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INDEX

|  |  |  |
| --- | --- | --- |
| Sr. No. | Content | Page Number |
| 1 | Introduction to Flow Cytometry | 1 |
| 2 | Historical Background | 1 |
| 3 | Components of Flow Cytometry | 1 |
| 4 | Specialized Components | 4 |
| 5 | Modifications of Flow Cytometry | 4 |
| 6 | Sample Preparation for Flow Cytometry | 5 |
| 7 | Special Considerations for Sample Preparation | 7 |
| 8 | Key Applications of Flow Cytometry | 8 |
| 9 | Advantages and Limitations of Flow Cytometry | 9 |
| 10 | Conclusion | 9 |
| 11 | References | 9 |

# Introduction to Flow Cytometry

Flow cytometry is a widely used analytical technique that allows for the measurement and analysis of multiple physical and chemical characteristics of individual cells or particles suspended in a stream of fluid. It combines fluidics, optics, and electronics to provide high-throughput, quantitative data on cell size, structure, and biomolecular markers. Flow cytometry is an indispensable tool in many fields, including immunology, hematology, cancer research, and drug discovery, offering the ability to analyze large numbers of cells rapidly, accurately, and with minimal sample preparation.

# Historical Background

Flow cytometry has evolved significantly since its inception in the mid-20th century. Early cell counting devices, such as the Coulter counter, were limited to measuring cell size and number. Flow cytometry, developed in the 1960s and 1970s, introduced the ability to measure more complex cellular properties, including the expression of surface and intracellular proteins using fluorescent dyes. As the technique matured, it became an essential tool for cell biology and clinical diagnostics, particularly in identifying and sorting specific cell populations, such as immune cells, and in diagnosing diseases like leukemia.

# Components of Flow Cytometry

Flow cytometers consist of several key components that work together to enable high-throughput, multiparametric analysis of cells or particles. d These components include:

1. Fluidics System
2. Optics System
3. Detectors
4. Electronics
5. Computer and Software

Each of these components contributes to the overall functioning of the flow cytometer, allowing for the precise and efficient analysis of cell populations.

## Fluidics System

The fluidics system is the backbone of flow cytometry, allowing cells or particles to be suspended in a fluid and passed through the detection area in single file. The sample, which typically contains cells, is injected into the center of a sheath fluid that surrounds the sample and hydrodynamically focuses it into a narrow stream. This ensures that cells pass through the laser beam one by one, allowing for accurate measurements of individual events.

### Key Elements of the Fluidics System:

1. Sample Input: The sample, containing cells or particles, is loaded into the flow cytometer. This sample is then combined with a carrier fluid known as the sheath fluid.
2. Sheath Fluid: A saline-based solution that helps focus the cells into a narrow stream. This hydrodynamic focusing ensures that cells or particles are arranged in single file, so they pass through the laser beam one at a time.
3. Flow Cell: A chamber where the sample and sheath fluids meet and are focused into a single stream. This area is critical as it ensures that cells align properly with the laser beam for consistent and accurate detection.

## Optical System

The optical system in flow cytometry comprises lasers and optical filters that allow for the illumination of cells and the collection of scattered and fluorescent light. When cells pass through the focused laser beam, they scatter light in different directions, and fluorescent dyes attached to the cells emit light at various wavelengths. These optical signals provide valuable information about the physical and chemical properties of the cells.

### Components of Optical System

1. Lasers: The primary light source in a flow cytometer, lasers emit a highly focused beam of light at specific wavelengths. Lasers are used to illuminate the cells as they pass through the flow cell, exciting fluorescent markers that have been bound to the cells.
   1. Multicolor Lasers: Modern flow cytometers often use multiple lasers with different wavelengths (e.g., blue, red, violet) to excite a variety of fluorochromes, enabling multicolor detection.
2. Beam Shaping Optics: Lenses and mirrors are used to focus the laser beams and direct them precisely onto the stream of cells.
3. Scattered Light:
   1. Forward Scatter (FSC): Light scattered in the forward direction (0 degrees) is detected by a photodiode and is primarily related to the size of the cell.
   2. Side Scatter (SSC): Light scattered at a 90-degree angle to the laser beam provides information about the cell’s internal complexity, such as granularity.
4. Fluorescence Emission: Cells labeled with fluorescent dyes or antibodies emit light when excited by the laser. This emitted light is collected by detectors that are tuned to specific wavelengths, allowing for the detection of multiple fluorescent markers simultaneously.
5. Optical Filters: These filters are used to selectively pass light of specific wavelengths. In flow cytometry, filters are critical for separating the various fluorescence signals from different dyes. Two types of filters are commonly used:
   1. Bandpass Filters: Allow light of a specific range of wavelengths to pass through.
   2. Dichroic Mirrors: Reflect some wavelengths of light while allowing others to pass through, ensuring that the light is directed toward the correct detector.

## Detectors

Detectors, including photodiodes and photomultiplier tubes (PMTs), capture the light scattered and emitted by cells. The data is then converted into electronic signals that are processed to provide quantitative information on cell characteristics.

### Types of Detectors:

1. Photodiodes: These are used to detect forward scatter (FSC). Since forward scatter is related to cell size, the photodiode captures the intensity of the forward-scattered light to determine cell size.
2. Photomultiplier Tubes (PMTs): PMTs are used to detect side scatter (SSC) and fluorescence. PMTs are highly sensitive to low levels of light and can detect a wide range of fluorescence intensities. PMTs are tuned to specific wavelengths to ensure that the correct fluorescent signal is detected and quantified.

### Detection of Signals:

1. Light Scatter Detection: Forward and side scatter provide information about the physical characteristics of the cell, such as size (FSC) and granularity or internal complexity (SSC).
2. Fluorescence Detection: When fluorochrome-conjugated antibodies or dyes on the cell are excited by the laser, they emit light at specific wavelengths. PMTs capture this fluorescence, and the intensity of the emitted light correlates with the amount of the target molecule (e.g., surface proteins or intracellular components) on or in the cell.

## Electronics

The electronics system processes the signals received from the detectors. These signals, initially in the form of light, are converted into electrical signals, which are then digitized for analysis.

### Signal Processing:

Signal Amplification: The electronic signals generated by the detectors are often very weak, so they are amplified to make them suitable for processing.

Pulse Shaping and Discrimination: Each signal is converted into a pulse, where the height of the pulse corresponds to the intensity of the signal. The electronics system differentiates between valid cellular events and background noise, ensuring that only data from single cells are recorded.

Pulse Height, Width, and Area: Flow cytometers typically measure three characteristics of each pulse:

1. Height: The peak intensity of the signal.
2. Width: The time it takes for the cell to pass through the laser.
3. Area: The total fluorescence or scattered light detected as the cell moves through the laser beam.

### Compensation

Compensation is an important process in flow cytometry when using multiple fluorochromes. Due to spectral overlap, where different fluorochromes emit light at similar wavelengths, compensation is required to ensure accurate data. The electronics system subtracts the overlapping signals to correct for this, ensuring that each fluorescent marker is measured accurately.

## Computer and Software

The computer and software components of a flow cytometer are critical for data acquisition, analysis, and visualization. Once the data has been processed by the electronics, it is transferred to a computer for further interpretation and presentation.

### Functions of the Computer and Software:

1. Data Acquisition: The computer captures and stores the signals detected by the flow cytometer. During this phase, the user can set up the flow cytometer to collect data on specific cell populations or characteristics, such as fluorescence intensity or light scatter.
2. Data Analysis: Flow cytometry software provides the ability to analyze the data in real time or post-acquisition. The data is typically represented as:
   1. Histograms: A plot that shows the distribution of a single parameter (e.g., fluorescence intensity) across the cell population.
   2. Dot Plots: A two-dimensional graph that plots two parameters on the X and Y axes, allowing the researcher to identify and quantify different cell populations based on their characteristics.
   3. Contour Plots: Display the density of cells in different regions of the dot plot, showing areas where more cells are present.
3. Gating: Software tools enable the user to draw gates around cell populations of interest. Gating is essential for identifying and quantifying specific subsets of cells, such as distinguishing between different immune cell populations (e.g., T cells, B cells, and NK cells).
4. Data Export: Once analyzed, the data can be exported into various formats for further statistical analysis, sharing, or inclusion in research reports and publications.

# Specialized Components

In addition to the standard components mentioned above, some flow cytometers include specialized components for more advanced applications:

## Fluorescence-Activated Cell Sorting (FACS)

A specialized form of flow cytometry, FACS allows for the physical separation of cells based on their characteristics. In addition to measuring cell properties, FACS uses an electrostatic deflection system to sort and collect specific cell populations for further research.

Electrostatic Deflection: Cells are charged based on their fluorescence or scatter characteristics, and these charged cells are deflected into different collection tubes by an electric field.

## Acoustic Focusing

Some flow cytometers use acoustic focusing instead of or in addition to hydrodynamic focusing. Acoustic waves align the cells in the flow stream, which can improve focusing accuracy and enable higher-throughput analysis.

# Modifications of Flow Cytometry

## Fluorochromes

Fluorochromes, also known as fluorescent dyes, are essential for flow cytometry as they emit light when excited by a laser. Each fluorochrome has a distinct excitation and emission spectrum, allowing for the simultaneous detection of multiple markers on a single cell.

Common Fluorochromes: FITC (fluorescein isothiocyanate), PE (phycoerythrin), APC (allophycocyanin), and PerCP (peridinin chlorophyll protein) are frequently used in flow cytometry.

Fluorochrome Conjugation: Fluorochromes are often conjugated to antibodies that target specific proteins on the surface or inside cells.

## Multicolor Flow Cytometry

Multicolor flow cytometry refers to the use of multiple fluorochromes in a single experiment, enabling the analysis of many different markers on each cell. Advances in flow cytometer design, including the development of lasers that excite different fluorochromes and detectors that can capture light at different wavelengths, have made it possible to conduct experiments with 10, 20, or more colors.

Panel Design: Designing multicolor panels requires careful selection of fluorochromes to avoid spectral overlap, where the emission spectra of two fluorochromes overlap, leading to inaccurate data. Compensation is used to correct for this overlap.

High-Throughput Screening: Multicolor flow cytometry allows for the simultaneous analysis of multiple cellular parameters, making it invaluable for high-throughput screening applications in drug discovery and immunology.

# Sample Preparation for Flow Cytometry

Processing a sample for flow cytometry is a crucial step in ensuring accurate and reliable results. Flow cytometry analyzes single cells or particles in suspension, so preparing the sample involves steps to isolate, label, and filter cells to make them suitable for analysis. Here's a detailed overview of how samples are processed for flow cytometry:

## Sample Collection

The first step in flow cytometry is to collect the sample, which can come from a variety of sources, such as:

1. Peripheral Blood: Blood samples are commonly used in immunology and hematology studies.
2. Bone Marrow: Used in clinical analysis, particularly for diagnosing blood disorders.
3. Tissue Samples: Tumor biopsies or organ tissues may be dissociated into single-cell suspensions.
4. Cell Cultures: Cells grown in vitro for research purposes, such as cancer cells, stem cells, or immune cells.

The source of the sample determines the method of isolation and processing. For example, solid tissues must be dissociated into single cells, while blood samples are typically diluted or lysed to remove red blood cells (RBCs) before analysis.

## Cell Isolation

For flow cytometry, it is essential to have a single-cell suspension, where each cell is separated from others and suspended in a liquid medium. Depending on the sample type, different techniques are used for isolating cells:

1. Blood Samples: Red blood cells (RBCs) are abundant in blood samples and may need to be removed to improve the analysis of other cells like lymphocytes, monocytes, or neutrophils. Common methods include:
   1. Red Blood Cell Lysis: Reagents such as ammonium chloride are used to lyse RBCs selectively, leaving the white blood cells intact for analysis.
   2. Density Gradient Centrifugation: Ficoll or Percoll gradients can be used to separate mononuclear cells (e.g., lymphocytes) from other blood components based on their density.
2. Tissue Samples: Tissues such as tumors, lymph nodes, or other organs need to be mechanically or enzymatically dissociated into single-cell suspensions. Techniques include:
   1. Enzymatic Digestion: Enzymes like collagenase, trypsin, or dispase are used to break down extracellular matrix proteins, releasing individual cells.
   2. Mechanical Dissociation: Tissue grinders, cell strainers, or specialized instruments (e.g., GentleMACS) can be used to physically break down tissues.
3. Adherent Cell Cultures: Adherent cells growing in culture are typically detached from the substrate using trypsin or EDTA to create a single-cell suspension for flow cytometry.

## Cell Counting

Once the cells are isolated, it is often necessary to count them to ensure the correct cell concentration for flow cytometry. A typical flow cytometry sample requires cells at a concentration of around 1 x 10^6 cells per mL.

1. Manual Counting: Cells can be counted manually using a hemocytometer under a microscope.
2. Automated Cell Counters: Devices like automated cell counters (e.g., Coulter counters or flow-based counters) can provide fast and accurate cell counts.

Knowing the exact cell concentration is critical because flow cytometry requires a suspension where the cells flow one by one through the detection system. Too many cells can clog the system or result in cells passing through in clumps, while too few cells can yield insufficient data.

## Staining (Fluorescent Labeling)

To detect specific cellular markers, the cells are labeled with fluorescent antibodies or dyes. Staining is one of the most critical steps in flow cytometry, as the fluorescent markers provide the data for multiparametric analysis. The choice of stain depends on the cellular features or molecules being analyzed.

### Types of Stains:

1. Fluorescent Antibodies (Immunofluorescence): Fluorescently labeled antibodies are used to target specific surface or intracellular proteins. For example:
2. Surface Markers: Antibodies targeting proteins like CD4, CD8, or CD19 are used to identify immune cell subsets.
3. Intracellular Stains: Some flow cytometry protocols require the cells to be permeabilized so that antibodies can stain intracellular proteins, such as transcription factors or cytokines.
4. DNA-binding Dyes: Dyes like Propidium Iodide (PI) or DAPI are used to measure DNA content, commonly for cell cycle analysis or apoptosis detection.
5. Functional Dyes: Some dyes are used to measure cell function, such as mitochondrial membrane potential (e.g., JC-1) or intracellular calcium levels (e.g., Fluo-4).

### Staining Procedure:

1. Incubation with Antibodies: The sample is incubated with the fluorescent antibodies for a specified amount of time, typically 15-30 minutes, in the dark to prevent photobleaching of the fluorophores.
2. Direct vs. Indirect Staining:
   1. Direct Staining: In this method, the fluorescent marker is directly conjugated to the antibody.
   2. Indirect Staining: A secondary antibody, conjugated to the fluorophore, binds to the primary antibody, amplifying the signal but requiring more steps.
3. Blocking Reagents: Blocking agents (e.g., Fc block) may be used to prevent nonspecific antibody binding, which can interfere with data interpretation.

## Washing and Resuspension

After staining, the cells are washed to remove unbound antibodies or dyes that could increase background fluorescence and reduce the accuracy of the analysis. Washing typically involves:

1. Centrifugation: The sample is spun down at low speeds to pellet the cells, allowing the removal of the supernatant containing excess dye.
2. Resuspension: After washing, the cell pellet is resuspended in flow buffer, which is usually a balanced salt solution (e.g., PBS with BSA or FBS) to keep cells in suspension and prevent clumping.

The final resuspension volume is adjusted to achieve the desired cell concentration for flow cytometry analysis

## Filtering

Flow cytometry requires that cells flow smoothly in single file through the laser beam for accurate detection. Clumped cells or debris can cause blockages in the fluidics system or lead to inaccurate data.

Cell Straining: Before loading the sample into the flow cytometer, the cell suspension is passed through a cell strainer (typically 40-70 µm mesh) to remove clumps and debris. This is a critical step, especially when working with tissue samples or adherent cell cultures that may form aggregates.

## Instrument Setup and Sample Loading

Before analyzing the sample, the flow cytometer must be set up to ensure optimal performance and accurate data collection. This includes:

1. Laser and Detector Calibration: The flow cytometer must be calibrated using control beads or known standards to ensure that the lasers and detectors are functioning correctly.
2. Compensation Setup: If multiple fluorochromes are being used, compensation controls are necessary to correct for spectral overlap between different fluorophores. This involves adjusting the detector settings to accurately separate the emission spectra of the fluorophores.

Once the instrument is calibrated, the prepared sample is loaded into the flow cytometer. The cells are drawn into the fluidics system, and data collection begins.

# Special Considerations for Sample Preparation

1. Cell Viability: It is important to assess the viability of cells, particularly when analyzing live cells. Dead cells can bind nonspecifically to antibodies and increase background fluorescence, leading to false-positive results. Viability dyes such as PI or 7-AAD are often used to exclude dead cells from analysis.
2. Fixation: In some cases, samples may need to be fixed with reagents like paraformaldehyde to preserve the cells and fluorescent signals. Fixation is commonly used when analyzing intracellular markers or when samples cannot be analyzed immediately.
3. Permeabilization: For intracellular staining, cells must be permeabilized using detergents such as saponin or Triton X-100. This step is critical when staining for intracellular proteins like cytokines or transcription factors.

# Key Applications of Flow Cytometry

## Immunophenotyping

One of the most common applications of flow cytometry is immunophenotyping, a technique used to analyze the types and frequencies of immune cells in a sample. By labeling cells with fluorescently tagged antibodies that bind to specific surface markers, researchers can identify and quantify different populations of immune cells, such as T cells, B cells, and natural killer (NK) cells. Immunophenotyping is critical in diagnosing immune disorders, monitoring the immune system, and conducting basic research in immunology.

1. Clinical Use: Flow cytometry is extensively used in clinical settings to diagnose diseases such as leukemia and lymphoma by determining abnormal cell populations.
2. Research Use: In immunology research, flow cytometry helps scientists study the immune response to infections, cancer, and autoimmune diseases.

## Cell Cycle Analysis

Cell cycle analysis using flow cytometry enables the measurement of DNA content in individual cells, allowing researchers to determine the distribution of cells in different phases of the cell cycle (G1, S, G2, M). Cells are stained with DNA-binding dyes, and their fluorescence intensity is measured as they pass through the flow cytometer. This technique is invaluable for studying cell proliferation, cancer biology, and the effects of drugs on the cell cycle.

1. Cancer Research: Cell cycle analysis helps in understanding how cancer cells proliferate and how different drugs impact cell division.
2. Drug Discovery: In pharmaceutical research, flow cytometry is used to screen compounds that influence cell cycle regulation and proliferation.

## Apoptosis Detection

Apoptosis, or programmed cell death, is a crucial process in maintaining cellular homeostasis. Flow cytometry is widely used to detect apoptosis by measuring changes in cell membrane integrity, mitochondrial function, and the activation of apoptotic pathways. By staining cells with specific dyes or antibodies, researchers can distinguish between live, apoptotic, and necrotic cells.

1. Annexin V Staining: Annexin V binds to phosphatidylserine, a phospholipid that translocates to the outer leaflet of the plasma membrane during early apoptosis. This is often combined with propidium iodide (PI) to exclude necrotic cells.
2. Mitochondrial Membrane Potential: Disruption of mitochondrial membrane potential is an early marker of apoptosis, which can be detected using fluorescent dyes like JC-1.

## Cell Sorting (FACS)

Fluorescence-activated cell sorting (FACS) is a specialized application of flow cytometry that enables the physical separation of specific cell populations based on their characteristics. FACS is used to isolate rare cell types, such as stem cells or cancer cells, for further analysis or therapeutic purposes. The sorted cells can be collected and used in downstream experiments, such as gene expression analysis or functional assays.

1. Stem Cell Research: FACS is used to isolate and enrich populations of stem cells for regenerative medicine and research.
2. Cancer Research: Researchers use FACS to separate cancer cells from normal cells for molecular analysis and drug testing.

# Advantages and Limitations of Flow Cytometry

## Advantages

1. High Throughput: Flow cytometry allows for the analysis of thousands of cells per second, providing statistically robust data.
2. Multiparametric Analysis: It enables the simultaneous measurement of multiple characteristics of each cell, such as size, granularity, and the expression of various proteins.
3. Quantitative Data: Flow cytometry provides precise, quantitative data on cell populations, making it ideal for diagnostics and research.
4. Cell Sorting Capability: FACS enables the physical separation of specific cell populations for further analysis.

## Limitations

1. Cost and Complexity: Flow cytometers are expensive, and their operation requires specialized training.
2. Spectral Overlap: The use of multiple fluorochromes can result in spectral overlap, leading to the need for compensation and complex data analysis.
3. Sample Preparation: Proper sample preparation is critical for accurate results, and some cell types may require special handling.

# Conclusion

Flow cytometry is a powerful and versatile tool for analyzing the characteristics of individual cells in a population. Its ability to measure multiple parameters simultaneously, combined with the high-throughput and quantitative nature of the technique, makes it invaluable in both research and clinical settings. From immunophenotyping and cell cycle analysis to apoptosis detection and cell sorting, flow cytometry has transformed our understanding of cell biology and contributed significantly to advances in medicine. Despite its complexity, the continued development of more user-friendly and affordable flow cytometers promises to broaden its application across various fields.

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