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Flow cytometry in veterinary science: A comprehensive review

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Abstract

Measurement of the physical and chemical characteristics of cells is called cytometry. Flow cytometry is a simple and rapid method for the quantitative and qualitative analysis of single-cell suspensions. Flow cytometry is a laser-based technology whereby cells are suspended in a fluid stream and stimulated by a laser; the cells scatter and/or emit light which is then detected by Photomultiplier-Based Tubes (PMTs). All parameters measured 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 related to fluorescence.

These are associated with the presence of one or more fluorochromes inside the cell or attached to the cell surface membrane, either naturally (autofluorescence) or artificially (e.g., using fluorochrome conjugated monoclonal antibodies). Flow cytometers are composed of three main systems: fluid system, optical system and electronic system. There are two different types of flow cytometry - named as non-sorting and sorting. Non-sorting type can perform light scattering and fluorescence emission while the sorting type has the ability to sort particles as well. Fluorescent Activated Cell Sorters (FACS) are flow cytometers that have the capacity to sort fluorescent-labeled cells from a mixed cell population. With the flexibility of flow cytometry, and the reliability of results, this technology has become an essential veterinary research instrument with important clinical diagnostic capabilities, particularly in the fields of immunology and hematology. It is possible to perform functional tests, biological evaluations of compounds, purify cell populations even when they are present in a low proportion in the sample. The most common use of flow cytometry is in the identification of markers on cells, particularly the immune system or immunophenotyping. It is one of the most popular and versatile techniques for studying apoptosis, and cell viability. It can also be used to evaluate cell proliferation, metabolic activity and intracellular signalling. Flow cytometry has been extensively used to study mammalian sperm in the areas of reproductive toxicology (to monitor effects from environmental, occupational and therapeutic exposures), veterinary science (to preselect the gender of offspring by sorting X- and Y-chromosome-bearing sperm) and clinical andrology (to assess individual fertility potential). Flow cytometry is most often applied in veterinary oncology to diagnose lymphoid neoplasia and leukemia, and can also be used to assess neoplastic and non-neoplastic marrow conditions.

Keywords: Flow cytometry, Fluorescence-Activated Cell Sorting (FACS), Hydrodynamic Focusing, Forward Scatter (FSC), Side Scatter (SSC), fluorochromes, immunophenotyping, cell viability, apoptosis, cell cycle analysis, fluorescence compensation, veterinary diagnostics, cancer research, sperm sorting, spectral flow cytometry

1. Introduction

Flow Cytometry (FC) is a technique used to detect and measure physical and chemical characteristics of a population of cells or particles (Givan, 2011) ^[17]. In this process, a sample containing cells or particles is suspended in a fluid and injected into the flow cytometer instrument. The sample is focused to ideally flow one cell at a time through a laser beam, where the light scattered is characteristic to the cells and their components. Cells are often labelled with fluorescent markers so light is absorbed and then emitted in a band of wavelengths. Tens of thousands of cells can be quickly examined and the data gathered are processed by a computer (Erotocritou *et al.*, 2007) ^[12].

First developed flow cytometry was a single parameter instrument detecting only the size of cells. Currently, highly sophisticated instruments have evolved with the capability of detecting more parameters simultaneously (Adan *et al.*, 2017) ^[1]. The main components of a flow cytometer are a flow chamber; a light source; a detector and a digital analogical converter that generates fluorescent parameters of size, complexity and signals; a system of amplification of linear or logarithmic signal; and a computer for the signal analysis (Errante, 2015) ^[13]. The technology is moving rapidly from the polychromatic flow cytometry that has been the go-to technology for 45 years to spectral flow cytometry, which is now the most significant change in nearly half a century of flow cytometry (Robinson, 2022) ^[36]. There are two different types of flow cytometry - named as non-sorting and sorting. Non-sorting type can perform light scattering and fluorescence emission while the sorting type has the ability to sort particles as well. Fluorescent activated cell sorters (FACS) are flow cytometers that have the capacity to sort fluorescent-labelled cells from a mixed cell population (Wilkerson, 2012) ^[49].

Flow cytometry is a powerful tool with applications in multiple disciplines such as immunology, virology, molecular biology, cancer biology, and infectious disease monitoring. For example, it is very effective for the study of the immune system and the immune response to infectious diseases and cancer. It allows for the simultaneous characterization of mixed populations of cells from blood and bone marrow as well as solid tissues that can be dissociated into single cells such as lymph nodes, spleen, mucosal tissues, solid tumors, etc. In addition to analysis of populations of cells, a major application of flow cytometry is sorting cells into uniform populations to be used for further downstream analysis (McKinnon, 2018) ^[28]. Additionally, whole cells and cellular components such as organelles, nuclei, DNA, RNA, chromosomes, cytokines, hormones and protein content can also be investigated by flow cytometry. Analysis of cell proliferation and cell cycle, measurements of calcium flux and membrane potentials are the commonly used examples of methods developed for flow cytometry (Adan *et al.* 2017) ^[1].

2. Historical aspect

Flow cytometry emerged from the convergence of microscopy, automated cell counting, and fluid-handling technologies, each contributing essential concepts to the development of modern single-cell analytical systems (Givan, 2001) ^[16]. This integration transformed static cellular observation into rapid, quantitative analysis of cells in suspension.

Microscopy provided the earliest foundation for cellular studies. Since the seventeenth century, microscopes have enabled visualization of cells and tissues, while the introduction of histological stains in the late nineteenth century enhanced cellular differentiation. The development of fluorescence microscopy in the mid-twentieth century represented a major advance, allowing specific cellular components to be visualized using fluorescent dyes (Errante, 2015) ^[13]. A critical breakthrough was the conjugation of fluorescent molecules to antibodies, pioneered by Albert Coons. The subsequent development of monoclonal antibody technology by Köhler and Milstein greatly expanded the range of detectable cellular markers, substantially increasing the analytical potential of fluorescence-based methods and later flow cytometry (Givan, 2001) ^[16].

The transition from static microscopy to dynamic single-cell

analysis began in 1934 when Andrew Moldavan described a system for detecting cells flowing through a glass capillary using photoelectric sensors (Moldavan, 1934) ^[30]. Although technically limited, this work established the concept of analyzing cells in motion. A pivotal innovation was introduced by Crosland-Taylor in 1953, who demonstrated hydrodynamic focusing by centering cells within a sheath fluid under laminar flow conditions, allowing cells to pass individually through the detection region (Crosland-Taylor, 1953) ^[52]. This principle became fundamental to all subsequent flow cytometric systems (Melamed, 2001) ^[29]. Parallel advances occurred in automated blood cell counting. Wallace Coulter introduced electrical impedance-based cell counting in the 1950s, enabling rapid and automated measurement of blood cells as they passed through a small orifice (Givan, 2001) ^[16]. Coulter counters became essential tools in clinical hematology laboratories and demonstrated the feasibility of high-throughput, flow-based cellular analysis.

During the 1960s, optical and fluorescence detection technologies advanced rapidly. Dittrich and Göhde demonstrated fluorescence-based DNA quantification in flowing cells, generating fluorescence intensity histograms and enabling quantitative cell cycle analysis (Givan, 2001) ^[16]. These developments led to the first fluorescence-based flow cytometers incorporating hydrodynamic focusing with orthogonal configurations of fluid flow, illumination, and signal detection. The introduction of laser light sources, particularly argon lasers, significantly improved sensitivity and precision (Givan, 2001) ^[16]. These innovations enabled multiparametric analysis by combining fluorescence, light scatter, and cell volume measurements within a single instrument. The addition of cell sorting capabilities marked a major milestone, allowing physical separation of defined cell populations. By the late 1960s, flow cytometric sorting permitted the purification of leukocyte subsets, including lymphocytes and granulocytes (Givan, 2001) ^[16]. In 1974, the first commercial flow cytometer capable of detecting light scatter and multiple enzyme-derived fluorescence signals was introduced, further expanding clinical and research applications. The incorporation of monoclonal antibodies into flow cytometry revolutionized immunophenotyping, particularly for identifying T- and B-cell subsets based on surface antigen expression (Errante, 2015) ^[13].

Currently, flow cytometry continues to evolve along two complementary trajectories. On one hand, highly sophisticated instruments provide ultra-sensitive, high-throughput multiparametric analysis and rapid cell sorting for advanced research and clinical diagnostics. On the other hand, the development of compact, user-friendly systems has made flow cytometry widely accessible, establishing it as an indispensable tool across biomedical research, clinical laboratories, and biotechnology industries (Errante, 2015) ^[13].

3. Principle

Flow cytometer is an instrument that analyzes individual particles or cells by employing a hydrodynamic, optical, electronic and computational system (Tung *et al.*, 2004) ^[45]. Flow cytometers capture light scatter properties and light emitted by individual cells that innately fluoresce or that are labelled with fluorescent antibodies or probes and then exposed to light. The basic principles of a flow cytometer are that

- 1) Cells or particles in single file in a fluid medium are interrogated by light from one or several lasers of defined wavelength, and

2) Light scatter and emission signals are captured (Ibrahim and Van den Engh, 2007) [22].

Thus, by necessity, specimens suitable for FC have to be sampled as a fluid (blood, bone marrow, or body fluids) or be transformed from a solid tissue into a cell suspension through digestion of extracellular matrix by proteases or through physical/chemical disruption of cellular adhesion by mincing, filtration, or chelation (Givan, 2001) [16].

The test cell suspension is then ejected into the center of a sheath fluid (Fig. 1), which comprises the support medium carrying cells through the cytometer. The sheath fluid circulates under pressure and is generally composed of a buffered saline solution. Cells join the sheath fluid in the flow cell and, through fluid mechanical design for achieving laminar flow, are placed in the center of the moving fluid stream, narrowed to single file, evenly spaced, and uniformly illuminated as they are exposed to the light beam. Physical factors influencing this process include the size, orientation, and concentration of cells or particles; the width of the orifice of the flow cell; the velocity of both the injected sample and the sheath fluid; the diameter of the center of the moving stream containing cells of interest relative to that of the sheath fluid; and many others (Reggeti & Bienzle, 2011) [35].

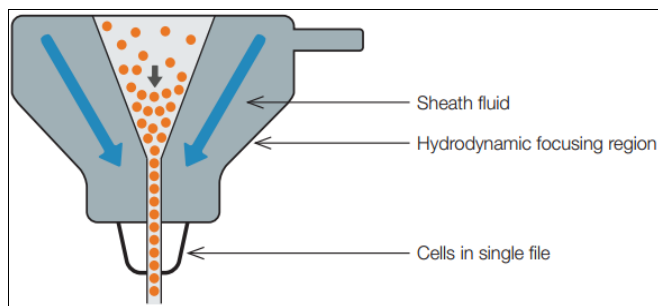


Fig 1: Hydrodynamic focusing produces a single stream of particles

Cells being illuminated at the analysis point scatter light or, if labeled with suitable fluorescent probes, emit light at specific wavelengths. These optical signals are captured by photodetectors and converted from photons to electrical impulses by photodiodes (that have limited signal amplification) or photomultiplier tubes, which increase the electron output per photon input. The first photodiode capturing low-angle forward scatter (FSC, usually at 0.5°) sits in the path of the light beam and is partially obscured by a metal strip to capture only light that has been diffracted at low angles (Fig. 2). The light beam is further partitioned toward other photodetectors through dichroic mirrors that partly

reflect and transmit light. One photodetector is placed to capture light scattered by cells at high angle (usually 90°), also termed side scatter (SSC) or orthogonal scatter (Fig. 2). SSC is proportional to the internal complexity and surface texture of cells, as readily illustrated by higher SSC resulting from granulocytes than lymphocytes (Sanchez-Torres *et al.*, 2022) [37]. Additional photodetectors are equipped with filters that capture specific wavelengths, such as green light emission and yellow-orange light emission, measured as fluorescence 1 and fluorescence 2, respectively, by some types of cytometers. Commonly used fluorochromes, such as Fluorescein Isothiocyanate (FITC) and Phycoerythrin (PE), absorb light at wavelengths of 488 nm and emit light at approximately 500 and 575 nm, respectively, which can be individually captured through use of appropriate bandpass filters (Givan, 2001) [16].

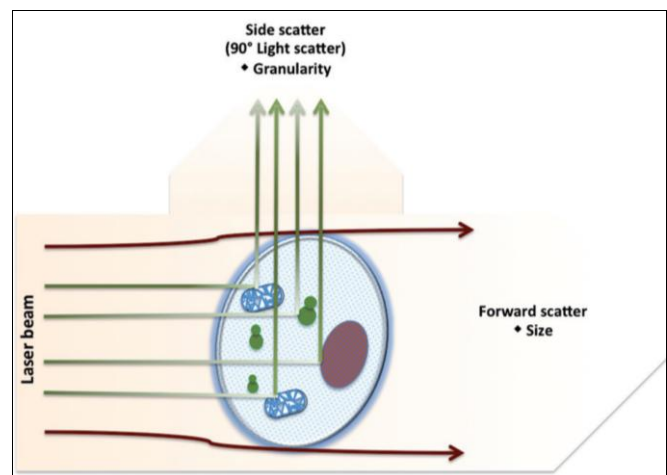


Fig 2: Light scattering. FSC is proportional to size while SSC is proportional to cell granularity or internal complexity

Electrical signals generated from photons are either directly captured on a linear scale, as typical for FSC and SSC, or amplified on a linear or logarithmic scale. Signals are proportional to light intensity and converted from analog to digital to assign intensity values. Signal analysis is multifactorial; it depends on factors such as the type of signal amplification and the number of channels into which the light intensity range is divided. Thus, although a numerical readout is generated for light intensity, in most instances, the value is relative, dependent on parameters adjusted by the user, and incomparable between instruments and between experiments (Shapiro, 2005) [39].

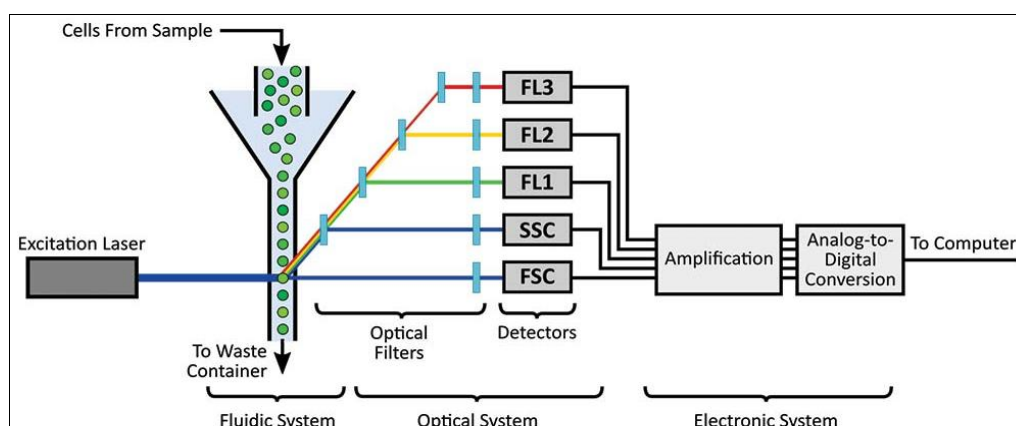


Fig 3: Schematic of a common flow cytometer, illustrating the fluidic, optical, and electronic systems

4. Hardware

4.1 Fluidics/fluidic system

The fluidic system transports the cells from a solution through the instrument to obtain the data includes two components; sheath fluid and pressurized lines. Sheath fluid is a diluent (commonly Phosphate-Buffered Saline (PBS)), which is injected into the flow chamber located at the heart of the instrument by pressurized lines. A pressurized airline also injects the suspended cells in the sample tube into the flow chamber (Givan, 2001) ^[16]. The sample stream becomes a central core in the sheath fluid stream called as a coaxial flow that is based on a pressure difference between the sheath fluid and sample stream. The sample pressure is always greater than the sheath fluid pressure, making the cells align in a single file fashion through the laser beam. Therefore, this event allows uniform illumination of a cell called hydrodynamic focusing (Wilkerson, 2012) ^[49]. So, the single cell at a time interact with the laser at interrogation point. The injection rate of the cells into the laser beam can be manipulated by the flow cytometer user based on the purpose of the analysis. For instance, high flow rates are common for qualitative measurements such as immunophenotyping of mammalian cells, whereas slow flow rates are appropriate for applications requiring higher resolution such as DNA content analysis (Adan *et al.*, 2017) ^[1].

4.1.1 Acoustic focusing

Hydrodynamic focusing for flow cytometry uses relatively high volumetric sheath flow that confines injected sample fluid to a small “core” that is accelerated to meters-per-second velocities. This has long been the standard method in flow cytometry for positioning particles within the optical interrogation region defined by a tightly focused laser beam. Acoustic focusing as applied in flow cytometry mimics this process without requiring sheath flow. It exploits physical differences between cells or particles relative to the background carrier medium to position the particles or cells into a single, focused line along the central axis of a flow channel. Acoustic waves (>2 MHz) pre-focus the sample before introduction to sheath fluid. The pre-focused sample is then injected into the hydrodynamic core and flowed through the instrument (Cossarizza *et al.*, 2019) ^[5]. The ability to tightly focus particles or cells over an extensive range of flow rates, with or without sheath, places a tremendous amount of power in the hands of the user by allowing for optimization of performance and throughput as is suited to each assay. The specific benefits that provide this power are as follows:

- 1) High throughput for dilute samples without resorting to centrifugation;
- 2) High precision focusing combined with high particle rates over a broad range of initial sample concentrations; and
- 3) High sensitivity and resolution from slower flow velocity combined with tight focus (Ward *et al.*, 2018) ^[48].

4.2 Optical System

A flow cytometer has an optical bench that holds the excitation, which includes the laser and lenses and collection optics in fixed positions. The lenses are used to shape and focus the laser beam. Meanwhile, the laser produces light by energizing electrons to high energy orbitals with high voltage electricity. Photons of light are produced when these energized electrons fall back into their lower energy orbitals (Wilkerson, 2012) ^[49]. Light is deflected around the edges of the cell after the laser

strikes the cells, also called as light scattering. Two types of light scatter occur named as Forward Scatter (FSC) and Side Scatters (SSC). The factors affecting total light scatter include the membrane, nucleus, granularity of the cell, cell shape and surface topography. Generally, the size of a cell or a particle and its internal complexity specify the type of scatter. FSC light is a result of diffraction collected along the same axis as the laser beam. FSC is proportional to cell-surface area or size and suitable for detecting particles that makes it the most commonly used method for immunophenotyping. On the other hand, SSC light is a measurement of mostly refracted and reflected light, which is collected at approximately 90° degrees to the laser beam (Sanchez-Torres *et al.*, 2022) ^[37]. SSC is proportional to cell granularity or internal complexity as important as the fluorescent light derived from fluorescent-labeled antibodies or dyes such as propidium iodide (PI) are reflected at the same angle as SSC. In order to differentiate the cell types in a heterogeneous population correlated measurements of FCS and SSC can be used (Reggeti & Bienzle, 2011) ^[35].

There is a variety of laser configurations in flow cytometers based on the type of fluorochromes being excited. Lasers employed for flow cytometers are mainly solid-state, continuous wave lasers. Such lasers have a small footprint and a typical output power range from 20 to 100 mW. Modern cytometers are equipped with up to seven different lasers in a typical laser line ranging from 355 to 650 nm. This gives high flexibility in choosing the fluorophores. The argon laser (a common laser with an excitation wavelength of 488 nm) is used to excite many synthetic dyes such as fluorescein isothiocyanate (FITC) and natural fluorochrome dyes including algae and phytoplanktons, resulting in the emission of light at a higher wavelength. Many flow cytometers and sorters have ultraviolet laser that excite UV (300-400 nm) sensitive fluorochromes or the red diode which excites fluorochromes of the far red (630 nm) range (Cossarizza *et al.*, 2019) ^[5].

Collection optics consists of a collection of the lens to collect light emitted from the particle-laser beam interaction and a system of optical mirrors and filters to separate and then direct specified wavelengths of the collected light to the appropriate optical detectors (Errante *et al.*, 2015) ^[13]. The specificity of a detector for a particular fluorescent dye is determined by placing a suitable filter that can be long pass, short pass and band pass filters. Band pass filters allow only a narrow range of wavelengths. For example, if the BP is named as 660/20, this means that light between 650 and 670 nm will pass through the filter to the photomultiplier tube and all other wavelengths will be reflected to the next filter set within the specified laser configuration (Sanchez-Torres *et al.*, 2022) ^[37]. Short pass filters transmit wavelengths of light equal to or shorter than a specified wavelength whereas long pass filters transmit wavelengths of light equal to or longer than a specified wavelength (Adan *et al.*, 2017) ^[1].

Sometimes mirrors (usually silver mirrors) are used in the optical bench of a flow cytometer in order to deflect light for geometrical or constructive reasons. These filters are >99%, reflective over a wide range of wavelengths. In contrast, a dichroic mirror deflects light of a certain wavelength while the rest pass-through. The effect of the dichroic is dependent on the operating angle (Cossarizza *et al.*, 2019) ^[5].

4.3 Electronic System

As a flow cytometer measures the biological information of a particle (e.g., a cell) via photons, this light needs to be

converted to electrons and processed by an amplifier, filter, Analog to Digital Converter (ADC), and baseline restorer in order to visualize and store the biological information of the cells or other particles. Light signals generated as particles passing through the laser beam in a fluid stream are converted to voltages by photodetectors. Two types of detectors, Photodiodes (PDs) and photomultiplier tubes (PMTs), can be preferred based on their sensitivity (Reggeti & Bienzle, 2011; Snow, 2004) [35, 41]. From a technical point of view, the detection of cell related light is difficult due to

- i) The low light level,
- ii) The high analysis rate, and
- iii) The high dynamic range of the light level (Cossarizza *et al.*, 2019) [5].

PMTs are vacuum tubes containing a photocathode, electron focusing electrodes, and a series of dynodes for electron multiplication. The photocathode converts photons to photoelectrons which are then multiplied by a series of dynodes driven by a high voltage. In many applications, PMTs are increasingly being replaced, e.g. by photodiodes due to their higher quantum efficiency. However, in flow cytometry, only one commercial instrument (CytoFlex, Beckman Coulter) employs APDs in order to improve the sensitivity for wavelengths >700 nm (Snow, 2004) [41].

4.3.1 Signal processing

The light signals captured by PMT or the PD are converted into a proportional number of electrons to create an electrical current. The electrical current travels to the amplifier and is converted to a voltage pulse. The maximum amount of scatter or fluorescence is obtained as the particle is in the center of the beam. When the particle leaves the beam, the pulse comes back down to the baseline (Snow, 2004) [41]. Analog signal measured as an analog quantity is generated after the conversion of the initial light signal into an electrical current by the detectors. After that, this electrical current is amplified by two types of amplifiers, which are linear and logarithmic. The obtained analog signal must be converted into digital signal by an analog to digital converters for computer processing. Then, the signals become a digital data that can be displayed as plots or histograms. Although the data obtained by using the two mentioned amplifiers is exactly the same, the distributions look different based on the approach used to convert an analog signal into digital data (Adan *et al.*, 2017) [1].

4.3.2 Electrostatic cell sorting

Electrostatic cell sorting is responsible for capturing and separation of the cells with predefined features. Once the cells of interest are collected, they can be used for further analysis such as microscopic, biochemical and functional studies. A single parameter or combination of several parameters can be used for cell sorting. To achieve cell sorting, cell sorters are commonly used. The general principle of flow cell sorters is based on the electrostatic deflection of charged droplets, some of which contain cells. In this method, the cells are injected through a nozzle to form a stream of regular droplets by applying a vibration to the nozzle. Then, these droplets pass through one or more laser beams and are charged by a charging electrode at the same time. Droplets can be deflected from the mainstream based on their given charges. Positively charged droplets are deflected toward a platinum plate of negative charge, negatively charged droplets are deflected toward the positively charged platinum plate and uncharged droplets are collected into a waste container (Davies, 2007) [10].

FACS is a specialized type of flow cytometry providing a method for sorting a heterogeneous mixture of fluorescent tagged cells into two or more containers, one cell at a time, based on the specific fluorescent characteristics of each cell (Davies, 2007) [10]. In this case, the operator selects the fluorescent cell of interest using a computer as they are passing through the laser beam. The tagged cells in the drop will be charged, which will be used to separate the cells into different collection vessels (Wilkerson, 2012) [49].

5. Common fluorochromes used in flow cytometry

Fluorochromes used in flow cytometry are classified into several groups including fluorochromes used to label proteins covalently, fluorochromes for nucleic acids and reporter molecules. In order to label proteins covalently, the probe is commonly selected as an antibody. However, other proteins such as a lectin, hormone, avidin or streptavidin, or even c-DNA may be labeled using various fluorochromes. The most widely used fluorochromes for labeling proteins include FITC (fluorescein isothiocyanate), Phycoerythrin (PE) and Allophycocyanin (APC) (Ormerod, 2009) [31]. The selection of the most suitable fluorochrome is an important issue and depends on the laser to be used. If an argon-ion laser including flow cytometer is going to be used, the first choice will be FITC, since there are numerous FITC-labeled antibodies available. PE is the second choice of color for a similar reason. FITC easily reacts with the amino groups on the lysine residues in the protein and produces moderately stable conjugates. FITC (excitation/emission maxima approx. 495/ 520 nm) is a good fluorochrome for single-color staining since its maximum absorbance near 490 nm. On the other hand, its emission with longer wavelengths makes it inappropriate for multicolor applications. Moreover, its fluorescence is highly pH-sensitive and subjected to photobleaching with a high rate. To solve these problems, various FITC derivatives have been developed such as AlexaTM series with greater photostability and increased fluorescence (Givan, 2001) [16]. PE (excitation/emission maxima 495-565/578 nm) and APC (excitation/emission maxima 650/660 nm) are called phycobiliproteins that are the components of photosynthetic systems. They have good light absorption and high fluorescence intensities. Although their fluorescence is about 30-fold greater than that of fluorescein, in practice, cells labeled with phycobiliprotein antibodies have fluorescence intensities between five- and 10-fold greater than those labeled with FITC-labeled antibody. Although using an argon laser excites FITC and PE, the excitation of APC needs helium-neon laser due to its higher (650 nm) absorption maxima. The major drawback of using phycobiliproteins is related to their higher molecular weight, causing steric changes when conjugated to proteins (Telford *et al.*, 2012) [43].

The development of tandem dyes, containing two fluorochromes, has increased the number of labeled proteins to be used. Examples include conjugates of PE and APC with various cyanine dyes, for instance, PE-Cy5 and APC-Cy7 (Hulspas *et al.*, 2009) [21]. In tandem dyes, when the first dye is excited and reaches its maximal absorbance, it transfers all its energy to the second dye located in close proximity. As a result, this second fluorochrome is activated and produces the fluorescence emission. This process is called Fluorescence Resonance Energy Transfer (FRET). It is a good way to obtain higher Stokes shifts that increase the number of colors analyzed from a single laser wavelength (Leavesley *et al.*, 2013) [27].

Fluorochromes used to stain nucleic acids in flow cytometry have several uses, such as measuring ploidy, cell cycle analysis, evaluating membrane integrity, and quantifying DNA. Ethidium Bromide (EB) and PI are two of the many dyes that are currently available that can stain both RNA and DNA. While measuring DNA, RNA must be removed using RNase. EB and PI intercalate between the bases in double-stranded nucleic acids. PI is excited by blue light and gives red fluorescence. If an argon laser is set to 488 nm, PI can be used in conjunction with FITC to assess DNA content and antibody binding simultaneously. 7-Aminoactinomycin D (7-ADD) characterized by its preferential binding to GC-rich regions of DNA is generally removed by live cells and used to stain DNA of dead cells. It can be excited by an argon laser and used with FITC- and PE-labeled antibodies. There are also UV-excited dyes such as Hoechst and DAPI that bind to AT-rich regions of DNA. Although they are membrane permeable dyes, dead cells can uptake them more easily (Adan *et al.*, 2017) [1].

Reporter molecules are also used in flow cytometric analysis. For instance, Green Fluorescent Protein (GFP) is excited at 488 nm and fluoresces green. GFP and its mutant forms are used in various applications such as sorting of GFP transfected cells (Telford *et al.*, 2012) [43]. Another reporter molecule is JC-1 dye used to measure mitochondrial membrane potential. JC-1 dye accumulates in mitochondria of healthy cells by forming red JC-1 aggregates with emission at 590 nm. In addition, there are several other reporter molecules to detect several changes in experimental cells such as production of the reactive oxygen species, measurement of intracellular calcium ions and changes in intracellular pH (Adan *et al.*, 2017) [1].

5.1 Fluorescence compensation

Each fluorochrome has a broad fluorescence spectrum. When more than one fluorochrome is used, the overlap between fluorochromes can occur. This situation is called spectrum overlap. This situation needs to be overcome. For example, the emission spectrum for FITC and PE is that the light emitted by the fluorescein overlaps the same wavelength as it passes through the filter used for PE. This spectral overlap is corrected by removing a portion of the FITC signal from the PE signals or vice versa. This process is called color compensation, which calculates a fluorochrome as a percentage to measure itself (Ormerod, 2009) [31].

Compensation is the mathematical process by which spectral overlap of multiparameter flow cytometric data is corrected. Since fluorochromes can have wide-ranging spectrum, they can overlap, causing the undesirable result of confusion during the analysis of data. This overlap, known as spillover and quantified in the spillover coefficient, is usually caused by detectors for a certain fluorochrome measuring a significant peak in wavelength from a different fluorochrome. Linear algebra is most often used to make this correction (Telford *et al.*, 2012) [43]. In general, when graphs of one or more parameters are displayed, it is to show that the other parameters do not contribute to the distribution shown. Especially when using the parameters which are more than double, this problem is more severe. Currently, no tools have been discovered to efficiently display multidimensional parameters. Compensation is very important to see the distinction between cells (Ormerod, 2009) [31].

6. Data analysis

Flow cytometry data analysis has been considered as the most critical parameter for biological experiments. It may be

necessary to formulate a strategy for the data acquisition, which includes the use of gates or acquisition gate to define positive/negative cell populations for a particular marker, or the proportion of positive cells (Herzenberg *et al.*, 2006) [20].

The major principles of data analysis are to selectively show the cells of interest and to find out more about those cells. This method is called “gating” in flow cytometry as well as a gate can be defined as one or more regions combined. A region can be defined as a set of points carefully selected by the user that determines an area on a graph. Several regions can be defined on the same graph. These parameters can provide you with an opportunity to eliminate results from the unwanted particles such as dead cells and debris. The most common application of gating strategy is to use FSC and SSC plots. The different physical characteristics of white blood cells, granulocytes, monocytes and lymphocytes, allow them to be distinguished from each other.

6.1 Gates and regions

A subset of data can be defined by gating methods. Additionally, a gate is a numerical or graphical boundary that can be used to define the characteristics of particles for further analysis. For example, in a blood sample containing a mixed population of cells, you might want to restrict your analysis to only the lymphocytes. Based on FSC or cell size, a gate can be set on the FSC versus SSC plot for the analysis of lymphocytes. The resulting display would reflect the fluorescence properties of only the lymphocytes. The data can be displayed by several different plot types. These range from histograms to 2-D plots such as dot plots, contour and density plots, to 3-D plots such as a tomogram plot (Adan *et al.*, 2017) [1].

6.2 Single parameter analysis

A histogram is a single parameter plot where the x-axis represents the parameter's signal value in channel numbers and the y-axis represents the number of events per channel number. Each event placed in the channel corresponds to its signal value. Signals with identical intensities accumulate in the same channel. Brighter signals are displayed in channels to the right of the dimmer signals. Ideally, flow cytometry will produce a single distinct peak. However, flow analysis is sometimes marked on a mixed population of cells resulting in several peaks on the histogram (Adan *et al.*, 2017) [1].

6.3 Two parameter analysis

Alternate methods of data display are available for two parameter plots. These are graphs that display two measurement parameters, one on the x-axis and one on the y-axis and the cell count as a density (dot) plot or contour map. A dot plot provides a two-parameter display of data and each dot represents one or more events (particles) while density plot displays two parameters as a frequency distribution. The color is used to code the different frequencies of events in density plot. The contour plot is the same as a density plot, except the density of the cells is displayed as contour lines (Adan *et al.*, 2017) [1].

A quadrant marker divides two-parameter plots into four sections to discriminate populations as negative, single positive or double positive. The upper-right quadrant of the plot indicates cells positive for both fluorescent marker or double positive. The lower-left quadrant displays events that are negative for both markers. The upper-left quadrant of the plot indicates cells only positive for the y-axis parameter. The lower-right quadrant contains events that are positive for the

x-axis marker but negative for the y-axis fluorescent marker (Herzenberg *et al.*, 2006) [20].

7. Applications of flow cytometry

As mentioned above, Flow cytometry is an extremely versatile technology that evaluates several parameters simultaneously in each cell, allowing correlations to be established between them. Not only can Flow cytometry analyze the presence of molecules on the surface or inside cells: it can also quantify them. It is possible to perform functional tests, biological evaluations of compounds in both eukaryotic and prokaryotic cells, purify cell populations even when they are present in a low proportion in the sample, or quantify soluble molecules and evaluate cell proliferation, cell viability, cell death, metabolic activity and intracellular signalling, just to mention some of them; Another very important aspect is that, by being able to evaluate many of these characteristics simultaneously, the sample volume required is reduced. The techniques described below have application in both human and veterinary medicine, and within the latter, both in domestic animals and wildlife (Thomason *et al.*, 2014) [44]. It is worth mentioning that, on some occasions, the limitation is the availability in the market of antibodies that recognize specific molecules of the different animal species, but given the importance of the results that can be obtained by FC, the availability of antibodies with different specificities has grown in recent years, facilitating the work in the veterinary area both in research and in the clinic, especially for diagnosis. In veterinary research, measurement of cell fluorescence may be employed for immunophenotyping, ploidy analysis, functional cell assays, or determination of transfection efficiency with genes encoding fluorescent proteins (Drescher *et al.*, 2021) [11].

7.1 Cell viability assay

Cell viability assays using flow cytometry are commonly used to quantify the number of viable cells in a sample and to assess the effects of various treatments on cell survival (Crowley *et al.*, 2016) [7]. Flow cytometry-based cell viability assays typically involve staining cells with fluorescent dyes that selectively bind to specific cellular components, such as nucleic acids, membrane proteins, or enzymes, that are indicative of cell viability (Al-Rubeai *et al.*, 1997) [2].

One of the most commonly used viability assays is the Propidium Iodide (PI) assay. PI is a fluorescent dye that binds to DNA, but is excluded from intact cells due to the presence of an intact cell membrane. In contrast, PI can penetrate cells with compromised membranes, such as dead or dying cells, and bind to their DNA, resulting in an increase in fluorescence intensity. To perform the PI assay, cells are harvested and stained with PI, either alone or in combination with other dyes that distinguish live cells from dead cells or debris. The cells are then analyzed by flow cytometry, and the percentage of PI-positive cells is calculated, which is indicative of the proportion of non-viable cells in the sample (Crowley *et al.*, 2016) [7].

Another commonly used viability assay is the 7-Aminoactinomycin D (7-AAD) assay. 7-AAD is a fluorescent dye that binds to DNA, similar to PI. However, 7-AAD has a higher affinity for DNA and has a narrower excitation and emission spectrum, which makes it more specific and less susceptible to interference from other fluorescent dyes. 7-AAD is also excluded from live cells due to their intact cell membranes, but can enter dead or dying cells with compromised membranes and bind to their DNA. The cells

are then analyzed by flow cytometry, and the percentage of 7-AAD-positive cells is calculated, which is indicative of the proportion of non-viable cells in the sample (Schmid *et al.*, 2007) [38].

Cell viability assays can also be performed using fluorescent dyes that selectively stain live cells, such as calcein-AM and SYTOX Green. Calcein-AM is a non-fluorescent dye that is converted to a fluorescent compound by intracellular esterases, which are active in live cells but not in dead cells. SYTOX Green is a fluorescent dye that selectively stains dead cells with compromised membranes, but is excluded from live cells due to their intact cell membranes. The cells are then analyzed by flow cytometry, and the percentage of live cells is calculated based on the fluorescence intensity of calcein-AM or the absence of SYTOX Green staining (Peperzak and Brussaard, 2011) [33].

In addition to these dyes, viability assays can also be performed using fluorescent substrates that are selectively cleaved by viable cells, such as resazurin and fluorescein diacetate. Resazurin is a non-fluorescent dye that is converted to a fluorescent compound by mitochondrial enzymes in viable cells, but not in dead cells (Crowley *et al.*, 2016) [7]. Fluorescein diacetate is a non-fluorescent dye that is cleaved by intracellular esterases in viable cells to generate a fluorescent compound. The cells are then analyzed by flow cytometry, and the percentage of viable cells is calculated based on the fluorescence intensity of resazurin or fluorescein (Al-Rubeai *et al.*, 1997) [2].

In conclusion, flow cytometry-based cell viability assays are powerful tools for assessing the number and proportion of viable cells in a sample and for quantifying the effects of various treatments on cell survival. The choice of dye or substrate will depend on the specific experimental question, the cell type, and the experimental conditions.

7.2 Apoptosis detection

Apoptosis, or programmed cell death, is a highly regulated process that plays a critical role in normal development and tissue homeostasis. It plays a key role in tissue and organ development during embryogenesis as well as in adult tissues during cell turnover. Dysregulation of apoptosis can contribute to a variety of diseases, including cancer and autoimmune disorders. Assays used to measure apoptosis include detection of morphologic changes by light microscopy, or electron microscopy. However, quantification of apoptosis by microscopy is cumbersome. Flow cytometry-based apoptosis detection can provide valuable insights into the mechanisms and regulation of apoptosis in various biological systems (Vermees *et al.*, 2000) [46]. Flow cytometry-based apoptosis detection typically involves staining cells with fluorescent dyes that selectively bind to different cellular components, such as DNA, membrane phospholipids, or cytoplasmic proteins (Vermees *et al.*, 2000) [46]. Apoptotic cells can be identified and quantified based on specific changes in their cellular characteristics, including alterations in cell size, shape, and granularity, as well as changes in the expression and localization of surface markers, intracellular proteins, and nucleic acids (Koopman *et al.*, 1994) [25]. One of the most commonly used assays for flow cytometry-based apoptosis detection is the Annexin V assay. In normal cells plasma membranes exhibit significant phospholipid asymmetry, with phosphatidylcholine and sphingomyelin predominantly on the external leaflet, and most of the membrane's phosphatidylethanolamine and phosphatidylserine on the inner leaflet. Entry into apoptosis was shown to lead to a loss of phospholipid asymmetry, with exposure of

phosphatidylserine on the outer leaflet. Annexin V is a calcium-dependent phospholipid-binding protein that specifically binds to Phosphatidylserine (PS), a phospholipid that is translocated from the inner to the outer leaflet of the plasma membrane during early apoptosis. Cells are stained with Annexin V conjugated to a fluorescent dye, such as FITC or APC, and a viability dye, such as propidium iodide (PI) or 7-aminoactinomycin D (7-AAD). Annexin V-positive cells are considered to be in the early stages of apoptosis, while Annexin V-positive and PI/7-AAD-positive cells are considered to be in the late stages of apoptosis or necrosis (Koopman *et al.*, 1994) [25]. In addition to Annexin V, flow cytometry-based apoptosis detection can also be performed using a variety of other assays, including TUNEL assays, caspase activation assays, mitochondrial membrane potential assays, and DNA content analysis. (Wlodkowicz *et al.*, 2009) [50].

In conclusion, flow cytometry-based apoptosis detection is a powerful technique that can provide valuable insights into the mechanisms and regulation of apoptosis in various biological systems. The choice of assay will depend on the specific biological question, the cell type, and the experimental conditions. Careful optimization and validation of the assays are necessary to ensure accurate and reproducible results.

7.3 Cell cycle analysis

Cell cycle analysis using flow cytometry is a widely used technique that allows for the quantification of cells in different phases of the cell cycle (Gray *et al.*, 1986) [18]. The cell cycle consists of four phases: G1, S, G2, and M, each of which is characterized by specific cellular events and changes in DNA content (Rabinovitch, 1994) [34]. Cell cycle analysis using flow cytometry involves labeling cells with a DNA-binding dye, such as Propidium Iodide (PI), which binds to DNA and emits fluorescence when excited by a laser. By measuring the intensity of PI fluorescence in individual cells, researchers can determine the DNA content of each cell and identify which phase of the cell cycle it is in (Gray *et al.*, 1986) [18]. The cell cycle is divided into four phases: G1, S, G2, and M. G1 is the first gap phase, during which cells grow and prepare for DNA synthesis. S is the synthesis phase, during which cells replicate their DNA. G2 is the second gap phase, during which cells prepare for mitosis. M is the mitosis phase, during which cells divide into two daughter cells (Rabinovitch, 1994) [34]. During cell cycle analysis using flow cytometry, cells are typically first fixed with a chemical such as ethanol or paraformaldehyde to preserve their DNA content. Next, cells are permeabilized to allow the DNA-binding dye to enter the cells and bind to the DNA. The labeled cells are then analyzed by a flow cytometer, which detects and quantifies the PI fluorescence emitted by each cell (Kim and Sederstrom, 2015) [24]. The resulting data can be analyzed using flow cytometry software to determine the percentage of cells in each phase of the cell cycle. For example, cells in the G1 phase typically have a lower DNA content than cells in the S or G2/M phases, and can be identified based on their position on a DNA content histogram generated by the flow cytometer (Gray *et al.*, 1986) [18]. In addition to PI, other DNA-binding dyes such as DAPI or Hoechst can be used for cell cycle analysis. (Darzynkiewicz, 2001) [8].

7.4 Anti-malarial drug screening and drug susceptibility test

Malaria caused by the parasitic protozoan *Plasmodium falciparum* is highly virulent and a leading cause of mortality

in children compared to other *Plasmodium* species (Janse and Van Vianen, 1994) [23]. Drug-resistant *Plasmodium* is a frequent global threat in malaria eradication programmes, highlighting the need for new anti-malarial drugs and efficient detection of treatment failure (Francisco, 2017) [15]. Conventional *Plasmodium falciparum* culture is essential in drug discovery and resistance surveillance. Despite its standardization and conventional use, microscopic assays to enumerate and differentiate various stages of intraerythrocytic *Plasmodium* parasites are tedious and time-consuming. Moreover, interrater variability among the microscopists largely affects the interpretation, emphasizing a need for rapid and simpler assays that retain accuracy (Kulkeaw, 2021) [26]. Flow cytometry has been used extensively in malaria research to investigate the biology of the parasite and to develop new drugs. In antimalarial drug screening, flow cytometry can be used to evaluate the efficacy of compounds against the parasite at different stages of its life cycle. The technique can also be used for susceptibility testing to determine the sensitivity of the parasite to a given drug. The use of flow cytometry in these applications has several advantages over traditional methods, including increased speed, sensitivity, and specificity. This is because flow cytometry can detect and measure changes in individual cells, rather than relying on bulk measurements (Janse and Van Vianen, 1994) [23]. One of the main advantages of flow cytometry is its ability to rapidly analyze large numbers of cells. This is particularly useful for screening compounds against the parasite at different stages of its life cycle. For example, the parasite has different physiological properties during its asexual replication in the erythrocyte and during its sexual reproduction in the mosquito. By using flow cytometry, researchers can easily distinguish between these different stages and screen compounds against them separately (Kulkeaw, 2021) [26].

Flow cytometry based antimalarial drug screening and assessing drug susceptibility involves the use of nucleic acid binding fluorochrome. Given that mature erythrocytes, host cells of blood-dwelling *Plasmodium*, lack nuclear DNA, detection of malarial DNA is thus able to distinguish parasitized erythrocytes from non-infected erythrocytes, especially those in a leukocyte-free culture of *Plasmodium* parasites (Kulkeaw, 2021) [26]. At present, many DNA- and RNA-binding fluorochromes are commercially available, and they are divided into two types: cell membrane permeable and non-permeable. A major change in asexual development of *Plasmodium* parasite is an increase in DNA synthesis and the number of nuclei. Thus, the use of nucleic acid-binding fluorescent chemicals is speculated to discriminate all asexual stages of *Plasmodium*. The use of FSCs and SSCs allows the exclusion of cell debris that is small; thus, quantifying a large number of cells is accurate, especially in the examination of parasitaemia post drug exposure. Upon excitation, the emitted fluorescence of nucleic acid binding fluorochromes can be displayed based on their fluorescence intensities: high, intermediate and low depending on the resolution of fluorescence intensity. Similar to the Giemsa-based microscopy data, the flow cytometer data indicate the proportion of *Plasmodium*-infected erythrocytes, known as parasitaemia (Francisco, 2017) [15].

In drug susceptibility assays, a highly synchronized culture of *Plasmodium* parasites (laboratory-adapted strains or field-isolated strains) is first prepared and subsequently treated with various doses of drug. Mostly, synchronized cultures of ring-like *Plasmodium* parasites are incubated with drugs, and their growth is assessed mostly at the schizont stage. Hence, prior

drug treatment and heterogeneity in *Plasmodium* parasites may confound the interpretation (Francisco, 2017) ^[15]. After drug treatment, flow cytometry could provide information about not only parasitaemia but also the proportion of each developmental stage. The quantifiable data can be displayed as a dose-response curve. Moreover, a change in the proportion of each asexual stage provides the stage-specific effects of the tested compound or anti-malarial drug, allowing further investigation of a drug mechanism. By measuring the percentage of cells that are inhibited at each concentration, researchers can determine the drug concentration required to inhibit growth by 50% (IC50). This is a widely used measure of drug susceptibility in malaria research and is used to compare the efficacy of different drugs and to monitor the emergence of drug resistance (Kulkeaw, 2021) ^[26].

7.5 Immunophenotyping

Immunophenotyping refers to the characterization of cell populations based on the molecules expressed by a cell, allowing their identification through the use of antibodies. In order to perform an immunophenotyping study, the antibodies used recognize particular molecules in the cells; based on their presence or absence, or on their level of expression, the identification or characterization is performed. It must keep in mind that there are molecules that are expressed in several cell types, while the expression of others is exclusive to certain cell populations or subpopulations, which implies that a correct selection of antibodies must be made. The detection of the different molecules of interest is performed using antibodies labeled with fluorochromes, so if a beam of light that excites the fluorochrome is shone on it, it will emit fluorescence at a certain wavelength that will be captured by a detector which will then identify the fluorochrome and, consequently, the molecule to which the antibody was bound (Craig and Foon, 2008). Flow cytometry technique is widely used in immunology research, and is particularly useful for identifying and characterizing subsets of immune cells based on their surface marker expression. This process is known as flow cytometry-based immunophenotyping (Chen and Luu, 2017) ^[3]. Immunophenotyping is a critical step in the diagnosis and monitoring of hematological malignancies, immunodeficiencies, and autoimmune diseases. By analyzing the expression of specific surface markers on immune cells, researchers can identify and quantify different cell populations in a sample, determine their activation status, and monitor changes in response to various stimuli or therapies (Papenfuss, 2017) ^[32]. Flow cytometry-based immunophenotyping involves labeling cells with fluorescent antibodies specific for different cell surface markers. The cells are then passed through a flow cytometer, which detects and quantifies the fluorescent signal from each cell (Chen and Luu, 2017) ^[3]. The resulting data can be used to generate histograms, scatter plots, and other visualizations that provide information about the frequency, distribution, and characteristics of different cell populations (Papenfuss, 2017) ^[32].

For example, flow cytometry can be used to identify and quantify subsets of T cells, B cells, natural killer cells, and myeloid cells based on their expression of different surface markers. This can help researchers understand the immune response to infections, tumors, or autoimmune disorders, and identify potential therapeutic targets. Additionally, flow cytometry can be used to monitor the effectiveness of immunotherapies, such as checkpoint inhibitors or CAR-T cells, by tracking changes in the frequency and activation

status of immune cells in response to treatment (Papenfuss, 2017) ^[32].

7.6 Cancer stem cell analysis

Cancer Stem Cells (CSCs) are a small population of cells within tumours that have the ability to self-renew and differentiate into heterogeneous tumour cells. CSCs are thought to play a critical role in tumour progression, metastasis, and recurrence, making them an important target for cancer therapy (Spillane and Henderson, 2007) ^[42]. Analysing CSCs using flow cytometry has become an important tool in cancer research, allowing for the identification and characterization of these cells. In flow cytometry assay the cells are labeled with fluorescent antibodies that specifically bind to cell surface markers that are characteristic of CSCs. By analyzing the fluorescence intensity of the labeled cells, flow cytometry can distinguish CSCs from other cells in the tumor (Greve *et al.*, 2012) ^[19].

One of the primary advantages of flow cytometry in CSC analysis is the ability to identify and isolate CSCs based on specific cell surface markers. Various markers have been used to identify CSCs in different types of tumors, such as CD44 and CD133 in breast cancer, CD24 and CD44 in pancreatic cancer, CD44 and CD117 in ovarian cancer and CD133 and CD166 in colon cancer. These markers can be labeled with fluorescent antibodies and analyzed using flow cytometry to identify and quantify CSCs within a tumor (Greve *et al.*, 2012) ^[19].

In addition to identifying CSCs, flow cytometry can also be used to characterize the properties of these cells. For example, CSCs have been found to have high expression levels of drug resistance-related genes and efflux pumps, which contribute to their resistance to chemotherapy. Flow cytometry can be used to analyze the expression levels of these genes and pumps in CSCs, providing important information for developing targeted therapies (Greve *et al.*, 2012) ^[19].

Another important application of flow cytometry in CSC analysis is the evaluation of CSCs in patient samples. By analyzing CSCs in patient samples, researchers can determine the prognostic value of CSCs and evaluate the efficacy of CSC-targeting therapies. For example, studies have shown that high levels of CSCs in patient samples are associated with poor prognosis, highlighting the importance of CSC analysis in cancer (Spillane and Henderson, 2007) ^[42]. By identifying and characterizing CSCs, flow cytometry provides valuable information for developing targeted therapies and improving cancer diagnosis and treatment.

7.7 Sperm cell analysis and sorting

In the field of reproductive biology, flow cytometry is used to analyze semen samples to determine sperm concentration, motility, morphology, and DNA fragmentation. Additionally, flow cytometry is used for sperm sexing, where spermatozoa are separated into X and Y-bearing populations based on the presence of X or Y-chromosomes, respectively (Cordelli *et al.*, 2005) ^[4].

Semen analysis using flow cytometry involves the labeling of sperm cells with fluorescent dyes that bind to specific components of the sperm. The sperm cells are then passed through a flow cytometer, which measures the fluorescence intensity of the cells as they pass through a laser beam. The intensity of the fluorescence corresponds to the amount of the labeled component present in the cell (Cordelli *et al.*, 2005) ^[4]. Sperm concentration is one of the most important parameters analyzed in semen analysis. By using flow

cytometry, sperm concentration can be accurately measured in a relatively short amount of time. Additionally, flow cytometry can be used to assess sperm motility. Sperm cells that are labeled with a fluorescent dye that binds to the mitochondrial membrane potential can be analyzed using flow cytometry to determine the proportion of motile sperm (Cordelli *et al.*, 2005) [4].

Another parameter analyzed in semen analysis is sperm morphology. Abnormal sperm morphology is associated with reduced fertility and increased risk of miscarriage. Flow cytometry can be used to assess sperm morphology by labeling sperm cells with fluorescent dyes that bind to the acrosome, nucleus, or tail. This allows for the detection of abnormalities in the shape and size of the sperm (Cordelli *et al.*, 2005) [4]. DNA fragmentation is another important parameter analyzed in semen analysis. High levels of DNA fragmentation are associated with reduced fertility and increased risk of genetic abnormalities in offspring (Evenson *et al.*, 2007) [14].

Sperm sorting is a technique used to separate sperm cells into X-bearing (Female) and Y-bearing (Male) populations, allowing for the selection of offspring of a desired sex. Flow cytometry is used in sperm sorting to sort sperm cells based on DNA content difference. The sorting of X- and Y-chromosome bearing sperm on the basis of DNA content differences is most reliable as well as repeatable technique for producing sex preselected animals. Difference in DNA content for most mammals are in the range of 3-4.2% with X-Chromosome contain more DNA than Y chromosome. The sorting process involves labeling the sperm cells with a fluorescent dye that binds to DNA. The labeled cells are then sorted based on their fluorescence intensity, with high-intensity cells being sorted into a separate sample (Yadav *et al.*, 2018) [51].

8. Conclusions

In conclusion, Flow Cytometry (FC) is a technique for qualitative and quantitative assessment of multiple parameters of individual cells or particles in complex cell suspensions. Flow cytometers capture light scatter properties and light emitted by individual cells that innately fluoresce or that are labeled with fluorescent antibodies or probes. Three parts constitute a flow cytometer: first, the fluidic system permits hydrodynamic focusing; second the excitation source and collection optic constitute the optical part and finally the electronic system digitalizes the signal to be analyzed with specific computer software. With the flexibility of flow cytometry, and the reliability of results, this technology has become an essential veterinary research instrument with important clinical diagnostic capabilities, particularly in the fields of immunology and hematology. It is possible to perform functional tests, biological evaluations of compounds, purify cell populations even when they are present in a low proportion in the sample. The most common use of flow cytometry is in the identification of markers on cells, particularly the immune system or immunophenotyping and evaluation of canine platelets. It is one of the most popular and versatile techniques for studying apoptosis. It can be applied as a rapid, precise and objective tool for routine cell viability measurement, cell proliferation, metabolic activity and intracellular signalling. Flow cytometry has been extensively used to study mammalian sperm in the areas of reproductive toxicology (to monitor effects from environmental, occupational and therapeutic exposures), veterinary science (to preselect the gender of offspring by

sorting X- and Y-chromosome-bearing sperm) and clinical andrology (to assess individual fertility potential). Flow cytometry is most often applied in veterinary oncology to diagnose lymphoid neoplasia and leukemia, and can also be used to assess neoplastic and non-neoplastic marrow conditions.

9. Future prospects

Flow cytometry has a bright future because it is the only viable technology for comprehensive and multiplexed analysis of single cells. Furthermore, with the latest spectral cell sorting technology, a single cell from a multicellular mixture, identifiable only by a complex algorithm, can be separated for cloning or sequencing. The next generation will function entirely in the digital domain, have features currently available only on custom-built instruments and have the capacity for potentially 150-200 parameters, which will engage metabolic, structural and functional probes, extending the field for several decades.

References

1. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. *Crit Rev Biotechnol*. 2017;37(2):163-176.
2. Al-Rubeai M, Welzenbach K, Lloyd DR, Emery AN. A rapid method for evaluation of cell number and viability by flow cytometry. *Cytotechnology*. 1997;24(2):161-168.
3. Chen W, Luu HS. Immunophenotyping by multiparameter flow cytometry. *Methods Mol Biol*. 2017;1633:51-73.
4. Cordelli E, Eleuteri P, Leter G, Rescia M, Spanò M. Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity. *Contraception*. 2005;72(4):273-279.
5. Cossarizza A, Chang HD, Radbruch A, Acs A, Adam D, Adam-Klages S, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur J Immunol*. 2019;49(10):1457-1973.
6. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood*. 2008;111(8):3941-3967.
7. Crowley LC, Scott AP, Marfell BJ, Boughaba JA, Chojnowski G, Waterhouse NJ. Measuring cell death by propidium iodide uptake and flow cytometry. *Cold Spring Harb Protoc*. 2016;2016(7):pdb.prot087163.
8. Darzynkiewicz Z. Cell cycle analysis by flow cytometry. *Methods Cell Biol*. 2001;63:373-380.
9. Darzynkiewicz Z, Galkowski D, Zhao H. Analysis of apoptosis by cytometry using TUNEL assay. *Methods*. 2008;44(3):250-254.
10. Davies D. Cell sorting by flow cytometry. In: *Flow cytometry: principles and applications*. Totowa (NJ): Humana Press; 2007. p. 257-276.
11. Drescher H, Weiskirchen S, Weiskirchen R. Flow cytometry: a blessing and a curse. *Biomedicines*. 2021;9(11):1613.
12. Erotocritou P, Arya M, Shukla SN, Patel HR. Flow cytometry. In: *Basic science techniques in clinical practice*. London: Springer; c2007. p. 38-47.
13. Errante PR, Ebbing PC, Rodrigues FSM, Ferraz RRN, Silva NPD. Flow cytometry: a literature review. *Rev Bras Hematol Hemoter*. 2015;37(1):45-51.

14. Evenson DP, Kasperson K, Wixon RL. Analysis of sperm DNA fragmentation using flow cytometry and other techniques. *Soc Reprod Fertil Suppl.* 2007;65:93-113.
15. Francisco DR. Optimization of a flow cytometric assay to measure antimalarial drug effects. *Exp Parasitol.* 2017;32:76-81.
16. Givan AL. Flow cytometry: first principles. New York: John Wiley & Sons; c2001. p. 34-41.
17. Givan AL. Flow cytometry: an introduction. *Methods Mol Biol.* 2011;699:1-29.
18. Gray JW, Dolbeare F, Pallavicini MG, Beisker W, Waldman F. Cell cycle analysis using flow cytometry. *Int J Radiat Biol Relat Stud Phys Chem Med.* 1986;49(2):237-255.
19. Greve B, Kelsch R, Spaniol K, Eich HT, Götte M. Flow cytometry in cancer stem cell analysis and separation. *Cytometry A.* 2012;81(4):284-293.
20. Herzenberg LA, Tung J, Moore WA, Parks DR. Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol.* 2006;7(7):681-685.
21. Hulspar R, Dombkowski D, Pfeffer F, Douglas D, Kildew-Shah B, Gilbert J. Flow cytometry and the stability of phycoerythrin-tandem dye conjugates. *Cytometry A.* 2009;75(11):966-972.
22. Ibrahim SF, van den Engh G. Flow cytometry and cell sorting. *Adv Biochem Eng Biotechnol.* 2007;106:19-39.
23. Janse CJ, Van Vianen PH. Flow cytometry in malaria detection. *Methods Cell Biol.* 1994;42(Pt B):295-318.
24. Kim KH, Sederstrom JM. Assaying cell cycle status using flow cytometry. *Curr Protoc Mol Biol.* 2015;111:28.6.1-28.6.11.
25. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 1994;84(5):1415-1420.
26. Kulkeaw K. Progress and challenges in the use of fluorescence-based flow cytometric assays for anti-malarial drug susceptibility tests. *Malar J.* 2021;20(1):57.
27. Leavesley SJ, Britain AL, Cichon LK, Nikolaev VO, Rich TC. Assessing FRET using spectral techniques. *Cytometry A.* 2013;83(10):898-912.
28. McKinnon KM. Flow cytometry: an overview. *Curr Protoc Immunol.* 2018;120:5.1.1-5.1.11.
29. Melamed MR. A brief history of flow cytometry and sorting. *Methods Cell Biol.* 2001;63:3-17.
30. Moldavan A. Photo-electric technique for the counting of microscopical cells. *Science.* 1934;80(2069):188-189.
31. Ormerod MG. Flow cytometry: a basic introduction. 5th ed. Redhill (UK): De Novo Software; 2009.
32. Papenfuss TL. Flow cytometry and immunophenotyping in drug development. In: *Immunopathology in toxicology and drug development.* Vol 1. Cham: Springer; c2017. p. 343-369.
33. Peperzak L, Brussaard CP. Flow cytometric applicability of fluorescent vitality probes on phytoplankton. *J Phycol.* 2011;47(3):692-702.
34. Rabinovitch PS. DNA content histogram and cell-cycle analysis. *Methods Cell Biol.* 1994;41:263-296.
35. Reggeti F, Bienzle D. Flow cytometry in veterinary oncology. *Vet Pathol.* 2011;48(1):223-235.
36. Robinson JP. Flow cytometry: past and future. *Biotechniques.* 2022;72(4):159-169.
37. Sanchez-Torres LE, Espinosa-Bonilla A, Diosdado-Vargas F. Flow cytometry, a universe of possibilities in the veterinary field. *Rev Mex Cienc Pecu.* 2022;13(3):763-786.
38. Schmid I, Uittenbogaart C, Jamieson BD. Live-cell assay for detection of apoptosis by dual-laser flow cytometry using Hoechst 33342 and 7-amino-actinomycin D. *Nat Protoc.* 2007;2(1):187-190.
39. Shapiro HM. Practical flow cytometry. 4th ed. New York: John Wiley & Sons; c2005. p. 27-35.
40. Sharpe JC, Evans KM. Advances in flow cytometry for sperm sexing. *Theriogenology.* 2009;71(1):4-10.
41. Snow C. Flow cytometer electronics. *Cytometry A.* 2004;57(2):63-69.
42. Spillane JB, Henderson MA. Cancer stem cells: a review. *ANZ J Surg.* 2007;77(6):464-468.
43. Telford WG, Hawley T, Subach F, Verkhusha V, Hawley RG. Flow cytometry of fluorescent proteins. *Methods.* 2012;57(3):318-330.
44. Thomason J, Archer T, Mackin A, Stokes J, Pinchuk L. Applications of flow cytometry in veterinary research and small animal clinical practice. *J Vet Med Res.* 2014;1(1):1004.
45. Tung JW, Parks DR, Moore WA, Herzenberg LA. New approaches to fluorescence compensation and visualization of FACS data. *Clin Immunol.* 2004;110(3):277-283.
46. Vermes I, Haanen C, Reutelingsperger C. Flow cytometry of apoptotic cell death. *J Immunol Methods.* 2000;243(1-2):167-190.
47. Wang C, Smith BR, Ault KA, Rinder HM. Reticulated platelets predict platelet count recovery following chemotherapy. *Transfusion.* 2002;42(3):368-374.
48. Ward MD, Kaduchak G. Fundamentals of acoustic cytometry. *Curr Protoc Cytom.* 2018;84(1):e36.
49. Wilkerson MJ. Principles and applications of flow cytometry and cell sorting in companion animal medicine. *Vet Clin North Am Small Anim Pract.* 2012;42(1):53-71.
50. Wlodkowic D, Skommer J, Darzynkiewicz Z. Flow cytometry-based apoptosis detection. *Methods Mol Biol.* 2009;559:19-32.
51. Yadav HP, Sahu SK, Lone SA, Shah N, Singh A, Verma UK, Mohanty TK. Advances in sperm sexing in bovines. *J Exp Zool India.* 2018;21(1):1-9.
52. Crosland-Taylor PJ. A device for counting small particles suspended in a fluid through a tube. *Nature.* 1953 Jan 3;171(4340):37-38.

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