FLOW CYTOMETRY

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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 Igf 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, e.g. passage through nylon wool, passage through an affinity column, and 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 function(s).

FACS-based cell isolation methods, in contrast, are well suited to this task, since they provide the potential for sorting and testing viable subpopulations. Furthermore, they provide a wide array of characteristics that can be used, individually or in combination, to define subpopulations, e.g. 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 of other cells.

B CELL SUBPOPULATIONS

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.

T CELL SUBPOPULATIONS

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 anti-Ig 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, since 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 end of the 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 man 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 cel surface antigens to recognize lymphocyte (and other) subpopulations and to sort these subpopulations to chart their functions.

Initial studies with this monoclonal-based methodology 1) defined the murine Ly-1 CD5) and Lyt-2 (CD8) lymphocyte surface antigens; 2) characterized the expression of these antigens on T cell subpopulations in mouse spleen, lymph node, and thymus; 3) used these FACS characterizations to recognize monoclonal antibodies that detected the human Leu-1 (CD5) and Leu-2 (CD8) homologs of the murine Ly-1 and Lyt-2 (see Fig. 1); and 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 AIDS.

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

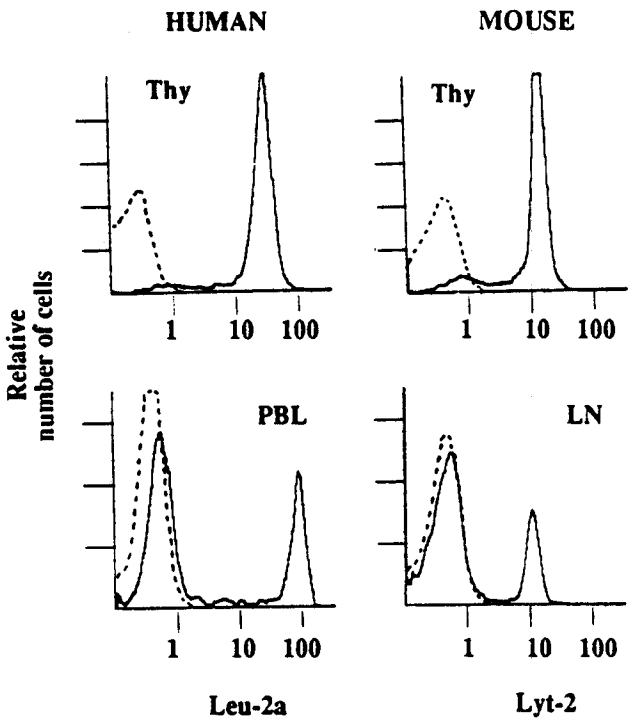

Figure 1. Leu-2a and Lyt-2 expression on human and mouse lymphoid cells. Early single-color FACS analysis, in conjunction with biochemical studies, demonstrated that human Leu-2a, which is found only on suppressor/cytotoxic cells, is very similar to the murine Lyt-2 surface antigen. The Leu-2a antigen was detected by indirect staining with SK1 monoclonal antibody supernatant followed by fluoresecein-conjugated goat anti-mouse 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 thymocytes (Thy) and 20-40% of the peripheral T cells (LN, lymph node; PBL, peripheral blood lymphocytes).

subpopulations on the basis of qualitative or semi-quantitative 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 phycobilliprotein dyes, 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. The light scatter measurement on this initial instrument was intended to signal the arrival of a cell-sized object in the light path; however, studies with cell populations from lymphoid organs soon showed that light scatter signals could also be used as a meaningful measure of cell size. Furthermore, these signals were shown to be useful for discriminating live cells from dead and for excluding ('gating out') dead cells during analysis and sorting. Thus, as the FACS came into use initially for biological studies, two parameters—light scatter and fluorescence—were routinely measured and recorded for individual cells.

The introduction of methods for measuring two additional parameters—large angle light 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. However, the current definition also rests on the expectation that the data will be analyzed by methods that take at least two of these measurements into account at a time, e.g. to produce dot plots, contour plots, gated histograms, or other displays of simultaneous measurements taken, stored, and processed for individual cells (i.e. in list mode). The early FACS instruments were capable of making the simultaneous fluorescence and scatter mesurements; however, computer methods for acquiring and processing these measurements to permit true multiparameter studies were not developed for FACS studies until relatively recently, when dual-laser FACS instruments that take up to four fluorescence and two scatter measurements per cell were introduced.

The power of these dual-laser instruments is evident from the unsuspected subdivisions their use has revealed in

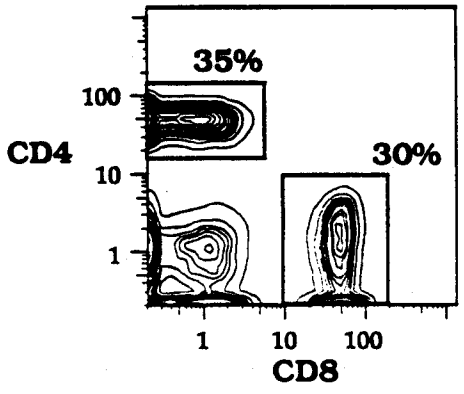

Figure 2. T cell analysis of C57B1/6 lymph node. Two-color FACS analysis is routinely used to quantitate $CD4^+$ helper/inducer T cells and $CD8^+$ cytotoxic/suppressor T cells in mouse and man. The 5% probability plots, which are also shown in Figs. 3 and 4, contain an equal number of cells between each pair of contour levels. The number of lines in each region of the map is essentially proportional to the number of cells in that region.

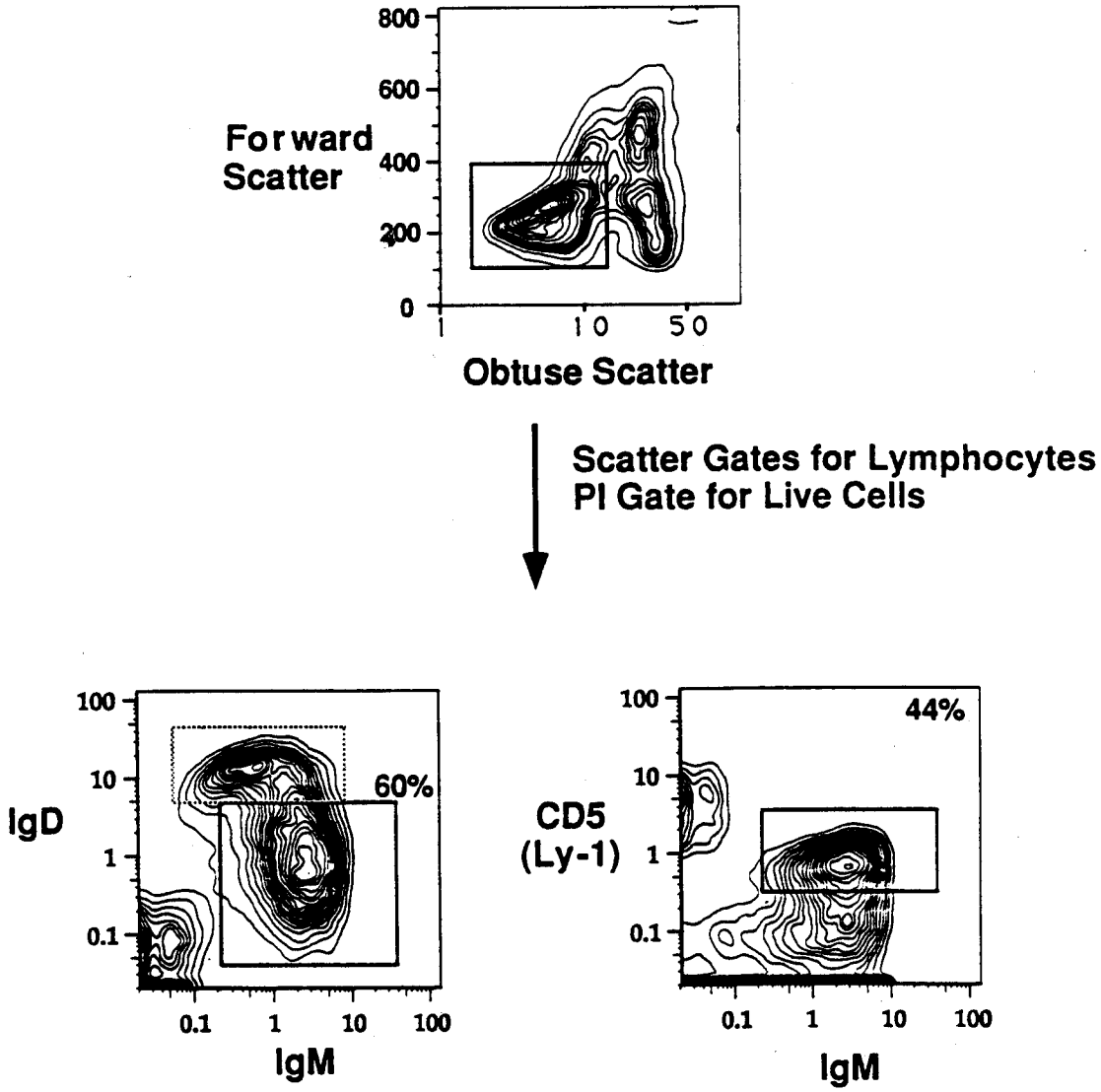

Figure 3. Six-parameter analysis of Balb/c peritoneal cavity cells. State-of-the-art dual-laser FACS analysis permits the simultaneous measurement of six or more parameters. The top plot shows the forward v obtuse scatter profile. The data, which are recorded in list mode, can be gated for live lymphocytes as shown. Cell surface staining profiles in the bottom 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 bottom left panel shows the characteristic IgM v 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 bottom right plot distinguishes the original $CD5^+$ Ly-1 lineage B cells (solid box) from the $CD5^-$ 'sister' Ly-1 B cells. Percentages are reported in terms of the total live lymphocytes, as determined by the scatter and propidium iodide gates.

biological systems. B cells, for example, were thought to be relatively homogeneous. However, initial multiparameter FACS studies conducted with the 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 man.

Unlike the major T cell subpopulations, which are recognizable by the expression of unique surface markers (CD4 or CD8 (see Fig. 2), none of the B cell subpopulations/lineages are as yet recognizable by the presence, absence, or surface level of an individual surface marker. Distinguishing these subpopulations requires measurement of at least two surface molecules (detected by differently colored antibodies)) see Fig. 3). 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.

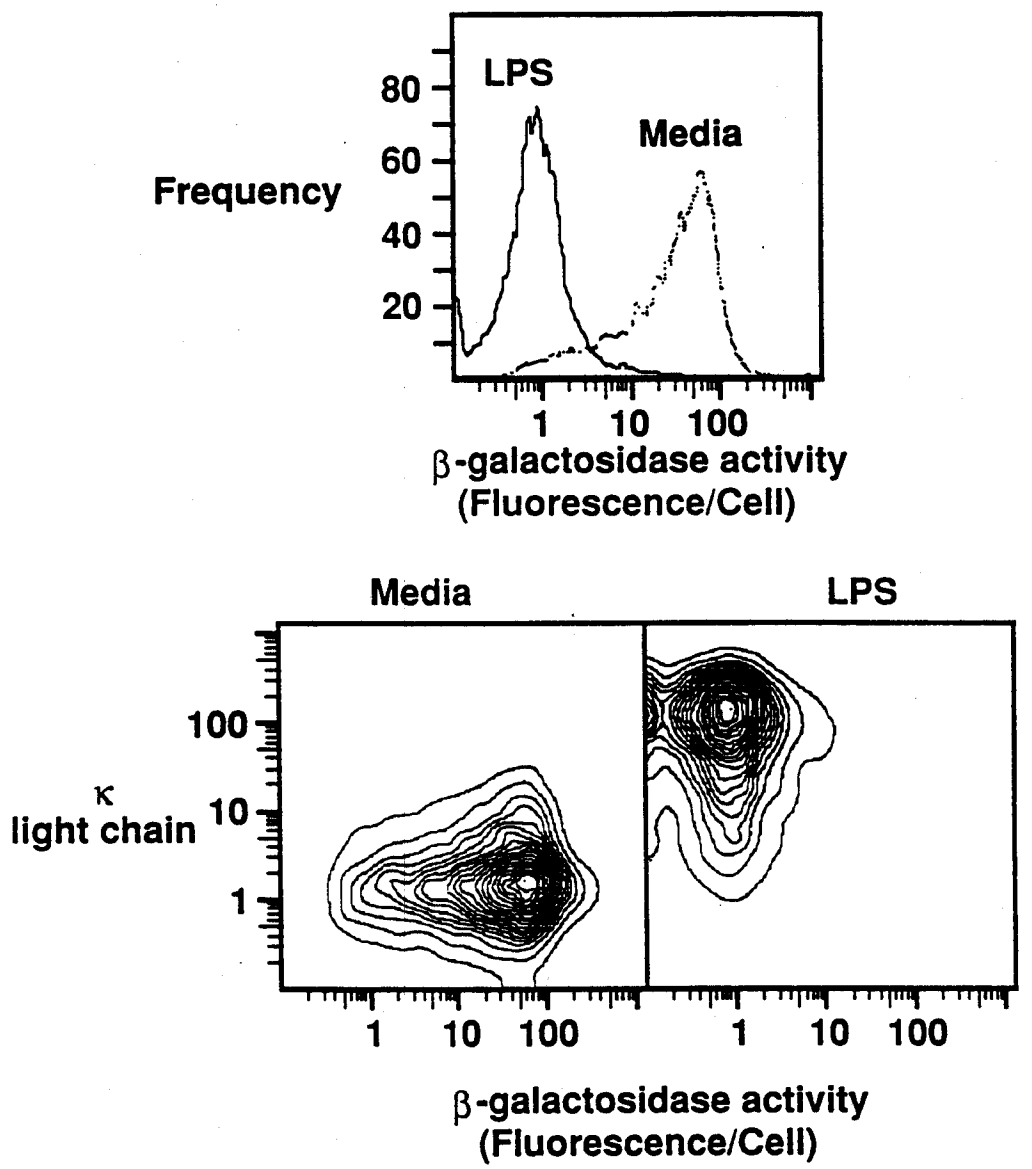

Figure 4. Analysis of intracellular gene expression. The histogram (top) demonstrates that LacZ (*E. coli* β-galactosidase) expression is reduced following LPS stimulation of 70/Z3 pre-B cell enhancer search clone 7e17-17. The enzyme activity is measured by introducing a fluorogenic substrate, fluorescein digalactoside, into the cells. The contour plots demonstrate a concomitant increase in κ light chain expression

Lymphocyte subpopulation studies such as those described above probably represent the most common and well known use for FACS instruments; however, the FACS is also used widely for cell cycle analysis and for measuring calcium uptake and other physiologic response indicators. In immunology, these kinds of measurements have been particularly important for interleukin (growth factor) studies and for activation and surface receptor studies with normal and neoplastic cell populations.

MOLECULAR STUDIES

The FACS has been used in a wide variety of immunologically relevant molecular studies. These range from multiparameter analyses charting the expression and developmental influences of Ig and T cell receptor transgenes in mice to the selection and cloning of cultured cells that express mutated genes or genes introduced with recombinant technology. Studies in this latter category include: the isolation of clones producing variant MHC molecules; the selection of Ig switch variants and antibody affinity variants; the introduction of genetically engineered constructs into cell lines; and the cloning of genes encoding lymphocyte surface antigens.

In recent years, the FACS molecular tool-kit has been extended by the development of methods for measuring the intracellular expression of the LacZ (*E. coli* β-galactosidase) gene in memmalian 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. 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, when introduced with the appropriate regulatory elements, to provide a quantitative indicator of shifts in the activation or differentiation status of individual cells (see Fig. 4).

In what may prove to be a very important application of this LacZ reporter gene technology, a series of recent studies has shown that intracellular thiols (particularly glutathione) regulate the lymphokine-stimulated activation of the NF-κB transcription factor and thereby control the expression of the HIV-1 (AIDS) retrovirus. These studies provide a model system for investigating conditions that influence HIV expression. In addition, they provide the first evidence that intracellular thiols play a central role in the signal transduction pathway responsible for activating the widely studied transactivating nuclear transcription factor, NF-κB. Thus, the LacZ reporter gene system, like FACS technology in general, can be expected to continue to produce key insights into the molecules, cells, and mechanisms operating in the immune system.

See also: B Lymphocytes; CD Antigens; Cell Separation Techniques; Fluorochrome Labeling; T Lymphocytes.

Further reading

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