How to Find Needles in Haystacks: Fluorescence-Activated Cell Sorting

A new instrument in Braun Laboratories is a dual-laser fluorescence-activated cell sorter — an irreplaceable tool for isolating rare cells from mixed populations or for determining which properties are correlated in populations of cells that differ in many ways

by Ellen Rothenberg

THE TISSUES of higher organisms are composed of extremely heterogeneous populations of cells. Within one organ, the developmental origins of various cells can be quite distinct. A good example is skin, which is peppered throughout with surveillance cells of bone marrow origin, and which is colored by pigment cells that are related to nerve tissue. Even when the cells in an organ are closely related, they may carry out very different functions involving distinct biochemical pathways. Thymus-derived lymphocytes, for example, are strictly specialized, either to secrete growth-stimulating polypeptide hormones or to bind and kill target cells. Finally, the cells from a single clone, although developmentally equivalent, may show different properties in different phases of their growth. Actively dividing cells not only have more stringent nutritional requirements than resting cells but also become vulnerable to a variety of poisons and serve frequently as preferred targets for viral infections. Most dramatically, early cancer cells may differ from their normal homologues in very few properties other than growth rate.

To understand the mechanism by which an organ carries out a particular function, we must be able to dissect out the key subpopulation of cells and distinguish its responses from those of other cells. It is to make this possible that fluorescence-activated cell sorting has been developed.

The cell sorter carries out two tasks. First, it records differences among individual cells in a population on the basis of one or several correlated properties. Second, it physically isolates the cells with properties of interest from the cells in the rest of the population. While its ability to deliver purified cell preparations is its most dramatic tour de force, the cell sorter is also an analytical instrument of unparalleled power and flexibility.

The basis on which the sorter discriminates between cells is optical. Individual cells intercept a slit-focused laser beam and scatter its light. The intensity of light scattered "forward," in a halo around the laser target, is mainly dependent on the cross-sectional area of the cell. Light scattered at a 90° angle is more a function of the refractive index of a cell, of its granularity or quantity of intracellular organelles. Simply using the intensities of forward and 90° light scatter makes it possible to discriminate between lymphocytes, monocytes, and granulocytes in human peripheral blood samples.

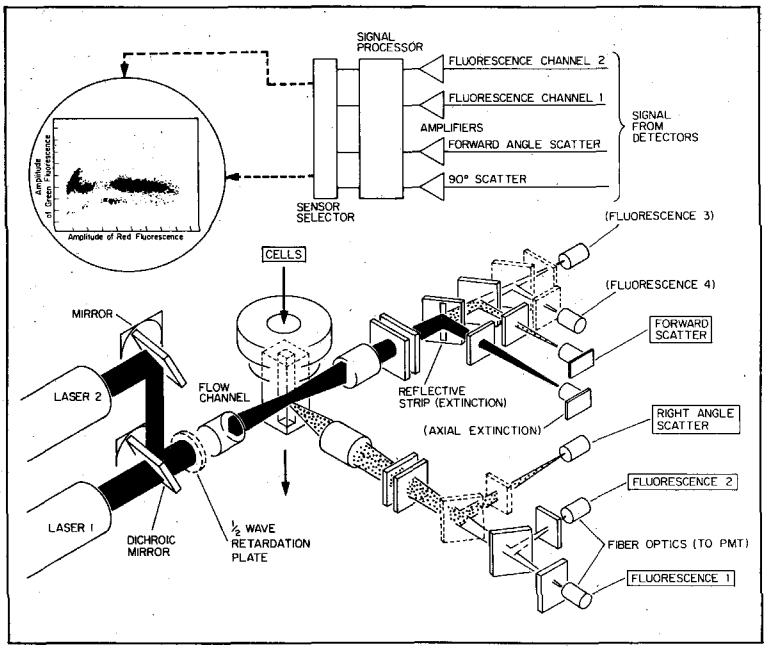
By using lasers tuned to appropriate wavelengths, it is possible to gather much more information. Cellular enzyme activities can be assayed with fluorogenic substrates, and cell surface molecules can be bound by fluorochrome-conjugated antibody molecules. The laser can emit at an excitation optimum for the fluorochrome (488 nanometers to excite green fluorescein, 568 nanometers for red tetramethyl rhodamine), so that the fluorescence intensity provides a fairly quantitative measure of enzyme activity or surface antigen expression in each cell. In the system installed in the Braun Labs, every cell is identified by up to four parameters, so that forward light scatter, 90° light scatter, and reaction with both green and red fluorochromes can all be considered simultaneously. In practice, this means that surface antigen A can be measured with a green-fluorescent antibody to A and surface antigen B with a red-fluorescent antibody to B. Then we can focus, for instance, on the largest, most granular cells in a sample and determine what fractions of that subpopulation are A+B+, A+B−, A−B+, or A−B−.

The sorter can analyze cells individually because of its fluidic system. Cells in dilute suspension are pumped from a reservoir into an optical flow cell, where they are funneled into a narrow stream and held centered by the flow of surrounding sheath fluid. This ensures that each cell will cross the path of the laser in the flow cell and makes it unlikely that two cells will coincide unless they are bound together. The light scatter and fluorescence signals from each cell are amplified, digitized, and fed immediately to the multichannel analyzer, so that within 40 to 120 microseconds a decision can be made on whether or not to collect that cell.

After traversing the optical flow chamber, the stream of cells and sheath fluid emerges through a vibrating nozzle that breaks it into droplets. Only one droplet in ten is likely to contain any cell at all. The droplets then pass between two deflection plates which are charged transiently if the command is given to sort. Two sorted populations can be collected by deflecting to the left or to the right, while unsorted droplets fall into a mass collection vessel in the center. The system can process up to 2000 cells per second without compromising the purity of the selection. Furthermore, living cells usually emerge from the instrument with undiminished viability and functional potential. Thus, in a typical preparative run of a little over an hour, we can collect a million cells of a type that make up one-tenth of the starting population.

The Cell Sorter In Genetics

One straightforward application of the sorting technology is to isolate extremely rare types of living cells. If the purified cells can maintain their properties and proliferate extensively in culture, then only relatively small numbers need to be collected initially. This application has been most useful in cases where the differences between the desired cells and the majority are genetic, and hence are differences that are maintained through many cell divisions. Classic somatic cell geneticists have used sorting to isolate rare mutant cells that express a new surface antigen. More recently, sorting has become a promising adjunct to recombinant DNA technology. If a cloned gene encodes a surface antigen or an enzyme that can use a fluorogenic substrate, then expression of the cloned gene can be measured by fluorescence. Thus, when the gene is introduced into cells under different conditions, the sorter can provide a quantitative comparison of the resulting levels of expression. Moreover, live cells expressing the gene can be isolated for further analysis and manipulation. This approach has been valuable both for studies of gene regulation and for identification of genes that encode particular cell-surface glycoproteins.



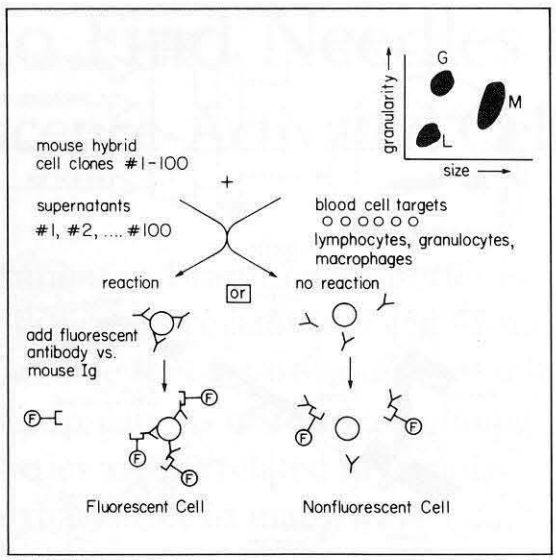
Optics of the cell sorter. Light scattered in a forward direction indicates the size of the cell, while the intensity of light scattered at a right angle from the incident laser beam depends on the cell's granularity. Green or red fluorescence detectors can measure the excitation of fluorescein and rhodamine, respectively, and can be used in either position.

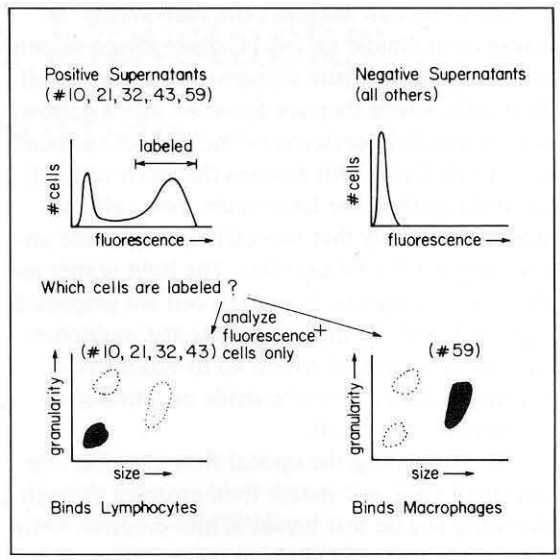
The Cell Sorter In Immunology

A different area where cell sorting has played a prominent role is the characterization of monoclonal antibodies. Monoclonal antibodies are homogeneous immunoglobulin molecules that bind specifically to one antigenic target. They are secreted by individual spleen lymphocytes that have been immortalized by fusion with lymphoid tumor cells. To make monoclonal antibodies against a desired cell type or a desired protein molecule, mice or rats must first be immunized with the cell or protein antigen. Spleen cells from the immunized animals are then fused en masse with the tumor cells, and hundreds of clones of fused hybrid cells are grown in culture. Each clone secretes some type of monoclonal antibody, but only a few will secrete antibody that binds to the target of interest. Finding those clones requires a massive and sophisticated screening program.

Cell sorting has provided two kinds of useful screens. First, a protein antigen can be used to coat fluorescent microspheres, which then bind only to those hybrid cells that make antibody to the protein. The hybrids with bound fluorescent microspheres can be isolated directly by the sorter. Second, when the antigen of interest is a particular cell type, the sorter can be used analytically to measure binding of each of a panel of monoclonal antibodies to that kind of cell. The antibodies secreted by different hybrid clones are separately reacted with the target cells, and

Identifying antibodies reactive with a particular target cell type. The monoclonal antibodies secreted by 100 different hybrid mouse cells are each allowed to react with blood cell targets. Fluorescein-conjugated antibody against mouse antibody makes a fluorescent "sandwich" wherever a mouse antibody succeeded in binding. The cells bearing the fluorescent complexes are analyzed in the sorter. Antibodies 10, 21, 32, and 43 all bind lymphocytes. Antibody 59 binds to macrophages. If anti-lymphocyte antibodies are desired, antibody 59 is discarded.



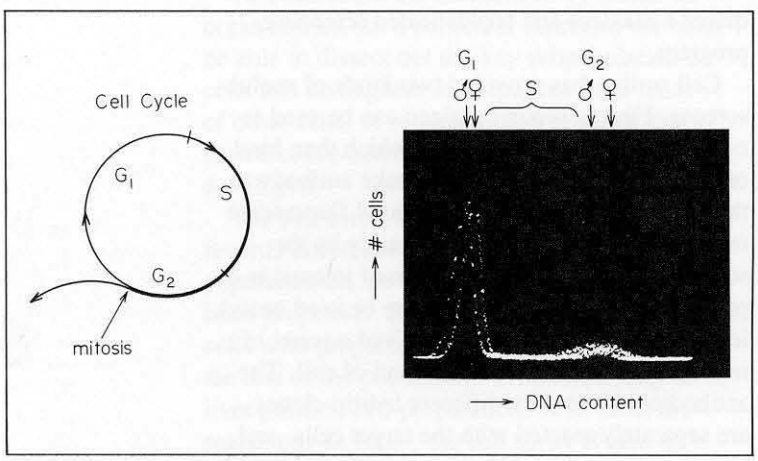


binding is detected by adding a fluorochrome-conjugated rabbit antibody against mouse or rat immunoglobulin (illustrated above). Because the sorter can measure additional properties of the target cells, it can discriminate between binding to the targets of interest and binding to some other cell type or to debris that may contaminate the target sample.

The Sorter in Cell Biology and Physiology

In addition to these applications, the cell sorter provides a unique approach to studies of growth control. This stems from the availability of fluorescent dyes that bind stoichiometrically to DNA in chromatin. The fluorescence recorded from stained cells is directly proportional to the DNA content of the cells. Thus, in histograms of cell number vs fluorescence intensity, one can readily distinguish the peak of cells that have replicated their DNA prior to mitosis (G_2) from the cells that have not yet begun DNA synthesis (G_1) since their last division. As shown in the figure below, even subtler differences are discernible, including the difference between male and female mammalian cells. The Y chromosome of the male is much smaller than the X chromosome, so that each male (XY) cell nucleus contains slightly less DNA than the female (XX) nucleus at a corresponding stage of the cell cycle.

Distinguishing male and female cells by DNA content. The ordinate shows the number of cells with a given intensity of fluorescence. The abcissa shows increasing fluorescence intensity, a direct measure of DNA content. G_1 cells have recently divided and have not begun to replicate their DNA for the next division; G_2 cells have just finished copying their DNA prior to division. Male cells at each stage have less DNA than female cells because the Y chromosome is smaller than the X chromosome.



Most determinations of cellular DNA content are carried out on fixed, permeabilized cells, because most of the DNA-binding dyes do not penetrate viable cells. One of the most interesting recent developments, however, is that new DNA-binding dyes have been found that enter live cells without serious toxic effects. This has opened up the possibility of sorting cells on the basis of their behavior.

Most cells in mammalian organs are in a resting state, and a crucial determinant of their function is often the type of stimulus they require to initiate DNA synthesis. The cells that respond to a particular signal in this way can be isolated by sorting on the basis of their increased DNA content. Even if the cells are fixed, the sorter can provide correlated information on their surface antigens and size. Since responsiveness is essentially a behavioral characteristic, though, it is most interesting to preserve these cells' viability, to be able to establish them in culture where their responses can be analyzed in greater detail.

These examples illustrate the cell sorter's broad range of analytical and preparative applications. In addition to the statistics it provides on the characteristics of cell populations, it can physically isolate rare cells defined by unusual combinations of those characteristics. Almost any property that can be measured optically may be exploited to distinguish between subclasses of cells. The limits of the sorter's versatility are mainly determined by our ingenuity in devising fluorescence and colorimetric assays and by our imagination in using them. □