17th Annual Meeting of the German Society of Cytometry

DGfZ

Oct 10th - Oct 13th 2007

Hosted at the University Hospital Regensburg, Germany

Organized by Gero Brockhoff

in cooperation with Marietta Bock, Simone Diermeier-Daucher, Angelika Graf, Andrea Sassen, Elisabeth Schmidt-Brücken, Silvia Seegers, Arabel Vollmann-Zwerenz

DGfZ Conference 10th thru 13th Oct. 2007

- Final program -

General remark:
Please find all abstracts submitted before the early registration deadline (June 30th) printed in Cytometry Part A Sept. issue, page 737 ff.
Abstracts submitted after the early registration deadline are printed in this booklet.

Wednesday, Oct 10th

Time frame		
	Basic Tutorials Chair: Beisker	
14:00 – 16:30	**Elmar Endl** **Ulrich Sack** **Wolfgang Beisker** **Susann Müller** **Wim Corver**	**(T 1)** Flow Cytometry: Instrumentation, Setup, Adjustment **(T 2)** Bead Based Cytometry: Selected Applications **(T 3)** Flow Cytometric Analysis of Apoptosis **(T 4)** Bacterial Activity Analysis: Proliferation vs. Viability Tests **(T 5)** Multiparametric DNA Flow Cytometry of Human Carcinomas
16:30 – 17:00	**Break**	
17:00 – 17:30	**Official Opening Ceremony; Welcome note by Brockhoff and the Dean of Faculty of Medicine, University of Regensburg**	
	Key note lectures Chair: Schmitz	
17:30 – 18:00 **18:00 – 18:30** **18:30 – 19:00**	**Mario Roederer** **Bob Murphy** **Robert M. Zucker**	**(KL 1)** The Multicolored World in Flow Cytometry: Prospects and Visions **(KL 2)** Proteome-Wide Determination of Subcellular Location by Automated Microscopy **(KL 3)** Confocal imaging of solid tissues
19:00 - :-) (-:	**Get together – Industry Opening**	

Thursday, Oct 11th

Time frame	Advanced Tutorials Chair: Tarnok	
08:30 – 11:00	**Attila Tarnok**	**(T 6)** Advances in Quantitative Slide Based Cytometry: Towards Cytomics
	Mario Roederer	**(T 7)** The Multicolored World in Flow Cytometry: Prospects and Visions
	Peter Nagy	**(T 8)** FRET in Flow and Image Cytometry
	Thomas Ried	**(T 9)** Multiparametric Fluorecence-in-situ-Hybridization (FISH) and Spectral Karyotyping (SKY) in Diagnosis of Human Malignancies
	Johannes Wessels	**(T 10)** Introduction into small animal imaging techniques - a comparative overview
11:00 – 11:30	**Break**	
11:30 – 13:00	**Industrial Presentations (I)** Chairs: Viergutz, Beisker **Large Lecture Hall**	**Small Lecture Hall** **Cytometry in Microbiology, Biotechnology, and Plants** Chairs: Müller, Obermayer
	(L 1) AxioVision SFM - Cell Image Associated Data for Flow Cytometry Results *Malkusch W (Carl Zeiss Imaging Solutions GmbH, Hallbergmos, Germany)* **(L 2) Optical filters - essential tools in cytometry** *Sommerauer M (AHF Analysentechnik AG, Tübingen, Germany)* **(L 3) Cell Proliferation Assay using Click Chemistry** *Bradford J (Flow Cytometry Section, Molecular Probes / Invitrogen)* **(L 4) Strategies to Improve siRNA Tools for Large Scale Library Screens** *Scory S (ThermoFisher Scientific)*	**(L 5) Physiology of living individual Saccharomyces cerevisiae cells - investigation on cellular and molecular level** *Achilles J, Repenning C, Harms H, Stahl F, Müller S* **(L 6) Correlation between GC content and genome size in plants** *Bures P, Šmarda P, Hralová I, Fuentes-Soriano S, Lysák M, Epka R, Helanová K, Rotreklová O, Procházková J, Úradníek L, Krová J, Masaryk* **(L 7) A high-throughput system for rapid ploidy analyis and seed screening for reproductive pathways in plants** *Aliyu OM, Sharbel TF* **(L 8) Evidence of flocculation in brewing yeast strains by flow cytometry, proteome and mRNA profiling** *Heine F, Wiacek C, Benndorf D, Sträuber H, Stahl F, von Bergen M, Harms H, Müller S* **(L 9) GC content and average chromosome size evolution in grasses** *Šmarda P, Bureš P, Horová L* **(L 10) Investigations of Plant in vitro cultures by Flow cytometry** *Weber J, Gerogiev V, Pavlov A, Ilieva M, Bley B*
13:00 – 14:00	**Lunch**	
14:00 – 14:30 **14:30 – 15:00**	**Key note lectures** Chair: Knuechel	
	Leoni Kunz-Schughart	**(KL 4)** Multicellular spheroids: An underestimated tool is catching up again
	Thomas Ried	**(KL 5)** Causes and consequences of chromosomal aneuploidy in cancer cells
15:00 – 16:30	**Get in contact with the industrial exhibitors**	**Break** **Poster Session** Chair: Viergutz, Endl
16:30 – 17:00	**Key note lectures** Chair: Tarnok	
	Paul Robinson	**(KL 6)** Cytometry and the dawn of the cytomics generation
17:00 – 17:45	**Distinguished Lecture** Chair: Jovin	
	Stefan Hell	**(DL 1)** Breaking Abbe's barrier: Diffraction unlimited resolution in far-field microscopy
	Closing	
19:30 - :-) (-:	**Banquette downtown for participants who have registered for**	

Friday, Oct 12th

Time frame		
	Key note lectures Chair: Weiss	
09:00 – 09:30	**Thomas M. Jovin**	**(KL 7)** Live cell microscopy of growth-factor dependent signal transduction pathways with a Programmable Array Microscope (PAM)
09:30 – 10:00	**Otto Wolfbeis**	**(KL 8)** Applications of Fluorescent Functional Microbeads and Nanobeads
10:00 – 10:30	**Break**	
10:30 – 11:30	**Industrial Presentations (II)** Chairs: Friedlaender, Nagy **Large Lecture Hall**	**Small Lecture Hall** **Special Instrumentation and Applications** Chairs: Wolfbeis, Jovin
	(L 11) Principles and Applications of Imaging Flow Cytometry *David B (Amnis Corporation, Seattle, WA, USA)* **(L 12) Looking for the xTreme? MoFloTM XDP Cell Sorter - the most powerful cell sorter on the planet** *Petkau K,(Dako Hamburg, Germany)* **(L 13) The Power of 40 Years Flow Cytometry - From the worldwide first fluorescence-based FCM instrument ICP 11 to the newest FCM Technologies for dedicated applications** *Göhde R (1), Overton WR(2), Köhler D (1) ([1]Partec, Munester, Germany ,[2]GCAT Inc, USA)*	**(L 14) State of the art Slide Based Cytometry (SBC) systems for Cytomics** *Tarnok A* **(L 15) Automated Multi-colour FISH Analysis Workstation for Lung Pharmacogenomics: Identifcation of Clonally Related Cells** *Dubrowski P (to be presented by MacAulay C)* **(L 16) MALDI-Imaging – a combination of histology with mass spectrometry for discovery of protein patterns with potential clinical impact** *Schwamborn K, Wellmann A, Knuechel R, Krieg R* **(L 17) Validating MELC-Technology for Clinical Diagnostics by Comparing Measurements of a Disease-Specific Combinatorial Molecular Phenotype on Peripheral Blood Mononuclear Cells with Flow Cytometry Measurements** *Bartsch S, Böckelmann R, Malykh Y, Karcher P, Pommer AJ, Gollnick H, Bonnekoh B*
11:30 – 12:30	**DGfZ special themes** Klaus-Goerttler-, Poster-Price Awarding, K-G-Winner Presentation, Special Tribute*	
12:30 – 13:30	**Lunch**	
13:30 – 15:00	**DGfZ General Public Meeting** (Chair: Brockhoff, Müller) Vice President and Council member elections, Official Matters, Announcements, Miscellaneous	
15:00 – 15:30	**Break**	
15:30 – 17:00	**Stem Cell Biology and Clinical Cytometry** Chairs: Rothe, Sack	
	(L 18) Green Fluorescent Protein (GFP) marking for the study of host and graft participation in the foetal intestine ectopic growth *Delreé P, Coulic V, Lallemand MC, Duprez L* **(L 19) Options and limitations in determination of bacterial contaminations in platelet concentrates (PC). A study, using flow cytometry and transmission electron microscopy** *Neumüller J, Renz R, Meißlitzer-Ruppitsch C, Neumüller-Guber S, Pavelka M* **(L 20) Cytometric monitoring of transplanted patients** *Sack U, Borte S, Hoppe A, Wegmann C, Luderer C, Oppel C, Bauer K, Emmrich F, Hauss J, Fangmann J* **(L 21) T cell subsets controlled for demographic and biomedical variables in an industrial sample of blue- and white-collar employees** *Fischer JC, Nguyen XD, Fischer JE* **(L 22) Extracting more information from routine immunophenotyping using quantitative multiparametry of flow cytometry: two triple staining combinations as examples** *Grunwald U* **(L 23) Biomarkers of T-cell function after cardiopulmonary bypass surgery** *Ferrari-Kuehne K, Kiehntopf M, Renner A, Hornung K, Gruen K, Deufel T, Gummert JF Barten MJ*	
17:00 – 17:30	**Break**	
	Key note lectures Chair: Nagy	
17:30 – 18:00	**Dieter Weiss**	**(KL 9)** Imaging of the Living Cytoskeleton and the Associated Organelle Motility
18:00 – 18:30	**Johannes Wessels**	**(KL 10)** Molecular imaging *in vivo* – a comparative overview
	Core Facility Managers Workshop - Refreshments Chair: Endl, Davies	
18:30 – closing	**E. Endl, D. Davies**	**(T 11)** Forum open for discussion – suggestions - criticism, etc.
20:00 - :-) (-:	**Special event down town for those who have registered for / or evening on your own**	

** Cytometry Part A, Sept issue, page 639 ff.*

Saturday, Oct 13th

Time frame	Key note lectures Chair: Kunz-Schughart	
09:00 – 09:30 **09:30 – 10:00**	**Janos Szöllösi** **Herbert Stepp**	**(KL 11)** Molecular Interactions of the erbB2-Receptor-Tyrosin-Kinase: Implications in Trastuzumab Resistance **(KL 12)** Photodynamic Therapy and Fluorescence Diagnosis with 5-aminolevulinic acid
10:00 – 10:30	**Break**	
10:30 – 12:15	**Cancer Biology and Therapy** Chairs: Brockhoff, Hemmer	
	(L 24) Circulating tumor cells in the metastatic pathway. *Pachmann K* **(L 25) Characterization of the proliferating chronic lymphocytic leukemia cells in an in vitro model for Pseudofollicles** *Plander M, Seegers S, Ugocsai P, Schwarz S, Orsó E, Diermeier-Daucher S, Knüchel R, Iványi J, Brockhoff G* **(L 26) The impact of Trastuzumab, Pertuzumab, and Cetuximab on cell proliferation of breast cancer cell lines** *Diermeier-Daucher S, Heckel B, Schmidt-Brücken E, Plander M, Hofstaedter F, Brockhoff G* **(L 27) Differential influence of chemoresistance on radiosensitivity in human normal and tumour cells** *Bartkowiak D, Bottke D, Wiegel T* **(L 28) Loss of FHIT and p16 are early events in tumorigenesis of oral squamous cell carcinoma and characteristically occur in simple keratosis** *Bier J, Schwarz S, Driemel O, Reichert T, Hauke S, Brockhoff G* **(L 29) Subpopulations of human CD4+CD25+ regulatory T cells identified by multicolour flow cytometric analysis: purity and suitability for in vitro expansion** *Hoffmann P, Eder R, Boeld TJ, Albrecht J, Doser K, Stahl J, Andreesen R, Edinger M* **(L 30) Assessment of DNA ploidy in salivary gland tumors** *Bauer R, Driemel O, Hemmer J*	
12:15 – :-) (-:	**Farewell**	

DGfZ Conference 10th thru 13th Oct. 2007

Poster Presentations

Cytometry in Systems Biology

(P 1) Bovine ovarian granulosa cells respond to the platelet-activating factor (PAF) with intracellular calcium mobilization via the PAF receptor
Viergutz T, Krüger B, Löhrke B

(P 2) Flow-cytometric measurement of respiratory burst in rat polymorphonuclear granulocytes: Comparison of four cell preparation procedures, and concentration-response evaluation of chemical stimulants
Bitzinger D, Schlachetzki F, Lindner R, Trabold B, Dittmar MS

Cancer Biology and Therapy

(P 3) The effect of cisplatin on the structure of chromatin in tumor cells
Gloushen G, Anikanov G, Khliabko P

(P 4) HER3 and HER4 gene amplification have prognostic impact in breast cancer
Sassen A, Rochon J, Wild P, Hartmann A, Hofstädter F, Schwarz S, Brockhoff G

(P 5) Generation of reactive oxygen species and induction of a cell cycle arrest by epoxy and acrylate monomers
Schweikl H, Hiller KA, Stich A, Bolay C, Brockhoff G, Eckhardt A, Schmalz G

(P 6) Flow cytometric DNA ploidy in brush biopsies of oral lesions
Dowejko A, Driemel A, Schwarz S, Reichert TE, Brockhoff G

(P 7) Application of fluorescence bar coding to multicolor flow cytometric quantification of ErbB receptor-driven intracellular signaling
Friedländer E, Diermeier-Daucher S, Vereb G, Brockhoff G

(P 8) Cooperation between two TNF receptors in the U937 in necessary for efficient cytocidal response to transmembrane TNF whereas protective response is not
Pierzchalski A, Banach K, Perycz M, Bigda J

Clinical Cytometry and Advances in Diagnostic Immunophenotyping

(P 9) 4-color immunohistochemical quantification of FoxP3+ regulatory T cells in transplanted kidney biopsies
Stoelcker S, Kryvoshey D, Ellmann S, Banas B, Kraemer BK

(P 10) Innovative concepts for Absolute Immunophenotyping by Slide-Based Cytometry
Laffers W, Mittag A, Tárnok A, Bootz F, Gerstner AOH

(P 11)The Core Unit Fluorescence-Technologies in the IZKF Leipzig
Lösche A, Grosche J

(P 12) Alteration of immune phenotype following protein losing enteropathy after total cavopulmonary connection by cytomics
Bocsi J, Lenz D, Sauer U, Wild L, Hess J, Schranz D, Hambsch J, Schneider P,Tarnok A

(P 13) Re-evaluation of the function of CCR6 on effector T cells by multi-colour flow cytometry
Pötzl J, Botteron C, Männel DM, Lechner A

(P 14) Continuous Ca2+ dependent shedding of CD163 from macrophages determine soluble CD163 level
Ugocsai P, Wolf Z, Paragh G, Schmitz G

Cytometry in Microbiology, Biotechnology, and Plants

(P 15) Populations profiles of the BTEX degrading enrichment culture L-D1 under balanced and non balanced growth conditions
Ramig S, Vogt C, Kleinsteuber S, Hübschmann T, Harms H, Müller S

(P 16) Genome size variation in species with holokinetic chromosomes (Cyperaceae)
Hralova I, Bures P, Rotreklova O, Smarda P, Grulich V, Zedek F, Smerda J, Horova L, Hroudova Z, Repka R

(P 17) Flow cytometry, a suitable method for detection of ploidy level and reproductive variability within the hawkweeds populations, Hieracium subgen. Pilosella
Rotreklova O, Krahulcova A, Krahulec F

(P 18) Flow-sorted nuclei are valuable subjects to investigate the structural and functional nuclear architecture in Arabidopsis
Fuchs J, Pecinka A, Lysak M, Schubert V, Lermontova I, Watanabe K, Jovtchev G, Schubert I

(P 19) Flow cytometric and phytochemical investigations with plant cell suspension cultures of sunflower (Helianthus annuus)
Haas C, Georgiev M, Weber J, Ludwig-Müller, Bley T

(P 20) Diploid and polyploid cytotypes distribution in the white-rayed complex of Melampodium (Heliantheae, Asteraceae)
Obermayer R, Reich D, Rebernig CA, Weiss-Schneeweiss H, Stuessy TF

Novel Instrumentation and Applications

(P 21) Comparing the effect of the thymidine analogues EdU and BrdU on cell cycle progression
Diermeier-Daucher S, Clarke S, Bradford J, Hill D, Brockhoff G

(P 22) Quantum Dots as Replacements for Tandem Dyes in Flow Cytometry
Buller GB, Zhang YZ, Godfrey WL

(P 23) Is quantitative cytometric 3D analysis of tissue possible?
Mittag, A

(P 24) On-chip and label-free cell characterization with an impedance spectroscopy flow cytometer
Schade-Kampmann G, Hebeisen M, Huwiler A, Hessler T, Di Berardino M

(P 25) Analysis of Cell Cycle Blockers using Click Chemistry Catalyzed EdU Detection
Bradford JA, Clarke ST, Buck SB, Gee KR, Agnew B, Salic A

(P 26) Photoinduced electron transfer (PET)-probes for the detection of cancer-related nucleases
Henkenjohann S, van de Linde S, Doose S, Wittig R, Schubert P, Coy JF, Sauer M

(P 27) Optimization of the hypoosmotic loading method of aequorin, an intracellular Ca2+ concentration indicator, into cells of follicular lymphoma
Skopalik J, Klabusa M,Borsky M

DGfZ Conference 10th thru 13th Oct. 2007
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(alphabetical order)

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Abstracts

from the

17th Annual Meeting of the German Society of Cytometry (DGfZ)

10–13 October 2007

Hosted at
the University Hospital Regensburg, Germany

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Hosted at the
University Hospital, Regensburg
Franz-Josef-Strauss-Allee 11
93053 Regensburg

Scientific Topics

- ✓ Biosensoric Applications and Nanotechnologies
- ✓ Cytometry in Systems Biology
- ✓ *in-vivo* and *in-situ* Imaging
- ✓ Stem Cell Biology
- ✓ Cancer Biology and Therapy
- ✓ Novel Instrumentation and Applications
- ✓ Analysis of Tissues and Tissue Related Systems
- ✓ Cytometry in Microbiology, Biotechnology, and Plants
- ✓ Clinical Cytometry and Advances in Diagnostic Immunophenotyping

Tutorials

- ✓ Basics in Flow and Image Cytometry (e. g. Instrumentation, Calibration, Single- and Multicolor Analysis, Compensation)
- ✓ Advanced Cytometry (e. g. Dynamic Cell Proliferation Assessment, Analysis of Apoptosis, FRET in Flow Cytometry and Imaging, Bead Based Applications, *in-vivo* and *in-situ* Imaging)
- ✓ Slide Based Cytometry and Tissue Analysis
- ✓ Selected Applications in Microbiology
- ✓ Core Facilities and Networking
- ✓ Company Tutorials

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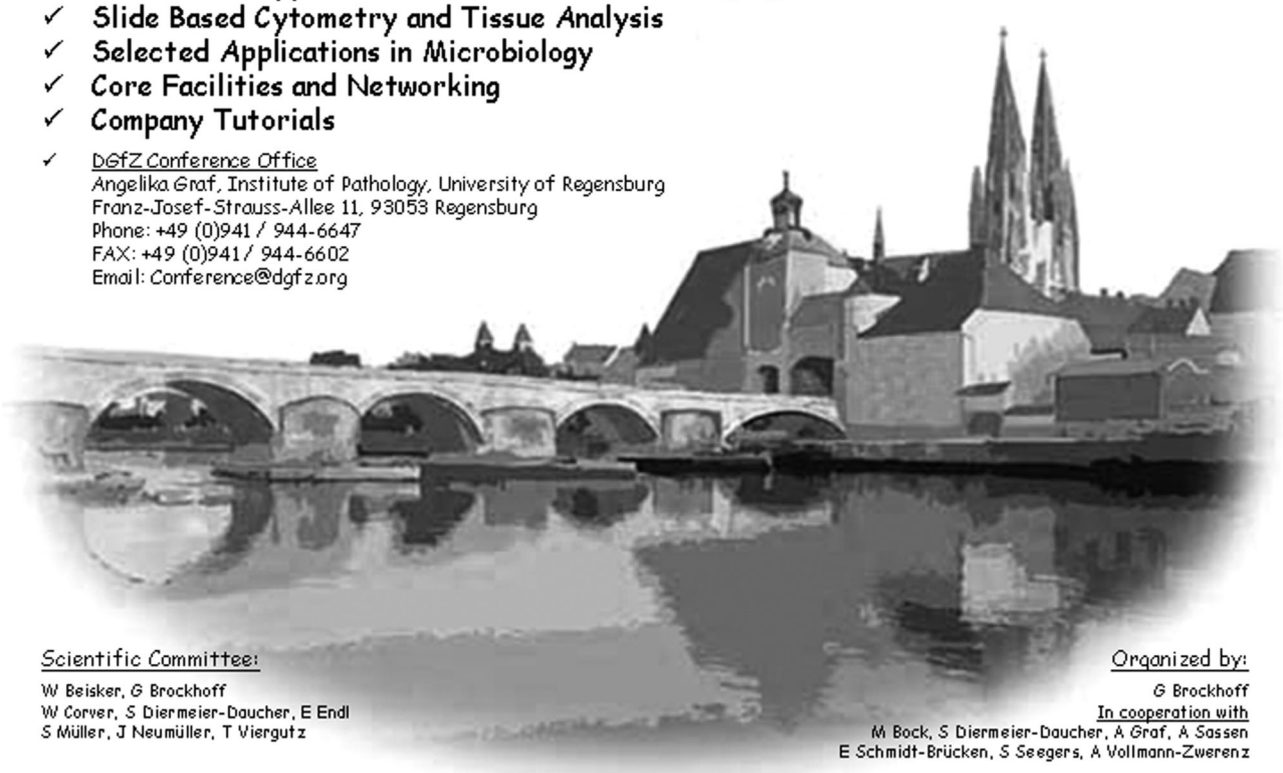

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W Beisker, G Brockhoff
W Corver, S Diermeier-Daucher, E Endl
S Müller, J Neumüller, T Viergutz

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Cytometry PART A
Journal of the International Society for Analytical Cytology

ISAC
International Society for Analytical Cytology

17th Annual Meeting of the German Society of Cytometry (DGfZ) October 10th to 13th, 2007

Gero Brockhoff

President of the German Society of Cytometry, Institute of Pathology, University of Regensburg, Germany

Welcome Note

In the capacity as president of the German Society of Cytometry, I would like to warmly welcome you to our 17th annual meeting. This year the society's meeting is being held for the very first time in Regensburg and is hosted by the University of Regensburg Medical Center. After 14 meetings traditionally held at the German Cancer Research Center (DKFZ) in Heidelberg, we moved the conference site to Leipzig where we had two successful meetings hosted at the Helmholtz Center for Environmental Research (UFZ). Since then our society has undergone a modification in both content and form. Over the last couple of years new ideas have influenced and reshaped the society and this modified body of thought has been reflected in the scientific programs presented in 2005 and 2006. Now in 2007, we have moved to Regensburg and intend to continue this trend of successful events by presenting a stimulating and rewarding scientific meeting held in charming, historic Regensburg.

This Year's Venue

This year the annual meeting takes place for the very first time in Regensburg (Bavaria, Germany) and is hosted by the University of Regensburg Medical Center. I am convinced that our society has found a very appropriate and likewise extraordinarily attractive venue for the 17th annual meeting.

The natural scientific landscape comprises the communities of the University, the Technical College and the University Medical Center with its associated Tumor Center, as well as the Archaea Center, the Technical School for Boundary Layer Chemistry and the WHO Collaborating Center, all situated on one common campus with a park-like character. The Technology Transfer Sites, the Data Processing Center and BioPark Regensburg, completed in the year 2000, are all within walking distance. The University Medical Center itself is constantly being enlarged and complemented by additional departments and modern research facilities.

In 2006 Regensburg was selected by UNESCO as a World Cultural Heritage Site. Located on the Danube River, the medieval town contains many buildings of exceptional quality that testify to its history as an important trading center and to its influence in the region dating back to as early as the 9th century. It has a notable number of preserved historic structures spanning some two millennia, including ancient Roman, Romanesque and Gothic buildings. Regensburg's 11th–13th century architecture, including the market, City Hall and St. Peter's Cathedral, still defines the character of the town marked by tall towers, charming narrow lanes, and strong fortifications. The buildings include medieval Patrician houses and towers, a large number of churches and monastaries as well as the famous Stone Bridge, which dates back from the 12th century. The town is also remarkable for the vestiges that testify to its rich institutional and religious history as one of the centers of the Holy Roman Empire. However, Regensburg is anything but a museum! It is a lovable, livable and vital town in the center of Bavaria.

Self Conception of the Society — A Brief History of the DGfZ

Our society was originally founded by Cess Cornelisse, Georg Feichter, Wolfgang Goehde, Klaus Goerttler, Holger Hoehn, Andreas Radbruch, Peter Schwarzmann, and Günter Valet in 1989 and designated as the Society of Cytometry (Gesellschaft fuer Zytometrie, GZ). An association was born dedicated to providing an interdisciplinary platform for scientists principally interested in the field of flow and image cytometry. Founding members were scientists whose personal scientific development was, and still is, closely intertwined with the development and advancement of cytometric technologies in Europe. Since its foundation, annual meetings have been organized to provide a platform for interdisciplinary exchange in basic research, as well as clinical and industrial developments. In 1994 the original name of the society was changed to the Deutsche Gesellschaft fuer Zytometrie (DGfZ, German Society of Cytometry), however, over time the society has expanded to include increasing international participation and in the mid-nineties the conference language was changed to English.

Since its foundation, the DGfZ has offered an organized platform and structure for the growing science of

Cytometry. The focus of the scientific interest of DGfZ activities is the analysis of genetic, physiological and structural processes in cells. Ab initio, the DGfZ has aimed to promote methodological and technical innovations in flow cytometry (FCM), image cytometry (ICM) and slide based cytometry (SBC), which furthers understanding of the cell and its integration into multi-cellular systems. The spectrum ranges from Oncology, Immunology and Pathology, to Cytogenetics, Microbiology and Plant Culture as well as Ecology, and includes all areas where biological cells and tissues are the primary focus. The DGfZ is dedicated to providing a platform for interdisciplinary and scientific exchange, and to facilitate communication as well as the sharing of knowledge. The society has continuously engaged in innovative science and the development of cutting-edge technologies. Examples are the first commercially available flow cytometer as well as methods for dual laser flow cytometry, cell separation and sorting, among others.

Even if the name "German Society of Cytometry" might suggest a technically oriented community, the society is consistently evolving. After extensive discussions in the mid-nineties, the council board decided to retain the original name in order to give shape to a society with a historical origin. Keeping pace with the times, however, a new way of thinking was reflected by the scientific topics the council board compiled and - needless to say - by the scientific presentations contributed by the conference attendees. Stem cell research, chip and array technology, and systems biology all became a vital part of past meetings representing cutting-edge research over a wide range of scientific fields. As a guiding force, the DGfZ provides a unique platform for a variety of scientists working in different areas of research and having different scientific interests, but all sharing the utilization of cytometric-based technologies and approaches to address scientific questions of critical importance.

A Tribute to Günter Valet

Günter Valet (previously affiliated to the Max Planck Institute, Martinsried, Germany, Dept. of Cell Biochemistry), officiated as the second president of the society from 1992 thru 1994 and has been deeply involved in the DGfZ since its foundation and is one of the pioneers who aptly realized that cytometric analysis had the potential for utilization in higher levels of research. He led cytometrists to the idea of cytomics by suggesting the application of the multimolecular analysis of cells and the heterogeneity of cell systems in combination with exhaustive bioinformatic knowledge extraction. Cytomics links proteomics and genomics to cell and tissue function by integrating the morphology and architecture of cell systems. In clinical applications, it opened the way towards predictive and preventive medicine in terms of individualized medicine. The idea of cytomics can be realized today by taking advantage of highly sophisticated cytometric technologies and, insofar, this innovative way of thinking has significantly contributed to a modified self-concept of the DGfZ.

We very much appreciate the valuable and vital input given by Günter Valet to the DGfZ over many years. He has consistently offered his expertise in helping to reshape and reform the society, helping it to achieve its current status. Hence we would like to appoint Günter Valet this year as an honorary member of our society.

Scientific Program

This year we have again successfully compiled a very interesting scientific program covering:

- Cancer Biology and Therapy
- *in vivo* and *in situ* Imaging
- Stem Cell Biology
- Analysis of Tissues and Tissue Related Systems
- Clinical Cytometry and Advances in Diagnostic Immunophenotyping
- Cytometry in Microbiology, Biotechnology, and Plants
- Cytometry in Systems Biology
- Novel Instrumentation and Applications
- Biosensoric Applications and Nanotechnologies

The last topic listed has been integrated as a first-off topic this year from which we can expect innovative input for our meeting.

I am extremely delighted about the acceptance of my invitation to the following keynote speakers (listed in alphabetical order):

- **Robert F. Murphy,** Biological Sciences, Biomedical Engineering, and Machine Learning Director, Center for Bioimage Informatics Director, Joint CMU-Pitt Ph.D. Program in Computational Biology President-elect, International Society for Analytical Cytology Carnegie Mellon University, Pittsburgh, PA 15213, USA
- **Thomas M. Jovin**, Dept. of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany
- **Leoni Kunz-Schughart**, Technical University of Dresden, Faculty of Medicine, OncoRay, Dresden, Germany
- **Thomas Ried**, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
- **Mario Roederer,** Vaccine Research Center, NIAID, National Institutes of Health, Bethesda, MD, USA
- **Herbert Stepp,** Laser Research Center, University Hospital Großhadern, LMU Munich, Germany
- **János Szöllõsi,** Medical and Health Science Center, Dept. of Biophysics and Cell Biology, University of Debrecen, Hungary
- **Dieter Weiss**, Dept. for Animal Physiology, University of Rostock, Germany
- **Johannes Wessels**, Center for Internal Medicine, Dept. of Nephrology and Rheumatology, University Hospital, Göttingen, Germany
- **Otto Wolfbeis**, Institute of Analytical Chemistry, Chemo- and Biosonsors, University of Regensburg, Germany
- **Robert Zucker**, Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711, USA

The abstracts submitted by the keynote speakers (and participants who have registered early) are published in this issue. (We expect more contributions due to the option of late registration by the end of August which could not be printed in this issue.) I am convinced that their presentations will significantly contribute to a high quality and rewarding scientific program. The complete compilation of abstracts will appear in the local program booklet. Some of the keynote speakers will also offer an additional advanced tutorial. Tutorials are scheduled as follows:

Basic Tutorials:

- Flow Cytometry: Instrumentation, Setup, Adjustment
- Bead Based Cytometry: Selected Applications
- Flow Cytometric Analysis of Apoptosis
- Bacterial Activity Analysis: Proliferation vs. Viability Tests
- Multiparametric DNA Flow Cytometry of Human Carcinomas

Advanced Tutorials:

- Advances in Quantitative Slide Based Cytometry: Towards Cytomics
- Designing, Implementing, and Analyzing Multicolor Flow Experiments
- FRET in Flow and Image Cytometry
- Multiparametric Fluorescence-in-situ-Hybridization (FISH) and Spectral Karyotyping (SKY) in Diagnosis of Human Malignancies
- Introduction into small animal imaging techniques - a comparative overview

Distinguished Lecture by Stefan Hell

It is a great pleasure for me to announce a distinguished lecture that will be given by Stefan Hell, MPI for Biophysical Chemistry, Dept. of NanoBiophotonics (Goettingen, Germany). Stefan Hell is laureate of the "German Annual Technology and Innovation Future Award 2006" which was conferred for the tenth time last year. The award is given in recognition of projects that not only have revolutionary implications for science but are also ready for application and marketing. Stefan Hell was the first to find a way of overcoming the 130-year-old Abbe limit in the fluorescence microscope, the most important microscope in biomedical research. Ever since the 17th century the light microscope has been one of the main symbols of scientific progress – particularly in biology and medicine. Harnessing Stimulated Emission Depletion (STED) microscopy, molecules can now be imaged with far greater definition than ever before.

I cordially invite you to attend Stefan Hell's distinguished lecture entitled: "Breaking Abbe's barrier: Diffraction unlimited resolution in far-field microscopy" (probably on Thursday afternoon; please check the final program).

Industrial Exhibition

It is a long-standing tradition that DGfZ meetings integrate industrial exhibitors. The DGfZ conference could not be held without the participation and sponsorship of industrial companies. Thus, in advance I would like to express my gratitude to the industry for participating in and thereby making our meeting possible. I am sure all will greatly benefit from the industry exhibitions and invite all participants to keep abreast of current state-of-the-art technological developments by visiting the booths and encourage everyone to attend the informative industrial tutorials.

Poster Session and Poster Prize Award

No DGfZ meeting is complete without a poster session! It is an essential part of the conference and an excellent opportunity to establish new contacts and interact and discuss important research issues with the presenter of the scientific work in person. Take advantage of this valuable opportunity and visit the poster presentation in a relaxed atmosphere. At the end of the meeting a poster prize will be awarded to an extraordinary presentation both in terms of scientific quality and poster layout.

Klaus-Goerttler-Prize Award

The prestigious Klaus-Goerttler prize was established in 1996 and since then has been awarded for an outstanding Ph.D. thesis or an equivalent work on the occasion of the meeting. I am looking forward to the awarding of this year's prize based on the review and decision by the society's council board.

Special Thanks

Finally, I would like to express my special gratitude to the local organizing group: Marietta Bock, Simone Diermeier-Daucher, Andrea Sassen, Angelika Graf, Silvia Seegers, Elisabeth Schmidt-Brücken, and Arabel Vollmann-Zwerenz. They have all contributed significantly to the preparation of this year's meeting. Thanks so much.

Again, welcome to Regensburg and welcome to the 17th Annual Meeting of the German Society of Cytometry (DGfZ) hosted by the University of Regensburg Medical Center. I hope you will have a fruitful meeting and enjoy the conference as well as the special social program.

Cordially yours
Gero Brockhoff
(President of the German Society of Cytometry)

DISTINGUISHED LECTURE

1

BREAKING ABBE'S BARRIER: DIFFRACTION-UNLIMITED RESOLUTION IN FAR-FIELD MICROSCOPY

Stefan W. Hell

Max Planck Institute for Biophysical Chemistry, Department of NanoBiophotonics, Goettingen, Germany

In 1873, Ernst Abbe discovered that the resolution of focusing ('far-field') optical microscopy is limited to $d = \lambda/(2n\sin\alpha) > 200$ nm, with $n\sin\alpha$ denoting the numerical aperture of the lens and λ the wavelength of light. While the diffraction barrier has prompted the invention of electron, scanning probe, and x-ray microscopy, in the life sciences 80% of all microscopy studies are still performed with lens-based (fluorescence) microscopy. The reason is that the 3D-imaging of the interior of (live) cells requires the use of focused visible light. Hence, besides being a fascinating physics endeavor, the development of a far-field light microscope with nanoscale resolution would facilitate observing the molecular processes of life.

In this talk, I will discuss novel physical concepts that radically break the diffraction barrier in focusing fluorescence microscopy. They share a common strategy: exploiting selected molecular transitions of the fluorescent marker to neutralize the limiting role of diffraction. More precisely, they establish a certain, signal-giving molecular state within subdiffraction dimensions in the sample [1].

The first viable concept of this kind was Stimulated Emission Depletion (STED) microscopy. In its simplest variant, STED microscopy uses a focused beam for fluorescence excitation, along with a red-shifted doughnut-shaped beam for subsequent quenching of fluorescent molecules by stimulated emission. Placing the doughnut-beam on top of its excitation counterpart in the focal plane confines the fluorescence near its central zero where stimulated emission is absent. The higher the doughnut intensity, the stronger is the confinement. In fact, the spot diameter follows $d \approx \lambda/\left(2n\,\sin\alpha\sqrt{1 + I/I_s}\right)$, with I denoting the intensity of the quenching (doughnut) beam and I_s giving the value at which fluorescence is reduced to I/e. Without the doughnut ($I = 0$) we have Abbe's equation, whereas for $I/I_s \rightarrow \infty$ it follows that $d \rightarrow 0$, meaning that the fluorescence spot can be arbitrarily reduced in size. Translating this subdiffraction spot across the specimen delivers images with a subdiffraction resolution that can, in principle, be molecular! Thus, the resolution of a STED microscope is no longer limited by λ, but on the perfection of its implementation. We will demonstrate a resolution down to $\lambda/45 \approx 15$–20 nm with nanoparticles and biological samples, i.e., 10–12 times below the diffraction barrier.

The concept underlying STED microscopy can be expanded by employing other molecular transitions that control or switch fluorescence emission, such as (i) shelving the fluorophore in a metastable triplet state, and (ii) photoswitching (optically bistable) marker molecules between a 'fluorescence on' and a 'fluorescence off' conformational state. Examples for the latter include photochromic organic compounds, and fluorescent proteins which undergo a photoinduced cis-trans isomerization or cyclization reaction. Due to their optical bistabilty/metastabilty, these molecules entail low values I_s, meaning that the diffraction barrier can be broken at low I. A complementary approach is to switch the marker molecules individually and assemble the image molecule by molecule. By providing molecular markers with the appropriate transitions, synthetic organic chemistry and protein biotechnology plays a key role in overcoming the diffraction barrier.

Finally, I discuss more recent work of my group showing that the advent of far-field 'nanoscopy' has already solved fundamental problems in (neuro)biology, such as the fate of synaptic vesicle proteins after synaptic transmission. Besides, the emerging far-field 'optical nanoscopy' also has the potential to advance nanolithography, the colloidal sciences, and to help elucidate the self-assembly of nanosized materials.

Reference

[1] S.W. Hell, Far-field optical nanoscopy, *Science* 316 (2007) 1153.

INVITED LECTURES

2

MULTICELLULAR SPHEROIDS: AN UNDERESTIMATED TOOL IS CATCHING UP AGAIN

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Monolayer cell-based assays have become an integral component in many stages of anti-tumor drug testing. However, they still represent a highly artificial cellular environment. 3-D cultures better reflect the pathophysiological *in vivo* situation in tumor tissues and are increasingly recognized as sophisticated tools for evaluating therapeutic intervention. Multicellular spheroids, one of the classical and well-established 3-D culture systems, reflect some phenomena in tumor tissues that are known to critically affect therapeutic efficacy, such as 3-D cell-cell interactions, development of hypoxic areas and proliferation

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gradients. Accordingly, they have frequently been applied to anti-tumor therapy testing throughout the past four decades. Nonetheless, their implementation into mainstream drug screening operations is still limited for various reasons. Technological progress with the use and scale-up of the spheroid model in experimental therapeutics include the validation of a reliable tool to rapidly analyze cell viability in tumor spheroids of different sizes. We explored a panel of standard assays to finally provide an easy-handling, standardized protocol that is applicable for single spheroids in 96-well plates, does not require spheroid dissociation, and is linear and highly sensitive for various tumor cell line spheroid types as a basis for a "Spheroid-based Screen". The intriguing observation that some primary tumor cell populations must be maintained in 3-D culture in order to retain certain tumor initiating (stem) cell properties, adds an additional fascinating challenge for future therapeutic campaigns but of course also requires further evidence and extended studies.

3

PHOTODYNAMIC THERAPY AND FLUORESCENCE DIAGNOSIS WITH 5-AMINOLEVULINIC ACID

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Laser Research Center, University Hospital Großhadern, Ludwigs-Maximilian-University of Munich, Germany

5-Aminolevulinic Acid (ALA) is a precursor of heme in its intracellular biosynthesis. As an intermediate product, the fluorochrome and potent photosensitizer Protoporphyrin IX (PpIX) is produced and can be accumulated under certain conditions. In a number of inflammatory or malignant tissues, this accumulation can be stimulated selectively compared to normal surrounding tissue by topical or systemic delivery of ALA or suitable derivatives.

Photodynamic Therapy (PDT) exploits the generation of Singlet-Oxygen by excited PpIX-molecules. For this purpose, intense but non-thermal visible light is irradiated to the sensitized target-tissue. Fluorescence Diagnosis (FD) exploits the low but sufficient fluorescence yield of PpIX to detect and localize otherwise invisible (early) malignant changes. Its great clinical advantage is its applicability during surgery.

Preclinical research on PDT and FD has started in 1990. Clinical approval is currently granted in Dermatology for PDT of actinic keratosis and basal cell carcinomas, FD for bladder cancer, and is pending for FD of malignant glioma (fluorescence guided resection).

The main steps of preclinical research and current clinical experience will be presented, with special emphasis on developments initiated in Munich. This covers FD and PDT for bladder cancer as well as for malignant glioma, cervical and ovarian cancer and cancer in the oral cavity.

Significant clinical experience has already been generated for FD of bladder cancer, proving its superior detection of early high grade cancer. Therapy of bladder cancer guided by intraoperative FD results in a significant reduction of residual tumor rate and an increase of recurrence free survival. The same is apparently true for FD of malignant glioma. A promising current achievement is stereotactic interstitial PDT of recurrent gliomas. A phase I study is showing rather unexpected longterm survival of some of the patients.

Further research and development will have to focus on elucidation of PDT-mechanisms (e.g. role of immune response), light delivery instrumentation, consideration of patient individual drug accumulation and optical tissue properties, optimized protocols for PDT and their integration into clinical routine.

4

IMAGING OF THE LIVING CYTOSKELETON AND THE ASSOCIATED ORGANELLE MOTILITY

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Two steps led to our present view of the cytoskeleton as a highly dynamic structure that is actively involved in force generation for various kinds of cell motility and is a highly dynamic structure itself.

1. The introduction of video microscopy, especially of the Allen Video Enhanced Contrast-Differential Interference Contrast Microscopy (AVEC-DIC), which allows the visualization of cellular structures in the light microscope that are up to 10 times smaller than the limit of resolution. This enables one to see images of unfixed, unstained, native or purified microtubules and actin bundles, and their interaction with membrane-bounded organelles. Additional information is obtained by applying Dynamic Phase Microscopy in the form of a Linnik type interference microscope and by particle tracking analyses.

2. The discovery of a system exceptionally well-suited to study microtubule and organelle movements, namely, the extruded axoplasm of the squid giant axon. From this axon the cytoplasm can be extruded free from surrounding plasma membrane, and individual microtubules and organelles can be separated from the bulk axoplasm.

These techniques which are best suited to study the living cytoplasm are presented together with some of the major results obtained, especially the microtubule-based and the actin filament-based motor enzymes, the dynamic instability of microtubules and a classification of the various types of organelle motility. Emphasis will be laid on the aspect that, except for the Dynamic Phase Microscopy, these techniques which allow us to extend the microscope's ability well beyond the classical limitations of resolution, visualization, brightness and contrast can be retrofitted to regular research microscopes.

References

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5

THE IMPACT OF FLOW CYTOMETRY ON HIV VACCINE DEVELOPMENT

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Vaccine development to protect against HIV development is actively proceeding on two fronts: generation of a sterilizing (neutralizing) humoral response, and generation of an effective cellular response. To date, it has been impossible to generate an antibody response of sufficient potency that sterilizing vaccination is possible. Nonetheless, a vaccine can be considered successful on a global scale if the induced cellular response is sufficient to dampen viral loads (reducing transmission) as well as reducing morbidity and mortality after infection.

Using different vaccine regimens, we can induce a variety of T cell responses. Using animal models, we hope to identify the kinds of responses that are best correlated with protection against challenge. As we move forward through phase I and II clinical trials in humans, and prepare for phase III, we are keen to determine whether or not these types of responses are induced in humans as well.

Our primary tool for determining the quantity and quality of the T cell response is flow cytometry. We routinely measure five or more different functional responses simultaneously from each cell (e.g., cytokine profile, cytotoxic potential, proliferative capacity). These complex combinations of functions reveal that there are a number of distinct "flavors" of T cell responses present in either naturally infected or vaccinated subjects; the task now is to identify which of these flavors is most suited to protection from challenge. In addition, by studying rapid HIV progressors vs. long term nonprogressors, we can identify differences in T cell responses correlated with disease pathogenesis, perhaps pointing us towards desirable types of vaccine responses.

These analyses have revealed that a potential correlate of effective T cell responses may be the capacity of T cells to be "polyfunctional", i.e., to simultaneously effect multiple functions. Polyfunctional T cell responses are correlated with nonprogression in HIV disease, as well as effective viral control of CMV, EBV, and other viruses. In addition, the level of polyfunctional T cells induced by different vaccine regimens directly predicts control of a L. Major infection in mice.

The mechanisms accounting for better protection by polyfunctional T cells remain to be elucidated; we are actively characterizing these cells and their differentiation capabilities. In addition, we have identified vaccine regimens that can elicit these cells. Together, these are important tools for the development of novel T-cell based vaccines against pathogens.

6

MOLECULAR INTERACTIONS OF THE ErbB2-RECEPTOR-TYROSIN-KINASE: IMPLICATIONS IN TRASTUZUMAB RESISTANCE

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The ErbB2 (HER2) protein is a member of the EGF receptor (ErbB) family of transmembrane receptor tyrosine kinases. Although no direct ligand has yet been assigned to ErbB2, recent biochemical and biophysical evidence suggests that this protein operates as a shared receptor subunit with other ErbB proteins. Its medical importance stems from its frequent overexpression in breast and other cancers. Humanized antibodies against ErbB2 (i.e. Herceptin or trastuzumab) have been introduced into clinical practice and were found to have cytostatic effect in ~40% of ErbB2 positive breast tumors. We used tratuzumab resistant (JIMT-1, MKN-7) and sensitive (SKBR-3, N-87) cell lines in order to demonstrate the importance of association pattern ErbB molecules with each other and with integrins, CD44 and lipid rafts. ErbB2, CD44 and beta1-integrin showed significant colocalization with each other and with lipid rafts regardless the cell lines. Trastuzumab-sensitive cell lines expressed more ErbB2 and fewer beta1-integrin and CD44 molecules on their surface than their resistant counterparts. We have found that in the resistant cell lines active ErbB2 homodimers that bind Herceptin with high affinity are scarce. We examined the role of antibody mediated cellular cytotoxicity (ADCC) using JIMT-1 cells that are ErbB2 positive but intrinsically resistant to trastuzumab in vitro. Unexpectedly, trastuzumab was able to inhibit the outgrowth of macroscopically detectable xenograft tumors for up to 5–7 weeks. The effect is likely to be mediated via ADCC, since trastuzumab-F(ab′)2 was ineffective in this model. These results suggest that ADCC may be the predominant mechanism of trastuzumab action on submacroscopic tumor spread. Thus, measuring the ADCC activity of patient's leukocytes against the tumor cells may be a relevant predictor of clinical trastuzumab responsiveness in vivo.

7

MORPHOLOGY AND APOPTOSIS IN THICK TISSUES USING A CONFOCAL MICROSCOPY

Robert M. Zucker

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Confocal laser scanning microscopy (CLSM) is a technique that is capable of generating serial sections of whole-mount tissue and then reassembling the computer-stored images as a virtual 3-dimensional structure. In many ways CLSM offers an alternative to traditional sectioning

approaches. However, the imaging of whole-mount tissues presents technical problems of its own. One of the major problems using CLSM to image whole organs and embryos is tissue penetration of laser light. High quality morphological images begin by optimizing the sample preparation technique [1, 2]. Additional factors include evaluating CLSM performance by optimizing the acquisition variables (i.e. objective lens, averaging, pinhole size, bleaching, PMT voltage, laser excitation source, and spectral registration.) of the confocal microscope [3, 4].

Confocal microscopy has been used by our laboratory to study cell death and morphology in embryos, ovaries, eyes, ears, kidneys lungs and limbs [1, 2]. The technique has revealed structural morphology and the initiation of cell death by the uptake LysoTracker dye into acidic cells. LysoTracker Red (LT) is fixable by paraformaldehyde and concentrates in acidic compartments of cells. In whole tissues, this accumulation indicates regions of high lysosomal activity and phagocytosis. LT staining is an indicator of apoptotic cell death and correlates with other standard apoptotic assays [1, 2]. LT staining revealed cell death regions in mammalian limbs, neonatal ovaries, fetuses and embryos. The mammalian samples were stained with LT, fixed with paraformaldehyde/glutaraldehyde, dehydrated with methanol (MeOH), and cleared with benzyl alcohol/benzyl benzoate (BABB). The use of BABB matches the refractive index of the tissue within the suspending medium. BABB helps increase the penetration of laser light during CLSM by reducing the amount of light scattering artifacts and allows for the visualization of morphology in thick tissue. Following this treatment, the tissues were nearly transparent. This sample preparation procedure, combined with the optimization of confocal laser scanning microscopy instrument factors, allowed for the detection and visualization of apoptosis in fetal limbs and embryos which were approximately 500 microns thick. Recently, spectroscopic imaging capacity has been incorporated into confocal microscopes. The LT spectra had a maximum peak around 610nm while the fixative, glutaraldehyde (Glut), had a maximum peak around 450nm. Glut was added primarily to preserve the tissue morphology, but also provided molecules emitting in the green fluorescence range that helped to visualize the morphology of the tissue. The understanding of the spectra derived from the tissue was extremely useful in optimizing the staining protocol. We have continued to incrementally improve the tissue staining and preparation techniques to achieve better quality images in 3D.

References

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[3] Zucker RM Cytometry 2006 69 A 659–676.
[4] Zucker RM Cytometry 2006 69A 677–690.

This abstract of a proposed presentation does not necessarily reflect EPA policy.

8

VISUALIZING CELL TRAFFIC OF SINGLE TUMOR CELLS LIVE AND IN VIVO BY A NEW INTRAVITAL MICROSCOPY APPROACH

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[3]Paediatric Surgery, University Hospital Tuebingen, Germany
[4]Core Facility Molecular & Optical Live Cell Imaging (MOLCI), University Hospital Goettingen, Germany

Recent advances in photonics, multi-photon microscopy, intravital microscopy, near infrared fluorescence based imaging and new molecular and genetic tools are empowering scientist from many different fields to answer longstanding unresolved questions in small living animals. To noninvasively image for example cancer cell/blood vessel interaction and drug response at the cellular and subcellular level in live mice new imaging systems and stick objectives now become possible. Sophisticated systems like the OV110 (Olympus) offers a wide range of magnification by the use of parcentred and parfocal lenses enabling both macroimaging the whole animal and microimaging single cells down to resolutions of less than 1μm. Using these approaches we could recently show in vivo cell trafficking of xenotransplanted human tumour cells within the vessels of mice at the single cell level. Animals underwent single injection of transfected tumor cells (approximately 10^6 tumor cells / 100μl) into the tail vein. The mice were anesthetized and an arc-shaped incision was made in the abdominal skin in order to prepare a skin flap. The skin flap was spread and fixed on a flat stand. Cancer cell trafficking was carried out real time within the skin flap down to resolution of single cells. Using this approach we are able to localize single cells within the blood flow, measure size, diameter, motility, nuclear-plasma ratio and fluorescence. Right now this approach can not claim to be a cytometric approach. We have therefore started to optimize and modify the system to additionally readout cellular information like granularity.

9

LIVE CELL MICROSCOPY OF GROWTH-FACTOR DEPENDENT SIGNAL TRANSDUCTION PATHWAYS WITH A PROGRAMMABLE ARRAY MICROSCOPE (PAM)

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We have developed an optical sectioning structured illumination microscope denoted *Programmable Array Microscope* (PAM) targeted primarily at live cell studies. The key design feature is the placement of a spatial light modulator (SLM) at an image plane of a conventional fluorescence microscope in order to generate arbitrary patterns of conjugate structured illumination and detection. The major advantages of the PAM are: (1) simple, inexpensive design with no moving parts; (2) 1–2 orders of magnitude speedup

in optical sectioning using pixel illumination duty cycles of up to 50%; (3) optimal detection sensitivity with electron multiplication CCD cameras; (4) arbitrary, continuously programmable, and adaptive optical sectioning modes between and/or within images based on dot, line, or pseudo-random (Sylvester) sequence patterns; (5) efficient and sensitive optical sectioning due to simultaneous detection and processing of both conjugate (in-focus) and non-conjugate (out-of-focus) images; (6) flexible light sources: LEDs, lasers, lamps; (7) generation and detection of arbitrarily patterned polarized states; (8) compatibility with hyperspectral and lifetime-resolved imaging; (9) incorporation of light sources for photochemical activation, destruction, and/or transformation, modes compatible with FRAP, FLIP, and FCS/ICS protocols; (10) minimal photobleaching due to widefield distribution of the excitation energy; and (11) compatibility with schemes for achieving spatial superresolution.

This report will describe a 2nd generation commercially available PAM created by the combined efforts of our Department (MPIbpc) and Cairn Research Ltd. (UK). The PAM module, including the light source(s) and detector(s), is incorporated in a stand-alone assembly featuring a ferroelectric liquid-crystal-on-silicon (LCoS) SLM. It can be attached to an exit port of a conventional, unmodified fluorescence microscope. The current MPI-Cairn prototype system incorporates an Andor iXon emCCD camera and is mounted on an Olympus IX71 fluorescence microscope equipped with 60–150x objectives, dual excitation/emission filter wheels, and a Prior Scientific ProScan *x/y/NanoScanZ-Piezo* stage/focusing system. Further enhancements include hyperspectral detection and lifetime imaging (FLIM) based on Lambert Instruments phase-modulation modules. Optical sectioning and display can be implemented with single 16 ms exposure cycles and at overall 20 Hz full-field frame rates.

The PAM is currently being applied to studies of signal transduction, e.g. based on the use of quantum dot-conjugated ligands. Tracking of individual diffusing and /or trafficking nanoparticles on and within cells is readily achieved. The combined use of a 405 nm diode laser and LED/ion laser sources permits arbitrary patterns and cycles of photoconversion (e.g. DRONPA) and thus high-speed multiloci determinations of molecular 3D movement.

10

CAUSES AND CONSEQUENCES OF CHROMOSOMAL ANEUPLOIDY IN CANCER CELLS

Thomas Ried, Kundan Sengupta, Jordi Camps, Amanda B. Hummon, Hesed M. Padilla-Nash, Marian Grade, B. Michael Ghadimi, and Michael J. Difilippantonio

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Chromosomal aneuploidies are observed in essentially all sporadic carcinomas. Aneuploidy results in tumor specific patterns of genomic imbalances that are acquired early during tumorigenesis, continuously selected for and faithfully maintained in cancer cells. In order to characterize patterns of global transcriptional deregulation in primary colon carcinomas, we performed gene expression profiling of 73 tumors (UICC stage II, n=33 and UICC stage III, n=40) using oligonucleotide microarrays. For 30 of the tumors, expression profiles were compared to those from matched normal mucosa samples. We identified a set of 1,950 genes with highly significant deregulation between tumors and mucosa samples ($P < 1e\text{-}7$). A significant proportion of these genes mapped to chromosome 20 (P=0.01). Finally, we established a relationship between specific genomic imbalances, which were mapped for 32 of the analyzed colon tumors by comparative genomic hybridization, and alterations of global transcriptional activity. Previously, we had conducted a similar analysis of primary rectal carcinomas. The systematic comparison of colon and rectal carcinomas revealed a significant overlap of genomic imbalances and transcriptional deregulation, including activation of the Wnt/-catenin signaling cascade, suggesting similar pathogenic pathways.

These results were confirmed experimentally: we generated artificial trisomies in a karyotypically stable diploid, yet mismatch-repair deficient, colorectal cancer cell line using microcell mediated chromosome transfer. We then used global gene expression levels to determine what effect chromosome copy number increases have on the average expression levels of genes residing on the trisomic chromosomes. Our results show that, regardless of chromosome or cell type, chromosomal trisomies result in a significant increase in the average transcriptional activity of the trisomic chromosome. We therefore postulate that the genomic imbalances observed in cancer cells exert their effect through a complex pattern of transcriptional deregulation. Finally, we could show that aneuploid chromosome assume a nuclear position that is conserved and similar to the one in diploid cells. This might indicate that active transcription requires conservation of the 3D position of chromosome territories.

11

PROTEOME-WIDE DETERMINATION OF SUBCELLULAR LOCATION BY AUTOMATED MICROSCOPY

Robert F. Murphy

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Systems Biology requires comprehensive, systematic data on all aspects and levels of biological organization and function. In addition to information on the sequence, structure, activities, and binding interactions of all biological macromolecules, the creation of accurate, predictive models of cell behavior will require detailed information on the distributions of those molecules within cells and the ways in which those distributions change over the cell cycle and in response to mutations or external stimuli. Current information on subcellular location in protein databases is limited to unstructured text descriptions or sets of terms assigned by human curators. These entries do not permit basic oper-

ations that are common to other biological databases, such as measurement of the degree of similarity between the distributions of two proteins, and they are not able to fully capture the complexity of protein patterns that can be observed. The field of location proteomics seeks to provide automated, objective, high-resolution descriptions of protein location patterns within cells. The initial foundation for the field was our demonstration that automated classifiers could be trained to recognize all major subcellular patterns in fluorescence microscope images. The very high accuracy (over 98% on single 3D images) of these systems, and their ability to discriminate patterns that visual examination could not, gave confidence that the numerical features used to describe location patterns could form a basis for extending the methods to unsupervised learning of patterns. Thus, we have described large-scale collection of images of randomly-tagged proteins following by grouping of the proteins into statistically-indistinguishable location patterns using consensus clustering methods. The resulting clusters, or subcellular location families, are analogous to clusters found for other domains, such as protein sequence families.

12

APPLICATIONS OF FLUORESCENT FUNCTIONAL MICROBEADS AND NANOBEADS TO SENSING AND IN BIOASSAYS

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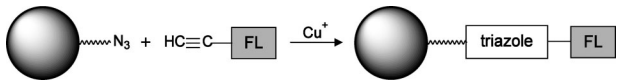
Fluorescent microbeads are widely used in bioanalytical sciences including fluorescence activated cell sortins (FACS). Fluorescent nanobeads, in turn, find applications as labels for biomolecules and even cells. Our research is focused on fluorescent beads that have a chemical functionality. For example, we have prepared microbeads of 1 to 10 μm in diameter that are loaded with a fluorescent indicator probe and then respond to various chemical parameters including pH value, oxygen partial pressure, hydrogen peroxide, and the like. They also can be made responsive to enzyme substrates by incorporating the respective enzyme. Beads can be made from various materials such as cellulose, polyurethane hydrogels, or polyacrylonitrile, but also from hydrophilic polymers such as ethyl cellulose or organically modified silicates which renders the materials to posses substantial mechanical strength. These materials resulted in various biosensory applications including imaging of oxygen distribution on skin.

The surface of such ("plastic") microparticles can be modified with biomolecules. In a typical application, fluorescence resonance energy transfer is demonstrated when fluorescently labeled streptavidin binds to fluorescent particles whose surface is coated with biotin.

We also make use of fluorescent nanoparticles in various forms. Silica nanobeads (3–5 nm i.d.) were labeled with conventional fluorophores such as fluorescein by a new and rather versatile method called "click chemistry" that is schematically outlined below. It enables room temperature fluorescent labelling of particles if it carries an alkyne group (–C≡CH) and the fluorophore carries an azido group (N_3), or vice versa.



In a final section, nanoparticles will be presented that are capable of converting near infrared light into visible light and whose surface can be modified so to make them amenable to bioassays. The nanoparticles usually are based on lanthanide oxides and the surface is usually modified using silyl reagents or click chemistry.

Selected articles: (1) Indicator-Loaded Permeation-Selective Microbeads for Use in Fiber Optic Simultaneous Sensing of pH and Dissolved Oxygen, G. S. Vasylevska et al., *Chem. Mat.* 2006, *18*, 4609; (2) The Click Reaction: Fluorescent Probing of a Metal Ions Using a Catalytic Reaction, and Its Implications to Biolabelling Techniques, O. S. Wolfbeis, *Angew. Chem. Intl. Ed.* 2007, *46*, 2980; (3) Novel Temperature-Sensitive Europium(III) Probes and Their Use for Simultaneous Optical Sensing of Temperature and Oxygen. S. M. Borisov et al., *Anal. Chem.* 2006, *78*, 5094.

BASIC TUTORIALS

13

MULTIPARAMETER DNA FLOW CYTOMETRY OF HUMAN CARCINOMAS

Willem E. Corver

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One of the first widely used flow cytometric applications was the characterization of cell cycle kinetics of human solid tumors and experimental leukemia. This included the measurement of DNA aneuploidy, proliferation rates and drug effects on cell cycle progression. Since this pioneering work of Göhde, Dittrich and others during the late sixties and early seventies, numerous scientists addressed the potential clinical relevance of flow cytometric DNA content (DNA ploidy) measurements. Especially the development of robust preparation and staining methods for flow cytometric DNA content measurements of fresh/

frozen (Vindelov method, Cytometry. 1983 Mar;3(5):317–339.) and even formalin-fixed, paraffin-embedded (FFPE) clinical samples (Hedley method, J Histochem Cytochem. 1983 Nov;31(11):1333–5.) boosted the number of studies. However, the literature showed to be controversial and in 1992 the DNA consensus conference held in Prout's Neck, Maine, USA, demonstrated that the impact of DNA ploidy measurements on patients clinical management had been limited (Cytometry. 1993;14(5):471–500.). Although the authors addressed several factors, amongst poor standardization and great lack of quality assurance that might have caused the DNA index to fail as an independent prognostic marker using multivariate analysis, they also made suggestions for further improvements. Especially the use of additional biomarkers, e.g. CD45 and/or keratin antibodies, was highly recommended. At present, more than a decade after the DNA consensus new protocols have been developed that do allow multiparameter DNA flow Cytometry of fresh/frozen or FFPE clinical samples. Normal cells and carcinoma cells can now be identified simultaneously in the same sample. The normal cell fraction can be used as an internal DNA reference (J Pathol. 2005 Jun;206(2):233–41.). Antigen retrievel technology markedly enhanced the quality of DNA histograms of FFPE samples. DNA ploidy analysis is highly improved. Simultaneously, S-phase estimates can be obtained without the presence of normal cells. Intra-tumor heterogeneity can now be studied in more detail. In addition, tumor cells can be flow-sorted for further molecular genetic analysis. A combination of DNA ploidy analysis, flow-sorting and the identification of specific molecular genetic changes can have clinical implications (Nat Genet. 2006 Apr;38(4):468–73.).

This basic tutorial will focus on techniques that allow the disaggregation, multi-color staining and flow cytometric analysis of fresh/frozen or FFPE samples from solid tumors.

14

BACTERIAL ACTIVITY ANALYSIS: PROLIFERATION VS. VIABILITY TESTS

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Current knowledge concerning the diverse multitude of complex bacterial communities and capacities is based mainly on studies on pure microbial cultures. Yet, pure cultures per se are highly artificial because ecosystems always consist of different taxa that generally use different strategies to gain energy and survive. However, most bacteria present in nature are not culturable in pure culture by means of classic cultivation methods. As a result, almost nothing is known about the complexity, diversity and activity of ongoing bacterial interactions in many ecosystems, which are very difficult to determine. Attempts to elucidate microbial interactions have often been made in the past by studying defined mixed laboratory cultures or natural communities by using phylogenetic techniques and simultaneous application of labelled substrates. Although for a long time the majority of bacteriologists were not aware of the possibilities that flow cytometry offers, in the meantime the technique has been shown to be able to analyse single-cell characteristics within communities. A greater insight is obtained into the activities of the members of a community and more informative data are acquired than would be obtained using measurements of the average properties on an entire consortium.

The tutorial will present techniques for viability assessment of bacteria, since it is of outstanding relevance in medicine, biotechnology and environmental microbiology. Besides using conventional cultivation techniques, viability is often determined with commercially available fluorescence kits that rely on propidium iodide (PI)-based assessment of membrane integrity. However, many users are unaware that the reliability of such tests has been demonstrated for a very limited number of bacterial species. On the other hand, analyses of DNA pattern provide a most excellent tool to determine activity states of bacteria. Bacterial cell cycle behaviour is generally different from the eukaryotic one and is predetermined by the bacteria's diversity within the phylogenetic tree and their metabolic traits. As a result every species creates its specific proliferation pattern which is divers from every other one. Only few bacterial species are investigated up to now, only little information is available about DNA cycling even in already known species. This prevents the understanding of the complexity and diversity of ongoing bacterial interactions in many ecosystems. However, some of these patterns are already known and will be presented within the tutorial.

15

FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS

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Programmed cell death or apoptosis is a natural process, occuring in embryonal development as well as in normal life to ensure tissue homeostasis. Because of its importance in many aspects of biology and medicine, the study of apoptosis in combination with other cellular factors can largely enhance our knowledge of the life cycle of cells. Cells, with the fate of dying, are pushed onto a molecular, biochemical and morphological path, which finally leads to a process dissolving the cellular integrity.

Different techniques, such as visual and electron microscopy, electrophoresis and flow cytometry have been applied to determine certain stages of the apoptotic fate. Not all apoptotic markers are recognized on every apoptotic cell, the complex cellular signaling pathways, finally leading to apoptosis, had to be discovered over the last ten years, giving different possibilities to access apoptosis and its processes.

During apoptosis, the most important features accessible to flow cytometry are 1) plasma membrane changes, 2) changes of mitochondria, 3) caspase activation and 4) DNA loss and fragmentation. Each of these features will be addressed, either by its own or in combination with others. Selective immunological markers can be used simultaneously with the markers mentioned above. A single criterion by itself is not enough in many cases to clearly identify apoptosis. If done so, it may be called a scientific malpractice. Different apoptotic signals can be measured at different time-points, from the very fast calcium reaction a few seconds after induction to the final disintegration of the cell hours later.

The combination of different markers in the same sample needs a careful selection of colors. One goal is to avoid as much as possible the so called compensation, a "black hole" for many users of flow cytometry. Not the flow instrument used to measure the sample, but the knowledge and expertise of the experimentator plays a key role. In this tutorial the trial will be undertaken, to overview many of these factors, to give insight in one of the most powerful techniques of cell functional analysis.

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BEAD BASED CYTOMETRY: SELECTED APPLICATIONS

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Bead based arrays provide a technological platform that allows multiplexed analysis of immunoassays in a miniaturized way. In contrast to protein chips, bead based technologies allow individualized labelling of a variety of ligands and thereby optimized assays even for proteins with highly diverging characteristics. In principle, bead based immunoassays represent modified sandwich immunoassays, commonly including a fluorescent solid phase and a fluorescent detection antibody. This technology allows not only miniaturized and/or multiplexed assays but also detection of soluble proteins by help of a cytometer, an improved sensitivity and detection range, and a one-platform combination of cellular and soluble parameters.

Currently, multiplex bead assays for infectious diseases, tumours, inflammatory and autoimmune diseases, cardiac or pulmonary diseases, and for toxicology are available, sometimes even as in vitro diagnostica for human use. Besides platform-dependent solutions, flexible assays that can be modified according to special requests are provided by several companies.

During this tutorial, current applications and solutions for multiplexed bead arrays will be presented. This includes the preparation and handling of the assays as well as the cytometric measurement equipment and software solutions for data analysis and quality control.

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FLOW CYTOMETRY: INSTRUMENTATION, SETUP, ADJUSTMENT

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Developments in multi laser instrumentation, optical devices and software have increased the potential of current flow cytometers,. This improvements are accompanied by changes in the way researchers setup their experiments and collect, analyze and present their data. Although instruments have evolved rapidly during the last years, some of the basic principles of flow cytometry remain the same.

The tutorial is intended to bridge the knowledge gap between already established procedures and recently discussed modifications to extend the scope and broaden the applications of this technology. We will focus on how the flow cytometer instrument specifications can be understood and utilized to result in a flexible platform yielding meaningful data.

The availability of an arsenal of new fluorescent probes, working in a digital world, offline compensation, base line adjustment, biexponential displays are just a few topics to be mentioned.

Overall the tutorial will discuss new challenges that follow the transitions from the older to the newer technologies.

ADVANCED TUTORIALS

18

FLOW AND IMAGE CYTOMETRIC FRET MEASUREMENTS

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Fluorescence resonance energy transfer (FRET) is a phenomenon in which an excited fluorescent molecule (donor) non-radiatively transfers its energy to an acceptor if the two molecules are within 10 nm from each other provided a couple of other requirements are met. These conditions include the overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor. The usefulness of FRET stems from the inverse sixth power dependence of the FRET rate on the distance between the donor and the acceptor. By determining the FRET efficiency, i.e. the fraction of excited donor molecules transferring their energy to an acceptor, biologists can estimate the proximity of fluorescently labeled molecules. FRET is manifested in several measurable phenomena including but not limited to decreased fluorescence intensity and lifetime of the donor (quenching) and enhanced fluorescence intensity of the acceptor (sensitized emission). In the tutorial we will give a detailed description of the most widely used FRET techniques, and briefly touch upon less widespread applications.

Donor quenching can be measured in flow cytometry by comparing the fluorescence intensity of the donor in the presence and absence of the acceptor. In microscopy quenching of the donor can be quantified by comparing the donor intensity before and after photobleaching the acceptor. Since bleaching photochemically destroys the acceptor, it can no longer accept energy from the donor, and the increase in donor fluorescence intensity (dequenching) is the measure of the FRET efficiency. Donor quenching can also be measured by comparing the fluorescence lifetime of the donor in the presence and absence of the acceptor. The major drawback of this approach is the expensive instrumentation required. Another manifestation of donor quenching is the FRET-induced decrease in the rate of donor photobleaching, which can be easily measured by most fluorescent microscopes.

The combined measurement of three fluorescent intensities in the donor, FRET and acceptor channels of a flow cytometer or microscope makes the determination of cell-by-cell or pixel-by-pixel FRET values possible. This method is computationally intensive, and requires the accurate determination of spectral correction factors.

Fluorescent labeling of molecules is usually achieved by fluorescent antibodies or green fluorescent protein derivatives. In addition to discussing the pros and cons of different labeling techniques, we will present exciting new developments, like the investigation of large-scale protein clustering by homo-FRET measurements or the detection of two different FRET interactions at the same time.

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DESIGNING, IMPLEMENTING AND ANALYZING MULTICOLOR FLOW EXPERIMENTS

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Flow cytometry-based immunophenotyipng assays have become increasingly multiparametric, concomitantly analyzing several different phenotypic and functional markers. In order to maximize the quality of the information obtained, antibody conjugate panels need to be developed with care, including requisite controls at all steps. The optimization procedure for such multicolor assays is extremely time-consuming, but the depth of the information content resulting from their application justifies the investment.

The requirement for multicolor assays has become evident in the past decade. Simply resolving important subsets of T cells requires at a minimum five or more measurements (e.g., lineage markers as well as differentiation markers). Assigning a functional description to each cell also requires multiple distinct measurements – for example, we recently demonstrated that cells capable of secreting multiple cytokines were far more protective against an infectious agent than cells making only single cytokines. However, identifying these cells required the measurement of three cytokines independently, in addition to the phenotypic markers.

I will discuss the general approach to the design and implementation of multicolor immunophenotyping panels. This process is iterative, building from a basic set of experiments to create the final, complex panel that will be used on large numbers of samples. There are a series of controls that must be designed at each step in the process that will verify that the panel is functioning as desired.

Finally, the problem of analysis of the resulting data sets will be briefly addressed. This topic is one that still requires significant research – the automation, batch analysis, and interpretation, along with the statistical validity of the results. While all of the difficulties may seem overly daunting, there is no doubt that the value of the information makes it worthwhile. And, given recent results, there is no doubt that these types of experiments will be required to optimally evaluate vaccines, pathogenesis, and the immune system in general.

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MOLECULAR IMAGING IN VIVO – A COMPARATIVE OVERVIEW

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From patient specific visualisation to advanced microscopic imaging techniques - Modern optical and molecular tumour imaging includes many different photonic technologies. To answer questions accumulated by specific tumour research fluorescence based imaging is a powerful tool providing an insight into molecular pathways and targets. Likewise, fluorescence and/or bioluminescence of defined structures can also be imaged in-vivo using intravital, confocal or multiphoton microscopy. The Introduction of novel fluorescent dyes emitting in the near infrared range (NIR) combined with the development of sensitive detector systems and monochromatic powerful NIR-Lasers first permits the quantification and imaging of fluorescence and/or bioluminescence in deeper tissues. This new technique of fluorescence based in-vivo imaging in whole animals is called Fluorescence Molecular Tomography (FMT) or Fluorescence Reflectance Imaging (FRI) and can either be used to study tumour growth e.g. via subcutaneous injected labelled cells or to study effects of pharmacological treatment on tumours using labelled drugs.

Techniques particularly in the NIR-range offers superb signal to noise ratios and thus the potential to detect molecular targets in-vivo. In combination with the technique of animal Volume Computer Tomography (VCT) questions dealing with tumour angiogenesis /vascularization could be answered non-invasive using the same animal. Different techniques like FRI, diffuse optical tomography (DOT) beneath others are currently being evaluated for clinical

applications and a combination of these molecular and optical imaging techniques with novel contrast agents and/or dyes may greatly facilitate molecular target recognition in patients in the near future. The workshop will summarize the main in vivo molecular imaging techniques and compare these particularly with regard to resolution, penetration depth, costs and practicability.

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SLIDE BASED CYTOMETRY SYSTEMS

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In the recent years multi-parameter and multicolor cytometry of biologicalmaterial fixed on slides (slide based cytometry) was made available by the development of new fast microscopic systems. These technologies allow therapid quantitative analysis of samples ranging from bacteria, over individual cells to tissue sections. The tutorial will introduce different fluorescence based microscopic systems for slide based cytometry that rely on laser or on Mercury-arc-lamp excitation. The technologies will be explained with respect to hardware and software and typical examples for various applications including hands-on experimental protocols for: immunology, drug discovery, tumor and cell biology.

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MULTIPARAMETRIC FLUORESCENCE-IN-SITU-HYBRIDIZATION (FISH) AND SPECTRAL KARYOTYPING (SKY) IN DIAGNOSIS OF HUMAN MALIGNANCIES

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SKY refers to the simultaneous visualization of 24 differnentially labeled chromosome painting probes, fluorescnce microscopy and spectral imaging. SKY has proven exceedingly valuable for the comprehensive analysis of cytogenetic abnormalities associated with malignant disease and has been applied to a large series of samples derived from hematological malignancies and solid tumors. The systematic analysis of chromosomal abnormalities in cancer cells using SKY allows for the characterization of novel and hidden chromosomal translocations, identification of complex rearrangements and reconstruction of clonal evolution events during cancer progression, and it has revealed the role of unstable chromosome rearrangements, such as jumping translocations, occurring as tissue-specific genomic imbalances. In fact, since its invention in 1996, more than 500 papers have been published that applied SKY for the analysis of various chromosomal preparations. SKY results can now be submitted to a interactive database, which facilitates data analyses (SKY, M-FISH and CGH), storage and retrieval of recurrent chromosome breakpoints found in many cancers and constitutional disorders (http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi). SKY greatly assists in identifying chromosomal regions involved in homogenous staining regions and double minute chromosomes, regardless of size and numbers. However, with respect to homogenous staining regions and double minute chromosomes, again, SKY has its limitations, in that these aberrations often contain multiple genes and/or DNA regions that are tightly linked; resolving these details therefore often requires additional FISH hybridizations with either gene-loci probes or specific chromosome or chromosome-arm paints. The application of SKY to murine chromosome has assisted in the elucidation of novel and critical mechanisms of chromosomal instability. We will discuss essentials of chromosome preparations, in situ hybridization, image analysis and interpretation. Lastly, we will discuss how chromosome based SKY analyis and multicolor interphase FISH can be intergrated with array based analyses, such as comparative genomic hybridization on BAC or oligonucleotide arrays.

COMPANY TUTORIALS

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AXIOVISION SFM - CELL IMAGE ASSOCIATED DATA FOR FLOW CYTOMETRY RESULTS

Wolf Malkusch

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AV SFM (Scanning Fluorescence Microscope) proved to be a reliable alternative method, providing results comparable to LSC (Laser Scan Cytometer) and FCM (Flow Cytometer). Slide based cytometry (SBC) proved to be more suitable for rare-cell detection than FCM. SFM with digital slides may prove an acceptable adaptation of conventional fluorescent microscopes in order to perform rare cell detection.

Based on the results of flow cytometry statistically sufficient cells from cytospin specimens will be acquired automatically in multiple fluorescence channels (Axio-Vision MosaiX) with a digital camera (AxioCam MRm) using an upright fluorescence microscope (AxioImager). Using appropriate scripts these images will then be measured with AxioVision image analysis software.

The scripts may be adapted to all evaluation needs. AxioVision offers all currently available functions and parameters for image processing and image analysis. All single steps may be combined in any order to receive reasonable extraction parameters for the single cell analysis. The measurement results of all cells are finally integrated in a single data table. The AxioVision SFM module is finally used to link the data of the results table with the original multichannel image. From these data various distribution types may be created (histogram, scatter plot, gallery, gated data table), in order to isolate "rare event" cells as an image with the connected data.

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OPTICAL FILTERS - ESSENTIAL TOOLS IN CYTOMETRY

Michael Sommerauer

AHF Analysentechnik AG, Tuebingen, Germany

This talk gives a survey on optical filters in modern fluorescence applications. Beginning at the basics of fluorescence we will proceed to advanced filtersystems in slide based cytometers and flow cytometers. New high transmissive filters maintain compensation and push the detection limits. Good signal/noise ratios paired with strong signals do not cancel out each other any more.

CORE FACILITIES MANAGER'S WORKSHOP

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CORE FACILITIES AND NETWORKING – DISCUSSION FORUM

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Approaches to managing a flow cytometry core facility vary widely depending on many factors that are unique to a given research environment. However a common factor among core facility managers is that every day seems to bring new challenges. One must consider several elements when determining the organizational structure that will be most appropriate for meeting the needs of the institution. In parallel the role of the core manager expands beyond traditional maintenance to include more developmental and management tasks as well as interaction with users.

This workshop is intended to give an overview on new techniques and tools to keep life and work in the Core as friendly and easy as possible. Facility requirements, budget calculations, funding, management and organisation of users, principal investigators and faculty, knowledge resources and networks, are just a few of the topics to be discussed.

But most importantly this workshop should be a venue for people to meet and share their experiences in running a flow cytometry core facility, regardless of whether they just started to build up their own facility or if they are considered to be experts in the field.

CANCER BIOLOGY AND THERAPY

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CHARACTERIZATION OF THE PROLIFERATING CHRONIC LYMPHOCYTIC LEUKEMIA CELLS IN AN IN VITRO MODEL FOR PSEUDOFOLLICLES

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Introduction: The reasons for the heterogeneous clinical behaviour of patients with chronic lymphocytic leukemia (CLL) are not well-known. The defective apoptosis of CLL cells is considered to be the main cause for accumulation of CD5+ B-cells, however, many evidence exist about a proliferating pool; proliferation centres (pseudofollicles) can be found in bone marrow and lymph node biopsies and proliferation of resting CLL cells in vitro is inducible. Our aim was to establish an in vitro culture similar to proliferation centres in order to investigate and to find differences in the behaviour of CLL cells. Methods: Sorted CLL cells from 21 patients were bred on bone marrow stromal cells or in medium under the stimulation of different T-cell derived cytokines (sCD40L/sCD40L+IL4/sCD40L+IL2+10) for 84 hours. On the harvested cells proliferation, apoptosis, changes in the immunophenotype were studied by flow cytometry and immunohistochemistry. Results: The CLL cells in the peripheral blood do not proliferate. The proliferation of CLL cells was inducible in co-culture with stromal cells by sCD40L, IL2 and IL10. CLL cells, exclusively from cases with lower platelet and red blood cell counts, were able to proliferate. The proliferating CLL cells regulated antigens important in the T-B-cell contact, as CD40, MHC II and adhesion molecules, as CD18, CD11c up. IL2 in combination with IL10 induced significantly different immunophenotype as IL4. The stromal cells protected the CLL cells from apoptosis. The CLL cells from clinically indolent cases showed far worse survival in medium than the cells from clinically progressive cases. Conclusions: In this co-culture system we could detect different behaviour patterns of CLL cells regarding proliferation and survival capacity. On the basis of these findings, we suppose that not only resistance to apoptosis, but proliferation contribute to the progression of the disease resulting in bone marrow failure with thrombocytopenia and anaemia. Supported by the Bayerische Forschungsstiftung PIZ-107-06.

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Her3 AND Her4 GENE AMPLIFICATION HAVE PROGNOSTIC IMPACT IN BREAST CANCER

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Her2 gene amplification or receptor protein overexpression is a globally accepted indicative parameter for Her-

ceptin therapy in metastatic breast cancer and is detected via (fluorescence)-in-situ-hybridization (FISH/ISH) or immunohistochemistry (IHC). However, in more than 50% of cases no therapeutic benefit from Herceptin can be observed, strongly indicating that Her2 alteration is indeed a prerequisite for initiating Herceptin therapy but insufficient for predicting therapy response. The Her2 related erbB receptors have been shown to directly interact with Her2 receptor and thereby mutually affect their activity and subsequently malignant growth potential. However the clinical outcome with respect to total erbB receptor state remains largely unknown. Here, we investigated Her1–Her4 both on DNA and protein level using FISH probes targeted to all four receptor loci and immunohistochemistry in tissue samples derived from 278 breast cancer patients. We retrospectively found Her3 gene amplification with an univariate negative impact on disease free survival (HR=2.35 (95% CI [1.08–5.11]), p=0.031) whereas Her4 amplification set a positive trend in overall, disease and recurrence free survival. Protein expression appeared inconsistent and in contrast to FISH bears ambiguous prognostic statement. Nevertheless, the simultaneous investigation of all erbB receptor genes reveals additional prognostic information for breast cancer patients and might help to stratify patients for individualized targeted therapy. Her3 and Her4 receptor status alterations have additional impact on course of disease in Her2 amplified and non-amplified patients and may represent worthwhile targets in breast cancer therapy. Supported by the Bayerische Forschungsstiftung AZ 585/03.

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THE IMPACT OF TRASTUZUMAB, PERTUZUMAB AND CETUXIMAB ON CELL PROLIFERATION OF BREAST CANCER CELL LINES

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Background: The potential of epidermal growth factor receptor (EGFR-) and Her2-targeted antibodies Cetuximab, Pertuzumab and Trastuzumab, used in combination to inhibit cell proliferation of breast cancer cells in vitro, has not been extensively investigated. It is anticipated that there would be differences between specific erbB receptor coexpression profiles that would affect tumour cell growth. Materials and methods: We have examined the effects of Cetuximab, Pertuzumab and Trastuzumab, applied separately or in combination, on cell proliferation of BT474 and SK-BR-3 breast cancer cell lines. Cell cycle progression of BT474 and SK-BR-3 cells was statically and dynamically assessed using flow cytometry. In order to discover a potential influence of differential EGFR co-expression on sensitivity to antibody treatment, EGFR was down-regulated by siRNA in SK-BR-3. An annexinV/propidium iodide assay was used to identify potential induction of apoptosis. Results: Treatment with Pertuzumab and Trastuzumab, both targeted to Her2, resulted in a reduced fraction of proliferating cells, prolongation of G1 phase and a great increase in quiescent BT474 cells. Cetuximab had no additional contribution to the effect of either Pertuzumab or Trastuzumab when administered simultaneously. Treatment with the antibodies did not induce an appreciable amount of apoptosis in either BT474 or SK-BR-3 cells. In contrast to SK-BR-3, the BT474 cell line appears to be more sensitive to antibody treatment due to low EGFR content besides Her2 overexpression. Conclusion: The extent of decelerated or blocked cell proliferation after antibody treatment that is targeted to EGFR and to Her2 depends both on EGFR and Her2 co-expression and on antibody combination used in the treatment setting. Cetuximab did not enhance any inhibitory effect of Trastuzumab or Pertuzumab, most probably due to the dominant overexpression of Her2. Cell susceptibility to Trastuzumab/Pertuzumab, both targeted to Her2, was defined by the ratio of EGFR/Her2 co-expression. Supported by the Else-Kröner Fresenius Stiftung eksvrt161.

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APPLICATION OF FLUORESCENCE BAR CODING TO MULTICOLOR FLOW CYTOMETRIC QUANTIFICATION OF ErbB RECEPTOR-DRIVEN INTRACELLULAR SIGNALING

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ErbB2, a ligandless member of the ErbB family of receptor tyrosine kinases is often overexpressed in invasive breast cancer; the level of overexpression is correlated with poor clinical outcome. For therapeutic purposes, ErbB2 can be targeted both extracellularly with antibodies (trastuzumab, Herceptin®; pertuzumab, Omnitarg®) and intracellularly with specific small molecule agents competitively inhibiting ATP binding. Unfortunately ErbB2 overexpression frequently does not correlate with the outcome of these therapies. Primary trastuzumab resistance occures in 60–70% of ErbB2 overexpressing invasive breast cancers, and resistance can also develop during treatment. Possible causes of varying sensitivity could be the differences in the Ras/Raf/MAPK/ERK1/2, PI3K/Akt and Stat3 signaling patterns of trastuzumab sensitive and resistant breast cancer cell lines SKBR-3, BT474 and JIMT-1. To analyse the signaling activity of these pathways in large populations of adherent cells, we have designed and optimized a protocol for the specific labeling and flow cytometric measurement of phosphorylated Stat-3, ERK1/2 and Akt, using phospho-specific antibodies. While high throughput array systems are already available for flow cytometry, these do not provide highly multiplexed excitation and detection. A tool, fluorescence bar coding, allows labeling different samples together in the same tube, decreasing the tube-to-tube variation of labeling intensity. We have set up a bar coding protocol for

adherent cells by using Alexa Fluor 350 and Pacific Blue dyes. In spite of the high overlap between the emission spectrum of these dyes, up to 9 differently stained populations could be well discriminated after proper compensation. This allows for applying diverse treatment protocols to several different cell lines in parallel, and then quantitatively comparing the specific signaling outcomes in multicolor flow cytometry from a single sample. Supported by the Bayerische Forschungsstiftung PIZ-108-06.

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THE EFFECT OF CISPLATIN ON THE STRUCTURE OF CHROMATIN IN TUMOR CELLS

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Cisplatin, or cis-diamminedichloroplatinum (II), is widely used for the treatment of tumors. Many cellular components react with cisplatin but its antitumor effect is based mainly on the damage of nuclear DNA. A variety of adducts that include interstrand and intrastrand DNA crosslinks and DNA-protein crosslinks are forming during this process. However, the mechanism whereby these DNA adducts kill tumor cells is not exactly understood. Image cytometry gives a possibility of integral estimation of molecular effects of cisplatin on the cellular level. For this purpose tumor lymphoid cells were incubated with cisplatin (5 mg/ml, 1 hour), fixed in ethanol (30% v/v) and stained with methylene blue (1%, 30 min). Ultraviolet light (365 nm) induced breaking of DNA was analysed by comet assay. Cell imaging was performed on a microscope Nikon Eclipse 50i equipped with a cooled CCD camera DS-5Mc; the number of cytometric features computed from images of cell nuclei includes Area, Mean Brightness, Integral Brightness, Energy, Entropy, Contrast, Homogenity, Corellation, Variance, Shade and Promenance. We have found that under influence of cisplatin it is observed significant increase such features of cell nuclei as Mean Brightness, Contrast, Variance, Shade and Promenance. On the contrary, Area of cell nucleus decreases. If Integral Brightness of cell nucleus does not alter when Area decreases and Mean Brightness increases it may be interpreted as a sign of cisplatin evoked compaction of chromatin. On the other hand increase of Contarst and Variance means a transition of euchromatin to heterochromatin. To our mind observed increase of Shade and Promenance reflects specific for cisplatin changes of chromatin microstructures on the level of optical resolution (300–400 nm). These findings are in accordance with notions that cisplatin represses gene activity, stabilizes supramolecular structure of chromatin and prevents relaxation of broken DNA in tumor cells.

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DIFFERENTIAL INFLUENCE OF CHEMORESISTANCE ON RADIOSENSITIVITY IN HUMAN NORMAL AND TUMOUR CELLS

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Background: Despite the clinical importance of combined radio-chemotherapy, the mutual modulatory capacity of the two treatment modalities is poorly understood. We analysed the radiosensitivity of wild type cell lines and their chemoresistant derivatives and conducted experiments to point out mechanisms which may underlie the observed modifications. Methods: A549 and Calu1 lung cancer, SW620 colon carcinoma, PC3 prostate cancer and the immortalised TK6 lymphoblastoid cell lines were tested. Chemoresistance was induced by intermittent exposure to doxorubicin, taxol or cisplatin, respectively. Radiosensitivity was determined by colony forming assay. An MDR-phenotype was tested for by Rhodamine-123 efflux experiments. The cell cycle response to radiation and cell death kinetics after serum depletion were measured by flow cytometry. Results: Doxorubicin resistant A549 cells were significantly more radiotolerant than wild type A549. Inversely, taxol selected PC3 cells were more sensitive to X-rays. In SW620, Calu1 and TK6 cells, no unequivocal change in their radioresponse was observed after chemoselection. Of the five chemoresistant cell lines, only SW620 had a clearly improved Rhodamin-123 efflux. Cell cycle arrest after 2 Gy, indicating ongoing DNA-damage repair, was differentially prolonged in the slightly radiosensitized cisplatin-selected SW620 cells. After 5 Gy, the massive arrest reactions did not allow to distinguish parental and chemoresistant daughter cell lines. Cell death (apoptosis) kinetics after serum withdrawal appeared accelerated in Calu1 but delayed in TK6, both of which exhibited a uniform radioresponse, irrespective of their chemotolerance. Only in taxol selected PC3, an increased loss of viable cells corresponded to their radiosensitized phenotype. Summary: Two out of five cell lines had an altered radiosensitivity after long term selection with cytostatic drugs. Neither MDR associated effect nor altered cell cycle response and DNA-repair nor early cell death kinetics could unambiguously explain the observed reactions. Structural resistance resulting from modified glutathione content is a further candidate parameter to be investigated.

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COOPERATION BETWEEN TWO TNF RECEPTORS IN THE U937 IS NECESSARY FOR EFFICIENT CYTOCIDAL RESPONSE TO TRANSMEMBRANE TNF WHEREAS PROTECTIVE RESPONSE IS NOT

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TNF exerts its wide range of activities via two receptors TNF-R55 and TNF-R75. The latter is efficiently activated by transmembrane TNF (tmTNF), but not by soluble TNF (sTNF), while TNF-R55 can respond fully to both TNF ligands. Very little is known about cooperation of TNF receptors on the level of initiation of activation by tmTNF. It is also of interest how cell death and life decisions are made on the level of receptors as it may be crucial for treatment of cancer and autoimmune diseases. We observed a distinct

pattern of cytocidal response to tmTNF in a model of U937 cell variants characterized by different TNF sensitivity. U937M cells were sensitive to sTNF and tmTNF, while U937ATCC cells were resistant to both forms of TNF. Surprisingly, CHX did not enhance cytocidal effect of tmTNF in U937M cells suggesting the impaired cytotoxic mechanism probably dependent on TNF-R75 function or its cooperation with TNF-R55. Blocking of TNF-R55 by antibodies in U937M cells was not efficient enough to inhibit cytocidal effect of sTNF and tmTNF. However it was possible to fully block the cytotoxic effect exerted by the p55-specific TNF mutein in U937M cells. This effect could suggest that TNF-R75 may transduce cytotoxic effect itself; however it was not confirmed by direct activation by p75 mutein or by different agonistic antibodies. The effect of sTNF and tmTNF was blocked effectively by anti-TNF-R55 antibodies in U937ATCC cells. Mobilization of NF-kB from cytoplasm to nucleus was estimated to measure pro-survival response in U937 cells. Activation of NF-kB was blocked by anti-TNF-R55 antibodies but not by anti-TNF-R75 antibodies upon stimulation with sTNF in U937M and U937ATCC cells. On the other hand tmTNF activation was blocked effectively by anti-TNF-R55 antibodies but not anty-TNF-R75 antibodies in U937ATCC cells. In U937M cells blocking by anti-TNF-R55 antibodies was weak. Similar mild effect was exerted by anti-TNF-R75 antibodies. It was also possible to activate NF-kB by TNF-R75 specific TNF mutein in the presence of stabilizing antibodies 80M2 in U937M cells. Taken together, we suggest that cooperation between TNF receptors in exerting cytotoxic effect by tmTNF in U937 cells is required. While TNF-R75 seems to have no effect on NF-kB mobilization in U937ATCC it is involved in induction of this activity by tmTNF in U937M cells.

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GENERATION OF REACTIVE OXYGEN SPECIES AND INDUCTION OF A CELL CYCLE ARREST BY EPOXY AND ACRYLATE MONOMERS

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Triethylene glycol dimethacrylate (TEGDMA) is a monomer of current dental resin materials, whereas epoxy cyclohexyl methyl-epoxy cyclo-hexane carboxylate (K-126) is an epoxy monomer included in experimental materials. Both chemicals caused genotoxicity in mammalian cell cultures. We hypothesized that the underlying mechanism of the genotoxic effects was the production of reactive oxygen species (ROS) that contribute to DNA damage, finally leading to a cell cycle delay. The generation of ROS by TEGDMA and K-126 in normal human fibroblasts (N1) was measured after various exposure periods by flow cytometry (FACS) using the oxidation-sensitive fluorescent probe 2′7′-dichlorodihydrofluorescin diacetate (H2DCF-DA). The distribution of N1 cells between the phases of the cell cycle (G1, S, G2) in untreated cell cultures and those treated with TEGDMA and K-126 was also analyzed by flow cytometry after PI staining. Both substances increased the amounts of ROS in N1 cells after a 2 hour exposure period, but the epoxide K-126 was more effective than TEGDMA. The production of ROS was enhanced about four-fold by 0.5 and 1.0 mmol/l K-126 compared with a 2–3-fold increase caused by 1.0 and 3.0 mmol/l TEGDMA. Furthermore, TEGDMA and K-126 modified the cell cycle differently. 3 mmol/l TEGDMA increased the numbers of cells on G1 phase to almost 80% after a 2, 6, and 24h exposure period compared with about 65% in untreated cell cultures. A statistically significant change was not found with cells in G2. On the contrary, 0.1 mmol/l K-126 reduced the number of cells in G1 to about 55% but increased the cell number in G2 from 15% to 25%. While 0.3 mmol/l K-126 had a similar effect, higher concentrations of K-126 were severely cytotoxic. Our results suggest that TEGDMA and K-126 support the intracellular formation of ROS but the modification of the cell cycle induced by both monomers is caused by different mechanisms. Supported by the Deutsche Forschungsgemeinschaft (DFG).

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CIRCULATING TUMOR CELLS IN THE METASTATIC PATHWAY

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Tumor cells from the primary tumor grow invasive forming supplying blood vessels and thus are supposed to enter the blood stream. Probably more frequently cells enter the interstitial fluid after down regulation of intercellular bonds, and are washed into the adjacent lymph nodes. Dependent on their (re)expression of adhesion molecules some of them may be retained in the lymph nodes while most of the cells might migrate further via the ductus thoracicus into the blood stream. We have analysed these ways of cell dissemination in breast cancer patients before treatment, during neoadjuvant systemic therapy, before and after surgery and during adjuvant therapyusing laser scanning cytomertry. We observed a correlation between tumor size and circulating tumor cell number before intended neoadjuvant treatment, indicating continuous release of cells during tumor growth. There was a reduction in cell number during the subsequent initial cycles of neoadjuvant treatment which was highly predictive of final tumor size reduction, indicating an identical behaviour of the circulating cells as the tumor itself. This initial response was also predictive for relapse free survival in spite of a subsequent increase in circulating cell numbers during the following chemotherapy cycles. This increase was supposed to be due to tumor tissue disintegration and the impact for further development of metastases needs to be evaluated. There was a release of epithelial cells also during surgery, part of which were re-eliminated rapidly, but the (probably malignant) cells could be observed even over prolonged times in the circulation. Adjuvant chemotherapy was able to reduce the circulating tumor

cell number correlating with relapse free survival, but there was a reincrease in part of the patients, preferentially with lymph node positive disease, highly predictive for early relapse. Dependent on the expression of adhesion molecules, tumor cells may be trapped in the microcirculation of organs like bone, liver or lung and disappear from the circulation. Others without adhesion molecules may be able to recirculate for long times. If the settled in the respective organs such cells may start growing. Most cells will have restricted growth potential and these micrometastases may disappear again, but cells with "stem cell like" properties may be able to divide into life restricted daughter cells and self renewing cells and thus sustain indefinite growth. Cells in the lymph nodes may be a surrogate marker for the tumor cells ability to adhere. We, here, present data on longitudinal observation of circulating tumor cells in breast cancer patients, sustaining the hypothesized the different steps of metastasis formation.

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LOSS OF FHIT AND p16 ARE EARLY EVENTS IN TUMORIGENESIS OF ORAL SQUAMOUS CELL CARCINOMA AND CHARACTERISTICALLY OCCUR IN SIMPLE KERATOSIS

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Background: Tumorigenesis of oral squamous cell carcinoma (OSCC) has been postulated to represent a multistep process driven by the accumulation of carcinogen-induced genetic changes. Alterations of tumor suppressor genes FHIT and p16 characteristically occur in oral leukoplakia, a known precursor of OSCC, and are considered to mark the transition from simple keratosis (hyperplasia) to dysplasia. Aim of the study was to examine the value of Fluorescence in-situ hybridization (FISH) as a tool for the detection of losses of FHIT and p16 in this process. Material and Methods: We studied 67 leukoplakias (24 hyperplasias, 33 dysplasias, 10 in-situ carcinomas) using FISH probes targeting FHIT gene and p16 gene and the respective centromeres 3 and 9. Control tissues of oral mucosa from infants and adults, invasive carcinomas and normal epithelia of tumor patients were included. Results: Subject to the definition of cut off values, losses of FHIT gene and p16 gene were present in up to 22 simple keratoses (92%), 25 dysplasias (76%) and 7 in-situ carcinomas (70%). Likewise, most normal epithelia of tumor patients showed loss of FHIT and p16 (62% and 92%, respectively), whereas in normal epithelia of non-tumor-patients these genetic alterations were almost absent (1/20, 5%). Polysomy 3 was much more frequent than polysomy 9 and occurred in 1 hyperplasia (4%), 10 dysplasias (30%) and 4 in-situ carcinomas (40%) marking the critical step from benign simple keratosis to obligatorily premalignant dysplasia. Conclusions: FISH is a highly sensitive method in the detection of genetic alterations on the level of single cells and tumor subclones and can clarify our understanding of the stepwise process of oral squamous cell carcinogenesis. In contrast to current tumor progression models losses of FHIT and p16 do not mark the transition from hyperplasia to dysplasia but are already present in simple keratosis.

CLINICAL CYTOMETRY AND ADVANCES IN DIAGNOSTIC IMMUNOPHENOTYPING

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OPTIONS AND LIMITATIONS IN DETERMINATION OF BACTERIAL CONTAMINATIONS IN PLATELET CONCENTRATES (PC) - A STUDY USING FLOW CYTOMETRY AND TRANSMISSION ELECTRON MICROSCOPY

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PC obtained from thrombapheresis are stored at room temperature. In this condition, the storage time is limited to few days. Blood collection and further processing of the PC is usually provided under strict precautions to avoid bacterial contamination. PLT are usually suspended in plasma which provides an ideal growth environment for bacteria in the case of bacterial contamination. Transfusion of a contaminated PC can cause severe sepsis in the recipient. Several methods have been developed to quickly demonstrate bacteria in PC. One of these techniques is flow cytometry which is quick and relatively simple. In the literature several problems with sensitivity and accuracy in respect to the linearity in dilution series have been reported which we also could find using high concentrations of PLT, spiked with Escherichia coli, strain DH-5 alpha. For comparison, bacteria were fluorolabeled with DNA stains such as DRAQ5TM, SYTO 9® and SYBR® Gold or with BacLightTM Green (which stains only the bacterial wall. Staining of bacteria was performed without and in the presence of PLT and quantified by flow cytometry using standard reference beads. In order to evaluate the accuracy of the measurements, we checked the linearity of data in 10-fold dilution series of bacteria used for spiking. The linearity was proofed in spiked PC or with bacteria alone. The comparison of the different stains showed that SYBR®-Gold was the most sensitive dye. DRAQ5TM did not provide a sufficient

staining while SYTO 9® exhibited a strong fluorescence but also a high background stain of PLT. In 10-fold dilution series of bacteria, linearity was exact over 5 decades and also sensitivity was good (less than 10 bacteria per μl could be clearly detected). However, in combination with PLT, linearity was not sufficient by measuring less than 1000 bacteria per μl. The reasons for this were problems to discriminate completely bacteria from PLT according to their fluorescence although appropriate gating strategies were performed. In order to see, whether bacteria can be attached or engulfed by PLT we used transmission electron microscopy to check such possibility in PC spiked with bacteria. We could demonstrate that PLT are able to attach and ingest bacteria which are deposed in the open canalicular system of PLT. Nevertheless, the engulfment of bacteria did not induce a significant activation of PLT as shown by flow cytometric determination of CD62 expression on PLT. In conclusion, we think that appropriate lysing procedures for PLT which dissolve them completely without causing injury to the fluorolabeled bacteria would be necessary in order to provide more accurate determination of bacterial contamination in PC.

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INNOVATIVE CONCEPTS FOR ABSOLUTE IMMUNOPHENOTYPING BY SLIDE-BASED CYTOMETRY

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Background: Absolute cell count and differential immunophenotyping of leukocytes is essential for diagnosis and follow-up, e.g. in HIV, cancer and neonates [1]. Routine analysis requires a minimum of 250 μl blood, and neither an absolute cell count nor a detailed immunophenotyping are available. We present two concepts using slide-based cytometry in order to overcome these boundaries. Materials and Methods: 10 μl EDTA blood of patients suffering from Head-Neck-Carcinomas, other otorhinolaryngological diseases or cardiologic patients were stained with DRAQ5 and either CD3-PE, CD4-Alexa Fluor 488, and CD8-PE-Cy5 or CD45-FITC, and CD14-PE, respectively. A 'no-lyse-no-wash' versus a 'lyse-no-wash' method (Quicklysis) was performed. 20 μl of the suspension were applied to a Neubauer chamber. Leukocytes were analyzed by LSC. Another aliquot of blood was taken for routine analysis. Erythrocyte count was performed after another dilution. Results: Brévais' regression coefficient showed a good internal correlation of LSC ($r > 0.85$) with no significant difference between routine analysis and LSC ('lyse-no-wash': $r = 0.9$, $p=0.009$, $\alpha = 0.05$; 'no-lyse-no-wash': $r = 0.98$, $p < 0.0001$, $\alpha = 0.05$). Doublet and Tripletts were relocalized, gated and calculated separately. Immunophenotyping by LSC (CD3/CD4/CD8, CD45/CD14) was unequivocal. Conclusion: An absolute cell count and detailed immunophenotyping is feasible by using the Neubauer chamber for slide-based analysis. Minimal amounts of blood are required, the method is very cost-efficient, and data show no significant difference to routine laboratory. This concept might prove versatile in patients with low blood volume and where absolute cell count is essential, e.g. monitoring during chemotherapy and in terms of cancer screening [2].

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THE CORE UNIT FLUORESCENCE-TECHNOLOGIES IN THE IZKF LEIPZIG

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The Core Unit Fluorescence-Technologies in the Interdisciplinary Centre for Clinical Research (IZKF) Leipzig provides instrumentation and technical and professional assistance in the fields of high-resolution fluorescence microscopy and analytical and preparative cytometry. Currently the Core Unit houses two confocal Laser Scanning Microscopes, a confocal multiphoton Laser Scanning Microscope, flow cytometric analysers (FACScan, LSR II), a FACSVantage SE high-speed cell sorter, and a Laser Scanning Cytometer. In recent years newly developed instruments and new fluorochromes extend the range of applications, offering researchers completely novel opportunities. So the trend is to acquire as much as possible parameters of the same cell at the same time. With our confocal Laser Scanning Microscopes six laser lines (364 nm, 405 nm, 458 nm, 488 nm, 543 nm, and 633 nm) for excitation are available. The flow cytometers are equipped with up to four lasers (UV, 405 nm, 488 nm, and 633 nm) and 12 parameters (10 fluorescence and 2 scatter parameters) can be measured at the same time. The Core Unit offers access to these techniques in a user-friendly manner by establishing services such as the maintenance of a permanent operational readiness of the equipment, instruction and training of PhD students and co-workers, optimisation of the analysis and the presentation of results and ensuring optimal utilisation by time scheduling. A further goal of the Core Unit is to introduce microscopic and cytometric methods into new research areas and to offer further training.

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ALTERATION OF IMMUNE PHENOTYPE FOLLOWING PROTEIN LOSING ENTEROPATHY AFTER TOTAL CAVOPULMONARY CONNECTION BY CYTOMICS

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Protein-losing enteropathy (PLE), the enteric loss of proteins, is a potential late complication after Fontan-type surgery (total cavopulmonary connection, TCPC). PLE etiology is poorly understood, but immunological factors seem to play a role. This study was aimed to gain insight into immune phenotype alterations following post-TCPC PLE. Patients were studied over a period of up to 5yrs after surgery. During routine follow-up, blood samples of TCPC patients without (n=21) and with manifest PLE (n=12) and age matched healthy children (control, n=22) were collected. Routine laboratory, immune phenotype and serological parameters were determined as earlier [1]. Following PLE the immune phenotype dramatically changed with signs of acute inflammation (increased neutrophil and monocyte count, CRP, serum IL-8 and complement activation). In contrast, lymphocyte count (NK-cells, abTCR+CD4+, abTCR+CD8+ cells) decreased (60–80%, $p<0.001$). The residual T-cells had elevated CD25 (IL-2R) and CD69 expression. In PLE-patients unique cell populations with CD3+ab/gdTCR- and abTCR+CD4-8- double negative phenotype were present in increased frequencies. Our studies show for the first time dramatically altered leukocyte phenotype, appearance of double-negative T cells and alteration of serum compounds after PLE in TCPC patients. These alterations resemble to changes in autoimmune diseases like systemic lupus erythematosus and celiac disease. We conclude that autoimmune processes may play a role in the etiology and pathophysiology of PLE. (This work was supported by the Maximilian research Award 1997 and Research grants of the Herzkind e.V. (D. Lenz)).

Reference

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CONTINUOUS CA^{2+} DEPENDENT SHEDDING OF CD163 FROM MACROPHAGES DETERMINE SOLUBLE CD163 LEVEL

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The scavenger receptor CD163 is selectively expressed human blood monocytes and tissue macrophages. CD163 is responsible for the clearance of hemoglobin and regulation of cytokine production. In the presence of calcium surface CD163 is cleaved by matrix metalloproteinases resulting in soluble CD163 (sCD163). This shedding can be further induced by proinflammatory cytokines. Here we investigated the influence of different extracellular calcium concentrations on monocyte surface CD163 expression, shedding and reproduction with flow cytometry and ELISA techniques using EDTA- and Li_Heparin anticoagluated peripheral blood, based either on binding extracellular calcium or on blocking heparan-sulfate binding sites with preserving physiologic Ca concentration, respectively. A strong upregulation of membrane associated CD163 and a significant decrease in plasma sCD163 was observed in blood samples without peripheral Ca (EDTA) compared to samples with physiologic Ca (Li-Heparin). Time kinetic experiments revealed a continuous increase in sCD163 for up to 4 hours after taking blood under physiologic Ca concentrations. Membrane CD163 on monocytes in Ca-free material showed an immediate large scale increase in surface CD163 compared to normal Ca controls, which further elevated up to 4 hours after blood withdrawal. A combined use of both anticoagulation methods resulted in a significantly elevated surface CD163 expression, which was restored after rising Ca to physiologic levels manually. Overdosing of Ca and also the addition of inflammatory cytokines (IL-1beta or TNFalpha) resulted in an elevated shedding of CD163. We could show that calcium is necessary for appropriate shedding of CD163 and that CD163 is continuously produced and cleaved on the surface of peripheral blood monocytes in order to maintain physiologic sCD163 levels, even after blood withdrawal. Furthermore we could show that ETDA anticouaglueted blood samples show abnormally high levels of surface CD163 and low sCD163, therefore making it unsuitable for the evaluation of physiologic surface CD163 expression and shedding.

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4-COLOR IMMUNOHISTOCHEMICAL QUANTIFICATION OF FOXP3+ REGULATORY T CELLS IN TRANSPLANTED KIDNEY BIOPSIES

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Despite the availability of HLA matching, routine cross-matching, and potent immunosuppressive drugs, allograft rejection remains one of the most common problems after kidney transplantation. The underlying mechanisms are still incompletely understood. Acute renal allograft rejection is characterized by infiltration of T and B lymphocytes, but also recruitment of monocytes/macrophages resulting in a rapid deterioration of renal function. The importance of regulatory T cells (Treg) in general immune homeostasis is well established. Additionally, increasing evidence points to an important role of Treg for survival of transplanted organs, as Treg are able to infiltrate an allograft. Despite significant advances in understanding the function and development of Treg, the relevance of Treg in the setting of human renal transplantation remains unclear. Although preliminary work showed mRNA expression of FoxP3, a classical marker of Treg, in renal allograft tissue, there is still a lack of clear data characterizing the phenotype of allograft infiltrating Treg. Classically, Treg are defined as staining positively for CD4, CD25 and FoxP3. But recent work has shown immune regulatory function of CD8+ and CD4CD8 double negative Treg. The main obstacle for an immunohistochemical characterization of infiltrating Treg is the limited amount of biopsy material available from transplanted patients. We therefore developed a method to simultaneously stain CD4, CD8 and FoxP3 on paraffin sections and to quantify the amount of infiltrating Treg in biopsies

from patients without rejection and unaltered morphology, with antibody-mediated acute rejection, with acute interstitial rejection and with acute vascular rejection to study the role of infiltrating CD4+ or CD8+ Treg for allograft survival. Data from 50 biopsies studied so far clearly indicate, that in acute allograft rejection a lower number of infiltrating CD4+ FoxP3+ Treg can be found compared to infiltrating CD4+ FoxP3+ Treg in non-rejected allografts. CD8+ FoxP3+ Treg could be found only occasionally in biopsies. Although we can demonstrate that reduced numbers of Treg correlates with allograft rejection, the mechanism by which infiltrating Treg could support allograft tolerance remains unclear.

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RE-EVALUATION OF THE FUNCTION OF CCR6 ON EFFECTOR T CELLS BY MULTI-COLOR FLOW CYTOMETRY

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Chemokines are small chemotactic cytokines that guide leukocytes through the body. The chemokine CCL20 is expressed in mucosal and dermal tissues and its only known receptor is CCR6, which is a trait of effector and memory T cells. CCR6 is upregulated on T cells after allogenic stimulation. Yet, its function on T cells in seems to be elusive. To clarify the function of CCR6 on activated T cells in the immune response, we analysed T cells at different time points after immunisation. In mice, CCR6 positive T cells show co-expression of CD127 and CD44 and are negative for the lymph node homing receptor CD62L. To pursue phenotypical changes during T cell activation, we combined analysis of T cell surface markers with a CFSE proliferation assay. The proliferating fraction of T cells consists mainly of CCR6 positive cells. However, we could not detect an upregulation of the receptor on T cells upon proliferation. Furthermore the phenotype of CCR6 expressing T cells does not alter significantly after immunisation. Regarding the expression profile of the CCR6 positive T cell subset, these cells show characteristics for long-lived effector memory T cells. In conclusion, by using multi colour flow cytometry, we defined a stable subset of effector T cells in mice, which is generated early in the immune response and survives for a long time.

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CYTOMETRIC MONITORING OF TRANSPLANTED PATIENTS

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Background T cells, dendritic cells (DC) and natural Killer Cells (NK) play key roles in the immune reaction against allografts. Little is known about DC or NK fate and about immune reactivity following transplantation. Material and methods Blood samples were taken from patients undergoing transplantion. At days 0 (pre-operative), 1, 3, 7, 10, 14, 21, and 28 venous blood samples were taken between and analysed by flow cytometry. DC were categorised by CD123+/HLA-DR+ as plasmoide (pDC) and by CD11c+/HLA-DR+ as myeloide DC (mDC). Function markers CD62L, CD80, CD83, and CD86 allowed the identification of DC subsets. NK cells were characterized by CD16 expression and regulation of NC-Receptors 1–3. T cell reactivity to recall antigenes was detected by elispot. Results DC and NK populations declined dramatically immediately after transplantation showing only partial recovery after 28 days. The myeloid CD86+ DCs and NKp44+ NK-cells were diminuished at day 1, and did not recover completely until day 28. In the expression of functional molecules on mDCs, CD62L was upregulated, and CD86 was permanently downregulated. Importantly, activation patterns of both NK and DC indicate rejection episodes. T cell reactivity to antigens was independent on antibody levels. Discussion and conclusion DCs are known to be crucial in allograft recognition and rejection. NK cells are beside T cells the major column of graft rejection. The measured dramatic loss of both populations following organ transplantation with standard immunosuppressive therapies suggest, that effect of immunosuppression on these cells contributes to graft survival. Changes in costimulatory molecule patterns indicate active processes regulating immune recognition of the transplant also by these cells. Further strategies for immunosuppression and potential tolerance development must consider these populations. Furthermore, specific T cell reactivity indicates persisting immune protection.

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T CELL SUBSETS CONTROLLED FOR DEMOGRAPHIC AND BIOMEDICAL VARIABLES IN AN INDUSTRIAL SAMPLE OF BLUE- AND WHITE-COLLAR EMPLOYEES

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We report here normative values in a large sample of healthy human adults, evidence supporting the influence of age and health behavioral on the composition of lymphocyte subsets. The study population comprised 686 apparently healthy employees of an airplane manufacturing plant in Southern Germany within 8 working days. The sample spanned the entire age of the work force (18–63 years) and all levels of socioeconomic status (from the general manager to unskilled workers). Predictors and possible confounders (smoking, physical activity, alcohol intake, age, gender, body mass index) were regressed against those lymphocyte subsets. For immunophenotyping several multicolor assays were used, covering regulatory T cells, T

Helper cells and cytotxic T cells. Different factors influencing the composition were identified on several, but not all cell subsets: summarizing the observed associations e.g. for cytotoxic T cells, early cells were related to health behavior and biological factors whereas late cytotoxic T cells were not. Preliminary ongoing analysis of a follow up study on the same cohort (March 2007) are confirming these results of the previous study.

CYTOMETRY IN MICROBIOLOGY, BIOTECHNOLOGY AND PLANTS

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PHYSIOLOGY OF LIVING INDIVIDUAL SACCHAROMYCES CEREVISIAE CELLS - INVESTIGATION ON CELLULAR AND MOLECULAR LEVEL

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The yeast Saccharomyces cerevisiae is a widely used microorganism in biotechnological processes. Hence, understanding the metabolism of S. cerevisae under changing micro-environmental conditions is of high importance for scientific and economic reasons. We established a three colour technique allowing the analysis of the affinity of individual S. cerevisiae cells to glucose using 2-NBDglucose, the analysis of cell proliferation activities using Hoechst 33342 and the dead cell amount using PI simultaneously. During continuous cultivation, S. cerevisiae showed different affinities to glucose, as was observed by variable fluorescence intensities of 2-NBDglucose. The affinity of the cells to glucose was high at low dilution rates and low at high dilution rates. Additionally, the affinity to the substrate was also different in subpopulations growing at the same growth rate. To understand these phenomena on the molecular level, microarray analysis was involved. Therefore, subpopulations with different affinities to the substrate were sorted. The analysis of differential gene expression requires the isolation of intact RNA. We want to present an efficient method which was used to obtain high yields of intact RNA from relative low numbers of vital, stained and sorted yeast cells ($5x10^7$).

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POPULATIONS PROFILES OF THE BTEX DEGRADING ENRICHMENT CULTURE L-D1 UNDER BALANCED AND NON BALANCED GROWTH CONDITIONS

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Man-made groundwater pollution by hazardous compounds is a global problem. It is our intention to investigate the dynamics of ingenious bacterial communities in order to understand the ecological principles of pollutant biodegradation in contaminated aquifers. This would be facilitated by the establishment of rapid methods to identify and observe pollutant-metabolizing single bacterial cells in enrichment cultures or even in natural systems. Multiparametric flow cytometry is a single-cell based method that was applied to determine emerging proliferating subcommunities and, when combined with 16S rRNA gene sequencing, to identify the phylotypes dominating microbial communities. We show the potential of this high speed detection tool for an oxic system, which is highly BTEX contaminated.

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FLOW-SORTED NUCLEI ARE VALUABLE SUBJECTS TO INVESTIGATE THE STRUCTURAL AND FUNCTIONAL NUCLEAR ARCHITECTURE IN ARABIDOPSIS

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Somatic tissues of Arabidopsis thaliana (0.32pg/2C) reveal extensive endoreduplication, resulting in tissues that comprise mixtures of polyploid cells. Flow-sorting of nuclear suspensions derived from these tissues allow the separation of nuclei according to their different ploidy levels. Sorted onto slides, the isolated nuclei are excellent subjects to study their structural and functional architecture. We were using this approach to investigate (i) the positioning and association of chromosome territories, (ii) the somatic pairing of distinct loci, (iii) the influence of repetitive transgenes on the local chromosome arrangement, (iv) the sister chromatid alignment, (v) the positioning and loading of the centromeric histone variant CENH3 and (vi) the global distribution of histone methylationmarks. From the obtained results we could draw the following conclusions: (i) The side-by-side arrangement of distinct homologous and heterologous chromosome territories is random. Only the homologous NOR-chromosome arms associate more often than expected at random. (ii) Positional homologous pairing along the chromosome arms is likewise mostly random. (iii) Homologous tandem repetitive transgenes associate more often with each other and with endogenous heterochromatin than normal euchromatic regions. (iv) Sister chromatids are not continuously aligned along chromosome arms. This alignment is positionally variable and decreases with endopolyploidy level. (v) The loading of the centromeric histone variant CENH3 to the centromeres occurs mainly during late G2. (vi) The heterochromatic chromocenters are enriched in H3K9me1,2; H3K27me1,2 and H4K20me1.

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EVIDENCE OF FLOCCULATION IN BREWING YEAST STRAINS BY FLOW CYTOMETRY, PROTEOME AND mRNA PROFILING

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Background Flocculation ability of brewing yeast is an important feature for making a qualtitive good beer. In the course of serveral reuses of the yeast during the brewing processes the flocculation features may change. Flocculation involves two main cell wall structures, which are flocculation proteins (flocculins) and the mannans to which the flocculins bind. Up to now no reliable techniques are available to predict changed flocculation characteristics of the yeast cells.

Methods Yeast strains were cultivated in batches or harvested from tanks of volumes of 300 m^3. Flow cytometry was involved to analyse lectin coupled glucose and mannose structures of the cell surface. Microarray techniques and tyramid staining was used to validate flocculine expression. 2D-gel electrophoresis and ESI-MS was involved to isolate and determine yeast cell flocculins.

Results Mannan labelling and differentiation with fluorescent lectins enabled for powdery and flocculent yeast cell differentiation only under laboratory conditions. Using microarray techniques and proteomics the 4 flocculins LgFlo1, Flo1, Flo5, Flo9 were identified. Their expression was several times higher in flocculent yeasts than in powdery ones.

Conclusion Flow cytometry offered a precise, fast and simple method to quantify the amount of powdery and flocculent yeast in a defined suspension. Differentiation of powdery from flocculent yeast cells under industrial conditions needs however the involvement of mRNA and protein expression profiles.

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A HIGH-THROUGHPUT SYSTEM FOR RAPID PLOIDY ANALYSIS AND SEED SCREENING FOR REPRODUCTIVE PATHWAYS IN PLANTS

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Flow cytometry is a powerful tool for the investigation of many parameters at the single cell level, and has the advantage of rapidly processing a high number of individual cells. In apomixis studies, the identification of apomictic individuals has relied on the evaluation of DNA ploidy and/or the screening for embryo to endosperm C-value ratios in a large number of seeds. Such screening methods using current nuclei preparation protocols can be labour and time-intensive, and represent a bottleneck which is not readily amenable to automation. We are optimising and automating this procedure through the use of a genogrinder in conjunction with a two-step buffer supplemented with a reducing agent for the rapid isolation of sufficient nuclei for FCM analysis. The protocols have produced reliable results for both dicots (Hypericum perforatum and Boechera spp) and monocots (Poa parentesis) for DNA content (ploidy) determination, and have enabled us to measure quantitative variation in apomictic and sexual seed productions between individuals. These methods have the potential for large scale application in the seed industry, apomixis research and crop improvement programmes.

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GC CONTENT AND AVERAGE CHROMOSOME SIZE EVOLUTION IN GRASSES

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Grasses represents one of the most species-rich flowering plant families dominating in many biomes over the whole globe. Because of their extreme economically importance grasses became a very detailed studied group and a well known genomic models, which enable to better understand the processes related to global quantitative (genome size) and qualitative (GC content) genome evolution. Using flow cytometry, and available phylogenetical and karyological data, we compared the GC content and average chromosome size evolution in over 120 Eurasian grasses of subfamily Pooideae, and further in over 100 taxa of Eurasian species of Festuca and their close relatives. Within both groups studied, GC content and average chromosome sizes were considerably phylogenetically conditioned and separates among groups of taxa of different taxonomic levels. Within Pooideae the main variability was associated with the average chromosome size and the ancient massive genome amplification by non-coding DNA seems to have predated the divergence of all modern representatives of this subfamily, including important cereal and forage crops, e.g., Triticum, Hordeum, Secale, Avena, Poa, Festuca. Within Festuca GC content and average chromosome size were positively correlated. Comparison with the present phylogeny of the genus revealed a long-term cyclic character of the changes in both parameters. Similarly as in the present studies in Oryza, we assume that this cycle may be in a consequence with the long-tern dynamics of GC rich transposable elements amplification and removal. The comparison of the close diploid polyploidy pairs in Festuca and allies revealed two possible trends in polyploid genome formation which seems to be influenced by parental genome size or by differences in allo- versus

auto-polyploidisation processes. Both our studies indicate high potential of general quantitative characters of the genome for understanding the long-term processes of genome evolution, testing evolutionary hypotheses, and their usefulness for large-scale genomic projects. Our research was supported by the Czech Ministry of Education (projects MSM0021622416 and LC06073).

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FLOW CYTOMETRY, A SUITABLE METHOD FOR DETECTION OF PLOIDY LEVEL AND REPRODUCTIVE VARIABILITY WITHIN THE HAWKWEEDS POPULATIONS, HIERACIUM SUBGEN. PILOSELLA

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Hieracium subgen. Pilosella group has a high diversity of species, subspecies and hybridogenous types which is based on the reproductive diversity (clonal growth and sexual and/or apomictic seed reproduction). For understanding of selective processes during the evolution of new types, the detailed studies of populations are necessary. Flow cytometry screen of plants and seeds was used to find variability in ploidy level and reproductive modes within the model mixed populations of coexisting facultatively apomictic and sexual biotypes. (1) Flow cytometry of leaf tissue was used for detection of ploidy level variability in plants and seedlings. (2) Flow cytometry seed screen (FCSS) was used to detect ploidy level and reproductive origin of couples fresh seeds. The method is based on different embryo/endosperm ploidy ration in seeds of sexual or apomictic origin (Matzk et al., Plant J 21:97–108, 2000). (3) A modified flow cytometry seed screen (Krahulcovã & Suda, Biol Plant 50:457–460, 2006) of ten seeds (one year kept dry to reduce of endosperm) was used for fast detection of seeds ploidy level variability. The study found that (1) Apomicts produced more variability than that of sexuals. Whereas progeny of sexual mothers was formed almost exclusively by hybridization via fusion of reduced gametes (n+n hybrids), the progeny of facultatively apomictic mothers has originated via four different pathways, i.e. apomixis (somatic parthenogenesis, 2n+0), hybridization via fusion either of reduced (n+n hybrids) or unreduced (2n+n hybrids) gametes and haploid parthenogenesis (n+0). (2) The high-polyploid hybrid biotypes (heptaploids, octoploids) generated a much variable progeny. (3) Higher ploidy level variability within the seeds than that within the seedlings confirmed the selection of specific progeny classes (namely polyhaploids, n+0) during germination and early development of seedlings. This study was supported by the Ministry of Education, Youth and Sports (projects MSM 0021622416 and LC 06073) and by the Grant Agency of the Academy of Sciences of the Czech Republic (project 206/07/0059).

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DIPLOID AND POLYPLOID CYTOTYPES DISTRIBUTION IN THE WHITE-RAYED COMPLEX OF MELAMPODIUM (HELIANTHEAE, ASTERACEAE)

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The genus Melampodium comprises 39 recognized species distributed throughout Mexico, Central America and the southwestern US. All species have yellow rays except for three shrubby, xerophytic taxa, M. leucanthum, M. cinereum and M. argophyllum, which form the so-called white-rayed complex and mark the northern limits of the genus range. These species are clearly separated by their distributions and ecology and are well supported in molecular analyses (AFLPs and haplotype analyses). Previous studies revealed the presence of three different ploidy levels within the white-rayed complex: diploids and tetraploids (plus occasional triploids) in M. cinereum and M. leucanthum, and exclusively hexaploids in M. argophyllum. In both M. cinereum and M. leucanthum diploid cytotypes prevail in the western part of their distribution area, while tetraploids are dominating in the east. M.argophyllum was hypothesised to be of allopolyploid origin, involving M. leucanthum and M. cinereum as parents. The current study presents the data on the ploidy levels estimations for 91 populations (1110 individuals) of M. leucanthum, for 29 populations (447 individuals) of M. cinereum and for 2 populations (36 individuals) of M. argophyllum. For ploidy determination, flow cytometry of DAPI stained silica gel-dried material has been applied, using Glycine max 'Merlin' as the internal standard. 62 populations of M. leucanthum are diploid (1 and 6 contain sporadically 4x and 3x individuals, respectively) and 29 populations are tetraploid (1 contains a 3x individual, 1 contains a 6x individual). 16 populations of M. cinereum are diploid (one contains a 3x individual) and 13 populations are tetraploid (three contain sporadically 5x individuals, and one contains a 6x individual). Both populations of M. argophyllum are hexaploid. The data obtained in this study agree well with the reports on ploidy level distribution (on the smaller scale) in the white rayed complex performed 40 years ago, suggesting that the polyploid establishment and maintenance is connected to ecological and perhaps historical biogeographical factors. The correlation of ploidy level distribution in the three taxa with the molecular, cytogenetic and ecological data will allow us to address the questions of the origin (single vs. recurrent), maintenance and the evolution of tetraploid cytotypes in M. leucanthum and M. cinereum, as well as on the origin of hexaploid M. argophyllum.

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CYTOMETRY IN SYSTEMS BIOLOGY

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STATE OF THE ART SLIDE BASED CYTOMETRY (SBC) SYSTEMS FOR CYTOMICS

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Cytomics, high-content analysis of cell systems [1], requires quantitative and stoichiometric analysis on the single cell level. SBC has become and important analytical technology in drug discovery, diagnosis and research and is an emerging technology for systems analysis [2]. It enables to perform high-content high-throughput measurement of cell suspensions, cell cultures and tissues. In the last years various commercial SBC instruments were launched that are in principle enabled to perform similar tasks. However, technical realisations are very divers (different light sources, detectors, colour detection, optical paths, confocal or non-confocal detection etc.), the residual image formats, the image analysis used to recognise single cells (cell detection modes) and resulting data formats. Presently most of the instruments are unique. Standardisation as well as comparability of different instruments is a major challenge. Typical examples will be shown and the key aspects for standardisation (sample handling, data analysis and output) of the different instruments enabling cross-system validation will be discussed.

References

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BOVINE OVARIAN GRANULOSA CELLS RESPOND TO THE PLATELET-ACTIVATING FACTOR (PAF) WITH INTRACELLULAR CALZIUM MOBILIZATION VIA THE PAF RECEPTOR

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The rupture of the ovarian follicle involves inflammatory-like process. PAF is a potent pro-inflammatory phospholipid which acts via the PAF receptor (PAFR). Recently, we detected PAF in the follicle fluid and PAFR in preovulatory follicular tissue. In other tissues, Ligand activation of PAFR triggers intracellular increase in cytosolic calcium, a criterion of functionality. This response is unknown in follicle cells. To elucidate functionality of PAFR, mural granulosa cells were incubated with the membrane-permeable calcium indicator fura-2. Change in fluorescence ratio was imaged by fluorescence microscopy. Exposure to PAF induced a pulsating change, indicating intracellular calcium release. Response was inhibited by WEB2086, a PAFR blocker. WEB2086 did not prevent response to thapsigargin that releases Ca2+ by inhibiting endoplasmic reticular Ca^{2+}-ATPase. These results indicate that granulosa cells from the follicle wall respond to PAF via PAFR with a calcium mobilization. In the follicle wall, preliminary data suggest that this PAFR-mediated signal transduction may modulate steroidogenesis by cross-talk with luteinising hormone signalling.

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GENOME SIZE VARIATION IN SPECIES WITH HOLOKINETIC CHROMOSOMES (CYPERACEAE)

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Holokinetic chromosomes occur in different organisms including insects (Lepidoptera, Heteroptera), nematoda (Caenorhabditis) and some plants (Cyperaceae, Juncaceae). Sedge family (Cyperaceae) contains about 4000 species in 70 genera world-wide distributed. Genus Carex is the most species-rich genus of Cyperaceae. 119 species of Carex (2/3 of all European Carex-species) and 43 taxa of genera Bolboschoenus, Cladium, Cyperus, Eleocharis, Eriophorum, Holoschoenus, Isolepis, Kobresia, Scirpus, Schoenoplectus, Schoenus, and Trichophorum were measured using flow cytometry during last 2 years. Genome size and average chromosome size were estimated in all taxa and chromosome number in 50% of all samples (for counting of average chromosome size the literary data were used in the rest of species studied). Genome size variation in the type subgenus of Carex ranges from c=0.27 pg in Carex hordeistichos and C. secalina to c=1.85 pg in C. serrulata (6.85-fold). In Carex subgen. Vignea, the genome size variation is lower ranged in the most of species from c=0.32 pg in Carex bohemica to c=0.48 pg in C. disticha (1.5-fold); just in dioecious species, i. e., in C. dioica and C. davalliana genome size is somewhat larger: c=0.57 pg, and c=0.6 pg, respectively. No genome size difference was found between males and females in dioecious taxa. The average chromosome size is also more variable in the subgenus Carex (7.96-fold) than in subgenus Vignea (2.09-fold or 3.24-fold if dioecious species are included). In the rest of Cyperaceae the smallest average chromosome size 4.7 Mbp was detected in Bolboschoenus yagara; the smallest genome size within the rest of Cyperaceae was detected in Cyperus fuscus (c=0.25 pg). In the genus Eleocharis, there is the largest contrast in chromosome size between E. quinqueflora (8.1 Mbp per chromosome) and E.austriaca (296 Mbp per chromosome). The genome size and average chromosome size are useable species specific characters, which are well correlated with the evolutionary distance between studied taxa. Our research was supported by the Czech Ministry of Education, projects LC06073 and MSM0021622416.

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CORRELATION BETWEEN GC CONTENT AND GENOME SIZE IN PLANTS

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Guanine and cytosine (GC) content is an important species specific character of prokaryotes, but its role in plant systematics and evolution is still poorly understood. Although GC content is positively correlated with genome size in bacteria and vertebrates (Vinogradov, Cytometry 16: 34–40, 1994) there is debate about how those two factors are related in plants. Barow and Meister (Cytometry 47: 1–7, 2002) estimated base composition and genome size in 54 taxa of angiosperms and gymnosperms, and their findings did not support Vinogradov's (l.c.) proposition stating that there is a positive correlation between GC content and genome size. More recently, Barow and Meister (in Doležel et al., Flow Cytometry with Plant Cells, pp. 177–215, 2007) confirmed that base composition and genome size were not correlated using an expanded sampling of 215 plant species measured by numerous previous authors. Using flow cytometry methods (FCM) with PI and DAPI, we estimated base composition in closely related species of Apiaceae (16 spp.), Brassicaceae (30 spp.), Cyperaceae (135 spp.), Onagraceae (15 spp.), Poaceae (132 spp.) and Salicaceae (20 spp.), and found a significant positive correlation between GC content and either genome size or monoploid genome size. Sequence data in BACs of 11 Oryza species (Ammiraju et al., Genome Research 16: 140–147, 2006) independently supported our FCM results and suggested that GC and genome size correlation is explained by genomic repeats and interspecific genome size variation found within closely related taxa. Conclusions:

1. No apparent correlations exist between GC content and genome size across major lineages of angiosperms.

2. A significant positive correlation was found between GC content and genome size in closelx related taxa.

3. The relationship between GC content and genome size are probably hidden in events of polyploidization.

4. Changes in genome size and GC content in closely related taxa may be constrained by similar selective pressures. Although plant genome size and GC content might respond predictably to similar parameters over short phylogenetic predictability might be influenced by genome dynamics, phylogenetic constraints, or by a number of speciation events the plants studied. Our research was supported by the Czech Ministry of Education, projects LC06073 and MSM0021622416.

NOVEL INSTRUMENTATION AND APPLICATIONS

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VALIDATING MELC-TECHNOLOGY FOR CLINICAL DIAGNOSTICS BY COMPARING MEASUREMENTS OF A DISEASE-SPECIFIC COMBINATORIAL MOLECULAR PHENOTYPE ON PERIPHERAL BLOOD MONONUCLEAR CELLS WITH FLOW CYTOMETRY MEASUREMENTS

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MELC (Multi-Epitope-Ligand-Cartography) technology comprises consecutive cycles of in situ fluorescence detection measured by Toponome Imaging Cycler®. A sample is incubated with a fluorochrome-labelled ligand, and after taking a fluorescence image followed by photobleaching, the cycle is repeated with a new labelled ligand. MELC technology enables to detect up to 100 epitopes and creates a stack of periplanar images. Colocalizations of multiple epitopes are analyzed after binarization in a pixel- or cell-related manner named combinatorial molecular phenotypes (CMPs). The current study established an innovative four-step strategy for biomarker identification in peripheral blood mononuclear cells (PBMC). As a clinical example, blood samples of patients with atopic dermatitis (AD) were compared with samples from healthy controls (HC). In the first data-mining step we applied a MELC library of 48 antibodies to compare AD patients with HC for the frequency of cell-related CMPs by TopoMiner search statistics. In a second confirmatory step, the most prominent CMP motifs which highly significantly discriminate both skin conditions were re-evaluated by another series of MELC measurements of a new set of specimens from the same individuals. In a third validating step the CMP motifs with the highest discriminative power were re-tested within an investigator-blinded study in additional cohorts of independent AD patients and HC. These steps filtered out a CMP motif indicative for a distinct subpopulation of CD2+/Ki67+ T lymphocytes which are also CD30+. The up-regulation of this CMP motif was found to be discriminating AD from HC. Finally, the samples from the same patients were re-measured using flow cytometry. The results showed a concordance of R2=0.84 compared to the MELC measurements. Thus, MELC technology is on its way to clinical diagnostics and offers the perspective for the identification of a new class of highly informative combinatorial biomarkers.

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MALDI-IMAGING – A COMBINATION OF HISTOLOGY WITH MASS SPECTROMETRY FOR DISCOVERY OF PROTEIN PATTERNS WITH POTENTIAL CLINICAL IMPACT

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The proteome is the collectivity of proteins expressed from a single cell simultaneously. The vision of clinical proteomics consists of a clinical specimen (tissue, serum, urine, body fluid), which is analyzed using proteomic techniques. The data obtained are of clinical significance – for diagnosis, documentation and therapy of disease states. MALDI-TOF mass spectrometry (MS) is an important method in contemporary proteomic approaches: Following microdissection and tissue lysis a MS spectrum can be acquired. MALDI-Imaging however is a technology to circumvent limitations of sample availability resulting in proteomic information with spatial resolution, which is correlated with histologic specimen details. Cryostat sections of tissue are mounted onto conductive glass slides (Bruker Daltonics, Bremen, Germany), rapidly fixed in ethanol and evenly spray coated with MALDI-matrix solution (sinapinic acid/acetonitrile/TFA). A virtual grid of measuring points (spot raster 50 – 200 μm) is created and overlaid with a microscopic image of the specimen prepared. Mass spectra are acquired from every raster position using a Reflex IV MALDI TOF (Bruker) and FlexImaging software (Bruker). Following MS the matrix is removed by organic solvents and the section H&E stained to be examined by a board certified pathologist for histological classification. The H&E image can be overlaid with a color encoded depiction of the MS-data obtained. To that localization of proteins expressed can be pinpointed with high resolution. For further analysis regions of interest are defined e.g. normal and cancerous tissue. Spectra obtained within these regions are exported and analyzed using ClinProTools 2.1 software (Bruker) for differential protein expression using e.g. a 5-dimensional genetic algorithm. The mathematic model obtained is able to distinguish between normal and cancerous tissue (pattern profiling). Establishing these patterns by using a well defined collective (= training set) of samples (healthy vs. diseased) enables future diagnosis of unknown samples (= test set) by just using the algorithm for new spectra obtained. Using this technique described we were able to detect cancerous glands in prostate tissue (n=11/11, specificity 90.74%, sensitivity 85.21 %) as well as lymphoma cell clusters within lymph nodes (n=22/32, specificity 89.37%, sensitivity 83.92%). MALDI Imaging correlates protein expression values with immediate histologic data. In contrast to immuno histochemistry unlimited proteins are scanned in parallel on a molecular scale.

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IS QUANTITATIVE CYTOMETRIC 3D ANALYSIS OF TISSUE POSSIBLE?

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Quantitative tissue analysis is essential for automated histopathology and experimental biology. To this end, appropriate standardized in situ tissue cytometry is required [1]. Analysis of tissue means always interference of autofluorescence and sectioning artifacts of not intact cells as well as fluorescence overlap of cells above or below the focus. In addition, for a quantitative cytometric and stoichiometric analysis the fluorescence information of whole single cells is required. Confocal microscopy only yields the information of a small part of the cell and multiple scans and modeling is necessary to reconstruct the total cell volume. Slide based cytometry provides the opportunity to measure the fluorescence of the whole cell due to high focal depth of the objectives but is it possible to detect the cells in focus and ignore all others? Here, a 3D model of fluorescent beads is presented which outlines the difficulties and possible methods of resolution for quantitative 3D analysis. Indeed, it is possible to detect different layers of beads but emission fluorescences of different wavelength seem to have variable focus. This means that for exact quantitative measurements multiple scans would be necessary to obtain quantitative fluorescence information of every used fluorochrome. Nevertheless, this won't solve the problem of fluorescence interference from cells out of focus. For this purpose a stable fluorescence signal equal to all cells within the tissue seem essential. For analysis of not dividing cells in G1 phase a DNA staining could be an appropriate way. Slide based cytometric analysis combined with confocal scanning could than be the verification of the cell location.

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ON-CHIP AND LABEL-FREE CELL CHARACTERIZATION WITH AN IMPEDANCE SPECTROSCOPY FLOW CYTOMETER

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We present here a refined on-chip-flow-cytometer for cell characterization and discrimination, whose technology is based on impedance spectroscopy. The impedance signal of cells is dependent on the applied frequency and provides information about cell volume, membrane capacitance and cytoplasm conductivity. The measurement occurs on a non-invasive and label-free way in a micro technologically fabricated chip. No extensive preparation or cell labelling with costly markers are necessary. Several applications in the field of cell physiology and dif-

ferentiation, haematology and microbiology were already presented. Here we will discuss some more experiments of cell discrimination in detail. 1. blood cells as lymphocytes, monocytes and derived cell lines as well as activation with LPS/PHA, 2. treated erythrocytes (DMSO, Gramicidin), 3. yeast cell growth and 4. detection of cells infected with parasites. Conclusions: Our Impedance Flow Cytometer determines the electrical impedance of single cells at multiple frequencies simultaneously. The cells do not need to be labelled for the analysis simplifying therefore sample preparation and providing near-online results. Studies on different cell models show promising results in the discrimination of the various cell types. The measurements provide a basis for addressing further potential applications in the fields of oncology, pre-diagnostics and diagnostics, stem cell research, parasitology and apoptosis and show that the impedance flow-cytometer can be used as a valuable complement to the known cell detection instruments.

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ANALYSIS OF CELL CYCLE BLOCKERS USING CLICK CHEMISTRY CATALYZED EDU DETECTION

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Changes in cell proliferation, as measured by the incorporation of nucleoside analog into actively synthesizing DNA have been the basis for assessing treatments which alter or block phases of the cell cycle. S-phase incorporation has classically been assayed with either radioactive thymidine, or 5-bromo-2′-deoxyuridine (BrdU) incorporation which requires a cumbersome antibody staining protocol. We introduce the novel use of click chemistry for the labeling and detection of DNA synthesis. This labeling strategy uses the incorporation of the thymidine analogue EdU (5-ethynyl-2′-deoxyuridine) followed by chemical coupling of the analogue with a dye-azide conjugate for detection. The protocol uses a simple aldehyde fixation and surfactant permeabilization to access the DNA, avoiding the need for the denaturation steps used with BrdU labeling. In this study, the efficacy of EdU labeling is demonstrated on three cell lines: A549 human lung carcinoma, HeLa human cervical carcinoma, and Jurkat human T-cell leukemia cells. DNA incorporation is shown in a dual parameter plot of click labeled DNA and cell cycle. In addition, cell cycle arrest is demonstrated with three drugs known to block G2M: etoposide, colchicine, and nocodazole. Expected changes in cell cycle and S-phase fraction (SPF) are seen. Click chemistry detection of incorporated EdU provides accurate and consistent performance using standard fixation and permeabilization protocols, without the need for denaturation of DNA. The click chemistry based EdU cell proliferation assay can be easily adapted to screen cells for proliferation anomalies.

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QUANTUM DOTS AS REPLACEMENTS FOR TANDEM DYES IN FLOW CYTOMETRY

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Qdot® nanocrystals provide fluorescent labels that can be excited with UV or violet light sources, but can also be used with longer wavelength excitation. In flow cytometry, investigators achieve greater multiplexing of cellular markers by using tandem fluors, long-wavelength organic dyes coupled to fluorescent donor proteins, R-phycoerythrin (R-PE) or allophycocyanin (APC), to enable far red emission using 488 and 633 nm laser excitation. Tandem fluors suffer from poor stability, batch variability, and donor dye spectral bleed-through. Qdot nanocrystals have long effective Stokes shifts and relatively narrow emission peaks. Even with sub-optimal excitation at 488 and 633 nm, they provide better population resolution than is achieved with most tandem fluors. For example, Qdot 705 and Qdot 800 secondary antibody reagents allow more effective resolution of CD4-positive populations in human blood than RPE and APC tandem fluors in far red emission regions. No emission is observed in the tandem fluor donor dye region, but fluorescence in the target emission region is observed with excitation off every laser. Nanocrystal reagents resolve tandem fluor issues with stability and donor bleed-through, but require cross-laser correction. Qdot nanocrystals can be used in place of tandem fluors in antibody staining, allowing detection of more targets over the same spectral range.

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COMPARING THE EFFECT OF THE THYMIDINE ANALOGUES EdU AND BrdU ON CELL CYCLE PROGRESSION

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Nucleoside analogue incorporation - mainly with BrdU (5-bromo-2′-deoxyuridine) - is frequently used for cell cycle analysis. Recently, a novel thymidine analogue EdU (5-ethynyl-2′-deoxyuridine) has been demonstrated in a simplified method of labeling S-phase cells using azido-labeled fluorochromes (click chemistry). Short pulses (0.5–3.0 hours) of either analogue have had minimal effect on cell cycle progression and have demonstrated no obvious impact on cell viability across a number of cell lines.

In this study, we compared two nucleosides, BrdU and EdU, for their effect on cell cycle and toxicity in two breast cancer cell lines, BT474 and SK-BR-3. Both cell lines were continuously labeled with analogues over a range of concentrations. Cells were then labeled for viability with a fixable dead cell reagent, followed by permeabilization and labeling with propidium iodide/RNase to detect the cell cycle profile.

The two cell lines demonstrated different responses to long-term incorporation (48 to 96 hours) of either analogue. BT474 cells showed moderate response differences between continuous exposure to BrdU and EdU. BrdU reduced the percentage of cells in the S-phase fraction (SPF) relative to the untreated controls at low BrdU concentrations, but increased SPF at 50 - 100 μM BrdU; EdU had no significant effect on SPF. Neither analogue affected the percentage of dead cells in BT474. In contrast, EdU showed

a dose dependent effect on SK-BR-3 cells after 96 hours of labeling, including reduced SPF and increased dead cells. SPF in SK-BR-3 cells also decreased with BrdU treatment, while cell viability was largely unaffected.

These results show that reagents with similar activities can demonstrate dramatic differences between cell lines, and highlight the need for validation of assays on individual cell lines.

STEM CELL BIOLOGY

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GREEN FLUORESCENT PROTEIN (GFP) MARKING FOR THE STUDY OF HOST AND GRAFT PARTICIPATION IN THE FOETAL INTESTINE ECTOPIC GROWTH

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After syngenic implantation into an adult animal ear, foetal organs first undergoes a destruction process but soon begin to grow and resume their ontogenetic development up to adult morphology and function. Is the origin of this growth to be found in the remaining viable precursor cells of the graft or in host stem cells or both? Aim of the work: to identify host and graft cells in the ectopically growing foetal intestine and to evaluate the reliability of the use of "green" transgenic mice for this purpose. Material and methods. 50 C57Bl "savage" and 50 green fluorescent protein (GPF) transgenic mice were used. Intestine was withdrawn from mice foetus aged from 15 -19 days and introduced into a subcutaneous pouch in the ear pavilion of adult mice. Biopsies of the grafts and host organs were performed up to day 30 after implantation. Tissues were fixed in 10% formol, embedded in paraffin and HE stained or GFP immunostained.. Results. Preliminary studies show a significant mosaic staining pattern in the "green" mice host. Among combinations of donors and recipients, the most informative one, is implanting "savage" graft into "green" host, so that presence of GFP positive cells in the graft sign their host provenance. In the first period after the operation, a great amount of marked cells was noted in the implant site. Later, the participation of host cells diminished: the growing intestine was made mainly of no marked cells but many capillaries and other blood vessels clearly came from the host. Conclusion. The chosen method of marking seems to be not absolutely reliable, that may be due to the fact that the "green" mice are hybrids (heterozygocy) but also because the GFP gene is transcribed to very low level in intestinal epithelium (epigenetic factor). Nevertheless, we can conclude to the participation of both host and graft, in the re-formation of the graft, with different respective percentage depending on the observation delays. Probably graft committed cells are involved rather than graft stem cells. It is also possible that the presence of the graft tissue may mobilize host stem cells and specify their evolution.. This could explain why the graft developed only as an intestine without any deviation.

DGfZ Conference 10th thru 13th Oct. 2007

Poster Presentations

Cytometry in Systems Biology

(P 1) Bovine ovarian granulosa cells respond to the platelet-activating factor (PAF) with intracellular calcium mobilization via the PAF receptor
Viergutz T, Krüger B, Löhrke B

(P 2) Flow-cytometric measurement of respiratory burst in rat polymorphonuclear granulocytes: Comparison of four cell preparation procedures, and concentration-response evaluation of chemical stimulants
Bitzinger D, Schlachetzki F, Lindner R, Trabold B, Dittmar MS

Cancer Biology and Therapy

(P 3) The effect of cisplatin on the structure of chromatin in tumor cells
Gloushen G, Anikanov G, Khliabko P

(P 4) HER3 and HER4 gene amplification have prognostic impact in breast cancer
Sassen A, Rochon J, Wild P, Hartmann A, Hofstädter F, Schwarz S, Brockhoff G

(P 5) Generation of reactive oxygen species and induction of a cell cycle arrest by epoxy and acrylate monomers
Schweikl H, Hiller KA, Stich A, Bolay C, Brockhoff G, Eckhardt A, Schmalz G

(P 6) Flow cytometric DNA ploidy in brush biopsies of oral lesions
Dowejko A, Driemel A, Schwarz S, Reichert TE, Brockhoff G

(P 7) Application of fluorescence bar coding to multicolor flow cytometric quantification of ErbB receptor-driven intracellular signaling
Friedländer E, Diermeier-Daucher S, Vereb G, Brockhoff G

(P 8) Cooperation between two TNF receptors in the U937 in necessary for efficient cytocidal response to transmembrane TNF whereas protective response is not
Pierzchalski A, Banach K, Perycz M, Bigda J

Clinical Cytometry and Advances in Diagnostic Immunophenotyping

(P 9) 4-color immunohistochemical quantification of FoxP3+ regulatory T cells in transplanted kidney biopsies
Stoelcker S, Kryvoshey D, Ellmann S, Banas B, Kraemer BK

(P 10) Innovative concepts for Absolute Immunophenotyping by Slide-Based Cytometry
Laffers W, Mittag A, Tárnok A, Bootz F, Gerstner AOH

(P 11)The Core Unit Fluorescence-Technologies in the IZKF Leipzig
Lösche A, Grosche J

(P 12) Alteration of immune phenotype following protein losing enteropathy after total cavopulmonary connection by cytomics
Bocsi J, Lenz D, Sauer U, Wild L, Hess J, Schranz D, Hambsch J, Schneider P,Tarnok A

(P 13) Re-evaluation of the function of CCR6 on effector T cells by multi-colour flow cytometry
Pötzl J, Botteron C, Männel DM, Lechner A

(P 14) Continuous Ca2+ dependent shedding of CD163 from macrophages determine soluble CD163 level
Ugocsai P, Wolf Z, Paragh G, Schmitz G

Cytometry in Microbiology, Biotechnology, and Plants

(P 15) Populations profiles of the BTEX degrading enrichment culture L-D1 under balanced and non balanced growth conditions
Ramig S, Vogt C, Kleinsteuber S, Hübschmann T, Harms H, Müller S

(P 16) Genome size variation in species with holokinetic chromosomes (Cyperaceae)
Hralova I, Bures P, Rotreklova O, Smarda P, Grulich V, Zedek F, Smerda J, Horova L, Hroudova Z, Repka R

(P 17) Flow cytometry, a suitable method for detection of ploidy level and reproductive variability within the hawkweeds populations, Hieracium subgen. Pilosella
Rotreklova O, Krahulcova A, Krahulec F

(P 18) Flow-sorted nuclei are valuable subjects to investigate the structural and functional nuclear architecture in Arabidopsis
Fuchs J, Pecinka A, Lysak M, Schubert V, Lermontova I, Watanabe K, Jovtchev G, Schubert I

(P 19) Flow cytometric and phytochemical investigations with plant cell suspension cultures of sunflower (Helianthus annuus)
Haas C, Georgiev M, Weber J, Ludwig-Müller, Bley T

(P 20) Diploid and polyploid cytotypes distribution in the white-rayed complex of Melampodium (Heliantheae, Asteraceae)
Obermayer R, Reich D, Rebernig CA, Weiss-Schneeweiss H, Stuessy TF

Novel Instrumentation and Applications

(P 21) Comparing the effect of the thymidine analogues EdU and BrdU on cell cycle progression
Diermeier-Daucher S, Clarke S, Bradford J, Hill D, Brockhoff G

(P 22) Quantum Dots as Replacements for Tandem Dyes in Flow Cytometry
Buller GB, Zhang YZ, Godfrey WL

(P 23) Is quantitative cytometric 3D analysis of tissue possible?
Mittag, A

(P 24) On-chip and label-free cell characterization with an impedance spectroscopy flow cytometer
Schade-Kampmann G, Hebeisen M, Huwiler A, Hessler T, Di Berardino M

(P 25) Analysis of Cell Cycle Blockers using Click Chemistry Catalyzed EdU Detection
Bradford JA, Clarke ST, Buck SB, Gee KR, Agnew B, Salic A

(P 26) Photoinduced electron transfer (PET)-probes for the detection of cancer-related nucleases
Henkenjohann S, van de Linde S, Doose S, Wittig R, Schubert P, Coy JF, Sauer M

(P 27) Optimization of the hypoosmotic loading method of aequorin, an intracellular Ca2+ concentration indicator, into cells of follicular lymphoma
Skopalik J, Klabusa M,Borsky M

Cancer Biology and Therapy

Subpopulations of human CD4+CD25+ regulatory T cells identified by multicolor flow cytometric analysis: purity and suitability for in vitro expansion

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CD4+CD25+ regulatory T (Treg) cells are pivotal for the maintenance of peripheral self-tolerance and they prevent graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (BMT) in murine disease models. Thus, the adoptive transfer of human Treg cells seems a promising strategy for the treatment of autoimmune diseases and the prophylaxis of GVHD after BMT. For clinical trials requiring large Treg cell numbers for repetitive treatments, we described in vitro culture conditions that permit a more than 3-log expansion of Treg cells (Blood 104:895; 2004 & Blood 108:4260; 2006). Those studies showed that the purity of the Treg cell product at the end of the expansion process was critically dependent on the nature and composition of the starting population. In contrast to CD4+CD25- T cells, the majority of expanded CD4+CD25high T cells maintained expression of the lymph node homing receptors CD62L and CCR7. Multiparameter analysis revealed that it was only this subpopulation with sustained CD62L and CCR7 co-expression during in vitro expansion that also showed all phenotypic and functional characteristics of natural Treg cells, such as constitutive intracellular expression of CTLA-4 and FOXP3, the only molecular Treg cell-specific marker known today, lack of cytokine secretion and potent suppression of responder T cell proliferation. Further analysis revealed that these cells originated from CD45RA+ naïve cells within the CD4+CD25high T cell compartment (RA+ Treg). In contrast, cell lines derived from CD45RA- memory-type CD4+CD25high T cells lost expression of FOXP3, CCR7 and CD62L more rapidly, contained more IL-2, IFN-gamma and IL-10 secreting cells and showed less suppressive activity upon in vitro expansion. A recently proposed alternative approach for the isolation of pure Treg cells relies on the exclusion of CD127+ cells, as activated, CD25+ conventional T cells express high levels, while CD4+CD25+ Treg cells show no or only weak expression of this marker. However, also cultures initiated with FACS-purified CD4+CD25+CD127low/neg T cells (CD127- Treg) gradually lost FOXP3 expression during in vitro expansion while containing increasing numbers of cytokine-producing FOXP3- cells over time. Further subdivision into CD45RA+ and CD45RA- subpopulations before expansion revealed that this was mainly due to CD45RA- cells within the starting population, whereas CD45RA+ CD127- Treg behaved similar to the RA+ Treg described above. Based on these findings, we suggest that isolation and expansion of CD45RA+CD4+CD25high T cells at present represents the best strategy for adoptive cell therapies requiring in vitro expanded Treg cells.

Flow cytometric DNA ploidy in brush biopsies of oral lesions

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Background: Oral brush biopsies have proved to be a promising new non-invasive methodology in the diagnosis of oral lesions. However the limited sensitivity and specificity of conventional brush biopsies make it necessary that the analysis is assisted by additional procedures like imaging analysis (Oral CDx), immunocytochemistry and DNA-image-cytometry. Flow cytometry is a widely and well-established method for the detection of DNA aberrations with sufficient sensitivity under high resolution conditions.

Purpose: This study was conducted to analyze whether the assessment of DNA ploidy by flow cytometry may assist conventional evaluation of oral brush biopsies in discriminating benign from malignant oral lesions.

Patients and Methods: 52 consecutive oral brush biopsies with histopathological diagnosis were analyzed by high resolution flow cytometry with DAPI staining of fresh samples: 5 brush biopsies of normal oral mucosa, 14 inflammatory lesions, 14 hyperproliferative lesions, 17 primary oral squamous cell carcinomas (OSCC) and 2 recurrent OSCCs. High resolution flow cytometry for correlative analysis was performed for scalpel biopsies of all 19 OSCCs.

Results: 96 % (50/52) of the samples prepared with the brush biopsy procedure contained cellular material that was quantitatively as well as qualitatively suitable for flow cytometric analysis. All 31 benign lesions were diploid while all 19 OSCC showed cell populations with aneuploid DNA content in oral brush biopsies as well as in scalpel biopsies. The mean coefficient of variation was 7.8 % (3.3-20.2 %) for diploid lesions and 6.3 % (4.3-13.1 %) for aneuploid lesions.

Conclusion: DNA flow cytometry in oral brush biopsies contributes to assist conventional cytology in identifying aneuploid and therefore clearly malignant lesions and recommends this quick and effective technique as a first diagnostic step in monitoring of mucosal lesions. High CV-values indicate the requirement of an alternative technique for cell preparation.

Assessment of DNA ploidy in salivary gland tumors

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Background: While DNA ploidy and S-phase fraction (SPF) are well established prognostic factors in oral squamous cell carcinoma, little is known about the clinical value of DNA flow cytometry in salivary gland tumors.

Purpose: This prospective flow cytometric study was conducted to examine the potential of DNA ploidy and SPF to assist routine histopathology in discriminating benign from malignant salivary gland tumors.

Patients and Methods: A total number of 229 benign tumors including 164 pleomorphic adenomas, 51 cystadenolymphomas and 14 others as well as 50 malignant tumors consisting of 18 adenoid cystic adenomas, 10 mucoepidermoid carcinomas, 5 acinic cell carcinomas, 5 carcinoma in pleomorphic adenoma and 12 others were the cohort of this study. All lesions were analyzed by high-resolution DNA flow cytometry with DAPI staining of fresh tissue samples.

Results: All 229 benign neoplasms were diploid while 12 of 50 malignant tumors showed cell populations with aneuploid DNA content. There was no significant relationship between DNA ploidy and histopathological grading, lymph node metastasis and local recurrence. In three cases which initially were taken for pleomorphic adenomas by routine histological examination, aneuploid cell sup-populations identified by DNA flow cytometry gave rise to an additional inspection of the suspicious lesions. Examination of consecutive slides revealed small assemblies of carcinoma cells. Thus the final diagnosis was a non-invasive carcinoma in pleomorphic adenoma. For diploid malignancies, SPF-values were significantly higher (mean 3.9 %) than in pleomorphic adenomas (mean 2.7%, $P < 0.01$)) but did not differ from cystadenolymphomas.

Conclusion: Despite the huge number of diploid cases in the malignant group, aneuploidy turned out as a unambiguous marker for malignant salivary glands. High SPF-values may contribute to discriminate diploid malignant lesions from benign lesions.

Stem Cell Biology and Clinical Cytometry

Extracting more information from routine immunophenotyping using quantitative multiparametry of flow cytometry: two triple staining combinations as examples

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A major problem in diagnostic flow cytometry is the broad inter-individual heterogeneity of cell parameters in the normal population and their often relatively low amplitude of change in patients – particularly in those with chronic diseases. One way to improve this situation is to switch from measuring single parameters to generating an overall picture of the system. For this a critical number of input elements is required. However with increasing pressure on budgets it will not be possible to achieve this by using larger panels of antibodies in routine diagnostics. However, many of the antigens commonly used in flow cytometry are expressed in a characteristic fashion on cells other than their designated targets. In this way much extra information can be collected without incurring increasing costs. This strategy is demonstrated with two triple stainings: CD4/CD8/CD3 detects conventional helper and cytotoxic effector T cells and, in addition a population of which appear as CD3+, CD4-, CD8- cells which are largely gamma delta T-cells. In some patient groups this third population is strinkingly elevated and/or activated. CD31/CD45RA/CD4 is used to stain direct thymic emigrants but much more information can be derived from it for it detects “stressed” monocytes, “blood macrophages”, plasmacytoid DC and immature neutrophils. In addition it detects a genetic defect in CD45RA splicing which is overrepresented in various autoimmune diseases.

Biomarkers of T-cell function after cardiopulmonary bypass surgery

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(2) University Hospital Jena, Institue of Laboratory Medicine, Germany

Background: Impairment of T-cell function after cardiopulmonary bypass surgery (CPBS) is associated with an increased risk for post-operatively complications. To further evaluate the mechanism of T-cell dysfunction, we developed whole-blood assays to analyze expression of biomarkers of T-cell function in patients undergoing CPBS. Methods: Blood was obtained from patients undergoing CPBS (n=11) pre-operatively and at days-3 and -7 postoperatively (POD). Blood was stimulated with four different concentrations of concanavalin A (ConA). As cyclosporine (CsA) and sirolimus (SRL) inhibit different target enzymes in different stages of the cell cycle (G0- and G1-phase, respectively), we added clinical relevant concentrations of both drugs to whole-blood. Flow cytometric analysis was done to assess T-cell expression of both CD25, required for activation, and CD95, involved in apoptose. Results: In untreated blood expression of CD25 and CD95 significantly increased with higher ConA concentrations ($p<0.05$), but regardless the stimulation expression of both biomarkers decreased over time with maximum at POD-7 compared to pre-operatively values ($p<0.05$). Interestingly, the comparison of all time points showed that inhibition of CD25 and CD95 expression was significant higher at POD3 for CsA and pre-operatively for SRL, respectively, regardless of the degree of stimulation ($p<0.05$). At all time points inhibition of CD25 and CD95 expression was significantly higher after CsA compared to SRL treatment regardless the ConA stimulation ($p<0.001$). Conclusion: Our results showed that different pathways of T-cell activation are impaired after CBP surgery over time. Future studies are needed to show the predictive value of biomarkers of T-cell function with the clinical outcome after CBP surgery and the therapeutic consequences.

Industrial Presentations

Principles and Applications of Imaging Flow Cytometry

Basiji D
Amnis Corporation

Multispectral imaging flow cytometry combines the statistical power of flow cytometry with the morphometry and signal localization capabilities of quantitative microscopy. This talk will describe the technology and applications of the ImageStream multispectral imaging flow cytometer developed by Amnis Corporation.
The ImageStream system can produce over 100,000 cell images per minute with fluorescence sensitivity better than flow cytometry. Six images per cell are generated simultaneously, including three distinct modes of microscopy: brightfield, darkfield, and fluorescence. Over 250 parameters can be calculated from the image set associated with each cell, which makes possible a wide variety of applications that are difficult or impossible to perform reliably using other techniques. A partial list of applications includes:

1. Accurate mitotic indices that measure the fraction of cells in prophase, metaphase, anaphase and telophase
2. Robust quantitation of the translocation of NF-kB and other transcription factors and signaling molecules
3. Co-localization of internalized antibodies to the endosomes, lysosomes, golgi, and other cellular compartments
4. Characterization of the apoptotic fraction in cell samples using nuclear and cellular morphology, without the need for Annexin Vstaining, caspase probes, BRDU incorporation, or other specific probes
5. Characterization of infection by virus and parasites
6. High throughput fluorescence in situ hybridization

These and other applications can be performed either alone or in combination, resulting in a high degree of multiplexing. Data illustrating several of these applications will be shown.

Cell Proliferation Assay using Click Chemistry

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Flow Cytometry Section, Molecular Probes/Invitrogen

The measurement of cellular proliferation is fundamental to the assessment of cell health, genotoxicity, and in the evaluation of anticancer drugs. The most accurate method of doing this is by directly measuring DNA synthesis. Initially this was performed by incorporation of the radioactive nucleoside 3H-Thymidine, which has largely been replaced by antibody-based detection of the nucleoside analog BrdU (5-bromo-2´-deoxyuridine). The Click-iT™ EdU Cell Proliferation Assay is an innovative alternative to the BrdU assay. EdU (5-ethynyl-2´-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction, where copper catalyzes a covalent reaction between the incorporated EdU alkyne and a fluorochrome-conjugated azide. Standard methods of fixation and permeabilization can then be used in conjunction with Click-iT labeling and detection with no need for harsh DNA denaturation steps. An overview of click chemistry and how it applies to this application will be presented. Comparison of the Click-iT EdU method and the BrdU antibody method will be discussed. Examples of flow cytometry and imaging data using the Click-iT method will be shown.

Strategies to Improve siRNA Tools for Large Scale Library Screens

Scory S
Thermo Fisher Scientific

siRNA-mediated RNA interference (RNAi) has become a key tool in functional genomics. In this presentation we will address the critical challenges associated with the use of siRNAs in large scale library screens. We will touch upon the delivery principles, outline the solutions for the on-target activity issues and focus on currently the most troublesome problem - specificity. Finally, we will discuss approaches to define criteria for robust siRNA library screens.
Lit.:Reynolds et al.: Rational siRNA Design for RNA Interference. Nature Biotechnol. 2004, Mar, 22 (3), 326-330. Birmingham et al.: 3`-UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nature Methods 2006, 3 (3). Jackson et al.: Position-Specific Chemical Modification increases Specificity of siRNA mediated gene silencing. RNA, 2006 12 (7),1197-1205. Li et al.: Defining Optimal Parameters for hair-pin based knock-down constructs. RNA, Aug 2007, 10.261/rna 599107

The Power of 40 Years Flow Cytometry - From the first fluorescence-based FCM instrument, ICP 11, to the newest FCM Technologies for dedicated applications

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The first fluorescence-based flow cytometry device (ICP 11) was developed in the year 1968 by researchers of the University of Münster in Germany (Patent No. DE1815352) and first commercialized in 1969 by Partec in Münster through Phywe in Göttingen at a time in which absorption methods were still favored by other scientists over fluorescence methods (e.g. Kamentsky, Proceedings of the Conference „Cytology Automation“ in Edinburgh, 1970).
The original name of the flow cytometry technology was pulse cytophotometry (German: Impulscytophotometrie). Only 10 years later in 1978, at the Conference of the American Engineering Foundation in Pensacola, Florida, the name was changed to "flow cytometry“, a term which quickly became popular, but this change in nomenclature causes literature searches, using only that term, to retrieve information just since 1978, while older references usually are not found. Fluorescence-based flow cytometry, as it is used today as a standard method for automated rapid cell analysis, in fact is a European invention which, has been used for cancer cell detection from the beginning.
Rapidly, the ICP 11 became very popular in a wide-spread field of various applications. By 1972, it was used for research in pathology, quantitative cytology, gynaecology, haematology, radiology, cancer research, pharmacology, biophysics & molecular biology, oncology, dermatology, cell and proliferation kinetics, and in the clinical field, e.g. for differentiation of normal and neoplastic cell populations, prescreening of vaginal and cervical smears, leukemia, and therapy control of skin tumours. Between 1969 and 1972, pulse cytophotometry by the ICP 11 was presented at 54 conferences and congresses in Europe, the US, Japan, and Brazil. In 1971, the instrument was awarded the Gold Medal at the internationally renown Leipzig Trade Fair. It is interesting to note that already in 1973, this instrument was successfully used for plant flow cytometry and, in 1974, by ETH Zurich, as a reference method for milk quality control in Switzerland, applications which then surprisingly have not been continued in the FCM field for almost 20 years until taken up again in the early 1990s, thus serving as examples for flow cytometric solutions which obviously have been introduced almost too much ahead of time.
Subsequently introduced flow cytometry instruments have been the Cytograph (1970) and the Cytofluorograph (1971) from Bio/Physics Systems Inc. (later: Ortho Diagnostics), the PAS 8000 (1973) from Partec, the first FACS instrument from Becton Dickinson (1974), the absorption and scatter based Hemalog D (1974) from Technicon (later: Bayer Diagnostics), the ICP 22 (1975) from Partec/Phywe and the Epics from Coulter (1977/78). Using a sublicense from Phywe, Ortho introduced the ICP 22 A in 1977. The PAS-II from Partec followed in 1979. Despite the significant potential of flow cytometry, only a relatively small number of manufacturers entered the field of which the majority exited relatively soon, including Ortho, Leitz, Skatron, BioRad, Heka, Bruker, and Showa Denko. The major FCM companies therefore remain BD, Partec, and Beckman Coulter, while Guava (1998) and Dako (acquiring Cytomation in 2001) joined the field of flow cytometry instrumentation later on.
In general, flow cytometers have been traditionally designed intending to cover with one and the same instrument type a wide range of research and clinical applications which made sense for large institutions using these varied applications while leading to a high production efficiency for the manufaturers. However, this functional versatility adds to the complexity and cost of the instrument and not all fields require the full configurations of such devices (number of optical parameters and fluorescence channels). Consequently, in the year 2000, Partec introduced the portable CyFlow SL for dedicated applications in HIV monitoring, microbiology, and industry. Due to the developments in modern component technology for laser modules, electronics, optoelectronics, and fluidics, FCM instruments nowadays can be made significantly more compact and robust. The modularity and flexibility of this new generation of flow cytometers permits the configuration of specifically tailored system solutions, e.g. for quality control in food industry and dairy products, as well as for increasingly important or new niche fields including plant and animal breeding, quality control in the beverage industry (breweries, wineries, distillieries, fruit juice), paper industry, etc.
A general tendency can be observed that, in the future, highly dedicated instrument types will be offered which exactly address the requirements of specific applications. It can be expected that in many fields, the names of upcoming instruments may be defined by their targeted application: Terms like "wine analyser“ or "wood pitch analyser” may be more widely used than "flow cytometer“, as is already the case for “CD4 counter” and “ploidy analyzer”. Development of a wide range of highly dedicated instruments may become more important for the future of flow cytometry than further increasing the number of fluorescence parameters of high-end systems, beyond the current 14 (Partec CyFlow ML) or 18 colours (BD LSR-II).

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Cytometry in Microbiology, Biotechnology, and Plants

Investigations of Plant in vitro cultures by Flow cytometry

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(2) Institute of Microbiology, Bulagarian Academy of Sciences, Bulgaria

The plant in vitro system is a promising technology both for obtaining of biological active substances and for basic research of the molecular biology and biochemistry of the plant cell. Bioactive compounds, currently extracted from plants, are used as food additives, pigments, dyes, insecticides, cosmetics, perfumes, and fine chemicals. However, decreased plant resources, especially natural habitats for medicinal plants, together with environmental problems have prompted industries, as well as scientists, to consider the possibilities to investigate in vitro systems as alternative systems for the production of these substances. The major problem concerning industrial implementation of plant in vitro systems are variable yields of the target secondary metabolites, which could be due to the genetic instability. Therefore investigations of the genome are a base for evaluation of algorithms for assessment and selection of high producing genetically stable plant in vitro culture lines. In this study the ploidy patterns of intact plants, calli, and hairy roots of the higher plants Beta vulgaris and Datura innoxia were analyzed. It was published that in vivo plant and the in vitro cultures obtained from them have the same genome size. However, for Datura innoxia we found differences in ploidy of the investigated plant systems: the hairy roots cultures undergo one endoreduplication cycle and therefore consisted mainly of tetraploid cells, while the callus undergo two endoreduplication cycles. The obtained data showed the same endoreduplication pattern for all 10 investigated transformed root lines, which is in contradiction with published opinion that transformed root cultures have identical genome size as the plants from which they were obtained. For Beta vulgaris again significant differences in the ploidy pattern of intact plants and plant in vitro cultures were found.

Flow cytometric and phytochemical investigations with plant cell suspension cultures of sunflower (Helianthus annuus)

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Plants are an unbounded source of valuable secondary metabolites used from centuries as pharmaceuticals, food additives, fragrances, dues and agrochemicals. Production of plant derived metabolites through classical technologies lead to several difficulties, resulting mainly from seasonal, geographical and soil features. Furthermore the isolation of such metabolites (usually in small amounts) from huge plant mass is labour- and time- consuming and makes the process more expensive. Plant cell, tissues and organ cultures offer an alternative opportunity for production of biological active substances. A critical step in the creation of industrial process is a development of on-/off-line methods for determination of cell's growth and the physiological behaviour of the cells during their growth in different cultivation systems. Recently flow cytometry became a popular method for ploidy screening, detection of mixoploidy and aneuploidy, cell cycle analysis, estimation of absolute DNA amount or genome size and etc. Cell suspension cultures of Helianthus annuus possess stable growth and morphological characteristics and were found to produce biologically-active substances (immunologically-active polysaccharides and Vitamine E) and therefore could be used as a good model system for determination of kinetics of growth and metabolite(s) production. The results from flow cytometry measurements (cell cycle analysis) and Vitamine E production obtained during the cultivation of Helianthus annuus cell suspension culture in shake-flasks and 5-L stirred tank reactor are presented and discussed.
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Cytometry in Systems Biology

Flow-cytometric measurement of respiratory burst in rat polymorphonuclear granulocytes: Comparison of four cell preparation procedures, and concentration-response evaluation of chemical stimulants

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Background: Polymorphonuclear neutrophils (PMNs) contribute to organ injury in sepsis, stroke, and other diseases. Evaluation of the oxidative burst by flow cytometry is frequently applied to examine PMN status in humans, but rarely in rats. However, methods established for human PMN analysis are hardly transferable to other species since blood cell counts, erythrocyte volumes, leukocyte specific weights differ considerably, and no systematic dose finding for PMN stimulating agents exists. Thus, we established a method to assess granulocyte activation in rats by means of FACS analysis of oxidative burst.
Methods: Two methods for blood cell isolation involving Histopaque separation were investigated, and additionally two whole blood techniques. The effectiveness of each method was assessed taking into account the facilities required, time of manipulation, purity of the PMN population yielded, viability, PMN activation by the procedure itself, and influence on the chemically stimulated ROD production. In addition, the concentration-response relation of the stimulants fMLP, PMA, TNF-alpha and LPS has been determined, both alone and in combination. The generation of reactive oxygen derivates (ROD) was quantified based on a hydrogen-peroxide formation assay, using the indicator dye dihydrorhodamine 123.
Results: A new technique with diluted rat whole blood proved to be most appropriate for PMN preparation. The systematic evaluation of the concentration-response relation showed that 1 µM PMA and fMLP, respectively, are the best concentrations for PMN stimulation in rat whole blood. Combined with 1 µM fMLP, 0.1 µg/ml TNF-alpha; and 1 µg/ml LPS, respectively, gave optimal additional stimulation.
Conclusions: This study defined appropriate conditions for evaluating reactive oxygen derivate production in rat PMNs by flow cytometry: The rapid, simple, and reliable cell preparation procedure of whole blood dilution preserves cell integrity and requires only small sample quantities. This is the first systematic dose-response evaluation of chemical stimulants of neutrophil respiratory burst in rats.

Biosensoric and Novel Applications / Nanotechnolgies

Photoinduced electron transfer (PET)-probes for the detection of cancer-related nucleases

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Over the last few decades research in oncology has exhibited a connection between various types of cancer with strongly changing nuclease levels in several domains of the human body. It has been reported, that several types of cancer correspond to high nuclease activities in human blood serum as well as in urine. Previous studies determined this enzyme to belong to the DNaseI-family. Recently it was shown however, that in cancerous tissue the expression of DNaseI is diminished while the closely related nuclease DnaseX is expressed. Therefore, specific and sensitive identification of DnaseX might pave the way for rapid early-stage detection of malignant diseases so that survival rates of patients can be substantially enhanced. We introduce a detection system fulfilling these demands by employing a novel design of fluorescent probes: an oligonucleotide is linked to a fluorophore, whose fluorescence is quenched by guanosine residues (G) via photo-induced electron transfer (PET) at close proximity between the electron donor and acceptor. This approach circumvents unspecific probe enzyme interactions and affinity problems, higher background signal and high costs associated with the use of two extrinsic labels in commonly used fluorescence resonant energy transfer- (FRET-) probes. As in FRET-probes the spatial contact between the fluorophore and guanosine residues gets lost upon cleavage by a nuclease. Thus fluorescence quenching is prevented and results in a distinct rise of fluorescence intensities. We demonstrate that our novel probes can be used advantageously to detect specific enzyme characteristics of nucleases, e.g. substrate specificities, temperature optimum and enzyme velocities. Furthermore, we present first results towards the design of a specific and sensitive PET-probe ideally suited for the fast detection for DnaseX even in natural environments.

Optimization of the hypoosmotic loading method of aequorin, an intracellular Ca^{2+} concentration indicator, into cells of follicular lymphoma

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Aequorin belongs to the group of Ca^{2+} indicators called Ca^{2+} regulated photoproteins. Aequorin has several advantages in comparison to widely used Ca^{2+} fluorescent indicators (Fura-2, Indo-1, Fluo-3), e.g. high signal to background ratio or resistance to motion artifacts. Incorporation of this indicator into cells is the most difficult technical problem, because aequorin is 22 kDa protein. Hypoosmotic solution was used to incorporate aequorin into cytoplasm of follicular lymphoma cells. Different incubation times and concentrations were tested in order to find both good cell survival of the procedure and sufficient amount of aequorin incorporating into the cytoplasm. The measurement of fluorescence was performed in a home-made apparatus using photomultiplier (Hammamatsu). The quantification of intracellular calcium concentration was done by the L/Lmax calculation and Ca^{2+} - effect concentration curve published elsewhere [1,2]. Effectiveness of the hypoosmotic solution to incorporate the indicator into cells was also checked with GFP protein, which is approximately the same size. The analysis of GFP fluorescence signal was performed on flow cytometer (Becton Dickinson FACSVantge SE). The improved hypoosmotic loading method proved to be effective for aequorin loading into follicular lymphoma cells in vitro. It could be anticipated that this method could be used for intracellular Ca2+ concentration measurement in other types of cultured cells. [1] Blinks J. R., Alen D. G. et all (1978) Life Sciences 22, 1237-1244. [2] Johnson P. C. et. all (1984) J Biol Chem 260, 2069-2076

Tissue analysis – Special Applications

Automated Multi-color FISH Analysis Workstation for Lung Pharmacogenomics: Identifcation of Clonally Related Cells

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We have developed an automated multicolour high-throughput Fluorescence in-situ Hybridization (FISH) scanning system for examining Non-Small Cell Lung Cancer (NSCLC) 5-10 um thick tissue specimens and analyzing their FISH spot-signal at the cell by cell level quantifying their architectural relationships (spatial distribution) with an aim to identify clonal groups of cells likely resistant to cis-platinum/vinorelbine chemotherapy treatments. Based on recent array comparative genomic hybridization (aCGH) results that determine genetic amplification and deletion profiles for chemoresistant tumors, loci specific FISH probes are created via nick translation using BAC clones deemed significant to resistance. These FISH probes are then used to automatically inspect gene copy number in cytological and histological patient samples. Multicolour labeling is attained simultaneously with 5-6 fluorophores including diethyl-aminomethyl-coumarin (DEAC), SpectrumGreen, SpectrumOrange, TexasRed, Cy5 or Cy5.5. Tissue scanning is performed using a fully automated Zeiss AxioImager Z1 microscope and images are acquired in 5-7 focal plane stacks with automatic focus and exposure control. Cell nuclei are segmented using an 'Enhanced Edge Detection' algorithm and spot detection is achieved with TopHat filtering and maxima detection. Using the segmentation and individual nuclei spot counting results, an architectural score based on either Delaunay triangulation or Voronoi tessellation of the tissue was implemented, measuring the spatial distribution and connectivity of cells with specific gene profiles. Preliminary results show success in delineating cancerous from non-cancerous regions in tissue samples. Evaluation experiments are underway to characterize performance and future work will focus on examining archived patient samples with known outcomes for presence of chemo-resistant tumor cell subpopulations and ultimately, play a role in customizing chemotherapy treatments.

Key notes
(supplemented / revised)

Morphology and Apoptosis in thick tissues using a Confocal Microscope (abstract revised)

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Confocal laser scanning microscopy (CLSM) is a technique that is capable of generating serial sections of whole-mount tissue and then reassembling the computer acquired images as a virtual 3-dimensional structure. In many ways CLSM offers an alternative to traditional sectioning approaches. However, the imaging of whole-mount tissues presents technical limitations of its own. One of the major problems using CLSM to image whole organs and embryos is tissue penetration of laser light. High quality morphological images begin by optimizing the sample preparation technique (1-2). Additional factors include evaluating CLSM performance by optimizing the acquisition variables (i.e. objective lens, averaging, pinhole size, bleaching, PMT voltage, laser excitation source, and spectral registration) of the confocal microscope. (3, 4)
Confocal microscopy has been used by our laboratory to study cell death and morphology in embryos, ovaries, eyes, ears, and limbs (1-2). The technique has revealed structural morphology and visualized areas of cell death by the uptake of the LysoTracker dye into phagolysomes. LysoTracker Red (LT) is fixable by paraformaldehyde and concentrates in acidic compartments of cells. In whole tissues, this accumulation indicates regions of high lysosomal activity and phagocytosis. LT staining is an indicator of apoptotic cell death and correlates with other standard apoptotic assays (1-2). LT staining revealed cell death regions in mammalian limbs, neonatal ovaries, fetuses and embryos. The mammalian samples were stained with LT, fixed with paraformaldehyde / glutaraldehyde, dehydrated with methanol (MeOH), and cleared with benzyl alcohol/benzyl benzoate (BABB). The use of BABB matches the refractive index of the tissue to that of the suspending medium. BABB helps increase the penetration of laser light during CLSM by reducing the amount of light scattering artifacts and allows for the visualization of morphology in thick tissue. Following this treatment, the tissues were nearly transparent. This sample preparation procedure, combined with the optimization of CLSM instrument factors, allowed for the detection and visualization of apoptosis in fetal limbs and embryos which were approximately 500 microns thick. Recently, spectroscopic imaging capacity has been incorporated into confocal microscopes. The LT spectra had a maximum peak around 610nm while the fixative, glutaraldehyde (Glut), had a maximum peak around 450nm. Glut was added primarily to preserve the tissue morphology, but also provided molecules emitting in the green fluorescence range that helped to visualize the morphology of the tissue. The understanding of the spectra derived from the tissue was extremely useful in optimizing the staining protocol. We have continued to incrementally improve the tissue staining and preparation techniques to achieve better quality 3D images.
References
Zucker RM and Jeffay SC. Cytometry, 2006 69A, 930-939; Zucker RM. Cytometry, 2006 69A, 1143-1152; Zucker RM. Cytometry, 2006 69 A, 659-676; Zucker RM. Cytometry, 2006 69A, 677-690. (This abstract of a proposed presentation does not necessarily reflect EPA policy.)

Cytometry and the dawn of the cytomics generation
(abstract supplemented)

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Cytometry has for many years focused on traditional techniques that are well understood and provide a quality of information on single cells, not possible with other technologies. However, as all technologies change, so to is cytometry. There is a gently but strong move toward tools that are driven by the demands of more complex systems biology approach. The result is a more comprehensive field of cytomics broadly defined as the systematic study of biological organization and behavior at the cellular level has begun to mature and establish itself as an integral component in cell biology. The necessary tools for integration of cytomics into the fundamental nature of cell systems analysis are maturing but new tools are demanded to achieve our goals. While there is a long way to go before we have tools that can perform true cytomics analysis, cytometry is a subset of tools that is tremendously powerful and from which we can extract a significant subset of information about many biological systems.

It is important therefore to realize that new technologies must be developed for cytomics to become a reality. For example there will be a need for essential development of new sensor technologies that provide both sensitivity and selection in the visible and near IR spectrum. Secondly, a better integration between different measurement and detection tools will be needed. We simply cannot make independent measurements and hope to integrate these tools easily. Thirdly, in order to analyze the complex data sets resulting from new technology integration a major advance is needed to accommodate analysis of these data sets. Fourthly, chemistries must advance to permit greater selectivity of tracking tools. These will most likely expand beyond fluorescence to accommodate enhanced scatter analysis as well as chemical composition.

Together, these advances place the Cytomic opportunity into a new dimension for understanding metabolic responses in single cells and ultimately defining new functional populations of cells. The result will be new research tools as well as a toolset for clinical and diagnostic utility.