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Annual Report of the Biological and Medical Research Group (H-4) of the LASL Health Division

January through December 1972

Compiled by

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Contributions from the staff are indicated by section.

Experimental animals used in work presented in this report were maintained in animal care facilities that are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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INTRODUCTION

A significant historical event which altered the course of mankind occurred on February 23, 1941, when Dr. G. T. Seaborg and colleagues discovered the element plutonium in room 301 of Gilman Hall at Berkeley. The isotope of mass-238 and not the more familiar mass-239 was first discovered at that time; the plutonium isotope of mass-239 was not isolated until the spring of 1941, and element 94 remained unnamed until March of the following year. On March 28, 1941, 0.5 μg of plutonium-239 was fissioned by thermal neutrons, and the enormous effort to produce plutonium-239 in quantity for military purposes was begun. The potential toxicity of plutonium was recognized soon after its discovery and availability in quantities for biomedical research.

It is worth recalling that only extremely small quantities of this precious material were available for experimentation during the early 1940's. As an example, the following memorandum written to the Director of the Health Division of the Metallurgical Laboratory is indicative of the early concern about the potential toxicity of plutonium. The memorandum states in part: "It has occurred to me that the physiological hazards of working with plutonium and its compounds may be very great. Due to its alpha radiation and long life it may be that the permanent location in the body of even very small amounts, say 1 milligram or less, may be very harmful. The ingestion of such extraordinarily small amounts as some few tens of micrograms might be unpleasant, if it locates itself in a permanent position. In the handling of the relatively large amounts soon to begin here and at site Y (Los Alamos), there are many conceivable methods by which amounts of this order might be taken in unless the greatest care is exercised. In addition to helping set up measures in handling so as to prevent the occurrence of such accidents, I would like to suggest that a program to trace the course of plutonium in the body be initiated as soon as possible. In my opinion, such a program should have the very highest priority." The writer of this memorandum was Dr. Glenn T. Seaborg, and the date was January 1944.

The biomedical research program at Los Alamos has evolved from its conception in 1943 as a small Health Group established to protect the health of the workers, to develop safe working procedures, and to establish tolerance levels for exposure to radioactivity, plutonium, and other radionuclides. In 1944, once significant amounts of plutonium began to accumulate at Los Alamos, the Laboratory Director, Dr. J. Robert Oppenheimer, at the request of the Health Group, authorized the temporary establishment of a group of four people to initiate a research program designed to develop tests for setting exposure limits for plutonium. Several months later, this small group was absorbed by the Health Group as a Biochemistry Section, and the Laboratory's biomedical research program was born. In 1945, the Section moved into a small building of its own, and its members established the urine assay procedure for diagnosing exposure of Laboratory personnel to plutonium. Experiments were conducted which led to the first successful labeling of a biologically important compound (nicotinic acid) with reactor-produced carbon-14. The first measurement of carbon-14 by scintillation counting procedures was accomplished here and formed the basis for the present generation of commercially available liquid scintillation counting systems.

By 1948, the Health Group was a Division in the Laboratory, and the Biochemistry Section became Group H-4, the Biomedical Research Group. In

Fig. 1. Makeshift building used for biomedical research activities during the war years (photographed in 1946 showing three additions to the original structure).

October 1952, the group moved from temporary wooden structures (Fig. 1) into its present building and by the end of that decade had established itself in both national and international circles as an authority on the effects of radiation from nuclear weapons, worldwide fallout, and the physiology and toxicology of tritium and plutonium.

The Biomedical Research Group pioneered in and became a recognized authority on liquid scintillation counting, synthesis of isotopically labeled organic compounds, use of radioactive tracers in biology and medicine, and whole-body counting techniques and applications to biomedical research. By utilizing the development of large-volume liquid scintillation detectors, the group contributed significantly to the field of anthropometry through its capability to measure total-body potassium by quantitating the natural level of potassium-40 within the human body. By exploiting the whole-body counting systems designed for both research animals and man and making use of the Laboratory's significant computer capabilities, the Biomedical Research Group contributed significantly to the field of radiation protection by conducting studies on the uptake, distribution, and excretion of radioisotopes by animals and man. The interest in metabolic kinetics was also applied to the emerging field of nuclear medicine during the late 1950's.

Shortly after the discovery in 1955 of the presence of cesium-137 in man from worldwide nuclear fallout, measurements were begun on a controlled population of subjects residing in the Los Alamos area. These studies have continued to the present time and represent perhaps the most meaningful documentation of the temporal changes in man of a radioactive material released to the environment.

Beginning in the early 1960's, more emphasis was placed on the fundamental research aspects of the biomedical research program. Although investigations continued in the Mammalian Metabolism and Mammalian Radiobiology Sections related to the response of higher organisms to ionizing radiations and radioactive materials, a major emphasis was directed toward research in the fields of molecular and cellular biology.

The late 1960's also marked the start of a research program related to the question of the probable biological effects resulting from nonuniform dose distribution of alpha-emitting particulates in the lung. Interestingly, this very difficult problem has received considerable attention but little resolution since the mid-1940's. This particular problem is now one of the highest priority because of the vast potential applications of the element plutonium as regards breeder reactors, space nuclear power systems (radioisotopic thermoelectric generators), medical applications such as the heart pacer and artificial heart, as well as production, transportation, and deployment of this material for national defense. It is interesting in a sense that part of the group is now actively working on the problem which relates to plutonium efforts conducted some 20 years previously at LASL.

The 1970's have witnessed the emergence of an interest in the stable isotope program designed to exploit the use of stable elements in the general field of biomedical research. In 1971, an Isotope Applications Section was formed within the group to concentrate on the biomedical aspects of the ICONS (Isotopes of Carbon, Oxygen, Nitrogen, and Sulfur) program which involves several groups within the Los Alamos Scientific Laboratory.

The Molecular Radiobiology Section is engaged in a variety of organic synthesis procedures to assemble polynucleotides having known base sequences. Certain enzymes that catalyze polynucleotide synthesis are not only being isolated and purified but are also being studied as biofunctional proteins participating in information transfer reactions. The structure, function, and metabolism of both acidic and basic nuclear proteins, believed to be involved in readout of genetic specifications and thus differentiation, are being investigated.

Biologists and biochemists in the Cellular Radiobiology Section are investigating the temporal sequence of a variety of cellular processes in relation to specific phases of the cell life cycle using synchronized cultures of mammalian cells. A method has been developed in this Laboratory for producing relatively large quantities of cultured mammalian cells synchronized with respect to period in the life cycle. Using these cultures, mechanisms controlling synthetic processes, energy metabolism, recovery from ionizing radiation, cell-surface phenomena, and mathematical methods of cell growth are

being investigated. Several members of the Molecular Radiobiology Section are using synchronized cultures to examine the synthesis, turnover, and structural alterations of nuclear and cytoplasmic basic proteins. In addition to studies on animal cells, investigations are in progress on survival of microorganisms exposed to ultraviolet and ionizing radiations and the biochemistry of bacterial genetic transformation.

The Biophysics Section is mainly concerned with development and improvement of instrumentation for cell biology research. In collaboration with the Cellular Radiobiology Section, electronic instruments have been developed for high-speed electronic cell counting and cell sizing. A high-speed sorter has been invented which can physically separate living cells in suspension according to cell volume. A fluorescent cell spectrometer has been developed which utilizes a laser to provide the high intensity and collimation of the light of excitation required to determine rapidly and quantitatively the DNA content of individual cells of a cell population. Both cell sensing and sorting efforts are currently being extended to other optical and multiparameter methods. The Biophysics Section also provides general electronics, mechanical engineering, radiological physics, and computer science support for the group.

On 1 July 1972, the Pion Radiobiology Group (H-9) was disbanded and incorporated into the Biomedical Research Group (H-4) to consolidate the research effort on the applications of negative π mesons to radiation therapy and pretherapy radiobiological research. This action added four people to H-4.

In September 1972, an Agricultural Biosciences Group (H-6) was formed within the Health Division, resulting in the loss of one person from the Biomedical Research Group.

cells as is feasible with a rapid, digitizing system capable of numerical analysis in real time.

A computer-oriented technique has been developed for rapid, routine, karyotypic examination that can be performed by technicians. A chromosome spread is projected with a projection microscope onto a special table. The arms of individual chromosomes are then traced with a digitizing pen, which transfers the X-Y coordinates of points along the chromosome to a readout device.* The coordinates are read directly into a PDP-8/E computer. Using these data, the computer calculates the position of the centromere, lengths of individual arms of the chromosomes, total length of all chromosomes, and average length, all in real time. Efforts are now underway to incorporate a classification technique into the computer program and to extend the method to analysis of human chromosomes.

ELECTRONICS DEVELOPMENT

(M. T. Butler, L. J. Carr, and J. H. Larkins)

Cell Separator and Counting Logics

Continuing developments in the "cell separator" program within the Biophysics Section have necessitated development of new electronic systems. The earlier system consisted of many separate modules, none of which was designed specifically for the task to which it was assigned. The system was slow and inefficient, permitted only single-channel separating in one direction, and had dead times of 1000 or more μsec.

The new system, designed and built by the Biomedical Research Group, is contained in a chassis 11-3/4 in. by 17 in. by 19 in. and consists of 3 fast single-channel analyzers, 3 scalers, a 20,000-volt high-voltage supply, positive and negative charging amplifiers, logics for 2 separating channels with up to 2050 μsec of adjustable delay, and low-voltage power supplies. All circuits use solid-state TTL logics except for the charging amplifier, which was transistorized.

This system has increased the speed of cell separating by a factor of 100 by using retriggerable one-shots in the logic module and shift registers to store a stream of cell pulses. Built-in anticoincidence circuits prevent the charging plates from trying to charge both positive and negative at the same time. Both separating channels are synchronized by a crystal-controlled clock operating at a frequency of 40 kHz. The front panel contains controls for setting the separation criteria (single-channel analyzers), selecting the separation channel, coarse and fine delay switches giving 700 to 2050 μsec delay, high-voltage meter and control allowing voltages from 2000 to 20,000 volts continuously variable, deflection plate voltage control allowing 25, 50, and 75 volts, and readout of three 6-decade scalers.

A second unit is now being fabricated by the LASL's Electronics Division, using drawings provided by the Biomedical Research Group. Figure 4 is a block diagram of the unit which has operated very successfully to date.

$V^{2/3}$ Generator

There is interest in the Biophysics Section for obtaining correlations of fluorescence light emission with cell-surface area. Therefore, a signal was required which was proportional to surface area. We decided to obtain this by modifying the Coulter volume signal, since the surface area of a sphere is proportional to $V^{2/3}$. The signal was obtained by connecting logarithmic elements (diode-connected transistors) in the feedback circuits of 2 operational amplifiers. The output of the first amplifier is proportional to the logarithm of the input voltage. This output signal is reduced by means of a voltage divider to 2/3 of its full value, and this latter signal is applied to the input of the second amplifier whose output current is proportional to the anti-logarithm of the input. A third amplifier serves as a current-to-voltage converter.

All the diode-connected transistors are part

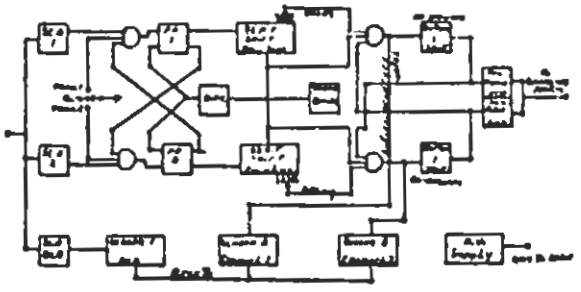


Fig. 4. Flow chart for cell separator with circuit.

*This instrument, called a Graf-Pen, is manufactured by Science Accessories Corporation.

of a monolithic integrated circuit; thus, they are very nearly identical electrically and, because of the common substrate, very close thermal coupling exists. LM-301 amplifiers were used because of low cost. The rise time of 5 μsec and dynamic range of over one decade were adequate for the experiment. Greater range can be obtained by choosing an amplifier with lower input current.

Amplifier with Base-Line Restoration

Most detectors used to drive electronic amplifiers have a DC offset in terms of voltage or current upon which the real signal is superimposed. In some cases, this steady-state value may be many times larger than the signal and, in addition, may vary enough to make simple subtraction impractical. The usual solution is capacitive coupling, which blocks the DC effectively but introduces base-line shift as the repetition rate increases. Passive clipping circuits using diodes eliminate most of an "undershoot" but have an offset of several hundred millivolts. In addition, the amplitude of a closely following pulse will be reduced because recovery is not complete.

An active base-line restorer, using an operational amplifier with fairly high open loop gain, overcomes both problems. The base line can be adjusted for zero offset, and the recovery time is reduced to a very small value because the total output of the amplifier is available to "jerk" literally the base line back to zero.

Two amplifiers were required for the cell separator unit. The signals to be detected consist of a small change in aperture current (from a Coulter counter) and a change in photomultiplier anode current (fluorescence detector). For successful operation at high counting rates, base-line restoration is required. One amplifier uses 301 operational amplifiers for rise times of about 5 μsec, and the other uses 715 operational amplifiers for rise times of less than 0.5 μsec. The latter will handle repetition rates up to 3×10^5 pulses per second or paired pulses separated by only 1.5 μsec.

PION BEAM CHARACTERISTICS

(C. Richman and W. D. Jinks)

Small silicon detectors (lithium-drifted and surface-barrier) are being tested for possible use in studying contamination and properties of the pion beam at the LAMPF. Electronics used with these detectors are conventional NIM linear and logic modules with pulse-height analysis. However, because of various types of radiations occurring in the pion beam (components of varying linear energy transfer, LET), it will be necessary to have a detailed knowledge of the pulses being produced in the detectors. By choosing the thickness of the detectors appropriately, various properties of the beam can be brought to the foreground. Thus, in thin detectors of 100 μ or less, the large pulses will be due to stars. By this means, mapping of the stars will be determined which will be a direct measurement of the momentum spectrum of pions.

To study the pulses from electrons, a ΔE-E arrangement has been built with 2 silicon detectors. A ^{207}Bi test source is used, and the E detector selects the 2 high-energy conversion electrons (974 keV and 1047 keV) in the spectrum. The ΔE detector (300 μ thick) then measures energy loss of these electrons. Because these electrons produce minimum ionization, they give pulses that would be produced closely by 200-MeV contamination of the pion beam.

A clear line at around 110 keV is obtained for energy loss in this detector system, and the Landau width does not appear to be very serious. The Landau spread will be studied with different detector thicknesses. At present, we feel that electrons will be distinguishable from pions.

To prepare for the tumor therapy program, phantom materials in the form of slabs are being made in the LASL shops and in the Materials Technology Group (CMB-6). These specially formulated plastic compounds would approximate bone, lung, muscle, soft tissue, and fat. A movable probe with location readout also is being designed so that mapping of the beam can be studied in these mock tissues. We also plan to study transition regions from one tissue to another. At present, this aspect of the pion dosimetry program is funded from non-AEC sources.

REFERENCE

1. C. R. Richmond and G. L. Voelz, eds., Annual Report of the Biological and Medical Research Group (H-4) of the LASL Health Division, January through December 1971, Los Alamos Scientific Laboratory report LA-4923-PR (1972), pp. 116-118.

BIOPHYSICS SECTION

INTRODUCTION

During the past year, the Biophysics Section has been reorganized because of the formation of a new section, Physical Radiobiology, within the group. This new section assumed the responsibility for physical radiobiology, computer applications, and electronics support formerly charged to the Biophysics Section, and these activities are described elsewhere in this report. The Biophysics Section is now solely concerned with research and development programs in the general area of biophysics and instrumentation with the current major emphasis on development and application of rapid methods for cell analysis and sorting. This major effort is divided into three main areas: (1) physical investigations of the light scattering and other properties of mammalian cells that might be measurable by our instrumentation, (2) development of instrumentation for cell sorting and analysis, and (3) applications of these methods to biological problems. Our success, particularly in this latter area, has been possible because of excellent cooperation with members of the Cellular Radiobiology Section.

Flow microphotometry, the method of making rapid optical measurements on individual biological cells, is an exciting and important emerging technology in cell research and has been pioneered by personnel of the Biophysics Section at Los Alamos. This development grew from our early efforts in quantitating Coulter counting techniques which resulted in improved Coulter volume spectrometry and cell sorting based on cell volume. Flow microfluorometry (FMF) is our most important contribution to date in the area of optical measurements on single cells.

During 1972, FMF was used routinely by LASL personnel as well as by investigators from other laboratories who visited LASL (Salk Institute, Karolinska Institute, Cold Spring Harbor Laboratory, University of Houston, University of Texas at Austin, University of New Mexico, University of Colorado, and University of California at Berkeley). In most of these experiments, cellular DNA was the important parameter measured. In such cases, FMF measurements were made on cells created by the fluorescent Feulgen method.

A two-color fluorescence sensor has been incorporated into our multiparameter cell sorter. Use of the bi-color fluorescence detector has allowed us to study acridine orange-stained human leucocytes that exhibit bi-color fluorescence. We have used cell sorting techniques to show that the cells, based on their red fluorescence, can be classified as lymphocytes, monocytes, and granulocytes. We have also initiated work designed to obtain nuclear-to-cytoplasmic ratios based upon nuclear and cytoplasmic fluorescence; ethidium bromide-stained DNA yields red fluorescence, and fluoroisothiocyanate-stained protein produces green fluorescence.

A dual-parameter cell analysis photometer was also designed and constructed during 1972. This device measures fluorescence and small-angle light scattering simultaneously on each cell as it passes through the laser light in a flow photometer. Our present application uses light scattering to gate electronically for fluorescence analysis, permitting an electronic "clean up" of the signal in those cases where the signal would not be discernible from noise present in debris-ladened biological samples.

More extensive theoretical studies on light scattered by biological cells were conducted during 1972. High-speed computers were used to calculate light scatter from coated spheres to simulate mammalian cells, the core representing the nucleus and the coating the cytoplasm. Results of the computer calculations indicate that the forward light scattering is dominated by gross size of the cell. Outside the forward direction, the scattering pattern is a function of both nuclear and cytoplasmic optical properties. Experimental scattering patterns also were obtained for suspensions of various cell types. In cases where cell

olume distribution, ratio of nuclear-to-whole cell iameter, and other properties are well defined .e.g., G_1 phase CHO cells), a high degree of cor-elation was obtained between experimental and heoretical scattering patterns. To our knowledge, his is the first time that such a complete physical escription of light scattering from mammalian cells as been obtained. These studies hold promise for ell identification on yet another parameter meas-rable with our flow systems.

In addition to the experiments mentioned above, iological applications of our physical methods have een pursued with other personnel at the LASL and ith others as part of our mutual-interest programs ith the U. S. Department of Agriculture and the ational Cancer Institute. These collaborative fforts include effects of stressing agents such as adiation, temperature, and chemicals on the life ycle of mammalian cells; ploidy studies on trans-ormed mammalian cells; comparison of chromosome anding techniques with DNA determinations by FMF echniques; DNA determinations on several trans-lantable tumors; applications of cell sorting to uman cervical material; improved methods of cell nd tissue preparation for FMF applications; fluo-escent antibody techniques; investigation of light cattering techniques as a possible indication of iral infection of mammalian cells; and binding of lant lectins such as fluorescein-tagged concana-alin A to cell surfaces.

The unique cell-analysis methods developed ere at the LASL are receiving increasing attention ithin the scientific community. In this respect, ne of us (M. A. Van Dilla) has been invited to articipate in the establishment of a similar effort t the Lawrence Livermore Laboratory of the Univer-ity of California. Dr. J. D. Watson of the Cold pring Harbor Laboratory invited two of us (L. S. ram and H. A. Crissman) to participate in the ummer program at Cold Spring Harbor. These two cientists and an FMF unit were sent there to demon-trate the FMF technique. In addition to the nstitutions listed above, investigators from both he United States and abroad have sent us samples or FMF analysis as part of mutual-interest studies.

We are also pleased to report that A. Brun-ting, an Associated Western Universities pre-octoral fellow from the Physics Department at the University of New Mexico, successfully completed the Ph.D. requirements in August 1972. During his stay at the LASL, Dr. Brunsting participated in the Biophysics Section's effort on the theoretical and experimental aspects of light scattering.

CELL ANALYSIS AND SORTING INSTRUMENTATION DEVELOPMENT

(A. Brunsting, J. R. Coulter, L. S. Cram, J. L. Horney, J. C. Martin, P. F. Mullaney, J. A. Steinkamp, M. A. Van Dilla, and W. T. West)

Differential Light-Scattering Signatures of Mammalian Cells

As we reported in the 1971 annual report,[1] mammalian cells have been simulated in computer models as coated spheres, the core representing the nucleus and the coating cytoplasm. Exact electromagnetic theory calculations[2] for individual particles with cell-like optical parameters indicate that light scattered in the forward direction is dominated mainly by the gross silhouette of the particle and contains information on particle size and that beyond the forward lobe the scattering pattern from a coated particle is significantly different from a simple homogeneous sphere. In the latter case, the scattering pattern is dependent upon optical properties of both the core and coat.

During 1972 some of our theoretical predictions have been tested with suspensions of mammalian cells. A simple but unique light-scattering photometer[3] was constructed for this purpose and is shown schematically in Fig. 1. The photometer is housed in a 2.5-foot diameter cylinder. Control of light from a 5-mW helium-neon laser (Spectra Physics Model 120) into the photometer is by shutter. The light then passes through a variable aperture to reduce extraneous nonlasing light and is reflected from a front surface mirror through a specially designed cuvette. The main laser beam and some forward scattered light are collected in a Rayleigh horn beam dump. Light scattered by the particles in the cuvette is recorded on high-speed, red-sensitive film (Kodak 2479 RAR ASA 400, 16-mm x 125-foot rolls) which is held in a track on the inside surface of the photometer. Approximately 34 exposures can be made from each roll of film. Fiducial markers on the film holder cast shadows on the film at 30°

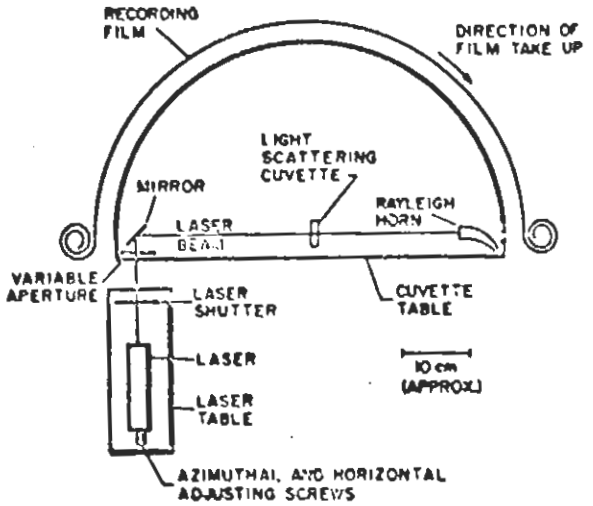


FIG. 1. Schematic diagram of the film photometer. The laser provides incident light to the scatterers in the cuvette. Most of the laser light is dumped into the Rayleigh horn while the film records the scattered light.

intervals, permitting film dispersion to be measured on each exposure. After exposure, the film is developed in a Kodak Versamat Model 11c using type B chemistry. This procedure produces a typical dynamic range of three decades, which can be extended by allowing some overlap between exposures and reducing intensity in the forward direction by use of neutral density filters. Developed film is read with a microdensitometer. Through the use of suitable calibration, film density as a function of distance on the film can be converted into scattering intensity as a function of scattering angle. Using these techniques, we have obtained scattering patterns for the angular range of 2.5 to 77°.

It is well known that angular positions of the maxima and minima in the scattering pattern are important functions of particle size.[4] For this reason, in a suspension of particles which are similar in composition but not identical in size, some smearing out of the scattering pattern should be expected. Therefore, it is important that the mean size, size distribution, and refractive index of the particles are well characterized. One also needs to establish that the photometer has sufficient resolution to record the essential features of the scattering pattern of interest. This last consideration was accomplished by a calibration procedure which uses extremely uniform diameter polystyrene microspheres produced at the Los Alamos Scientific Laboratory.[5] The refractive index of the spheres was measured by an immersion technique and found to be 1.562 ± 0.002 (with respect to air). Optical microscopy and Coulter volume spectrometry measurements of the spheres provided information on mean diameter (10.5 microns) and coefficient of variation of volume (3 to 4 percent).

Light scattering measurements made at concentrations of 5×10^4 particles/ml are shown in Fig. 2. At this concentration, the particles behave as independent scattering centers (i.e., increasing the concentration produces a proportional signal increase with no change in shape of the scattering curve).

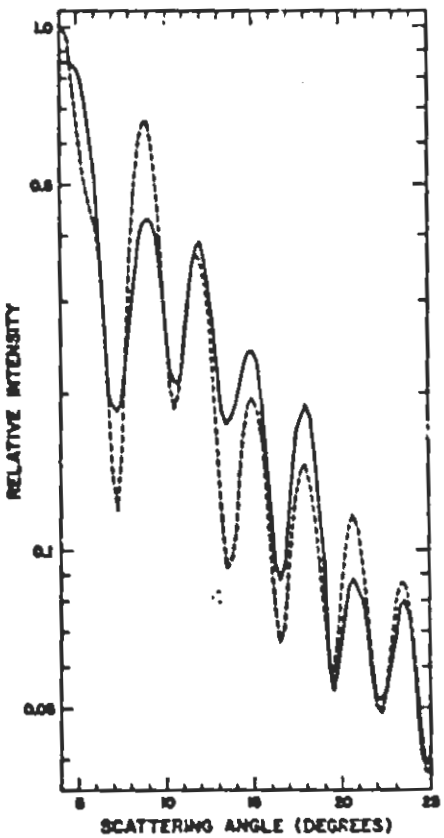


FIG. 2. A comparison of theory and experiment for the microsphere scatterers. A semi-log scale of the relative intensity, computed (thin) and measured (thick), versus scattering angle, θ, in degrees. Theory accounted for the size distribution of the spheres and angular resolution of the photometer. The experimental curve was corrected for photometer scattering by transmission properties of the cuvette. Theoretical curves are given by dashed lines and experimental by a solid line.

Theoretical results are shown by the dotted curve and experimental results by the solid curve. Although the curves do not match exactly in shape, their maxima and minima agree quite well (Table 1). The agreement between theory and experiment confirms the accuracy of measured size distributions for the spheres based upon microscopic and Coulter volume spectrometric measurements. Light-scattering measurements yield a mean diameter of 10.3 microns as compared with 10.5 microns as determined by other methods.

The difference in relative intensity between the two curves (Fig. 2) is caused by a number of factors. Although uniform in diameter, the spheres are not smooth, and optical microscopy shows them to have a golfball-like surface. Theoretical calculations assumed a perfectly uniform sphere. In addition, there is an uncertainty of about 5 percent in measurement of film density response characteristics which is not a linear function of intensity. The fact that the maxima and minima agree so well demonstrates that the film photometer has sufficient resolution to measure the main characteristics in the scattering pattern obtained from particles with the dimensions of mammalian cells.

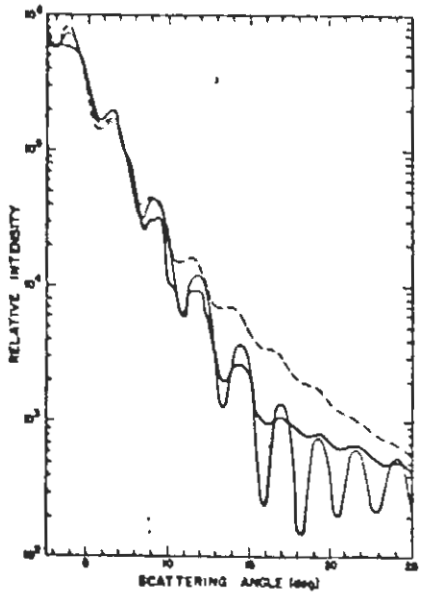
For all particles studied by this method, mean size, size distribution, and refractive index were measured. The computer codes written for individual particles were modified to account for size distributions. Thus, at each scattering angle, we could obtain the calculated intensity contribution from each particle in the distribution. These results were then weighted according to the measured diameter distribution and integrated across this distribution function to yield the theoretical curves shown in this report. Details of this approach are given elsewhere.[6]

TABLE 1. LOCATIONS OF MAXIMA AND MINIMA FOR THEORY AND EXPERIMENT IN THE CASE OF PLASTIC MICROSPHERES (see Fig. 2)

Theory (± 0.15°)		Experiment (± 0.2°)	
Maxima	Minima	Maxima	Minima
4.25	7.25	4.5	7.2
9.00	10.50	9.0	10.5
11.75	13.50	11.9	13.6
15.00	16.50	15.0	16.5
18.00	19.50	17.9	19.3
20.75	22.25	20.8	22.1
23.50	24.75	23.5	25.0

Measurements were made on several tissue culture cell lines and results for Chinese hamster cells (line CHO)[7] are reported here. Using the technique of Tobey et al.,[8] cells were synchronized in the M stage of the life cycle. Scattering measurements were made for these cells and for cells allowed to enter early G_1. Experimentally determined light-scattering curves then were compared with theoretically derived curves for coated spheres of the appropriate parameters and equivalent homogeneous spheres.

Mean cell volume and volume distribution information on both CHO cells and spheres was obtained with a laminar flow Coulter volume sensor.[9] CHO cells in G_1 were found to have a mean diameter of 11.5 microns and a volume coefficient of variation of 13.6 percent. Photomicrographs of several hundred of these cells were made, and the ratio of nuclear diameter to whole cell diameter was calculated to be 0.73 ± 0.08. Using a phase microscopy technique,[10] the refractive index of the nucleus and cytoplasm (with respect to air) was found to be 1.392 and 1.3703, respectively. However, cells are immersed in a water-like medium; in which case, these figures become 1.030 (cytoplasm) and 1.047 (nucleus). Barer[11] has shown that the refractive index (m) of cells and their density (d) are related by $(m - 1) \cdot d^{-1} = k$. From the work of Anderson,[12] which demonstrated that the density of CHO cells is quite constant, we infer that the refractive index is not very variable among these cells. This information was used to calculate theoretical scattering curves for CHO cells in the G_1 stage of the cell cycle. The computer program treats the refractive indices of both nucleus and cytoplasm as being constant for the values stated above. The ratio of nuclear diameter to whole cell diameter was also assumed to be constant and equal to 0.73. Mean volume and volume distribution of these cells were also incorporated into the computer program. We also calculated light scattering from an equivalent homogeneous sphere in which the mean refractive index was equal to the volume-averaged refractive index of the nucleus and cytoplasm.[6] A comparison of these two theoretical results with experimental data is shown in Fig. 3.



G. 3. Two theoretical plots and corresponding experimental results for the differential scatter patterns for G_1 Chinese hamster ovary cells. The coated sphere (thin solid line), the equivalent homogeneous sphere (thin dashed line) whose refractive index has been volume-averaged from the coated sphere, and the experimental results (thick solid line) are shown.

e log of the scattering intensity is plotted as a nction of scattering angle. Experimental results e given by the heavy solid curve, coated sphere eoretical results by the light solid curve, and uivalent homogeneous sphere theoretical results by e dashed curve. Experimental results were ob-ined with cells in a concentration of 10^5 cells/ml. low 7 to 8° all three curves agree quite well, dicating that gross size effects primarily domi-te the scattering response in this angular region. yond 8° the light scattering from the coated here is considerably less than that from the equiv-ent homogeneous sphere. The position of the perimental maxima and minima agrees quite well th theoretical results for coated spheres, al-ough there is some wash-out beyond roughly 15°, obably because of heterogeneity not accounted for the model for CHO cells. The main feature of g. 3 is that actual cells behave much more like ated spheres than homogeneous spheres. Beyond the rward direction, the scattering curve reflects clear properties of the cell.

Figure 4 shows experimental scattering from CHO lls in M as compared with an equivalent homo-neous sphere. Unlike G_1 cells, these cells show

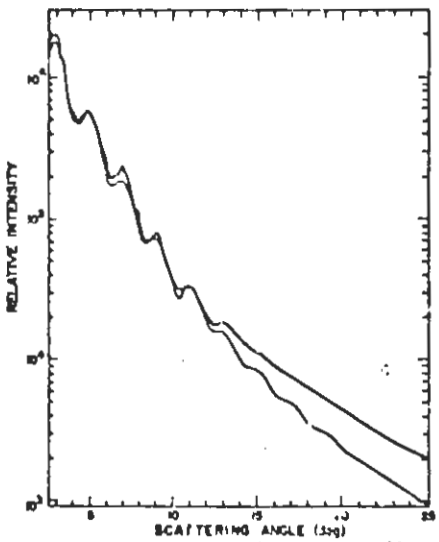


FIG. 4. Theoretical plot and corresponding experimental results for the differential scatter patterns for M Chinese hamster ovary cells in M. The equivalent homogeneous sphere (thin solid line) and experimental results are shown.

no well-defined nucleus. In this case, CHO cells in M are quite well modeled as homogeneous spheres over the angular range of interest. We should note that virtually all the scattering by cells is contained in the first 25°.

These preliminary studies were conducted on suspensions of living cells. Perhaps the most interesting result is that light scattering at larger angles is influenced by the internal structure of the cells. We plan to incorporate a wide-angle light-scatter sensor on our flow photometers to investigate the possibility of wide-angle scattering as another parameter for cell identification in flow-system analysis.

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Multiparameter Cell Sorter

A multiparameter cell analysis and sorting system for use in cell biology research and possible clinical applications has been developed. Cell samples stained with a fluorescent dye that specifically labels biochemical components of interest are suspended in physiological saline and introduced into a flow chamber where optical and electrical sensors measure cell volume (Coulter method), single- or two-color fluorescence, and scattered light. Signals from the sensors are electronically processed in a variety of ways to provide optimum cell discrimination and are displayed as frequency distribution histograms. Processed signals are also compared with preselected standards, and this triggers sorting of the desired cells. Populations of mammalian tissue culture cells, human leucocytes, and other samples have been analyzed and sorted.

Figure 1 illustrates a cut-away sectional view of the multiparameter cell sorter. Fluorescently stained cells dispersed in saline are introduced (1,000 cells/sec) into a dual sheath flow chamber via the sample inlet tube. Flowing coaxially around the inlet tube is a particle-free sheath fluid (sheath No. 1) of saline. Because the flow is laminar, the cell stream and surrounding sheath do not mix but move together through a Coulter volume sensing orifice, where cell volume is measured electronically. The flow next enters a fluid-filled viewing region where it intersects an argon-ion laser beam, causing light scattering and fluorescence. Both these

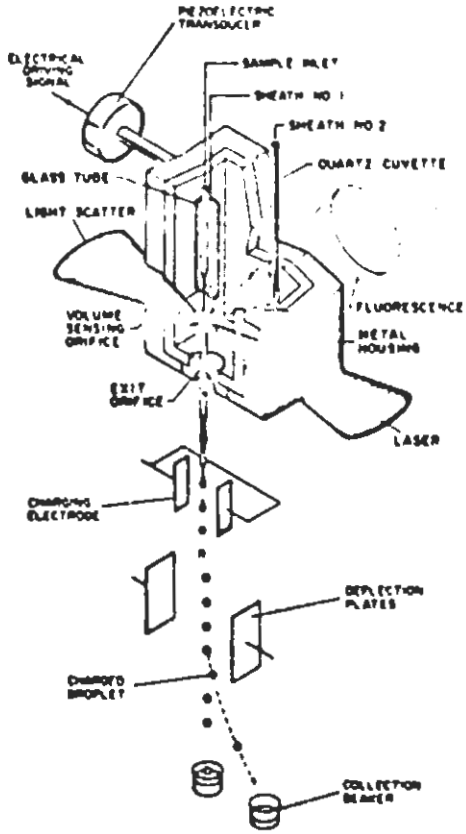

FIG. 1. The multiparameter cell sorter.

signals are electro-optically measured; the fluorescence sensor is a dual photomultiplier array which permits measurements of red and green fluorescence. Light scatter is measured in the forward direction by blocking the laser beam with an optical stop and focusing the forward scattered light onto a photodiode.

A second sheath fluid (sheath No. 2), also of saline, flows coaxially around the cell stream-sheath No. 1 flow. The total flow jets out into the air from an exit nozzle. A piezoelectric transducer mechanically coupled to the flow chamber and electrically driven at 45 kHz produces uniform liquid droplets (45,000/sec) by regularly disturbing the emerging jet. Cells are isolated effectively into single liquid droplets in this manner.[1] A group of droplets, one of which will contain a cell to be sorted, is electrically charged at the point of droplet formation (charging electrode) and is then deflected by a static electric field into a collection vessel.

Signals from the cell sensors are routed to a hard wired multiparameter analog signal processing

unit. Processed signals are then routed to a multichannel pulse-height analyzer, where frequency distribution histograms of cell volume, fluorescence, light scatter, or a combination of these parameters can be displayed. Processed signals also trigger cell sorting by comparing the amplitude of each processed signal pulse to a preset standard (i.e., if the signal amplitude falls within a preselected range, an appropriate electronic delay is activated which triggers a droplet charging pulse, causing the droplet containing the cell to be charged and subsequently deflected). A group of droplets, usually about 9, is then sorted from the main stream. Those cells failing to meet the criteria of the preset standards do not trigger sorting and are allowed to pass to a waste vessel. In a typical experiment 10^4 to 10^5 cells are sorted in a few minutes.

Figure 2a shows the volume and fluorescent Feulgen DNA histograms of Chinese hamster (line CHO) cells growing asynchronously. These cells are stained with the fluorochrome acriflavine using the fluorescent Feulgen[2] procedure. DNA distributions show two peaks: the first represents the cells with diploid DNA (G_1 phase) having a coefficient of variation of 4 percent, and the second peak represents cells with tetraploid DNA content (G_2 and M phase). The region between the peaks represents cells synthesizing DNA (S phase). The ratio of G_1 and G_2 + M modal fluorescence intensities of the two peaks is 2.04, very close to the expected value of 2.00. Cell volume distribution is broad, unimodal, and typical of a cell population in exponential growth. The DNA-to-cell volume ratio for this cell population, shown in Fig. 2b, is unimodal with a coefficient of variation of 15 percent. Volume distribution of G_1 cells (Fig. 2c) was obtained by analyzing only those Coulter sensor signals associated with the fluorescence signals indicating the G_1 amount of DNA (see Fig. 2a). Similarly, the volume distribution of G_2 + M cells was obtained by analyzing only those Coulter sensor signals associated with the G_2 + M fluorescence peak of the DNA distribution. Modal volume ratio $(G_2 + M)/G_1$ is 1.7, less than the total volume increase over the life cycle of the cell (double) because the instrument is measuring average cell volume distributions of the G_1 and G_2 + M phases.

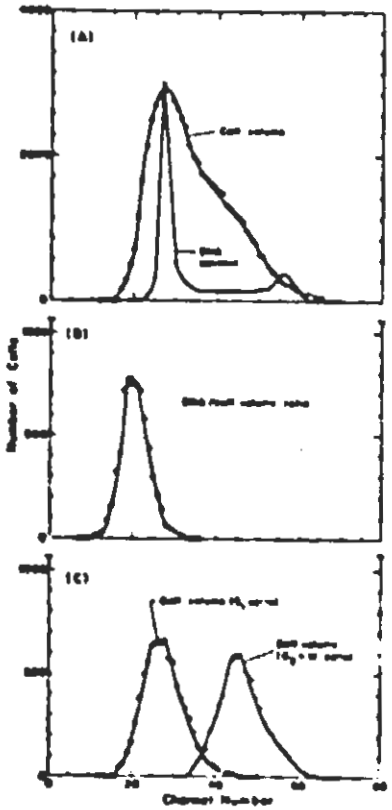

FIG. 2. Volume, DNA, and DNA-to-cell volume distribution histograms of Chinese hamster ovary cells growing asynchronously in suspension culture: (A) volume and DNA distribution; (B) DNA-to-cell volume distribution, and (C) volume distribution of G_1 and G_2 + M cells.

The red and green fluorescence of human leucocytes supravitally stained with the metachromatic fluorochrome acridine orange[3] is shown in Fig. 3. When leucocytes of diluted whole blood are stained according to this procedure, cytoplasmic granules exhibit red fluorescence whereas the nucleus fluoresces green. Erythrocytes do not take up the acridine orange stain. The bi-color fluorescence sensor was set to measure green and red fluorescence, and the cell sorting logic was adjusted to separate leucocytes having a red fluorescence corresponding to region 1. Differential microscopic counts on sorted leucocytes show that approximately 95 percent are lymphocytes. When the sorting logics are adjusted to separate cells lying within regions 2 and 3, subsequent microscopic counts show that 90 percent of the cells separated from region 2 are monocytes and that 95 percent separated from region 3 are granulocytes.

Because of the large ratio of erythrocytes to

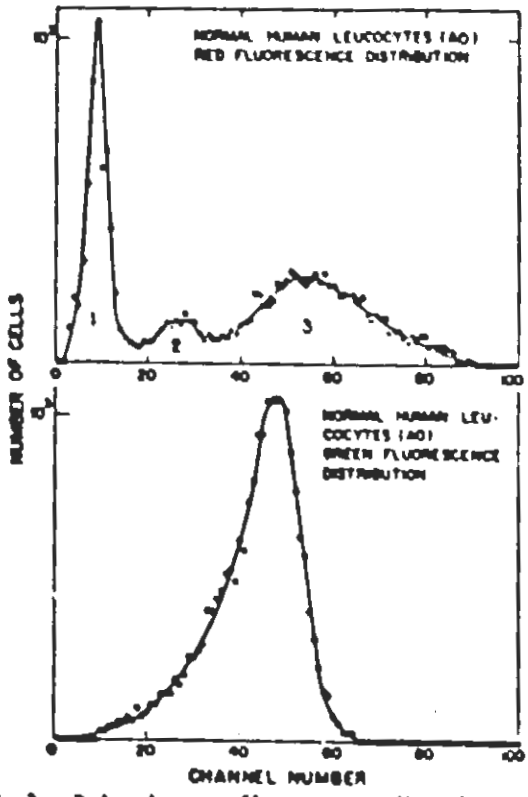

FIG. 3. Red and green fluorescence distribution histogram of normal human leucocytes supravitally stained with acridine orange.

leucocytes in normal human blood, leucocyte volume distributions cannot be obtained without prior saponin hemolysis of erythrocytes. This difficulty can be overcome by sorting all green fluorescing cells, thus producing a leucocyte-enriched sample. This sample is then reintroduced into the sorter, and the volume of cells showing only green fluorescence is measured.[4] This method has the advantage of yielding a leucocyte volume distribution without requiring saponin hemolysis.[5]

A second multiparameter cell sorter is under construction presently, with a completion date of early 1973. This unit is intended to be dedicated to biological experiments, while the present sorter system continues to be used for some limited biological experiments coupled with instrument development (i.e., new cell sensing methods and determination of optimal detection parameters). Current experiments underway on the present cell sorter include ethidium bromide-fluorescein isothiocyanate (FITC) staining for DNA/cell protein studies, fluorescein-conjugated concanavalin A bound to membrane surface sites, continued study of human and animal (hamster) leucocyte characterization with the metachromatic fluorochrome acridine orange, and work on cells from solid tumors and exfoliative material.

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A Dual-Parameter Cell Microphotometer

During 1971, a dual-parameter microphotometer was developed and subjected to initial testing. The dual-parameter instrument described here represents a combination of the fluorescent microphotometer[1] and light-scattering photometer developed earlier.[2] Each cell stained with an appropriate fluorescent dye produces a pulse of fluorescent light, as well as a pulse of scattered light as it crosses a beam of blue light from an argon laser. These signals occur simultaneously and can be used as two descriptors for each cell. After detection, each signal is amplified and fed through a dual-parameter processing unit. If certain logical conditions are met, these two signals are then available for pulse-height analysis. The net result is a frequency histogram of fluorescence or light scatter of the cells of interest.

A schematic diagram of the dual-parameter photometer is shown in Fig. 1. The flow chamber is basically the same as that described previously.[2] Laminar flow is established within the chamber, and the cells are injected as the core of the main flow. Just prior to entering the viewing area, the entire

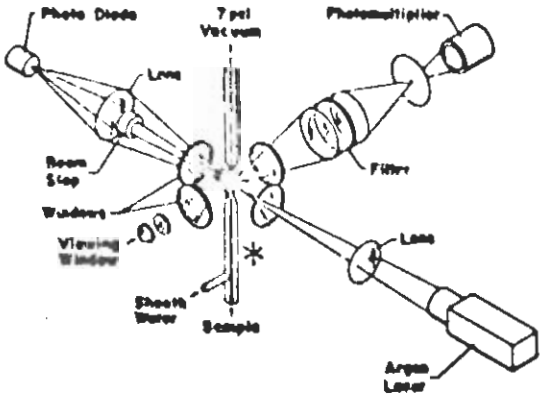

FIG. 1. Schematic of the dual-parameter flow microfluorometer. The argon-ion laser beam (lower right corner) is focused with a 20-cm lens to a 50-micron spot at the center of the flow chamber. Fluorescent light is detected at 90° to the incident direction. Light scattered in the forward direction between the central beam stop and outer stop is collected with a 10-cm lens and focused onto a photodiode.

flow moves through a constriction orifice which narrows the cell stream to a column about 20 microns in diameter. The cells are then lined up much as beads on a string as they intersect the laser light.

The light source for this photometer is a Coherent Radiation Laboratory Model 52GA argon laser operating at 1 watt at 488 nm. Laser light is focused with an 18-cm convex lens to form a 50-micron spot at the intersection with the cell stream at the center of the flow chamber. After passing through the laser beam, the cells exit out the top of the chamber and go to a waste vessel.

As each cell passes through the laser beam, it produces a 10-microsecond pulse of fluorescent and scattered light. The main laser beam is eliminated in a small trap. The cone of light scattered about this trap (approximately 0.7 to 2.0°) is collected with a 10-cm convex lens and focused on a photodiode. The resulting signal is then amplified.

The fluorescence signal is collected at 90° to the direction of the incident beam. Light then passes through a yellow barrier filter and is focused onto a 300-micron diameter pinhole. The fluorescent light emerging through the pinhole is viewed with an RCA Model C7164R photomultiplier tube selected for its extended red sensitivity. This signal is also amplified.

A box diagram of the dual-parameter signal

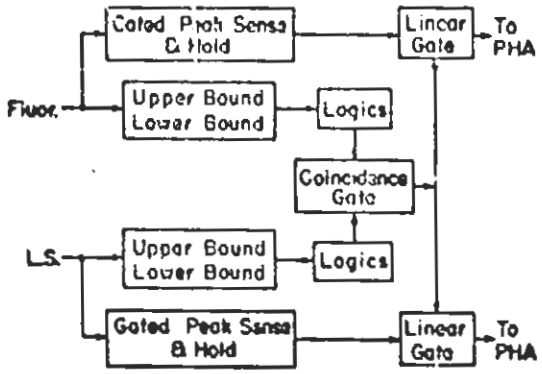

FIG. 2. Block diagram of dual-parameter signal-processing unit. When the conditions of coincidence and proper signal amplitude are met, both analog signals are passed for pulse-height analysis (PHA).

processing unit is shown in Fig. 2. In the present application, one signal is used to gate the second. After amplification, the signal from each of the two detectors is fed to separate adjustable discriminators. If both signals are within the appropriate amplitude range, they are then tested for coincidence. If both tests are positive, the analog signals are passed through a linear gate, and either or both signals are available for pulse-height analysis. The logics of Fig. 2 can be bypassed; in which case, the instrument functions as a single-parameter photometer.

A particularly useful application of the dual-parameter photometer is the analysis of fluorescence from weakly stained samples. Typically, 5×10^4 cells/min pass through the photometer, with each cell spending about 5 microseconds in the light beam. The total time that cells spend in the beam is then 5×10^{-1} seconds out of every minute; the photometer duty cycle is 0.83 percent. The remaining 99.17 percent of the time the photomultiplier is measuring noise due to fluorescence of non-cellular material in the cell stream. This noise may be from stained cellular debris or fluorescent dye in solution in the cell suspending medium.

Figure 3 shows the light-scattering frequency distribution obtained with Chinese hamster cells (line CHO) supravitally stained with 10^{-8} M acridine orange. When viewed in the fluorescence microscope, these cells exhibit a weak green nuclear fluorescence and no detectable cytoplasmic fluorescence. The light-scattering signal is seen to be free from any debris which is comparable in size to

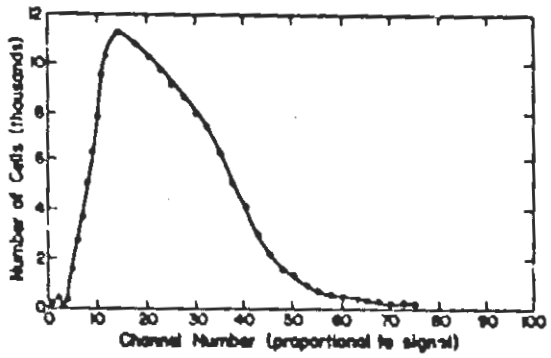

FIG. 3. Light-scattering distribution for random CHO cells stained with 10^{-8} M acridine orange.

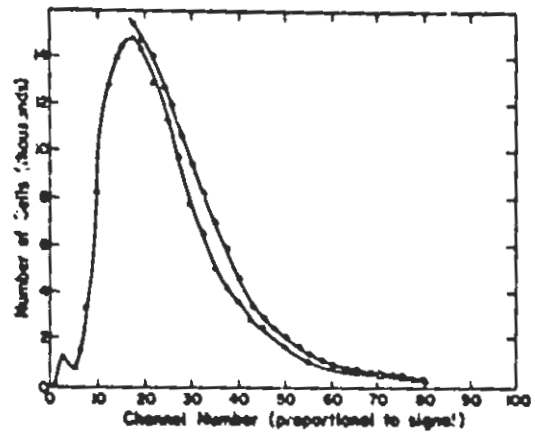

FIG. 4. Fluorescence distribution obtained for CHO cells stained with 10^{-8} M acridine orange: (-0-0-) when the instrument is used as an FMF; and (-Δ-Δ-) fluorescence gated on the light-scattering signal from each cell.

the cells. Figure 4 shows the fluorescence frequency distribution. When the dual-parameter photometer is operated as a single parameter, one obtains the upper curve which has the appearance of an exponential decay typical of a noise spectrum. However, when the fluorescence signal is gated by the light-scattering signal from the same cells, one obtains the lower curve. In this case, only the fluorescence of objects in the size range of cells is analyzed. This lower fluorescence frequency distribution is similar to that obtained for greater acridine orange concentrations ($> 10^{-5}$ M) where detection is no problem.

The instrument may be used also to identify particles based on the presence or absence of these two parameters. In Fig. 5, the light-scattering and fluorescent frequency distributions of paper mulberry pollen stained with acridine orange are shown. Both signals are free of noise.

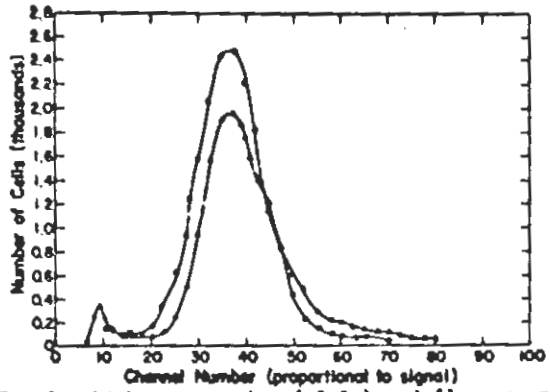

FIG. 5. Light scattering (-0-0-) and fluorescence (-Δ-Δ-) distributions for paper mulberry pollen stained with acridine orange.

Next, paper mulberry pollen was mixed with unstained CHO cells, and the light-scattering distribution of the ensemble was measured as shown in Fig. 6. Both particles are the same size (approximately 12 microns), but CHO cells show a much broader size distribution (cf. Fig. 3), hence broadening the distribution obtained from the combination. However, if light scattering from the combination is gated by the fluorescence of paper mulberry pollen, one obtains the scatter curve shown in Fig. 6, which is identical to that of paper mulberry pollen alone.

This instrument offers the possibility of improving the signal-to-noise ratios on samples that fluoresce poorly because of preparational difficulties and distinguishing one biological particle from another on the basis of differences in light-scattering and/or fluorescent properties. The

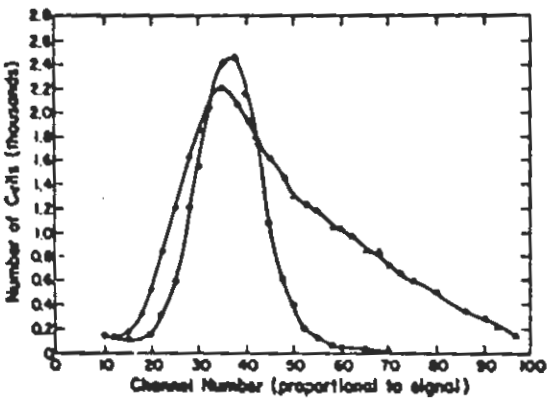

FIG. 6. Light-scattering distribution (-Δ-Δ-) obtained from a mixture of stained paper mulberry pollen and unstained CHO cells. Light-scatter distribution (-0-0-) obtained when the scattering signals from the mixture were gated by simultaneous fluorescent signals from the pollen.

first attribute permits measurements on samples not previously amenable to FMF analysis. The second feature greatly extends the FMF concept of cell identification.

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BIOLOGICAL APPLICATIONS OF CELL ANALYSIS AND SORTING

(L. S. Cram, H. A. Crissman, J. C. Forslund, P. K. Horan, P. M. Kraemer, D. F. Petersen, M. R. Raju, A. Romero, R. A. Tobey, and T. T. Trujillo)

Fluorescent DNA Distributions in MCA-1 Tumor Cell Populations

The tumor cell line MCA-1 was derived in C3H mice by the application of the carcinogen methylcholanthrene to the skin. The resulting tumor was classified as a squamous cell carcinoma. Because of our interest in developing and separating squamous cell carcinoma for the cervical and uterine regions of humans, we felt that this tumor would serve as a good working model. For example, use of the MCA-1 tumor would allow us to have sufficient cells which are easily dissociated to determine the parameters which are most suited for cancer cell identification. Information gained with this model system would then be applied to an investigation of the identification of cancer in humans. One parameter or cell property considered acceptable for identifying abnormal cells in any cell population is the presence and extent of polyploid cells when analyzed for DNA content per cell. After working out a satisfactory single-cell suspension technique prior to fixation, fluorescent DNA distributions could be made from several animal tumors. The cells were stained by the acriflavine Feulgen reaction method[1] and measured by FMF techniques.[2] The fluorescent distribution histograms of these samples (Fig. 1) show three distinct peaks in a channel ratio of 1:2:4. The first peak corresponds to the normal diploid cells present with tumor cells. The position of this peak corresponds to that of diploid cells obtained from kidney and spleen from the same

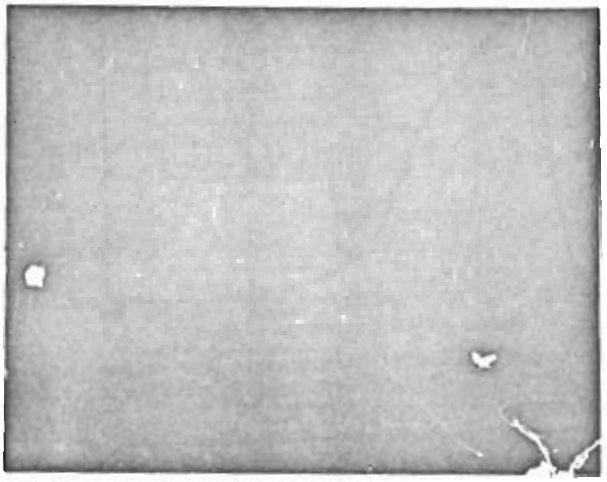

FIG. 1. DNA distribution of MCA-1 tumor cells.

animal and to CHO cells in G_1. The MCA-1 cell distribution pattern indicated the presence of cells with 2N, 4N, and 8N amounts of DNA. The second population (4N) appeared to be the most numerous. The three peaks seen in a single sample may also indicate the presence of two cell lines growing together: (1) normal diploid cells with the 2N amount of DNA, and (2) proliferating tumor cells as indicated by the G_1 (4N DNA) and G_2 + M (8N DNA) peaks. We thought it would be interesting to investigate whether MCA-1 tumor cells could be grown in culture; this would not only provide a ready source of cells but might also lead to a selective line more suitable for the model system described above. This has been done with quite a degree of success. During the early passages (1 through 5), the fluorescent DNA distribution resembled the original tumor cells (Fig. 1); however, in later passages (6 through 10), we observed the disappearance of the first peak (2N) while the cell population was still at a nonconfluent state of growth (Fig. 2). However, a confluent population displayed only one peak at the 4N DNA level (Fig. 3).

From these preliminary data, we conclude that animal MCA-1 tumor tissue contains both normal diploid cells and polyploid tumor cells, with the former unable to survive and proliferate after a few passages in culture media. Also, when cultured MCA-1 tumor cells are allowed to grow to a confluent state, we see a typical G_1 arrest as observed by others[3] in other mammalian cell lines. Because normal vaginal and cervical specimens produce typical diploid (2N DNA) histograms, it seems

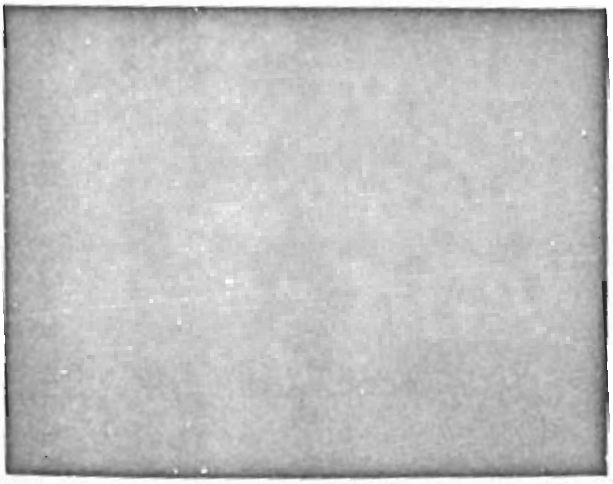

FIG. 2. DNA distribution of MCA-1 cultured cells (nonconfluent).

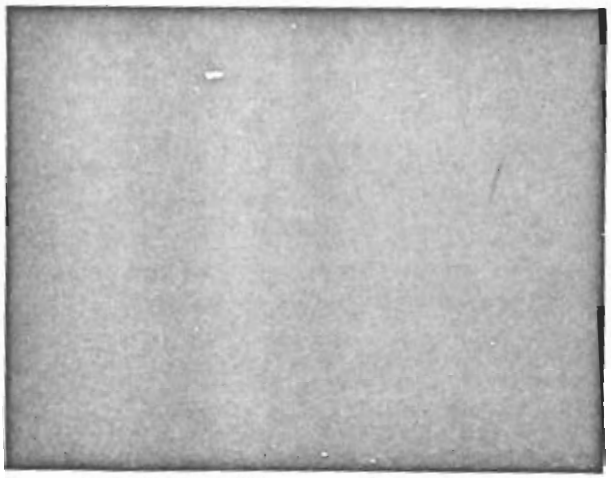

FIG. 3. DNA distribution of MCA-1 cultured cells (confluent).

obvious that addition of varying amounts of G_1 (4N) tumor cells to these specimens will aid in determining the minimal levels of polyploid cells detectable with the currently used sensing and sorting equipment.

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Tumor Cell Identification and Separation

A squamous cell carcinoma (MCA-1) growing in C3H mice was obtained from Dr. R. Malmgren of the National Cancer Institute. Tumors from these animals were removed using sterile procedures, minced, and placed into Ham's F-10 medium supplemented with serum and antibiotics. The resulting cells are epithelial in morphology and, when implanted in C3H mice, produce tumors in approximately one-third the time required for the original line to produce tumors in these animals. The purpose of this work was to determine if the multiparameter cell sorter[1] could distinguish normal tissue from neoplastic tissue based on cellular DNA content measurements and distinguish malignant cells from clumps of lymphocytes. Cell identification instruments designed for cancer screening have failed because of their inability to distinguish malignant cells from clusters of lymphocytes.[2] A great deal of lymphocyte activity is found in many tumors; therefore, it is of paramount importance to be able to distinguish tumor cells from white cells.

To test the capability of the instrument to distinguish these two cell types from each other, an artificial mixture was made from spleen cells and MCA-1 tumor cells grown in tissue culture. These cells were subjected to trypsinization, fixation, and acriflavine-Feulgen staining.[3] Figure 1a and 1b shows that the modal channel of the G_1 spleen cells is less than the modal channel of the G_1 tumor cells, suggesting that it is indeed possible to distinguish the DNA distribution of tumor and spleen cells. A mixture of spleen and MCA-1 cells is shown in Fig. 1c. The mixed cells were then sorted and, as seen in Fig. 2, peak 1 is indeed representative of spleen cells, peak 2 is comprised mostly of tumor cells, and peak 3 is only tumor cells.

To check our capability to detect tumor cells *in vivo*, MCA-1 tumor cells grown in tissue culture were trypsinized and approximately 10^6 tumor cells inoculated subscapularly into each of 6 C3H mice. After 1.5 weeks, the animals were sacrificed and the tumors removed. The tumors were subjected to trypsinization, fixation, and acriflavine-Feulgen staining.[3] Resulting DNA distributions of tumor cells are shown in Fig. 1d. The distribution is comprised of three peaks representing two

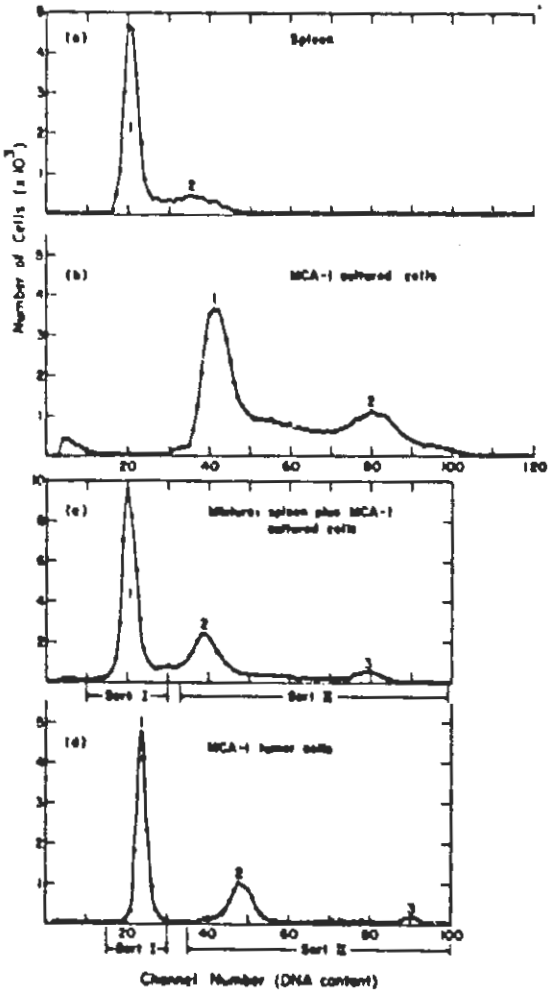


FIG. 1. Modal channel of G_1 spleen cells and G_1 tumor cells: (a) Mouse spleen cells which were trypsinized and fixed in formalin. The cells were stained with acriflavine-Feulgen. Cells in channel 20 represent G_1 spleen cells and those in channel 40 G_2 + M cells. (b) MCA-1 cultured tumor cells, stained in the same fashion as the spleen cells. Note that the G_1 modal channel is 45, indicating that the DNA content of tumor cells is ≈2 times greater than diploid spleen cells. (c) Artificial mixture of cells from (a) and (b). The LASL cell sorter was adjusted to sort cells in channels 15 to 30 into one beaker and those in channels 35 to 100 into another beaker. (d) MCA-1 tumor cells taken from a C3H/Hej mouse. Preperation was the same as in Fig. 1a.

overlapping bimodal distributions. The first peak on the left represents normal diploid cells, either fibroblasts or lymphocytes, within the tumor. The second or middle peak is representative of the G_1 tumor cells and the G_2 + M diploid population, and the third peak is representative of G_2 + M tumor

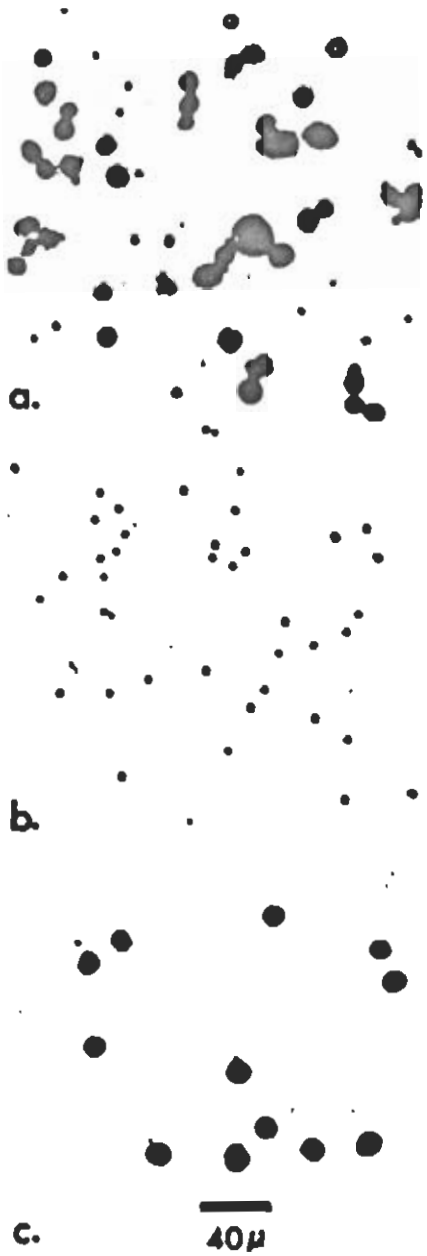
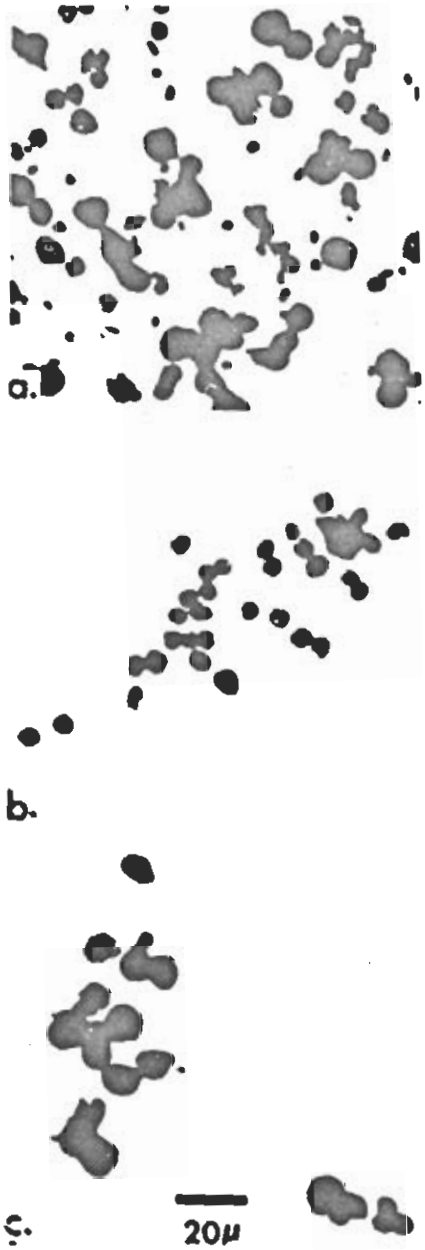


FIG. 2. Photomicrographs of cells described in Fig. 1c: (a) Unsorted mixture of cultured MCA-1 tumor cells and mouse spleen cells. (b) Sort I from distribution shown in Fig. 1c. (c) Sort II from distribution shown in Fig. 1c.

cells. From Fig. 3 it is obvious that tumor cell enrichment is possible if one uses the LASL cell sorter. It is also clear from Sort I (Fig. 3) that diploid cells within the tumor are lymphocytes and not fibroblasts.

While these preliminary studies suggest that measurement of DNA content alone is sufficient to detect tumor cells from normal diploid cells, many



G. 3. Photomicrographs of cells described in Fig. 1d: (a) Unsorted tumor cells dispersed with trypsin. (b) Sort I from distribution described in Fig. 1d. (c) Sort II from distribution described in Fig. 1d.

ber parameters must be investigated. Parameters ch as nuclear-to-cytoplasmic ratio and tumor mem-ne antigens currently are of interest. Perhaps e use of multiparameter analysis (i.e., DNA con-t versus nuclear-to-cytoplasmic ratio[4] might make quivocal tumor cell detection a reality.

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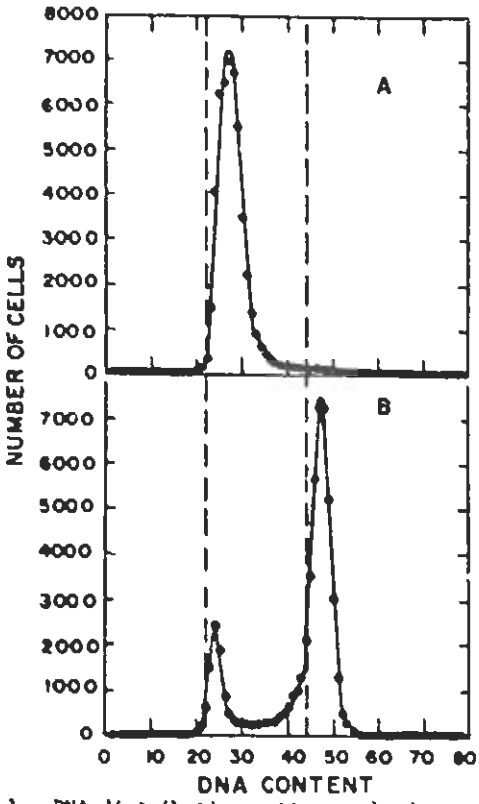
Use of Flow Microfluorometry for Analysis and Evaluation of Synchronizing Protocols and Drug Effects on Cell-Cycle Traverse

A number of cooperative studies with the Cellular Radiobiology Section were undertaken to determine the effects of various protocols on DNA synthesis. Flow microfluorometry (FMF) analysis offers the unique advantage of providing DNA distribution patterns of large numbers of cells, thereby revealing the relative number of cells in the various phases of the cell cycle under a variety of experimental conditions. FMF analysis, coupled with cell enumeration and autoradiography, provides a powerful method for analyzing the effects of various synchronizing protocols or drugs on cell-cycle traverse.

In a collaborative study with Drs. R. A. Tobey and P. M. Kraemer, the effects of three commonly used synchronizing methods, isoleucine deprivation,[1] double-thymidine blockade, and mitotic selection,[2,3] were analyzed and evaluated with respect to their effects on subsequent DNA replication. In each case, we found a given fraction of cells was unable to complete genome replication following synchronization. The term "traverse perturbation index" was designated for the fraction of cells converted to a noncycle-traversing state because of experimental manipulation.[4] Traverse perturbation indices for double-thymidine blockade, isoleucine deprivation, and mitotic selection were 17.0, 12.4, and 5.5 percent, respectively. A typical DNA distribution pattern revealing the noncycle cells following release from double-thymidine blockade is shown in Fig. 1. A knowledge of the traverse pertubation index will permit a direct comparison



IG. 1. DNA distribution patterns showing non-traversing fraction of cells after reversal of the double-thymidine blockade synchronizing technique. Broken lines represent values for G_1 and G_2 + M DNA calculated from controls. Cells were prepared via the double-thymidine blockade technique. FMF patterns in the culture at time of removal of the second thymidine blockade (DNA pattern shown in A) and at 6 hr later, immediately before the first increase in cell number (shown in B). The numbers of cells examined in (A) and (B) were 51,000 and 52,000, respectively.

f the effects of various synchrony-induction protocols on cell-cycle traverse.

In another study conducted with Dr. R. A. obey, FMF techniques were used to evaluate a new rotocol for preparing large quantities of synchronized mammalian cells in late G_1 of the pre-DNA eplication phase of the cell cycle.[5] This technique, a modification of the method described by obey and Ley,[1] employs hydroxyurea (to 10^{-3} M) or ytosine arabinoside (to 5 µg/ml) for 10 hr following release of cells from isoleucine deprivation. ells that are then washed and resuspended in fresh edium without drugs will initiate DNA synthesis nd begin dividing within 7 hr. DNA distribution atterns for cells synchronized by this technique re shown in Fig. 2. This protocol offers the

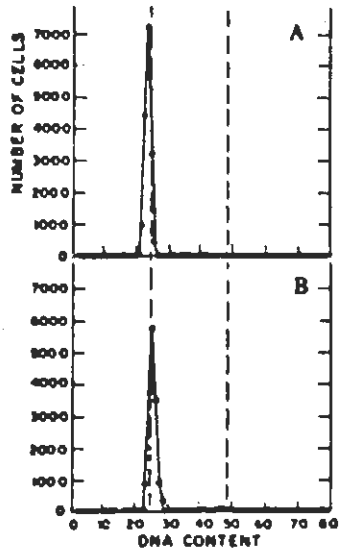


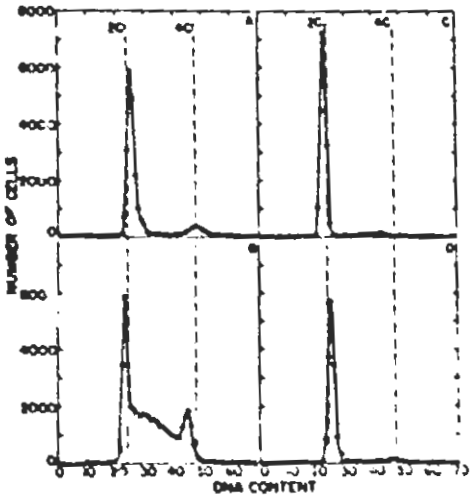
FIG. 2. DNA distribution patterns of various synchronized cell populations: (A) Cells were prepared by cultivation for 30 hr in isoleucine-deficient medium, then resuspended in fresh isoleucine-containing medium containing hydroxyurea to 10^{-3} M; the sample for FMF analysis was removed at 10 hr after resuspension of isoleucine-deficient cells in normal medium plus hydroxyurea. (B) Cells were prepared by cultivation for 30 hr in isoleucine-deficient medium, then resuspended in fresh isoleucine-containing medium containing cytosine arabinoside to 5 µg/ml; the sample for FMF analysis was removed from the culture at 10 hr after resuspension of isoleucine-deficient cells in normal medium plus cytosine arabinoside. The number of cells examined in each culture was 19,000 (isoleucine-deficiency and hydroxyurea) and 17,000 (isoleucine-deficiency and cytosine arabinoside). Broken lines represent values for G_1 and G_2 + M DNA peak values calculated from controls.

advantage of providing large quantities of cells near the G_1/S boundary suitable for studies of biochemical events associated with completion of interphase and initiation of genome replication.

In another cooperative study with Dr. R. A. Tobey, FMF techniques were used in experiments designed to determine effects of several chemotherapeutic agents on cell-cycle traverse.[6] Four agents with differing effects on cell-cycle progression were examined: hydroxyurea, cytosine arabinoside, bleomycin, and camptothecin. Both hydroxyurea (10^{-3} M) and cytosine arabinoside (5 µg/ml) grossly decreased the rate of progression of cells into S phase, resulting in accumulation of cells at the G_1/S boundary. Neither agent

completely prevented cells from initiating DNA synthesis. Bleomycin (100 µg/ml) allowed initiation and completion of genome replication to occur at a early normal rate, but cells accumulated in G_2 and ost cells lost the capacity to enter mitosis. amptothecin (1 µg/ml) reduced the overall rate of ycle progression and allowed a few cells to replicate a complete complement of DNA.

The DNA distribution patterns for cultures released from isoleucine-deficient G_1-arrest and aintained in the various drugs are shown in Fig. 3.



FG. 3. DNA distribution patterns for cultures released from isoleucine-deficient G_1-arrest and maintained for 10 hr in 100 µg/ml bleomycin (B); 10^{-3} M hydroxyurea (C), or 5 µg/ml cytosine arabinoside (D). (A) Control. The culture was maintained for 30 hr in isoleucine-deficient medium, at which time an aliquot was examined via FMF analysis. (B) Bleomycin. The culture was maintained for 30 hr in isoleucine-deficient medium; then the cells were resuspended in fresh, complete (isoleucine-containing) medium supplemented with bleomycin to 100 µg/ml, and after 10 hr a sample was removed for FMF analysis. (C) Hydroxyurea. The culture was maintained in isoleucine-deficient medium for 30 hr; then the cells were resuspended in fresh, complete medium containing hydroxyurea to 10^{-3} M, and after 10 hr a sample was removed for FMF analysis. (D) Cytosine arabinoside. After 30 hr in isoleucine deficient medium, the cells were resuspended in fresh, complete medium containing cytosine arabinoside to 5 µg/ml; 10 hr later an aliquot was removed for FMF analysis. The broken lines represent G_1 and G_2 + M DNA peak values calculated from the exponential culture, which was the source of all cells used in these experiments. The number of cells examined in each culture was (A) 23,000; (B) 47,000; (C) 19,000; and (D) 17,000.

The combination of FMF, cell enumeration, and autoradiographic techniques provides a new approach to studying the effects of chemotherapeutic agents on cell-cycle traverse and provides valuable information to the clinician regarding drug dosage and times of application.

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Application of Biophysical Instrumentation to Animal Disease Diagnosis

The Hog Cholera PK-15 System.--Investigative effort since last year's annual report describing our successful "proof-of-principle" experiments[1] has concentrated on adaptation of this system to fresh tissue techniques. The specific aim is one of direct detection of viral antigen from field submissions.

New problems were encountered in preparing heterogeneous cell samples (pig blood and tissue biopsy material) for flow microfluorometry (FMF) measurements. The very low fluorescence of cells labeled with fluorescently tagged antibodies requires monodisperse cell suspensions that are free of small debris and homogeneous. Procedures were developed for isolation, fixation, and conjugation

>leen and white blood cell suspensions to
re compatibility between these procedures and
:enance of good single-cell suspensions. Nu-
is anticoagulants, red blood cell lysing agents
>rocedures, and several fixation methods were
iated to provide procedures compatible with
:aining single-cell suspensions with minimal
.s and still provide cell permeability to
rescein-labeled antibody molecules. Buffy coat
cultures were also considered. Although satis-
ory protocols were developed, nonspecific bind-
of conjugate to uninfected cells decreased the
tivity of FMF analysis below that of standard
iques. The present level of sensitivity re-
s that 10 percent of the cells in a sample be
ted. Improved conjugate purity would increase
ignal-to-noise ratio and make FMF a useful tool
tudying virus replication in mammalian cells;
is being attempted.

Basic support experiments using the PK-15 cell
m to determine if other areas of FMF applic-
ty exist will continue. For the first time, a
itative evaluation of conjugates can be ac-
ished using techniques developed for the proof-
inciple project. Quality evaluation of com-
ally produced conjugates is important for USDA
sing procedures and for evaluating our own
gates. Conjugate evaluation is based on two
eters: (1) relative cell brightness or the
t of fluoresceinated antibody bound to a cell
mpared with a "standard" conjugate, and
pecificity as evidenced by the ratio of spe-
ally to nonspecifically bound conjugate. Both
eters can be quantitated easily using the FMF.
Although instrumental development on this
ct has been reduced, new techniques of rapid
identification and information processing
>ped in the section have been applied to the
rscence analysis of randomly growing PK-15
infected with hog cholera virus. Recent
>pments permit measuring the ratio of cell
rscence (total amount of fluorescent antibody
) to cell volume. The resulting distributions
1) for control and infected cells are both
rr (coefficient of variation decreases by
timately 2.0), indicating that larger, more
: cells do not produce more virus per cell
maller cells. The improved coefficient of

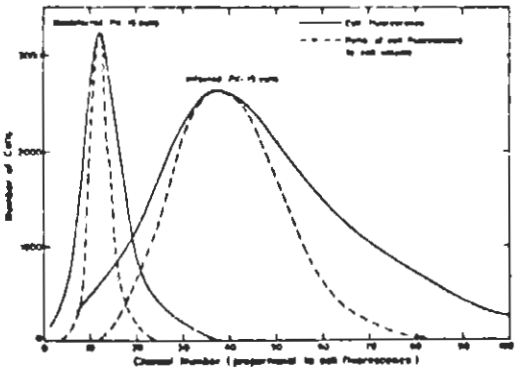

FIG. 1. Distributions for control and infected cells.

variation of the peaks is a result of cancelling the contribution of nonspecific binding which is proportional to cell volume.

Differences in light-scattering patterns were observed for hog cholera infected and noninfected PK-15 cells.[2] Light-scattering measurements were made with a new photometer[3] described elsewhere in this report which uses high-speed film as the detector. Intensity of scattered radiation from suspensions of live infected and noninfected cells is shown in Fig. 2. In the angular range of 2.5 to 4.0°, overlap of the two curves is consistent with light-scatter theory and Coulter volume data that indicate the two cell populations are very similar in volume. At larger angles (4 to 25°), infected

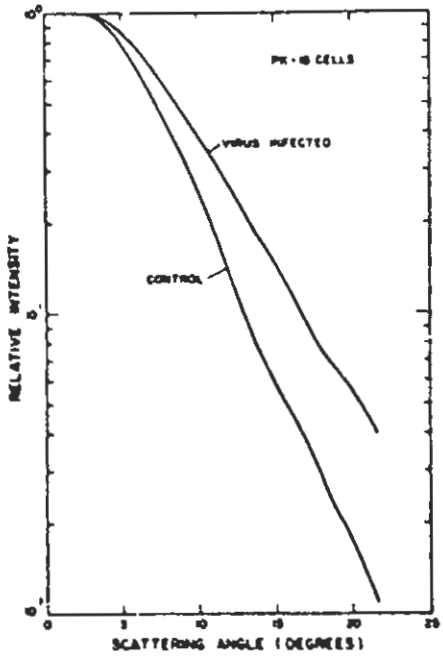

FIG. 2. Scattered radiation intensity for infected and noninfected cells.

cells scatter much more light (about 2.1 times at 20°) than noninfected cells. This difference in large angle light-scatter pattern is believed to be due to some change in internal structure between the two cell populations.

In response to a request for help from the Emergency Disease Task Force of the USDA, an effort is underway to investigate the applicability of our biophysical instrumentation for rapid differential diagnosis of Newcastle disease virus. The outbreak of a velogenic strain of the virus has resulted in the disease being declared a national emergency. The degree of polyploidy in chicken embryo fibroblasts after Newcastle infection is reported[4] to correlate with the virulence of the virus. Therefore, a rapid, quantitative measurement of polyploidy with FMF should provide a rapid screening technique to identify differentially strains of Newcastle disease virus.

Effects of Temperature on the Mitotic Cycle of CHO Cells.--The purpose of this study is to make a detailed investigation into the effects of temperature on different phases of the CHO cell life cycle using FMF techniques for cell-cycle analysis.

Chinese hamster cells (CHO) in suspension culture were grown through two exponential growth cycles at three temperatures: 34, 37, and 40°C. Preliminary data suggest that, during the first cycle of cell growth after a 3° temperature change, most of the effects are on reaction rates. During the second cycle, when a steady state situation has been achieved, the effects are a consequence of changes in concentration of chemical reactants. This is evidenced by an apparent change in cell composition which was measured as a change in cell size. CHO cells growing at other than their optimum temperature were found to increase in volume.

Of particular interest is the observation that, upon a decrease in temperature (37 to 34°C), the percent of CHO cells in the G_1 phase of their life cycle increases from 46 to 61 percent, which is just the reverse for HeLa cells which show a decrease from 48 to 36 percent.[5]

Investigations with the Unique Hixoploid Cell Line, PK-15.--The unique DNA distribution of PK-15 cells, a mixoploid cell line, was reported in last year's annual report.[1] Clones that were diploid and tetraploid in DNA content (i.e., they differ by a factor of 2 in DNA content but not in chromosome number) were isolated from the cell line. Because these two PK-15 clones were isolated from the same culture, we believe they either had exactly the same doubling time or their generation times were not equal but intercellular metabolic factors were being produced that differentially regulate cell doubling. Cells from the two clones were mixed in different ratios and allowed to grow through 6 passages. Their Feulgen-DNA distributions were then measured to determine if the two populations were growing at the same rate and maintaining the same ratios. Because the mixing ratios remained constant, we conclude that the two clones have the same doubling times and respond similarly to cell density. Further experiments are planned to compare these clones with regard to their chromosome banding pattern, response to temperature, and radiation.

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Normal and Tumor Cell Kinetic Studies Using Flow Microfluorometry (FMF)

Methods for studying the kinetics of proliferative cells from both normal and tumor tissues in vivo are of fundamental interest in addition to their possible application to the areas of chemotherapy and radiotherapy. Cell analysis and sorting instrumentation currently under development at the Los Alamos Scientific Laboratory can be used with

relative ease for kinetic studies. Basically, the procedure consists of (a) preparing single-cell suspensions from the tissue of interest using trypsinization procedures; (b) fixing single cells in methanol and formaldehyde mixture; and (c) staining fixed cells with acriflavine (fluorescent-Feulgen) for specific staining of cellular DNA. Other available techniques include the use of (a) the Coulter principle to determine the volume distribution of cells; (b) a signal from scattered light to gate the electronic system which measures fluorescence, thereby eliminating noise signals generated by debris in cell preparations; (c) a multiparameter cell separator to isolate cell populations with different DNA distributions; and (d) a combination of the above systems to measure directly the DNA content per unit cell volume. These techniques are described in detail in our annual report for 1971.[1]

Our preliminary studies indicate that cell preparations for mouse tissues such as skin, intestinal epithelium, liver, spleen, and kidney that are considered important in radiotherapy can be made and their DNA distributions measured by FMF instrumentation. Figure 1 shows DNA distributions for different normal tissues that are considered to be some of the limiting tissues in radiation therapy. The noise caused by debris in the cell preparation and appearing in low channel numbers was not electronically gated out in these measurements. The DNA content of all these normal tissues is about the same and shows that a large fraction of cells is in the G_0 or G_1 state.

Dr. R. F. Kallman and associates[2,3] have concluded from their studies with KHT sarcoma and EMT6 (mammary carcinoma) that a dose of 300 rads induces synchrony in the tumor cells, thereby causing cyclically fluctuating radiosensitivity changes as a function of time after exposure. Although it will be difficult to correlate results of DNA distribution with results of radiation sensitivity, it will be of interest to study the changes in DNA distributions in tumor cells *in vivo* with time after radiation exposure. KHT is a sarcoma that arose spontaneously at the base of the ear of a C3H/KM mouse in 1962 in Dr. Kallman's laboratory at Stanford University. This tumor line can be maintained by serial subcutaneous passage and has been studied extensively.[4,5] A tumor of about 12 mm in

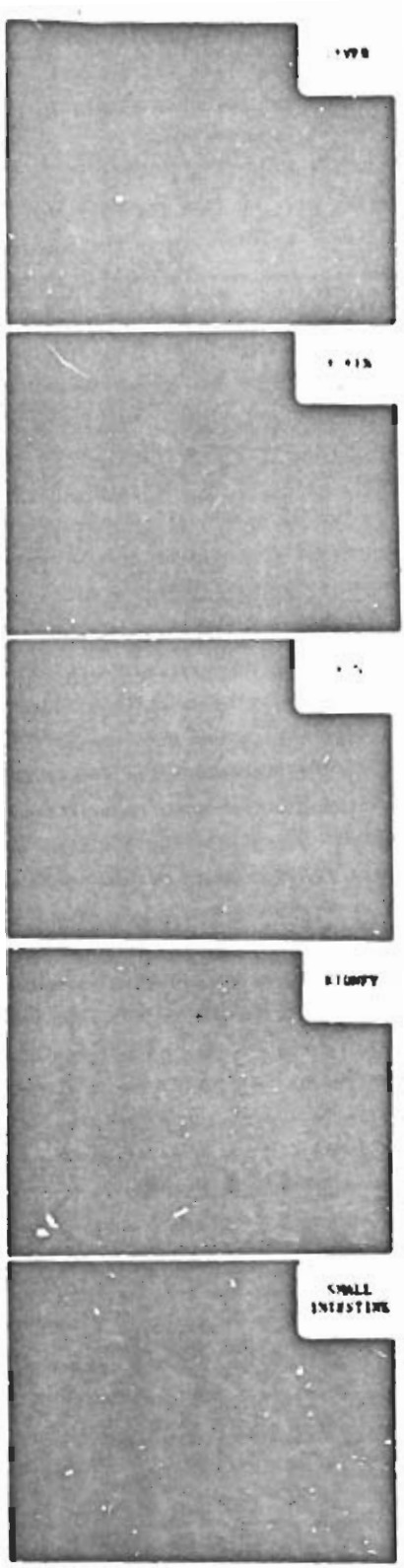

FIG. 1. DNA distribution of normal tissues in mice. The X axis is the channel number (proportional to DNA content in the cells), and the Y axis is the relative number of cells per channel.

diameter from a donor mouse is excised, placed in normal saline, and minced into small pieces about 1 mm^3 in volume. Tumor pieces are then transplanted onto the back of C3H mice via a small incision in the skin of the freshly shaven back. The tumor grows to about 1 cm in diameter on about the 12th day after transplantation. When the tumors were about 1 cm in diameter, the mice were anesthetized (0.1 ml/g body weight of Nembutal), and the tumors were exposed to X rays by shielding the remainder of the mouse. Mouse tumors exposed to 300 rads of 250-KVP X-rays were sacrificed at 0, 8, 12, 16, 22, 24, 26, and 30 hr after X-ray exposure, and cell preparations were made to measure DNA distribution. A few tissues were also exposed to 1500 or 3500 rads of X rays and were sacrificed at 24 hr after exposure. Tumor growth measurements suggest that 3500 rads is a curative dose. Two mice were used for each dose level and fixation time after exposure.

A scattered light signal was used to gate the noise signal arising from debris when the cells were used in the FMF instrumentation. All samples were measured to a total count of 50,000 cells. Figure 2 shows the results of DNA distributions at different times after 300 rads of exposure, where there are significant differences in DNA distribution. Microscopic examination showed that most of the cells were single and that the doublets were less than 5 percent in all samples. The first peak in the DNA distribution represents normal diploid cells in the tumor. The second peak, corresponding to the amount of DNA nearly twice that of normal diploid cells, is due to tumor cells in the G_1 phase. The third peak, corresponding to twice the amount of tumor cells in the G_1 phase, is due to tumor cells in the G_2 + M phase. DNA distributions corresponding to cells in between G_1 and G_2 + M tumor cells are due to cells in the S phase. When the cells in a tumor control sample were separated according to the DNA content in the cells using the cell separator, we found that the first peak in DNA distributions is due to leucocytes. It can be seen from Fig. 2 that the DNA distribution of tumors 8 and 12 hr after exposure to 300 rads, when compared to zero hour after exposure, has more cells in the G_2 + M phase. This could be due to G_2 block caused by radiation. However, it can be seen that 12 hr

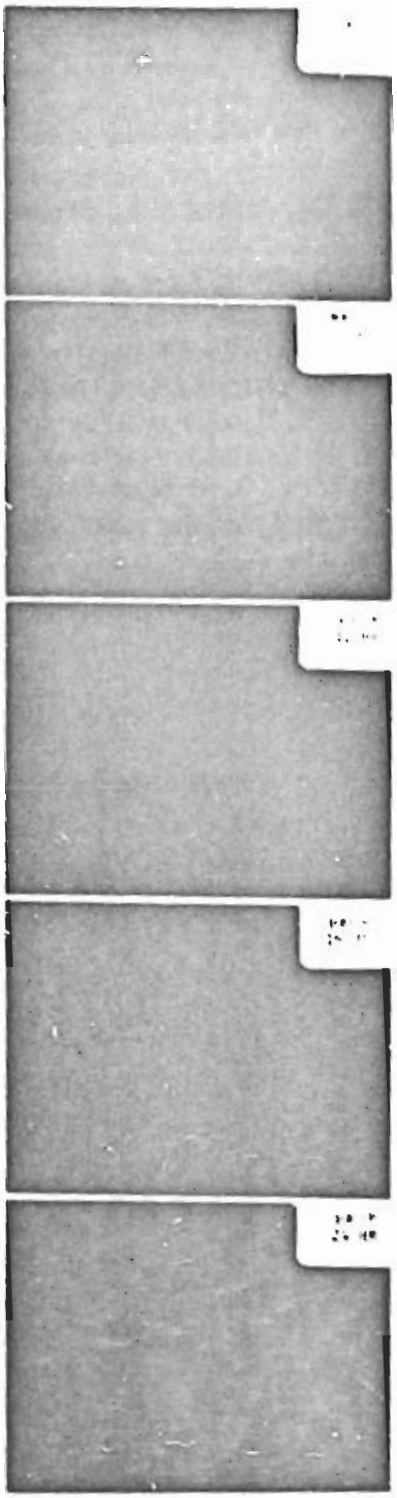

FIG. 2. DNA distribution of KHT sarcoma tumor cells at different times after exposure to 300 rads of X-rays.

after exposure the cells are beginning to divide and that at 24 hr after exposure to 300 rads DNA distribution looks very similar to that of the control. Figure 3 shows that when the cells are exposed to 1500 rads there still seems to be a small increase in cells in the G_2 + M phase at 24 hr after exposure, whereas with 3500 rads nearly 50 percent of the cells are still in the G_2 + M phase. One would expect this trend because with increasing dose the mitotic delay is increased. These results are in agreement with results of Kal[6] using Rhabdomyosarcoma cells with impulse photometer instrumentation. Variations in DNA distribution among any two tumor samples treated in the same way were found to be remarkably similar. Thus, the difference in DNA distribution with dose and time after exposure are real. It can be concluded from these preliminary studies that FMF is a very good tool

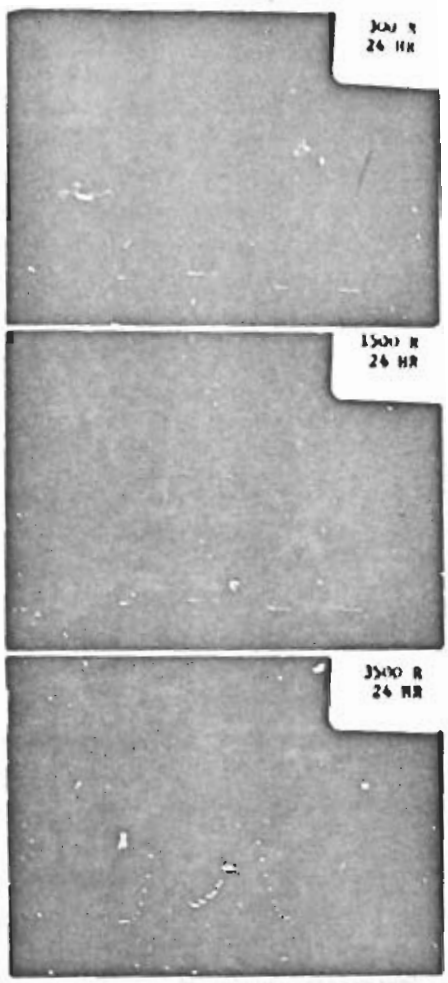

FIG. 3. DNA distribution of KHT sarcoma tumor cells after 24 hr of exposure to 300, 1500, and 3500 rads of X-rays.

to obtain quantitative information on tumor as well as normal cell kinetics after radiation treatment.

We propose to study the progression of normal and tumor cell populations in experimental mammals in addition to cells in culture when they are exposed to ionizing radiations such as X rays and later with negative pions. DNA distributions in patients treated with conventional radiations and later with negative pions could also be made whenever tumor biopsies are available. This type of measurement in experimental animals and possibly in some patients could give important information that may lead to optimum fractionation in radiotherapy and could also be used as a diagnostic modality to judge radiation effects after a given treatment and to plan future exposure.

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