Chapter 24

Monitoring Telomere Maintenance During Regeneration of Annelids

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Abstract

Telomere shortening is a hallmark of aging and eventually constrains the proliferative capacity of cells. The protocols discussed here are used for monitoring telomeres comprehensively in *Aeolosoma viride*, a model system for regeneration studies. We present methods for analyzing the activity of telomerase enzyme in regenerating tissue by telomeric repeat amplification protocol (TRAP) assay, for comparing telomere length between existing tissue and newly regenerated tissue by telomere restriction fragment (TRF) assay, as well as for visualizing telomeres by fluorescence in situ hybridization (FISH).

Key words *Aeolosoma viride*, Telomere maintenance, Regeneration, TRAP assay, TRF assay, Telomere FISH

1 Introduction

Regeneration aids in repairing or rebuilding damaged tissue or entire body parts in animals. The ability to regenerate is not uniform among animals. For instance, metazoans like planarians can regenerate entirely with symmetry and proportion from tiny fragments of their existing body using adult somatic stem cells called neoblasts [1]. Advanced mammals like humans can only regenerate certain body parts like liver after partial resection [2]. Salamanders like axolotls and newts can regenerate several body parts including limbs or tail by activating progenitor cells [3]. While axolotls lose the ability to regenerate eye lens around 2 weeks after birth, newts can regenerate lens without being limited by age [4]. Observations akin to this implicate aging as one of the factors that affect regeneration potential. Although the real mechanism of how age affects wound healing and regeneration is not clear, most people accept that wound healing is affected by aging.

This raises questions about the aging situation of the regenerated tissues. Are the regenerated tissues similar to the existing tissue or younger than them? Does regeneration evoke rejuvenation?

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Aging is a complicated life phenomenon, and it is quite difficult to evaluate the age of animals especially that of invertebrates. Length of telomere, one of characteristics to realize aging in animals, was chosen as the parameter to figure out the relationship between aging and regeneration. The inability of DNA replication mechanism to fully replicate the lagging strand of chromosomes causes progressive shortening of telomeres [5].

Telomerase is a ribonucleoprotein composed of TERC (RNA component) and TERT (reverse transcriptase) to lengthen telomeres. Expression of telomerase is varied among organisms. Remarkably, certain species of animals with good regenerative ability have upregulated telomerase expression [6, 7]. Also, regenerative ability waning with age supports the notion that shorter telomeres contribute to diminished proliferation.

Established model animals for regeneration studies, such as amphibians and reptiles, usually have long life spans, which disallows the exploration of age as a factor restricting regeneration. To circumvent this problem, we used *Aeolosoma viride*, a freshwater annelid as a new model for regeneration and aging-related research. Annelids are well known for their ability to restore anterior and posterior ends from few of their body segments. Lifespan of *A. viride* is approximately 2 months, making it an appealing model for aging-related study.

A. viride of varying age groups when observed for regeneration showed that regeneration declines as age of the worm increases [8]. Telomere length was maintained at the regeneration sites after amputation of the head [8].

Here, we describe protocols to monitor the telomere maintenance in *A. viride* during tissue regeneration. We discuss three main strategies: (1) PCR-based telomeric repeat amplification protocol (TRAP) assay for analyzing the activity of telomerase in regenerating sites (2). Telomere restriction fragment (TRF) assay (3). Telomere fluorescence in situ hybridization (FISH).

2 Materials

All solutions are prepared using filtered and deionized ultrapure water at room temperature (RT) and analytical grade reagents (unless otherwise indicated).

2.1 Telomeric Repeat Amplification Protocol (TRAP) Assay

1. Regenerating *Aeolosoma viride*.
2. Artificial spring water (ASW): 48 mg/L $NaHCO_3$, 24 mg/L $CaSO_4 \cdot 2H_2O$, 30 mg/L $MgSO_4 \cdot 7H_2O$, 2 mg/L KCl in ddH_2O.
3. Telomerase detection kit (e.g., TRAPeze, Millipore).

4. 1× CHAPS lysis buffer: 10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% (v/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10% (v/v) glycerol. Store at −20 °C.
5. Total protein concentration kit (e.g., Bradford reagent).
6. 10× TRAP reaction buffer: 200 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 630 mM KCl, 0.5% (v/v) Tween 20, 10 mM EGTA. Store at −20 °C.
7. 50× dNTP mix: 2.5 mM each of dATP, dTTP, dGTP, dCTP. Store at −20 °C.
8. TS primer. Store at −20 °C (*see* **Note 1**).
9. TRAP primer mix: reverse primer (RP), internal control forward primer (e.g., FP K1 in TRAPeze, Millipore), internal control template (e.g., TSK1 in TRAPeze, Millipore) (*see* **Note 2**). Store at −20 °C.
10. PCR grade water: protease, DNase and RNase free deionized water. Store at −20 °C.
11. DNA polymerase (e.g., Platinum™ Taq, Invitrogen). Store at −20 °C (*see* **Note 3**).
12. Acrylamide solution: 40% acrylamide/bis-acrylamide 19:1 (e.g., Bio-Rad).
13. Tetramethylethylenediamine (TEMED).
14. 10% (w/v) Ammonium persulfate (APS).
15. 0.5 M ethylenediaminetetraacetic acid (EDTA): 186.1 g disodium EDTA in 800 mL ddH_2O. Adjust pH to 8.0 with NaOH to completely dissolve EDTA.
16. 5× TBE solution: 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA, pH 8.0 in 1 L ddH_2O. Adjust to pH 8.3 using NaOH.
17. DNA fluorescent loading dye (e.g., FluoroDye™, SMOBIO).
18. 10% (w/v) non-denaturing polyacrylamide gel: 3.75 mL acrylamide solution, 3 mL 5× TBE solution, 200 μL 10% APS, 10 μL tetramethylethylenediamine (TEMED). Bring final volume to 15 mL with ddH_2O.
19. Polyacrylamide gel visualization setup (e.g., FluorChem M system, Protein Simple).

2.2 Terminal Restriction Fragment (TRF) Assay

1. Nuclei lysis buffer (e.g., Wizard Genomic DNA Purification Kit Promega).
2. 4 mg/mL RNase solution.
3. Protein precipitation solution (e.g., Wizard Genomic DNA Purification Kit Promega).

4. Nucleic acid quantification setup (e.g., NanoDrop ND-1000, Thermo Scientific).
5. 50× TAE stock: 242 g Tris base, 600 mL ddH_2O. Add 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA. Bring final volume to 1 L with ddH_2O.
6. TAE buffer: 20 mL 50× TAE stock, 980 mL ddH_2O.
7. 1% (w/v) agarose gel: 1 g agarose, 100 mL TAE buffer, melt and cool at 60 °C. Add nucleic acid stain according to the manufacturer's instructions. Pour the gel into a gel tank and insert a comb.
8. 6× DNA loading buffer: 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue.
9. *Rsa*I restriction endonuclease.
10. *Hinf*I restriction endonuclease.
11. Restriction enzyme buffer.
12. Depurination solution: 0.25 N HCl.
13. Denaturing solution: 0.5 N NaOH, 1.5 M NaCl.
14. Neutralizing solution: 1.5 M NaCl, 1 M Tris–HCl.
15. Nylon membrane (e.g., Immobilon-Ny + Membrane; Millipore).
16. 10× SSC buffer: 1 M NaCl, 300 mM sodium citrate. Adjust pH to 7.0 by gradually adding HCl, dropwise. Autoclave.
17. UV cross-linker.
18. Hybridization oven.
19. Hybridization tube.
20. Church buffer: 0.5 M $NaPO_4$, 1 mM EDTA, 7% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) bovine serum albumin (BSA) in nuclease-free water.
21. $(TTAGGG)_4$ and $(CCCTAA)_4$ primers to generate double-stranded $(TTAGGG)_n$.
22. RadPrime™ DNA labeling system (Invitrogen).
23. 20% (w/v) SDS in nuclease-free water.
24. 4× SSC/0.1% SDS: 400 mL 10× SSC, 5 mL 20% SDS, 595 mL ddH_2O.
25. 2× SSC/0.1% SDS: 200 mL 10× SSC, 5 mL 20% SDS, 795 mL ddH_2O.
26. Phosphor screen.

1. 0.02% (w/v) colchicine in ASW.
2. Carnoy's fixative: 750 mL methanol, 250 mL glacial acetic acid.

2.3 Telomere Fluorescence In Situ Hybridization (Telomere FISH)

3. 60% (v/v) acetic acid.
4. 10× phosphate-buffered saline (PBS) stock: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 800 mL ddH_2O. Adjust pH to 7.4 using HCl and bring volume to 1 L with ddH_2O. Autoclave.
5. 1× PBS: 100 mL 10× PBS stock, 900 mL ddH_2O.
6. 4% paraformaldehyde (PFA): 40 g PFA in 800 mL 60 °C heated 1× PBS. Stir, add 1 N NaOH dropwise until PFA is dissolved. Bring volume of solution to 1 L with 1× PBS. Cool the solution and filter sterilize.
7. Hybridization mix: 70% (v/v) formamide, 0.5% (v/v) blocking agent (e.g., Roche), 250 nM $(CCCTAA)_3$ Peptide Nucleic Acid (PNA) probe (e.g., Panagene), 10 mM Tris–HCl, pH 7.5.
8. Wash buffer I: 70% (v/v) formamide, 10 mM Tris–HCl, pH 7.5.
9. Wash buffer II: 150 mM NaCl, 0.08% (v/v) Tween-20, 100 mM Tris–HCl, pH 7.5.
10. Counterstain: 0.3 μM DAPI.
11. Antifading mounting medium (e.g., ProLong® Gold, Molecular Probes).

3 Methods

3.1 Protein Extraction from Regenerating Tissues of Aeolosoma Viride

1. Bisect the anterior most segment of the regenerating site with needles from 100 regenerating annelids.
2. Pool the bisected tissue in ice-cold 1× CHAPS lysis buffer.
3. Homogenize the tissue by pipetting.
4. Incubate on ice for 30 min.
5. Centrifuge at 12,000 rcf for 20 min at 4 °C.
6. Collect the supernatant.
7. Measure the concentration of the total protein extract using the kit.
8. Store protein extract at −80 °C.

3.2 Telomeric Repeat Amplification Protocol (TRAP) Assay

1. Heat inactivate control samples at 85 °C for 10 min (*see* **Note 4**).
2. Set up TRAP reaction with 200 ng of protein extract.
3. Each TRAP reaction contains 2.5 μL of 10× TRAP reaction buffer, 0.5 μL of 50× dNTP mix, 0.5 μL of TS primer, 0.5 μL of TRAP primer mix, 0.2 μL of DNA polymerase and ddH_2O to make up volume to 25 μL.

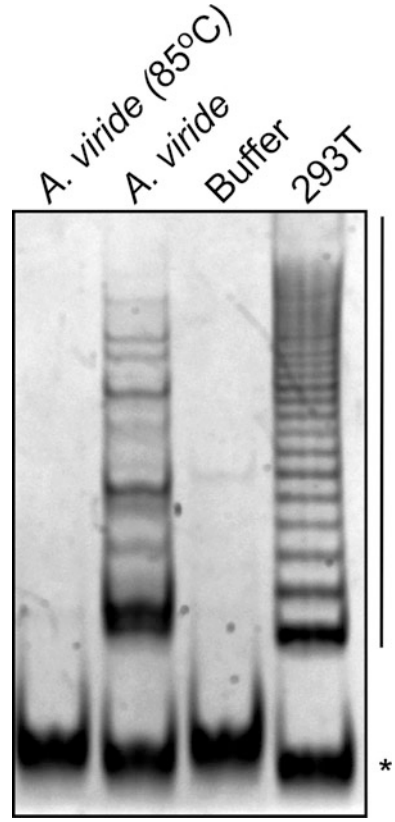

Fig. 1 TRAP assay of *A. viride* extract. Crude and heat-inactivated (85 °C) *A. viride* extracts were used for TRAP reaction. Lysate from human 293 T cells was used for positive control reaction and lysis buffer for negative control reaction. A ladder pattern indicates telomerase-extended products. Asterisk indicates internal control products. (Adapted from [8]).

4. Incubate the reaction mixtures for extension at 30 °C for 30 min.
5. Set up PCR with telomerase extended products by following these conditions: 95 °C for 2 min, 30 cycles of 94 °C for 15 s, 59 °C for 30 s, and 72 °C for 1 min.
6. Mix the PCR products with the DNA loading dye.
7. Resolve the products for 2 h on the 10% polyacrylamide gel at 150 V.
8. Visualize the products by imaging the gel (Fig. 1).
9. Analyze TRAP products (*see* **Note 5**).
10. Use the internal control band as normalization to quantify relative telomerase activity between samples.

3.3 Genomic DNA Extraction from Regenerating Tissues

1. Bisect the most anterior segment from 200 amputated worms with needles at desired time points after amputation.
2. Pool the tissues in 600 μL ice-cold nuclei lysis solution.
3. Store at −20 °C until extraction.
4. Homogenize the tissue in the nuclei lysis buffer with a micropestle.
5. Incubate at 65 °C for 5 min.
6. Mix the tissue lysate with 3 μL RNase solution.
7. Incubate at 37 °C for 5 min.
8. Cool down the sample to RT.
9. Add 200 μL of protein precipitation solution.
10. Mix thoroughly by vortexing.
11. Chill on ice for 5 min.
12. Centrifuge at 13,000 rcf for 4 min to precipitate the proteins.
13. Transfer the supernatant to a new tube.
14. Add an equal volume of isopropanol.
15. Mix by inverting the tube until thread-like aggregates of DNA form.
16. Centrifuge at full speed for 1 min at RT.
17. Discard the supernatant.
18. Add 1 mL of 75% ethanol to wash the DNA pellet.
19. Centrifuge at full speed for 1 min.
20. Remove the supernatant carefully.
21. Air-dry the DNA pellet.
22. Rehydrate by adding 100 μL nuclease-free water.
23. Measure concentration and quality of the isolated DNA.
24. Check DNA integrity by running a 1% agarose gel (*see* **Note 6**).

3.4 Terminal Restriction Fragment (TRF) Assay

1. Digest 700 ng of genomic DNA with 1 U/μL of *Rsa*I and 1 U/μL *Hinf*I at 37 °C overnight.
2. Resolve the digested DNA overnight on 1% agarose gel at 40 volts.
3. Stain the gel with the DNA staining dye.
4. Confirm complete digestion under UV source (*see* **Note 7**).
5. Soak the gel in depurination solution for 15 min.
6. Rinse the gel with ddH_2O to remove HCl.
7. Soak the gel in denaturing solution for 15 min.
8. Rinse with ddH_2O.
9. Soak the gel again in denaturing solution for 15 min.

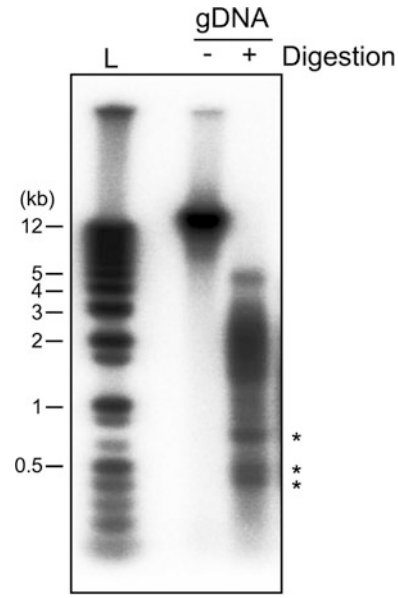

Fig. 2 TRF assay of *A. viride* genomic DNA. Intact (−) and restriction digested (+) genomic DNA were resolved by agarose gel electrophoresis for southern blotting. Restriction fragments of telomeric DNA shows a span of 0.2–5 kb in length. Asterisks indicate internal repetitive sequences. Sizes of selected bands of DNA ladder (L) are indicated on the left. (Adapted from [8]).

10. Rinse with ddH_2O.
11. Soak the gel in neutralizing solution for 15 min, twice.
12. Blot the DNA fragments onto a nylon membrane overnight by capillary transfer in 10× SSC buffer (*see* **Note 8**).
13. Rinse the blotted membrane briefly with 10× SSC buffer.
14. Air-dry the membrane until completely dry.
15. Crosslink by exposing it to 120 mJ/cm^2 UV light.
16. Place the membrane into a hybridization tube containing 45 °C pre-warmed Church buffer.
17. Pre-hybridize at 45 °C for 1 h.
18. While the membrane is incubating, generate double-stranded $(TTAGGG)_n$ DNA by self-priming PCR using $(TTAGGG)_4$ and $(CCCTAA)_4$ primers (*see* **Note 9**).
19. Generate ^{32}P-labeled probes with [α-^{32}P] dCTP using the DNA labeling system.
20. Column purify the probes.
21. Heat the probes for 10 min.
22. Chill on ice.
23. Add 500 µL of fresh Church buffer.

24. Discard the used Church buffer after pre-hybridization.
25. Add 50 mL fresh Church buffer containing double-stranded TTAGGG probe.
26. Hybridize overnight at 50 °C.
27. Discard the used Church buffer and wash the membrane with 4× SSC at 45 °C for 15 min.
28. Discard and wash membrane twice with 4× SSC/0.1% SDS at 45 °C for 15 min.
29. Discard and wash membrane twice with 2× SSC/0.1% SDS at 45 °C for 15 min.
30. Briefly rinse the membrane with 4× SSC and expose membrane to a phosphor screen overnight.
31. Image the phosphor screen (Fig. 2).

3.5 Telomere Fluorescence in Situ Hybridization (Telomere FISH)

1. Treat the regenerating worms with 0.02% colchicine overnight.
2. Place the colchicine-treated worms in distilled water for 20 min to cause hypotonic shock.
3. Harvest the regenerating tails in ice-cold Carnoy's fixative.
4. Incubate on ice for 1 h to fix them.
5. Replace used fixative with fresh fixative.
6. Store the sample at 4 °C overnight.
7. Dissociate the fixed tissue in 60% acetic acid with needles on a coverslip.
8. Place the coverslip on a heating plate at 70 °C for 1 min.
9. Air-dry the coverslip at RT overnight.
10. Rehydrate the sample briefly in 5 mL 1× PBS.
11. Fix in 4% PFA at RT for 10 min.
12. Wash the sample twice in 1× PBS for 30 s.
13. Dehydrate the sample using an ethanol series (70%, 95%, and 100%) for 5 min in each.
14. Air-dry the sample thoroughly.
15. Turn the coverslip upside down and place it over a drop of hybridization mix on a slide.
16. Place the slide on a heating plate at 80 °C for 3 min.
17. Incubate the slide at RT overnight in darkness (*see* **Note 10**).
18. Pick up the coverslip containing the cell spreads and wash by immersing it in wash buffer I twice at RT for 5 min (*see* **Note 11**).
19. Wash the coverslip with wash buffer II thrice at RT for 5 min.
20. Counterstain the sample with 0.3 μM DAPI.

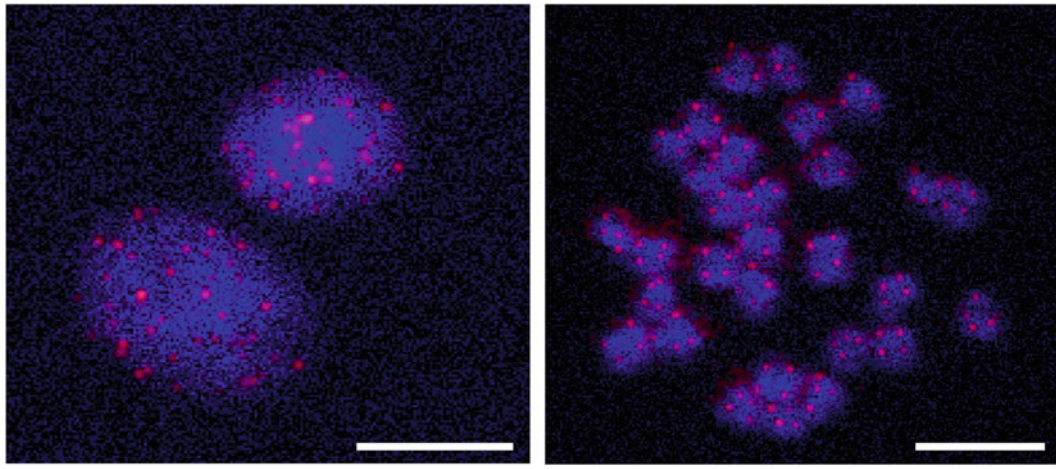

Fig. 3 FISH images of *A. viride* interphase nuclei (left) and metaphase spread (right) stained with telomeric PNA probes (red) and DAPI (blue). Scale bars: 10 μm. (Adapted from [8]).

21. Dehydrate the sample in ethanol series (70%, 95%, and 100%) for 5 min each.
22. Air-dry the sample thoroughly.
23. Mount the sample on a slide in antifade mounting medium.
24. Visualize telomeres with a fluorescence microscope equipped with 63× or 100× objective (Fig. 3) (*see* **Note 12**).

4 Notes

1. TS primer is non-telomeric synthetic oligonucleotide that is recognized by telomerase. If the prepared sample has active telomerase, new "TTAGGG" telomere repeats will be added to the 3′ end of the TS primer. Sequence of TS primer is 5′-AATCCGTCGAGCAGAGTT-3′.
2. Reverse primer (RP) is used in the downstream PCR to amplify the products of extension reaction. Reverse primer is complementary to telomere repeat sequence.

   Additionally, each reaction benefits from the addition of internal controls. This is done by adding a forward primer (e.g., K1in TRAPeze Telomerase Detection Kit) and an internal control template (e.g., TSK1 in TRAPeze Telomerase Detection Kit). Together with the TS primer, K1 and TSK1 amplify an internal control standard (a 36 bp amplicon in TRAPeze Telomerase Detection Kit). The internal control band is an indicator of PCR amplification efficiency.
3. TRAP reaction in its classical form uses a wax barrier to prevent CX primer from mixing with the other reaction components.

This was later simplified by the use of "hot start" Taq DNA-polymerase such as Platinum™ *Taq* DNA polymerase. The "hot start" Taq DNA-polymerase enzyme is activated when the sample is heated to 95 °C for 2 min before PCR cycling.

4. Heat-inactivated controls: Prepare heat-inactivated controls by heating 2 μL of the extracted test samples at 85 °C for 10 min. Add this to the reaction mix in place of the protein extract. Since telomerase is heat sensitive, heat-inactivated samples serve as negative controls.
5. ImageJ [9] is used to analyze TRAP products. Bands on positive control lanes are designated the standard, and intensity of bands from other reactions are compared to the standard (Fig. 1).
6. Evaluating the integrity of DNA must not be skipped prior to digestion. A quality DNA sample when visualized after agarose gel electrophoresis will appear as a single compact band. If the sample presents as a smear, it is an indication of possible degradation, and the sample is unsuitable for TRF assay. DNA integrity is vital in obtaining reliable telomere length, as a degraded sample may provide inaccurate measurements.
7. TRF assay relies on telomeric DNA being unaffected by restriction digestion with *RsaI/HinfI* due to lack of restriction sites, while genomic DNA is completely digested. After digestion with restriction enzymes, genomic DNA runs as a smear below 800 bp marker. Anything above 800 bp points to incomplete digestion of the genomic DNA. If incomplete digestion is observed, prolong the duration of digestion.
8. A tank is filled with 1 L 10× SSC buffer, and a platform to support the blot setup is placed in the tank. Wet the platform with 10× SSC buffer and place a filter paper (e.g., Whatman™ 3MM) on the support. Gently pour more 10× SSC buffer over the filter paper and remove air bubbles. Place the gel on top. Wet a nylon membrane (big enough to completely cover the gel) with ddH_2O and place it over the gel taking care to remove air bubbles in between. Wet few filter papers with 10× SSC buffer and place them over the nylon membrane. Lay a stack of paper towels over the filter paper and place a weight over it. Capillary transfer at RT overnight.
9. Preparation of ^{32}P-labeled TTAGGG probes is described from **steps 19** to **24** of Subheading 3.4. These steps can be performed during the incubation period of the prehybridization. There is ample time for the probe preparation during this time.
10. All the steps following hybridization are to be performed in a dark environment.

11. Post hybridization washes are performed by simply immersing the coverslips in the wash buffers for the specified amount of time without agitation or shaking. When wash time is over, the coverslip is picked up and placed into the next wash.
12. Interphase nuclei are identified as circular DAPI-stained areas in ~7.5–15 μm diameter (Fig. 3, left). Metaphase chromosomes (~1–2 μm) containing four telomeres are clustered together (Fig. 3, right).

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