

Flow Cytometry Overview

Introduction:

Flow cytometry is a method for quantitating components or structural features of cells, primarily by optical means. Although it makes measurements on one cell at a time, it can process thousands of cells in a few seconds. Since cell types can be distinguished by quantitating structural features, flow cytometry can be used to count prokaryotic or eukaryotic cells of different types in complex mixtures.

How Does It Work?

Cells may be alive or fixed at the time of measurement, but must be in *monodisperse* (single cell) suspension. They can be stained directly using metabolic fluorescent labels or indirectly using fluorescently labelled antibodies. They are passed single-file through a laser beam by continuous *flow* of a fine stream of the suspension. Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser. The cytometer typically measures several parameters simultaneously for each cell:

- low angle forward scatter intensity, approximately proportional to cell diameter
- orthogonal (90 degree) scatter intensity, approximately proportional to the quantity of granular structures within the cell
- fluorescence intensities at several wavelengths

Light scatter alone is often quite useful. It is commonly used to exclude dead cells, cell aggregates, and cell debris from the fluorescence data. It is sufficient to distinguish lymphocytes from monocytes from granulocytes in blood leukocyte samples. Light scatter has been used in our Facility to quantitate aggregation of living cells.

Fluorescence intensities are typically measured at several different wavelengths simultaneously for each cell. Fluorescent probes are used to report the quantities of specific components of the cells. Fluorescent antibodies are often used to report the densities of specific surface receptors, and thus to distinguish subpopulations of differentiated cell types, including cells expressing a transgene. By making them fluorescent, the binding of viruses, bacteria or hormones to surface receptors can be measured. Intracellular components can also be reported by fluorescent probes, including total DNA/cell (allowing cell cycle analysis), newly synthesized DNA, specific nucleotide sequences in DNA or mRNA, filamentous actin, and any structure for which an antibody is available. Flow cytometry can also monitor rapid changes in intracellular free calcium, membrane potential, pH, or free fatty acids.

Flow cytometers involve sophisticated fluidics, laser optics, electronic detectors, analog to digital converters, and computers. The optics deliver laser light focused to a beam a few cell diameters across. The fluidics hydrodynamically focus the cell stream to a small

fraction of a cell diameter, and, in sorters, break the stream into uniform-sized droplets to separate individual cells. The electronics quantitate the faint flashes of scattered and fluorescent light, and, under computer control, electrically charge droplets containing cells of interest so that they can be deflected into a separate test tube or culture wells. The computer records data for thousands of cells per sample, and displays the data graphically.

What is Flow Sorting?

Sorting can be defined as the physical separation of a cell or particle of interest from a heterogeneous population.

How Does Sorting Work?

In general, flow cytometers use a principle involving the electrostatic deflection of charged droplets similar to that used in ink-jet printers. Cells are aspirated from a sample and ejected one by one from a nozzle in a stream of sheath fluid.

All streams are unstable with respect to time and will eventually break up into droplets. It is possible to stabilise this break-off point by applying a stationary wave of vibration of known frequency and amplitude to the stream.

As the cell intercepts with the laser beam, scattered light and fluorescence signals are generated and the sort logic boards make a decision as to whether the cell is to be sorted or not (according to user-defined criteria). By applying two different levels of charge to the left or the right streams it is actually possible to sort two streams either side.

The theory and the practicalities of cell sorting are rather more complicated than this simple overview. However, it is also useful to know that different modes of sorting can be altered to give **maximum purity**, **maximum recovery** (if a small, precious population is required) or **maximum count accuracy** (for cloning). Another important point to make is that good sorting is dependent on three main factors: good **operator**, good **instrument** and good **samples**.

Lasers and Fluorochromes

There are several laser types available that can be used in flow cytometers. They differ in the gain medium that is used to amplify light. Lasers can be gas lasers (eg Helium-Neon, Argon, Helium-Cadmium); solid state lasers (eg NdYAG); dye lasers or semi-conductor lasers.

The choice of fluorochrome to be used is influenced both by the application and the excitation wavelengths available. The following table is by no means exhaustive, but lists the major fluorochromes, their excitation and emission wavelengths together with their common applications. Any core facility would have to have the ability to assess a good proportion of these fluorochromes to accommodate the wide range of investigators found within the Conway Institute and to be useful into the future.

FLUOROCHROME	EX.	EM.	APPLICATION
Indo-1 (unbound)	335	490	Calcium Flux
Indo-1 (Bound to Calcium)	335	405	Calcium Flux
Hoechst 33342	350	470	DNA analysis
DAPI	359	462	DNA analysis
Alexa350	350	442	Phenotyping
PerCP	470	670	Phenotyping
R-Phycoerythrin	480	578	Phenotyping
Green Fluorescent Protein (GFP)	488	510	Reporter molecule
YO-PRO-1	488	510	Apoptosis analysis
Fluorescein diacetate	488	530	Cell viability
Alexa488	488	530	Phenotyping
Sytox Green	488	530	DNA analysis
SNARF-1	488	530-640	pH measurement
Fluo-3	488	530	Calcium flux
dsRED	488	588	Reporter molecule
PE-Cy5 (TriColor, Cychrome)	488	670	Phenotyping
PE-Cy7	488	770	Phenotyping
Propidium Iodide	495	637	DNA analysis
Rhodamine 123	515	525	Membrane potential
Yellow Fluorescent Protein (YFP)	519	534	Reporter molecule
LDS-751	543	712	Nucleated cell detection
7-Aminoactinomycin D	546	655	DNA analysis
Alexa 546	546	573	Phenotyping
Cy3	550	565	Phenotyping
CMXRos (Mitotracker Red)	560	610	Mitochondrial membrane potential
Texas Red	596	615	Phenotyping
TO-PRO-3	643	661	DNA analysis
Alexa 647	647	667	Phenotyping
APC-Cy7	647	774	Phenotyping
Allophycocyanin (APC)	650	660	Phenotyping