Fig. 1. A LASL flow cytometer chamber showing a liquid jet emerging from the bottom of the chamber and breaking into droplets. The chamber is illuminated by blue (488 nm) and red (647 nm) lasers. A piezoelectric crystal vibrates the flow chamber so that the jet breaks up into small uniform droplets that are illuminated by a 3-mm-diameter strobed yellow light. The charging collar silhouetted by the yellow light makes it possible to place a positive or negative electrical charge on a few droplets. An individual cancer cell located within a droplet can be sorted by charging the droplet and passing it through a static electric field. White reflections indicate where the laser beam passes through the flow cell.

Flow Cytometry

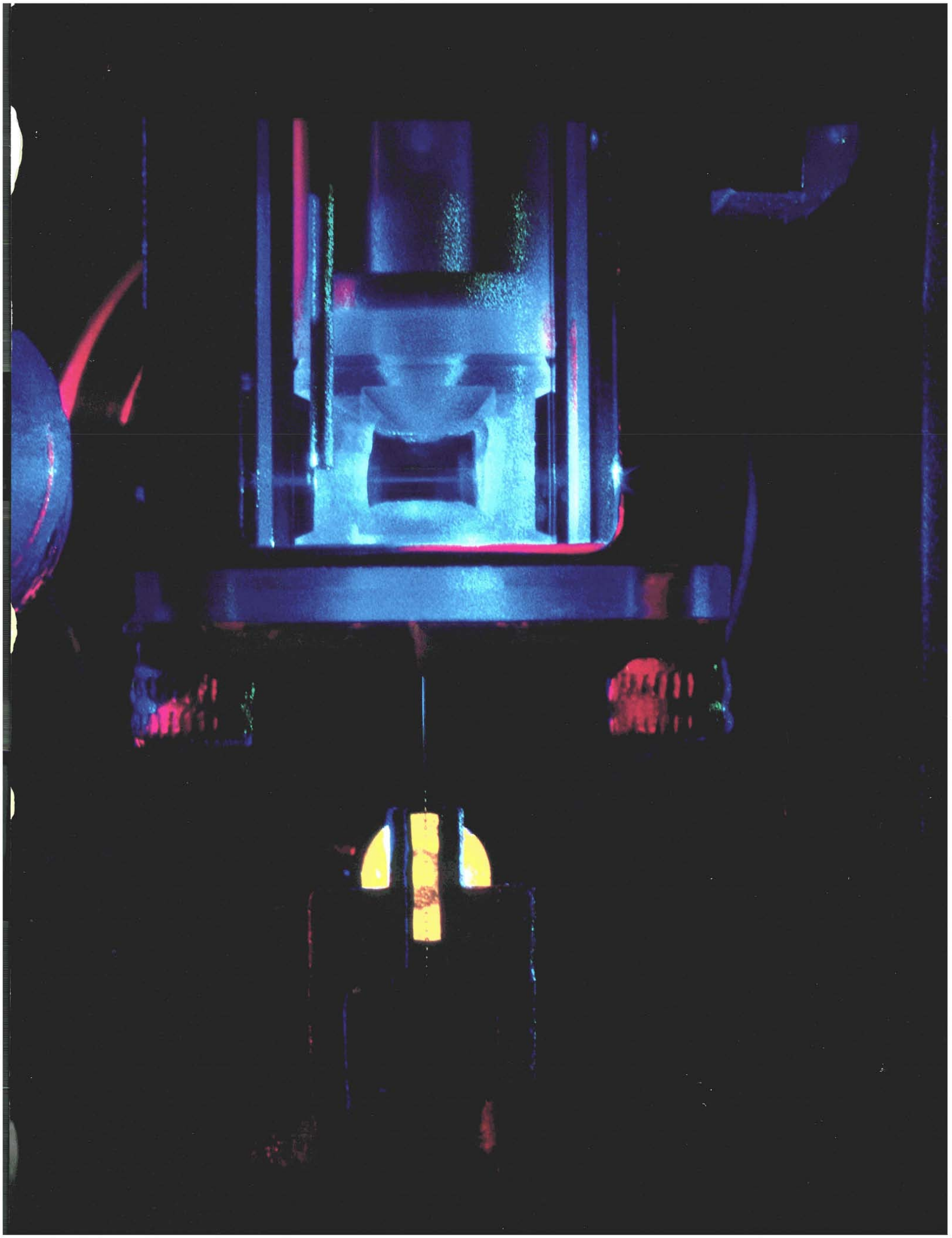
A New Tool for Quantitative Cell Biology

by L. Scott Cram, Dale M. Holm, and Paul F. Mullaney

The intense light of a laser beam illuminates a single cell stained with fluorescent dye. The cell fluoresces and if the signal is large, it indicates the presence of an elevated complement of DNA. Perhaps the cell is malignant. It is sorted automatically into a separate container for later study. In less than a millisecond, another cell passes through the laser beam to be analyzed quickly and effortlessly. The *flow cytometer* can examine and sort these cells as they flow single file through its narrow passageway at an average rate of 3000 cells per second.

This revolutionary tool (Fig. 1) was developed because biologists needed to analyze and sort individual cells according to specific characteristics.

The flow cytometer allows us to measure cellular properties and the dynamics of changes in those properties accurately in large populations of cells. At present, we can measure cell size, DNA content, the presence of specific antibodies, the permeability of cell membranes to particular molecules, the migration of specific receptors on a cell surface, certain chemical reaction rates within cells, and the shapes and sizes of individual chromosomes—and the list grows longer every year. We can detect rare events occurring at frequencies of 1 in 1000 or 1 in 10,000 cells and can determine small differences in cell size, DNA content, or other properties among different sample populations of cells. Moreover, the sophisticated instrumentation of flow cytometry allows all these measurements to be made with great precision and high statistical accuracy. Most of these applications depend on tagging specific biological molecules in a cell with a fluorescent dye and measuring the fluorescence signal generated as the cell passes through the flow cytometer. A combination of hydrodynamic, optical, and electronic design ensures that cells are measured one at a time. The cells are illuminated uniformly by the laser beam so that the intensity and duration of fluorescence signals reflect the concentration and, in some instruments, the location of stained



molecules within the cell.

Just as molecular biologists must be able to isolate and purify different biochemicals from the complex mixtures collected from disrupted cells, cell biologists must be able to obtain pure populations of cells from a heterogenous tissue or organ. Current techniques for separating viable cells include electrophoresis, centrifugation, and flow sorting. The first two are bulk isolation techniques. The third, when coupled to flow cytometry, can sort individual cells based on the variables measured on a particular cell. Thus flow sorting (Fig. 2) is a more precise method of separating closely related but functionally distinct cell types than either electrophoresis or centrifugation.

Flow cytometers were developed in the 1960s at Los Alamos Scientific Laboratory (LASL) and independently by Göhde in Germany. Although the first instruments lacked resolution, scientists soon recognized their potential for monitoring the growth pattern of cells, the transformation of cells from normal to malignant, and the function of the immune system. Several groups pioneered the early development of flow cytometry and its application to major problems in biomedicine. This extraordinary technique has been applied to problems in cancer diagnosis and treatment and to studies of basic cellular processes in normal and abnormal cells. Among early expectations was the possibility that this technique could be used for automated cancer detection and thus perhaps for mass cancer screening. This possibility still exists, but we must find new measurement variables that more clearly differentiate normal from malignant cells before it can be realized.

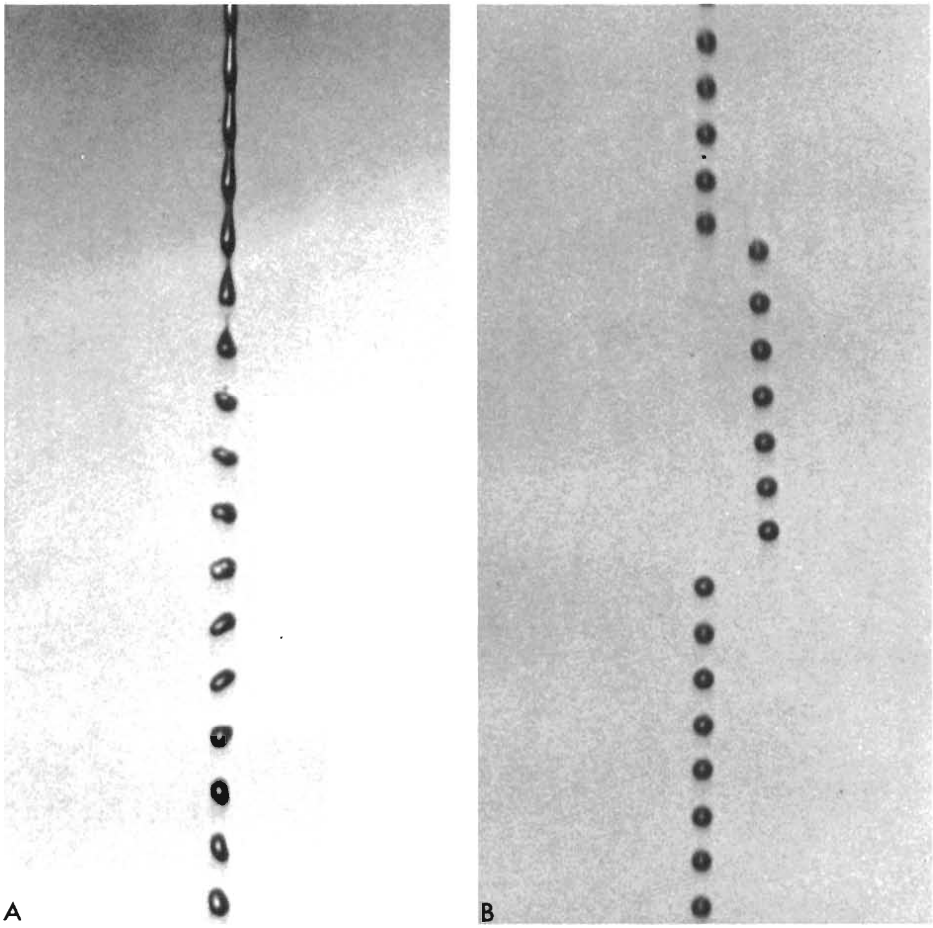


Fig. 2. A stroboscopic light appears to stop the movement of droplets as they jet out of the flow cytometer chamber. (A) Experimenters can program the flow cytometer electronics to separate a particular biological cell from the rest of the sample by charging a group of droplets as they break off from the solid stream of electrically conducting fluid. An electric field separates the charged droplets containing the cell of interest. (B) A group of 7 droplets is separated from the main stream. Since the average concentration of cells is about 1 cell per 50 drops, only one cell will normally be in the 7 droplets.

Early Staining and Measurement Techniques

The ability to stain DNA and other specific biochemical constituents of cells, the cornerstone of flow cytometry, dates back more than 50 years to the work of Feulgen and Rossenbeck, who developed chemical procedures that allowed stoichiometric staining of DNA, the genetic material in cells. For the first time, the presence of DNA and its localization in the cell nucleus could be seen through a microscope. The procedure, called the Feulgen reaction, has been used widely to locate DNA in its various configurations including its condensation into chromosomes. A modification of the Feulgen reaction with fluorescent staining, developed in the 1960s, still serves as a standard for other staining procedures.

The first attempt to determine the quantity of DNA in a cell nucleus by optical means was made by Casperson in 1936. He developed a microscope-photometer to measure the amount of light absorbed by DNA. More absorption corresponded to more DNA. The correspondence was not exact, but it was useful nevertheless.

In the 1950s, Barder, Atkins, Mellors, Tolles, and others, using the early microscopic techniques, observed that elevated DNA levels are characteristic of cells derived from a large number and variety of human tumors. Thus the detection of malignant cells and, hence, clinical diagnosis might be based on the recognition of populations of cells with abnormal DNA distributions. However, microscope techniques are very slow and painstaking. Atkins, a prodigious worker in this field, spent years gathering data that can be acquired in minutes with flow cytometry.

The Beginning of Flow Cytometry

When biophysicists at LASL developed the first flow cytometer, they were studying the effects of radiation on cells. The biophysics group had been concerned with monitoring the effects of radiation on whole organisms. How large a dose, they were asking, is required to affect life span measurably or to change tumor incidence? In 1965, the Atomic Energy Commission changed the direction of the biology program to the cellular level. At that time, the primary analytical tool available at LASL for monitoring cells was the Coulter counter, an electronic device that counts cells by measuring changes in electrical resistance. To use the Coulter counter, cells, immersed in a conducting medium, are passed through an insulating orifice. Because biological cells are quite good insulators, they decrease the conductivity across the orifice as they pass through it. The Coulter counter converts the decrease in conductivity to a voltage pulse for counting the number of cells per unit volume.

At that time, physicists who had transferred from nuclear reactor work to biophysics were using the techniques of gamma-ray spectroscopy to detect the presence and character of radioactive materials in humans and animals by counting and measuring the number of gamma rays emitted from an organism. By adapting the techniques of pulse-height analysis to the analysis of voltage pulses from the Coulter counter, they converted the Coulter counter into a device that quantitated cell volume. Now, volume distributions of large populations of cells could be measured. The desire to examine the cells corresponding to a specific volume led to another important development, the design by Fulwyler of the automatic *cell sorter.* The group used this device to sort individual cells with a specific volume into a separate container.

The volume-sorting instrument soon was applied to monitoring the life cycle of multiplying cells. LASL scientists considered cell volume a useful parameter to measure because a cell's DNA content doubles during the life cycle to insure proper transfer of genetic information when the cell divides, and an increase in cell volume must accompany the increase in DNA content. Detailed studies led to the conclusion that cell volume is not a unique marker to differentiate cells at different stages of the life cycle. Fortunately, another parameter, DNA content, is unique. In 1966-67 Mullaney and Van Dilla constructed the progenitor of the LASL flow cytometers, an instrument that measures the fluorescence of a single cell as it passes through a laser beam. This device allows us to measure the DNA content of each cell in a population, if the cells have been stained with a dye chemically specific for DNA. The measurement allows us to follow the normal growth of cells or their abnormal growth caused by a perturbation of the cell's environment or as occurs in diseases.

Almost immediately, the biologists in the group were interested in using the flow cytometer to analyze the life cycle of exponentially growing cell populations by measuring the DNA distribution in cells exposed to various experimental conditions. The National Cancer Institute saw flow cytometry with its high accuracy and precision in measuring large populations of cells as a possible tool for early diagnosis of cancer, when the frequency of malignant cells is very low. Several groups including the LASL scientists, Wheeless and his group at the University of Rochester, Sweet and Bonner at Stanford University, and others improved the instrumentation and pioneered the application of flow cytometry to cancer diagnosis and to broader studies of cancer and the immune system.

How the Instrument Works

All flow cytometers have three basic components: (1) a flow chamber in which cells are aligned for measurement; (2) a system for optical measurements consisting of a light source (usually a laser), beam-shaping and collection optics, and a light-detection device; and (3) electronics for signal acquisition, analysis, and display. The entire instrument is shown in Fig. 3 and details of the flow chamber, the optics, and the electronics are shown in Figs. 4, 5, and 6, respectively.

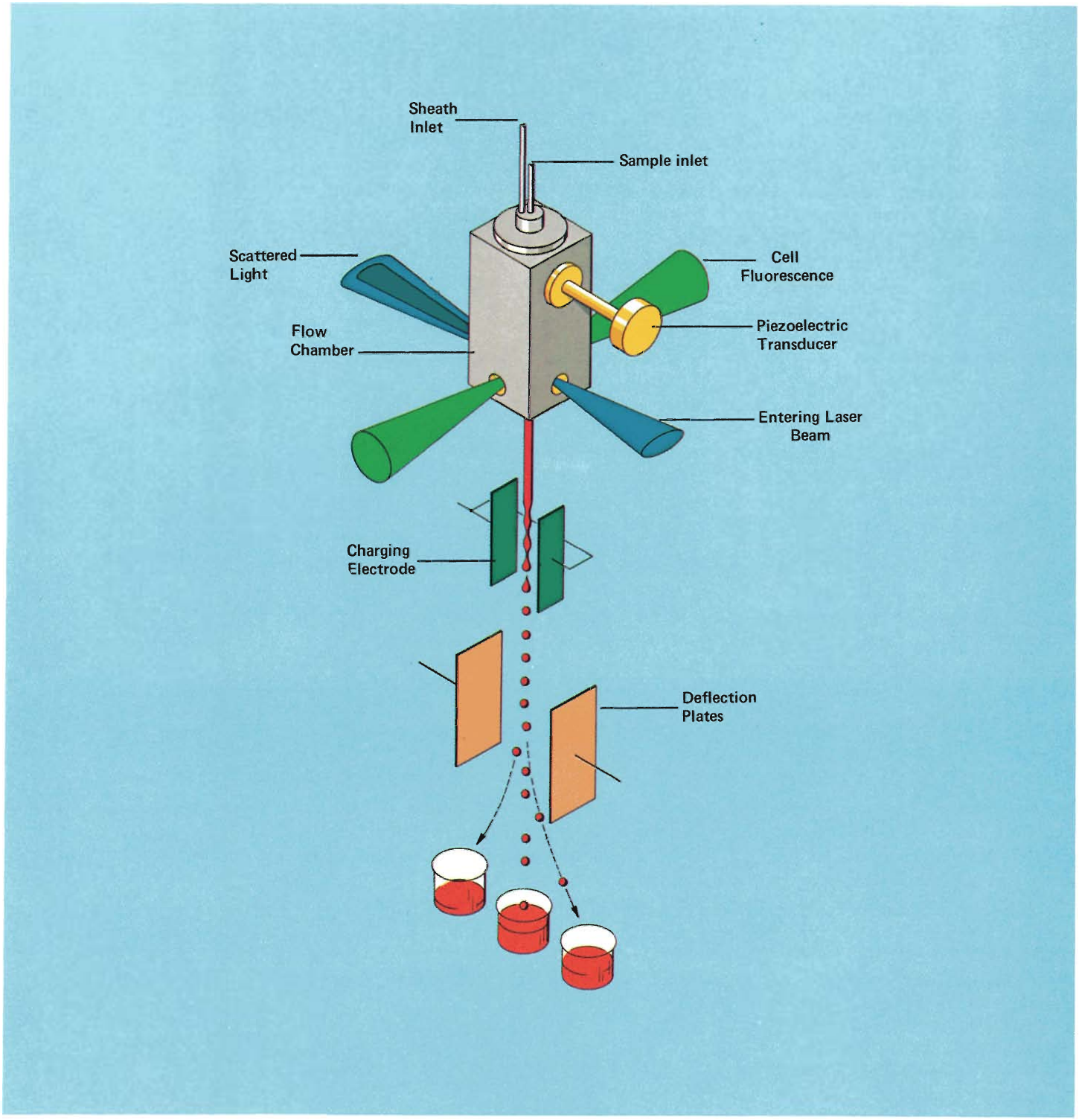


Fig. 3. A schematic diagram illustrates how a flow cytometer works. The sample consisting of cells stained to identify a particular cellular property, such as the amount of DNA, enters at the top. The cells are dispersed into a single-cell suspension in the conducting medium (normally a saline solution). An electrically conducting sheath fluid is added at the top to ensure precise sample location in the flow cell (see Fig. 4). The laser beam enters the chamber from the right and is focused into a elliptically shaped slit of light to excite each fluorescently stained cell as it passes through the laser beam. The fluorescent light is analyzed with sophisticated electronics to quantitate the amount of fluorescent dye in each cell. As each cell passes through the laser beam, it also causes a scattering of the laser light that can provide additional information on cellular properties. The piezoelectric transducer is coupled mechanically to the flow chamber and tuned to about 40,000 hertz to vibrate the chamber and break the emerging stream into uniform droplets at a rate of about 40,000 droplets per second. The electrode can be charged rapidly to 75 volts so that droplets can be electrically charged as they break off from the main stream. The charged droplets are deflected by an electrical field supplied to the deflection plates. Thus, a group of droplets can be charged either positively or negatively and separated from the uncharged stream.

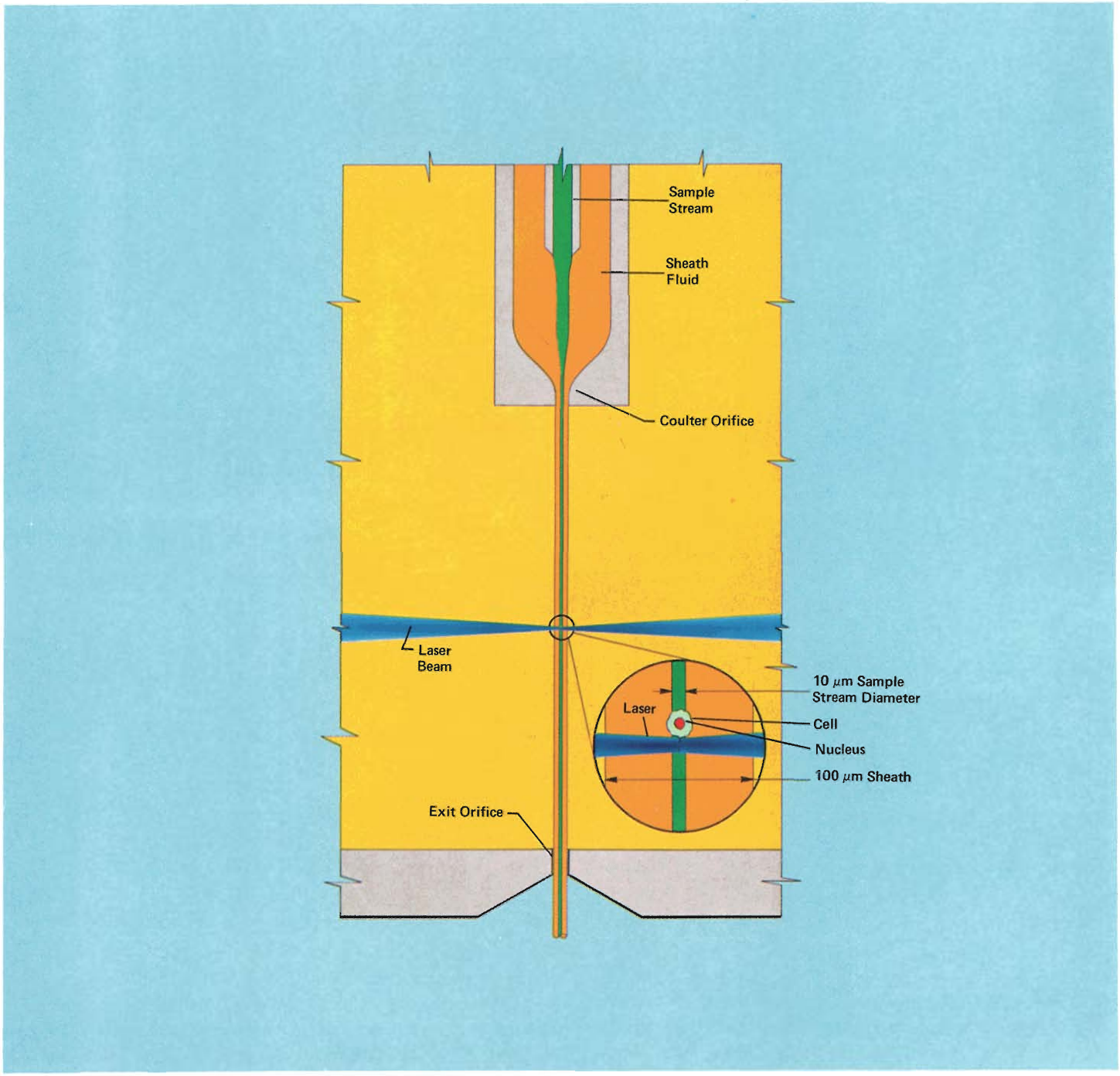


Fig. 4. The hydrodynamic design of the flow chamber is critical because cells must flow single file in a narrow path through the center of the Coulter orifice and then through the focused region of the laser beam. Most chambers currently in use are variations of a flow chamber originally designed by Crosslyn-Taylor to count particles in air. The three fluids are colored in this diagram to show their boundaries and to aid discussion. The sample inlet tube is located concentric with the sheath flow stream to ensure accurate cell stream positioning. The sheath container is shaped with a smooth transition region tapering from a few millimeters down to 100 microns, the size of the Coulter volume orifice. The shape causes a large increase in sample velocity to about 10 meters per second. The dc voltage across the Coulter orifice is applied to the sample inlet tube and to another electrode in the quiescent liquid. Because the sample stream is small, all cells pass through the same electrical field.

As the combined sample stream and sheath flow jet from the Coulter orifice across to the 100-micron-diameter exit orifice, the cells are illuminated by the highly focused laser beam (shown in the blowup of the inner section). With typical flow rates, the sheath volume is more than 100 times the sample stream flow rate, and the average diameter of the sample stream is only 10 microns. Thus, a 15-micron-diameter cell appears as a bulge in the sample stream. Cells are lined up much like beads on a string as they pass through the flow chamber. At a typical cell concentration of about 500,000 cells per milliliter, the average separation between cells is about 2 milliliters, so the likelihood of two cells passing through the laser beam simultaneously is small.

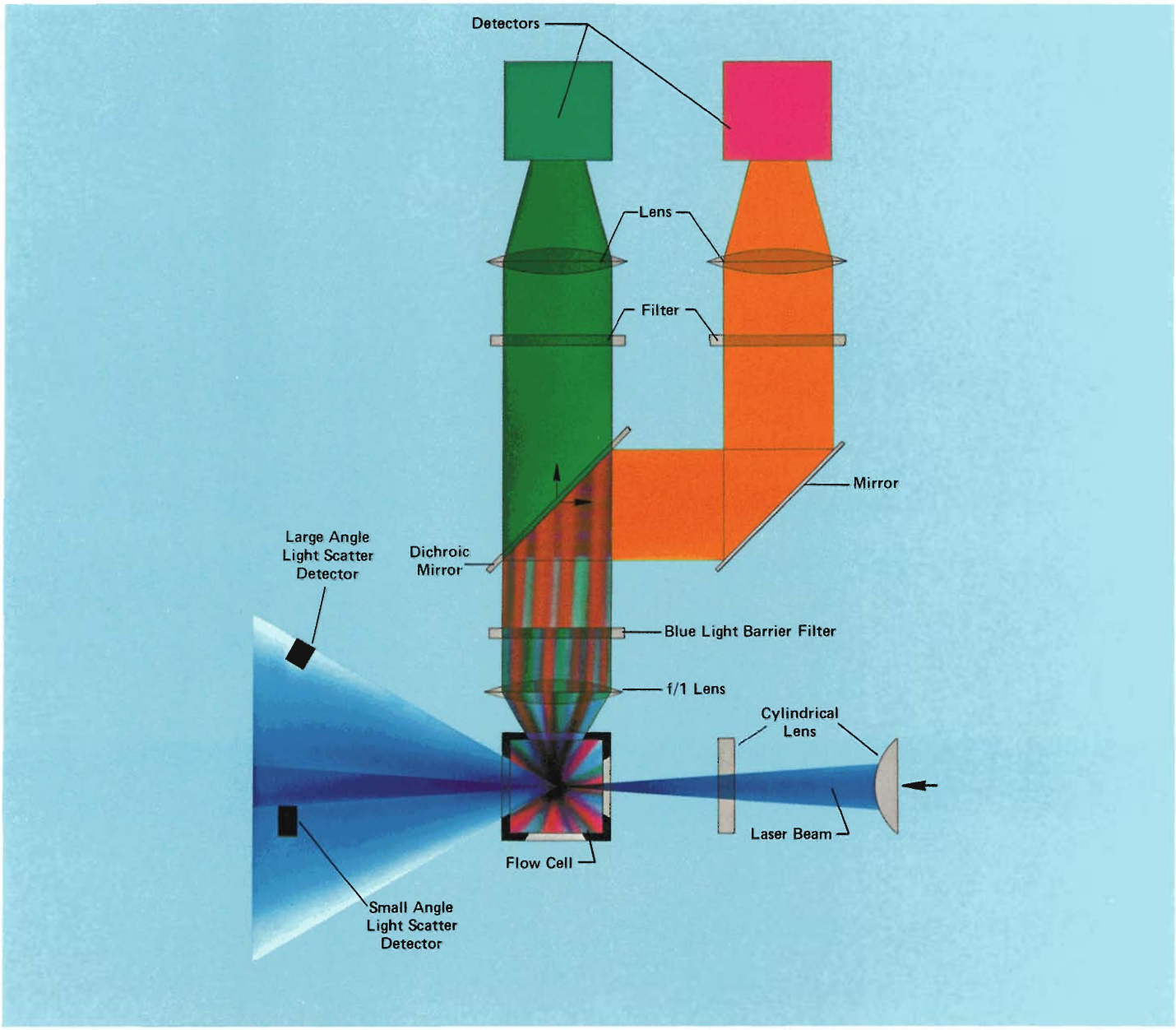


Fig. 5. Flow cytometer optics. Cylindrical lenses of different focal lengths focus the laser beam into an ellipse at the center of the flow cell. As a stained cell passes through the laser beam, a pulse of fluorescent light is generated and a portion of it is collected by f/1 light-collection optics. An interference "barrier filter" prevents scattered laser light from entering the highly efficient photomultiplier detectors. In the model shown, there are two photodetectors to measure green and red fluorescence originating from different parts of the cell. A dichroic mirror reflects the longer wavelength red fluorescence and transmits the shorter wavelength green fluorescence.

Concurrent with the emission of cell fluorescence, the blue laser light is scattered. The scattered light provides a means of obtaining information concerning the structure (morphology) of the scattering object. Since the size, shape, and mass (hence the refractive index) of a cell are the morphological features usually desired, an analysis of the light-scatter pattern produced by a cell may permit cell identification by providing a "signature" related to these physical properties. If light scatter signals from two detectors at different angles are compared, a signal can be generated that will discriminate fluorescent signals from debris and thereby enhance the purity of the signals from cells.

Two general types of laser illuminating light beams have been used. Earlier flow cytometers used spherical lenses to focus the laser beam into a spot of about 75-micron diameter at the cell stream intersection point to give light pulses about 40 microseconds long. The system shown here uses cylindrical lenses with different focal lengths to form an elliptical beam about 5-7 microns across its minor axis and about 100 microns across its major axis. Because the intensity distribution of the laser beam is Gaussian, these dimensions correspond to the points where the intensity of the laser beam is about 1/10th the intensity in the center of the laser beam. Cells traverse this laser beam in about 3-4 microseconds, so standard electronic circuitry for gamma ray spectroscopy can be used.

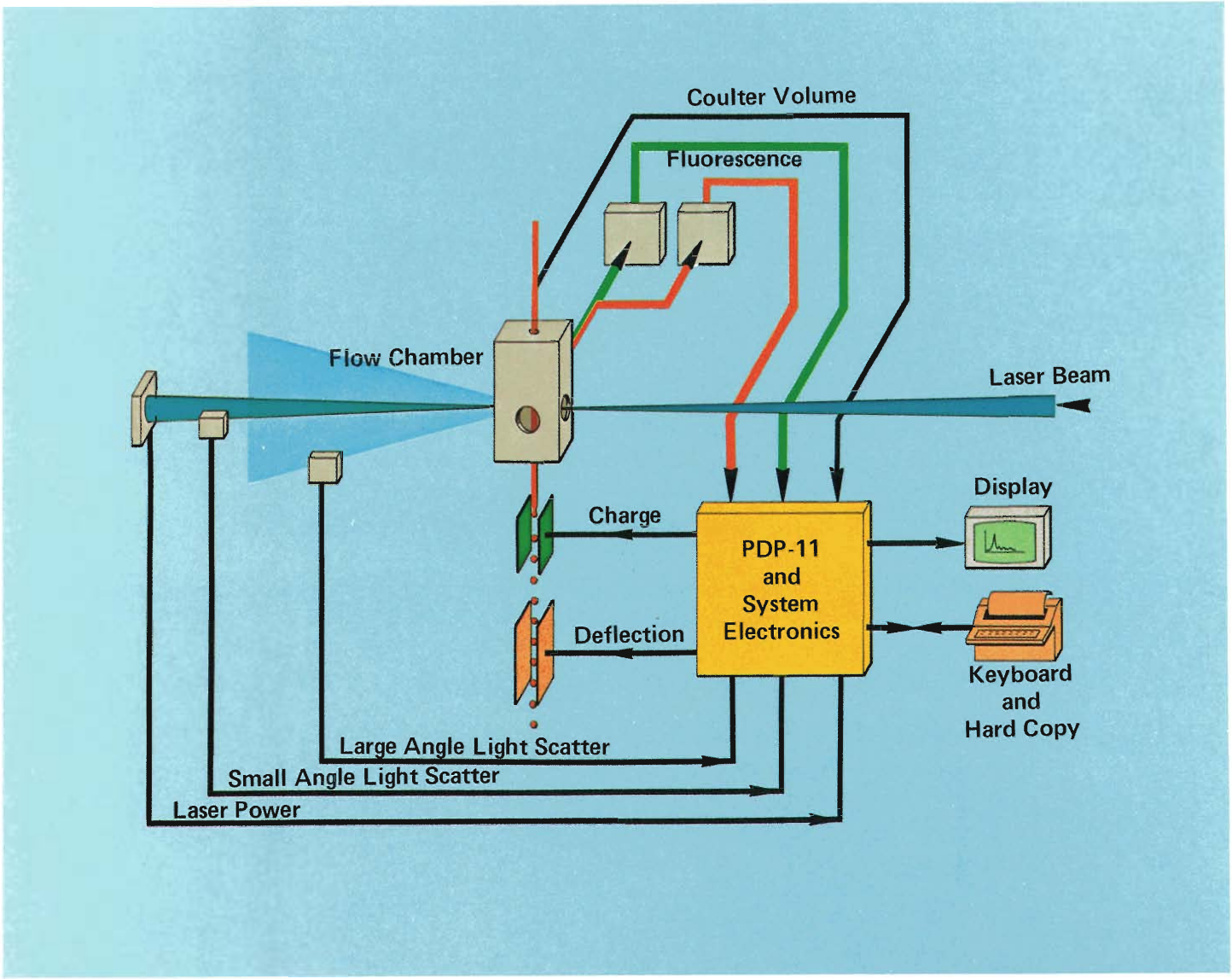


Fig. 6. This diagram represents the various signals that are handled by the electronics and the PDP-11 computer. The time between signals is calibrated and the signals are held so that all appropriate signals from the same cell can be compared and decisions can be made based on their values. This is all done before the cell arrives at the droplet break-off point. An induced charge can be placed on the few droplets most likely to contain the cell of interest, and these can be sorted. With this system purities exceeding 90% have been achieved. The operator interacts with the PDP-11 through a keyboard terminal and a cathode ray display.

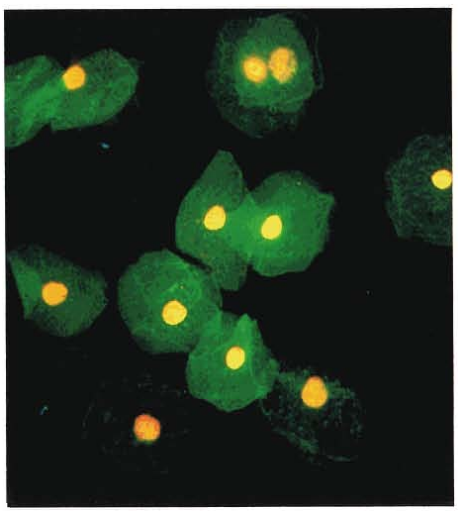


Fig. 7. Normal squamous cells stained with fluorescein isothiocyanate (green) and mithramycin (yellow). The cellular cytoplasm is green and the nucleus is yellow/red. The picture was taken through a fluorescent microscope with dark-field illumination.

Basic Fluorescence Measurements

Most applications of flow cytometry involve measurement of the fluorescence induced in a cell stained with a fluorescent dye, as the cell passes through a laser beam. Analysis of the light pulse determines the concentration and location of the stained biological molecules within the cell.

To illustrate, we consider a cell stained with two fluorescent dyes, a yellow stain specific for the DNA in the cell nucleus and a green stain specific for the protein in the cytoplasm (Fig. 7). Exciting the cells with the 448-nm line of an argon laser generates two fluorescent signals, a red/yellow signal from the nucleus and a green signal from the cytoplasm.

The flow chamber ensures that each cell passes through the center of the laser beam and is illuminated uniformly by the laser light. In most LASL flow

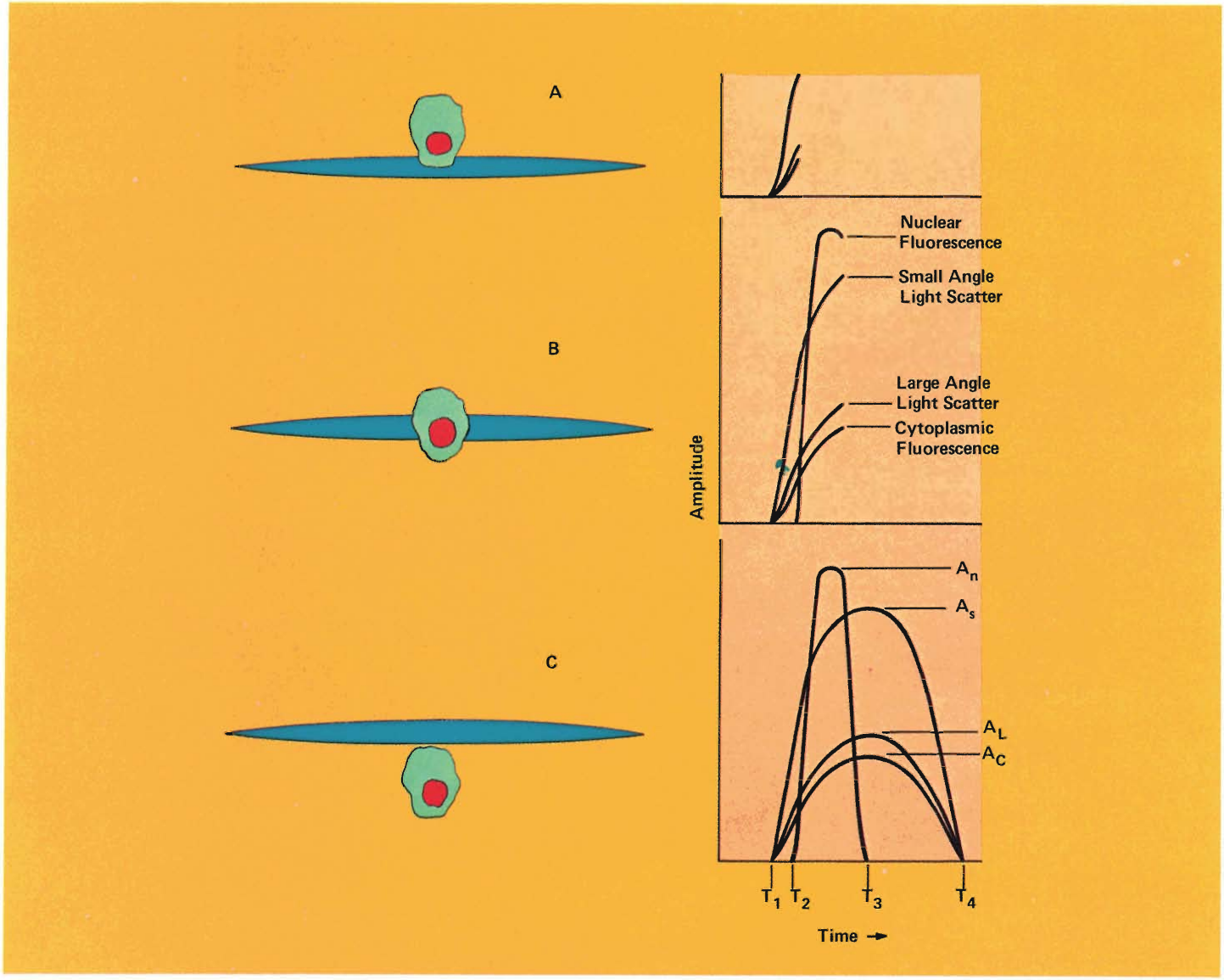


Fig. 8. The origin of electrical pulses that are generated as a two-color stained cell passes through the laser beam is illustrated here. In A, as the cell enters the laser beam, three signals are initiated: small- and large-angle light scatter and the excitation of green cytoplasm fluorescence. In B, the yellow nucleus fluorescence has been excited. In C, the complete pulse shapes from the four detectors can be seen. Several useful signals can be obtained from these voltages pulses. The amplitude of each signal (A_L, A_S, A_C, A_N) gives a measure of the density of the stain within the cell and the amount of light scattered. The length of the pulses above the threshold setting ($T_4 - T_1$ and $T_3 - T_2$) gives a measure of the cell and nuclear diameters, and the area under each curve gives a quantitative measure of the total amount of fluorochrome and the total amount of light scattered. The experimenter can decide which signals are best for analysis. In reality, the light-scatter signals are considerably larger than the fluorescence signals. Adjustment of amplifier gains allows one to display the signals overlayed on the same scale. Nuclear and cytoplasmic diameters can be obtained from the length of the pulses

cytometers the laser beam is shaped to an elliptical cross section at the intersection with the sample stream. The slit of laser excitation light provides a low-resolution scan of each cell as it passes through the laser beam. The fluorescent signals (Fig. 8) are measured at 90° from the laser beam optic axis to minimize the background light in the fluorescent light detectors (that is, to give dark-field illumination). The fluorescence signal intensities are proportional to the amount of yellow and green fluorescent stain and thus to the amount of DNA and protein. The use of interference filters to separate the colors of light into the light detectors produces separate signals. The signals are analyzed individually by a two-dimensional pulse-height analyzer. The integrated intensity of a fluorescent pulse yields the DNA content (or protein content) of the cell, and the duration of the pulse yields the nuclear diameter (or cytoplasmic diameter).

A precise determination of DNA content was not possible with the first flow cytometers because of their poor resolution. For example, all normal, nonreplicating cells should have the same DNA content. However, the DNA distribution for a population of such cells measured with early flow cytometers

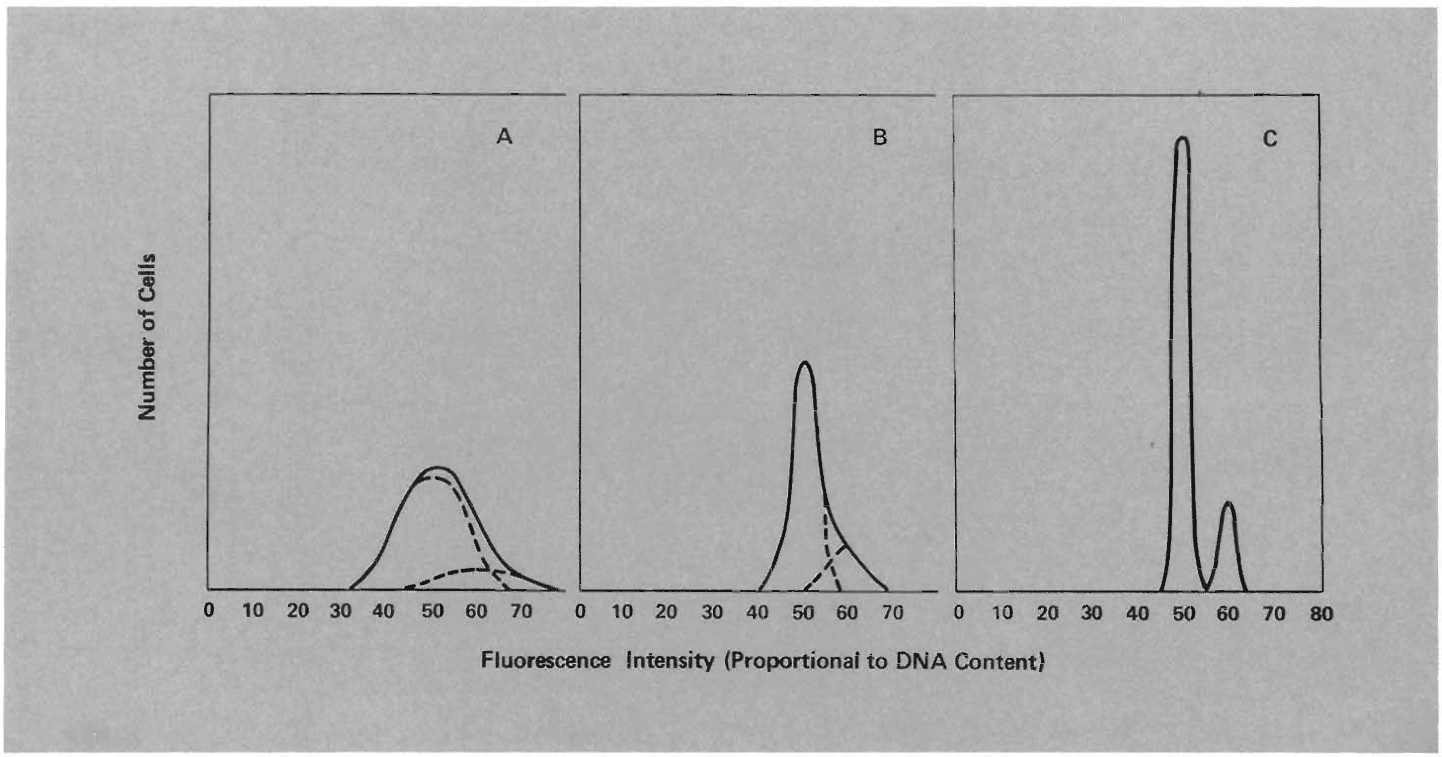


Fig. 9. Pulse-height distributions that might be obtained from two populations of cells stained for DNA content. The two populations are present in a ratio of 5:1 and their pulse-height distributions measured before mixing are shown by dashed and dotted curves. Cells in the less prevalent population have 20% more DNA. The solid lines in A, B, and C represent the pulse-height distribution of the populations mixed together. The distributions in A were measured by a low-resolution flow cytometer (14% cv) and in B by one having twice the resolution (7% cv). The resolution obtained with current instruments, shown in C, was four times the resolution in A. Here the two populations can be resolved completely and measured easily.

had a coefficient of variation (cv) of 14%. A number of possible causes for the poor resolution were examined: nonuniform laser output, low laser beam intensity, low-efficiency photomultipliers, noisy electronics, poor staining procedures, and low-brightness dyes. We concluded that more powerful and stable lasers, brighter dyes, and better staining techniques were needed. With these improvements we routinely obtained a 3-4% cv, and by using two DNA fluorochromes of differing specificity we obtained 1.5-3% cv.

The curves in Fig. 9, representing histograms obtained on a pulse-height analyzer, illustrate how instrument resolution influences data quality and the ability to see small differences in the DNA content of mixed cell populations. The histograms show the number of cells vs DNA content per cell.

The motivation for improving instrument resolution came from the desire to monitor the growth of replicating cells and from the need to produce safe human vaccines from cell lines maintained in the laboratory. At the time, it was proposed that only those mammalian cells that faithfully maintain their normal numbers of chromosomes should be used for vaccine production. By assumption, any increase in the number of chromosomes would be reflected by a corresponding increase in DNA content. Consequently, if flow cytometers could measure DNA content more accurately, they could detect abnormal cells with increased DNA content and thus monitor the early stages of chromosome instability. We improved the resolution of our instruments for this purpose and found that a change in the number of chromosomes *does not necessarily* correspond to a change in DNA content. This highly significant result indicates that in some instances a cell can conserve its DNA while repackaging it into a different number of chromosomes.

The need to distinguish cell doublets (two cells stuck together) from a single

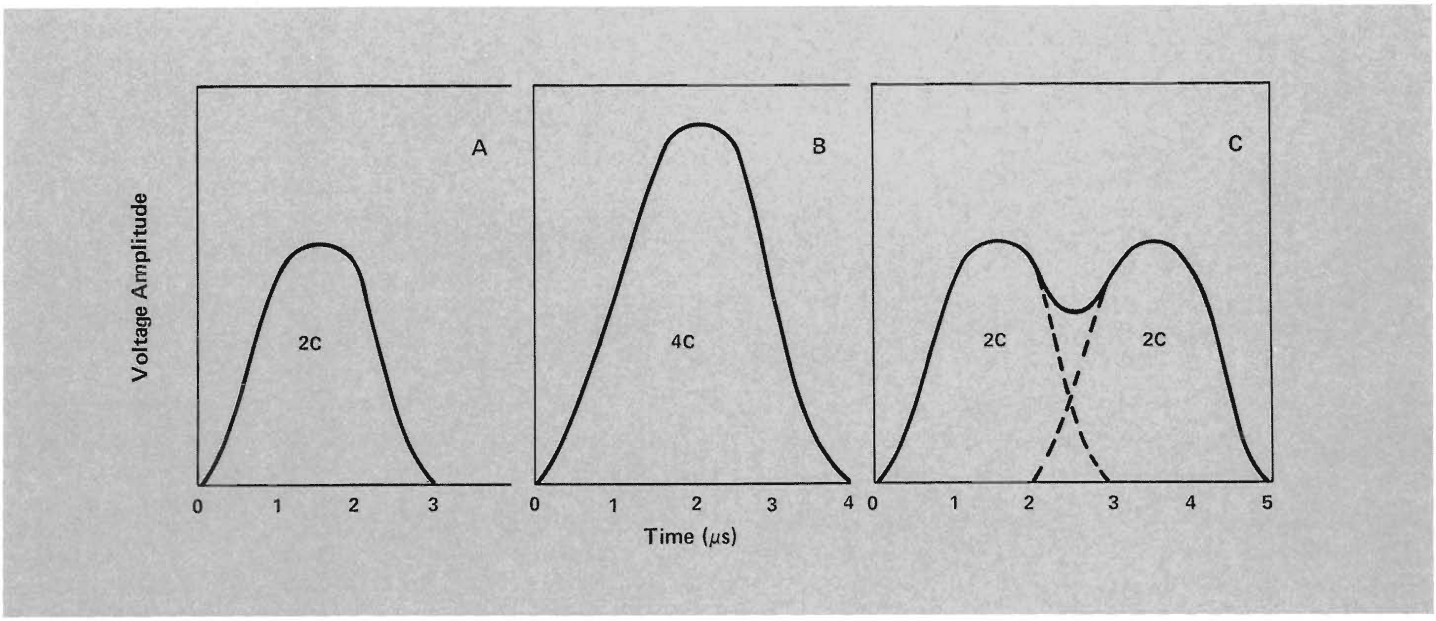


Fig. 10. Pulse shape profiles. A. Pulse from a nucleus of a single cell (2c DNA content) as it passed through a laser beam that is small (7 microns) compared to the diameter of the nucleus. By definition, 1c is the DNA content of a germ cell. B. Pulse from the nucleus of a single cell having twice the amount of DNA (4c). C. Pulse from two cells (each having 2c DNA content) that are stuck together. Since the electronics normally integrates the area of the pulses to get the total amount of fluorescent light, both the one cell in B and the two cells in C would be recorded as a single cell with twice the normal amount of DNA. In reality, the profile in C arises from two cells. The narrow width of the laser beam causes the pulses from the two cells to overlap (dashed part of curve C) to produce a saddle in a single pulse. Electronic circuitry has been designed to detect the saddle between the two peaks so that pulses of this shape can be discriminated from pulses of equal area such as shown in B. Thus, a single cell having twice the amount of DNA can be distinguished from a doublet.

cell that has doubled its DNA content before cell division or contains double the normal amount of DNA led to another important improvement in instrument resolution. The original spherical optics that produced an illumination area much larger than a single cell were changed to optics that shaped the laser beam into an elliptical slit (as described above) much smaller than the cell nucleus. Figure 10 illustrates the difference in pulse shape obtained using a 7-micron laser beam for a single cell with double the DNA content of a nonproliferating cell and two nonproliferating cells stuck together. Electronics were incorporated to discriminate between the two pulse shapes.

The ability of flow cytometers to measure DNA content with high statistical accuracy has been crucial for many biological studies, in particular, those related to the nature of cancer and its diagnosis and treatment.

Nature of Cancer

Cancer is a general term used to describe what is probably a number of diseases. The common manifestation is growth of the cancer cell beyond the condition of simple repair and replacement to a condition that leads to interference with normal biological processes and eventual death. It appears certain that several steps are required for a cell to become malignant. Some of the steps involve growth characteristics, and others involve immunological alterations. Although most normal cells in a mature organism are not growing (that is, dividing to produce new cells) there is some continuing amount of cell proliferation. Therefore, finding a very slight increase in cell proliferation in inappropriate cells (for early cancer detection) is a very difficult problem. However, prevalent opinion holds that there must be at least one, and perhaps more, unique and measureable properties of cells that are specific in cancer. A great deal of effort has been made to find such properties.

Because tumors can arise from a single cancer cell, all of the information that defines a particular cancer probably is contained within a single cell. This is one rationale for performing cellular

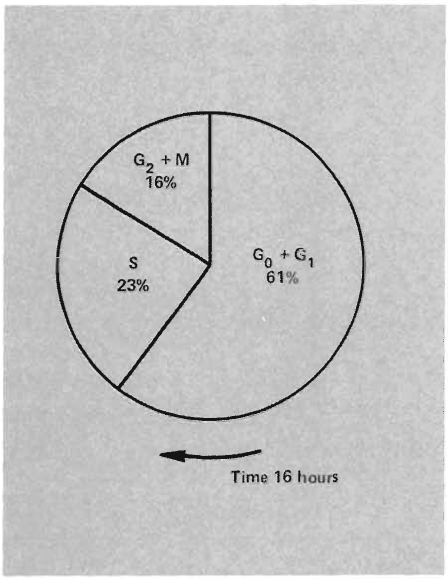


Fig. 11. A conceptual diagram of how Chinese hamster ovary cells (CHO), in exponential growth, are distributed throughout their life cycle.

research to find an unequivocal marker for a cancer cell. A suitable marker could be any of the following: the presence of specific antibodies or enzymes, an unknown chemical substance, a change in electrical properties, a change in the DNA, a loss of chromosome stability, or some presently unimagined feature. Experiments with DNA have high priority because the central cancer problem is thought to be one of altered gene expressions.

The Cell Cycle

To define an abnormal or cancerous condition, we must understand the range of normal conditions. Therefore it is not truly meaningful to separate cancer research from basic research directed to the understanding and quantification of normal biological processes. Undoubtedly a detailed understanding of cellular growth throughout the life cycle of a cell holds the key to an understanding of where the process goes astray and produces uncontrolled tumor growth. One gross change that can be monitored during the life cycle is DNA content. Each phase of a cell's life cycle (Fig. 11) is characterized by the amount of DNA present in the cell nucleus. DNA is measured in units of c, the number of picograms of DNA in the sperm or egg cell of a particular species. All other cells in that species contain double this amount, or 2c. In Figs. 11 and 12, G_0 represents the stage of a mature cell that is not multiplying (for example, a lymphocyte). If the cell receives a signal to replicate (the presence of a foreign material might initiate such a signal), it goes into stage G_1, in which several biochemical activities including the production of protein in the cytoplasm take place. Upon completion of these activities, the cell goes into stage S, in which the DNA replicates itself and the amount of DNA increases from 2c to 4c. Stage S is followed by G_2, a resting stage, and then by M, the mitosis stage, in which the DNA condenses into chromosomes and the cell divides.

Figure 12 shows the distribution of DNA content for a population of normal multiplying cells measured with a flow cytometer. The large peak, containing most of the cells at 2c DNA content, corresponds to stage G_1. The smaller peak around 4c corresponds to cells in stages G_2 and M. The region between the peaks contains the cells in stage S, undergoing DNA synthesis. In the normal life cycle, cells remain between 2c and 4c. However, when things go astray as in malignant growth, abnormal amounts of DNA are sometimes observed. As an organism ages or is subjected to certain chemicals or ultraviolet light and other environmental stresses, both the amount of DNA and the way it is packaged into individual chromosomes may change.

The measurement of DNA distributions therefore is extremely useful for tracking cells through the life cycle and for assaying the effects on the cell cycle of radiation, environmental conditions, and chemical drugs. The technique also

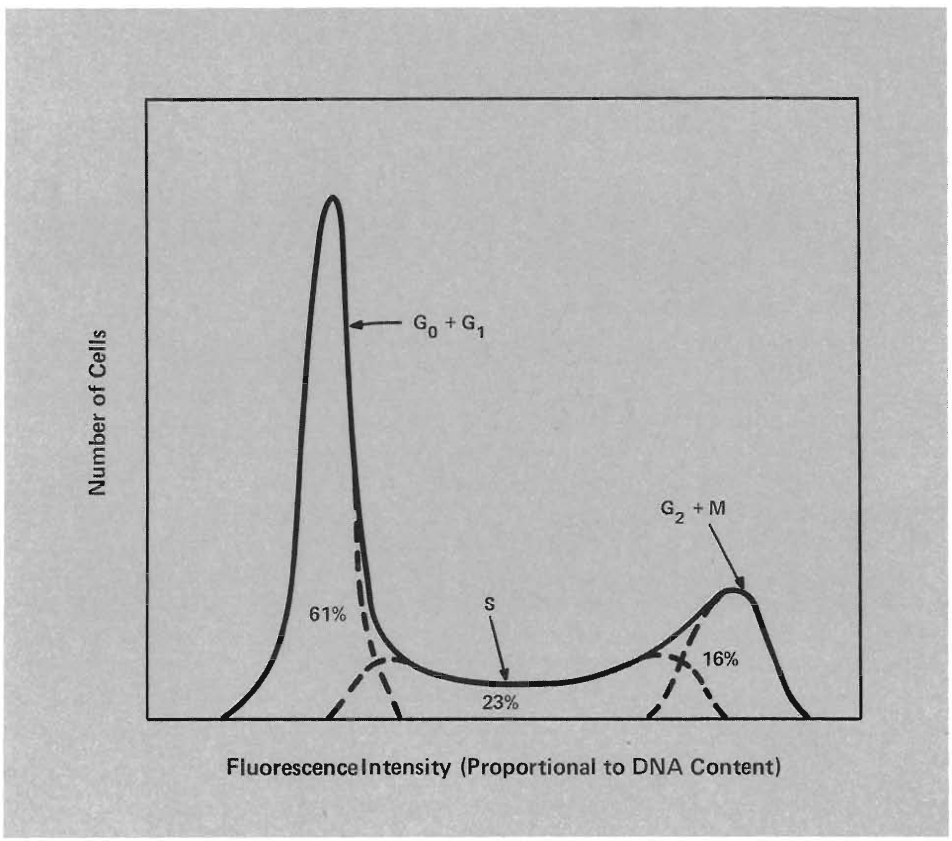


Fig. 12. The pulse-height distribution from exponential growing cells showing how the integral curve can be unfolded into its component parts to determine the percentage of cells in G_0 + G_1, S, and G_2 + M.

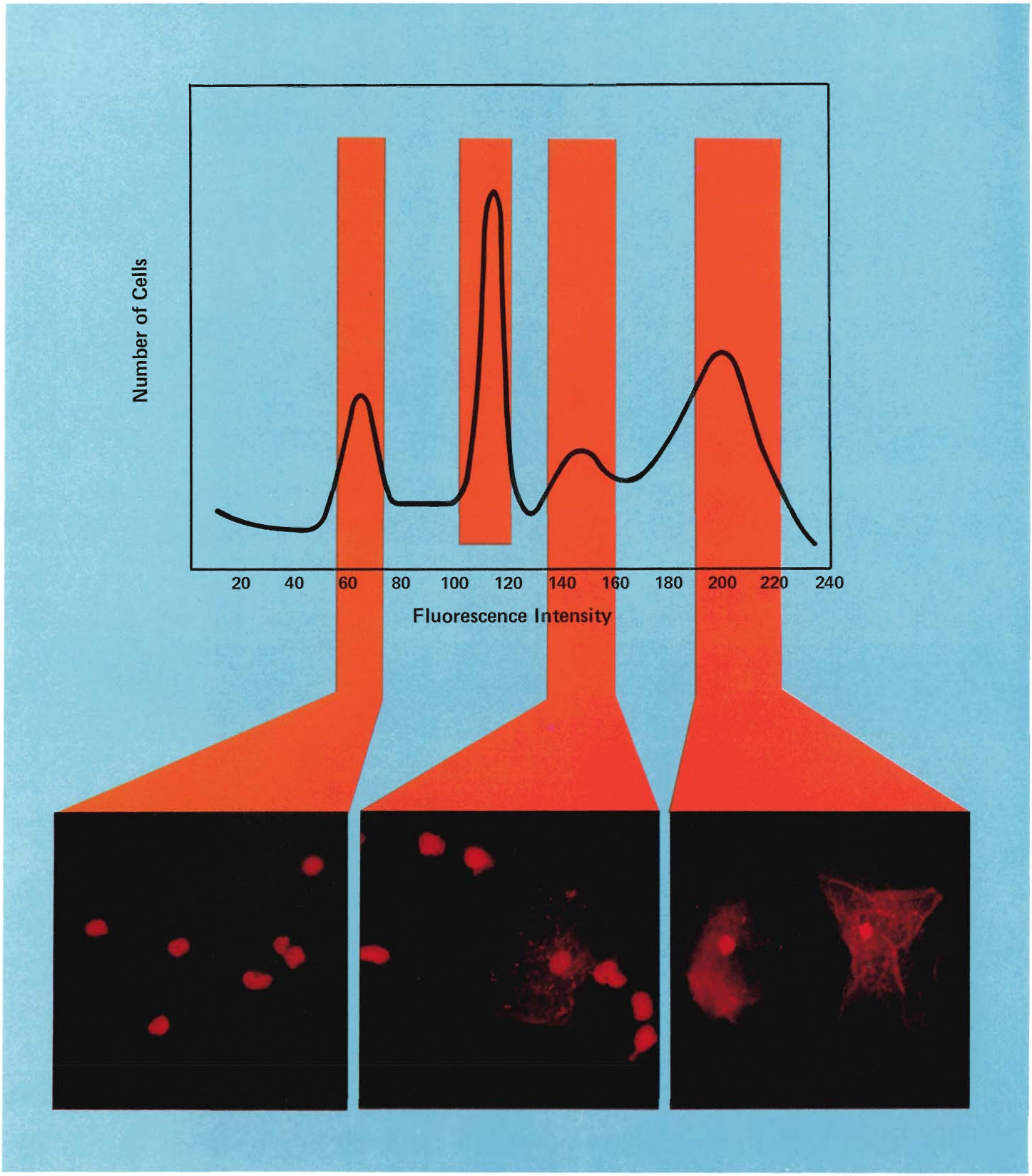


Fig. 13. Intensity distribution of three cell types artificially mixed before fixation in 70% ethanol and stained using a nonspecific propidium iodide procedure. Fluorescence analysis was made using 488-nm laser illumination. Photomicrographs of cells sorted from each of the peaks are shown in the lower part of the figure. Based on cell morphology the cells from the indicated sorting windows were identified as: sort region 1, human white blood cells, magnification of 438 X; sort region 2, cells derived from a methylcolanthrene-induced skin tumor of mice (not shown); sort region 3, methylcolanthrene tumor cells with a few contaminating squamous cells from region 4, magnification of 276 X; region 4, normal human buccal cells (squamous cells) obtained from the lining of the oral cavity, magnification of 276 X. (Figure courtesy of Z. Svitra and John Steinkamp, LASL.)

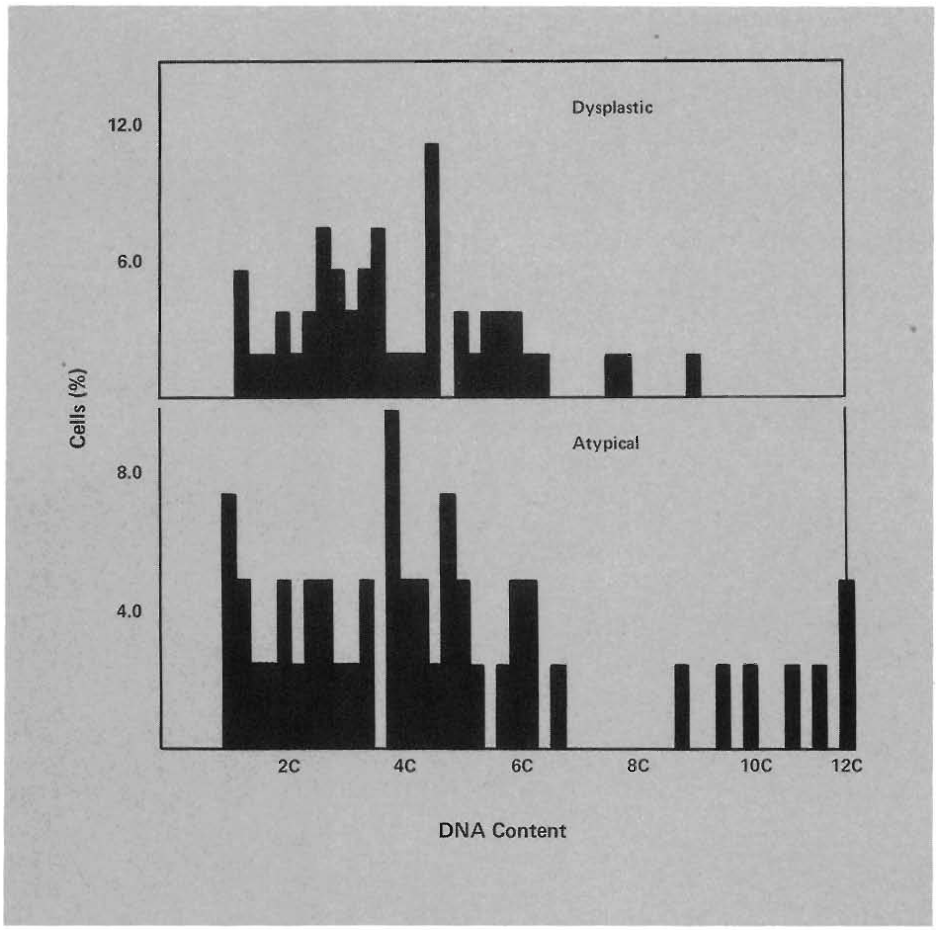


Fig. 14. DNA distributions obtained from abnormal clinical cervical samples —(A) Dysplasia, a recognized precancerous, but temporarily benign condition, and (B) frank cancer (carcinoma in situ). Both conditions have a similar characteristic, that is, cell proliferation as indicated by DNA densities at 4c, 6c, 8c, etc. Resting or mature cells display a DNA value of 2c.

has been used in attempts at cancer diagnosis and in monitoring the effects of chemotherapy on malignant cells. In one application, the cell sorter unscrambled a complex mixture of malignant cells and two types of normal cells as illustrated in Fig. 13. In this instance, the fluorescent staining procedure was only partially specific for the DNA of each cell type. Although the procedure is empirical, it produced clear resolution of the three cell types in the intensity distribution. If a stoichiometric staining procedure had been used, this resolution would not have been achieved. To verify which cells constitute each peak, several thousand cells were sorted from each peak; the results are illustrated in Fig. 13.

Attempts to Automate Cancer Diagnosis

To the cytopathologist, malignant cells have certain distinguishing features that can be observed through the microscope. Because some of these features can be measured with a flow cytometer, we can hope that this instrument may one day be used as an inexpensive automated method for mass screening for cancer. As an example, consider the case of cervical cancer. This disease represents a major worldwide public health concern. In the United States alone, there are approximately 90 million women in the "at risk" age group. Moreover, clear evidence based on case histories tells us that early and accurate detection is the key to a good prognosis. For women who are screened regularly with the "Pap test," survival from this disease is greater than 95% if the disease is detected. However in the United States, mainly for socioeconomic reasons, the majority of the female population never undergoes this screening. Thus for the general, infrequently screened population, survival is more like 50% if the disease is detected. For these reasons, cervical cancer detection has long been a prime candidate for automation.

Under a microscope, the stained nucleus of a malignant cell looks irregular compared to the nucleus of a normal cell. Frequently the nucleus is larger than normal, and it may be less symmetrical. Sometimes the cell too is larger and more irregular and may have a granular appearance. These qualitative differences between normal and malignant cells are fairly easily detected by a trained eye.

The question is, can an instrument do as well? Detailed pattern recognition is slow and expensive, but a flow cytometer can measure several relevant parameters with good accuracy. These are DNA content, nuclear diameter, protein content, and cytoplasmic diameter. On the average these parameters change from normal to malignant cell populations as shown in Table I. The size features (nuclear and cytoplasmic diameters) overlap considerably between normal and malignant cell types. DNA content may increase significantly in malignant cells and the ratio of nuclear diameter to cytoplasmic diameter also tends to increase. The DNA distribution for cells from a frank cervical tumor is shown in Fig. 14. These cells can have DNA

TABLE I

Morphological Characteristics of Normal and Malignant Cells

Feature	Noncycling Cells	Malignant Cells
DNA (G_1)	2c	>2c
Nuclear diameter	<12 microns	>12 microns
Nuc/Cyto diameter	<0.5	>0.5
Cytoplasmic diameter	10-80 microns	20-40 microns

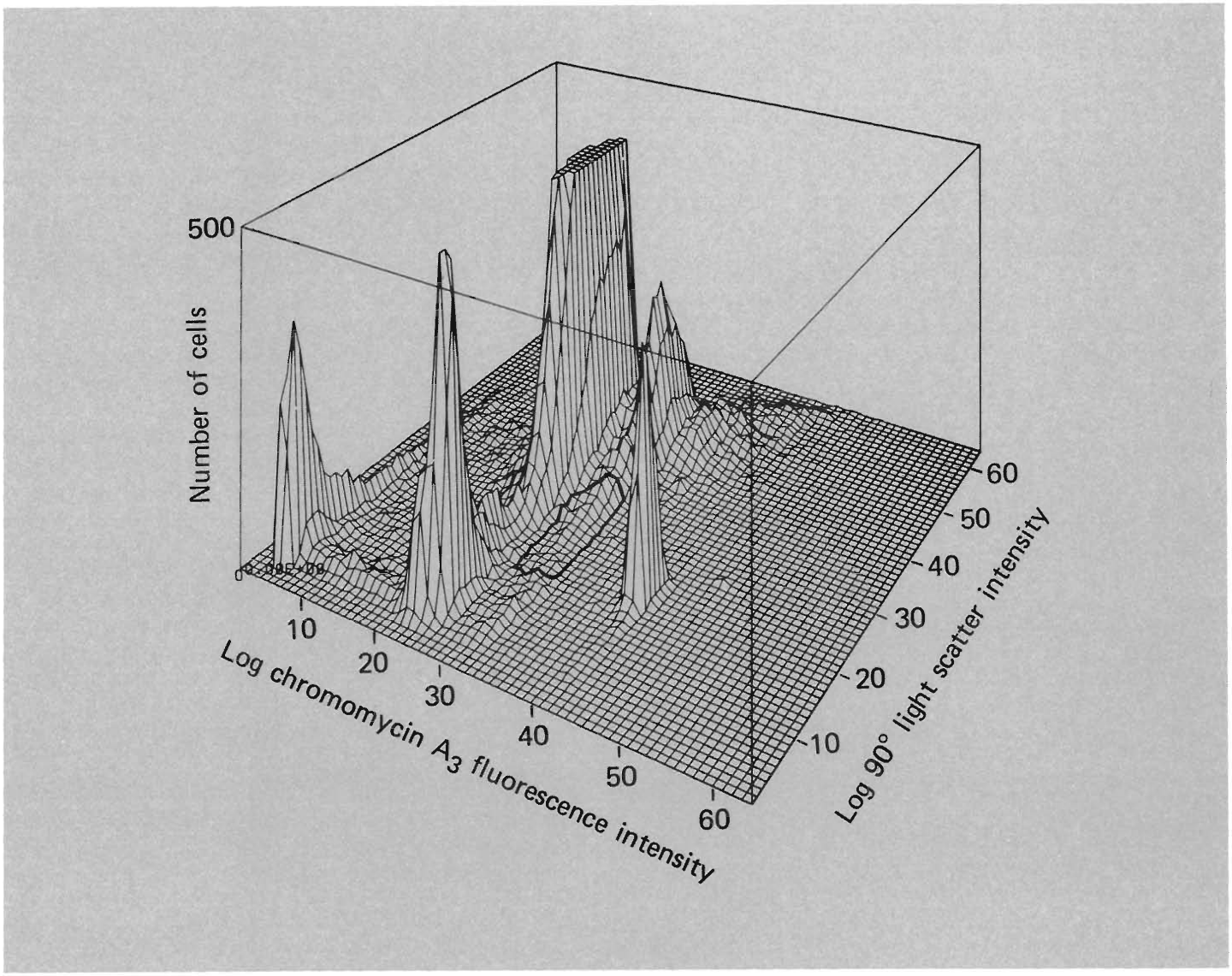


Fig. 15. A two-parameter histogram of cervical and vaginal cells measured by flow cytometry. The histogram is generated by determining chromomycin A3 fluorescence intensity as a measure of cellular DNA content (abscissa in the plane) and 90-degree light-scatter intensity as a measure of cell size (ordinate in the plane) simultaneously on each cell. Thus the location on the plane shows DNA content and cell size, while the height above the plane shows the relative frequency of occurrence. The intensities of the fluorescence and scatter signals are measured and presented logarithmically. An increase in seven channels in either direction corresponds to a doubling of intensity. With the cell sorter, it is possible to identify the cell types that give rise to histogram features. The peak and shoulder at the far left corner of the histogram (the lowest values of fluorescence and light-scatter) are due to cell debris and bacteria. The sharp peak in the foreground with a higher fluorescence intensity and low light-scatter is due to white blood cells; the large peak at equal fluorescence intensity but higher scatter values is due to intermediate and superficial epithelial cells. The small shoulders to the right of the white cell and epithelial cell peaks are due to cell aggregates. The sharp peak at even higher fluorescence is due to fluorescent microspheres that were added for machine calibration. The identities of the cells responsible for each feature have been confirmed by cell sorting and morphological analysis. (Figure courtesy of R. Jensen, Lawrence Livermore Laboratory)

values as high as 12c, or 6 times the normal noncycling value. Since the geometric features have been shown clinically to have diagnostic value, sets of parameters such as DNA and size have been investigated for use in automated diagnosis of cervical cancer by flow cytometry.

At the Lawrence Livermore Laboratory, clinical material has been examined for its DNA content and cell size. The DNA was measured by fluorescence, and the cell size was determined from light-scattering measurements. The data result in three-dimensional histograms of the two variables, as shown in Fig. 15 from an abnormal specimen.

Malignant and premalignant cells contain elevated amounts of DNA and tend to be intermediate in size. A large fraction of the abnormal cells in Fig. 15 show fluorescence and light-scatter signals that are localized to the right of the main peaks (in the heavily outlined area). A 20- to 30-fold enrichment of abnormal cells can be obtained for

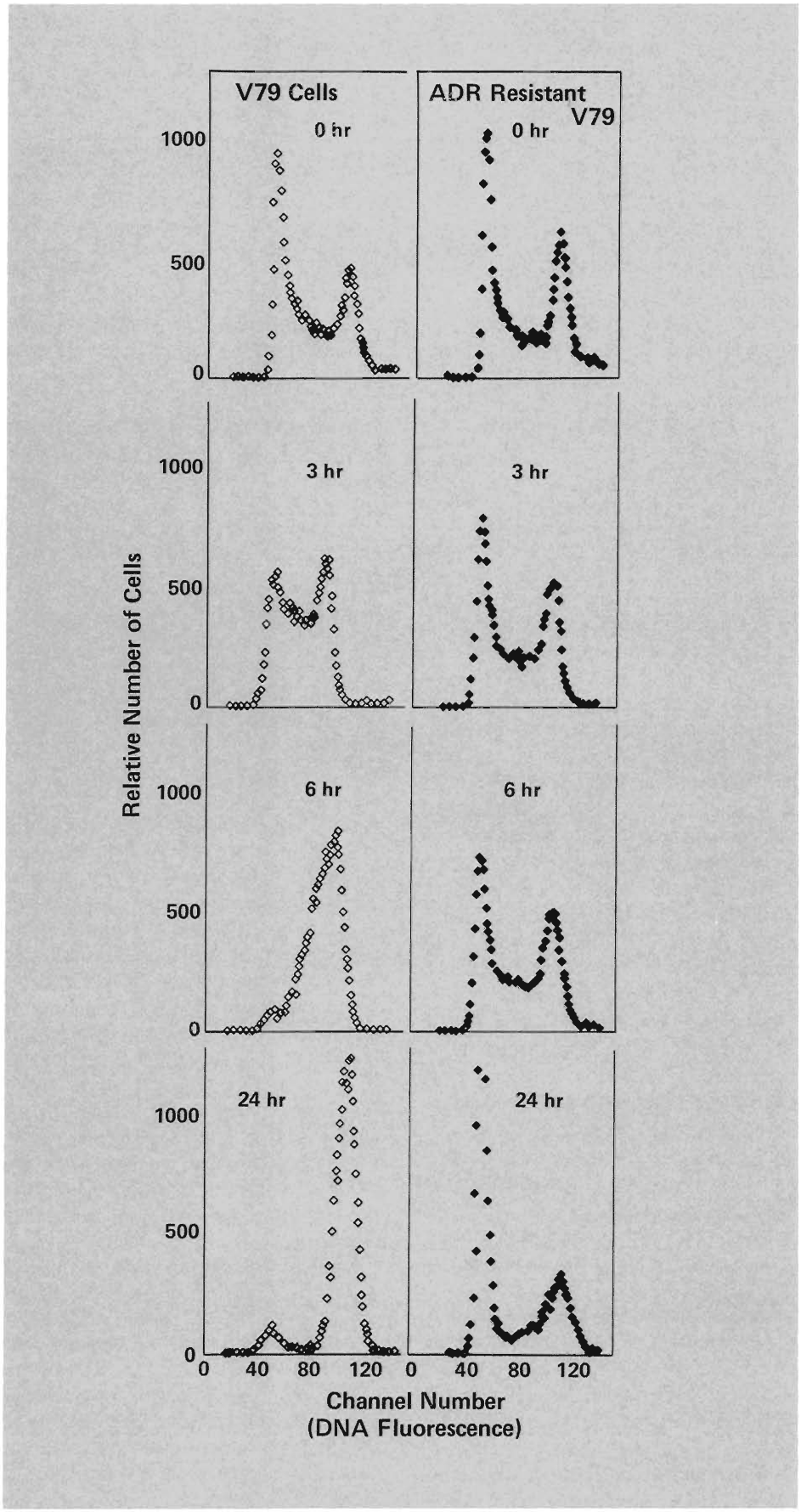


Fig. 16. Comparison of DNA distributions of normal cell line V79 and Adriamycin-resistant V79b cell line following exposures to Adriamycin (0.5 mg/ml, 1 h). (Courtesy of N. Tokita, LASL)

microscopic analysis by sorting cells from this area. Because some signals from normal cells and aggregates also appear in the area, we cannot use histogram analysis directly for automated diagnosis of cervical cancer. To increase the potential accuracy of automated diagnosis, a new independent marker correlated with cellular abnormality must be included as a third parameter.

Recently a group at the National Cancer Institute Division of Cancer Biology and Diagnosis used the LASL multivariable cell sorter in combination with the two-color DNA/protein staining protocol. A double-blind clinical trial was conducted so that experimenters were not informed of the clinical diagnosis of the samples. That is, each sample was divided into two fractions, one screened by cytopathology and the other screened by the machine. Upon completion of the trials, the instrument results were compared with the cytopathology results, which were assumed to be correct. With this parameter set the accuracy was 80%. Accuracy depends on normal variation and on how one sets the criteria for defining an abnormal cell. Because of variations between normal cells, it may be impossible to improve the accuracy by measuring only DNA and protein. It appears that additional variables must be measured to improve definition of a cancerous cell.

Errors are expected in any diagnosis. The two important errors are the false negative rate (rate of abnormal cases misclassified as normal) and the false positive rate (rate of normal cases classified as abnormal). In the trial just described, the false negative rate was 10%, a serious error because it means that disease is missed in 10% of the incidents. The false positive rate, 27% in the trial, is less serious. In any mass screening program, the false positive error increases both work and costs because sampling must be repeated. However, a 27% false positive rate would be acceptable, if the false negative rate could be brought down to about 5%. Thus, although these results do not justify a clinical technique at present, they are encouraging.

Perhaps as we learn more about the nature of cancer, we can identify more definitive parameters that will make

automated screening more accurate than the cytopathologist. Work continues with this goal in mind.

Monitoring Radiotherapy and Chemotherapy Effects

Flow cytometry is used extensively to study the effects of radiation and chemotherapeutic drugs on cultured cells, rodent tumors, and human tumors. Goals include an understanding of the damage in terms of cell-killing and the application of this information to radiotherapy and chemotherapy problems.

After cells are exposed to radiation or to chemotherapeutic drugs, the cell's DNA distributions and other cytometric parameters are measured at different times after exposure to complement the data obtained by other methods. For example, two cell lines of Chinese hamster origin differ considerably in their sensitivity to Adriamycin, a drug commonly used for the treatment of human cancer. When M. R. Raju (LASL) studied the DNA distributions for the two cell lines after treatment with 0.5 milligram per milliliter Adriamycin for 1 hour, he found that one cell line was more sensitive than the other (Fig. 16). Changes in DNA distributions in the normal (V79) cell line were dramatic, but the changes in the Adriamycin-resistant cell line (V79b) were small. Cell survival data obtained by colony formation indicated that cell survival of the normal (V79) cell line was 3%, but cell survival of the Adriamycin-resistant (V79b) cell line was 80%. Therefore, the magnitude of the cell cycle DNA distribution perturbations measured by flow cytometry was related to the cell killing. Since Adriamycin resistance is due to a decreased drug uptake, he measured the amount of Adriamycin per cell using flow cytometry. The measurements confirmed that the drug resistance of the V79b cell line was due to lower drug uptake. This study, together with others, indicates that DNA distributions can be used to measure and predict the drug sensitivity of tumor cells rapidly. DNA distributions also may be useful for studying drug-induced perturbations of cells from a patient biopsy as a prognostic test for cancer patients.

Search for Key Malignancy Parameters

Because it is likely that a single cell (a stem cell) is the origin of every cancer, the isolation and characterization of such a cell remains an important problem. A stem cell should be inherently different from other cells of the population in one or more characteristics. The differences probably originate in an altered expression of the cell's DNA. It is unrealistic to expect that initial changes in a cell's gene expression will be manifested in DNA changes large enough to be detected in the presence of the cell's total DNA content. In humans, the packaging of cellular DNA into chromosomes during a portion of the cell cycle (mitosis) provides a natural subdivision of cellular DNA into 46 chromosomes, which can be isolated and studied individually with flow cytometers and sorters. That chromosome changes are important in the etiology of tumors has long been recognized, but the number of chromosomes we can study using a microscope is too small for us to detect the rare chromosome changes that occur when a tumorigenic stem cell first begins to propagate.

Lawrence Livermore Laboratory and Los Alamos Scientific Laboratory in cooperation with the Max-Planck-Institut in Goettingen, West Germany, have demonstrated the capability to analyze isolated individual chromosomes by flow cytometry. Figure 17 is an example of such a high-resolution measurement in which all but one of the expected chromosome types isolated from Chinese hamster M3-1 cells were resolved. In addition, three "homologue" pairs (1, 1; 7, 7; and 9,9) had small differences in DNA content. Chromosome changes reflecting the appearance of a new stem cell in the population might be manifested by changes in the position or area of one of the peaks.

More subtle changes in chromosome morphology can be quantitated by using a unique chromosome-imaging flow sorter. In contrast to conventional flow cytometers that measure total fluorescence without regard to its spatial distribution, our chromosome-imaging sorter maintains the optical image as the object passes through the laser beam. The chromosome image is formed in front of a mechanical slit placed at the image plane; the slit scans the chromosome as it flows through the observation region. The intensity profile of a chromosome contains valuable additional information as illustrated in Fig. 18. A unique intensity profile is recorded for each chromosome at the rate of 1,000 per second. Now much more subtle features associated with chromosome aberrations can be sought, and when chromosomes with these features are found, they can be sorted for verification and further analysis. Chromosomes with aberrations such as breaks, some types of translocation of chromosome pieces, fragments, and dicentrics (two chromosomes joined at their ends) should be detectable. The search for additional parameters that will define chromosome types uniquely is continuing.

The role of chromosome changes in cancer is not well defined. In certain types of cancer, such as chronic myelocytic leukemia, a specific and fairly consistent chromosome abnormality has been identified. In advanced cancers, one observes a large variety of chromosome aberrations but not much consistency. These observations are in stark contrast with the remarkable process of DNA replication and cellular mitosis that results in exact maintenance of the amount of DNA per cell and the stability of chromosome number and

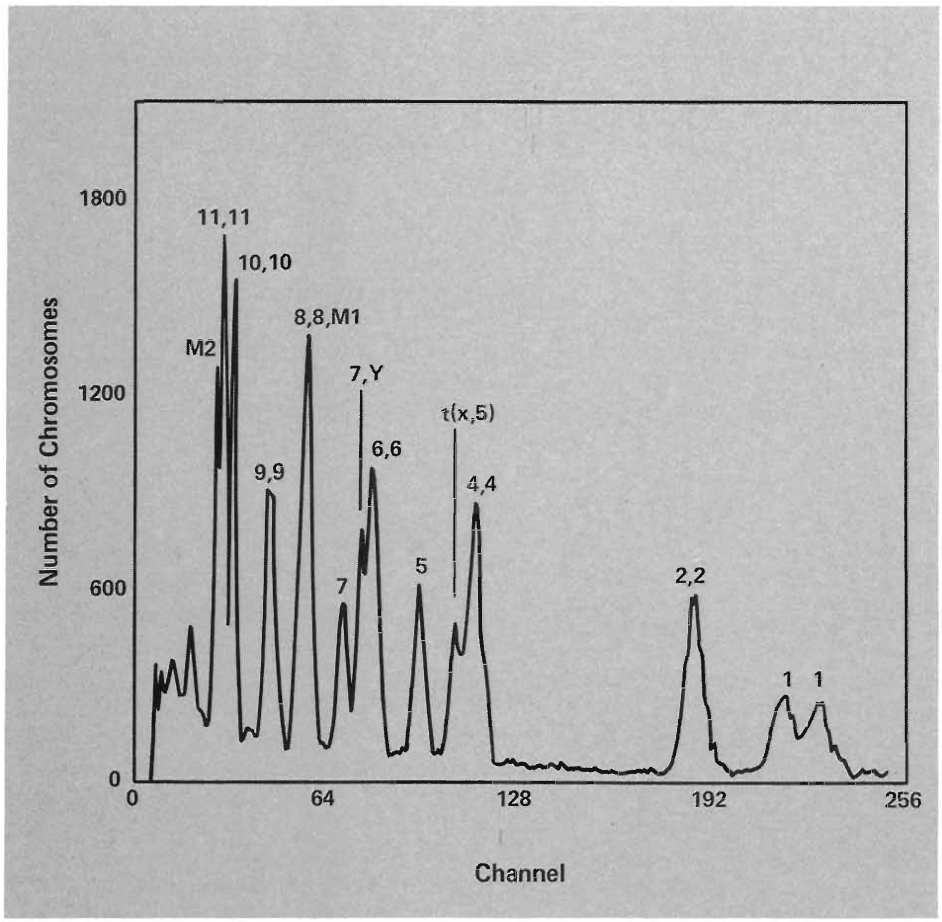


Fig. 17. The flow karyotype of chromosomes isolated from Chinese hamster M3-1 cells and stained with the bis-benzimadazole dye Hoechst 33342 (10 micromolar). The mean fluorescence intensity of a peak reflects stain content and hence DNA content for chromosomes of that type. The area of each peak is proportional to the number of chromosomes of that type. Approximately 30,000 chromosomes were analyzed. The type of chromosomes constituting each peak was verified by sorting and visual identification. The longest chromosomes (No. 1) have the most DNA, and the smallest chromosomes (M2 or markers) have the least. Each chromosome type and its corresponding peak are identified.

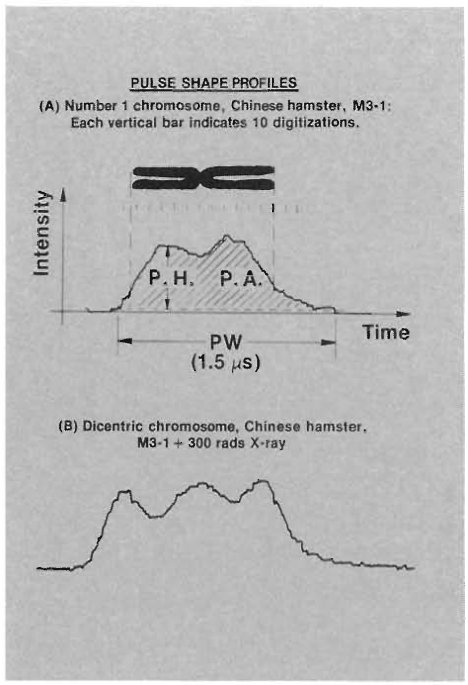


Fig. 18. Pulse shape profiles of individual chromosomes isolated from Chinese hamster M3-1 cells. (A) Pulse shape profile of a single No. 1 chromosome. The intensity of fluorescence across the length of each chromosome is analyzed by a waveform recorder, which rapidly digitizes the fluorescence intensity as a function of time. This information, recorded for each chromosome, can be used to make sorting decisions. Pulse shape profiles provide information on pulse area (P.A.) or total DNA content, pulse width (P.W.) or total chromosome length, and pulse height (P.H.) or fluorescence density per unit length. (B) Pulse shape profile of what appears to be a dicentric chromosome (two chromosomes attached end to end) isolated from the cells used in A but having received 300 rads of x ray 16 hours before chromosome isolation. The time axis is the same for both profiles.

morphology in all normal cells. It is this very contrast that makes chromosome stability appear to be of extraordinary importance. With full implementation of the imaging flow cytometer/sorter, we will be able to characterize 100,000 or more chromosomes—a truly remarkable accomplishment that has been equated to taking that first look at the back side of the moon.

The development of flow cytometry is due to the combined efforts of many people throughout the world. Many members of the LASL Life Sciences Division and groups at Stanford University, Lawrence Livermore Laboratory, Memorial Sloan-Kettering Cancer Center, and the University of Rochester have contributed to this work. The manufacture of commercial equipment is a strong indication that the technology has reached a fairly mature state. The instruments now can be used by a large variety of biologists throughout the world and the potential biological applications appear to be very great.

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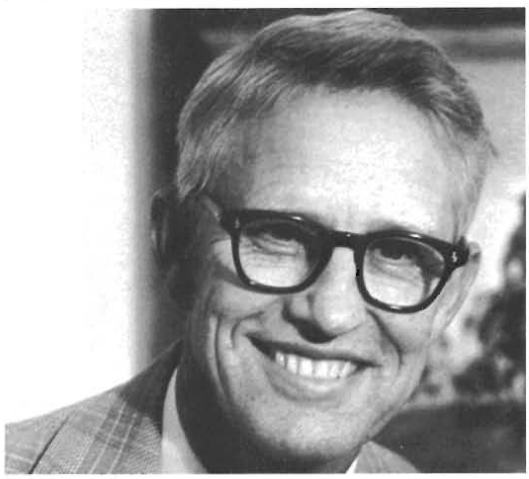
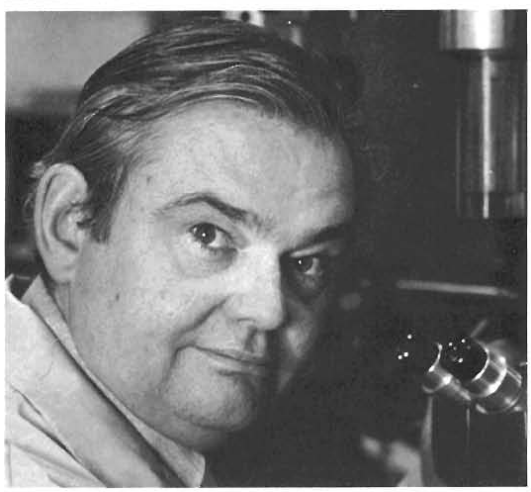
THE AUTHORS





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Dale M. Holm is Alternate Group Leader of LASL's Biophysics Group, USDA Program Manager at LASL, and LASL/USDA Liaison Officer. He joined LASL in 1952, and since then, he has been involved in nuclear physics experiments; reactor design, construction, and operation; activation analysis; bomb testing; biophysics; and agricultural bioscience. He holds a patent on flow cytometry, for which he also helped to develop instrumentation. At present, his work involves application of LASL technology to many problems of the USDA, including detection of tuberculosis in cattle using flow cytometry. He earned his bachelor of science degree in physics from Lewis and Clark College, and his Ph.D. in physics from Oregon State College in 1955.

Paul F. Mullaney, Group Leader of LASL's Biophysics Group, received his bachelor of science degree in physics from Iona College in 1959 and his Ph.D. in physics and biophysics from the University of Delaware in 1965. He is president-elect of the Society for Analytical Cytology and serves on the editorial board of the Journal for Analytical Cytology. A Fellow of the Alexander von Humboldt Stiftung Foundation, Bad Godesberg-Bonn, FRG, Mullaney earned their Senior American Scientist prize in 1978. His work has resulted in patents issued for dual-parameter flow photometric apparatus and method, a potential-sensing cell analyzer, and an ellipsoidal cell flow system.