

Development and Application of a Rapid Cell Sorter

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We have further developed our rapid cell analyzer and sorter, so that we can now identify and separate functionally distinct groups of viable cells that have, or can be made to have, either different fluorescence intensities, different light-scattering characteristics, or different combinations of these two variables. In this instrument, cells are observed individually in suspension in the central stream of a very small coaxial liquid jet, as they pass through two laser beams. The jet is later broken into uniform droplets, and those droplets containing the desired cells are charged electrically and then deflected in an electric field. Several thousand cells can be processed per second. Enrichment factors of up to 500 and final purities and viabilities of 90% or more can be routinely achieved. Cell populations present in fractions as small as 1 in 10^5 or less can be identified. Radioisotope analytical techniques indicate that only a few thousand fluorescent molecules need be present on a cell for it to be detected. The instrument is described and various modes of operation and biological and clinical applications are discussed briefly.

Additional Keyphrases: *cell analyzer • fluorescence • light scattering • immunofluorescence • laser • tool for study of the immune system • screening prenatally for genetic defects*

In the same way that the spectacular advances in molecular biology were impossible until biochemists could separate functionally different molecules by such means as electrophoresis and ultracentrifugation, advances in cell biology have awaited development of instruments able to separate large numbers of functionally different viable cell types. Many workers have attempted to do this by bulk methods, but the resolution of such methods is limited. It appeared to us that a better approach to the problem was to inspect the cells individually and sort them on the basis of their individually measured characteristics. The characteristics we chose to use were fluorescence, which can be used to detect functional differences after reacting the cells with appropriate fluorescent-labeled proteins, and small-angle forward light scattering, a function of particle size. A previous version of our separator, in which only fluorescence was used as the basis of separation, was described in the article by Bonner et al. (1).

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Materials and Methods

Instrument Description

A simplified block diagram of the present unit is shown in Figure 1. Cells in liquid suspension are forced under pressure through a micronozzle into the center of a stream of cell-free fluid, and then out an effluent nozzle, 50 μm 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 40,000 hertz, breaking the jet into 40,000 uniform droplets per second. Immediately below the nozzle, before droplet formation occurs, the jet is illuminated by two lasers. Low-angle scattered light from one, a helium-neon unit operating at 632.8 nm, falls on a red-sensitive photodetector, producing a signal related to cell volume. The second laser, an argon ion unit operating at one of a number of lines between 454 and 514 nm, excites fluorescence in cells tagged with appropriate fluorescent material. Some of the fluorescent light falls on a second photodetector, providing a signal proportional to the number of fluorescent residues on the cell. Another fluorescence channel, operating in a different wavelength band, is available if desired. Signals produced in the scatter and (or) fluorescent channels are processed, delayed, and combined as required to produce electrical pulses, which are used to charge the liquid stream at the time the droplet containing the desired cell is forming. Droplets broken off while the stream is charged retain their charge. Further downstream, the droplets pass through an electric field between

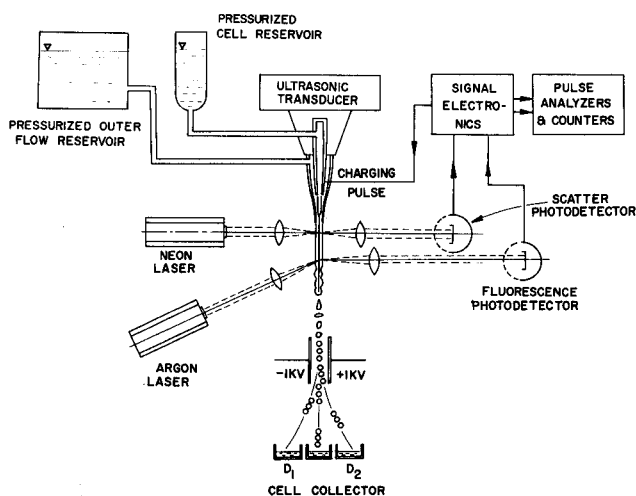


Fig. 1. Simplified block diagram of the cell sorter

two charged plates. Charged droplets are deflected appropriately, while uncharged droplets continue on their original course. To ensure that the droplet containing a desired cell is charged—and thus deflected—the charging pulse lasts for three droplet periods, centered on the time the cell is expected to enter a droplet. The charging pulses are synchronized with the ultrasonic droplet generator so as to ensure that all drops formed are equally charged. Further details of basic instrument operation can be found in the article by Bonner et al. (1).

Modes of Operation

Most present separations involve use of both scatter and fluorescent signals. A lower scatter threshold is set at a signal level corresponding to the size of the smallest cells desired. An upper scatter threshold can also be set. Corresponding thresholds are set for the fluorescent signal channel. Unless a signal within the recognized scatter limits is received, no drops will be deflected in either direction. If only a scatter signal of correct magnitude is received, the appropriate string of three droplets is deflected in one direction (D_1). If both scatter and fluorescent signals are received the droplets are deflected in the opposite direction (D_2). If a second particle with different fluorescent or scatter classification than the first one passes through the beam within 75 μ s (3 droplet periods), the deflection circuits are disabled during the time the deflection pulses would overlap, and any droplets generated during this time will not be deflected.

When this procedure is used, the D_2 fraction will consist almost exclusively of fluorescent cells and the D_1 fraction almost exclusively of nonfluorescent cells in the appropriate size range. The undeflected fraction will consist of droplets for which no charging pulse was generated, either because they contained no cells of appropriate characteristics or because there was a high probability that they contained both D_1 and D_2 cells.

This mode is not used with concentrated samples containing a relatively low proportion of fluorescent (D_2) cells, because much of the time under these conditions the train of droplets containing the fluorescent cell will also contain a second nonfluorescent cell, and the D_2 deflection circuits will be disabled. Often such samples are run through the separator with only the fluorescence channel providing deflection signals. This initial pass provides a suspension enriched in the desired cells by factors of 10 to 50, depending on initial proportions and separation parameters. A second pass with the usual mode will then provide the desired high purity. Even dual passes seem to have little effect on cell viability, which usually is in the 80–90% range both before and after passing through the instrument, as determined by trypan blue dye-exclusion tests.

Additional modes of operation can be used if de-

sired. Four single-channel analyzers, each capable of providing both an upper and a lower limit, are available. It is thus possible to deflect into the D_2 fraction only cells within relatively narrow scatter and fluorescent limits, and into the D_1 fraction only cells below a much lower fluorescent limit (and if desired within different scatter limits). A variety of other combinations can be used, if desired, for special separations.

Separation of Doubly-Labeled Cells

A second fluorescent channel was provided to make possible simultaneous separation of different fluors excited by the same input radiation. However, we have found in the separations we have actually made—involving cells tagged with fluorescein and (or) with tetramethyl rhodamine (TMR)—that we can get a more distinct separation if we use different exciting wavelengths and stain the cells sequentially. We find the optimum procedure to be an initial stain with TMR followed by a separation with use of a 514-nm laser line. The separated fractions can then be tagged with a different molecule that carries fluorescein, and run through the sorter, with use of a 488-nm laser line and a barrier filter that passes only those wavelengths between about 510 and 560 nm. With this procedure the fluorescent output of TMR is only about $\frac{1}{40}$ as much on the second pass as on the first. No fluorescein is present on the first pass, so it causes no interference in determining TMR.

Results and Discussion

Sample Purity and Enrichment

The purity of the D_2 fraction is limited by the time constants of the electronic channels. We are presently able to differentiate two signals about 15 μ s apart in either channel. At our usual separation rates of a few thousand cells per second, this means that a few percent of the time the sensing circuits are essentially disabled. Because the D_1 cells are distributed randomly, a few percent of these cells will thus wind up in the D_2 receptacle. Routinely, more than 90% of the cells in the D_2 fraction are fluorescent (as determined either by microscopic examination or analysis on the separator), and less than 0.1% of the cells in the D_1 fraction are fluorescent.

Total enrichment depends on initial concentration and the separation parameters chosen. We have produced an enrichment of over 500 in a single pass (2).

Minimum Detectable Number of Molecules

The minimum detectable numbers of residues of TMR and of fluorescein have been determined, taking advantage of the affinity of concanavalin A, a protein from jack bean meal, for carbohydrate residues on the cell surface (3). The concanavalin A was labeled with both ^{125}I and with one of the fluorochromes in the form of the isothiocyanate. After labeling, the number of TMR or fluorescein residues

per protein molecule was determined spectrophotometrically, and the number of ^{125}I atoms per molecule by use of a well-type scintillation (gamma) counter. The cells were then incubated with the concanavalin A in appropriate concentrations, washed, counted, and the number of concanavalin A molecules (and thus of fluor molecules bound) was determined by a second gamma count. We then calculated the average number of fluor molecules per cell. The cells were also run through the separator and pulse height analyses taken, giving the number of pulses as a function of the pulse amplitude. A typical result is shown in Figure 2, where the abscissa represents pulse amplitude, proportional to channel number, and the ordinate represents number of pulses or cells in a given channel or amplitude increment. The relative number of pulses increases rapidly at low pulse amplitudes because of background noise. The average signal amplitude, produced by fluorescent cells, corresponds approximately to the channel containing the peak number of signal pulses. The effective noise threshold was taken as the channel in the noise-produced portion of the distribution that contained half that many noise pulses. There were about 20 times as many signal as noise pulses above this threshold. While this choice of threshold may appear conservative, a threshold allowing half as many noise as signal pulses would only have been about 20% lower in amplitude.

The first tests were made utilizing only the fluorescent signal. Considerable improvement of signal-to-noise ratio resulted when the usual dual-channel mode of operation was used. In this mode the fluorescent channel is not turned on unless a scatter signal indicates that a cell is present, so far fewer noise pulses occur in the noise amplitude spectrum. The effective noise threshold is about $\frac{2}{3}$ of that in the mode in which only fluorescence is measured.

Several samples were run with various concentrations of fluor on their surfaces. The ratios of the sig-

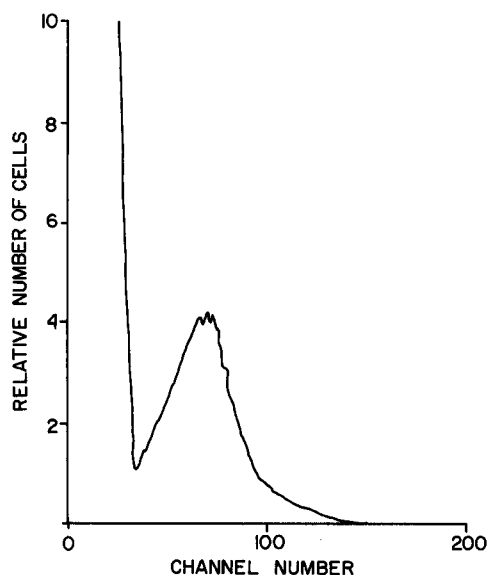


Fig. 2. Pulse height analysis

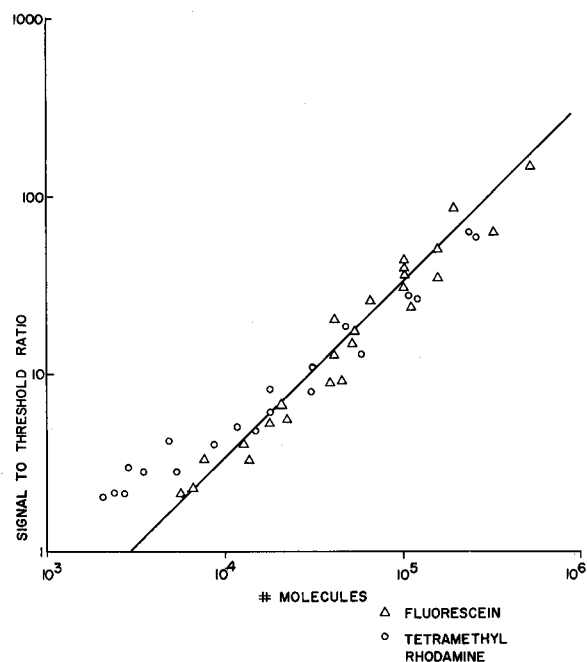


Fig. 3. Ratio of signal to minimum detectable signal as a function of the average number of fluorescent residues per cell

nal found to the minimum detectable signal were calculated for each sample. Figure 3 shows these ratios as a function of the number of molecules bound per cell.

As shown in Figure 3, a few thousand molecules of either fluorescein or TMR on a cell should be detectable. When it is considered that several molecules of fluor can be conjugated with each protein molecule, and that a further amplification factor can be obtained by using "sandwich" immunofluorescence techniques, it appears that cells with a few hundred active sites on their surface may be separable under ideal conditions by using this equipment. Preliminary experiments in which we used cells reacting with radioactive T, G, A-L, a synthetic protein, and then with a fluoresceinated anti-T, G, A-L, confirm this number.

Biological and Clinical Applications

A number of interesting experiments have been performed by using the equipment to study the immune system. Many of these involve the relative roles of "B" cells and "T" cells. B cells are cells originating in bone marrow, which are believed to have large numbers of antibody molecules on their surfaces and to be precursors to antibody producing cells; T cells are thymus-derived cells believed to have smaller amounts of surface antibody and to act as needed cooperators with the B cells in immune response. In early experiments we obtained suspensions of spleen cells from mice that had been primed (i.e., previously injected) with human serum albumin. B cells in these suspensions should be capable of combining with much human serum albumin, while the T cells should combine with very little.

The suspensions were incubated with human serum albumin, and then with fluorescein labeled goat anti-human serum albumin, and the fluorescent (presumably B) cells were separated by the machine. Neither the D₁ (T and other cells) or D₂ (B cells) fraction proved able to give rise to antibodies to human serum albumin when injected into an irradiated host mouse, but when the two fractions were combined, or when the fluorescent (B cell) fraction was combined with thymus (T) cells from an unprimed mouse, the response was restored. These data accord with the hypothesis stated above. Similar findings have been obtained with other antigens (2).

In another series of experiments we used the ability of the instrument to distinguish between two flours to obtain, from heterozygous rabbits, populations of lymphocytes with one or the other or both of two cell-surface immunoglobulin allotypes. We found the lymphocytes showing both allotypes were probably artifacts, because when these cells were cultured after surface proteins were removed by treatment with proteolytic enzymes (Pronase), one or the other allotype reappeared on each cell, but not both. When the separated cells were injected into irradiated rabbits of a third allotype, cells bearing a given allotype appeared to give rise to cells producing only that allotype, thus providing evidence that cells are programmed or committed to produce the allotype that they bear (4).

A series of experiments was conducted to determine the minimum detectable leakage of Rh⁺ fetal cells into an Rh⁻ maternal circulation. In these tests Rh⁺ antibody was added to suspensions of Rh⁻ cells containing various proportions of Rh⁺ cells, and the suspension was then treated with fluorescent goat antihuman gamma globulin, rendering fluorescent those cells with Rh⁺ antibody on their surface. Results showed that Rh⁺ cells could be detected easily by the separator at dilutions of 10⁻⁵, and possibly at dilutions of 10⁻⁶.¹

Some difficulties were initially encountered in achieving sufficiently aseptic operation to permit culture of separated fractions, but we have now had a dozen sequential aseptic separations without contamination. Treatment of separated cells with mitogens such as phytohemagglutinin and pokeweed mitogen shows that these substances can successfully stimulate division in T cells free of B cells but not in

B cells free of T cells.² However, monocytes (about 10%) are required for T cell stimulation. Interferon is also produced in T but not B cell cultures. This work paves the way towards characterization of human leukemias as to T and B type.

Work has started on using the separator to fractionate fetal lymphocytes from maternal peripheral blood, using HL-A³ antigens as markers. There are reports that a usefully large proportion of such fetal lymphocytes (in the range of tenths of a percent) are found early in pregnancy (5). Preliminary experiments indicate that a sandwich technique, in which maternal lymphocyte suspensions are treated with antiserum to the father's HL-A antigens, then with fluoresceinated rabbit anti-human gamma globulin, and run through the separator, should be able to provide considerable enrichment of the fetal cells. It may be possible to use this technique eventually to provide fetal cell cultures for use in prenatal diagnosis of chromosome abnormalities, permitting use of maternal blood samples as a substitute for amniocentesis, and early abortion when a fetus exhibits Down's syndrome or other chromosome defects.

We conclude that the cell separator we have developed can be a very useful tool in a number of biological and clinical areas, ranging from basic research in the immune system and its role in such problems as transplant rejection, to routine use as a substitute for amniocentesis in screening for such defects as Down's syndrome.

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² Kreth, W., and Epstein, L. B. (in preparation).

³ HL-A is the designation for the major gene locus for the system of leukocyte antigens related to human histocompatibility.