Chapter 12

Analysis of Meiotic Telomere Behavior in the Mouse

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Abstract

Linear eukaryotic chromosomes are capped by the telomeres, which consist of highly repetitive nucleotide sequences bound by several telomere-specific proteins. While the general role of telomeres is to protect chromosomes from degradation and end-to-end fusion, during meiosis they are assigned with a distinct and without doubt highly fascinating function. During meiosis, telomeres attach to the nuclear envelope and mediate characteristic chromosome movements, essential for correct haploidization of the genome. Here, we provide elaborate tools to study telomeres in mammalian meiotic germ cells, which include (co-) immunofluorescence staining procedures on cell spreads and paraffin-embedded tissues. We provide detailed procedures for fluorescence labeling of telomeric DNA (Telo-FISH) to visualize telomeres at the light microscopic level, which we often use in combination with immunofluorescence staining of meiotic proteins. We also present a protocol for detection of telomeric DNA at the electron microscopic level (EM-ISH). We finally describe how meiotic telomeres can be visualized by common electron microscopic methods and how they can be analyzed at the ultrastructural level by immunogold labeling of telomere components or associated structures.

Key words Telomere, Meiosis, Nuclear envelope, Immunofluorescence analysis, FISH, EM-ISH, Electron microscopy

1 Introduction

Telomeres define the ends of linear eukaryotic chromosomes. They consist of multiple tandem repeats of short, highly conserved nucleotide sequences, such as TTAGGG in vertebrates, and a number of telomeric DNA-binding proteins. Telomeres protect chromosomes from end-to-end fusion and are pivotal for chromosome maintenance and genome stability [1]. In recent years, it turned out that during meiosis telomeres hold a very special but utmost important function. In early meiotic prophase I, telomeres move to the nuclear periphery and firmly attach to the nuclear envelope. During meiotic progression, the attached telomeres mediate the characteristic movements of meiotic chromosomes, which are required for efficient pairing, synapsis, and recombination of the homologs [2–4]. In this chapter, we give a cumulative detailed

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overview about a set of microscopy techniques that we use to analyze mammalian meiotic telomere behavior and function. This includes immunofluorescence techniques on cell spreads and paraffin-embedded tissues as well as combinatory approaches with co-detection of telomeres by fluorescence in situ hybridization. We also present a protocol for visualization of telomeric DNA at the electron microscopic level. Lastly, we provide short methods for morphological and immunocytological investigation of telomeres by conventional electron microscopy.

2 Materials

2.1 Animals and Tissues

For standard investigation of telomeres and chromosomes in male meiosis, testis tissue is obtained from young adult, 30–40 days old wild-type mice. To analyze female meiotic prophase, ovaries are taken from female embryos (day 17–19 postfertilization) and prepared for paraffin embedding or spreading. We study time-related telomere behavior during meiotic progression on sequential testes samples from juvenile mice of increasing age (days 10–15 postpartum; *see* **Note 1**).

2.2 Meiotic Chromosome Spreads

1. Fresh mouse testis or embryonal ovary tissue.
2. Hypotonic buffer for chromosome spreads: 30 mM Tris–HCl, 17 mM Na-Citrate, 5 mM EDTA, 50 mM Sucrose, 5 mM DTT (dilute from 1 M aqueous stock solution), pH 8.2.
3. 100 mM sucrose solution in water; filter through a 0.2 μm filter and store at −20 °C until use.
4. 1 % PFA solution: dissolve 1 % (w/v) paraformaldehyde in H_2O at 58 °C; cool to room temperature (RT) and adjust to pH 9.2 with NaOH; add 0.15 % (v/v) Triton X-100 diluted from a 10 % aqueous stock solution.

2.3 Paraffin Embedding of Testes and Ovaries, Sectioning and Preparing Paraffin Samples for Immunofluorescence Microscopy

1. PBS (phosphate-buffered saline): 140 mM NaCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.6 mM KCl; adjust to pH 7.4 with NaOH.
2. 2 % PFA solution: dissolve 2 % (w/v) paraformaldehyde in PBS at 58 °C; after cooling to RT, adjust to pH 7.4 with NaOH.
3. Increasing ethanol series: 50, 60, 70, 80, 90, and 100 % (diluent: H_2O).
4. Tert-butyl alcohol (2-methyl-2-propanol).
5. Paraffin pellets (melting point 57–60 °C).
6. Glass slides (Superfrost Plus; Menzel Glas, Braunschweig, Germany).

7. Rotihistol (xylene substitute).
8. Unmasking Solution (Vector laboratories, Burlingame, CA, USA); diluted 1:100 in H_2O before use.

2.4 Components for Immunofluorescence Staining

1. PBS, pH 7.4.
2. Blocking Solution: 5 % dry milk powder, 5 % fetal calf serum (FCS), in PBS, pH 7.4. Can be stored at –20 °C; before use, remove insoluble material by centrifugation at 16,000 × *g* for 10 min.
3. 0.1 % Triton X-100 in PBS, pH 7.4.
4. PBT: 1.5 % BSA, 0.1 % Tween 20, in PBS, pH 7.4.
5. Affinity-purified polyclonal or monoclonal antibodies against proteins of interest, such as SUN1, SYCP3, lamin C2 [5, 6], or digoxigenin.
6. Appropriate fluorochrome-labeled secondary antibodies.
7. Hoechst 33258 (15 μg/mL).
8. Mounting medium: 50 % glycerol in PBS.

2.5 Telomere Fluorescence In Situ Hybridization Components

1. Telomere oligonucleotides to be used for end-labeling with digoxigenin can be obtained from a commercial source as oligonucleotides with sequences $(TAACCC)_7$ and $(GGGTTA)_7$.
2. Reagents for digoxigenin end-labeling: 100 pmol telomere oligonucleotide, Dig-ddUTP (1 mM stock solution), terminal deoxynucleotidyl transferase (TdT; 15 U/μL), 5× TdT buffer (1 M potassium cacodylate, 0.125 M Tris–HCL, 0.05 % (v/v) Triton X-100, 5 mM $CoCl_2$, pH 7.2).
3. 2× SSC: 0.3 M NaCl, 0.03 M Na-Citrate, pH 7.4.
4. Hybridization solution: 30 % formamide, 10 % dextran sulfate, 250 μg/mL *E. coli* DNA, in 2× SSC.
5. TBS: 150 mM NaCl, 10 mM Tris–HCl, pH 7.4.
6. TBS/BR: 0.5 % Roche blocking reagent in TBS, pH 7.4.
7. TBST: 150 mM NaCl, 10 mM Tris–HCl, 0.05 % Tween 20, pH 7.4.
8. Mouse anti-digoxigenin antibody.
9. Appropriate fluorochrome-labeled secondary antibodies.

2.6 Electron Microscopy (EM) Components

1. Cacodylate buffer: 50 mM cacodylate, 50 mM KCl, 2.5 mM $MgCl_2$, pH 7.2.
2. 2.5 % glutaraldehyde solution: dissolve 2.5 % (v/v) glutaraldehyde in cacodylate buffer.
3. 2 % osmium tetroxide in cacodylate buffer.
4. Increasing ethanol series 30, 50, 70, 90, 96, and 100 % (diluted in H_2O).

5. Epon 812.
6. Propylene oxide/Epon 1:1.
7. Gelatine capsules.
8. Formvar-coated copper grids.
9. 2 % uranyl acetate in H_2O.
10. Reynold's lead citrate [7]; right before use, dilute 1:1 in outgassed H_2O.

2.7 EM In Situ Hybridization Components

1. PBS, pH 7.4.
2. 4 % PFA solution: dissolve 4 % (w/v) paraformaldehyde in PBS as described above; adjust to pH 7.4 with NaOH.
3. Blocking solution: 50 mM NH_4Cl in PBS, pH 7.4.
4. Increasing ethanol series: 30, 50, 70, 90, and 100 % (diluent: H_2O).
5. LR White resin, hard grain; right before use, add 0.1 g benzoyl peroxide (catalyst for LR White polymerization) to 5.05 mL of LR White resin.
6. EtOH/LR White (1:1).
7. Gelatine capsules.
8. Formvar-coated nickel grids.
9. 2× SSC (*see* Subheading 2.4).
10. RNase A (100 µg/mL in 2× SSC).
11. 0.5 mg/mL Proteinase K in 20 mM Tris–HCl, 2 mM $CaCl_2$; pH 7.4.
12. Hybridization solution, TBS, TBS/BR, TBST (*see* Subheading 2.5).
13. Mouse anti-digoxigenin antibody.
14. Appropriate gold-labeled secondary antibodies.
15. 2 % uranyl acetate in H_2O.
16. Reynold's lead citrate; right before use dilute 1:1 in outgassed H_2O.

2.8 EM Immunogold Components

1. PBS, pH 7.4.
2. 2 % PFA solution: dissolve (w/v) paraformaldehyde in PBS; adjust to pH 7.4 with NaOH.
3. Blocking solution: 50 mM NH_4Cl in PBS, pH 7.4.
4. Increasing ethanol series: 30, 50, 70, 90, 96, and 100 % (diluted in H_2O).
5. Epon 812.
6. Propylene oxide/Epon 1:1.
7. Gelatine capsules.

8. Formvar-coated copper grids.
9. Appropriate gold-labeled secondary antibodies.
10. 2 % uranyl acetate in H_2O.
11. Reynold's lead citrate; right before use dilute in outgassed H_2O.

3 Methods

3.1 Preparation of Chromosome Spreads

Chromosome spreads are essentially prepared according to a slightly adapted protocol developed by de Boer and colleagues [8].

1. For spreads of spermatocytes, remove the tunica surrounding the freshly prepared testes using fine tweezers. Transfer single tubules into a drop of hypotonic buffer on a slide; leave tubules to swell for 30–60 min. To spread oocytes, place freshly prepared ovaries in hypotonic buffer and incubate for 30–60 min.
2. Place a 20 μL droplet of the sterile 100 mM sucrose solution onto a new slide and transfer small seminiferous tubule pieces or the swollen ovaries (one per slide) from the hypotonic buffer. In this solution, wash germ cells out of the tubules/ovaries by thoroughly tearing the tissues with fine tweezers and subsequent careful resuspension with a 10 μL pipette. Immediately remove remaining larger pieces of debris before proceeding.
3. Cover a new slide with 1 % PFA solution and tilt so that the entire volume of the solution accumulates in one corner of the slide. Transfer 20 μL of the sucrose solution containing the individualized spermatocytes or oocytes in this droplet.
4. Tilt the slide in all directions to mix the solutions and allow cells to disperse evenly over the slide.
5. Place the slides in a moisture chamber and incubate in the closed chamber for 2 h to allow cells to settle down. Slightly open the lid and incubate for another 30 min. Remove the lid and dry specimen in the open moisture chamber for another 2–4 h at RT; make sure the fluid dries out entirely.
6. In case that spreads are not used immediately, wrap the dried slides in tinfoil and store at –70 °C until use.

3.2 PFA Fixation and Paraffin Embedding of Testis and Ovary Tissue Samples

1. For paraffin embedding, fix testes or ovary tissue samples in 1 % PBS-buffered formaldehyde for 3 h.
2. After washing in PBS for 1 h, dehydrate tissues in an increasing ethanol series by subsequently incubating in 50, 60, 70, 80, 90, and 100 % ethanol for at least 1 h each.
3. Immerse the tissues in tert-butyl alcohol at 25 °C for 1 h.
4. Transfer tissue samples into preheated (melted) liquid paraffin in an oven at 60 °C; incubate in the warm paraffin for another

24 h and transfer tissues into a fresh plastic mold containing melted paraffin (*see* **Note 2**).

5. While the paraffin is still liquid, adjust if necessary the position of the tissue within the paraffin using a pre-warmed needle. Once the tissue is at the desired position, remove the mold from the oven and cool the paraffin down slowly to RT. Store the paraffin blocks at 4 °C until use.

3.3 Immunofluorescence Staining on Chromosome Spreads of Spermatocytes and Oocytes

1. Thaw the slides with chromosome spreads at RT, keeping them still wrapped in tinfoil to avoid condensation of water on the slide surface. Once the slides have reached RT, remove the tinfoil and wash the slides three times 5 min in PBS.
2. During this time, thaw the dry milk-containing blocking solution and centrifuge 30 min at 16,000 × *g* and 4 °C to remove insoluble material. Use the supernatant for all subsequent blocking steps to dilute the antibodies, provided that they are compatible with this kind of blocking (*see* **Note 3**).
3. Transfer slides to a moisture chamber, cover spreads with 500 μL of the blocking solution, and block by incubating at RT for 30–60 min.
4. Apply the appropriate first primary antibodies (diluted to working concentration in blocking solution) and incubate for 1 h at RT or overnight at 4 °C (*see* **Note 4**).
5. Wash slides twice for 5 min each in PBS and re-block with blocking solution for another 30 min. To co-stain for a second structure/epitope, incubate spreads with second primary antibodies raised in a different species.
6. Wash twice 5 min in PBS and block for another 30 min in blocking solution.
7. Incubate slides with appropriate fluorescently labeled secondary antibodies for 30 min. In case of co-immunofluorescence, apply both secondary antibodies (coupled to different fluorochromes) in one solution.
8. To counterstain DNA, add in last 10 min of incubation two to three drops of Hoechst (from a 15 μg/μL stock solution) by pipetting directly onto the slides.
9. Finally, wash slides three times 5 min in PBS and mount with glycerol/PBS.

3.4 Immunofluorescence Staining on Paraffin Sections to Analyze Three-Dimensionally Preserved Meiotic Nuclei

1. Prepare 2–15 μm thick cross sections of paraffin-embedded testes or ovaries with a standard sliding microtome (*see* **Note 5**).
2. Transfer sections with a small brush onto a water droplet, placed on Superfrost Plus slides.
3. To remove tissue foldings, stretch the sections on top of the water droplets by gently heating to 58 °C on a heating panel.

4. As soon as the sections appear fully stretched, immediately take off the slides from the plate and remove the water underneath the paraffin section by slanting the slide and absorbing the water with a paper towel. Residual water between the paraffin section and the slide may be removed by rigorous shaking off.
5. Dry the tissue sections over night at RT.
6. Incubate the slides twice in Rotihistol for 10 min each to remove the paraffin from the tissues.
7. Transfer the slides to 100 % ethanol and allow infiltration for 10 min.
8. Rehydrate tissue samples in a decreasing ethanol series by incubating in 90, 80, 70, and 60 % ethanol, for at least 2 min each. Place slides in 50 % ethanol and incubate for 10 min to ensure complete exchange with 50 % ethanol.
9. Collect the sections in distilled water and leave in water for at least 5 min.
10. While the tissue is rehydrated, place the diluted unmasking solution in a closed container and preheat the solution to 125 °C at 1.5 bar in a table autoclave. Once the unmasking solution has reached the final temperature, release pressure of the autoclave and open the autoclave.
11. Immediately transfer the slides from water into hot unmasking solution (*see* **Note 6**), close the autoclave, and heat again until the autoclave reaches 125 °C and 1.5 bar; incubate specimen for 12–30 min depending on the needs (*see* **Note 7**).
12. When unmasking is completed, switch off the autoclave and open the valve to slowly evaporate. When the pressure is down, open the autoclave and place the slides immediately in PBS at RT (*see* **Note 8**).
13. Incubate sections in PBS containing 0.1 % Triton for 10 min.
14. Wash twice for 5 min in PBS and block samples for at least 60 min in PBT.
15. Incubate tissue sections with primary antibodies or a combination of primary antibodies for 1 h at RT or overnight at 4 °C.
16. Wash three times 5 min in PBS and incubate sections with an appropriate combination of secondary antibodies for 30 min.
17. To counterstain DNA, add Hoechst in the last 10 min of incubation.
18. Wash slides three times 5 min in PBS and mount with glycerol/PBS.

3.5 Telomere Fluorescence In Situ Hybridization in Combination with Immuno-fluorescence Staining

In most of our studies, Telo-FISH on meiotic cells was mainly correlated with antibody staining against synaptonemal complex components and/or proteins involved in DNA double-strand repair and recombination (see e.g. [5, 6]). To co-stain telomere DNA and meiotic proteins, we first do the in situ hybridization before proceeding to the immunofluorescence staining procedure.

1. Prepare telomere-specific probes for Telo-FISH by 3′-end-labeling of the two 42mer synthetic telomere oligonucleotides (each of the two oligonucleotides in a separate reaction): add 100 pmol oligonucleotide to 4 μL 5× TdT buffer, 1 μL Dig-ddUTP (1 mM stock) and fill to 18 μL with H_2O. Add 2 μL TdT enzyme, mix gently, and incubate at 37 °C for 20 min. Store at −20 °C until use.
2. Prepare either cell spreads or paraffin sections as described above: for cell spreads follow **steps 1–6** of Subheading 3.1; for preparing paraffin tissue sections, follow **steps 1–9** of Subheading 3.4, which includes the entire antigen unmasking procedure.
3. Wash the specimen in 2× SSC for at least 5 min.
4. Preheat the hybridization solution and both labeling reactions, all separately, to 95 °C for approx. 15 min.
5. Right before hybridization, add 2 μL of each of the two labeling reactions to 40 μL of the preheated hybridization solution, mix, and pipette onto the slides in a moisture chamber (*see* **Note 9**).
6. Close the chamber; tightly seal with Parafilm to avoid evaporation of the hot fluids.
7. Immediately place the chamber in an oven preheated to 95 °C and denature for 20 min (*see* **Note 10**).
8. Let the slides cool down and hybridize overnight at 37 °C.
9. Remove unbound telomere probes by washing twice for 10 min in 2× SSC at 37 °C.
10. Cover sections/cells with TBS/BR and incubate for 30 min at RT.
11. Apply 100 μL of diluted mouse anti-digoxigenin antibodies (1:50 in TBS/BR) for 1 h at RT.
12. Wash specimen twice in TBST for 10 min each.
13. In case of co-immunofluorescence staining for proteins on cell spread preparations, proceed with **step 3** of Subheading 3.3; in case of co-staining on tissue sections, continue with **step 11** of Subheading 3.4.

3.6 Telomere In Situ Hybridization on Electron Microscopic Samples (EM-ISH)

Analyzing meiotic telomeres on the ultrastructural level requires a sophisticated protocol that warrants accessibility for probes on structurally well-preserved electron microscopic samples. Testing and modifying different proceedings, we some years ago developed a protocol which enabled us to detect telomere DNA by in situ hybridization on structurally preserved LR-White-embedded material [9]. We here describe in detail the method of LR White embedding and the detection of telomere DNA on ultrathin sections.

1. Fix small testes tissue pieces in 4 % PBS-buffered formaldehyde for 3 h at 4 °C.
2. Wash five times 3 min each in PBS.
3. Incubate samples in 50 mM NH_4Cl (in PBS) for 15 min at RT.
4. Wash the tissue pieces five times 3 min each in H_2O.
5. Dehydrate samples in an increasing ethanol series by incubating twice in 30 % EtOH (4 °C) for 15 min each, twice in 50 %, 70 %, 90 % EtOH (−20 °C) for 30 min each, and twice in 100 % EtOH (−20 °C) for 1 h.
6. Supplement LR White resin with catalyst benzoyl peroxide (*see* **item 5** of Subheading 2.7).
7. Infiltrate samples overnight with EtOH/LR White (ratio 1:1) at 4 °C (*see* **Note 11**).
8. Transfer samples into pure LR White and infiltrate for 1 h at 4 °C.
9. Substitute with fresh LR White, incubate for another 4 h (or overnight), and repeat this step twice.
10. Transfer the tissue samples into LR White containing gelatine capsules.
11. Incubate at 40 °C for at least 3 days for complete polymerization of the resin.
12. Cut ultrathin sections of the LR-White-embedded samples on an ultramicrotome and transfer the sections to Formvar-coated nickel grids.
13. Prepare a moisture chamber and place a piece of Parafilm inside.
14. Pipette a small droplet of RNase A solution onto the Parafilm and place the nickel grid upside down on top of the droplet (*see* **Note 12**), close the chamber, and incubate at 37 °C for 1 h.
15. Wash twice in 2× SSC for 5 min each and incubate in Proteinase K solution for 8 min at RT, then wash twice in 2× SSC.
16. Place the grid on a droplet of hybridization solution containing the Dig-labeled probes (*see* **step 5** of Subheading 3.5), close the chamber, and seal with Parafilm to avoid evaporation.

17. Immediately place the chamber in a preheated oven at 93 °C for 20 min to denature the probes.
18. Hybridize overnight at 37 °C.
19. Rinse the grids in 2× SSC twice for 10 min at 37 °C to remove unbound probes.
20. To block unspecific binding, incubate samples in TBS/BR for 30 min at RT.
21. Incubate with mouse anti-digoxigenin antibodies (1:20 in TBS/BR) for 1 h at RT.
22. Wash the grids twice in TBST and incubate 1 h with appropriate gold-labeled secondary antibodies (*see* **Note 13**).
23. Wash samples twice in TBST and twice in H_2O for 10 min each.
24. For contrasting, place grids on a droplet of 2 % uranyl acetate and incubate for 4–5 min.
25. Wash two times in H_2O by shaking for 10 s in a coplin jar.
26. Counterstain with Reynold's lead citrate (diluted 1:1 in outgassed H_2O) for 4–5 min.
27. Wash twice in H_2O by shaking for 10 s in a coplin jar.
28. Dry the grids at RT before inspecting them with a transmission electron microscope.

3.7 Electron Microscopy (EM) and Immunogold EM

Standard EM and immunogold EM are well-established techniques, and protocols including minor modifications are used by the different groups worldwide. Here, we describe the protocols routinely used in our labs.

3.7.1 Standard EM

1. Small testes pieces (1 mm^3) are fixed in cacodylate buffer containing 2.5 % glutaraldehyde for 1 h. Glutaraldehyde fixation as well as the following steps are at 4 °C.
2. Wash five times for 3 min each in cacodylate buffer.
3. Fix samples in cacodylate buffer containing 2 % osmium tetroxide for 2 h.
4. Wash five times for 3 min each in H_2O.
5. Incubate the tissue pieces with 0.5 % uranyl acetate overnight.
6. Wash five times for 3 min each in H_2O.
7. Dehydrate samples in an increasing ethanol series by incubating in 30, 50, 70, 90, and 96 % EtOH for 30 min each.
8. Dehydrate samples two times in 100 % EtOH for 30 min each.
9. Incubate samples three times for 30 min each in propylene oxide at RT.
10. For embedding, infiltrate samples overnight with propylene oxide/Epon (ratio 1:1) at RT.

11. Infiltrate samples two times for 2 h each in Epon at RT.
12. Transfer the samples into Epon-containing molds.
13. For polymerization of the resin, incubate at 60 °C for 48 h.
14. Cut ultrathin sections on an ultramicrotome and transfer the sections to Formvar-coated copper grids.
15. Place the grids on a droplet of 2 % uranyl acetate for 20 min at RT.
16. Wash two times in H_2O by shaking for 10 s in a coplin jar.
17. Counterstain sections in Reynold's lead citrate for 10 min.
18. Wash in H_2O by shaking for 10 s in a coplin jar.
19. Dry the grids at RT and inspect them at the electron microscope.

3.7.2 Pre-embedding Immunogold EM

1. Cryosections (6–10 μm thick) are obtained from frozen testes pieces using a cryomicrotome.
2. Sections are placed on coverslips and air-dried.
3. Sections are fixed in PBS containing 2 % formaldehyde for 1 h at RT.
4. After washing five times 3 min each in PBS, samples are blocked in PBS containing 50 mM NH_4Cl for 15 min at RT.
5. Wash the tissue pieces five times 3 min each in H_2O.
6. Coverslips are transferred to a moisture chamber and placed on a Parafilm sheet.
7. Incubate sections with 50 μL of the desired primary antibodies for 1 h.
8. Wash three times for 10 min each.
9. Incubate sections in the moisture chamber with 50 μL of the corresponding secondary antibodies conjugated with gold particles for 1 h (*see* **Note 13**).
10. Wash three times for 10 min each.
11. Fix the sections in cacodylate buffer containing 2.5 % glutaraldehyde for 1 h. Glutaraldehyde fixation as well as the following steps are at 4 °C.
12. Wash five times for 3 min each in cacodylate buffer.
13. Refix samples in cacodylate buffer containing 2 % osmium tetroxide for 2 h.
14. Wash five times for 3 min each in H_2O.
15. Incubate with 0.5 % uranyl acetate overnight.
16. Wash five times for 3 min each in H_2O.
17. Dehydrate the tissue sections in an increasing ethanol series by incubating in 30, 50, 70, 90, and 96 % EtOH for 30 min each.
18. Dehydrate samples two times in 100 % EtOH for 30 min each.

19. Incubate samples three times for 30 min each in propylene oxide at RT.
20. For embedding, infiltrate samples overnight with propylene oxide/Epon (ratio 1:1) at RT.
21. Infiltrate samples two times for 2 h each in Epon at RT.
22. Place the coverslips on a slide covered with a tin foil and cover the section with a gelatine capsule containing 100 % Epon.
23. For polymerization of the resin, incubate at 60 °C for 48 h.
24. Detach the coverslip from tin foil.
25. Expose the gelatine capsule with the attached coverslip to liquid nitrogen to crush the glass into small pieces.
26. Remove the glass pieces from the surface of the sample with a razor blade.
27. Cut ultrathin sections on an ultramicrotome and transfer the sections to Formvar-coated copper grids.
28. Place the grids on a droplet of 2 % uranyl acetate for 20 min at RT.
29. Wash two times in H_2O by shaking for 10 s in a coplin jar.
30. Counterstain sections in Reynold's lead citrate for 10 min.
31. Wash in H_2O by shaking for 10 s in a coplin jar.
32. Dry the grids and inspect the samples at the electron microscope.

4 Notes

1. The first wave of pubertal spermatogenesis, starting approximately at 8–9 days postpartum (dpp), offers an ideal time frame for the investigation of telomere movements because the first prophase I of meiosis progresses almost synchronously within the testis of young mice. Leptotene stages are commonly enriched in mice aged 9–10 dpp, zygotene spermatocytes are mostly found in mice of approximately 11–12 dpp, whereas spermatocytes in mice older than 12 dpp are usually in the transition into pachytene or in early pachytene. Mice aged 14 dpp show enrichment of mid-pachytene spermatocytes, with meiotic progression continuing as the age of the mice increases [5, 6, 10].
2. Use warm, preheated tools to transfer the tissues in order to prevent hardening of the paraffin. Assure that the tissues are completely submerged in the liquid paraffin rather than floating on top of it. During paraffin infiltration, temperature must not exceed 60 °C; if so, tissues may become brittle and unusable.

3. In case that antibodies are not compatible with milk-containing blocking solutions, PBT (*see* Subheading 2.4) or 100 mM glycine in PBS pH 7.4 could be alternatively used for efficient blocking; in this event incubation time has to be 1 h at the least.
4. In our co-immunofluorescence studies, we used antibodies that in some cases required different conditions of incubation. While on paraffin sections, for example, antibodies against MLH1 are usually incubated overnight at 4 °C; with our antibodies against SUN1, we obtained good results when incubating for only 1 h at RT [5]. To obtain best results, conditions have to be pretested separately for each of the primary antibodies.
5. Required thickness of cross sections depends on the actual application: 4–7 μm thick sections comply with most standard immunofluorescence studies on testis tissue; for localization of telomeres and telomere-associated proteins within three-dimensionally preserved entire nuclei, sections should be thick enough to include whole nuclei (i.e., 10–15 μm).
6. At this step, the unmasking solution has approx. 100 °C. Handle with care. To avoid burning, use heatproof glassware and wear heat-resistant gloves.
7. Duration of unmasking depends on the antibody target and the thickness of the section. To get optimal results, pretest your antibodies on serial sections unmasked with increasing time and select the most promising conditions.
8. To prevent floating off the tissue sections, bubbling of the unmasking solution has to be strictly avoided during and after the unmasking procedure.
9. Compared to the ISH on tissue sections, a slightly larger volume of the hybridization solution (and thus of the labeled probes) is needed for hybridization of chromosome spreads on slides. For successful ISH of a reasonably sized central region on the slide, roughly 80–100 μL of hybridization solution is used. It may help to mark a central region on the slide using a grease pencil to be able to subject this same region to the following immunofluorescence procedure.
10. Working quickly and smoothly during this procedure greatly increases the success of the protocol. The hotter the hybridization solution containing the heated telomere probes is when it is placed onto the slides, the more efficient the hybridization reaction is.
11. Use glassware during all incubation steps with LR White resin to avoid potential plastic solvation.
12. All incubation steps starting with RNase A treatment till contrasting with 2 % uranyl acetate are performed within a moister chamber on droplets of approx. 20 μL placed on a Parafilm.

Watch out the orientation of the grids to warrant that the samples always face the solution.

13. In many cases we use goat anti-mouse antibodies coupled with 12- or 6 nm gold (Jackson ImmunoResearch). Before use, dilute the antibodies 1:7.5 in TBS/BR for EM-ISH (Subheading 3.6) or 1:5 in PBS for pre-embedding immunogold EM (Subheading 3.7.2).

References

1. Blackburn EH (2001) Switching and signaling at the telomere. Cell 106:661–673
2. Zickler D, Kleckner N (1998) The leptotene-zygotene transition of meiosis. Annu Rev Genet 32:619–697
3. Sherthan H (2001) A bouquet makes ends meet. Nat Rev Mol Cell Biol 2:621–627
4. Alsheimer M (2009) The dance floor of meiosis: evolutionary conservation of nuclear envelope attachment and dynamics of meiotic telomeres. Genome Dyn 5:81–93
5. Link J, Jahn D, Schmitt J et al (2013) The meiotic nuclear lamina regulates chromosome dynamics and promotes efficient homologous recombination in the mouse. PLoS Genet 9: e1003261
6. Link J, Leubner M, Schmitt J et al (2014) Analysis of meiosis in SUN1 deficient mice reveals a distinct role of SUN2 in mammalian meiotic LINC complex formation and function. PLoS Genet 10:e1004099
7. Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212
8. de Boer E, Lhuissier FG, Heyting C (2009) Cytological analysis of interference in mouse meiosis. Methods Mol Biol 558:355–382
9. Liebe B, Alsheimer M, Höög C et al (2004) Telomere attachment, meiotic chromosome condensation, pairing, and bouquet stage duration are modified in spermatocytes lacking axial elements. Mol Biol Cell 15:827–837
10. Bellve AR, Cavicchia JC, Millette CF et al (1977) Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol 74:68–85