Flow Cytometry

Karpaga Preethitha .S

Department of Pathology, University of Utah, ARUP Laboratories, Inc., Salt Lake City, UT 84132

Abstract: ***Flow cytometry is defined as a laser based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in basic research, clinical practice and clinical trials. A common variation is to physically sort particles based on their properties, so as to purify populations of interest. [1 http://en.wikipedia.org/wiki/Flow_cytometry]. Here we will discuss the principle, working and few applications of flow cytometry in various fields.***

Keywords: Laser Based, Fluid, Multiparametric

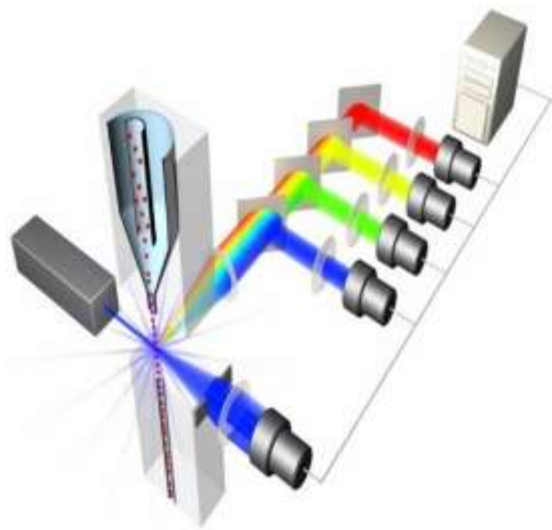
1. Introduction

The first fluorescence-based flow cytometry device (ICP 11) was developed in 1968 by Wolfgang Göhde from the University of Münster, Germany and first commercialized in 1968/69 by German developer and manufacturer Partec through Phywe AG in Göttingen. At that time, absorption methods were still widely favored by other scientists over fluorescence methods. The original name of the flow cytometry technology was pulse cytophotometry (German: Impulszytophotometrie). Only 10 years later in 1978, at the Conference of the American Engineering Foundation in Pensacola, Florida, the name was changed to flow cytometry, a term that quickly became popular. Soon after, flow cytometry instruments were developed, including the Cytofluorograph (1971) from Bio/Physics Systems Inc. (later: Ortho Diagnostics), the PAS 8000 (1973) from Partec, the first FACS instrument from Becton Dickinson (1974), the ICP 22 (1975) from Partec/Phywe and the Epics from Coulter (1977/78) [2 https://www.beckmancoulter.com]

2. Principle of Flow Cytometry

All forms of cytometry depends on the basic laws of physics, including that of fluidics, optics & electronics. It is a system for sensing cells or particles as they move in a liquid stream through laser/light beam past a sensing area. The related light scattering and colour discriminated fluorescence of the microscopic particles are measured. Analysis and differentiation of cells are based on size and granularity & whether the cell is carrying the fluorescent molecule in the form of antibodies or dye. As the cell passes through the laser beam, the light is scattered in all directions, the light scattered in forward direction at low angles (0.5-10˚) from the axis is proportional to the square of the radius of sphere and so to the size of the cell or particle. Light may enter the cell and be reflected and refracted by the nucleus and other contents of the cell. Thus the 90˚ light scattered can be considered proportional to the granularity of the cell. The cells may be labeled with fluorochrome-linked antibodies or stained with fluorescent membrane, cytoplasmic, or nuclear dyes. Thus, differentiation of cell types the presence of membrane receptors & antigens, membrane potential, pH, enzyme activity and DNA content may be facilitated. [3 http://books.google.co.in, Springer, 01-Jan-2007 - Medical , edited by Marion G. Macey]

3. Working of Flow Cytometry



The tissue sample is broken up into single cells and held in a test tube, which is placed into the flow cytometer. The liquid containing the cells is drawn up from the test tube and pumped into the flow chamber.

1) **Flow chamber** – Cells flow through the flow chamber one at a time very quickly, about 10,000 cells in 20 seconds or 500 cells per second. This animation shows the cells moving in slow motion.
2) **Laser** – A small laser beam of very bright light hits the cells as they pass through the flow chamber. The way the light bounces off each cell gives information about the cell’s physical characteristics. Light bounced off at small angles is called **forward scatter**. Light bounced off in other directions is called **side scatter**.
3) **Light detector** - The light detector processes the light signals and sends the information to the computer. Forward scatter tells you the size of the cell. Side scatter tells you if the cell contains granules. Each type of cell in the immune system has a unique combination of forward and side scatter measurements, allowing you count the number of each type of cell.
4) **Filters** – The filters direct the light emitted by the fluorochromes to the color detectors.

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5) **Color detectors** - As the cells pass through the laser, the fluorochromes attached to the cells absorb light and then emit a specific color of light depending on the type of fluorochrome. The fluorochromes on the cells act like the bar code on groceries as the cashier passes them over the scanner. In this case, there are two types of fluorescent markers: yellow and green. Any one cell can have one, both or none of the markers on its surface. The color detectors collect the different colors of light emitted by the fluorochromes. The fluorochrome data signal is also sent to the computer.
6) **Computer** – The data from the light detector and the color detectors is sent to a computer and plotted on a graph called a histogram. [4 http://www.unsolvedmysteries.oregonstate.edu]

4. Immunophenotyping Applications in Hematology

The distributed nature of the hematopoietic system makes it amenable to flow cytometric analysis. Many surface proteins and glycoproteins on erythrocytes, leukocytes, and platelets have been studied in great detail. The availability of monoclonal antibodies directed against these surface proteins permits flow cytometric analysis of erythrocytes, leukocytes, and platelets. Antibodies against intracellular proteins such as myeloperoxidase and terminal deoxynucleotidyl transferase are also commercially available and permit analysis of markers.an increasing number of intracellular [5] **Michael Brown**1 **and Carl Wittwer**[a],1

Erythrocyte Analysis

The use of flow cytometry for the detection and quantification of fetal red cells in maternal blood has increased in recent years. Currently in the United States, rhesus D-negative women receive prophylactic Rh-immune globulin at 28 weeks and also within 72 h of delivery (6 Hartwell EA. Use of Rh immune globulin: ASCP practice parameter. American Society of Clinical Pathologists [see comments]. *Am J Clin Pathol* 1998; **110**:281-292. **Medline Order article via InfotrieveWeb of Science**). The standard single dose is enough to prevent alloimmunization from ∼15 mL of fetal rhesus D+ red cells. If feto-maternal hemorrhage is suspected, the mother's blood is tested for the presence and quantity of fetal red cells, and an appropriate amount of Rh-immune globulin is administered. The quantitative test most frequently used in clinical laboratories is the Kleihauer-Betke acid-elution test. This test is fraught with interobserver and interlaboratory variability, and is tedious and time-consuming (7 Polesky HF, Sebring ES. Evaluation of methods for detection and quantitation of fetal cells and their effect on RhIgG usage. *Am J Clin Pathol* 1981;**76**:525-529.**MedlineOrder article via InfotrieveWeb of Science**). The use of flow cytometry for the detection of fetal cells is much more objective, reproducible, and sensitive than the Kleihauer-Betke test (8 Bayliss KM, Kueck BD, Johnson ST, Fueger JT, McFadden PW, Mikulski D, et al. Detecting fetomaternal hemorrhage: a comparison of five methods [see comments]. *Transfusion* 1991;**31**:303-307. **CrossRef Medline Order article via InfotrieveWeb of Science**) (9 Bromilow IM, Duguid JK. Measurement of feto-maternal haemorrhage: a comparative study of three Kleihauer techniques and two flow cytometry methods.*Clin Lab Haematol* 1997;**19**:137-142.**CrossRefMedlineOrder article via InfotrieveWeb of Science**)(10 Davis BH, Olsen S, Bigelow NC, Chen JC. Detection of fetal red cells in fetomaternal hemorrhage using a fetal hemoglobin monoclonal antibody by flow cytometry. *Transfusion* 1998; **38**:749-756.**CrossRefMedlineOrder article via InfotrieveWeb of Science**). Fluorescently labeled antibodies to the rhesus (D) antigen can be used, or more recently, antibodies directed against hemoglobin F (8) (9) (10) (11 Navenot JM, Merghoub T, Ducrocq R, Muller JY, Krishnamoorthy R, Blanchard D. New method for quantitative determination of fetal hemoglobin-containing red blood cells by flow cytometry: application to sickle-cell disease. *Cytometry* 1998;**32**:186-190. **CrossRef Medline Order article via InfotrieveWeb of Science**)(12 Campbell TA, Ware RE, Mason M. Detection of hemoglobin variants in erythrocytes by flow cytometry. *Cytometry* 1999; **35**:242-248. **CrossRef Medline Order article via Infotrieve Web of Science**)(13 Johnson PR, Tait RC, Austin EB, Shwe KH, Lee D. Flow cytometry in diagnosis and management of large fetomaternal haemorrhage . *J Clin Pathol*1995;**48**:1005-1008.) (14 Oosterwijk JC, Knepfle CF, Mesker WE, Vrolijk H, Sloos WC, Pattenier H, et al. Strategies for rare-event detection: an approach for automated fetal cell detection in maternal blood. *Am J Hum Genet* 1998;**63**:1783-1792.) (15 Oosterwijk JC, Mesker WE, Ouwerkerk-van Velzen MC, Knepfle CF, Wiesmeijer KC, Beverstock GC, et al. Fetal cell detection in maternal blood: a study in 236 samples using erythroblast morphology, DAB and HbF staining, and FISH analysis.*Cytometry* 1998;**32**:178-185) (16 Nance SJ, Nelson JM, Arndt PA, Lam HC, Garratty G. Quantitation of fetal-maternal hemorrhage by flow cytometry. A simple and accurate method. *Am J Clin Pathol* 1989;**91**:288-292). This intracellular approach, which uses permeabilization of the red cell membrane and an antibody to the γ chain of human hemoglobin, is precise and sensitive (10). This method has the ability to distinguish fetal cells from F-cells (adult red cells with small amounts of hemoglobin F. Although the flow cytometry method is technically superior to the Kleihauer-Betke test, cost, instrument availability, and stat access may limit its practical utility.

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal stem cell disorder that leads to intravascular hemolysis with associated thrombotic and infectious complications. PNH can arise in the setting of aplastic anemia and may be followed by acute leukemia. The disease is caused by deficient biosynthesis of a glycosylphosphatidylinositol linker that anchors several complement and immunoregulatory surface proteins on erythrocytes, monocytes, neutrophils, lymphocytes, and platelets (17 Fores R, Alcocer M, Diez-Martin JL, Fernandez MN. Flow cytometric analysis of decay-accelerating factor (CD55) on neutrophils from aplastic anaemia patients. *Br J Haematol* 1995;**90**:728-730) (18 Hall SE, Rosse WF. The use of monoclonal antibodies and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria. *Blood* 1996;**87**:5332-5340) (19 Yamada N, Miyata T, Maeda K, Kitani T, Takeda J, Kinoshita T. Somatic mutations of the PIG-A gene found in Japanese patients with paroxysmal nocturnal hemoglobinuria. *Blood* 1995; **85**:885-892) (20 Rotoli B,

Boccuni P. The PIG-A gene somatic mutation responsible for paroxysmal nocturnal hemoglobinuria. *Haematologica* 1995; **80**:539-545) . On erythrocytes, deficiencies of decay-accelerating factor and membrane-inhibitor of reactive lysis render red cells susceptible to complement-mediated lysis (18)(19). Conventional laboratory tests for the diagnosis of PNH include the sugar water test and the Ham's acid hemolysis test (21 Elghetany M, Davey FR. Erythrocytic disorders. Henry JB eds. *Clinical diagnosis and management by laboratory methods, 19th ed* 1996:617-663 WB Saunders Philadelphia). Problems associated with these tests include stringent specimen requirements and limited specificity. Antibodies to CD55 and CD59 are specific for decay-accelerating factor and membrane-inhibitor of reactive lysis, respectively, and can be analyzed by flow cytometry to make a definitive diagnosis of PNH. In affected patients, two or more populations of erythrocytes can be readily identified, with different degrees of expression of CD55 and CD59 (5)

Leukocyte Analysis

Immunologic monitoring of HIV-infected patients is a mainstay of the clinical flow cytometry laboratory. HIV infects helper/inducer T lymphocytes via the CD4 antigen. Infected lymphocytes may be lysed when new virions are released or may be removed by the cellular immune system. As HIV disease progresses, CD4-positive T lymphocytes decrease in total number. The absolute CD4 count provides a powerful laboratory measurement for predicting, staging, and monitoring disease progression and response to treatment in HIV-infected individuals. Quantitative viral load testing is a complementary test for clinical monitoring of disease and is correlated inversely to CD4 counts (24 Saag MS, Holodniy M, Kuritzkes DR, O'Brien WA, Coombs R, Poscher ME, et al. HIV viral load markers in clinical practice. *Nat Med* 1996;**2**:625-629) (25 Mellors JW, Rinaldo CR, Jr, Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma [published erratum appears in Science 1997;275:14]. *Science* 1996;**272**:1167-1170). However, CD4 counts directly assess the patient's immune status and not just the amount of virus. It is likely that both CD4 T-cell enumeration and HIV viral load will continue to be used for diagnosis, prognosis, and therapeutic management of HIV-infected persons.

Perhaps the best example of simultaneous analysis of multiple characteristics by flow cytometry involves the immunophenotyping of leukemias and lymphomas. Immunophenotyping as part of the diagnostic work-up of hematologic malignancies offers a rapid and effective means of providing a diagnosis. The ability to analyze multiple cellular characteristics, along with new antibodies and gating strategies, has substantially enhanced the utility of flow cytometry in the diagnosis of leukemias and lymphomas. Different leukemias and lymphomas often have subtle differences in their antigen profiles that make them ideal for analysis by flow cytometry. Diagnostic interpretations depend on a combination of antigen patterns and fluorescence intensity. Flow cytometry is very effective in distinguishing myeloid and lymphoid lineages in acute leukemias and minimally differentiated leukemias. Additionally, CD45/side scatter gating often can better isolate the blast population for more phenotyping than is possible with forward scatter/side scatter gating. Although most acute myeloid leukemias are difficult to classify by phenotype alone, flow cytometry can be useful in distinguishing certain acute myeloid leukemias, such as acute promyelocytic leukemia (26 Erber WN, Asbahr H, Rule SA, Scott CS. Unique immunophenotype of acute promyelocytic leukaemia as defined by CD9 and CD68 antibodies. *Br J Haematol*1994; **88**:101-104) (27 Stone RM, Mayer RJ. The unique aspects of acute promyelocytic leukemia. *J Clin Oncol* 1990; **8**:1913-1921). Flow cytometry can also be used to identify leukemias that may be resistant to therapy (28 Maslak P, Hegewisch-Becker S, Godfrey L, Andreeff M. Flow cytometric determination of the multidrug-resistant phenotype in acute leukemia. *Cytometry*1994; **17**:84-93).

5. Clinical Applications

There are seven major applications in the clinical laboratory:
1) Monitoring AIDS patients
2) Immunophenotyping Leukemia and Lymphomas and Monitoring Residual Disease
3) Determining CD34 counts for Hematopoietic Reconstitution
4) Monitoring Organ Transplant Patients for Rejection
5) Reticulocyte Counts
6) Diagnosis of Paroxymal Nocturnal Hemoglobinuria(PNH)
7) DNA analysis of S-phase fraction of solid tumors

Two new potential clinical applications are monitoring solid tumor metastasis in blood and bone marrow and the use of microspheres to multiplex immuno and oligo transcription assays.(22 http://members.tripod.com/~buff_flow/applications.htm)

General Cell Biology
General cell biology is rapidly expanding its use of flow cytometry as a tool to examine pathways and propose biologically-relevant mechanisms of action in pathologies such as cancer. As shown below, many new applications are currently available to aid research in this area.(23 http://www.millipore.com)

Cell Health & Quality – Mitochondrial Analysis
Mitochondria are critical cellular organelles that produce 90% of cellular energy, control cell survival by regulating apoptosis, and produce reactive oxygen species (ROS). Mitochondrial superoxide generation results in oxidative stress, damage and cell death by apoptosis or to cellular energetic decline. Therefore, mitochondrial dysfunction caused by disease or compound treatment has dire consequences that can result in cell death. Monitoring the impact on mitochondria and related cell health markers is an important part of drug screening programs, pathway mapping, apoptosis, and disease research.

Flow cytometry detects multiple markers simultaneously at various stages of apoptosis, making it a powerful technique for studying pathways governing cell health and cell death. A variety of FlowCellect Kits can now be employed to assess changes in mitochondrial membrane potential,

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apoptosis as measured by Annexin V binding, mitochondrial oxidative stress, and cell death. (22)

Immunology

The immune system, which mediates the body's response to the introduction of foreign material, is made up of multiple cell types collectively called lymphocytes. Lymphocyte subtypes include B cells (which secrete antibodies), cytotoxic T cells, helper T cells (which secrete cytokines), and natural killer (NK) cells. Characterization of lymphocyte subtypes and cytokine signaling is essential for understanding the complex nature of the immune system.

Guava easycyte™ systems offer rapid interrogation of a variety of common immunologic assays, with up to 8 parameters of detection. Amnis imaging flow cytometers can readily analyze more complex assays, with up to 5-12 parameters, including visual verification of specific cells in suspension and rare events.

Multiparametric phenotypic analysis by flow cytometry allows researchers to study the dynamics of immune signaling in intact cells, and offers many unique capabilities, such as:

- Distinguish one subpopulation of cells within a heterogeneous mixture
- Evaluate impact of activation by antigens
- Detect suppression of normal immune activation or impact of disease state on phenotypes(22)

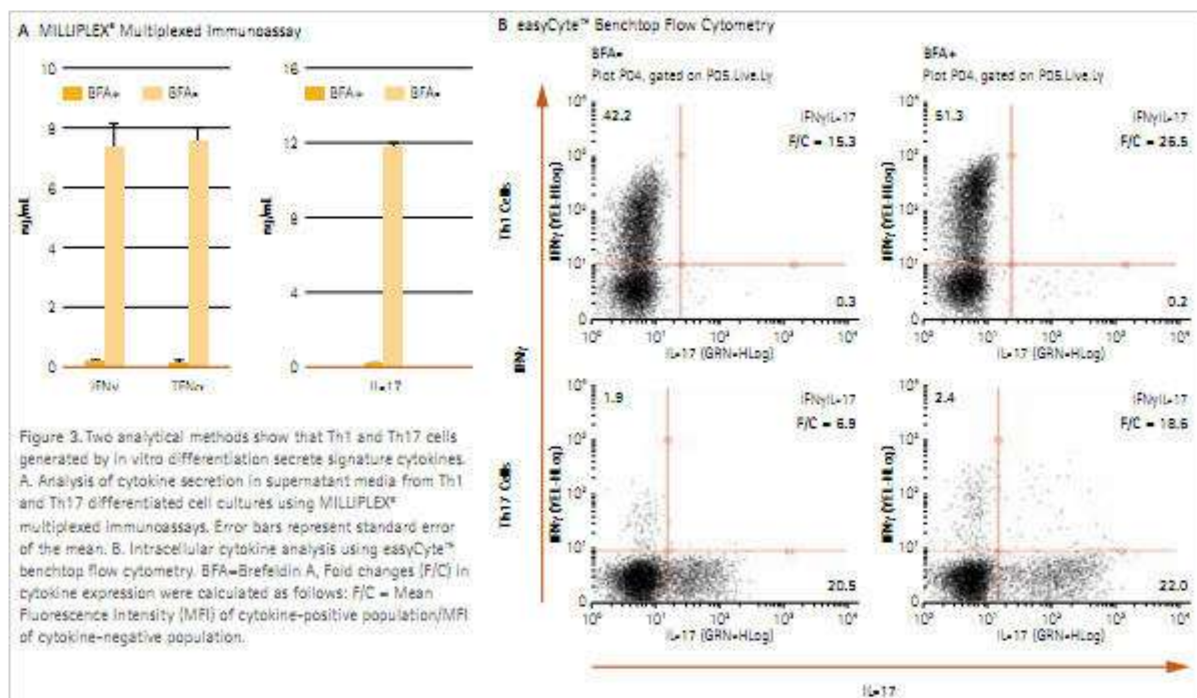

Figure 3. Two analytical methods show that Th1 and Th17 cells generated by in vitro differentiation secrete signature cytokines. A. Analysis of cytokine secretion in supernatant media from Th1 and Th17 differentiated cell cultures using MILLIPLEX® multiplexed immunoassays. Error bars represent standard error of the mean. B. Intracellular cytokine analysis using easyCyte™ benchtop flow cytometry. BFA=Brefeldin A, Fold changes (F/C) in cytokine expression were calculated as follows: F/C = Mean Fluorescence Intensity (MFI) of cytokine-positive population/MFI of cytokine-negative population.

6. Conclusion

Flow cytometry is one of the latest and growing method of analysis of various cells and an important method of diagnosis is various types of cancers like leukemia, lymphoma ,etc. Flow cytometry is a powerful technique for correlating multiple characteristics on single cells. This qualitative and quantitative technique has made the transition from a research tool to standard clinical testing. Applications in hematology include DNA content analysis, leukemia and lymphoma phenotyping, immunologic monitoring of HIV-infected individuals, and assessment of structural and functional properties of erythrocytes, leukocytes, and platelets. Smaller, less expensive instruments and an increasing number of clinically useful antibodies are creating more opportunities for routine clinical laboratories to use flow cytometry in the diagnosis and management of disease.(5)

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[5] **Michael Brown**1 **and Carl Wittwer**[a]**,**1 Department of Pathology, University of Utah, ARUP Laboratories, Inc., Salt Lake City, UT 84132. Address correspondence to this author at: Department of Pathology, University of Utah, 50 North Medical Dr., Salt Lake City, UT 84132. E-mailcarl_wittwer@hlthsci.med.utah.edu.)
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