An integrated system for synchronous culture of animal cells under controlled conditions

Elena Mendoza-Pérez¹, Vanessa Hernández², Laura A. Palomares², and José A. Serrato¹
¹Departamento de Bioquímica, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, Mexico and ²Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Mexico.

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The cell cycle has fundamental effects on cell cultures and their products. Tools to synchronize cultured cells allow the study of cellular physiology and metabolism at particular cell cycle phases. However, cells are most often arrested by methods that alter their homeostasis and are then cultivated in poorly controlled environments. Cell behavior could then be affected by the synchronization method and culture conditions used, and not just by the particular cell cycle phase under study. Moreover, only a few viable cells are recovered. Here, we designed an integrated system where a large number of cells from a controlled bioreactor culture is separated by centrifugal elutriation at high viabilities. In contrast to current elutriation methods, cells are injected directly from a bioreactor into an injection loop, allowing the introduction of a large number of cells into the separation chamber without stressful centrifugation. A low pulsation peristaltic pump increases the stability of the elutriation chamber. Using this approach, a large number of healthy cells at each cell cycle phase were obtained, allowing their direct inoculation into fully instrumented bioreactors. Hybridoma cells synchronized and cultured in this system behaved as expected for a synchronous culture.
few milliliters with experimental limitations (22). As a consequence, the Zeng group (20,21) had to centrifuge cells and perform several repeated separations in order to obtain sufficient cells to inoculate bioreactor cultures of 250 mL.

Another issue when using centrifugal elutriation is chamber instability. In our experience, the pump traditionally used to introduce flow into the elutriation chamber is an important source of chamber instability when high cell concentrations and/or low centrifugal forces are used, reducing performance and the purity of the synchronization.

To overcome these limitations, we present here an integral system designed to introduce cells directly from an exponentially growing controlled bioreactor culture into the elutriation chamber, without centrifugation or further manipulation of the cells. A low pulsating peristaltic pump was used to increase chamber stability, with a consequent increase in separation efficiency and purity. The system integrates a fully-controlled minibioreactor culture where unsynchronized cells are grown, with an injection loop that allows the stress-free injection of the complete culture into the centrifugal elutriation chamber using the low pulsation peristaltic pump, where cells are separated based on their size and density. Cells are then introduced directly into minibioreactors, where synchronized cells in a specific cell cycle phase are cultured under controlled conditions. The system presented here allowed us to characterize the behavior of synchronized cells in the absence of confounding effects caused by stressful conditions during traditional separation methods.

Materials and methods

The murine hybridoma cell line BCF2 (23) was cultured in CD Hybridoma medium 1× (Cat No. 11279–023; Gibco/Life Technologies, Grand Island NY). Cell concentration and viability were measured in a hemocytometer by trypan blue (Cat No. T-0776; Sigma-Aldrich Co., St. Louis, MO) exclusion (2). Cell size was measured in a Coulter Multisizer 3 (Beckman Instruments, Brea, CA). Cultures were performed in 500 mL (unsynchronized) and 250 mL (synchronized) instrumented minibioreactors (Applikon Biotechnology, Delft, The Netherlands) with working volumes of 200–300 mL and 50–100 mL respectively, and agitated at 200 rpm or 130 rpm, respectively. All cultures were maintained at 37°C, pH 7.1 with a dissolved oxygen tension of 50% (with respect to saturation with air at 2410 m altitude). The concentrations of glucose, lactate, and glutamine were measured in a YSI 2900 biochemical analyzer (Yellow Springs Instruments, Yellow Springs, OH) according to the manufacturer’s instructions.

Cell cycle analyses were performed by measuring the DNA content of cells by flow cytometry. Briefly, cells were fixed and incubated in 70% ice-cooled ethanol for at least 1 h; after washing with cooled PBS, cells were incubated for 30 min in a mixture of RNAse and propidium iodide for RNA elimination and DNA staining, (Cat No. P4170; Sigma-Aldrich). A FACSCalibur cytometer (Becton Dickinson, San José, CA) was used (24). The relative abundance of cells in each cell cycle phase was estimated by adjusting DNA content data to mathematical models (Dean Jett Fox and Watson Pragmatic) specific for...
the cell cycle using the software Flow Jo (Tri Star Inc., Ashland, OR).

Design of the integrated system for synchronizing and culturing animal cells
The integrated system we designed is depicted in Figure 1. It is composed of four elements:

(i) Counter-flow centrifugal elutriator. Cells are separated in this device based on their size and density, which correlates with cell cycle phase. An ultracentrifuge J2–21 equipped with a JE-6B rotor (both from Beckman Instruments) with a standard elutriation chamber. Banfalvi (25,26) and the manufacturer of the ultracentrifuge and rotor provide a detailed description of the elutriator chamber and its operation. Cells are separated in the elutration chamber according to their sedimentation rate under a countercurrent flow. Each cell will be positioned where its sedimentation rate is in equilibrium with the countercurrent flow. Larger cells will be positioned at the chamber entrance and smaller cells at the elutration boundary (chamber exit). By increasing the countercurrent flow, cells cross the elutration boundary and exit the chamber allowing fractions containing homogeneous cells to be collected. A digital peristaltic pump with low pulsation (Gilson Inc., Villiers le Vel, France) that delivers a flow of elutriation buffer or culture medium through the elutriation chamber was used. Flow was changed as needed. Equation 1 represents the simplified Stokes’ law formula, which was used to calculate either the flow rate at which cells of particular diameters are elutriated out of the elutration chamber or the cell diameter of cells elutriated at a particular flow rate:

\[
F = Xd^2 \left( \frac{rpm}{1000} \right)^2
\]

[Eq. 1],

where \( F \) is the flow rate (mL/min), \( X \) is a chamber constant (dimensionless), \( d \) is the cell diameter, and \( rpm \) is the rotor speed. Dorin (Technical paper T-1785A. Developing elutriation protocols. Beckman Instruments) provides detailed procedures for calculating the required flow rate as well as the values of the chamber constants. In this work, a standard chamber with a constant of 5.11 \( \times 10^{-2} \) was used.

(ii) Cell injector. An injection loop was connected to an array of four three-way polycarbonate valves using Luer lock connections. The injection loop is a cylindrical 300 mL glass column with a conical bottom. The column has at its top part a venting port and an entry fluid port through which unsynchronized cells or elutriation buffer are introduced. An exit port is located at the bottom of the column. The cell injector allows the direct introduction of cells growing in a controlled bioreactor into the elutriation chamber, without the stress and cell damage often observed when cells are centrifuged and introduced into the system with a syringe, as is common practice (27).

(iii) Bioreactor for the culture of synchronized cells. A 250 mL bioreactor is used to receive and culture synchronized cells. If desired and available, several bioreactors can be set up for the collection of fractions at different cell cycle phases. The working volume of bioreactors depends on the population that is selected for further characterization and is normally 50–100 mL.

The system was set up and operated in a laminar flow hood under sterile conditions. The laminar flow hood was modified to allow a direct connection with the centrifuge and a direct flow into and from the rotor.

Results and discussion
Cell growth kinetics of BCF2 hybridoma cells were characterized to determine the best timing and conditions for separating cells according to their cell cycle using our system. Cell growth in the controlled bioreactor occurred at a maximum growth rate of 0.031 ± 0.001 h\(^{-1}\). A concentration of 1.5 \( \times 10^{6} \) cells/mL\(^{-1}\), typically obtained after 96 h of culture and a viability > 90% were set as the criteria for harvesting unsynchronized cells from the bioreactor for cell separation. A typical cell-size distribution obtained from harvested cells is shown in Figure 2. Cell-size distributions of unsynchronized cells ranged from 12 to 18 µm.

The system we designed was used to introduce the cells into the elutration rotor under aseptic conditions. The use of a controlled and monitored bioreactor culture ensured that cells were under balanced growth conditions, exponentially growing, and without nutrient limitations. Cells to be introduced into the elutration chamber are expected to be homogeneous as a result of balanced growth. The use of a bioreactor culture also provided sufficient cells to conduct further experiments after their synchronization. To characterize the separation of an unsynchronized population of BCF2 hybridoma cells into their corresponding cell cycle phases, 3 \( \times 10^{6} \) cells in a 200 mL volume were transferred from the 500 mL bioreactor into the cell injector and thus into the centrifugal elutriator.

Increasing the flow rate as indicated in Table 1, 19 cellular fractions were collected. Each fraction had cells with diameters within 0.3 µm, in a range of 12.9

![Figure 2. Typical cell-size distribution of the murine hybridoma cell line BCF2 in exponential growth.](image-url)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Recovered cells (%)</th>
<th>Flow rate (mL/min)</th>
<th>Diameter* (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7</td>
<td>19–20</td>
<td>12.9–13.2</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>20–21</td>
<td>13.2–13.5</td>
</tr>
<tr>
<td>3</td>
<td>2.7</td>
<td>21–22</td>
<td>13.5–13.8</td>
</tr>
<tr>
<td>4</td>
<td>5.7</td>
<td>22–23</td>
<td>13.8–14.1</td>
</tr>
<tr>
<td>5</td>
<td>6.3</td>
<td>23–24</td>
<td>14.1–14.4</td>
</tr>
<tr>
<td>6</td>
<td>9.7</td>
<td>24–25</td>
<td>14.4–14.7</td>
</tr>
<tr>
<td>7</td>
<td>7.0</td>
<td>25–26</td>
<td>14.7–15.0</td>
</tr>
<tr>
<td>8</td>
<td>4.3</td>
<td>26–27</td>
<td>15.0–15.3</td>
</tr>
<tr>
<td>9</td>
<td>4.0</td>
<td>27–28</td>
<td>15.3–15.6</td>
</tr>
<tr>
<td>10</td>
<td>5.7</td>
<td>28–29</td>
<td>15.6–15.9</td>
</tr>
<tr>
<td>11</td>
<td>3.7</td>
<td>29–30</td>
<td>15.9–16.2</td>
</tr>
<tr>
<td>12</td>
<td>4.0</td>
<td>30–31</td>
<td>16.2–16.4</td>
</tr>
<tr>
<td>13</td>
<td>2.7</td>
<td>31–32</td>
<td>16.4–16.7</td>
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<td>2.3</td>
<td>32–33</td>
<td>16.7–16.9</td>
</tr>
<tr>
<td>15</td>
<td>3.0</td>
<td>33–34</td>
<td>16.9–17.2</td>
</tr>
<tr>
<td>16</td>
<td>4.7</td>
<td>34–35</td>
<td>17.2–17.4</td>
</tr>
<tr>
<td>17</td>
<td>2.7</td>
<td>35–36</td>
<td>17.4–17.7</td>
</tr>
<tr>
<td>18</td>
<td>2.7</td>
<td>36–37</td>
<td>17.7–17.9</td>
</tr>
<tr>
<td>19</td>
<td>1.3</td>
<td>37–38</td>
<td>17.9–18.2</td>
</tr>
</tbody>
</table>

* Calculated with Equation 1.
to 18.2 µm. Of the total cells introduced into the system, 77% were recovered. The DNA content of cells in each fraction was determined (Figure 3A). While an exponentially growing unsynchronized culture had approximately 45% of cells in the G1/G0 phase, 40% in the S phase, and 15% in the G2/M phase, elutriated fractions contained up to 90% of cells synchronized at a specific cell cycle phase with high viability. The first fractions, with the smaller size range, contained cells mainly in the G1/G0 cell cycle phase. Cells of the sub-G0 population were detected in the first two fractions. As larger cells were recovered, a higher DNA content per cell was detected, corresponding to cells in the S phase and, finally, cells in the G2/M phase.

Figure 3B shows the population distribution obtained in each fraction. Fractions 2, 3, and 4 contained cells from the G1/G0 phase at a high purity. Fraction 8 and 9 contained mostly cells from the S phase, while fractions 18 and 19 contained mostly cells at the G2/M phase.

Cells from specific fractions were used to seed the second bioreactor with synchronized cells. To demonstrate the utility of the proposed system, a culture performed with cells from Fraction 2, corresponding to cells in the G1/G0 phase (Figure 3B) is shown in Figure 3C, D, and E. As can be observed in Figure 3C, the use of an instrumented and controlled bioreactor allowed tight control of pH and dissolved oxygen, providing the optimal environment for cell growth of synchronized cultures and preventing the effects of changing conditions. Its growth kinetics is shown in Figure 3D. As expected for a synchronized culture, stepwise growth was observed for two cycles (18,28). Cell concentration remained constant for 22 h. Cell concentration then increased for the next 4 h. At 26 h, the cell concentration had doubled from 0.4 × 10^6 to 0.8 × 10^6 cells·mL⁻¹. The doubling time of the culture was between 22 and 24 h, similar to that observed in unsynchronized cultures. Viability remained >90% during this time. As can be seen in Figure 3E, glucose and glutamine concentrations remained above their limiting concentrations during the culture, with specific consumption rates of 0.22 and 0.11 pmol·cell⁻¹·h⁻¹ respectively, similar to those reported for the same hybridoma cell line during exponential growth in asynchronous cultures (29–31). Interestingly, in respect to lactate accumulation, the synchronous culture had a high specific lactate production rate of 0.63 pmol·cell⁻¹·h⁻¹, 60% higher than the production rate observed in exponentially growing asynchronous cultures (31). In terms of the yield of lactate from glucose, synchronized cells had a higher yield (2.9 mol/mol) in comparison with asynchronous cultures (1.6 mol/mol), exceeding the maximum theoretical ratio of 2 moles of lactate produced per mole of glucose consumed, indicative that other carbon sources, such as glutamine or alanine, were catabolized into lactate.

Here, we present an integrated system for the separation and controlled culture of synchronized cells according to their cell cycle phases. Our system allowed the use of a controlled and monitored bioreactor...
culture in conjunction with an injection loop to directly insert cells into an elutriation chamber, without the use of intermediate procedures that can damage cells, and the direct transfer of the synchronized cells of interest into a second controlled and instrumented bioreactor. This strategy yielded sufficient cells to perform a bioreactor culture of synchronized cells, which were seeded directly from the elutriation chamber without further manipulation. As a proof of concept, a synchronous cell culture that started with G1 cells was performed. Its growth kinetics had two phases, as would be expected from a synchronous culture. In comparison with exponentially growing cells, a similar metabolic behavior for glucose and glutamine consumption was observed; however, that was not the case for lactate production; synchronous cultures exhibited a higher lactate production rate and yield.

A tight control of culture conditions was possible in both the seeding and harvesting bioreactors.

The integrated system presented here had several advantages over the common method for separation of cells by centrifugal elutriation. The use of an injection loop allowed the separation of cells in a single step, without the need to centrifuge cells or perform multiple elutriation separations. Moreover, cells were not stressed, as no centrifugation was performed. The time needed to introduce cells into the separation chamber from the well-controlled seed bioreactor was greatly reduced, further reducing stress. A highly stable elutriation chamber operation increased stability, efficiency, and purity. The system presented here allowed for the first time the integration of controlled cultures in bioreactors with a non-deleterious cell synchronization method to readily perform synchronous culture of animal cells and further experimentation under tightly controlled and monitored conditions.

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Competing interests

The authors declare no competing interests.

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Address correspondence to José A. Serrato, Departamento de Bioquímica, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Calzada de Talpan 4502, Colonia Sección X VI, C.P. 14080 Del. Talpan, D.F. E-mail: serratorjm@gmail.com

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