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IDENTIFICATION OF CELL ASYMMETRY AND ORIENTATION BY LIGHT SCATTERING[1]

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Light scattering from chicken red blood cells has been used as a model system to identify the asymmetry of cells. The histogram for forward angle light scattering for these cells is bimodal, the signal size being dependent on the cell orientation. A dual orthogonal scatter system is used to conclusively demonstrate this orientational variation in signal. A third scattering system, using a single incident beam with two orthogonal detectors, is used to further characterize the orientational variation of the scatter signal. In this third system it is shown that the signal in a detector set 90° from the incident beam collects light reflected from the cell surface. The optical selection of cells in specific orientations using these systems may circumvent the need to physically orient cells in flow systems.

The scattering of light by cells in flow systems has been used to determine their size (4, 6, 13, 14), to differentiate viable from nonviable cells (7, 11) and to distinguish between cell types (12, 15, 16). Cells with axial symmetry about the direction of flow will yield the same signal upon rotation around that axis; however, cells without such symmetry, such as epithelial cells, may produce a scatter signal that is dependent upon their orientation (10). The detection in cervical smears of a few cancerous cells among a normal population (10, 16) may be greatly hindered by the effects of asymmetry of the cells being studied. Fluorescence has also been shown to be affected by asymmetry since the fluorescence signal from bull sperm cells, stained to determine their deoxyribonucleic acid content, is related to cell orientation (3). Some cells change their shape when exposed to shear forces and become axially symmetrical even though they are quite asymmetrical in a static state (8, 9). Calculations based on the static shape of these deformable cells may be quite different from what is observed while they are flowing.

This article describes a model system for the detection of asymmetry and for the determination of cell orientation in a flow system. Once the orientation of the cell has been determined, other optical parameters, such as light scattering or fluorescence, can be obtained from cells with the proper orientation. Using an optical selective process eliminates the need to physically orient the cells in the flow system.

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MATERIALS AND METHODS

The asymmetric model cells used in this study were glutaraldehyde-fixed chicken red blood cells (CRBC). Although these cells have bilateral symmetry, they do not have axial symmetry as they flow past a detector. Their axial asymmetry and orientation were studied using the light scattering signal detected by three different optical systems. The first light scatter detector system utilizes a single laser beam and a single detector. This system, which collects light scattered from a cell at a forward angle, has been described previously (11). The second system utilizes two complete light scattering detector systems set orthogonally to each other. The asymmetry of a cell as it passes this dual detector system is determined by the difference in the signal observed between one detector and a similar detector observing the cell 90° from the first system. The third optical system consists of only one incident beam and two detectors set 90° apart. Detectors collecting light at 0° and 90° observe different scattering parameters of the particle. Calculations for homogeneous spheres show that the diffractive and refractive components predominate at narrow angles, whereas the reflective component becomes more significant at larger angles (5).

All of the optical systems described in this paper utilize the flow components of a fluorescence-activated cell sorter (FACS) (11). Briefly, cells are forced under pressure through a nozzle where they are centered in a 60 μ diameter effluent jet by means of a coaxial cell-free sheath fluid. This jet flows vertically downward at a velocity of 12 m/sec. The incident laser beam or beams from an argon ion laser (488 nm) intersect the flow stream outside the

nozzle approximately 0.5 mm from the tip. The detectors are focused on the intersection of the illuminating light and the stream. Cells identified by their optical properties can be separated by preselected criteria using the separation capabilities of the FACS (1).

RESULTS

Single scatter detector: A glutaraldehyde-fixed CRBC can be described as a flattened ellipsoid with a round nucleus in the center (Fig. 1). Since the fixing process (10% glutaraldehyde for 15 min at 0°C) stabilizes the cell shape, these cells are excellent for use in a model system to study asymmetry. Figure 1 illustrates two possible orientations for CRBC passing a scatter detector, assuming that the long axis is aligned with the flow of the stream (8, 9). A third orientation, an end view looking down the long axis, is not drawn. The glutaraldehyde fixation process also makes the cells fluorescent so that they have been used as fluorescence and light-scattering standards for flow systems (7, 11).

The asymmetry of fixed CRBC was first noted in a flow system using a single forward angle light-scattering detector. The distribution of scatter signals for fixed CRBC observed in a detector collecting all the light from 0.5–13° is shown in Figure 2. It is readily apparent that at least two populations are present, one that gives signals averaging 35 units and another, more populous group, with the peak centered at 125 units. A continuous distribution of signals lies between these modal values. Changing the rate of flow of the cells relative to the sheath fluid, and thereby increasing the volume in the fluid jet to which the cells are confined (2) did not change the shape of this distribution. This

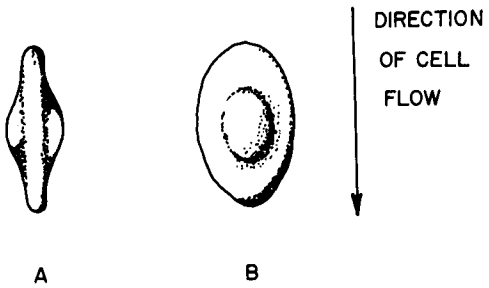

FIG. 1. Drawing of chicken red blood cells showing two possible orientations. A third perspective, looking down the long axis, is not shown. These cells tend to align themselves as they pass through the nozzle orifice as shown by Kachel (8, 9).

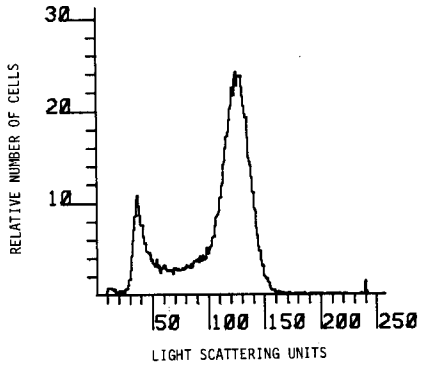

FIG. 2. Histogram of forward angle light scattering for CRBC. Although the scattering histogram is bimodal, the fluorescence histogram of these cells is unimodal. The cells comprising the peaks at 35 and 120 units can be separated by the FACS. Reanalysis of these separated populations yields the original bimodal histogram.

indicates that the position of the cells within the stream is not causing the bimodality.

The fluorescence distribution of these cells as observed through appropriate optical filters (11) is unimodal. Since fluorescence emission is less anisotropic than light scattering, the difference between the scatter and fluorescence histograms is attributed to cell asymmetry, *i.e.*, to the orientation of the cell with respect to the scatter detector.

The conclusion that the bimodality of the scatter histogram is related to cell orientation is supported by separation of the cells comprising the two peaks. Using the sorting capabilities of the FACS, cells from either the smaller or the larger peak were separated and reanalyzed for the light scattering profile. The original bimodal distributions were obtained upon reanalysis using cells separated from either the small or large peak. This indicates that the two peaks are not a result of a separable population of cells but rather are due to orientation of the cells flowing through the system. Assignment of these different signals to specific cellular orientations has been studied by two other optical systems described in the following sections.

Two orthogonal scatter systems: In order to confirm that the bimodality observed in the scatter histogram is a result of the asymmetry of the cells, a second scattering detector and incident beam was added to the previous system. This second complete scatter system was orientated orthogonally to the first system as shown in Figure 3. Detector 1 collected light

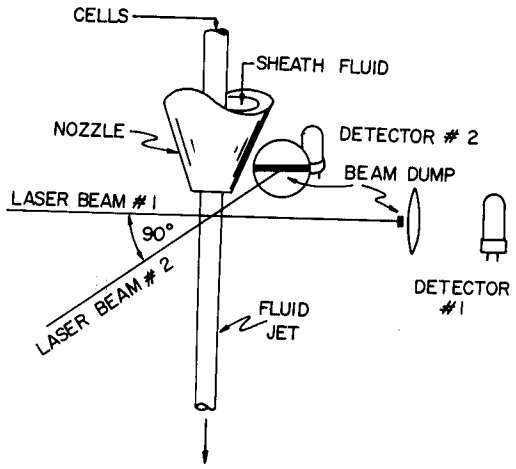

FIG. 3. Schematic drawing of the dual orthogonal, forward angle scattering systems. Scatter detector 1 collects light from 0.5–13° while detector 2 collects light from 0.5–30°.

from 0.5–13° while detector 2 accepted light from 0.5–30°. The scatter histograms from the individual detectors were essentially the same as that shown in Figure 2. Both were bimodal with a continuous distribution of signals between the two peaks.

The correlation of the signals from one detector with those generated in the other detector can be observed by displaying the signals on an X-Y storage oscilloscope. Each cell is represented by a dot whose position is determined by the relative amplutide of the signals generated by that cell in the two detectors. Fig. 4A shows this correlation plot for fixed CRBC as observed by the two orthogonal scatter systems. The signals from detector 1 are plotted on the ordinate while signals from detector 2 are plotted on the abscissa. It is apparent that the signals that produce the small peak in the histograms from either detector (Fig. 2) are always correlated with a large signal in the other detector. This means that what is observed as small in one detector is always large in the other detector in this orthogonal scatter system; there are no small-small signals.

These results can be explained by assuming that the fixed CRBC are oriented with their long axis aligned with the flow of the stream. This alignment has been observed for cells flowing inside an orifice of dimensions similar to those of the nozzle orifice (8, 9). If the orientation of the cells just outside the orifice is the same as that inside the orifice the possible orientations observed by the scatter detector are reduced to rotation about the long axis of the cell. Referring to Figure 1, where the incident laser beam is coming out of the page and the reader becomes the scatter detector, cells in orientation A will give a small signal while cells in orientation B will yield a larger signal. Rotating the cell from orientation A to B will cause an increase in scattering cross-section and, therefore, cause an increase in the signal generated by that cell.

Signal incident beam, dual detectors: The assumption that the CRBC's are oriented with

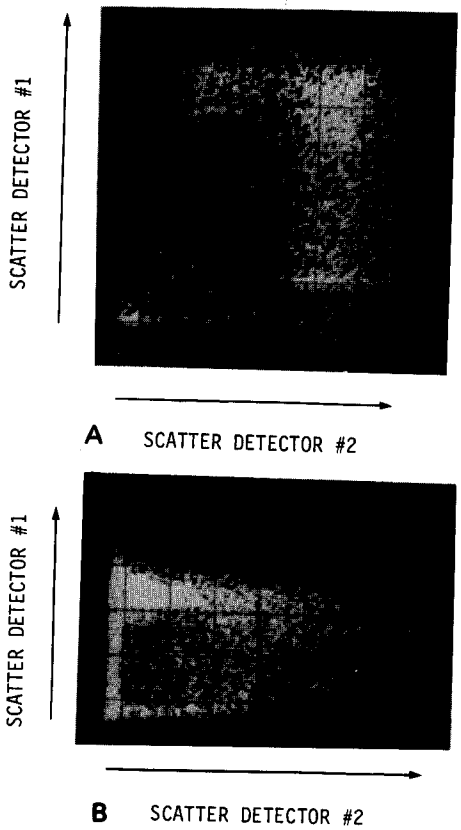

FIG. 4. A, Correlation plot of two orthogonal forward angle scatter systems (Fig. 3). Each detector yields a histogram essentially the same as that shown in Figure 2. Those signals that are small in one detector are always large in the other detector indicating that the forward angle signal is orientation dependent. The dots in the lower left-hand corner denote the origin. B, Correlation plot to a forward angle scatter detector and one set 90° from the first. The histogram of the signals in the forward angle detector, 1, is shown in Figure 2. The histogram of the signals in the 90° detector, 2, is shown in Figure 5A. The shape of this plot is consistent with the CRBC being aligned with the flow of the stream and the 90° detector observing primarily reflected light.

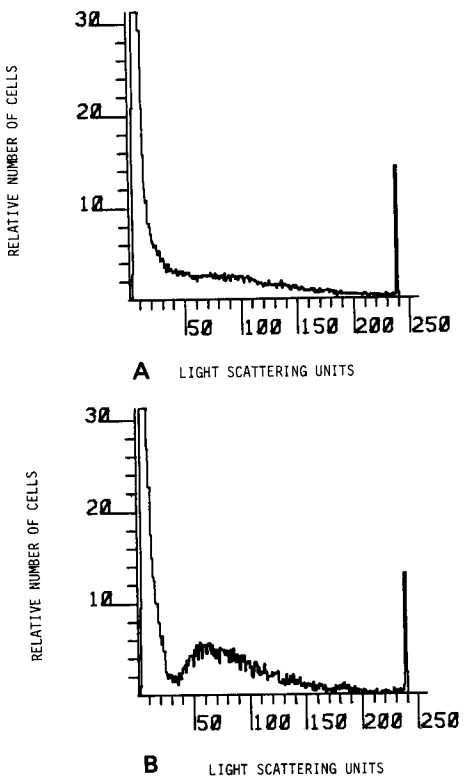

FIG. 5. A, Histogram of scatter signals from CRBC observed in a detector set 90° to the incident beam. The included angle of this detector is from 60–120°. Thirty-five per cent of the cells yield signals greater than 35 units. The peak at channel 240 is a result of all cells that are off-scale being counted in the last channel. B, Histogram of scatter signals in a 90° detector that have been gated on signals from the forward angle scatter system. Only signals from cells with forward scattering between 50 and 100 units (Fig. 2), were collected. Forty-five per cent of the cells have signals greater than 35 units.

their long axis in the direction of flow is supported by using an optical system that contains two orthogonal detectors and only one incident beam. The system is identical to that illustrated in Figure 3 except that the laser beam 2 has been removed. Detector 1 collects light from 0.5–13° while detector 2 now collects light from 60–120°.

The histogram obtained with detector 1 is the same as shown in Figure 2 since the optics of this detector are unchanged. The histogram obtained with detector 2 in this optical configuration is shown in Figure 5A. The majority of cells yield a small signal in detector 2 while a significant portion of cells yields a signal larger than this modal population. This histogram was obtained by gating detector 2 only when detector 1 observed a cell, *i.e.*, signals greater than 25 units in Figure 2. All the signals shown in Figure 5A are well above system noise which occurred in channel 1. The cells which give rise to these large signals in detector 2 can be identified by looking at the correlation plot between the signals from the two detectors (Fig. 4B) The cells that yield signals in detector 2 greater than 50 units correspond to the cells which lie primarily between the modal values in detector 1 (cells with 50–110 units in Figure 2). There are, however, some cells that yield signals between the modal values in detector 1 that produce small rather than large signals in detector 2. The lack of dots in the center of Figure 4B indicates that this is an "all or nothing" event. These cells either give a small signal or a large signal in detector 2, but very few give a signal between these extremes.

This correlation plot can be explained by referring to Figure 6. This diagram is drawn with the cells flowing into the page so that their long axis points toward the reader. If detector 2 observes primarily reflected light, only those cells in position C will produce a large signal in this detector. Cells in positions A and B reflect little light toward detector 2, while the reflected light in position D is directed away from this detector. The reflection of light from the surface of fixed CRBC's can be observed visually by dark field microscopy. When fixed CRBC's tumble past the area of observation, cells in certain orientations reflect a large amount of light into the objective. In this manner only those cells with orientation C will reflect light strongly into detector 2. The unique shape of the correlation plot is a combination of reflection in detector 2 and forward light scattering observed in detector 1. From the conclusions of the earlier experiments, cells in orietation A will yield small signals in detector 1 and will also yield small signals in detector 2. Cells with orientation B will give rise to large signals in detector 1 but will again give small signals in detector 2. Cells in position C will give large signals in detector 2 but only medium signals in detector 1. Because an equivalent proportion of cells can assume either orientation C or D, only half of the cells giving medium signals in detector 1 will give large signals in detector 2. In this way, cells which yield 50–100 units in Figure 2 can give either a small or a large signal in the 90° detector.

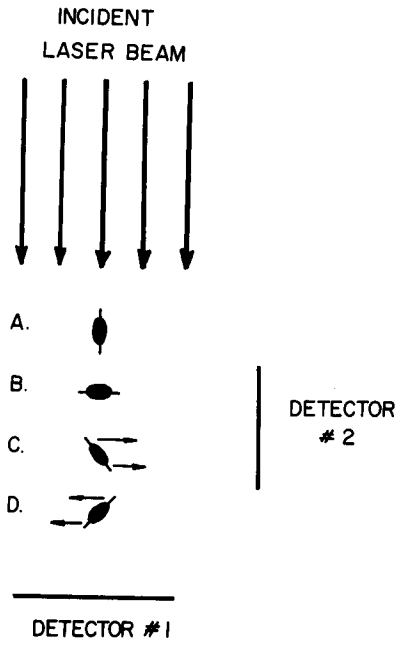

FIG. 6. Diagram of different orientations that cells can assume as they rotate around their long axis. The cells are flowing into the page so the view is end-on. Detector 1 observes forward angle scatter while detector 2 observes 90° scatter. Only cells in orientation C will reflect light into detector 2 resulting in a large signal. These cells will yield medium size signals in detector 1. The other orientations will give relatively small signals in the 90° detector.

If cell rotation is completely random in the stream, cells in orientations C and D should be equally probable. This can be determined by gating the electronics so that only cells giving intermediate signals in detector 1 will be recorded by detector 2. The histogram of signals in detector 2 for cells from 50–100 units of signal in detector 1 (Fig. 2) is shown in Figure 5B. Integration between channels 35 and 250 yields 45%, indicating that approximately half of the cells are appearing in each orientation. This shows that there is little, if any, preferential orientation as the cells flow through the nozzle. This type of detection system could be used to assess the degree of orientation in a nozzle that physically orients the cell.

DISCUSSION

The data from the three optical systems indicate that the asymmetry of fixed CRBC can be detected using light scattering. The bimodal histogram of light scattering signals in the forward angle detector is due to the orientation of the cells relative to the scatter detector. The largest signals are due to the orientation with the greatest scattering cross-section, while the smallest signals result from a smaller cross-section being exposed to the detector. The data from all three detector systems are consistent with an alignment of the long axis of the cells along the direction of flow. The data from both the dual incident beam-dual detector system and the single incident beam-dual detector system are readily explained by having the CRBC aligned with the stream. Two identical scatter systems set orthogonally to each other can then be used to observe cell asymmetry. A detector set 90° from the incident beam can be used to detect reflected light from scattering particles.

The bimodality in the forward angle light scattering histogram is not due to a separable cell population since the original distribution is obtained when either the small or large cells are separated and reanalyzed in the system. Separation of cells and reanalysis is an excellent method for detecting flow or orientational artifacts which may hinder the discrimination between two truly different cell types. If reanalysis of the separated fractions does not yield histograms similar to those expected from the sorting criteria, a flow or orientational artifact may be involved.

By optically selecting asymmetric cells in particular orientations it is not necessary to physically orient the cells. Other parameters, such as fluorescence or multiangle light scattering (12, 16) can then be assessed for these cells and correlations can be made for the effect of asymmetry on the various optical parameters. The usefulness of this technique is limited to situations where an adequate number of cells is available to overcome the inefficiency of analyzing only the small fraction of cells occurring in a particular orientation.

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