Immunofluorescence Assay for S Phase Entry Using BrdU Incorporation

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[Abstract] Bromodeoxyuridine (BrdU) is thymidine analogue and can incorporated into the newly synthezised DNA of S-phase cells, the antibody specific for BrdU can then be used to detect the incorporated BrdU, thus indicating cells that were actively replicating their DNA and estimating for the percentage of cells in S-phase. The immunocytochemical detection of BrdU incorporated into DNA is a powerful tool to study the cytokinetics of normal and tumor cells. *In vitro* or *in vivo* labeling of tumor cells with BrdU and the subsequent detection of incorporated BrdU with specific anti-BrdU monoclonal antibodies is an accurate and comprehensive method to quantitate the degree of DNA-synthesis.

Materials and Reagents

1. BrdU (Sigma-Aldrich, catalog number: B5002)
2. Phosphate buffered saline (PBS)
3. Methanol (Thermo Fisher Scientific, catalog number: BP1105-4)
4. BSA (Sigma-Aldrich, catalog number: A3803)
5. HCl
6. NaN_3
7. Triton X-100
8. Monoclonal anti-BrdU (Sigma-Aldrich, catalog number: B2531)
9. Alexa 488-conjugated goat anti-mouse immunoglobulin G antibody (Life Technologies, Molecular Probes®/Alexa Fluor® 488, catalog number: A-11008 or A-11034)
10. DAPI (make 1 mg/ml stock, 1,000x) (Boeringer Manheim 236 276)
11. Coverglasses (12 mm) (Thermo Fisher Scientific, catalog number: 12-545-82)
12. Fluormount-G™ Mounting solution (SouthernBiotech, catalog number: 0100-01)
13. PBS-BT solution (see Recipes)

Equipment

1. Centrifuges (Beckman Falcon, model: TLS-55)
2. Parafilm

3. 6-well plate
4. 24-well plate

Procedure

1. Split cells onto tissue culture dishes containing coverglasses or chambered slides.
   Notes:
   a. *We usually put one coverglass in one well of the 24-well plate; or no more than 2 coverglasses in one well of the 6-well plate, or no more than 5 coverglasses in a 60 mm dish.*
   b. *Pipette up and down or shaking the dish to make sure cells are not concentrating in the center of the dish well. Make sure there are no air bubbles between the coverglasses and the tissue culture dish.*
2. Grow cells to 70-100% confluency.
3. Add 30 μM BrdU to cells. Incubate for 30-60 min.
   Note: BrdU is light sensitive and should be added in the dark. Cells pulsed with BrdU may be photosensitive -- incubations should be in the dark as well. Time of pulse and BrdU concentration variable with cell type and doubling time. Ranges from 15 min to 2 h, and from 10 μM to 100 μM. For example: We treat mouse embryonic fibroblast cells at 30 μM for 60 min.
4. Transfer the coverglasses or chambered slides into another tissue plate containing sufficient methanol (-20 °C stock), fix for 10 min or a couple of weeks.
5. Carefully transfer the coverglasses or chambered slides from the plates and place cell side up onto secured Parafilm. Wash the coverglasses or chambered slides with 100 μl PBS immediately after transfer, never dry the cells.
6. Block with 100 μl PBS-BT for 30 min.
7. Incubate 100 μl 2 M HCl, 30 min.
8. Wash 2x with 100 μl PBS-BT, each wash for 5 min
9. Incubate with 100 μl mouse anti-BrdU (1:100 dilution in PBS-BT), 30 min.
10. Wash 3x with 100 μl PBS-BT, each wash for 5 min.
11. Incubate with 40 μl second antibody (1:200 dilution in PBS-BT), 30 min.
12. Wash 3x with 100 μl PBS-BT, each wash for 5 min.
13. Cells were incubated in 40 μl DAPI (1 μg/ml final concentration, 1:1,000 dilute in PBS) for 2 min, and then wash with PBS once.
14. Add 5-10 μl mounting solution to a clean microscope slide for each coverglass, place stained coverglass cell side down onto mounting solution from one edge; allow mounting solution to cover the entire surface of the coverglass, avoiding air bubbles.

15. Let the mounting solution dry and self-seal for 30 min at RT.

Recipes

1. BrdU stock in PBS (pH 7.4) or ddH_2O, filter sterile (MW = 307.1, 20 mM = 0.006 g/ml)
2. PBS-BT solution
   10 ml 10x PBS
   3 g BSA (to 3%)
   1 ml 10 % Triton X-100 (to 0.1%)
   1 ml 5% NaN_3
   ddH_2O to 100 ml, store at 4 °C.

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References

1. Zhu, H., Coppinger, J. A., Jang, C. Y., Yates, J. R., 3rd and Fang, G. (2008). FAM29A promotes microtubule amplification via recruitment of the NEDD1-gamma-tubulin complex to the mitotic spindle. *J Cell Biol* 183(5): 835-848.

