Cell Sorting: Automated Separation of Mammalian Cells as a Function of Intracellular Fluorescence

#48

Abstract. *A system for high-speed sorting of fluorescent cells was able to sort mouse spleen cells from Chinese hamster ovarian cells after development of fluorochromasia. Highly fluorescent fractions separated after similar treatment from mouse spleen cells immunized to sheep erythrocytes were enriched in antibody-producing cells by factors of 4 to 10.*

Separation of large numbers of functionally different cell types from the complex mixtures found in such organs as spleen, bone marrow, lymph nodes, liver, or kidney would be useful in biological and biochemical investigations. Direct fractionation methods, such as differential or isopycnic centrifugation, column fractionation, electrophoresis, and so forth, allow separation of large numbers of cells but produce only limited resolution of functionally different types. We are exploring a directed cell-sorting method where various parameters or criteria for separation can be used with a common sorting mechanism based on electrostatic deflection of charged droplets containing the cells. Here we report the use of intracellular fluorescence developed after exposure to the fluorogenic esterase substrate, fluorescein diacetate (FDA), as the parameter for directed sorting of cultured Chinese hamster ovarian (CHO) tumor cells from mouse spleen cells, and for concentration of antibody-producing hemolytic plaque-forming cells from spleens of mice sensitized to sheep red blood cells.

Live cells incubated with FDA accumulate fluorescein intracellularly since FDA can enter the cells and be hydrolyzed by intracellular esterases, but the fluorescein thus produced cannot readily leave through intact cell membranes. This phenomenon has been called "fluorochromasia" (*1*).

In these experiments a suspension of cells, after FDA treatment, passes through a small glass nozzle, forming a liquid stream. This stream is darkfield illuminated by an exciting beam of blue light from a mercury arc source, and the yellow-green fluorescence emitted by the cells is focused by a microscope on a photomultiplier tube through a yellow barrier filter. When a fluorescent cell generates a signal of designated amplitude at the photomultiplier a charging voltage pulse is applied to the stream. The stream breaks into droplets at the frequency of a low-power ultrasonic vibration applied to it, and those droplets formed while the charge is applied, including the one containing the fluorescent cell, remain charged after separation from the stream. These are deflected by an electric field between a pair of statically charged deflection plates and collected in a separate container from that collecting the undeflected droplets. The droplet-separating system is modified from that described by Sweet (*2*) and adapted by Fulwyler (*3*) for another cell-sorting system. Observation of fluorescence and other optical characteristics of cells in flow systems has previously been reported by Kamentsky *et al.* (*4*) and Van Dilla *et al.* (*5*).

The cell suspensions used were diluted to about 10^7 cells per milliliter, in Eagle's minimal essential medium containing 5 percent fetal calf serum. To develop fluorochromasia, 0.2 percent of a 0.5 percent solution of FDA in acetone was added and the samples were incubated at room temperature (23°C) for periods of 5 to 30 minutes (longer periods were found to be necessary for full development of fluorochromasia in small cells). The samples

Table 1. Sorting of Chinese hamster ovarian (CHO) tumor cells from mouse spleen cells by fluorescence intensity, compared with sorting such cells by volume.

Cells	Cells exceeding threshold (percent)	
	Fluorescence* (cell sorter)	Volume† (electronic counter)
Mouse spleen	2	13
CHO	90	70
1 : 1.2 mixture of spleen and CHO tumor (before sorting)	47	48
Low-fluorescence fraction	2	23
High-fluorescence fraction	71	66

* These data were taken at the threshold used for detection. † The threshold was chosen to discriminate optimally between the signals generated by spleen cells and CHO tumor cells.

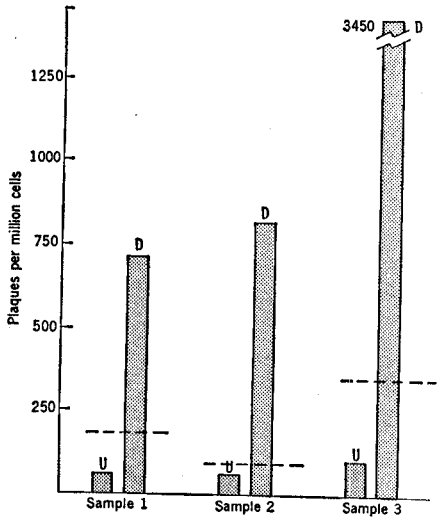

Fig. 1. Plaques per million cells in deflected and undeflected fractions, compared to original. *U*, Undeflected fraction; *D*, deflected fraction; ---, original. Ratio of *D* to *U* is 14 for sample 1, 16 for sample 2, and 34 for sample 3.

were then diluted in the same medium to a concentration of about 10^5 cells per milliliter and kept in the cold throughout the sorting operation. Flow rates were of the order of 1 ml/min.

In Table 1 we compare the efficiency of discrimination of mouse spleen cells from CHO tumor cells by volume and fluorescence intensity and show the efficiency of sorting by fluorescence intensity. Thresholds of volume and fluorescence intensity were chosen to best discriminate between the two cell types. The best fluorescence threshold was exceeded by the fluorescent signals given by 90 percent of the CHO tumor cells and by only 2 percent of the mouse spleen cells. The corresponding volume measurements were 70 percent and 13 percent respectively. A mixture of the two cell types was sorted into fractions containing cells exceeding and not exceeding the chosen threshold of fluorescence intensity. Analysis of the fluorescence intensity of the cells in the two fractions, presented in Table 1, showed efficient sorting. About 80 percent of the fluorochromatic signals were still detectable on a second passage through the instrument, indicating little cell damage. This was confirmed by a trypan blue dye exclusion test which showed that over 80 percent of the cells in both the initial sample and the deflected fraction were viable.

The poor correlation of volume with fluorescence intensity is also shown in Table 1. Although there is relatively good sorting of the small mouse spleen cells from the large CHO tumor cells in this experiment, the intensity of fluorochromasia is not a function of size alone. This has been demonstrated in several separations. For example, a suspension of spleen cells was sorted, deflecting only those cells giving signals above an arbitrary threshold (about 15 percent of the population). There was relative enrichment of the largest cells, but the concentration of even the smallest cells in the deflected sample (as measured by the Coulter counter) was actually two and one-half times as great as that in the control. Most of these small cells must have been among the most fluorescent 15 percent and thus more fluorescent than many larger ones, indicating increased esterase activity or different membrane characteristics than average cells.

In a search for other biological activity associated with high fluorochromatic ability, cell samples from spleens of mice immunized with sheep erythrocytes were prepared at times from 4 to 9 days after injection and run through the instrument, which deflected the most fluorescent 8 to 10 percent of the cells. The enrichment of highly fluorescent cells in the deflected sample, and their depletion in the undeflected sample, were confirmed on a rapid cell spectrophotometer similar to that described by Kamentsky *et al.* (*4*, *6*). The ability of the cells to form hemolytic complement dependent plaques was then assayed by using a modification of the Jerne hemolytic plaquing technique (*7*). Results are shown in Fig. 1. All deflected fractions contained much higher proportions of plaque-forming cells than the original samples, while the undeflected samples were correspondingly depleted. Apparent recovery of plaque-forming cells in the two sorted fractions varied from 60 to 110 percent of the number in the unseparated control. While there were more than ten times as many cells in the undeflected samples as in the deflected samples, the former always contained fewer of the plaque-formers than the latter. Since on the 4th day after immunization most of the plaque-forming cells produce 19*S* antibody while on the 9th day most produce 7*S*, cells producing antibody of both types were enriched in the deflected sample.

Although the present version of the sorter has only two sorting channels, sequential passes allow multiple fractions to be obtained. Simple modifications will permit a simultaneous collection of several fractions. Use of fluorochromasia from FDA as the sorting parameter in this cell sorter may have other applications in selecting functionally different groups of cells. The possibility of substituting other fluorogenic substrates for FDA should be considered as well as the use of other fluorescent dyes and of fluorescent antibody techniques.

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References and Notes

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