RAPID, SIMULTANEOUS MEASUREMENT OF DNA, PROTEIN, AND CELL VOLUME IN SINGLE CELLS FROM LARGE MAMMALIAN CELL POPULATIONS

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INTRODUCTION

The advent of high speed, cell analysis systems (1–5) for rapid measurement of physical and biochemical properties in single cells has provided new and useful techniques for performing a wide variety of biological experiments. Such systems recently have been used in studies related to DNA content per cell as a function of chromosome number (6, 7), effects of chemotherapeutic agents on cell cycle traverse (8), and quantitation of cell surface binding of the plant lectin concanavalin A (9) as well as immunofluorescent detection of antigen-binding cells (10). These investigations have involved quantitative fluorescent staining of monodisperse cell populations in liquid suspension by techniques specific for biochemical components of interest and subsequent rapid analysis of the fluorescence emission signal obtained as each cell traverses a laser beam. General operating characteristics of this flow system, as well as validation of the methodology, have been presented in detail elsewhere (7, 11).

These earlier studies involved measurement of only a single cellular property. In this report we describe a method for measurement and simultaneous analysis of DNA, protein, and cell volume in the same cell by use of a newly developed multiparameter cell analysis system which incorporates several pre-existing analytical techniques and allows all the various measurements to be performed *on the same cell* under conditions where large populations can be analyzed rapidly and a high degree of statistical precision obtained. Electronic processing of fluorescence and volume signals from each cell provides a direct method for establishing relationships between various cellular properties reflecting specific phases of the cell cycle.

MATERIALS AND METHODS

HeLa and L-929 cells were grown on monolayers in double-strength Eagle's basal medium supplemented with 20% fetal calf serum, streptomycin, and penicillin. Chinese hamster cells (line CHO) were grown in suspension culture as previously described (8). Cells were doubly stained for DNA and protein by a modification of the techniques of Dittrich et al. (12) substituting propidium iodide (PI) (13) for ethidium bromide (EB) (14) as the DNA stain. Cells were dispersed with trypsin (15), fixed on ice in 70% ethanol for 30 min, and then treated with RNase (1 mg/ml, pH 7.0) for 30 min at 37°C. After incubation, cells were rinsed with distilled water, stained on ice first with PI (0.05 mg/ml in 1.12% sodium citrate) for 20 min, rinsed once with distilled water, and then stained for 30 min with cold fluorescein isothiocyanate (FITC) (0.03 mg/ml in 0.5 M sodium bicarbonate). Cells were rinsed several times with water to remove all excess stain and were finally resuspended in 0.85% saline for analysis. In the excited state, PI-stained cells emit a reddish-orange fluorescence (emission peak 590 nm), while FITC emits a greenish-yellow fluorescence (emission peak 530 nm). Although some overlapping of the two colors occurs, adequate resolution is obtained by use of appropriate color filters. Preliminary experiments revealed that the DNA distribution patterns obtained with PI are equivalent to patterns obtained utilizing Feulgen procedures. Validation of FITC staining is presented in this report.

Cells stained for DNA and protein are introduced at a rate of approximately 1,000 cells/s into a specially designed flow chamber where electrical and optical sensors measure cell volume and single or two color fluorescence. Cells first traverse a 75-μm diameter orifice, producing an electronic signal pulse which is proportional to cell volume (16). Approximately 135 μs later, the cells intersect an argon ion laser beam (488 nm) which excites the fluorochromes. The cell volume signal is amplified and the peak amplitude stored electronically until associated amplitudes of each fluorescence signal are measured and stored. Signals from each cell are then processed electronically in a variety of ways for obtaining single parameter analyses, ratios of parameters, or gated single parameter analysis. Distributions obtained for a given cell population are accumulated and displayed as histograms using a multichannel pulse height analyzer. Complete two parameter analysis of cellular properties also is permitted by using a dual parameter multichannel pulse height analyzer for displaying

three-dimensional pulse amplitude distributions. Details concerning instrument and flow chamber design will be discussed elsewhere (17, 18).

RESULTS AND DISCUSSION

Fig. 1 shows pulse amplitude distributions obtained from DNA, protein, and cell volume measurements made on PI/FITC-stained Chinese hamster, HeLa, and L-929 cells. DNA distributions (Fig. 1 *a*) obtained by analyzing only red fluorescence are bimodal, typical for exponential populations. The large peak represents cells in G_1. The peak at approximately twice the modal channel number of G_1 cells represents cells in $G_2 + M$ (mitosis). Distributed between the two peaks are cells in S phase with varying degrees of DNA replication completed. Computer analysis of the DNA content of exponentially distributed CHO cell populations has been used to demonstrate that the fraction of cells in each major phase of the cell cycle was comparable to fractions deduced by biochemical cell cycle analysis.[1, 2] The relative protein content and cell volume distributions (Fig. 1 *b*) were obtained by measuring either green fluorescence or volume signals separately. Preliminary studies showed that ethanol fixation decreased the volume signal by a relative factor of approximately 7.5 as compared to viable cells. However, the volume distribution curves for fixed and viable cells were essentially identical. The similarity of protein and cell volume curves in Fig. 1 *b* derives from the fact that protein comprises approximately 70–80% of the dry weight of cells and further reveals that FITC yields reliable protein content data. Ratios of protein content-to-cell volume (Fig. 1 *c*) were obtained by processing simultaneously the green fluorescence and cell volume peak signal amplitudes in a manner to form a ratio signal for each individual cell. The ratio histograms are narrow, unimodal distributions which vividly demonstrate a high degree of correlation between these two cellular properties. In comparison, the ratios of both DNA-to-protein and DNA-to-cell volume (Fig. 1 *d*) are not as well correlated as protein and cell volume. This is not surprising in view of the continuous rate of increase in both protein mass and cell volume during progression from G_1 to M (19, 20) coupled with the discontinuous increase in DNA content, with genome replication occurring only during the S phase of the cell cycle. Computer analysis of the ratio of DNA-to-protein through the S period will reveal information relating to the rate of DNA synthesis.

Fig. 2 *a* shows frequency distributions of DNA and protein for CHO cells obtained as described for Fig. 1. Figure 2 *b* and *c* represent the protein content distributions and DNA-to-protein ratios, respectively, for populations in G_1, S, and $G_2 + M$ which were obtained by gated single parameter analysis techniques. This method permits selective analysis of various cellular parameters based on criteria set by another cellular parameter. For example, the protein distribution of G_1 cells was obtained by analyzing electronically only those green fluorescence signals (i.e., protein content) associated with cells whose red fluorescence signal amplitude (i.e., DNA) fell within the G_1 range of the DNA distribution. DNA-to-protein ratio distributions (Fig. 2 *c*) were obtained in a similar manner by using both the red and green fluorescence signals to produce ratio displays for the G_1, S, and $G_2 + M$ phases. Mean values of the protein content distributions (Fig. 2 *b*) were calculated, and ratios of S to G_1 and $G_2 + M$ to G_1 were compared to those obtained from a theoretical model of cell growth (21). The theoretical model neglects dispersion and assumes all cells have divided with identical protein content, grow exponentially in this quantity, and divide with an exactly doubled protein content. The model also assumes that duration of the phases of the cycle are identical for all cells. Comparisons of observed-to-theoretical values were: S to G_1, 1.39 vs. 1.33; $G_2 + M$ to G_1, 1.73 vs. 1.55, respectively. Agreement for the S to G_1 ratio is good, while that for the $G_2 + M$ to G_1 ratio shows the deviation expected from neglect of dispersion in the theoretical model. By assuming that DNA content increases linearly across S, a similar model prediction can be made for the DNA-to-protein ratio for S and for $G_2 + M$ normalized to the G_1 value (Fig. 2 *c*). Comparisons of observed-to-theoretical ratios are: S to G_1, 1.11 vs. 1.13; $G_2 + M$ to G_1, 1.24 vs. 1.29, respectively. In this case, agreement is excellent and demonstrates further that electronic gated analysis is particularly useful for quantitative investigation of biochemical processes occurring at specific phases of the cell cycle.

[1] H. A. Crissman, J. H. Jett, and D. F. Petersen. Life-cycle analysis of mammalian cells by flow microfluorometry and labeled DNA precursors. In preparation.

[2] P. N. Dean, and J. H. Jett. Computer analysis of flow microfluorometrically determined DNA distributions in mammalian cell populations. In preparation.

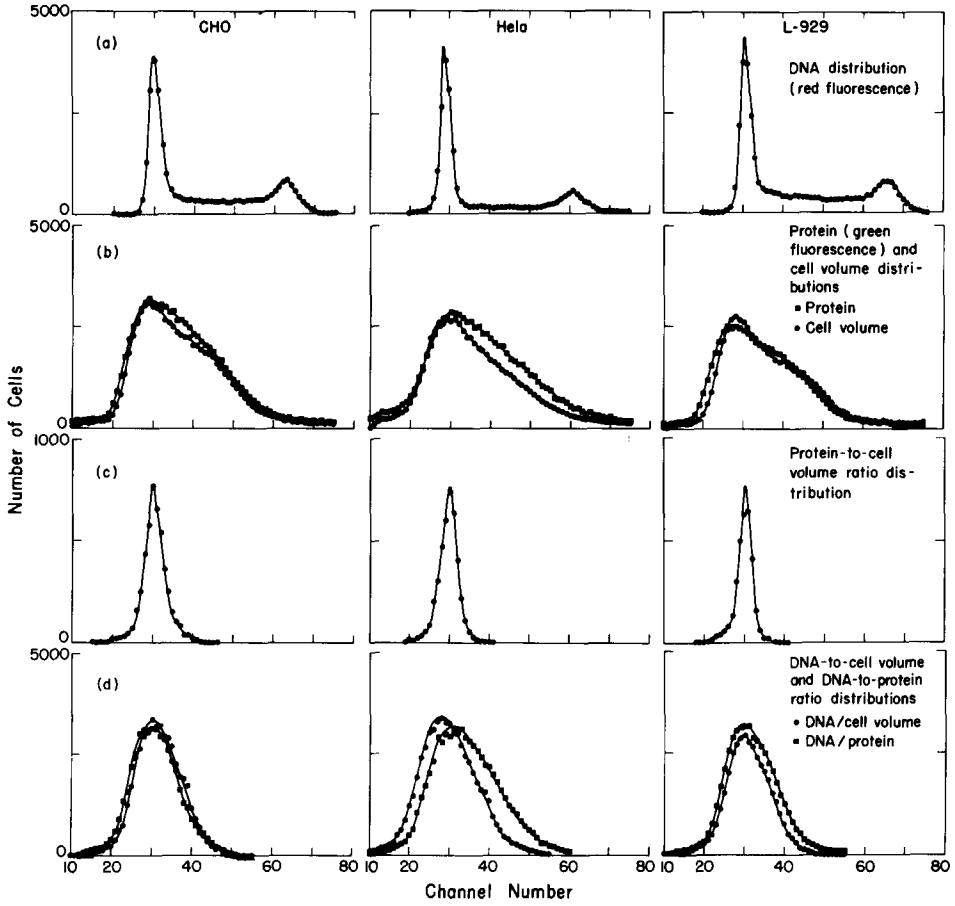

FIGURE 1 DNA, protein, and cell volume distributions (*a* and *b*) for CHO, HeLa, and L-929 cells. Protein-to-cell volume ratios (*c*) were obtained by utilizing both the green fluorescence and cell volume signals from each cell to produce electronically a ratio with an analog divider system. The coefficients of variation (standard deviation divided by the mean) of the ratio distributions are 6.8%, 6.5%, and 5.3%, respectively, for CHO, HeLa, and L-929 cells. DNA-to-protein ratios (*d*) were obtained similarly by analysis of both the red and green fluorescence signals for each cell. The abscissal scales (channel number) have a depressed zero but otherwise are proportional to either DNA content, protein content, cell volume, or respective ratios. The constants of proportionality are different for the various cell types.

Complete two parameter cell analysis can be performed also by using a dual parameter, multichannel, pulse height analyzer. The accumulated data then may be displayed as isometric or contour views. Fig. 3 shows an example of the additional fine structure which can be obtained from this analysis. In particular, the contour data display reveals wide variations in protein content of both G_1 and G_2 + M cells. However, in any given region in S, these variations are notably diminished. Two parameter analysis also permits three-dimensional sectioning of a portion of the total population to select cells contained within a narrow range having a given property (e.g., protein content) and then quantitating the dependence of another property (e.g., DNA) to the initial preselected property. Computer analysis of data obtained in this manner will reveal information related to cell size and cell division (22), effects of various drugs on both DNA and protein synthesis at various times during the cell cycle, as well as the rate of DNA synthesis of different cell types under a variety of experimental conditions. Numerous combinations of two parameter data such as DNA-to-protein ratios vs. either protein, DNA, or cell volume also can be generated and displayed.

The techniques of simultaneous cell volume and

two color fluorescence measurement coupled with multiparameter processing methods add a new dimension to biological investigation. In addition to DNA and protein analyses presented here, the multiparameter analysis system will be equally useful for investigating the cell cycle dependence of antigen production using PI in combination with FITC conjugated to a given antibody. The

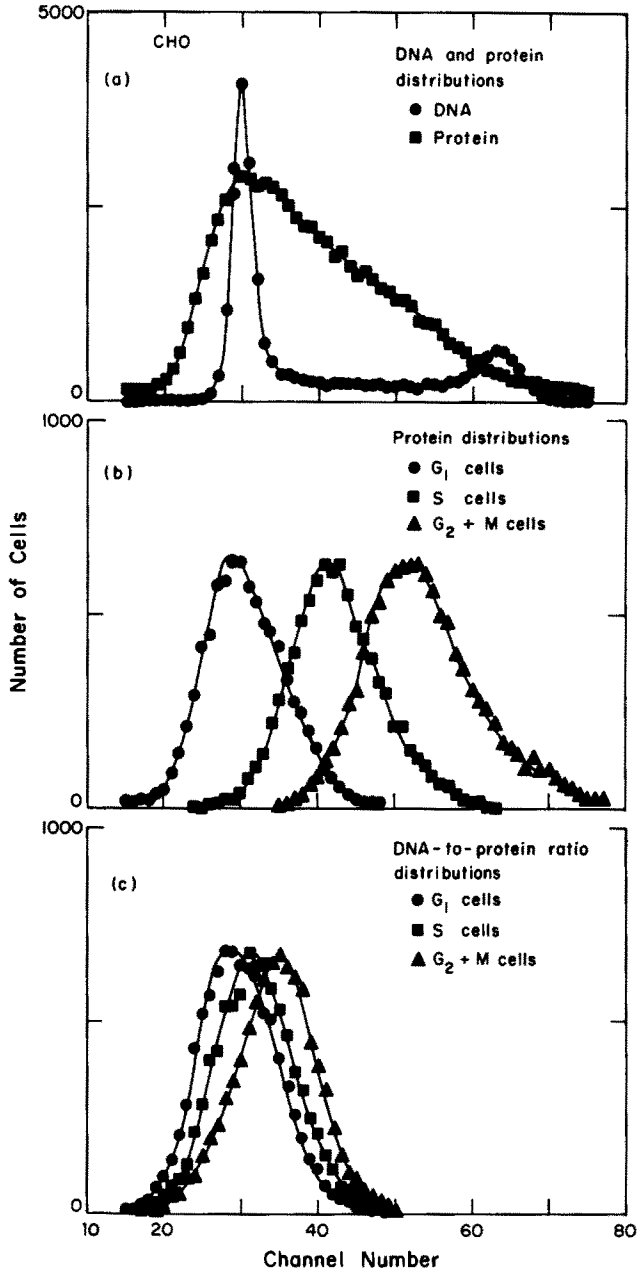

FIGURE 2 Gated single parameter analysis of PI/FITC-stained CHO cells. DNA and protein distributions (*a*) of stained CHO cells were obtained by measuring either the red or green fluorescence signal separately. Protein distributions (*b*) and DNA-to-protein ratios (*c*) for the G_1, S, and G_2 + M phases of the cell cycle were obtained by electronic gated analysis, as explained in the text. The abscissal scale (channel number) has a depressed zero.

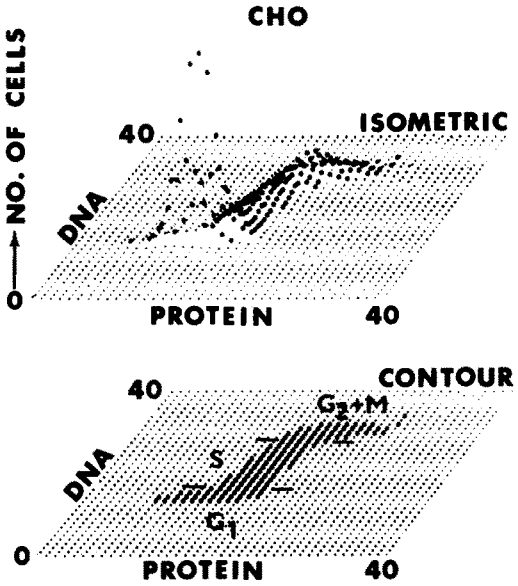

FIGURE 3 Complete two parameter analysis display of DNA and protein content of stained CHO cells. The top figure is an isometric display which better shows cell density in particular regions. The lower figure, which is a contour display of the same distribution, is delineated to show various regions of the cell cycle.

study of two antigens in cells also can be achieved by using two different antibodies, one conjugated to a red fluorescent dye (e.g., rhodamine B isothiocyanate) and the other to FITC (23), and the ratio of the two antigens relative to cell size can be obtained. Other studies involving plant lectins such as concanavalin A and wheat germ agglutinin conjugated to FITC (9) can be made to study quantitatively cell surface interactions with respect to cell size and cell cycle. The methods described here have great potential in medical and biological research, and the range of applications will expand as other staining methods become available.

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