

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

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

### THE EIGHTH ANNUAL FLOWDOWN

“CRAZY EIGHT TACKY PARTY”

THE PARK EAST HOTEL

MILWAUKEE, WISCONSIN

OCTOBER 1 - 3, 1999

visit our web site:

<http://www.cyto.purdue.edu/flowcyt/glifca/gliifca.htm>.

Local Site Organizer: Wendy Kopinski

Meeting Organizers: Alex Nakeff, Sigie Stewart

Program Chairs: Jonni Moore,

Kathy Muirhead and Betsy Ohlsson-Wilhelm



#### CORPORATE MEMBERS

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(100 years of Cancer Treatment)

GLIFCA EIGHT – OCTOBER 1 – 3, 1999 PARK EAST HOTEL, MILWAUKEE, WI - PROGRAM

**FRIDAY Evening - October 1**

Registration	4 pm – 11 pm
Reception/Exhibitor Session/Poster Set-Up	8 pm – 11 pm

**RECEPTION SPONSORED BY BECKMAN COULTER CORPORATION**

**SATURDAY - October 2**

Breakfast	7:30 – 8:30 am
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**PLENARY SESSION I**

(times include 5 minutes for questions)

**Current Applications- What are we doing now?**

**Chair – Betsy Ohlsson-Wilhelm**

**Welcoming Remarks: Carl Stewart**

8:30 – 8:35 am

**Featured Speaker: Phil McCoy**, Camden, NJ, “The Dog in Dogma”

A discussion of what has changed in our approach to clinical flow cytometry

8:35 – 9:10 am

**Plenary Talks:**

“ASRs” – **Helene Paxton**, Baltimore, MD

9:10 – 9:35 am

“How to Do Four Color Compensation” – **Carl Stewart**, Buffalo, NY

9:35 – 10:00 am

“Immunofluorescent Standardization in Flow Cytometry: 3 applications”

– **Lori Charie**, Miami, FL

10:00 – 10:25 am

**Coffee Break**

**SPONSORED BY CALTAG**

10:25 – 10:40 am

“Single vs. Dual Platform Absolute Counting” – **Michele Bergeron**, Ottawa, Ontario

10:40 – 11:05 am

“Principles of Quantitation in Image Analysis: Practical Considerations for Drug Discovery”

– **Mike Esterman**, Indianapolis, IN

11:05 – 11:30 am

**Platform Presentations**

“ TGFβ/IFNγ Affect Survival of CLL B Cells by Multiple Pathways”

– **Mohamed Zaki**, Philadelphia, PA

11:30 – 11:45 am

“Effect of LY335979 on Efflux Activity of AML Bone Marrow Specimens”

- **Lisa Green**, Indianapolis, IN

11:45 – 12:00 pm

**Luncheon Roundtables**

**SPONSORED BY PHARMINGEN**

12:00 – 1:30 pm

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

Sign up for the following Luncheon Workshops at the Registration Desk: **Core Facility Management**, co-chairs, Kristi Harkins, Julie Auger, **Platelet Flow Cytometry**, co-chairs, Vince Shankey, Phil Marder, **Cell Cycle Regulation and Apoptosis**, co-chairs, Jake Jacobberger, David Hedley, **Cell Sorting and Instrument Maintenance**, co-chairs, Hank Pletcher, Kathy Schell, **Cytokine Networks**, co-chairs, Jonni Moore, Mohammed Zaki, **Image Analysis**, chair, Paul Robinson, **Leukemia/Lymphoma**, chair, Carl Stewart, **Clinical Cytometry**, co-chairs, Phil McCoy, Chuck Goolsby

**PLENARY SESSION II**

(times include 5 minutes for questions)

**Emerging Applications–What is just around the bend?**

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

**Featured Speaker: -**

**Carolyn Kever-Taylor**, Milwaukee, WI “Progenitor Cell Graft Analysis”

1:30 – 2:05 pm

**Plenary Talks:**

“HIV Detection by in situ hybridization” – **Victoria L. Mosiman**, Mountain View, CA

2:05 – 2:30 pm

“Quantitation of Donor and Recipient Cells in Bronchial Lavages from Lung Transplant Patients” - **Lynn DiVito-Haynes**, Madison, WI

2:30 – 2:55 pm

**Coffee Break**

**SPONSORED BY PHARMINGEN**

2:55 – 3:15 pm

**Platform Presentation:** Flow Cytometric Intracellular Ionized Calcium Analysis of Peripheral Blood Mononuclear Cells in Periparturient Dairy Cattle - **Kayoko Kimura**, Ames, IA

3:15 – 4:00 pm

“Involvement of Ca<sup>2+</sup> Compartmentalization in Thapsigargin-induced Apoptosis of Human Colon Carcinoma SW 620 Cells” - **Tao Fu**, Chicago, IL

4:00– 4:15 pm

**Plenary Talks:**

“PKH2 for Detection of Antigen Specific Lymphocyte Proliferation” - **Ray Waters**, Ames, IA

4:15– 4:40 pm

“Application of Laser Scanning Cytometry for the Evaluation of DNA Ploidy in Routing Cytologic Specimens” – **Eva Wojcik**, Maywood, IL

4:40 – 5:05 pm

“Instrumentation, Reagents, Standards: What is coming? - **Alan Stall**, San Diego, CA

5:05 – 5:30 pm

**Wine and Cheese Happy Hours & Poster Session in Exhibit Area**

**5:30 – 7:30 pm**

**SPONSORED BY CYTOMATION, SPHEROTECH AND VERITY SOFTWARE HOUSE**

**“TACKY PARTY” FLOWDOWN**

**8:00 - 12 am**

**SPONSORED BY BECTON DICKINSON BIOSCIENCES**

**SUNDAY - October 3**

**Steering Committee Meeting**

**7:30 – 9:00 am**

**Breakfast**

**8:00 – 9:00 am**

**PLENARY SESSION III**

**(times include 5 minutes for questions)**

**Future Applications- What is on the Horizon?**

**Chair, Kathy Muirhead**

**Featured Speaker: – Carl June, Philadelphia, PA,**

**9:00 - 9:35 am**

“Cell and Gene Therapy: Where are We on the Road from Hype to Hope?”

**Plenary Talk:**

“Analysis of Ad-p53 Mediated Gene Therapy in Ovarian Carcinoma”

- **Jake Jacobberger, Cleveland, OH**

**9:35 – 10:00 am**

**Platform Presentations:**

“A Role For Cell Division in T Cell Fate Decisions?” - **Andrew D. Wells, Philadelphia, PA**

**10:00 – 10:15 am**

“Use of Primary Hepatocytes And Hepatoma Cells to Assess Drug-Induced Intracellular

Oxidative Stress by Flow Cytometry”, - **Donna M. Williams, King of Prussia, PA**

**10:15 – 10:30 am**

**Coffee Break**

**SPONSORED BY PHARMINGEN**

**10:30 – 10:50 am**

**Plenary Talk:**

"Wide Field, Multicolor Fluorescence Image Analysis Techniques for Studying

Functional Cancer Biology" – **David Hedley, Toronto, Ontario**

**10:50 – 11:15 am**

**Panel Discussion – “Getting it all Together: How to Avoid Information Overload (data management)”**

Chair – **Paul Robinson, Indianapolis, IN**

**11:15 – 11: 50 am**

**AWARD/ TRAVEL STIPEND PRESENTATIONS**

**11:50 – 11:55 am**

**SPONSORED BY FLOW CYTOMETRY STANDARDS CORPORATION,**

**Closing Remarks, Carl Stewart**

**11:55 - 12 noon**

**STEERING**

**Canada**

**David Hedley, Frank Mandy**

**Illinois**

**Julie Auger, Charles Goolsby,**

**Maurice R. G. O’Gorman, T. Vincent Shankey**

**Indiana**

**J. Paul Robinson, Philip Marder**

**Michigan**

**Bruce Davis, Alexander Nakeff**

**Minnesota**

**Waclaw Jaszcz, Wendy Walters**

**New York**

**Zbigniew Darzynkiewicz, John M. Lehman,**

**Carleton C. Stewart**

**Ohio**

**Charles L. Hitchcock, James W. Jacobberger**

**Pennsylvania**

**Jonni Moore, Katharine Muirhead,**

**Stanley E. Shackney**

**Wisconsin**

**Wendy Kopinski, Kathleen Schell**

**COMMITTEE**

**CLINICAL FLOW CYTOMETRY: THE DOG IN DOGMA**

**J. Philip McCoy, Jr., Ph.D.**  
**Cooper Health System**  
**RWJ-Univ. of Medicine & Dentistry of N.J.**  
**Camden, NJ**

**Abstract**

As we focus our attention on the practice of our profession in the next millennium, it is appropriate to reflect on the evolution of clinical flow cytometry over the past several decades. Beginning as a technological achievement research laboratories, flow cytometry quickly demonstrated tremendous potential for application in the clinical laboratory. This application was a learning process starting from square one. Not only did the demanding technical aspects of flow cytometry instrumentation need to be adapted to the clinical laboratory, but also entirely new areas of expertise, such as in depth knowledge of monoclonal antibodies and fluorochromes, needed to be acquired. As with any continually evolving process, the standards, or dogma, change as more knowledge is acquired. In clinical flow cytometry one might consider examples of changing dogma such as: density gradient separation of specimens prior to analysis; isotype controls; light scatter gating; DNA analysis of paraffin-embedded tissue; analysis of partially nonviable specimens; and more. Other concepts have changed very little over several decades and remain the gold standard today. This lecture will review the changing standards of practice in clinical flow cytometry. Clearly standardization and consensus guidelines continue to be needed in this field, but so does the need to challenge the dogma as our knowledge and technology evolves.

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**SUCCESSFUL USE OF ASR'S IN CLINICAL DEVELOPMENT FOR IVD'S**

**Helene Paxton, MS, M.T.(ASCP)**  
**Baltimore MD 21227**

Analyte Specific Reagents: Are they an Effective Solution to the Regulatory Dilemma?

There are 3-GENERAL CATEGORIES:

Commercial (FDA cleared/approved), for Research Use, and for Investigational Use

WHY THE NEED FOR AN ASR RULE?

- Many reagents placed into RUO category with the advent of monoclonal antibodies.
- FDA concerned with the lack of regulatory authority over the use and manufacturing of these reagents.
- Manufacturer's concerned about the expense of Class III reagents clearance through PMA's. Definition of "intended use" sometimes cloudy.
- Laboratories concerned over the term "research use" and the impact on Medicare and insurance reimbursement.
- Impact of Biotechnology with the development of nucleic acid hybridization technologies and new diagnostic markers made increased demand for novel tests by the medical community.
- "Home brew" practice left many labs facing inspection hurdles under CLIA-88, subpart K.

Before the ASR regulation companies responded to the needs of the medical community with IUO and RUO labeling and...

- This led to overt commercialization and misleading claims  
FDA'S RESPONSE WAS THE 1992 draft Compliance Policy Guide (CPG) which....
  - Stated FDA's intention to require pre-market applications for all applicable RUO/IUO reagents within 30 months and increased enforcement scrutiny.
  - Did create some exceptions for medically critical RUO/IUO in wide use. So.....REACTION to the CPG CAUSED GREAT CONCERN TO THE.....MEDICAL COMMUNITY and to the IVD INDUSTRY.

The industry wanted to provide product to its clients, but faced incredible regulatory hurdles.

BETWEEN THE '92 CPG AND THE '97FDA MODERNIZATION ACT and the new ASR rule, the IVD industry and the Medical Societies worked through existing regulations for down classification of certain Classes of reagents, such as the Immunohistochemistry (IHC's). Clearly the benefits of this approach led to better communications between FDA, the IVD industry and the end users of the products. The ASR regulation resulted from these interactions...

Went into effect November 28th, 1998

- is defined as:"antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reaction with substances in a specimen, are intended for use in a Diagnostic application for the identification and quantification of an individual chemical substance or ligand in biological specimens."

**HOW TO DO FOUR COLOR COMPENSATION**

**Carleton C. Stewart**

**Roswell Park Cancer Institute**

**Buffalo, NY 14263**

**Abstract**

Currently, there are two procedures for performing Four-Color Immunophenotyping. In the first method, introduced by Beckman-Coulter, a single laser is used to excite FITC, PE, PE-TR and PE-CY5 or PerCP. In the second method, introduced by Becton Dickinson Biosciences, two lasers are used. The first excites APC or CY5 and the second excites FITC, PE and PE-CY5 or PerCP. In both approaches, tandem complexes of either PE-TR or PE-CY5 are used. While Beckman Coulter is currently the only supplier of antibodies directly conjugated with PE-TR (called ECD), there are several companies that supply PE-CY5 antibody conjugates. These reagents exhibit a high degree of inter- and intra-batch variation in the amount of PE leakage from the construct. This requires that each fluorochrome-antibody conjugate must be individually compensated to prevent acquisition of erroneous data.

In both systems, it is also very easy to either over- or under-compensate the instrument because single labeled particles are used and this is inadequate to obtain the correct compensation. By using a bright PE conjugated antibody like PECD8, a bright PE-TR antibody like PE-TR (ECD) CD4 and a dim PE-CY5 antibody, such as PE-CY5-CD2, for labeling lymphocytes, correct compensation can be verified between the separate CD8+ and CD4+ populations. Since both are CD2+, and there is an additional population that is CD2+ CD4- CD8-, correct compensation between PE, PE-TR and PE-CY5 can be verified. For the dual laser system, compensation between FL3 and FL4 is also very sensitive to over- or under-compensation. A very bright APC reagent, such as CD45, can be combined with a PE-CY5 conjugated Ab, such as CD8 and with a PE conjugated antibody such as CD4, to verify the correct compensation.

Because of the variation in CY5 excitation in the PE-CY5 construct (or PerCP) by the diode laser, it is also necessary to compensate each PE-CY5 conjugated antibody uniquely. To perform this task conveniently, we recommend using software compensation for FL2-%FL3 and FL4-%FL3. Careful attention to the details of compensation can provide reliable data for any combination of fluorochromes using any instrument to measure 4-colors of fluorescence. Supported by CCSG # 5P30CA16056-22 and 5RO1CA602006 from NCI.

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**IMMUNOFLUORESCENT STANDARDIZATION IN FLOW CYTOMETRY: 3 APPLICATIONS**

**Lori Charie**

**Beckman Coulter Corporation**

**Miami, FL**

**Abstract**

Recognizing the growing need for standardization in flow cytometry, several companies have developed and market particles for instrument setup and calibration. Manufacturers of flow cytometers, such as Becton Dickinson Immunocytometry Systems and Coulter Corporation, and reagent manufacturers like Flow Cytometry Standards Corporation have all developed their own commercial standards for instrument setup. The development of the CELLQUANT technology, a powerful approach that is targeted towards low density expressed antigens, heralds the second generation of BioCytex products. The calibrator is unique for each application and the measuring scale is designed to reflect the expression level of the antigen of interest. Three applications of this technology are demonstrated in the measurement of CD38 on CD8+ cells in HIV, CD55/CD59 expression on erythrocytes and granulocytes in PNH, and GpIIb/IIIa receptor occupancy on platelets.

**A UNIVERSAL TEMPLATE FOR SINGLE PLATFORM METHODOLOGY**

**Michèle Bergeron, Tao Ding, Sieglinde Phaneuf, Tracy Minkus, Francis Mandy. National Laboratory for Analytical Cytology, Bureau of HIV/AIDS, STD & TB, Laboratory Center for Disease Control, Health Protection Branch, Health Canada, Ottawa, Canada**

**Abstract**

The National Laboratory of Analytical Cytology provides a Quality Immunophenotyping Assessment Program (QAP) for the laboratories of the Canadian HIV trials network (CTN). Its primary role is to ensure accurate T-cell subset measurements and facilitate transfer of biotechnologies to provide the best available surveillance data.

Traditionally, the method for reporting an absolute cell count for T cell subsets relied on the combination of two technologies. The results from both the hematology analyzer (absolute lymphocyte count) and the flow cytometer (cell subset lymphocyte percentage) were combined for the enumeration of T cell subsets. This is what constituted the double platform technology. Two years ago, manufacturers began to market a retrofit technology that allowed the reporting of absolute numbers directly on the flow cytometer: the single platform methodology was born.

The first objective of this study was to ensure minimal inter-laboratory variation for reporting both relative and absolute cell count by the single platform methodology. The second goal was to develop a universal template to assist the CTN with the implementation of this new technology.

The universal template was adapted to the clinical environment with regard to instrumentations, lysing agents, monoclonal antibody combinations and calibration beads. In the past two years a total of 8 single platform trials have taken place with the participation of 30 to 40 laboratories. Both the Trucount and FlowCount calibrator beads were used.

Single platform methodology improved significantly the precision of inter-laboratory variation compared to the traditional double platform approach, and confirmed the usefulness of the universal approach in the context of a Quality Assessment Program.

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**PRINCIPLES OF QUANTITATION IN IMAGE ANALYSIS:  
PRACTICAL CONSIDERATIONS FOR DRUG DISCOVERY**

**Michail A. Esterman  
Information Technology, Discovery  
Lilly Research Labs  
Indianapolis, IN 46285**

**Abstract**

The traditional disciplines of science are grounded in the observation and measurement of object properties, static and dynamic. The development of imaging tools such as the microscope, telescope and photographic film extended the range of natural vision and enabled scientist to observe, record and measure otherwise inaccessible object properties by means of their images. In the last several years, the development of computer based imaging systems has resulted from technological advances in computer science and light detectors. These technologies have exponentially increased the number of object properties that can be observed as well as the accuracy and reproducibility with which they can be measured.

Quantitative imaging is concerned with the principles, strategies and methods of image formation and the measurement of object properties which can be characterized by their numerical values. This presentation will examine the principles involved in quantitation of microscopic images including reproducibility and accuracy of measurement, which depends upon the quality of segmentation of the images. Various strategies for application of filters and measurement algorithms will be discussed. Examples of medium throughput cell and tissue based assays designed for drug discovery will be used to examine the practical considerations involved in the extraction of numerical data from images.

**PLATFORM PRESENTATIONS – AWARD COMPETITION**

**TGF $\beta$ /IFN $\gamma$  AFFECT SURVIVAL OF CLL B CELLS BY MULTIPLE PATHWAYS**

**M. Zaki, N. Patten, M. Bachinsky, P. Nowell, and J. Moore**  
Dept of Path & Lab. Med., Univ. of PA  
Philadelphia PA

**Abstract**

Defects in host cytokine networks may contribute to the survival of the neoplastic population in chronic lymphocytic leukemia (B-CLL). We focused on TGF $\beta$  and IFN $\gamma$  networks in CLL. We showed that CLL B cells, while expressing relatively normal levels of type II TGF $\beta$  receptors, are resistant to the apoptotic effects of TGF $\beta$ . CLL B cells also exhibit enhanced response to anti-apoptotic effects of IFN $\gamma$  along with elevated levels of IFN $\gamma$  receptors. Both of these cytokines are detected at increased levels in CLL patients. To further understand how survival of CLL B cells may be related to these altered cytokine network responses, we studied the effects of TGF $\beta$  and IFN $\gamma$  on caspase-3 activation. Using multiparameter flow cytometry, we showed that the enhanced anti-apoptotic effects of IFN $\gamma$  were associated with an inhibition of activation of caspase-3. TGF $\beta$ , consistent with its lack of effect on CLL B cells, failed to induce active caspase-3 beyond the spontaneous level in these cells. The phosphorylation of STAT-1 is critical to IFN $\gamma$  signaling and can be detected using a sensitive flow cytometric assay. In preliminary studies, we showed that TGF $\beta$  inhibited the phosphorylation of STAT-1 in response to IFN $\gamma$  in normal B cells while in CLL B cells, TGF $\beta$  enhanced phosphorylation of STAT-1 subsequent to IFN $\gamma$ . Our findings support the hypothesis that alterations in the TGF $\beta$  and IFN $\gamma$  networks, as well as in their interaction, may play a major role in the survival of CLL B cells.

**EFFECT OF LY335979 ON EFFLUX ACTIVITY OF AML BONE MARROW SPECIMENS**

**L. Green, P. Marder, L. Cripe, and C. Slapak**  
Lilly Research Laboratories and Indiana University Medical Center, Indianapolis, IN

**Abstract**

Human cancers can exhibit resistance to chemotherapy via overexpression of the multi-drug resistance protein known as P-glycoprotein (Pgp). Pgp can function as a pump to efflux cytotoxic drugs. New therapeutic agents such as LY335979 are being developed that block this activity and restore drug sensitivity to resistant tumors. Using flow cytometry, we previously demonstrated that LY335979 blocked Pgp function of surrogate target cells (CD56<sup>+</sup> lymphocytes) in the Phase Ia subjects. In the present study, we describe how we have adapted this method to study efflux function of acute myeloid leukemia (AML) cells from human bone marrow. We measured CD33, CD34 and Pgp expression and function on cells from 10 subjects. Our results showed significant intra-subject heterogeneity. We identified some cases where efflux was blocked by *in vitro* LY335979 treatment. We plan to use this assay to evaluate LY335979's effects during efficacy studies.

**PROGENITOR CELL GRAFT ANALYSIS.**

**Carolyn A. Keever-Taylor, Ph.D. Director**  
Cell Processing Laboratories,  
Medical College of Wisconsin, Bone Marrow Transplantation Program  
Milwaukee, Wisconsin

**Abstract**

Pluripotent progenitor cells capable of restoring hematopoiesis after marrow ablative therapies may be obtained from several sources, including: bone marrow, mobilized peripheral blood, and umbilical cord blood. In some cases the donor of the progenitor cells may be the patient (autologous transplants) while for other disorders, the donor must be another individual (allogeneic transplants). The progenitor cell-containing component may undergo processing to remove specific cell subsets so as to make allogeneic transplants safer, or to enrich for the progenitor cells themselves to reduce the risk of infusing unwanted cell subsets (such as tumor cells in autologous components). While in theory a single pluripotent progenitor cell can ultimately restore hematopoiesis, in practice the restoration of hematopoiesis is dose dependent and may require cell types other than the pluripotent stem cell alone. It becomes important for the clinician to know the dose of progenitor cells administered with the graft and the dose of target cells remaining after processing in order to determine if additional collections or post transplant patient treatment is required. Additionally it is important for the Cell Processing Laboratory to monitor the various stages of processing to ensure that the desired outcomes are achieved. For most situations multi-parameter flow cytometry can be used alone or together with other techniques to monitor cell subset content. This presentation will discuss variables that need to be considered in determining the method(s) to use for graft monitoring, and will focus on the analysis of bone marrow grafts that have been processed to remove mature T lymphocytes using complement-mediated lysis of antibody-coated T cells. Analysis of grafts treated with two different antibodies, T<sub>10</sub>B<sub>9</sub>.1A-31 (T<sub>10</sub>B<sub>9</sub>) and Muromonab-Orthoclone OKT3 (OKT3) specific for T lymphocytes will be compared. Potential problems associated with the analysis of progenitor cell content in mobilized peripheral blood from patients versus normal donors will also be presented. Selection of optimal monitoring method(s) is dependent on the specific characteristics of the progenitor cell source and of the processing technique used. For most techniques multi-parameter flow cytometry can give rapid and accurate results, for other systems functional assays may be required.

**FLUORESCENT IN SITU HYBRIDIZATION AND HIV DETECTION**

**Victoria L. Mosiman  
Assay Development  
Biometric Imaging, Inc.  
Mountain View, CA**

**Abstract**

The combination of enhanced technologies and sophisticated techniques is greatly impacting the methods we use for cellular investigation. The merging of two or more seemingly different scientific fields has spawned new techniques as well as methods of analysis. One example of this marriage, termed molecular cytometry, adapts molecular techniques for use in the field of flow cytometry. In one instance, this merging resulted in the development of solution-based fluorescent in situ hybridization (FISH), which enables investigators to detect and measure specific nucleic acid sequences within intact cells by flow cytometry. Historically, techniques that detected or measured nucleic acid sequences such as PCR or direct hybridization required disruption of the cellular membrane, whereby all information regarding cellular morphology and immunologic phenotype was lost. By keeping cellular integrity intact, one can measure not only the external, but also the internal characteristics of cells. This advancement can facilitate either exploration of nucleic acid sequences of cellular subpopulations defined by immunologic phenotype, or conversely, promote the examination of cellular heterogeneity in populations identified by nucleic acid sequence expression.

This presentation will serve as a brief introduction to some of the techniques and approaches used in solution-based fluorescent in situ hybridization for flow cytometry. Next will be a discussion to highlight some of the challenges and pitfalls commonly found in applying these techniques. Finally, we will explore some potential applications of this technique in the field of virology, and specifically for HIV detection.

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**QUANTITATION OF DONOR AND RECIPIENT CELLS IN BRONCHIAL LAVAGES  
FROM LUNG TRANSPLANT PATIENTS**

**Lynn DeVito-Haynes  
University of Wisconsin  
Department of Surgery  
Madison, WI**

**Abstract**

Peripheral microchimerism has been found in patients with kidney and liver allograft tolerance, but the biologic significance of this microchimerism is still controversial, partly due to the lack of knowledge of the donor leukocyte phenotype associated with tolerance. In addition, the role of persisting intragraft donor leukocyte chimerism, especially antigen presenting cells, has also been controversial.

Sampling of the intragraft space of transplanted human kidney, heart, and liver organs is only achievable by the traumatic technique of biopsy. However, in the case of lung transplantation, the bronchoalveolar space is routinely sampled by lavage (6-8 times during the first year post-transplant) and the cells that patrol this environment are readily available for analysis. We have used the technique of 4-color flow cytometry (2 directly coupled mAbs against leukocyte antigens, a biotinylated mAb against donor HLA proteins, and PI) on bronchoalveolar lavage (BAL) derived cells from lung transplant recipients. We have analyzed the phenotypes of the cells (both donor and recipient) and the rate of donor cell loss from this space, and have correlated these with the patient's clinical status.

We have found that patients with donor cell persistence have better graft function than those with rapid donor cell depletion. In addition, acute rejection is not necessarily the mechanism of donor cell loss as some patients lose all donor cells prior to their first clinically diagnosed rejection, while the rest retain donor cells more than 30 days beyond diagnosis. And finally, data from two patients, indicates a potential misdiagnosis of acute rejection as these patients had high numbers of CD8<sup>+</sup> donor rather than recipient cells within the BAL at the time of diagnosed rejection.

We think that 4-color flow cytometry to quantitate donor vs. recipient cells while still allowing for leukocyte differentiation and the potential for sorting or intracellular cytokine analysis offers a unique tool to analyze chimerism in transplant recipients.

PLATFORM PRESENTATION – AWARD COMPETITION

**FLOW CYTOMETRIC INTRACELLULAR IONIZED CALCIUM ANALYSIS OF PERIPHERAL BLOOD MONONUCLEAR CELLS IN PERIPARTURIENT DAIRY CATTLE**

**Kayoko Kimura, Jesse P. Goff, Timothy A. Reinhardt.**  
National Animal Disease Center  
Ames, Iowa

**Abstract**

Last year, we demonstrated that Fluo-4/Fura-Red ratio is a useful tool to measure the changes in intracellular ionized calcium ( $[Ca^{2+}]_i$ ) in cattle. We have applied this method to the periparturient dairy cows. Impaired immune function in periparturient dairy cows is believed to contribute to the increased susceptibility of dairy cows to infectious disease during the periparturient period. We hypothesized that this periparturient immunosuppression is due to the impaired ability of peripheral blood mononuclear cells (PBMC) to increase in  $[Ca^{2+}]_i$  after stimulation. To test this hypothesis, we measured the changes in  $[Ca^{2+}]_i$  after stimulation by CD3 and secondary antibody, and  $H_2O_2$  using 13 multiparous Jersey cows. The ability to stimulate  $[Ca^{2+}]_i$  was significantly decreased around the time of parturition and required 1-2 weeks to recover. Periparturient immunosuppression is associated with reduced ability to increase  $[Ca^{2+}]_i$  in response to stimulation.

**INVOLVEMENT OF  $Ca^{2+}$  COMPARTMENTALIZATION IN THAPSIGARGIN-INDUCED APOPTOSIS OF HUMAN COLON CARCINOMA SW 620 CELLS**

**T. Fu<sup>1,2</sup>, S. Zeng<sup>1</sup>, H.E. Soriano<sup>2</sup>, X. Huang<sup>2</sup>, D. Guo<sup>2</sup>,  
M.R. O'Gorman<sup>2</sup>, S.E. Crawford<sup>2</sup> and W. Fan<sup>1</sup>.**  
<sup>1</sup> Medical University of South Carolina, Charleston, SC 29425,  
<sup>2</sup> Northwestern University Medical School, Children's  
Memorial Hospital, Chicago, IL 60614

**Abstract**

Although alterations of calcium homeostasis have been reported to be involved in the regulation of apoptosis in several biological systems, the precise mechanisms are still unclear. Here, we demonstrated that thapsigargin (TG), an inhibitor of the endoplasmic reticular calcium pump induced apoptosis of human colon carcinoma SW620 cells. However, another  $Ca^{2+}$  mobilizing compound, ionomycin (from 10 nM to 10  $\mu$ M) could not induce apoptosis. Both TG and ionomycin triggered increase of intracellular free  $Ca^{2+}$  concentration in Fura-2-loaded SW 620 cells. In the presence of EGTA (1mM) in the  $Ca^{2+}$ -free buffer solution, intracellular calcium response to sequential addition of two stimulants exhibited the homologous desensitization of either TG or ionomycin, but no heterologous desensitization between TG and ionomycin, indicating the release of  $Ca^{2+}$  from different intracellular  $Ca^{2+}$  stores. In Rhod-2-loaded cells, ionomycin produced decrease of fluorescence intensity, but TG generated increase of fluorescence intensity and the increase could be suppressed by pretreatment with antimycin A, plus oligomycin, agents that are known to inhibit mitochondrial  $Ca^{2+}$  import by dissipating the mitochondrial membrane potential. Further, treatment of these cells with combination of TG (100 nM) and ionomycin (1 $\mu$ M) did not produce apoptosis. These results suggest that increase of  $Ca^{2+}$  concentration in mitochondria could be involved in the initiation of apoptosis during TG treatment.

**PKH2 FOR DETECTION OF ANTIGEN SPECIFIC LYMPHOCYTE PROLIFERATION**

**Ray Waters**  
Ames, IA

**Abstract**

In vitro lymphocyte proliferative responses are frequently used for the determination of previous exposure of individuals to a particular antigen. Staining cells with the green fluorescent vital dye, PKH2, prior to in vitro culture provides a means to track proliferation of individual lymphocyte subsets. Using this assay with three unrelated studies, it was determined that B cells from cattle with subclinical *Mycobacterium avium* subsp. *paratuberculosis* infection proliferate vigorously upon in vitro antigen stimulation whereas B cells from cattle with clinical disease respond poorly. Additionally, the proliferative response of lymphocytes from *Mycobacterium bovis*-infected deer is primarily due to MHC class II-restricted  $CD4^+$  T cells. And, vaccination with a protective bacterin for swine dysentery induces a  $CD8\alpha\alpha$  T cell proliferative response. These examples demonstrate the utility of this assay in disease pathogenesis and protective immunity studies.

**APPLICATION OF LASER SCANNING CYTOMETRY FOR THE EVALUATION  
OF DNA PLOIDY IN ROUTINE CYTOLOGIC SPECIMENS**

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**Abstract**

The Laser Scanning Cytometer (LSC) is a new instrument which combines the features of both flow and image cytometry. The LSC is capable of measuring multicolor fluorescence, light scatter, and the location of cells fixed to a microscopic slide, thus, enabling relocation of the cells of interest for morphologic examination and classification. Although this technology became commercially available just a few years ago, an extensive literature describing principles, capabilities and applications of LSC has been already published. The major applications of LSC include immunophenotyping and analyses of cell cycle, apoptosis, enzyme kinetics and drug uptake, and FISH. The majority of institutions apply the LSC for research purposes and only a few centers utilize this instrument in their routine service work. The most commonly used service application is immunophenotyping.

The purpose of this presentation is to examine the application of LSC for the evaluation of DNA ploidy in routine cytologic specimens, mainly bladder washings. Our quality control program will be discussed and the optimal specimen preparation for LSC will be presented. The performance of the LSC will be compared with other methods commonly used for evaluation of DNA ploidy in cytologic specimens.

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**INSTRUMENTATION, REAGENTS, STANDARDS: WHAT IS COMING?**

**Alan Stall, San Diego, CA**

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**CELL AND GENE THERAPY: WHERE ARE WE ON THE ROAD FROM HYPE TO HOPE?**

**Carl H. June, M.D., University of Pennsylvania, Philadelphia, PA**

**Abstract**

Bellingham, Brent and Medawar first coined the term ‘adoptive immunotherapy’ in 1954 to describe the transfer of lymphocytes to mediate an effector function. Yet as we complete nearly 50 years of research into adoptive immunity, no forms of cellular therapy for tumors or infections have been approved by the United States Food and Drug Administration. After the first initial apparent successes at tumor elimination, enthusiasm waned as it was learned that the initial tumor rejections were actually due to allogeneic effects rather than to specific anti-tumor immunity. Indeed, skepticism regarding the possibility of effective T-cell mediated anti-tumor immunity held sway for more than a decade.

Results from ongoing trials using *ex vivo* T cell costimulation and expansion to treat patients with HIV infection and in separate studies, patients with chemotherapy-resistant B cell NHL will be presented. In patients with HIV infection, we have observed a dose-dependent increase in CD4 counts in patients treated with autologous CD4 T cell infusions. In a second study carried out in collaboration with Cell Genesys, we have infused autologous gene-modified T cells into patients with HIV infection. For the first time, high level and sustained engraftment has been observed. In the third study, escalating doses of *ex vivo* CD3/28 costimulated T cells are being given as an immunotherapeutic adjunct to dose-intensive chemotherapy for patients with chemotherapy-resistant B cell NHL. Analysis of T cell activation responses in these patients pre- and post-adoptive costimulated T cell therapy demonstrated that this therapeutic approach could correct T cell activation defects that were present in these patients prior to therapy. In conclusion, enthusiasm is rebuilding for the prospects of harnessing T cell mediated anti-tumor immunity as it has been clearly demonstrated that tumors are antigenic yet poorly immunogenic. Similarly, there are now promising preclinical and clinical data from a variety of novel forms of adoptive immunotherapies for a number of chronic viral diseases.

**ANALYSIS OF Ad-p53 MEDIATED GENE THERAPY IN OVAIAN CARCINOMA**

**Jake Jacobberger, Case Western Reserve University, Cleveland, OH**

**Abstract**

We have developed a cytometric approach for detection of p53 and mdm2 to evaluate adenoviral mediated p53 gene therapy of ovarian cancers. Three prostate cancer cell lines (PC-3, LNCaP, DU 145) that are null, wild-type, and mutant for p53, respectively, and two ovarian cancer cell lines (PA1, MDAH 2774) that are wild-type and mutant for p53, respectively, were tested for immunoreactivity and lack of cross-reactivity with the monoclonal antibodies, DO-7 (anti-p53) and IF2 (anti-mdm2). Optimal dual staining conditions for a flow cytometric assay employing saturating levels of antibody were developed and tested by infection of PC-3, PA1, and MDAH 2774 with Ad-p53 or a control virus, Ad-luc. Dual staining with DO-7 and propidium di-iodide was used to determine any biological effect of the transferred gene. Neither DO-7 nor IF2 showed appreciable cross-reactions by Western blot analysis of representative prostate or ovarian cell lines. By flow cytometric titration, DO-7 appears to be a high avidity antibody (saturation staining of  $10^6$  DU 145 cells with 0.5ug) whereas IF2 appears less so (optimum signal to noise ratio at  $1\text{ug}/10^6$  cells). Infection with Ad-p53 was detected at 6 to 48 hours post infection as a uniform relative increase in p53 levels over background p53 levels. Coincident increases in mdm2 immunoreactivity were also detected. DNA content measurements of PA1 and MDAH 2774 cells indicated that G1 arrest and/or apoptosis occurred subsequent to Ad-p53 infection. p53 and mdm2 levels and DNA content distributions for Ad-luc infected cells were equivalent to uninfected cells. When combined with anti-CD45, the non-immune component of ascites can be evaluated for p53 expression and, by mdm2 levels, p53 activity after treatment of with p53 encoding adenoviral vectors.

**PLATFORM PRESENTATIONS – AWARD COMPETITION**

**A ROLE FOR CELL DIVISION IN T CELL FATE DECISIONS?**

**Andrew D. Wells, Matthew C. Walsh, Hrefna Gudmundsdottir and Laurence A. Turka**

**Department of Medicine, University of Pennsylvania, Philadelphia, PA**

**Abstract**

The events that drive the development of naïve T cells into effector and/or memory cells are unclear. We have observed a large degree of heterogeneity in T cell proliferative behavior during a primary response, suggesting that this could serve as a source of functional heterogeneity within the activated T cell pool. To address this question, we labeled T cells with the fluorescent dye CFSE to track cell division during an immune response. Activated T cells were then separated into discrete division populations via cell sorting, and secondary effector function (cytokine production and proliferation) was assessed as a function of previous division history. Our data demonstrate that the integrity of TCR-coupled MAPK activation in response to antigen re-encounter, as well as the frequency of cytokine-producing and proliferative cells in each pool, are quantitatively tuned to the number of mitotic events achieved during primary stimulation. We therefore propose that the process of cell division may shape T cell fate decisions during an immune response.

**USE OF PRIMARY HEPATOCYTES AND HEPATOMA CELLS TO ASSESS DRUG-INDUCED INTRACELLULAR OXIDATIVE STRESS BY FLOW CYTOMETRY**

**D. M. Williams, P. Narayanan, C. Zhang, T. K. Hart. Safety Assessment, SmithKline Beecham Pharmaceuticals R&D, King of Prussia, PA 19406**

**Abstract**

The liver is a primary organ for drug and chemical-induced toxicity that is frequently believed to result from oxidative stress. Hepatotoxicity encountered *in vivo* during preclinical drug development can be evaluated *in vitro* to dissect the underlying mechanisms of the injury. Towards this end, primary rat hepatocytes and rat hepatoma cells (Novikoff) exposed to menadione were utilized for monitoring the onset of intracellular oxidative stress on the FACSVantage-SE flow cytometer. In contrast to earlier studies that used parinaric acid, a 325 nm excitable dye, we examined the feasibility of using the loss of fluorescence of the 488 nm excitable 5-dodecanoylamino fluorescein (C11-fluor) for estimating lipid peroxidation in combination with UV-excitable monochlorobimane (modulation of glutathione (GSH) content) and the 637 nm excitable dye chloromethyl-X-rosamine (mitochondrial membrane potential ( $\Psi_{mit}$ )). Cellular viability was concurrently assessed using the vital dyes, propidium iodide or JOJO (595 and 544nm). Menadione, which binds covalently to cellular thiols, induced a time-dependent and concentration-dependent loss of C-11 fluorescence, depletion of GSH content, and loss of  $\Psi_{mit}$ . Pretreatment of the cells with the antioxidant N-acetylcysteine (1 mM), reduced the rate of cellular injury associated with the oxidative stress parameters. This multiparametric flow cytometric approach provides a method for the simultaneous examination of metabolic capacity, oxidative homeostasis and viability end points that are useful for the *in vitro* dissection of drug-induced cytotoxicity.

**WIDE FIELD, MULTICOLOR FLUORESCENCE IMAGE ANALYSIS TECHNIQUES  
FOR STUDYING FUNCTIONAL CANCER BIOLOGY**

**David W. Hedley, Ontario Cancer Institute/Princess Margaret Hospital, Toronto, Ontario**

**Abstract**

The ability to measure and correlate multiple markers has major advantages in image analysis, and is achieved using fluorescence microscopy. The use of an autostage and software allows the creation of composite images covering up to several square millimeters. This gives an overview of tissue architecture and intra-tumoral heterogeneity similar to that obtained with immunohistochemistry, but with correlated information about multiple markers similar to that obtained by flow cytometry. Our laboratory is applying this technique to study complex, regulated processes occurring solid tumours that determine treatment sensitivity. Examples include understanding the interrelations between micro blood vessel formation, blood flow, and tissue hypoxia, and how these affect the expression of genes involved in cell survival and drug resistance. This branch of cytometry (or “histometry”) is relatively new and unexplored, with few existing rules or guidelines for data analysis.