Mid Anglia Cytometry Club, basic flow cytometry theory,handout
Introduction to Flow Cytometry

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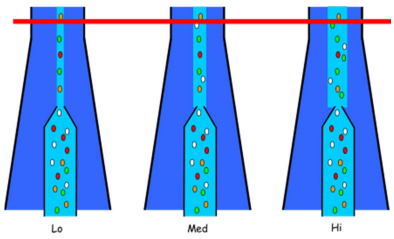
Flow Cytometry is an important quantitative technique in which every single cell (in a suspension) is measured and the technique is increasing in popularity and complexity due to the growth in the technologies behind lasers, cytometry machines and fluorochromes. Flow Cytometry is also incorrectly referred to as FACS (Fluorescent Activated Cell Sorter) and cytometers can be either analyser or sorters. They use the same principles of fluidics, optics, event detection and electronics however with sorters there is an additional sorter module.

Flow cytometers contain 1 or more lasers lines (e.g. 488nm, 561nm, 635nm) and these laser beams are used to excite fluorochromes.

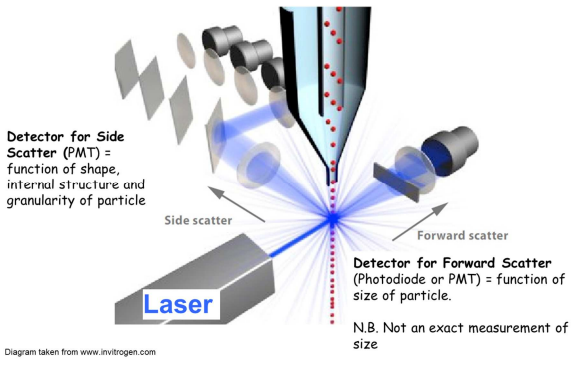
Fluorescence is the emission of light by a fluorochrome that has absorbed light of a different wavelength. All fluorochromes have an excitation and emission maxima and it is best to match laser excitation lines and emission band pass filter to the maxima (e.g.530/30 bandpass filter allows light of 530nm +/- 15nm through it). Some fluorochromes are excited by more than one laser.

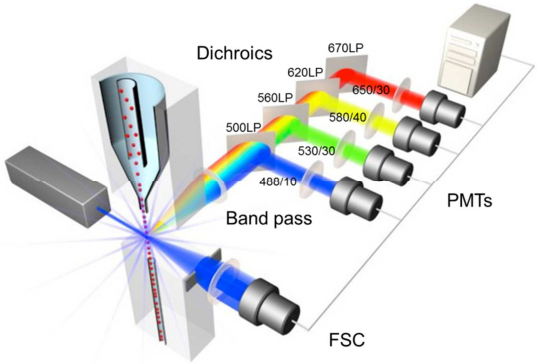
Sheath fluid used to align and accelerate sample into flow cell by hydrodynamic focussing for interrogation by lasers. Increasing the sample throughput on the machine increases core diameter.



Each event that goes through the machine creates a pulse, with information about height, width and area of that pulse. Using this we can discriminate against doublets.

Parameters Detected:





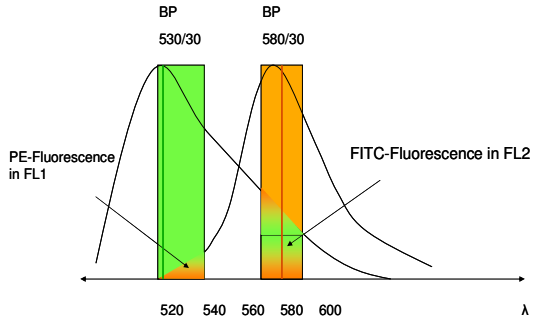
Filters separate the emitted fluorescent light into towards individual detectors. PMT (photomultiplier tube) = convert light energy into electrical voltage. PMT sensitivity can be increased/decreased by changing voltage on the cytometer software.

Data is created as a listmode file and stored in the FCS format (Flow Cytometry Standard 2.0 or 3.0)

Fluorescence and Compensation

Fluorescence process can be understood as emission of photons. The emission wavelength of any fluorochrome will always be longer than its excitation wavelength. The difference between $E_{excitation}$ and $E_{emission}$ is called Stokes Shift. Fluorochromes with large Stokes Shift are better for fluorescence studies, especially for multicolour experiments.

It is important to select a fluorochrome with excitation maximum close to one of the laser lines

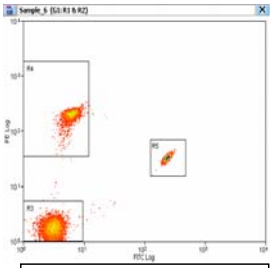


FL2-%FL1= corrected FL2

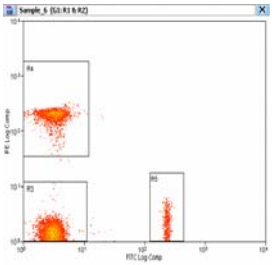
FL1-%FL2= corrected FL1

Every fluorescent molecule emits light with a particular spectrum unique to that molecule. To simultaneously measure the emissions from multiple fluorochromes, we can choose optical filters ("bandpass filters") which only transmit specific wavelengths of light. For simultaneous detection of emissions, whole spectra must be considered rather than only emission maxima. These emission spectra overlap for multiple fluorochromes, in some cases very significantly.

Compensation is the process by which we correct for "spillover". This can be performed in a digital or analogue way (corrective and subtractive), latter affecting the raw data.



uncompensated



compensated

Practical compensation

(1) Run unstained and singly-stained controls, adjusting, if necessary, PMT voltages to ensure the optimal positive signal and minimal overlap (remember to re-run controls in this case).

(2) Align the populations by adjusting/matching the medians of fluorescence in negative and single-stained populations (using the statistics in the instrument's analysis software). Only cells with identical autofluorescence characteristics must be analysed.

(3) Repeat this procedure pair-wise with all controls.

(4) Always remember i) to check all single-stained samples pair-wise, ii) re-run them if adjusted voltage AND iii) perform compensation prior to acquisition if using analogue instrument.

To reduce/avoid compensation: i) use fluorochromes excitable by different lasers, ii) and/or fluorochromes with markedly different Stokes Shifts iii) use optimal filters for optical separation compensation

Experimental design guidelines

- Use fluorochromes suited to the instrument you want to use .
- Choose the brightest fluorophore for the least expressed marker and the dimmest fluorophore for the best expressed marker.
- Choose fluorophores with the least spectral overlap.
- Avoid strong makers spilling over into channels requiring high sensitivity.
- Some tandem dyes may be susceptible to degradation .

Other points to remember:

- pH of sample can affect fluorescence of some dyes e.g. FITC.
- Fixation can also have an effect.
- Wash away unbound antibody - otherwise high background signal.
- Use Dump Channels where you need to exclude several markers.
- Can use a colour that has a lot of spectral spillover into other channels i.e. PE-Cy5 or PE-Cy7.
- Use a live/dead marker to exclude non-specific staining from dead cells (some live/dead markers are fixable).

Controls

- unstained – to set voltages on the instrument.
- single stained (positive and negative) to set compensation/spillover – can be cells or antibody capture beads – live/dead markers and fluorescent proteins will have to be cells.
- FMO – fluorescence minus one, cells stained with everything except one marker to set sample positive/negative gates.

Talk to your flow cytometry core facility, or one nearby, for any help and advice!

Sample Preparation

Flow Cytometry requires a suspension of single cells, or other particles, with a minimum of aggregates and debris. The tissue architecture and any information about the spatial relationship between different cells are lost when single cells are prepared.

Good sample preparation is key to getting reliable data.

- Titrate your antibodies / dye to find the optimal concentration for use
- Dissociate cells with appropriate reagents
- Filter samples that are aggregated through a nylon mesh to remove clumps. Remember that a 51μm particle will block a 50μm nozzle.
- Know your cells and how they behave
- Be consistent

Always include controls. You will need a control to set your background value, and single colour controls to set the position of your positive cells on scale and to adjust the colour compensation.

Choose the dyes used in your experiments wisely. Use the brightest dye on the least expressed antigen. Choose dyes with least spectral overlap, or which use different lasers for excitation.

Useful references are:

- Flow Cytometry A Practical Approach. MG Ormerod
- Practical Flow Cytometry. Howard M Sharpiro (free pdf) http://www.beckmancoulter.com/wsrportal/wsr/research-and-discovery/products-and-services/flow-cytometry/practical-flow-cytometry/index.htm
- FLOW CYTOMETRY A basic introduction. Michael G. Ormerod http://flowbook.denovosoftware.com/

Key Points on Data Analysis

Data sets in flow cytometry are often very large (>5000 events). They consist of discontinuous variables with a large number of possible values (data bins) and the distributions of the values are rarely truly Gaussian (normal), even after logarithmic or other transformation. Median or geometric mean fluorescent intensity should generally give a more reliable measure of the sample average.

Considerable care needs to be taken in using statistical methods to compare even simple single parameter datasets (histograms) as established comparison methods, e.g. Kolmogorov-Smirnov, will often suggest that replicate datasets from the same sample are non-identical at low p value ($p<10^{-6}$). If histograms do not look obviously different it is safer to assume they are not; conversely those that are clearly different will always be 'statistically' different.

Biological replicates are the most important tool and ideally groups of replicate samples should be compared by simple non-parametric tests (since these make few assumptions about underlying distribution). However, for large multiple-treatment comparisons these methods may underestimate the difference between groups and parametric methods, e.g. Anovar, may be better reflection of our confidence that groups are different where this is justified by the separation of the distributions.

Presentation of flow cytometric data

Minimum Information about a Flow Cytometry Experiment (MIFlowCyt)

http://www.mibbi.org

A consistent presentation style ensures better communication of data to readers and listeners (common language, faster interpretation)

Guidelines do not define how to do science or how to analyze and interpret the data. In most cases, they are not requirements but make sure to check journal's peer-review policies).

Minimum Information consist of:

Experimental overview	experimental variables, conclusions, QC
Samples	material, source, treatment, reagents, analyte, detector/reporter
Instrument details	instrument identification, fluidics organization, optical and electronic configuration
Data analysis	list mode data, compensation, gating, statistics

Principles of graphical presentation:

- Choose one graph type and be consistent.
- Graph axis labels should include (at a minimum) the reagent being measured. In case of antibody labeling, both the specificity and the fluorochrome should be indicated.
- The number of events displayed in any graph should be indicated.
- Show an illustration of the gating process. A backgating display is highly recommended.

Be aware of different publication types in the field (e.g. OMIPs - Optimized Multicolor Immunofluorescence Panel)

Links:

http://www.mibbi.org

http://flowcyt.sourceforge.net

http://www.isac-net.org

Cytometry Part A: http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291552-4930

Publications:

Am J Physiol Lung Cell Mol Physiol. 2010 Feb;298(2):L127-30.

Publishing flow cytometry data.

Alvarez DF, Helm K, Degregori J, Roederer M, Majka S.

Methods Cell Biol. 2004;75:241-56.

Guidelines for the presentation of flow cytometric data.

Roederer M, Darzynkiewicz Z, Parks DR.