Light Scattered at Two Wavelengths Can Discriminate Viable Lymphoid Cell Populations on a Fluorescence-activated Cell Sorter

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A fluorescence-activated cell sorter was modified to simultaneously detect two different colors of light scattered by cells as they passed through the instrument. A single argon ion laser with output from 351 to 488 nm was used as the light source. The signals from two light scattering detectors (using the same collection optics but having different optical filters) were compared. In the analysis of glutaraldehyde-fixed chicken erythrocytes the histograms collected for ultraviolet (uv) and 488 nm scattered light were similar in shape. However, the correlation plot between the two signals indicated that a simple linear transformation could not be made between uv and 488 nm light scattering signals. The analysis of mouse bone marrow indicated that two distinct nucleated cell populations could be identified by 488 nm light scattering, whereas, only one population was identified by uv light scattering.

Erythrocytes could be distinguished from nucleated cells by both uv and 488 nm light scattering. By correlating the two colors of light scattering a third population of bone marrow cells, predominantly large lymphocytes, was identified. Among splenic lymphocytes, T cells could be distinguished from B cells based on the differences in scattering of these two wavelengths. These data indicate that light scattering patterns change drastically when different colors of light are used to analyze the population. In addition, the correlation of light scattered at two different wavelengths can aid in distinguishing populations of cells that could not be identified by a single wavelength of scattered light.

KEY WORDS: Light scattering; T and B lymphocytes; Bone marrow; Fluorescence-activated cell sorter; Automated differential analysis; Standards.

Introduction

Light scattering has been used in flow systems to discriminate live from dead cells (14), to identify cell size (18–20), to distinguish cell types (22,16), and to identify cell orientation (15). This method of analysis and cell discrimination has the advantage that there is no requirement for fixing or staining the cells. Theoretical calculations for the amount of light scattered from regularly shaped objects at any particular point in space are complex and exact theory becomes intractable for irregularly shaped objects or for objects with complex internal structure (23). For flow systems designed to detect the signals with the stream in air rather than in a cuvette the calculations become even more difficult (24). Because of the lens effect of the stream-air interface the outer collection angle of forward scattered light must be increased to 10° or more in order to minimize positional differences in particle trajectories (14). Although modeling efforts may provide guidance as to which physical parameters can be identified by light scattering techniques, an empirical approach may prove to be the most direct method of obtaining information from the light scattering signals.

In all light scattering calculations a critical parameter is the object size, which is usually expressed as a dimensionless parameter \( \alpha = \frac{m d}{\lambda} \) where \( d \) is the particle diameter and \( \lambda \) is the wavelength of light in the medium. The refractive index component in the calculations is also dependent upon the wavelength of light used to study the object (7,11). Since two commercial flow instruments (Becton-Dickinson, Sunnyvale, CA and Coulter Electronics Inc., Hialeah, FL) use a single laser for both the fluorescence excitation and light scattering, a change of the incident wavelength to excite different cell bound chromophores may also affect the light scattering signal generated by those cells.

In the experiments presented in this article the scatter detector of a fluorescence-activated cell sorter (FACS) was modified in order to investigate the effect that changes in wave-

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length have on the light scattered by live cells. A single laser operating in a multiple wavelength mode produced concentric beams of ultraviolet (uv) and visible light. A single lens was used to collect the light scattered by the cells. A beam splitter directed this light onto two detectors fitted with appropriate optical filters so that the resulting signals in the two detectors could be compared.

Materials and Methods

Modification of the instrument. The light scattering optics of a FACS II (Becton-Dickinson, Sunnyvale, CA) were modified (Figure 1) so that two detectors could be used to monitor the light scattered by a cell. An argon-ion laser, model 164-05 (Spectra-Physics, Mountain View, CA), was fitted with a special set of mirrors (Spectra-Physics 83128A and 83198A/83199, on loan from Becton-Dickinson) so that the laser operated with all wavelengths of light between 351 and 488 nm (13). Short pass filters 420 sp and 440 sp (Diric Optical Co., Marlboro, MA) were placed in front of one detector while a 488 nm narrow band filter (Diric) was placed in front of the other detector. Two filters in succession in front of the uv detector were needed because of the inefficiency of each filter alone. Two photo diodes (PIN 10, United Detector Technology, Santa Monica, CA) were used in this system. For some experiments a photo tube (9924B, EM1Gencom, Inc., Plainview, NY) was used to detect the uv scattered light.

In order to test whether the two optical paths were identical, the optical filters were interchanged. The correlation plots shown in Figures 3, 5 and 6 were unaffected by this switch in filters. As a further test of the system identical filters, either two 420 sp or two 488 narrow band filters, were placed in front of the two detectors. Cells analyzed using this arrangement gave live, highly correlated plots indicating that the two optical paths were the same.

The detection of fluorescence emitted by the cells utilized the optical filters described previously (14). In each of the histograms (Figures 2, 4) 108 cells were analyzed. Viable and nonviable nucleated cells were discriminated by analyzing the cells in the presence of propidium iodide (12). An 80 μm nozzle was used in all experiments.

Calibration of scatter detector. The rear lens of the scatter detector was removed and a translucent light diffuser was placed adjacent to the rear iris. The stream was then focused onto the plane of the rear iris. The distance from the stream to the front of the scatter detector was measured and the rear iris was closed down to a pinhole. The entire scatter detector was then removed from the optical bench and placed such that the laser beam could be directed onto the translucent light diffuser in the rear of the detector. Light then traveled backwards through the light scattering detector optics. The light entering the rear of the scatter detector, was focused onto a point in front of the detector, and was then projected onto a wall. The size of the circle of light on the wall was then dependent on the front iris of the scatter detector. By measuring this projected circle the outer collection angle of the scatter detector could be calculated using trigonometry. In a similar manner the angle subtended by the obscuration bar could be determined by measuring the shadow cast by the bar. In these experiments, the light scattering detector was set to collect light from 0.7° out to 15°. (These calculations ignore the effect of the refractive index difference in the stream-air interface (21).)

Cell preparation and staining. Chicken erythrocytes were collected in heparin, were washed three times in buffered saline and were resuspended in 0.1% glutaraldehyde (25 times the packed red cell volume). After 15 min at 0°C the cells were pelleted and were resuspended in 0.1 M glycine. After 2 hr in the cold the cells were washed three times in distilled water and were stored at 4°C.

Spleen and bone marrow cells were obtained from 2-month-old BALB/c mice (Cumberland View Farms, Clinton, TN). The bone marrow cells were washed twice in buffered saline and analyzed in the presence of 200 ng/ml propidium iodide. Spleen cells were stained with rabbit anti-mouse immunoglobulin (3) conjugated with fluorescein isothiocyanate (4) following the techniques previously described (14).

The production and characterization of monoclonal antibody DNL1.9 has been described (5). This antibody was coupled with biotin-streptavidine following the protocol of Bayer and Wildcheck (2). Fluorescein-conjugated avidin (Vector Laboratories, Burlingame, CA) was used as a second step in indirect immunofluorescence.

Cell populations isolated from bone marrow were pelleted onto glass slides and the morphology identified using Wright's stain. Identical slides were also stained with peroxidase (9) to identify monocytes and granulocytes.

Results

Analysis of Chicken Erythrocytes

Glutaraldehyde-fixed chicken erythrocytes have been used as a both fluorescent and light scattering standards in flow systems (6). The typical histogram for forward angle light scattering is bimodal (Figure 2), which results from the asymmetry of the cell (15). Histograms collected for light scattered in the uv (Figure 2A) or light scattered at 488 nm (Figure 2B) are similar. The coefficient of variation for cells identified as being edge on with respect to the scatter detector (lower peak in the histogram) is less for the uv scattered light.
The correlation plot for the two detectors is shown in Figure 3. A linear correlation does not exist between the amount of light scattered at 488 nm and in the uv. This means that there is not a simple transformation between the intensity of light scattered in uv to that intensity expected at 488 nm.

**Analysis of Mouse Bone Marrow**

The cells in murine bone marrow can be subdivided into four groups using visible forward angle scattered light (16). The histogram for forward angle 488 nm scattered light for mouse bone marrow is compared to the histogram for uv scattered light in Figure 4. Only two distinct populations can be observed using uv scattered light, while three peaks are apparent in the 488 nm light scattering histogram. This indicates that less discrimination between unixed mouse bone marrow cell populations can be made using uv light as compared with 488 nm light.

The relationship between the signals generated by the two colors of scattered light is shown in the correlation plot in Figure 5. Only viable cells that excluded propidium iodide were analyzed in this figure. The cluster of dots with the smallest signals are erythrocytes (Figure 5, E) as determined by morphological analysis of cells isolated using the sorting capabilities of the FACS. These cells can be distinguished from the nucleated cells by both visible and uv scattered light.

Two other populations (Figure 5, L and G), which are easily distinguished by visible light scattering, are not separated by the uv light scattering signals. However, by correlating the two signals a third population of cells (Figure 5, LL) lying between the two major clusters of cells can be identified. These populations were separated for morphological and histochemical studies. The group of cells lying within the box in Figure 5 (LL) were separated from the cells lying directly below...
cell in population G were granulocytes and monocytes. In order to better characterize these purified populations the slides were stained for peroxidase, since monocytes and granulocytes are distinguished from large lymphocytes by their ability to be stained by this procedure (10). The percent of positively stained cells in the purified populations are presented in Table 1. The cells that contain the peroxidase are found in cluster G. This suggests that the cells in LL are primarily lymphoid rather than myeloid in origin.

In order to further study the nature of these large cells the bone marrow cells were stained with monoclonal antibody DNL1.9 (5). (This antibody identifies all murine lymphocytes of the B lineage but not thymocytes or mature T cells. This antibody binds to 60–80% of the small cells identified by forward angle visible light scattering and 10–15% of the large nucleated bone marrow cells (5).) Bone marrow cells were stained by this antibody and then were analyzed by the two color light scattering system. By gating on the immunofluorescence the cells that bound this monoclonal antibody were found in populations L and LL.

**Analysis of Mouse Spleen Cells**

The histograms of uv and 488 nm scattered light from mouse spleen cells are very similar. Two populations can be identified, one which is predominantly erythrocytes and the other is comprised primarily of small lymphocytes. The correlation plots between uv and visible scattered light, shown in Figure 6, indicate some surprising differences. The correlation plot of all cells including erythrocytes and both viable and nonviable lymphocytes is shown in Figure 6A. Erythrocytes (E in Figure 6) can be identified by either uv or by 488 nm light scattering. Nonviable nucleated cells (N in Figure 6) can be distinguished from viable cells using 488 nm light scattering but overlap in

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**Figure 4.** Light scattering histograms of mouse bone marrow cells: (A) uv light scattering; (B) 488 nm light scattering.

**Figure 5.** Correlation plot of uv vs. visible light scattering signals for viable mouse bone marrow cells. Dead cells, which stained with propidium iodide, were gated out of this plot. The cells represented by the clusters were isolated for morphological analysis: (E) erythrocytes; (L) lymphocytes; (LL) large lymphocytes; (G) granulocytes and monocytes.
Table 1. Peroxidase staining of bone marrow cells isolated using two color light scattering

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Peroxidase positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unseparated</td>
<td>48</td>
</tr>
<tr>
<td>L</td>
<td>0</td>
</tr>
<tr>
<td>LL</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>98</td>
</tr>
</tbody>
</table>

*Bone marrow cells were analyzed using two color light scattering. Populations L, LL, and G (Figure 3) were isolated and were centrifuged onto slides. These slide preparations were then stained for peroxidase (9).

scattering intensity with viable lymphocytes (L in Figure 6) when uv light is used.

The viable lymphocytes in spleen are comprised of two functionally distinct populations of cells, T and B cells. By staining the cells with immunofluorescence, these cells can be distinguished. The two color light scattering correlation plot for cells that express cell surface immunoglobulin (i.e., B cells) is shown in Figure 6B, while the correlation plot for immunoglobulin-negative cells is shown in Figure 6C. It is clear that the cells which express cell surface immunoglobulin scatter light differently than those which lack this determinant. This difference is not simply a result of having antibodies bound to the cells. In other experiments the same discrimination by light scattering could be obtained using Thy 1.2 as a marker for T cells. Lymphocytes that stained with a monoclonal anti-Thy 1.2 (AT83A from F.W. Fitch) produced a scattering pattern similar to Figure 6C, while those cells that did not express Thy 1.2 gave a scattering pattern like Figure 6B.

Discussion

The differences in light scattering signals that are observed with different wavelengths in a flow system are not unexpected. A change in wavelength, in effect, changes the size of the cell as measured by light scattering (7,11). The relationship between the light scatter signal and particle size changes as the size of the object changes (25) and may, in some instances, decrease as the particle increases in size (25). By collecting the scattered light using the same collection optics, different fractions of the two wavelengths of scattered light are blocked by the obscuration bar. This places different emphasis on the diffraction and refraction components of the scattered light with the two detectors (7). In other words, the cellular parameters observed using one wavelength of light may be quite different from the cellular parameters identified with a different wavelength of light. The light scattering data from the fixed chicken erythrocytes indicates that even for cells with the same refractive index but which differ in apparent size, a linear transformation can not be made between the visible and uv light scattering signals.

The ability to discriminate between T and B lymphocytes in the spleen and between different cell types in the bone marrow may be a result of the emphasize of the refractive index and size differences between cells as detected by two different colors of scattered light. It has been shown that T and B cells differ in size (8). In addition it has been shown that the nuclei of Fuelgen-stained B cells are slightly smaller than those of T cells (1). The B cells stain more densely and contain coarser chromatin granules (1). These subtle differences, which can not be discerned by visual analysis, may be amplified by the two color light scattering analysis. It might be expected that these differences may be further enhanced by comparing the...
light scattering in uv with that observed in the far red or even infrared portion of the spectrum.

It should be emphasized that the discrimination observed in this article was performed on viable cells that could be isolated for further functional characterization. This potential of studying the cells for functional differences after analysis by two color light scattering provides a new method for identifying subpopulations of normal cells in a heterogeneous group. The discrimination between large lymphocytes and granulocytes in the bone marrow could only be done using two wavelengths of light.

It is interesting that population LL (Figure 5) binds the hybridoma DNL I.9. This monoclonal antibody identifies all lymphocytes of the B cell lineage and may recognize very early precursors of this cell type. Large pre-B cells have been described which do not have detectable cell surface immunoglobulin but which synthesize and secrete monomeric IgM (17,21). Functional studies are in progress to determine if the large DNL I.9 binding cells, which can be identified by this dual wavelength technique, correspond to those large pre-B cells. By correlating the cell surface antigens with cell populations identified by physical differences, a better discrimination between different lineages of cells can be made.

The two color light scattering technique also provides a method for rapidly screening newly formed hybridomas. By staining spleen cells with hybridoma supernatants and analyzing the lymphocytes using this two color light scattering technique, monoclonal antibodies that bind to all lymphocytes can be distinguished from antibodies that bind to predominantly B cells or to mature T cells. No prior separation of the normal spleen into T and B enriched fractions is necessary in this assay procedure. The cells that are stained can be identified simply by gating on the two light scattering signals. Likewise the staining of minor populations of bone marrow can be recognized using these procedures so that particularly interesting hybridomas can be readily identified for further characterization.

The change in light scattering profile with a change in wavelength has direct practical implications. Two commercially available instruments (from Becton-Dickinson and from Coulter) use a single laser for generating both fluorescence and light scattering signals. Changing the wavelength for the excitation of different dyes produces a concomitant change in the light scattering pattern. Cell populations that were identified by light scattering at one wavelength may not be the same as those identified at other wavelengths. In order to use light scatter as a consistent parameter for cell identification a second laser should be used in these instruments that does not change when different wavelengths are used to excite different fluorochromes.

Literature Cited


