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Incorporation of Thymidine Analogs for Studying Replication Kinetics in Fission Yeast

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Abstract

Summary—Labeling DNA during in vivo replication by the incorporation of exogenous thymidine and thymidine analogs has been a mainstay of DNA replication and repair studies for decades. Unfortunately, thymidine labeling does not work in fungi, because they lack the thymidine salvage pathway required for uptake of exogenous thymidine. This obstacle to thymidine labeling has been overcome in yeast by engineering a minimal thymidine salvage pathway consisting of a nucleoside transporter to allow uptake of exogenous thymidine from the medium and a thymidine kinase to phosphorylate the thymidine into thymidine monophosphate, which can be used by the cell. This chapter describes the labeling of fission yeast, *Schizosaccharomyces pombe* , with the thymidine analog BrdU in order to identify sites and determine kinetics of DNA replication.

Keywords

Thymidine; BrdU; IdU; CldU; Thymidine kinase; hENT1; DNA replication; Fission yeast; Schizosaccharomyces pombe

1. Introduction

In vivo labeling with thymidine requires that the cell be able to take up thymidine (TdR, thymine deoxyriboside) via a cell surface nucleoside transporter and to phosphorylate the nucleoside to create thymidylate (dTMP, deoxythymidine monophosphate) via a thymidine kinase. To allow exogenous thymidine utilization in fission yeast, two groups have created strains that express both the human equilibrative nucleoside transporter, hENT1, and the herpes virus thymidine kinase, tk (1,2). Cells expressing tk, but not hENT1, also incorporate exogenous thymidine, albeit at a greatly reduced rate (1).

Since neither hENT1 nor tk discriminate against the halogenated thymidine analogs, 5-bromo-2′-deoxyuridine (BrdU), 5-chloro-2′-deoxyuridine (CldU) or 5-iodo-2′-deoxyuridine (IdU) can also be used for in vivo labeling. These analogs, which differ from thymidine by replacement of the 5-methyl group with the appropriate halogen, are fairly well tolerated by cells; bromine, in particular, is sterically and isoelectrically similar to the methyl group, making BrdU the preferred thymidine analog. They also make useful DNA labels because they cannot be incorporated into RNA without loss of the halogen label on the 5-carbon.

There are two general approaches to identifying sites of DNA replication using in vivo incorporation of thymidine analogs. The first approach is to use a differential DNA isolation strategy, such as density centrifugation or immunoprecipitation, to purify the analog-labeled DNA from the unlabeled DNA and then use a label independent method, such as southern

blotting or PCR, to determine the extent to which a given locus partitions into the label, and thus replicated, population. The second approach is to directly visualize the incorporated label in the replicated DNA by antibody staining, either in fixed cells, which allows one to determine the location of replication within the nucleus at fairly low resolution, or on isolated, combed DNA molecules, which allows one to determine the location of replication along the chromosome at relatively high resolution. This chapter presents one of these approaches to the use of thymidine analog labeling in fission yeast – CsCl density gradient centrifugation; whole-cell immunofluorescence and DNA combing are covered in Chapters "Microscopy Techniques to Examine DNA Replication in Fission Yeast" and "Use of DNA Combing for Studying DNA Replication In Vivo in Yeast and Mammalian Cells," respectively.

Density centrifugation is useful in studying the kinetics of replication of one or many loci (for example, *see* **refs**. 3,4). The advantage of BrdU over the traditional density labels, ^{13}C and ^{15}N, is that BrdU is much less expensive. Density shift experiments using ^{13}C and ^{15}N-labeled cells achieve a maximum molecular weight difference of 27 Da/bp between heavy and light DNA. For CldU, BrdU, and IdU, the difference is 10, 33, and 56 Da/bp, respectively, assuming 50% G + C content.

The basic outline of the experiment is to synchronize cells, add BrdU, and follow the synchronous culture through S-phase, taking timepoints every 5 min. From each timepoint, the replicated and unreplicated DNA is separated by CsCl density gradient centrifugation and the timing of replication of a specific locus is determined by measuring when it moves from the unreplicated to the replicated fraction. A more detailed treatment of density shift labeling can be found in Chapter "Density Transfer as a Method to Analyze the Progression of DNA Replication Forks."

2. Materials

2.1. BrdU Incorporation

1. *hENT1 tk* fission yeast strains (*see* Note 1).
2. *YES medium (Yeast Extract + Supplements)*. 5 g/L yeast extract, 30 g/L glucose, 75 mg/L leucine, 75 mg/L uracil, 75 mg/L adenine, 75 mg/L histidine, autoclaved, stable for years at room temperature (*see* Note 2).
3. 1 mM 5-bromo-2′-deoxyuridine (BrdU) in water, filter sterilized, stable at 4°C for years.

2.2. Density Gradient Centrifugation

1. *TE*. 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, stable at room temperature for years.
2. *Lysis Buffer*. 1% SDS in TE, stable at room temperature for years.
3. 0.5-μm glass beads.
4. TE saturated 1:1 phenol:chloroform, stable at 4°C for years.
5. 100% ethanol.
6. 1× *Eco*RI or other appropriate restriction digest buffer.

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[1] *hENT1 tk* cells are available from the Rhind (nick.rhind@umassmed.edu) and Forsburg (forsburg@usc.edu) labs. Depending on your needs you may be able to use the strains as is, or you may have to cross them in to a particular genetic background, e.g., to study replication in a specific mutant.

[2] Labeling works equally well in EMM2, a defined fission yeast medium (5) , and at temperatures between 20 and 35°C.

7. 20 unit/μL *Eco*RI.
8. 20 mg/mL RNase A in water, stable for years at −20°C.
9. Refractometer such as the Reichert Abbe Mark II refractometer.
10. $T_{10}E_{100}$. 10 mM Tris–HCl, pH 7.5, 100 mM EDTA with a refractive index of 1.3329 (adjusted by adding 10 mM Tris–HCl, pH 7.5)
11. CsCl solution of refractive index 1.4021 (approximately 1.28 g/mL CsCl) prepared in T_{10} E_{100} with a refractive index of 1.3329
12. 5-mL Beckman quickseal centrifuge tube and heat sealer or equivalent.
13. NVT90 rotor and Beckman L8-55M Ultracentrifuge or equivalent.
14. 20 mg/mL glycogen.
15. 70% ethanol.

3. Methods

3.1. BrdU Incorporation

1. Grow *hENT1 tk* cells to mid-log (OD_{600} 0.1–1.0) shaking in YES at 25° (*see* Note 2).
2. Synchronize cells using one of the techniques described in Chapter “Cell Cycle Synchrony for Analysis of *S. pombe* DNA Replication” (*see* Note 3).
3. Reinoculate 250 OD units of synchronized cells (*see* Note 4) into 250 mL YES at 30°C containing 5 μM BrdU and return to shaking at 25°C.
4. Take 20 ODs of cells every five minutes from the beginning of S-phase (*see* Note 5).
5. Pellet cells by centrifugation for 3 min at 2,000 × *g* in a tabletop centrifuge.
6. Decant supernatant and resuspend pellet in 1 mL of ice-cold water and transfer to a 1.5-mL screw-cap tube (*see* Note 6).
7. Spin 10 s at maximum speed in a microfuge, aspirate supernatant, and freeze samples at −80°C or proceed directly to DNA isolation.

3.2. Density Gradient Centrifugation

1. Resuspend cell pellet in 250 μL of Lysis Buffer at 4°C.
2. Add 0.5 μm glass beads to the meniscus of the Lysis Buffer.
3. Add 250 μL of phenol:chloroform.

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[3]The method of synchronization will depend upon the details of the experiment. We generally use centrifugal elutriation, but this technique requires a specialized centrifuge. Cdc25 -ts block and release, which blocks cells in G2 and releases them into a synchronous M-G1-S progression, is a convenient alternative that gives a high degree of synchrony. Cdc 10 -ts G1 block and release does not work well, presumably because arresting cells for long periods of time in G1 by inactivating the G1/S transcription factor compromises the subsequent S phase.

[4]OD units are a measure of cell number calculated as the optical density of the culture at 600 nm times the volume of the culture in milliliters. Thus, a 20-mL culture at an OD_{600} of 0.5 contains 10 OD units of cells. 1 OD unit is about 2×10^7 cells.

[5]S phase takes about 20 min in fission yeast, but because of perfect synchronization is impossible, it takes about 40 min for bulk replication of a well-synchronized culture. Therefore we generally take 12 timepoints to ensure we cover the whole of S phase. For cells synchronized in G2, S phase begins about 20 min before the peak of septation, which can be monitored by phase-contrast microscopy. The best way to assay bulk replication and determine when S phase is occurring in your timecourse is by flow cytometry, as described in Chapter “Measuring DNA Content by Flow Cytometry in Fission Yeast.”

[6]It is important to spin down the cells and resuspend them in cold water as quickly as possible in order to arrest replication in the cells.

4. Vortex 5 min (*see* Note 7).
5. Spin at maximum speed in a microfuge (about 15,000 × *g*) for 2 min.
6. Transfer upper aqueous phase to a new microfuge tube and add 250 μL TE.
7. Add 250 μL of phenol:chloroform, vortex 5 s, spin 2 min, and transfer upper aqueous phase to new microfuge tube.
8. Repeat organic extraction until interface is relatively clear (for a total of about three extractions).
9. Transfer upper aqueous phase to new microfuge tube, add 1 mL of 100% ethanol, and mix well to precipitate DNA.
10. Spin at maximum speed in a microfuge for 5 min.
11. Aspirate supernatant and wash DNA pellet with 500 μL 70% ethanol.
12. Aspirate most of the ethanol, spin 5 s at maximum speed in a microfuge, and carefully aspirate residual ethanol.
13. Resuspend pellet in 200 μL of 1× *Eco* RI buffer.
14. Add 1 μL 20 unit/ μL *Eco* RI and 1 μL 20 μg/mL RNase; incubate 4 h at 37°C (*see* Note 8).
15. Add DNA to a 5-mL Beckman quickseal centrifuge tube, fill the remaining volume with about 5 mL of CsCl solution of refractive index 1.4021 (it is not necessary to mix the two solutions), and seal.
16. Spin 20 h at 50 krpm (about 200,000 × *g*) in an NVT90 rotor in a Beckman L8-55M ultracentrifuge or equivalent.
17. Fractionate gradients into approximately 250 μL fractions by dripping out of the bottom of the punctured tube into fresh microfuge tubes (*see* Note 9).
18. Measure refractive index using 100 μL of each fraction. These data are used to compare DNA positions on different gradients (*see* Note 10).
19. Dilute remaining DNA samples to 500 μL with water, precipitate by addition of 1 μL of 20 mg/mL glycogen and 1 mL 100% ethanol, and pellet by centrifugation for 5 min at maximum speed in a microfuge.
20. Wash pellets with 70% ethanol, carefully aspirate residual ethanol, and resuspend in 10 μL TE.
21. Identify the position of the DNA loci of interest among the gradient fractions (*see* Note 11).

[7]Vortexing is generally done using some sort of tube-holding head on a vortexer, especially when multiple samples are being processed. Screw-cap tubes are used to prevent phenol leaking during the vortexing. Snap-cap tubes can be used for subsequent steps.
[8]The restriction digest fragments the genome, so that replicated and unreplicated regions can equilibrate separately on the gradient. If *Eco* RI does not digest your loci of interest into reasonable (0.5–5 kb)-sized fragments, another enzyme should be chosen.
[9]A commercially available gradient puller makes this step easier and more reproducible, but is not required to get good data.
[10]Instead of plotting the distribution of your DNA of interest versus fraction number, plot it versus the refractive index of each fraction. This approach allows comparisons to be made between gradients even if the fraction number or volume varies between experiments.
[11]There are several ways to localize your locus of interest on the gradient, the more sophisticated being qPCR and slot blotting. However, southern blotting is cheap, easy, and requires no specialized equipment. Simply run a standard 1× TAE 1% agarose gel, blot it to a nylon membrane, and hybridize it with a probe that recognizes your locus of interest. It is also possible to monitor multiple loci that produce fragments of distinct sizes by hybridizing with multiple probes.

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