides the final outcome for **septically admitted ICU patients** already on the admission day under the presently optimal therapy. The increased lead time provided by data pattern analysis may permit to specifically improve the therapy for early identified risk patients.

**Postoperative Capillary Leak (CLS)** patients in children cardiac surgery can be identified quite reliably by data pattern analysis on preoperative humoral blood parameters. For reasons of time, amount of required blood for analysis and analysis costs, it was investigated whether the analysis of flow cytometric parameters of blood leukocytes could improve the practicality of preoperative predictions. A first, hand evaluated flow cytometric data set shows that the purely cytometric approach is, indeed, promising. Short analysis time, low required blood volume and overall cost reduction are attractive.

Duke's stageing has a recognized prognostic value in **colorectal cancer** patients at surgery. Although useful, the sensitivity/specificity between 65-70% with positive/negative predictive values in the order of 60-65% is insufficient in many instances for practical consequences. The photometric measurement of G6PDH activity in conjunction with superoxide dismutase (SOD) and lipid peroxidation analysis on cancer and normal areas in frozen tissue sections increases the positive predictive value to 100% at a sensitivity of around 65%. This model example indicates that the pathologist may develop a significant predictive potential as soon as the diagnostically relevant standard stains are supplemented by biochemically specific molecular indicator stains or reactions.

# Microorganisms

## THE CHARACTERIZATION OF PHYTOPLANKTON POPULATIONS USING FLOW CYTOMETRY AND LASER SCANNING CYTOMETRY

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The flow cytometric identification of different phytoplankton populations by chlorophyll pigment analysis allows a rapid identification of different phytoplankton groups. Either with two laser excitation using 528nm and 632nm and an emission wavelength of longer than 650nm for each laser or a single laser of 488nm and different emission wavelength bands ranging from green to the near infrared, a detailed study of associated chlorophyll pigments for identification of various phytoplankton groups is possible. This allows to give a rapid overview of the plankton inventory of lakes and rivers as well. However, if it is necessary to visualize different plankton groups for detailed taxonomic determination, flow cytometry is a relatively slow process. Each population has to be gated out and sorted onto a microscopic slide, which has to be looked at by visual microscopy and/or image analysis.

For the taxonomic identification by microscopy the new technology of laser scanning cytometry (LSC) gives a more rapid approach. Using either two laser(488nm and 632nm) or single laser (488nm) scanning, a dotplot to identify chlorophyll pigment groups (as well as Phycoerithrin) is created and the population can be directly visualized after scanning. Phasecontrast objectives in combination with a phasecontrast condenser can be added to the LSC to give detailed images of the algal cells for a taxonomic analysis. For LSC measurement the slide preparation is facilitated using a cytospin centrifuge with a volume of 0.5ml for eu- or mesotrophic lakes and of 6ml for oligotrophic lakes with a very low cell density.

In our report we compare data from the pigment analysis by flow cytometry and laser scanning cytometry for different lakes around Berlin and for three lakes with and without artificial aeration near Heilbronn. In order to calibrate the chlorophyll pigment definition, cultures of phytoplankton (*chlorella v.*, *Synechococcus leonis* and *navicula*) have been used.

## IN-SITU MIKROSKOPIE BEI DER KULTIVIERUNG VON MIKROORGANISMEN

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Digitale Bildaufnahme- und Bildanalysesysteme werden in zunehmendem Maße in den unterschiedlichsten Aufgabengebieten eingesetzt. In diesem Vortrag wird die Entwicklung eines preiswerten, online-fähigen Bildanalysesystems für die Überwachung eines biotechnologischen Kultivierungsprozesses vorgestellt. Als Meßobjekte werden Tier- und Hefezellen (BHK, CHO, *S. cerevisiae*) verwendet, deren Zustand während des Kultivierungsverlaufs am Ort ihres Entstehens innerhalb eines Bioreaktors beobachtet wird. Dem Einsatz eines automatischen Bildanalysesystems kommt dabei große Bedeutung zu, weil dadurch Kontamination des Reaktors bei der Probenahme, Meßfehler durch Überlastung des auswertenden Personals oder Subjektivität beim Auszählen von Zellproben vermieden werden. Eine kontinuierliche Beobachtung des Kultivierungsprozesses bei geringem Personalaufwand wird möglich. Zur Bildaufnahme innerhalb des Bioreaktors wird ein spezielles Lichtmikroskop, ein sogenanntes In-situ Mikroskop, entwickelt, das in Verbindung mit einer angeschlossenen CCD-Kamera und einem Framegrabber die Aufnahmeeinheit darstellt. Anforderungen an das In-situ Mikroskop sind u.a. Miniaturisierbarkeit, Sterilisierbarkeit und leichte Handhabbarkeit. Nach der Aufnahme wird das Bild unter Verwendung von selbst entwickelten Bildverarbeitungsroutinen automatisch ausgewertet. Das Bild wird auf Zellzahl, -größenverteilung und Zellmorphologie (Einzelzellen, Doppelzellen, Zellcluster) untersucht. Untersuchungen mit der In-situ Mikroskopie ergaben, daß die erhaltenen Daten gut mit denen klassischer Analysemethoden, wie Zellzählung mit der Thomakammer oder Messung der optischen Dichte, korrelieren. Zellkonzentrationen bis 2E8 Zellen/mL können bestimmt werden. Weiterhin wird eine Online-Erfassung der Zellmorphologie durch die In-situ Mikroskopie erst möglich.

## FLOW CYTOMETRY ALS METHODE ZUR PROZEßIDENTIFIKATION AM BEISPIEL DER SYNTHESE VON POLY- $\beta$ -HYDROXY-BUTTERSÄURE (PHB) DURCH *METHYLOBACTERIUM RHODESIANUM* MB126

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Bakterielle Syntheseprozesse, bei denen gewünschte mikrobielle Leistungen an bestimmte Zustände im Wachstums- und Vermehrungszyklus gekoppelt sind, erlangen heutzutage immer größeres wissenschaftliches und wirtschaftliches Interesse. Um diese Prozesse steuern zu können und damit Stabilität und Reproduzierbarkeit zu erreichen sowie bestimmte Zielfunktionen (Produktivität, Produktqualität) zu optimieren, sind Informationen über den physiologischen Zustand der mikrobiellen Population erforderlich.

Integrale Messungen (Bestimmung des CO<sub>2</sub>-Gehalts in der Abluft, Ermittlung des intrazellulären Produktgehalts) liefern nur Mittelwerte über die Population, eine Verteilung über verschiedene Zustände der Einzelzellen kann nur über lokale Verfahren ermittelt werden.

Zur lokalen Untersuchung der PHB-Synthese durch den Bakterienstamm *Methylobacterium rhodesianum* MB126 wurden Informationen über unterschiedliche Zellzustände innerhalb einer mikrobiellen Population mit Hilfe der Methode der Flow Cytometry gewonnen.

Die Steuerung bzw. Regelung eines biotechnologischen Prozesses erfordert vorzugsweise online-Informationen über die inhärenten biologischen Zustände. Da aber die Flow Cytometry für online-Analysen nicht oder nur beschränkt einsetzbar ist, muß eine diesbezügliche Zustandserkennung durch den Einsatz von Methoden der Informationsverarbeitung (Prognosefilter) und der experimentellen Prozeßanalyse (Erweitertes Kalman Filter) realisiert werden.

Dazu wurden einerseits die lokalen Informationen mit den Ergebnissen der Standardverfahren (integrale Untersuchungen) korreliert und andererseits ein mathematisches Modell entwickelt, welches die flow-cytometrisch gewonnenen Informationen über die Zustandsverteilung innerhalb der mikrobiellen Population enthält.

Auf dieser Grundlage lassen sich Prozeßführungsstrategien entwerfen, die zustandsabhängige Leistungen berücksichtigen und somit zur Optimierung der oben angesprochenen Zielfunktionen beitragen können.

# DURCHFLUßZYTOMETRISCHE BESTIMMUNG DER FLUIDITÄT ZUR CHARAKTERISIERUNG DER STRUKTUREN BAKTERIELLER MEMBRANEN

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In biotechnologischen Prozessen ist es von großem Interesse, die Lebendigkeit und die damit verbundenen Stoffwechselleistungen von Bakterienzellen abschätzen zu können. Der Ausdruck "Lebendigkeit" beschreibt eine Vielfalt physiologischer Zustände von bakteriellen Einzelzellen. Die meisten Methoden, die zur Bestimmung der Lebendigkeit angewandt werden (z.B. die Messung des Membranpotentials und die Bestimmung des DNA Gehaltes) eignen sich vorrangig zur Charakterisierung stoffwechselintensiver Zellzustände. Es sollten deshalb Methoden angewendet werden, die auch Zellen mit geringer Stoffwechselaktivität oder im Ruhezustand befindliche erfassen und beschreiben können.

Die Zusammensetzung der Membranlipide ändert sich charakteristisch im Verlauf des Zellzyklus sowie in Abhängigkeit der externen Wachstumsbedingungen. Diese Änderungen beeinflussen die Fluidität der Membran. Unter Verwendung des Fluoreszenzfarbstoffes 1,6 - Diphenyl-1,3,5-hexatrien (DPH) wurde eine Methode entwickelt, die für die flow cytometrische Untersuchung der Fluidität bakterieller Zellen geeignet ist. DPH interkaliert zwischen die Kohlenwasserstoffketten der Membranlipide. Die Menge des eingelagerten Farbstoffes ist abhängig von Struktur und Beweglichkeit der Membranmoleküle und wurde als Fluiditäts-bezogene Fluoreszenzeintensität bestimmt.

Die Methode wurde an einem häufig genutztem Modellorganismus, *Acinetobacter calcoaceticus* 69-V, der unter verschiedenen äußeren Bedingungen wuchs, getestet.

## Poster Exhibition

**EVALUATION DER QUANTIFIZIERUNG DER CD38-EXPRESSION  
(ANTIBODY BINDING CAPACITY) AUF T-SUPPRESSORZELLEN UNTER  
DEN BESONDEREN BEDINGUNGEN DES EINSENDELABORS**

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Publikationen der Gruppe um J. Giorgi zeigen, dass die Quantifizierung der CD38 Expression prognostische Bedeutung hinsichtlich des Krankheitsverlaufs bei HIV-Infizierten haben kann (J Acquir Immune Defic Syndr Hum Retrovirol 18:332-340, 1998). Im Routinelabor und speziell im Einsendelabor werden ärobuste Methoden mit Hinblick auf Praktikabilität, Reproduzierbarkeit, Probenalter und Stabilität benötigt. Wie die Arbeit von S. Iyer (Cytometry 33:206-212, 1998) zeigt, sollte die Quantifizierung der CD38-Expression bei Verwendung von QuantiBrite® Beads und ärepurified PE-Konjugaten des CD38-Antikörpers (Becton Dickinson) die Kriterien einer Routinebestimmung erfüllen. In den zitierten Arbeiten wurde das Blut innerhalb 6 bzw. 4 h aufbereitet. Im Einsendelabor ist ein Probenalter von etwa 24 h anzunehmen. Die für die Lymphozytentifferenzierung etablierte Aufarbeitung von Vollblutproben (fixierende Erythrozytenlyse) wurde hinsichtlich der Inkubationszeiten an die für CD38 publizierten Werte angepasst. Bei über 70 Blutproben (überwiegend HIV-Infizierte, aber auch andere Patienten und Normalpersonen) bei denen innerhalb von 6 h nach Blutentnahme eine Erstpräparation durchgeführt werden konnte, wurde 24 h später eine 2. Präparation aus dem Restblut vorgenommen. Bei ca. 100 präparierten Proben wurde vor der Messung ein Aliquot abgetrennt, 24 h im Kühlschrank aufbewahrt und dann gemessen. Durch parallele Ansätze wurden die Variabilität der Methode und die Variabilität zwischen zwei Konjugationschargen abgeschätzt. Der Vergleich zwischen Erst- und Zweitpräparation (24 h später) zeigt eine leichte Reduktion der CD38-Antikörperbindung. Die klinische Aussage wird jedoch nicht beeinflusst, sofern der Parameter als dynamischer und nicht als statischer Wert verstanden wird. Die Ergebnisse der Parallelansätze und die Untersuchung zur Stabilität gefährbarer Proben zeigen eine ausreichend hohe Reproduzierbarkeit, so dass die Methode im Einsendelabor durchführbar ist.

**IMMUNOLOGICAL DIFFERENCES BETWEEN IDIOPATHIC CD4+ T LYMPHOCYTOPENIA (ICL) AND HLV-INFECTED**

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The cause of idiopathic CD4+ T-lymphocytopenia (ICL) is still unclear. In a 61-year-old male patient we found CD4+ T cell counts below of 300/ $\mu$ l (range 111 - 296/ $\mu$ l). HIV-tests for both type 1 and 2 by using ELISA, western blot, HIV-core antigen (p24) analysis and PCR were repeatedly negative. Moreover, other viral infections such as herpes simplex, varicella zoster, HHV-6, EBV, CMV, hepatitis B and C could be excluded. In order to get detailed insight into immunological interactions, we monitored a variety of immunological parameters in the patient with ICL over a period of more than 5 years and compared the results to those obtained from HIV-infected patients ( $n = 256$ ) in different stages. Our data show that: (i) contrary to HIV-infection CD3+CD4+ cell counts remained stable during long-term observation in ICL; (ii) ICL had mainly CD4+ cells with Th1-cytokine pattern and HIV mainly Th2 as detected by flow cytometry and intracytoplasmatic anticytokine staining and by mRNA determination after PMA and ionomycin in vitro stimulation; (iii) Ig-bearing CD4+ cells could be detected only in HIV-patients by a special assay (Immun Lett 41:163-167); (iv) CD95 expression on CD3+ subsets was normal in ICL and increased in HIV; (v) only ICL had a profound deficiency of CD4+CD45RO+ and CD4+CD45 RA+ cells, while T-cells expressing the intermediate stage CD4+CD45RO+ CD45RA+ were increased; (vi) CD3+CD8+ cell counts were normal in ICL and increased in HIV ( $p < 0.00001$ ); (vii) HLA-DR expression on CD3+ cells was increased in HIV and normal in ICL ( $p < 0.05$ ); (viii) hypergammaglobulinaemia was a prerequisite of HIV-infection in different stages. Surprisingly, despite of normal folic acid levels (serum and intraerythrocytic) a substitution led to significantly increasing CD4+ T-cell counts (mean  $\pm$  SEM:  $342.4 \pm 15.8$  vs.  $283.3 \pm 13.0$ ,  $p < 0.008$ ) in the patient with ICL. Our data show profound immunological differences between HIV-infection and ICL. Although the pathomechanisms of both ICL and glossopyrosis are still unclear, based on recent data from the literature it seems likely that they represent heterogeneous conditions. Taken together these findings indicate rather a disturbed folic acid utilization than an underlying unknown viral infection in the pathogenesis of ICL.

## DNA CONTENT IN HUMAN SPERMATOZOA AND IN VITRO FERTILISATION EFFECTS

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Over the past ten years the diagnosis and treatment of man infertility has advanced rapidly, with the introduction of new technology such as in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). Consequently there is much interest in developing laboratory tests of sperm to predict the final results of assisted reproduction. The aim of the study was to determine the correlation between DNA content in human spermatozoa and fertilisation rates in vitro. Semen samples were obtained from 31 men from couples undergoing IVF treatment. Ejaculated spermatozoa were selected by Percoll gradient centrifugation. The DNA content in spermatozoa was estimated by computer imaging technique and flow cytometry. Cells were stained by Feulgen method (for image analysis) or by acridine orange (for flow cytometry). As an internal standard the human lymphocytes were used. The histograms of DNA content in spermatozoa obtained from each patient were tested for normality using W Shapiro-Wilk test. We found identifiably marked deviations in DNA distribution (aneuploid content, haploid content with additional peaks, asymmetric distribution) in cases where fertilisation of oocytes in an IVF program had been not observed. The results suggest that evaluation of DNA content in spermatozoa can be used in diagnostic of human semen classified for in vitro fertilisation program.

## MIKRONUKLEI UND ZELLZYKLUSVERZÖGERUNG ALS PARAMETER ZUR BESTIMMUNG DER INDIVIDUELLEN STRAHLENEMPFINDLICHKEIT

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Die individuelle, genetisch festgelegte Empfindlichkeit von Personen gegenüber ionisierender Strahlung kann stark variieren. Idealerweise müßte dies bei Strahlentherapiepatienten berücksichtigt werden, da trotz identischer physikalischer Dosis das Risiko für Komplikationen unterschiedlich sein könnte. Mit prädiktiven Testverfahren ließen sich besonders strahlenempfindliche Personen von einer möglicherweise zu sehr belastenden Therapie ausschließen.

Ein Maß für die Strahlenempfindlichkeit von Patienten ist die SF2, die Überlebensrate von Zellen nach einer Bestrahlung mit 2 Gy. Da mit herkömmlichen Untersuchungsmethoden, wie dem Koloniebildungstest, Ergebnisse erst nach ca. 1 Woche feststehen, ist es sinnvoll, neue Testverfahren zu etablieren, deren Ergebnisse schneller zur Verfügung stehen. Eine schnelle und sensitive Testmethode ist die Bildung von Mikrokernen. 72 Stunden nach der Bestrahlung können die Mikrokernmessungen mit dem Durchflußzytometer mit einer Rate bis zu 10000 Zellen pro Sekunde durchgeführt werden.

Vollblutkulturen wurden 24 h nach PHA-Stimulierung mit unterschiedlichen Dosen röntgenbestrahlt (Müller MG150, 100 kV, 10 mA, 1 mm Al-Filter, FHA 31 cm). 72 h nach der Bestrahlung wurden die Lymphozyten über einen Ficollgradienten isoliert und ihre Zellkerne mit DAPI herausgelöst, gefärbt und im Durchflußzytometer bei zwei Wellenlängen gemessen. Die Dosiseffektcurven mehrerer Normalprobanden zeigen ähnliche Verläufe. In den mit 2 Gy bestrahlten Proben befanden sich nach Abzug der Kontrollwerte zwischen 10 und 12 % aller erfaßten DNA-Signale als Mikrokeme. Mit steigender Dosis wurde nach 72h auch eine Abnahme der Zellen im 2. Teilungszyklus beobachtet: Während bei Kontrollsätzen 70 % der Zellen im 2. Zyklus waren, nahm diese Population bei 2 Gy auf 60 % und bei 5 Gy auf 30 % ab. Beide Parameter, die relative Anzahl der Mikrokerne und das verzögerte Eintreten in den 2. und 3. Teilungszyklus, scheinen für die Beurteilung der individuellen Strahlenempfindlichkeit geeignet zu sein und sollen in Zukunft an Patientenproben in Kombination mit klinischen Daten verglichen werden.

## **INDICATION FOR AN ALTERED TRANSFER OF LYMPHOCYTE SUBSETS FROM BLOOD INTO CEREBROSPINAL FLUID (CSF) IN AGING HUMANS**

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Increased leakage of human blood-brain barrier (bbb) was found in aging normal humans with respect to proteins, e.g. IgG and albumin. As transfer of lymphocytes from blood into CSF takes place at the same barriers, e.g. at the blood/CSF barrier and the bbb, transfer of lymphocyte subsets from blood into CSF was studied here in aging control individuals.

Flow cytometry with FITC- and PE-conjugated monoclonal antibody reagents (Becton Dickinson) was performed in venous blood after lysis and, adapted to low cell counts, in CSF using LDS-751 to restrict the collected data to nucleated cells only.

Adaptation of flow cytometric procedure proved to be sufficiently precise, sensitive and accurate to analyse low lymphocyte counts in CSF. Blood/CSF ratio analysis of 4 major and 6 minor lymphocyte subset counts indicated with control individuals that

-- transfers of NK cells (CD16+56+3-) and their CD8+3- subset, as well as of B cells were barred to central nervous system (CNS) compared to that of T cells;  
-- transfer of immature CD8+4+ T cells was facilitated.

Results with aging human control individuals indicate that

-- transfer of CD3+ HLA-DR- and CD4+3+ T subsets decreased significantly with age;

-- transfer of CD8+3+, NK cells (CD16+56+3-), and B cells showed no significant dependency upon age of individuals studied here.

Conclusion: The data indicate an altered immune surveillance of the aging human brain with respect to the induction of cellular immune responses in the elderly e.g. by an reduced supplying with helper/inducer T subsets and/or with non-activated T cells.

Further experiments are needed to confirm and support the results.

## **DIFFERENT BLOOD/CEREBROSPINAL FLUID (CSF) RATIOS OF HUMAN LYMPHOCYTE SUBSETS**

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Immune surveillance of the human central nervous system (CNS) is controlled by at least two barriers: endothelial blood/brain barrier and choroidal blood/CSF barrier. Blood/CSF barrier for total lymphocytes averages  $\approx 2000 : 1$  with adult control individuals.

To analyse lymphocytes subsets, flow cytometry was performed with FITC- and PE-conjugated monoclonal antibody reagents (Becton Dickinson) in venous blood after lysis; it was adapted to low cell counts in CSF using LDS-751 to restrict the collected data to nucleated cells only.

Adaptation of flow cytometric procedure proved to be sufficiently precise, sensitive and accurate to analyse low lymphocyte counts in CSF. 4 major and 6 minor lymphocyte subsets were detected. With control subjects, blood/CSF ratios increased in the order: CD8+4+ << CD3+4+ o CD3+ HLA-DR- o CD3+8+ < CD3+ HLA-DR+ < CD3+16+56+ < CD16+56+3- < CD8+3- << CD19+3- subsets. The data indicate a selecting effect of the barriers on lymphocyte trafficking into CNS; transfer of T lymphocytes was prefered. - The ratios were altered in CSF from patients suffering from various CNS inflammation disorders: blood/CSF ratios of the subsets were lower, but arrangement of the ratio pattern (see above) was similar like that found with control subjects in lumbar CSF; it was altered to some extent in ventricular CSF of patients suffering from different inflammatory disorders. The data indicate different leakage effects of the barriers to lymphocyte subsets in CNS during various inflammation disorders.

Conclusion: Cellular immune surveillance of human CNS is investigated with adapted flow cytometry of lymphocytes by evaluating blood/CSF ratios of 9 subset cell counts. Transfer of T lymphocytes is prefered through the barriers with controls and with diverse inflammation disorders of CNS, too.

## THE FLOW CYTOMETRIC CROSSMATCH IN RENAL TRANSPLANTATION

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Prevention of antibody-mediated rejection by the pretransplant crossmatch is a firmly established concept in renal transplantation. Several retrospective studies have reported that a positive FCXM (flow cytometry crossmatch) and CDC (complement-dependent cytotoxicity) negative is associated with rejection episodes and graft failure.

Flow cytometry (FCXM) is a more sensitive method, which detects complement-fixing antibodies as well as non-complement-fixing antibodies. Identification of type of cells detectable by flow cytometry was determined by two-color fluorescence. Flow cytometry was performed utilizing a FACSCalibur (Becton Dickinson) and data was analyzed using CellQuest software. The study was performed in patients crossmatched with cells from living donors by both FCXM and CDC.

The results showed that when the T cell crossmatch was negative by FCXM it was always negative by CDC. Some of patients with negative CDC B cell crossmatch had positive FCXM. Flow cytometry not only can detect subliminal levels of presensitization, but can also readily distinguish between anti-T and anti-B lymphocyte antibodies. Our preliminary data indicates that the FCXM offers an additional approach for identifying sensitized patients at risk of early renal allograft loss.

## STUDY ON ASSOCIATION BETWEEN DNA CONTENT AND SPERM MORPHOLOGY OF MALES WITH UNEXPLAINED INFERTILITY.

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In the recent years the image cytometry (ICM) is used to measure nuclear DNA content in human cells. It seems that aneuploid DNA content in human spermatozoa can influence their biological value. The study aimed to evaluate the DNA content in human spermatozoa in association with their morphology. The test group comprised 72 males with oligozoospermia and unexplained infertility during at least 2 years. The control group comprised 7 healthy donors whose semen parameters were within the normal range established by World Health Organisation (WHO, 1992). Sperm morphology was observed using H+E staining. The Feulgen reaction was used to stain nuclear DNA in spermatozoa. As an internal standard the human lymphocytes were used. DNA content was estimated by Computer Image Analyser MagiCal. The DNA index (DI) related the measured values of DNA ploidy in spermatozoa to standard (diploid) cells. The results for control group showed the haploid DNA content with symmetric and nearly symmetric histograms. In the test group, the 77% of patients with oligozoospermia showed the abnormal DNA content with DI demonstrating the values characteristic of aneuploidy. There was significant negative correlation ( $P<0.05$ ) between spermatozoa with aneuploid DNA content and normal morphology and a small positive correlation relating to spermatozoa with big head and aneuploid DNA content. Our conclusion is that for patients with increased percentage of spermatozoa with abnormal morphology the estimation of DNA ploidy is advisable.

## OPTICAL TOOLS IN LASER SCANNING MICROSCOPY OF CULTURED MAMMALIAN CELLS

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In order to study structures in living mammalian cells it is often necessary to fix the object of interest spatially for a defined time. In a LSM this is especially necessary because the image acquisition time is comparably long (up to the minute range).

As an example the change of the pH-value of vesicles in pancreatic acinar cells was investigated. Those cells segregate preliminary stages of digestion enzymes that are collected in vesicles. The vesicles pass through a maturation process, while the vesicle interior is strongly acidified to hold the enzymes inactive. The vesicles approaches a specific place near the cell wall where exocytosis can take place.

The fluorescence dye BCECF shows a pH-dependent increase in fluorescence intensity in the range of pH 6 to pH 8 (excitation 488 nm with an argon ion laser, emission via 505 nm long pass filter). The acidic vesicles are therefore dark gaps in the cytoplasm. Using an optical tweezers, one can hold such vesicles during scanning the cell in the LSM. For this purpose a Nd:YAG laser was coupled into a Zeiss LSM 510.

## FLOW CYTOMETRIC TECHNIQUES TO CHARACTERISE PHYSIOLOGICAL STATES OF ACINETOBACTER CALCOACIDUS

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Monitoring biotechnological processes involves acquiring information about key metabolic events and, ideally, single cell states should be determined to obtain comprehensive data on the physiological status of the measured population. Growth stages of the strain *Acinetobacter calcoacidus* were characterised at the single cell level using flow cytometry. Flow cytometric analysing bacterial cellular characteristics by fluorescence were compared with respect to their sensitivity to changes in the physiological status induced by changing micro-environmental conditions. DNA analysis was considered to be highly informative with regard to the multiplication activity of the population. Measuring the membrane potential related fluorescence intensity (FRFI) and the rRNA content were found to be useful for describing high-activity cells. A method for the measurement of the fluidity related fluorescence intensity (FRFI) was developed, since it allowed changes in the fluidity of the bacterial membrane to be detected, and thereby provided a valuable measure of the early adaptation of the population to micro-environmental deviations from standard growth conditions.

**INTERAKTIONEN ZWISCHEN MONOZYTEN AUS DEM PERIPHEREN BLUT  
VON PATIENTEN MIT ARTHRITIS PSORIATICA UND KULTIVIERTEN  
HUMANEN DERMALEN MIKROVASKULÄREN ENDOTHELZELLEN.**

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**Zielsetzung:** Die Adhäsion und Migration von Monozyten (MON) von Patienten mit Arthritis psoriatica (PsA) im Vergleich zu Kontrollpersonen (KONTR) wurde mittels *in vitro*-Kokultivationsmodellen mit humanen dermalen mikrovaskulären Endothelzellen (HDMEC) untersucht.

**Methodik:**

4. Bestimmung der Oberflächenexpression von Adhäsionsmolekülen und Aktivierungsmarkern von MON aus dem peripheren Blut (kurz nach Isolation sowie nach 24-stündiger Aktivierung mit Lipopolysaccharid (LPS)) bei PsA und KONTR. mittels quantitativer Flußzytometrie.
5. Kokultivierungsuntersuchungen zur Bestimmung der Adhäsion von MON an sowie der Transmigration durch ruhende oder TNF- $\alpha$ -aktivierte HDMEC.
6. Nachweis der Zytokine IL-6 und IL-8 im Überstand der kokultivierten Zellen mittels ELISA.

**Die Ergebnisse erbrachten**

4. eine signifikant verminderte Expression von CD-11b an nicht-aktivierten MON von PsA im Vergleich zu KONTR-MON.
5. eine signifikante Erniedrigung der Adhäsion an und der Migration durch aktivierte HDMEC bei PsA gegenüber KONTR.
6. eine signifikant erhöhte Produktion von IL-6 und IL-8 nach Aktivierung der HDMEC durch LPS, unabhängig davon, ob die MON von PsA oder KONTR stammten. Die IL-8-Produktion im Überstand von Kokulturen der MON von PsA mit HDMEC war im Vergleich zur IL-8-Synthese in Kokulturen von nicht-aktivierten MON von KONTR mit HDMEC signifikant vermindert.

Diese Ergebnisse unterstützen die Vorstellung, daß bei PsA systemische Veränderungen des Immunsystems an der Pathogenese dieser Erkrankung beteiligt sein könnten.

**DURCHFLUßZYTOMETRIE IN DER MIKROBIOLOGISCHEN DIAGNOSTIK**

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Infektionen mit multiresistenten Keimen erfordern in der Klinik eine möglichst schnelle Erfassung dieser Erreger. Dabei kommt einer Verkürzung der Analysenzeit durch die direkte Verwendung von Nativmaterial besondere Bedeutung zu. Auch spielt die Reduktion der für die Analyse benötigten Keimmenge eine Rolle, da zeitintensive Vermehrungszyklen auf ein Mindestmaß verringert werden. Zur Wahl der geeigneten Antibiotika ist eine rasche Information über das Resistenzverhalten des Bakterienstamms notwendig. Eine frühzeitige gezielte Therapie hat für den Patienten fundamentale Bedeutung, da so Sekundärschäden durch die Dauer der Infektion und eine mögliche nosokomiale Ausbreitung der Infektion vermieden werden.

Eine Möglichkeit zur Identifizierung der Keime in Direktmaterial ist der Nachweis der Erreger über spezifische Membranbausteine, Antigene oder spezifische Reaktionen.

Mit Hilfe des membranpotentialsensitiven Farbstoffes DiBAC<sub>4</sub>(3) kann innerhalb 90 Minuten die Empfindlichkeit eines Erregers für verschiedene Antibiotika getestet werden. Für die am häufigsten vorkommenden humanpathogenen Keime wurden bereits zuverlässige Antibiogramme erstellt. Ein solches Verfahren zur Identifizierung und Empfindlichkeits-testung von multiresistenten Keimen direkt aus Patientenmaterial wurde am Beispiel methicillinresistenter *Staphylococcus aureus*-Stämme (MRSA) erarbeitet. In einem Analysenansatz werden am Durchflußzytometer gleichzeitig Identifizierung und Oxacillin-resistenzbestimmung durchgeführt. Dabei zeigt sich eine gute Korrelation mit konventionellen Nachweismethoden wie Latexagglutinationstests und dem Nachweis des *mec A*-Genproduktes. Durch die Schnelligkeit dieser Methode wird am selben Tag ein Resultat erzielt und eine gezielte Therapie kann begonnen werden. **Kosmetische** Isoliermaßnahmen für die betroffenen Patienten können somit auf ein Minimum beschränkt werden.

## CARDIAC CALCIUM HOMEOSTASIS ENDS UP IN OSCILLATIONS: MICRO-CYTOMETRIC MEASUREMENTS AND NUMERIC SIMULATIONS

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The oscillation of the cytosolic calcium concentration in cardiac myocytes resembles the rhythmic behavior of the entire heart on the subcellular level. The parallels confirm the behavior of the healthy heart very well. But the onset of arrhythmias and fibrillation of the entire heart muscle is not resembled on the level of the single cell. This problem arises as a prognostic lack together with the application of cell based antiarrhythmic compounds which are proarrhythmic if given to the patient. To address this problem we have developed an experimental setup for microbeam supported real time calcium imaging on the one hand and a partial differential equation system on the other hand. The reaction of monolayered tissue patches is investigated. They are confronted with irregular high cytosolic calcium concentrations as known from ischemic tissue. The recorded reaction of the cells in the patch can be numerically simulated with a partial differential equation.

Following the artificially increase of the calcium concentration optically restricted to a region of ten micrometer diameter the cellular calcium homeostasis starts. Because of the homeostatic activity and the diffusion of the calcium ions in the cytosol the calcium concentration in the influenced region decreases. After trespassing a certain value the calcium concentration starts to oscillate. In the beginning the oscillations are restricted to small parts of the cell and do not share any common frequency or amplitude. Together with the onset of subcellular calcium waves the regions melt together and a homogeneously beating cardiac myocyte in the still resting tissue patch results. Together with the first oscillation which occupies the whole cardiac myocyte a pacemaking activity can be seen. The newly beating cell stimulates cytosolic calcium oscillations in the surrounding cells. We are able to generate a pacemaker of cellular dimensions inside resting cardiac tissue patches and to record the spread of the new irregular beats.

The solutions of an equation of the FitzHugh-Nagumo-type resemble elementary events of exponential calcium homeostasis, change to oscillation, and the development of pacing activity. The microscope derived results are interpreted on a comparative semi-quantitative basis.

## EXAMINATION OF INNER MITOCHONDRIAL TRANSMEMBRANE POTENTIAL (DYM) USING CHLORMETHYL-X-ROSAMINE DYE

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The disruption of inner mitochondrial transmembrane potential (DYM) is thought to be the first irreversibly apoptotic event. In our study we used Chloromethyl-X-Rosamine (CMXRos) to assess it during hydrocortisone induced apoptosis in cell lines and lymphocyte cultures from patients with B-CLL. The biggest advantages of this dye are ability to fix cells and to apply three-colour staining technique. In our study we observed lower expression of CMXRos in apoptotic line cells than in non-apoptotic ones. Cultured neoplastic B-CLL lymphocytes showed decreased mitochondrial transmembrane potential comparing to the potential of fresh isolated ones. Moreover, time and degree of decrease in hydrocortisone induced apoptotic B-CLL cells vary one another in different patients. This phenomena could be useful in predicting B-CLL patient outcome in the future.

## ON-LINE MONITORING AN BIOPROZESSEN MITTELS FLIEßINJEKTIONSCYTOMETRISCHER MESSUNGEN

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Die Prozeßsteuerung- und optimierung in der Biotechnologie erfordert eine moderne Analytik, um einen möglichst schnellen Überblick über Wachstum und Stoffwechselvorgänge im Reaktor zu erhalten. On-line Messungen bieten die Möglichkeit in kurzer Zeit Informationen über den Status der Kultivierung zu gewinnen, um bei Bedarf in den Prozeßablauf eingreifen zu können. Hierfür wurde ein Durchflußcytometer (Coulter Epics XL) mittels eines Fließinjektionsanalysen-(FIA)-systems für On-line Messungen am Bioprozeß erweitert. Ein Probenstrom wird aus dem Reaktor zusammen mit Propidiumiodid-Färbelösung in eine Reaktionsschleife geleitet, welche die gefärbte Zellsuspension direkt in die Probenahmezone des Cytometers weiterführt. Durch vollautomatisierte Regelungstechnik mit Hilfe des FIA-Steuerprogramms CAFCA (Computer Assisted Flow Control & Analysis), kann eine Messung sowohl von Zellzahl und Vitalität als auch von Zellgröße und -größenverteilung erfolgen. Die kontinuierliche Bestimmung dieser prozeßrelevanten Parameter erfolgt in Zeitintervallen ab 5 Minuten und kann somit in kürzester Zeit durchgeführt werden. Durch einen vor der Reaktionsschleife implementierten Selektor kann das Fließinjektionscytometrie-System sowohl vollautomatisch für die Zellzahlbestimmung kalibriert als auch zur Datenaufnahme von bis zu 6 Reaktoren eingesetzt werden. Kultivierungen mit verschiedenen Bakterien- und Säugerzelllinien dienen als Modell zur Optimierung der Prozeßsteuerungsstrategie. Die hieraus resultierende Transparenz der Fermentation ermöglicht es, jederzeit regulierende Eingriffe in die Prozeßsteuerung durchführen zu können und erlaubt weiterhin eine rechtzeitige Detektion von Kontaminationen oder anderen Störungen im Prozeßablauf.

## THE EFFECT OF CHEMICALS ON THE DURATION OF MALE MEIOSIS IN MICE DETECTED WITH LASER-SCANNING-CYTOMETRY (LSC)

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Chemical mutagens are characterized by a differential spermatogenic response, in that they can affect different developmental stages of male germ cells. Aneuploidy studies in germ cells require a precise knowledge of the duration of the meiotic stages. The effect of colchicine, diazepam, griseofulvin and vinblastine on the duration of male meiosis was measured in mice. With thymidine analogues, such as bromodesoxy-uridine (BrdU) it is possible to label cells at S-phase during preleptotene of meiosis. During meiosis I and II, 13 days later, the mice were treated with the test chemi-cals. In a time frame of 20-25 days after treatment, BrdU containing sperm were identified with a fluorescence-labeled anti-BrdU antibody. Laser-Scanning-Cytometry (LSC) allows to score sperm with fluorescence signals on slides normally used for microscopic ana-lysis. Preliminary data showed no significant differences between the frequencies of BrdU labeled sperm determined by manual fluorescence microscopy and by LSC. After colchicine treatment, the peak of BrdU labeled epididymal sperm occurred significantly later than in the control group (day 24 vs. day 23). On days 21 and 22, the control frequencies of BrdU-labeled sperm were 28.4% and 30.6%, the corresponding colchicine-group frequencies were 11.7% and 9.4% ( $p>0.01$ ). These data indicate that colchicine prolonged the duration of the meiotic divisions in mouse spermatocytes. Diazepam, griseofulvin and vinblastine showed no significant effect on meiotic cell cycle progression. It can be concluded that LSC analysis has the potential to measure the effect of chemicals on the duration of male meiosis which facilitates the studies of aneuploidy induction in male meiosis by aneugenic chemi-cals.

Research funded by EU-contract ENV4-CT97-0471

**FLOW CYTOMETRY FOR ASSESSING BIOMATERIALS COMPATIBILITY OF TYPE I  
COLLAGEN-COATED TITANIUM ALLOY**

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A number of studies have demonstrated the pivotal role of collagen in modulating cell growth and differentiation. In bone, where the extracellular matrix comprises approximately 85 % type I collagen, cellular interaction with matrix components has been shown to be important in regulation of the osteoblast phenotype. Preservation or enhancement of normal osteoblast function and appositional bone formation after implant placement represents a strategy that can be useful for the purpose of improving osseointegration. In order to improve the biocompatibility, we have coated the common orthopaedic implant metal, titanium alloy Ti6Al4V with type I collagen. In the present study, we have assessed *in vitro* behaviour of primary osteoblasts grown on both fibrillar collagen-coated and tropocollagen-coated titanium in comparison with uncoated titanium. As parameters of biocompatibility, a variety of processes including cell attachment, spreading, cytoskeletal organization, focal contact formation, proliferation and expression of a differentiated phenotype were investigated. Flow cytometry was used as a possible procedure for measuring *in vitro* the biocompatibility of implant materials.

Concerning the parameters of cell adherence, our results demonstrated that in comparison to uncoated titanium, collagen-coated titanium enhanced spreading and resulted in a more rapid formation of focal adhesions and their associated stress fibers. Using flow cytometry, osteoblasts grown on collagen-coated titanium had a higher proliferative capacity compared with cells on uncoated titanium. In addition, we have demonstrated by flow cytometry, that collagen caused upregulation of the intracellular expression of osteopontin, a marker of osteoblastic cell differentiation.

In this study we have demonstrated that type I collagen-coated titanium alloy exhibits favorable effects on the initial adhesion and growth activities of osteoblasts, encouraging its use as a bone graft material.



**dkfz**

**Announcement**

**13<sup>rd</sup>**



**Heidelberg Cytometry Symposium**

**Annual Conference of the  
German Society of Cytometry e.V.**

**Heidelberg, 19<sup>th</sup> - 21<sup>st</sup> October 2000**

**Location**

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