

GREAT LAKES INTERNATIONAL IMAGING AND FLOW CYTOMETRY ASSOCIATION, INC.



GLI²FCA “CellEFTA” 20 YEARS OF IMMUNOPHENOTYPING “FROM OKT-4 RECEPTORS TO CYTONOMICS”

**HOTEL ST REGIS
DETROIT, MICHIGAN
OCTOBER 4 - 6, 2002**

**Site Organizer: Alex Nakeff
Program Chairs: Frank Mandy and David Hedley**

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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.

It is the 20th anniversary of the introduction of clinical flow cytometry. To celebrate, let us go back to the early 1980's and let the Ghostbusters take the dance floor because if you will not flashdance with us, you will be relegated to solve the Rubik's cube and figure out cellefta all by yourselves.

FRIDAY Evening - October 4

Registration/ Poster Set-Up 1700 - 1930

Steve Graves, Los Alamos, NM.

“FLOW CYTOMETRY AS A PLATFORM FOR ANALYSIS AND DISCOVERY OF MOLECULAR ASSEMBLIES” 1930 - 2000

George Lustyik, Madison, WI.

“SOFTWARE TO HARNESS FUTURE SUSPENSION ARRAYS TECHNOLOGY” 2000 - 2030

Reception/Poster Session **SPONSORED BY BD BIOSCIENCES** 2030 - 2330

SATURDAY - October 5

Breakfast 0730 - 0800

THE PAST Chair – Jan Nicholson, Atlanta, Georgia

Carl Stewart, Buffalo, NY.

“THE EARLY DAYS OF CANCER RESEARCH AND THE ROLE OF FLOW CYTOMETRY” 0800 - 0840

George Janossy, London, UK.

“IMMUNOPHENOTYPING FROM THE BEGINNING” 0840 - 0920

Break **SPONSORED BY CALTAG** 0920 - 1000

THE PRESENT I Chair – Frank Mandy, Ottawa, Ontario.

Jan Nicholson, Atlanta, Georgia

“CAN FLOW CYTOMETRY HELP US WITH ANTI-BIOTERRORISM?” 1020 - 1100

Frank Mandy, Ottawa, Ontario

“REFLECTIONS ON TWENTY YEARS OF CD4 T-CELL COUNTING” 1100 - 1130

Platform Speaker Award Competition Speakers

Steve Kaiser, Marshfield, WI

“BIOMARKERS OF OXIDATIVE STRESS IN MURINE EMBRYONIC STEM CELLS.” 1130 - 1145

T.Milovanova, Philadelphia, PA,

”A FLOW CYTOMETRIC TEST FOR BERYLLIUM SENSITIVITY” 1145 - 1200

Luncheon Roundtables 1200 - 1300

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.

THE PRESENT II Chair – Chuck Goolsby, Chicago, Illinois.

David Hedley, Toronto, Ontario

"INTEGRATING FLOW CYTOMETRY AND IMAGE ANALYSIS INTO ANTICANCER DRUG DEVELOPMENT PROGRAMS"

1300 - 1340

Vince Shankey, Miami, FL.

"MULTIPARAMETER ANALYSIS OF SIGNAL TRANSDUCTION PATHWAYS"

1340 - 1420

Jake Jacobberger, Cleveland, OH

"MULTIPARAMETRIC CELL CYCLE ANALYSIS: THE G2→M TRANSITION"

1420 - 1500

Platform Speaker Award Competition Speaker

Kayoko Kimura, Ames, Iowa,

"INTRACELLULAR BOVINE CYTOKINE ANALYSIS WITH ANTI HUMAN CYTOKINE MABS" 1500 – 1515

Break SPONSORED BY DAKOCYTOMATION

1515 – 1545

Keynote Address: Chair: David Hedley, Toronto, Ontario

Chuck Goolsby, Chicago, Illinois "CYTONOMICS"

1545 - 1645

Wine and Cheese Happy Hours SPONSORED BY VERITY AND SPHEROTECH

& Poster Session in Exhibit Area

1700 - 1930

THE FLOWGADOWN

"The Ghostbusters go Flashdancing" SPONSORED BY BD BIOSCIENCES

2000 - 2400

SUNDAY - October 6

Steering Committee Meeting

0730 – 0900

Breakfast

0800 – 0900

THE FUTURE Chair – Alex Nakeff, Detroit, MI.

Chuck Hitchcock, Cleveland, OH.

"ROUTINE CLINICAL USE OF TELEPATHOLOGY – SYSTEMS AND APPLICATION"

0900 - 0940

Tim Barder, Chicago, IL. "2-D LIQUID PROTEIN MASS MAPPING"

0940 - 1020

Dan Farkas, Pittsburgh, PA, " THE FUTURE OF CYTOMETRY BY THE NUMBERS:

3 (Rs), 20/20 (Vision), 50-50 (Distribution), 100 (% Relevance), 1M (\$)"

1020 - 1100

Break SPONSORED BY SIGMA ALDRICH INC

1100 – 1115

Chair, Maurice O’Gorman, Chicago, IL.

Fred Koller, San Diego, CA,

"HIGH-THROUGHPUT CELL IMAGING AND LASER-BASED MANIPULATION ON THE LEAP™ PLATFORM"

1115 - 1130

Jay Pink, - Agilent Technology, Chicago, IL. "LAB-ON-A-CHIP MICROFLUIDIC CELL ASSAY: A NEW TECHNOLOGY FOR FLOW CYTOMETRY"

1130 - 1200

Bo Wang, Somerville, MA, "A LARGE PARTICLE FLOW SORTER"

1200 - 1215

AWARD PRESENTATION

SPONSORED BY SOUTHERN BIOTECHNOLOGY ASSOCIATES

TRAVEL STIPEND PRESENTATION(S)

SPONSORED BY CELL SIGNALING AND JACKSON IMMUNORESEARCH LABS

Education Chair: Jonni Moore, Philadelphia, PA

1215 – 1230

Abstracts: Friday Evening:

FLOW CYTOMETRY AS A PLATFORM FOR ANALYSIS AND DISCOVERY OF MOLECULAR ASSEMBLIES

Steve Graves, Los Alamos, NM

A primary goal of biomedical research is to understand how the multitudes of proteins encoded in the genome assemble into multiprotein "molecular machines". Realization of this goal will require new technologies to assay protein assembly and function. Ideally these technologies will provide high sensitivity, homogenous analysis methods, multiple detection modalities, millisecond kinetic resolution, and high throughput analysis. While no current technology has all these features, flow cytometry is an excellent beginning point for further development, as it offers, to varying degrees, most of the above requirements. I will discuss new instrumentation providing stopped flow mixing, dynamic temperature control and improved resolution of low affinity complexes. Furthermore, I will illustrate application development using microsphere based protein-protein and enzyme-substrate interactions. Finally, the implications of these technological developments and how they effect the use of flow cytometry in the wider fields of molecular assembly analysis and proteomics will be presented.

SOFTWARE TO HARNESS FUTURE SUSPENSION ARRAYS TECHNOLOGY

George Lustyk, Soft Flow Inc. Madison, WI

The future potential for suspension array technology (SAT) lies in its unique capacity to measure molecular interactions simultaneously on solid phase surface, on microfluorospheres, in a multiplexed suspension environment. SAT performs numerous discrete homogeneous assays simultaneously from a single 50 to 100 μ L sample with the help of advanced digital signal processors. Can innovative data analysis and management software be generated for SAT to assure progress to meet the requirements of research and diagnostic medicine of the future?

In the past cost-effective assay platforms had to combine only high throughput, sensitivity, and specificity. In the future SAT must also provide flexibility in rapidly rearranging assay combinations. The multiplexed assay combination selection must provide the capacity for disease speciation or deferential diagnosis of a condition.

The ability to cope with multiplexed data from a variety of assays performed simultaneously must be cost effective. The presentation will focus on data management from sandwich type immunoassays. How the software interface and algorithms help with multiplexed data implementation.

Saturday Morning THE PAST:

THE EARLY DAYS OF CANCER RESEARCH AND THE ROLE OF FLOW CYTOMETRY

Carl Stewart, Roswell Park Cancer Institute, Buffalo, NY.

The first flow cytometer was built in 1934 by Moldavan and was used to count erythrocytes. It did not work well. In 1949, Wallace Coulter built a flow cytometer to count and size cells using an impedance principle in which cells passing through a small orifice with a voltage difference on either side produced a pulse proportional to the volume displaced. In 1965, Mac Fulwyler at Los Alamos built the first cell sorter in which droplets were generated by rapidly vibrating the flow cell. As the droplet broke away from the cell stream it becomes charged and falls through two deflection plates held at a high potential difference where it is deflected into a collection vessel. An instrument using a laser to illuminate the cell as it passed by the laser beam had the advantage that the scatter properties could be measured. Cells could be labeled with fluorescent dyes, which could be quantitatively measured. DNA dyes were the first application for the measurement of aneuploid cells in cancer. Later, a combination of lasers and cell sorting technology to measure immunofluorescence was developed by Hertenberg, who was credited with the development of the Fluorescent Activated Cell Sorter, as we know it today. These instruments have had the highest impact in hematology for routine blood counts with automated differential. With the development of monoclonal antibodies to cellular constituents that are conjugated with fluorochromes of different colors, it is now possible to rapidly count and identify specific subsets of hematopoietic cells. The use of many antibodies with different fluorochromes makes possible multiparameter evaluation for the explicit resolution of cell populations of any size. This has had a major impact on the classification of hematopoietic malignancy.

IMMUNOPHENOTYPING FROM THE BEGINNING. DEVELOPING CONCEPTS FOR IMMUNODIAGNOSIS BY FLOW CYTOMETRY – A EUROPEAN PERSPECTIVE

George Janossy, HIV Immunology, Royal Free and University College Hospital, London, UK.

The early history of these events, leading to the development of the immunodiagnostic platform for immunodiagnosis started to roll in 1970, an Olympian year for immunology at the National Institute of Medical Research, Mill Hill, UK. The influence of Medawar was in full swing collaborating 'immunology-oriented' local groups. Dr. Mitchison was working on the T and B cell story. Drs. Raff and Cantor introduced the 'markers' for discriminating between T and B lymphocytes. E-rosetting and immunoglobulin, as respective markers for human T and B cells, were described and a new chapter in lymphocyte physiology was on its way.

During the period of 1970-1974 the T/B cell concept was amply applied for leukaemia differential diagnosis and the study of immunodeficiencies. Seligman and Prud'homme were in competition with Max Cooper's group in Alabama, for creating reagents in order to establish the B cell origin and monoclonality of lymphomas and myelomas. Drs. Flandrin and Catovsky had found T cell

malignancies, both chronic and acute, the latter shown to be of thymic origin by Borella and co-workers. Mel Greaves developed his antiserum, with Geoff Brown, against the common form of acute lymphoblastic leukaemia. The flow cytometry obtained its unquestionable dominance in leukaemia analysis with the documentation of normal, infant and fetal bone marrow, e.g. in the studies performed by Civin, Loken, Terstappen and their colleagues in the Becton Dickinson laboratories. For such a detailed work monoclonal antibodies were essential. The explosion of immunodiagnostic activities, by the introduction of monoclonal antibodies linked to the spread of flow cytometry. These included the simplification of protocols by the 'lyse-no-wash' technology and the establishment of extremely precise absolute counting procedures using both the volumetric and microsphere-related technologies.

Current clinical tests, including CD4 T cell counts can be performed elegantly in a more precise manner without costing too much – leading to a spread of the basic technology to resource poor countries where AIDS takes its toll. The platform had just become far more all-embracing. The flow cytometers of tomorrow will perform not only research and CD4 and CD8 T cell counting, but will also be the standard quality controlling techniques for white blood cell differentials, reticulocyte counts, for differential diagnosis of infectious diseases including active tuberculosis and malaria, and also for quantitative ELISA-related immunoassays in a vast area of medicine. It appears that the excitement and clinically oriented innovation, so characteristic for the early days, is back with a vengeance.

THE PRESENT I:

CAN FLOW CYTOMETRY HELP US WITH ANTI-BIOTERRORISM?

Jan Nicholson, Centers for Disease Control and Prevention, Atlanta, GA.

The intentional release of anthrax through letters sent to New York, Washington, DC, and (likely) Florida put into action a response plan developed by the Centers for Disease Control and Prevention (CDC) in 1999. This plan includes the creation of a network of laboratories throughout the country that have been trained to identify agents considered to have the highest probability of biological or chemical terrorism. The CDC has partnered with other Federal agencies and subject-matter experts to develop laboratory protocols for detecting bioterrorism (BT) agents. These protocols have been provided to a tiered group of laboratories, beginning with Level A laboratories (local hospitals, etc.), and including Level B, C, and D laboratories. Level B and C laboratories are state public health labs, and Level D laboratories are at CDC and in the Department of Defense, where biosafety level (BSL)-4 labs are found.

Research monies are being made available for the development of new diagnostics and evaluating the pathogenesis of agents of bioterrorism. New developments in flow cytometry for use in the area of agent detection using beads and characterization of pathogenesis of infection may hold promise. Novel applications for diagnostic tests will need proper evaluation and validation before use in diagnostic and clinical laboratories. Methods to evaluate pathogenesis will further our understanding of the immune response to infection and may provide insights into new therapies or prophylactic treatments.

REFLECTIONS ON 20 YEARS OF CD4 T-CELL COUNTING

Frank Mandy, Ottawa, Ontario

Flow cytometry impacted HIV disease monitoring more than any other clinical condition. Therefore, it seems appropriate to review the evolution of immunophenotyping in the context of following the fight against AIDS over the past 20 years. Contrary to some of the original expectations, it was AIDS, not some frequently performed oncological test that was responsible for the massive and rapid worldwide mobilisation of flow cytometers into clinical immunology laboratories. In the early 80's, reports appeared from various parts of the USA about young gay men who had unusual immunosuppression manifesting as opportunistic infections. Soon it was the hallmark of this new disease, acquired immunodeficiency syndrome (AIDS), was a decrease in numbers of CD4 T-cells in peripheral blood. Next, the causative viral agent for AIDS was isolated, the human immunodeficiency virus (HIV). It was established that the T-helper cell is the primary target for HIV and the CD4 receptor on the surface of these cells is the principal means for viral entry. The current cocktails of anti-retroviral drug combinations (three or more) can suppress viral replications for years, hence disease monitoring of patients will be required for decades as none of current therapies can completely eliminate the virus from the host. Quantitative viral load tests are also often used, however their reproducibility is limited to within half of a log. Therefore, T-cell subset is the test followed throughout the course of disease, as it is the best surrogate marker for assessing the patient's immune status. Normal levels of T-helper cells are about 1000 cells/ml of blood (with a range from 600 to 1400); generally, levels below 500 cells/ml indicate that the virus has damaged the immune system. The overall level of T-cells remains constant until late stages of the disease. For most of the disease course the CD8 T-cell numbers increase as CD4 T-cells diminish. CDC in Atlanta developed a definition for AIDS based on a CD4 T-cell count of less than 200 cells/ml. Current HAART (Highly Activated Anti-retroviral Therapy) adds years of quality life to patients living with HIV; however, the virus is never eliminated from the patient. Eventually, opportunistic infections prevail and are usually the direct cause of death. In the quest to eradicate the AIDS pandemic, perhaps the next generation of multilaser, multiparameter instruments will accelerate discoveries in cellular immunology and that in turn will lead to breakthroughs in the fundamental understanding of events in adaptive and innate immunity. It is predicted that cytometers will continue to shrink in size and cost and that engineers will develop instruments to comply with visions of cytonomics. Low cost instruments will be available to deal with the challenges confronting us in Africa and Asia. With a one-tear system for diagnostic tests for both resource rich and poor regions of the globe we will destroy this evil dragon with our laser weapon of choice.

Platform Speaker Competition

BIOMARKERS OF OXIDATIVE STRESS IN MURINE EMBRYONIC STEM CELLS.

Steve Kaiser, Tamara Kronenwetter-Koepel, Anne R. Greenlee

Pleuripotent mouse embryonic stem cells (ESCs) show promise as a substrate for developmental toxicology studies. Here, the expression of heat shock protein 72 and active Caspase-3 in ESCs following 16 hr exposure to 7.5 – 60 μ M sodium arsenite, an oxidative stressor, were evaluated. 10^5 ESCs were fixed with Cytifix/CytopermTM and incubated with 1 μ g PE- rabbit anti-mouse active Caspase-3, and/or 1 μ g rabbit anti-mouse Hsp-72 and 1 μ g FITC- goat anti-mouse Ig. Percentages of cells stained were determined using a MoFloTM. The percentages of Caspase-3 expressing cells rose with increasing concentrations of arsenite, declining at 60 μ M. More than 80% of ESCs expressed Hsp-72 regardless of arsenite concentration. Dual staining confirmed both findings. Our results suggest that Caspase-3 activity may serve as an indicator of oxidative stress in mouse ESCs and that Hsp-72 expression occurs independently of Caspase-3 activity and arsenite concentration.

Platform Speaker Competition

A FLOW CYTOMETRIC TEST FOR BERYLLIUM SENSITIVITY

Tatyana Milovanova, J.Moore, M.Rossman, University of Pennsylvania

Testing (³H-Be-LPT) for beryllium sensitivity is used in industry to screen current and former workers for Chronic Beryllium Disease-lung granulomatosis disorder, consisting predominantly CD4+T-cells. However, surveys have shown that 30-70% of individuals with BH could have negative blood responses. Since a positive Be-LPT means, that a patient will undergo the bronchoscopy, a better screening test for BH would be useful. We utilized CFSE and multicolor surface staining flow cytometry to identify CD4+ T-cells that specifically proliferate after beryllium stimulation to confirm BH.

THE PRESENT II:

INTEGRATING FLOW CYTOMETRY AND IMAGE ANALYSIS INTO ANTICANCER DRUG DEVELOPMENT PROGRAMS

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

Treatment of human cancers is being radically transformed through the introduction of molecular targeted anticancer agents. This field is evolving extremely rapidly, and promises major improvements in patient outcome. Due to the complexity of molecular control mechanisms and the problems of tumour heterogeneity, it is likely that the rational use of molecular targeted cancer therapies will need to be individualized, based on the use of sophisticated analytical methods for treatment planning and monitoring. Flow cytometry and image analysis are well suited to this, because they are quantitative, address the problems of cellular heterogeneity, and are capable of analyzing biological complexity. Our group has become particularly interested in the use of wide field, multicolour fluorescence image analysis techniques that can be applied to tissue sections. Laboratory protocols can be developed and validated using human tumour xenografts treated with the appropriate drug, with the xenografted tissues subsequently used for laboratory standards and quality control with the patient biopsies. In combination with flow methodology, this has the potential to become a major new application of clinical cytometry.

MULTIPARAMETER ANALYSIS OF SIGNAL TRANSDUCTION PATHWAYS

Vince Shankey, Advanced Technology Center, Beckman Coulter, Inc., Miami, FL

The analysis of signal transduction proteins is currently providing important insight into cell biological processes that regulate cell proliferation, apoptosis and differentiation. In general, these pathways involve activation of a cell surface receptor complex and subsequent signaling through a cascade of membrane bound or cytosolic proteins which frequently terminate with nuclear localization and transcriptional activation of specific targeted genes. One common factor in signal transduction cascades is the generation of specific tyrosine, serine, or threonine kinases, which after activation by upstream proteins, subsequently phosphorylate specific downstream proteins at specific residues. One tool that has proven of significant value to studies of signal transduction pathways is the availability of antibodies that can recognize specific phosphorylation sites on specific signal transduction proteins.

For the most part, studies of signal transduction proteins or pathways have thus far been limited to Western blot analyses, and imaging studies (to determine localization of activated proteins). Very few studies (1,2), thus far, have utilized flow cytometry. However, these studies have demonstrated the unique insight provided from flow cytometry, including the ability to analyze the status of signal transduction proteins in subpopulations of cells, and the ability to study specific inhibitors of these pathways in complex mixtures of responding and non-responding cells.

In this presentation, a number of critical factors will be discussed which are important for the successful utilization of flow cytometric analysis of signal transduction pathways. These include validation of antibody specificity, titration of antibodies to obtain optimal S/N, the use of different controls and standards, and the need to rapidly stabilize phospho-specific epitopes to prevent artifactual results. Choices of fluorochrome-antibody pairs will be discussed, as well as complications arising from signal compensation from multiple fluorochromes. Finally, specific examples of signal transduction pathways analyzed by flow cytometry will be discussed.

1. Chow S, Patel H, and Hedley DW. Measurement of MAP Kinase Activation by Flow Cytometry Using Phospho-Specific Antibodies to MEK and ERK: Potential for Pharmacodynamic Monitoring of Signal Transduction Inhibition. *Comm. Clin Cytometry*. 46: 72-78, 2001.

2. Perez OD, and Nolan GP. Simultaneous Measurement of Multiple Active Kinase States Using Polychromatic Flow Cytometry. *Nature Biotech*. 20: 155-162, 2002.

MULTIPARAMETRIC CELL CYCLE ANALYSIS: THE G2→M TRANSITION

Jake Jacobberger, Cleveland, Ohio

Pines and Rieder (*Nature Cell Biol.*, 2001, **3**:E3-E6) have argued that somatic cell mitosis might be viewed as 5 phases defined by the activity of cell-cycle regulators. These are termed transitions 1 through 5, and are defined by a dominating (as we currently understand it) role for (1) Cyclin A/CDK, polo-like kinases, and aurora B kinase; (2) Cyclin B/Cdk1, (3) APC-Cdc20, BubR1/Mad2; (4) fully active APC-Cdc20; and (5) APC-Cdh1. Transition 1 is reversible, morphologically visible, but a precise beginning is not well-defined. It is also termed “preparation for mitosis”. Transition 2 is characterized by the nuclear translocation and activation of peak levels of cyclin B1/Cdk1. After peaked cyclin B1/Cdk1 has been activated, mitosis has not been observed to be reversible. This is viewed as the “true” onset of mitosis. Transition 3 is the mitotic spindle checkpoint(s). At least one mechanism through which this occurs is mediated by BubR1 and Mad2 and targets APC through cdc20. APC is prevented through Cdc20 from degrading securin and cyclin B as chromosomes attach to the mitotic spindle. Cyclin A, however, is degraded. This is equivalent to prometaphase. Transition 4 includes chromatid separation and is characterized by fully active APC-Cdc20, which normally results in the rapid degradation of cyclin B and loss of Cdk1 activity; Transition 5 includes interphase nucleus formation and resetting APC to its interphase form – i.e., degradation of Cdc20 and replacement with Cdh1.

At least part of the usefulness of this model is that it is based on what are perceived to be central molecular mechanisms driving time-dependent changes in function (or purpose). An additional feature is that, theoretically, these Transitions can be identified by measurements of the activity of the identifying cell cycle regulators. As an intermediate step, we are having some success with measurements that are surrogates for activity (the cyclins) or substrates of specific kinases. For fixed cell populations, cytometric immunofluorescence measurements of cyclin B1, cyclin A, phospho-(serine 10)-histone H3 (pH3), coupled with DAPI fluorescence provides enough information in unperturbed cells to identify states similar to the 5 described above. Remarkably, we have found that some of these transitions can be identified as discreet populations in proliferating cells – i.e., they can form clusters demarked by multidimensional Gaussian distributions. Although this is largely work-in-progress, Transition 1 is tentatively identified by intermediate chromatin condensation and intermediate levels of pH3 immunoreactivity. Transition 2 is characterized by peak levels pH3, cyclin B1, and cyclin A. Transition 3 is represented by cells with peak pH3 and cyclin B1 but downward transitional immunoreactivity for cyclin A. Transition 4 is composed of cells with peak pH3, low cyclin A, and downward transition immunoreactivity of cyclin B1. Transition 5 is represented by cells with low cyclin A, low cyclin B1, and downward transitional immunoreactivity for pH3. This last state is the least well characterized. We have done a variety of inhibitor, cell sorting, and kinetic experiments to support this model.

Platform Speaker Competition

INTRACELLULAR BOVINE CYTOKINE ANALYSIS WITH ANTI HUMAN CYTOKINE MABS

Kayoko Kimura, Jesse P. Goff, Judith Stabel. USDA-ARS, National Animal Disease Center, Ames, IA

Flow cytometric analysis of intracellular cytokines has proven useful for assay of human and rodent immune cell function. Limited availability of commercial mAbs to bovine cytokines has prevented application of this method to bovine immune cells. We have used anti human IFN- γ and IL-8 mAbs to develop a method to study intracellular cytokines in bovine immune cells. PBMC expressing IFN- γ and PMN expressing IL-8 increased by 11 and 86 %, respectively, following culturing with pokeweed mitogen for 20 hours. Ab to human IL-8 is known to cross react with bovine IL-8. To validate the specificity of anti human IFN- γ mAb against bovine IFN- γ , we incubated the mAb with recombinant bovine IFN- γ and demonstrated nearly complete blockage of mAb binding to cells. These results demonstrate anti human cytokine mAbs can be used for intracellular bovine cytokine analysis. In the course of these studies we also demonstrated bovine neutrophils are positive for IFN- γ production as reported in human cells.

Keynote Address:

CYTONOMICS

Chuck Goolsby, Northwestern University Medical School, Chicago, IL

Since Cytometry's beginnings as a fledgling research tool some 40 years ago, it has mushroomed into an integral component of modern hematopoietic malignancy diagnosis and a standard, widely used research tool in a wide range of disciplines. Questions of direction for the society and the science of cytometry as the field matured have arisen, but the term "cytonomics" is coming to crystallize the enthusiasm of cytometrists for this science as we move into, in my mind, perhaps the most exciting time since the inception of this family of technologies. Probes, dyes, and instrumentation to facilitate highly complex, multiparametric (6, 7, 8, color) cell based measurements are now becoming readily available. Forays into array analyses in the genomics/ proteomics initiatives and exciting developments in both cell based and tissue based image analysis are occurring. Within the genomics/proteomics programs, complex cell based measurements may be key to understanding the role in cell function and human disease of the genes and proteins identified. But perhaps, no where more than in the evolving role of clinical cytometry is the exciting future revealed. A move of clinical cytometry from merely serving as a diagnostic adjunct to a technology intimately involved in patient therapeutic decision making and monitoring is occurring. This is a fundamental, major change in the role of this technology in the clinical setting. In this presentation, these transitions will be discussed with emphasis on the later, hopefully, relaying the enthusiasm for the exiting future that lies ahead for cytometry.

THE FUTURE

ROUTINE CLINICAL USE OF TELEPATHOLOGY – SYSTEMS AND APPLICATION

Chuck Hitchcock, Department of Pathology, The Ohio State University, Columbus, OH

Problem: A free standing breast center, located 11 miles from the OSU Medical Center, requires a full-time pathologist for immediate assessment of fine needle aspirates (FNA), frozen sections, and outside slide consults. There were insufficient pathologists to provide the needed service.

Solution: After several non-technical possibilities were tested, the decision was made to establish a telepathology system.

Hardware System: Several factors dictated the basic system requirements. These included: need for robotic control, diagnostic images, rapid transmission time, simultaneous multi-user capability, and use of a gross-station. Added hardware considerations included: microscope, camera, transmission and network capabilities, and desktop computers and monitors.

Personnel System: Pathologist training was required in order to improve diagnostic accuracy and comfort level, and to decrease the time needed to make a diagnosis. Cytotechnologists were trained to process all samples and to operate the sending station. Telepathology was incorporated into the daily workflow in cytology and surgical pathology. All slides from the telepathology and telet cytology cases were reviewed prior to sending out a final report.

Applications: During an 18-month period a total of 169 frozen section biopsies (FS-Bx) and 179 fine needle aspirates (FNAs) were submitted for possible telepathology. Of the 169 FS-Bx submitted a microscopic telepathology diagnosis was rendered 98 cases with a diagnostic concordance of > 95%. A telepathology diagnosis was rendered in 175 of the 179 FNA cases with a diagnostic concordance of 98%.

Conclusions: Telepathology was a cost-effective solution to providing "on-site" pathology services to a distant surgical center and was easily incorporated into the daily workflow of cytology and surgical pathology. Telepathology is an accurate diagnostic tool for providing initial assessment of gross biopsy material, FNAs, and frozen sections.

ProteoSep™: AN ALL-LIQUID PHASE ALTERNATIVE TO 1D AND 2D GELS.

Tim Barder, Eprogen, Inc., Chicago, IL

ProteoSep™ is a novel protein mapping technology and a new and powerful gel-free technique to analyze complex protein mixtures. **ProteoSep** is an all-liquid phase alternative to 2D PAGE that uses standard HPLC instrument technology to produce high resolution 2D maps of complex protein systems such as whole cell lysates, plasma and sera. A unique high performance chromatofocusing (CF) column has been developed to provide the pI information biologists need in the first dimension analysis of complex protein systems. The use of HPLC provides these pI fractions in the liquid phase containing the intact proteins. Subsequent analysis of these pI fractions with proprietary reverse phase *NPS*[®] columns provides for the second dimension separation information based on hydrophobicity or MW, depending on whether you use UV or MS detection or both. A "2D protein map" is produced using the **ProteoSep Software Suite**, which provides either UV/pI or Mass/pI maps or both, displaying the proteins present in bands like that presented in a 2D PAGE gel. The all-liquid phase format provides for easy collection of sample fractions either at the pI stage or after the *NPS* analysis using conventional automation and multi-well plate fraction collectors and autosamplers without the need for

complicated gel extractions. Typical analyses of whole cell lysates yield >1000 proteins with detection limits at or better than silver stained gels.

**THE FUTURE OF CYTOMETRY BY THE NUMBERS:
3 (Rs), 20/20 (Vision), 50-50 (Distribution), 100 (% Relevance), 1M (\$)
Dan Farkas, Carnegie Mellon University and University of Pittsburgh**

Cells are the basic *units* of life, richly deserving our interest, especially when intact. Optical methods yield most versatile approaches, with studies conducted in 0-4 dimensions (by flow and imaging), non-invasively. Microscopy - an icon of the sciences and long the dominant technique - has enjoyed a veritable renaissance recently, with current technology allowing dynamic 3-D study of molecules in action, within *living* cells.

Relying on improvements in image acquisition, further potentiated by digital analysis, great progress has been made [1], and will be reviewed briefly, emphasizing some of our contributions: automated microscopy workstations allowing live monitoring of subcellular events [2], enhanced spectrally and temporally by acousto-optic tunable filters [3-5]. Microscopic imaging with simultaneous spatio-temporal resolution an order of magnitude better than current standards, as well as selected applications [6,7] will be discussed, with emphasis on the enabling technological advances and their engineering underpinnings.

In spite of these achievements, a very old cautionary urging from Francis Bacon [8] still holds true: “...but the inadequacy of these microscopes, for the observation of any but the most minute bodies, and even those if part of a larger body, destroys their utility; for if the invention could be extended to greater bodies, or the minute parts of greater bodies, so that...the latent minutiae and irregularities of liquids, urine, blood, wounds, and many other things could be rendered visible, the greatest advantage would, without doubt, be derived.” In view of this, we developed extensions of the methods described, in order to move closer to the ultimate in cytometry: cellular/molecular detection, resolution and quantitation at the tissue, organ and whole body level [9-13], preferably in *living* animals and humans. The potential of such mesoscopic imaging for addressing important medical challenges, including neurobiology, tissue engineering and cancer [14-21] will be illustrated with relevant results.

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**HIGH-THROUGHPUT CELL IMAGING AND LASER-BASED MANIPULATION ON THE LEAP™ PLATFORM
Fred Koller, Cyntellect, San Diego, CA**

Numerous methods have been developed that rely on lasers to study and manipulate cells and tissues. Examples include inactivation of specific proteins or genes (e.g., chromophore-assisted laser inactivation), optoinjection of genes or macromolecules, activation of photosensitive agents (e.g., uncaging), photo-bleaching (e.g., motility/diffusion studies), and killing (e.g., cell purification). However, these potentially powerful techniques have been developed on low-throughput manual microscope systems, hampering their widespread use. To fully reap the benefits of these techniques, a novel automated imaging and laser-based processing technology for the analysis and manipulation of individual cells in a high-throughput manner has been developed. The technology, called the Laser-Enabled Analysis and Processing (LEAP™) platform, has a number of unique attributes. The LEAP platform incorporates a

moveable stage for plate handling, LED and xenon lamp illumination and excitation, adjustable magnification (2.5X - 20X), dual CCD cameras for imaging in brightfield, darkfield, phase contrast, or multi-color fluorescence, a pulsed laser with galvanometer steering for rapidly manipulating individual cells, and software for image/data analysis and process control. The LEAP platform rapidly captures images of cells *in situ* (e.g., in a well plate) through a combination of galvanometer and stage motions, thereby limiting stage movement (and cell displacement), while still achieving throughputs over 100,000 cells/sec. This speed enables reading of cell-based assays (e.g., live/dead, apoptosis, etc.) in 1536 well plates in <5 minutes. If desired, the laser can be fired at individual cells with specified criteria to achieve various cellular manipulations. The LEAP platform has been applied in several research settings to analyze, purify, and optoinject various macromolecules into, different non-adherent and adherent cell types. For example, LEAP has been used in experiments to address the controversy surrounding the phenotype of the hematopoietic stem cell. In this case, LEAP was used to detect and eliminate the 0.01 – 0.30% of CD34⁺ cells that were left behind after two-pass flow cytometry sorting for CD34⁻ cells, yielding a more accurate putative stem cell population that was used for *in vivo* stem cell assays. The more highly purified CD34⁻ cells led to a different *in vivo* engraftment profile, indicating their more primitive status than CD34⁺ cells. Additional examples of cell manipulations, such as optoinjection of cells with cell impermeable dyes, GFP-encoding plasmids, oligos, and 3 - 70 kD dextrans with high efficiency and yield, will be discussed. These capabilities make LEAP a powerful new cell analysis and processing technology with many potential applications.

LAB-ON-A-CHIP MICROFLUIDIC CELL ASSAY: A NEW TECHNOLOGY FOR FLOW CYTOMETRY

Jay Pink, Agilent Technology

The main concepts of Lab-on-a-chip technology are to achieve a drastic reduction of sample and reagent volume, reduction of analysis time and automation of lab processes. Here we present the new implementation of cell assays on disposable microfluidic chips. These applications are based on the controlled movement of cells by pressure-driven flow inside networks of microfluidic channels. Cells are hydrodynamically focused to a portion of the microchannel before passing the fluorescence detector in single file. Dual light sources enable two-color detection of fluorescently labeled cells within the channels. Specific advantages of a chip-based microfluidic system are the low number of cells required for analysis and the ease-of-use. This is of great importance when working with small samples of homogenous cells derived from tissue or other precious primary cells. Initial applications of the technology are the antibody staining for surface and intracellular protein expression, determination of cell transfection efficiency and apoptosis detection. Transfection data were obtained from cells expressing green fluorescent protein (GFP) and from antibody-stained cells. Counterstaining with a live cell dye facilitates the accurate determination of transfection efficiency. As an apoptosis parameter, we studied the externalization of phosphatidylserine (PS) to the outer cell membrane leaflet. In this assay, calcein staining was used to differentiate live apoptotic cells from dead cells. For all applications preparation of fluorescent-labeled cell samples can be done in the same way as in conventional flow cytometry. Importantly, we developed staining procedures working ‘on-chip’, eliminating time-consuming washing steps. These procedures require only 30,000 cells per sample and nicely demonstrate the advantages of the microfluidic technology by reducing sample and reagent volumes dramatically. Results obtained with the microfluidic chips showed good correlation with data obtained using a standard flow cytometer.

A LARGE PARTICLE FLOW SORTER

Bo Wang, Applications Engineer and Biologist, Union Biometrica, Inc.

Until now, limited flow sorting capabilities have been available to researchers studying gene function or toxicology in model organisms such as *C. elegans*, *Drosophila*, and zebrafish, primarily due to the larger size and viability requirements of these researchers. COPAS instruments automate the analysis, sorting, and dispensing of objects from 40 - 1000 microns using object size, optical density, and intensity of fluorescent markers as sorting criteria. The specially designed gentle pneumatic sorting mechanism located after the flow cell does not harm or change objects, making the instrument suitable for live biological organisms or even sensitive chemistries such as those used in Bead-based Combinatorial Chemistry Libraries. COPAS instruments allow for multiple fluorescence excitation and emission wavelengths with sensitivity for GFP, YFP, DsRed, as well as other commercially available fluorophores.

UNION BIOMETRICA INC. COMPANY DESCRIPTION

Union Biometrica offers large particle flow-sorters (COPAS) for particles from 40 - 1000 microns for analysis, sorting, and dispensing of combinatorial chemistry beads, seeds, or viable small model organisms such as *C. elegans*, *Drosophila*, and zebrafish. Objects are sorted by optical density, size, and two-colors of fluorescence. The specially designed gentle pneumatic sorting mechanism located after the flow cell does not harm or change objects, making the instrument suitable for live biological materials or sensitive chemistries. We also offer ultra-sensitive microscopic readers (MIAS-2) and imaging applications (eaZYX software) for High Information Content Screening assays using cellular, tissue and small animal model assays.

POSTERS

Novel DcR3 (TR6) Analog Inhibits Fas ligand Induced Apoptosis of Target Cells in Whole Blood. **Lisa Green and Philip Marder, Lilly Research Laboratories**

1

The binding of Fas ligand (FasL) to cell surface Fas receptor (Fas) is an important pro-apoptotic initiator in normal physiology and in varied disease states. Fas/FasL induced apoptosis has been shown to contribute to the elimination of virus-infected and tumor cells as well as in hepatocyte apoptosis in liver disease. Recently, other researchers have shown that Fas/FasL interactions may be modulated by members of the TNF receptor family, such as the Decoy Receptor 3 (DcR3), also known as TR6. DcR3, a naturally secreted TNF receptor family member has been shown to inhibit FasL induced apoptosis *in vitro* and *in vivo*. Colleagues in our institution have prepared a molecularly engineered analog of the native DcR3 structure that we term Fas Ligand INhibitory proTein or FLINT analog (FLINT_a). Our data show that FLINT_a inhibits soluble Fas ligand-induced Jurkat cell apoptosis in tissue culture medium and in 100% human plasma. Our results show that, unlike Jurkat cells, human peripheral blood lymphocytes are not readily susceptible to FasL-induced apoptosis. So, in order to study the activity of FLINT_a in a whole blood milieu, we developed a heterologous cell assay system. First, we constructed a Green Fluorescent Protein- (GFP) expressing Jurkat cell line by transfection and cell sorting. When these cells were spiked into human whole blood, they were readily distinguishable from normal leukocytes after erythrocyte lysis and flow cytometric analysis. In our heterologous assay, GFP Jurkats were suspended in heparinized whole blood containing soluble FasL (25 ng/ml). After 5 hours we determined that the GFP fluorescence signal disappeared. Using an absolute count technique (Coulter Flow-Count™), we demonstrated that the Jurkat cell disappearance was due to a combination of cell lysis and GFP quenching or leaching. Addition of FLINT_a to the sFasL-containing blood protected the GFP-Jurkats in a concentration dependant manner. When GFP Jurkats were added to EDTA whole blood and treated with sFasL for shorter periods (1 – 3 hours) most of the Jurkat cells remained intact and exhibited a strong GFP signal. Up to 90%, of these cells, however, also stained positive for the active form of the apoptotic effector enzyme caspase 3, using two-color flow cytometric analysis of the blood samples. Our results showed that FLINT_a inhibited the generation of active caspase 3 in these cells with an IC₅₀ ~ 100 ng/ml. In summary, our flow cytometric method demonstrates that FLINT_a can interrupt Fas/FasL -induced apoptosis of target cells suspended in human whole blood.

A Simple, Remotly Accessable, Database to Track Experiments in a Flow Cytometry Core Laboratory.

2

Richard D. Schretzenmair, William Murphy, Charles H. Pletcher, Jonni Moore, Abramson Cancer Center Flow Cytometry and Cell Sorting Shared Resource of the University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania

A simple and inexpensive searchable electronic database was developed to maintain experiment setup for each cell sort performed in our laboratory. This database replaces the previous paper records which were organized by date. It was developed in Filmaker Pro and uses the Filemaker's Web Companion to make the database available over the internet via web browsers to our remote locations. Web Companion allows up to ten differernt connections (determined by TCP/IP address) in a rolling 24 hour period. Records for all experiments performed on our three cell sorters are stored in a single location and are available at any of our laboratory locations. Access to data is protected by password. Most simple functions like data entry and search functions are avaiable through the web browsers, some more complex functions are only available using the Filemaker interface. This allows us to easily search the database and duplicate instrument setup in future experiments. The utility of this approach is further strengthened by interfacing this database with our general usage database. With this combination, we can generate billing as well as usage demographics and workload statistics.

FLOW CYTOMETRIC MEASUREMENT OF CELL PROLIFERATION: Characterization of PTIR271 as a Long Red Cell Division Tracker.

3

A. Bantly¹, **K. Muirhead**², **B. Gray**³, **E. Breslin**³, **B. Ohlsson-Wilhelm**², **J. Moore**¹. ¹Dept. of Pathology, Univ. of PA, Philadelphia, PA. ²SciGro, Inc., Malvern, PA. ³PTI Research, Inc., Exton, PA.

Goal: CFSE and PKH26 are used to track cell division history in lymphocyte subsets but are difficult to compensate due to high intensity signals and overlap with other visible fluors. Our goal was to determine whether PTIR271, a long red emitting PKH26 analog (ex. 626 nm, em. 647 nm), could simplify color compensation for multicolor cell proliferation studies.

Methods: Human PBMC were isolated; stained with CFSE, PKH26 or PTIR271; cultured with either CD3 + IL-2 or no stimulus for 3-5 days; harvested; counterstained with CD4 and CD8 antibodies; and analyzed on either a FACScan or FACSCalibur.

Results/Conclusions: PTIR271 was excited by both the 488 nm laser (~1% efficiency) and the 635 nm laser on the FACS-Calibur. By taking advantage of the 488 nm excitation, we were able to demonstrate that PTIR271 can be used in combination with FITC and PE in a multiparameter analysis of lymphocyte subset proliferation. This strategy offers an alternative to the PKH26 and CFSE protocols that may provide more flexibility in the analysis of proliferating subsets.

COST EFFECTIVE QUALITY CONTROL SOLUTION FOR T-CELL SUBSET ABSOLUTE ENUMERATION BY FLOW CYTOMETRY

4

Bergeron M, Lobo S, Ding T, Phaneuf S, **Mandy F**
National HIV Immunology Laboratory, Health Canada, Ottawa, Ontario

Introduction: CD4 T-cell numbers remain the best surrogate marker for monitoring immune status of HIV infected individuals. Single-platform flow cytometry technology is being used increasingly for the determination of absolute T-cell counts. Implementations of rigorous quality control measures are essential to ensure both precision and accuracy of the CD4 T-cell counts. Commercial stabilized blood products are available, however some more cost-effective solution were considered. The suitability of frozen whole blood specimens were evaluated as potential quality control material for monitoring the intra-laboratory variation of both relative and absolute lymphocyte measurements.

Objective: To study the suitability and stability of frozen whole blood preparations as quality control products for T-cell subset immunophenotyping.

Method: 2 HIV+ and 2 HIV- EDTA whole blood specimens were aliquoted within 2 hours of venipuncture and stored at -80°C. Immunophenotyping was performed at various time point using a four-color mAb combination Cyto-Stat tetraCHROME , CD45 FITC/CD4RD1/CD8ECD/CD3PC5. The stained preparation was then lysed with ImmunoPrep lysing reagent and processed ON a TQ-Prep workstation. Flow-Count fluorospheres were added as a calibrator bead to calculate the absolute values. All samples were analysed on an Epics- XL-MCL instrument. The results obtained from fresh specimens for both absolute and relative T-cell subset were used as baseline values. The aliquots were tested every week for 1 month. Mean, SD and %CV were generated for each time study.

Result: % CV's obtained with all four specimens were below 10% for absolute counts and below 5 % for relative lymphocyte measurements.

Conclusion: This preliminary study suggested that frozen whole blood retains sample integrity for at least one month-supporting its use as a suitable quality control material for HIV immunophenotyping.

MODIFYING A BENCHTOP FLOW CYTOMETER FOR INFRARED EXCITATION AND DETECTION

5

Ed Podniesinski and Carl Stewart, Roswell Park Cancer Institute, Buffalo, NY

Recent developments in solid state Laser technology have provided alternative excitation sources for the Flow Cytometry industry to choose from. Compact solid state lasers are being produced with wavelengths beyond the 635nm Laser pointers, at reasonable pricing. A small infrared laser diode is being produced which is the size of a "C" cell battery. A Becton Dickinson FACScan normally equipped with an argon-ion 488nm Laser was modified by removing this stock source and replaced with a 785nm laser diode at 44-mw-output power for the fraction of it's cost, size and power consumption. Scatter parameter measurements, florescence detection, and hardware challenges will be presented. Supported by DOE SBIR #DE-FG02-01ER83134

NEW LIFE FOR A FACSTAR CELL SORTER

6

Ed Podniesinski and Carl Stewart, Roswell Park Cancer Institute, Buffalo, NY

A single Laser FACStar cell sorter has been neglected due to it's antiquated computer acquisition hardware and operating system. New life has been given to this sorter of long service by a merge with current sorter technology. A discarded FACS Vantage card cage electronics and some interface modifications have turned our FACStar into a modern 3 color single Laser cell sorter. The modified FACStar operates like a Vantage running Cell Quest software within the Macintosh operating environment. Pictures and data will be displayed to show the results of the transformation. Supported by DOE SBIR #DE-FG02-01ER83134

NEW SOLID STATE LASERS TO REPLACE CURRENT AIR COOLED SYSTEM USED BY BENCH FLOW CYTOMETERS

7

Ed Podniesinski and Carl Stewart, Roswell Park Cancer Institute, Buffalo, NY

New solid-state laser sources of comparable wavelengths have appeared to replace current air-cooled models. A 488nm and 532nm diode laser (both capable of producing 20mw output power) was installed separately in a standard bench flow Cytometer as the interrogating laser. Installation photos and data acquired using these sources will be displayed. Supported by DOE SBIR #DE-FG02-01ER83134