Fluorescence Activated Cell Sorting*

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An instrument has been developed for sorting biological cells. The cells are rendered differentially fluorescent and incorporated into a small liquid stream illuminated by a laser beam. The cells pass sequentially through the beam, and fluorescent light from the cells gives rise to electrical signals. The stream is broken into a series of uniform size drops downstream of the laser. The cell signals are used to give appropriate electrostatic charges to drops containing the cells. The drops then pass between two charged plates and are deflected to appropriate containers. The system has proved capable of providing fractions containing large numbers of viable cells highly enriched in a particular functional type.

INTRODUCTION

Cells found in such organs as spleen, bone marrow, lymph nodes, liver, or kidney are generally complex mixtures of functionally different types. Separation of specific cell types along functional lines would be useful in many biological and biochemical investigations. We have developed an instrument which separates cells on the basis of fluorescence. It is thus capable of separating cells which have been labeled by different fluorescent markers.

The instrument is based on a separation technique first used by Fulwyler,[1] in a cell volume sorter. This unit, in turn, uses drop deflection methods developed by Sweet.[2] As in Fulwyler's instrument, our separator generates a stream of droplets containing the cell mixture and electrostatically charges and deflects those droplets containing cells to be separated from the mixture. In our instrument, the sorting parameter is cell fluorescence, produced by fluorescent stains or reactions with fluorescent tagged antibodies. Fluorescent compounds used include fluorescein, acridine orange, or quinacrine mustard. Preliminary work on a simpler, less efficient instrument has been reported previously.[3]

DESCRIPTION OF THE INSTRUMENT

A photograph of the instrument is shown in Fig. 1, and a simplified block diagram is shown in Fig. 2. As indicated there the cells are observed in a column of liquid illuminated by a laser immediately after emerging from a small glass nozzle. A variation of the Crossland–Taylor coaxial flow system[4] is used to confine the cells to the stream axis. This keeps particulate material away from the walls, minimizing both clogging and any optical variations which might arise as a result of cell position in the stream. Cells are confined to approximately the central 15 μ of the 50 μ diam jet. While this small an area would be plugged frequently if defined by a rigid orifice, we have been able routinely to conduct cell separations lasting up to 10 h without problems.

The cell suspension is injected through the inner nozzle (diam 50 μ) of a coaxial nozzle assembly. Cell-free sheath fluid flows around the inner nozzle, accelerating and narrowing the inner stream. The fluids emerge coaxially from the 50 μ exit diameter outer nozzle.

The relative flow rates of sheath and sample fluids are determined by the differential pressure established by the relative heights of their respective supply reservoirs, both pressurized from a common compressed air source. Typically, an average pressure of 0.84 kg/cm^2 (12 lb/in.^{2}), and an excess head of 30 cm for the sheath fluid, produce a jet velocity of 10 m/sec, a sheath flow rate of 0.02 ml/sec, and a sample flow rate of 0.003 ml/sec.

The cylindrical liquid stream is illuminated immediately after emergence by an argon laser (Coherent Radiation, model 54) which can be tuned to any one of a number of wavelengths between 454 and 514 nm. Output at 488 nm, the most frequently used line, is 300 mW. A combination of spherical and cylindrical lenses focuses the beam to a narrow ellipse at the intersection with the stream axis. The laser beam intersects the stream axis at an angle of 45°, to prevent the direct beam from reaching the detector.

A cell crossing the laser beam on the stream axis is illuminated for several microseconds, during which time it emits a fluorescent pulse. Some of this light is collected by a microscope objective (positioned to minimize entrance of direct laser light) and directed to the cathode of a photomultiplier tube after passing through a GG14 and a Wratten No. 16 barrier filter. Thus a signal pulse is generated at the photomultiplier output whenever a fluorescent cell crosses the laser beam. If the signal pulse from a cell is within predetermined amplitude limits, a charging pulse is generated electronically, after a delay corresponding to the transit time of the cell from the laser beam to the point where the drops break off. This charging pulse (of the order of 50 V) is applied to an electrode which makes contact with the electrically conducting (isotonic) fluid in the nozzle. The pulse induces a charge on the stream proportional to the applied voltage. Drops which break off from the stream while the pulse is applied, including the drop containing the desired cell, carry a sample of the charge existing at the instant they separate.

After being selectively charged in this way the droplet stream passes through a transverse electrostatic field established by two parallel deflection plates having dc potentials of ±1000 V. Charged drops are deflected transversely by an amount proportional to their charge. After traversing 10 cm of deflecting field, the trajectories of charged and uncharged drops are separated by a few millimeters. They may then be easily collected in separate collecting tubes positioned in the stream path.

Droplet size and spacing are made precisely uniform, and the point at which drops separate from the parent stream is stabilized by vibrating the nozzle assembly along its axis at a frequency of 40 kHz with a piezoelectric transducer. The resulting velocity modulation of the stream produces small variations in stream diameter. These are further amplified by surface tension forces to decompose the jet downstream from the observation point into very uniform droplets which form in synchrony with the applied vibration. Modulation of the stream diameter at the observation point modulates both the incident light and the fluorescent signal. To minimize this effect, the vibration amplitude is made as small as possible, consistent with stable and uniform drop formation, and the stream is illuminated soon after it leaves the nozzle.

Because the droplet geometry is uniform, the charge carried away by each droplet and thus its subsequent deflection are precisely determined by the charging voltage. The remarkable uniformity and stability of this process are shown in Fig. 3, a stroboscopic photograph of deflected droplets in flight.

Each charging pulse must arrive at the appropriate time to separate the droplet containing the selected fluorescent cell. Experiments have shown that the time delay between observation of a cell and its capture by a separating droplet
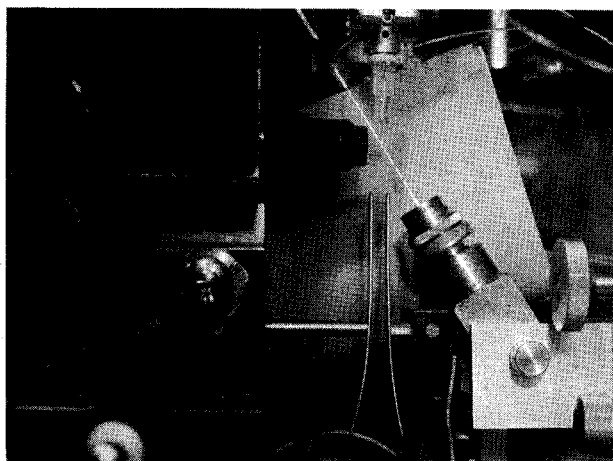

FIG. 1. Photograph of cell fluorescence sorter.

(typically 150 μsec) is predictable to within one drop period. A charging pulse width of 75 μsec, sufficient to charge three droplets, is used to ensure separation of the wanted cell. The deflection pulse generator is retriggerable so that cells following one another at less than 75 μsec intervals will result in the generation of an appropriately longer pulse.

Figure 4 is a block diagram of the signal processing electronics system. Signal pulses from a 1P21 photomultiplier tube are amplified (by an Ortec model 113 preamplifier and model 450 amplifier) to peak amplitudes between 0.1 and 10 V. The amplified signal feeds a "single channel analyzer" (Ortec model 406A) that generates a trigger for each input pulse between selectable amplitude limits, and a pulse height analyzer system (Ortec model 442 linear gate stretcher followed by Nuclear Data model 110 analyzer).
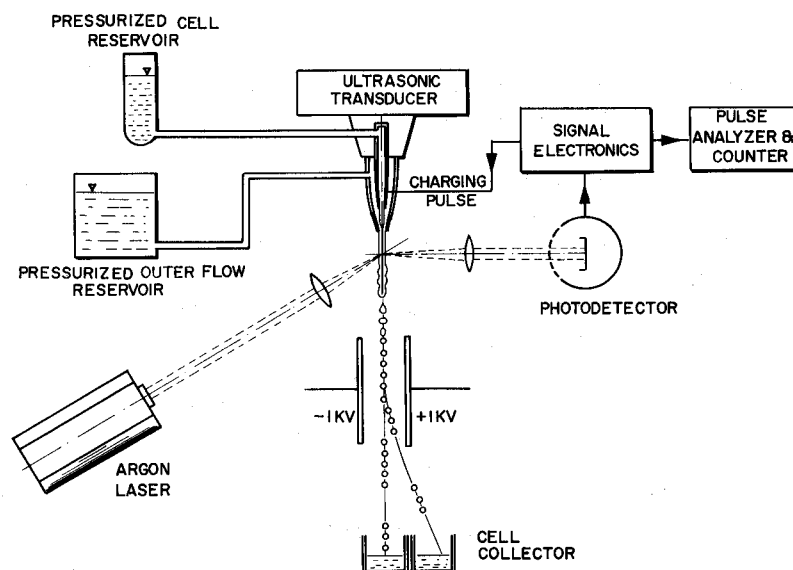

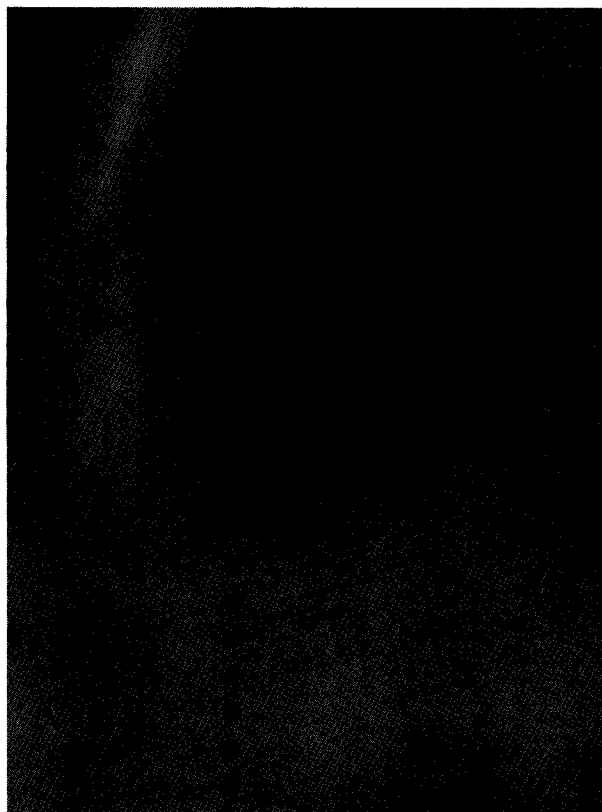FIG. 2. Simplified block diagram of cell sorter.

Fig. 3. Stroboscopic photograph of deflected and undeflected drops in flight.

Each trigger from the single channel analyzer, delayed by up to $400\,\mu$sec in a 0–64 bit shift register clocked at 160 kHz, initiates a drop charging pulse. A 40 kHz synchronizing signal permits on or off transitions of the charging pulse to occur only midway between formation of successive drops, ensuring that each drop that forms during the pulse is charged to the same value.

Efficiency of Separation

Unwanted cells from the input mixture divide between the deflected and undeflected output fractions in the ratio of deflected to undeflected droplet rates. The required purity of the deflected fraction sets a limit to the rate at which cells can be processed and makes a charging pulse of minimum width desirable. For example, if fluorescent cells are separated from a mixture at the rate of 2000 cells/sec, 6000 droplets/sec (neglecting the small number of cells spaced at less than $75\,\mu$sec intervals) must be charged. Since this is 15% of the 40 000 droplets generated per second, the deflected fraction will contain, in addition to the desired cells, 15% of the unwanted (passenger) cells from the input mixture. On the other hand if only 200 cells/sec are separated, only 1.5% of the passenger cells should be deflected.

scattering properties, and which will inhibit drop charging if an unwanted cell would otherwise be deflected along with a fluorescent cell.

Operational Considerations

The cells passing through the machine must retain both viability and function in order to be useful for many biological experiments. We have found it desirable to operate with cell and sheath flow suspensions and containers for the emerging streams in an ice bath. Under these conditions there appears to be little if any damage to the cells from passing through the unit. In common operation, we substantially reduce the time required to separate large numbers of cells by using two passes, processing the first time at high rates—several thousand per second—and then running the processed fractions through a second time to achieve the necessary purity. Even in this case there is no indication of cell damage.

Typical cell concentrations are in the range from 10^5 to 10^7 cells/ml, although up to 10^9 has been used without undue trouble from blockage. However since flow rates of the cell suspension are about 3×10^{-3} ml/sec, concentrations much higher than 10^7 will average over one cell in each drop generated.

Our experiments thus far have not required completely sterile conditions, but the unit has been designed so that it can be autoclaved for sterility if necessary.

We have experienced no difficulty with any of the commonly used cell suspending media. Obviously the ionic strength must be high enough to conduct the charging current, but this is always the case for living cell media.

Some work has been done to try to determine the minimum detectable number of fluorescing molecules. The simplest way to do this is to measure the weakest concentration of the free fluor which can be detected above the noise. In the case of fluorescein a 5×10^{-10} M solution in the inner stream gives a signal clearly above noise. Since the volume of solution observed is a cylinder roughly $50\,\mu$ long by $15\,\mu$ in diameter it contains about $10^4\,\mu^3$ or 10^{-11} liters,
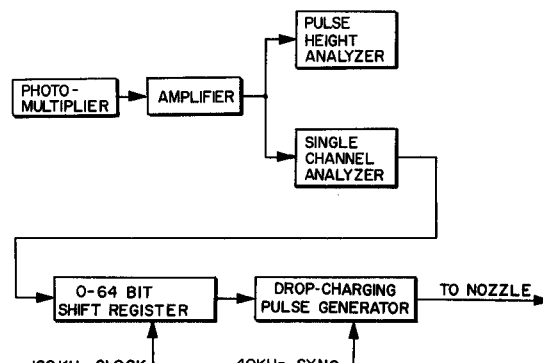
with some 5×10^{-21} moles or 3000 molecules. This number will usually be larger when the fluors are incorporated in a cell as there is almost always some fluorescent quenching.

Sensitivity and Coefficient of Variation of Cell Detection

Fixed quinacrine mustard stained chicken erythrocytes have been used as test objects in this system. Cells were fixed by diluting fresh chicken blood approximately 100/1 in cold 0.1% glutaraldehyde solution in normal saline. After 15 min the fixed cells were spun down and washed twice with distilled water, then resuspended in normal saline at a concentration of 10^8/ml. They were stained by spinning down 2 ml of this saline suspension, decanting the supernatant, adding 1 ml of 10^{-4} M quinacrine mustard to the pellet, stirring, allowing to stand overnight in the refrigerator, and then spinning down and washing twice with 5 ml normal saline.

A representative fluorescence amplitude distribution is shown in Fig. 5. The coefficient of variation is about 15%. In an effort to determine if this variation is inherent in the cells themselves or results from system variance we attempted to separate the cells giving signals above the lower half-amplitude point from the remainder. Fluorescence amplitude distributions from this experiment are shown in Fig. 6. It can be seen that the distribution for the original (curve A) the separated fraction (curve B) and the remainder (curve C) were somewhat different, indicating that at least some of the variations were inherent in the cells.

A sample of fluorescent beads of very uniform fluorescence was kindly provided by Dr. Scott Cram and Dr. Mack Fulwyler of Los Alamos Scientific Laboratory. We used these beads in order to explore the instrumental
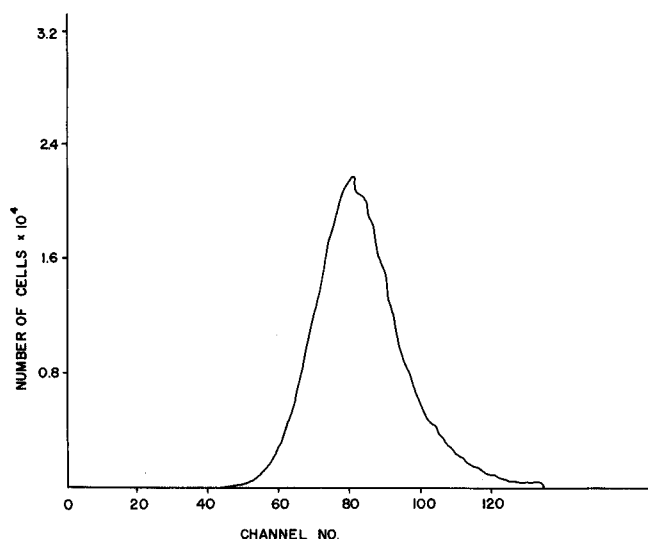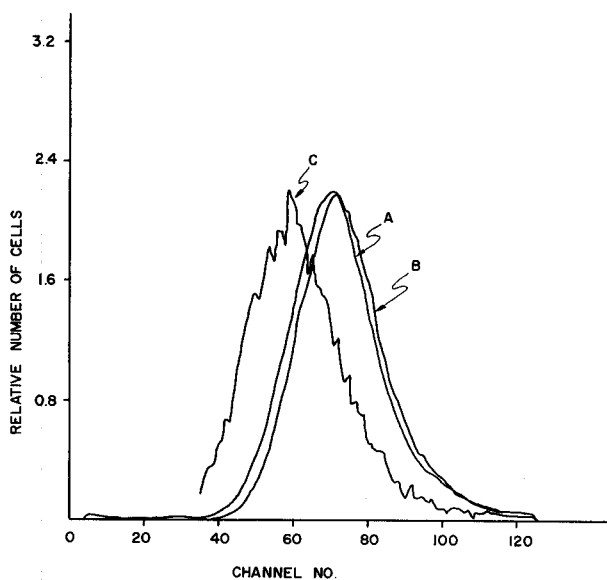

FIG. 6. Fluorescence amplitude distributions of quinacrine mustard stained chicken erythrocytes before and after separation. Curve A—original suspension; curve B—deflected fraction; curve C—undeflected fraction.

variations further. Their fluorescence amplitude distribution is shown in Fig. 7. The coefficient of variation of the main peak is less than 9%. This is probably primarily instrumental. This instrumental spread is small enough so that it has not been a major problem.

Figure 8 shows the theoretically attainable purity of separation (defined as the ratio of the fraction of desired to the undesired cells giving signals above the chosen threshold) as a function of the separation between two cell distributions assuming the following: (i) gaussian distribution of instrumental spread, (ii) equality of the intrinsic signals from a given cell population, (iii) no cell coincidence (as discussed above) during separation. Two threshold

FIG. 5. Fluorescence amplitude distribution of quinacrine mustard stained chicken erythrocytes.
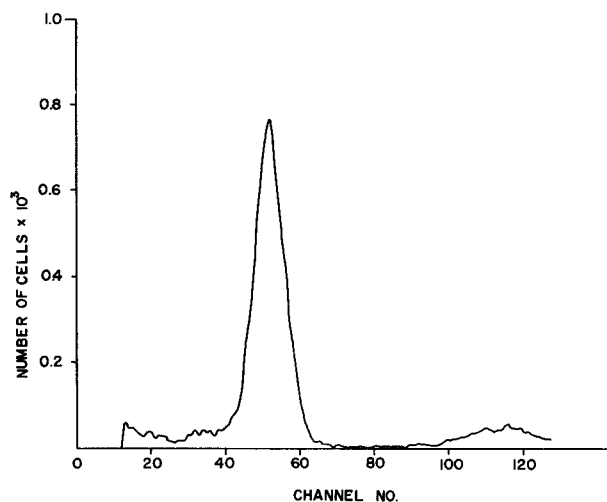

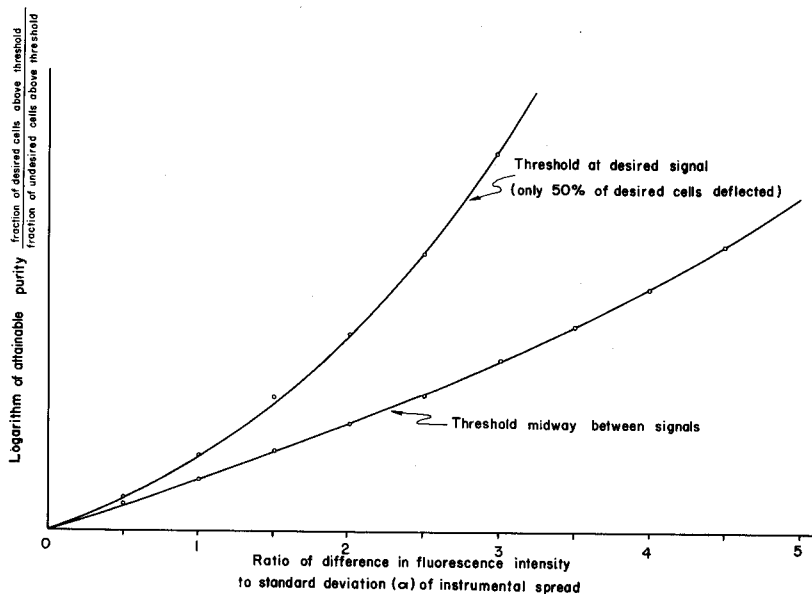FIG. 7. Fluorescence amplitude distribution of uniform beads.

FIG. 8. Attainable purity as a function of signal strength differences.

conditions were used for sample calculations—one halfway between the desired and undesired signals, the other at the desired signal. In the latter case only 50% of the desired cells will be separated but purity is much higher. Even in the first case, however, and even if the 9% coefficient of variation noted above were all instrumental, about 10 times as large a fraction of desired as undesired cells will give signals above threshold for an intrinsic signal difference of about 25%. This ratio becomes over 100 at 50%. We are usually looking for much greater signal differences than this.

The instrument has been applied to a number of separations of fluorescent from nonfluorescent cells. One of the most interesting uses the indirect immunofluorescent sandwich technique, whereby cells with surface sites binding particular antigens are rendered fluorescent by reacting first with the antigen, and then with a fluorescent tagged antibody[5] to the antigen. After labeling in this way,
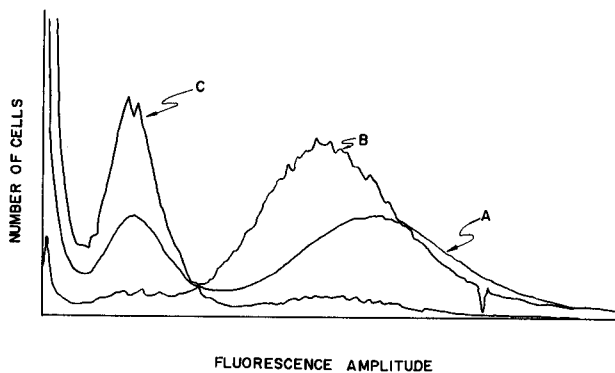

FIG. 9. Fluorescent amplitude distributions of concanavalin stained human leucocytes before and after separation. Curve A—original suspension; curve B—deflected fraction; curve C—undeflected fraction.

cells possessing particular immunological properties can be separated, a goal of immunologists for many years.

In a typical experiment an input sample containing 50×10^6 mouse spleen cells is divided into fluorescent and nonfluorescent fractions which are then injected into irradiated mice to evaluate immunological properties. As mentioned above we have found that the time required to separate such large numbers of cells can be reduced substantially by processing initially at high rates, several thousand/sec, and then achieving the necessary purity by reprocessing both the deflected and undeflected fractions. Even with two passes through the instrument there is practically no loss of cell viability or function. Experiments using this technique will be described more fully elsewhere.[6,7]

A somewhat similar technique uses fluoresceinated concanavalin A, a protein which binds to specific carbohydrates groups on cell surfaces, particularly chain end αD glucopyranosyl residues.[8] In this case separations can be made on the basis of the number of such sites which bind the fluoresceinated protein.

A preparation of human leucocytes treated in this way gave the pulse height distribution shown in curve A, Fig. 9. A separation was made, with the separation threshold set to the minimum between the two peaks. The efficiency of separation is shown in curves B and C, where B is the distribution of the deflected (large amplitude) cells and C is the distribution of the undeflected cells. The separation was obviously quite good. Microscopic examination showed that the deflected cells were over 90% granulocytes, the undeflected cells 90% lymphocytes, while the original suspension was about 75% granulocytes and 25% lymphocytes.

Other experimental and clinical applications of the separator are being investigated.

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