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# Flow Cytometry and Its Diagnostic Application in Animal Health: A Review

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**Abstract:** Flow cytometry has emerged as a major new technology in veterinary clinical laboratories. Flow cytometers in current use include stand-alone instruments and cytometers incorporated into hematology analyzers. Flow cytometers offer rapid and quantitative analysis of a variety of cell types based on cell size, molecular complexity and antigenic composition. The use of flow cytometry in veterinary diagnostics is becoming a valuable clinical tool with a broad range of applications. Physical characteristics of cells can be determined by the flow cytometer laser and electronics through the measurement of changes in light scatter properties. The techniques of flow cytometry are becoming more and more important for the clinical application (diagnosis)and laboratory. Therefore, flow cytometry complements and extends the knowledge that can be obtained by light microscopy. Flow cytometry has often been proposed for the differentiation of milk cells and diagnosis of diseases.

**Key words:** Animal Health · Diagnosis · Diseases · Flow Cytometry

## INTRODUCTION

Flow cytometry (FCM) can be used to identify physical and functional properties of cells from blood, fluid and tissues of human and veterinary patients. It was first used in medical sciences such as oncology (e.g., for diagnosis of cancer, chromosomal defect diagnosis) and hematology. Veterinary medicine and clinical applications of flow cytometry still account for the vast majority of publications on this technique, but during the past few years it has also become a valuable tool in biology, pharmacology, toxicology, bacteriology, virology, environmental sciences and bioprocess monitoring.

The recent success of flow cytometry is based on commercially available flow cytometry equipment that is both robust and versatile, together with modern data acquisition and interpretation software and tremendous successes in the development of various specific staining assays [1]. FCM can be used to count and even distinguish cells of different types in a mixture by quantitating their structural features. In addition, cell sorting with flow cytometers has been a powerful tool for diverse fields in research, clinical applications and clinical trials [2].

FCM is a rather recent and innovative technology by means of which different cell characteristics are simultaneously analyzed on a single cell basis. This is achieved by means of hydrodynamic focusing of cells that pass aligned one by one in front of a set of light detectors; at the same time, they are illuminated by the flow cytometer light source, which is usually a laser beam. All cell parameters measured in this way can be divided into two main groups: 1) those related to light scattering, which mainly reflects the size of the cell and its internal complexity and 2) those associated with the presence of one or more fluorochromes inside the cell or attached to the cell surface membrane [3].

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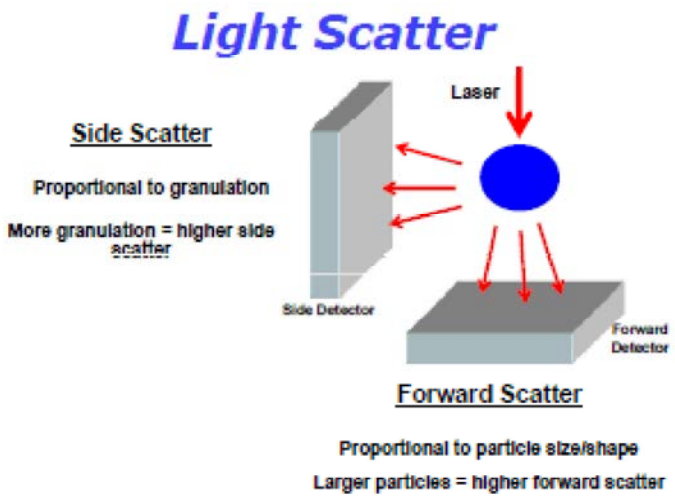


Fig. 1: Forward scatter intensity (FSI) and Side scatter intensity (SSI).
Source: Givan [7]

Therefore, this review is to give a short introduction into the principles of flow cytometry and its application for different areas in veterinary diagnosis with the following objectives:

- To know the challenges and limitation of flow cytometric application.
- To create awareness for veterinarians how to apply in practically.

**Flow Cytometry:** FCM is a methodology for determining and quantitating cellular features, organelles or cell structural components primarily by both optical and electronic means [2]. Although it measures one cell at a time, the newest equipment is able to process up to several hundred thousand cells in a few seconds [4].

FCM involve sophisticated handling of fluids and pressure, complex laser beams and optics, very sensitive electronic detectors, analogue to digital converters and high capability computers. It enables measurement of multiple characteristics of a single cell/particle simultaneously. FCM is a general method for rapidly analyzing large numbers of cells individually using light-scattering, fluorescence and absorbance measurements [5]. The power of this method lays both in the wide range of cellular parameters that can be determined and, in the ability to obtain information on how these parameters are distributed in the cell population [6].

**Methods and Principles of Flow Cytometry:** The cells prepared in a mono disperse (Single cell) suspension may be alive or fixed at the time of measurement. They are passed through a chamber as single cells. The fine stream containing the cellular suspension is passed through the chamber as a continuous flow. The cells inside the chamber are excited by the beam of the laser (s) light. Each cell scatters some of the laser light, as well as emitting fluorescent light following excitation by the laser. The signal of the scattered light and the signal of the emitted fluorescence are collected for analysis [7]. The FCM typically measures several parameters simultaneously for each cell (Figure 1): I) forward scatter intensity (FSI) is approximately proportional to cell diameter. ii) Side scatter intensity (SSI) or orthogonal (90 degrees) scatter is approximately proportional to the quantity of granular structures within the cell. iii) Fluorescence intensities are measured at several wavelengths [6].

FSI alone is often quite useful. It is commonly used to exclude dead cells, cell aggregates and cell debris from the fluorescence data. It is sufficient to distinguish lymphocytes from monocytes or from granulocytes in blood laboratory to assess granularity of living cells such as dendritic cells. Fluorescence intensities are typically measured at several different wavelengths simultaneously for each cell. Fluorescent probes, generally coupled to antibodies, are used to report the quantities leukocyte samples. Side scatter has been used in of specific components of the cells. Fluorescent antibodies are often used to report the densities of specific surface receptors and thus to distinguish subpopulations of differentiated cell types, including cells expressing a transgenic or cells expressing a unique marker [7].

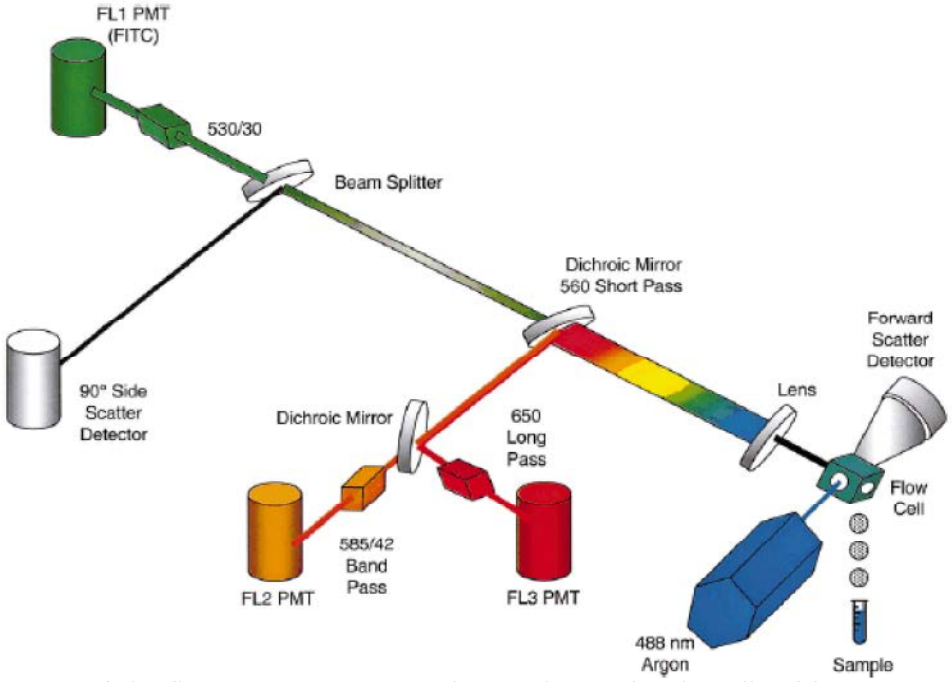


Fig. 2: Components of the flow cytometer system. The sample contains the cells of interest. Lens filters and a photodiode and photomultiplier (PMT) tubes collect the diffracted light and flourescent emission
Source: Davis *et al*. [8]

The binding to surface receptors of viruses, or diverse proteins such as hormones, can also be measured by making them fluorescent. The fluidics hydro dynamically focus the cell stream within a margin of a small fraction of a cell diameter and, even in sorters, break the stream into uniform-sized droplets in order to separate individual cells [5]. The electronics quantitative the faint flashes of scattered and fluorescent light and, under computer control, select the electrically charged droplets containing cells of interest so that they can be deflected into a separate test tube or culture wells. The computer is able to record data for thousands of cells per sample and displays the data graphically [7].

**Components of Flow Cytometers:** Major components of a flow cytometer include the laser light source, a fluidics system, an optics system and an electronic detection system interfaced with a computer. It is essential that fluorochromes that absorb the 488 nm blue light emitted by the argon laser are utilized, However, some cytometers have additional lasers such as the red-diode laser and the ultraviolet laser, which provide light emissions at 635 nm or in a 360 to 400nm range, respectively. These dual and triple laser flow cytometers expand the fluorescent parameters and fluorochromes that can be used for cell analyses. The fluidics system is pressurized to produce a laminar stream in which cells pass through the laser beam one at a time. The optics system consists of a series of dichroic mirrors that reflect light to the appropriate filters and detection system. A beam splitter separates the orthogonal light scatter from fluorescent light signals [8].

**Diagnositic Application of Flow Cytometry**

**Detection of Mastitis in the Bovine Mammary Gland at Early Stage:** Subclinical mastitis is a costly disease and its diagnosis is difficult. Besides the somatic cell count (SCC) and bacteriology, the differential inflammatory cell count (DICC) is a meaningful tool for mastitis detection. As microscopy is very subjective because of the low number of events to be counted, flow cytometry has often been proposed for the differentiation of milk cells to identify subclinical mastitis in cattle at an early stage by a simple and fast flow cytometric method.

The FCM identifies main leucocyte populations in flow cytometric dot plot (Polymorphonuclear (PMN) neutrophils, lymphocytes and macrophages) and with these, mastitis prognostics has been made. The milk cells were incubated with different specific antibodies that bind to different cell types and also to propidium iodide (PI), which differs between viable and non-viable cells.

This procedure made it possible to localize cell types in a flow cytometric dot plot and to differentiate between viable and non-viable PMN. Percentage of viable PMN was determined by a procedure consisting of a simple centrifugation, incubation with PI and flow cytometric measurement. So FCM enabled to quickly determine the stage of the inflammation even in quarters with a low SCI [9]. On the other hand, the differential inflammatory cellcount (DICC), which distinguishes between granulocytes and mononuclear cells and enumerates the concentration of granulocytes, makes it possible to detect mastitis in its initial phase, when the SCC is still low and to evaluate the success of antibiotic treatments [10].

**Detection of Rabies Virus Antigen or Antibody:** Flow cytometry has been applied in the detection of intracellular rabies virus antigen or antibodies. It was suggested by these authors that more such studies comparing flow cytometry with other established methods and the use of cells would be beneficial before more extensive application of this new approach in clinical laboratories also called for the application of computer-automated detection of fluorescence methods for rapid and efficient titration of anti rabies antibodies in immunized hosts to reduce the work time required to undertake. Hence, this study was undertaken to evaluate the application of flow cytometry for the detection of rabies virus antigen in cells and predictive quantification of rabies virus antibodies in field sera samples. Diagnosis of rabies virus antigen is conventionally done by Negri body examination or by direct using fluorescent antibody test rabies ant nucleocapsid antibody conjugate on brain impression smears. Fluorescent antibody test is considered to be a standard rabies virus diagnostic test against which other diagnostic approaches need to be compared. Cultivation of virus in cell culture allows the replication of the rabies virus originally present in the sample and thus would enhance the sensitivity of virus detection over direct fluorescent antibody test done on impression smears [11].

**Identifying Marek's Disease Virus:** Identification of Marek's disease virus (MDV) infected lymphocytes is essential for a full understanding of the pathogenesis of Marek's disease; describes the development of a simple, quantitative and reproducible flow cytometric technique for the identification of the phenotype of Marek's disease virus-infected lymphocytes.

The method is based upon the detection of the Marek's disease virus-specific phosphoprotein (pp38), in saponin-permeabilized lymphocytes, using the monoclonal antibody and the identification of the phenotype of pp38+ lymphocytes using monoclonal antibodies against lymphocyte surface markers. Pp38 expression in the spleen was demonstrated in the cytolytic phase of infection [12].

**Detection of Mycobacterium Tuberculosis Infection:** By measuring the T-cell response to early secretory antigenic target-6 (ESAT-6) is a promising method for identifying individuals infected with M. tuberculosis. In most of the published work so far, secreted cytokines were measured or Elispot was performed after stimulation of whole blood or peripheral blood mononuclear cells (PBMCs) with recombinant protein or peptides in, published that a pool of overlapping peptides covering the complete sequence of ESAT-6 was equivalent to recombinant ESAT-6 as antigen for T-cell stimulation. The potential usefulness of a diagnostic test measuring the T-cell response to ESAT-6 indicated that apart from establishing the presence or absence of a T-cell response to ESAT-6, it is useful to determine the frequencies of ESAT-6-specific T cells. However, we believe that that cytokine flow cytometry in combination with T-cell stimulation by a single protein-spanning ESAT-6 peptide pool is a preferable alternative to currently used approaches, such as measuring cytokines in culture supernatant .The workload for technicians may be roughly, so that this novel approach will be used primarily in research [13].

**Detection Normal Canine and Normal Feline Blood Lymphocytes:** Flow cytometry used to assess the progression of FIV and FeLV infections, determine virus activation and pathogenesis, measure apoptosis, evaluate the response to transplanted organs in canine models and used characterize the immune response during infections [14].

**Detection of Apoptosis:** Programmed cell death (Apoptosis) is the genetically controlled destruction of cells. Apoptosis can be distinguished from necrosis by the biochemical and morphological changes that occur. In contrast to necrotic cells, apoptotic cells are characterized morphologically by a reduction of the cytoplasm, compaction of the nuclear chromatin and production of membrane-bound apoptotic bodies.

Table 1: Major Characteristics of Flow Cytometry as Compared to Conventional Microscopy.

| Parameter | Flow cytometry | Conventional microscope |
|---|---|---|
| Simplicity | High | Moderate |
| Speed | High | Low |
| Number of cell analyzed | High | Low |
| Absolute cell counts | Accurate | Inaccurate |
| Sensitivity | High | Moderate/Low |
| Specificity | High | High |
| Information of antigen expression | Quantitative | Qualitative |
| Information on Antigen localization | Limited | Detailed |

Source: Coligan [3]

Biochemically, apoptosis is distinguished by fragmentation of the genome and cleavage or degradation of several cellular proteins. Several methods have been developed to distinguish between living cells, early or late apoptotic cells and necrotic cells [15]. Fluorescent annex-V in conjugates can be used for studying the externalization of phosphatidyl serine, one of the earliest indicators of apoptosis. The anticoagulant annex-V is a phospholipid-binding protein with a high affinity for phosphatidyl serine. In normal viable cells, phosphatidyl serine is located on the cytoplasmic surface of the cell membrane. In contrast, the phosphatidyl serine in apoptotic cells is trans located from the inner to the outer leaflet of the plasma membrane. The measured difference in fluorescence intensity of annex -V-phosphatidyl serine complexes between apoptotic and non-apoptotic cells can be detected by flow cytometry. An annex -V affinity assay has been used for the detection of apoptosis in mammalian cells with flow cytometry and automatic image analysis [16].

**Detection of Intracellular Calcium Concentration:** The intracellular calcium concentration plays an important role in a broad range of cellular functions, including enzyme activity. Many biotechnological processes that are based on a high rate of cell growth are influenced both by the concentration of calcium and by that of magnesium. The interactive effects of calcium and magnesium have been intensively investigated [17] and extended information and assay descriptions are available [18]. Over the past 20 years, many dyes for calcium measurements have been developed, mostly for use in clinical applications. For biotechnological purposes, was used to monitor the effect of hydrogen peroxide on CHO cells in culture. It has been determined that an elevated intracellular calcium concentration may protect cells against $H_2O_2$induced mitochondrial damage and cytotoxity [19]. Calcium concentrations also vary under the influence of fluid forces that cause shear stress in cell cultures [20].

**Advantage of Flow Cytometry:** Since the early 1970's, flow cytometers that do not employ fluorescence have been commercially available. They were initially used for complete blood cell counts, in clinical laboratories and veterinary diagnosis. Their ease of handling and reliability of results increased and popularized their use. The newest and most versatile research instruments the world-wide utilization of flow cytometry is demonstrated by the occurrence of flow cytometric data [2]. Therefore, flow cytometry has great advantages compared to conventional microscopy since it permits the analysis of a greater number of cells in a fraction of the time (Table 1)

**Limitations of Flow Cytometry:** The extensive use of flow cytometry in the field of veterinary is still limited. This is mainly due to the high cost of the instruments and the need for well trained personnel for operating the system and for sample preparation. Another limiting factor is the demand for single cell suspensions. If cell aggregates are present in the suspension, false results will be obtained. In the presence of cell tissue, adherent cells or cell clusters, mechanical or enzymatic methods have to be applied to separate the cells from one another prior to measurement. When fluorescent dyes are used, an additional permeabilization step is often required to allow the fluorescent dyes or antibodies to enter the cells. For measurements of intracellular components, several washing steps between different staining steps must be included. This pretreatment of the probes is time-consuming [21].

Furthermore, size measurements are influenced by the refraction indices of calibration and sample particles, which can be a source of error. Limitations concerning the sensitivity of the method have been overcome by the continuously introducing number of new fluorescent dyes [19].

## CONCLUSION AND RECOMMENDATIONS

Flow cytometric analysis will probably be indispensable for a rapid and objective assessment of the cell surface and different intracellular characteristics and, when combined with other clinical laboratory. Flow cytometers are becoming more user friendly and there is much interest in the development of fluorochromes for multi parametric analysis and the clinical application of new clusters of monoclonal antibodies. The next step will be to see that all clinical laboratories adhere to such rigid standards and likewise stringent, in order to verify the reproducibility of results obtained in the above mentioned fields. Thus FCM are a more conclusive of the challenges and limitations of the method is presented along with a future in veterinary diagnosis. For instance the automated flow cytometry assay was found to be useful in assessing the exact percentage of rabies virus infected cells and allowed predictive quantification of rabies virus antibodies from a single serum dilution without observer bias. Flow cytometry has the great advantages of detecting mastitis before clinical signs appear and testing the success of antibiotic treatment. Analysis of percentages of viable PMN makes it possible to determine the age of the inflammation. Based on this conclusion the following recommendations are forwarded:

- The government should be give attention research centers to investigate the application of flow cytometry.
- Multidisciplinary researcher /veterinarians/ should be providing evidence-based information on the application of flow cytometry.
- The sample size measurement should be well prepared.
- The researcher /veterinarians/should be create the awareness to the government to be implementing, planning and formulating of basic instruments and create opportunity to be trained.

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