# Flow Cytometry Data Analysis

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## INTRODUCTION

As the name suggests, flow cytometry (FCM) is a technique that conducts measurements (metry) on cells (cyto) in a flow system. The technique was originally known as "pulse cytophotometry," though the term "flow cytometry" was adopted in 1976 at the 5th American Engineering Foundation Conference on Automated Cytology in Pensacola (Florida). The first flow cytometer was designed by Wallace H. Coulter in 1953 and employed the impedance method. The first cell sorter to utilize a FCM technique was invented by Mack Fulwyler in 1965. This was rapidly followed by the first fluorescence-based flow cytometer (ICP 11), developed by Wolfgang Gohde at the University of Munster (Germany) in 1968. This device was the first system to use fluorescence rather than absorption to characterize cells.

To date, new systems and new applications continue to be developed. Most improvements aim to increase the number of parameters that can be analyzed, decrease equipment price, reduce equipment size, and increase the speed of cell sorting all while maintaining a good manufacturing grade suitable for clinical applications. FCM presents a vast array of potential applications and will continue to play an important role in academic and clinical research, clinical diagnostics, and disease treatment. This book article will present basic and advanced definitions of FCM data analysis while demonstrating some clinical and biomedical applications, which include analysis of HIV contamination, cancer cells, and stem cells.

## BACKGROUND

### What is FCM?

FCM analyses cellular characteristics in a manner similar to immunocytochemistry techniques. In immunocytochemistry, cells are evaluated while fixed to a slide, while FCM analyzes cells suspended in a solution. Therefore, the principle of FCM is similar to immunocytochemistry and the flow cytometer is similar to a microscope.

For microscope-based immunocytochemistry techniques each cell is observed and evaluated by human eyes. This presents several limitations. For example, cells are fixed in 2D, making some shape characteristics hard to evaluate. Additionally, the number of cells that can be observed and evaluated is restricted owing to the processing capacity limitations of human eyes and the number of cells on each slide. FCM does not possess these limitations. For FCM, cells are suspended in fluid to retain their three-dimensional structure while their continuous flow across an electronic optical detector increases the rate of data recording.

### FCM Principle

How does a flow cytometer evaluate cell characteristics? Electronic detectors record signals from cells and convert them to computerized data, facilitating classification of cells into subgroups with similar characteristics. Signals are produced by focusing a beam of light (usually laser light) of a single wavelength on cells in a stream of fluid. This light is modified by cells and this modified light is recorded by detectors. Usually, there are three kinds of signals recorded: forward scatter (FSC), side scatter (SSC), and fluorescence. Therefore, a flow cytometer usually has at least three detectors collecting signals for FSC, SSC and fluorescence.

FSC correlates with cell volume, while SSC correlates with cellular content or granularity (such as shape of the nucleus, presence of cytoplasmic proteins). Hence, FSC gives information about cell size while SSC gives information about cell complexity. By combining these signals, cells that differ in their size and complexity can be recognized. A fluorescence signal only

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is created by fluorescent dyes when stimulated by the appropriate wavelength of light. In FCM, fluorescent dyes are typically used to stain nucleic acids or label antibodies that will identify specific proteins.

## The Flow Cytometer

There are three major components of a flow cytometer: fluidics, optics, and electronics. Fluidics create a stream of fluid that permits cell flow. Cells flow across a beam of exciting light that is created by the optical components. Exciting light is modified by cells to generate the emitted light. Electronics collect emitted light and convert signals to digitized values that are stored on a computer. These digitalized values can then be displayed and analyzed by computer software.

The optics of the flow cytometer include light sources, filters and detectors. In all flow cytometers, the light source is a laser. Depending on the number of signals required, a system can have a single laser or multiple lasers. An argon laser, which produces blue light, is the most commonly used in FCM. The lasers create light beams that pass through the center of a flow chamber. This chamber is designed to deliver cells in single file at the point of measurement. To achieve this, the flow chamber makes a stream of liquid called the sheath fluid. The sheath fluid will make a "cover" to maintain single file cell flow at the center of the stream. Two types of flow chamber exist: one for analysis only, and another for analysis and sorting. In contrast to analysis-only chambers that are fully closed, chambers allowing both analysis and sorting are opened to allow collection of cells of interest. To enhance detection of fluorescence and scattered light in the chamber, collecting lenses are located in the chamber walls.

As there are many wavelengths of light that can be scattered from a cell, flow cytometers are equipped with various optical filters that split light into specific wavelengths in order to detect them independently. At present, there are four classes of optical filters: long-pass, short-pass, band-pass, and dichroic. Fluorochrome molecules typically fluoresce over a large range of the spectrum (100 nm or more). Consequently, a detector may receive more than one fluorescence signal from more than one fluorochrome. To appropriately quantify signals from each dye the instrument must be "compensated," so that one detector registers signals from a single fluorochrome.

Fluidics facilitate the flow of cells one-by-one for single-cell analysis. In any system, there are two sources of fluid: the sheath fluid, and the specimen. Sheath fluid contains buffered saline while the specimen contains the cells to be analyzed suspended in a fluid of choice. Initially, cells from the specimen tube are injected into the sheath stream. Some systems use pressure from a pump to push cells into the sheath fluid, while others use a pump to suck cells into the sheath fluid.

## Signal Converter

There are two major types of photo detectors used in FCM: photodiode detectors and photomultiplier tube (PMT) detectors. Photodiode detectors are usually used to detect strong signals such as FSC or SSC. PMT detectors are used to detect weaker signals such as fluorescence because PMTs are more sensitive. Both photodiode and PMT detectors collect photons from emitted light and convert them to current. The electronics process and convert the current to a digitized value that can be analyzed and displayed on a graph by a computer.

PMTs contain a series of plates to efficiently collect photons. However, even with this high efficiency the signal from fluorescent dyes produces a low–level electric current. Unlike FSC and SSC signals, all fluorescent signals need amplification to increases the current voltages. Depending on the strength of the signal, current amplifiers will amplify current range from 0 to 10 V. Furthermore, the voltage signal is an analog signal that cannot be recognized by a computer, making an analog-to-digital converter (ADC) essential. ADCs use a channel range of 0–1024 channels. All signals of a specific voltage are counted in a channel corresponding to the strength of the signal.

The amplifier can amplify the signals in either linear (LIN) or logarithmic (LOG) fashions. LIN amplification is typically used to amplify the scatter signal (FSC and SSC) and some strong signals from nucleic acids, while LOG amplification is used for fluorescence. Similar to fluorescence-based techniques, the electronics must be set to a defined signal threshold. That means the amount of light scattered must exceed a threshold value to activate a set window of time for signal detection. Usually the threshold value is set on FSC.