A Review and Applications of Flow Cytometry

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Abstract

A description and applications using flow cytometry are presented herein. Cytometry uses fluorescence and scattering to analyze a population of cells, organelles, or other similarly sized particles quantitatively. Flow cytometry can examine a multitude of biological parameters, such as particle size, cell type, DNA content, and enzymatic function at up to 7000 cells per second. New applications, such as the characterization of cell apoptosis, as well as the function of drug-delivery devices, are also examined.

Introduction

Cytometry is a set of methods to quantitatively examine a population of cells and the analytes inside of them. It exploits fluorescent-labeling techniques such as immunohistochemistry in order to obtain information about a large number of cells. Fluorescence microscopy, the standard practice for analyzing cellular components via the use of a large number of chromophore probes, is preferred when observing a small number of cells is adequate, or when solely qualitative information is required. Cytometry is the fluorescence microscope’s quantitative counterpart.
Cytometry can aid in the analysis of cell types, organelles, nucleic acids, enzymes, and other moieties in a cell population. As in microscopy, fluorescent probes are used to bind to the species under investigation. For instance, attaching probes to particular cell surface antigens on the membrane allows cytometry to quantify cell types in a sample. Intracellular antigens are used for applications such as measuring enzymes, organelles and inclusion bodies, monitoring mitosis, as well as analyzing nucleic acid content in cells. The most popular cytometric technique is flow cytometry of living cells. This involves the movement of suspended cells flowing through a thin orifice, sequentially probed by a light source to excite the attached fluorescent label. Flow cytometry is typically used in configurations for either quantitative analysis or physical cell sorting. The most common type of quantitative analysis using cytometry data involves creating a histogram of fluorescence events to count the number of cells with the attached probe. This effectively creates a set of data which gives a ratio of the cells in a population with a particular surface protein, enzyme, or other analyte. Multiple analytes can be quantified simultaneously. Cell sorting involves the physical separation of two cell populations by creating tiny droplets of detected cell media which are separated according to the droplet’s fluorescence. The end-result of such separations can be over 99% accurate.

Cytometry developed from the need to count blood cells as counts of both red and white cells were of considerable interest to medical researchers by the 1930’s. Hemocytometers, which allow microscopists to count cells via a microscope slide containing a ruled grid, giving a rough approximation of the number of cells per volume of blood, were known to be imperfect. In the 1950’s, the Coulter counter was developed
to supplant the hemocytometer in high-volume applications. This device pushed cells single-file through a device which measured their resistance. Since cells possess a higher amount of resistance than a saline solution in which the cells were suspended, an increase in electrical impedance in the device would signal the presence of a cell. By counting such events, the accuracy and speed of cell counts were improved. Fluorescence was combined with flow cytometry in the 1960’s because one could measure more than the number of cells. Cellular DNA content was quantified with fluorescence-based cytometry by researchers in Germany and Los Alamos. Physical cell sorting was developed by Kamentsky at IBM in the 1960’s using ink jet printer technology. He then started his own company, Bio/Physics Systems, which sold the first fluorescence-based cytometer with an argon ion laser, instead of an arc lamp, as its excitation source. In the early 1980’s, flow cytometers became especially popular when it was found that the new mysterious disease, AIDS, could be characterized by a decline in T-helper lymphocytes. The fast quantification of flow cytometry was invaluable to early AIDS researchers.

**Method and Analysis Techniques**

Cytometry must be conducted on a free suspension of single cells sans large aggregates or debris. Protocols for preparation of blood cells usually involves suspending in buffer and centrifugating. Dense tissues are often disassociated by enzymatic digestion. Fluorescent labels are then applied to tag a desired cell population or other cell-related moiety such as nucleic acid or protein. Antibody-labeled dyes are popular for tagging cell surface antigens, enzymes, the cytoskeleton, and organelles. Intercalating dyes are often used to measure DNA content in cells, which can be used to detect cancer. The dyes
fluorescein isothiocyanate (FITC) and R-phycoerythrin (R-PE) are especially popular for differentiating cells. Dye sets are chosen so they can be excited with the same laser and have fluorescence maxima which are significantly different in order to minimize crosstalk.

![Optical layout of a typical flow cytometer.](image)

**Figure 1.** Optical layout of a typical flow cytometer.

The typical flow cytometer uses sheath flow to force cells into a capillary by the process of hydrodynamic focusing. A narrowing channel of the fluid forces cells into the center of the chamber and into a capillary through which cells flow single-file. The capillary usually ends in a flow cuvette where the sample is then illuminated by an arc lamp or laser. Collection optics (Figure 1) split the emission into separate channels for detection of multiple fluorophores by separate photomultiplier tubes. Optical filtration is used to
separate the emission into separate detectors, often via dichroic mirrors and bandpass filters. Certain analysis techniques require scattered light instead of fluorescence. Flow cytometers carry an extra detector for forward-scattered light. Orthogonal (90°) scattered (side-scattered) light is also measured, often simultaneously with forward-scattered light during an experiment.

When a sample of cells stained for the presence of one or more species is run through a cytometer, data is usually displayed as either a histogram or a cytogram (dot plot). A histogram records the number of events versus their intensities. Cells which stain positive for a species will emit at a higher intensity than negative cells (Figure 2a). Usually displayed logarithmically, a typical data set will contain two major populations of events denoting stained and unstained samples. The second common type of display, the cytogram, plots the intensity of events hitting one detector versus the intensity at a second

![Figure 2](image.png)

**Figure 2.** (a) Histogram of FITC-labeled CD8 cells showing unlabeled and labeled cell populations. (b) Cytogram of bi-labeled cells showing four discrete populations divided into regions and quantified as a percentage of the total.
detector, where each event is represented by a dot. This produces a set of clusters which correspond to the populations within the sample (Figure 2b). The cytogram is analyzed via computer: boxes are drawn around regions of the plot and the percentage of total fluorescence events is calculated in each of the regions.

Scattered light is often measured along with fluorescence. The forward-scatter detector can be used to determine the ratio of dead cells, which are more permeable to certain fluorophores. These cells have lower forward scatter than live cells. The amount of forward (small angle) scatter is also dependent upon particle size, refractive index of the cells with respect to the medium, and the absorbance of the cells. Researchers can use forward scatter to measure these and other parameters.

The total amount 90° (side) scatter from a sample is also used for characterization. It is theorized that the amount of side scatter is related to its cytoplasmic granularity of a cell. Although this is not confirmed, the difference in side scattering between granulocytes, lymphocytes, monocytes, and other cell types is clear and can be used for measuring their

Figure 3. Cytogram of light scattering off a sample of canine bone marrow cells. Investigators divided these into populations of mature erythroid cells (R2), immature erythroid cells (R3), metamyelocytes (R4), immature myeloid cells (R5), megakaryocytes (R7), and neutrophils (R8). Lymphocytes cannot be separated in this plot because they overlap with mature erythroid cells.
ratios without the need for a stain. Figure 3 shows a plot of side scatter versus forward scatter, allowing the quantification of many cell types simultaneously.

Flow sorting, the physical separation of cells or organelles, or other particles, is one of the primary uses of flow cytometers (Figure 4). Sorting is accomplished by equipment placed after the flow cuvette used for detection. Using technology derived from ink-jet printers, the fluid exiting the cuvette is divided into equally-spaced droplets by a piezoelectric transducer which applies a regular vibration to the flow chamber. As each droplet is forming, it is given a positive, negative, or neutral charge by a voltage pulse. The charged droplets then fall between two high voltage plates which can deflect them to the left or right of the original stream. The charge given to each droplet is controlled by circuitry which gives each falling drop the correct charge based on parameters set by the user. The sorting may be accomplished by telling the computer to look for droplets with a particular fluorescence intensity, scattering property, or other directive decided by the investigator.

**Figure 4**. Flow sorting scheme. Droplets are charged as they break off from the stream and deflected by high voltage plates into receptacles for collection.
Researchers at the University of Illinois Flow Cytometry Facility are studying apoptosis, or programmed cell death. This is a particular kind of degradation in which cells deteriorate into several remnants with intact membranes. Flow cytometry can be used to study this process during an experiment via several methods. For instance, forward scatter, which can detect particle sizes in a flow cytometer, is employed to measure the decrease in sizes of apoptotic cells (Figure 5a). Additionally, granularity, which can be detected by side scattering, increases in apoptotic

**Application: Apoptosis**

Figure 5. Healthy and apoptotic cells characterized by (a) forward scatter measurements to show changes in size and (b) side scatter measurements to show changes in granularity.

Figure 6. Healthy and apoptotic cells characterized by Hoeschst dye and forward light scattering. (a) Healthy cells appear in a defined grouping. (b) Apoptotic cells form a second population as they become more permeable to the dye and their decreased particle size attenuates forward scattering intensity.
cells. This is shown in Figure 5b, which illustrates the increase in orthogonal scatter caused by apoptotic cells.

The plasma membranes of apoptotic cells become more permeable to certain fluorescent dyes. This is similar to the measurements conducted dead (necrotic) cells. The Hoechst dye is used to illustrate the transformation of healthy cells to those which are undergoing apoptosis. Figure 6a shows a dot plot of Hoechst dye fluorescence versus forward light scatter. This gives a relatively localized region of dots which illustrates the distribution of fluorescence intensities of the dye versus the relative range of sizes of the cells.

![Figure 7](image)

**Figure 7.** (a) Fluorescence histogram showing populations of unlabeled (M1) and FITC-BSA tagged water/oil/water droplet emulsions. (b) Dot plot of forward and side scattering of droplets showing the size (forward scattering) and complexity (side scattering) distributions. (c) Micrograph of the droplets.
It is apparent that fluorescence intensity increases monotonically with particle size, which is to be expected because larger cells naturally contain more dye molecules. Figure 6b illustrates the change in size and fluorescence intensity of apoptotic cells, which become smaller but increase in permeability to the Hoechst dye.

**Application: Oil Emulsions**

Water/oil/water immersions are droplets of oil, immersed in water, which contain smaller droplets of water in them. They are under investigation for biomimetic mineral formation and as drug delivery devices. As delivery devices, a useful compound could be stored in the water phase which is entrapped by oil. It is important to understand the homogeneity of the droplets as well as the release rate of the compound contained in this phase. Water/oil/water immersions containing a fluorescent biomarker (BSA-FITC) trapped in the internal water phase were made to study these phenomena via flow cytometry. To study the yield of preparation of fluorescent droplets, a blank and tagged emulsion were fed through a flow cytometer. After two minutes, 100,000 droplets (events) were measured and a histogram was generated which shows logarithmic fluorescence intensity versus count number (Figure 7a). The M1 region is defined as the background, and contains all events which produced a fluorescence intensity of under 100 arbitrary units. The M2 region is defined as having high enough intensity to make the determination that the droplet contained the FITC-BSA label. Using the histogram, one can determine that, as 27,353 of 100,000 measured events are contained in the M2 region, that the yield of marked droplets is 27.35%.
Forward and side scatter are used to show the droplet complexity and size inhomogeneity. The forward scatter axis (x-axis) in Figure 7b shows the relative range of sizes of the droplets. The side scatter axis (y-axis) is related to droplet complexity (granularity if it were a cell).

The plot illustrates that the larger oil droplets are more complex—they contain a larger number of internal water droplets. A micrograph of the emulsion droplets is shown in Figure 7c.

**Figure 8.** Release of the fluorescent marker FITC-BSA from emulsion droplets over time.

Release of the FITC-BSA marker from the droplets is studied over a period of days by repeatedly running the droplets through a flow cytometer. The release fraction of the fluorescent marker from the droplet is defined as $R = \frac{(Y_0 - Y_t) \times 100}{Y_0}$, where $Y_0$ is the yield of the marker at time 0 and $Y_t$ is the yield of the marker at time t in days. Figure 8 shows the increase of the release fraction over 7 days after preparation for two different initial marker concentrations.
References


