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ABSTRACT

Understanding the molecular and biochemical basis of cellular growth and division involves the investigation of regulatory events that most often occur in a cell-cycle phase-dependent fashion. Studies examining cell-cycle regulatory mechanisms and progression invariably require cell-cycle synchronization of cell populations. Thus, many methods have been established to synchronize cells at specific phases of the cell cycle. Several of the common methods involve pharmacological agents, which act at various points throughout the cell cycle. Because of adverse cellular perturbations resulting from many of the synchronizing drugs used, other synchrony methods that involve less perturbation of biological systems, such as serum deprivation, contact inhibition, and centrifugal elutriation have a significant advantage. The advantages and disadvantages of these cell synchronization methods are discussed in this review.

INTRODUCTION

Gene expression, translation, and post-translational modifications occurring in a cell-cycle-dependent manner are crucial for the regulation of cell-cycle progression. Using synchronous populations of cells from distinct cell cycle phases allows for the study of molecular and biochemical events and their consequences during cell division. Examples include the inactivation of the retinoblastoma tumor suppressor protein (pRB) by hyperphosphorylation at the restriction point in late G_1 (4,36), expression of cyclin A at the late G_1 to S phase transition (64,65), and the onset of cyclin B/Cdc2 activity at the G_2 to M phase transition (12,13,66).

Pharmacological agents that arrest cells at specific phases are commonly used for synchronization purposes. For instance, the prevention of DNA initiation at origins of replication by mimosine results in an early G_1 arrest (37,44). Release of early G_1 arrest by the removal of mimosine has been cited to elevate p53 and p21 protein levels; therefore, these findings should be taken into account when using this drug for synchronization (26). Arrest in early G_1 is also achieved by treatment with lovastatin, a mevalonate synthesis inhibitor (20,25,30,35); however, lovastatin has recently been demonstrated to effectively induce apoptosis in a variety of cell lines (27,49,53,55). Hydroxyurea or thymidine treatment arrests cells in S phase by targeting ribonucleotide reductase (56). When studying later cell-cycle phases, drugs used to synchronize cells from the G_2/M phases can be advantageous. Microtubule-disrupting agents, such as nocadozole and colcimid, arrest cells in M phase (41). Following their removal, these drugs provide synchronous cell populations, but metabolic perturbations and toxicity are likely to occur. The manipulation of cells using synchronizing agents has been demonstrated to causally promote side effects, such as dissociation of nuclear and cytoplasmic cell-cycle processes, disruption in the metabolic state of the cell, and cell death (32–34,51).

In contrast, several drug-independent methods have been established for synchronizing cells from asynchronous cell populations. Among these methods include G_0 quiescence by serum deprivation (50,57), isolation of early G_1 cells by cell contact inhibition (7,9), and centrifugal elutriation of cells in any phase of the cell cycle. Mitotic shake-off is also a nondisruptive method of isolating mitotic cells (2,19,24). Monolayer cells entering mitosis group together and become loosely attached to the dish so that they can be isolated by shaking the dish. Medium is subsequently transferred to another dish, and cells are allowed to attach upon G_1 entry. However, the limitation of this method is that the yield of cells is very low and, thus, cannot be used for several types of experiments. Because of potential adverse cellular consequences of synchronizing cells by pharmacological agents, we have chosen to focus on three cell synchrony methods involving minor cell manipulation: serum deprivation, contact inhibition, and centrifugal elutriation. However, when choosing a particular cell synchronization method, cell type, the cell-cycle phase/event to be studied, the doubling time of the cell cycle, and the duration of each cell-cycle phase should be considered.

SERUM DEPRIVATION

The transition between G_0 quiescence and early G_1 is regulated by growth-stimulatory and growth-inhibitory factors present in the extracellular environment. Control of the G_0 quiescence-early G_1 transition is, in part, mediated through mammalian D-type cyclins that are upregulated in the presence of growth factors and facilitate early G_1 progression (42,62,61). Cell-cycle exit into G_0 quiescence can thus be achieved by removing serum, which contains mitogenic factors from the cell culture medium (1,39). Cell-cycle synchrony is achieved following the addition of serum back to the medium to stimulate cell-cycle entry into the early G_1 phase. However, the effectiveness of this method relies on the susceptibility of cells to exit into G_0 quiescence following the serum withdrawal. G_0 quiescence is often difficult to achieve in transformed cells, but primary and immortalized cells may exhibit decreased viability in low-serum conditions. It must be noted that serum deprivation induces cell exit into the G_0 compartment, which results in the transcriptional repression of several cell-cycle regulatory genes, such as cyclins and cyclin-dependent kinases (Cdks) (43). Therefore, entry into the G_1 phase of the cell cycle from G_0 quiescence does not necessarily reflect the G_1 progression following the completion of the M phase, which would be applicable for studying the early G_1 phase of cycling cells, such as tumor cells. Factoring in the eventual loss of cell synchrony as cells progress through the later phases of the cell cycle is also of importance.

To achieve a G_0 arrest, cells are seeded at subconfluent conditions in high serum-containing medium (5%–10% serum). Following 18–24 h, cells are washed multiple times with PBS, and medium containing low serum amounts is added back to the cultures (60,68). The exit into G_0 quiescence by the majority of cells requires different periods of time in reduced-serum conditions, depending on cell type. For example, G_0 quiescence is achieved by NIH3T3 cells when they are maintained in the presence of 0.2% serum for 24 h (60). Studies have demonstrated that G_0 quiescence occurs in at least 95% of diploid fibroblasts (68) within 48 h of being in 0.1% serum; however, longer periods (48–72 h) under serum deprivation conditions are often required for many cell types. To determine the percentage of cells exiting quiescence and progressing through the cell cycle, cells entering S phase can be monitored by BrdU incorporation (5,17) or staining with propidium iodide for DNA content, followed by analysis using a flow cytometer (58,59,67). Distinguishing quiescent cells from G_1 cells can be achieved by examining Cdk4:cyclin D activity (15) and the proliferation marker Ki67 (30).

It is important to note that, when G_0 quiescent cells enter early G_1 upon serum stimulation, synchrony occurs only to a certain degree depending on the cell type. Not all cells enter into early G_1 from G_0 quiescence, and those cells that do enter early G_1 do not necessarily progress through the cell cycle at the same rate (Figure 1A) (14,45). In fact, variability of cell-cycle kinetics is inherent from cell to cell and occurs to some extent using any synchronization method. After the addition of serum back to the medium, a significantly greater number of normal cells in comparison to tumor cells may remain behind in G_0 quiescence. As the cells continue to progress through the cell cycle into late G_1 phase and S phase following serum addition, the differential rates of cell-cycle progression within the cell population continue to increase and thereby generate unsynchronized cells (Figure 1A). This is an important factor that should be taken into consideration when analyzing and interpreting data from experiments using this method of synchrony.

CONTACT INHIBITION

The phenomenon of contact inhibition in cell culture conditions occurs as cells continually divide, resulting in high cell density and cell-to-cell contact. At this point, cells undergo an arrest in the early G_1 phase of the cell cycle; this is unlike serum deprivation, which results in G_0 quiescence (47,69). Cell type determines the degree to which the cells arrest in early G_1 as a result of high cell density conditions. Normal cells and some transformed cells exhibit contact inhibition, but a subpopulation of cells may exist that are resistant to early G_1 arrest under these conditions. Contact inhibition, in conjunction with serum withdrawal, may yield an increased number of cells arrested in early G_1, which may aid in the arrest of tumor cells in particular. Although contact inhibition-mediated early G_1 arrest may involve p27, a Cdk2 inhibitor, as indicated by several studies reporting the accumulation of p27 in contact inhibited cells (3,54), fibroblasts from p27 knockout mice strongly suggest the involvement of additional unknown mechanisms during the contact inhibition process (46). Similar to synchronization by serum deprivation, processes mediated by contact inhibition may affect the protein of interest and should be considered when choosing this method of synchrony.

Contact inhibition can be achieved in human diploid fibroblasts by allowing cells to reach 89% confluency. The fibroblasts are then replated at high-density conditions (6×10^6 cells/10-cm plate) that have been experimentally predetermined to elicit an early G_1 arrest. Reduction of serum (from 5% to 0.5%) in the medium may aid contact inhibition of certain cell types. The degree of early G_1 arrested cells within a population can be distinguished from those progressing through S, G_2, and M phases by the analysis of DNA content using flow cytometry (58,59,67). In our laboratory, greater than 90% of cultured human diploid fibroblasts arrest in early G_1 when they are maintained at confluency for 48 h (A.H. and S.F.D., personal observations). To release cells from contact inhibition, cells are replated at low density (1×10^6 cells/10-cm plate), and the progression of the cells into subsequent phases is monitored by DNA content analysis using flow cytometry and BrdU incorporation. Further analysis can be carried out to determine which cells are progressing through the restriction point in late G_1 and entering S phase. At the restriction point, hyperphosphorylation of pRB occurs, which can be detected by slower migration using SDS-PAGE (15,18, 40). Greater than 50% of cells traverse into S phase 25 h after replating (A.H. and S.F.D., personal observations).

Replating contact-inhibited cells at low density results in continued cell-cycle progression from early G_1. While the majority of cells will be synchronous at this time, some cells may progress at different rates and have a delayed response to cell-cycle progression from early G_1 (Figure 1B). The degree of cell-cycle synchrony will lessen with time after low density replating of the contact inhibited cells. In addition, the differential rates of progression by cell populations become more significant as cells enter later phases of the cell cycle (Figure 1B). An important consideration when designing and analyzing experiments is the fact that, in most instances, only the majority of the cell population will be synchronous at a given time following release from contact inhibition. Synchronization of fibroblasts by contact inhibition can allow examination of early G_1-specific events (Figure 2). In our laboratory, we used synchronized fibroblasts following contact inhibition measurements of cyclin D:cdk4/6 activity, pRB phosphorylation, and cyclin E:cdk2 activity. As fibroblasts progress from early to late G_1, cyclin D:Cdk4/6 activity is maintained throughout G_1, while cyclin E:Cdk2 activation occurs concurrent with pRB hyperphosphorylation as cells traverse into late G_1 phase (Figure 2).

CENTRIFUGAL ELUTRIATION

In theory and in practice, cells in a particular phase of the cell cycle can be isolated based on size, using the method of centrifugal elutriation. For instance, early G_1 cells are approximately half the size of cells in the late G_2 or M phase, while S phase cells exhibit an intermediate size. To ensure successful isolation of phase-specific populations by elutriation, it should be noted that some cell types may not exhibit significant size variability as they progress through the cell cycle. Although the centrifugal elutriation setup requires specially designated equipment, this method offers several significant biological advantages compared with other methods of cell-cycle synchronization. First, centrifugal elutriation can be used to isolate almost all cell types, adherent or suspension, including Rat1 fibroblasts (22), Swiss 3T3 cells (23,72), NOSE-1 epithelial cells (70), NB41A3 neuroblastoma cells (73), primary dipoloid fibroblasts (22,23), Jurkat leukemia cells (38), and HT-29 adenocarcinoma cells (31). Second, this method does not require exposing the cells to pharmacological agents or maintaining them in stress-inducing environments, such as low-serum and high-density conditions. Third, elutriation can be used to acquire cell populations from any phase of the cell cycle and yield a large population of phase-specific cells rapidly isolated for subsequent analysis. Finally, once the elutriation parameters are established for a given cell type and cell-cycle position, centrifugal elutriation can be consistently repeated.

The method of centrifugal elutriation consists of a specially designed centrifuge rotor in which the centrifugal force on the cell population is countered by medium flowing in the opposite direction (Figure 3). Cells are injected by the use of a pump into the elutriation rotor, which is spinning at a constant *g* force. When the centrifugal force of the rotor (proximal to distal; see Figure 3) is balanced with the opposing force of the flow rate (distal to proximal), cells float at a specific position within the elutriation chamber,

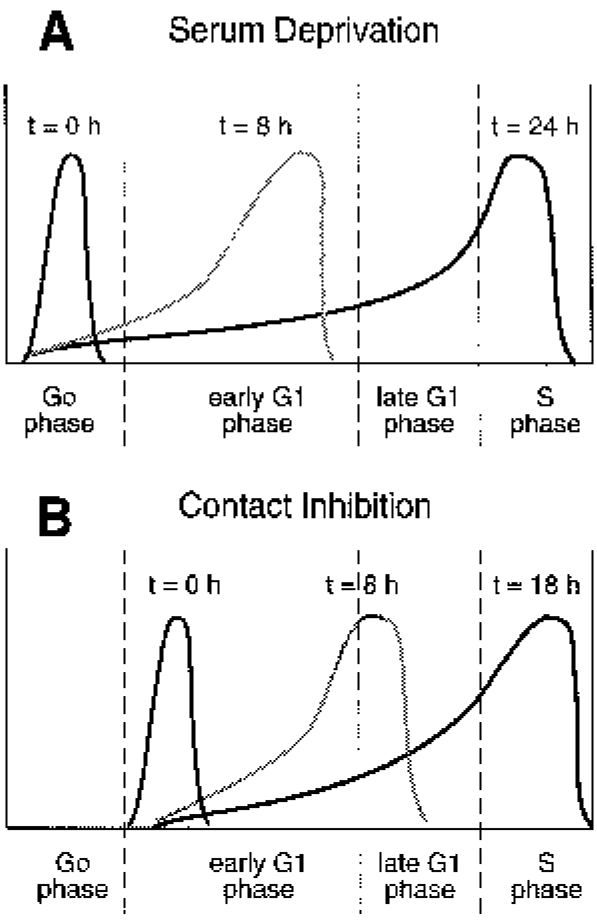

Figure 1. Comparative depiction of relative starting points for cells synchronized by serum deprivation versus contact inhibition. (A) Serum deprivation results in cell-cycle exit into G_0 quiescence, whereas (B) contact inhibition leads to an early G_1 arrest-like position. Like most cell synchrony methods, cells following the release of cell-cycle arrest or G_1 entry from quiescence will not progress through the subsequent phases at the same rate. Note: upon restimulation or replating, not all cells traverse equally efficiently through the subsequent phases of the cell cycle; some cells become more unsynchronized in the later phases of the cell cycle.

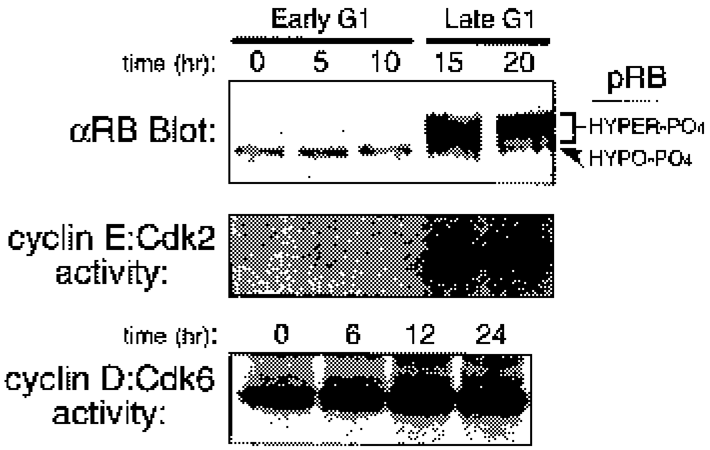

Figure 2. Analysis of contact-inhibited cells. Human (HaCaT) keratinocytes were contact inhibited for 48 h at high density in 10% serum, released by replating at low density, and then assayed at the indicated times. Immunoblot analysis was performed as described (15) with anti-pRB (BD Biosciences, San Diego, CA, USA). Anti-Cdk2 and anti-Cdk6 (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) immunoprecipitation kinase assays were performed using GST-pRB C terminus or histone H1 as the substrate in vitro, according to Ezhevsky et al. (15).

based on their size. Hence, small, early G_1 phase cells are proximal, while the larger G_2/M phase cells are distal. As the flow rate is increased, the proximal early G_1 cells are elutriated or pushed out of the chamber and collected. By further increasing the flow rate, the late G_1 phase cells can then be elutriated and collected, followed by the S phase and then G_2/M phase cells. Importantly, throughout the entire process, cells are maintained at 34°C–37°C in the presence of serum-containing medium and, thus, are exposed to minimal biological perturbations. Parameters such as rotor speed, number of cell injected, flow rate, and volume of the cell fractions collected must be taken into account when establishing elutriation conditions for a particular cell type. Determining the cell-cycle phase (i.e., G_1, S, and G_2/M phases) of a population can be carried out by analyzing the DNA content by flow cytometry (Figure 4). The analysis of cell viability within each elutriated fraction can also be carried out using light-scatter techniques with flow cytometry.

The entire elutriation typically takes 1–2 h and can yield as many as 107 pure early G_1 cells. We have successfully elutriated multiple cell types, both adherent and suspension. Elutriation of several cell types yields highly enriched, phase-specific cell populations (10,21,23,29). As with the previous methods discussed, the phase-specific cell population may be contaminated to some extent with cells from other phases. Following the isolation of phase-specific cell population, the cells can either be replated and followed through various phases of the cell cycle or be directly examined for phase-specific expression profiles (Figure 4).

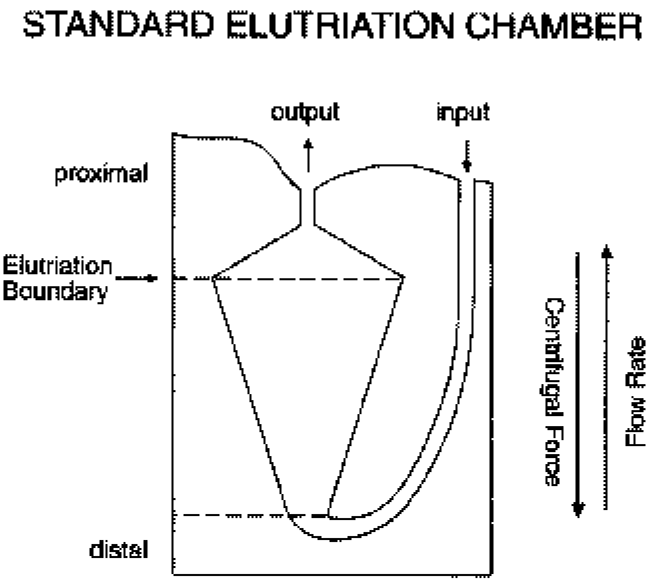

Figure 3. Standard centrifugal elutriation chamber. Cells become balanced in the chamber by the countering centrifugal and flow forces. Small (G_1 phase) cells are forced into the proximal end of the chamber, whereas the larger (G_2/M phase) cells remain at the distal end of the chamber. Once cells cross the elutriating boundary, the flow rate dramatically increases because of the narrowing of the chamber, and the cells become captured and exit the chamber.

DISCUSSION

Here, we describe that the most important parameters to be considered when choosing a method of cell synchrony are the cell-cycle events/phases to be studied and the properties of the cells used. Methods that induce cell-cycle arrest in a particular phase of the cell cycle may involve different cellular mechanisms that can potentially affect the cellular event, protein studied, and/or the biology of the cell. This was illustrated in a study that reported differences in regulatory molecules during G_0 quiescence of human diploid fibroblasts induced by serum deprivation versus contact inhibition in the presence of serum (9). While the accumulation of p27 and p16 Cdk inhibitors occurs in contact-inhibited early G_1 phase fibroblasts, these proteins remain low in G_0 quiescence serum-deprived cells (9).

In contrast, centrifugal elutriation offers a significant advantage over serum deprivation and contact inhibition because elutriation provides cell-cycle phase-specific cells from cycling cells, including normal and tumor cells. The importance of selecting synchrony methods involving alterations of cells compared to unaltered elutriated cells is demonstrated by studies examining D-type cyclins. Studies using G_0 quiescent serum-deprived cells exhibited a mid-G_1 expression pattern of D-type cyclins

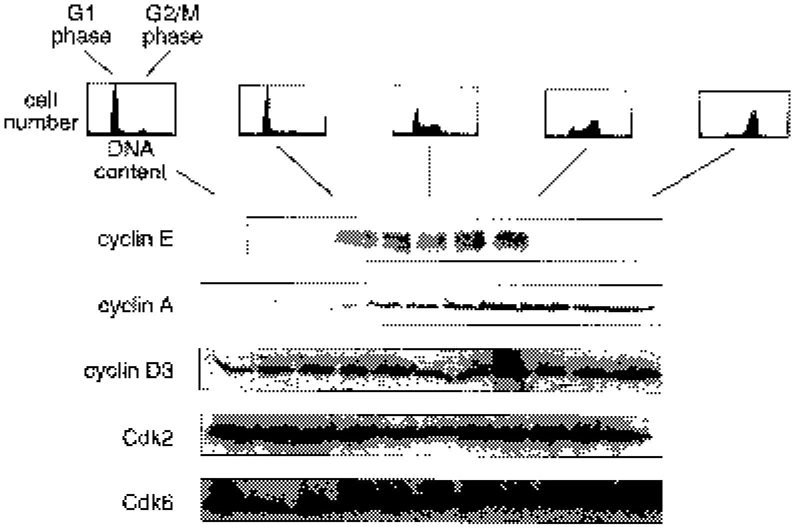

Figure 4. Analysis of centrifugally elutriated cells. Human Jurkat T cells were separated, based on size by elutriation and the various fractions of cell-cycle phase-specific populations collected. Cell-cycle position was analyzed by DNA content with propidium iodide using flow cytometry (top) (54,55,61). To further demonstrate the temporal cell-cycle position of the cells, Rb protein was examined by immunoblot analysis using anti-Rb (BD Biosciences) (36). In early G_1, Rb becomes hypophosphorylated, but as cells progress through the restriction point approaching late G_1, Rb becomes hyperphosphorylated (noted by an upward shift in mobility using SDS-PAGE).

(63). Indeed, this result supported a hypothesis that D-type cyclins, in conjunction with their Cdk partners, Cdk4 and Cdk6, performed the initial inactivating hyperphosphorylation of pRB at the late G_1 restriction point. However, analysis of cyclin D expression and activities from elutriated cycling cells and contact-inhibited cells demonstrated a constitutive cell-cycle pattern of cyclin D:Cdk4 expression and kinase activity (11) throughout the cell cycle (Figures 2 and 4). These observations directly challenge the notion previously held from serum-deprived experiments. Therefore, the use of unaltered synchronized cells resulting from elutriation may have a significant impact on previous findings from studies that relied on methods involving cell stress.

Phase-specific cells can also be obtained by the use of flow cytometry and cell sorting using DNA content analysis (an indicator of cell cycle phase) and cell size (an indicator of cell viability) as the sorting criteria (48,52). Depending on the study, cells can be fixed for phase-specific markers, such as cyclin B (71) and AF-2 protein (8), before cell sorting by flow cytometry to acquire very pure and specific subpopulations of phase-specific cells (28).

Finally, synchronous populations of cells can be achieved by stimulating naturally G_0 quiescent cells to enter the cell cycle. Primary peripheral blood lymphocytes exist in a G_0 quiescent state until stimulated to undergo cell division, which can be done by treatment with polyclonal mitogens, such as phytohemagglutinin and concanavalin A for T cells and lipopolysaccharide for mouse B cells. For example, treatment of G_0 quiescent primary peripheral blood lymphocytes with phytohemagglutinin drives these cells into early G_1 phase of the cell cycle, with subsequent progression into S phase at approximately 36 h after stimulation (6,16).

While several methods of synchrony cells exist, the consequences of these methods on the overall physiology of the cell and the impact that the chosen method may have on the particular experiments to be carried out should be kept in mind. If possible, preliminary experiments using different methods of synchrony should be conducted to address these issues.

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