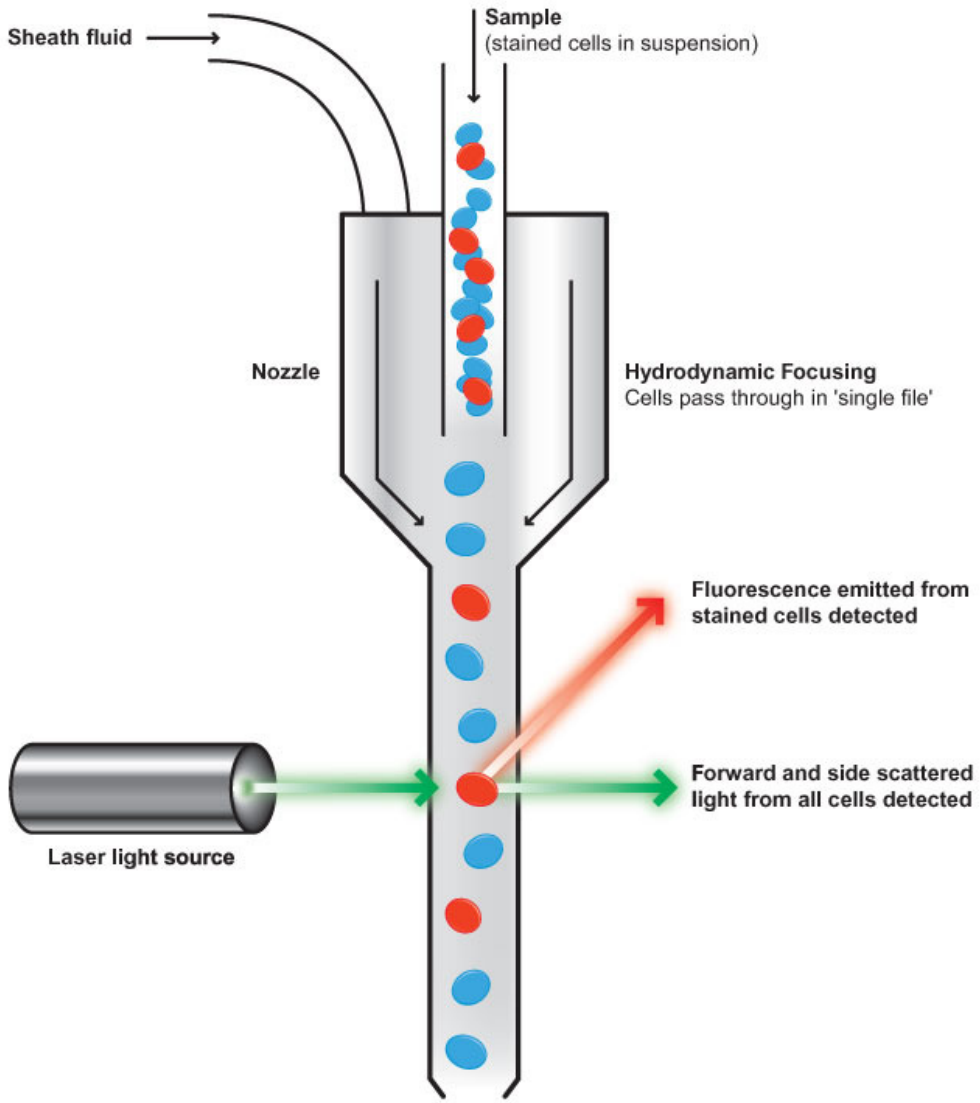
FLOW CYTOMETRY

Flow cytometry is now a widely used method for analyzing expression of cell surface and intracellular molecules, characterizing and defining different cell types in heterogeneous cell populations, assessing the purity of isolated subpopulations, and analyzing cell size and volume. It allows simultaneous multi-parameter analysis of single cells. It is predominantly used to measure fluorescence intensity produced by fluorescent-labelled antibodies detecting proteins or ligands that bind to specific cell-associated molecules, such as DNA binding by Propidium Iodide .

The staining procedure involves making a single-cell suspension from cell culture or tissue samples. The cells are then incubated in tubes or microtiter plates with unlabeled or fluorescent-labelled antibodies. Cells are then analysed on the flow cytometer.

The Flow Cytometer:



When the stained cell sample in suspension buffer is run through the cytometer, it is hydrodynamically focused, using sheath fluid, through a very small nozzle. The tiny 'stream' of fluid takes the cells past the laser light one cell at a time. There are a number of detectors to detect the light scattered from the cells/particles as they go through the beam. There is one in front of the light beam (Forward Scatter or FSC) and several side on to it (Side Scatter or SSC). Fluorescent detectors are used for the detection of fluorochromes themselves.

Particles/cells passing through the beam will scatter the light, which is detected as forward scatter and side scatter. The combination of scattered and fluorescent light is detected and analyzed. Forward Scatter correlates with the cell size and Side Scatter depends on the density of the particle/cell (i.e. number of cytoplasmic granules, membrane size), and in this manner cell populations can often be distinguished based on their difference in size and density. Fluorochromes used for detection/staining will emit light when excited by a laser with the corresponding excitation wavelength. These particles/cells can be detected individually and the data analyzed.

Direct Staining:

In direct imunofluorescence staining, cells are incubated with an antibody directly conjugated to a fluorochrome (e.g. FITC). This has the advantage of requiring only one antibody incubation step and eliminates the possibility of non-specific binding from a secondary antibody. It is particularly useful for intracellular staining, where large antibody-fluorochrome complexes including secondary antibodies can become trapped causing non-specific binding, or even fail to enter the cell and prevent primary antibody detection.

Indirect staining:

In indirect staining, the primary antibody is not fluorochrome labelled but is detected by a second fluorochrome-labelled antibody. This second reagent may be an antibody with specificity for the first antibody. Alternatively, the avidin-biotin system can be used, whereby an antibody is conjugated to biotin and detected with fluorochrome-labeled avidin. With the wide range of conjugated secondary antibodies now available, this method means that unconjugated primary antibodies raised against many different targets can be used in conjunction with a labelled secondary antibody for FACS analysis. This widens the choice of target proteins for the researcher.

Intracellular staining:

Staining of intracellular antigens for flow cytometry depends on various fixation and permeabilisation methods to allow access of antibodies to internal cellular proteins.

A successful staining procedure in all cases is dependent on optimization of experimental conditions through titering of antibodies, use of appropriate controls to set up the flow cytometer correctly, and optimised fixation and permeabilisation methods if necessary.

SELECTING A FLUOROCHROME CONJUGATE

The ability of a given antibody to resolve a positive from a negative often depends on which fluorochrome conjugate is used.

A general guideline for the relative intensities of the various fluorochromes is, from brightest to dimmest, PE, PE-Cy7, PE-Cy5, APC > APC-Cy7, Alexa Fluor 47, Alexa Fluor 700 > FITC, Pacific Blue, Alexa fluor 488 . This is a general pattern when using signal/noise ratio. Some differences are seen for individual antibody.

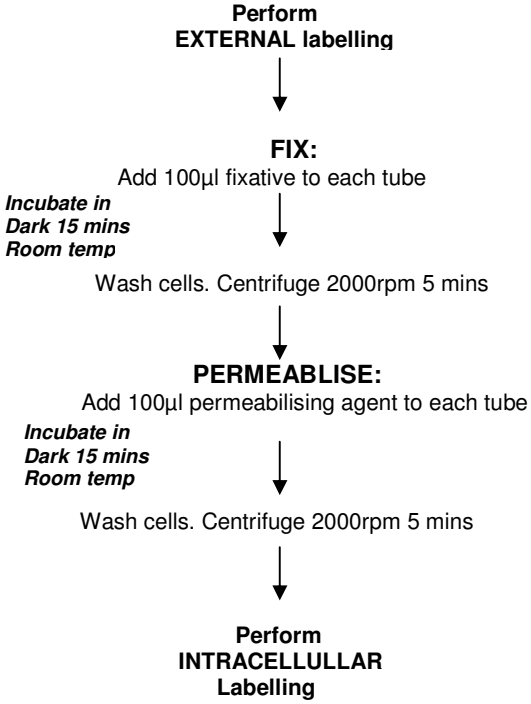
A highly expressed antigen will usually be resolved with almost any fluorophore. An antigen expressed at lower density might require the higher Signal/background ratio provided by a brighter PE or APC conjugate to separate the positive cells adequately from the unstained cells.

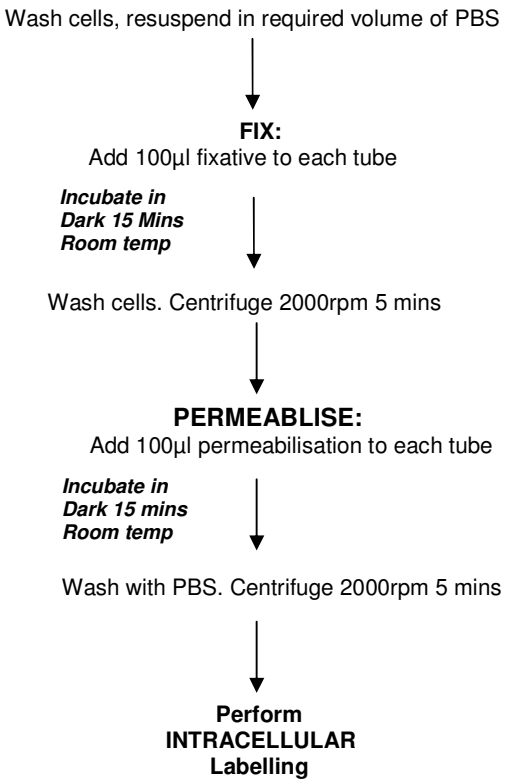
The relative fluorochrome intensity depends on the instrument. This is because of differences in the laser and filter combinations used on the different instruments. Be sure to use the appropriate FACS instrument.

FLOW CYTOMETRY ANTIBODY LABELLING

Internal and external:

Internal only (fix first):





For simultaneous analysis of cell-surface and internal antigen, cells that have been stained for expression of cell surface antigens can also be stained for expression of intracellular antigens.