LA-3848-MS
UC-48, BIOLOGY
AND MEDICINE
TID-4500

LOS ALAMOS SCIENTIFIC LABORATORY
of the
University of California
LOS ALAMOS • NEW MEXICO

Report written: September 1967
Report distributed: January 23, 1968

Biological and Medical Research Group (H-4) of the Health Division--Annual Report July 1966 through June 1967

Compiled by

D. G. Ott

This LA...MS report presents the status of biological and medical research in Group H-4 at LASL. Previous Annual Reports in this series, all unclassified, are:

LAMS-2780
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Printed in the United States of America. Available from Clearinghouse for Federal Scientific and Technical Information National Bureau of Standards, U. S. Department of Commerce Springfield, Virginia 22151

Price: Printed Copy $3.00; Microfiche $0.65

CHAPTER 1

INTRODUCTION

This report summarizes the past year's activities of the Biological and Medical Research Group at the Los Alamos Scientific Laboratory. The arrangement is by sections; however, there is considerable overlap in many instances. Work which has been published or submitted for publication has not been rewritten. Abstracts of these journal articles and manuscripts are given at the end of each section's chapter and constitute most important parts of this report.

The Biology and Medicine program includes those types of investigations which take advantage of the Laboratory's unique facilities and talents and which are of fundamental scientific significance in radiation biology. The staff and facilities are sufficiently flexible to assume without delay programmatic problems as they may arise within the Laboratory or elsewhere. Over the past few years increased emphasis has been placed on studies at the molecular and cellular levels, and interesting results from these new projects are appearing at an increasing rate.

During the next year most of these projects will continue with the total group effort remaining at about the present level. Consideration will be given to initial specific planning of facilities and experiments for the proposed biomedical research addition to the Los Alamos Meson Physics Facility.

The previous annual report of the Biological and Medical Research Group for the period July 1965 through June 1966 appeared as Los Alamos Scientific Laboratory Report LA-3610-MS (1966).

The current group organization and personnel are shown in the following table.

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BIOMEDICAL RESEARCH GROUP

W. H. Langham, Ph.D., Group Leader
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E. M. Sullivan, Secretary
J. H. Montague, Clerk-Typist

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W. D. Currie, Ph.D.*
J. M. Machinist, Ph.D.**

ARMU Doctoral Candidate

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N. J. Basmann

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S. Cordova
L. Ortiz
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F. Benavidez
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J. H. Larkins, B.S.
P. F. Mullaney, Ph.D.
J. D. Perrings
T. T. Trujillo, B.S.

Electronics Technicians

M. T. Butler
L. J. Carr

*Terminated 9/66.

**Began 1/67.

***On leave 9/66-6/67.

****Terminated 5/67.

THE FLUORESCENT CELL PHOTOMETER: A NEW METHOD FOR THE RAPID MEASUREMENT OF BIOLOGICAL CELLS STAINED WITH FLUORESCENT DYES

(M. A. Van Dilla, P. F. Mullaney, and J. R. Coulter*)

Introduction

Fluorescent staining is a widely used technique in many types of investigation of both normal and pathological cells and subcellular components. The usual visual detection method with the fluorescence microscope is slow and often quite subjective. A fluorescent cell photometer is being developed which quantitatively measures the fluorescent light emission of individual dyed cells at high speed, typically 10^4 to 10^5 per minute. It is expected also to provide cell size information via small angle light scattering and, in addition, act as a new sensor for cell sorting. Preliminary results have been obtained with inert particles and mammalian cells treated with the fluorescent dyes fluorescein and acridine orange. Fluorescence intensity distributions have been compared with volume distributions taken with a Coulter spectrometer. Cell counts on the same sample obtained by both methods agree within a few percent, indicating that every fluorescent particle is being sensed.

Methods

The fluorescent cell photometer is, in principle, similar to a conventional photoelectric fluorometer with the exception that instead of a d.c. measurement of a fluorescent solution we line up cells stained with a fluorescent dye, flow them one at a time at high speed across the exciting light beam, and measure the amount of fluorescent light emitted by each cell. The arrangement is shown in Fig. 1.

The light source is a PEK-110 compact arc mercury vapor lamp mounted in a Tech/Ops Model 580-12 lamphouse. Two Bausch and Lomb microscope objectives (f/1, 32 mm, f/3) form a condenser of unity magnification; a second pair of similar Gaertner objectives form a projector, again of unity magnification. Light from the mercury arc is imaged on a slit 125 μ wide by the condenser. The slit image in turn is projected on the cell stream in the flow chamber. A Zeiss BG-12 blue filter transmits light in the 3500- to 5000-Å band only; this is the exciting light. Fluorescent emission at longer wavelengths from a cell is collected by a pair of Simpson f/1.6 projection lenses at unity magnification and transmitted by a yellow filter (Corning 3-69) to the photocathode of an ITT multiplier phototube (type FW-130). The light pulse thus generated is

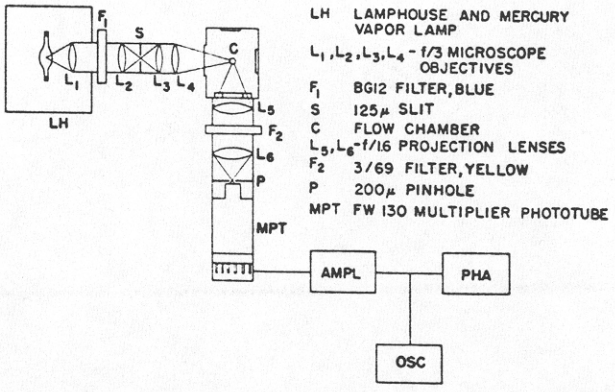

Fig. 1. Block diagram of fluorescent cell photometer.

*LASL Shops Department.

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amplified and analyzed for pulse height by a Victoreen SCIPP multichannel analyzer, the result being the distribution of fluorescent light intensity for the cell population examined (i.e., distribution of dye content).

One of the crucial parts of this system is the flow chamber (Fig. 2), which follows the design used in the Vickers Instruments J12 cell counter which, in turn, follows the earlier design of Crosland-Taylor (1); the basic idea is found in a paper on laminar and turbulent fluid flow by Reynolds (2) published in 1883. A laminar flow of any convenient liquid (i.e., water, saline, sucrose solution) is established in the cylindrical bore section of the flow chamber [labeled (1) in Fig. 2, diameter 3.2 mm]. The flow then enters a smooth transition region to a short cylindrical section of smaller bore [labeled (3) in Fig. 2, diameter 0.5 mm]. The fine hypodermic tubing (internal diameter 0.5 mm) on the axis of the large cylindrical bore serves to introduce the cell suspension smoothly into the faster flowing fluid.‡ In this way, the sample stream flows along the axis of the chamber without mixing with the main or "sheath" flow. The Reynolds number in section (1) of the flow chamber is typically 200 and well below the critical value of about 2000 which is the "boundary" between laminar and turbulent flow. In the small bore region of section (3), the Reynolds number is typically 1000 and is thus closer to the value at which turbulence can develop. The effect of the constriction is to decrease the diameter of the cell suspension stream to about 75 μ. At a typical cell concentration of 30,000/ml, the average separation of cells is 1 cm, and the chance of two cells passing a given point simultaneously is very small. Thus, the cells have been lined up for exposure to the exciting light one at a time.

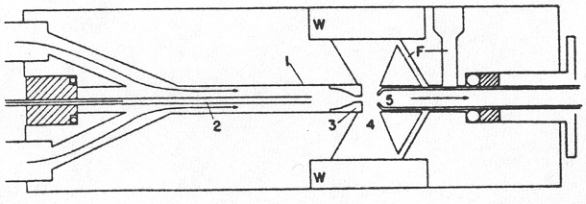

Fig. 2. The flow chamber (scale 4 times actual). W = window; F = flushing system for bubble removal; 1 = sheath flow; 2 = sample injection; 3 = nozzle; 4 = quiescent (viewing) region; and 5 = exit tube.

The flow jets out of the nozzle (3) across a relatively quiescent region (4) and exits via the tube (5), of the same internal diameter as the nozzle outlet. Laminar flow is maintained across the quiescent region. The flow chamber is of square cross section with 4 windows, allowing a perpendicular view of the fluid flow. Exciting blue light enters through one window, and the fluorescent emission at 90° is viewed through another window; the cone angles allow use of f/1 optics.

In this way a cell stream of very small diameter can be generated with much larger tubes, virtually eliminating plugging problems. With the windows far from the cell stream, light scattered by dirt on windows cannot enter the detector, and the effect of multiple reflections and window fluorescence is minimized.

A pressure differential of 5 in. of mercury is maintained across the chamber, producing a sheath flow of 30 ml/min. The sample rate of 0.5 to 1 ml/min is produced by gravity flow. The cell suspension is replaced with ink for alignment purposes.

The image of the arc produced by the illumination system is focused on the sample stream as it crosses the quiescent region. The illuminated and sensed volume (sensitive volume) is then a cylinder of diameter 75 μ and height 125 μ. Cells pass through this sensitive volume one at a time at a rate of 10^4 to 10^5 cells/min with a transit time of 30 μsec. Typical cells

‡In Reynolds' classic work, he introduced colored water into clear water to visualize the onset of turbulence.

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have diameters of 10 to 20 μ and, therefore, an individual cell occupies less than 1 percent of the sensitive volume. Other fluorescent materials contained within the sensitive volume, in addition to the cell of interest, can give rise to a noise signal. It is, therefore, advantageous to reduce the sample stream diameter and to narrow the optical slit used in the illuminating system, cellular dimensions being the lower limit. Part of the present effort is in this direction.

The multiplier phototube must be sensitive to long wavelength light (> 5000 Å), suggesting the S-20 type photocathode. In addition, low noise is crucial because of the low light levels being sensed. Tests of several multiplier phototubes indicated that the ITT Model FW-130 was best. The very small effective diameter of the photocathode (2.5 mm) greatly reduces thermionic and sidewall noise. In addition, the effective photocathode can be moved about on the actual (and larger) photocathode by magnetic deflecting coils, greatly aiding lineup of the system. Fluorescent light from the sample stream is focused by unity magnification optics on a 200-μ pinhole just in front of the photocathode. In this way the field of view of the FW-130 is limited so that stray light from outside the 75 x 125-μ sensitive volume is minimized.

The dyes used (acridine orange and fluorescein) absorb strongly between 4000 and 5000 Å with an absorption maximum near 4900 Å. The fluorescence emission is contained in a wide band extending from 5000 to 6000 Å. The brightest conventional sources of 4000 to 5000 Å light available are the short arc mercury vapor lamps such as the PEK-110 with an electrode separation of 300 μ and a brightness of 140,000 candles/cm^2. Since image brightness cannot exceed object brightness (3), this represents an upper limit on cell stream illumination with conventional sources. If f/1 optics are used to collect the light from a point source placed at the focus, 1/16 of the emitted light will be collected, and this represents a practical upper limit on aperture.

Results

The first positive results were obtained with ragweed pollen stained with acridine orange. These particles are relatively uniform in size and pick up the dye quite effectively. They are about 20 times as bright as CHO cells treated with fluorescein diacetate (see below). With this relatively crude first experimental arrangement, fluorescent particle signals were barely 2 times noise level. With the improved current apparatus, the same fluorescent particles give signals about 1000 times above instrumental noise. The pulse height distribution of the fluorescent ragweed pollen is shown in Fig. 3, along with the light scatter distribution (BG-12 and 3/69 filters out) and the volume distribution (measured with a Coulter spectrometer). The amount of light scattered per particle is much greater than the fluorescent emission per cell under these conditions. All three distributions are similar in shape -- approximately Gaussian. The widths of these distributions halfway down from the peak expressed as percent of the mode are given in Fig. 3. Note that the scatter and the

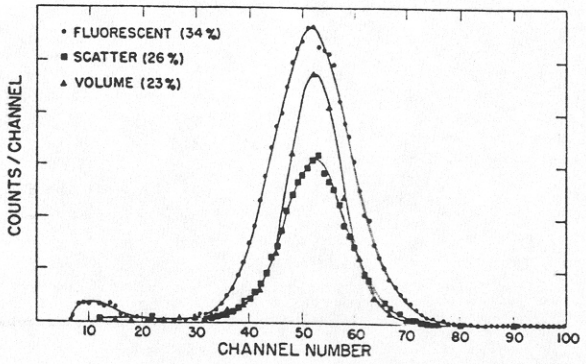

Fig. 3. Fluorescent, scatter, and volume spectra of acridine orange-stained ragweed pollen. Values in parentheses are width halfway down from peak as percent of mode.

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fluorescent distribution are broader than the volume distribution; they are expected to be narrower by close to 33 percent if scatter and fluorescent light intensities are proportional to particle surface area and if the particles are spherical. The broadening may be due in part to instrumental factors (like non-uniform illumination of the particle stream) and in part to the knobby surface of the ragweed pollen. Microscopic examination of sections shows that the acridine orange remains near the pollen surface.

From sample flow rates and areas under the curves of Fig. 3, particle concentrations have been calculated and are listed in Table 1. All these values are within a range of ± 3 percent, showing that all methods agree and all particles pick up the dye and fluoresce.

In addition to these results on pollen, we have measured fluorescence distributions of Chinese hamster ovary (CHO) cells showing fluorochromasia (4). CHO cells were added to saline solutions of 10^{-5} M, 2 x 10^{-6} M, and 5 x 10^{-7} M fluorescein diacetate (FDA) and remained in these solutions during the course of an experiment. The FDA, which is not fluorescent, is quickly absorbed into the cells where it is hydrolyzed to fluorescein by enzyme activity. Fluorescein, the fluorescent product of this reaction, accumulates within the cells. Cells treated in this manner appear green against a dark background in the fluorescent microscope. Their fluorescence is weaker than the ragweed pollen stained with acridine orange by a factor of 15 to 50, but is sufficient to yield the distributions shown in Figs. 4 and 5. These fluorescent intensity distributions were taken under different electronic gain, and hence the three modal channels do not fall as closely together as indicated in the figures. If the modal channel of the 10^{-5} M FDA sample is taken as 28, then the modal channels for the other two samples at the same gain are 16 (2 x 10^{-6} M FDA) and 11 (5 x 10^{-7} M FDA).

TABLE 1. CONCENTRATION OF A SUSPENSION OF RAGWEED POLLEN STAINED WITH ACRIDINE ORANGE AND MEASURED BY FLUORESCENCE, SCATTER, AND VOLUME SENSORS

Method	Particles/ml
Fluorescent cell spectrometer	10,200
Light scatter	10,700
Coulter volume spectrometer	10,070
Coulter counter	10,300

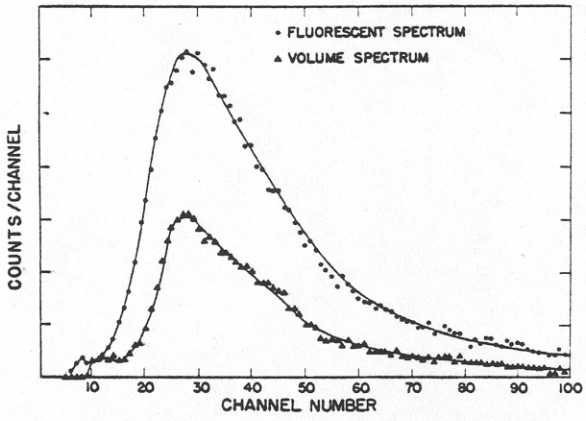

Fig. 4. Fluorescent and Coulter spectra of CHO cells treated with 10^{-5} M fluorescein diacetate.

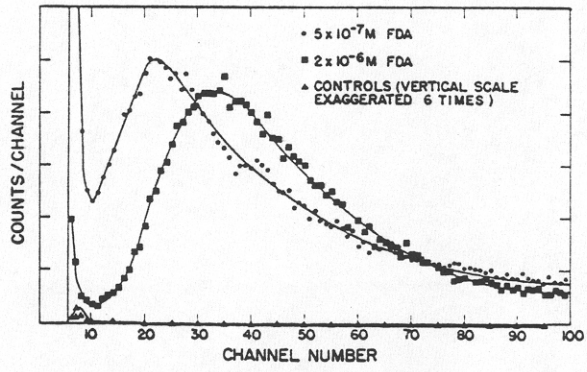

Fig. 5. Fluorescent spectra of CHO cells treated with 2 x 10^{-6} M and 5 x 10^{-7} M fluorescein diacetate and controls (see text for explanation of electronic gain used).

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Thus, the fluorescent signal strength is not proportional to FDA concentration but tends to saturate. If no FDA is added to the cells, there is virtually no fluorescent signal present. At all three FDA concentrations, there was low channel fluorescent noise present which increased with time and which was more prominent relative to cell fluorescence at the lower FDA concentrations. If the fluorescent CHO cell suspensions are centrifuged at 3×10^3 RPM for 5 minutes in a clinical centrifuge and the supernatant liquid examined with the fluorescent cell photometer, the low channel noise persists and the cell signal is absent. Thus, the noise appears to be a fluorescence of approximately constant intensity, possibly from debris saturated with fluorescein or fluorescein in solution.

A typical volume distribution is shown in Fig. 4. Both volume and fluorescence distributions show little or no change in shape or modal value over a 50-minute period, but both show a similar drop in area (i.e., cell concentration) with a half-life of about 2 hours. This indicates no loss in fluorescence, rather a loss of cells by sticking to glass, disruption, or some other mechanism. The similarity in shape of the volume and fluorescence spectra indicates that cell fluorescein content is proportional to cell volume.

Discussion

The ragweed pollen results show that the fluorescent light distribution and fluorescent particle count can be measured at high speed (about 10^4/min) for brightly fluorescing particles with good accuracy. The fluorescent distribution may be somewhat too wide due to instrumental broadening effect, but this can be investigated and may actually be real. The CHO cells containing fluorescein are probably as bright as most biological cells treated with fluorochromes; they yield signals which are well above instrumental noise, although background fluorescence (probably from fluorescein in solution or fragments) is a problem. CHO cells stained with acridine orange show a similar background fluorescence. Two approaches to this problem are being tried: better staining methods and improvement in instrumental design. The latter approach includes reduction in sensitive volume and pulsing the fluorescence sensor.

As the sensitive volume is reduced, solution fluorescence noise riding on a cell signal is reduced. Cell stream diameter can be reduced by increasing sheath flow at constant sample flow while maintaining laminar flow. These factors are being investigated. Light beam diameter can be reduced by reducing slit width with no change in light intensity. This represents an advantage but an increasing waste of light, something inherent in conventional light sources and optical elements (i.e., lenses and mirrors). A better solution would be use of a laser beam, because the entire light output of a laser can be focused down to image dimensions equal to (and even below) minimum useful cell stream diameter. Fortunately, argon ion lasers with large outputs (1 to 2 watts) in the 4000- to 5000-Å band have recently become available. This power is actually comparable to the output of the PEK-110 mercury vapor lamp in the same band; the difference lies in the ability to concentrate it. We plan to use such a laser (Spectra-Physics Model 140) as the light source. Two to three orders of magnitude more cell stream illumination is expected. The output beam (diameter about 1.2 mm) can be focused to a "slit" image with a pair of cylindrical lenses. Although the light intensity on a cell is very large under these conditions, it can be shown equivalent to a 30-second exposure in a Zeiss fluorescence microscope using 200-watt mercury vapor lamp illumination. Theoretical and experimental evidence indicates no dye saturation.

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Since cells are present in the sensitive volume about 1 percent of the time, only noise pulses are counted during the remaining 99 percent of the time. These can be eliminated and two additional advantages gained by converting to pulsed operation in which the system is turned on only when a cell is in the slit. We plan to use light scattered at small angles as a trigger signal to open a normally closed gate to the analyzer, thus collecting fluorescence pulses only when a cell is present. In this way we hope to reduce noise pulse accumulation in early channels, generate a time-coincident scatter signal which should yield information on cell size, and use this signal to permit fluorescent signal retrieval from noise by averaging.

It is also possible that the present rather inefficient fluorescent light collection may be improved by use of reflecting chambers and appropriate optics in place of the present f/1.6 optics. Several designs, including parabolic, elliptical, and spherical reflectors, have been investigated with the aid of lens-design computer codes. Image quality is very poor for the first two, fair for the spherical case. Due to uncertainty at present as to allowable image deterioration and also to fabrication difficulties, this approach is postponed until it is clear that more efficient light collection is necessary.

Crude spectral analysis is also contemplated for the future. A second FW-130 multiplier phototube at the other 90° window would sense the same fluorescence signal as the present FW-130. With appropriate filters one could sense part of the emission band, and the other could sense the rest of the emission band.

Acknowledgments

We thank B. Brixner (Group GMX-9) for valuable assistance in optical design and computer analysis; R. Hiebert (Group P-1) and L. Carr and M. Butler (Group H-4) for similar help in electronic design.

References

(1) P. J. Crosland-Taylor, Nature 171, 37 (1953).
(2) O. Reynolds, Phil. Trans. Royal Society (London) 174, 935 (1883).
(3) M. Born and E. Wolf, Principles of Optics, MacMillan, New York (1964), p. 189.
(4) B. Rotman and B. Papermaster, Proc. Natl. Acad. Sci. 55, 134 (1966).

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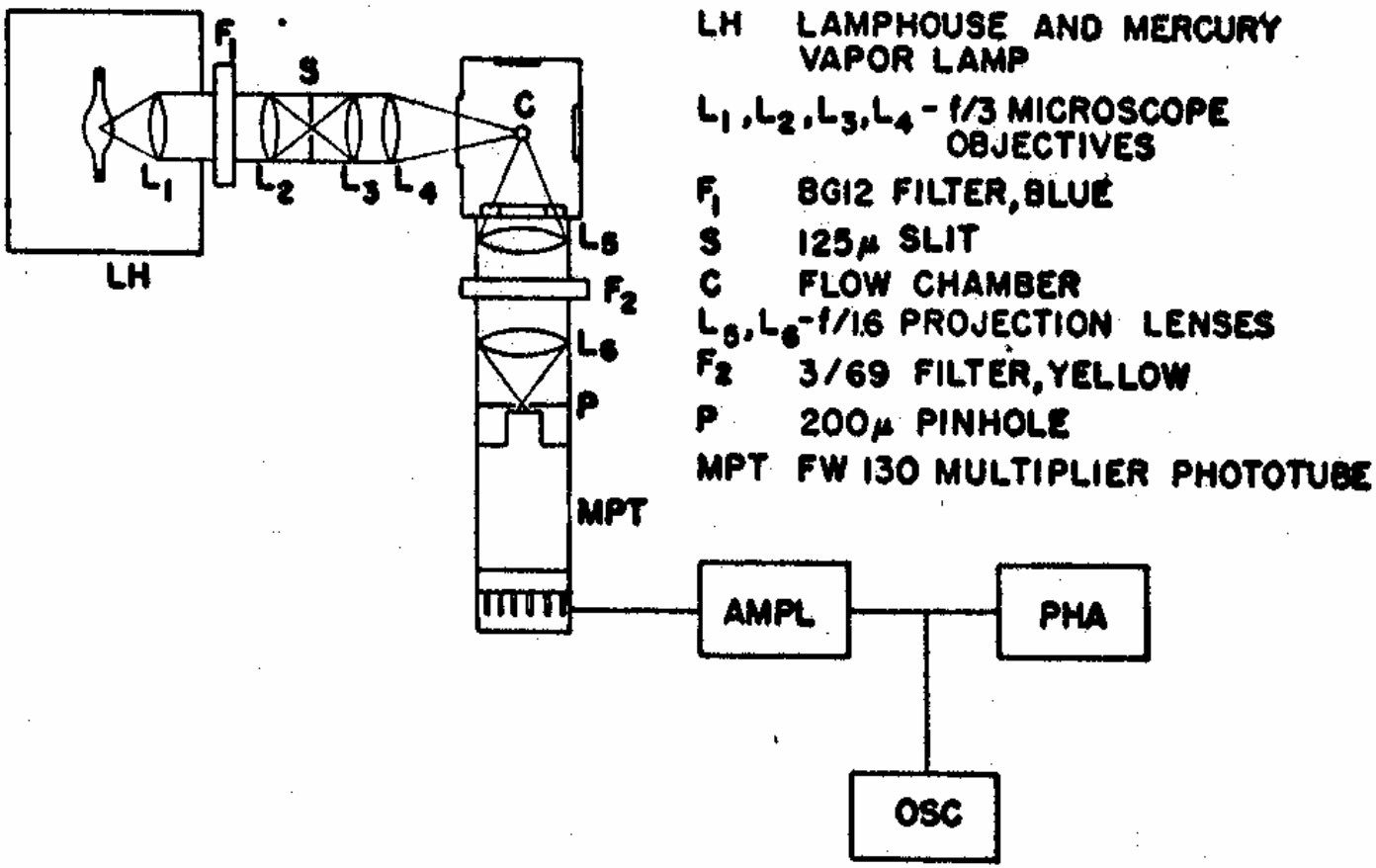

Fig. 1. Block diagram of fluorescent cell photometer.

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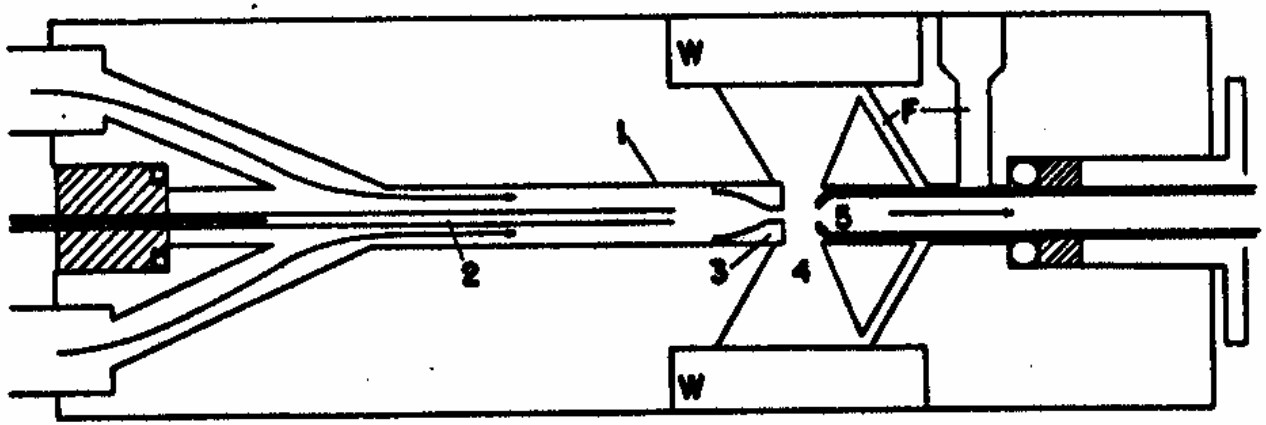

Fig. 2. The flow chamber (scale 4 times actual). W = window; F = flushing system for bubble removal; 1 = sheath flow; 2 = sample injection; 3 = nozzle; 4 = quiescent (viewing) region; and 5 = exit tube.

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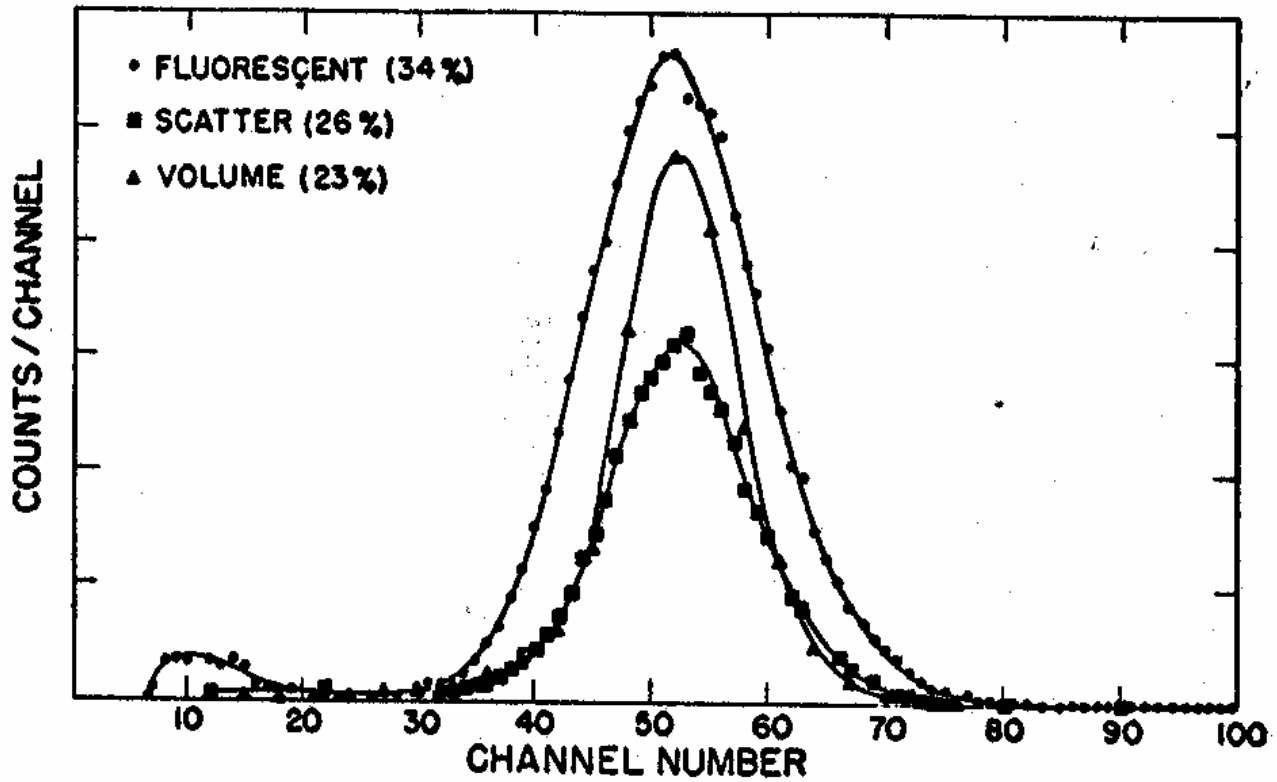

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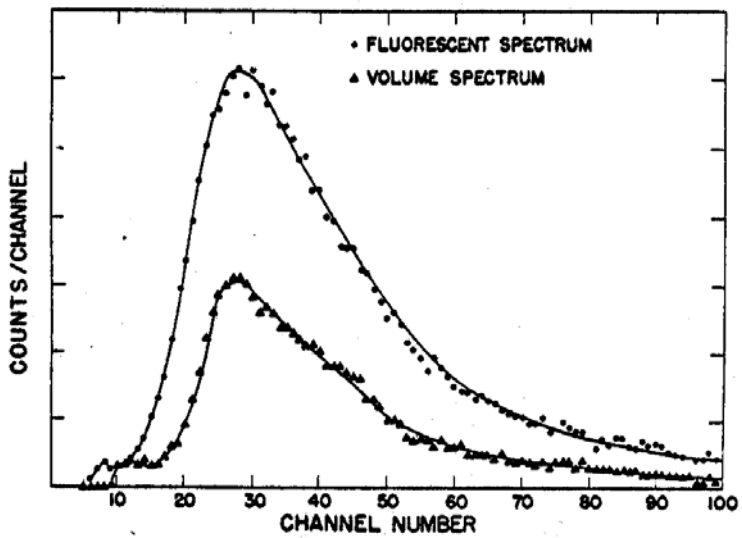

Fig. 4. Fluorescent and Coulter spectra of CHO cells treated with 10^{-5} M fluorescein diacetate.

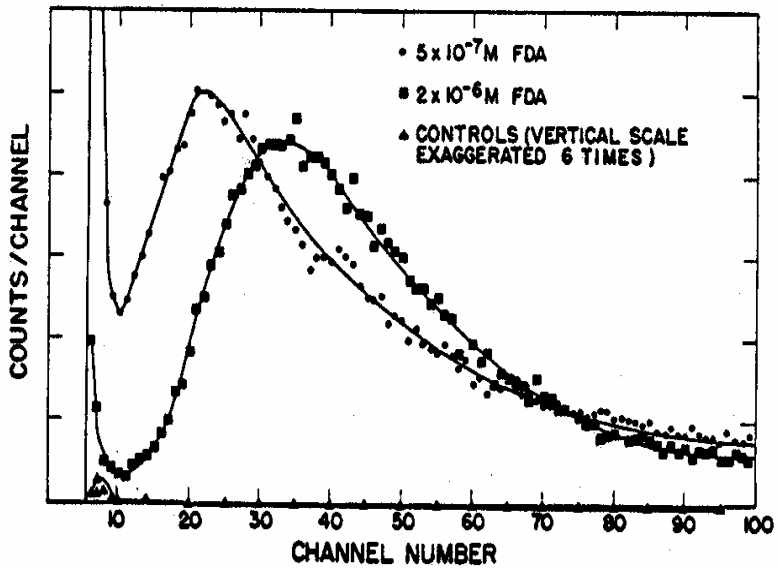

Fig. 5. Fluorescent spectra of CHO cells treated with 2×10^{-6} M and 5×10^{-7} M fluorescein diacetate and controls (see text for explanation of electronic gain used).

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