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Publication Date
1977

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Submitted to the Proceedings of the
National Academy of Sciences of the
United States of America

LBL-6170 c.1
Preprint

# QUANTITATIVE DETERMINATION OF TRANSFORMED CELLS IN A MIXED POPULATION BY SIMULTANEOUS FLUORESCENCE ANALYSIS OF CELL SURFACE AND DNA IN INDIVIDUAL CELLS.

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January 1977

Prepared for the U. S. Energy Research and
Development Administration under Contract W-7405-ENG-48

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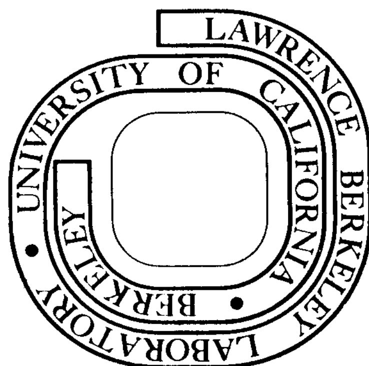


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Cell Biology

# Quantitative determination of transformed cells in a mixed population by simultaneous fluorescence analysis of cell surface and DNA in individual cells.

(flow microfluorometry / SV40 / MSV/MLV / benzo[a]pyrene / fluorescamine)

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Abbreviations: saline GM, 1.5 mM $Na_2HPO_4$, 1.1 mM $KH_2PO_4$ (pH 7.4) containing 1.1 mM glucose, 0.14 M NaCl, and 5 mM KCl; borate buffer, 0.2 M $H_3BO_4$ (pH 9.0).

## ABSTRACT

Cell surface labeling with fluorescamine indicates that the fluorescence of Balb 3T3 A31 cells is considerably decreased after both viral and chemical transformation. This phenomenon coupled with the technique of flow microfluorometry enabled non-transformed and transformed cells to be distinguished. A second fluorescent probe, propidium iodide, which intercalates into DNA, was used in combination with fluorescamine in order to obtain a ratio of cell surface labeling to DNA content. This manipulation allowed enhanced resolution of the two populations and the detection of small numbers of transformants in a predominantly normal population.

INTRODUCTION

The advent of flow microfluorometry has created new potential for the development of techniques for malignant cell detection. The high speed and convenience of the systems allow statistically significant populations of cells to be analyzed, one cell at a time, on the basis of various parameters. These include measurements of cell volume to differentiate human leukocytes (1), the use of nuclear fluorochromes to determine differences in DNA content of chemically induced mouse tumor cells and normal cells (2,3), measurements of uv absorption and light scatter to separate abnormal cells from uterine cervical carcinomas (4) and the measurement of nuclear to cytoplasmic ratios to detect maglignant cells in gynecological specimens (5,6).

The basis of the distinction between normal and malignant cells in most of the above studies is a difference in DNA and total protein content. Promising new approaches are directed more at the level of the plasma membrane. For example, the determination of membrane microviscocities by fluorescence emission anisotropy allows some normal and leukemic lymphocytes to be separated (7). Fluorescently labeled antibodies directed at cell surface antigens, like those used to distinguish between T and B lymphocytes (8), also offer a possible means of detecting malignant cells. However, the usefulness of this approach will not be realized until specific tumor antigens are purified.

Recently a more general technique (9,10) which was developed for labeling the outer surfaces of tissue culture cells with fluorescamine, offered a possible method of detecting transformed cells. Fluorescamine forms a fluorescent product with primary amines exposed at the cell surface and has been shown to label both proteins and

lipids. With this method it has been demonstrated that on the basis of protein content the fluorescence of chick embryo fibroblasts was decreased by a factor of three after transformation with Rous sarcoma virus (9).

The present report describes the adaptation of the method of fluorescamine labeling for use in single cell analyses by flow microfluorometry. This technique has been used to extend the study to lines of mouse cells transformed both by viruses and a chemical carcinogen and has demonstrated that they also differ considerably from non-transformed cells with respect to the fluorescence intensity of surface bound fluorescamine. This property can be used as the basis for distinguishing the two types of cells. Additional resolution is provided by the incorporation of a second fluorescent probe, propidium iodide, which binds to DNA (11). Analyses of the ratio of cell surface to DNA fluorescence decreases dispersion of data due to cell-cycle variations in fluorescamine labeling and therefore enhances the resolution of non-transformed and transformed cells in a mixed population.

## Materials and Methods

### Cells

Balb 3T3 A31 HYF cells were derived by clonal selection from Balb 3T3 A31 mouse fibroblasts (12). The continuous line of MSV/MLV Balb 3T3 A31 HYF transformed cells was produced by infection of the non-transformed line with the Moloney strain of Murine sarcoma virus (MSV/MLV). Twenty-four hours after seeding, Balb 3T3 A31 HYF cells ($2 \times 10^5$) were infected with $6 \times 10^5$ focus forming units of MSV/MLV. When the cells reached saturation density ($1.1 \times 10^7$ cells/100 mm tissue culture dish) they were transferred a total of four passages by seeding at $2 \times 10^5$ cells/100 mm dish. They were then frozen at -70°C in Dulbecco's modified Eagle's medium (DME), from Gibco, Grand Island, N.Y., containing 20% newborn calf serum and 10% DMSO. Cells in these experiments were used 1-6 passages beyond frozen stocks.

Simian virus 40(SV40) transformation of Balb 3T3 A31 HYF cells was carried out according to the methods of Todaro (13). The cells were cloned six passages after infection and transferred a further six times before use. At that time they were producing T antigen as demonstrated by the procedure of Pope and Rowe (14).

Benzo[a]pyrene-transformed Balb 3T3 A31 cells (BP3T3) were originally derived by DiPaolo and further cloned by Holley (15).

All cells were carried in 100 mm tissue culture dishes (Falcon, Oxnard, California) and incubated at 37°C and 10% $CO_2$ in DME containing 10% newborn calf serum. They were considered to be free of mycoplasma by lack of cytoplasmic incorporation of [$^3$H]thymidine after a 24 hr pulse (0.5 µCi/ml, 20.1 Ci/mM; New England Nuclear, Boston, Masschusetts).

Labeling Procedure

Monolayer cultures of non-transformed and transformed cells were labeled 2-3 days after seeding 7 x $10^5$ cells/100 mm dish, at which time they were both actively growing. After removal of the medium the cells were washed with warm saline GM and borate buffer. A solution of fluorescamine (Hoffman-La Roche, Inc., Nutley, New Jersey) in acetone was added to borate buffer to a final concentration of 0.5% acetone and 500 μg/ml fluorescamine and immediately applied to the cells. After 30 seconds the cells were washed with warm borate buffer and saline GM and removed from the tissue culture dishes by gentle scraping with a rubber policeman. The cells were fixed at 4°C for one hour in saline GM containing 15 mM $MgCl_2$ and 25% ethanol. Ribonuclease (5 x crystallized, Calbiochem, San Diego, California) was added to a final concentration of 1 mg/ml, and the cells were incubated for one hour at 37°C. The ribonuclease was removed by two washes in saline GM before staining with propidium iodide (Calbiochem, A grade) at 50 μg/ml in saline GM. After 30 minutes at room temperature the cells were washed once in saline GM and filtered through a 37 micron mesh Nitex filter (Tetko, Inc., Los Angeles, California) before analysis.

Flow Microfluorometry

Analysis was performed using an instrument constructed basically according to the design of Steinkamp *et al*. (16) with modifications to eliminate the sorting mode of operation. The instrument is represented diagrammatically in Figure 1. Cells stained with propidium iodide and/or fluorescamine traversed a flow chamber at approximately 500 cells/second in a stream which intersected the beam of an argon ion laser tuned to 351.1 and 363.8 nm. Emitted light, collected at 90° from the laser beam

and cell stream, was passed through a series of filters which allowed excellent resolution of fluorescence above 580 nm into the RED photomultiplier and below 560 nm into the BLUE photomultiplier. The optical system was constructed as follows: barrier filter--Corning 3-73, dichroic beam splitter--Bausch and Lomb 45-1-580, short pass filter--Corning 1-64 and long-pass filter--Corning 2-63. Signals from each cell were then processed electronically to allow single parameter analysis (either red of blue fluorescence), ratios of the two signals or gated singled parameter analysis. Processed signals for a given cell population were accumulated and displayed as pulse amplitude frequency distribution histograms on a multichannel pulse height analyser. The data was stored and processed by a Sigma-2 computer (Xerox Corp. Rochester, New York).

## Results and Discussion

Balb 3T3 A31 HYF and MSV/MLV Balb 3T3 A31 HYF cells labeled with either propidium iodide or fluorescamine were examined by fluorescence microscopy. Fluorescamine labeled cells yielded a blue fluorescence at the cell surface due to the reaction of the reagent with surface components and the nuclei of propidium iodide labeled cells yielded a red fluorescence.

Single parameter flow microfluorometer analyses of these cells is shown in Figure 2. Cell surface distributions obtained by analyzing the blue fluorescence of fluorescamine labeled cells were unimodal with non-transformed cells yielding an average fluorescence intensity 2.5 times greater than transformed cells (Figure 2, C and D). The difference in the fluorescamine fluorescence per cell which enabled the two populations to be distinguished was not simply due to a change in cell size upon transformation as Coulter volume measurements of both cell types indicated

identical cell volumes (data not shown). The factors which determine the difference in fluorescence intensity are currently under investigation.

DNA distributions obtained by analyzing red fluorescence of propidium iodide stained cells were bimodal (Figure 2, A and B). The large peak represents cells in the $G_1$ phase of the cell cycle and the second peak at approximately twice the fluorescence intensity of the first represents $G_2$ + M cells. Distributed between the two peaks are cells at various stages of DNA replication in the S phase.

In order to determine whether fluorescamine labeling was uniform throughout the cell cycle, it was desirable to make simultaneous measurements of surface fluorescence and DNA in individual cells. For this purpose it was necessary to label cells with both fluorescamine and propidium iodide. The spectral qualities of these compounds render them particularly suitable for use in combination, as shown in Figure 3. The excitation spectra of the two fluorfors overlap such that both can be excited by the 351.1 and 363.8 nm lines of an argon ion laser whereas their emission spectra are widely separated, with propidium iodide fluorescing mainly in the red (560-720 nm) and fluorescamine in the blue-green (400-580 nm) region of the spectrum. Under the conditions described, fluorescence from propidium iodide was not detected by the blue photmultiplier and fluorescamine did not contribute to the measurement of propidium iodide, as the signal from the former was extremely small and was eliminated electronically.

In a given population, the distribution of fluorescamine labeled cells was found to be, in part, related to the cell cycle distribution. Figure 4 shows that when the surface fluorescence measurements of a rapidly growing population of MSV/MLV Balb 3T3 A31 HYF cells were gated to DNA

content, as determined by propidium iodide fluorescence, the amount of fluorescamine signal increased throughout the cell cycle. Non-transformed Balb 3T3 A31 HYF cells showed similar cell cycle variations (data not shown). For both cell types distribution of fluorescamine signals in each of the cell cycle phases was broad and presumably reflects the spread of cells throughout that phase.

Since the purpose of this study was to devise a means of discriminating between non-transformed and transformed cells, it was evident that any manipulation which would narrow the fluorescamine distributions would enhance the resolution of the two populations of cells. Thus, in order to compensate for variations in cell surface fluorescence associated with different stages of the cell cycle, an analog divider was incorporated into the processing unit so that the ratio of fluorescamine to propidium iodide (F/PI) could be calculated. Separate analyses of blue fluorescence of non-transformed and transformed cells labeled with both fluorescamine and propidium iodide allowed fairly good resolution of the two types of cells although superimposition of the two histograms indicates some degree of overlap (Figure 5A). As expected, the use of the ratio mode enhanced the separation of the two histograms (Figure 5B) not only by decreasing cell cycle variations but also probably by minimising dispersion of the data due to presence of debris and clumps of cells in the sample and to minor variations in laser power. In four trials the F/PI for non-transformed cells was 3.65 ± 0.50 times the corresponding value for tranformed cells.

In order to test whether this technique would be useful for detecting transformed cells in a mixed population, Balb 3T3 A31 HYF cells and their MSV/MLV transformed counterparts were labeled with fluorescamine and propidium iodide as described and subsequently mixed in various proportions. Analysis of the mixtures by the ratio mode indicated that a small

percentage of transformed cells could indeed be detected within a predominantly non-transformed population (Figure 6). This data suggests that it should be possible to detect the presence of less than 1% transformed cells.

To check that the observed differences in fluorescamine labeling were not restricted to chick embryo fibroblasts transformed by RSV and mouse cells transformed by MSV/MLV, the study was extended to other transformed lines of Balb 3T3 A31 cells. In a single experiment in which cells were seeded at $1 \times 10^6$ cells/100 mm dish the F/PI for non-transformed mouse cells was found to be 13 times greater than the ratio for cells transformed with Simian virus 40 (SV40), Murine sarcoma virus (MSV/MLV) and benzo[a]pyrene. The increased difference of the F/PI for the non-transformed and transformed cells (which was previously 3.65:1), in this instance was due to increased levels of cell surface fluorescence of the non-transformed cells. In this experiment cells were seeded at a higher density than previously and the observation may therefore be a reflection of density-dependent increases of surface proteins similar to those reported by Hynes and Bye (17). It should be stressed that the experiments reported in Figures 2,5, and 6 were performed with actively growing cells and therefore the basic difference between the non-transformed and transformed cells was not related to different states of growth. However, as mentioned above, the magnitude of the fluorescence signals from non-transformed cells was apparently affected by cell density. Cells, whose growth rate was slowing down at high density were more fluorescent than actively growing cells. In contrast, the fluorescence intensity of transformed cells was unaffected by seeding at high density but remained at a low level. The exact effect of density upon these measurements requires further examination. These preliminary observations do, however, suggest that growth conditions could be manipulated to allow even greater resolution of the two populations.

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In conclusion, this technique can be used to distinguish between non-transformed and transformed cells from both primary cultures, in the case of chick embryo fibroblasts reported earlier (9), and established lines of Balb 3T3 cells. Furthermore, this distinction is applicable to cells transformed with an RNA virus (MSV/MLV), a DNA virus (SV40) and a chemical carcinogen (benzo[a]pyrene) and therefore appears to be a general property of transformation. It is hoped that these observations of cells in culture can be applied for use as a possible means of malignant cell detection. The availability of such a general technique would not only facilitate early detection of malignancies but would also aid in monitoring the effectiveness of chemotherapeutic agents.

Acknowledgement:

We would like to thank Dr. Melvin Calvin for his encouragement and useful discussion, Ms. F. Suzuki and Mr. H. Yokota for excellent technical assistance in culturing the cells, Mr. B. Turko and Mr. B. Leskovar for assistance in the construction of the flow microfluorometer and Dr. R. Holley for his generous gift of cloned BP3T3 cells. We would also like to thank Dr. J. Burki for use of the Coulter volume analyzer. This work was supported, in part, by NCI Grant No. 2 P01 CA 14828. S.P.H. is a Fellow of the Elsa U. Pardee Foundation.

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Legends

Figure 1. Diagram of flow microfluorometer. B, beam shaping lenses; FC, flow chamber; L. lens; PMT, photomultiplier tube.

Figure 2. Histograms representing distributions of fluorescence intensity among cells in the population after staining with propidium iodide (A and B) or fluorescamine (C and D). The ordinate represents the number of cells for which a given fluorescence intensity was measured and the abscissa represents the relative fluorescence intensity of propidium iodide (proportional to DNA content) or fluorescamine (proportional to cell surface components). The cells were Balb 3T3 A31 HYF (A,C) and MSV/MLV transformed Balb 3T3 A31 HYF (B,D).

Figure 3. Fluorescence spectra of fluorescamine (—·—·—) and propidium iodide (————) labeled Balb 3T3 A31 HYF cells in suspension in saline GM. Spectra were recorded on a spectrofluorometer (Perkin-Elmer, model MPF-3, Norwalk, Connecticut) with pathlengths, excitation 10 mm and emission 3 mm and absorbance <0.1 at all excitation wavelengths. Fluorescamine scale 3.33x propidium iodide scale. – – – – represents 351.1 and 363.8 nm lines of argon ion laser; |————| represents effective wavelength range detected by blue (I) and red (II) photomultiplier tubes.

Figure 4. Histograms representing distributions of fluorescence intensity among MSV/MLV Balb 3T3 A31 HYF cells in the population after labeling with both fluorescamine and propidium iodide. A, DNA distribution indicating the phases of the cell cycle ($G_1$, S and $G_2$ + M) for which electronic gates were established. B, cell surface distribution. C, cell surface

0 0 0 0 4 7 1 0 0 1 6



distributions of cells at different stages in the cell cycle obtained by gated analysis against the DNA signal gates established in A. For the purpose of visual display the gain setting for fluorescence intensity in panels B and C was increased relative to all other data shown.

Figure 5. Flow microfluorometric analysis of non-transformed Balb 3T3 A31 HYF cells (NT) and their MSV/MLV transformed counterparts (T) labeled with both fluorescamine and propidium iodide. Panel A represents histograms of the fluorescence intensity of the fluorescamine signal, panel B represents histograms of the ratio of the fluorescamine signal to the propidium iodide signal.

Figure 6. Flow microfluorometric analyses of non-transformed and MSV/MLV transformed Balb 3T3 A31 HYF cells labeled with fluorescamine and propidium iodide. The results are expressed as the ratio of fluorescamine to propidium iodide fluorescence. A, transformed cells; B, non-transformed cells; C-E, mixtures of transformed and non-transformed cells containing 8, 35, and 95% transformants respectively.

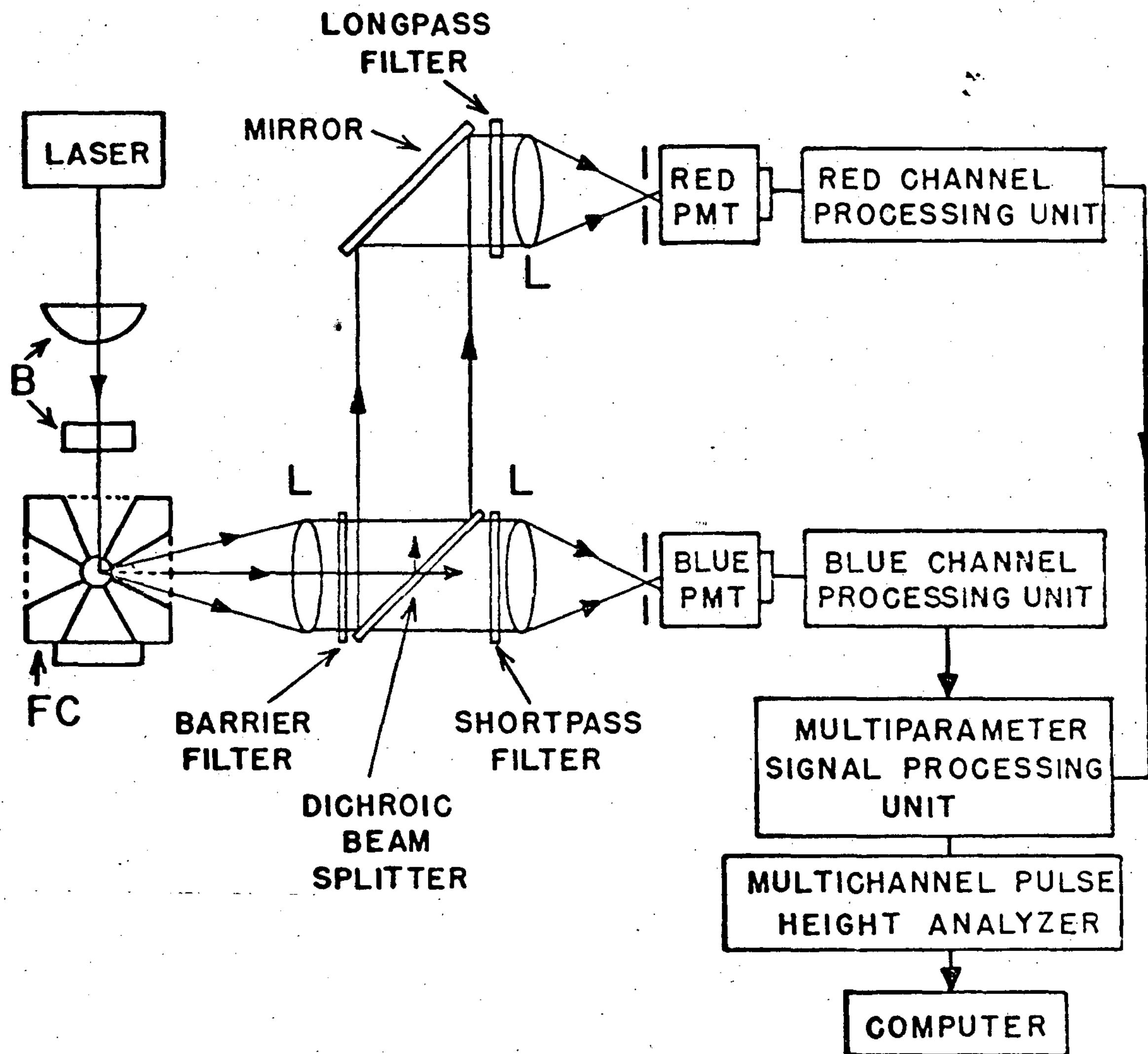


Fig. 1

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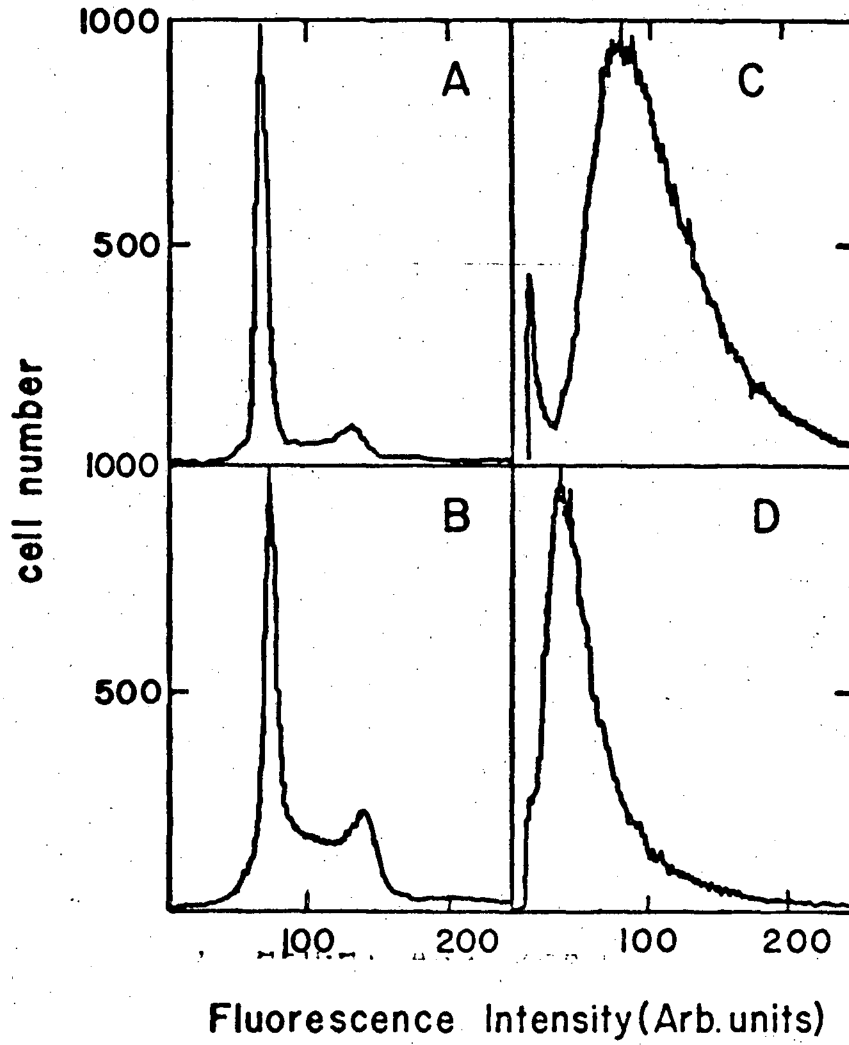


Fig. 2

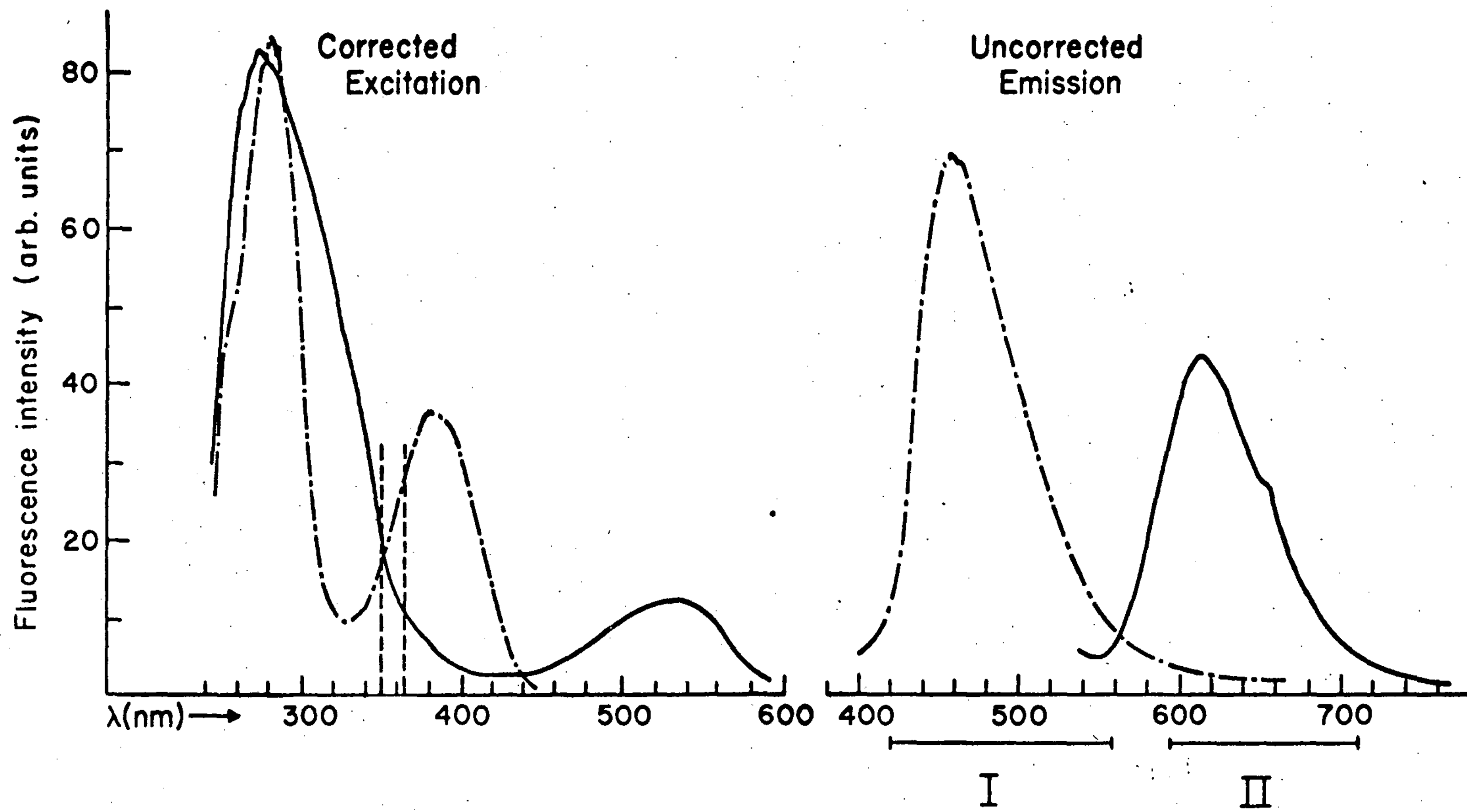


Fig. 3

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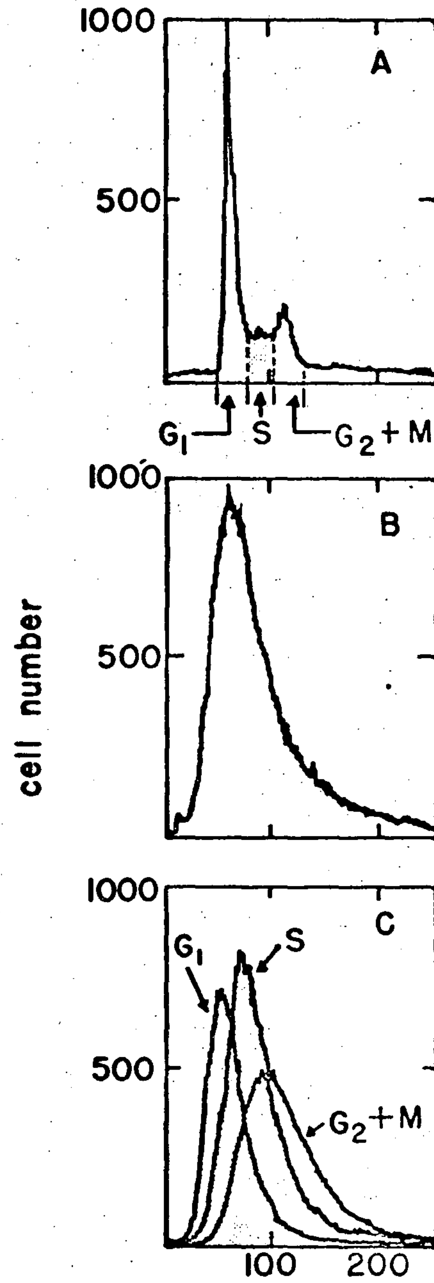


Fig. 4

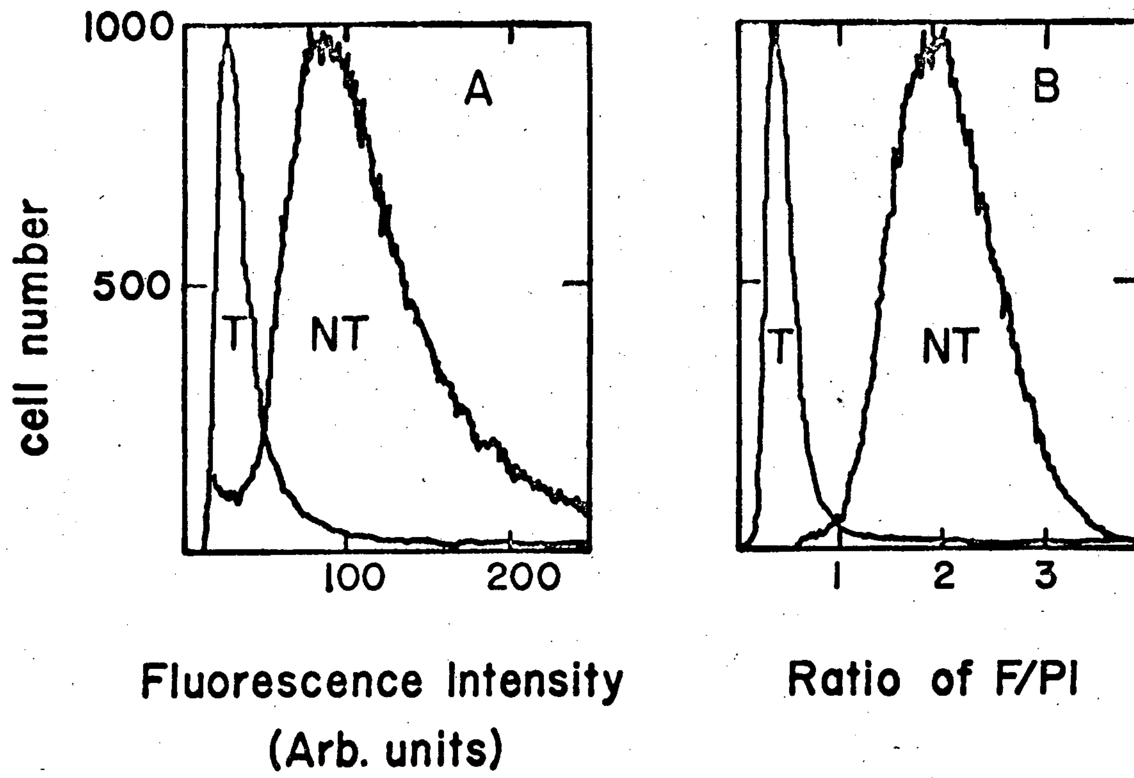


Fig. 5

0 0 0 0 4 7 1 0 0 1 9



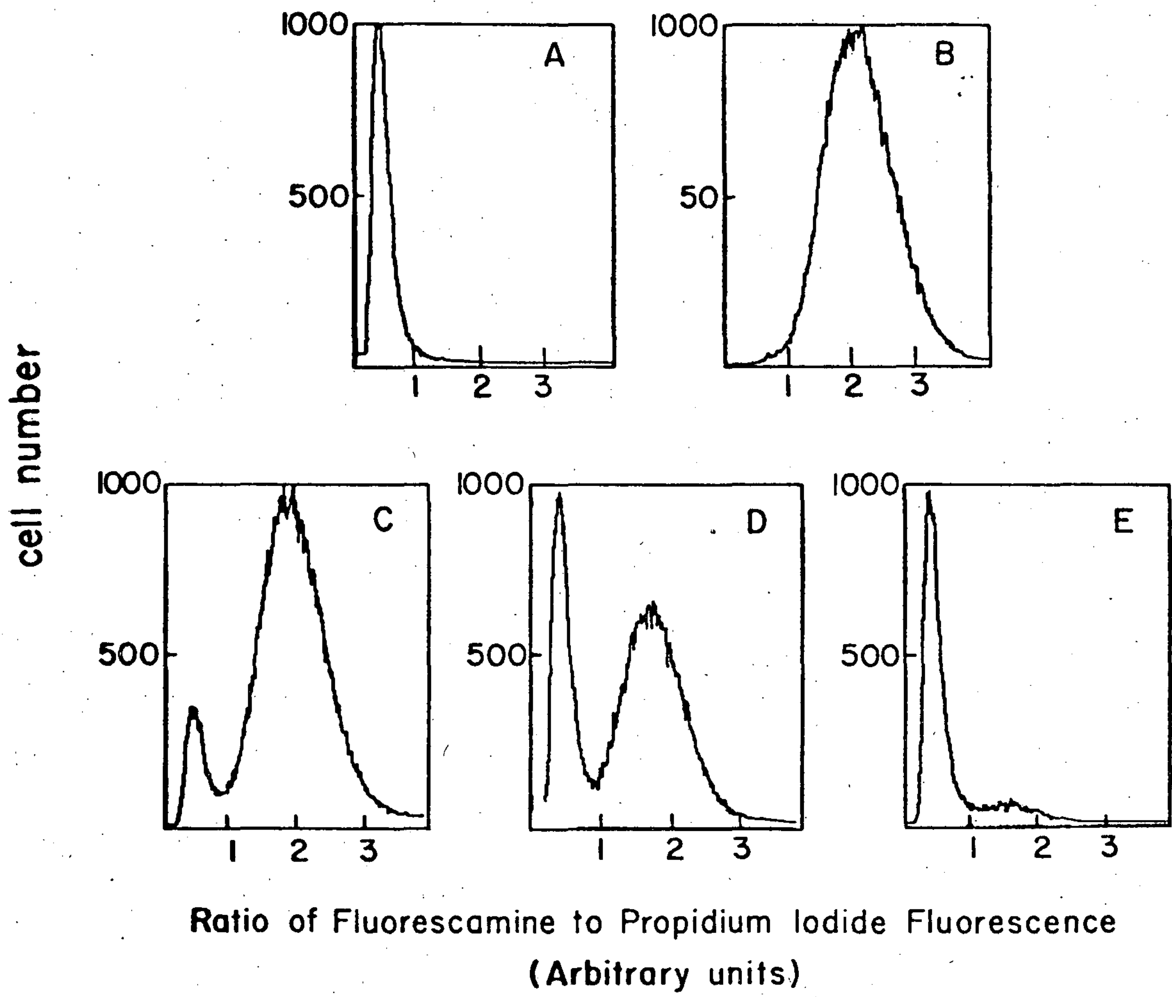


Fig. 6

This report was done with support from the United States Energy Research and Development Administration. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the United States Energy Research and Development Administration.

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