PRACTICAL FLOW CYTOMETRY

Fourth Edition

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polarizing filters (see p. 157). The technique requires no reagents, and represents the easiest way to count and sort eosinophils from a wide range of species (L. Terstappen, personal communication). The sorter manufacturers won't tell you about it, because it is the subject of a patent now held by Abbott and used in that company's Cell-Dyn hematology instruments. The birefringence of eosinophil granules, which is responsible for the cells' higher depolarized scatter signals, was noted decades ago; one could very likely use polarized and depolarized extinction signals to count or sort eosinophils without infringing on anybody's patent.

In 1999, Mendelow et al[2536] reported that a population of cells with relatively low polarized side scatter values and high depolarized side scatter values appeared when the peripheral blood of malaria patients was analyzed in a Cell-Dyn instrument. It was suggested that these cells were monocytes containing the malaria pigment hemozoin, which is known to be birefringent. Nordström et al[2537] subsequently confirmed this by cell sorting.

Many bacteria have birefringent cell walls, which suggests that polarized and depolarized scatter measurements might be useful in discriminating among genera or species. However, depolarized scatter signals, even from eukaryotic cells, are typically of much lower intensity than polarized scatter signals, suggesting that depolarized signals from bacteria could be too weak to permit precise measurements.

Multiple Wavelength Scattering Measurements

Any apparatus that incorporates a lamp instead of a laser as a light source for light scattering measurements will, obviously, measure light scattered over a larger range of wavelengths than will a laser source instrument. Technicon's older blood cell and differential counters[84,85], the Cytomat arc source instrument[88], the apparatus (Skatron Argus, Bio-Rad Bryte, etc.) developed by Lindmo and Steen[100-3], and the old B-D FACS analyzer all fell into this category.

The Technicon Hemalog D apparatus was noteworthy for its utilization of measurements of light scattering at different wavelengths for cell classification. In this system, basophil leukocytes were stained with a blue basic dye, which imparted a blue color to their specific granules. The granules therefore absorbed red light; however, their absorption was not high enough to permit unequivocal discrimination between basophils and other cells on the basis of absorption, especially since the number of granules per cell is highly variable. Satisfactory discrimination was achieved by making separate measurements of the scattering of red and near-infrared light by the cells. Since basophils, which contained the blue dye, absorbed more red light than infrared light, the ratio of red scatter amplitude to near-IR scatter amplitude was lower for the basophils, which could thus be discriminated from other cell types in the two-dimensional measurement space.

While developing the Block differential counter, we measured forward and 90° scatter signals from fixed and unfixed, unstained leukocytes at different laser wavelengths (325 and 441 nm from a helium-cadmium laser, 488 and 515 nm from an argon ion laser, and 633 nm from a helium-neon laser), and were somewhat surprised to find that ratios of scatter intensities at different wavelengths were different for different cell types, to an extent that allowed us to obtain differential counts of lymphocytes, monocytes, neutrophils, and eosinophils in unstained blood samples. Our enthusiasm for further work along these lines was tempered when we found that slight alterations in the geometry of the optical system used for forward scatter measurements could produce large changes in the relative positions of cell clusters. I was thus not surprised to read others' reports[156,161,180] that laser light scattering at different wavelengths (351/363, 457, 488, and 515 nm, all from argon lasers) provided information that could discriminate different types of blood cells.

A principal problem with studies of this type lies in the difficulty of making sure that the optical geometry is the same at the different wavelengths used. Even in the case in which UV and visible light are emitted in the same collinear beam by the same argon laser, the beam diameters are apt to be different, and the focusing lens, unless custom made for the purpose, will not have the same focal length at both wavelengths. This makes it hard to determine the extent to which apparent differences in scattering at different wavelengths may be due to small but significant differences in such factors as beam size and collection angle. Others have reported that UV scatter signals, obtained from the B-D FACS using an argon laser source, were less satisfactory than signals at 488 nm for cell discrimination. Using the Block instruments, we usually obtained better discrimination from the UV scatter signals than from signals at 488 nm. This may represent another instance among many in which each of several groups gets its best results when using the methodology with which it is most familiar.

From Russia with Lobes

A few pages back, I said we'd get back to the complex stuff about scatter; by now, you probably think the Mueller matrix has been sent to Siberia. Well, it has, quite literally, and I am happy to report that it is alive and well there, in Valeri Maltsev's lab at the Institute of Chemical Kinetics and Combustion.

Over the years, a few attempts have been made to apply relatively sophisticated multiangle scattering measurements to cell sizing and characterization. Since Mie's theory of scattering, which forms the basis for attempts to measure cell size measurement by forward scatter, uses a model in which particles are spherical and homogeneous, we might reasonably expect that, however bad our results might be with spherical cells, they would get worse when we tried to size asymmetric cells. This presented a problem to people concerned with the most common and most profitable type of cell volume measurements, i.e., the manufacturers of blood cell counters for clinical laboratories, because erythrocytes are normally not spherical. Technicon's H-1 hematology

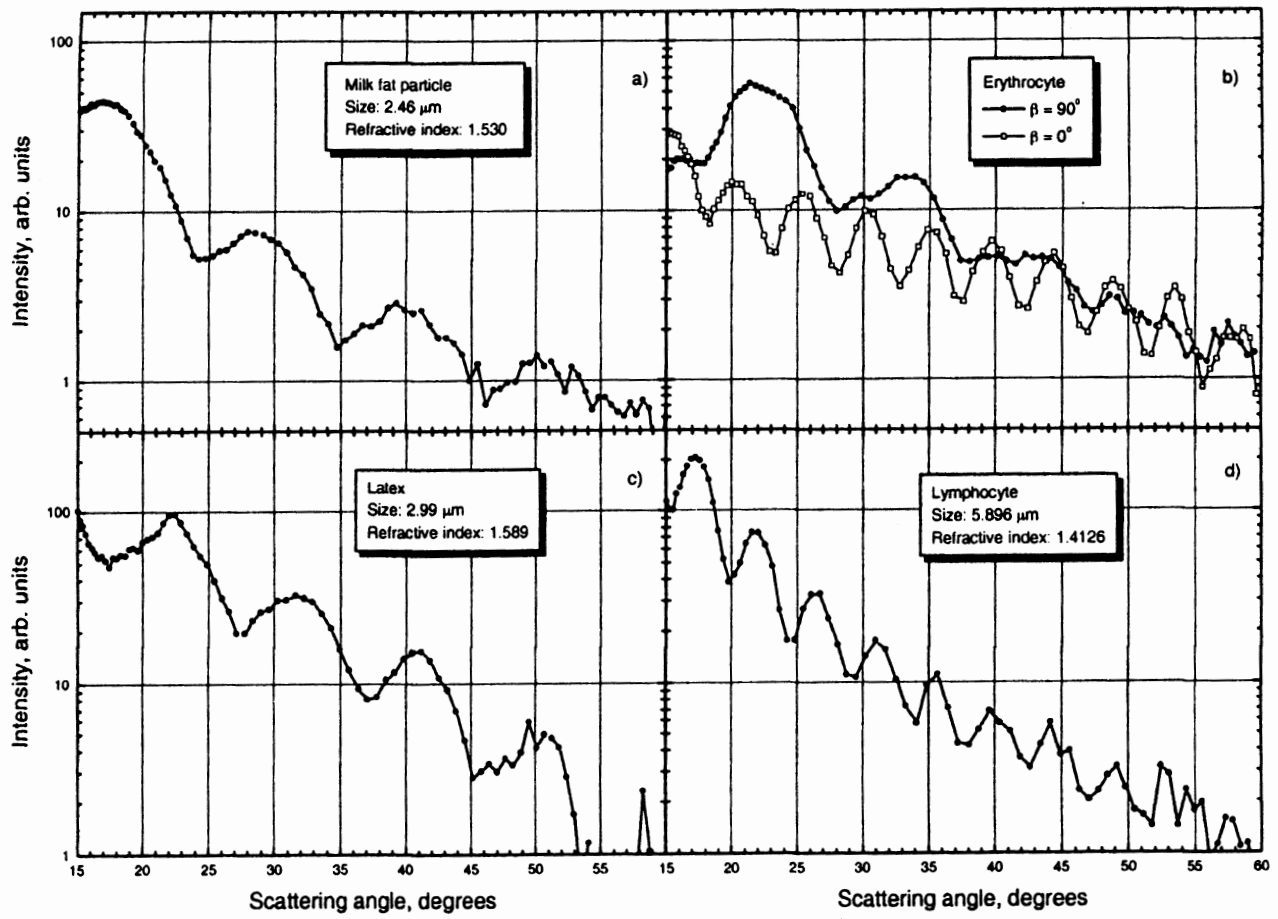

Figure 7-3. Indicatrices (plots of intensity vs. scattering angle) of a milk fat globule, a latex bead, a lymphocyte, and two erythrocytes. From: Shvalov AN et al, Cytometry 37:215-220, 1999 (Reference 2539), © John Wiley & Sons, Inc., used by permission.

instruments, and their successors (Technicon is now Bayer Diagnostics; see Chapter 8), employ a couple of ingenious maneuvers to deal with the problem. The discoid erythrocytes are converted, without changing their volumes, to spheres, using a procedure described by Kim and Ornstein[166]. This eliminates artifacts due to cell asymmetry; however, one still encounters substantial variation in the refractive indices of individual cells due to cell-to-cell differences in hemoglobin concentration. A measurement procedure devised by Tycko et al[684] meets this problem head-on; since the variation of refractive index with hemoglobin concentration is linear, it is possible, by making measurements of light scattered at two angles, to determine hemoglobin concentration and obtain volume from tables calculated using Mie theory. The system can be calibrated in absolute units of volume, using droplets of water-immiscible oils of different refractive indices as standards. This elegant methodology is demonstrably successful for its clinical hematologic purpose; it is unlikely to be adaptable to sizing other cell types.

Maltsev et al have come up with a simpler way of sizing sphered red cells in the course of extensive investigations on multiangle light scattering by cells and beads. They have devised a unique scanning flow cytometer in which particles are illuminated by a laser beam directed along the axis of flow; a fixed spherical mirror is used to collect scattered light. As the particle moves down the flow stream, the scattering angle from which light reaches the mirror changes. Thus, it is possible to derive a plot of intensity vs. scattering angle, known as an **indicatrix**, for each individual particle traversing the system. Indicatrices for a milk fat particle, a latex bead, a lymphocyte, and two (unsphered) differently oriented erythrocytes appear in Figure 7-3.

Indicatrices are uniquely characteristic of particle types; those for beads agree closely with calculations according to Mie theory. The sizes and refractive indices of spherical and near-spherical particles can be determined from their indicatrices, and, in at least some cases, it is possible to specify two or three angles for which light scattering measurements can be used for sizing. However, as Murphy's Law would predict, these angles are different for different cell types.

In cytometry, as in many other fields, there is little new under the sun; Loken, Sweet, and Herzenberg made measurements of multiangle light scattering in a modified FACS in the 1970's[155]; they did not, however, pursue the theoretical side of their investigations far enough to yield practical results. A commercial application of the Maltsev group's

work to hematology is now being pursued, but it still seems unlikely that the high-speed cell sorter manufacturers will be rushing to put indicatrix measurement capability into their products any time soon.

Optical Parameters: Absorption

Since, even in this day and age, a lot of quantitative chemical and biochemical analyses are done with absorption measurements, we might consider why we don't make more use of absorption in cytometry. Absorption measurements are somewhat more demanding than scatter and fluorescence measurements. In any microscope, light scattered at larger angles than can be collected by the microscope objective used cannot readily be distinguished from light absorbed by the specimen; accurate absorption measurements therefore require light collection over a relatively large angle, necessitating use of a lens with a high N.A. It's not that hard to build a flow cytometer with high-N.A. lenses, oriented along the same axis, for illumination and collection, suitable for absorption measurements; this configuration is used in blood cell counters. However, most laser source fluorescence flow cytometers incorporate a high-N.A. collection lens placed at right angles to the illuminating beam, making it impossible to mount a high-N.A. lens along the beam axis, and use low-N.A. optics to form an illuminating beam, which is not effective for absorption measurements.

To maximize precision in absorption measurements, it is also necessary to match the refractive indices of the object under study and the medium in which it is suspended, this maneuver minimizes apparent absorption due to scattering. However, in work with intact cells, it is generally not possible to completely avoid scattering by internal cellular structures, e.g., cytoplasmic granules, even when the indices of sheath, sample, and cell membranes are matched.

The major problem with absorption measurements arises from the fact that, in most cases, cells, even when stained, do not absorb more than a small fraction of the light passing through them. Most of the light reaching the detector is unaffected by interaction with the specimen; it is, therefore, background, and, as Johnnie Cochran might summarize the Wood-Hoffman model discussed on pp. 221-3, "If there's too high a B, you just can't see." Fluctuations in the intensity of illumination therefore have larger effects on absorption measurements than on measurements of scattered light and fluorescence. In the latter, signal intensity is proportional to source intensity; a 1% change in illumination intensity produces a 1% change in a scatter or fluorescence signal. Suppose, however, that an absorption measurement is made of a particle that removes 10% of the light from the incident beam. A 1% increase in illumination intensity occurring while the particle passes through the observation region would result in an apparent light loss of 9% instead of 10%, while a 1% decrease in illumination intensity would result in an apparent light loss of 11%. In this case, a 1% change in illumination intensity produces a 10% change in the amplitude of an absorption signal; the less light the particle removes from the beam, the larger the effect of source intensity fluctuations. The intensity fluctuations of the laser sources most commonly used in fluorescence flow cytometers, while small (a few percent peak-to-peak), are sufficient to make absorption measurements unacceptably imprecise. Because of the problems just discussed, the use of absorption measurements in flow cytometry is somewhat restricted; they are most commonly employed for semiquantitative detection of strongly absorbing substances in apparatus designed for blood cell counting and sizing, employing lamps rather than lasers as light sources.

Absorption Effects on Light Scattering

I noted on p. 279 that the presence of an absorbing (colored) substance in cells may be inferred from differences in intensity of scattering signals at wavelengths at which the material does and does not absorb strongly. A dramatic illustration of this, shown in Figure 7-4, comes from the work of Ost et al[2449], who measured red and violet light scattering by leukocytes and erythrocytes in unstained dilute whole blood.

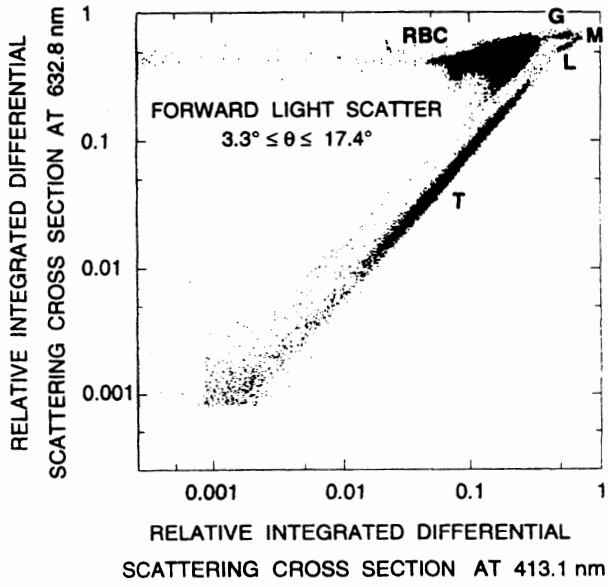

Figure 7-4. Erythrocytes scatter less light at a wavelength at which hemoglobin exhibits strong absorption. G: granulocytes; L: lymphocytes, M: monocytes, RBC: erythrocytes, T: thrombocytes. From: Ost V et al, Cytometry 32:191-197, 1998 (Reference 2449), © John Wiley & Sons, Inc., used by permission.

Erythrocytes contain high concentrations of hemoglobin, which absorbs strongly in the violet spectral region but only weakly in the red spectral region. If more of the photons incident on a cell are absorbed, fewer remain to be scattered. Their relatively low forward scatter intensities at 413.1 nm (violet) allow the erythrocytes (RBC) to be distinguished from leukocytes (G, M, L) and thrombocytes (T) on the two-dimensional dot plot of violet vs. red forward scatter.