

Flow Cytometry Instrumentation and techniques; keeping up with the Changes

The BioPark Hertfordshire, Welwyn Garden City, AL7 3AX: 19th November 2010

An eclectic range of applications will be discussed covering numerous areas of biology, including new developments in instrumentation. Meeting Chair: *Dr Gary Warnes*, Flow Cytometry Manager, Flow Cytometry Core Facility, London University, UK

This event has CPD accreditation and will have a troubleshooting panel session.

On registration you will be able to submit your questions to the panel that will be asked by the chair on the day of the event

9:00 – 9:45 **Registration**

9:45 – 10:00 **Introduction by the Chair:** *Dr Gary Warnes*, Flow Cytometry Manager, Flow Cytometry Core Facility, Blizard Institute of Cell & Molecular Science, Barts and The Royal London School of medicine and Dentistry, London University, UK

10:00 – 10:30 **Current challenges in practical flow cytometry**

Dr. Anna Petrunkina, Cambridge Institute for Medical Research, Cambridge, UK

Besides obvious technical challenges associated with the development of new instruments and techniques, there are major challenges in practical flow cytometry related to experimental design and operating procedures. Working with heterogeneous biological material, optimising optics, combining fluorescent dyes for simultaneous use and applying a variety of gating strategies to select populations of interest all require careful consideration to avoid generation of artefacts. Basing my remarks on the daily running of a core facility operating in a research environment, I will focus on reviewing major challenges of practical flow cytometry with respect to experimental planning and preventive troubleshooting. Myths, prejudices, and doubtful practices such as identifying viable cells by light scatter or misinterpreting data due to morphological and technical artefacts will be considered.

10:30 – 11:00 **Flow Cytometry of Micro & Nanovesicles**

Dr Paul Harrison, **Oxford Haemophilia & Thrombosis Centre**, Oxford

Circulating micro & nanovesicles (MV) are becoming recognised as potentially important biomarkers of disease and their measurement is becoming increasingly popular. MV are heterogeneous (derived from platelets, leucocytes, erythrocytes and the vascular endothelium), small (between 50 nm and 1.0 microns), express antigens from their cell of origin and are often procoagulant (with expression of anionic phospholipid). There are now a number of different methods available to measure MV within whole blood and/or platelet free plasma (PFP). One problem is that there is considerable variation in methods of blood sampling, centrifugation (number of steps, time, g values etc) and storage of samples for MV analysis all of which can significantly affect their measurement.

Although flow cytometry is the most widely used technique for measuring MV there are a number of significant limitations and standardization issues. Although different MV can be easily identified by their small size and phenotype, the detection of particles < 0.5 microns is often difficult as this is close to the wavelength of the most commonly used lasers (i.e 488 nm). The resolution of MV from electronic and background noise can also be variable between instruments depending upon instrument settings (e.g. flow rate and FS gain), filtration of sheath fluid, instrument age and manufacturer coupled with the level of maintenance (e.g. laser alignment, replacing instrument tubing and cleanliness of the flow cell). However, not all MV are positive for an individual marker (e.g. PS). One approach around the standardization problems is to use a commercial mixture of beads (Megamix, Biocytex) to standardize the gating of MV between 0.5 and 1.0 microns with the controlled elimination of electronic and background noise. This helps to control for within and between instrument variation and has been shown to be successful for standardising MV measurements in a recent large international study by the ISTH Vascular Biology SSC.

Application of alternative measurement techniques (e.g. Atomic Force Microscopy and Nanoparticle Tracking Analysis, Nanosight) has recently shown that there are a very large number of smaller MV that potentially remain undetectable by conventional flow cytometry. However, some of the new generation cytometers now exhibit improvements in their ability to improve light scatter collection of Forward Scatter e.g. the W2 (wide angle) option on the Navios/Gallios (Beckman Coulter). This not only allows the discrimination of 0.3 and 0.1 micron beads but facilitates detection of a much smaller population of MV down to 0.3 microns. Other potential improvements include the capture of more light with a numerical aperture microscope lens (e.g. the small particle option on the Influx sorter, Becton Dickinson), aperture impedance flow cytometry (e.g. Quanta, Beckman Coulter) and an instrument designed for measuring small particles (e.g. A50 system, Apogee).

Flow cytometry still remains the most popular method for measuring MV but has some significant limitations that can be controlled to an extent by good instrument maintenance, optimal settings and the use of Megamix to improve standardization. Newer generation cytometers are now beginning to be used and emerging data suggests that the total pool of circulating MV may be significantly greater in number and smaller in size than previously recognised. The ISTH vascular biology SSC is now addressing some of the standardization issues and it is envisaged that optimal standardized protocols for preparing and measuring plasma samples will become available in the future. This, coupled with the application of existing and new technologies to the measurement of MV within health and disease, could lead to some very exciting developments including accurate identification and quantification of novel biomarkers in various diseases.

11:00 – 11:10 **Speakers photo**
11:10 – 11:30 **Mid-morning break**

11:30 – 12:00 **Microbial flow cytometry**
Dr. Roy Bongaerts, Institute of Food Research (IFR), Norwich Research Park, Colney

12:00 – 12:30 **Strategies for Single Cell sorting**
Dr Guglielmo Rosignoli, Flow Cytometry Core Facility, Queen Mary University Of London
Single cell sorting analysis is increased in demand in conjunction with the development of sensitive single cell analysis molecular biological techniques for both RNA and DNA analysis. Single cells can be sorted in different collection devices. Microscope slides with microarray reagent embedded are commercially available One issue related to single cell-sorting analysis is validation of the single cell sort. We developed a staining and gating strategies which enable us to confirm the presence of a single cell in the slide reaction well to support the validity of the array.

12:30 –13:30 **Lunch and Poster Viewing**

13:30 - 14:30 **Question and Answer Session**
Delegates will be asked to submit questions to a panel of experts. Questions can be submitted before the event or on the day

14:30 - 14:45 **Automated multi-dimensional analysis of differentiation phenotypes of Tregulatory cells**
Irfan Zaidi, MRC Laboratories, The Gambia
This talk will discuss the automatation of the identification of naïve and memory subsets of CD4⁺FOXP3⁺ from healthy controls and HIV infected patients.

14:45 - 15:15 **Point of Care Blood Cell Analysis**
Dr Judith Holloway, University of Southampton School of Medicine, UK
Miniature high speed label-free cell analysis systems have yet to be developed, but could potentially deliver fast, inexpensive and simple full blood cell analysis systems for routine clinical practice. We demonstrate a microfluidic single cell impedance cytometer that performs a white blood cell differential count. The device consists of a microfluidic chip with micro-electrodes that measure the impedance of single cells at two frequencies. Human blood flows through the device and a complete blood count is performed in a few minutes. Verification of cell dielectric parameters was performed by simultaneous fluorescence-based immunophenotyping. Tests with patient samples correlated with commercial blood analysis equipment, demonstrating potential clinical utility of the impedance microcytometer for point-of-care blood analysis.

15:15 – 15:45 **Afternoon Tea/Coffee and Last Poster Viewing**

15:45– 16:15 **Autophagy detection using the Image stream**
Dr Katja Simon, Group Leader and Head of Translational Immunology Core lab, Oxford University
Autophagy is one of the cell's main degradation pathways for which molecular players have just been identified. Autophagy is a very novel and exciting field and plays a role in neurodegeneration, autoimmunity, cancer, liver and muscle diseases and ageing. Detection of autophagy is difficult, especially at high throughput and on primary cells, as it relies on intracellular localisation and co-localisation. We established a robust and quantitative technique using the Image stream, an imaging flow cytometer, detecting autophagy on peripheral blood mononuclear cells. This technique is currently being applied to samples of patients suffering from diverse diseases by Oxford researchers.

16:15 – 16:45 **Flow FISH: the pro's and con's of telomere length estimations**
Dr Gary Warnes, Flow Cytometry Manager, Flow Cytometry Core Facility, Blizard Institute of Cell & Molecular Science, Barts and The Royal London School of medicine and Dentistry, London University, UK
The Dako Telomere PNA Kit/FITC uses a synthetic DNA/RNA analog specific for the TTAGGG nucleotide sequence of telomeres and is optimised so that fluorescence intensity of the PNA signal is directly proportional to the length of the telomere and has a good correlation to the Southern Blot determination of telomere lengths. After performing in situ hybridization of the PNA probe into a mixture of control cells of known telomere length and cells in which the telomere length is unknown cells are stained with a sub-optimal amount of propidium iodide. Flow cytometry performed for cell cycle analysis of G0/1 cells and the PNA-FITC signals compared to cells without the PNA-FITC probe. From median fluorescence values of PNA-FITC and background signals of control and test cells the relative telomere length (RTL) can be determined.

16:45 - 17:00 **Chairman's summing up**

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About the chair

Gary Warnes interest in flow cytometry started at St. Mary's in 1986, analysing T-cell subsets. Then set up a new flow cytometric T-cell subset service at St. Thomas' Hospital. Completed a PhD investigating the immunosuppression of HIV-ve haemophiliacs at St. Thomas' Hospital. Post-doctoral position, investigated the regulation of Tissue Factor expression by immune costimulatory molecules in sepsis. Then managed the Flow & Imaging Core Facilities at the MRC Clinical Science Centre at Hammersmith Hospital. Worked with Derek Davies at Cancer Research UK. Currently managing the Flow facility at the Blizard Institute of Cell & Molecular Science, Queens Mary's College.

About the Speakers

Anna Petrunkina started as theoretical physicist in St-Petersburg, then moved into cell biology. She conducted research in Germany, at Veterinary School Hanover, where she holds an associate Readership in Bioengineering and Reproductive Biology. Anna's interest in flow cytometry originated at Babraham Institute in 1997, while studying mechanisms which regulate sperm function and are crucial for fertility. Then, back to Hanover, she set up a first departmental cytometry facility. Currently she is Head of Flow Cytometry at the Cambridge Institute for Medical Research. She retains an active research interest in reproductive biology and serves on Editorial Board of 'Reproduction, Fertility and Development'.

Guglielmo Rosignoli, - 2000-2005 Research Assistant William Harvey Research Institute, working on rheumatoid arthritis and inflammation. 2005-2009 PhD on T-cell immunology Department of Immunology Chelsea & Westminster Hospital Imperial College focusing on T-cell memory subsets, T cell receptor co-stimulatory molecules and anti-HIV-specific responses characterization in HIV-1 infected individuals.

2010-present: Lab manager for the Flow Cytometry Core Facility for the William Harvey Research Institute and the Institute of Cancer at St Bartholomew's Hospital Queen Mary University of London.

Katja Simon is a group leader at the Weatherall Institute of Molecular Medicine in Oxford, and she is also the head of the Translational Immunology lab funded by the BRC. Her research group is interested in autophagy in the haematopoietic system. In preclinical models her lab established that autophagy is important for the removal of mitochondria during red blood cell maturation. In the absence of autophagy, anaemia develops which strongly resembles human myelodysplastic syndrome (MDS). The detection of autophagy at high throughput in primary cells using the Image stream will enable her team to observe functional changes in human MDS samples.

Judith Holloway completed her doctorate in 1999 developing flow cytometric methods for the identification and isolation of dendritic cells from peripheral blood and their application to study these in atopic asthma. Following this, Judith undertook postdoctoral studies of gestational immunology, investigating the development of the human immune system, in particular dendritic cells, during pregnancy and the first year of life. More recently, in collaboration with Professor Hywel Morgan (School of Electronics and Computer Science), Judith helped to develop a micro-flow cytometer (μ FACS) capable of performing impedance measurements on human leukocytes giving a 3-part leukocyte discrimination system. In addition to analysis of blood using label-free technology, fluorescent events linked to cells and bioparticles can also be measured. In 2007 Judith was appointed Lecturer in Allergy and Programme Director of the MSc Allergy.

Paul Harrison is a Clinical Scientist within the Oxford Haemophilia & Thrombosis Centre at the Churchill Hospital in Oxford in the UK. He is the current secretary of the British Society of Haemostasis & Thrombosis and a Fellow of the Royal Society of Pathologists. Paul obtained his PhD in 1988 and since then his scientific research has focussed on haemostasis and thrombosis with an emphasis on platelet biology and platelet function testing. He is a member of the editorial boards for the Journal of Thrombosis and Haemostasis, Platelets and the International Journal of Laboratory Haematology. He has published over 100 papers and book chapters/reviews in his field. Recently he has become interested in new technologies for the detection and measurement of microparticles. He was the winner of the Sysmex Outstanding Science Award in 2009 for his work on immature platelets. Paul has recently been featured as one of the top 100 healthcare scientists in the UK by the Department of Health.

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THE USE OF DRAQ5 TO MONITOR INTRACELLULAR DNA IN *ESCHERICHIA COLI* BY FLOW CYTOMETRY

F. Silva, O. Lourenço, C. Pina-Vaz, A. G. Rodrigues, J. A. Queiroz, F. C. Domingues

Postal Address: CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Avenida Infante D. Henrique, 6200-506 Covilhã, Portugal

Flow cytometry provides a rapid and high-content multiparameter analysis of individual microorganisms within a population. In the past years, several fluorescent stains were developed in order to monitor DNA content distribution and cell-cycle phases, mainly in eukaryotic cells. Recently, due to its low detection limits, several of these fluorescent stains were also applied to prokaryotic cells. In this study, the ability of a novel far-red fluorescent stain DRAQ5 in assessing intracellular DNA content distribution in *Escherichia coli* DH5 α was evaluated. The results showed that a DRAQ5-labelled live *E. coli* suspension can be obtained by incubation of 1×10^6 cells/mL with 5 μ M DRAQ5 in PBS buffer supplemented with EDTA (pH=7.4) during 30 min at 37 °C. Flow cytometric analysis of fixed *E. coli* cells revealed that ethanol should be used in detriment of glutaraldehyde for DRAQ5 labelling. After the analysis of RNase and DNase digested samples, DRAQ5 was proven to be a specific DNA labelling stain. The present study demonstrates that the use of DRAQ5 as a DNA-labelling stain provides an easy assessment of intracellular DNA content and cell-cycle phases in Gram-negative bacteria such as *E. coli*.

BACTERIOSTATIC VERSUS BACTERICIDAL ACTIVITY OF CIPROFLOXACIN IN GRAM NEGATIVE BACTERIA ASSESSED BY FLOW CYTOMETRY USING A NOVEL FAR-RED DYE

F. Silva, O. Lourenço, J. A. Queiroz, F. C. Domingues

Postal Address: CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Avenida Infante D. Henrique, 6200-506 Covilhã, Portugal

The global emergence of antibacterial resistance among pathogens over the last decade lead to a more rationalized and strategic application of antimicrobial agents. The use of bactericidal rather than bacteriostatic agents as first-line therapy is recommended because the eradication of microorganisms serves to limit the development of bacterial resistance. However, even when bactericidal drugs such as the fluoroquinolones are used, one should verify if the dose is sufficient to cause bacterial killing, because depending on the dosage, they could either have a bacteriostatic or a bactericidal activity. Flow cytometry is becoming a powerful technique for the analysis of microorganisms, namely to study cell cycle in bacterial population. This technique enables the quantification of the distribution of cells at several cell cycle stages which is highly important to study cell growth and to monitor and model cell division. Since common microbiology methods for the assessment of bacteriostatic or bactericidal activities are very time-consuming, in this work we describe the use of a novel far-red fluorescent stain, Vybrant® DyeCycle™ Ruby (DCR) for the flow cytometric analysis of a fluoroquinolone (ciprofloxacin) bacteriostatic and bactericidal activity in Gram-negative bacteria. DCR proved to be specific for bacterial DNA and, after ciprofloxacin exposure, DNA distribution analysis was achieved using a 5 μ M DCR concentration to stain 5×10^5 ethanol-fixed bacterial cells. The analysis of the bacterial DNA histograms obtained for the ciprofloxacin concentrations tested, enabled the distinction between ciprofloxacin bacteriostatic and bactericidal activities

AUTOMATED MULTI-DIMENSIONAL ANALYSIS OF DIFFERENTIATION PHENOTYPES OF T REGULATORY CELLS

I. Zaidi, M. Holland, D. Jeffries*

MRC Laboratories, Atlantic Road, Fajara, The Gambia, izaidi@mrc.gm

* Corresponding author djeffries@mrc.gm

Natural T regulatory cells (Tregs) are an important subset of CD4 T cells that have been shown to modulate responses to self and foreign antigens. These cells are typified by the constitutive expression of the transcription factor Forkhead Box Protein 3 (FOXP3) but it has recently become evident that CD4⁺FOXP3⁺ cells represent a heterogeneous population of naïve and memory cells as judged by the expression of differentiation markers CD45RO, CD27 and CD28.

Currently such populations are categorized by cross-hairs for 2-D marker distributions. This leads to highly subjective classifications with poor reproducibility and is limited to multiple pair wise combinations of markers. These procedures can be onerous for high throughput studies and are often performed un-blinded, which can lead to bias.

Automation procedures have focused on clustering algorithms, but due to the lack of structure in the event distributions of differentiation phenotypes of Tregs, they have limited application for this type of data. The approach in this paper uses a maximum likelihood clustering metric, but applies it to separation by cut-off, rather than classification into an unknown number of clusters of arbitrary shape. The likelihood is a function of the within cluster covariance matrices, which have the same order as the number of markers and is applicable to any number of dimensions.

The algorithm is easy to integrate into an automatic processing tool, already published by the authors and provides full automation of the procedure from lymphocyte identification through to statistical analysis. The data driven approach to cut-off location leads to a reproducible and consistent solution. Latin Hypercube Sampling, with intervals around the chosen cut-offs, allows sensitivity to be quantified using partial correlation coefficients (measures the relationship between one marker cut-off and the outcome population count, allowing for the effect of the other markers). The automation allows the Latin Hypercube samples to be incorporated into the statistical analysis of patient cohorts, allowing rapid identification of both marker and subject outliers.

Using the above outlined approach it was possible to automate the identification of naïve and memory subsets of CD4⁺FOXP3⁺ from healthy controls and HIV infected patients. A 3-D analysis simultaneously yields unique cut-offs for each differentiation marker. The results show with a robust inference, that the proportion of Tregs with a naïve phenotype is similar in HIV infected individuals compared to healthy controls.

PLASMA MEMBRANE-DERIVED VESICLES CARRY TGF- β 1 AND PROMOTE ADHESION AND DIFFERENTIATION OF THP-1 MONOCYTES TO MACROPHAGES.

Ephraim A. Ansa-Addo and Jameel M. Inal

Microvesiculation is a ubiquitous cellular mechanism, which occurs as a result of exocytosis, to release exosomes (between 50-100nm) or by direct release of vesicles from the cell plasma membrane (PM), referred to, in this study as Plasma Membrane-derived Vesicles, PMVs (0.1-1 μ m). Plasma Membrane-derived Vesicles are small, intact membrane vesicles released from cells and involved in intercellular communication. We have examined the role of PMVs, distinct from exosomes, in the differentiation of THP-1 monocytes. These PMVs caused THP-1 cells to enter G0/G1 cell cycle arrest and induced terminal monocyte-to-macrophage differentiation. Use of the transforming growth factor β (TGF- β) receptor antagonist, SB-431542, showed that this was due to TGF- β 1 and its concentration on PMVs. Although TGF- β 1 levels have been shown to increase in cell culture supernatants during macrophage differentiation, and dendritic cell maturation, its presence in PMVs is yet to be reported.