A PROPOSAL FOR AN AUTOMATIC MULTIPARAMETER ANALYZER FOR CELLS (AMAC)

Robert C. Leif

DEPARTMENT OF CHEMISTRY AND INSTITUTE OF MOLECULAR BIOPHYSICS, FLORIDA STATE UNIVERSITY, TALLAHASSEE, FLORIDA

I have been given the privilege of writing both this chapter on flow systems for automated cell analysis and another on buoyant density separations of cells (*1*). The partial or complete physical resolution of mixed cell populations into homogeneous populations will greatly facilitate the programming of automated cell analyzers. Conversely cell analyzers will serve as one of the main means of monitoring the results of cell purification experiments.

The major disadvantage of a flow system for automated cell analysis when compared to one based on pattern recognition is that in the former case there is no direct way to correlate the digital data with the observations of a trained cytologist. In the case of white blood cells this is apparently not insurmountable because Kamentsky (*2*) has already demonstrated that leukemic blood containing an excess of one type of cell can serve to identify the various populations described by the electronic data profile. However, in the case of bone marrow, other tissues, and even blood more direct procedures will be required.

One direct way to solve this problem is to employ a flow system with its own separator (*3–5*). The drawback to this solution is that this greatly limits the types of permissible analysis as the cells are required to end up in a form suitable for subsequent microscopic analysis. It is also possible to identify the cells by phase contrast and to perform the same optic measurements as are utilized in the flow system in a conventional static system. This has been employed with some degree of success by O'Brien (*6*) in collaboration with Kamentsky. However, this would be of no use in a system of morphologically identical cells such as described by Wied *et al.* (*7*).

The heterogeneity of complex mixtures of cells from tissues such as bone

marrow can be considerably reduced by separating the cells by physical techniques. It should then be possible to correlate the digital data obtained with an automated cell analyzer with the limited number of morphological types present in these purified or partially purified populations. In turn, an automated cell analyzer will be utilized to test the purity of the separated cells. Such reciprocal analysis, although not absolute, can at least serve to establish internal consistency.

The technique of centrifugal cytology (*1*, *8*) permits conventional microscopic analysis of the dilute cell suspensions previously obtained by cell separation techniques.

It is probable that an automated analysis system can analyze cells in sufficient numbers to permit the identification of a biologic activity with a given cell at a much lower level of purification than is possible with conventional cytologic techniques. The distribution of the activity even if it is due to only one cell in a thousand can then be correlated with the spectra generated by the cell analyzer.

In this book are discussed the choice and utilization of various descriptors that have been successfully employed to discriminate between various types of cells in recognition studies. For this purpose the descriptors can be any properties which with the aid of a single multipurpose transducer or compatible array of transducers can each be converted into a numeric value.

These numbers can be arranged in an order to form a computer word. If, as has already been shown by Prewitt and Mendelsohn (*9*), this word is large enough, i.e., contains enough nondegenerate measurements or descriptors; it will uniquely characterize the cell. Quite obviously, each cell of a given type is not going to be represented by exactly the same series of digits, but by a set of numbers which does not intersect with the set of numbers which represents the other cell types.

The proposed multiparameter flow system, which is designed for computer intelligence, describes the cells as number words based on physical measurements. In contrast, the current pattern recognition system employed by human intelligence describes the cells according to multiparameter words based primarily on analog descriptors of morphologic criteria. These latter include quantities such as cell size, shape, nuclear-to-cytoplasmic ratio, nuclear arrangement granularity of the chromatin, reactivity of the nucleus and cytoplasm with acid and basic dyes, and the presence of nucleoli and cytoplasmic granules.

In general, flow systems rely on either the direct measurements of single parameters or simple functions derived from single parameters and pattern recognition studies on derived measurements. In the case of pattern recognition studies, as many as 1000 sequential measurements must be reduced

by computation to approximately five parameters. These measurements in order to provide the necessary resolution are performed over small areas, thus restricting the photon flux and consequently lengthening the time necessary for each individual measurement. Since the parameters of flow systems are based on single measurements of the entire cell, the photon flux available is greatly increased and thus the time required for each measurement can be proportionally decreased.

Therefore, if many cells are to be analyzed, it will be more economic and very much faster to utilize flow systems which require of the order of five rapid measurements and minimal computation rather than 1000 slower measurements and significant computation.

Kamentsky and co-workers (*10*) have already demonstrated that flow systems based on optic parameters can detect cells with increased concentrations of nucleic acid at rates of the order of a 1000 cells/sec and at a sensitivity which has permitted them to detect 10 abnormal cells per 100.000 cells (*11*).

Even though Kamentsky has been successful in achieving quadrapartite discrimination between white blood cells with only two parameters, it will be necessary to employ four or five for analysis of complex tissue such as bone marrow. In addition to optic parameters, a flow system should include electronic cell volume measurement, the Coulter effect. It will be demonstrated that the addition of electronic cell volume analysis, besides providing another independent parameter, will also permit the utilization of flashable light sources. These sources, because they only operate when the cell is present, can produce a much greater photon flux than conventional continuous light sources.

The rest of this chapter will consist of a brief review of both the Coulter effect and the optic parameters and a description demonstrating the feasibility and advantages of combining these measurements into an automatic multiparameter analyzer for cells (AMAC).

In contradistinction to the efforts of Fulwyler (*3*, *4*, *12*), no attempt at both the simultaneous analysis and separation of cells is to be undertaken. The AMAC system is designed solely for analysis. In order to provide the greatest possibility for success, the AMAC system cannot be limited to nondestructive analysis procedures. Toxic procedures such as fluorochroming with acridine dyes or the employment of lethal flashes of ultraviolet light will have to be employed. Later, if it is necessary, the AMAC system can be modified for cell separation. Most probably it would then serve as a final step in a sequence of separations achieved by combined density gradient and liquid curtain electrophoreses (*1*, *13*). These latter two procedures are inherently capable of processing much greater quantities of materials than an electronic cell sorter.

I. The Coulter Effect

When a cell or other small nonconductive particle traverses an orifice channel containing a conducting medium, the resistance of the orifice channel will be increased. If a potential is applied to the orifice, then this resistance increment can be measured as an electric pulse (*14, 15*).

The amplitude of this pulse is proportional to the volume of the cell. Both the measurement of these pulses and the application of this technique to obtaining volume distribution spectra of bacteria have been excellently described by Kubitschek (*16*) and Harvey (*16a*) in separate review articles.

The initial theoretical relationship between cell volume and the amplitude of the resistance increment was first derived by Kubitschek (*16b*). Later Gregg and Steidley (*16c*) using a more extensive theoretical treatment derived an expression which corrected for the fields at the boundaries of the spherical cells.

These authors also calculated the shape factors for right circular cylinders and verified their results utilizing models and a tank system with a scale factor of about 500. They also demonstrated that the artifact due to the conformation of the erythrocytes in the aperture was quite significant. This treatment will be utilized in this article. Hurley (*16d*) calculated the effect of shape for oblate and prolate spheroids. Very recently an elegant theoretical treatment for both spheres and elliptical particles has been given by Grover *et al.* (*16e*). These authors also calculated the effect that the gradient of the field at the entrance and exit of the orifice has on the Coulter pulse. They also calculated the effect of profiles of the liquid as it traverses the orifice. The two most common nonideal observed pulse shapes are characterized by, respectively, a rounded and saddle-shaped top. These authors demonstrated that these deviations from the ideal square wave form could be quantitatively explained by assuming trajectories along different radial distances from the axis of the cylindrical orifice at the ends of the orifice where the differences in field gradients occur.

The amplitude of this resistance increment, ΔR, is given by Gregg and Steidley's Eq. (7) (*16c*). The right side has been multiplied by the factor 3/2 in order to correct for the approximations for the fields at the boundaries of a sphere

$$\Delta R = \frac{3}{2}\frac{\delta\zeta_1}{\pi^2 r_2^4}\frac{[1 + 0.3(r_1/r_2)^4 + \ldots]}{[1 - (r_1/r_2)^2]^{1/2}} \tag{1}$$

where ΔR is the resistance increment, δ is the volume of the cell, ζ_1 is the resistivity of the suspending medium, and r_1 and r_2 are, respectively, the radius of the particle and the radius of the orifice. If the value of r_1/r_2 is small, then Eq. (1) reduces to

$$\Delta R = \frac{3}{2}\frac{\delta_{21}}{\pi^2 r_2^4} \tag{2}$$

which is equal to Gregg and Steidley's Eq. (8) except for the presence of the 3/2 term.

These authors have calculated an effective time constant for the capacitance of the cell to be equal to 4×10^{-7} sec.

Presently, the most common instrument available based on the effect is manufactured by Coulter, Inc. primarily for the counting of cells in a clinical laboratory. Brecher *et al.* (*17*) demonstrated the utility and the improved reproducibility of this instrument compared to manual counting with a hemacytometer. The transducer of this instrument consists of an orifice of approximately 100 μ in diameter and length. The liquid column containing the cells is pulled through the orifice by the action of a vacuum which is applied through a mercury manometer.

The first reported mating of a Coulter-Counter with a multichannel analyzer was by Kubitschek (*16b*). This author utilized this pair of instruments to obtain the volume distributions of *E. coli* and *B. megaterium* spores as well as calibrated polystyrene spheres. Lushbaugh and co-workers (*18*, *19*) were the first with similar instrumentation to obtain volume distributions of cells. At Los Alamos they mated the commercially available instrument with a multichannel analyzer in order to obtain cell volume distribution functions. They were evidently the first to utilize a presettable counter in order to obtain a normalized distribution function. Since the area of these functions is constant, it is possible to compare directly the results of different experiments.

The pulse amplitude is measured in terms of volts. A conversion factor of volts or channel number into cubic microns was determined from the packed mean erythrocyte volume of several animals.

These authors also reported a bimodal distribution of human erythrocyte volumes (*20*, *21*). The bimodality of this distribution became more apparent, with increasing aperture current. The smaller cells could be selectively removed by lysis with saponin and if younger cells were labeled with radioactive iron, the label was released at the higher concentrations of saponin, which were lytic for the larger cells.

Van Dilla and co-workers (*22*), also at Los Alamos, were extremely dubious of these bimodal distributions. A bimodal distribution had already been demonstrated by Brecher *et al.* (*23*) to be due to the effect of placing the cells in sodium chloride. Erythrocytes placed in Brecher's version of Eagle's saline were unimodal. Van Dilla and co-workers (*22*) indicated that this bimodality was an artifact generated by the high aperture currents employed by Lushbaugh to increase the resolution of the instrument (*24*). Van Dilla

and co-workers (*25*) as well as Kubitschek (*26*) have stated that this loss of resolution was the result of the limited period in which the cells traversep the orifice compared to the rise time (~8–10 μsec) of the amplifier. The transit time could be varied from 8 μsec with a 56 μ long orifice to 78 μsec with a 400 μ long orifice. Van Dilla and co-workers (*27*) obtained almost perfect Gaussian distributions with a 225-μ orifice and low currents.

These authors (*25*) showed that by lengthening the orifice they could obtain an unimodal sharp distribution of polystyrene spheres. The broadening of this distribution was almost entirely due to the noise broadening of 2.1 channels. The peak is composed of 10 channels with a midpoint at about channel 40. Of these channels, only 4 contained a significant number of counts. This distribution is, however, still wider than the authors' best estimate of the standard deviation of the diameter distribution of the spheres, which was about 0.5%. In a companion paper (*27*) of this series these authors demonstrated that human erythrocytes possessed a unimodal distribution which was skewed to larger volumes.

Additional evidence against the biomodality of the erythrocyte volume distribution was given by Leif and Vinograd (*28*). They demonstrated the similarity of the breadth and profiles of the various volume distribution curves obtained with buoyant density fractions of both young and old erythrocytes, as well as the unfractionated cells. The differences in the average volumes for these distributions was approximately half due to the loss of water, which would occur if the density change was based entirely on dehydration, and half due to unknown reasons. These differences were certainly not of the order to agree with the bimodal distribution described by Lushbaugh *et al.* (*19*).

Adams, Voelker, and Gregg (*29*) report studies with a low-impedance preamplifier and low-noise power supply system. The noise level was, according to these authors, equal to a 3-μ particle. They do not state whether this is the volume or diameter of the particle. However, it is most probably the diameter. The noise level was given as 7×10^{-9} amp. The current was 400×10^{-6} amp and the resistance increment due to the presence of an average cell of 100 μ^3 volume in the orifice was very approximately 1/10.000 that of the orifice. The current decrement due to the presence of the cell in the orifice would then be 40×10^{-9} amp. This ratio of 40 to 7 for amplitude-to-noise level agrees with the distribution given by authors. The minimum between the noise and the cells occurred at about channel 18. The edge of the noise was at channel 15. The rise time of the low noise amplifier was stated to be 5 μsec. These authors state that this is a compromise between minimizing the noise level and maximizing the flow rate. The theoretic duration of the cell in the orifice was 16 μsec. However, in order to minimize the coincidences, the sample had to be diluted so as to yield a counting rate of 330 particles/sec. This corresponds to an effective orifice stay of 40 μsec

and was in good agreement with the effective orifice length calculated from the resistance of the aperture. These authors obtained an agreement within 20% for the optically measured diameters of pollen grains when compared with the pulse amplitude spectra.

Anderson and Petersen (*30*) obtained results similar to those of Adams, Voelker, and Gregg (*29*). The electronic volumes averaged 8% greater than the optic volumes determined by phase-contrast microscopy and 12% lower than the volumes measured of stained cells. Chinese hamster cells and pollen grains were used for this study. The pollen grains could not be utilized for phase-contrast studies.

The shapes of the volume distributions and the volume distributions derived from diameter measurements, Price-Jones (*31*) curves, were in good agreement. The integrating time constant of their amplifier was 5 μsec.

Grover and co-workers in a second paper (*31a*) established the validity of their theoretical approach utilizing polystyrene latex spheres. The mean size of the particles measured by utilizing the Coulter effect and by electron microscopy were, respectively, 11.17 μ^3 and 11.01 μ^3. The coefficients of variation were, respectively, 4.2% and 4.1%. These authors also state that they obtained better agreement between the expected value for the mean volume of ragweed pollen and that measured utilizing the Coulter effect than that obtained by Gregg and Steidley (*16c*).

Spielman and Goren (*31b*) have described the use of a capillary director positioned 2–4 mm from the apertures of the Coulter orifice. These authors claimed to have focused all the particles along the same trajectory through the Coulter orifice with a resultant sharpening of the distribution of polystyrene latex particles.

In a talk at the Biophysical Society Meeting in 1968, Adams (*32*) demonstrated that the shape of the Coulter pulse was dependent on the trajectory of the cell through the orifice. By introducing the cells with a director capillary at the orifice periphery, he succeeded in generating a much flatter pulse than those injected at the orifice center. Previously, the theoretically square, clean, flat-topped pulse had not been published. The pulses usually have sloping leading and trailing edges, as well as, very rough plateaus indicating considerable noise. In another presentation Buchold *et al.* (*33*) indicated that the erythrocytes are deformed as they traverse the orifice. If the cells are fixed in glutaraldehyde and the study performed at 4°C, a unimodal distribution of erythrocytes is obtained.

The effect of coincidences has been analyzed by Princeton and Kwolek (*34*), who demonstrated that the apparent count, n, can be corrected to the actual count by means of the following equation:

$$n = N - (A/2V)(N^2) \tag{3}$$

where V is equal to the total volume of pumped cell suspension, A is the

sensing volume. and N, the true number of particles. These authors also differentiated between vertical and horizontal coincidences. In the first case, the pulse amplitude is equal to the sum of the pulse heights of the two particles; in the second, it is equal to the amplitude of the larger particle.

James and Anderson (*35*) have described a flow transducer for monitoring cultures which automatically mixes the cells with a constant ratio of diluent. This transducer is not suitable for simultaneous optical measurements.

II. General Description of AMAC

The AMAC system will utilize the pulse generated while the cell traverses the Coulter orifice for both the first parameter and to serve to initiate a sequence of fluorescence and light-scattering measurements while the cell is located at a known position in the orifice.

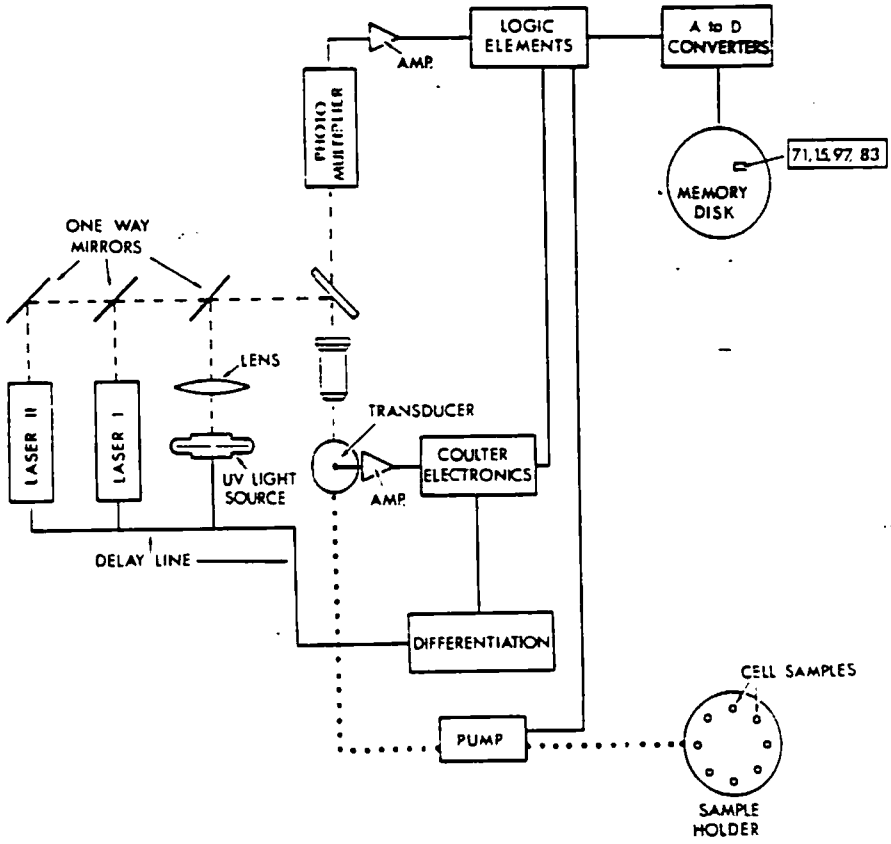

FIG. 1. Block diagram of the proposed AMAC system. The circuits for electricity (—), light (– – –), and liquids (. . .).

Figure 1 is a block diagram of the proposed system. The circuits for electricity, light and liquids are shown. The cells are automatically diluted at the transducer. A feedback from the computer acts as a rate meter and controls the peristaltic pump which delivers the sample. The Coulter pulse controls the pump and the timing of the light sources. The pulse is transmitted via a series of delay lines to the various light sources. The ultraviolet nanosecond flash lamp is shown first and then a pair of lasers. The period between flashes must be greater than 30 nsec. All of the light sources are directed down the microscope tube and through the objective (episcopic illumination) to the Coulter orifice. A simple semitransparent mirror can be utilized; but in order to maximize the energy recovered, a stack of dichroic mirrors such as was employed by Kamentsky and Melamed will most likely be utilized.

The series of pulses from the photomultiplier are digitized by the computer. Probably, several analog-to-digital converters will be necessary as the pulses are too close together to be analyzed with one unit by current techniques. The first descriptor in this system is the amplitude of the Coulter pulse, the second would be the ultraviolet-induced emission, tryptophan fluorescence, and the next two would be, for instance, the acridine orange green and red fluorescences. If each of these measurements were to cover only an energy span of 128, then a 28-bit word would be stored on the memory disk. The contents of the disk would subsequently be ordered as a monotonic series to permit data analysis. Since the feasibility of this entire system depends on the existence of high-intensity light sources, these sources will be described below.

In order to fully utilize the Coulter effect, it will be necessary to employ a dual-beam system composed of two orifices. Diluted cells flow through one (the sample orifice) and diluent through the other (the reference orifice). The problem of the time constant of the orifice due to its capacitance is mitigated by two facts. First, the presently measured time constant includes that of the measuring electrodes and the surface ion double layer (*36*). The current-carrying function can be separated from that of measurement by introducing a pair of charge-sensing electrodes which would eliminate the effects of electrode capacitance (*37*). Second, by decreasing the surface area across which the Coulter potential is applied to solely that of the orifice, it has been found that the body of the present Coulter transducer is the major source of the capacitance and that the orifice only contributes of the order of 20 picofarads.

A sapphire orifice of ~ 102-μ length and diameter was attached with epoxide to the bottom of a glass tube (Fig. 2). This modified Coulter probe assembly was filled with filtered phosphate-buffered saline containing 1% bovine serum albumin and immersed approximately 3 cm in a beaker

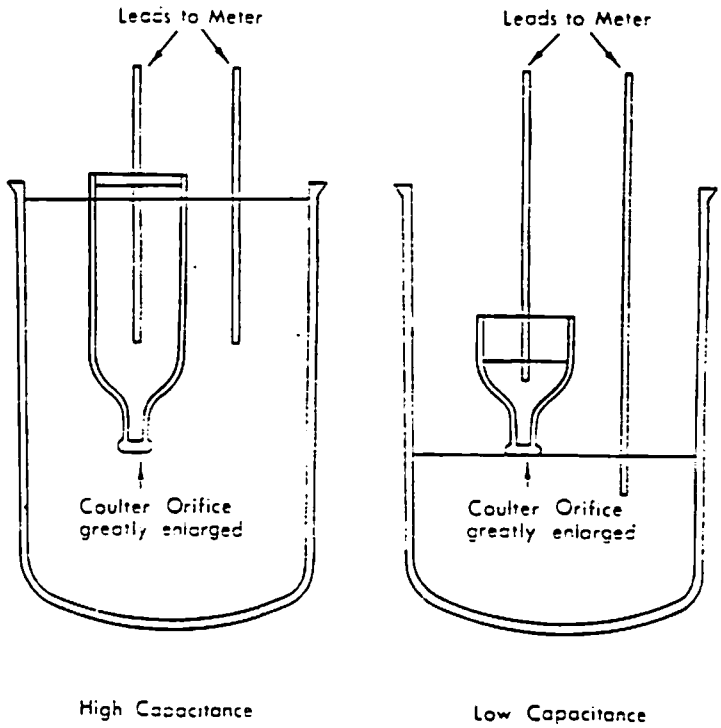

FIG. 2. Arrangement for measurements of the capacitance of the Coulter orifice.

containing the same solution. The initial measurements (*38*) were performed with a Hewlett-Packard 4800A Vector impedance meter, which has a range from DC to 500 kHz. The frequency for a 45° phase angle where the real and imaginary values of the impedance are equal was measured to be 170 kHz. The impedance was measured to be 10,800 ohms. In a second measurement, this frequency was 195 kHz and the impedance was 9000 ohms. The capacitance was calculated to be 150 picofarads. If an equivalent resistor was substituted for the orifice and the leads shorted out in the salt solution, the phase angle was 6° at 195 kHz. These results would preclude any attempts at building the high-speed Coulter transducer necessary for the AMAC, but fortunately they are an *artifact*.

When the measurements were repeated with the orifice just touching the surface of the liquid in the beaker, the phase angle was found to be 12° at 500 kHz and the impedance 11,500 ohms. Since 550 kHz is the end of the range of the vector impedance bridge that had been utilized, the next series of measurements were performed with a Hewlett Packard 481 SA RF H.D. These results are shown in Table I.

The capacitance calculations were performed with the Hewlett Packard Capacitance Inductance Reactance Calculator Slide Chart. These results are by no means quantitative. The only error which has been substantially

TABLE I
RESULTS FOR LOW CAPACITANCE ARRANGEMENT OF COULTER ORIFICE

Frequency (MHz)	Measured impedance (ohms)	Phase angle (degrees)	Capacitance (picofarads)
0.5	15,700	28	22
1.5	11,700	37	11
2.28	10,500	45	10
3.00	9,400	50	6
4.5	6,400	57	10
14	2,900	72	11
35	1,240	74	11
100	1,600	70	24

reduced is that due to the capacitance of the assembly. Even if the worst case assumption is made that the capacitance is 22 picofarads and the resistance is ~20,000 ohms, the frequency response of the orifice is still 2 mHz.

Figure 3 is a block diagram of the AMAC transducer and bridge circuit. Two orifices are utilized for the sample and reference solutions, respectively.

The integral built-in rapid mixer on the sample side of this transducer automatically dilutes the cell suspension. The diluent is delivered at a constant rate by a syringe pump through a 0.22-μ sterile filter. This filtrate is then conveyed via the buffer inlet of the transducer into the dispersing channel which empties into the nine tangential diluent channels. The cavitation of the rapid mixing chamber is maintained by this flow. The cell suspension is delivered at a variable rate by a peristaltic pump and particles capable of blocking the 100-μ orifice are removed with an 80-μ polypropylene filter. After filtration, the suspension will be conveyed via the sample inlet into the sample channel.

In order to maintain a constant counting rate, the velocity of the peristaltic pump is controlled by a feedback loop of the computer. The counting rate will be adjusted to maintain a constant low percentage of coincidences. The effect of coincidences will then be the same for each analysis and the counting rate will be a maximum possible for this level of coincidences. A minimum predetermined number of cells consistent with obtaining a desired statistic analysis will be analyzed. This permits the distribution functions of the various parameters to be compared and the period of each analysis to be minimized. An integral electromechanical counter will measure the volume of liquid metered by the variable speed peristaltic pump during

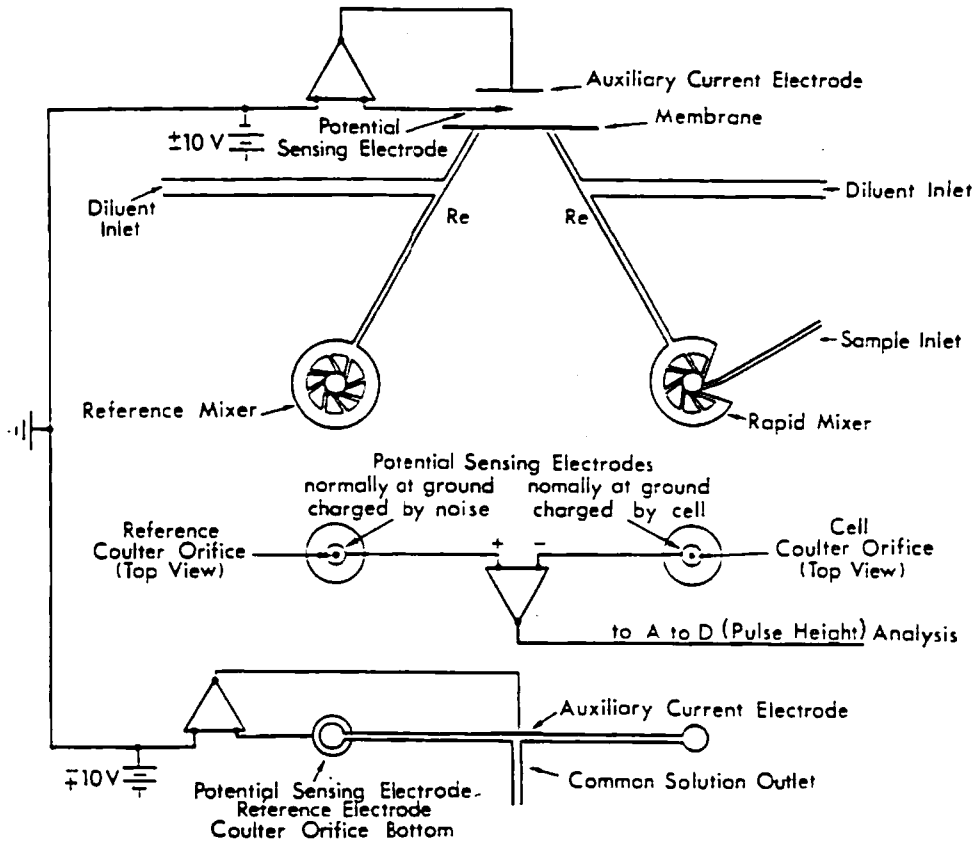

FIG. 3. AMAC bridge circuit and transducer.

each predetermined count of the cells. The concentration of the cell suspension is the predetermined count divided by the volume of the cell suspension delivered to the transducer.

The diluting solution also cavitates in the reference orifice; however, as is shown in Fig. 3 no sample is added. The two buffer channels besides delivering diluent serve as the equivalent resistors of the bridge circuit. The electronic resistance of each of these channels is adjusted so that the sensing electrodes located on top of both orifices are at ground. This balances the bridge circuit; as the resistance of the equivalent resistor Re would now equal that of its orifice. The electrical resistance of the two buffer inlets is increased by addition of a tapered needle. By suitable experimentation with the tapers, it has been possible to build a fairly sensitive equivalent of a precision potentiometer (*38a*).

On the inlet side of the transducer, both the current and the sensing electrodes are isolated from the buffer inlet channels by a membrane. The sensing electrode will be maintained at a potential of 10 volts.

On the common outlet side of both orifices an electrode, most likely to be located on the reference orifice, controls the other current electrode to maintain a potential also of 10 volts but of the opposite polarity to that of the input electrode. The two sensing electrodes are located right at the periphery of the orifice on the buffer inlet side. These electrodes are to be joined by a high-impedance differential amplifier such as the Textronics P6046. These electrodes are not polarized as they have been adjusted to be at ground. They carry a negligible current of $\sim 10^{-9}$ amp only when a cell is in the orifice. This permits these electrodes to be much smaller than the conventional current-carrying electrodes and thus to possess a negligible capacitance. By reducing this quantity, the resistance capacitance time constant of the orifice will be reduced and advantage can be taken of the fast response of the P6046 preamplifier, 50 MHz.* If a particle traverses the reference orifice, it will generate a pulse of the opposite sign to that generated by cells as they traverse the sample orifice. Noise generated in the reference orifice imbalances the bridge circuit in the opposite direction to a real pulse. These noise pulses can be recorded simultaneously with the real data, however, in a separate part of the computer memory. This noise spectrum when corrected for slight differences in the diameter of the orifices can be subtracted from the sample volume distribution.

These sensing electrodes because of their small size will be located at the periphery of the orifice. This location will ensure a steep leading edge for the Coulter pulse.

The swirling motion should direct the cells through the center of the orifice at right angles to the sensing electrode. This geometry is just shifted 90° from that employed by Adams (*32*) when he did obtain an approximately flat-topped wave form. The relative geometry is the same.

In order to reduce the noise to a level of less than 1% of the pulse amplitude with a conventional Coulter system, the ripple in the power supply must be regulated to approximately one part in one million in the frequency range of the Coulter pulse. Although this is probably presently possible with the available low-noise power supplies and filtering techniques, it is by no means the simplest approach. The proposed double-beam system should be more stable and hence much more precise than a conventional system. A differential bridge circuit will be utilized in order to obtain the advantages of a common mode rejection ratio of one thousand to one at 50 MHz of the Textronics P6046 differential probe and amplifier.

The voltage developed across the preamplifier of the AMAC transducer (Fig. 3). ΔE, is the voltage difference between E_c, the voltage drop across

* Instruction Manual P6046 Probe Tektronix, Inc., Oregon (1967).

the Coulter orifice, and half of the drop across the other leg of the differential bridge $E/2$. Since

$$\Delta E = E_c - \tfrac{1}{2}E = \left(\frac{R_e + \Delta R}{2R_e + \Delta R}\right) E - \frac{E}{2}$$
$$\therefore \frac{\Delta E}{E} = \frac{\Delta R}{4R_e + 2\Delta R} \tag{4}$$

The term $2\,\Delta R$ in the divisor is negligible, being of the order of 1/20,000 of $+\,R$.

The common mode rejection capability of the preamplifier will decrease any residual power supply noise a thousandfold.

If Eq. (4) for the proposed bridge-type circuit is combined with Eq. (7) of Gregg and Steidley (*16c*) and all negligible terms are neglected, therefore substituting $L/\pi r_2^2$ for R_e, the resistance of the particle-free orifice, and solving for δ, the cell volume, then,

$$\delta = \frac{8\Delta E \pi r_2^2 L}{3E} = \frac{8\,EV}{3E} \tag{5}$$

where L and V are, respectively, the length and volume of the orifice.

The value of E, the voltage across the orifice, can be measured with a precision digital voltmeter and the value of ΔE is known in terms of channels of the pulse-height analyzer from previous measurements with a precision pulse generator.

The substitution of square-wave AC for the applied DC voltage which is employed in the present commercial version of the instrument will eliminate any possible residual effects of polarization, contamination by electrode products including the evolved gases, and enable a very considerable reduction in the size of the instrument. Laboratory conductivity measurements are routinely performed with AC currents of 60 and 1000 Hz. No gas under these conditions is evolved nor are significant amounts of electrode products or degree of polarization shown to be generated or to occur. One thousand cycle square waves of equal amplitude should still permit the measurement of the Coulter pulse. Some minimal dead time will result from the switching. The pulses which are initiated during the switching operation can be eliminated or, preferably, the switching can be programmed to occur just as the cell leaves the orifice during the dead time of the analysis circuit. The dead time due to switching will certainly not be significant, because in order to limit coincidences to the level of 1%, the aperture must be free of cells for 98% of the time (see below).

The usual orifice for cell counting and obtaining volume distributions is a cylinder of 0.01-cm diameter and length. This corresponds to a volume of $\sim 8 \times 10^{-7}$ cc. However, in order to limit the coincidences to a level of 1%,

this volume must be multiplied 50-fold, or 4×10^{-5} cc, which is 25,000 cells cc. The pulse length which corresponds to the period for the cell to traverse the effective orifice is approximately 50 μsec. The Coulter-Counter is usually set to operate at between 3% and 10% coincidence. The data are then corrected. No consideration of electronic dead time was made in the above calculation. Electronic dead time and the artificial lengthening of the orifice due to the geometry of the applied field are significant in the present Coulter Counter and RIDL* systems.

It has been shown that the limitations in counting rate imposed by electronic circuitry can be greatly relaxed and that the limitation will then be the flow rate of the cells across the orifice. In order to increase this flow rate, the dilute cell suspension will be pushed through the orifice. If a flow rate of 10 ml/min can be achieved and the orifice length halved, it may be possible to count as many as 500,000 cells in 1 min. The acceptable cell concentration for an orifice of given cross-sectional area is simply inversely related to the orifice length, which is directly related to the pulse length.

A pulse of less than 10 μsec duration will permit the elimination of the monostable and any other pulse shaping which must presently be interposed between the amplifier and the analog-to-digital converter of a pulse-height analyzer system. Since a 50 MHz amplifier is to be employed, amplifier rise time is no longer a limiting factor, as it was in the studies of Van Dilla.

The rapid rise time of the amplifier, 9 nsec, and the alteration in field shape described above should result in a pulse which more nearly approximates the ideal square wave. The much steeper leading edge, when differentiated, should produce a sharp peak. By monitoring the pulse duration (time-of-flight measurements) it should be possible to detect and eliminate coincidences. The duration of the Coulter pulse does not significantly depend on the electrophoretic mobility of the cell (see below).

The elimination of extraneous noise from the pulse peak may render possible the detection of high-frequency oscillations due to the difference in resistive increments added to the orifice by various conformations of a nonspherical rotating cell. This rotation could be natural or induced by the swirling effect of the rapid mixer. This ripple effect should be most noticeable in the case of erythrocytes which are biconcave disks. If this ripple is found, it will provide a second parameter in the AMAC system.

It would be of great utility to determine the electrophoretic mobility of the cells as they traverse the orifice by time-of-flight measurements with the analyzer system. Electrophoretic mobility has been shown by microelectrophoresis measurements to be directly related to cell type (*39*). However, the electrophoretic contribution to the total cell velocity through the orifice is

* RIDL Division Nuclear Chicago, Des Plaines, Illinois.

only of the order of 0.1%. Electrophoretic mobility of the cells due to their negative surface charge is of the order of 1 μ/sec per volt-centimeter. The customary 10 volts applied to the 100 μ orifice result in an electric potential of 1000 volts/cm. The velocity of the cells then is approximately 1000 μ/sec. However, as shown above, the cells traverse by fluid flow 100 μ in the range of 15–50 μsec; thus, the contribution of the electrophoretic mobility to the total mobility is at most 0.05%. The present lack of an ultrastable fluidic power supply mitigates against this approach.

These proposed improvements in the electronics for the determination of cell volume, besides improving the accuracy of the measurement of this parameter, will permit the accurate triggering of the proposed optical measurements. In order to ensure reproducibility of these measurements, they must be performed at always the same focal depth in the orifice. Because of the improvement in pulse shape, detection and discrimination against coincident pulses will be greatly improved. These pulses result from two cells being in the orifice at the same time. If the pulse is either a vertical or horizontal coincidence as described by Princeton and Kwolek (*34*), then the AMAC number word produced would not belong to any of the sets which describe normal cells. These odd cells are always suspect. For instance, in the simplest case, a vertical coincidence would simulate a tetraploid cell. As the electronic cell volume analysis is the rate-limiting step in the AMAC system, the suggested improvements in the electronics will serve to greatly increase the rate of analysis by the entire system.

III. Optical Parameters

The selection of the optical parameters to be concurrently analyzed is limited by the necessity for a rapid measurement, of the order of 10 or less μsec, and the fact that the cell only occupies approximately 1/100 of the total illuminated area of the orifice. These considerations argue against any optical systems based on the direct measurement of transmitted light. The signal-to-background ratio would be high and would necessitate complications such as a dual-beam system or the very stable light source and detector system employed by Kamentsky.

Furthermore, absorbance is a logarithmic quantity. Thus the true integrated absorbance cannot be measured directly from the integrated value of the transmittance because of the distributional error (*40*). This error occurs whenever measurements are made at larger than the theoretical limit of optical resolution over a field of varying absorbance, especially in the case where the cell orientation can change. The two-wavelength system of Ornstein (*40*) and Patau (*41*) is not applicable because of the extremely high background described above, nor is the two-area system of Garcia and

Iorio (*42*) which would either require a dual-beam system or at least necessitate the lengthening of the duration of the light pulse. Absorbance measurements have been made with flow systems and are the basis of the rapid cell spectrophotometer system of Kamentsky for cell analysis. Decreasing the size of the orifice would improve the signal-to-noise ratio of the absorbance measurements; however, it would ruin the Coulter measurements and most probably increase the occurrence of orifice clogging. It is possible (*43*) to flow the cells through about a 10-μ area of a 100-μ stream by utilizing a Crosland-Taylor flow cell (*44*) with a slowly tapering constriction which will sharply focus the inner stream. However, very little is gained by this procedure if conventional light sources are employed. The photon flux from a conventional source can not be increased by focusing at less than unity magnification. If lasers are utilized, then the energies available are so great as perhaps not to warrant this extra focusing step. Since Van Dilla (*45*) has demonstrated that the amplitude of light scattered is not equivalent to that of the Coulter pulse, there is no reason to eliminate the latter. Because the proposed transducer automatically dilutes the cell suspension, artifacts due to the lack of reproducibility of treatments with toxic substances as acridine orange can be minimized. The automatic dilution of the cell suspension just before the cells enter the orifice will permit a great diminution in the concentration of unbound dye and consequently the background fluorescence due to the dye.

It would be possible to obtain a parameter related to absorbance by monitoring the dark field emission at various wavelengths. This might be particularly useful for determining the presence and concentration of hemoglobin. However, dark field condensers are generally of low numerical aperture and approximately the same order of magnitude of light would be available to the photomultiplier as in a fluorescence measurement.

In general fluorescence measurements are the most practicable because, first, as has been described in great detail by Udenfriend (*46*) for macroscopic spectrophotometry, fluorescence measurements are usually several orders of magnitude more sensitive than comparable absorbance measurements. According to Ornstein (*47*), for microscopic measurements this is not always the case, as statistical fluctuations due to the paucity of photons can become a major consideration. However, if the laser light sources described below are utilized, this shot noise effect will not be significant. In the case of the ultraviolet fluorescence, where an arc lamp is to be utilized, statistics of photon flux will render the measurement somewhat marginal but still significant.

Second, cellular fluorescence is a directly additive quantity. No scanning procedure to integrate small areas of the cell is necessary as the position of the cell in the cross section of the orifice does not effect the emission by the

cell, nor should the conformation, providing absorbance quenching does not occur. Since fluorescence decay times are of the order of nanoseconds and nanosecond sources are to be employed, many successive measurements will be possible during the microsecond period while the cell is traversing the orifice. The fluorescence decay time itself can be utilized as a parameter or the fluorescence emission pulse can be divided up into segments as it decays in order to differentiate between components with differing decay times. Thirdly, the close relationship between ultraviolet-stimulated fluorescence and cell type has already been established by Brumberg and co-workers (see below).

Since Ruch (*48*) and others (*49*, *50*) have so ably described many fluorescent measurements, only a very incomplete review will be given of several types of measurements which may be of particular use in automated flow systems. This review will be limited to only the fluorescence that can be obtained with unfixed cells (*46*).

Udenfriend (*46a*) in his classic monographs has described and discussed the mechanism of the fluorescence in solution of many cellular substituents. Chen, Vurek, and Alexander (*51*) have performed fluorescence live time measurements of numerous compounds of biologic interest. Chance and co-workers (*52*, *53*) have observed fluorescence of, presumably, NADH in mitochondria. The emission wavelengths were between 400 and 600 mμ. These spectra would not overlap the tryptophan spectra to be described below. The intensity of the fluorescence depended very greatly on the respiratory state of the mitochondria.

Konev (*54*) in his monograph has extensively reviewed the Russian studies of ultraviolet-induced fluorescence of cells. Brumberg and co-workers (*55*) have indicated differences in the ultraviolet-induced fluorescence of the various cell types present in blood. They also claim increased emission from leukemia cells (*56*). Most of these measurements were made from photographs. The fluorescence was stimulated by a quartz-mercury lamp which was filtered by a chlorine–bromine filter to obtain light in the region of 250–280 mμ. Fluorescence changes were also noted in the cells of the bone marrow and the peripheral blood after radiation injury (*57*, *58*). Brumberg *et al.* (*59*) have shown in the case of dividing cells that the fluorescence is increased as compared to that of nondividing cells and that it increases from early prophase and finally is at its highest intensity in the middle of the division process in metaphase and then the fluorescence slowly decreases. They state that not only the nucleus, but also the entire cytoplasm of the dividing cell, fluoresces. The chromosomes are non fluorescing.

Brumberg and co-workers (*55*) originally ascribed this fluorescence to free nucleotides. Konev (*54*, *60*) demonstrated that this fluorescence is due to emission by tryptophan which is fluorescent, itself, and accepts excitons

from phenylalanine and tyrosine. Both the Russian (*54, 61*) and English school (*62, 63*) are evidently in agreement that most, if not all, of the ultraviolet-stimulated emission spectra of proteins is that of tryptophan. There is evidently disagreement about the mechanism of exciton transfer. Brumberg's school (*64*) apparently seem to accept tryptophan as the emitting substance, but argue that coenzymes are involved in quenching this fluorescence and thus it is still coupled to the state of the small molecules in the cell. If this is correct, then the fluorescence of these small molecules should be enhanced.

Brumberg and co-workers (*55*) have demonstrated that the erythroid cells have a quenched fluorescence, which is due to the presence of hemoglobin. The exciton is transferred from the tryptophan to the heme moiety (*54, 62, 65*). Hemoglobin has been reported not to fluoresce (*65*). However, the form of hemoglobin, whether it is oxy or reduced, without ligand or ferri, is not discussed in either Teale's (*62*) or Konev's (*54, 60*) papers.

Very recently Barenboim, Domanskii, and Turoverov (*65a*) described and provided photographs of the ultraviolet fluorescence of cells which is very intense in the mitochondria and nucleoli.

The differential fluorescence of acridine orange due to the nucleus staining green and the cytoplasm red (*66*) has been utilized with fixed cell preparations as a clinical tool (*67, 68*). Brumberg *et al.* in their ultraviolet studies (*59*) have utilized the green fluorescence of the nucleus and red fluorescence of cytoplasmic particles to identify the cells. They claim that the ultraviolet and visible fluorescences do not interfere. In the case of living cells stained with acridine orange, Zelenin and Lyapunova (*69*) have demonstrated that the protoplasm of amniotic epithelium produces a faint dark green fluorescence, upon which flame red granules, mainly distributed in the perinuclear zone, stand out sharply. The nuclei appear as roundish, bright green homogeneous bodies in each of which there are several even brighter light green nucleoli. These studies were made with tissue culture cells. These authors claim that the brightness of the fluorescence of nuclei of dividing cells is considerably greater than that of interkinetic resting nuclei. They also state that the chromosomes lightly fluoresce green.

Wolf and Aronson (*70*) in studies of chick embryo heart fibroblasts, epithelium from newborn rabbits, and choridodal melanocytes from adult rabbits observed that the fluorescence of these cells was limited to the nucleus and was green; however, they report a photoinduced appearance of red granules with concomitant photo-killing of the cells. After sufficient illumination, the center of the nucleus became orange and the entire cytoplasm fluoresced intense, diffuse orange.

Hill, Bensch, and King (*71*) report that the nuclei of L-strain fibroblasts fluoresced green and that red particles are apparent in the cytoplasm. In

HeLa cells. Robbins, Marcus and Gonatas (*72*) have observed that treatment with high concentrations of dye for short time intervals, as little as 1½ min, induced the entire cytoplasm to fluoresce red. If the cells are incubated, dye was concentrated into particles, which Koenig (*73*) suggests are lysosomes.

Van Dilla *et al.* (*43*) have described elegant results with an automated Feulgen fluorescence system which analyzes 50,000 cells/min. The distributions were very sharp with a low coefficient of variation. The system was based on a continuous argon laser and a focusing lens. No microscope optics were necessary for these studies.

Kamentsky (*5*) has already demonstrated the utility of light-scattering studies in flow cell analysis. Since light will be scattered by the cell any time an optical measurement is performed, this parameter can be added to the system without the addition of any extra light source. It is possible that this parameter may be related to morphology if the wide-angle scattering by the organelles and granules of the cells is detectable.

In "Light Scattering by Small Particles" (*74*) Van de Hulst describes the scattering distribution expected for transparent cells in solution with a refractive index which does not differ very much from that of the media. He terms this the anomalous region because the scattering intensity is small, even though the particles are many times larger than the wavelength of light. The scattering intensity depends on the reciprocal of the square of the wavelength, rather than on the fourth power as occurs with small particles. This scattering by cells is limited to much smaller angles, as has been demonstrated by Verveen (*75*), than that observed with macromolecules.

Van Dilla (*45*) has obtained light-scattering distribution functions of cells by means of pulse-height analysis of the output of a Vicker's cell counter. This unit, which is based on the design of Crosland-Taylor (*44*), counts the light pulses induced by the dark field scattering due to individual cells, which are contained in a 10 μ wide flowing stream. The light-scattering distributions of ragweed and paper mulberry pollen were practically superimposible, in contrast to the volume distributions, which are distinct. The distance between the scattering distribution of leukocytes and erythrocytes was considerably reduced compared to the volume distributions.

Mullaney and Dean (*76*) reported on a theoretical study which indicated that the scattering of light by single cells at low angles, below 2.00°, is due to diffraction and should be proportional to cell volume.

Mullaney *et al.* (*77*) reported on experiments with a Crosland-Taylor instrument illuminated with a laser to measure the lowangle light scattering of the cells as they flow through the instrument. The small-angle scattering by polystyrene latex spheres does agree with theory as does fixed unstained and stained cells. However, the scattering by unfixed cells is greater than that

predicted. Van Dilla also indicated the possibility of combined electronic volume measurement and light scattering utilizing the Crosland-Taylor instrument.

The light source requirements for the AMAC are rather stringent and, in fact, up until recently were thought to be an impossibility. Firstly, a repetition rate of at least 1000 and preferably 10,000 cycles/second will be necessary. Secondly, each individual flash must supply at least 4×10^5 photons to the cell, in order that at a quantum efficiency of 10% and a 25% efficiency of the microscope light-gathering system for 10,000 photons to be available to the photomultiplier. This number of photons is necessary to perform the measurement with 1% accuracy. Thirdly, the flash should be of less than 5 nsec duration.

In the case of the tryptophan fluorescence of protein, no laser described in Table II emits the proper wavelength except the double argon, which

TABLE II
LASER PROPERTIES

	Neon ion		N_2	Doubled	Doubled
	Pulsed	Mode-locked	Super-radiant	Ar ion	ruby
Pulse width	100 nsec	100 psec	1–10 nsec	100 psec	50 nsec
Repetition rate (pps)	10^3	10^8	10–10^3	10^8	5
Peak power (watts)	10	10	10^3–10^5	0.10	10^5
Wavelength (Å)	3324	3324	3371	2572	3471

has insufficient output. Table III lists the various types of light sources with relevant characteristics. Since no laser is available, the choice lies between the lamps. The high-pressure spark lamps because of the very narrow luminous gap width, 0.3–0.7 mm, would be most suitable; as focusing these incoherent light sources to anything below unity magnification does not increase the photon flux. The spectral distribution from one of these light sources is shown in Fig. 4. The number of watts per steradian emitted is given at the top of each block. Approximately 18 watts are available in the protein excitation band (the first two blocks). According to Dr. S. A. Pollack of TRW who made this lamp and supplied these tables, this lamp emits 10^{10} photons per steradian in the wavelength band of interest.

In order to estimate the light available to the cell, the following worst case assumptions will be made: Ninety percent of the light will be lost by the optical components. Only the light from one steradian will be available.

TABLE III
LIGHT SOURCE PROPERTIES

	Arc lamps		High-pressure spark lamps	Q-Switched and/or TVR solid state lasers	Mode-locked gaseous lasers	Superradiant lasers
	Low pressure	Medium pressure				
Rise time (10% to 90%)	3–4 nsec	0.05–0.1 μsec	0.5–1 nsec	2–3 nsec	100–300 psec	A few nanosec
Fall time (10% to 90%)	3–10 nsec	0.1–2 μsec	2–5 nsec,	2–3 nsec	100–300 psec	
Pulse half-width	4–20 nsec	0.1–1 μsec	1–3 nsec	5–50 nsec	100–300 psec	0.5–10 nsec
Overall peak power (watts)	20–350	$10^3 - 10^4$	100–500	10^6	10–300	$10^3 - 10^6$
Repetition rate (pps)	$10–10^4$	$10–10^3$	$10^3–10^6$	1–10	10^8	$10–10^3$
Luminous gap length or beam width (mm)	5–10	5–10	0.3–0.7	10	2–3	1–5
Spectral composition	Lines and/or bands which can be concentrated in a desired region between 2000 and 10,000 Å		Continuum from UV to IR	Single line	A few single lines	Single line

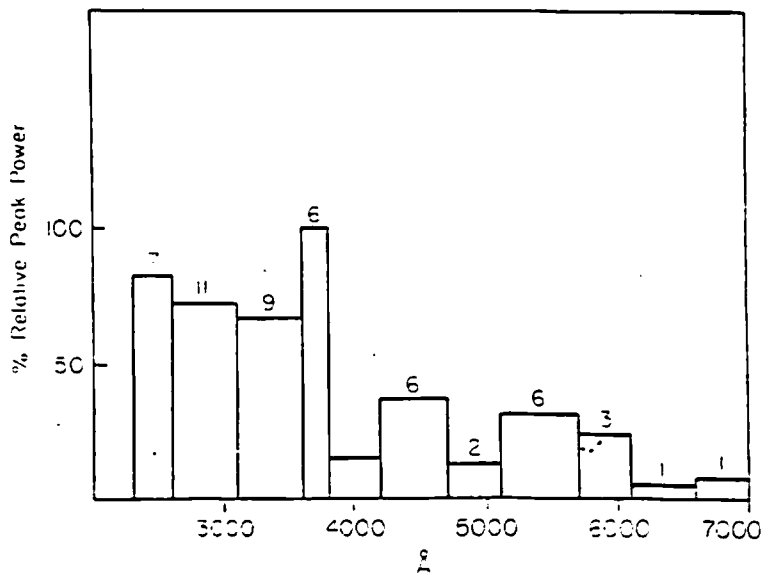

FIG. 4. Spectral distribution of the TRW high-pressure arc lamp.

The image of the gap of the spherical light source will be focused at unity magnification. An average cell of 10 μ diameter will occupy 1/100 of the area of the orifice, or 1/2500 of the area of the image of the light source. The photon flux then available to the cell will be 4×10^5 photons. The microscope objective should gather approximately one-fourth of the emitted photons so that a quantum efficiency of 10% will yield 10^4 photons.

Dr. W. Simmons of TRW (Thompson-Ramo-Wooldridge) gives a much higher estimate of light available to the orifice, namely, one-fourth of the total light from two steradians. Utilizing his estimate, an average cell occupying 1/100 of the orifice area would receive $\sim 10^7$ photons.

In the case of longer wavelengths, the meeting of light source requirements for AMAC no longer requires the maximum capacity of the present state of the art. Table IV describes various types of lasers. In the case of Super-Radiant lasers, all the photon flux can be focused on the orifice. The 90% light loss in the optics becomes very pessimistic at longer wavelengths; however, even using this figure, of the order of 10^{13} photons will be available to the orifice and 10^{11} photons to a 10-μ cell. A quantum efficiency of 4×10^{-7} would still permit the proposed measurements.

The energies available have become so great that by utilizing an episcopic illumination system similar to the one proposed for AMAC the laboratory of D. Glick (*78*) succeeded in vaporizing small sections of cells for spectroscopic elemental analysis.

TABLE IV

TYPICAL PULSED LASER CHARACTERISTICS

Laser type	λ (Å)	Typ. peak power (watts)	Typ. pulse Length	Energy per pulse (photons)		Comments
Ion Lasers						
Neon	3324	0.3	1 μsec	3×10^{-7} joules	(5×10^{11} photons)	True diffraction-limited point sources
Argon	5145	4.0	1 μsec	4×10^{-6} joules	(1×10^{13} photons)	
	4880	2.0	1 μsec	2×10^{-6} joules	(5×10^{12} photons)	
Krypton	5682	0.3	1 μsec	3×10^{-7} joules	(8.5×10^{11} photons)	
	6471	0.5	1 μsec	5×10^{-7} joules	(1.6×10^{12} photons)	
Metal Vapor Ion Lasers						
Cadmium	4416	These lasers are presently still in a research and development state. They are typically very low power devices. Principal difficulty lies in their high ambient operating temperatures (>500°C)				
Lead	5372					
Mercury	6150					
Superradiant Lasers						
Nitrogen	3371	100 kW	20 nsec	2×10^{-3} joules	(3.4×10^{15} photons)	Not true lasers. All light can be focused into 0.01 mm² area spot. Wavelength spread typically a few Å
Krypton	8104	1 kW	5 nsec	5×10^{-6} joules	(2×10^{13} photons)	
Neon	5401	10 kW	10 nsec	1×10^{-4} joules	(2.7×10^{14} photons)	
Copper	5106	10 kW	16 nsec	1.6×10^{-4} joules	(4×10^{14} photons)	
Solid Lasers						
Nd^{3+}:YAG	10,600	2 kW	0.25 μsec	5×10^{-4} joules	(2.5×10^{15} photons)	Can be doubled to $\lambda = 5300$ Å, losing 2–3 orders of magnitude in power
Semiconductor Lasers						
GaAs	~8500	1 watt	0.3 μsec	3×10^{-7} joules	(1.3×10^{12} photons)	Similar to superradiant lasers. Must be cooled to liquid N_2 temperature

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