

RAPID FLOW CYTOFLUOROMETRIC ANALYSIS OF MAMMALIAN CELL CYCLE BY PROPIDIUM IODIDE STAINING

AWTAR KRISHAN. From the Sidney Farber Cancer Center and Harvard Medical School, Boston, Massachusetts 02115

In mammalian cell cycle studies, one of the prime objectives is to identify cells in the process of genome replication, and, consequently, incorporation of radioactive DNA precursors such as tritiated thymidine has been one of the main tools for such analysis. Whereas scintillation spectrophotometry provides quantitative information on the amount of precursor incorporated into DNA of the replicating cells, autoradiography by both light and electron microscopy helps to localize this incorporation and thus identifies the part of the replicating population (labeling index).

A number of other chemical and physical methods are available for determination of the DNA content per cell and involve the use of either a quantitative chemical determination of the amount of DNA per number of cells, or a determination of the DNA content per cell by Feulgen DNA absorption microspectrophotometry. Al-

though many of these analytical methods have been used alone or in combination, their widespread use in clinical cytokinetic or related studies has been hindered by the requirements of large sample size and by the time-consuming nature of the procedures. The recent introduction of laser-based flow microfluorometry (9) obviates some of these difficulties and makes it possible to do rapid determinations of DNA per cell on monodisperse cell populations. A number of preparative techniques have been used for cytofluorographic analysis and involve the use of either fluorescent dyes such as acriflavine, auromine-o, etc. after acid hydrolysis by the fluorescent Feulgen procedure (5, 7-10), or the use of DNA intercalating fluorescent compounds such as ethidium bromide and propidium iodide (4, 6). In both of these methods, a minimum of 2 h is required for processing of the specimens. Due to extensive cell loss incurred in

fixation, repeated centrifugation, and hydrolysis (Feulgen method) or RNase digestion (propidium iodide), large samples are needed for processing. Although in experimental studies ample specimens can be procured, the use of cytofluorometry in clinical studies has been hampered by the nonavailability of large samples and the difficulty of preparing single cell suspensions after fixation and repeated centrifugations of the clinical samples.

A simpler and quicker staining method for the analysis of DNA per cell by flow microfluorometry has been described by Crissman and Tobey (2) and involves the use of mithramycin as a fluorescent DNA intercalating label. Maximum excitation of the DNA-mithramycin complex fluorescence is obtained by a laser beam wavelength of 390–400 nm, and, since the commercially available Cytofluorograf (model 4801, Bio/Physics Systems, Inc., Mahopac, N. Y.) has only the 488-nm wavelength available for excitation, the fluorescence distribution patterns obtained from this instrument after mithramycin staining are not useful for cell cycle analysis.

In some of our recent cytokinetic studies, we have used propidium iodide staining as described by Crissman and Steinkamp (1), and by careful sample preparation we have analyzed samples containing a total of 1×10^6 cells. However, a minimum processing time of 1 h is still needed for this technique. The need for speedier results and the availability of rather very small biopsy samples has led us to compare the DNA per cell distribution histograms obtained after direct staining of cells with propidium iodide with those of cells analyzed after fixation in methanol and digestion in RNase. The present report describes some of these results.

MATERIALS AND METHODS

Human leukemic lymphoblasts of the CCRF-CEM cell line were grown in suspension cultures and nourished with Eagle's minimal essential medium (for spinner cultures) supplemented with 10% fetal calf serum, penicillin, and streptomycin. To obtain cell populations with different proportions of cells in G_1 , S, and G_2 -M of the cell cycle, cultures were blocked in G_1 early S of the cell cycle by exposure to thymidine (0.04 mM) or 0.5 mM 2,3-dihydro-1-H-imidazo[1,2-b]pyrazole (IMPY)¹ (3) for 18 h and were washed and reincubated in fresh

¹ *Abbreviation used in this paper:* IMPY, 2,3-dihydro-1-H-imidazo-[1,2-b]pyrazole.

medium. Aliquots of cells were removed at various time intervals from these blocked cultures.

Human peripheral blood was obtained either from a finger prick or by collection of 10 ml of peripheral venous blood in a heparinized syringe. Human bone marrow samples were collected in tubes containing sodium citrate as an anticoagulant.

CCRF-CEM cells (1×10^6) were centrifuged at 150 g for 5 min and the cell pellets resuspended in propidium iodide solution (0.05 mg/ml in 0.1% sodium citrate). For peripheral blood and bone marrow samples, 1–2 drops of the specimen were mixed with 1 ml of propidium iodide solution. Samples were examined after 5–10 min of staining at 4°C.

Cell pellets retrieved after centrifugation (150 g) of cells in culture were mixed with 50% methanol and fixed for 30 min at 4°C. Osmotic lysing of the red blood cells in the peripheral blood and the bone marrow samples was achieved by threefold dilution of the sample (1 ml) with glass-distilled water (3 ml) followed after 1 min by addition and mixing of 1 ml of 4.6% sodium chloride solution. Cells retrieved after centrifugation were resuspended and fixed in 50% methanol at 4°C for 30 min. Rehydration of the sample through a graded alcohol series was followed by incubation of cells in 1 mg/ml solution of RNase (in 0.2 M phosphate buffer, pH 7.0) for 30 min at 37°C. Subsequent centrifugation of the samples was followed by a wash in glass-distilled water and staining with propidium iodide solution at 4°C for 30–60 min.

Cells stained with propidium iodide directly and after fixation in methanol and digestion in RNase were analyzed in a Cytofluorograf (model 4801, Bio/Physics Systems, Inc.). In this instrument, single cells from a suspension are exposed to a focused argon ion laser beam (488 nm), and the fluorescence resulting from the laser excitation of the dye-DNA complex in each cell is collected and quantitated through a system of filters and photomultipliers. The resulting electrical pulses are stored in the memory unit of a pulse height analyzer and displayed as a histogram. The abscissa of the histogram is divided into 100 channels of increasing linear value from 0 to 100, while the height of the bars indicates the number of cells recorded in each channel. Data on the number of cells recorded in each channel were also obtained on paper tape.

RESULTS

In Fig. 1, DNA per cell distribution histograms of cells stained with propidium iodide after fixation in methanol and RNase digestion are shown on the left-hand side (A, C, E, G, and I) while on the opposite side (B, D, F, H, and J) are histograms of identical samples stained directly with propidium iodide for 5–10 min. Both sets of samples were analyzed under identical settings of laser power,

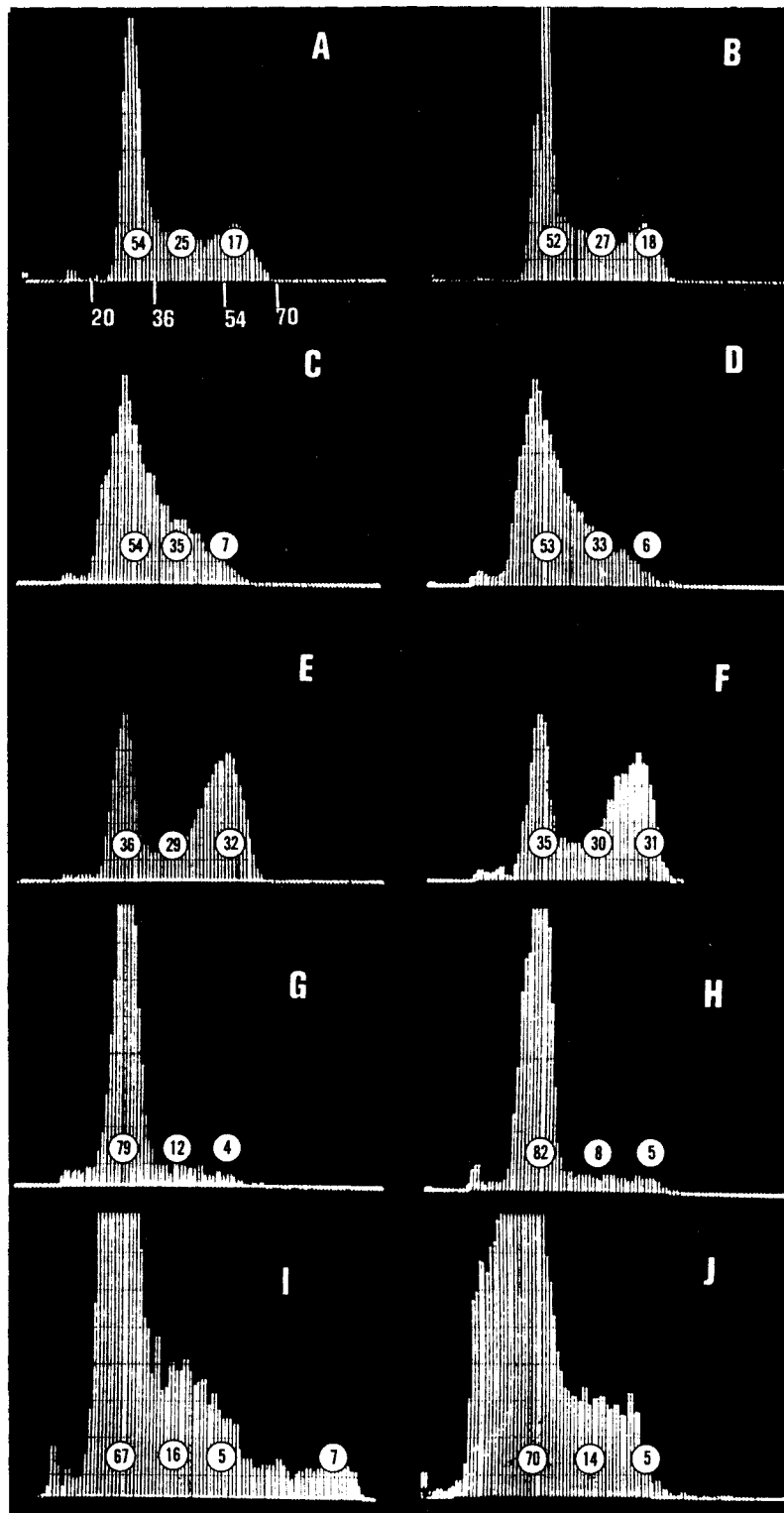


FIGURE 1 Shows DNA per cell frequency distribution histograms of various synchronized populations of CCRF-CEM lymphoblasts in culture (Fig. 1 A-F), and human bone marrow samples (Fig. 1 G-J). Histograms on the left are of samples stained with propidium iodide after fixation in methanol and digestion in RNase, while those on the right side are of identical samples after 5 min of incubation with propidium iodide. Numbers at the bottom of histogram 1 A record the position of the channels, while those recorded in circles on the histograms represent percentage of cells between channels 20-35 (G_1 -early S DNA content), 36-53 (S), and 54-70 (late S- G_2 -M).

electronic amplification and optics. The numbers (20, 36, 54, and 70) recorded at the bottom of the histogram in Fig. 1 A indicate the position of the channels, while those on the histograms (in circles) indicate the percentage of cells recorded between channels 20–35 (G_1 -early S), 36–53 (S), and 54–70 (late S, G_2 , and M).

Histograms in Fig. 1 A and B show the DNA per cell frequency distribution of log-phase CCRF-CEM cultures. A large peak is recorded between channels 20 and 35 and contains cells with G_1 and early S DNA content. These cells represent approximately 54% of the total population. In between channels 36 and 53, cells with the DNA content intermediate between that of G_1 and G_2 are recorded (S) while a smaller peak seen between channels 54–70 represents cells with the DNA content of cells in the late S, G_2 , and M part of the cell cycle.

A comparison of the histograms in Fig. 1 A and B along with the cell counts recorded between the three major channel groups shows that no significant difference is seen between DNA per cell distribution histograms of cells stained directly with propidium iodide (Fig. 1 B) and those stained after fixation with methanol and digestion in RNase (Fig. 1 A).

Similarly, in Fig. 1 C and D which show DNA per cell distribution histograms of a population of CCRF-CEM cells blocked in G_1 and early S part of the cell cycle by exposure to 0.04 mM thymidine for 18 h, no significant differences are seen between the two histograms from cells stained directly (Fig. 1 D) and those stained after fixation and RNase digestion (Fig. 1 C).

Histograms in Fig. 1 E and F show DNA frequency distribution of a CCRF-CEM culture blocked in G_1 -S for 18 h with 0.5 mM IMPY and subsequently incubated in fresh medium for 8 h. Approximately one-third of the population in these cultures had the DNA per cell content of cells in late S and the G_2 -M part of the cell cycle. A comparison of histograms of cells stained after fixation and RNase digestion (Fig. 1 E) with those stained directly with propidium iodide (Fig. 1 F) again confirms the overall similarity of the results from the two different staining procedures.

In a second series of experiments, we have compared the DNA per cell distribution histograms of peripheral blood, bone marrow, and cell suspensions from tumor biopsies stained with the direct method with those stained after fixation in methanol and digestion in RNase.

Histograms in Fig. 1 G and H show DNA distribution per cell from a bone marrow specimen stained after fixation and RNase digestion (Fig. 1 G) and after direct staining with propidium iodide (Fig. 1 H). A major proportion of the cells from this sample (approximately 80%) had the G_1 DNA content, and only a small proportion of the cells (13–16%) were recorded between channels 36 and 70 (S, G_2 , and M). As in the case of the CCRF-CEM cultures, identical results were obtained by the two different staining methods.

Fig. 1 I and J show DNA per cell distribution histograms from a second bone marrow sample analyzed after staining with propidium iodide directly (Fig. 1 J) and after fixation and digestion with RNase (Fig. 1 I). In these histograms, the height of the vertical bars has been amplified fourfold (compared to that of histograms 1 G and H) to give a better comparison of channels 54–100. Although an apparent similarity is noted between the two histograms, in the sample stained after fixation and digestion in RNase (Fig. 1 I), a significant number of cells (7% of total) are recorded with the DNA per cell content greater than that of G_2 -M (channels 71–99). In contrast to this, in samples stained directly with propidium iodide (Fig. 1 J), only 0.3% of the total population is recorded in this part of the DNA per cell distribution histogram. We presume that this population represents clumps of cells formed in the preparative manipulation of the sample (fixation and centrifugation) rather than representing cells with the hyperdiploid DNA content. Light sonication of cell suspensions prepared after fixation and centrifugation often reduces the number of these cell clumps, with a consequent decrease in the percentage of cells recorded between channels 70 and 99. As seen in Fig. 1 J, direct staining of bone marrow specimens with propidium iodide avoids formation of cell clumps and thus gives a DNA per cell distribution histogram without the artifacts caused by sample preparations.

DISCUSSION

Propidium iodide, an analog of ethidium bromide, intercalates with DNA and RNA and this binding markedly enhances the fluorescence of the dye (4, 6). In cells incubated with propidium iodide dissolved in an isotonic solution, dye entry into the cell is prevented by the cell membrane, and laser-excited fluorescence is seen only in nuclei of dead cells. Thus, propidium iodide dissolved in isotonic saline can be used for rapid fluorometric

quantitation of dead cells in a population. However, by incubation of cells in a hypotonic solution of propidium iodide, the structural ability of the plasma membrane for dye exclusion is lost and a brilliant fluorescent staining of the dye-nuclear DNA complex is observed within a few minutes of staining. Examination of these nuclei after 5 min of staining shows that hypotonic treatment with citrate results in isolated nuclei which do not lose their integrity or staining intensity for up to 60 min of incubation at 4°C.

Although the present communication is illustrated with DNA per cell distribution histograms of three different synchronized CCRF-CEM populations and two bone marrow samples, we have compared a large variety of experimental and clinical samples to confirm the observation that staining of live cells with propidium iodide, as used in the present study, is not only convenient and time saving but also as accurate as staining of cells after the lengthy procedure of fixation and digestion in RNase. The other great advantage of the direct propidium staining method lies in the fact that very small samples are required for analysis, and this is illustrated by the use of 1–2 drops of blood from a finger prick or a bone marrow sample. Although in experimental studies large samples are readily available, advantages of the direct staining are obvious in human punch or needle biopsy specimens where sample size is often limited. However, the only exceptions where direct staining of cells with propidium iodide could not be used are (a) cells with fluorescent constituents which may interfere with the DNA-propidium fluorescence and (b) cells with unusually large amounts of double-stranded RNA in the nucleus. One prime example of the former would be the chlorophyll-containing organisms such as *Euglena* where excitation of chlorophyll by the 488-nm laser wavelength results in a large amount of autofluorescence which masks the fluorescence emanating from the DNA-propidium iodide complex (personal unpublished observations).

The earlier published techniques of staining by acriflavine-Schiff's procedure (7, 8, 10) cause excessive cell loss and clumping from the hydrolysis and centrifugation, and they consequently require a large sample size. These procedures also require more than 2 h of processing, and in order to get proper discrimination between the peaks of the DNA distribution histograms repeated washing and centrifugation is required to remove the unbound dye. Another drawback of this procedure

is the extensive loss of material by adhesion to the glass or plastic tube walls, especially noted after acid hydrolysis. The propidium iodide method of Crissman and Steinkamp (1) obviates some of these problems and is an improvement over the acriflavine method. However, repeated centrifugation of the sample in this procedure is also time consuming and often leads to formation of cell clumps. The mithramycin staining technique (2) would be ideal for the processing of small samples in alcoholic solution of mithramycin, but it is limited by the nonavailability of the appropriate excitation laser wavelength in the commercially available Cytofluorograf. In comparison to these techniques, the method of staining with propidium iodide in hypotonic solutions offers the following advantages: (a) very small amounts of sample, $0.5\text{--}1.0 \times 10^6$ cells, or 1–2 drops of blood are enough for doing a DNA per cell distribution analysis. This advantage is of particular value in the handling of clinical samples and tumor biopsies. (b) The analysis can be done on the commercially available Cytofluorograf and does not need the special excitation wavelength of the mithramycin technique. (c) The cell loss by adhesion to tubes in centrifugation and by formation of cell clumps is completely avoided by incubation of the sample in a sodium citrate-containing solution of propidium iodide. (d) Rapidity of the staining method which normally requires only 5 min of staining recommends it for analyses where the time saved in the processing of the specimens and the availability of the results is of primary concern. (e) Compared to mithramycin, propidium iodide is easily available from commercial sources.

SUMMARY

A rapid method for the flow microfluorometric determination of the DNA content per cell is described. Incubation of cells in a hypotonic solution of propidium iodide results in disruption of the cell membrane and rapid staining of nuclear chromatin. DNA distribution histograms generated from cells stained by this method are identical to those generated after fixation and RNase digestion. In contrast to some earlier described methods, the present technique is rapid (5 min of processing), requires a minimal amount of material, and avoids formation of cell clumps.

The excellent technical assistance of Ms. Penelope Rivers and Mr. John Sullivan is gratefully acknowledged.

The present investigations were supported by Re-

search Grant CA-06516 from the National Cancer Institute.

Received for publication 27 January 1975, and in revised form 6 March 1975.

REFERENCES

1. CRISSMAN, H. A., and J. A. STEINKAMP. 1973. Rapid, simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. *J. Cell Biol.* **59**:766-771.
2. CRISSMAN, H. A., and R. A. TOBEY. 1974. Cell-cycle analysis in 20 minutes. *Science (Wash. D. C.)*. **184**:1297-1298.
3. ENNIS, H. L., L. MOLLER, J. J. WANG, and O. S. SELAWRY. 1971. 2,3-dihydro-1H-imidazol[1,2-b]pyrazole. A new inhibitor of deoxyribonucleic acid synthesis. *Biochem. Pharmacol.* **20**:2639-2646.
4. HUDSON, B., W. B. UPHOLT, J. DEVINNY, and J. VINOGRAD. 1969. The use of an ethidium analogue in the dye-buoyant density procedure for the isolation of closed circular DNA: the variation of the superhelix density of mitochondrial DNA. *Proc. Natl. Acad. Sci. U. S. A.* **62**:813-820.
5. KASTEN, F. H. 1959. Schiff-type reagents in cytochemistry. 1. Theoretical and practical considerations. *Histochemie.* **1**:466-509.
6. LEPECQ, J.-B., and C. PAOLETTI. 1967. A fluorescent complex between ethidium bromide and nucleic acids. *J. Mol. Biol.* **27**:87-106.
7. TOBEY, R. A., and H. A. CRISSMAN. 1972. Use of flow microfluorometry in detailed analysis of effects of chemical agents on cell cycle progression. *Cancer Res.* **32**:2726-2732.
8. TRUJILLO, T. T., and M. A. VANDILLA. 1972. Adaptation of the fluorescent Feulgen reaction to cells in suspension for flow microfluorometry. *Acta Cytol.* **16**:26-30.
9. VANDILLA, M. A., T. T. TRUJILLO, P. F. MULLANEY, and J. R. COULTER. 1968. Cell microfluorometry: a method for rapid fluorescence measurement. *Science (Wash. D. C.)*. **163**:1213-1214.
10. YATAGANAS, X., A. STRIFE, A. PEREZ, and B. D. CLARKSON. 1974. Microfluorimetric evaluation of cell kill kinetics with 1- β -D-arabinofuranosylcytosine. *Cancer Res.* **34**:2795-2806.