Optimizing Cell Separation with Beckman Coulter's Centrifugal Elutriation System

Introduction to Cell Separation Techniques

In many applications of cell biology, separation of cells from a heterogenous population to an enriched population of specific target cells is necessary to answer relevant scientific questions. At times, this is a difficult task as cells different in function often times are similar in size, morphology, and other physical traits. Development of cell sorting methodologies that are simple, fast, high throughput, and non-invasive are needed for a range of cellular applications including cell-based therapy and gene expression analysis.[1] Currently, the most common cell separation techniques involve separating populations based on cell size, density, electric charge, and antibody-dependent, magnetic or fluorescence activation.

Centrifugation is one of the most commonly used methods of cell separation, especially rate-zonal density gradient centrifugation, which relies on centrifugal force to sediment cells through a linear density gradient. The process is relatively time-consuming and small scale. Excessive time is required to set up the gradient, spin the cells, and fractionate after sedimentation. The scale is limited based on the number of centrifuge tubes and rotor type. Continuous flow centrifugation is often used to scale up the process but involves pelleting cells rather than isolating in a gradient, and is typically seen in large industrial processes such as vaccine development.

Magnetic- or fluorescent-activated cell sorting (MACS or FACS) is increasing in popularity but relies on cell labeling and transformation steps. A highly accurate technique, MACS or FACS typically utilizes antibodies conjugated to cell receptors which are then tethered to a fluorescent or magnetic marker for separation purposes. Cell sorters are expensive pieces of equipment, and although efficient, require manipulating cells and long sample preparation times.

Free-flow electrophoresis (FFE) uses similar principles as both capillary electrophoresis and gel electrophoresis but is a relatively unused technology, especially for cell separation. Pressure is applied to a sample to drive flow through a separation chamber. Simultaneously, an electric field is applied perpendicular to the direction of flow, directing particles of varying charge to separate isolation chambers. FFE is a higher throughput technology than rate-zonal centrifugation and MACS or FACS, but is limited in separation capabilities for most cell types as variance in cellular electric charge is miniscule.

A less common but highly effective technique called centrifugal elutriation can also be used to isolate cells of interest. In elutriation, a sample of heterogenous cells is passed into a triangular-shaped chamber embedded in a centrifuge rotor/chamber while the rotor is being spun. Centrifugal force pushes cells away from the wider end of the chamber, whereas counterflow produces an opposing force toward the smaller end with sedimentation toward an inlet located at the wider end of the chamber (Figure 1). Higher throughput than many other techniques, centrifugal elutriation can achieve high-resolution separations after optimized methods. The technique is quite inexpensive compared to others in that the major cost after initial equipment costs is cell buffer, as opposed to antibodies and magnetic beads required in MACS or FACS. Furthermore, cell modification and/or manipulation is low, as cell morphology and viability has been shown to be similar before and after separation using elutriation.[2]

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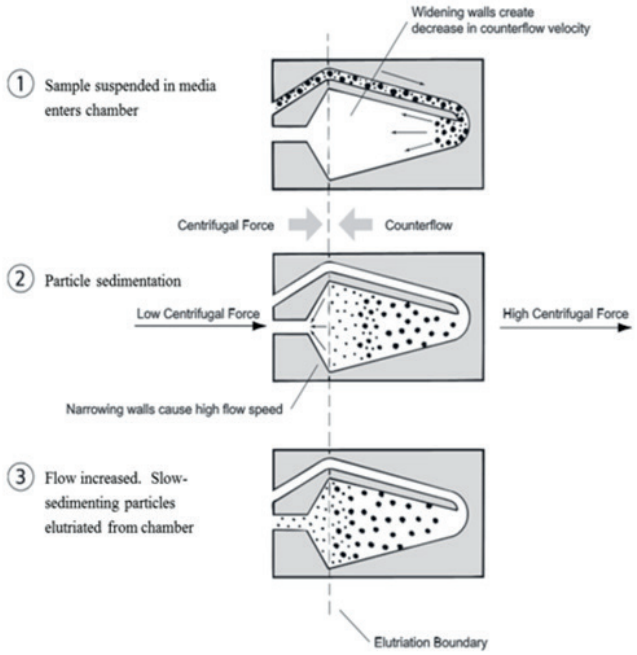

Fig. 1. Principles of elutriation. Centrifugal counterflow elutriation separates particles based on density and size. Centrifugal force opposes force generated from a flow pump which pushes cells and other particles out of the chamber and into a collection flask. By varying flow and/or centrifugal force, separations can be achieved.

Centrifugal Elutriation

The principle of elutriation has been around for hundreds of years, mainly seen in industrial applications; however, centrifugal elutriation is a historically newer process. Described in 1948 by Per Eric Lindahl,[3] the original idea was termed "counterstreaming centrifugation." Dr. Lindahl was characterizing differently sized yeast cells and proposed the elutriation technique to manage heterogeneous cell populations. In the early 1960s, Beckman Coulter, Inc. began work to develop a simplified device that could readily be used in research labs around the world. Modification of several designs led to the evolved elutriation system called the JE-6, which contained one separation chamber and a counterbalance on the opposite side. In this model, the chamber is visible through a centrifuge window, transparent chamber materials, and a synchronized strobe light which allows researchers to monitor cell transport during a run. A modified chamber was devised years later by Richard Sanderson (deemed the Sanderson chamber) who determined mathematically and then experimentally that an extra taper layer for cells to become trapped would help in separating physically similar cells (Figure 2).

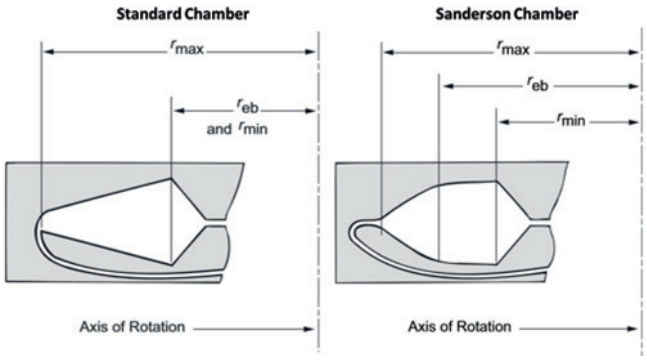

Fig. 2. Beckman standard versus Sanderson chamber. The Sanderson design has a second tapered layer which allows for cells of similar size to be better separated.

Researchers have discovered several applications over the years[4-6] and the technique is continuing to evolve to meet research demands. Beckman Coulter centrifuges are also often evolving. To promote versatility, Beckman Coulter's JE-6B elutriator rotor is compatible with older high-performance centrifuge models such as the J2 and J6, whereas the JE-5.0 is compatible with the newer Avanti J-26 XP, Avanti J-26S XP, and J6-MI centrifuges.

Mathematical Modeling for Cellular Separation

Many variables determine the sedimentation of cells under a centrifugal force and flow rate inside an elutriation chamber: 1) the g-force provided by the centrifuge; 2) the counterflow velocity; 3) the diameter of cells in solution; 4) the densities of both the cells and the elutriation buffer; and 5) the cross-sectional area of the chamber. Stokes' Law does the best at explaining the relationship between these variables.

[EQ.1]

$$SV = \left(\frac{d^2(\rho_p - \rho_m)}{18\eta}\right)\omega^2 r$$

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; r = radial position of the particle; and ω = angular velocity in radians/second. The two terms in the above equation, dictated by the properties of the cell, include the density and diameter of the cell. It is apparent that since the diameter variable is raised to the second power, it contributes more significantly to sedimentation velocity than the particle

density. Furthermore, since there is little variation in cell population density, counterflow centrifugal elutriation separates almost solely on the diameter or size of cells.

In counterflow centrifugal elutriation, particle sedimentation in a radial direction is balanced by the velocity of fluid flowing in the opposite direction. The flow velocity (V) at any point is equal to the flow rate (F), divided by the cross-sectional area at that point (A), V = F/A. Since the flow rate is the same at every point in the chamber, only changes in the cross-sectional area produce changes in the flow velocity. Thus, at chamber positions with small cross-sectional area (for example, at r_{max}), flow velocity is highest, and vice versa. Through chamber design, a velocity gradient is formed in the elutriation chamber using constant flow.

In a similar manner, a gradient in centrifugal force is introduced along the radial direction of the chamber, as centrifugal force is related to the rotor radius or distance from the center of the rotor. At r_{max}, the force of centrifugation is greatest; however, the flow velocity is also greatest at this point as the cross-sectional area of the chamber is smallest. As we move closer to the center of the rotor, both the centrifugal force and flow velocity decrease as *r* is shortened and *A* increases across the chamber, respectively. When the opposing forces are equal, the system is said to be in equilibrium—a state where smaller cells stay at rest near the elutriation boundary (closest to the center of the rotor) and larger cells remain stationary near the flow inlet (r_{max}) (Figure 1). Thus, separations are the result of cells of different sedimentation velocities being in equilibrium at different radial positions in the chamber. When the flow rate is increased (or the speed is decreased), cells that were in equilibrium near the elutriation boundary are washed out of the chamber first and the distribution of cells at equilibrium shifts toward the center of rotation.

Deriving Stokes' Law with normal cellular run conditions (assuming that $\rho_p - \rho_m = 0.05$ g/mL, $\eta = 1.002$ mPa/s), a relationship between flow rate *F*, cell diameter *d*, and centrifugal speed (*RPM*) can be expressed as:

$$F = Xd^2\left(\frac{RPM}{1000}\right)^2 \quad \text{[EQ.2]}$$

where, *F* = flow rate and *X* is a constant reflective of the geometry of the chamber. Chamber constants for various Beckman Coulter, Inc. elutriation chambers can be found in Table 1. Using this equation, a chart called a nomogram (Figure 3) allows you to determine flow rate and speed combinations at which cells of a given size will either be retained or swept out of the chamber.

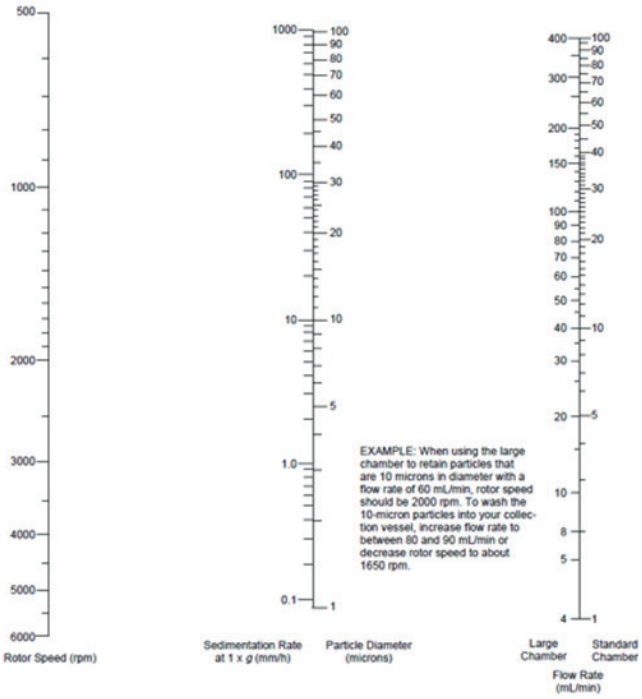

Fig. 3. Rotor speed and flow rate nomogram. Use a straight edge to connect flow rate and rotor speed so that the line intersects the particle diameter axis at a point corresponding to the smallest, lightest particles to be retained for the specific chamber of use. For example, to retain all particles 10 microns and larger in the large chamber at a rotor speed of 2,000 rpm, a flow rate less than 70 mL/min. should be used. To collect the particles, either increase the flow rate or decrease the speed below 2,000 rpm.

Table 1. Chamber Constants.

Chamber Type	X, Chamber Constant
40 mL large chamber	1.73×10^{-1}
4 mL standard chamber	5.11×10^{-2}
5.5 mL Sanderson chamber	3.78×10^{-2}

Run Procedures

1) Prepare buffers and cell solutions. Determine the appropriate number of cells to introduce into the rotor at one time using Table 2. With the standard and Sanderson chambers, the volume of buffer normally required for separation is approximately 100 mL times the number of fractions to be collected. With the large chamber, the volume of buffer normally required is 1,000 mL times the number of fractions. Be sure to prepare enough extra buffer to flush the system at the beginning of a run.

2) Assemble rotor, tubing, buffer reservoirs and elutriation chambers as seen in Figure 4.

3) Consult the nomogram (Figure 3) or a published method using similar cell types and/or buffer system to determine the initial flow rate for elutriation. If an appropriate method cannot be determined, find an appropriate rotor speed on the nomogram (use less than 3,000 rpm for most cell types to avoid lysing). Locate the approximate size of the smallest particle in solution (as deemed by literature search or particle sizer, such as COULTER COUNTER or Beckman Coulter Vi-CELL). With a straight edge, connect the rotor speed with the particle size. The point at which the line crosses the flow rate column indicates the pump flow rate required to retain the smallest particle in the chamber.

4) Set initial flow rate such that the smallest desired particle or cell is retained.

5) Turn on the centrifuge and set the appropriate speed, time and temperature.

6) After the system is thoroughly flushed, load the cell sample into a syringe with a luer fitting and slowly press the plunger to inject the sample. After all cells are in the reservoir, turn injection valve back again. Subsequently, turn the bypass valve to allow buffer to wash the cells into the rotor. Other sample injection methods are available. Consult manual for more information.

7) Vary flow or rotor speed to elute cells out of the chamber based on application (see the following for example separations). Fractionate and collect samples of interest for downstream analysis.

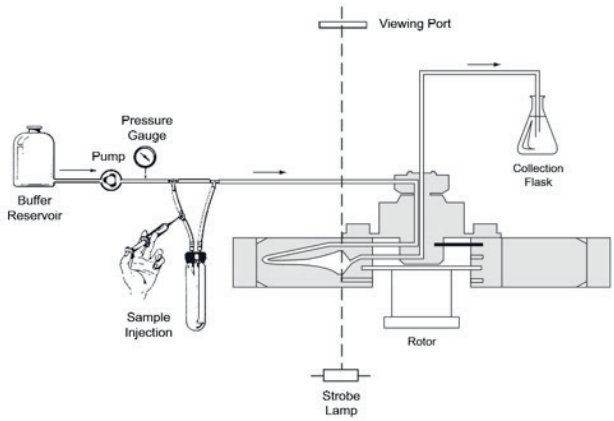

Fig. 4. Typical elutriation setup. A buffer supply is attached to a spinning elutriation chamber spanned by a pump, pressure gauge, and sample injection inlet. Sample is injected into reservoir and then pushed into elutriation chamber by buffer flow. A strobe lamp provides light for a researcher to view cells passing through a viewing port. Fractions are collected through outlet valve of elutriation chamber and recovered for subsequent use.

Table 2. Cell Introduction Specifications.

	Large Chamber	Standard Chamber	Sanderson Chamber
Sample volume: *Minimum* *Maximum*	2×10^8 cells 1×10^{10} cells	2×10^7 cells 1×10^9 cells	2×10^6 cells 1×10^9 cells
Amount of buffer required to elutriate one fraction	~500 to 1,000 mL	~75 to 250 mL	~75 to 250 mL
Air purge parameters: Pump rate (then spin rotor to 1,000 rpm)	200 mL/min.	20 mL/min.	20 mL/min.
Pump calibration	Up to 400 mL/min.	Up to 250 mL/min.	Up to 250 mL/min.

Example Separations

Let's take a look at a few specific examples using Equation 2 (see previous page) on how to separate particular cell populations.

Case 1

A researcher wishes to derive a set of elutriation parameters for the separation of two particular cell species in a heterogeneous mixture (e.g., lymphocytes and monocytes) derived from a Ficoll density gradient separation of peripheral blood. The gradient separation yields a mixture having the following components: 32% monocytes, mean cell diameter 9.0 µm; and, 68% lymphocytes, mean cell diameter 4.5 µm. The researcher wants to use PBS as the elutriation buffer and run the elutriation at a constant speed of 2,500 rpm in a 5 mL Sanderson chamber. The flow rates can be calculated as such:

$$F = 0.0378 * (4.5)^2 * \left(\frac{2500}{1000}\right)^2 = 4.78 \; mL/\text{min. } for \; lymphocytes$$

$$F = 0.0378 * (9.0)^2 * \left(\frac{2500}{1000}\right)^2 = 19 \; mL/\text{min. } for \; monocytes$$

By setting the initial buffer flow to 5 mL/min., the researcher will establish elutriation boundary conditions for the lymphocytes when they are loaded into the elutriation chamber. Increasing the flow rate by 1 or 2 mL/minute after the elutriation boundary is established will then wash the lymphocytes out of the chamber but retain cells whose diameter exceeds 4.5 µm. After collection of a 150 mL fraction of the 4.5 µm cells, the researcher will then increase the buffer flow rate to 20 to 21 mL/min. and collect a 150 mL fraction of the 9.0 µm monocytes.

Case 2

A second researcher wishes to separate 6.0 µm to 7.5 µm cells from a mixture with a range of cell sizes from 2.5 µm to 10.3 µm. By using the formulas for flow rate as in Case 1, at 2,500 rpm the flow rate for eluting 5.9 µm cells is 8.0 mL/min., and for 7.5 µm cells it is 13 mL/min. Therefore, the researcher loads at 8.0 mL/min. and discards a fraction of 150 mL. The buffer flow will then be increased to 13 to 14 mL/min. and a second fraction of 150 mL is collected. This fraction contains the 6.0 µm to 7.5 µm cells. What remains in the chamber is washed out by stopping the rotor and allowing the buffer flow to continue. This fraction is also discarded.

The effect of increasing rotor speed is to increase the range of flow rates required to elute cells of differing size. For example, to elute the same 6.0 µm to 7.5 µm cell population at 3,400 rpm would require flow rates of 16 mL/min. to 25 mL/min. (versus 8 mL/min. to 13 mL/min. for 2,500 rpm). This fact can be used to researchers' advantage if separating cells whose size differences are small; that is, the higher the rpm, the easier to resolve the 2 cell lines due to the greater difference in flow rate to wash the cells free of the chamber. However, higher speeds may cause damage to some cell types and cell shearing should be monitored.

Specific Case from Literature

In a recent article in the prestigious journal *Cell*, Tsubouchi described a method[5] for separating mouse embryonic stem cells into different cell cycle stages, adapted from Banfalvi.[7] A flow rate of 6 mL/min. and rotor speed of 1,800 rpm at 4°C was initially used during and after sample injection in a Sanderson chamber. Cell subpopulations were then eluted by gradually increasing the flow rate 1 mL/min. For one specific cell type, enrichment of cells at stages G1, S, and G2/M was achieved by setting flow rates at 8 to 9 mL/min, 12 to 14 mL/min, and 16 to 17 mL/min. Solving for diameter, *d*, in Equation 2, we can estimate the expected upper limit cell size of G1, S, and G2/M cell stages at 8.6 µm, 10.7 µm, and 11.8 µm, respectively. At least 150 mL was collected per fraction and the cells were subsequently analyzed for gene expression by quantitative RT-PCR, Western blot, immunofluorescence, and fluorescent *in situ* hybridization (FISH) revealing critical information related to epigenetic reprogramming.

Notes

1) For any individual protocol evolution, consideration must be given to ease of using the protocol, survival rates of cells subjected to constant handling, effects of time and elutriation buffer on cell functions, and so on. In general, the less the cells are handled and the less time they spend out of culture conditions, the better the cell survival rates and the less the normal cell function is disrupted. It is important to control all variables as closely as possible when confirming flow rate and speed parameters. For example, buffer temperature must be constant from reservoir to elutriation chamber if an accurate chamber temperature is to be derived for elution parameters. The centrifuge must maintain accurate temperature and speed for the rotor, and this should

be calibrated periodically. All air should be purged from the rotor before loading cells into the chamber (verified by a "0" reading on the in-line pressure gauge). Elutriation chambers and rotors must be kept clean and free of endotoxins and contamination. Pumps need calibration routinely to ensure accurate flow rates, and electronic particle counters and sizers need routine calibration to ensure proper readout.

2) The sedimentation velocity (SV) scale of the nomogram assumes that the sedimentation velocity of the cell was measured in a fluid having the same density and viscosity as the fluid being used for elutriation. If this is not the case, a more detailed formula can be used. Please refer to the rotor manual for this specific case.

3) When the particle diameter scale is used instead of the sedimentation velocity scale, flow rates from the graph may require adjustment, if the viscosity of the elutriation medium and the difference in density between the particle and the medium differ significantly from the assumptions made to construct the nomogram (1.002 mPa/s and 0.05 g/mL, respectively). Use Stokes' Law (Equation 1) to determine appropriate flow rate and speed or determine experimentally.

Summary

Centrifugal elutriation has been around for years and remains one of the most gentle and efficient techniques for separating cells based on size. Applications behind cell separation include, but are not limited to, cell cycle synchronization, removal of cellular debris, and enrichment of certain cell populations. Many biochemical assays require a substantial number of cells for downstream analysis. Generating large quantities of homogenous, live cells is critical to determining answers to many scientific questions. Often times, counterflow centrifugal elutriation is seen coupled with another sorting or analysis technique. In flow cytometry, for example, removal of cellular debris prior to analysis generates higher effective sorting speeds, increases efficiency due to coincident events, and decreases the potential for system clogging. Centrifugal elutriation, using Beckman Coulter's well-established line of centrifuges and rotors, offers ideal solutions to current difficulties associated with separating cells into homogenous populations for downstream analysis.

References

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