Chapter 8

Cell Synchronization Techniques to Study the Action of CDK Inhibitors

Beatriz Pérez-Benavente and Rosa Farràs

Abstract

Cell synchronization techniques have been used for the studies of mechanisms involved in cell cycle regulation. Synchronization involves the enrichment of subpopulations of cells in specific stages of the cell cycle. These subpopulations are then used to study regulatory mechanisms of the cell cycle such as DNA synthesis, gene expression, protein synthesis, protein phosphorylation, protein degradation, and development of new drugs (e.g., CDK inhibitors). Here, we describe several protocols for synchronization of cells from different phases of the cell cycle. We also describe protocols for determining cell viability and mitotic index and for validating the synchrony of the cells by flow cytometry.

Key words Cell cycle, DNA content, Flow cytometry, Mitotic index, Synchronization

1 Introduction

A precise control of all stages of the cell cycle is essential for normal cell division. Two major processes characterize the cell cycle: DNA replication and segregation of duplicated chromosomes in two daughter cells [1]. It consists of two main periods: the interphase and mitosis. The interphase comprises (1) the G1 phase, which extends from previous mitosis until the S phase, (2) the S phase during which the cell duplicates its DNA, and (3) the G2 phase during which the cell prepares for mitosis. Mitosis (M) is the cycle period during which take place (1) the segregation of duplicated chromosomes and (2) cytokinesis resulting in two daughter cells. The transitions from one phase to another take place in an orderly and sequential manner and are controlled by various kinases called cyclin-dependent kinases (CDK). The activity of these kinases is regulated by mechanisms such as phosphorylation/dephosphorylation, but also by the spatiotemporally regulated expression of cyclins (Cyc) and cyclin kinase inhibitors or CKIs.

Mar Orzáez et al. (eds.), *Cyclin-Dependent Kinase (CDK) Inhibitors: Methods and Protocols*, Methods in Molecular Biology, vol. 1336, DOI 10.1007/978-1-4939-2926-9_8,

The periodic variation in the abundance of cyclins and CKI_S depends on their synthesis and degradation by the ubiquitin-proteasome system (UPS).

Synchronization involves the enrichment of subpopulations of cells in specific stages of the cell cycle. Protocols for synchronizing cells are based on (1) physical properties of the cells and include physical fractionation (mitotic shake-off, flow cytometry, dielectrophoresis, cytofluorometric purification), (2) selective nutrient depletion, and (3) chemical blockade by the addition of pharmacological agents [2, 3]. Methods based on physical characteristics have the advantage that the cells are not exposed to pharmacological agents; however many of these methods require specific equipment. Several chemicals may reversibly block cells from cycling resulting in a homogeneous population of cells at a particular stage of the cell cycle. Cells that are synchronized are artificially induced to cycle in a homogeneous manner. However, the chemicals may alter gene expression and posttranslational modifications after the blockade. Synchronization using chemicals is achieved immediately after release of the drugs but it is gradually lost after cells divide. Synchronized cells can be used to study the activity of new drugs that target the cell cycle checkpoints responsible for the control of cell cycle progression. These drugs may either increase or decrease the degree of checkpoint arrest and may slow growth and induce cell death.

The protocols presented here describe procedures used to synchronize NIH 3T3, HeLa or U2OS cells [4–6], in various stages of the cell cycle (*see* Fig. 1). Protocols will be presented for synchronizing cells in the G1 by serum withdrawal [4], G1/S by feedback control through addition of excess nutrients (e.g., thymidine, [7, 8]) or by the use of chemical agents (e.g., aphidicolin, [9]), and in M by morphological differences (mitotic shake-off, [10]) or by the addition of chemical agents (e.g., nocodazole [11, 12]) (*see* Fig. 2).

For some cell types, serum starvation is not a viable method of synchronization either because cells arrest permanently in G_0, do not arrest (e.g., HeLa) or undergo apoptosis. To block cells in G1/S a double-blockade (thymidine/thymidine or thymidine/aphidicolin) procedure is generally used to block DNA synthesis. The addition of thymidine induces feedback inhibition of DNA replication and arrests cells throughout S phase [8]. After releasing cells from the thymidine blockade they cycle throughout S phase and mitosis and then are trapped in G1/S by a second block with thymidine or aphidicolin. Aphidicolin is an inhibitor of DNA polymerase-α and DNA polymerase-δ. This method is used to monitor S- and G2/M-phase progression [6, 8]. The procedures described below are for the synchronization of cells with a doubling time of 24 h (e.g., HeLa and U2OS). Synchronization of cells by mitotic shake-off or nocodazole (which prevents microtubule assembly [13]) is useful to study M-G1-S-phase

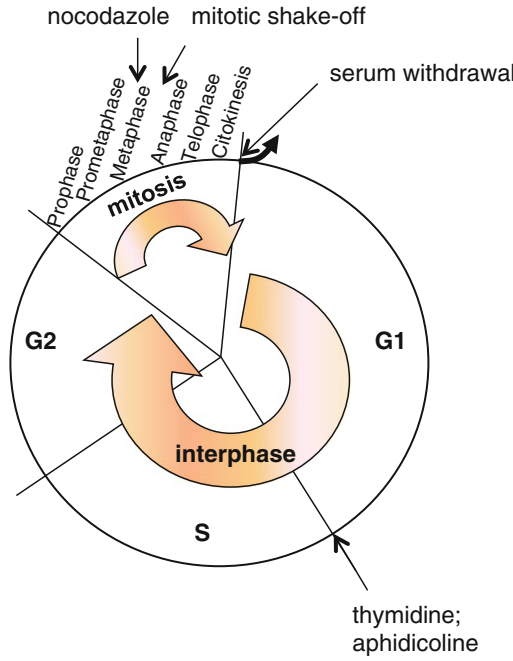

Fig. 1 Common methods for mammalian cell cycle synchronization. *Arrows* indicate the phase of the cell cycle at which cells are arrested

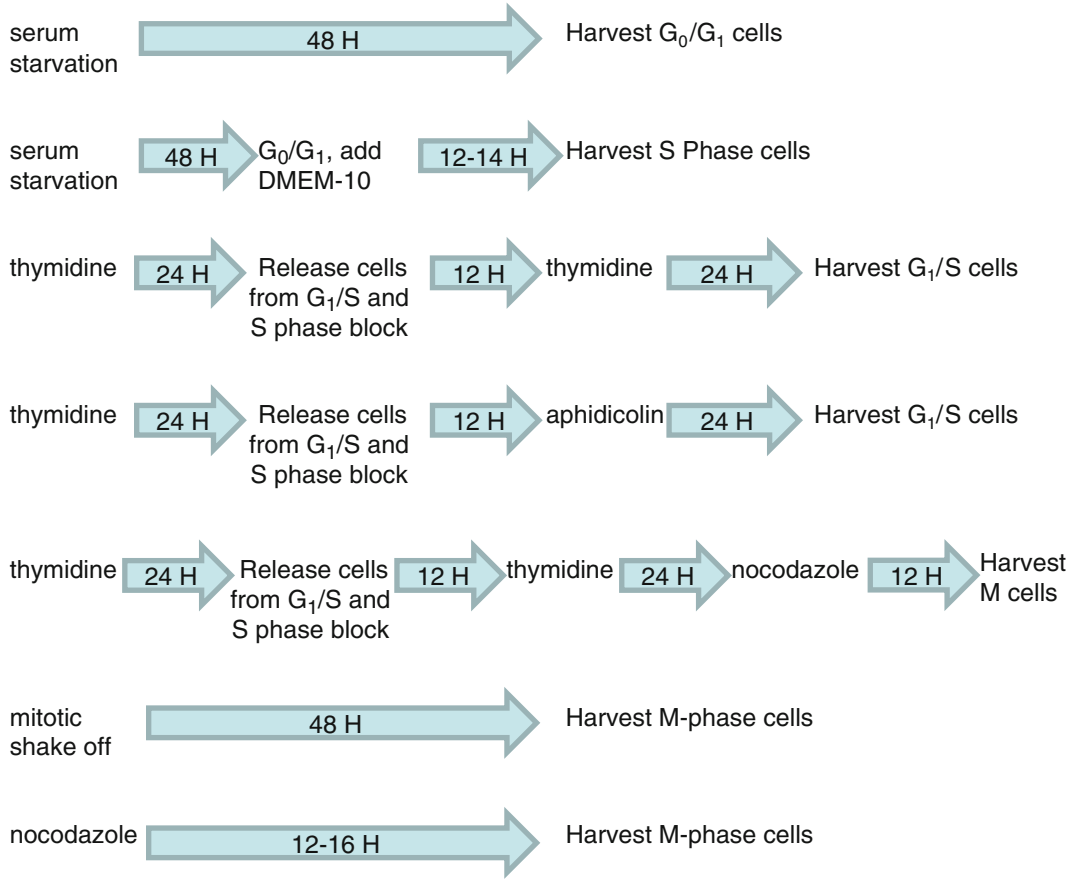

Fig. 2 Outline of the different synchronization methods described in this chapter

transition. Synchronization is achieved immediately after release of the drugs but after division cells lose synchrony rapidly. Methods are provided for determining cell viability and for measuring DNA content and mitotic index by flow cytometry.

2 Materials

1. NIH 3T3 cells, HeLa cells, U2OS cells.
2. DMEM prewarmed to 37 °C.
3. DMEM-1 supplemented with 1 % (v/v) fetal bovine serum (FBS) prewarmed to 37 °C.
4. DMEM-10 supplemented with 10 % (v/v) FBS prewarmed to 37 °C.
5. PBS.
6. 1× Trypsin.
7. CO_2 cell culture incubator.
8. Centrifuge.
9. 15 mL polypropylene V-bottomed tubes.
10. 50 mL polypropylene V-bottomed tubes.
11. Nocodazole.
12. Thymidine.
13. Aphidicolin.
14. Hemocytometer for cell counting.
15. Trypan blue 0.4 %.
16. Propidium iodide.
17. RNase A.
18. Ethanol 70 %.
19. 1 % BSA in PBS.
20. pS10-Histone H3 antibody.
21. FITC-conjugated secondary antibody.
22. 0.25 % Triton X-100 in PBS.

3 Methods

3.1 Method Enrichment of Cells at G0/G1 by Serum Starvation

1. Trypsinize exponentially growing NIH-3T3 cells and plate in prewarmed DMEM-1 at 30–40 % of their confluent density in a 100 mm tissue culture plate (*see* **Note 1**).
2. Incubate the cells for 48 h at 37 °C.

3. Remove DMEM-1 and add 10 mL DMEM-10 prewarmed at 37 °C (*see* **Note 2**). Incubate at 37 °C.
4. Approximately 12–14 h after stimulation cells progress into S phase.

3.2 Cell Synchronization at the Onset of S Phase by Double-Thymidine Block (See Note 3)

1. Trypsinize exponentially growing U2OS cells and plate in prewarmed DMEM-10 at 3×10^5 per 60 mm, 6×10^5 per 100 mm, or 1.5×10^6 cells per 150 mm tissue culture plates (*see* **Note 1**).
2. After 24 h, replace medium with new DMEM-10 prewarmed to 37 °C, add 1/40 volume of 100 mM thymidine in DMEM (*see* **Note 4**), and incubate cells for 25 h at 37 °C (*see* **Note 2**).
3. Release thymidine block by removing the thymidine-containing medium, and rinse the dishes twice with 5 mL prewarmed DMEM.
4. Add 10 mL prewarmed DMEM-10.
5. After 12–14 h add 1/40 volume of 100 mM thymidine in DMEM and incubate cells for 24 h at 37 °C.
6. Release thymidine block as above and add 10 mL prewarmed DMEM-10.
7. Monitor the progression of cells into S, G2/M, and M/G1 phase by flow cytometry (*see* Subheadings 3.8 and 3.9).

3.3 Cell Synchronization at the Onset of S Phase by Thymidine-Aphidicolin Block (See Note 3)

1. Trypsinize exponentially growing HeLa cells and plate in prewarmed DMEM-10 at 3×10^5 per 60 mm, 6×10^5 per 100 mm, or 1.5×10^6 cells per 150 mm tissue culture plates (*see* **Note 1**).
2. After 24 h, replace medium with new DMEM-10 prewarmed to 37 °C and add 1/40 volume of 100 mM thymidine in DMEM (*see* **Note 4**) and incubate cells for 25 h at 37 °C (*see* **Note 2**).
3. Release thymidine block by removing the thymidine-containing medium, and rinse the dishes twice with 10 mL fresh prewarmed DMEM.
4. Add 10 mL prewarmed DMEM-10.
5. After 12–14 h add 1/1000 volume of 5 mg/mL aphidicolin in DMSO (*see* **Note 5**) and incubate cells for 24 h at 37 °C.
6. Release as above and add 10 mL prewarmed DMEM-10.
7. Monitor the progression of cells into S, G2/M, and M/G1 phase by flow cytometry (*see* Subheadings 3.8 and 3.9).
8. If enrichment of M-phase cells is needed, add 1/1000 volume of 40 μg/mL nocodazole (*see* **Note 6**) 4 h after aphidicolin release. Cells will reach mitosis after 10–12 h. Harvest mitotic cells by gently pipetting off dish.

3.4 Cell Synchronization at M by Thymidine-Nocodazole Block

1. Plate log-phase cells, 2×10^6 per 150 mm tissue culture plates.
2. After 24 h add 1/40 volume of 100 mM thymidine.
3. Block cells for 25 h.
4. Release block by washing cells with prewarmed serum-free DMEM, and replace with complete medium (*see* **Note 2**).
5. After 4 h add 1/1000 volume of 40 μg/mL nocodazole in DMSO (*see* **Note 6**).
6. Block for 12 h and harvest mitotic cells by gently pipetting off dish (*see* **Note 7**).
7. Release cells by centrifugation for 5 min at $400 \times g$ and wash twice with 10 mL DMEM prewarmed at 37 °C.
8. Resuspend cells in 10 mL prewarmed DMEM-10 and replate cells in a 100 mm tissue culture plate; cells take 1–2 h to reattach.
9. Measure the cell cycle-related events during the M/G1/S-phase transitions.

3.5 Enrichment of Mitotic Cells by Mitotic Shake-Off

1. Trypsinize exponentially growing HeLa cells and plate in prewarmed DMEM-10 at 2.5×10^6 cells per 150 mm tissue culture plate.
2. After 24 h replace medium with new prewarmed DMEM-10 (*see* **Note 2**).
3. After 24 h harvest round mitotic cells by gently tapping the flask on the bench to shake off any loosely rounded cells and then collect mitotic cells by gently pipetting off the medium contained in the dish (*see* **Note 7**).
4. Transfer cell suspension to 50 mL centrifuge tubes and centrifuge for 5 min at $400 \times g$ at room temperature.
5. Discard supernatant, resuspend pellet in 20 mL DMEM-10 prewarmed at 37 °C, and replate in a 150 mm tissue culture plate.
6. Cells take 1–2 h to attach.
7. Measure the cell cycle-related events during the M/G1/S-phase transitions.

3.6 Enrichment of Mitotic Cells by Nocodazole Arrest

1. Trypsinize exponentially growing HeLa cells and replate in prewarmed DMEM-10 at 2.5×10^6 cells per 150 mm tissue culture plate.
2. After 24 h replace medium with new DMEM-10 prewarmed to 37 °C and add 1/1000 volume of 40 μg/mL nocodazole in DMSO (*see* **Note 2**).
3. Block for 12–18 h and harvest mitotic cells by gently pipetting off dish (*see* **Note 7**).

4. Transfer cell suspension to 50 mL centrifuge tubes and centrifuge for 5 min at 400 × *g* at room temperature.
5. Discard supernatant, and resuspend pellet in 20 mL fresh, prewarmed DMEM. Repeat centrifugation and addition of 20 mL prewarmed DMEM. Repeat centrifugation.
6. Resuspend cells in 20 mL prewarmed DMEM-10 and replate cells in a 150 mm tissue culture plate.
7. Cells take 1–2 h to reattach, and 2 h approximately to exit mitosis.
8. Measure the cell cycle-related events during the M/G1/S-phase transitions.

3.7 Cell Counting and Viability Measurement Using Trypan Blue

Trypan blue is a vital dye used to selectively stain dead cells. Reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable.

1. Add 50 µL of 0.4 % trypan blue solution to 350 µL of media.
2. Add 100 µL of the cell suspension and mix well. This makes a final 1:5 cell suspension dilution. This will be referred to as the "dilution factor" in the formula below.
3. Fill a hemocytometer as for cell counting.
4. Under a microscope, observe if nonviable cells are stained and viable cells excluded the stain.
5. Determine cell viability using the formula below:

   Viable cell count/quadrants counted × dilution factor* × hemocytometer factor* = viable cells/mL.

   *Dilution factor in this protocol is 5

   *Hemocytometer factor is 10,000.

3.8 Quantification of DNA Content by Flow Cytometry

1. Harvest cells by trypsinizing and prepare single-cell suspension in PBS.
2. Wash cells and resuspend at 1–2 × 10^6 cells/mL.
3. Aliquot 1 mL cells in a 15 mL polypropylene V-bottomed tube, centrifuge, and resuspend in 200 µL PBS.
4. Add 1 mL cold 70 % ethanol, dropwise, while vortexing to prevent cell clumping.
5. Fix cells for at least 2 h at –20 °C. (*see* **Note 8**).
6. Wash cells in PBS.
7. Add 1 mL of propidium iodide/RNase A staining solution (*see* **Note 5**) to cell pellet, mix well, and incubate for 30 min at 37 °C.
8. Store samples at 4 °C until analyzed by flow cytometry.

3.9 Determination of Mitotic Index by Flow Cytometry

Mitotic cells are indistinguishable from G2 cells when quantifying DNA content by flow cytometry. Since histone H3 is phosphorylated exclusively during mitosis the specific detection of this modification can be used to identify mitotic cells [14].

1. Harvest cells by trypsinizing and prepare single-cell suspension in PBS.
2. Wash cells with PBS and resuspend at $1–2 \times 10^6$ cells/mL.
3. Aliquot 1 mL cells in a 15 mL polypropylene V-bottomed tube, centrifuge, and resuspend in 200 μL PBS.
4. Add 1 mL cold 70 % ethanol, dropwise, while vortexing to prevent cell clumping.
5. Fix cells for at least 2 h at −20 °C (*see* **Note 8**).
6. Wash cells in cold PBS.
7. Add 1 mL 0.25 % Triton X-100 in PBS and incubate for 15 min on ice.
8. Wash cells in cold PBS.
9. Resuspend cells in 100 μL of 1 % solution of BSA in PBS containing an antibody that specifically recognizes the phosphorylated form of histone 3 at serine 10. Incubate for 1–3 h at room temperature.
10. Wash cells twice with 1 % BSA in PBS.
11. Resuspend cells in 100 μL of 1 % solution of BSA in PBS containing the appropriate FITC-conjugated secondary antibody. Incubate for 1 h at room temperature in the dark.
12. Wash cells twice with 1 % BSA in PBS.
13. Add 500 μL of propidium iodide/RNase A staining solution (*see* **Note 9**) to cell pellet, mix well, and incubate for 30 min at 37 °C.
14. Analyze samples by flow cytometry.

4 Notes

1. Density of cells is important to ensure cell cycle progression and to avoid reaching confluency during the assay.
2. To prevent losing synchronization is important to use cell culture media prewarmed at 37 °C.
3. The protocols for synchronization of cells at the onset of S phase indicated here are only convenient for cells with up to 24 h doubling time.
4. Prepare a 100 mM stock solution of thymidine in DMEM and store at −20 °C.
5. Prepare a 5 mg/mL stock solution of aphidicolin in DMSO and store at −20 °C.

6. Dissolve nocodazole in DMSO to a concentration of 4 mg/mL and store at −20 °C. Dilute this solution to 40 μg/mL nocodazole in DMSO and store at −20 °C.
7. Mitotic shake-off method is based on the observation that as cells progress into metaphase during mitosis they become rounded and have fewer points of attachment with the culture vessel [15].
8. Cells may be stored at −20 °C for 2–3 weeks prior to propidium iodide staining and flow cytometric analysis.
9. Propidium iodide/RNase solution working concentration contains propidium iodide 50 μg/mL in PBS and RNase A 0.1 mg/mL. This solution should be stored at 4 °C in the dark.

References

1. Morgan DO (2007) The cell cycle: principles of control, Primers in biology. New Science Press Ltd in association with Oxford University Press, London
2. Banfalvi G (2011) Overview of cell synchronization. Methods Mol Biol 761:1–23
3. Rosner M, Schipany K, Hengstschlager M (2013) Merging high-quality biochemical fractionation with a refined flow cytometry approach to monitor nucleocytoplasmic protein expression throughout the unperturbed mammalian cell cycle. Nat Protoc 8(3):602–626
4. Campisi J, Morreo G, Pardee AB (1984) Kinetics of G1 transit following brief starvation for serum factors. Exp Cell Res 152(2):459–466
5. Masters JR (2002) HeLa cells 50 years on: the good, the bad and the ugly. Nat Rev Cancer 2(4):315–319
6. Farras R, Baldin V, Gallach S, Acquaviva C, Bossis G, Jariel-Encontre I, Piechaczyk M (2008) JunB breakdown in mid-/late G2 is required for down-regulation of cyclin A2 levels and proper mitosis. Mol Cell Biol 28(12):4173–4187. MCB.01620-07 [pii]
7. Stein GS, Borun TW (1972) The synthesis of acidic chromosomal proteins during the cell cycle of HeLa S-3 cells. I. The accelerated accumulation of acidic residual nuclear protein before the initiation of DNA replication. J Cell Biol 52(2):292–307
8. Harper JV (2005) Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. Methods Mol Biol 296:157–166
9. Heintz N, Sive HL, Roeder RG (1983) Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Mol Cell Biol 3(4):539–550
10. Pines J, Hunter T (1989) Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell 58(5):833–846, 0092-8674(89)90936-7 [pii]
11. Kohn KW, Jackman J, O'Connor PM (1994) Cell cycle control and cancer chemotherapy. J Cell Biochem 54(4):440–452
12. Perez-Benavente B, Garcia JL, Rodriguez MS, Pineda-Lucena A, Piechaczyk M, Font de Mora J, Farras R (2013) GSK3-SCF(FBXW7) targets JunB for degradation in G2 to preserve chromatid cohesion before anaphase. Oncogene 32(17):2189–2199
13. Ferrero M, Ferragud J, Orlando L, Valero L, Sanchez del Pino M, Farras R, Font de Mora J (2011) Phosphorylation of AIB1 at mitosis is regulated by CDK1/CYCLIN B. PLoS One 6(12), e28602
14. Xu B, Kim S, Kastan MB (2001) Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation. Mol Cell Biol 21(10):3445–3450
15. Elvin P, Evans CW (1983) Cell adhesiveness and the cell cycle: correlation in synchronized Balb/c 3T3 cells. Biol Cell 48(1):1–9