Chapter 1

Flow Cytometry in Clinical Haematopathology: Basic Principles and Data Analysis of Multiparameter Data Sets

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Introduction

Flow cytometry (FCM), as indicated by its name, is a semi-automated method, which combines two basic approaches, cytometry and flow, respectively.

Cytometry is by essence the measurement of cell characteristics. More broadly, it can be applied to various types of particles. In fact, the very first application of automated cytometry was invented by Wallace and Joseph Coulter with the first objective of counting paint particles [1]. Before that era, cell counts were performed manually under a microscope with specific calibrated slides called haemocytometers usually bearing the names of their inventors (Malassez, Thoma, Neubauer, Nageotte and others) [2]. Such devices also allow us to recognise some cell types based on their size and granularity, detectable without any staining, by simple phase contrast.

Other properties of cells can be examined with an optical bright field microscope after preparing smears or cytospins where the cells are spread in a thin monolayer fixed on a slide. Such preparations are then stained, most frequently with May Grünwald Giemsa (MGG) or Wright stains [3]. These panoptic stains contain eosin and methylene blue, plus azure for MGG, and thus make acidic components appear blue and basic components appear orange-red to violet. Smears or cell suspensions can also be labelled with antibodies conjugated to fluorochromes and examined under UV light in specially equipped microscopes.

The flow component of FCM is a liquid sheath that allows to convert a cell suspension in a narrow linear flux individualizing cells/particles. The flow part of the instrument performs what is called hydrofocusing, i.e. single cell alignment. Hydrodynamic focusing is achieved by injecting the cell suspension in the core of sheath fluid at a slightly higher pressure and at a point when the channel becomes smaller. The acceleration of the fluids through this narrow channel and the different speed of the sheath and the cell suspension result in cell alignment. Of note, there is no mixing of the cells/particles suspension with the sheath liquid. The latter can therefore very well be pure water, its main characteristic being to be devoid of any particle. The flux of cells/particles is guided in a specific device called a flow cell, through which a source of light will illuminate each cell as it passes in front of it.

The major advantages of FCM, compared to the methods briefly mentioned above, are that larger numbers of cells will be counted and that many parameters including immunological characterisation of cells will be examined. Moreover, all results will be electronically stored, remaining available for analysis at any time after data acquisition.

Cell Counting in FCM

Haemocytometers allow us to count cells in a well-defined chamber of 0.1 mm^3 using unmanipulated suspensions (i.e. cerebrospinal fluid) or diluted samples where red blood cells have been lysed. Data are then converted by calculation in the usual measurement units of events per mm^3 or per litre. For stained cells, typically, between 100 and 500 cells are counted manually when performing cell differentials. Both these methods are prone to errors linked to the small number of events actually taken into account and thus lack precision. This has been well established by Rümke, who designed a table displaying the decreasing level of incertitude associated with larger numbers of events counted [4].

Flow cytometry, that examines several thousands of events in a few minutes, provides a high level of sensitivity and exactitude. The relative numbers (proportions) of each cell subsets acquired will therefore be accurate. However, for exact absolute counting, flow cytometers require the use of standardised bead suspensions with a known number of beads per microlitre mixed volume/volume with the cells/particles preparation. When this known number of beads has been recorded by the instrument, it can therefore be concluded that 1 µL of sample has been examined. This can

then be extrapolated to the other cells recorded during the same time. Another possibility is to use calibrated volumetric systems built in the flow cell. Because of these large numbers, it also allows to identify minute populations, likely to be missed with smaller counts.

FCM and Light

Besides counting particles, FCM allows to appreciate their physical, chemical or biological properties. The major physical properties of particles/cells exploited by FCM are their ability to diffract, reflect and refract the coherent monochromatic light of a laser beam. Different types of lasers are available. Flow cytometers were initially equipped with gas lasers using argon, krypton, helium-neon or helium-cadmium, some requiring a cooling system. Solid-state lasers are crystals [ruby, yttrium aluminium garnet (YAG)] or ions such as titanium or chromium. More recent instruments use laser diodes based on semi-conductors and similar to light-emitting diodes (LEDs) [5].

When the narrow and focused coherent light of the laser encounters a cell/particle (so-called event), its diffraction intensity is proportional to the size of the 'event'. Flow cytometers are equipped with photodiodes collecting the light diffracted by the cell in the path of the laser beam, of forward scatter (FSC). The instruments are also equipped with a device (mask) blocking the laser light from the FSC photodiode when no particle crosses it. The beam is widened proportionally to the size of the cell as one enters the laser's path and the FSC photodiode can collect and transform it in an electronic signal proportional to the size of the cell/particle (Figure 1.1).

Concomitantly, a second detector (photodiode or photomultiplier tube (PMT)) collects the light reflected by the surface of the cell/particle as well as by any surface inside it (i.e. organelles, vesicles, granules, etc.) at a defined angle, lateral to the path of the laser beam. The intensity of this side scatter (SSC) signal will thus be proportional to the granularity of the cell. Typically, in a blood sample, the small erythrocytes with no nuclei will provide very small signals while those generated by the larger granulocytes will be more intense. The pattern of scatter signals will differ slightly between instruments, depending on the angle of the SSC detector and the number of display channels (see below in signal acquisition).

The voltage applied to the detector will also modify the intensity of light collected. It must be adapted to the type of cells investigated, i.e. it will have to be higher to see small particles such as platelets and lower to see larger cells such as granulocytes.

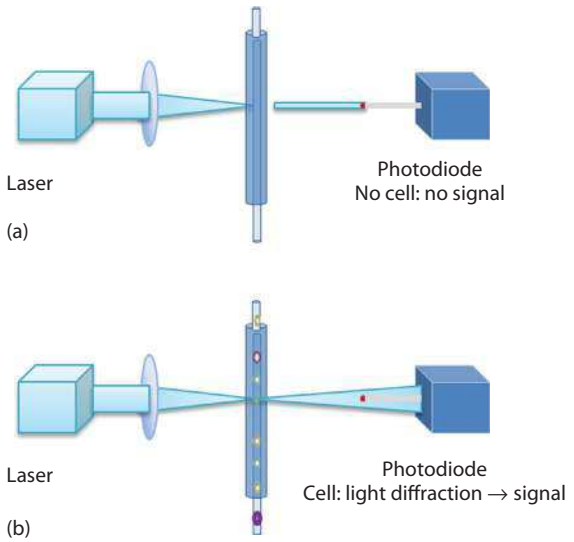

Figure 1.1 Forward scatter measurement of cell size. (a) The laser light is blocked by the mask when no cell passes through the beam. (b) Each cell, by diffracting light, allows for a signal proportional to its size to be collected on the detector around the mask.

Chemical parameters can also be measured by flow cytometers, typically based on the properties of fluorochromes. The latter are chemical substances able to absorb light at a defined wavelength and re-emit it at a higher and defined wavelength. This is based on the fact that, in these molecules, absorption of a photon will result in a modification of electrons, moving from a ground state to an excited state. When electrons return to their ground state, they restore the energy by going through transition stages resulting in the emission of a quantum of light with a higher energy and thus higher wavelength than the emission light [6]. Basically, in FCM, lasers provide excitation light and fluorochromes emission light. To collect emitted light from each fluorochrome, flow cytometers are equipped with dichroic mirrors and bandpass filters before each signal is registered by a dedicated PMT. Dichroic mirrors reflect light at a specific wavelength while letting all other light pass through. They are positioned at an angle from the emission source so that reflected beams make a 90° angle to the mirror and get directed towards the relevant PMT. Just before PMTs, filters of a specific wavelength will narrow the beam of light collected, ideally at the level of peak fluorescence.

Table 1.1 Selection of commonly used fluorochromes

Fluorochrome	Excitation wavelength	Laser	Emission wavelength
Pacific Blue	405	Violet	455
Brilliant Violet421™	405	Violet	421
BD HorizonV450™	405	Violet	455
Alexa Fluor405™	405	Violet	421
BD HorizonV500™	405	Violet	500
Krome Orange	405	Violet	528
Pacific Orange™	405	Violet	551
DAPI	405	Violet	461
R-Phycoerythrin (PE)	488	Blue	575
PE-Texas Red/ECD	488	Blue	620
PE-Cy5/PC5	488	Blue	670
PE-Cy5.5/PC5.5	488	Blue	692
PerCP-Cy5.5™	488	Blue	695
PE-Cy7/PC7	488	Blue	770
GFP	488	Blue	510
Fluorescein isothiocyanate (FITC)	488	Blue	520
Alexa Fluor 488™	488	Blue	520
PI	540	Blue	637
7-AAD	550	Blue	647
APC	643	Red	660
APC-Cy7	650	Red	667
Alexa Fluor 700™	650	Red	720
APC-Alexa Fluor 700™	643	Red	720
APC-Alexa Fluor 750™	650	Red	774

Cy = Cyanin, APC = Allophycocyanin, PerCP = Peridinin chlorophyll.

All of the above is valid for instruments with one laser. However, the development of multiparameter FCM has led to build flow cytometers with multiple lasers, thus broadening the possibilities of staining by using fluorochromes excitable at different wavelengths. Although the basic FSC and SSC parameters are usually measured on the 488 nm blue laser, separate pathways are then used to channel the emitted lights generated by the different lasers [7].

Fluorochromes

Fluorochromes (Table 1.1), also called fluorophores, are aromatic polycyclic carbohydrates, which can be found naturally in some algae (i.e. phycoerythrin or rhodamine) or organic synthetised compounds (i.e. fluorescein isothiocyanate) [8]. Some proteins are also fluorescent, such as the green fluorescent protein (GFP), which will stain cells transfected with its gene [9]. Some fluorochromes are used individually, such as propidium iodide (PI), which will fluoresce in red once intercalated in the hydrophobic environment of deoxyribonucleic acid (DNA) and is widely used together with annexinV to study cell death and apoptosis [10], or thiazole orange, which will stain both DNA and RNA and is widely used for the analysis of reticulocytes and platelets [11, 12].

Nowadays, fluorochromes are mostly used conjugated to monoclonal antibodies, allowing visualisation of the structures specifically recognised by the latter. Most of such conjugates (monoclonal antibody/fluorochrome combination) use single fluorochromes.

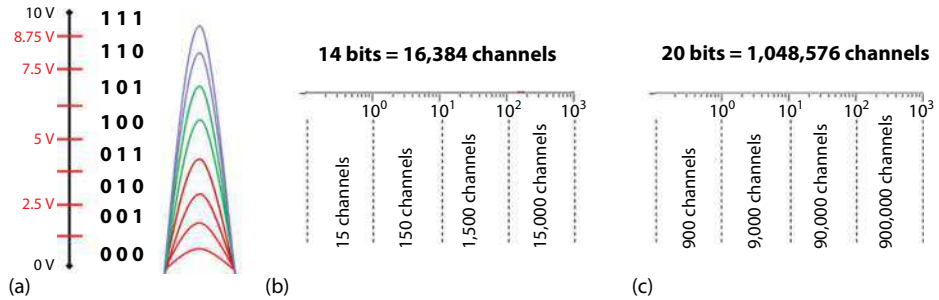

Figure 1.2 Principle of digital voltage partitioning and resulting sensitivity of fluorescence expression. (a) Example of a 3 bit partitioning of the signal between 0 and 10 V. (b) Number of channels obtained by using 14 or 20 bits digitalisation.

In some combinations, the properties of two different compounds are used in 'tandem' fluorochromes. This allows detection of emitted light from a fluorochrome, which cannot be excited by the available laser. The principle of such tandems is to use the light emitted by the first molecule to excite the second fluorochrome tightly bound to it and then measure the second emitted fluorescence. The principle used is also called fluorescence resonance energy transfer (FRET) [13].

A general very important property of fluorochromes is their sensitivity to light. For this reason, fluorochrome conjugates are provided in light-proof dark vials. Great care must be taken while manipulating these reagents to avoid exposure to direct light at all stages of the experiment. Some fluorochromes are also sensitive to pH, yet stable in the neutral conditions associated with most biological applications of FCM [14]. Finally, tandems can be unstable and get degraded. In this case, the fluorescence collected with them will thus erroneously be that of the first fluorochrome [15].

Among the numerous fluorochromes available, the major properties to examine are the wavelengths of their excitation and emission lights (Table 1.1), assuring that they are adapted to the parameters of the flow cytometer to be used. It is also important to know their level of brightness to choose a conjugate best adapted to the level of expression of the molecule to be stained.

Signal Acquisition

The signals sent to PMTs are very low and need to be amplified, hence the name of these collectors which increase the signal provided by collected photons. Amplification is performed by increasing the tension applied from 100 to 1,000 V [16]. Linear amplification can also be obtained by applying a gain value, usually for FSC and SSC. Each signal is transformed electronically by analogic/digital converters (ADC), which generate binary signals of 0 or 1. Typically, no or a low signal is 0 and a higher signal is 1. The sensitivity of the signals collected can be improved by increasing the number of bits, which are the combinations of 0 and 1 that are used to partition the range of voltage of the signal. For instance, a signal between 0 and 10 V can be dichotomised as 0 between 0 and 5 V and 1 between 5 and 10 V in a 1 bit digitalisation. Three bits digitalisation (i.e. all permutations between 000 and 111) will divide this voltage range in eight levels (2^3). Typically, flow cytometers use either 14 or 20 bits combinations, which yield 16,384 or 1,048,576 channels. The second combination provides a better discrimination for weak signals. Indeed, since the graphic representation of these signals generally uses logarithmic scales, on a four decades scale the first configuration will provide ~16 channels in the first decade and the second one ~900 channels (Figure 1.2). The difference will be minimal in the high voltages but will be visible in the low values.

These technical considerations may impact the settings of the instruments. When lower numbers of bits are used, the dynamic range of the logarithmic scale can be limited and the choice of PMT values can impact the resolution of the signals at low intensities.

Signals Displays

A number of software packages have been developed to analyse the signals provided by flow cytometers, some being incorporated in the instrument and used during acquisition and others (or portable versions of the same) used to perform analyses at distance [17].

The simplest way to analyse FCM signals is a monoparametric histogram where the signal intensity is displayed in the abscissa and the number of events in the ordinate. Because most applications of FCM are

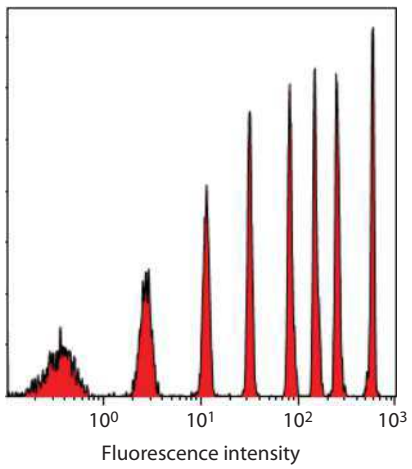

Figure 1.3 Monoparametric representation of the eight Rainbow® beads of different fluorescence intensities. Note that the signal gets narrower in the high fluorescence channels. Abscissa: fluorescence intensity. Ordinate: particle count.

to biological systems, this usually results in Gaussian peaks, the breadth of which depends on the variability of expression of the marker investigated. For fluorescent beads used to check the proper alignment of the laser, this peak should be very narrow, especially for bright fluorescence, and reflects the sensitivity of the instruments at low fluorescence (see above in Signal Acquisition; Figure 1.3). In a properly aligned instrument, the coefficient of variation of each peak (standard error divided by the mean) should be as low as possible, independently of the apparent width of the peak which will logically appear larger at low fluorescence values.

Biparametric histograms also called scattergrams or dot plots are used to display events based on two of their properties. They will thus appear as dots (each event resulting in a dot at the intersection between its *X* and *Y* values) forming one or more clusters depending on the populations present in the sample. The most basic biparametric histogram to check for proper selection of FSC and SSC settings (Figure 1.4a) uses these two parameters, most frequently on linear scales. As mentioned above, this is also used by other instruments such as cell counters and does not depend on fluorescence.

Displaying each fluorescence against SSC (Figure 1.4b) allows for a good appreciation of autofluorescence versus specific signals in a given population. With such a display for the analysis of examined samples (such as peripheral blood (PB), bone marrow (BM) or lymph node cell suspension), unstained cells provide an excellent internal control.

Biparametric histograms are also used to examine the relationship between two different fluorochromes (Figure 1.4c). The results can become extremely pertinent to small subsets if a succession of selection of relevant subsets is performed by drawing gates. Such gates can be coloured/painted and the use of a homogeneous colour code on a single platform greatly facilitates data interpretation.

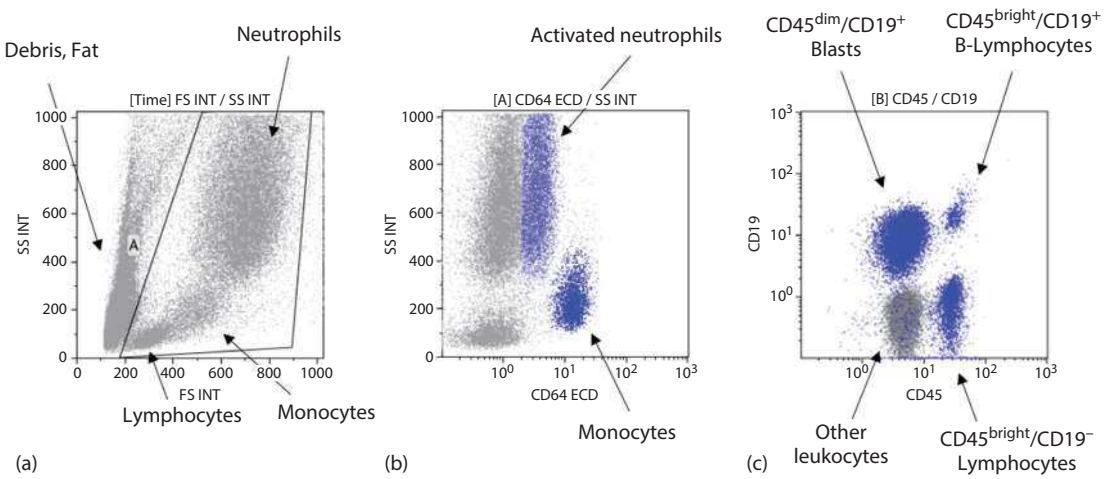

Figure 1.4 Flow cytometry displays: (a) biparametric plot of FSC and SSC, i.e. light diffraction without considering any fluorescence; leukocyte subsets are easily distinguishable and debris can be gated out from gate A; (b) biparametric plot of FL1 against SSC allowing for an easy distinction of positive subsets, here CD64+ monocytes (blue) and a subset of activated neutrophils (lighter blue), by comparison to unstained other subsets blood and (c) relationship between two fluorochromes, here CD45 and CD19 in a sample containing B-blasts, T and NK lymphocytes, B-lymphocytes and neutrophils.

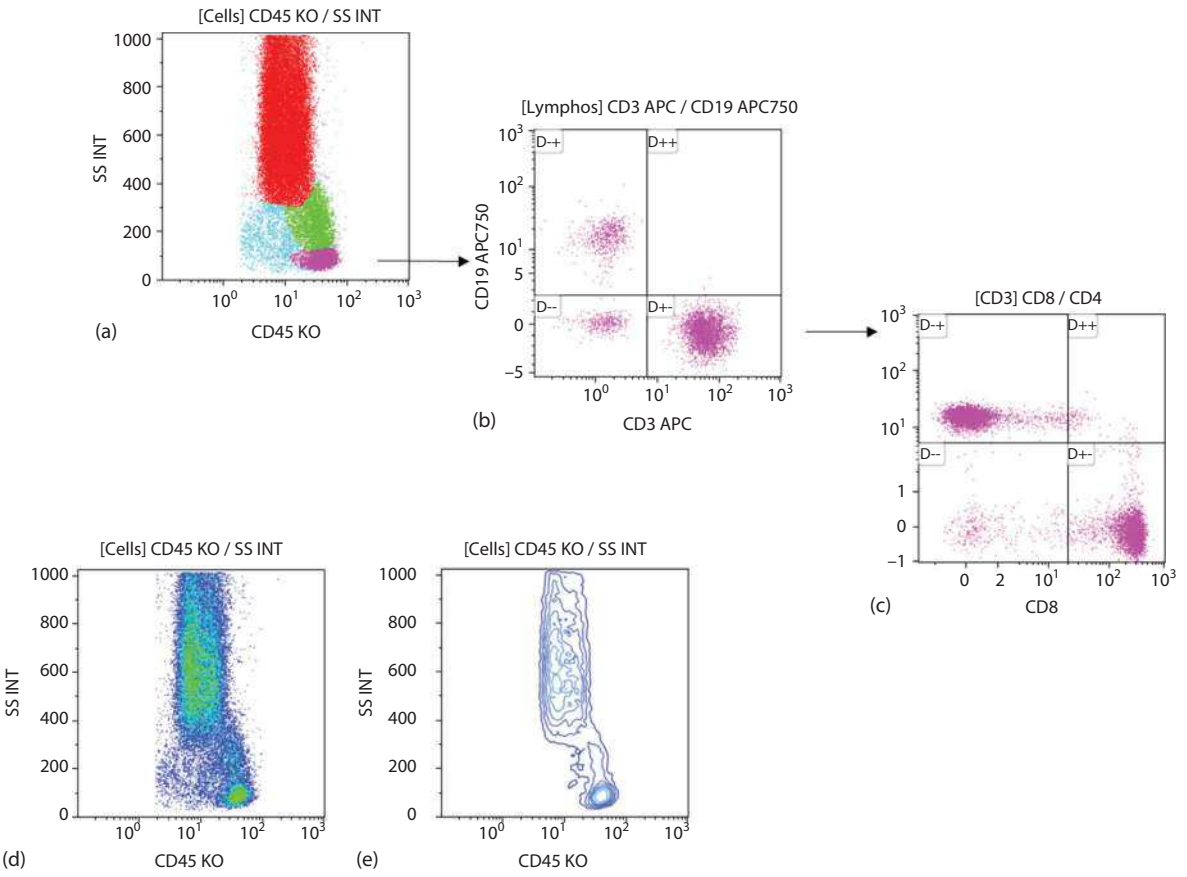

Figure 1.5 (a) Backgating of coloured leukocyte subsets on a CD45/SSC 'cartography' of BM; (b) gating on lymphocytes allows to distinguish CD19+ and CD3+ lymphocytes; (c) further gating on the CD3+ population allows to display T-cell subsets CD4 and CD8; (d) density plot representation of the same plot as in (a) and (e) contour representation of the same plot as in (a).

In the example provided in Figure 1.5, the specific staining of leukocytes by a monoclonal antibody directed to CD45 is displayed (see also Chapter 3). Gating strategies have been used in this example to colour granulocytes in red, monocytes in green and lymphocytes in magenta. These subsets have then been backgated on the CD45/SSC scattergram. Selecting only the lymphocyte gate, and a biparametric histogram based on CD3 and CD19 expression, the major lymphocyte subsets are displayed. Gating on CD3+ lymphocytes and displaying CD4 and CD8 allow for analysis and visualisation of the main T-cell subsets.

The gating hierarchy must be kept in mind when performing complex analyses. Most software allows for a visualisation of this hierarchy and labels histograms based on the parameters used. Although software will basically provide letters to identify gates, it is advised to design and save protocols where subsets are more precisely named.

Counts and percentages (called gate statistics), based on the chosen reference population, can be obtained by various means. For monoparametric histograms, integration cursors can be placed encompassing the Gaussian peak of interest. For bi- or multiparameter histograms, gates of different shapes (polygons, squares, rectangles, circles, freehand…) will provide their *X* and *Y* coordinates as well as the number and percentage of events they contain. For well-separated subsets, quadrants can be used, dividing each histogram into four regions the size and shape of which can be adapted to best delineate the various subsets visualised (Figure 1.5b and c).

It is important to consider populations and subpopulations as Gaussian clusters and not to divide

them either by too short integration cursors of quadrants bisecting clusters. A frequent mistake is to rely too strictly on controls such as irrelevant isotype antibodies stained with the same fluorochrome and consider the brightest signal provided by the Gaussian of such a negative control as the beginning of significant fluorescence. Although this may be the case for brightly stained subsets, often the peak of the population of interest presents with a shift overlapping the 'negative' peak [18].

In the examples of biparametric histograms shown so far, individual dots are only seen on the periphery of coloured clusters. A better idea of the density of events in such clusters can be provided by monoparametric histograms but also by variations of biparametric representations such as density or contour plots (Figure 1.5d and e). This can prove very useful to properly delineate cell subsets. The number of cells displayed can also be chosen or the resolution of the plots modified.

Some software packages also provide various approaches of multiparameter representations in spaces with more than one dimension. This can be modelled by mathematical calculation of principal component analysis or drawn by moving the length and angle of the various vectors to best individualise populations of interest (see also Chapter 14).

Finally, for samples assayed with the same antibody combinations, the merge function of some software allows for a direct comparison of different conditions (i.e. diagnosis and relapse) or for the concomitant analysis of larger numbers of events. For example, merging six different samples of normal BM will provide a good idea of what normal is, by smoothing out individual variations (Figure 1.6). An important parameter for such displays is the time of acquisition, which must be systematically recorded. This allows, during merge analysis, to always individualize each sample.

Compensation

Emission spectra of fluorochromes appear as semi-Gaussian curves with a maximal peak that dictates the choice of the bandpass filters placed before the PMTs (Figure 1.7a). Yet, there is frequent overlap of these emission spectra and it may be necessary to eliminate the contaminating signal [19]. This is achieved mathematically by subtracting a percentage of the overlapping signal(s) in each fluorescence channel (Figure 1.7b).

Basically, compensations will be calculated by performing single staining of cells or beads with each of the antibodies intended to be used in a panel. Each preparation will then be examined in each possible emission fluorescence channel, and the compensation percentage will be adjusted to remove overlapping signals. It is important to have in the preparations a negative control which will provide a reference baseline signal. Currently, beads coated with antibodies to mouse immunoglobulins allow compensation of settings for any antibody/fluorochrome combination [18]. If different conjugates are obtained from the same manufacturer, using the same fluorochromes, a compensation matrix adequate for different panels can be used. The beads stained with the antibodies are mixed with uncoated beads, which will provide the baseline signal. Each biparametric histogram combining the fluorescence tested versus each other channel will be examined to provide for the absent fluorochromes the same signal as that of the negative beads. It is also possible to perform or check for proper compensation by using the 'fluorescence minus one' or FMO method. This technique consists of testing a relevant sample in a series of tubes where, for each, one of the markers intended to be used in the panel is missing. This allows us to visualise/check the overspill or spreading of other emitted fluorescences in the fluorescence of interest where it is known that no antibody has been added [20].

When checking for proper compensation, or building a compensation matrix with cells, it is wise to use a biexponential or logical representation of low signals since some staining will lead to an unavoidable spreading or trumpet effect (Figure 1.7c). This means that light from labelled cells will spill over in a different channel with a broader yet symmetrical display, which is also called signal distortion or spreading [21–23].

Compensations could initially be performed 'manually' with specific tools provided in the software of the instrument or in external software. Nowadays, with the development of multiparameter FCM, the interferences are too complex and most software provides a 'wizard tool' that automatically performs compensations using the acquisition file of each single labelling.

FCM Settings

For any given experiment, the parameters of the instruments must be defined beforehand. The PMTs must be adjusted in such a way that the signals recorded are well defined yet do not saturate in the brightest decades.

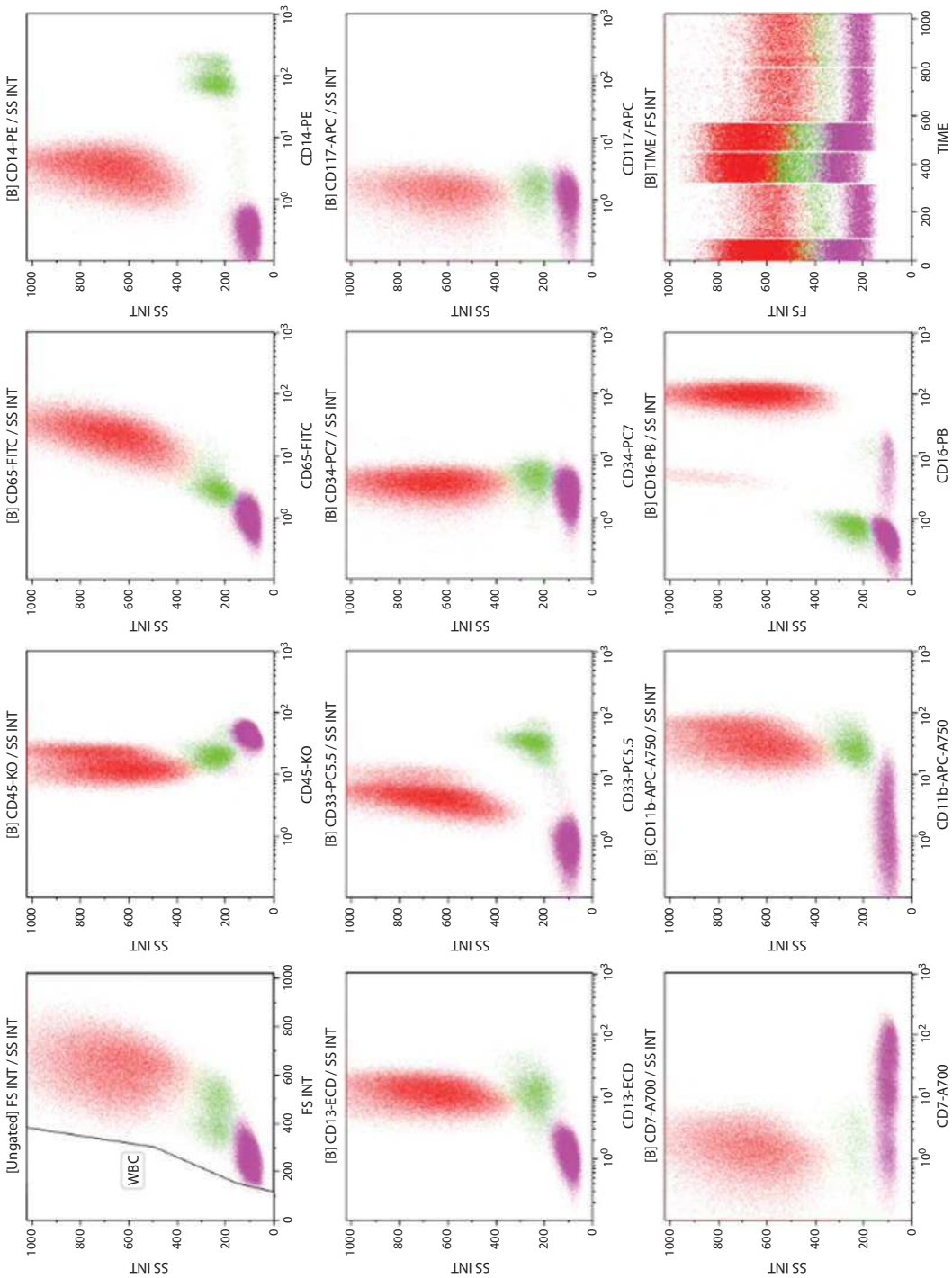

Figure 1.6 Example of the merge of six PB samples. The bottom right histogram shows the merged samples according to a 'time' abscissa that allows to discriminate each sample as a single column. All cells in this histogram (i.e. merged samples) have been used then to examine the florescence of each of the markers in this common tube using SSC as ordinate. The top left histogram allows for eliminating debris on a FSC/SSC display. The second top histogram is the 'cartography' of CD45/SSC display with backgating of neutrophils in red, monocytes in green and lymphocytes in magenta. The third histogram shows the strong positivity of neutrophils for CD65 and intermediate labelling of monocytes. The fourth histogram shows the strong positivity of monocytes for CD14. On the second row, histograms show CD13-positive monocytes and neutrophils, strongly CD33-positive monocytes with lower staining of neutrophils and, in the last two histograms, absence of immature myeloid cells expressing CD34 or CD117, respectively. The three bottom left histograms show CD7-positive lymphocytes, CD11b-positive monocytes and neutrophils and CD16^{bright} neutrophils and finally CD16^{intermediate} NK-lymphocytes.

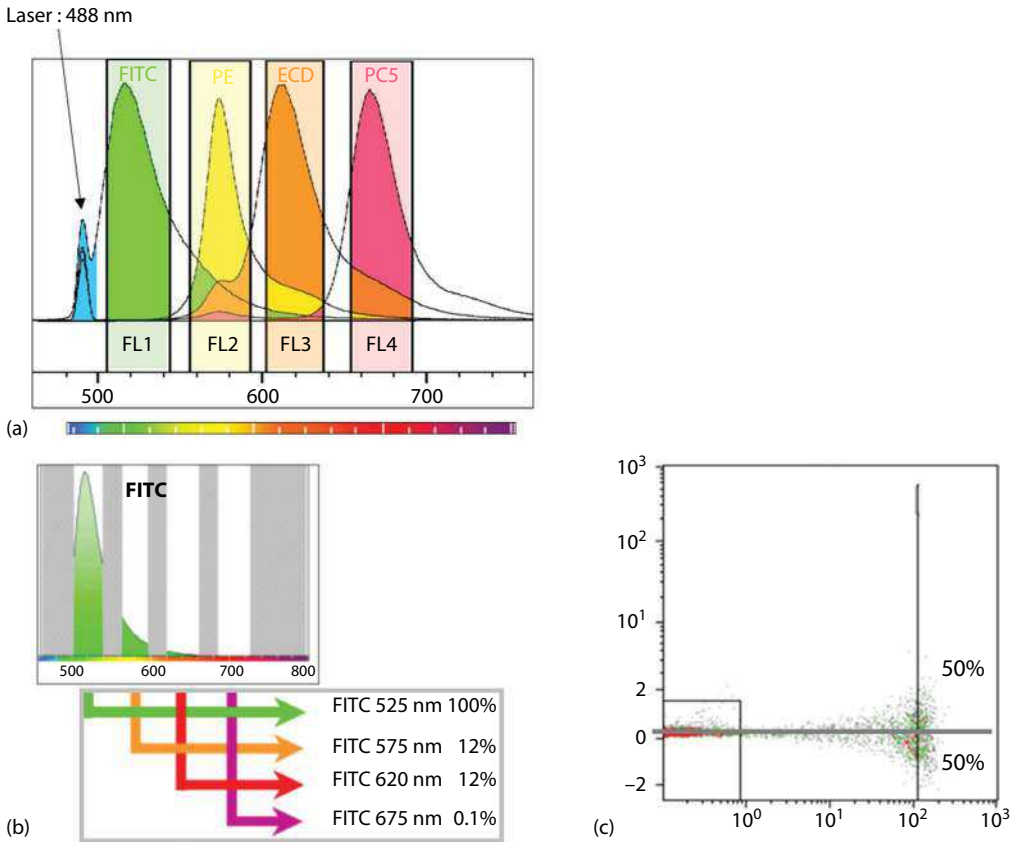

Figure 1.7 (a) The spectral overlap of four commonly used fluorochromes, ordered by emission wavelength (abscissa and spectrum below); (b) the strategy for compensation calculations, indicating the percentage of overspill of FITC in higher wavelengths and (c) distortion or spreading or 'trumpet effect': compensations are perfect with 50% of the spread on both sides of the negative signal for the fluorescence on the ordinate.

A good way of defining this is to use unstained lysed blood and adjust all PMTs so that over 85% of the cells can be seen in the first decade, i.e. above the first channel. Such adjustments can then be used to identify the fluorescence channels where a specific batch of beads fluorescing in all channels will appear. This strategy, adopted in the Harmonemia initiative, allows for excellent reproducibility between instruments, new channels being recorded at each change of beads batch [24].

Lasers' alignment with the flow cell must be checked regularly, although current instruments are usually extremely stable. This is performed by recording the fluorescence emitted by specific beads, with target limits of coefficients of variation of the peaks obtained [25]. Such controls belong to the daily assessment of laser alignment and result in the generation of traceable data in the form, most frequently, of Levy-Jennings graphs. The mandatory pre-analytical precautions to ensure that proper data are generated have been extensively described by an expert working group [25].

The sheath fluid is usually an isotonic solution, but since there is no contact between this liquid channel and the cell suspension it guides, water can also be used. The mandatory condition is that this fluid is completely devoid of any particle that could generate a light signal. Depending on the number of cells that have to be analysed and the precision of the detection, the pressure of the sample fluid can be adjusted between low for the more precise measurements and intermediate or high for less sensitive signals.

The tubing of the instrument must be kept extremely clean, and regular cleansing cycles with chlorine and several tubes of distilled water are recommended

on top of the automatic cleanses performed by most machines between samples.

Panel Design

Multiparameter FCM presents the great advantage of being able to test numerous characteristics of a suspension at the same time. Besides FSC and SSC, 8 and 10 colours flow cytometers now very frequently provide up to 10 or 12 parameters. This has proven to be extremely valuable in immunophenotyping for the diagnosis of haematological malignancies especially when only small samples such as fine-needle aspirates (FNA) or cerebrospinal fluid are available.

Two types of considerations can be taken into account when designing a panel [23]. One is to use a variety of markers allowing characterisation of a maximum of different subsets, such as markers for granulocytes, monocytes, lymphocytes and progenitors. The other option is to investigate the co-expression of several markers on the same cells. This is applied if the goal is to define the maturation stage or activation status of a given subset. In that case, it is important to choose wisely the fluorochromes associated with the various monoclonal antibodies used.

In all cases, bright fluorochromes must be preferred for antigens expressed at low levels, and dimmer fluorochromes for densely expressed antigens. When co-expression is expected, the overlap of fluorochromes must be taken into account to avoid the necessity of too much (or impossible) compensation. Several rules apply for this case. The first channel of the blue laser (488 nm excitation), generally used for FITC conjugates, is never impacted by other fluorochromes. The same is true for the first channel of the violet laser (405 nm excitation) used for Pacific Blue® or Brilliant Violet 421®. These two channels can be used for weakly expressed antigens, since no compensation needs to be applied. The second channel of the violet laser conversely never overlaps other channels. It can thus be used for bright markers and/or 'parent' markers that will contain subsets. This is for instance the case for the pan-leukocyte CD45. The degree of spillover will then vary between fluorochrome combinations which should also guide the choice of combinations. 'Parent' and 'children' relationships between markers characterise the fact that all 'children' are also stained by the 'parent' marker. Spillover can then be accepted from a 'child' to the 'parent', since the primary staining will prevail. Conversely, 'parents' should have no or low spray on 'children' to avoid erroneous appreciation or fuzzy images [26]. It is also important to know that co-expressed markers can lead to dot plots with an angled shape suggesting poor compensation although it is just due to the dual staining.

Compensations are usually easily done for lights emitted after excitation with blue and violet lasers. They are more complicated for excitation with the red laser and mutually exclusive markers can be preferred for these channels, where spillover will not interfere.

Sample Handling

Flow cytometry by definition requires cells in suspension, implying that PB or BM are collected on anticoagulated tubes [27]. Depending on local habits and on other tests liable to be performed on the same sample, ethylene diamine tetraacetic acid (EDTA) or heparin can be used. It is also recommended to perform FCM analyses as rapidly as possible after collection. For fragile samples such as cerebrospinal fluid, it may be interesting to add a specific preservative solution to collect more accurate information and avoid cell loss in the inhospitable low-protein content of this liquid [28]. Because staining and acquisition are relatively rapid, this allows rapid answers to the clinicians, nearly at the same time as morphologic analyses. When transport is needed, the time to processing for samples other than cerebrospinal fluid should not exceed 72 h [27]. Of note, the characterisation of leukaemic cells in a heavily infiltrated sample usually will not be modified by some delay possibly leading to some apoptosis. Conversely, the search for minute subsets in the context of minimal residual disease can be impacted by extended delays. Also, some tumour cells such as high-grade B-cell lymphoma or plasma cells in myeloma may be more prone to apoptosis.

Another pre-analytical aspect of possible impact is the haemodilution of BM samples [29]. It is important to remember that this will not impact the characterisation of malignant cells which can be isolated by gating strategies. However, the great quality of FCM to be able to provide exact counts is lost in haemodiluted samples. It therefore cannot be used to make a diagnosis of leukaemia or myelodysplasia where blast percentages impact the result. However, this is well put to use to enumerate mobilised CD34+ progenitors in PB before stem-cell transplantation [30].

The choice of the panel of antibodies to test will depend on clinical information provided when sending the sample. Screening or disease-specific panels