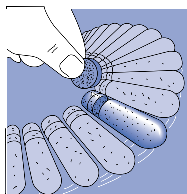

Application Information

General Purpose Centrifugation

Synchronizing Cultured Cells by Centrifugal Elutriation

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The series of ordered processes that cells undergo as they grow and divide is referred to as the cell cycle. Studies of the cell cycle require methods for isolating cells at specific positions in this series of processes, or for determining where in the cycle they reside. Here we describe a method that takes advantage of the progressive increase in cell size for isolating large numbers of cells at any cell cycle position.

Descriptions of the cell cycle divide it into a series of stages as shown in Figure 1. Immediately after cell division, cells undergo an apparently quiescent stage referred to as G1. This is followed by a period of DNA synthesis (S phase), and by a second period of apparent quiescence (G2). The final stage of the cycle consists of chromosome condensation and the events of mitosis (M stage). The duration of the cycle differs considerably from one cell type to another, but for cultured mammalian cells it lasts typically for 7-12 h.

In addition to cycling cells, most cell populations contain one or more classes of cells that have withdrawn from the cycle. For example, cells deprived of growth factors may arrest early in G1 and enter a state called G0. Immature oocytes may enter a similar resting phase near the G2/M boundary. Other cells in a culture may have been damaged and stopped cycling at other positions. These will eventually degrade. Since these noncycling cells have sizes and DNA contents that are indistinguishable from those of cycling cells, it is important to minimize their occurrence in the culture.

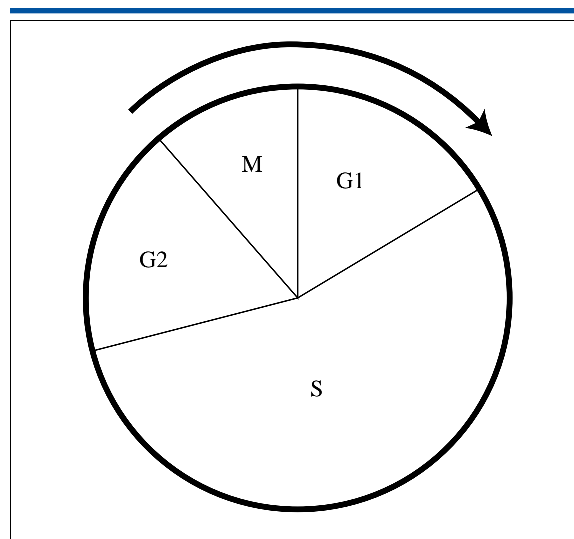

Figure 1. *The cell cycle.*

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Methods of Cell Synchrony

Forward and Retroactive Protocols

Two types of protocol are in general use for studies of cell populations that are synchronous with respect to the cell cycle. In a *forward synchrony* experiment, a synchronous population is first isolated and then resubmitted to culture. The effects of the experimental treatment are then observed as the population continues to grow. This protocol is suitable if behavioral or morphological changes at a particular point in the cycle are expected. The alternative protocol, *retroactive synchrony,* is more suitable for biochemical studies, as it avoids most potential artifacts of the synchronization procedure. In this approach, tracers are added to an exponentially growing, asynchronous cell population, and a synchronous subpopulation is isolated later on. From a knowledge of the duration of the cell cycle stages of the cell line, the stage during which the tracer was administered can be deduced.

Chemical Methods

Several agents are available that block specific processes in the cell cycle and cause cells to arrest at particular stages. For example, high concentrations of thymidine prevent the entry of cells into the S phase, and hydroxyurea causes them to accumulate at the G1/S boundary. Such methods are advantageous in that 1) large quantities of cells are readily treated, and 2) very tight synchrony can be obtained. However, it is difficult to ensure that no drug artifacts affect the experimental observations, and in many cases, there is evidence suggesting that the cycle is perturbed by their use. For example, there are indications of an increase in the rate of DNA synthesis after release from a hydroxyurea block.

Physical Methods

Cell Sorting

Direct selection of cells based on their DNA content is possible using a fluorescence-activated cell sorter and an appropriate dye. Dyes are available that bind DNA quantitatively in living cells. This powerful method is ideal for studies of cells at specific positions within S, or in G1 or G2/M. Cells generally undergo extensive manipulation at room temperature, so that the method is best suited for studies with retroactive synchrony (see below). A further limitation is the very low capacity of the method: high-resolution sorts are generally limited to several hundred cells per second, making the method unsuitable for most biochemical applications.

Mitotic Detachment

Substrate-adherent cells tend to round up during mitosis and to loosen their attachment to the culture surface. If cultures are gently shaken, mitotic cells will release into the medium, and may be recovered by decanting. This method can provide very pure populations of cells in mitosis, and is useful for studying events into G1 when the cells are replated. Some loss of synchrony occurs during late G1, presumably as the population passes the restriction point, a regulatory event at which the decision to enter G0 may be taken. A limitation of the technique is the modest recovery; usually only 5% or less of a culture is in mitosis at any time.

Elutriation

Counterflow centrifugal elutriation (CCE) is a method for isolating cellular subpopulations on the basis of their sedimentation coefficient, itself a function of cell volume and density. In a cultured cell line, individual cells will vary only slightly in density, so that CCE will classify such cells on the basis of size. Figure 2 shows the excellent correlation between size and DNA content found for a typical cultured cell line. A major advantage of CCE is that it permits direct selection of cells from any point in the cell cycle, since cells continue to grow from one cell division to the next. CCE also permits very high throughputs and recoveries of living cells.

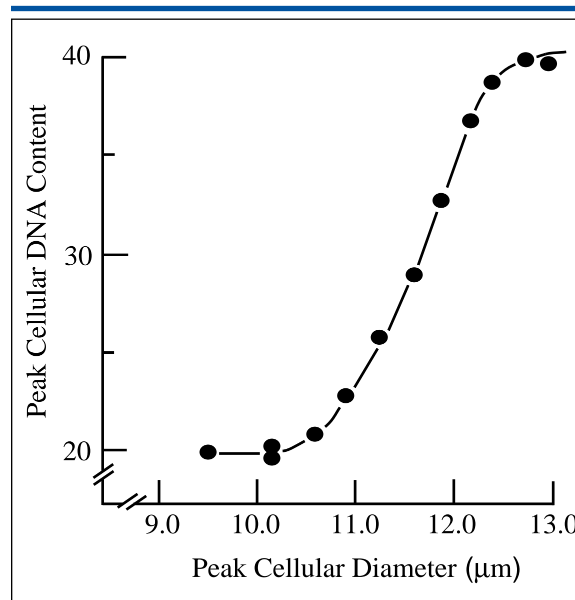

Figure 2. *Correlation of cell size with DNA content was determined for cultured MEL cells. Details are given in the legend to Figure 3.*

Unit gravity

Similar to elutriation in principle, unit gravity devices sort cells on the basis of sedimentation coefficient by allowing them to settle through a viscous gradient. Although the method is slow (separations require 3-10 h), it can provide some enrichment of cells in G1.

Fractionating Cell Populations by Elutriation

Cell Culture

Elutriation is a highly reproducible separation technique. In order to obtain similar separations from one run to the next, several factors must be attended to in addition to the operation of the instrument. The most important of these is the preparation of the cells to be fractionated.

For cell cycle separations, it is essential that the cell population be maintained in the early log phase of growth for several generations prior to fractionation. Although this can be costly, it provides the greatest proportion of cycling cells and clearly leads to the best results. As a cell population reaches saturation, or even late log phase, an increasing proportion of cells leaves the cycle and enters the G0 resting state. Other cells may be damaged due to anoxia or to less-than-optimal conditions in the culture flask. These cells will co-isolate with cycling cells of comparable size, leading to an apparent broadening of the DNA distribution for each fraction.

Nearly all suspension cell lines give good results under these conditions. Cell lines that attach to the culture substrate are more difficult to handle, and many cannot be studied by elutriation. Procedures required to release cells from the substrate may lead to damage and cause them to behave anomalously in the elutriator rotor. Persistence of cytoskeletal elements may prevent uniform rounding of the cells prior to fractionation. In any case, pilot experiments are recommended for substrate-adherent cells before deciding upon elutriation as the fractionation method of choice.

Rotors and Pumps

Rotors

Two elutriator rotors are available from Beckman. The JE-6B rotor is capable of speeds to 6000 rpm (considerably higher than required for most cell cycle separations) and can be run in any Beckman high-performance centrifuge. The JE-5.0 rotor provides the additional capability for running the larger-volume chambers, but its 16-inch diameter requires that it be run in a centrifuge with a large rotor chamber, such as the Beckman J-6.

Pumps

A media pump must be attached to the system to provide flow through the chamber. Nearly any peristaltic pump with the appropriate flow rates (2-100 mL/min for the small chambers; 5-400 mL/min for the larger chamber) may be used, although minimal pulsation is desirable to maintain a steady flow of medium. Although not necessary for routine operations, computer-controlled pumps are available to provide a predetermined program of flow rates. Such pumps may be interfaced with a fraction collector and electronic balance in order to increase the flow rate once a given volume has been collected. They may also be used to provide a gradually changing rate of flow (see below).

Chamber Shapes and Sizes

Two chamber styles are available for use with Beckman elutriator rotors. While either may be used for cell cycle separations, the best results have been obtained using the *standard chamber.* This chamber is available in two sizes: a small version with a capacity of approximately 4.5 mL, suitable for fractionating between 2×10^7 and 1×10^9 cells, and a larger, 40-mL version with approximately ten times the capacity. The actual cell load that a chamber can accommodate is dependent upon the mean cellular volume and will vary from one cell line to another. The high capacity of these chambers makes them the best choice for experiments in which biochemical operations will be performed after isolation of particular cellular components, such as DNA.

A second chamber style, the *Sanderson chamber,* is also available. This geometry gives very good resolution with small cells and is preferred by some investigators. Its limited cell capacity, about 1×10^6 to 1×10^8 cells, renders it less suitable for most cell cycle work.

Media

Separations may be performed in essentially any elutriation medium. For a forward synchrony, the usual practice is to select a fluid as similar as possible to the culture medium. This is often limited by the cost of fetal calf serum. Furthermore, a very high protein content may lead to foaming during the separation. A frequently seen compromise is to use culture medium with the serum content reduced to 0.1%-1%. For a retroactive synchrony, it is sufficient to use an isotonic saline at 4°C. Many investigators add a small quantity of protein, such as 1% BSA, to prevent cells or debris adhering to the chamber walls, but this is generally not necessary.

Temperature

Elutriation may be performed at any temperature that the centrifuge will maintain. However, care must be taken to avoid exposing cells to significant changes of temperature once the separation has begun. Such temperature shifts may result in changes to cellular size or to the rigidity of the cell membrane and thereby alter the elutriation profile.

For experiments where cell fractions are to be subjected to immediate biochemical studies, the simplest solution is to work at 4°C. In this case, it is necessary to chill both the cells and a sufficient volume of the separation medium before the run. This is generally accomplished by putting them on ice.

If cells are to be cultured after the fractionation, one may work at either 37°C or at room temperature. In the latter case it is desirable to perform a control experiment to see how rapidly cells resume cycling after being held at the lower temperature. This may be done by isolating an early-S phase fraction from the elutriator and using flow cytometry to follow motion of the DNA peak with time, or by pulse-labeling an unfractionated culture with ^{3}H-thymidine and using autoradiography to track the resumption of DNA synthesis.

Rotor Speeds and Flow Rates

With a knowledge of the size distribution of cells in the population to be fractionated, the nomograms in the elutriator manuals may be used to get a first estimate of the combination of rotor speeds and flow rates to be used. In general, it is necessary to confirm this estimate with one or two pilot experiments. Depending on the resolution sought, a cycling cell population may be subdivided into 10-20 fractions using regular increases in flow rate.

Rotor speeds for cell cycle fractionations of yeast or mammalian cell populations are usually in the 2000-3600 rpm range. In general, higher speeds give somewhat better resolution, but since the flow rates will have to be greater, the cells will be subjected to more shear.

Fractions can be incremented by decreasing the rotor speed or by increasing the flow rate. Of the two methods, the latter is preferred since the pump's speed is easier to control than that of the centrifuge. It is worth noting that an improvement in resolution can be obtained by avoiding abrupt changes in flow rate. The flow should be increased gradually, or in small steps, until the next desired rate is attained. Alternatively, a computer-controlled pump may be set to ramp the flow rate linearly to the next desired rate.

Loading the Sample

A critical factor in elutriation is control of the number of particles to be separated. Once a separation protocol has been established, it is essential that the same number of cells be loaded every time. A change in the cell load will alter the sizes of cells that elute with a particular flow rate, since the effective flow rate within the chamber varies with the cell load.

Two options are available for loading cells into the chamber. The first is to make use of the Beckman sample loading reservoir, and is described in the rotor manual. It requires that the cells be pelleted first with a low-speed spin in a preparative centrifuge, typically 10-15 min at about 1500 rpm. The cells are then injected into the sample loading reservoir in a small volume of buffer, and flushed into the rotor using the flow from the pump. Most cell lines are robust enough to show no effects from such a procedure but, for a forward synchrony protocol, a control experiment such as the one described above should be performed.

An alternative loading procedure avoids the stresses inherent in pelleting cells. A large volume of cells, in their culture medium, is drawn into the elutriator chamber with the rotor at speed, and the centrifugal force is used to concentrate the cells in the rotor. When the sample has been loaded, a valve is used to switch the fluid influx to a separate reservoir containing buffer from the run. This procedure takes longer than the other, since the flow must be maintained at a rate low enough to prevent cells from being eluted.

Fractions and Volumes

The number of fractions to be collected will depend upon the cell population and upon the resolution desired. Most mammalian suspension cultures can be separated into 10-20 distinct size fractions. A recommended procedure is to perform a pilot experiment in which, once the cells are loaded and held in the chamber, the flow rate is raised in 20% increments. Flow rates can then be adjusted based upon the results of this initial study, and will provide reproducible results if the cell load is held constant.

The fraction volume required to deplete a particular size of cell is dependent upon the population resident in the chamber. Thus, the best way to determine fraction volume is to monitor the effluent, and to increase the flow rate only when the rate of particle outflow drops off. This may be done with an in-line turbidity monitor, or by diverting a small volume of the outflow from time to time and monitoring the content with a particle counter.

Once appropriate fraction volumes are determined, it is not necessary to continue this monitoring procedure, and a simple volumetric collection can be done. It should be emphasized that, to maintain reproducibility between fractionations, the cell load and the fraction volumes should not be changed.

Monitoring Results by Cell Size and DNA Content

Correlation of Cell Size and DNA Content

Since elutriation is an indirect method of cell synchrony, it will be necessary to monitor the results to ensure 1) that the classification of cells according to size by the rotor is indeed successful, and 2) that the correlation between cell size and DNA content holds up for the cell line under study. Figure 3 illustrates such a correlation for the murine erythroleukemia (MEL) cell line.

Size Monitoring Methods

Several methods are available for monitoring the particle size and count in each elutriated fraction. The cheapest method is to employ a hemocytometer. This can give accurate counts and a qualitative appreciation of the size of particles in each fraction, but it is tedious to use. Nevertheless, it is the only method that can distinguish between large particles and aggregates of smaller ones. Visual observation of the fractions is recommended to control against cell clumping.

A variety of particle counters are available to automate the counting procedure, and most of these can provide the size distribution as well. These devices generally function by monitoring changes in conductivity while a volume of fluid is drawn through an orifice. The size distributions they provide are subject to errors from coincidence, aggregation, and nonspherical particles, but with appropriate dilutions and visual monitoring, they are ideal complements to an elutriator installation.

Flow cytometry can also be used to monitor the size distribution if a detector is set up for low angle forward scatter. This allows both the particle size and DNA distributions to be monitored in a single step.

DNA Monitoring Methods

Use of a flow cytometer or fluorescence-activated cell sorter is recommended for monitoring the cellular DNA content. Several dyes are available to stain DNA quantitatively in either living or fixed cells. The most commonly used are Hoechst 33258 and propidium iodide. The DNA distributions should be compared to that of a population in logarithmic growth to determine the cell cycle position of each fraction. Distributions from living cells should be compared to those for fixed material with and without treatment with RNase, to ensure the specificity of the staining.

Other Controls

In addition to direct monitoring of the DNA content, the tightness of synchrony obtained can be characterized by additional controls. The most straightforward is to introduce an aliquot from a fraction into cell culture, and to monitor the cell count as a function of time. Cells isolated from S phase or G2 should double in number during a very narrow interval.

As a second control, cells actually in S phase can be quantified after reculturing by pulse labeling with ^{3}H-thymidine and observation by autoradiography.

Advantages and Disadvantages of CCE

As the example above demonstrates, CCE can provide synchronous cell populations of very high resolution at nearly any point in the cell cycle. In particular, it is capable of subdividing the S phase into a large number of sequential stages, a critical capability for studies of the temporal nature of gene replication or transcription. Very large numbers of cells can be processed in reasonable times, and potential artifacts from drug treatments are avoided. The technique is applicable to nearly all suspension-grown cell lines, but is not as universally applied to substrate-adherent lines.

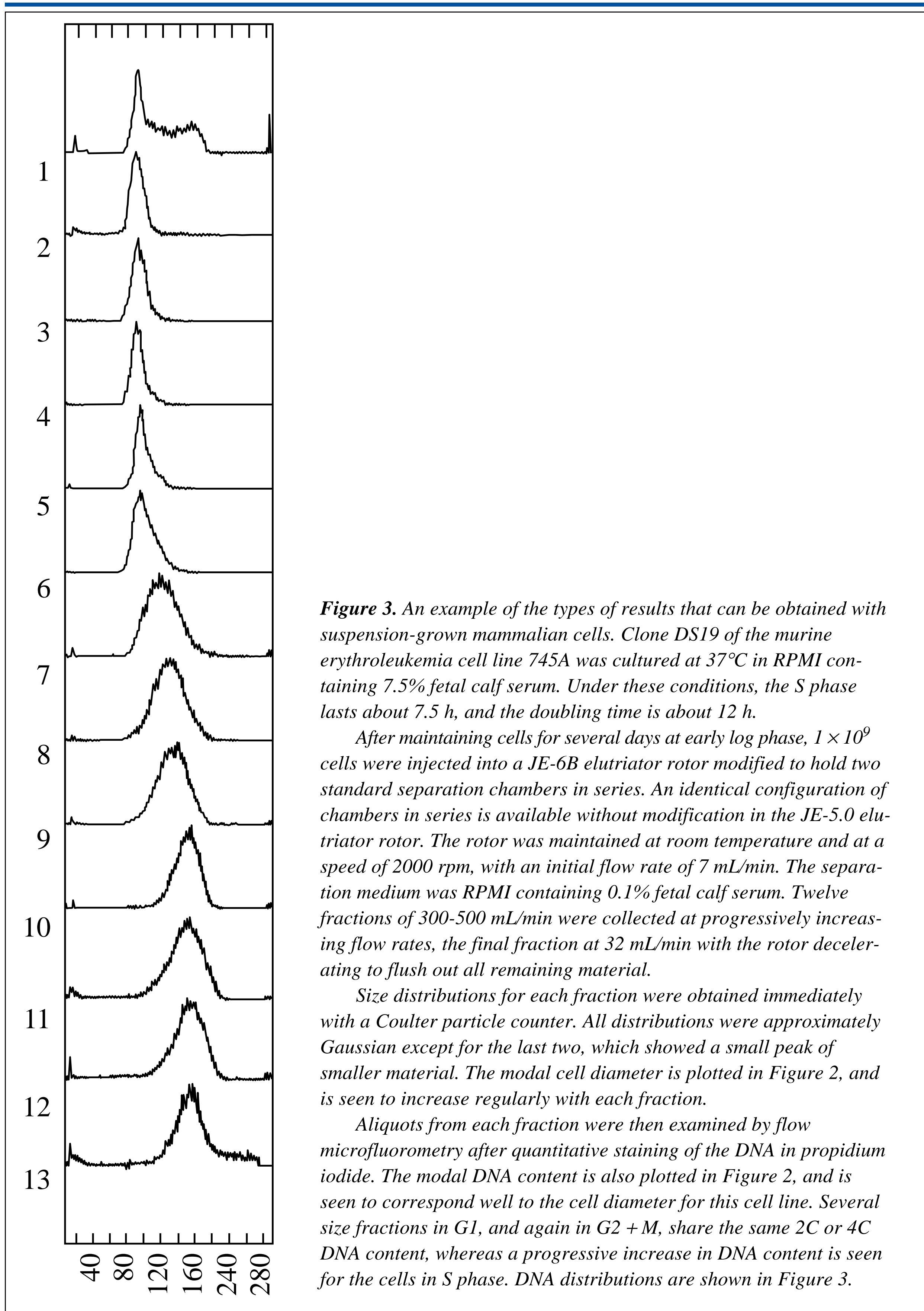

Figure 3. *An example of the types of results that can be obtained with suspension-grown mammalian cells. Clone DS19 of the murine erythroleukemia cell line 745A was cultured at 37°C in RPMI containing 7.5% fetal calf serum. Under these conditions, the S phase lasts about 7.5 h, and the doubling time is about 12 h.*

After maintaining cells for several days at early log phase, 1×10^9 cells were injected into a JE-6B elutriator rotor modified to hold two standard separation chambers in series. An identical configuration of chambers in series is available without modification in the JE-5.0 elutriator rotor. The rotor was maintained at room temperature and at a speed of 2000 rpm, with an initial flow rate of 7 mL/min. The separation medium was RPMI containing 0.1% fetal calf serum. Twelve fractions of 300-500 mL/min were collected at progressively increasing flow rates, the final fraction at 32 mL/min with the rotor decelerating to flush out all remaining material.

Size distributions for each fraction were obtained immediately with a Coulter particle counter. All distributions were approximately Gaussian except for the last two, which showed a small peak of smaller material. The modal cell diameter is plotted in Figure 2, and is seen to increase regularly with each fraction.

Aliquots from each fraction were then examined by flow microfluorometry after quantitative staining of the DNA in propidium iodide. The modal DNA content is also plotted in Figure 2, and is seen to correspond well to the cell diameter for this cell line. Several size fractions in G1, and again in G2 + M, share the same 2C or 4C DNA content, whereas a progressive increase in DNA content is seen for the cells in S phase. DNA distributions are shown in Figure 3.

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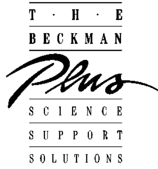

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