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Protocol

Analyzing DNA Replication III: Antibody Labeling of Incorporated Bromodeoxyuridine (BrdU) in Tissues and Cells

Dean Jackson and Peter R. Cook

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. After fixation to preserve nuclear morphology, the DNA duplex is denatured to allow antibodies access to the BrdU. 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. This protocol describes two commonly used methods of denaturation, as well as techniques for antibody labeling of mounted tissues and encapsulated cells.

RELATED INFORMATION

Methods for loading and processing animals, tissues, and cells with nonisotopic nucleotide precursors are described in **Analyzing DNA Replication I: Labeling Animals, Tissues, and Cells with Bromodeoxyuridine (BrdU)** (Jackson and Cook 2008a) and **Analyzing DNA Replication II: Fixation and Processing of Tissues and Cells Labeled with Bromodeoxyuridine (BrdU)** (Jackson and Cook 2008b).

Note that samples loaded with fluorescently tagged triphosphate precursors generally can be mounted and examined with minimal additional processing.

MATERIALS

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

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Reagents

Antibody, primary (e.g., mouse anti-BrdU, mouse anti-digoxigenin, goat anti-biotin)

Although commercially available anti-BrdU antibodies react poorly with BrdUMP in native DNA, denaturation increases antigenicity 10-20-fold. Commercial anti-BrdU antibodies are usually mouse monoclonals. Sera-Lab supplies a rat monoclonal that is useful for double labeling.

Antibody, secondary, fluorochrome- or enzyme-conjugated (e.g., fluorescein isothiocyanate [FITC] goat anti-mouse, alkaline phosphatase goat anti-mouse)
Bovine serum albumin, Fraction V (BSA; Sigma)
Chemicals for developing enzyme-coupled reagents (see Step 6)
<**!**>DAPI (4′,6-diamidino-2-phenylindole) (for antibody labeling of encapsulated cells; see Step 16)
DNase I (Sigma) (for nuclease-dependent denaturation; see Step 1.viii)
<**!**>HCl (2 N) (for acid denaturation; see Step 1.ii)
<**!**>$MgCl_2$ (for nuclease-dependent denaturation; see Step 1.viii)
Mounting medium (e.g., Vectashield, Vector Laboratories)
$Na_2B_4O_7$ (0.1 M) (sodium tetraborate; for acid denaturation; see Step 1.iii)
<**R**>PBS containing 0.1% (v/v) Tween 20 (PBT)
<**R**>PBS containing 0.1% (v/v) Tween 20 and 0.5% (w/v) BSA (PBT/BSA) (ice-cold for Steps 8, 11, and 14)
<**R**>Phosphate-buffered saline (PBS)
<**R**><**!**>SSC (2X) containing 50% (v/v) deionized formamide (optional; see Step 1)
Tissue or cells of interest (mounted or encapsulated in agarose microbeads, fixed)
Tris-buffered saline (if using alkaline phosphatase-labeled antibodies in Step 3; as alternative to PBS in Step 5)

Equipment

Chamber (humidified)
Coverslips
Incubator (humidified) preset to 37°C (65°C if using SSC-formamide denaturation; see Step 1)
Microcentrifuge
Nail polish
Tubes (microcentrifuge)
Slides, glass

METHOD

Denaturation

There is no need to denature DNA after incorporating nonhalogenated precursors (e.g., biotin-16-dUTP, digoxigenin-11-dUTP, etc.).

1. Treat samples to denature the DNA:

   DNA can also be denatured by incubating samples with 2X SSC containing 50% (v/v) deionized formamide for 10 min at 65°C.

   For acid denaturation

   Loss of morphology accompanies acid denaturation. Use nuclease-dependent detection systems if morphological considerations are critical.

   i. Rinse slides or coverslips in H_2O.

   ii. Denature DNA in 2 N HCl for 1 h at room temperature.

   iii. Neutralize DNA in 0.1 M $Na_2B_4O_7$ in H_2O.

   iv. Rinse samples twice in PBS.

   v. Add primary antibody diluted 1/50-1/500 (determined empirically) in PBT/BSA.

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vi. Incubate in a humidified chamber for 1-2 h at room temperature.

For nuclease-dependent denaturation

Nuclease-dependent detection systems improve morphology at the expense of sensitivity.

vii. Rinse slides or coverslips in PBS.

viii. Prepare PBT containing 0.5% BSA, 50 µg/mL DNase I, and 2.5 mM $MgCl_2$. Dilute primary antibody 1/50-1/500 (determined empirically) in this solution and add the diluted antibody to the slides or coverslips.

ix. Incubate in a humidified chamber for 1-2 h at 37°C.

Antibody Labeling of Mounted Samples

2. Wash the samples three times for 10 min at room temperature each in PBT/BSA.
3. Prepare fluorochrome- or enzyme-labeled secondary antibody diluted 1/50-1/1000 in PBT/BSA and add it to the samples.
   Dilute alkaline phosphatase-labeled antibodies in Tris-buffered saline.
4. Incubate the samples in a humidified chamber at room temperature for 1-2 h.
5. Wash the samples with PBS (or Tris-buffered saline) three times, 15 min each, at room temperature.
6. Develop enzyme-coupled reagents using standard protocols.
7. Prepare samples for microscopy using an appropriate mounting medium.
   Specimens are now ready for microscopic examination.
   See Troubleshooting.

Antibody Labeling of Encapsulated Cells

8. Wash agarose microbeads containing fixed cells twice in ice-cold PBT/BSA.
9. Mix 100 µL of the beads with 400 µL of primary antibody diluted 1/400 in PBT/BSA.
10. Incubate for 2 h at 0°C with periodic mixing.
11. Wash three times, 10 min each, in 10 volumes of ice-cold PBT/BSA.
12. Mix the bead pellet with 400 µL of fluorochrome-coupled secondary antibody diluted 1/400 in PBT/BSA.
13. Incubate for 2 h at 0°C with periodic mixing.
14. Wash three times, 10 min each, in 10 volumes of ice-cold PBT/BSA.
15. Wash once for 5 min at room temperature in 10 volumes of PBS.
16. Prepare PBS containing 0.02 µg/mL DAPI. Wash once for 5 min at room temperature in 10 volumes of this solution.
17. Wash once for 5 min at room temperature in 10 volumes of PBS.
18. Mix 5 µL of beads with an equal volume of mounting medium on a glass slide.
19. Apply coverslip with gentle pressure to eliminate excess fluid. Seal with nail polish.
    Specimens are now ready for microscopic examination.
    See Troubleshooting.

TROUBLESHOOTING

Problem: There is nonspecific antibody binding.
[After Steps 7 and 19]
Solution: Incubate samples with 5% normal serum (from the animal species in which the secondary antibody was generated) diluted in PBS prior to primary antibody labeling.

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Problem: Sensitivity is low.
[After Steps 7 and 19]
Solution: Amplify the signal by using a biotinylated secondary antibody followed by streptavidin coupled to alkaline phosphatase or a fluorochrome.

DISCUSSION

Fluorochrome-coupled secondary antibodies provide high-resolution detection and can be analyzed using confocal laser scanning microscopes or sensitive, cooled, charge-coupled-device cameras. Fluorescence-based detection systems also allow convenient multiple labeling (see, e.g., O'Keefe et al. 1992). Enzyme-dependent detection systems are generally sensitive but give poor resolution; the different and characteristic S-phase replication patterns (Fig. 1) are usually obscured. Similar sensitivity, but with higher resolution, can be achieved using 1-nm gold-conjugated secondary antibody and silver enhancement (Meyer et al. 1989) or post-embedding immunogold labeling, which requires no DNA denaturation step (O'Keefe et al. 1992).

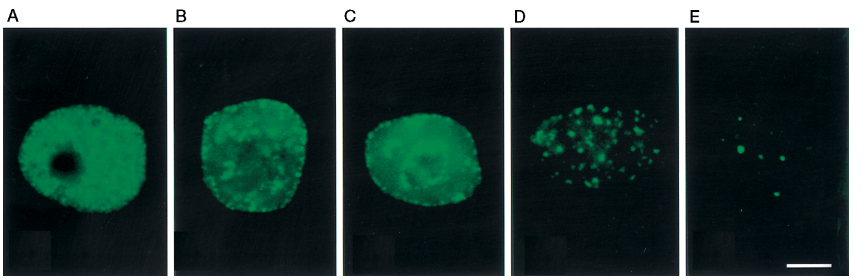

FIGURE 1. HeLa cells display five distinct patterns of DNA replication. Early in S-phase, numerous foci of replication are observed scattered throughout the nucleoplasm (*A*). Later in S-phase, larger clusters of DNA appear to replicate (*B-E*) with chromosome-specific α-satellite DNA sequences replicating in mid S-phase (*C*). Bar, 10 μm. (Reprinted from O'Keefe et al. 1992 with permission from Rockefeller University Press © 1992.)

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot5033

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