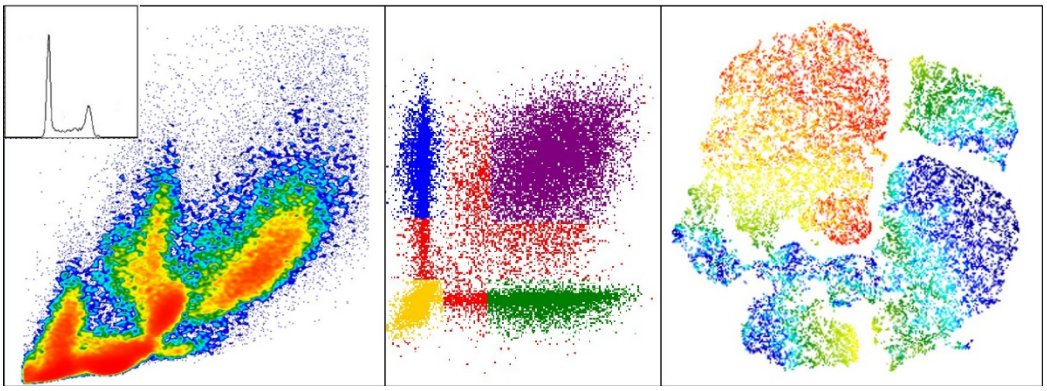

THE UNIVERSITY OF MELBOURNE

MELBOURNE CYTOMETRY PLATFORM

Introduction to Flow Cytometry

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Melbourne Cytometry Platform

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INTRODUCTION TO FLOW CYTOMETRY

Preface

To explain flow cytometry to someone who knows little about it, imagine it to be like doing a big shop at the supermarket. You select a variety of products and take them to the checkout. You place them onto a conveyor belt and the sales assistant scans a barcode on each product, one at a time. Once identified, similar products are packed together e.g. fresh fruit and veg go into one shopping bag, food in jars or cans into a second bag and cleaning items into yet another. Now replace shopping with biological cells; the conveyor belt with sheath fluid, the barcode with cellular markers, the scanner with lasers and detectors, and shopping bags with sample collection tubes – welcome to the world of flow cytometry and cell sorting!

Introduction

When you break down the term "Flow Cytometry", the definition jumps out at you: it is the measurement (metry) of cells (cyto) in a fluid (flow). With flow cytometry, we detect and measure the physical, chemical and fluorescent characteristics of cells (or particles), one at a time, by passing them through laser beams and collecting the light that is emitted or scattered. If you can dissociate your samples into 'single' cells or particles, you can analyse them with flow cytometry.

Flow cytometry is an extremely powerful and versatile technology for most biological and materials studies because:

- It is very FAST! – you can measure tens of thousands of particles per second.
- The large amount of data points collected can allow robust statistical analysis.
- There is a LOT of information to be gained. Many physical and fluorescent parameters are measured simultaneously allowing for the resolution of distinct populations and cellular functions in complex and heterogeneous mixes.
- Data is qualitative, quantitative and unbiased.
- (optional) Pure, sterile, live cells of interest can be retrieved for downstream applications such as culture, functional assays, transplant, imaging and "omics" including DNA and RNA sequencing, proteomics, metabolomics, etc…

Here you will learn about the three major subsystems shared by all flow cytometers: fluidics, optics and electronics (signal processing/data display). We will briefly cover light scatter and fluorescence, basics of data analysis and commonly used cytometry applications, while highlighting important concepts on experimental design, sample preparation, controls and instrument setup and operation.

Figure 1 **Particles analysed by flow cytometry**

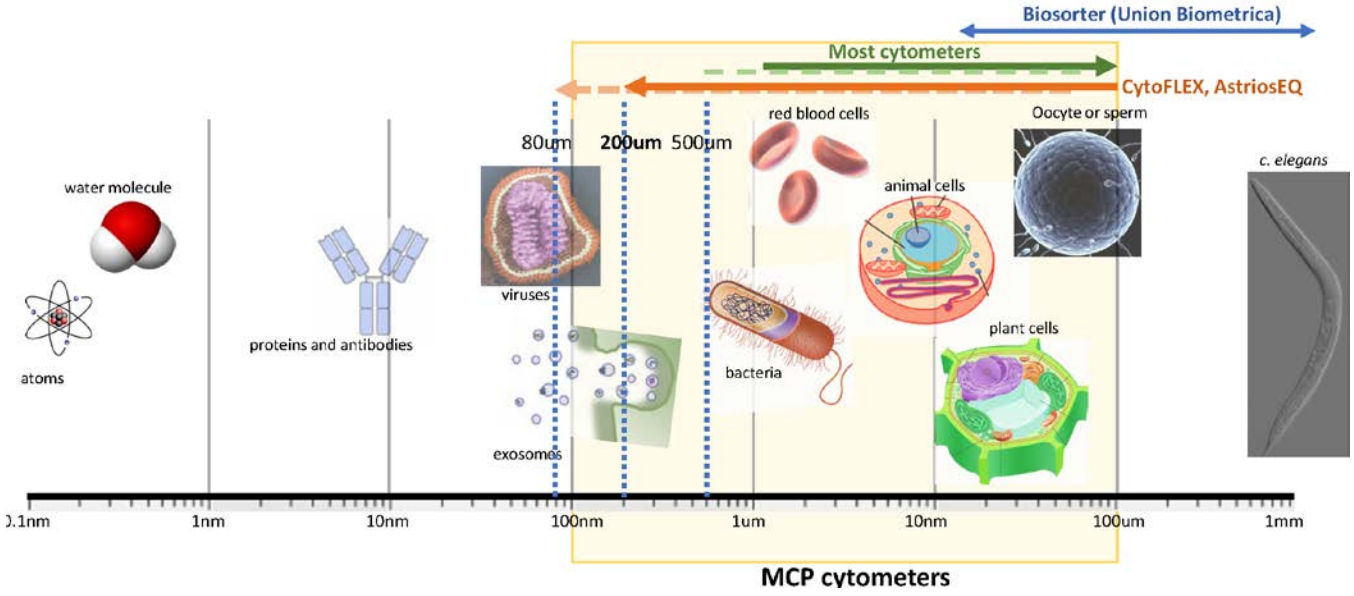
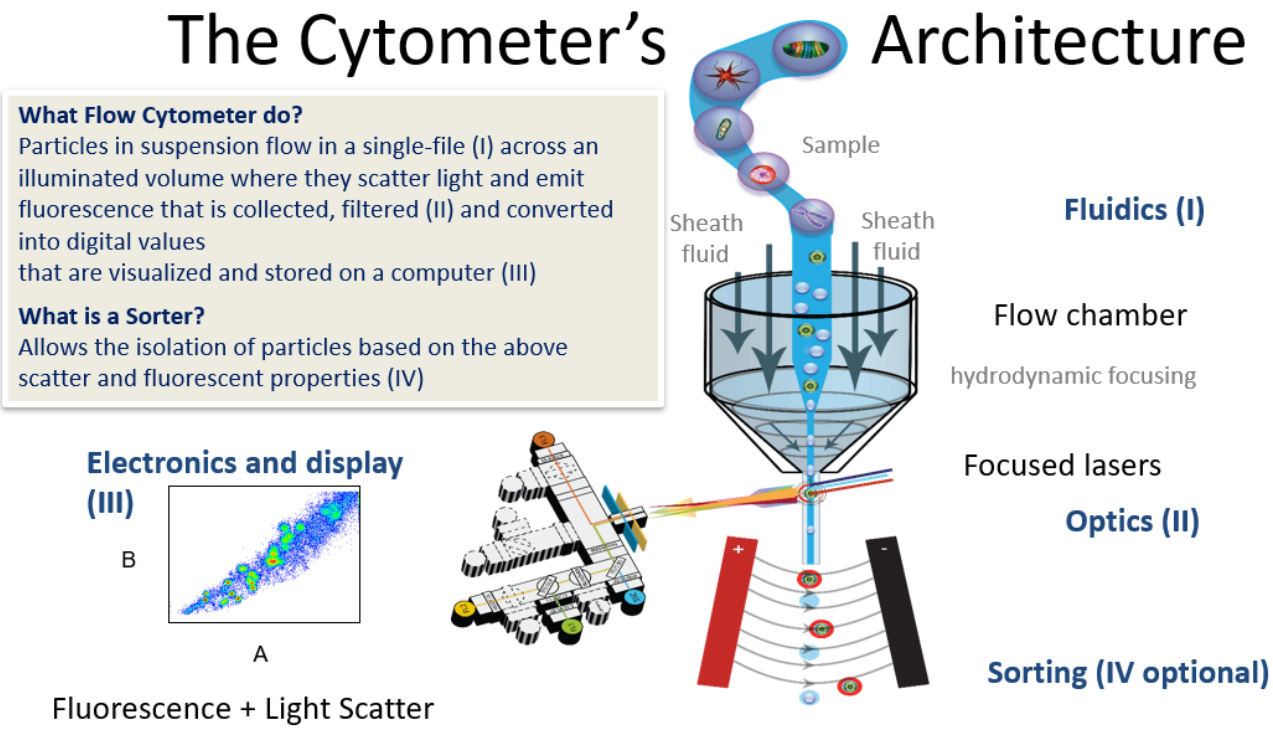


Figure 2 **Basic components of a cytometer**



1. Fluidics system

One of the fundamentals of flow cytometry is the ability to consistently measure properties of individual particles as they flow at high speed across focused laser beams. When a solution of particles is injected into a cytometer's flow cell, the particles are initially randomly distributed in three-dimensional space. To achieve measurement consistency, these randomly arranged particles must first be aligned into a single file before arriving at the laser interrogation points where they are illuminated and measured by the machine's detection system. Precise particle alignment and consistent delivery is handled in cytometers by their fluidics subsystem, taking advantage from the properties of laminar flow to guarantee consistent, predictable motion and delivery and hydrodynamic focusing that accelerates and orientates particles in a single file.

2. Laminar flow

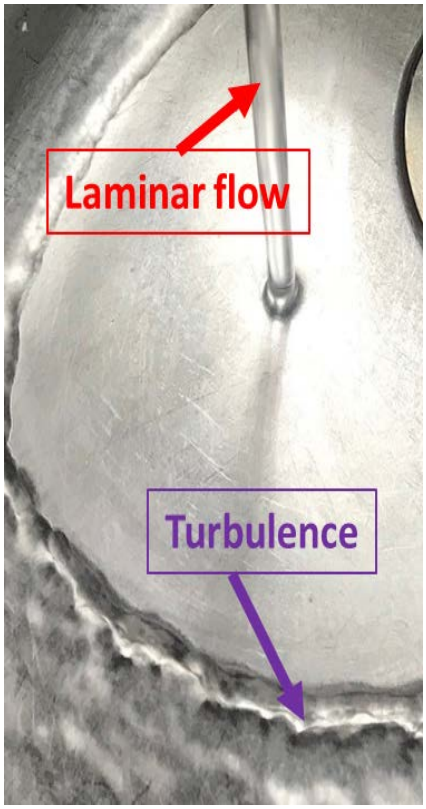


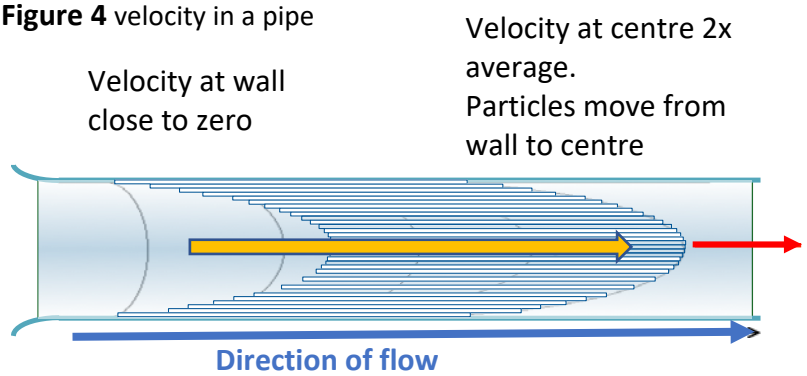
Figure 3 In a sink, the stream coming from the tap is laminar. The water sloshing around in the basin is turbulent.

Laminar flow is characterized by fluid flowing in parallel layers with no mixing between them. Within the bounds of laminar flow, the path of fluid is consistent and predictable. Turbulence is the opposite of laminar and is characterized by chaotic and inconsistent fluid motion.

Liquids and gases can flow as either laminar or turbulent fluids, with transitions between these flow patterns triggered by defined physical changes (liquid viscosity and density, linear velocity, pipe dimensions, physical disruption). Flow cells and cytometers fluidics systems are designed to maintain laminar flow conditions.

When liquid runs laminar in a pipe, such as a cytometer's flow cell, it eventually develops a parabolic velocity profile, where the liquid at the centre of the pipe travels approximately at twice the average fluid velocity and where liquid near the pipe's walls has a velocity close to zero. This generates an inverse pressure gradient, with the lowest pressure at the pipe's centre and maximum pressure at the walls (Bernouille's Principle). Because of this, particles are forced to relocate to the faster moving centre.

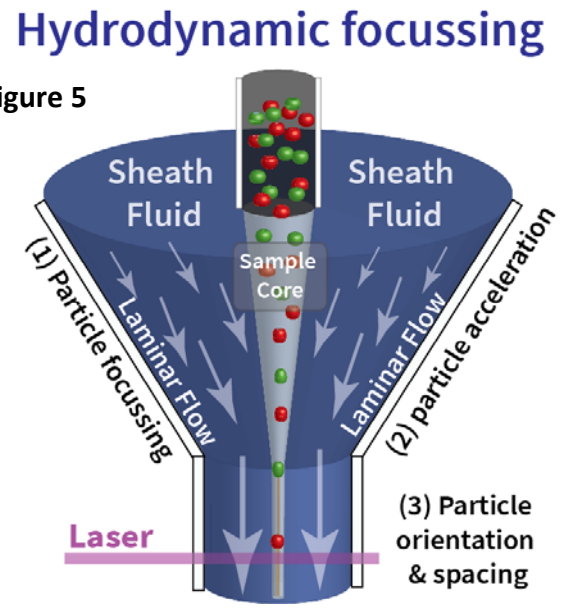
Figure 4 velocity in a pipe



3. Flow cell and hydrodynamic focusing

Flow cells are designed to accelerate, space out, focus and align particles. This is achieved by funneling (narrowing) the flow cell's inner cross section lengthwise with the narrowest part at the point where particles exit on their way to the lasers. Narrowing of the flow space confines the particles carried by the fluid into a progressively smaller area with ever increasing velocities. This is the basis for hydrodynamic focusing.

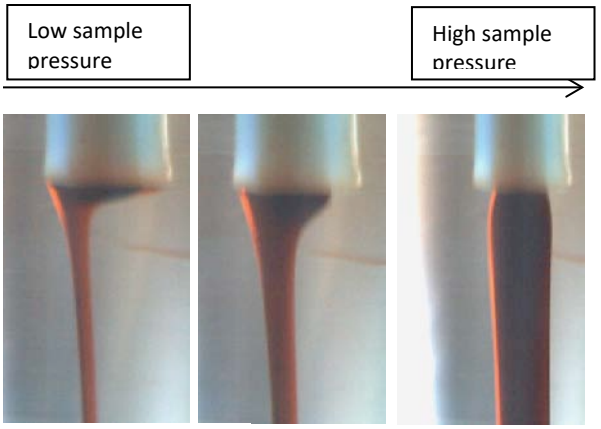
Figure 5



Soon after exiting the hydrodynamic focusing flow cell segment, a thin core of single file particles surrounded and carried by sheath fluid, travels at a constant velocity across the focused laser beams for illumination and photon capture by the optics.

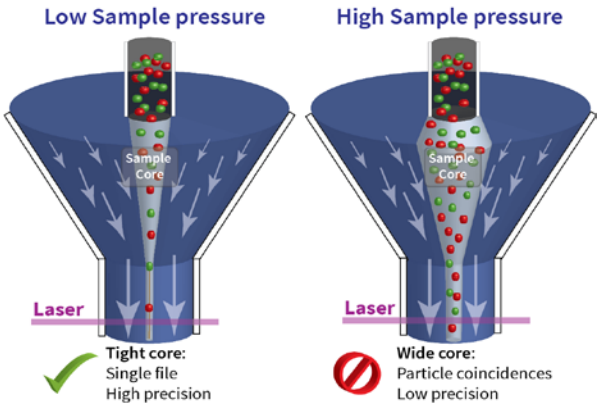
4. Sample pressure

The volumetric speed at which samples are run through the cytometer is controlled by altering the sample-to-sheath pressure differential. When running at low speed (low pressure), the sample core fluid forms a narrow cross section within the sheath fluid boundary right upon entry into the flow cell. Samples can be run at higher speeds (higher pressure) and wider core cross-section, but the trade-offs are lower stability and potentially compromised illumination. Since the final dimension of the sample core upon exiting the flow cell hydrodynamic focusing element is a function of the ratio of the entry and exit inner cross section areas, which is fixed by design, injecting samples with low pressure (or small volumetric speed) will guarantee the narrowest core path and the most consistent laser interrogation and particle measurement. As pictured below, at low pressure, the final cross-sectional area of the sample core is narrow, carrying cells one at a time with it while travelling through the laser spots. This gives the most accurate illumination for single particles while limiting particle coincidences. At high pressure, the sample core is wider resulting in frequent particle coincidences. Precision is diminished because cytometers can't always distinguish the separate particles. It is critical for measurements with small fluorescence differences such as cell cycle to run at the lowest possible sample pressure. In these cases, if you want to reduce acquisition time, it is preferable to achieve higher sample flow rates by increasing the particle concentration. Using the smallest possible sample pressure and a low particle density are crucial strategies when evaluating nanoparticles via cytometry, since due to their very small dimensions, hydrodynamic focusing alone cannot prevent n-particle coincidences from crossing the laser spots or in the worst case scenario (at high particles concentrations) prevent the electronics from saturating. Particularly in nanoparticle analysis, high pressure and high particle concentration will lead to erroneous measurements and artefacts- the equivalent in Cytometry to "fake news".



A Riddell. EMBO

Figure 6



Increasing the sample pressure (core fluid, red ink) will expand the final cross-section of the cells path (the core fluid), leading to illumination inconsistencies.

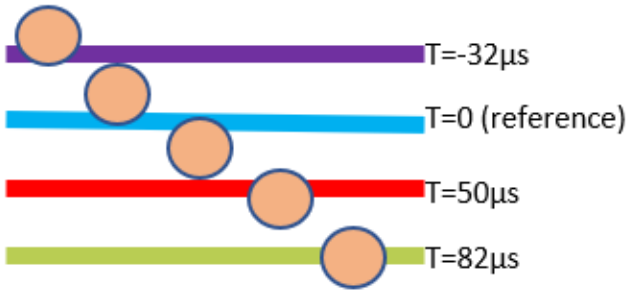
5. Sample Preparation and Stability

The fluidic stability provided by laminar flow and hydrodynamic focusing ensure that fluorescence and light scatter signals from each particle are accurately measured and that each particle is exposed to the same amount of laser photons. Stable fluidics also ensure consistency in the laser crossing time or Time of Flight (TOF) of each particle- the equivalent in cytometry to photography's exposure time.

Consistent linear velocity is especially important when working with multi-laser cytometers and cells labelled with multiple fluorochromes. In, multi-laser cytometers, the distance between laser interrogation points is fixed, and so the particle travel time between each laser needs to be consistent. The electronics that read signals from each laser interrogation point are calibrated to activate at fixed times. A disruption in fluidic stability can result in severe disruptions/ inconsistencies or the complete loss of signals from certain lasers.

Figure 7

Electronics send signals from each laser for processing at precise times (T=x µs). These times are set based on QC setups and are dependent on laminar flow



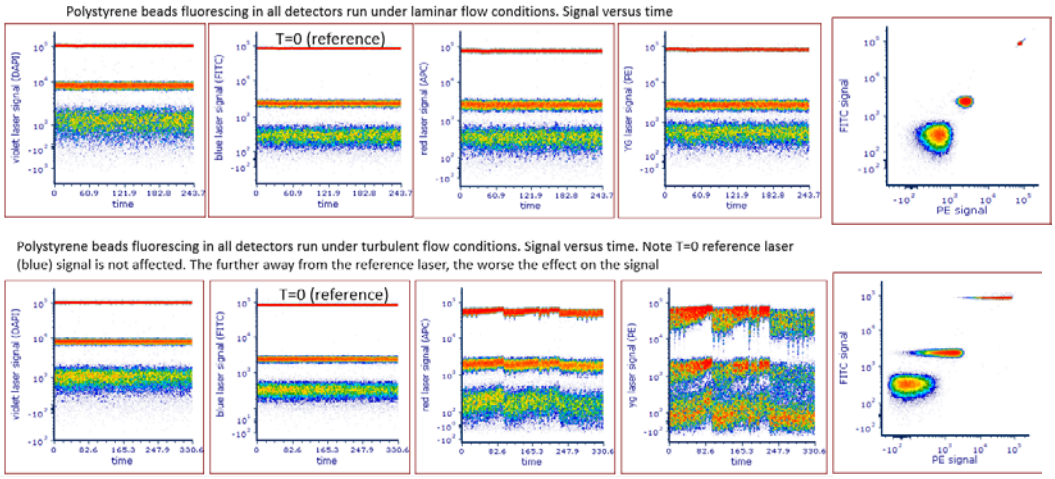
Many of the factors that contribute to fluidic instability are associated with sample quality and preparation, including cellular clumps, poorly-prepared samples of solid or hard tissues (extracellular-matrix components, corals, exoskeleton, bones), or even extensive cell death leading to large cell and DNA aggregates. In addition, poor preparation of the cytometer, allowing samples or sheath fluid to run dry, or even insufficient cleaning after a run can introduce or accumulate air bubbles and dirt in the fluidics system and at the flow cell walls, disrupting any (or all) of the laser beams', fluorescence or

scatter's travelling paths, or worse, result in transient or permanent clogs and the turbulence patterns and backpressure that result from them.

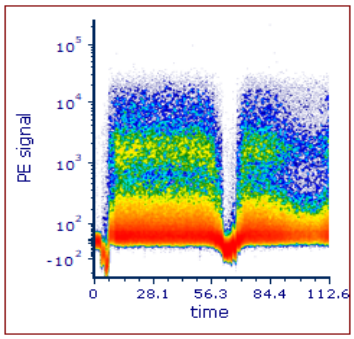
6. To prevent flow instabilities:

- Cytometer-specific fluidics start-up must be performed;
- All cell suspensions must be filtered right before acquisition;
- Tubes/wells should never be allowed to run dry -this introduces air bubbles into the system. These take time and effort to fully remove!

Figure 8 fluidics instability



PE signal severely affected. Impacts on resolution of your PE+ populations producing 'spread' and possibly even false-negative events



Real life sample – disruption of PE signal. Unstable fluidics. Possibly caused by a blockage or air bubble that resolves itself

In order to detect instabilities as they happen, you must always include as many signal vs. time plots as lasers needed to excite the fluorochromes in your samples. Preferably, you should do this during sample acquisition (so that you could report systemic issues to platform staff) or during post-acquisition analysis to assess the quality of your acquired data and exclude compromised segments from the analysis.

7. Signals Measured in Cytometry

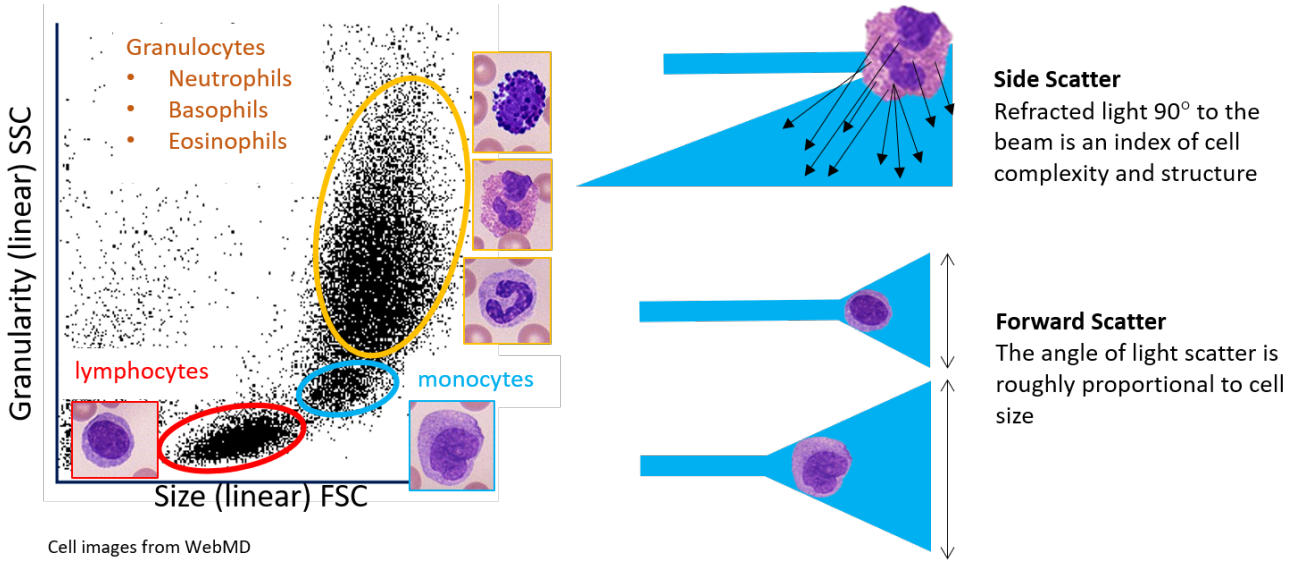
After hydrodynamic focusing, each particle passes through one or more focused beams of light. Lasers are the most common light source in flow cytometry and consist of photons of pure single wavelength (a laser line) in phase with each other (coherent light). Lasers in cytometry typically have higher power than those used in microscopy because of the limited amount of time particles spend at the illumination points (from 2 µs in jet-in-air-cell sorters to several milliseconds in bench-top analysers and some cell sorters).

Light scattering and fluorescence emission provide information about the particles' physical and biological properties. Particle-derived scatter and fluorescence photons are collected by optical lenses and transmitted to light detectors via fiber-optic cables and in most cases, across several optical filters. To amplify weak cell-derived photon pulses cytometers use highly efficient light detectors such as silicon photodiodes, photomultiplier tubes (PMTs), and most recently avalanche photodiodes (APDs) and silicon photomultipliers. The least sensitive- silicon photodiodes - are used to measure forward scatter (relative particle size) in most instruments, since this signal is typically the strongest (except for nanoparticles and bacteria, where FSC signals are too small to be detected from background in most instruments). PMT and APD detectors are far more sensitive and thus ideal for side scatter and fluorescence measurement.

8. Scatter

As soon as a particle hits the laser, laser light is scattered in every possible angle, mostly around the laser travelling plane. However, most cytometers measure two information-rich scatter components: forward scatter (FSC) and side scatter (SSC). Light that is scattered in the forward direction, typically up to 20º offset from the laser beam's horizontal axis, is measured by FSC detectors. The FSC intensity is influenced by factors such as particle size and membrane integrity and can be used to distinguish between cellular debris and living cells. Light measured orthogonal (around to 90º angle) to the laser's horizontal axis, is measured by SSC detectors. SSC provides information about cells' complexity, granularity and membrane roughness. Both FSC and SSC are unique for every cell, affected by differentiation, proliferation or activation status. Combined, these two scatter detectors are frequently used in cytometry to coarsely distinguish unique populations in heterogeneous samples (1) to visualize and acquire all particles of interest regardless of fluorescence content, (2) to define acquisition threshold and trigger and (3) to remove cellular aggregates for downstream analysis.

Figure 9 Scatter measurements in flow cytometry. Example of peripheral blood leukocytes

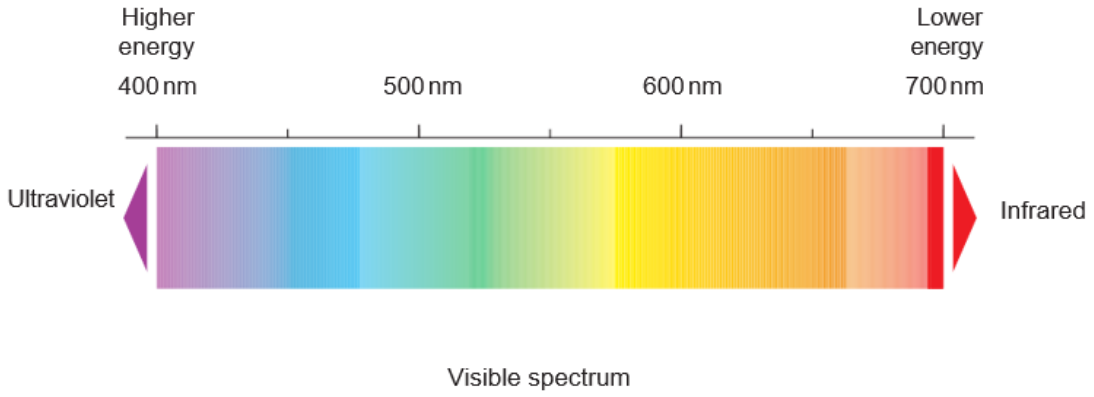


9. Light, Fluorochromes and Fluorescence

Fluorochromes (fluors) are molecules that absorb light energy (e.g. from a laser) at given wavelengths and emit a portion of this energy at longer wavelengths. These two processes are called fluorescence excitation and emission. The process of emission follows absorption extremely rapidly, commonly in the order of 1-10 nanoseconds, which makes fluorescence a valuable tool for almost instantaneous readings. There are hundreds of fluorochromes available to flow cytometry. Before we consider some classical examples, it is important to understand some of the general principles of light absorbance and emission when it comes to fluorescence.

Light is an electromagnetic wave that travels as photons of defined quantum energies. These waves can be described by either their frequency or wavelength. The light that can be visualized by the human eye represents a narrow wavelength band (380–700 nm) between ultraviolet (UV) and infrared (IR) radiation. Sunlight, for example, contains UV and IR light that, although invisible to the eye, can still be felt as burning or warmth on the skin and can be measured scientifically using photon detectors. The visible spectrum can be further subdivided according to the perceived colour of light. In wavelength increasing order, light can appear as violet, blue, green, yellow, orange, red with all transitions in between. Red light is at the longer wavelength end of the spectrum (photons of lower energy) and violet light is among the shortest visible wavelengths (photons with higher energy).

Figure 10 Light Spectrum

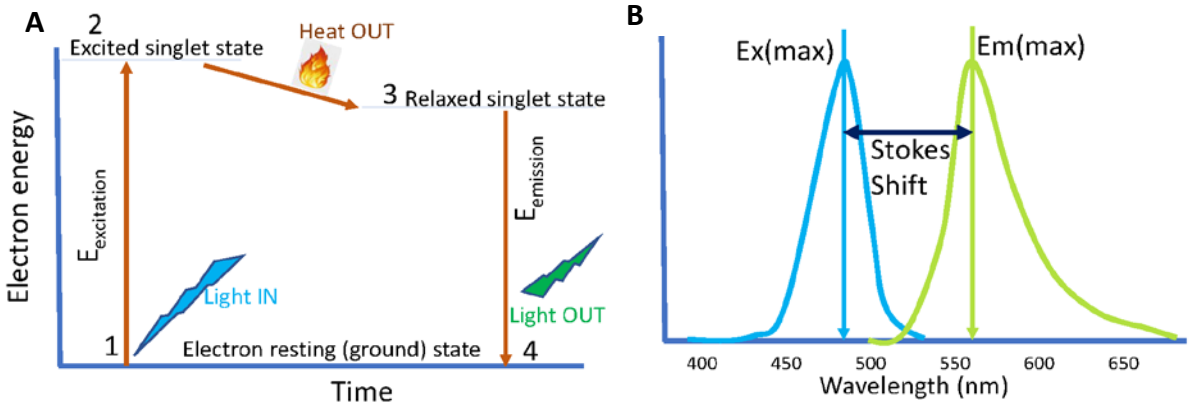


10. Fluorescence and Stokes Shift

When fluorochromes absorb photons of specific wavelength, electrons in the fluorochrome become excited and move from a resting state (1) to a maximal energy level called the 'excited electronic singlet state' (2). The amount of photon energy required for this energy jump ($E_{photon} = hf = hc/\lambda$) differs for each fluorochrome and is depicted below as $E_{excitation}$. The excited state only lasts for 1–10 nanoseconds as the fluorochrome undergoes internal conformational changes and, in doing so, releases some of the absorbed energy as heat. The electrons fall to a lower, more stable, energy level called the 'relaxed electronic singlet state' (3). As electrons move back to the ground state, they release the remaining energy ($E_{emission}$) as fluorescence (4).

The emission wavelength of any fluorochrome will always be longer (lower energy) than its excitation wavelength. The difference between $E_{excitation}$ and $E_{emission}$ is known as the Stokes Shift and the difference in wavelength value determines how good a fluorochrome is for fluorescence studies. It is imperative that the light produced by emission can be distinguished from the light used for excitation. Fluorescence is easier to detect when fluorescent molecules have a large Stokes Shift.

Figure 11 A Fluorescence and **B** stokes shift



11. Absorbance, Emission and Spectra Viewers

The excitation wavelength is critical as it determines the probability of photons being absorbed by a fluorochrome. FITC (fluorescein isothiocyanate), for example, will absorb light within the range 400–550 nm but the closer the wavelength is to 496nm (its peak or maximum absorption wavelength), the greater the absorbance is. In turn, the more photons absorbed, the more intense the fluorescence emission will be. When planning your cytometry experiment, always ensure that your cytometer is equipped with laser lines that best fit your sample fluorochromes. The Melbourne Cytometry Platform has cytometers equipped with some or all these lasers: 350nm (UV), 405nm (violet), 488nm (blue), 561nm (Yellow/green), 640nm (Red) and 808nm (Infrared) see Appendix 1. Importantly, you don't need (and won't always be able) to perfectly match your fluorochrome's absorption maximum, you just get close enough to it to guarantee the best possible excitation, for example for some fluorochromes such as DAPI, 405nm laser light that excites at 4% efficiency is sufficient to generate a very bright signal. In the case of phycoerythrin (PE), the 488nm and 561nm lasers both excite electrons but at 40% and 76% efficiency respectively. While both laser lines are routinely used for PE excitation, use of the 561nm laser for PE excitation is preferable when looking for very dim antigen expression because this will deliver the best fluorescent signal.

The excitation and emission spectral traces of a fluorochrome are extremely useful to determine its maximal (optimal) excitation and emission wavelengths. The excitation spectral trace is used to define the laser line best used for excitation. In the case of FITC (below), its maximum excitation wavelength falls within the blue spectrum. Therefore, the blue 488nm laser line, close to FITC's absorbance peak of 496nm is used. Using the excitation trace, it is apparent that the 405nm (violet) will only weakly excite FITC electrons, while the 561nm (yellow/green) will not excite them at all. FITC emits fluorescence over the range 475–610nm peaking at 525nm, which falls in the green emission spectrum. If filters are used to screen out all light other than that measured at the maximum via channel (530/30), FITC will appear predominantly green. Hence, 'fluorescence colour' usually refers to the colour of the light a fluorochrome emits with the highest probability. However, if FITC fluorescence is detected only via channel 582/15, it will appear orange and be much weaker in intensity. Choosing the right laser excitation line and the best emission spectral bandwidth for each fluorochromes will have a massive impact on your ability to collect a measurable signal. There are many spectral viewers available online that will help you to visualize absorption and emission maximum for fluorochromes whenever you are in doubt. Type "spectra viewer" into a search engine to access them. Companies that sell fluorescent reagents usually give you access to online spectral viewer tools showcasing their proprietary products (Biolegend, BD Biosciences, Cytek, Beckman Coulter, Thermofisher, etc).

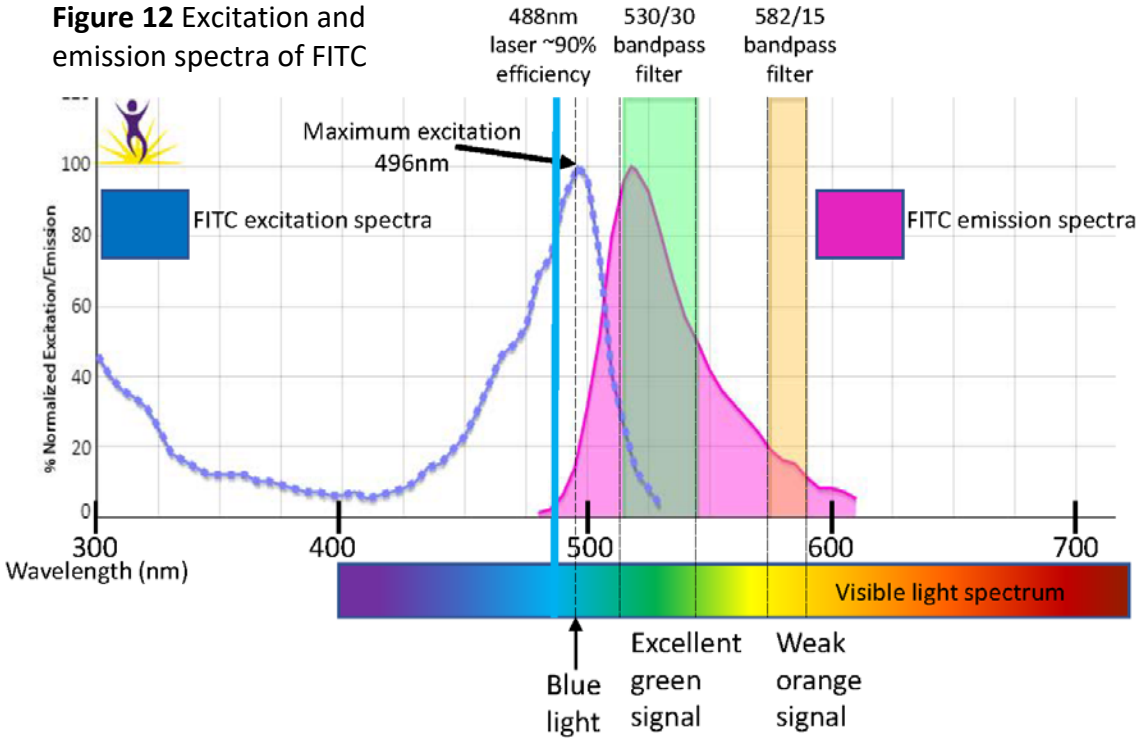


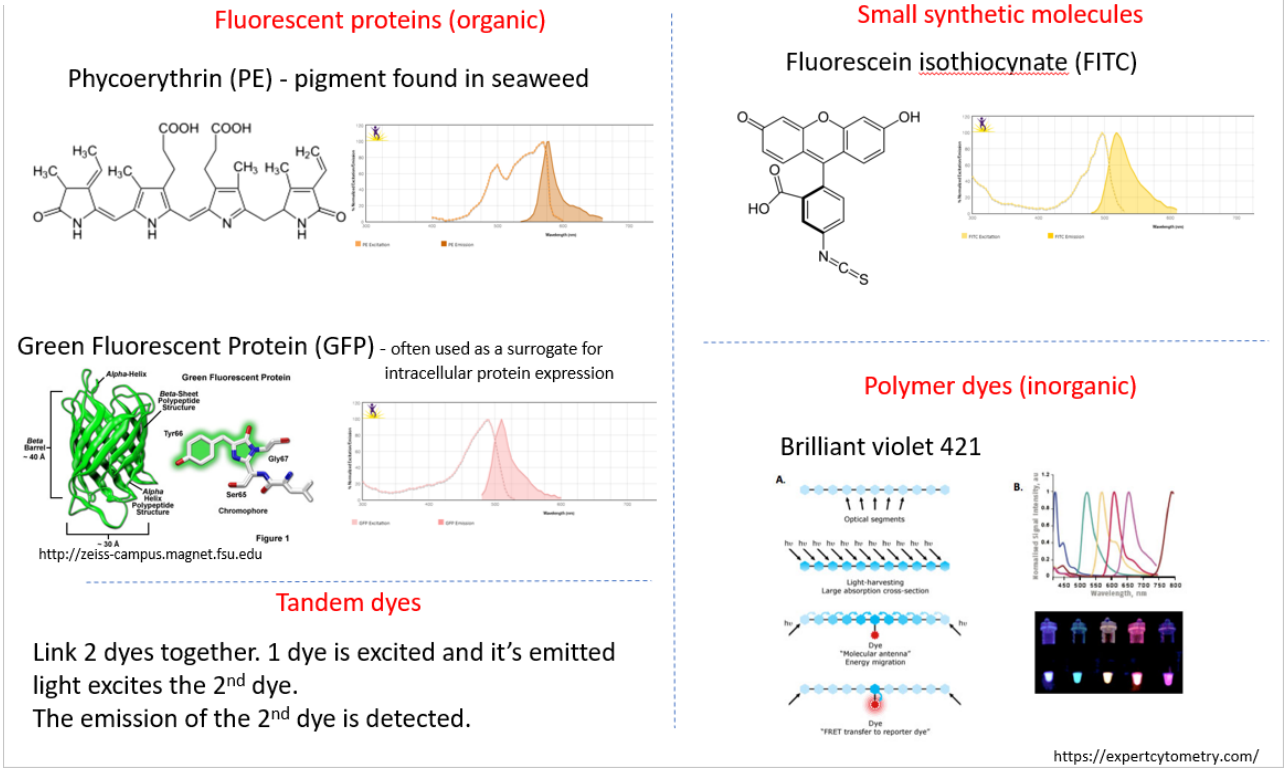
Figure 12 Excitation and emission spectra of FITC

12. Fluorescent Molecules

Fluorescent probes, such as structural and functional dyes, fluorochrome-conjugated antibodies and fluorescent proteins, are used in cytometry to directly label molecules (proteins, DNA, other moieties) of interest when studying the biological and biochemical properties of particles. Fluorescent probes are useful in a wide range of applications including identifying and quantifying distinct populations of cells, cell surface receptors or intracellular organelles, cell sorting, immunophenotyping, calcium influx, nucleic acid content, enzymatic activity, and apoptosis to name a few.

By combining more than one fluorochrome tag, it is possible to achieve a deeper understanding of complex cellular populations via the analysis of co-expression and correlation between cellular components. This forms the basis of multi-colour fluorescence studies. The maximum number of fluorochromes that can be identified and measured varies among cytometers and depends on the number of lasers and detectors and the procedure used to spectrally resolve fluorochromes from each other. MCP instruments can handle up to 40 fluorescent molecules within the one sample.

Figure 13 Sources of common fluorescent molecules



13. Single Fluorophores

Single fluorophores are typically synthesised or naturally occurring molecules that when excited by an energy source undergo conformation changes and fluoresce. Single fluorophores include small synthetic molecules as FITC, organic molecules derived from biological sources, such as phycoerythrin (PE) and allophycocyanin (APC) and inorganic polymers such as Brilliant violet 421 and Brilliant violet 510. They have unique structures with specific excitation and emission maximum and can be used as molecular blocks to build tandem fluorophores of extended Stokes Shift.

14. Tandem Fluorophores

In a tandem fluorophore, tandems of two or more single fluorochromes "blocks" are covalently linked. Typically, a small fluorochrome 'piggy-backs' onto another larger fluorochrome. When the laser excitable dye portion of the tandem (known as the "donor") is excited and reaches its maximal singlet state, all its energy transfers to the second dye (the "acceptor"). The "acceptor" molecule, whose absorption spectra overlaps with the emission spectra of the first dye, produces the fluorescence emission. The process is called FRET (Forster resonance energy transfer). It's a way to achieve higher Stokes Shifts and, therefore, increase the number of colours that can be analysed from a single laser wavelength. Examples of tandem dyes are: PerCP-Cy5.5, PE-Cy7, APC-Fire750 and Brilliant violet 650.

15. Fluorescent Proteins

Fluorescent proteins (FPs), such as green fluorescent protein (GFP), mCherry or cyan fluorescent protein (CFP) are typically used as surrogate indicators of endogenous protein expression or protein over-expression. Fluorescent proteins are transcribed/translated by cells either from transduced, transformed or transfected plasmids or after integration into the genome either by random insertion or targeted homologous recombination. FPs can be detected and measured by flow cytometry, even when expressed intracellularly.

When suitable pairs of FPs are introduced into cells, they can be used to measure protein-protein interactions or binding through measurement of FRET. In a common FRET pairing, CFP/YFP, the CFP, Ex 405nm (violet), Em 485nm (green)/ and YFP Ex 488nm (blue), Em 527nm (yellow). If YFP is in close enough proximity to CFP (within 10nm), the emission energy of CFP will be transferred to YFP and the FRET emission will be detected in the violet 525/20 detector. If there is no interaction, CFP will be detected in its primary detector (violet 450/40) and YFP will be detected in its primary detector (blue 530/30), and there will be no signal in the FRET detector (violet 525/20).

16. Fluorescent Dyes

Fluorescent dyes are synthetic chemicals that enter cells in various circumstances. Some fluorescent dyes can cross the membrane of a live cell (membrane permeable) such as Celltrace violet, CFSE, Hoechst 33342, JC1 and DRAQ9. Other dyes are not able to enter live cells, and only cross the membranes of compromised cells. These include nucleic acid binding dyes DAPI (A-T regions), 7AAD and propidium iodide (G-C regions) that fluoresce once bound to nucleic acids. These dyes are commonly used to distinguish between live and dead cells, and to measure cell cycle.

Amine-reactive dyes are also used in cytometry often to distinguish live and dead cells in experiments where cells are fixed and permeabilised. Amine-reactive dyes bind proteins. A dead cell has more exposed proteins (amine groups) than a live cell because its cytoplasmic proteins are also exposed. Staining cells with Live/Dead amine-reactive dyes prior to fixation and permeabilisation for intracellular staining allows you differentiate the starting dead cells from live permeabilised cells: live cells have less amine-reactive dye signal than dead cells. Examples of amine-binding dyes for live/dead discrimination include the Zombie dyes (BioLegend) and LIVE/DEAD (ThermoFisher).

17. Optics and Fluorescence Detection

Scattered and emitted light originating at the laser interrogation points is collected by optical lenses and delivered to the cytometer detectors assembly. Here, an array of light-dissecting optical filters block or reflect certain wavelengths while transmitting (passing) limited bandwidths of photons to each detector. There are three major classes of optical filters used in Cytometry:

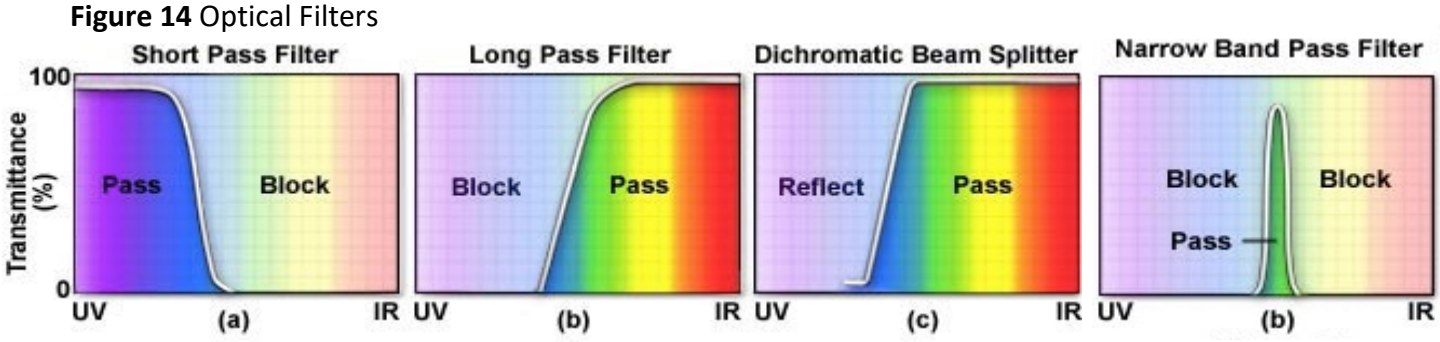
‘Long pass’ (or LP) filters allow through light above a cut-off wavelength,

‘Short pass’ (or SP) filters transmit light below a cut-off wavelength and

‘Band pass’ (or BP) filters transmit light within a specified narrow range of wavelengths.

All these filters block light by either absorption or reflection. Dichroic mirrors/ filters placed before BP filters perform two functions: (1) to pass specified wavelengths in the forward direction and (2), to deflect non transmitted light. As Cytometers detect multiple fluorescence signals simultaneously, optical layout design arranges LP, SP, DC and BP in precise order to dissect sample spectral components and guide the right fluorescence bandwidth to the appropriate detector.

Figure 14 Optical Filters

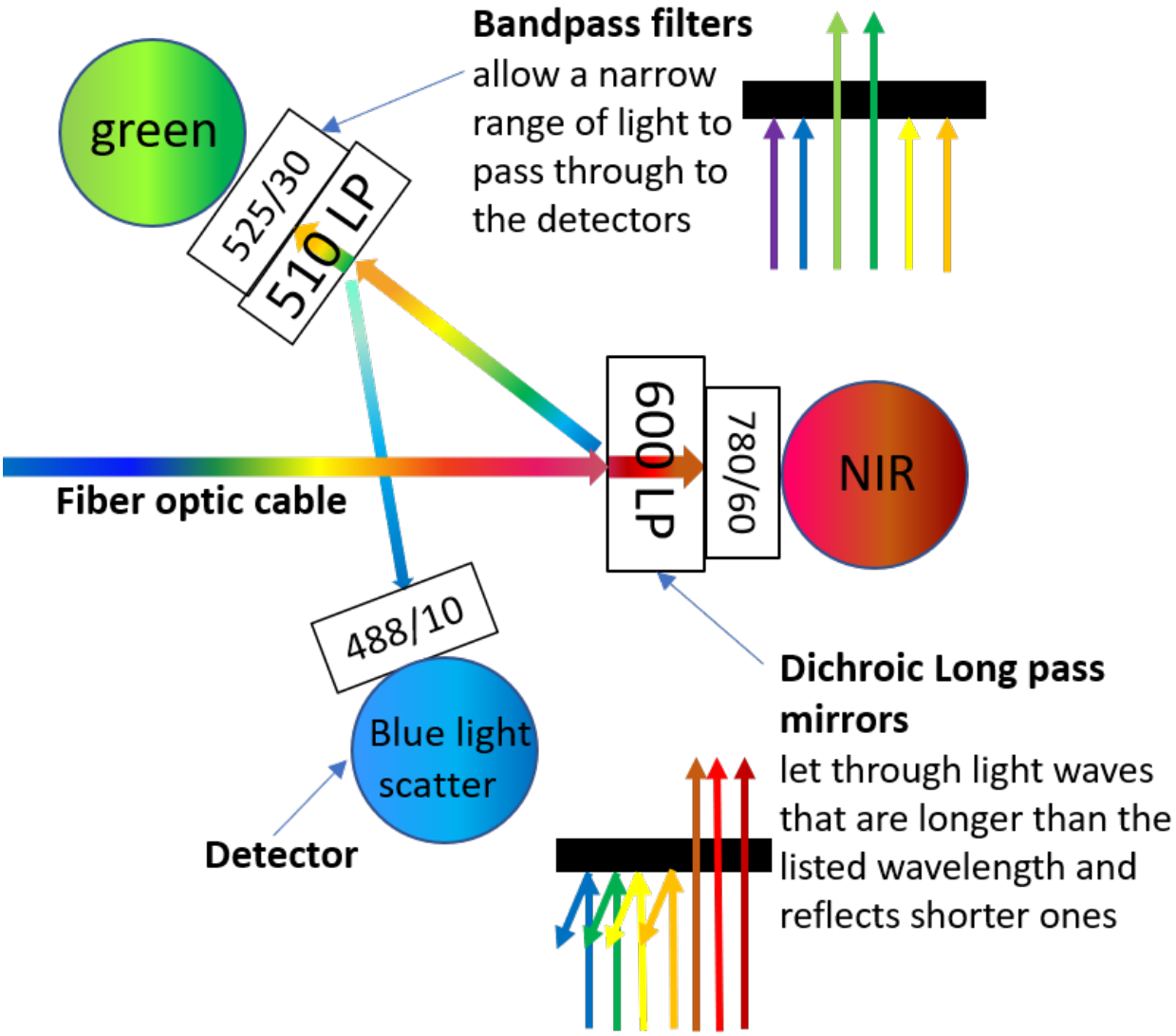


https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/fluorescence/filters/

18. Fluorescence Detection in Conventional Cytometers

Traditional flow cytometers at the Melbourne Cytometry Platform including the BD FACS Aria III, FACS Fusion, CantoII and LSR Fortessa assign each of their fluorescence detectors to measure unique fluorochromes around their emission maximum. In this one fluorochrome = one detector model, the number of fluorochromes that can be measured by a traditional cytometer matches the number of fluorescence detectors it possesses. The number of detectors (or channels) and fluorescent dyes traditional cytometers can accommodate varies according to instrument, model, and manufacturer.

Figure 15 Optical trigon in a FACS Aria III cell sorter



A typical trigon array found in a BD FACS Aria III cell sorter. It utilizes dichroic long-pass mirrors and bandpass filters to separate light through reflection and, transmission to optical detectors

19. Conventional Cytometer Fluorescence Detection (Newer Technology)

Technology in the optics space is fast evolving. Newer cytometers such as the CytoFLEX analysers available at the Dental School and Melbourne Brain Centre do away with dichroic mirrors that transmit light. In CytoFLEX instruments, light arriving at the detector array is sequentially reflected between bandpass filters by a mirror that efficiently reflects all wavelengths of light until a filter with a permissive wavelength bandwidth is encountered. Reflection of light is a much more efficient process than transmission, reducing photon loss and hence the sensitivity for very dim signals is enhanced.

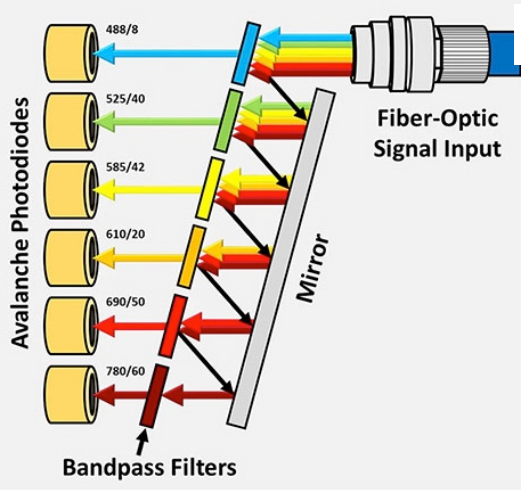


Figure 16 Optical configuration in a CytoFLEX analyser

Brittain GC 4th et.al. (2019) A Novel Semiconductor-Based Flow Cytometer with Enhanced Light-Scatter Sensitivity for the Analysis of Biological Nanoparticles. Sci Rep 9(1): 16039

20. Spectral cytometer fluorescence detection

While conventional cytometers measure a flurochrome's abundance via its maximum emission in 1 detector from its primary laser excitation line, spectral cytometers (Cytek Aurora 5L available at the Doherty Institute flow cytometry node) detect a fluorochrome's entire light emission spectrum across every detector associated with each laser. The recording of the light intensity across each detector of each laser results in a spectral 'trace'. Emission peak at a certain detector is still recorded for each fluor and this determines the fluor's overall intensity (bright fluor versus weak fluor).

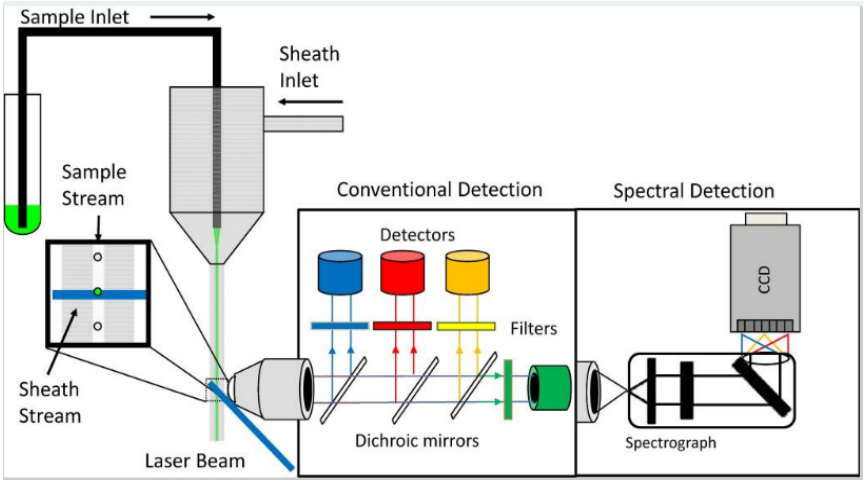
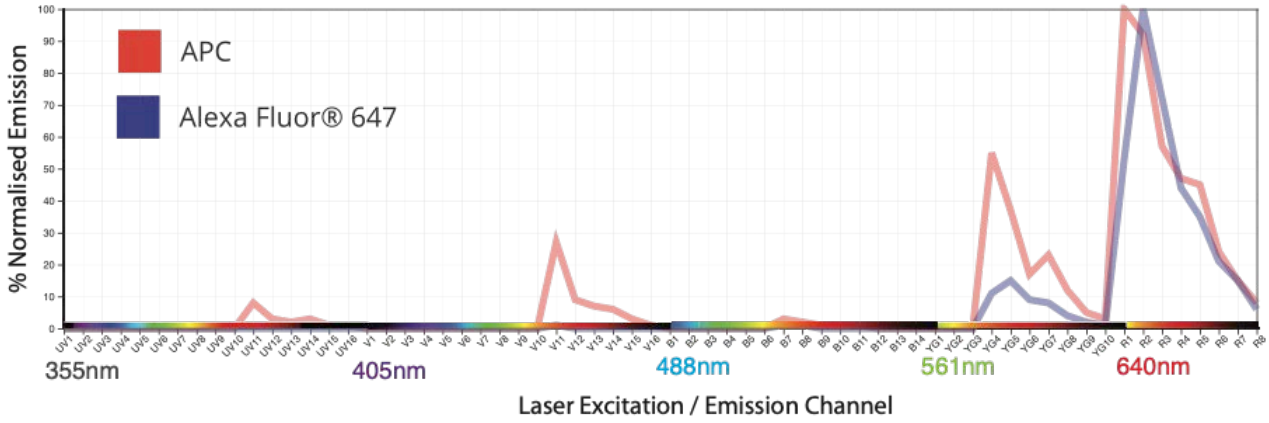


Figure 17

Schematic overview of a conventional or spectral detection. Nolan JP, Condello D. Spectral flow cytometry. *Curr Protoc Cytom*. 2013;Chapter 1:Unit1.27.

For example, APC produces maximal emission at 660nm when excited by a red laser. So, in a conventional cytometer, the amount of APC signal is based on measurements made at the red 660/20 detector. In a spectral cytometer APC's emission peak still identified within the red-excitation detectors, but the APC emission is measured across all the lasers (ultraviolet, violet, blue, yellow/green and red) and all associated detectors. This produces a fluorescence 'trace' unique to APC (or whichever fluorescent molecule is being measured) allowing a spectral cytometer to distinguish between fluorochromes with similar or even identical maximum emission wavelengths, such as APC and Alexa Fluor 647 (AF647). Both APC and AF647 are detected by the red 660/20 detector on a conventional cytometer and thus you cannot use them together in an immunophenotyping panel. With a spectral cytometer such as the Aurora, you can distinguish between APC and AF647 and thus use them in the same panel because, in spite of their close emission maximum, their fluorescence profile signatures when read across all detectors and lasers, is unique. Below is the emission spectra for APC (red) and AF647 (blue) on a 5-laser Cytek Aurora spectral cytometer. Emission is recorded across 16 UV laser detectors, 16 violet laser detectors, 14 blue laser detectors, 10 yellow/green laser detectors and 8 red laser detectors. Note the unique fingerprint across the non-red detectors, and even the differences in emissions within the red detectors.

Figure 18 Spectral "trace" of APC and Alexa Fluor 647

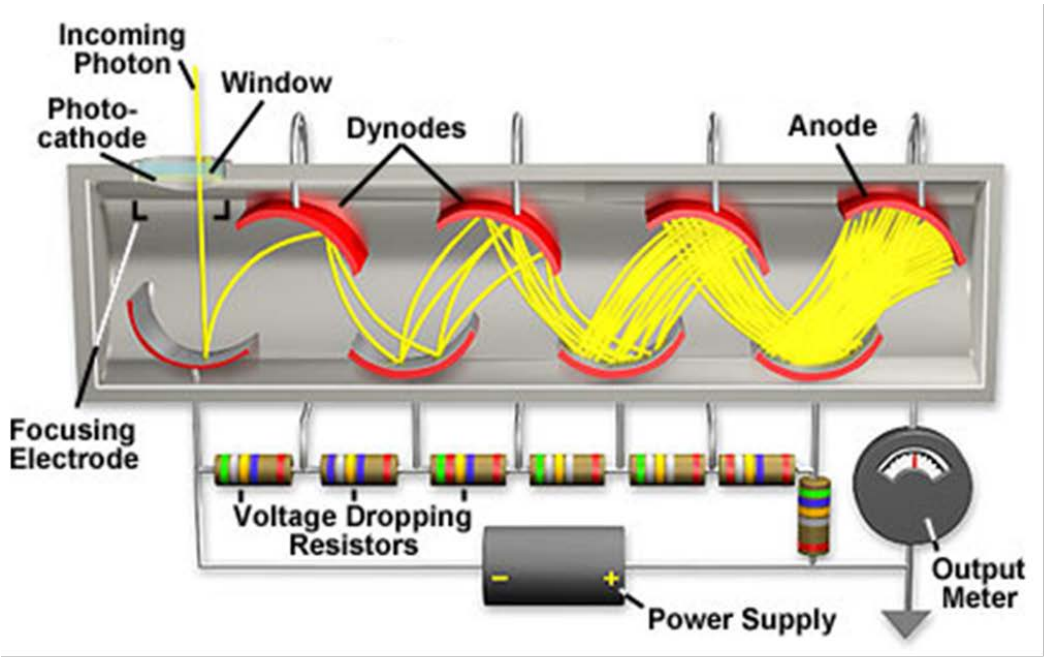


Be aware though, that some fluorescent molecules that have different names are indistinguishable in emission trace. For example, Alexa Fluor 488, FITC, VioBright FITC, Zombie Green and Live/Dead Green all have the same trace so cannot be used together in a panel on a spectral cytometer. However, BB515 and any of the above fluors can be used in combination because BB515 has a spectral trace that is sufficiently different. On a conventional cytometer, these would all be excited and detected by blue laser, 530/30 detector.

21. Signal processing

When light hits a photodetector a small current (a few microamperes) is generated. Its associated voltage has an amplitude proportional to the total number of light photons received by the detector. This voltage is amplified by a series of linear or logarithmic amplifiers, and by analogue to digital convertors (ADCs), into electrical signals large enough (5–10 volts) to be plotted graphically.
Log amplification is normally used for fluorescence studies because it expands weak signals and compresses strong signals, resulting in a distribution of large dynamic range of fluorescence intensity distributions that is easy to display on a histogram. Linear scaling is preferable whenever signal distributions have low dynamic range, e.g. in cell cycle analysis.
The measurement from each detector is referred to as a 'parameter' e.g. forward scatter, side scatter or fluorescence (1..n). Every detectable particle and its associated parameter values is known as an 'event'.

Figure 19 Signal amplification in a photomultiplier tube and what an actual PMT looks like



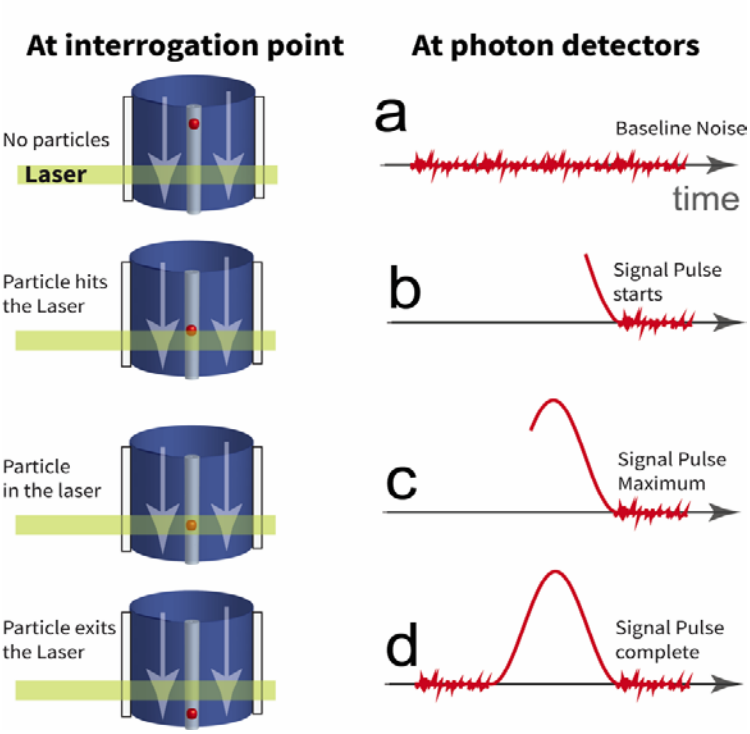
https://micro.magnet.fsu.edu/primer/digitalimaging/concepts/photomultipliers.html

In a PMT (the most often used detector), photons strike a photocathode and electrons are released a discharged towards a dynode. There is an exponentially increasing cascade of electrons that move toward a photoanode that produces a measurable photocurrent (signal)

22. Signal Pulses: Height, Width and Area

Fluorescence and scatter signals are collected as electric pulses, generated at the detectors as soon as a particle hits the lasers. Several properties of these pulses are captured and stored as part of the attributes of a particle's associated signal. For instance, a FSC pulse can be described in terms of:

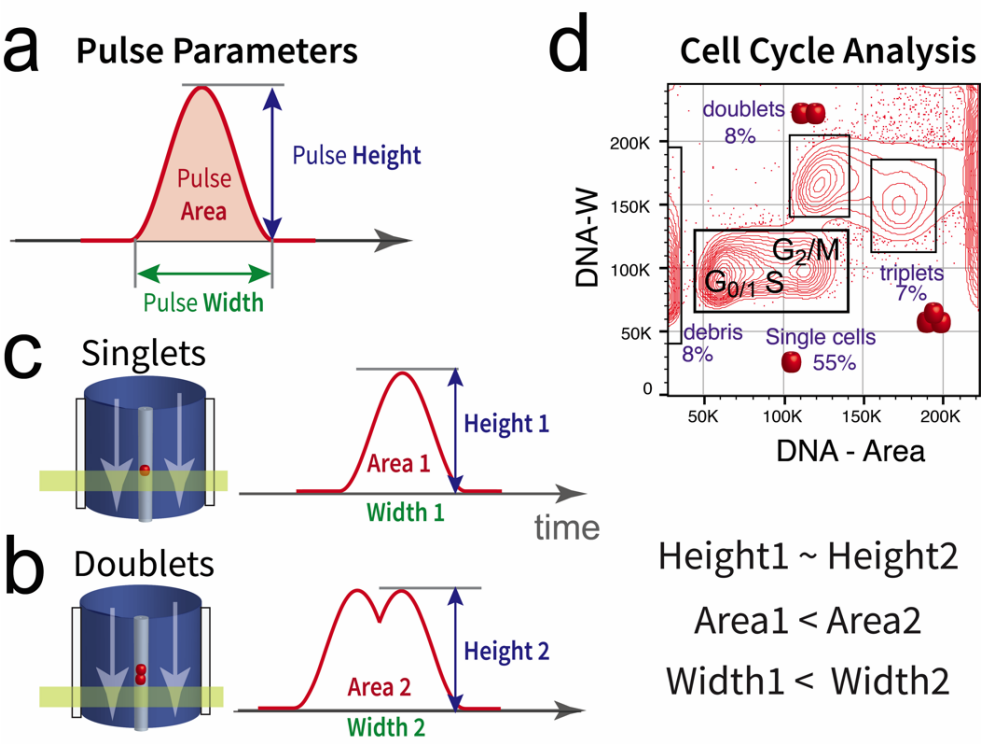
Figure 20 Pulse generation



Height (H-FSC), or the difference between the maximum FSC signal scored by a pulse as a particle crosses a laser and the baseline noise between pulses; Area (A-FSC), approximates the total scatter signal (~photons) gathered during the laser interrogation; Width (W-FSC), or a measure of the pulse duration (Time of flight). For instruments with constant flow velocity, and fixed dimensions of the laser beam spot, the width of a pulse is a function of the particle length/size.

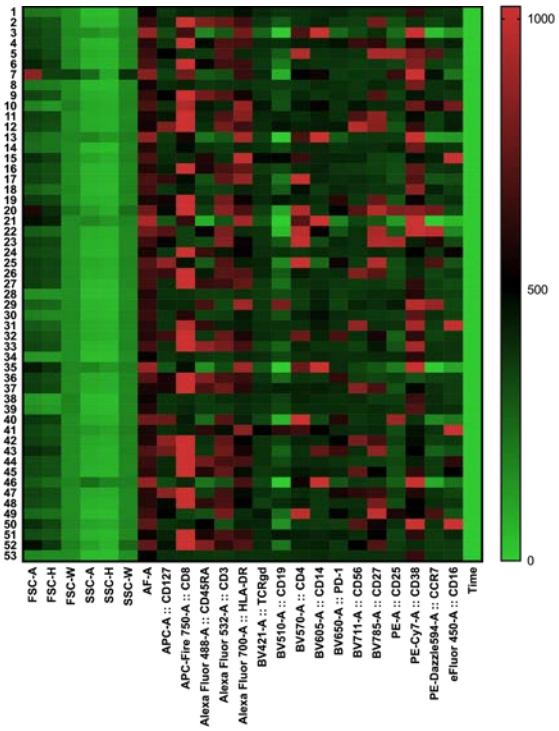
The evaluation of Height, Area and Width values for pulses associated with a given parameter have many uses in Cytometry. The most frequent application is the distinction between single particles and aggregates as a preliminary step during data analysis and during cell cycle analysis. Although cells, depending on their cell cycle stage, differentiation or activation status, will show variations in scatter Height, Width and Area and values, these will be lower than the values associated with doublets and n-particle aggregates. In cell cycle analysis based on DNA-bound fluorescence, it would be impossible to distinguish, in terms of total DNA content (Fluorescence pulse Area), single cells in G2/M from aggregates consisting of 2 cells in G0/G1 (both have the same total amount of Fluorescence-tagged DNA or Pulse Area). These two cases can however be resolved via the analysis of DNA fluorescence pulse Width, that will be larger in G0/G1 doublets than in single G2/M cells.

Figure 21 Pulse measurements



23. Cytometry Data Files and Data Visualisation

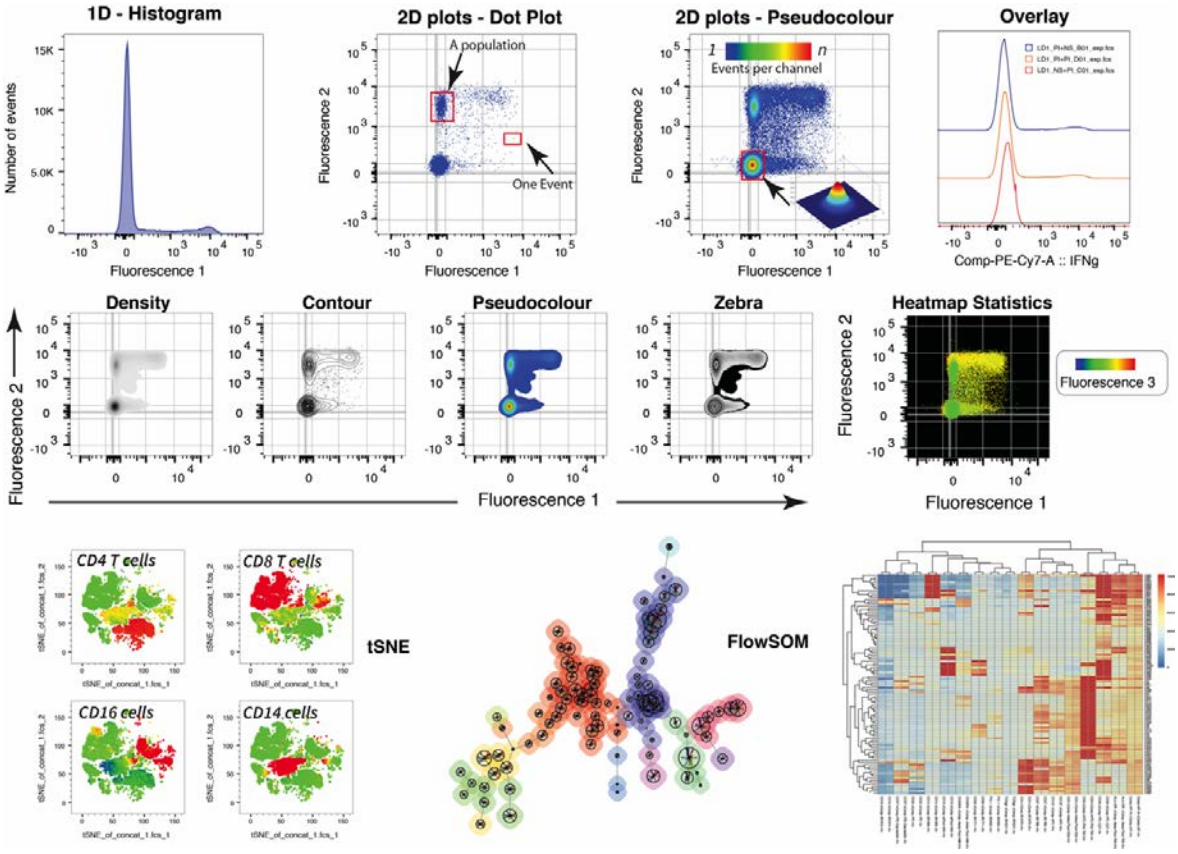
Figure 22 FCS file information



Data files generated by all cytometers are known as FCS files (Flow Cytometry Standard), an array of all parameter (column) intensities associated with each measured event (rows), in addition to ASCII text string keywords and metadata headings listing information on the instrument, the instrument QC/QA status, the detector gains or voltages, date for the acquisition, acquisition beginning and end time, etc. FCS files can be analysed using instrument embedded proprietary software used during the acquisition of samples (FACS Diva, Summit, CytExpert, SpectroFlo), or post-acquisition with advanced software like FCS Express and Flowjo, both of these available at competitive prices to all researchers at the University of Melbourne via Site licenses. FCS files can also be analysed with software packages like R and MATLAb. Data can be visualised as histograms, two dimensional plots of different formats, 3D plots and Heatmaps. Advanced

visualisation tools allow you to reduce all measured markers into two dimensions (tSNE), enable the thorough definition of cellular subsets or clusters (FlowSOM) in highly complex multicolour panel studies.

Figure 23 Data visualisation plots



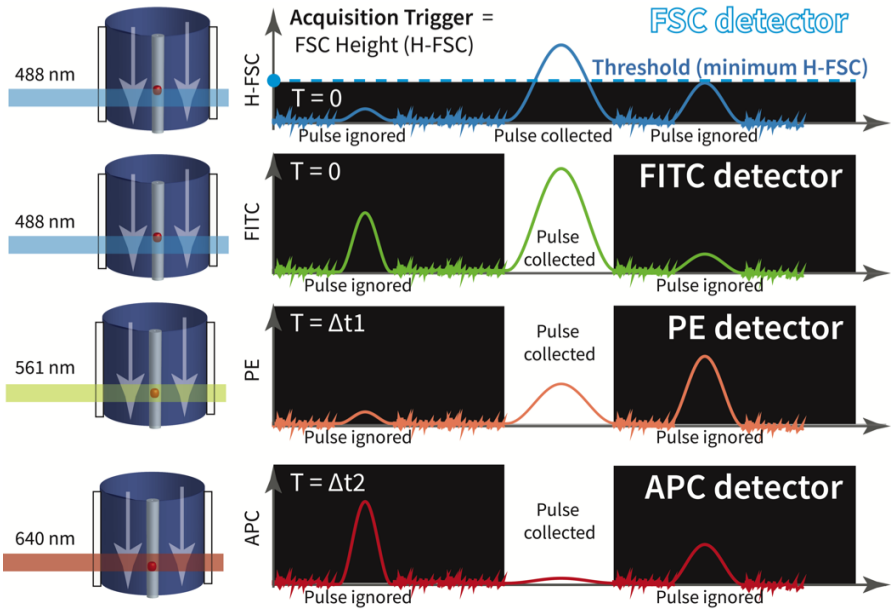
24. Acquisition: Trigger and Threshold

Not all pulses generated at the laser interrogation point make it into the FCS file, nor you would like this to be the case! Due to the pulse processing speed limit associated with each cytometer's electronics, we need to ensure that debris and other suspended contaminants are excluded from the FCS file right at the point of acquisition so as not to saturate the electronics or end up with massively large FCS files.

Blinding the electronics to unimportant events is achieved via acquisition Triggers and signal Thresholds. Typically, one trigger parameter is chosen to define a signal intensity level (Threshold) above which events will be deemed relevant for visualisation, analysis and storage. Due to their generic nature, FSC or SSC are usually chosen as trigger parameters. Scatter threshold values are typically defined empirically in combination with detector gain adjustments and as part of the

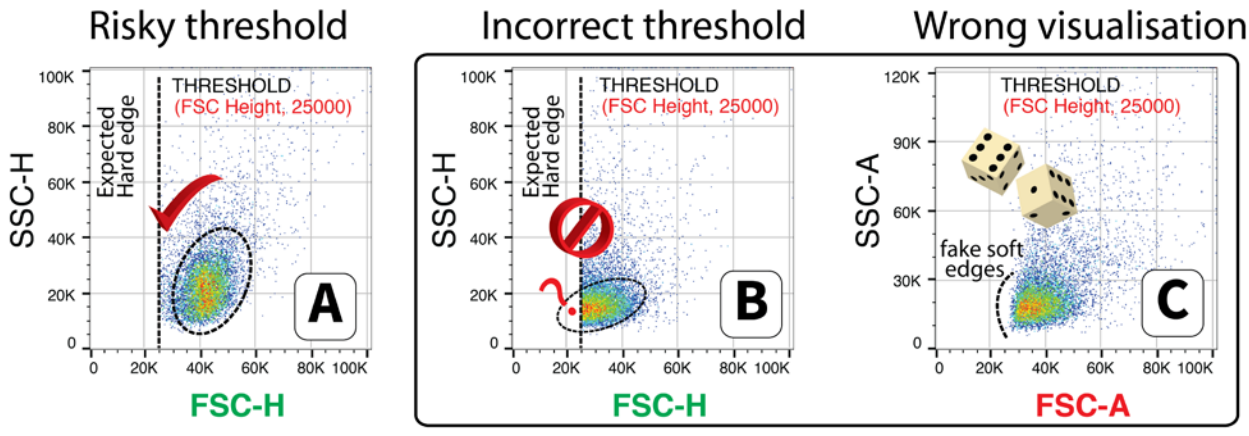
preliminary analysis required during acquisition setup. Applying safe and correct threshold values is critical, since this will define which events are captured and saved, vs. which ones won't. Only the events that fall over the defined threshold are visualised, acquired and saved!

Figure 24 Various parameter acquisition triggers



Cell scatter is affected by sample handling, membrane integrity, proliferation status and viability, so you must define a threshold that will accommodate all your experiment's samples: unstimulated vs. stimulated, differentiated vs. undifferentiated. A threshold value good enough for some samples (A), could prevent the full visualisation of the scatter distribution of populations of interest in others (B). Also, if your trigger parameter is FSC Height, you must evaluate the effect of threshold on rendered scatter via FSC-Height (B, with expected hard edge set at 25,000), not FSC Area (C, fake soft edge boundaries). Height is the true measure of a pulse's signal. Pulse Area in most digital instruments is approximately calculated based on Height, "Area scaling" factors and pulse width values.

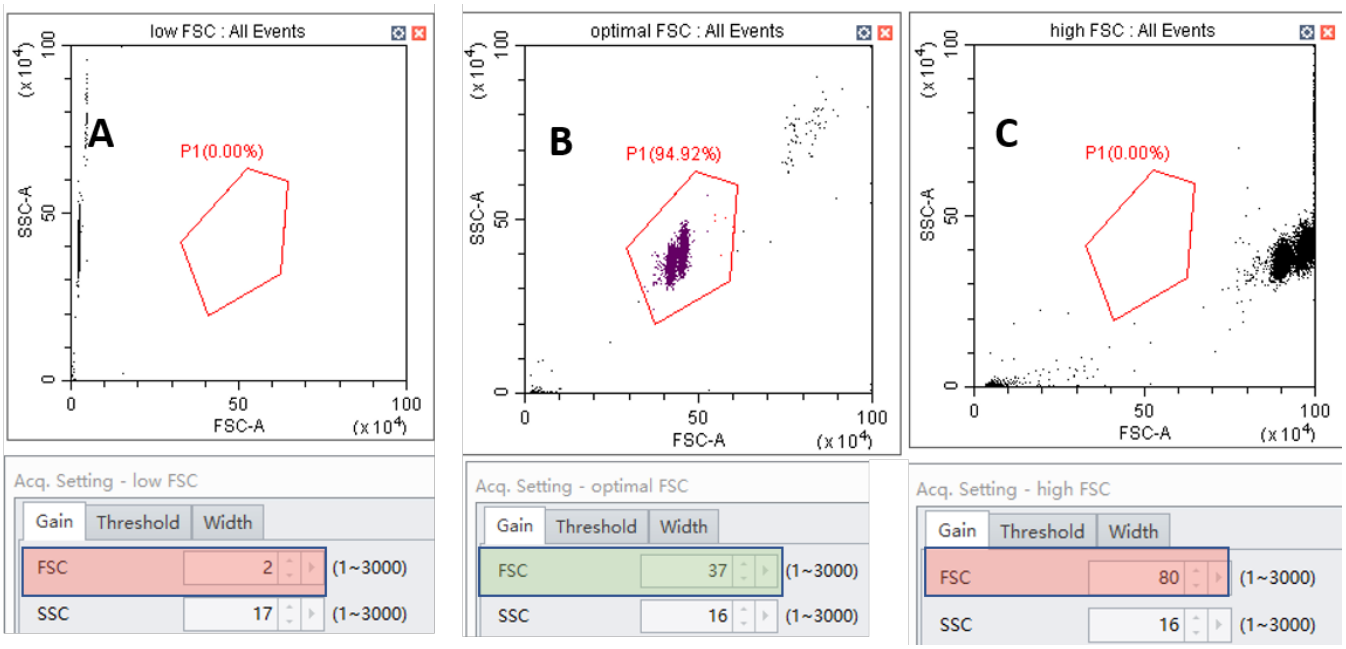
Figure 25 Threshold cautions



25. Setting Gains (Voltages) – a practical example

Gain (or voltage) is the amount of power applied to and required for a detector to resolve the population of interest from background (electronic) noise. Gain values also have an impact in the dynamic range and the resolution of the signals collected at each detector and as such must be adjusted by the user *while the sample is running* for every scatter and fluorescence detector as part of the initial acquisition setup. Figure 26 shows the effect of different CytoFLEX FSC gain settings on the visualisation of single polystyrene beads, sample debris and particle doublets.

Figure 26 Demonstrating "Gain" or "voltage" using forward scatter (FSC) measurement

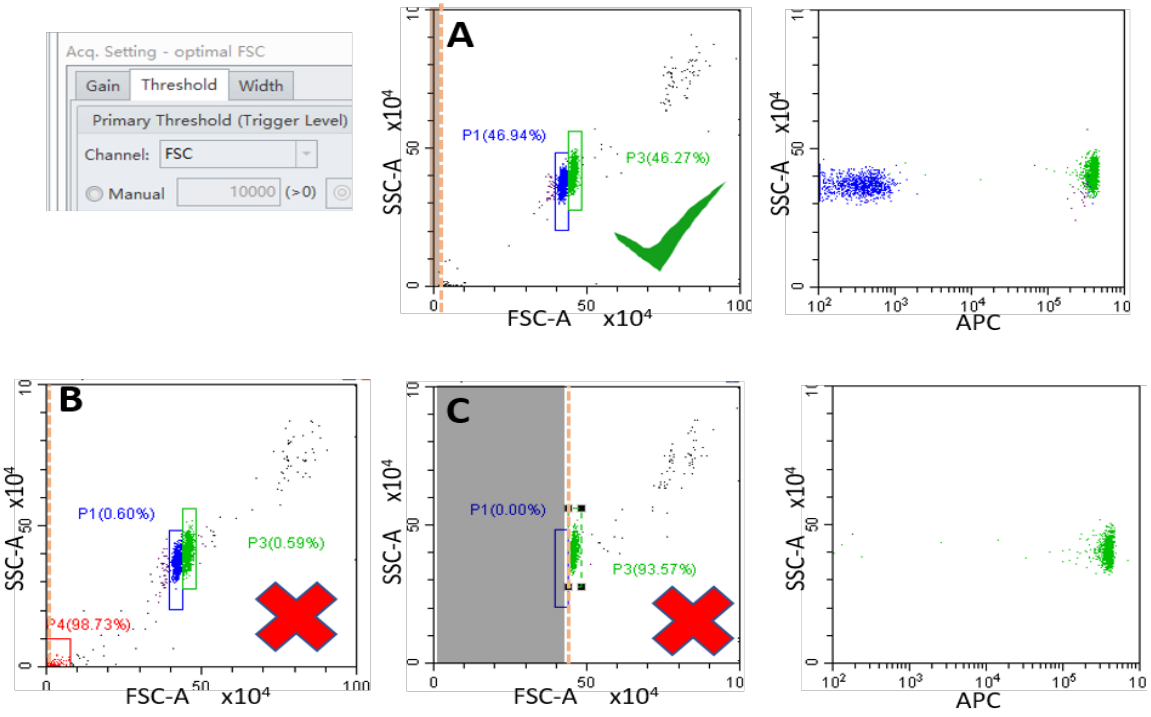


A. Too little power is applied to the FSC detector (gain = 2). The main bead population is squashed against the left-hand y-axis, and the sample shows very little detail.
B. A higher gain is applied to the FSC detector (gain = 37). The polystyrene bead population (within the red region) is placed in the centre of the plot and is well resolved from debris (bottom left-hand corner) and from aggregates (top right). This FSC gain of 37 (or gains aground this value) could be classified as optimal, since it reveals relevant details and subsets within the sample FSC scatter profile.
C. Too much FSC gain is applied (gain = 80) and the main population of beads is now against the right-hand axis. Debris in the bottom left-hand corner is more resolved.
Every time you setup an experiment for the first time you'll need to reserve a few minutes for some initial real time evaluation of the effect of gains on the visualization of your scatter and fluorescence signals. Are all relevant populations visualized? Are my plots encompassing both high and low signals? Am I allowing too much debris to make it into my .fcs files?"

26. Setting Thresholds – a practical example

"Thresholds" can be applied to any channel, including fluorescence to ignore events/particles that do not meet a scatter, size or fluorescence minimum. They are most often (default) set in the triggering FSC detectors to eliminate unwanted small particles (such as debris or red blood cells) that can interfere with collection of meaningful data. When analysing small particles and bacteria, trigger and threshold are set on SSC either from the blue (488nm) or -in instruments that have this feature- from the violet laser (405 nm, which is best than 488 nm in small particle scatter detection sensitivity). Like gains, thresholds must be set in real time *while the sample is running*.

Figure 27 Demonstrating "Threshold" using forward scatter (FSC) channel



A. Polystyrene beads (a mixture of unstained (blue) and APC+ (green)) are run at an optimal FSC gain defined in Figure 26 (gain = 37) at a default threshold setting of 10,000. On the FSC x-axis, the blue and green beads are sitting between 400,000-500,000, and collectively make up 93% of all events in the plot. At 10,000 FSC threshold (orange dotted line), there is a small amount of visible debris in the bottom left-hand corner.

B. FSC threshold has been now reduced to 1,000. The green and blue beads are still visible but now make up for just ~1% of total events, because the instrument now detects debris and noise that was hidden when the FSC threshold was higher. This threshold is suboptimal, since 98% of your FCS file events will be debris without analytical use, with the files becoming extremely large for this reason.

C. The threshold has been set at 450,000 (orange dotted line). Everything that falls to the left of the orange dotted line is ignored (represented as a greyed-out box). While the debris is no longer visible, neither is the blue population of beads that were sitting at around 400,000 on the x-axis. The green (APC+) beads are still visible because those polystyrene beads are slightly larger (460,000) than the unstained (blue) beads. This is an extreme example to demonstrate that while thresholds are extremely useful to eliminate unwanted small signals, caution must be exercised not to lose data by setting thresholds too high or too close to important event clusters. Always remember that what falls under the threshold value is altogether ignored by the instrument electronics and can't be recovered. A wrongly defined threshold can bias your analysis and render the whole experiment useless."

Gains and threshold values for a trigger parameter work in combination and are defined together as part of the initial instrument setup. A good threshold X for a detector gain value of 10 may be too little if the gain value is increased to 100. Whereas gains impact on signal visualization and dynamic range, the threshold allows you to blind the instrument to the lower end of the signal spectrum, containing meaningless debris, for instance. Adjustment in gain can be followed by adjustments to threshold until a combination of both best fit your samples.

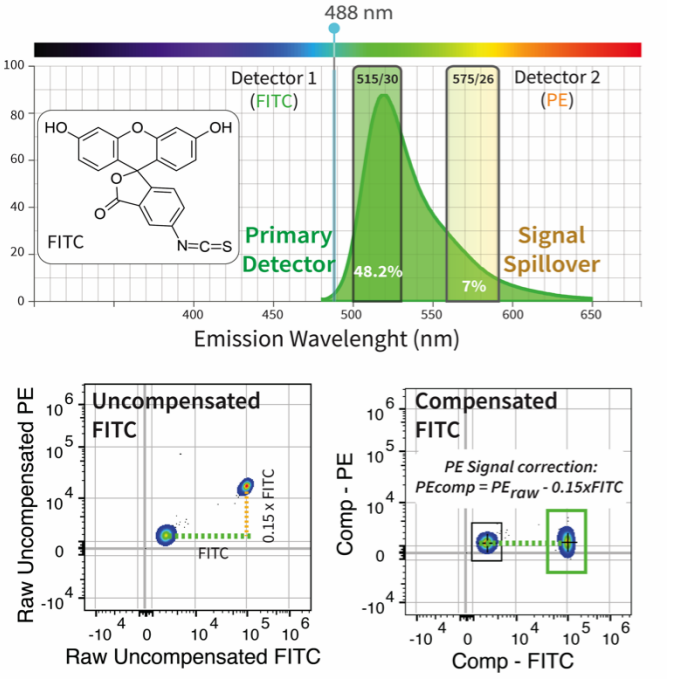
27. Fluorescence Compensation

When two or more fluorochromes are used in combination, chances are that their emission fluorescence spectra will overlap. Due to the broad emission typical of most fluorochromes, some of the fluorescence photons of let's say fluor A will spill into fluor B primary detector and vice versa. If these spillover contributions were not corrected, signals acquired at each fluorescence detector will in most cases be a mix of primary dye photons and spillover fluorescence out of the overlapping emission from other dyes in the panel. Although this can be avoided by using fluorochromes at very different ends of the spectrum e.g. BV421 and APC, this choice is not always practical or possible. All traditional cytometers correct detector measurements from unwanted fluorescence spillover via a process called compensation.

Compensation can be applied right before sample acquisition or during post acquisition data analysis in order to remove unwanted fluorescence spillover and thus isolate pure fluorescence specific signal components. The process requires the acquisition of unstained and single colour stained sample controls, which are used as references in the estimation of the degree of signal interference contributed by each individual fluorochrome emission spectrum as it spills into non-primary detectors.

During compensation, unwanted spillover is mathematically subtracted from the total particle signal measured at each non-primary detector until single colour population median matches the median value of unstained cells carrying the autofluorescence baseline. Modern flow cytometry analytical software applies fluorescence compensation mathematics automatically, which simplifies matters considerably. Due to their importance, we will be focusing on sample control requirements for a successful compensation later in this document **Single-Colour Controls (Conventional Cytometers)**.

Figure 28 Compensation: FITC out of PE channel

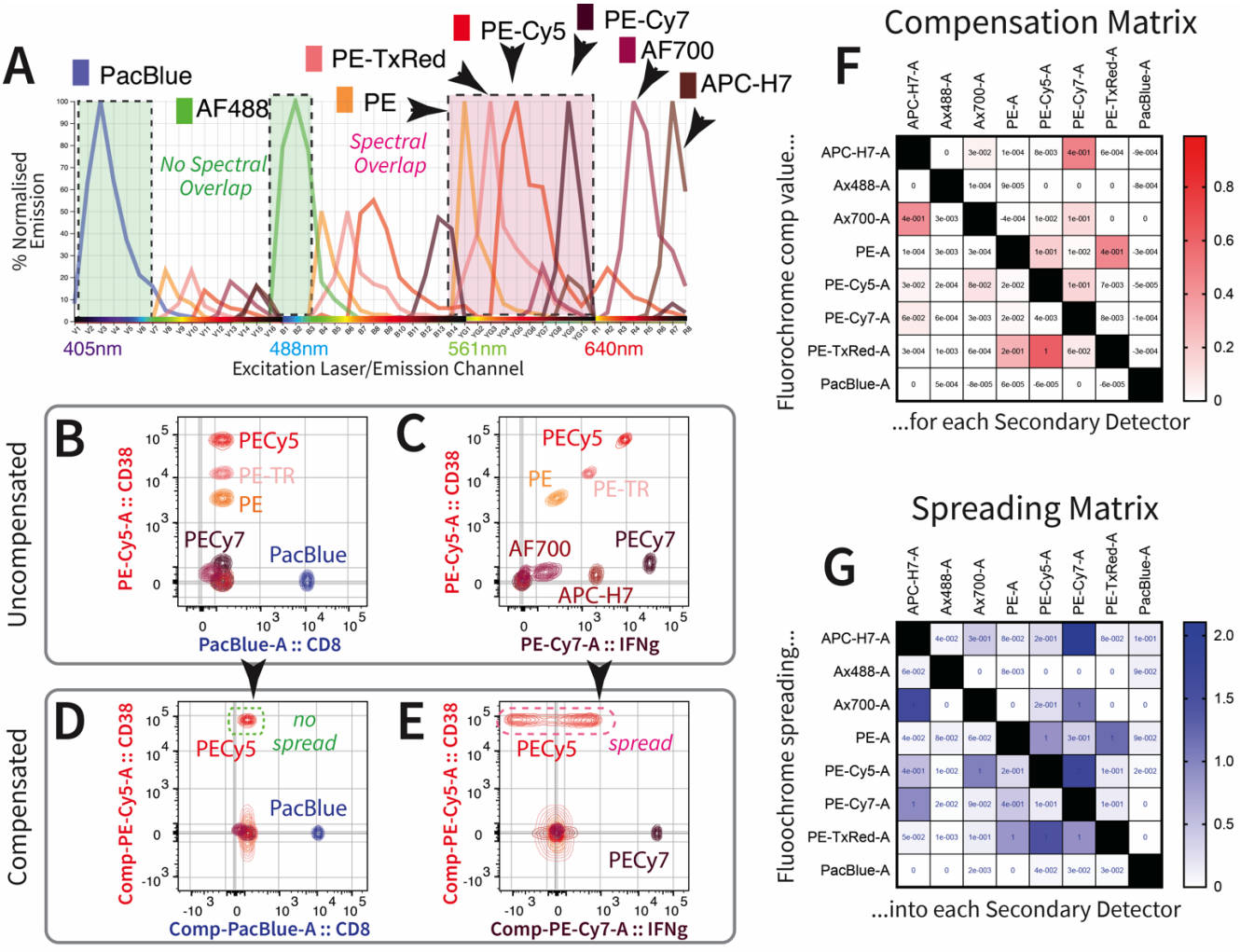


28. Fluorescence Compensation in Multi-Colour Panels

As the number of measured fluorochromes per sample increases, so does the complexity of the signals gathered at each detector as a result of emission spectra overlap. Figure 29 shows an example of one of such cases, a small panel of 8 fluorochromes including spectrally non-overlapping dyes such as Pacific Blue and FITC (A), and overlapping dyes including PE, PE tandem dyes (PE-Texas Red, PE-Cy5 and PE-Cy7), Alexa Fluor 700 and APC-H7. In the absence of compensation, spectral overlap leads to substantial fluorescence spillover into non primary detectors (See Plot B, C). Multicolour panel compensation removes this spillover to guarantee only pure fluorochrome signals are associated with each primary detector (D, E). Compensation is summarised via matrixes (F) showing comp values for all panel fluorochromes (rows) at each non-primary detector (columns).

One of the drawbacks of compensation is the increase in signal spreading around compensated negative values (E). This spreading correlates with spectral overlap, antigen abundance, fluorochrome brightness and detector gains (these increase signal intensity and variance in primary and non-primary detectors). Spreading can be observed for instance after compensating PE-Cy5+ single colour populations (bright signal) out of the heavily overlapping PE-Cy7 detector (Plot E). Bright PE-Cy5+ events when compensated show a noticeable increase in dispersion around the baseline PE-Cy7- channel values (PE-Cy7 channel median ~ Autofluorescence). This dispersion is far greater than what's observed in unstrained populations. In contrast, PE-Cy5 and Pacific Blue, with non-overlapping spectra, require minimum compensation and produce almost no spreading into each other's detector (D). Why is this important? Compensation-derived spreading can sometimes be so high as to compromise a detector's ability to resolve very dim signals from baseline dispersion or noise. Preventing spreading conflicts by carefully associating specific dyes with rare markers is a key strategy in multi-colour panel design. For instance, in the panel discussed above, using PE-Cy7 for a rare marker A that will be co-expressed with an abundant marker B labelled with PE-Cy5 should be avoided at all cost. PE-Cy7 will be best used to tag a marker C which is not co-expressed with the PE-Cy5 labelled B maker. We won't extend any further into panel design here, just to mention that several offline software packages generate spreading matrixes (G), which are extremely useful references to best plan your multi-colour panels.

Figure 29 Compensation considerations in multi-colour panels



29. Data Analysis: Gates and Regions

An important tool in flow cytometry data analysis called 'gating' allows you to selectively isolate cellular subsets or combinations of interest while eliminating unwanted events e.g. dead cells, debris, cell aggregates and artefacts. Gating also allows the use of Boolean logic (and, or, not) gating rules. In a simple gating strategy as displayed below, cells can be gated according to physical and biological characteristics in 2D data plots (shown) and histograms. Subcellular debris and clumps can be distinguished from single cells by their distinct scatter profiles and intensities or the relationships between signal Height, Area and width values for pulses recorded at a given scatter detector. Necrotic cells for instance, have lower forward scatter and higher side scatter than living cells. Dead cells can be distinguished from live cells via Live/Dead discrimination dyes. Leukocytes can be resolved from erythrocytes for subsequent subset analysis via the expression of CD45 pan- leukocyte surface marker and so on.

Figure 30 Acquired data: ungated versus and sequentially gated

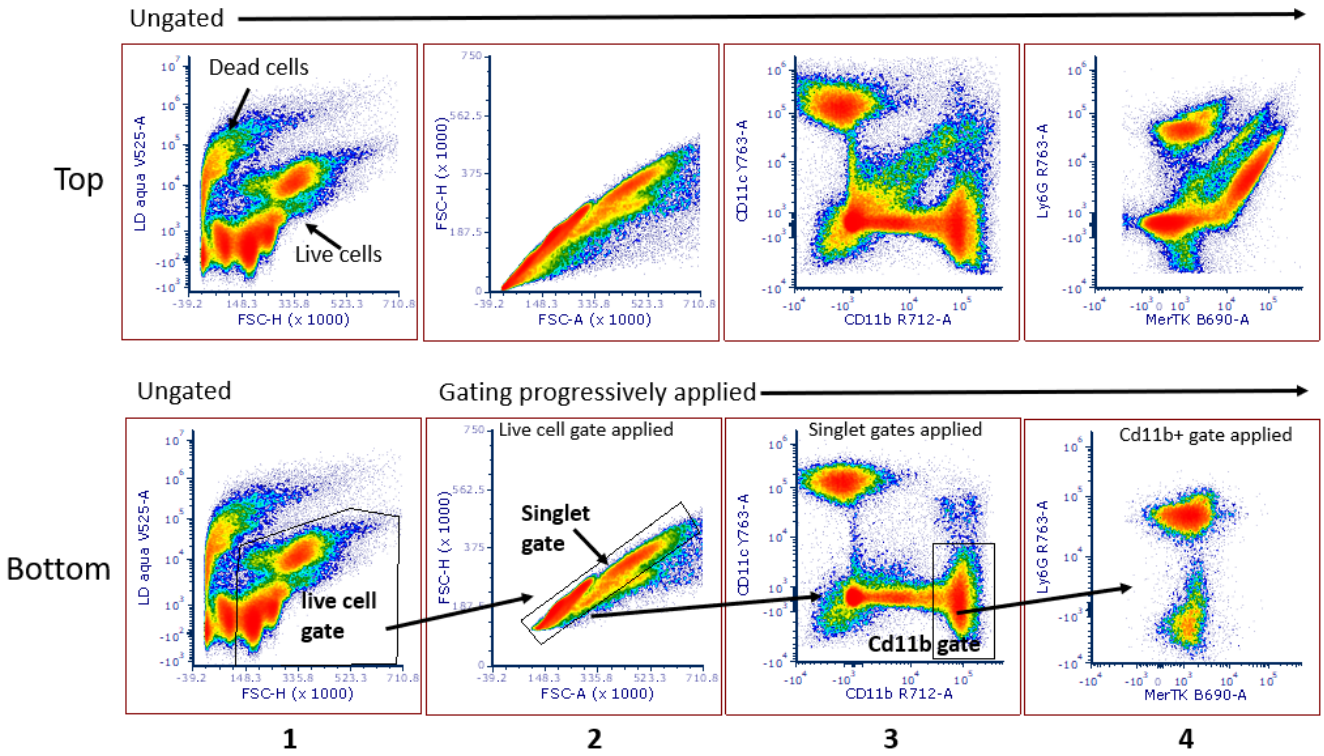


Figure 30 shows the same acquisition file across different measurements. Plots at the top show all acquired events ungated. The same plots are shown on the bottom row, this time with sequential gating been applied:

Plot 1. FSC (x-axis) vs live/dead marker (y-axis). A "live cell gate" region. simultaneously excludes dead cells (FSC low, LD aqua high) and cellular debris (low FSC, LD aqua negative)

Plot 2. FSC-A (x-axis) vs FSC-H (y-axis). Data here shows only events contained in the above defined "live cells gate". The bottom-left-hand corner events are no longer there on the bottom plot compared to the top plot. A new "Singlet gate" region is drawn around single cells, excluding aggregates from further analysis.

Plot 3. CD11b-AF700 (x-axis) vs CD11c-PE-Cy7 (y-axis). The lower plot shows only live single cells (only events which are simultaneously inside the "live cell" and Singlet gates). Compare to top plot 3 where no gating has been applied. Draw a gate around CD11b+/CD11c- events (CD11b gate).

Plot 4. MerTK-PerCPCy5.5 (x-axis) vs Ly6G-APC-Cy7 (y-axis) and only events in the CD11b gate (Live & Singlet & CD11b+/CD11c- cells). See 2 distinct populations along the y-axis. Compare to top plot where no gating has been applied.

30. Back-gating

Gating strategies aim to sequentially isolate populations of interest in an orderly and logical fashion, from broader properties such as scatter > singlets > viability, followed by general pan-markers (like CD45+ for leukocytes), down to specific, sometimes rare subpopulations (CD3+ CD4+ IFNg+ cells).

But what can you do when you have no idea of the scatter properties for your cells of interest? Or you are overwhelmed with random scatter from dead cells in tumour tissues or debris in dissociated, fixed solid organs? Or dealing with whole blood samples where the majority of events are red blood cells? In all these cases, the best strategy is to reverse the traditional gating order and start your analysis looking first at specific fluorescence markers. Once the particles of interested have been identified via specific fluorescence tags, you can apply regions around these fluorescence+ clusters and visualise where they fall in a scatter plot. A useful strategy is to use 'colour dot plots' when back-gating. In colour dot plots, events within a region are colour-coded and they can be easily visualised among non-gated events that usually appear black. It is then easy to alter scatter settings based on the coloured events as shown below.

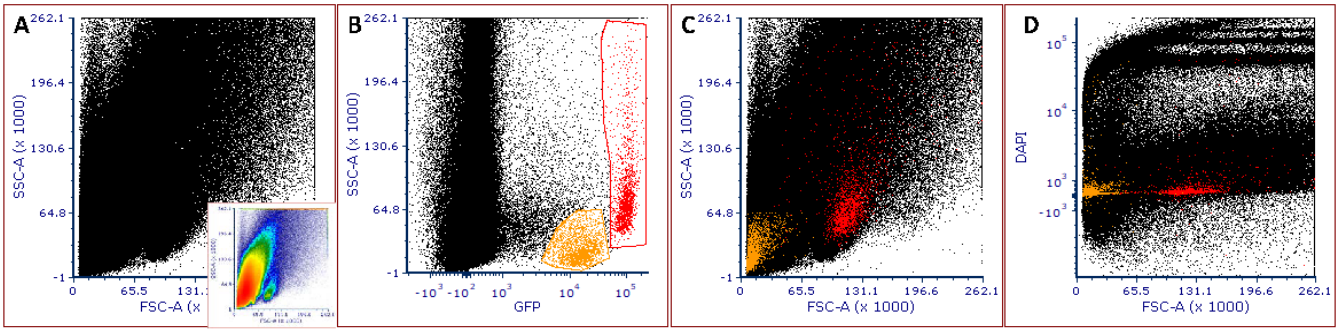


Figure 31: Back-gating example

Using fluorescence and live/dead back-gating to confirm scatter settings of GFP+ microglia in the eye

Solid tissues are made up of cells with various scatter profiles and it can be difficult to distinguish discrete populations of interest via scatter. Enzymatic and physical dissociation cause cellular damage and can produce a lot of debris, while incomplete digestion and extracellular matrix components give the appearance of very large scatter particles. Without a fluorescent tagged guide (live/dead or marker of interest), it is very difficult to orient yourself around the sample to determine the correct scatter settings for the cells of interest.

A, FSC vs SSC dot-plot (inset also is a pseudo-colour plot) of dissociated retina (eye) cells brought for cell sorting. The researcher was interested in collecting live GFP+ microglial cells.

B Acquisition software dot plots colour-code particles in specific gates. In this back-gating example, we used a dot-plot of GFP (x-axis) versus SSC (y-axis) and gated GFP+ particles. The GFP bright, higher SSC cluster was gated red and GFP dimmer lower SSC cluster was gated orange.

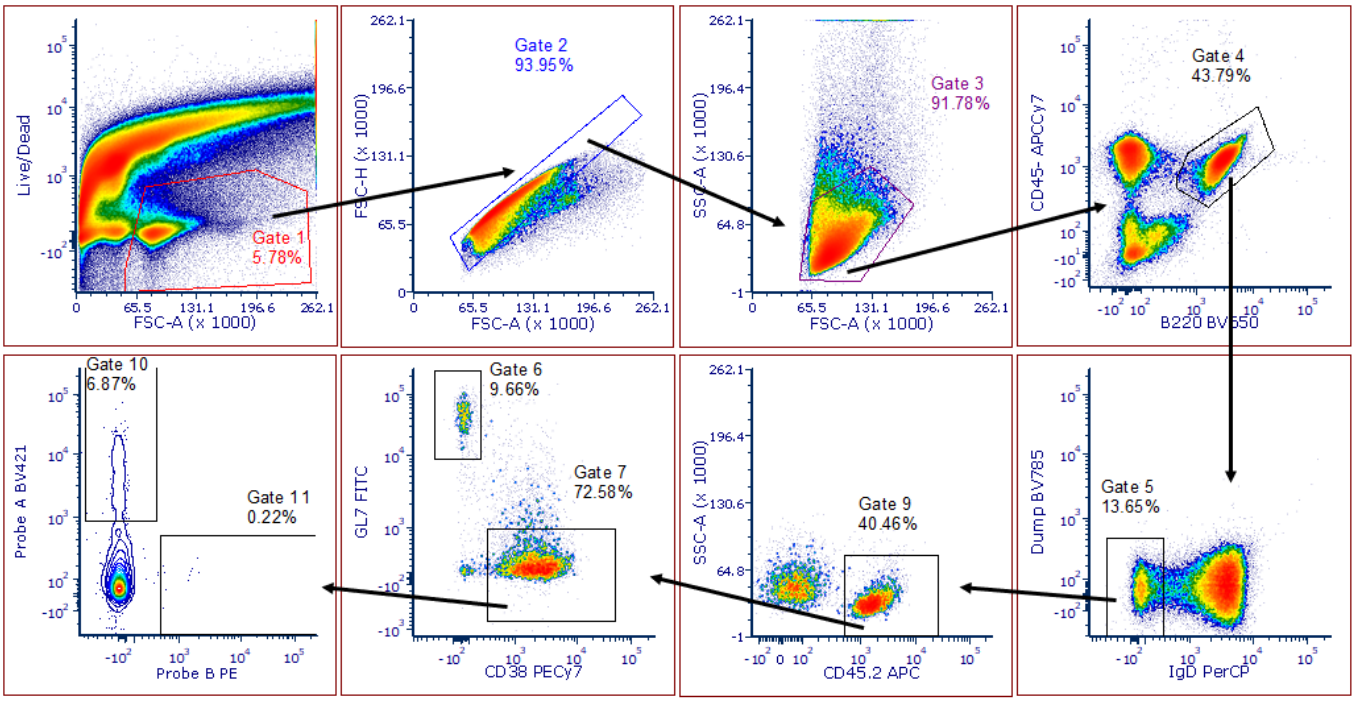
C The colour-coding showed the location of the red and orange cells in an FSC vs SSC plot, and we were able to adjust our scatter settings to more accurately resolve these cells by their physical properties. Interestingly, the orange cluster appeared smaller than the red cluster (tucked in at the bottom, left corner of the plot).

D Importantly, by back-gating onto an FSC vs live/dead (DAPI) plot, it became clear that the orange-coloured cluster were as small or smaller than DAPI+ dead cells and scattered within the debris field by size. Subsequent sorting followed by RNA extraction/ microscope visualisation of both the orange and red cell regions confirmed that the orange particles lacked a nucleus, and were instead, GFP+ cell fragments damaged during tissue dissociation.

31. Immunophenotyping

Cells express a variety of cell-surface and intracellular proteins, lipids and carbohydrate moieties, conferring functionality and reflecting maturation status. Flow cytometry is often used to identify and study discrete populations within heterogeneous mixtures, by exploiting these unique cell-surface and intracellular marker expression signatures, via a technique known as 'immunophenotyping'. Here is an example of immunophenotyping in a mouse lung using 10 fluorescent molecules - fluorophore-conjugated antibodies against cell-surface antigens and an amine-binding dye (to exclude dead cells) - to identify 3 discrete cell populations following a transplant experiment. Gating strategies were applied sequentially to narrow down to the populations of interest. Immunophenotyping is often used in discovery research or to compare frequencies of different cell types between experimental groups.

Figure 32 Immunophenotyping example



The labels on each axis show the name of each parameter (scatter or fluorescence). The fluorescent labels include the name of the antigen (such as IgD) and the name of the fluorochrome (such as PerCP)

32. Other applications for flow cytometry

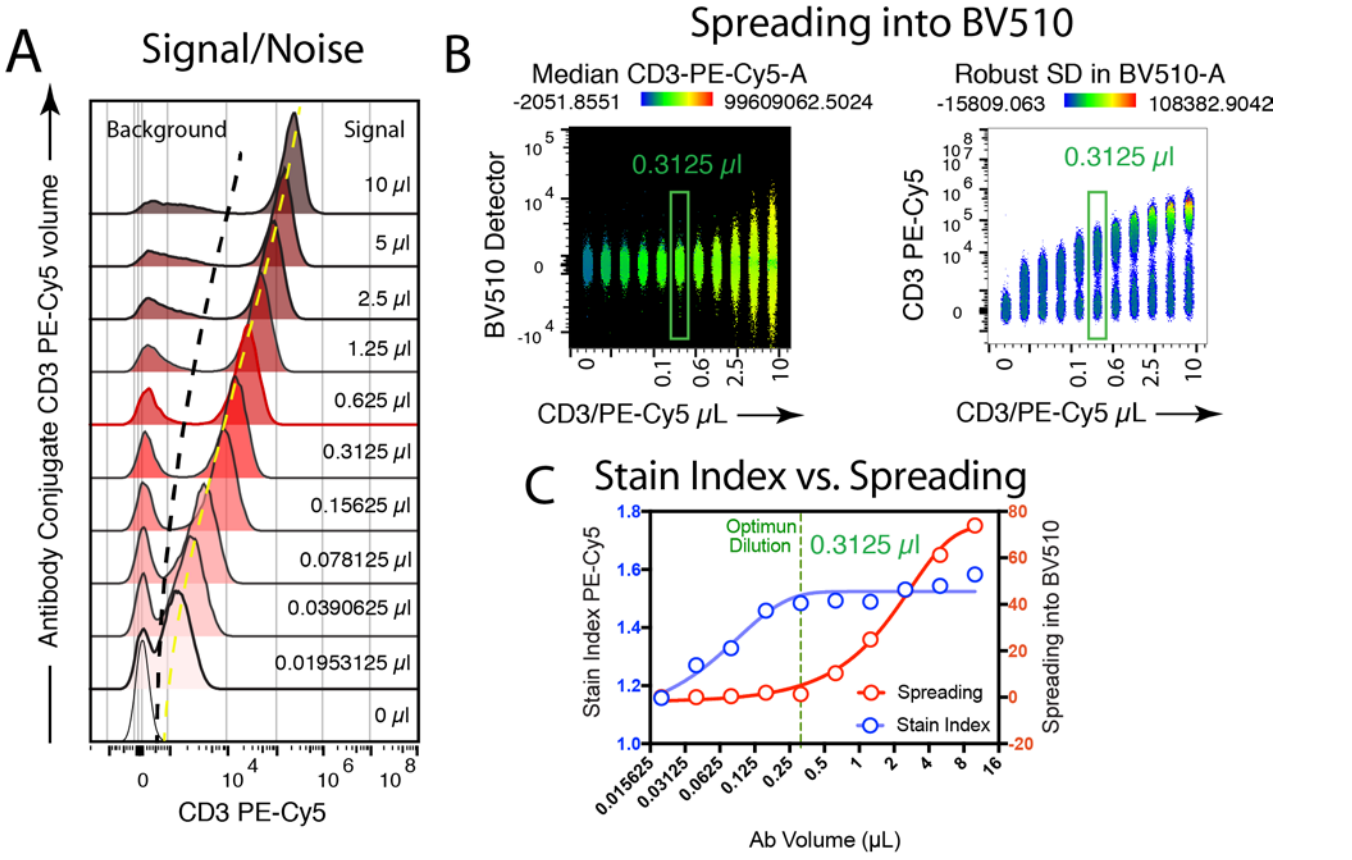
Flow cytometry is not limited to immunophenotyping. There are many assays that can be performed by flow cytometry including (but not limited to):

- Cell kinetics
    - Division/ generations
    - Metabolism
    - Calcium flux
- Intracellular antigens
- Protein expression (fluorescent proteins)
- Cell viability, membrane integrity
- Apoptosis
    - Plasma membrane changes (early)
    - Mitochondrial membrane depolarization using JC1 (early)
    - Caspase activation (early)
    - Chromatin condensation (late)
    - DNA fragmentation (late)
- Cell cycle
    - DNA content (combined with cyclins or histones)
- Protein-protein interaction (FRET)
- Cytokine expression (cytometric bead arrays)
    - Measure a variety of soluble and intracellular proteins (cytokines, chemokines, growth factors)
- Nanoparticles, bacteria, whole blood analysis

33. Optimisation: Titration, Saturation, Signal-to-Noise and Spreading

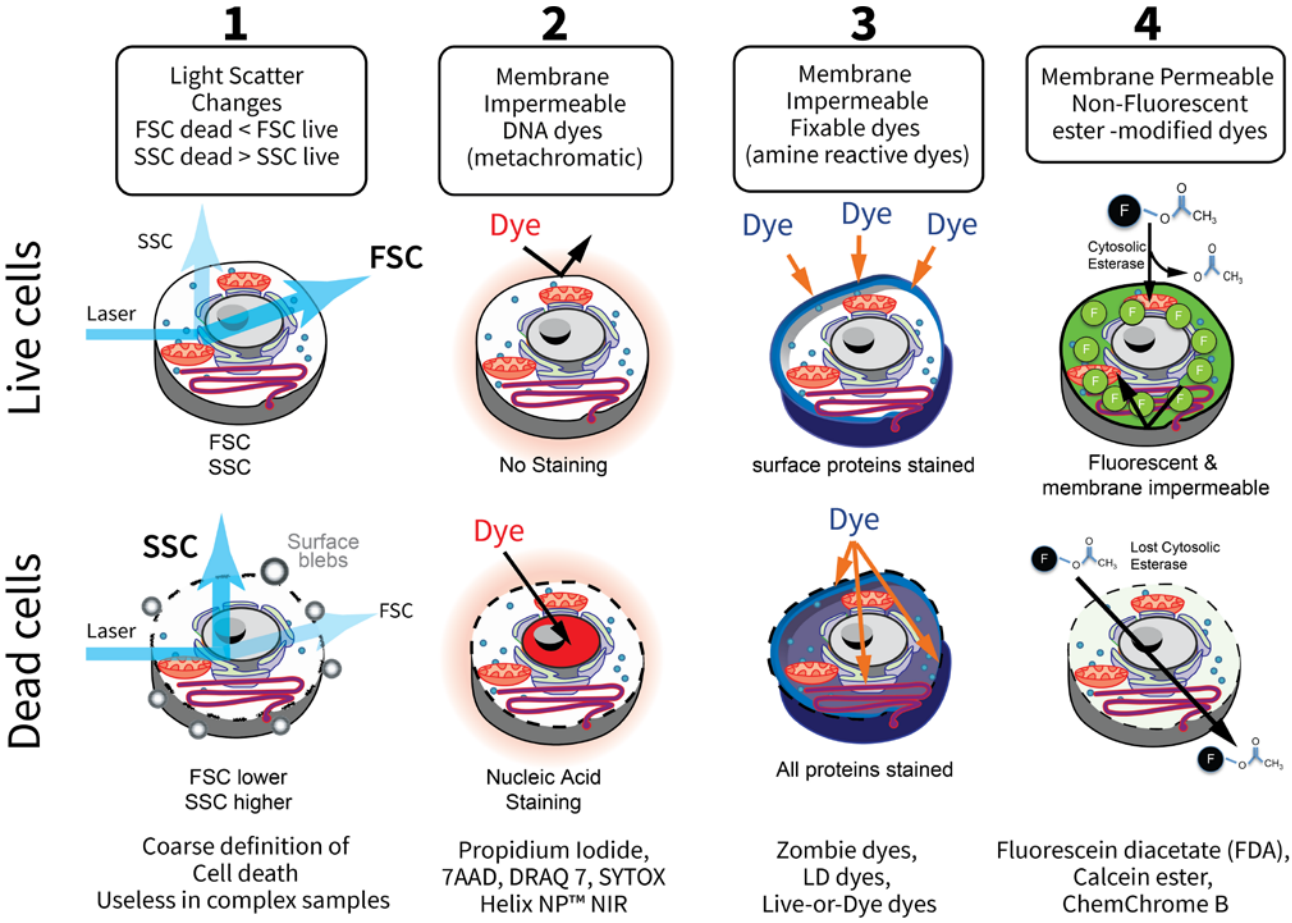
To get the best out of your cytometry experiments, optimisation of staining is critical. Staining efficiency is a function of several factors: incubation time, temperature and crucially, dye-to-particle ratio. As with any other instrument or technique, cytometers have detection limits. There needs to be enough fluorescent antibody conjugate or dye per cell in order to achieve proper resolution of signal from background noise. This is achieved via titration: the evaluation of data derived from samples exposed to serial dilutions (2- or 3-fold) of the dye/conjugate, while keeping cell numbers, temperature and incubation time constant (A). Optimum conditions are achieved when signal to noise ratio, i.e. the resolution of negative and positive populations, is maximised (A, C). In multi-colour experiments, it is important to determine spreading behaviour of fluors into non-primary detectors (a function of spillover signal intensity and can be attenuated by titration) (B, C). Sometimes, saturating dye concentrations, optimal in terms of achieved resolution in the primary channel, lead to increased compensation-derived spreading into critical non-primary detectors. Choosing the dye/conjugate dilution that best fit your experiment will sometimes call for a compromise in resolution for the sake of reducing spreading. Figure C is an example of Stain index and Spreading as functions of PE-Cy5 conjugated antibody volume. In this case, 0.3125 µl of PE-Cy5 antibody per sample is the optimum staining condition, since it achieves a resolution close to maximum while showing minimum levels of spreading into BV510 (~0).

Figure 33 Optimising resolution: signal and spread



34. Dead or Alive: Scatter and Fluorescent Dyes

Figure 34 Live and dead cell separation strategies



Dead cells bind non-specifically to many particles including dyes and conjugated antibodies, producing artefacts that compromise results. As such, identifying and removing dead cells from your analysis is essential. Although dead and live cells can be distinguished via scatter plots (1), more resolution of live/dead cells is required especially when dealing with complex samples where scatter profiles are not clear. There are several fluorescence-based approaches to distinguish live from dead cells (2-4). The appropriate live/dead stain depends on the nature of your experiment.

2. Metachromatic membrane impermeable nucleic acid dyes are hydrophilic and cannot pass through intact cell membranes, and therefore only enter cells with compromised membranes (dead or dying cells). Nucleic acid dyes show very little or no fluorescence until they bind to dsDNA and/or RNA. They CANNOT be used as live/dead distinguishing markers in experiments where cells are permeabilised. They can be used on permeabilised cells for cell cycle analysis only. Because binding of these dyes is not covalent, it is possible to lose some nucleic acid staining if the dyes are diluted.

3. LIVE/DEAD fixable viability dyes distinguish live cells from dead cells based on the availability of amines groups, to which they bind covalently. By adding these dyes before fixation/ permeabilization,

researchers can distinguish which cells were alive and which were dead before fixation. All cells will be stained with LIVE/DEAD stains, however live cells will have less staining than dead cells. This is because dead cells have more available amine groups to bind (both cell surface and intracellular), while live cells only have cell-surface amine binding groups available.
4. Another type of Live/Dead dye can be passively loaded into cells as hydrophobic non-fluorescent esters that are converted into hydrophilic fluorescent dyes by ubiquitous cytosolic esterase enzymes. These cell-permeant esterase substrates serve as viability probes that measure both enzymatic activity, which is required to activate their fluorescence, and cell membrane integrity, which is required for intracellular retention of their fluorescent products.

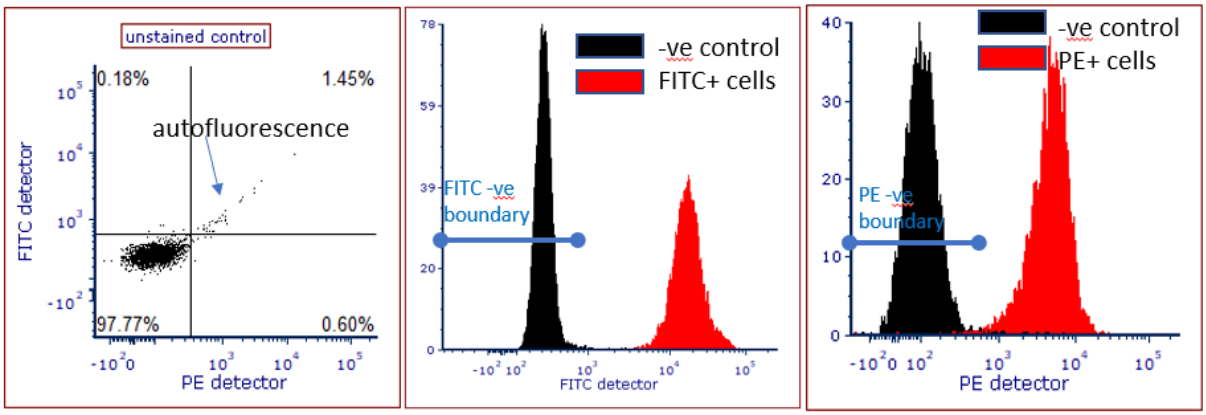
35. Sample Preparation and Acquisition: Controls

The are no absolute negative measurements in cytometry. The mean fluorescence intensity of any "negative" population will change depending on intrinsic autofluorescence, detector gain settings and background. Autofluorescence varies depending on cell type (i.e., lymphocytes vs granulocytes vs cell lines, etc), activation status (resting vs effector), sample preparation/handling (fixed vs unfixed, transfected vs non-transfected), etc. This is the primary reason why controls are crucial for the optimal setup of cytometers and the correct interpretation of generated data. Real positive events can only be revealed and defined in comparison to appropriate reference controls.

36. Negative/ Unstained/ Parental Control

"Negative" controls are particles or cells that have had no external fluorescence added to them and include unlabeled/ non-transduced parental cell lines, unstained tissue, or unstained tissue from an equivalent wild-type animal (where a transgenic FP-expressing animal is being studied). In the case of a differentiating iPSC or embryonic stem cells with a fluorescent protein maturation marker, a negative control would be the same parental cell line, having undergone the same differentiation conditions and for the same amount of time. In spectral cytometers, unstained controls are essential, and needed in the calculation of normalised fluorescence spectra for each reference fluorochrome without autofluorescence contribution. This negative, sometimes called an 'autofluorescence' parameter can be subtracted when measuring dim signals from fluorescent molecules to get an appreciation of the signal from the fluorescent molecule without interfering background fluorescence contribution.
Due to their critical value, 'negative' controls should always be included in your experiment and it is wise not to skimp on the amount of 'negative' control sample brought for a setup.
During instrument setup, we use negative controls to set the amount of power (voltage or gain) given to each scatter and fluorescence detector that will place the particle-derived signals away from electronic noise-rich regions. In this way, events showing signal intensity values beyond the negative population-defined will be considered positive for fluorescence in that detector.

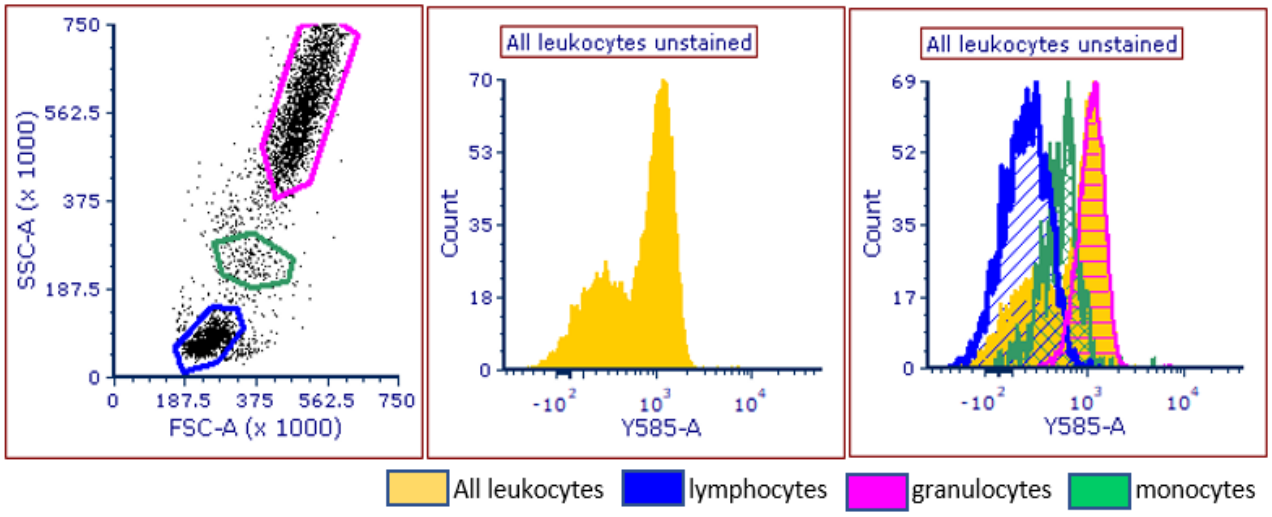
Figure 35 Negative cell boundaries



Negative controls can also be useful to detect dead cells and exclude artefacts in a sample. Dead cells typically exhibit a higher autofluorescence footprint than live cells across several detectors and can be gated out to get a cleaner estimation of true fluorescence positive events

When setting voltages using negative controls, it is also important to draw a scatter-based region around the cells that you will be analysing in order to use them as references to setup the values of voltage (or gain) applied to each fluorescence detector. Debris, dead cells and cells that you are not interested in will have different levels of background fluorescence. A classic example of different background fluorescence levels in heterogeneous cell mixes is among leukocytes subsets in peripheral blood. Leukocytes (lymphocytes, monocytes and granulocytes) have distinct levels of background fluorescence. Higher autofluorescence correlates with cell size and granularity. This can be appreciated in the example below, showing unlabeled, lysed blood autofluorescence levels at the Y585-A (PE) fluorescence detector for whole ungated cells (yellow) vs. lymphocytes (blue), monocytes (green) and granulocytes (pink) gated based on their FSC vs SSC profiles. Ungated (total) cells shows a double peak of “fluorescence” intensities in the PE detector, reflecting the variations in autofluorescence carried by lymphocytes, monocytes and granulocytes and their relative population frequencies.

Figure 36 Autofluorescence differences between leukocytes



37. Single-Colour Controls (Conventional Cytometers)

When staining your samples with more than 1 fluorochrome, fluorescence spill-over contribution into non-primary detectors needs to be corrected before fully stained samples can be analysed (see **Fluorescence Compensation**). To account for this spillover resulting from spectral overlap, we use single-colour stained controls. By running acquired data from single-colour controls through an inbuilt algorithm, the cytometer can calculate the exact amount of signal they contribute into non-primary detectors. This contribution is then removed from each event measured to make sure intensity values scored by particles at each detector correspond to single "pure" primary fluorochromes.
Single-colour controls must comply with the following rules:

1. There must be a negative (-ve) population serving as a reference for the single-colour fluorochrome either within the same tube, or as a universal reference in a separate tube. The -ve reference MUST match the positive (+ve) control in autofluorescence properties. For example, if the +ve control is on lymphocytes, your -ve control must be lymphocytes. If you are using monocytes for your +ve control, your -ve control must be monocytes. If your +ve control is polystyrene beads, your -ve control must be the same polystyrene beads. If your +ve control is on HEK293T cells, your -ve control must be on HEK293T cells.
2. The +ve control flurochrome MUST match the flurochrome in your experiment. For example, even though GFP and Alexa Fluor 488 both require blue laser excitation and emit green (detected in the 530/30nm detector), their emission spectra and spillover into non-primary detectors are different. As such, if you use GFP in your experiment, your single-colour control must also be GFP.
3. Your single-colour control must be clearly resolvable from the negative population, being at least as bright as the brightest fluorochrome+ population in your experiment. For example, if you use a low-abundance marker for your single-colour control that generates a dim signal, but

you use a high-abundance marker in your experiment that generates a bright signal, the experiment will be under-compensated and the spill-over into the non-primary detectors will not be fully corrected. Antibody capture (compensation) beads can be used in many cases where your experimental cells have low-abundance markers or rare antigen expression.

4. You must collect enough +ve and -ve stained samples to allow for statistically relevant spillover calculations, which are based on accurate estimations of population medians. If you are using cells with a very rare marker for your single-colour controls, there may be insufficient events in the +ve fraction to calculate compensation. If this is the case, antibody capture beads are recommended to get enough +ve and -ve events.
5. Tandem flurochromes used in single-colour controls must be batch matched to the ones used in your experiment. Tandem dyes are produced by conjugating 2 fluorochromes and this process is variable in its efficiency and thus there is variability in FRET and emission spectra between batches. If using CD4-PECy7 for your single-colour control, you must use the exact same product (including batch/lot number) in your antibody cocktail.
6. Some dyes change their emission spectra when bound to beads. For instance, if using Brilliant Violet 605 in your antibody panels, it is important that your single-colour controls are on same particles as used in your experiment.

38. Single-Colour "Reference" Controls (Spectral Cytometers)

The rules for conventional cytometer single-colour controls described in **Single-Colour Controls (Conventional Cytometers)** equally apply to spectral cytometer "reference" controls. In addition, spectral cytometers calculate the autofluorescence fingerprint produced by each sample. To calculate the correct level of autofluorescence for each sample, unstained and single-colour controls MUST be treated EXACTLY the same as the experimental samples. If analysing fixed and permeabilised cells in your experiment, you must also include a fixed/permeabilised unstained sample, etc… If the fluorochromes in your experimental samples have been exposed to fixation/ permeabilization agents, your single-colour controls must also be exposed to the same agents.

39. Isotype Controls

Isotype controls are not considered as best practice in cytometry. Isotype controls are useful only to show how effective blocking steps have been and should NOT be used as boundaries for +ve staining. Insufficient blocking of Fc regions or sticky molecules in cells will result in isotype control antibodies sticking or binding to cells that have these regions available. Below is an example of cells fixed and permeabilised before staining with mouse-anti-human collagen II primary antibody, then a goat-anti-mouse IgG-AF488 secondary antibody. Note that the isotype control AF488 signal is ~ ½ log brighter than the secondary-antibody only control, and its staining tail extends as far as the specific collagen staining. The isotype control here is an example demonstrating why isotype controls should not be used to set boundaries for specific staining.

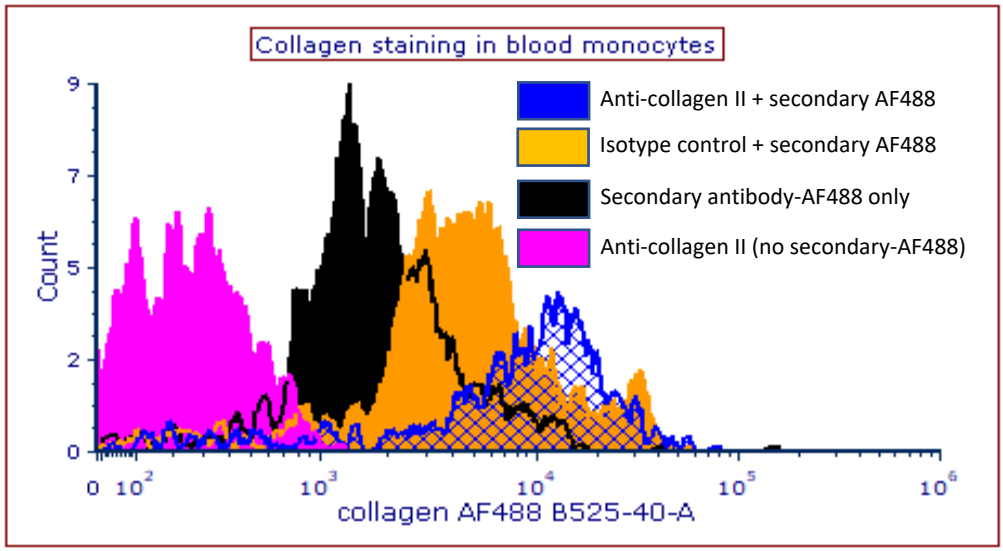


Figure 37 Isotype control deceptive positive staining boundaries

N. Rajab (2019)

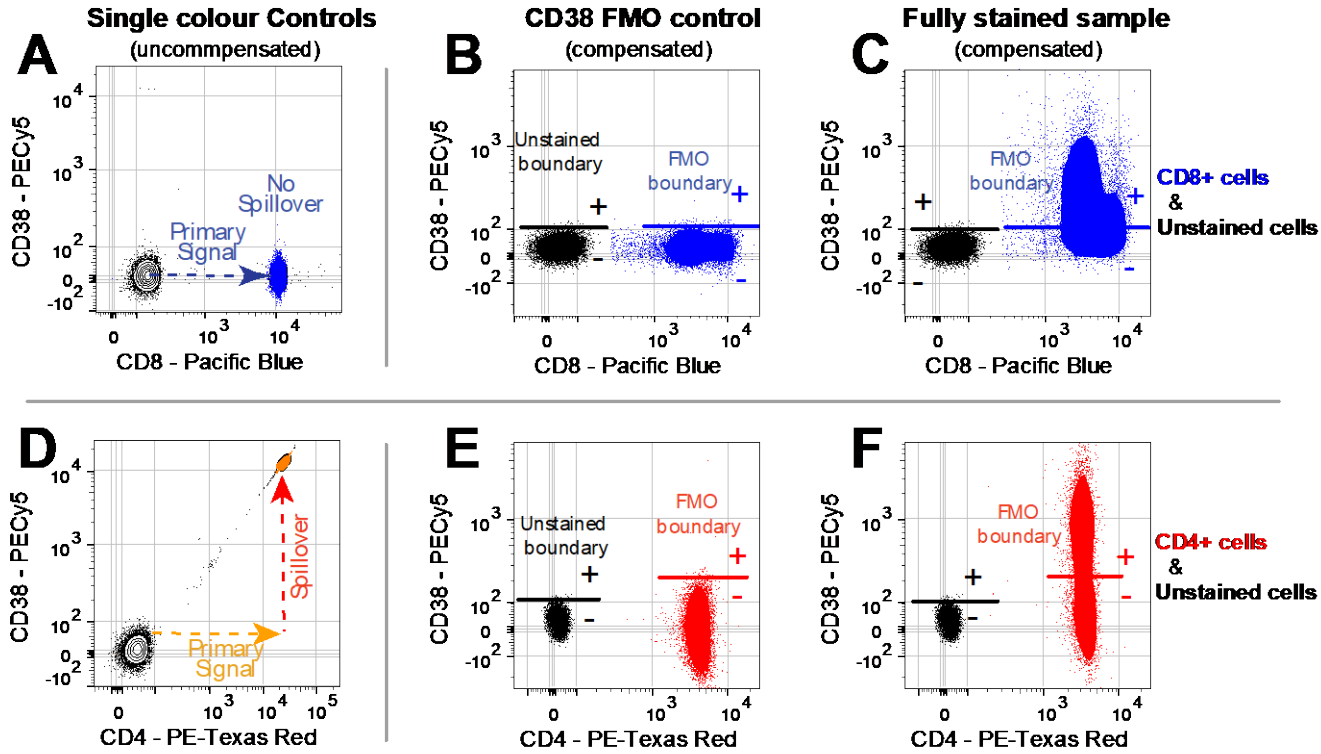
40. Fluorescence Minus One (FMO) Controls

In an ideal world, you would expect unstained controls to be sufficient when defining boundaries between negative and positive at any given channel. This is not always the case, especially after compensation or spectral unmixing of multi-colour samples. One of compensation's drawbacks is the increase in population spreading, compromising the resolution of dim fluorescence signals (see **Titration, Saturation, Signal-to-Noise and Spreading**). The spreading of the negative boundaries after compensation is one of the reasons behind the need for another type of control "Florescence minus one" (FMO). FMO controls are considered best practice for setting positive/ negative boundaries in multi-colour flow cytometry experiments, particularly when looking at antigens where expression is low or variable (across a continuum, from very low to medium or high intensities). As the name suggests, an FMO control consists of cells stained with all the fluorochromes used in a panel except one, that's purposely excluded. The aim is to assess the effect of the spreading of all the other panel fluorochromes on the negative boundary definition at the channel where the excluded colour will be measured. For example, FMO controls for a panel with 5 fluorochromes would require an additional 5 control tubes

	BV421	BV510	BB515	BB700	APC
BV421 FMO	-	Y	Y	Y	Y
BV510 FMO	Y	-	Y	Y	Y
BB515 FMO	Y	Y	-	Y	Y
BB700 FMO	Y	Y	Y	-	Y
APC	Y	Y	Y	Y	-

Figure 38 shows an example of FMO controls to define the boundary for a marker with very low expression: CD38 (PE-Cy5) within the CD8+ (stained with Pacific Blue, plots B & C) and CD4+ (stained with PE-Texas Red, plots E & F) T cell subsets in a 9 colour panel. In the fully stained sample plots gated on CD4+ (F) and CD8+ (C) cells, CD38+ cells overlap with CD38- cells, with low expression appearing as a continuum from very low to medium levels of intensity (1 log decade over the negative cells, or ~10x brighter).

Figure 38 FMO control strategies



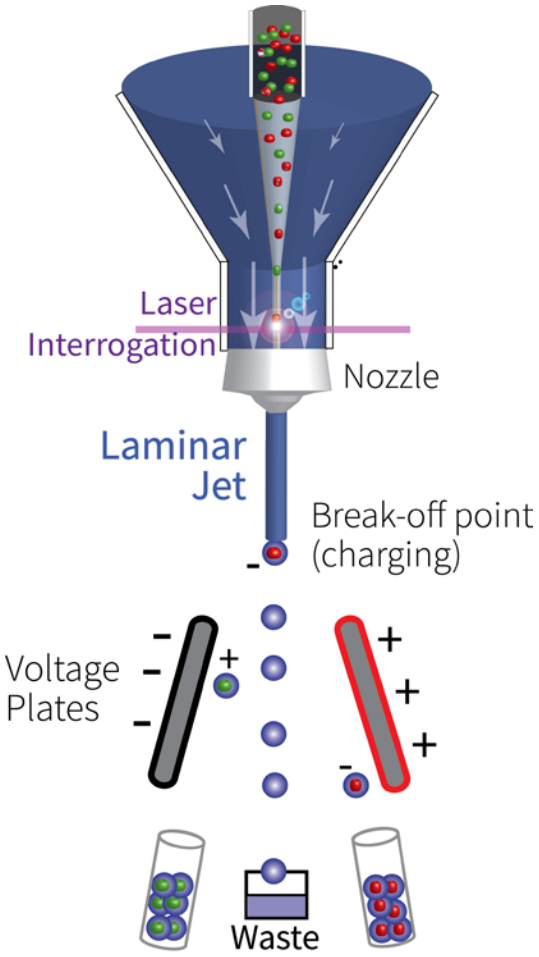
The boundaries between CD38 negative and CD38 positive events in the CD4+ and CD8+ populations differ in relationship to what's suggested by the unstained cells. These cell type-specific CD38-/+ boundaries in CD4+ and CD8+ populations - otherwise identical in terms of autofluorescence- are best defined with the analysis of the CD38 FMO control (B and E). The discrepancy in CD38-/+ boundaries between unstained and single colour CD4+ populations is due to PE-Texas Read spreading into the PE-Cy5 detector after compensation. This compensation-derived spreading results from the high spillover of PE-Texas Red into the PE-Cy5 detector (both dyes are heavily overlapping), shown in the uncompensated single colour PE-Texas Red control (plot D). In the CD8+ cells, the CD38-/+ boundary matches that defined by unstained cells, with ~ 0 spreading of Pacific Blue into the PE-Cy5 channel after compensation, due to the absence of spectral overlap and signal spillover between these two dyes (A).

41. Biological/ experimental controls

These controls are important for answering various biological questions, such as differences between healthy and sick patient samples, wildtype versus knockout or transgenic animals, activated and non-activated lines etc… When analysing fixed, or activated cells, where the scatter and fluorescence profiles will be altered, the controls used to set up the experiment need to be performed on fixed or activated parental cells. Unfixed, non-permabilised sample controls used as a reference for fixed/permeabilised intracellular analysis is asking for problems!

Figure 39 Cell sorting schematic

42. Electrostatic cell sorting



A major advantage of flow cytometry is the ability to separate specific viable cells according to their subtype or epitope expression for further biological studies. This is achieved via cell sorting, also known as by the Becton Dickinson trademark name “fluorescence activated cell sorting” (FACS).

In jet-in-air sorters, a fine jet of liquid carrying the cells is expelled from a nozzle (a funnel-like device that accelerates the flow and produces consistent laminar liquid jets) coupled to a piezo crystal, that expands or contracts as a function of incoming electric inputs. A precise electrical sine wave is imposed on the piezo crystal causing a uniform sonic wave (~30-100 kHz) to be imprinted onto the jet, which under strict conditions, will break from the jet into droplets with a defined frequency and at a fixed location (known as the jet’s break-off point). As with analysers, particles for analysis and sorting are injected into the core of the nozzle and confined to a single file through hydrodynamic forces. Once in the jet’s core, particles will ideally move one at a time through a focused laser beam spot, allowing light scatter and fluorescent measurements to be taken from each particle. Classification of particles can be made (i.e. which ones are GFP+), and because the break-off point (BOP) of the jet is stable in time, drops containing desired target particles can be selectively charged as soon as they arrive at the BOP, deflected by a powerful electrostatic field at precise angles, and finally collected in a container (culture flasks, tubes of any format, multi-well plates, microscope slides, etc).

43. Cell sorting FAQs

Adapted from https://med.virginia.edu/flow-cytometry-facility/resources/faqs/faqs-for-cell-sorting/

What are the advantages of sorting by Flow Cytometry compared to other isolation techniques (i.e. magnetic bead separation, panning, etc…)?

a) Higher purity
b) Can separate populations of cells based on their level of fluorescence intensity
c) Better separation of populations using multiple antibodies
d) Higher recovery of cells of interest
e) Can eliminate dead cells
f) Can sort based on internal staining (DNA, cytokine expression, GFP, etc.)
g) Can sort four-six populations simultaneously (depending on the sorter)

Which cells can I sort?

Most cell types that can be prepared and kept as single cell suspension can be sorted. However, some limitations across platform instruments apply based on cell size, physical and biological fragility, as well as the cell type (infectious, live bacteria & yeast). During cell sorting cells are accelerated and aligned via shear forces that increase towards the nozzle exit, with large cells being most susceptible to damage unless special sorter setup strategies are used. In terms of size, cells need to be much smaller than the nozzle output orifice (from 50 to 120 µm) in order not to clog it and also not to interfere with the droplet formation process. It is important to minimise cell clumping, via careful sample preparation and the definition of optimum suspension buffers. Avoiding a disruption of the sort process by aggregates will improve the purity of your sort products, as well as increase your sort yield and recovery. Primary human samples or samples infected with PC2 pathogens require special handling and engineering precautions. Please contact our Platform staff for special instructions prior to scheduling these samples.

What do I need to do prior to scheduling a sort?

You must first have an account in Calpendo. It is advisable to set up a consultation with one of the core staff via phone or email (staff contact details are available at biomedicalsciences.unimelb.edu.au/cytometry) to discuss the specifics of your experiment prior to scheduling your sort.

What effect will the sorting process have on my cells' viability?

The effects of sorting on cells is dependent on several factors:

- cell type – some cell types are more "fragile/labile" than others. Any type of cell that has structure on the outside of the cell membrane is more likely to be damaged during the sorting process.

- condition of the cells prior to being sorted – cells that have been through physical and enzymatic dissociation from solid organs or have been activated or treated with drugs may be more susceptible to the effects of sorting.
- pressure/jet velocity at which cells are sorted – the faster you want to sort, the higher the pressure. Some cells (such as DCs) activate under high pressure conditions. Faster is not necessarily better.
- the buffer the sample is to be sorted into – most cells will not tolerate significant mixing of buffers. Carbonate buffers when mixed with phosphate buffers (sorting fluid) can cause precipitates to form on cell membranes and compromise integrity.

How many cells do I need to bring?

This will depend on a) How many cells you would like to isolate; b) What's the frequency of the target population of interest in the original sample and c) What's the expected percentage of sort yield (typically between 75-90%, depending on sample quality, droplet frequency and sort speed). For example, if the target cells you are interested in sorting are 10% of your unsorted cells and you need to recover 1 x 10^6 target cells, you would need 2.0 x 10^7 as a starting cell number: [2.0 x 10^7 x 0.10 x 0.50 = 1 x 10^6]. It is always best to calculate the number of input cells based on the worst-case scenario of a 50% yield rate so you will be sure to have an ample number of cells to start.

What kind of information do you need to provide sort operators with?

Due to its complexity, cell sorting is assisted by platform dedicated staff in most cases. Ahead of your first ever sort, it is highly recommended you contact platform or node managers to discuss experimental details, sample requirements, etc to define the best instrument for the job and the appropriate sort strategy to best accommodate your experimental needs. Let our staff know the following information in advance or at the time of reservation via Calpendo booking fields:
Type of cells (species, tissue, primary or cell line); this is important for establishing instrument settings and pressures.
Are these samples infectious or derived from human tissues?
What's the largest cell size in your samples; this is very important in selecting the nozzle size to be used. If you already know this, select the appropriate nozzle size in the booking window corresponding field.
Which fluorochromes have been used to label the cells; this is important in selecting lasers and optical filters and in setting compensation for spectral overlap. It is very important to discuss these issues with the operator prior to setting up your experiment to ensure the instrument can excite, detect and compensate your fluorochrome combination.
Are you looking for high purity or would you just like to enrich your target populations? Do you require to get as much the target cells as possible, because your starting sample is small/or your population is rare? Are you happy to compromise some yield for the sake of sort processing speed?

What's the fate of the target cells post-sort (short-term culture, long-term culture, PCR, functional assays, genomics, proteomics, advanced microscopy, etc); this will help determine sample preparation, acquisition setup, the sterility conditions, as well as jet velocity.
Vessel type you wish to sort into (i.e. 12x75mm tube, 15ml Conical Tube, plate (including number of wells), microscope slide).
Number of populations to be sorted (1-6 populations, depending on the instrument). Note: Only one population can be sorted at a time for plate and slide sorting.

What concentration and in what medium should my cells be in?

This depends on your cell type. Some cells tend to clump at higher densities. In general, 5-10 X 10^6/ml in PBS or HBSS with 1-2% BSA will be fine. If your cells are happiest in something like RPMI, you should use RPMI that is phenol and biotin free (HyClone Laboratories). Cells should be brought tubes: 1ml 'bullet tubes', 5ml 'FACS tubes' or 15ml Falcon tubes (preferably polypropylene).

How can I keep my cells from clumping?

Keeping cells in single-cell suspension during the entire sort is critical to the success of a sort. Cell preparations with large amounts of cellular aggregates can cause the nozzle to clog and necessitate the re-establishment of instrument settings. Nozzle clogs can also increase the possibility of sort contaminations. Cell clumping can be a problem with adherent cells, activated cells, or samples with a high percentage of dead cells. Firstly, samples to be sorted must be passed through a nylon mesh to remove as many clumps as possible prior to the sort. Raising the concentration of EDTA to 5mM in the sample buffer may help with reducing cation-dependent cell-to-cell adhesion. For adherent cells, there are commercially prepared cell detachment products, such as Accutase, or the use of cation-free FBS buffer as a Trypsin inhibitor and can help reduce clumping. If there are many dead or damaged cells in the sample, soluble DNA can cause the cells to become "sticky" and start to clump. The addition of DNAase II (10U/ml) to the buffer solution can help eliminate the clumping due to free DNA. If, in spite of these efforts, clumping still occurs the operator will pause the sort to re-strain the sample through nylon mesh.

How long to sort? Factors impacting good sort? End application of sorted cells etc….

The speed of sorting depends on several factors including particle size and the output diameter of the nozzle used to accommodate cells of different size (70, 85, 100µm are typical nozzle sizes), the events rate, the starting frequency of cells to be sorted and the rate or frequency of droplet formation, which matches the piezo-imposed electric sine wave controlled by the instrument electronics. The maximum possible value of the droplet generation frequency is ultimately a function of nozzle size and jet speed (function of sheath tank pressure). Nozzles, depending on their diameter, can produce 30,000–100,000 droplets per second (the smaller the nozzle orifice diameter, the higher the maximum droplet generation frequency), which is ideal for high-speed sorting.

If you are sorting for rare populations of cells, it will generally take much longer than if you are sorting a population that is 30-50% of your original sample. 2.0 x 10^7 cells can take anywhere from 1.0-2.0 hours to run through a sorter, depending on the pressure, quality of sample, and size of the cells (larger cells require a larger nozzle which necessitates lower pressures). Set up time for a sort is variable and depends on the cytometer. From start-up cytometer takes 60-90 minutes to warm up, stabilise and to run QC- this is usually done well in advance of a sort starting. Depending on the complexity of the sort, 10-60 minutes are required to establish detector settings and compensations, 5-10 minutes for regions and sort gates, and 10-15 minutes for post sort analysis.

How many populations can I sort simultaneously?

Up to four cell populations can be sorted simultaneously on FACS Aria cell sorters, up to 6 in Astrios cells sorters. Half of these populations are sorted left and right around the central laminar jet containing non-sorted cells captured in the waste container. Each population can be identified with multiple parameters; i.e. multiple fluorescent probes, size and internal complexity. It is often useful to sort a negative (not the cells of interest) and positive populations (cells of interest) to have an internal control for whatever assay is performed on the sorted sample.

What should I sort my samples into?

Droplets carrying sorted cells have very little volume and a net positive or negative charge. When these charged droplets are sorted into empty vessels, the quickly dry or eventually bounce away from large sorted volumes due to electrostatic repulsion. All collection vessels must contain an appropriate collection media. For example, 12x75mm Falcon polypropylene tubes should have approx. 1ml of support media which contains 20% FBS (or some other source of protein such as BSA) and antibiotic (pen/strep or gentamicin). When sorting larger cells (i.e. using a larger nozzle size), the droplets sorted are larger and therefore generate a larger volume of sorted sample. In this case it may be necessary to bring two 15ml conical tubes with 3mls support media to accommodate the larger volume. The same applies when the sorted sample contains more than 4×10^6 cells. Use this Droplet Volume Estimate to estimate how much volume will be deposited into your collection tube for a 70µm nozzle.

How should I treat my sorted sample to ensure the best recovery and viability?

Cells can be a bit compromised by the trauma of the sort. The cells should be spun down as soon as possible following the sort and re-suspended in their usual medium at a concentration that they are most happy and cultured under their usual conditions. Keep in mind that the condition of the cells prior to the sort will greatly impact their viability in the end. The healthier they are going into the sort the better the post sort viability.

What is yield and why is it only 75-90%?

Yield is defined as:

{[number of cells in the collection tube] * [the % purity]} / {[the number of cells you started with] * [% of the target population]}.

The factors that affect the actual yield of the sorted sample include:

Electronic aborts – Cells which arrive in the laser beam too close to one another; with each electronic abort, more than one cell is thrown away and not processed

Sort conflicts – A target and non-target cell occurring within the same or overlapping sort envelopes; both target and non-target cells are not sorted.

Sample quality – Loss of target cells due to cell death (pre and post sort) or adherence to tube walls. Increasing sample rate (#cells/second analysed) and the presence of cell aggregates can increase these losses and ultimately reduce your yield.

Appendix 1 -Application-specific comparison of Melbourne Cytometry Platform analysers

Extensive cytometer testing was performed by MCP experts to generate the table below. Researchers looking to perform specialist procedures should refer to this table and contact our managers to discuss the optimal cytometers for their needs

Application	Instrument ranking - the more stars, the better the performance							
	Aurora	CytoFLEX		LSR Fortessa		LSRII and Canto II		Verse
	5L	6L	4L	5L	4L	3L	2L	2L
high dimensional (35 colour) immunophenotyping	★★★★★	NA	NA	NA	NA	NA	NA	NA
spectral cytometry	★★★★★	NA	NA	NA	NA	NA	NA	NA
autofluorescence extraction	★★★★★	NA	NA	NA	NA	NA	NA	NA
multi-colour phenotyping	★★★★★	★★★★	★★★	★★★★	★★★	★★	★	★
resolve very dim signals	★★★	★★★★★	★★★★★	★★	★★	★★	★★	★
sub-cellular analysis	★★★★	★★★★★	★★★★★	★★★★	★★★★	★★★★	★★★★	★★★★
nanoparticle analysis	NA	★★★★★	★★★★★	NA	NA	NA	NA	NA
fluorophore-free autofluorescence measurements	★★★★★	★★★★★	★★★★★	NA	NA	NA	NA	NA
violet scatter for whole blood analysis	★★★★★	★★★★★	★★★★★	NA	NA	NA	NA	NA
signal dynamic range	★★★★	★★★★★	★★★★	★★★	★★★	★★★	★★★	★★★

Lasers
blue, red
blue, red, violet
blue, red, violet, yellow/green
blue, red, violet, yellow/green, UV
blue, red, violet, yellow/green, UV, IR

NA	not applicable