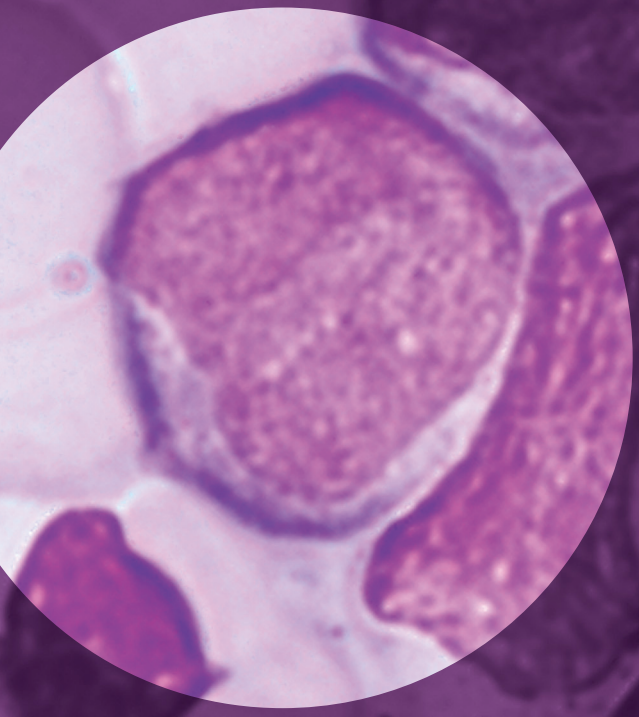
Practical Flow Cytometry in Haematology Diagnosis

Mike Leach, Mark Drummond and Allyson Doig



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Practical Flow Cytometry in Haematology Diagnosis

This book is dedicated to Dr Norman Lucie, pioneer of flow cytometry in Glasgow.

Practical Flow Cytometry in Haematology Diagnosis

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Contents

Preface

The accurate diagnosis of haematologic malignancies is a complex and challenging task. It routinely involves morphologic, molecular, cyogenetic and flow cytometric expertise; how these results are integrated, interpreted and reported will ultimately determine what treatment protocol will be followed. Flow cytometry is central in this diagnostic pathway.

When the authors began to develop their interest in flow cytometry there were few texts available to assist in the day-to-day interpretation of results from clinical samples. The laboratory texts in most frequent use were technically orientated to assist the cytometrist in providing quality data for subsequent reporting. Excellent morphologic and haematopathology texts were available, but a readable, clinically orientated flow cytometry text was notably absent. Things have certainly changed, with a plethora of textbooks describing the application of flow cytometry in the routine diagnostic laboratory. These have taken a varied approach, with cased-based, pattern-based and traditional pathological entity-based approaches having appeared over the years. However the authors consider something still to be missing, namely the textbook they would have wanted to pick up 15 years ago as enthusiastic but inexperienced flow cytometrists who wanted to better learn how to interpret the data to inform their haematology practice. Two of the authors of this book are practicing haematologists who between them routinely diagnose and treat all the malignancies described herein. There is therefore a clinical emphasis throughout, as to how flow cytometry results should be interpreted and applied to optimize patient care. In the real world samples arrive from a range of body fluids, from both malignant and benign conditions and are of variable quality. We have endeavored to reflect this. This book is therefore not a technical manual, but instead it is aimed at those who instigate, perform, interpret or act upon flow cytometry on patient material. This might include clinical scientists, pathologists, haematologists and research laboratory staff. It has therefore been written to encompass the varied background and knowledge of these individuals. Although it has been written to be read from cover to cover it will be a useful laboratory reference text, and we hope of use to both novice and expert alike.

The approach the authors have taken is to start with the basics in each chapter and 'work up'. We have deliberately kept the number of data plots and histograms on display to a useful minimum to allow the text to flow, and inserted carefully chosen real-life cases in each chapter where relevant. High quality morphological images captured especially for this book are used throughout to illustrate these cases. While there is a necessary emphasis on haematological malignancy, flow cytometry of normal populations is not neglected and somewhat uniquely we emphasize the abnormalities that may be encountered as reactive phenomenon, as well as the challenges encountered across a variety of body fluids. That said, this textbook has a traditional structure in terms of the pathological entities described. This is for the simple reason that in most clinical situations, be it based on clinical features, blood indices or initial morphology the investigating clinician or pathologist will have some idea as to what type of disorder they are likely dealing with from the outset. We hope that the readers of this text will enjoy it as much as we enjoyed writing it.

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Mark Drummond
Allyson Doig
Glasgow

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CHAPTER 1
Introduction

The field of Clinical Haematology is a rapidly evolving specialty. The clinical entities encountered in everyday practice have basically not changed, but our understanding of them and the biological and molecular processes that drive them are undoubtedly much better understood. Our classification of these diseases has been continually refined over the decades, with the WHO classification of tumours of haematopoietic and lymphoid tissues now being firmly established as our current working reference manual [1]. The introduction of the WHO classification of haematological malignancy provides a structure for the development of integrated haematopathology laboratories, with its emphasis on definition of disease entities based on clinical, morphological, phenotypical and molecular features.

The real fascination of diagnostic clinical haematology lies in the diversity of the entities that we encounter. The whole spectrum of abnormal haematological parameters triggered by a variety of reactive and neoplastic processes are studied in the diagnostic laboratory. Flow cytometers are no longer a technical novelty confined to a few highly specialized diagnostic institutions. The flow cytometer methodology is well known, cytometers are readily available and whilst its applications are constantly diversifying its contribution to routine diagnosis is widely appreciated. This technology provides objective, rapid, sensitive and accurate measurement of a broad range of cell characteristics.

Accurate diagnosis is clearly of utmost importance in making decisions on patient management. The cognitive pathways to explain the processes of achieving a diagnosis, have in our opinion, however, been somewhat neglected in the haematological literature. There are many excellent texts which describe the clinical, haematological, immunophenotypic, cytogenetic and molecular characteristics of a wide variety of neoplastic disorders. These texts are ideal reference manuals to study once a diagnosis has been achieved. The missing element, however, is a systematic approach to immunophenotypic diagnosis, taking into account all available clinical and laboratory data. In laboratory diagnostics, we are often left with the chore of finding the missing piece to the jigsaw puzzle. This should not be through a process of aimless searching but should be achieved through a logical carefully considered approach. What this publication aims to achieve is to generate a readable text to clearly explain the principles by which these diagnoses can be achieved, how immunophenotypic data can be analysed in clinical context and how meaningful conclusions can be drawn. We focus on clinical flow cytometry analysis of normal and malignant cells, not just in blood and bone marrow, but also cells in extramedullary sites such as effusions and cerebrospinal fluid. Each of these specimens needs to be handled, analysed and interpreted in a specific way. Of utmost importance is the assessment of features of the clinical history, physical examination, biochemical, immunological and radiological findings of a clinical case in relation to the current haematological parameters. We make no apology for this, as we believe this is of ultimate importance and this principle will be encountered repeatedly throughout this text. This approach, as recommended by the National Institute of Clinical Excellence [2], suggests that the diagnosis of leukaemia and lymphoma should take place in a specialist laboratory and in most cases this should be organized on a regional basis with full access to, and interaction with, diagnostic histopathology, cytogenetic and molecular laboratories with expertise in this field.

Flow cytometry analysis, used to determine the nature, origin and behaviour of cells if carefully directed can be a magnificent diagnostic tool and is exquisite in

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categorizing cell populations present at low levels through the analysis of cell size, complexity and granularity in relation to the expression of surface, cytoplasmic and nuclear antigens. Many thousands of cells can be so categorized over a short timeframe. It cannot, however, be applied in isolation and flow cytometry, if poorly directed, can lead to erroneous conclusions. For example, identification of the diseased cell population is usually easy in a bone marrow aspirate in a patient presenting with pancytopenia due to acute leukaemia. The disease cells may not be apparent in peripheral blood but are abundant in bone marrow and are identified and categorized using a carefully chosen myeloid panel. In contrast, the disease cells in hairy cell leukaemia may form a minority population in peripheral blood in a second patient with pancytopenia. The bone marrow aspirate is often dry and uninformative. Immunophenotyping using a mature lymphoid panel will provide a diagnosis. Unless the material is carefully scrutinized and the clinical presentation taken into account, then immunophenotyping can be misdirected and the wrong conclusions might be drawn.

We aim to explain from those most basic steps, how to approach clinical flow cytometry analysis of a variety of clinical specimens, to highlight the strengths and pitfalls, and how to safely embark on this fascinating diagnostic process in a variety of clinical circumstances. We aim to cover reactive phenomena, which in our opinion have not been well covered in the world literature. We aim to illustrate to the student that there is clear logic to explain the immunophenotype of any clonal condition and that they should understand this basis and not attempt to remember an immunophenotype as a random set of CD numbers. This latter approach is bound to fail. The former approach will establish a firm understanding and foundation on which to build and will assist in suggesting additional immunophenotypic studies or ancillary investigations in situations where the diagnosis is not immediately apparent. In addition to the diagnosis of leukaemia and lymphoma we look at flow cytometric applications to response assessment and quantification of minimal residual disease. Finally, we also look at FCM analysis with respect to the diagnosis of red cell, granulocyte and platelet disorders.

This text is written for trainee and practising haematologists, haematopathologists and biomedical scientists with a specific interest. It should assist in preparation for FRCPath UK in Haematology and is intended as a working manual for diagnostic laboratories throughout the world. The chapters are illustrated with morphology images, scatter plots, cytogenetic and molecular data from real clinical cases – often a carefully chosen image will illustrate a principle much more succinctly than a thousand written words.

The authors have done their utmost to ensure the accuracy of data presented in this text. In fact, one of the driving forces in undertaking this exercise was to provide a practical handbook that would assist in the safe and accurate use of flow cytometry as a diagnostic tool. The content is extensively researched and referenced but also relates to personal experience of the authors who interpret flow cytometry on a daily basis in a regional reference centre. We sincerely hope that our readers will find it of interest and of practical value when applied to haematology diagnosis. We cannot, however, accept any responsibility for any error or misinterpretation which might, in any way, arise from its use.

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CHAPTER 2
Principles of Flow Cytometry

Introduction

Over the years, flow cytometry technology, with its multiple applications, has had a significant impact on our understanding of cell biology, immunology and haemopoietic ontogeny, allowing its application to the diagnostic challenges of clinical medicine.

The basic principle of flow cytometry is inherent in the ability to analyse multiple characteristics of a single cell within a heterogenous population, in a short period of time [1, 2]. The term heterogenous is very important. In many clinical scenarios it might be far from clear whether a cellular proliferation is reactive or, indeed, neoplastic. Furthermore, these populations do not exist in isolation but within a milieu of other cell types. The diversity of diseases afflicting the cells of the body compartments is vast. Clinico-pathological correlation is at the very core of accurate diagnosis. Ignore the characteristics of either, at your peril.

Modern flow cytometers have the capability to analyse several thousands of cells per second. Cells in suspension pass through a beam of light (usually a laser beam) in single file; signals generated are related to the size of the cell and the internal complexity or granularity of the cell, enabling the cytometer to identify different cell populations depending on these characteristics. There are a wide range of applications for flow cytometry in a number of different disciplines. However, in haematology it has become an important tool in the identification of haematological disorders from a wide range of diagnostic samples, such as peripheral blood, bone marrow, CSF, pleural effusion, ascitic fluid and lymph node aspirates [1]. For the analysis of solid tissue a cell suspension must first be made.

There are many books and articles available outlining the principles of flow cytometry in great detail [1–7]. This chapter is written with the view of giving a simplified overview of flow cytometry, the gating strategies and data analysis applied in diagnostic flow cytometry applied to haematological disorders. It is aimed at laboratory staff and junior medical staff, and even more senior staff who have not had the privilege to experience clinical flow cytometry in all its diagnostic applications.

Sample preparation

At the outset we begin with a sample, usually of blood or bone marrow. We need to interrogate the cells in the sample as to their nature by determining their surface, cytoplasmic or nuclear immunophenotypic characteristics. As an example (clearly sample management will vary from one institution to another) the following is a simplified version of a sample preparation for flow analysis in our institution.

Direct staining method for surface immunophenotyping of peripheral blood or bone marrow (lyse – no wash method)

1 If sample white cell count is normal, sample may be used undiluted. If white cell count is raised, dilute sample in phosphate-buffered saline (PBS) to within normal value. (For example, if WBC twice normal then dilute 1:2.)
2 Label FACS tubes with sample ID and antibody combinations according to panel being run.
3 Add sample (100 µl) to required number of tubes.

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4 Add antibody conjugates to sample in volumes as recommended by manufacturer and mix.
5 Incubate at room temperature for 20–30 min in the dark.
6 Add appropriate volume of ammonium chloride RBC lysing solution and vortex.
7 Allow to lyse for 10 min.
8 Analyse on flow cytometer.

Surface immunoglobulin staining

1 Adjust sample white cell count to within normal limits if it is raised.
2 Add 100 µl sample to FACS tube.
3 Wash ×3 in warm (37 °C) PBS to remove excess proteins from the sample.
4 Continue with surface staining as previously described.

Intracellular Staining

1 Carry out surface staining as described in steps 1–5 above, if required.
2 Wash ×1 in warm (37 °C) PBS.
3 Using Fix and Perm® Cell permeabilization reagents add 100 µl Reagent A (fixing reagent) and incubate for 15 min.
4 Wash ×1 in warm (37 °C) PBS, centrifuge for 5 min at 300–500 g.
5 Add 100 µl Reagent B (permeabilizing reagent) and appropriate volume of antibody conjugate.
6 Incubate 20–30 min in the dark
7 Add appropriate volume of ammonium chloride RBC lysing solution and vortex.
8 Allow to lyse for 10 min.
9 Analyse on flow cytometer.
PBS; phosphate buffered saline, RBC; red blood cell solution.

The flow cytometer

There are three main components to the flow cytometer [8]:
1 The Fluidics System
Presentation of the sample to the laser.
2 The Optical System
Gathering information from the scattered light of the analysis.
3 The Computer/Electronic System
Conversion of optical to digital signals for display.

The fluidic system

The aim of the fluidics system is to present cells (or particles) in suspension to the laser interrogation point (the point at which cells pass through the laser light beam) one cell at a time. This is achieved by a process known as 'hydrodynamic focusing' (Figure 2.1). In the flow cell (flow chamber) the sample stream is injected into a faster moving stream of sheath fluid (usually phosphate buffered saline). Differences in the pressure, velocity and density of

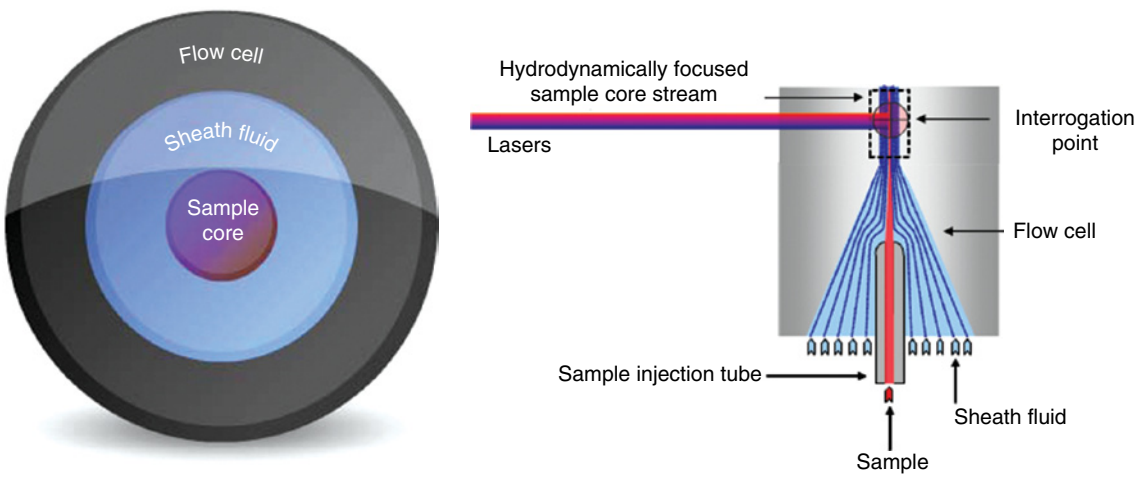

Figure 2.1 Hydrodynamic focusing and interrogation point. The sample core is a narrow coaxial stream (stream within a stream) surrounded by a wider stream of sheath fluid. The shape of the flow cell helps minimize turbulence while ensuring the sample core is focused in the centre of the stream for presentation to the laser. Courtesy of Becton Dickinson.

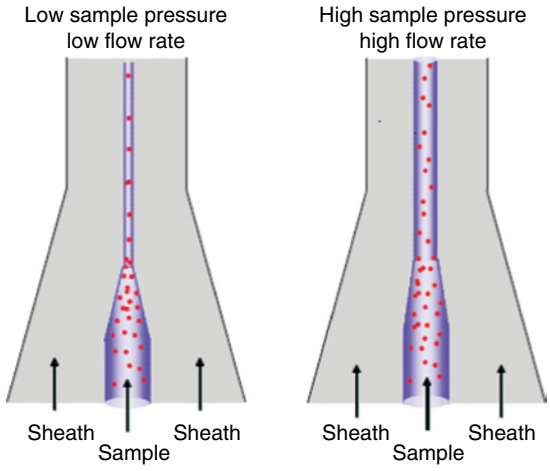

Figure 2.2 Flow rate and sample pressure. Low flow rate = low sample pressure = narrow sample stream = cells pass beam in single file. High flow rate = high sample pressure = wider sample stream = more than one cell passes through the beam at a time. Courtesy of Becton Dickinson.

the two fluids prevent them from mixing. The flow cell is designed so that at the laser interrogation point the two streams are under pressure, focusing the sample stream in the centre of the sheath fluid, forcing the cells into single file before passing through the laser beam [3, 5, 6, 8].

Sample pressure is always greater than the sheath pressure. Altering the rate at which the cell suspension is injected into the centre of the sheath fluid will have a direct effect on the width of the sample stream and the number of cells passing through the interrogation point. The higher the sample pressure the wider the coaxial stream, resulting in more cells passing the interrogation point in a less than optimal position. By lowering the sample pressure this narrows the coaxial stream, resulting in cells passing the interrogation point in single file (Figure 2.2).

It is important that the correct flow rate is applied for the application being used. For immunophenotyping, measurements can be acquired quickly and therefore a high flow rate can be applied. DNA analysis, for example, requires a much higher resolution so a narrow sample core is necessary to ensure single cells pass through the laser beam at any given time; here a low flow rate should be applied.

The optical system

The optical system of the flow cytometer comprises excitation optics and collection optics. The excitation optics are made up of the laser with focusing lenses and prisms, whilst the collection optics lenses, mirrors and filters all gather and direct the scattered light to specific optical detectors (Figure 2.3). The intercept point of laser light and cells must be constant so the laser is held in a fixed position.

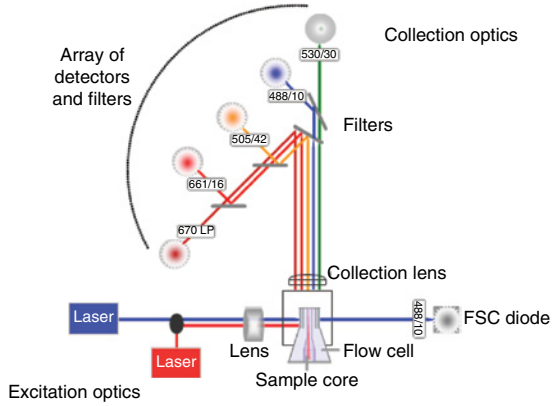

Figure 2.3 Example of the optical bench in the FACSCalibur flow cytometer. Courtesy of Becton Dickinson.

Light scatter

As a cell passes through the interrogation point, light from the laser beam is scattered in forward and 90° angles. The amount of light scattered is dependent on the physical properties of the cell, such as, cell size, nuclear complexity and cytoplasmic granularity. These light scattering signals are gathered by specific detectors, converted to digital signals and finally displayed as dot plots for analysis.

Light diffracted at narrow angles to the laser beam is called forward scattered light (FSC) or forward angle light scatter (FALS). The amount of FSC is proportional to the surface area or size of the cell. The forward scattered light is collected by a detector placed in line with the laser beam on the opposite side of the sample stream. Some light will pass through the cell membrane and is refracted and reflected by cytoplasmic organelles or nucleus of the cell. This light is collected by a photodiode positioned at approximately 90° to the laser beam and is known as side scattered light (SSC). Side scattered light is proportional to the granularity or internal complexity of the cell (Figure 2.4).

Together, FSC and SSC signals provide information on the physical properties of the cells allowing differentiation of cells within a heterogeneous population, for example the differentiation of white blood cells (Figure 2.5).

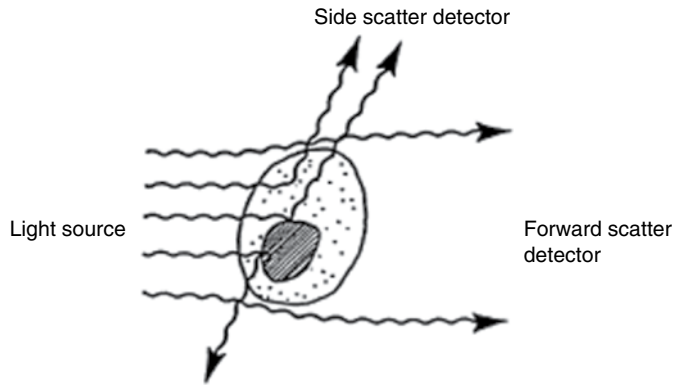

Figure 2.4 Simple illustration of forward and side light scatter properties of a cell. Courtesy of Becton Dickinson.

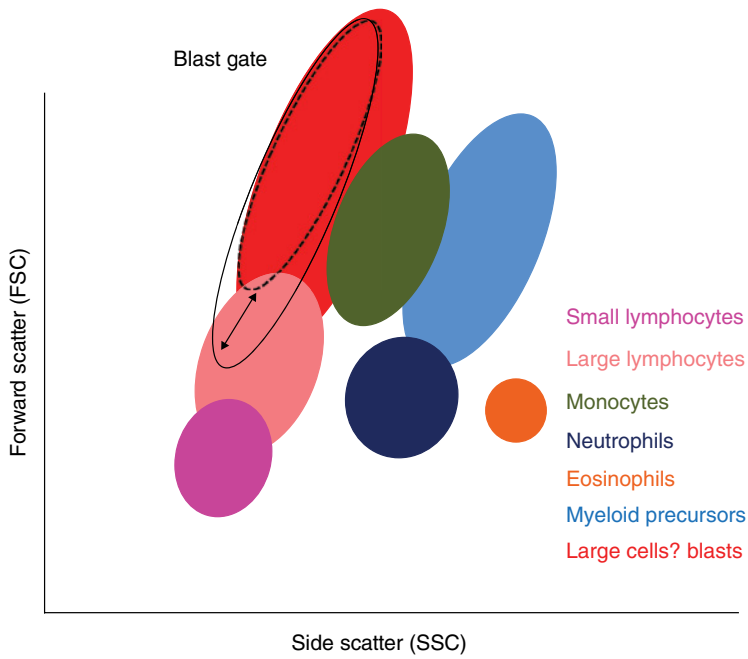

Figure 2.5 Schematic diagram of differentiation of peripheral blood leucocytes according to forward and side scatter characteristics.

Fluorescence

The forward and side scatter light signals are emitted at a 488 nm wavelength and are of the same colour as the laser light [3]. These signals can therefore be determined without the need for a dedicated fluorescent probe.

To determine the specific biochemical properties of a cell, dyes that can bind directly to the cell or fluorochromes that are bound to ligands, for example monoclonal antibodies, are used. The dyes or fluorochromes are excited by light of a wavelength that is characteristic for that molecule. It will absorb the light, gaining energy, resulting in the excitation of electrons within the molecule; on returning to its unexcited state this excess energy is released as photons of light resulting in fluorescence.

The wavelength range at which a fluorochrome absorbs light and becomes excited is known as its excitation (or absorption) wavelength. The wavelength range of the emitted light is termed its emission wavelength. The emitted wavelength range will be longer than that of the absorption wavelength range; this difference is referred

to as Stoke's Shift [5] (Figure 2.6). As laser light is of a fixed wavelength, it is essential that the fluorochromes or dyes to be used have excitation wavelengths compatible with the flow cytometer (Table 2.1).

If a fluorochrome or dye can be excited sufficiently by light of a specific wavelength and their emission wavelengths are sufficiently different from one another, more than one fluorescent compound may be used at one time. However, if the flow cytometer uses only a single laser then the absorption spectrum of each fluorochrome would have to be similar.

In the flow cytometer the fluorescent signals are collected by photomultiplier tubes. To optimize these signals, optical filters specific to a wavelength range are placed in front of the photomultiplier, allowing only a narrow range of wavelength to reach the detector. These are as follows: bandpass (BP) filters which transmit light within a specified wavelength range (Figure 2.7)

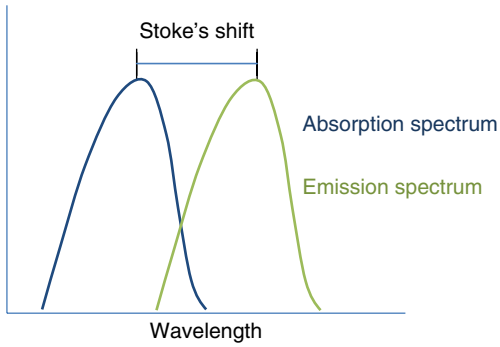

Figure 2.6 Stoke's shift. Courtesy of Becton Dickinson.

Table 2.1 Maximum excitation and emission wavelengths for some of the common fluorochromes used in flow cytometry.

Fluorochrome	Abbreviation	Excitation max (nm)	Emisson max (nm)
Cascade blue		380, 401	419
Cascade yellow		399	549
Pacific blue		410	455
Alexa 488*		495	519
Fluorescein isothiocyanate*	FITC	494	519
Phycoerythrin*	PE	496, 546	578
Texas red*	ECD	595	615
PE-cyanine 5*	PC5/PE-Cy5	496, 546	667
PE-cyanine 5.5*	PC5.5/PE-Cy5.5	495, 564	696
PE-cyanine 7*	PC7/PE-Cy7	495, 564	767
Peridinin-chlorophyll*	PerCP	482	678
PerCP-cyanine 5.5	PerCP-Cy5.5	482	678
Allophycocyanin*	APC	650	660
APC-cyanine 7	APC-Cy7	650	785

(*most commonly used).

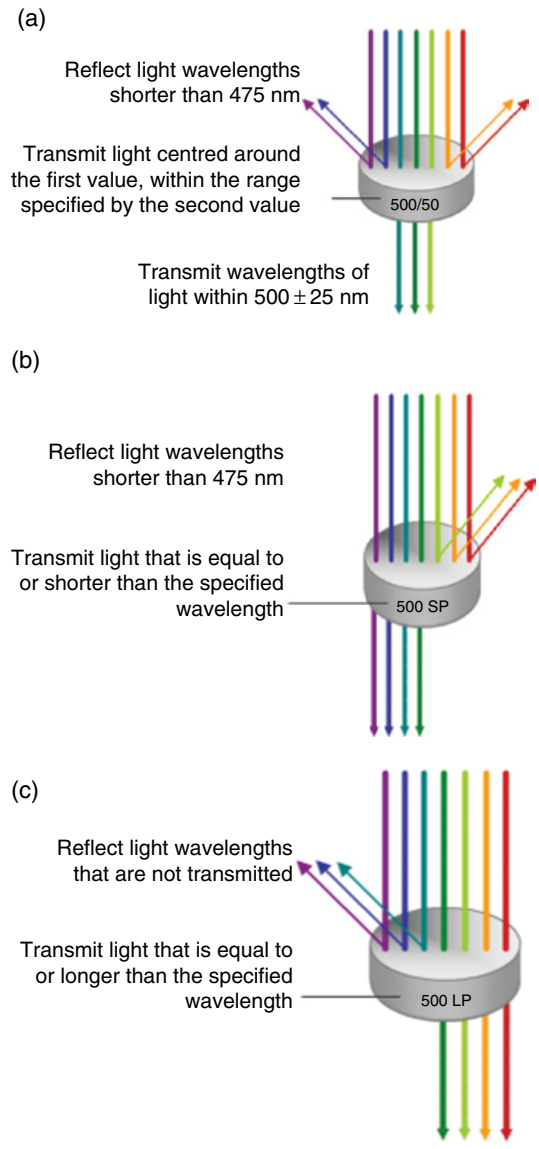

Figure 2.7 Bandpass filters allowing only specific wavelengths of fluorescence to pass through. Courtesy of Becton Dickinson.

(a); shortpass (SP) filters (b) which transmit light with wavelengths equal to or shorter than specified; longpass (LP) filters (c) which transmit light with wavelengths equal to or longer than specified.

Fluorescence intensity

The brightness or fluorescence intensity of any captured event for a particular fluorochrome is recorded by the cytometer for that channel. When many events have been captured it is possible to derive a mean fluorescence value; this is known as the mean fluorescence intensity (MFI) and is a very important characteristic that should be assessed in routine diagnostic practice (Figure 2.8a). It relates to not only the presence of the relevant antigen, but also the strength of expression or integrity of that antigen in a given cell population. It can carry great significance when used in the differentiation of neoplastic from reactive and normal cell populations. Fluorescence intensity can also be used to identify dual populations and allow subsequent directed gating strategies (Figure 2.8b). Some cell populations can show a spectrum of expression of a given antigen, for example CD13 expression in acute myeloid leukaemia, so care has to be used when using MFI data without paying attention to the plot (Figure 2.8c). These patterns of expression can be important in diagnosis.

It is clearly important to maintain consistency in fluorescence intensity data, both on a single cytometer over time and between different cytometers in the same laboratory. The means of achieving this are explained in the calibration section below.

Spectral overlap

Although a detector is designed to collect fluorescence from a specific wavelength, the emission spectra for a given fluorochrome can cover a range of wavelengths, allowing fluorescence spill over to a detector designed for a different fluorochrome. This is referred to as spectral overlap (Figure 2.9). For accurate analysis of data, the spectral overlap between fluorochromes must be corrected. This correction is done by compensation (Figure 2.10). In simple terms, compensation is carried out by correcting for inappropriate signals generated by the fluorochrome responsible for the overlap. With the use of compensation controls, this can be corrected manually and visually set, so minimizing interference from this phenomenon. However, when using multiple

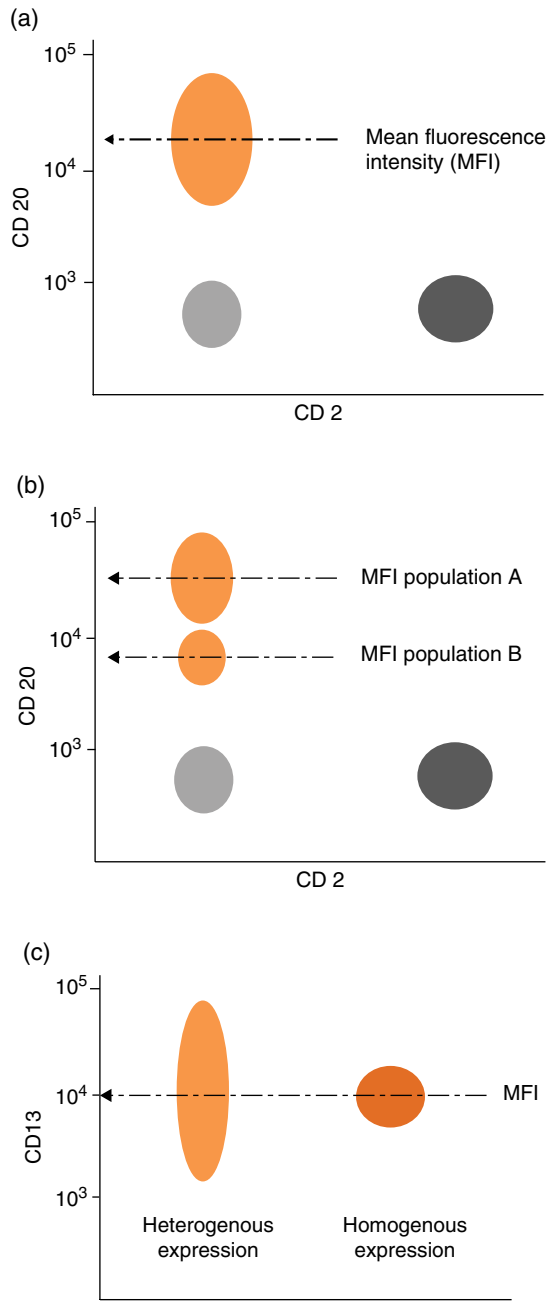

Figure 2.8 (a) Deriving a CD20⁺ MFI value for a B-cell population in blood. (b) Differences in CD20 MFI values for two different B-cell populations in blood can assist in diagnosis. (c) In addition to MFI, the spectrum of fluorescence intensity for a given antigen is important. Here, although the MFI is the same for the two myeloid populations, the variation in intensity (heterogenous versus homogenous expression) is different and is valuable in diagnosis.

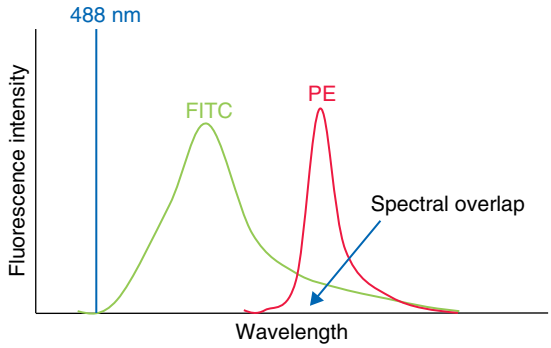

Figure 2.9 Spectral overlap – the emission wavelengths of FITC and PE overlap. In order to ensure accuracy, compensation has to be applied to subtract the overlapping signals. The high wavelength emissions from FITC will be captured by the PE detector and vice versa. If compensation is not applied then inappropriate dual positive events (Q2) will be captured. Courtesy of Becton Dickinson.

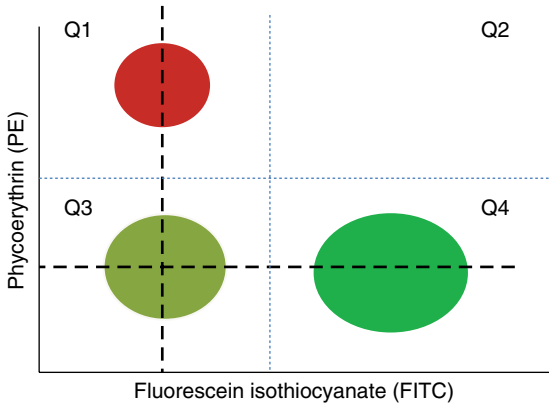

Figure 2.10 Compensation for spectral overlap of fluorochromes. Schematic diagram: this illustrates that the mean fluorescence intensity (MFI) for each fluorochrome should be equivalent for both positive and negative events so compensating for spectral overlap.

fluorochromes in modern flow cytometers this process is much more difficult to carry out manually. Therefore, current four and six colour flow cytometers usually have a software programme to aid with this potentially complex compensation set up process (Figure 2.11).

The electronic system

The electronic system of the flow cytometer allows the light signals to be converted into numerical data for analysis. As light hits the photodetectors, the incoming photons are converted to electrons, resulting in a current [3, 7, 8]. This current passes through an amplifier and a voltage pulse is generated that is proportional to the number of photons detected. The voltage pulse is created as soon as a cell or particle passes through the laser beam, with its highest point achieved when the cell or particle is in the centre of the beam (Figure 2.12).

Two types of photodetectors are used, termed photodiodes and photomultiplier tubes (PMTs). Photodiodes are less efficient with lower sensitivity than PMTs and generally used to collect the forward light scatter which produces a strong signal. Photomultiplier tubes are very efficient and used to collect the weaker side scatter and fluorescent signals. By applying a voltage to the photodetectors the electrical signals can be amplified. For the amplified signals to be displayed by the computer for analysis they require to be digitized. A numerical value is generated for the pulse height, width and area, and assigned a channel number by the analogue-to-digital convertor (ADC). The channel number is then transferred to the computer and displayed as a point on an analysis plot. The signals can be applied linearly or logarithmically for analysis (Figure 2.13).

Threshold

Whenever a particle or cell passes through the laser beam a voltage pulse is generated. To prevent interference from background noise or debris, a threshold can be set. By setting a threshold signal value, processing only occurs when a voltage pulse signal is above this limit. Signals below threshold are not processed (Figure 2.14). It is important to set the threshold limit so that the highest numbers of cells of interest are detected: if the threshold limit is set too low or too high there is the risk that cells of interest are missed [9].

Data display

Once signals have been assigned channel numbers the computer processes and stores these values. Once stored the data is saved in a standard format developed by the Society for Analytical Cytology [10]. These files of raw data are generally referred to as 'listmode files'. The stored data files can then be displayed in a number of ways depending on the analysis software application and reanalysed over and over again. The most common data displays for analysis in immunophenotyping are described below.

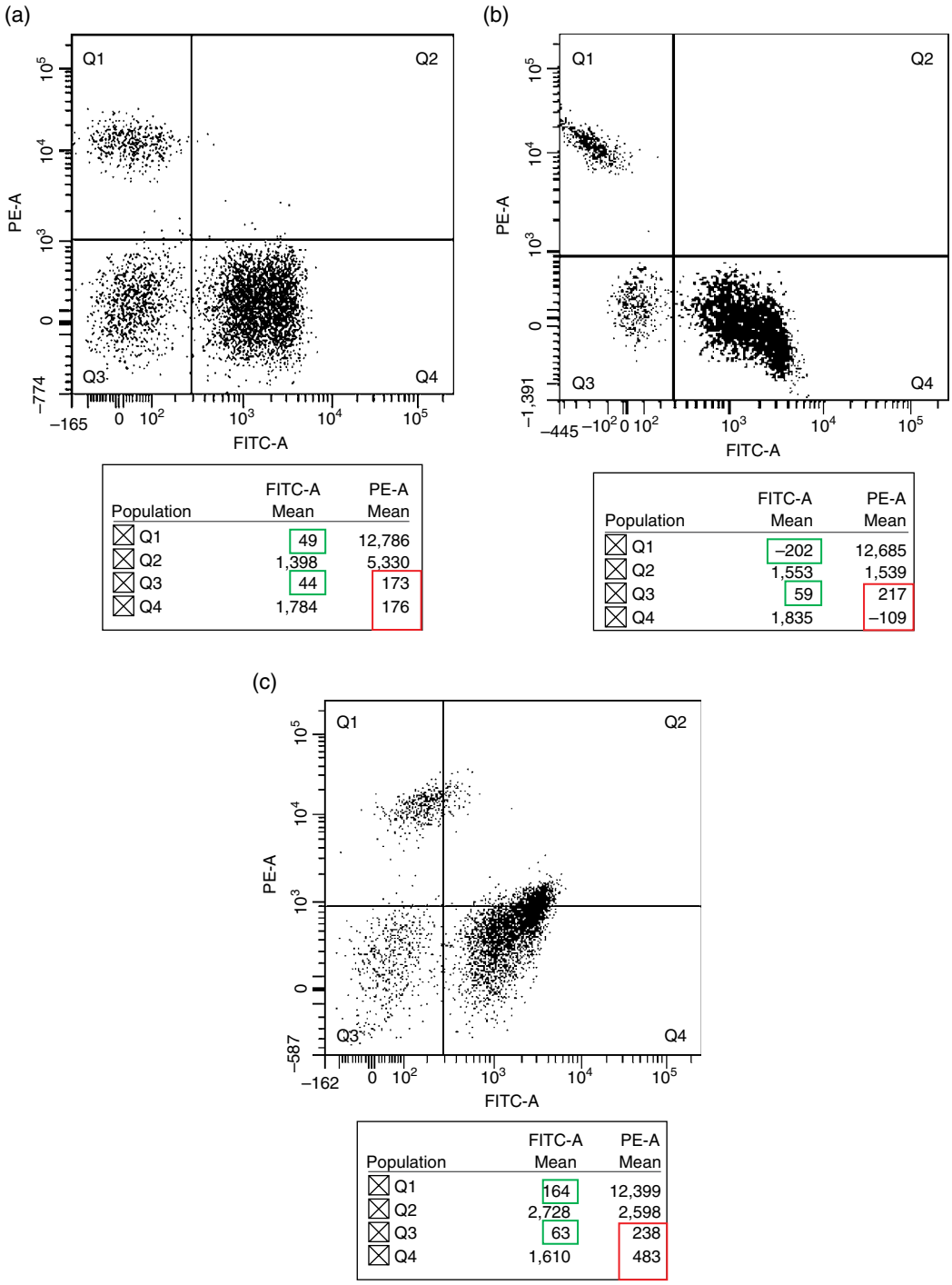

Figure 2.11 Compensation for spectral overlap of fluorochromes: illustrating a worked example.

(a) Correct compensation
Accurate compensation allows for spectral overlap and generates discrete cell populations with equivalent MFI for both FITC (green values) and PE (red values) channels.

(b) Over compensation
This encourages populations to be pulled down the axis for the relevant fluorochrome – too much subtraction collapses/skews the events downward and the MFI values are different. If this is not corrected dual positive population may be missed.

(c) Under compensation
This encourages populations to be pulled up the axis for the opposing fluorochrome – too little subtraction collapses/skews the events upward and the MFI values are different. This may lead to false dual positivity being reported.

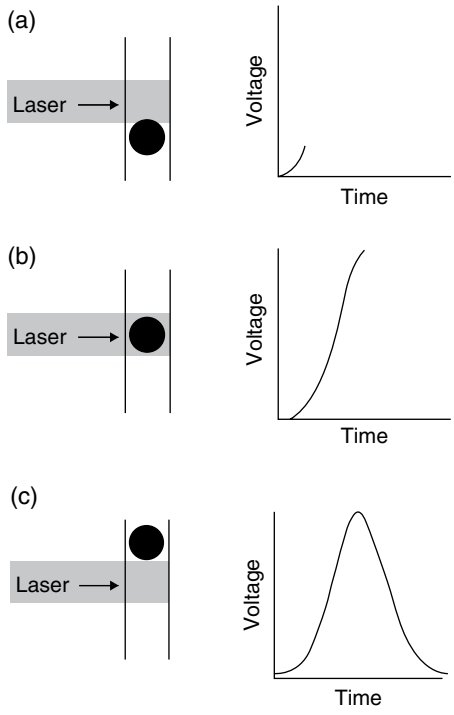

Figure 2.12 (a) The cell enters the laser beam and the voltage pulse is created. (b) The pulse reaches its peak as the cell is in the centre of the beam. (c) The pulse returns to baseline as the cell has left the beam.

Histogram

The histogram represents a single dimension and is used for displaying a single parameter. The Y axis shows the number of events counted and the X axis shows the fluorescence intensity in Figure 2.15(a).

Dot plot

The dot plot is used to display two parameters where each dot represents a cell/particle. The stronger the signal the further along each scale the data is displayed. The forward versus side scatter plot is a frequently used dot plot for peripheral blood analysis and subsequent gating as in Figure 2.15(b). Forward scatter correlates with cell size and nuclear/cytoplasmic complexity, whereas side scatter correlates with cytoplasmic granularity as noted in Figure 2.5.

Other plots for displaying data are density and contour plots but these are less commonly used. Both display two parameters but provide a third dimension and are able to quantify and give a visual representation of multiple events occurring in the same zone of the plot. The contour plot joins x and y coordinates with similar event counts and looks similar to a topographical map. Density and contour plots are not generally used for routine analysis but mainly used for displaying data in publications (Figure 2.15(c)).

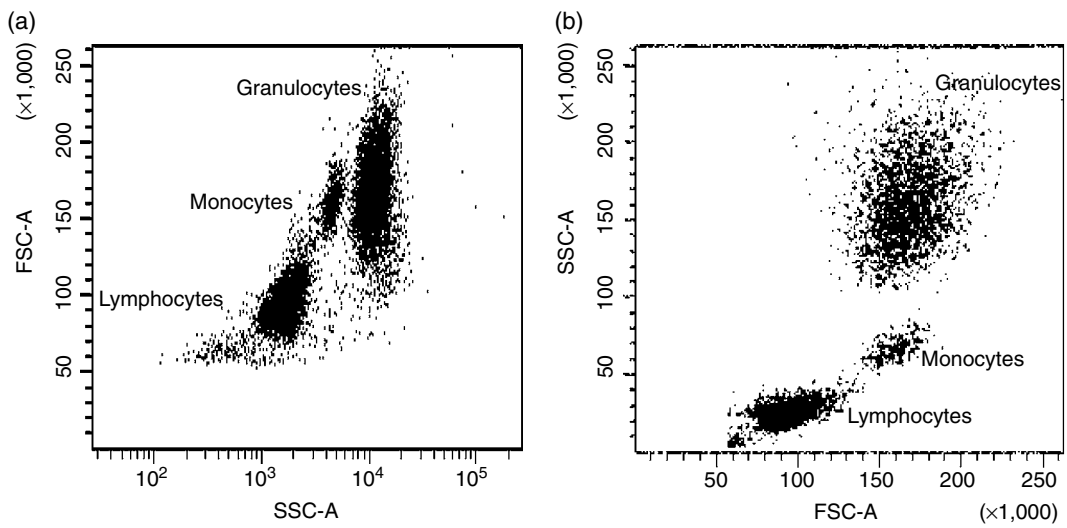

Figure 2.13 Leucocyte differentiation from FSC and SSC signals. (a) Dot plot using logarithmic scale. (b) Display using linear scale. Courtesy of Becton Dickinson.

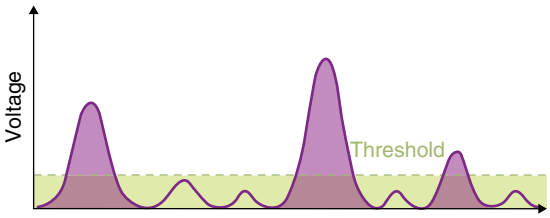

Figure 2.14 The principle of setting a threshold voltage. Courtesy of Becton Dickinson.

Gating

Gating refers to the ability to isolate single populations of interest within a heterogeneous sample. As flow cytometers are capable of analysing thousands of cells per second, gating allows analysis to be restricted to a subpopulation of cells without having to isolate them from a mixed sample prior to analysis [5]. As previously shown in Figure 2.5, subpopulations of leucocytes can be identified according

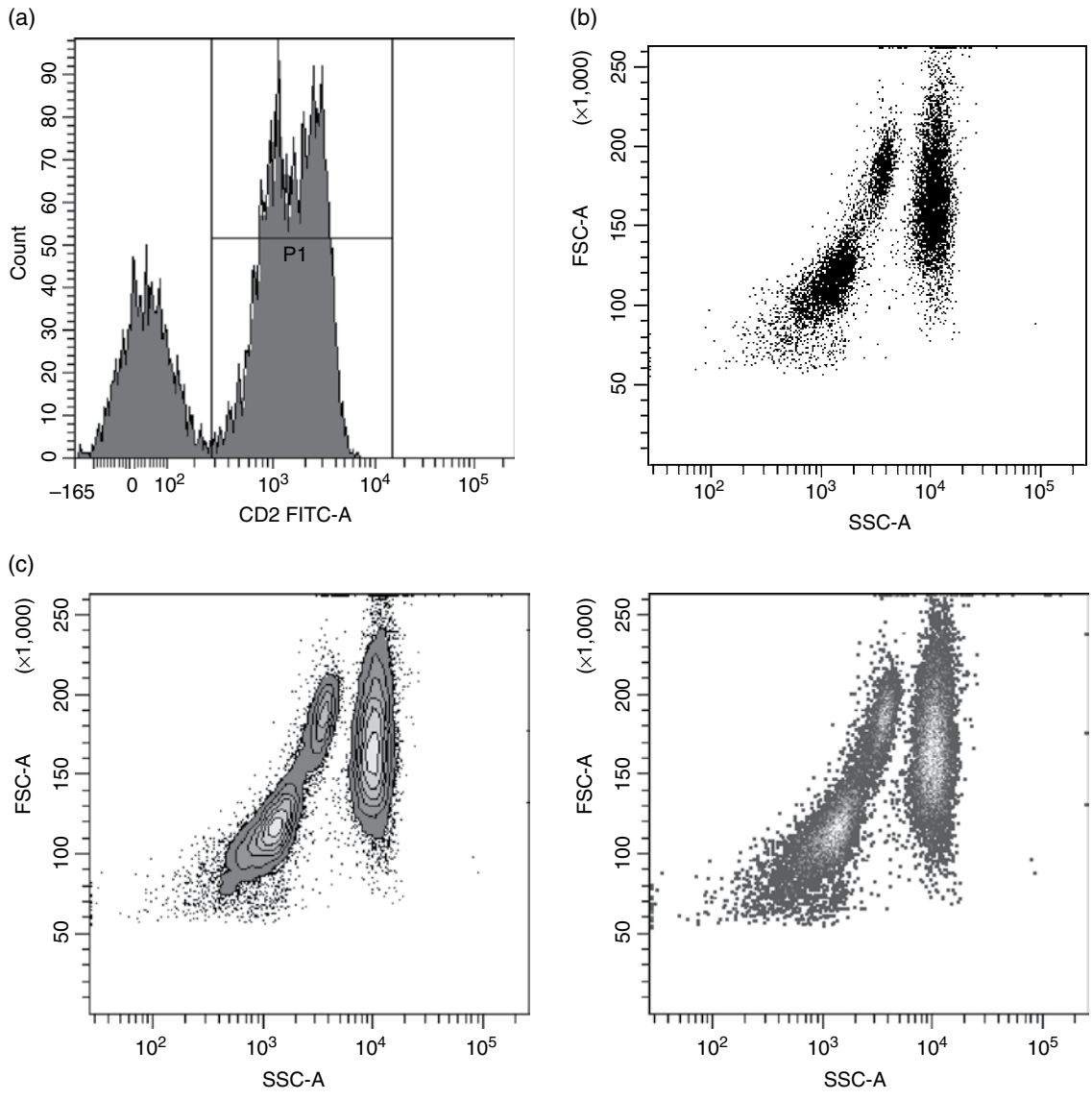

Figure 2.15 (a) Histogram plot showing CD2 expression of peripheral blood lymphocytes. (b) Dot plot using a forward scatter (FSC) versus side scatter (SSC) analysis. (c) Contour plot (left) and density plot (right) of the same forward scatter (FSC) versus side scatter (SSC) analysis.

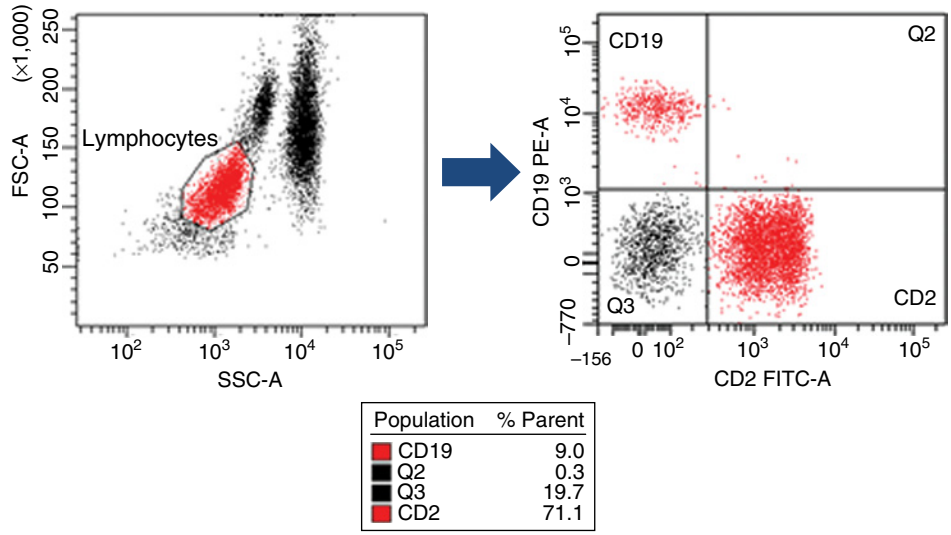

Figure 2.16 A FSC/SCC plot showing gate around lymphocytes and a dot plot showing only parameters related to the gated region.

to their light scatter properties. If left ungated, then non-specific fluorescence from monocytes and neutrophils will be seen on the analysis plot. However, by drawing a gate (or region) around the lymphocyte population the fluorescent properties of only lymphocytes can be displayed, making analysis much more specific (Figure 2.16). The orientation of populations of cells in a FSC versus SSC light scatter plot is the simplest method for gating. It has the advantage that dead cells or debris can be excluded from the analysis but the disadvantage of being unable to discriminate between cells with similar light scatter properties.

Lack of discrimination according to light scatter behaviour can be overcome by 'back gating'. This is a strategy that can be used to identify a population of cells for analysis while excluding contamination by other cell populations, for example monocytes being included in the lymphocyte gate. This was first described in 1990 [11]: by staining cells with CD45 (all normal leucocytes are $CD45^+$) and CD14, (monocytes are $CD14^+$, lymphocytes $CD14^-$) a dual parameter dot plot of CD45 versus CD14 is created. By drawing a gate around the brightest $CD45^+$ $CD14^-$ populations it is then possible to identify the lymphocytes with minimal monocyte contamination (Figure 2.17).

With the introduction of more monoclonal antibodies in multiparameter flow cytometry it has become common to use specific cell surface antigen expression as a gating parameter as a means of identifying the cells of interest within a heterogeneous population. By using fluorescence along with side scatter as a gating parameter this can provide the interpreter with a specific phenotype of these cells. For example, if CD19 was used to isolate B lymphocytes from the T lymphocytes the resulting information would only be relevant to the B-lymphocyte population within the sample. This same principle can be applied to identifying blasts within a leukaemic sample (Figure 2.18).

Another common gating strategy utilizes CD45 as the fluorescent parameter. CD45 is a common surface antigen expressed on leucocytes with differing levels of fluorescence intensity, but is not expressed by erythroid or non-haematological cells. Lymphocytes can be easily identified by their higher CD45 staining intensity than that of monocytes and granulocytes. Due to this difference in fluorescence intensity between cell types, along with side scatter properties, CD45 can be a useful gating parameter for identifying leucocytes of different lineages and degrees of maturation [12] (Figure 2.19).

It is possible to apply multiple gates; several sequential gates can be set on different parameters and analysed simultaneously. In the clinical setting examples of this include the enumeration of $CD34^+$ cells in peripheral blood or peripheral blood stem cell collections, and

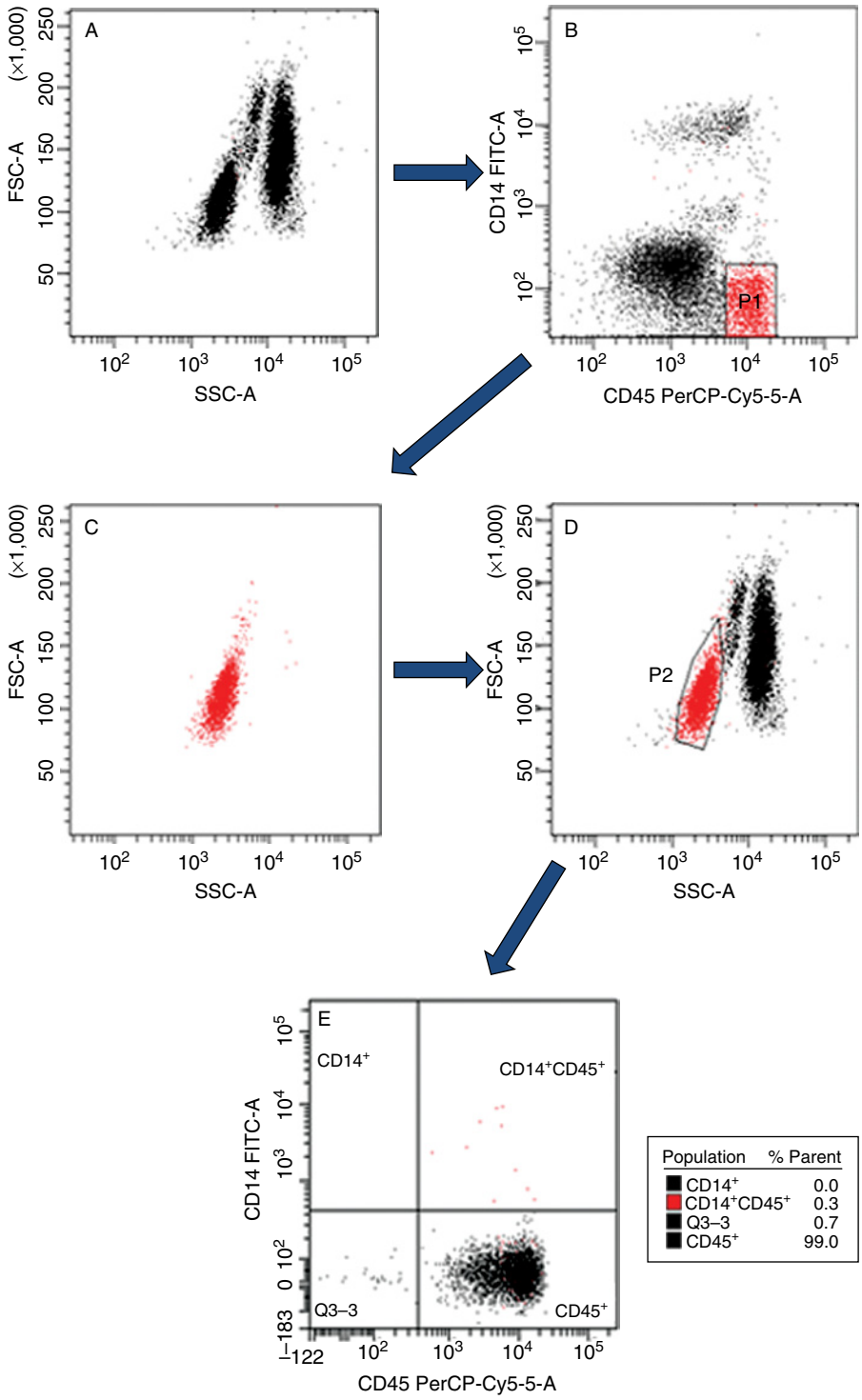

Figure 2.17 Back gating strategy (according to [11]). Plot A shows useful but incomplete separation of peripheral blood leucocytes on the FSC versus SSC plot. Plot B shows the clear identification of lymphocytes in population P1 ($CD45^{bright}$ $CD14^{-}$). Plot C shows back gating of the P1 lymphocytes indicating where they reside in the original FSC versus SSC plot. Plot D shows subsequent gating on these lymphocytes in population P2. Plot E shows subsequent CD45 and CD14 analysis showing a virtually pure lymphocyte population with minimal monocyte (0.3%) contamination.

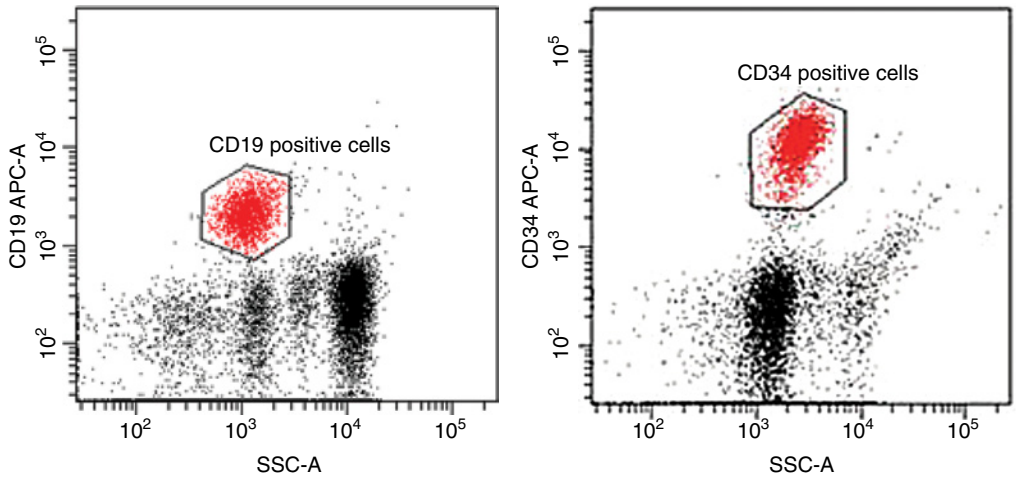

Figure 2.18 Illustration of gating strategy using fluorescence versus SSC to isolate the cells of interest. B lymphocytes are identified using CD19 (left) whilst CD34 (right) is used to identify blasts.

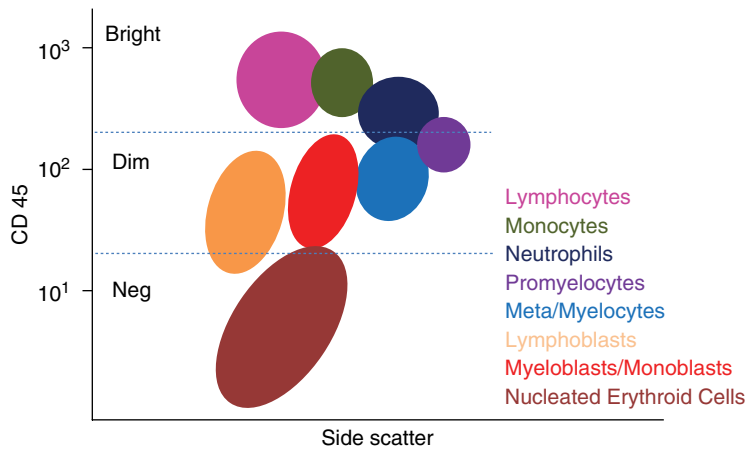

Figure 2.19 CD45 plot leucocyte lineage and maturation.

CD4⁺ helper T-cell numbers from the total T-lymphocyte population [13, 14] (Figure 2.20).

Instrument set-up and quality control

To ensure the accuracy and precision of the data generated, particularly in the clinical laboratory, the performance of the flow cytometer must be rigorously monitored and controlled. Although most instrument calibration is carried out during installation, it is good laboratory practice for the flow lab to establish a robust internal quality control (IQC) programme. This will ensure that the flow cytometer performs to its expected standard. This can be done through the use of commercial standards and control materials. The term 'standard' usually refers to a suspension of microbeads/particles which do not require further preparation and are generally used to set up or calibrate the instrument. Control material is different in that they are usually analytes, for example fixed whole blood cells that have pre-determined values and require preparation in a similar way to patient samples [15].

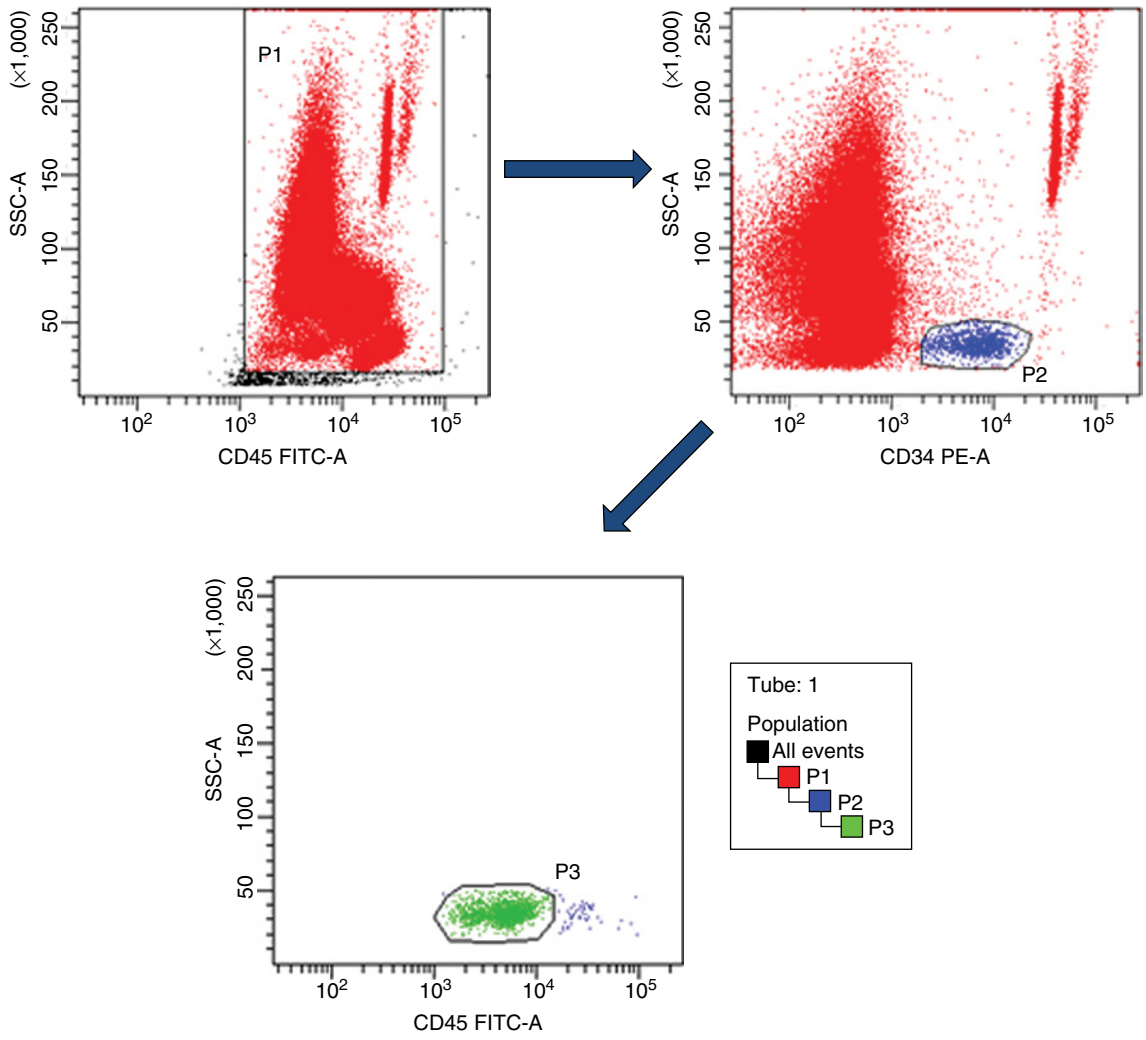

Figure 2.20 Simplified example of sequential gating used in the ISHAGE protocol for absolute CD34 enumeration. A plot displaying SSC versus CD45 FITC fluorescence has a gate P1 drawn around all positive cells excluding noise/debris. Everything within P1 is displayed on a second plot, SSC versus anti-CD34 PE. A gate is drawn around the positive cells in gate P2 which in turn are displayed on another SSC versus CD45 FITC and a gate drawn around the discrete group of cells with moderate FITC fluorescence intensity and low SSC properties, gate P3. By following the population hierarchy the gating sequence can be understood. The cells within the P3 gate are also found in P1 and P2 regions. This sequential gating process effectively purifies the $CD34^+$ population and excludes rogue cells. Gates can be set during acquisition (real time) or post acquisition. Either way since the computer saves the files (listmode data), repeated analysis can be carried out with different gating strategies being applied an infinite number of times without affecting the original file.

The microbead standards can be classified into four categories [15]:

Type 0 (certified blank): these beads are approximately the same size as a lymphocyte, with no added fluorescent dye. They have a fluorescence signal lower than the autofluorescence of unstained peripheral blood lymphocytes, helping to set the threshold level so that there is no interference from noise in the immunophenotyping assay.

Type I (alignment beads): these are designed for optical alignment of the flow cytometer. They are subclassified according to size into type Ia (smaller than a lymphocyte)