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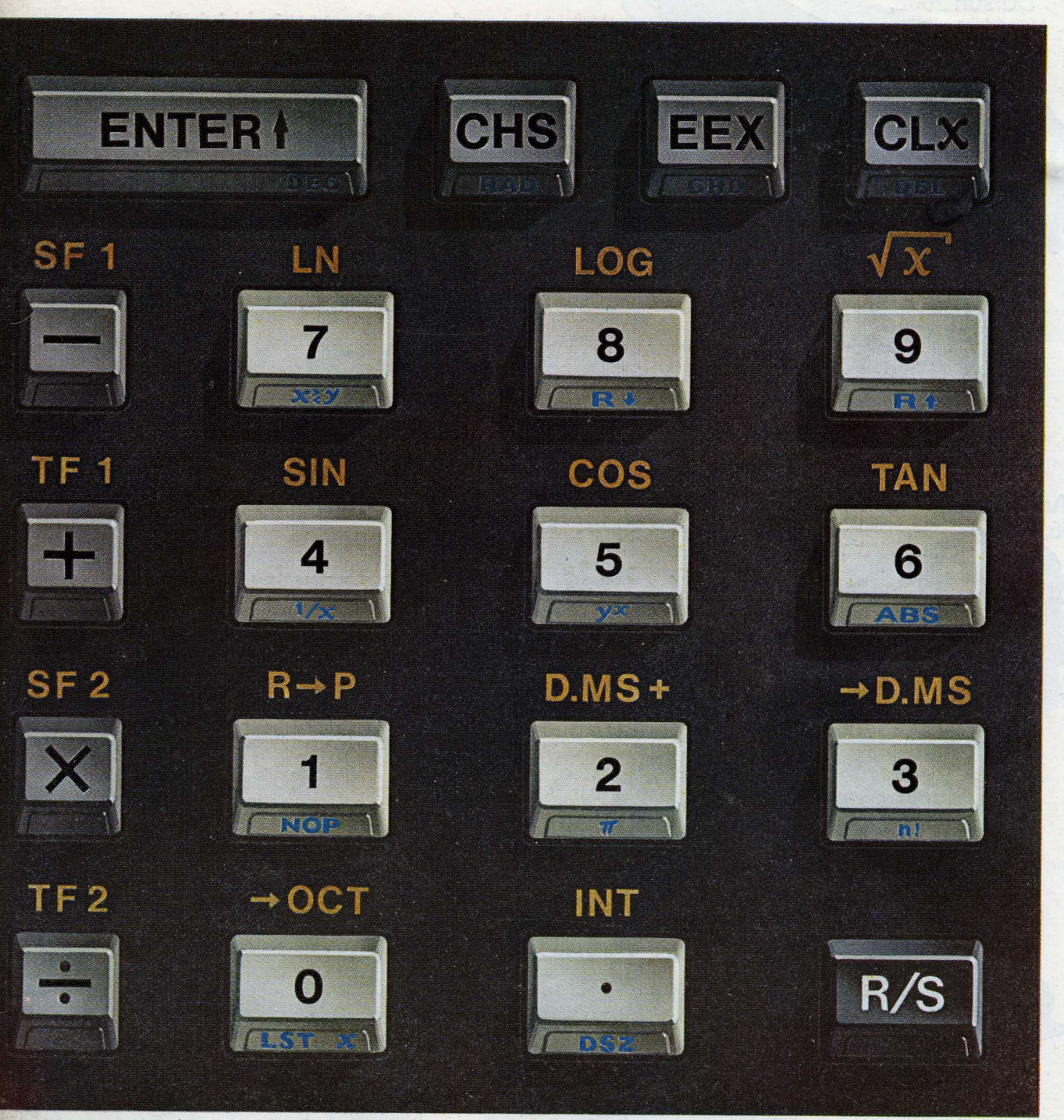

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Fluorescence-activated Cell Sorting

This new technique, which is capable of identifying and isolating closely related types of animal cells at rates of up to 5,000 cells per second, holds much promise for investigations of cell biology

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Perhaps the chief obstacle to the study of cell biology today is the problem of obtaining pure populations of different kinds of living cells from a given organ. Imagine molecular biology without tools such as ultracentrifugation, column chromatography and electrophoresis for the isolation of DNA, RNA, enzymes and other constituents from the molecular "soup" obtained by the disruption of cells. Detailed descriptions of the mechanisms involved in the metabolism of sugar and the synthesis of protein—two outstanding achievements of recent years—would still be a gleam in the biochemist's eye. An important prerequisite for studying the components of any biological system, be they the molecules in a cell or the cells in an organ, is the ability to isolate those components from one another so that they can be characterized and recombined under controlled conditions.

Of course, cell biology is not entirely lacking in techniques for isolating and studying living cells. Several methods, including centrifugation and electrophoresis, have played a key part in fundamental discoveries. As knowledge in the field expands, however, so does the quest for a more refined method to separate closely related yet functionally distinct types of cells. Here we shall describe a promising new approach to the problem of cell separation, pursued mainly in our laboratory at Stanford University.

The common starting point for all cell-separation techniques is the breakdown of the organ (or organism) to obtain a suspension of viable dissociated cells floating free in a suitable medium. That is fairly easy to accomplish with many tissues, where it is generally adequate to crush the excised tissue against a fine wire mesh. Other tissues may call for enzymes or other chemicals to sever the intercellular connections that bind the cells together. From some organs only one or a few of the cell types present can be recovered, whereas for other organs no techniques have yet been found for obtaining viable cell suspensions in good yields.

The development of techniques for preparing suspensions of dissociated cells was in itself a major advance in cell biology. Much of what we know today about cell functions comes from studies in which suspensions of cells are either injected back into animals or cultured for longer or shorter periods in the laboratory. As the cells obtained from a given organ were found to be responsible for a given function, however, questions inevitably arose: Which cells in the suspension were actually doing the work? Was an interaction between two or more different cell types required?

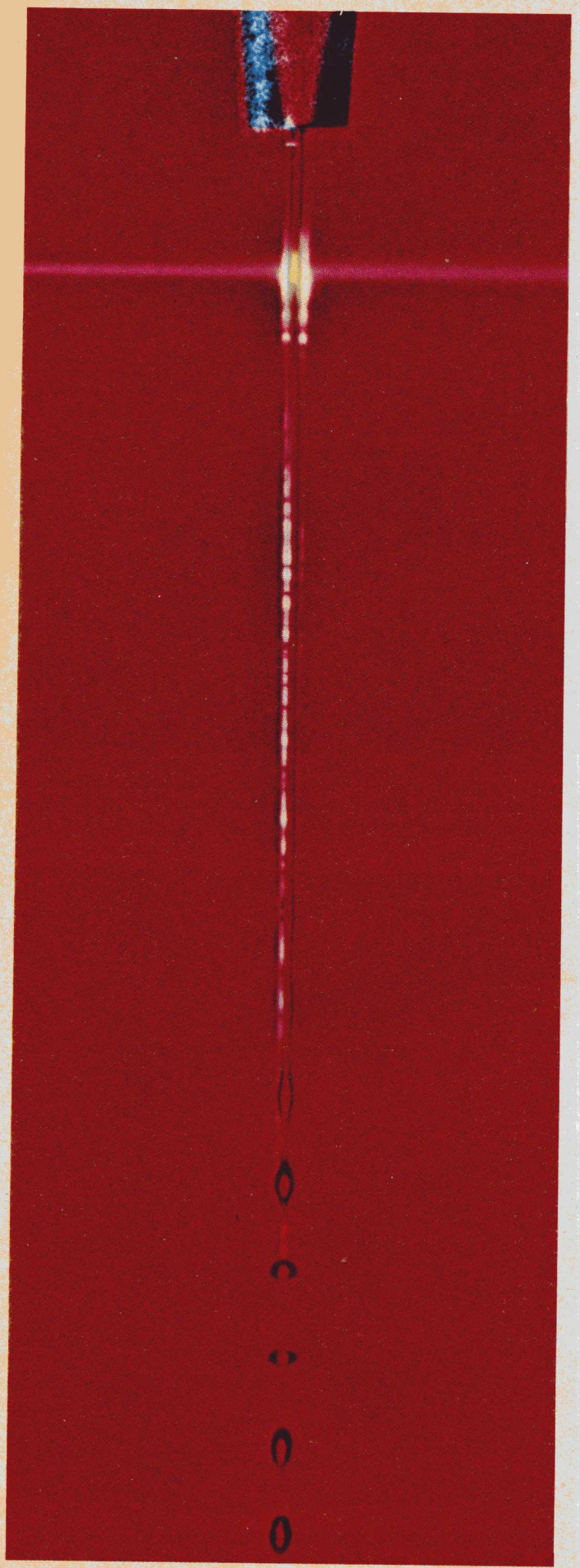
The existing methods for separating cells grew out of the need to answer such questions for cell suspensions from a variety of organs being studied for a variety of functions. Some of those methods depend on the selective killing of unwanted cells in order to enrich the suspension in the surviving type (or types) of cells. Other techniques aim for selective enrichment based on physical or chemical differences between the cells in the suspension. In either case the method of choice is dictated by the cells under study, their susceptibility to damage by various treatments, the methods used to assay cell function and, most important, by the nature of the differences between the cells that must be separated from one another.

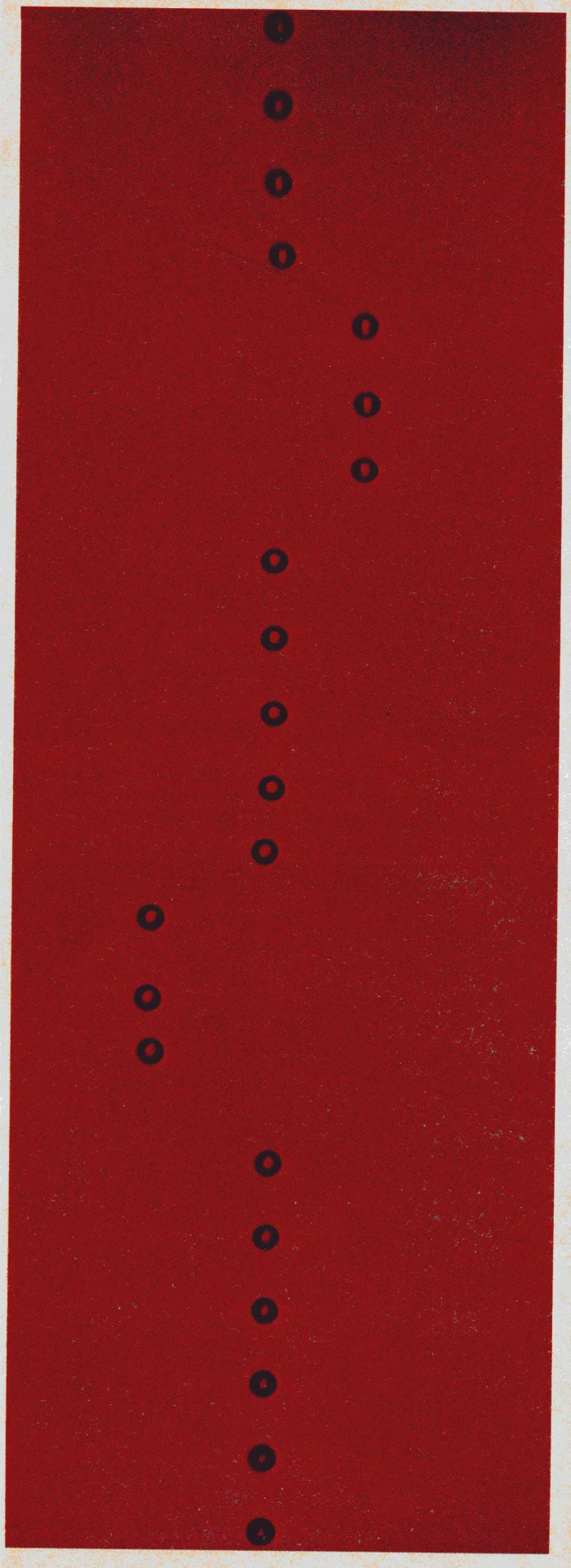
Cell-separation methods can take advantage of differences in the physical properties of the cells (such as size or density) or they can exploit differences in the properties of the surface membranes of the cells (such as their electric charge or their ability to adhere to glass or plastics). Because functionally different cells may differ from one another only in the amount or type of a single surface membrane constituent, however, many laboratories, including our own, have sought techniques with more specificity than is afforded by physical criteria alone.

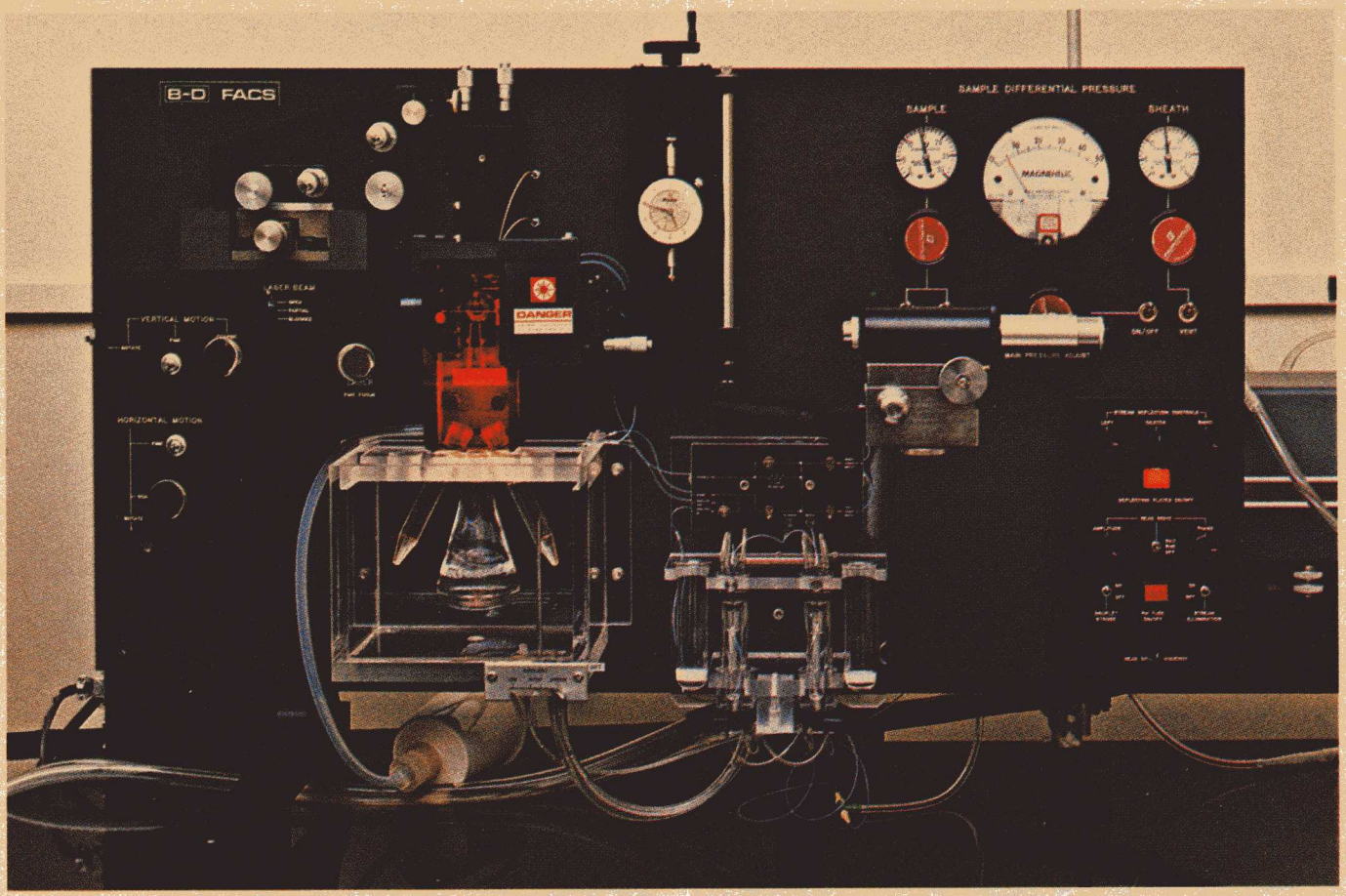
Several years ago we were searching for methods for isolating variants that appeared infrequently in cultured animal-cell lines. We wanted variants that differed only slightly from the parent cell line, perhaps only by the gain or loss of a single structure on the surface of the cell. We were struck by the apparent ease with which certain structures on the cell surface could be coated with a specific fluorescent substance and by the almost infinite variety of surface structures that could be labeled in this way to distinguish the cells from one another. It was clear that we could identify the variants we wanted with the aid of the fluorescent-marker method and the fluorescence microscope. Other investigators had already done so in similar experiments, but what we needed for our experiments was a method not only for identifying fluorescence-tagged cells but also for isolating them.

We were at that time also becoming interested in the interactions of lymphocyte cells in the immune response and in the genetics and biology of lymphatic tumors. Again we recognized the need for a method for isolating various kinds of lymphocytes, many of which deviate from other lymphocytes only by small surface differences. And again be-

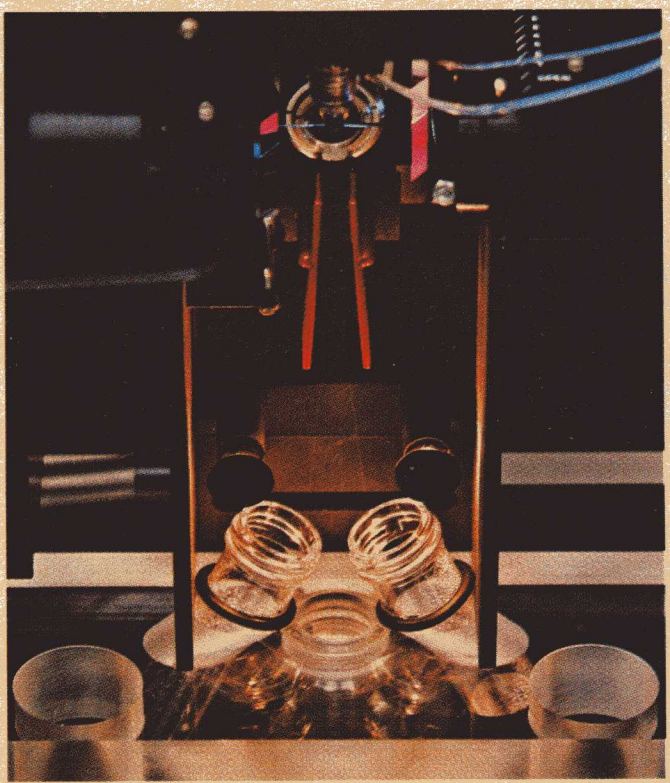
FLUID JET containing a mixture of different cells, some "tagged" with a fluorescent dye, is shown emerging from a rapidly vibrating nozzle and breaking into a uniform stream of tiny droplets in the left-hand photograph on the opposite page. Just below the nozzle, before the droplets form, the jet is illuminated with the focused blue light of an argon-ion laser, which excites a yellow-green fluorescence in the tagged cells. The fluorescent light is detected, along with light scattered forward out of the illuminating beam by the passing cell, and the resulting electrical signals are used to charge the liquid stream exactly when the droplet containing a desired cell is forming. Farther downstream the droplets, which retain their charge after they separate from the stream, pass through a constant electric field across their path. As the right-hand photograph shows, charged droplets are deflected toward appropriate collecting reservoirs, whereas uncharged droplets continue on their original course. Both of these demonstration photographs are multiple exposures made with the aid of a microscope and a stroboscopic lamp timed to flash synchronously with the procession of falling droplets; thus the image of each droplet seen in the photographs is actually made up of thousands of identical droplet images. Here the fluorescence was enhanced by adding extra fluorescent dye to cell suspension.



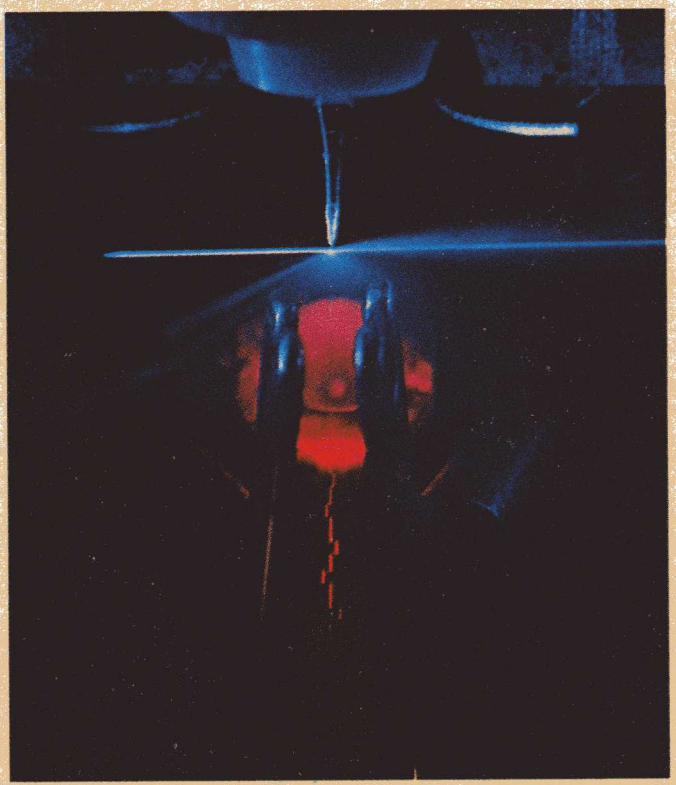




CURRENT MODEL of the fluorescence-activated cell sorter, the FACS-II, was photographed in the authors' laboratory at Stanford University, where it was originally developed. The machine, which is now manufactured by the Becton Dickinson Electronics Laboratory, is capable of separating as many as 90 million cells, differing by as little as 5 percent in their fluorescence, in a typical run of five hours.



CLOSEUP PHOTOGRAPH shows the portion of the Stanford cell sorter where the fluid droplets are actually formed and separated. The deflected streams of droplets are barely visible in this normal exposure as faint lines curving toward the collecting vessels at bottom.



STROBOSCOPIC-FLASH PHOTOGRAPH similar to photographs on the preceding page but at a macroscopic scale manages to capture the deflected droplets in mid-flight near the electrically charged plates. The laser beam was made visible by blowing smoke in its path.

cause these differences could be detected by the fluorescence microscope, we wanted a method of isolating cells tagged with a fluorescent marker.

Those needs led us to become intrigued with the idea of building a fluorescence-activated cell-sorting machine that would enable us to do the desired experiments. We contacted Louis A. Kamentsky of the International Business Machines Corporation, who had already built a machine that could separate fluorescent cells. The goals set for his instrument, however, were different from our own. Kamentsky was concerned with isolating small numbers of potential cancer cells—not necessarily viable after isolation—and depositing them on a slide for microscopic study by a pathologist, whereas what we wanted was to obtain large numbers of isolated cells, both viable and suitable for a variety of functional biological studies.

We would probably have gone no further than dreaming about a cell sorter that met our needs if it had not been for the fortunate proximity in another laboratory at Stanford of a team of investigators engaged in the National Aeronautics and Space Administration's Exobiology Program. Over coffee and lunch we talked with our engineer neighbors about the feasibility of jointly building a cell sorter. Eventually we got some initial funding from various sources and decided to attempt to build such a machine. Several years later a prototype model of a fluorescence-activated cell sorter, designed principally by William A. Bonner and H. Russel Hulett, went into operation in our laboratory.

The fluorescence-activated cell sorter, or FACS, separates cells according to how much fluorescent dye is bound to each cell. The current model, FACS-II, detects cells with as few as 3,000 molecules of fluorescein (a common fluorescent dye) on each cell. In addition, by means of correlated light-scattering, the device simultaneously measures the sizes of all cells; hence it can be set to separate those cells that fall within desired ranges of both size and fluorescence. The sorter can operate efficiently at rates of up to 5,000 cells per second, which means that in a typical five-hour run 90 million cells can be processed for various experimental purposes.

Briefly FACS-II works as follows. As cells emerge from the starting reservoir they are rapidly passed single file across a small area intensely illuminated by a laser beam; they are then isolated in tiny droplets that are electrostatically charged and deflected to different collecting reservoirs [*see illustration on next page*]. As the cells flash across the laser beam, the optical signals generate electric pulses, which are rapidly compared with preset criteria so that the droplet containing the observed cell can be deflected into the appropriate reservoir.

The techniques we rely on for generating and controlling fluid droplets have their origin in the work of the 19th-century physicist Félix Savart, who showed that a small fluid jet would break up into a procession of droplets having remarkable uniformity and regularity if the nozzle or orifice that formed the jet was vibrated at the proper frequency. Savart's work, published in 1833, stimulated research by other investigators, including Lord Rayleigh, Chichester Bell (Alexander Graham Bell's cousin) and C. V. Boys. In Boys's well-known book on soap bubbles and other surface-tension phenomena there appears a photograph of a water jet made with the light of an electric spark. A musical note, acoustically coupled to the jet nozzle, produced minute bulges in the cylindrical column of fluid as it emerged from the nozzle, and surface-tension forces amplified those regular disturbances until they severed the jet. By deflecting droplets generated in this way with the field produced by a piece of electrified sealing wax Boys showed that processions of identical droplets could be made to follow precise curved trajectories.

Those early studies were limited to jets with a diameter on the order of a millimeter and a repetition rate of less than 1,000 droplets per second. In 1961, after preliminary experiments had verified that much higher drop frequencies could be obtained with smaller jets, that the drops could be independently and precisely charged and that useful deflections could be produced with available electrostatic technology, a project was initiated by one of us (Sweet) at Stanford's Applied Electronics Laboratory to utilize those techniques as the basis of a high-speed recorder that would write with electrostatically deflected droplets of ink. The Stanford project developed techniques for accurately and independently deflecting ink droplets at rates of up to 200,000 per second, a capacity that has since led to the production of high-speed ink-jet computer printers, mail addressers and other printing equipment.

In 1964 the Stanford work on ink-jet recording was noticed by Mack J. Fulwyler and Marvin A. Van Dilla of the Los Alamos Scientific Laboratory. Fulwyler had been studying individual cells with a Coulter counter, an instrument that determines the volume of a cell by flowing an electrically conducting saline solution containing suspended cells through a tiny aperture and measuring the change in the electrical resistance of the fluid in the aperture as each cell or other insulating particle passes through it. Fulwyler successfully combined this counting principle with the Stanford droplet generator in an instrument that initially was capable of sorting cells at rates of up to about 1,000 per second. In describing their apparatus Fulwyler and Van Dilla noted that measurements of the optical properties of cells (such as their fluorescence) might also serve as the basis of a sorting technique.

The present Stanford fluorescence-activated cell sorter is an outgrowth of that work. Cells in liquid suspension are forced under pressure through a micronozzle into the center of a stream of cell-free fluid and then out through an effluent nozzle 50 micrometers in diameter. This design creates a coaxial flow that keeps the cells near the axis of the effluent jet. The nozzle assembly is vibrated axially at an ultrasonic frequency of 40,000 cycles per second, breaking the jet into 40,000 uniform droplets per second.

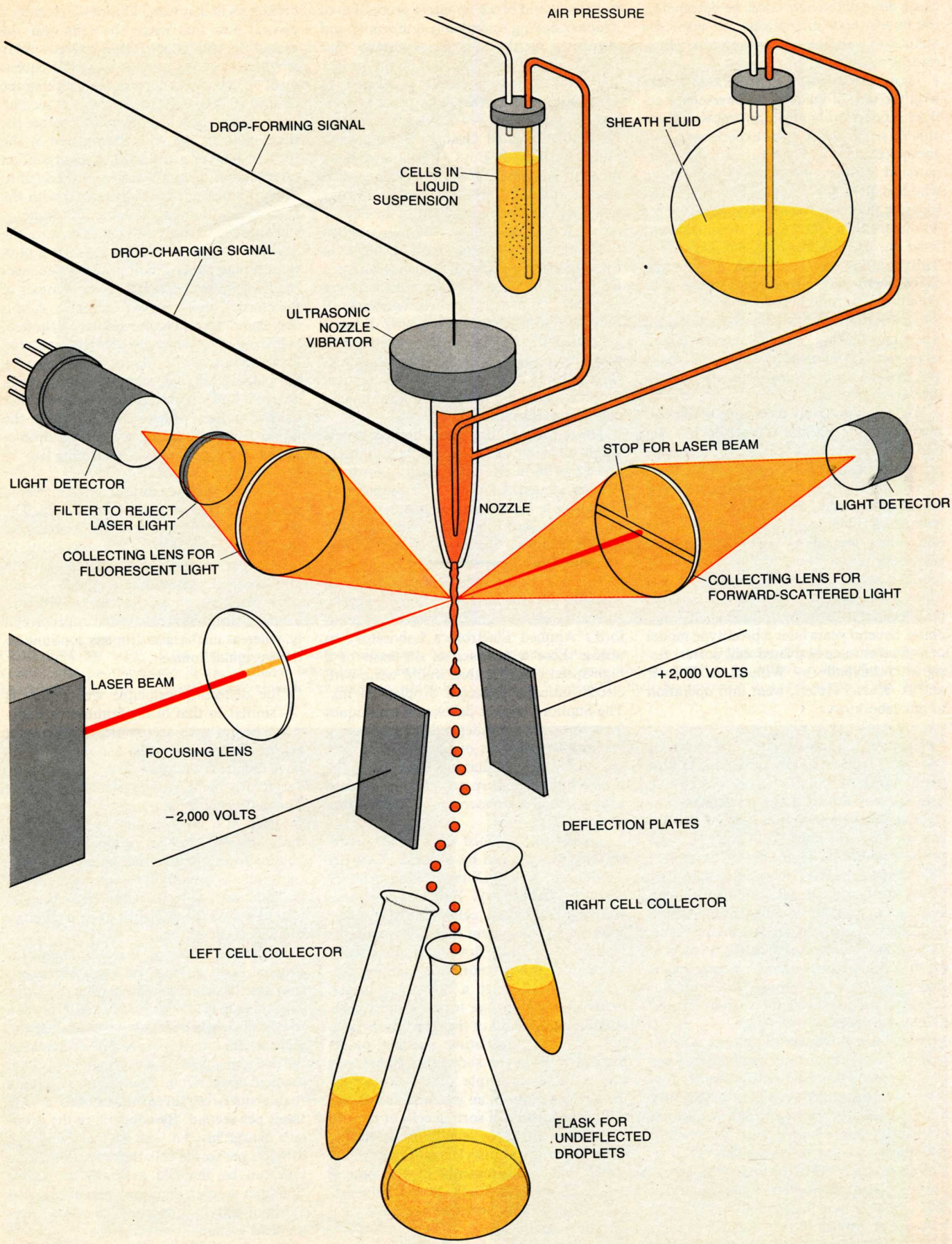
Immediately below the nozzle, before the droplets form, the jet is illuminated by the focused blue or green light of an argon-ion laser, operating at a wavelength selected to excite fluorescence in cells tagged with the appropriate fluorescent material. Some of the fluorescent light, filtered to remove the exciting wavelength, is focused onto a photomultiplier tube, which generates an electrical signal proportional to the number of fluorescent molecules on each cell. A second signal, related to the volume of the cell, is generated by detecting the light scattered forward out of the illuminating beam by the passing cell. The signals are processed, delayed and combined to produce other electric pulses, which serve to charge the liquid stream exactly when the droplet containing a desired cell is forming [*see illustration on page 113*].

Droplets that separate from the stream while it is charged retain their charge. The droplets, still traveling at the velocity of the jet from which they formed (about 10 meters per second), are directed between two charged plates that establish a constant electrostatic field across their path. Charged droplets are deflected appropriately, whereas uncharged droplets continue on their original course.

The deflection principle we employ is similar to that of the familiar cathode-ray tube but with an important difference. In the cathode-ray tube the electrons all have identical charges and are deflected by varying the field through which they pass. The deflecting force on all the electrons in the field at a given instant is in the same direction, and neighboring electrons cannot be directed along different paths. That factor limits the minimum response time of the cathode-ray tube to the comparatively short time required for electrons to pass through the deflection system.

In the cell sorter, on the other hand, the droplets have different charges. Moreover, they also have a much lower ratio of charge to mass than electrons, and hence if they are to be sufficiently deflected, they must spend a longer time (about three milliseconds) in the deflecting field. In a varying-field system the field could not be changed more rapidly than about every three milliseconds, or 333 times per second. By controlling the droplet's charge instead, and by specifying two droplets per sorted cell, the deflection direction can be changed as many as 20,000 times per second, an improvement in speed of about sixtyfold over a high-speed varying-field system.

The electrical signals generated by the two light detectors are processed by comparing the signals with preset amplitude limits that define cells by class. Upper and lower limits for the amplitudes of both signals can be set independently in order to

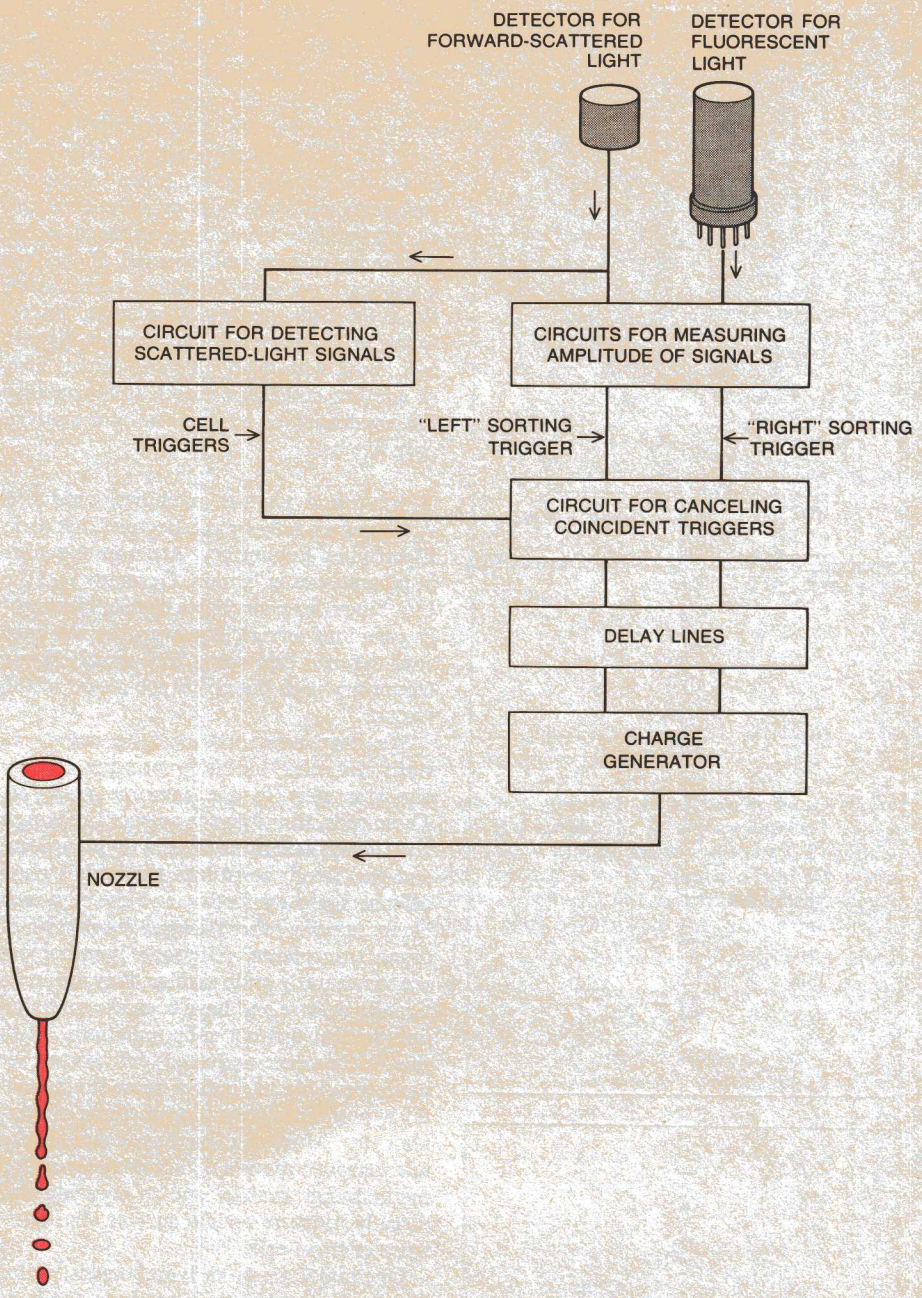


MAIN COMPONENTS of the fluorescence-activated cell-sorting machine are depicted in this schematic diagram. The cells are kept near the axis of the effluent jet by forcing them under pressure through a nozzle into the center of a stream of cell-free sheath fluid. The nozzle assembly is vibrated axially at 40,000 cycles per second, breaking the jet into 40,000 droplets per second. The detected fluorescent light generates an electrical signal that is proportional to the number of fluorescent molecules on each cell. The signal that is generated by detecting the forward-scattered light is related to the volume of the passing cell. The drop-charging signal is triggered by means of an electrical system that processes the signals that are received from two light detectors (*see illustration on opposite page*).

define two cell fractions to be sorted from the input sample.

Each cell that satisfies the criteria for sorting actuates a "left" or "right" sorting trigger. To ensure that only the desired cells are deflected a control device checks for adequate time separation between the cells that actuate sorting triggers and the unwanted cells, and cancels those triggering events that correspond to cells too closely spaced to separate. The triggering events are then delayed until the cell arrives at the point where droplets form. The charge generator is switched on just before the desired cell enters a droplet, charging the entire stream, and is switched off after the droplet separates, marking the droplet with a trapped positive or negative charge, which remains unchanged during the droplet's journey to the collecting tube. To be certain of charging the right droplet, at least two droplets are charged for each cell to be sorted. A third reservoir, the contents of which are usually discarded, collects undeflected droplets (which are empty or contain unwanted cells, debris and cells too closely spaced to separate).

At the typical sorting rate of 5,000 per second (approximately 18 million cells per hour) the fluorescence-activated cell sorter yields separated samples with a purity of between 90 and 99 percent. The viability of the cells is not affected by their passage through the instrument; in fact, the percent of living cells in the input sample can actually be increased by setting the scattered-light threshold to exclude dead cells. The temperature of the input and output reservoirs is controlled, and all components of the instrument that are in contact with cells can be sterilized. The duration of a run, which is determined by the number of separated cells required and the stability of the biological material, often lasts for up to eight hours.



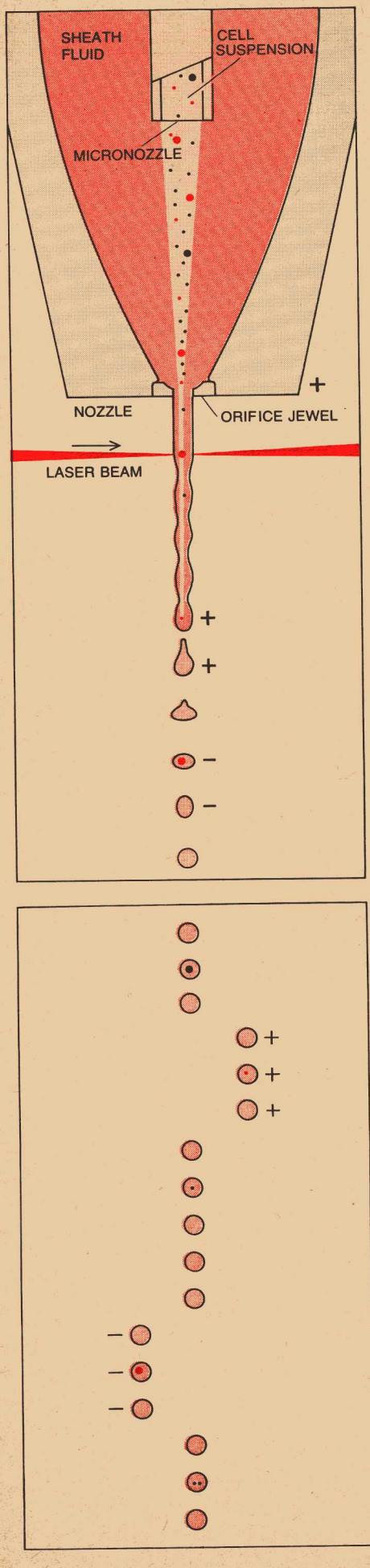
SIGNALS FROM LIGHT DETECTORS are processed and combined to produce electric pulses called sorting triggers, which are used to charge the fluid jet exactly when the droplet containing a desired cell is forming. Preset amplitude limits can be established for both signals in order to define two cell fractions to be sorted from the input sample. Triggering events that correspond to cells too closely spaced to separate are canceled. To make certain of charging the droplet containing the cell, at least two droplets are charged for each cell to be sorted.

The analytical capabilities of the fluorescence-activated cell sorter make it a powerful tool for measuring the distribution of cells in a given sample according to either size or fluorescence. The range for fluorescence detection extends well below the usual detection level in the fluorescence microscope. Cells that differ by as little as 5 percent in fluorescence can be differentiated. Furthermore, by appropriate manipulation of the thresholds it is possible to determine the size of cells characterized by a certain level of fluorescence or the level of the fluorescence of cells within a particular size range. These analyses have been greatly facilitated by coupling the cell sorter to a computer that stores, manipulates, displays and plots the accumulated data.

Of course, analysis and separation go hand in hand. Without the analytical ability of the cell sorter the choice of separation thresholds would be an excursion into the unknown; conversely, without the ability to separate subgroups of cells identified by analysis the correlation of function with the identified characteristics would be extremely difficult if not impossible.

By examining the computer-generated record of the distribution of cells as a function of the amount of fluorescence per cell or the amount of light scattered per cell the investigator can often tell whether the cell suspension under study consists of more than one resolvable population of cells. In addition, because the fluorescence measurements and the forward-scattered light measurements are made simultaneously for each cell, the investigator can selectively determine the fluorescence distribution of those cells that fall above or below a given size threshold. Alternatively he can determine the size distribution of those cells that generate fluorescence signals above or below a given fluorescence threshold. Thus, for example, the existence of a population of "medium-sized bright cells" can be recognized by appropriate manipulations of upper and lower thresholds. Once such a population is recognized it can be separated and studied functionally.

This "gating" technique also makes it possible to identify and remove fluorescent artifacts caused by the presence of odd-sized bits of fluorescent "junk" and dead cells in the suspension. Dead cells, it turns out, scatter less light than live cells, and hence the dead cells can be resolved into a separate peak in the curve representing the forward-scattered light distribution [*see bottom illustration on page 115*]. This finding might be just a curiosity if it were



not for its great practical significance in actual sorting experiments. Dead cells often bind the fluorescein-tagged protein reagents we use to stain the surface structures of cells. Therefore the almost unavoidable presence of a small percentage of dead cells in a suspension increases "noise" in the experiment, and such cells must be eliminated, particularly when the number of viable fluorescent-tagged cells is small. If the forward-scattered light threshold is set above the range associated with dead cells, the cell sorter will ignore all fluorescence signals associated with dead cells and direct them to another collection vessel, thus enabling the experimenter to separate and analyze only live cells.

FACS-I and FACS-II machines, produced commercially by the Becton Dickinson Electronics Laboratory, are now installed in laboratories in Britain, Germany and the U.S. Since those machines have only recently gone into service, however, the work we shall describe below was done mostly in our laboratory with the prototype model of the FACS-I.

We have used our machine mainly to study the roles that different types of lymphocytes play in the immune response. These cells, found in the spleen, the lymph nodes, the bone marrow, the thymus gland and the blood, constitute the body's chief defense against infection and are responsible for, among other things, the rejection of organ transplants. Although lymphocytes are structurally quite similar, they are functionally diverse and have been shown to fall into several distinct subpopulations. With the fluorescence-activated cell sorter we have been able to confirm some fundamental hypotheses about the functions of lymphocytes. Moreover, we have been able to add new information obtainable only with a method that enables one to separate and directly measure certain surface characteristics of these cells.

One major group of lymphocytes, called *B* cells (because of their origin in the bone marrow), includes precursor cells that may be triggered by an invading pathogen (or some other foreign substance) to divide and differentiate, forming cells that release large numbers of antibody molecules, which in turn react with the triggering antigen. The triggering process usually also requires the participation of lymphocytes belonging to another group, called *T* cells (because they originate in the thymus gland).

ENLARGED VIEWS of tip of nozzle (*top*) and region downstream near deflection plates (*bottom*) reveal how cells can be sorted into two classes depending on their size and their fluorescence. At instant shown in top drawing nozzle and liquid stream have positive charge, since "small bright cell" in droplet that is about to break off is to be sorted into "right" fraction. The undeflected droplets are either empty or contain unwanted cells, debris or cells too closely spaced to separate. Relative differences in cell sizes have been exaggerated in these drawings. The diameter of a cell is about a tenth of the diameter of a droplet.

In a typical immune response to an antigen an animal will produce a large number of different kinds of structurally similar antibody molecules, which differ only in the amino acid sequence of a small part of the antibody molecule. These structurally unique regions constitute the combining sites that make antibodies capable of reacting specifically with the triggering antigen. In general the antibody is highly specific for the antigen and will combine poorly if at all with other molecular structures. Similar antigenic structures do "cross-react" with antibodies elicited by one or the other structure, but even a very small modification of an antigen, such as the substitution of a single amino acid or a single sugar in a large protein or carbohydrate molecule, can be distinguished by antibody populations.

The potential for responding to antigens by producing antibody molecules with a wide range of combining-site structures has been traced to the precursor *B*-cell population. Each mature antibody-producing cell gives rise to only a single species of antibody molecule with a unique combining site that is reactive with the stimulating antigen. Therefore the interaction of the antigen and the precursor cells must be responsible for determining what antibody molecules will be produced.

According to current theory, that decision results from the selective triggering of precursor *B* cells, each of which is already committed to the production of a unique combining site. The combining-site structure, incorporated in an antibody molecule, is manufactured in small amounts by the committed precursor and is exposed on the surface membrane of the cell. When the appropriate antigen comes in contact with the surface antibody, it is firmly bound, and the precursor cell is triggered (with the help of a cooperating *T* cell) to divide and differentiate.

The combining site is at one end of the antibody molecule; the other end defines a group of physiologically important characteristics of the antibody. During differentiation the progeny of a given *B* cell remain committed to the production of the combining-site end of the molecule found on the precursor cell, but they may switch the other end to one of several different structures.

The concept that antibody production results from the combination of an antigen with a specific combining site on a precursor cell was first postulated by Paul Ehrlich around the turn of the century. Succeeding generations of immunologists have refined and modified the concept to include the commitment of the precursor cell to a unique specificity that reflects the structure of the combining-site end of the molecule and the selective triggering by the antigen. By isolating specific precursor cells with the fluorescence-activated cell sorter we have now been able to obtain direct evidence supporting the predictions of this commitment theory.

Before we did this work a number of laboratories had established that precursor cells with a given kind of surface-antibody specificity could be depleted from lymphoid-cell populations by allowing the cells to react with the appropriate target antigen. Since the precursor cells could not be recovered and tested directly in these studies, however, we decided to attack the problem with the FACS-I.

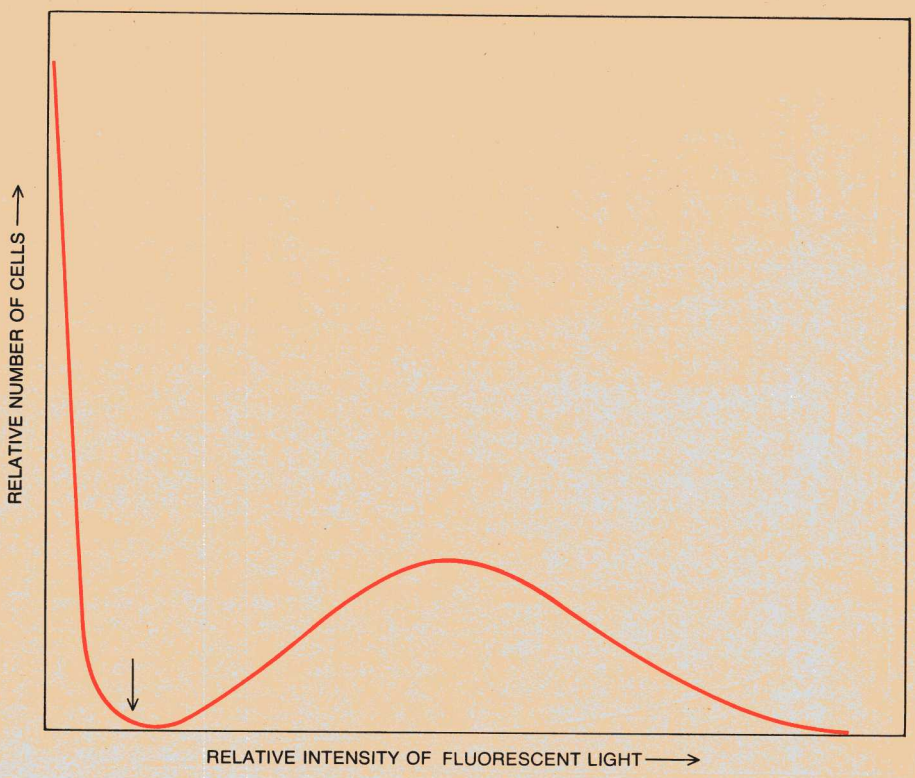
Michael Julius and Tohru Masuda, then working in our laboratory, isolated a subpopulation of mouse-spleen cells that bound a given antigen. They showed that this population of cells was highly enriched for the precursors of cells that produced antibody reactive with the antigen.

For the experiment the particular antigen chosen (a hemocyanin obtained from the keyhole limpet, a marine gastropod) was tagged with fluorescein and incubated with the cells. The cells were then washed free of excess antigen and passed through the cell sorter to separate those cells that bound the hemocyanin from those that did not. The two resulting cell fractions, labeled fluorescence-positive and fluorescence-negative, were injected into mice along with the appropriate cooperative *T* cells and fresh unlabeled antigen to allow the precursor cells to mature to form antibody-producing cells. An unrelated antigen was also injected into some of the mice to provide a control for testing the specificity of the separated precursor cells.

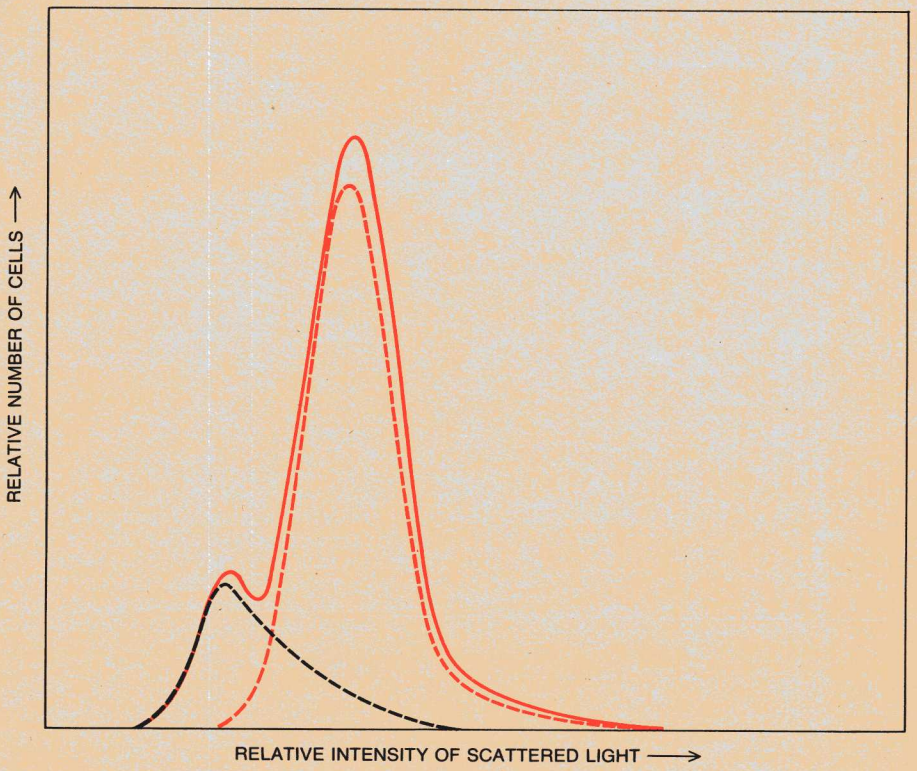
The results showed that the mouse recipients of the hemocyanin-binding, or fluorescent, cells manufactured antibody reactive with hemocyanin, whereas the recipients of the nonfluorescent cells did not. The latter mice, however, did produce antibody to the unrelated antigen, whereas the fluorescent cells could not. Thus the specificity of the antigen-combining site produced by the precursor cell determined the specificity of the progeny's antibody-producing cells.

Another study supporting the commitment hypothesis was done as a joint project involving our laboratory and workers in the laboratory of John J. Cebra at Johns Hopkins University. Working with genetically determined structural variations found in rabbit antibodies near the part of the molecule that incorporates the combining site, Patricia Jones of Johns Hopkins discovered a strict correlation between the antibody type of the precursor cell and that of the producer cell. The structural differences she worked with, called allotypes, are easily detectable by antibodies that react specifically with the allotypic structure of the antibody molecule.

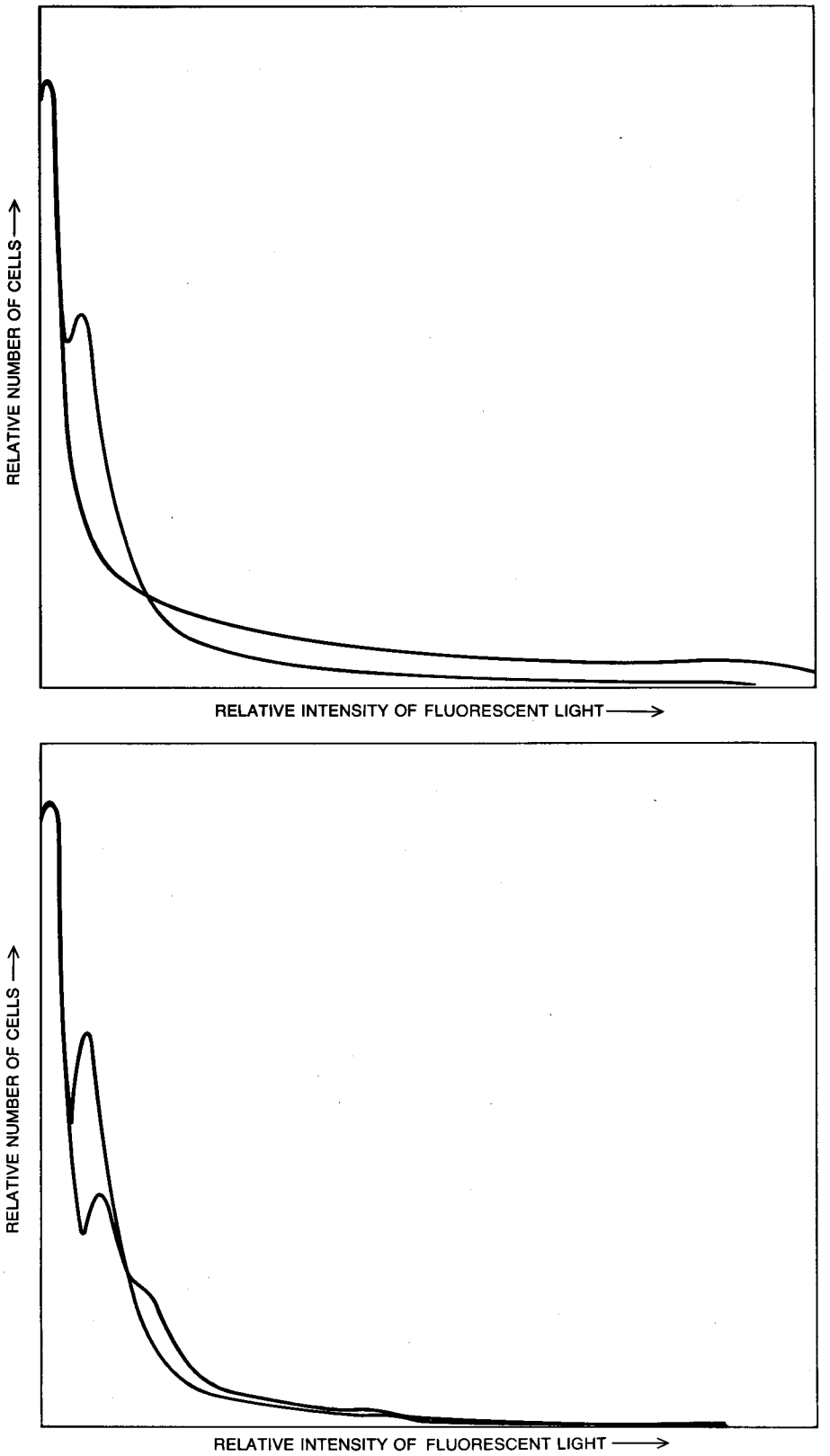
In studies of this kind the separation of lymphocytes was based on minute qualitative differences in the structure of surface-membrane components. The fluorescence-activated cell sorter has also been brought into play to separate cells where quantitative differences in the amount of surface antigens distinguish the subpopulations. A good example of that ability is the definition of thymus-derived-lymphocyte (*T*-cell) sub-



ONE WAY to distinguish live cells from dead cells in a given cell population by means of the fluorescence-activated cell sorter is represented by this graph. The data were obtained from a study in which a sample of human spleen cells was treated with fluorescein diacetate, a nonfluorescent substance that is converted to fluorescent fluorescein after entering a cell. Since live cells retain fluorescein but dead cells do not, the two fractions can be separated by adjusting a threshold setting (*arrow*) that divides the "bright" cells (*right*) from the "dark" ones (*left*).



ANOTHER WAY to distinguish live cells from dead cells is illustrated here for the same fluorescein-diacetate-stained spleen-cell suspension. The solid colored curve shows the distribution of all the cells in the sample according to how much light they scatter forward out of the laser beam. The broken colored curve shows the "fluorescence-gated" scattered-light distribution, which is composed only of fluorescent, or live, cells. The broken black curve shows the scattered-light distribution for nonfluorescent, or dead, cells. Obviously dead cells scatter less light and can be resolved into a separate peak in the overall scattered-light distribution curve.



NEW ANTISERUM found by workers at the Roswell Park Memorial Institute in Buffalo to contain antibodies that are reactive with antigens on the surface of certain cells in the thymus gland and spleen of mice was analyzed by Robert Stout at Stanford, using the FACS-I. He was able to show that the antiserum actually contained antibodies reactive with two independent cell-surface antigens, one of which (called *ThB*) was present on two cell types (spleen cells and thymus cells), whereas the other (called *ML2*) was present on only one of the two types (spleen cells). The FACS-I compared the amount of fluorescence on spleen cells incubated either with the intact serum, containing both anti-*ThB* and anti-*ML2* antibody (*black curves*), or incubated with a specific anti-*ML2* reagent prepared by absorbing the intact serum with thymus cells that remove the anti-*ThB* antibody (*colored curves*). The findings, which are charted here for three-week-old animals (*top graph*) and three-month-old animals (*bottom graph*), showed that the amount of *ThB* found on the spleen cells decreased progressively as the animal aged.

populations. In experiments exploiting the fluorescence-activated cell sorter workers in our laboratory have developed major evidence for the sequence of *T*-cell development in the thymus gland and for the identification of *T*-cell subgroups in the spleen and lymph nodes.

So far we have discussed the fluorescent-antibody preparations used to detect the various cell-surface antigens as if immunized animals normally make antibody only against the desired determinant structure. Actually antiserums prepared against such antigens frequently contain several populations of antibodies, each of which reacts with an independent antigenic structure. Therefore each antiserum employed was carefully examined for irrelevant antibodies and if necessary was purified so that the final reagent was specific for the antigen under study.

Occasionally, in spite of the multitude of tricks developed by immunologists over the years, important antiserums defy resolution into specific antibody populations and must be regarded as mixed reagents. We shall discuss one such case, where the FACS-I, because of its ability to detect the amount of fluorescent antibody bound to cells, was able to measure quantitative and qualitative differences in surface antigens of lymphocyte populations during development even though the antiserum detecting the antigens was only partially resolved.

Robert Stout, working in our laboratory, used the FACS-I to study an interesting antiserum obtained from M. Yutoku, Allan L. Grossberg and David Pressman of the Roswell Park Memorial Institute in Buffalo. The Roswell Park workers had shown that the antiserum in question contained antibodies reactive with a new type of antigen present on the surface of about half of the lymphocytes in the thymus gland (thymocytes) and also on some spleen cells in mice. Stout confirmed the original observation and went on to show that the antiserum contained antibodies reactive with two independent surface antigens, one of which (called *ThB*) was present on thymocytes and *B* cells, whereas the other (called *ML2*) was present only on *B* cells. (Neither antigen was present on peripheral *T* cells.) Furthermore, he showed that the amount of *ThB* found on *B* cells decreased progressively as the animal aged.

In order to demonstrate the developmental changes, Stout compared the amount of fluorescence on spleen cells incubated either with intact serum, containing anti-*ThB* and anti-*ML2*, or incubated with a specific anti-*ML2* reagent prepared by absorbing the intact serum with *T* cells that remove the anti-*ThB* antibody. The findings showed that spleen cells from three-week-old animals and three-month-old animals bind about the same amount of fluorescent antibody when they are incubated with the specific anti-*ML2* serum. Incubation of cells with the intact serum, containing antibody to both *ThB* and *ML2*, was found to in-

crease the amount of fluorescence per cell, indicating the presence of *ThB* and *ML2* on the same cells. The increase, however, is much greater with cells taken from the three-week-old animals. The broad, flat distribution of fluorescence seen with these cells suggests that they carry variable amounts of *ThB*, sometimes up to five times the amount present on cells from older animals [*see illustration on opposite page*]. With this method of analysis we are now examining the developmental patterns for cells carrying these antigens.

The applications of our technique that we have cited have been chosen to give some idea of the kinds of problem a fluorescence-activated cell sorter can be expected to solve. Similar investigations are in progress in a number of laboratories on a wide range of cell types, including normal and leukemic blood cells, cells in the nervous system, cultured normal cells and cultured tumor cells.

Recent work on human leukemias done with the FACS shows promise for substantially improving the diagnosis and treatment of this group of diseases. Investigators at University College London and the Harvard Medical School report that with the aid of a newly developed battery of fluorescent reagents they can now quickly identify many of the different types of leukemia from small samples of blood taken from patients. Because FACS analysis requires only a blood sample rather than the costlier and more painful bone-marrow sample currently used to identify leukemia, the new approach may enable the physician both to diagnose the disease more rapidly and to monitor therapy at frequent intervals, watching for the disappearance or reappearance of leukemic cells. It may also lead to the development of new methods for treating specific types of leukemia and even "tailor-made" treatments designed to suit each individual patient.

Fluorescence sorting and analysis is also being used to attack other problems of immediate clinical relevance, such as measuring the DNA content of individual chromosomes in suspension with the aim of developing rapid ways to define the chromosome constitution (karyotype) of cells for genetic analysis and attempting to automate the counting of different types of white blood cells, a very common test in the clinical laboratory. At Stanford we are now working on a safe screening method for prenatal diagnosis of genetic disease based on isolating and testing the small number of fetal cells reportedly present in maternal blood quite early in pregnancy.

As these few examples show, the potential applications of a technique for separating fluorescence-labeled cells range over virtually all biology and medicine. Future directions will be determined by the ingenuity of investigators not only in finding ways to put fluorescent labels on cells but also in finding ways to make use of the separated cells.