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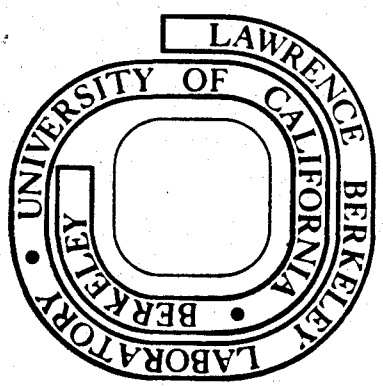

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FLOW MICROFLUOROMETRY OF ISOLATED NUCLEI

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Brief Note

INTRODUCTION

The technique of flow microfluorometry(FMF) has contributed new insights into the areas of cell cycle analysis(2,3), cell surface labeling(5,13,14) chromosomal analysis (9,10,20), and tumor cell detection (1,12,18). The hardware necessary for these analyses as described by Holm and Cram(11), Steinkamp, et al.(19), and Van Dilla, et al.(22,23) has been developed to a high level of sophistication. One difficult area in FMF analysis that remains is sample staining.

The most important criterion of a stain for FMF analysis is that it be selective for the cell property being investigated. In cell cycle analyses the approach has been either to use a stain which binds specifically to DNA, or to eliminate from the cells components other than DNA which bind the stain. An example of the former approach is the successful use of the antibiotic mithramycin(7,21). Mithramycin staining of fixed cells is rapid, and DNA histograms with quite good coefficients of variation(CV'es) can be obtained. However, the antibiotic is not readily available, and substitutes such as chromomycin are expensive. Also, the argon-ion laser line used to excite mithramycin stained cells (457 nm) is realtively weak in most commercially available lasers. Propidium iodide staining of cells has been used in cell cycle analyses(6), but successful application of this dye requires the prior removal of RNA by treating the fixed cells with ribonuclease. Krishan(15) eliminated the RNAase procedure by isolating nuclei from cytoplasmic RNA and subsequently staining them with propidium iodide. Hypotonic lysis by Krishan's technique yields with some cell systems DNA histograms superior to other staining procedures particularly when cells grown in suspension are used. However, nuclei cannot be satisfactorily

isolated from many cell types by this technique, especially those cells which grow in monolayers(8). We have modified a standard nuclear isolation technique(17) and have included a ribonuclease treatment to yield nuclei from fibroblasts which can be readily stained with propidium iodide. Our technique greatly improves the resolution when compared with DNA histograms of stained whole cells grown in monolayer. In addition, if replicate samples of stained whole cells and nuclei are examined using this technique a numerical evaluation of the proportion of cells in G_2 and in mitosis can be obtained.

MATERIALS AND METHODS

Cell Culture Techniques

The cells used in this study were mouse fibroblasts, Balb 3T3 A31 HYF. The properties and culturing of these cells have been described previously(2). For these experiments density inhibited cells were obtained from cultures that had been seeded at 1×10^4 cells/cm^2 and allowed to grow for 5 days in Dulbecco's modified Eagles medium(24; GIBCO, Grand Island, N.Y.) containing 10% newborn calf serum (Flow Laboratories, Rockville, Md.). Growing cells were from cultures seeded a 1×10^3 cells/cm^2.

Nuclear Isolation

Cell monolayers were washed 3 times with cold phosphate buffer saline (PBS) and centrifuged at 900 rpm for 5 min in an International PR-2 centrifuge. Nuclei were isolated in a hypotonic buffer(10 mM Hepes pH 7.5, 10 mM KCl 1.5 mM $Mg(OAc)_2$) containing 0.1% Triton X-100. The cell pellet was resuspended in 1 ml hypotonic buffer, and aspirated gently. Density inhibited cells were allowed to swell in this buffer for 5 min at 4^o, growing cells required 20 to 30 min. Cells were homogenized in a Dounce glass homogenizer 10 times with the B pestle. Nuclei were judged free of cytoplasmic contamination at this point by phase contrast microscopy. Nuclei were then pelleted by spinning at 400 rpm for 5 min in an International PR-2 centrifuge. The pellet was then gently resuspended in 1 ml of hypotonic buffer containing 10 µg/ml propidum iodide(Calbiochem, San Diego, Calif.). Nuclei were kept at 4^o prior to analysis.

Cell Staining Technique

Cell monolayers were washed 2 times with cold Saline GM (1.5 mM $Na_2HPO_4.7H_2O$, 1.1 mM KH_2PO_4, pH 7.4 containing 1.1 mM glucose, 0.14 M NaCl,

and 5 mM KCl) and removed from the dishes by treating for 10 min at 37° with DISPO(Saline GM containing 0.5 mM EDTA and 0.1% trypsin). The trypsin was neutralized by treating the suspension with an equal volume of NEUT (Saline GM containing 0.63 mM $MgSO_4.7H_2O$, 0.11 mM $CaCl_2.2H_2O$, 2% soybean trypsin inhibitor, and 0.1% DNAase I). The cells were pelleted by centrifuging for 5 min at 720 x G in an International IEC HN-S centrifuge, and were fixed for at least 30 min at 4° in 25% ethanol containing 15 mM $MgCl_2$. The fixed cells were incubated at 37° for 1 hr with 1 mg/ml RNAase (Calbiochem, San Diego, Calif.), pelleted as above, and washed 2 times in Saline GM. Propidium iodide(Calbiochem, San Diego, Calif.) was added to a final concentration of 50 µg/ml. The cells were stained for 30 min at room temperature, washed 1 time in distilled water, and resuspended in water for analysis.

Flow Microfluorometry

The stained cells were analyzed by FMF as described previously(2). The instrument used for these analyses was constructed according to the specifications of Steinkamp, et al.(19).

RESULTS AND DISCUSSION

Whole cells and isolated nuclei from monolayer cultures of Balb 3T3 A31 HYF cells both actively growing and density inhibited were stained. The analysis of these samples was essentially identical in all cases except that the amplifier gain used for the nuclei was 1.67 times that used for the whole cells. Figure 1 presents the DNA histograms obtained with these samples. When the signal amplitudes were corrected for the different gain settings it was determined that the cells were approximately 1.30 times brighter than the isolated nuclei. Previous experiments indicated that with neither staining technique were the samples saturated with dye; and therefore, the intensity of the signal was a function of how much dye was used in the staining reaction. Both the nuclear and whole cell samples were stable at 4^{o} for at least 24 hr.

The coefficient of variation(CV) for these histograms was calculated using the following equation.

[1] $$CV = \frac{(W_{1/2h})\ 0.426}{\text{mode channel}} \quad X\ 100$$

where: $W_{1/2h}$ = the number of channels encompassing the top half of the G_1 peak

The CV'es calculated for the four samples in Figure 1 are listed in Table 1. In all cases, the isolated nuclei had CV'es much lower than those exhibited by whole cells. Also in Table 1 are listed the proportions of the analyzed populations in the various phases of the cell cycle. These values were obtained by fitting the data using a computer program based on the mathematical model of the DNA histograms described by Fried, et al.(8). In both the density inhibited and growing populations the stained nuclei gave a greater proportion of the original population in G_1 than did the whole cells. This increase was primarily at the expense of a decrease in the the proportion of cells in S. As described below the

majority of this redistribution can be accounted for by the loss of mitotic cells from the nuclear preparation; however, undoubtedly some of this effect is due to the improved fit to the data made possible by the decreased CV and a closer approach to a Gaussian distribution in the compartments from the DNA histograms of the isolated nuclei.

The proportion of the whole cell preparation that is mitotic cells was calculated from the DNA histograms of the isolated nuclei and whole cells. This calculation is based on the observation that the mitotic cells are lost during the nuclear isolation procedure. Therefore, the histogram from the isolated nuclei represents those cells in G_1+S+G_2; whereas, the whole cell histogram is of cells from all phases of the cell cycle. Since each histogram represents 100% of the particles analyzed for that particular sample, and the proportions calculated by the fitting model for the various phases are proportions of that histogram, it was not possible to subtract the G_2 value obtained from the nuclei from the G_2+M value obtained from the whole cells and get the proportion of cells in M. Instead, the proportion of cells in M was calculated by use of equations [2] and [3].

[2] $$\frac{G_2^c+M^c}{G_1^c+S^c} - \frac{G_2^n}{G_1^n+S^n} = \frac{M^c}{G_1^c+S^c}$$

[3] $$\frac{G_1^c + S^c(M^c)}{G_1^c + S^c} = M^c$$

where: the superscript c refers to the value obtained from the whole cell analysis and n refers to the value from the nuclei analysis

This calculation assumes that the ratio of G_2 to G_1+S cells is not changed by the two staining techniques. When a value of 16 hr was used for the doubling time of Balb 3T3 A31 HYF cells grown as described in MATERIALS AND METHODS(2), the length of the various phases was calculated

(Table 1). The length of M for these cells(0.37 hr) is in good agreement with the value reported for other systems using other analysis techniques(4).

During the course of these studies we observed that the RNAase treatment did not alter the histograms obtained from nuclei of density inhibited cells; however, this procedure was essential for the analysis of nuclei from growing cells. Without the RNAase treatment nuclei from growing cells gave histograms with greatly decreased resolution of G_1, S, and G_2 (large CV'es). Presumably, this difference in the need for the RNAase treatment is due to the presence of more RNA in the nucleus of growing cells vs density inhibited cells. This observation may also be due to a greater contamination of nuclei from growing cells with cytoplasmic RNA. For routine cell cyle analysis the RNAase step is recommended to eliminate artifacts due to changes in RNA content during the experiment.

Summary

A simple method is described for obtaining nuclei suitable for flow microfluorometry from cells grown in monolayer. The technique involves detergent lysis of cells in hypotonic buffer followed by a brief homogenization. For some samples, treatment of the nuclei with RNAase was necessary to achieve maximum resolution. The method in combination with whole cell analysis allows an estimation of the proportion of cells in M. For the Balb 3T3 A31 HYF cells used in these experiments, the length of M was calculated to be 0.37 hr.

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Table 1. Cells Cycle Distributions of Density Inhibited and Growing Balb 3T3 A31 HYF Cells Analyzed by Flow Microfluorometry of Propidium Iodide Stained Nuclei or Whole Cells.

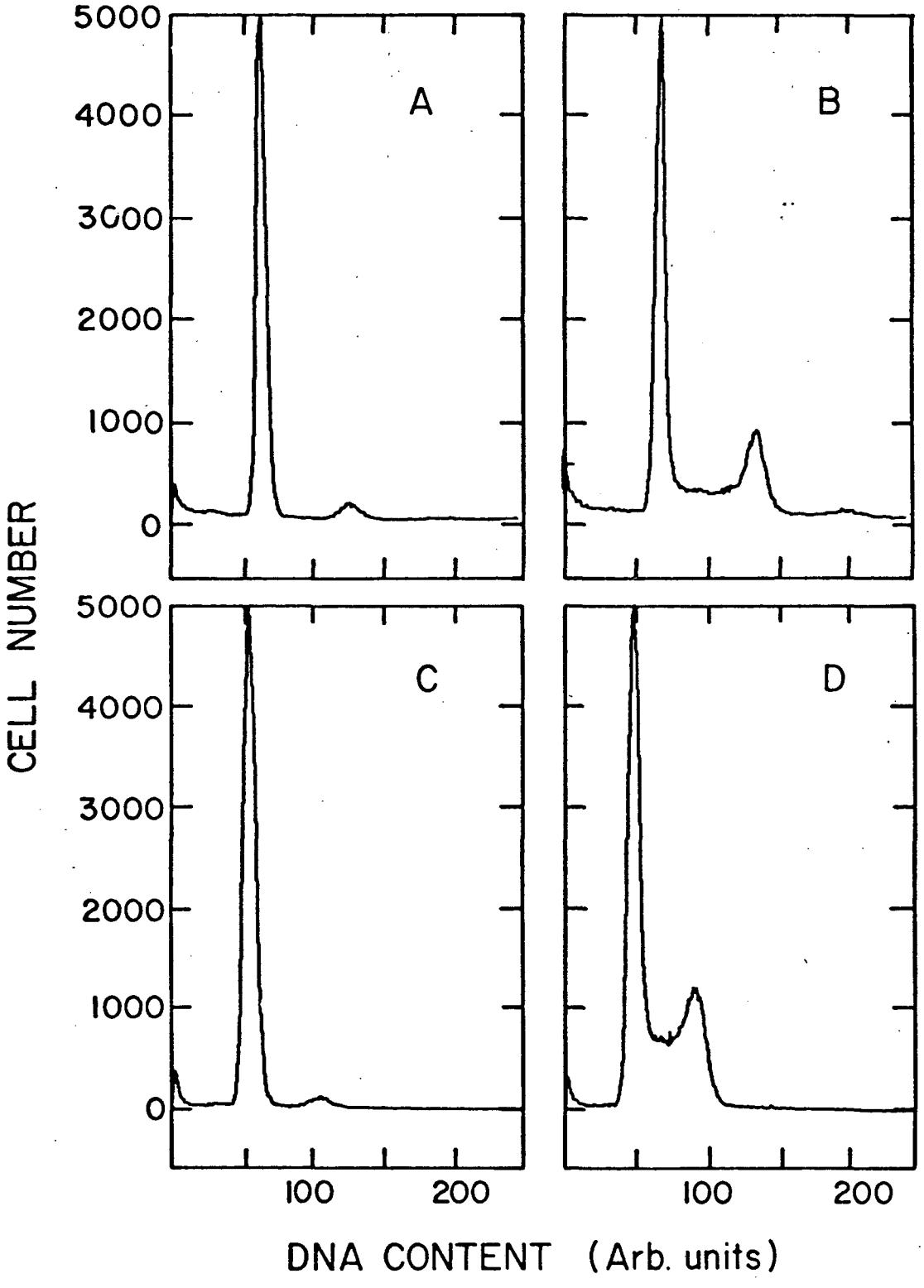
Sample	CV[a]	Fraction of Cells in Each Phase G_1	S		G_2[b]	M[b]
Density Inhibited						
Nuclei	5.01	0.900	0.046	0.050	-	-
Cells	8.07	0.861	0.087	0.052	-	-
Growing						
Nuclei	4.60	0.549	0.266	0.186	-	-
Cells	8.03	0.513 (6.83)	0.276 (3.44)	0.211	0.180 (2.17)	0.031 (0.37)

The numbers in parentheses are the values calculated for the length in hours of the cell cycle phases using the fraction of cells in that phase and a doubling time of 16 hours(2), and the formulation described by Mak(16).

[a]Coefficient of Variation.

[b]Calculated as described in the text.

Figure 1. DNA histograms of isolated nuclei and whole cells stained with propidium iodide: (A) Nuclei from density inhibited cultures; (B) Nuclei from growing cultures; (C) Whole cells from density inhibited cultures; (D) Whole cells from growing cultures.


5000
4000
3000
2000
1000
0
A
B
C
D
CELL NUMBER
100
200
DNA CONTENT (Arb. units)

XBL 771-4102

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