Cell Proliferation Assay by Flow Cytometry (BrdU and PI Staining)

Hui Zhu*

Department of Genetics, Stanford University, Stanford, USA
*For correspondence: huizhu@stanford.edu

[Abstract] Cell Proliferation assays include an important set of fluorescence-based tests that can monitor cell health and cell division by evaluating DNA synthesis through thymidine incorporation. Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is a synthetic nucleoside that is an analogue of thymidine. BrdU is commonly used in the detection of proliferating cells in living tissues. BrdU can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle), substituting for thymidine during DNA replication. Antibodies specific for BrdU can then be used to detect the incorporated chemical, thus indicating cells that were actively replicating their DNA. Binding of the antibody requires denaturation of the DNA, usually by exposing the cells to acid or heat. The incorporation of BrdU is normally analyzed in flow cytometry by labelling with a conjugate anti-BrdU antibody and DNA dyes Propidium Iodide (PI) to perform cell cycle analysis.

Materials and Reagents

1. BrdU (Sigma-Aldrich, catalog number: B5002)
2. RNase A (Sigma-Aldrich, catalog number: R4642)
3. Tween 20 (Thermo Fisher Scientific, catalog number: BP337-500)
4. Triton X-100 (Sigma-Aldrich, catalog number: T9284)
5. BSA (Sigma-Aldrich, catalog number: A3803)
6. Propidium iodide (PI) (Sigma-Aldrich, catalog number: P4170)
7. Monoclonal anti-BrdU (Sigma-Aldrich, catalog number: B2531)
8. Goat-anti-Mouse IgG (Whole molecule) FITC conjugate (Sigma-Aldrich, catalog number: F0257)
9. Sodium Tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) (Sigma-Aldrich, catalog number: B9876)
10. 0.05% Trypsin-EDTA (Life Technologies, Gibco®, catalog number: 25300-062)
11. Phosphate buffered saline (PBS)
12. EtOH
13. HCl
14. Sodium tetraborate (see Recipes)
15. PBS/ 1% BSA (see Recipes)
16. PI stock solution (see Recipes)

Equipment

1. Centrifuges (Beckman Falcon, TLS-55)
2. Fluorescence Activated Cell Sorting (FACS) machine
3. FACS tubes (BD Biosciences, Falcon®, catalog number: 352054)

Procedure

1. Culture cells under optimum growing conditions (log growth phase at critical cell density). Add BrdU at a final concentration of 30 μM. 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.
2. Remove BrdU media. Rinse once with PBS.
3. Trypsinize and harvest cells.
4. Permeabilize cells: Rsuspend pellet in 0.3 ml PBS, agitate gently, then add 0.7 ml ice-cold 100% EtOH slowly. Mix gently with a 1 ml glass transfer pipette. Store at 4 °C at least 1 h.
   Note: The cell concentration following permeabilization should be approximately 10^6 cells/sample. Samples can be stored in EtOH for up to 2 weeks at 4 °C.
5. Pellet cells. Aspirate supernatant completely.
   Note: All pellet cells below at 4,000 rpm, 2 min.
6. Add 0.5 ml 2 N HCl/0.5% Triton X-100 and incubate 30 min at room temperature (RT).
7. Pellet cells and remove supernatant. Resuspend cells in 0.5 ml 0.1 M sodium tetraborate for 2 min. Pellet cells.
8. Wash cells once with 150 μl PBS/ 1% BSA.
9. Resuspend cells in 50 μl 0.5% Tween 20/1% BSA/PBS. Add 10-20 μl (1 μg/10^6 cells) α-BrdU (mAb) and incubate for 1 h at RT.
10. Pellet cells and wash cells once with 150 μl PBS/ 1% BSA.
11. Pellet cells and resuspend in 50 μl 0.5% Tween 20/1% BSA/PBS. Add 5 μl (1 μg/10^6 cells) Goat anti-Mouse IgG-FITC. Incubate 30 min at RT.
12. Pellet cells and transfer to FACS tubes.
13. Resuspend pellet in 0.5 ml PBS containing 10 μg/ml RNase A and 20 μg/ml PI stock solution.
14. Leave samples at RT for 0.5 h in the dark.
15. FACS immediately or store at 4 °C.

Notes: Flow cytometry setup controls needed:

a. *Cells stained with PI alone.*

b. *Cells with no BrdU pulse, and only the antibodies added.*

Recipes

1. 0.1 M sodium tetraborate (pH 8.5)
   2 N HCl
   0.5% Trition X-100 (0.05 ml)
   1.67 ml HCl in 8.28 ml ddH_2O
2. PBS/ 1% BSA
   0.1 mg BSA in 10 ml PBS
3. 0.5% tween 20/1% BSA/PBS
   0.05 ml Tween 20
   0.1 mg BSA in 9.5 ml PBS
4. 1 mg/ml PI stock solution
   10 mg PI
   10 ml ddH_2O
5. BrdU stock in PBS pH 7.4 or ddH_2O, filter sterile (MW = 307.1, 20 mM = 0.006 g/ml)

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References

1. Hoy, C. A., Seamer, L. C. and Schimke, R. T. (1989). Thermal denaturation of DNA for immunochemical staining of incorporated bromodeoxyuridine (BrdUrd): critical factors that affect the amount of fluorescence and the shape of BrdUrd/DNA histogram. *Cytometry* 10(6): 718-725.

