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FLOW CYTOMETRY

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History

By the time the prototype of the fluorescence-activated cell sorter (FACS) went into operation (at Stanford in the early 1970s), immunofluorescence studies with the light microscope had already demonstrated that lymphocytes could be broadly subdivided according to whether they expressed surface immunoglobulin (Ig) or surface Thy-1. These visually-defined subpopulations, which we now know as B cells and T cells, were presumed to be responsible for different lymphocyte functions; however, the lack of methods for cleanly separating one subpopulation from the other confounded attempts to establish this point.

During the next few years, while the FACS was undergoing its initial metamorphosis into a commercially-available single-laser instrument, a variety of physical and immunocytotoxic methods were developed that could selectively deplete B cells, T cells or T cell subpopulations from a cell suspension; for example, passage through nylon wool, passage through an affinity column, or complement-dependent killing with antibodies to distinctive cell surface molecules. These depletion methods have proven extremely useful; however, they are mainly restricted to depleting unwanted cell types from a population and thus do not provide for the specific isolation of subpopulations, which is necessary for definitively characterizing their functions.

FACS-based cell isolation methods, in contrast, are well suited to this task, as they provide the potential for sorting and testing viable subpopulations. A significant advantage over mechanical separation methods is that the populations isolated by FACS are usually highly pure. Whereas mechanical separations usually result in purities of 60–90% (with the

remaining cells being unwanted subsets), single FACS-separation runs can achieve over 99% purity – with re-sorting resulting in essentially 100% purity. In addition, the electronic control of the sorting in FACS gives the researcher the ability to sort only a single, viable cell – the quickest and most efficient cell cloning technology extant.

The major advantage of FACS-based separations, however, resides in the multiparametric nature of the measurement. A wide array of characteristics can be used, individually or in combination, to define subpopulations. For example, cell size, surface phenotype, mitotic stage, and intracellular levels of enzymes and metabolites. Thus, as the capabilities of this newly-developed instrument became known, it found increasing use in studies of the functional and developmental relationships among subpopulations of lymphocytes, as well as other cells.

In the last decade, the power of the FACS has been utilized for a much greater range of samples than leukocytes. All kinds of unicellular organisms, from bacteria to yeast to paramecia, have been separated by FACS. This technology has also been applied to the separation of subcellular particles: for instance, sorting endosomes, lysosomes or mitochondria. In addition, the FACS has been used to separate individual eukaryotic chromosomes – a technique that has been pivotal in the establishment of chromosome-specific DNA libraries, an important tool in the human genome project.

In recent years, the application of FACS to biology has taken another significant turn – that of single-cell functional characterization. A variety of assays have been developed to quantitate the functional capacity of individual cells by flow cytometry. These include assays for proliferation or cell cycle progression (e.g. total DNA, RNA and protein contents, as well as number of divisions measured by dyes permanently retained by DNA or cellular membranes), activation (e.g. expression of activation markers on the surface of cells), cytokine expression, apoptosis, calcium mobilization, acidification of internal compartments, changes in reduction–oxidation potential, metabolic activity and various enzymatic activities.

The power of these assays by FACS comes through two unique capabilities. First, since the assay is single-cell based, the resulting measurements provide a view of the *distribution* of activities within a population, rather than an average across the entire population. Since few biological processes are homogeneous across all cells, this view can be critical in assessing the true functional nature of cells. Second, the functional assays can be combined with standard immunofluorescence assays to phenotype the cells; thus, the functional quantitation can be restricted to any desired subpopulation of cells.

While such functional assays are still reserved primarily for the basic researcher, more and more of them are finding clinical utility. As the instrumentation and data analysis becomes routinely automated, these assays progress into clinical settings, such as prognosis in human immunodeficiency virus (HIV) disease. Over the next 10 years, many of these single-cell FACS-based functional assays will become routine in the clinic because they provide information that cannot be obtained by standard bulk assays.

Elucidation of lymphocyte subpopulations by FACS

Much of the early work centered around the characterization of B cell phenotypes, functions and precursor–progeny relationships. The first significant FACS studies in these areas, published in 1972, demonstrated that antigen-binding cells are precursors of cells that secrete antibodies reactive with the bound antigen. Other studies, begun shortly thereafter, demonstrated that the surface Ig molecules expressed on B cells are restricted to individual allotypes and isotypes and that these surface Ig molecules reflect the allotype and isotype commitment of the cell and its antibody-producing (plasma cell) progeny.

By 1976, sorting and transfer studies with Ig allotype congenic mice placed this basic description of 'allelic exclusion' in its current context by showing that all Ig heavy chains expressed by an individual B cell or its progeny are encoded on the same Ig heavy chain chromosome (haplotype). These findings, which provided the operational and theoretical framework for a large series of subsequent B cell development studies, also laid the groundwork for current molecular and FACS studies defining the genetic organization of the Ig heavy chain (IgH) chromosome region and the mechanisms involved in IgH gene rearrangements and class (isotype) switching.

FACS contributions to T cell studies during this period were less dramatic, largely because of difficulties encountered in obtaining antibodies (and hence FACS reagents) that specifically detected individual cell surface antigens. At this time, antibodies for use in FACS (and other) studies were prepared from conventional antisera, which typically contain a mixture of antibodies reactive with the target antigens and an abundance of irrelevant Ig that can stick nonspecifically to cells. Anti-Ig reagents, such as those used in the B cell studies cited above, were relatively easy to prepare because Ig-specific antibodies can be bound to (and eluted from) secreted Ig

coupled to an insoluble matrix. Specific reagents for detecting surface determinants other than Ig, however, were substantially more difficult to produce, as they could not readily be isolated by binding and elution methods. By and large, these reagents had to be produced from antisera (or fractionated Ig) from which contaminant antibodies were removed by absorption with appropriate cells. Thus, FACS T cell work proceeded slowly until the late 1970s, when monoclonal antibodies were introduced as FACS staining reagents.

The development of monoclonal reagents for detecting the major T cell surface determinants in mouse and humans went hand in hand with the development of FACS methods for identifying and sorting the major T cell subpopulations. In essence, the availability of highly specific and easily purified antibodies that could be readily coupled with fluorochromes made it possible to use the quantitative expression of cell surface antigens to recognize lymphocyte (and other) subpopulations and to sort these subpopulations to chart their functions.

Initial studies with this monoclonal-based methodology defined the murine Ly-1 (CD5) and Lyt-2 (CD8) lymphocyte surface antigens and characterized the expression of these antigens on T cell subpopulations in mouse spleen, lymph node and thymus. These FACS characterizations were used to discover monoclonal antibodies that detected the human Leu-1 (CD5) and Leu-2a (CD8) homologs of the murine genes (**Figure 1**). In addition, these early studies used these (and other) monoclonal antibodies to identify and characterize the so-called 'helper/inducer' and 'suppressor/cytotoxic' subsets of human T cells.

At present, nearly all FACS studies characterizing the expression of cell surface antigens are conducted with monoclonal antibody reagents. The shift to this virtually exclusive use of these reagents occurred extremely rapidly and was accompanied by an equally rapid expansion in FACS utilization, particularly in studies of human and murine lymphocytes. Surface phenotypes for helper and suppressor T cell subsets were further defined and new subpopulations within these subsets were identified. B cell FACS studies also prospered and marked advances were made in clinical FACS application. In one striking example, leukemia phenotyping opened the way to a rationalization of treatment protocols that substantially improved patient survival. In another dramatic instance, measurement of helper and suppressor subpopulation frequencies in normal human adults laid the groundwork for the current widespread use of such measurements as an index of disease progression in acquired immune deficiency syndrome (AIDS).

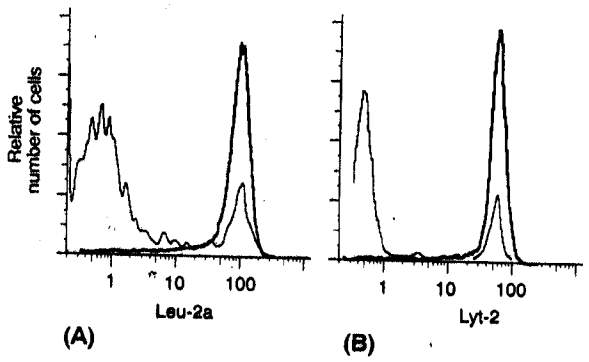

Figure 1 Two-parameter FACS: Leu-2a and Lyt-2 expression on (A) human and (B) mouse lymphoid cells. Early single-color FACS analysis, in conjunction with biochemical studies, demonstrated that human Leu-2a, which is found only on suppressor/cytotoxic cells, is expressed in a very similar pattern as the murine Lyt-2 surface antigen. The Leu-2a antigen was detected by indirect staining with SK1 monoclonal antibody supernatant followed by fluorescein-conjugated goat antimouse IgG1. The Lyt-2 antigen on mouse cells was detected by direct staining with fluorescein-conjugated 53-6.7 monoclonal antibody. Both of the determinants are found on 80–90% of the thymus cells (light lines) and 20–40% of the spleen T cells (dark lines). These antigens are now known as CD8.

Multiparameter FACS studies

Methods for labeling antibodies with different fluorochromes (e.g. fluorescein and rhodamine) were developed while the FACS was still in its infancy. Fluorescence microscope studies demonstrated that these 'differently-colored' antibodies could be independently detected on the same cell and thus could be used to distinguish subpopulations on the basis of qualitative or semiqualitative correlations in the expression of two or more surface antigens. In essence, these studies showed that lymphocyte subpopulations distinguished initially by the expression (or nonexpression) of one surface marker could be further subdivided according to the expression (or nonexpression) of a second marker, etc.

Developing an array of fluorescent dyes for reagent labeling and efficient FACS systems that could capitalize on the availability of differently-colored antibody reagents produced with these dyes took some time. Early FACS instruments were equipped with a basic two-color capability that allowed quantitative evaluation of the expression of pairs of cell surface markers on individual cells; however, these initial multiparameter studies were severely restricted by the limited dye combinations, the single-laser FACS instruments and the simplistic computer support available at the time. In fact, studies with these early

instruments basically provided a tantalizing glimpse of how valuable multiparameter FACS methods could be; relatively little was actually accomplished with these methods until the introduction of the dual-laser FACS, the phycobiliproteins (phycoerythrin (PE) and allophycocyanin (APC)) and the sophisticated FACS software that together characterize the multiparameter analysis and sorting methods in use today.

Strictly speaking, multiparameter FACS studies (defined as the measurement of more than one parameter per cell) began with the prototype FACS, which was built with two sensors: one for fluorescence, and the other for low-angle light scatter ('forward scatter'). The forward scatter measurement on this initial instrument was intended solely to signal the arrival of a cell-sized object in the light path; however, studies with cell populations from lymphoid organs soon showed that the forward scatter signal could also be used as a meaningful measure of

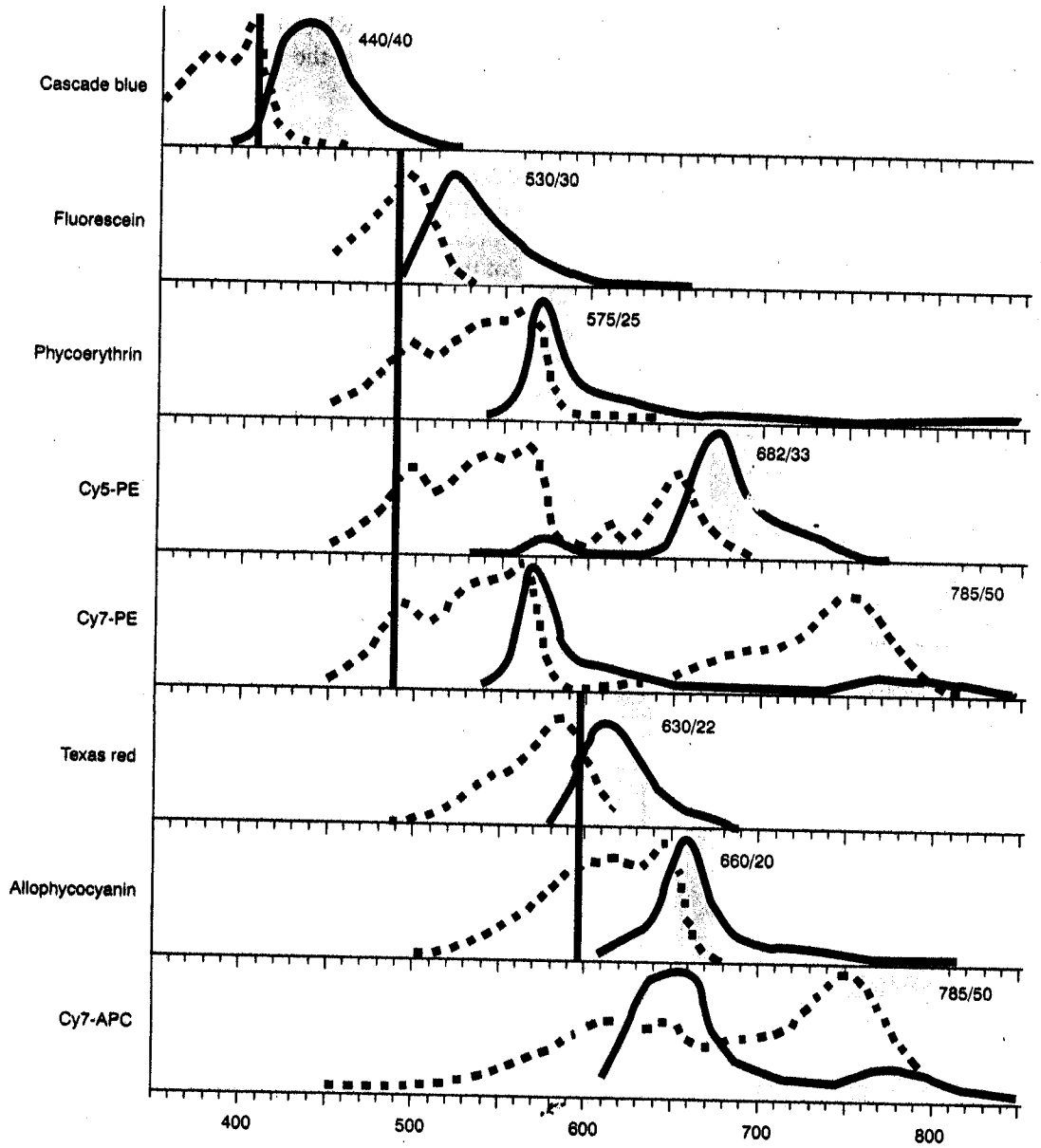

Figure 2 Fluorescence spectra of molecules conjugated to antibody reagents. The excitation and emission spectra of eight fluorescent molecules that can be conjugated to immunoglobulins and used in immunofluorescence studies are shown. All eight can be simultaneously and independently measured. Dashed lines, excitation spectra; solid lines, emission spectra. Spectra are uncorrected for detector sensitivity and are scaled for presentation purposes. Also shown are the excitation lines from the 406 nm krypton line, the 488 nm argon line, and the 595 nm dye laser line. Finally, the stippled boxes represent the transmission areas for each of the filters used to detect that fluorescent molecule by FACS.

cell size and could distinguish, for instance, lymphocytes from monocytes. In addition, the forward scatter signals were shown to be useful for discriminating live cells from dead cells, and for excluding ('gating out') dead cells during analysis and sorting. Thus, as the FACS came into use initially for biological studies, two parameters – forward scatter and fluorescence – were routinely measured and recorded for individual cells.

The introduction of methods for measuring two additional parameters – large-angle light scatter ('side scatter') and a second fluorescence color – moved FACS measurements closer to the current definition of multiparameter studies, i.e. those in which at least two fluorescence measurements and one light scatter measurement are taken per cell. Most flow cytometers are now capable of measuring forward and side scatter as well as at least three fluorescence colors. State of the art instrumentation at Stanford is capable of detecting the two scatter signals as well as eight different, independently-quantifiable fluorescence measurements. The advance to such ten-parameter, three-laser instrumentation has required development efforts at a multitude of levels: 1) chemistry – the continuing development of fluorescent dyes with spectra suitable for flow cytometry (e.g. excitation by commonly available lasers and emission spectra that do not overlap excessively) has made such advances possible (Figure 2, see p. 935); 2) optics – the construction and optimization of a complex array of filters and photomultiplier tubes that can separate the emitted light into its components and accurately measure those components; 3) electronics – the simultaneous collection and processing of ten or more analog signals, digitization, decisions for sorting, as well as distribution to various computer elements for data storage and analysis; and 4) computer software – analysis of this highly multidimensional data requires 'expert' systems software which can automate many of the tasks that are beyond the capacity of the human mind.

Power of multiparameter analysis

The power of these multiparametric analyses is evident from the unsuspected subdivisions their use has revealed in biological systems. Early on, the division of the two major T cell subsets (helper/inducer versus suppressor/cytotoxic) was shown to be possible by the simultaneous measurement of CD4 (expressed on

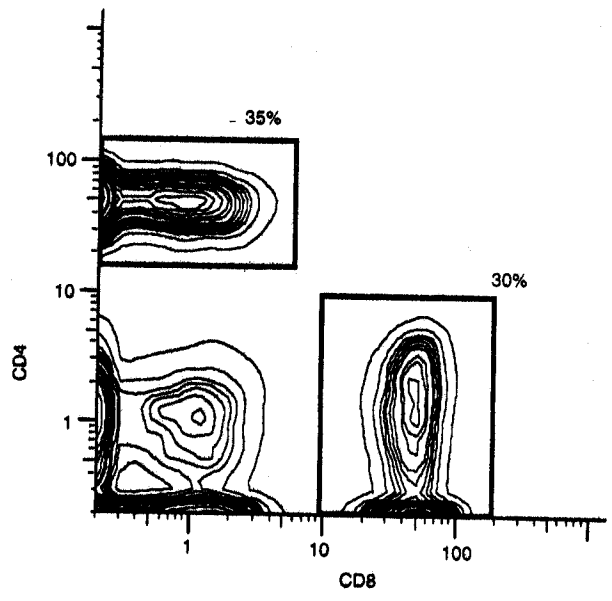

Figure 3 Four-parameter FACS: T cell analysis of a mouse lymph node. Two-color FACS analysis is routinely used to quantitate helper/inducer T cells and cytotoxic/suppressor T cells in mouse and humans. The 5% probability plots, which are also shown in other figures, contain an equal number of cells between each pair of contour levels. In this figure, three types of cell can be distinguished: those expressing CD4 (helper/inducer); CD8 (cytotoxic/suppressor); or neither (B cells, NK cells and other leukocytes).

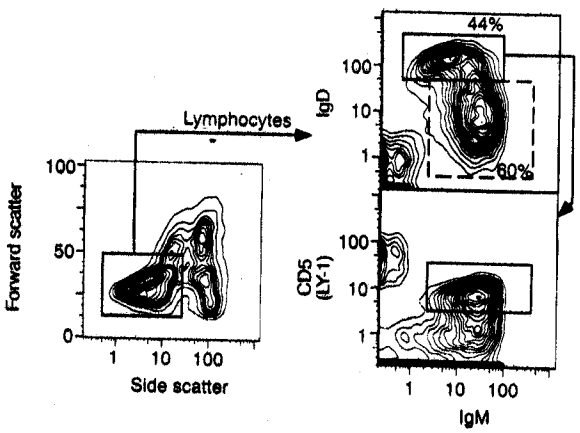

Figure 4 Six-parameter FACS: analysis of murine peritoneal cavity cells. Dual laser FACS analysis permits the simultaneous measurement of six or more parameters. The left plot shows the forward versus side scatter profile. The data, which are recorded in list mode, can be 'gated' for live lymphocytes as shown (i.e. only those cells which fall within the box drawn on the forward scatter versus side scatter plot are viewed in subsequent analyses). Cell surface staining profiles in the right panels do not include dead cells or large live cells such as macrophages. The cell surface analysis of the peritoneal cavity reveals an abundance of Ly-1 lineage B cells. The upper panel shows the characteristic IgM versus IgD phenotype: conventional B cells are indicated by the dashed box and the sum of the $CD5^+$ and $CD5^-$ Ly-1 B cell lineages are indicated by the solid box. The right bottom plot, which is gated as shown by the solid box in the upper panel, distinguishes the $CD5^+$ Ly-1 lineage B cells (solid box) from the $CD5^-$ 'sister' Ly-1 B cells (i.e. by the expression of CD5). Percentages are reported in terms of the total live lymphocytes, as determined by the forward and side scatter gates. (Courtesy of Dr Aaron Kantor.)

the helper/inducer) and CD8 (expressed on the suppressor/cytotoxic) populations (Figure 3). These distinctions are relatively easy to make, being primarily 'positive' versus 'negative' in terms of expression of a surface molecule. However, many lymphocyte subsets cannot be distinguished so easily. B cells, for example, were thought to be relatively homogeneous. However, initial multiparameter FACS studies conducted with a dual-laser FACS system in our laboratory revealed a series of murine B cell subpopulations and lineages with distinctive functions, localizing behavior, developmental patterns, mitogen and lymphokine responsiveness, etc. Furthermore, potentially homologous B cell subpopulations with similar FACS and functional phenotypes have been identified in rat and in humans.

None of the B cell subpopulations/lineages are as yet recognizable by the presence, absence or surface

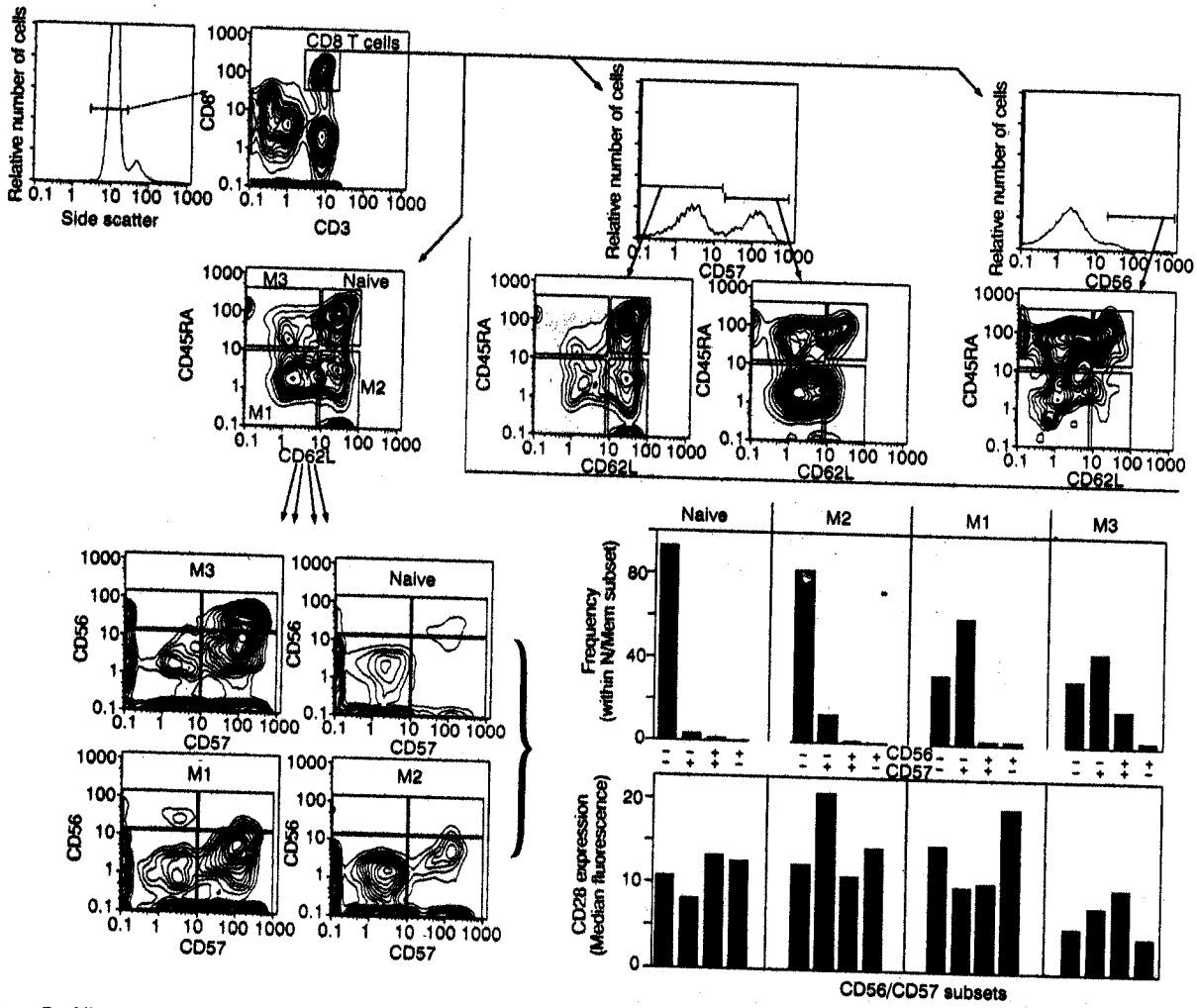

Figure 5 Nine-parameter FACS: analysis of human peripheral CD8 T cells. The complex heterogeneity of peripheral CD8 T cells in humans is exemplified by a seven-color staining together with forward and side scatter collection. PBMC were simultaneously stained with CD3, CD8, CD28, CD45RA, CD56, CD57 and CD62L. Shown is progressive 'gating' or restriction of the sample by these parameters. Lymphocytes are selected based on the size parameter (top left histogram); CD8 T cells are selected from this subset by coexpression of CD3 and CD8. The second line of plots shows CD45RA versus CD62L expression for (left to right): total CD8 T cells $CD57^-$ CD8 cells, $CD57^+$ CD8 cells, and $CD56^+$ CD8 cells. Based on functional studies, 'naive' versus 'memory' CD8 T cells can be distinguished by CD45RA and CD62L, as shown in the leftmost panel of this series. $CD56^+$ and $CD57^+$ cells, which are themselves functionally distinct memory subsets, have heterogeneous expression of these markers – some of these cells fall into the 'naive' gate. Thus, unique identification of naive CD8 T cells requires the simultaneous measurement of (at least) six parameters: $CD3^+$, $CD8^+$, $CD45RA^+$, $CD62L^+$, $CD56^-$, $CD57^-$. Another evaluation of the expression of CD56 and CD57 on the naive and memory subsets is shown in the four plots on the lower left. The bar charts represent analyses of these 16 subsets: the frequency within the naive and memory (N/Mem) subsets (upper) and the relative expression of CD28 on cells of each subset (lower). Data sets such as this single sample are so complex that it becomes impossible to satisfactorily analyze by conventional methods (i.e. 'by hand'). Computer programs which can provide automated 'expert'-type analysis combined with nonparametric cluster algorithms will become invaluable, if not necessary, analysis tools.

level of an individual surface marker. Distinguishing these subpopulations requires measurement of at least two surface molecules (detected by differently-colored antibodies) (**Figure 4**, see p. 936). In fact, some subpopulations are even difficult to distinguish with three fluorescence and two scatter measurements. Thus, although B cell surface marker expression had been studied extensively in single-color analyses, the introduction of multiparameter FACS methods opened an entirely new perspective on the heterogeneity within the overall B cell population.

Nowhere is the power of multiparametric analysis more evident than in studies of peripheral T cell subsets in humans. Similar to the case in mouse, it was recognized early that T cells could be divided into helper/inducer and suppressor/cytotoxic subsets by the surface antigens CD4 and CD8, respectively. However, each one of these subsets is richly heterogeneous, and can be further subdivided by more than a dozen other antigens. The functional roles of these different subsets is poorly understood, primarily because studies have been performed so far using only three- or four-color separations, which does not result in truly homogeneous populations.

Figure 5 (see p. 937), for example, shows how human CD8 T cells can be subdivided into a number of different subsets based on the simultaneous quantitation of six different antigens. The need for this differentiation is exemplified by recent studies of immunopathogenesis in HIV disease (AIDS). Until the early 1990s, the understanding of the changes in the T cell compartment accompanying progression of this disease was rather simplistic: CD4 T cells declined continuously; CD8 T cells initially expanded, stayed high until very late in disease, and then declined somewhat.

However, there were dozens of publications detailing many changes within the CD8 T cell compartment based on two- or three-color approaches (i.e. using one or two colors to identify uniquely the CD8 T cells, and then measuring a single other parameter for those cells). These include increases in HLA-DR, CD38 and CD57 (taken as indications of activation); or decreases in CD26, CD28, CD31, CD45RA, CD62L, and increases in CD45RO, CD11b (taken as indications of a change in the state of differentiation). However, these changes by themselves were usually relatively minor.

Multiparametric analysis began to reveal that, in fact, there are major and consistent changes in the CD8 T cell population that accompany HIV disease – changes that could not be observed by the two-color approaches. For example, naive CD8 T cells are virtually obliterated during progression of HIV disease. This subset of CD8 T cells is required for any new immune response; it is identified by the coexpression of CD26L and CD45RA. The loss of this important subset was missed for many years because many memory T cells express one or the other (but not both) of these markers; thus, three- or four-color phenotyping was required to reveal this loss. Indeed, when CD8 T cells are carefully phenotyped, it became evident that all 'normal' CD8 T cells were disappearing during HIV progression – exactly like CD4 T cells. The expansion of the bulk population was due entirely to the presence of activated CD8 T cells. Such studies profoundly impact the views of immunopathogenesis of disease.

Multiparametric studies in the near future will resolve much of the confusion surrounding the immunopathogenesis of diseases. These studies will reveal those populations that it is most relevant to quantitate. In addition, the sorting capability of the FACS allows the isolation of pure and homogeneous subsets of cells on which functional studies can be

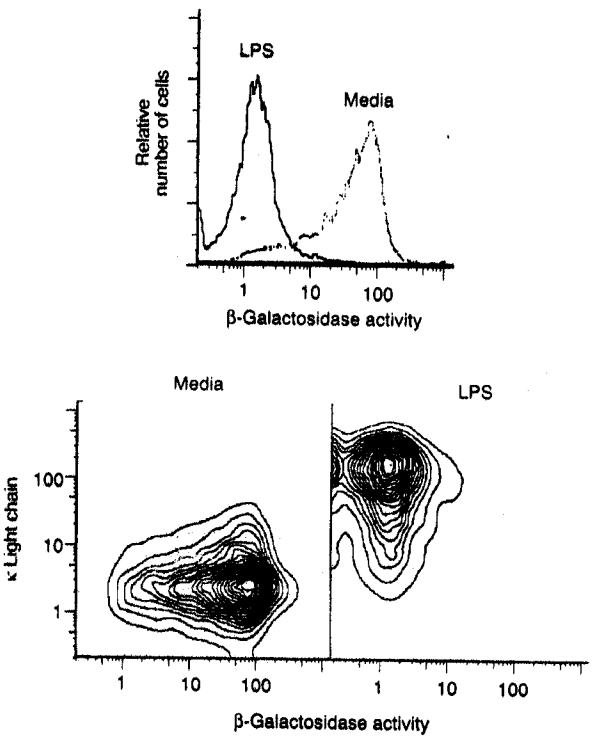

Figure 6 Analysis of intracellular gene expression. A B-cell line was generated, as a model of differentiation, which upon stimulation expresses κ light chain and turns off the expression of DNA elements controlling the artificially introduced bacterial *lacZ* gene. The histogram (top) demonstrates that *lacZ* (*E. coli* β-galactosidase) expression is reduced following lipopolysaccharide (LPS) stimulation. The β-galactosidase enzyme activity is measured by introducing a fluorogenic substrate, fluorescein digalactoside, into the cells. The contour plots demonstrate a concomitant increase in κ light chain expression, as detected by surface immunofluorescence. (Courtesy of Dr William Kerr.)

performed. These studies are necessary for understanding the pathogenic consequences of changes in the representation of fine lymphocyte subsets.

Molecular and functional studies

More and more frequently, the FACS is used in a wide variety of immunologically relevant molecular studies. These range from measuring changes in the concentration of intracellular small molecules (physiological state or signal transduction events) to quantitating gene expression to chart activation or differentiation events.

In the late 1980s, the FACS molecular tool kit was extended by the development of a technique for measuring the commonly-used reporter gene *lacZ* (β-galactosidase) within the intracellular compartment of mammalian (as well as bacterial and insect) cells. Depending on how it is introduced, this gene can be used either as a marker for lineage and migration studies or as an insertion probe that can reveal differentiation-dependent enhancer and promoter sites (**Figure 6**, see p. 938). Furthermore, when introduced as a reporter gene under the control of particular promoter and enhancer elements, it can be used to investigate the mechanisms that regulate gene expression or provide a quantitative indicator of shifts in the activation or differentiation status of individual cells (**Figure 7**).

Recent developments using inherently fluorescent proteins have provided another tool in the arsenal of FACS-based reporter gene studies. The first of these proteins, termed green fluorescent protein (GFP), has spectral characteristics marginally suitable for quantitation by flow cytometry. An enormous effort has gone into the generation of mutants of the original GFP which have not only increased brightness, but different spectral properties as well. Currently, there are two versions of the GFP which can be simultaneously and independently quantitated. This brings

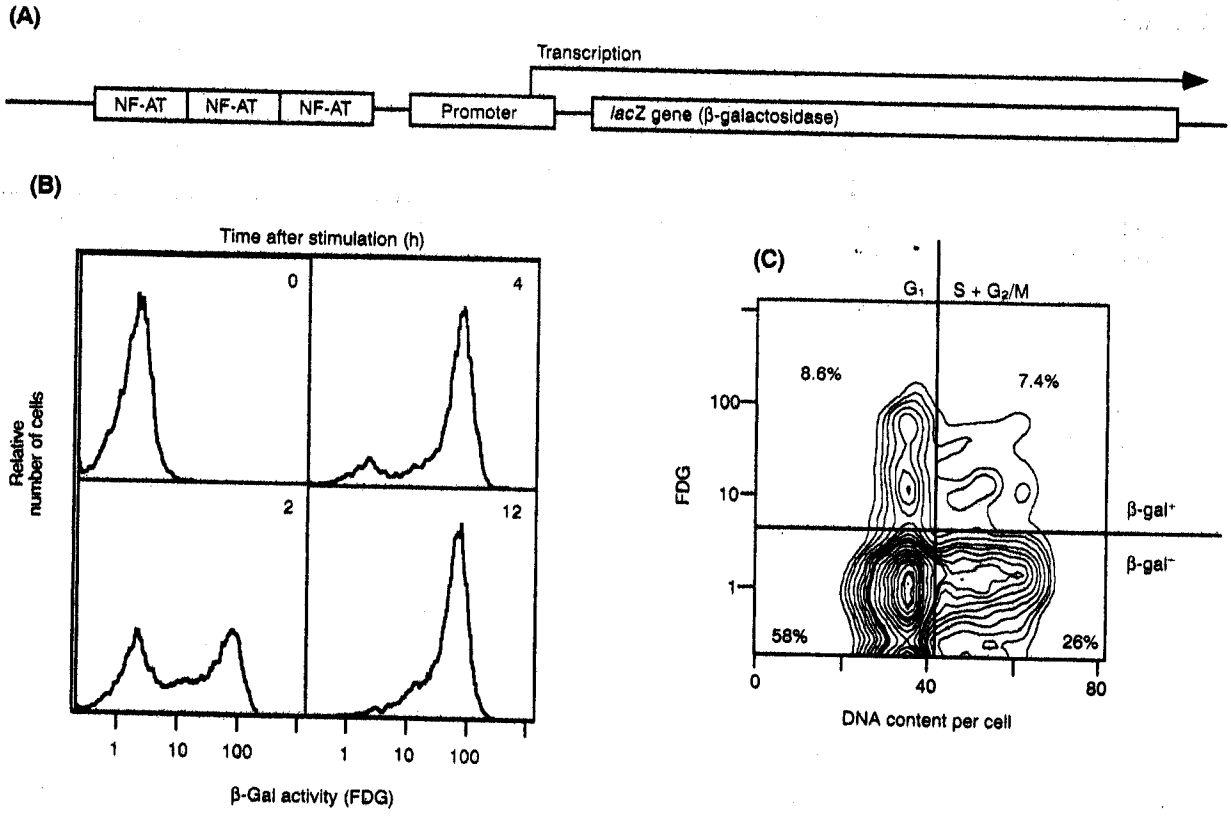

Figure 7 Correlation of gene expression with cell cycle progression. (A) The β-galactosidase gene expression in a Jurkat T cell line is controlled by DNA enhancer elements bound by a nuclear transcription factor termed NF-AT. Stimulation of T cells results in a signal transduction cascade, including the activation of this nuclear factor. (B) After stimulation for various periods of time up to 12 h, the *fraction* of cells expressing β-galactosidase activity (and thus having activated NF-AT) increases. β-Galactosidase activity is measured using the fluorogenic substrate fluorescein di-galactoside (FDG). The activation signal is a threshold event: after activation, no further increase in the production of β-galactosidase occurs (compare the positive cells at 2 h with those at 12 h). (C) Simultaneous quantitation of the activation of NF-AT (as revealed by β-galactosidase activity) and cell cycle progression (as revealed by the DNA content of cells) after 2 h of stimulation. Note that cells that have activated NF-AT are more likely to already be in cell cycle progression (S + G_2/M) than unactivated cells. (Courtesy of Dr Stephen Fiering.)

to gene expression analysis the power of multiparameter FACS; that is, the quantitation of different gene elements within individual cells, providing a correlated distribution of expression of those genes in a population (for example, see Figure 8).

The FACS is also a powerful tool for measuring the physiological state of cells. A commonly measured physiological response is the cytoplasmic calcium concentration. Calcium is mobilized from internal stores as well as the extracellular milieu in response to mitogenic signals. Thus, measuring the change in the internal calcium concentration after signaling is an important indicator of the responsiveness of cells, and a predictor of their ultimate proliferative capacity. Figure 9 shows an example of such an experiment. This experiment demonstrates that peripheral blood T cells have a heterogeneous responsiveness in terms of the ability to flux calcium – a heterogeneity which is only evident by combining this experiment with antibody stains to subdivide the populations.

It is very common in flow cytometric analysis to quantitate live versus dead cells. In general, this is most easily accomplished using propidium iodide (PI). PI binds avidly to DNA, but is excluded from DNA in live cells because it cannot cross the membrane. Dead cells, on the other hand, have permeable membranes that readily allow PI to enter and bind to the DNA. Thus, inclusion of a small amount of PI in all samples allows dead cells to be excluded from further analysis (or at least separately enumerated). Recently, much interest has focused on pathways by which cells die, in particular that of programmed cell death (or apoptosis). Several assays exist for the quantitation of apoptosis; such an assay is shown in Figure 10. In this example, the cells that have been signaled to die are shown to progress through several distinct stages.

In addition to these examples, assays that measure intracellular pH, reduction–oxidation (redox) potential, mitochondrial activity, RNA content, membrane area, membrane potential and endocytic activity have been developed for use with the multiparametric paradigm of flow cytometry. Changes in these measurements reveal signal transduction events, stress responses, mitogenic responses, etc. – important parameters of the functional capacity of cells. While most of these are used primarily in basic

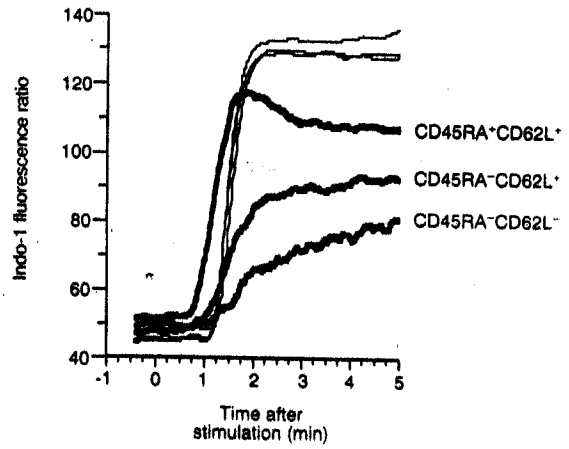

Figure 9 T cell signal transduction measured by calcium translocation. T cells isolated from a healthy individual were 'loaded' with Indo-1, a fluorescent molecule whose spectrum depends on calcium concentration. By measuring and calculating the ratio of two different Indo-1 fluorescences, one which declines and one which increases with increased calcium concentration, an estimate of intracellular calcium content can be made. In this experiment, T cells were also surface stained to determine their differentiation state. They were then stimulated through the T cell receptor, the primary signal for activation of these cells. The figure shows the relative calcium content after stimulating CD4 T cells with antibody to CD3 (dark lines). Cells that coexpress CD45RA and CD62L (and are 'naive' T cells) have a greater capacity to translocate calcium into the cytoplasm, compared with the memory subsets. This observation correlates with the greater capacity of naive T cells to proliferate after such a stimulation. The heterogeneous calcium flux is due to a difference in the CD3 signal transduction pathway: when the cells are stimulated with low concentrations of ionomycin (light lines), the three subsets have indistinguishable calcium mobilization profiles.

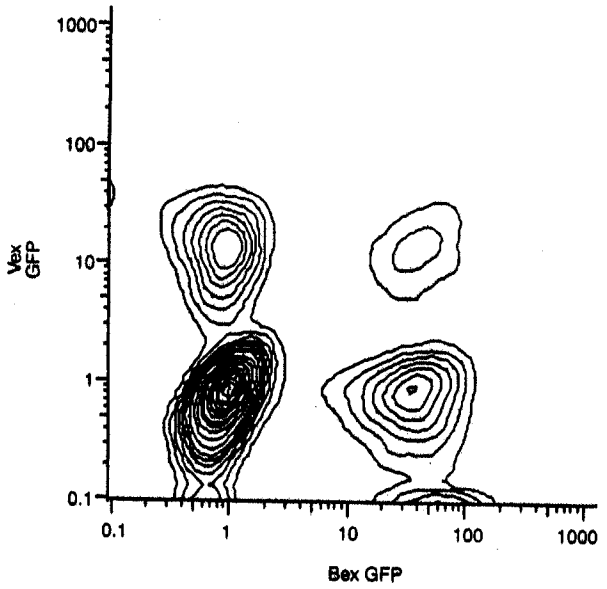

Figure 8 Simultaneous detection of two mutants of green fluorescent protein. Two different mutants (Bex and Vex) of the inherently-fluorescent GFP were selected so that they could be simultaneously and independently measured. In this experiment, cells were infected with a mixture of two different viruses, each expressing one of the mutant GFPs. Most cells were uninfected (expressing neither GFP); some cells were singly infected; and some cells were infected by both viruses (and show expression of both the Bex and Vex mutant GFPs). (Courtesy of Dr Michael Anderson.)

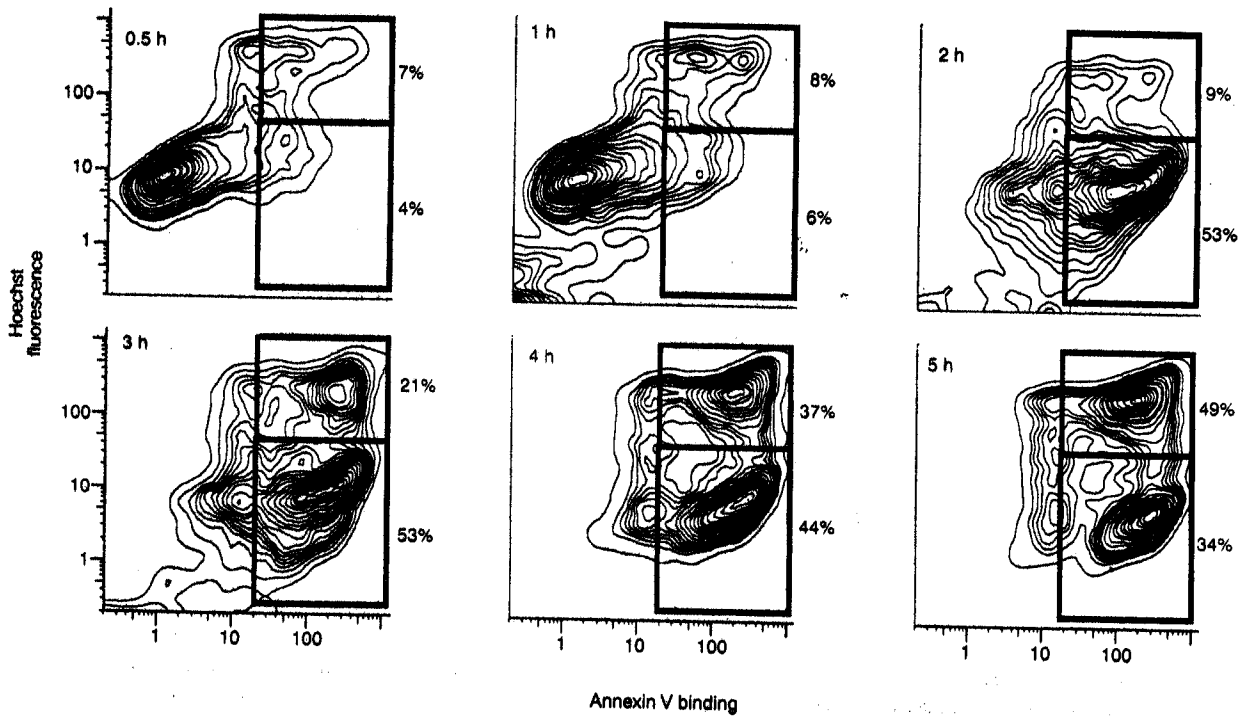

Figure 10 Discrimination of dying and apoptotic cells. Annexin V is a protein which binds to phosphatidylserine (PTS). PTS is normally not expressed on the outside of cellular membranes, but only on the inside. When cells commit to apoptosis (programmed cell death), one of the earliest events is the loss of this membrane asymmetry in PTS expression. Using fluorescently-conjugated Annexin V, cells early in the apoptosis commitment phase can be identified. During later stages of cell death, the chromatin condenses and binds Hoechst 33342 to a very high degree (note this use in distinction to **Figure 7**, where quantitative binding reveals DNA content); in addition, the Hoechst dye itself is no longer actively removed from the cells. In this figure, Jurkat T cells were induced to apoptosis; after various times they were assayed for Annexin V binding and Hoechst 33342 uptake. There is a clear progression of cells from double-negative, to Annexin V-positive (apoptosis-committed), to double-positive (dead). (Courtesy of Dr Peter Katsikis.)

research, some assays are beginning to find clinical relevance.

For example, oxidative stress has been shown to accompany HIV disease. A FACS assay which can measure intracellular glutathione (the major intracellular antioxidant) showed that lymphocytes in persons with HIV disease are depleted for glutathione – and that this depletion was a predictor of survival in advanced stage disease (**Figure 11**, see p. 942). The combination of surface immunophenotyping and a physiological measurement therefore provides a powerful, clinically relevant parameter. **Figure 11** also shows an example of the *in vivo* quantitation of gene expression of FACS. In this case, the number of CD38 molecules per CD8 T cell (i.e. proportional to the fluorescence of cells stained with phycoerythrin-conjugated anti-CD38 monoclonal antibody) also provides a significant predictor of progression to death in AIDS. Using these two parameters simultaneously is the most significant predictor of lifespan in AIDS patients known to date – far more significant that CD4 T cell counts, the most commonly used surrogate marker.

Clinical relevance has also been ascribed to the quantitative determination of DNA content in lymphocytes. In this case, lymphocyte membranes are disrupted to allow free entry of dyes such as Hoechst 33342 or PI. Quantitative fluorescence measurements easily reveal 2N or 4N DNA content (akin to the example in **Figure 7**). Clinical relevance comes from the observation that many leukemias and lymphomas have aberrant DNA content (aneuploidy). There is an extensive literature on typing such cancers based on DNA quantification.

Finally, the FACS can be used to perform powerful functional assays. FACS-based assays exist which can measure activation, proliferation, cytotoxic activity, apoptosis and cytokine production. Often, the FACS assays are considerably more sensitive than bulk assays. For instance, cytokine production assays can be performed in cell populations stimulated for only 6 h (for example, see **Figure 12**, see p. 942). Bulk assays, which measure cytokine production in supernatant, are technically easier to perform but yield considerably less information, together with greater potential artifact. Bulk assays for cytokine

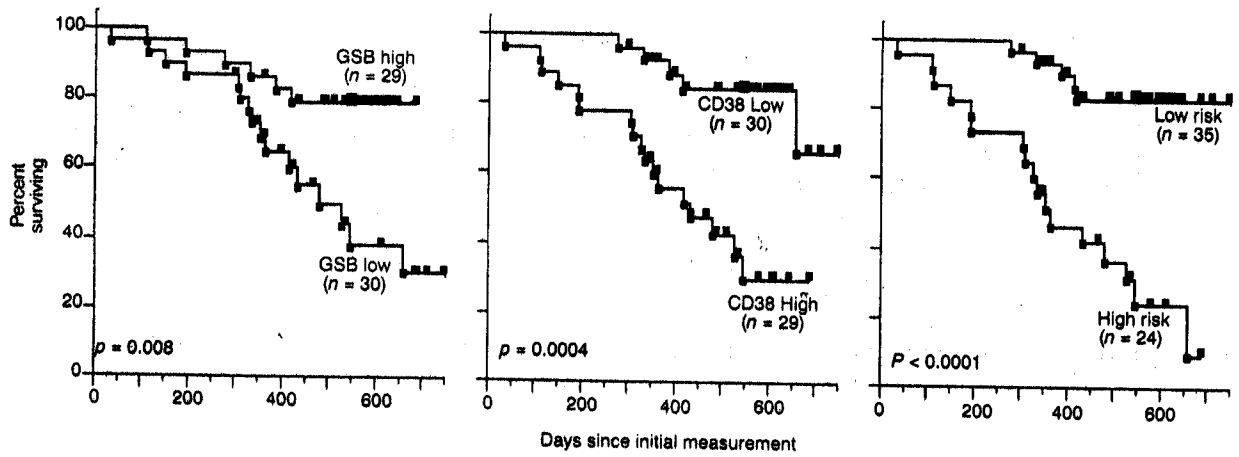

Figure 11 FACS-measured quantities are powerful prognostic variables in AIDS. Shown are Kaplan–Meier survival plots, which predict the survival of a group of individuals based on a measurement made at an initial time (day 0). (Left) The amount of glutathione (GSB) (the major intracellular antioxidant) was measured in CD4 T cells in 59 HIV-infected adults with advanced disease. Those with high glutathione were far more likely to survive 2 years than those with low glutathione. (Middle) The same individuals were divided on the basis of CD38 expression on CD8 T cells. Quantitating the absolute expression of this molecule, which may reflect the activation state of the immune system, also provides a powerful prognostic of death. (Right) The glutathione measurement and CD38 measurement were correlated to provide an index of individuals who are at high risk for progression to death (i.e. low glutathione and high CD38) versus those who are at low risk. The combination of these two parameters provides the most powerful prognostic value for predicting survival in AIDS patients.

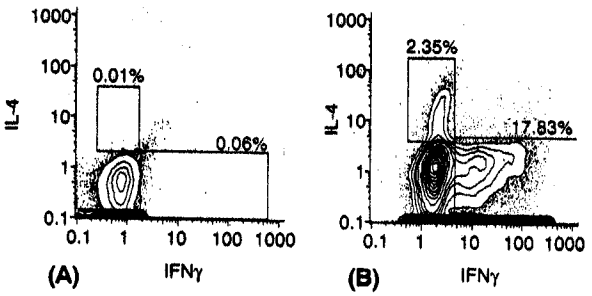

Figure 12 Cytokine profiles of human peripheral T cells. Human T cells were either resting (A) or stimulated with phorbol ester and ionomycin – a powerful activating combination (B). After 6 h, cells were stained for the presence of intracellular cytokines (i.e. the cytokines that are just synthesized and are about to be secreted for effector functions). Essentially no resting T cells are synthesizing cytokines; stimulated T cells will make either interleukin 4 (IL-4) or interferon γ (IFNγ) but very rarely both. Note that a bulk measurement of cytokine production would reveal that both cytokines are made in the population, but would not demonstrate the exclusivity of production on a cell-by-cell basis.

production are typically performed after at least 24 h of culture and provide no information about the distribution of cytokine expression among the cells (e.g. are all cells making the same amount of cytokine, or are only a small fraction of the cells each making a lot?). In the example of Figure 12, it would be impossible to deduce from a bulk assay that some cells make only IL-4, while others make only IFNγ.

Again, the real utility of the FACS for making functional assays comes with the combination of these assays with each other or with immunophenotyping. Thus, not only is it possible to measure how many cells make a particular cytokine, but also what the phenotype of those cells is, and, for instance, what their proliferative capacity is.

Summary

Flow cytometry is a platform upon which dozens of very different, highly-specialized assays can be performed, and provides the capability for performing many of these assays simultaneously. It is this multifactorial nature of the analysis which makes the FACS a unique tool in immunobiology today. The extremely high information density of the data derived from FACS assays is an absolute requirement to begin to elucidate the extremely complex immune system. FACS technology continues to evolve at a fast pace, as it has for a quarter of a century. This evolution includes a continuous infusion of applications into the clinical environment – indeed, the power of FACS for clinical diagnosis and prognosis is already evident from the fact that virtually every modern hospital has at least one of these instruments. The assays being developed in basic research laboratories today, such as those described above, will become routine clinical assays in hospital settings over the next decade.

See also: **B lymphocytes; CD antigens; Cell separation techniques; Fluorochrome labeling; T lymphocytes.**

Further reading

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FLUOROCHROME LABELING

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Fluorochrome-labeled reagents are an important tool in the analysis of cell surface antigens, both for use in fluorescence microscopy and in flow cytometry. The development of the hybridoma technique for the generation of monoclonal antibodies that can then be employed as fluorescent reagents has greatly aided the delineations of numerous functional subpopulations now known to constitute the immune system. Furthermore, the workhorses of microscopy, fluorescein isothiocyanate (FITC, fluorescing green) and tetramethyl rhodamine isothiocyanate (TRITC, fluorescing red) have been joined in recent years by new dyes, the photosynthetic accessory proteins known as phycobiliproteins, possessing intense fluorescence and useful excitation/emission spectra. The techniques used for generating fluorescent