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**Single Particle Analysis with a
360° Light Scattering Photometer**

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Single Particle Analysis with a 360° Light Scattering Photometer

Marty Frank Bartholdi

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SINGLE PARTICLE ANALYSIS WITH A 360° LIGHT SCATTERING PHOTOMETER

by

Marty Frank Bartholdi

ABSTRACT

Light scattering by single spherical homogeneous particles in the diameter range 1 to 20 μm and relative refractive index 1.20 is measured. Particle size of narrowly dispersed populations is determined and a multi-modal dispersion of five components is completely analyzed.

A 360° light scattering photometer for analysis of single particles has been designed and developed. A fluid stream containing single particles intersects a focused laser beam at the primary focal point of an ellipsoidal reflector ring. The light scattered at angles $\theta = 2.5^\circ$ to 177.5° at $\phi = 0^\circ$ and 180° is reflected onto a circular array of photodiodes. The ellipsoidal reflector is situated in a chamber filled with fluid matching that of the stream to minimize refracting and reflecting interfaces. The detector array consists of 60 photodiodes each subtending 3° in scattering angle on 6° centers around 360° . 32 measurements on individual particles can be acquired at rates of 500 particles per second.

The intensity and angular distribution of light scattered by spherical particles are indicative of size and relative refractive index. Calculations, using Lorenz-Mie theory, of differential scattering patterns integrated over angle corresponding to the detector geometry determined the instrument response to particle size. From this the expected resolution and experimental procedures are determined.

First, measurements of light scattering over the angular range of 0 to 180° and 0 to 360° from single particles of 9.5, 12.5, 15.6, and 19.5 μm are compared with the calculated responses. The excellent agreement in both the shape and dynamic range of the integrated patterns, and the symmetry of the 360° patterns, confirms the proper operation of the photometer. Second, particle size of narrowly dispersed populations is determined by measurement of scattered light intensities at 3 angular positions between 27 and 42° . Experimental data for eight samples with particle diameters of 1.1, 2.7, 5.0, 7.9, 10.0, 12.5, 15.6, and 19.5 μm are consistent with calculated size-response curves. Third, a multi-modal dispersion consisting of a mixture of five samples with particle diameters

of 1.1, 5.0, 10.0, 15.6, and 19.5 μm is prepared with measured proportions from stock solutions of known particle number concentrations. The correct fraction of each component is then determined from an analysis of a combination of measurements of light scattered intensities between approximately 5 and 50°. By a particle by particle examination this dispersion is completely analyzed.

Ultimately, the photometer will be utilized for identification and discrimination of biological cells based on the sensitivity of light scattering to size, shape, refractive index differences, internal granularity, and other internal morphology. This study has demonstrated the utility of the photometer and indicates potential for application to light scattering studies of biological cells.

I. INTRODUCTION

I-1. Objective

The objective of this work was the measurement of light scattering by individual particles over 360° . The light scattering was then to be analyzed for the ability to discriminate among classes of particles and to identify particle size.

The measurements were to be done on particles suspended in liquid medium so some manner of flow system was required to allow examination of individual particles. A small, well defined illumination spot necessitated use of a laser light source. To collect the light scattering over a wide angular range and yet still resolve the features of the scattering pattern, required a light scattering flow chamber and detector array design which could be joined to produce a photometer capable of measurements over 360° . In order to achieve statistical significance in the analysis of particle populations, a large number of particles must be examined, and, to fully exploit the light scattering, a large number of data must be collected for each particle. This required a speed and reliability of data collection and storage which could only be achieved by computer operations. Finally, a range of particle size over an extent and magnitude not usually examined by light scattering techniques was sought for analysis.

The work to accomplish this goal proceeded in two parts. First, was the design and development of the photometer guided by analysis of established light scattering measurement procedures and knowledge of the nature of light scattering gained from the vast store of theoretical studies. The measurement objectives were ambitious, and limitations of technology and fabrication constrained the complete fulfillment of these.

Second, was the demonstration of the performance of the photometer for the measurement of light scattering from individual particles. The photometer response to particle size, refractive index, and detector geometry was modeled by calculations based on Lorenz-Mie theory of the scattering of electromagnetic radiation by homogenous spherical particles. Experiments were performed in which 180° and 360° scattering patterns were measured for particle diameters in the range of 1.1 to 19.5 μm , as well as multi-modal dispersions extending over this entire size range. The results were compared with the calculated responses with agreement that establishes the accuracy of the measurements and provides a calibration for analysis of unknown particle populations.

I-2. Purpose

The purpose for developing the technology and procedures for these light scattering measurements, was not only for the

identification of well defined spherical particles, but ultimately for the analysis of light scattering from single biological cells. Indeed, the measurement objectives were set to meet the demanding requirements of this application. This photometer and its use represent an extension of existing technology and a search for new measurements in the automated analysis of biological cells.

The goals of automated cytology (1) are cell measurements for biological research and automation of clinical laboratory tests. The potential for flow instrumentation in biomedical research is enormous and is being actively exploited.

Morphology is the most important feature for classification of a biological cell (2). Cells are typically 5 to 20 μm in diameter so that visible light should be an excellent non-perturbative probe for cellular structure. Optically, biological cells can be considered as a complicated distribution of refracting, absorbing, and fluorescing matter. When these properties vary over distances on the order of the wavelength of visible light, measurements of light scattering, absorption and fluorescence may provide information needed for cell identification. The technology of flow cytometric instrumentation enables measurement of a large number of cells on a cell by cell basis.

Flow cytometry seeks to determine distinguishing cell

attributes. These include, among many others, cell size, relative size of the nucleus to the cytoplasm, and DNA content. Each of these particular cell properties can be presently measured by various techniques in flow cytometry. Fluorescence intensity (3) measured from cells stained with a DNA specific fluorescent dye is indicative of DNA content. One dimensional slit scanning in which cells are sequentially illuminated as they flow through a slit of laser illumination has yielded nuclear to cell diameter ratios (4). Cell size can be obtained electronically by the Coulter principle (5), or optically by low angle light scattering (6).

Due to the physical nature of the interaction of light and a heterogenous particle like a biological cell, it is certain that information of internal structure resides in the scattered radiation at potentially any scattering angle. The challenge is to first accurately measure the angular light scattering from individual biological cells and then invert these measurements to determine the properties of the scatterer.

This work is concerned with the initial aspect of this challenge, the accurate measurement of light scattering from individual latex particles. No biological material was examined. That step remains to be taken with potentially very rewarding possibilities.

I-3. Résumé

Chapter II examines the application of light scattering to automated cytology. A brief summary of the physical basis of light scattering is presented. A search of the literature then provides the historical development of flow instrumentation and past and present uses of light scattering in flow cytometry. The justification for undertaking this work is then appreciated.

Chapter III deals with the design and development of the photometer. The optics to capture the 360° light scattering pattern are described as well as each component in the configuration of the instrument. These are the scattering chamber, flow system, detector array, and signal processing electronics. Then the technical aspects of operating the photometer, alignment, polarization of the scattered radiation, the effects of reflections, the illumination optics, and flow properties, are accounted in turn.

Chapter IV examines the photometer response to particle size, refractive index and detector geometry by calculations of the scattering patterns of homogenous spheres, corrected for the effects of the measurement process. It is here that the difficulties and limitations in the measurement of light scattering as can be done on this instrument are exposed. But, the capabilities of the photometer are determined and

the experimental procedures for particle discrimination and sizing are outlined.

In chapter V, three sets of experimental results are reported. The first is the measurement of 180° and 360° scattering patterns from individual particles. The second is the quantitative measure of particle size. The third is the resolution of a pentamorous mixture with particles ranging in diameter from 1.1 to 19.5 μm . The relative concentrations of the particles resolved agrees well with the predetermined number concentrations of single particle samples.

II. LIGHT SCATTERING AND FLOW CYTOMETRY

II-1. Introduction

A brief consideration of the physical basis of light scattering is presented in order to exhibit the achievements and potential of light scattering in flow cytometry. This is followed by an historical background and survey of past and present techniques. The formulation of the solution to the scattering of electromagnetic radiation from a spherical homogenous particle of arbitrary size and refractive index is outlined. Further, recent theoretical advances in predicting the interaction of the penetrating radiation with the internal structure of heterogenous particles are discussed with interesting consequences for back angle light scattering being indicative of cellular morphology.

The history of flow instrumentation is fresh, with the major developments in technology occurring within the last ten years. Even though the physical basis of the measurements of cell attributes has seen little progress, the achievements and potential applications of these techniques to biological problems are remarkable with new and important developments occurring at an accelerating and exciting pace. Four instruments, including the first use of light scattering to properly quantify a cellular property (size), are then described.

II-2. Light Scattering

II-2-1. Spherical Homogenous Particles

When light is incident on a small particle of refractive index different from the surrounding medium, light is scattered into all directions. The processes of diffraction from the edges of the particle, absorption, transmission with refraction, specular reflection, and surface wave propagation at the particle medium boundary, interact to produce the light scattering phenomena. Since these processes are sensitive to size, shape, and refractive index of the scattering particle, these properties can be determined from the angular distribution of the scattered light. It is possible, using Lorenz-Mie theory (7), to calculate the intensity, polarization, and angular distribution of the light scattered from isotropic spherical homogenous particles of arbitrary size and refractive index.

The basis for formulation of problems of electromagnetic nature is Maxwell's equations which predict that light is an electromagnetic phenomenon. When a plane light wave encounters a small dielectric particle, waves are generated both inside (the internal wave) and outside (the scattered and incident waves) the particle boundary. The electric and magnetic fields satisfy boundary conditions that their tangential components are continuous across the particle surface.

For simple shapes such as spheres, infinite cylinders and spheroids both the scattered and internal waves can be determined analytically. Computations are presently feasible on a routine basis only for spheres and infinite cylinders.

The properties of a spherical homogenous particle are its diameter and refractive index. Two parameters which are used in the formulation of the scattering problem are $\alpha = \pi d/\lambda$ (size parameter) and $m = n(1-i\kappa)/n_0$ (relative refractive index). The size is scaled by the wavelength of the incident light and it is the ratio, and not just the diameter of the particle, which determines the scattering. Usually scattering particles are immersed in a medium of refractive index n_0 , and the relative refractive index, m , is used rather than the refractive index of the particle in vacuum. The imaginary part of the refractive index contains κ , the absorption index.

The geometry of the scattering problem is sketched in figure 1. A monochromatic, collimated, plane light wave propagates along the positive z axis with the electric field linearly polarized along the x axis. An isotropic, homogenous sphere is centered at the origin. The coordinates of a scattered ray are the polar angle θ and azimuthal angle ϕ . In the scattering plane defined by the propagation vector of the incident light and the scattered ray, the amplitude of the

Figure 1. Polar coordinate system with a sphere centered at the origin. The incident radiation propagates along the positive z axis. A scattered ray has coordinates θ, ϕ .

electric field vector can be resolved into parallel and perpendicular components. The solution for the scattered wave in this plane is then comprised of two angular intensity functions $i_1(\theta)$ and $i_2(\theta)$. The intensity function i_1 is the scattered component polarized perpendicular to the scattering plane and i_2 is the parallel component. In figure 1 the azimuthal angle, ϕ , is 0 at the x axis, and the incident electric field vector is polarized along this axis. In the scattering plane shown, the intensity of the scattering ray is then,

$$I(\theta, \phi) = \frac{\lambda^2}{4\pi^2 r^2} \{i_1(\theta)\sin^2\phi + i_2(\theta)\cos^2\phi\}.$$

If the scattering plane is in the plane defined by the x-z axes, with the incident electric vector still linearly polarized parallel to this plane, then for homogenous spheres, the intensity function i_2 is measured. If the plane of polarization is perpendicular to the scattering plane then i_1 is measured. Further, at supplementary ϕ angles the scattering from homogenous spheres is symmetrical.

The intensity functions i_1 and i_2 are $|S_1|^2$ and $|S_2|^2$, where S_1 and S_2 are the amplitude functions given by,

$$S_1 = \sum \frac{2n+1}{n(n+1)} \{a_n \tau_n(\cos \theta) + b_n \tau_n(\cos \theta)\} (-1)^{n+1},$$

and

$$S_2 = \sum \frac{2n+1}{n(n+1)} \{a_n \tau_n(\cos \theta) + b_n \pi_n(\cos \theta)\} (-1)^{n+1}.$$

The angular dependence resides in the terms π_n and τ_n which are expressed in Legendre polynomials and the size and refractive index dependence in the terms a_n and b_n which are expressed in Ricatti-Bessel and Hankel functions. The terms a_n and b_n are coefficients of the superposition of the solutions to the wave equation, and are related to the moments of the multipoles representative of the charge distribution of the scatterer. The terms π_n and τ_n are functions of scattering angle which appear on deriving the tangential field components. The intensity functions i_1 or i_2 can be plotted on a polar diagram showing the scattering pattern for the particle (see section IV-3-1). The scattering pattern is characteristic of the particular size parameter and refractive index.

II-2-2. Particles with Internal Structure

Particles with a structure in which the internal refractive index distribution varies only in the radial direction can be represented as two or more concentric spheres. The light scattering from spheres consisting of concentric layers, each of constant refractive index, but different from the others, or by a sphere with a continuously varying radially symmetric refractive index, can be exactly determined by an extension of Lorenz-Mie theory (8). The light scattering from a biological cell, modeled as a nucleus concentric within an outer membrane

(coated sphere), has been investigated by the concentric sphere formulation (9). Diffraction from the edges of the outer surface and also the inner core was found to predominate the scattering at forward angles. The fine lobe structure of the scattering pattern depended on the whole cell size and the envelope lobe structure on the relative nucleus size. This model considers an ideal cell and ignores a number of important cell attributes which affect the light scatter pattern. In particular, cells are not usually spherical, the nucleus is not centered within the cell, and all cells have subcellular organelles.

An elegant formulation of the light scattering by particles of unrestricted shape or internal structure is evolving. Light scattering by spheres with internal structure is presently being studied (10) by integration of the internal electric field over an internal distribution of refractive index. Progress to-date has led to an approximation which is useful when the variations of the refractive index within the particle are small. For concentric spheres it produces results in good agreement with exact calculations. This approximation shows that the effect of non-concentric internal structure on the differential scattering pattern is greatest at back angles.

Both theoretical studies, involving scattering from concentric spheres, and scattering from arbitrary internal

structures, indicate that the entire scattering pattern might contain information about cell morphology. However, light scattering measurements from biological cells are particularly difficult to interpret. The large size parameter yields a complicated scattering pattern and the effect of the internal structure of real cells further complicates the pattern making its interpretation more difficult. Yet, the importance of the biological problems justifies the efforts to measure the light scattering from single biological cells.

II-3. Historical Background

The earliest use, 1934, of flow cytology was for counting individual cells (11,12). A liquid suspension of cells flowed through a capillary positioned on a microscope stage. The cells were illuminated by bright or dark field optics. A light scatter flash indicated the passage of a cell and was counted. A blood cell counter (13) was then developed based on this technique in 1955.

An electrical system (14), the Coulter principle, for counting and sizing blood cells became the basis for the first automated flow cytometer in 1956. The individual cells flowed through a small (100 or 50 μm) orifice across which an electric current flowed. The passage of the cell produced an impedance change in the orifice which was determined by the size of the cell. The instrument counted individual cells and provided a

cell size histogram.

A flow cytometer (15) developed in 1965 measured both the light scattered at small forward angles at 410 nm and absorption at 254 nm. A mercury arc lamp was the light source. Nucleic acids have an absorption maximum near 254 nm. The absorption signal was an estimate of the nucleic acid content of each cell, and the light scatter signal an indication of cell size.

Two important new technical developments were incorporated into an instrument (16) which is the basis for the majority of flow cytometers presently in use. Firstly, the use of a laser facilitated focusing the incident illumination at the cell stream and its increased intensity improved fluorescence measurements. Secondly, a laminar sheath flow technique (17) was employed which confines cells to the center of the flow stream and reduces clogging due to clumps. In this instrument the fluorescence is detected at right angles to both the cell stream and laser beam by a photomultiplier.

Many flow cytometers have been designed to make several measurements on each cell (18). Electronic cell volume, fluorescence at two wavelengths, and low angle light scatter are incorporated into one instrument (19), that also has a sorting capability. The general development of this technology has lead to applications in cancer biology, cancer

therapy, cancer screening, somatic cell genetics, and immunobiology (20).

II-4. Applications of Light Scattering in Flow Cytometry

II-4-1. Cell Sizing by Small Angle Light Scattering

Both an experimental and theoretical investigation of determining cell size by small angle light scattering was undertaken by Mullaney et al (21,22). The essence of the theoretical study was that scattering at 0.5° is approximately proportional to volume for homogenous particles with relative refractive index 1.05. The contribution of transmission with refraction for this refractive index ranges from 29 to 7% of the total signal at 0.5° . The remainder is due to diffraction which is independent of refractive index and is a strong function of size. Accordingly a scattering measurement within the diffraction dominated region should yield size information.

A flow system photometer was then developed to measure the light scattering between 0.5 to 2.0° from individual particles. The response of the instrument was found to be proportional to volume within about 15% based on results with cells and particles of known diameter. This measurement has since found wide use in flow cytometry. It is preferred over electronic volume sensing due to instrumental problems,

such as poor electronic shielding or errant salt paths introduced by that technique.

An alternative method of measuring cell size depends on the duration of a light scatter pulse produced as a flowing particle enters and exits a sharply focused beam. Especially if the beam size is less than that of the particle, then, as the particle traverses the narrow beam, the time between the rising and falling edges of the light scatter pulse depends on the length of the particle in the direction of flow, the width of the beam, and the flow velocity. For example, with one detector on either side of the beam in the direction of flow, the time difference between the pulse on the upstream detector as the particle enters the beam and the pulse on the downstream detector as the particle exits, was also found to be dependent on particle size (23).

II-4-2. Particle Size by Ratios of Scattered Intensities

A method and instrumentation to determine particle size by simultaneous measurement of light scattering at two angles was developed by Jovin et al (24). A ratio function of light scatter signals resolved particle size even when the intensity distributions showed severe overlap. Mixtures of up to three sizes of particles, in the range of 0.48 to 2.02 μm in diameter were separated. The results were verified by sorting, with

separated fractions of 95% purity. No results were given for particles larger than 2.02 μm .

II-4-3. Multiangle Light Scatter - Single Detector

A unique design, developed by Loken et al (25), uses the motion of the particles in the flow stream moving past a single detector to scan the light scattering pattern. As a particle traverses an expanded illumination beam, the scattered light passes through an aperture with an 1° acceptance angle onto a photomultiplier. The angle from which the scattering reaches the detector varies continuously from nearly 0 to 49° . The detector output versus time is related to the scattered light intensity versus scattering angle. The output requires corrections for the nonuniform incident illumination and variations in collection efficiency with particle position. The fluorescence measured from a dyed particle with assumed uniform emission directly proportional to the incident illumination, was used to normalize the output to approximate the actual scattering pattern.

This instrument does resolve the maxima and minima of the scattering pattern between 0 and 49° even though their relative magnitudes are probably not accurate. Scattering patterns from mouse thymocytes showed differences from cells in the population that are functionally different. No analysis was made to correlate the details of the scattering

patterns with morphological characteristics of the cells.

II-4-4. Multiangle Light Scatter - Detector Array

A multiangle flow system instrument has been developed by Salzman et al (26) which employs 32 detectors over an angular range of 0 to 30° . The detector array consists of 32 concentric semicircular rings of photodiode material. The inner rings subtend 0.11° in scattering angle which increases to 1.8° for the outermost ring. Each ring spans 180° in azimuthal angle. The area of ring 32 at 30° is about 2000 times greater than that of ring 1 at 0.1° , which roughly compensates for the wide range of intensities which occur between 0 and 30° . Consequently, the magnitudes of the signals at each ring are of the same order of magnitude. The light scattering signals from a single particle are sensed simultaneously at each of the 32 detectors and stored in a computer in 250 μ sec.

Light scattering signals from 8 and 10 μ m diameter polystyrene spheres were well separated between 1° and 2° but overlapped in the remainder of the angular range with this detection scheme. A gynecological specimen diagnosed as invasive carcinoma was distinguished from a normal specimen based only on the angular distribution of the light scattered by single cells in each specimen.

The 32 ring detector was replaced (27) by an 128 element photodiode array which detected the light scattered between 7.5° and 21.5° with 0.1° resolution over that entire angular range. The maxima and minima of the scattering from 12.5, 15.7 and 19.8 μm diameter polystyrene spheres were well resolved though the magnitudes of the signals were not compared. No biological cells were examined with this detector.

II-4-5. Inversion of Light Scattering from Biological Cells

The inversion of light scattering measurements from biological cells is complicated by a number of factors not encountered in the conventional analysis of homogenous spheres. However, since individual particles are measured instead of suspensions the light scattering signals are not averaged over the particle size distribution. This is crucial for studies on biological cells. Yet, determination of the size and the details of internal structure of cells presents enormous difficulties.

In light of the theoretical and experimental work to-date, it is evident that in order to provide the optimum possibility for inversion of light scattering measurements, investigation capability over an unrestricted range of angle is required. While it will not be necessary to make measurements at all

angles for each and every cell, it is unpredictable at which angle a cell or cell attribute can best be identified. For this reason a photometer capable of making light scatter measurements over almost 360° from single particles has been developed.

III. PHOTOMETER DESIGN AND OPERATION

III-1. Introduction

In this chapter the design of the photometer is presented with the relative arrangement of the components and the operation procedure. The fabrication of the scattering chamber and detector array represent sophisticated technical achievements. The optics, hydrodynamics, mechanics and electronics are each described.

The operation of the photometer is straightforward but care must be exercised to analyze the consequences of the interaction between components and the measurement process. Alignment, the polarization of the scattered radiation, and reflections of both the incident illumination and scattered radiation are accounted. The proper detector array position is determined from a ray tracing of the light paths and an adjustment must be applied to the distance between the chamber and detectors due to the refraction of the emerging rays. The use of a mask to reduce stray light incidence on the detector array is illustrated. The primary source of instrumental error is seen to arise from the nature of the intersection of the laser beam and flow stream.

III-2. Photometer Design

III-2-1. Configuration

The measurement of light scattered over 360° by

individual particles suspended in liquid in a sufficiently rapid time to provide analysis of particle populations required design of a photometer which incorporated a flow system for control of single particles, a scattering chamber and detector scheme with measurement capability over 360° and very fast data acquisition rates. A 360° scattering photometer had previously been developed (28) for single aerosol particle measurement in which an aerosol stream intercepts a laser beam at one of the foci of an ellipsoidal mirror. A photomultiplier at the second focus accepts the scattered light limited by a rotating aperture to a 5° segment. The aperture rotates at about 50 rev/sec so that a 360° scan is completed in 20 msec.

For potential analysis of cells a liquid environment is required. Also, to characterize populations with possibly very small but interesting subpopulations, rates of at least 500 events/sec are needed. A light scattering multiangle flow system instrument has been developed (29) to process data at the rates required but is restricted to forward angles. Thus the photometer for this work was designed to combine the principles of both these instruments into one photometer with the unique capability of ultra rapid light scattering measurements over 360° from single particles.

The optical principle of the photometer is shown in figure 2. A focused laser beam intersects the single particle stream at the primary focus of the ellipsoidal mirror. If a particle scatters light while it is located at the primary focus of an ellipsoidal reflector, the secondary focus will collect the scattered light which has been reflected. The circular array of discrete photodiodes placed between the two focal points detects the scattering and preserves the angular distribution. The size and position of the photodiode elements determines the resolution of the differential scattering pattern. The scattering angles θ and ϕ in the photometer are shown in figure 3. Figure 1 in section II-2-1, depicted the polar coordinate system for the analytical solution of the scattering problem. By rotating the x axis of that figure to the vertical and the y axis normal to the page the scattering angles can be shown in relation to ellipsoidal reflector. The laser beam propagates along the positive z axis and is linearly polarized in the x direction. The scattering plane in the photometer is the x-z plane and is vertical with respect to the laboratory bench. For an incident vertical plane of polarization the parallel polarized component $i_2(\theta)$, is measured.

This arrangement of the laser beam and particle stream

Figure 2. The basis of the photometer is the intersection of the laser beam and single particle stream at the primary focus of an ellipsoidal reflector. A circle of discrete photodiodes intercepts the scattered light before it is focused at the secondary focal point. The scattered rays illustrate how the angular distribution of the scattering is preserved.

Figure 3. The scattering angles in the photometer orientation. The ellipsoidal mirror reflects the scattering between 2.5° and 177.5° at $\phi = 0^\circ$ and $\phi = 180^\circ$.

was chosen to maximize the usable reflector surface area. The laser is designed to emit a horizontal beam so this direction was selected as the z axis. Less reflector area needed to be removed for holes cut for the laser beam than if the flow nozzles were mounted in the reflector. The particle stream flows between two nozzles each positioned 0.75 mm from the primary focus. The exit stream is brought out by tubing through the center of the detector array.

A schematic of the photometer is shown in figure 4 and a photograph in figure 5. The laser and optical components, flow chamber, flow system, detector array seen in the photograph and the signal processing electronics are now detailed in the following sections.

III-2-2. Optical Components

A laser is used as a convenient light source for its power, monochromaticity, and collimated output. The laser (Model 54 Ion Laser System, Coherent Radiation, Palo Alto, CA) has a total power of 500 mW, with a minimum of 200 mW in each of the two principle argon lines, 514.5 nm (green) and 488.0 nm (blue). The laser operates with a continuous wave output in the TEM₀₀ mode. The beam diameter is 1.1 mm at the $1/e^2$ points and the beam divergence is 0.9 milli-radians. The output is linearly polarized to 1:100 with the plane of polarization vertical. For all light scattering experiments

Figure 4. Schematic of the photometer with the laser, scattering chamber, flow system, detector array and signal processing electronics.

Figure 5. A photograph of the photometer. The detector array was moved out of position to show the scattering chamber.

the single 514.5 nm wavelength was used. The laser output radiant flux is measured with a power meter (Lexel Corp. Palo Alto, CA). The laser head is mounted on stands with the beam propagating horizontally at a height of 33 cm above the table. A vibration isolated table (Model RS-46-12, Newport Research Corp. Fountain Valley CA) supports the instrument.

A 30 cm long optical bench holds a shutter for the laser beam, a series of neutral density filters (Melles Griot, Irvine, CA), and a carrier for the spherical focusing lens (Melles Griot, Irvine, CA).

III-2-3. Scattering Chamber

The ellipsoidal reflector (Product number 02 REM 001, Melles Griot) is mounted in a chamber, (see figures 6 and 7) designed to be filled with fluid to reduce reflecting and refracting interfaces, and to allow precise adjustments of the flow stream trajectory. The reflector, as purchased, consisted of half the total surface area of a total ellipsoid. The semimajor axis is 115 mm with the primary and secondary foci 12.7 mm and 102 mm from the vertex respectively. The reflector was cut to a ring extending 10 mm on either side of the primary focus. Two 2 mm diameter holes were cut for the laser beam. Even though only a narrow (about 1 mm) ring about the focus is required for the reflection of the desired

Figure 6. Cross sectional and front views of the scattering chamber.

Fig. 7. A photograph of the front view of the scattering chamber. The outside dimensions are 100 x 100 mm. The inlet tubes on either side of the inlet nozzle are reflected from the ellipsoidal mirror. The chamber is filled with distilled water.

light scattering, a 2 cm ring was used for strength and prevention of deformations of the elliptical shape.

The production process for these reflectors begins with the electrolytic deposition to a thickness of 0.5 mm, of a nickel substrate on a master surface. The nickel is then electrolytically coated with rhodium. While the reflectance of rhodium is close to 80% throughout the visible region of the electromagnetic spectrum, 400 nm to 700 nm wavelength, compared to 90% for aluminum and 95% for silver, rhodium is exceptionally hard and durable. The reflectance of rhodium is 79% at 514.5 nm for normal incidence. It adheres well to nickel substrates and is resistant to oxidation and tarnish. In the chamber the reflector surface is in direct contact with a 0.9% saline solution. Along with the need for periodic cleaning rhodium is the optimum coating for the reflector.

The dimensions of the positions of the ellipsoid focal points listed by the manufacturer are given with an uncertainty of 0.13 cm. They state that the foci are best located experimentally (30). Since the size of the scattering objects of interest is about 10 μ m a means of precise alignment of the flow stream with the focal point of the ellipse is needed. The inlet and outlet nozzles are mounted separately on circular plates in the chamber (see figure 6) which can be adjusted

by opposing micrometers and thumbscrews. The plates are sealed with o-rings to maintain a water tight compartment. The outlet nozzle is mounted in a quartz plate transparent to the scattered light. The reflections and refraction at the surfaces of the quartz plate are discussed in section III-3-3. The inlet nozzle is mounted in a plate of Kel-F, a machinable plastic with an inert, slippery surface. The sensitivity of the micrometers (Catalog number BM 1704, Klinger Scientific Apparatus Corp. Jamaica NY) is about 1 μm . In practice, the sliding plates move in increments of about 10 μm . After adjustments the opposing thumbscrews are tightened. The alignment procedure is discussed in section III-3-1.

The body of the chamber is machined from Rexolite, a plastic chosen for its machining characteristics, inertness, and lightness. The reflector is fixed with epoxy to a piece of Rexolite machined to match roughly the outer elliptical surface. This piece then fits in the main body with the holes for the laser beam aligned. A CaF_2 window for the beam entrance is sealed with an o-ring and a Rayleigh horn dumps the beam at the exit. The plates holding the flow nozzles are held in place with brass retainers with tolerance to allow movement of the plates.

The outside dimensions of the chamber are 102 mm by 102 mm by 60 mm.

The distance between the laser beam holes in the reflector is 47 mm. The inlet and outlet nozzle tips are 1.5 mm apart.

The brass retainers holding the quartz plate can be removed for access to the reflector for cleaning. A dilute detergent cleanser (Isoterge, Coulter Electronics, Hialeah, FL) is sprayed on the reflecting surface followed by an ethanol rinse and a distilled water rinse. The entrance laser window can also be removed for cleaning.

III-2-4. Flow System

The method used to examine rapidly flowing individual biological cells requires control of a fluid stream of sufficiently small diameter with hydrodynamic properties such that the cells flow in a single file manner in a well defined line. Hydrodynamic focusing is the term given to the process of controlling the sample stream by an outer concentric sheath stream. The sheath stream serves both to stabilize the sample stream and control the flow rate, the number of particles passing through the orifice per second. The flow rate depends on the concentration of the sample and the volume of sample flow through the orifice. The volume of the sample flow is controlled by adjusting the volume of the sheath flow while maintaining a constant pressure on the sample reservoir.

The flow nozzle is made of Rexolite (see figure 8).

Figure 8. The inlet flow nozzle. The sheath fills the inside of the nozzle and flows through the guiding star shown below in cross section. The sample flow is surrounded by the sheath flow in the stream.

The sheath and sample are brought into the back wall by 0.89 mm outside diameter platinum tubing. The sheath tube terminates inside the back wall while the sample tube passes down the center. A guiding star centers the sample tube as well as radially distributing the sheath flow. The sample tube terminates 9.5 mm from the orifice. The body of the nozzle smoothly tapers from an inner diameter of 8 mm to the orifice.

The jeweled orifice (Richard H. Bird & Co. Waltham, MA) is made of sapphire. Its diameter is 100 μm and its length is 300 μm . The outside diameter of the jewel is 2.2 mm with a thickness of 1.2 mm. It is fitted and secured to the end of the tapered nozzle. Great care was taken in the fabrication process to ensure that the stream flowed straight and smoothly from the orifice.

The Reynolds number for the flow stream in the orifice is 425 assuming laminar flow. The maximum flow velocity of the flow profile is measured to be 8.5 m/s in a manner described in section III-3-5. Concentric cylindrical surfaces have constant velocities and the individual cylindrical laminae slide over each other in laminar flow.

The volume of sheath flow constricts the sample flow in the region before the orifice. Increasing the volume of the sheath constricts the sample more tightly and reduces its flow. Under operating conditions the sample stream has a

20 to 30 μm diameter concentric with the 100 μm sheath stream. The stream is injected into the fluid which fills the chamber and enters the 100 μm exit orifice 1.5 mm away. The exit orifice is aligned to catch the stream by using colored water in the sample.

The flow system, (see figure 9) operates by pressurizing a reservoir causing the fluid to flow. Two sheath reservoirs, one for distilled water and one for physiological saline are under 10,500 Pascal and the sample holder is under 5,250 Pascal. The isotonic buffered saline (Scientific Products, McGraw Park IL) is a solution of sodium, 180 meq/l, potassium 5 meq/l, chloride 153 meq/l and EDTA 1.0 mmol/l, ethylenediamine tetracetic acid, a cell anti-clumping agent, and is generally used to suspend cells. This saline solution is used to both fill the scattering chamber and dilute the particle samples. The distilled water is used to flush the system and for filling the chamber when it is not in operation. The pressures on the sheath reservoirs and the sample holder are regulated independently from a compressed N_2 gas cylinder.

The saline and distilled water are filtered through a 0.4 μm pore size membrane filter with a 0.45 μm glass fiber prefilter (Nuclepore, Pleasanton CA) before filling the reservoirs. Each reservoir also has an in-line filter

Figure 9. A schematic of the flow system.

with the same filter combination.

The volume of sheath flow is controlled by a double pattern fine metering valve (Nupro Co. Willoughby OH). The inlet valve can be set at the maximum desired flow. The outlet valve is then used for fine flow control in the range up to the preset maximum. The flow controls are adjusted by observing the shape of a colored sample stream inside the flow nozzle before the orifice. The properties of the flow are examined in section III-3-6.

The chamber is filled by two inlets made of 0.89 mm outside diameter platinum tubing which enter the flow chamber on either side of the inlet nozzle. A drain is located at the bottom and a vent at the top of the chamber. At the entrance laser window and at the Rayleigh horn, drains remove air bubbles which might be trapped in the holes cut for the laser beam. All drains can be placed under vacuum. A single panel holds all the valves necessary for controlling the flow operation and the filling and draining procedures.

When draining or filling the chamber the top vent is opened. The chamber can be entirely drained and refilled in about 30 seconds. When not in use the chamber is kept filled with distilled water to maintain a wetted surface on the reflector preventing formation of adhering air bubbles. During operation the filling inlets and all drains and vents are

closed. The volume flowing through the sample inlet is removed by an exit orifice thereby maintaining a constant fluid level in the chamber.

III-2-5. Electronic Particle Sensor

An electronic volume sensor is incorporated into the instrument. An electric current passing through the inlet orifice is altered by the passage of a particle. A constant voltage is maintained across the orifice. The change in current detected as the particle passes through is due to the differing resistivity of the particle from that of the conducting saline.

In the chamber the sample inlet tube is held at a positive potential of 4 volts. The saline solution in the orifice and in the chamber conducts the current to the grounded fill inlet tubes. Platinum tubing is used because its resistance to corrosion. A 0.1 mamp current flows through the orifice which presents a resistance of 40,000 ohms. 5 μ m diameter polystyrene latex spheres are the smallest size detectable in this manner.

This sensor was not used in any of the experiments in this work but is an added feature intended for application to cells.

III-2-6. Detector Array

The detector array consists of 60 photodiodes centered on a 2 cm circle (see figures 10 and 11). The photodiode detectors (Opto-chip MRDC7 Motorola Phoenix AR) are silicon PIN type with a response time of nanoseconds. The spectral response of silicon extends from 400 to 1100 nm with a peak at 800 nm. The sensitivity of an individual silicon photodiode depends primarily on its active area. Square optochips 0.76 mm on edge were selected for this application since this small size fits a reasonable number of chips into the available space and still provides sufficient active area for sensitivity. The active area of these chips is 0.5 mm square with 2.6 μ amp bare chip radiation current from a 5.0 mW/cm² tungsten lamp source at 2870 K.

These chips are mounted on a ceramic substrate with a common 15 volt conductor to the cathodes of all photodiodes. This common bias line has bypass capacitors to ground every fifth opto-chip to assure freedom from pulse cross talk. Anodes are wire bonded to leads which feed signals to individual hybrid microcircuit preamplifiers. These circuits are configured as transimpedance amplifiers, optimized for high signal to noise ratio with good bandwidth. The transimpedance of these preamplifiers is 1 megohm and the rise time is about 0.5 μ s. Typical output noise

Figure 10. Schematic of the detector array showing arrangement and numbering of the elements with the subtended angle.

the center of the wheel, and the outer rim. The
inner rim is made of a material that is
resistant to wear and tear, and the outer rim
is made of a material that is resistant to
corrosion.

is 500 μ V rms.

The resolution in polar angle (θ) is set by the arc length subtended by a single detector on the circumference. Each detector subtends an equal angle of 2.91° . A schematic of the array and numbering of the elements is shown in figure 10. The photograph in figure 11 is a close up of the array showing the individual detector chips. Table 1 lists the angular limits subtended by each chip.

The output of one photodiode chip is shown for a three order of magnitude range of incident intensity in figure 12. This was measured by focusing the laser beam onto one chip. The incident radiant flux was decreased in steps by neutral density filters and the corresponding voltage output recorded. The response was found to be linear within measurement error. The dynamic range of the detector extends only a small range at either end of the graph so that the detector array is accurate over a three order of magnitude range of incident intensity.

The uniformity of response among the chips was determined by focusing the laser beam on to one chip and then moving the array so that the beam fell on an adjacent chip. The output signals were compared for equal illumination. The output of the detectors was uniform within the small uncertainties of monitoring the laser power and adjustment

Table 1. Angular limits subtended by each
detector chip.

Detector Number	$\Delta\theta^\circ$	Detector Number	$\Delta\theta^\circ$
1	4.37-7.28	16	91.68-94.60
2	10.19-13.10	17	97.51-100.42
3	16.01-18.92	18	103.33-106.24
4	21.83-24.74	19	109.15-112.06
5	27.65-30.56	20	114.97-117.88
6	33.47-36.38	21	120.79-123.70
7	39.29-42.20	22	126.61-129.52
8	45.11-48.03	23	132.43-135.34
9	50.94-53.85	24	138.25-141.17
10	56.76-59.67	25	144.08-146.97
11	62.58-65.50	26	149.90-152.81
12	68.40-71.31	27	155.72-158.63
13	74.22-77.13	28	161.54-164.45
14	80.04-82.95	29	167.36-170.27
15	85.41-88.32	30	172.72-175.63

Figure 12. Photodiode voltage output for a three order of magnitude range of incident intensity. The response is linear over the entire range.

of the chip for maximum signal in the beam.

III-2-7. Signal Processing Electronics

The same electronics employed by the 32 detector forward angle instrument (31) at the Los Alamos Scientific Laboratory, Biophysics group, are shared to process the signals for this photometer. This system (see figure 13) can process up to 32 signals for each scattering event. Each signal is logarithmically amplified and its magnitude held by a peak sense and hold circuit. Any one of the signals can be chosen as an event trigger to indicate the presence of a particle in the laser beam. When the trigger signal exceeds an adjustable threshold the master control unit activates all 32 peak sense and hold circuits which are then locked so subsequent pulses do not enter the system until processing is complete. The 32 signals are then multiplexed to a fast 8-bit analog to digital converter (ADC) (Model ADC-G10B-2A, Datel Systems, Canton MA). The digitized output of the ADC is transferred to the computer memory (Model PDP 11/45 Digital Equipment Corp., Maynard MA) by the master control and a direct memory interface (DMA). When the 32 signals have been transmitted to memory the peak sense and hold circuits are cleared and another event can be accepted. About 250 μ s are required to accept an event and to transfer its scatter

Figure 13. Schematic of signal processing electronics.
The system has 32 parallel logarithmic amplifiers and peak sense and hold circuits to acquire 32 measurements for each scattering event.

pattern to the computer. The events in the computer memory are periodically transferred to the magnetic disc for storage and subsequent access.

Three orders of magnitude of photodiode output signal (see figure 14) are compressed to a voltage range of ten volts by the logarithmic amplifiers. Each of 32 signals is processed in parallel by the 32 amplifiers and peak sense and hold circuits. The amplifiers are designed to achieve accurate pulse logarithmic amplification even in the presence of a high DC background from ambient light or detector leakage currents. The amplifier rise time is 0.5 μ s. The minimum rise time of the measured signal pulses is 2 μ s. The peak sense and hold circuit stores the maximum value of the signal pulse for a long enough period to allow readout through the multiplexer. Only the pulses which arise simultaneously from the detector array are sensed and held. The analog voltage level is converted to an eight bit digital output by the ADC. The discrete outputs are identified by binary code in the range 1 to 2^8 (256). The continuous analog input is quantized and coded to discrete levels with an irreducible error dependent on the number of quantization levels. To minimize this error a 10-bit converter is used with the output truncated to 8 bits. The conversion time for this ADC is 1 μ s.

Figure 14. Calibration graph for logarithmic amplifier No. 2. V_{in} is the input voltage to the amplifier from photodiode No. 2. V_{out} is the compressed voltage output.

The signal gating, signal multiplexing, and synchronization with the computer are handled by the master control (See figure 15). One of the light scatter signals is selected as the event signal. The signal level which triggers the discriminator can be adjusted to reject lower level noise pulses. Noise pulses can arise from electrical noise in the circuits which are of a very low level and stray particles or debris in the flow stream or flow chamber. Care must be taken not to set the discriminator too high or significant pulses might be rejected. The output of the discriminator triggers the G1 (sample) pulse generator which initiates the subsequent chain of action. The leading edge of G1 triggers the G2 (hold) pulse generator and the trailing edge triggers the dead time gate generator (not shown on the timing diagram) to block the system from accepting another event for analysis until the computer has completed acquisition of the data from the initial trigger.

The trailing edge of the sample pulse also initiates a start convert pulse for the ADC. The leading edge of this pulse clears out the previous contents of the ADC and the trailing edge initiates digitization of the analog level being fed to the ADC from the multiplexer. The multiplexer address is derived from the DMA word count register (DRWC).

Figure 15. Timing diagram for the signal processing system.

The DRWC is preset to 31 and the first signal level to be digitized will be the one connected to that address in the multiplexer. When the conversion for a detector signal is complete (end of conversion, EOC) the DRWC counts down one digit for the next detector to be read out. When the DRWC counts down to zero (underflows) the reset generator is triggered. This pulse clears the analog holds and the dead time gate returning the system to a ready state for another event.

III-3. Photometer Operation

III-3-1. Alignment

For proper operation of the photometer the intersection of the laser beam and flow stream must be at the primary focal point of the ellipsoidal reflector, and the detector array must be positioned to subtend the predicted angular limits. The alignment process first requires the adjustment of the flow stream so that it passes through the ellipsoid primary focal point. This position was determined by scattering the focused beam from the sheath stream in air. The stream is a cylinder of 100 μm diameter. Adjustment of the plate holding the inlet orifice moves the flow stream with respect to the focal point of the ellipse. By iterative adjustment of the translators the intersection of the laser beam and flow stream could be positioned at the focus of the ellipse. The ring of scattered light from the cylindrical stream was used as the indicator for proper alignment. When the intersection was not at the focal point the reflected ring of scattered light was not circular and not focused to a sharp point at the secondary focus. It assumed various distorted configurations. The flow stream was then adjusted a slight amount and the entire reflector and flow assembly moved to intersect the

laser beam. A circular ring of light which decreased to a sharp point at the proper distance and then expanding while still circular indicated proper alignment. The adjustment of the scattering chamber is provided by an xyz translator combination with a rotating mount about the vertical axis. The laser beam propagating horizontally, fixed at 33 cm above the laboratory bench, served as the base line. The rotating mount is used to align the flow stream perpendicular to the laser beam. The translators (Ardel Kinematic, Jamaica NY) have a resolution of 1 μm and a repeatability of 5 μm .

The detector array is mounted on a separate translator combination and is not mechanically connected to the flow chamber. The proper position of the detector array can be set by aligning the center of the array with the primary focus of the ellipsoid at the same height and lateral position. However, because of the refraction of the light at the scattering chamber window the detector array must be positioned closer to the chamber than if there were no intervening refracting interfaces. A ray tracing of the light, in figure 16, (see also figure 17) illustrates the effect. The correct distance of the array from the chamber was determined by a scale diagram of the ray tracing in figure 16 to be $x = 24 \text{ mm}$. In practice the detector array

Figure 16. Cross section of the scattering chamber showing the azimuthal acceptance angle, $\Delta\phi$, and the detector array position relative to the scattering chamber. Because of the refraction of the emerging rays the detector array must be positioned closer to the chamber than if the rays were focused at the secondary focus.

can be quickly positioned to within 0.25 mm of this predetermined position and then more finely aligned by examination of the scattering patterns of particles.

The individual detector elements also extend radially and thus subtend a section of the reflector which collects light over a finite range of azimuthal angle, ϕ . This angle was also determined from a scale diagram of the figure 16 by tracing the paths of the rays from the inner and outer edges of the detector chip accounting for the refraction at the quartz window and back to the primary focus. The angle between the ray at $\phi = 0^\circ$ which strikes the center of the detector and the rays at either edge is 1.5° .

For the purposes of ray tracing the refractive indices n_1 , n_2 , and n_3 used were 1.34, 1.46 and 1.00, corresponding to the saline filled flow chamber, quartz window and air.

In the geometry of this photometer with the incident plane of polarization parallel to the scattering plane at $\phi = 0^\circ$, the contribution of the cross polarized component to the scattered intensity at this outer limit of the 1.5° interval is $i_1 \sin^2(\phi)$ compared to that of $i_2 \cos^2(\phi)$. Even if i_1 were an order of magnitude greater than i_2 its contribution to the signal would still be less than 0.5%. For all the experiments reported the incident plane of polarization was parallel to the scattering plane and consequently the

scattering at the detectors in this position was considered to be comprised of only the parallel intensity function, i_2 .

III-3-2. Polarization

The angular intensity function i_2 is linearly polarized with the electric vector parallel to the scattering plane. In general, linearly polarized light reflected from a metallic surface is elliptically polarized. At other than normal incidence the plane polarized incident light can be resolved into two components, one perpendicular to the reflecting surface and one parallel. These components are reflected with a phase difference giving rise to elliptical polarization. The particular values of the amplitude and phase of each reflected component depend upon the angle of incidence as given by Fresnel's equations.

The angle of incidence of the scattered light on the reflector is 37.5° for all polar angles. The vibrations of the linearly polarized scattered light are perpendicular to the plane of incidence at the reflector, defined by the lines coincident with the incident ray and the normal to the reflecting surface, and are parallel to the reflecting surface so that linear polarization of the incident light is preserved upon reflection. This is true in this instrument for all polar scattering angles. The percentage of light

loss by reflection is then also the same at each polar angle. The reflectance of rhodium is 79% for 514.5 nm wavelength light at normal incidence, and is probably slightly greater than this for incidence at 37.5° for this polarization component.

III-3-3. Reflections

We now account for the effects of reflected light, both of the incident illumination and the scattered radiation. The incident laser beam if allowed to reflect from any surface after passing the scattering point can re-impinge on a particle. If the reflecting surface is a glass-air interface, for example, the reflected beam will have about 4% of the original intensity. Since the forward scattered light for particles in this size range is significantly more intense than the back scattered light, the scattering by the reflected beam can be comparable to that of the original beam. The angular patterns from the two beams are exactly reversed from each other so that the forward scatter from the reflected beam is superimposed on the back scatter of the original beam. A Rayleigh horn is placed at the beam exit hole in order to avoid this artifact. The horn is curved and coated with black paint to trap and absorb the beam. The horn is connected directly to the chamber

so there are no reflecting interfaces.

It is also possible that scattered light diffusely or specularly reflected can not only reduce the signal strength at the appropriate detector, but more seriously contaminate the response at other detectors. Use of an ellipsoidal reflector (or alternatively, a paraboloidal reflector with the property that rays arising from a focal point are collimated), reduces the problem of specular reflection. Figure 17 shows the trace of a scattered ray reflected from the ellipsoidal reflector and then transmitted through the 4.0 mm fused quartz window as well as the secondary rays reflected from the saline-quartz and the quartz-air interfaces.

The angle of incidence at the reflector is $\phi_1 = 37.5^\circ$ and at the saline-quartz interface it is $\phi_2 = 15.0^\circ$. Application of Snell's law, yields the angles of refraction at the saline-quartz-air interfaces to be 13.7° and 20.3° . The effect of these refractions is to focus the reflected rays at a point inside the secondary focal point, F_2 , which is taken as if the entire ellipse were filled with saline.

The paths for the reflected rays are drawn in figure 17 satisfying the law of reflection. These reflected rays are again reflected by the ellipsoidal reflector and would follow

Figure 17. Cross section of the scattering chamber showing the light path and reflections. The light scattering event occurs at F_1 with light radiated in all directions. The path of the detected ray is illustrated. ϕ_1 is the angle of incidence on the ellipsoidal reflector, ϕ_2 is the angle of incidence at the saline (n_1) quartz window (n_2) interface, ϕ_3 is the angle of incidence at the quartz window-air (n_3) interface, and ϕ_4 is the angle of refraction of the emerging light. The reflections from each interface would be stopped by the mask.

a path that leads them out of the flow chamber, but not usually incident on the detectors. However, these rays will be diffused upon striking the inlet flow nozzle which is made of a white, semi-transparent plastic. Some of this diffusely reflected light would hit the detectors. This has been minimized by use of a mask, with a circular slot, which allows only the scattered light reflected once from the ellipsoid to fall on the detectors, and blocks the diffusely or specularly reflected light as well as the light scattered that could directly hit the detectors. The mask is made of black anodized aluminum and fits on the outer surface of the quartz window.

Further reduction of spurious reflections could be attained by placing the detector array beyond the point where the light converges. Any stray light could be stopped by a pinhole. However, the angular resolution of the scattered light is not as well preserved here as before the focus. The ring of light scattered from a glass cylinder in air appeared less circular beyond the focus, where the effect of slight misalignment of the laser beam-particle stream intersection with the primary focus of the ellipsoidal reflector becomes exaggerated. The detector array is also more difficult to align farther from the flow chamber.

III-3-4. Illumination Optics

The incident laser beam is focused to a small spot to increase the intensity incident on the scattering particles. The distribution of intensity across a laser beam in the TEM_{00} mode is Gaussian and the beam radius is defined as the radial distance at which the light intensity is $1/e^2$ (13.5%) of its value on the beam axis. The diffraction limit of spot size formation by a simple lens is given by (32):

$$r = (4/3)1.22 \lambda f/D,$$

where r is the radius of the spot, λ the wavelength, f the focal length of the lens, and D the diameter of the limiting aperture. The factor of $4/3$ is from the manufacturer's specification for the performance of the lens. The $1/e^2$ diameter of the laser beam is 1.1 mm at 514.5 nm. The $1/e^2$ points on the Gaussian distribution have a 86.5% throughput and the $1.5 (1/e^2)$ points a 99% throughput of the total radiant flux of the laser beam. The beam radius is self limiting and no aperture is necessary to define the beam. However, a 2 mm diameter aperture is placed 75 mm before the bi-convex focusing lens to block two divergent, low power (less than 1% of the main beam) beams which arise from defects in the mirrors in the laser head. The 1.1 mm beam diameter is used for the calculation of the spot size. A 100 mm focal length lens which gives a minimum beam waist diameter of 150 μm

was used for the light scattering experiments. Although a focal length lens as short as 75 mm can be used with the flow chamber, substitution of a 75 mm lens for the 100 mm one resulted in greater variation in the signal amplitude from monodisperse particles.

A correction for the position of the minimum beam diameter must be applied due to the refraction of the beam at the entrance beam window. The beam will not converge at the focal length of the lens, but at a point beyond due to the higher refractive index medium in which the beam is focused. The path length of the beam from the entrance window to the primary focal point is 40 mm in a medium with refractive index 1.3375. A tracing of the path of a ray from a 0.55 mm radius at the lens, obeying Snell's law at the air (window) saline interface indicates that the lens should be placed 113 mm from the primary focal point of the reflector, 13 mm beyond its focal point. Rays originating from intermediate radial distances on the lens are focused at the same point in the flow chamber.

This 150 μm diameter spot, though small, is not as sharply focused as the incident illumination in some flow cytometers which can be as sharp as 5 μm in one dimension. However, in this case we utilize the electromagnetic solution of the

scattering problem which assumes an infinite parallel plane wave as the incident illumination. The results of analysis of Gaussian beams by diffraction theory indicate that the wavefronts are plane at the waist of a focused beam (33). Solutions of light scattering by spherical particles in a converging beam (34) show that there is little deviation from the results of the parallel plane wave solutions if the particles are small compared to the beam diameter. In these experiments where the largest scattering objects studied were 19.5 μm diameter spheres the criteria for the standard plane wave solutions are satisfied and these can be used to model the scattering in this photometer.

III-3-5. Flow

The characteristics of the flow and its intersection with the laser beam profile can significantly contribute to variations in signal amplitude from a sample of monodisperse particles. These instrumental errors were investigated and minimized by examining the pulse shape and duration of the light scatter signals and the timing between these and the electronic volume sensor. The flow system controls are adjusted to make the light scatter pulses as uniform and consistent as feasible. A single photodiode (SGD- 040-B, EG &G, Salem MA) with a fast rise time of 0.1 μsec was used to monitor the pulses with an oscilloscope. The pulse observed

is the time dependence of the light scattered due to the passage of a particle through the beam.

The fast rise time of this photodiode allows interpretation of the pulse shape as representative of the laser beam profile. If the beam were bimodal, or suffering severe distortion, the intensity falling on the particle as it traversed the beam would be irregular in time. For a Gaussian beam profile the pulse shape should also be Gaussian. In this case the pulse width can be related to the actual beam width and the width of the peak of the pulse related to the region of uniform illumination.

The major cause of signal variation from the photometer (besides gross misalignment) is due to particles passing through sections of the laser beam profile of varying intensity. Either the central particle trajectory can intersect the edge of the beam, or the particles can follow different paths through the beam. The edges of the Gaussian beam profile where the intensity rises (or falls) from 10% to 90% of its maximum intensity are about 60 μm . The peak width of the beam where the intensity distribution is flat is estimated to be only 30 μm , since the $1/e^2$ diameter is 150 μm . The diameter of the sample stream is about 25 μm , encased in a 100 μm sheath stream. Particle diameters are in the range of 20 μm to 1 μm . Precise alignment, within at least 5 μm of the

flow stream with the laser beam is therefore required.

When the particle trajectory is consistent, that is, if all the particles follow the same flow path, then this alignment is not difficult. The translators with 1 μm resolution upon which the chamber is mounted can be adjusted so that the light scatter pulses are uniform. If the particle stream is only in the edge of the beam, the pulses are reduced in amplitude and vary considerably in both width and amplitude. When the particles are passing through the center of the beam the pulses are all of the same width with the slightest variation in amplitude. The proper position also produces pulses of maximum height.

The pulse produced by the detector is the result of the intensity of the light scattered as the particles flow through the laser beam. The characteristic of the pulse which is taken to represent the scattered light as if the particle were fixed in the beam is its maximum height. The maximum height of the pulse depends on the maximum illumination incident on the particle.

The performance of the instrument can be seriously limited by an erratic flow stream. Using a concentric sheath stream to control the sample, either the flow is stable and laminar, or is turbulent due to an obstruction such as an air bubble. Turbulence is obvious and the steps needed to

restore laminar flow can be taken.

The inherent limitation is in the nature of the laminar flow. Although the flow stream is not directly visible (even with the aid of a microscope), the view being obstructed by the outlet nozzle, a good criterion of the quality of flow is the constancy of the transit time from the orifice to the laser beam. If this time is very uniform all the particles are traveling the same path to the laser beam. Erratic paths would vary the arrival time at the beam. The electronic volume sense signal is produced during the time the particle is in the orifice, and its falling edge occurs just as the particle leaves the orifice. The light scatter pulse is produced a time later depending on the distance between the orifice and the laser beam as well as the flow velocity. Under proper running conditions it is observed that the transit time differences among particles are less than $0.5 \mu\text{sec}$. This indicates that the particles are following closely the same path through the beam. The effect of flow variations which cannot be eliminated by adjustment of the flow properties or alignment are seen in section V-3-1.

The flow velocity can be measured by translating the chamber and thus the orifice a known distance from the beam and measuring the change in the transit time. When the orifice

was moved 0.85 mm from the beam the transit time changed by 100 μ sec. The particle velocity is then 8.5 m/sec (μ m/ μ sec). Other settings of the pressures on the sheath tank and sample holder gave velocities up to 10.0 m/sec. For all the light scattering experiments the settings were such that the flow velocity was 8.5 m/sec.

The beam width can be determined from the duration of the light scatter pulse and flow velocity. The pulse width is 18 μ sec which gives a beam diameter of 153 μ m in agreement with the spot size calculation. The duration of the electronic volume signal is 36 μ sec indicating an orifice length of about 306 μ m consistent with the manufacturers specification.

With a particle flow rate of 500 particles per second, a particle flows through the laser beam about every 2 msec. The intervals between scattering events is actually somewhat random. However, only 250 μ sec are required to process and store 32 signals from a single particle. It is unlikely that many arrive during the processing dead time and coincident arrival of two particles is highly unlikely unless the particles are joined together in a doublet.

IV. CALCULATIONS FOR PHOTOMETER RESPONSE

IV-1. Introduction

In this chapter we calculate the effects of particle size, refractive index, and detector geometry upon the photometer response. First, the manner in which the computations were done is explained. The drastic effect of the angular integration by the detector array is then illustrated by comparing differential light scattering patterns resolved to an angular resolution of 0.1° with those integrated over angle by the detector array. These integrated scattering patterns are analyzed to determine their sensitivity to size and refractive index. It will be seen that the forward scattering region can be used to discriminate among different size samples and that the back scattering region is very sensitive to refractive index differences. In all figures showing integrated patterns, the scales are the same so that the amplitudes of the patterns can be directly compared among the figures. Only the intensity function i_2 is considered.

IV-2. Computational Methods

The computational problem is to evaluate the scattering amplitudes S_1 and S_2 . The program, which was originally written for use on IBM SYSTEM 360 computers (35), has been modified to run on a PDP 11/45 computer (Digital Equipment Corp.). The modification which runs in single precision did not change the computational algorithm. Results in all cases tested agree to at least five decimal places when compared to the original program output. This is sufficient for the applications in this work. The program modified for use on the PDP 11/45 can accept α values up to 900. Calculation time, for output of i_1 and i_2 with an angular resolution of 0.1° from 0° to 180° was 90 sec for $\alpha = 21.9$ and 220 sec for $\alpha = 127.5$.

IV-3. Calculated Light Scattering Patterns

IV-3-1. Differential Scattering Patterns

Two differential light scattering patterns, for $\alpha = 21.9$ ($d = 2.68 \mu\text{m}$) and $\alpha = 102.1$ ($d = 12.5 \mu\text{m}$), calculated with an angular resolution of 0.1° , are shown in figures 18 and 19. The logarithm of the intensity function i_2 is plotted on a polar angular scale. The relative refractive index for both is 1.20.

Two outstanding characteristics of the patterns that might serve as discriminators of particle diameter (with known

Figure 18. Differential scattering pattern for $\alpha = 21.9$ with angular resolution of 0.1° and relative refractive index of 1.20.

Figure 19. Differential scattering pattern for $\alpha = 102.1$ with angular resolution of 0.1° and relative refractive index of 1.20.

refractive index) are the number and position of the maxima and minima in each, and the relative intensities of each pattern. In general, the number of minima can be approximated by α (36). There are 22 minima in figure 18 for which $\alpha = 21.9$, and about 100 minima in figure 19 for which $\alpha = 102.1$. Potentially, resolution and precise angular location of these extrema represent information for precise and absolute size determination (37,38). Unfortunately, it is not practical to resolve an appropriate number of extrema for such large values of α in this photometer.

The magnitudes of the intensities of these two patterns can be compared by noting that the 0° scattering is three orders of magnitude greater for $\alpha = 102.1$ than that for $\alpha = 21.9$. The relative intensity for the large particle then decreases to only one order of magnitude greater at 60° , and at 90° they are of about the same order of magnitude. The extrema extend over greater than two orders of magnitude for $\alpha = 102.1$ in the wide angle region. The backscattered intensity for the larger particle is again greater by nearly three orders of magnitude.

IV-3-2. Integrated Scattering Patterns

In any light scattering photometer the photodetector subtends a finite solid angle. A relatively large solid angle will accept a larger fraction of the scattered radiation but

will limit the angular resolution of the scattering pattern. The magnitude of solid angle accepted determines to a significant extent the amount of information in the signal. With the photodiode array used in this photometer the size and position of each detector are fixed. The detector size used is a compromise between practicality in alignment and signal strength with resolution of the angular pattern. No attempts were made to alter the angular position of any detector, since this would not significantly affect the nature of the measurement, except perhaps at the most forward angles.

With the high α values associated with the particles used here, it is not technically feasible, and perhaps not even advantageous to resolve the angular pattern in detail. Figure 20 shows, on one graph, the transformation of the differential patterns in figures 18 and 19, by the 30 detectors in the array between 0° and 130° each subtending 3° intervals. (For more precise angular locations see table 1, section III-2-6). The logarithm of the intensity function, i_2 , integrated over the angles subtended by the detector is plotted versus a linear angular scale. The transformation is severe, as only the slope and envelope of the differential patterns are preserved. The dynamic range of the pattern for $\alpha = 102.1$, defined by the difference between the highest maxima and lowest minima, is compressed from eight orders of

Figure 20. Integrated scattering patterns for $\alpha = 102.1$
and 21.9 with relative refractive index of 1.20.

magnitude to just four. Since the most forward detector is at 4° , the main forward diffraction lobe whose minima is within 2° , is not sensed. This accounts for the loss of two orders of magnitude of dynamic range. Two more orders of magnitude are lost by integrating over the deep narrow minima in the wide angle region.

This compression of dynamic range by the detector array is experimentally advantageous since the dynamic range of the photodiode array and signal processing electronics is only three orders of magnitude. A neutral density filter mask is used over the forward angular region to compress the intensity range to the detectable range of three orders of magnitude.

It is evident from the integrated patterns that none of the actual maxima or minima are detected. What is preserved is the envelope of the scattering pattern and it is this which must be used to distinguish among different populations. Figure 21 shows the patterns from two particles with $\alpha = 81.7$ and 102.1 corresponding to a $2.5 \mu\text{m}$ difference in diameter. The patterns are plotted on a logarithmic scale so that they are quite distinct at most angles even though the differences appear small.

The integrated scattering patterns for the photometer

Figure 21. Integrated scattering patterns for $\alpha = 102.1$
and 81.7 with relative refractive index of 1.20.

response were computed by averaging the differential scattering pattern over the angular intervals corresponding to those accepted by the detector array. The differential pattern was first calculated with 0.1° resolution. The values between and including the angular limits for each detector element (table 1, section III-2-6) were added and the average value used to represent the signal response produced by that detector. In addition to the simple average, the trapezoid rule for numerical integration produced similar results. Also, the ϕ angular integration over 1.5° was included in a test run with negligible effect as expected.

The averaged values, for each of the 30 detectors, are then plotted as a continuous curve over 180° . The scattering patterns presented in the remainder of this chapter have been averaged, and represent the scattering patterns that are expected from this photometer. Other instrumental designs will produce different results.

IV-4. Size Effects

IV-4-1. Large Size Differences

Scattering patterns calculated for five particle sizes, with diameter differences of about $5\text{ }\mu\text{m}$ are plotted in figure 22. The five particle diameters are; 19.5, 15.6, 10.0, 5.0 and $1.1\text{ }\mu\text{m}$, with corresponding α values of 159, 128, 82, 41 and 9. The five patterns are well separated in a regular

Figure 22. Five integrated scattering patterns showing the effects of large diameter differences.

manner with size in the angular range of 4° to about 60° . The differences here are quite large. This region is the best candidate for size discrimination by comparison of signal pulse heights. In the lateral and back angular regions the patterns are not clearly separated. Even for these large diameter differences there is some overlapping among the patterns. At particular angles certain sizes may be well separated but the patterns in the wide and back angles are more erratic and irregular than in the forward region. The pattern for the $1.1 \mu\text{m}$ diameter particle fell below the intensity scale in the back angle region. Since these values were found to be below the sensitivity of the photometer the scale was not extended to include the entire pattern.

IV-4-2. Pattern Comparison

The following numerical experiment was performed in order to determine whether a particular scattering pattern as produced by this detector scheme might uniquely determine the particle size. A library of integrated patterns was calculated for α values of 80 to 180 with a step size of 1. The patterns were integrated over angle to correspond to the detector response. One calculated response pattern was selected and compared with the library, by computing a deviation D as,

$$D = \sum_{i=0}^{30} (X_i - Y_i)^2,$$

where X_i and Y_i are the calculated response at the same detector for the selected and library patterns. A difference is determined for each of the 101 members of the library and these differences are plotted against the corresponding α .

Two sets of tests were done. In one series of comparisons the differences were computed with the responses unnormalized. Here the combined effects of both the relative magnitude and shape of the response patterns are compared. Figure 23 shows the results of comparing a pattern with $\alpha = 110.0$ with the library. The difference is zero when the pattern is compared with itself and this is, of course, determined to be the best α value.

In the second series of tests, each pattern was normalized to its own maximum value, which is always the most forward detector. Here only the shapes of the response patterns are compared. Figure 24 shows the differences computed for the pattern of α value 110 in this manner. In both figures 23 and 24, there is no unique deep depression in the difference plot for the calculated pattern selected for comparison. It is evident that with a measured pattern from a particle of unknown size, depending on the uncertainties added from the measurement process, a number of α values might be assigned to characterize it. An attempt was made to compare experimentally measured patterns from particles of known size with

Figure 23. Comparison of a calculated integrated scattering pattern with $\alpha = 110$, unnormalized.

Figure 24. Comparison of a calculated integrated scattering pattern with $\alpha = 110$, normalized.

the library of calculated response patterns. The results were very ambiguous in all cases tried with α values from the entire range of 80 to 180 assigned to particles from the same narrowly dispersed sample. The conclusion was reached that due to the uncertainties in the measurement process the shapes of the scattering patterns alone, as measured by this photometer, cannot be used to determine particle size.

IV-3-4. Small Size Differences

Any narrowly dispersed sample of latex particles has actually a distribution of particle sizes within it. This distribution may be quite narrow, but light scattering measurements on suspensions of such samples can be sensitive to these small size differences. In many cases, a size distribution characterized by a mean and standard deviation can be fit to such experimental data (39,40).

If the mean particle size is large, or if the sample has a distribution of sizes, or if the population is multimodal, then the light scattering from such a suspension is averaged over particle size and inversion of the data is not feasible. If the scattering from single particles can be measured with sufficient precision to resolve such small differences in diameter then any such distribution could be characterized (41). Indeed, it is this possibility which has provided some of the motivation for developing this and other

flow instruments which measure scattering by single particles.

To analyze the sensitivity of this photometer to small size differences, the light scattering pattern for particle diameters differing by $\alpha = 1.0$, or only $0.12 \mu\text{m}$, were calculated taking account of the angle integration at the detectors. Two sets of such calculations are shown in figures 25 and 26. In each are five scattering patterns representing the range which might be expected in a typical narrowly dispersed sample.

The patterns in figure 25 are centered about that for $\alpha = 159$. They are all quite similar except for $\alpha = 157$ which differs significantly especially for $\theta > 45^\circ$.

The patterns in figure 26, centered about $\alpha = 102$, show considerably more variation. All patterns are coincident in the forward angle region. This is a feature common to other size dispersions analyzed as well. In this example, the patterns begin to spread at about 45° with significant variation at the lateral and back angles. At first glance it would appear that since the patterns are well separated at 90° , this should provide a sensitive measure of particle size. However, the response here is not monotonic with size. The response of the pattern for $\alpha = 104$, which is the largest size represented in the dispersion, is the second lowest at

Figure 25. Five integrated scattering patterns showing the effects of small size differences about $\alpha = 159$.

Figure 26. Five integrated scattering patterns showing the effects of small size differences about $\alpha = 102$.

90°. The pattern for $\alpha = 100$ is the lowest response and the rest of the patterns do increase in a somewhat regular way. But increasing particle size from $\alpha = 103$ to $\alpha = 104$ produces a drastic drop in response at 90°. As α increases or decreases slightly, the maxima and minima of the scattering (see figure 19, section IV-3-1) shift in angular position. With a detector element subtending an angle of 3° about 2 minima are spanned. Apparently, shifting extrema over the boundaries of the detector can result in such large amplitude variations for small size differences. The detector response to size here is very erratic. This detection scheme then is not capable of resolving small size differences. The forward angular range is not sensitive to the degree of size variation expected within a narrowly dispersed sample, but can discriminate large size differences on the basis of the relative scattered intensities.

IV-4-4. Pulse Height Distributions

Since comparison of the shapes of the light scattering patterns as produced by this photometer has been found insufficient for data analysis, resort must be made to the response of the signal pulses at the discrete detectors, particularly those in the forward angular range.

To analyze the data in this way pulse height distributions can be constructed at each detector. The entire dynamic range of pulse heights is divided into a number of discrete channels. Then the number or frequency of pulse heights falling in each channel is determined. A distribution of pulse heights at a

detector is obtained from a plot of signal frequency versus channel number.

Others have observed that multimodal pulse height distributions occur from light scattering measurements of particle samples of supposedly uniform characteristics (42). Complex and apparently anomalous pulse height distributions were obtained for a monodisperse sample of particles. There was no resemblance between the variation of the particle size distribution and the variation in the light signal distribution.

To examine this effect, the coefficient of variation was calculated for the pulse height distributions at each of the 30 detector elements that would be expected for the responses to the model particle size distribution shown in figure 26. The coefficient of variation is defined as the sample standard deviation divided by the sample mean expressed in percentage. It is a relative measure of variation in contrast to the standard deviation which is in the same units as the observation.

The model particle size distribution with a coefficient of variation of 2% was constructed using an α step size of 1.0 centered about $\alpha = 102$. This distribution would not represent a real particle population but served to explore the effect of slight size variation.

In figure 27, the calculated coefficients of variation of

Figure 27. Coefficients of variation of the calculated pulse height distributions at the 30 detector elements for a model particle population centered at $\alpha = 102$ with a diameter coefficient of variation of 2%.

the pulse height distributions at each detector are shown for this model particle population. Detector 1 is located at the most forward position. The values range from less than 1% in the forward region to over 15% at detector 14, near 90° . The calculated pulse height distributions from the same model particle population were observed to be different at each detector. Where small size changes produce large changes in the light scatter signals a large coefficient of variation in the light signal results. This calculated variation indicates the minimum variation that can be expected from measurements of particles in a narrowly dispersed population. Superimposed on this are contributions from each source of experimental error. This is evident in the measurements in section V-3-1. Consequently it is not feasible to relate measured coefficients of variation directly to the coefficient of variation of the particle size distribution. This exercise illustrates how certain light scattering measurements can exhibit a bewildering variation, even from a uniform sample.

IV-5. Refractive Index Effects

In addition to the optical size α , the scattering patterns of spherical particles depend upon refractive index. Some effects of refractive index are shown in figures 28 and 29 for $\alpha = 81$. The range in figure 28 $m = 1.04, 1.05, 1.07, 1.09$

Figure 28. Five integrated scattering patterns showing the effects of refractive index differences in the range of live and fixed biological cells. The patterns are for homogenous spheres with $\alpha = 81$ (10 μm diameter).

Figure 29. Five integrated scattering patterns showing the effects of refractive index differences in the range of latex particles. The patterns are for homogenous spheres with $\alpha = 81$ (10 μm diameter).

and 1.11 is similar to the relative refractive index of living biological cells in aqueous suspension ($m = 1.04$ to 1.07) and to fixed cells treated with ethanol ($m = 1.10$). The range in figure 29 $m = 1.13$ to 1.21 includes the relative refractive index of the latex particles, used in this study ($m = 1.20$).

For the range of refractive index corresponding to living cells the patterns as in figure 28 are clearly separated at the lateral and back angles. The most distinctive separation is at about 100° . The patterns for $m = 1.09$ and 1.11 exhibit a broad maximum whose peaks are just below or above 90° respectively. This maximum is a property of the envelope of the actual differential scattering pattern. The maximum can be seen to emerge from the patterns for the lower refractive indices at lower angles which then shift backward as refractive index increases. This trend can be seen to continue in figure 29 where the maximum shifts to still larger angles with increasing refractive index.

These patterns make clear that the effect of refractive index must be taken into account when interpreting light scattering data. Although these figures are restricted to one α value at some angles scattering is very sensitive to refractive index, with potential for particle characterization.

Another interesting feature of figures 28 and 29 is that at the lowest angles, less than 10° , the intensity varies inversely with refractive index.

V. EXPERIMENTAL RESULTS

V-1. Introduction

Three sets of experiments are reported. The first consists of measurements of light scattering patterns for individual particles of four sizes. The corresponding calculated patterns integrated for detector response are compared with the measurements with good agreement. The measured 360° patterns, which are 180° patterns measured at supplementary azimuthal angles, are examined for symmetry. These measurements evaluate the optical performance of the photometer.

The second set of experiments consists of a quantitative calibration of particle sizes as can be done with this instrument. The signal pulse heights from particles from eight samples ranging in diameter from 1.1 to 19.5 μm at certain forward detectors are calibrated by a theoretical size-response curve.

The potential of this photometer is revealed in the third set of experiments. Mixtures of five samples, with particle diameters of 1.1, 5.0, 10.0, 15.6, and 19.5 μm are prepared with known concentrations, except for the 1.1 μm diameter particles which are treated as an unknown fraction. This pentamorous distribution is resolved completely by examining the distribution of signal amplitudes at detectors 1 through 8. At no one

detector are all the components satisfactorily resolved, but each can be successfully distinguished at particular detectors, with different detectors serving for each. Not only are the proper relative concentrations obtained for the components of known proportions, but an estimate is given for the concentration of the 1.1 μm diameter particle sample. Considering the fact that the scattering cross section of the largest particle in the dispersion is over 300 times that of the smallest, it is a significant achievement to resolve their relative concentrations. Such a measurement has not been demonstrated with any other present flow instrument employing either optical or electronic means, though with certain modifications other instruments might be capable of it.

Taken together, these experiments confirm the utility of the photometer for the analysis of individual particles and constitute a quantitative technique upon which further work with biological material can proceed.

V-2. Particle Samples

The nine samples used, each purchased commercially, are listed in table 2. The nominal diameter is that provided by the manufacturer. The corresponding value of α is given for a 514.5 nm wavelength in a medium of refractive index 1.3375, used in all experiments.

Table 2. Particle Samples

Sample number	Nominal diameter, μm	α	Material	Commercial source	Lot number
1	19.52	159.4	PSL ^a	CEI ^d	1040
2	15.61	127.5	PSL	CEI	0003
3	12.50	102.1	PSL	CEI	0002
4	10.0	81.7	PSL	PTI ^e	42C
5	7.9	64.5	PSL	PTI	1C
6	5.0	40.8	PSL	PTI	11C
7	2.68	21.9	PVT ^b	Dow ^f	642-6
8	1.09	8.9	PSL	Dow	654C
9	9.55	78.0	PMMA ^c	CEI	Special

a Polystyrene, $m = 1.20$

b Poly vinyltoluene, $m = 1.19$

c Poly methylmethacrylate, $m = 1.12$

d Coulter Electronics, Inc., Hialeah, FL

e Particle Technology, Inc., Los Alamos, NM

f Dow Chemical Co., Midland, MI

The particle samples are diluted to within the concentration suitable for data acquisition rates and the flow system hydrodynamics. A quite wide range of concentration is acceptable, from about 10^6 particles/ml to 10^3 particles/ml, consequently for most work no precise record of the sample concentration is needed. The dilution medium is isotonic buffered saline consisting of; sodium 180 meq/l, potassium 5 meq/l, chloride 153 meq/l, and EDTA 1.0 mmol/l. It is filtered through 0.4 μ m pore size membrane filters. This medium is also used to fill the flow chamber. Generally, a working sample of 5 ml or less is used, and is mechanically mixed and/or ultrasonically agitated before measurement.

The refractive index relative to the dispersing medium for each material is polystyrene, $m = 1.20$, poly vinyltoluene, $m = 1.19$ (43), and poly methylmethacrylate, $m = 1.12$ (44). Each particle sample was assumed to have a mono-modal narrow size distribution (monodisperse) about the mean nominal diameter provided.

V-3. Measured Light Scattering Patterns

V-3-1. Experimental Photometer Response

To compare the light scattering measurements with calculated scattering patterns, the raw data, as stored in the

computer, must be processed to remove the effects of the signal processing system. Just as the calculated differential patterns must be integrated over angle to yield the calculated detector response, the data must be corrected for the effects of the signal processing electronics, computer storage, the incident illumination, and the neutral density mask, to yield the experimental detector response. The purpose of comparing the scattering patterns, in spite of the determination that they contain little discriminatory information, is to demonstrate the performance of the photometer. The feature of the patterns which is of interest is the relative measure of the light scattering intensity at each detector. If both the shape and dynamic range of the measured patterns agree with that of the calculated patterns, then the optics, alignment, signal processing electronics, and operation of the photometer can be assumed to be functioning as designed.

The radiant intensity, arising from a scattering particle, impinges on a detector photodiode element, which produces a proportional electrical signal. Uncertainties can be introduced both before the light reaches the detector array and in the processing of the resulting signal. Misalignment of the ellipsoidal reflector and detector array, or of the laser beam and flow stream, stray light from reflections, or optical

defects in any part of the photometer would not only degrade the precision of the measurements, but also destroy the accuracy. These effects must be minimized in the experimental procedure. Due to the range and amount of data generated in a very short time processing and computer acquisition and storage of the data is necessary. The data are compressed, quantized and digitized with an error of about 0.2%. The available dynamic range in storage is small, so the range of the detector signals must be logarithmically compressed requiring use of a 32 channel parallel signal processing system.

The following procedure was used to process the data into graphical form to account for the processing of the signals. For each of the 10,240 patterns recorded in one run of a particle sample, 32, 8-bit bytes are stored. The data are in binary code in the range of 1 to 2^8 , or effectively each datum is an integer in the range of 1 to 256. Consider the data for just one pattern consisting of a sequence of 32 integers.

The first step is to convert the data from digital to analog form, reversing the effect of the analog to digital converter, resulting in a series of numbers in the range of 1 to 10 volts, representing the output of the logarithmic amplifiers. Each amplifier was individually calibrated with a calibration graph (see figure 14, section III-2-7). The corresponding voltage input to the amplifiers, which is the signal output of the detector elements can then be found from

the proper calibration graph.

Since the output of the elements of the detector array was found to be uniform and the response linear, no corrections were applied for the array itself. When the entire 180° patterns were measured, a 1.0 neutral density filter mask was placed on the circular slot mask over 0 to 60° . The first 10 values of the patterns, corresponding to detectors 1 to 10, were increased by a factor of 10. Additionally, if the amplitudes of the patterns were to be compared between runs, the values were divided by the incident radiant flux. The pattern is then plotted on a logarithmic intensity scale. If needed, the numerical values can be listed for quantitative comparisons.

In both figures 30 and 31, 10 measured scattering patterns, each pattern from an individual particle, are shown. The corrected measured intensity is plotted on a logarithmic scale. The α values refer to the nominal diameters provided by the manufacturer for the particles samples used in each case. These figures illustrate the degree and manner of variation from measurements on individual particles from a monodisperse sample. In figure 30, the patterns are quite similar in shape with the major variation due to an overall amplitude offset among the patterns. Figure 25, in section IV-4-3, showed that for particles in this high range of α values, the patterns exhibit little sensitivity to the degree of size variation

Figure 30. 10 measured scattering patterns, each from an individual particle in a narrowly dispersed population, sample 1.

Figure 31. 10 measured scattering patterns, each from an individual particle in a narrowly dispersed population, sample 3.

expected within a particle sample. The parallel amplitude offset variation can then be attributed to instrumental errors, primarily the effect of flow path deviations through the Gaussian shaped laser beam.

In figure 31, not only is this parallel offset variation evident, but there is also greater variation in the shapes of the patterns, especially in the lateral and back angular region. Since figure 26 in section IV-4-3 shows this same type of variation for calculated patterns corresponding to this size range, it can be attributed to the slight particle size differences in the population of the particle sample itself. This figure shows how both types of variation, that from the measurement process, and that from the sample variability, combine in the data.

V-3-2. 180° Patterns

Despite the degree of variation in the measured patterns due in part to the distribution of particle size, a search of the 10,000 patterns measured for a particle sample did yield a number of patterns that compared well to the response pattern calculated for the α value corresponding to the nominal diameter of the sample. The match of a measured pattern was determined by a visual search, which could be done rapidly on an interactive graphics terminal. About 200 patterns were examined in each of the following cases.

Figures 32, 33, 34 and 35 each show one pattern selected

Figure 32. A selected scattering pattern from a single particle from sample 9 compared with the representative calculated pattern.

Figure 33. A selected scattering pattern from a single particle in sample 3 compared with the representative calculated pattern.

Figure 34. A selected scattering pattern from a single particle from sample 2 compared with the representative calculated pattern.

Figure 35. A selected scattering pattern from a single particle from sample 1 compared with the representative calculated pattern.

to compare with the calculated response pattern representative for that particle sample. The measured patterns were adjusted such that the amplitude in the forward region overlaid the calculated pattern. The dynamic range of the patterns extend over three orders of magnitude and in general each selected pattern matches the calculated pattern well over the entire angular range. The poorest agreement is in the lowest range of measured values, around 90° . In each case the measured values are somewhat higher than the calculated patterns. There are two effects which can cause an over estimation of the scattering here. The first is that this region, since the intensities are lowest here, is the most susceptible to contamination by stray light arising from reflections of the much more intense scattered light at other angles. The second is that the limits of the range of the scattering intensities must be adjusted by controlling the incident radiant flux to fall within the detectable range. If the incident power is high, the signals at the forward detectors will be above the saturation level. If it is too low the signals at the 90° region will be below the detector sensitivity. The neutral density filter mask does reduce the range of the signals and helps to alleviate this problem. In figure 34, the incident power was evidently too low, and the signals around 90° are at the sensitivity limit of the detectors. The backscattered measured values in each case appear to be

in good agreement, at least in amplitude, indicating that the laser beam is not reflected back on the scattering particle. The variation in the back region is due to the fact that the patterns are very sensitive to small size differences here.

V-3-3. 360° Patterns

The detector array consists of 60 elements in a full circle, any 32 of which can be used in one experiment. To illustrate the potential of the array and the ellipsoidal reflector scattering chamber together, measurements were made over the entire 360° angular range. The differential patterns for spherical homogenous spheres are symmetrical at supplementary azimuthal angles. Biological material is, however, generally neither spherical nor homogenous. Possibly measurements at supplementary azimuthal angles might be used to examine the orientation in flow and morphology of cells.

In figures 36 and 37 measured patterns over 360° from single particles are shown. The 15 even numbered elements and their 15 opposite mates were used. The α values refer to the sample diameter for each particle.

The 360° patterns show excellent symmetry about 180° . This is good evidence that the optics and alignment of the photometer are capable of resolving the scattering from individual particles at an almost complete selection of angles.

Figure 36. A measured 360° scattering pattern of a particle from sample 3.

Figure 37. A measured 360° scattering pattern of a particle from sample 2.

V-4. Particle Size Measurement

V-4-1. Experimental Method

In this experiment the instrument was calibrated for particle size analysis by measuring the response of detector elements 5 (27.6° to 30.5°), 6 (33.5° to 36.4°) and 7 (39.3° to 42.2°) for each of eight samples with nominal diameters ranging from 1.1 to 19.5 μm . The mean of the signals measured from about 10,000 individual particles in each sample was analyzed and plotted against the nominal size. Also plotted were the calculated responses for α values 5.0 (1.0) 175.0. This procedure provided both an empirical and a theoretical response curve which could then be compared for consistency.

Before data acquisition was initiated the signal pulse height at detector 1 was adjusted to the upper limit of the amplifier response by controlling the incident radiant flux with the neutral density filters. The maximum laser power of 200 mW was required for the 1.1 μm diameter particle sample, with less power needed for the larger samples. Only 20 mW was used for the 19.5 μm particle sample. No additional neutral density masking was used over the forward angle detectors, and the signal strength at the wide angles was generally below the sensitivity limit of the detectors. For each run the storage file name, sample, laser radiant flux, neutral

density filter, number of events acquired, particle flow rate, and discriminator setting, were recorded. The particle flow rate was usually about 200 particles/sec and the discriminator setting determined the lowest level signal to be acquired. This eliminated triggering the processing system on low level signals due to random noise or small debris such as dust. 10,240 events were stored in a run time of less than one minute. The sample input tubing can be rapidly cleaned by draining, and the system readied for another sample. After the photometer is aligned and all systems determined to be functional, eight samples can be processed in less than 30 minutes. The subsequent analysis requires somewhat more time.

V-4-2. Data Analysis

The experimental quantity to be determined is the ratio I_{θ}/ϕ_0 . I_{θ} is the radiant intensity of the scattered light at angle θ . ϕ_0 is the incident radiant flux. The radiant flux is used instead of irradiance, radiant flux/area, since a laser emits a unidirectional, collimated beam which if completely subtended allows direct measurement of the radiant flux of the source. This ratio is analogous to the Rayleigh ratio $H_{\theta,h}$. The scattering plane in this photometer is vertically oriented as opposed to the usual horizontally oriented plane, so this is the proper ratio to use here.

What is being measured is the polarized component of scattering parallel to the scattering plane.

A pulse height distribution for the signals at detector 6 from sample 1, $\alpha = 159.4$, is shown in figure 38. The channel number is the value as stored in the computer and is proportional to $\log(I_\theta)$. The distribution can be limited to eliminate any outlying values which clearly are not members. These signals are due to either large debris above the discrimination level, or to data dropped by the signal processing electronics or computer storage devices. In this example the lower limit was set at channel 157, and the upper limit at channel number 194. 9384 values remain between the limits with mean channel number 177, and coefficient of variation 2.8%. The mean channel number is proportional to the logarithm of the mean scattering intensity and will be taken to represent the signal from the mean particle size. This is not strictly valid as the pulse height distribution is not an accurate description of the actual particle population. But, since the resolution of the measurements exceeds the range of sizes within a narrow population, this value is used as characteristic of the mean size.

The incident radiant flux is determined from a measurement of the radiant flux emitted at the laser head and the transmittance of the neutral density filter. The ratio I_θ/Φ_0 ,

Figure 38. Pulse height distribution at detector 6 (35.5° to 36.4°). The data are for sample 1, $d = 19.5 \mu\text{m}$. The short vertical lines limit the distribution for analysis.

can then be evaluated for each sample at each detector. Table 3 lists the data at detector 6 for the eight samples. The mean channel number is reduced by a factor of 100 for the computations.

V-4-3. Calibration

The calculated calibration curves for detector response consist of values of i_2 computed with an angular resolution of 0.1° , integrated over angle with limits corresponding to the angle subtended by the detector element. The range of α is 5.0 (1.0) 175.0, which is a diameter range of 0.6 to 21.4 μm . The relative refractive index is 1.20. (The calculated response for refractive index of 1.19 for sample 7 at the detectors was not significantly different from that for refractive index 1.20). The values are plotted on a logarithmic scale to compress the dynamic range.

The responses at detectors 5, 6 and 7, in figures 39, 40 and 41, increase in a somewhat regular manner with size, but exhibit a rough ripple structure. This ripple structure limits the precision to which size can be measured, and the resolution between particles of different size, to no better than 1 μm . Measurement at several detectors can remove some of the uncertainty, but no improvement over the size provided by the manufacturer can be expected.

The small dispersion in size within a sample will cause

Table 3. Data at Detector 6 for Particle Size Analysis

Sample number	α	Mean channel number	ϕ_o	$\log (I_{\theta}/\phi_o)$
1	159.4	177	20.0	0.47
2	127.5	150	20.0	0.20
3	102.1	134	19.0	0.06
4	81.7	149	38.3	-0.09
5	64.5	145	58.5	-0.32
6	40.8	138	185.	-0.89
7	21.9	115	185.	-1.12
8	8.9	54	195.	-1.75

Figure 39. Comparison of measured and calculated photometer response to particle size at detector 5 (27.7° to 30.5°). The plotting increment is 3.80. The measurements span the size range of 1.1 to 19.5 μm .

Figure 40. Comparison of measured and calculated photometer response to particle size at detector 6 (33.5° to 36.4°). The plotting increment is 3.78.

Figure 41. Comparison of measured and calculated photometer response to particle size at detector 7 (39.5° to 42.4°). The plotting increment is 3.78.

the experimental response to partially span an oscillation in the response curve. The effective result is that a curve drawn through experimentally determined points only will yield a smooth response curve which is misleading in that the inherent limitation in resolution would not be apparent. This would also occur if the calculated response curve were determined with a larger step size.

The calculated response curves at detectors 4 through 8 indicate that this is the optimum region for size discrimination in this photometer. The response at detectors 1, 2 and 3 showed more pronounced and wider oscillations with an apparent resolution of 3 μm . The response of detector elements at larger angles is wildly oscillatory. There are instances, where, over an α range of 10, the response oscillates once or twice over an order of magnitude. Although the general response is poor over this broad size range, and cannot be used to measure size, there are specific areas of heightened discrimination.

To place the experimentally measured quantities on the calculated response, a plotting increment was determined. This is the amount, the same for each measured point, by which it is to be incremented to fall on the calculated curve. This is equivalent to shifting the vertical axis of a plot of the experimental points over the response curve.

For the data from the eight samples at one detector, the value of $\log (I_{\theta}/\Phi_0)$ determined for a sample is subtracted from the logarithm of the calculated value of the detector response at the nominal diameter of that sample. The mean of the differences for the eight samples is used as the plotting increment for each measured point at that detector response curve. The data for the computation of the three plotting increments at detectors 5, 6 and 7, are shown in table 4. The three plotting increments are nearly equal. Also, there is a consistency for the particle samples considered individually at each of the three detectors. A systemic deviation as seen for samples 4 or 6 could be attributed to using the nominal diameter provided by the manufacturer as the mean size of the sample, or to a failure of the mean signal to be representative of the mean size.

The results of the measured points plotted with these increments at detectors 5, 6 and 7 are shown in figures 39, 40 and 41. There are eight measured points on each curve. Each was plotted at the nominal α value for the sample. It is evident that assignment of a precise α value to a sample of unknown size would be difficult based on the intensity alone at one detector. The error bars at each point are one standard deviation, calculated from the coefficient of variation of the pulse height distributions.

This analysis depends on an independent value of the

Table 4. Plotting Increments at Detectors 5, 6 and 7

Sample number	α	Detector 5	Detector 6	Detector 7
1	159.4	3.79	3.72	3.77
2	127.5	3.83	3.81	3.84
3	102.1	3.78	3.77	3.78
4	81.7	3.75	3.66	3.67
5	64.5	3.82	3.77	3.79
6	40.8	3.82	3.92	3.92
7	21.9	3.95	3.74	3.79
8	8.9	<u>3.66</u>	<u>3.72</u>	<u>3.62</u>
Mean		3.80	3.78	3.78

Note: The plotting increment is the difference of the logarithm of the calculated detector response and the logarithm of I_{θ}/Φ_0 .

size for the particles measured. Since only the intensities of the signal are compared, and not the differential scattering patterns, and because the size response increases in an irregular fashion, it is not possible to determine the size absolutely. Neither is it feasible to account for all the experimental effects to evaluate the absolute value of the scattered radiant intensity. As a recourse, the experimental response is compared to the calculated response by means of the plotting increment. This is more reliable than an experimental response curve only, since the basis of the measured discrimination between the particle sizes can be established. Calibration of the experimental response with the calculated response does allow an unknown sample to be characterized quantitatively, rather than be interpolated between measurements from knowns.

These measurements of particle size agree well with the calculated response and are consistent and reproducible. This analysis has not only illustrated the ability of the photometer to discriminate between particles over a very large range, but also establishes the measurements quantitatively.

V-5. Measurement of a Multi-modal Dispersion

V-5-1. Experimental Method

The purpose of this experiment was to resolve the concentrations of five components of a mixture. The components

mixed were samples 1, 2, 4, 6 and 8, with diameters of 19.5, 15.6, 10.0, 5.0 and 1.1 μm . Stock solutions were prepared and the number concentration determined on a Coulter counter. This instrument counts the number of particles in a 0.5 ml volume by electronic means. The particular orifice used was not able to sense the 1.1 μm diameter particles. (Smaller orifices are available to detect particles as small as 0.6 μm by this principle. However, no one combination of orifice size, resistance, and impedance match on present instrumentation of this type is capable of resolving a mixture with components as widely dispersed as used here). The number concentration of the 1.1 μm stock solution was treated as an unknown.

The concentrations of the stock solutions, including that for sample 8, were all in the range suitable for processing in the photometer. The pulse height distributions from each of the five components were measured individually. Measured volumes of the five stock solutions were then mixed.

The difficulty in measuring the light scattering from a distribution this broadly dispersed, is that the same radiant flux is incident on each particle. Figure 22 in section IV-4-1 shows the calculated patterns for each of these five particle sizes. The calculated response patterns for the particle of 19.5 μm diameter is 1-5 orders of magnitude

greater than that for the 1.1 μm diameter particles. The dynamic range of the detector elements is limited to only 3 orders of magnitude.

In the forward angle range up to about 50° , the response patterns decrease significantly in amplitude in a more or less parallel manner. Using maximum incident power it was expected that all the signals at detector 1 (5°) would exceed the saturation level except those from the smallest particles. At detectors 2 (11°) and 3 (17°) the next smallest particle sample would appear, and at detector 5 (29°) the next. By detector 8 (47°) signals from each particle size would have dropped below the saturation level. Those from the smaller samples may well be in the noise here, but at certain detectors between 1 and 8, depending on the range of scattered intensities for that particle size, each component would be resolvable from the other four. This is indeed the response measured from these multi-modal dispersions.

From the pulse height distributions at detectors 1 through 8 the number of particles in each peak can be counted. The relative proportion of the particles counted can then be compared with the proportion determined from the Coulter counts. A certain amount of trial and error was needed in the experimental procedure before this distribution was successfully resolved. The characterization of the

components, and four mixtures from these, of slightly different proportions, are reported. The number concentration of the 1.1 μ m particle stock solution is also determined.

V-5-2. Components

For the five components, 15 ml stock solutions were prepared by a 1:100 dilution, in saline medium, of particle samples 1, 2, 4 and 6, and an approximate dilution of 1:10⁶ for sample 8. No attempt was made to determine the number concentration of the concentrated commercial samples.

The number concentrations of the stock solutions were determined on a Coulter counter (Model ZB, Coulter Electronics, Inc., Hialeah, FL). The particles, suspended in an electrolytic solution are counted by passing them through an orifice through which a current is flowing. As the particles pass through the orifice the resistance in the path of the current changes. The number of current impulses which are counted as a specific amount of sample volume is drawn through the orifice represents the number of particles within the suspension.

The stock solutions for samples 1, 2, 4 and 6, were diluted 1:5 before counting and 0.5 ml volume counted. The counts for each sample and number concentration of each stock solution are listed in table 5. The orifice diameter used

Table 5. Number Concentration of Stock Solutions Determined
by Coulter Counter

Sample number	Raw counts/0.5 ml	$n_{\text{coincidence}}$	$N, 10^4$ Particles/ml
1	1748 2175 2052 2006 <u>1850</u>		
	$\bar{n} = 1964 \pm 170$	9	$1.96 \pm .17$
2	6828 7797 7196 7855 <u>7291</u>		
	$\bar{n} = 7394 \pm 432$	132	$7.52 \pm .43$
Saline	$n_{\text{background}} = 10$		
4	10286 10265 <u>10090</u>		
	$\bar{n} = 10213 \pm 107$	256	10.4 ± 0.1
6	12415 11667 12410 <u>13064</u>		
	$\bar{n} = 12374 \pm 571$	378	12.7 ± 0.6
Saline	$n_{\text{background}} = 80$		

$N = (\bar{n} - n_{\text{background}} + n_{\text{coincidence}}) \times 10/\text{ml}$; the factor of 10 accounts for the 1:5 dilution of the stock solution before counting and the 0.5 ml volume counted.

was 100 μm which is capable of sensing the nominal particle diameter range of 5 to 20 μm . An amplifier setting of 1/8 was used for samples 1 and 2 and setting 1/2 for samples 4 and 6. Samples of saline alone were run to determine the level of background counts at each setting. Also, there is a probability that two particles might flow through the orifice simultaneously producing only one count. This coincidence count factor can be calculated by (45), $\bar{n}_{\text{coincidence}} = (n/1000)^2$, where $\bar{n}$ is the average of two or more raw counts. The number concentration of the stock solutions is then, $N = (\bar{n} - n_{\text{background}} + n_{\text{coincidence}}) \times 10/\text{ml}$. The factor of 10 accounts for the 1:5 dilution and 0.5 ml volume counted.

In order to determine the particle components responsible for each peak in the pulse height distribution of the mixture, each sample was first run individually with the identical incident radiant flux (200 mW) and discriminator setting to be used to analyze the mixture. The pulse height distributions at detectors 1 through 8 for the samples consisting of particle diameter, 1.1, 5.0, 10.0, 15.6 and 19.5 μm are shown in figures 42 to 46 respectively.

The pulse height distributions for the 1.1 μm diameter particles, figure 42, are narrowly dispersed. The mean channel number of the distributions decreases from detector 1 to 8. These detectors are located in the angular range of

Figure 42. Pulse height distributions at detectors 1 through 8 for sample 8 with particle diameters about 1.1 μm .

Figure 43. Pulse height distributions at detectors 1 through 8 for sample 6 with particle diameters about 5.0 μm .

Figure 44. Pulse height distributions at detectors 1 through 8 for sample 4 with particle diameters about 10.0 μm .

Figure 45. Pulse height distributions at detectors 1 through 8 for sample 2 with particle diameters about 15.6 μm .

Figure 46. Pulse height distributions at detectors 1 through 8 for sample 1 with particle diameters about 19.5 μm .

4° to 50° where the calculated response pattern (figure 22, section IV-4-1) decreases smoothly. The signals at detector 8 are at the sensitivity level of the detector.

The distributions from sample 6 consisting of $5.0\text{ }\mu\text{m}$ particle diameters, figure 43, are more broadly dispersed than those from the $1.1\text{ }\mu\text{m}$ particles indicating a broader diameter distribution. For this incident radiant flux the signals at detector 1, which is at the lowest angle, are just below the saturation level of the detector and the pulse height distribution here is compressed in the upper channel numbers. At detector 3 the higher range of scattering signals from these particles is below the saturation level and the distribution is discernable.

In the next figures, 44, 45, 46, showing the distributions from the $10.0\text{ }\mu\text{m}$, 15.6 and $19.5\text{ }\mu\text{m}$ diameter particles, the location of the detector at which the signals fall below the saturation level and the distribution is resolvable, increases in scattering angle. For the $10.0\text{ }\mu\text{m}$ diameter particles it is detector 5, for the $15.6\text{ }\mu\text{m}$ particles it is detector 6, and for the $19.5\text{ }\mu\text{m}$ particles it is detector 7. At the detectors located at angles forward of these detectors the entire distributions are in channel number 256. For this incident radiant flux the scattering signals are above the saturation level here. The distributions at the detectors

where the signals are in the measurable range for each particle sample are narrow and mono-modal. But, in each of these three figures there is a distribution of lower level signals. These are probably from dust, or possibly contamination within the samples. The number of signals of this nature is very much reduced in the distributions for the 1.1 μm diameter particles purchased from a separate commercial source than the other samples. The debris is most evident in the 19.5 μm diameter particle sample, that of the largest particles.

In the measurement of the samples individually these debris signals can be both discriminated against in the signal processing or eliminated from the analysis. Here the discriminator was set at a very low level to allow acquisition of the signals from the 1.1 μm diameter particles, hence many of these extraneous signals were acquired when measuring the larger particle samples. Initially the 1.1 μm particles were obscured if these particles were mixed with any other size sample. To minimize the contamination resulting from preparing the samples for measurement, the diluting medium was repeatedly filtered, all glassware thoroughly cleaned, and all tubing in the flow system replaced with cleaned tubing. Also, no ultrasonic agitation was used in any step of the particle processing procedure.

However, since the number concentration of the stock solutions was determined on a Coulter counter an electrolytic dilutant was required. These precautions, as well as careful adjustment of the discriminator, reduced the unwanted signals to the levels shown in the figures. The signals from the 1.1 μm diameter particles were now resolvable at detectors 1, 2 and 3 from these spurious signals.

V-5-3. Mixture Analysis

Four mixtures were prepared. The volumes of each stock solution and the resulting particle number of each component, are listed in table 6. The relative proportions of MIX1 were selected so that each component would have equivalent particle numbers. The proportions of the next three mixtures were variations on this mixture. The particle numbers listed are the products of the predetermined number concentrations and volumes of the stock solutions mixed.

The pulse height distributions at detectors 1 through 8 for MIX1 are shown in figure 47. At detector 1 the peak at channel number 200 is from the 1.1 μm particles. The lower signals are from debris. The signals from all other particles are in channel 256. At detector 2 the peak corresponding to the 5.0 μm particles becomes evident and is

Table 6. Four Mixtures

Sample	Volume mixed, ml	Particle number, 10^4 particles	
1	4.0	7.84 + 0.68	MIX1
2	1.3	9.78 $\pm$ 0.56	
4	0.6	6.24 $\pm$ 0.06	
6	0.3	3.81 $\pm$ 0.18	
8	0.5	unknown	
1	4.0	7.84 + 0.68	MIX2
2	1.3	9.78 $\pm$ 0.56	
4	2.0	20.8 $\pm$ 0.2	
6	2.0	25.4 $\pm$ 1.2	
8	4.0	unknown	
1	3.0	5.88 + 0.51	MIX3
2	3.0	22.6 $\pm$ 1.29	
4	1.0	10.4 $\pm$ 0.1	
6	1.0	12.7 $\pm$ 0.6	
8	1.0	unknown	
1	3.0	5.88 + 0.51	MIX4
2	1.2	9.02 $\pm$ 0.52	
4	0.5	5.20 $\pm$ 0.05	
6	0.5	6.35 $\pm$ 0.36	
8	0.6	unknown	

Figure 47. Pulse height distributions at detectors 1 through 8 from MIX1. The mixture consisted of roughly equal proportions of 19.5, 15.6, 10.0, 5.0 and 1.1 μm diameter particles. The individual peaks were identified from pulse height distributions of the single samples. Signals from debris account for the spread between peaks and the sixth peak at detectors 7 and 8.

resolvable at detectors 3 and 4. At detector 5 the peak from the 10.0 μm particles comes out, and if figure 44 is compared to this figure, it can be seen that this peak is from this sample alone. Each peak in the figure can be identified with the proper particle size by a similar comparison of the distributions from the single sample measurements. At detector 6 the peak from the 15.6 μm sample first emerges. At both detectors 7 and 8 there are six peaks. The peak at the second lowest channel number is due to debris. If the debris could be further reduced, all five samples could be resolved at either of these two detectors.

The number of signals falling in each peak were counted by determining the lower and upper limits of each peak as is demonstrated in figure 48. Counts were obtained for the 1.1 μm particles at detectors 1, 2 and 3, for the 5.0 μm particles at 3, 4 and 5, for the 10.0 μm particles at 5, 6, 7 and 8, and for the 15.6 and 19.5 μm particles at detectors 7 and 8. The means of these counts for each component size and the percentage of the total each comprises are listed in table 7 for the four mixtures.

For example, in figure 48, the count at detector 5 of 10.0 μm particles is 6088, at detector 6, 6316. at 7, 6366 and at detector 8, 6387. The mean of these four counts is 6289 and is listed in table 7 as the mean count for sample 4 in

Figure 48. Pulse height distributions at detectors 5 through 8 from MIX1. The peaks from the 10.0 μm particles are limited by the short vertical lines and the number of signals in this range is counted.

Table 7. Mean Counts of Relative Particle Number as Determined by Light Scattering Pulse Heights Distributions for Four Mixtures.

Sample number	Mean counts				
1	7385	+	10	23.9%	MIX1
2	9541	+	36	30.9%	
4	6289	+	137	20.3%	
6	3985	+	88	12.9%	
8	<u>3679</u>	+	454	11.9%	
total = 30879					
1	2226	+	127	8.5%	MIX2
2	2836	+	58	10.8%	
4	5631	+	76	21.5%	
6	7695	+	56	29.4%	
8	<u>7802</u>	+	172	29.8%	
total = 26193					
1	3057	+	81	10.5%	MIX3
2	10655	+	113	36.7%	
4	4688	+	204	16.0%	
6	6804	+	242	23.4%	
8	<u>3886</u>	+	224	13.4%	
total = 29670					
1	5120	+	296	18.8%	MIX4
2	7762	+	213	28.4%	
4	4459	+	172	16.3%	
6	5465	+	38	20.0%	
8	<u>4479</u>	+	157	16.4%	
total = 27285					

MIX1.

In the measurement of a mixture, 37,886 scattering events were acquired. This required over 1.2 million bytes of computer storage. As can be seen from the total particle counts in table 7, over 20% of the stored events were from debris. However, the pulse height analysis procedure eliminated the gross effect of these signals. This capability is needed for the analysis of biological material since debris in the cell preparations is unavoidable.

Because the number concentration of the 1.1 μm sample was unknown, the initial percentage of each component in the mixtures could not be predetermined. To evaluate the accuracy of the light scatter counts, all ratios of counts that could be formed for two components, excluding the 1.1 μm particles, were compared with the ratios of the component particle number determined by the Coulter method and volumes mixed. These comparison ratios are listed in table 8. The agreement is good, especially for MIX4.

The internal consistency of the light scattering counts, in MIX1 and MIX2, may be checked by considering that the volumes mixed for the 19.5 and 15.6 μm particles were the same, while those for the 10.0 and 5.0 μm particles were varied. The number of 10.0 and 5.0 μm particles counted relative to the 19.5 and 15.6 μm particles should change in proportion to the volumes. These ratios are listed in table 9. The agreement is 10% in

Table 8. Comparison of Ratios of Relative Component Particle Number Determined by Light Scattering Distributions Versus Coulter Number Concentrations of Stock Solutions.

Component ratio	Mixture	Light scattering	Coulter number
19.5/15.6	MIX1	0.774 + .004	0.802 + .024
	MIX2	0.785 $\pm$.029	0.802 $\pm$.024
	MIX3	0.287 $\pm$.005	0.260 $\pm$.008
	MIX4	0.660 $\pm$.020	0.652 $\pm$.019
19.5/10.0	MIX1	1.17 + .024	1.26 + .090
	MIX2	0.395 $\pm$.017	0.377 $\pm$.029
	MIX3	0.655 $\pm$.011	0.565 $\pm$.044
	MIX4	1.15 $\pm$.022	1.13 $\pm$.087
19.5/5.0	MIX1	1.85 + .048	2.05 + .079
	MIX2	0.289 $\pm$.019	0.309 $\pm$.043
	MIX3	0.449 $\pm$.029	0.463 $\pm$.065
	MIX4	0.937 $\pm$.061	0.926 $\pm$.071
15.6/10.0	MIX1	1.52 + .037	1.57 + .050
	MIX2	0.504 $\pm$.017	0.470 $\pm$.032
	MIX3	2.28 $\pm$.065	2.17 $\pm$.027
	MIX4	1.74 $\pm$.060	1.73 $\pm$.055
15.6/5.0	MIX1	2.39 + .068	2.57 + .095
	MIX2	0.368 $\pm$.011	0.385 $\pm$.042
	MIX3	1.57 $\pm$.071	1.78 $\pm$.074
	MIX4	1.42 $\pm$.050	1.42 $\pm$.017
10.0/5.0	MIX1	1.58 + .007	1.63 + .075
	MIX2	0.732 $\pm$.015	0.819 $\pm$.050
	MIX3	0.686 $\pm$.056	0.819 $\pm$.050
	MIX4	0.816 $\pm$.037	0.819 $\pm$.050

Table 9. Comparison of Relative Numbers of Particles Counted
for Light Scattering Distributions to Proportions
of Volumes Mixed

Mixture	Components	Count ratios	Volume ratios
MIX1 MIX2	$\frac{19.5/10.0}{19.5/10.0}$	$\frac{1.17}{0.395} = 2.96$	$\frac{6.67}{2.00} = 3.33$
MIX1 MIX2	$\frac{19.5/5.0}{19.5/5.0}$	$\frac{1.85}{0.289} = 6.40$	$\frac{13.3}{2.00} = 6.65$
MIX1 MIX2	$\frac{15.6/10.0}{15.6/10.0}$	$\frac{1.52}{0.504} = 3.02$	$\frac{2.17}{.650} = 3.34$
MIX1 MIX2	$\frac{15.6/5.0}{15.6/5.0}$	$\frac{2.39}{.368} = 6.50$	$\frac{4.33}{.650} = 6.67$

comparison with the 10.0 μm component and 4% in comparison with 5.0 μm component. This is not an unreasonable deviation.

The light scattering counts have yielded the fraction in each mixture comprised by the 1.1 μm particles. To determine the number concentration of the stock solution for these particles, first the total number of particles of every size in the whole volume of the mixture can be found from the component particle number as determined by the Coulter counts and volumes mixed and its fraction of the total as determined by the light scatter counts. Then, the number of 1.1 μm particles can be evaluated in each mixture, and with the volume of the stock solution mixed, its number concentration. Table 10 lists the results of this analysis. The precision is relatively low since the resolution of the 1.1 μm particles suffered most from extraneous signals.

It should be noted that, even though this photometer can count particles, either electronically or optically, there is no provision for measuring the volume of a sample measured. Due to the hydrodynamic focusing by a sheath stream and the extent of tubing used to introduce the sample it is not presently possible to measure accurately the volume of sample consumed and to correlate this with the number of events acquired by the signal processing system.

Table 10. Determination of the Number Concentration of the
1.1 μm Diameter Particle Stock Solution

	MIX1	MIX2	MIX3	MIX4
$N_{\text{total}}^{\text{a}}$	31.2	91.5	59.2	31.7
% 1.1 μm particles ^b	11.9%	29.8%	13.4%	16.4%
Number of 1.1 μm particles ^c	3.71	27.3	7.93	5.19
Volume mixed, ml	0.5	4.0	1.0	0.6
N^{d}	7.42	6.81	7.93	8.66

$$\bar{N} = 7.70 \pm .78 \times 10^4 \text{ particles/ml}$$

a total number particles of every size in the mixture in
unit of 10^4 particles

b determined by light scattering distributions

c in units of 10^4 particles

d in units of 10^4 particles/ml

VI. CONCLUSION

Light scattering from single particles has been measured and analyzed to determine particle size and to characterize particle populations on a particle by particle basis. Measurements of 180° light scattering patterns from single particles agreed with calculated responses in both shape and dynamic range. Eight narrowly dispersed populations of particles with mean diameters ranging from 1.1 to 19.5 μm were sized by forward angle, (27° - 30° , 33° - 36° , 39° - 42°), scattering measurements. The experimental and theoretical response curves agreed with excellent consistency. A mixture of particles with diameters of 1.1, 5.0, 10.0, 15.6 and 19.5 μm was analyzed. The number concentration of each sample was predetermined and measured volumes mixed. The samples were first analyzed individually so that the data from the mixture could be interpreted accurately. Each component in the mixture was resolved from the other components at particular detectors depending on its range of scattered intensities. The relative number of particles of each component determined by light scattering agreed well with the predetermined number concentrations of the single samples.

To carry out these measurements a photometer capable of acquiring 32 separate light scattering measurements, in the angular range of $\theta = 2.5^\circ$ to 177.5° at $\phi = 0^\circ$ and 180° ,

from single particles for up to 500 particles per second was designed and developed. The optical principle of an existing aerosol measurement device and the electronics of an existing flow cytometer were combined to produce a unique photometer consisting of an ellipsoidal reflector to collect the scattered light and a circular array of photodiodes for measurement. The positions and subtended angles of the detector array employed determined the resolution of the differential scattering pattern. It was demonstrated by calculations using the Lorenz-Mie theory that the effect of the angular integration by this detector array limited measurement of the details of the differential scattering pattern. The response of the detectors to the intensity of the scattered light from the range of particle diameter 1 to 20 μm was sufficient for size determination with resolution of 1 μm .

The successful operation of this photometer has been demonstrated by the excellent agreement between the experimental results and calculated responses. The interactions between the apparatus and the measurement procedure were analyzed to account for alignment, reflections, polarization and the nature of the intersection of the particle stream and laser beam. The use of an ellipsoidal mirror aided in the reduction of stray light and reflected

the scattered light in a way that it could be detected by an array of photodiodes. The technology and fabrication of the detector array was the primary limitation of the photometer performance for more precise light scattering measurements even though it is a significant achievement in itself. Improvement of the hydrodynamics of the flow stream and illumination optics so that each particle is illuminated to a more uniform extent would also aid the performance.

This photometer and its use represent a method of obtaining size and population information of particle dispersions in the range of 1 to 20 μm . The precision for sizing homogenous spheres might be improved by finer angular resolution of the differential scattering pattern but would require a substantial investment in technological development. It might prove less expensive, but just as fruitful, to examine alternative detector geometries, in particular subtending a wider solid angle. This might smooth the response to size especially for particles of lower refractive index.

Examination of single particles in a rapid manner as is done in this photometer is an excellent means of obtaining information about populations of a small total number, but containing many subpopulations. A sufficient number of

particles are counted so that statistical sampling errors are negligible which is not the case with counts obtained by direct microscopic examination. Also, a small total sample volume is consumed which is an added advantage over batch processes such as sedimentation, for example.

This photometer has a particularly rich potential for analysis of biological cells by light scattering, and also fluorescence. Light scattering is a non-perturbative technique for examination of cellular size, shape, refractive index differences, internal granularity and other internal morphology. The design of this photometer allows access to the entire range of scattering angle and further it could be applied as a fluorescence detection instrument by placing a single detector with the appropriate filters at the point where the reflected light is focused. A potential biological problem for which this photometer is well suited is the analysis of red blood cell morphology in which shape is the primary feature for classification.

VII. REFERENCES

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