Preface

The field of cell cycle regulation is based on the observation that the life cycle of a cell progresses through several distinct phases, G1, M, S, and G2, occurring in a well-defined temporal order. Details of the mechanisms involved are rapidly emerging and appear extraordinarily complex. Furthermore, not only is the order of the phases important, but in normal eukaryotic cells one phase will not begin unless the prior phase is completed successfully. Checkpoint control mechanisms are essentially surveillance systems that monitor the events in each phase, and assure that the cell does not progress prematurely to the next phase. If conditions are such that the cell is not ready to progress—for example, because of incomplete DNA replication in S or DNA damage that may interfere with chromosome segregation in M—a transient delay in cell cycle progression will occur. Once the inducing event is properly handled—for example, DNA replication is no longer blocked or damaged DNA is repaired—cell cycle progression continues. Checkpoint controls have recently been the focus of intense study by investigators interested in mechanisms that regulate the cell cycle. Furthermore, the relationship between checkpoint control and carcinogenesis has additionally enhanced interest in these cell cycle regulatory pathways. It is clear that cancer cells often lack these checkpoints and exhibit genomic instability as a result. Moreover, several tumor suppressor genes participate in checkpoint control, and alterations in these genes are associated with genomic instability as well as the development of cancer.

Cell Cycle Checkpoint Control Protocols is designed to augment the growing field and, through detailed descriptions of cell cycle-related methodologies using mammalian, yeast, and frog model systems, aid in the performance of experiments that bear on furthering the understanding of cell cycle checkpoint control. Chapters include descriptions of methods to induce cell cycle checkpoints, detect changes in cell cycle progression, identify and analyze genes and proteins that regulate the process, and characterize chromosomal status as a function of cell cycle phase and progression. The list of protocols is by no means complete, yet is comprehensive enough to at a minimum describe major methodologies used by investigators in the field.

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Howard B. Lieberman

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Methods for Synchronizing Mammalian Cells

Michael H. Fox

1. Introduction

When studying cell cycle checkpoints, it is often very useful to have large numbers of cells that are synchronized in various stages of the cell cycle. A variety of methods have been developed to obtain synchronous (or partially synchronous) cells, all of which have some drawbacks. Many cell types that attach to plastic culture dishes round up in mitosis and can then be dislodged by agitation. This mitotic shake-off method, originally discovered by Terasima and Tolmach ***(1)***, is useful for cells synchronized in metaphase, which on plating into culture dishes move into G1 phase in a synchronous manner. A drawback to the mitotic shake-off method is that only a small percentage (2–4%) of cells are in mitosis at any given time, so the yield is very small. Also, cells rapidly become asynchronous as they progress through G1 phase, so the synchronization in S phase and especially G2 phase is not very good. The first limitation can be overcome by plating multiple T150 flasks with cells, using roller bottles, or blocking cells in mitosis by inhibitors such as Colcemid or nocodazole ***(2)***. Mitotic cells that are collected can be held on ice for an hour or so while multiple collections are done to obtain larger numbers of cells.

To obtain more highly synchronous populations of cells in S phase, the mitotic shake-off procedure can be combined with the use of deoxyribonucleic acid (DNA) synthesis inhibitors, such as hydroxyurea (HU) or aphidicolin (APH), to block cells at the G1/S border (but probably past the G1 checkpoint). APH inhibits DNA polymerase α ***(3–5)***, whereas HU inhibits the enzyme ribonucleotide reductase ***(6)***, though it may operate by other mechanisms also ***(7)***. On release from the block, cells move in a highly synchronized fashion through S phase and into G2 phase ***(8)***. In terms of number of synchronized cells, this method has the same limitation as discussed above, because the starting cell population

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derives from the mitotic shake-off procedure. In addition, the block of cells with drugs can cause unbalanced cell growth, so one cannot necessarily conclude that all biochemical processes are also synchronized.

Large numbers of synchronous cells can be obtained using centrifugal elutriation ***(9)***. This method requires the use of a special rotor in a large floor centrifuge and separates cells into the cell cycle based on cell size. Cells may be obtained in early or late G1 phase, or primarily in S phase. However, the cell populations are not highly synchronous in S phase but instead have significant populations of G1- and G2-phase cells included. Nevertheless, it is possible to synchronize very large numbers of cells using this method, and biochemical processes are not perturbed.

Another method that results in highly synchronous populations is based on labeling cells with a viable dye for DNA (Hoechst 33342) ***(10)***. Cells stained with this dye can then be sorted by cell cycle phase. Sorted G1 cells will be distributed throughout G1, however. Cells in S phase can be sorted into a small window in S phase and thus will be highly synchronized, but only a small number of cells can be obtained. G2 phase cells will be contaminated with late S phase cells. Furthermore, some cell types do not stain well with Hoechst 33342, so sufficiently good DNA histograms cannot be obtained.

The protocols presented in this chapter are based on the mitotic shake-off procedure optimized to obtain large numbers of cells. Procedures for obtaining highly synchronized cells in G1 phase, various stages in S phase, and G2 phase are described, along with DNA histograms demonstrating the quality of results that can be obtained.

2. Materials

2.1. Cell Culture

1. Attached cell lines, such as Chinese hamster ovary (CHO) or HeLa.
2. Appropriate medium, such as Ham's F12 or minimum essential medium (MEM).
3. Fetal bovine serum (10–15%).
4. T75 or T150 tissue-culture flasks, or both.

2.2. Stock Solutions and Reagents

1. HU (2 m*M* in medium).
2. APH (1–3 μg/mL in medium; *see* **Note 1**).
3. Trypsin (*see* **Note 2**).

2.3. Equipment

1. Variable speed shaker with platform to hold T75 or T150 tissue-culture flasks (available from Fisher Scientific, VWR, Daigger, ISC Bioexpress, etc.).
2. Flow cytometer for analysis of synchronized cell populations.

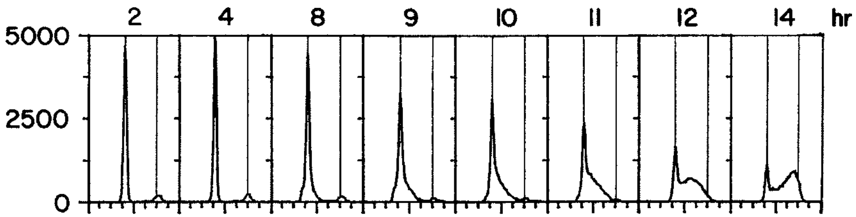

Fig. 1. Flow cytometry histograms at various times after mitotic selection synchrony procedure. The vertical lines show locations of the G1 and G2 peaks. The time after mitotic selection is shown at top of histograms. This figure is reproduced in part from **ref.** *8*.

3. Floor centrifuge to spin down large volumes of cells and medium or tabletop centrifuge that can handle 50-mL centrifuge tubes for smaller experiments.

3. Methods

3.1. Mitotic Shake-Off for G1 Synchrony

1. Culture cells in T75 or T150 flasks (*see* **Note 3**).
2. Trypsinize and replate 3–5 × 10^6 cells in 25 mL medium in T150 flasks.
3. Grow cells in incubator for 24–48 h to increase cell numbers (*see* **Note 4**).
4. Select mitotic cells by placing flasks on shaker tray suitable for holding 96-well trays and shake for 30 s to 1 min at 150–200 rpm (*see* **Note 5**).
5. Collect the medium containing mitotic cells in 500-mL centrifuge bottles and put on ice.
6. Add 25 mL prewarmed medium to flasks and incubate for 10 min.
7. Select mitotic cells by repeating **steps 4–6**. This can be done sequentially for 1–2 h to collect sufficient numbers of mitotic cells (*see* **Note 6**).
8. After sufficient numbers of cells have been collected and held on ice, pool the collections and centrifuge them in a floor-model centrifuge to concentrate the mitotic cells.
9. Plate appropriate numbers of cells (1–5 × 10^5) into T25 flasks.
10. Add 4 mL prewarmed medium.
11. Incubate flasks in a 37°C incubator for desired time to get cells in early-, mid-, or late-G1 phase (*see* **Note 7** and **Fig. 1**).
12. Process parallel samples for cell cycle analysis to monitor cell cycle progression. Fix cells with 70% ethanol on ice for 20–30 min, stain with propidium iodide for 5–10 min, and analyze by flow cytometry (*see* Chapter 4 in this book for details on flow cytometry cell cycle analysis).

3.2. Mitotic Shake-Off Plus HU for S- and G2-Phase Synchrony

1. Follow **steps 1–8** in **Subheading 3.1**.
2. Plate appropriate numbers of cells (1–5 × 10^5) into T25 flasks containing 4 mL medium with 2 m*M* HU.

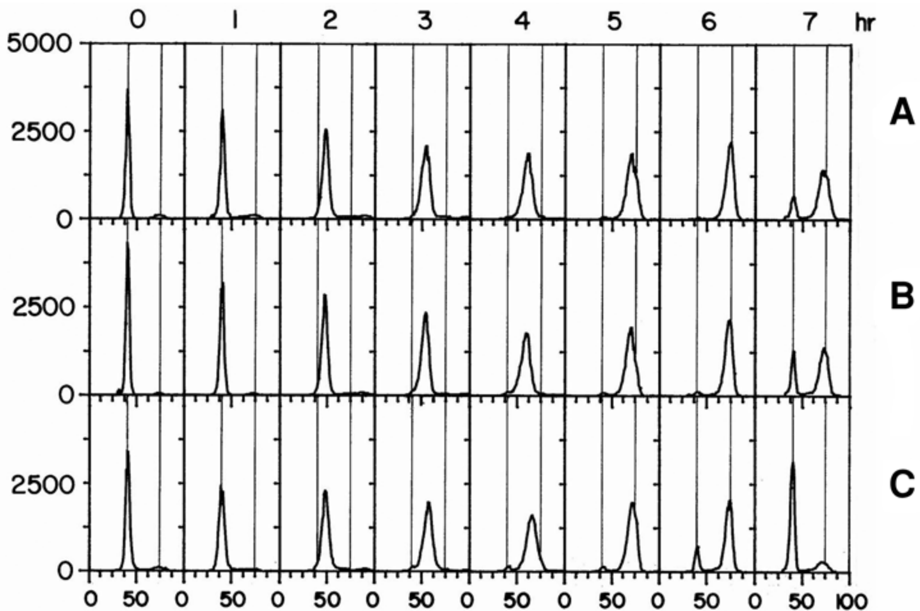

Fig. 2. Flow cytometry histograms obtained after releasing cells from mitotic selection plus 12 h of drug. **(A)** 2 m*M* HU. **(B)** 3 μg/mL APH. **(C)** 1 μg/mL APH. This figure is reproduced from **ref. *8***.

3. Incubate for approx 1 cell cycle time (approx 12 h for CHO cells, 24 h for human cells; *see* **Note 8**).
4. Aspirate off medium; rinse once with 4 mL of warm (37°C) medium.
5. Add 4 mL of warm medium and put flasks in incubator.
6. Remove flasks at various times after removing medium to get cells synchronized at various points in S phase or G2 phase (*see* **Note 9** and **Fig. 2A**).
7. Process parallel samples for flow cytometry analysis to determine the exact location of cells in S phase. To determine the G2 phase, it is best to use BrdU uptake and dual-parameter flow cytometry analysis as described in **Chapter XX**.

3.3. Mitotic Shake-Off Plus APH for S and G2 Synchrony

1. Follow **steps 1–10** in **Subheading 3.1.**
2. Add 4 μL APH from 10 mg/mL dimethyl sulfoxide (DMSO) stock to flasks for a final concentration of 1 μg/mL.
3. Follow **steps 3–7** in **Subheading 3.2.** (*see* **Note 10**, and refer to **Fig. 2B,C** for examples of synchronized populations obtained by this method).

4. Notes

1. APH is made in a stock solution of 10 mg/mL DMSO because it is not water soluble. At a final concentration of 3 μg/mL APH, the DMSO concentration is only 0.03% and should have little effect on cells.

2. Optimum trypsin concentration can vary for different cell types. We typically use 0.03% but sometimes use 0.25%. One m*M* ethylenediaminetetraacetic acid (EDTA) can also be used.
3. Make sure that cells are in exponential growth, not approaching confluence, so that the mitotic index will be as high as possible. The limiting concentration of cells in the flask will depend on cell type. The number of plates needed will depend on how many cells need to be synchronized.
4. Because about 3–4% of cells are in mitosis at any given time, the number of synchronized cells needed will govern how many flasks and how many cells are needed. The time for incubation to increase cell numbers will also depend on the cycle time for the cells. CHO cells have a cycle time of 12–14 h, whereas human cells have a cycle time of approx 24 h. The final concentration in a T150 should not exceed 1×10^7 cells to assure a high mitotic index. This will yield about $3–4 \times 10^5$ mitotic cells in a shake-off.
5. The exact conditions to shake the flasks will depend on the cell type. Typical conditions would be 1 min at 200 rpm. If shaking is too vigorous, the mitotic selection window will not be as narrow. In the absence of a mechanical shaker, it is possible to manually shake the cells off by firmly banging the flasks against your hand. This will work for a small number of cells but is not practical for a large synchrony experiment.
6. Discard the first 3–5 shakes to eliminate loosely attached cells that are not in mitosis. It is a good idea to quickly make a slide of collected cells and get a mitotic index. This can be done by swelling the cells in water for a minute, spinning them down, resuspending and adding a few drops of ice-cold methanol:acetic acid (3:1), then dropping the cells onto a microscope slide. The mitotic index should be above 95% to get highly synchronized cells.
7. It will take about 1 h for cells to attach to the plastic and move into G1 phase. Different stages of G1 can be studied by waiting different time periods before analyzing or treating the cells. Cells will become desynchronized as they move through G1, however, because this is a heterogeneous phase for transit time. Cells can also be allowed to move into S phase and G2 phase, but the synchronization is degraded substantially (*see* **Fig. 1A**).
8. It is important to hold cells at the G1/S border with HU for approx 1 cell cycle time because some cells take much longer to traverse G1 than others. One cell cycle time will be sufficient for >95% of the cells to block at the G1/S border. HU may become toxic to cells after about 12 h, however ***(8)***. This is not the G1 checkpoint because HU allows cells to initiate DNA synthesis ***(7)***.
9. There will be a slight delay for cells to begin progression into S phase. However, by 1 h about 98% of cells should be in early S phase in a tight distribution (*see* **Fig. 2A**). It is hard to predict the time when the maximum population will be in G2 phase. It is possible to quickly fix and analyze a sample of cells by flow cytometry as they progress through S phase and then predict more accurately when the maximal concentration will be in G2 phase.

10. APH at 1 μg/mL is not toxic to G1 cells and is not very toxic to S-phase cells *(8)*. One μg/mL APH does not delay cells in moving through S phase, but 3 μg/mL causes a slight delay.

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