Chapter 2

Synchronization of Mammalian Cells and Nuclei by Centrifugal Elutriation

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Abstract

Synchronized populations of large numbers of cells can be obtained by centrifugal elutriation on the basis of sedimentation properties of small round particles, with minimal perturbation of cellular functions. The physical characteristics of cell size and sedimentation velocity are operative in the technique of centrifugal elutriation also known as counterstreaming centrifugation. The elutriator is an advanced device for increasing the sedimentation rate to yield enhanced resolution of cell separation. A random population of cells is introduced into the elutriation chamber of an elutriator rotor running in a specially designed centrifuge. By increasing step-by-step the flow rate of the elutriation fluid, successive populations of relatively homogeneous cell size can be removed from the elutriation chamber and used as synchronized subpopulations. For cell synchronization by centrifugal elutriation, early log S phase cell populations are most suitable where most of the cells are in G1 and S phase (>80%). Apoptotic cells can be found in the early elutriation fractions belonging to the sub-Go window. Protocols for the synchronization of nuclei of murine pre-B cells and high-resolution centrifugal elutriation of CHO cells are given. The verification of purity and cell cycle positions of cells in elutriated fractions includes the measurement of DNA synthesis by [^{3}H]-thymidine incorporation and DNA content by propidium iodide flow cytometry.

Key words Counterstreaming centrifugation, Cell separation, Velocity sedimentation, Elutriator, Resolution power

1 Introduction

1.1 Definition of Terms: Elution, Elutriation, Centrifugal Elutriation

Elution is an analytical process by which chemicals are emerging from a chromatography column and normally flow into a detector. The material obtained with the carrier (eluent) is the mobile phase and known as the eluate. In liquid column chromatography, the eluent is a solvent (Fig. 1a). Elutriation is a technique to separate small particles suspended in a fluidized bed into different size groups by passing an increasing flow rate through the elutriation chamber. In elutriation, particles are suspended in a moving fluid which can be liquid or gas. In vertical elutriation, smaller particles move upward with the fluid. Large particles will not be driven out of the elutriation chamber and tend to settle out on the walls or at

Gaspar Banfalvi (ed.), *Cell Cycle Synchronization: Methods and Protocols*, Methods in Molecular Biology, vol. 1524,
DOI 10.1007/978-1-4939-6603-5_2,

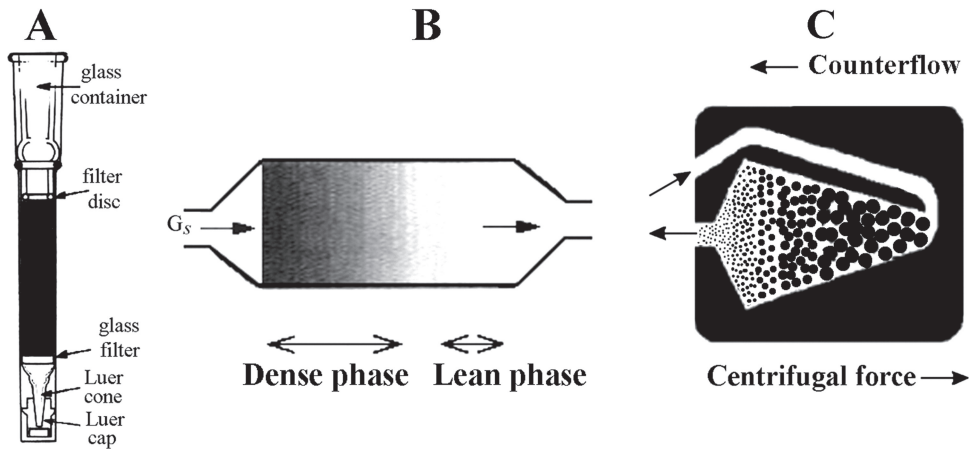

Fig. 1 Schematic views of elution, elutriation, and centrifugal elutriation. (**a**) For elution in column chromatography, a liquid eluent is used. (**b**) Horizontal elutriation in a fluidized bed of particles with flux G_s. (**c**) Fractionation of cells by centrifugal elutriation. Cells are introduced with an initial lower flow rate which is opposed be the centrifugal force. By increasing the flow rate, the floating cells in the elutriation chamber can be driven out and separated into 10–30 distinct size fractions

the bottom of the elutriator due to the higher gravity exerted on their larger size. In horizontal position, the suspended particles are passed through the elutriation chamber. By increasing stepwise the fluid velocity, the particles can be separated into fractions (Fig. 1b). The principle of centrifugal elutriation is similar to elutriation except that the fluid is pumped into a rotating separation chamber that converges into an outlet tube (Fig. 1c).

1.2 Development and Application of Centrifugal Elutriation

The first apparatus that exploited differences in velocity sedimentation for the separation of cells by counterstreaming centrifugation was described by Lindahl in 1948 [1]. The Beckman Instrument Company modified this instrument and named it elutriator and termed the process centrifugal elutriation [2]. Further refinement and a second chamber style know as the Sanderson chamber was introduced giving a better resolution especially with small cells [3]. Centrifugal elutriation has been applied to separate hemopoietic cells, mouse tumor cells, testicular cells, and a variety of other specialized cells from other cells and cells in particular phases of the cell cycle reviewed first by Pretlow in 1979 [4]. Several further applications have been described [5–9]. The construction of the commercially available centrifugal elutriator resulted in a rapid purchase of these instruments especially in the United States in the second half of the 1970s, with high expectations and significantly lower outcome than originally expected. The major reason of insufficient or lacking data is the missing technical skill necessary for the assembling and operation of these instruments and the use of such heterogeneous batch cultures that made the separation irreproducible. It is thus important to discuss first the

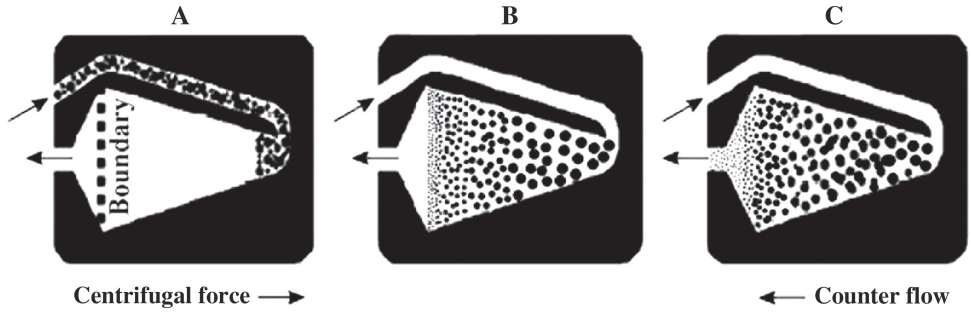

Fig. 2 Cell synchronization by centrifugal elutriation based on different cell size. The centrifugal force and the counter stream act in opposite directions in the elutriation chamber. (**a**) Early S phase cells enter the elutriation chamber. (**b**) Size gradient is balanced by centrifugal force and counterflow of elutriation keeping cells floating inside the chamber. (**c**) Increasing flow rate elutes smaller cells first followed by larger ones. Reproduced with permission of Banfalvi [11]

circumstances that may cause experimental failures during the centrifugal elutriation.

1.3 Principles of Centrifugal Elutriation

Among the methods used to monitor cell cycle changes, two distinct strategies prevailed [10]. One is the chemical "arrest-and-release" approach that arrests cells at a certain stage of the cell cycle and then by the removal of the blocking agent synchronized cells are released to the next phase. In the alternative approach, cells are separated by physical means such as mitotic shake-up, gradient centrifugation, cell sorting, and centrifugal elutriation. These methods have been overviewed in Chapter 1.

Counterflow centrifugal elutriation separates subpopulations of cells on the basis of cell volume and density. As there is only a slight change in cell density during the cell cycle, the principle of cell separation during elutriation is based on cell size (Fig. 2).

Before going to the point-by-point elutriation protocol, the following precautions are advised to avoid unnecessary mistakes and problems during the execution of this cell synchronization technique:

Culture medium. For test run and pilot experiments, saline can be used to find out the right conditions (centrifugal force, flow rate) for cell fractionation. Although any medium can be used for elutriation, it is advised that the elutriation fluid should be the physiological culture medium. To limit the cost of elutriation, the fetal calf serum concentration can be reduced to 1%. Some investigators use cost-efficient 1% bovine serum albumin. The protein content prevents cells from clumping and the cellular debris from adhering to the chamber wall. Cell aggregates normally accumulate at the entrance of the elutriation chamber and may clog it. In such case, the chamber has to be disassembled, whipped out with a soft paper towel, cleaned with a mild detergent, and reassembled

taking care of the right position of the plastic insolation. High protein concentration may cause foaming and cell lysis. The culture medium should contain only soluble materials, any particle left in the medium may contribute to aggregation or will appear in one of the elutriated fractions obscuring the cell cycle profile.

Cells. Most significant cell growth takes place in S phase; it is thus not surprising that nearly 100% of G1 cells and more than 80% of S cells could be recovered by centrifugal elutriation [12]. Best separation can be achieved by using cell populations maintained in early S phase of growth for several generations. This will reduce the number of aging and dying cells due to anoxia, the formation of artifacts, cell debris, and will prevent clumpiness. Early S phase cells contribute to the reproduction of the results causing only small shift in the elutriation profile that can be attributed to minor differences in cell distribution.

Any suspension cell line can be used for centrifugal elutriation. Cells that tend to stick together or attach to the culture substrate (e.g., Chinese hamster ovary cells) need more attention. These cells should be kept in suspension before synchronization by growing them in spinner flasks continuously stirred with a magnetic stirrer at speed high enough to prevent sedimentation and low enough to avoid cell breakage (Fig. 3). The resuspension of the cell suspension is also recommended by passing it several times through an 18-gauge needle right before loading in the elutriation chamber to ensure monodispersion. Once cells are in the chamber, the flotation of cells keeps them separated.

1.4 Components of the Centrifugal Elutriation System

The counterflow elutriation system (earlier Beckman, now Beckman-Coulter Inc.) consists of a centrifuge, elutriation rotor containing the elutriation chamber, stroboscop, peristaltic pump, manometer, sample mixing tube, and the flow system (gauge for monitoring back pressure, injection and bypass valves, T-connector, rubber stoppers, and silanistic tubing) (Fig. 4). Additionally, the pump may be interfaced with a fraction collector. The cell separation is carried out in a specially designed centrifuge hosting either the standard JE-6B rotor or the larger JE-5.0 rotor. The standard elutriation chamber is available in two sizes. The small elutriation chamber has a capacity of approximately 4.5 ml and is suitable for the fractionation of 10^7–10^9 cells. The large chamber is able to separate ten times more volume and cells. The geometry of the Sanderson chamber gives a better resolution for the separation of small cells in the range between 10^6–10^8 cells. The peristaltic pump is providing a gradually changing flow through the chamber with flow rates between 2 and 400 ml/ml depending on the size of the chamber and the cells. The elutriation system uses either a constant centrifugal force and gradually increasing counterflow rate or constant flow rate and reducing rotor speed to yield high resolution of synchronization. As the fine control of rotor speed reduction is not

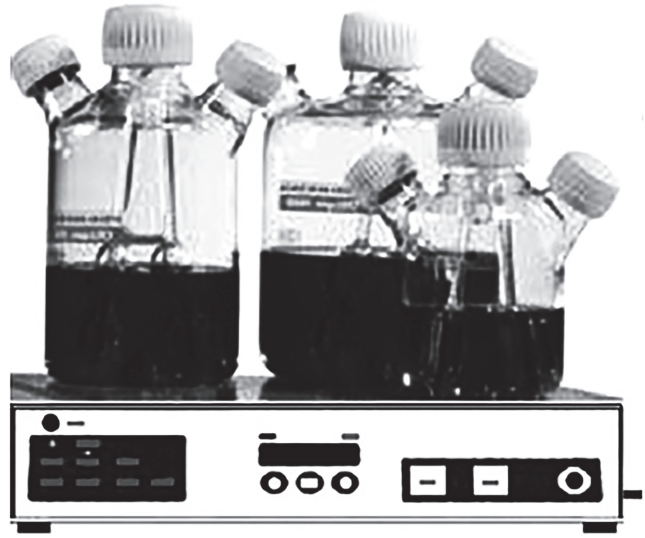

Fig. 3 Spinner flasks for suspension cultures. Cell cultures (suspension or monolayer) are started in T-flasks containing 25 ml medium. After reaching confluency, the cells of two flasks are combined in a smaller (200 ml) spinner flask that is sitting in the CO_2 incubator. Cells in suspension are grown under constant stirring in 100 ml medium starting at ~10^5 ml/ml density. Logarithmic growth is maintained by scaling up daily from 100 to 200 ml medium, then switching to a larger spinner flask (2 L) and doubling the volume of the medium to 400, 800 and if necessary 1600 ml. Up to 3–4 × 10^8 cells can be grown in one week before starting elutriation

possible, constant speed and increasing flow rate are preferred. The rotor speed may vary between 1800 and 3500 rpm (~550–1700 g relative centrifugal force) depending on cell size. Although higher speed gives a better resolution, the pump speed will also go up increasing the shearing force and the amount of cell debris. The relationship between cell size and centrifugal force is demonstrated in Table 1.

1.5 Monitoring the Efficiency of Centrifugal Elutriation

Due to the close correlation between cell size and DNA content, the effectiveness of the separation can be tested by monitoring the results by cell number, cell size, and DNA content. The most simple method is to count the cells and estimate their size in a hemocytometer. This indispensible method visualizes cells and distinguishes among small and large cells as well as cell aggregates. Particle counters generally monitor changes in conductivity and help to automatize the counting procedure. Most of these devices provide information with respect to particle size and volume distribution. However, size distributions do not exclude errors generated by aggregation, coincidence, and by nonspherical particles. Coincidence can be taken into consideration by counting the same cell population several times in a Bürker chamber and by the

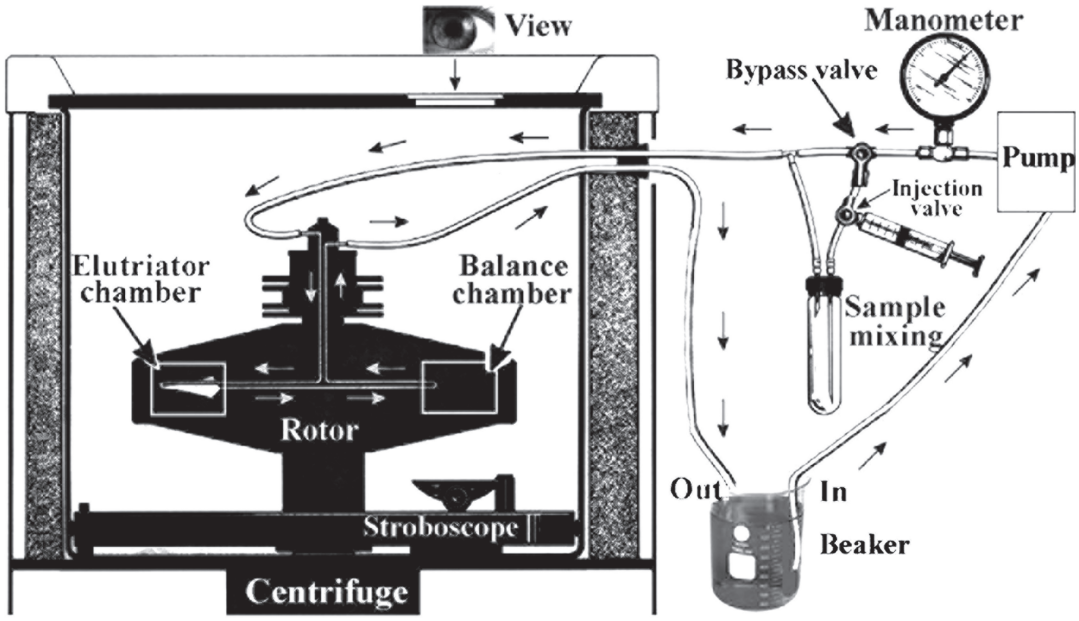

Fig. 4 Counterflow centrifugal elutriation system. The stroboscope is placed and fixed at the bottom of the centrifuge. The centrifuge is hosting the rotor that is running with constant speed. The pump is turned on and sterilization takes place by running 70 % ethanol through the system followed by physiological buffer to remove traces of ethanol. The physiological buffer is then replaced by the elutriation fluid at an initial flow rate. The preliminary run also serves to remove bubbles from the system. The loading chamber serves as a sample mixer and in its bypass position traps small remaining bubbles and compensates pump pulsation. The bypass valve helps to remove residual bubbles from the sample mixer chamber. Reproduced with permission of Banfalvi [11]

Table 1
Elutriation conditions for *Drosophila* and mammalian cells and murine preB nuclei

			Centrifugal force of elutriation		
Cell type	**Cell diameter (μm)**	**Flow rate (ml/min)**	**Rpm**	**Rcf average (g)**	**Rcf maximum (g)**
CHO	11.9	12–49	2000	569	753
CHO	11.9	19–52	2200	575	683
Indian muntjac	11.4	12–50	2200	575	683
Murine preB	10.2	11–73	2200	688	911
Drosophila S2	8.1	13–53	2500	889	1176
PreB nuclei	5.0	14–77	3500	1742	2305

Temperature was 20 °C for each elutriation
Cell diameter refers to an average value of exponentially growing cells and average size of nuclei before synchronization, measured by multiparametric particle size counting
Rotational speed is given as revolution per minute (Rpm), relative centrifugal force (Rcf) in *g*. Reproduced with permission of Banfalvi [11]

particle counter. The ratio of cell count obtained by the cell number counted by the Bürker chamber over the cell number obtained by the particle counter serves then as a factor which is used to multiply the counter number to get the real cell number.

Flow cytometry is used to asses the quality of synchronization by monitoring size distribution with forward scatter analysis and concomitantly the DNA content of each elutriated fraction after staining with propidium iodide. Flow cytometry is also generally accepted for the evaluation of cellular processes [13, 14] for calculating proliferative parameters [15] to identify apoptotic cells by nuclear staining [16–18] or by the appearance of phosphatidylserine on cell surface [19] and monitor other cellular changes [20–22].

2 Materials

2.1 Disposables

1. 1.5 ml microcentrifuge tubes.
2. 15 and 50 ml centrifuge tubes.
3. 15 and 50 ml conical centrifuge tubes.
4. Polystyrene test tubes.
5. 25 and 75 cm^2 flasks for cell culture.
6. 5 ml, 12 × 75 mm FACS tubes.

2.2 Media and Solutions

1. RPMI 1640 growth medium.
2. Fetal bovine serum (FBS).
3. *Ham's medium*: Measure 10.64 g l-glutamine containing F12 powder for 1 L of 1× solution. Add 1.176 g/L of $NaHCO_3$ powder. Adjust pH to 7.1–7.2, as it will rise to 0.2–0.3 pH units during filtration. Filter-sterilize it immediately using a membrane with a porosity of 0.22 μm. Aseptically dispense into 100-ml sterile bottles. Store them in a cold room or in a refrigerator under 5 °C until use. Before use, add 11 ml FBS albumin to each 100-ml F12 medium. F12 can be ordered as 1-, 5-, 10-, and 50-L liquid medium containing l-glutamine, without sodium hydrogen carbonate. Sterilized medium can be stored at 5 °C for several weeks if unopened.
4. *Elutriation medium* (for 30 elutriation fractions): Prepare fresh 3.5 L of F12 culture medium in a 4-L flask containing 1% FBS. Omit filter sterilization, as fractionated cells are processed immediately after elutriation. Larger particles originating from F12 medium or from cell aggregates can be removed by passing the medium and cell suspension through a 100-mesh stainless steel sieve.

5. *Phosphate-buffered saline (PBS)*: Instead of culture medium plus 1% FBS, Ca^{2+}- and Mg^{2+}-free PBS with 0.01% EDTA or without EDTA is successfully used as elutriation buffer. PBS contains 2.7 mM KCl, 4.3 mM sodium phosphate dibasic (Na_2HPO4), 1.8 mM potassium phosphate monobasic (KH_2PO4), 137 mM NaCl, pH 7.2. Sterilize in an autoclave.
6. *Saline*: Dissolve 9 g of NaCl in 1 L of distilled water and sterilize in an autoclave.
7. *Trypsin/EDTA solution*: Make up from 0.25% (wt/vol) trypsin, 1 mM EDTA in phosphate-buffered saline (PBS). Filter-sterilize. Stored at –20 °C.
8. *PI solution for flow cytometry*: Dissolve 50 mg/ml of PI in 0.1 M ammonium citrate solution (not sterile).

3 Methods

3.1 Cell Growth

1. Keep the epithelial-like CHO-K1 cells (ATCC, CCL61) in suspension culture in spinner flasks using F12 Ham's medium supplemented with 10% heat-inactivated FBS at 37 °C and 5% CO_2. Grow the murine pre-B cell line 70Z/3-M8 [23] in suspension culture at 37 °C in RPMI 1640 medium supplemented with 10% FBS, 2 mg/ml mycophenolic acid, 150 mg/ml xanthine and 15 mg/ml hypoxanthine and 2×10^{-5} M β-mercaptoethanol.
2. Maintain CHO cells as either monolayers in 75 cm^2 tissue culture flasks when smaller quantities of cells (10^6–10^7) are needed or in suspension in 250 ml and in 1-L spinner flasks if many cells are required, such as in elutriation ($2–3 \times 10^8$).
3. Maintain cells in logarithmic growth and high viability by culturing at densities between 1×10^5 and 4×10^5.
4. Replace media by splitting cells in the ratio of 1:2 daily. Grow CHO cells in suspension culture in 1 L spinner flasks containing a final volume of 800 ml F12 Ham's medium supplemented with 10% FBS. The suspension culture should initially contain $1–2 \times 10^5$ cells/ml[1] at 37 °C in 5% CO_2.
5. Grow cells for 24 h to a final concentration of $2–4 \times 10^5$ cells/ml for fractionation by centrifugal elutriation. Harvest cells by centrifugation at $600 \times g$ for 5 min at 5 °C and resuspend in 10 ml of F12 medium containing 1% FBS. To avoid the presence of dead and fragmented cells in elutriated fractions, *see* **Note 1**. Remove cell aggregates by passing the cell suspension through a 100-mesh stainless steel sieve.

3.2 DNA Isolation

Isolate high molecular weight DNA from 10^6 cells of elutriated fractions, determine the amount of DNA by a DyNA Quant fluorimeter (Hofer Scientific Instruments) using Hoechst 33258 dye,

which binds to the minor groove of double-stranded DNA. Excite the bound dye with long UV light at 365 nm and measure its fluorescence at 458 nm with calf thymus as DNA standard.

3.3 Determination of DNA Content

1. Isolate DNA from elutriated fractions by phenol extraction from an equal number of cells (10^6) from each elutriated fraction.
2. Stain the DNA. The amount of DNA can be determined by specific dyes (e.g., Hoechst 33258).
3. Excite the bond dye with UV light and measure DNA content. Use calf thymus DNA as DNA standard.

3.4 Measurement of Cell Number and Cell Size

Confirm the synchrony of elutriated fractions by measuring cell size in a Coulter Channelyzer or Coulter multisizer. The measurement of gradual and simultaneous increase both in cell size and cell volume as well as the correlation between cell size and DNA content confirm that synchrony is maintained not only at low, but also at increased and higher resolution.

3.5 Flow Cytometry

1. Stain cells with 50 mg/ml of PI in 0.1 M ammonium citrate for 15 min at 0 °C.
2. Add an equal volume of 70% ethanol.
3. Perform cell cycle analysis in a fluorescence-activated cell sorter. Use the flow cytometric profiles of elutriated fractions to calculate the nuclear DNA content expressed in average C-values [24]. The C-value increases from 2C to 4C as the cell progresses through the S phase, providing quantities measure of cell growth. When many fractions (>20) have to be processed within the same day, stained and fixed cells can be stored overnight in refrigerator (4 °C) and flow cytometry can be performed next day.

3.6 Conditions Used for Centrifugal Elutriation

The elutriation system (earlier Beckman, now Beckman Coulter Inc.) is an advanced device that uses either a constant centrifugal force and gradually increasing counterflow rate or constant flow rate and reducing rotor speed to yield high resolution of synchronization. The cell separation is carried out in a specially designed centrifuge and rotor. This elutriator system was designed exclusively for research, not for diagnostic or therapeutic use.

3.6.1 Hardware

The hardware of the JE-6 elutriator system (JE-6B Rotor, elutriator, 6000 rpm, 5080 × *g*, recoverable cells per run 10^9) consists of the following subassemblies: centrifuge, rotor assembly, stroboscope, flow system, modified door for the Model J-21/J-21B or J2-21 centrifuges as well as various tools and lubricants. In our elutriation system, we used a J2-21 centrifuge and a JE-6B separation chamber (Beckman Coulter Inc.), in which the elutriation

fluid was circulated at an initial flow rate of 16.5 ml/min using a MasterFlex peristaltic pump (Cole-Parmer Instrument Inc.) presterilized with 70% ethanol. Elutriation was performed at a rotor speed of 2200 rpm ($683 \times g$) and temperature of 20 °C for 4–5 h. Subpopulations of cells were eluted in F12 medium containing 1% FBS. We have also used the JE-5.0 elutriation system (JE-5.0 Rotor, Elutriator, 5000 rpm, $4704 \times g$, retrievable cells per run 10^{10}), including a Sanderson elutriation chamber (5 ml) and a J-6 M1 centrifuge (Beckman Instruments Inc.) [23]. When the JE-5.0 elutriation chamber is used with the Avanti J-25 and Avanti J-26 XP series and with J6-MI centrifuges equipped with viewport door and strobe assembly, accelerated throughput, shorter run times can be achieved. The JE-6B low-volume elutriator rotor is excluded from the JE-5.0 elutriation system.

The protocol presented here has been developed to enable cell cycle studies in a variety of cell lines [25–27]. The elutriation of $1–2 \times 10^8$ cells in a JE-6B rotor and a J2-21 centrifuge was used most frequently in our experiments [28]. The small JE-6B elutriation system was assembled according to the manufacturer's instructions. Only the flow system (gauge for monitoring back pressure, two three-way valves, T-connector, rubber stoppers, and several feet of Silanistic tubing) was regularly disassembled after elutriation and reassembled before the next elutriation, as the centrifuge was normally used in several other experiments. For shaft, rotor, chamber, pressure ring, stroboscope, and flow system assembly and disassembly, see the instruction manual of the Beckman JE-6 or JE-5.0 elutriator rotor. The rotor speed and flow rate monogram in the manual give the necessary preliminary information on rotor speed (rpm), sedimentation rate, particle diameter (mm), and flow rate (ml/min).

3.6.2 Calibration of Peristaltic Pump

Among the preliminary experiments, the relationship between pump speed and flow rate has to be established. The calibration of the peristaltic pump is done by measuring the flow rates at different pump setting using elutriation buffer. It is recommended to do the calibration before the day of elutriation to lighten the burden of elutriation. The pump can be used within the linear range. In our case, linearity was maintained up to 80 ml/min.

3.6.3 Particle Size Counting

Particle size counters are recommended that produce a particulate size profile and average particle diameter for a given culture. The size limit is set for cells to count them above the limit and cell debris below the limit. The unit is cleaned and first a sample of solution (used for dilution) is analyzed, then the sample of the culture is profiled. The solution profile is subtracted from the culture profile to eliminate solution debris. This yields a debris count and a cell count. More sophisticated units include measurement of the mean particle diameter, which, for cell cultures above the size limit, equates to the average cell diameter.

We have used different counters to determine the sizes of the cells in each fraction. The counter is based on the principle of electronic particle counting, where the amplitude of a voltage pulse, caused by a change in impedance when a particle suspended in an electrolyte is drawn through an electrical field, is proportional to the volumetric size of the particle [29]. From the change in impedance, the diameter and surface area of the particle are calculated, assuming that the particles are spherical.

3.7 Installation of Elutriation System

To operate the elutriation system, install the stroboscope first, as its chamber unit has to be placed at the bottom of the centrifuge. Turn the stroboscope unit in such a way that the flashlight will be under the observation port when the door of the centrifuge is closed. Rotate the wheels on the casting to tighten the chamber of the stroboscope. Run the cables of the stroboscope through the ports of the centrifuge inside the chamber and secure them with split rubber stoppers installed from outside. Connect the cables to the control unit of the stroboscope outside the centrifuge. Turn on the power switch and verify that the lamp of the photocell is lit (*see* **Note 2**).

3.7.1 Installation of Stroboscope

3.7.2 Installation of Elutriator Rotor

Open the door of the centrifuge and install the assembled rotor containing the installed chambers (elutriator and bypass chambers). Place the rotor in the centrifuge (*see* **Note 3**).

3.7.3 Installation of Flow Harness

In addition to the port for the strobe control unit, there are two more ports in the centrifuge chamber, one for the tubing coming from the pump and the other for the outlet tubing. One can use only centrifuges that have these ports. Make sure that the tubing running from the pump to the rotor is placed in the upper (inlet) fitting and the outlet tubing in the lower fitting of the elutriator rotor (*see* **Note 4**). Connect the liquid lines. Secure tubings firmly with split rubber stoppers outside the centrifuge.

3.7.4 Installation of Control Units

The preliminary run serves to test whether the control units are functioning properly and the centrifuge is running smoothly. For the test run, check the control units of the centrifuge and set the elutriation speed of the centrifuge. When the rotor is at speed, reset the counter and push START button (*see* **Notes 5** and **6**). The rotor speed will be displayed in tens of rpm on the counter. Put the timer at "Hold" position to avoid untimely interruption of elutriation, the brake at "Maximum" and set the temperature at 20 °C, and limit its change within ±1–2 °C. On the stroboscope control, turn the FLASH DELAY potentiometer counterclockwise to 0 position and slowly turn it clockwise to bring the image of the elutriation chamber into view, seen through the viewport door of the centrifuge. If you have an old JE-6 rotor, the bypass chamber will come into view first. Keep turning the potentiometer till the

separation chamber is seen. The separation chamber has two screws in it that make it distinguishable from the bypass chamber. Watch the pressure gauge in the flow harness (*see* **Notes 7** and **8**). Rise in pressure indicates the presence of air in the system (*see* **Note 9**). Stop the pump and/or decelerate the rotor. Note that not all the cables and ports are visualized in the scheme (Fig. 4). When the pressure is zero, the rotor speed is set at elutriation speed.

3.8 Synchronization of CHO Cells

1. Prepare 3500 ml of fresh F12 elutriation medium in a 4000-ml flask.
2. Fill up a 3000-ml flask with elutriation medium to the rim. Use this as the permanent medium reservoir. Cover it with aluminum foil until further use.
3. Pour 400 ml of elutriation medium into a 500 ml beaker and use it as a temporary medium reservoir.
4. Set up your centrifugal system (Fig. 4). Pump 100 ml of 70% ethanol through the system. Then pump through PBS solution (200 ml) to remove the 70% ethanol used to provide sterilization. Start the centrifugation to remove bubbles. Remove PBS and the bubbles by pumping 100 ml of elutriation medium through the elutriation system and discard this fraction (*see* **Note 9**).
5. Replace the temporary medium reservoir with the permanent medium reservoir. Fill the cell reservoir (sample mixer) with medium using a large (30 ml) syringe. To flush all the air from the sample-mixing chamber, retract the short needle so that the end is flushed with the inside of the rubber stopper. When the sample chamber is full and no more air is seen leaving the exit flow line, close the bypass valve to make sure that the liquid does not enter the sample chamber. Set the initial flow rate of the peristaltic pump at 16.5 ml/min. Keep the elutriation fluid circulating until the cells are introduced into the sample reservoir. Make sure once more that the bypass valve is closed, the speed of the centrifuge is correct (2200 rpm, $683 \times g$), the refrigeration of the centrifuge is on and set at 20 °C and the timer of the centrifuge is set at HOLD (endless) position. Turn the flash delay potentiometer of the stroboscope clockwise slowly from 0 position to bring the image of the separation chamber into view. Number empty culture bottles (or tubes) for collecting elutriation fractions (100 ml each) and stand in ice. Use the scale on the culture bottle as the volume measure.
6. Load the sample reservoir by passing the concentrated cell suspension ($1–2 \times 10^8$ cells in 10 ml of Ham's medium containing 1% FBS in a 50-ml screw cap tube) through an 18-gauge needle three times just before loading to ensure monodispersion. Use a 10-ml Luer-lock syringe for injecting cells into the loading chamber. This also keeps the needle from jumping off the

hypodermic syringe. The needle attaches to the body of the syringe via a screw-type twist. Remove the needle from the syringe. Close the bypass valve, as elution liquid is not allowed to enter the sample reservoir during loading. Attach the syringe directly to the Luer fitting on the injector valve for sample injection. Open the injector valve and inject resuspended cells into the sample reservoir. After injecting cells slowly into the sample reservoir, close the injection valve. Air is not allowed to enter the system. As cells cannot be completely removed from the 50-ml tube and filled in the sample reservoir, those suspended cells that are left in the tube (~0.5 ml) serve as nonelutriated control.

7. Load cells into the elutriation chamber. To load cells from the sample reservoir to the elutriation chamber, turn the sample chamber upside down and open the bypass valve. Start to collect the first 100 ml fraction flushed from the centrifuge. About 100 ml of liquid is required to remove one subpopulation of cells from the 4.5-ml separation chamber. View the loading of cells through the observation window of the door of the centrifuge. The appearance of a darkening cloud indicates the introduction of cells into the elutriation chamber. After loading, no more cells are allowed to enter the elutriation chamber. Close the bypass valve and turn back the loading chamber to its original position (*see* **Note 10**). The fraction collected during loading is discarded. This fraction contains cell debris and dead cells (*see* **Notes 11** and **12**).
8. To obtain the first elutriation fraction, increase the pump speed from 16.5 ml/min to 19 ml/ml slowly by ~1 ml/min increments and collect the next 100 ml media. Gradually increase the flow rate (or decrease rotor speed) to permit the elutriation of further fractions. Collect consecutively 100 ml effluent volumes from the centrifuge. Immediately after a fraction is collected, take 2×1-ml samples and add 9 ml of saline to each sample for Coulter counting and cell-size measurement. Keep elutriated fractions in ice until further use and shake them gently from time to time to avoid cohesive sedimentation and loss of cells (*see* **Note 13**). We have obtained reproducible experiments by performing centrifugal elutriation at low, increased and high resolution of elutriation and collecting 9, 16, and 30 elutriation fractions, respectively (Fig. 5). Only as many samples should be collected as the small team (2–3 persons) carrying out the elutriation can process on the same day (*see* **Note 14**).
9. To finish elutriation, turn off rotor speed and let the high flow rate drive out residual particles from the elutriation chamber. Let the system run and collect 100 ml more liquid to remove all the remaining particles. Remove the medium at high flow rate and rinse the system with 200 ml of distilled water to lyse

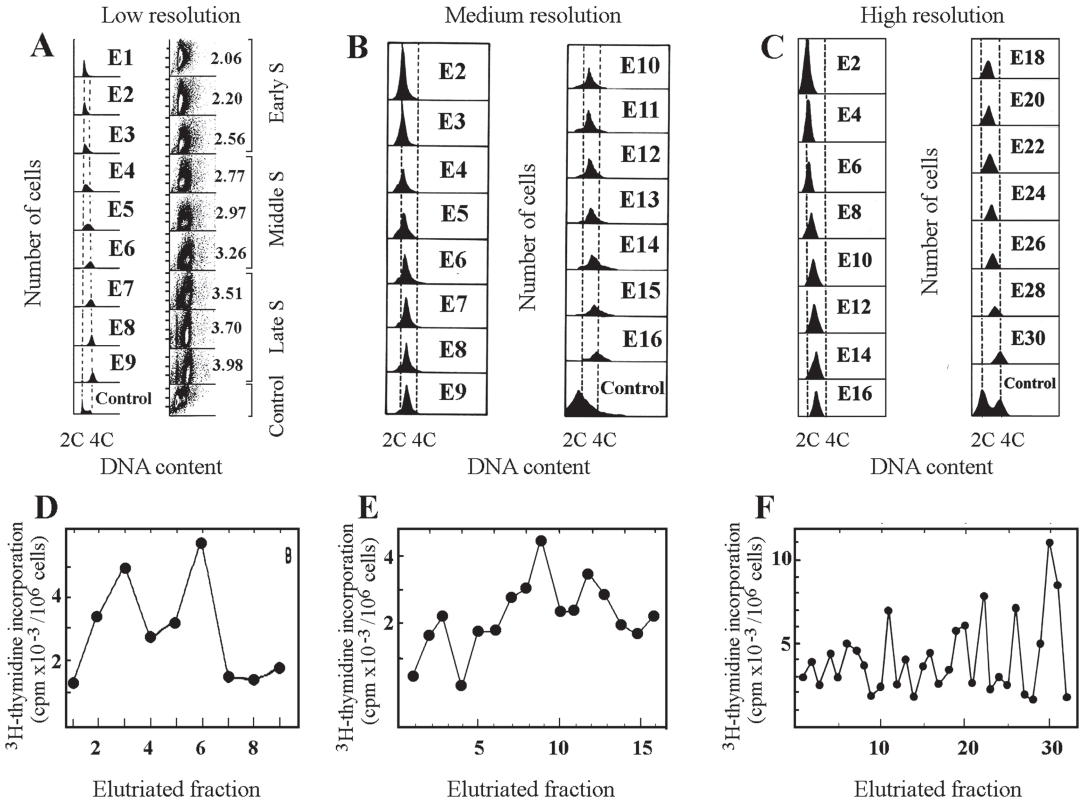

Fig. 5 Centrifugal elutriation at low, medium, and high resolution. Exponentially growing CHO cells were synchronized by counterflow centrifugal elutriation. (**a**) Flow cytometric profiles of DNA content at low (**a**), medium (**b**), and high resolution of elutriation. The cell number is indicated in the ordinate and the DNA content on the abscissa. The average C value (haploid genome content) of elutriated fractions at low resolution (9 fractions, E1–E9) was calculated. For medium resolution 16 fractions (E2–E16) and at high resolution 30 elutriation fractions (E2–E30) were collected. (**b**) DNA synthesis in elutriated fractions at low (**d**), medium (**e**), and high resolution (**f**). ^{3}H-thymidine incorporation is shown in the ordinate and fraction number on the abscissa. 2C- and 4C-values were calculated from the flow cytometric profiles of elutriated fractions [24]. Reproduced with the permission of Banfalvi et al. [30, 31]; Szepessy et al. [32]

any cell left in the system. Cells left in the elutriator chamber may cause pellet formation (*see* **Note 11**).

10. Although almost all parts of the elutriator rotor can be autoclaved, we rinse the system with 70% ethanol to sterilize it. Remove the ethanol by pumping and dry the passages with clean compressed air and leave the rotor assembled. Disconnect the liquid lines and remove the rotor and stroboscope chamber from the chamber. In case of longer storage, remove the elutriation chamber and the bypass (balance) chamber, but store the rest of the rotor assembled.
11. Check the status of your elutriator system by a test run before doing cell separation (*see* **Note 15**).

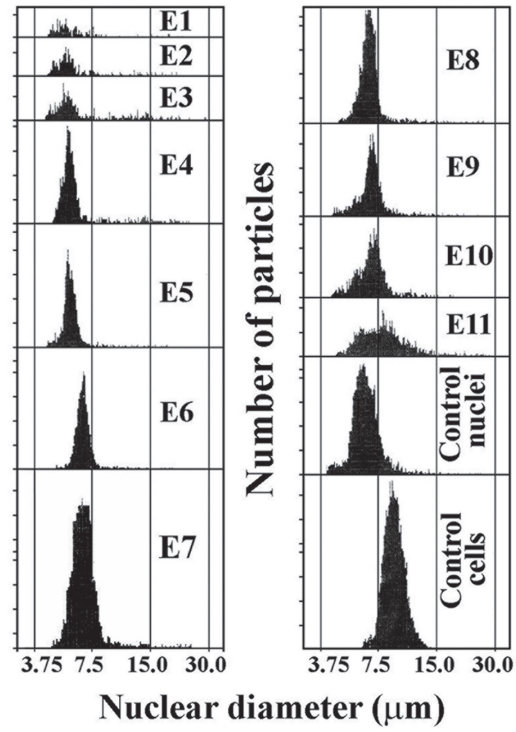

Fig. 6 Centrifugal elutriation of nuclei isolated from murine pre-B cells. Nuclear size is indicated on the abscissa, nuclear number or cell number is given on the ordinate. Cells (1.15×10^8) were subjected to the isolation of nuclei. Elutriation of 6.86×10^7 nuclei was carried out in a JE-5.0 elutriation system equipped with a 5 ml Sandetson chamber and a MasterFlex (Cole-Parmer Instruments) peristaltic pump and a J-6 M1 centrifuge (Beckman Instruments Inc.). Elutriation took place at 20 °C and 3500 rpm ($2305 \times g$). Eleven fractions (E1–E11) were collected, 100 ml each in RPMI medium 1640 supplemented with 1 % FBS. After separation, nuclei were washed with PBS and counted by Coulter multisizer, and nuclear size analysis was carried out. Control nuclei and control cells were not subjected to elutriation. Reproduced with permission of Banfalvi [11]

3.9 Synchronization of Nuclei of Murine Pre-B Cells

1. For the synchronization of isolated murine pre-B nuclei (average diameter 6.8 μm), use higher centrifugal force (3500 rpm, $2305 \times g$).
2. Use a flow rate between 14 and 77 ml/min for murine pre-B cells (average diameter 10.2 μm) and apply a similar flow rate (11–73 ml/min), but a lower centrifugal force (2200 rpm, $683 \times g$) [23, 33]. The synchronization of murine preB nuclei is demonstrated in Fig. 6.

Due to the closer correlation between nuclear size and DNA content than between cell size and DNA content, it could be of potential interest to elutriate subcellular particles to study the fine-tuning of nuclear control. The synchronization of nuclei is expected

to open new vistas for the synchronization of protoplast preparations. The synchronization is based on the nuclear size that is increasing during DNA synthesis and nuclei can be separated by centrifugal elutriation. The introduction of a density gradient during elutriation is not recommended (*see* **Note 16**). To elutriate smaller particles either the centrifugal force has to be increased, or the flow rate decreased. A lower flow rate would limit the resolution, while maximal pump speed (~500 ml/min) should also be avoided as the linearity between the pump speed and flow rate could not be maintained. Turbulence can be minimized by increasing the flow rate slowly from fraction to fraction (*see* **Note 17**).

3.10 Verification of Synchronization

1. Assay the quality of synchronization after centrifugal elutriation in each elutriated fraction. Among the methods used for the isolation and detection of homogeneous populations of cells, several flow cytometric methods are generally accepted. As its introduction, the PI flow cytometric assay has been widely used for the evaluation in different cellular processes in animal models [14] and plant cells [34].
2. Determine the DNA content by fluorometry (*see* Subheading 3.3).
3. Measure simultaneously the cell number and the increasing cell size. This option offers the fastest measurement and is recommended when many fractions are collected and several measurements have to be carried out within a short period of time. A reliable combination is to choose at least two of the three options.

3.11 Synchronized Apoptotic Cells Detected by Forward Scattering

The estimation of cellular DNA degradation during programmed cell death is closely related to the particle size that can be amplified and shows more convincingly the decrease in the cell size of apoptotic cells (Fig. 7a). The quasi 3D representation of forward light scatter analysis in Fig. 7b shows the progression of gamma irradiation-induced apoptosis during 24 h from elutriated fractions 1–9. By means of flow cytometric data, the DNA content of distinct apoptotic cell fractions was estimated and found to be increased from ~0.05 to ~1 C-value [35]. Flow cytometric forward scatter was used to determine the proportion of apoptotic cells relative to unirradiated ones after 24 h incubation. Apoptosis was low in unirradiated cells (~1%) and increased significantly in irradiated cells from 5% to 13–14% (Fig. 7c). To estimate the average value of apoptosis, non-elutriated control populations were also subjected to γ-irradiation and grown in culture medium for 2 and 24 h, respectively. Not only the increase of apoptotic cells from 4.8% to 15.6% were seen in the sub-G_1 marker window, but also the increase in G_1/G_0 and G_2/M phase and the decrease of S phase cells were observed (Fig. 7d). These changes also confirmed the

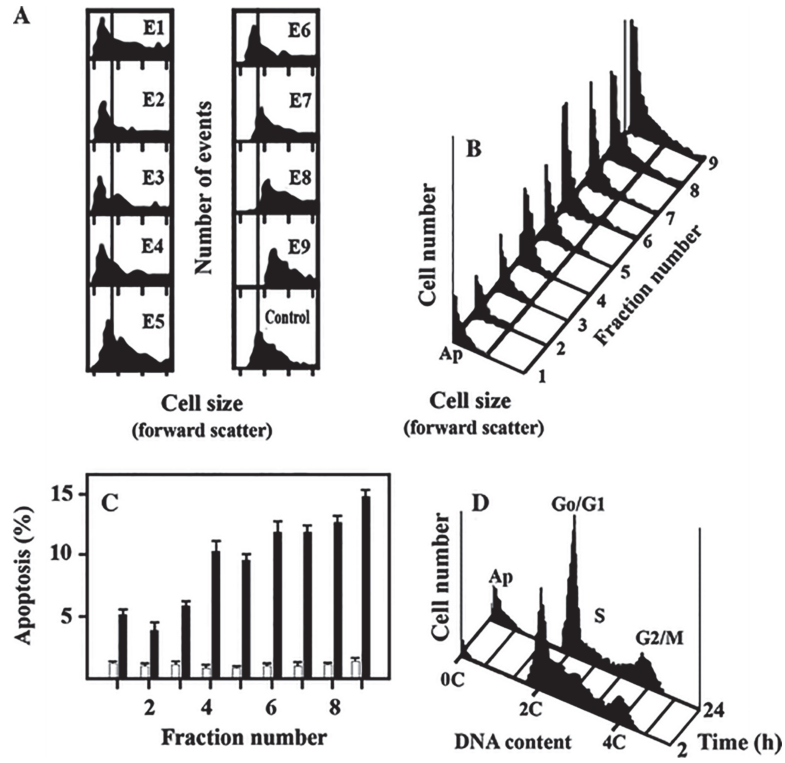

Fig. 7 Apoptotic changes detected by the forward scattering during the cell cycle. Gamma irradiated K562 human erythroleukemia cells subjected to elutriation followed by propidium iodide staining and forward light scatter analysis and found as shrinked cells in the sub-G_1 marker window. (**a**) To follow the apoptotic cell size, a vertical line was placed on the main peak of the irradiated, non-elutriated control. (**b**) After irradiation and incubation for 24 h, apoptotic cells were detected by forward scatter analysis. (**c**) Percentage of apoptotic cells of non-treated (*open square*) and of γ-irradiated (*filled square*) cell fractions. (**d**) Gamma-irradiated cells from non-elutriated populations were cultured at 37 °C. One aliquot of cells was incubated for 2 h, the other for 24 h, then stained with PI and was subjected to forward light scatter analysis. The C-values and major phases of the cell cycle are indicated. *Ap* apoptotic cells. Reproduced with permission of Banfalvi et al. [35]

radiation-induced cell cycle arrest near the G_1/G_0 and at the G_2/M checkpoints [35]. Cells representing different stages of apoptosis can be collected by choosing a somewhat lower centrifugal force for elutriation than for nuclei. By fine-scale elutriation, apoptotic cells can be separated from undamaged healthy cells (*see* **Note 18**). Cold-shock-induced apoptosis has been used without the addition of toxic compounds or antibodies, to synchronize homogeneous apoptotic cell populations [36].

4 Notes

1. *Dead cells*. Avoid the presence of dead and fragmented cells in elutriated fractions. The cell culture has to be kept constantly in logarithmic growth. Best results are obtained when cells are recultivated every day.
2. *Installation of stroboscope*. Make sure that the cables run close to the wall of the centrifuge so that the rotor cannot touch them. In case the photocell of the stroboscope does not work replace the flash lamp.
3. *Placing rotor in the centrifuge*. Be sure that the rotor is properly seated. If the pin of the rotor is improperly resting on one of the castellations, it will lift off when the drive starts. The centrifuge should be perfectly balanced and run smoothly. If you notice any vibration, the balance is imperfect. When the speed of the centrifuge is fluctuating, flow forces are not precisely balanced against centrifugal force to allow proper segregation. Check speed constancy and find the balance between centrifugal force and flow rate in preliminary test runs.
4. *Leaks*. These usually occur when (a) the chamber gasket is put backward; (b) the chamber screws are not tight; (c) tubings and connectors are not properly fitted; (d) there are nicks, scratches, irregularities on O-rings; and (e) permanently lubricated sealed bearings are washed with detergents, which can leach out the lubricant, resulting in a shortened bearing time.
   - *Seal inspection*. Clean the stained stationary seal housing (made of alumina) with soap and water or mild detergent. Abrasive materials should be used neither for cleaning nor for elutriation. Reassemble the bearing assembly (note that the seal mount has a left-handed thread). The seal should be replaced when any damage is visible.
   - *Cross leakage*. The most common problem in centrifugal elutriation is caused by defective or missing O-rings in the shaft assembly. Frequent disassembling and assembling of the elutriator system may result in defective O-rings and leaks. Make sure that all O-rings on the seal screw and transfer tube are in right place, lightly greased. Note, however, that overgreasing may clog the system. Once the subassemblies, as well as the flow system have been assembled and the system proved to be completely sealed without any leakage in the preliminary run, it is not recommended to disassemble it after each elutriation.
   - *High back pressure*. Insufficient spring pressure, which holds the rotating seal against the stationary seal, can be overcome by the back pressure, causing cross leakage. But the most common causes of high back pressure are the

opening of liquid lines in the wrong direction and inadequate de-aeration.

5. *Rotor speed*. As the flow rate relative to rotor speed can be regulated on a much broader and finer scale, it is recommended that during elutriation the rotor speed is held constant rather than using decreasing rotor speed and constant flow rate.
6. *Oscillations in rotor speed*. To avoid temporary changes in rotor speed, constant electric voltage (110 or 220 V) can be secured by plugging the centrifuge to a stabilizer.
7. *Manometer*. Keep the manometer under constant observation at the beginning of elutriation to avoid pressure formation. In the elutriation system, the presence of bubbles is indicated by the sharp increase of the pressure. In such cases, quickly turn off the speed of the centrifuge, which will drive out the bubbles. Repeatedly lower the speed of the centrifuge to remove all the bubbles before elutriation.
8. *Tightness of tubing*. Do not make the connection between the tubing and the rotor too tight; leave some movement for the tubing. As the speed of the elutriation rotors is not high (maximum 6000 rpm, $5080 \times g$), vacuum in the centrifuge is not required and could not be maintained due to the liquid lines and electric cables.
9. *Removal of bubbles*. Be sure to fill all parts of the flow harness with liquid until all the bubbles have been removed. Preworm the solutions and the centrifuge to the temperature where elutriation will be performed with the exception of the cells before their loading and the collected fractions which are kept in ice.
10. *Loading cells*. During loading cells into the elutriation chamber, one can line up cells exactly at the boundary inside the elutriation chamber (Fig. 1a): (a) a small increase of the flow rate will push forward the cell population to the boundary; (b) a small decrease of the flow rate will bring the population back to the boundary. Alternatively, the rotor speed could also be changed, but has an opposite effect as changing the flow rate. Such corrections during elutriation are recommended only for advanced users of the system. Basic principle: Do not change conditions during elutriation.
11. *Pellet formation*. Those particles that are not driven out from the separation chamber by the pumping will be pelleted at the entrance of the elutriation chamber as soon as the peristaltic pump stops. The peristaltic pump should not be turned off during elutriation. To remove the sticky pellet, the elutriation chamber has to be disassembled and cleaned.
12. *Damaged cells*. These cells release DNA and tend to stick together. Clumping of cells leads to nonhomogeneous cell populations especially at the final stage of elutriation. Solution:

Avoid rapid pipetting and fast resuspension of pelleted cells, which results in a significant loss of cell recovery, viability, release of chromosomal DNA, and stickiness of damaged cells. We have avoided DNase and EDTA treatment by the gentle treatment and loading of cells.

13. *Cell loss during elutriation*. The possible reasons are as follows: (a) cells are lysed (the system was not properly set up, check solutions for hypotony); (b) cells were left in the mixing chamber; (c) overloading the elutriation chamber results in the loss of smaller (G0/G1 and early S phase) cells; (d) the size gradient was not balanced and cells were not loaded in the elutriation chamber; (e) the centrifuge was stopped or decelerated. The particles concentrate near the increased cross-sectional area of the chamber. Flow velocity is greatest adjacent to the entrance port, the particle concentration is reduced in this area. This effect can be reduced by lowering the centrifugal force and the correspondent flow rates.
14. *High resolution of elutriation*. The danger of collecting too many elutriated fractions is that they cannot be processed. High resolution of elutriation needs the processing of many samples within the same day. Careful planning, the involvement of 2–3 persons in this highly coordinated work, some technical skill and experience are needed.
15. *Unable to judge*. This problem almost always arises when elutriation has not been done before. Consult the JE-6 or JE-5 instruction manual carefully before doing any or further installation. Carry out your first elutriation with an expert and/or discuss the details. Clarify the condition of your rotor by calling your nearest service office and ask for inspection.
16. *Application of density gradient*. Introduction of a density gradient would require a second reservoir. It is not recommended as a second reservoir would make the system too complex to handle. Growth medium with minimum FBS (1%) maintains cell viability and is dense enough to avoid contamination.
17. *Coriolis jetting effect*. Turbulence of particles inside the elutriation chamber can be minimized by increasing the flow rate slowly from fraction to fraction. The collection of many fractions (i.e., small increment in flow rate) also reduces turbulence and contributes to the increased resolution power of elutriation. A small shift (1–2 fractions) in the elutriation profile can be attributed to minor differences in cell growth. This difference can be reduced by strict cell culture conditions and corrected by calculating the C-value (haploid genome content) of each elutriated fraction from the corresponding flow cytometric profile [24].

18. The fine-scale synchronization of apoptotic cells needs specific elutriation conditions to be adapted to cell type and apoptotic treatment.

Acknowledgement

This work was supported by the OTKA grant TO42762 (G.B.).

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http://www.springer.com/978-1-4939-6602-8

Cell Cycle Synchronization
Methods and Protocols
Banfalvi, G. (Ed.)
2017, XV, 354 p. 71 illus., 14 illus. in color., Hardcover
ISBN: 978-1-4939-6602-8
A product of Humana Press