Application of this approach involves first sensitizing the spores to heat by converting them to the hydrogen form by acid treatment. The substrate containing live but heat-sensitized spores is then neutralized back to its original *p*H before the lethal heat treatment. Since the rate of the heat-sensitizing conversion of the spore to the hydrogen form varies inversely with *p*H but positively with temperature, a wide range of conditions of *p*H and temperature are available for the sensitizing step. A choice would be guided by such factors as the time available, the stability of the substrate, and the metal form of the spores. Spores in the divalent (calcium) form require a lower *p*H than do spores in the monovalent form.

To demonstrate this new approach to heat severity reduction (Fig. 1) two complex biological mixtures were chosen, liver and tryptone culture medium, each inoculated with a large amount of the heat-resistant spores of *Bacillus stearothermophilus*.

With this approach, heating time advantages for a given degree of survivor reduction are large, amounting to several-fold. For example, the normal heating times without such a sensitizing treatment were longer by 8.5-fold for liver and 11-fold for the culture medium at a 100,000 × survivor reduction.

Because an acid and a base are added in sequence, the net additive in this approach is a small amount of a salt of an acid, in this case sodium chloride. The added sodium chloride amounted to 0.15 percent for the culture medium and about 1.0 percent for the liver on an undiluted basis. Inasmuch as this approach to heating severity reduction does not involve the use of antimicrobial additives, the attendant questions of additive persistence, toxicity, and ecological effects are avoided.

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5. We thank Mrs. P. A. Thompson for plating and counting the tests.

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Cell Microfluorometry: A Method for Rapid Fluorescence Measurement

Abstract. *A high-speed flow system for quantitative determination of fluorescence of cells containing fluorochrome has been developed. Feulgen-DNA distributions in populations of tissue culture cells and human leukocytes have been measured at a rate of 10^4 to 10^5 cells per minute and compare well with results of other independent methods.*

Fluorescent dyes have found widespread use in biology and medicine, examples being nucleic acid cytochemistry, fluorescent antibody studies, exfoliative cell diagnosis, cancer cell detection, and fluorochromasia (*1*). A method for rapid, quantitative measurement of fluorescent light emission from cells containing fluorochrome has been developed. A flow system is used; cells in aqueous suspension are measured at a rate of 10^4 to 10^5 cells/min. When stained by the fluorescent Feulgen procedure, normal human leukocytes show a Feulgen-DNA distribution with a single peak, while Chinese hamster ovary (*2*) cells growing asynchronously or synchronously show distinctive bimodal distributions which depend upon cell distribution around the life cycle.

The principle of the method is simple. Cells containing fluorochrome flow in a narrow stream (diameter 50 μ) across a beam of exciting light (diameter 100 μ); the resultant fluorescent light pulses are viewed perpendicularly to both cell stream and light beam by a photomultiplier (that is, dark field illumination). The cell stream is formed in a laminar flow chamber developed from a design by Crosland-Taylor (*3*); the light source is an argon ion laser (Spectra-Physics model 140) operated at 488 nm and a beam power of about 1 watt. No microscope and a minimum of optics are used. A simple lens of 20 cm focal length focuses the laser beam to the desired 100-μ diameter at the cell stream, and a pair of *f*1.6 movie projection lenses transfer an image of the cell stream-laser beam intersection at unity magnification to a low-noise photomultiplier (ITT type FW-130) with enhanced long wavelength response (S-20). A yellow filter (Corning CS 3/69) between the projection lenses is the barrier filter. A pinhole 400 μ in diameter in the image plane of the projection lenses serves as a limiting aperture to reduce optical noise. A cell takes about 15 to 20 μsec to cross the light beam, which is also the duration of the fluorescent light pulse. The resulting electrical pulses from the photomultiplier have this same duration, and their amplitude is proportional to fluorescence intensity. They are amplified, analyzed for amplitude, and stored in the memory of a multichannel pulse-height analyzer (SCIPP 1600, Victoreen Instrument Co.) at a typical rate of 50,000 per minute. The contents of analyzer memory are thus a frequency distribution histogram of fluorescent light emission per cell which is displayed and read out for further analysis.

The fluorescent Feulgen reaction described by Kasten and by Ruch (*4*) is used to stain for DNA. Modifications involving centrifugation were necessary to permit the several steps in the staining procedure to be carried out with cells in suspension rather than on slides. The procedure takes 2 hours. When viewed in a fluorescence microscope, the stained cells show little or no cytoplasmic fluorescence, while nuclei (or chromosomes in mitotic cells) fluoresce bright green. Centrifugation is conducive to cell clumping, an undesirable effect which is reduced by shearing at each step and sonication at the end of the procedure. A method which avoids the possibility of clumping would be advantageous.

Figure 1 shows both Feulgen-DNA and volume distributions for Chinese hamster ovary cells growing asynchronously. The volume distribution (before staining) measured with a modified Coulter volume spectrometer and a long aperture [diameter 80 μ, length 260 μ (*5*)] is broad, unimodal, and typical of a cell population in exponential growth with cells of all ages present. The DNA distribution as indicated by fluorescence shows two peaks: the first represents cells with diploid DNA content (G_1 phase cells), and the second represents cells with tetraploid DNA content (G_2 and M phases). The region between peaks represents cells synthesizing DNA (S phase). The coefficient of variation

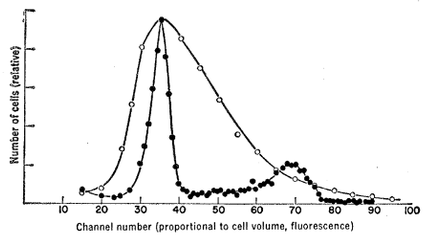

Fig. 1. Volume and Feulgen-DNA distributions of Chinese hamster ovary cells growing asynchronously in suspension culture. ○, Volume distribution before staining; ●, nuclear fluorescence distribution (coefficient of variation = 6.4 percent) after staining.

of the G_1 peak is about 6 percent, which is about as narrow a distribution for cells of equal DNA content as has been observed by conventional two-wavelength cytophotometry (*6*). We do not know at present whether this variation is instrumental or due to lack of stoichiometry of our modification of the Feulgen method, but results of Alpen and Johnston (*6*) suggests the former. The ratio of the modal fluorescence intensities of the two peaks is 1.97, very close to the expected value of 2.00. The second peak may be contaminated with some G_1 cells stuck together as doublets, but the effect is not large, as shown by studies using sonication to break up doublets. Our Feulgen-DNA distributions are also quite similar to the results of Zetterberg and Killander (*7*) on mouse fibroblasts grown in vitro as monolayer cultures in the exponential phase of growth and measured for Feulgen-DNA by an absorption scanning microspectrophotometer of the Caspersson type. The noteworthy differences between the two histograms are the improvement in statistical precision evident in our result on about 50,000 cells, as compared with the microspectrophotometer result on 594 cells, and the reduction in measurement time (1 minute for our result compared with many hours for the microspectrophotometer result). The time-course of DNA synthesis and duration of the G_1, S, and $G_2 + M$ phases of the life cycle are derivable from such distributions.

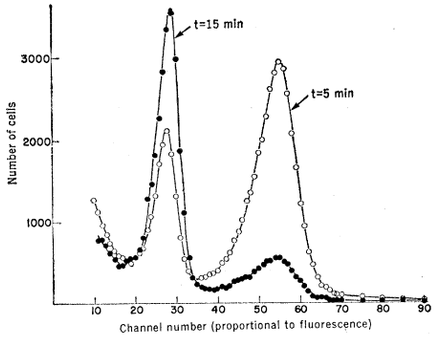

Fig. 2. Feulgen-DNA distribution of Chinese hamster ovary cells synchronized in metaphase by shaking off a glass monolayer culture. One fraction (○) then grew in suspension culture for 5 minutes and another (●) for 15 minutes before fixation.

Figure 2 shows the Feulgen-DNA distribution of Chinese hamster ovary cells synchronized by shaking metaphase cells off a glass monolayer culture (*8*). One fraction was allowed to grow in suspension culture for 5 minutes and another for 15 minutes before staining. The cell population distribution and fluorescence spectrum of the two fractions differ from each other and from that of the randomly growing population. After 5 minutes of growth, the cell population consists mostly of mitotic cells and some which have just divided and are in the G_1 phase. The first peak in the fluorescence spectrum represents G_1 cells and the second peak M cells. This observation was confirmed by visual fluorescence microscopy. A cell count showed about 75 percent of cells in M and 25 percent in G_1, in close agreement with the result obtained from peak areas. The ratio of the modal channel numbers is 1.96, very close to the expected value of 2.00 if DNA halves on cell division and if the different DNA distribution (chromosomes contrasted with diffuse chromatin) has no effect. After 15 minutes of growth, most of the cells have divided so that peak areas are reversed. The first peak (G_1) now dominates, again the modal channel ratio is approximately 2.00, and again visual fluorescence miscroscopy confirms the conclusion from peak areas that 23 percent of the cells are in M and 77 percent in G_1.

Volume and Feulgen-DNA measurements were also made on normal human leukocytes. Cells were obtained from freshly drawn whole blood by centrifugation, removal of the buffy coat, and minimum treatment with saponin to lyse remaining erythrocytes (*9*). The volume distribution was determined, and the cells were subsequently stained. The volume and Feulgen-DNA distributions are quite different. The volume distribution shows the expected bimodality with the first peak representing small lymphocytes and the larger second peak representing granulocytes and other large cells (*5*). The fluorescence distribution shows a single peak of small dispersion (coefficient of variation = 6 percent) to be expected if all leukocytes have the same DNA content and bind equal amounts of dye. Although there is not complete agreement in the cytophotometric literature over Feulgen-DNA results on leukocytes, these values do not differ by more than 10 percent for the abundant types (small lymphocytes and neutrophils). Our results are consistent with this observation.

We anticipate that extension of this method is possible and of potential value. Other possibilities include (i) staining with other fluorochromes such as acridine orange for DNA and RNA and brilliant sulfaflavine for protein; (ii) application to fluorescent antibody studies; (iii) application to cancer cell identification; (iv) optical spectral analysis by use of more than one photomultiplier and appropriate filters; (v) multi-parameter analysis by combination with small-angle light scatter (*10*) or Coulter orifice; and (vi) cell sorting (*11*) on the basis of these optical sensors.

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