

Phenotypic and Functional Measurements on Circulating Immune Cells and their Subsets

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... Whatever you think
It's more than that, more than that ...

Robin Williamson, *Job's Tears*, 1967

INTRODUCTION

Current flow cytometry as applied to the detection of biomarkers is defined by several attributes: application of quality control measures, use of multiple-color measurements, use of the quantitative capacities of flow cytometry (inferring number of molecules from fluorescence, measuring the absolute number of events of interest) and most importantly, measuring function on defined subpopulations of cells within a heterogeneous mixture. The purpose of this chapter is to trace the development of these advances and provide examples and, hopefully, practical suggestions for their implementation in basic and translational research.

IMMUNE BIOMARKERS BEFORE FLOW CYTOMETRY

By the early 1970s it was well appreciated that the morphologically homogeneous peripheral blood lymphocytes could be divided into several subpopulations on the basis of differing surface structures that serve as population markers (Winchester and Ross, 1976). In mice, lymphocyte subpopulations were divided into functionally distinct subsets by alloantisera. In humans, rosetting techniques

were used to quantify T cells and B cells. T cells form complexes, known as E-rosettes, with neuraminidase-treated sheep red blood cells and B cells were identified by forming rosettes with antibody-coated or sensitized, sheep red blood cells. B cell rosetting was known to occur because of the presence of Fc receptors on B cells. Although the mechanism for T cell rosetting was obscure at the time that this assay was developed, it is now appreciated that they form because red cells bind avidly to CD2, a signaling molecule that induces costimulation when bound to its natural ligand, the adhesion molecule CD58 (LFA-1) on antigen presenting cells (Arulanandam et al., 1994). Although rosette assays were technically challenging, they became widely adopted by clinical immunology laboratories and were used for diagnosis of lymphoid malignancies, congenital immune deficiency states and studies on the pathogenesis of autoimmunity. An early but classic example of immune monitoring is the study of Noel et al., in which the kinetics of peripheral T and B cell reconstitution was followed in allogeneic bone marrow transplant patients with and without acute graft versus host disease (Noel et al., 1978). The data and normal ranges are still valid today.

FLOW CYTOMETRY AND THE FIRST MONOCLONAL ANTIBODIES

Characterization of lymphocyte subsets was one of the earliest applications of flow cytometry. In the mid-1970s the Herzenberg laboratory combined standard

immunofluorescence staining techniques with their newly developed single color 2-parameter fluorescence activated cell sorter and identified and separated murine B cells (Loken and Herzenberg, 1975) and T cells (Cantor et al., 1975). These studies used polyclonal antisera, which were well developed in the murine system because of strain-specific polymorphisms. The description of two-color analysis by the same group followed, using fluorescein and rhodamine tagged polyclonal antibodies (Loken et al., 1977). Despite the rapid progress of flow cytometry, its application to human immunology and medicine appeared to be at an impasse. Unlike the murine and rat systems, where strain specific genetic differences could be exploited, surface molecule specific anti-human antisera were difficult to produce. As late as 1984, Kametsky, a pioneer of analytical cytology, lamented, 'Although there are now flow cytometers in clinical laboratories, their applications remain limited by a lack of specific markers' (Kametsky, 2003). The creation of monoclonal antibodies by Milstein and colleagues in 1977 (Pearson et al., 1977) gradually changed the field of flow cytometry, providing an inexhaustible supply of reagents of exquisite specificity. Within a year after Milstein's invention, monoclonal antibody specific for rat (White et al., 1978) and murine (Trowbridge, 1978) helper T cells and murine MHC antigens (Hammerling et al., 1978) were described. Shortly thereafter, the description of three murine monoclonal antibodies specific for human T cell surface determinants, designated OKT1, OKT3 and OKT4 (Kung et al., 1979) paved the way for human studies. Today these hybridomas are recognized as producing antibodies against the CD5, CD3 and CD4 determinants, respectively. In 1982 the first Human Leukocyte Differentiation Antigen (HLDA) Workshop was held in Paris, France and the principle of classifying monoclonal antibodies by clusters of differentiation (CDs) was formulated and a total of 15 CDs were defined. To date, 247 CDs have been identified. The eighth HLDA workshop, initiated in 2000 and scheduled for completion in 2004, is an international effort involving several hundred laboratories.

BENCHTOP ANALYTICAL FLOW CYTOMETERS

Advances in instrumentation also played a large role in bringing flow cytometry into routine use in clinical and less specialized research laboratories. The first flow cytometers were designed as cell sorters, with data analysis being a secondary consideration. On some models, data were recorded by taking Polaroid photographs of events plotted on an oscilloscope screen. By the late 1970s small, expensive computers produced by Tektronix, Hewlett-Packard and Digital Equipment Corporation permitted rudimentary computer-assisted data analysis. In these early days, internal random access memory was often limited to 64 kb and low capacity magnetic tapes and giant 256 kb 8-inch floppy disks were the only devices available

for data storage. By 1984, desktop computers and data storage on hard-drives had advanced to the stage that a Flow Cytometry Standard (FCS) for data storage was proposed (Murphy and Chused, 1984), allowing offline reanalysis of 'listmode' data for the first time. At about the same time, affordable benchtop cytometers became available. These instruments had air-cooled argon lasers and fixed optics, eliminating water cooling systems, special power requirements and daily optical alignment. Analytical software also progressed rapidly and analytical tools geared toward offline data exploration, such as Becton-Dickinson's revolutionary Paint-A-Gate (capable of color-eventing, which will be discussed later) and Verity's WinList and ModFit became available. In the mid-1990s, Coulter introduced the Elite XL 4-color analytical cytometer with digital electronics. After analog pulse-processing, signals were digitized at high-resolution, eliminating non-linear log amplifiers and paving the way for correct multi-color compensation. The newest generation of Beckman-Coulter, Becton-Dickinson and Dako-Cytomation cytometers all have provisions for listmode storage of uncompensated high-resolution data, extending to offline analysis the ability to recalculate color compensation.

THE HIV EPIDEMIC AND THE RISE OF FLOW CYTOMETRY IN CLINICAL IMMUNOLOGY

The history of the CD4 T-cell assay and its role in the fight against AIDS has been ably recounted by Mandy (Mandy et al., 2002). The finding that CD4 loss was associated with immunodeficiency in this newly described syndrome (Reinherz et al., 1981) led to the adoption of CD4 count as a biomarker of disease progression, standardization of CD4 analysis (Centers for Disease Control, 1992) and, most recently, the development of a bead-calibrated single-platform absolute count assay (Mandy et al., 2003) (discussed below). In the early days of AIDS clinical trials, the AIDS Clinical Trial Group (ACTG) and the National Institutes of Allergy and Infectious Disease Division of AIDS confronted the need for inter-laboratory standardization, ultimately developing protocols incorporating sample send-outs and other measures of quality assessment now widely accepted by the flow community (Paxton et al., 1989; Gelman et al., 1993; Bergeron et al., 1998).

The multi-center AIDS cohort study (MACS), a prospective study of almost 5000 gay men, proved to be an early triumph of standardization (Giorgi et al., 1998). The study, launched in 1984, followed the entire MACS cohort on a twice-yearly basis collecting clinical data, biological specimens and, of course, measuring CD4+ T cells. When inconsistencies were noted in the results from the first two visits, MACS investigators recognized the need for standardization and greatly improved assay agreement across centers during subsequent visits. An important aspect of the MACS study is that gay men were recruited without prior knowledge of their HIV status. More than 600 MACS

participants converted from HIV negative to HIV positive while on study, allowing the precise relationship between CD4 count and HIV infection to be defined (Margolick et al., 1993). Follow-up studies demonstrated that total T cell numbers (CD4 plus CD8) are conserved until the onset of clinical AIDS, providing evidence for the homeostatic regulation of T cell generation (Margolick et al., 1995; Margolick and Donnenberg, 1997). Another early MACS innovation was the discovery that assessing activation markers in combination with T cell subset markers could yield additional prognostic information (Giorgi et al., 1993). The MACS, which is still active 20 years since its inception, still relies heavily on flow cytometry for its studies on the immunologic monitoring of HIV+ cohort members who are now on anti-retroviral therapy. In retrospect, the HIV epidemic was a major driving force for the transition of flow cytometers from temperamental research instruments into ubiquitous clinical tools.

CONTEMPORARY FLOW CYTOMETRY

Quality control

Perhaps the least glamorous, but most important aspect of modern flow cytometry is the emphasis on quality control (QC). Like those pesky experimental controls, essential quality control measures are necessary for the valid interpretation of flow cytometric data. The instrument-related elements of a quality control program are shown in Table 20.1. The College of American Pathologists

(CAP), which administers a quality assurance program for clinical flow cytometry laboratories, states that quality assurance measures must cover specimen and result integrity throughout pre-analytical, analytical and post-analytical processes (D'hautcourt, 1996; Gratama et al., 1999). CAP provides a helpful checklist covering the areas that must be addressed by a quality assurance program. As a part of their accreditation process, laboratories also participate in surveys conducted twice or three times per year, in which blood samples are aliquoted and sent by express mail to participating laboratories. The results are tabulated and compared to the results of other laboratories using similar instrumentation and methods.

Although the level of quality control required in clinical laboratories may be overkill in the research setting, the careful investigator would do well to review the CAP checklist and decide which standards should be adopted.

The items listed in Table 20.1 are suggested for laboratories involved in human investigation to confirm consistent operation of the cytometer and performance of the reagents. Other QC issues such as sample integrity and consistency of data analysis are beyond the scope of this discussion, but should be considered as well. The first two steps of QC, optics and PMT setting verification, are performed with standard beads such as Beckman-Coulter FlowCheck and FlowSet, respectively. Fluidics/optics verification is performed on a linear scale and the objective is to ensure that the beads give a coefficient of variation (CV, standard deviation divided by the mean) below a predetermined value. Tight CVs indicate proper laser alignment and good hydrodynamic focusing in the flow cell.

Table 20.1 Recommended elements of flow cytometry quality control

Element	Procedure	Frequency	Comments
Fluidics/optics	Check CV of all parameters with standard beads	Daily	Poor CV can result from partial obstruction, drifting optical alignment, failing laser
Electronics	Set gain for all PMTs to place standard beads in target channels	Daily	Assures day-to-day consistency in fluorescence measurement. Assures that a balance between PMTs is maintained (important for compensation)
Color compensation	Stain single color control samples	Variable	Stained controls may be cells or beads and may be reagent-specific or fluorochrome-specific
Internal process/reagents	Concurrent staining of normal or preserved standard cells	Daily	For standard cells, mean per cent positive and absolute count with upper and lower limits are published by the vendor
Linearity/sensitivity	Check fluorescence intensity of multi-peak beads	Yearly. More often if quantitative fluorescence measurements are made	Beads of graded fluorescence intensity are run and the expected and observed fluorescence are compared

When a partial obstruction occurs, the most sensitive parameter is forward light scatter and this parameter will fall out of tolerance. Daily assessment of PMT gain is also important and easy to do. During the assay development phase, PMT settings (voltage and gain) will have been chosen appropriate to the test. For each fluorochrome, settings will have been chosen such that cell populations known to be negative are usually placed within the first decade and the brightest positive populations are on scale. At that time, calibration beads, such as Beckman-Coulter FlowSet would have been run and the target channel (channel of bead mean fluorescence intensity) would have been noted for each PMT. During daily calibration, the same calibration beads are run again and PMT gain is adjusted, if necessary, to place the beads at their target channel. This helps ensure that quantitative determination of fluorescence is consistent from day to day. The other ingredients necessary for quantitative fluorescence measurement are linearity of detection and calibration relative to a known standard. Linearity can be measured with multi-peak beads and antibody binding can be calibrated to molecules of equivalent soluble fluorochrome (MESF) using beads of known antibody binding capacity.

The topic of color compensation and how often and by what method it should be confirmed, is controversial. In the two-color world, where fluorescein isothiocyanate (FITC) and phycoerythrin (PE) were the only fluorochromes used, the required amount of color compensation did not vary from day to day, providing that the instrument had been calibrated as described above. Correct two-color compensation can be verified daily, weekly, or even less often, using beads dyed with the fluorochromes of interest, such as Becton-Dickinson CaliBRITE beads. The introduction of tandem dyes such as PE-Texas Red (also known as ECD), PE-Cyanine5 (PC5), PE-Cyanine7 (PC7), Allophycocyanine-Cyanine5 (APC-Cy5) and Allophycocyanine-Cyanine7 (APC-Cy7), opened up the world of polychromatic flow cytometry, but also introduced a twist to compensation. The emissions spectra of single fluorochrome dyes are always the same, regardless of the antibody to which it is conjugated, given that other relevant parameters (such as pH for FITC) are constant. This is not always the case for the tandem dyes, which can vary from manufacturer to manufacturer, lot to lot and, over time, within the same vial. Most of this variability is explained by the amount of free PE, PE that behaves as if it were not part of a tandem dye. Tandem dyes are especially light sensitive and the amount of apparent free PE can increase upon exposure to ambient light. Thus, the simple approach used for two-color cytometry, arriving at a single compensation solution to be used for all cells stained with the same fluorochromes, is not optimal in every setting. A method, advocated by some vendors, is to stain preserved or freshly isolated cells singly, with brightly staining antibodies representing each fluorochrome to be used in the multi-color combination. CD8 or CD45 are frequently used for this purpose.

However, the assumption that the compensation required for the CD8-PC7 in your single stained standard is identical to that required by the CD4-PC7 in your multicolor stained sample may not always be correct. The alternative, to stain cells singly with each antibody used in your panel, is not ideal either, since some of the markers will stain dimly and therefore will yield imprecise compensation settings. A solution advocated by some laboratories is the use of anti-Ig capture beads, which bind all murine monoclonal antibodies equally well, regardless of their specificity or fluorochrome. Such standards could be run with each assay, or even acquired after the fact to confirm that correct compensation settings were used. As mentioned above, many new cytometers have the ability to save uncompensated high-resolution listmode data (16 or 20 bits), allowing compensation to be performed or corrected after acquisition.

Identifying T cell subsets

Lymphocyte subsetting, the idea that immune cells at different stages of development or with distinct functions can be identified and quantified on the basis of expression of cell surface markers, has been one of the major applications to drive the development of current multi-parameter flow cytometry. The other major application, the classification and detection of leukemias and lymphomas, is beyond the scope of this article. In Western medicine, the notion of immunologic naiveté and memory can be traced to Jennerian vaccination in the late eighteenth century and the observation that exposure to a pathogen can lead to subsequent resistance to reinfection. Although this insight has led to the eradication of smallpox and the control of polio and many other infectious diseases of major public health significance, the molecular events and changes in lymphocyte populations responsible for the phenomenon of immune memory are still incompletely understood and controversial (Ahmed and Gray, 1996). The first inkling that immune memory was mediated by lymphocytes came from Landsteiner and Chase in 1942, when they showed that transfer of delayed-type hypersensitivity (DTH) from an immune to a naive guinea pig required leukocytes. I was fortunate to hear Dr Merrill Chase, who died at the age of 98 in January of 2004, recount the tale of this discovery and it is worthy of a short digression.

Dr Chase was a young assistant working on this problem at the Rockefeller University in the laboratory of the Nobel Laureate Karl Landsteiner. Since it was well appreciated that immunity to a variety of agents could be transferred from immune to non-immune animals by passive transfer of antibodies, their goal was to identify the humoral factor responsible for DTH. Guinea pigs were immunized with guinea pig stromal cells that had been haptenated with picryl chloride. This is a powerful DTH antigen and upon intradermal challenge, immunized animals developed characteristic erythematous reactions. In an attempt to

transfer this response, peritoneal exudates were induced and harvested from immune animals, clarified by centrifugation and injected into naive animals. It was the winter holiday season and the experiments thus far had been entirely negative. Rushing to get out of the laboratory, Dr Chase elected to save a few minutes by skipping the centrifugation step. The naive animals inoculated with cloudy cellular peritoneal exudate fluid responded vigorously to antigen challenge. The good news was that Dr Chase had opened a new field of enquiry. The bad news was that he had to admit to the demanding Dr Landsteiner precisely what he had done. The experiment was repeated with appropriate negative controls and the rest is history.

Decades later, the existence of memory T cells participating in B-cell mediated antibody responses (Mitchell et al., 1972) and in the generation of cytotoxic effector functions (Kedar and Bonavida, 1975) were also demonstrated in adoptive transfer models. The next advance awaited the development of several anti-human monoclonal antibodies that could be used to separate T cells into recall antigen responsive and unresponsive populations. These antibodies included 4B4 (CD29, the adhesion molecule beta-1 integrin) (Morimoto et al., 1985a), HB-11 (CD38, an ectoenzyme ADP-ribosyl cyclase) (Tedder et al., 1985), 2H4 (CD45RA, a high molecular weight isoform of the leukocyte tyrosine phosphatase) (Morimoto et al., 1985b) and UCHL1 (CD45RO, the low molecular weight isoform of the leukocyte tyrosine phosphatase) (Smith et al., 1986). The Shaw laboratory was the first to frame this problem in terms of a dichotomy between naive and memory T cells (Sanders et al., 1988), demonstrating that umbilical cord blood T cells (prototypically naive) expressed low levels of LFA-1 (CD11a/CD18, adhesion and signaling), LFA-3 (CD58, adhesion and costimulation), CD29 and UCHL1 (CD45RO) and high levels of CD45RA, compared to adult peripheral T cells. They also showed that mitogen stimulation of cord blood resulted in upregulation of CD45RO, CD58 and CD29. CD45, the leukocyte common plasma membrane-associated tyrosine phosphatase, has proved to a particularly useful marker of T cell differentiation and activation (Trowbridge and Thomas, 1994). CD45 contains three exons (A, B and C), which can be differentially spliced to produce isoforms of distinct molecular weight. Isoform switching takes place during T cell maturation and activation and multiple isoforms can be expressed in the same cell. As mentioned above, recall-antigen reactive memory T cells were found within the population expressing the lowest molecular weight isoform (CD45RO) in which all three exons have been spliced out. Naive cells, again defined by their predominance in umbilical cord blood, were found within the population retaining the A exon (CD45RA) (Sanders et al., 1988; Bradley et al., 1989). However, the advent of multiparameter cytometry revealed both phenotypic and functional heterogeneity within T cell populations, whether defined on the basis of CD45 isoform expression, or expression of any other single marker. Using four-color

cytometry, then a great novelty, Picker and colleagues integrated the findings of Shaw and definitively mapped the phenotypic changes accompanying the transition from naive to memory T cell (Picker et al., 1993). Among the many important findings in this report was that CD45RA+/CD45RO- naive cells proceed through a CD45RA+/CD45RO+ intermediate on the way to becoming CD45RA-/CD45RO+ and expressing high levels of CD58, CD11a and CD54 (ICAM-1). They also demonstrated that CD62L (L-selectin) was critically involved in T cell homing, being high in peripheral lymph nodes, even during activation, but downregulated in the tonsils and appendix during activation or *in vitro* after stimulation. This study also set a benchmark for the analysis of multiparameter data, making excellent use of *color-eventing* (demonstrated later in this chapter) to reveal the relationship of four parameters in a single bi-variate plot.

Chemokine receptor 7 (CCR7) is also important in lymphocyte trafficking. Once CD62L has allowed the lymphocyte to dock on the high endothelial venule, engagement of CCR7 with its endothelial cell ligand SLC, allows lymphocyte to transmigrate into the lymph node (Campbell et al., 2001). Sallusto et al. (1999) looked at the functional properties of CD4+ and CD8+ T cells sorted on the basis of CCR7 and CD45RA expression. Among CD4+ T cells, sort purified CD45RA-/CCR7- cells secreted the highest levels of interferon-gamma, IL-4 and IL-5 upon subsequent polyclonal stimulation. In contrast, all CD4 populations secreted IL-2. For CD8+ T cells, the double negative population and the CD45RA+/CCR7- populations were strong interferon-gamma producers, whereas CD45RA+/CCR7+/CD62L+ cells produced the highest levels of IL-2. They theorized a T cell maturational pathway in which CD45RA+/CCR7- naive T cells (capable of lymph node homing) give rise to CD45RA-/CCR7+ central memory cells (also capable of lymph node homing). These in turn give rise to CD45RA-/CCR7- effector memory cells. The CD45RA+/CCR7- population was too rare to study in CD4+ T cells, but comprised the most powerful effector population, as measured by cytokine secretion and granzyme and perforin expression among CD8+ T-cells.

Appay and colleagues (2002) used MHC-class I tetramer technology to examine the differentiation of antigen specific CD8+ T cells obtained during acute and chronic infections with Epstein Barr virus, hepatitis C virus, human immunodeficiency virus and cytomegalovirus. Defining effector cells as activated, virus specific cells found during acute infection and memory cells as resting virus specific cells found during periods of low viral load (chronic infection), they disputed the hypothesis that antigen specific CD8+ T cells transition from naive cells, to memory cells and then to effector cells. Instead, they proposed that antigen-primed T cells could be divided into three subsets according to CD28 and CD27 expression, evolving unidirectionally with time after antigen exposure from CD28/CD27 double positive, to CD28-/CD27+ and

finally to CD28⁻/CD27⁻. The early cells in this continuum have greater proliferative potential and later cells greater cytotoxic potential. Depending on the specific virus, memory cells (antigen experienced resting cells) could 'accumulate' at any stage of this continuum.

Approaching the problem of T cell subsetting with the current state of the art in cytometry, DeRosa and colleagues (2001) used 11-color cytometry to define unambiguously the phenotype of naive T cells. In their example, they used a highly modified hybrid cytometer (Becton-Dickinson and Cytomation) fitted with violet, blue and red lasers, succeeding in detecting five colors off the blue laser, four colors off the red laser and two colors off the violet laser. Examining CD4⁺ and CD8⁺ T cells simultaneously, they concluded that naive T cells, defined as incapable of producing interferon-gamma, were CD45RA⁺/CD62L⁺/CD11a dim/CD27⁺. Minor cell populations discordant for expression of these markers secreted interferon-gamma and therefore were interpreted as being contaminating memory T cells.

PRACTICAL CONSIDERATIONS AND EXAMPLES

Rare event problems and multiparameter flow cytometry

Discriminating individual cell populations in a heterogeneous mixture always comes down to a signal to noise problem: how to separate the populations of interest from other populations in multiparameter space. Autofluorescence, non-specific antibody binding, multimodal distribution of specific antibody binding and shared antibody specificities (e.g. CD5 is present on T cells, but also a population of B cells, CD8 is present on subsets of T and NK cells), all contribute to this problem. By careful choice of antibodies and fluorochromes, populations of interest can be pulled into unique locations in multiparameter space. This is especially important for populations that are present at low frequency. The advent of polychromatic flow cytometry has given us the unprecedented ability to identify subsets on the basis of multiple parameters and perform functional measurements simultaneously in well-defined cell subsets. Such functional determinations include cytokine secretion (Brosterhus et al., 1999), T-cell receptor specificity (Altman et al., 1996), cytotoxicity (Liu et al., 2002), proliferation (Drach et al., 1989; Li et al., 1995; Nordon et al., 1997; Shapiro, 2003), apoptosis (Guedez et al., 1998), calcium flux (June et al., 1986), viability (Darzynkiewicz et al., 1982), multiple drug resistance pump activity (Donnenberg et al., 2004), mitochondrial membrane potential (Cossarizza et al., 1993), kinase activity (Perez and Nolan, 2002), telomere length (Baerlocher and Lansdorp, 2003) and many others. In this section, we will provide several practical examples from our laboratory of flow cytometry techniques, many of which exemplify the synthesis of phenotypic and functional determinations.

The challenge of rare event problems: dendritic cells in the peripheral circulation

The detection of circulating monocytoïd and lymphoid dendritic cells (DC1 and DC2, respectively) requires strategies that are common to all 'rare event' problems in flow cytometry. Technical aspects of rare-event detection have been reviewed by several authors (van den Engh, 1993; Rosenblatt et al., 1997; Donnenberg and Meyer, 1999; Baumgarth and Roederer, 2000; Donnenberg et al., 2003). What follows is a practical discussion of rare event detection strategies as applied to the DC problem, which we have previously discussed in some detail (Donnenberg and Donnenberg, 2003).

Sample concentration and flow rate

Although event frequency is an intrinsic property of the sample, the proportion of events of interest can be greatly increased in the analytical window. For example, knowing that DC1 and DC2 do not express lineage markers present on most peripheral mononuclear cells, it becomes possible to increase greatly the proportion of DCs in the analysis by eliminating irrelevant or interfering cell populations. In the measurement of DC, this virtual depletion is accomplished by staining T cells, monocytes and B cells with a cocktail of lineage markers (CD3, CD14, CD19), all labeled with the same fluorochrome. Cells expressing any of these markers are logically removed from the analysis using what has been called a *dump gate* (Donnenberg et al., 2001). Through judicious use of the threshold parameter or live gating, irrelevant events can be made invisible to the cytometer or eliminated from the list-mode file (an example of the latter will be provided in Figure 20.4). However, it is the absolute frequency of the event of interest which dictates how many total cells must be processed. Obviously the lower the frequency of the events of interest, the more events it will be necessary to acquire. Although the rate of sample acquisition on the flow cytometer can be manipulated by increasing the sample flow rate, best results are obtained when the flow rate is not unduly rapid and the cell concentration of the sample is optimized for the particular instrument.

Signal to noise

Successful detection of rare events depends on maximizing the signal to noise ratio. Noise comes from many sources including the non-specific binding of a fluorochrome of interest, cellular autofluorescence, disruptions in fluidics and other electrical or mechanical problems. One very important aspect of rare event analysis is to characterize the total noise using an appropriate negative control. Sometimes, it is possible to devise a control sample that is identical to the experimental sample in all respects, except that it does not contain the rare event of interest (Barratt-Boyes et al., 2000) or, it may be

necessary to use control reagents such as isotype-matched fluorochrome-conjugated antibodies (Donnenberg et al., 2001). In the latter case, a few caveats apply: in addition to being matched for isotype, the control reagent must be similar to the experimental reagent with respect to the ratio of fluorescent dye to protein and it must be used at the same concentration. Additionally, staining must be designed so that the same gating strategy used to detect the rare event can be applied to the negative control sample. Mario Roederer has termed this strategy FMO, or fluorescence minus one (Roederer, 2001), although when we have two outcome parameters (see Figure 20.7) FMT may be more appropriate. It is also important to point out that an identical number of events must be acquired for the negative control sample as for the experimental sample, because it is the frequency of false positive events that determines the lower limit of detection. For example, if the frequency of spuriously positive events is 1 in 5000 in the FMO negative control, it is impossible to use this assay to detect cells present at a frequency of 1 in 10 000, no matter how many events are acquired. It follows that there is a point of diminishing return beyond which acquiring a greater numbers of events will increase the precision of the rare event frequency estimate, but will not increase the sensitivity of the assay.

Two important factors bear on the signal to noise ratio: the difference in mean fluorescence intensity between negative and positive populations and their variances (usually expressed as the CV). Some membrane or cell-associated dyes give very bright signals and therefore place cells far from noise. When using combinations of fluorochrome-conjugated antibodies, we often reserve PE for the most critical measurement, because the absorption and emissions spectra are widely separated, and the extinction coefficient and quantum yield are high compared to other fluorochromes (Shapiro, 1995).

When autofluorescent myeloid cells, cellular debris or red blood cells interfere with rare event detection it is often possible to *move them out of the way* by targeting them with a specific antibody (e.g. CD14 for monocyte/macrophages). We have used this strategy to identify DC subsets that comprise a small proportion (1 in 10 000 to 1 in 100 000) of bronchoalveolar lavage cells (Donnenberg and Donnenberg, 2003). Cell doublets, either cells physically adherent to each other, or two or more cells erroneously detected as a single pulse, are also a source of concern for rare event detection. These can be gated from the analysis using doublet discrimination, a comparison of pulse height and pulse integral, or pulse height and pulse width (Wersto et al., 2001). An example of doublet discrimination will be given in Figure 20.4.

Another important aspect of the overall signal to noise is the number of parameters used to define the rare event of interest. Modern multi-laser, multi-PMT instruments permit detection strategies that make use of multiple parameters to define the rare event. In the case of DC1, the rare event of interest should be positively identified

by more than one fluorescence parameter (CD4+, HLA-DR+ and CD11c+). Not quite so obvious is the importance of including one or more fluorescence parameters for which the rare event is negative (lineage negative, CD123 negative). In rare event detection, it is almost as important to specify where the rare event is *not*, as to specify where it is. This is especially helpful for defining a set of compound logical gates that assign the population of interest a unique location in multiparameter space. Such analyses are performed after the fact, on listmode datafiles. Compound gating strategies that maximize detection of events in the positive control while minimizing false positive events in the negative control can be determined empirically and applied to the experimental data. It is often helpful to use *color-eventing* to determine where the events of interest fall with respect to all of the measured parameters. From the clustering of colored events, it sometimes becomes apparent which are genuine and which are artifactual. Examples of *color-eventing* will be presented in Figures 20.1, 20.7 and 20.9.

Cellular autofluorescence, due primarily to the presence of native fluorescent intracellular molecules such as flavins are excited by the 488 nm laser line and can contribute to noise (Aubin, 1979). As long as the rare event of interest is not itself highly autofluorescent, autofluorescent cells can be eliminated from the analysis by acquiring an unstained or irrelevantly stained fluorescence parameter and including this in the dump gate. We are currently investigating the use of a green laser line to mitigate autofluorescence in cells stained with PE and PE-tandem dyes.

The use of a time parameter, the time that each event was acquired, will be illustrated in Figures 20.2, 20.3 and 20.4. Time, either saved as a listmode parameter or calculated offline, can be used to identify and eliminate episodic noise encountered during long sample acquisitions.

Reproducibility

The results of rare event experiments are often visually unimpressive. Against a denominator of a million or more acquired events are a small number of positive events that have been filtered through a series of logical gates defining the population of interest. Sometimes the events form a tight cluster in a two-parameter scatterplot (e.g. CD123 versus CD11c in Figure 20.1). Sometimes they are diffuse in two-parameter space but are unique in multiparameter space. The credibility of such results can be enhanced by performing replicate determinations. This will be illustrated in Figure 20.3, where six replicate determinations are used in the detection of T cells in a radically T-depleted hematopoietic stem cell graft. The frequency of rare events can then be reported as the mean value and the associated confidence interval and the CV can be calculated. For example, when we measured DC2 in the peripheral blood in triplicate, the intra-subject CV was only 6.9 per cent, indicating high reproducibility

despite the relative rarity of the event being measured (Donnenberg and Donnenberg, 2003). The frequency of false positive events detected in the negative control sample (also collected in replicate with the same number of events as the experimental sample) should also be reported and a lower limit of detection may be calculated as the upper 99th percentile of negative control. In our DC studies the lower limits of detection for pDC was 0.0003 per cent (<1/300 000) (Donnenberg et al., 2001), whereas in our MHC tetramer studies it was only 0.0125 per cent (1/8000) (Hoffmann et al., 2000). This illustrates the critical importance of the FMO negative control for the interpretation of rare event data.

Example 1: Five-color simultaneous detection of DC1 and DC2

Immature dendritic cells are present in the peripheral blood at low frequency, but are easily visualized and resolved into myeloid and lymphoid subsets using multi-parameter cytometry, the gating strategy shown in Figure 20.1 (Donnenberg et al., 2001). A dump gate was used to actively define what the event of interest is not. In this example a *lineage cocktail* of antibodies directed against

CD3, CD14 and CD19 was used to eliminate T cells, monocytes and B cells during the first stage of analysis. Limiting the analysis to cells that coexpress CD4 and HLA-DR, a phenotype shared by myeloid and lymphoid DC, further narrowed the field. Finally, these subsets were distinguished by the expression of CD11c, unique on myeloid DCs, and CD123, unique on lymphoid DCs. Backgating the resulting DC1 and DC2 populations onto forward by side light scatter allowed these physical parameters to be compared between the two DC subsets.

Example 2: Cellular product monitoring

Flow cytometry plays a critical role in assessing the purity and, to some extent, the potency of products manufactured for cellular therapy. Release criteria for cellular products include limits for these two parameters, plus some assessment of product safety (often sterility and absence of endotoxin). In a recent NIH-sponsored trial conducted at the University of Pittsburgh, USA, patients with severe scleroderma, a systemic autoimmune disease, were treated with high dose anti-lymphocyte therapy followed by rescue with autologous hematopoietic progenitor cells. In order to avoid reintroduction of T and

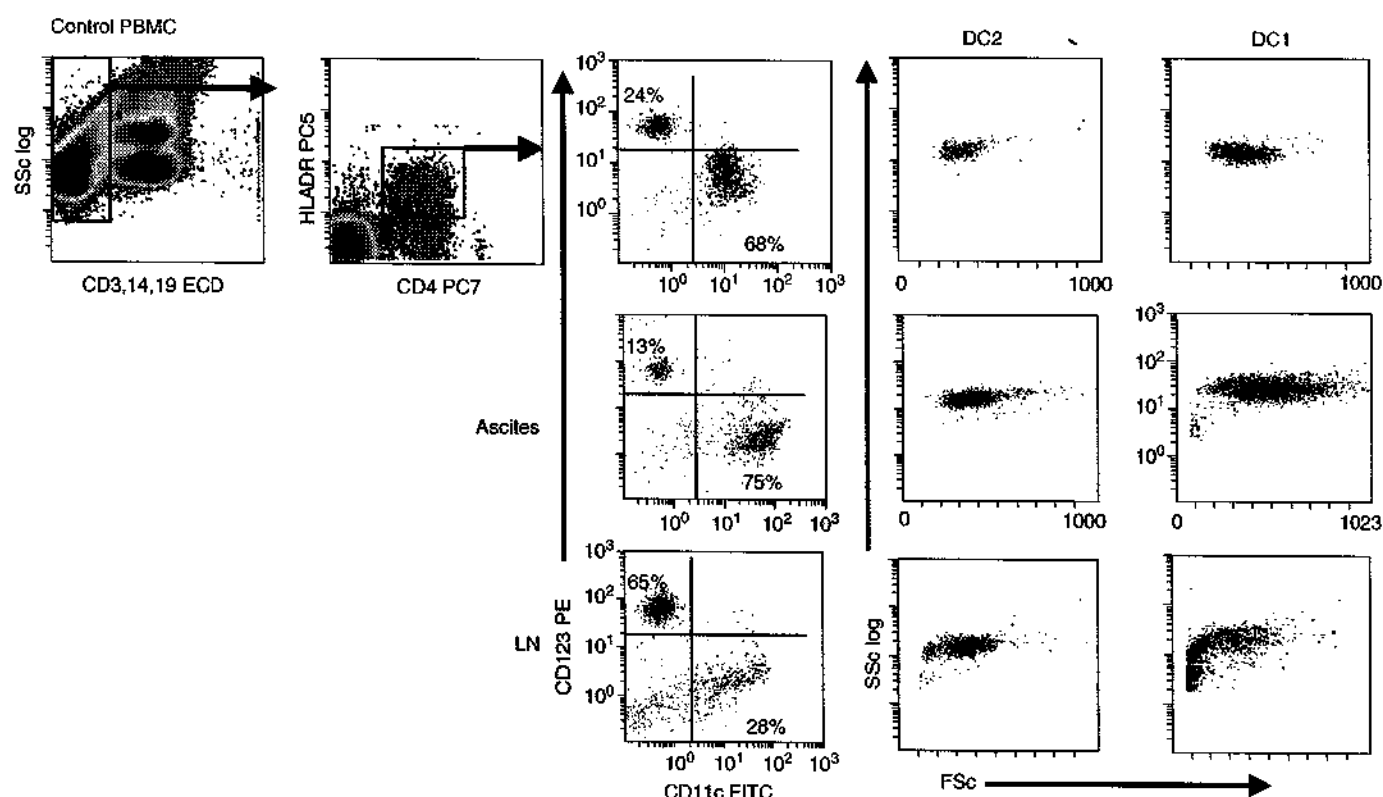


Figure 20.1 Detection of DC1 and DC2. DCs in the peripheral blood of a healthy subject are contrasted with ascitic fluid and peritoneal lymph node (LN) from a patient with newly diagnosed untreated ovarian cancer. Cell suspensions were stained with antibodies CD11c-FITC, CD123-PE, a cocktail of CD3, CD14 and CD19-PE, HLADR-PC5 and CD4-PC7. Cells were acquired on a Beckman-Coulter FC500 cytometer. In this analysis, lymphoid DCs have been color-evented red and myeloid DCs have been colored blue. The differences in scatter properties of these two populations are shown in the last column. In ovarian cancer, myeloid DCs are larger in ascites, compared to control peripheral blood. In LN, where lymphoid DCs predominate, the myeloid DC evidence a population with low forward and side scatter, consistent with apoptosis. See colour plate 20.1.

B lymphocytes that could reinitiate the disease process, peripheral blood progenitor cells were radically depleted in a two-step immunomagnetic separation process that included the positive selection of CD34+ progenitor cells followed by the negative selection of CD3+ T cells. In the starting product, T cells represent anywhere from 10 to 20 per cent of nucleated cells, whereas CD34 progenitor cells usually comprise 1 per cent or less. According to the release criteria of the study, a pure, potent and safe T-depleted product would have an adequate progenitor cell dose (5×10^6 CD34/kg), a 5-log or greater reduction in T cells and be negative for bacterial or fungal

contamination. A single platform, bead-calibrated assay was used to assess CD34 purity, absolute CD34 content and CD34 viability (Figure 20.2), using the landmark gating strategy of Sutherland (Sutherland et al., 1996). Absolute white cell count (WBC) was also measured using CD45 to identify leukocytes. CD34 and WBC viability was determined by exclusion of the fluorescent agent 7-amino-actinomycin D (Donnenberg et al., 2002). This assay was modified for quantification of residual T cells in the product as rare events (Figure 20.3). The modifications included substitution of CD3 for CD34, an increase in the amount of product added per tube (from 100 μ l to 200 μ l)

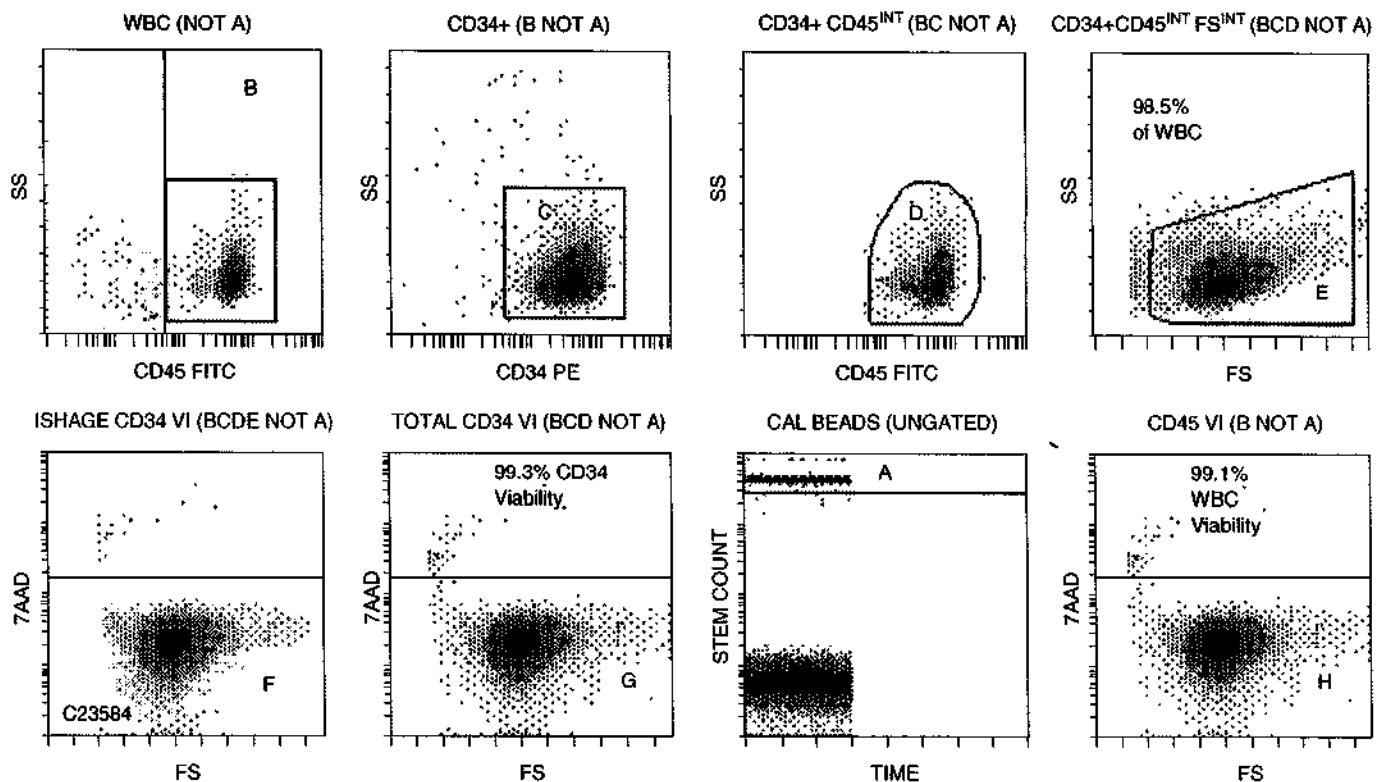


Figure 20.2 Single platform determination of CD34 content and viability of a CD34 selected, CD3 depleted peripheral hematopoietic progenitor cell graft. Graft aliquots (100 μ l) were stained in duplicate with anti-CD45-FITC and anti-CD34-PE, in a bead-calibrated lyse/no wash assay (Beckman-Coulter StemKit). A sample stained with the same reagents plus great excess of unlabeled anti-CD34 was run as a negative *isoclonic* control. Cells were acquired on a Beckman-Coulter XL cytometer. StemCount beads are identified by their high fluorescence in FL4 versus time (Gate A). These are removed from subsequent analyses using a *not* gate. Collecting *time* as a parameter facilitates identification of fluidic and other instrument problems during sample acquisition. White blood cells are identified by CD45 expression (gate B). The WBC gate, compounded with the *not bead* gate is passed to a histogram of anti-CD34 versus side scatter. CD34+ events with low side scatter are identified (gate C). The compound gate of WBC, *not bead* and CD34+ is passed to a histogram of CD45 versus side scatter, where cells of intermediate CD45 expression are identified (gate D). The compound gate of WBC, *not bead*, CD34+ and intermediate CD45 expression is passed to a histogram of forward scatter versus side scatter (gate E), which is used to eliminate events with low forward scatter. The number of CD34+ cells obtained in gate E, taken as a percent of WBC identified in gate B, represents the percent CD34+ cells as defined by the International Society of Hematology and Graft Engineering (ISHAGE, now the International Society of Cellular Therapy). For determination of the viability of CD34+ cells, as defined by ISHAGE, the compound gate of WBC, *not bead*, CD34+, intermediate CD45+ expression and intermediate to high forward scatter is passed to a histogram of forward scatter versus intracellular 7AAD concentration (FL3). Because 7AAD is excluded by viable cells, viable CD34+ cells are detected in region F. Even in samples with low overall viability, the proportion of dead cells within the F region is invariably low. This is because the majority of dead and dying cells have low forward scatter and are eliminated by gate E. It is for this reason that we measure and report CD34 viability using a compound gate of WBC, *not bead*, CD34+ and intermediate CD45 expression, where viable CD34+ cells are detected in region G and reported as a per cent of cells in gate D. Similarly, viable WBC are detected in region H and reported as a per cent of cells in gate B. Absolute WBC and CD34 counts are obtained by dividing the events in gates B and E, respectively, by the known StemCount bead concentration. In this sample, CD34+ cells represented more than 98 per cent of CD45+ events. The viability of total WBC (CD45+) and CD34+ cells exceeded 99 per cent. Prior to CD34 selection/CD3 depletion, CD34+ cells comprised 2.95% of CD45+ cells.

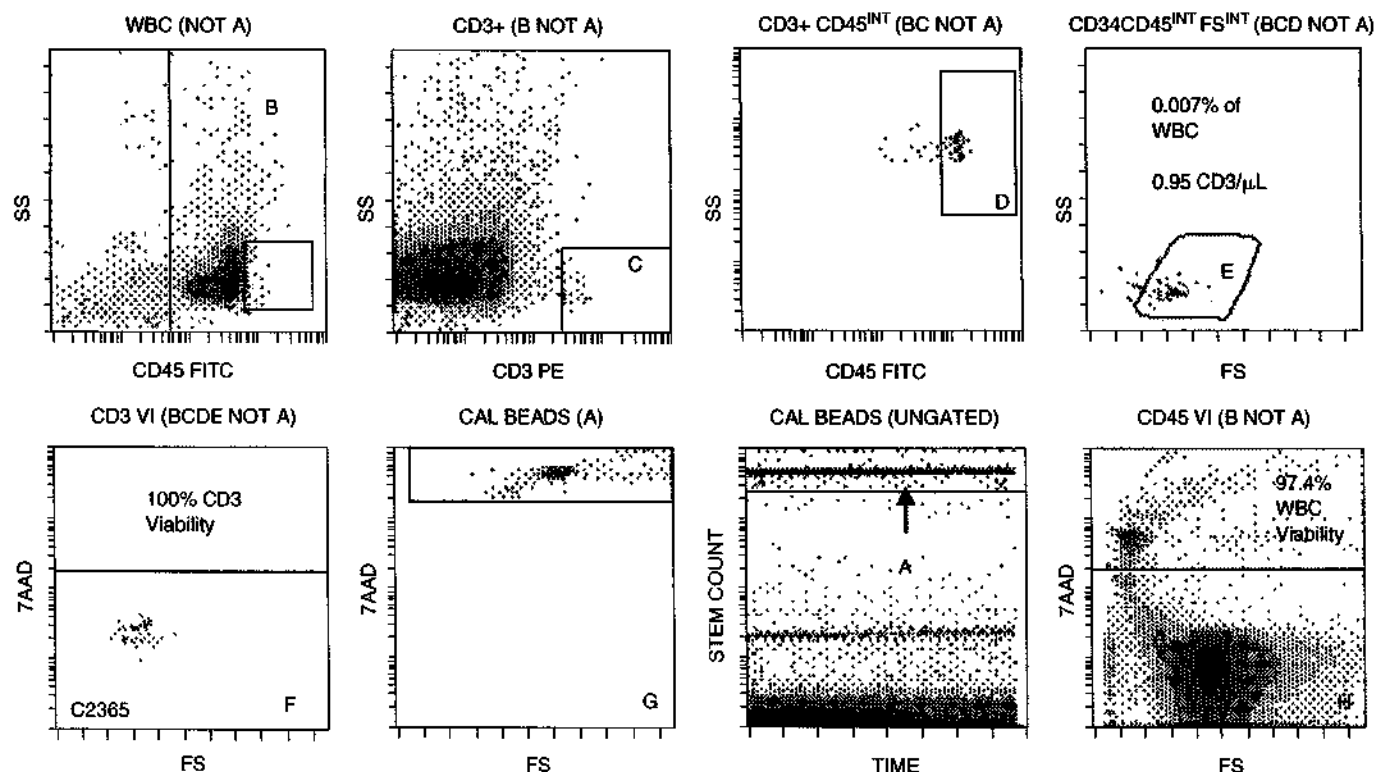


Figure 20.3 Detection of CD3+ T cells as rare events in a CD34 selected, CD3 depleted peripheral hematopoietic progenitor cell graft. The data are from the same product shown in Figure 20.2. In order to achieve maximum sensitivity, graft aliquots (200 μ L) were stained with anti-CD45-FITC and anti-CD3-PE in 6-plicate and cells were acquired to exhaustion. Cells were acquired on a Beckman-Coulter XL cytometer. Analysis was similar to that used in Figure 20.2. CD3+ T cells were distinguished from noise by their bright expression of CD45 (Gate D). No events fitting these criteria were seen in the FMO control (not shown). CD3+ cells comprised only 0.007 ± 0.001 per cent (mean $\pm$ SEM) of nucleated cells in this sample and were present in the graft at a concentration of 0.950 ± 0.148 cells/ μ L, for a total of 1.3×10^5 T cells in the entire product. Prior to CD34 selection/CD3 depletion, CD3+ cells comprised 13 per cent of CD45+ cells for a total of 9.9×10^9 T cells in product. This represents a 5-log depletion.

and an increase in the number of replicate tubes from two to six. The samples were exhaustively acquired, such that every cell in 1.2 ml of product was counted, yielding a lower limit of detection of <500 T cells/ml, several orders of magnitude lower than that of conventional assays. The replicate determinations also permit confidence intervals to be determined about the mean absolute count and per cent positive.

The *single-platform* nature of these assays also plays an important role in reducing the variability of this assay and bears some explanation. The first widely used absolute count application was the measurement of circulating CD4+ T cells. As recounted above, the accurate determination of CD4 count proved very useful for monitoring patients with HIV infection. The first efforts required dual platforms: the absolute lymphocyte count was determined by a hematology analyzer and the proportion of CD4+ cells, where the denominator was events falling within a lymphocyte light scatter gate, was determined by the flow cytometry. The absolute count (CD4 cells/ μ L of blood) was derived by multiplying these two values. This approach suffers from two faults: both assays contribute independent sources of variance and the two assays are not entirely consistent in identifying lymphocytes, especially in

abnormal samples. Single platform assays include those using a volumetric approach (O'Gorman and Gelman, 1997) and those that are calibrated with reference to beads of a known concentration. The latter approach was used in Figures 20.2 and 20.3. Undiluted product, added as precisely as possible using a positive displacement pipette, is incubated with antibodies. Red cells, if present, are lysed by addition of ammonium chloride. Before acquisition, a precise volume of uniform beads of known concentration (StemCount, Beckman-Coulter) is added. Since the sample is never washed, there is no possibility of cell loss. The number of events of interest counted (CD34+ cells or T cells) is compared to the number of beads counted. Since the concentration of the beads is known, the concentration of the events of interest can be inferred with accuracy and precision not attainable in dual platform assays.

Example 3: Measuring antigen specificity and T cell receptor repertoire in T cell subsets

MHC class I/peptide tetrameric complexes (tetramers) represent a powerful tool for detecting, quantifying and separating peptide-specific CD8+ T cells. Theoretically,

cells binding a particular tetramer should be pauci-clonal (i.e. consisting of few clones), because they represent only those cells with T-cell receptors able to bind with sufficient avidity a particular nine-mer peptide in the context of a particular MHC class I allele. In this example, we used a strain-common influenza matrix protein peptide (GILGFVFTL) as a prototype recall peptide, because virtually all adult human subjects have experienced multiple exposures to influenza virus naturally or by vaccination. As part of our validation studies, we examined the V-beta usage in flu tetramer+ and flu tetramer negative CD8+ T cells. In unselected CD8+ T cells, individual V-beta families represent anywhere from 10 to 0.1 per cent. Even in highly immune individuals, flu tetramer positive cells represent 0.1–0.2 per cent of CD8+ T cells (Hoffmann et al., 2000). Evaluation of V-beta usage on tetramer+ T cells is therefore a quintessential rare event problem because the events of interest are the product of the tetramer and V-beta frequencies.

This analysis illustrates several principles of rare event analysis:

- 1 bulk staining
- 2 doublet discrimination
- 3 the use of a dump gate
- 4 live gating
- 5 the use of time as a gating parameter
- 6 high-speed sample acquisition.

To accomplish this, we stained approximately 200 million peripheral blood mononuclear cells obtained from an HLA A201+ volunteer donor under an IRB approved protocol. The cells (200×10^6) were first stained in bulk with APC conjugated Flu tetramer (Beckman-Coulter T20135), followed by anti-CD8 PE-Cy7 and anti-CD4 ECD (an internal negative control). When determining the amount of tetramer to use, it is important to remember that it is the final concentration of tetramer in the staining mixture and not the number of cells, that is the critical variable. The sample was then divided into 10 tubes (8 V-beta tubes representing 24 specificities, Beckman-Coulter BetaMark), plus alpha-beta/CD3 and isotype controls) and stained for V-beta. Each tube therefore contained about 20 million cells. The V-beta reagents occupied the FITC and PE channels, but covered 3 V-beta specificities per tube. This is possible because only one V-beta specificity is expressed on any given T cell. Thus for a mixture of antibodies to V-beta families X, Y and Z, X was FITC conjugated, Y was PE conjugated and Z was a mixture of FITC and PE conjugated antibodies. We resuspended the cells at approximately 30×10^6 /ml and acquired the data on a Dako-Cytomation MoFlo cytometer. In order to keep the size of the data files under 2 megabytes each, 10–15 million events were collected and saved to a listmode file using a live gate that included only tetramer+ events (Figure 20.4). This reduced the number of events saved to the listmode file to about 50 000. For analysis tetramer+ events were

further gated on single cells, followed by lymphoid light scatter, CD8+, CD4– (Figure 20.4).

This reduced the number of true tetramer+ CD8+ T cells to approximately 2000 per tube, a number sufficient to perform V-beta analysis. At a rate of approximately 25 000 total events per second, acquisition required about 10 minutes per tube, or about 2 h for the entire data set. In order to compare V-beta usage in tetramer+ CD8+ T cells to that in total CD8+ T cells, we also acquired approximately 1 million events without the use of a live gate. The data clearly show that flu tetramer positive cells are heavily skewed toward the V-beta 16 family (Figure 20.4, panel H). Whereas only 1.2 per cent of total CD8+ T cells used this T-cell receptor sequence, fully 51.4 per cent of tetramer+ CD8+ T cells were V-beta 16 positive.

Example 4: Kinetic analysis of the multiple drug resistance transporter P-gp in T cell subsets

An aspect unique to multi-parameter flow cytometry is the ability to make functional measurements over time in defined subsets within a heterogeneous sample. The measurement of calcium flux in T cells immediately after T cell receptor binding (June et al., 1986) is an early and well-known application. In this present example we measured activity of the multiple drug resistance (MDR) transporter P-glycoprotein (P-gp) in freshly isolated peripheral blood CD8+ central memory/memory effector T cells. The probe, rhodamine 123 (R123) is a green fluorescent cationic lipophilic dye and a P-gp substrate. Cells can be pre-stained with monoclonal antibodies, so that P-gp activity can be determined individually in lymphocyte subsets. Conventionally, cells are loaded with R123 at 37°C in an incubator or water bath. The cells are washed and the R123 fluorescence is measured to determine the maximum brightness immediately after loading with the dye. The cells are then returned to the incubator for 30 min to 3 h and R123 fluorescence is determined again. R123 is not passively lost in this time frame and only cells actively transporting R123 from the plasma membrane will show a decrement in R123 fluorescence (Donnenberg et al., 2004). In the present example, we extended this assay by performing real-time measurement of R123 uptake and efflux in four circulating T cell populations subsetted by CD4, CD8 and CD45RA expression. Using a water-heated sample station, cells were maintained at 37°C while cells were acquired on the flow cytometer. Baseline data were acquired for 2 min, after which the cells were loaded with R123 and acquired continuously for 15 min. The sample was removed from the cytometer, washed at 4°C and returned to the cytometer for continuous measurement of R123 efflux for an additional 30 min. Kinetics of dye uptake and efflux were determined for each T cell subset and optimal loading and efflux times were determined. During uptake, R123 fluorescence increased rapidly and approached plateau by 10 min. The data for the

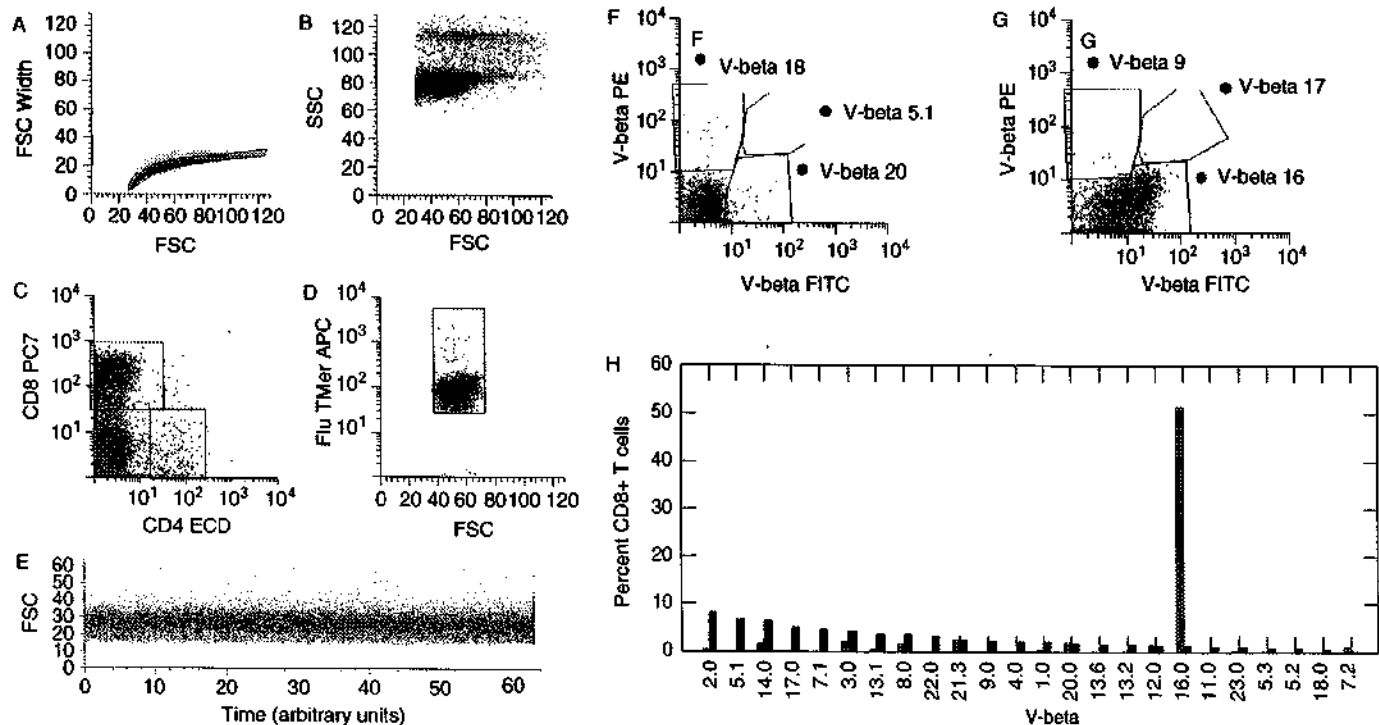


Figure 20.4 Rare event detection of TCR V-beta usage among Flu tetramer+ CD8+ T cells. In panel A, a gate is created on forward scatter pulse height and forward scatter pulse width, excluding doublets (high pulse width for a given pulse height). In panel B the analysis is limited to events displaying lymphoid forward and side light scatter properties. Panel C (*dump* gate) is used both to positively identify CD8+ T cells and to eliminate CD4+ T cells (and anything else appearing positive in the ECD channel). Panel D is the *live* gate on tetramer+ events. Only tetramer+ events falling within this gate were saved in the listmode file, greatly reducing the size of the datafile and increasing the speed of analysis. Forward scatter is shown versus Time (panel E), a calculated parameter that is used to identify and eliminate any spurious event bursts (none were detected here). Finally, panels F and G (from separately stained tubes) show the results of the application of this compound gating strategy. Panel F shows the usage of V-beta 18 (0.04 per cent), 5.1 (0.0 per cent) and 20 (1.9 per cent) among Flu tetramer+/CD8+/CD4- cells. Panel G shows that the majority of Flu tetramer+ T cells (51.4 per cent) are V-beta 16+. Panel H shows the final result, the V-beta repertoire of flu tetramer- and flu tetramer+ CD8+ T cells. Data from tetramer+ events were collected for 24 V-beta specificities. Data were acquired separately for determination of V-beta usage in tetramer negative T cells (without the use of the live gate D). The results are plotted in the order of frequency of V-beta usage in tetramer negative CD8+ T cells (blue bars). Common specificities such as V-beta 2, 5.1, 14 and 17 were very underrepresented in flu tetramer+ T cells (red bars), whereas V-beta 16, which comprised only 1.2 per cent of total CD8+ T cells, accounted for 51.4 per cent of flu tetramer+ T cells. These results are consistent with the clonality (or pauci-clonality) of the majority of influenza matrix peptide specific T cells in this sample. See colour plate 20.4.

CD8+ CD45RA- population, the population with the highest P-gp activity, is shown in Figure 20.5. All cell subsets tested achieved similar plateau levels. R123 efflux exhibited a biphasic profile. The initial slope over a 10-min interval (log-fluorescence channels/min) differed markedly among T cell subsets with CD8+ CD45RA+ evidencing the greatest slope. These results extend our previous static observations that the CD8 memory subset exhibits the greatest P-gp activity, which may have important pharmacological implications during cell-specific immune responses.

Example 5: Caspase 3 activity and plasma membrane phospholipid composition in early T cell apoptosis

Late apoptotic events can be visualized by flow cytometry in a variety of ways, including changes in light scatter (DC1 in Figure 20.1) and DNA laddering (Lyons et al., 1992). Many physical and biochemical changes take place

in the cell as it follows its program from initiation of apoptosis through cell death and, as Mario Roederer has commented, it is inadvisable to try to measure anything on a dead cell. If one wishes to perform analyses such as determination of apoptosis on specific subsets, it is therefore important to focus on the earliest phases of the apoptotic process, when cells are still technically alive by many criteria. In this example we combine two assays reported to reveal early aspects of apoptosis: activation of the protease caspase 3 (Hirata et al., 1998) and loss of plasma membrane phospholipid asymmetry (Aupeix et al., 1996). We were able to make inferences concerning the temporal sequence of these early events by the simultaneous analysis of changes in light scatter, a late apoptotic event. Caspase 3 activity was measured using the substrate Phi Phi Lux (Oncolmmunin), which fluoresces green when cleaved. Plasma membrane phospholipid asymmetry was measured using PE-labeled Annexin V (BD-Pharmingen) binding to phosphatidyl serine. Phosphatidyl serine is

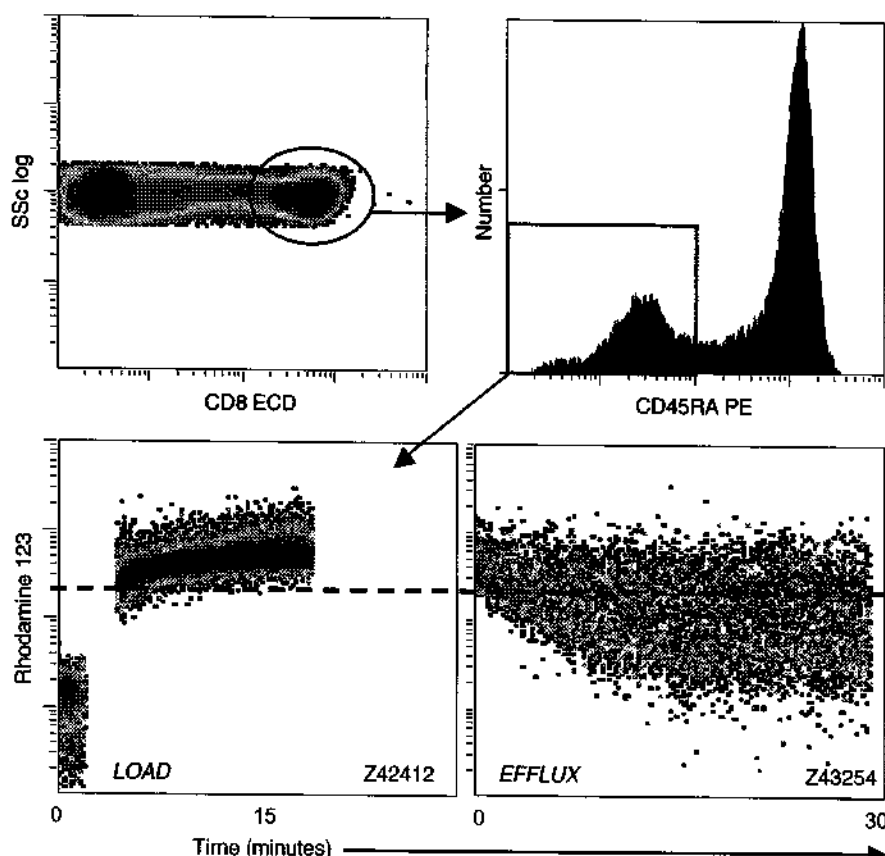


Figure 20.5 R123 load and efflux in CD45RA negative central memory/effector memory peripheral CD8+ T cells. Peripheral blood mononuclear cells were obtained from a normal healthy volunteer. Cells were stained with antibodies CD45RA-PE, CD8-ECD and CD4 plus CD3 PE-Cy5. Cells were acquired on a Beckman-Coulter XL cytometer. Cells were gated on CD3+ events (not shown). Low side scatter CD8+, CD45RA- T cells were identified using a compound gate (top panels). The lower panels show R123 fluorescence as a function of acquisition time. During the first 2 min of acquisition and prior to addition of R123, baseline autofluorescence was determined. R123 LOAD: Acquisition of R123 unstained sample was paused, R123 was added (0.13 μ M final concentration) and acquisition was immediately resumed; cells were acquired continuously for an additional 15 min. R123 EFFLUX: Loaded cells were chilled and washed 2 times with 10 ml of ice cold PBS, resuspended in culture medium at 37°C and returned to the cytometer. Cells were maintained at 37°C and data acquired continuously for 30 min. R123 loading and efflux were also determined for CD3+ CD8+ CD45RA+ cells and for the analogous CD4+ T-cell subsets (not shown). See colour plate 20.5.

normally confined to the cytoplasmic side of the plasma membrane in T cells, but is exposed during the membrane perturbations associated with apoptosis. Exposure of phosphatidyl serine on the surface of T cells is a signal for phagocytosis by macrophages (Dini et al., 1996) and clearance from the circulation. Figure 20.6 illustrates the simultaneous measurement of caspase 3 activity, annexin V binding and forward light scatter in CD4+ CD45-CD27- effector memory cells in the ascitic fluid of an ovarian cancer patient. This subset is illustrated because it displayed the highest degree of early apoptosis among the subsets defined by CD45RA and CD27. A compound gate on CD4+, low side and intermediate side scatter, CD27-, CD45RA- (not shown) was applied to the data. We used color-eventing to identify temporal sequence of caspase activation and membrane perturbation, using loss of forward light scatter as an indication of late apoptosis. The data indicate that the earliest phase of apoptosis detectable with these parameters is marked

by a coordinate increase in caspase activity and annexin V binding.

Example 6: Measuring antigen-driven T cell activation and proliferation *in vitro*

One of the great puzzles in the dawning age of cellular immunology was the seemingly inert nature of circulating lymphocytes. With bland nuclear features and scant cytoplasm, they seemed ill suited for any real immunologic work. In 1960, Peter Nowell made a discovery that facilitated routine karyotyping when he determined that peripheral blood lymphocytes could be driven to mitogenesis *in vitro* when stimulated with the plant lectin phytohemagglutinin. In addition to solving an important practical problem, this observation was a milestone in the development of biomarkers of cellular immunity, paving the way for the development of the current battery of *in vitro* assays (Hirschhorn et al., 1963). Today, we recognize that

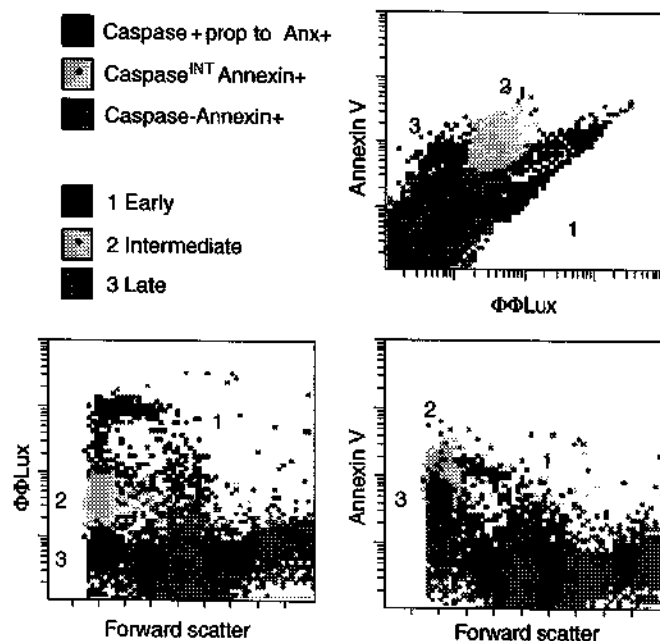


Figure 20.6 Early events in T cell apoptosis. Ficoll/Hypaque separated mononuclear cells recovered from the ascitic fluid of an untreated ovarian cancer patient were stained with antibodies CD45RA-ECD, CD27-PC5 and CD4-PC5. Cells were washed and incubated with Phi Phi Lux-G1D2, washed again, resuspended in high calcium buffer and incubated with Annexin V-PE. Cells were acquired immediately on a Beckman-Coulter FC500 cytometer. CD4+ effector memory cells were identified by gating on CD4+, low and intermediate side scatter, CD27- and CD45RA- events (not shown). Color eventing was used to identify three populations of cells based on caspase 3 activity and annexin V binding. In the first population (blue), caspase activity and annexin V binding increase coordinately, forming a diagonal in the bivariate scatter plot. In the second population (yellow) annexin V binding remains high, but caspase activity, while still present, is reduced. In the third population (red), annexin binding is positive and somewhat decreased, but at this point caspase activity is lost. The temporal sequence of apoptotic events was confirmed by backgating on Phi Phi Lux versus forward light scatter, using color eventing to identify the three populations. Here, it can be seen that the first population (blue) forms an arc as it gains and then loses caspase activity as it collapses in forward scatter. The second population already has low light scatter and further loses caspase activity. The red population, the last in the sequence, consists of small annexin V low, caspase 3 negative cells. Note that a few red colored events have normal forward scatter characteristic. These represent annexin V- cells that were misclassified when the red color event gate was created in the top right panel. See colour plate 20.6.

T and B lymphocytes are far from inert. Ligation of their antigen receptors in conjunction with appropriate costimulatory signals results in striking physiologic alterations that can be measured by changes in the expression of a variety of physical, cell-surface and intracytoplasmic markers. One of the earliest assays to be applied to immune monitoring was the lymphocyte blast transformation assay (Curtis et al., 1970), later referred to as the lymphoproliferation assay. This was performed by stimulating isolated peripheral blood mononuclear cells (lymphocytes, monocytes and immature DC) with mitogens or soluble protein antigens. Proliferation was usually quantified

by scintillation counting after a short pulse with tritiated thymidine. Our example is a modern day multiparameter variation in which Ficoll/Hypaque separated peripheral blood mononuclear cells are cultured for 5 days with a protein recall antigen (diphtheria toxoid) and proliferation is measured by expression of the transferrin receptor, CD71 (Shapiro, 2003). The multiparameter nature of flow cytometry allows us to measure an additional outcome, expression of the growth factor receptor IL-2 alpha (CD25), simultaneously on several subsets. In the present example, these outcomes have been measured on CD4+ cells subsetted into naive, memory and effector compartments based on CD45RA and CD27 expression (Figure 20.7). The gating strategy is first to determine which parameters are to be used to classify the cells into subsets, and then to measure outcome parameters in each subset.

In this example, we begin by plotting CD4 by log side scatter, rather than with the customary lymphocyte light scatter gate. This allows us to immediately resolve CD4+ cells. Had these been freshly isolated cells (see Figure 20.9), or unstimulated cultured cells, a monocyte population would have been clearly distinguishable and easily eliminated from the CD4+ T cell gate. In these activated cells, the CD4 bright cells with high log side scatter are activated T cells and if this were the only parameter that we wished to measure we could stop right here. Our sequential gating next proceeds to the scatter gate, which reveals much cleaner populations than we would have detected had we started with this gate. Late apoptotic cells, if present, can be identified as events with low forward scatter and relatively high log side scatter and eliminated at this step. Continuing with our classification parameters, we next divide non-apoptotic CD4+ T cells into four subsets based on CD45RA and CD27 expression. Here we used quadrant cuts based on unstimulated cells, but quadrants A and C seem to form a continuous population (this will be resolved in the next figure). Finally, we measured the outcome parameters, CD71 and CD25 expression in each subset. The majority of CD71/CD25 double positive cells are clearly in the CD45RA- population, but span CD27 negative and positive populations (quadrants A and C, respectively). A small proportion of responding cells can be seen in the naive (CD45RA+, CD27+) population, but many are positive for CD25 alone and have not entered the cell cycle as measured by CD71 expression. Once we have performed this analysis, we can now turn it inside out, starting with the outcome variable and proceeding to the classification parameters. Through the magic of color-eventing we will be able to visualize four parameters simultaneously in one histogram (Figure 20.8).

Using the quadrants determined in Figure 20.7 on the CD71 by CD25 histogram, we assign a unique color to the events in each quadrant. Thus, resting cells are green, CD25+/CD71- cells are red, CD25/CD71 double positive cells are gold and the rare CD25-/CD71+ cell is blue. These color-gated events are then projected to bivariate

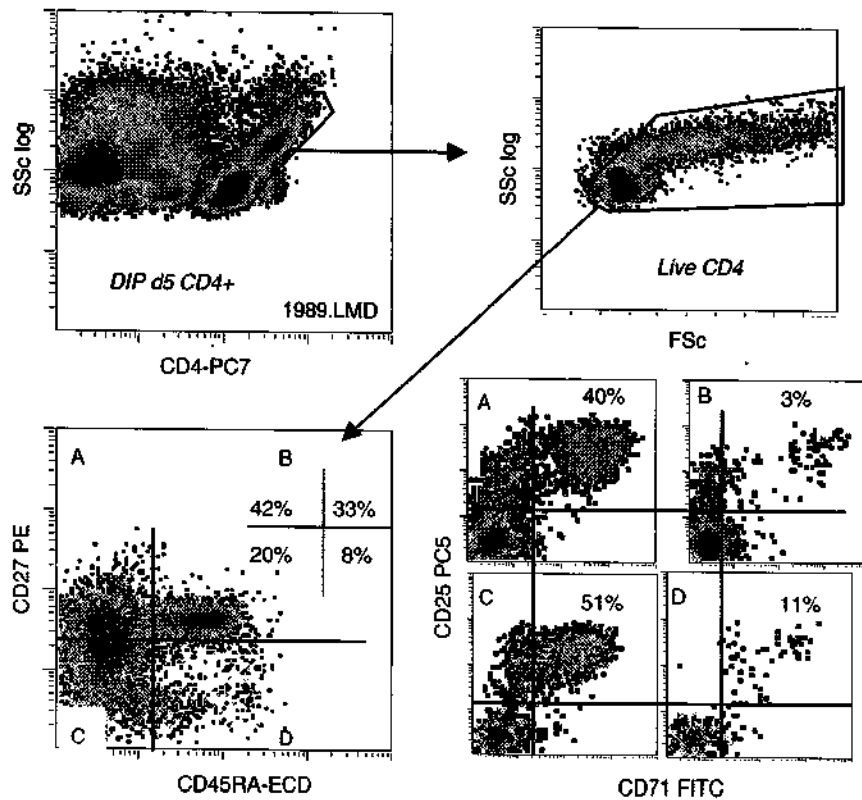


Figure 20.7 Antigen-driven upregulation of CD25 and CD71 in CD4+ T cell subsets. Peripheral blood mononuclear cells were obtained from a healthy subject and cultured for 5 days in the presence of diphtheria toxoid (2 µg/ml). Cultured cells were harvested and stained with antibodies CD71-FITC, CD27-PE, CD45RA-ECD, CD25-PC5 and CD4-PC7. Cells were acquired on a Beckman-Coulter FC500 cytometer. In the top left panel, responding cells can be visualized as CD4+ T cells with high log side scatter. CD4+ cells were filtered through a scatter gate (top right panel) used to eliminate late apoptotic cells, if present and subsetted on the basis of CD45RA and CD27 expression (A, B, C, D). The outcome parameters, CD71 and CD25 were determined in each subset. See colour plate 20.7.

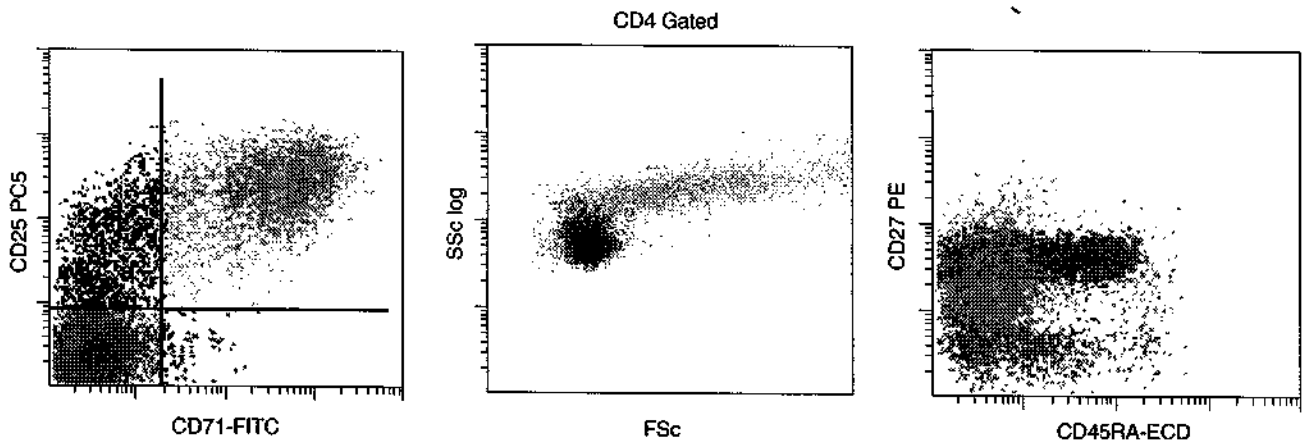


Figure 20.8 The use of color-eventing simultaneously to view outcome and classification parameters. The data are the same as those displayed in Figure 20.7. Here the outcome parameters CD71 and CD25 have been divided into quadrants and each quadrant used as a color-evented gate. For example, CD71/CD25 double positive cells were colored gold. These events are then displayed in bivariate scatter plots of forward by log side scatter and CD45RA by CD71 expression. Proliferating cells (gold) can clearly be visualized as CD45RA negative, CD27 intermediate cells with high forward scatter and high log side scatter. See colour plate 20.8.

scatter plots showing light scatter or CD4 subset. The particular colors that we have chosen create what we have termed the *flaming olive* pattern in forward by log side scatter. Resting cells (the olive) and CD25 single positive cells (the pimento) are small lymphocytes with low forward and side scatter. Proliferating CD25+/CD71+ cells (the flame) are heterogeneous with respect to forward scatter and high in side scatter. The subset distribution of

these populations can also be clearly delineated with color eventing. Resting cells (green) occupy all quadrants and look very similar to unstimulated cells (not shown). CD25+/CD71- cells (red) are confined to the CD27+ populations but span CD45RA expression, suggesting a transition in CD45 isoforms. Proliferating cells (gold) occupy a unique place in CD45RA/CD27 space, being CD45RA- but CD27 intermediate.

Example 7: Polychromatic flow cytometry

Recent improvements in cytometers and cytometry software have made multicolor (polychromatic) cytometry a reality for many laboratories (De Rosa et al., 2001, 2003). The advantages of polychromatic flow cytometry go beyond the convenience of multiplexing several determinations

in a single tube. Most important is the resolution afforded by polychrome cytometry. Figure 20.9 illustrates the use of eight-color cytometry to resolve T cell subsets. Three solid-state lasers (violet 405, blue 488 and red 633) were used for this analysis of naive/memory markers on CD4+ and CD8+ cells. Five fluorochromes were detected off

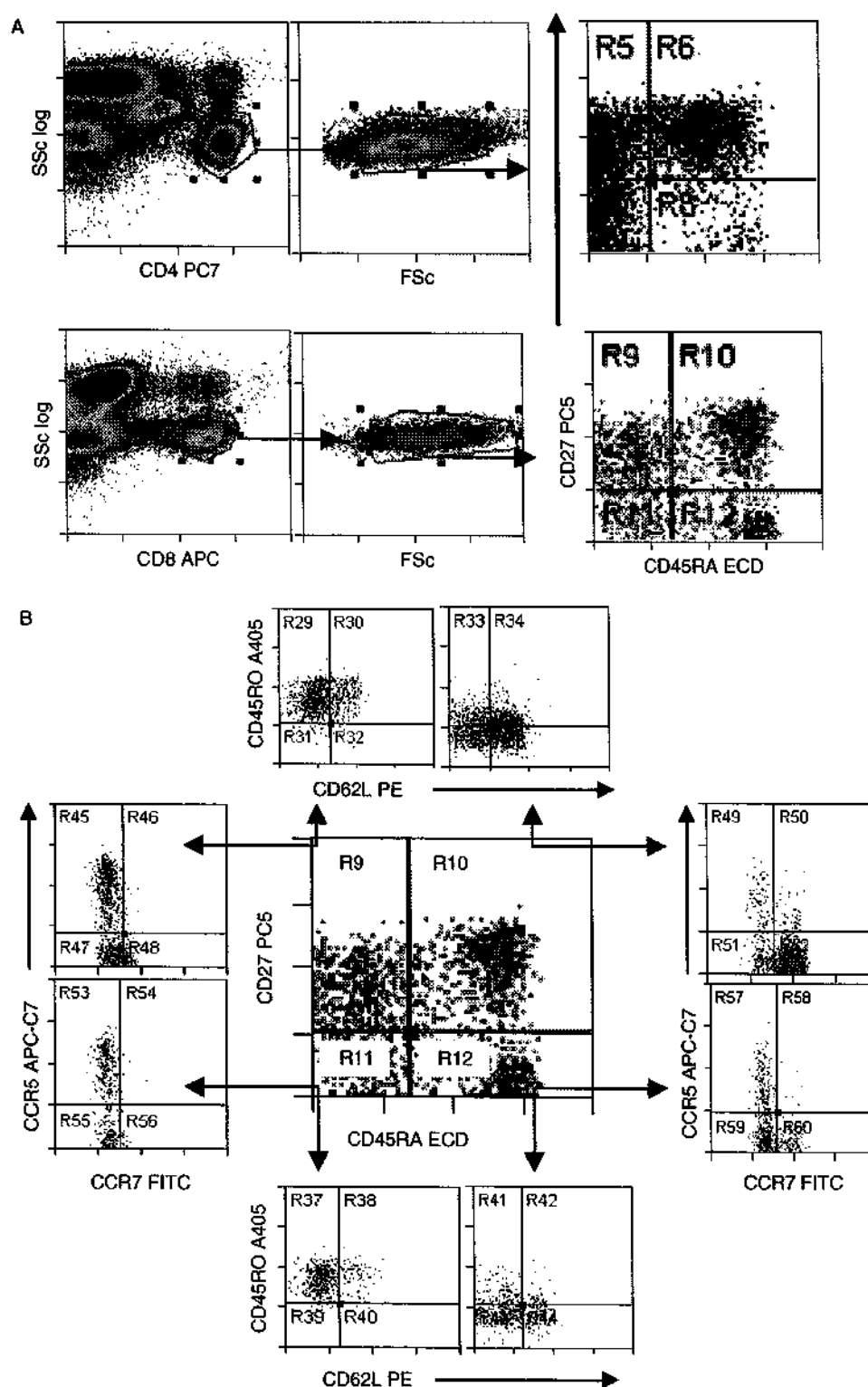


Figure 20.9 Polychromatic visualization of differentiation markers on CD4+ and CD8+ T cells. The gating strategy for this analysis begins in A in the same way as in Figure 20.7, this time with CD4+ and CD8+ populations identified and subsetted on CD45RA and CD27 in parallel. From here the 4 subpopulations each give rise to two bivariate plots (CD62L versus CD45RO and CCR7 versus CCR5). The data for the CD8+ population is shown in 9B. Note that CD8+ naive cells comprise a homogeneous population of CD45RA+, CD45RO-, CCR7+, CCR5- cells, the majority of which are CD62L+. See colour plate 20.9.

the blue laser (FITC, PE, ECD, PE-Cy5 and PE-C7), two by the red laser (APC and APC-Cy7) and one by the violet laser (Alexa 405). The matching of fluorochromes to antibodies was largely a matter of convenience and commercial availability, but care was taken to match a relatively dim marker in PE-Cy5 (CD27) with a bright marker in APC (CD8). This is advisable because PE-Cy5 is excited twice, once by the blue laser and again by the red laser. The latter signal emits at the same wavelength as APC and must be removed by cross-laser compensation. Once the data are acquired, the analysis begins by first identifying populations CD4+ or CD8+ T cells versus log side scatter. Note how beautifully the CD4 and CD8 populations stand out. This example shows freshly isolated PBMC, but in stimulated cells, the activated populations are very apparent with this gating strategy, owing to their higher side scatter and, in the case of CD4+ T cells, brighter CD4 expression (Figure 20.9). The gating proceeds as in Figure 20.7, except that this time, CD4+ and CD8+ are gated in parallel. Application of the *lymphocyte scatter gate* is far more informative when applied after the first classification gate (i.e. CD4 or CD8). Apoptotic cells having low forward scatter can be removed in this step and large activated cells, if present, can be gated separately for further analysis. Following this, the first subsetting cut is taken on the basis of CD45RA and CD27 expression. So far so good, but this is only four colors. If we want to look at all possible subsets in this panel, there are potentially 128 populations (counting the empty ones like CCR7+/CCR5+). This is clearly unfeasible and a strategy is required. In the example shown, we are using our primary subsetting antibodies (CD45RA and CD27) to divide our CD8+ T cells into central memory, naive effector/memory and effector populations and looking at CD62L, CD45RO, CCR7 and CCR5 expression on those four subsets. As an example, in naive CD8 T cells (CD45RA+/CD27+), we see that they are CD45RO-, largely (but not exclusively) CD62L+, CCR7+ and CCR5-. We could do the same sort of analysis for CD4+ T cells (not shown). As was shown in Figure 20.7, it is useful to think of markers as denoting either classification, or outcome. Outcome markers are determined on all meaningful permutations of classification markers.

FUTURE PROSPECTS

Surveying the newest cytometers and the voluminous catalogs of available reagents, it is clear that polychromatic cytometry will be available to a wide community of investigators by the time the currently deployed generation of four and five-color cytometers have finished their useful service lives. Other advances, such as the increasing speed of computers and the decreasing cost of mass data storage, will also remove barriers to multiparameter cytometry. As a result, we can expect to see more laboratories combine functional and phenotypic determinations

and increasingly apply these methods to rare events problems. As the number of parameters that can be measured increases, flow cytometry approaches cytomics, the integration of genomics, transcriptomics and proteomics, all at the level of the single cell. Like proteomics (the study of the entire protein complement in a cell, tissue or organism), cytomics encompasses the activity, modification, localization and interaction of proteins. However, in cytomics the task of data reduction is even more daunting because the unit of analysis is the individual cell.

Among the areas for future applications identified by flow cytometry pioneer Leonard Herzenberg are, biochemical analysis of rare cells, multiparameter high-throughput screening and multidimensional assessment of cell signaling networks (Herzenberg et al., 2002). To this list, Howard Shapiro has added the use of flow cytometry to identify and characterize microorganisms (Shapiro, 2003). New analytical strategies and tools for exploratory data analysis will certainly be required to realize these goals. Although it is difficult to picture exactly what these tools will look like, they will help us cluster cell populations in multidimensional space better to identify and understand progenitor-progeny relationships, functional attributes and disease states.

REFERENCES

- Ahmed, R. and Gray, D. (1996). Immunological memory and protective immunity: understanding their relation. *Science* 272, 54–60.
- Altman, J.D., Moss, P.A., Goulder, P.J. et al. (1996). Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274, 94–96.
- Appay, V., Dunbar, P.R., Callan, M. et al. (2002). Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 8, 379–385.
- Arulanandam, A.R., Kister, A., McGregor, M.J., Wyss, D.F., Wagner, G. and Reinherz, E.L. (1994). Interaction between human CD2 and CD58 involves the major beta sheet surface of each of their respective adhesion domains. *J Exp Med* 180, 1861.
- Aubin, J.E. (1979). Autofluorescence of viable cultured mammalian cells. *J Histochem Cytochem* 27, 36–43.
- Aupeix, K., Toti, F., Satta, N., Bischoff, P. and Freyssinet, J.M. (1996). Oxysterols induce membrane procoagulant activity in monocytic THP-1 cells. *Biochem J* 314, 1027–1033.
- Baerlocher, G.M. and Lansdorp, P.M. (2003). Telomere length measurements in leukocyte subsets by automated multicolor flow-FISH. *Cytometry* 55A, 1–6.
- Barratt-Boyes, S.M., Zimmer, M.I., Harshyne, L.A. et al. (2000). Maturation and trafficking of monocyte-derived dendritic cells in monkeys: implications for dendritic cell-based vaccines. *J Immunol* 164, 2487–2495.
- Baumgarth, N. and Roederer, M. (2003). A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Methods* 243, 77–97.
- Bergeron, M., Faucher, S., Minkus, T. et al. (1998). Impact of unified procedures as implemented in the Canadian Quality

- Assurance Program for T lymphocyte subset enumeration. Participating Flow Cytometry Laboratories of the Canadian Clinical Trials Network for HIV/AIDS Therapies. *Cytometry* 33, 146–155.
- Bradley, L.M., Bradley, J.S., Ching, D.L. and Shiigi, S.M. (1989). Predominance of T cells that express CD45R in the CD4+ helper/inducer lymphocyte subset of neonates. *Clin Immunol Immunopathol* 51, 426–435.
- Brosterhus, H., Brings, S., Leyendeckers, H. et al. (1999). Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. *Eur J Immunol* 29, 4053–4059.
- Campbell, J.J., Murphy, K.E., Kunkel, E.J. et al. (2001). CCR7 expression and memory T cell diversity in humans. *J Immunol* 166, 877–884.
- Cantor, H., Simpson, E., Sato, V.L., Fathman, C.G. and Herzenberg, L.A. (1975). Characterization of subpopulations of T lymphocytes. I. Separation and functional studies of peripheral T-cells binding different amounts of fluorescent anti-Thy 1.2 (theta) antibody using a fluorescence-activated cell sorter (FACS). *Cell Immunol* 15, 180–196.
- Centers for Disease Control. (1992). Guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus infection. *MMWR* 41(No. RR-8).
- Cossarizza, A., Baccarani-Contri, M., Kalashnikova, G. and Franceschi, C. (1993). A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). *Biochem Biophys Res Commun* 197, 40–45.
- Curtis, J.E., Hersh, E.M., Harris, J.E., McBride, C. and Freireich, E.J. (1970). The human primary immune response to keyhole limpet haemocyanin: interrelationships of delayed hypersensitivity, antibody response and in vitro blast transformation. *Clin Exp Immunol* 6, 473–491.
- Darzynkiewicz, Z., Traganos, F., Staiano-Coico, L., Kapuscinski, J. and Melamed, M.R. (1982). Interaction of rhodamine 123 with living cells studied by flow cytometry. *Cancer Res* 42, 799–806.
- De Rosa, S.C., Herzenberg, L.A., Herzenberg, L.A. and Roederer, M. (2001). 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. *Nat Med* 7, 245–248.
- De Rosa, S.C., Brenchley, J.M. and Roederer, M. (2003). Beyond six colors: a new era in flow cytometry. *Nat Med* 9, 112–117.
- D'hautcourt, J.L. (1996). Quality control procedures for flow cytometric applications in the hematology laboratory. *Hematol Cell Ther* 38, 467–470.
- Dini, L., Ruzittu, M.T. and Falasca, L. (1996). Recognition and phagocytosis of apoptotic cells. *Scanning Microscop* 10, 239–251.
- Donnenberg, V.S. and Donnenberg, A.D. (2003). Identification, rare-event detection and analysis of dendritic cell subsets in broncho-alveolar lavage fluid and peripheral blood by flow cytometry. *Frontiers Biosci* 8, s1175–1180.
- Donnenberg, A.D. and Meyer, E.M. (1999). Principles of rare event analysis by flow cytometry: detection of injected dendritic cells in draining lymphatic tissue. *Clin Immunol Newslett* 19, 124–128.
- Donnenberg, A.D., Donnenberg, V.S. and Shen, H. (2003). Rare-event detection and analysis in flow cytometry. *The Connection* 5.
- Donnenberg, A.D., Koch, E.K., Griffin, D.L. et al. (2002). Viability of cryopreserved BM progenitor cells stored for more than a decade. *Cytotherapy* 4, 157–166.
- Donnenberg, V.S., O'Connell, P.J., Logar, A.J., Zeevi, A., Thomson, A.W. and Donnenberg, A.D. (2001). Rare-event analysis of circulating human dendritic cell subsets and their presumptive mouse counterparts. *Transplantation* 72, 1946–1951.
- Donnenberg, V.S., Wilson, J.W., Burckart, G.J., Zeevi, A., Iacono, A. and Donnenberg, A.D. (2004). Measurement of basal, substrate induced and total P-gp activity in broncho-alveolar lavage T-cell subsets. *Cytometry*. In press.
- Drach, J., Gattlinger, C., Glassl, H., Schwarting, R., Stein, H. and Huber, H. (1989). Simultaneous flow cytometric analysis of surface markers and nuclear Ki-67 antigen in leukemia and lymphoma. *Cytometry* 10, 743–749.
- Gelman, R., Cheng, S.C., Kidd, P., Waxdal, M. and Kagan, J. (1993). Assessment of the effects of instrumentation, monoclonal antibody, and fluorochrome on flow cytometric immunophenotyping: a report based on 2 years of the NIAID DAIDS flow cytometry quality assessment program. *Clin Immunol Immunopathol* 66, 150–162.
- Giorgi, J.V., Cheng, H.L., Margolick, J.B. et al. (1990). Quality control in the flow cytometric measurement of T-lymphocyte subsets: the multicenter AIDS cohort study experience. The Multicenter AIDS Cohort Study Group. *Clin Immunol Immunopathol* 55, 173–186.
- Giorgi, J.V., Liu, Z., Hultin, L.E., Cumberland, W.G., Hennessey, K. and Detels, R. (1993). Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. *J Acq Immune Defic Synd* 6, 904–912.
- Gratama, J.W., Bolhuis, R.L. and Van 't Veer, M.B. (1999). Quality control of flow cytometric immunophenotyping of haematological malignancies. *Clin Lab Haematol* 21, 155–160.
- Guedez, L., Stetler-Stevenson, W.G., Wolff, L. et al. (1998). In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. *J Clin Invest* 102, 2002–2010.
- Hammerling, G.J., Lemke, H., Hammerling, U. et al. (1978). Monoclonal antibodies against murine cell surface antigens: anti-H-2, anti-Ia and anti-T cell antibodies. *Curr Top Microbiol Immunol* 81, 100–106.
- Herzenberg, L.A., Parks, D., Sahaf, B., Perez, O., Roederer, M. and Herzenberg, L.A. (2002). The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford (Historical Article. Lectures). *Clin Chem* 48, 1819–1827.
- Hirata, H., Takahashi, A., Kobayashi, S. et al. (1998). Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. *J Exp Med* 187, 587–600.
- Hirschhorn, K., Bach, F., Kolodny, R.L., Firschein, I.L. and Hashem, N. (1963). Immune response and mitosis of human peripheral blood lymphocytes in vitro. *Science* 142, 1185.
- Hoffmann, T.K., Donnenberg, V.S., Friebe-Hoffmann, U. et al. (2000). Competition of peptide-MHC class I tetrameric complexes with anti-CD3 provides evidence for specificity of peptide binding to the TCR complex. *Cytometry* 41, 321–328.
- June, C.H., Ledbetter, J.A., Rabinovitch, P.S., Martin, P.J., Beatty, P.G. and Hansen, J.A. (1986). Distinct patterns of transmembrane

- calcium flux and intracellular calcium mobilization after differentiation antigen cluster 2 (E rosette receptor) or 3 (T3) stimulation of human lymphocytes. *J Clin Invest* 77, 1224–1232.
- Kamentsky, (2003). In *Practical Flow Cytometry*, 3rd edn, Shapiro, H.M., ed. Hoboken, NJ: Wiley Liss, p. xivii.
- Kedar, E. and Bonavida, B. (1975). Studies on the induction and expression of T cell-mediated immunity. IV. Non-overlapping populations of alloimmune cytotoxic lymphocytes with specificity for tumor-associated antigens and transplantation antigens. *J Immunol* 115, 1301–1308.
- Kung, P., Goldstein, G., Reinherz, E.L. and Schlossman, S.F. (1979). Monoclonal antibodies defining distinctive human T cell surface antigens. *Science* 206, 347–349.
- Landsteiner, K. and Chase, M.W. (1942). Experiments in transfer of cutaneous sensitivity to simple compounds. *Proc Soc Exp Biol Med* 49, 688–690.
- Li, X. and Darzynkiewicz, Z. (1995). Labelling DNA strand breaks with BrdUTP. Detection of apoptosis and cell proliferation. *Cell Prolif* 28, 571–579.
- Liu, L., Chahroudi, A., Silvestri, G. et al. (2002). Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. *Nat Med* 8, 185–189.
- Loken, M.R. and Herzenberg, L.A. (1975). Analysis of cell populations with a fluorescence-activated cell sorter. *Ann NY Acad Sci* 254, 163–171.
- Loken, M.R., Parks, D.R. and Herzenberg, L.A. (1977). Two-color immunofluorescence using a fluorescence-activated cell sorter. *J Histochem Cytochem* 25, 899–907.
- Lyons, A.B., Samuel, K., Sanderson, A. and Maddy, A.H. (1992). Simultaneous analysis of immunophenotype and apoptosis of murine thymocytes by single laser flow cytometry. *Cytometry* 13, 809–821.
- Mandy, F., Nicholson, J., Autran, B. and Janossy, G. (2002). T-cell subset counting and the fight against AIDS: reflections over a 20-year struggle. *Cytometry* 50, 39–45.
- Mandy, F.F., Nicholson, J.K. and McDougal, J.S. (2003). Guidelines for Performing Single-Platform Absolute CD4+ T-Cell Determinations with CD45 Gating for Persons Infected with Human Immunodeficiency Virus. *MMWR* 52(No. RR02): 1–13.
- Margolick, J.B., Donnenberg, A.D., Munoz, A. et al. (1993). Changes in T and non-T lymphocyte subsets following seroconversion to HIV-1: stable CD3+ and declining CD3– populations suggest regulatory responses linked to loss of CD4 lymphocytes. The Multicenter AIDS Cohort Study. *J Acq Immune Defic Synd* 6, 153–161.
- Margolick, J.B., Muñoz, A., Donnenberg, A.D. et al. for the multicenter AIDS cohort study. (1995). Failure of T-cell homeostasis preceding AIDS in HIV-1 infection. *Nat Med* 1, 674–681.
- Margolick, J.B. and Donnenberg, A.D. (1997). T-cell homeostasis in HIV-1 infection. *Sem Immunol* 9, 381–388.
- Mitchell, G.F., Chan, E.L., Noble, M.S., Weissman, I.L., Mishell, R.I. and Herzenberg, L.A. (1972). Immunological memory in mice. 3. Memory to heterologous erythrocytes in both T cell and B cell populations and requirement for T cells in expression of B cell memory. Evidence using immunoglobulin allotype and mouse alloantigen theta markers with congenic mice. *J Exp Med* 135, 165–184.
- Morimoto, C., Letvin, N.L., Boyd, A.W. et al. (1985a). The isolation and characterization of the human helper inducer T cell subset. *J Immunol* 134, 3762–3769.
- Morimoto, C., Letvin, N.L., Distaso, J.A., Aldrich, W.R. and Schlossman, S.F. (1985b). The isolation and characterization of the human suppressor inducer T cell subset. *J Immunol* 134, 1508–1515.
- Murphy, R.F. and Chused, T.M. (1984). A proposal for a flow cytometric data file standard. *Cytometry* 5, 553–555.
- Noel, D.R., Witherspoon, R.P., Storb, R. et al. (1978). Does graft-versus-host disease influence the tempo of immunologic recovery after allogeneic human marrow transplantation? An observation on 56 long-term survivors. *Blood* 51, 1087–1105.
- Nordon, R.E., Ginsberg, S.S. and Eaves, C.J. (1997). High-resolution cell division tracking demonstrates the FLT3-ligand-dependence of human marrow CD34+CD38– cell production in vitro. *Br J Haematol* 98, 528–553.
- Nówell, P.C. (1960). PHA: An initiator of mitosis in cultures of normal human leukocytes. *Cancer Res* 20, 562.
- O’Gorman, M.R. and Gelman, R. (1997). Inter- and intrainstitutional evaluation of automated volumetric capillary cytometry for the quantitation of CD4- and CD8-positive T lymphocytes in the peripheral blood of persons infected with human immunodeficiency virus. Site Investigators and the NIAID New CD4 Technologies Focus Group. *Clin Diag Lab Immunol* 4, 173–179.
- Paxton, H., Kidd, P., Landay, A. et al. (1989). Results of the flow cytometry ACTG quality control program: analysis and findings. *Clin Immunol Immunopathol* 52, 68–84.
- Pearson, T., Galfre, G., Ziegler, A. and Milstein, C. (1977). A myeloma hybrid producing antibody specific for an allotypic determinant on ‘IgD-like’ molecules of the mouse. *Eur J Immunol* 7, 684–690.
- Perez, O.D. and Nolan, G.P. (2002). Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. *Nat Biotechnol* 20, 155–162.
- Picker, L.J., Treer, J.R., Ferguson-Darnell, B., Collins, P.A., Buck, D. and Terstappen, L.W. (1993). Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selectin on T cells during the virgin to memory cell transition. *J Immunol* 150, 1105–1121.
- Reinherz, E.L., Geha, R., Wohl, M.E., Morimoto, C., Rosen, F.S. and Schlossman, S.F. (1981). Immunodeficiency associated with loss of T4+ inducer T-cell function. *N Eng J Med* 304, 811–816.
- Roederer, M. (2001). Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry* 45, 194–205.
- Rosenblatt, J.I., Hokanson, J.A., McLaughlin, S.R. and Leary, J.F. (1997). Theoretical basis for sampling statistics useful for detecting and isolating rare cells using flow cytometry and cell sorting. *Cytometry* 27, 233–238.
- Sallusto, F., Lenig, D., Forster, R., Lipp, M. and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708–712.
- Sanders, M.E., Makgoba, M.W., Sharrow, S.O. et al. (1988). Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. *J Immunol* 140, 1401–1407.
- Shapiro, H.M. (1995). *Practical flow cytometry*, 3rd edn. New York: Wiley-Liss, p. 276.
- Shapiro, H.M. (ed.) (2003). *Practical Flow Cytometry*, 4th edn. Hoboken, NJ: John Wiley and Sons, pp. 565–566.
- (2003). In *Practical Flow Cytometry*, 4th edn, Shapiro, H.M., ed. Hoboken, NJ: John Wiley and Sons, p. 460.

- Smith, S.H., Brown, M.H., Rowe, D., Callard, R.E. and Beverley, P.C. (1986). Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. *Immunology* 58, 63–70.
- Sutherland, D.R., Anderson, L., Keene, Y.M., Nayar, R. and Chin-Yee, I. (1996). The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *International Society of Hematotherapy and Graft Engineering. J Hematother* 5, 213–226.
- Tedder, T.F., Cooper, M.D. and Clement, L.T. (1985). Human lymphocyte differentiation antigens HB-10 and HB-11. II. Differential production of B cell growth and differentiation factors by distinct helper T cell subpopulations. *J Immunol* 134, 2989–2994.
- Trowbridge, I.S. (1978). Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. *J Exp Med* 148, 313–323.
- Trowbridge, I.S. and Thomas, M.L. (1994). CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol* 12, 85–116.
- van den Engh, G. (1993). New applications of flow cytometry. *Curr Opin Biotechnol* 4, 63–68.
- Wersto, R.P., Chrest, F.J., Leary, J.F., Morris, C., Stetler-Stevenson, M.A. and Gabrielson, E. (2001). Doublet discrimination in DNA cell-cycle analysis. *Cytometry* 46, 296–306.
- White, R.A., Mason, D.W., Williams, A.F., Galfre, G. and Milstein, C. (1978). T-lymphocyte heterogeneity in the rat: separation of functional subpopulations using a monoclonal antibody. *J Exp Med* 148, 664–673.
- Winchester, R.J. and Ross, G. (1976). Methods for enumerating lymphocyte populations. In *Manual of Clinical Immunology*, Rose, N.R. and Friedman, H., eds. Washington DC: ASM Press, pp. 64–76.