LA-UR-73-1594

24,097

CONF-731007--1

TITLE:

MULTIPARAMETER CELL SORTING AND ANALYSIS

AUTHOR(S):

John A. Steinkamp

SUBMITTED TO:

To be published in the Proceedings of the First Annual Life Sciences Symposium on "Mammalian Cells: Probes and Problems," to be held at the Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico (October 17-19, 1973); AEC Symposium Series, Technical Information Center, Oak Ridge, Tennessee

los alamos
scientific laboratory
of the University of California
LOS ALAMOS, NEW MEXICO 87544

Form No. 836
St. No. 2629
1/73

UNITED STATES
ATOMIC ENERGY COMMISSION
CONTRACT W-7405-ENG. 36

MULTIPARAMETER CELL SORTING AND ANALYSIS

John A. Steinkamp

Biophysics and Instrumentation Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544

ABSTRACT

Improved multisensor cell sorting instrumentation for quantitative analysis and sorting of cells has been developed. Cells stained with fluorescent dyes enter a flow chamber where cell volume, fluorescence, and light scatter sensors simultaneously measure multiple cellular properties. Cells then emerge in a liquid jet that is broken into uniform liquid droplets. Sensor signals are electronically processed in one of several ways and are displayed as pulse-amplitude frequency distribution histograms using a multichannel pulse-height analyzer. Processed signals activate cell sorting according to preselected parametric criteria by electrically charging droplets containing cells and electrostatically deflecting them into collection vessels. Illustrative examples of multiparameter cell analysis and sorting experiments using cultured mammalian cells, a model mouse tumor cell system, and animal leukocytes are presented.

INTRODUCTION

The development of high-speed, flow-system, cell-sorting methods based on physical and biochemical measurements of single cells has provided a new dimension to biological investigation by being able to isolate physically cells with particular properties from a heterogeneous mixture.[1-3] Such systems have been used to differentiate and sort human leukocytes based on volume measurement,[4] to isolate antigen-binding cells which are precursors to antibody-producing cells by use of immunofluorescence,[5] and to analyze and separate cells based on ultraviolet absorption and visible light scatter.[2] Improved instrumentation for separating cells based on simultaneous measurement of cell volume, total or two-color fluorescence, and light scatter has recently been developed[6] and has been utilized to analyze and separate acridine orange-stained human leukocytes on the basis of red fluorescence of cytoplasmic granulation.[7] This system incorporates several existing analytical techniques, permitting multiple measurements to be performed on the same cell. Coupled with cell sorting, multiparameter analysis methods provide unusual versatility in experimental cell research by measuring and processing several cell characteristics simultaneously and separating cells according to selected combinations of parameters. Cell sorting permits the correlation of machine-measured cell properties with cell morphology and cell enrichment (i.e., concentration of cell subclasses).

Presented in this report is a description of the cell analysis and sorting techniques made possible with this instrument. Validation of the methodology is discussed with illustrative examples of analysis and sorting on the basis of single parameters, ratios, and two parameter combinations of cell volume, cell surface area, and fluorescence. Multiparameter analysis of

(a) CHO cells reacted with fluorescein isothiocyanate conjugated concanavalin A and (b) HeLa cells doubly stained for protein and DNA contents is presented, and cell sorting is demonstrated using mouse tumor cells according to DNA content of (c) dog leukocytes into lymphocytes, neutrophils, and eosinophils on the basis of cytoplasmic granulation and (d) HeLa cells based on DNA-cell volume relationships.

INSTRUMENTATION

A comprehensive description of the instrumentation has been described elsewhere.[6] Cells stained with fluorescent dyes that label specific biochemical components are suspended in normal saline and introduced into the flow chamber (Fig. 1) at approximately 500 cells/sec. Cells first pass centrally through a 75-μm diameter cell volume-sensing orifice (Coulter principle) and then across a fluid-filled viewing region intersecting an argon laser beam (488 nm wavelength), causing fluorescence and light scatter. Cell volume pulses also can be raised to the two-thirds power, producing signals proportional to cell surface area.[8] Both fluorescence and light scatter are electro-optically measured, the fluorescence sensor being a dual photomultiplier tube array which measures total (above 520 nm wavelength) or two-color fluorescence of selectable color separation regions. Light scatter also can be measured by optically focusing the forward scattered light onto a photodiode. After optical measurement, the liquid stream carrying suspended cells emerges into air as a jet and is broken into uniform droplets (45,000/sec). Thus, cells are isolated into droplets with approximately 1% of the droplets containing a cell.

Parametric signals from the cell sensor amplifiers are processed on a cell-by-cell basis as single parameters, ratios (analog pulse divider), and

gated single parameters using a LASL-developed multiparameter signal processing unit.[6] A multichannel pulse-height analyzer accumulates and displays processed signals as pulse-amplitude frequency distribution histograms. Gated single parameter analysis permits the examination of particular subclasses of cells within selectable ranges on different cellular property values. For example, the volume distribution for G_1 cells can be obtained by analyzing only those processed volume signals from cells yielding G_1 DNA content as determined by fluorescence measurement. That is, if the DNA content signal falls within the G_1 DNA content range as determined by adjustable signal amplitude gates, only then will the associated volume signal be accumulated and displayed. Gated single parameter techniques also permit distributions to be obtained from weakly fluorescing signals (e.g., fluorescent antibody) by requiring electronic coincidence with cell volume, surface area, or light scatter signals. In this manner, fluorescence signals from debris are eliminated. Complete two parameter analysis also is available by selecting two processed signals as inputs to a dual parameter pulse-height analyzer and displaying subsequent three-dimensional pulse-height distributions and two-dimensional contour views.

Processed signals activate cell sorting if the amplitudes fall within preselected ranges of single-channel, pulse-height analyzers. An electronic time delay is activated which subsequently triggers a positive or a negative droplet charging pulse, causing a group of droplets containing a cell to be charged and deflected by a static electric field into a collection vessel. Those cells not satisfying the sorting criteria pass undeflected into a separate collection vessel. The sorted suspension is introduced into a cytocentrifuge and deposited onto microscope slides for counterstaining and microscopic examination.

EXAMPLES OF MULTIPARAMETER ANALYSIS AND SORTING

An example of cell surface fluorescence and surface area measurements being combined as a surface density function (ratio) to study lectin binding per unit cell surface area is shown in Fig. 2. Gated single parameter analysis methods are also utilized to eliminate signals created by fluorescing debris. Chinese hamster cells (line CHO) reacted with fluorescein isothiocyanate conjugated concanavalin A (Con A-F)[9] are used to demonstrate these techniques. Figure 2A shows the single parameter cell volume and cell surface area distributions for randomly grown CHO cells. Dispersion of the cell surface area distribution is less than that of the cell volume plot. This is due to the surface area being proportional to cell diameter squared, whereas cell volume is proportional to cell diameter cubed. Figure 2B shows both the single parameter and gated single parameter cell surface fluorescence distributions. Small fluorescent debris is responsible for the high number of background counts to the left side of the ungated fluorescence distribution. These counts are virtually eliminated by requiring coincidence of cell fluorescence signals with cell surface area signals using gated single parameter methods. This is accomplished by displaying only those fluorescence signals having cell area signals between channels 23 and 40 of Fig. 2A, as illustrated by the shaded region. The resulting gated cell surface fluorescence distribution is essentially free from background interference but contains dispersion due to cell size variability. The correlation of lectin binding sites to cell surface is illustrated in Fig. 2C using ratio analysis methods. The gated cell surface fluorescence-to-surface area ratio distribution provides new methodology for studying the surface density of lectin binding sites per unit cell surface area.[8]

Figure 3 shows single parameter DNA and protein content distributions of HeLa cells doubly stained with propidium iodide (DNA) and fluorescein isothiocyanate (protein),[10] illustrating two-color fluorescence analysis. The cell volume and protein-to-cell volume ratio distributions are also shown. The DNA content distribution (Fig. 3A) shows two peaks: the first peak representing G_1 cells and the second peak G_2 + M cells having twice the DNA content. Cells synthesizing DNA (S phase) lie in the region between the peaks. The coefficient of variation for G_1 phase cells is about 4.5%. The protein and cell volume distributions (Fig. 3B) are broad, unimodal, and typical of a cell population in exponential growth. The protein-to-cell volume ratio distribution of Fig. 3C is unimodal (coefficient of variation near 6%) and shows the high degree of correlation between protein content and cell volume as measured on a cell-by-cell basis.

Complete two parameter analysis of DNA-protein content relationships is demonstrated in Fig. 4, showing a three-dimensional frequency distribution histogram (isometric view) and a two-dimensional contour view which is a base-plane sectional view of the isometric display. The contour view illustrates the correlation of DNA and protein around the cell cycle, with additional fine structure capable of being observed with this type of analysis. Two parameter analysis also provides a method to quantitate the number of cells within a given range of two cellular properties and permits cell cycle-dependent events to be studied. This methodology has recently been used to demonstrate these relationships on other cultured mammalian cell lines.[10]

Figure 5 shows the DNA content distributions for normal mouse spleen and methylcholanthrene-induced mouse tumor (MCA-1) cells[11] prepared and stained with acriflavine using the fluorescent-Feulgen procedure.[12] The mouse spleen cell DNA distribution (Fig. 5A) provides an indication for normal 2C diploid

LASL AEC-OFFICIAL

DNA content. The DNA distribution of MCA-1 tumor cells (Fig. 5B) shows three peaks, the first peak being cells having a normal 2C diploid DNA content and the second and third peaks representing cells having 4C and 8C DNA contents, respectively. The tumor cell population was sorted on the basis of DNA content into two groups, designated by sort regions 1 and 2 (Fig. 5B). Figure 5C is a photomicrograph of dispersed and counterstained tumor cells prior to sorting. Cells having normal DNA content from sort region 1 are leukocytes (Fig. 5D), whereas cells containing an elevated or abnormal DNA content from sort region 2 are tumorigenic cells (Fig. 5E), the two subpopulations being morphologically distinct. The second and third peaks of Fig. 5B are G_1 and G_2 + M phase tumor cells, with S phase cells contained between the peaks. These results have been verified for MCA-1 tumor cells cultured *in vivo* and *in vitro*.[11]

The red and green fluorescence pulse-amplitude distributions of normal dog (beagle) leukocytes vitally stained with acridine orange[13] are shown in Fig. 6. The green fluorescence distribution (Fig. 6A) is unimodal, illustrating uniform nuclear staining, whereas the red fluorescence distribution (Fig. 6B) shows three distinct peaks, characterizing dog leukocytes primarily into three subpopulations on the basis of cytoplasmic granules which exhibit red fluorescence. Leukocytes having red fluorescence signal amplitudes corresponding to sort regions 1, 2, and 3 (Fig. 6B) were separated, counterstained, and identified by microscopic examination as principally consisting of lymphocytes, neutrophils, and eosinophils, respectively. Figures 6D, 6E, 6F, and 6G show photomicrographs of the blood smear and sorted leukocyte fractions. Eosinophils are characterized by coarse cytoplasmic granules compared to finer (dust-like) granulation in lymphocytes and neutrophils as exemplified in the red fluorescence distribution. Dog leukocytes were also characterized on the

basis of green-to-red fluorescence ratio measurements (Fig. 6C). Leukocytes having ratio signal amplitudes corresponding to sort regions 4, 5, and 6 were separated and identified as eosinophils, neutrophils, and lymphocytes.

Cell analysis and sorting on the basis of two parameters are demonstrated using acriflavine-Feulgen stained HeLa cells.[12] Single parameter DNA content and cell volume distributions were first recorded (Fig. 7A) as was the DNA-cell volume two parameter plot (Fig. 7C). The DNA content and cell volume distributions are similar to those of Fig. 3. Figure 7C shows a three-dimensional DNA-cell volume distribution (isometric view) and a two-dimensional contour view. The contour display illustrates the correlation of DNA content and cell volume around the cell cycle.

Using gated single parameter analysis methods, the individual G_1 and G_2 + M cell volume distributions were next recorded (Fig. 7B) by analyzing the volume of cells having G_1 and G_2 + M DNA content, respectively (channels 25 to 33 and 58 to 70 of the DNA distribution). Equal numbers of cells were then sorted into two groups, designated by sort regions 1 and 2 (Fig. 7B), resulting in small volume G_1 and large volume G_2 + M cells being collected. The two subpopulations were mixed together and rerun through the cell sorter, recording the DNA-cell volume two parameter distribution (Fig. 7D). The two parameter DNA-cell volume distribution of the combined sorted populations vividly illustrates this method of cell sorting and provides additional capability to measure quantitatively and to characterize cell properties on the basis of two or more parameters.

CONCLUSIONS

We have presented several examples of analysis and sorting of mammalian cells on the basis of single and two parameter combinations of cell volume,

cell surface area, and total or two-color fluorescence. Multiparameter signal processing coupled with cell sorting provides a versatile and extremely useful method for cell biology research with the range of applications expanding as new methods of obtaining information on cells become available. In principle, other cell-sensing and analysis methods may give meaningful information on physical and biochemical cellular characteristics as both instrumental development and biological applications are in their initial stages of growth.

ACKNOWLEDGMENTS

This work was performed at the Los Alamos Scientific Laboratory, Los Alamos, New Mexico, under the joint sponsorship of the U. S. Atomic Energy Commission and the National Cancer Institute. I thank Drs. H. A. Crissman and P. M. Kraemer for cell preparation and staining; Dr. P. K. Horan for growing of MCA-1 tumor cells and subsequent staining; Ms. A. Romero for cell counterstaining; Mrs. Julia Grilly for photomicrography of sorted cells; and L. M. Holland, D.V.M., for obtaining animal blood samples. MCA-1 tumor was obtained from the Laboratory of Pathology, National Cancer Institute of the National Institutes of Health, Bethesda, Maryland.

LASL-AEC-OFFICIAL

REFERENCES

1. M. J. Fulwyler, Electronic Separation of Biological Cells by Volume, Science, 150: 910-911 (1965).

2. L. A. Kamentsky and M. R. Melamed, Spectrophotometric Cell Sorter, Science, 156: 1364-1365 (1967).

3. H. R. Hulett, W. A. Bonner, J. Barrett, and L. A. Herzenberg, Cell Sorting: Automated Separation of Mammalian Cells as a Function of Intracellular Fluorescence, Science, 166: 747-749 (1969).

4. M. A. Van Dilla, M. J. Fulwyler, and I. U. Boone, Volume Distribution and Separation of Normal Human Leucocytes, Proc. Soc. Exp. Biol. Med., 125: 367-370 (1967).

5. M. H. Julius, T. Masuda, and L. A. Herzenberg, Demonstration that Antigen-Binding Cells are Precursors of Antibody-Producing Cells after Purification with a Fluorescence-Activated Cell Sorter, Proc. Natl. Acad. Sci. U.S.A., 69: 1934-1938 (1972).

6. J. A. Steinkamp, M. J. Fulwyler, J. R. Coulter, R. D. Hiebert, J. L. Horney, and P. F. Mullaney, A New Multiparameter Separator for Microscopic Particles and Biological Cells, Rev. Sci. Instr., 44: 1301-1310 (1973).

7. J. A. Steinkamp, A. Romero, and M. A. Van Dilla, Multiparameter Cell Sorting: Identification of Human Leukocytes by Acridine Orange Fluorescence, Acta Cytol., 17: 113-117 (1973).

8. J. A. Steinkamp, J. H. Larkins, L. J. Carr, and P. M. Kraemer, Flow Microfluorometric Studies of Lectin Binding to Mammalian Cells. II. Estimation of the Surface Density of Receptor Sites by Multiparameter Analysis (manuscript in preparation).

9. P. M. Kraemer, R. A. Tobey, and M. A. Van Dilla, Flow Microfluorometric Studies of Lectin Binding to Mammalian Cells. I. General Features, J. Cell. Physiol., 81: 305-314 (1973).

10. H. A. Crissman and J. A. Steinkamp, Rapid Simultaneous Measurement of DNA, Protein, and Cell Volume in Single Cells from Large Mammalian Cell Populations, J. Cell Biol. (1973), in press.

11. P. K. Horan, A. Romero, J. A. Steinkamp, and D. F. Petersen, Detection of Heteroploid Tumor Cells, J. Natl. Cancer Inst. US (1973), submitted.

12. R. A. Tobey, H. A. Crissman, and P. M. Kraemer, A Method for Comparing Effects of Different Synchronizing Protocols on Mammalian Cell Cycle Traverse. The Traverse Perturbation Index, J. Cell Biol., 54: 638-645 (1972).

13. L. R. Adams and L. A. Kamentsky, Machine Characterization of Human Leukocytes by Acridine Orange Fluorescence, Acta Cytol., 15: 289-291 (1971).

LASL-AEC-OFFICIAL

FIGURE LEGENDS

Fig. 1. Multiparameter cell separator illustrating the flow chamber; laser illumination; droplet generation, charging, and deflection scheme; and sample collection.

Fig. 2. Frequency distribution histograms of CHO cells reacted with Con A-F: (A) cell volume and cell surface area distributions; (B) ungated and gated cell surface fluorescence distributions; and (C) gated cell surface fluorescence-to-area ratio distribution.

Fig. 3. Frequency distribution histograms of HeLa cells doubly stained with fluorescein isothiocyanate (protein) and propidium iodide (DNA): (A) DNA content distribution; (B) protein and cell volume distributions; and (C) protein-to-cell volume ratio distribution.

Fig. 4. Two parameter DNA-protein frequency distribution histogram (isometric view) and contour view of HeLa cells stained with fluorescein isothiocyanate (protein) and propidium iodide (DNA). The contour view was recorded near the zero cell number level.

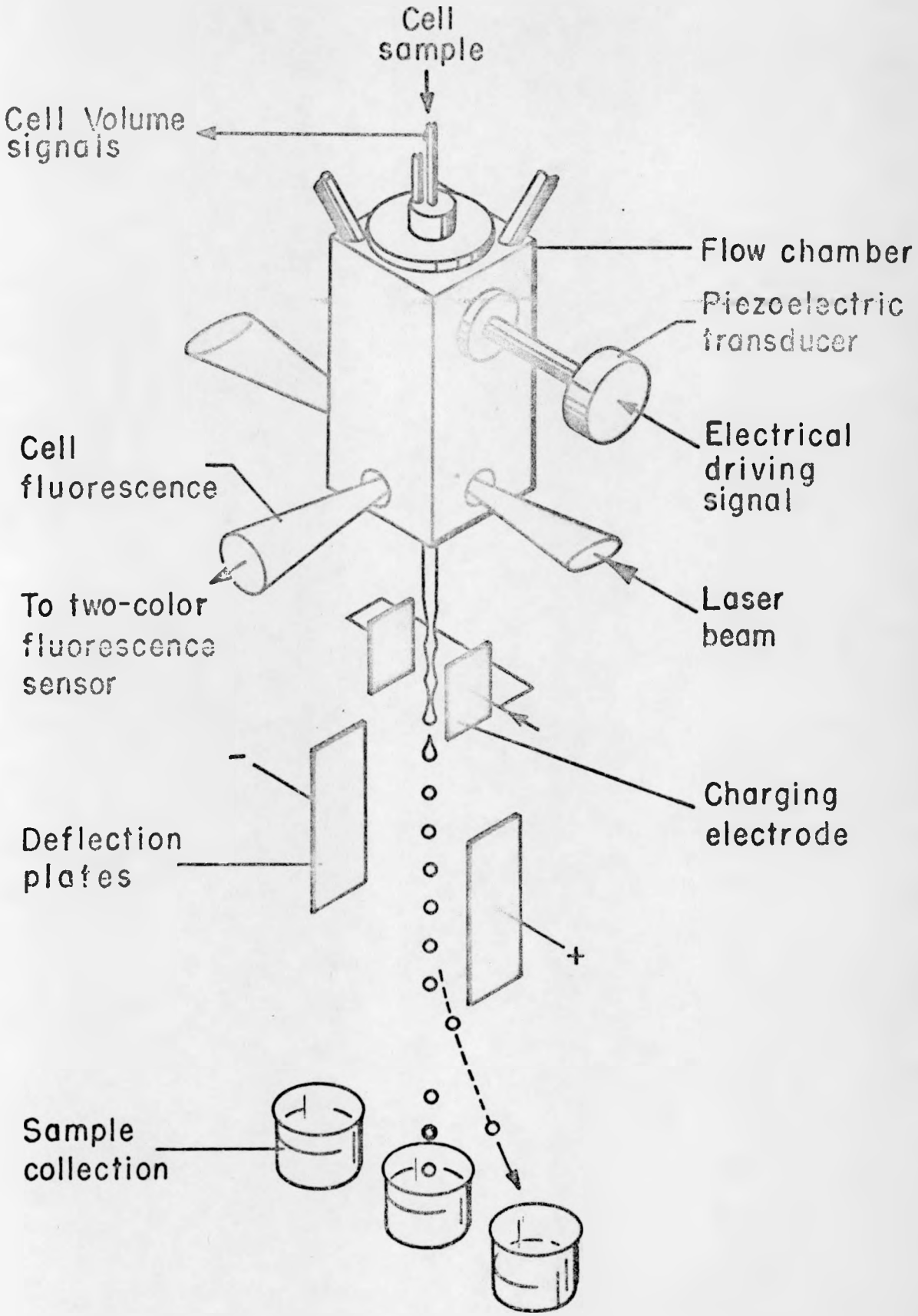
Fig. 5. Frequency distribution histograms of acriflavine-Feulgen DNA stained mouse (C3H/Hej) spleen and MCA-1 tumor cells: (A) DNA content distribution of normal spleen cells; and (B) DNA content distribution of MCA-1 tumor cells. Photomicrographs of (C) dispersed tumor cells prior to sorting; (D) sorted mouse leukocytes of sort region 1; and (E) sorted tumorigenic cells of sort region 2. Both DNA content distributions (A) and (B) were measured at the same fluorescence channel amplifier gain setting.

Fig. 6. Frequency distribution histograms of acridine orange-stained normal dog (beagle) leukocytes: (A) green fluorescence distribution; (B) red fluorescence distribution; and (C) green-to-red fluorescence ratio distribution. Photomicrographs of (D) blood smear prior to sorting; (E) sorted

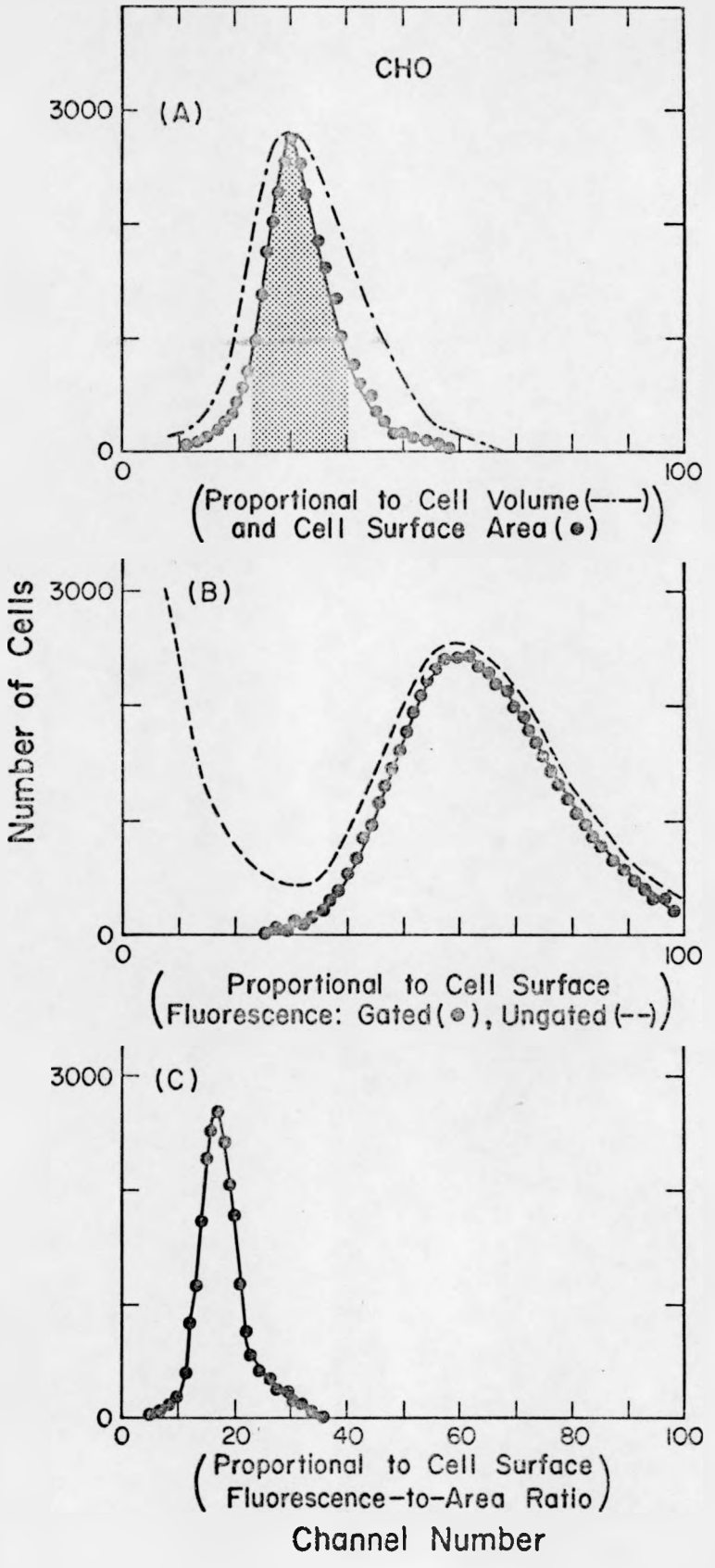
LASL·AEC·OFFICIAL

lymphocytes; (F) sorted neutrophils; and (G) sorted eosinophils. Erythrocytes contained in the photomicrographs were separated along with selected leukocytes.

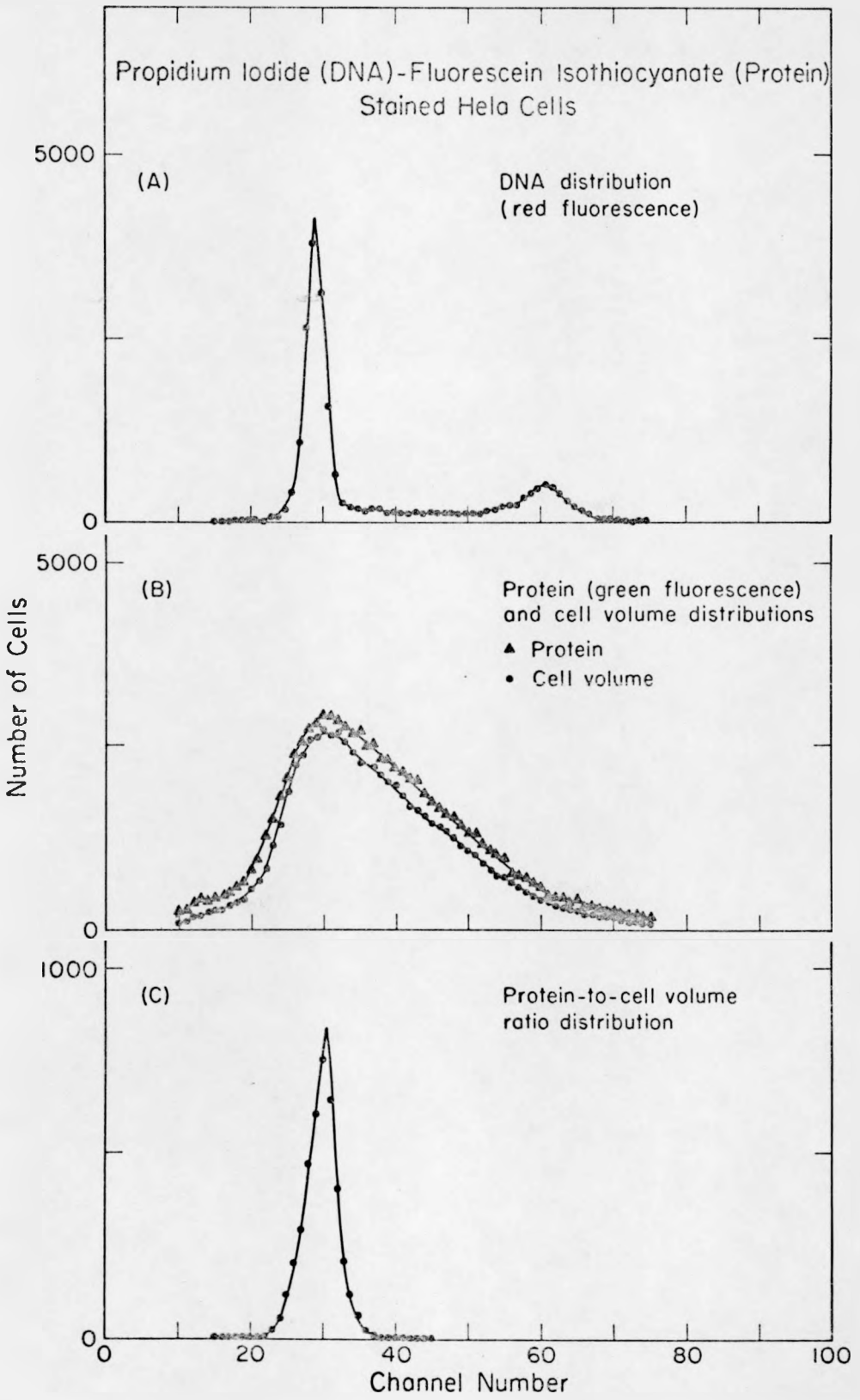
Fig. 7. Frequency distribution histograms of acriflavine-Feulgen DNA stained HeLa cells: (A) DNA content and cell volume distributions; and (B) cell volume distributions of G_1 and G_2 + M cells obtained by gated single parameter analysis. Two parameter DNA-cell volume plots illustrating (C) three-dimensional isometric and two-dimensional contour views of cells prior to sorting; and (D) cells reanalyzed after sorting. The contour views were recorded near the zero cell number level.



LASL-AEC-OFFICIAL

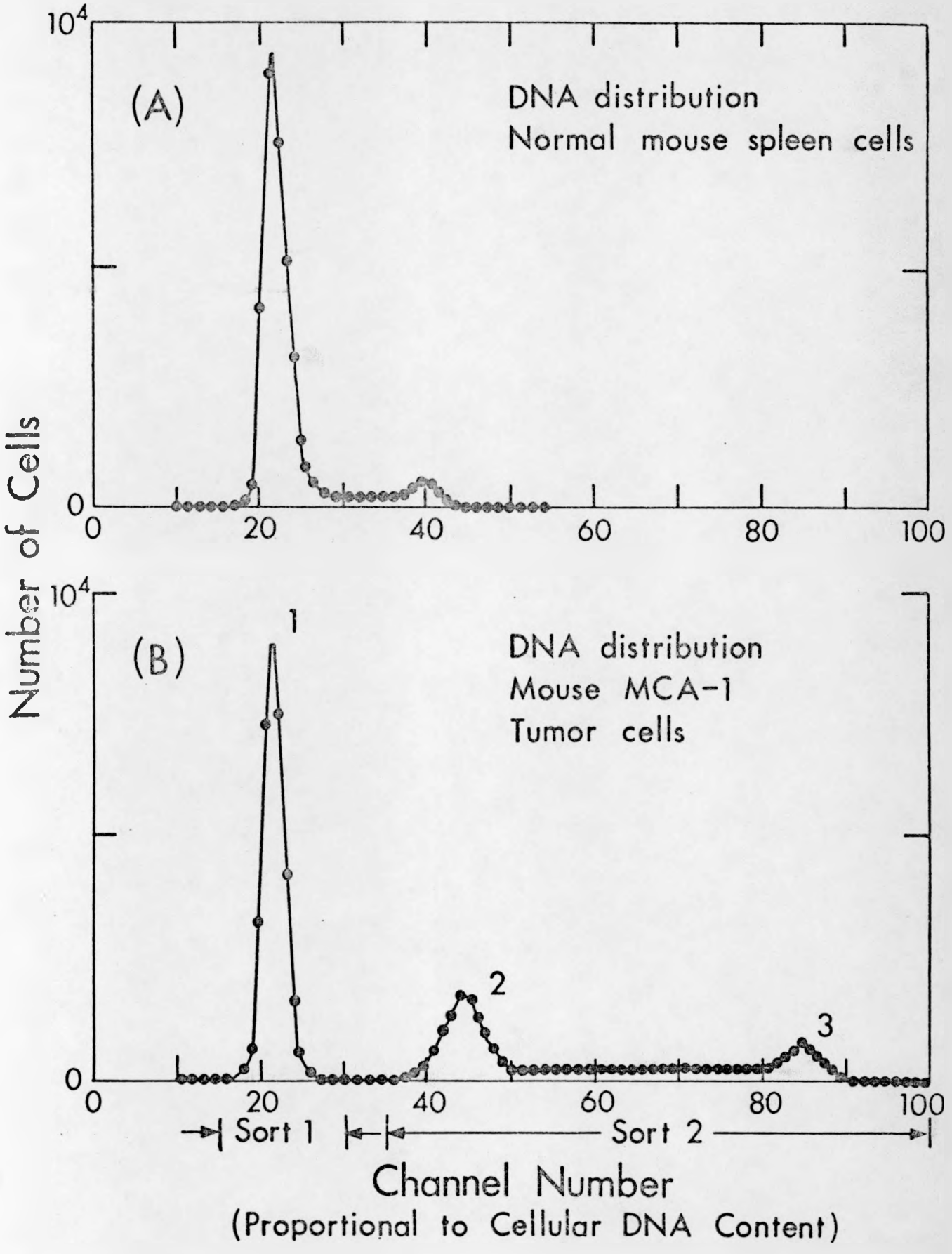


LASL-AEC-OFFICIAL



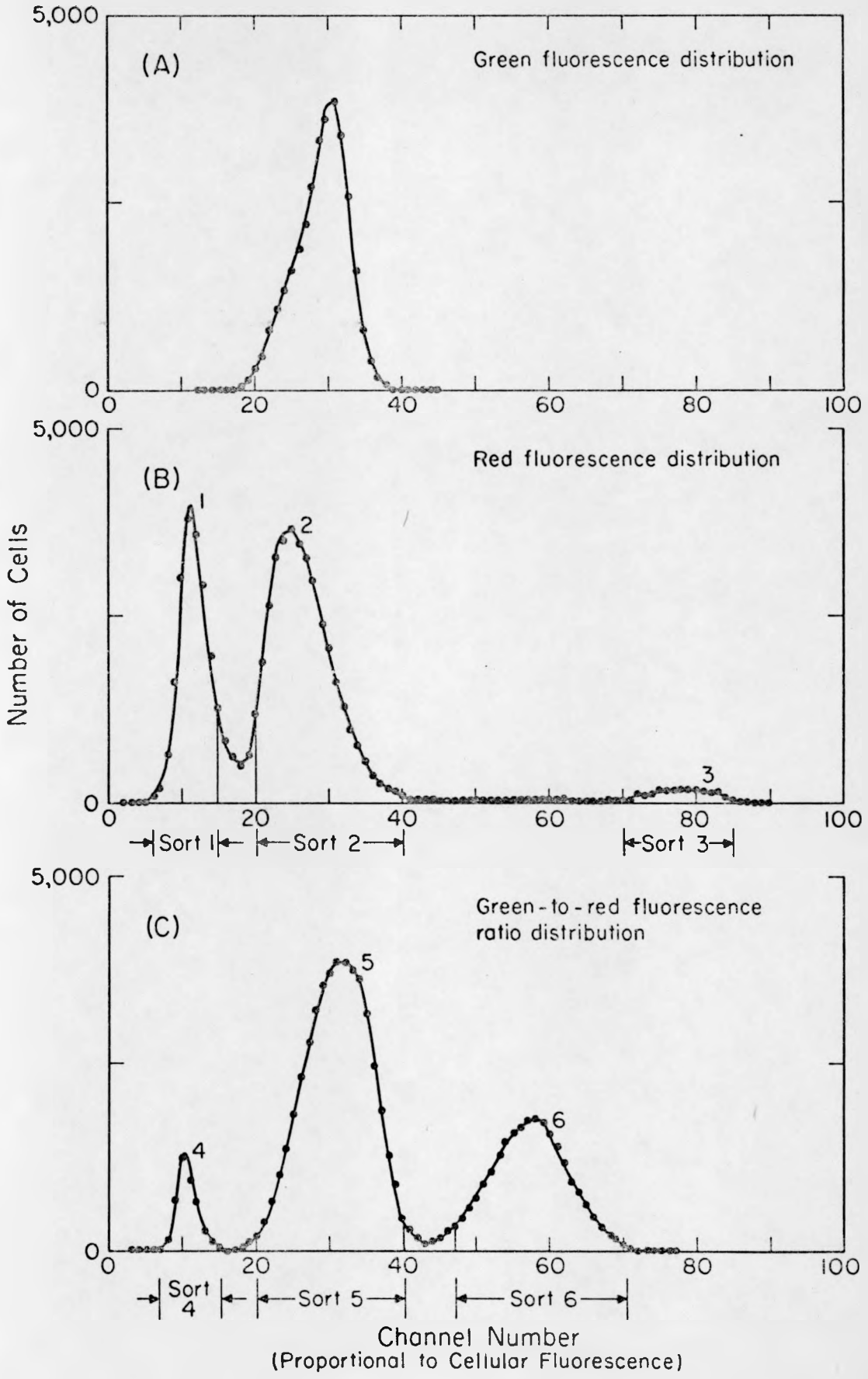
LASL·AEC·OFFICIAL

HELA
ISOMETRIC
CONTOUR
NO. OF CELLS
0
40
DNA
PROTEIN
G1
S
G2+M
LASL-AEC-OFFICIAL
LASL-AEC-OFFICIAL



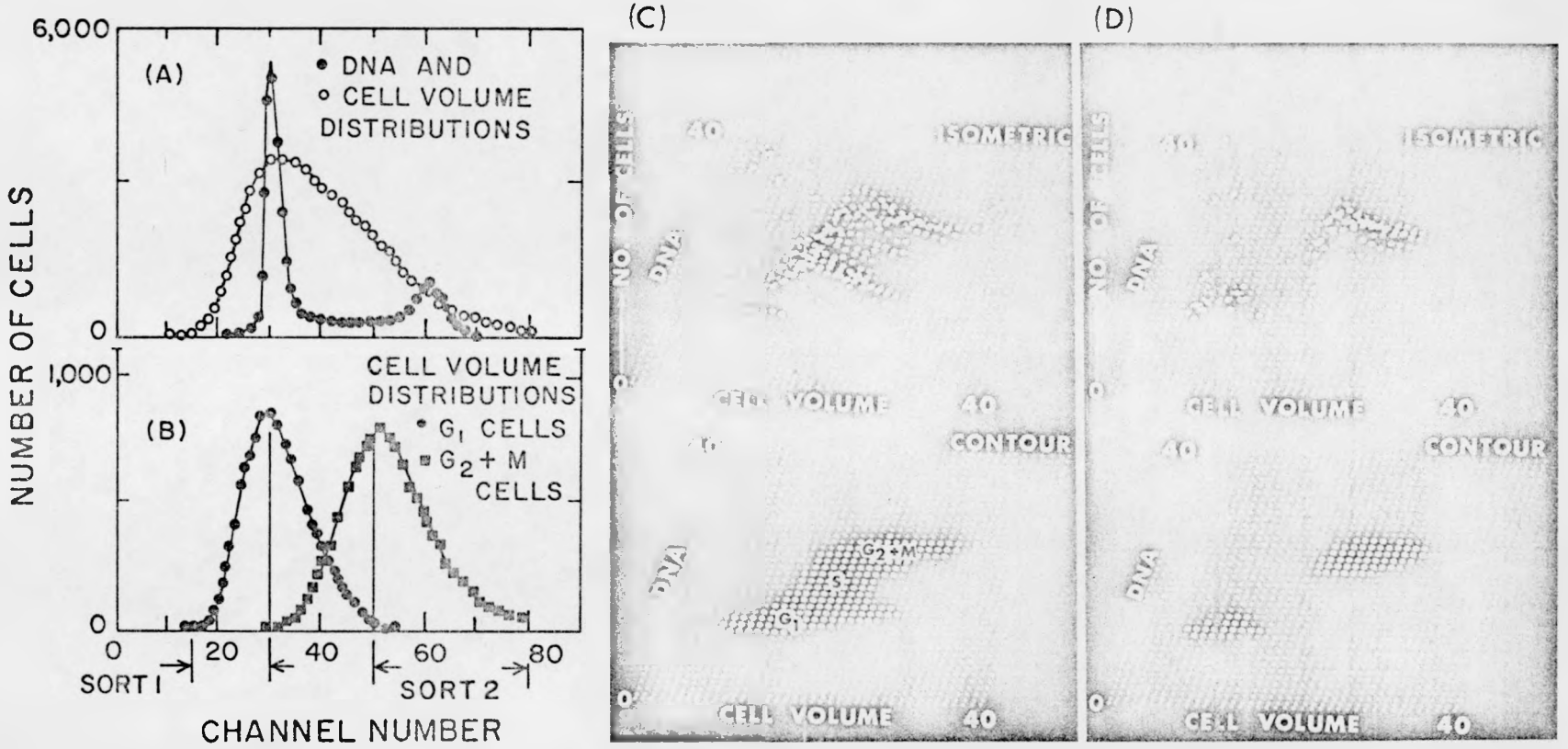
LASL AEC-OFFICIAL

LASL-AEC-OFFICIAL



LASL AEC-OFFICIAL

LASL AEC-OFFICIAL



LASL-AEC-OFFICIAL