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Assessing G₁-to-S-phase progression after genotoxic stress

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Abstract

Maintenance of genomic integrity is critical for the survival of organisms. Thus, mammalian cells employ a complex DNA damage response that can sense and repair DNA damage. One important aspect of the cellular DNA damage response is the activation of checkpoints that result in cell cycle arrest. In this chapter we present methods for the induction of genotoxic stress. Additionally, we describe methods for studying the progression of cells from G₁- to-S-phase after genotoxic stress.

Keywords

Cell cycle; checkpoint; DNA double-strand breaks; G₁; S-phase; propidium iodide; bromodeoxyuridine; γ -H2AX; immunofluorescence

1. Introduction

The success of an organism requires the ability to transmit an intact copy of its genome to its progeny. This task is complicated by the sheer number of genotoxic insults that assail organisms from exogenous as well as endogenous sources including ultraviolet and ionizing radiation, oxidative metabolic byproducts, chemotherapeutics, and errors in replication (1–6). It is thus necessary for organisms to employ complex mechanisms of DNA surveillance and repair. These processes are coupled to a number of cell cycle checkpoints which allow for proliferative arrest, DNA repair, and, in cases of extreme genetic insults, apoptosis. Breakdown of these checkpoints can result in a number of human disorders often characterized by immunodeficiency and a predisposition to certain types of cancer (3–4).

The generation of DNA double-strand breaks (DSBs), such as those caused by ionizing radiation or radiomimetic drugs, potently activates cell cycle checkpoints. In G₁-phase, DSBs are typically recognized by the multiprotein MRN (MRE11, Rad50, and NBS1) complex, which is responsible for activating the signaling kinase ATM (Ataxia Telangiectasia Mutated) (1). After activation, ATM is able to phosphorylate a number of targets including the histone H2A variant H2AX, producing γ -H2AX, and the signal transduction kinase CHK2 (1, 7). ATM and CHK2 cooperate to activate and stabilize the tumor suppressor p53 by directly targeting p53 as well as targeting MDM2, the ubiquitin ligase that is normally responsible for the rapid turnover of p53 protein (8–12). Among the transcriptional targets of p53 is the cyclin dependent kinase (CDK) inhibitor p21^{CIP1/WAF1}, which inhibits the activity of the S-phase promoting cyclin E/Cdk2 complex (1, 3–4). The inhibition of cyclin E/Cdk2 results in cell cycle arrest at the G₁/S phase boundary, as well as the down-regulation histone gene expression (13).

Notes:

Experimentally, activation of the G₁ checkpoint can be studied by monitoring the inhibition of cell cycle progression from G₁ to S-phase after genotoxic insults. These insults are commonly introduced using radiation (ionizing or ultraviolet) or radiomimetic drugs. After genotoxic insults, progression from G₁ to S can be monitored by flow cytometry to assay for DNA content or the incorporation of 5-bromo-2-deoxyuridine (BrdU), a synthetic nucleotide that is incorporated into cellular DNA during replication.

Here we provide a number of protocols for studying the progression of cells from G₁-to-S-phase after genotoxic stress. In these protocols, genotoxic insults are introduced by γ -irradiation or the use of the radiomimetic drug, bleomycin. These sources of genotoxic insults produce DNA double-strand breaks in addition to other genotoxic lesions. The induction of DNA double-strand breaks by these treatments can be assessed by the immunofluorescent staining of γ -H2AX, a marker for DSBs (Figure 1) (14–15). Genotoxic insults can be introduced into asynchronously growing cells. Alternatively, cells can be synchronized in G₀ first and then treated with DNA damaging agents. This synchronization allows for monitoring progression from G₁-to-S-phase in a majority of cells after genotoxic insults. Cell cycle progression is assessed by monitoring DNA content with the fluorescent DNA-binding dye propidium iodide (Figure 2) or BrdU incorporation (Figure 3).

2. Materials

2.1 Cell Cycle Synchronization by Serum Starvation

1. Normal growth medium: Cells should be cultured in the appropriate medium for the cells of interest. Growth medium is usually supplemented with 10% fetal bovine serum and antibiotics.
2. Starvation medium: The appropriate medium for the cells of interest supplemented with 0.1% FBS

2.2 Induction of DNA breaks

1. Cesium-137 irradiator in a shielded room. Use extreme caution when operating the irradiator. Be sure to read and follow all safety precautions for your instrument.
2. Bleomycin: A stock solution is prepared by dissolving bleomycin in phosphate-buffered saline (see 2.3.4) at 10 mg/mL. This solution is filter sterilized and stored at –20°C for several months.

2.3 Assessment of DNA breaks by immunofluorescent staining of γ -H2AX

1. 12mm round glass coverslips
2. 24-well cell culture dish
3. Dumont No. 5 Jewelers Forceps
4. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4
5. Paraformaldehyde diluted to 4% (v/v) in PBS
6. Phosphate-buffered saline with Tween-20 (PBS-T): PBS containing 0.2% (v/v) Tween-20
7. Permeabilization solution: PBS-T containing 0.5% (v/v) Triton X-100
8. Blocking solution: PBS-T with 5% (v/v) horse serum, 5% (v/v) goat serum, 0.1% (v/v) fish gelatin.

9. Primary antibody: anti-phospho-histone H2A.X (Ser139), clone JBW301 (Millipore, Billerica, MA).
10. Secondary antibody: Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA)
11. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI): A stock solution of 2 mg/mL DAPI in PBS is stored at -20°C and diluted 1:40000 when needed.
12. Vectashield mounting medium (Vector Laboratories, Burlingame, CA)
13. Glass microscope slides
14. Clear nail polish

2.4 Propidium iodide staining and flow cytometric analysis of DNA content

1. EDTA/PBS: 0.1% EDTA in PBS
2. 80% Ethanol
3. PBS with 0.1% FBS
4. Propidium iodide (PI): A 50x stock solution is prepared by dissolving PI at 0.5 mg/mL in 38mM sodium citrate pH 7.0. The stock solution is then stored at 4°C in the dark. A working solution is obtained by diluting the stock solution 1:50 in PBS.
5. RNase A: A 40x stock solution is prepared by dissolving RNase A at 10 mg/mL in 10mM Tris-HCl pH 7.5, 15 mM NaCl. Boil for 15 minutes. After cooling to room temperature, the solution is aliquoted and stored at -20°C . The working solution is prepared by diluting the stock solution 1:40 in 1x PI solution.
6. 40 μm cell strainer
7. 5 mL polystyrene round-bottom tubes
8. Flow cytometer

2.5 BrdU labeling and propidium iodide staining

1. 5-Bromo-2'-deoxyuridine (BrdU): A stock solution is prepared by dissolving BrdU in PBS at a concentration of 10 mM. This solution is filtered through a 0.2 μm filter, aliquoted, and stored at -20°C .
2. PBS containing 0.5% bovine serum albumin (BSA)
3. 2N HCl with 0.5% (v/v) Triton X-100
4. Neutralization Buffer: 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$
5. PBS containing 0.5% BSA and 0.5% (v/v) Tween-20
6. Anti-BrdU (Becton Dickson, Franklin Lakes, NJ)
7. Fluorescein-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA)
8. Propidium iodide (PI): A 50x stock solution is prepared by dissolving PI at 0.5 mg/mL in 38mM sodium citrate pH 7.0. The stock solution is then stored at 4°C in the dark. A working solution is obtained by diluting the stock solution 1:50 in PBS.
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10. 40µm cell strainer
11. 5 mL polystyrene round-bottom tubes

3. Methods

3.1 Cell Cycle Synchronization by Serum Starvation

1. Grow cells to 40–50% confluence in normal growth medium.
2. Wash cells twice with PBS to completely remove serum.
3. Add starvation medium containing 0.1% serum.
4. Incubate cells in starvation medium for 48–72 hours depending on the cells of interest.
5. After starvation, cells can be stimulated to reenter the cell cycle by replacing the starvation medium with normal growth medium.
6. Harvest or treat the cells at the desired time.

3.2 Induction of DNA breaks by ionizing irradiation

1. Grow cells to 70–80% confluence in normal growth medium. Alternately, synchronized cells prepared as described in 3.1 can be used.
2. Irradiate the cells with the desired dose (see Note 1), using a Cesium-137 irradiator. An unirradiated control should be included in all experiments.
3. Incubate the cells until the desired time point for analysis.

3.3 Induction of DNA breaks by Bleomycin

1. Grow cells to 70–80% confluence in normal growth medium. Alternately, cells prepared as described in Section 3.1 can be used.
2. Aspirate medium and replace with medium containing the desired concentration of bleomycin (see Note 2).
3. Incubate the cells with bleomycin for 30 minutes then wash the cells with PBS and add normal growth medium.
4. Harvest the cells at the desired time point for analysis.

3.4 Assessment of DNA double-strand breaks by immunofluorescent staining of γ-H2AX

1. Grow cells to 70–80% confluence or as described in Section 3.1 on round glass coverslips in plates.
2. DNA breaks are induced by the methods described in Section 3.2 or 3.3.
3. Transfer each coverslip to individual wells of a 24 well tissue culture plate using jewelers forceps (see Note 3). When transferring coverslips it is important to keep the side on which the cells are growing facing up. This allows for proper exposure of the cells to solutions added to the wells.
4. Wash once with PBS.

¹It may be helpful to test a range of doses in initial experiments. We generally use doses in the range of 3–20 Gy.

²We suggest testing a range of doses initially. In general, we use doses between 5–50 µg/mL.

³Twenty-four well tissue culture plates are convenient because they allow for removal of solutions from wells by decanting without removing the coverslips. This is especially important for cells that are poorly adherent and may be lost during aspiration.

5. Fix the cells with 4% paraformaldehyde for 10 minutes at room temperature. Remove the fixing solution.
6. Wash the coverslips twice with PBS.
7. Permeabilize the cells by incubating with permeabilization solution for 10 minutes at room temperature. Remove the permeabilization solution.
8. Wash the coverslips twice with PBS-T.
9. To prevent non-specific binding of antibodies, incubate the cells with the blocking solution for 20 minutes at room temperature. Remove the blocking solution.
10. Add primary antibody diluted in blocking solution to each well (see Notes 4 and ⁵). Incubate for 1 hour at room temperature.
11. Wash coverslips three times for 5 minutes each with PBS-T.
12. Add secondary antibody diluted in blocking solution to each well (see Notes 5, ⁶, and ⁷). Incubate for 1 hour in the dark at room temperature.
13. Wash coverslips three times for 5 minutes each with PBS-T.
14. Dilute the DAPI stock solution 1:40,000 in PBS and add the this solution to each well. Incubate 10 minutes in the dark at room temperature.
15. Wash coverslips two times for 5 minutes each with PBS.
16. Place a small drop of Vectashield mounting medium on a glass microscope slide.
17. Remove a coverslip from the plate using jewelers forceps.
18. Carefully remove excess solution from the coverslip by tapping the edge of the coverslip on a paper towel.
19. Mount the coverslip cell-side down in the drop of mounting medium.
20. Remove excess mounting solution by gently pressing down on the coverslip with a paper towel.
21. To prevent drying, seal the coverslips by applying clear nail polish to the edges.
22. View the slides on an epifluorescence microscope.

3.5 Propidium iodide staining and flow cytometric analysis of DNA content

1. Harvest cells by incubation with 0.1% EDTA/PBS for 5 minutes at 37°C.
2. Resuspend the cells by pipetting up and down several times (see Note 8).
3. Centrifuge the suspended cells at 250g for 5 minutes. Discard the supernatant.
4. Wash once with PBS.
5. Resuspend the cell pellet in 0.5 mL PBS

⁴We typically use a dilution of 1:5000 for the primary antibody, but you may wish to test a range of concentrations.

⁵Be sure to add enough antibody to completely cover the coverslips.

⁶We typically use a dilution of 1:1000 for the secondary antibody.

⁷After addition of the secondary antibody it is important to minimize exposure to light. This prevents fading of the fluorescent signal. This can be accomplished by covering the plates with aluminum foil during washes or placing the plates in a drawer during incubations.

⁸In each resuspension step of the flow cytometry protocols it is important to pipette cells thoroughly in order to obtain a single cell suspension.

6. Fix the cells by adding 6 mL of 80% ethanol dropwise while gently vortexing to prevent cell clumping.
7. Incubate the cells in ethanol for at least 15 minutes at 4°C. Cells can be stored this way for several weeks.
8. Centrifuge the fixed cells at 250g for 5 minutes. Discard the supernatant.
9. Wash the cells once with PBS containing 0.1% serum.
10. Resuspend the cells in propidium iodide/RNase A solution at a final concentration of approximately 10^6 cells/mL.
11. Pass the cell suspension through a 40µm cell strainer and collect the strained suspension in a 5 mL round bottom tube.
12. Incubate the cells in the dark for 30 minutes at 37°C.
13. Analyze the cell cycle distribution of the cells by flow cytometry.

3.6 BrdU labeling and Propidium iodide staining

1. Add 30 µM BrdU to the medium 30 minutes before harvesting cells.
2. Remove the medium and wash the cells twice with PBS.
3. Harvest the cells by incubating with 0.1% EDTA/PBS and incubate for 5 minutes at 37°C.
4. Resuspend the cells by pipetting thoroughly (see Note 8).
5. Centrifuge the cells at 250g for 5 minutes and discard the supernatant.
6. Wash twice with PBS.
7. Resuspend the cells thoroughly in 0.5 mL PBS.
8. Fix the cells by the dropwise addition of 6 mL of 80% ethanol while gently vortexing.
9. Incubate the cells on ice for at least 15 minutes.
10. Wash once with PBS containing 0.5% BSA.
11. Denature cellular DNA by incubating the cells in 0.5 mL of 2N HCl/0.5% Triton X-100 for 20 minutes at room temperature.
12. Centrifuge the cells at 250g for 5 minutes and discard the supernatant.
13. Resuspend the cells in 1 mL of neutralization buffer (0.1 M $\text{Na}_2\text{B}_4\text{O}_7$) and incubate for 2 minutes at room temperature.
14. Centrifuge the cells at 250g for 5 minutes and discard the supernatant.
15. Wash the cells once with PBS containing 0.5% BSA and 0.5% Tween 20.
16. Centrifuge the cells at 250g for 5 minutes and discard most of the supernatant. Leaving 30–50µL of wash solution can help in resuspending the pellet in the next step.
17. Tap the tube to loosen the cell pellet and add 10 µl of anti-BrdU antibody per 10^6 cells. Tap again to mix. Incubate for 30 minutes at room temperature.
18. Wash the cells twice with PBS containing 0.5% BSA and 0.5% Tween 20.

19. Centrifuge the cells at 250g for 5 minutes and discard most of the supernatant. Leaving 30–50µL of wash solution can help in resuspending the pellet in the next step.
20. Tap the tube to loosen the cell pellet and add 1.5 µl fluorescein-conjugated anti-mouse IgG per 10⁶ cells. Tap the tube again to mix. Incubate for 30 minutes at room temperature.
21. Wash twice with PBS containing 0.5% BSA and 0.5% Tween 20.
22. Resuspend the cells at a concentration of approximately 10⁶ cells/mL in propidium iodide/RNase A solution.
23. Pass the cell suspension through a cell strainer and collect the strained suspension in a 5 mL round bottom tube.
24. Incubate the cells in the dark for 30 minutes at 37°C.
25. Analyze the cell cycle distribution of the cells by flow cytometry.

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Figure 1.

U2OS cells were γ -irradiated with the indicated dose. 30 minutes after irradiation the cells were fixed and accumulation of γ -H2AX foci was examined by immunofluorescent staining. DAPI was employed to label the nucleus.

Figure 2.

WI38 cells were first arrested at G₀ by serum starvation and then stimulated to enter the cell cycle by serum addition. At 4 hours after the addition of serum the cells were harvested (4hr, control), treated with IR (28h, irradiated), or left untreated (28h, control). The cell cycle profile of the cells is shown. (from Su C., et al (2004) *EMBO* **23**, 1133–1143.)

Time after irradiation:	0 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12 hr	14 hr
S phase (%):	38.9	41.8	42.6	39.8	34.2	27.3	18.5	13.7

Figure 3. Asynchronously growing HCT116 cells were γ -irradiated (12Gy) and assayed at the indicated time points for DNA content and BrdU incorporation. (from Su C., et al (2004) *EMBO* **23**, 1133–1143.)