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

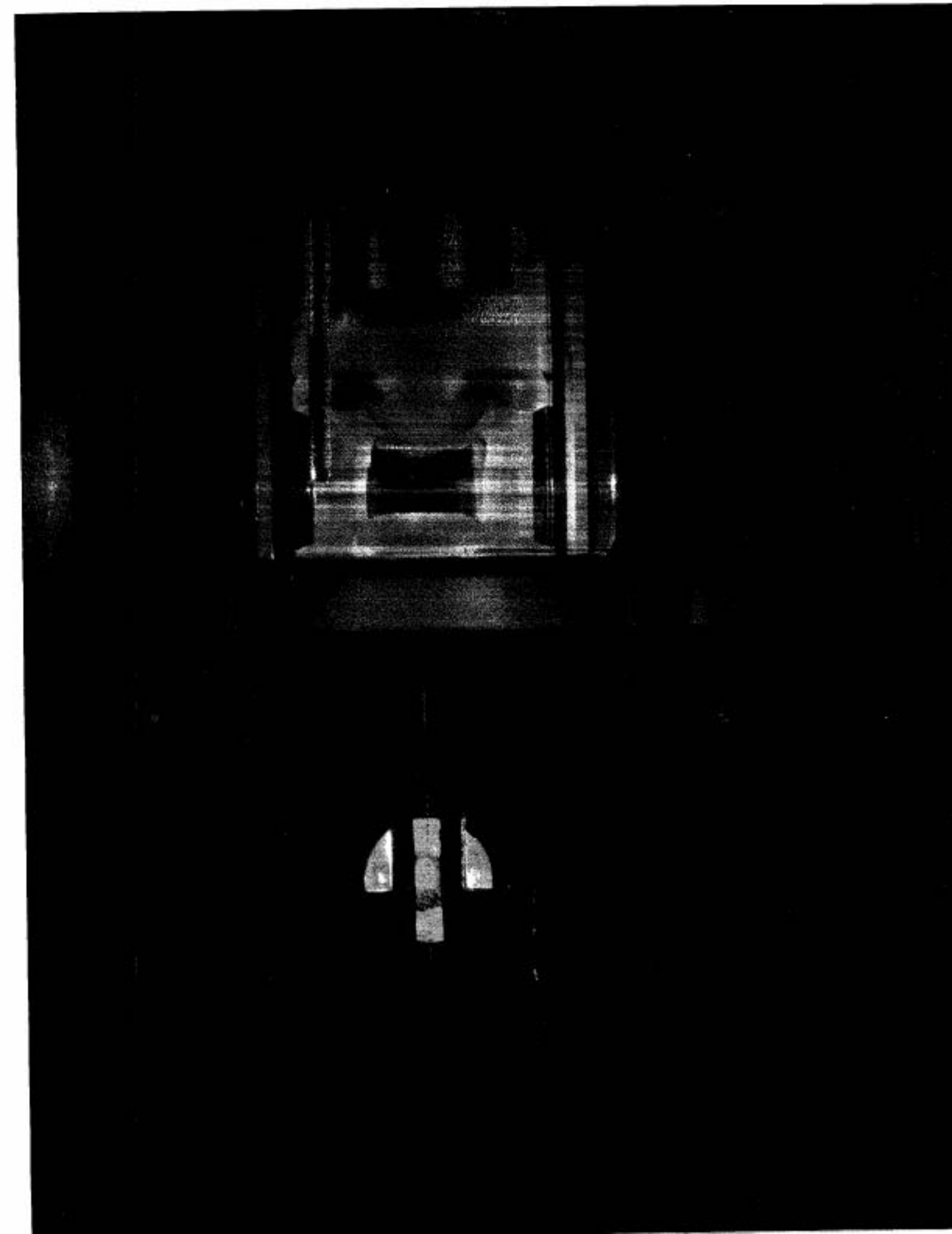
A New Tool for Quantitative Cell Biology

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 Gohde 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.

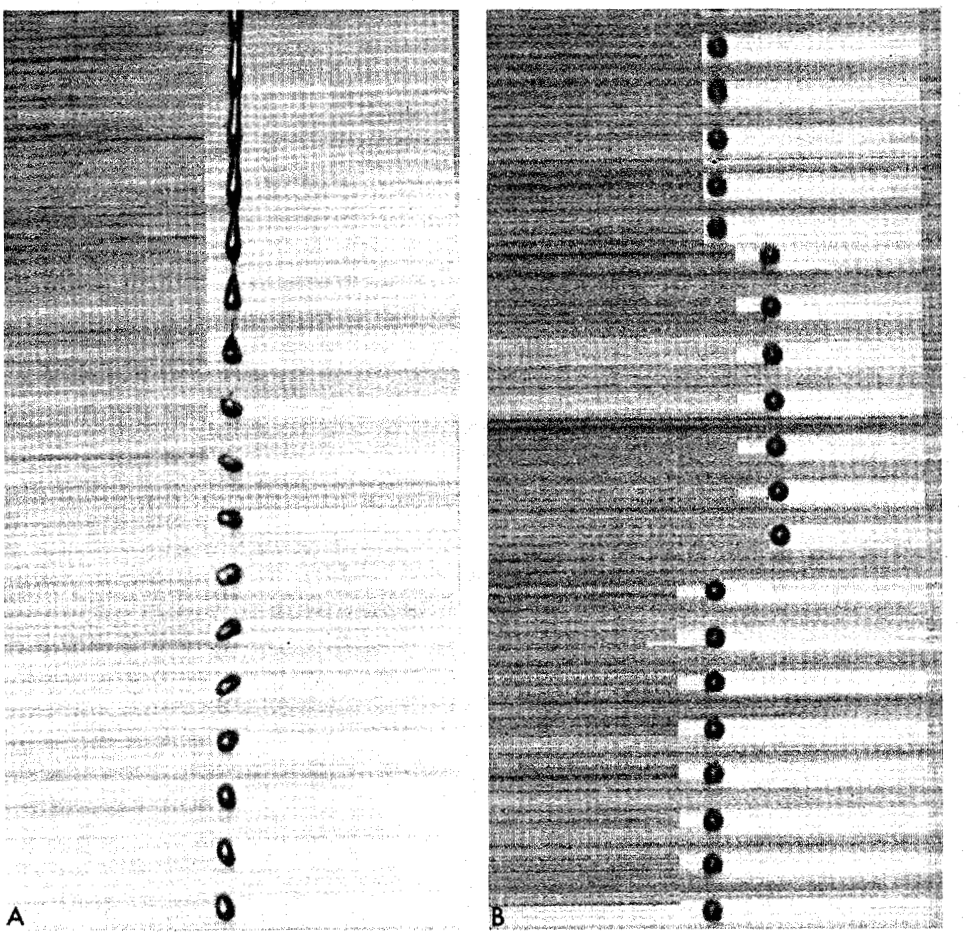


Fig. 2. A stroboscopic light appears to stop the movement of droplets as they jet out of the flow cytometer chamber. (A) Experimenters can program the flow cytometer electronics to separate a particular biological cell from the rest of the sample by charging a group of droplets as they break off from 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.

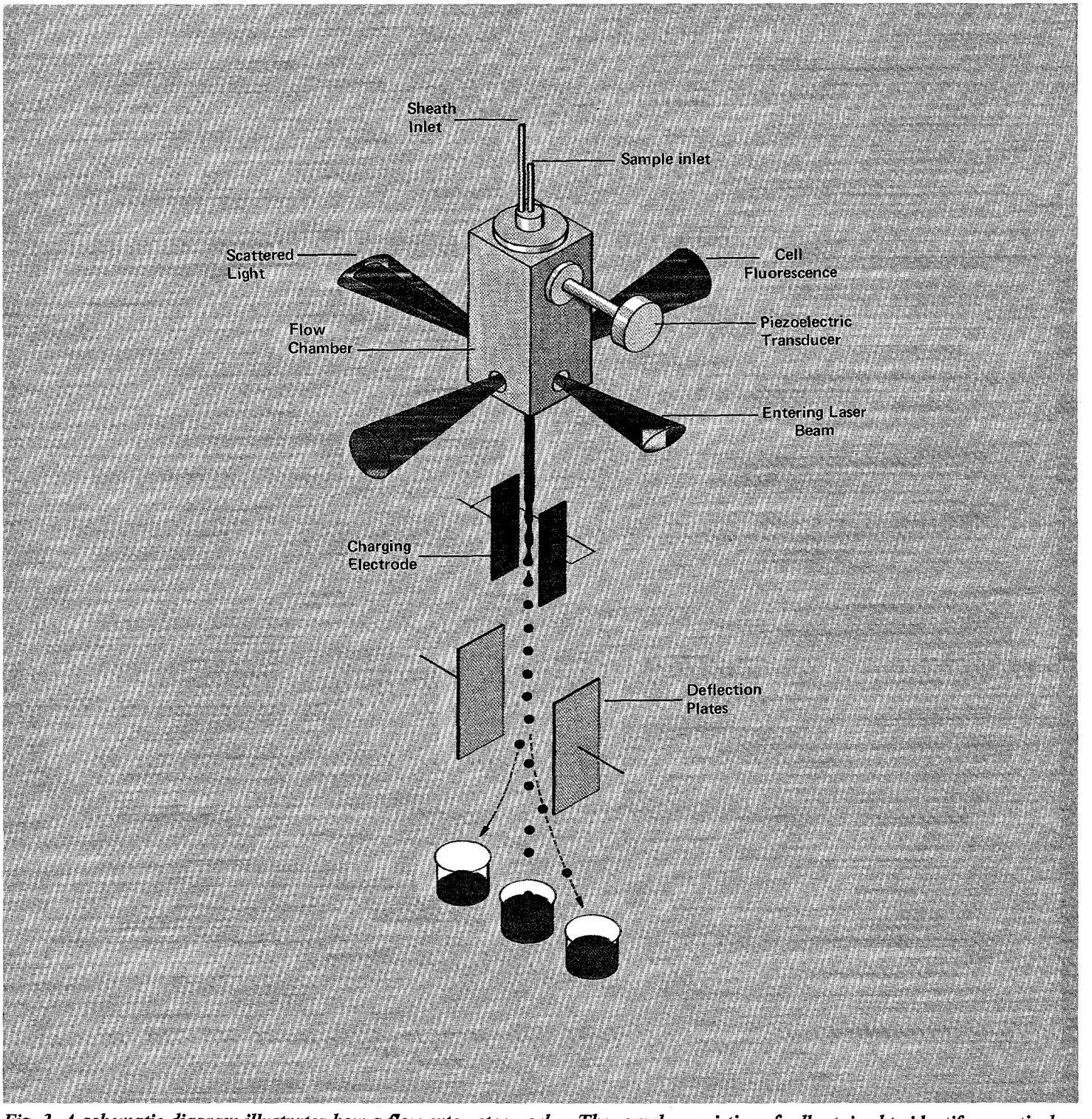


Fig. 3. A schematic diagram illustrates how a flow cytometer works. The sample consisting of cells stained to identify a particular cellular property, such as the amount of DNA, enters at the top. The cells are dispersed into a single-cell suspension in the conducting medium (normally a saline solution). 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.

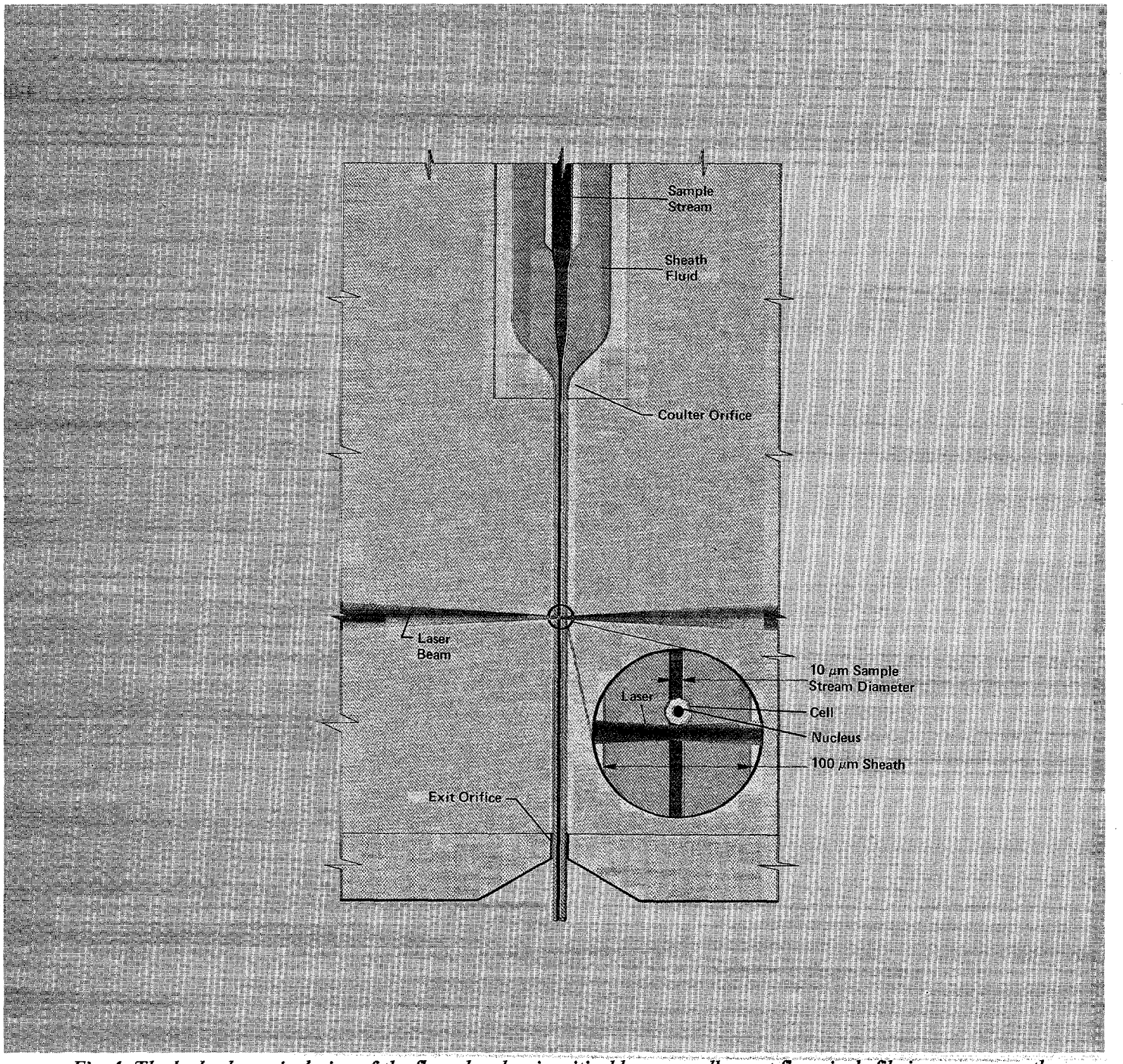


Fig. 4. The hydrodynamic design of the flow chamber is critical because cells must flow single file in a narrow path through the center of the Coulter orifice and then through the focused region of the laser beam. Most chambers currently in use are variations of a flow chamber originally designed by 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.