Flow cytometry techniques and instrumentation: keeping up with the changes

An eclectic range of flow cytometry applications was covered when experts and researchers came together at a recent meeting organised by EuroSciCon. Clearly, in addition to its role in haematology and immunology, flow cytometry has application in diverse biological processes, as Ruth Challis reports.

Flow cytometry is used to investigate a wide range of biological processes and was the subject of a recent meeting organised by EuroSciCon, chaired by Dr Gary Warnes (Blizard Institute of Cell and Molecular Science, Barts and The Royal London School of Medicine and Dentistry, London, UK).

CURRENT CHALLENGES

The first speaker of the day was Dr Anna Petrunkina (Cambridge Institute for Medical Research, UK) who provided a comprehensive review of the main challenges in flow cytometry. In her view most of these challenges may be overcome with care and attention to experimental design and good operating procedures. She drew her examples from a wide range of applications working with heterogeneous biological material based on the experience of running a core facility within a research environment.

'Most challenges in flow cytometry can be overcome with care and attention to experimental design and good operating procedures'

Optic optimisation, compatibility and stability of fluorochromes together with gating strategy techniques in multiparameter flow cytometry were all considered to minimise artefacts and therefore maximise the generation of useful and complete data.

Further challenges considered included discrimination of doublets and the elimination of dead cells using viability stains rather than morphological interpretations based on forward/side scatter parameters alone. Preventative troubleshooting based on optimal sample preparation and good use of appropriate antibodies combined with the technical specification of the flow instrumentation were also considered and discussed.

MICRO AND NANOVESICLE INVESTIGATIONS

The second speaker, Dr Paul Harrison (Oxford Haemophilia and Thrombosis Centre, UK) discussed the growing field of circulating micro and nanovesicle particle (MV) investigation and their detection methods. The most common of these methods is flow cytometry, but Dr Harrison also described alternative measurement techniques (eg atomic force microscopy and nanoparticle tracking analysis [Nanosight]).

Circulating MVs are increasingly recognised as functionally active particles

The EuroSciCon meeting covered a wide range of flow cytometry applications.

with roles in a wide range of biological processes, as well as potentially important biomarkers of disease. They are heterogeneous in origin (ie derived from platelets, leucocytes, erythrocytes and the vascular endothelium) and very small (between 50 nm and 1 μm). One of the current major challenges in positively identifying MVs is their small size, which remains very close to, or, for the smallest particles, just below, the limit of detection by most flow cytometers. This is because the resolution is starting to be affected by electronic and background noise. While this limitation varies to some extent between individual flow cytometers, general factors that can affect resolution were presented and discussed, including filtration of sheath fluid and the level of maintenance of the cytometer (eg laser alignment, cleanliness of the flow cell and artefacts related to replacements of instrument tubing).

Despite these limitations, flow cytometry remains the most popular method for measuring MVs. Therefore, efforts are being made to standardise techniques for sample preparation, which has varied considerably in blood sampling, centrifugation (eg number of steps, time, ***g*** values etc) and storage of samples for MV analysis. Another approach that improves detection is the use of commercial standard bead mixtures (eg Megamix, Biocytex).

A recent large international study by the ISTH Vascular Biology SSC has shown improvements in the standardisation of MV measurement by these approaches. Therefore, it is envisaged that optimal standardised protocols for preparing and measuring plasma samples will be available in the future.

MICROBIAL FLOW CYTOMETRY

The theme of micro flow was continued in the presentation by Dr Roy Bongaerts (Institute of Food Research, Norwich Research Park, Colney, UK), who described the challenges facing detection of bacterial particles by flow cytometry and covered techniques and approaches to overcome these problems.

Microbial cells range in size from approximately 1–2 μm (compared to 5 μm for a red blood cell) and therefore push the limits of detection or resolution by flow cytometry. Certain factors were revisited including the need to filter the sheath fluid, although Dr Bongaerts also warned of additional complicating artefacts arising from this step (eg post-filtration bubbles). Again, tubing replacement was addressed and the need to recalibrate subsequently. However, techniques to overcome these challenges were presented, such as the use of bead standards and alternative sample preparation tactics including the sonication of samples to help prevent cell aggregation or doublet formation. Challenges specific to the analysis of microbial cells included an awareness of the influence of their cellular morphology which, unlike beads, can be far from spherical and therefore can distort their apparent size in relation to the beads.

> 'Circulating micro and nanovesicle particles are increasingly recognised as functionally active with roles in a wide range of biological processes'

Flow-activated cell sorting of microbial cells was also covered and Dr Bongaerts highlighted the many applications of this technique, particularly for studying microbial systems biology. In this area, cell sorting is particularly useful for functional genomic studies, as an aid to screening large libraries, and to aid verification of viability assessments. Dr Bongaerts concluded that, despite the many challenges, flow cytometric analysis of microbes remains a vital tool to gain greater biological understanding of this class of organism.

SINGLE-CELL SORTING

Dr Guglielmo Rosignoli (Flow Cytometry Core Facility, Queen Mary University of London, UK) described single-cell sorting analysis which was used to enable sensitive single-cell analysis of RNA or DNA, or monoclonal cell production of B-cell subsets. Using a FACS Aria, single cells were sorted on to different devices according to the downstream application. Cells sorted into 96-well plates, followed by DNA extraction, allowed the presence of single cells to be confirmed by the production of a DNA standard curve to quantify the amount of DNA in each well. In this way, DNA extraction from single cells in a 96-well plate was found to exceed 60% of the wells. For SNP allele discrimination, cells were sorted directly on to commercially available microscope slides with microarray reagent embedded on the slides for direct RT-PCR amplification of the DNA. In this case, the presence of single cells was confirmed by light microscopy.

AUTOMATED ANALYSIS

In recent years, flow technology and fluorochrome availability has meant that the number of parameters used to analyse cells has increased, but analysis techniques for gating the cell populations have remained relatively static. Most populations are still gated using sequential manually assigned two-dimensional cut-offs, which can be highly subjective and show poor reproducibility. In addition, these procedures can be time-consuming for high-throughput studies and are often performed unblinded, which can lead to bias.

The next speaker, Banky Ahadzie (MRC Laboratories, The Gambia), described his group's development of an automated multidimensional analysis program. It has been applied to polyfunctional cytokine responses in mice models, and differentiation of phenotypes of T-regulatory cells. According to Mr Ahadzie, many automated procedures have focused on clustering algorithms, but, due to the lack of structure in the event distributions of certain biological data sets, they have limited application in such settings. The approach described used a maximum likelihood clustering metric, but applied 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 automatic processing tool developed has been shown to generate reproducible, consistent and biologically relevant data that compare well with manual researcher-led analysis. The need for such automated analysis tools is becoming necessary because of the amount of flow data generated and the need for consistency between multicentre studies, particularly in an increasingly regulated environment.

MICROCHIP FLOW CYTOMETRY

At present, miniature high-speed, label-free cell analysis systems are not available commercially, but applications for such a device are virtually limitless. Dr Judith Holloway (University of Southampton School of Medicine, UK) described a microfluidic single-cell impedance cytometer that performs a white blood cell differential count with unlabelled human blood within minutes. Her vision is that such a device could transform blood cell analysis by allowing point-of-care (POC) assessments in GP surgeries or in the wider community setting.

The device consists of a microfluidic chip with micro-electrodes that measure the impedance of cells at two frequencies. Fingerprick blood is applied, it flows through the device by capillary action and a complete blood count is performed. The cell dielectric parameters have been verified by simultaneous fluorescence-based immunophenotyping using conventional flow cytometers, where tests with patient samples correlate with the commercial blood analysis equipment. This demonstrates the clinical utility of the impedance microcytometer for

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POC blood analysis. The device is due to be used in veterinary practice early this year and should be ready for clinical use in 2014.

AUTOPHAGY DETECTION

Dr Katja Simon (Translational Immunology Core Laboratory, Oxford University) described her research interests in autophagy detection using the ImageStream imaging flow cytometer (Amnis, Seattle, USA). The ImageStream instrument blends microscopy and flow cytometry by acquiring up to 15,000 cells per minute with brightfield, darkfield and four fluorescent parameter images of individual cells. Thus, the capture rate broadens the application of traditional imaging techniques to permit discrimination of rare cell populations, while the applications of traditional flow cytometry are broadened to include signal localisation and morphological analysis.

This technique is currently applied in different disease settings by Oxford researchers in a number of different ways, including autophagy detection in Parkinson's and Crohn's disease, for fluorescence *in situ* hybridisation (FISH) analysis of DNA or chromosomal changes, and for phagocytosis assays. The combination of imaging and flow capabilities of the ImageStream enable the analysis of new mechanistic targets that may prove challenging using imaging or flow cytometric analysis alone. For example, inherent challenges in the detection of autophagy, especially at high throughput and on primary cells, are due to the reliance on intracellular localisation and co-localisation of specific markers.

Dr Simon gave an interesting review of autophagy, describing its role in the development and normal functioning of the immune system as well as its involvement in certain disease settings. She illustrated how the investigation of autophagocytic cells has been aided by analysis using the ImageStream.

FLOW CYTOMETRY AND *IN SITU* HYBRIDISATION

The final talk of the day, given by Dr Gary Warnes, described telomere length estimations by FISH using flow cytometry (flow-FISH). Telomeres contain non-coding DNA repeats at the end of the chromosome, are essential for chromosomal stability and are implicated in regulating the replication and senescence of cells. The gradual loss of telomere repeats in cells has been linked to ageing and tumour development, and methods to measure telomere length are of increasing interest.

At least three methods for measuring the length of telomere repeats have been described: Southern blot analysis and quantitative FISH using either digital fluorescence microscopy (Q-FISH) or flow cytometry (flow-FISH). Dr Warnes described his experience of flow-FISH analysis.

The main advantage of flow-FISH for estimating telomere length is that it is significantly less time-consuming than either Southern blot or Q-FISH and can be completed in a single day. Further advantages include a considerable reduction in the number of cells needed for analysis, as well as the fact that data on telomere length can be acquired from individual cells and subsets of cells (eg lymphocytes and granulocytes) in the same sample. However. one limitation of flow-FISH is the need to compare test cells to a cell type of known telomere length (for which Southern blotting is needed to make this initial estimation).

FLOWING OUT OF THE LABOARTORY?

Flow cytometry remains a fast and valuable methodology for the analysis of cells. As the speakers at this meeting demonstrated, the different applications of flow cytometry are expanding to encompass new and diverse techniques to enhance our biological understanding of a wide range of processes. In addition, exciting new developments in technological hardware may transform the way we see flow cytometry used, from a laboratory-based discipline into a tool for use in the community in further new applications. ■

Speakers at the meeting provided a diverse range of expertise.

FURTHER READING

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