Video Article

Preparation of Primary Acute Lymphoblastic Leukemia Cells in Different Cell Cycle Phases by Centrifugal Elutriation

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Abstract

The ability to synchronize cells has been central to advancing our understanding of cell cycle regulation. Common techniques employed include serum deprivation; chemicals which arrest cells at different cell cycle phases; or the use of mitotic shake-off which exploits their reduced adherence. However, all of these have disadvantages. For example, serum starvation works well for normal cells but less well for tumor cells with compromised cell cycle checkpoints due to oncogene activation or tumor suppressor loss. Similarly, chemically-treated cell populations can harbor drug-induced damage and show stress-related alterations. A technique which circumvents these problems is counterflow centrifugal elutriation (CCE), where cells are subjected to two opposing forces, centrifugal force and fluid velocity, which results in the separation of cells on the basis of size and density. Since cells advancing through the cycle typically enlarge, CCE can be used to separate cells into different cell cycle phases. Here we apply this technique to primary acute lymphoblastic leukemia cells. Under optimal conditions, an essentially pure population of cells in G1 phase and a highly enriched population of cells in G2/M phases can be obtained in excellent yield. These cell populations are ideally suited for studying cell cycle-dependent mechanisms of action of anticancer drugs and for other applications. We also show how modifications to the standard procedure can result in suboptimal performance and discuss the limitations of the technique. The detailed methodology presented should facilitate application and exploration of the technique to other types of cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/56418/

Introduction

Cultured cells typically grow asynchronously and individual cells are present in different phases of the cell cycle. Some organisms exhibit naturally synchronous cell cycles or can be synchronized by specific physiological stimuli. For example, nuclei within giant plasmodia of the slime mold *Physarum polycephalum* divide in a highly synchronous fashion[1], and cells of the green algae *Desmodesmus quadricauda* can be synchronized by alternating light and dark periods[2]. While such organisms offer unique experimental attributes, they inadequately model the complexities of mammalian cells. The ability to artificially synchronize mammalian cell populations has been central to advancing our understanding of cell cycle regulation and the molecular basis of cell cycle checkpoints. Common methods include serum deprivation, chemical block and release, or exploiting physical characteristics[3,4]. Withdrawal of serum often causes cells to enter quiescence, and the re-addition of serum promotes cell cycle reentry into the G1 phase[5]. Chemical inhibitors include agents such as excess thymidine or hydroxyurea which block cells at the G1/S boundary, or microtubule inhibitors which typically arrest cells in the M phase[3,4]. Approaches that exploit physical characteristics include mitotic shake-off which can be used to enrich for mitotic cells since they are less adherent than interphase cells[6]. However, all of these techniques have potential drawbacks. For example, not all cell types maintain viability in the absence of serum or the presence of chemical inhibitors, and the yield of cells after mitotic shake-off is limited without prior synchronization with mitotic inhibitors.

With the exception of early embryonic cell cycles, where cells progressively decrease in size as they divide[7], most cells advancing through the cycle undergo growth phases and become larger. This property is exploited in the technique of counterflow centrifugal elutriation (CCE), which can be used to separate cells differing in size and hence in cell cycle phase[8,9]. During CCE, cells are under the influence of two opposing forces: centrifugal force, which drives the cells away from the axis of rotation, and fluid velocity (counterflow), which drives the cells towards the axis of rotation (**Figure 1**). Cells in the elutriation chamber reach an equilibrium position where these forces are equal. The key factors dictating the equilibrium position are cell diameter and density. As the flow rate of the buffer solution is increased and the counterflow drag force outweighs the centrifugal force, a new equilibrium is established, causing a change in position of cells inside the chamber. All cells are shifted toward the chamber exit, resulting in smaller ones leaving the chamber first, whereas the larger cells stay within the chamber until the counterflow rate is increased sufficiently to promote their exit. The cells escaping the elutriation chamber with consecutive increases in counterflow rate can be collected in specific fractions and each fraction contains cells of sequentially increasing sizes. Instead of conducting elutriation with incremental increases in counterflow rate, sequential decreases in centrifugal force by decreasing centrifugation speed will accomplish the same result. The

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process of elutriation requires optimization depending on cell type, elutriation buffer employed, and the specific apparatus used. The rate of sedimentation of cells under these conditions is best described by Stokes Law: $SV = [d^2(\rho_p-\rho_m)/18\eta].\omega^2 r$, where SV = sedimentation velocity; d = diameter of the particle; ρ_p = density of the particle; ρ_m = density of the buffer; η = viscosity of the buffer; ω = angular velocity of the rotor; and r = radial position of the particle. Thus, SV is proportional to cell diameter and density, and since diameter is raised to the second power, it contributes more significantly than density which is generally constant through the cell cycle.

An important advantage of CCE is that cells are not subjected to harsh conditions of chemical treatment or nutritional deprivation and can be recovered in excellent yield essentially unperturbed. A requirement is that the cells in question undergo a significant increase in diameter, of at least 30%, as they traverse the cell cycle. The main disadvantage is the cost of the specialized centrifuge, rotor, and accessories. Nonetheless, the ability to prepare cells enriched in specific cell cycle phases by CCE has greatly facilitated cell cycle research in organisms from yeast to mammalian cells[9,10]. Moreover, recent advances are expanding uses to the separation of cells from healthy versus cancerous tissue, the separation of different cell types in heterogeneous mixtures, and to the production of specific cell types for immunotherapy[9].

Our major interest has been in understanding the mechanism of action of microtubule targeting agents (MTAs) such as vinca alkaloids and taxanes. These drugs were thought to act exclusively in mitosis, blocking spindle microtubule function[11,12], but recent evidence suggests they may also target interphase microtubules[13,14]. We recently reported that primary acute lymphoblastic leukemia (ALL) cells undergo death in either the G1 phase or in the M phase when treated with vincristine and other MTAs in a cell cycle-dependent manner[15]. The ability to separate ALL cells into different cell cycle phases by CCE was instrumental in reaching this conclusion. In this article, we describe the technical details and offer practical tips for the application of CCE to the preparation of primary ALL cells in different cell cycle phases.

Protocol

NOTE: This protocol has been optimized for primary B-ALL cells which range in diameter from approximately 8 µm (G1 phase) to 13 µm (G2/M phases). Thus, the specific centrifuge speeds and pump flow rates may not be applicable to cells with different size ranges. Nevertheless, appropriate elutriation parameters can be estimated using the sedimentation rate equation. Cells are cultured in a defined serum-free medium as described[16] at an optimal density of 1 - 3 x 10^6 cells/mL. With the exception of the collection of fractions which is conducted at 4 °C using ice-cooled tubes, all steps are conducted at room temperature and the centrifuge set at 20 °C with a maximum of 24 °C.

1. Preparation of Elutriation Buffer and Materials

1. In order to reduce clumping of cells, prepare an elutriation buffer of Hank's balanced saline solution (HBSS) with phenol red supplemented with 2% fetal bovine serum (FBS), to protect cells from mechanical stress during the elutriation process, and 1.6 g/L of 2-naphthol-6,8-disulfonic acid dipotassium salt (NDA), which renders the cell exterior negatively charged and reduces clumping.
    1. Add 1.6 g of NDA to a 50 mL conical tube.
    2. Under sterile conditions, add 35 mL of HBSS from a 1 L bottle to the 50 mL conical tube containing NDA. Swirl the tube until the NDA has dissolved.
    3. Using a 0.2 µm filter, transfer the dissolved NDA into a new sterile 50 mL conical tube. Then add the NDA to the remaining 1 L of HBSS.
    4. Finally, add 21 mL FBS to the HBSS to obtain a final concentration of 2% (v/v).
    NOTE: The elutriation buffer can be prepared and stored at 4 °C until the expiration date on the HBSS or until there is a major change in the pH as indicated by a yellowing of the indicator dye.

2. Label 50 mL conical tubes for collection of fractions so that there are tubes labeled wash 1 and 2 (W1 and W2), fractions 1 - 20 (F1 - F20), and STOP.
    1. Pre-chill these tubes on ice before collecting fractions.

2. Assembly of the Elutriation System

1. First, install the strobe assembly into the centrifuge so that the power cord is fed out of the chamber through the port on the left side. Ensure that the strobe flash lamp is at the front of the chamber so it will line up with the viewing window when the centrifuge is closed.
    1. Secure the assembly in place by first tightening two Phillips-head screws into the two holes at the top of the each bracket and then by tightening the thumbscrews at the bottom of each bracket.
    2. Plug the power cord into the strobe power port at the back of the centrifuge.

2. Next, set the rotor straight down onto the centrifuge drive hub and secure with a T-handle hex wrench.
NOTE: If the centrifuge is not needed for non-elutriation purposes, the strobe assembly and the rotor can be kept in the centrifuge between runs.
3. Once the rotor is secured, place the quick-release assembly containing the elutriation chamber, counter balance, rotating seal assembly, and a mounting plate into the rotor.
    1. Push down evenly with one hand on the elutriation chamber and the other hand on the counter balance until the bolts in the rotor snap into the holes on the mounting plate.
    2. Finally, attach the anchoring cable by placing the pin on one side of the cable into the hole in the rotating seal assembly and securing the eyehole on the other side of the cable with one of the Phillips-head screws (mentioned in 2.1.1) on the bracket on the left side of the chamber.

4. Next, assemble the flow system using a variable speed pump and tubing to pump buffer into the elutriation chamber and collect buffer as it flows out of the elutriation chamber.

1. Insert the size 16 input tubing coming from the variable speed pump through the port at the top left of the centrifuge chamber so that it enters into the chamber.
2. Attach a fitting to the free end of the input tubing and insert the fitting into the input hole on the side of the rotating seal assembly.
3. Insert the size 16 output tubing through the port and attach it to the transfer tube at the top of the rotating seal assembly.
   NOTE: The flow system can also remain assembled between elutriation runs.

5. Lastly, turn the centrifuge on and set the specifications necessary for the experiment.
   1. Change the rotor ID.
   2. If the centrifuge requires a time input, set for 2 h, which is more than sufficient to complete an elutriation run.
   3. Set the temperature to 20 °C with a max temp of 24 °C.
   4. Set the acceleration for max and the deceleration for slow.
   5. Finally, set the speed to 867 x g.

3. Priming of the Elutriation System

1. First in order to sterilize the elutriation system, place the reservoir tubing into a 500 mL plastic bottle containing 5% bleach and place the output tubing into an empty 1 L plastic waste bottle.
   1. Turn the pump on and set the speed to 25 mL/min and allow the 5% bleach to flow all the way through the elutriation system until it is pumped out of the output tube into the waste bottle.
   2. Turn the centrifuge on and allow it to come to the maximum designated speed (867 x g) in order to release bubbles and backpressure.
   3. Stop the centrifuge. Once the rotor has stopped rotating, open the lid and inspect the chamber for any leaks.
   4. If there are no leaks, let the bleach solution flow through the system for at least 5 min.

2. Next, flush the system with autoclaved water as bleach can lead to formation of precipitates from the elutriation buffer which may lead to clumping of the cells, as well as cause harm to the cells.
   1. Place the reservoir tubing into a 1 L bottle of autoclaved water and allow it to flush through for at least 10 min.

3. After flushing with water, introduce elutriation buffer into the system. Use a 150 mL glass bottle which has been autoclaved to create a reservoir for the elutriation buffer and cells.
   1. Add 100 mL of elutriation buffer to the bottle and move the reservoir tubing to the glass bottle so that the tube is at the very bottom.
   2. Start the centrifuge and allow the buffer to flow through the system to flush out the water.
   3. Once the reservoir bottle is almost empty and the water is completely flushed out of the system, move the output tubing from the waste bottle to the reservoir so that the buffer is being recycled through the system.

4. Finally, adjust the viewing window so that the elutriation chamber can be seen at the current speed.
   1. Press the strobe lamp power button on the outside of the centrifuge so that the lamp turns on. Turn the elutriation knob also on the outside of the centrifuge all the way to the left.
   2. While looking through the viewing window, slowly turn the knob to the right until the elutriation chamber comes into view and the strobe light is in time with the rotation of the rotor.
      NOTE: The elutriation system can be left in this state until the cells are prepped.

4. Preparation of the Cell Sample

1. Count cells and aliquot 300 to 400 million in growth medium into several 50 mL conical tubes. Spin down the cells at 1210 x g for 3 min.
2. Aspirate off growth media and resuspend cells in 25 mL of elutriation buffer by pipetting up and down gently.
3. In order to reduce cell clumping, pass cells twice through a 25 gauge needle.
   1. Using a 10 mL syringe draw up the cells and then attach 25 gauge needle and pass the cells through onto the inside wall of a sterile 50 mL conical tube. Repeat this until all 25 mL have passed through the needle.
   2. Obtain a new needle and syringe and repeat the process above once.
      Caution: Syringe needles should be placed in sharps containers.
      NOTE: It is critical that the cells are pushed through the needle immediately before introducing them to the elutriation system to keep cell clumping to a minimum.

5. Introduction of Cell Sample into the Elutriation System and Collection of Fractions

1. Introduce the cell sample into the elutriation system by pouring the cell sample into the reservoir tank which has the remaining elutriation buffer recycling through.
   1. Let the cells enter into the system and reach equilibrium within the elutriation chamber.
      NOTE: This process will take about 5 min and progress can be tracked by watching the cells as they enter the chamber via the viewing window. To ensure all cells are in the chamber, take a drop of eluent, place on a glass slide, and view under a microscope. If the sample is free of intact cells, this confirms their retention in the chamber.

2. Start collecting fractions once all the cells have entered into the chamber and there is a distinct boundary at the widest part of the chamber in which the cells are in equilibrium.
   1. Begin by moving the output tube to the 50 mL conical tube labeled W1. Collect 50 mL of elutriation buffer for this fraction, making sure to keep an eye on the reservoir tank and replenish with elutriation buffer when necessary.

NOTE: In order to make sure that all cells have entered into the system, allow the reservoir tank to become almost empty before refilling the reservoir the first time. After the first refill it will not be necessary to wait for the reservoir to become almost empty. Also, DO NOT ever allow the tank to become empty as this will create bubbles and back pressure in the system.

2. Once 50 mL have been collected for W1, simultaneously change the speed to 821 x g and move the output tube to the conical tube labeled W2 and collect 50 mL more at this speed.
3. Continue changing the speed and collecting fractions as indicated in Table 1. Make sure there is media in the reservoir tank and keep the conical tubes on ice at all times. Finally, adjust the viewing window by slowly turning the knob to the right as the speed decreases.
4. Once all 20 fractions have been collected, stop the centrifuge and collect 50 mL in the conical tube labeled STOP.

6. Flushing of the Elutriation System

1. After the centrifuge has stopped completely flush the system by putting the reservoir tube in the 1 L bottle of autoclaved water and the output tube back in the waste bottle.
    1. Turn the pump speed up to 50 mL/min and allow the water to run through the system until it is depleted.

2. Turn off the pump, remove the input and output hose from the quick-release assembly, and pull out the pin from the anchoring cable.
    1. Remove the quick-release assembly from the rotor.
    NOTE: Depending on what the centrifuge will be used for, the elutriation system can now be left as is. Otherwise, the whole assembly can be taken down in the opposite order as described above.The elutriation chamber can be removed from the quick-release assembly and cleaned between runs using an ultrasonic water bath. This removes debris and contaminants from the chamber wall which may negatively affect later runs.

7. Analysis of Fractions and Collection of the G1 or G2/M Pools.

Note: In order to demonstrate the effectiveness of elutriation, each fraction is analyzed for cell count, cell diameter, and DNA content, and pools are created of cells in the G1 or G2/M phases for immunoblot analyses, as described in Representative Results. This protocol section will describe how to create these pools and prepare them for experimental use.

1. Spin each fraction at 2000 x g for 5 min at 4 °C and aspirate off elutriation buffer.
2. Combine fractions together in order to obtain both a G1 phase pool and a G2/M phases pool.
    1. For the G1 phase pool, resuspend fractions which were collected in the speed range of 788 x g to 661 x g (F1 - F5) in a total 1 mL of phosphate buffer saline solution (PBS) and transfer to a 15 mL conical tube. For the G2/M phases pool, resuspend fractions which were collected in the speed range of 387 x g and 333 x g (F17 - F20).
    2. Spin down cells at 2000 x g for 5 min at 4 °C and aspirate off PBS.

3. Resuspend cells in 10 mL of growth media and take a cell count.
4. Take an aliquot from each pool to be analyzed for DNA content via propidium iodide staining and flow cytometric analysis[15].
NOTE: The remainder of each pool can be used for further experiments.

Representative Results

Primary ALL cells (3 - 4 x 10^8) were subjected to centrifugal elutriation and collected into two wash fractions and twenty main fractions, as described in the protocol. Table 1 shows representative data where the total number of cells in each fraction as well as the corresponding rotor speed are presented. Overall yield was typically over 80%. Measurement of cell diameter in individual fractions confirmed that cell diameter increased with advancing elutriation (**Figure 2**). These results represent averages of two independent determinations and reflect the high degree of data reproducibility. Fractions were next subjected to propidium iodide staining and flow cytometry to determine DNA content (**Figure 3**). Early fractions (F1 - F5) contained almost exclusively cells with 2N DNA content representing G1 phase. A mixture of cells in G1 phase and S phase was observed in intermediate fractions (F6 - F9) and cells with 4N DNA content were observed starting in F10. Later fractions had mainly cells with 4N DNA content representing G2/M phases. These data together with the results of **Figure 2** confirmed a relationship between cell size and cell cycle phase.

Based on these results, fractions F1 - F5 were combined to create a pool of cells in G1 phase, and fractions F17 - F20 were combined to create a pool of cells in G2/M phase. The proportion of cells with 2N DNA content in the G1 phase pool was typically 99%, and the proportion of cells with 4N DNA content in the G2/M pool was typically 85%, as determined by propidium iodide staining and flow cytometry. Under asynchronous conditions, 60 - 70% of primary ALL cells are in G1 phase and 15 - 20% are in G2/M phases[15], thus the values obtained after elutriation reflect high levels of enrichment. To further authenticate the two pools with respect to cell cycle phase, extracts from each were subjected to immunoblotting with markers of either G1 or G2/M phases. First, we utilized a phospho-specific antibody which recognizes retinoblastoma (Rb) protein phosphorylated on either Ser-807 or Ser-811, since it is well established that Rb becomes increasingly phosphorylated as cells advance through the cell cycle[17]. As shown in **Figure 4**, levels of phospho-Rb were much higher in cells in the G2/M pool versus the G1 phase pool, consistent with expectations. We also probed for cyclin B1, which is expressed most highly in G2/M phase cells, and for cyclin D1, which is most highly expressed in G1 phase cells[18] (**Figure 4**). The G2/M pool had much higher levels of cyclin B1 compared to the G1 pool, consistent with expectations. Cyclin D1 gave only a weak signal in these preparations, but nonetheless was detectable in the G1 pool and undetectable in the G2/M pool. GAPDH was used a control to confirm equal protein loading.

Modifications to the standard protocol were performed to assess their effects and to establish conditions for optimal performance. First, the number of fractions collected was decreased, from the standard twenty to only three, using speeds determined from Table 1 to represent the terminal points for collection of cells in the G1, S or G2/M phases, as shown in Table 2. Analysis of the DNA content of fractions F1 and F3 are shown in **Figure 5A**. Fraction 1 contained cells highly enriched in G1 phase (95%) and was thus of similar purity to that obtained under standard conditions (**Figure 3**). However, fraction 3, ostensibly cells highly enriched in G2/M phase, gave a mixed population, with cells in G1 and S phases also present. G2/M cells represented only 51% of the total, compared to 85% under standard conditions. These results indicate that attempts to simplify the procedure by reducing the number of fractions compromises sample quality. Next, we tested the effect of reducing fraction volume, as a means to reduce reagent consumption. A standard elutriation was performed except that 40 mL instead of 50 mL fractions were collected. Fractions F1 and F20 were analyzed for DNA content. As shown in **Figure 5B**, fraction F1 was highly enriched in G1 phase cells (98%), similar to that obtained under standard conditions. However, fraction F20 contained cells in all three phases, with cells in G2/M phase only representing 64% of the total, compared to 85% under standard conditions. These results indicate that reducing fraction volume also compromises sample quality.

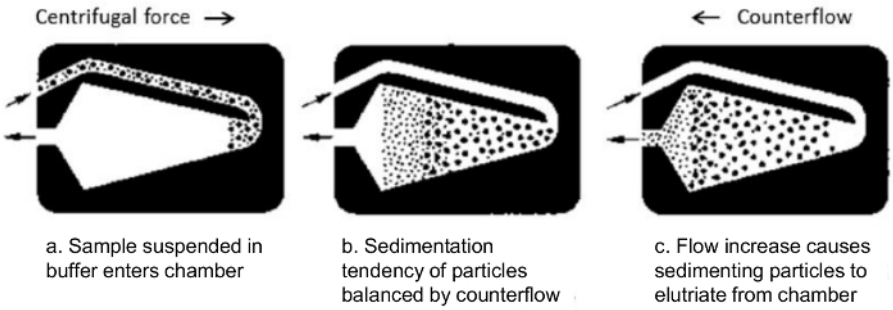

Figure 1. Principle of counterflow centrifugal elutriation.
Note that elutriation of cells can be achieved either by increasing the rate of counterflow as indicated here or by decreasing rotor speed. Adapted by permission from Macmillan Publishers Ltd: [Nature Protocols] (Ref 8.), copyright (2008)[8]. Please click here to view a larger version of this figure.

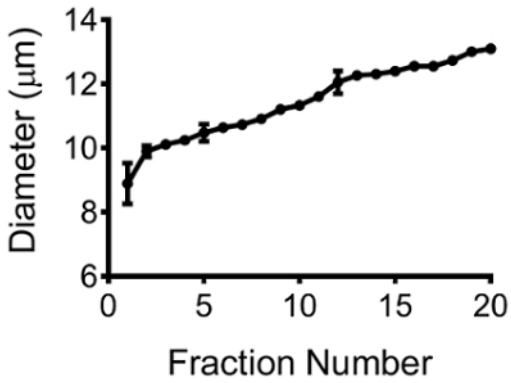

Figure 2. Diameter of primary ALL cells increases with elutriation fraction.
The average diameter was determined for each fraction using a cell analyzer which records data from 500 - 4,500 individual cells per sample. The data shown are mean ± S.D. from two independent experiments. Note that error bars for many points are smaller than the symbol and were omitted for clarity. Please click here to view a larger version of this figure.

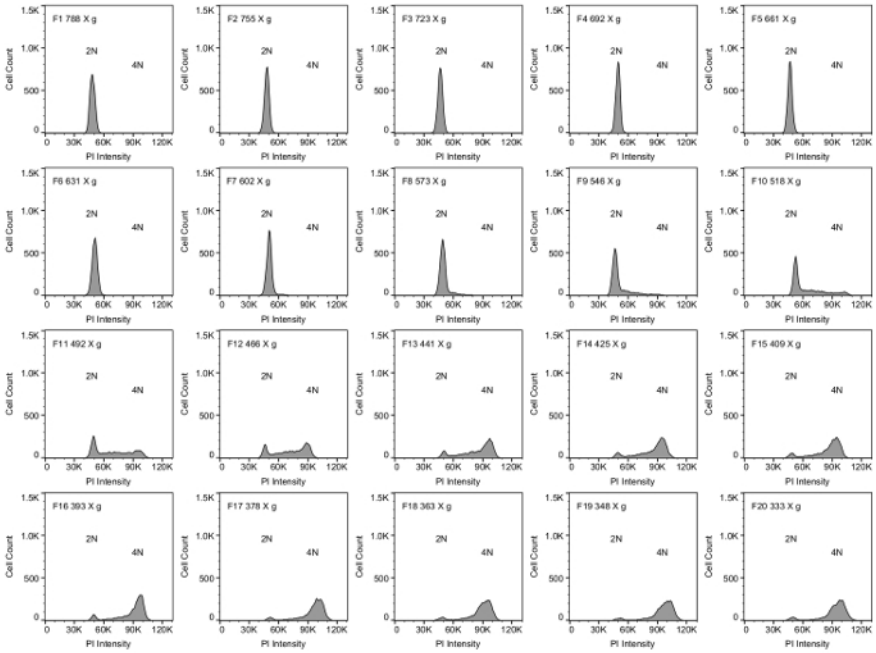

Figure 3. Analysis of DNA content confirms successful separation of primary ALL cells in different cell cycle phases.
10 mL of each fraction was fixed in 70% EtOH, stained in propidium iodide and analyzed by flow cytometry, as described[15]. Propidium iodide integrated intensity (x-axis) was plotted versus cell count (y-axis) to determine the portion of cells in each phase, where 2N DNA content indicates G1 phase and 4N DNA content indicates cells in either G2 or M phases. The data shown are from a representative experiment and essential identical results have been obtained in four repeats. Please click here to view a larger version of this figure.

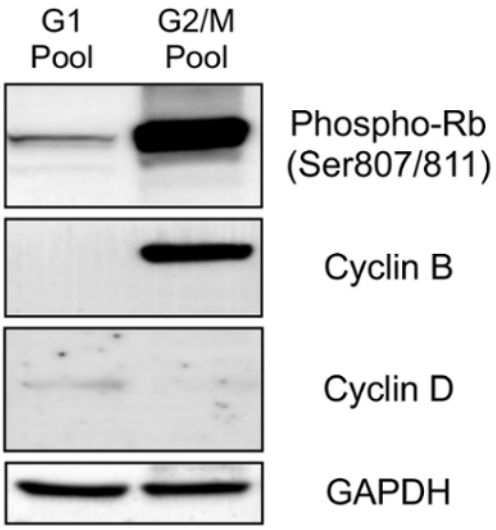

Figure 4. Immunoblot analysis of cell cycle-related proteins in pooled fractions.
Following a standard elutriation of primary ALL cells, fractions F1 - F5 were combined to create a G1 phase pool and fractions F17 - F20 were combined to create a G2/M phase pool. Extracts were prepared and 40 µg/lane subjected to immunoblot analysis, as described previously[15], with antibodies to phospho-Rb (Ser807/811), cyclin B1, or cyclin D1, all used at 1:2,000 dilution. GAPDH (1:10,000) was used as a loading control. Please click here to view a larger version of this figure.

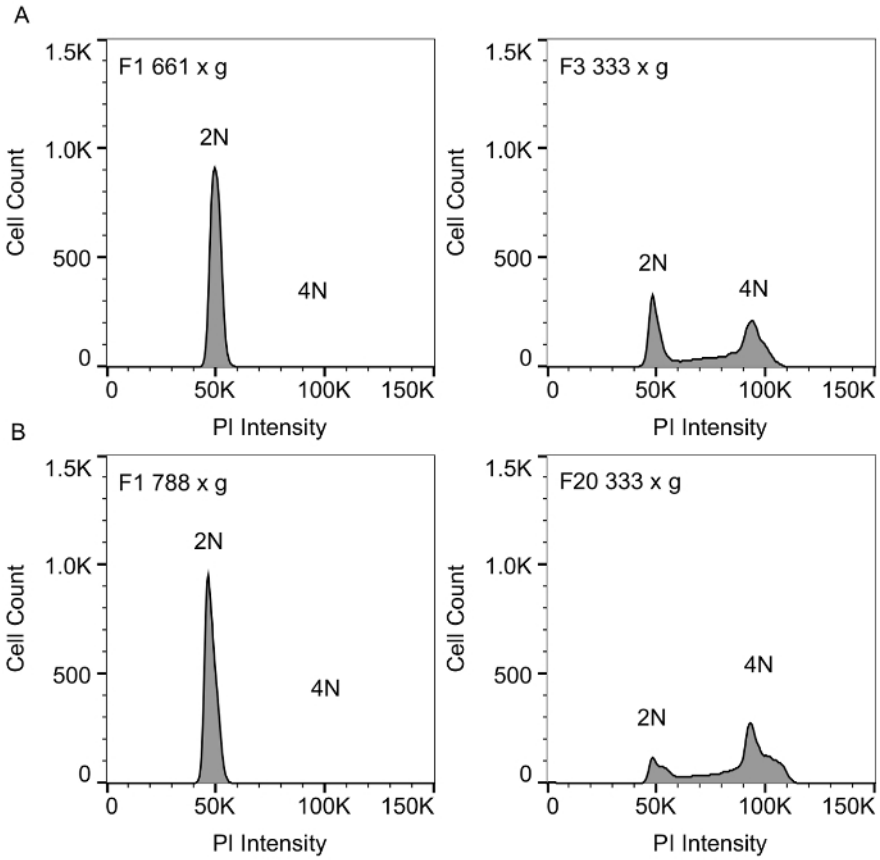

Figure 5. Illustrative data from suboptimal elutriations.
A) Effect of decreasing the number of fractions collected. Only three main fractions (F1 - F3), instead of the standard twenty, each of 100 mL, were collected, at speeds as indicated in Table 2. Fractions F1 and F3 were analyzed for DNA content as in **Figure 3**. B) Effect of reducing fraction volume. Twenty fractions were collected, under the same conditions as in Table 1, but only 40 mL of elutriation buffer instead of the standard 50 mL was used for each. Fractions F1 and F20 were analyzed for DNA content as in **Figure 3**. Please click here to view a larger version of this figure.

Fraction	Speed (rpm)	G-Force	Total Cells (x 10^6)
W1	3000	867 x g	17.75
W2	2920	821 x g	7.00
F1	2860	788 x g	4.60
F2	2800	755 x g	10.75
F3	2740	723 x g	15.25
F4	2680	692 x g	22.50
F5	2620	661 x g	26.75
F6	2560	631 x g	25.00
F7	2500	602 x g	22.75
F8	2440	573 x g	18.00
F9	2380	546 x g	14.00
F10	2320	518 x g	10.75
F11	2260	492 x g	7.43
F12	2200	466 x g	8.63
F13	2140	441 x g	6.63
F14	2100	425 x g	4.98
F15	2060	409 x g	3.46
F16	2020	393 x g	3.43
F17	1980	378 x g	2.63
F18	1940	363 x g	3.17
F19	1900	248 x g	2.78
F20	1860	333 x g	1.66
STOP	0	0 x g	5.19
Total Yield			245.06
Percent Yield			81.69

Table 1. Cell yield and corresponding rotor speed for each fraction.
Cell count was determined using a automated cell counter. Total yield was determined by the sum of individual fractions versus input (3 x 10^8 cells). Data are an average of two representative experiments. Centrifugal force and corresponding rotor speed are given.

Fraction	Speed (rpm)	G Force
W1	3000	867 x g
W2	2920	821 x g
F1	2620	661 x g
F2	2020	393 x g
F3	1860	333 x g
Stop	0	0 x g

Table 2. Speed parameters for suboptimal elutriation.
Shown are centrifugal forces and corresponding rotor speeds for the washes and three fractions collected in a compressed suboptimal elutriation. See **Figure 5A** for corresponding DNA content and text for details.

Discussion

We have described a method for obtaining primary ALL cells in different phases of the cell cycle using CCE. Under optimal conditions an essentially pure population of cells in G1 phase and a highly enriched population of cells in G2/M phases could readily be obtained in excellent yield, and cells highly enriched in S phase can also be obtained if desired. The results presented here were obtained using one primary ALL culture (specifically, ALL-5), and essentially identical results have been obtained with an independent culture, ALL-2[15]. In addition, all other primary ALL cultures examined to date have similar density ranges during asynchronous growth, and will likely perform similarly when subjected to CCE. Our prior studies have also shown that other leukemia cell lines, including HL60 and K562, can be separated into different cell cycle phases using CCE[19,20]. Critical steps in the procedure include ensuring that the cells are mono-dispersed and not clumped at the start of the run; optimization of the conditions of counterflow rate and rotor speed; ensuring the components of the system are clean and free of debris;

and avoiding the introduction of air bubbles into the flow lines. CCE works best with cells that significantly increase in cell size as they advance through the cell cycle. For a given population of cells in suspension, this can be established initially and easily by examining the relationship between DNA content and side-scatter, the latter dependent on cell size. Both parameters can be measured simultaneously by flow cytometry after propidium iodide staining, as we have described[15]. A linear relationship between side-scatter and DNA content provides confidence that cell size increases with cell cycle advance and thus that CCE has the potential to separate cells based on cell cycle phase.

As we have shown (**Figure 5**), deviations from the optimized procedure result in compromised sample quality, especially for cells in G2/M phases. However, reduction in the number of fractions or fraction volume did not appreciably affect the purity of cells in G1 phase (**Figure 5**). Thus, shortcuts to the procedure can be tolerated if only purified G1 phase cells are needed. Although CCE is readily amenable to suspension cell cultures, it can also be applied to subcellular fractions. For example, nuclei from murine pre-B cells have been successfully size- separated using CCE[8]. Adherent cell types pose potential problems due to their propensity for clumping and possible adherence to the surfaces of the tubing and elutriation chamber and thus should be investigated with caution.

The main limitation of CCE is the cost of the equipment; a possible solution is to collaborate with a lab which has the necessary apparatus. Note however that while the rotor is a dedicated instrument, the centrifuge can be used for general centrifugation purposes, thus reducing the cost invested solely in CCE. Another limitation is that the scheduling of experiments is more problematic compared to the flexibility of other methods such as serum starvation or chemical synchronization. Elutriation runs typically take 5 - 6 hours, and if the cells obtained are subsequently needed for short-term or multi-time point experiments, this can result in scheduling inconveniences. The biggest advantage is that cells obtained by this method are unperturbed and can be cultured for many days without any signs of distress, as we have shown[15]. This contrasts with cells synchronized by chemical means which may harbor drug-induced damage. A recent study, using NB4 cells derived from acute myeloid leukemia blasts, directly compared CCE and other methods, including arrest with serum starvation, hydroxyurea, or treatment with a cyclin-dependent kinase 1 inhibitor, RO-3306[21]. Elutriated cells and cells arrested at comparable phases appeared similar with respect to DNA content and cyclin expression. However, when the proteome was examined, major changes in protein abundance, particularly stress-related and arrest-specific proteins, were observed in the arrested cells and not in the elutriated cells at equivalent cell size positions. These results highlight the utility of CCE for the production of unperturbed cells and emphasize that caution should be used when interpreting data from cells synchronized by other means.

Disclosures

Anisha Kothari is currently at the Department of Cell and Molecular Biology at St. Jude Children's Research Hospital.

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