Reprinted from
ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Volume 254, Pages 163–171
June 30, 1975

ANALYSIS OF CELL POPULATIONS WITH A FLUORESCENCE-ACTIVATED CELL SORTER

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INTRODUCTION

Cell biologists have long been concerned with morphologic and functional characterization of the different cell types present in various tissues. Immunofluorescence has been applied to this problem to differentiate cell populations according to their various antigenic characteristics. Visual methods of detecting the fluorescence have limitations, because classification of a cell as fluorescent or nonfluorescent can be subjective. In addition, fluorescence microscopy is not readily adapted to the quantitative analysis of fluorochromes on different cells. A fluorescence-activated cell sorter (FACS), designed to characterize and isolate viable cells, can be used to obviate some of the analytic difficulties encountered with visual methods.

The FACS analyzes a cell preparation with respect to two parameters in a quantitative rather than qualitative manner. It also permits physical separation of these cells for further study. Individual viable cells, stained by conventional immunofluorescence techniques, are analyzed for both fluorescence and low-angle light scattering as they pass through a laser beam. The frequency distributions of these two parameters are used to characterize the cell population. The criterion for denoting a specific subpopulation is obtained from these histograms, so that the classification of cells into one or another category is more objective compared with visual techniques. Subpopulations of cells identified by this type of analysis can then be isolated with the FACS and assayed for various functional activities.

MATERIALS AND METHODS

Instrument Description

The instrument used in these experiments was built by Becton-Dickinson Electronics Laboratory (Mountain View, Calif). It is essentially the same as that described previously;[1] however, a single argon ion laser is used for both the scatter and fluorescence channels.

Cells to be analyzed or separated are prepared and stained in suspension by conventional immunofluorescence techniques. This suspension of cells is forced under pressure through a micronozzle, in which the cells are centered in the

* Supported by a grant from the Jane Coffin Childs Memorial Fund for Medical Research and by National Institutes of Health Grant GM 17367.

† Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

5 mg/ml. Two λ of this solution were added to 2×10^7 cells in 1 ml of Dulbecco's phosphate-buffered saline solution[9] that contained 5% heat-inactivated fetal calf serum. This suspension was incubated for 15 min at room temperature and then washed three times. Cells to be stained with R α M Fab^F were pelleted and resuspended in the reagent with 100 μl/2×10^7 cells. After a 15-min incubation at 0°C, the cells were pelleted through fetal calf serum and washed once more with phosphate-buffered saline.

Sheep red blood cells (SRBC) were obtained from W. T. Bennet Ranch Laboratory (Woodland, Calif.) and washed three times with phosphate-buffered saline. Two $\times 10^7$ cells were incubated in 1 ml of a 1 : 100 dilution of mouse anti-SRBC produced in BALB/cN mice. After a 15-min incubation at 0°C, the cells were washed twice and stained with [^{125}I] R α M Fab^F in the same manner as the spleen cells.

Red cell concentrations were determined by lysing the cells with distilled water and comparing the absorbance at 414 nm to a standard curve obtained with the same preparation of SRBC.

Histogram Comparison

Prior to each experiment, the FACS was adjusted to give the same scatter and fluorescence profiles with a standard preparation of glutaraldehyde-fixed chicken red blood cells. Because the SRBC are smaller than spleen cells, the amplification used to collect the curve in FIGURE 4 was twice that used in FIGURES 1 and 3. To place them on the same scale, the channel numbers in FIGURE 4 should be divided in half.

The standardization curve in FIGURE 6 was calculated in terms of a fluorescence amplifier gain of 50. The curve in FIGURE 2 was collected at a gain of 20. To compare this curve with the standardization, the channel numbers must be multiplied by 2.5. The curve in FIGURE 5 was collected at a gain of 10, so these channels must be multiplied by five.

ANALYSIS OF CELLS WITH LOW-ANGLE LIGHT SCATTERING

The amount of light scattered by a particle is related to the size and shape of that particle. For spheres with physical characteristics similar to cells, the amount of light scattered in a forward direction is a function of both volume and refractive index.[10,11] Although the low-angle-scattered light increases with cell size, the relationship between cell volume and amount of scattered light collected from 0 to 12° is not linear. Thus, the light-scattering channel does provide cell size information, but it may not always be directly proportional to cell volume. Additional information can be obtained from the light-scattering channels, because it can be used to distinguish viable from nonviable cells and to distinguish certain cell types.

Preparations of lymphocytes from murine spleen, thymus, and lymph node all yield scatter histograms that have at least two peaks (FIGURE 1,A). The two populations have been identified as viable and nonviable cells.[12] When incubated with the fluorogenic compound, fluorescein diacetate, only live cells will become fluorescent.[13] The scatter histogram of viable cells can then be generated by recording only the scatter signals of cells made fluorescent by this compound.

A comparison of the scatter profile of the total cell population with that of the fluorescent cells alone is shown in FIGURE 1. The heights of the peaks have

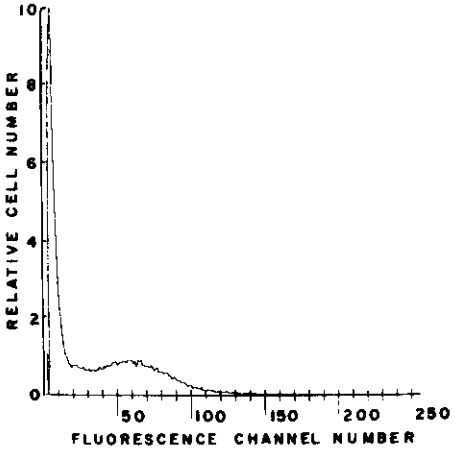

FIGURE 2. Fluorescence distribution of spleen cells stained with fluoresceinated rabbit antimouse Fab serum. More than half (51%) of the cells have fluorescence less than channel 25. The fluorescence at channel 50 is equal to 8×10^6 molecules of fluorescein.

channel 25, comprise 49% of the total. These numbers are in agreement with other methods of quantitating immunoglobulin-positive cells.[15]

There is an obvious overlap between the fluorescence distributions of stained and unstained cells. The threshold over which a cell is said to be fluorescent is arbitrarily set. The shape of the distribution does indicate where the threshold should be; however, a mathematical separation of the two curves would give an accurate count of fluorescent and nonfluorescent cells.

Although a fluorescent population can be observed with the FACS, no fluorescence of this sample could be detected by fluorescence microscopy. It is evident that the FACS is more sensitive than the microscope in detecting these fluorescent cells. The lower limit of detection with the FACS is discussed in the next section.

In addition to a fluorescence difference, the scatter profiles of the immunoglobulin-positive and- negative populations also differ. The scatter profile of cells that fluoresce from channels 0 to 20 (FIGURE 2) is shown in FIGURE 3,B. The immunoglobulin-positive cells, which have fluorescence greater than channel 30, have the scatter profile shown in FIGURE 3,A. It is evident that those cells that bear immunoglobulin on their surface are somewhat smaller than the immunoglobulin-negative cells. The same results have been obtained when the staining was accomplished with a multispecific rabbit antimouse immunoglobulin serum and an anti-Thy-1.2.[12] These results agree with the data of Howard *et al.*,[16] who used unit gravity sedimentation to separate lymphocyte populations. Combination of the scatter and fluorescence signals provides additional information about the cell populations.

QUANTITATION OF THE FLUORESCENCE SIGNAL

To use the FACS in a quantitative manner, it is necessary to know if a linear relationship exists between the fluorescence signal and the number of emitting fluorochromes. For an absolute standardization, this relationship must be quantitated. With these data, it is also possible to estimate the minimum number of molecules that can be detected above noise.

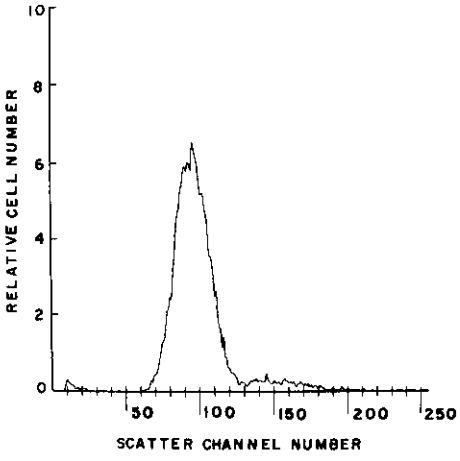

FIGURE 4. Scatter distribution of SRBC stained with mouse antisheep red blood cell serum and fluoresceinated rabbit antimouse Fab. The singlet peak occurs at channel 96, whereas the doublet peak is at channel 150.

were collected over a 6-day period with two different preparations of the [^{125}I]R α M Fab^{F}. In two experiments, the radioactive reagent was diluted with cold reagent. There was no significant difference between these experiments, which demonstrates that the specific activity of the whole reagent is the same as that of the antibodies that bind to the cells. Iodination of the reagent did not destroy antibody activity. The line drawn through the data points is the least-squares analysis with a calculated intercept at the position of the peak due to noise.

The data are plotted as a function of position of the singlet peak. The standardization can therefore be extended by a factor of two, because doublets give a signal that is twice the amplitude of singlets. This extends the standardization through the full range of amplification of the instrument.

The minimum number of molecules that can be detected with fluorescein is estimated to be between 3000 and 5000. This finding is in agreement with the

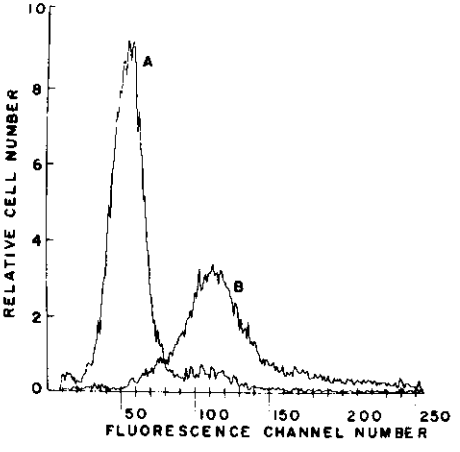

FIGURE 5. Fluorescence distribution of the cells described in FIGURE 4. Curve A is the total fluorescence. Curve B is the fluorescence of cells with scatter greater than channel 125 (FIGURE 4). The fluorescence of the doublets is twice that of the singlets.

Absolute standardization also permits the determination of the amount of antigen on cell surfaces. With knowledge of the number of antibodies that bind to an antigenic site and the molar dye to protein ratio, the fluorescence observed by the FACS can be related to the number of antigens on the cells. The distribution of the antigen among the cell population can be ascertained from the fluorescence histogram.

ACKNOWLEDGMENT

The authors appreciate the expert technical assistance of Mr. F. K. Assisi.

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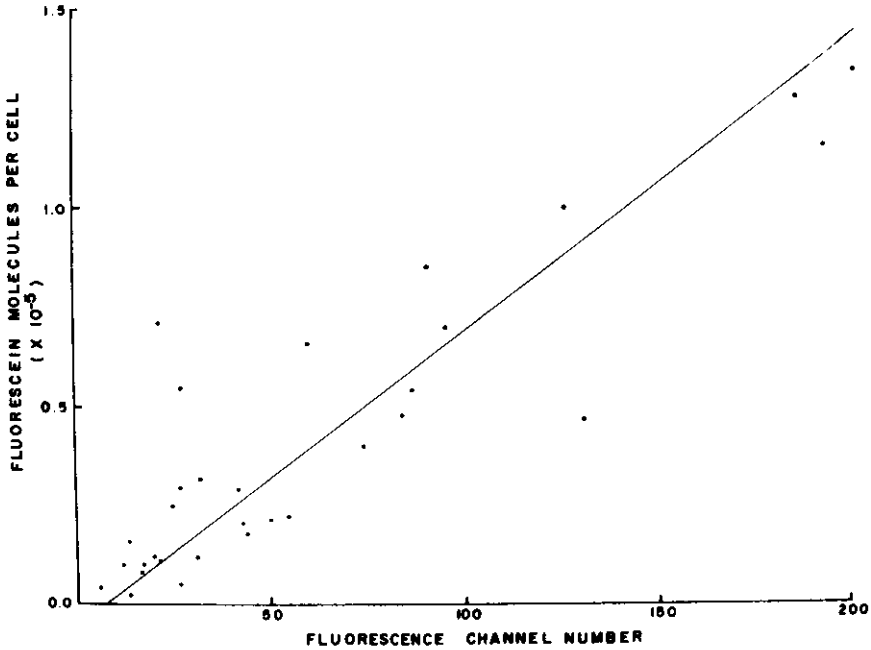

FIGURE 6. Standardization of the fluorescence channel with a reagent doubly labeled with ^{125}I and fluorescein. The fluoescence channels are calculated for an amplifier gain of 50. The least-squares analysis of the data has an intercept the same as the peak due to noise.

data of Hulett *et al.*,[1] who used the binding of concanavalin A labeled with fluorescein and ^{125}I for their standardization.

The instrumental noise is only partly due to electronics and randomly scattered light. At high amplification, unstained cells give a signal that is higher than background noise. This low-level signal, which has been attributed to autofluorescence, appears with all cell types studied so far. This signal can be eliminated by fixation of the cells with 0.1% OsO_4 for 5 min. These fixed cells no longer exhibit autofluorescence and can be stored for months without change in their optical or physical characteristics. These cells are used as a nonfluorescent standard.

Fluorescence standards have been prepared by taking osmium-fixed SRBC and coupling fluorescein to them with FITC at various concentrations. These samples, calibrated during the standardization of the instrument, can be used to check the alignment and functioning of the machine prior to analysis of other cell populations.

Because the fluorescence is linearly related to the number of dye molecules, the FACS can be used to titrate sera. Osmium-fixed cells can be coupled with the hapten dinitrophenyl. Anti-dinitrophenyl antibody can then be coupled to these cells in various dilutions. Total immunoglobulin bound to the cells is determined with the R α M Fab^F. Specific classes or allotypes can also be determined with class- or allotype-specific fluoresceinated antisera for the second step.

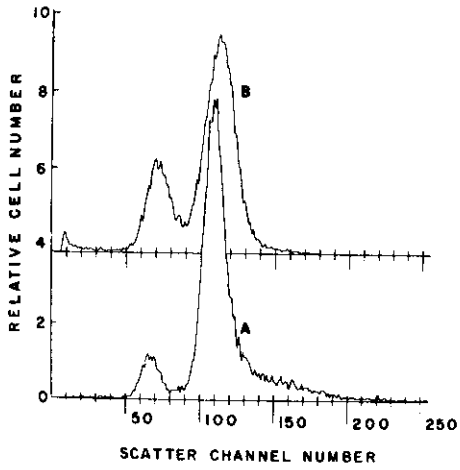

FIGURE 3. Scatter histograms of immunoglobulin-positive cells (A) and immunoglobulin-negative cells (B).

The method chosen for standardization of the instrument involves the independent determination of the number of fluorochromes in a sample with a reagent that has been labeled with both fluorescein and ^{125}I. The reagent used in this standardization is a routinely used serum, R α M Fab^F, described in the preceding section. SRBC are incubated with mouse anti-SRBC serum to coat the cells with mouse immunoglobulin. These coated cells are then incubated with varying concentrations of the doubly labeled reagent [^{125}I] R α M Fab^F. The radioactivity per cell is determined and the sample analyzed by the FACS. From the specific activity of the serum, the average number of fluorescein molecules per cell is calculated. This number is plotted as a function of peak position as determined by the FACS.

The scatter distribution for a typical sample is shown in FIGURE 4. The major peak is due to single cells, and a second peak results from two cells attached together. An unstained sample of SRBC does not contain this second peak. The position of the doublet peak is not twice that of the singlet peak, which indicates that the amount of scattered light is not directly proportional to the particle volume.

The fluorescence distribution for this sample is shown in FIGURE 5,A. The fluorescence profile is bimodal, with the second peak in a channel two times that of the first peak. The position of the second peak can be more easily determined by looking only at doublets. Setting the threshold of the scatter at channel 125 (FIGURE 4) allows the fluorescence of only the doublets to be analyzed. The scatter-gated fluorescence distribution of those cells is shown in FIGURE 5,B. The doublets yield a fluorescence signal twice that of singlets, which indicates that the fluoresence signal, unlike the scatter signal, increases linearly. This linearity between singlets and doublets was observed for all samples, regardless of staining concentration or amplification needed to observe the fluorescence.

One of the criteria for valid standardization has been met, in that the fluorescence is unimodal and the position of the peak can be accurately determined. This position can be checked by comparing it to the position of the doublet peak.

The relationship between fluorescence signal and number of fluorescein molecules, as determined by radioactivity, is shown in FIGURE 6. These data

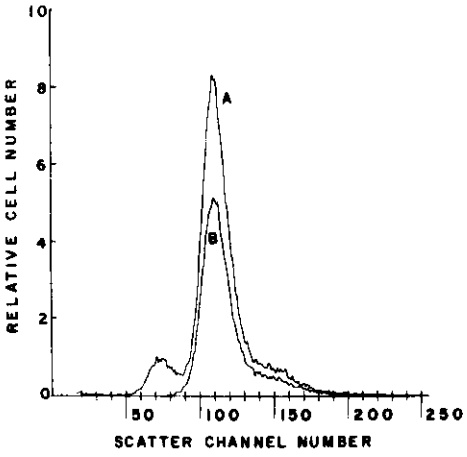

FIGURE 1. Scatter profile of murine spleen cells. A, scatter distribution of total cells; B, scatter distribution of fluorescein diacetate-fluorescent (viable) cells.

been normalized differently for better visualization. The subpopulation to the left in FIGURE 1,A is absent in the population gated by fluorescence (FIGURE 1,B), which indicates that nonviable cells scatter less light. This characteristic dead cell scatter profile is obtained from cells of all lymphoid organs studied thus far.

The separation of these two peaks is dependent on the angles over which the scatter signal is obtained. As the angle of collection is decreased, the two peaks merge. The best separation of these two peaks occurs on the FACS when the collection angle for scattered light is set from 0.5 to 12°.

Contamination of the sample by other cell types can be observed by light scattering. Red blood cells scatter even less light than nonviable lymphocytes and yield a peak to the left of the main lymphocyte population. Granulocytes, monocytes, and polymorphonuclear leukocytes scatter more light than small lymphocytes, so that these, too, can be identified in a lymphocyte population. Because nonviable lymphocytes and other types of cells can be differentiated on the basis of scatter alone, appropriate threshold settings enable all analyses and separations to be accomplished on the basis of viable small lymphocyte content.

ANALYSIS OF CELLS WITH BOTH FLUORESCENCE AND LIGHT SCATTERING

The lymphoid cells from mice can be divided into two major subpopulations that have different functional and antigenic characteristics. One population, the B cells, bears detectable immunoglobulin on its surface.[15] This population can be visualized by staining a cell suspension with a directly fluoresceinated rabbit antibody directed against the Fab fragment of mouse immunoglobulin. This reagent (R α M Fab^F) will stain all classes of immunoglobulins present on cell surfaces.

The fluorescence distribution of spleen cells stained with R α M Fab^F is shown in FIGURE 2. There are two distinct populations present. The unstained cells, which extend from channel 0 to approximately channel 25, comprise 51% of the total population. The stained cells, which have fluorescence greater than

effluent jet by an outer coaxial flow of cell-free fluid. The stream, which is approximately 60 μm in diameter, is illuminated by an argon-ion laser (model 164, Spectra-Physics, Palo Alto, Calif.) immediately after it leaves the nozzle. The laser beam is focused on the stream with a 124-mm focal length spherical lens.

The noise in the fluorescence channel is decreased with a dual-channel mode of operation. In this mode, the fluorescence channel is activated only when a scatter signal indicates that a cell is present. This is called scatter-gated fluorescence. The reciprocal operation, fluorescence-gated scatter, can be performed, so that the scatter signal is not accepted unless the fluorescence pulse is greater than a certain threshold.

Light for the scattering channel is collected at a forward angle of 0.5–12° from the incident beam. This setting yields the sharpest and most reproducible scatter profiles. An eight-times neutral density filter is used to attenuate the light that enters the scatter detector, a PIN-10 photodiode (United Detector Technology, Santa Monica, Calif.).

The fluorescence detector is a photo multiplier tube (no. 9524 EMI Co., Plainview, N.Y.). The filters used in the fluorescence channel for fluorescein emission include two cut on filters, 520.0 nm and 530.0 nm, series D (Ditric Optics Inc., Marlboro, Mass.), and a Wratten 15 filter (Eastman Kodak Co., Rochester, N.Y.). Rhodamine can be observed by adding a 3-68 filter (Corning Glass Works, Corning, N.Y.). Fluorescein is excited by the 488 nm line of the laser, which is operated at 400 mW output. The 515-nm laser line is used for rhodamine excitation.

Cells are analyzed at a rate of 500–1000/sec, although rates of up to 5000 cells/sec can be attained. The higher flow rates are normally used when cells are to be separated.

A detailed description of the sorting capabilities of the FACS has previously been presented.[1,2]

Reagents

The rabbit antimouse Fab (R α M Fab) was made against the Fab fragment of the myeloma protein MOPC 141. The 45% saturated ammonium sulfate precipitate of this serum was conjugated with fluorescein isothiocyanate.[3] The R α M Fab^F was fractionated by gradient elution from a DE-52 column.[4,5] Fractions with molar fluorescein to protein ratios greater than 2 were pooled.

A lactoperoxidase method[6] was used for radioiodination of the fluorescein conjugate. Free isotope was removed by passing the reagent through Dowex-1X8. Radioactive counts were determined with a gamma counter (Nuclear-Chicago Co., Des Plaines, Ill.). The final preparations of two samples contained 6.9×10^7 and 2.5×10^7 trichloroacetic acid precipitable counts per minute per milligram of protein. The concentration of fluorescein was determined from an absorbance measurement at 495 nm with an extinction of 0.2077.[7] The molecular weight of the coupled fluorochrome was assumed to be 389.

Cell Staining

Spleen cells were obtained from 4-month-old BALB/cN mice. Single-cell suspensions were made as previously described.[8] Fluorescein diacetate (Swartz-Mann, Orangeburg, N.Y.) was dissolved in acetone to a final concentration of