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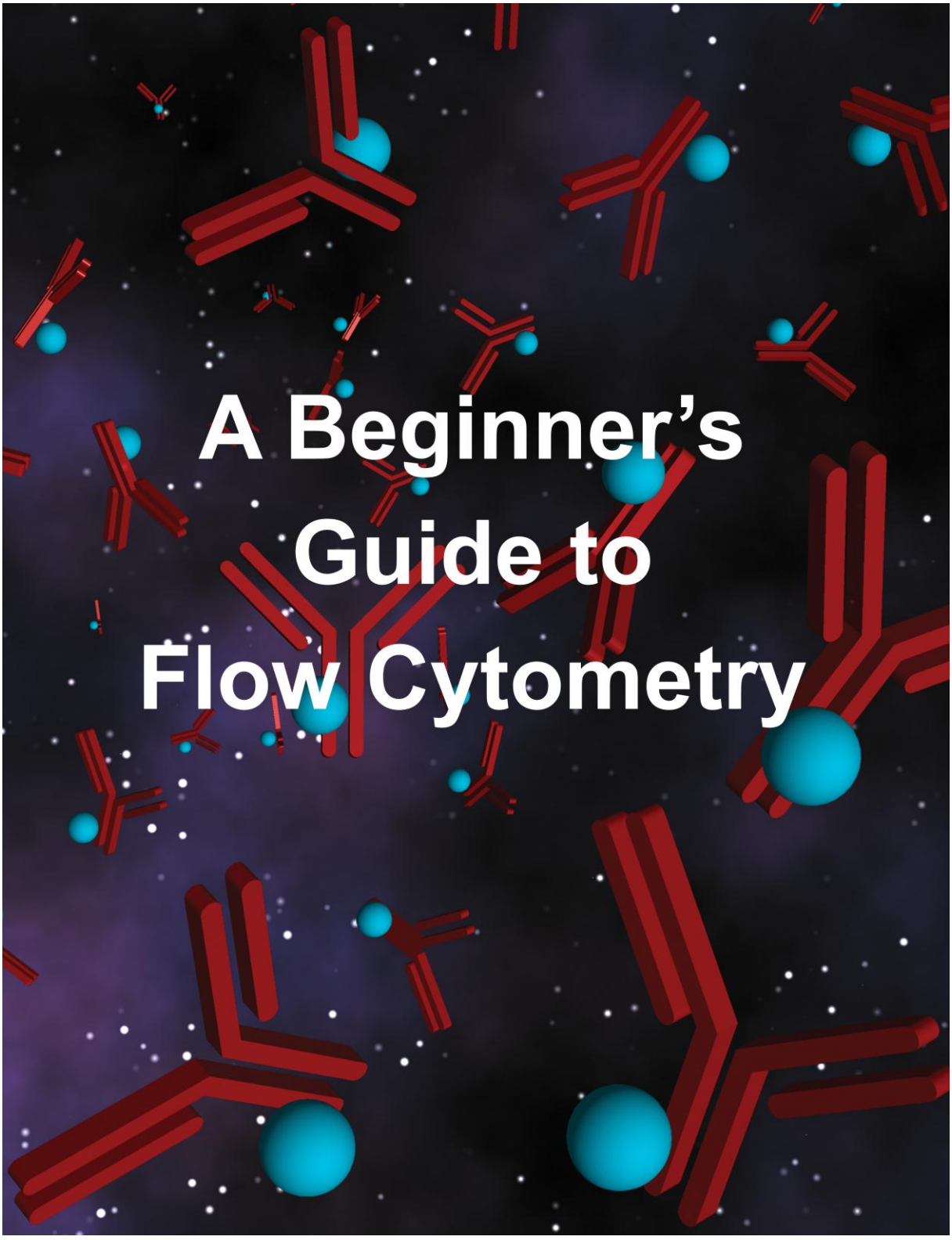


A Beginner's Guide to Flow Cytometry

Introduction

Flow Cytometry is a widely used method for cell analysis which, for the novice, can appear daunting due the complexity of the experimental approach and data analysis. This simple introductory guide has been written with such individuals in mind. It is intended to provide an overview of:

- Flow Cytometry and its associated applications
- The basic components of the flow cytometer
- The main complexities of flow cytometry
- The benefits of Lightning-Link™ - a 30 seconds hands on, simple antibody and protein labelling technique

What is flow cytometry and what can it do?

With respect to cellular analysis, the underlying principle of flow cytometry is that a cell suspension is focussed into a single cell stream which passes through a light source (typically a laser beam). The scattered and emitted fluorescent light (if the cells are fluorescently labelled) is subsequently measured using a range of detectors and these measurements are used to generate multi-parameter data sets that describe the physical characteristics of the cells and their fluorescent properties. The size and granularity of cells can be identified on the basis of their forward and side light scatter characteristics (FSc and SSc respectively). Their characteristics and/or expression of different proteins can be further defined by pre-staining with fluorescently-labelled antibodies or molecules that identify cellular components and / or integrity (viability) or, indeed, fluorescently-labelled proteins.

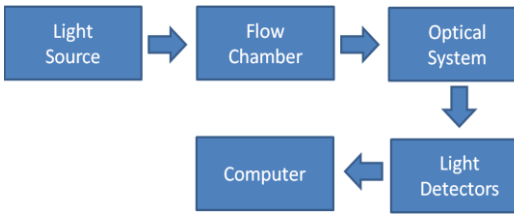
Fluorescent dyes accept light energy at a given wavelength (excitation) and re-emit at a longer wavelength (emission). The fluorescent light which is emitted by these dyes when they are excited by the appropriate light source is channelled via appropriate filters and the signals are collected by an array of detectors. Linking such fluorochromes to an antibody or using fluorescently-labelled molecules that bind to cellular components allows profiles of proteins that are expressed by these cells to be identified and characterized by flow cytometry. With appropriate instrumentation, it is possible to identify particular cell subpopulations on the basis of their physical and / or fluorescent characteristics and isolate or 'sort' these.

Some applications of flow cytometry

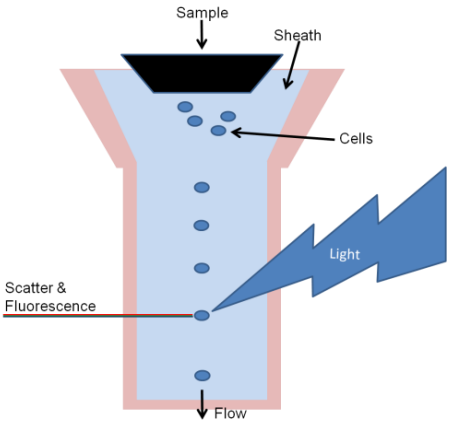
- DNA/Cell Cycle analysis
- Cell proliferation
- Multicolor phenotyping (cell surface)
- Monocyte oxidative burst
- Neutrophil oxidative burst
- Microbiological analysis
- Cellular and antibody or complement-mediated cytotoxicity
- Cell viability
- Intracellular ionic (e.g. Ca^{2+}) fluxes
- Multicolor phenotyping (intracellular)
- Monocyte phagocytosis
- Neutrophil phagocytosis
- Cell trafficking
- Sorting on the basis of morphology (FSC or SSc) and/or fluorescent characteristics

Flow cytometers can evaluate cells at an extremely rapid rate (up to several tens of thousands of events (cells) per second) and can detect as few as 500 molecules per cell. The speed and sensitivity of flow cytometry therefore makes it ideally suited to the analysis of minor cellular populations.

The Flow Cytometer – Basic Components



The **Flow Chamber** (also known as fluidics; please see diagram to the right) is at the heart of the instrument system and is designed to deliver a single cell stream at the point of measurement. This is achieved by focussing cells into the centre of a narrow stream of liquid called sheath fluid such that only single cells pass through the illuminating beam of light in the flow chamber. Although the majority of instruments achieve a single cell stream using fluidics, instruments which achieve this using acoustic focussing are now being developed.



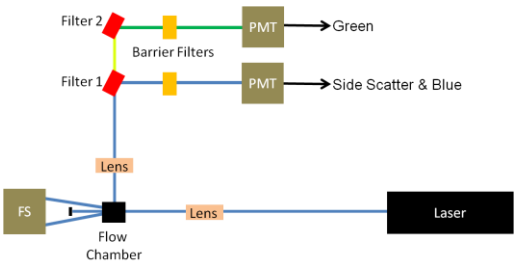
The flow rate of sheath fluid will determine how rapidly the stream of material will move, and thus reach the laser(s) of the cytometer. In an ideal situation, material will pass through the laser one-by-one, and thus data from each individual call ('event') will be recorded. This will provide the most accurate results and it is therefore very important to understand the capabilities of your cytometer.

Some cytometers have a peak detection rate of 900 events per second. Should the flow rate deliver cells at a rate which is faster than the recommended maximum of the instrument being used, then 2 or more could pass through the laser simultaneously ('coincidental events'). This will only result in only one event being recorded and a loss of data. Although a warning message such as a 'data rate warning' might occur on the cytometers software, it is important to understand the consequences should the sheath flow be set too high.

The **light source** can be a laser, arc lamp or light emitting diode (LED). Arc lamps (e.g. mercury, xenon-mercury) are bright and powerful light sources that emit light at multiple wavelengths. They are typically found in light microscopes. Although this broad excitation spectrum is an advantage in some instances, it can also be a disadvantage. A far more common light source in flow cytometers is the laser. These provide a bright and coherent light source at well-defined and specific narrow wavelengths. A number of different lasers are currently available and the range of these is continually expanding. Typical lasers include the Argon ion (351, 454, 488, 514 nm), Krypton (488, 532, 630 nm), Helium neon (632 nm), Helium cadmium (325, 441 nm) and Yag (532 nm) lasers.

The Optical System

Light which is emitted from the cells that have been illuminated in the flow chamber is directed to an array of detectors using a complex system of filters and mirrors called the **optical system**. A simple configuration for a single laser instrument is shown below.



In this example, the light source originates from a blue laser which has passed through a focusing lens and entered the flow chamber. As stated earlier, the physical and fluorescent characteristics of the cells are directly related to the characteristics of the emitted light signals.

A bar placed on the opposite side of the flow chamber blocks the laser beam. The forward light scatter (FSc) detector sits behind the blocker bar and this detects light scatter at angles in a forward direction. Forward light scatter is an indication of cell size.

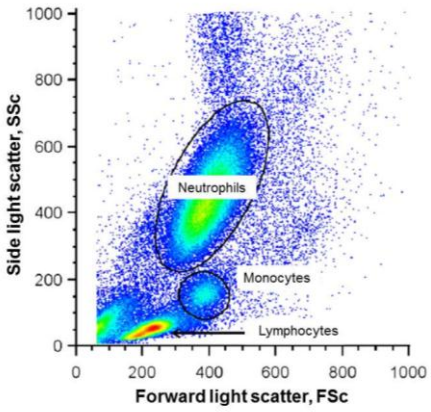
A second focusing lens is used to direct light on to a series of filters that are used to select out light of different wavelengths. **Long pass filters** allow light above a certain wavelength to pass through and **short pass filters** allow light below a certain wavelength to pass through. In the above diagram, Filter 1 selects light at wavelengths less than 500nm (blue) which gives rise to the side scatter data (cell granularity) and Filter 2 selects light below 540nm which passes through a green barrier filter.

The light (photons) which passes through an optical system must be converted into an electrical signal in order for the data to be acquired and analysed. This is achieved using photodiodes (for forward scatter, i.e. size detection) and photomultiplier tubes (PMTs) for the fluorescent signals, and signals that are associated with the detection of side-scattered light (i.e. for the measurement of cellular granularity); such components are also referred to as light detectors. Current flow cytometers can have as many as 12 PMTs and this number will no doubt increase as the technology develops.

Computer - Data Analysis

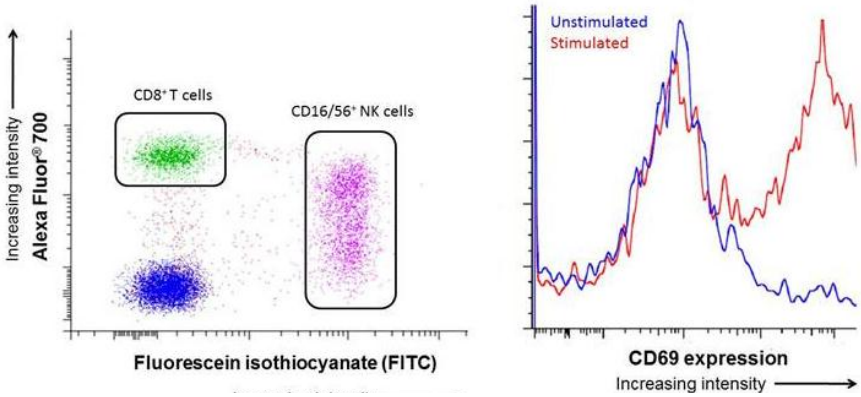
The electronic signals that are generated by the photodiodes and PMTs are collected and processed using software programs, some of which are dedicated to the acquisition and analysis of data from specific instruments, and others that are free-standing and can analyse data from any instruments which have been collected in the flow cytometry standard (.fcs) file format.

The forward and side light scatter (FSc and SSc respectively) characteristics can be used to identify different cells in a mixed population on the basis of cell size and granularity. In the example to the right, neutrophil, monocytes and lymphocyte populations can be identified on the basis of their light scattering characteristics alone (Data courtesy of Dr Jason Boland, University of Sheffield).



Cellular integrity (viability), the presence of cellular components and the expression of different proteins (amongst other parameters) can be defined by staining cells with appropriate fluorescently labelled molecules and antibodies. It is also possible to investigate the interactions of cells with different molecules by incubating them with fluorescently labelled molecules and proteins. The fluorescent light which is released by these dyes when they pass through the laser excitation line is collected and directed through the optical system.

The signals are collected by the array of detectors and processed to provide information on the fluorescent characteristics of the cell population which is being investigated. Staining of human peripheral blood mononuclear cells with an Alexa Fluor® 700 conjugated mAb reactive with CD8 and fluorescein isothiocyanate (FITC)-conjugated mAbs reactive with CD56 and CD16 (identifies natural killer (NK) cells) clearly defines the two cell populations when the Alexa Fluor® 700 and FITC fluorescent characteristics of cells are plotted against each other in what is known as a 'dot plot' (see below left; Data courtesy of Dr Jason Boland, University of Sheffield). In addition to being able to define whether a particular cell is reactive with a fluorescent probe, flow cytometry can provide information on the intensity of the signal and hence an indication of the amount of probe that has bound. As an example, stimulating $CD8^+$ T cells via the T cell receptor activates them and induces the expression of the early activation antigen CD69 (see below right; Data courtesy of Hannah Cussen/Gemma Foulds (left panel) and Dr Jason Boland (right panel), University of Sheffield). It is therefore important to consider both the proportion of cells that are exhibiting the fluorescence of interest and the intensity of that fluorescence.



The Complexities of Flow Cytometry

The range of flow cytometers which is available is expanding, and these instruments can be configured to suit the particular needs of the User. The laser and filter configurations can be customised either by the original manufacturer, or specialised suppliers. When first developed, flow cytometers typically had a single laser and were capable of detecting five parameters (forward light scatter, side light scatter plus 3 different fluorescent wavelengths). However, the complexity of these instruments has increased considerably, with the latest models having up to 5 lasers and the capability of measuring 20 parameters (forward light scatter, side light scatter plus 18 different fluorescent wavelengths). These advances have made the design of experiments and analytical approaches far more difficult, as a number of issues must be considered (see below). Although the new 'bench top' instrumentation which is currently available renders the physical practice of flow cytometry relatively straightforward, the acquisition of meaningful results remains a challenge and it is essential that the investigator has a full understanding of the strengths and potential weaknesses of the technique and the limitations of the data that are generated. This is especially the case with respect to the inclusion of appropriate controls.

The combination of fluorescent labels to be used is critical, as each one must be selected on the basis of their own characteristics, as well as their compatibility with the other fluorescent molecules that are to be used and the instrument which is to be used for the sample analysis.

Additional issues that must be taken into consideration include:

1. **Stain Index:** In its simplest terms, the Stain Index is a parameter which reflects the ability to resolve a dim positive signal from background. In the design of multi-parameter experiments, it is better to use a fluorochrome with a low Stain Index for measuring parameters that are expressed at high levels and a fluorochrome with a high Stain Index for measuring parameters that are expressed at low level.

2. **Spectral Overlap:** Spectral Overlap occurs when the light emitted from one fluorochrome 'leaks' into the channel which detects the fluorescent signal which is being emitted by another fluorochrome. The potential consequence of this is a false positive signal. Although it is possible to eliminate this by electronically removing this signal (a process called 'compensation), it is best avoided/minimised if possible. The concept of compensation remains one of the aspects of flow cytometry which continues to mystify new users. Although many instruments and software packages can perform the compensation for experimenters (assuming that the correct samples have been included in the assay), it is still important to understand the basic principles. For a two colour experiment, the easiest way to approach compensation is to stain a sample with one fluorochrome which is known to give a signal in its 'own' channel and look for fluorescent signals in the other channels that are being used. If no signal is present, then there is no spectral overlap and hence no need to compensate. If there is a fluorescent signal in a channel which should not see one, then spectral overlap exists and should be

eliminated by subtracting the 'inappropriate' signal in this channel from the measurements made using the controls of the flow cytometer. One can imagine that cross-checking spectral overlap in all possible channels becomes progressively more challenging as the number of fluorochromes increases.

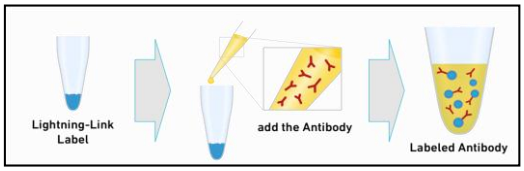
3. **Appropriate Controls:** Flow cytometry is crucially dependent on the inclusion of appropriate controls which enable Spectral Overlap and non-specific binding of reagents to be evaluated and considered. Incubating samples with immunoglobulins that are labelled with the same fluorochrome as the antibodies being used, but which are known not to react with the particular cell and species under investigation (a so called 'isotype' control) provides insight into any potential non-specific binding problems. Although this is not necessarily definitive, it is a control which is widely utilised. ***It is essential to fully appreciate the potential artefacts that might be present in a particular experiments and include the necessary controls as appropriate*.**

4. **Reagent Sourcing and Availability:** The number of products and suppliers of reagents for flow cytometry is rapidly increasing and it is becoming progressively more difficult to identify the appropriate combination of reagents for a particular flow cytometry experiment and source these from the plethora of manufacturers that are now supplying antibodies. Furthermore, it is not always the case that the labelled antibody which is required is available and approaches that can generate these 'in house' are therefore needed. In the vast majority of cases, it is not financially practical to generate a monoclonal antibody for a particular application if this is already available from another. It is therefore important to have the capacity to label commercially sourced antibodies in a cost effective manner whilst simultaneously overcoming the difficulties and sample loss that are associated with traditional labelling techniques.

Lightning-Link™ - Simple to Use Antibody Labelling Kits

The simplification of the antibody labelling process (most notably the elimination of column separation steps) circumvents many issues that have beset traditional procedures for years, i.e. loss of material, sample dilution during column chromatography, batch-to-batch variation and difficulties in scaling up.

The Lightning-Link™ process is summarized in the Figure below. The purified antibody to be labelled is transferred into a vial of lyophilized mixture containing the label of interest. Dissolution of the vial contents activates the chemicals that mediate the antibody labelling reaction. As there are no purification or separation steps (byproducts of the reaction are completely benign), antibody recovery is close to 100%. The simplicity of the approach means that the labelling procedure can be completed in less than thirty seconds. A timed demonstration of the antibody labelling process can be seen here (Lightning-Link video). The labeling chemistry involves the free amine groups found on lysine amino acids, and so any antibody or protein, irrespective of isotype or species, can be labeled.



Although the antibody labelling procedure is very simple, the chemical approach is sophisticated – allowing the formation only of antibody-label conjugates, in a gentle and controlled process.

These simple antibody labelling kits are of special interest to the flow cytometry community, as there are over 40 different labels including fluorescent proteins, dyes and tandems, which span the whole light spectrum from UV to far infrared. It is therefore now possible for the flow cytometry user to design and develop a unique set of labelled antibodies for use in the flow cytometer using commercially sourced unlabelled antibodies. Furthermore, there are many benefits associated with direct labelling of a primary antibody:

- Eliminates the need for the use of secondary antibodies, thus reducing the number of incubation and wash steps - saves both time and money.

- In experiments which use several antibodies simultaneously, cross-species reactivity is not an issue as the primary antibodies can be labelled with different dyes. With indirect detection, cross-species reactivity of secondary antibodies is often a problem.
- Commercial sources often do not have the correct antibody conjugated to the required label. By direct labelling of the antibody of interest yourself, this hurdle is easily overcome.

Additional information can be read in the 'Guide to Antibody Labelling and Direct Detection.'

Summary

Flow cytometry is a powerful technique for cellular analysis which can rapidly generate complex datasets that can provide insight into cellular status, processes and events which would be difficult, if not impossible, to achieve using other approaches. If the reader has been inspired by this simple guide, then you are encouraged to delve deeper into the subject by reviewing the selected resources in order to increase your knowledge base and enable the mastering of this powerful and adaptable technique.

Bibliography and Resources

- Dean PN, Bagwell CB, Lindmo T, Murphy RF and Salzman GC. Data File Standard for Flow Cytometry. Cytometry 1990, 11:323-332.
- Murphy RF, Chused TM:A proposal for a flow cytometric data file standard. Cytometry 1984 5:553-555.
- Robinson PJ. Mack Fulwyler in his own words. Cytometry Part A 2005, 67A:61–67
- Flow Cytometry - A Basic Introduction – Wicki Version (Michael G. Ormerod) http://flowbook-wiki.denovosoftware.com/
- Consolidated Resource for Flow Cytometry Antibodies, Instrumentation, Software & Suppliers: http://www.chromocyte.com