COMPUTER-CONTROLLED CELL (PARTICLE) ANALYZER AND SEPARATOR. USE OF LIGHT SCATTERING

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1. Introduction

Analytical flow systems enable the rapid characterization of a large number of individual cells or particles by spectroscopic and electronic means [1–6]. So-called sorters or separators incorporate the additional feature of separation on the basis of the measured properties [7–12]. Flow systems have been used to investigate such diverse biological problems as tumor cell identification [13–15], leucocyte distributions [10,16–18], DNA content and cell cycle analysis [19–24], lymphocyte expression and antibody production [10,25,26], intracellular enzyme concentration and activity [27], cell surface properties and lectin binding [26,28], and in our laboratory, cellular organization of *Hydra*, cell fusion, and the analysis of bacteria and submicron particles. These studies have exploited the use of fluorochromic stains for nucleic acids and proteins, fluorogenic substrates for intracellular enzymes, fluorescent antibodies, Coulter volume, and light scattering. The simultaneous use of multiple sensors for fluorescence, light scattering and other signals in various combinations forms the basis of separation and thus a meaningful resolution of complex biological systems. The process of separation itself serves two functions, first the morphological, biological, or biochemical identification of components in a distribution, and second the physical isolation of homogeneous material for further study.

In another communication we have described the features of a computer-controlled multiparameter analyzer and separator [12]. The present report deals primarily with preliminary studies on cells and particles, 0.3 to 30 μm in size, using light scattering simultaneously at two freely selectable angles. To our knowledge these data are the first reported for bacteria and submicron particles using a flow instrument capable of separation.

2. Materials and methods

2.1. *Beads and cells*

Dow Chemical Corp. polystyrene beads of 0.481 ± 0.0018 μm and 0.357 ± 0.0056 μm diameter were centrifuged and filtered through 0.6 and 0.45 μm Millipore filters respectively and resuspended in water filtered through 50 Å Sartorius membranes. Sheath fluid for these experiments was filtered to 50 Å as well. *E. coli* strain B was grown in minimal medium at 37°C. Live cells were centrifuged and filtered through 8 μm Millipore filters, or cells were fixed in 90% EtOH at −40°C, centrifuged, filtered and resuspended in water. Mammalian cells were continuous Chinese hamster fibroblast lines or Friend-virus-transformed erythropoietic cells (the kind gift of Dr W. Ostertag). Suspension cultures were harvested by centrifugation, surface adhering cultures by trypsinization, and filtered through 25 μm steel mesh filters in glucose-supplemented buffered saline. *Hydra attenuata* (provided by Dr A. Gierer) was disrupted in 70 mosmolar medium [29], filtered sequentially through a glass G1, and 45 and 28 μm steel mesh filters.

2.2. *Instrument for analysis and sorting*

A schematic representation of the analyzer-sorter is shown in fig. 1 and the general functional features of the device are given in table 1. We have operated to date in single and multiparameter modes with both fluorescence and light scatter detectors at particle frequencies up to 10 kHz. In the current version of system software, various analysis and deflection algorithms are selectable from the keyboard or console.

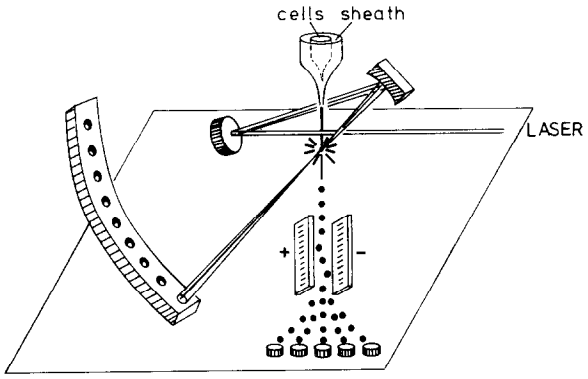

Fig. 1. Schematic diagram of multiparameter cell-particle analyzer–sorter. In common to devices reported by other groups [10], the stream of cells or particles is confined to the central portion of a narrow liquid column in air by the interaction of coaxial flows of the sample suspension and a sheath fluid. A focused laser beam intercepts the liquid column at one point and the individual particles emit a characteristic luminescence spectrum consisting in the general case of fluorescence, light scattering, and absorption components. Two photomultipliers (not shown) capture fluorescence signals at different wavelengths, a third (also not shown) monitors the laser beam intensity. Other detectors implemented with fiber optics are used for light scattering and absorption. These can be set independently with precision to any desired angle with respect to the laser beam. The focusing of the beam with mirrors renders the system substantially achromatic and permits the easy adjustment of the focused beam geometry. Furthermore, the laser intersects the liquid stream obliquely leading to three advantages: a) minimization of front surface and internal reflections (at Brewster's angle of incidence: 53°); b) a conical distribution of the light scattered circumferentially from the stream itself, thereby reducing the background noise signal to the horizontally mounted fluorescence detectors; and c) an observation angle larger than the corresponding true scattering angle, thus reducing unwanted contributions from the main transmitted laser beam. The fluid drops generated precisely by a crystal mounted on the nozzle assembly (not shown) are charged before break-off according to the sorting program and are electrostatically deflected into 5 chambers. Additional features include the systems for control of flow and electronic signal processing.

3. Results and discussion

3.1. *Cells, 5–30 μm*

Although fluorescence is one of the most powerful spectroscopic parameters to be used with flow systems for distinguishing cell types, the method of vital staining often results in the loss of viability while fixation introduces artifacts and precludes further growth. Furthermore, fluorescence detection alone is not likely to resolve complex mixtures of cells. Light scattering, however, can give additional information concerning the size, shape, and internal structure of cells and particles [30–36].

Table 1
Features of computer-controlled cell (particle) analyzer–separator

Flow system:	selection of 6 thermostated samples and 3 sheath fluids.
	semiautomatic flushing system: sterile operation.
Excitation source:	argon laser, visible and UV lines (alone or simultaneous).
Signal sources:	cell (particle): inherent or derived fluorescence, light scattering, absorption. Single or multiple detectors in variable combination.
	laser intensity reference (for signal normalization); system monitors.
Signal conditioning:	5 channels for 5 sources, producing both a peak and integral.
	ac, dc coupling; scaling (amplification); filtering
	simultaneous digitization of 10 analog signals.
Separation:	drop charging: 2 polarities and 2 levels, thus giving 4 deflected and 1 undeflected categories.
	programmable number and timing of drops charged for each cell (particle).
	maximal throughout rate of 10–20 kHz.
Computer control:	setup and testing prior to analysis and separation.
	real-time data acquisition and processing.
	real-time single or multiple parameter pulse-height analysis, display, and drop charging using freely programmable functions of all signals.
	data storage, analysis, and plotting subsequent to experiment.

The laser is a Model 53 (Coherent Radiation) and the computer a PDP 11/45 (Digital Equipment Corp.).

Light scattering in flow system analyzers and sorters has been utilized previously only at single fixed angles and with mammalian cells or spherical particles of like dimension [4, 11, 37]. We have attempted to extend the usable size range and provide for variable angles and apertures in studies on the following systems. In cooperation with Dr A. Gierer, Tübingen, we have analyzed the disaggregated cells of *Hydra* after maceration [29] using both fluorescence and light scattering detection, and have reconstituted live animals after passage of the cells through the instrument, thus establishing their viability. We have separated the interstitial cells from the remaining cell types using light scattering detection alone at 10°. In other studies of mammalian cell fusion induced by a variety of chemical agents, we have combined fluorescence detection of live cells using their enzymatic cleavage of fluorescein diacetate [38] and light scattering at 10° as a measure of the total cell number [39].

3.2. *Bacteria*

There are in the literature a number of theoretical treatments of light scattering from particles with the size and refractive index of bacteria [31–33]. Most experimental studies have been conducted with devices which do not measure the properties of individual bacteria but rather those of a large population possibly

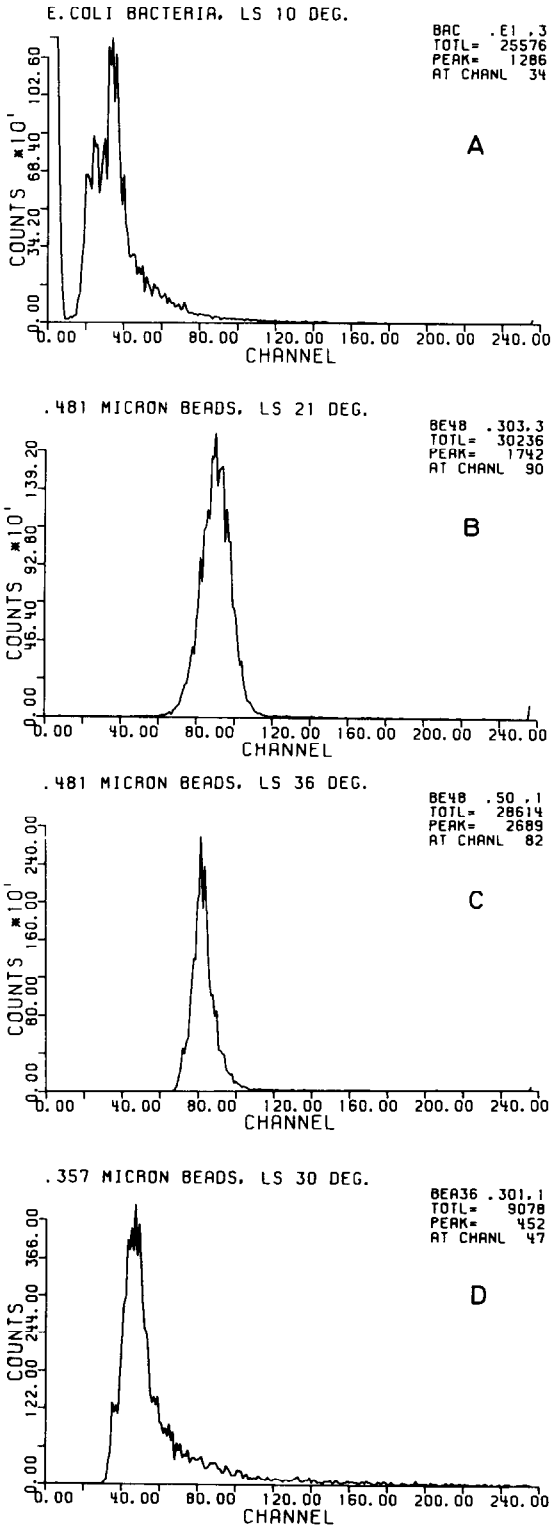

Fig. 2. Light scattering frequency distributions of polystyrene beads and bacteria measured with the flow analyzer–separator. Computer generated and unprocessed pulse height analyses of light scattering signals measured at the indicated angles from fixed *E. coli* bacteria (A) and 0.481 μm (B, C) and 0.357 μm (D) polystyrene beads. In all cases, the peak height of each signal was acquired and digitized. The total counts, peak position, and peak height of each distribution are indicated below the file name. In the case of the bacteria, low amplitude noise possibly due to debris can be seen in the very low channels and the ascending limb of the peak. It was found possible to perform a blank run in a separate experiment and subtract out its contribution (not shown here). The distributions are not particularly smooth due to the relatively low number of counts collected and the finite differential non-linearity of the analog-to-digital converters. The 488 nm (B, C, D) and 514 nm (A) argon laser lines were used at power levels of about 0.5 watts. The beam was focused with a 46 mm focal length mirror to a spot 25–30 μm in height and an elliptical axial ratio of about 5 (long axis horizontal to the stream) measured at the $1/e^2$ intensity contour. The incidence angle was 53° (A, D) and 43° (B, C). The detectors had an acceptance angle of 0.5°. In these preliminary experiments, separation was not attempted and thus the drop generator was not activated.

heterogeneous in size, shape, and structure. Possible exceptions are experiments done on aerosolized bacteria which, however, are dehydrated and would be expected to differ markedly from cells in liquid suspension [40]. Furthermore, these devices are not capable of performing separations. With our flow system, however, we have succeeded in measuring the light scattering properties of individual fixed log-phase *E. coli* (fig. 2A) and live bacteria have been analyzed as well with the instrument. We are presently investigating the effects of orientation factors and metabolic state upon the distributions and the use of light scattering in combination with fluorescence for the detection and isolation of mutants.

3.3. *Submicron particles*

Many biological particles are of dimensions smaller than 1 μm. The actual *counting* of such structures for the estimation of biochemical composition and content per particle as with vesicles, or for the determination of plaque forming units to particle ratios as in the case of viruses remains a technical problem. In addition, the determination of the physical size and shape of a biological particle in its normal aqueous environment is difficult. One has to rely in general upon electronmicroscopic measurements after sample dehydration or classical light scattering determinations which integrate over the entire population.

Spherical particles smaller than 1 μm can be treated by the electromagnetic theory of scattering developed by Mie [41]. We have thus conducted measurements upon uniform submicron beads so as to make quantitative correlations with the theoretically predicted scattered intensities. Typical distributions are shown in fig. 2(B–D). The observed coefficients of variation (6–11%) are larger than those given by the manufacturer and reflect as yet unresolved instrumental factors as well as the non-linear dependence of scattered intensity upon particle diameter. The angular dependence of the measured signals was determined in the range of 10–50°. A comparison between theory and experiment for 0.48 μm beads is shown in fig. 3. The measured points fall between the theoretical curves for particles with diameters of 0.46 and 0.53 μm in a surprisingly good correspondence with the known diameter. It thus

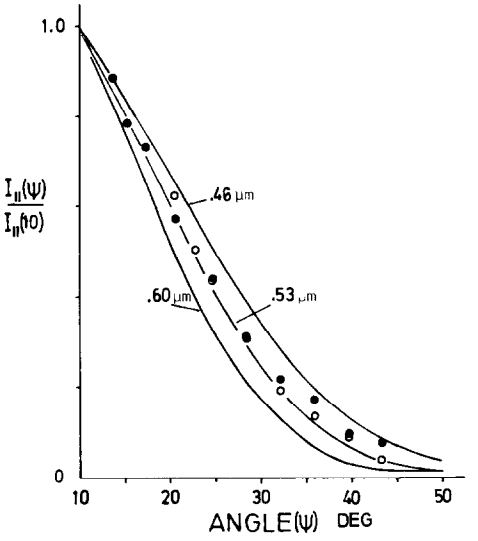

Fig. 3. Angular dependence of the forward scattering from 0.48 μm polystyrene beads measured with the flow system. The ordinate represents the scattered intensity at various angles, normalized to values at 10°. The laser beam was polarized in the plane of incidence (and observation) and intersected the stream at an angle of 43°. The angles have been corrected for refraction effects due to the air–water interfaces. Two detectors with an acceptance angle of 0.5° and separated by a constant angle of 10° were used. The values for the outermost detector (○) are normalized to those of the innermost detector (●) at 17.5°. The theoretical curves (solid lines) are derived from Denman et al. [42] and correspond to the laser wavelength of 488 nm, the refractive index of the beads (1.59) and the indicated particle diameters in microns.

appears that *determinations of absolute size using light scattering with flow systems* may be possible under given conditions.

It is evident from fig. 3 that data are required at two or more angles in order to generate the functions of *relative* scattered intensities which can be correlated with size. Our instrument incorporates two detectors which allow the simultaneous detection of signals from individual particles and thus enable, in principle, the fractionation of mixed populations on the basis of size. The optimal angles and algorithms are yet to be determined although the use of simple ratios appears very promising in initial experiments with synthetically mixed populations of defined beads.

4. Conclusions

We have demonstrated the ability to use light scattering signals from biological samples smaller than mammalian cells as one of the criteria for analysis and thus separation in an automated flow instrument. This feature opens new areas of biological investigation. At the micron size level subcellular structures such as nuclei and mitochondria become accessible as well as a whole range of bacteria, yeasts and other organisms. Since the scattering at higher angles reflects internal structures, and external inhomogeneities as well as overall shape, one can hope to separate classes of functionally different subcellular particles and perhaps bacterial mutants or sporulating bacterial cells.

It is obvious from fig. 4 that the signal-to-noise ratios in the case of submicron particles are not yet limiting factors and we are presently investigating the limits of sensitivity and resolution. It already appears feasible to examine biological particles such as viruses, chromaffin granules [43], and metaphase chromosomes (projects currently under investigation in our laboratory) and with instrumental modifications it may be possible to extend the range to vesicles and larger macromolecules.

It is important to stress the enormous advantage of a multiparameter approach to analysis and separation using flow systems. The use of a computer is almost a prerequisite for the full utilization of available features, especially with respect to the large amount of data that is generated within the course of an experiment.

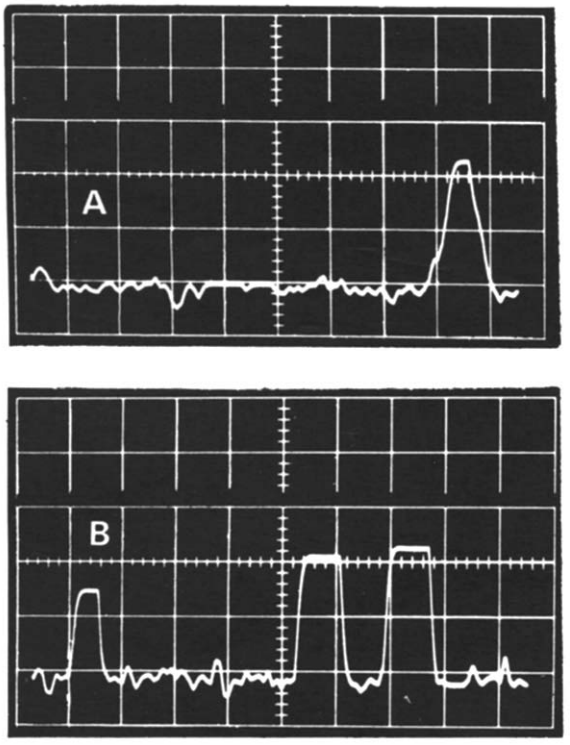

Fig. 4. Light scattering signals derived from 0.357 μm polystyrene beads. (A) Actual signal from the photomultiplier receiving 0.5° of light at 13.7° from the 488 nm laser beam intersecting the liquid stram at 43°. The scale is 2 μsec/division and −0.25 V/division. (B) Peak sample-hold signals produced from individual light scattering signals such as shown in (A). The scale is 50 μsec/division and + 1 V/division.

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