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A PARABOLOIDAL FLOW CYTOMETER FOR IMPROVED FLUORESCENCE ANALYSIS OF SINGLE CELLS

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A paraboloidal flow cytometer
for improved fluorescence analysis of single cells

by

Mary Jane Skogen Hagenson

A Dissertation Submitted to the
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Major: Biomedical Engineering

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DEDICATION

for Randy, Leigh, and Lara
CHAPTER I. INTRODUCTION AND BACKGROUND

Flow cytometers measure properties of individual particles which flow in a well-defined trajectory through an illumination area. The most commonly measured optical parameters are fluorescence and scattered light.

Flow cytometry is well-established in many areas of biological, medical, and clinical research, and its application to new areas is rapidly increasing. Excellent collections of papers covering all aspects of flow cytometric theory, design, and biological applications can be found in the Proceedings of the Engineering Foundation Conferences on Automatic Cytology (The Journal of Histochemistry and Cytochemistry, 1977; The Journal of Histochemistry and Cytochemistry, 1979).

Cells to be analyzed by flow cytometry must be dispersed as a suspension of single cells, so that each cell can be independently sensed. For fluorescence measurements, cells must be stained with a fluorochrome specific for the material to be quantitated. Cells flow through an illumination area, most commonly a focused laser beam, where fluorescence excitation occurs and light is scattered. Appropriately placed lenses collect a portion of each optical signal and focus the light onto photodetectors. Cell analysis rates on the order of 500-1000 cells per second are common.

The major design factors influencing the fluorescence measurement by flow cytometers are: 1) the light source and associated focusing optics, which determine the illumination geometry; 2) the flow trajectory of particles through this area and their orientation at the point
of analysis; and 3) the means of collecting the resultant fluorescence for measurement.

Ideally, particles to be analyzed by flow cytometers would receive uniform illumination from all angles, thereby minimizing the effect of illumination geometry on fluorescence excitation and subsequent fluorescence measurement. Non-uniform excitation is caused by several factors which include: 1) inherent particle asymmetry; 2) orientation of particles when they are illuminated; and 3) the light source and associated focusing optics. Factors 2 and 3 can be dealt with by effective system design. Since non-uniform excitation results from a combination of these factors, it is difficult to isolate each for characterization, and if possible, quantitation.

Most flow cytometers use either lasers or mercury arc lamps for fluorescence excitation. The most obvious differences between these light sources are their spectral output, and the spatial distribution of the radiation. Lasers emit monochromatic light in a highly collimated beam with a typical diameter less than 2 mm. The primary advantage of the laser is the high output power, often between 1 and 10 W. The beam is easily focused to a small intense spot coinciding with the particle stream. The major disadvantage is the limitation in available wavelengths, and the use of one wavelength at any given time, thus limiting the number of useful fluorochromes to those with excitation spectra overlapping a laser line. Some systems have eased this restriction by using a dual-laser excitation (Steinkamp et al., 1979; Stöhr et al., 1977). In such systems, spatially separated beams from an argon and a krypton or helium-cadmium laser illuminate two areas, each with a
unique wavelength. This greatly increases the number of dye combinations which can be used in multiparameter analysis.

In contrast to the laser, the mercury arc lamp emits light in a broad spectral band, with several regions of high intensity over a large solid angle. This type of spectral output allows the use of fluorochromes with different excitation characteristics. A major disadvantage of the arc lamp is the relatively low power which is ultimately focused onto the particle stream. The arc, being of finite spatial extent and producing incoherent light, is most useful when focused to a relatively broad spot of low, but nearly uniform, intensity. The intensity from a 100 W high pressure mercury arc is on the order of $10^3$ less than that achieved with a focused 2 W laser line. Therefore, the flow systems incorporating the mercury arc lamp can only be used to analyze relatively bright fluorescent objects. A recent article by Peters (1979) compares in greater detail the laser and mercury arc lamp as sources of illumination in flow cytometers.

No information on the spatial distribution of fluorescing material within a particle is obtained by zero-resolution systems in which the illumination area is larger than the cells being analyzed. The first experimental flow cytometers were of this type (Van Dilla et al., 1969, Holm and Cram, 1973), as are most of the current commercial machines (FACS I, II, III, and IV,¹ TPSI and II,² Impulse Cytophotometer,³ and the ICP Flow Cytometer⁴).

¹Becton-Dickenson and Co., 506 Clyde Ave., Mountain View, Ca. 94040.
²Coulter Electronics, Inc., 590 W. 20th St., Hialeah, Fla. 33010.
³Phywe, A. G., POB 665, D-3400 Göttingen, West Germany.
⁴Ortho Instruments, 400 University Ave., Westwood, Mass. 02090.
Limited spatial resolution is obtained with slit-scan cytometers which focus the beam to a waist smaller than the cell diameter (Wheeless and Patten, 1973; Gray et al., 1979), or which scan images of particles through a slit located at the image plane of the objective lens (Cram et al., 1979). The resolution which can be obtained depends upon the width of the "slit" of light relative to the diameter of the cell. As the particle traverses the beam, fluorescence is scanned by a fast photomultiplier tube and electronics, yielding information on the spatial distribution of fluorescent material. This technique is not without orientation problems (Cambier and Wheeless, 1979; Wheeless et al., 1979), but it yields information not present in zero-resolution measurements. Arc lamps have not been used in a slit-scan system due to problems with focusing the arc output to create a slit of light with sufficient intensity to get detectable fluorescence signals.

The typical optimal schemes used to focus the light from lasers and arc lamps onto a particle stream are quite varied, as diagrammed in Figure 1. Flow cytometers using lasers for excitation focus the beam to a small waist, typically less than 100 μm wide in the plane perpendicular to the flow stream. Often, crossed cylindrical lenses are used to give independent focusing along the horizontal and vertical axes. Because the laser intensity falls off rapidly with distance away from
Figure 1. Typical illumination geometry for fluorescence excitation featuring a mercury arc lamp (top) and a laser (bottom).
KOHLER ILLUMINATION

MERCURY ARC

FILTER

DICHROIC FILTER

OIL IMMERSION LENS

PARTICLE FLOW

FLUSH

LASER

F/1.6 OPTICS

FILTER

TO DETECTOR
the optical axis, the sample stream trajectory must be well-controlled to avoid variations in excitation for each particle. These focused-beam systems are also sensitive to the orientation of particles as they flow through the beam, since illumination is uni-directional.

In microscope-based flow cytometers, light from a mercury arc filament is focused by special systems to produce Köhler illumination (Göhde, 1973). The intensity of illumination is nearly uniform over an angle approaching 2π steradians, although it is often a factor of 10^3 less than that achieved with lasers. Such systems are not as sensitive to particle orientation or trajectory in the illumination area, due to the wide illumination angle.

To minimize the problem of particle asymmetry in focused-beam systems, several investigators (Fulwyler, 1977; Kachel et al., 1977; Stovel et al., 1978) have attempted to force alignment of particles hydrodynamically with modified entrance nozzles. Highly asymmetric particles naturally align with their longest axis parallel, or nearly so, with the axis of flow. Problems with the approach have been noted for high flow rates. These nozzles, forcing a uniform lateral orientation, were proven successful with fixed chicken erythrocytes, by direct observation of orientation with special photography. Their usefulness in the fluorescence analysis of highly asymmetric cells, mammalian sperm, has been reported (Pinkel et al., 1979).

---

1 R. G. Sweet, Department of Genetics, Stanford University School of Medicine, Stanford, California. Private communication. 1978.
Another approach to the problem of cell asymmetry is detection of fluorescence from asymmetric cells only when they are in a particular orientation, as determined by light scatter (Loken et al., 1977). It has been demonstrated that this approach, with glutaraldehyde-fixed chicken erythrocytes, can significantly improve fluorescent resolution.

All flow cytometers collect only a fraction of the fluorescence emitted by each particle for analysis. Improved fluorescence collection in flow cytometers benefits both fluorescence resolution and sensitivity. The statistical variation in particle measurements is inversely proportional to the number of photons measured (Barton, 1976). Thus, better photon statistics result in improved fluorescence resolution. The notable exception is when the fluorescence emission is so bright that photon limiting is dominated by other factors influencing resolution. A second benefit is increased signal-to-noise (s/n) ratio which improves the systems' sensitivity. Noise in the electronically amplified optical signals has several components: the "baseline" electronic noise from the photomultiplier tube; the amplifiers and other electronics; "added" noise resulting from increased amplification; biological noise; and optical noise caused by imperfect blockage of scattered laser light. Fluorescence measurements of dim sources, such as cell surface antigens, require improved s/n ratios because of low signal intensities relative to the inherent biological and system noise. The biological noise

component can be minimized by improved sample preparation and staining. But to increase the s/n ratio in order to detect fluorescence from dim but carefully prepared samples, signal collection rather than amplification must be increased, as accomplished by specialized, high efficiency fluorescence collectors. Although more efficient systems may increase the "biological noise" which is collected, due to the greater signal level less electronic amplification and lower laser powers are needed, thus decreasing the added noise components. This is demonstrated by signal-to-noise measurements reported in Chapter IV. Finally, more efficient fluorescence collection will minimize the orientation and asymmetry effects which have been recognized as problems in flow cytometers using small-angle collection optics.

Only recently has theoretical modeling of particle fluorescence been attempted. Chew et al. (1976a) modeled the fluorescence from molecules uniformly dispersed in a sphere embedded in a homogenous medium. They later extended the theory to molecules embedded in N concentric spheres (Chew et al., 1976b). Important particle parameters in these studies are the size, refractive index, and shape. Such a model can be extended to biological cells by considering the nucleus, cytoplasm, and the cell membrane to be three concentric spherical layers, keeping in mind that the effects of particle asymmetry are not considered. Lee et al. (1978) have experimentally determined the angular distribution of fluorescence from dye dissolved in a liquid, and from fluorescent dye contained by spheres which are dispersed in the same liquid. Differences in the angular distribution of fluorescence are reported.
Most flow cytometers using focused-beam illumination collect fluorescence over a small solid angle (from 0.1π steradian with f/1.6 optics up to about 1.2π steradians with larger numerical aperture lenses) at 90° to both the optic axis and the particle stream. The sensitivity of these orthogonal systems to particle asymmetry is most dramatically illustrated with the fluorescence distributions obtained from mammalian spermatozoa stained with DNA-specific dyes (Van Dilla et al., 1977). Such a distribution is shown in Figure 2. The right lateral extension is a consequence of the orthogonal system geometry.

In some of these systems the particle stream, surrounded by a water or saline sheath, intersects the laser beam in air. This arrangement is mechanically simpler than causing the intersection to occur in a fluid environment, but it does have disadvantages. As the fluorescence rays travel from the sheath fluid into the surrounding air, with a lower refractive index, they are refracted away from the horizontal plane, as diagrammed in Figure 3. The net result is less fluorescence collected by orthogonal systems than is predicted by simple solid angle calculations which take into account the numerical aperture of the collection optics.

Cytometers which feature Köhler illumination with arc lamps collect fluorescence with the same optics which determine illumination, thus collecting over a wide angle. Overall, these systems are less sensitive to asymmetry effects than the orthogonal cytometers, although fluorescence from less than 2π steradians is actually collected. The disadvantage here is the relatively low signal intensity which is collected, due to low intensity excitation.
Figure 2. DNA pulse height distribution from bull sperm. The counts in channels 100 through 250 are a consequence of cellular asymmetry in an orthogonal flow system.

Figure 3. The refraction of a fluorescence ray at a sheath-air boundary is schematically shown. The path of a ray in a water environment is shown by the dotted line.
Some flow cytometers have maintained the focused laser beam as the high intensity illumination source, while increasing fluorescence collection with specialized optics. One such system uses a spherical mirror to increase collection efficiency by about an order of magnitude (Arndt-Jovin and Jovin, 1974). An ellipsoidal reflector was designed which collects fluorescence over a solid angle of approximately $3\pi$ steradians (Skogen-Hagenson et al., 1977). This system was used to detect weak bacterial fluorescence signals which could not be detected with orthogonal systems (Hagenson, 1976).

In this dissertation, the development, design, and biological application of a new paraboloidal flow cytometer are described. A specialized flow chamber, a paraboloid of revolution, was designed to incorporate some of the beneficial aspects of previous flow cytometers, with particular emphasis placed on improvement of fluorescence excitation, flow hydrodynamics, and fluorescence collection, with the goals being: 1) improved fluorescence resolution; 2) increased fluorescence sensitivity; and 3) insensitivity to orientation artifacts. In the paraboloid, fluorescence is collected from a solid angle greater than $3.5\pi$ steradians, with the overall collection efficiency equal to about 80%. Chapters II and III describe the design logic and the system design for the paraboloidal flow cytometer, and Chapter IV gives the results of system characterization with standard particles.

In this dissertation, the biological usefulness of the paraboloidal flow cytometer is demonstrated by the fluorescence analysis of the DNA content of mouse testicular cells. A new staining protocol is
reported which, when combined with the paraboloid's wide-angle fluorescence collection, results in the resolution of elongated, elongating, and round spermatids, as well as diploid and tetraploid cells. Chapter V describes the biological materials, and the results of paraboloidal analysis of mouse testicular cells are given in Chapter VI.
CHAPTER II. DESIGN BASIS FOR THE PARABOLOIDAL FLOW CYTOMETER

Fluorescence Excitation

The paraboloidal flow cytometer presented here uses an argon-ion laser for particle excitation and can provide either focused-beam or wide-angle illumination. In conventional flow cytometers with orthogonal geometry, focused-beam illumination is used. With the paraboloidal flow chamber it is possible to use the paraboloidal surface to focus laser light onto a particle stream at the focal point, providing high intensity illumination from a solid angle greater than \(3.5\pi\) steradians. The goals of this illumination geometry are minimization of fluorescence artifacts caused by particle asymmetry (see Figure 2), and minimization of broadening of fluorescence distributions due to non-uniform excitation. To produce wide-angle illumination, the beam is diverged, recollimated, and reflected into a paraboloid through a quartz plate at the aperture. The paraboloidal surface reflects the beam, bringing it to a focus at the paraboloid's focal point. Assuming a perfectly parallel input beam, the spot size, or blur-circle, produced at the focal point is primarily a function of the surface quality of the paraboloid. Although the spot size is difficult to measure directly, a numerical value can be assigned by comparing the length of the fluorescence pulses from a given type of standard spheres produced by wide-angle illumination, and the pulses produced by conventional lenses with calculable spot sizes. Wide-angle illumination was used to analyze fluorescence from standard particles, and the results are given in Chapter IV.
Focused-beam illumination is produced with two crossed-cylindrical lenses. This lens arrangement allows independent horizontal and vertical focusing, and permits the user to tailor the beam size to the particular application. In the paraboloidal flow cytometer, the incoming beam passes through a quartz window, and comes to a focus in water. Even though the beam is refracted at these interfaces, the beam size at the focal point is determined by the focusing lenses, which were experimentally positioned so that the point of focus coincided with the sample stream. For this reason, the following calculations for in-air-focusing were used directly.

When focusing an ideal Gaussian beam in air, the intensity distribution along the optical axis and in the focal plane (perpendicular to the optical axis) can be expressed as (Innes and Bloom, 1966):

\[ I(u,v) = \frac{I_0}{\pi(1+u^2)} \exp \left[ -v^2/(1+u^2) \right] \]  

where \( I_0 \) is total beam power and \( u \) and \( v \) are dimensionless quantities,

\[ u = \frac{\pi \Delta^2 z}{2 \lambda f^2} \]  

\[ v = \frac{\pi r \Delta}{\lambda f} \], where \( \Delta \) is the aperture (beam diameter at the \( 1/e^2 \) intensity points), specified to be 1.4 mm, the wavelength, \( \lambda \), equals 488 nm, and \( f \) is the focal length of the lens used. The variables \( z \) and \( r \) are actual physical dimensions parallel and perpendicular to the optic axis, respectively, measured from the focal point. In the focal plane \( u=0 \) and \( I(u,v) \) becomes:
while along the optic axis \( v=0 \) and

\[
I(u) = \frac{I_0}{\pi(1+u^2)} .
\]

These equations are applied independently for horizontal and vertical focusing lenses, yielding the horizontal and vertical beam intensity profiles in the focal plane, and the intensity distribution along the optic axis, i.e., depth of focus information. The results of the calculations are plotted in Figures 4 and 5 for the various lenses used in these experiments.

The amount of light intercepted by the particle as it flows through the illumination area affects the accuracy of the fluorescence measurement, since a variation in illumination is reflected in the emitted fluorescence. The effect of cell position on the intercepted power can be approximated for a given particle trajectory, a specified pair of crossed cylindrical lenses, and a given cell size, small in comparison to the horizontal beam in diameter. The reduction in relative intensity along the optic axis (see Figure 5) is maximum for the vertical focusing lens, where a variation in particle trajectory of \( \pm 10 \mu m \) yields a reduction in intensity from 1.0 to 0.989. This change is negligible when compared to the corresponding change in intensity along the \( x \) or \( y \) axes. It is therefore assumed that, in the region of interest (the sample stream area), the illumination area is an elliptic cylinder, shown in Figure 6, and only movement of particles in the focal plane will be considered in the calculations. In this plane, for an
Figure 4. Intensity profile produced by each lens in the focal plane plotted as a function of distance perpendicular to the optic axis. Focal lengths range from 24.7 mm to 300 mm.

Figure 5. Intensity profile produced by each lens along the optic axis plotted as a function of distance along that axis. Focal lengths range from 24.7 mm to 300 mm.
elliptical beam spot, the intensity equation can be written as:

\[ I = \frac{2P}{\pi ab} \exp[-2(x^2/a^2 + y^2/b^2)] \], where \( \frac{2}{\pi ab} \) is a normalization factor, \( a \) and \( b \) are semimajor and semiminor axes of the ellipse, diagrammed in Figure 7, and \( P_0 \) is the total beam power. Because the beam is collimated in the area of particle illumination, the total power intercepted by a cell as it flows through the laser beam can be approximated by integrating the Gaussian beam profile in the focal plane (Figure 7) over the area intercepted by the cell. The cell is modeled as a square box, 4.3 \( \mu m \) on a side, with a face normal to the beam direction (the cross-sectional area is equivalent to that of a spherical cell 4.8 \( \mu m \) in diameter). It is assumed that the intensity variation over the width of the box, and the edge effects as the particle enters and leaves the beam, are negligible. It is reasonable to assume that the cell does not rotate or change trajectory in the illumination area. Then, the total power, \( P_c \), intercepted by the cell is given by:

\[ P_c = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \frac{2P_0}{\pi ab} \exp[-2(x^2/a^2 + y^2/b^2)] \, dx \, dy \]

\[ = \int_{-\infty}^{\infty} \frac{2P_0}{\sqrt{2\pi}a} \exp[-2(x^2/a^2)] \, dx \].

This cannot be integrated as a simple function of \( x \), but must be integrated numerically. The upper and lower limits on \( x \) correspond to the \( x \) coordinates of the sides of the square cell, and reflect cell position along the \( x \) axis. Probability tables for normal distributions were used to calculate \( P_c \) for a cell with its center located at \( x=0 \),
Figure 6. The laser beam in the area of the focal point is modeled as an elliptic cylinder because of the relatively constant intensity along the optic axis.

Figure 7. Intensity profile in the focal plane modeled as an ellipse with semimajor and semiminor axes equal to a and b. Three possible particle positions within the beam are shown.
PARTICLE STREAM

LASER

PARTICLE STREAM

Diagram showing a laser and a particle stream in a 3D coordinate system.
5, 10, 15, and 20 µm, for various horizontal lenses. The results are plotted in Figure 8. To check the validity of the assumptions made regarding intensity variations, the calculations were also done for a 1.0 µm square cell with a 100 mm lens, the lens for which the intensity falls off most quickly with x. In this case, $P_c$ was normalized to the same power at $x=0$, as obtained with the 4.3 µm cell and the 100 mm lens, and the results were plotted in Figure 8. It was found that the curves are nearly identical, and therefore the assumptions made do not negate the validity of the calculations for cells up to 4.3 µm wide.

The following conclusions can be made regarding particle excitation with various lenses: 1) if the system has a relatively wide particle stream, a longer focal length lens should be used to produce a relatively broad, although less intense illumination area, resulting in enhanced fluorescence resolution at the expense of signal intensity; 2) on the other hand, if a very fine particle stream is obtained, it is most practical to take advantage of this by using a shorter focal length lens which will focus most of the power onto the particle stream with no loss in fluorescence resolution. Noting how quickly the power decreases with distance from the 100 mm lens (see Figure 8), one can conclude that the particles must follow nearly identical trajectories if such short focal length lenses are used for horizontal focusing. Experimental results using the four lens combinations are reported in Chapter IV.

Flow Hydrodynamics

With proper design of the fluid entrance assembly, particles
Figure 8. The effect of cell position in the focal plane on the intercepted power is plotted for each of the horizontal focusing lenses ranging in focal length from 100 mm to 300 mm.
flow in a narrow, well-defined trajectory though the illumination area at the paraboloid's focal point where optical interrogation occurs. Ideally, each particle intercepts exactly the same portion of the focused laser beam. Artifacts resulting from non-uniform excitation, and variation in position of the fluorescing particle with respect to the collection optics, are thereby minimized. The paraboloid's entrance assembly is designed to approximate this ideal condition within practical limits.

Since the flow hydrodynamics of an entrance assembly is exceedingly difficult to model theoretically, practical considerations and prior art play a major role in determination of a particular design. Once constructed and operational, the performance is evaluated experimentally by characterization of the flow rate, flow velocities, and fluid resistances. The most important parameters, the diameter and spatial stability of the particle stream at the focal point, can be measured directly. Hydrodynamic theory is then applied to explain experimental results, and ultimately to lead to improved entrance assembly design.

The principal design parameters to be determined are shown schematically in Figure 9. The system is designed to allow flexibility in these parameters whenever possible so that an optimum value can be experimentally determined. Variation in the height of the sample tube above the tapered nozzle, parameter c, is possible because the sample tube is snug-fit into the main assembly, rather than being epoxied into place. A snug-fit is water-tight while permitting vertical movement of the tube. Since the tapered nozzle is the most critical portion of the assembly it is desirable to have this component machined separately,
Figure 9. Schematic of the principal design parameters for a fluid entrance assembly. A cross-section of the guiding star at AA is shown.
threading into the main assembly. Thus several nozzles can be machined if the first design is inadequate.

Distilled water is used as the sheath fluid, while distilled water or phosphate-buffered saline (PBS) is the common fluid medium for the sample. These fluids can be considered incompressible at 30 psia (pounds per square inch absolute), the maximum pressure applied, "in view of the very slight compressibility of liquids" (Nernst, 1923, p. 239). Increasing the pressure from 14.7 psi to 30 psi causes a 0.2% change in compressibility, while a temperature increase from 25°C to 30°C at constant pressure, causes a corresponding change of 2.4%. Once the flow of these fluids through the entrance assembly and the paraboloid has reached equilibrium, the flow is considered to be steady, and the rate at which fluid flows past any two arbitrary points must therefore be equal. This is stated by the continuity equation,

\[ Q = A_1 V_1 = A_2 V_2 = A_n V_n = \text{Constant} \]  

(8)

where \( Q \) is the flow rate, \( A \) is the cross sectional tube area at point \( n \), and \( V \) is the average flow velocity at \( n \). At several points within the entrance assembly, \( Q \) can be measured experimentally and the average flow velocities calculated using the cross-sectional area at the corresponding points.

Laminar flow conditions must exist at all times; as the sample is injected into the sheath, as the flow is constricted, and as the fluid is ejected from the nozzle, forming a jet which must pass through the quiescent volume of distilled water which fills the paraboloid. A well-defined trajectory is impossible if turbulence is present. The degree
of turbulence is indicated by the Reynolds number, $Re$, a dimensionless quantity used to characterize flow conditions (Reynolds, 1884) and is given by the equation

$$Re = \frac{vdp}{\mu}$$  

(9)

where $v$ is the average flow velocity, $d$ is the tube diameter, $\rho$ is the fluid density, and $\mu$ is the fluid viscosity. It is generally accepted that for $Re < 2000$, the Reynolds lower critical number for pipe flow, laminar conditions always exist. $Re$ can be calculated for any point at which $v$ can be experimentally determined.

Laminar flow in tubes is characterized by a parabolic velocity profile which is established as a real fluid flows through a tube, encountering resistance at the tube boundary. Starting with a linear velocity profile at the inlet of a circular tube, the fluid must flow a distance $x_e$, given by (Langhaar, 1942)

$$x_e = 0.06d \ Re$$  

(10)

before a parabolic profile (within 1%) is established. It is therefore important in entrance assembly design to minimize constrictions in tube diameter, which increase fluid resistance and perturb laminar flow, and to allow sufficient tube length for laminar flow to become established.

The sheath fluid initially flows in the annulus between the sample tube and the main bore. It is assumed that the flow velocities are equal where the sample is injected into the sheath, resulting in no shearing between the fluids. Laminar conditions must exist as the fluid enters the constricting portion of the nozzle; otherwise magnification
of any pre-existing turbulence will result. Optimum positioning of the sample tube will minimize Re prior to constriction.

The orifice at the end of the straight-tip nozzle ejects the fluid into the paraboloid. The orifice is the smallest-diameter portion of the entrance tubing, and correspondingly produces the highest flow velocities and Re. The submerged jet continues to converge for a short distance beyond the orifice, usually about 0.5d from the opening, where d is the orifice diameter (Daugherty and Franzini, 1965). The region of minimum jet diameter is called the vena contracta, where flow is parallel. Beyond the vena contracta the jet diverges due to friction at the jet-water boundary. The length of the relatively parallel flow region is influenced by the flow conditions prior to the orifice, as determined by the entrance assembly design.

Ideally the nozzle would extend into the paraboloid so that the vena contracta coincides with the focal point. In the actual design, the orifice is 150 μm in diameter, placing the vena contracta roughly 75 μm beyond the orifice. In this particular application, placing the orifice in such close proximity to the focal point is not desirable, since much of the fluorescence emitted by the particles would be blocked by the nozzle assembly, never reaching the paraboloid's surface for collection. As a compromise, the orifice is positioned 1 mm from the focal point, as described in detail in Chapter III, with a resultant sample stream of about 6 μm diameter at the focal point.
Fluorescence Collection

The paraboloid of revolution used in these experiments increases the fluorescence collection to over $3.5\pi$ steradians. Solid angle losses at the apertures required for fluid and laser transmission have been minimized. Since a paraboloidal reflector will collimate only light emanating from the focal point, refraction at a water-air boundary would severely distort the optical output. It is therefore essential that the paraboloid be kept full of distilled water at all times. Figure 10 simply illustrates the consequences of a stream-in-air geometry. Cylindrical symmetry exists about the flow axis for sheath and sample flow, so only one plane through this axis is considered in the illustration. The critical angle, $\theta_c$, at the interface is $48.75^\circ$, hence all fluorescence emitted by the particle at an angle greater than $\theta_c$ is internally reflected and is not available for collection. For angles less than $\theta_c$ the refraction is most severe at larger angles, as plotted in Figure 11. At the surface of the paraboloid, the angle of incidence equals the angle of reflection, so that any angular deviations of the incident ray due to refraction equals the deviation of the reflected ray from the ideal collimated ray. The optical train at the output of the paraboloid eliminates all rays which deviate from parallel by more than $0.25^\circ$. The net effect is elimination of any ray which is refracted at the water-air boundary by more than $0.25^\circ$. Thus, only a ring of fluorescence in the horizontal plane would be collected, and the benefits of a paraboloidal collection chamber negated.
Figure 10. The consequences of stream-in-air geometry for the paraboloid are shown by comparing the divergence of the refracted ray with the ideal ray in a water-filled paraboloid.
Air, $n = 1.0$

Water, $n = 1.33$

Ideal Ray

Reflected Ray

Parabolic Cross-Section

Sheath

Sample

Focal Point

$\theta_I$

$\theta_R$

$\theta_1$

$\theta_2$

$n = 1.33$

$n = 1.0$
Figure 11. Angle made by a fluorescent ray after refraction at the sheath-air boundary ($\theta_2$) plotted versus the original angle ($\theta_1$). The critical angle at the boundary is 48.75°. No refraction occurs for submerged sample streams, where $\theta_1 = \theta_2$. 
CHAPTER III. SYSTEM DESCRIPTION

Overview

The flow system used in these studies, diagrammed in Figure 12, incorporates a paraboloid of revolution as the flow chamber for wide-angle light collection. Fluorochrome-stained cells suspended in fluid are injected into the water-filled paraboloid and intersect a focused laser beam at the paraboloidal focal point, yielding fluorescence and scattered light. The optical signals are collimated after one reflection from the parabolic surface, and pass out of the paraboloid through a quartz plate. After optical filtering, the fluorescence and light scatter signals are optically separated and passed to photodetectors, yielding two signals per cell. After amplification, the electrical signals are digitized and stored in a computer for analysis.

Paraboloidal Chamber

To improve the collection of fluorescent light from particles analyzed by flow cytometers, a specialized flow chamber was designed, shown in Figure 13. The chamber, a paraboloid of revolution, is described by the equation:

\[ y^2 = 4fx, \text{ vertex at origin and depth along x axis.} \] (11)

The focal length, \( f \), is equal to 2.0 ± 0.05 mm. A depth of 40.0 mm was chosen, with a resulting aperture of 35.77 mm, to maximize the amount of surface area while keeping the output light beam (equal to the aperture size) small enough to focus with conventional convex lenses.
Figure 12. An overhead cross-sectional view of the paraboloidal flow cytometer showing the optical separation and detection of scattered and fluorescent light. Focused-beam illumination is used.
Figure 13. Cross-sectional views through the focal point of the paraboloidal flow chamber in (A), the plane of the optic axis, and in (B), the plane of the laser beam.
The paraboloid was numerically machined in a solid block of #6061 aluminum. This metal was chosen due to its relative ease of machining. The paraboloidal surface was hand polished to a finish of about 100/80 (scratch and dig, dimensions of surface scratches, in μm) in preparation for optical coating. Due to the small size and relatively large depth of this chamber, polishing near the vertex and applying uniform evaporated coatings were especially difficult. An electroplated layer of Canogen nickel followed by an evaporated layer of chromium, was deposited on the aluminum surface to provide some protection from water and saline solutions. An evaporated coating of gold less than 5 μm thick served as the final coating. Gold was chosen because its reflectivity increases from 48% to 92% (normal incidence) between 500 nm and 600 nm, shown in Figure A1, thereby enhancing the collection of fluorescence, which has longer wavelengths than scattered laser light.

The continuity of the paraboloidal surface is interrupted by five apertures (Figure 13) which allow for transmission of the flow stream and laser beam, and removal of air bubbles from the top of the chamber. Figure 14 diagrams the method of calculating solid angle losses, and Table 1 lists each aperture, its radius, its distance from the focal point, and the solid angle each subtends with respect to the parabolic focal point. The diameter of each aperture was minimized to increase the amount of surface available for fluorescence collection.

1 Special Optics, Box 163, Little Falls, NJ, 07424.

2 Chemistry-Materials Science Division, Los Alamos Scientific Laboratory, Los Alamos, NM 87545.
b = distance from the primary focus to the center of the orifice

\( r = \text{radius of the inscribed sphere, equal to } \frac{\sqrt{a^2 + b^2}}{r} \)

The solid angle, \( \Omega \), is equal to

\[
\Omega = \frac{A}{r^2} = \frac{2\pi rh}{r^2}
\]

Figure 14. Geometry for calculations of solid angle loss at each orifice.
The total solid angle loss is found by summing the values for $\Omega$ in Table 1, and is equal to $0.506\pi$ steradians. Therefore, approximately $3.49\pi$ steradians, or about 87% of the spherical area, is available for light collection. Since the reflectivity of gold is 92% at 600 nm, (for normal incidence), the overall collection efficiency is about 80% at this wavelength.

A 3.0 mm thick circular quartz plate seated against an O-ring, forms a water-tight seal at the aperture of the paraboloid. Fused quartz is a suitable window material since it transmits visible light efficiently, but will not fluoresce when irradiated with intense scattered light. To remove bubbles from the water-filled chamber, a small hole was drilled from the top of the aluminum block through the paraboloid surface near the aperture. Stainless steel hypodermic tubing was joined to the hole at the surface of the block, allowing connection of a vacuum line. Vacuum is applied only when bubbles are present, and is never on during particle analysis.

Flow through the paraboloidal chamber is determined by two specially designed brass assemblies, one each for fluid entrance and exit, positioned directly above and below the focal point, respectively. The fluid is composed of the sample (particles dispersed in liquid) surrounded by one concentric distilled-water sheath. The sheath serves two major functions: it aids in hydrodynamic focusing of the sample stream to a small diameter at the optical analysis region, and it restrains the sample flow so that no mixing occurs with the quiescent water in the paraboloid.
Table 1. Dimensions and the solid angle loss for each aperture in the paraboloid.

<table>
<thead>
<tr>
<th>Orifice</th>
<th>a (mm)</th>
<th>b (mm)</th>
<th>r (mm)</th>
<th>Ω (steradians)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laser entrance</td>
<td>0.75</td>
<td>4.00</td>
<td>4.07</td>
<td>0.034π</td>
</tr>
<tr>
<td>Laser exit</td>
<td>0.75</td>
<td>4.00</td>
<td>4.07</td>
<td>0.034π</td>
</tr>
<tr>
<td>Stream entrance</td>
<td>0.25</td>
<td>1.00</td>
<td>1.03</td>
<td>0.058π</td>
</tr>
<tr>
<td>Stream exit</td>
<td>0.75</td>
<td>4.00</td>
<td>4.07</td>
<td>0.034π</td>
</tr>
<tr>
<td>Nozzle blockage(^1)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.156π</td>
</tr>
<tr>
<td>Aperture</td>
<td>17.89</td>
<td>38.00</td>
<td>42.00</td>
<td>0.190π</td>
</tr>
</tbody>
</table>

\(^1\)Nozzle blockage is a shadow phenomenon. The fraction of the cross-sectional area of the paraboloid through the focal point which was occluded by the nozzle, multiplied by 0.5 (since 1/2 of isotropically emitted fluorescence is reflected by the surface between the vertex and the focal point cross-sectional plane), and converted to an equivalent solid angle loss.

The entrance assembly, diagrammed in Figure 15, is attached to the aluminum block with four small screws. The sample tubing (stainless-steel, 0.035 inch outer diameter) was snug-fit through a bore into the 0.125 inch diameter central bore, extending through the guiding-star into the nozzle. This snug-fit arrangement is water tight, while allowing for vertical movement of the sample tubing for hydrodynamic tests. The distilled-water sheath flows into the main bore through a stainless-steel tube (0.035 inch outer diameter, OD), joining the bore at an angle of approximately 5°. This angle was minimized to decrease flow resistance. The guiding star centers and supports the sample tubing, and enhances the hydrodynamic stability of the sheath.
Figure 15. Mechanical drawing of the entrance assembly and nozzle. All dimensions are given in inches.
DRILL & C'BORE FOR #6 SOCKET HD. SCREWS — 4 PLACES

GUIDING STAR PRESS FIT

150/U ORIFICE

SCALE: 4=1
The nozzle threads into the entrance assembly at the end of the bore, with a silicone gasket maintaining a water-tight seal. The interior of the nozzle is electroformed in brass, and the outer surface is machined to the desired wall thickness, approximately 20 μm. A converging angle of 28° over a distance of 3.5 mm causes gradual hydrodynamic focusing of the sample and sheath fluids. This tapered portion of the nozzle extends 3.0 mm into the paraboloid chamber; the 150 μm orifice (0.5 mm long) discharging the fluid jet 1.0 mm above the focal point. Positioning of the orifice near the focal point minimizes divergence of the jet before optical analysis; the divergence is caused by loss of forward momentum due to friction with quiescent water filling the chamber. This positioning produces a 6 μm diameter sample stream at the paraboloidal focal point. A microscope with a calibrated eyepiece was used to measure sample stream diameter, and its spatial stability with respect to the focal point. The stream showed no oscillations about the focal point with time.

The fluid jet exits the chamber through a 200 μm orifice in the exit assembly, diagrammed in Figure 16. The bore diameter increases in two steps through this assembly, resulting in a gradual reduction of flow resistance and velocity. The entire assembly threads into the bottom of the paraboloid block, the orifice being flush with the surface curvature of the paraboloid. At the output of the exit assembly plastic tubing carries the fluid to a waste collector.

Window assemblies are designed to transmit the focused laser beam through the paraboloid while maintaining a water-tight seal, and to
Figure 16. Mechanical drawing of the exit assembly. All dimensions are given in inches.
200 μ ORIFICE
GASKET SURFACE
$\frac{3}{8}$ 32 THD.
THD. RELIEF
KNURLED

SCALE: 4 = 1
minimize the size of the orifice required in the parabolic surface. Fused quartz was chosen as the window material since, unlike ordinary glass, it will not fluoresce when irradiated with an intense laser beam. As shown in Figure 17, the window assemblies thread into the block and are recessed from the surface of the paraboloid. This design allows for relatively large quartz windows (5.0 mm diameter, 3.0 mm thick), which can be removed from the assembly for cleaning or replacement, while requiring an aperture in the paraboloid surface only 1.5 mm in diameter.

A 1.5 mm diameter clearance path drilled in the aluminum block, allows transmission of the laser beam between the quartz windows and the interior of the paraboloid. To avoid distortion of the beam by air bubbles present in the passages, a small hole was drilled from the top of the aluminum block into each clearance path, and connected to a vacuum via a 15 gauge needle epoxied into each hole at the block's surface. By briefly applying the vacuum independently to each clearance path, all bubbles are removed.

Fluid Flow

Fluid flow through the paraboloidal chamber is controlled by a specially designed pressure system, diagrammed in Figure 18. The basic components of the system are a sheath fluid reservoir, sample holder, pressure control panel, flow control panel, and appropriate tubing for air and fluid. Both the sheath reservoir and the sample holder have air-tight seals allowing pressurization.
Figure 17. Mechanical drawing of the laser window assemblies. All dimensions are given in inches.
0.528Ø

0.400

0.200

0.202 Ø

0.198

.052 - #55 DRILL 2 HOLES

THD. RELIEF

3/8-32 THD.

1/4-40 THD.

GASKET

QUARTZ DISK 5MM Ø, 3MM THK.

3/12 Ø

0.312

1/4-40 THD. .137 DEEP

GASKET

0.313

0.310

0.136

0.137

100

105

0.25

0.137

.262

.137

1180

0.313

0.136

1180

SCALE: 4 = 1
Air lines

Fluid lines

Pressure gauge

Pressure regulator

Needle valve.

All fluid valves are Hamilton Inert valves

All pressures are given in pounds per square inch gauge (psig).

Figure 18. Diagram of the pressure system used for fluid control.
Pressurized air is applied to the system via needle valve control, and is regulated to about 25 psi (pounds per square inch), the maximum pressure tolerated by the regulators which follow. The incoming air then branches to regulators in the sheath and sample lines, decreasing the pressure to approximately 9.8 and 5.6 psig (pounds per square inch gauge), respectively. Visi-float flowmeters\(^1\) are used to bleed a small, constant amount of pressure from each line to decrease recovery time after a pressure change. Pressure gauges read the final pressure which is applied to the sheath and sample containers.

The metal sheath tank is lined with a cylindrical glass jar to isolate the water from the metal surface. A filter holder\(^2\) in the sheath fluid line forces the water through a 0.8 \(\mu\)m pore-size filter shortly after exit from the sheath container. This insures the absence of small particles which could plug the orifice within the paraboloid's entrance assembly, and cause high background noise levels if passed through the laser beam. Sheath flow into the paraboloid is controlled by a Hamilton\(^3\) valve.

Hamilton valves are also used to control application of pressure to the sample holder and flow out of the sample tube. The sample must

\(^1\)Dwyer Instruments, Inc., P.O. Box 373, Michigan City, Indiana 46360.

\(^2\)Millipore Corporation, Bedford, Massachusetts 01730.

\(^3\)Hamilton Co., P.O. Box 10030, Reno, Nevada 89510.
never flow in the absence of sheath flow. Violation of this requirement results in high optical noise levels due to particles dispersed throughout the chamber. To backflush the sample line, the sample pressure must be vented and the sample valve turned "ON" while the sheath is flowing. Sheath fluid is thereby forced through the sample line, back into an empty sample tube. The system is flushed in this way between samples. The length of the tubing between the sample holder and the paraboloid was minimized, to decrease flushing and sample delivery time.

Vacuum is applied to the waste container, which collects all fluid exiting the paraboloid. The vacuum is also fed to a four-way Hamilton valve, which selectively removes air bubbles from either of the laser window passages, or the top of the paraboloidal chamber.

**Illumination, Source and Optics**

Biological cells which are to be analyzed by this flow cytometer are dispersed in a single-cell suspension and flow in single file through the focal point of the paraboloid, where they are illuminated. Two methods of illumination have been used in these experiments: focused-beam illumination is the principal means of illumination, while wide-angle illumination\(^1\) was used for analysis of standard spheres.

The light source for both types of illumination is an argon-ion laser\(^2\) with a total power output of 4.0 Watts in the TEM\(_{00}\) mode.

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\(^1\)Illumination angle > 2\(\pi\) steradians.

(transverse electric and magnetic mode in which there are no nodal lines). The most frequently used spectral lines are 488 nm and 457 nm, yielding 1.3 W and 0.25 W, respectively.

To achieve wide-angle illumination at the parabolic focal point, diagrammed in Figure 19, the paraboloidal surface is used to focus collimated light, entering through the aperture, onto the sample at the focal point. The laser beam with a $1/e^2$ diameter of 1.4 mm, is first diverged by a -1.7 cm focal length (f.l.) bi-concave lens. A 22.2 cm f.l. bi-convex lens, focused on the rear focal point of the concave lens, collimates the diverging laser beam with a diameter of 18 mm. This expanded beam is reflected by a dichroic mirror (reflection vs. wavelength given in Figure A3) through the lens-aperture combination, which further expands the beam to a diameter of approximately 36 mm. The beam passes through the quartz plate at the aperture of the paraboloid, is reflected by the paraboloidal surface, and comes to a focus at the parabolic focal point.

For focused-beam illumination, shown in Figure 12, the laser output is focused by two crossed-cylindrical lenses with unequal focal lengths, and passed through the parabolic focal point via two quartz windows, previously described. Since each cylindrical lens is focused onto the parabolic focal point, the beam waist cross-section is elliptical at that point, the axes of the ellipse depending on the focal lengths of the lenses used. This lens arrangement allows independent focusing of the beam in two dimensions, and allows for variation in the spot shape with the use of lenses with different focal lengths, as discussed in detail in Chapter II.
Figure 19. A schematic of the paraboloidal flow chamber in the wide-angle illumination mode.
PHOTOMULTIPLIER

FLUORESCENCE

OPTICAL FILTERS

DICHROIC FILTER

f - 17mm

LASER

PARABOLIC FLOW CHAMBER
Vertical focusing of the laser beam is done with a 24.7 mm f.l. cylindrical lens with a corresponding $1/e^2$ vertical waist diameter of about 7.8 μm. To experimentally determine the effect of the horizontal waist diameter on fluorescence resolution and intensity, several cylindrical lenses were used, including 100 mm, 200 mm, 250 mm, and 300 mm focal lengths; corresponding $1/e^2$ horizontal waist diameters are 31 μm, 63 μm, 78 μm, 94 μm, respectively. The 250 mm f.l. lens is used for all routine particle analysis.

Fluorescence and Scattered Light

When fluorochrome-stained particles flow through the illumination area at the paraboloidal focal point light is scattered by the particle and fluorescence excitation occurs. These rays emanate in all directions from the focal point, the degree of anisotropy varying from particle to particle. It is generally assumed that the fluorescence emission from an ideal spherical particle is quite isotropic, while scattered light from spherical particles is very anisotropic, with most of the light scattered in the forward direction.

The paraboloidal surface reflects all radiation incident upon it, with varying efficiency, depending on the wavelength, and angle of incidence. By virtue of its geometry, all rays reflected by the paraboloidal surface exit the paraboloid as a collimated beam. The cone of light which emanates from the focal point in the solid angle subtended by the aperture is not reflected by the surface, and therefore is not collimated at the output.
At the aperture of the paraboloidal chamber a quartz plate forms a water-tight seal against an o-ring for the internal fluid, and efficiently transmits the optical output without itself fluorescing. A large plano-convex lens (40.0 mm diameter, 50.0 mm f.l.) then focuses all parallel rays onto a 500 μm pinhole aperture. Rays which deviate from parallel by less than 0.25° at the output of the paraboloid will be passed through the pinhole, thereby insuring that only light originating at the focal point will be collected for optical analysis.

Collimation of the optical beam is essential for proper operation of filters used to separate the fluorescence and scatter wavelengths. To achieve this, a bi-convex lens (20.0 mm diameter, 22.0 mm f.l.) is focused on the pinhole, collimating the light diverging from the pinhole to a diameter of 16.3 mm. This diameter is chosen since a beam of this size will illuminate approximately 90% of the photomultiplier cathode area.

A dichroic filter, which separates light on the basis of wavelength, is used in these experiments to optically separate the scattered laser light from the fluorescence. The optical characteristics of the filter are in Figure A2. As diagrammed in Figure 20, the dichroic filter is mounted at an angle of 45° to the collimated beam, reflecting the scattered light to a photodiode detector while transmitting the fluorescence to the photomultiplier tube (PMT).

A 20 mm focal length bi-convex lens focuses the scattered light onto a silicon photodiode, which is described in the next section. The fluorescent light transmitted by the dichroic filter passes through
Figure 20. Dichroic filter box for optical separation of fluorescence and scattered light. The relationship of the dichroic filter to the rest of the system is shown in Figure 12.

An interference filter, insuring that only fluorescence wavelengths are detected by the photomultiplier tube. Spectral transmission curves for all optical filters used are shown in Figures A2, A3, and A4.

Electronics

An RCA\textsuperscript{1} 4526 photomultiplier tube (PMT) was used for fluorescent light detection and signal amplification. The multalkali photocathode has a high quantum efficiency between 400 nm and 650 nm, with peak spectral response at approximately 530 nm, as shown in Figure A5.

\textsuperscript{1}RCA Electro-Optics and Devices, New Holland Avenue, Lancaster, Pennsylvania 17604.
The 4526 PMT is a Dormer-window type, with a projected photocathode area of 1.65 cm by 1.52 cm. The tube is mounted with its long axis perpendicular to the flow system's optical axis, and with the window centered about the optical axis. About 90% of the photocathode is illuminated by the fluorescence beam. The base socket assembly for the 4526 PMT establishes equal potential difference between each of the ten dynodes with a negative voltage between -400 V and -900 V, applied to the cathode. Typical gain in this voltage region is $10^4$, with an approximate anode pulse rise time of 2.0 nsec. The electrical signal collected at the anode is directly proportional to the incident fluorescent light intensity. This signal is fed to the PMT preamplifier circuit.

The preamplifier consists of a FET (field-effect transistor)-input operational amplifier in a transimpedence configuration. The transimpedence is 200 kΩ, in parallel with a frequency compensating capacitor with a range of 1.5 to 7.0 picofarads, needed to eliminate oscillations and to optimize rise time for pulses of various lengths.

The linearity and gain curves of the PMT with the preamplifier, and the preamplifier alone (using test-pulse input) are shown in Figures 21 and 22. The typical fluorescence output pulse (Figure 23) is symmetric, about 4 μsec in duration (for 10 μm diameter uniform particles), and 50 mV to 100 mV in magnitude. The preamplifier output

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Figure 21. Peak pulse-heights at the preamplifier output as a function of PMT voltage, for standard fluorescent particles.
Figure 22. Gain-linearity curve for the fluorescence preamplifier.

Figure 23. Typical fluorescence output voltage pulse at the 8124 amplifier. Horizontal scale, 2 μsec per division.

Figure 24. Gain-linearity of the 8124 amplifier/integrator, gain equal to 10.
is fed to the model 8124 amplifier\textsuperscript{1}, and the signal then branches two ways. One path drives a d.c. amplifier which measures the average anode current. A meter mounted on the front panel of the amplifier continually gives a reading of the anode current in the sensitivity ranges, 0 to 10 nA or 0 to 100 nA. High anode currents usually imply optical "noise" in the chamber, often due to fluorescent debris or air bubbles in the sample flow. High currents in the absence of sample flow indicate contaminated sheath fluid.

The other path is for 2-stage signal amplification and integration. The first amplifier has a fixed gain of ten, while a second stage amplifier adds a gain factor of one to ten. Therefore, the overall gain of the 8124 amplifier varies from 10 to 100, and is linear over that range as shown in Figure 24. The output of the second-stage amplifier branches to a cable connector at the rear of the chassis, where the signal is monitored, and also feeds the integrator portion of the circuit. The "amplifier-out" signal is one of the data parameters collected during particle analysis.

In addition to the amplifier-output signal, the manually set trigger threshold level is fed to the integrator. Only portions of the input pulses exceeding the threshold voltage will be integrated.

The integrator gain can also be adjusted to compensate for pulses of varying lengths. It is most desirable to set the gain so that the

\textsuperscript{1}Model 8124 Amplifier/Integrator, Drawing No. 4Y-89839. 1974. Los Alamos Scientific Laboratory, Los Alamos, New Mexico 87545.
peak voltage of the "integrator-out" signals are smaller as it would result in serious errors if the output of the amplifier were saturated.

An additional variable in the integration process is the gate duration, which sets the period of time over which integration can occur. It is most desirable for the gate duration to be set longer than the input pulse, resulting in integration of the entire pulse above the threshold level. Circuitry associated with the gate insures that the peak integrated level will be held for at least 1 μsec, guaranteeing proper operation of the peak sense and hold (PS&H) circuit which follows. The integrator output is the second data parameter collected during particle analysis.

A silicon photodiode\textsuperscript{1} with high spectral sensitivity, low noise, and wide spectral response, is mounted in a specially designed preamplifier\textsuperscript{2} for light scatter detection. Neutral density filters can be positioned in front of the photodiode to avoid electronic saturation. The photodiode has a spectral sensitivity of about 0.2 A/W at 488 nm (a commonly used laser line), and has guard ring construction to minimize shot noise (EG&G, 1975). The circular detector area is equal to 0.82 mm\textsuperscript{2}. The preamplifier has been designed to minimize stray capacitance effects on circuit sensitivity and bandwidth (Hiebert, 1974).

\textsuperscript{1}Type SGD-040B, EG&G Inc., 35 Congress St. Salem, Massachusetts 01970.

\textsuperscript{2}Model 74 Photodiode Preamplifier, Drawing No. 4Y-89748. 1974. Los Alamos Scientific Laboratory, Los Alamos, New Mexico 87545.
The entire scatter detection unit, consisting of the bi-convex lens, the photodiode, and the preamplifier circuitry, is housed in one half of the dichroic filter box, diagrammed in Figure 20.

The box is designed in a modular fashion so that it can be removed from the system, allowing analysis of fluorescence only. The light scatter detection circuitry and lens can be removed from the box to allow entry of the expanded laser beam for wide-angle illumination, as described earlier in this chapter.

Typical scatter preamplifier output (Figure 25) is a positive pulse, a few volts in magnitude, with a short rise time and a relatively long fall time (~1usec and ~6usec, respectively, for 10 μm diameter uniform particles). The preamplifier output is fed to either a linear or a logarithmic amplifier, depending on the degree of heterogeneity of the pulses being analyzed. Special features of the logarithmic amplifier include selection of two logarithmic ranges: 3 decades or 1.5 decades, allowing signals differing in amplitude by factors of 1000 or about 32, respectively, to be fitted into a 0 to 10 V output range. An attenuator at the input of this amplifier provides linear scaling of pulses prior to logarithmic amplification. After amplification, whether it be linear or logarithmic, the light scatter signals are fed to a PS&H circuit.

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1Model 1415 Linear Amplifier, Canberra Industries, 45 Gracey Avenue, Meriden, Connecticut 06450.

2Model 8139 Logarithmic Amplifier and PS&H, Drawing No. 4Y-168329. Los Alamos Scientific Laboratory, Los Alamos, New Mexico 97545.
The three data parameters, the amplified fluorescence, the integrated fluorescence, and the amplified light scatter, each feed an identical PS&H circuit.\(^1\) One of the optical parameters is used as an external trigger for each of the PS&H circuits, and is fed through a discriminator to a connector at the rear of each chassis. When the trigger pulse is received at each PS&H circuit, the three corresponding electrical pulses (i.e., all three from the same particle) are accepted by the PS&H's. The peak voltage level of each pulse is held until it has been multiplexed, digitized, and moved to computer storage, as discussed in the next section. Once the signals are stored in computer memory, a clear-hold pulse is sent back to each PS&H from the computer. This pulse readies the circuits to accept another trigger signal.

\(^1\)Model 8139 Log amp and PS&H, Drawing No. 4Y-168329. Los Alamos Scientific Laboratory, Los Alamos, New Mexico. 97545.
Thus, as a prerequisite for computerized data acquisition, at least one optical signal, the one used as the trigger signal, must be present for each particle. If the light scatter signal is chosen to be the trigger, the optical signals from every cell will be stored in the computer for analysis, since every particle scatters light. If however, a fluorescence signal is the trigger source, only optical signals from cells which yield fluorescence will be analyzed. If it is important to quantitate the total number of particles interrogated, or to calculate the fraction of fluorescent cells, light scatter should be used as the trigger. To accurately analyze the data, the experimenter must be aware of the particles not included in data acquisition as a result of external trigger selection.

Computerized Data Acquisition and Analysis

A PDP-11/45 minicomputer with 80K words of core memory is used for data acquisition, analysis, and display. The operating system for the PDP-11/45 is RSX-11M V02. This multi-user operating system allows the use of many peripherals. The peripherals used in these experiments are the 4010-1 Tektronix Computer Display Terminal, a model 1110 Versatec Line Printer, two Control Data Corporation (CDC) model 9427H cartridge disk drives, and a RX11 Flexible Disk Controller.  

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1 Digital Equipment Corporation, Maynard, Massachusetts 01754.
2 Tektronix, Inc., Beaverton, Oregon 97005.
3 Versatec, Inc., Cupertino, California 95014.
4 Control Data Corporation, Minneapolis, Minnesota 55401.
The computer interface used is the DRll-B, a direct-memory access (DMA) interface to the PDP-11 Unibus. The DRll-B moves the data between the Master Control Unit (Figure 25) and the core memory, rather than using program controlled transfers. A specialized device driver, DRDRV, was written for the DRll-B, to accommodate peculiar interactions of the interface with the Master Control Unit (Crowell, 1977a). DRDRV and MLS, the data acquisition code (Crowell, 1977b), are written in assembly language to minimize data acquisition time.

A block diagram of the hardware involved in data acquisition is shown in Figure 26, and the corresponding time diagram is shown in Figure 27. The initialization of the appropriate bit patterns in each of the four DRll-B registers is set up by the DRll-B device driver interacting with MLS.

The signal chosen to be the external trigger is fed to the discriminator input. A signal exceeding the discriminator threshold represents an actual event which should be accepted by the computer as data, and serves as the external trigger to the PS&H circuits. The PS&H sampling (Gl Sample in Figure 27), the held multiplexer (MUX) levels, and the analog-to-digital conversion are under direct control of the Master Control Unit, which interacts with the DRll-B interface. The Go causes the PS&H output to be sampled, held, and fed to the MUX. The first MUX level (representing the amplified fluorescent signal)
Figure 26. Block diagram of data acquisition hardware.
Figure 27. Timing diagram for the Master Control Unit shown in Figure 26.
Event signal

Total count out

G1 Sample

G2 Hold

Multichannel analog hold levels to MUX

Start convert

End of conv (EOC)

Cycle request

End cycle

MUX settle time

DRWC overflow

Reset

Conv time
eq

Computer storage

n th Cycle

DRWC decrements

Inhibits start con
is then digitized by the fast analog-to-digital (ADC) converter. At the end of conversion, the DRll-B takes control of the computer bus via a non-processor request (NPR) and moves one byte of data to core memory. The DRWC (the DRll-B word count register) is then decremented. By the same procedure, the second and third MUX levels (integrated fluorescence and amplified light scatter) are digitized and moved to memory via NPR's. The third transfer is represented as the \( n^{th} \) cycle in Figure 27, after which the DRWC overflows, causing the interrupt to begin execution of the MLS data acquisition service routine. This routine checks to see if the core memory buffer is full or if any errors have occurred. If neither is true, the DRWC is reloaded and data acquisition is re-initiated by the interface. This causes three more NPR data transfers to occur, moving one more event to core memory. This cycle continues until one core buffer is filled with data.

Core data storage is double-buffered in units of 512 events, allowing one buffer to fill while the second is written to a raw data file on the magnetic disk. Disk storage is divided into blocks of 512 bytes. The disk files created by MLS are contiguous, and four blocks are required to store the contents of one core memory buffer. Again, the advantage is reduction in data acquisition time since contiguous files are more rapidly accessed than non-contiguous files. During data acquisition, the number of blocks or events already on the

\(^1\) Datel Systems, Model ADC-G10B-2A.
Data analysis is accomplished with a FORTRAN code called STAT4 (Salzman et al., 1978). This code operates on four-parameter data, generating pulse height distributions of specified disk raw data files. Each pulse height distribution is a plot of the number of counts versus channel number. For each distribution, the channel number is proportional to the signal intensity of the optical parameter being measured. Since the gain-linearity characteristics of the electronics components have been determined, one can state that the integrated fluorescence output and the linearly amplified light scatter signals are directly proportional to channel number.

Such a direct relationship does not necessarily exist for the amplifier output. For example, two particles may have the same peak fluorescence value, but because of differences in cell size, the total fluorescence yield is not equal. Thus, interpretation of amplifier output distributions must be done carefully, and for routine analysis of fluorescence from a variety of particles, the integrator output should be used as the truly proportional measure of total cellular fluorescence. If the logarithmic amplifiers have been used for the light scatter signals, the channel number and the signal intensity can be correlated since each amplifier has been characterized.

Features of the routine STAT4 include selection of full scale count, integration of counts between two channels marked by a set of terminal cursors, and also gated analysis, in which a region of
interest is selected on one of the four distributions by marking the channel on either side with a cursor. Then the raw data are reprocessed; and only events which include counts in the region of interest of the selected distribution contribute to the generation of four new pulse height distributions, allowing correlation of multi-parameter data.
CHAPTER IV. SYSTEM CHARACTERIZATION WITH STANDARD PARTICLES

Commercial standard fluorescent 10 μm diameter polystyrene spheres\(^1\) were used for system alignment, characterization, and optimization. The spheres are excited at 488 nm and fluoresce in a band from 520 to 620 nm.

Four cylindrical lenses with focal lengths of 100 mm, 200 mm, 250 mm, and 300 nm were used independently for horizontal focusing of the laser beam during fluorescence measurements of spheres, to determine the effect of various beam intensity profiles on fluorescence intensity and resolution. The corresponding theoretical data are given in Chapter II. Table 2 summarizes the experimental results. The coefficient of variation\(^2\) (c.v.) of the fluorescence distribution was used as a measure of resolution of fluorescence intensity. As the resolution improves the c.v. decreases. The c.v. obtained when no horizontal-focusing lens was used is 35% greater than that produced when a lens is inserted, due to a low power density and photon limiting. The mean channel of the fluorescence peak increased with decreasing focal length, due to higher power densities at the illumination point, and the fluorescence resolution improved relative to the no lens case. The c.v. did not improve as the focal length decreased, presumably

\(^1\)Lot 32C, Coulter Electronics, Inc., 590 W. 20th Street, Hialeah, Florida 33010.

\(^2\)Coefficient of variation = \(\frac{\text{Full width at half maximum}}{\text{Mean channel} \times 2.35}\).

See Appendix C for derivation.
Table 2. Effect of horizontal-focusing lenses on fluorescence intensity and resolution.

<table>
<thead>
<tr>
<th>Focal length (mm)</th>
<th>Fluorescence mean (channel number)</th>
<th>c.v. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>134</td>
<td>3.7</td>
</tr>
<tr>
<td>100^a</td>
<td>178</td>
<td>2.4</td>
</tr>
<tr>
<td>200</td>
<td>241</td>
<td>2.6</td>
</tr>
<tr>
<td>250</td>
<td>228</td>
<td>2.4</td>
</tr>
<tr>
<td>300</td>
<td>173</td>
<td>2.4</td>
</tr>
</tbody>
</table>

^aThe 100 mm lens was placed 120 mm from the paraboloidal focal point because of space limitations near the chamber.

because the fluorescence emission was intense, so that photon statistics no longer played a dominant role in determining the c.v. The 100 mm lens was out of focus during use, due to space limitations near the paraboloidal chamber, and the particles flowed through the beam beyond its focal point. Here the power density was less than maximum, but the c.v. did not increase. Two conclusions can be drawn from these results. First, with a horizontal focusing lens, the paraboloid is not photon limited when analyzing brightly fluorescent spheres, because the resolution did not improve with increasingly intense fluorescence. Second, the particle stream must be narrow and spatially well-defined, because the c.v. did not increase with shorter focal length lenses. (See Chapter II for a discussion of these principles.) The second conclusion agrees well with particle stream measurements, reported in the next section.
Fluid discharge, $Q$, in units of volume per time, was measured so that flow velocities and Reynolds numbers could be calculated, at several points in the system. $Q$ for the assembled paraboloid was determined by measuring fluid discharge from the exit tubing, just prior to the waste collector. An average $Q$ of 0.09 m$^3$/sec was obtained, for ten measurements. The smallest constriction in the entire fluid system is the 150 μm diameter orifice in the fluid entrance assembly, and by the continuity equation, $Q$ at the 150 μm orifice is also equal to 0.09 m$^3$/sec. The resultant flow velocity and Reynolds number at the orifice are 5.3 m/sec and 795. All calculations were carried out assuming an ambient temperature of 20°C (68°F). This value for Re is well within the region of laminar flow.

Flow through the entrance assembly while detached from the paraboloid block was characterized by measurements of $Q$ at 3 points, labeled 1-3 in Figure 28. All measurements were performed at unchanged pressure settings, and at the original heights so that there would be no change in hydrostatic pressure. Table 3 summarizes the results. The flow rate, $Q$, decreases from points 1 through 3. The most severe reduction in $Q$ occurs between points 2 and 3 with the addition of the orifice. This indicates that the orifice itself is the most resistant factor in this fluid system, offering more resistance to flow than even the quiescent water in the paraboloid (compare points 3 and 4). For the same flow rates, Re is much greater at the orifice than within the entrance tubing (point 1), although all values for Re are well within the laminar range.
Figure 28. Flow through the entrance assembly in ml/sec is measured while detached from the paraboloid block at the following points: 1, the sheath inlet tubing; 2, the main bore, without orifice attached; and 3, the orifice.
DRILL & C'BORE FOR
#6 SOCKET HD. SCREWS
- 4 PLACES

GUIDING STAR
PRESS FIT

SCALE: 4 = 1
The effect of sample tube height within the entrance assembly, on fluorescence intensity resolution was determined by acquiring fluorescence data from standard spheres for various tube positions. The sample tube was moved manually between experiments in vertical increments of 3 mm. During this process, the sample flow was maintained and no perturbation on fluorescence pulses (viewed on the oscilloscope) was observed, indicating laminar conditions. The results are given in Table 4. A 250 mm focal length lens was used for horizontal focusing for all experiments. The effect of sample tube height was usually observed through a microscope focused on an ink stream. Even during sample tube movement, no perturbation of the ink stream was seen. These results indicate that resolution is independent of tube height for a variation in height of 9 mm. It can be deduced that flow is laminar in the area of the nozzle, otherwise the resolution would have decreased as the tube was extended into the nozzle. Extending the sample tube further into the converging portion of the nozzle without altering operating conditions.
Table 4. Effect of sample tube height on fluorescence resolution.

<table>
<thead>
<tr>
<th>Tube Height Above Orifice (mm)(^{a})</th>
<th>c.v. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0(^{b})</td>
<td>2.5</td>
</tr>
<tr>
<td>6.0</td>
<td>2.5</td>
</tr>
<tr>
<td>9.0</td>
<td>2.5</td>
</tr>
<tr>
<td>12.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\(^{a}\)Measured from the inside edge of the 0.5 mm long orifice.

\(^{b}\)This is 0.5 mm into the converging portion of the nozzle.

pressures caused flow problems due to increased blockage of sheath fluid. During normal operation, the sample tube is kept at a height of about 6.0 mm.

Measurements of the sample stream diameter at the paraboloid's focal point were made with a calibrated reticle in the microscope's eyepiece. Once again ink was used as the sample so that it was optically visible. A sample stream diameter of 6.0 ± 0.5 μm was measured for sample tube heights of 3 mm through 12 mm. The spatial positioning of the sample did not change with time, and the stream diameter was stable even after one hour, at which time flow was shut off.

The paraboloidal flow cytometer has a very high collection efficiency, compared to all other types of flow systems. The real advantage of such a highly efficient system in measuring faintly fluorescent particles was determined by signal-to-noise measurements made
for various PMT and amplifier gains at several levels at laser power, with and without sample flow. The output of the 8124 amplifier was fed to an RMS (root mean square) voltmeter to determine the electronic "noise," and to the oscilloscope where the peak "signal" voltage could be observed and measured.

With no laser beam through the paraboloid, and for an 8124 gain setting of 10 (minimum), the RMS noise was measured for PMT voltages of 100 V through 1000 V. The RMS noise was 0.00125 V ± .00005, independent of PMT voltage, indicating that the PMT is a good means of signal amplification. For a PMT voltage setting of 500 V, the RMS noise was measured as a function of 8124 amplifier gain. The results, given in Table 5, show that the noise increases linearly from 0.0012 V to 0.010 V for an increase in gain from 10 to 100. These measurements show that the noise is amplified at the same rate as the signal, and clearly can not be used to improve the s/n ratio.

With the beam passing through the paraboloid, the effect of laser power on the s/n ratio was determined by making several measurements at laser power levels of 3, 50, 110, 240, 420, and 780 mW. These power levels were arbitrarily chosen to give data throughout the power range of the laser. The 488 nm line was used for all measurements, and the laser was in the light regulated mode during each experiment. Laser power was measured with a power meter\(^1\), calibrated at each power range.

\(^1\)Model 36-0001, Scientech, Inc., 5649 Arapahoe, Boulder, Colorado 80303.
Table 5. RMS noise as a function of 8124 amplifier gain. Laser off, PMT voltage = 500 V. The RMS noise increases linearly with 8124 gain.

<table>
<thead>
<tr>
<th>8124 Gain</th>
<th>RMS Noise (V)</th>
<th>8124 Gain</th>
<th>RMS noise (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.0012</td>
<td>60</td>
<td>0.0062</td>
</tr>
<tr>
<td>20</td>
<td>0.0020</td>
<td>70</td>
<td>0.0073</td>
</tr>
<tr>
<td>30</td>
<td>0.0032</td>
<td>80</td>
<td>0.0083</td>
</tr>
<tr>
<td>40</td>
<td>0.0042</td>
<td>90</td>
<td>0.0093</td>
</tr>
<tr>
<td>50</td>
<td>0.0052</td>
<td>100</td>
<td>0.010</td>
</tr>
</tbody>
</table>

using an equivalent resistive circuit \((P = I^2R)\). The results are given in Table 6, and the signal-to-noise ratios, as a function of PMT voltage, are plotted in Figure 29. Signals are from the 10 μm spheres.

No definite trend in s/n is seen for increasing laser power, because of antagonistic effects. First, an increase in power results in increased signal magnitude for given PMT voltage, due to increased fluorescence excitation energy. However, with increased laser power, the intensity of light which is scattered by undesired particles and collected by the paraboloid also increases, as well as background scattering from water molecules and debris in the sheath fluid. Since the 488 nm blocking filters are not perfect, the amount of background "noise" generated, and subsequently amplified, by even a small amount of transmitted 488 nm light, increases with laser power. For the standard spheres, an optimum s/n value of 2500 was obtained at 50 mW for a PMT voltage of 600 V. It is believed that for faintly fluorescent
Table 6. Noise and signal measurements for various laser power levels and PMT voltages. 8124 amplifier gain = 10 for these experiments.

<table>
<thead>
<tr>
<th>Laser Power (mW)</th>
<th>PMT (V)</th>
<th>No Flow (V)</th>
<th>Sheath (V)</th>
<th>Sample (V)</th>
<th>Signal (V)</th>
<th>s/n</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>400</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0014</td>
<td>0.04</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0014</td>
<td>0.30</td>
<td>214</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0017</td>
<td>1.1</td>
<td>647</td>
</tr>
<tr>
<td></td>
<td>700</td>
<td>0.0013</td>
<td>0.0012</td>
<td>0.0018</td>
<td>3.2</td>
<td>1778</td>
</tr>
<tr>
<td>50</td>
<td>400</td>
<td>0.0016</td>
<td>0.0016</td>
<td>0.0040</td>
<td>9.0</td>
<td>2250</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0015</td>
<td>1.3</td>
<td>867</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0020</td>
<td>5.0</td>
<td>2500</td>
</tr>
<tr>
<td>110</td>
<td>400</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0018</td>
<td>0.4</td>
<td>222</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0024</td>
<td>2.0</td>
<td>833</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0070</td>
<td>7.6</td>
<td>1086</td>
</tr>
<tr>
<td>240</td>
<td>400</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0013</td>
<td>0.9</td>
<td>692</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0023</td>
<td>4.4</td>
<td>1913</td>
</tr>
<tr>
<td>420</td>
<td>400</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.004</td>
<td>1.3</td>
<td>325</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.005</td>
<td>6.2</td>
<td>1240</td>
</tr>
<tr>
<td>780</td>
<td>400</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.010</td>
<td>2.0</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.015</td>
<td>9.0</td>
<td>600</td>
</tr>
</tbody>
</table>
Figure 29. The signal-to-noise ratio at the fluorescence amplifier obtained at various laser power levels is plotted as a function of PMT voltage.
biological particles, until an improved means of blocking scattered light is implemented, relatively low laser power levels should be used, with signal amplification obtained by increased PMT voltage.

Several conclusions can be made based on the s/n measurements. The laser beam itself does not increase "noise." It is when light is scattered and efficiently collected by the parabolic surface, that the noise levels increase. A solution is improved blockage of the laser wavelength prior to the PMT. When faintly fluorescent pulses which have a low s/n ratio must be amplified, the PMT voltage should be increased rather than the 8124 gain level. This is because the 8124 amplifier amplifies the signal and the noise by the same factor, thus not improving the s/n ratio obtained. As shown in Figure 29, it is possible to improve the s/n ratio, by carefully choosing optimum PMT and laser power levels.

Unfortunately, no signal to noise measurements have been reported for other flow cytometers, making it difficult to assess the s/n enhancement due to the paraboloid's high collection efficiency. It is obvious that since the paraboloid is more efficient, less electronic amplification of signals and less intense laser light is needed to obtain detectable pulses, thus lowering these noise components relative to less efficient cytometers.

Two independent collaborative studies have used the paraboloidal chamber to detect low levels of intracellular adriamycin within tumor cells (Tokita et al., 1979), and to detect direct and indirect
immunofluorescence from specific antigens on mouse testicular cells. Both studies have demonstrated and relied upon the enhanced s/n capability of the paraboloid, since in some cases the signals could not be resolved from the noise with orthogonal systems.

Standard spheres were analyzed by the paraboloid in the wide-angle illumination mode. No modifications were made in the fluorescence collection optics, and only one combination of lenses was used for divergence and recollimation of the laser beam. Fluorescence pulses about 6 μsec in duration were obtained, as measured on the oscilloscope screen, indicating that the illumination "spot" was comparable in diameter (vertically) to the spot used in the focused-beam case. With more care in alignment of the beam diverging and recollimation lenses, spread in signal intensities did decrease, but the signal to noise ratio was approximately 2. The major problem was high background light levels present at the PMT due to the intense laser beam exiting the chamber through the aperture, after excitation of particles at the focal point. It is believed that this approach can improve fluorescence resolution, and should be attempted again when improved blockage of the laser wavelength can be achieved. A monochromator or more effective optical filtration between the paraboloid and the PMT are possible solutions. Due to the low s/n ratio, this method of illumination was not attempted with biological particles.

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CHAPTER V. BIOLOGICAL MATERIALS AND METHODS

The paraboloidal flow cytometer with focused-beam illumination was used in these experiments to measure the DNA content of mouse testicular cells and isolated testicular cell nuclei. This chapter describes the biological system and cell preparation for flow analysis. Results are given in Chapter VI.

Introduction

A better understanding of male infertility, contraceptives, animal breeding, mutagenesis (Allen and Latt, 1976; Meistrich et al., 1978a), and the effects of drugs and radiation, can be obtained through increased knowledge of spermatogenesis. In addition, spermatogenic cells serve as a model for the study of cell differentiation, because of the unique and rapid progression of cells through mitosis, meiosis, differentiation and maturation (Lam et al., 1970; Meistrich and Eng, 1971).

Testicular cells are conventionally studied by microscope observations of fixed sections, stained by the hematoxylin-eosin or the periodic acid-Schiff (PAS) reactions. Based on cellular morphology, location within the tubule, and nuclear shape, the frequency of occurrence of specific cell types can be determined. However, these studies are time consuming, and qualitative, rather than quantitative, in nature. Biochemical studies of enriched samples of
specific testicular cell types were made possible by separation of dispersed testicular cells on the basis of differential sedimentation at unit gravity (Go et al., 1971; Lam et al., 1970; Loir and Lanneau, 1977; Meistrich et al., 1973).

The first applications of flow cytometry to the analysis of testicular cells were the fluorescence measurements of DNA stained by the acriflavin-Feulgen procedure (Meistrich et al., 1978b, using an orthogonal flow cytometer), ethidium bromide and mithramycin (Zante et al., 1977, using a wide-angle flow system), or propidium iodide (Libbus and Schuetz, 1978, using an orthogonal system). Three fluorescence populations, corresponding to haploid, diploid, and tetraploid cells, were obtained in each case. Zante et al. (1977) first reported the use of enzymatic treatments (pepsin, pronase and papain) to improve the stainability of maturing spermatids in testicular cell preparations. Researchers at Lawrence Livermore Laboratory (Meistrich et al., 1978b) were the first to measure the DNA content of isolated testicular nuclei, obtaining resolution of elongated from round spermatids, as well as diploid and tetraploid cells. The orthogonal geometry of their flow cytometer, however, introduced artifacts when measuring the highly asymmetric elongated spermatids.

The techniques presented in this dissertation to prepare mouse testicular cells for flow analysis are modifications of the previously discussed methods. It was found that by enzymatically treating isolated testicular nuclei, resolution of elongated, elongating, and round spermatids, as well as diploid and tetraploid nuclei, is possible with the paraboloidal flow cytometer.
Mouse Testicular Cells

Testes are the site of spermatogenesis, the production of male germ cells. In mice, testes are normally located in the scrotum, an extension of the peritoneal cavity (Figures 30 and 31). Each testis is covered by a fibrous capsule, the tunica albuginea, from which thin septa project into the organ, dividing it into lobules, each supporting several convoluted tubules within which spermatogenesis occurs. The interstitial stroma contains blood and lymph vessels and the cells of Leydig, which secrete testosterone. Excellent reviews dealing with testicular histology are given by Dym (1977) and Bloom and Fawcett (1968, Chapter 31).

Convoluted tubules are lined with seminiferous epithelium, composed of Sertoli cells and spermatogenic cells. The Sertoli cells give structural support to the proliferating spermatogenic population, and are thought to play a role in their nutrition.

Spermatogenesis is the sequence of events by which spermatogonia produce spermatocytes, which divide meiotically, yielding early spermatids. Spermiogenesis refers to the subsequent stages of spermatid maturation. The cell sequence is diagrammed in Figure 32. In tissue section the cell types can be identified by their shape, nuclear staining, and location within the seminiferous tubules. A photomicrograph of a mouse testicular section is shown in Figure 33. In order of occurrence from the periphery to the lumen of the tubule are spermatogonia, spermatocytes, and increasingly mature spermatids. In rodents, eight types of spermatogonia have been described (Dym, 1977) each
Figure 30. Male reproductive organs in the mouse (Cook, 1965, p. 75).
Figure 31. The right mouse testis and epididymis (Cook, 1965, p. 76).
Figure 32. Cells of the mouse testis. Mitotic and meiotic divisions are indicated by m and M, respectively (Meistrich, 1977, p. 16).
Figure 33. This mouse testicular cross-section is stained by the PAS-hematoxylin method described in this chapter. Portions of three tubules are seen in cross-section. Spermatogonia, spermatocytes, round spermatids, and elongated spermatids are visible.

resulting from the mitotic division of the preceding type. All spermatogonia are diploid cells, containing the normal somatic complement (2C) of DNA. The intermediate (In) and all successive cell types are differentiated and dedicated to the production of spermatocytes.

The preleptotene spermatocytes (Pl) do not divide mitotically, but move away from the periphery of the tubule, accumulating cytoplasm. The successive spermatocytes correspond to the stages of meiosis, and are characterized by their chromatin structure. The first meiotic
division results in secondary spermatocytes, which have a haploid (1C) DNA content, and are physically smaller than preceding spermato­
cytes. Secondary spermatocytes are short-lived, proceeding to the second meiotic division which gives rise to the earliest spermatids.

The division of cells in spermatogenesis is unusual in that there is not complete cytokinesis, so that the daughter cells remain connected by intracellular bridges (Fawcett et al., 1959; Krishan and Buck, 1965). This phenomenon is first exhibited in the division of type B sperma­
togonia and persists through the divisions of spermatocytes. The exact function of the bridges is not fully understood, nor is the process by which mature spermatids become separated and released into the lumen. During mechanical dispersal some cells remain connected by intracellular bridges. A higher than normal number of multinucleated cells and cell clumps can also be observed in such preparations. Their occurrence precludes individual measurements of nuclear DNA content and inter­
eres with quantitation of normal diploid and tetraploid cells. One solution to these problems is analysis of isolated testicular nuclei. More accurate results would be obtained because the fluorescence measured is a true indicator of DNA content per nucleus.

In the mouse, spermiogenesis can be divided into sixteen steps (Figure 32). Four groups are easily distinguished based on nuclear and cytoplasmic shape. (Meistrich et al., 1973). Early spermatids (steps 1-8) are roughly spherical, and contain a relatively small, round, lightly staining nucleus. Elongating spermatids (steps 9-10) exhibit nuclear asymmetry, and the nucleus may protrude from the cyto­
plasm. Increasing asymmetry is seen in steps 11-13, characterized by less
cytoplasm, and a hook-shaped nucleus, similar to a mature spermatozoa. In steps 14-16, maturation of the spermatid continues with little change in nuclear shape. Step 16 spermatids closely resemble fully mature spermatozoa, having a hook-shaped head and flagellum.

The percentages of various cell types occurring in mechanically dispersed mouse testicular suspensions were determined on the basis of cellular morphology, by Meistrich et al. (1973). Many cells were damaged during dispersal, resulting in 53% cytoplasmic fragments and residual bodies, and about 2% multinucleated cells. Taking into account only cells which were intact, about 2% spermatogonia, 4% spermatocytes, 27% round spermatids (steps 1-8), 19% elongating spermatids (steps 9-13), 40% elongated spermatids, 5% multinucleated cells, and 3% non-spermatogenic cells were observed. A significant loss of spermatogonia, primary spermatocytes, Sertoli, and Leydig cells, relative to spermatids, in the suspensions was noted.

Hacker et al. (1979), classifying suspended mouse testicular cells with respect to DNA content, found that about 64-72% are haploid (~ 40% round spermatids and 24-32% other spermatids), 12-17% are diploid, 3-7% are in S phase, and 10-13% are tetraploid. These data serve as a basis of comparison for the results given in Chapter VI.

DNA Fluorochromes

The DNA of dispersed mouse testicular cells and nuclei was stained with two fluorochromes, propidium iodide (PI) and mithramycin (MT), for quantitation by the paraboloidal flow cytometer. PI is an analog of
ethidium bromide (Hudson et al., 1969), binding specifically to double-stranded nucleic acids by intercalation between adjacent base pairs (Le Pecq and Paoletti, 1966; Pakroppa et al., 1975; Waring, 1970). MI is an antibiotic drug with high specificity for DNA, but not RNA (Ward et al., 1965), and is thought to bind on the surface of the DNA helix (Waring, 1970). Therefore, successive staining with PI and MI yields efficient use of internal and surface binding sites. A good review of these, and other DNA fluorochromes for flow cytometry, is given by Crissman et al. (1979).

Laser wavelengths used for excitation of MI and PI, are 457 nm and 488 nm, respectively. Emission spectra for each dye alone, in combination, and with calf thymus DNA or albumin are shown in Figure 34. (An Aminco-Bowman Spectrophotofluorometer\(^1\) was used to obtain all spectral data.) The dyes in a Triton X-100 solution were also analyzed, since Triton X-100 was used in stain solutions to obtain isolated nuclei in some of the experiments.

Spectral results show no significant binding to protein for any of the dye combinations. When PI was bound to DNA the emission maximum was shifted from about 640 nm (unbound dye) to 620 nm. This was identical to the results with PI and MI in combination. When MI alone was bound to DNA no spectral shift in the emission was seen. These results are consistent with the belief that energy transfer takes place when the dyes are used together (Zante et al., 1976; Barlogie et al., 1976).

\(^1\)American Instrument Company, Silver Spring, Maryland.
Figure 34. Spectral emission curves of propidium iodide (PI), mithramycin (MI), or both (PI + MI), with (left), or without (right), Triton X-100 present, in combination with DNA (A), protein (B), or the dye alone (C).
When excited at 457 nm, MI fluoresces in a spectral region which overlaps the PI excitation band. The result is enhanced fluorescence emission from nuclear DNA, where PI and MI are bound in close proximity so that energy transfer takes place. Thus fluorescence resulting from non-specific staining, such as PI stained RNA, is eliminated, thereby enhancing the resolution of DNA measurements.

**Preparation for Flow Analysis**

Mature male mice (Simonson, strain BDF₁) between 6 and 18 months of age were euthanized by cervical dislocation. For each experiment, two mice of the same age were used. The testes and spleens were excised and mechanically dispersed through coarse wire mesh into chilled saline GM + SDTA (composition of this solution is given in Appendix B). The cell solution was transferred to a conical tube, and centrifuged at 350 g for five minutes. The pellet was resuspended in 1.5 ml chilled saline GM + EDTA, and bulbed (repeatedly drawn into and ejected from a pipette) until no clumps were seen. Samples were withdrawn from this "stock" solution for staining and flow analysis.

After the stock sample and freshly prepared pepsin¹ (5 mg/1 ml in HCl, pH = 1.8) were warmed to 37° C, 1.5 ml of the pepsin solution was added to the stock tube, and the mixture was stirred frequently. One of the four following procedures was used to enzymatically treat and stain the testes samples:

¹Serva, Feinbiochemica, Heidelberg, West Germany.
Method 1: Ten drops of the stock sample were withdrawn after 0, 5, 10, 15, 20, and 30 minutes in pepsin, and placed in 4.0 ml of MI (100 mg/ml MI, 15 mM MgCl₂, in normal saline).

Method 2: Ten drops of the stock sample were withdrawn after 0, 8, and 10 minutes in pepsin, and placed in 2.5 ml PI (25 μg/ml PI, 0.1 M Tris, 0.1 M NaCl, in distilled H₂O). After at least 5 minutes, 2.5 ml MI (50 μg/ml MI, 7 mM MgCl₂, and 12.5% EtOH, in Normal saline), and about 3 drops of RNAse (1 mg/ml, pH = 7.4) were added to each sample. This staining procedure is a slightly modified version of Meistrich et al. (1978c), where ethidium bromide rather than PI was used, and pepsinization was performed at room temperature rather than 37°C, thus requiring longer treatment times.

Method 3: Isolated nuclei were obtained using Triton X-100 in a hypotonic solution which will hereafter be called the Triton X-100 solution (5 mM MgCl₂ • H₂O, 5 mM NaH₂PO₄ • H₂O, and 0.25% Triton X-100 (v/v) in distilled water, Meistrich et al., 1978b). Ten drops of stock sample were withdrawn at one minute intervals, from 0 to 10 minutes in pepsin, and placed in 2.5 ml PI in Triton X-100 (25 μg/ml PI in Triton X-100 solution). After at least five minutes, 2.5 ml MI

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1 Pfizer, Inc., 235 East 42nd Street, New York, New York 10017.
2 Calbiochem, San Diego, California.
4 Lot 17C-0241, No. T-6878, Sigma Chemical Co., P. O. Box 14508, St. Louis, Missouri 63178.
(50 µg/ml MI in Triton X-100 solution), and three drops of RNase were added to each tube. Triton X-100 affects the nuclear membrane, in addition to its role in cell lysis. Membrane permeability, and therefore the stainability of the condensed spermatids is probably increased.

Method 4: Four drops of stock sample were placed in 2.5 ml of each of the following staining solutions, without prior pepsinization: 25 µg/ml PI in Triton X-100 solution (the amount of MgCl₂·6H₂O was increased to 15 mM); 50 µg/ml MI in Triton X-100 solution; equal volumes of 25 µg/ml PI and 50 µg/ml MI in Triton X-100 solution; 25 µg/ml PI in a solution (15 mM MgCl₂·6H₂O and 5 mM NaH₂PO₄ in distilled H₂O) which henceforth will be called the hypotonic solution; 50 µg/ml MI in hypotonic solution; and equal volumes of 25 µg/ml PI and 50 µg/ml MI in hypotonic solution. The hypotonic solution is the same as the Triton X-100 solution, except that the Triton X-100 is deleted. All samples are filtered (44 µm diameter pore size), held at 0° C, and analyzed by the paraboloidal flow cytometer within four hours of staining.

Identification of Subpopulations

Flow measurement of the DNA content of testicular cell nuclei yielded multi-peaked distributions (see Chapter VI). Confirmation of the cellular composition of the various haploid peaks was essential,

1M. L. Meistrich, Section of Experimental Radiotherapy, M.D. Anderson Hospital, Houston, Texas. Private communication. 1978.
and was accomplished by the fluorescence analysis of enriched spermatid populations.

Enriched populations of round spermatids (steps 1-8), elongated spermatids (steps 9-13), and mature spermatids (steps 14-15) were obtained by velocity sedimentation through linear 1 to 4% bovine serum albumin¹ (BSA) gradients. This technique is well-established for mouse testicular cells (Meistrich, 1977; Loir and Lanneau, 1977; Lam et al., 1970), although each investigator uses slightly different gradient compositions, cell densities, and separation times.

In the experiments reported in this thesis, gradients were formed in nitrocellulose, one-inch diameter tubes with a linear two-chamber gradient former.² The left and right (exit) chambers were filled with 18.4 ml of 1% BSA in phosphate buffered saline (PBS), and 18.0 ml of 4% BSA in PBS, respectively. After each gradient was formed, 2.0 ml of mechanically dispersed testes cells in chilled saline GM + EDTA (density = 40x10⁶ / ml) was layered on top of the BSA. Each gradient was fractionated after three hours of separation at 4° C.

A density gradient fractionator³ divided each gradient into 30, 1.2 ml fractions. The fractions were numbered consecutively, starting at the top of the gradient tube. Separation was caused by puncturing

¹BSA from Fraction V Albumin. SIGMA Chemical Co., P. O. Box 14508, St. Louis, Missouri. 63178.

²Buchler Instruments, Fort Lee, New Jersey.

³Model 640, Instrumentation Specialties Company (ISCO), P. O. Box 5347, Lincoln, Nebraska.
the bottom of the gradient tube, and pumping in a 50% sucrose solution (w/w in distilled H₂O) at the rate of 3 ml/min. To insure a high density of cells per fraction, similar numbered fractions from two gradients were pooled.

A cytocentrifuge was used to deposit 0.2 ml of each fraction onto slides for microscopic identification. The slides were fixed in 95% ethanol (EtOH), and stained with periodic acid–Schiff (PAS) and counterstained with hematoxylin. (See Appendix B for details of the staining procedure). PAS is the most commonly used histological stain for testicular sections, or dispersed cells on slides, and the staining characteristics of spermatids and other testicular cells are well-documented (Meistrich et al., 1973). The depth of blue nuclear staining is indicative of the degree of condensation, and fine chromatin detail can be seen in primary spermatocytes. The hematoxylin-eosin staining procedure was used to stain one set of fractions, but was discarded due to the resultant poor nuclear detail. Photomicrographs of several fractions stained with PAS-hematoxylin are shown in Chapter VI.

Even though most types of spermatids were easily identified by their nuclear and cytoplasmic shapes, cellular morphology was affected to some extent by the methods of cell dispersal and separation. These effects are considered by Barcellona and Meistrich (1977), and Romrell et al. (1976).

The remaining volume of each fraction (2.2 ml) was prepared for flow DNA measurements with the paraboloidal chamber. About 5 ml of chilled saline GM + EDTA was added to each fraction tube, and then
centrifuged at 350 g for five minutes. Each pellet was resuspended in 1.0 ml chilled saline GM + EDTA and warmed to 37° C. Then, 0.6 ml pepsin (5 mg/ml in HCl, pH = 1.8) was added to each tube and stirred frequently. The enzymatic reaction was halted after nine minutes by adding 5 ml chilled saline GM + EDTA to each tube. All samples were centrifuged at 350 g for five minutes.

Nuclei were isolated and stained by resuspending each pellet in 0.5 ml PI (25 μg/ml PI in 0.25% Triton X-100, 5 mM MgCl₂ · H₂O, 5 mM NaH₂PO₄ · H₂O in distilled H₂O). After five minutes, 0.5 ml MI (50 μg MI in 0.25% Triton X-100, 5 mM MgCl₂ · H₂O, 5 mM NaH₂PO₄ · H₂O in distilled H₂O) and ~5 drops of RNAse were added. All samples were filtered (44 μm diameter pore size) and held at 0° C until analysis by the paraboloidal flow cytometer.
CHAPTER VI. BIOLOGICAL RESULTS AND DISCUSSION

The DNA histograms resulting from analysis of testicular cells or isolated nuclei by the paraboloidal flow cytometer are given. Each histogram is a plot of the number of cells versus fluorescence intensity, for one sample. Four different methods of sample preparation were used to determine as much as possible about the effect of pepsinization on MI or PI stained cells and nuclei, so that the method allowing the best discrimination of testicular cell subpopulations could be selected.

Fluorescence distributions from testicular cells prepared by Method 1 (pepsinization followed by MI staining) using 6 month old mice are shown in Figure 35. In each histogram haploid (1C), diploid (2C), and tetraploid (4C) populations are present, and are comprised of spermatids, G0 and G1 phase cells and clumps of two spermatids, and G2+M phase cells plus clumps of two G1 cells, respectively. The peak channels show the expected ratio 1:2:4. The data are summarized in Table 7. Cells in the S phase of mitosis account for the small population between the 2C and 4C peaks. In the control sample (0 minutes in pepsin), a peak is present with the mean in channel 28, about 72% as intense as round spermatids (mean in channel 39). This population is thought to be comprised of elongating spermatids. This agrees well with results of Zante et al. (1977) and Gledhill et al., (1966) which showed that maturing spermatids yield from 50% to 77% of the fluorescence intensity of round spermatids due to increased nuclear
Figure 35. Fluorescence distributions from testicular cells prepared by Method 1. The time in pepsin is indicated in the upper right corner of each histogram. Haploid, diploid, and tetraploid peaks are marked 1C, 2C, and 4C, respectively. The PMT voltage and 8124 amplifier gain settings were 650 V and 50, respectively. The laser output was 300 mW at 457 nm.
Table 7. Summary of data using Method 1.

<table>
<thead>
<tr>
<th>Min. in Pepsin</th>
<th>Total&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1C</th>
<th>% Cells in Population</th>
<th>C.V.&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Peak Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1C</td>
<td>2C</td>
<td>4C</td>
</tr>
<tr>
<td>0</td>
<td>35,000</td>
<td>25,200</td>
<td>21, 51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>35,000</td>
<td>26,250</td>
<td>75</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>35,000</td>
<td>26,967</td>
<td>77</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>15</td>
<td>35,000</td>
<td>26,912</td>
<td>77</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
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<td>35,000</td>
<td>27,376</td>
<td>78</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>30</td>
<td>35,000</td>
<td>27,117</td>
<td>77</td>
<td>14</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup>The total number of cells is normalized to 35,000.

<sup>b</sup>These numbers are for the two haploid populations.

<sup>c</sup>C.V. = coefficient of variation = full width at half maximum/(mean x 2.35).

<sup>d</sup>Bimodal distribution.
condensation, depending upon which enzymes and fluorochromes are used. Mature spermatids do not contribute to this population as evidenced by microscopic observations using blue excitation, which showed no detectable fluorescence from mature (elongated) spermatids in the control sample. What appears to be a population of cells at the far left side of the histogram, is actually "noise" which falls above the discriminator cut-off voltage. The percentages of elongating spermatids, round spermatids, diploid, and tetraploid cells (Table 7) in the control sample are 21, 51, 18, and 8%, respectively. These numbers are consistent with the findings of Hacker et al. (1979) and Meistrich et al. (1973) for dispersed testicular cells, given in Chapter V, after elongated spermatids are removed from the total cell count. After pepsinization, the elongating spermatids stain stoichiometrically but the mature spermatids remain unstained and do not contribute to the fluorescence histograms, evidenced independently by microscopic observations and the integrated cell counts in Table 7. The total number of cells in each subpopulation was normalized to give a total cell count of 35,000 for each histogram. The number of cells in the haploid population increased by only 3% from 0 to 5 minutes in pepsin and was constant thereafter, indicating that the elongating spermatids are still present in this population, and must therefore fluoresce as intensely as round spermatids after pepsinization. The increase in the haploid population equals the loss in the diploid and tetraploid populations due to reduced occurrence of cell clumps in the latter groups. In addition to inhibiting cell clumping, pepsin treatment significantly
reduced the noise level. These results show that elongated spermatids, which normally account for about 25% of dispersed testicular cells, were not fluorescent and thus were not measured, even after 30 minutes in pepsin.

Fluorescence resolution, indicated by the c.v. of the haploid peak, decreased up to ten minutes in pepsin, and then increased slightly. The relative intensity of MI staining also increased up to ten minutes pepsinization. Therefore, for Method 1, the ten minute treatment is optimum, but this method is not particularly useful in testicular cell analysis because the elongated spermatids are not analyzed.

Method 2 (pepsinization, and staining with PI followed by MI in 12.5% EtOH, plus RNAse) was used in an attempt to stain the DNA of elongated spermatids, and to improve fluorescence resolution. The resulting DNA histograms are given in Figure 36. Haploid and diploid populations are present, while tetraploid cells are off scale. Mice 7 months of age were used in this experiment. As for Method 1, the pepsinization eliminated fluorescent debris, and in combination with ethanol fixation (12.5%) improved the stainability of elongated spermatids, because fluorescence was observed with the microscope. But, the staining was not stoichiometric, as evidenced by the fluorescent population forming an extension to the left of the true haploid peak. The resolution, however, was much improved, relative to Method 1, the c.v. of the haploid peak being only 3.2% for 8 minutes in pepsin. The same sample analyzed with an orthogonal system (Holm and Cram, 1973), resulted in a haploid c.v. of 3.9%. The use of RNAse in
Figure 36. Fluorescence distributions from testicular cells prepared by Method 2. Time in pepsin is given in the upper right corner of each histogram. The PMT voltage and 8124 amplifier gain settings were 647 V and 28.5, respectively. The laser output was 300 mW at 457 nm.
Method 2 eliminated fluorescence from PI bound to RNA, and excitation of the MI-PI combination at 457 nm enhanced nuclear fluorescence due to energy transfer. Although this method is superior to Method 1, with respect to resolution, good stainability of mature spermatids is still not obtained.

Method 3, using Triton X-100 in a hypotonic solution to isolate testicular nuclei from 7 month old mice, did achieve stoichiometric staining of mature spermatids after pepsin treatment. The fluorescence histograms are shown in Figures 37 and 38. Six peaks were obtained for the control sample. Round spermatids (1C), diploid cells (2C), and tetraploid cells (4C) account for the peaks in channels 60, 120, and 230, respectively (the last population was partially off scale due to saturation of system electronics at the voltage corresponding to channel 232). The populations centered about channels 87 and 185 are thought to correspond to clumps of mature spermatids, and diploid cells with round spermatids, respectively. The peak centered at channel 45 is thought to consist of elongated spermatid nuclei. This was confirmed by the fluorescence analysis of populations enriched in elongated spermatids (see the next section). Microscopic observations showed brightly fluorescent mature spermatid nuclei in all samples analyzed.

The results presented above for the control sample are similar to those obtained by Meistrich et al. (1978b), using the acriflavine-Feulgen staining procedure, where the contents of each peak were identified by 1) sorting nuclei from each population during flow analysis and then observing them microscopically, and 2) by analyzing enriched
Figure 37. Fluorescence distributions from testicular cells prepared by Method 3. Time in pepsin, from 0 to 5 minutes, shown in the upper right corner of each histogram. Haploid, diploid, and tetraploid peaks are marked 1C, 2C, and 4C, respectively. The PMT voltage and 8124 amplifier gain settings were 700 V and 10. The laser output was 250 mW at 457 nm.
Figure 38. Fluorescence distributions, continued from Figure 37.
populations with a flow cytometer. His results confirm the contents of
the six peaks in the control sample (although orientation artifacts were
present for asymmetric spermatids) and support the assumptions made
regarding the populations in Figures 37 and 38.

Figures 37 and 38 show that for 0 to 10 minutes in pepsin, striking
changes occur in the fluorescent intensities of non-round spermatids,
while the intensity of the round spermatids is unchanged. It has been
shown that untreated spermatid nuclei stain less intensely with in­
creasing maturation and nuclear condensation, and that pepsinization
increases the stainability by causing decondensation. It is reasonable
to assume that the time required for decondensation to occur is directly
related to the initial state of condensation. Therefore, elongating
spermatids should stain stoichiometrically before elongated spermatids.
These relationships permit interpretation of the changes seen in the
histograms with pepsinization.

After one minute in pepsin, three major peaks are evident in the
haploid population in channels 30, 40, and 50, and are believed to
correspond to elongated, elongating, and round spermatids, in order of
increasing intensity. What may be taken for a peak in channel 10 is
actually noise (non-nuclear fluorescence) just above the discriminator
cut-off. The contents of the small peak in channel 22, comprising a
small fraction of the total population, is unknown. At 2 and 3 minutes,
the location of the elongated peak remains in channel 30, while the
elongating population, which responds more quickly to pepsinization,
has increased in fluorescent intensity, and falls under the round
spermatid peak in channel 50. The intensity of the mature spermatid population steadily increases in intensity from 5 to 10 minutes in pepsin, and this population is seen as a shoulder on the left of the round spermatid peak after 10 minutes.

The fluorescence intensity of all populations linearly decreases with increasing time in pepsin. The mean channel of the round spermatid population decreases from 60 to 50 to 40, for the control, 5 minute, and 10 minute histograms, respectively. This is thought to be due to an inhibition of PI staining by pepsin treatment, an effect in addition to decondensation, and has been observed by Krishan\(^1\) for other cell types.

Mouse spleen cell nuclei were used as a marker of the fluorescence intensity from normal diploid cells and served as basis of comparison of sample preparation. The diploid and tetraploid peaks occur at channels 72 and 144, respectively, and the c.v. of the haploid peak is 4.5%. Microscopic observations showed that the spleen sample consisted of white blood cell nuclei. Standard fluorescent spheres were also analyzed by the paraboloidal flow cytometer as an indicator of performance. A resolution of 2.4% c.v. was obtained. The same spheres analyzed by orthogonal systems (Holm and Cram, 1973) yield at best 3% c.v.

These results obtained by using Method 3 are significant in that for the first time the three major classes of spermatids can be

\(^{1}\)A. Krishan, Comprehensive Cancer Center for the State of Florida and Dept. of Oncology, University of Miami Medical School, Miami, Florida. Private communication. 1979.
resolved by flow cytometry. This provides a sensitive biological system to study spermatogenesis, and the effects of age, drugs, radiation, contraceptives, and other agents which alter the spermatogenic process.

Method 3 was also used to prepare testicular cells from older mice for analysis by the paraboloid. Figures 39, 40, and 41 are fluorescence and corresponding light scatter histograms from 10 month old mice, and Figure 42 shows similar distributions for 15 month old mice. For all samples, the PMT voltage was 550 V, the 8124 gain was 20, the laser output was 800 mW at 488 nm, and 1 1/2 decades of logarithmic amplification was used for the scattered light.

The light scatter distributions for all control samples show two broad populations. Light scatter intensity measured over a wide angle by the paraboloidal flow cytometer is primarily a function of cell size for cells of similar refractive index (Husar, 1974). Addition of pepsin decreases the number of cells in the high-intensity scatter population, and thereafter the distributions are relatively unchanged through ten minutes in pepsin. Gated analysis (Chapter 3) was performed on subpopulations in the fluorescence distributions to determine the corresponding scatter distributions. The results of gating on fluorescence distributions in histograms 0 and 2 of Figure 39 are shown in Figure 43. Gating independently on the round (top histogram) and elongated (center histogram) spermatids in the control sample showed that the high and low intensity scatter peaks correspond to round and elongated nuclei, respectively. This reflects the cell size, since round spermatids have more cytoplasm and are expected to be
Figure 39. Fluorescence (left) and light scatter (right) distributions from 10 month old mice treated for 0-3 minutes in pepsin, and stained by Method 3.
Figure 40. Same as for Figure 39, but for 4-7 minutes in pepsin.
Figure 41. Same as for Figure 39, but 8-10 minutes in pepsin, plus
distributions from spleen cells.
Figure 42. Fluorescence (left) and light scatter (right) distributions from 15 month old mice treated for 0, 5, or 10 minutes in pepsin, and stained by Method 3.
larger than elongated spermatids. There is some amount of overlap in
the scatter distributions, which may be partially due to the fact that
the elongating spermatids were present in both fluorescent groups,
and thus contributed to both scatter distributions. Results of gating
on the round and elongating spermatid peak for 2 minutes in pepsin,
are shown at the bottom of Figure 43. These nuclei have the same
scatter distribution as the entire ungated population, indicating that
pepsin treatment has altered cellular morphology. This was confirmed
by microscopic observations which showed much cytoplasm adhering to
nearly all nuclei in the control sample, but after one minute pep-
sinization, most nuclei were free from cytoplasmic debris, and by 2
minutes only isolated nuclei were observed. Therefore, round sperma-
tids had higher light scatter values in the control sample due to
the presence of cytoplasm, which was removed by subsequent pepsiniza-
tion. Pepsin treatment has little effect on the scatter properties
of elongated spermatids, since they have relatively no cytoplasm.

Fluorescence of nuclei was observed microscopically with blue ex-
citation. In all samples, mature spermatids were brightly fluores-
cent. Round spermatids were the most frequently occurring cell type
as was expected, but many other spermatids were also observed in all
samples.

Spleen cells were again used as a diploid marker. The resolution
obtained was 4.7%, quite similar to the 4.5% reported for the former
experiment, indicating the preparations are of similar quality.
Figure 43. Fluorescence (left) and light scatter (right) distributions for the control sample (0) and for two minutes in pepsin. Gating was based on the shaded portion of the fluorescence distribution. The same cell population comprises the shaded portion in each pair of histograms.
In general, the fluorescence distributions from older mice (Figures 39, 40, 41, and 42) are very similar to those obtained from young mice (Figures 37 and 38). This implies that the mice at ages up to 15 months (oldest mice used) are still fertile, with elongated and round spermatids shown in Figure 42. These results disagree with those of Zante et al. (1977), which stated that "aged" mice have round spermatids but no elongating or elongated spermatids, present in testicular preparations. Their results are inconsistent with the known pathway of maturation in the testes, where round spermatids mature into elongating and finally elongated spermatids. The time course of this maturation process does not become altered with age of the animal, and if round spermatids were present, there should have been increasingly mature spermatids also.

Method 4 was used to determine the effect of Triton X-100 on fluorescence results, and to compare DNA fluorescence from the three dye combinations. Figure 44 shows that although the basic shapes of the histograms are the same for a given stain, samples treated with Triton X-100 are better resolved than samples prepared in the hypotonic solution only. Microscopic observations revealed that only isolated nuclei were present in all samples, but that the samples without Triton X-100 contained more cytoplasmic debris, both in association with nuclei, and throughout the suspension.

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1 D. W. Fawcett, Hersey Professor of Anatomy, Harvard Medical School, Boston, Massachusetts. Private communication. 1979.
Figure 44. Fluorescence distributions from testicular cells prepared by Method 4. BOTH implies the MI-PI combination. Histograms resulting for Triton X-100 treatment (left), as well as hypotonic treatment (right) are shown.
Samples stained with MI alone yielded only 3 peaks, corresponding to haploid, diploid, and tetraploid DNA content. However, when PI was used alone, the results were essentially identical to those obtained for the MI-PI combination, except that the spermatids are not as well-resolved. MI, PI, and the MI-PI combination were excited at 457 nm, 488 nm, and 457 nm, respectively. Therefore, the MI-PI combination should be used preferentially over MI or PI for staining isolated testicular nuclei, when resolution of the spermatid sub-populations is required. At this time, it is not known why MI and PI give different results, but a probable explanation can be made.

Mithramycin binds to the surface of the DNA helix and appears to bind stoichiometrically to isolated mature spermatid nuclei, even though nuclear condensation has occurred. The results indicate that the intensity of mithramycin fluorescence is directly proportional to DNA content, and independent of the degree of nuclear condensation.

In contrast, propidium iodide, the intercalating dye, yields fluorescent intensity from isolated testicular cell nuclei which is inversely proportional to the degree of nuclear condensation. When used in combination with mithramycin, the fluorescence resolution improves slightly because of the higher specificity of MI for DNA, and the energy transfer which takes place. This characteristic of PI binding allows differentiation of the elongated, elongating, and round spermatids, after pepsinization.

The relative fluorescence intensities of mature, elongating, and round spermatid nuclei were obtained by paraboloid analysis of samples
enriched in each cell type. Figures 45 through 49 are photomicrographs of specific fractions, resulting from BSA gradient separation, which show enrichment of various cell types. Fractions 1 through 3 consist purely of elongated spermatids; fractions 4 through 6 contain primarily elongating spermatids; and fractions 7 through 10 contain a majority of round spermatids. Diploid cells, which are not of particular interest in this experiment, sediment out in higher-numbered fractions. Chromatin structure is highly visible in spermatocytes prior to the first meiotic division, shown in Figures 48 and 49.

To determine the fluorescence intensities of the various spermatid classes, only fractions 1 through 9, resulting from the same gradient separation, were analyzed by the paraboloidal flow cytometer because all spermatids were contained in these fractions. Microscopic observations confirmed the contents of each fraction. The best enrichments of elongated, elongating, and round spermatids were obtained in fractions 3, 5, and 7, respectively. Photomicrographs of these fractions are shown in Figures 50 - 52. DNA fluorescence measurement of these same fractions yielded mean fluorescence channels of 35, 42, and 46, respectively. The fluorescence distributions are shown in Figure 53, along with the total testes preparation. These data confirm the assumption that the fluorescence intensity is inversely related to the state of nuclear condensation, and agree well with all multi-peaked histograms from isolated testicular nuclei.
Figure 45. Photomicrograph of Fraction 3. Only elongated (mature) spermatids are present. X400.

Figure 46. Photomicrograph of Fraction 6. The dominant cell type is elongating spermatids. X400.
Figure 47. Photomicrograph of Fraction 10. Predominantly round spermatids are present. A few elongating spermatids can be seen. X400.

Figure 48. Photomicrograph of Fraction 15. Spermatocytes in various stages of meiotic division are present. Chromatin structure is visible in many cells. There are some contaminating spermatids and multinucleated cells. X400.
Figure 49. Photomicrograph of Fraction 20. Many spermatocytes, along with multinucleated cells, and a few elongating spermatids spermatids are present. X400.

Figure 50. Photomicrograph of Fraction 3 showing elongated spermatids, which yielded a mean fluorescence channel of 35. X400.
Figure 51. Photomicrograph of Fraction 5, showing many elongating spermatids, which yielded a mean fluorescence channel of 42. X400.

Figure 52. Photomicrograph of Fraction 7, showing predominantly round spermatids, which yielded a mean fluorescence channel of 46. X400.
Figure 53. Fluorescence distributions from samples enriched in elongated spermatids (A), elongating spermatids (B), round spermatids (C), and an unseparated sample containing all types of testicular cells (D). Large dots mark every tenth channel, beginning with channel 10.
A new flow cytometer featuring a paraboloidal flow chamber for optical collection has been designed to improve fluorescence analysis of single cells. The improvement is achieved by increasing the fluorescence collection efficiency, permitting measurement of weakly fluorescent particles, and by improving fluorescence intensity resolution, thereby making it possible to differentiate cells with smaller differences in fluorescence intensity.

The paraboloid has a focal length of 2.0 mm and a depth of 40.0 mm. A specially designed entrance nozzle extending into the water-filled chamber, injects the sample just 1.0 mm above the focal point. A sample stream approximately 6 μm in diameter is obtained at the focal point, where illumination occurs, resulting in increased uniformity of fluorescence excitation and emission. The internal surface of the paraboloidal chamber is coated with gold, and the size of all surface apertures minimized, to increase fluorescence collection efficiency to 80%, as compared to about 2.5% for most orthogonal systems.

The paraboloidal flow system has been evaluated with standard spheres, mouse testicular cells and nuclei, and weakly fluorescent cells. It has been demonstrated that the paraboloid: 1) improves fluorescence intensity resolution relative to orthogonal systems by nearly 20% for standard particles and round spermatid nuclei, 2) increases the fluorescence signal-to-noise ratio, allowing measurement of fluorescence which was not resolved from background noise in orthogonal systems, and
3) is relatively insensitive to particle orientation with respect to illumination and collection optics.

Several staining methods have been reported herein, to prepare mouse testicular cells for DNA analysis. The goal of this application of the paraboloidal flow cytometer was improved differentiation of elongated, elongating, and round spermatids on the basis of DNA fluorescence. With mithramycin, poorly stained elongating spermatids, unstained elongated spermatids, and a coefficient of variation of about 6% on the round spermatid distribution were obtained. The mithramycin-propidium iodide combination, with 12.5% EtOH fixation and pepsin treatment, significantly improved fluorescence resolution (3.2% c.v. for the round spermatid peak), but mature spermatids were poorly stained.

The best staining procedure was Method 3 (Chapter V), in which isolated nuclei were stained with the mithramycin-propidium iodide combination, and treated with pepsin. This new method of testicular cell preparation resulted in the resolution of elongated, elongating, and round spermatids, on the basis of their response to pepsinization, reflecting the increasing nuclear condensation of maturing spermatids. This provides a sensitive biological system to study cellular differentiation, spermatogenesis, and the effects of maturation, aging, drugs, radiation, and contraceptives on the spermatogenic process.


Reynolds, O. 1884. An experimental investigation of the circumstances which determine whether the motion of water shall be direct or sinuous, and of the law of resistance in parallel channels. Philosophical Transactions of the Royal Society of London 174:935-982.


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Most affectionately, I thank my parents, my husband Randy, and daughters Leigh and Lara, for their encouragement and understanding without which this work could never have been accomplished.
This research was performed under the auspices of the National Cancer Institute and the U. S. Department of Energy.
Figure A1. Gold reflectivity (in %) as a function of wavelength (99.9 % Au) for normal incidence (Gray, 1972).
Figure A2. Spectral transmittance of the 45° dichroic filter (Filter number 45-2-520, Bausch and Lomb, Rochester, New York).
Figure A3. Spectral transmission of the Corning 3-69 filter. (Glass color filters, Corning Laboratory Glassware, Corning, New York, 1965.)

Figure A4. Spectral transmittance of the 488 nm barrier filter (Stock Filter Catalog Visual Section, Optical Coating Laboratory, Inc., Santa Rosa, California, 1975.)
Figure A5. Spectral response of the 4526 RCA PMT. (Photomultiplier tubes, photodiodes, electron multipliers, RCA Technical Publication PIT-700A, Harrison, New Jersey, 1971.)
APPENDIX B: BIOLOGICAL REAGENTS

**Saline GM + EDTA (Crissman et al., 1977)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.1</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$ • 12 H$_2$O</td>
<td>0.39</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.15</td>
</tr>
<tr>
<td>EDTA (50 mM)</td>
<td>0.186</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.0012</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.0004</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>Q. S. to 1.0 liter</td>
</tr>
</tbody>
</table>
PAS - Hematoxylin Reaction\(^1\) (Luna, 1968)

1) Successive rinses in 95%, 80%, 50%, EtOH.
2) Tap \(\text{H}_2\text{O}\) rinse.
3) Distilled \(\text{H}_2\text{O}\) rinse.
4) Periodic acid, 0.5% for 5 minutes.
5) Schiff's Reagent for 15 minutes.
6) 3 changes of Sulfurous acid rinse, 2 minutes each.
7) Tap \(\text{H}_2\text{O}\) wash for 5-10 minutes.
8) Harris's Hematoxylin for 3 minutes.
9) Tap \(\text{H}_2\text{O}\) wash.
10) 50% EtOH, dip.
11) 80% EtOH dip.
12) 95% EtOH, 2 dips.
13) Absolute EtOH, 2 dips.
14) Xylene, 3 dips.
15) Slides are covered by synthetic resin and cover slips.

\(^1\) Modifications by Robert H. Wood, HT (ASCP). Chief, Histopathology Lab, Los Alamos Scientific Laboratory, Los Alamos, New Mexico.
APPENDIX C: DERIVATION OF COEFFICIENT OF VARIATION IN TERMS OF FWHM

The coefficient of variation (c.v.) of a normal distribution is a measure of the spread in relative terms, and is expressed as:

\[ \text{c.v.} = \frac{\sigma}{\mu} \]  \hspace{1cm} (C1)

where \( \sigma \) is the standard deviation, and \( \mu \) is the sample mean. When making c.v. measurements from a pulse height distribution it is much more convenient to measure the c.v. in terms of the full width at half maximum (FWHM), in which case the following relationship is used:

\[ \text{c.v.} = \frac{\text{FWHM}}{2.35 \mu} \]  \hspace{1cm} (C2)

The equivalency of the two forms is shown below.

The equation for the normal probability curve is

\[ f(x) = \frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}} \]  \hspace{1cm} (C3)

At \( f(x) = \frac{1}{2} \text{MAXIMUM} \),

\[ \frac{1}{2} = e^{-\frac{(x-\mu)^2}{2\sigma^2}} \]  \hspace{1cm} (C4)

But, at \( \frac{1}{2} \text{MAXIMUM} \), \( x-\mu = \text{HWHM} \) (half width at half maximum)

\[ \text{therefore, } \frac{1}{2} = e^{-\text{HWHM}^2/2\sigma^2} \]  \hspace{1cm} (C5)

Solving for \( \sigma \) gives:

\[ \sigma = \frac{\text{HWHM}}{1.177} = \frac{\text{FWHM}}{2.35} \]  \hspace{1cm} and so,
\[
\frac{2.35 \text{FWHM}}{\text{FWHM}} = \frac{1}{D} = \Phi \cdot A.\]

(6)