

# GREAT LAKES INTERNATIONAL IMAGING AND FLOW CYTOMETRY ASSOCIATION, INC.



## GLI<sup>2</sup>FCA TEN

### “DECAFLODOWN”

**CLARION HOTEL CONFERENCE CENTER  
MILWAUKEE WISCONSIN  
OCTOBER 12 - 14, 2001**

**Site Organizers: Alex Nakeff, Wendy Kopinski, Kathy Schell, Bruce Pesch  
Program Chairs: Jake Jacobberger, Tom Sawyer, Karen Domenico**

#### STEERING COMMITTEE

##### Canada

David Hedley, Frank Mandy

##### Illinois

Julie Auger, Charles Goolsby,  
Maurice R. G. O’Gorman, T. Vincent Shankey

##### Indiana

J. Paul Robinson, Philip Marder

##### Iowa

Bruce Pesch

##### Michigan

Alexander Nakeff

##### Minnesota

Waclaw Jaszcz, Wendy Walters

##### New York

John M. Lehman, Carleton C. Stewart

##### Ohio

Charles L. Hitchcock, James W. Jacobberger, Tom Sawyer

##### Pennsylvania

Jonni Moore, Katharine Muirhead,  
Betsy Ohlsson-Wilhelm

##### Wisconsin

Wendy Kopinski, Kathleen Schell, Robert Truitt

#### CORPORATE MEMBERS AND MEETING SPONSORS

Bangs Laboratories

Beckman-Coulter Corporation

BD Biosciences

BIOErgonomics, Inc.

CALTAG Laboratories

Cell Signaling Technology

Cytomation, Inc.

DAKO Corporation

Guava Technologies

Jackson Immunoresearch Labs

Linco, Inc.

NPE Systems

Phoenix Flow Systems, Inc.

R&D Systems, Inc.

Serotec Ltd.

Spherotech, Inc.

Tree Star, Inc.

Verity Software House, Inc.

Roswell Park Cancer Institute

visit our web site: <http://www.cyto.purdue.edu/flowcyt/glifca/gliifca.htm>

This continuing medical laboratory education activity is recognized by the American Society of Clinical Pathologists as meeting the criteria for 12 hours of CMLE credit.

Announcing the 10<sup>th</sup> year Anniversary Meeting, GLIIFCA 2001 or, Cytometry and the Golden Age of Biomedicine.

The theme for the Flowgadown will be "Decafdown", dress for the perfect 10!

**FRIDAY Evening - October 12**

**Registration/ Poster Set-Up** **1700 - 1900**

**SHORT PRESENTATIONS, New Directions, Chair – Tom Sawyer, Toledo, OH**

**Dan Collins**, St. Paul, MN "Misregulation of iron in neurodegenerative disease" **1900 - 1930**

**Roy Overton**, Fort Collins, CO "Automated flow cytometry breakthrough for High Throughput Screening" **1930 - 2000**

**Frank Mortari**, Minneapolis, MN "A new paradigm to intracellular detection of proteins and RNAs" **2000 - 2030**

**Ben Verwer**, San Jose, CA "Multicolor analysis and cell sorting with the FACSDiVa digital electronics" **2030 - 2100**

**Reception/Poster Session**

**SPONSORED BY BECKMAN COULTER**

**2030 - 2230**

**SATURDAY - October 13**

**Breakfast** **0730 - 0800**

**PLENARY SESSION I**

**Biological Correlates and Therapeutic Monitors** **(times include 5 minutes for questions)**

**Chair – Jonni Moore**, Philadelphia, PA

**David Hedley**, Toronto, Canada "Cytometric analysis of MAP kinase signal transduction" **0800 - 0840**

**Keith Shults**, Nashville, TN, "Simultaneous measurement of p53, p21<sup>waf1</sup> and mdm2 provides non-redundant information on the downstream effects of p53 expression following treatment with adenoviral vectors and DNA damaging agents ". **0840 - 0920**

**Albert Donnenberg**, Pittsburgh, PA, "Interpretation of MHC-tetramer data: Avoiding the pitfalls" **0920 -1000**

**Break**

**SPONSORED BY SPHEROTECH**

**1000 – 1025**

**MID-SESSION**

**Leukemias & Lymphomas**

**Chair – Carl Stewart**, Buffalo, NY

**Chuck Goolsby**, Chicago, IL, "Bcl-2 Apoptosis regulatory pathway is functional In Vitro in B cell CLL". **1025 - 1105**

**Carl-Fredrik Bassøe**, Bergen, Norway, "Phagocyte function in acute myeloid leukemia" **1105 - 1145**

**Platform Competition:**

**Connie Yuan**, University of Penn, "'Adjusting' to the new role of DNA analysis in breast cancer prognosis. " **1145 - 1200**

**Luncheon Roundtables**

**1200 - 1330**

**Chair- Julie Auger**, Chicago, IL

A variety of round table discussions will be offered covering a wide range of flow cytometry and image topics. Some topics will include cytokine networks, leukemia/lymphoma phenotyping, cell cycle regulation, cell sorting, management issues etc. Someone experienced in the field will lead each discussion. A sign-up sheet will be available Friday night for roundtable selection. There is no charge.

**PLENARY SESSION II**

**Immunology, Microbiology, Virology** **(times include 5 minutes for questions)**

**Chairs –Karen Domenico**, Toledo, OH

**Bruce Patterson**, Chicago, IL, "Quantification and antiretroviral drug susceptibility testing of HIV-1 using molecular flow cytometry". **1330 - 1410**

**Mo O’Gorman**, Chicago, IL, "The use of flow cytometry for diagnosis of primary immunodeficiency disease" **1410 - 1450**

**Platform Competitions:**

**Chad Ray**, Indianapolis, IN "Application issues for clinical multiplexing immunoassays of cytokines" **1450 – 1505**

**Kayoko Kimura**, Ames, IA, "Hypocalcemia blunts immune responses dependent on release of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) stores" **1505 - 1520**

**Arumugham Raghunathan**, New Haven, CT, "Rolling circle amplification – A new approach to increase sensitivity for flow cytometry." **1520 -1535**

Break  1535 – 1600

**Keynote Address: Chair, Chuck Goolsby**, Chicago, IL 1600 - 1700  
**Peter Rabinovitch**, Seattle, WA, “Genomic Instability, Cytometry and Progression to Gastrointestinal Cancer”.

**ANNIVERSARY COMMENTS – 10 years later**  
**Carl Stewart**, Buffalo, NY

Wine and Cheese Happy Hours & Poster Session in Exhibit Area SPONSORED BY CYTOMATION and  
VERITY SOFTWARE HOUSE 1700 - 1715  
1715 - 1930

**“THE DECAFLODOWN”**  
**SPONSORED BY**  
**BD BIOSCIENCES** 2000 – 2400

**SUNDAY - October 14**  
**Steering Committee Meeting** 0730 – 0900  
**Breakfast** 0800 – 0900

**PLENARY SESSION III** 0900 - 1100  
**Imaging and Technology**  
**Chair, David Hedley**, Toronto, Ontario  
**Vojislav Vukovic**, Toronto, Ont. “Multiparametric Characterization of Solid Tumor Microenvironments”. 0900 - 0940  
**Carl Stewart**, Buffalo, NY, “Breast Ductal Lavage ”. 0940 - 1020

**Break** 1020 – 1045

**Commercial Talks,**  
**Chair, Alex Nakeff**, Detroit, MI  
**Jeff Clapper**, Beckman-Coulter  
“Updates in Beckman Coulter Flow Cytometry Automation: The PrepPlus II and CellPrep” 1045 –

1115  
**Ken Murchison**, BD Biosciences, San Jose, CA  
"The Latest Information on New Product Releases from Becton-Dickinson" 1115 - 1145  
**Mark Munson**, Verity Software House, Topsham, ME, “and now, for something completely different...” 1145 - 1215

**AWARD/ TRAVEL STIPEND PRESENTATIONS-**  
**Julie Auger**, Chicago, IL 1215 - 1230

**Travel Stipends supported by Cell Signaling Technology.**  
**Plenary Speaker Award supported by Jackson ImmunoResearch Laboratories.**

## **MISREGULATION OF IRON IN NEURODEGENERATIVE DISEASE**

**Daniel P. Collins, BioErgonomics, Inc., St Paul, MN 55127**

Alzheimer's Disease (AD) is the end result of an array of biochemical changes in the neurons of patients including over-expression of  $\beta$ -amyloid precursor protein ( $\beta$ APP), heightened expression or activity of presenilins that cleave  $\beta$ APP into  $A\beta$ , reduced activity of  $A\beta$  digestive enzymes like neprilysin, clogging and overloading of the ubiquitin/proteosome system, inhibition of repair mechanisms associated with the APOE4 isotype, creation of tau protein tangles and altered activity of iron-regulatory proteins which overload the cells with toxic levels of iron contributing to the creation of reactive oxidative species and neuronal cell death. The main genetic markers that are being studied in association with AD (presenilin1 and 2, APOE4, etc.) have been related with rare cases of early onset familial AD but have little if any association with sporadic and late-onset AD. Currently, no test exists that is diagnostic of AD other than post mortem pathology. This study is investigating the role of Iron Regulatory Protein 2 (IRP-2) in the misregulation of iron by neurons and the development of AD and its potential use as an ex-vivo marker for AD as a collaborative effort between BioE, Loma Linda University, Washington University, and the NIH. We have demonstrated the presence of IRP-2 in circulating leukocytes and preliminary evidence of up-regulation of IRP-2 in leukocytes from AD patients. The role of site-specific mutations, alterations of the ubiquitin/proteosome system and iron metabolism in AD will be discussed. In addition, the use of peripheral blood leukocytes as a model for altered iron metabolism by neurons and the use of flow cytometry and image analysis to develop an AD-specific diagnostic will be discussed.

## **AUTOMATED FLOW CYTOMETRY BREAKTHROUGH FOR HIGH THROUGHPUT SCREENING**

**W. Roy Overton, Cytomation, Fort Collins, CO**

For more than two decades, flow cytometry has been a powerful tool in many areas of medical and biological research. Fields such as immunology, genetics, and oncology would not have advanced as quickly as they have without flow cytometry and many advances in the future probably will come as a result of this technology. However, other fields of research and product development have not been able take advantage of the rapid, single cell analysis that flow cytometry provides because today's cytometers lack the type of automation that is needed. High Throughput Screening (HTS) is one of those areas that can benefit from the automation of flow cytometry.

Performing flow cytometry has two major components. The first component is the boring, tedious task of loading samples into the cytometer. The second component is the challenging job of analyzing and interpreting the data that is collected by the cytometer. Since HTS requires the analysis of thousands, even millions of samples per day, it is not practical to load each sample by hand with the conventional fluidics systems of our traditional instruments. With that kind of sample load, the volume of data would be overwhelming with typical flow cytometry software. Clearly, traditional cytometers can not handle the demands of HTS.

Cytomation, Inc. has taken the modular, ultra-high-speed cytometer, MoFlo<sup>®</sup>, and built new modules that enable it to meet the demands of HTS and other applications for drug discovery. The first module for HTS is MoSkeeto<sup>®</sup> which permits the rapid uptake of samples from microtiter plates. MoSkeeto can feed 96 samples from a microtiter plate into a MoFlo in less than 90 seconds. MoFlo's high-speed electronics detect and process the light scatter and fluorescence data from these cells at event rates exceeding 100,000 per second. This data is stored, organized and analyzed using Cytomation's Summit software. The second module developed for HTS is Cytomation's new CytoBorg<sup>®</sup> tray movement system which moves microtiter trays between MoSkeeto, CyCLONE<sup>®</sup> (MoFlo's single cell deposition device) and either incubators or hotels, using a computer-controlled robotic arm.

These advances in automation enable MoFlo to perform a variety of HTS applications for drug discovery and other product development. Custom configurations of MoFlo HTS are currently being used by biotechnology and drug discovery companies including Rigel Pharmaceuticals, Inc., Lynx Therapeutics, and Diversa Corp.. Some of the HTS applications that have been developed for or adapted to flow cytometry include postgenomics combinatorial biology (Rigel), Megaclone™ technology (Lynx) and SingleCell™ technology (Diversa). Since High-Throughput Screening laboratories are just beginning to discover the power and potential of flow cytometry, flow cytometric HTS has immense potential for growth in the future.

### **A NEW PARADIGM TO INTRACELLULAR DETECTION OF PROTEINS AND RNAS**

**Frank Mortari, R&D Systems, Inc., 614 McKinley Place N.E., Minneapolis, MN 55413.**

Cells respond to environmental signals through cell surface receptors that mediate their action through the engagement of accessory intracellular molecules. These molecules activate a cascade of events that ultimately can impact RNA transcriptional activity. The presence of these intracellular molecules has traditionally been monitored through such assays as Western blotting, gel-shift assays and Northern blotting. These assays require a certain level of expertise but more importantly they lack in quantitative power.

Enzyme Linked Immunosorbent Assays (ELISA) have been in use for multiple decades and have found greatest acceptance in the monitoring of soluble proteins such as cytokines. We wondered if ELISA type assays could be used for the detection of other intracellular molecules. As proof of principle we applied this assay format to the detection of three model molecules: active caspases, molecules involved in the apoptosis process like cytochrome C and survivin, and mRNAs for various cytokines.

We generated suitable reagents to detect these molecules and designed the assays for a 96-well format. The mRNA assay is based on the ability to detect mRNA through the use of biotinylated oligomers that capture the specific mRNA and while digoxigenin conjugated oligomers act as specific detection molecules. The assay is calibrated with mRNA standards. The results show the mRNA assay to be very comparable in sensitivity to Northern blotting techniques however with greater range and linearity.

The active caspase assay is capable of specifically detecting active caspase-3 and 7 in a quantitative manner using the biotinylated fluoromethylketone caspase inhibitors as an intermediate reactive agent. Lastly, we established that intracellular molecules involved in the apoptosis, like cytochrome C and survivin, can be also detected and accurately quantitated in various cell types using a paired antibody strategy.

We conclude that ELISA type assays can be developed for a variety of intracellular molecules traditionally not thought to be detectable with this technology and can yield quantitative data without the need for expensive equipment or highly trained technical staff. Furthermore the 96-well plate format and the time saving feature of these assays lend themselves well to high-through put screening scenarios.

### **MULTICOLOR ANALYSIS AND CELL SORTING WITH THE *FACSDiVa* DIGITAL ELECTRONICS.**

**Ben Verwer. Becton Dickinson, San Jose, CA**

The FACSDiVa uses 10 MHz, 14-bit A/D converters to continuously digitize flow signals. The digitized signals stream into memory, upon which the system extracts area, height and width. For area, the pulse is integrated, giving the system an effective dynamic range of 18-bits for typical pulse widths. After extraction of area, height and width, the system can compensate between any two parameters, take the ratio between any two parameters or calculate the logarithm of a parameter. For compensation it employs matrix inversion such that the user can directly enter spillover coefficients instead of the more traditional compensation coefficients. Logarithmic amplifiers are no longer used. Continuous digitization of pulses eliminates electronic dead time. There still can be electronic aborts, but only when window gates are extended or the event rate gets too high. The elimination of electronic dead time leads to improved sorting over systems which have peak-and-hold circuits to digitize pulses. A novel way of making tradeoffs between purity, yield, counting accuracy and side streams is shown.

**Speaker Abstracts, Plenary Session I****CYTOMETRIC ANALYSIS OF MAP KINASE SIGNAL TRANSDUCTION.  
David W. Hedley, Princess Margaret Hospital/Ontario Cancer Institute, Toronto.**

Oncogenic mutations that result in increased signaling via MAP kinase occur in a high proportion of human cancers. Pharmaceuticals that target these abnormalities are now being developed in large numbers. Some have already shown clinical activity, suggesting that the rational deployment of molecular targeted agents will have a major impact on cancer over the next few years. Because of the complexity of signal transduction mechanisms, and the problems of tumour heterogeneity, laboratory measurements are needed to identify patients likely to respond to specific drugs or drug combinations, and for monitoring therapeutic response. Development of these analytical techniques is facilitated by the availability of antibodies that specifically label the phosphorylated (activated) forms of signaling elements. We recently published a flow cytometry method for pharmacodynamic monitoring of a raf kinase inhibitor, based on the ability to detect phosphorylated ERK in peripheral T-cell following PMA activation (Chow et al., *Cytometry* 2001;46:72-78). This general methodology is now being extended using both flow and fluorescence image cytometry techniques, as will be discussed.

**SIMULTANEOUS MEASUREMENT OF P53, P21<sup>WAF1</sup> AND MDM2 PROVIDES NON-REDUNDANT INFORMATION ON THE DOWNSTREAM EFFECTS OF P53 EXPRESSION FOLLOWING TREATMENT WITH ADENOVIRAL VECTORS AND DNA DAMAGING AGENTS****Keith Shults<sup>1</sup> and James W. Jacobberger<sup>2</sup>****Esoterix Center for Innovation, Brentwood, TN<sup>1</sup> and Case Western Reserve University, Cleveland, OH<sup>2</sup>**

**Introduction:** Previous studies have demonstrated that MDM2 is upregulated in ovarian cancer cell lines and cytokeratin positive cells in ascites following transduction of wild-type p53 with an adenovirus vector (Ad-p53, INGN201) indicative of both transduction and function (*Cytometry*, 38, 201-213, 1999, and unpublished). Since p53 regulates the transcription of many genes, we wished to determine whether measuring the expression of additional p53 regulated genes would increase the sensitivity of the assay as well as add any functional information. **Materials & Methods:** As a model for the adenoviral vector studies, we used the ovarian line MDAH 2774 and multiparametric flow cytometry to study the kinetics of expression of p53, MDM2, and p21 following Ad-p53 treatment *in vitro*. As a model for the DNA damaging agents, we used the breast line MCF7 (wild type p53) treated with cis-platinum in a series of kinetic studies. Once the assay was devised, we also tested a series of prostatic cell lines in an attempt to elucidate p53 function. **Results:** In MDAH cells, p53 transduction resulted in a 3-fold increase of MDM2 over baseline whereas the p21 expression increased 17 fold 12 hours post infection. Subpopulation analysis defined three relevant populations post infection (p21<sub>b</sub>/mdm2<sup>↑</sup>, p21<sup>↑</sup>/mdm2<sub>b</sub>, p21<sup>↑</sup>/mdm2<sup>↑</sup>) in the p53 induced population (b = baseline, ↑ = elevated). Thus, p53 mediated p21 and MDM2 expression appeared to be mutually exclusive. Related cell cycle experiments (simultaneous measurement of p53, [p21 or MDM2], and DNA content) showed an absence of S phase cells in induced p21 expressing subpopulations whereas MDM2 positive cells were enhanced for the S-phase fraction. Additionally, p21 was the most sensitive marker for Ad-p53 transduction and activity of the wild-type p53 gene. Quantitative RT-PCR of sorted MDAH cell populations revealed a 15-fold increase of the Ad-p53 mRNA in the p53<sub>b</sub>/p21<sup>↑</sup> population. Finally, by correlating light scatter measurements with those of p21 and MDM2, p21 expression was significantly associated with G1 arrest and cell viability whereas expression of MDM2 was associated with advance to S phase and cell death. The patterns of expression determined for transduced MDAH cells and cis-platinum treated MCF7 cells were similar. **Conclusions:** These data suggest the following: (1) the combined measurements of p53, p21, and MDM2 add information to the system at the cellular level, i.e., the multiparametric measurements by flow cytometry are not redundant, (2) in cells expressing mutant p53 at high levels, p21 is a more sensitive marker for p53 transduction and activity than either p53 and/or MDM2 and (3) p53 function may be studied using the downstream products of active p53 transcription.

**INTERPRETATION OF MHC-TETRAMER DATA: AVOIDING THE PITFALLS**

**Albert D. Donnenberg<sup>1</sup>, Thomas K. Hoffmann<sup>2</sup> and Vera Donnenberg<sup>1</sup>**

**University of Pittsburgh School of Medicine, Pittsburgh, PA**

**and Heinrich-Heine-University, Duesseldorf, Germany<sup>2</sup>**

Major histocompatibility complex (MHC)-peptide tetrameric complexes (tetramers) provide a unique analytic tool for detection and quantification of antigen specific T cells. Despite the fact that the technology is almost five years old, interpretation of tetramer data is still difficult. In the best of all possible worlds, detecting a tetramer-binding T-cell means that the cell bears a receptor capable of binding the peptide of interest presented in the context of self MHC. Presumably this interaction is of sufficient avidity to ensure that same peptide/MHC complex, encountered *in vivo*, would initiate a receptor-mediated response. The purpose of this talk is to discuss sources of error and experimental strategies to evaluate the accuracy and precision of the assay.

Detection of tetramer positive cells is often a rare event problem; large numbers of events are acquired and even a small proportion of nonspecifically binding (NSB) cells (or cells that read as positive without binding tetramer) can result in both poor specificity and low sensitivity. Investigators have shown that interaction between tetramer and CD8 can result in NSB. Reaction temperature, and of course, tetramer concentration are also important parameters. Strategies to increase the signal to noise ratio (get positive events far away from negative events in multiparameter space) include: Using labeled anti-CD14 as a “dump parameter” to eliminate monocytes and autofluorescent events; Using a tight side scatter gate (with freshly isolated T cells); and Using a fluorochrome with high quantum efficiency (PE) or that excites at a wavelength that does not induce autofluorescence (APC).

Specificity can be determined in a variety of ways. Expanded peptide-specific T cell lines or clones are a good positive control, although they are rarely 100% tetramer positive. Paired pre- and post immunization samples are also instructive, but are usually not available in human research. Tetramer may compete with anti-CD3 binding, giving tetramer positive events a unique signature as CD3<sup>dim</sup>. Unlabeled tetramer may be used in excess to show competition with binding of labeled tetramer, or two tetramers loaded with the same peptide but labeled with different fluorochromes can be used simultaneously to determine whether the same cell binds both tetramers. Finally, tetramer positive cells can be purified, expanded and retested for specificity.

Negative controls may include an irrelevant MHC correct T-cell clone, but these may not be ideal if they have increased autofluorescence compared to resting cells. In our studies using A2.1 tetramers, individuals who were A2 negative were valuable, not only as negative control subjects, but also for establishing the limits of detection of the assay (defined as the 99<sup>th</sup> percentile of the frequency distribution for a group of A2 negative subjects).

**Speaker Abstracts, Plenary Session I, Mid-session, Leukemias and Lymphomas**

**APOPTOSIS DYSREGULATION IN B CHRONIC LYMPHOCYTIC LEUKEMIA-  
THE Bcl-2 REGULATORY PATHWAY IS FUNCTIONAL**

**Charles Goolsby, Ph.D., Department of Pathology, Northwestern University Medical School**

B chronic lymphocytic leukemia (B-CLL) is a malignancy characterized by the accumulation of clonal B cells co-expressing CD5 and CD23. The malignant B cells demonstrate high expression levels of Bcl-2 as compared to normal. This coupled with low expression levels of the pro-apoptotic proteins of the Bcl-2 family, such as Bax, implies that in vivo the B cells have an anti-apoptotic phenotype. Indeed, the accumulation of abnormal B cells is due to altered apoptosis regulation rather than increased proliferation, however, it is unclear whether there are inherent defects in the Bcl-2 apoptotic pathway. These same B cells rapidly undergo apoptosis with in vitro culture. To investigate alterations in apoptosis regulation in B-CLL, Bcl-2, Bax, mitochondrial membrane potential (MMP) (CMXRosamin), Annexin V staining and caspase activation were simultaneously monitored during in vitro apoptosis. With in vitro culture, 30-50% of the B cells in cultures established from B-CLL samples were apoptotic (annexin V<sup>+</sup>/low CMXRosamine) at 24 hours as compared to <10% of the T cells. In cultures established from normals, <10% of either T or B cells were apoptotic at 24 hours. Apoptosis in the B-CLL B cells, as in the B-CLL T cells and in normal cells, was accompanied by a dramatic upregulation of Bax and slight decreases in Bcl-2 expression. Further, these apoptotic cells showed reduced MMP and significantly increased caspase-3 cellular protein levels. Measurement of caspase activation in cellular lysates was assessed using fluorescent substrates for caspase-3, -8, and -9. Caspase-3 and caspase-9 activity was increased 18-50 fold and 6-11 fold, respectively, following 24 hours of in vitro culture as compared to activity levels at initial culture. Caspase-8 showed only limited or no activation (<4 fold). These data show that in vitro apoptosis of the B cells in B-CLL occurs through a well characterized Bcl-2 regulatory pathway. This implies that the Bcl-2 regulatory pathway is functional and that the anti-apoptotic phenotype of these cells is dependent on the in vivo environment, potentially involving paracrine and/or autocrine interactions with other cells in vivo. Potential autocrine interactions with the clonal/oligoclonal T cells seen in all B CLL patients will be discussed.

**PHAGOCYTE FUNCTION IN ACUTE MYELOID LEUKEMIA.**

**Carl-Fredrik Bassøe, MD, PhD, MA, Hematology Section, Medical Department, N-5021 Haukeland, University Hospital, Bergen, NORWAY**

Surprisingly few studies have been performed on phagocyte functions in acute leukemia. Due to mixtures of cell types and inability to discriminate active and inactive leukemic cells, the results of most of the earlier studies are difficult to interpret. Recent flow cytometric investigations show that blast cells have limited phagocytic capacity, and that the phagocytic capacity of FAB types M4&M5 is significantly higher than that of M1&M2. The phagocytosis cytograms of AML is very different from the normal counterparts, and may aid the diagnosis of AML. Mature neutrophils from most AML patients seem to have normal phagocytic capacity. Phagocytosis by AML blasts and more mature cells in the neutrophil cell line depends on Fcγ- and complement receptors. Diminished total phagocytic capacity may increase the risk of serious infections. Normalization of the diminished phagocyte function in AML may serve as a marker of differentiation in both experimental and clinical situations.

**“ADJUSTING” TO THE NEW ROLE OF DNA ANALYSIS IN BREAST CANCER PROGNOSIS.**  
**Yuan CM\*, Clevenger C, McCarthy T, McCoy CS, Herbert D, Yoo J, Bagwell B, Moore JS.**

Evaluating the malignant potential of breast neoplasms remains a clinical challenge. Although DNA ploidy analysis with S-phase fraction (SPF) provides a measure of cell turnover, its prognostic significance in breast neoplasms is controversial. Recently Bagwell et. al. (Comm. Clin. Cytom 46: 121, 2001) reveal that DNA ploidy and SPF measurements are not only reproducible, but demonstrate tremendous prognostic utility when a set of specific interpretive adjustments are used. By combining this data with clinical information, a relative risk index is derived (Baylor model). This study compares some criteria of the Baylor model versus traditional SPF stratification. We examined 200 DNA histograms derived from fresh breast tissue. All listmode files were evaluated using ungated analysis with Modfit 3.0 and 2.0, and our traditional gated analysis with Modfit 2.0. Using the Bagwell guidelines, each case was evaluated for histogram quality, SPF and consistency in SPF categorization vs. the Baylor model. Our study showed: 1) 83% of fresh cases met quality criteria (while 80% of paraffins failed); 2) SPF correlated best in ungated analysis (R=0.81) but poorly when aggregates were gated out (R=0.59); 3) Gated analysis tended to overestimate SPF when stratified as low, intermediate, or high; 4) Ploidy classification by the Baylor model was consistent, demonstrating 100% and 92% concordance in ungated and gated analysis of aneuploid cases, respectively; 5) Traditional SPF stratification was less consistent than the Baylor model. Ultimately, these results, combined with clinical outcome data, would allow application of the Baylor model to our own patient population as well as development of more powerful strategies for “adjusting” DNA analysis for prognostic significance.

**Speaker Abstracts, Plenary Session II, Immunology, Microbiology, Virology**

**ANTIRETROVIRAL DRUG SUSCEPTIBILITY TESTING USING MOLECULAR FLOW  
CYTOMETRY**

**Bruce Patterson, Northwestern University Medical School, Chicago, IL**

Highly active antiretroviral therapy (HAART) is the cornerstone of treatment for HIV/AIDS. Despite the success in decreasing the prevalence of AIDS in industrialized countries; compliance, toxicity and multidrug resistance diminish the effectiveness of this treatment strategy. Monitoring HAART using molecular techniques such as genotyping and phenotyping assays has improved patient care. These techniques involve time consuming, labor intensive, and expensive technology such as gene sequencing, cloning, and quantitative viral culture. Taken together, the turnaround time of these assays are commonly 14 days or more. Most importantly, recent evidence suggests that antiretroviral drugs suppress viral replication to grossly different levels in T-cells compared to monocytes/macrophages and other cellular reservoirs. Here, we present a new technology using in cell HIV-1 quantification (ViroTect, Invirion Inc, Frankfort, MI) and flow cytometry that can determine the inhibitory concentration of antiretroviral drugs (IC<sub>50</sub>) in T-cells and monocytes/macrophages from HIV-1 infected individuals in 5 days. We show that AZT, 3TC, and amprenavir are far less effective against HIV-1 replication in monocytes/macrophages compared to T-cells while abacavir is equally effective in both T-cells and monocytes/macrophages. Use of an abacavir-containing regimen more effectively cleared virus from monocytes/macrophages (in both blood and lymphoid tissue) in a clinical trial involving 5 patients monitored at baseline, 24 weeks and 48 weeks. This technology will allow clinicians to tailor antiretroviral regimens in HIV-1 infected individuals based on the predominantly infected cell type.

**Speaker Abstracts, Plenary Session II, Immunology, Microbiology, Virology**  
**ROLE OF FLOW CYTOMETRY IN THE DIAGNOSIS OF**  
**PRIMARY IMMUNODEFICIENCY DISEASE.**

**Maurice R. G. O’Gorman,**

**Northwestern University Medical School and The Children’s Memorial Hospital.**

Primary immunodeficiency disease can be broadly categorized into i/ B cell or Humoral Immunodeficiency, ii. Combined B and T cell immunodeficiency and iii. phagocytic immune deficiency. Currently there are greater than 95 unique primary immunodeficiency diseases (Buckley, NEJM ‘00 and the IUIS, Clin and Exp. Immunol ’99) which fall into these three groups. Many of the diseases in each group will be reviewed in terms of: 1/ The individual entities and their molecular etiologies. 2/ criteria which included flow cytometry in the diagnosis of definite, probable or possible disease as published in the consensus document on the “Diagnostic Criteria for Primary Immunodeficiencies” (Connely et. al. Clin. Immunol. ’99) and 3/ our laboratory’s own experience in the development and utilization of flow cytometry for the diagnosis of primary immune deficiency disease including histograms of specific cases.

**Platform Competition Abstract**

**APPLICATION ISSUES FOR CLINICAL MULTIPLEXING IMMUNOASSAYS OF CYTOKINES**

**C. A. Ray, Eli Lilly and Company, Indianapolis, IN, USA.**

**Purpose:** Quantification of biomarkers can provide important information about the safety and efficacy of candidate drugs. Unfortunately, limited volume of patient specimen and the costs of analytical reagents and labor often preclude determination of multiple biomarkers during drug development. Recently introduced flow-based multiplex immunoassays represent one approach to overcome these limitations. We developed, optimized, validated, and implemented a flow-based multiplex assay for simultaneous measurement of multiple circulating cytokines in patients from a range of clinical trials. This effort was initiated to define the relationship between circulating cytokine concentrations and the natural history and response to therapy for various inflammatory diseases.

**Methods:** Multiplex immunoassays were performed using the Luminex LabMAP instrument. Microspheres provide the solid support for a “sandwich” immunoassay. Cytokine-specific capture antibodies were covalently bound to microsphere subsets and the latter are gated based upon dye ratios. The concentration of the individual cytokine was determined by measuring orange fluorescence produced by a complex of a biotinylated cytokine-specific antibody and streptavidin-phycoerythrin. The validated multiplex immunoassay simultaneously measured the following cytokines: IL-1b, TNF $\alpha$ , IL-6, IL-8, and IL-10.

**Table of analytical results.**

Analyte	Pre-study Validation	In-study Performance		
Interassay Precision (CV)	Recovery (%)	Interassay Precision (CV)	Recovery (%)	
IL-6	5.7-15.9	86.7-99.0	11.3-17.7	96.4-109.0
IL-8	11.2-18.4	83.7-95.3	17.2-24.2	90.8-113.3
IL-1b	7.5-22.3	94.3-107.8	12.5-16.3	97.5-124.6
IL-10	7.5-16.9	88.4-97.4	12.3-18.5	89.8-101.7
TNF $\alpha$	9.3-17.1	97.2-108.8	11.3-13.0	96.6-119.2

The lower limit of quantitation for all assays was 20 pg/mL with the exception of IL-8 which was 100 pg/mL. In a linearity assessment, a 15,000 pg/mL multi-analyte control could be diluted 1:50 and maintain expected accuracy. We measured the cytokine concentrations in more than 2000 serum samples from patients with sepsis.

Results for IL-6 were compared to a conventional commercially available ELISA kit. The two methods were judged to perform equivalently ( $r^2$  value of 0.952 and a slope of 0.512).

**Conclusions:** The flow-based multiplex format provided simultaneous measurement of circulating cytokines using 20% less patient specimen compared to traditional approaches and at a significantly decreased cost. Efficient use of this platform requires process improvements in the form of an integrated sample management, data reduction, and data reporting device to fully maximize the positive impact of multiplex assays in clinical drug development.

### Platform Competition Abstract

#### **HYPOCALCEMIA BLUNTS IMMUNE RESPONSES DEPENDENT ON RELEASE OF INTRACELLULAR $Ca^{2+}$ ( $[Ca^{2+}]_i$ ) STORES**

**Kayoko Kimura, Jesse P. Goff, Timothy A. Reinhardt.**

**National Animal Disease Center, Ames, Iowa**

2

Plasma Ca often decreases in dairy cows at parturition. We hypothesized that hypocalcemia affects  $[Ca^{2+}]_i$  stores and therefore blunts the  $[Ca^{2+}]_i$  response for activating stimuli in immune cells. Using blood samples from Jersey cows,  $[Ca^{2+}]_i$  stores were estimated by analysis of  $[Ca^{2+}]_i$  released after stimulation by pervanadate and ionomycin in the absence of extra-cellular Ca.  $[Ca^{2+}]_i$  was measured by ratiometric analysis of Fluo-4/Fura-Red ratios using flow cytometry.  $[Ca^{2+}]_i$  response to stimuli tended to decrease as calving approached, but significantly increased following calving.  $[Ca^{2+}]_i$  stores followed a similar pattern suggesting that the hypocalcemia in periparturient dairy cows may reduce  $[Ca^{2+}]_i$  stores of immune cells, reducing their ability to respond to activating stimuli.

### Platform Competition Abstract

#### **ROLLING CIRCLE AMPLIFICATION – A NEW APPROACH TO INCREASE SENSITIVITY FOR FLOW CYTOMETRY.** **Arumugham Raghunathan, Martin Sorette, Harley Ferguson Jr., and Vanessa Wheeler.** **Molecular Staging, Inc., 300 George Street, Suite 701, New Haven, CT 06510.**

Rolling circle amplification (RCA) generates a localized signal via an isothermal amplification of an oligonucleotide circle. The application of this approach to flow cytometry could extend the utility of this and various other methods to include a more complete set of immunological and molecular probes. RCA-mediated signal amplification has been successfully applied to the sensitive and specific detection of a variety of cell surface antigens (CD3 and CD4) within a variety of cells. RCA technology is a robust and simple procedure that can provide a universal platform for the localization of a wide variety of molecules as a function of either antigenicity or nucleic-acid sequence. The use of RCA in this way could make use of the current wealth of antibodies that are not being used due to lack of sensitivity, as well as permit the integration of information emerging from genomics and proteomics into cell- and tissue-based analyses.

**Keynote Address:**

**GENOMIC INSTABILITY, CYTOMETRY AND PROGRESSION TO GASTROINTESTINAL  
CANCER**

**Peter S. Rabinovitch, University of Washington, Seattle, WA.**

Patients with long-standing gastrointestinal (GI) diseases such as Ulcerative Colitis (UC) and Barrett's Esophagus (BE) are at increased risk for developing adenocarcinoma in the affected organ. Our work, and that of others, has previously shown that gross genomic instability, as evidenced by DNA aneuploidy and/or elevated 4N fractions, usually precedes the development of cancer. These flow cytometric findings can be used to detect a subset of patients that are at increased risk of cancer, and can therefore be provided more intensive clinical surveillance. Recently, it has become clear that earlier stages of these GI diseases are associated with more subtle evidence of genomic instability. This can be detected by application of interphase FISH, which shows that the extent of this instability can be very widespread. One mechanism of chromosomal instability arises as a consequence of telomere shortening; the loss of the protective telomeric sequence on chromosome ends permits end-end chromosome fusion, with subsequent chromosome breakage at mitosis. To study telomeres in GI epithelium we have used *in situ* quantitative FISH using peptide nucleic acid telomere probes and confocal microscopy. Telomere shortening is found in non-dysplastic epithelium in both UC and BE. In UC, the extent of telomere shortening is directly correlated to the extent of chromosomal instability, the first time this has been demonstrated in a human tissue. These results suggest that dysplasia and cancer in UC and BE arise through a process of genomic instability, and that cytometric methods can be used to detect this instability and may be useful in assessing and managing patient risk.

---

**Speaker Abstracts, Plenary Session III**

**MULTIPARAMETRIC CHARACTERIZATION OF SOLID TUMOR MICROENVIRONMENTS**

**Vojislav Vukovic, Toronto, Ontario**

Multiparameter imaging of large tissue areas/volumes at microscopic resolutions is uniquely suited for characterization and quantification of molecular processes at individual cell, tissue and organ levels, thus bridging the existing gap between microscopy and non-invasive imaging (e.g., ultrasound, CT, NMR). Biomedical applications of this technology include structure/function relationship analysis, study of gene expression profiles in preserved tissue context, micropharmacokinetic and micropharmacodynamic studies, and establishment of surrogate clinical drug efficacy endpoints.

Characterization of heterogeneous solid tumor microenvironments will be presented as an illustration of the concept and implementation of wide field multi-parameter fluorescence microscopy. Tumor oxygenation status was determined using the nitrimidazole EF5, an established hypoxia marker, and by immunostaining of the intrinsic hypoxia marker Hypoxia-Inducible Factor 1 alpha (HIF-1 $\alpha$ ) in cervical carcinoma xenografts. The relationship of vasculature and tumor oxygenation was studied by mapping tumor hypoxia as a function of distance to blood vessels. As an example of adaptive tumor responses to hypoxia, tissue non-protein sulfhydryl (NPSH) levels were determined using spatial image mapping techniques and semiquantitative colocalization analysis. Micropharmacokinetic and micropharmacodynamic properties of Buthionine Sulfoximine, a potent glutathione *de novo* synthesis inhibitor, were analyzed in relation to tumor oxygenation.

**Speaker Abstracts, Plenary Session III**

**BREAST DUCTAL LAVAGE**

**Carleton C. Stewart, Jan Hoffmann, Steven Edge\*, Deborah Wirtfeld\***  
**Laboratory of Flow Cytometry and Department of Surgery\*,**  
**Roswell Park Cancer Institute, Buffalo, New York**

The evaluation of solid tumors by flow cytometry has been difficult and results are often quite variable among laboratories primarily because of the need to dissociate cells. This process can result in sampling errors where the specimen is biased in unknown ways. Flow cytometry is the method of choice, however, for any specimens wherein the cells are naturally dispersed. In patients with high risk for breast cancer or those with active disease, fluid within the ducts are laden with cells that can be captured for analysis by lavage. In preliminary investigations lavage specimens from the contralateral breast of six patients with active breast cancer were collected for flow cytometric evaluation. Using a combination of CD45 and anti-cytokeratin 8,18 and utilizing a single platform counting procedure, the yield of cells ranged from a low of 9000 to over  $5 \times 10^6$  with a median of  $10^5$ . Four populations could be found, one population composed of lymphocytes (CD45+CK-), one of epithelial cells (CD45-CK+), one of probably stromal cells (CD45-CK-) and one of dead cells. The frequency of each population was highly variable and ranged from a dominance of leukocytes to a dominance of epithelial cells. These results have led us to develop a more extensive antibody combination to better characterize the specimens.

Supported by the Roswell Park Cancer Institute Alliance

---

**Commercial Talks, Sunday October 15**

**Beckman-Coulter: "Updates in Beckman Coulter Flow Cytometry Automation: The PrepPlusII and CellPrep", presented by Jeff Clapper**

No abstract

**Becton Dickinson: "The Latest Information on New Product Releases from Becton-Dickinson", presented by Ken Murchison, San Jose, CA**

No abstract

**Verity Software House: "And now, for something completely different...", presented by Mark Munson, Topsham, ME**

Some time ago, Verity began a comprehensive effort to explore the underlying reasons for the literature-reported variability regarding the prognostic importance of DNA ploidy and S-phase for breast cancer. This project was a collaborative effort, involving many US and European laboratories and resulting in a publication outlining a series of ten adjustments to obtain significant and reproducible results. Before undertaking this study, Verity needed to establish an analysis strategy that would allow different operators modeling that same DNA histograms to get the same results. This was not a trivial undertaking. The result was a set of very specific DNA analysis rules. Our new Rule Based Training System is designed to teach others this new method of DNA analysis.

### COMPARISON OF CELLULAR IMMUNE RESPONSE INDUCED BY ATTENUATED AND WILD-TYPE PRRS VIRUS

**Janutenaite, J.<sup>1</sup>, Lager, K.<sup>2</sup>, Stabel, T.<sup>2</sup>, Pesch, B.<sup>2</sup> Brockmeier, S.<sup>2</sup>.**

<sup>1</sup>National Veterinary Laboratory, Vilnius, Lithuania, <sup>2</sup>National Animal Disease Center, USDA-ARS, Ames, Iowa

An attenuated porcine reproductive and respiratory syndrome virus (PRRSV) and its wild-type parent virus (strain NADC-8) were given to 4-5 week-old-pigs free of PRRSV and PRRSV-specific antibody. Experiment design: a sham-inoculated control group, an attenuated virus-inoculated group and a wild-type parent virus inoculated group. Pigs were inoculated intramuscularly with their respective virus (about  $2 \times 10^5$  TCID<sub>50</sub> virus) on day 0. Blood samples were collected on days -7, -4, 0, 3, 6, 9, 13 and 20. A panel of monoclonal antibodies was used to analyze peripheral blood leukocytes by two-color flow cytometric analysis. The percentages of CD8+ and CD3+ T cells was significantly increased on days 6 to 13 and on day 9 for the wild type virus group, respectively. After an initial decrease, the percentage of cells expressing Class II marker increased with time. Total white blood cell counts decreased shortly after infection then increased with time. No significant changes were found in peripheral blood leukocytes of pigs inoculated with attenuated PRRSV or sham inoculum. Results indicate that the peripheral blood leukocytes response to PRRSV infection mainly involves CD8+ T cells.

### DETECTION OF EPSTEIN-BARR VIRUS LATENTLY INFECTED B CELLS BY A FLOW CYTOMETRY BASED FISH ASSAY

**M Paniagua\*, E Lopez Presas, L Peterson, and C Goolsby.**

Northwestern University Medical School, Robert H. Lurie Comprehensive Cancer Center, Chicago, IL.

Epstein-Barr virus (EBV) latent infection has been implicated in the pathogenesis of a number of B cell lymphoproliferative disorders and B-cell lymphomas in the context of immunosuppression including post transplant lymphoproliferative disorders (PTLD) as well as HIV related lymphomas. A flow cytometry-based fluorescence in situ hybridization assay (FISH) for the detection of EBV latently infected B cells was characterized using a cell line model system. EBV latently infected B cell lines (Raji, B95-8, Nawalma), an EBV negative B cell line (Ramos), and EBV negative peripheral blood mononuclear cells (PBMC) were employed. A 5-probe oligonucleotide cocktail (Stowe et al, J Virol Meth 75: 83, 1998) having homology to non-overlapping regions within the EBV nuclear RNA transcript, EBER1, was employed. Each 20 basepair probe was labeled with 5(6)-carboxyfluorescein and hybridization followed that of Patterson et al (Cytometry 31:265, 1998). Mixing experiments were performed by spiking varying concentration of Raji cells into either EBV- Ramos cells or into EBV seronegative PBMC. A greater than 50-fold increase in hybridization fluorescence intensity was seen in the FISH results for the Raji (EBV positive) cells as compared to the EBV-cells, either Ramos or PBMC. The mixing experiments demonstrated the sensitivity for detecting EBV+ cells to be <0.01%. Preliminary application of these techniques to patient material has been done.

Analysis of an involved lymph node from an EBV+ lymphoma patient demonstrated the clonal B cells to be EBV+, however, the polyclonal B cells also present were EBV-. In analysis of PBMC from 7 PTLD patients (peripheral blood not involved), no EBV+ B cells were seen. This flow cytometry based FISH assay provides a rapid and specific method for detecting and quantifying EBER1+ B cells with a sensitivity of <0.01% positive cells in a heterogeneous sample. This may provide a useful tool to evaluate EBV+ cells in blood, lymph node, or other tissues in patients with EBV related lymphomas.

**FLOW CYTOMETRIC METHOD TO MEASURE ACTIVATION, PROLIFERATION, APOPTOSIS  
AND DEATH OF BOVINE T CELLS**

**T.E. Rahner, D. Cheng, M.V. Palmer, D.L. Whipple, W.R. Waters;  
National Animal Disease Center and Iowa State Univ., Ames, IA**

5

The goal of this study was to develop an assay to concurrently examine phenotype, activation, proliferation, apoptosis and death of antigen- or mitogen-stimulated bovine lymphocytes. Peripheral blood mononuclear cells (PBMC) were isolated from either non-infected or *Mycobacterium bovis*-infected calves. Once isolated, PBMC were labeled with PKH67 dye and cultured with no stimulation, *M. bovis* purified protein derivative or pokeweed mitogen. Cells were evaluated for activation (CD62L, CD44, CD25), phenotype (CD4, CD8,  $\gamma\delta$ TCR), apoptosis (Annexin V and 7AAD), viability (7AAD) and proliferation (PKH67). Upon stimulation the mean fluorescence intensity (mfi) of CD62L decreased whereas the mfi of CD44 and CD25 increased on CD4<sup>+</sup> cells within the live gate. In general, activation marker expression was negligible on Annexin V<sup>+</sup> cells. This assay allows for a more detailed determination of the cellular immune response of cattle.

**LONG-TERM RETENTION OF ANTIGEN IN FIXED CELLS.**

**Phyllis S. Frisa and James W. Jacobberger.**

**Cancer Research Center, Case Western Reserve University, Cleveland OH**

6

A primary goal of fixation is to preserve cellular antigens quantitatively; a secondary goal is to maintain that level during storage. Antigens we fixed in formaldehyde/methanol and kept at -20°C had decreased immunofluorescence with time as measured by cytometry. We have quantified this loss, characterized it and attempted to circumvent it.

We examined SV40 large T antigen (Tag), cyclin B1 and vimentin. Tag began to degrade by 13 weeks at -20°C. This change was seen by cytometry and by Western blots using extracts of the fixed cells. The rate of loss of cyclin B1 was similar to Tag while vimentin degraded more slowly. Degradation was not slowed by the addition of protease inhibitors or DTT. Mg, NaCl and EDTA promoted degradation. Western blots of fixed cell supernatants indicated that diffusion from fixed cells is not a major mechanism of loss.

Lyophilization of the fixed cells appears to be a feasible means of preserving antigen levels. Our data on long term stability of the lyophilized cells are still tentative but promising.

**CD95 EXPRESSION AND FAS LIGAND INDUCED APOPTOSIS OF JURKAT T CELLS AND  
HUMAN LYMPHOCYTES.**

**Lisa Green and Philip Marder. Lilly Research Laboratories, Indianapolis, IN.**

7

Apoptosis of peripheral blood lymphocytes is thought to be an important mechanism of immune regulation. The Fas Receptor/ Fas Ligand expression system is believed to regulate uncontrolled expansion of specific antigen reactive lymphocytes by triggering lymphocyte apoptosis. Activated lymphocytes may express both FasR and FasL on their cell surface. Triggering the apoptotic pathway requires the cross-linking of FasR with either FasL (soluble or membrane-bound) or with antibodies to FasR. In the present study we examined the relationship between CD95 (FasR, APO-1) expression of Jurkat T cells and peripheral blood lymphocytes and susceptibility to FasL-induced apoptosis. We probed for apoptosis using intracellular immunostaining for active caspase 3. We found that CD95 was constitutively expressed at high levels on Jurkat cells and was upregulated on lymphocytes following overnight incubation with a combination of anti-CD3 and anti-CD28 or with phytohemagglutinin. Our results indicated that whereas Jurkat cells were highly sensitive to FasL-induced apoptosis, CD95 upregulation on human lymphocytes was not sufficient for priming FasL-induced apoptosis. Longer culture periods increased susceptibility of lymphocytes to FasR/FasL-triggered apoptosis. This suggests that additional factors cooperate for regulation of human lymphocyte apoptosis.

**TGF $\beta$  CYTOKINE NETWORK ALTERATIONS IN B-CLL: OVER-EXPRESSION OF TGF $\beta$ RII ASSOCIATED WITH DECREASED SPF IN PATIENT T CELLS.**

**J.Moore, A.Bantly, M.Zaki, Dept. of Pathology, Univ. of PA, Philadelphia, PA.**

8

B cell chronic lymphocytic leukemia (B-CLL) is a multifactorial disease where defects in cytokine networks may contribute to the progression and complication of the malignancy. We have focused on the role of the elevated levels of active TGF $\beta$  produced by CLL B cells. Because the CLL B cells are resistant to the apoptotic effects of TGF $\beta$ , we asked whether the target of action of this tumor-produced cytokine might be regulatory cells in the tumor environment. TGF $\beta$  can induce growth arrest in mid/late G1 phase of the cell cycle in lymphocytes, so we investigated the ability of T cells from CLL patients to enter the cell cycle following in vitro stimulation with anti-CD3 and IL-2. We found a significantly lower level of s-phase fraction (SPF) as compared to T cells from normals (41+/-6 vs 17 +/-3, p<0.0001). Hypothesizing, that this might result from enhanced response of the CLL T cells to the tumor-produced TGF $\beta$ , we measured the level of TGF $\beta$ RII on the CLL T cells. When compared to normal T cells, we demonstrated significantly higher levels of TGF $\beta$ RII on the T cells from CLL patients (p<0.001). These results suggest that the significantly lower SPF of the CLL T cells may be related to the elevated level of TGF $\beta$ RII on these T cells making them more responsive to cell cycle inhibition by active TGF $\beta$  produced by malignant B cells. This could lead to multiple alterations in T cell function affecting both the expansions of the tumor and the host immune environment.

**FLOW CYTOMETRIC IMMUNOPHENOTYPING TEST FOR STAGING/MONITORING NEUROBLASTOMA PATIENTS**

**MJ Warzynski\*, DM Graham, RA Axtell, JV Higgins, YA Hammers  
Spectrum Health, Grand Rapids, MI, 49503.**

9

About four years ago we made an incidental flow cytometric observation while immunophenotyping lymph node/marrow samples from children suspected to have leukemia/NHL which were subsequently proven to have neuroblastoma. The patients' sample contained neoplastic CD45<sup>-</sup> cells that had an extremely bright CD56<sup>+</sup> (beyond fourth decade) population distinguishable from CD45<sup>+</sup> CD56<sup>"usual density"</sup> NK lymphocytes as well as other CD45<sup>-</sup> CD56<sup>"usual density"</sup> non-hematopoietic tumors such as small cell carcinoma or melanoma. Following the "rare event" philosophy of selecting one negative and two positive antigens, we initially tried an unsuccessful "cocktail" of CD45<sup>-</sup> CD56<sup>very bright+</sup> neuron specific enolase<sup>cytoplasmic+</sup> which we switched to a more applicable "lysed whole blood" CD45<sup>-</sup> CD56<sup>very bright+</sup> ganglioside GD2<sup>+</sup> "cocktail" to increase the specificity and sensitivity of the assay. The poster will compare the I.S.S. stage, histologic subtype, urine catecholamine levels, karyotype, N-MYC amplification, and immunophenotyping results of the patients. The "three-color" GD2 assay has successfully detected cells in marrow samples down to 0.002% (1 per 10<sup>6</sup> cells) using patient samples (not artificially spiked material). We now use this "rare event" clinical test to help stage and monitor all patients with neuroblastoma.

**Poster Abstracts:**

**D. V. Soni and J. W. Jacobberger**

**School of Medicine and Cancer Center, Case Western Reserve University, Cleveland, Ohio.**

G2/M transit is regulated by cdk1-cyclin B1 complex (maturation promoting factor/MPF). Cdk1 is in excess throughout the cell cycle while the level of cyclin B1 rises in S and peaks at G2/M transit. The current G2/M transition model is that cdk1 initiates mitosis at a threshold activity set by the level of cyclin B1. The aim of this study was to determine if (i) average and threshold cyclin B1 levels vary as a function of G2 rate (ii) MPF activity covaries with cyclin B1 level. Methods: HeLa cells were grown asynchronously under conditions designed to manipulate cell cycle time without activating cell cycle checkpoint controls. Cyclin B1 content of cells was measured by flow cytometry and western blot. MPF activity was quantified by histone H1 kinase assay. Results: Cyclin B1 content varied as a function of cell density and serum. The average and threshold levels of cyclin B1 were proportional to G2 time. The MPF activity covaried with cyclin B1 level. Conclusion: The threshold activity for transition from G2 to M appears to be a variable function.

### **MULTIPARAMETRIC AND DNA ANALYSIS OF CORE BIOPSIES**

**Megan Gottlieb, James Jacobberger**

**Case Western Reserve University School of Medicine, Cleveland, OH 44106**

**11**

Core biopsies from cancer patients provide small amounts of tissue for processing single cell suspensions for cell cycle and apoptosis measurements. We wished to test whether processing inflammatory breast core biopsies would provide a sufficient number of intact cells for DNA and multiparametric analysis through flow cytometry.

Methods- Core biopsies from inflammatory breast patients were segmented and snap frozen in liquid nitrogen and then transferred to -80° C freezer. Tissue fractions were again segmented at approximately 1mm in size, and were incubated in Purified Collagenase. RPMI was added, and the samples were disaggregated by pressing tissue through a 150um metal screen. To clear the mesh, a small amount of RPMI was added, and the samples were gently resuspended. Samples were washed with PBS and then resuspended in PBS and fixed with MeOH. After fixation, the samples were washed with PBS, PBS-BSA and incubated with anti-cytokeratin monoclonal antibody. Following incubation, the samples were washed with PBS-BSA. The final pellet was resuspended in PBS with RNase A. After a short incubation period, propidium iodide was added. DNA and cytokeratin content were measured through flow cytometry. Negative controls for DNA ploidy and cytokeratin were obtained through the use of normal peripheral blood mononuclear cells. DU145 cells were used as cytokeratin positive controls. DNA modeling was performed on all events.

Results- Of forty-six samples obtained, thirty-seven were processed for flow cytometry. Thirty of the samples provided sufficient cell for analysis (1000 or more detectable events). Through repeat analysis, it was shown that reproducibility was adequate. There is, however, a substantial amount of sample to sample biological variation, e.g. tumor heterogeneity.

Conclusion- Multiparametric analysis of core biopsies is possible, and the results are reproducible. We can detect cytoplasmic cytokeratin, which leads us to believe that nuclear markers and other proteins can also be detected. In a clinical setting, measurement of apoptosis and quantitation of tumor cells through flow cytometry would be useful.