Flow cytometry – Getting the most information from cells.

Most high throughput or ELISA based assays measure compounds or metabolic products that are released or processed into a liquid matrix, such as serum, urine, or culture media. One main drawback of these assays is that they are several steps removed from the native cellular process, i.e. they do not give information about the source of the compounds or metabolic products. This is all the more significant when working with heterogeneous cell populations (e.g. water samples from marine or freshwater sources; peripheral blood mononuclear cells; disaggregated organ or biopsy samples). The method of choice for high throughput investigation of biological phenomena at the cellular level is flow cytometry

Flow cytometry is a laser-based fluorescence detection technology that allows high speed multi-parameter measurement of markers on large populations of cells. Cells are labeled with fluorescent reagents and are passed through a laser beam (see Figure 1).

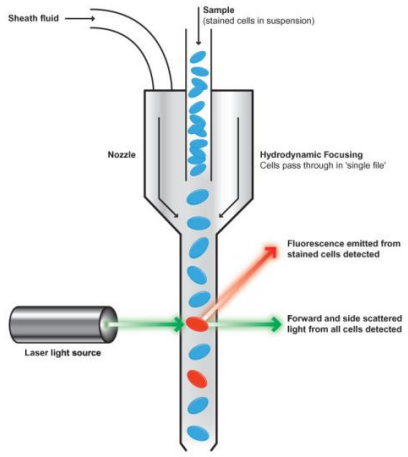

Figure 1[(a)]

The fluorescence elicited by the laser is captured and amplified by sensitive detectors called photomultiplier tubes (PMTs). The number of different PMTs in a given flow cytometer can range from 3 to as high as 18. This means, for high end flow cytometers, we can detect and measure up to 18 parameters on each cell! As a point of comparison, immunofluorescence microscopy can measure at most 3 fluorochromes.

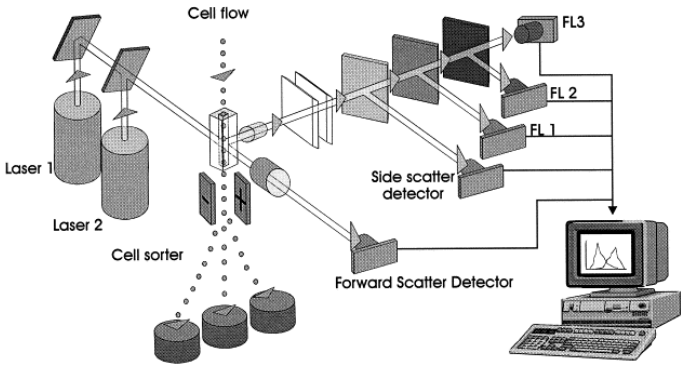

Figure 2[(b)]

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One of the distinct advantages of flow cytometry is that each individual cell is interrogated. In contrast to standard ELISAs where the analyte is measured from a given matrix such as blood, serum, urine, etc., flow cytometry allows the researcher to detect the analyte in a specific cell or a population of cells and to determine the cell's corresponding phenotypic characteristics and functional capabilities. A few examples of the extensive list of parameters that can be measured by flow cytometry are:

- Total cellular nucleic acid content (DNA or RNA)
- Cellular pigments (Chlorophyll or phycoerythrin)
- Chromosome composition
- Protein expression and localization
- Cell surface antigens or cluster of differentiation (CD) markers
- Intracellular molecules (cytokines, kinases, effector molecules)
- Apoptosis markers

Another advantage is the ability to rescue live cells after analysis when using flow cytometers specifically equipped for Fluorescence Activated Cell Sorting or FACS. This is achieved by encapsulating each cell in a fluid droplet. Once the laser has detected a particular fluorescence from a target cell, an assigned electrical charge is applied to the droplet containing the target cell. Charged plates generate an electric field that moves the droplet to a targeted location. A positive, negative or neutral charge can be applied to the droplets allowing several populations to be "sorted". In more specialized machines, single cells can be sorted directly into each well of an ELISA plate, where subsequent experiments can be performed at the single cell level. This reduces the amount of work needed in functional cell isolation studies (e.g. limiting dilution assays for specific T cell activity).

Flow cytometry has applications in many fields such as molecular biology, pathology, immunology, plant and marine biology and oncology. It is used widely in the medical field, e.g. transplantation, tumor immunology, cancer diagnosis.

In Marine Biology, flow cytometry is used to measure and monitor bacteria in picoplankton:

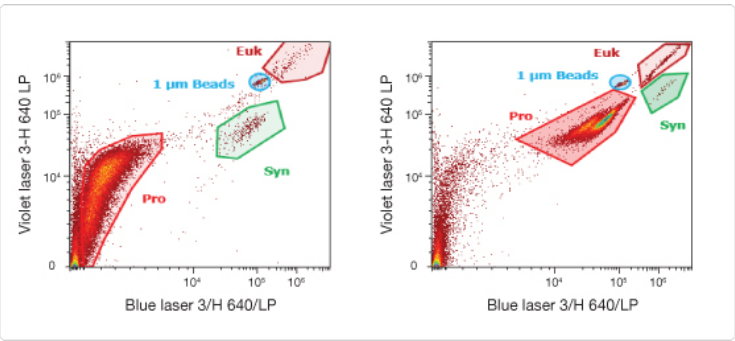

Figure 3[(c)]

Flow cytometry is critical in the monitoring of disease progression in HIV by measuring CD4 T cell counts in patients.

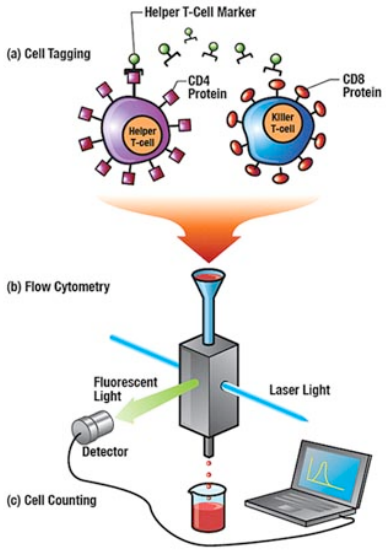

Figure 4a. [d]

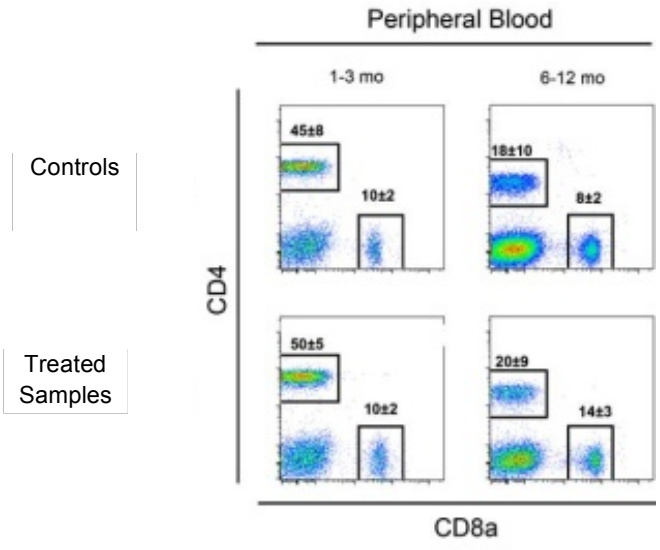

Figure 4b [e]

Although morphologic assessment of blood smears, bone marrow smears, and tissue sections remains the cornerstone of lymphoma and leukemia diagnosis and classification, immunophenotyping by flow cytometry is a very valuable and important complementary tool. Flow cytometry is routinely used in the differential diagnoses of the varied forms of leukemia. The example below (Figure 5) shows the use of

the CD20 antigen to isolate the B cells against all other bone marrow cells. The clonality of the B cell population can then be further tested by kappa or lambda light chain expression. The patient was diagnosed with low grade B cell lymphoma using these flow cytometry results.

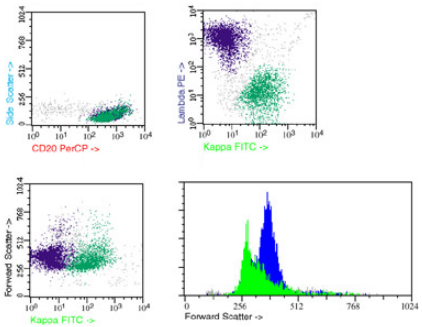

Figure 5[f]

Technological advances in digitization of emissions data from PMTs and fluorochrome design have led to increased accuracy and consistency of data acquisition and to the number of measurable parameters for flow cytometry. This has had a tremendous impact in biological research, particularly in the field of immunology. As more immune-related diseases and deficiencies are discovered, and as more of the highly intricate interactions of the human immune system are revealed, there is a corresponding increase in the need for complex cell based functional assays. As mentioned earlier, there are flow cytometers that can measure up to 18 colors. This allows for specific identification of differentiation sets and subsets of cell populations in a complex mixture, e.g. PBMC, bone marrow cell extracts.

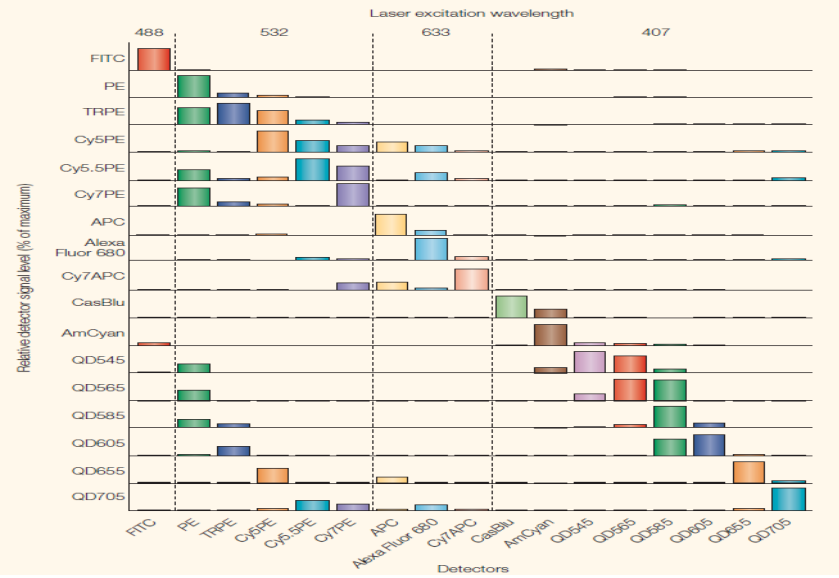

Figure 6[g]

As the number of parameters increase, the acquisition of data becomes exponentially more complicated. Part of the complexity is due to the nature of using fluorescence as the signal. The incident laser light triggers the fluorochrome to emit a range of wavelengths that can overlap with light emitted by other fluorochromes. The process by which we account for the different combinations of overlap between all the fluorochromes in a given experiment is called compensation. Calculating compensation for 18 colors is much more complicated than for 4 or 6 colors. This increasing complexity has a direct consequence on validating experiments for multi-color flow cytometry. There are many adjustable controls on a flow cytometer. These allow the user to obtain data that is easy to interpret and analyze. The laser and PMT voltages are adjustable in the optics assembly of a flow cytometer. Although these give the user flexibility in manipulating the display of the output data, these present unique problems when validating experiments performed on flow cytometers, and more so for the more recent models with 3 lasers and 18 PMTs. For cutting edge research, 18 colors from 4 lasers is the method of choice (e.g. BD Fortessa) where flexibility is the main criterion. For clinical applications, 4-6 colors from 2 lasers, with reduced user-adjustable controls, are desirable (e.g. BD FACSCanto II).

As the number of fluorochromes and corresponding PMTs increase, there are less available wavelengths in the visible electromagnetic radiation spectrum that can be used for new fluorochromes. Mass cytometry is a new technology that makes use of heavy metals as reporter molecules and mass spectrometry as the readout instrument. The CyTOF, a mass cytometer developed by the University of Toronto and DVS Sciences, is considered the next generation of multiplexed cytometers.

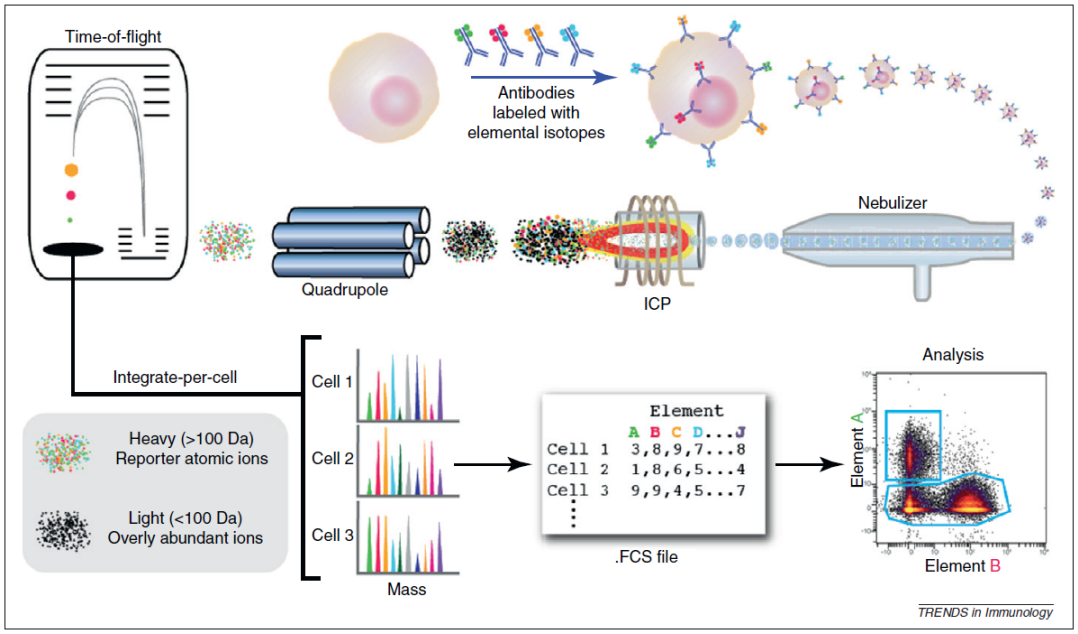

Figure 2. Mass cytometry allows single-cell atomic mass spectrometry of heavy elemental (>100 Da) reporters. Schematic of ICP-MS-based analysis of cellular markers. An affinity product (e.g. antibody) tagged with a specific element binds to the cellular epitope. The cell is introduced into the ICP by droplet nebulization. Each cell is atomized, ionized, overly abundant ions removed, and the elemental composition of remaining heavy elements (reporters) is determined. Signals corresponding to each elemental tag are then correlated with the presence of the respective marker and analyzed using conventional cytometry platforms.

Figure 7[(h)]

Conclusion

Advances in flow cytometry technology allow researchers and clinicians to ask deeper and more nuanced questions and understand biological phenomena at the molecular and cellular level. Flow cytometry plays a critical role in research studies on cancer, autoimmune and infectious diseases, and the results from these studies eventually lead to better diagnostic, preventive and therapeutic methods for all.

[a] http://www.abcam.com/index.html?pageconfig=resource&rid=11446

[b] **Brain Research Protocols**, Volume 4, Issue 3, December 1999, Pages 280-287

[c] http://www.invitrogen.com/site/us/en/home/References/Newsletters-and-Journals/BioProbes-Journal-of-Cell-Biology-Applications/BioProbes-Issues-2011/BioProbes-66-October-2011/Flow-Cytometry-Environmental-Microbiology.html

[d] Picture taken from Los Alamos National Laboratory. http://www.lanl.gov

[e] **Blood** April 9, 2009 vol. 113 no. 15 3461-3471

[f] Flow Cytometry of Leukemia. http://162.129.103.34/cgi-win/front.exe/Instr?17

[g] **Nature Review Immunology** 4 (8), 648-655.

[h] **Trends Immunology** 2012 Jul;33(7):323-32.

Flow cytometry – Getting the most information from cells.

Maloy Mangada is a Research Associate in the Research and Development group at Cambridge

Biomedical. He was trained as T cell immunologist and molecular virologist and has more than a decade's experience in flow cytometry. He has broad experience in assay development, optimization and troubleshooting. Maloy has expertise in most aspects of immunology and virology such as cell culture, handling of whole blood and blood components, plaque and neutralization assays, cytotoxicity and proliferation assays, ELISA, nucleic acid sequencing. Maloy received his Masters degree in Molecular Biology and Biotechnology from the University of the Philippines. He performed research activities at the Institute of Tropical Medicine, Nagasaki, Japan and the Center for Infectious Diseases and Vaccine Research at the University of Massachusetts Medical School.