Replication Labeling with Halogenated Thymidine Analogs

DNA replication is essential for cell proliferation. Genetic information contained in the genome must be duplicated before each cell division (Gilbert, 2002; Goren and Cedar, 2003). Because the genome of eukaryotes consists of long, linear chromosomes, DNA replication must initiate at many positions along the length of the entire chromosome in order to complete replication in a timely fashion. DNA replication proceeds via the synchronous firing of clusters of about six replication origins that together form coordinate replicated domains of several hundred kilobases (Berezney et al., 2000). These coordinate-replicated “replication domains” appear as punctuate foci when sites of DNA synthesis are pulse-labeled. Despite the fact that replication domains derive from many different chromosomal sites, the same cohort of replication foci replicate synchronously from cell cycle to cell cycle, implying a tight coordination of the temporal order of replication domains throughout S-phase (Ma et al., 1998; Dimitrova and Gilbert, 1999; Zink et al., 2004). Although the biological function of this temporal order is not understood, it has been shown that domains that replicate at particular times are localized to distinct regions of the nucleus, suggesting a relationship between replication domains and nuclear organization (McNairn and Gilbert, 2003). Mounting evidence suggests that replication domains form structural and functional units of chromosome organization (Bornfleth et al., 1999; Edelmann et al., 2001; Sadoni et al., 2004). Because replication is regulated at the level of large chromosomal domains, studies relating DNA replication to nuclear structure and function can be performed with the light microscope.

In higher eukaryotes, the molecular factors that dictate where and when replication will initiate are poorly understood. Replication origins do not appear to consist of any particular DNA sequence (Gilbert, 2001, 2004). Furthermore, replication origin activity is regulated both during development and in response to DNA damage or the metabolic state of cells (Anglana et al., 2003; Gilbert, 2005; Norio et al., 2005). Hence, studies of replication origins must be performed without any bias regarding where they may appear under different conditions. The distribution and spacing of origins throughout the genome, as well as the rate of elongation of replication forks, can reveal important information regarding the physiological state of cells without knowledge of the specific initiation sites. In addition, observing the positions of replication origins along the length of specific DNA fibers can reveal the positions of initiation sites to within a few kilobases.

In this unit, several conventional protocols to visualize replication foci in mammalian cells are described. Basic Protocol 1 describes visualizing DNA replication sites with bromodeoxyuridine (BrdU); this is the most basic procedure to visualize replication foci by identifying the sites of DNA synthesis. Newly synthesized DNA can be labeled with halogenated thymidine analogs such as BrdU. After immunostaining, replication foci containing BrdU-labeled replication forks can be observed using fluorescence microscopy. Basic Protocol 2, a combination of replication labeling and fluorescence in situ hybridization, is a typical application to examine the replication and subnuclear location of specific sequences, revealing the positional relationship between DNA synthesis and specific DNA regions. Basic Protocol 3, dual replication labeling with chloro- and iododeoxyuridine, provides an opportunity to examine the dynamics of DNA synthesis without the need for cumbersome cell-synchronization methods. This protocol distinguishes two different stages of S-phase by separating two different pulse labels with increasing chase times. Finally, Basic Protocol 4, replication labeling of DNA fibers, is
a convenient method to observe replication sites on stretched DNA fibers, rather than on tangled DNA strands in the nucleus. Methods to examine the positional relationship between DNA synthesis and specific proteins or histone modifications have been described elsewhere (Wu et al., 2005b).

**VISUALIZING DNA REPLICATION SITES WITH BROMODEOXYURIDINE (REPLICATION LABELING)**

This protocol describes replication labeling. BrdU is added to culture medium to label replication sites (BrdU incorporation). Cells are treated with fixatives and detergents as necessary (fixation and permeabilization). BrdU incorporated in the genome is recognized by incubation with an appropriate antibody (immunostaining). Fluorophore-conjugated antibodies are observed by fluorescence microscopy (visualization). Anticipated images are shown in Figure 22.10.1.

**Materials**

- Adherent tissue culture cells and appropriate medium
- 10 mg/ml bromodeoxyuridine (BrdU) stock in sterile tissue culture-grade water (store indefinitely at −20°C)
- Phosphate-buffered saline (PBS; APPENDIX 2A)
- 2% (w/v) paraformaldehyde in PBS
- 0.4% (v/v) Triton X-100
- 1.5 N HCl
- 5% (w/v) BSA in PBST (0.5% v/v Tween 20 in PBS)
- Primary antibody: monoclonal mouse anti-BrdU antibody (Becton Dickinson)

![Figure 22.10.1](http://www.currentprotocols.com) (A) Replication patterns visualized by BrdU incorporation during S-phase. Images are shown by courtesy of The American Society For Cell Biology. (Wu et al., 2005a). (B) DNA fibers visualized by CldU incorporation (F. Li and D.M. Gilbert, unpub. observ.). For the color version of this figure go to [http://www.currentprotocols.com](http://www.currentprotocols.com).
Secondary antibody: Alexa Fluor 495-conjugated goat anti-mouse antibody (Molecular Probes)
PBST: 0.5% (v/v) Tween 20 in PBS
1 μg/ml DAPI in Vectashield (Vector Labs) mounting medium
100-mm tissue culture dishes for plating cells on coverslips
12-mm-diameter round glass coverslips, sterilized by flaming after dipping in 70% ethanol or by autoclaving
24-well tissue culture plate
Glass microscope slides
Clear nail polish
Fluorescence microscope (UNIT 4.2) with appropriate color filters for fluorophores used, e.g., blue (DAPI), green (FITC, Alexa Fluor 488), and red (e.g., Texas Red, Alexa Fluor 594)

Additional reagents and equipment for cell culture (UNIT 1.1) and fluorescence microscopy (UNIT 4.2)

Prepare cells labeled with BrdU

1. Plate 5 × 10^6 adherent cells in a 100-mm tissue culture dish containing several round coverslips. Incubate overnight to allow cells to attach to coverslips.

   The authors use mammalian cells in their laboratory but believe that any tissue culture cells can be used in this protocol. The size of the dish and number of cells plated may vary depending upon the density desired. If cell density is too low, the yield in terms of cell number will be compromised; if density is too high, BrdU incorporation is decreased. Typically use 0.5–2 × 10^6 cells/ml.

   Basic cell culture techniques are described in UNIT 1.1.

2. Add sufficient 10 mg/ml BrdU stock solution for a final concentration of 10 μg/ml. Incubate the dish 30 min at 37°C.

3. Transfer the coverslips into a new container (24-well tissue culture plate recommended) containing prewarmed growth medium. Rinse the cells on the coverslip twice, each time with 1 ml PBS.

   Use of a 24-well culture dish as a container is convenient for handling many coverslips. Coverslips can be transferred to the 24-well dish one at a time prior to the BrdU labeling step to allow multiple time points with the same cell population.

4. Aspirate PBS. Add 0.5 ml/well of 2% paraformaldehyde solution and incubate for 10 min at room temperature. Wash twice with 1 ml/well PBS and remove the medium. Add 0.5 ml/well of 0.4% Triton X-100 and incubate for 10 minutes to permeabilize the cells. Wash twice with 1 ml/well PBS.

   If 3-D structure of the nucleus is not important, simply add 70% ethanol to fix the cells and incubate for 10 min at room temperature. The fixed cells on the coverslip can be stored in the 70% ethanol solution at 4°C for 1 week.

5. Aspirate PBS. Add 0.5 ml/well of 1.5 N HCl and incubate for 30 min to denature DNA.

6. Wash twice with 1 ml/well PBS and remove the medium.

7. Add 5 ml/well of 5% BSA and incubate for 10 min at room temperature. Remove the medium.

Immunostain cells

The antibody dilution factors below are those typically used in the authors’ laboratory, but these should be determined empirically, and they depend on the cells and culture conditions used.
8. Dilute primary antibody (i.e., anti-BrdU mouse monoclonal antibody) 1:10 to 1:100 (depending on affinity of secondary antibody) in PBST. Add 30 µl of the diluted antibody to the cells on each coverslip. Incubate 30 min at room temperature.

9. Wash three times with 1 ml/well PBST. Aspirate PBST.

10. Dilute fluorophore-conjugated secondary antibody (i.e., Alexa Fluor 495–conjugated goat anti-mouse antibody) 1:100 in PBST. Add 30 µl of the diluted secondary antibody to the cells on each coverslip. Incubate 30 min at room temperature.

11. Wash three times with 1 ml/well PBST. Aspirate PBST.

**Mount cells for imaging**

12. Pipet 5 µl Vectashield containing 1 µg/ml DAPI on a glass microscope slide. Place the round coverslip with the labeled cells, cell-side-down, over the drop and seal with clear nail polish.

   *Avoid air bubbles. If trapped air bubbles are a problem, Vectashield can be pipetted directly onto the coverslip first; the coverslip is then placed on the glass slide with the cell side facing down.*

13. Observe the cells using fluorescence microscopy (UNIT 4.2) with appropriate filters for the fluorophore.

**COMBINATION OF REPLICATION LABELING AND FLUORESCENCE IN SITU HYBRIDIZATION (FISH)**

This protocol describes how to simultaneously visualize replication sites and specific sequences in the genome by fluorescence in situ hybridization (FISH). Cells are swollen in hypotonic buffer before being fixed using methanol/acetic acid (3:1). The DNA region of interest—gene locus, promoter region, or functional domain—is visualized by in situ hybridization with a specific DNA probe, while replication domains are visualized by BrdU labeling. Up to eight glass slides can be processed in one Coplin jar using this procedure.

**Materials**

- Adherent tissue culture cells and appropriate medium
- 10 mg/ml bromodeoxyuridine (BrdU) stock (store indefinitely at −20°C)
- Phosphate-buffered saline (PBS; **APPENDIX 2A**)
- Trypsin solution (see recipe)
- Fixative: 3:1 (v/v) methanol/acetic acid (prepare fresh and store on ice)
- 75 mM KCl
- 0.0625 µg/µl probe DNA sequence in TE buffer (see **APPENDIX 2A** for TE buffer)
- Roche Nick Translation Mix (for digoxigenin or biotin, depending on desired labeling)
- 0.5 mM disodium EDTA, pH 8.0
- Hybridization mix (see recipe)
- 2× SSC (**APPENDIX 2A**) containing 0.1 mg/ml RNaseA
- 2× and 0.1× SSC (**APPENDIX 2A**)
- 70%, 90%, and 100% (v/v) ethanol
- Clear nail polish
- 2× SSC (**APPENDIX 2A**) 70% (v/v) formamide
- 2× SSC (**APPENDIX 2A**) 50% (v/v) formamide
- SSCT: 4× SSC (**APPENDIX 2A**) 0.1% (v/v) Tween 20
- Blocking solution: SSCT (see above) containing 5% (w/v) nonfat dry milk
Primary antibody solution: cocktail in blocking solution of sheep anti-digoxigenin, FITC-conjugated (Molecular Probes) and mouse anti-BrdU (Becton Dickinson), each at a dilution of 1:10
Secondary antibody solution: cocktail in blocking solution of rabbit anti-sheep IgG, FITC-conjugated (Vector Labs), diluted 1:10, and goat anti-mouse IgG, Alexa Fluor 594–conjugated (Molecular Probes), diluted 1:500

Vectashield mounting medium (Vector Labs)

100-mm tissue culture dishes
15-ml conical centrifuge tubes
Centrifuge
Glass microscope slides
15°C water bath (kept in cold room)
45°, 65°, and 80°C water bath
Coplin jars
Vacuum desiccator with desiccant (e.g., silica gel)
18 × 18-mm square glass coverslips, sterile
Slide warmer (Precision Scientific)
Humidified chamber (e.g., covered Tupperware container with moistened paper towels on the bottom)
22 × 40-mm glass coverslips
Fluorescence microscope (UNIT 4.2) with appropriate color filters for fluorophores used, e.g., blue (DAPI), green (FITC, Alexa Fluor 488), and red (e.g., Texas Red, Alexa Fluor 594)

Additional reagents and equipment for cell culture technique including counting cells (UNIT 1.1) and fluorescence microscopy (UNIT 4.2)

Grow cells and treat with BrdU

1. Grow the cells in a 100-mm tissue culture dish to the appropriate cell density (UNIT 1.1).

   For overnight incubation seed 5 × 10⁶ cells.

   If cell density is too low, the yield in terms of cell number will be compromised; if density is too high, BrdU incorporation is decreased. Typically, use 0.5–1 × 10⁶ cells/ml.

2. Add 10 mg/ml BrdU to a final concentration of 10 µg/ml. Incubate the dish for 30 min at 37°C.

3. Rinse the cells twice with 10 ml PBS.

Permeabilize cells by hypotonic shock

4. Dissociate the cells into a single-cell suspension using 2 ml trypsin solution (also see UNIT 1.1), add 6 ml of serum-containing growth medium to stop the reaction, and collect them in a 15-ml conical tube. Determine cell number (UNIT 1.1).

5. Centrifuge cells 1 min at 200 × g, room temperature. Remove the supernatant. Resuspend the cells gently with 10 ml of 75 mM KCl at 1 × 10⁶ cells/ml (total 1 × 10⁷ cells) and incubate for 15 min at room temperature to swell the cell bodies.

   Cell concentration is critical for swelling.

   Prepare fresh fixative solution (3:1 methanol/acetic acid) during this time and keep it on ice.

Fix cells

6. Add 300 µl methanol/acetic acid fixative per 10 ml of KCl solution, dropwise, while gently vortexing the cell suspension. Leave the mixture 5 to 10 min at room temperature.

   During this incubation period, do not agitate the cell suspension, as this causes cells to aggregate.
7. Centrifuge the cells 1 min at 200 × g, room temperature. Remove the supernatant.

8. Add sufficient methanol/acetic acid fixative to adjust the cell density to 2 × 10⁶ cells/ml. Incubate cells at −20°C for least 1 hr or keep this cell suspension overnight in −20°C freezer.

9. Transfer 1 × 10⁷ cells into a new 15-ml conical tube. Centrifuge the cells and resuspend the pellet with 5 ml methanol/acetic acid fixative.

   Note that the final cell density should be ~ 2 × 10⁶ cells/ml.

   This step is essential to remove unnecessary salt that may interfere with clear imaging on the microscope. The cell suspension after this step can be stored at −20°C for up to 3 months.

Prepare mitotic spreads on glass slides
10. Dilute the fixed cell suspension 1/2 to 1/20 in methanol/acetic acid fixative.

11. Place or drop 10 μl of the diluted cell suspension on the glass microscope slide. Dry the specimen at room temperature for 10 min.

   At this stage, slides can be stored in the dark at room temperature indefinitely.

Prepare DNA probe labeled with digoxigenin or biotin and prepare probe mix
12. Mix 16 μl of 0.0625 μg/μl probe DNA solution (total amount 1 μg DNA) and 4 μl of Roche Nick Translation Mix (for digoxigenin or biotin, depending on desired labeling). Incubate for 90 min at 15°C in a water bath kept in a cold room.

13. Add 1 μl of 0.5 mM disodium EDTA pH 8.0, to stop the reaction. Heat the mixture at 65°C for 10 min. Combine 1 μl of this labeled probe DNA with 12 μl hybridization mix to prepare the probe mix. Mix well and denature 5 min at 95°C, then immediately place on ice and let stand 5 min.

Perform in situ hybridization
14. In Coplin jars, prewarm 40 ml 2× SSC containing 0.1 mg/ml RNase A at 37°C and 40 ml 2× SSC/70% formamide at 80°C.

15. Incubate the slides in 2× SSC containing 0.1 mg/ml RNase A in the first Coplin jar at 37°C for 30 min.

16. Transfer the slides to a second Coplin jar containing 2× SSC and wash briefly.

17. Dehydrate slides in Coplin jars containing 40 ml of each of the following ethanol solutions at room temperature for the times indicated:

   70% ethanol for 4 min
   90% ethanol for 4 min
   100% ethanol for 4 min.

18. Desiccate the slides under low pressure in a vacuum desiccator containing desiccant (e.g., silica gel) for at least 10 min.

19. Denature the slides in 40 ml 2× SSC/70% formamide at 80°C for 2 min.

20. Immediately dehydrate slides in Coplin jars containing 40 ml of each of the following ethanol solutions at room temperature for the times indicated:

   70% ethanol for 2 min
   90% ethanol for 2 min
   100% ethanol for 2 min.

21. Desiccate the slides in a vacuum desiccator for at least 10 min.
22. Warm the slides, probe mix (from step 13), and 18 × 18-mm coverslips on a slide warmer at 37°C. Place a humidified chamber in a 37°C incubator.

23. Pipet 12 µl probe mix on a prewarmed coverslip, then put it upside down on the slide. Seal with clear nail polish and place in the prewarmed humidified chamber. Incubate in 37°C incubator for 6 to 12 hr.

*Placing probe mix solutions on coverslips first before placing on the glass slides helps avoid trapping air bubbles under the coverslip.*

24. Prewarm at least 120 ml of 2× SSC/50% formamide and at least 120 ml of 2× SSC at 45°C. Prewarm at least 120 ml of 0.1× SSC at 45°C to 80°C (optimal temperature determined empirically, see annotation to step 25).

25. Remove the nail polish from around the coverslip and wash the slides in Coplin jars for 5 min with 40 ml each of the washing solutions shown below:

- 2× SSC/50% formamide at 45°C, three washes
- 2× SSC at 45°C, three washes
- 0.1× SSC at 45° to 80°C (optimized empirically), three washes.

*Every probe has unique hybridization properties. To achieve better signal-to-noise ratio, conditions for the final washing step (with 0.1× SSC) should be determined empirically. If background proves too high in a pilot experiment, use a higher temperature of 0.1× SSC (i.e., more stringent washing conditions; 80°C); if background is low and signal is low, use a lower temperature of 0.1× SSC (more relaxed washing conditions; 45°C).*

26. Wash briefly in 40 ml SSCT in a Coplin jar at room temperature. Incubate the slides in a Coplin jar with 40 ml blocking solution at room temperature for >30 min.

**Immunostain cells**

27. Pipet 30 µl primary antibody solution onto the slide and cover with a 22 × 40-mm coverslip. Incubate the slide for 40 min at room temperature.

*It should be noted that the primary antibodies used here to detect the BrdU and FISH (digoxigenin) signals must be produced in different animal species in order to prevent cross-reactation of the secondary antibodies.*

*Note that although the primary and secondary antibodies in this protocol are applied as a cocktail, in some cases this is not possible owing to the incompatibility of antibodies. For instance, if one of the secondary antibodies (e.g., rabbit anti-sheep IgG-FITC) were mouse anti-sheep IgG-FITC, it would not be possible to use the cocktail method because another secondary antibody (goat anti-mouse IgG-Alexa 594) could have a cross-reaction. In that case, the secondary antibodies would have to be applied sequentially, e.g., goat anti–mouse IgG–Alexa 594 first, then mouse anti–sheep IgG–FITC second.*

*If biotin was used to label the probe (see step 12) only an anti-BrdU primary antibody is used, and the FISH signal is detected by fluorochrome-labeled avidin.*

28. Wash the slides in Coplin jars three times, each time with 40 ml SSCT for 2 min.

29. Repeat steps 27 and 28 for the secondary antibodies.

*Signal intensity can be increased by sequential “sandwich” method if necessary (e.g., use avidin-Texas red as a first layer. Then use goat anti-avidin antibody conjugated to biotin as the second layer. Repeat this cycle to increase the signal).*

**Mount cells for imaging**

30. Add 5 µl Vectashield mounting medium per slide and cover with 18 × 18-mm coverslip. Seal with clear nail polish and observe using a fluorescence microscope with appropriate filters for the fluorophores used *(UNIT 4.2).*
DUAL REPLICATION LABELING WITH CHLORO- AND IODODEOXYURIDINE

This section describes the procedure for visualizing replication sites using two halogenated thymidine analogs such as chlorodeoxyuridine (CldU) and iododeoxyuridine (IdU). It should be noted that the choice of primary antibodies is the key in this protocol. CldU is recognized by rat anti-BrdU antibody (Accurate Chemical), while IdU is recognized by mouse anti-BrdU antibody (Becton Dickinson). Anti-rat and anti-mouse IgG antibodies are used as secondary antibodies, respectively.

Materials

Cell culture on 12-mm round coverslips (Basic Protocol 1) in culture dish
10 mg/ml chlorodeoxyuridine (CldU) stock solution (store indefinitely at −20°C)
10 mg/ml iododeoxyuridine (IdU) stock solution (store indefinitely at −20°C)
Phosphate-buffered saline (PBS; APPENDIX 2A)
70% (v/v) ethanol
100% methanol
1.5 N HCl
PBST (0.5% v/v Tween 20 in PBS) containing 5% (w/v) BSA
PBST: 0.5% (v/v) Tween 20 in PBS
Primary antibody for CldU: rat anti-BrdU (Accurate Chemical)
Secondary antibody for CldU: FITC-conjugated goat anti-rat Ig (Molecular Probes)
Primary antibody for IdU: mouse anti-BrdU (Becton Dickinson)
Secondary antibody for IdU: Texas Red–conjugated donkey anti–mouse Ig
(Jackson ImmunoResearch)
High-salt buffer (see recipe)
Vectashield mounting medium (Vector Labs)
Clear nail polish
12-mm-diameter round glass coverslips, sterilized by flaming after dipping in 70% ethanol or by autoclaving
100-mm petri dishes and 24-well plates
Humidified chamber (e.g., covered Tupperware container with moistened paper towels on the bottom)
Glass microscope slides
Fluorescence microscope (UNIT 4.2) with appropriate color filters for fluorophores used, e.g., blue (DAPI), green (FITC, Alexa Fluor 488), and red (e.g., Texas Red, Alexa Fluor 594)
Additional reagents and equipment for fluorescence microscopy (UNIT 4.2)

Pulse-chase-pulse label with CldU and IdU

1. Culture the cells on 12-mm round coverslips as in Basic Protocol 1.

2. Add the first halogenated thymidine analog (e.g., CldU) to the culture from 10 mg/ml stock solution for a final concentration of 10 µg/ml, to incorporate CldU in the genome. Incubate 30 min at 37°C.

   This is the first pulse step.

3. Change the medium to remove the analog and incubate the cells for the desired length of chase time.

   This is the chase step. The chase time depends on the objective of dual labeling and the properties of the cells (length of S-phase). To visualize the transition of replicating foci, the chase time can be omitted. To visualize the early and late replicating foci through the S-phase, the chase time should be 6 to 10 hr, depending on the length of the entire S-phase.
4. Add the second halogenated thymidine analog (e.g., IdU) to the culture from 10 
mg/ml stock solution for a final concentration of 10 μg/ml. Incubate the dish for 30 
min at 37°C.

   This is the second pulse step.

5. Rinse the cells on the coverslip twice with 10 ml PBS.

Prepare fixed cells on coverslips after double labeling

6. Immerse the coverslip in 10 ml of 70% ethanol in a 100-mm petri dish for 5 min at
   room temperature.

   Alternatively, transfer coverslips individually into wells of a 24-well plate at this step. In
   that case, use 1 mlwell of following reagents: 100% methanol (step 7), PBS (step 8), and
   1.5 N HCl (step 9) per coverslip directly in the wells.

   The specimens can be stored indefinitely until use.

7. Incubate with 10 ml of 100% methanol for 5 min at room temperature.

8. Wash with 10 ml PBS twice.

9. Incubate with 10 ml 1.5 N HCl for 30 min at room temperature.

10. Wash with 10 ml PBS twice.

Stain with antibodies against CldU and IdU

Perform CldU staining

11. Block by incubating with 10 ml of 5% BSA in PBST for 20 min at room temperature.
    Transfer the coverslips to wells of a 24-well plate or a humidified chamber. Aspitate
    blocking solution.

    If the glass coverslips are kept inside a 24-well plate, the humidified chamber is not
    necessary; otherwise all staining steps should be done inside the humidified chamber.

12. Pipet 30 μl primary antibody (rat anti-BrdU antibody; diluted 1:10 in 5% BSA/PBST)
    onto the coverslip. Incubate 1 hr at room temperature.

13. Wash the coverslip three times, each time with 1 ml PBST for 5 min.

14. Pipet 30 μl secondary antibody (goat anti–rat IgG conjugated to FITC; diluted 1:10
    in 5% BSA/PBST) onto the coverslip and incubate 1 hr at room temperature.

15. Wash three times with 1 ml PBST, each time for 5 min.

Perform IdU staining

16. Block by incubating with 10 ml of 5% BSA in PBST in a 100-mm petri dish for
    20 min at room temperature. Transfer the coverslips to wells of a 24-well plate or a
    humidified chamber.

    If the glass coverslips are kept inside a 24-well plate, the humidified chamber is not
    necessary; otherwise all staining steps should be done inside the humidified chamber.

    When CldU and IdU staining are performed sequentially, this blocking step can be
    omitted, but be sure to prepare the antibody dilutions in blocking solution.

17. Pipet 30 μl primary antibody solution (mouse anti-BrdU antibody; diluted 1:10 in
    5% BSA/PBST) onto the coverslip. Incubate for 1 hr at room temperature.

18. Wash the coverslip with 1 ml PBST three times, each time for 5 min at room
    temperature, then wash with 1 ml high-salt buffer for 15 min at room temperature.

    The wash with high-salt buffer is essential to avoid cross-reactivity of antibodies.
19. Pipet 30 μl secondary antibody (donkey anti–mouse IgG conjugated to Texas Red; diluted 1:10 in 5% BSA/PBST) onto the coverslip and incubate for 1 hr at room temperature.

20. Wash three times, each time with 1 ml PBST for 5 min.

**Mount coverslips for imaging**

21. Pipet 5 μl Vectashield mounting medium on a glass microscope slide. Place the coverslip with the labeled cells, cell-side-down, over the drop and seal with clear nail polish. Observe by fluorescence microscopy (UNIT 4.2) with appropriate filters for the fluorophores.

**REPLICATION LABELING OF DNA FIBERS**

This section describes the procedure to visualize replication sites on DNA fibers. BrdU-labeled cells are lysed in detergent-containing buffer on a glass slide. Tilting the glass slide extends DNA fibers along the surface of the slide. The fibers are fixed with methanol/acetic acid and are then immunostained.

**Materials**

Adherent tissue culture cells and appropriate medium
10 mg/ml bromodeoxyuridine (BrdU) stock (store indefinitely at −20°C)
Phosphate-buffered saline (PBS; APPENDIX 2A)
Lysis buffer (see recipe)
Fixative: 3:1 (v/v) methanol/acetic acid (prepare fresh and store on ice)
Blocking buffer: 1% (w/v) BSA in PBST (0.5% v/v Tween 20 in PBS)
PBST: 0.5% (v/v) Tween 20 in PBS
Primary antibody solution: mouse monoclonal anti-BrdU (Becton Dickinson),
diluted 1:10 in blocking buffer (see above)
Secondary antibody solution: Alexa Fluor 594–conjugated goat anti–mouse IgG
(Molecular Probes) diluted 1:500 in blocking buffer (see above)
Vectashield mounting medium (Vector Labs)
100-mm culture dishes
15-ml conical centrifuge tubes
Centrifuge
Glass microscope slides
Diamond pen (optional)
Coplin jars
Humidified chamber (e.g., covered Tupperware container with moistened paper
towels on the bottom)
Fluorescence microscope (UNIT 4.2) with appropriate color filters for fluorophores
used, e.g., blue (DAPI), green (FITC, Alexa Fluor 488), and red (e.g., Texas
Red, Alexa Fluor 594)
Additional reagents and equipment for cell culture (including trypsinization and
cell counting; UNIT 1.1) and fluorescence microscopy (UNIT 4.2).

**Grow and label cells**

1. Grow the cells in a 100-mm culture dish to the appropriate cell density (UNIT 1.1).

   For overnight incubation seed 5 × 10^6 cells.

   If cell density is too low, the yield in terms of cell number will be compromised; if density
   is too high, BrdU incorporation is decreased. Typically, use 0.5–1 × 10^6 cells/ml.
2. Add 10 mg/ml BrdU stock for a final concentration of 10 μg/ml. Incubate the dish 30 min at 37°C. Prepare cells without BrdU treatment under the same conditions at the same time.

Alternatively, CldU and/or IdU can be used.

3. Trypsinize the cells (UNIT 1.1) and collect them in a 15-ml conical tube.

4. Centrifuge 1 min at 200 × g, room temperature, and remove supernatant. Add 10 ml PBS to the pellet, centrifuge again as before, and remove the supernatant.

5. Count the cells (UNIT 1.1). Prepare a single-cell suspension at 2 × 10⁶ cells/ml in PBS.

Mixing 20 μl labeled cells and 80 μl unlabeled cells can help to reduce the density of BrdU-incorporated DNA fibers on the slide, yielding clearer images. Unlabeled cells are also used as a negative control for immunostaining.

Prepare fibers

6. Place 2 μl of the cell suspension onto the edge of a glass slide.

7. Add 10 to 50 μl lysis buffer and mix briefly on the slide with a pipet tip. Incubate the slide for no more than 10 min at room temperature.

The smaller the volume of lysis buffer, the better the result. However, it might be a bit difficult to handle a 10-μl volume of lysis buffer; therefore, beginners should try 20 to 50 μl.

8. Tilt the slide gradually to a 15° to 30° angle (e.g., by laying the slide on the edge of a test tube rack so that the one side of the slide is raised ~2 cm).

The cell lysate deposits DNA fibers as the cell suspension flows down the surface of the glass slide.

It is useful to mark the extent of flow of the cell lysate on the opposite side of the slide with a diamond pen.

9. Pipet 400 μl freshly prepared fixation solution directly onto the slide. Incubate for 2 min, then remove the fixative and allow the slide to air dry.

At this step, the glass slide can be stored in the dark at room temperature indefinitely.

Immunostain DNA fibers

10. Add sufficient blocking buffer (1% BSA in PBST) to cover the glass slide (~1 ml) and incubate 20 min at room temperature.

Large numbers of glass slides can be processed at the same time by using Coplin jars. Otherwise, pipet the solutions directly onto the glass slide.

11. Pipet 30 μl primary antibody solution onto the slide. Cover the slide with an 18 × 18-mm glass coverslip. Incubate the slide for 1 hr at room temperature in a humidified chamber.

12. Wash slides three times by immersion in 40 ml PBST in Coplin jars, each time for 3 min.

13. Add 30 μl secondary antibody and incubate for 1 hr at room temperature in a humidified chamber.

14. Wash slides three times by immersion in 40 ml PBST in Coplin jars, each time for 3 min.

Mount cells for imaging

15. Add 5 μl Vectashield mounting medium per slide and cover with 24 × 50-mm coverslip. Seal with clear nail polish and observe with a fluorescence microscope (UNIT 4.2) using the appropriate filters for the fluorochromes.
REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

High-salt buffer

Phosphate-buffered saline (PBS; APPENDIX 2A) containing:
0.5 % (v/v) Tween 20
0.4 M NaCl
0.2% (v/v) Nonidet P-40 (NP-40)
Prepare fresh

Hybridization mix

2 × SSC (APPENDIX 2A) containing:
50% (v/v) formamide
1% (v/v) Tween 20
10% (w/v) dextran sulfate
Prepare fresh

Lysis buffer

0.5% (w/v) sodium dodecyl sulfate (SDS; see APPENDIX 2A for 20% stock)
50 mM disodium EDTA
200 mM Tris-Cl, pH 7.4 (APPENDIX 2A)
Prepare fresh

Trypsin solution

Hanks’ balanced salt solution (HBSS; APPENDIX 2A) without Ca, Mg, or bicarbonate, containing:
0.01% (w/v) trypsin
0.1 mM disodium EDTA

COMMENTARY

Background Information

Since the late 1960s, a variety of cytochemical methods have been developed to understand DNA metabolism in the nucleus. These techniques include autoradiography using tritium as well as dye staining using, e.g., Giemsa. Considering the sensitivity required to measure small amounts of newly synthesized DNA, the identification of proliferating cells has usually been accomplished by demonstrating [³H]thymidine incorporation into DNA by autoradiography (Cleaver, 1967; Baserga and Malamud, 1969). The problem with tritium incorporation is that autoradiographic studies are time consuming. In addition, the radiation hazard posed by [³H]thymidine has been a major barrier to its application in the field of clinical diagnostics and treatment, including cell kinetics and histopathology of human patients. Thus, investigators have been seeking a faster and less hazardous technique.

In the early 1970s, fluorescence quenching of DNA-specific fluorochromes by incorporated bromodeoxyuridine was developed (Latt, 1973; Stubblefield, 1975). Fluorescence quenching techniques using BrdU were first applied to flow cytometric studies of DNA replication (Dutrillaux et al., 1973). In the mid-1970s, antibodies raised against BrdU made possible an immunochemical method for detection of BrdU incorporated into DNA (Grazigner et al., 1975, 1976, 1978). In particular, the development of monoclonal anti-BrdU antibody significantly advanced microscopic methods, including image analysis, in the study of DNA synthesis (Grazigner, 1982). In combination with the use of fluorophore-conjugated secondary antibodies, immunochemical staining of incorporated BrdU and other halogenated thymidine analogs has been applied both to single cells and to tumor tissues, to estimate the labeling index of individual specimens (Raza et al., 1984; Nagashima and Hoshino, 1985).

Critical Parameters and Troubleshooting

Obviously, the most critical parameters common to all protocols described in this unit
are the concentration and incubation time for labeling with halogenated thymidine analog. Low concentrations result in lower signal-to-noise ratio, while high concentrations may affect cell viability because the thymidine analogs are toxic to cells. Short incubation periods yield low signal, whereas long incubation times result in larger regions being labeled, and, hence, lower resolution. In mammals, each replication focus takes 45 to 60 min to complete synthesis (Ma et al., 1998; Dimitrova and Gilbert, 1999), so periods longer than that will label several cohorts of foci. While the final concentration of 10 μg/ml and 30-min incubation time typically gives good results with most mammalian cells, the dose and period should be determined empirically. Different cell types may differ in their permeability or in the levels of endogenous thymidine pools, which affect the final intracellular concentration of labeled nucleoside. Thus, optimization may need to be performed if unexpected images are obtained.

It is feasible to employ a combination of the protocols in this unit to further analyze DNA replication. For example, it should be noted that the replication foci visualized by BrdU incorporation can be either replication origins or replication forks. To measure the density of active origins, IdU and CldU double labeling (Basic Protocol 3) should be employed in the DNA-fiber method (Basic Protocol 4). Cells are briefly labeled with IdU, followed by a longer CldU chase to allow forks to progress and to label the remainder of the long DNA fiber. DNA is then stretched on glass slides and stained with differentially labeled antibodies to highlight the sites of IdU and CldU incorporation. Short IdU stretches flanked on either side by long CldU stretches identify replication origins. The distances between these short IdU stretches can be measured under each experimental condition to evaluate the effects on the density of active replication origins (Pasero et al., 2002; Anglana et al., 2003; Norio et al., 2005).

Anticipated Results

Typical replication patterns visualized by BrdU incorporation are shown in Figure 22.10.1A. Cells were labeled with BrdU followed by anti-BrdU antibody immunofluorescence staining. Sites of BrdU incorporation (replication pattern) were shown in the order of appearance from early-S to late-S (left to right; Wu et al., 2005a). The results of dual replication labeling have been published (Dimitrova and Gilbert, 1999; Dimitrova et al., 1999; Li et al., 2003). DNA fibers labeled with CldU and visualized as per Basic Protocol 4 are shown in Figure 22.10.1B (F. Li and D.M. Gilbert, unpub. observ.). It should be noted that DNA fibers are not necessarily a single stretch of double-stranded DNA.

Time Considerations

Typically, replication labeling with one halogenated thymidine analog takes 30 min. After the labeling, an additional 1 hr is required to process the specimen. For immunostaining, typically 1 to 2 hr is required. Since FISH involves a hybridization step of 6 to 12 hr, 2 working days may be required. Preparation of DNA fibers usually takes <1 hr.

Literature Cited


Contributed by Tomoki Yokochi and David M. Gilbert
Department of Biological Science
Florida State University
Tallahassee, Florida