Protocol

Analyzing DNA Replication I: Labeling Animals, Tissues, and Cells with Bromodeoxyuridine (BrdU)

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This protocol was adapted from "Analyzing DNA Replication: Nonisotopic Labeling," Chapter 13, in *Basic Methods in Microscopy*, (eds. Spector and Goldman). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2006.

INTRODUCTION

The number of cells traversing the cell cycle and the rate of progression through it provide important indices of cell growth and tumorigenicity. S-phase cells can also be identified by their high content of DNA polymerase and proliferating cell nuclear antigen, a component of the leading-strand polymerase. Although both these markers can be detected rapidly and conveniently using the appropriate antibodies, neither are found exclusively in S-phase cells. Immunolabeling after incorporation of modified DNA precursors (e.g., 5-bromodeoxyuridine [BrdU, bromodeoxyuridine]) allows more rapid and precise detection of cells in S-phase of the cell cycle. BrdU is phosphorylated by cells to give BrdUTP, and this precursor is incorporated into DNA instead of deoxythimidine triphosphate. In living cells, BrdU is incorporated into replication sites that can then be detected using fluorochrome or enzyme-coupled antibodies. Alternatively, DNA synthesis sites can be labeled at high resolution by incubating cells with analogs of the natural precursors of DNA. The cells are then fixed and the incorporation sites are detected using fluorochrome- or enzyme-tagged antibodies. Cells labeled in this way either in vivo or in vitro display a few hundred discrete nuclear sites early in S-phase, with distinct patterns of DNA replication that are characteristic of different stages of S-phase. "Foci" labeled after very short incubations correspond with sites where many replicons are duplicated simultaneously within massive protein complexes. This protocol provides details for incorporating DNA precursors into tissues in whole animals in vivo, isolated tissue in vitro, and cultured cells.

RELATED INFORMATION

Procedures for the processing and detection of samples loaded with incorporated precursors are described in **Analyzing DNA Replication II: Fixation and Processing of Tissues and Cells Labeled with Bromodeoxyuridine (BrdU)** (Jackson and Cook 2008a) and **Analyzing DNA Replication III: Antibody Labeling of Incorporated Bromodeoxyuridine (BrdU) in Tissues and Cells** (Jackson and Cook 2008b). Because antibodies are available that have different affinities for different incorporated halogens, cell cycle parameters can also be assessed by double-labeling using iododeoxyuridine (IdU) and chlorodeoxyuridine or IdU and BrdU (Manders et al. 1992; Yanik et al. 1992).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Agarose microbeads (for cultured cell labeling; see Step 10.v)
<!>Bromodeoxyuridine (BrdU; Sigma)
Cell culture medium, appropriate for cell type to be examined (for tissue labeling and cultured cell labeling; see Steps 6.ii-8 and Step 10)
Collagenase (optional; see Step 6.ii)
<R>Initiation mix (10X; IM) (for labeling in vitro with phosphorylated precursors; see Step 14)
Material to be labeled (e.g., whole animal, cultured cells, etc.)
<R>Phosphate-buffered saline (PBS) (ice-cold for Steps 9 and 11)
<R>Physiological buffer (PB) (ice-cold for Step 15)
<!>Triton X-100 (for labeling in vitro with phosphorylated precursors; see Step 12)

Equipment

Coverslips (gelatin- or poly-L-lysine-coated) (for tissue labeling; see Step 7)
Coverslips (glass, 22-mm^2) (for cultured cell labeling; see Step 10.i)
Dishes (Petri, 35-mm diameter) (for cultured cell labeling; see Step 10.i)
Dissection equipment (for whole animal and tissue labeling; see Steps 3, 5, and 6)
Incubator preset to 33°C or 37°C
Filters (0.22-µm)
Minipump (osmotic) (optional; see Step 3)
Needles (for whole animal labeling; see Step 2)
Syringes (for whole animal labeling; see Step 2)

METHOD

Whole Animal Labeling (Mouse/Rat)

1. Dissolve 20-50 mg of BrdU in PBS. Filter-sterilize.
2. Inject BrdU at 5-50 mg/kg of body weight intraperitoneally.
   An intravenous dose of 100-200 mg/m^2 BrdU is sufficient for unambiguous labeling of S-phase cells in human tumors after 1-2 h (Hoshino et al. 1986; Yanik et al. 1992).
3. After 1-5 h (usually 1-2 h), sacrifice the animal and recover the tissue of interest.
   For continual labeling of cells in situ (over 2-7 d), low doses of BrdU can be administered with an osmotic minipump.
4. Rinse tissue in PBS.
   Samples are now ready to be fixed and immunolabeled.

Tissue Labeling

5. Sacrifice the animal. Remove the tissue of interest.
6. Mince or otherwise disperse the tissue:
   i. Cut into convenient pieces, e.g., 1-mm slices or 2-mm cubes. Return to medium.
   ii. Disperse tissue mechanically.
   Treat with collagenase if necessary; the conditions will depend on the tissue.
   iii. Collect the single-cell suspension. Resuspend in medium.
7. Seed cells on coverslips coated with gelatin or poly-L-lysine.
   Nonadherent cells can be purified and transferred to appropriate medium or encapsulated in agarose microbeads (see Step 10.v).

8. Prepare cell culture medium containing 20 µM BrdU. Incubate cells in this medium for 15 min at 37°C.

   Adjust concentration and time of BrdU incubation as required.

9. Rinse the cells in ice-cold PBS.

   Samples are now ready to be fixed and immunolabeled.

Cultured Cell Labeling

10. Grow cultured cells logarithmically.

    It is sometimes informative to use synchronized S-phase cells.

    For adherent cells

    Adherent cells can also be labeled after encapsulation in agarose microbeads; this is particularly useful for morphological analysis or if many subsequent manipulations are required (see Step 10.v).

    i. Place a clean 22-mm square glass coverslip in a 35-mm diameter Petri dish.

    ii. Seed ~ 2×10^5 cells in 2 mL of fresh medium.

    iii. Grow cells for 24-48 h, until cells are ~50% confluent.

    To label cells with phosphorylated precursors, proceed to Step 12.

    iv. Prepare fresh medium containing 20 µM BrdU. Replace growth medium with this fresh medium. Incubate for 15 min at 37°C.

    For nonadherent cells

    v. Encapsulate cells in agarose microbeads at a density of ~2×10^6/mL.

    vi. Regrow cells in fresh medium for 1 h.

    To label cells with phosphorylated precursors, proceed to Step 12.

    vii. Add BrdU to a final concentration of 20 µM. Regrow for 15 min.

11. Rinse cells in ice-cold PBS.

    Samples are now ready to be fixed and immunolabeled.

Labeling In Vitro with Phosphorylated Precursors

This procedure can be used on cells prepared on coverslips (Steps 10.i-10.iii) or encapsulated in agarose microbeads (Steps 10.v-10.vi).

12. Prepare PB containing 0.1%-0.2% (v/v) Triton X-100 and permeabilize cells in this solution for 5 min.

13. Incubate coverslips or microbeads in PB for 5 min at 33°C.

    If inhibitors are to be used, incubate cells in them for 15 min at 0°C and for 5 min at 33°C prior to addition of 10X IM.

14. Add 0.1 volumes of 10X IM and mix. Incubate for 2-60 min at 33°C.

    The concentration of modified precursor and duration of labeling can be adjusted to suit individual requirements. See Discussion.

15. Wash three times in >10 volumes of ice-cold PB.

    Samples are now ready to be fixed and immunolabeled.

DISCUSSION

For many applications, labeling in vitro with phosphorylated precursors provides an appealing versatility and has a number of advantages. After permeabilization, precursor pools can be depleted by washing. Elongation rates can be modified at will by adjusting precursor concentrations. A range of

different precursors is available. Some precursors label sites of DNA synthesis directly and so can be used without fixation, and others do not require DNA denaturation during subsequent detection; this allows better preservation of nuclear morphology. Note that incorporated BrdU can only be detected after the DNA is denatured, unless thin sections are immunolabeled for electron microscopy. Modified precursors incorporated into DNA can be removed by the repair pathway, but such repair is inefficient in permeabilized HeLa cells.

For labeling in vitro with biotin- or digoxigenin-coupled precursors (e.g., 20 µM), 15-min incubations give good indirect immunofluorescence signals, and longer incubations give correspondingly stronger signals. With 100 µM biotin-16-dUTP, 5- and 2-min incubations allow detection by light and electron microscopy, respectively, using standard detection protocols. With 20-µM fluorescent precursors, incorporated label can be detected after incubation for 30 to 60 min.

REFERENCES

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