Cell-Cycle Analysis in 20 Minutes

Abstract. *Mithramycin added to mammalian cells fixed in aqueous ethanol is bound to DNA and fluoresces in direct proportion to the cellular DNA content. Quantitative fluorescence measurement by means of a high-speed flow system instrument provides a rapid method for cell-cycle analysis and for the first time permits continuous monitoring of cell-cycle kinetics during ongoing experiments.*

High-speed flow system analysis of Feulgen-stained cells yields data on DNA distributions that accurately reveal the fraction of cells in the various phases of the cell cycle (*1*). This technique has been employed to study the effects of synchrony induction protocols (*2*), chemotherapeutic drugs (*3*), and radiation (*4*) on cell-cycle progression. Although analysis is rapid (approximately 5×10^4 cells per minute), cell preparation (including dispersal, fixation, and staining) generally requires many hours; therefore, the analytical data are not available immediately during an experiment. Although the vital dye acridine orange can be employed to stain cells rapidly, the fluorescence patterns obtained are unsatisfactory for detailed cell-cycle analysis (*5*). In this report we describe a simple, rapid technique that yields data on distributions of DNA contents of single cells in large cell populations within 20 minutes after removal of a sample from a culture vessel. This new method permits population kinetics to be monitored continuously throughout an experiment.

Ward *et al.* (*6*) reported that the drug mithramycin selectively fluoresces when bound to DNA but fails to interact with RNA. Following a suggestion by Reich, we exploited this phenomenon for rapid analysis of DNA contents of single cells by using the Los Alamos flow microfluorometer (FMF) (*7*). Human diploid WI-38 cells and HeLa cells were grown in monolayer culture in double-strength Eagle's basal medium supplemented with 20 percent fetal calf serum. Line CHO Chinese hamster cells were grown in suspension culture in F-10 medium supplemented with 10 percent calf serum and 5 percent fetal calf serum. After trypsin treatment of monolayer cultures, the cells were centrifuged and resuspended in 25 percent aqueous ethanol containing mithramycin (100 μg/ml) (*8*) and 15 m*M* $MgCl_2$. Cells were harvested from suspension culture by centrifugation and resuspended directly in the drug solution without prior trypsin treatment. After 15 to 20 minutes, the cells in drug solution were examined directly in the FMF with a laser wavelength setting of 457 nm. Duplicate samples were treated with trypsin fixed in saline containing 4 percent formalin and stained by the acriflavine-Feulgen technique, and FMF analysis was performed with a laser wavelength of 488 nm as described (*2*). For all samples, the fraction of cells in each phase of the cell cycle was obtained by a computer fit of the fluorescence (which is proportional to DNA) distribution patterns obtainable in both mithramycin-treated and acriflavine-Feulgen–stained cells (*1*). Design and operation of the Los Alamos FMF, as well as validation of the acriflavine-Feulgen method, are described elsewhere (*4*).

Figure 1 shows the DNA distribution patterns from mithramycin-treated and acriflavine-Feulgen–stained cultures of WI-38 and HeLa cells in exponential growth. The larger peak represents cells in G_1 phase (prior to DNA replication); the smaller peak represents cells in G_2 (following DNA replication) or M (mitosis), with cells in S phase (during DNA replication) lying between the two peaks. The coefficients of variation (CV) (100 times the standard deviation divided by the mean) for the G_1 distributions are indicated for each population, along with the fraction of cells in the various phases of the cell cycle as derived from computer analysis.

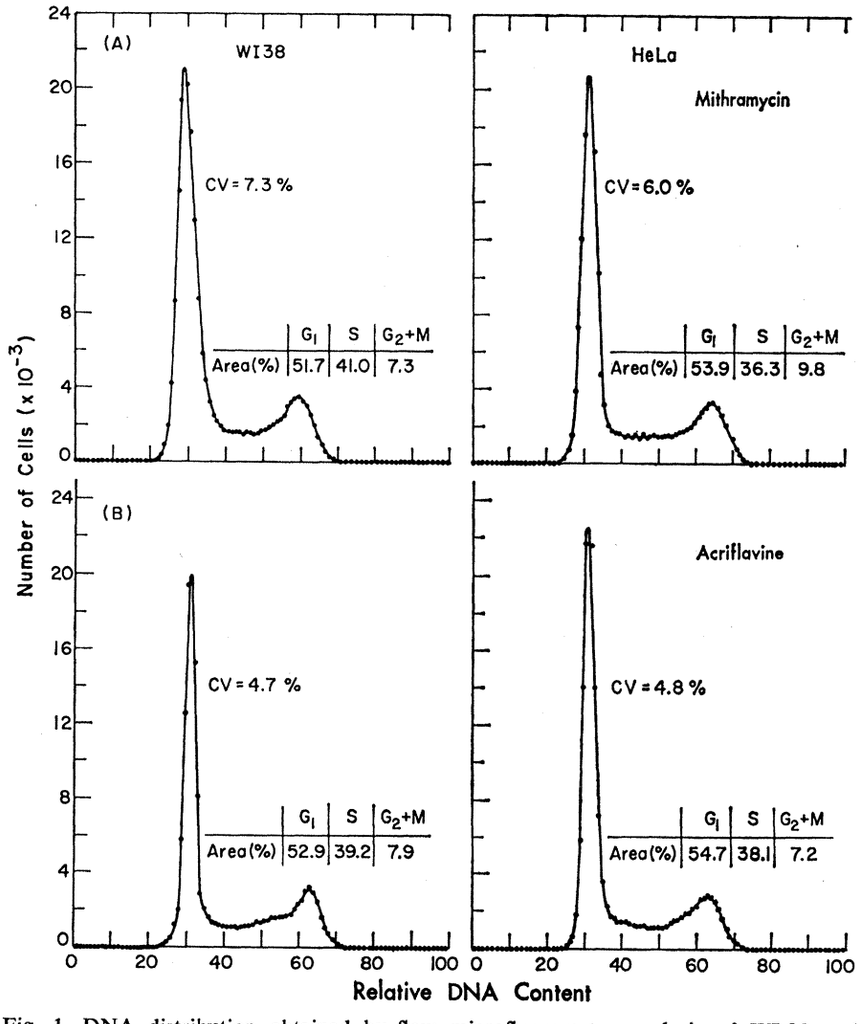
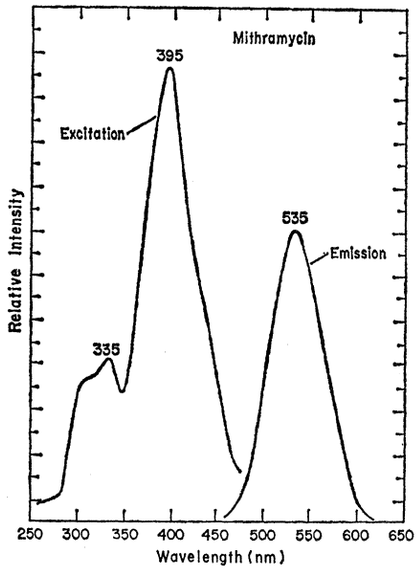


Fig. 1. DNA distribution obtained by flow microfluorometer analysis of WI-38 and HeLa cells stained with either (A) mithramycin, as described in the text, or by the (B) acriflavine-Feulgen procedure (*2*). Coefficients of variation (CV) for the G_1 distributions and the percentage of cells in G_1, S, or $G_2 + M$ were obtained by computer-fit analyses of the DNA distribution curves.

Fig. 2. Excitation and emisson spectra of mithramycin-stained Chinese hamster cells (line CHO) measured with an Aminco-Bowman spectrophotofluorometer.



Although the CV were somewhat larger in the mithramycin-treated cultures, the two staining protocols yielded essentially equivalent values for the fraction of cells in various phases of the cell cycle. The CV for suspension cultures of CHO cells treated with mithramycin or acriflavine were 5.8 and 4.0 percent, respectively; the fractions of cells in G_1, S, and G_2 or M were 51.4, 41.2, and 7.4 percent, respectively, in mithramycin-treated samples and 48.4, 44.4, and 7.2 percent, respectively, in cultures treated with acriflavine (not shown).

Initial experiments indicated that stable fluorescence patterns were obtained only after 15 to 20 minutes of exposure of cells to mithramycin solution; patterns remained stable for at least 1 hour thereafter. Lower concentrations of mithramycin than the 100 μg/ml used in the experiment shown in Fig. 1 could be employed, but the amount of fluorescence was decreased and the time required for drug equilibration was greatly increased. Treatment of cells with mithramycin in phosphate-buffered saline or in water was unsatisfactory because of an apparent inability of the cells to incorporate sufficient quantities of drug to yield reproducible fluorescence patterns. Subsequent experiments indicated that cells stored in cold 70 percent ethanol for a least 1 week prior to staining yielded distribution patterns comparable to those of cells stained immediately after harvesting. Treatment of fixed cells with ribonuclease (1 mg/ml) for 30 minutes at 37°C had no effect on the fluorescence pattern, while treatment with deoxyribonuclease (1 mg/ml) for 2 hours at 37°C prior to exposure to mithramycin rendered the cells virtually nonfluorescent.

Figure 2 shows the excitation and emission spectra for mithramycin-stained Chinese hamster cells. The lowest available wavelength for exciting the drug with the laser employed in the FMF (argon ion, Coherent Radiation model 52) is 457 nm. Although the drug is suboptimally excited at 457 nm, this wavelength was superior to other available laser lines of higher wavelength (461, 476, 488, and 514 nm). In particular, the fluorescence distribution patterns obtained for mithramycin-treated CHO cells excited at 488 or 514 nm proved unacceptable for cell-cycle analysis. We would expect a significant improvement in CV values if laser lines of substantial power were available for excitation of mithramycin in the 390- to 400-nm range.

The fluorescence patterns in mithramycin-treated cells agree well with patterns obtained with acriflavine-Feulgen–stained cells, and staining by the latter method has been shown to correlate with DNA content by standard cell-cycle analytical techniques with [^{3}H]thymidine (*1*). We therefore conclude that mithramycin is bound and fluoresces in direct proportion to the amount of cellular DNA. The lack of fluorescence in deoxyribonuclease-treated cells exposed to mithramycin solution is further support for the specificity of interaction between mithramycin and cellular DNA.

Among the advantages of this technique is its applicability to the following kinds of studies: (i) populations of cells devoid of DNA-synthesizing or mitotic cells; (ii) slowly or nonprogressing populations; and (iii) isotopically labeled cells in which standard [^{3}H]thymidine autoradiographic techniques are difficult or impossible. However, the unique features of the mithramycin technique are rapidity and simplicity, which permit monitoring of population kinetic properties in ongoing experiments, with the added option of altering an experiment in progress in response to a population change.

HARRY A. CRISSMAN
ROBERT A. TOBEY
Biophysics and Instrumentation Group and Cellular and Molecular Radiobiology Group,
Los Alamos Scientific Laboratory,
University of California,
Los Alamos, New Mexico 87544

References and Notes

1. P. N. Dean and J. H. Jett, *J. Cell Biol.* **60**, 523 (1974).
2. R. A. Tobey, H. A. Crissman, P. M. Kraemer, *ibid.* **54**, 638 (1972).
3. R. A. Tobey and H. A. Crissman, *Cancer Res.* **32**, 2726 (1972).
4. P. M. Kraemer *et al.*, *Adv. Cell Mol. Biol.* **2**, 47 (1972).
5. R. A. Tobey, *Methods Cell Biol.* **6**, 85 (1973).
6. D. C. Ward, E. Reich, I. H. Goldberg, *Science* **149**, 1259 (1965).
7. M. A. Van Dilla, T. T. Trujillo, P. F. Mullaney, J. R. Coulter, *ibid.* **163**, 1213 (1969);
8. D. M. Holm and L. S. Cram, *Exp. Cell Res.* **80**, 105 (1973).
9. Mithramycin (NSC 24559) was obtained through the Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland.
10. Supported by contract NIH-CR-(71)-56 from the Division of Cancer Treatment, National Cancer Institute, under interagency agreement with the Atomic Energy Commission.

26 December 1973; revised 4 March 1974

Selective Destruction of the Pigmented Epithelium in the Ciliary Body of the Eye

Abstract. *Perfusion of the internal carotid artery with hypertonic solution selectively destroys most of the pigmented epithelium of the ciliary body of the monkey eye, converts fenestrated endothelium of capillaries to continuous endothelium, and transiently breaks down the blood-aqueous barrier. The nonpigmented epithelium regenerates and the intraocular pressure returns to normal even though the pigmented epithelium is permanently destroyed.*

The mechanism and site of aqueous formation remain major questions in eye physiology and are important to understanding the pathogenesis of glaucoma. It has been proposed that aqueous humor is secreted by the ciliary epithelium into the posterior chamber of the eye after plasma ultrafiltration through fenestrated capillaries of the ciliary body (*1*). The ciliary epithelium is double-layered, having a pigmented layer facing the stroma and blood vessels and a nonpigmented layer whose basal surface faces the posterior cham-