Separation of developing cells into fractions of differing stages of maturity is critical to effective biochemical study of the process of cellular differentiation. Density gradient techniques utilizing rate-zonal or isopycnic separations have permitted partial separations based on cell mass or cell density. In this study the separation of various rabbit marrow cells was improved by high-speed flow analysis and sorting in a Coulter Two-Parameter Cell Sorter. After preliminary isopycnic separation of marrow cells in Ficoll-Hypaque, cells were sorted into lymphoid and myeloid elements, utilizing light-scatter (LS) profiles to determine sorting. Characteristic LS patterns were present for erythrocytes, lymphocytes, devitalized cells, and granulocytes. When erythrocytes and their precursors were removed by hypotonic lysis, the remaining granulocytes could be sorted to give samples with much greater purity with respect to developmental stage than was possible with density gradients alone. Thus the combination of techniques represents a significant improvement in the ability to study the mechanisms of normal or altered cellular maturation.

Improved techniques for separating individual cell types from a mixed population are necessary to distinguish biochemical or functional characteristics of the various differentiated cells in a tissue or organ. In addition, study of the process of cell differentiation requires effective separation of cells of a given lineage into fractions containing roughly similar stages of maturation. Centrifugation techniques involving separation in density gradients have been tried on bone marrow with some success. Cells of blood and bone marrow lend themselves to studies of this kind because of their ready availability as single-cell suspensions, and the presence of readily defineable stages of maturation makes them attractive cells for study of the process of differentiation. Young marrow precursors are less dense than the mature cells, and density gradient analysis displays a spectrum of development stages of granulocyte precursors.1,2,3

By utilizing a second discriminating cell parameter, it should be possible to make further improvement in the ability to separate cell fractions limited to narrow ranges of developmental stages. In this report we have investigated the value of using light-scatter (LS) intensity profiles of marrow cells to improve the efficiency of the separation process. Using a combination of iso-

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pycnic sedimentation followed by LS analysis of the gradient fractions, we were able to sort fractions of increased purity. This was accomplished with a Coulter Two-Parameter Cell Sorter, which analyzes the LS distribution of cells flowing in a liquid stream intersecting a laser beam. LS profiles of the cell suspensions are generated and cells with various LS intensities can be sorted, thereby producing fractions containing narrower ranges with respect to stage of differentiation.

In this report this two-step separation procedure is detailed. LS characteristics of normal rabbit marrow cells are illustrated and the successful sorting of various fractions of leukocyte precursors is demonstrated.

**MATERIALS AND METHODS**

Preparation of rabbit marrow suspensions and separation of marrow precursors by isopycnic sedimentation was described in detail in a previous publication. Briefly, rabbit marrow was removed from the long bones, filtered through sterile gauze, and centrifuged to separate fat particles. Except as indicated, each sample was subjected to brief hypotonic lysis of erythrocytes and most erythrocyte precursors using distilled water as described by Fallon et al. Continuous density gradients of Ficoll-Hypaque were formed in a Beckman gradient former from starting solutions with densities of 1.0478 and 1.1579 g/cc. Approximately 150 x 10⁶ marrow cells were added to Beckman SW41 tubes (13 ml) and centrifuged at 4300 g for 40 min. Gradients were collected in ten equal fractions for flow analysis using an ISCO gradient fractionator. The cells were centrifuged and suspended in buffered balanced salt solution with 15% fetal calf serum at a concentration of 5 x 10⁶/ml. Cell viability was determined by Trypan blue dye exclusion.

Each gradient fraction was analyzed with a Coulter Electronics TPS-I Cell Sorter. This instrument is a modification of a design described by Steinkamp et al. and was used to produce a LS intensity histogram of the marrow cells from which portions were chosen for sorting and subsequent morphologic analysis.

In the cell sorter cells are caused to flow in coaxial fashion in a stream of buffer through the path of an argon-ion laser beam (488 nm). The light scattered by each cell through an angle of 1°-20° from incidence is measured by a detector whose output is digitized and recorded as a count in one channel of a 128-channel digital memory. The particular channel in which each event is recorded is proportional to the digitized intensity of LS of the individual cell intersecting the laser beam. The stored data can be displayed on a cathode ray tube or digital plotter as the histogram of LS intensity (128 channels) versus number of cells in each channel. The display can then be used to set electronic windows that pass only LS signals of certain intensities to generate sort commands. In this way distinct populations of cells with respect to LS intensity can be selected.

The sorting is accomplished by ultrasonically dividing the sample stream into small droplets subsequent to the laser intersection point. Each droplet containing a cell that generates a sort command is given an electrostatic charge. The droplets then pass between charged plates that selectively deflect the charged droplets into one of two containers depending on the sign of the droplet charge. The main stream of uncharged droplets is not deflected and falls into another container. An anticoincidence circuit rejects sort pulses if more than one cell is detected within the interval of collection.

When marrow cells were analyzed, the resulting histogram of LS intensity versus number of cells was divided into regions on the basis of apparently distinct populations and by regular increments of intensity (channel numbers) across the plot. A sort window was set on each region (two at any one time), and the TPS-I was operated in the sort mode to yield 100,000 cells having LS intensity corresponding to that particular region.

Sort windows were located according to specific features of the LS pattern; i.e., a peak, left, right, or center of a peak, or a skewed region. A number of alternate window locations were tested, including taking five-channel cuts across the LS pattern, with no significant increase in resolution of fractions.
The sorter was operated at a flow rate of 1000-3000 cells/sec with the anticoincidence circuit on to prevent contamination by simultaneous sorting of more than one cell. The rate of sorting of the cells of interest varied directly in each case with their proportion of the total cell population.

The instrument was calibrated daily with 9.5 µm beads to ensure the constancy of modal peak channel response. While peak channels may change over a long period of time, or following machine adjustment, they are reproducible during any given set of experiments. Reproducibility of the LS pattern for gradient fractions was checked and found to be stable during the course of an experiment. In the event of any evidence of laser instability during an experiment, data from that experiment were discarded. All experiments were repeated several times, all with the same results.

Sorted cells were collected in fetal calf serum and centrifuged onto microscope slides in a Shandon cytocentrifuge. Cells were fixed with methanol and stained with Wright’s stain prior to differential counting. One hundred cells were counted on each fraction. Cellular morphology was not appreciably changed after centrifugation in Ficoll-Hypaque gradients or passage through the cell sorter. The stages of erythroid and myeloid development in rabbits are generally similar to those of humans. The most striking difference is the size and staining of the granules of the heterophil leukocytes, which are analogous to human neutrophils. In the rabbit the specific granules are large and eosinophilic.

RESULTS

Light scatter profiles of crude marrow suspensions before and after lysis of young erythrocytes. Bone marrow from normal rabbits was removed as described in Materials and Methods and suspended in buffer at a concentration of 5 × 10⁶/ml. These samples were then analyzed with the Coulter TPS-1, and LS histograms were obtained (Fig. 1, curve A). The computer-generated histogram shows the number of cells on the ordinate and the intensity of scattered light recorded in the 128 channels of the instrument on the abscissa. Under these circumstances, where the suspension contained numerous erythrocytes and red cell precursors as well as granulocytic precursors and lymphocytes, the LS profile was overshadowed by the very large numbers of cells near the origin, indicative of less light scatter. Cells sorted from this large LS peak near the origin in Fig. 1, curve A proved to be primarily erythrocytes. As can be seen, very little discrimination was obtained from crude marrow containing erythrocytes. However, as shown in Fig. 1, curve B, if a hypotonic lysis step

![Image of LS histogram with curves A and B]
were included prior to analysis, a quite different LS profile was obtained. In this instance there are four primary peaks, labeled 1-4. Peak 1 represents subcellular debris and is not considered for sorting.

The cell sorter capability of the TPS-1 was then utilized to obtain samples of the cells represented by the various LS peaks for morphologic analysis. By electronically “gating” on these peaks, cells representing these peaks could be obtained in suspension and spun in a cytocentrifuge to obtain smears for Wright staining. Cells were also obtained for viability testing by Trypan blue dye exclusion. Cells sorted utilizing LS window set on peak 2 (Fig. 1, curve B) consisted of 1% young neutrophils, 6% lymphocytes, and 93% devitalized or

![Graph](image-url)

**Fig. 2.** LS histograms and differential counts of sorted cell fractions from samples of marrow first fractionated on density gradients. Abscissa, increasing LS intensity designated by increasing instrument channel number. Figure illustrates gradient fraction 1 from the top of the gradient. Recovered cells were then subjected to flow analysis producing the LS histogram shown. Areas under peaks (shown by vertical bars) were sorted electronically and cytocentrifuge preparations were made for differential counts of stained cells, shown in the bottom part of the figure.
Fig. 3. Density gradient fraction V (midgradient). Recovered cells subjected to flow analysis and sorting of cells with varying LS properties.

"smudged" nuclei. Control experiments showed that "smudged" nuclei corresponded to the nonviable cells present in the original suspensions. Gating on peak 3 yielded 2.0% juvenile neutrophils (or heterophils in the rabbit), 1.8% metamyelocytes, 25.9% lymphocytes, 3.2% monocytes, and 65% "smudged" nuclei. A gate window set on peak 4 yielded 21% mature heterophils, 54% juvenile heterophils, 14% metamyelocytes, 6% myelocytes, no promyelocytes, 3% blasts, 1% lymphocytes, 1% monocytes, and no "smudged" nuclei.

The cells responsible for peak 2 (Fig. 1B) proved to be mostly nonviable (Trypan blue dye uptake), as confirmed by their morphologic appearance as damaged cells, whereas approximately half of the cells in peak 3 were viable. This corresponded to the number of morphologically intact cells. The viability of cells in peak 4, containing granulocyte precursors, was 90%.

Effect of cell maturity on LS patterns of marrow suspensions. In order to
obtain better resolution of the LS scatter patterns of the granulocytic cell precursors remaining after lysis of the young erythrocytes, cell suspensions from which the erythrocytes were lysed were subjected to density gradient centrifugation. This technique, as described in a previous report, consists of isopycnic sedimentation in density gradients of Ficoll and Hypaque. The gradients are collected in ten equal fractions. The young granulocytic precursors are stratified at the top of the gradient, and cells of progressively increasing age are found extending to the bottom of the gradient. Each gradient fraction is then recovered and suspended at a concentration of $5 \times 10^6$ ml for LS analysis.

Fig. 4. Density gradient fraction IX (near bottom of ten-fraction gradient). Recovered cells subjected to flow analysis and sorting of cells with varying LS properties.
The upper portions of Figs. 2-4 show the LS pattern. The most striking initial feature of these histograms is the disappearance of the peak representing the dead cells or "smudged" nuclei (peak 2 in Fig. 1, curve B). In Ficoll-Hypaque these cells sediment more as nuclei than whole cells and have a much higher buoyant density than viable cells. They were thus found in the pellet at the bottom of the tube. The remaining cells in the density gradient were virtually all viable cells.

The gradient fractions represented in Figs. 2-4 are fractions I, V, and IX, respectively, of the ten-fraction density gradient. The first peak, then, in the LS profiles was generated predominantly by lymphocytes, and the second peak in each profile was due to granulocytic cells. Since the cellular composition of each gradient fraction differs, one would not necessarily expect the LS peaks (lymphocyte or granulocyte) to appear in exactly the same channels of the instrument in different gradient fractions. Nevertheless, morphology of the cells under the major peaks was shown to be lymphocytic and granulocytic cells, respectively, in all fractions. Profiles of fractions taken near the top of the gradient showed a very tall and narrow lymphocyte peak, whereas the granulocyte peak was smaller and skewed to the right (Fig. 2). In fraction V of the density gradient, the granulocyte precursor peak was larger than the lymphocyte peak (Fig. 3). Fraction IX (Fig. 4) was similar to fraction V, although a peak corresponding to the reduced LS of nonviable cells was present. This peak was slightly larger in the profile of the bottom gradient fraction (fraction X, not shown), suggesting minor contamination of this fraction with material from the pellet, which included large numbers of dead cells.

These data are readily reproducible with respect to cellular compositions of gradient fractions and sort fractions and to general features of the LS pattern upon which placement is based.

**Differential counts of sorted cells.** The lower portions of Figs. 2-4 show the differential counts of sorted cells. The gates on the cell sorter were set as indicated by the vertical bars in the diagram, and approximately 100,000 cells were sorted with each window. Two cytocentrifuge slides were made from each fraction.

The morphology of these cells is generally similar in rabbit and human marrow. The major difference is in the larger eosinophilic granules of the rabbit heterophil leukocytes, which are equivalent functionally to the neutrophil leukocytes of humans. Some eosinophil precursors could also be identified by the presence of somewhat larger and more densely packed eosinophilic granules but for this study they were included among the remaining granules from the heterophil line.

The differential counts of sorted cells from the top of the density gradient (gradient fraction I) are shown in the lower portion of Fig. 2. The tall narrow peak (sort fraction 1) contained 76\% lymphocytes and 24\% dead cells and comprised 45\% of the total cells in the gradient fraction. Three separate fractions were sorted by gating on different parts of the broad granulocyte peak. In sort fraction 4, which contained those cells with the greatest LS intensity, there is a preponderance of young granulocyte precursors, especially promyelocytes and myelocytes, which make up 68\% of the sorted cells. Fraction 3 contained more mature cells but was predominantly a myelocyte fraction. Sort fraction 2 con-
tained relatively more mature cells, although more blast cells were identified in this fraction. Monocytes also appeared in this fraction.

The lower portion of Fig. 3 illustrates the differential counts of sorted cells from fraction V of the density gradient. The lymphocyte peak (69% lymphocytes) now represents only 9% of the total number of cells (sort fraction 1). On the skewed right-hand side of the granulocyte peak (sort fraction 4) there are a number of myelocytes and metamyelocytes. The central portions and left-hand side of the granulocyte peak correspond primarily to metamyelocytes and band forms (sort fractions 2 and 3).

Figure 4 shows gradient fraction IX. There is a slight increase of dead cells in sort fraction 1, presumably a contamination from those dead cells that reached the pellet because of their very high density. A small population (sort fraction 2) with a predominance of lymphocytes was also present, but still 83% of the cells in gradient fraction IX were of the granulocytic variety. Sorting on the major portion of the granulocyte peak produced a fraction containing predominantly bands and polys (sort fraction 3). Only one sort is shown in this peak, since separate sorts of each side of the much narrowed peak were essentially identical. A sort window set on the right-hand leading edge of this peak still yielded some relatively immature polys (sort fraction 4). This skewed
portion of the granulocyte peak accounted for only 9.1% of the total peak, whereas the skewed region of gradient fraction 1 (Fig. 2, sort fraction 4) accounted for 20.0%. Thus the predominant cells in the lower part of the gradient are the most mature cells of the marrow, with very few early cells.

Figures 5-8 are photomicrographs illustrating several portions of the gradient and fractions sorted from their granulocyte peaks. Figure 5 shows cells sorted from the top of the gradient, gating on the right-hand side of the granulocyte peak. This fraction contained a number of immature granulocyte precursors, including blasts, promyelocytes, and myelocytes (and, in the illustration, one metamyelocyte). Figure 6 shows an intermediate fraction of the gradient sorted in the cell sorter, showing some myelocytes but more heavily granulated cells and a few mature polys. Figure 7 is a fraction from the bottom of the gradient obtained by gating on the granulocyte peak, containing almost entirely mature heterophils and band forms. Figure 8 is a fraction sorted by gating on peak 1 of the top of the gradient, containing various lymphocyte forms ranging from large to small lymphocytes, all of which appear to scatter light quite similarly despite their differences in size.
DISCUSSION

Light scatter can be defined as the deflection of light from an incident beam without appreciable change in wavelength. The various mechanisms involved in scatter include reflection from cell surfaces, refraction at various optical boundaries within the cell, shift in phase of the incident light passing through or around the cell, and diffraction of light by different parts of the cell.

The intensity of scattered light varies with the angle of scatter as a series of maxima and minima, the positions of which differ with various properties of the scatterer. For cells having the same shape, light scattered at low angles ($2^\circ$–$5^\circ$) is primarily related to size. Although larger angles of acceptance yield information on cell ultrastructure and refractive indices of various cellular constituents, LS intensity falls off rapidly as a function of increasing angle. For this reason, the $1^\circ$–$20^\circ$ angle integral detector of the TPS-1 used in this work would be expected to respond primarily to cell size. Loken et al., however, using unfractionated mouse marrow and similar instrumentation, noted that an acceptance angle of $2^\circ$–$8^\circ$ in fact yields other than size information.

This study was designed to investigate (1) LS properties of marrow cells in suspension, (2) the feasibility of sorting granulocyte precursors based on LS differences, and (3) the value of combining density gradient separation and cell sorting by flow analysis to obtain improved preparations of differentiating cells.

The LS pattern obtained from bone marrow distinguishes mature erythrocytes and lymphocytes from myeloid and erythroid precursor cells. Erythrocytes formed a peak with low LS intensity. In samples in which erythrocytes were destroyed by hypotonic lysis, it can be appreciated that lymphocytes (many of which are similar in size to erythrocytes) and the nuclei of devitalized cells also scattered light similarly. Granulocyte precursors produced a rather broad LS peak. The youngest (larger) granulocyte precursors scattered more light within the angle of detection than did the more mature granulocytes. Exceptions to this were myeloblasts and other cells identified morphologically as "blasts." They are a small proportion of the total, but are most often sorted from areas of less light scatter. This may reflect the relatively smaller size of the blast cell prior to becoming the next identifiable stage, the promyelocyte. In addition, these primitive cells have fewer internal organelles to scatter light. The myeloblast and pronormoblast are believed to arise from a lymphocytelike stem cell, and a continuum of cells would be expected, first with lymphocyte-like LS properties and then progressively more granulocytelike.

Monocytes also tended to scatter less light than granulocytes, which is unexpected if cell size were the sole determinant of LS. Monocytes are distinctly larger than mature heterophils (the rabbit equivalent of the human neutrophil) and might be expected to be found with the younger granulocytes, although in fact they tended to appear with the older granulocytes and lymphocytes. Likewise, the fraction sorted on the lymphocyte peak contained not only small lymphocytes but large ones as well (Fig. 8). If LS separation depended solely on cell size, these cells should be more widely distributed in the LS profile. Since the major morphologic similarity of lymphocytes of various size is the character of the nucleus, this suggests an important role of the cell nucleus in LS differentiation between cells.
LS patterns of crude marrow suspensions (Fig. 1, curve A) are dominated by large numbers of erythrocytes normally present, making the LS profile of little discriminating value. After hypotonic lysis of erythrocytes, the LS profile is quite changed, showing three characteristic cellular fractions (Fig. 1, curve B). The first peak represents mostly devitalized cells, the second mostly lymphocytes, and the third mostly granulocytes.

However, sorting multiple fractions through the granulocyte peak of crude marrow did not produce a clear separation of different degrees of cellular maturity. Supplementing flow analysis with the additional dimension of prior fractionation of the marrow cells in density gradients gave further definition to these fractions. In fractions from the Ficoll-Hypaque gradients, the most striking initial difference was the lack of the first peak of devitalized cells. The buoyant density of these cells (or nuclei of lysed cells) is essentially that of the dense DNA-rich nucleus, and they sediment into the pellet at the bottom. The LS profile then represents only those viable cells; since hypotonic lysis removes all erythrocytes and identifiable erythrocyte precursors, these remaining cells are lymphocytes and granulocytes of various stages of development.

Since the younger granulocyte precursors are found toward the top of the gradient, the LS profile of the granulocyte becomes progressively narrower moving down the gradient. This makes it possible to further fractionate these samples by sorting fractions on each successive granulocyte peak to obtain samples of greater uniformity than obtained either by sorting crude marrow or from density gradient fractionation alone.

These studies provide a framework for further investitigation of marrow cell development. We extended these studies of separation of developing granulocytes to characterize the inflammatory response in rabbit marrow. This allows collection of marrow in which early granulocytes have been stimulated to proliferate and also permits the collection of uniform populations of mature granulocytes from peritoneal exudates as well. These are being reported elsewhere.

The ability of LS phenomena to provide more than simply cell size measurement is an area that deserves further study. It may be possible, by using discrete angles of scatter, to characterize internal structure of various cells more clearly and provide a way of developing characteristic LS “signatures” of different cell types.

Already LS has been used to measure different cell shapes and virus-infected tissue culture cells have been distinguished from morphologically identical controls by their LS fine-structure changes. Using multiple-angle detectors and flow analysis, it may then be possible to screen large numbers of cells in search of a small population of pathologic cells. This would have great importance in the diagnosis of malignant disease or for seeking evidence of leukemia relapse at an earlier stage when amenable to therapy.

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Separation of rabbit marrow precursor cells by combined isopycnic sedimentation and electronic cell sorting

RB Scott, WM Grogan and JM Collins