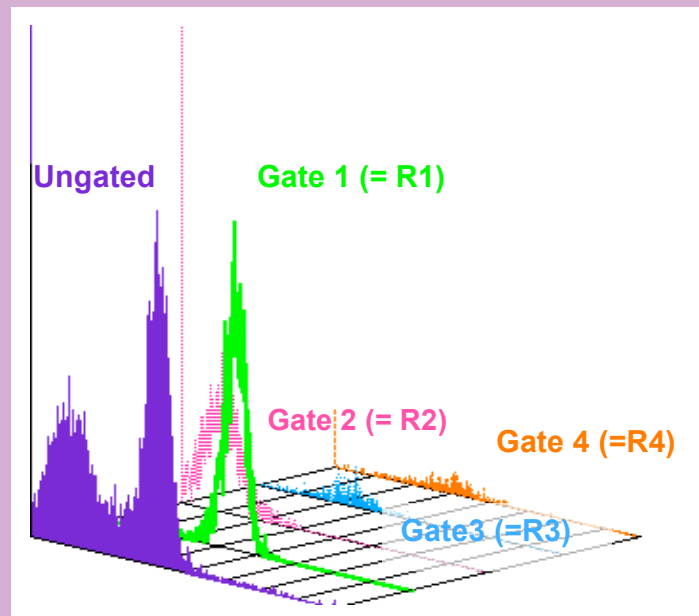
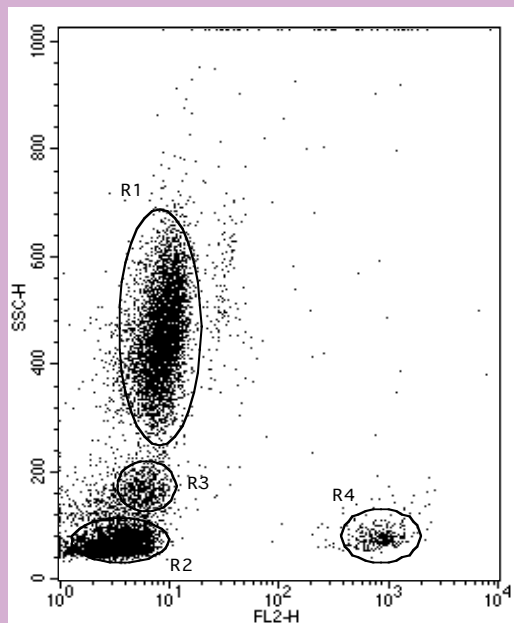


# Flow Cytometry

## A Basic Guide



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Cover Picture: Whole blood leukocytes stained with a FITC-conjugated mouse anti-human CD35 mAb and a PE-anti-human CD19 mAb. The FL-2 vs. SSc dot plot identifies four cell sub-populations (granulocytes, monocytes, B cells and non-B lymphocytes), while the overlay histogram displays the CD35 expression profiles of both the total cell population and each of the identified sub-populations. The cells were investigated using a BD FACScan flow cytometer.

## **Foreword**

The purpose of this guide is to introduce flow cytometry (FC) to those who are about to use the technique for the first time or those with limited previous experience of this approach. The guide by no means intended as a comprehensive instruction manual in the use of FC. It is,

rather, a description of the basic FC principles, with illustrations of their application, in such a way that readers acquire a feel for the technique, and its potential, which enables them to use FC for their own purposes.

*Graham Leslie*

*July, 2006*

## What Is Flow Cytometry?

*Measurements performed  
On individual cells  
In a liquid stream.*

One little haiku says it all! The essence of FC, which distinguishes it from almost all other techniques, is the provision of detailed quantitative information on *every single cell* in a larger cell population (anything from a few dozen to several

million cells). This is in contrast with traditional quantitative/semi-quantitative approaches (radioassays, ELISAs, Western Blots etc.), where the measurements are performed – as a bulk assay – on the entire cell population under investigation.

**Table 1 FC: A unique approach to cell investigation**

Assay type	Bulk Assay	Individual cell assay
Examples	Radioisotope Assay	Flow Cytometry
The essence	Total uptake of a given marker by n million cells used to estimate <i>mean</i> activity per cell.	Simultaneous measurements of 5 or more parameters on <i>each</i> of 1,000-1,000,000 cells is stored.
The Requirement	Cell preparations of >95 % purity. (Time consuming and potentially disturbing purification procedures)	A statistically significant number of <i>identical</i> cells (say 200) in the total cell population. (Frequencies as low as 0.1 % of the total)
A Prime Difference	Radioassay gives an "end result". No further work on the cells is feasible.	The cells can be purified by sorting on the basis of their phenotypic profile.

The difference between FC and traditional bulk assays has far-reaching implications (see Table 1).

1. With FC, *no prior purification of the cells is necessary*. The FC collects information on each cell in the total population under investigation and stores it as a database. Phenotypic data acquired, as part of an analysis, can be used to select, from the database, the particular cell subpopulation of interest and to obtain further information on these cells alone. We talk about "purification on the computer screen".
2. The lack of need for purification means that analyses can be performed on cells that have been subjected to minimal prior treatment. This can be of great

- advantage in studies of certain surface markers, which are readily up- or down-regulated by the physical or chemical changes involved in a purification procedure.
3. Assays investigating the transfer of ligands between cell sub-populations (e.g. distribution of soluble immune complexes between blood leukocytes) can readily be performed with FC, whereas this is virtually impossible using bulk techniques.
4. Whereas, in bulk assays, the cells are often destroyed in the process (and almost always discarded), the FC approach is essentially non-invasive and, indeed, can be used to physically purify the cells for subsequent culture and study (Cell Sorting).

## How does a Flow Cytometer work?

The basic principles of FC are illustrated with the cut-away diagram of a classic flow cytometer, the FACScan (over 20 years old and still going strong!) (Fig. 1).

The essential features are:

1. An air-cooled argon laser tuned to emit at a wavelength of 488 nm (blue light).
2. A system of prisms and lenses to focus the laser beam on a cuvette (Flow Cell) through which the cells are propelled in an annular fluid stream from the sample tube by air pressure (Sample and Sheath-fluid Pressure Pump).
3. Upon passage through the laser beam, the cells perturb the beam directly in two ways:
  - a) by acting as miniature lenses they deflect the beam (along its axis) to a degree that is

dependent on the size of the cell and the difference in refractive index between the cell contents and the surrounding sheath fluid. The deflected light is measured as "Forward Scatter" (FSc) by a suitably positioned detector (Forward Scatter Detector).

- b) Particulate elements (mitochondria, granules etc.) in the cell disperse the beam in all directions. For convenience, the light scattered at a right-angle to the path of the laser beam is normally relayed, via a set of filters and lenses, to a photomultiplier tube which registers the intensity of this Side Scatter (SSc) light.

*NB. The wavelength of the light recorded in both instances is that of the laser i.e. 488 nm.*

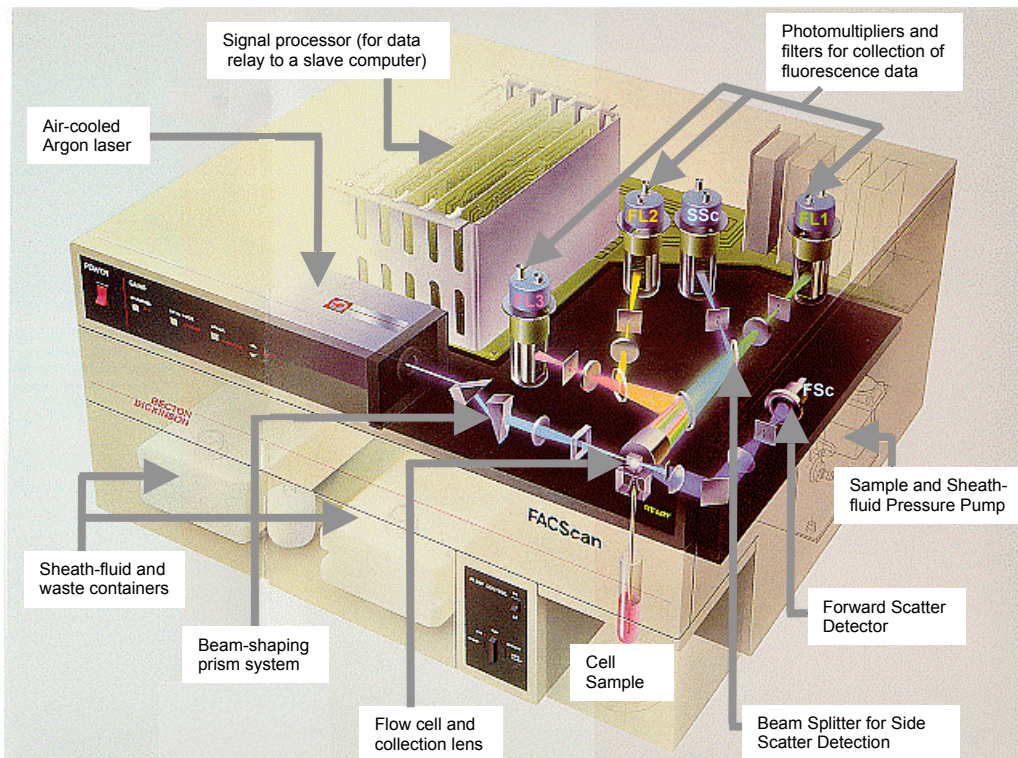


Figure 1. FACScan – an archetypal flow cytometer. (Courtesy of BD Biosciences)

4. The FC can be used to detect cell surface (or intracellular) components by attaching to these specific markers (usually antibodies) conjugated with fluorochromes,

which are optimally excited at the wavelength emitted by the laser. Various fluorochromes are available, which share this property but differ with respect to the light they

emit (green, yellow, orange or red). The different colours of emitted light are separated from each other by a system of filters and mirrors and then registered by the signal they generate in appropriately placed photomultiplier tubes (PMT).

*NB! The PMT signals generated are quantitative i.e. not only do they register the presence of a marker on the cell they also measure the intensity of the fluorescence emitted by the marker. With appropriate calibration, this information can be used to determine how many copies of the investigated component are present on each cell (see later).*

5. The signals from the detectors are passed via a signal processor to a slave computer, which has two functions:
  - a) To adjust the settings of the FC (PMT voltages, FSc detector gain and detection threshold etc.) for optimal investigation of the cell

sample and to register the data collected in a folder allocated for this purpose ("Acquisition Mode"), and

- b) To provide the software platform for subsequent analysis of the data, which may be recalled at any time ("Analysis Mode").
6. The rate of data collection is determined by the concentration of cells in the sample and a two-speed sampling switch (Hi or Lo), with a recommended maximum rate (for the FACScan) of 1000 cells/sec.

Newer FC supply a number of refinements on this basic theme, such as higher sampling rate, alternative optics for registering more than five parameters, additional lasers (red diode, violet or ultraviolet lasers) and measurement of signal width (or area) as well as signal height. However, the operating principles are basically the same.

## Data Presentation (One parameter or two?)

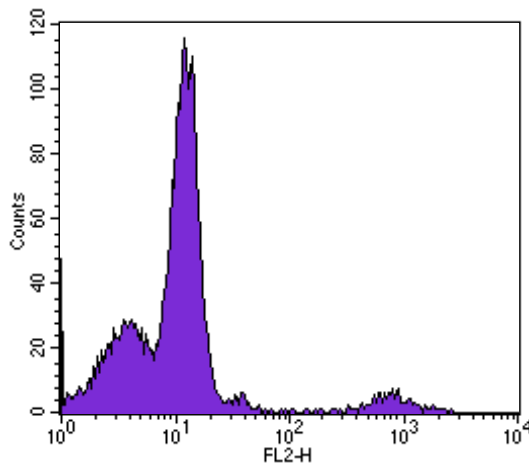
By far the most common ways of depicting FC data are with histograms to display a single parameter (usually fluorescence) or dot plots to show distribution of cells in terms of two parameters (e.g. FSC vs. SSC or 2 fluorescent markers).

The histogram displays the intensity of the signal recorded on the x-axis with the number of cells displaying a particular intensity on the y-axis, while the dot plot charts the coordinates for two parameters

(e.g. FSC and SSC) with each cell registered as a single dot (see Fig. 2).

Generally speaking, histograms are used to obtain detailed quantitative information about a particular marker (membrane or intracellular) associated with a defined cell sub population (see later) whereas dot plots are used to identify and quantify individual sub-populations within a cell mixture. Dot plots are often used to define cell sub-populations for subsequent histogram analysis.

**One parameter: Histogram**



**Two parameters: Dot plot**

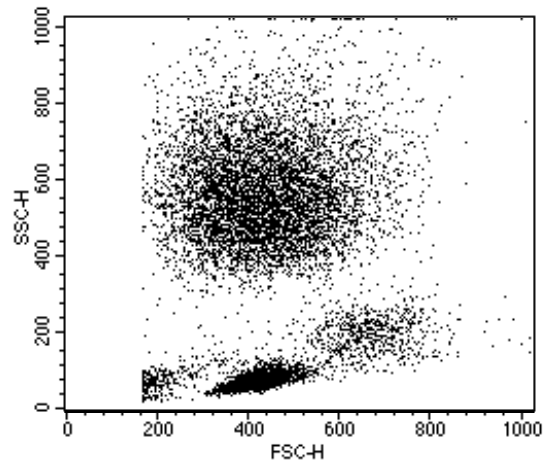


Figure 2. A histogram showing the fluorescence intensity pattern for blood leukocytes stained with the B cell marker, anti-CD19. The small population displaying high fluorescence (around  $10^3$  units) are the B cells. The dot plot displays the FSC vs. SSC characteristics of the leukocytes. (Note that the fluorescence data is presented on a log scale while the FSC and SSC axes are linear.)

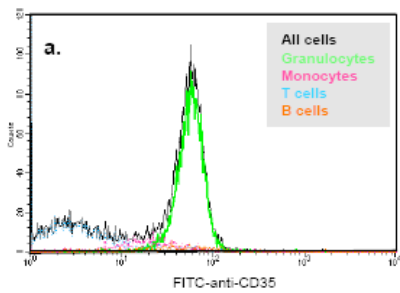
### Variations on the theme.

An illustrative way of presenting a series of histograms is through the use of an overlay, where the individual histograms are assigned different colours and then superimposed on each other. This may be shown in a 2-D format (Fig. 3a) or more effectively in 3-D (Fig. 3b).

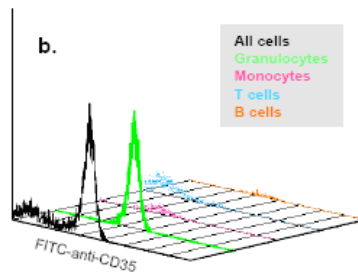
Dot data can also be transformed into contour plots delineating either the cell *density* at a particular set of coordinates, or

the *probability* that the next cell to be analysed will display these coordinates. Contour plots, especially when depicted with coloured contour lines, can be helpful in discriminating between two poorly separated sub-populations of cells (Fig. 3c).

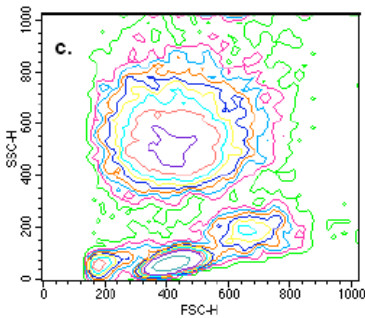
The 3-D dot plot (Fig. 3d), which can also be regarded as a 2-parameter histogram, is again useful for illustrative purposes, but not amenable to quantitation.



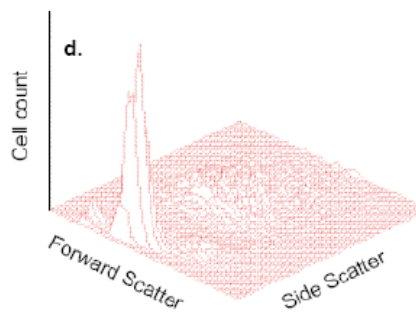
Overlay histogram



3-D overlay histogram



Contour plot (probability)



3-D dot plot

Figure 3 Other modes of data presentation.

a. Overlay Histogram,  
b. 3-D Overlay Histogram,  
c. Contour Plot (10 % probability contours)  
d. 3-D Dot Plot.

Note! All plots depict the same cell preparation i.e. whole blood leukocytes

### More than two parameters?

Software has been developed for the presentation of three parameters in a 3-D dot plot format: see Verity Software at

<http://www.vsh.com/Presentations/DisplayForFlow.pdf>

While such plots, in their basic form, are not readily interpretable, modifications involving the clustering of dots into spheres or clouds has improved their "user

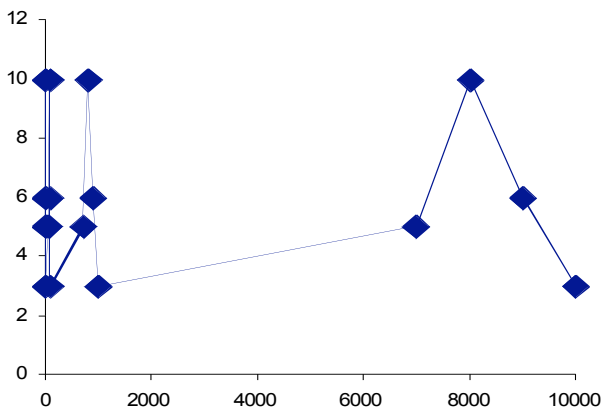
friendliness". However the large computer capacity needed to process this information renders this approach inaccessible to most FC practitioners. Instead the usual approach is to run through the parameters in sequence, using for example FSc and SSc and/or cell specific surface markers, as an initial step, to identify cell sub-populations for analysis using other parameters.



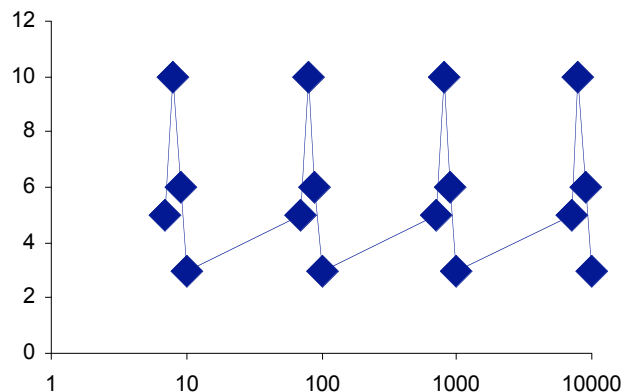
## Data Acquisition: Linear or Log?

The signal intensities from the various detectors are recorded by allocating them to one of 1024 channels (or factors thereof) in the database. This can be done

either in a linear or log mode. The question is "Which to use?" Fig 4 shows the results of recording the same data in each mode.



**Linear Scale**



**Log Scale**

The data:

Signal Intensity (x-axis)	Number of Events (y-axis)	Signal Intensity (x-axis)	Number of Events (y-axis)
7	5	700	5
8	10	800	10
9	6	900	6
10	3	1,000	3
70	5	7,000	5
80	10	8,000	10
90	6	9,000	6
100	3	10,000	3

Figure 4 Depiction of data in linear vs. log format.

As can be seen, the linear mode is not suitable for depicting data that spreads over a wide range, as is often the case with the fluorescence signals associated with cell surface markers. On the other hand the resolution in the linear mode is better over a single order of magnitude (here the 1,000

to 10,000 range). Generally, the linear scale is only used to depict Forward Scatter, Side Scatter and DNA content (Propidium Iodide staining), while the log scale is used for most other applications (incl. all fluorescent markers).

## Setting the Signal Intensity

The strength of the signal recorded by a PMT is directly related to the voltage applied to detector. Thus you can alter your peak at will! Generally, one chooses a voltage that sets the non-fluorescent cells or particles within the first decade of the

fluorescent scale (as is the case with 500 or 550 v in Fig. 5). The positive signals will then lie at anything from a few units up to 10,000 units, depending efficiency of the probe and on the degree of expression of investigated component by the cell.

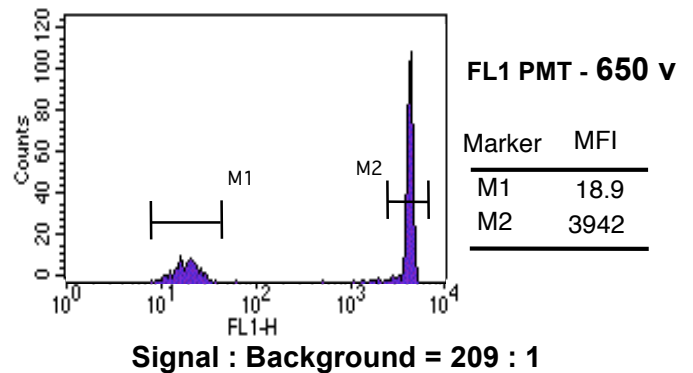
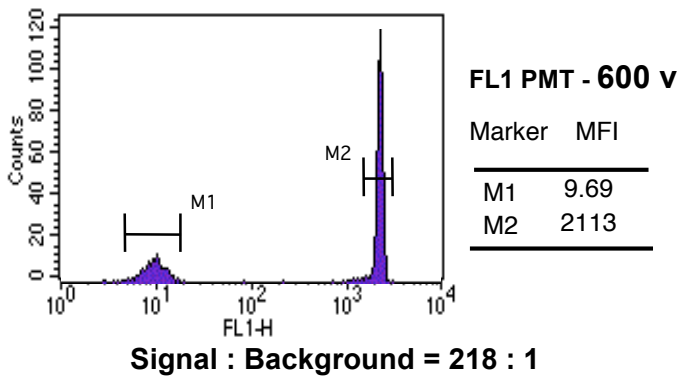
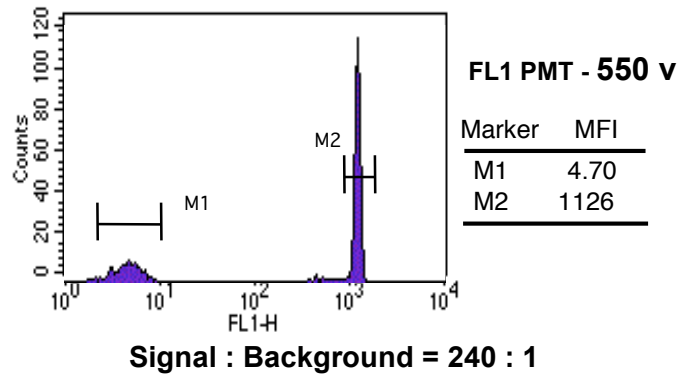
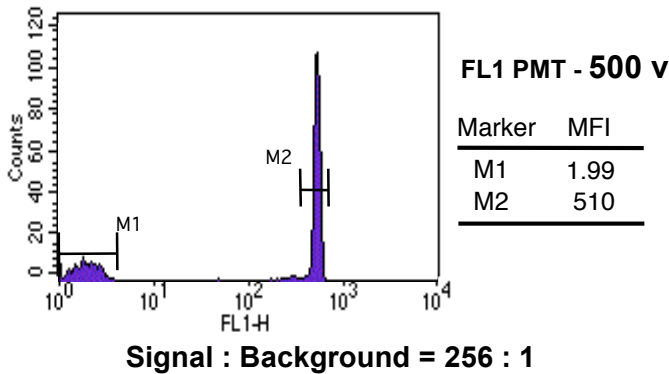


Figure 5. The signals recorded with FITC-conjugated and unlabelled latex beads (Calibrite, BD Biosciences) at 4 different PMT voltages on the FL1 detector. Note:

1. that an increase of 50 v results in an approximate doubling of the fluorescence signal, and
2. the ratio of the FITC : blank signals falls as the PMT voltage is increased.

In situations where the signal from the probed cell is very weak, and not readily distinguishable from the background, it might seem tempting to screw up the PMT voltage in order to improve the response. However, as can be seen from Fig. 5, this does not help because the background response is also increased, with the result

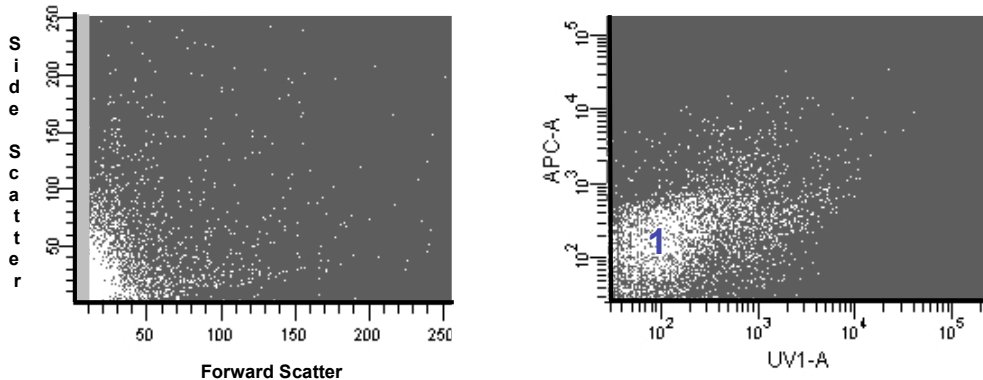
that the signal to background ratio remains unchanged or is even diminished (i.e. poorer discrimination!) In other words, you can't amplify your way out of the problem: the only solution is to find a more effective probe (e.g. by indirect labelling or use of biotin-streptavidin amplification).

## Setting a threshold.

The detectors in a FC are capable of picking up signals that lie way below the level of practical value in cell analysis. This background noise (arising from cell debris and other micro-particles in the buffer as

well as from low level electrical noise) can be a considerable nuisance, in that it can severely reduce the number of relevant events (i.e. cells) recorded in an acquisition.

### Low Threshold



### High Threshold

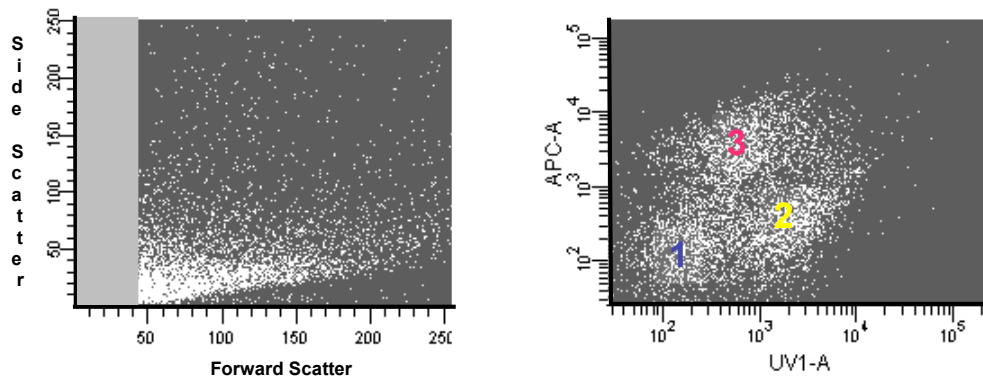


Figure 6 Use of a threshold to exclude background noise. (Plots by courtesy of Ditte Andersen)

To overcome this problem, a threshold is set on the FSc detector and the cytometer is, thus, instructed to ignore all events displaying signals under this level. As illustrated in figure 6 (upper panels), a low threshold setting (10 units,) results in the registering of extraneous signals from cell debris etc. which comprise the majority of the 10,000 events recorded. The APC-A/UV-1 fluorescent pattern, which is primarily of low intensity (area 1), reflects this fact. By setting the threshold up to exclude these events (to 40 units, lower panels), the flow cytometer is commanded to register only signals derived from the cells in the sample. The fluorescent pattern

resulting from this mode of acquisition clearly reveals the presence of two cell sub-populations (2 and 3). *NB! Some low-fluorescent events (1) are still recorded with the high threshold: these could either be from a third, negatively-staining, population of cells or from particles with forward scatter signals that exceed the threshold.*

While all FC provide for threshold setting on the FSc detector (as the best discriminator between cells and noise), some of the more sophisticated instruments, used for cell sorting, also allow for threshold setting on other parameters.

## Cell doublets and how to get rid of them

No matter how careful one is in preparing cells for FC analysis and irrespective of which types of cells are under investigation, there will always be a few events recorded by the FC which consist of cell doublets. These may either be the consequence of two cells becoming physically attached to each other, or arise because two cells have passed through the laser beam so close to each other that they have been "seen" as a single event.

Such an occurrence, involving two different types of cell, will result in the FC

recording that a cell displaying the characteristics of both cell-types was present in the sample (a "double-positive"), and, if one were seeking just such a rare cell-type, the existence of doublets could be highly misleading. Also in cell sorting, it is important to be able to distinguish between the single cells of interest and doublets comprised of the desired cell and a silent passenger (i.e. lacking any exclusion markers) to ensure the purity of the sort.

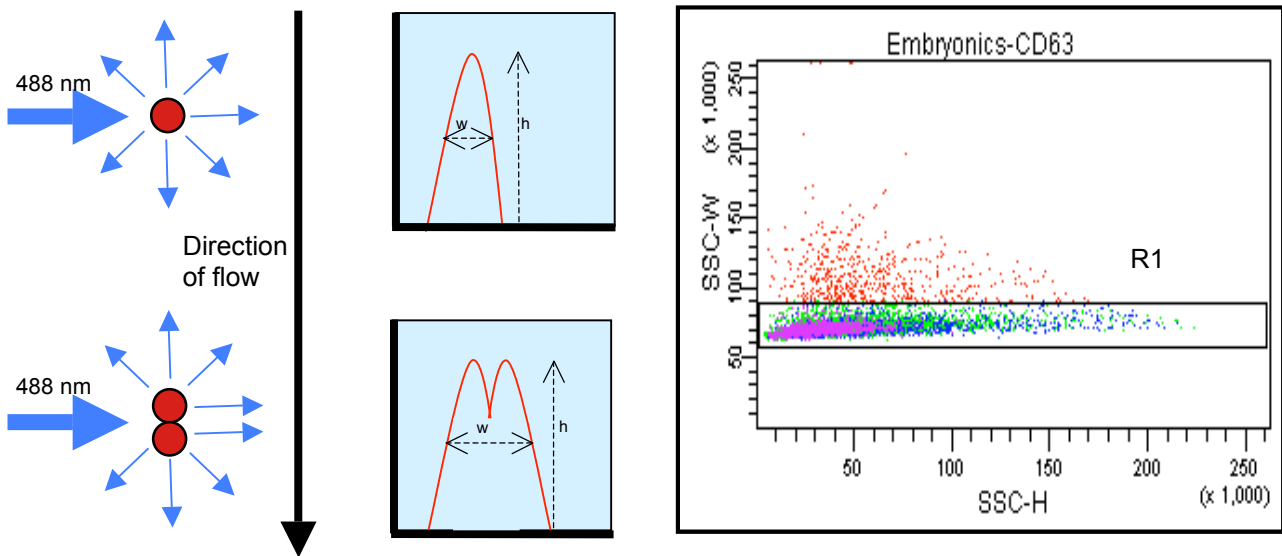


Figure 7 Doublet exclusion. Cells passing through the laser beam elicit a SSc signal the strength of which remains the same irrespective of whether the cell is alone or present as part of a pair. On the other hand, cell pairs – because they tend to align themselves with the direction of the flow – elicit a signal that lasts twice as long as single cells. This is reflected in the width of the signal peak. Thus doublets can be distinguished from single cells using a SSc-height vs. SSc-width dot plot (the red dots) and excluded from the data acquired. (Plot by courtesy of Ditte Andersen)

So how do we guard ourselves against such events? Fortunately the FC can distinguish between single cells and doublets; not by *strength* of signal they emit, but by *the length of time* the signal lasts. Physical doublets, at least, have a tendency to line themselves up with the direction of flow, so their signal lasts ca. twice as long as with single cells (see Fig. 7). The time that elapses during their passage through the

beam is registered as peak width. Thus, for example, a plot of SSc peak width against peak height provides for clear discrimination between single cells and doublets. A region (R1), set to encompass just the singlets (see section, "Regions and Gates", for details), can be used to eliminate doublets from the subsequent data acquisition or sort.

## Regions and Gates

Regions and gates are used to select - usually on the basis of two or more parameters - cell sub-populations for acquisition and sorting ("Live Gating") or for subsequent analysis with respect to further parameters.

### What is difference between a region and a gate?

A *region* is a tool used to identify a specific group of cells in a histogram or dot

plot on the basis of their signals in 1 or 2 parameters, respectively. Thus, on a histogram, a region consists of a bar spanning a signal intensity range, within which the cells of interest are to be found (Fig. 8a), while, on a dot plot, cells clustering within a particular coordinate range are identified by regions consisting rectangles, ellipses or freely drawn areas (Fig. 8b).

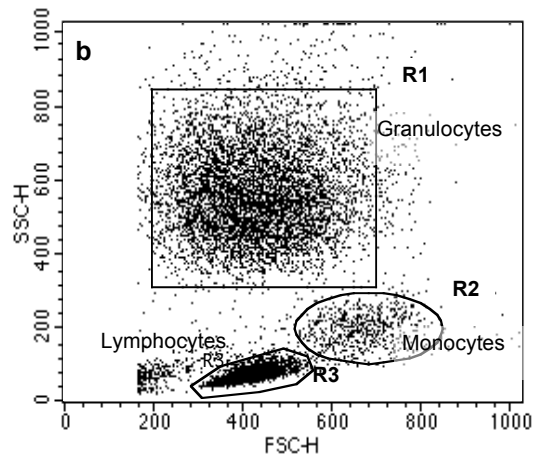
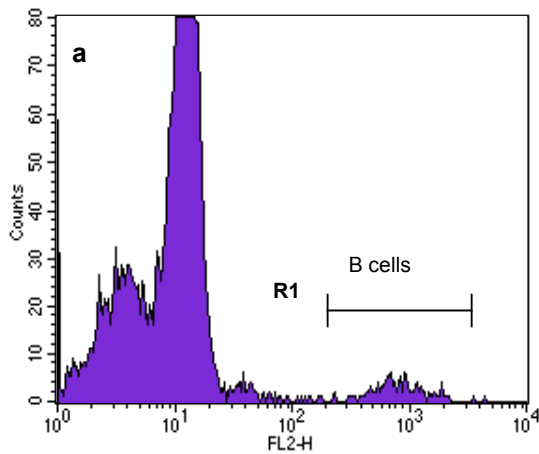
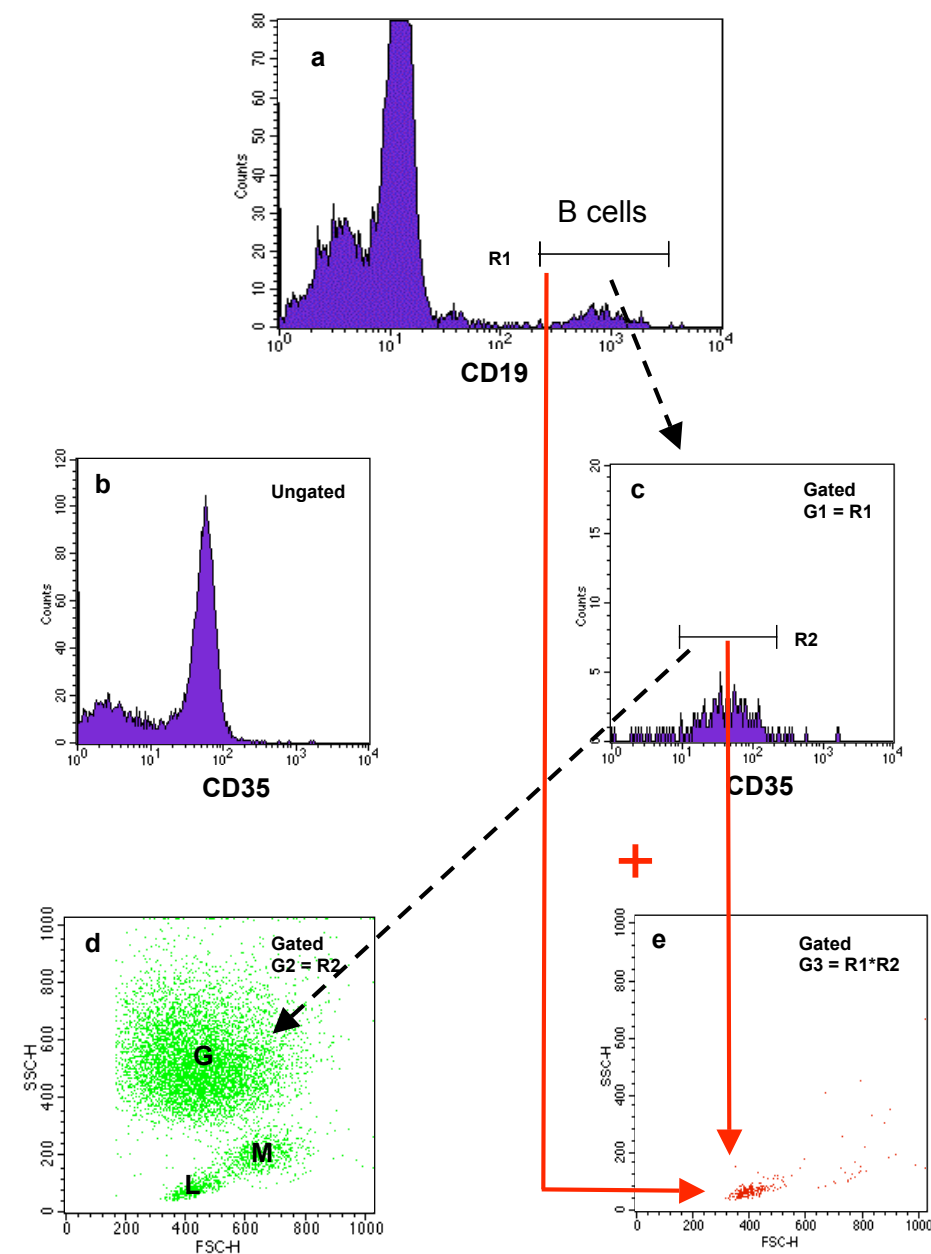


Figure 8 Setting regions to identify cell sub-populations. a) The B cell-specific marker CD19 is used to identify these cells in a leukocyte preparation using a region bar on a CD19-fluorescence histogram. b) Granulocytes, monocytes and lymphocytes are identified on a FSc vs. SSC dot plot using a rectangular, an ellipsoid and free-drawn region, respectively.

Gates are the selection operators one uses to command the computer to provide further information (or perform sorting) on the cell sub-population(s) identified by the regions. When a region is defined, the computer software automatically assigns it to a gate R1→G1, R2→G2 etc., and these can be used as such without further ado. However, in many cases, one will want to define the cells of interest by their inclusion in (or exclusion from) *more* than one region. Composite gates can be constructed ("G4 = R1 and R2", "G5 = R1 or R2", "G6 = R1 and *not* R2" etc) to fulfil these requirements. Thus, while a region can only include 1 or 2 parameters, a gate can encompass 3, 4 or even more.

### NB! Automatic Gates comprise just *one* region.

A common misconception is that a region drawn on a *gated* histogram or dot plot generates a new gate that automatically includes the restriction defined by the first gate. In the example shown here, CD19 is used to define the B cell population (see Fig 9a) for subsequent analysis of complement receptor type 1 (CD35) expression by these cells. CD35 is expressed by most blood leukocytes (granulocytes, monocytes etc.) and it is the signal from these cells that dominate the CD35-staining picture (Fig 9b).



plot.

On the other hand, the CD19 gate ( $G1 = R1$ ) efficiently selects the B cells for CD35 analysis (Fig. 9c). Should we be interested in examining the morphology of these CD35-expressing B cells, it might be tempting to make a new region, R2, on the B cell ( $G1$ -gated) CD35 histogram (Fig. 9c) and use this to re-examine the cells for their FSc vs. SSc pattern. However, the  $G2$  gate that is automatically created in this situation consists *only* of R2 (a fluorescence

range in the FL1 channel) and takes no account of the restriction under which this region was created in the first place (the  $G1$  gate on FL2). Thus, when  $G2$  is used to examine the FSc/SSc properties of cells showing this fluorescence all leukocytes, which fulfil the FL1 criterion, are included (Fig 9d). To examine the CD35-expressing B cells in this context a new gate,  $G3$ , including *both* R1 and R2, as selection criteria, has to be constructed (see Fig 9e).

Figure 9 Simple and composite gates. a) The histogram generated by probing whole blood leukocytes for expression of the B cell-specific marker, CD19. The B cells are identified by the region bar, R1. b) The fluorescent profile generated by probing whole blood leukocytes for complement receptor type 1 (CD35). The histogram shows a complex profile reflecting heterogeneity of expression of CD35 by the different leukocyte sub-populations. c) By gating on R1 ( $G1 = R1$ ), the CD35 signal from the B cells is revealed as a single broad peak (plus some peripheral signals). The region set on this histogram ( $R2$ ) identifies the CD35-positive B cells. NB! The automatic gate ( $G2$ ) derived from setting  $R2$  *only* specifies the CD35 fluorescent intensity range, which is shared by many other leukocytes in the preparation. d) Using just  $G2$  to investigate the morphology (i.e. FSc/SSc pattern) of the CD35-expressing cells reveals that granulocytes (G), monocytes (M) and some lymphocytes (L) all express CD35 to the same degree as B cells. e) To see the pattern for the CD35-positive B cells, a composite gate,  $G3$ , has to be constructed specifying that the cells of interest have to lie both in R1 and R2. Not surprisingly, these cells are to be found in the lymphocyte region of the dot

### **Live Gating**

Gating is not just an analytical tool! Gates can also be used to select a sub-population during the acquisition phase (a useful ploy if the cells of interest are present in the cell preparation at a very low frequency; e.g. 1 % or less). The gate is defined in the same way as gates used for analysis. Thereafter, there are two options for data acquisition:

a) the FC records the signals for *all* cells passing through laser beam while basing its count only on those cells registered within the gate (e.g. 1,000 B

cells are counted for determining the *size* of the acquisition file, which will also contain data on the 50,000 or so other leukocytes that have also passed through the FC) or

b) it will only register data on the 1,000 selected cells and throw away all information on the other cells passing through the system. This approach is particularly useful in the investigation of very rare cells, where the size of the data files would otherwise be enormous. *NB! Live gating is also essence of cell sorting, where cells, identified by the gate, are separated from all the others.*

## Multicolour Mode and Back-gating

Events located within a defined region (and thereby automatically assigned to a gate) can be displayed in a dot plot with a colour that indicates to which gate it belongs (in the so-called "Multicolour Mode"). Thus in the example in Fig. 10, blood leukocytes stained with anti-CD35 have been gated in the histogram in accordance with their degree of expression of this marker and these gates have been used to indicate

which cells bear these various levels of CD35. It is apparent that granulocytes express the largest amounts of CD35 and monocyte expression lies at an intermediate level while the lymphocytes (the majority of which are T cells) are registered as negative for this marker. (Note that B cells, which express CD35 at a similar level to granulocytes, are "drowned" in this form of display).

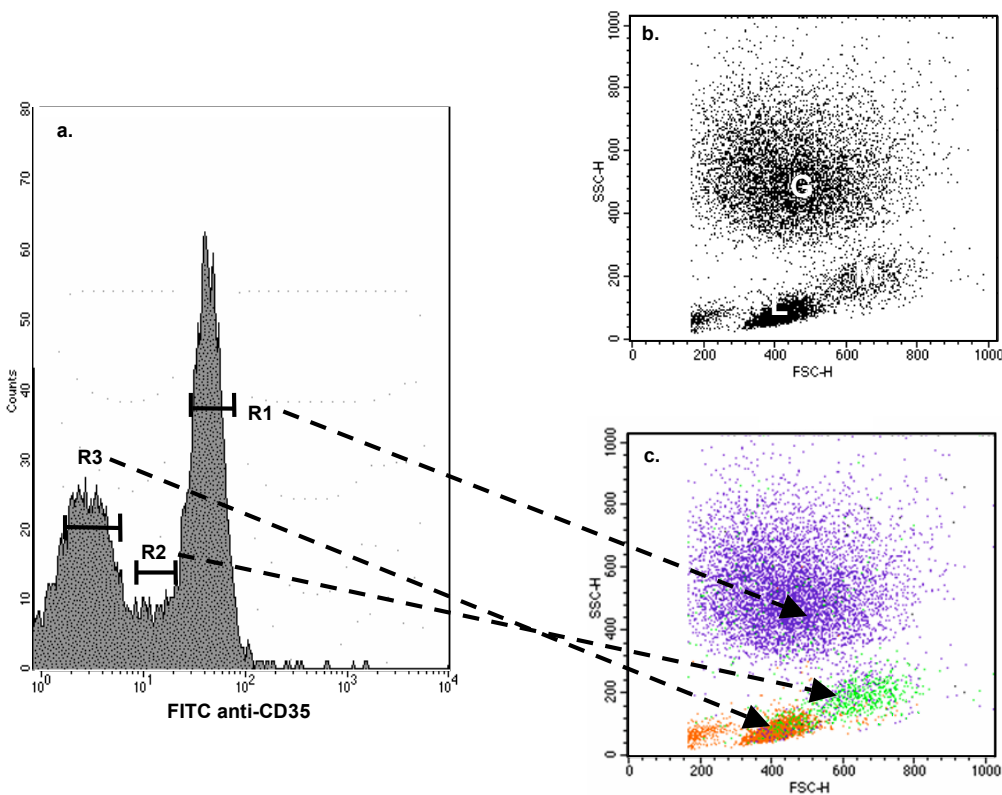


Figure 10 displays a) a histogram for the staining of blood leukocytes with FITC-anti-CD35, where the fluorescence profile has been divided into 3 regions corresponding to high, intermediate and low (negative) staining with the probe. On the right are two FSc vs. SSc, dot plots for the same cells in b) single colour and c) multicolour modes, respectively. Note that there is some mixing of the colours, which reflects overlap in CD35 expression by the various leukocyte sub-populations.

The advantage with this procedure is that it provides information about *all* the cells in a mixed population simultaneously (cf. Fig 6 where the use of gated dot plots displayed only cells positive for a particular characteristic). The disadvantage lies in its lack of sensitivity with regard to infrequent cells within the population.

### Back-gating

The normal gating strategy is to use gates defined on the basis of 2, 3, 4 or more parameters to select a cell sub-population for examination in terms of yet another parameter. This pre-supposes that one already knows a lot about the sub-population in question. There are situations, however, where the only prior information



one has is that the cell of interest bears a particular cell-specific marker (e.g. a stem cell in a primary culture) and one wishes to use this information to elicit data on a range of other properties of the cell (e.g. morphology and the expression - or absence - of shared cell markers). Here, a gate set on the known parameter can be used to inspect all the other parameters collected, either as gated histograms or as gated or multi-colour dot plots. The exercise illustrated in Figure 10 is, in fact, an example of the back-gating approach.

Back-gating has many applications, for example identifying stem cells for sub-

sequent sorting, comparing the morphology - or auto-fluorescence - of dividing vs. non-dividing cells or looking for correlation between cell-receptor expression and other functional parameters, such as ligand uptake.

Back-gating can also be used as a form of scanning, where a small region is made on one graph (histogram or dot plot) and then shifted around while keeping an eye on the changes arising in the correspondingly gated plots for other parameters. The "moving picture" obtained can often be an exceedingly effective and rapid way of acquiring new information.

## Fluorochromes

Fluorochromes are substances (usually aromatic compounds), which absorb light of one wavelength and emit light of another, longer wavelength. Physically speaking, the absorbed light excites an electron in the fluorochrome, which moves into a higher energy orbit around the nucleus. When it falls back again, the energy is released, partially as light and partially in other forms. The longer wavelength of the emitted light is simply a reflection of the reduction in the energy release as light.

The number of fluorochromes available for FC purposes has undergone an explosive growth in the course of recent years. This has partly been due to the development of FC with lasers capable of exciting fluorochromes at other wavelengths than 488 nm, and partly from the development of new fluorochromes. Of

particular importance, in this connection, has been the development of so-called tandem fluorochromes, where the light emitted by one fluorochrome (e.g. Phycoerythrin PE) is used to excite a covalently attached second fluorochrome (e.g. Texas Red), which then emits the light to be registered by the PMT. This development has expanded considerably the number of fluorescence emissions (i.e. colours) that can be obtained from the use of a single laser. *NB! It is most important that the transfer of energy from the first to second fluorochrome is highly efficient: otherwise the un-captured light from the first fluorochrome will interfere with the signal derived from markers conjugated only with the first fluorochrome.*

Some of the most commonly used fluorochromes are listed in Table 2.

**Table 2. Useful fluorochromes**

---

**Excitement at 488 nm (blue laser):**

**FITC, Alexa Fluor 488**

**PE, PI**

**PE-TX red, PE-Cy5, PerCP**

**PerCP-Cy5.5, PE-Cy7**

**Excitement at 633 nm (red laser):**

**APC**

**APC-Cy7**

**UV/Violet\* excitement:**

**Hoechst 33258** (viability), **Hoechst 33342** (side population sorting),

**Indo-1** (Ca<sup>2+</sup>-flux),

**Alexa fluor 405, Pacific Blue**

---

A new generation of fluorochromes is represented by the fluorescent semiconductor nanocrystals (e.g. Quantum Dots, Invitrogen), where the wavelength of light emitted is directly related to the size of the crystal. The main advantages of these fluorochromes, from an FC point-of-view, is their sharp emission peaks and the broad range of wavelengths at which they can be excited (i.e. they can all be activated by just one laser). Furthermore, they do not bleach, which means that they

can be excited repeatedly without loss in fluorescent intensity (a feature of more importance for fluorescence microscopy than FC). A number of FC probes are now available as nanocrystal conjugates.

*NB! Not all fluorochromes emit light with same intensity: FITC is a relatively weak light emitter whereas PE is highly fluorescent. One should try to ensure that the signals obtained with various markers are more or less similar by using a weaker fluorochrome to detect a highly expressed cell component (e.g. CD45) and vice versa.*

## Compensation – a thorny problem.

Fluorochromes, unfortunately, do not emit light at just a single discrete wavelength, but rather over fairly wide range, as shown in Fig. 11a. The light from the various fluorochromes is collected through band pass filters (the grey boxes) covering the wavelength range for maximum emission of each fluorochrome.

These will inevitably also register the leading or trailing tails of the emission spectra from neighbouring fluorochromes resulting in intensity signals that are virtually impossible to analyse with any accuracy. So how do we extricate ourselves from such a mess?

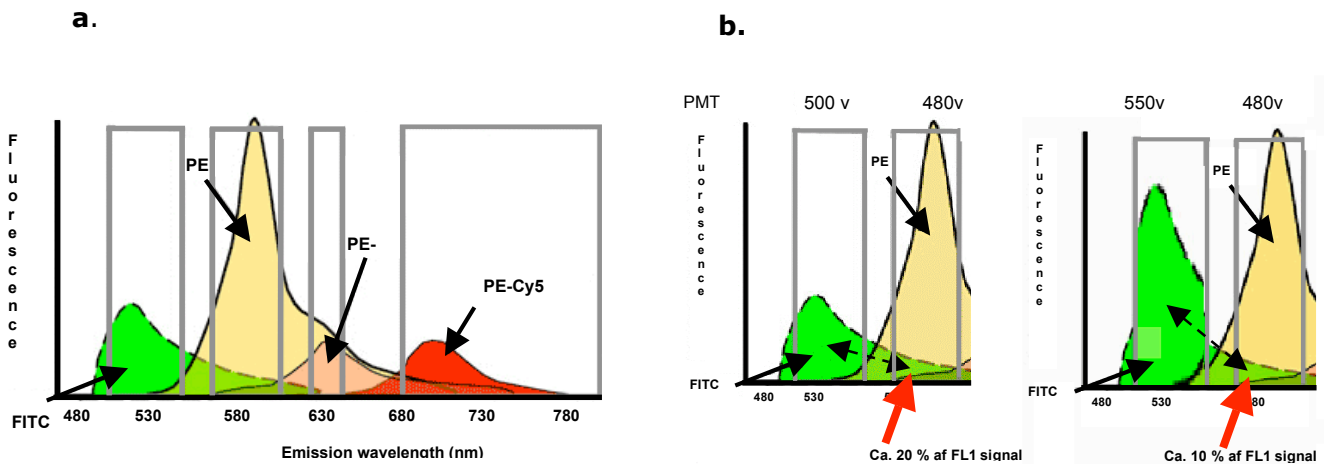


Figure 11 a) The fluorescence spectra of FITC, PE and two tandem conjugates of PE (PE-TR and PE-Cy5) illustrating the overlaps between these spectra and the degree of signal spill over between neighbouring detection channels (grey boxes).

b) The effect of changing the PMT voltage for one detector (FL1) on the signal intensity recorded by this detector and the degree of spill over to the neighbouring detector

The answer is in fact quite simple. A bead sample bearing just one of the fluorochromes is run through the FC and the fluorescent signals from these beads are recorded by all the fluorescence detectors in use. Since the signals in the peripheral detectors are directly proportional to the signal registered by the prime detector (i.e. the overlap signal is a constant % of the prime signal), it is possible to instruct the FC to automatically deduct the appropriate percentages of the signal registered in the prime detector from those recorded by the peripheral detectors. This process is termed *compensation*, and is performed for each of the fluorochromes used in a particular experiment.

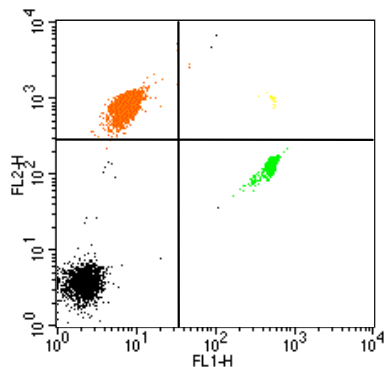
However, it is important to remember that a set of compensation values *only* applies to the particular comb -

ination of PMT voltages that have been chosen for a given acquisition. Any change in the voltages means that the compensation has to be set again. In the example shown in Fig. 11B, the initial voltage settings for the FL1 and FL2 PMT are 500 v and 480v, respectively. With these settings, the signal from FITC (a FL1 channel fluorochrome) registered by the FL2 detector is about 20 % of the signal registered by the FL1 detector itself (left hand panel). The compensation setting for removal of the FITC signal from the FL2 channel would therefore be "FL2 - 20 % FL1". If the voltage on the FL1 detector is increased by 50, this results in an approximate doubling of the PMT sensitivity (i.e. the FITC signal detected is twice as large). On the other hand, the signal size in FL2 remains unchanged and thus comprises

only 10 % of the new FL1 signal. Therefore the compensation has to be reduced to "FL2 - 10 % FL1. to maintain the proper signal removal from the FL2 channel. Fig. 12

portrays the actual procedure involved in such a compensation.

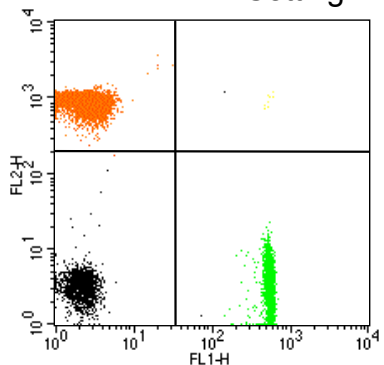
### Setting 1 - No Compensation



FL1 setting = 505 V  
 FL2 setting = 506 V  
 FL1 - % FL2 = 0 %  
 FL2 - % FL1 = 0 %

Quad	% Total	X Mean	Y Mean
UL	60.6	8.1	871
UR	0.20	429	1589
LL	18.1	2.2	4.33
LR	21.1	517	139

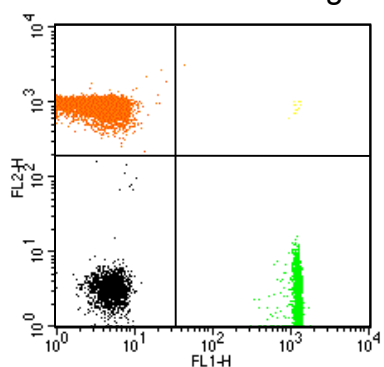
### Setting 1 - Compensated



FL1 setting = 505 V  
 FL2 setting = 506 V  
 FL1 - % FL2 = 0.6 %  
 FL2 - % FL1 = 26.3 %

Quad	% Total	X Mean	Y Mean
UL	61.3	2.5	876
UR	0.09	485	933
LL	17.5	2.23	3.42
LR	21.1	539	3.14

### Setting 2 - Compensated



FL1 setting = 556 V  
 FL2 setting = 506 V  
 FL1 - % FL2 = 1.5 %  
 FL2 - % FL1 = 11.7 %

Quad	% Total	X Mean	Y Mean
UL	61.4	3.65	872
UR	0.13	1091	1005
LL	17.8	5.13	3.66
LR	20.7	1226	2.61

Performing compensation between 2, 3 or even 4 colours can be achieved manually (i.e. by running single coloured beads and juggling with FLx - % FLY bars provided in the acquisition menu), although the complexity of the procedure increases

Figure 12 Calibration beads (Calibrite, BD), conjugated with FITC (green) and PE (orange) or unconjugated (black) were run on a FACScan FC using similar voltage settings for the FL1 and FI2 detectors without compensation (top panel). Quadrant analysis (see later) of the resulting FL1 vs. FL2 dot plot revealed that FITC registered with a mean fluorescence intensity of 517 units in the FL1 channel and 139 in the FL2 channel i.e. an spill-over into FL2 of ca. 25 %. Similarly the signals for PE were 871 and 8.06 in FL2 and FL1 (spill-over = <1 %).

The next run was performed after setting compensation values that brought the bead signals down to background values on the cross-over channels. This required a 0.6 % compensation for PE in FL1, and a 26.3 % compensation for FITC in FL2 (middle panel).

The voltage on the FL1 PMT was then increased by 50 volts. This resulted in FITC dots sinking below the x-axis of the plot (not shown) and it required a reduction of the FL1 compensation in FL2 to 11.7 % to restore the balance bottom panel. Note that this squares with a doubling of the FITC signal in FL1 channel (to 1227 units) while the signal for PE (and hence the FITC spill-over!) is unchanged.

*NB! Changing the PMT voltage does not only affect the FITC compensation in FL2, it also affects compensation in the reverse direction. The reason for this is that the signal for PE spill-over into the FL1 channel has also been doubled. Therefore, to return the PE dots to their proper position the FL2 compensation in the FL1 channel has to be correspondingly increased (to 1.5 %).*

exponentially with each additional colour. FC with the capacity to analyse larger numbers of fluorochromes usually include a compensation package in their software to perform this task.

## Autofluorescence – the bugbear of FC!

Autofluorescence is the term used to describe the fluorescent signals derived from the cells in the absence of fluorescent probes. For live, resting cells – such as freshly drawn blood leukocytes – the autofluorescent signal is generally low and does not seriously interfere with the signals derived from fluorescent probes. Dead, or dying, cells and cells in an activated or proliferating state (e.g. cell-lines) may, on the other hand, display auto-fluorescent

signals that are of a similar magnitude to those obtained with the weaker probes.

The problem with autofluorescence is the breadth of the emission peak: it is essentially white light that is detected in equal measure by all FL detectors. Thus, the signal pattern obtained in a two-fluorescence plot is a population spreading diagonally up the dot plot (Fig 13).

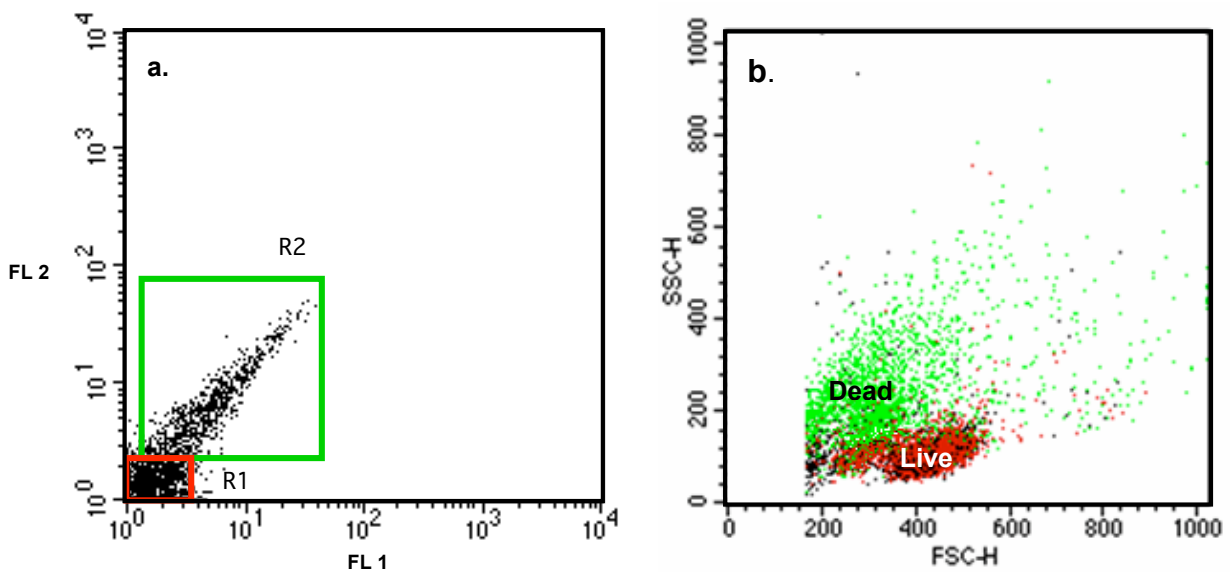


Figure 13. Autofluorescence arising from cell death. a. Blood mononuclear cells exposed to dexamethasone for 24 hours became autofluorescent, as indicated by the diagonal signal pattern. Regions, R1 and R2, were set to define low and high fluorescent sub-populations. b. Gating back on to the FSc vs. SSc dot plot revealed that these 2 populations displayed the morphology of live (red) and dead (green) cells, respectively (see next section).

Consequently, autofluorescence is not something that can be compensated away (the spectral overlap between one detector and the next is always ca. 100 %). You just have to live with it! Fortunately, cells that are positive for a particular marker, usually lie separated from the autofluorescence; either above or to the right of the diagonal in a 2 colour dot plot. Thus, at least, they can be readily identified (e.g. for sorting purposes). The major problem arises when quantitative data (e.g. marker expression)

is required from cells that are highly auto-fluorescent, in that the auto-fluorescence also contributes to the signal from the probe.

Here, the only option is to sacrifice one detector channel (e.g. FL2) to set compensation (ca. 100 %) for the autofluorescence in the neighbouring channels (i.e. FL1 and FL3). Not a particularly elegant solution, maybe, but at least it works.

## The FSc vs. SSc Dot Plot – more informative than you might imagine!

The FSc vs. SSc dot plot is the usual starting point for any cell analysis. It provides basic morphological information on the cells under study, enabling division of these cells into sub-populations, which can then be examined for their distinct properties with regard to other parameters (e.g. differential expression of surface markers). The use of this dot plot is, as we have already seen, particularly useful in the analysis of blood leukocytes, where the granulocytes, monocytes and lymphocytes segregate so clearly. The plot may also

prove of value in detecting morphological variability in a primary culture of tissue cells, while it is likely to be less informative in the investigation of cell lines, where by definition the population should be homogeneous (except, perhaps, with respect to expression of transfected or growth phase-related genes). Irrespective of the cell preparation under study, however, the FSc vs. SSc dot plot can prove informative about two other aspects of the cells' condition; their viability and their activation/proliferative state (Fig. 14).

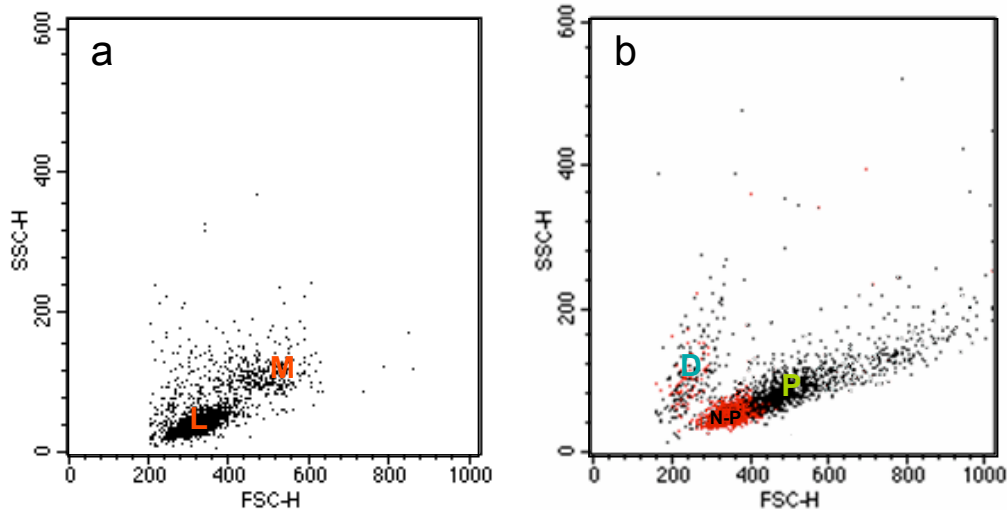


Figure 14 Dot plots of a) freshly prepared blood mononuclear cells i.e. Lymphocytes (L) and monocytes (M) and b) the same cells after 6 days *in vitro* culture with an antigen (Tetanus toxoid). Both proliferating (P) and dying (D) cells are present in the culture, as could be confirmed by staining with PI (to detect dead cells) and

the analysis of a proliferation marker (CFSE) – not shown. The red cell population consists of non-proliferating lymphocytes (N-P) that were *not* stimulated by the antigen. *NB!* By day 6 all the monocytes will have adhered to the culture plates and are, therefore, not to be seen in the dot plot.

### Cell death

Cell death is characterised by a fall in the FSc signal and an increase in SSc. The fall in FSc is popularly ascribed to a shrinking of the cells when they die. This may be part of the explanation but another influential factor here is that the cell membranes become porous allowing exchanges of ions etc. with the surrounding medium. This reduces the difference in refractive index within and outside the cells making them less effective as mini "lenses" to deflect the laser beam. Thus, irrespective

of any change in cell size, a fall in FSc would be observed. They change in SSc is less easy to explain.

### Cell activation and proliferation

Cell activation and, particularly, proliferation are accompanied by increases in both FSc and SSc. Again the FSc increase is generally more pronounced than that for SSc and is almost certainly a direct reflection of a change in cell in cell size. SSc increase in this case probably reflects an increase in the number of organelles in the active cells.

## Positive and Negative Controls

Irritating in their banality, controls are nevertheless the cornerstone on which all meaningful results are built. The controls we're talking about here are exclusively controls for the fluorescent signals derived from the cells (FSC and SSC cannot really be controlled, as such, though it may be of interest at times to compare the signals derived from cell under study with a reference cell population).

### Positive controls

When investigating a new system (a new cell-type or a new Ab conjugate) it is always advisable to include a positive control (e.g. a cell type *known* to express the antigen in question or an existing Ab conjugate with previously documented activity). A positive control is also of value in assessing hitherto uncharted characteristics of a particular cell type (e.g.

use of control cells that constitutively express a marker, which you wish to induce in the cell under study).

### Negative controls

Background fluorescent signals are obtained from *all* cells that pass through the laser beam. Various factors contribute to this signal; electrical "noise" in the system (probably only of significance where very high PMT voltages are used), the autofluorescence of the cells themselves and fluorescence arising from non-specific adherence of a probe, irrespective of the presence the corresponding cell marker. It is generally a good idea to check the autofluorescent contribution from the cells themselves by running them initially as an unlabelled preparation through the FC (see Fig 15).

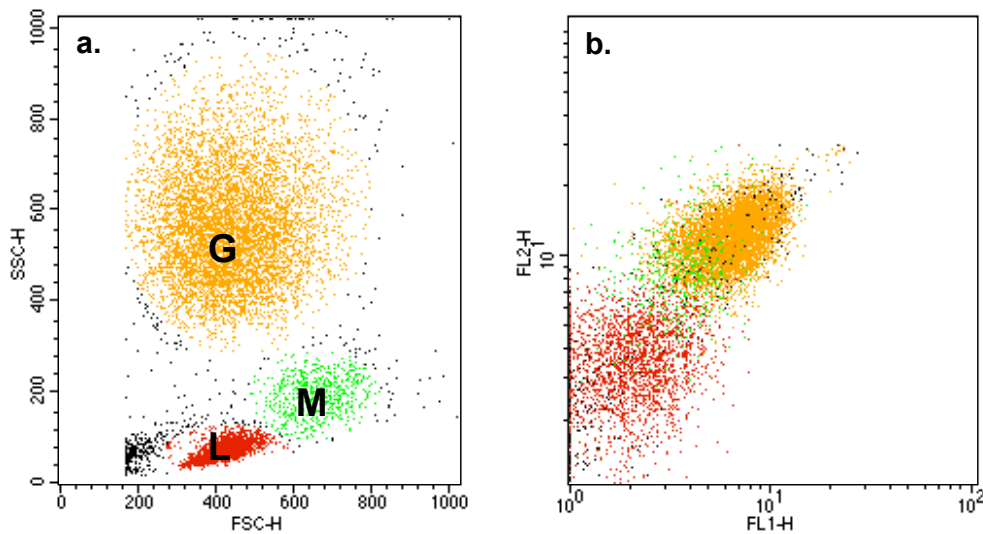


Figure 15 Autofluorescence. Unlabelled peripheral blood leukocytes examined at "normal" PMT settings (ca. 500 v on FL1 and FL2). Granulocytes (G), in particular, display a significant level of autofluorescence, which may amount to 10 % or more of the signal observed with the specific probe. Monocytes display an intermediate degree of autofluorescence, while lymphocyte autofluorescence is much lower. (Note that these signals correlate with the cytoplasmic complexity of the various cell types.) *NB! Any contribution from electrical noise will also be detected in this run.*

Non-specific binding of mAb-fluorochrome conjugates can arise from low-grade physicochemical interactions (charge, hydrophobicity etc.) between the probes and the cell surface or, especially in the case of leukocytes, interaction of the heavy chain C-terminal (Fc) domains of the antibody probe with so-called Fc-receptors

(FcR) on the cell surfaces. The contribution of these factors to the staining picture can be assessed with an "isotype" control i.e. an immunoglobulin preparation of the same class/sub-class as the probing antibody, which has been fluorescently labelled to precisely the same degree (Fig. 16).

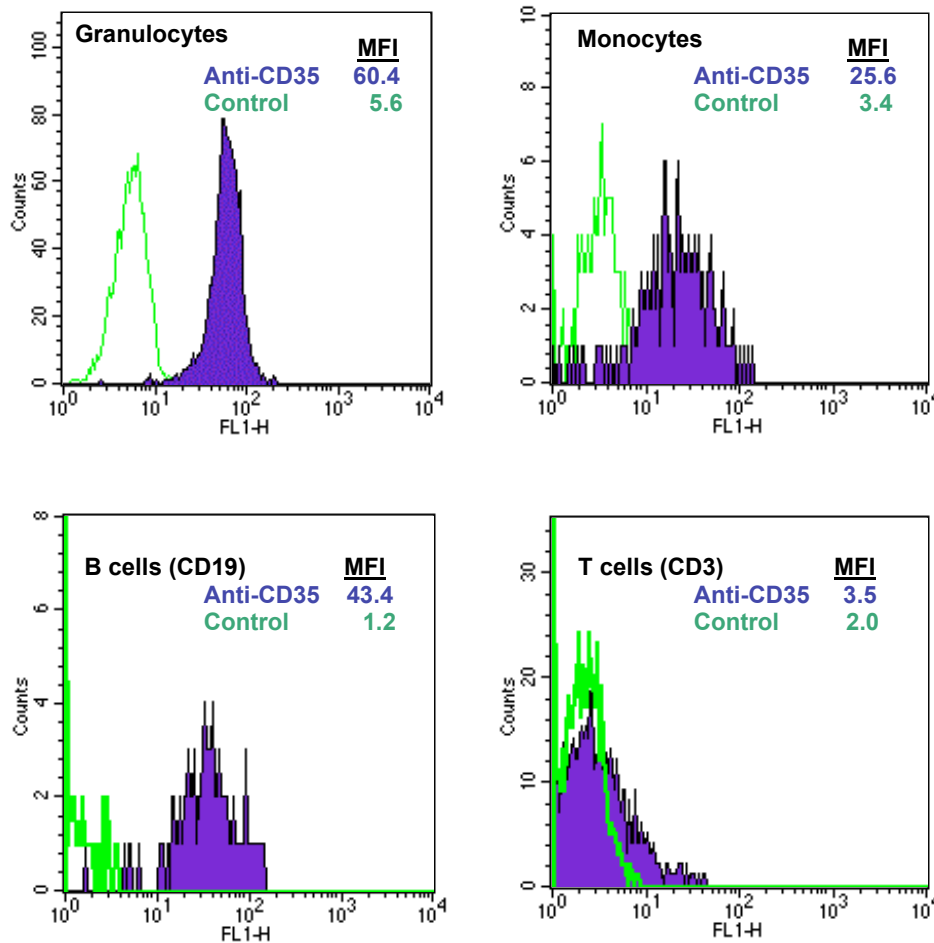


Figure 16. A freshly-drawn blood sample (with anti-coagulant) was diluted 1:1 with isotonic phosphate buffer and incubated with FITC-conjugated anti-CD35 (or isotype control), PE anti-CD19 and PerCP anti-CD3. After lysis of the erythrocytes with NH<sub>4</sub>CL, the leukocytes were analysed for expression of CD35, using their FSC vs. SSC characteristics, as well the presence of the markers CD3 or CD19 (to identify the T and B cell sub-populations, respectively). Note that the negative control reading for granulocytes (5.6 Mean Fluorescence Intensity units, MFI) amounts to almost 10 % of the total signal!!

### The Fc Receptor question.

When using the isotype control to assess signal background, it is *essential* that the contribution from Fc-FcR interactions is *blocked* (by the inclusion of at least 10 % homologous serum or 5 mg/ml unlabelled IgG) during the staining procedure. This is of particular importance if the cell sub-population of interest is also known to bear FcR.

The reason that this precaution is so vital is that a probing antibody will, in all likelihood, react with *both* the surface antigen *and* the FcR simultaneously ("cross-linking") and so the presence of FcR will not enhance the number of probe molecules binding but simply enhance their binding avidity (see Fig. 17). Since these two forms of binding are not additive, deduction of the signal derived from FcR-binding of the isotype control is not appropriate and would result in *underestimation* of the number of

probe antibodies binding to their cognate antigen!

*NB! Fc-FcR interactions are not likely to present a serious problem when probing directly with fluorochrome-conjugated monoclonal antibodies (mAb). This is because the antibodies in question are of very high affinity ( $K_d = 10^{-9}$  to  $10^{-12}$  M) and are 100 % pure, so the concentrations used to ensure saturation binding to the antigen (ca. 0.5-2.0  $\mu$ g/ml) are well below the those required to achieve major uptake of the isotype control by the low-affinity FcR ( $K_d = 10^{-5}$  to  $10^{-8}$  M). On the other hand when probing with polyclonal antibody preparations and/or polyclonal secondary antibody conjugates (indirect labelling), the higher working concentrations required (20-50  $\mu$ g/ml) may result in a disturbing "Fc effect".*



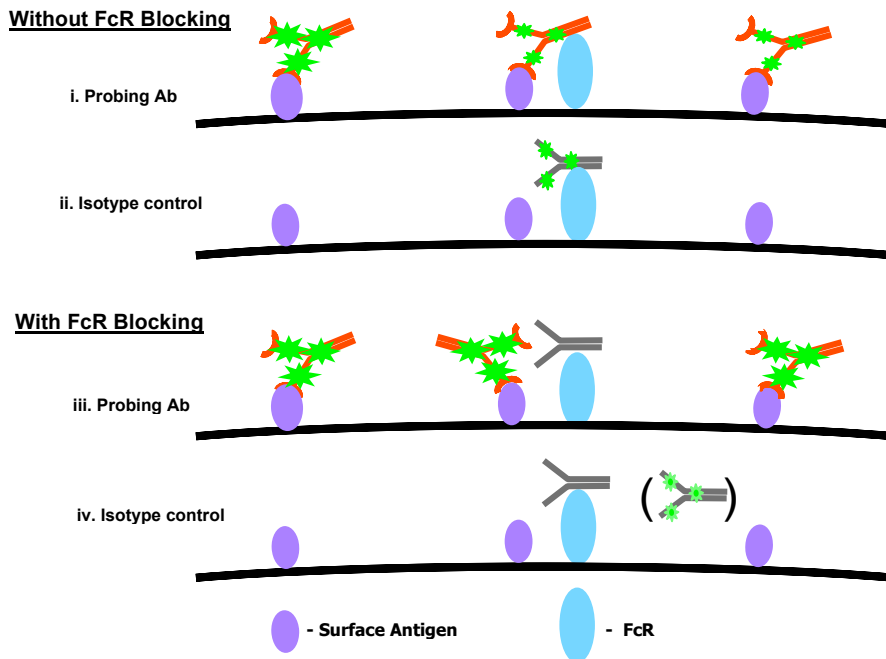


Figure 17 Isotype control – use and misuse. With cells bearing both Ag and FcR (i.), the probing Ab may bind to *both* entities simultaneously (cross-linking). Thus, the presence of FcR does not enhance the number of probing Ab taken up by the cell. Without FcR blockade, the isotype control binds to this receptor (ii), giving a falsely high estimate of non-specific binding. Blockade with unlabelled Ig prevents both the probing Ab (iii) and the isotype control (iv.) from binding to the FcR. Any isotype control uptake, now, represents the true background.

### Controlling for reproducibility

When performing a larger number of identical experiments on the FC (especially, for example, when examining cohorts of donors or patients for one or other characteristic of their cells) it is most important to ensure that the FC is

performing reproducibly. The best way of doing this is to ensure that all experiments are carried out using the same detector settings and running standard fluorescent beads (e.g. BD's Calibrite) before each run to check that the signals recorded from them remain the same.

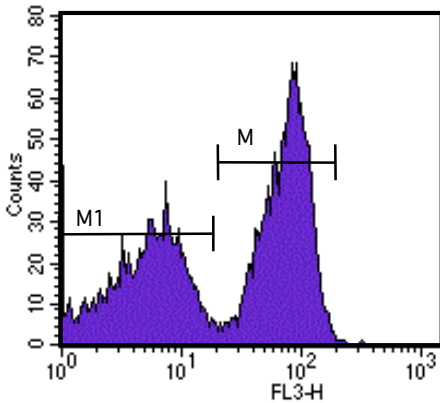
## Data Analysis

Analyses of data acquired by the FC can be regarded as falling into two main categories:

- cell-population size analysis and
- quantitative, or semi-quantitative, analysis of the fluorescent signals

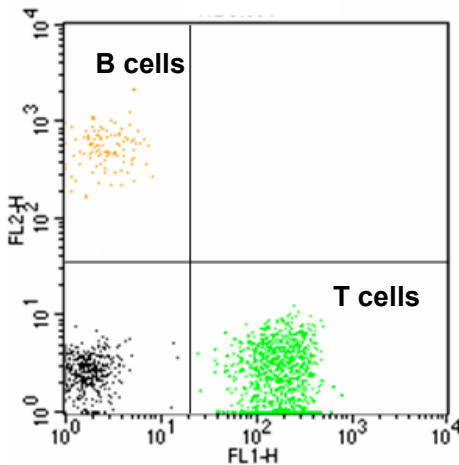
derived from individual cell sub-populations.

Dot plots are most commonly used for the first purpose, while quantitative data is derived, in greatest detail, from histograms.



### Histogram statistics

Marker	Events	% Gated	% Total	Mean	CV	Geo Mean	Median
All	9892	100.0	13.1	49.4	88.4	23.6	46.6
M1	4157	42.0	5.50	6.05	61.7	4.83	5.52
M2	5708	57.7	7.56	80.9	37.1	75.0	79.2



### Quadrant statistics

Total Events:  
Gate: G1 Gated Events = 1809

Quad	Events	% Total	x Mean	y Mean
UL	105	1.05	3.01	570
UR	0	0.00	***	***
LL	427	4.27	1.89	2.77
LR	1277	12.8	199	2.66

Figure 18 Histogram markers and dot plot quadrants: the two basic statistical tools. *NB! For the sake of clarity the statistics tables have been edited to show only the most relevant data.*

### Histogram statistics

Setting marker bars on a histogram rapidly provides a wealth of information about the cells thus selected (Fig. 18). For the first, they provide basic information about the number of cells present and what % they constitute of both the total cell population and of the gated sub-population selected to generate the histogram. In addition, quantitative information about the

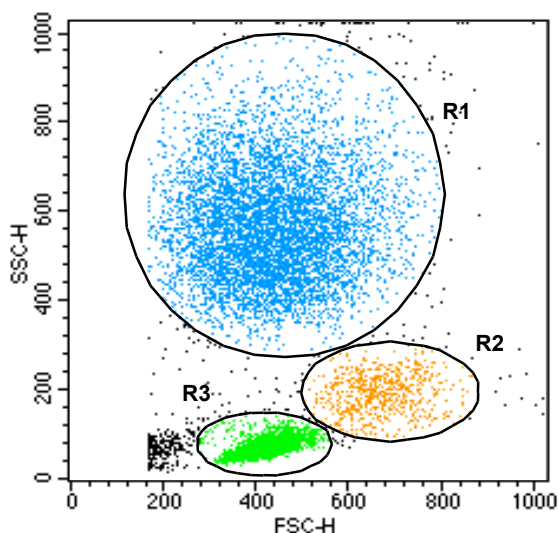
fluorescence emitted by the selected cells is provided in the form of Arithmetic (AM) or Geometric Means (GM) of the fluorescence intensity, as well as the Median value for the fluorescent peak. Finally, a statistical assessment of the spread of the selected fluorescence peak is presented as the Coefficient of Variance (CV) and/or Standard Deviation (SD) of the AM. Most FC software programmes also provide for

statistical comparison of populations represented in two or more histograms.

### Quadrant statistics

The quadrant statistics (Fig 18. package provides essentially the same degree information about the cell

population as well as a measure of signal intensities in the form of AM or GM (not shown here). Median values and measures of statistical variability, however, are not provided.



### Region Statistics

Total Events: 10000

Region	Events	% Total	x AM	x GM	y AM	y GM
R1	6421	64.2	434	417	566	554
R2	655	6.55	669	665	190	185
R3	2460	24.6	419	416	72.7	70.4

### Gate Statistics

Total Events: 10000

Gate	Events	% Total
G1	6421	64.21
G2	655	6.55
G3	2460	24.6
G4	9536	95.36

Where G4 = R1 + R2 + R3

Figure 19 A Fsc vs. SSc dot plot illustrating the use of Region and Gate statistics.

### Region Statistics and Gate Statistics

In situations, where the groups in a dot plot cannot be segregated using a quadrant (e.g. as in Fig. 19), region statistics provide a useful alternative. The information derived by choosing this option is essentially the same as that obtained with a quadrant. The information provided by choosing Gate Statistics, on the other hand, is limited to population size and distribution.

### AM, GM or Median?

The eternal question: which means of expressing fluorescence intensity should I use? The fluorescent signals from a single cell population are often widely spread and not necessarily distributed in a Gaussian

fashion. Therefore, GM or median values might seem the most obvious choice.

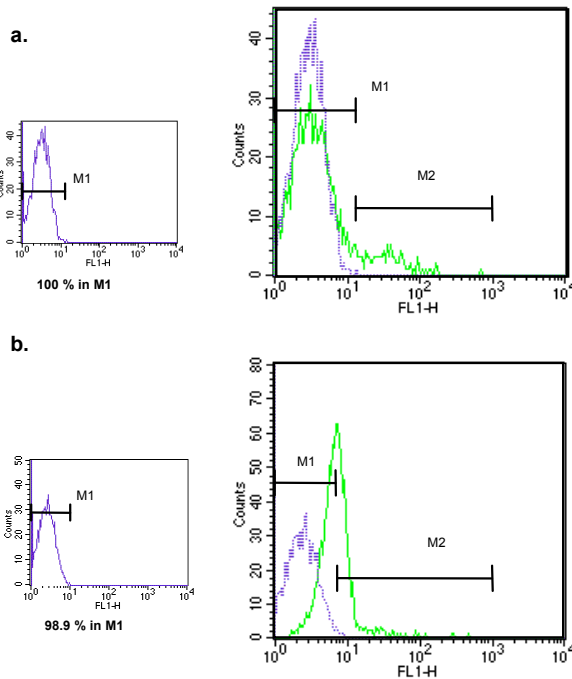
On the other hand, one has to take into account the type of analysis that is to be performed. For calculation of cell marker expression in absolute terms (i.e. as molecules per cell), AM is the logical choice. This also applies for any calculations involving subtraction (e.g. of a background). If data is to be expressed in the form of ratios (e.g. fold increase over an un-stimulated control), then GM values are most appropriate, while median values are the correct option for making non-parametric statistical comparisons (e.g. for comparing properties between patient and control groups).

## Estimating Population Size

Probably the most common use of flow cytometry is to estimate the size of a cell sub-population, or more precisely, to determine what % of the total cell population (or a sub-group) is represented by the cells under study. (NB! The FC was not designed to provide absolute cell numbers, although there are ways of making it do so – e.g. by inclusion of known numbers of fluorescent beads in the cell sample) Quadrant statistics are the easiest method of doing so, providing that the various cell groups are well separated from each other by the two phenotypic markers employed to construct the dot plot. If the

segregation is more complex (i.e. does not readily fit into quadrants), then region or gates statistics can be applied.

Histograms are also used for this purpose, especially to examine heterogeneity within a defined sub-population (e.g. dividing vs. non-dividing cells or cells displaying enhanced expression of a particular membrane component or synthesising a particular cytokine). Here, however, it is of prime importance that one distinguishes between genuine heterogeneity with regard to the response and a homogeneous, albeit weak, response by the whole sub-population.



Marker	Events	% Gated	Mean
All	3749	100	6.2
M1	3476	92.7	3.6
M2	273	7.3	39.1

% positive cells = 7.3 %  
**RIGHT!**

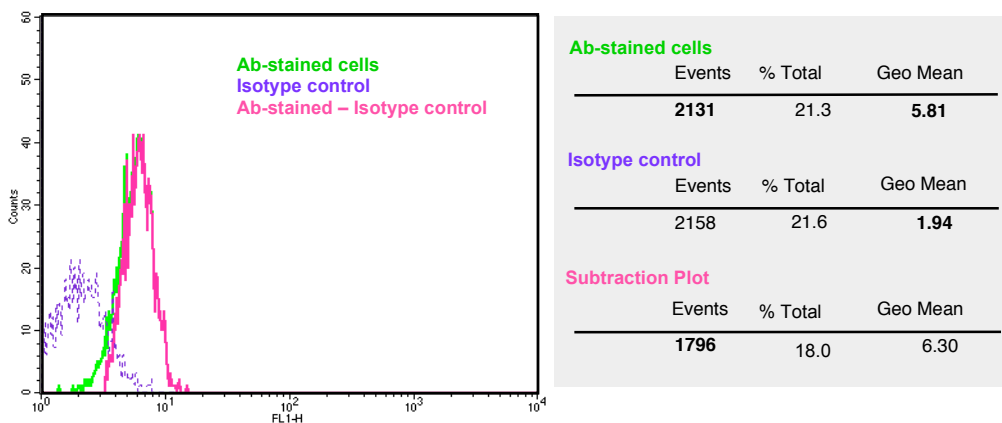
Marker	Events	% Gated	Geo Mean
All	4394	100	7.3
M1	2553	58.1	5.3
M2	1699	38.7	10.3

% positive cells = 38.7 %  
**WRONG!** (= ca. 100 %)

Figure 20 Appropriate (a.) and inappropriate (b.) determinations of population size. The isotype control (left-hand panels) has been used to set the marker (M1) that defines the negative cell population. This marker was copied to the overlap histogram where the Antibody-stained cells are displayed (in green). M2 was then set to indicate the positive cell population. The statistics shown are those for the cells incubated with the antibody.

An all-too-common misuse of population size estimation is illustrated in Fig. 20. Whereas, in Fig. 20a, the probing antibody clearly demonstrates that the cells under study segregate into two populations (a major, negative population, with the same fluorescence profile as the isotype control, and minor positive population showing up as a leading peak), the cells in Fig. 20b show a small, but uniform, displacement to higher fluorescence than the control. While

it is appropriate, in the first case, to use the isotype control signal, to determine a limit below which cells may be regarded as negative, this is not the case in Fig. 20b, where, by the threshold criterion, only 38.7 % are deemed to be as positive, although almost all the cells are giving a signal above background.



Option 1. % positive cells	= 1796/2131 x 100
	= <b>84.3 %</b>
Option 2. Signal : Background ratio	= 5.81/1.94 : 1
	= <b>3.06 : 1</b>

Figure 21 An "overlay and subtraction" histogram. An FL1 histogram for the Ab-stained cells was called up and the Isotype Control (IC) profile was added as an overlay. The cells from the IC registering in the same FL channels as the Ab-stained cells were then deducted from those registered by Ab-staining to give the subtraction plot. Statistics were requested for all three plots. The data employed in computing options 1 and 2 are shown in bold

So how do we get around this problem? One option would be to use a subtraction plot, where, for each fluorescent intensity channel, the number of cells registered using the isotype control is deducted from the number of antibody-stained cells (Fig. 21). However this does not take in account the degree of overlap that may exist between background and weak-positive fluorescence. The best option, in this case, would be to shift focus from population size to signal intensity for the *whole* population and express the activity as a ratio to the background fluorescence, using the geometric means for all the antibody-stained cells and the isotype control, respectively, in the determination.

*NB! The "percentage above threshold" approach can be employed with a uniform cell population when it is the probing antibody that is under study rather than the cell population itself. Thus, for example, it would appropriate to use the parameter "% cells registering as positive" in testing sera for the presence, say, of specified anti-microbial antibodies. One could, to this end, define the Ab content as the dilution of serum that registers a particular % of the target microbe (e.g. 50 %) as positive, in an indirect staining assay, or, alternatively, in terms of the % positive events observed at a pre-determined serum dilution. Testing sera in this way is likely to be less sensitive than measuring the intensity of the fluorescence signal generated by the serum antibodies but may, on the other hand, prove more robust*

## Quantifying the Fluorescence Signal

The signal registered, for a particular fluorochrome, is quantitative in the sense that it is directly proportional to the amount of fluorochrome bound to the cell under examination. Thus, if one cell expresses twice as much marker as another, its signal will be also twice as great. The only problem is that the signal registered is dependent on the voltage setting for detecting PMT, and this can be changed at the operator's will. Increasing or reducing the voltage by 50 v will, for example, double or halve the recorded signal, respectively (see section "Setting the Signal Intensity"). So how do we convert the semi-quantitative signal into an absolute value (i.e. the number of marker molecules expressed per cell)?

The answer is quite simple – calibration. By running beads bearing known amounts of fluorochrome (or probing antibodies) on their surfaces, a calibration curve can be constructed and the signals from the cells can then be converted to absolute numbers by interpolation on the curve. If the binding ratio of the probing antibody for the marker is known (usually 1 : 1 for monoclonal antibodies, mAb) then this information can be used to determine the number of marker molecules expressed by the cell. *(NB! This only applies when the beads are run on to the FC at the same voltage setting as used for the cells; any change in setting for the cells will also require a new calibration run.)*

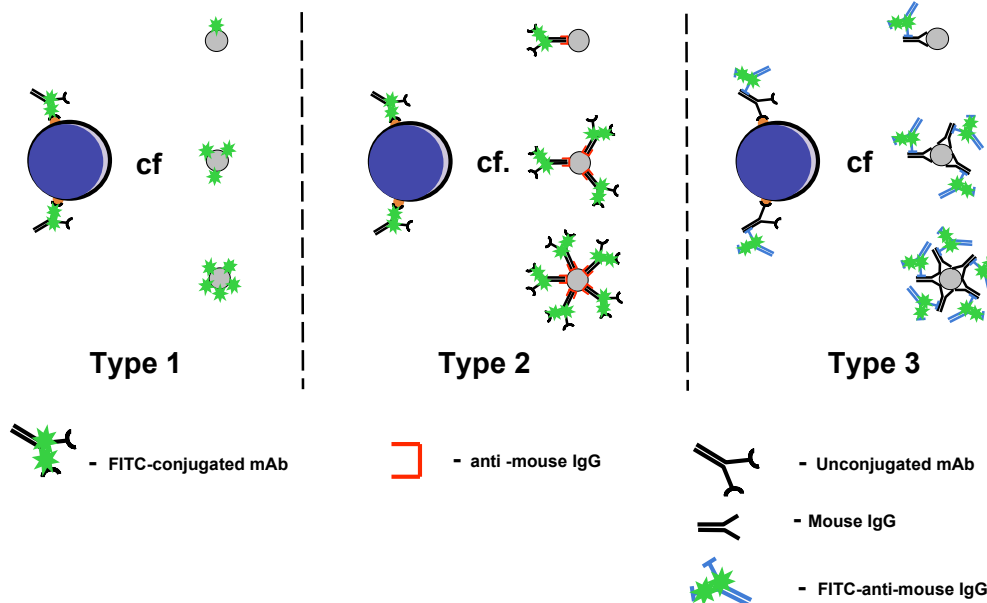


Figure 22 Three types of calibration beads. *Type 1* are coated with known numbers of fluorochrome molecules of the same type as used in the experiment. *Type 2* are beads coated with known numbers of Ab molecules recognising the fluorochrome conjugated mAb used in the experiment. *Type 3* beads bear known numbers of mouse IgG molecules, which are recognised by the fluorescent anti-mouse IgG Ab, used in indirect labelling of the cells

under study.

There are three general procedures for calibration for which the reagents are commercially available:

1. A series of beads (e.g. Quantum MESF beads, Bangs Laboratories, Inc. <http://www.bangslabs.com/flow/catalog.php>), which are coated with the fluorochrome in various amounts that have been measured as

corresponding to particular numbers of fluorochrome molecules in free solution (i.e. as Soluble Fluorescent Equivalents, SFE). If the specific fluorescence of the probing antibody is measured in the same way, then the cell signal can be converted first to SFE and then numbers of bound

antibodies in a simple two-step calculation.

2. Beads coated with known numbers of antibodies against the mouse mAb used as probe ("Simply Cellular" beads, Bangs Laboratories). If these beads are treated using exactly the same procedure as for cell labelling then the signal they emit can be used to convert the cell signal directly from the recorded Mean Fluorescent Intensity to number of probing antibodies bound per cell. *NB! This applies irrespective of whether the cells have been directly or indirectly labelled, but the primary antibody has to be a mouse mAb*
3. Beads coated with known numbers of mouse IgG molecules (QiFikit, DAKO, [http://www.dako.dk/index/prod\\_se\\_arch/prod\\_products.htm?productar\\_eaid=7&baseprodidver=A11112000g](http://www.dako.dk/index/prod_se_arch/prod_products.htm?productar_eaid=7&baseprodidver=A11112000g)) that are then incubated with the fluorochrome-conjugated secondary antibody (e.g. FITC-goat anti-mouse IgG) that is used in the cell study. Again this system enables direct conversion of the cell signal to the number of probing mAb bound per cell. *NB! The restriction with this kit is that it can only be used to calibrate indirect labelling studies.*

The first approach is the most reliable in that it does not involve any manipulation of the beads (they are just run on to the FC as they are). The disadvantages, though, are i) that it requires that the operator has access to a spectrofluorimeter to measure the specific activity of the probing antibody, in the same way as the beads were originally characterised and ii) that it is only applicable to direct-labelling studies. The other two approaches are simpler but assume that the labelling procedure used to

give optimal staining of the cells will also be appropriate for optimal labelling of the beads. Thus errors can arise if this not the case (if, for example, the saturation of the bead sites requires a higher concentration of probing antibody than is the case for the cells). In this respect, the Simply Cellular beads are most susceptible to error in that the binding reaction between the probing antibody and the beads (mouse mAb to anti-mouse IgG) is not the same as that for the cells (mAb to cell marker). Indeed, the mouse mAb/anti-mouse IgG interaction may be of considerably *lower* affinity. Thus, it is important, particularly with these beads, to check that mAb concentration used is sufficient to ensure saturation of the bead sites.

### **Quantifying CD35 expression on B cells**

The following example of a quantitative investigation using FC employs beads of the first type as the calibrating standard.

The initial stage in the procedure is to measure the specific fluorescence activity of the fluorochrome-conjugated antibody. This requires the use of a spectrofluorimeter which provides the same excitation wavelength (e.g. 488 nm for FITC excitation) and equipped with the same band-pass filter for collection of the emitted light as is used in the FC. The fluorescence signal that is obtained from a known concentration (or preferably two) of the conjugated mAb is then compared with the fluorescence signals measured from a serial dilution of the free fluorochrome. In the experiment shown in Fig. 22a, the two concentrations of the FITC-conjugated mAb employed (6.67 and 13.3 nM) emit light that corresponds to free FITC in solution at concentrations of 7.79 and 15.3 nM, respectively. The specific activity of the antibody is determined by dividing the free FITC concentration by that of the antibody resulting, in this case, in measured specific activities of 1.17 and 1.15 Soluble Fluorescein Equivalents/IgG, respectively, at the two FITC-mAb concentrations employed. The mean of these two values (1.16 SFE/IgG) is then used in the quantitation.

The next stage in the procedure is to calibrate the signal derived from the FC

at the PMT voltage chosen to investigate the cells (Fig 22b). Commercially available latex beads bearing various, known, amounts of FITC (expressed as SFE/bead and determined by the same procedure as used with the FITC-mAb) are run through the FC. A curve is then plotted of the fluorescence signals - obtained with each population of bead - against the stated SFE

values for these bead populations. *NB! In plotting the curve, the net FIU values are used (i.e. after deduction of the fluorescence signal observed with blank beads). This is important because the latex beads exhibit an autofluorescence quite different from that of the cells.*

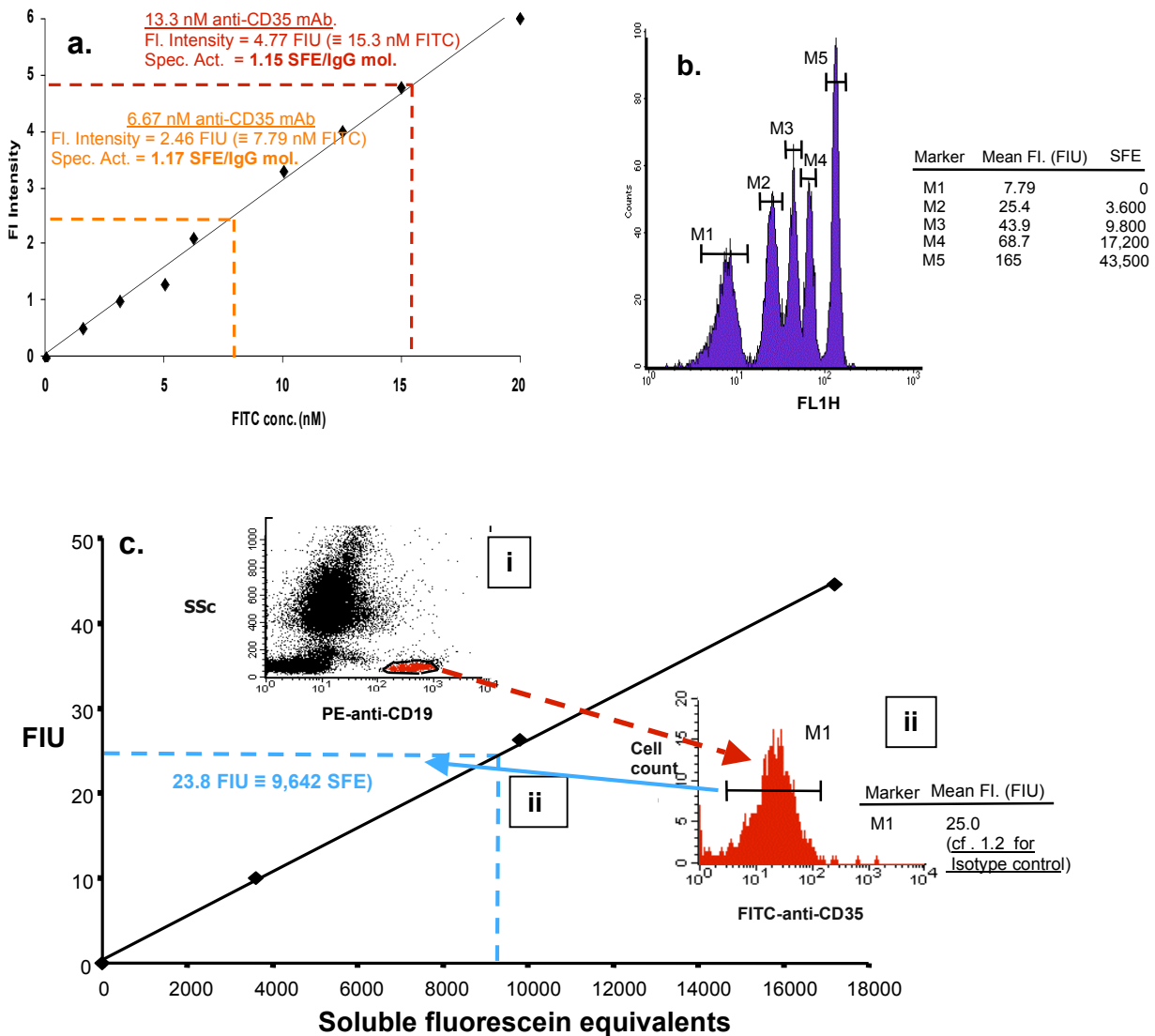


Figure 22 Quantifying CD35 expression on B cells in a whole blood preparation, using a FITC-coated latex bead standard. a. The specific activity of the FITC-conjugated anti-CD35 mAb (HB8592) is determined by comparing the light emitted from known concentrations of the conjugate with that from free FITC in solution. b. Calibrating the FC with commercially obtained latex beads bearing known amounts of FITC. c. The assay: whole blood (100  $\mu$ l) was diluted 1:1 with buffered saline and incubated with FITC-conjugated anti-CD35 Ab (2  $\mu$ g/ml) and PE-conjugated anti-CD19 (a B cell marker). The erythrocytes were removed by lysis and the leukocytes run on the FC. The data from two thousand B cells were acquired using a live gate based on their SSs vs. PE signal (red region in c.i). For analysis, an FL1 (FITC) histogram was used and the mean fluorescent intensity of the cells was determined using the Histogram statistics package (see c.ii). The net value for peak fluorescence (after deduction of the signal registered by the isotype control) was plotted into the calibration curve to convert the signal into SFE (c.iii).



The system to be quantified should, by now, seem quite familiar; CD35 expression by B cells. Whole blood was incubated with the FITC-labelled Anti-CD35 mAb at a concentration previously determined to be sufficient to achieve saturation binding (most important!). PE-anti-CD19 was used to identify the B cell and data was acquired using a live gate to ensure that the desired number of B cells (2,000) was examined. Using histogram analysis, the signals obtained with both the anti-CD35 antibody and the corresponding isotype control (FITC-conjugated mouse IgG1) were recorded. The calibration curve was then used to convert the net signal with the mAb from (the arbitrary) fluorescence intensity units (FIU) into absolute soluble fluorescence equivalents

(SFE). This value (9,642 SFE) was then divided by the specific activity of the mAb (1.16 SFE/IgG) to determine how many anti-CD35 mAb were bound, on average, per cell; in this case, 8,312 per B cell. Having previously shown that the mAb binds its antigen in a 1 : 1 ratio, we now have a measure of how many CD35 are expressed per B cell. *NB! This quantitation was performed on a sub-population that amounts to only 2-5 % of the total leukocyte population in a simple single-step procedure taking little over an hour. By using the live gate only for counting the B cells (while collecting data on all the cells passing the laser) we could have simultaneously performed quantitation on all the cell subpopulations in the blood with no extra time expenditure.*

## Postscript

The purpose of writing this guide has been to introduce you, the reader, to the basic principles of flow cytometry, to give an impression of its strengths as an analytical tool and to alert you, as new user, to some of the pit-falls that lie in wait to trap you.

Not covered by the guide are the wealth of applications to which FC can be put; investigative procedures for examining, in essence, every aspect of a cell's life and death. Table 3 lists just some of these.

### Table 3 FC applications in cell research

#### "Superficial" characterisation of the cells

1. Phenotypic characterisation and determination of cell sub-populations
2. Quantifying membrane proteins
3. Kinetic investigations of ligand-receptor interactions (including ligand distribution between different cell types in a mixed population).

#### Investigations of cell activation

4. Mobilisation of intracellular Ca<sup>2+</sup> ions
5. Tyrosine phosphorylation
6. Gene transcription
7. Expression of surface activation markers (CD69, CD25 on T cells; B7, MHC class II on B cells etc.)
8. Cellular proliferation

#### Investigation of cellular activities

9. Protein synthesis (e.g. Cytokines)
10. Phagocyte function (Phagocytosis/"Respiratory Burst")
11. Cell-mediated cytotoxicity
12. Cell death (Apoptosis and necrosis i.e. programmed and non-programmed death).

#### Stem cell investigations

13. Stem cell identification and sorting on the basis of specific phenotypic markers and/or DNA-binding dye expulsion ("Side population" sorting).
14. Studies of induced stem cell differentiation.

Not only are there many fields of investigation to which FC can be applied, there are also, within each field, numerous application options for the technique. For example, cell proliferation can be investigated in four or more different ways (e.g. DNA content, appearance of gene-regulating proteins, DNA-analogue incorporation and cytoplasmic dye dilution), depending on which aspect of the process (cell cycling, proliferation kinetics, generation numbers, precursor frequencies

etc.) is of prime interest. Another example is the study cell death, where a number of parameters can be examined (e.g. Propidium Iodide uptake, Annexin V binding, Caspase expression or so-called Terminal deoxynucleotidyl transferase UTP Nick End Labelling, TUNEL) to discriminate between programmed cell death (apoptosis) and indiscriminate cell killing (necrosis).

The diversity, and complexity, of the investigations possible, using FC, put the

subject beyond the scope of this basic review. Instead, I refer you to other

information sources that may be of help (Table 4).

#### **Table 4 Sources of Information on FC applications**

##### **Books:**

Ormerod MG (Ed.): Flow Cytometry - A Practical Approach. 3rd edition. Oxford, Oxford University Press, 2000 (*Both readable and sound – always a good place to start looking*)

Darzynkiewicz Z, Crissman HA, Robinson JP (Eds.): Flow Cytometry. 3rd Edition. Methods in Cell Biology, Volumes 63 (Part A) and 64 (Part B). San Diego, Academic Press, 2000 (*Very comprehensive and comprehensible*)

Shapiro HM: Practical Flow Cytometry. 3rd Edition. New York, Wiley-Liss, 1995. (*FC to the very last detail. "The Bible", but definitely not bed-time reading!*)

##### **Monographs:**

Robinson JP, Darzynkiewicz Z, Dean P, Dressler L, Rabinovitch P, Stewart C, Tanke H, Wheelless L, (Eds.): Current Protocols in Cytometry, New York, John Wiley & Sons (*Continuing updates*).

##### **Journals:**

Cytometry, Flow Cytometry Methods, Journal of Immunological Methods

##### **Web sites:**

###### *Core Facilities*

NCI ETI Branch Flow Cytometry Core Laboratory

<http://home.ncifcrf.gov/ccr/flowcore/protocols.htm>

The Janis V. Giorgi Flow Cytometry Laboratory, UCLA

<http://cyto.mednet.ucla.edu/protocol.htm>

Cancer Research UK

[http://science.cancerresearchuk.org/sci/facs/facs\\_major\\_apps/?version=1](http://science.cancerresearchuk.org/sci/facs/facs_major_apps/?version=1)

Baylor College of Medicine, Texas Medical Center

[http://research.bcm.tmc.edu/Research\\_at\\_Baylor/adv\\_labs/flow/protocols.htm](http://research.bcm.tmc.edu/Research_at_Baylor/adv_labs/flow/protocols.htm)

###### *Manufacturers*

BD Biosciences/PharMingen

<http://www.bdbiosciences.com/pharMingen/protocols/>

All the above sources should prove useful, although the range of protocols available on the web is somewhat limited. Ultimately, the best approach is to sift through research articles dealing with your own, or

a very closely related, area of research, and see what methods the authors have used.

*Wishing you all success with your FC experiments!*

*Graham Leslie*